Metabolomics of Endophytic Fungal Isolate Grown with and without Antibiotics to Identify Bioactive Metabolites and their *In-silico* Studies

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A thesis submitted in partial fulfillment of the requirements for the degree of

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بسم الله الرحمن الرحيم

DEDICATED

TO

My Grand Parents (Late) and my Parents

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LIST OF ABBREVATIONS

^{13}C	NMR Carbon NMR
1D	One dimensional
^{1}H NMR	Proton NMR
2D	Two dimensional
3D	Three dimensional
AMBER	Assisted model building with energy refinement
ATCC	American type culture collection
CFU	Colony forming unit
COSY	Correlation spectroscopy
DEPT	Distortion less enhancement by polarization transfer
DMSO	Dimethyl sulphoxide
EIMS	Electron impact mass spectrometry
ESIMS	Electrospray ionization mass spectrometry
EtOAc	Ethyl acetate
GNPS	Global natural product social molecular networking
HIV	Human immune deficiency virus
HMBC	Heteronuclear multiple bond correlation
HPLC	High profile liquid chromatography
HSQC	Heteronuclear single quantum coherence
IC_{50}	Concentration that reduced the effect by 50 %
Kcal/mol	Kilo calorie per mol
KD	Kilo Dalton
LCMS	Liquid chromatography mass spectrometry
m/z	Mass to charge ratio
MD	Molecular dynamics simulation
MeOH	Methanol

MMGBSA	Molecular mechanics generalize born surface area
MMPBSA	Molecular mechanics Poisson-Boltzmann surface area
MN	Molecular networking
MRL	Microbiology research lab
MS	Mass spectrometry
N.A	Nutrient agar
NMR	Nuclear magnetic resonance spectroscopy
NOESY	Nuclear overhauser effect spectroscopy
OSMAC	One strain many compounds
PDA	Potato dextrose agar
QAU	Quaid-i-Azam University
SDA	Sabouraud dextrose agar
SDB	Sabouraud dextrose broth
TCA	Trichloroacetic acid
TLC	Thin layer liquid chromatography
UV	Ultraviolet
WHO	World Health Organization

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ABSTRACT

Endophytes are the microbes resides inside plant without any apparent harm to the host. These microbes are potential source of new therapeutic compounds against many diseases especially against microbial infections. Resistance to antibiotics by bacterial pathogens is growing concern at present, antibiotics are becoming increasingly ineffective as drug-resistance spreads globally leading to more difficult to treat infections. According to WHO currently, at least 700,000 people die each year due to drug-resistant diseases. Therefore, the need of new potent compounds with broad spectrum antibacterial activity is of high time. Natural products have been as widely used as chemical drugs against clinical diseases. Studies revealed that currently, approximately 60 % of approved small molecule medicines are related to natural products, and 69 % of all antibacterial agents originate from natural products.

In the search towards discovery of novel drugs, endophytic fungi have appeared as an outstanding source of high metabolic versatility. This study describes the metabolic profiling, isolation, structure elucidation, bioactivity, and *In-silico* studies of the bioactive compounds obtained from the fungal isolates of *Taxus fauna* of West Himalayan region of Pakistan. The fungal isolates were grown in culture medium with and without addition of different antibiotics. The presence of antibiotics in culture medium may induce certain bioactive metabolites or the antibiotic themselves can be biotransformed into a different chemical structure comprising improved antibacterial activities. The aim of the study was to find potentially new compounds with antibacterial activities.

Seven endophytic fungal isolates of *Taxus fauna* including *Epicoccum* sp. NFW1, *Mucor hiemalis* NFW6, *Epicoccum nigrum* NFW7, *Chaetomium* sp. NFW8, *Fusarium oxysporum* NFW16, *Penicillium milleri* NFL1 and *Paraconiothyrium* sp. NFL6 were cultivated on Sabouraud dextrose medium with and without the addition of antibiotics Clarithromycin, Moxifloxacin, Ciprofloxacin, Cephradine and Cefixime. After incubation, the fermentation broth was extracted with ethyl acetate and the crude extracts were evaluated for antibacterial activities. The crude extracts of *Epicoccum* sp. NFW1, *Chaetomium* sp. NFW8 and *Fusarium oxysporum* NFW16 showed enhanced antibacterial effect when media was amended with clarithromycin and moxifloxacin, so these three fungal isolates were selected for metabolomics profiling and bioactive

compounds isolation under the stress of clarithromycin and moxifloxacin in the medium.

Metabolic profiling of the endophytic fungal isolates with and without antibiotics were carried out by screening the crude extracts with LC-ESIHRMS and data was analyzed and dereplicated by using global natural product social molecular networking (GNPS). Molecular networks were created using the online workflow at GNPS (http://gnps.ucsd.edu). The molecular networking revealed that the antibiotic in the medium changed the metabolic profiles of the fungal isolates by inducing certain metabolites as well as by overwhelming the production of few other metabolites which were produced without the antibiotic stress. The dereplication studies showed that several known bioactive metabolites were produced by these endophytic fungal isolates while many of the metabolites in molecular network were were putatively new or novel metabolites. *Epicoccum* sp. NFW1 among them showed strong metabolic profile in terms of unknown compounds.

The crude extracts were initially purified by normal phase silica gel column chromatography. The active fractions obtained from the column were pooled up and subjected to preparative HPLC for purification. The purified bioactive compounds were structurally elucidated by 1D, 2D NMR and HRESI-MS. A novel dimeric compound (8E,16E)-5,7,10,15,18,20-hexahydroxy-3,22-dimethoxy-5,20-dimethyl-1,24-diphenyltetracosa-8,16-dien-1,2,4,6,19,21,23,24-octaone with chemical formula $C_{40}H_{46}O_{16}$ was isolated and characterized from the *Epicoccum* sp. NFW1 grown in the medium under clarithromycin stress. Another new compound (1R,2R,3R,4S,5S,6R)-1-((1R,2S,3S,4R,5R,7R,Z)-6-(1-aminoethylidene)-8-(cyclopenta-2,4-dien-1-ylmethyl)-1,3,4,5,7-pentahydroxy-2 isopropoxynon-8-en-1-yl)cyclohexane-1,2,3,4,5,6-hexaol with chemical formula $C_{26}H_{43}NO_{12}$ was also obtained from the same fungal isolate when grown with the addition of moxifloxacin to the medium. Similarly, one novel compound 11-(tert-butyl)-11-(hydroxymethyl)-13,13-dimethyl-2,5,8-trioxa-1(1,4)-benzenacyclotridecaphan-10-ol ($C_{22}H_{36}O_5$) was isolated from *Chaetomium* sp. NFW8 grown in medium containing clarithromycin while a previously known compound 4-N-butylphthalic acid ($C_{12}H_{14}O_4$) was first time isolated from any fungal source in this study from *Chaetomium* sp. NFW8 when grown in SDB supplemented with moxifloxacin. A novel compound (S)-1,2,3,6-tetrahydrocyclopenta[d]azepine-6-

carboxylic acid ($C_{10}H_{11}NO_2$) was isolated from *Fusarium oxysporum* NFW16 grown in the presence of clarithromycin in SDB medium. These compounds showed moderate to strong antibacterial activities against test bacterial strains.

The structurally characterized compounds were tested for antibacterial activity but to get a better picture without losing the compound *in-silico* studies (docking, molecular dynamics simulation ad binding energies) were performed. A random screening was performed to check the binding affinity of reported compounds to antibacterial targets. It was unraveled that MurF enzyme of bacterial cell wall machinery might be the target to which these compounds bind with good affinity. It was found that the affinity is the attribute of both hydrophobic and hydrophilic interactions and binding was seen both at the ATP and UDP binding substrate of the MurF. All the reported compounds form stable complex with MurF protein.

This study showed that these endophytic fungal isolates can grow on medium added with high concentrations of antibiotics. The addition of antibiotics to medium will change the metabolic profile of endophytic fungal isolates. Metabolomics and dereplication studies revealed that the endophytic fungal isolates are reservoirs of many known and new therapeutically important compounds. Antibacterial activity and *In-silico* studies suggest that these compounds have significant inhibitory effect against several disease-causing bacteria.

CHAPTER 1

1 INTRODUCTION

Endophytes were introduced as '*organisms that grow within plant tissues'* by De Bary (1866). The most suitable definition, to date remains the one by Petrini (1991); according to which "any organism that resides some part of its life cycle to colonizing the internal tissues of plants without causing any harm to them is called endophyte". Various definitions evolved over the period of time (Bacon and White, 2000; Hallmann *et al*., 1997; Wilson, 1995). According to Nair & Padmavathy (2014) "the microorganisms colonizing healthy plant tissues inter or intra cellularly without observable symptoms of a disease are classified as endophytes". Since isolated from almost all plant species studied; endophytes have a ubiquitous occurrence. As per reports, out of nearly 300,000 plant species inhabiting earth only a few have been explored in context of their endophytic biology (Schulz & Boyle 2002). Therefore, profound avenues for both: the discovery of new endophytic species among plant diversity; and the isolation of novel products such as microbial biocatalysts (Strobel, & Daisy, 2003), are open to exploration.

As per phylogenetic evidence endophytes are broadly characterized as clavicipateous and non-clavicipateous. Clavicipateous colonize grasses while non-clavicipatious endophytes are associated with nonvascular plant, angiosperms, ferns, and conifers (Rodriguez *et al*., 2009). Among variety of microorganisms recognized as endophytes such as fungi, bacteria, actinomycetes and mycoplasmas; fungi are the most prevalent group (Wani *et al*., 2015) with members of Ascomycota and Basidiomycota found in abundance (Jalgaonwala *et al*., 2011; Bhardwaj and Agrawal, 2014). Endophytes colonize the roots, shoots, stem, petioles, leaf segments, fruit, buds, seeds more frequently, also the hollow hyaline plant cells (Hata and Sone, 2008; Specian *et al*., 2012; Stępniewska and Kuzniar, 2013). Endophytic fungi are particularly known for their frequent occurrence in plants and have potential to produce a plethora of bioactive metabolites, enzymes, pigments, and other products of biotechnological applications. Recent studies showed that several antibacterial compounds were isolated from endophytic fungi.

Antibiotics are antibacterial compounds that decrease or altogether halt the bacterial growth. Different mode of actions of antibiotics in bacteria include Inhibition of cell wall synthesis, proteins synthesis, nucleic acid synthesis such as the deoxyribonucleic

acid (DNA), ribonucleic acid (RNA) and bacterial cell membrane disruption (Alekshun and Levy, 2004). During past 60 years millions of metric tons of newer antibiotic classes have been produced. Due to the increased demand of antibiotics, less expensive off label drugs are in demand in many sectors (Chopra *et al*., 2003). As investigated by the researchers over a period of 16 years antibiotic consumption has increased over all by 6 percent in high-income countries such as the U.S., France, and Hong Kong with a 4 percent decrease on a per capita basis. Whereas in the low- and middle-income countries the antibiotic consumption has increased up to 114 percent overall, with per capita consumption contrastingly growing up to 77 percent (Aslam *et al*., 2018).

Antibiotic resistance is acknowledged as a major threat to economic development today impacting global health and food security. The increasing antibiotic resistance has impacted all areas of biomedicine leading to elevated medical costs, extended hospital stays, and higher mortality rates. Without necessary action, we are moving towards a post-antibiotic era, in which minor injuries and common infections will be fatal (WHO). According to an estimate by the year 2050 around 10 million deaths will be attributed to antimicrobial resistant bacteria globally. By 2030, highly adverse impact upon economy is predicted, as antimicrobial resistance is expected to force up to 24 million people into extreme poverty. If neglected for long, around 100 trillion USD of the world's economic outputs are budgeted on the verge of loss (WHO 29 April 2019 Joint News Release New York) hence substantial efforts should be promptly made to curtail this threat. Many bacterial pathogens developing resistance to current therapeutics among them World Health Organization (WHO) has also recently listed ESKAPE pathogens against which new antibiotics are urgently needed (Tacconelli *et al*., 2018). The term "ESKAPE" encompasses six such pathogens with growing multidrug resistance and virulence: *Enterococcus faecium, Staphylococcus aureus, Klebsiella pneumoniae, Acinetobacter baumannii, Pseudomonas aeruginosa*, and *Enterobacter* spp. (Rice, 2008). ESKAPE pathogens are associated with the highest risk of mortality thereby resulting in increased health care costs (Founou *et al*., 2017) and are capable of "escaping" the biocidal action of antimicrobial agents (Rice, 2008; Navidinia, 2016).

On 22 October 2015, WHO launched the Global Antimicrobial Resistance and Use Surveillance System (GLASS)**,** the first global collaborative effort to standardize AMR

surveillance countries collect and submit AMR data on select pathogens for four types of infection: BSIs caused by *Acinetobacter* spp., *Escherichia coli, Klebsiella pneumoniae, Salmonella* spp., *Staphylococcus aureus,* and *Staphylococcus pneumoniae*; UTIs caused by *E coli* and *K pneumoniae*; gastrointestinal infections caused by *Salmonella* spp. and *Shigella* spp.; and genital infections caused by *Neisseria gonorrhoeae*. The GLASS data for 2019 showed that, in UTIs caused by *E coli* and *K pneumoniae,* resistance to the first-line antibiotic co-trimoxazole was 54.4 % and 43.1 %, respectively, and resistance to ciprofloxacin, a broad-spectrum second option for UTIs, was also found to be consistently high (43.1% for *E coli* and 36.4% for *K pneumoniae*). The report notes that the high rate of resistance to ciprofloxacin is consistent with reports that the antibiotic is frequently being overused for UTI treatment. BSIs caused by MRSA and *E coli* with resistance to third generation cephalosporins have been identified by the WHO as two important AMR Sustainable Development Goal indicators. Another concern is the extremely high resistance to carbapenems found in BSIs caused by *Acinetobacter*. Persistent use of antibiotics has provoked the emergence of multidrug resistant (MDR) and extensively drug resistant (XDR) bacteria, which render even the most effective drugs ineffective. Extended spectrum β-lactamase (ESBL) and carbapenemase producing Gram negative bacteria have emerged as an important therapeutic challenge.

The concern of prevalent antibiotic resistance is compounded by the immense pressure that the finding of newer antibiotics is also limited, and occasional progress is made in this field after prolonged efforts and experimental validation. Majority of the antibiotics currently available in market were discovered, mid to late 20th century. There are ample chances that bacteria are simultaneously become resistant to numerous drugs at a time; hence there are limited options of drugs to combat resistant bacteria. To overcome these problems beside the appropriate use of antibiotics and all other precautionary measures there is a constant need to discover or develop efficient and safe antibacterial (Monserrat-Martinez *et al*., 2019).

The uncertainty around the process of drug discovery makes it a high-stake game. The pharmaceutical companies spend billions of dollars, each year to find the elusive new drug, a panacea against all the resistant infections. The conventional scheme for the detection of drug from natural products usually contains plants and microbes that are

thoroughly fermented and fractionated so, the extracted purified fractions can be processed for structural exposition and determining the extent of biological activities against test organisms. There are several hurdles in the whole process like inconsistency of source materials, difficulty in fractionating the active components, and the possibility of that active constituent, being identified as just another known compound (Tyers *et al*., 2019). As par varying estimates, generally only one compound in 10,000 succeeds as a new pharmaceutical; and it takes around 12 years for the discovered compound to reach market with an approximated cost of US$350 million (Okhravi, 2020). To enumerate the success rewards, most effective drugs have made business up to US$1 billion a year with incalculable benefits to society. Several technological advancements have improvised the process of product discovery from natural sources. Innovations in the field have resolved past challenges while re-establishing natural products as major sources for the eminent drugs. Among remarkable innovations made are enhanced screening procedure, improved sourcing of natural product, synthetic biology methods combining biosynthesis, organic synthesis and microbial-genomics and metabolomics (Tejesvi *et al*., 2018; Greco *et al*., 2019). Among myriad approaches to overcome the hurdle of antibiotic resistance one focuses upon modifying the existing antibiotics to combat emerging and re-emerging resistant pathogens globally (Davies and Davies, 2010). Such modifications can be achieved by chemical method, by biological approach or where necessary applying both simultaneously.

The method of chemical conversions, despite providing abundant products is limited by the selectivity constraints (‘regio’ and ‘stereo’). Though involvement of elaborate reaction steps generates overall low final product yields. However, bio-catalysis reactions involving well-established “green” techniques for chemo-regio and stereo-selective functionalization of several molecules, remains the most attractive option as the drug development process (Richter, 2017). There are many filamentous fungi that can perform complex biotransformation which are otherwise difficult to attain by chemical transformation methods. For instance, the exemplary endophytic fungus *Fusarium oxysporum* from *Catharanthus roseus* converts the sterile solution of Vinblastine to vincristine, upon incubation with the fungal mycelial mass at room temperature. The biological synthesis of beta lactam antibiotics is the example of bio-catalysis used in pharmaceutical industries. Among important enzymes of microbial origin used for these processes, penicillin V acylases, penicillin G acylases,

cephalosporin acylases, are significantly used (Prasad, 2018). Other side of microbial transformation of antibiotics can also degrade antibiotics under ambient conditions which is a powerful tool to remove environmental contamination due to antibiotics.

Endophytic fungi are the promising source in both the cases to degrade antibiotics contaminating environment as well as to transform them into more efficient and potential form. These endophytic fungi can be used in different environments and industry as a biological source of transforming antibiotics into desired compounds or molecules. However, nature have remarkable sources of therapeutic compounds among them microbes and especially endophytic fungi from medicinal plants turned out as promising source. So, to discover novel bioactive metabolites by overcoming hurdles we can integrate different techniques together and one of potential method is use of metabolomics and global molecular social molecular networking (GNPS) in the arena of natural product discovery along with purification and structure elucidation. Metabolomics is the investigation of entire metabolites existing in the organisms under precise time and conditions (Cuperlovic-Culf *et al.,* 2016; Wang *et al*., 2017). While molecular networking yields an MS/MS spectral similarity map that permits the visualization of structurally linked molecules. The core strength of this method is that it can be used for the investigation of millions of MS/MS spectra, without any former knowledge regarding the chemical composition of samples (Mohimani *et al*., 2017; Wang *et al*., 2017). Moreover, GNPS can perform dereplication of MS/MS data automatically by spectral-library search for well-known molecules in the molecular networks, if their MS/MS spectra are present in public MS/MS spectral-libraries (Wang *et al*. 2017). To understand the bioactive potential of a metabolite or compound by computer aided studies which are known as *in-silico* studies could be used as an alternative approach to reduce the time, labor, and resources to study the bioactivities of metabolites. There are several *in-silico* studies among them for bioactive metabolites, molecular docking, molecular dynamic simulation, and binding energies are crucial to understand the action of that metabolite. These *in-silico* studies help us in understand the binding of ligand (metabolite/compound) with target protein (receptor) and compare the receptor, ligand and complex in solvation and gas phase to determine the role of water molecules in the complex stability inside cellular environment (Sahihi, 2016; George *et al*., 2019; Gunalan *et al*., 2020).

To study bioactive metabolites from natural source we can get more out of it when we integrate multiple approaches. The present study is aimed to the isolation, screening and characterization of the bioactive metabolites produced by endophytic fungi of *Taxus fauna* under antibiotic stressed condition by integrating different strategies and approaches.

1.1 AIM AND OBJECTIVES

Aim of the study is identification and characterization of novel bioactive metabolites from endophytic fungal isolates grown with and without antibiotics.

Objectives

1) Screening of bioactive metabolites production by endophytic fungal isolates grown with and without antibiotic (antibacterial activity guided screening).

2) Production, evaluation and chromatographic identification and separation of bioactive fractions from extracts of selected fungal isolates.

3) Metabolomics of fungal isolates grown with and without antibiotics, dereplication studies by HRLCMS/MS using GNPs as dereplication strategy.

4) Isolation and characterization of bioactive compounds using HPLC, NMR and HRMS analysis and antibacterial activity.

5) *In-silico* studies of characterized metabolites for antibacterial potential using docking, binding energies, and molecular dynamics simulations.

CHAPTER 2

2 REVIEW OF LITERATURE

Since the beginning till now mankind is facing problems of bacterial infections, and these infections are spreading day by day. This is due to the capability of bacteria to become resistant against antibacterial representatives (Levy & Marshall 2004). Infectious organisms are developing resistance ("ability of bacteria to escape and oppose the effects of antibacterial agents that were previously effective to cure against that bacteria") against the commonly prescribed antibacterial drugs which results in extended infection and higher risk of mortality, due to which antibiotic resistance is considered a "global public health concern" (Cosgrove, 2006). Another disturbing aspect is that it has advanced over period, from resistance against single class of antibiotic drugs to multiple classes (multidrug resistance) and yet extreme drug resistance (Walsh, 2013), intensifying the challenge for the formulation/development of more effective drugs.

Due to issues concerning antibiotic resistance more than 0.7 million people died/year worldwide, which by 2050 is estimated to be around 10 million (O'neill, 2014). Conferring to the current data from the "European Medicines Agency" and "European Centre for Disease Prevention and Control" almost 25000 European nationals (5.1/100,000 individuals) die every year due to infections caused by antibiotic resistant bacteria (http://www.ecdc.europa. eu/). In the USA, approximately 12,000 (4.0/100,000 individuals) die from nosocomial infections every year (Zell and Goldmann, 2007), its estimated that in excess of 70 % bacteria causing these infections have acquired resistance to at least one of the commonly used antibiotics (Muto, 2005). According to an assessment the annual consumption of antibiotics worldwide lies from 100,000 to 200,000 ton/year, while China alone consumes in excess of 25,000 tons/year (Van Boeckel *et al*., 2015). Microbes are acquiring resistance against antibiotics by various molecular mechanisms which include limiting uptake of a drug, modification of a drug target, inactivation of a drug, and active efflux of a drug (Figure 2.1; Ferri *et al*., 2017; Blair *et al*., 2015). Therefore, time demands to exploit new novel classes of antibacterial compounds having unique mechanism of action against diseases. Growing demand of novel antibacterial therapeutic agents, helpful in controlling the resistance mechanism and spreading of emergent bacterial diseases, researchers are exploring both terrestrial and marine environment in search of new antibacterial compounds (Chaung *et al*., 2015).

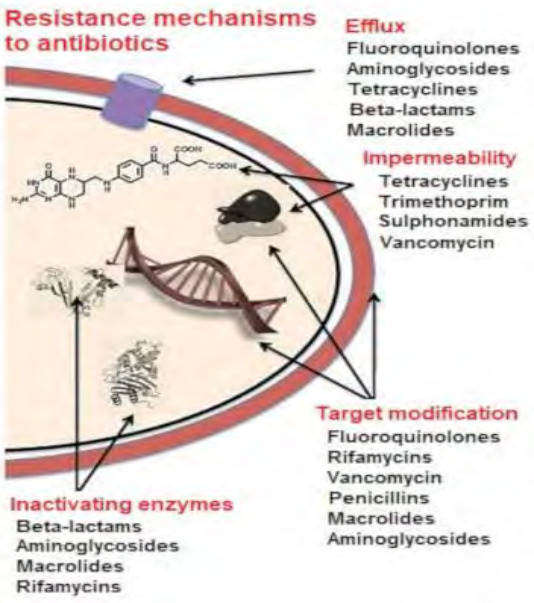


Figure 2.1 Antibiotic resistant mechanisms (Fig. Adopted from encyclopedia of environment)

These mechanisms of resistant may be native to the microorganisms or acquired from other microorganisms. Plasmid-mediated transmission of resistance genes is the most common route for acquisition of outside genetic material; bacteriophage-borne transmission is rare (Reygaert, 2018). The diagrammatic representation of bacterial resistant mechanism showed in (Figure 2.2).

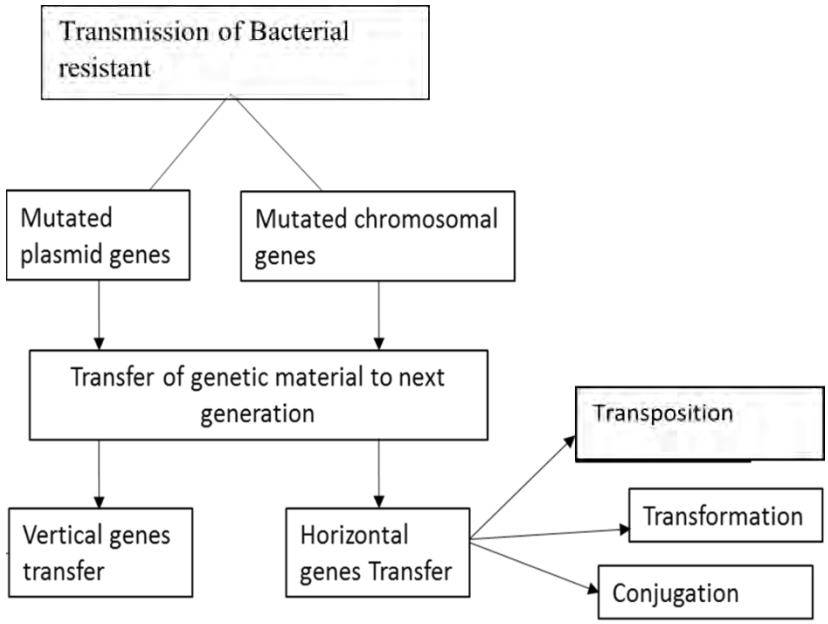


Figure 2.2 Transmission of resistance in bacteria

Serval strategies were employed for drug discovery, but natural products are likely to continue to be sources of new commercially valuable drug leads because the chemical novelty associated with natural products is higher than that of any other source. The bioactive compounds were achieved from plants till the discovery of penicillin. After the discovery of penicillin microbes were also considered as a potent source of bioactive compounds. As microbes are very rich in diversity and very a smaller number of microbes were explored for the production bioactive compounds. Microbes from unusual niches should explored for bioactive compounds and endophytes are one of the most important examples of microbes residing such habitats. There are several medicinal plants which are endangered and required specific condition and time for growth and insufficient to meet the needs of bioactive compounds that they produced. There are fair chances that these plants harbor some endophytic micro-organisms which may produce compounds with similar properties which can be cultivated in labs, and we can produce the desired bioactive compounds without facing the issues related with the plant species. Along with other bioactive compounds this global problem of antibiotic resistance led the scientists to feature endophytic fungi, predominantly isolated from the plants of medicinal importance, for their potential, as a source of novel antibacterial compounds (Radu & Kqueen, 2002; De Siqueira *et al*., 2011; Liang *et al*., 2012).

Some fungi hosted by medicinal plants are not only contributing to the co-production of active metabolites but also take part in biological activities that are exhibited by the host plants (Alvin *et al*., 2014). Endophytic fungi reside throughout their life or part of their life cycle in host plants tissues (Strobel & Daisy, 2003). Endophytic fungi bring mechanism of resistance against invading parasite by producing secondary metabolites to protect host plants. These secondary metabolites have antagonistic activity against pathogens. With all these characteristics endophytic fungi are thought to be as the hub, to produce secondary metabolites that leads to the production of novel drug production (Strobel, 2003; Owen & Hundley, 2004).

2.1 Types of Endophytes

There are different types of endophytes which are producing potential secondary metabolites as therapeutics and pharmaceutics. These endophytic groups include

bacteria, mycoplasma and actinomycetes and fungi where, the most frequent endophytes are fungi (Figure 2.1; Gouda *et al*., 2016).

2.1.1 Clavicipitaceous Endophytes (C-endophytes)

They are also recognized as class 1 endophytes. The C endophytes are also known as *Balansiaceous* fungi that only represents the phylogenetically related few numbers of clavicipitaceous species which are fast growing and only restricted to the grasses of warm and cool seasons (Bischoff & White 2005). These endophytes probably lives on the upper ground portion of the grass, and they make intercellular relationship with the host to spend their whole life in host. They are not only non-pathogenic towards their host, but they also provide resistance for herbivorous and drought impact which increases the host plant biomass. However, these benefits offered by these C-endophytes depend upon the host plant species, environmental factors and genotype (Faeth & Hamilton 2006). Primarily class 1 endophytes follow the vertical pattern of transmission within the host through which they invade from maternal parts of the plant to the offspring's through seed infections. This is the reason that mostly they colonized in the specific region of the host plant (Saikkonen *et al*., 2010). This fungal infection upsurges the growth of the host over a time lapse suggesting the adaptive benefit for the host. They defend their host against water stress and nitrogen starvation. Many studies revealed that epichloe endophytes (*N. uncinatum*, *Neotyphodium coenophialum*, and *N. lolii*) of the meadow fescue, tall fescue and perennial ryegrass respectively have fixed benefits for plant growth. Although these systemic grass fungi have been well understood about their interaction between the endophytes and the host plants in natural population have not been comprehend fully till now (Uzma *et al*., 2019).

2.1.2 Non-Clavicipitaceous Endophytes (N-C endophytes)

The NC-endophytes that are also known as non-balansiaceous have the polyphyletic-assemblage of *Ascomycetous* fungi and are highly diverse with unidentified ecological roles. These endophytes ranges from terrestrial to tundra regions and exists in almost agro-ecosystems and tropics. (Arnold & Lutzoni, 2007). They are classified into three functional classes 2, 3 and 4 which may differ from each other pattern of host colonization, transmission between host generation, ecological roles, and plant diversity. Although all the three classes possess the diverse range of host but among them class 2 have host in above ground and below ground plant parts. However, class

3 only resides in the above ground portion of the plant and class 4 is only limited to the roots of the plants. Pattern of colonization within the host is also the big difference between these classes as class 3 causes localized infection in the host plants while class 2 and 4 offer widespread tissue colonization.

2.1.2.1 Class 2 Endophytes

Class 2 endophytes basically encompasses, *Ascomycota* and *Basidimycota* but the dominant among them is Ascomycota. They are dissimilar from the other NC-endophytes as they resides in all portions of the plants like root, stem and leaves and causes extensive plant tissue infections. They normally spread through the seed coat or the rhizomes of the plants. They receive much attention as they have vast species diversity and their various ecological modes and the switching lifestyle abilities from endophytic to free living organism and having numerous potential applications (Rodriguez *et al*. 2009). This class is reflected as the largest symbiotic assembly of fungi that can be culturable on the synthetic media and robust in making colonization in host plant. The first identification of the endophytes from this class was in *Phoma sp*. of *Calluna vulgaris* (Rayner, 1915). They had a mutualistic relation with their host plants by giving them resistance for abiotic pressure and taking nutrients for their own growth and reproduction.

2.1.2.2 Class 3 Endophytes

The class 3 endophytes mostly representative of the group of *Ascomycota* and lesser number is belonged to the *Basidiomycota* group. They propagate in the host via hyphal fragmentation, and they can produce asexual and sexual spores on dead tissues of the host. These spores and hyphal fragmentation have been released through physical factors such as air, rain, or wind or by herbivores (Herre *et al*., 2007). They are mainly colonized in the shoots of the plants that make them different from other two classes. They harbor in the diversified range of plant kingdom which includes tropical trees, non-seed plants, non-vascular and seedless-vascular plants and woody, herbaceous angiosperms in the tropical-forests (Davis and Shaw, 2008). Beside all these hosts these endophytes are also present in the fruits, flowers, and inner bark. In moist tropical forest most of the healthy leaves acquired many independent infections as compared to systematic infections (Arnold, 2007).

2.1.2.3 Class 4 Endophytes

These are branded for the existence of dark melanized septa. These NC endophytes are mostly connected with the tissues of the roots of the angiosperms that are comprising of pigmented fungi that's why they are named as "dark septa endophytes" (DSE) that are only limited to the root region. They belong to *Ascomycotic* group of fungi and make both microsclerotia, inter and intracellular hyphae within the roots. Mostly their mode of transmission is conidial and mycelial fragmentation that transmitted horizontally in the host plant. The root colonization of *Phialocephala fortinii* is an excellent example of DSE. The colonization starts from the root system where loose individual hyphae is formed and root system play a vital role in its transmission through cortical cells and inside the depression of epidermal cells (Mandyam *et al*., 2012). They had been described in almost all type of ecosystems i.e., Arctic, tropical, Antarctic, and temperate. However, their role remains uncertain for mysterious fungal symbiosis.

Table 2.1 Classification of endophytes their host and mode of transmission

	C-endophytes	**N-C endophytes**		
	Class 1	**Class 2**	**Class 3**	**Class 4**
Host Plants	Grasses	Tropical and terrestrial plants	Vascular and non-vascular plants and trees	Angiosperms
Mode of transmission within host	Vertical and horizontal mode of transmission in upper ground portion of hosts	Vertical and horizontal Invasion in upper and lower parts of plants	Horizontal propagation through hyphae on dead plants specially in the shoots of the host plants	Invasion is horizontal through conidial and mycelia fragmentation in the roots of host plants
Representative fungi group	*Balansiaceous* fungi	*Basidiomycota* (dominant) and *Ascomycota*	*Ascomycota* (dominant) and *Basidiomycota*	*Ascomycota*

2.2 Fungi and Bioactive Metabolites

Since decades kingdom Fungi is very well known to produce bioactive molecules having therapeutic tendencies. Bioactive metabolites extracted from the endophytic fungi has many vital biological properties such as antibacterials, antioxidants, flavoring agents, pigments, medicines, recreational drugs, immunomodulators, anticancer, anti-parasitic, antiviral, anti-tuberculosis and insecticides (Craney *et al*., 2013; Hussain *et al*., 2014).

Discovery of the penicillin was the new revolution in the era of medicine and discovery of new therapeutic agents for bacterial infections and till now the research is going on in the search of bioactive compounds from different sources among them endophytic fungi is one of the promising a source. Due to the diversity occurrence of endophytic fungi, they are yet not studying extensively to explore the therapeutic potential of these fungal group. Many other studies on marine and terrestrial fungi revealed that they are noble source of producing exceptional bioactive molecules. Many antibacterial compounds are produced by fungi throughout the world and few of them are approved as drugs such as fusidic acid and cephalosporin (Wo *et al*., 1962; Newton and Abraham 1955).

Most of the pharmaceutically important compounds are derived from the fungi are sourced from Ascomycota and the most renowned species are *Penicillium* and *Aspergillus* (Bérdy, 2005). Whereas some of the pharmaceuticals are of industrial importance have been derived from Basidiomycota and some other groups of fungi as well (Bala *et al*., 2011). Among fungal species the most diverse studied specie is *Aspergillus* which is the most commonly present. One reason for extensive study and high number of isolated metabolites from *Aspergillus* is that they are tolerant to salt, easily obtained from any source or substrate and it is fast growing (Bugni and Ireland, 2004). Having broad structural array diversity and biological activities this genus continually attracted the interest of pharmacologist for the identification of wide range of secondary metabolites (Wang *et al*. 2017). *Aspergillus* is prolific basis of active secondary metabolites with fascinating biological accomplishments such as antibacterial activity (Perrone *et al*., 2007; Xu *et al*., 2015). Whereas filamentous fungi also represent an important group of microorganisms involved in the synthesis of wide

variety of bioactive natural compounds (Horgan and Murphy, 2011; Jansen *et al*., 2013; Bills and Stadler, 2014).

2.3 Role of Fungal Endophytes in Drug Discovery

According to Saha *et al* (2019) who spotted the importance of fungal metabolites as the next level generation of antibiotics. They demonstrated that endophytic fungi are the most important epitomes of many bioactive metabolites and natural compounds, which are important in the medical and industrial arenas of nowadays. As these endophytic fungi resides in the host plants, some contemporary endophytes and their host makes a unique relationship which can control the plant metabolites and affects the physico-chemical properties of plant based crude drugs. Now a days, human lifestyle has led to the increase in many chronic diseases such as asthma, pulmonary diseases, cancer, and cardiovascular diseases. Due to the introduction of several new drugs in the market there is increase in the antibiotic resistance in the patients suffering with chronic disease. Synthetic drugs have proved uncertain, so time demands to develop new drugs that give protection against harmful microorganisms with minimal side effects. In this regard natural sources have always been a choice for the identification and isolation of the novel bioactive compounds with diverse therapeutic applications. It has already been determined that almost 20,00,000 natural products are originated from microorganisms. Endophytic fungi produce secondary metabolites that have many biological potential properties which are helpful in the pharmaceutics to cure illness of various kinds. These properties include antimicrobial, anticancer, antiviral and many others (Figures 2.3).

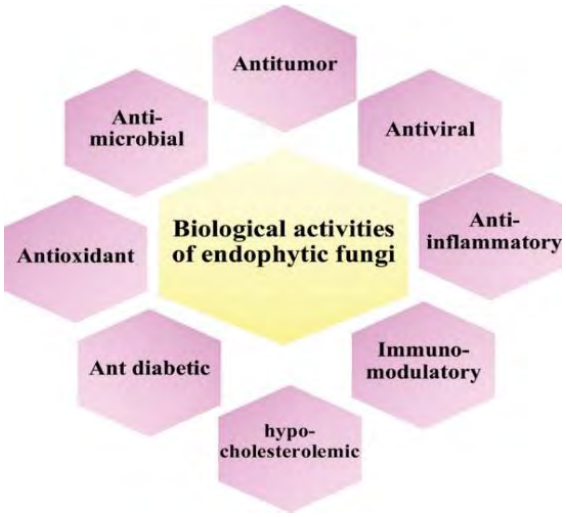


Figure 2.3: Biological activities of endophytic fungi

2.3.1 Antibacterial Compounds from Endophytic Fungi

Goutam *et al*., (2016), extracted metabolites from the endophytic fungi *Emericella qaudrilineata* (RS-5) and subjected to fractionation to identify the most active compound posing the antibacterial activity. The dominating compound was recognized as a Benzyl benzoate that exhibited the robust antibacterial activity against gram positive *Staphylococcus aureus* and gram negative *Aeromonas hydrophilla*. Medically this Benzyl benzoate is the active element of Ascabiol, which is commonly used in the treatment of various skin diseases such as scabies. Marcellano *et al*., (2017) studied the endophytic fungi isolated from the bark medicinal plant *Cinnamomum mercadoi.* All the endophytic fungi isolated possessed antibacterial activity. Two endophytic fungal strains among them showed broad-spectrum activity and restricted the growth of all test organisms. *Fusarium* sp. was recognized as the most potent candidate for antibacterial activity with MIC ranges between 2.-4.2 mg/L.

The ethyl acetate extracts of 44 endophytic fungi of Zingiber were checked for their antibacterial and antioxidant activities. The activity of the extract from *Arthrinium* sp. MFLUCC16-1053 displayed good activity against Gram negative and Gram-positive bacteria. The MIC against *E. coli* and *Staphylococcus aureus* was 7.81 and 31.25 µg/mL, respectively. GC-MS concluded that *Arthrinium* sp. MFLUCC16-1053 extract contains both antibacterial and antioxidant composites which are 3*E*-cembrene A, β-cyclocitral, laurenan-2-one, 2*Z*, 6*E*-farnesol, sclareol, β-isocomene, cembrene, and γ-curcumene (Pansanit *et al*., 2018). Praptiwi *et al*., (2018) reported antioxidant and antibacterial properties of the 40 endophytic fungi isolated from the 10 species of the medicinal plants collected from Palolo, Central Sulawesi. Bioautography screening showed that 30 extracts inhibited the *S. aureus* and 29 inhibited the *E. coli*.

Fungal extracts of 60 endophytic fungal isolates from *Sceletium tortuosum* were characterized and 15 % of the extracts showed narrow spectrum for bacterial strains. None of the fungal extract was able to inhibit the growth of *Enterococcus faecalis* and *Enterococcus gallinarum*, while the *Bacillus cereus* was the utmost susceptible strain against the activity of fungal extracts. *Fusarium oxysporum* possessed the best antibacterial activity due to the high levels of octadecanoic acid and 5-hydroxymethylfurfural (HMF) whose presence was identified through GC-MS (Manganyi *et al*., 2019). The extracted metabolite from fungal strain AE1 isolated from

the leaves of the *Azardirachta indica* was very thermostable and non-proteinaceous and showed very prominent zone of inhibition for Gram negative and Gram-positive bacteria with MIC values of 300-400 µg/mL (Chatterjee *et al*., 2019). Five different alkaloids compounds in which one was new compound (GKK1032C) and four were already known compounds (pyrrospirones E, F, GK1032B and A2) were extracted from the fungal endophytic strain *Penicillium* sp. CPCC 400817. The compound GKK1032C exhibited antibacterial activity against *Staphylococcus aureus* having MIC value of 1.6 $\mu g\ mL^{-1}$ (Qi *et al*., 2019).

The noticeable metabolites that were extracted as bioactive secondary metabolites by submerged fermentation from *Diaporthe schini* were found to be 2-hexadecene, 13-docosenamide, 9-octadecenamide, 3,7,11,15-tetramethyl; and 11-octadecenoic acid. Extracts exhibited high inhibitory effects for *Staphylococcus epidermidis, Staphylococcus aureus, Enterobacter aerogenes* and *Klebsiella pneumonia* (Dos Reis *et al*., 2019). A study was conducted for the extraction of antibacterial metabolites from the endophytic fungal isolate *Lasiodiplodia pseudotheobromae* IBRL OS-64 that harbors the leaves of *Ocimum sanctum Linn*, a medicinal herb. All the test strains were susceptible towards the extracts however, among the tested bacterial strains *E. coli, Pseudomonas aeruginosa and Proteus mirabilis* were the least susceptible strains (Taufiq & Darah, 2019).

Al-Fakih & Almaqtri, (2019) published the review on antibacterial studies from endophytic terrestrial fungal isolates of *Aspergillus*. They mainly focused on the antibacterial activities, structures, and mode of action of the extracts starting from 1942 to 2018. They inferred from the results published in the world that very large number of extracts showed antibacterial activities for so many Gram positive and Gram-negative bacteria. The most prominent result obtained was of CJ-17,665 that was isolated from *A. ochraceus*, which showed excellent activity against multi drug resistant *Staphylococcus aureus*. Antimicrobial activity of the 42 endophytic fungi taken from *Ficus elastic* leaves was checked in contradiction of five pathogenic microbes. Two strains Fes1711 (*Penicillium funiculosum*) and Fes1712 (*Trichoderma harzianum*) showed wide-spectrum bactericidal activities. Two new derivatives of isocoumarin were identified along with the three known compounds which exhibited the antibacterial activity against *E. coli* (Ding *et al*., 2019).

The antibacterial activity of the polysaccharide (GCP) extracted from the *Chaetomium globosum* was composed of arabinose, glucose, galactose, mannose, xylose, and glucuronic acid in a molar ratio of 0.64: 23.53: 2.47: 2.58: 0.90: 0.27 having molecular weight of 8.093×10^4 Da and polydispersity 1.014 (Mw/Mn). Antibacterial activity was checked against *E. coli* and *Staphylococcus aureus* and MIC value for both was 0.67 mg/mL and 1.75 mg/mL respectively. Results clearly demonstrated that GCP can be the promising source to produce antibacterial compounds used in food and pharmaceutical industries (Wang *et al*., 2019).

Ratnaweera *et al*., (2018) determined the antibacterial potential of endophytic fungi residing in the *Cyperaceae* family. In this regard 72 endophytic fungi of distinct morphology were isolated from the six plants and their antibacterial activities were examined against two gram positive and three Gram negative bacterial strains. Two of the antibacterial metabolites were isolated and their structures and MIC were elucidated during the study. Out of 72 fungal extracts 66 were active against the bacterium tested. 9-Epi viridol isolated from *Trichoderma virens* and Butyrolactone I isolated from the *Aspergillus terreus*, exhibited MIC values ranges from 128-256 µg/mL against *E. coli, Staphylococcus aureus*, *Bacillus subtilis* and MRSA. That study inferred that *Cyperaceae* plant is harboring endophytes that had potential for antibacterial activities.

Ola *et al*., (2018) isolated a new antibacterial polyketide palitantin that was isolated from the *Aspergillus fumigatiaffnis,* an endophytic fungus that lives in the medicinal plant *Tribulus terestris*. Its antibacterial activity was also checked against the *Enterococcos faecalis* and *Streptococcus pneumonia* where it showed activity against both the strains with MIC value of 64 µg/mL. Yang *et al*., (2018) studied three new natural compounds which includes, a new xanthone analog isosecosterigmatocystin, new anthraquinone derivative isoversicolorin C and a new amino acid derivative, glulisine, alongside six related metabolites that were extracted from *Aspergillus nidulans* MA-143, the mangrove-derived endophytic fungus culture broth and mycelia extracts by giving 0.1% ethanol stress. These compounds also showed potent activity against tested bacterium.

The most recently isolated antibacterial compounds form endophytic fungal strains are illustrated in Figure 2.4.

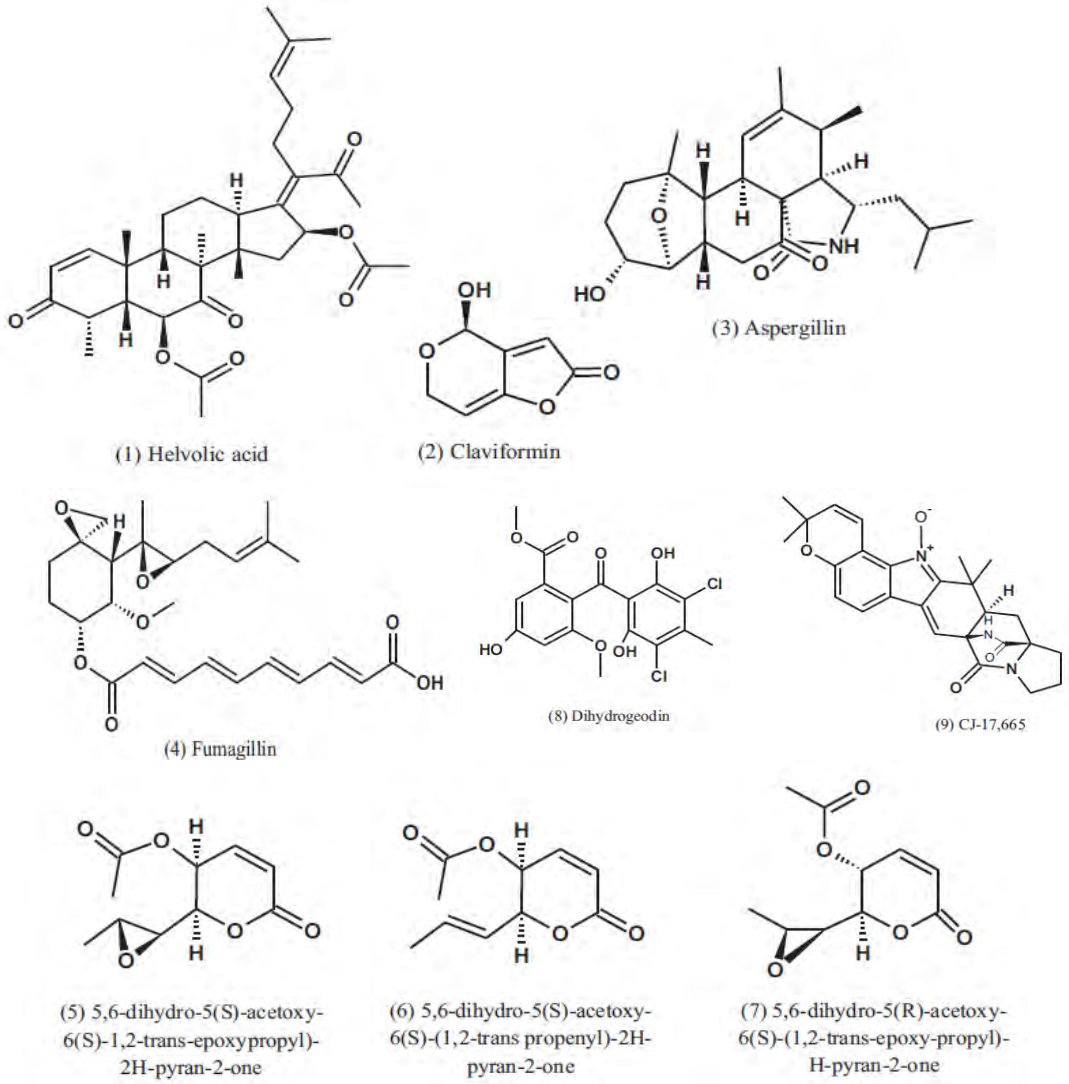


Figure 2.4 Structural presentation of recently identified anti-bacterial compounds from endophytic fungi.

2.3.2 Endophytes as Potential Source of Anti-Cancerous Compounds

Bioactive compounds attained from the endophytic fungi possess a very good characteristic to inhibit cancer through apoptosis, cytotoxicity, and disruption of cellular filaments. Till now several endophytic fungal strains has been identified to produce secondary metabolites that are proven to be effective in the cytotoxicity analysis and treatment of cancer (Joseph and Priya, 2011). Vincristine, etoposide, topotecan, irinotecan, taxol and vinblastine are few of the drugs that are derived from the plants and are in clinical/medical use to treat many human cancers, presently (Balunas and Kinghorn, 2005). The association between the endophytes and host plant

led to the discovery of taxol from the *Taxus brevifolia.* This achievement led to the identification of more endophytic fungi from the host plants to produce structurally and activity wise somehow similar kind of metabolites. The FDA approved drug Taxol ($C_{47}H_{51}NO_{14}$) has been used for the targeted treatment of cancers (Breast, lung, neck, head, ovarian and Kaposi's sarcoma) is highly functionalized diterpenoid which is considered as world first billion-dollar drug. After this discovery, the breakthrough was the production of taxol from the endophytic fungi*, Taxus brevifolia, Taxomyces andreanae* and *Pestalotiopsis microspora* from *Taxus wallichiana* has been reported *(*Aly *et al*. 2011). Due to these findings research is more focused towards the search of more novel paclitaxel byproducts/derivatives from the fungal endophytes of non-*Taxus* and *Taxus* species (Kaul *et al*. 2013). Moreover, the podophyllotoxin and its derivative analogs are very important in pharmaceutics as they acquired antiviral and cytotoxic activities (Abd-Elsalam and Hashim, 2013). Due to deforestation and overexploitation of the medicinal plants the natural source of not only podophyllotoxin but of many more therapeutic activity harboring plants have been limited and the artificial production of the compounds is still under its way to optimize the newer ways of production with quantifiable yields. For instance, the sustainable production from the plant tissue culturing technique is offering very consistent, reliable, and capacitated solution for these compounds (Majumder and Jha, 2009). Figure (2.5) shows the names and structures of the compounds having anticancer potential extracted from the endophytic fungi (Deshmukh, 2018).

2.3.3 Endophytes as Potential Source of Antiviral Compounds

Endophytes have not been well-studied microorganisms which gained importance in recent decades for their biological activities especially of anti-viral activities with high levels of structural variety (Selim *et al*., 2016). The antiviral prospective of endophytic fungi that were isolated from the medicinal plants was evaluated and 29 endophytic fungi isolates were recognized as *Ascomycetes*, excluding two strains that were identified as *Zygomycetes*. Out of 99 total 15 extracts restricted the reproduction of HSV-2 virus, while VSV-virus reproduction was stopped by 16 extracts. The promising anti-viral activity for both viruses was shown by *Plospora terda* strain that was isolated from *Ephedra aphylla*. Viral inhibitory activity was 40 % for HSV-2 and 15.2 % for VSV virus. These compounds were identified as alternariol and alternariol-9-methyl ether elucidated by NMR.

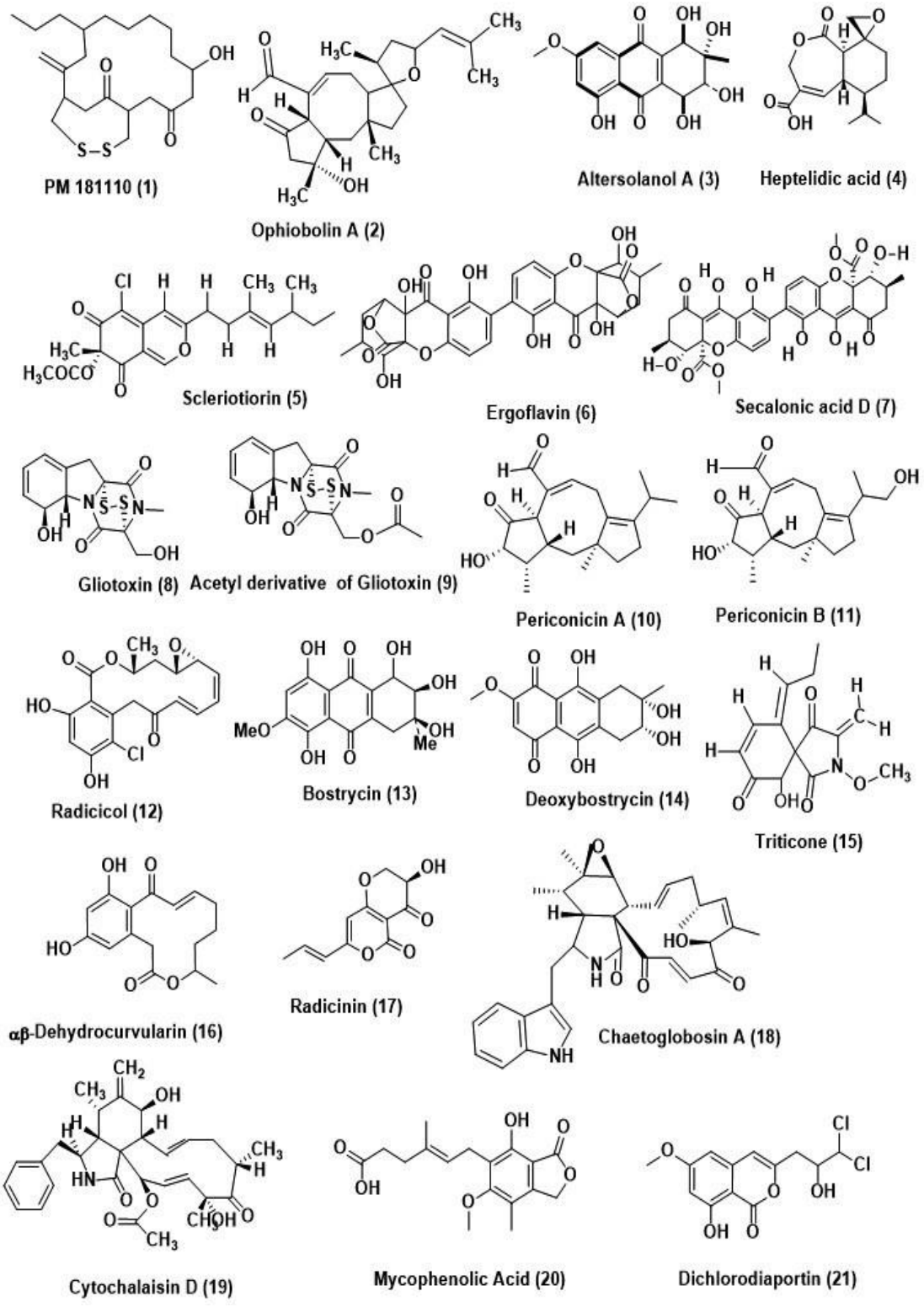


Figure 2.5 Names and structures of anticancer compounds isolated from endophytic fungi.

2.3.4 Endophytes as Source of Antioxidant Compounds

The prolific source of novel active metabolites having antioxidant properties were isolated from medicinal plants. These free radicals scavenging molecules are of diverse range such as lignans, tannins, vitamins, coumarins, flavonoids, phenolic acid, terpenoids, tannins, amines, alkaloids, quinones and other endogenous metabolites. Antioxidants are very effective in the treatment of the damage occurred due to reactive oxygen species (Huang *et al*. 2007). Hence, they have proved as promising source for the treatment of many ROS-linked diseases such as hypertension, cancer, diabetes, atherosclerosis, neurodegenerative diseases, and aging (Rukachaisirikul *et al*., 2008). The antioxidant compounds that were extracted from *Pestalotiopsis microspora* from *Papua* New *Guinea* are pestacin and isopestacin (Figure 2.6). The antioxidant activity of the pestacin was quite high as compared to the standard Trolox i.e. Vitamin E derivative (Harper *et al*. 2003). Despite many studies on antioxidant activities and phenolic contents in plants no relative assessment was done for their assemblage in endophytes. The preliminary investigations on *Eugenia jambolana* (endophytic fungi) demonstrated that the presence of flavonoids, saponins, terpenes, phenols, alkaloids are responsible for the lipid per-oxidation reduction and acted as primary and secondary antioxidants. The endophytic contents having high phenolic contents were considered as potent antioxidant producers.

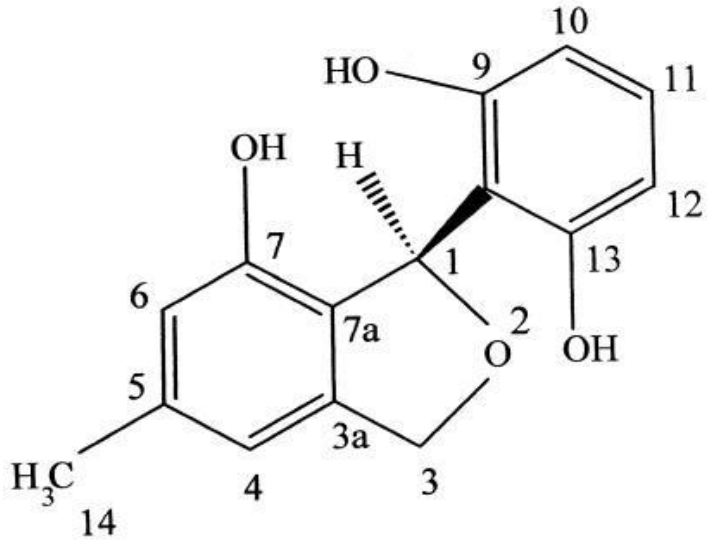


Figure 2.6 Chemical structure of Pestacin extracted from endophytic fungi.

Table 2.2 summarize some of the important compounds produced by endophytic fungi and their relative bioactivities.

Table 2.2 Compounds isolated from Endophytic fungi and their bioactivities

Serial #	**Name of Compound**	**Endophytic fungi**	**Biological Activity**	**Reference**
1.	Conidiogenone B Conidiogenol	*Penicillium chrysogenum*	**Anti-Bacterial**	(Gao *et al*., 2012)
2.	8 α-Acetoxyphomadecalin C Phomadecalin E	*Microdiplodia sp.*		(Hatakeyama *et al*., 2010)
3.	Diversonol Ergosterol Microdiplodiasol Microdiplodiasone Microdiplodiasolol	*Microdiplodia sp.*		(Siddiqui *et al*., 2011)
4.	Helvolic acid	*Pichia guilliermondii*		(Zhao *et al*., 2010)
5.	Phomosines A–C	*Phomopsis sp.*		(Krohn *et al*., 1995)
6.	Guanacastepene	*CR115*		(Singh *et al*., 2000)
7.	Diepoxin κ	*Dzf12*		(Cai *et al*., 2009)
8.	Alterporriol N and E	*Stemphylium globuliferuman*		(Debbab *et al*., 2009)
9.	Javanicin	*Chloridium sp.*		(Kharwar *et al*., 2008)
10.	Xananteric aids I and II	*Alternaria sp.*		(Kjer *et al*., 2009)

11.	Pestalone	*Pestalotia sp.*		(Cueto *et al*., 2001)
12.	Colletotric acid	*Colletotrichum gloeosporioides*		(Zou *et al*., 2000)
13.	Epicolactone, Epicoccolides A and B	*Epicoccum sp.*	**Anti-Fungal**	(Talontsi *et al*, 2013)
14.	Curvularides A–E	*Curvularia geniculata*		(Chomcheon *et al*., 2010)
15.	Phomenone	*Xylaria sp.*		(Silva *et al*., 2010)
16.	Altenusin	*Alternaria sp*		(Cota *et al*., 2008
17.	Isofusidienol A, B, C, and D	*Chalara sp.*		(Lösgen *et al*., 2008)
18.	Cycloepoxylactone Cycloepoxytriol B	*Phomopsis sp.*		(Hussain *et al*., 2009)
19.	Pestalachloride A	*Pestalotiopsis adusta*		(Li *et al*., 2008a)
20.	Trichodermin	*Trichoderma harzianum*		(Chen *et al*., 2007)
21.	Nodulisporins A-C Nodulisporins D-F	*Nodulisporium sp.*		(Dai *et al*., 2006; 2009)
22.	Cryptocin	*Cryptosporiopsis cf. quercina*		(Li *et al*., 2000)
23.	Pyrrocidines A and B	*Acremonium zeae*		(Wicklow *et al*., 2005)

24.	3,12-Dihydroxycadalene	*Phomopsis cassiae*		(Silva *et al*., 2006)
25.	Cryptocandin	*Cryptosporiopsis cf. quercina*		(Strobel *et al*., 1999)
26.	Mullein	*Pezicula livida*		(Schulz *et al*., 1995)
27.	Gamahonolide A and B	*Epichloe typhina*		(Hiroyuki *et al*., 1992)
28.	Chaetoglobosin A	*Chaetomium globosum*	**Anti-cancer**	(Li *et al*., 2014)
29.	Brefeldin A	*Aspergillus clavatus*		(Kharwar *et al*., 2011)
30.	Merulin A and C	*XG8D (Basidiomycete)*		(Chokpaiboon *et al*., 2010)
31.	Brefeldin A	*Paecilomyces sp.*		(Kharwar *et al*., 2011)
32.	Anthracenedione	*Halorosellinia sp. Guignardia sp.*		(Zhang *et al*., 2010)
33.	Pestaloficiol I Pestaloficiol J Pestaloficiol K Pestaloficiol L	*Pestalotiopsis fici*		(Liu *et al*., 2009)
34.	Cochliodinol Isocochliodinol	*Chaetomium sp.*		(Debbab *et al*., 2009)
35.	Camptothecin	*Neurospora crassa*		(Rehman *et al*., 2008)
36.	Alternariol Alternusin	*Alternaria sp.*		(Aly *et al*., 2008)

37.	Daldinone C Daldinone D	*Hypoxylon truncatum*		(Gu *et al*., 2007)
38.	Chaetopyanin	*Chaetomium globosum*		(Wang *et al*., 2006)
39.	Camptothecin	*Entrophospora infrequens*		(Puri *et al*., 2005)
40.	Chaetoglobosin U Chaetoglobosin C Chaetoglobosin F Chaetoglobosin E Penochalasin A	*Chaetomium globosum*		(Ding *et al*., 2006)
41.	Globosumone A Globosumone B	*Chaetomium globosum*		(Bashyal *et al*., 2005)
42.	Torreyanic acid	*Pestalotiopsis microspora*		(Lee *et al*., 1996)
43.	Cinnamic acid Kaempferol	*Strain M7226*	**Antioxidant**	(Yan *et al*., 2014)
44.	Flavipin	*Chaetomium globosum*		(Ye *et al*., 2013)
45.	4,6-dihydroxy-5-meth- oxy-7-methylphthalide.	*Cephalosporium sp.*		(Huang *et al*., 2012)
46.	Monocerin Fusarentin	*Colletotrichum sp.*		(Tianpanich *et al*., 2011)
47	Chlorogenic acid	*Sordariomycete sp.*		(Chen *et al*., 2010)
48.	Chaetopyranin	*Chaetomium globosum*		(Wang *et al*., 2006)

49.	Corynesidones A, B	*Corynespora cassiicola*		(Chomcheon *et al*., 2009)
50.	Graphislactone A	*Microsphaeropsis olivacea Cephalosporium sp*		(Hormazabal *et al*., 2005; Song *et al*., 2005)
51.	Pestacin Isopestacin	*Pestalotiopsis microspora*		(Harper *et al*., 2003)
52.	Subglutinol A	*Fusarium subglutinans*	**Immunosuppressive**	(Lim *et al*., 2015)
53.	Codinaeopsin	*CR127A*	**Anti-malarial**	(Kontnik and Clardy, 2008)
54.	Palmarumycin CP17 Palmarumycin CP18	*Edenia sp.*	**Anti-leishmanial**	(Martínez-Luis *et al*., 2008)
55.	Pestalotheols C	*Pestalotiopsis theae*	**Anti-Viral**	(Li *et al*., 2008a)
56.	Brefeldin A	*Paecilomyces sp.*		Wang *et al*., 2007;
57.	Cytonic acids A and B	*Cytonaema sp.*		(Guo *et al*., 2000)

2.4 Biotransformation of Drugs

Biotransformation can be elaborate as the use of biological systems (biocatalysts) for the introduction of chemical changes on compounds that are not their natural substrates. These biocatalysts could be either whole cells or enzymes. They are used under many diverse conditions like free enzymes, whole cells, immobilized enzyme/whole cells, aqueous and two-phase systems. Biocatalysts can be used for stereo and regio-selective reactions, or to introduce chirality in ways that would be very hard or even impossible for classical synthetic processes (Kebamo *et al.,* 2016). Fungal biotransformation is

divided into preparative and analytical applications. Analytical transformation is helpful in the modelling of the metabolic fate of the drugs in higher eukaryotes. On the other hand, preparative application is the generation of the derivatives of bio-active compounds along with the catalytic steps in their synthesis. In the fungal biotransformation the desired pharmaceutical compound are modified is such a way that is not easily achievable in chemical synthesis. Further, among these derivatives, the compounds that have the novel and enhanced bioactivity may be subjected towards the structural elucidation. The scope of the fungal biotransformation ranges from the milligram scale for research to high/large scale production.

To screen the ability for the Transformation of the anti-inflammatory drug meloxicam 26 fungal strains were screened during the study. A filamentous fungus *Cunninghamella blakesleeana* NCIM 687 transform the drug into three metabolites. Transformation was confirmed through HPLC. Further, LC-MS/MS, two metabolites were identified as 5-hydroxymethyl meloxicam and 5-carboxy meloxicam (Girisham & Reddy, 2009). Venisetty *et al*., (2011) studied the biotransformation of the Silibinin by using fungi. Out of 29 fungi 11 were able to transform Silibinin and produced two new metabolites which were confirmed from new peaks appeared in HPLC chromatogram that were absent in control. *Cunninghamella echinulata* NCIM 691 showed the maximum biotransformation of 29 % and transformed into hydroxylated (M1) and demethylated (M2) products. In mammalian metabolic studies these metabolites were already reported. Hence this study proved that *Cunninghamella echinulata* NCIM 691 is a potent candidate for biotransformation of drugs.

Screening of many fungi was done for the biotransformation of the fenofibrate drug to its active component fenofibric acid. Among all the tested fungi only *Aspergillus terreus* can carried out this biotransformation reaction (Vidyavathi *et al*., 2014). Structural biotransformation was done by endophytic fungi *Cunninghamella blakesleeana* and *Beauvaria bassiana* and a range of metabolites that were identified through spectroscopic techniques as*;* (3R, 4S)-1-(4-fluorophenyl)-3-(3-(4fluorophenyl)-3-oxopropyl)-4-(4-hydroxyphenyl) azetidin-2-one; (3R,4S)-1-(4-fluorophenyl)-3-((E)-3-(4-fluorophenyl) allyl)-4-(4-hydroxyphenyl) azetidin-2-one; (2R,5S)-N, 5-bis (4-fluorophenyl)-5-hydroxy-2-(4-hydroxybenzyl) pentanamide (5) and (2), (3), (3R,4S) 1-(4-fluorophenyl)-3-(3-(4-fluorophenyl) propyl)-4-(4-

hydroxyphenyl) azetidin-2-one. This study highlighted two important aspects of these fungi, viz., metabolic ability for halogenated compounds and the capacity to transform drugs that are the potential targets for the UDP-Glucuronyl transferase system, this phenomenon was not commonly observed (Pervaiz *et al*., 2014). Biotransformation of the flavones (apigenin chrysin, diosmetin, luteolin) and flavanones (pinocembrin, eriodictyol, naringenin, hesperetin) from fungal strains of *Absidia coerulea*, *Beauveria bassiana,* and *Absidia glauca* was studied. The *Beauveria bassiana* AM 278 catalyzed the methyl glucose attachment reaction was occurred at flavonoids skeleton at the position of C7 and C3. Whereas the application of the *Absidia* genus (*A. glauca* AM 177, *A. coerulea* AM 93) as a biocatalyst showed glucosides were formed with sugar molecule in the skeleton of the flavonoids. Nine of the products formed during that study have not been reported previously (Sordon *et al*., 2019).

An endophytic fungus *Fusarium oxysporum* isolated from the plant *Catharanthus roseus,* transform Vinblastine into Vincristine when incubated with the fungal mycelial mass in water without any media/nutrients at room temperature (Kumar and Ahmad 2013).

2.5 Metabolomics and Drug Discovery from Natural Products

Natural products are very important in the endeavor of drug discovery. The conventional and traditional approaches for in the detection of the natural product was too laborious, costly and time consuming that results in the loss of possible bioactive compound, as compared to the novel approaches for the drug discovery. There are so many novel methods for the detection of novel compounds discovered in last decade (Yuliana *et al.,* 2011). Among all these discoveries metabolomics is an emerging field that provides systematic approach for the exploration of complex mixtures and relations between the observations attained during testing with active compounds without any need of isolation. Metabolomics is the approach to insight all the metabolic compounds present in the organisms under precise time and circumstances (Yuliana *et al*., 2013). Metabolomics is the comprehensive study of the metabolite levels in biological sample (Figure 2.7).

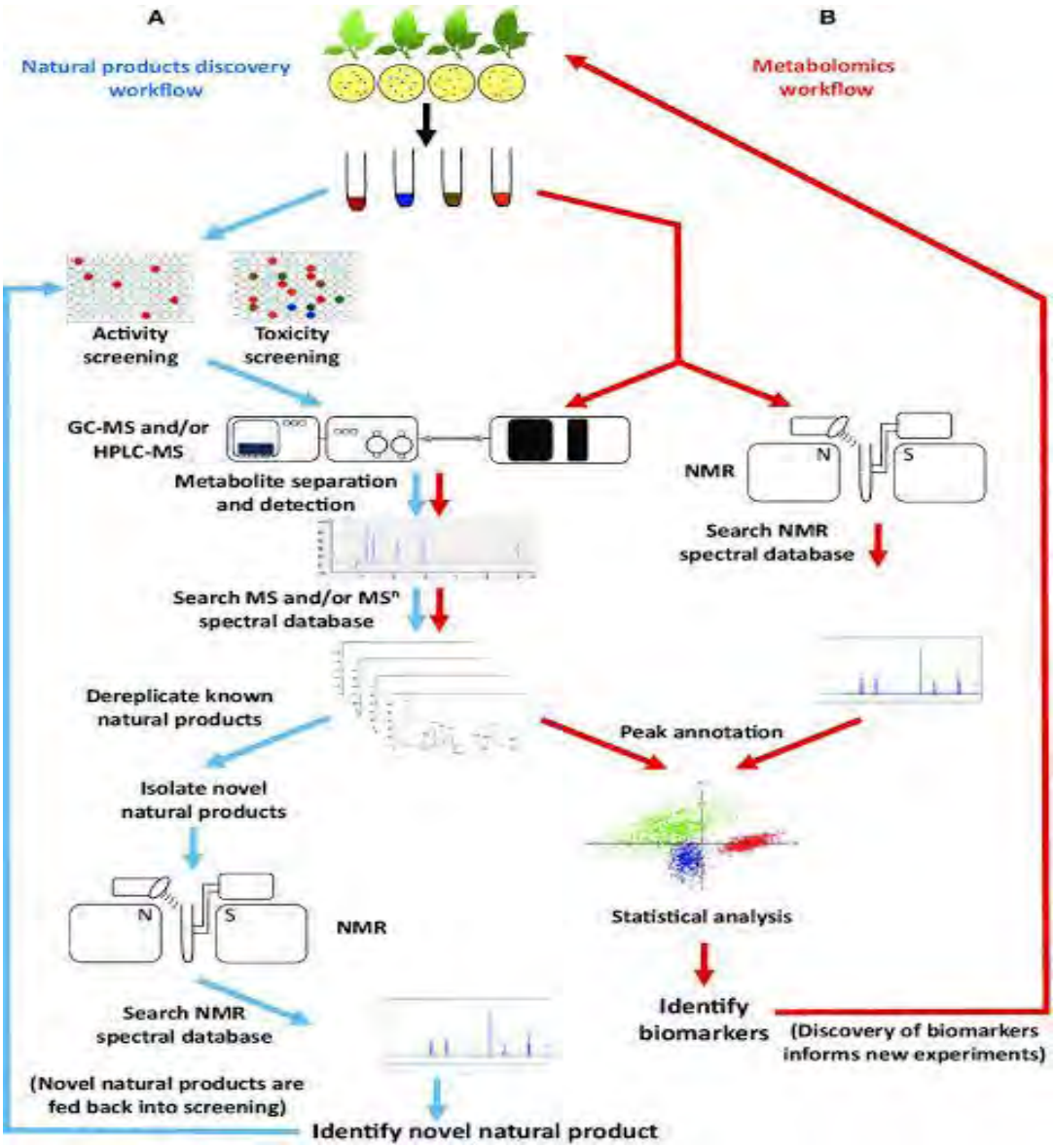


Figure2.7 Metabolomics workflow for novel natural product discovery

2.5.1 Metabolomics Analytical Platform

Metabolomics studies mainly relies on the analytical technique that permits the discovery of the wide-spectrum compounds with extraordinary sensitivity and selectivity. Secondary metabolites have the diverse range of chemical and physical characteristics such as polarity, solubility, volatility, and molecular weight (Yuliana *et al.,* 2013). Moreover, the concentration of the metabolites differ over 9 orders of magnitude, which makes its sampling, sample preparation, instrumentation, and analysis from metabolomics a challenging task (Xiong *et al.,* 2013). Among all the

analytical techniques tandem Mass spectrometry (MS/MS), Nuclear magnetic resonance (NMR) are the most used techniques.

2.5.2 Global Natural Products Social Molecular Networking (GNPS)

Global natural products social molecular networking (GNPS) also known as molecular networking (MN). This is a tandem mass-spectrometry (MS/MS) data organizational method which is newly introduced in the metabolomics, drug discovery and related fields of medicine. The molecules chemistry commands the fragmentation by MS/MS, so that, two correlated molecules can show similar fragment of the ion-spectra. MN manages the MS/MS data as a relational-spectral-network which can map the chemistry of the identified molecules in an MS/MS based metabolomics experiment. Molecular networking is used for the detection of therapeutic leads, monitoring of the drug transformation, clinical diagnostics, and other new applications in the field of precision medicine (Aksenov *et al*., 2017).

Molecular networking is being used to develop the world's major repository and data analysis tool for tandem mass-spectrometry (MS/MS) data, named Global Natural Products Social Molecular Networking (GNPS). GNPS is being used to explore the 'dark matter' of metabolomics of our world, starting from the extracts of the plants and cultures of microbes to the diversity of anthropological and environmental samples, by proliferating spectral library-based annotation and presenting the chemical relationships between several identified molecules across diverse types of the sample. Initiating from the assumption that interrelated molecules produced alike fragmentation patterns in MS/MS (Watrous *et al*., 2012), molecular networking creates an MS/MS spectral map that permits the visualization of structurally identical or related molecules. The major strength of this method is, it can be used for the search for millions of MS/MS spectra without any former knowledge of the composition of the samples (Garg *et al*., 2015). Moreover, if MS/MS data is publicly available in its spectral libraries than GNPS can easily perform a search for the spectral library for known molecules in the networks (Wang *et al*., 2016). This whole process is called process de-replication, which can also term as recognizing 'known unknowns' in metabolomics which is not only critical for the molecular annotation of MS/MS generated spectra, but also for the propagation of annotations through the molecular networks, permitting detection of analogs and the discovery of unique chemical product Figure 2.9 (Yang *et al*., 2013).

Node: presenting a cluster of highly identical MS/MS spectra, as determined by vector-based spectral-matching. **Edge**: is the connection between two metabolite nodes denoted by the presence of a significantly spectral resemblance (Aksenov *et al*., 2017).

Figure (2.8) represents nodes and edges from a molecular network image.

Figure 2.8 Molecular networking. Extracts of endophytic fungi grown with and without antibiotics are analyzed by liquid chromatography-tandem mass spectrometry (LC-MS/MS). Individual metabolites in the spectral network are colored according to their presence or absence under antibiotic stress and with antibiotic stress (red nodes present the metabolites produced without stress and blue and green nodes, are metabolites produced under the stress of clarithromycin and moxifloxacin respectively).

2.6 *In Silico* Studies of Fungal Metabolites

Conventional methods that are used for the screening and searching of the potential secondary bioactive metabolites are time consuming, tedious, and cost intensive task. Therefore, computational molecular docking technique can be adopted. This molecular docking process not only overlapped all the disadvantages of the conventional method, but it is also gaining the more attention in the research of natural compound screening and drug development stages (Figure 2.9). By using this technique, a very short time is required for the identification and analysis of the most important bioactive compound among the mass array of all the identified compounds (George *et al*., 2019). Molecular docking has been successfully applied in prediction of possible binding modes of ligand such as compound or any drug candidate to a given receptor e.g., nucleic acid or proteins involved in the biological processes (Meng *et al*., 2011).

Interaction between the pathogen target site and the bioactive compound is a very complex process starting from the exertion of antibacterial action of bioactive

compounds which includes, physical interaction between that target and the compound which results in, the alteration at the biochemical, ultrastructure and molecular level (Kohanski *et al*., 2010). By using the *in silico* approaches the most credible binding site of the chosen compound, to be encountered with could be interpreted effectively. The selection of the appropriate targets for preferred disease condition and selection of the more appropriate model from the protein bank are the most crucial conditions for in silico studies (Meng *et al*., 2011). According to many action mechanisms, the prevalent antibacterials are broadly classifying in four ways (i) Cell-wall synthesis inhibitors, (ii) DNA gyrase inhibitors, (iii) DNA-dependent RNA synthesis inhibitors and (iv) Protein synthesis inhibitors (Kohanski *et al*., 2010). Several studies has been done in which they targeted these sites along with many known target sites by using *in silico* studies that include Molecular docking, free binding energies, and Molecular dynamic simulations through modelers.

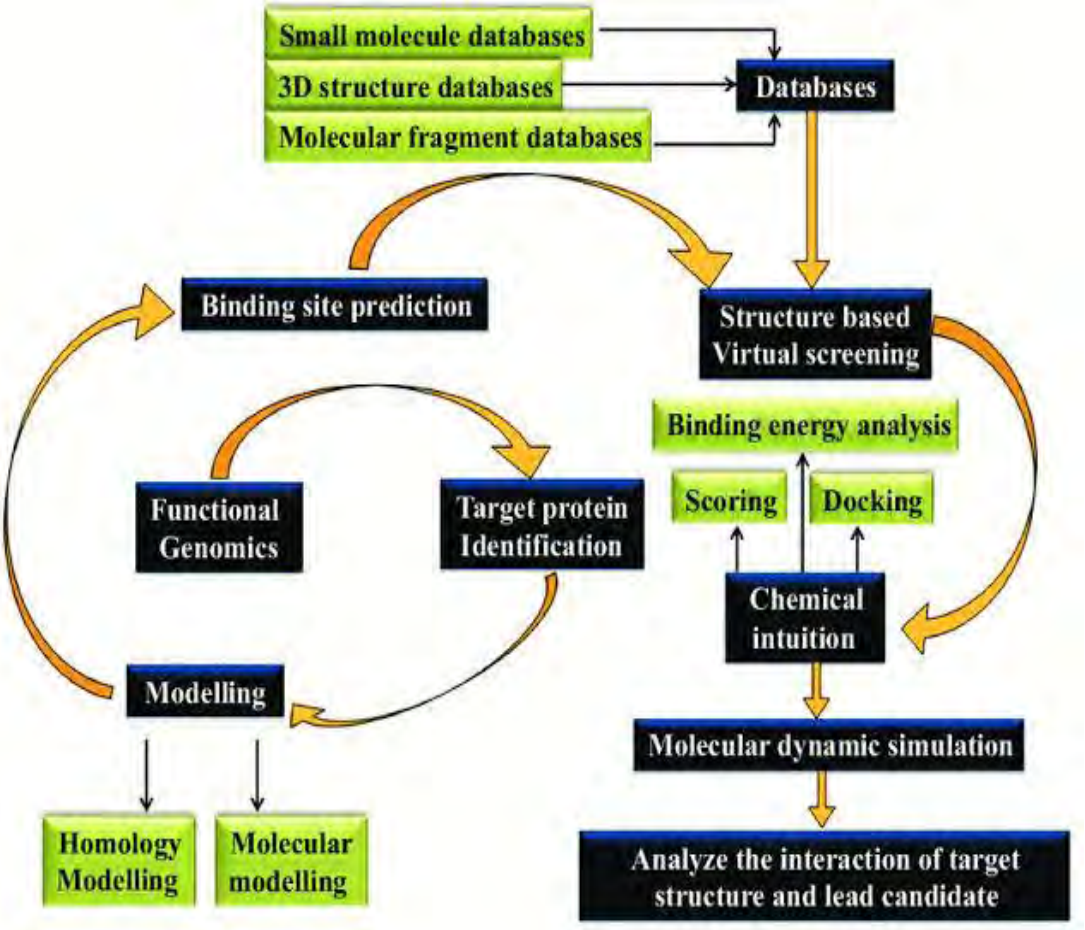


Figure 2.9 Flow chart is showing the steps for the structural analysis of novel compounds.

2.6.1 Molecular Docking

There are three different types of molecular docking programs mostly used worldwide i.e., AutoDock4, SwissDock and Surflex-Dock. Molecular docking is the approach to fit the ligand in 3D binding site. Basically, two approaches used during docking strategy. In the first operation, there is a search for the conformational space vacant for the ligand tailed by the scoring function that demonstrate the binding affinity. Despite the differences between the two operations the docking operates at the same algorithms for both. Some programs may include the ability to achieve the consensus scoring when a comprised sum of algorithms has been included. These algorithms may differ in the weight that is given to the non-covalent interactions that are producing a multiple set of results for the same ligand-database in the same target (Lohning *et al*., 2017).

2.6.2 Swiss Dock (SD)

It was developed by the molecular modelers group at the Swiss-institute for bioinformatics. It provides free service for academia who want to dock, set of ligands to a marked biomolecule by using a docking server. It works on the graphical user interface (GUI) that provides access to the general algorithms and few examples. It is mainly based on the dihedral space sampling (DSS) in EA Dock which depends on the target nature and ligand. It usually performs fast single-step process with less interference of user-controlled input. The results from the SD are work as a seed for further modelling and docking. CHARMM (Chemistry at HARvard Macromolecular Mechanics) is used for the energy measurements by using the forcefield on the vital IT cluster computer. These cluster groups of docked poses are scored, and these results can be downloaded. SD performs single ligand docking; each ligand is docked individually in ligand databases which consumes time and can be burdensome working with large ligand libraries. SD cannot dock the single ligand into multiple targets. SD performs the ‘Blind Docking’ in the absence of the grid box definition, where algorithms search for the thermodynamically favorable ligand binding site. However, since there are many binding sites are present in the channel and are therefore ignored by the system or program. By toggling between the accuracy levels of ‘very fast’ to ‘fast’ or ‘Accurate’ may marginally increases the likelihood of gaining the binding mode that can correctly predicts the conformational bindings (Lohning *et al*., 2017).

2.6.3 Autodock 4 (AD4)

Autodock4 is an automated docking software that is freely available, made by Scripps research institute and University of California, La Jolla, CA. It is the standalone program platform that is operating on Mac OS, Linux, Sun Solaris and Windows operating systems. ADT is the graphical frontend python molecular viewer for using AD4 and is included in the package known as MGL tools that is provided by the Scripps research institute. The key benefit of using AD4 is that it is of industry standard, cost free and very robust. It is also possible to complete the whole analysis in the GUI environment by using various frontend software's. Virtual can be done by using the software package known as PyRx (Dallakyan *et al*., 2015). By using AD4 multiple ligands can be visualized or screened by using PyRx frontend GUI (Lohning *et al*., 2017).

2.6.4 Surflex-Dock

With Surflex-Dock that is developed by Tripos, receptor-ligand docking, and virtual screening can be done by using the SYBYL-X suite of MM (molecular modelling) package (International, 2014). It provides a robust algorithm. Easing in target-ligand preparation, user friendly by providing comprehensive control over the docking program, consensus scoring, ring flexing, protomol guided docking, docking of large libraries of ligands and parallelization. With default settings of the Surflex is quite easy to run the docking. This program used protomol rather using grid box parameters to define the conformational space when the ligands that are bounded are available. During the process of docking each ligand is fragmented and they used to search available space after fragmentation. The ligands that scored higher are then retained and are again reconstructed to form the final ligand. In the absence of the bounded ligand the key target residues will be selected otherwise the software is only able to forecast the potential binding sites in the 3rd option (Lohning *et al*., 2017).

2.6.5 Molecular Dynamics Simulation

Molecular docking studies only provide one static snapshot of a docked ligand to the receptor (Pagadala & Tuszynski, 2017). This does not give the details about the real binding mode of a ligand and does not provide complete picture from medicinal chemistry point of view (Karplus & McCammon, 2002). The dynamics of the complex,

on contrary, not only affirm the docking predictions but at the same time allow users to list several snapshots superimposed over one another giving opportunity for many statistical calculations to probe important biological events vital in designing of novel leads (Durrant & McCammon, 2011). Computer simulations in computer aided drug designing are considered significant to decipher real cellular behavior of a ligand with respect to the receptor macromolecule (Kiani *et al*., 2019).

The chemical moieties responsible for chemical interactions to the receptor are microscopically investigated to guide further structure optimization of the compound to favor better fitting of the analogs in the pocket and formation of stable non-covalent interactions (Haq *et al*., 2017). Several biophysical parameters are evaluated including root mean square deviations (RMSD), root mean square fluctuations (RMSF), radius of gyration (Rg), and beta factor (β-factor) (Abbasi *et al*., 2016). RMSD is the carbon alpha deviation of complex protein vs time. This assay is vital in understanding the protein conformational transitions and resulting effect of such on global or local alterations on ligand binding. RMSF is the deviation of protein residues. Identification of flexible and stable residues upon ligand binding are key in underling hotspot residues true in binding and holding ligand at the docked site. Rg is overall descriptor of protein compactness and relax conformers. This yield structural data highlighting the role of local structure elements in allowing ligand stable binding or facilitate its detachment.

There are five most commonly used softwares for Molecular Dynamics Simulation calculations which were listed below.

1. GROMACS
2. CHARMM
3. AMBER
4. NAMD
5. LAMMPS

All these software have some common features along with some unique capabilities. Some of them are open-source (e.g, GROMACS, and LAMMPS) and rest are either proprietary or commercial.

2.6.6 Binding Free Energies in Drug Designing

The free energy liberated upon ligand binding to a particular target is a good parameter to disclose (Miller *et al*., 2012). The stable conformer of a ligand gives highly stable values. More negative the energy implies high stability of the pose and vice versa (Ahmad *et al*., 2017). Many different free energy approaches are available however, considering the appropriate estimation of the energy and usage of the moderate computational efforts, MMGBSA (molecular mechanics generalized born surface area) and MMPBSA (molecular mechanics Poisson Boltzmann) are the widely used techniques at present (Genheden & Ryde, 2015). Both methods compare the receptor, ligand and complex in solvation and gas phase to determine the role of water molecules in the complex stabilit

CHAPTER 3

3 SCREENING OF ENDOPHYTIC FUNGAL ISOLATES FOR ANTIBACTERIAL ACTIVITY GROWN WITH AND WITHOUT ANTIBIOTICS

3.1 INTRODUCTION

Endophytic fungi and bacteria produce compounds that poses a diverse range of pharmacological activities such as, antibacterial, antiviral, antifungal, antioxidant, proliferative and cytotoxic effects (Pal *et al*., 2020). Natural products from microbial sources are noteworthy since they contribute more than 2 million of the total compounds present today in the pharmaceutical industry (Saha *et al*., 2019). Compounds originating from endophytic microbial community have introduced a new era in the paradigm of natural product discovery. These hidden treasures offer unique chemical structures with exciting biological potential, withholding an immense possibility to develop a new library of novel compounds. Thus, this cherishable resource provides an alternative source for the development of new drugs and pharmaceutical compounds to combat the global issue of antibiotic resistance against existing synthetic drugs (Green, 2019).

Recent progress in the screening technologies for the endophytes revealed the great potential to produce unique bioactive compounds by them. However, bioprospecting the unexploited and unusual habitats of the plants with stringent selection criteria is essential to enrich the novel secondary metabolites from endophytes for medical, agriculture and industrial application (Ngashangva, 2019). These prime screening efforts could play a central role in the selection and prioritization of the potential candidate strains which could eventually lead to the extraction of pure novel compounds having pharmacological significant properties (Abdalla *et al*., 2020).

The stabilized production of desired compound from host microbes is overcome by employing different strategies. One of the recently adopted approach is the addition of unusual elicitors to the microbial growth medium. These elicitors alter or extend the metabolic profile of the microorganism either by triggering the production of new metabolites or by being transformed into a new chemical moiety (El-Bialy and El-Bastawisy, 2020). This change of metabolic profile is being investigated in fungi and is found to be dependent on fungal species as well as nature, concentration, and stage of addition of the elicitor to the cell culture (Salehi *et al*., 2019).

The current study was aimed at the isolation, screening, and production of bioactive compounds from endophytic fungi with and without the addition of antibiotic; as elicitors; to the growth medium.

This chapter describes the small-scale cultivation, extraction, and preliminary antibacterial investigation of endophytic fungi. Additionally, this chapter incorporates biotransformation of the antibiotics, by endophytic fungal isolate, into compounds which could be purified and structurally elucidated as an entity different from the parent drug. A total of seven endophytic fungi, previously isolated from the medicinal plant *Taxus fuana* of Western Himalayan region of Pakistan (Fatima *et al*., 2016), were selected. The isolates were cultivated in growth medium supplemented with and without the antibiotic. Five antibiotics from different classes were utilized for screening. Following cultivation, candidate strains were tested for their antibacterial potential in three different ways.

A. Crude extract obtained after cultivation of fungal endophytes in growth medium supplemented with and without antibiotics (elicitor) was subjected to antibacterial screening against bacterial pathogens.

B: Protein precipitate obtained from cultivation broth of the fungal endophyte grown without antibiotic supplementation was treated with antibiotic solution (1mg/mL). The same antibiotic was as elicitor.

C: Mycelial mass of the fungal endophyte grown without antibiotic supplementation was treated with antibiotic solution (1mg/mL).

This preliminary screening was helpful in selecting the most potent endophytic fungi from among a total of seven strains. This screened further facilitated prioritization of the antibiotics to be used as elicitors for follow up studies. The selected fungal endophyte would be later on cultivated for the large-scale production and purification of potential novel compounds.

3.2 MATERIAL AND METHODS

3.2.1 Chemicals, Media, and Solvents

All the chemicals and reagents used during the study were of analytical grade and secured from Oxoid, UK, Sigma Aldrich, and BDH. Antibiotics were gift from a local pharmaceutical company Focus & Rules pharmaceuticals (pvt) Ltd. Islamabad, Pakistan. The media solvents and antibiotics used in this study are as under.

Sabouraud Dextrose agar and broth (SDA and SDB) from Oxoid

Mueller-Hinton agar (MHA) from oxoid

Ethyl Acetate (EtoAc) from Merck

Ammonium Sulphate from Sigma Aldrich

Dimethyl Sulfoxide (DMSO) from Sigma Aldrich

Antibiotics include (Cefixime, Cephradine, ciprofloxacin, moxifloxacin, and clarithromycin) obtained from Focus & Rules pharmaceuticals (Pvt) Ltd. Islamabad

McFarland standard No.0.5 (turbidity standard)

Tris buffer

3.2.2 Collection and Maintenance of Endophytic Fungi

A total of seven endophytic fungi maintained on Sabouraud Dextrose agar (SDA) plates and slants were obtained from Applied and Environmental Microbiology Research Lab, Quaid-i-Azam University, Islamabad, Pakistan. These endophytic fungal isolates were isolated from a medicinal plant *Taxus fuana* from Nathia Gali which is located at west Himalayan region of Pakistan. They were previously identified as *Epicoccum* sp. NFW1 (JX402049.1), *Mucor hiemalis* NFW6 (JX854471.1), *Epicoccum* sp. NFW7 (JX838793.1), *Chaetomium* sp. NFW8 (KC797170.1), *Fusarium oxysporum* NFW16, *Penicillium milleri* NFL1 (KC812764.1) and *Paraconiothyrium* sp. NFL6 (KC797169.1); (Fatima *et al.,* 2016).

3.2.3 Cultivation of Fungal Isolates and Organic Extraction of Metabolites for Screening of Antibacterial Compounds

All the isolates were grown in SDB medium with and without supplementation of antibiotics. SDB (150 mL in 250 mL flask) were inoculated with three mycelial plugs (3 mm diameter) of freshly grown fungal isolates and incubated in shaking incubator at 30°C, 150 rpm for 21 days. Five different antibiotics (Cefixime, Cephradine, ciprofloxacin, moxifloxacin, and clarithromycin) in a concentration of 1mg/mL were added to the culture medium one by one. Each fungal isolates were grown on the medium containing antibiotics as well as control i.e. without adding antibiotics. After incubation, the fermentation broth from both the control and antibiotic added medium was filtered, and cell free fermentation broth 100 mL was subjected to organic extraction three times with ethyl acetate 1:1 as organic solvent. The organic layers were collected from separating funnel after two hours and dried by using rotary evaporator. The dried crude ethyl acetate extracts were collected for further studies. The experiments were performed in triplicate.

3.2.4 Assay for Antibacterial Activity

The crude ethyl acetate extracts dissolved in DMSO at a concentration of 4mg/mL were screened for antibacterial activity against gram positive and gram-negative bacteria by agar well diffusion assay (Haq *et al*., 2012). The test bacterial strains were obtained from Department of Microbiology, Quaid-i-Azam University, Islamabad which include *Bacillus* sp. (ATCC 6633), *Staphylococcus aureus* (ATCC25923), *Escherichia coli* (ATCC 15224), *Klebsiella pneumoniae* (ATCC 13883). A bacterial suspension was prepared from overnight fresh cultures of test bacterial strains containing 1.5×10^8 CFU/mL. using McFarland standard No.0.5 as turbidity standard This suspension was swabbed to prepare a uniform bacterial lawn on MHA plates. Wells were made on MHA plate by sterile cork borer (7mm diameter) at appropriate distance and loaded with 100µL sample (0.4mg of crude extract). Zone of inhibitions were measured in mm after 16-24 hours of incubation at 37°C. The experiments were performed in triplicate.

3.2.5 Protein Precipitation

Some of the extracellular enzymes may transformed the antibiotics into different structures. To check the bactericidal activity of these transformed structures

antibacterial activity was performed after treated the antibiotics solution in water with total proteins precipitated from cell free fermentation broth. Ammonium sulphate precipitation method (Wingfield, 2016) were used for this purpose. An 80 % saturation of ammonium sulphate at 4°C was achieved by dissolving 53.28g in 100mL of fermentation broth for total protein precipitation. The ammonium sulphate is added gradually to the fermentation broth kept at 4°C followed by shaking to enhance the solubilization. After dissolving the ammonium sulphate the broth was kept at 4°C overnight to precipitate down the extracellular proteins. The precipitated proteins were collected by centrifugation and kept them in Tris buffer pH (7.4).

3.2.6 Screening Strategies

In this study three different strategies were used to screen the antibacterial potential of endophytic fungal isolates grown with and without antibiotic stress in SDB medium.

3.2.6.1 Screening Strategy 1

In this strategy the fungal growth media emended with the above-mentioned antibiotics in concentration of 1 mg/mL and without antibiotics. The fungal isolates were similarly cultured as discussed earlier in the medium to study the effect of antibiotics on the production of bioactive metabolites. After incubation, the organic extraction and antibacterial assay was performed as mentioned earlier. Figure 3.2 showed the diagrammatic scheme of the methodology. Two controls were used to differentiate the antibacterial activity of antibiotics itself and fungal metabolites produced without antibiotic stress. This will help to study the effect of antibiotics stress on the production of antibacterial compounds from fungal isolates.

Control 1 Antibiotic solution in SDB media without inoculation were kept under the same conditions and extracted in the same procedure to rule out the possibility of antibiotic self-degradation and the antibacterial activity of antibiotic itself.

Control 2 Fungal extract obtained from SDB medium without antibiotics supplementation to check either the antibacterial compounds are produced by the fungal without antibiotic stress or not.

3.2.6.2 Screening Strategy 2

The fungal isolates were grown in SDB medium without antibiotic supplementation for 21 days. After incubation and fungal biomass was filtered and washed thoroughly to remove the extracellular residues. The washed fungal biomass was added to antibiotic solution in distilled water and incubated in shaking incubator under the conditions discussed in strategy 1 for seven days followed by organic extraction (Figure 3.2).

Control 1 Biomass added to fresh SDB without antibiotic supplementation to check the antibacterial activity of fungi without antibiotic stress.

Control 2 Antibiotic solution in SDB.

3.2.6.3 Screening Strategy 3

The fungal isolates were grown in SDB medium without antibiotic supplementation for 21 days and filtered to get cell free supernatant 100 mL which was subjected to protein precipitation by ammonium sulphate as mentioned under the heading of "protein precipitation" earlier in this chapter. The precipitated proteins were added to antibiotic solution in distilled water. An antibiotic solution was prepared in distilled water 100mL with 100mg of the five different antibiotics used in this study (mentioned earlier) individually and total protein precipitates from 100mL of fermentation broth (0.1mg) were incubated with antibiotic solutions. An average of three hours times were given in shaking incubator 150 rpm at 30°C to achieve the transformation reactions.

Control 1 Antibiotic solution in distilled water without addition of precipitated proteins
Control 2 Precipitated proteins to check either these proteins possess antibacterial activity or not (Figure 3.2).

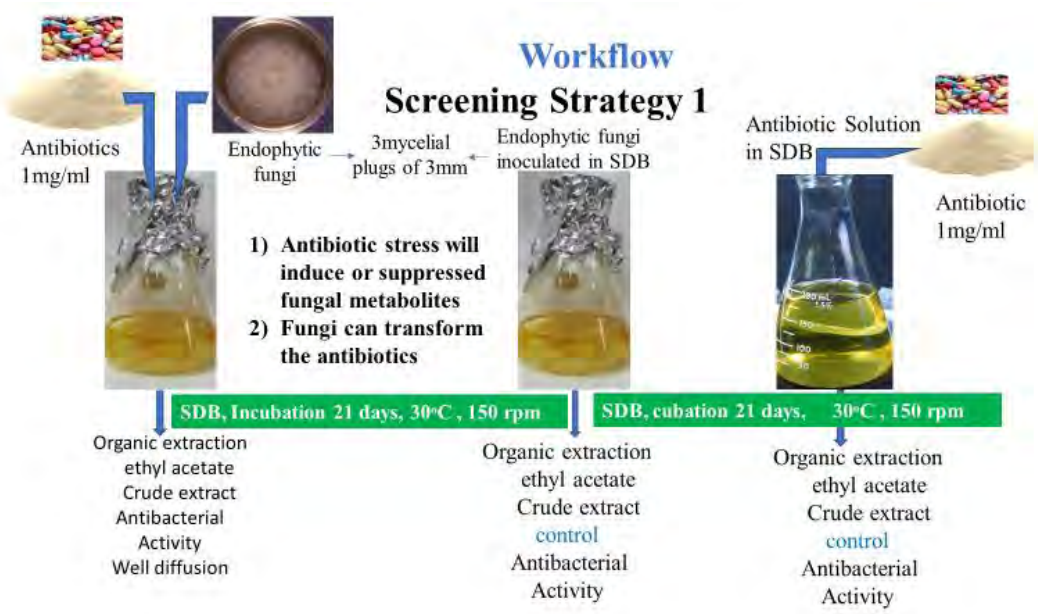


Figure 3.1 Graphical presentation of screening strategy 1 for antimicrobial production by endophytic fungal strains

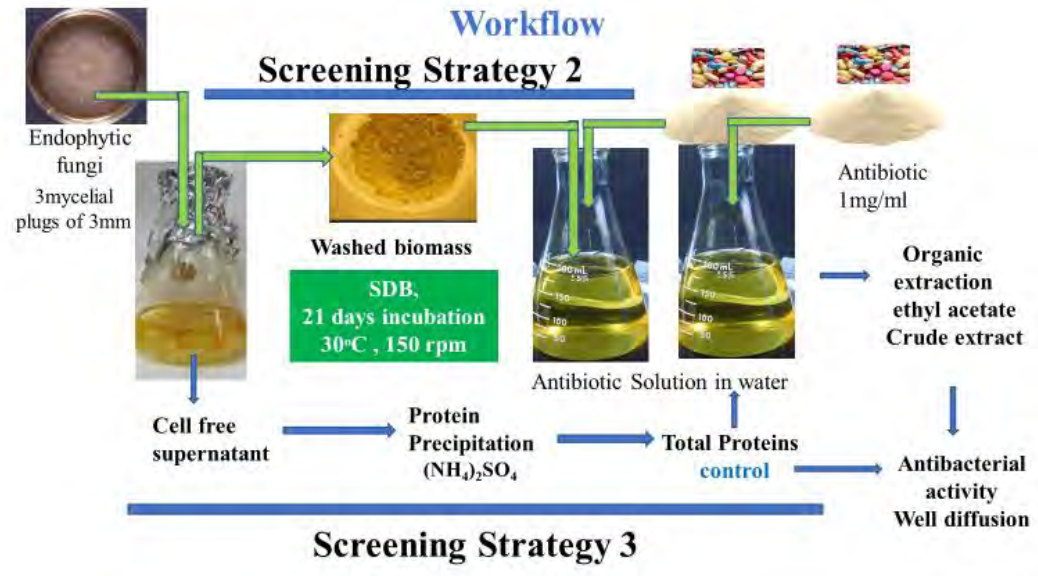


Figure 3.2 Graphical presentation of screening strategy 2 and 3 for antimicrobial production by endophytic fungal strains

3.3 RESULTS

3.3.1 Growth of Endophytic Fungal Isolates on Antibiotics Supplemented Medium

The growth of endophytic fungal isolates encoded as NFW1, NFW6, NFW7, NFW8, NFW16, NFL1, and NFL6 were screened against five different antibiotics including ciprofloxacin, moxifloxacin, clarithromycin, cefixime and cephradine supplemented to Sabouraud Dextrose Agar and broth (SDA and SDB). It was observed that only NFW7 growth was affected by ciprofloxacin and clarithromycin, but the growth was enough to carry on the experiments as the aim was to study bioactive metabolites produced by antibiotic stress or the active form of antibiotic transformed from its parent structure by the endophytic fungal isolates. All the other isolates showed no growth reduction at all or very low effect in terms of biomass production in broth or mycelia formation on agar plate in the media supplemented with antibiotics. Table 4.1 showed the growth of fungal isolates grown on the medium with and without antibiotic supplementation.

3.3.2 Antibacterial Activity of Fungal Proteins and Crude Extracts Grown without Supplementation of Antibiotics to the Medium

The crude extracts of fungal isolates grown on SDB medium without antibiotic supplementation grown at 30°C showed mild or no antibacterial activity as compared to when grown in the presence of antibiotic under the same conditions as because of the presence of antibiotic in extract and may be the production of bioactive metabolites due to antibiotic presence in medium or from the transformed products of antibiotics by fungi. Therefore, this control was not shown in the experimental results when fungi was grown under the presence of antibiotic in medium to make the results graph more convenient. Similarly, the precipitated extracellular proteins from fungal strains grown with and without antibiotic presence were also checked for antibacterial activity against all the test bacterial strain and observed that when grown on SDB at 30°C their proteins do not have any bactericidal effect on test bacterial strain, so they were also not shown in the graphical presentation of results.

Table 3.1 Growth of Fungal Isolates on Medium Supplemented with Antibiotics (**1mg/mL**)

Fungal Isolates	**Control SDA/SDB without Antibiotics**	**Growth on SDB and SDA with antibiotics**				
		Cefixime	**Cephradine**	**Ciprofloxacin**	**Moxifloxacin**	**Clarithromycin**
NFW1	+++	+++	+++	++	+++	+++
NFW6	+++	+++	+++	+++	+++	++
NFW7	+++	+++	+++	+	+++	+
NFW8	+++	+++	+++	++	+++	+++
NFW16	+++	+++	+++	++	+++	+++
NFL1	+++	+++	+++	++	+++	++
NFL6	+++	+++	+++	++	+++	++

Key: +++ = Normal growth, ++ = sufficient growth, + = minimal growth, - = no growth (biomass based) Control = fungal growth without antibiotic stress

3.3.3 Antibacterial Activity of *Epicoccum* sp. NFW1 gown with Antibiotics in SDB Medium

The crude extracts obtained from *Epicoccum* sp. NFW1 when grown under antibiotic stress showed increased antibacterial activity with ciprofloxacin, moxifloxacin, and clarithromycin against *E. coli*, *Klebsiella pneumonia*, *Bacillus sp*. and *S. aureus* strains, while under the stress of cephradine and cefixime not only the antibacterial activity reduced but degradation of antibiotic also observed because there was complete loss of antibacterial activity in case of cephradine (Fig 3.3).

The fungal biomass grown in SDB without the presence of antibiotics were filtered washed and then subjected to antibiotic solution. The antibiotic solutions were prepared in distilled water so there is no other carbon source for fungi. When the already developed biomass of fungal strain grown in antibiotic solution followed by organic extraction and the crude extract were used in antibacterial assay showed that the activity in case of ciprofloxacin, moxifloxacin and clarithromycin increased in the range of 30-46, 23-27 and 23-33% respectively against test bacterial strains whereas, the activity decreased when grown on cefixime and cephradine (Figure 3.4).

Precipitated Proteins (0.1mg) of *Epicoccum* sp. NFW1 when added to 100mL of antibiotic solution (1mg/mL) in water showed mild effect on the activity of moxifloxacin by slightly improving it up to 10, 6.9, 8 and 5.45% against *E. coli*, *Klebsiella pneumonia*, *Bacillus sp*. and *S aureus* strains respectively, whereas the activity of ciprofloxacin, clarithromycin, cefixime and cephradine decreased in the range of range of 7-11, 7-17, 20-35 and 10-18% after subsequent treatment with fungal proteins (Figure 3.5).

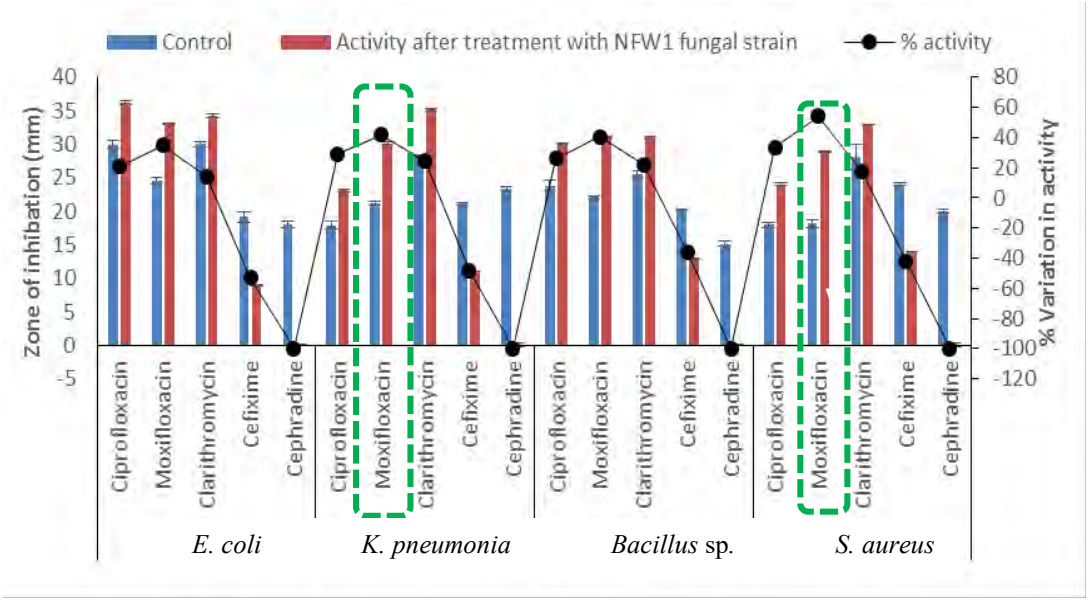


Figure 3.3 Antibacterial activity of crude extract of *Epicoccum sp.* NFW1 grown with antibiotic supplemented media.

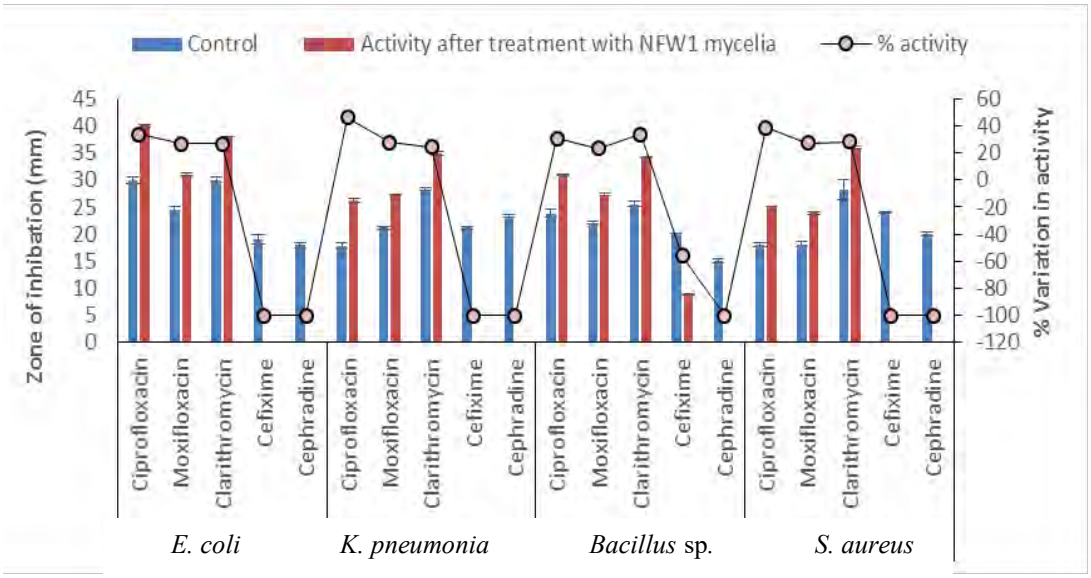


Figure 3.4 *Epicoccum sp.* NFW1 developed biomass incubated with antibiotic solution extracted

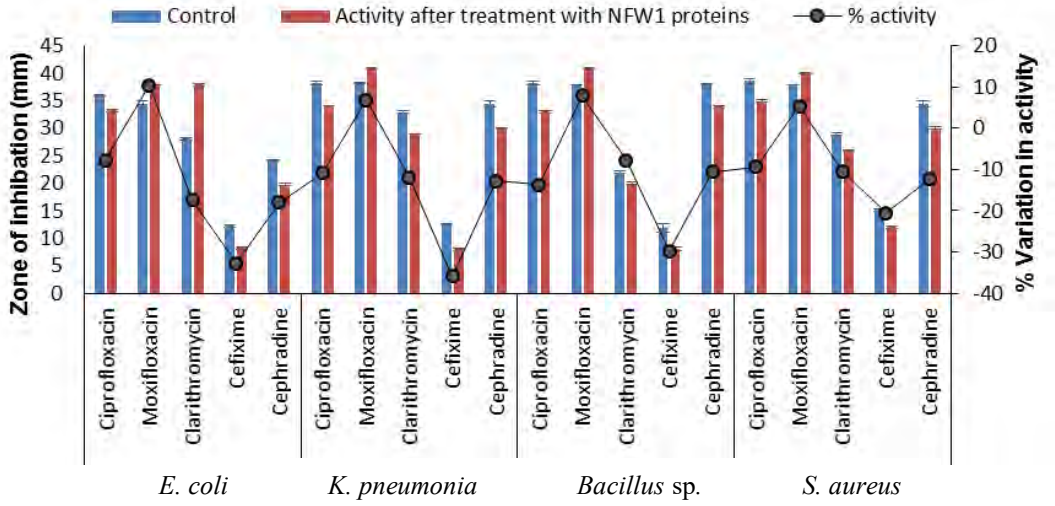


Figure 3.5 Effect of *Epicoccum sp.* NFW1 precipitated proteins on antibiotic solution

3.3.4 Antibacterial Activity of *Mucor hiemalis* NFW6 Grown with Antibiotics in SDB Medium

The antibacterial assay of crude extract of *Mucor hiemalis* NFW6 grown on media emended with antibiotics showed that antibacterial activity increased when grown with moxifloxacin and clarithromycin while the activity decreased when grown on media containing ciprofloxacin, cefixime and cephradine against all the test bacterial strains (Figure 3.6).

When already developed biomass *Mucor hiemalis* NFW6 on SDB medium without antibiotics addition of antibiotics subjected to antibiotic solution in water an increased activity was recorded in the case of moxifloxacin and clarithromycin whereas, the activity decreased than the control in case of ciprofloxacin, cefixime and cephradine (Figure 3.7).

In case of *Mucor hiemalis* NFW6 precipitated proteins, when added to the antibiotic solution in water followed by organic extraction the activity of crude extract obtained from moxifloxacin, clarithromycin and cefixime containing solutions marginally increased 10-16, 3-8 and 6-9 % respectively against all the test bacterial strains while that of ciprofloxacin and cephradine the antibacterial activity decreased considerably suggesting the transformation of antibiotics to less active form by fungal enzymes (Figure 3.8).

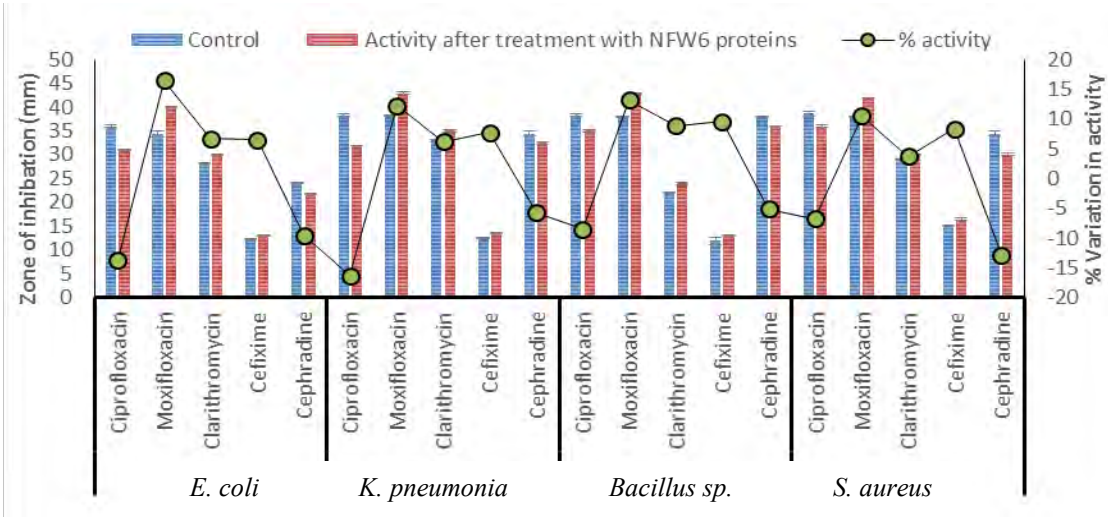


Figure 3.6 Antibacterial activity of crude extract of *Mucor hiemalis* NFW6 grown with antibiotic supplemented media

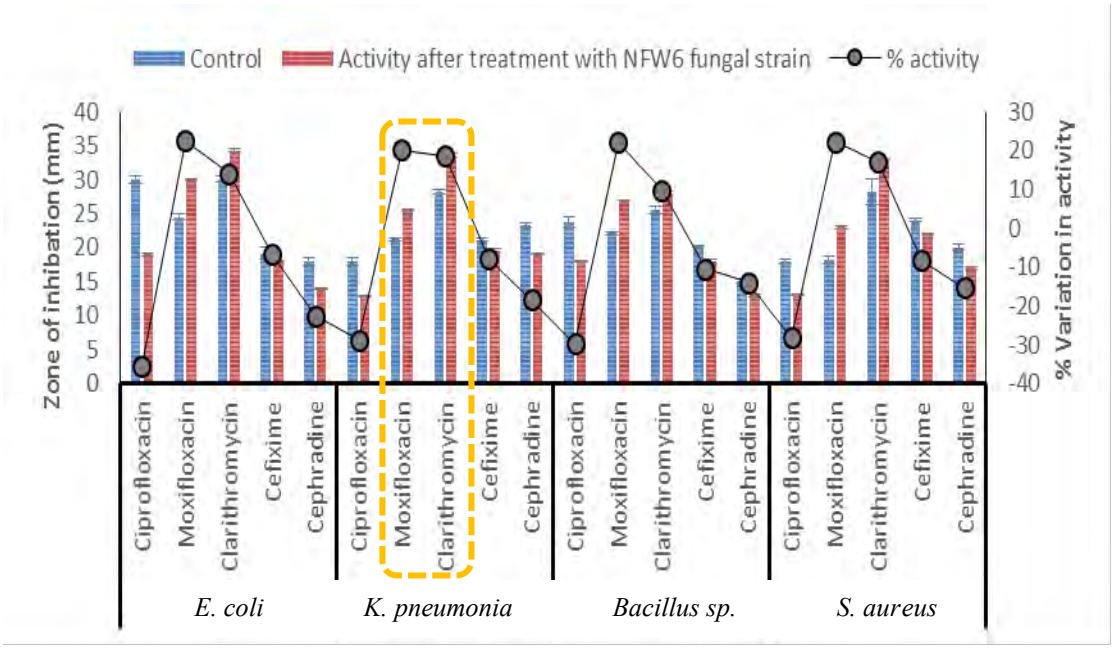


Figure 3.7 Antibacterial activities of *Mucor hiemalis* NFW6 developed biomass incubated with antibiotic solution

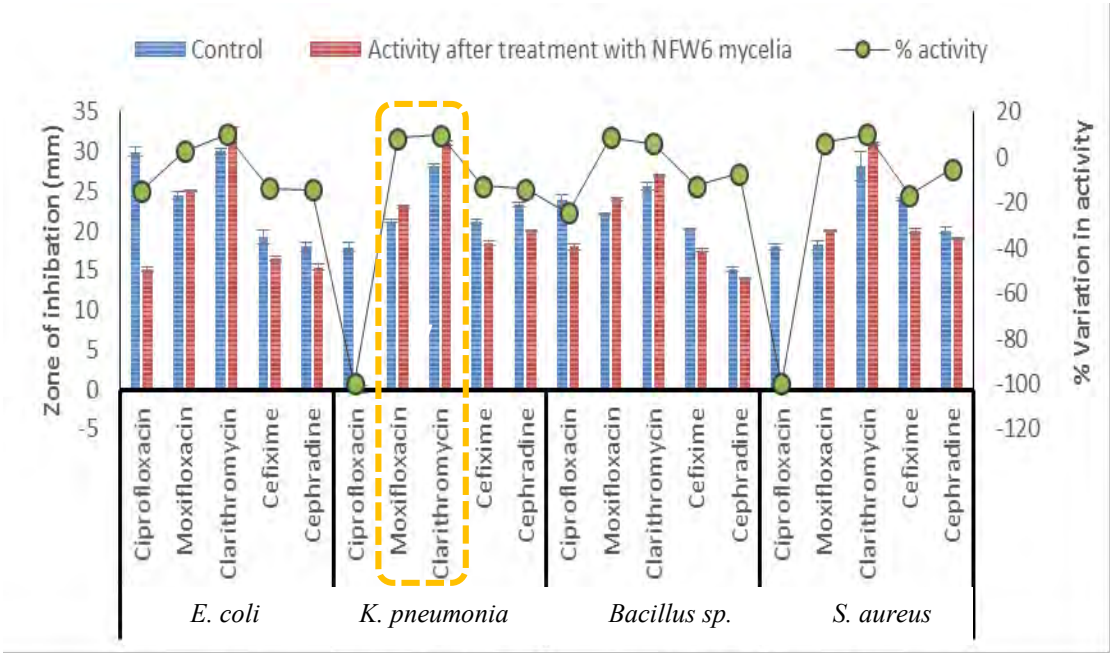


Figure 3.8 Effect of *Mucor hiemalis* NFW6 precipitated proteins on antibiotics solution

3.3.5 Antibacterial Activity of *Epicoccum* sp. NFW7 Grown with Antibiotics in SDB Medium

From antibacterial assay, it was revealed that when *Epicoccum* sp. NFW7 grown on medium augmented with antibiotics followed by organic extraction the crude extract of NFW7 grown in the presence of clarithromycin showed improved activity against *E. coli* (16.7 %), *Klebsiella pneumonia* (17.8 %), *Bacillus sp*. (24 %) and *S. aureus* (20.7 %) while the activity of *Epicoccum* sp. (NFW7 reduced when grown on the rest of the antibiotics as shown in (Figure 3.9).

When already developed biomass of *Epicoccum* sp. NFW7 in SDB added to antibiotic solution in water followed by extraction it was observed that the activity of NFW7 when grown on cephradine and clarithromycin solutions increased whereas, a remarkable reduction in antibacterial activity of ciprofloxacin, cefixime and moxifloxacin was reported as shown in Figure (3.10).

In case of *Epicoccum* sp. NWF7 fungal protein when added to antibiotic solution and extracted it was seen that 14-17 and 6-13% increase in the activity of clarithromycin and cefixime, respectively. While the activity of other antibiotics decreased significantly after treatment with fungal proteins as shown in Figure (3.11).

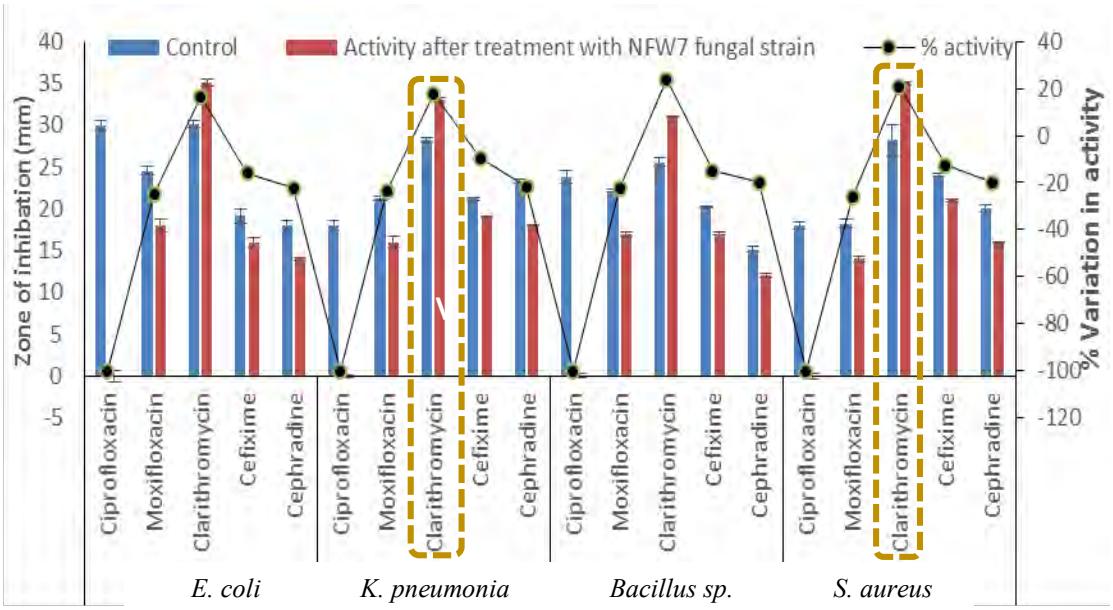


Figure 3.9 Antibacterial activity of *Epicoccum* sp. NFW7 grown with antibiotic supplemented media

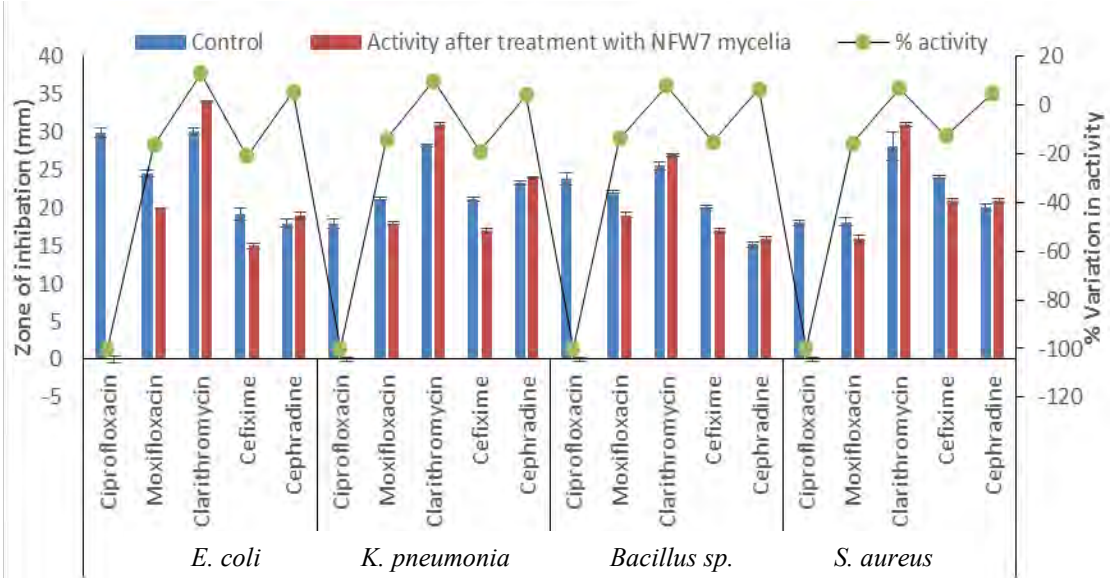


Figure 3.10 *Epicoccum* sp. NFW7 developed biomass incubated with antibiotic solution

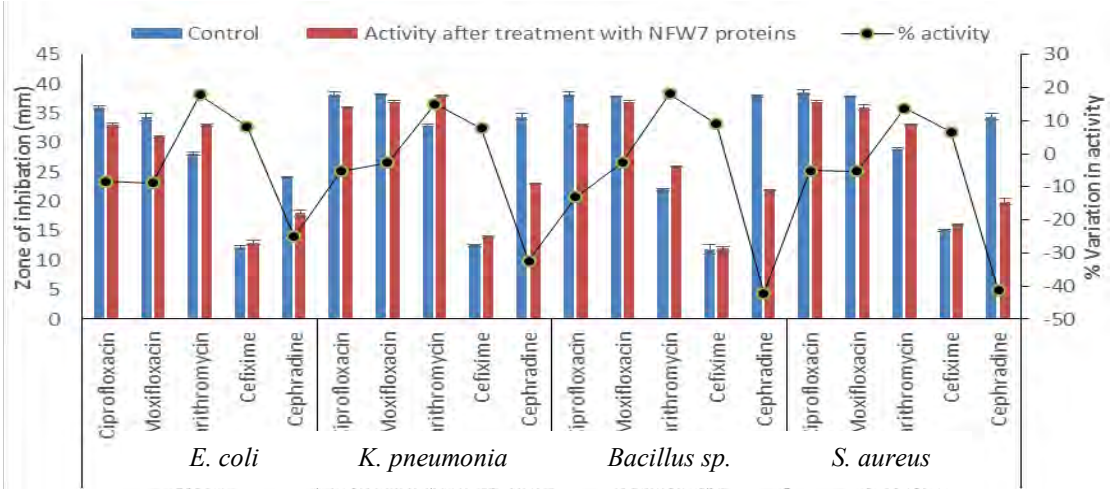


Figure 3.11 Effect of *Epicoccum* sp. NFW7 precipitated proteins on antibiotic solution

3.3.6 Antibacterial Activity of *Chaetomium* sp. NFW8 Grown with Antibiotics in SDB Medium

When *Chaetomium* sp. NFW8 grown on medium along with antibiotics it was revealed that the fungal isolate showed improved activity when clarithromycin and moxifloxacin were present in the medium while when the remaining three antibiotics were present in the medium the antibacterial activity of crude extracts of NFW8 reduced as shown in (Figure 3.12)

When developed biomass of *Chaetomium* sp. NFW8 was incubated on antibiotic solution in water containing moxifloxacin and clarithromycin the activity was enhanced while the biomass grown on ciprofloxacin, cefixime and cephradine showed less antibacterial potential than control. Figure (3.13) represents the antibacterial activities mentioned.

In case of *Chaetomium* sp. NFW8 fungal proteins incubated with antibiotic solution in water it was observed that the activity of moxifloxacin and cephradine increased up to 5-10 and 20-36% respectively against *E. coli*, *Klebsiella pneumonia*, *Bacillus sp.* and *S. aureus* bacterial strains. Moreover, reduction in the activity of ciprofloxacin, clarithromycin and cephradine antibiotics were observed after treatment with *Chaetomium* sp. NFW8 proteins as shown in Figure (3.14).

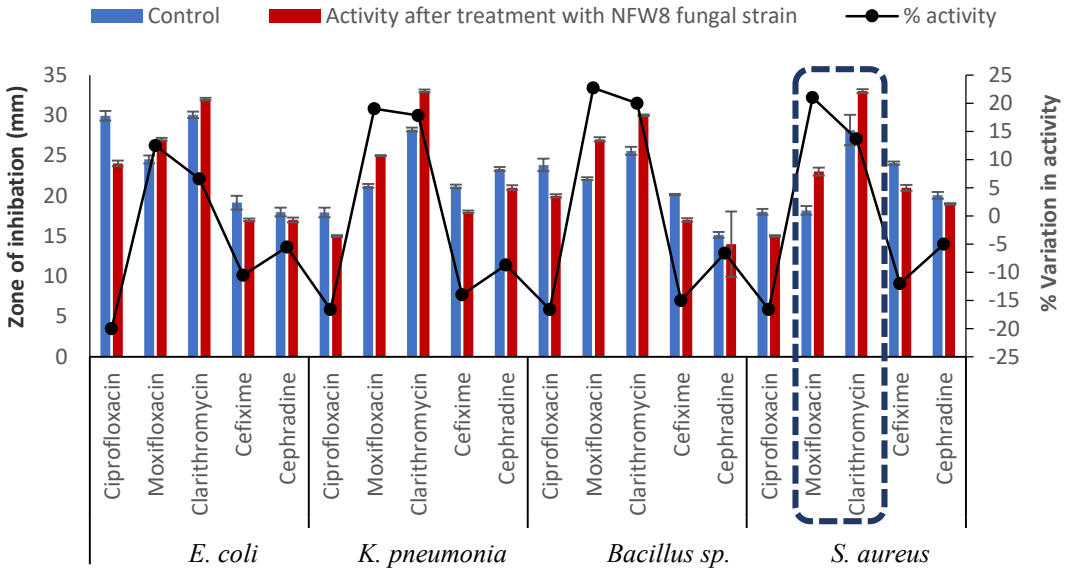


Figure 3.12 Antibacterial activities of *Chaetomium* sp. NFW8 organic extract grown with antibiotic supplemented media

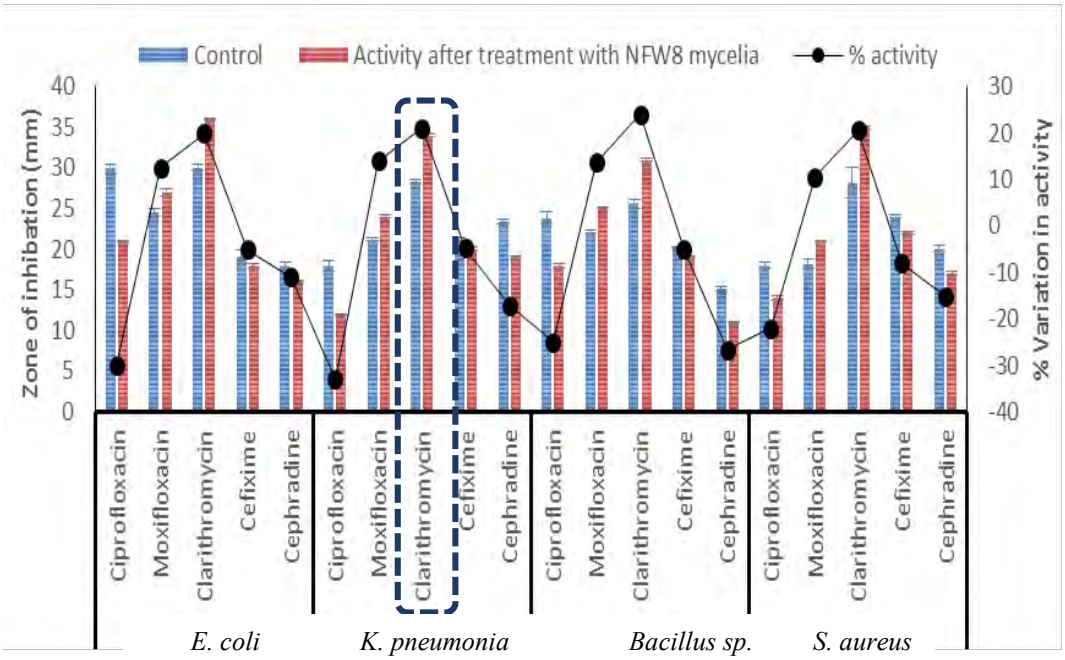


Figure 3.13 *Chaetomium sp*. NFW8 developed biomass incubated with antibiotic solution

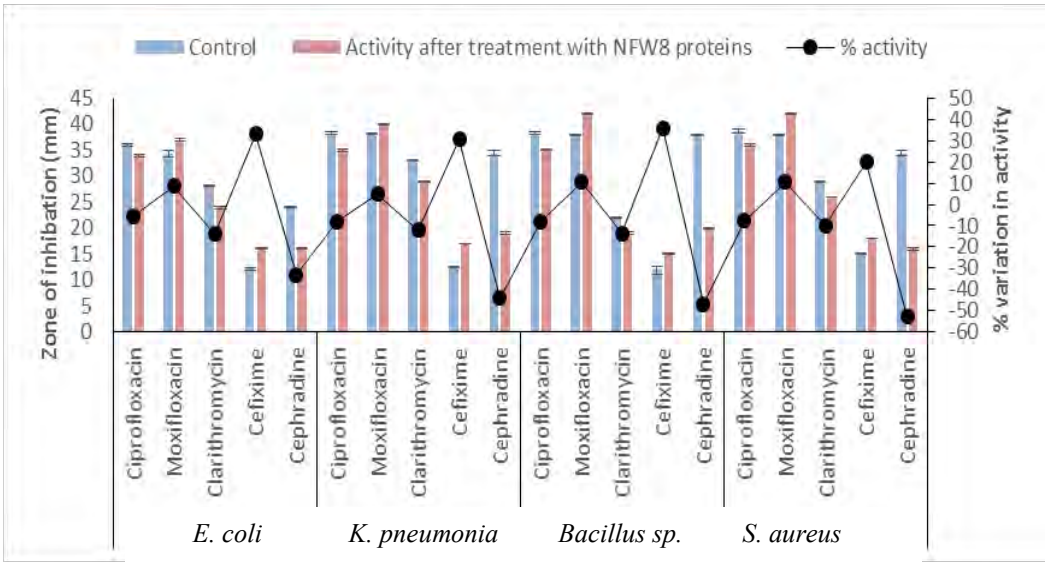


Figure 3.14 Effect of *Chaetomium* sp. NFW8 precipitated proteins on antibiotic solution

3.3.7 Antibacterial Activity of *Fusarium oxysporum* NFW16 Grown with Antibiotics in SDB Medium

From antibacterial assay, it was observed that *Fusarium oxysporum* NFW16 when cultured on medium containing antibiotics, the antibacterial activity increased in the presence of ciprofloxacin (10-40 %), moxifloxacin (44-64 %), cephradine (10-13 %) and clarithromycin (9-27 %) against *E. coli*, *Klebsiella pneumonia*, *Bacillus sp*. and *S. aureus* except in the presence of cefixime, where the activity decreased in the range of 31-49% against the test bacterial strains (Figure 3.15).

The already grown biomass of *Fusarium oxysporum* NFW16 when incubated with antibiotic solution followed by extraction the antibacterial activity of the extracts when incubated with ciprofloxacin and moxifloxacin increased up to 14-42 and 23-44% respectively whereas, the activity in the presence of clarithromycin, cephradine and cefixime decreased up to 3-24, 10-33 and 43-100% respectively against test bacterial strains (Figure 3.16).

After treatment of antibiotic solution with *Fusarium oxysporum* NFW16 proteins, it was observed that the activity of moxifloxacin, clarithromycin and cephradine increased as compared to their respective controls whereas the activity of ciprofloxacin and cefixime decreased (Figure 3.17).

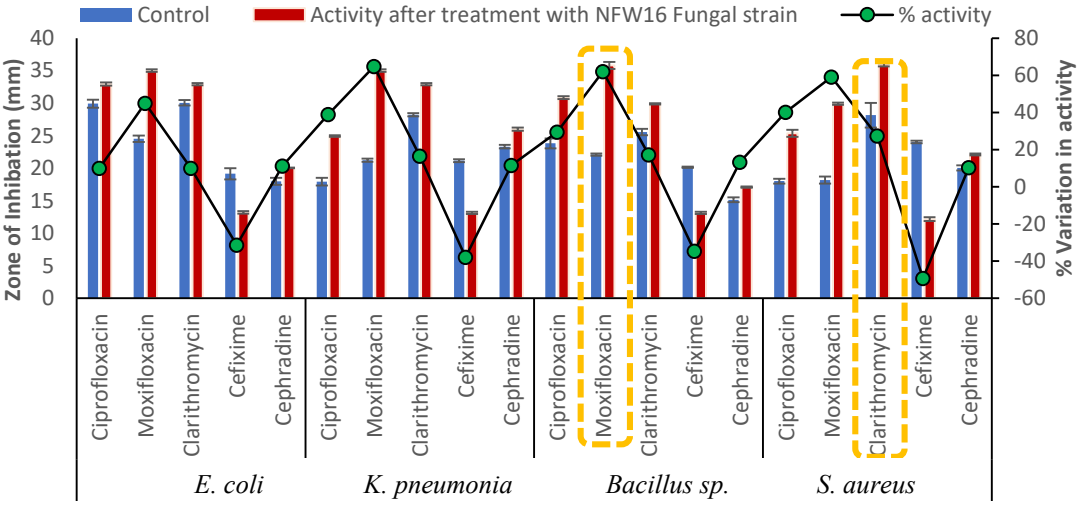


Figure 3.15 Antibacterial activities of *Fusarium oxysporum* NFW16 grown with antibiotic supplemented media against test strains

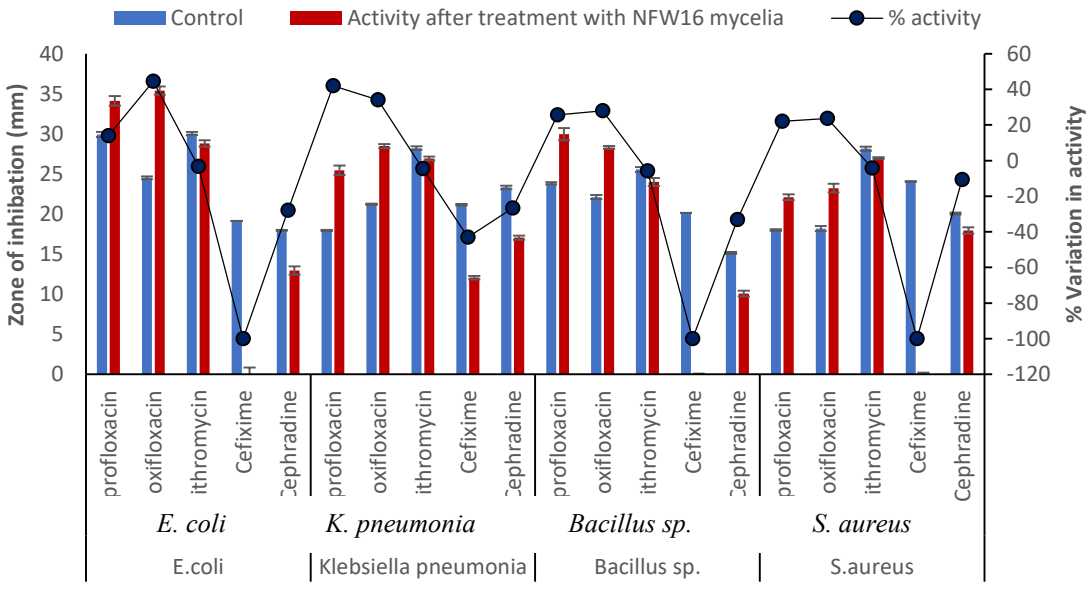


Figure 3.16 Antibacterial potential of *Fusarium oxysporum* NFW16 developed biomass incubated with antibiotic solution

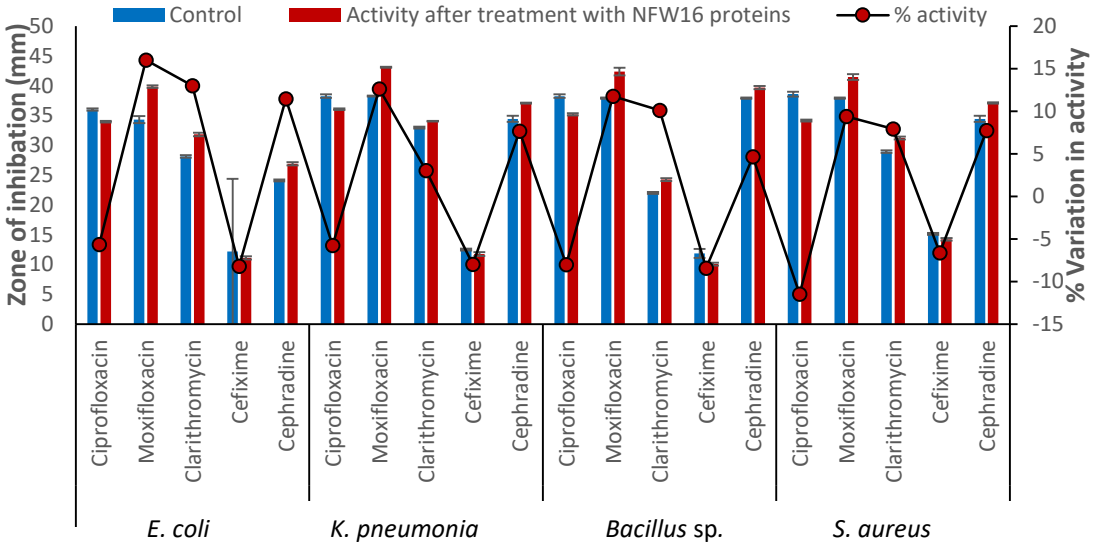


Figure 3.17 Effect of *Fusarium oxysporum* NFW16 precipitated proteins on antibiotic solution

3.3.8 Antibacterial Activity of *Penicillium milleri* NFL1 Grown with Antibiotics in SDB Medium

When *Penicillium milleri* NFL1 grown on medium along with antibiotics it was revealed that the isolate showed improved activity when grown on all the four antibiotics except cefixime against test bacterial strains (Figure 3.18)

Previously grown biomass of *Penicillium milleri* NFL1 when incubated with antibiotic solution followed by extraction, the antibacterial activity of the extracts from ciprofloxacin, moxifloxacin, clarithromycin and cephradine solutions showed more antibacterial activity than control while in case of cefixime the activity dropped as shown in figure 3.19.

Activity of antibiotics were considered against all bacterial strains after treatment with *Penicillium milleri* NFL1 proteins, it was observed that *Penicillium milleri* NFL1 proteins enhanced the activity of cefixime, clarithromycin and cephradine while, the activity of ciprofloxacin, moxifloxacin was decreased after treatment with the proteins of *Penicillium milleri* NFL1 (Figure 3.20).

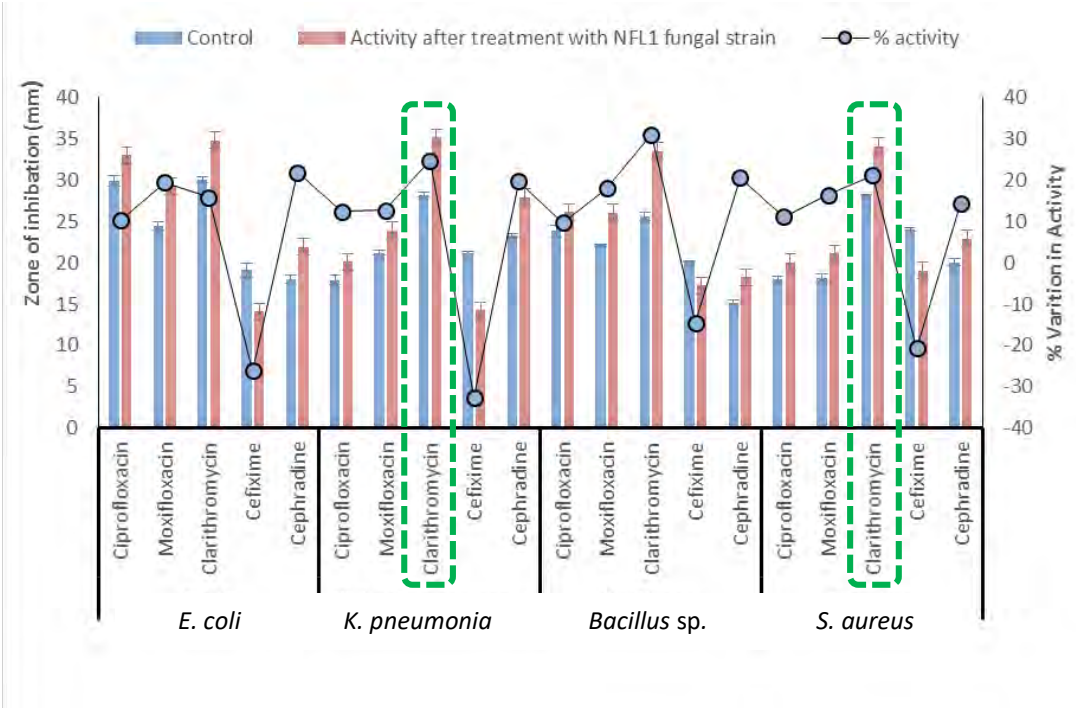


Figure 3.18 *Penicillium milleri* NFL1 grown with antibiotic supplemented media

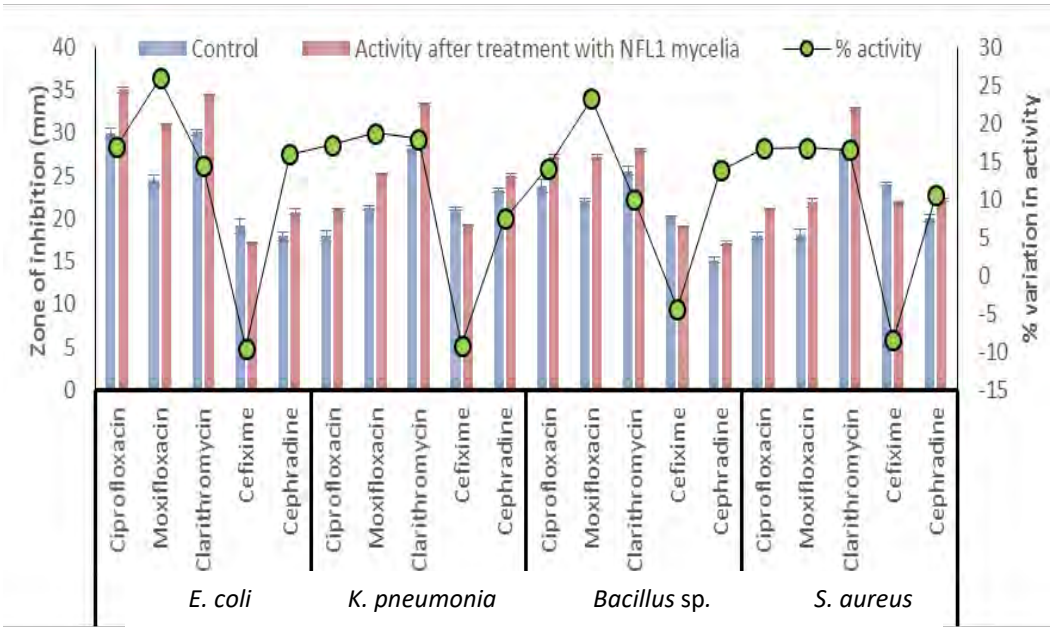


Figure 3.19 *Penicillium milleri* NFL1 developed biomass incubated with antibiotic solution

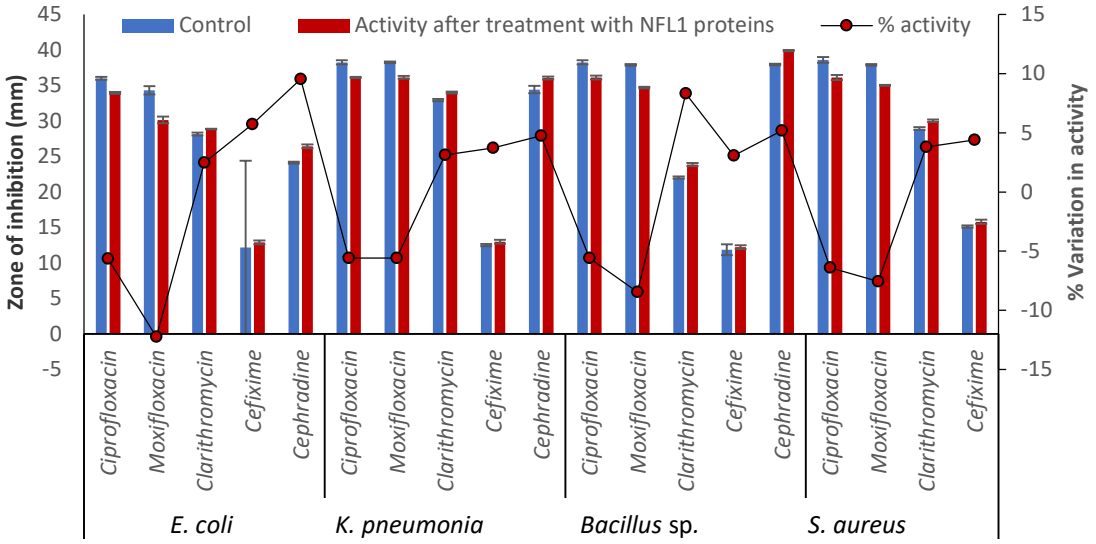


Figure 3.20 Effect of *Penicillium milleri* NFL1 precipitated proteins on antibiotic solution

3.3.9 Antibacterial Activity of *Paraconiothyrium* sp. NFL6 Grown with Antibiotics in SDB Medium

The crude extracts obtained from *Paraconiothyrium* sp. NFL6 grown under antibiotic stress showed better antibacterial activity under the stress of moxifloxacin (22-23%), clarithromycin (9-13%) and cephradine (4-8 %) against different bacterial strains whereas, the activity in the presence of ciprofloxacin and cefixime decreased shown in figure (3.21)

The already developed biomass of *Paraconiothyrium* sp. NFL6 when grown on only antibiotics in water the activity increased when grown on moxifloxacin 38% against *E. coli*, 32% against *Klebsiella pneumonia*, 31% against *Bacillus sp*. and 41% against *S. aureus* while when grown on the remaining four antibiotics the antibacterial activity reduced as shown in (Figure 3.22)

In addition to this, the results of antibacterial assay of protein treated antibiotics revealed that the activity of clarithromycin and cefixime increased while the remaining antibiotics attained declined in antimicrobial activity after treatment (Figure 3.23)

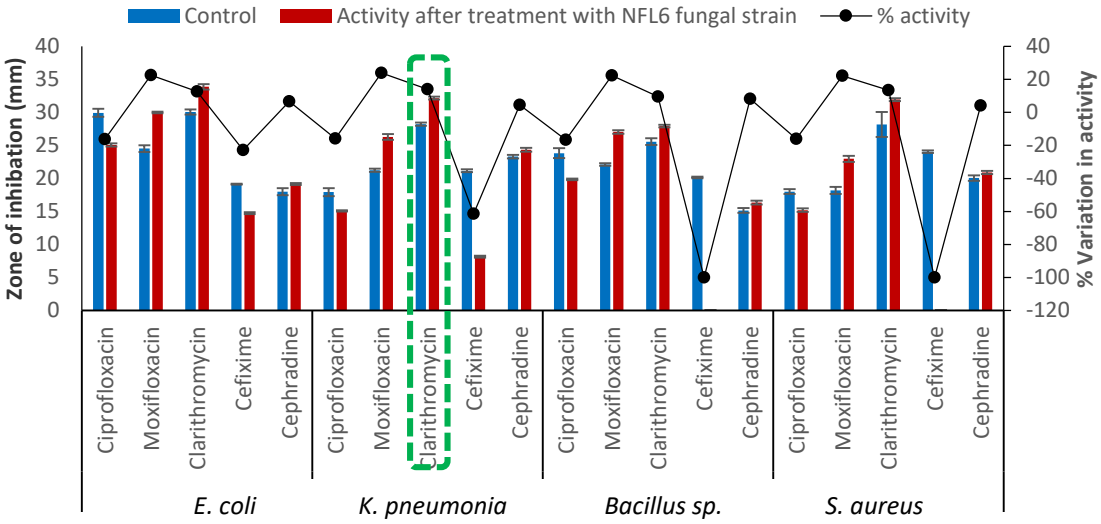


Figure 3.21 *Paraconiothyrium* sp. NFL6 *grown with antibiotic supplemented media*

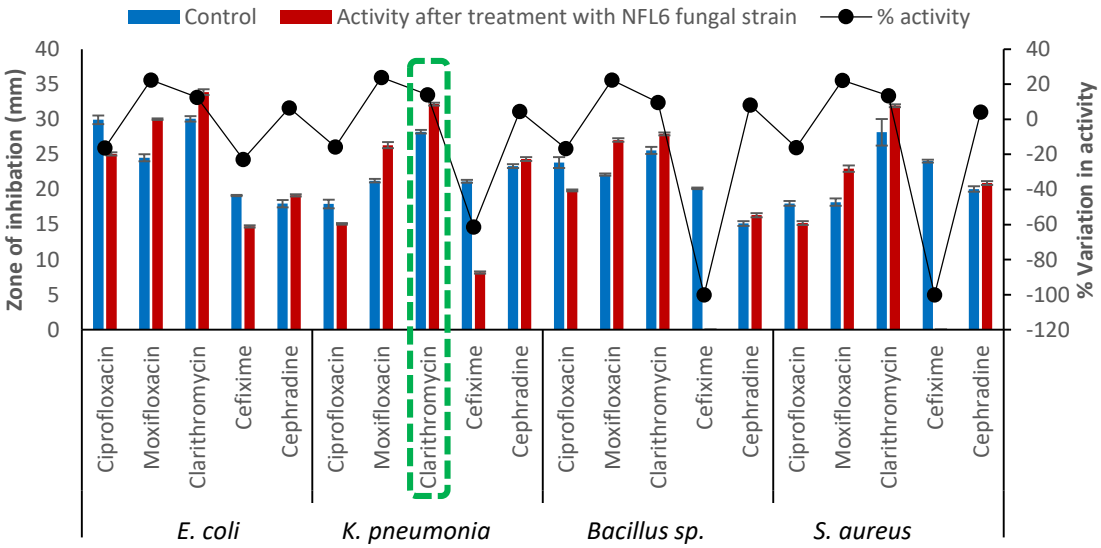


Figure3.22 *Paraconiothyrium sp.* NFL6 developed biomass incubated with antibiotic solution

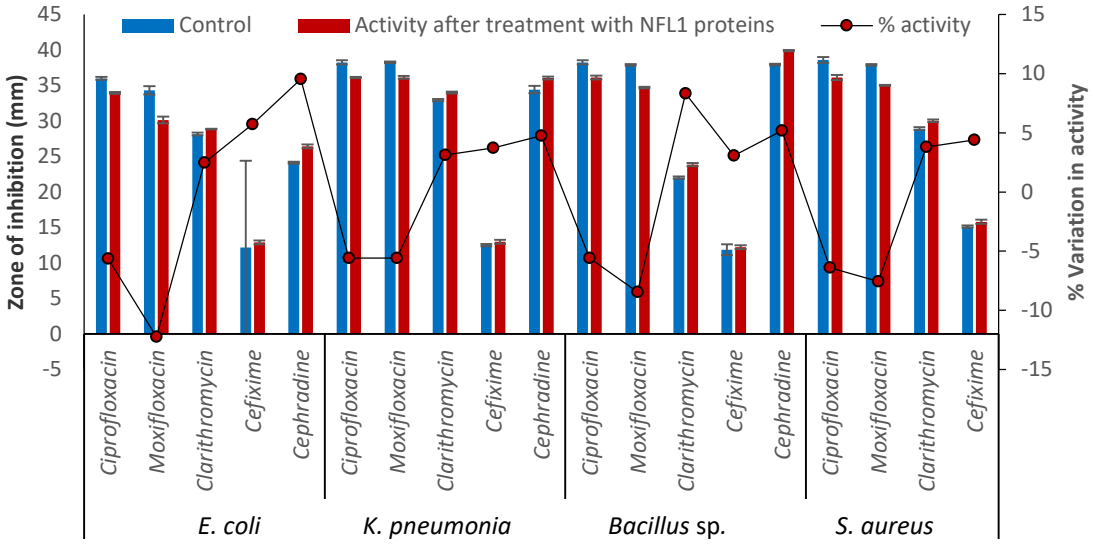


Figure 3.23 Effect of *Paraconiothyrium sp.* NFL6 precipitated proteins on antibiotic solution

3.4 DISCUSSION

Endophytic fungi have the potential to produce various bioactive metabolites under normal and stressed condition and can transform variety of substrates. In this chapter, seven endophytic fungal isolates previously isolated from *Taxus fauna* and identified (Fatima *et al*., 2016) are grown on SDB medium supplemented with antibiotic and without the addition of any antibiotic. The aim was to analyze the production of antibacterial compounds under both conditions as well as study the transformation of antibiotics by endophytic fungi. It was observed that the endophytic fungal isolates showed viable growth under a high concentration (1mg/mL) of antibiotics. Moreover, when the isolates were grown on medium supplemented with antibiotics, antibacterial activity of the extracts showed variations. In some cases, there was significant increase in antimicrobial spectrum, while in others either minor decline or no change altogether was observed. Since, the underlying rational is the production and isolation of potential novel or new lead biochemical compounds triggered in response to antibiotic supplementation. Therefore, initially, bioactivity guided screening of the extracts obtained from the isolates was conducted to select the isolates and antibiotics showing pronounced biological effects.

Endophytic fungi have been studied extensively for the production of antimicrobial compounds. The unique chemical moieties are known to provide survival benefits against pathogens inside plants (Rodriguez *et al*., 2009). The chemical compounds produced by endophytes display remarkable structural diversity (Aly *et al*., 2010). The ability to produce several pharmaceutically important metabolites from endophytic fungi isolated from *Taxus fauna* and their biosynthetic potential is well established (Heinig, 2012). Although endophytic fungi can grow on several media, studies suggest that the complexity of medium can enhance the production of unique metabolites (Aly *et al*., 2011). Nevertheless, medium suitable for some species can turn out to be unproductive for others (Vandergrmolen *et al*., 2013). These facts advocate the uniqueness as well as complexity of the dynamic metabolic mechanism within these ubiquitous organisms.

In the present study, endophytic fungi were grown in antibiotic supplemented medium and screened for change in metabolic profile by adopting three different screening strategies. The findings revealed that screening crude organic extract of the strains

grown in the presence of antibiotic could provide significant insight about the metabolic potential of the fungal endophyte. Therefore, this strategy could be prioritized for the production of new and novel biochemical compounds from endophytes. These findings are in close agreements with various recent studies (Parthasarathy *et al*., 2020; Aboobaker *et al*., 2019; Sharma *et al*., 2016; Santos *et al*., 2015).

Production of potent biochemical metabolites by endophytic *Epicoccum* species is well documented. This genus is known for the production of epiccocamide (Wright *et al*., 2003), epicocconigrones (El Amrani *et al*., 2014), epipyridone and epicoccarines (Wangun and Hertweck 2007). Similarly, studies describe significant antibacterial potential of endophytic *Mucor* sp. and *Chaetomium* sp., isolated from medicinal plants (Selim *et al*., 2011). *Fusarium* and *Aspergillus* species isolated from *Panax notoginseng* a Chinese medicinal herb showed the production of antibacterial saponins against test bacterial strains (Jin *et al*., 2017). Ding *et al*., (2019) isolated 42 endophytic fungi from *Ficus elastica* and identified them as seven different taxa based on ITS region sequencing. Two isolates *Penicillium funiculosum* and *Trichoderma harzianum* showed broad spectrum antimicrobial activities against all test microbes.

Recent studies analyzing metabolic profile of the endophytic fungi, describe the effect of media supplementation. Merrouche and colleagues amended the endophytic growth broth with sorbic acid which led to the production of a new dithiolopyrrolone derivative (Merrouche *et al*., 2019). Comprehensive biochemical characterization revealed that this iso-hexanoyl-pyrrothine, is a novel dithiolopyrrolone derivative and possess moderate to strong inhibitory effects against gram positive bacteria (Merrouche *et al*., 2019).Another study in which the medium is augmented with electors, to enhance the production of taxol from endophytes , revealed that the maximum production of taxol was achieved when 20 mg/L of salicylic acid is added to medium under other optimum conditions (Somjaipeng *et al*., 2016).

Mei *et al*., (2019) conducted a related study in which they isolated a strain Rhizopus oryzae that can transform sophoricoside into genistein by producing an enzyme β glucosidase. Under optimized conditions, the yield of genistein reached to 85.6 % in 24 hours shake flask experiment while the initial concentration of substrate was 1mg/mL. This process provides an alternative way to produce genistein and other pharmaceutically important compounds, using other strains and substrates.

Epicoccum species NFW1, NFW7 and *Fusarium oxysporum* NFW16 can completely diminish the antibacterial activity of cephradine, ciprofloxacin and cefixime antibiotics. In the present study, Epicoccum species NFW1, NFW7 and Fusarium oxysporum NFW16, when grown in medium supplemented with either ciprofloxacin, cefixime or cephradine, expressed loss of their antibacterial activity. This could be due to either biodegradation or biotransformation of the antibiotic into products which not only lack the biological activity but also affected the production of antibacterial compounds by the endophyte itself. Hence, a noticeable reduction in antibacterial spectrum of the endophytes was observed when grown in the presence of antibiotic. These observations are in accordance with the study of Mei *et al* (2019).

A similar study by Cyancarova *et al* (2013) studied the biotransformation of antibiotics Flumequine and fluroquinolone by using five different lignolytic fungi. The fungi Irpex lacteus, Dichomitus squalens and Trametes versicolor were more potent in transforming the drugs up to 90% after 6 days of incubation while some strain needs 14 days to transform 90% of drugs. The transformed metabolites were identified as 7-hydrooxy flumequine and flumequine ethyl ester, among eight other transformed products. The antibacterial activity of residual mixtures was reduced in many cases. However, in case of D. squalens although the parent antibiotic transform up to 90 %, the antibacterial activity remained unaltered, indicating that either the transformed products possess the antibacterial activity or the fungus itself has induced the production of antibacterial metabolites.

Likewise, Rusch and coworkers (2017) studied the biotransformation of difloxacin, marbofloxacin and enrofloxacin by a Xylaria fungal specie. The antibiotics were transformed by fungal mediated N-oxidation of fluroquinolones which reduces 77-90% activity of parent antibiotics, affecting its cytotoxicity significantly (Rusch *et al*., 2017). In order to utilize biotransformation potential of fungal endophytes, Holanda *et al*., (2019) analyzed the biodegradation of chloramphenicol by five fungal strains isolated from Bertholletia excelsa of Brazilian amzonia. The liquid culture medium showed the degradation of antibiotic by these fungal strains at different levels. These results are in accordance with the result of the present study since a reduction in antibacterial activity signifies chemical deformity.

In the present study, endophytic fungi grown in the medium amended with clarithromycin and moxifloxacin showed enhanced antibacterial activity as compared to those grown without these antibiotics. This could be attributed to the production of more active transformed product of the parent antibiotic or induced production of potential bioactive compounds by the microbial endophyte. These finding are supported by recent study by Sponchiado and colleagues who reported transformation of Rifampicin into rifampicin quinone and a novel metabolite by the fungus Cunninghamella elegans in 120 hours of incubation (Sponchiado *et al*., 2020).

3.5 CONCLUSIONS

This chapter concludes that endophytic fungi isolated from medicinal plant *Taxus fuana* can show variable antibacterial activities in response to supplementation of antibiotics as analyzed using three different screening strategies. The crude ethyl acetate extract of strains *Epicoccum* sp. NFW1, *Chaetomium* sp. NFW8 and *Fusarium oxysporum* NFW16, when grown in SDB supplemented with clarithromycin and moxifloxacin showed noticeable increase in antibacterial activity. Bactericidal effects vanished altogether when grown in the presence of cephradine, ciprofloxacin and/or cefixime antibiotics. Therefore, these three endophytes *Epicoccum* sp. NFW1, *Chaetomium* sp. NFW8 and *Fusarium oxysporum* NFW16, and two antibiotics clarithromycin and moxifloxacin were prioritized for the rest of studies.

CHAPTER 4

4 METABOLOMICS OF SELECTED FUNGAL ISOLATE FOR ANTIMICROBIAL METABOLITES PRODUCTION

4.1 INTRODUCTION

Natural products of microbial origins brought a drastic change in our living standards by providing an excellent source of compounds which revolutionized the world of medicine. In this regard, fungi are the most prolific source to produce diverse range of natural molecules. Recent advances in the field of genetics, synthetic biology and bioinformatics enhanced the efficiencies in the discovery of novel drugs (Greco *et al*., 2019). The traditional drug discovery approach is a major downfall in the discovery of novel drugs which may not only lead to the rediscovery of the compounds, but it is also a cumbersome process, to produce the desired metabolite. Therefore, adoption of the de-replication-based techniques and bioinformatics tools could reduce the possible uncertainties in the quest of the novel compounds (Vasundhara *et al*., 2016).

Recent advances in high throughput targeted and un-targeted metabolomics approach in microbial product discovery, provides many advantages over bioassay based traditional isolation methods. One major advantage is the effective de-replication of the compound prior to their isolation. Metabolomics approaches includes chemical de-replication methods and bioinformatics de-replication methods. The major problem in the compound isolation is to distinguish between the known and un-known molecules. The chemical de-replication process is helpful in avoiding the characterization of the already known compounds (Greco *et al*., 2019). Application of the advanced analytical techniques such as HPLC, LC-UV/MS and NMR and other recent techniques such as MS/MS, tandem spectrometry coupled with precision liquid chromatography link with statistical analysis for data elucidation are very helpful in the discovery of potentially valuable microbial compounds as well as their bioassays aided isolation and screening. Moreover, data generated from the NMR and MS/MS techniques are very supportive in elucidation of the molecular structure of the unknown compounds (Vasundhara *et al*., 2016). Furthermore, recent developments in separation of the molecules through liquid chromatography and their detection through HRMS linking with the use of database makes the chemical de-replication a bit faster (Nielsen *et al*., 2015). In the recent years, a new set of databases called Global Natural Products Social Molecular Networking (GNPS) was made, in which researchers can upload the raw processed data

taken from MS/MS. This database provides a platform for “molecular explorer” which have the information regarding a specific molecule or its analogs. Moreover, GNPS also provide molecular networking to the user to identify, visualize and correlate a set of spectra for similar compounds or molecules (Wang *et al*., 2017).

This chapter describes the comprehensive screening of metabolic profiles of endophytic fungal isolates grown with and without antibiotic presence in SDB medium. The aim of this experimentation was to elucidate any shift or change in the metabolic profile of the endophytes by adopting untargeted metabolic approach. Therefore, the crude ethyl acetate extract was subjected to LCMS/MS followed by dereplication and differentiation in metabolic profile under different conditions through Global Natural Products Social Molecular Networking (GNPs). This led to observe the change in metabolic profile under different conditions and the identification of known and unknown chemical moieties thereby facilitating the discovery of novel or new chemical lead compounds.

4.2 MATERIALS AND METHODS

4.2.1 Reagents

For LCMS analysis, the LCMS grade formic acid and organic solvents (methanol and acetonitrile) were purchased from Sigma (Sigma Aldrich USA). Water was obtained from Millipore Sigma Direct-Q 3 tap water purification system. This study was conducted in TM Lab Oregon State University USA.

4.2.2 Sample Preparation

The crude extract of endophytic fungal isolates grown on SDB medium without antibiotics and with antibiotics were obtained separately by using ethyl acetate as organic solvent for extraction as mentioned in chapter 3. For LC-HRMS/MS analysis the extracts were dissolved in concentration of 0.1mg/mL in LCMS grade methanol. The antibiotics separately were also included as controls.

4.2.3 Multivariate Statistical Analysis

Multivariate statistical analysis was performed using SIMCA-P V 13.0 (Umetric, Umea Sweden). The data was converted into a CSV file and exported to SIMCA-P V 13.0 for unsupervised statistical analysis Principal Component Analysis (PCA) and supervised statistical analysis Orthogonal Partial Least Square Discriminatory Analysis (OPLS-DA).

4.2.4 LCMS Analyses and Global Natural Product Social Molecular Networking GNPS

LC-MS spectral data were viewed using MZmine 2.53, Peak viewer, MassHunter and converted into “mzXML” files. High-resolution mass spectrometry (HR-MS) was obtained using an Agilent 1260 HPLC upstream of an Agilent 6545 Q-ToF. The separation was achieved using Infinity Lab Poroshell EC-C18 column (100 × 3.0 mm, 2.7 μm) at a flow rate of 0.4 mL/min and the following gradient. Line A was water with 0.1% (v/v) formic acid, and line B was acetonitrile with 0.1% (v/v) formic acid. The column was preequilibrated with 90% A/10% B. Upon injection, the mobile phase composition was maintained for 1 minute upon when the mobile phase was changed using a linear gradient to 0% A/100% B over the following 45 minutes. This concentration was held for 6 minutes followed by changing the mobile phase to 90%

A/10% B over 1 min. The Q-ToF mass spectrometer was operated in the Auto MS/MS mode with electrospray ionization (ESI+) HR-MS spectra with a resolution of 60 000 and a mass accuracy of <5 ppm were recorded. For positive peak findings, data-dependent high-resolution product ion spectra (HR-MS/MS) at a resolution of 7500 were produced. MassHunter software was used to operate Q-ToF machine, and data were processed using MassHunter Qualitative Analysis software. Compounds were identified for Global Natural Products Social Molecular Networking (GNPS) analysis using the "Compounds Discovery" workflow with the "Auto-Select Compound Mining" settings (Wang *et al*., 2016). MS/MS peaks and spectrum were chosen according to the following settings: average scans, 10% of peak height; exclude, if above 10% of saturation; no MS/MS spectrum background; height filters, absolute height, 10 counts, relative height, 1% of largest peak; charge state isotope model, unbiased; limit-assigned charge states to a range of 1-2. The files were then exported as both MGF and MzData formats and used for molecular networking.

4.3 RESULTS

4.3.1 Metabolic Profile of *Epicoccum* Sp. NFW1 in SDB with and without Antibiotics

The metabolic profile of the fungal isolate grown with and without antibiotics were analysed initially by comparing LCMS chromatograms followed by PCA analysis. For mre comprehensive analysis and dereplication studies GNPS were performed.

4.3.1.1. LCMS Chromatograms

The LCMS analysis revealed the major differences in the chemical profile of extracts obtained by cultivation *Epicoccum* sp. NFW1 on SDB media with and without antibiotics. LC-MS chromatogram shows that although some peaks are detected in all the conditions, but most of the compounds are produced only in the presence of specific antibiotic in the medium. majority of the compounds eluted before 35 minutes while few compounds along with clarithromycin eluted after 35 minutes so a range from less polar to polar compounds were present in the extracts. Moxifloxacin and clarithromycin added to the medium when eluted resulting a huge and broad peak because they were added in high concentration (1mg/mL) to the medium. Similarly, there are clear differences in the relative concentration of various metabolites, indicated by varying

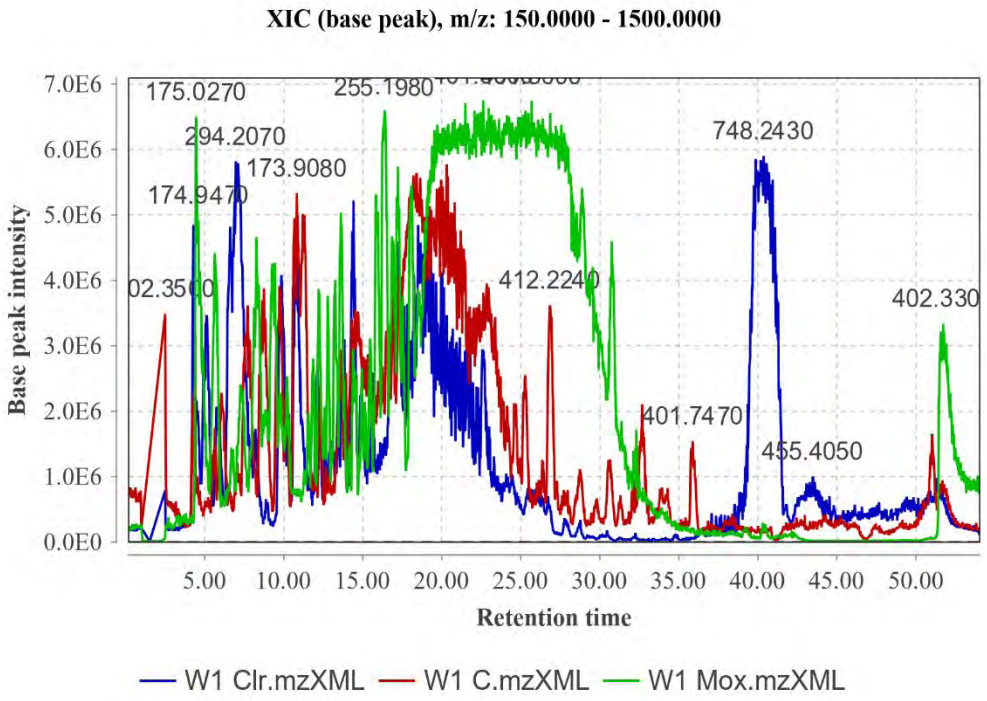


Figure 4.1 Comparison of the total ion chromatograms of the crude ethyl extracts of *Epicoccum* sp. NFW1 cultured on SDB medium. Red = without antibiotic addition to medium, Blue Clarithromycin supplementation to medium, Green Moxifloxacin supplementation to medium

peak intensities. To better understand the effect of change in culture condition the chromatograms were overlapped with different colours as shown in (Figure 4.1).

4.3.1.2 PCA Analysis

Processed data was subjected to unsupervised statistical analysis, Principal Component Analysis (PCA) using SIMCA-P (V 13.0). PCA identifies the differentiating features in the chemical profile of the extracts and helps in prioritizing the candidates with interesting metabolome. The PCA score scatter plot (Figure 4.2) is the graphical presentation of similarity or uniqueness of the extracts. PCA score scatter plot of *Epicoccum* sp. NFW1 in SDB medium with and without antibiotic addition showed that the addition of antibiotics to medium vastly effect the production of diverse metabolites hence they were lied at outside of different quadrant S-plot (OPLS-DA) showing distribution of the metabolites produced by *Epicoccum* sp. NFW1 in the presence and absence of antibiotics in the culture medium. the S-plot showed many compounds with unique chemistries were produced with the addition of antibiotics to the culture medium as they were clustered in different quadrants away from the center (Figure 4.3).

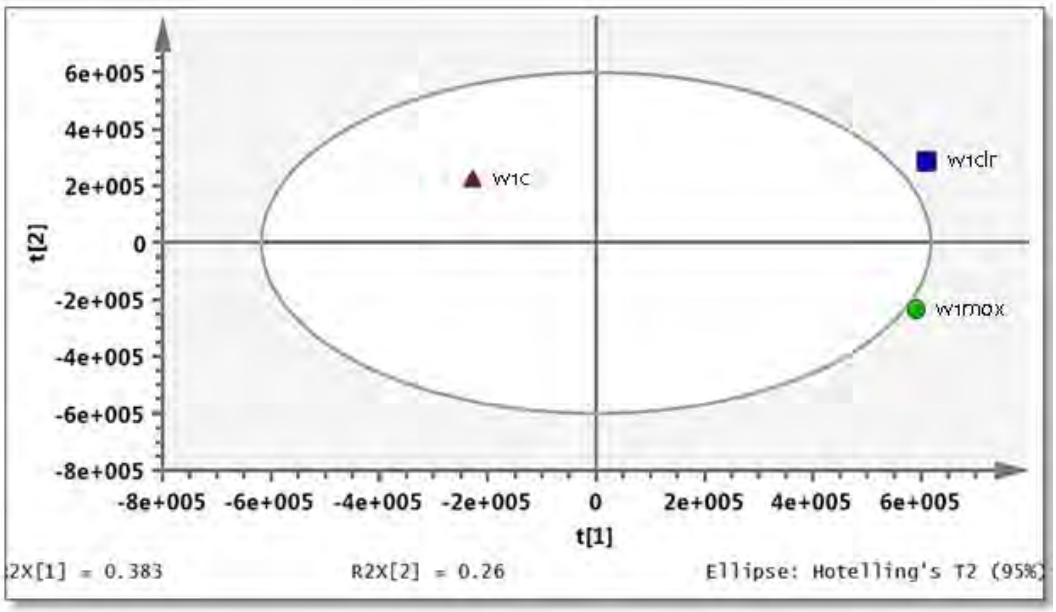


Figure 4.2 PCA score scatter plot for crude ethyl acetate extracts of *Epicoccum* sp. NFW1 (W1c=grown without antibiotic addition to culture media, W1clr= grown with the addition of clarithromycin and w1mox grown with the addition of moxifloxacin

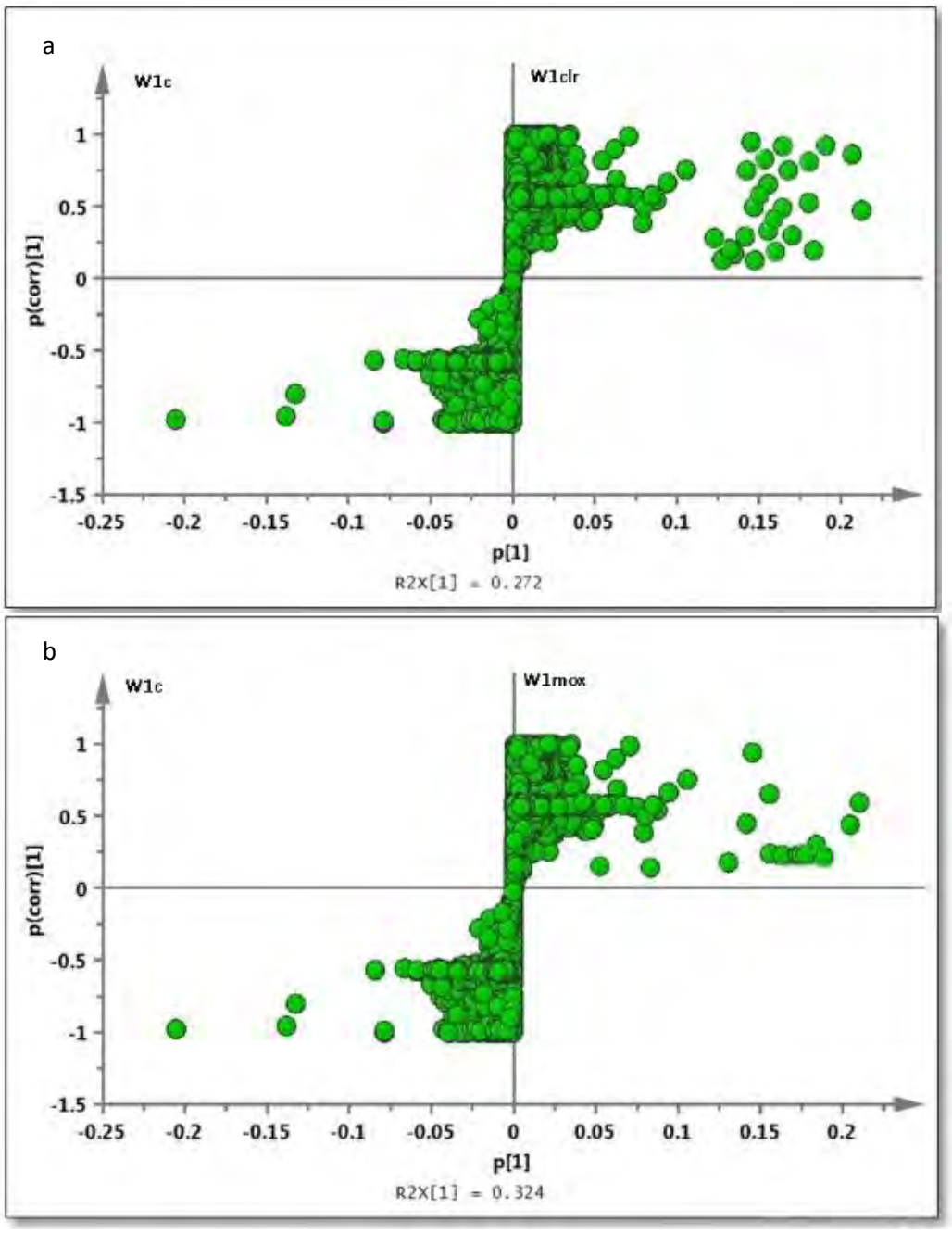


Figure 4.3 OPLS-DA S-plot for crude ethyl acetate extracts of *Epicoccum* sp. NFW1. a) S-plot showing distribution of the metabolites in the fungal isolate W1c = grown without antibiotic W1clr=grown with clarithromycin, b) W1c = grown without antibiotic W1moxifloxacin

4.3.1.3 Global Natural Product Social Molecular Networking GNPS to Differentiate and Dereplicate the Metabolome of *Epicoccum* sp. NFW1

The endophytic fungal isolate *Epicoccum* sp. NFW1 when analyzed for metabolic profiling under different conditions a total of 2414 hits were detected. Among these metabolites several metabolites were known as their spectral information were present in databases while some metabolites which were unknown but having structural similarities with the known metabolites as they were in the same clusters as the known metabolites were present. They may be new metabolites, or their spectral data is yet not included in databases. Some metabolites are present as single node either having no structure similarity with others or may be the new or novel metabolites (Figure 4.8) represent such nodes. Figure 4.4 shows the overall view of metabolites produced by *Epicoccum* sp. NFW1 with and without antibiotic stress.

The data can be interpreted in several ways but in this study, we majorly focused on the metabolites which were induced or suppressed by antibiotics. Figure 4.5 showed a targeted region in molecular network in which clusters of different metabolites were present in the form of colored nodes and labelled by their respective masses.

When the *Epicoccum* sp. NFW1 grown in SDB medium without antibiotics certain metabolites were produced which were suppressed by the presence of antibiotics in medium. These metabolites were present as red coloured node in molecular network. A total of 290 such metabolites appeared which were only present in the fungal isolate when there was no antibiotic in the medium. Among these metabolites the well-known metabolites were shown in Table (4.1) by using GNPS as dereplication method and one of the cluster of such metabolites is shown in (Figure 4.6a).

Under the presence of clarithromycin *Epicoccum* sp. NFW1 produced certain metabolites which were not produced otherwise as these metabolites were induced by clarithromycin. A total of 451 such nodes were detected. They were also dereplicated to find out the known ones (Table 4.2). Figure (4.6b) shows cluster of such metabolites with their respective mass which were only present when culture medium was supplemented with clarithromycin.

Moxifloxacin induced the production of 497 metabolites by *Epicoccum* sp. NFW1. They were also dereplicated by using GNPS as dereplication strategy and the well-

known metabolites were shown in table (4.3). Figure (4.6c) representing the cluster of these with few other structurally related metabolites. They were shown as green colour nodes along with their mass in molecular network.

Some metabolites are those which were produced in the presence of both antibiotic as well as without antibiotic. A total of 85 such nodes were detected. They have a node with three different colour red, blue, and green (Figure 4.7b). Table (4.4) summarized the already known metabolites which were produced under the said condition. Several metabolites are produced with both antibiotics and the nodes representing these metabolites are in the dual colour that is blue and green (Figure 4.7a) represent these metabolites. Among them already known metabolites were present in (Table 4.5). Numerous other metabolites were arising from the transformation of antibiotic itself either by addition or deletion of certain moieties upon antibiotics, they are in orange colour when clarithromycin is the parent compound or yellow when moxifloxacin is the parent compound shown in (Figure 4.5). Few contaminants were also detected as they have all the five colours in a single node supposed to be external or solvent contaminants (Figure 4.7c).

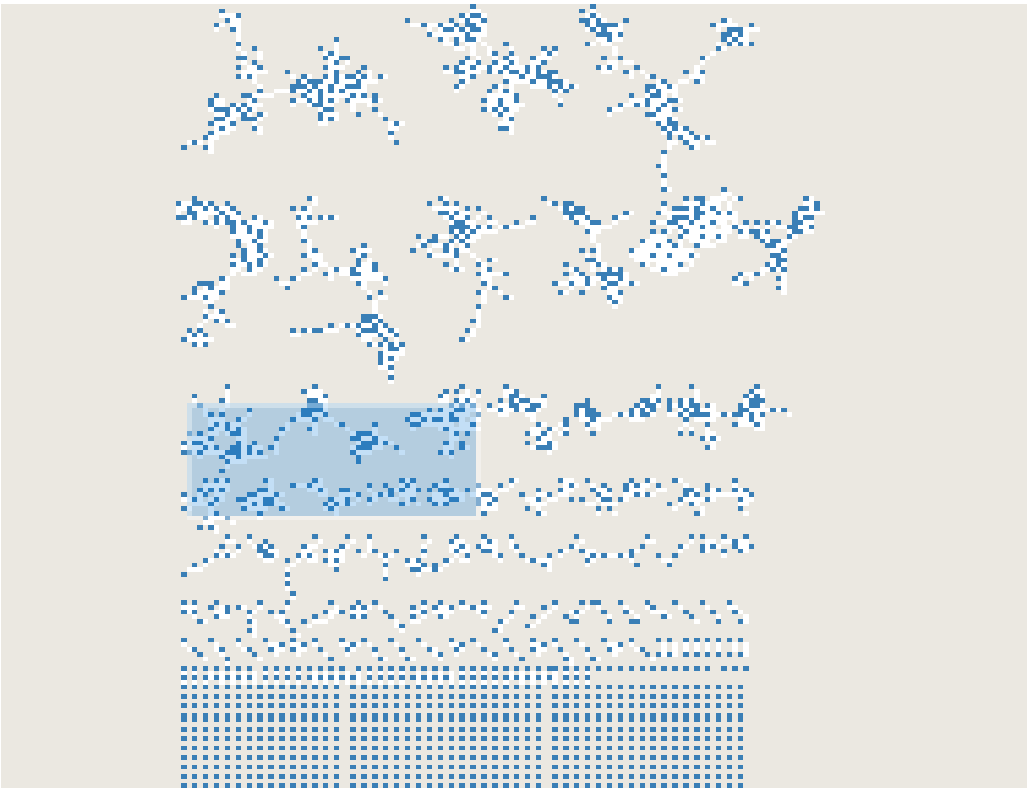


Figure 4.4 Over all view of GNPS showing metabolites with and without antibiotic by *Epicoccum* sp. NFW1

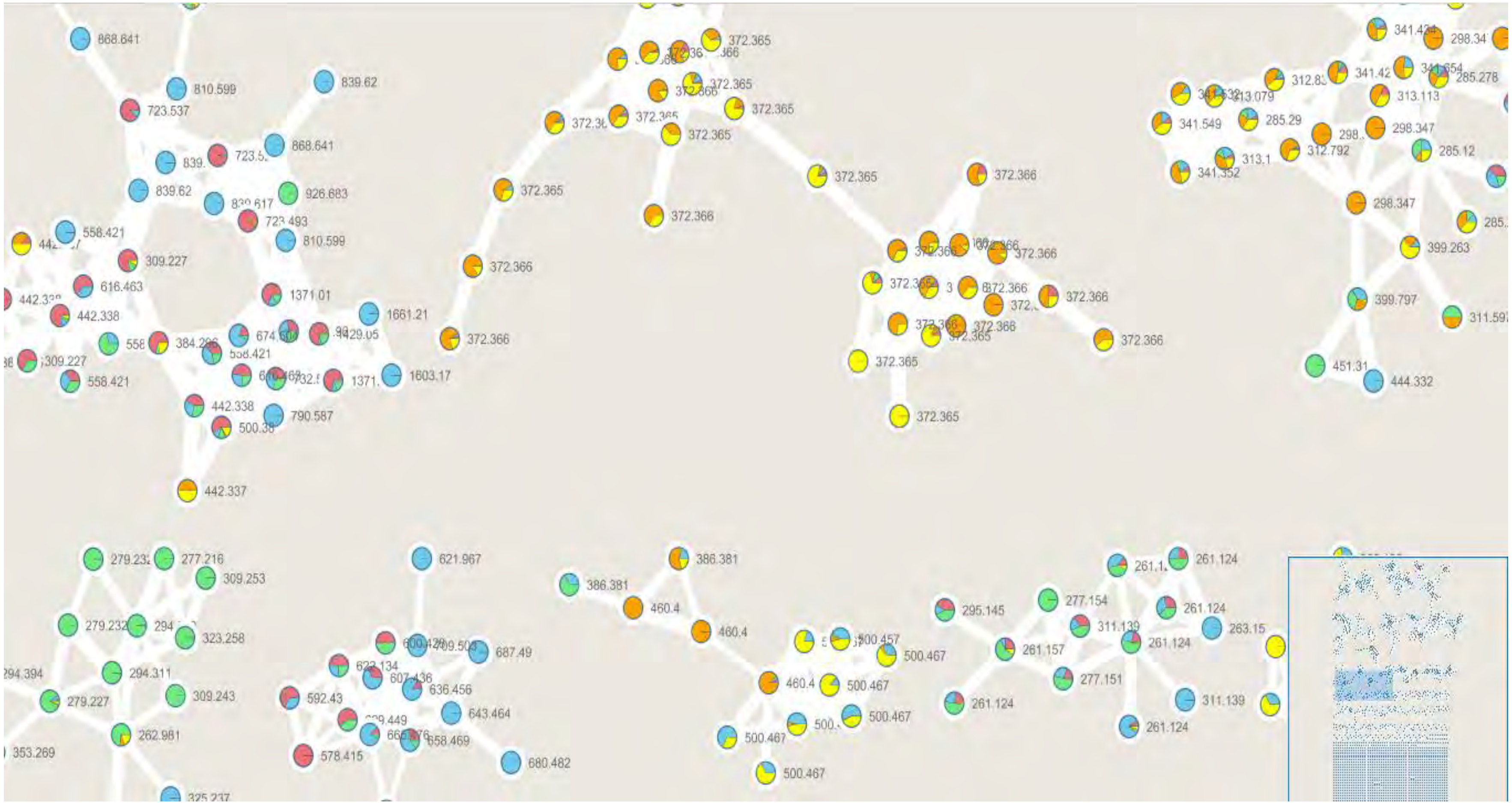


Figure 4.5 View of targeted area showing nodes of different colours representing the metabolites produced under different conditions by *Epicoccum* sp. NFW1

Red = without antibiotic: **Blue** = with clarithromycin: **Green** = with moxifloxacin: **Orange** = clarithromycin or transformed clarithromycin: **Yellow** = Moxifloxacin or transformed moxifloxacin

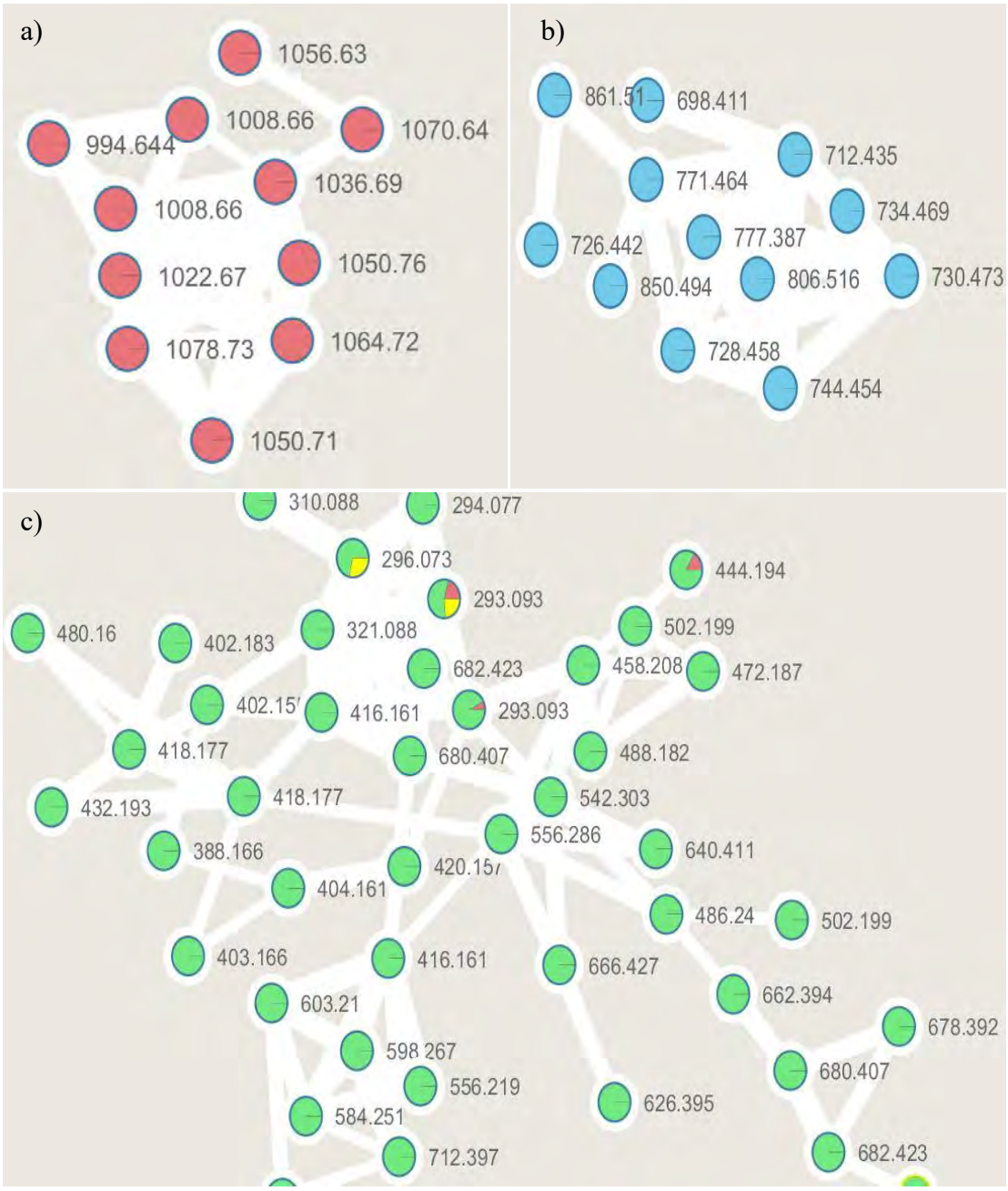


Figure 4.6 Metabolites produced by *Epicoccum* sp. NFW. a) node in red colour indicate metabolites produced in the presence of antibiotics in SDB, b); nodes in blue colour indicate metabolites produced in the presence of clarithromycin in SDB, c); nodes in green colour indicates metabolites produced in the presence of moxifloxacin in SDB.

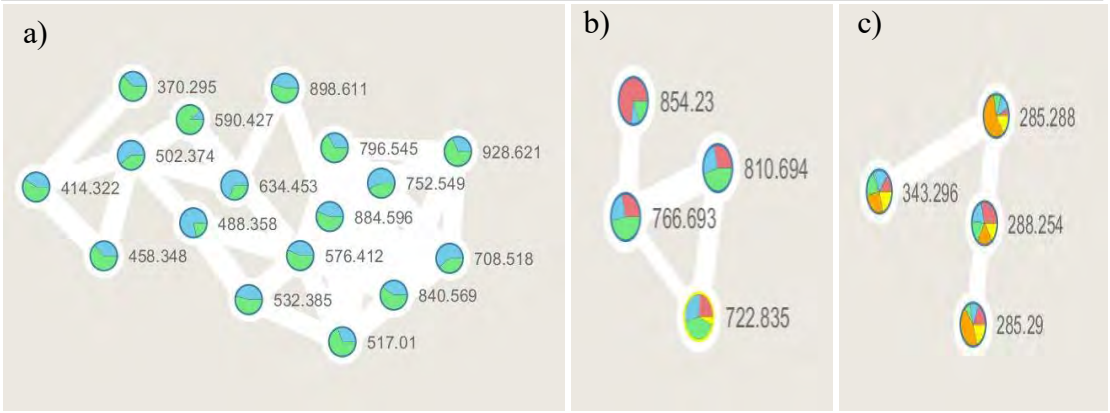


Figure 4.7 Metabolites detected from the extract of *Epicoccum* sp. NFW1 **a)** nodes representing metabolites produced under the presence of both clarithromycin and moxifloxacin **b)** nodes present with and without antibiotics **c)** nodes present in all the cases solvent contaminants

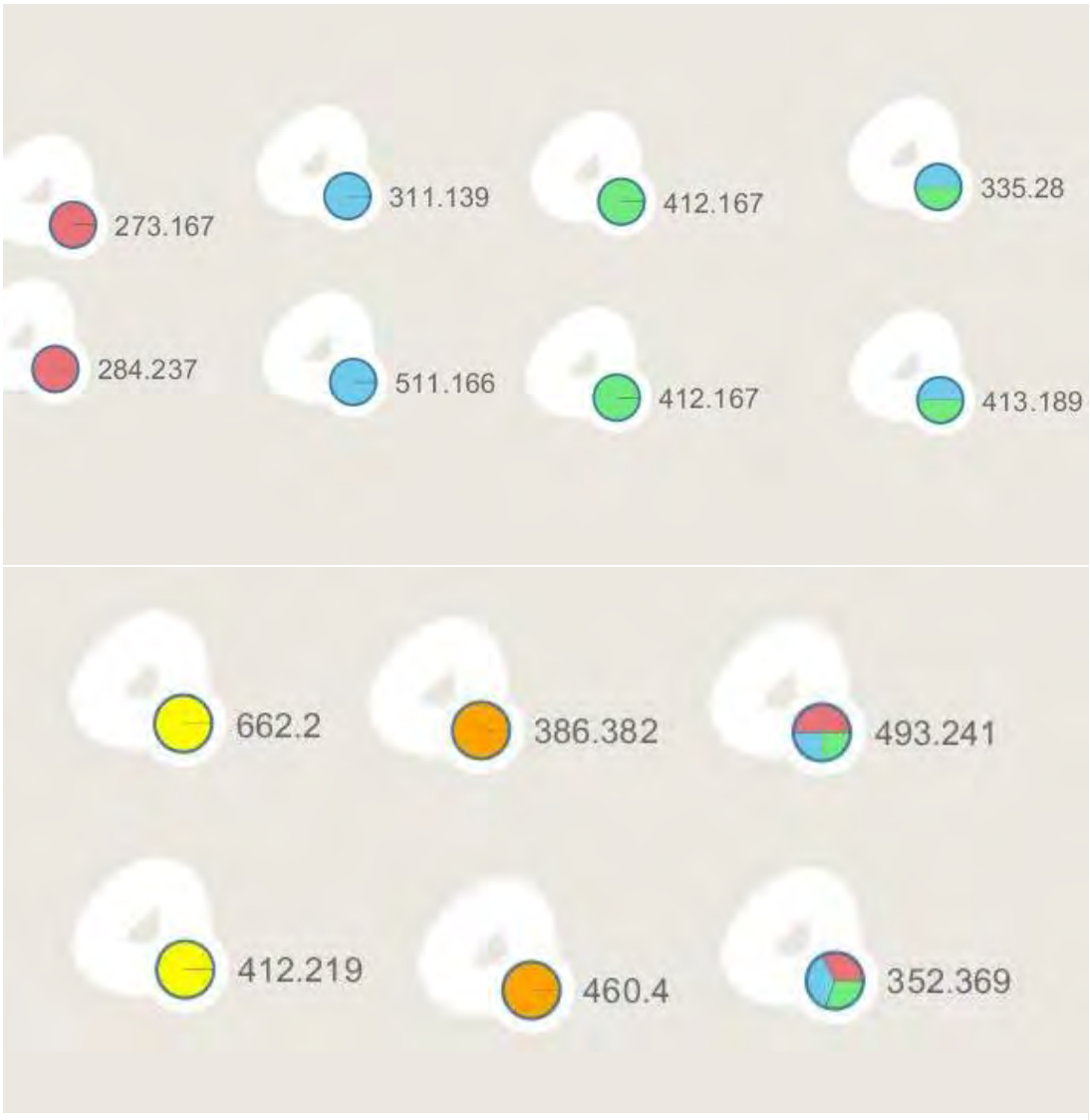


Figure 4.8 Some individual nodes in each case having no structural relevance with the rest of metabolites.

Table 4.1 Metabolites produced by *Epicoccum* sp. NFW1 without antibiotics and identified by spectral matches from databases using GNPs as dereplication strategy.

Adduct	**Compound name and database**	**Mass**	**∆PPM**
[M+H] +	[(2R,3R,4S,5R,6S)-6-[(3S,4S,5S,6R)-4,5-dihydroxy-6-(hydroxymethyl) oxan-3-yl] oxy-3,4,5-trihydroxyoxan-2-yl] methyl benzoate	432.239	2.52242
M+H	MLS000069620-01Nifuroxazide	274.217	1.30472
M+H	Lichenysin_A_38808_dereplictor_pv_8.81201e-29	1008.66	2.26249
M+H	Lichenysin-G5a_44121_dereplictor_pv_1.17877e-36	1022.67	2.42547
M+H	Pumilacidin_F_31271_dereplictor_pv_1.40196e-35	1050.71	2.63152
M+H	Surfactin	1036.69	3.20922
M+K	Surfactin	1074.65	2.37419
M+Na	Surfactin C14	1044.66	1.82077
M+Na	Surfactin C13	1030.64	2.50403
Unknown	Surfactin (Putative)	1072.69	1.82077

Table 4.2 Metabolites produced by *Epicoccum* sp. NFW1 in the presence of clarithromycin only, identified by spectral matches from databases using GNPs as dereplication strategy

Adduct	**Compound name and database**	**Mass**	**∆PPM**
M+Na	NCGC00380089-01_C22H25NO8_1-Oxa-7-azaspiro [4.4] non-2-ene-4,6-dione, 8-benzoyl-2-[(1S,2S,3Z)-1,2-dihydroxy-3-hexen-1-yl]-9-hydroxy-8-methoxy-3-methyl-, (5S,8S,9R)-	454.147	3.71772
M+Na	NCGC00381007-01_C21H23NO8_(5S,8S,9R)-8-Benzoyl-2-[(1S,2S,3Z)-1,2-dihydroxy-3-hexen-1-yl]-8,9-dihydroxy-3-methyl-1-oxa-7-azaspiro [4.4] non-2-ene-4,6-dione	440.132	3.85673
$M+H^{-}H_2O$	Spectral Match to cis, cis-9,12-Octadecadien-1-ol from NIST14	251.164	3.49374
M+H	Spectral Match to Dibutyl phthalate from NIST14	279.158	3.20922
M+H	Spectral Match to p-tert-Octylphenol tetraglycol ether from NIST14	383.279	3.85673
$[M-H]^{-}$	1-[2,4-dihydroxy-6-[(2S,3R,4S,5S,6R)-3,4,5-trihydroxy-6-(hydroxymethyl) oxan-2-yl] oxyphenyl]-3-(4-methoxyphenyl)propan-1-one	447.273	3.85673
$[M+NH_4]^{+}$	(E)-1-(2,4-dihydroxyphenyl)-3-[4-(3-methylbut-2-enoxy) phenyl] prop-2-en-1-one	341.222	4.65722
$[M+Na]^{+}$	2-(3,5-dihydroxy-4-methoxyphenyl)-5,7-dihydroxy-3-[(2S,3R,4R,5R,6S)-3,4,5-trihydroxy-6-methyloxan-2-yl] oxychromen-4-one	500.379	3.85673

$[M+K]^+$	(5S,8S)-8-benzoyl-2-[(Z,1S,2S)-1,2-dihydroxyhex-3-enyl]-9-hydroxy-8-methoxy-3-methyl-1-oxa-7-azaspiro[4.4]non-2-ene-4,6-dione	470.141	3.85673

Table 4.3 Metabolites produced by *Epicoccum* sp. NFW1 in the presence of moxifloxacin only, identified by spectral matches from databases using GNPs as dereplication strategy.

Adduct	**Compound name and database**	**Mass**	**ΔPPM**
[M+H]	ReSpect:PM014404 Cyclo(Tyr-Leu)	277.154	5.75364
$[M+H]^+$	Massbank: FFF00027 Coprostanone	388.166	5.34218
$[M-H]^-$	1,5,9-trihydroxy-5,7,7-trimethyl-4,5a,6,8,8a,9-hexahydro-1H-azuleno[5,6-c] furan-3-one	279.232	5.91364
M+H	Spectral Match to Tri-2-ethylhexyl trimellitate from NIST14	547.397	4.7816
M+H	Tetrodotoxin	321.088	5.30043
M+H	"MLS001049080-01!2-[6-(cyclohexylamino) purin-9-yl]-5-(hydroxymethyl) oxolane-3,4-diol"	352.162	5.35899
M+H	Spectral Match to 13-Keto-9Z,11E-octadecadienoic acid from NIST14	295.227	5.85977
$M+H^-H_2O$	Spectral Match to 9-Oxo-10E,12Z-octadecadienoic acid from NIST14	277.216	5.21507
$M+H^-H_2O$	Spectral Match to 13S-Hydroxy-9Z,11E,15Z-octadecatrienoic acid from NIST14	277.216	5.6433

Table 4.4 Metabolites produced by *Epicoccum* sp. NFW1 with and without antibiotics clarithromycin and moxifloxacin, identified by spectral matches from databases using GNPs as dereplication strategy.

Adduct	**Compound name and database**	**Mass**	**∆PPM**
$[M+H]^+$	Massbank: EA285513 Dronedarone|N-[2-butyl-3-[4-[3-(dibutylamino) propoxy] benzoyl]-1-benzofuran-5-yl]methanesulfonamide	558.421	3.10922
M+H	Spectral Match to Palmitamide from NIST14	256.264	3.39374
M+H	Spectral Match to Cyclohexasiloxane, dodecamethyl- from NIST14	889.255	2.70403
M+H	Spectral Match to Cyclohexasiloxane, dodecamethyl- from NIST14	445.12	2.92242
$[M+Na]^+$	Dehydroxynocardamine	608.384	2.33152
$[M+K]^+$	[5-hydroxy-3-(hydroxymethyl)-2-oxo-6-propan-2-ylcyclohex-3-en-1-yl] 3-methylpentanoate	338.468	4.78816
[M+H]	ReSpect:PM014404 Cyclo (Tyr-Leu)	277.151	5.90043
[M+H]	ReSpect:PM014406 Cyclo (Phe-Tyr)	311.139	1.92077
$[M+H]^+$	Cyclo (tyrosyl-prolyl)	261.124	1.72077
M+H	Spectral Match to Diisodecyl phthalate from NIST14	447.347	2.36249

Table 4.5 Metabolites produced by *Epicoccum* sp. NFW1 in the presence of both clarithromycin and moxifloxacin, identified by spectral matches from databases using GNPs as dereplication strategy.

Adduct	**Compound Name and database**	**Mass**	**∆PPM**
M+H	Spectral Match to 9-Octadecenamide, (Z)- from NIST14	280.689	3.19022
M+H	Spectral Match to Cyclopentasiloxane, decamethyl- from NIST14	371.101	3.33974
M+Na	Spectral Match to p-tert-Octylphenol pentaglycol ether from NIST14	449.287	2.09403
$[M+H]^+$	Cyclo (tyrosyl-prolyl)	261.124	2.93542
$[M+K]^+$	Lagochilin	396.332	2.34752
$[M+K]^+$	Astaxanthin	634.453	4.81716
$[M+H]^+$	Asiatic Acid	488.358	5.93443
M+H	Cyclo (L-Trp-L-Pro)	284.139	5.33152
M+H	p-tert-Octylphenol heptaglycol ether	1033.02	1.14218

4.3.2 Metabolic Profile of *Chaetomium sp*. NFW8 in SDB with and without Antibiotics

The metabolic profile of the fungal isolate grown with and without antibiotics were analysed initially by comparing LCMS chromatograms followed by PCA analysis. For mre comprehensive analysis and dereplication studies GNPS were performed.

4.3.2.1. LCMS chromatograms

Endophytic *Chaetomium* sp. NFW8 was cultivated on SDB medium with and without antibiotics and the metabolites produced were analyzed. LC-MS data shows different metabolic profile of the ethyl acetate extracts under different media conditions. The compounds eluted from 5 minute till 50 minutes suggest a wide range of polarities of these compounds. The differences manifest the robust influence of addition of antibiotics to medium on the metabolome of the *Chaetomium* sp. NFW8. Difference in retention time and intensities of peaks in the chromatogram among the three different conditions suggest the metabolic diversity of the extracts (Figure 4.9).

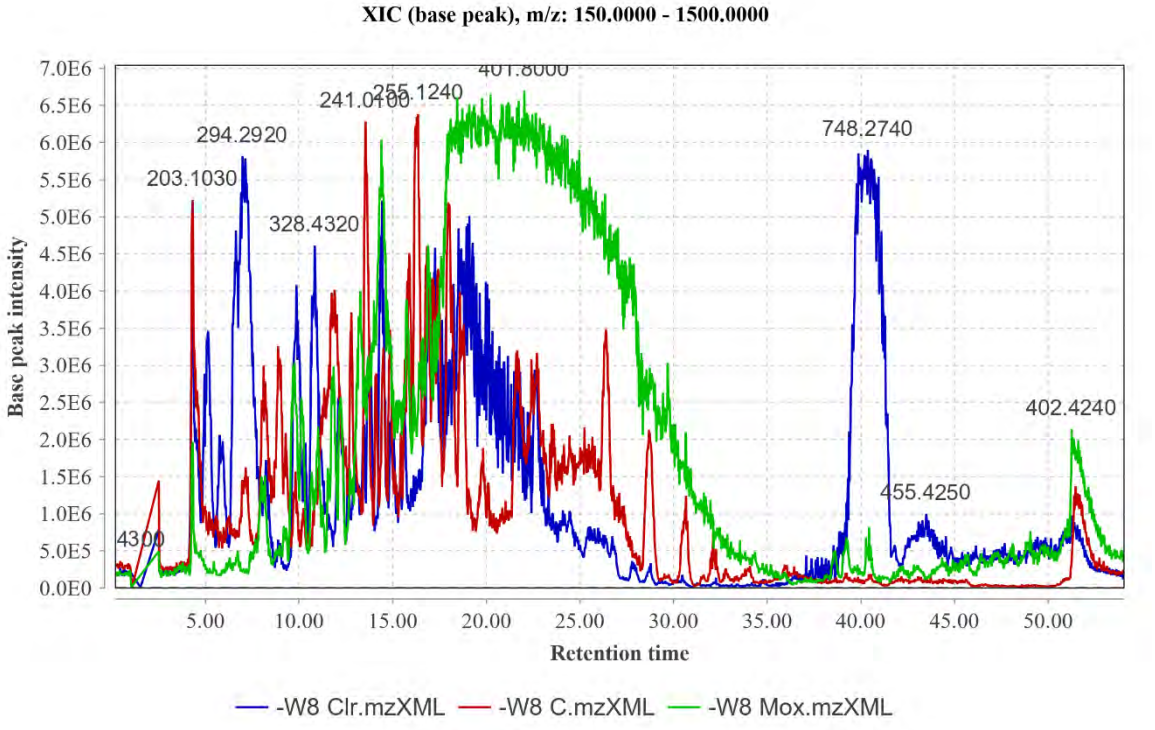


Figure 4.9 Comparison of the total ion chromatograms of the crude ethyl extracts of *Chaetomium* sp. NFW8 cultured on SDB medium. (Red = without antibiotic addition to medium, Blue Clarithromycin supplementation to medium, Green Moxifloxacin supplementation to medium

4.3.2.2 PCA Analysis

PCA score scatter plot clustered the fractions into different groups based on underlying metabolic diversity. *Chaetomium* sp. NFW8 grown on SDB medium with antibiotics have unique metabolic profile and appeared as an outlier of different quadrant in score scatter plot (Figure 4.10). To visualize the major differentiation among the samples, S-plot was generated. S-plots (OPLS-DA) of the fungal isolate advocate that there are numerous metabolites which were produced only when the culture medium was supplemented with antibiotics (Figure 4.11).

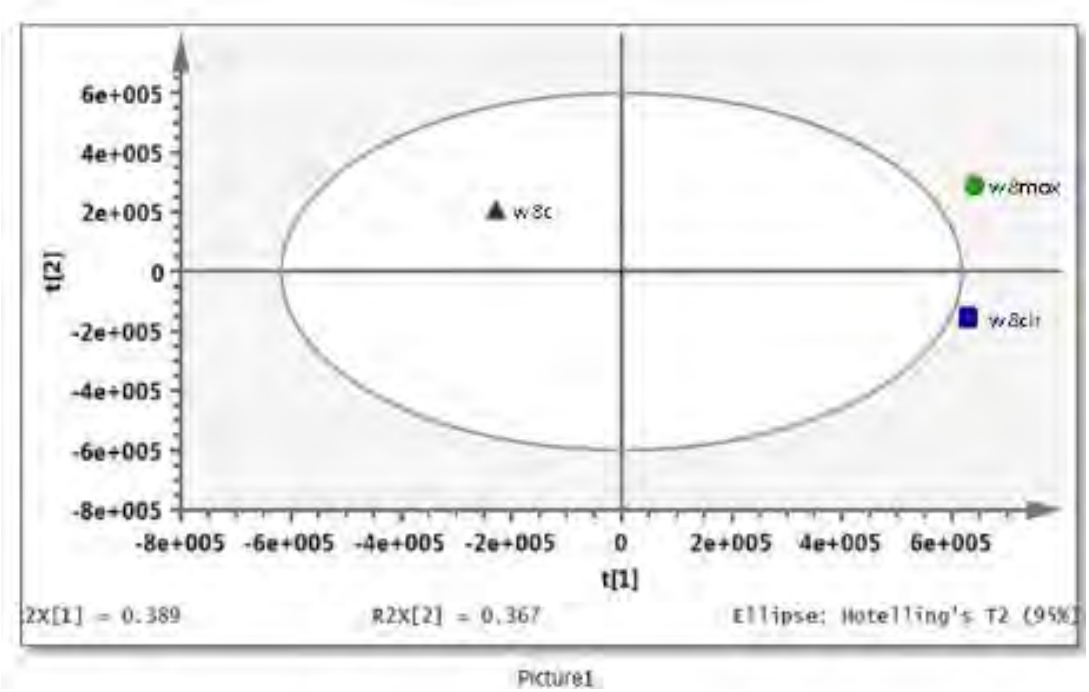


Figure 4.10 PCA score scatter plot for crude ethyl acetate extracts of *Chaetomium* sp. NFW8 (W8c=grown without antibiotic addition to culture media, W8clr= grown with the addition of clarithromycin and w1mox grown with the addition of moxifloxacin

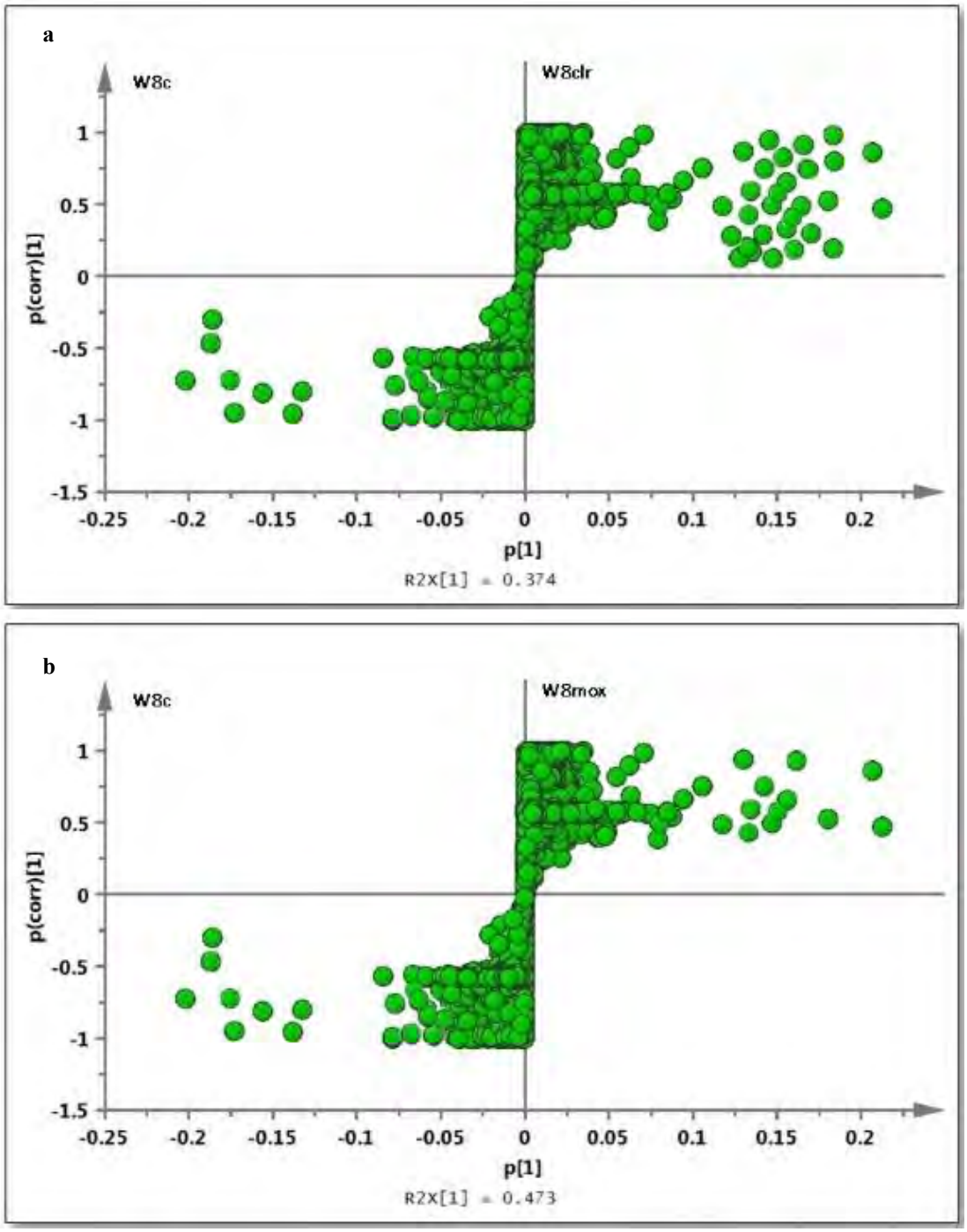


Figure 4.11 OPLS-DA S-plot for crude ethyl acetate extracts of *Chaetomium* sp. NFW8. a) S-plot showing distribution of the metabolites in the fungal isolate W8c = grown without antibiotic W8clr=grown with clarithromycin, b) W8mox= grown without moxifloxacin

4.3.2.3 Global Natural Product Social Molecular Networking GNPS to Differentiate and Dereplicate the Metabolome of *Chaetomium* sp. NFW8

The endophytic fungal isolate *Chaetomium* sp. NFW8 when analyzed for metabolic profiling a total of 2314 hits were detected. The molecular networking revealed that several metabolites were produced while several were up pressed due to the presence of antibiotic in the medium. Figure (4.12) showed the overall hits present in *Chaetomium* sp. NFW8. A targeted area where majority of nodes with all the colours were present which were produced under various condition by the fungi are shown in (Figure 4.13).

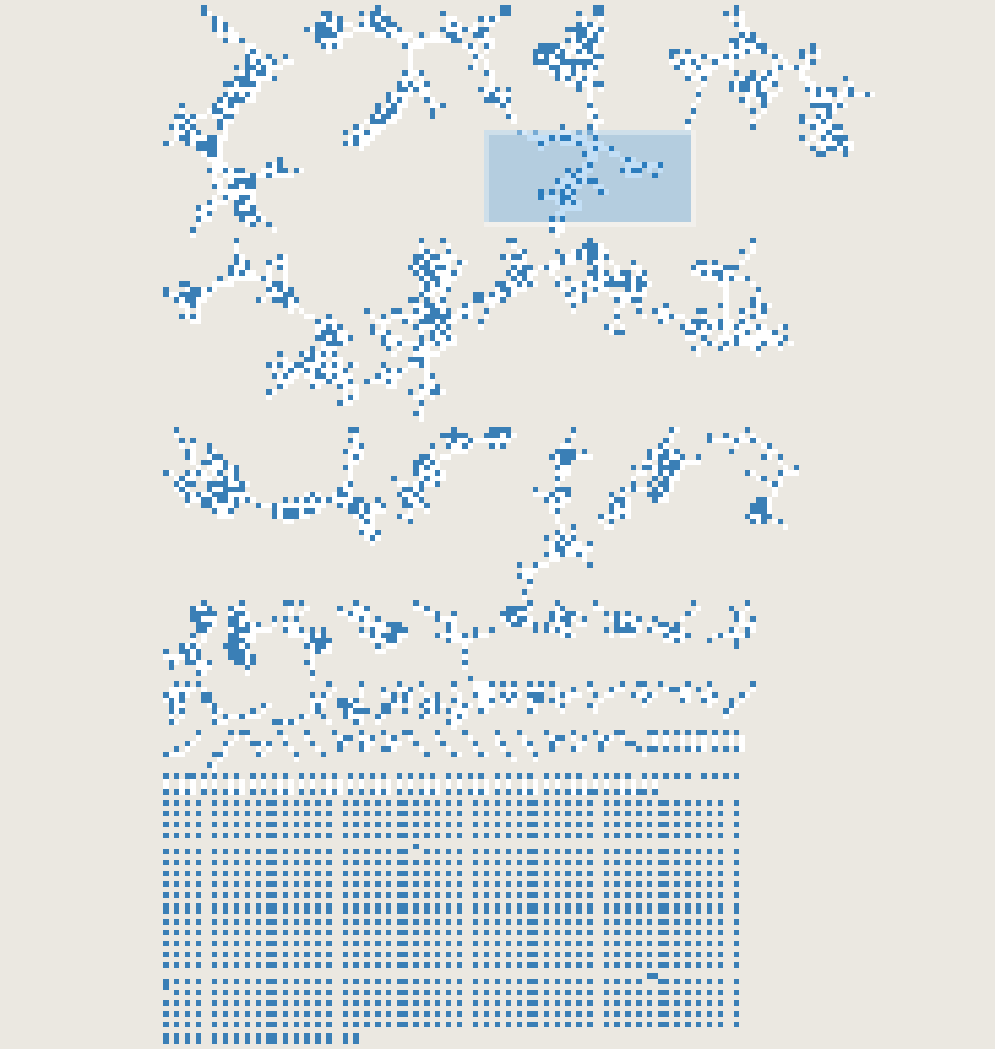


Figure 4.12 Over all view of GNPS showing metabolites with and without antibiotic by *Chaetomium* sp. NFW8

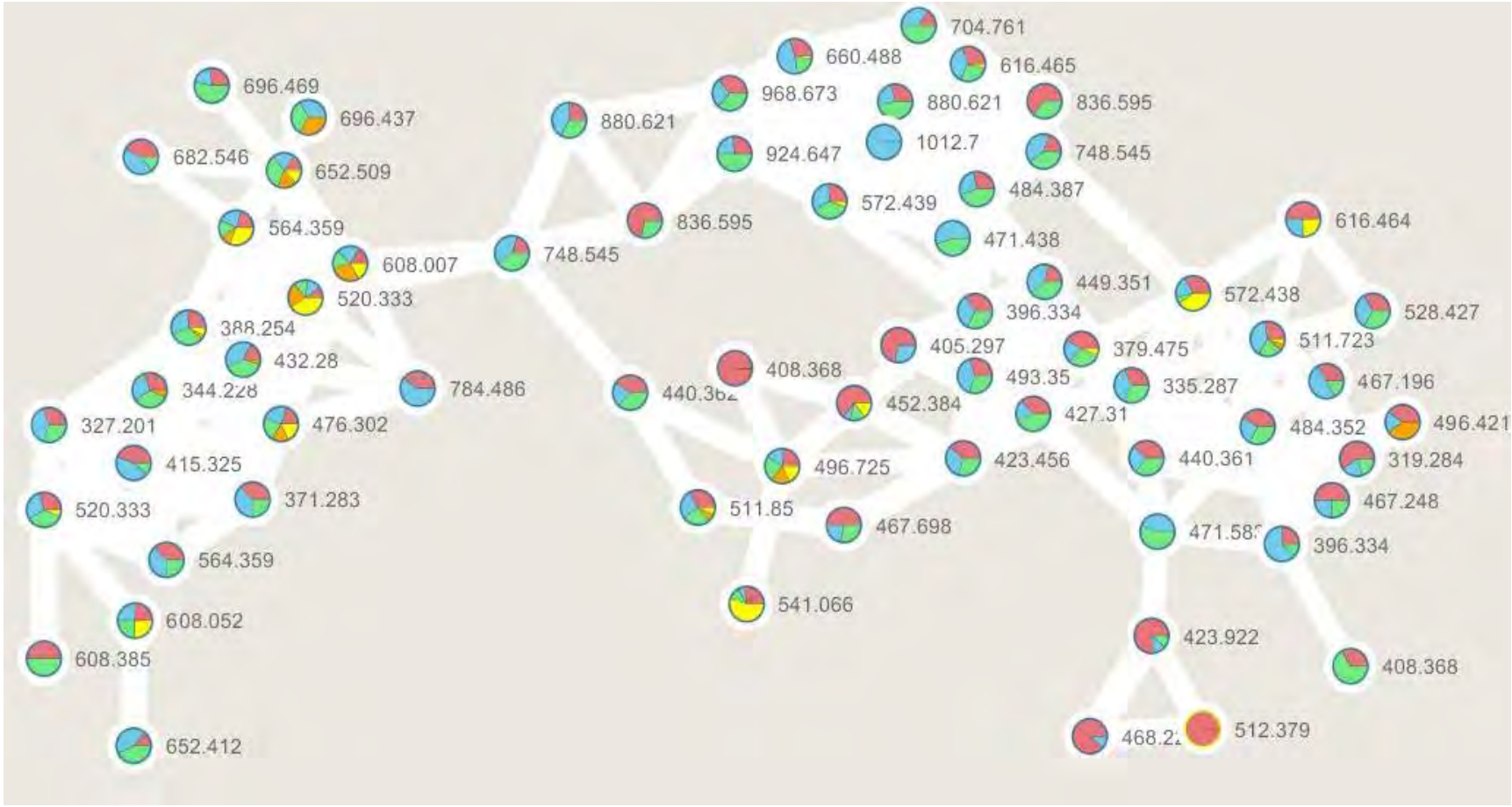


Figure 4.13 View of targeted area showing nodes of different colours representing the metabolites produced under different conditions by *Chaetomium* sp. NFW8

Red = without antibiotic: **Blue** = with clarithromycin: **Green** = with moxifloxacin: **Orange** = clarithromycin or transformed clarithromycin: **Yellow** = Moxifloxacin or transformed moxifloxacin

Chaetomium sp. NFW8 produced various metabolites which were not present when the fungus was grown on medium containing antibiotics. A total of 110 such metabolites were observed by molecular networking. A cluster of such metabolites as red nodes with respective masses shown in (Figure 4.14) while the well-known metabolites produced by *Chaetomium* sp. NFW8. are shown in (Table 4.6) by using GNPS as dereplication strategy

Clarithromycin induced metabolites produce by *Chaetomium* sp. NFW8 are shown as blue nodes in molecular network. A total of 155 blue nodes (Figure 4.15) were present in the molecular network produce by *Chaetomium* sp. NFW8 when grown in the presence of clarithromycin. They were also dereplicated to find out the known ones (Table 4.7). Figure (4.10) showed a cluster of those metabolites which were only produced in the presence of clarithromycin by *Chaetomium* sp. NFW8.

When the media was emended with moxifloxacin the *Chaetomium* sp. NFW8 produced several metabolites which were only present when there was moxifloxacin in the medium. There are 171 such hits in molecular network of the fungal isolate. Figure (4.16a) represent one of the clusters containing these metabolites shown as green nodes with their mass values. They were also dereplicated by using GNPS as dereplication strategy and the well-known metabolites are shown in (Table 4.8).

Several metabolites were produced in the presence of both antibiotics as well as they were also produced without the presence of antibiotic in the medium by *Chaetomium* sp. NFW8. These metabolites are not affected by the presence or absence of antibiotics and a total of 55 such nodes (single node with red, blue, and green colour) were present in molecular network. Figure (4.16b) represent one of the clusters containing these metabolites and Table (4.9) shows some of the known compounds among these metabolites by dereplication studies.

There were numerous metabolites which were induced by both antibiotics, and they were shown by double colour nodes i.e., blue, and green colour node in molecular networking. The known metabolites among them are enlisted in (Table 4.10).

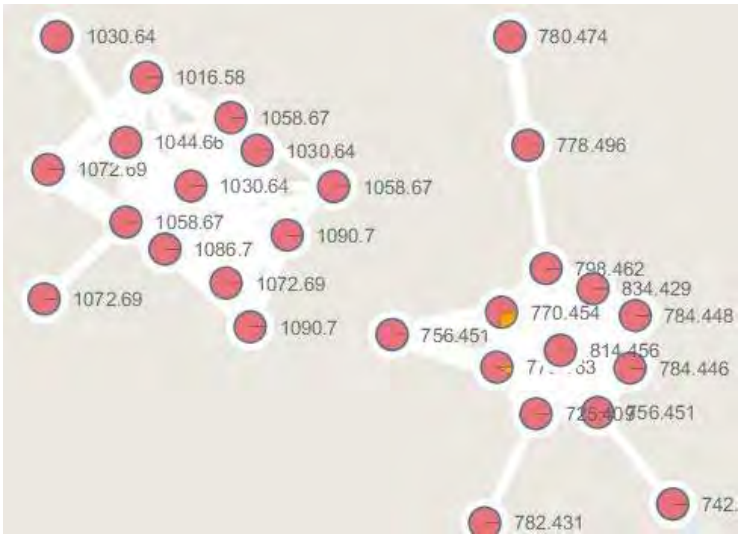


Figure 4.14 Metabolites produced by *Chaetomium* sp. NFW8, node in red colour without the presence of antibiotic in SDB

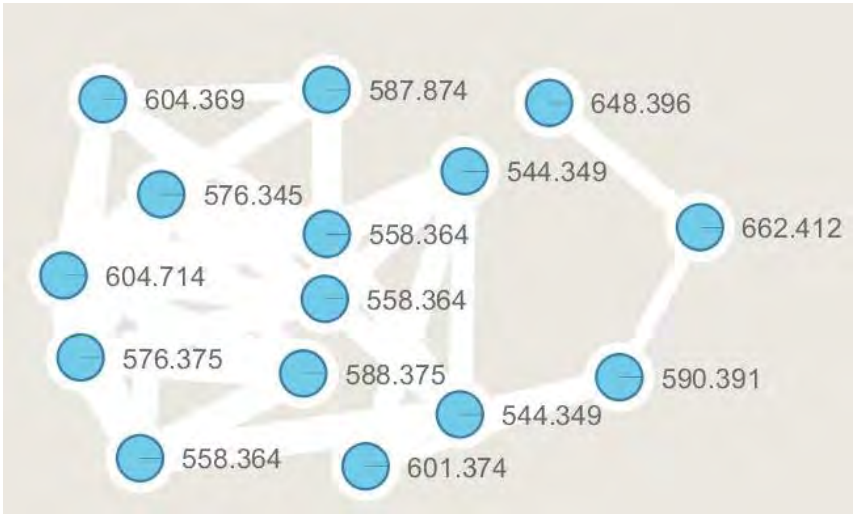


Figure 4.15 Metabolites produced by *Chaetomium* sp. NFW8 node in blue colour in the presence of clarithromycin in SDB

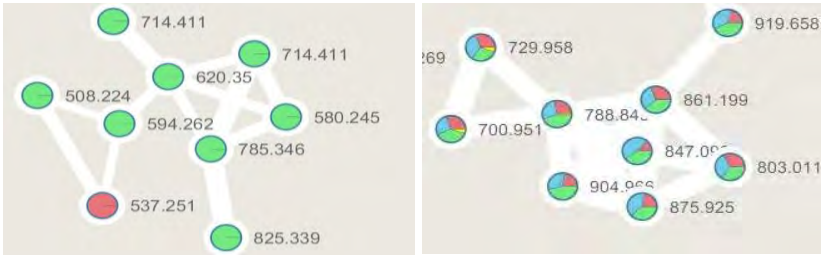


Figure 4.16 a) Metabolites produced by *Chaetomium* sp. NFW8, nodes in green colour indicate the metabolites produced in the presence of moxifloxacin in SDB **b)** metabolites produced under the stress of both antibiotics and without the presence of any antibiotic in medium.

Table 4.6 *Chaetomium* sp. NFW8 producing metabolites without antibiotics identified by spectral matches from databases using GNPs as dereplication strategy

Adduct	**Compound name and database**	**Mass**	**ΔPPM**
$[M-H]^-$	7-hydroxy-2-[4-[(2S,3R,4S,5S,6R)-3,4,5-trihydroxy-6-(hydroxymethyl) oxan-2-yl] oxyphenyl]-2,3-dihydrochromen-4-one	415.212	2.62136
$[M+H^+$	Asiatic Acid	488.359	5.66061
$[M+H^+$	Chaulmoogric Acid	280.276	2.73116
$[M+H^+$	Cyclo (tyrosyl-prolyl)	261.124	2.74367
$[M-H]^-$	Dehydrovariabilin	281.187	2.73127
M-H	Diploschistesic acid	331.165	3.85673
M-H	Diploschistesic acid	331.165	2.97117
$[M+N]^+$	Galanthamine hydrobromide	388.254	2.73316
M+H	Lichenysin_A_38808_dereplictor_pv_8.81201e-29	1008.66	2.83218
M+H	Lichenysin-G5a_44121_dereplictor_pv_1.17877e-36	1022.67	2.73116
$[M+H]^+$	Massbank: EA023210 Norfloxacin|1-ethyl-6-fluoro-4-oxo-7-piperazin-1-yl-1,4-dihydroquinoline-3-carboxylic acid	321.088	4.76333
M+H	MLS000830274-01! Ethmozine	426.306	2.73116

M+H	NCGC00380229-01_$C_{14}H_{15}N_3O_2$_2,5-Piperazinedione, 3-(1H-indol-3-methyl)-6-methyl-	256.133	2.73419
M+H	NCGC00381276-01_C22H34O14_Hexitol, 2,6-anhydro-, 5-[4-(beta-D-glucopyranosyloxy)-2-methylenebutanoate] 1-(4-hydroxy-2-methylenebutanoate)	524.416	4.61008
[M+N^+	quercetin 3-O-glucuronide	500.38	2.74234
[M+H]	ReSpect:PM014405 Cyclo (Tyr-Ile)	277.145	1.84489
[M+H]	ReSpect:PM014406 Cyclo(Phe-Tyr)	311.139	4.58382
[M+H^+	Sodium Tetradecyl Sulfate	316.321	5.66433
M+H	Spectral Match to Erythromycin from NIST14	734.439	2.93816
M+H	Spectral Match to Hexapropylene glycol from NIST14	367.269	2.65898
M+Na	Surfactin	1030.64	2.50403
M+H	Surfactin-D_40519_dereplictor_pv_9.12507e-42	1050.7	5.81705

Table 4.7 Metabolites produced by *Chaetomium* sp. NFW8 in the presence of clarithromycin only, identified by spectral matches from databases using GNPs as dereplication strategy

Adduct	**Compound name and database**	**Mass**	**ΔPPM**
$[M+H]^+$	Sarmentoside B	663.454	1.06652
$[M+NH_4]^+$	ACARBOSE	663.454	1.19871
$[M+H]^+$	Sarmentoside B	663.453	1.49131
M+H	Spectral Match to Erythromycin from NIST14	734.467	1.49131
M+H	MLS000877030-01! Nobiletin 478-01-3	403.138	2.68801

Table 4.8 Metabolites produced by *Chaetomium* sp. NFW8 in the presence of moxifloxacin only, identified by spectral matches from databases using GNPs as dereplication strategy**.**

Adduct	**Compound name and database**	**Mass**	**∆PPM**
M+H	Cyclo (Phe-4-hydroxy-Pro)	261.131	5.01694
[M+H]	Oxaprozin	293.094	1.06652
M+H	Spectral Match to 9-Octadecenamide, (Z)- from NIST14	282.279	1.40636
M+H	Moxifloxacin HCl	402.182	1.09131
[M+H]	Flunixin meglumin	296.073	1.49131
M+H	val-leu-pro-val-pro	652.407	2.00266
M+H	Spectral Match to Dioctyl phthalate from NIST14	391.284	1.18476
$[M+K]^+$	4-[5-(3,4-dimethoxyphenyl)-3,4-dimethyloxolan-2-yl] phenol	365.136	5.14191
$[M+H]^+$	Massbank: Cholesterol	387.193	1.46347

Table 4.9 Metabolites produced by *Chaetomium* sp. NFW8 without antibiotic as well as in the presence of both clarithromycin and moxifloxacin, identified by spectral matches from databases using GNPs as dereplication strategy.

Adduct	Compound name and database	Mass	ΔPPM
$[M+H]^+$	MassbankEU:SM876702 Lauryl diethanolamide|N,N-bis(2-hydroxyethyl)dodecanamide	288.269	1.22491
$[M+H]^+$	salicin	288.266	3.57984
[M+H]	Tripelennamine HCl	256.951	2.59312
$[M+N]^+$	2-(3,5-dihydroxy-4-methoxyphenyl)-5,7-dihydroxy-3-[(2S,3R,4R,5R,6S)-3,4,5-trihydroxy-6-methyloxan-2-yl] oxychromen-4-one	500.38	1.01961
$[M+N]^+$	D-Ehydroxynocardamine	608.189	4.94363
$[M+N]^+$	3-[(Z)-heptadec-10-enyl] benzene-1,2-diol	367.368	2.84363
$[M+K]^+$	(2Z)-4,6-dihydroxy-2-[(4-hydroxy-3,5-dimethoxyphenyl) methylidene]-1-benzofuran-3-one	367.368	5.44697
$[M+N]^+$	2-Chloro-1,3,8-trihydroxy-6-(hydroxymethyl) anthracene-9,10-dione	343.296	3.487001
$[M+N]^+$	2-Chloro-1,3,8-trihydroxy-6-(hydroxymethyl) anthracene-9,10-dione	341.336	1.54513
$[M+H]^+$	Sarmentoside B	663.453	3.66626

Table 4.10 Metabolites produced by *Chaetomium* sp. NFW8 in the presence of both clarithromycin and moxifloxacin, identified by spectral matches from databases using GNPs as dereplication strategy

Adduct	**Compound name and database**	**Mass**	**ΔPPM**
[M-H]-	2-[1-hydroxy-1-(4-methoxyphenyl) propan-2-yl] oxy-6-(hydroxymethyl) oxane-3,4,5-triol	341.353	3.28779
M+H	Spectral Match to Tris(2-butoxyethyl) phosphate from NIST14	399.278	1.29113
$[M+NH_4]^+$	ACARBOSE	663.108	5.36489
M+H	MLS000877030-01! Nobiletin478-01-3	403.138	2.43684
$[M+Na]^+$	3,7-Epoxycaryophyllan-6-Ol	262.216	5.43684
$[M+H]^+$	Massbank: Cholesterol	387.193	6.44308

4.3.3 Metabolic Profile of *Fusarium oxysporum* NFW16 in SDB with and without Antibiotics

The metabolic profile of the fungal isolate grown with and without antibiotics were analysed initially by comparing LCMS chromatograms followed by PCA analysis. For mre comprehensive analysis and dereplication studies GNPS were performed.

4.3.3.1 LCMS Chromatograms

Ethyl acetate extracts of endophytic fungal isolate *Fusarium oxysporum* NFW16 grown with and without antibiotics showed different metabolic profile when analyzed by comparing of the LC-MS spectra indicates major variation in the metabolome of different extracts. Peaks at different retention time with different intensities and m/z value in chromatogram of each sample were shown with different colours, advocate the diverse metabolome of each extract (Figure 4.17).

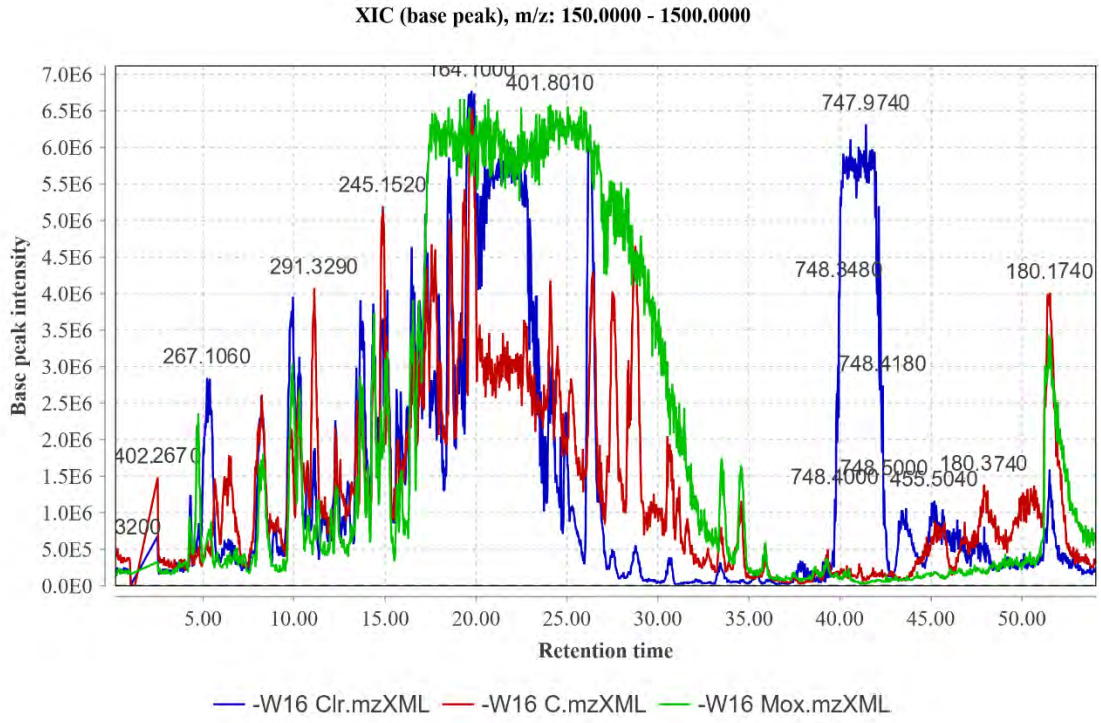


Figure 4.17 Comparison of the total ion chromatograms of the crude ethyl extracts of *Fusarium oxysporum* NFW16 cultured on SDB medium. (Red = without antibiotic addition to medium, Blue= Clarithromycin supplementation to medium, Green =Moxifloxacin supplementation to medium

4.3.3.2 PCA Analysis

The processed dataset was subjected to unsupervised principal component analysis (PCA) to visualize clustering trends among the extracts obtained. The score scatter plot shows that the extracts fo *Fusarium oxysporum* NFW16 grown in SDB medium with and without antibiotics show variations and positioned away from each other (Figure 4.18). The S-plot (OPLS-DA) suggest that the presence of antibiotics in the medium induced the production of certain metabolites which were not produced otherwise as they were clustered in different quadrant (Figure 4.19).

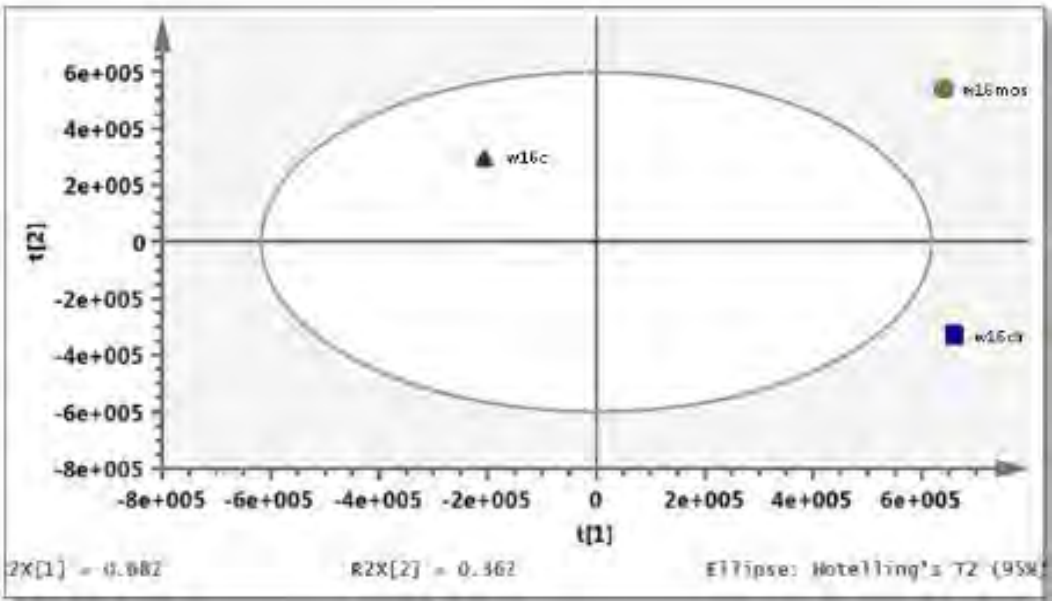


Figure 4.18 PCA score scatter plot for crude ethyl acetate extracts of *Fusarium oxysporum* NFW16 (W16c=grown without antibiotic addition to culture media, W16clr= grown with the addition of clarithromycin and w16mox= grown with the addition of moxifloxacin

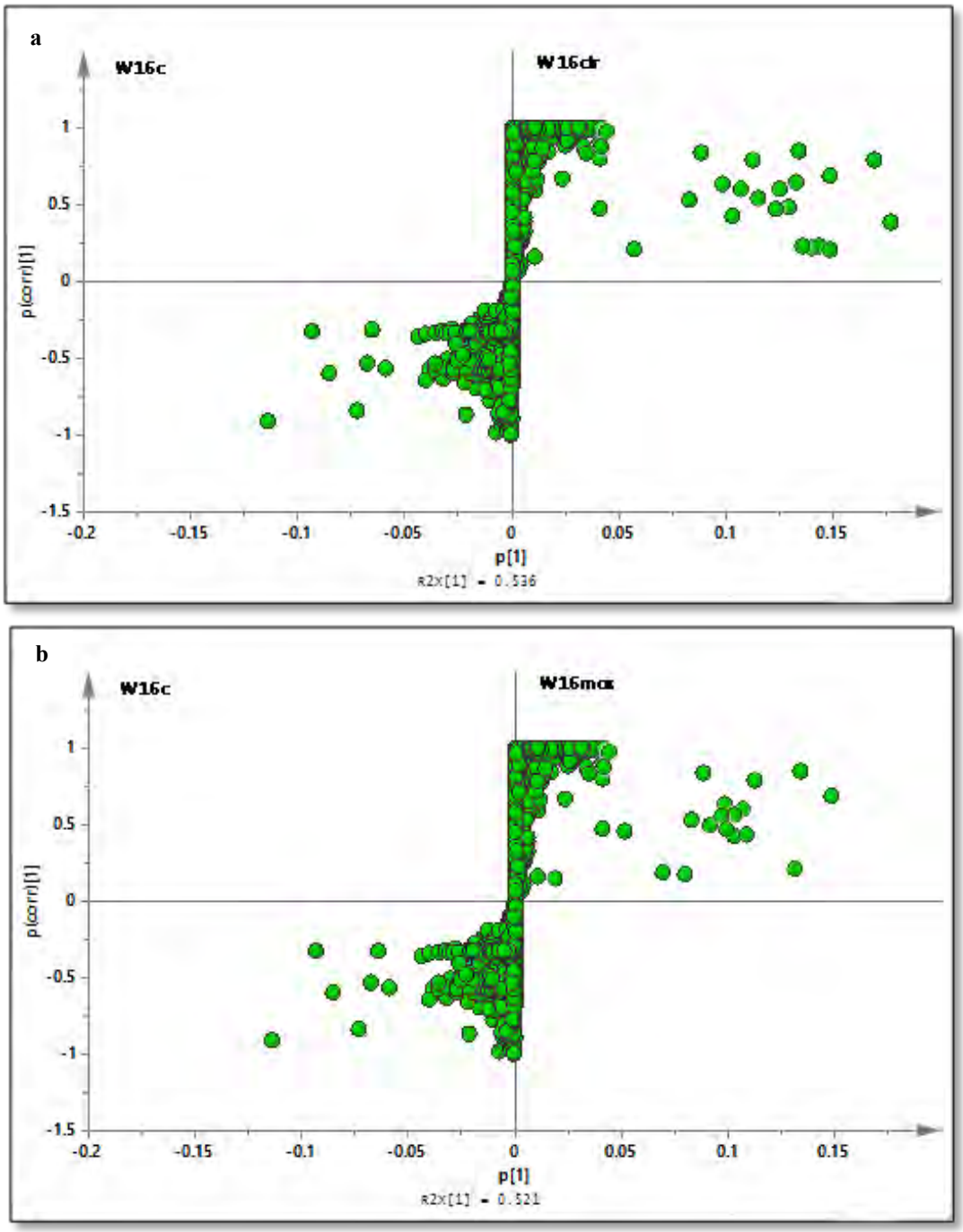


Figure 4.19 OPLS-DA S-plot for crude ethyl acetate extracts of *Fusarium oxysporum* NFW16. S-plot showing distribution of the metabolites in the fungal isolate **a)** W16c =grown without antibiotics w16clr=grown with clarithromycin **b)** w16mox=grown with moxifloxacin

4.3.3.3 Global Natural Product Social Molecular Networking GNPS to Differentiate and Dereplicate the Metabolome of *Fusarium oxysporum* NFW16

The endophytic fungi *Fusarium oxysporum*. NFW16 metabolic profile showed that a total of 2315 metabolites were found when analyzed and dereplicated by using GNPs (Figure 4.20).

Figure 4.20 Over all view of GNPS showing metabolites with and without antibiotic by *Fusarium oxysporum*. NFW16

The fungal isolate *Fusarium oxysporum* NFW16 produced 131 specified metabolites without the presence of antibiotic in the medium. These metabolites were not produced by the fungal isolates when the medium supplementesd with clarithromycin and moxifloxacin. A single cluster among several such clusters were presented in (Figure 4.21a) in which red nodes labelled with their masses showing these metabolites. The known metabolites among them are summarized in (Table 4.11).

When the clarithromycin was added to the medium it induced the production of numerous metabolites by *Fusarium oxysporum* NFW16 which were not produced otherwise. A total of 264 such metabolites were present in molecular network which were induced by clarithromycin. These metabolites were shown as blue colour nodes in molecular network. A cluster of such nodes is shown in (Figure 4.21b). Dereplication of the clarithromycin induce metabolites suggest the presenc of unknown, along with few already known metabolites which were enlisted in (Table 4.12).

When moxifloxacin is added to medium it induced the production of 281 metabolites from *Fusarium oxysporum* NFW16 grown in SDB. These metabolites wer not produced in the absence of moxifloacin in the medium. These metabolites shown as blue nodes in molecular network and a cluster of these nodes is presented (Figure 4.22). These 281 metabolites when dereplicated there were several known and unknown metabolites, some of the well-known metabolites are listed in (Table 4.13).

The metabolic profile of *Fusarium oxysporum* NFW16 showed that there are some metabolites which were produced with and without the presence of antibiotics. They were shown as a tri colour node (red, blue, green) in molecular network and a total of 307 such nodes were present in the molecular network. Figure 4.23 showed a cluster containing a small number of these metabolites with their respective m/z values. Among these 307 metabolites. the already known metabolites were presented in (Table 4.14) while (Table 4.15) represents the known metabolites which were produced by the fungal isolate under the presence of both antibiotics. Various metabolites having no structural similarities with other metabolites are shown as individual nodes in molecular network (Figure 4.24).

Figure 4.21 Metabolites produced by *Fusarium oxysporum* NFW16, **a)** nodes in red colour indicate metabolites without the presence of antibiotic in SDB **b)** nodes in blue colour indicate metabolites produced in the presence of clarithromycin in SDB

Figure 4.22 Metabolites produced by *Fusarium oxysporum* NFW16 node in blue colour in the presence of moxifloxacin in SDB

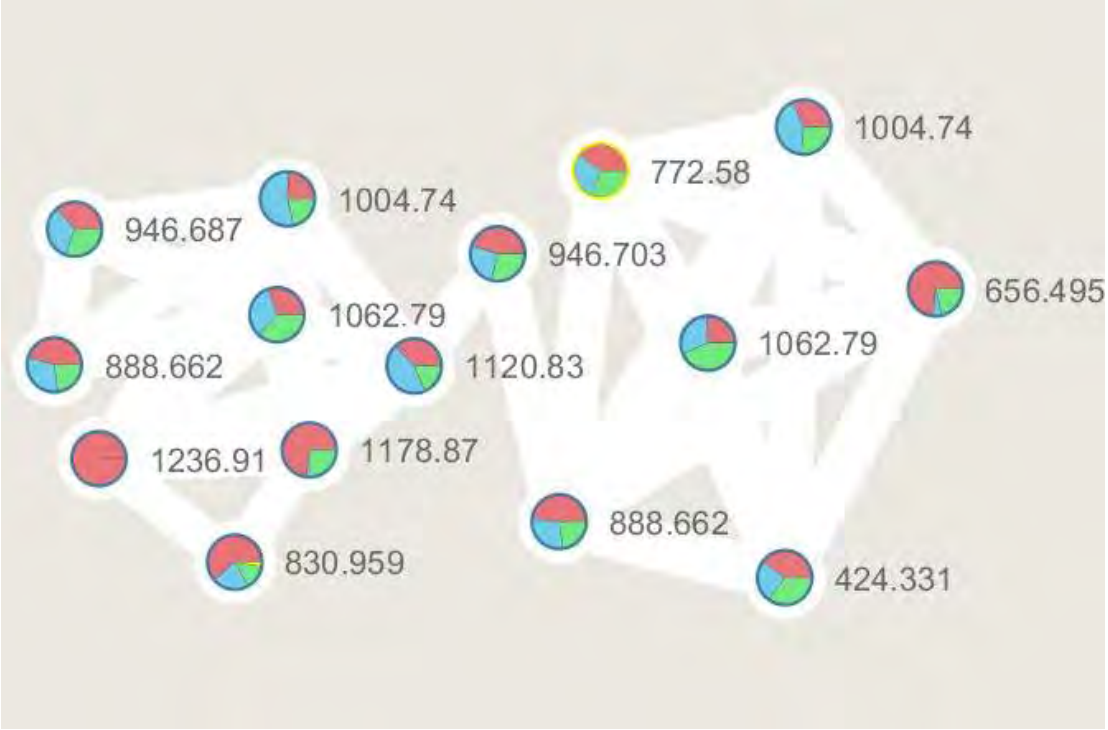


Figure 4.23 Metabolites produced by *Fusarium oxysporum* NFW16 under the stress of both antibiotics and without the presence of any antibiotic in medium

Figure 4.24 Some individual nodes in each case having no structural relevance with the rest of metabolites produced by *Fusarium oxysporum* NFW16

Table 4.11 Metabolites produced by *Fusarium oxysporum* NFW16 without antibiotics identified by spectral matches from databases using GNPs as dereplication strategy

Adduct	**Compound name and database**	**Mass**	**ΔPPM**
[M-H]-	Trehalose	339.238	3.30785
$[M+Na]^+$	CHOLESTEROL	411.289	2.74234
M+H	Smenospongiarine	411.289	3.09708
M+H	MLS001076677-01! Moban	278.196	4.7816
[M+H]	ANASTROZOLE	295.181	1.28671
$[M-H]^-$	3-Deacetylkhivorin	544.405	1.30472
$[M+Na]^+$	(1S,2R,4aS,6aR,6bR,10S,12aR,14bS)-1,2,6b,9,9,12a-hexamethyl-10-[(2R,3R,4S,5S,6R)-3,4,5-trihydroxy-6-methyloxan-2-yl]oxy-2,3,4,5,6,6a,7,8,8a,10,11,12,13,14b-tetradecahydro-1H-picene-4a,6a-dicarboxylic acid	656.226	3.6795
M+H	Spectral Match to Tri-2-ethylhexyl trimellitate from NIST14	547.399	3.30785

Table 4.12 Metabolites produced by *Fusarium oxysporum* NFW16 in the presence of Clarithromycin only, identified by spectral matches from databases using GNPs as dereplication strategy.

Adduct	**Compound name and database**	**Mass**	**ΔPPM**
M+H	Spectral Match to Bestatin from NIST14	311.139	3.49374
M+H	"MLS001159079-01! N-[2-(1H-indol-3-yl) ethyl]-2-(3,4,8-trimethyl-2-oxochromen-7-yl) oxyacetamide"	405.229	3.6795
$[M+H]^+$	MoNA:645294 Elimite (TN)	389.269	3.94048
$[M+NH_4]+$	Chaulmoogric Acid	297.242	3.99571
M+H	NCGC00384716-01!3-oxo-3-[[(2R,3S,4S,5R,6S)-3,4,5-trihydroxy-6-[3-(4-methoxyphenyl)-4-oxochromen-7-yl]oxyoxan-2-yl]methoxy]propanoic acid	515.196	4.7816
M+H	Spectral Match to 9-Oxo-10E,12Z-octadecadienoic acid from NIST14	295.227	6.15689
[M+H]	Nadifloxacin	359.221	4.6433
$[M+Na]^+$	2-(3,5-dihydroxy-4-methoxyphenyl)-5,7-dihydroxy-3-[(2S,3R,4R,5R,6S)-3,4,5-trihydroxy-6-methyloxan-2-yl] oxychromen-4-one	500.38	2.85977
$[M+Na]^+$	2-(7-oxo-2,3-dihydrofuro[3,2-g] chromen-2-yl) propan-2-yl 3-methylbut-2-enoate	352.321	3.09708
M+H	Spectral Match to Decaethylene glycol from NIST14	918.165	4.7816
[M-H]-	Trehalose	339.238	1.28671

Table 4.13 Metabolites produced by *Fusarium oxysporum* NFW16 in the presence of moxifloxacin only, identified by spectral matches from databases using GNPs as dereplication strategy.

Adduct	**Compound name and database**	**Mass**	**∆PPM**
$[M+H]^+$	MassbankEU:SM859002 Acetyl-sulfamethoxazole|N-[4-[(5-methyl-1,2-oxazol-3-yl) sulfamoyl] phenyl] acetamide	325.172	3.19237
$[M+Na]^+$	dehydroxynocardamine	608.385	4.27131
$[M+NH_4]^+$	5-[(E)-2-(3,5-dihydroxyphenyl) ethenyl]-2-methoxybenzene-1,3-diol	291.253	4.32548
$[M+NH_4]^+$	ACARBOSE	663.454	2.80022
$[M+H]^+$	MoNA:832715 Iodosulfuron-methyl	506.53	2.80022
$[M-H]^-$	7-hydroxy-2-[4-[(2S,3R,4S,5S,6R)-3,4,5-trihydroxy-6-(hydroxymethyl) oxan-2-yl] oxyphenyl]-2,3-dihydrochromen-4-one	415.212	4.01036
M+H	Spectral Match to Cyclopentasiloxane, decamethyl- from NIST14	371.101	1.19237
$[M+H]^+$	MassbankEU:SM855703 Verapamil|2-(3,4-dimethoxyphenyl)-5-[2-(3,4-dimethoxyphenyl) ethyl-methylamino]-2-propan-2-ylpentanenitrile	455.29	4.27131
M+H	NCGC00160219-01! METHYLHALFORDINOL	252.083	4.32548
M+H	Spectral Match to Dibutyl phthalate from NIST14	279.16	5.52252
$[M-H]^-$	5-hydroxy-3-(4-methoxyphenyl)-7-[3,4,5-trihydroxy-6-[(3,4,5-trihydroxy-6-methyloxan-2-yl) oxymethyl] oxan-2-yl] oxychromen-4-one	590.536	2.85051

M+H	Spectral Match to Palmitamide from NIST14	256.264	2.9612
M+H	Spectral Match to Cyclohexasiloxane, dodecamethyl- from NIST14	445.12	3.40641

Table 4.14 Metabolites produced by *Fusarium oxysporum* NFW16 with and without antibiotics identified by spectral matches from databases using GNPs as dereplication strategy

Adduct	**Compound name and database**	**Mass**	**ΔPPM**
[M+H]	ReSpect:PM014406 Cyclo (Phe-Tyr)	311.139	2.80316
$[M+Na]^+$	Cyclo (tyrosyl-prolyl)	283.168	1.85051
M+H	Spectral Match to Nonaethylene glycol from NIST14	415.325	2.9612
[M+H]	ReSpect:PM014404 Cyclo (Tyr-Leu)	277.151	3.40641
$[M+Na]^+$	GALANTHAMINE HYDROBROMIDE	388.254	1.42054
M+H	Spectral Match to Heptapropylene glycol from NIST14	425.308	3.43341
$[M+H]^+$	Cyclo (tyrosyl-prolyl)	259.138	4.45985
M+H	Spectral Match to Erythromycin from NIST14	734.471	3.46128
M-H	Spectral Match to D-(+)-Trehalose from NIST14	339.656	5.47538

Table 4.15 Metabolites produced by *Fusarium oxysporum* NFW16 in the presence of both clarithromycin and moxifloxacin, identified by spectral matches from databases using GNPs as dereplication strategy.

Adduct	Compound name and database	Mass	MZ Error PPM
$[M+H]^+$	Sarmentoside B	1327.91	3.6148 5
M+H	NCGC00380798-01_C45H57N3O9_(3S,6R,9S,12R,15S,18R)-3,9,15-Tribenzyl-6,12,18-triisopropyl-4,10,16-trimethyl-1,7,13-trioxa-4,10,16-triazacyclooctadecane-2,5,8,11,14,17-hexone	784.417	2.7475 6
M+Na	NCGC00380798-01_C45H57N3O9_(3S,6R,9S,12R,15S,18R)-3,9,15-Tribenzyl-6,12,18-triisopropyl-4,10,16-trimethyl-1,7,13-trioxa-4,10,16-triazacyclooctadecane-2,5,8,11,14,17-hexone	806.4	4.0103 6
M+H-H_2O	Spectral Match to 9(10)-EpOME from NIST14	279.096	4.1923 7

4.4 DISCUSSION

Chemical compounds produced by microorganism especially from fungi are of immense importance when it comes to therapeutics and other biotechnological applications. Although, large number of metabolites originating from microorganisms have been isolated and in daily use, still a great percentage with exciting biotechnological remains undiscovered. The chemical nature of natural products is complex, and quantities are often limited rendering their isolation a daunting task. Therefore, the developments of rigorous methodologies employing robust tools is deemed essential for the isolation and structural characterization of these unique biochemical entities. One such handful approach is the application of metabolomics and dereplication studies based on LC-HRMS/MS. The data generated, resultantly, is further challenging but the use of bioinformatics and chemoinformatic tools, software and databases could ease the processing and hence interpretation. One of such tools for natural product analysis and dereplication is GNPS which was extensively utilized for dereplication as described in this study.

This chapter describes metabolic profiling of endophytic fungal isolates *Epicoccum* sp. NFW1 *Chaetomium* sp. NFW8 and *Fusarium oxysporum* NFW16 when grown in SDB supplemented with clarithromycin and moxifloxacin. The addition of antibiotics to culture medium resulted in the production and suppression of particular metabolites as well as the biotransformation of antibiotics by the fungi. These metabolites may contain bioactivies therefore the crude extract obtained following incubation was subjected to LC-HRMS/MS and data was analyzed by using global natural product social molecular network GNPS as dereplication strategy.

Molecular networking and dereplication studies revealed that endophytic fungal isolates produced different metabolites under different conditions. If there is antibiotic in the medium, the metabolic profile of fungal isolates was liable to change compared to the medium without antibiotics, keeping other conditions constant. Dereplication studies using GNPS showed the production of several interesting, well known secondary metabolites as mentioned earlier in results section. In case of *Epicoccum* sp. NFW1, most notable were pumilacidin, astaxanthin asiatic acid, flunixin meglumine, dibutyl phthalate and surfactin while from *Chaetomium* sp. NFW8 Chaulmoogric acid, diploschistesic acid, dehydrovariobili, acarbose, sarmentoside B and salicin along with

many other metabolites were found to be present. *Fusarium oxysporum* NFW16 showed production of compounds such as trehalose, anastrozole 3-Deacetyl khivorin, mycophenolic, cyclo (tyrosyl-prolyl) galanthamine hydrobromide and ethmozine. Interestingly, a large number of secondary metabolites produced under antibiotic supplementation turned out to be unknown. This implies the potential for the production and hence isolation of novel or new biochemical entities. These findings are consistent with some recent investigations describing isolation of new biochemical entities from microbial sources. A comprehensive study by Macia *et al*., (2018) investigated natural products of 822 fungal strains by untargeted UPLC-ESI-MS/MS-based approach. They detected more than 17 thousand metabolites, almost eight thousand, of which were classified into different molecular families. Majority of the 191 annotated metabolites were found to be of fungal origin. The molecular networking, they performed showed that almost 61% of the metabolites were specific to a fungal order. This dataset could serve as an extensive reference and facilitate dereplication of natural products of fungal origin. Crude extract of the endophytic fungus isolated from *Sandwithia guyanensis* when analyzed using molecular networking, revealed the presence of 5 stephensiolides compounds, four of which exhibited antibacterial activity (Mai *et al*., 2020).

As in current study molecular networking was performed to study the metabolic profile change and known compounds produced by endophytic fungal isolates of *Taxus fuana*. These findings are supported by a recent study of Chinese researchers. The study describes targeted isolation of new antiviral sterigmatocystin derivatives, sterigmatocystins A–C, from a fungus *Aspergillus versicolour* resides in marine sponge, as a result of molecular networking (Han *et al.,* 2020).

The screening of crude extract of *Penicillium janthinellum* KTMT5, based on untargeted metabolic profiling by using LC-QTOF-MS/MS on the Global Natural Product Social molecular networking (GNPS) workflow, revealed the presence of several known metabolites and the relationship among them (Tapfuma *et al*., 2020). The findings as well as approach of Tapfuma and colleagues strongly correlates with those of the present study

Present findings show the production of known as well as appreciable percentage of undetected chemical entities by the endophytic fungi, when challenged by adding antibiotics to the growth medium. Comparable observations could be made when fungal

cultures are co-cultivated with pathogenic bacteria and fungi. Oppong-Danguah *et al*., (2018) co-cultivated marine fungi with pathogenic bacteria (*Ralstonia solanacearum* and *Pseudomonas syringae*) and pathogenic fungi (*Botrytis cinerea* and *Magnaporthe oryzae*) followed by UPLC–QToF–HRMS/MS-based molecular networking (MN) of the crude organic extract. Results showed that co-culturing led to a shift in metabolic profile as observed by generating molecular network map (Oppong-Danguah *et al*., 2018). Total eighteen clusters were annotated, of which nine were produced only in co-cultures. Some cluster cannot be annotated to any known metabolites which depicts that they can be putatively new compounds (Oppong-Danguah *et al*., 2018). These findings are in strong agreement with this study and advocate the potential for the discovery of new compounds from endophytic fungi.

In a related study, Fan *et al*., (2020) investigated the crude extract of an endophytic fungi *Pyrenochaetopsis* sp. FVE-001 based on molecular networking approach and rapidly isolated three new decalinoyl spirotetramic acid derivatives, pyrenosetins A–C (**1**–**3**) and a previously known decalin tetramic acid phomasetin (**4**). Compound 1 and 2 showed higher anticancer activities against tested cell lines as compared to the others. A cluster containing different nodes that were structurally related to diketopiprazine (**5**) was also detected in molecular network (Fan *et al*., 2020).

In another experiment, co-cultivation of *Aspergillus* species with phytopathogen *Botrytis cinereal,* lead to bigger cluster size of quinolones and echinulin type indole alkaloids with various putative unknown analogues in UPLC-QTOF-MS/MS-based molecular networking (Parrot *et al*., 2017). Similarly, compounds depsipeptides and cyclopeptides with potential against methicillin-resistant *Staphylococcus aureus* (MRSA) were discovered when a library of 197 endophytic fungi; isolated from Amazonian pal tree *Astrocaryum sciophilum*; was analyzed using combined UPHLC-HRMS/MS analyses and molecular networking data (Barthelemy *et al*., 2019).

4.5 CONCLUSIONS

Endophytic fungi from medicinal plants especially the fungal isolates used in this study are a potent source of many secondary metabolites with therapeutic and other biotechnological importance. The dereplication studies showed that the fungal isolates are great source of known and unknown bioactive metabolites. The metabolic profile showed variation when the medium was supplemented with antibiotics. Several metabolites were induced by the antibiotics in medium some of them are known bioactive metabolites together with many unknown metabolites. The study suggests that these endophytic fungal isolates showed enhance production of bioactive metabolites when they were challenged with antibiotic in liquid medium. Dereplication studies provide sufficient information about the known compounds produced by these fungal isolates with and without the supplementation of antibiotics so large-scale production of any targeted compound can be achieved.

CHAPTER 5

5 PURIFICATION AND STRUCTURE ELUCIDATION OF COMPOUNDS PRODUCED BY ENDOPHYTIC FUNGAL ISOLATES

5.1 INTRODUCTION

Bioactive metabolites obtained from natural sources are the effective therapeutics. Conventionally, the selection of the natural sources for the products are either random or based on the ecology and taxonomy of the source (Hou *et al.,* 2012). Endophytic fungi, reside within plant's micro ecosystem and have gained prime importance since over a decade. It has been reported that over one million endophytic fungi residing in plant tissues have diverse biotechnological applications (Firakova *et al.,* 2007). Though, remarkable discoveries have been made from these natural reserves in recent past, there still lies substantial potential to discover more. Therefore, need of the hour is to explore the biosynthetic potential of endophytic fungi to its maximum and isolate potential candidate compounds to produce new drugs. Although, specific function of secondary metabolites, in the organisms producing them, is largely unknown, their utility to human population and their specific significance in developing novel pharmaceuticals is exemplary (Yu and Keller, 2005).

This chapter describes the isolation and identification of bioactive secondary metabolites from endophytic fungi *Epicoccum* sp. NFW1, *Chaetomium* sp. NFW8 and *Fusarium oxysporum* NFW16. Liquid state fermentation was carried out using SDB medium supplemented with clarithromycin and moxifloxacin; followed by ethyl acetate extraction for further fractionation. Crude ethyl acetate extract of endophytic fungi grown on SDB without antibiotic supplementation served as control. Chromatographic techniques were used initially to isolate and purify compounds as described in chapter 3. In case of all three strains, fractions expressing antibacterial activity were pooled and HPLC based metabolic profiling was conducted. Based on retention time, peaks which were not found in control were collected using preparative HPLC. This was followed by antibacterial activity and characterization by 1D, 2D NMR and high-resolution mass spectrometry experiments.

5.2 MATERIALS AND METHODS

This study was conducted in TM lab Oregon State University USA.

5.2.1 Reagents

HPLC grade water and organic solvents (acetonitrile, and methanol) were used. Trifluoro acetic acid (TFA) was used in some mobile phases of HPLC. The solvents used for NMR analysis were deuterated water, deuterated chloroform ($CDCl_3$), deuterated methanol (MeOH- *d6*). All the chemical and reagents were manufactured by Sigma-Aldrich Co., Missouri, USA.

5.2.2 Antibacterial Activity

Antibacterial activity of peaks collected from semi preparative HPLC were analyzed by disk diffusion method against *E. coli* and *S. aureus*. Sterilized disk of 5mm diameter were loaded with 10uL of fraction containing 0.1mg of extract to bacterial plates containing test bacterial strains were prepared as described in chapter 3. Disk diffusion assay was performed by placing extract loaded disk carefully on test bacterial strains containing plates. Zone of inhibitions in mm were record against the compounds of every collected peak at different retention time appeared in HPLC.

5.2.3 Normal Phase Column Chromatography

Crude extract (1 gram for *Fusarium oxysporum* NFW16 and 3 grams for other two strains) were subjected to dissolution in ethyl acetate for moxifloxacin containing extracts and acetone was used clarithromycin containing extracts. It was then adsorbed on silica (70-230 mesh, Fluka Switzerland) maintaining the ratio at 1-gram extract on 1.5 grams silica. Pre-washed column (diameter: 5 cm, length: 30 cm, silica gel: 150 g) was embedded with cotton wool at the base and measured quantity of dry silica was poured above it to pack the column. Air bubbles were avoided, and extract adsorbed on silica was added to the leveled column. A protective layer of pure silica (1 cm) was again settled above the extract layer and blocked with the help of cotton wool at the top. Crude extract was eluted with 100 % n-hexane as starting solvent system. Then 2nd mobile phase was 80 % n-hexane with 20 % ethyl acetate and concentration was gradually increased with ethyl acetate till 100 % ethyl acetate. Methanol was introduced as third solvent with ethyl acetate by changing the ratios starting from 90:10 (ethyl

acetate: methanol) to 100% methanol, followed by washing column with methanol. Total volume of solvents 5 L to afford 25 fractions of 200 mL (code F1–F25).

Above 25 fractions were collected in glass vials and further processed for antibacterial activity of each friction by disc diffusion assay. All the active fractions were pooled to get sufficient concentration and to purify them on preparative HPLC.

5.2.4 High Performance Liquid Chromatography (HPLC)

All the extracts and pooled fractions were dissolved in methanol and filtered (0.2 micron) to remove suspended particles. The fractions were directly separated on two different semi-preparative HPLCs a Shimadzu LC-20AD with a SPD-M20A detector manufactured by Shimadzu Kyoto Japan and HPLC Agilent 1100 with G1315 DAD detector (Agilent Technologies, Santa Clara, CA, USA) An ACE C18-AR column (250 × 10 mm; 5 μm) and C_{18} YMC column (250 mm×10.0 mm 5 μm ID; Japan) were used for compound separation. flow rate: 2 mL/min and the column oven temperature was 30℃ constant for all the samples processed.

HPLC chromatogram of pooled antibacterial fraction obtained by column chromatography of the ethyl acetate extract of endophytic fungi grown on SDB supplemented with antibiotics, was acquired under the developed method. Another HPLC chromatogram of ethyl acetate extract of endophytic fungal isolate grown on SDB without antibiotic stress, under the same method was obtained. The chromatograms in each case were comparatively analyzed to identify peaks which appeared only in the chromatograms of active fractions from fungi grown on antibiotic supplemented media, were collected for compounds purification and structure elucidation. This will guide to the purification of compounds produced by the supplementation of antibiotics only.

HPLC chromatograms was obtained for *Epicoccum* sp. NFW1 the mobile phase constituted 100 % water (solvent A) and 100 % Methanol (solvent B). Gradient elution was performed from 10 % solvent B and (90 % solvent A (t=0 min) to 50 % solvent B at t=20 min to 100 % solvent B at t=35min and this mobile phase runs till 45 minutes where again the initial concentration 10% solvent B and 90% solvent A was established till 55 minutes to stabilize the column for next sample.

Purification of peak No. 6 from HPLC chromatograms of pooled active fractions *Epicoccum* sp. NFW1 grown on clarithromycin supplemented medium was further purified by subjecting it to HPLC using mobile phase 100 % water (solvent A) and 100% Acetonitrile (solvent B). Gradient elution was performed from 10 % solvent B and 90 % solvent A *t*=0 min to 50 % solvent B at *t*=5 to 60 % solvent B at t=20 min to 100 % solvent B at *t*=25 min and this mobile phase runs till 30 minutes.

The mobile phase for *Chaetomium* sp. NFW8 constituted 99.9% water and 0.1% TFA (solvent A) and 100% Methanol (solvent B). Gradient elution was performed from 10 % solvent B and (90 % solvent A (*t*=0 min) to 50 % solvent B at *t*=20 min to 100 % solvent B at *t*=40 min and this mobile phase runs till 50 minutes where again the initial concentration 10% solvent B and 90 % solvent A was established till 55 minutes to stabilize the column for next sample.

The mobile phase for *Fusarium oxysporum* NFW16 constituted 99.9 % water and 0.1% TFA (solvent A) and 100 % Acetonitrile (solvent B). Gradient elution was performed from 10% solvent B and (90% solvent A (*t*=0 min) to 50% solvent B at *t*=20 min to 100% solvent B at *t*=40min and this mobile phase runs till 50 minutes where again the initial concentration 10% solvent B and 90% solvent A was established till 55 minutes to stabilize the column for next sample.

5.2.5 Nuclear Magnetic Resonance Spectroscopy (NMR)

Samples for NMR analysis were prepared by dissolving pure compounds in NMR solvents i.e., deuterated water, and deuterated methanol (Cambridge Isotope Laboratories Inc.). Samples were analyzed for structure determination using ID and 2D experiments such as ^{1}H, ^{13}C, DEPT-135, COSY, HMBC, HSQC and NOESY where required. NMR spectrometer 700 MHZ (Bruker) a 2-channel spectrometer equipped with a 5 mm carbon observe cryoprobe with Z-axis gradient.

5.2.6 High Resolution Mass Spectrometry

Molecular weight of the pure compounds was confirmed by high resolution ESI mass spectrometry was performed in positive-ion mode on a 6230 TOF mass spectrometer (Agilent Technologies, Santa Clara, CA). HR-MS spectra with a resolution of 60 000 and a mass accuracy of <5 ppm were recorded. The sample was dissolved in filtered

LCMS grade methanol at a concentration of 1 µg/mL prior to injection in mass spectrometer.

5.3 RESULTS

5.3.1 Isolation of Compound 1 from *Epicoccum* sp. NFW1

The compound isolated from the ethyl acetate extract of *Epicoccum* sp. NFW1 grown in the presence of clarithromycin is given name of compound 1.

5.3.1.1 Purification of Crude Extracts Obtained from *Epicoccum* Sp. NFW1 Grown under the Stress of Clarithromycin

After purification by column chromatography 25 fractions were collected. Among all these, 16 fractions showed activities against test bacterial strains *S. aureus* and *E. coli.* The fraction number 22 showed maximum activity against *S. aureus* with respect to its control (clarithromycin) i.e. 11mm whereas fraction number 03 showed 19 mm zone of inhibition against *E. coli.* Most of the fractions exhibit antibacterial activities against test strain (Figure 5.1).

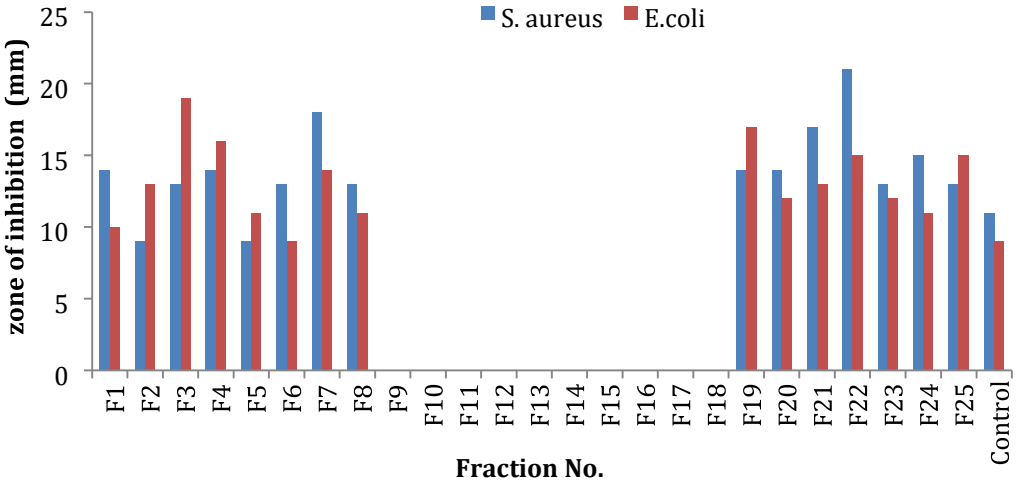


Figure 5.1 Antibacterial activity of fractions obtained from silica gel column chromatography of the crude extract of *Epicoccum* sp. NFW1 grown under the stress of clarithromycin

5.3.1.2 Preparative HPLC for Extracts of *Epicoccum* sp. NFW1

The HPLC chromatograms of pooled active fractions of *Epicoccum* sp. NFW1 grown in the presence of clarithromycin and ethyl acetate extract of *Epicoccum* sp. NFW1 grown without antibiotic were comparatively analyzed to identify peaks in each extract. Figure 5.2 shows the HPLC chromatogram of ethyl acetate extract of *Epicoccum* sp. NFW1 grown on SDB without antibiotics supplementation. Figure 5.3 is the HPLC chromatogram of pooled active fraction obtained from column chromatography of ethyl acetate extract of *Epicoccum* sp. NFW1 grown on SDB supplemented with clarithromycin. These chromatograms were compared, and the peaks present distinctly in the pooled active fractions were labelled as 1-9. Based on antibacterial activity, peak No. 6 was selected for further purification by developing method as mentioned in methodology to separate it and acquire maximum purity (Figure 5.4). The peak at retention time around 20 minutes was collected and based on its antibacterial activity (8mm against *S. aureus* and 7mm against *E. coli* in concentration of 0.1mg) and purity its structure was elucidated and named as compound 1.

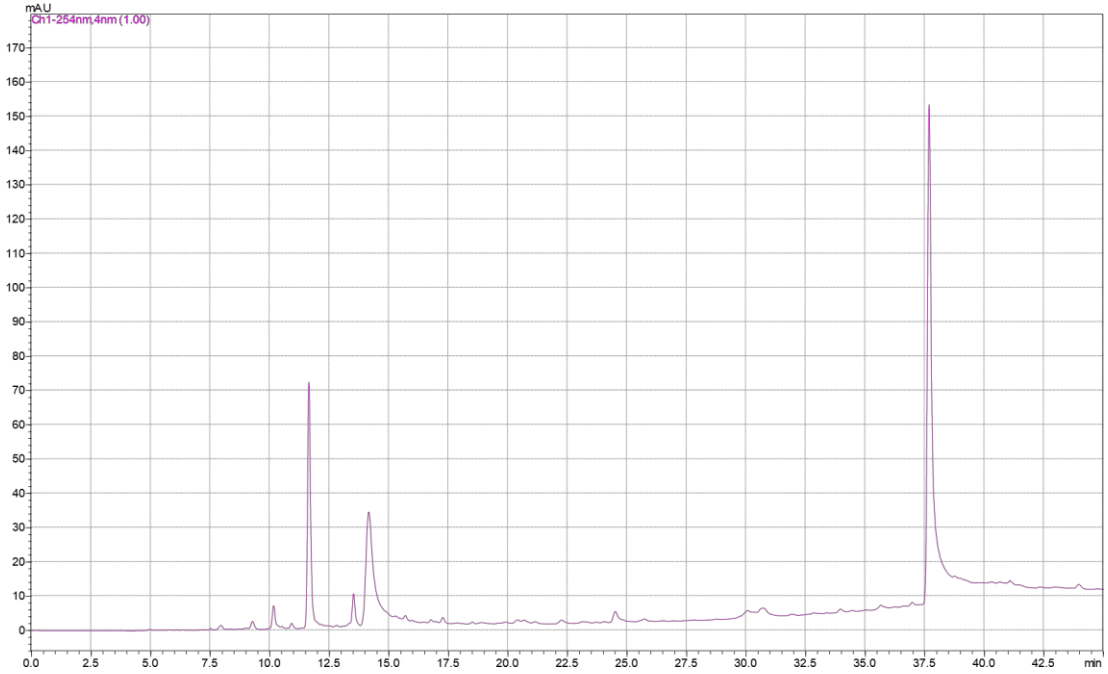


Figure 5.2. HPLC chromatogram of ethyl acetate extract of *Epicoccum sp.* NFW1 grown on SDB without antibiotics supplementation

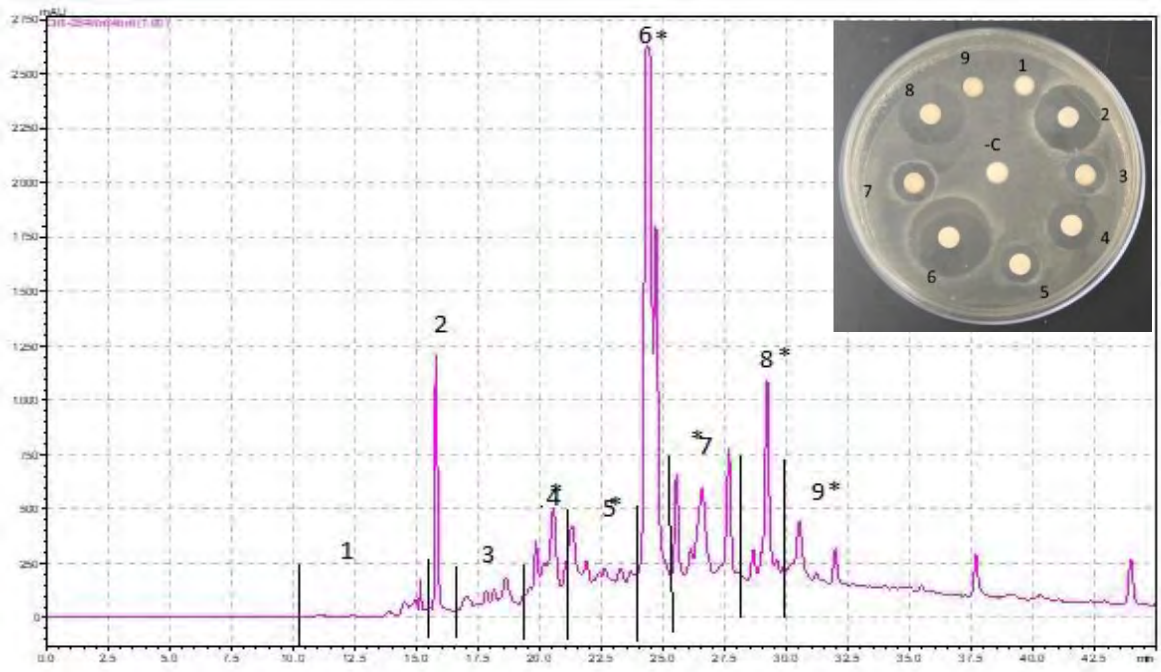


Figure 5.3 HPLC chromatogram of ethyl acetate extract of *Epicoccum* sp. NFW1 grown on SDB supplemented with clarithromycin

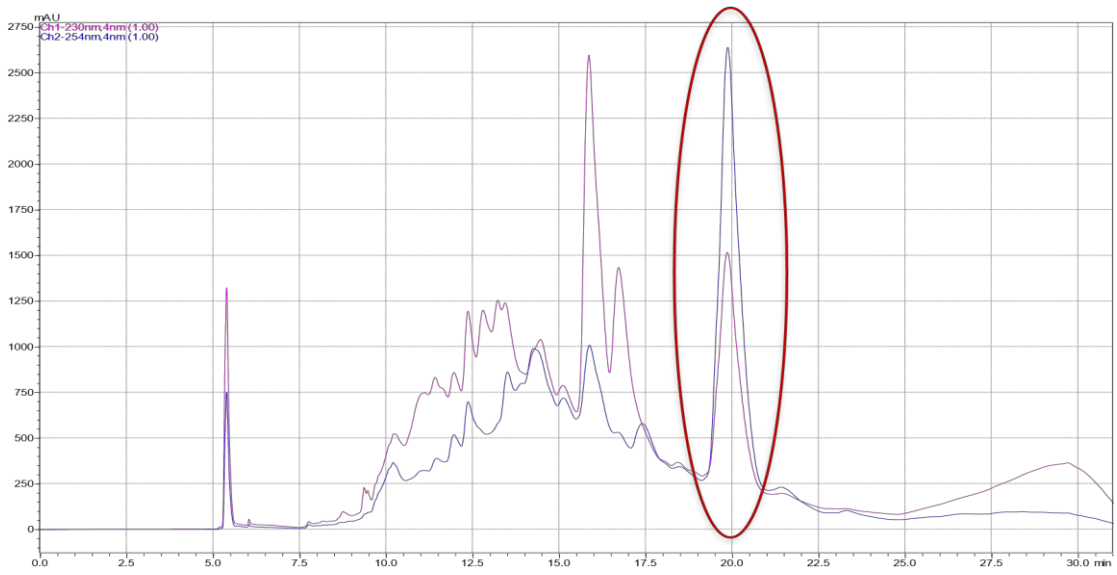


Figure 5.4 HPLC chromatogram for purification of peak 6 of Epicoccum sp NFW1 grown on SDB supplemented with clarithromycin

5.3.1.3 Structure Elucidation of Compound 1

Compound 1 (6 mg) collected as white powder when dried, after several runs of semi preparative HPLC. The compound was characterized using ESI-MS, 1D and 2D NMR. The structure of the compound was elucidated by careful of proton, carbon, dept-135, COSY, HSQC, HMBC and NOSY NMR as well as mass spectra, shown below.

The proton and carbon NMRs showed a characteristic mono substituted aromatic ring structure with chemical shifts at 8.35-7.48 and 137.1-128.1 ppm for δH and δC respectively. The chemical shifts for carbon between 169.3 and 199.4 results from 4 carbonyl groups and the chemical shifts for hydrogen between 4.53 and 3.34 ppm as well as the carbon shifts between 114.6 and 52.6 ppm results from oxygenated functional groups such as methoxy and hydroxyl groups. The peaks at 5.46 and 5.62 ppm for hydrogen and the peaks at 129.3 and 129.7 for carbon indicated the presence of a trans alkene.

This novel compound is chiral and contains 4 stereocenters. One of these chiral centers is the allylic chiral center that is near the 2 methylene protons and yields in 2 different chemical shifts for each methylene protons due to their enantiotopic feature protons. After evaluating dept-135, COSY, HSQC, NOSY and based on the HMBC correlations it was evident that the compound was dimeric compound (8E,16E)-5,7,10,15,18,20-hexahydroxy-3,22-dimethoxy-5,20-dimethyl-1,24-diphenyltetracosa-8,16-dien-

1,2,4,6,19,21,23,24-octaone) having molecular formula of $C_{40}H_{46}O_{16}$ with unique structure. Furthermore, the ESI-MS spectrum showed in the positive mode a [M^+ + 2K + Na] molecular ion peak at 885.3054 m/z with an addition of a 2 Potassium and 1 Sodium atoms and made the molecular ion peak 782.3 m/z with a molecular formula of $C_{40}H_{46}O_{16}$. We found that this peak was due to the dimeric nature of the compound 1 as the high intensity peak resulted at 454.1474 m/z resulted from [(M/2) + K + Na].

Therefore, the monomeric unit ‘s molecular weight was found to be 392.1 m/z and had a molecular formula of $C_{20}H_{24}O_8$. Thus, this unique structure displays a C_{2v} symmetry which was also confirmed based on NMR spectral evaluation.

Table 5.1: Chemical Shifts of Compound 1

Position	δ H (ppm)	δ C (ppm)
1-CH	7.64	135.3
2-CH	7.50	128.9
3-CH	8.34	137.5
4-C	-	132.4
5-CO	-	169.3
6-CO	-	188.9
7-CHOH	4.53 - s	94.0
8-CO	-	197.3
9-C-OH	-	114.6

10-CO	-	199.4
11-CHOH	4.50 – d	76.5
12-CH	5.62 – dd	129.7
13-CH	5.46 – t	129.3
14-CH-OH	3.54 – t	73.1
15-CH2a	2.17 – m	22.4
15-CH2b	2.12 – m	22.4
16-CH2a	1.51 – q	6.0
16-CH2b	1.39 - ddd	6.0
17-OCH3	3.34 - s	52.6
18-OH	-	-
19-CH3	1.76 - s	14.7
20-OH	3.66	-
21-OH	-	-

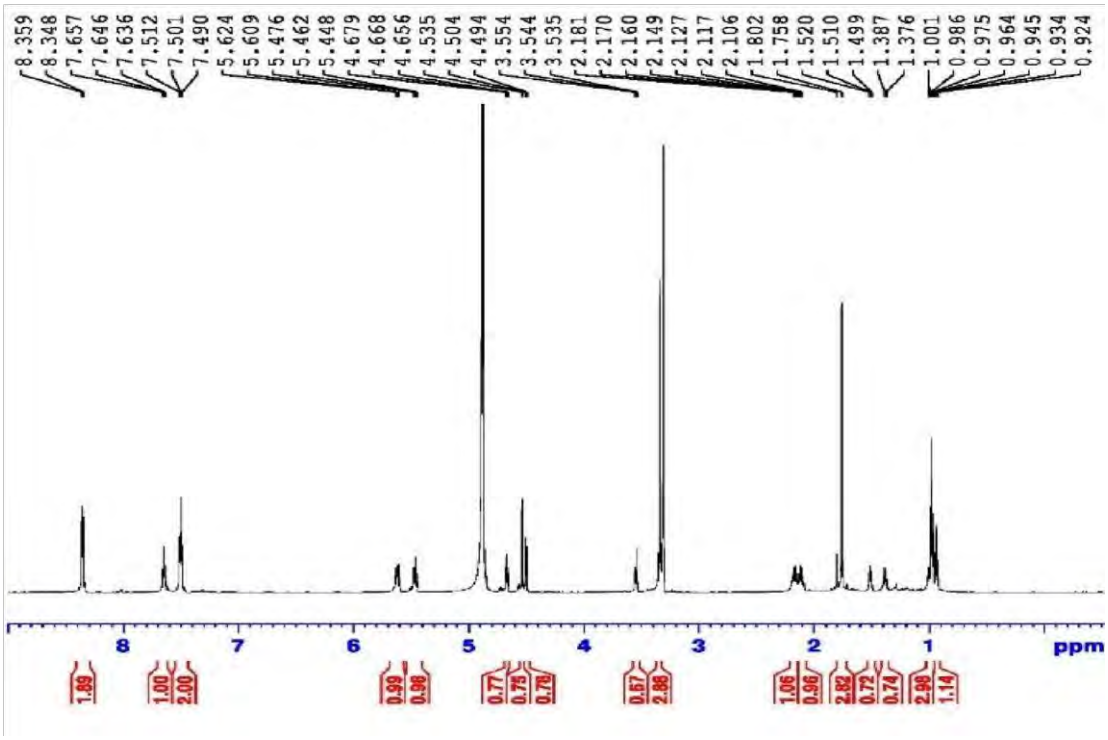


Figure 5.5 ^{1}H NMR spectrum of compound 1

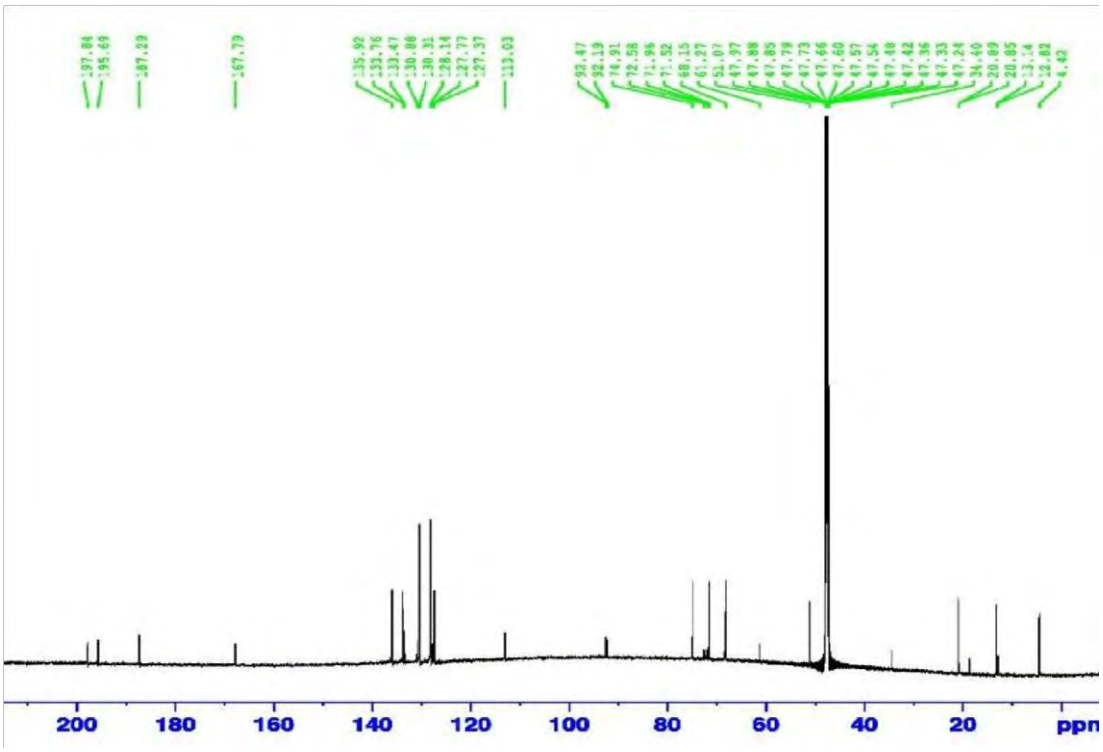


Figure 5.6 ^{13}C NMR spectrum of compound 1

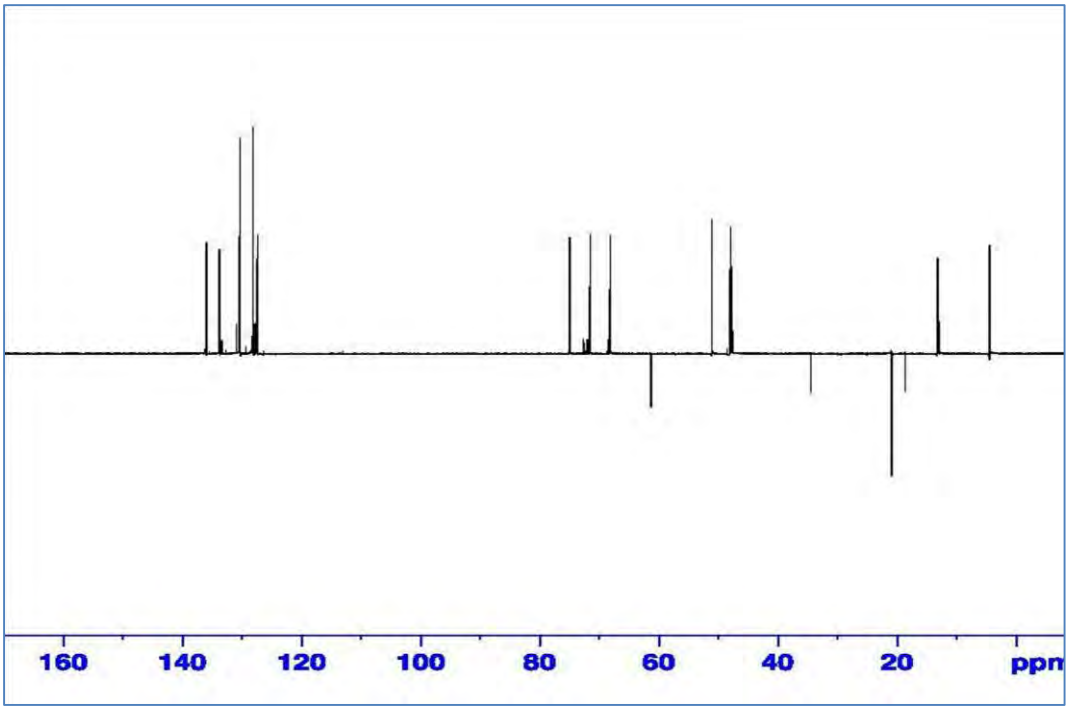


Figure 5.7 D PT -135 Of compound 1

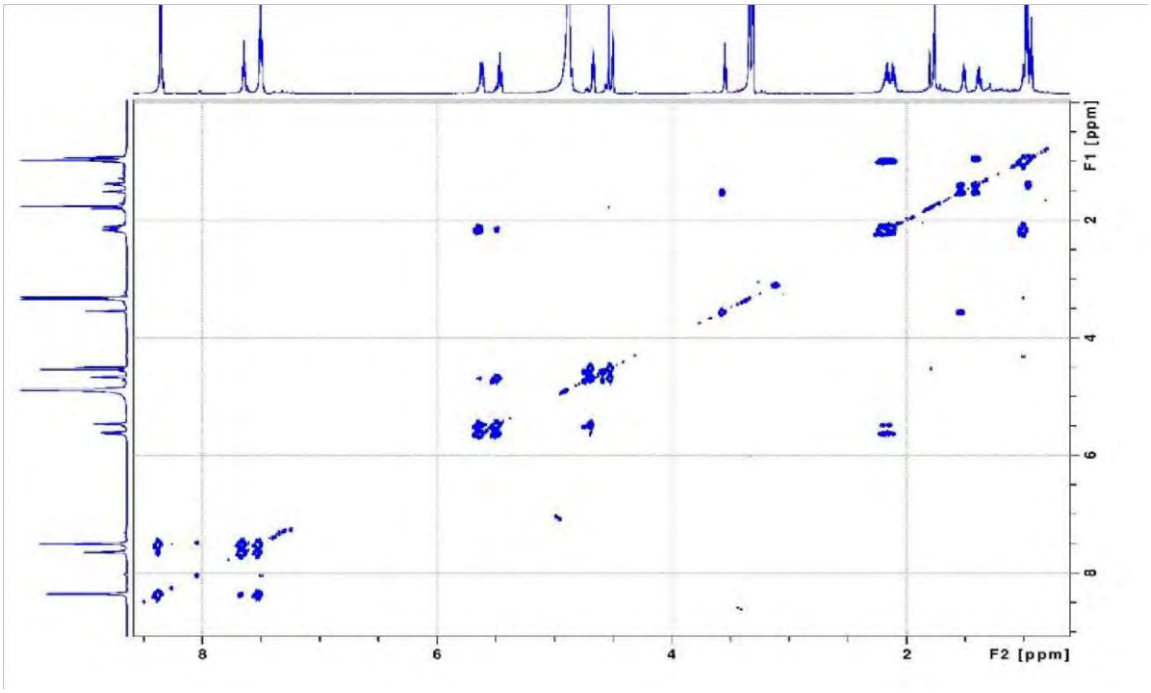


Figure 5.8 COSY of compound 1

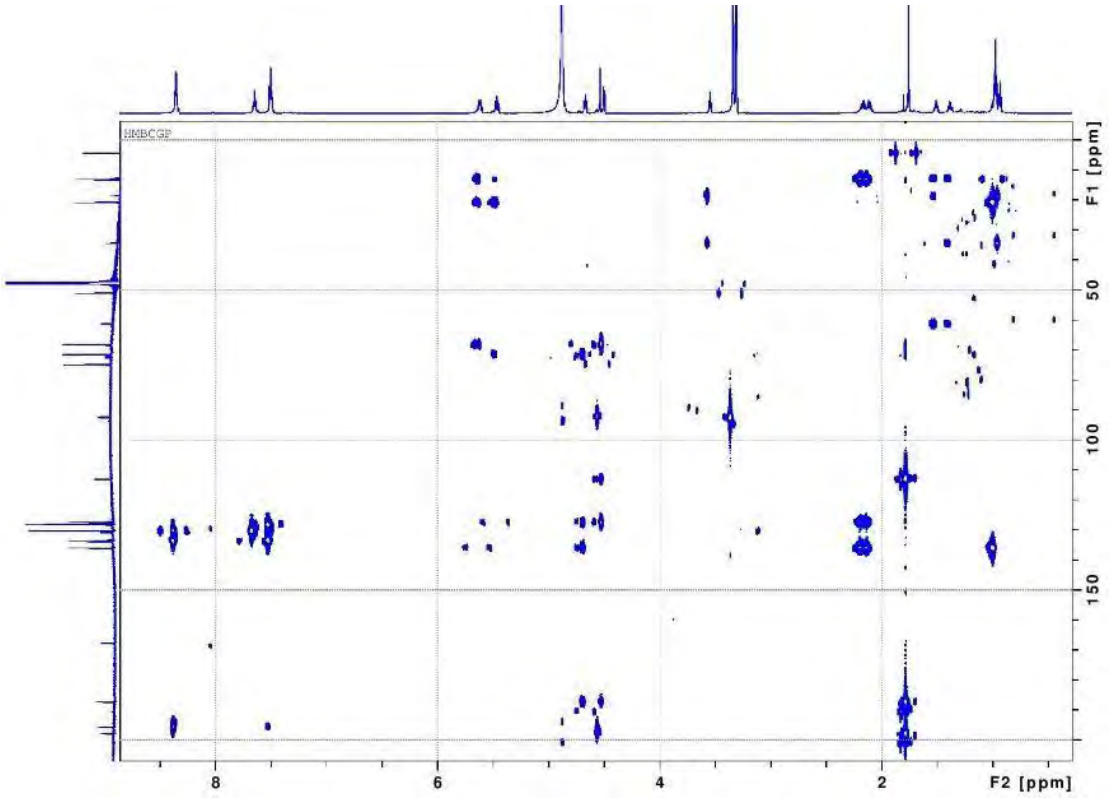


Figure 5.9 HMBC of compound 1

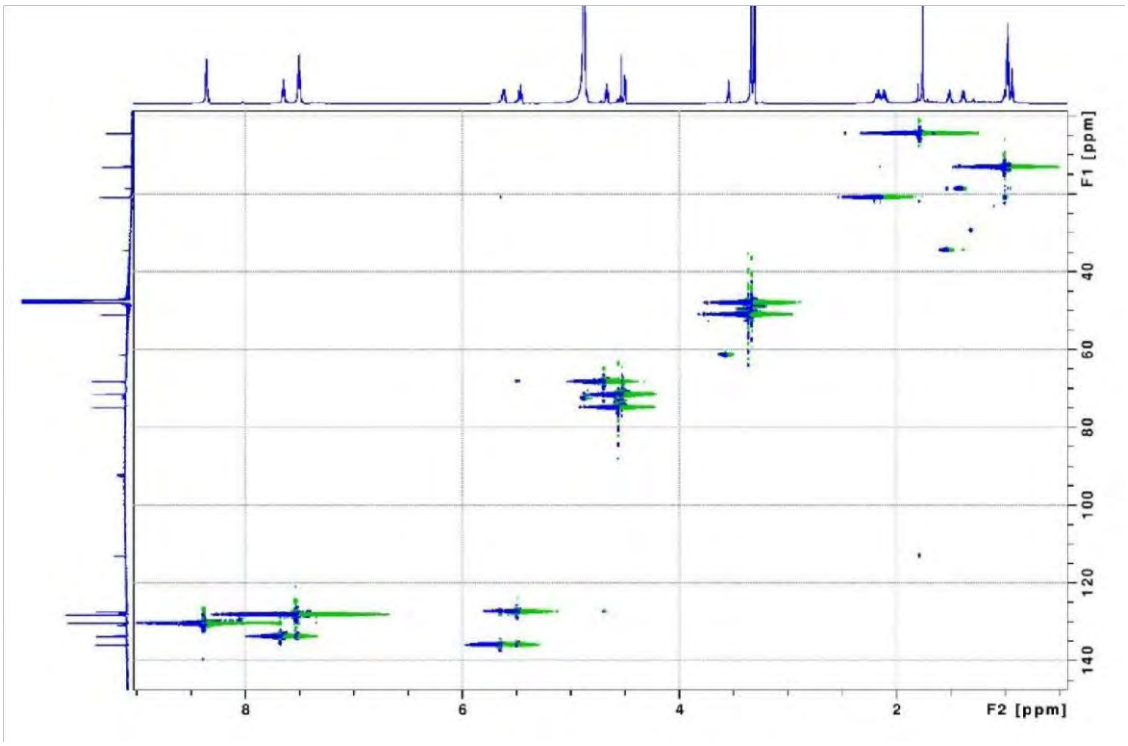


Figure 5.10 HSQC of compound 1

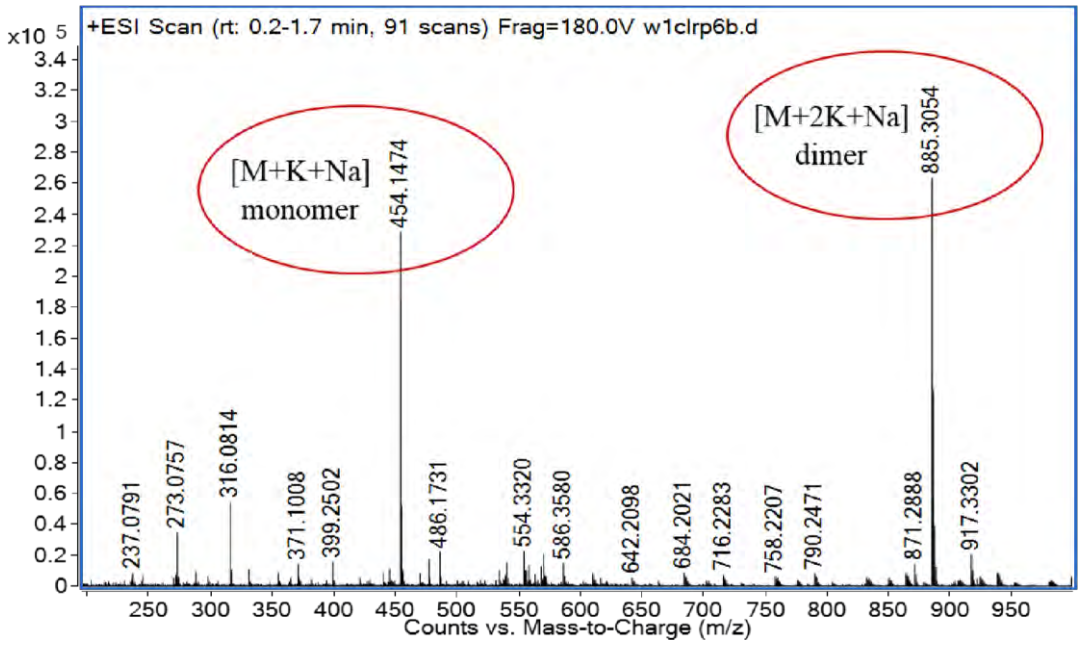
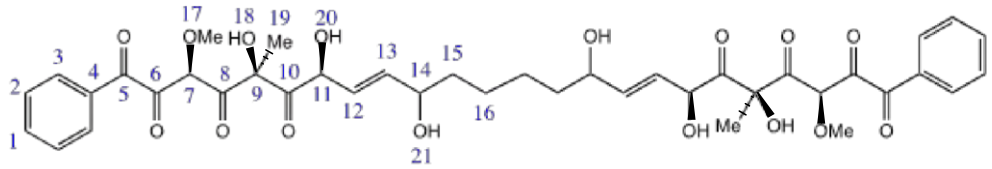
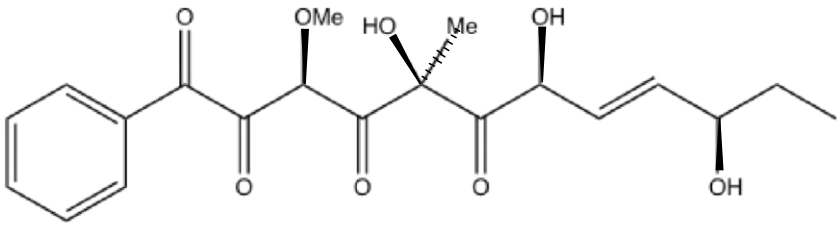


Figure 5.11 HRMS of compound 1



Chemical Formula: $C_{40}H_{46}O_{16}$
Exact Mass: 782.3
Molecular Weight: 782.8
m/z: 782.3 (100.0%), 783.3 (43.3%), 784.3 (9.1%), 784.3 (3.3%), 785.3 (1.4%)
Elemental Analysis: C, 61.38; H, 5.92; O, 32.70

Figure 5.12 structure of compound 1 (Dimer)



Chemical Formula: $C_{20}H_{24}O_8$
Exact Mass: 392.1
Molecular Weight: 392.4
m/z: 392.1 (100.0%), 393.2 (21.6%), 394.2 (2.2%), 394.2 (1.6%)
Elemental Analysis: C, 61.22; H, 6.17; O, 32.62

Figure 5.13 structure of compound 1 (Monomer)

5.3.2 Isolation of Compound 2 from *Epicoccum* sp. NFW1

The compound isolated from the ethyl acetate extract of *Epicoccum* sp. NFW1 grown in the presence of moxifloxacin is given name of compound 2.

5.3.2.1 Purification of Crude Extracts Obtained from *Epicoccum* sp. NFW1 Grown under the Stress of Moxifloxacin

A total of 25 fractions were collected; the fractions were subjected for antibacterial activity against *E. coli* and *S. aureus.* Among these 25 fractions, 12 fractions showed antibacterial activity. The fraction number 07 showed the maximum zone of inhibition 17 mm against *S. aureus* while fraction number 13 showed the maximum activity against *E. coli*. The antibacterial spectrum of fractions was presented in Figure 5.14.

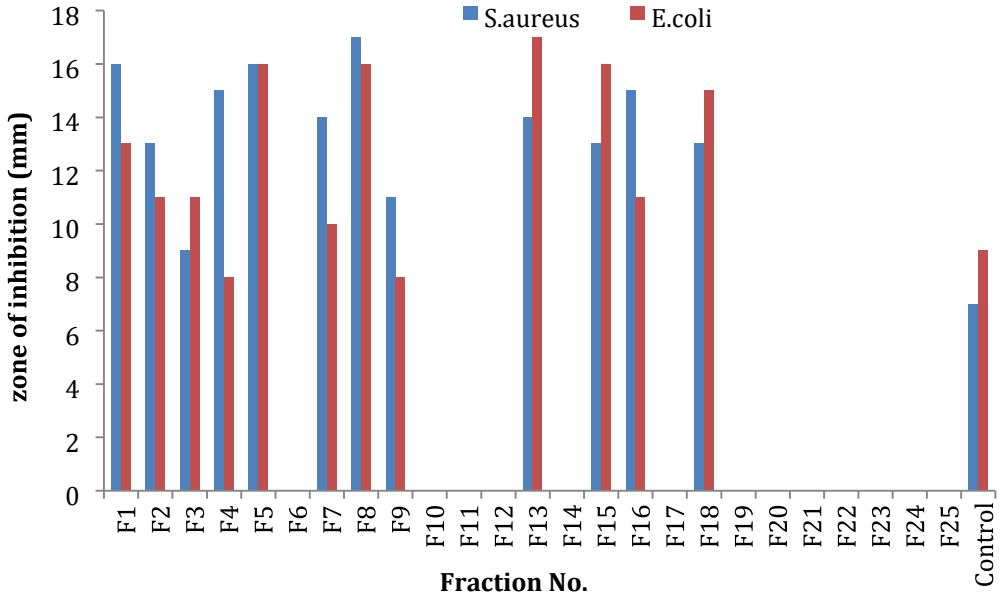


Figure 5.14 Antibacterial activity of fractions obtained from silica gel column chromatography of the crude extract of *Epicoccum* sp. NFW1 grown with moxifloxacin

5.3.2.2 Preparative HPLC for *Epicoccum* sp. NFW1 Grown on Moxifloxacin Emended Medium

HPLC chromatograms of pooled active fractions of *Epicoccum* sp. NFW1 grown in the presence of moxifloxacin and ethyl acetate extract of *Epicoccum* sp. NFW1 grown without antibiotic was obtained as mentioned in methodology. The chromatograms in each case were comparatively analyzed to identify peaks in each extract. Figure 5.15 showing the HPLC chromatogram of ethyl acetate extract of *Epicoccum* sp. NFW1

grown on SDB without antibiotics supplementation. Figure 5.16 is the HPLC chromatogram of pooled active fraction obtained from column chromatography of ethyl acetate extract of *Epicoccum* sp. NFW1 grown on SDB supplemented with moxifloxacin. These chromatograms were compared, and the peaks noticeably present in active fractions were labelled as 1 and 2. Following peaks were collected and checked for antibacterial activity (9 mm against *S. aureus* and 8 mm against *E. coli* in concentration of 0.1mg). Based on antibacterial activity and purity peak No. 1 was collected having retention time around 24 minutes for structure elucidation and named as compound 2.

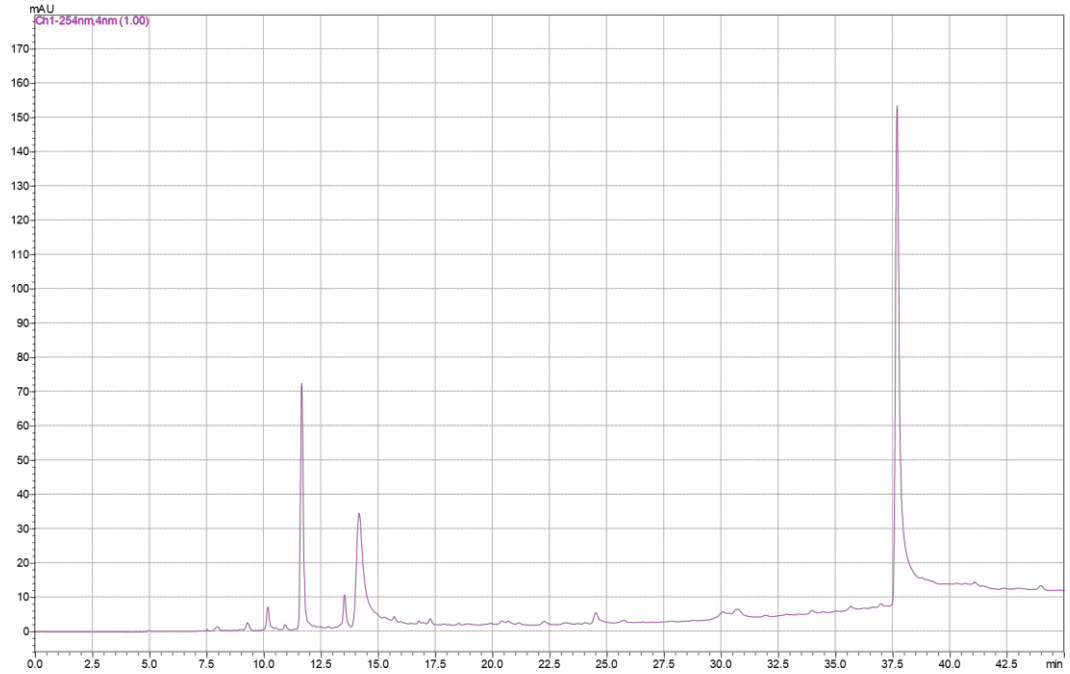


Figure 5.15 HPLC chromatogram of ethyl acetate extract of *Epicoccum sp.* NFW1 grown on SDB without antibiotics supplementation

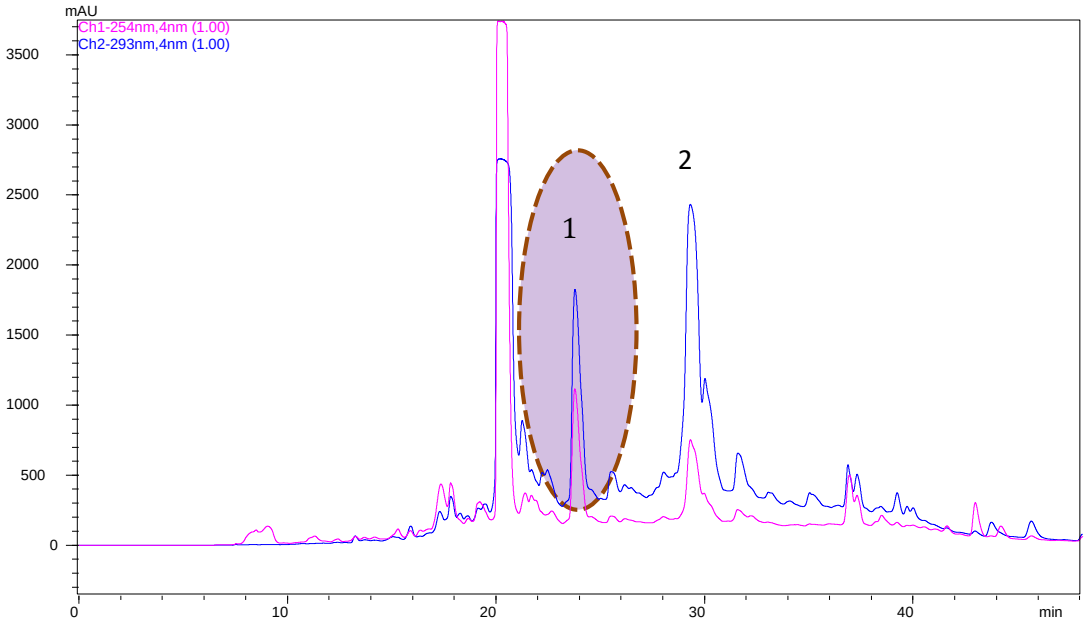


Figure 5.16 HPLC chromatogram of ethyl acetate extract of *Epicoccum* sp. NFW1 grown on SDB supplemented with moxifloxacin

5.3.2.3 Structure Elucidation of Compound 2

Compound 2 (5mg) was collected as light brownish powder when dried. To characterize compound 2, ESI-MS, 1D and 2D NMRs were used. The compound structure was elucidated according to the NMR spectra of proton, carbon, dept-135, COSY, HSQC, HMBC and NOSY as well as mass spectra. The corresponding spectra of the compound are listed in the following.

The down field peaks in the proton and carbon NMR resulted from unsaturation and showed a characteristic cyclopentadiene ring structure with chemical shifts at 6.696.68 ppm for δ H and 136 – 131 ppm for δ C. The other alkenes formed the hydrogen peaks at 5.19 and 4.61 ppm, whereas the carbon peaks were at 135.1-92.0 ppm. Furthermore, the relatively high number of downfield chemical shifts of carbon atoms between 75.9-51.0 ppm and for hydrogens between 4.22-3.26, indicated a high number of oxygenated functional groups such as hydroxyl groups and ether linkages in the molecule.

This novel compound is chiral and contains 12 stereocenters of which 11 are consecutive, the chiral center that is not consecutive is in a double allyl alcohol position. Another interesting feature of this molecule is its cyclohexyl carbons are all hydroxylated. After evaluating dept-135, COSY, HSQC, NOSY and based on the HMBC correlations it was evident that the compound 2 was a novel compound.

Furthermore, the ESI-MS spectrum showed in the positive mode a $[M^+ + K]$ molecular ion peak at 600.5241 m/z with an addition of a 1 Potassium atom and made the [M+] ion peak 561.5 m/z. This also indicated the presence of one nitrogen atom. Therefore, compound 2 had a molecular formula of $C_{26}H_{43}NO_{12}$ (1R,2R,3R,4S,5S,6R)-1-((1R,2S,3S,4R,5R,7R,Z)-6-(1-aminoethylidene)-8-(cyclopenta-2,4-dien-1-ylmethyl)-1,3,4,5,7-pentahydroxy-2 isopropoxynon-8-en-1-yl)cyclohexane-1,2,3,4,5,6-hexaol and its molecular weight was found to be 561.5. Thus, the unique compound's structure was determined as shown in Figure 5.26.

Table 5.2: Chemical Shift of Compound 2

Position	δH (ppm) - multiplicity	δC (ppm)
1-C	-	74.1
2-CHOH	3.70 - d	72.7
3-CHOH	3.73 - dd	71.4
4-CHOH	3.76 - dd	70.7
5-CHOH	3.78 - dd	75.7
6-CHOH	3.71 - d	69.2
7-CHOH	3.81 - d	60.7
8-CHO*i*Pr	3.54 - dd	63.1
9-CHOH	3.86 - dd	69.6
10-CHOH	4.22 - dd	69.5
11-CHOH	3.54 - d	60.5
12-C	-	95.8
13-CHOH	6.54 - s	97.5

14-C	-	135.1
15-CH2	1.39 - d	30.8
16-CH	3.26 - m	51.0
17-CH	6.69 - dd	136.0
18-CH	6.68 - dd	131
19-CHa	5.19 - d	92.0
19-CHb	4.61 - d	92.0
20-C	-	131.5
21-CH3	2.53 - s	17.0
22-CH	3.42 - m	62.4
23-CH3	1.43 - d	22.6

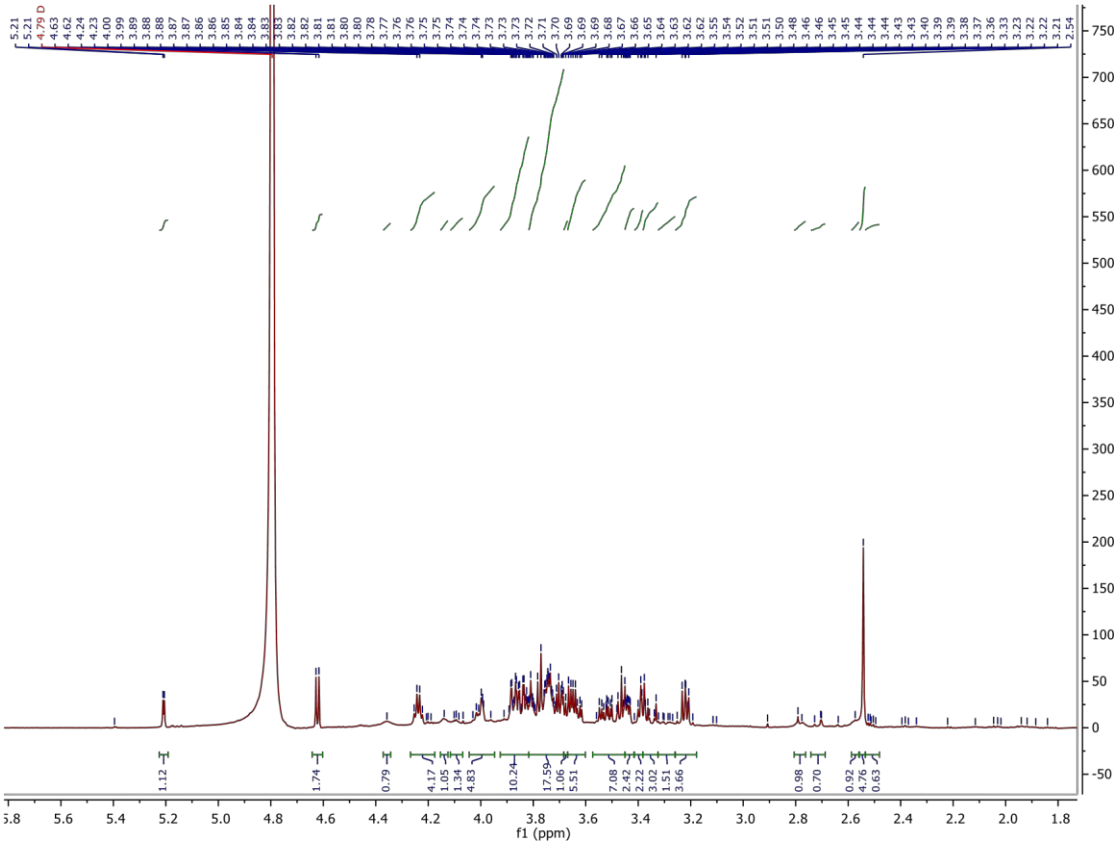


Figure 5.17 ^{1}H NMR spectra of compound 2

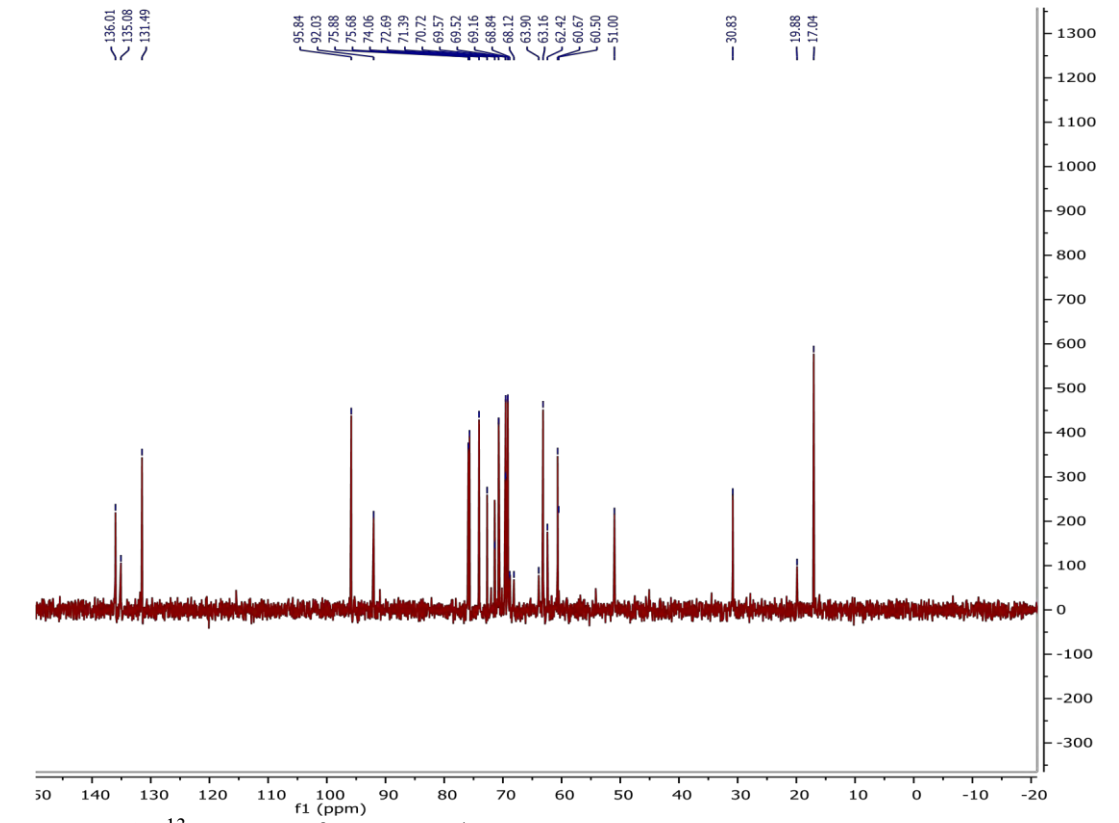


Figure 5.18 ^{13}C NMR of compound *2*

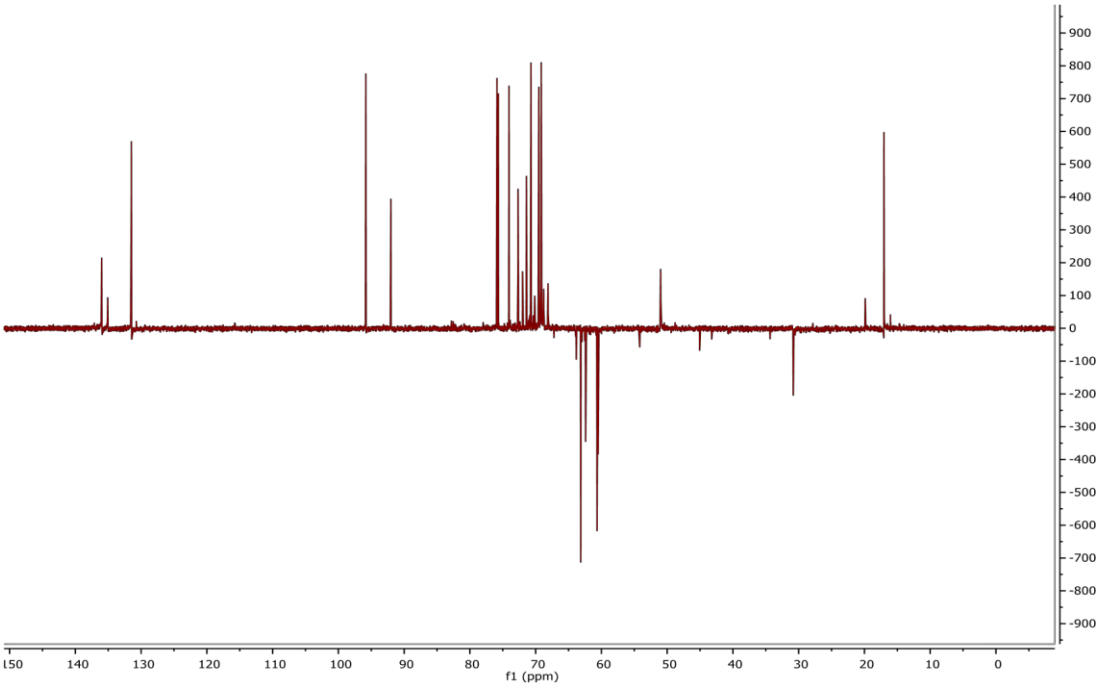


Figure 5.19 DEPT-135 of compound 2

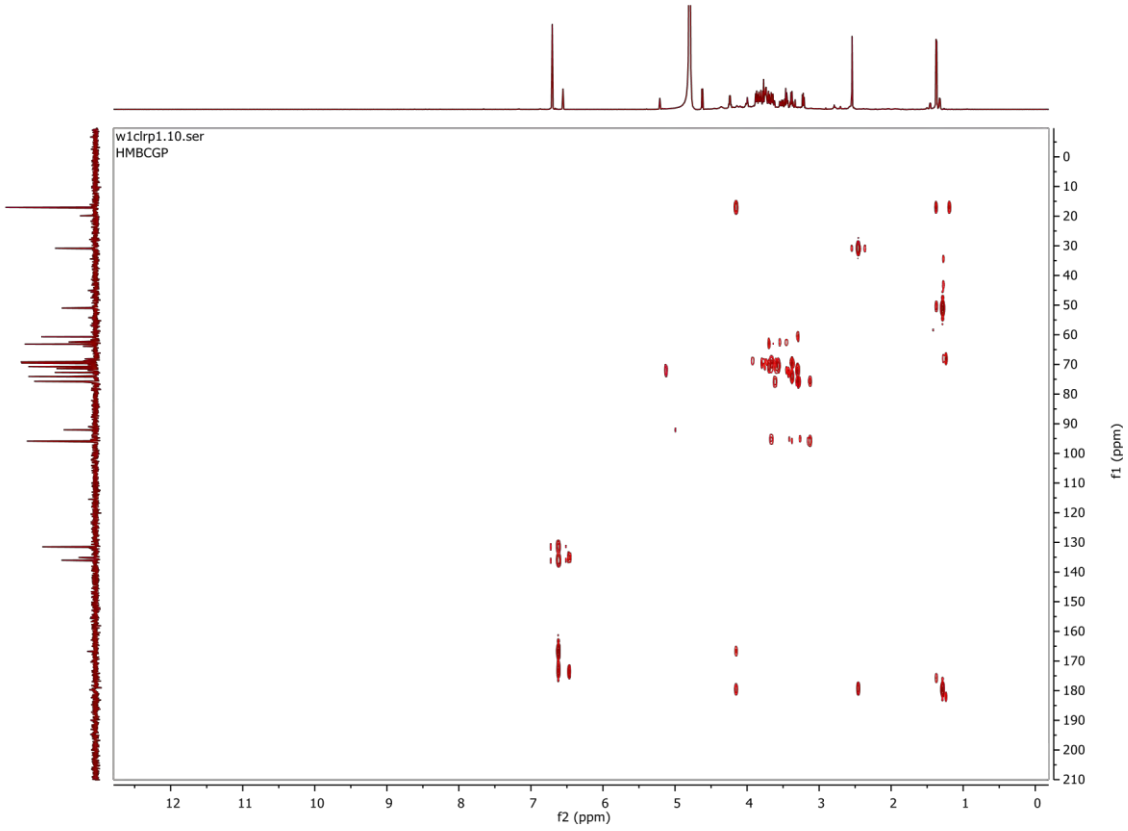


Figure 5.20 COSY of compound 2

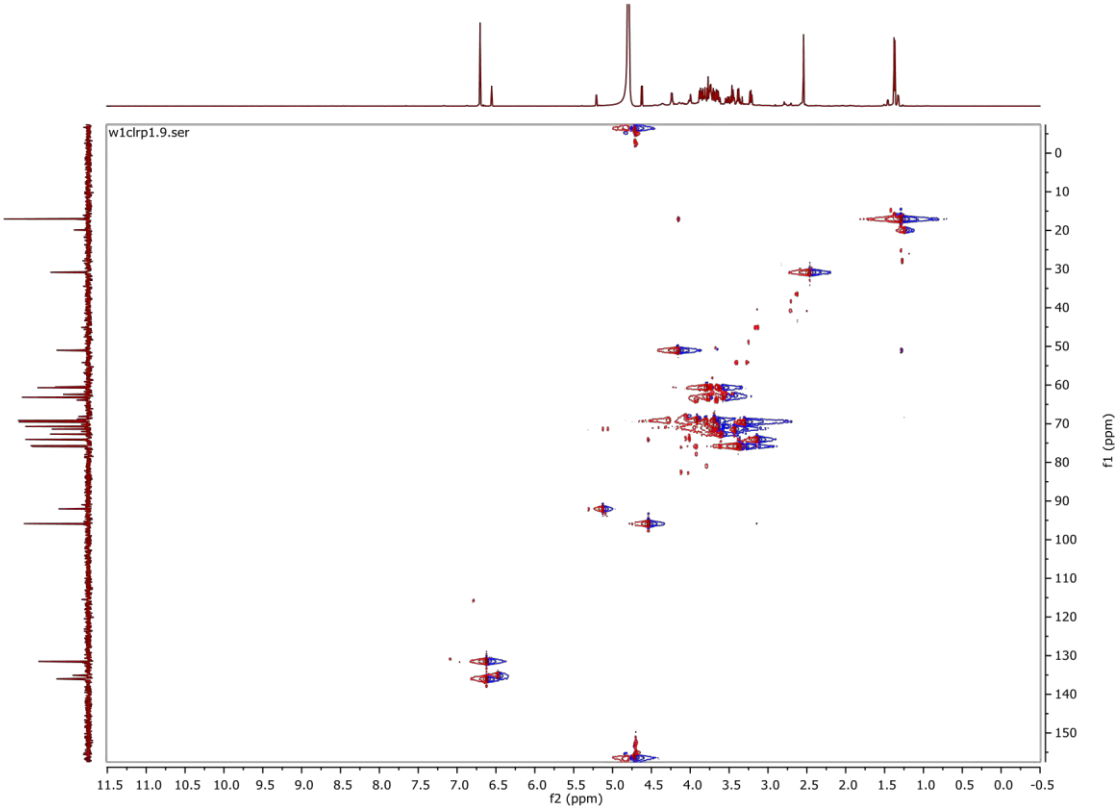


Figure 5.21 HMBC of compound 2

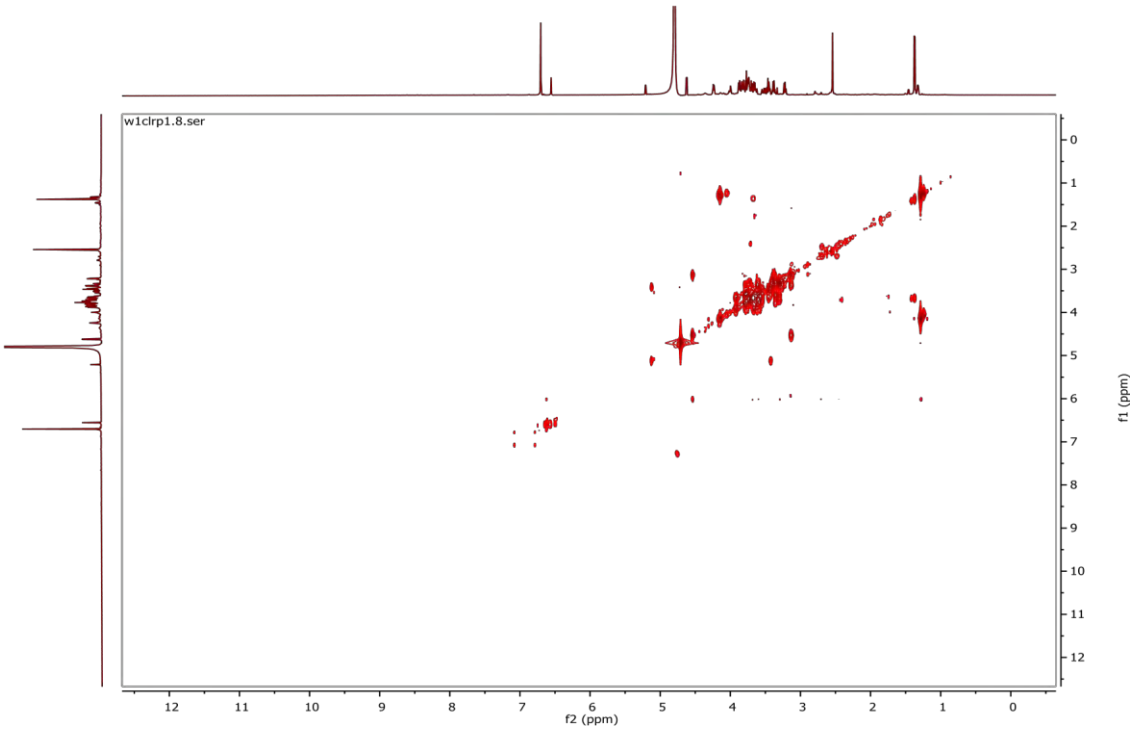


Figure5.22 HSQC of compound 2

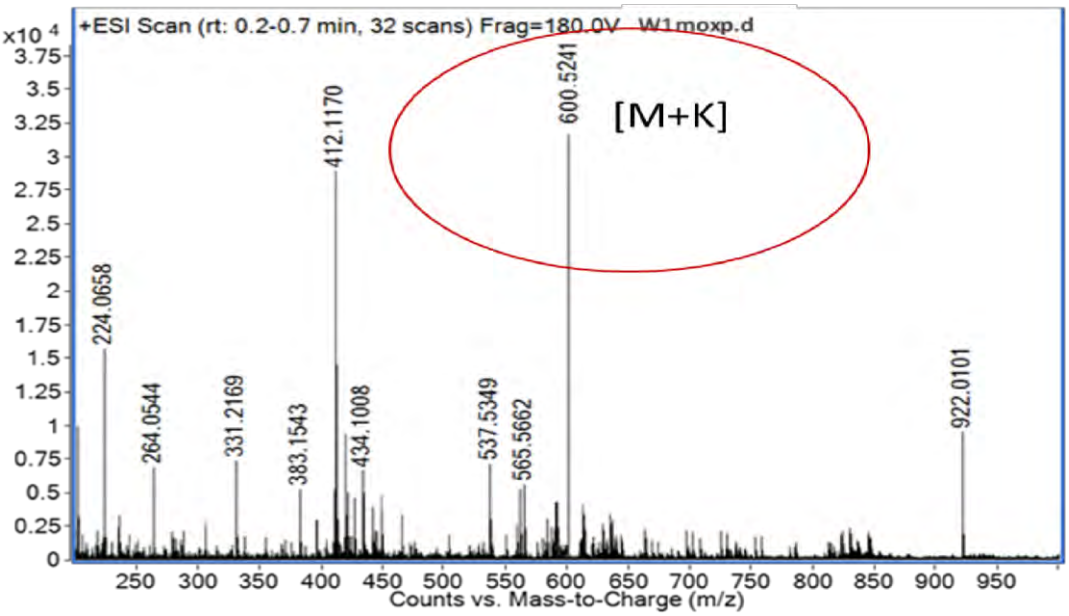
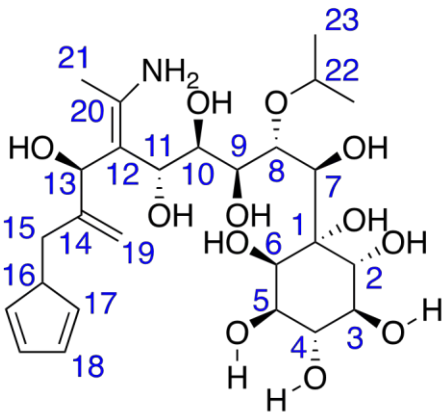


Figure 5.23 HRMS of compound 2



Chemical Formula: $C_{26}H_{43}NO_{12}$
Exact Mass: 561.3
Molecular Weight: 561.6
m/z: 561.3 (100.0%), 562.3 (28.1%), 563.3 (2.7%), 563.3 (2.5%), 563.3 (1.1%)
Elemental Analysis: C, 55.60; H, 7.72; N, 2.49; O, 34.18

Figure 5.24 Structure of compound 2

5.3.3 Isolation of Compound 3 from *Chaetomium* sp. NFW8

The compound isolated from the ethyl acetate extract of *Chaetomium* sp. NFW8 grown in the presence of clarithromycin is given name of compound 3.

5.3.3.1 Purification of Crude Extracts Obtained from *Chaetomium* sp. NFW8 Grown under the Stress of Clarithromycin

After purification of. Crude extract obtained from *Chaetomium* sp**.** NFW8 grown under Clarithromycin stress a total 25 fractions were collected, among these 05 fractions showed antibacterial activity against *S. aureus* and *E. coli.* The fraction 01 showed the maximum activity i.e. 17 mm against *E. coli* with respect to its control (i.e. 13 mm) whereas fraction number 01 showed the 21mm zone of inhibition with respect to its control i.e. 12 mm against *S. aureus.* Fraction number 2, 9 and 16 also showed antibacterial activities are presented in Figure 5.25.

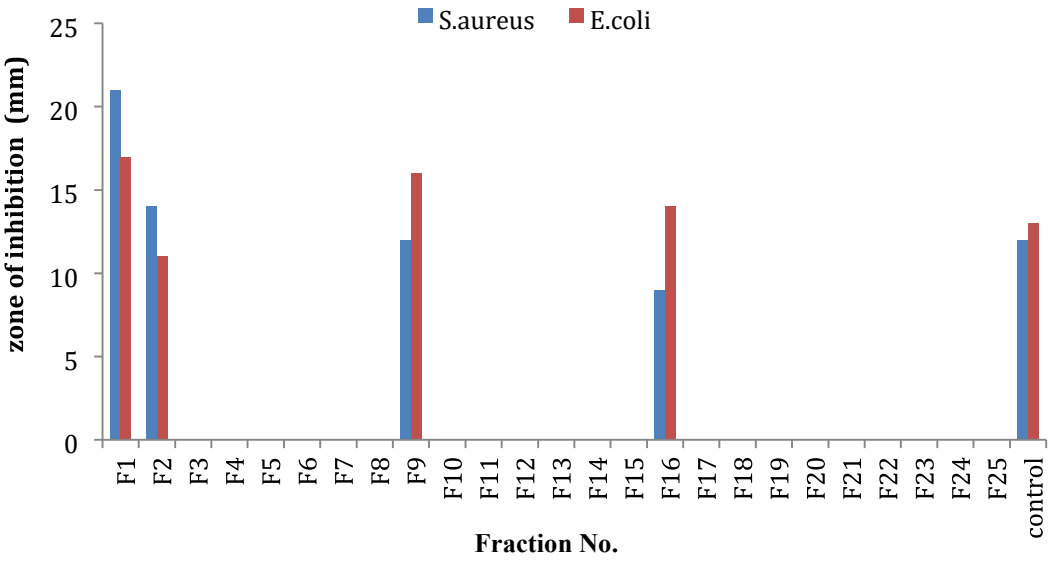


Figure 5.25 Antibacterial activity of fractions obtained from silica gel column chromatography of the crude extract of *Chaetomium* sp. NFW8 grown under the stress of clarithromycin

5.3.3.2 Preparative HPLC for *Chaetomium* sp. NFW8 Grown on Clarithromycin Emended Medium

HPLC chromatograms of pooled active fractions of *Chaetomium* sp. NFW8 grown in the presence of moxifloxacin and ethyl acetate extract of *Chaetomium* sp. NFW8 grown

without antibiotic was obtained as mentioned in methodology. The chromatograms in each case were comparatively analyzed to identify peaks in each extract. Figure 5.26 showing the HPLC chromatogram of ethyl acetate extract of *Chaetomium* sp. NFW8 grown on SDB without antibiotics supplementation. Figure 5.27 is the HPLC chromatogram of pooled active fraction obtained from column chromatography of ethyl acetate extract of *Chaetomium* sp. NFW8 grown on SDB supplemented with moxifloxacin. These chromatograms were compared, and a peak separately present in active fractions were labelled as 1 and collected and checked for antibacterial activity (10 mm against *S. aureus* and 11mm against *E. coli* in concentration of 0.1mg). Based on antibacterial activity and purity this peak at retention time around 35 minutes was elucidated for structure and name as compound 3.

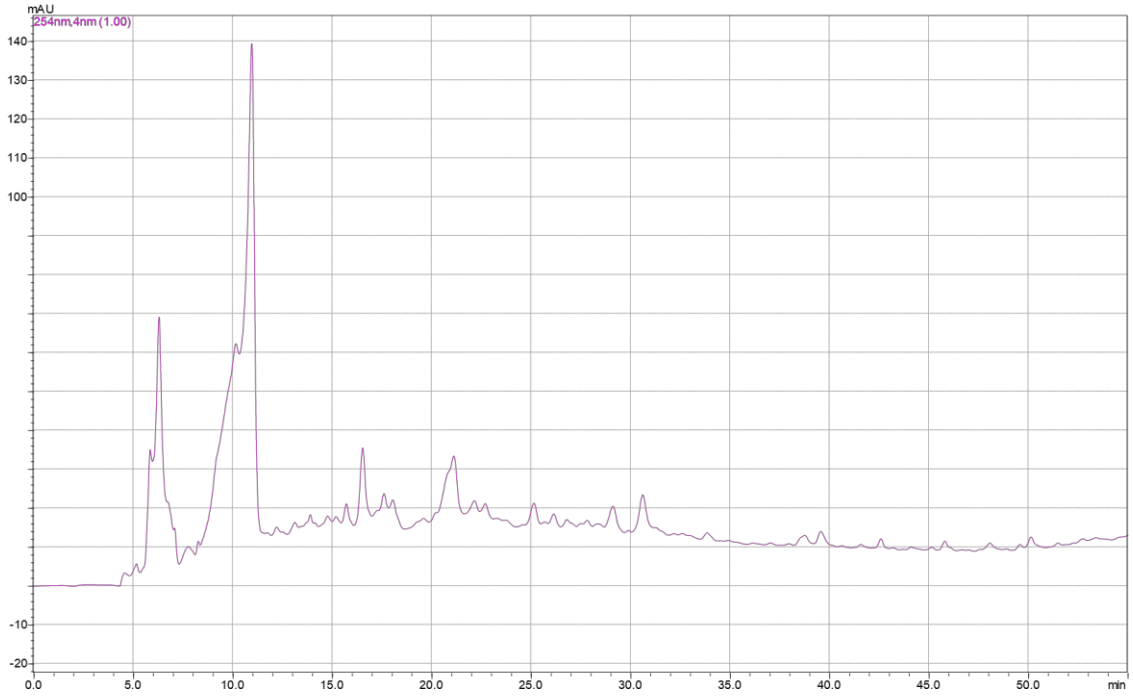


Figure 5.26 HPLC chromatogram of ethyl acetate extract of *Chaetomium* sp. NFW8 grown on SDB without antibiotics supplementation

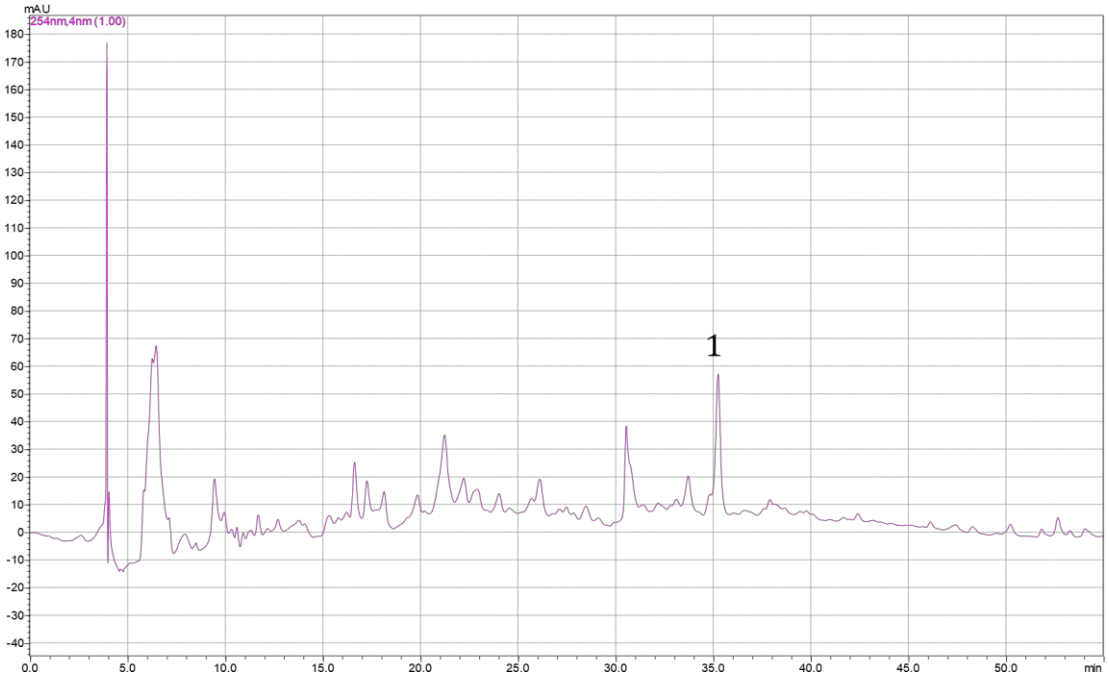


Figure 5.27 HPLC chromatogram of pooled active fractions of *Chaetomium* sp. NFW8 grown on SDB emended with clarithromycin

5.3.3.3 Structural Elucidation of Compound 3

Compound 3 was collected (4mg) and appeared as brown powder when dried. For characterization of compound 3, ESI-MS, 1D and 2D NMRs were taken. The compound was assembled according to mass spectra, proton, carbon, dept-135, COSY, HSQC, HMBC and NOSY NMR spectra, which are depicted below.

The ESI-MS spectrum in the positive mode showed a [M^+ + H] molecular ion peak at 381.3246 m/z with an addition of a hydrogen atom and made the [M+] ion peak 380.3 m/z. The proton and carbon NMR showed a characteristic para substituted aromatic ring structure with chemical shifts at 7.27-6.82 and 158.1-115.0 ppm for δ H and δ C respectively. The presence of the peaks between 4.09-3.54 for δ H and 73.8 and 62.4 ppm for δ C resulted from oxygenated functional groups such as hydroxyl or ether linkage. The evaluation of these peaks resulted in 6 methylene and 1 methine structural feature. This compound featured a quaternary dimethyl and a tertiary butyl moiety showing chemical shifts at 1.34 and 0.71 ppm for δ H and 32.5 and 32.4 ppm for δ C, respectively and was found to contain a primary and a secondary alcohol group.

After evaluating Dept-135, COSY, HSQC, NOESY and based on the HMBC correlation between carbon C-1 and 17-CH3 hydrogen and the corresponding correlations throughout the hydrocarbon chain as well as the correlation between the hydrogens of 6-CH_2 and the carbon C-4, it was evident that the compound was a novel cyclophane compound.

Furthermore, it contained 2 adjacent chiral centers and one of them was an all carbon quaternary chiral center. Thus, the molecular formula of compound 3 is $C_{22}H_{36}O_5$ and has a molecular weight of 380.3 g/mol. The structure determined as 11-(tert-butyl)-11-(hydroxymethyl)-13,13-dimethyl-2,5,8-trioxa-1(1,4)-benzenacyclotridecaphan-10-ol shown in Figure 5.39.

Table 5.3 Chemical Shifts of Compound 3

Position	δ H (ppm) - multiplicity	δ C (ppm)
1-C	-	144.3
2-CH	7.27 - d	128.4
3-CH	6.83 - d	115.0
4-C	-	158.1
5-O	-	-
6-CH2	4.10 - t	68.7
7-CH2	3.82 - t	71.1
8-O	-	-
9-CH2	3.69 - m	71.5
10-CH2	3.65 - m	71.9
11-O	-	-
12-CH2	3.54 - t	73.8
13-CH	3.62 - m	62.3

14-C	-	49.1
15-CH2	1.74 - s	58.2
16-C	-	39.0
17-CH3	1.34 - s	32.5
18-CH3	0.71 - s	32.4
19-C	-	33.3
20-CH2	3.66 - s	71.7
21-OH	-	-
22-OH	-	-

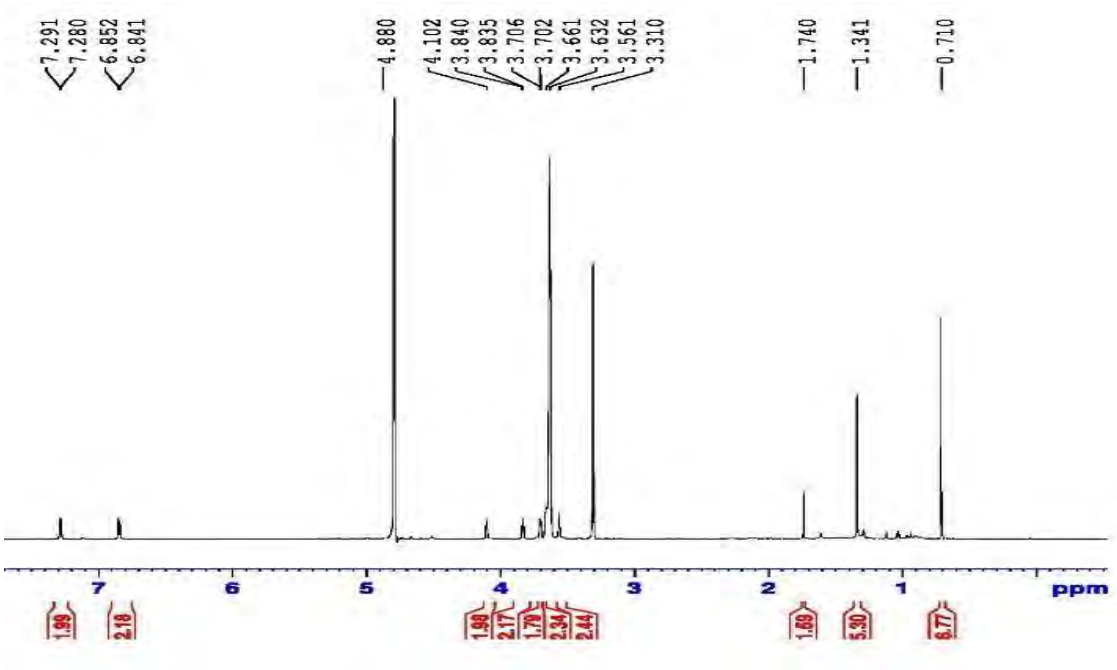


Figure 5. 28 ^{1}H NMR of compound 3

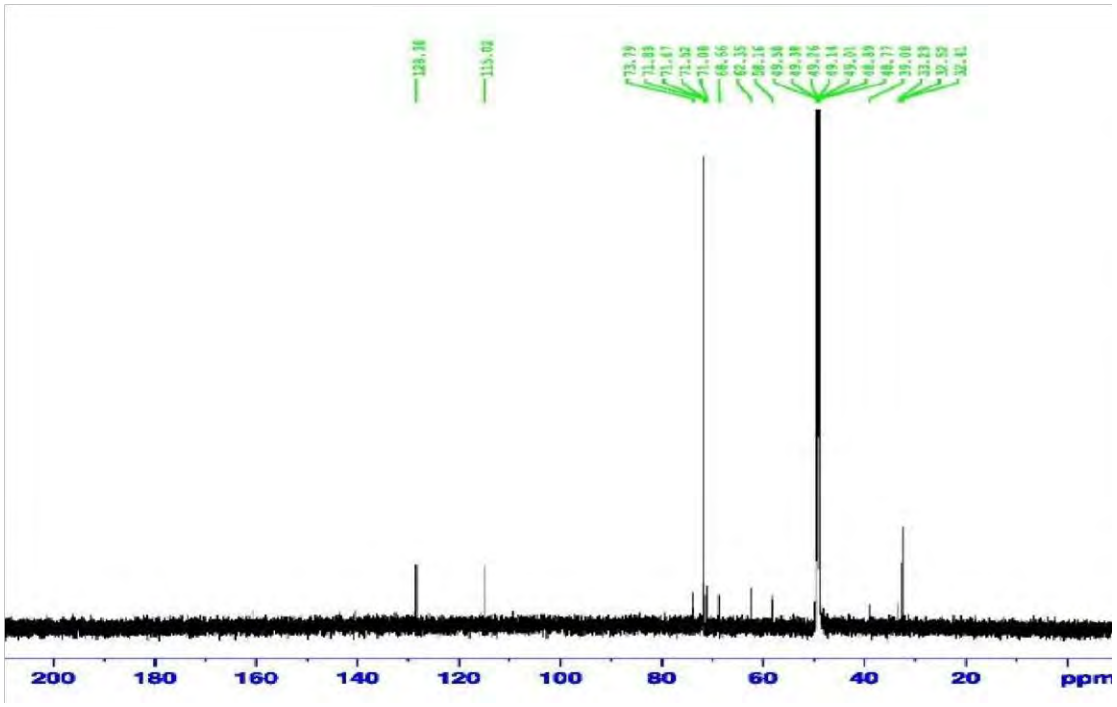


Figure 5.29 ^{13}C NMR of compound 3

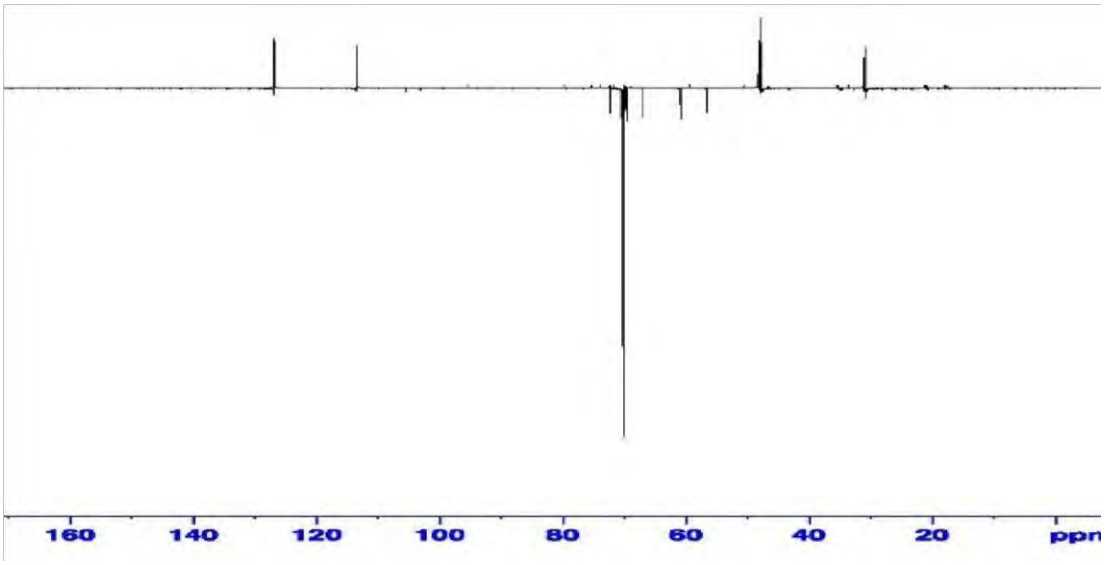


Figure 5.30 DEPT-135 of compound 3

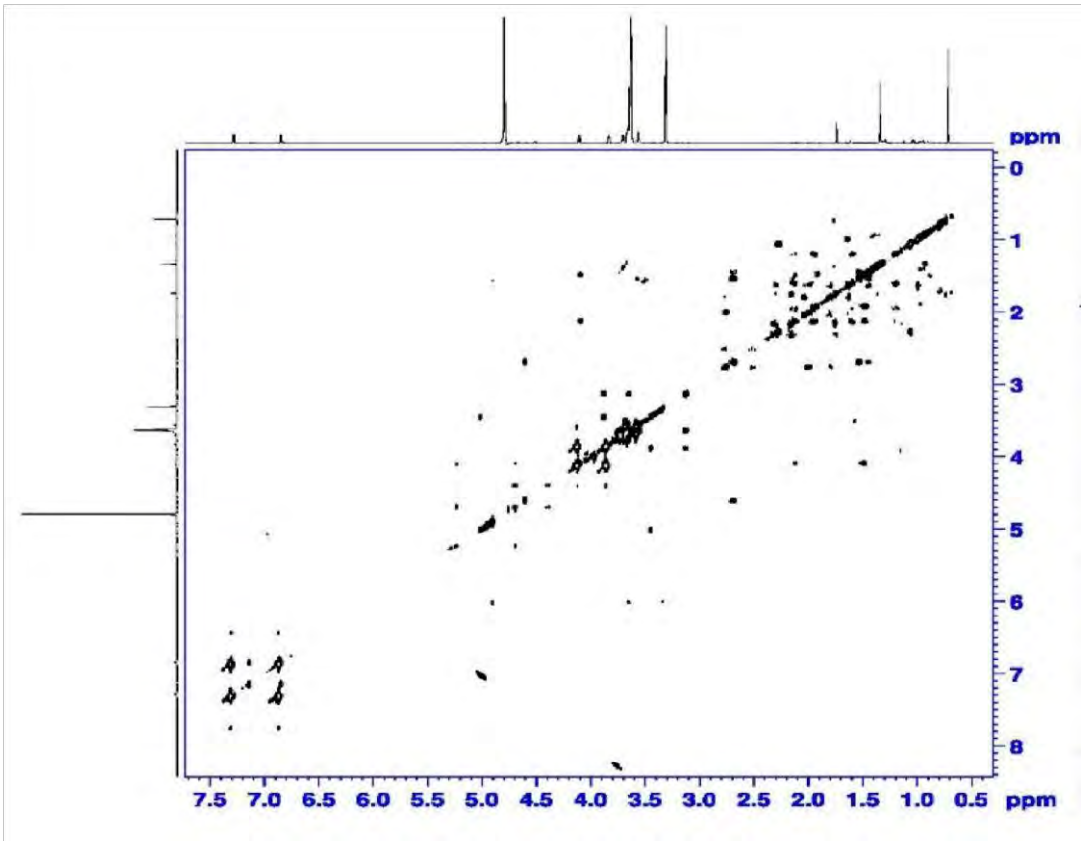


Figure 5.31 COSY of compound 3

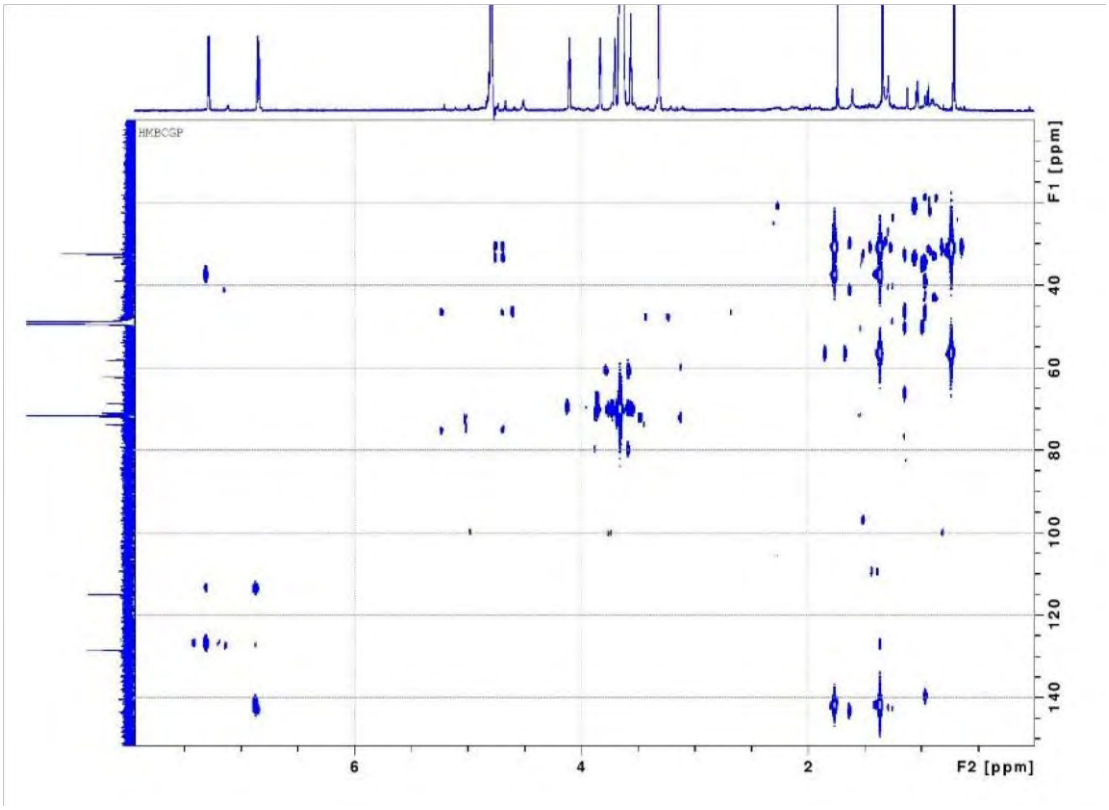


Figure 5.32 HMBC of compound 3

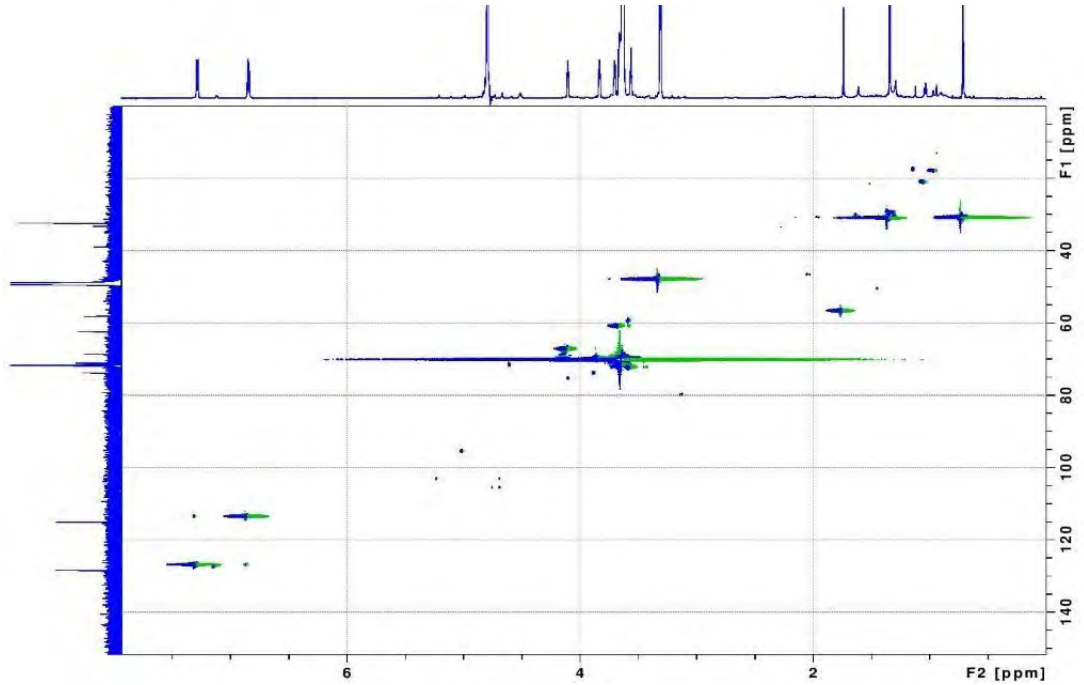


Figure 5.33 HSQC of compound 3

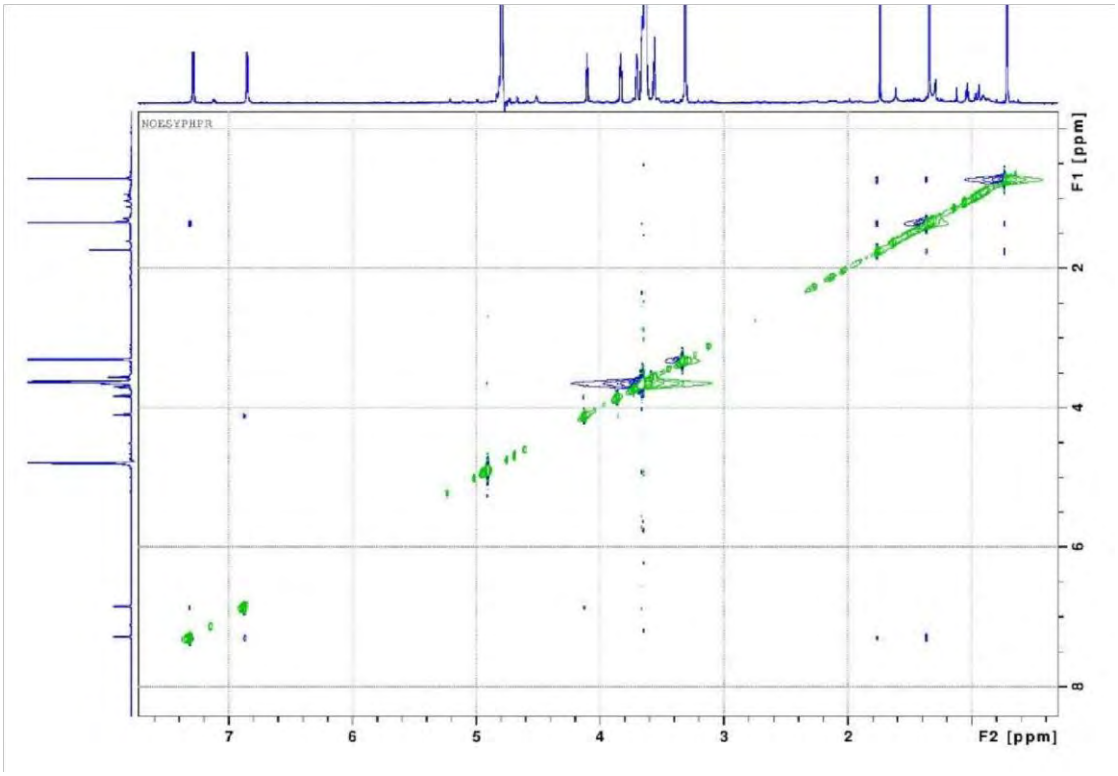


Figure 5.34 NOESY of compound 3

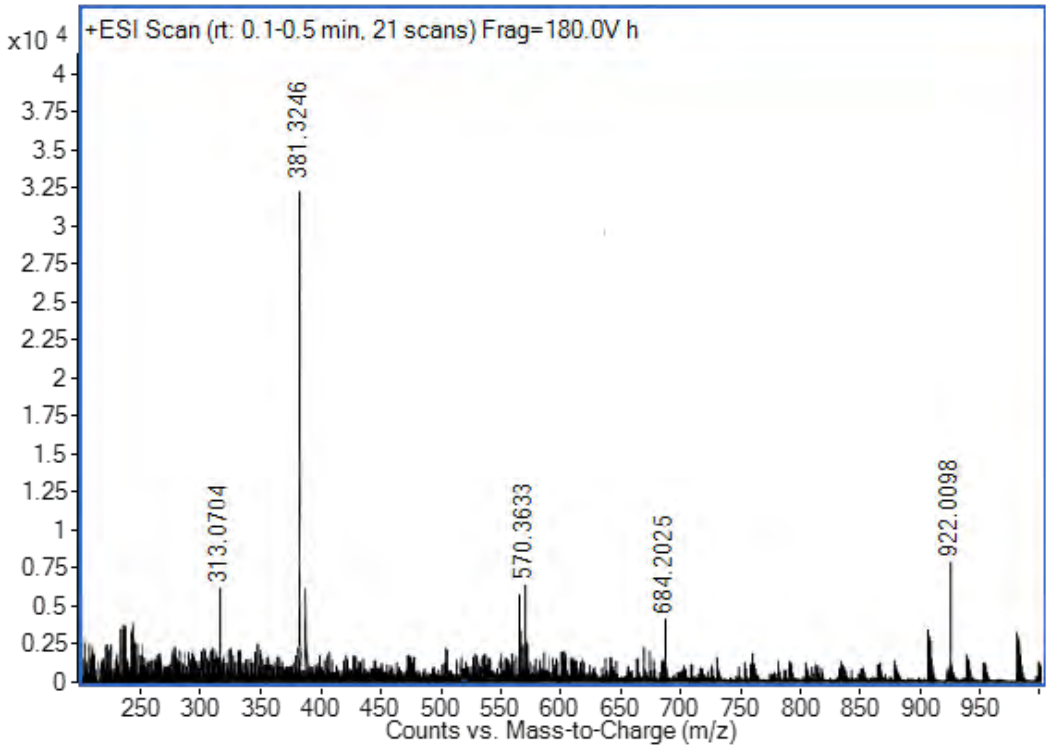
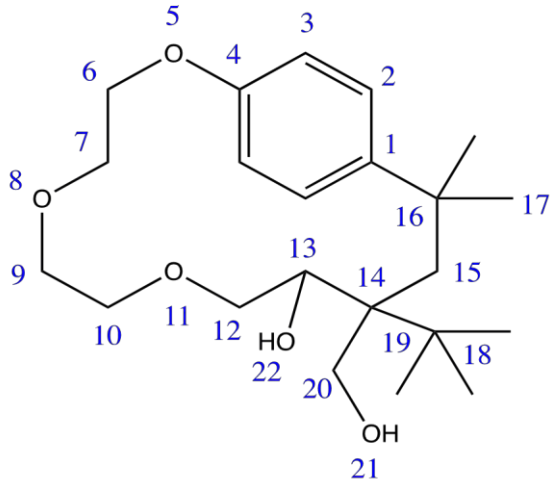


Figure 5.35 HRMS of compound 3



Chemical Formula: $C_{22}H_{36}O_5$
Exact Mass: 380.3
Molecular Weight: 380.5
m/z: 380.3 (100.0%), 381.3 (23.8%), 382.3 (2.7%), 382.3 (1.0%)
Elemental Analysis: C, 69.44; H, 9.54; O, 21.02

Figure 5.36 Structure of compound 3

5.3.4 Isolation of Compound 4 from *Chaetomium* sp. NFW8 Grown under the Stress of Moxifloxacin

The compound isolated from the ethyl acetate extract of *Chaetomium* sp. NFW8 grown in the presence of moxifloxacin is given name of compound 4.

5.3.4.1 Purification of Crude Extracts Obtained from *Chaetomium* sp. NFW8 Grown under the Stress of Moxifloxacin

A total number 25 fractions were collected after purification. Among all these fractions 04 fractions showed antibacterial activity against test bacterial strains i.e. *S. aureus* and *E. coli*. The fraction number 05 showed the maximum activity i.e. 18 mm against *S. aureus* with respect to its control (i.e. 9 mm) whereas fraction number 13 showed the 16 mm zone of inhibition with respect to its control (i.e. 11 mm) against *E. coli.* Fraction number 05 and 13 also exhibited antibacterial activities (Figure 5.37)

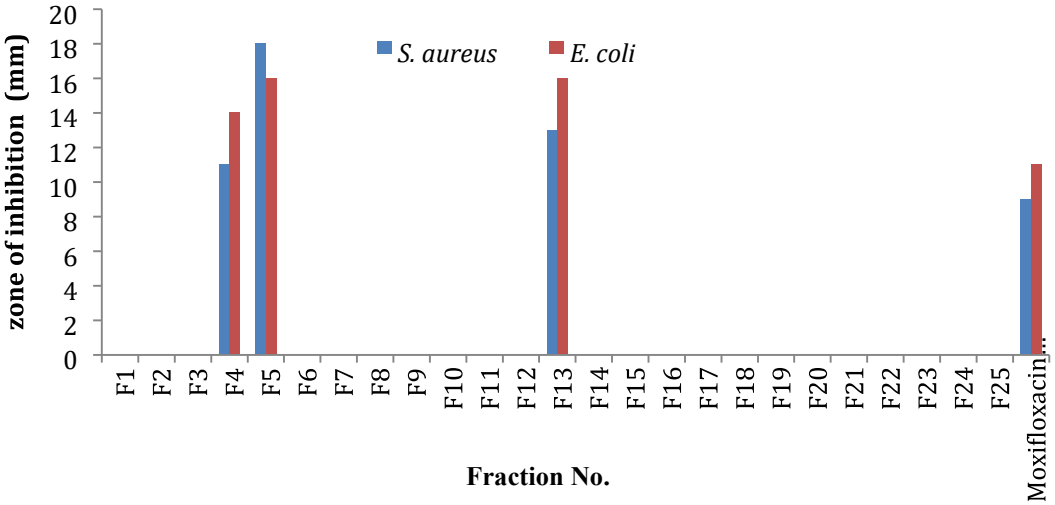


Figure 5.37 Antibacterial activity of fractions obtained from silica gel column chromatography of the crude extract of *Chaetomium* sp. NFW8 grown in the presence of moxifloxacin

5.3.4.2 Preparative HPLC for *Chaetomium* sp. NFW8 Grown in SDB Supplemented with Moxifloxacin

HPLC chromatograms of pooled active fractions of *Chaetomium* sp. NFW8 grown in the presence of moxifloxacin and ethyl acetate extract of *Chaetomium* sp. NFW8 grown without antibiotic was obtained as mentioned in methodology. The HPLC mobile phase and method for *Chaetomium* sp. NFW8 was the same as discussed in methodology. Figure 5.38 showing the HPLC chromatogram of ethyl acetate extract of *Chaetomium* sp. NFW8 grown on SDB without antibiotics supplementation. Figure 5.39 is the HPLC chromatogram of pooled active fraction obtained from column chromatography of ethyl acetate extract of *Chaetomium* sp. NFW8 grown on SDB supplemented with moxifloxacin and peak around retention time of 38 minutes collected based on antibacterial activity (7 mm against *S. aureus* and 7 mm against *E. coli* in concentration of 0.1mg).and purity was collected for structure elucidation and given the name as compound 4.

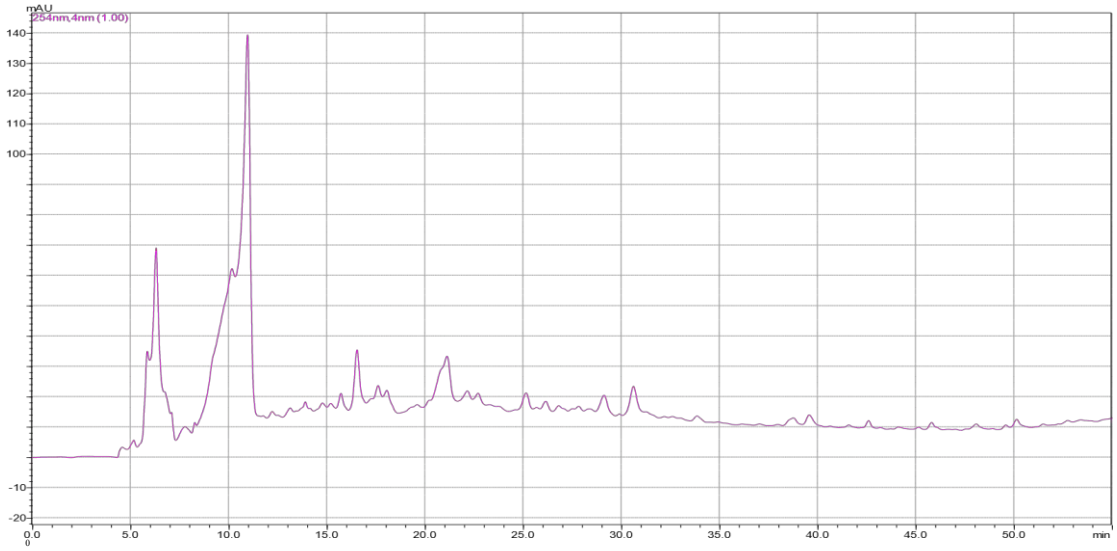


Figure 5.38 HPLC chromatogram of ethyl acetate extract of *Chaetomium* sp. NFW1 grown on SDB without antibiotics supplementation

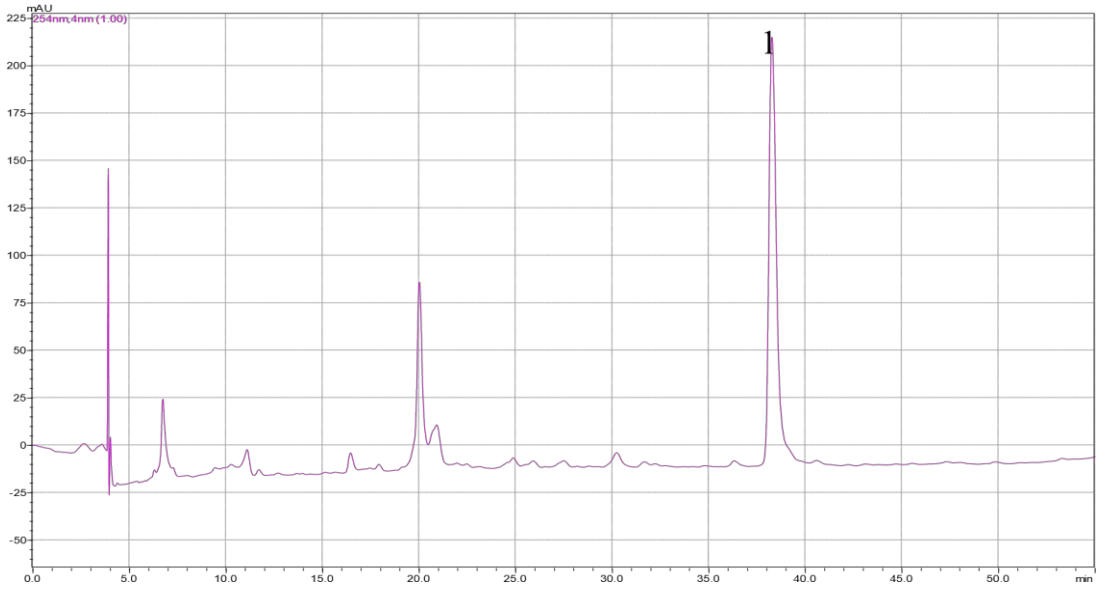


Figure 5.39 HPLC chromatogram of ethyl acetate extract of *Chaetomium* sp. NFW8 grown in the presence of moxifloxacin

5.3.4.3 Structure Elucidation of Compound 4

Compound 4 was collected (4mg) as white to off white powder when dried. For characterization of compound 4, ESI-MS, 1D and 2D NMRs were taken. The compound was assembled according to mass spectra, proton NMR, carbon NMR, dept-135, COSY, HSQC and HMBC NMR spectra, which are depicted below.

The ESI-MS spectrum in the positive mode showed a [M + H^+] molecular ion peak at 223.0932 m/z with an additional hydrogen ion, which makes the [M+] ion 222.09 m/z. The proton and carbon NMR showed a characteristic tri-substituted aromatic ring structure with chemical shifts at 8.50-7.90 and 124.8-147.9 ppm for δ H and δ C respectively. The presence of the peaks between 2.75-0.96 for δ H and 32.8 and 12.7 ppm for δ C resulted from the butyl aliphatic chain. Furthermore, this molecule has two carboxylic acid moieties in ortho position to each other and in ortho and para position relative to the aliphatic chain.

After evaluating dept-135, COSY and HSQC and based on the HMBC correlation between carbon C-1 and 8-CH hydrogen, the correlation between C-2 carbon and 5CH hydrogen, and the corresponding correlations throughout the hydrocarbon chain as well as the correlation between the C-6 carbon and 9-CH_2 hydrogen, it was evident that the compound was a compound 4-n-butylphthalic acid.

Thus, the evaluation of these peaks resulted in a compound with a tri substituted aromatic ring, having two carboxylic acids and a butane aliphatic rest as outlined in figure 5.52. The molecular formula of compound 4 is $C_{12}H_{14}O_4$ and has a molecular weight of 222.09 g/mol.

Table 5.4: Chemical Shifts of Compound 4

Position	δ H (ppm) - multiplicity	δ C (ppm)
1-CO_2H	-	165.8
2-CO_2H	-	163.3
3-C	-	124.8
4-C	-	124.8
5-CH	8.50 - d	145.4
6-C	-	147.9
7-CH	7.90 - dd	142.9
8-CH	8.11 - d	138.5
9-CH_2	2.75 - t	32.8
10-CH_2	1.65 - q	32.1
11-CH_2	1.39 - m	21.9
12-CH_3	0.96 - t	12.7

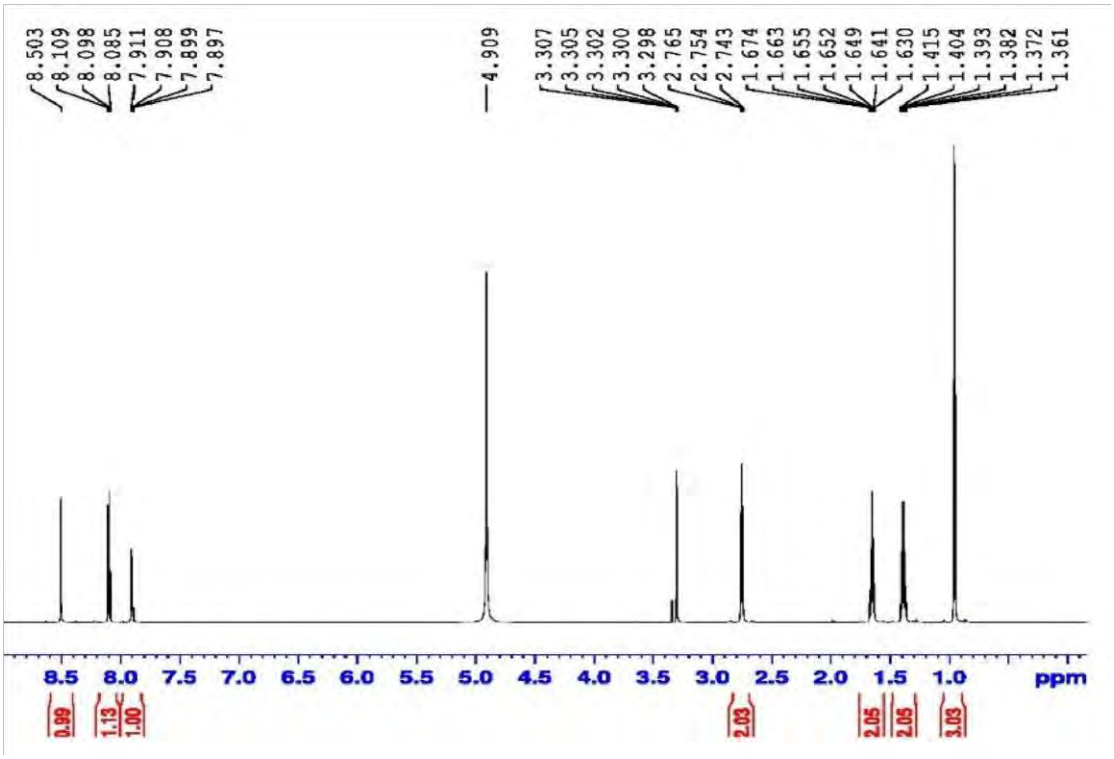


Figure 5.40 ^{1}H NMR of compound 4

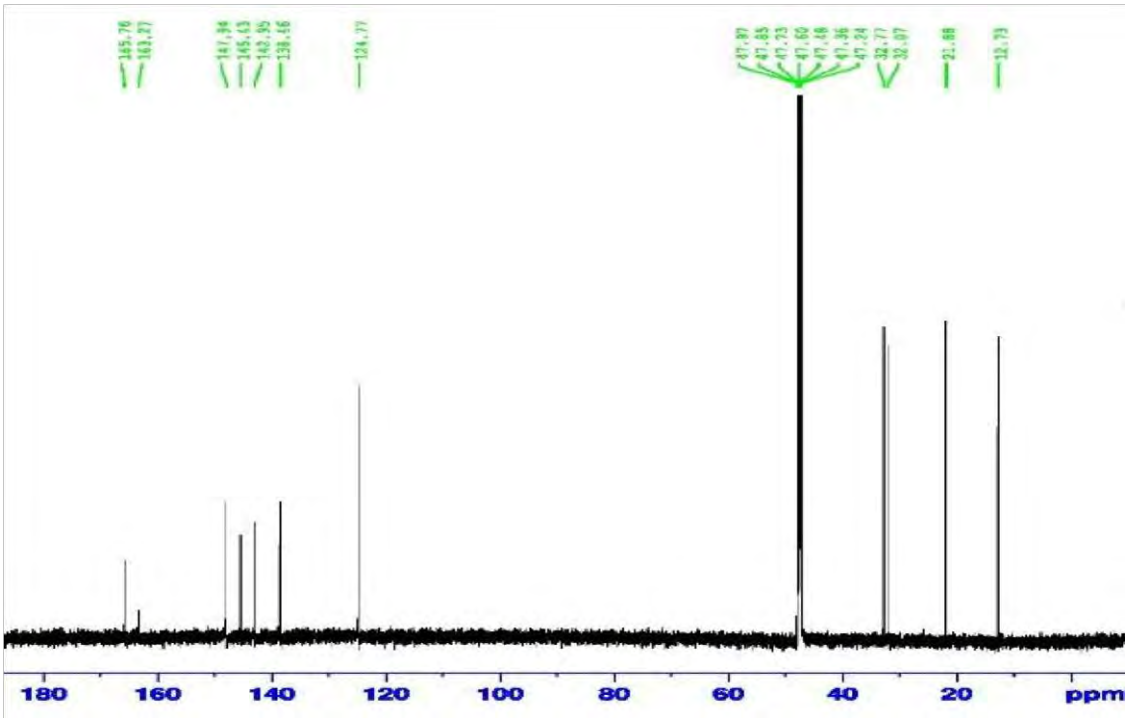


Figure 5.41 ^{13}C NMR of compound 4

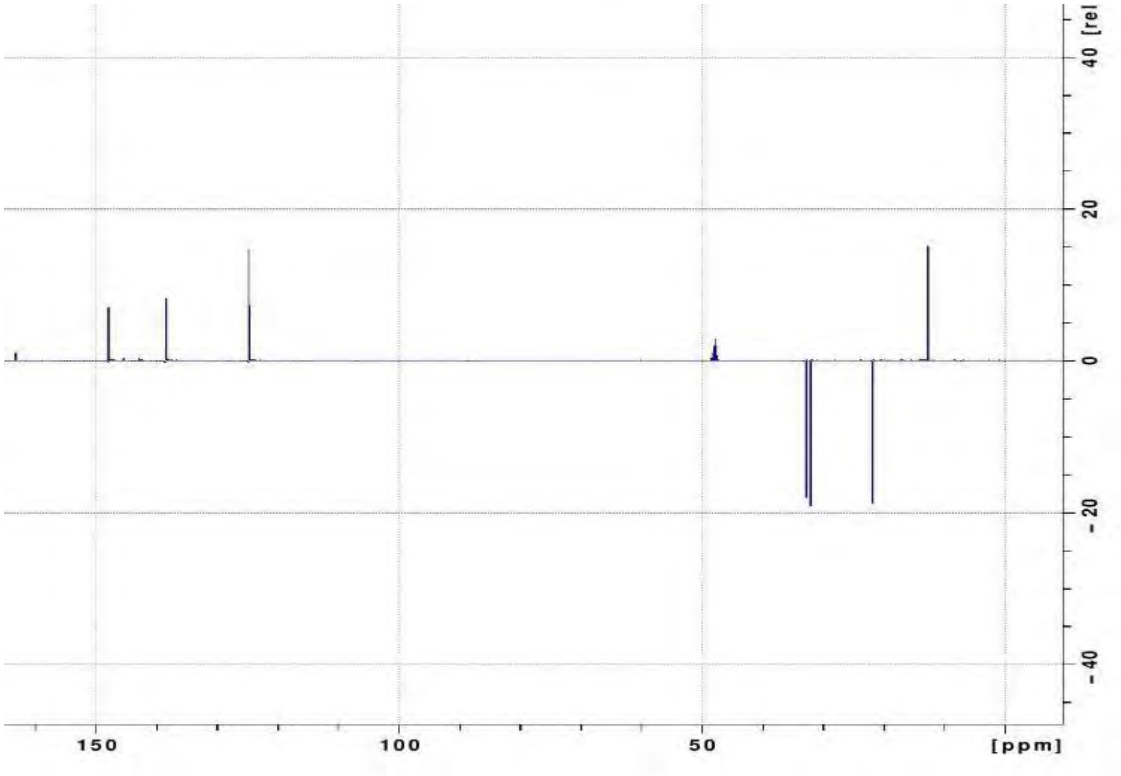


Figure 5.42 DEPT-135 of compound 4

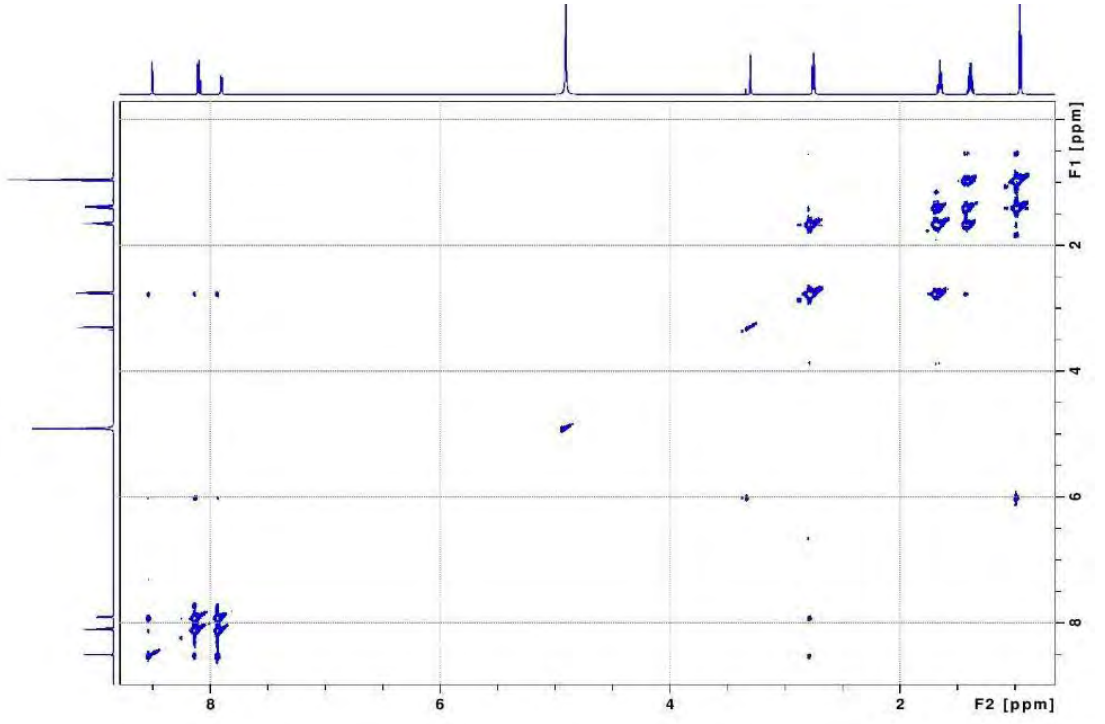


Figure 5.43 COSY of compound 4

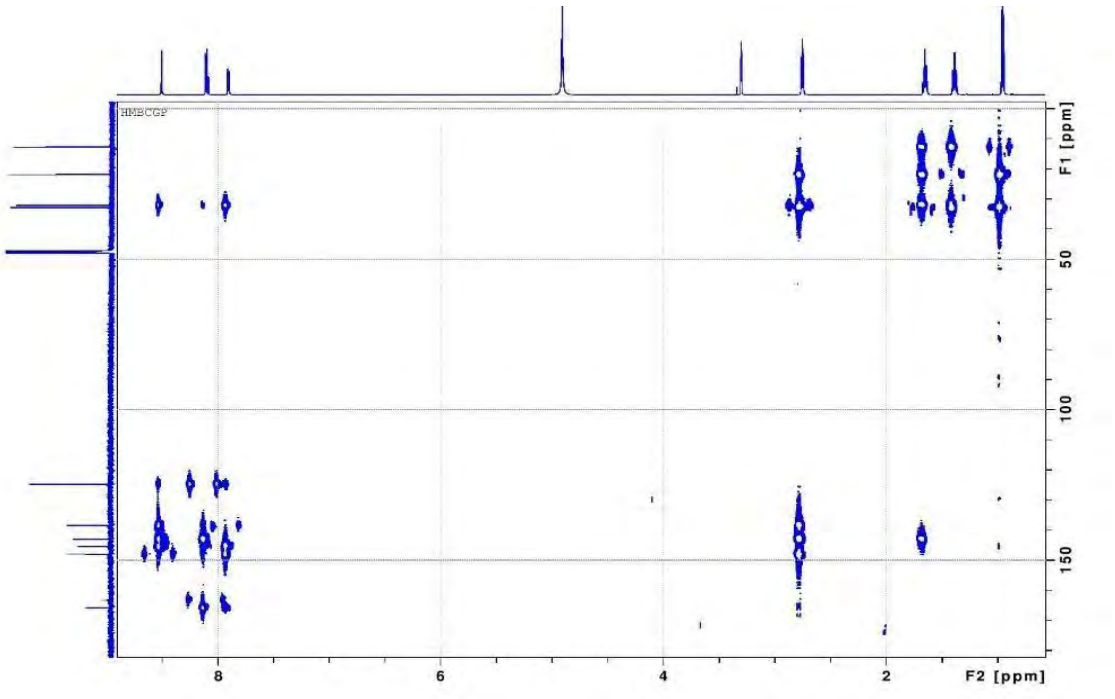


Figure 5.44 HMBC of compound 4

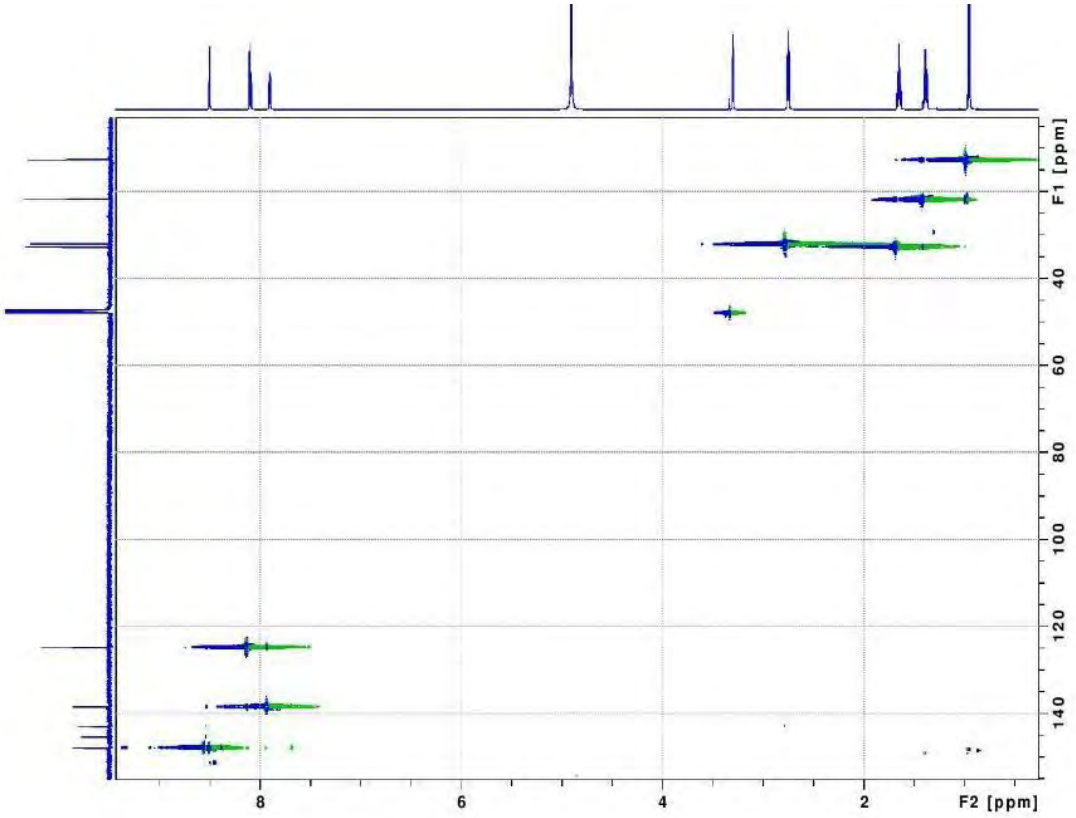


Figure 5.45 HSQC of compound 4

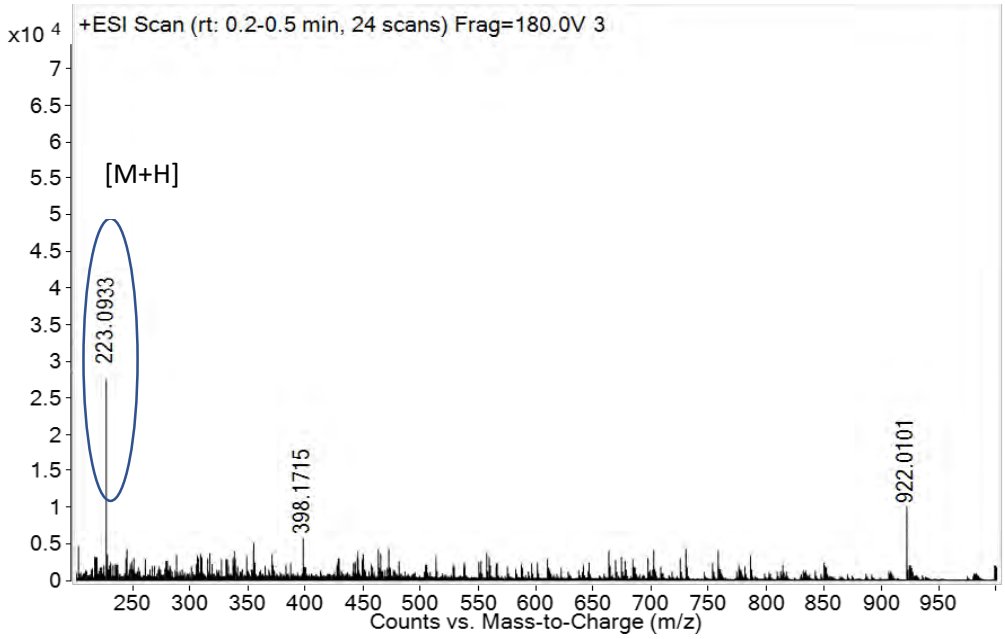
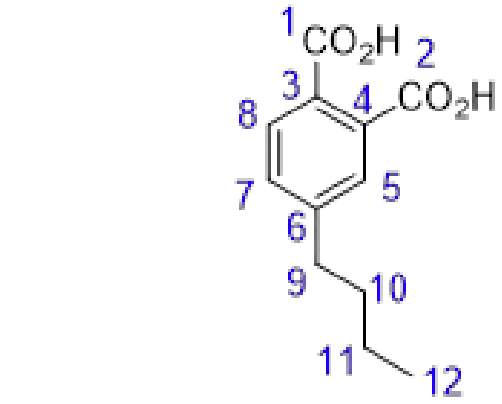


Figure 5.46 HRMS of compound 4



Chemical Formula: $C_{12}H_{14}O_4$
Exact Mass: 222.09
Molecular Weight: 222.24
m/z: 222.09 (100.0%), 223.09 (13.0%)
Elemental Analysis: C, 64.85; H, 6.35; O, 28.80

Figure 5.47 Structure of compound 4

5.3.5 Isolation of Compound 5 from *Fusarium oxysporum* NFW16

The compound isolated from the ethyl acetate extract of *Fusarium oxysporum* NFW16 grown in the presence of clarithromycin is given name of compound 5.

5.3.5.1 Purification of Crude Extracts Obtained from *Fusarium oxysporum* NFW16 Grown under the Stress of Clarithromycin

The crude extract obtained was purified using normal phase column chromatography using mobile phases from low polarity to high polarity. A total of 25 fractions were obtained and checked for their antibacterial activity against ATCC strains of *E. coli* and *S. aureus.*

Among the 25 fractions 04 fractions showed antibacterial activity against both the test strains. Fraction 01 showed the maximum activity i.e., 17mm zone of inhibition against *E. coli* with respect to its control (i.e.13mm) whereas it showed 21mm zone of inhibition with respect to its control (i.e. 12 mm) against *S. aureus.* Fraction number 2, 9 and 16 also have antibacterial activities are shown in Figure 5.48.

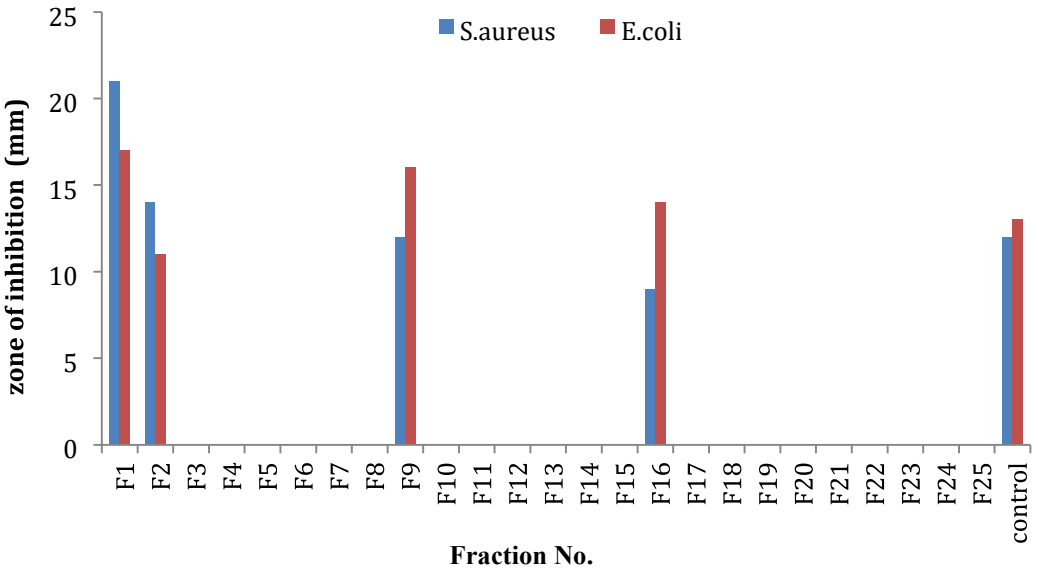


Figure 5.48 Antibacterial activity of fractions obtained from silica gel column chromatography of the crude extract of *Fusarium oxysporum* NFW16 grown under the stress of clarithromycin

5.3.5.2 Preparative HPLC for *Fusarium oxysporum* NFW16 Grown in SDB Supplemented with Clarithromycin

HPLC chromatograms of pooled active fractions of *Fusarium oxysporum* NFW16 grown in the presence of moxifloxacin and ethyl acetate extract of *Fusarium oxysporum* NFW16 grown without antibiotic was obtained as mentioned in methodology. The chromatograms in each case were comparatively analyzed to identify peaks in each extract. Figure 5.49 is showing the HPLC chromatogram of ethyl acetate extract of *Fusarium oxysporum* NFW16 grown on SDB without antibiotics supplementation. Figure 5.50 is the HPLC chromatogram of pooled active fraction obtained from column chromatography of ethyl acetate extract of *Fusarium oxysporum* NFW16 grown on SDB supplemented with clarithromycin. The peak number 4 collected based on purity and antibacterial activity (9 mm against *S. aureus* and 8 mm against *E. coli* in concentration of 0.1mg) was collected for structure elucidation and given the name as compound 4 having retention time around 27.5 minutes. This compound was initially named as compound 5.

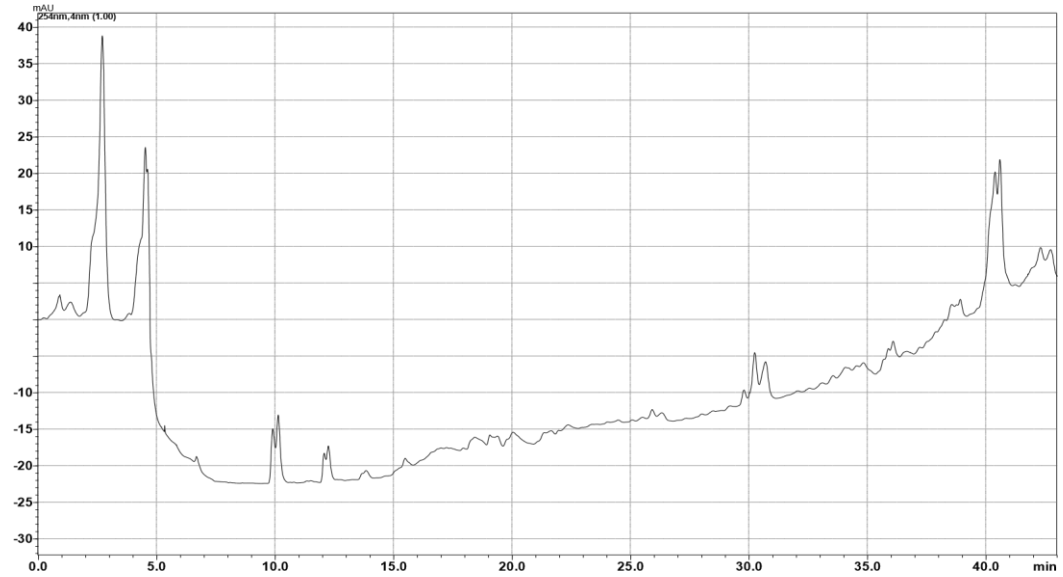


Figure 5.49 HPLC chromatogram of ethyl acetate extract of *Fusarium oxysporum* NFW16 grown on SDB without antibiotics supplementation

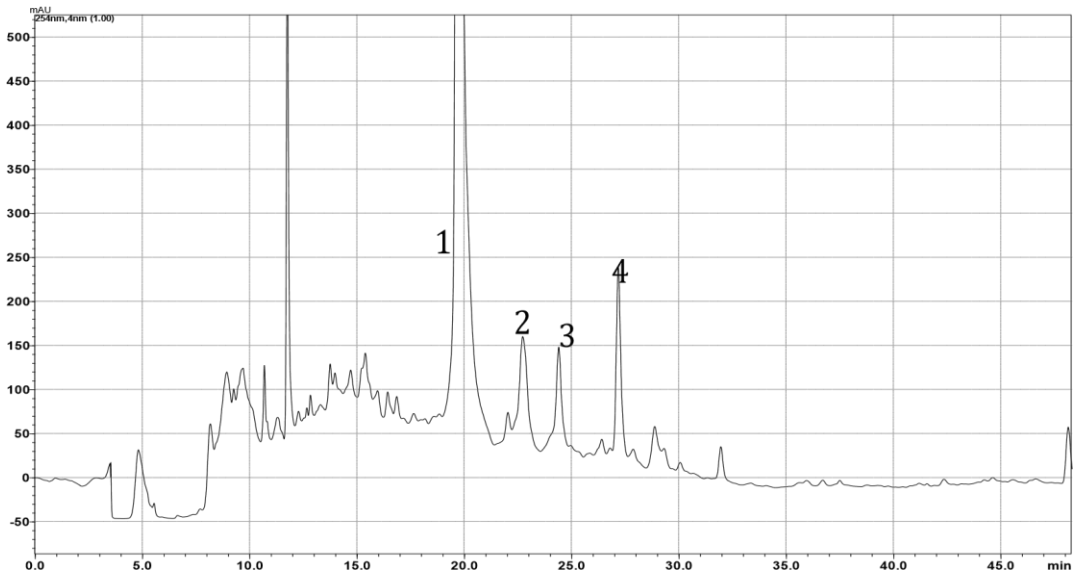


Figure 5.50 HPLC chromatogram of ethyl acetate extract of *Fusarium oxysporum* NFW16 grown on SDB without antibiotics supplementation

5.3.5.3 Structure Elucidation of Compound 5

Compound 5 was collected (5mg) as yellowish powder when dried. For characterization of compound 5, ESI-MS, 1D and 2D NMRs were taken. The compound was assembled according to mass spectrum, proton, carbon, dept-135, COSY, HSQC and HMBC NMR spectra, which are depicted below.

The ESI-MS spectrum in the positive mode showed a [M + $2Na^+$] molecular ion peak at 222.0497 m/z with two additional Sodium ions, which makes the [M+] ion 177.08 m/z. The proton and carbon NMR showed a characteristic conjugated tri unsaturated alkene pattern aromatic ring structure with chemical shifts at 8.10-5.01 and 149.6-126.0 ppm for δ H and δ C, respectively. The shift of the aliphatic and olefinic peaks at 2.85 and 5.84 for δ H and 35.8 and 147.2 for δ C resulted from nitrogen atom linking them together. Furthermore, this molecule has a stereo center at the α-position to the carboxylic acid. After evaluating Dept-135, COSY and HSQC and based on the HMBC correlation between carbon C-5 and 9-CH hydrogen, the corresponding correlations throughout the hydrocarbon chain as well as the correlation between the hydrogens of 6-CH_2 and the carbon C-10, it was evident that the compound was a unique compound.

Thus, the evaluation of these peaks resulted in a compound with a 5, 7 fused ring system, having one chiral center, one secondary nitrogen atom, three alkenes, 2 methylene and 1 carboxylic acid structural features as outlined in Figure 5.63. The molecular formula of compound 5 is $C_{10}H_{11}NO_2$ and has a molecular weight of 177.08 g/mol and chemically named as (S)-1,2,3,6-tetrahydrocyclopenta[d]azepine-6-carboxylic acid.

Table 5.5: Chemical Shifts of Compound 5

Position	δ H (ppm) - multiplicity	δC (ppm)
1-COOH	-	167.4
2-CH	4.98 - d	116.5
3-CH	7.90 - dd	143.2
4-CH	8.10 - d	139.9
5-C	-	149.6
6-CH_2	2.43 - dd	33.1
7-CH_2	2.85 - t	35.8
8-CH	5.84 - d	147.2
9-CH	5.01 - d	126.0
10-C	-	138.1

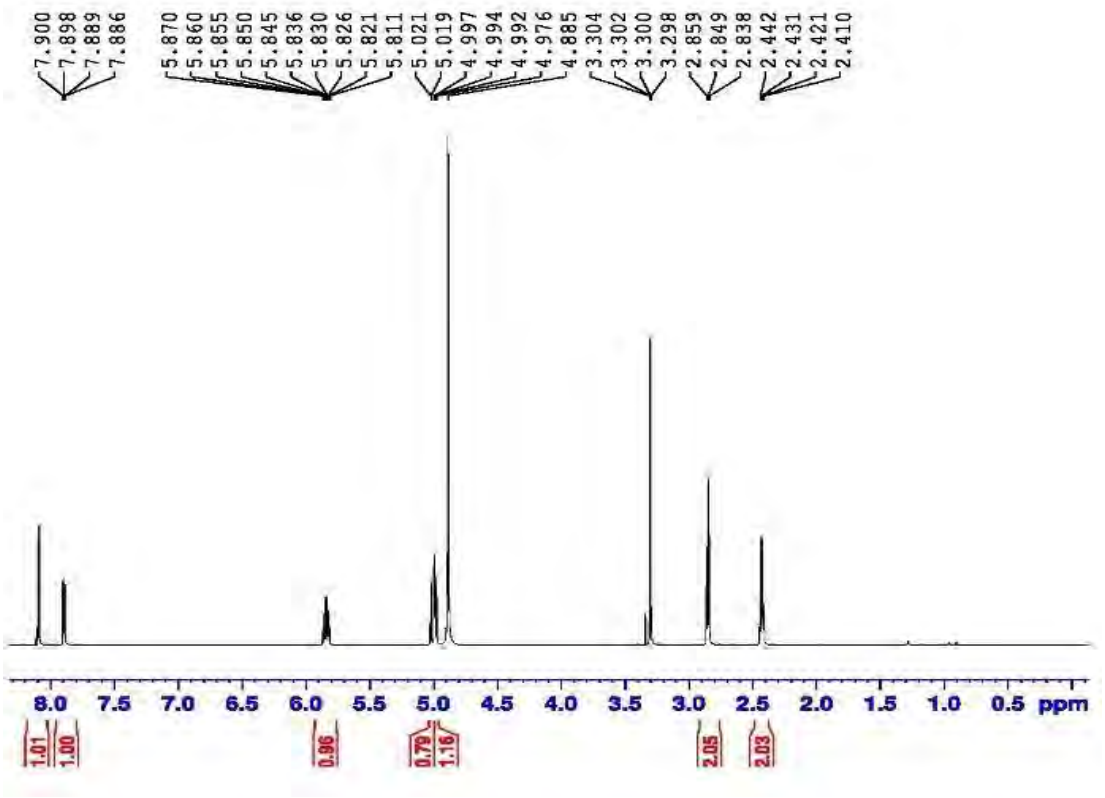


Figure 5.51 ^{1}H NMR of compound 5

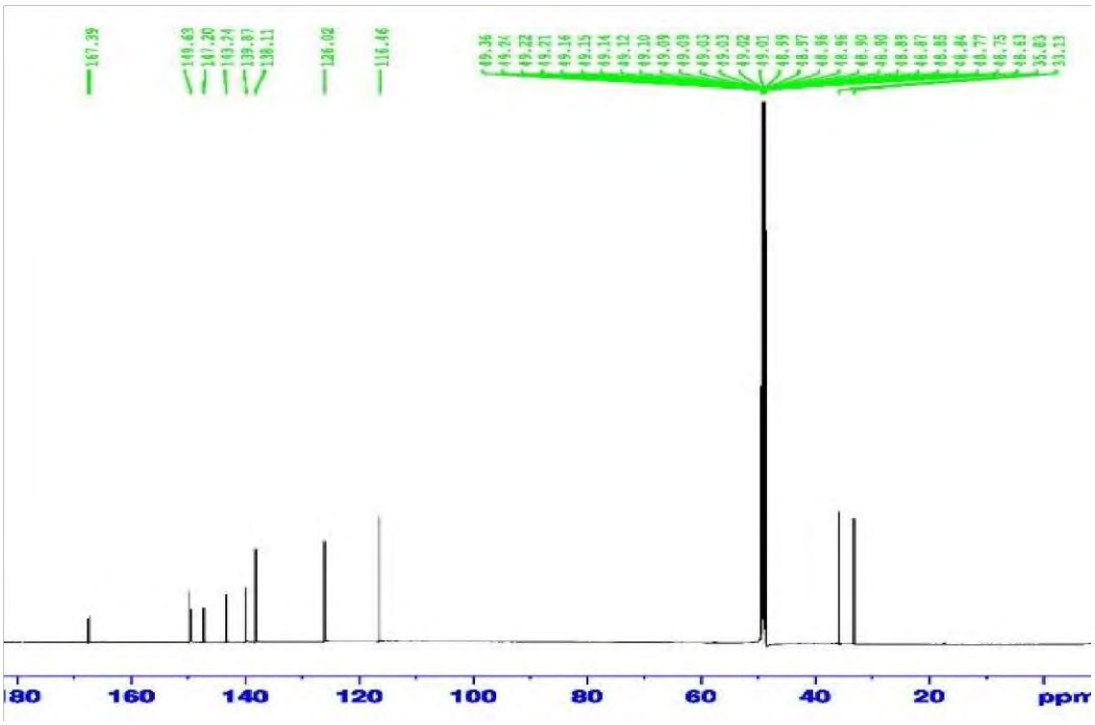


Figure 5.52 ^{13}C NMR of compound 5

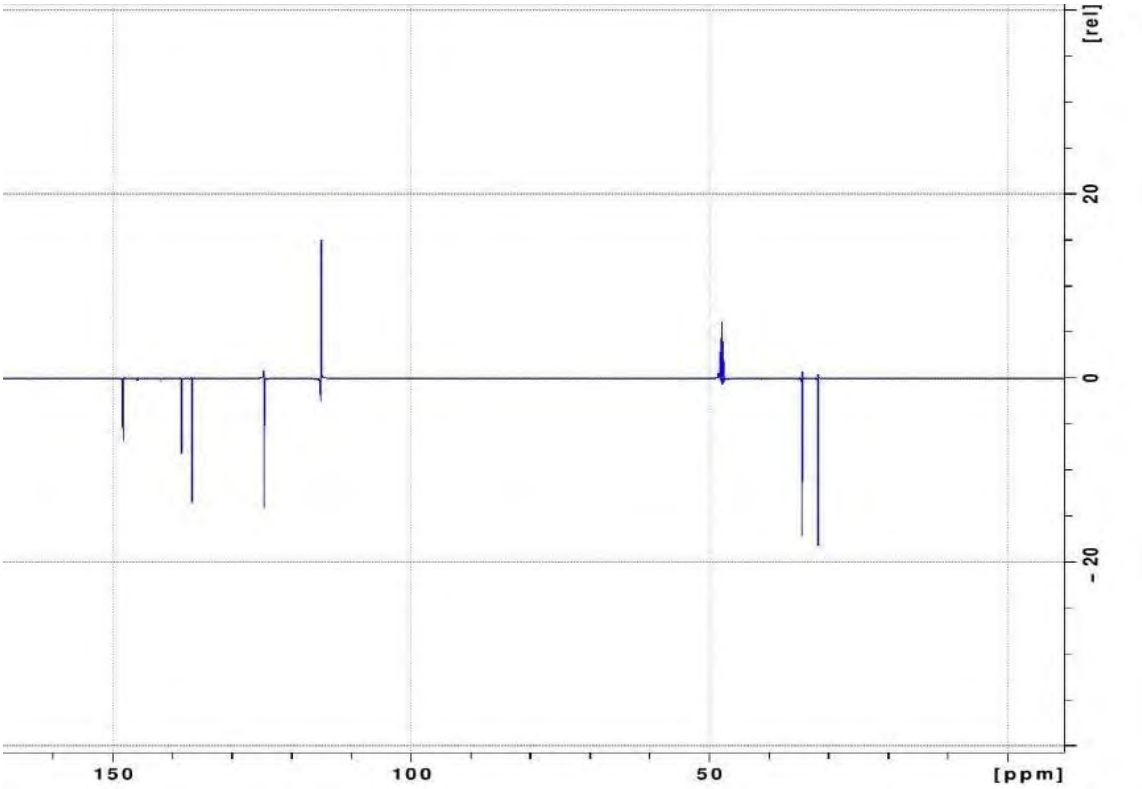


Figure 5.53 DEPT-135 of compound 5

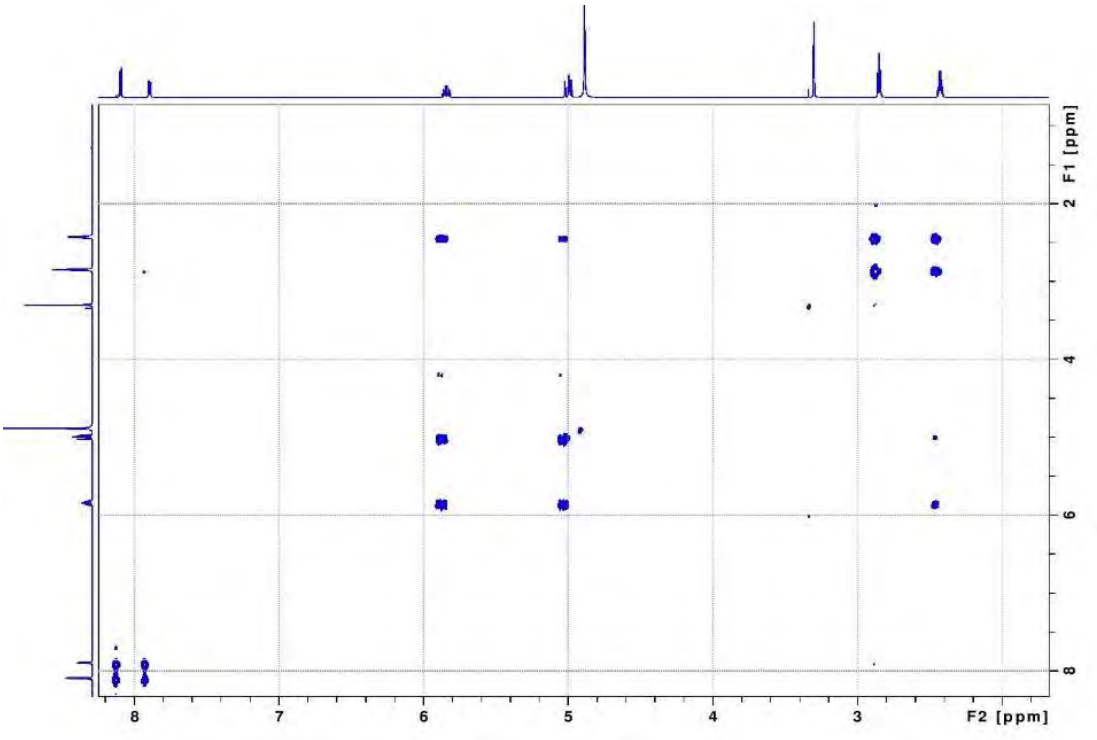


Figure 5.54 COSY of compound 5

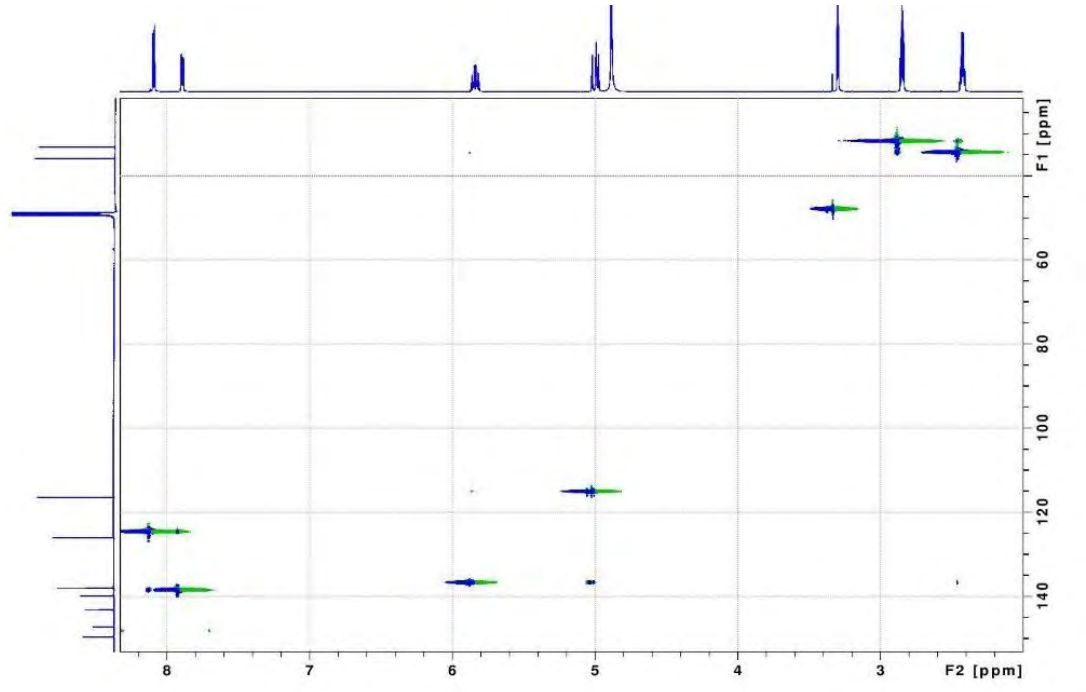


Figure 5.55 HMBC of compound 5

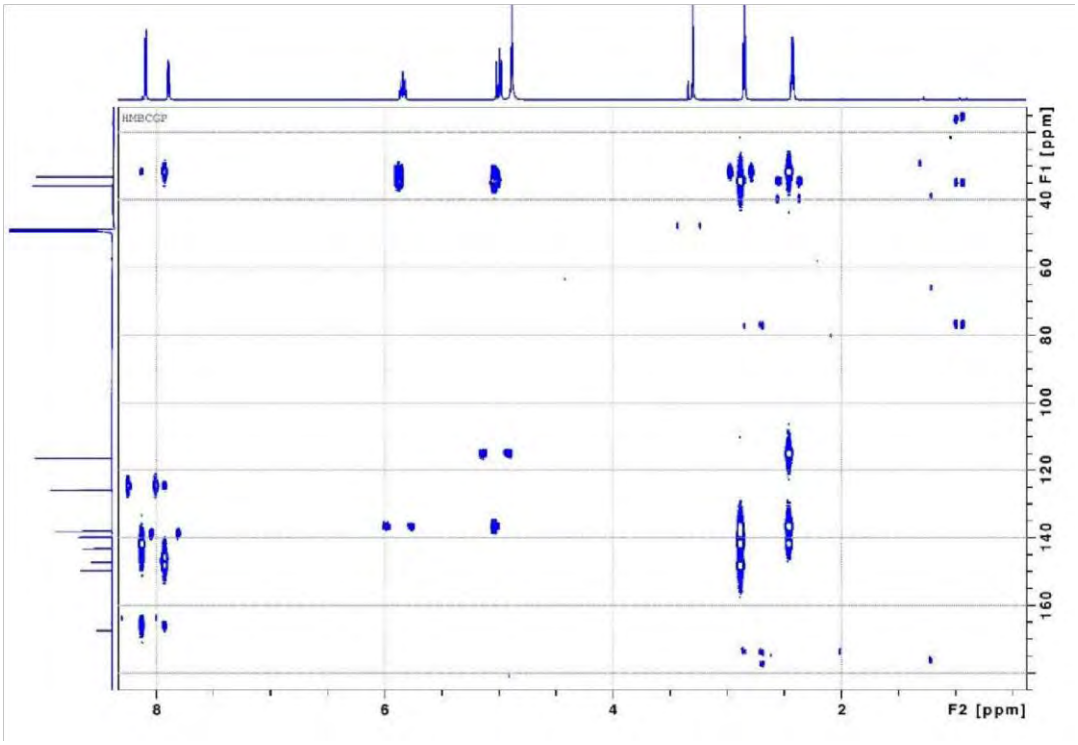


Figure 5.56 HSQC of compound 5

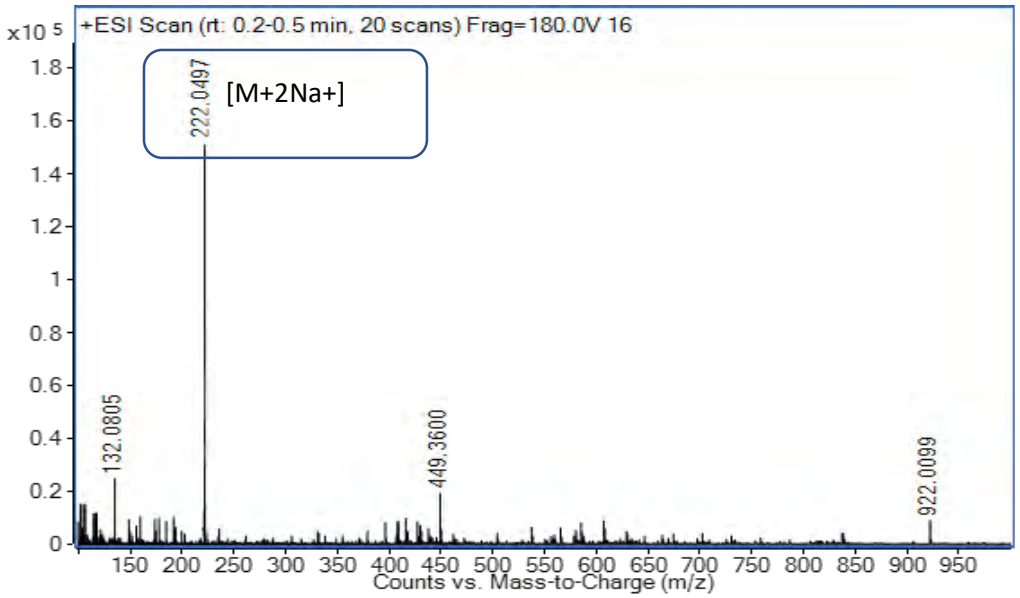
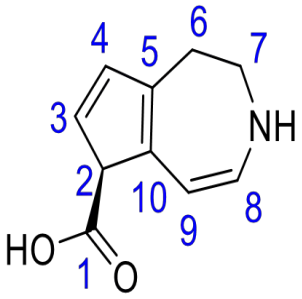


Figure 5.57 HRMS of compound 5



Chemical Formula: $C_{10}H_{11}NO_2$

Exact Mass: 177.08

Molecular Weight: 177.20

m/z: 177.08 (100.0%), 178.08 (10.8%)

Elemental Analysis: C, 67.78; H, 6.26; N, 7.90; O, 18

Figure 5. 58 Structure of compound 5

The compounds isolated and characterized in this study were summarised in the following (Table 5.6)**.**

Table 5.6 Summary of antibacterial natural compounds isolated and characterized in the present study

Sr. No.	**Compound Name**	**Chemical formula**	**Source**
1	(8E,16E)-5,7,10,15,18,20-hexahydroxy-3,22-dimethoxy-5,20-dimethyl-1,24-diphenyltetracosa-8,16-dien-1,2,4,6,19,21,23,24-octaone	$C_{40}H_{46}O_{16}$	*Epicoccum* sp. NFW1 grown on SDB medium supplemented with clarithromycin
2	(1R,2R,3R,4S,5S,6R)-1-((1R,2S,3S,4R,5R,7R,Z)-6-(1-aminoethylidene)-8-(cyclopenta-2,4-dien-1-ylmethyl)-1,3,4,5,7-pentahydroxy-2 isopropoxynon-8-en-1-yl)cyclohexane-1,2,3,4,5,6-hexaol	$C_{26}H_{43}NO_{12}$	*Epicoccum* sp. NFW1 grown on SDB medium supplemented with moxifloxacin
3	11-(tert-butyl)-11-(hydroxymethyl)-13,13-dimethyl-2,5,8-trioxa-1(1,4)-benzenacyclotridecaphan-10-ol	$C_{22}H_{36}O_5$	*Chaetomium* sp. NFW8 grown on SDB medium supplemented with clarithromycin
4	compound 4-N-butylphthalic acid	$C_{12}H_{14}O_4$	*Chaetomium* sp. NFW8 grown on SDB medium

			supplemented with moxifloxacin
5	(S)-1,2,3,6-tetrahydrocyclopenta[d]azepine-6-carboxylic acid	$C_{10}H_{11}NO_2$	*Fusarium oxysporum*. NFW16 grown on SDB medium supplemented with clarithromycin

5.4 DISCUSSION

Microbial metabolites are great source for therapeutics and have been extensively sought out over the decades. Since the discovery of penicillin, a significant number of microbial metabolites have been developed into effective therapeutics. However, the need for novel compounds to combat emerging diseases and recurring resistant in current disease-causing microbes is largely unmet and advocates the universal exploration of underexplored microbial domains.

5.4.1 Metabolites Isolated from *Epicoccum* sp. NFW1

Epicoccum sp. is known for its bioactive metabolites production. Several important therapeutic compounds were isolated from *Epicoccum* sp. and many more are yet to be discovered. In this study, two novel antibacterial metabolites (8E,16E)-5,7,10,15,18,20-hexahydroxy-3,22-dimethoxy-5,20-dimethyl-1,24-diphenyltetracosa-8,16-dien-1,2,4,6,19,21,23,24-octaone with chemical formula $C_{40}H_{46}O_{16}$ and (1R,2R,3R,4S,5S,6R)-1-((1R,2S,3S,4R,5R,7R,Z)-6-(1-aminoethylidene)-8-(cyclopenta-2,4-dien-1-ylmethyl)-1,3,4,5,7-pentahydroxy-2 isopropoxynon-8-en-1-yl)cyclohexane-1,2,3,4,5,6-hexaol with chemical formula $C_{26}H_{43}NO_{12}$ produced by *Epicoccum* sp. NFW1, were isolated and characterized. The strain was cultivated under selective modification of the cultivation media where antibiotics were added. This cost-effective modification was proposed to extend and reveal the biosynthetic potential of the endophytic fungi by activating cryptic biosynthetic gene clusters. These modifications were supported by Bode and colleagues who reported production of additional bioactive compounds by the microbial strain upon changing cultivating conditions (Bode *et al.,* 2002). Similar observation was made in case of endophytic fungus *Bulgaria inquinans* where changes in cultivation media led to the expansion in chemical diversity (Ariatari *et al*., 2019).

Epicoccum sp. are highly cherished to produce potent therapeutics. Production of novel antimicrobial compounds by endophytic *Epicoccum* sp. NFW1 is in close agreement with the findings of various research groups. A recent study reports production of new epidithiodiketopiperazine (ETP), pretrichodermamide and three already known (epi)dithiodiketopiparazines from endophytic *Epicoccum nigrum* associated with medicinal plant *Salix* sp. (Harwako *et al.,* 2019). These compounds were active against

Mycobacterium tuberculosis and plant pathogenic fungi. A related study reports production of flavipin-derived alkaloids azacoccones F-H along with known compounds Epicocconigrone A, epipyrone A and epicoccolids B, from similar endophytic strain (Harwoko *et al*., 2020). These compounds exhibit moderate antibacterial activity (Harwoko *et al*., 2020). Epicotripeptin, a new cyclic tripeptide along with four known cyclic dipeptides and one acetamide derivative were isolated from seagrass-associated endophytic fungus *Epicoccum nigrum* M13. These compounds were structurally elucidated by 1D, 2D NMR and HRESIMS and screened for antimicrobial properties (Qader *et al.,* 2021).

Guo and colleagues reported production of new diketopiperazines, epicoccins E-H 1 with two knowns diphenylalazines A and B with potential antiviral activity against HIV-1 from *Cordyceps* associated *Epicoccum nigrum* (Guo *et al.,* 2009). While searching for novel antibacterial metabolites, an *Epicoccum* sp. residing in *Theobroma cacao,* was found to produce three new polyketides which are polyoxygenated and named as epicolactone and epicoccolides A and B (Talontsi *et al.,* 2013).

Yan *et al.,* (2019) performed a similar study and isolated six new polyketides namely coumarin, two isocoumarins, one dihydroradicinin, derivatives of benzofuranone, along with seven different analogues. These compounds were isolated from an endophytic fungus *Epicoccum nigrum* SCNU-F0002 associated with mangrove. Some of these compounds showed different bioactivities like antioxidant, antifungal and antibacterial against test Gram positive and Gram Negative ATCC bacterial strains (Yan *et al.,* 2019).

Recently, Yan and colleagues reported isolation and characterization of new multicyclic polyketides with (±)-isoepicolactone, (±)-, and one new isobenzofuranone monomer and found known compounds from *Epicoccum nigrum* SCNU-F0002 associated with *Acanthus ilicifolius L*. (Yan *et al*., 2020). These compounds present complex conjugated structures and support findings of the present study.

An *Epicoccum* sp. YUD17002 isolated from *Gastrodia elata* when co-cultured with *Armillaria sp.* produced eight new metabolites, among them five are protoilludane-type sesquiterpenes and three aryl esters along with six known analogues. Few of the samples were active against different human cell lines (Li *et al*., 2020). A similar study

was conducted Wang *et al.,* (2010) to isolate and characterised compound form *Epicoccum* sp. They isolated and structurally characterized thirteen new thiodiketopiperazines, epicoccin I ent-epiicoccin G and epicoccins J-T and previously known six diketopiperazines from *Epicoccum nigrum.*

Another study on endophytic fungus *Epicoccum nigrum,* inhabiting sugar cane, revealed production of compounds mullein, epicolactone and 4,5-dimethylresorcinol by the endophyte. Characterization of epicolactone by MS, NMR and X-ray crystallography uncovered a new natural product with unusual carbon skeleton (Araujo *et al*., 2012). These reports strongly support production of novel compounds with complex structures and unusual configurations by *Epicoccum* sp. NFW1. Dzoyem *et al*., (2017) reported isolation four compounds produced by *Epicoccum nigrum*. Of these, Beauvericin expressed significant antibacterial activity against two strains of Gram-Negative bacteria and mild antioxidant activity.

5.4.2 Metabolites Isolated from *Chaetomium* sp. NFW8

In this study, two metabolites were isolated from *Chaetomium* sp. NFW8. One of these is a novel antibacterial metabolite having formula 11-(tert-butyl)-11-(hydroxymethyl)-13,13-dimethyl-2,5,8-trioxa-1(1,4)-benzenacyclotridecaphan-10-ol ($C_{22}H_{36}O_5$), while the other one is previously known compound 4-N-butylphthalic acid ($C_{12}H_{14}O_4$) isolated for the first time from any natural source. These compounds were produced when the fungal isolate *Chaetomium* sp. NFW8 was grown in medium supplemented with clarithromycin and moxifloxacin.

Tantapakul *et al*., (2020) conducted a similar study and reported production of a new xanthoquindodin B9, active against gram positive bacteria from *Chaetomium globosum* 7s-1, isolated from *Rhapis cochinchinensis* (Lour.). Compounds epipolythiodioxopiperazines, dethio-tetra (methylthio)chetomin, chaetocochin and chetomin along with alatinone, chrysophanol, emodin, ergosterol and two known xanthoquinodin A1 and xanthoquinodin A3 were also isolated from the same fungus.

Another study on *Chaetomium* sp., isolated from an Egyptian medicinal plant *Salvia officinalis,* reports the production of a new hydroperoxycochliodinol derivative with some known compounds including cochliodino, isocochliodinol, chaetomin, and two simple indole derivatives Mallouk *et al*., 2020. A related study describes isolation of a

novel compound chaetoglobol acid along with eleven known metabolites (chlorinated azaphiones and cytochalasin) from an endophytic *Chaetomium globosum* of *Ginkgo biloba* (Wang *et al*., 2020). Five compounds, including three new depsidone analogs, mollicellin S, mollicellin T, and mollicellin U, and two known compounds, mollicellin D and mollicellin H, exhibited significant inhibition against *Staphylococcus aureus* (Zhao *et al*., 2021).

Jin and colleagues reported isolation of two new bisindoles, chaetoindolone E and F and five others already known indole derivatives from endophytic *Chaetomium sp*. when grown in medium supplemented with 1,2-dimethylindole (Jin *et al*., 2020). This study is in close agreement with the present study and suggest enhancement of bioactive metabolite production by the microorganisms under the presence of unusual chemicals.

In a study by Song *et al.,* (2020), a new azaphilone, chaephilone E was reported form endophytic fugus *Chaetomium globosum.* Three chaetoglobosins and eight azaphilones derivatives were also reportedly produced by the fungus.

Yan *et al*., (2018) carried a related study on endophytic *Chaetomium globosum* CDW7 and isolated a new isocoumarin derivative, prochaetoviridin A and a new compound named indole alkaloid, chaetoindolin A. Few known compounds were also isolated and characterised as chaetoviridin A, chaetoglobosins R and T.

5.4.3 Metabolites Isolated from *Fusarium oxysporum* NFW16

In this study a novel metabolite (S)-1,2,3,6-tetrahydrocyclopenta[d]azepine-6-carboxylic acid ($C_{10}H_{11}NO_2$) with antibacterial effect, presenting molecular formula $C_{10}H_{11}NO_2$ was isolated from *Fusarium oxysporum* NFW16 when grown in the medium containing clarithromycin.

Fusarium oxysporum is filamentous fungi, found in plants as endophytes and habitats of soil as well. This sp. is a potent source of various interesting and novel metabolites (Wang *et al*., 2013). Kumar and colleagues reported production of potent therapeutics vinblastine and vincristine by *Fusarium oxysporum,* isolated from *Catharanthus roseus* (Kumar *et al*., 2013). Another study exploited *Fusarium oxysporum* to produce two new compounds oxysporidinone analogue and 3-hydroxyl-2-piperidinone derivative together with seven known compounds (Wang *et al.,* 2011). The compounds were

active against human cancer cell lines and methicillin resistant *S. aureus* and *B. subtilis* (Wang *et al.,* 2011).

A similar study reports production and isolation of Cyclic hexadepsipeptides, fusarihexin A and fusarihexin B from an endophytic fungus *Fusarium* sp. R5 (Zhu *et al*., 2018). These compounds were active against plant pathogenic fungi causing anthracnose in many vegetables and fruits (Zhu *et al*., 2018). Liu and co-workers investigated an endophytic *Fusarium oxysporum* from the fruits of *Drepanocarpus lunatus* and found eight new derivatives of fusaric acid along with four other known compounds (Liu *et al*., 2016).

In a recent study, bioactivity guided isolation led to two new angularly prenylated indole alkaloids (PIAs), amoenamide C and sclerotiamide B from *Fusarium sambucinum* TE-6L inhabiting *Nicotiana tabacum L* (Zhang *et al.,* 2019). These studies are coherent with the present study supporting production of unique biochemical moieties by the fungal endophytes. Ebrahim *et al*., (2020) studied the ethyl acteate extract of *Fusarium verticillioides* WF18 and found a new polyketide fusaisocoumarin A, with two other known compounds emodin and sesquiterpenoid phytohormone (+)-abscisic acid (ABA) All these compounds showed antifungal activity against many other endophytic fungal strains.

Xiao *et al*., (2018) conducted similar research and reported a novel pyrone derivative along with two new naphthalenome derivatives from an endophytic fungi *Fusarium* sp. HP-2 of "Qi-Nan" agarwood. While in search for novel secondary metabolites of endophytic *Fusarium avenaceum* SF-1502 and *Fusarium proliferatum* AF-04, Jiang *et al* (2019) reported production and isolation of alkaloid, a depsipeptide and five sesquiterpenoids, a sesterterpene and naphthoquinones. These findings are in accordance with the present findings and decipher the diversity cryptic metabolic functions in endophytes.

5.5 CONCLUSIONS

The endophytic fungal isolates of *Taxus fauna* were screened to produce new or novel metabolites by cultivating them in the presence of antibiotics supplemented liquid medium. It was revealed that the metabolic profile of the strain was changed as analyzed by bioactivity and HPLC chromatogram. The distinct peaks in the HPLC chromatogram of the extracts obtained from fungal isolates grown in the presence of antibiotics were collected and based on their antibacterial activity, purity ^{1}H-NMR, fractions were collected for structural elucidation. Following 1D, 2D NMR and HRMS, five unique compounds were characterized All of these compounds were produced only when the fungal isolates were grown in medium supplemented with antibiotics. Two novel compounds with moderate antibacterial activity having chemical nomenclature (8E,16E)-5,7,10,15,18,20-hexahydroxy-3,22-dimethoxy-5,20-dimethyl-1,24-diphenyltetracosa-8,16-dien-1,2,4,6,19,21,23,24-octaone with chemical formula ($C_{40}H_{46}O_{16}$), and (1R,2R,3R,4S,5S,6R)-1-((1R,2S,3S,4R,5R,7R,Z)-6-(1-aminoethylidene)-8-(cyclopenta-2,4-dien-1-ylmethyl)-1,3,4,5,7-pentahydroxy-2 isopropoxynon-8-en-1-yl)cyclohexane-1,2,3,4,5,6-hexaol with chemical formula $C_{26}H_{43}NO_{12}$ were isolated from *Epicoccum* sp. NFW1. Two other metabolites were isolated from *Chaetomium* sp. NFW8. The compound 11-(tert-butyl)-11-(hydroxymethyl)-13,13-dimethyl-2,5,8-trioxa-1(1,4)-benzenacyclotridecaphan-10-ol ($C_{22}H_{36}O_5$) is a novel entity produced when clarithromycin is present in medium. The previously known compound 4-N-butylphthalic acid ($C_{12}H_{14}O_4$) was isolated for the first time from any fungal source in this study. Its production was induced when moxifloxacin was added to the growth medium. A novel compound (S)-1,2,3,6-tetrahydrocyclopenta[d]azepine-6-carboxylic acid ($C_{10}H_{11}NO_2$) with antibacterial activities against both gram positive and gram-negative bacteria was isolated from *Fusarium oxysporum* NFW16 when grown in the presence of clarithromycin in the growth medium. Thus, this chapter concludes that endophytic fungi can lead to the production and isolation of novel biochemical moieties when challenged by the presence of antimicrobial substances. Production of unique biochemical entities from fungal endophytes under the effect of antibiotics; strongly advocates the activation of cryptic biosynthetic gene clusters and hence extension of biochemical spectrum of the isolates. This approach thus proves its efficacy in elucidating the hidden potential of

the endophytic microflora and could be a step towards fulfilling the global demand of promising bioactive moieties.

CHAPTER 6

6 *IN SILICO* STUDIES OF ISOLATED FUNGAL METABOLITES

6.1 INTRODUCTION

Over a century, pharmacological scientists has had enriched with the ability to build semi-qualitative or qualitative relationships between molecular structure and activity of the compounds. To test these relationships mostly they used traditional methods but from the last decade there is an increased use of computational methods commonly refered as "*in-silico"* studies. These *in-silico* methods comprises of quantitative structure and activity relations, databases, homology models, pharmacophores, machine learning, molecular modelling approach and data analysis tools. Primarily these methods are used along with the generation of the *in vitro* data both to create model and its verification. Such *in silico* methods have been frequently used in the discovery and optimization of the isolated novel compounds with affinity to the target, absorption, distribution and metabolism, excretion and toxicity analysis along with physico-chemical characterization as well (Ekins *et al*., 2007; Roy & Adhikari, 2018).

Target based virtual screening methods are mostly depends on the availability of the information about the target structure, that may either determined through experimentation or derived through computational studies by means of homology modelling. The aim of using these methods is to provide not only an approximation of the expected conformation, as well as the orientation of the ligand into the protein cavity called "*Docking*" (Trosset & Cavé, 2019). Molecular dynamics (MD) is a computer simulation method for analyzing the physical movements of atoms and molecules. The atoms and molecules can interact for a fixed period, giving a view of the dynamic evolution of the system. Simulations studies are significant in computer aided drug designing to unravel real cellular behavior of a ligand with respect to the receptor molecule (Kiani *et al*., 2019). Binding energies are commonly used to identify potential drug molecule stability and different interaction energy with respect to the target receptor. It is also used to validate docking and simulation analysis.

This chapter discusses the *in-silico* studies that include molecular docking studies, molecular dynamics simulation and MMGB/PBSA Energies Calculations of the endophytic fungal metabolites isolated and characterized in the study for the prediction of possible antibacterial targets.

Docking studies were performed for all the compounds while molecular dynamic simulation and biding energies were performed for the selected compounds with a sepicific taget protein MurF. This protein participates in the peptidoglycan manufacturing pathway, which is a component of cell wall biogenesis. UDP-N-acetylmuramyl-pentapeptide, the monomeric precursor of the peptidoglycan, is produced in the cytoplasm or at the inner surface of the cytoplasmic membrane, and the enzymes involved in its production are interesting targets. The addition of l-Ala, d-Glu, meso-diaminopimelate (m-DAP), or l-Lys and d-Ala-d-Ala dipeptide to UDP-N-acetylmuramic acid results in UDP-N-acetylmuramyl-pentapeptide (Van Heijenoort, 1994). The ATP-dependent cytoplasmic enzymes catalyse these processes. They're intriguing therapeutic targets because of their great specificity, distinctiveness, and presence exclusively in eubacteria (Cha *et al.,* 2014).

6.2 MATERIAL AND METHODS

6.2.1 Molecular Docking with Autodock

Prior to docking studies, compound's structure were drawn using chemdraw 12.0 and converted to pdb. The geometry of the compounds was clean using Discovery Studio 2017. Afterward, the compounds were minimized using UCSF Chimera first through steepest descent steps of 100, followed by 10 rounds of conjugate gradient steps. The steepest descent step size and conjugate gradient steps size were treated as default i.e. 0.02 Å. The crystal structure of multi-drug resistant *Acinetobacter baumannii* UDP-N-acetylmuramoyl-tripeptide-D-alanyl-D-alanine ligase (MurF) ligase enzyme (PDB ID: 4QDI) was employed as a receptor macromolecule. MurF structure after retrieval from RCSB PDB was subjected to a brief preparation phase where first the associated ATP and UDP molecules were removed The protein was then protonated and optimized for 750 rounds of steepest descent and conjugate gradient. The pdbqt files of both the compounds and the receptor MurF were generated using MGL tools. Blind docking calculations were performed using Autodock 4.2 to predict the preferred binding orientation and interaction of the compounds with the MurF. The search space was set as X; -43.8381 Å, Y; 18.1310 Å and Z; -9.8488 Å whereas the dimensions were set as X; 78.2688 Å, Y; 65.2830 Å and Z; 72.7632 Å. The docking procedure was evaluated first for reliability by docking the co-crystalized ligand molecules of MurF and the docking poses were investigated and compared to the respective crystalized structures. Once the protocol is validated, all the compounds were docked with MurF and 10 conformations for each compound were generated. The best docked complex conformation with lowest binding energy in kcal/mol and root mean square deviation (RMSD) was considered for visual interpretation of chemical interactions and binding mode analysis. The visualization of complexes was done through UCSF chimera and Discovery Studio 2017.

6.2.2 Molecular Dynamics Simulation Procedure

The conformational space and dynamics of the best characterized pose for each compound were evaluated through 100-ns run of MD simulations. The procedure was commenced with initial energy minimization of each complex by subjecting them to 1500 rounds (divided into 750 round of conjugate gradient and 750 steepest decent algorithm) in UCSF Chimera 1.13.1. The compounds force field were produced using

AM1-BCC charge method and topology files were created using antechamber module of AMBER. Each complex was solvated in TIP3P waterbox as such to allow a padding distance of 12 Å between the protein and waterbox edge. Protein and compound topology files were generated using ff03 and GAFF force field, respectively in tleap. Moving ahead, each system underwent different steps of minimization with constraint on different atoms. In the first minimization, hydrogen atoms of the systems were minimized for 500 rounds applying 100 kcal mol^{-1} $Å^{-2}$ of energy constraint on remaining of the complex atoms. Following, 1000 rounds of water molecules minimization was performed with restraint of 200 kcal mol^{-1} $Å^{-2}$ on the rest of systems atoms. Next, constraint of 100 kcal mol^{-1} $Å^{-2}$ were applied on systems and the water and ions were relaxed for 300 steps. Afterward, entire systems were minimized keeping a restraint of 5 kcal mol^{-1} $Å^{-2}$ on carbon alpha atoms. In heating phase, the systems were heated in NVT ensemble with periodic boundary conditions and steadily scaling temperature from 0 to 300 kelvin allowing 20 picoseconds restraint of 5 kcal mol^{-1} $Å^{-2}$ on carbon alpha atoms. Calculations were performed with Langevin dynamics with time step set to 2 at 2 femto second. Gradual equilibration in NPT ensemble was done at different intervals for adjusting system density. The system was equilibrated first with a constraint of 5 kcal mol^{-1} $Å^{-2}$ on carbon alpha atoms for 200 picoseconds, followed by reducing constraint of 1 kcal mol^{-1} $Å^{-2}$ with interval space of 100 picoseconds and lastly of 1 ns equilibration without any constraint. Simulations run were performed for 100-ns at time step of 2 femtosecond.

6.2.3 MMGB/PBSA Energies Calculations

The MMPBSA.py integrated in AMBER16 was used to estimate binding free energies of each complex system. The complex, receptor, and ligand prmtop files were generated through ante-MMPBSA.py. In total, 100 complex frames were extracted form simulation trajectories. The binding free energies were estimated using below equation,

$$\Delta G_{binding} = G_{complex} - (G_{protein} + G_{ligand})$$

G in the equation represents average free energy of the complex, receptor and ligand and calculated by,

$$G = E_{MM} + G_{solvation} - TS$$

where E_{MM} stands for internal energy (angle, bond, and dihedral), electrostatic and van der Waals interactions as shown in below equation,

$$E_{MM} = E_{internal} + E_{electrostatic} + E_{van\ der\ Waals}$$

TS is the entropy contribution and estimated by normal mode. The calculation of solvation free energy is done by,

$$G_{solvation} = G_{polar} + G_{non\text{-}polar}$$

The polar solvation energy is calculated by GB method, whereas the non-polar solvation energy calculation is based on solvent-accessible surface area (SASA) using the equation mentioned below,

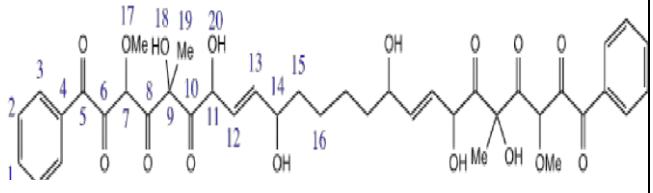
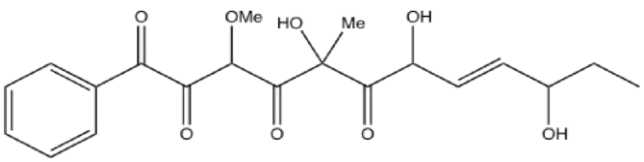
$$G_{non\text{-}polar} = \gamma SASA + \beta$$

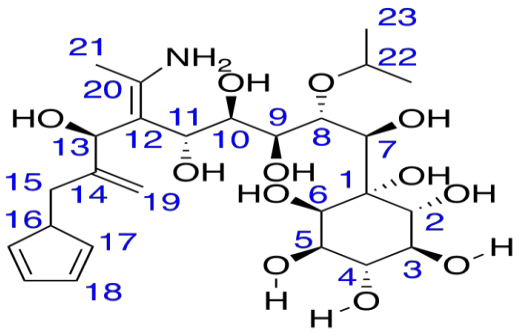
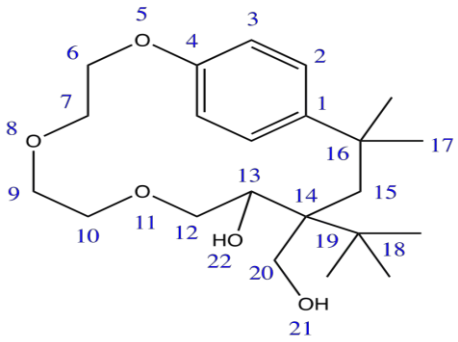
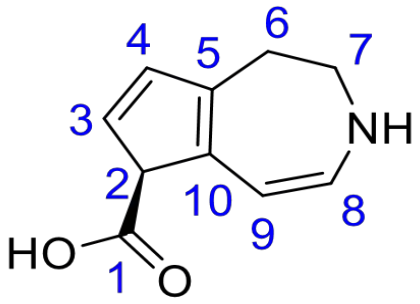
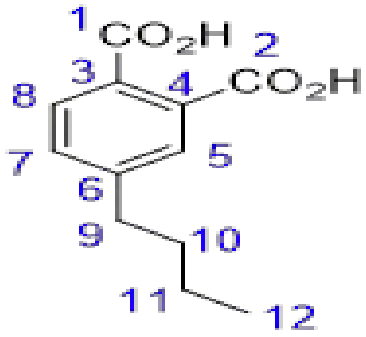
This is estimated through Linear Combinations of Pairwise Overlaps (LCPO) with water probe radius set to 1.4 Å. The standard constants set for γ and β used in PBSA are 0.0052 kcal/mol·Å^2 and 0.92 kcal/mol whereas in GBSA the values are 0.0072 kcal/mol·Å^2 and 0 kcal/mol, respectively.

6.3 RESULTS AND DISCUSSION

Molecular docking is a chemoinformatics approach applied commonly in drug discovery for hunting a lead molecule that is pharmacological and biological active and highly likely to be therapeutically useful. All the used compounds were docked with *A. baumannii* MurF ligase enzyme as a test case to decipher affinity of the compounds for bacterial cell wall biosynthesis enzymes machinery. MurF was selected based on serval reasons; (i) it has no counterpart in human, therefore, highly specific to bacteria and thus targeting this enzyme ensures no autoimmune responses, (ii) this enzyme is widespread in bacteria making it a broad-spectrum antibacterial target, (iii) it catalyzes the final cytoplasmic step of peptidoglycan synthesis and therefore easy to target compared to the rest of Mur ligases associated with membrane localized reactions, (iv) MurF is a promising target for antibiotic development. Several inhibitors against the MurF proteins of harmful bacteria such as *Streptococcus pneumoniae, Staphylococcus aureus*, and *Escherichia coli* have been discovered (Longenecker *et al*., 2005). MurF is essential to the growth and survival of the bacteria and as such can be used as a highly selective target. The binding affinity of the compounds in kcal/mol is tabulated in (Table 6.1).

Table 6.1 Predicted affinities of the used compounds

Compound	**Binding Affinity (kcal/mol)**	**RMSD**
$C_{40}H_{46}O_{16}$ 	-7.3	0
$C_{20}H_{24}O_8$ 	-6	0

$C_{26}H_{43}NO_{12}$ 	-7.9	0
$C_{22}H_{36}O_5$ 	-6.2	0
$C_{10}H_{11}NO_2$ 	-7.1	0
4-n- butyl Pathalic acid $C_{12}H_{14}O_4$ 	-7.2	0

6.3.1 Binding Mode and Interactions of Compound 1 Monomer ($C_{20}H_{24}O_8$)

Compound $C_{20}H_{24}O_8$ prefers binding to the central domain of MurF, interfering with the binding of ATP molecule through 3-methoxy-1-phenyl-propane-1,2-dione. The 1-methoxybutane-2,3-dione moiety of the mentioned ring seems to be more attracted towards the central domain α4 and β6, allowing the downward bending of the attached benzene ring fitting into the space surrounded by Ser122, Ser123, Gly124, Lys125 Thr126, Thr127, and Arg327 from the central domain and Tyr343, and Asn344 from the C-terminal domain. The O17 of this ring was seen in triple hydrogen bonding: one with each side chain hydrogen HZ1, HZ2, and HZ3 of α4 Lys125 at distance of 2.2 Å, 3.9 Å, and 3.5 Å, respectively. Similarly, the O22 of the same ring enjoys multiple binding to the same Lys125 residues. According to literature support the backbone nitrogen atoms of the N-terminal end of 4 (residues 124-127) in the core domain form numerous hydrogen interactions with the α- and β-phosphates. Furthermore, the triphosphate moiety interacts with Lys125's side chains in a number of charged or polar interactions (Cha *et al* 2014). The interacting distance seen with HZ1 is 2.2 Å, HZ2 is 2.8 Å, and HZ3 is 3.6 Å. The O20 of this ring is positioned downward as such to facilitate strong coordination with the MG ion at distance of 2.36 Å. The bending behavior of the benzene ring allowed long distance pi-cation interaction with Arg327 from the loop that connect the Central and C-terminal domain. Additionally, this ring was observed in multiple hydrophobic interactions with residues from both the central domain as well as from the C-terminal domain as illustrated in Figure 6.1. The opposite attached (E)-3,5,8-trihydroxy-3-methyl-dec-6-ene-2,4-dione chain was posed upward on the C11 base to favor binding with loop residues sandwiched between α5 and β7 of the central domain. The O13 and H13 of the base 3-hydroxy-3-methyl-pentane-2,4-dione favor weak hydrogen bonding with side chain HD2 and ND2 of loop Asn150 between α6 and β8 of the central domain with respective distance of 3.6 Å and 3.1 Å. Both atoms were additionally noticed in binding to MG at distance of 3.9 Å for O13 and 3.4 Å for H13. The correct and proximal placement of reactive groups in the active site appears to be the functional role of MG, which adds considerably to MurF's catalytic activity (Bertrand *et al*., 1999). The O15 of the said chain was allowed free in the space of the central and C-terminal domain with no interactions observed. The O4 and H14 of (E)-3,6-dihydroxyoct-4-en-2-one chain interact through hydrogen bond with O atom of the central domain Gly174 with distance of 3.0 Å and 2.8 Å,

respectively. Both these ligand atoms also interact with HN and N atoms of Asn176 at distance of 2.6 Å. Furthermore, O4 form a strong hydrogen bond of 2.3 Å with HD2 side chain hydrogen from Asn176. The H8 of O8 was found involve in weak hydrogen bond with ND2 of loop Asn150. The O10 of the chain was revealed to adjusted into the open cavity with no observed interactions. Likewise, 3-methoxy-1-phenyl-propane-1,2-dione, (E)-3,5,8-trihydroxy 3-methyl-dec-6-ene-2,4-dione chain was seen involved in multiple hydrophobic contacts with residues of the central domain.

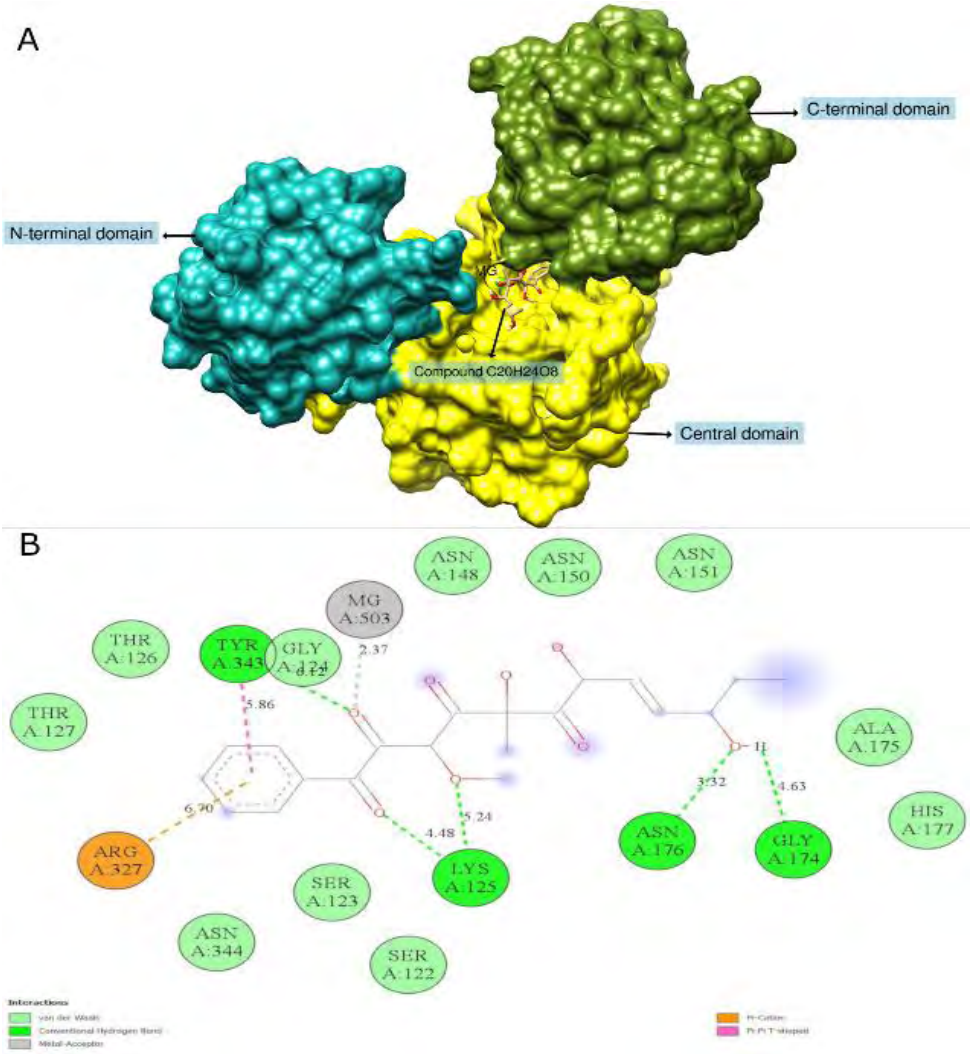


Figure 6.1 Compound $C_{20}H_{24}O_8$ docked pose (**A**), and binding interactions **(B)** with MurF

6.3.2 Binding Mode and Interactions of Compound 1 Dimer ($C_{40}H_{46}O_{16}$)

$C_{40}H_{49}O_{16}$ also occupy the ATP binding side and posed along the interface wall of the central and C-terminal domain (**Figure 6.3A**). The binding site of this compound is formed by α12 and loop between α12 and β15 from the C-terminal domain and α4 and loop between α4 and β6 from the central domain. Binding is as such to allow deep access of the central hexane;5-hydroxy-3-methoxy-1-phenyl-hexane-1,2,4-trione to the interface base, while both of the attached (E)-5,7,10-trihydroxy-3-methoxy-5-methyl-1-phenyl-dec-8-ene-1,2,4,6-tetrone are tilted towards the C-terminal domain. In normal condition ATP interacts with MurF residues on several levels at the interface between the central and C-terminal domains. The adenine base is surrounded in the central domain by Ser123, His292, and Asn296 and in the C-terminal domain by Ser349 and Ala352 (Cha *et al* 2014). Herein, the side chain amide of Asn296 forms polar contacts with N1 and NH2 at C-6.The central domain residues Gly124 and Lys125 from the α4, Ser123 from the loop between α4 and β6 and His292 from α4 were seen in both hydrophobic and hydrophilic interactions with the compound within distance of ≤ 3 Å. Similarly, the C-terminal domain residues: Thr348, Ser349, and Asn346 from α12 and Thr343, and Asn344 from the loop between α12 and β15 were reported in strong contacts with the compound. Additionally, Arg327 from the loop that connects the central and C-terminal domain is also noticed in a strong hydrogen bond of 2.4 Å with the compound base chain. Figure 6.2 B illustrated all the chemical interactions of the compound at the docked site.

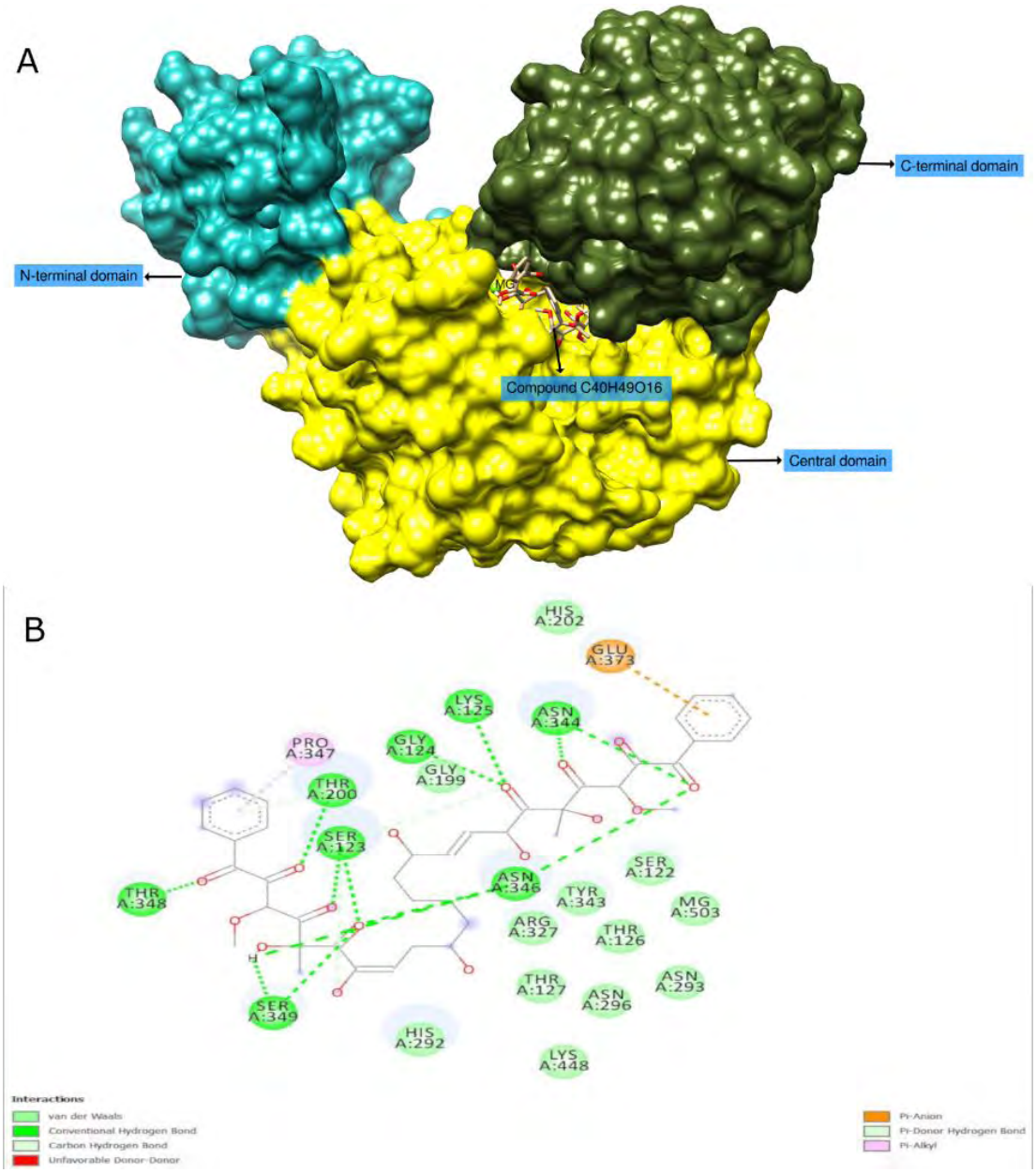


Figure 6.2. Compound $C_{40}H_{49}O_{16}$ docked pose (**A**), and binding interactions **(B)** with MurF

6.3.3 Binding Mode and Interactions of Compound 2 ($C_{26}H_{43}NO_{12}$)

Compound $C_{26}H_{43}NO_{12}$ was seen docked at the ATP binding region and sandwiched between the central and C-terminal domain (Figure 6.3A). The cyclohexane-1,2,3,4,5,6-hexol ring of the compound revealed to favor binding and positioning for α4 of the central domain and by doing so favor strong hydrogen binding to α4 and loop of α4 and β12 whereas the rest of (2R,3S,4R,5R,6Z,7R)-6-(1-aminoethylidene)-8-(cyclopenta-2,4-dien-1-ylmethyl)-2-isopropoxy-non-8-ene-1,3,4,5,7-pentol chain is

situated at the interface of the central and C-terminal domain. The interface is formed by α12 and β15 and β16 from the C-terminal domain and α4, α10 and loops from β12, β13 and β14 from the central domain. During routine process the conformational shift from a crescent-like conformation to an ATP-bound conformation is induced by ATP binding to the interface between the central and C-terminal domains, indicating that the ATP-bound conformation is required for MurF substrate binding (Anderson *et al* 1996). The cyclohexane-1,2,3,4,5,6-hexol ring was observed in rich hydrogen bond interactions with multiple residues of the central domain. These residues include Ser122, Gly124, Lys125, and Thr127 from the central domain within the distance of 3 Å. The O41 and H41 of the cyclohexane-1,2,3,4,5,6-hexol ring are also reported engaged in hydrogen bonding with MG at distance of 3.0 Å and 3.2 Å, respectively. The H33 of propan-2-ol was found tilted towards the residues of the loop residues between α12 and β16 from the C-terminal domain in particular to Asn344 to which it shows two hydrogen bonding: one with OD1 at distance of 2.1 Å and one with ND2 at distance of 3.8 Å. The (2R,3R,4R,5R,6Z,7R)-6-(1-aminoethylidene)-8-(cyclopenta-2,4-dien-1-ylmethyl) non-8-ene-2,3,4,5,7-pentol was reported in most of the hydrophobic contacts with residues from both the C-terminal and the central domain. All interactions of the compounds with the target can be seen in Fig 6.3B. These interactive changes block the site for ATP binding and reduces its catalytic activity during its metabolic pathway.

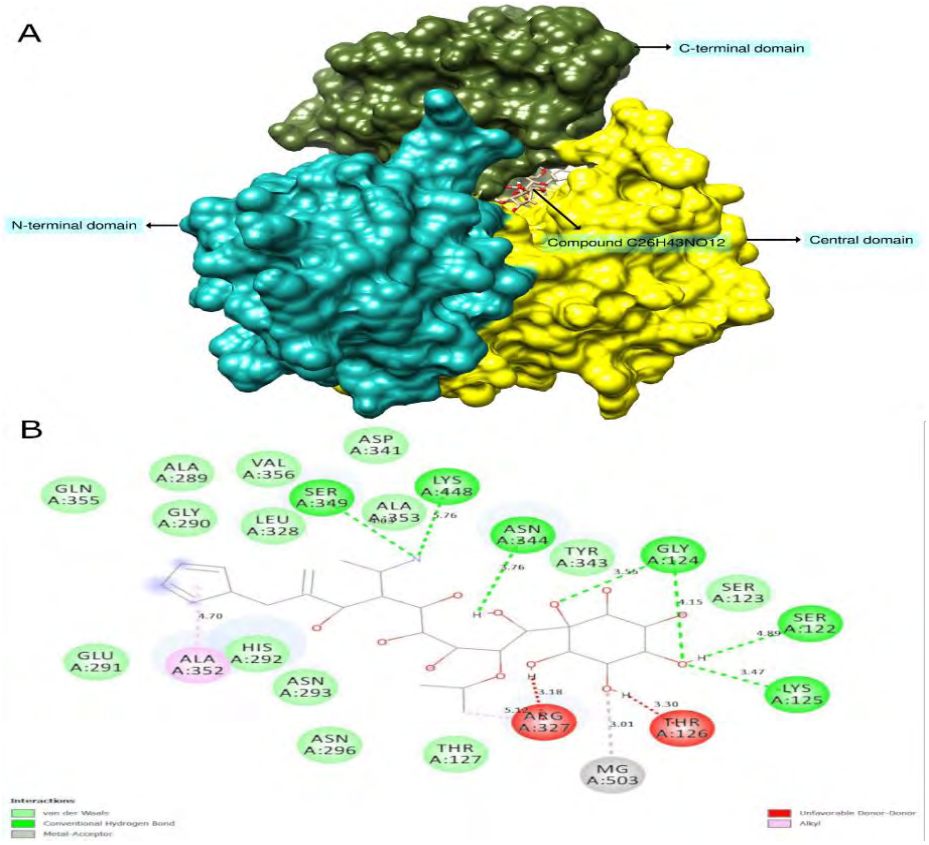


Figure 6.3 Compound $C_{26}H_{43}NO_{12}$ docked pose (**A**), and binding interactions **(B)** with MurF.

6.3.4 Binding Mode and Interactions of Compound 3 ($C_{22}H_{36}O_5$)

Compound $C_{22}H_{36}O_5$, in contrast to the rest of compounds, favors binding to the N-terminal domain predominantly to the binding region of uridine diphosphate (UDP). The uracil ring stacks between Arg29 and Tyr50 in MurF, which correspond to Arg45 and Phe66 (Cha *et al* 2014). Within 3 Å, the compound tend to interact with two important residues of the UDP binding region, namely Asp43 and Ser44. Both these residues previously reported as important for binding and stabilizing inhibitors at the docked site. The O25 and H25 of the compound were revealed in strong hydrogen bonding with HN of Ser44 (distance 2.4 Å) and OD1 of Asp43 (distance 2.8 Å), respectively. The docked conformation and interactions of the compound to the MurF are provided in **Figure 6.4.**

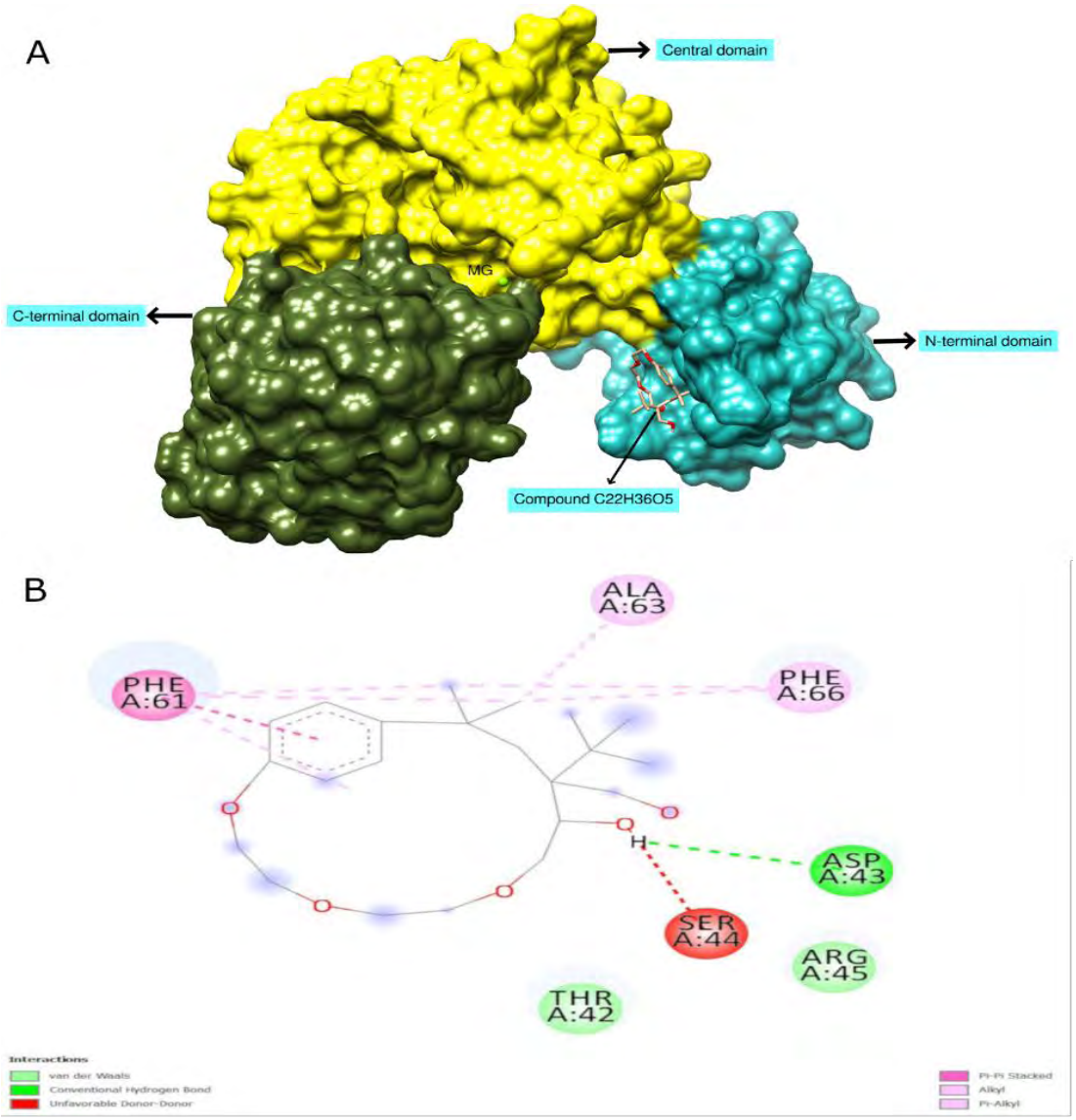


Figure 6.4. Compound $C_{22}H_{36}O_5$ docked pose (**A**), and binding interactions **(B)** with MurF.

6.3.5 Binding Mode and Interaction of Compound 4 ($C_{12}H_{14}O_4$)

A docked pose for a compound $C_{12}H_{14}O_4$ have been retrieved and examined for its binding interaction. Dynamically Intensified hydrogen bonds were measured among the profound inhibitor and Murf protein. Inhibitors bind deep inside the active pocket at ATP binding domain. Binding residues were examined UCSF Chimera and Discovery Studio as can be depicted in **Figure 6.5** strong interactions resulting in binding affinity of -7.3 kcal/mol were achieved. Ligand inside the cavity shows electronegative atoms “oxygen” of this heterocyclic ring to be involved in strong conventional hydrogen linkage to protein residues Lys125, Thr126 and Arg327

respectively. During natural condition the triphosphate moiety interacts with the side chains of Lys125, Asn150, and Arg327 in numerous charged or polar contacts in the central domain, (Nobeli *et al*., 2001). On other hand Mg+ comes was found in an active pocket position of hydrophilic region where it has made a clear unfavorable metal donating bond. Thus, such binding affinity was further investigated in Molecular simulation protocols, which affirms the complex stability in real time cellular environment.

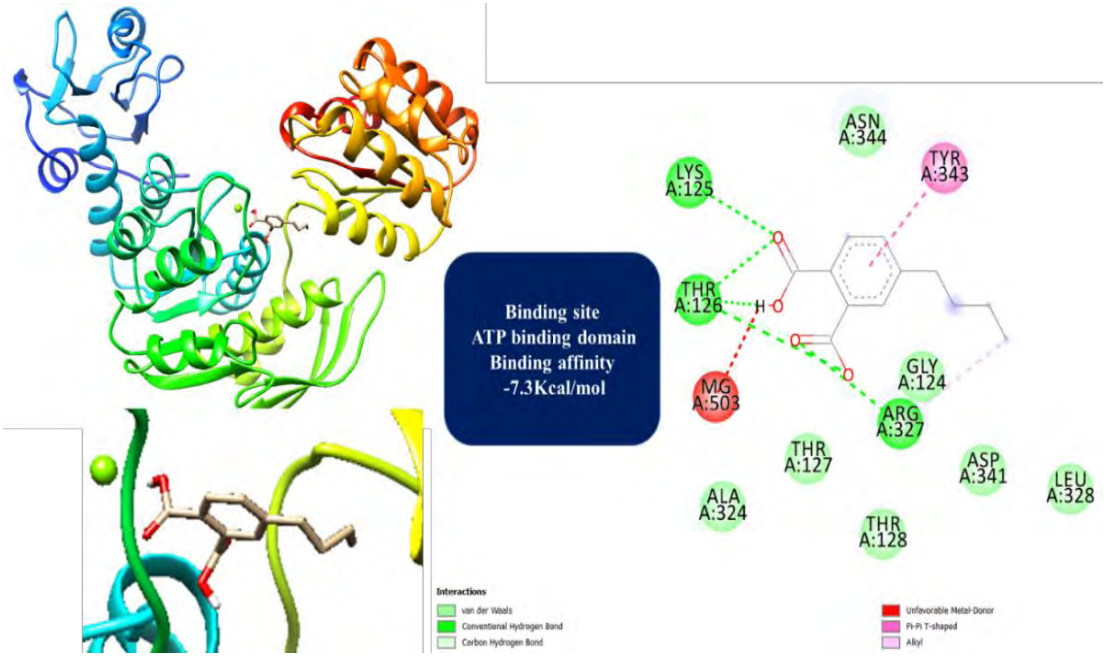


Figure 6.5. Binding mode (left) and interactions (right) of **$C_{12}H_{14}O_4$**

6.3.6 Binding Mode and Interaction of Compound 5 ($C_{10}H_{11}NO_2$)

The ATP binding domain was targeted with compound $C_{10}H_{11}NO_2$ during the c ourse of docking with Autodock-vina. This novel hit molecule showed a range of binding affinity -6.3 kcal/mol and found predominantly inside the pocket. Furthermore, binding residues were examined through a visualizing tool: UCSF Chimera and Discovery Studio as depicted in Figure. Here a clear image of residues have been explored that makes a strong hydrogen bonding among the atom of protein residues and ligand. Covalent bonds with two strong hydrogen linkages, alkyl and conventional bonds have been shown in 2D- Figure. Mainly, a protein residues Ala289 and Lys448 interactive the ligand atom with less distance have been investigated. However, it can be predicted

that these residues involved in this active domain will affect the binding affinity of side chain atom of the residues. Exactly, the same interactive residues has been captivated in the C-terminal domain, the ribose 3′–OH is in hydrogen bond distances between the side-chain carboxyl group of Asp341 and the amine group of Lys448 when ATP is binding (Nobeli *et al*., 2001). Moreover, examining the dynamics of this novel natural chemical entity inside the cavity it was seen that two-oxygen atom with a small ring has made a strong linkage both at central domain and N-terminal. The binding conformation and interactions are provided in Figure 6.6.

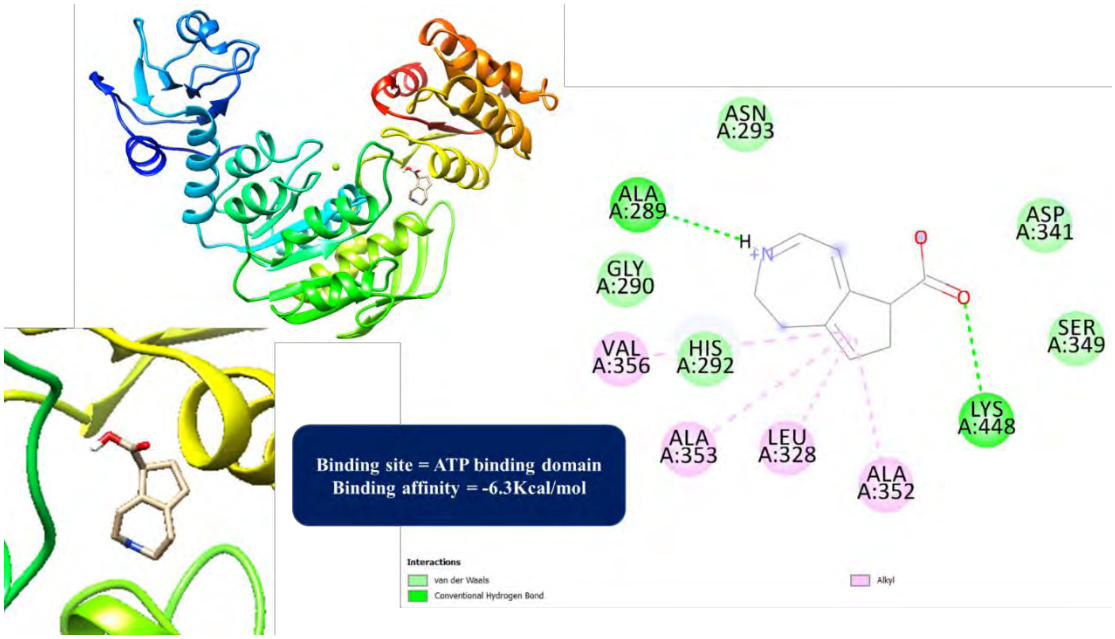


Figure 6.6. Binding conformation (left) and interactions (right)

6.3.7 Molecular Dynamics Simulation

The dynamics of complexes in aqueous solutions were deciphered through 100-ns production run and the trajectories were analyzed through root mean square deviations (RMSD), and root mean square fluctuations (rmsf). RMSD is a mean of measuring the atomic distance of superimposed models and is an effective way to probe structural deviations. RMSD is routinely employed in drug designing and molecular dynamics simulation to decipher whether a system of interest is in equilibrium or not. Stable systems are presented by a constant RMSD. A mean Cα RMSD estimated for 4QDI-$C_{20}H_{24}O_8$, 4QDI-$C_{26}H_{43}NO_{12}$, 4QDI-$C_{40}H_{49}NO_{16}$, and 4QDI-$C_{22}H_{36}O_5$ is 2.78 Å (max, 4.64 Å), 2.15 Å (max, 3.54 Å), 8.46 Å (max, 12.44 Å), and 3.11 Å (max, 4.79 Å), respectively (Figure 6.7). The three complexes i.e., 4QDI-$C_{20}H_{24}O_8$, 4QDI-

$C_{26}H_{43}NO_{12}$ and 4QDI-$C_{22}H_{36}O_5$ were found quite stable throughout the simulation with no global conformation changes observed and the inhibitors binding mode remained stable. The 4QDI-$C_{40}H_{49}NO_{16}$ complex was seen highly unstable with constant fluctuating RMSD and thus thoroughly investigated for structural dynamics. Representative snapshots at 0-ps, 2500-ps, 5000-ps, 7500-ps and 10000-ps were extracted from the trajectories and superimposed in UCSF Chimera to look for structural changes acquired by the receptor and responsible for constantly increasing RMSD in simulation (Figure 6.8). The overall RMSD of these superimposed snapshots is 1.07 Å. Major conformational changes and structural deviations were found that can be explained according to specific protein domains. First, the N-terminal domain was evaluated (Figure 6.9). The initial seven residues (Val1 to Thr7) of the pattern less loop illustrated high flexibility upon inhibitor binding, the β1 sheet (Arg31 to Leu33) seen transformed to loop at 2500-ps, 5000-ps, 7500-ps and afterward seen stable as sheet at 10000-ps. The central domain revealed less local structure interconversion exception to frequent Leu188-Gly199 loop to coil transitions (Figure 6.10). The C-terminal domain depicted high distant morphs as illustrated in Figure 6.11.

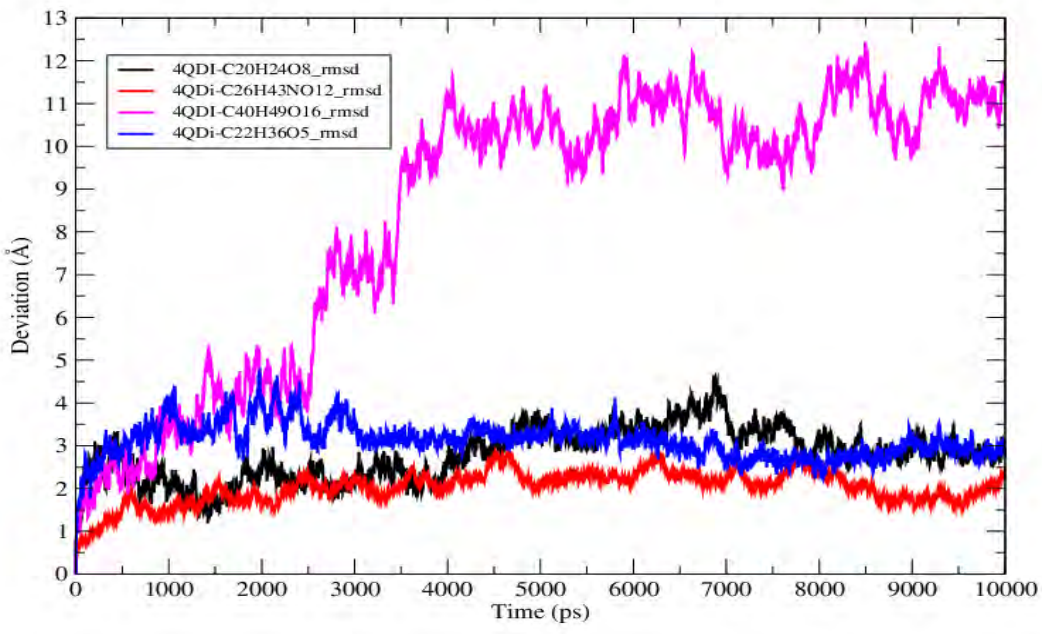


Figure 6.7. RMSD plots of the MurF in the presence of compounds used

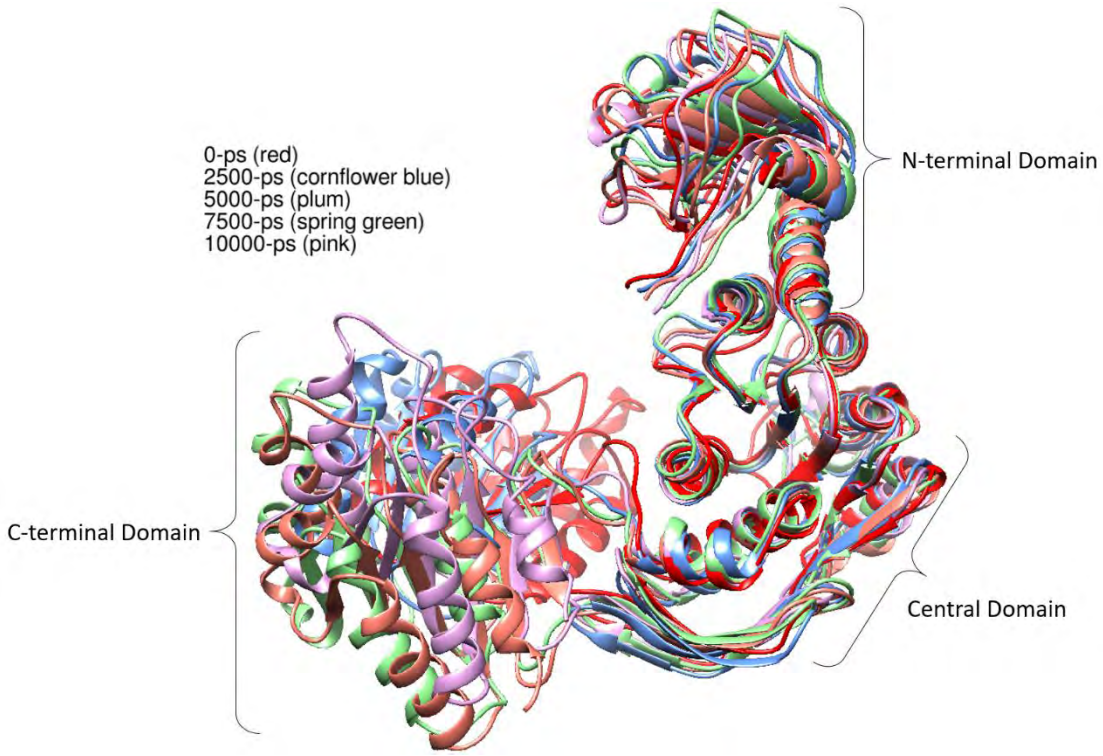


Figure 6.8. Superimposed snapshots of 4QDI-$C_{40}H_{49}NO_{16}$ – inhibitor complex at different time intervals

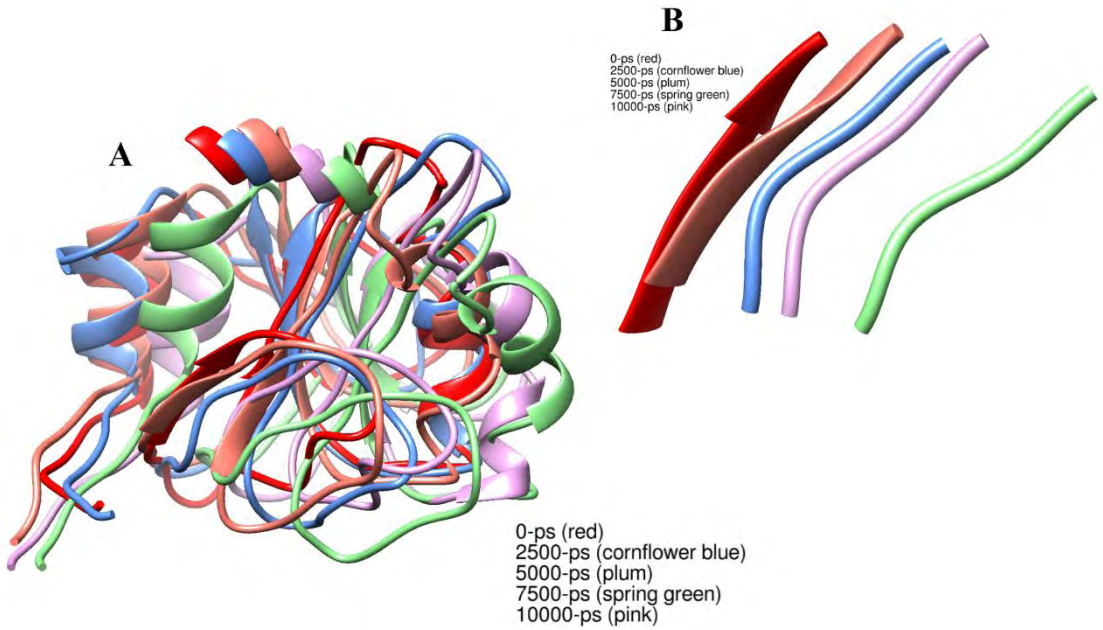


Figure 6.9. (A) Superimposed N-terminal domain of 4QDI-$C_{40}H_{49}NO_{16}$ – inhibitor complex at different time intervals, (B) Closer view of the β1 sheet to loop conversion at different snapshots.

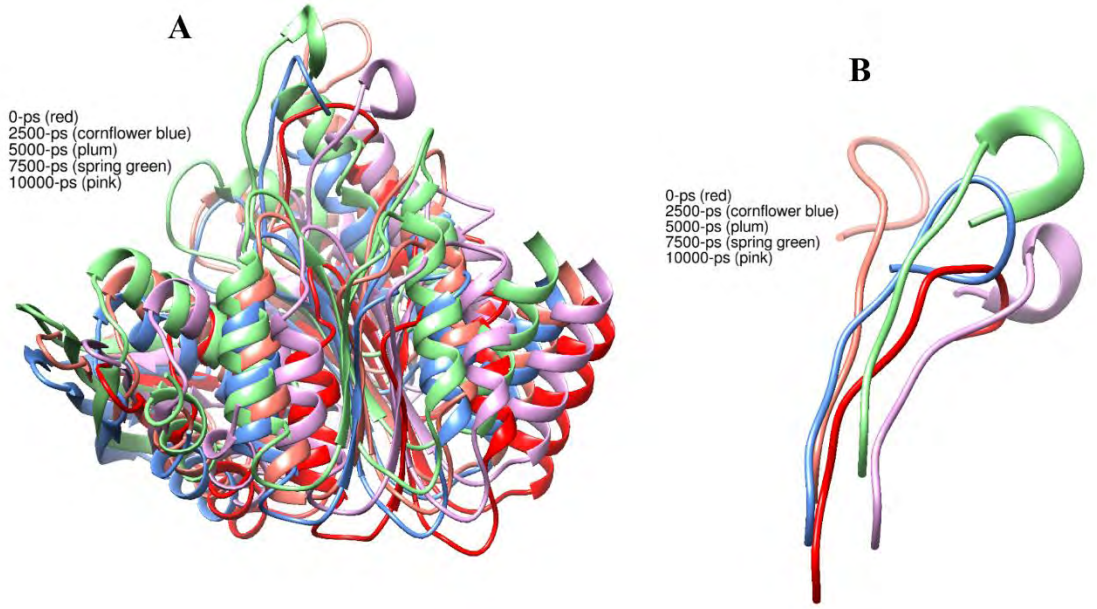


Figure 6.10. (A) Superimposed central domain of 4QDI-$C_{40}H_{49}NO_{16}$ – inhibitor complex at different time intervals, (B) Closer view of Leu188-Gly199 loop conversion to coil at different snapshots

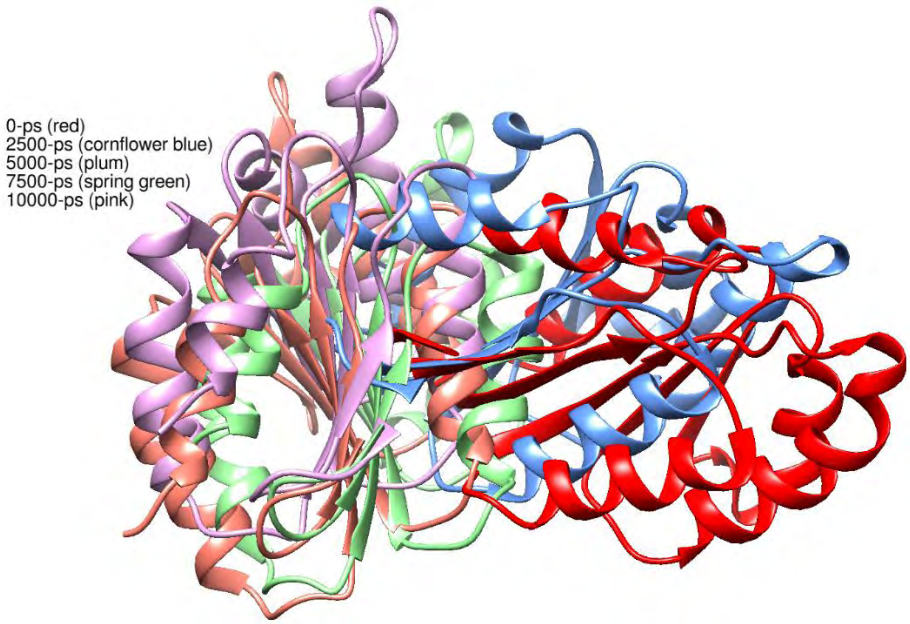


Figure 6.11. Superimposed C-terminal domain of 4QDI-$C_{40}H_{49}NO_{16}$ – inhibitor complex at different time intervals illustrating several different distinct morphs

6.3.8 Root Mean Square Fluctuations (RMSF) Analysis

RMSF is used to identify flexible regions of protein and measures average atomic fluctuations versus time (Figure 6.12). The average RMSF for 4QDI-$C_{20}H_{24}O_8$, 4QDI-$C_{26}H_{43}NO_{12}$, 4QDI-$C_{40}H_{49}NO_{16}$, and 4QDI-$C_{22}H_{36}O_5$ is 1.71 Å (max, 5.98 Å), 1.41 Å (max, 3.79 Å), 3.86 (max, 12.30 Å), and 1.10 Å (max, 7.13 Å). The loop of the central

domain that is involved in direct interactions with the inhibitor 4QDI-$C_{20}H_{24}O_8$For 4QDI-$C_{26}H_{43}NO_{12}$, The high fluctuation of the receptor residues of 4QDI-$C_{40}H_{49}NO_{16}$ complex observed in the central and C-terminal domain and the outcome of constantly moving inhibitor at the interface of the mentioned domains.

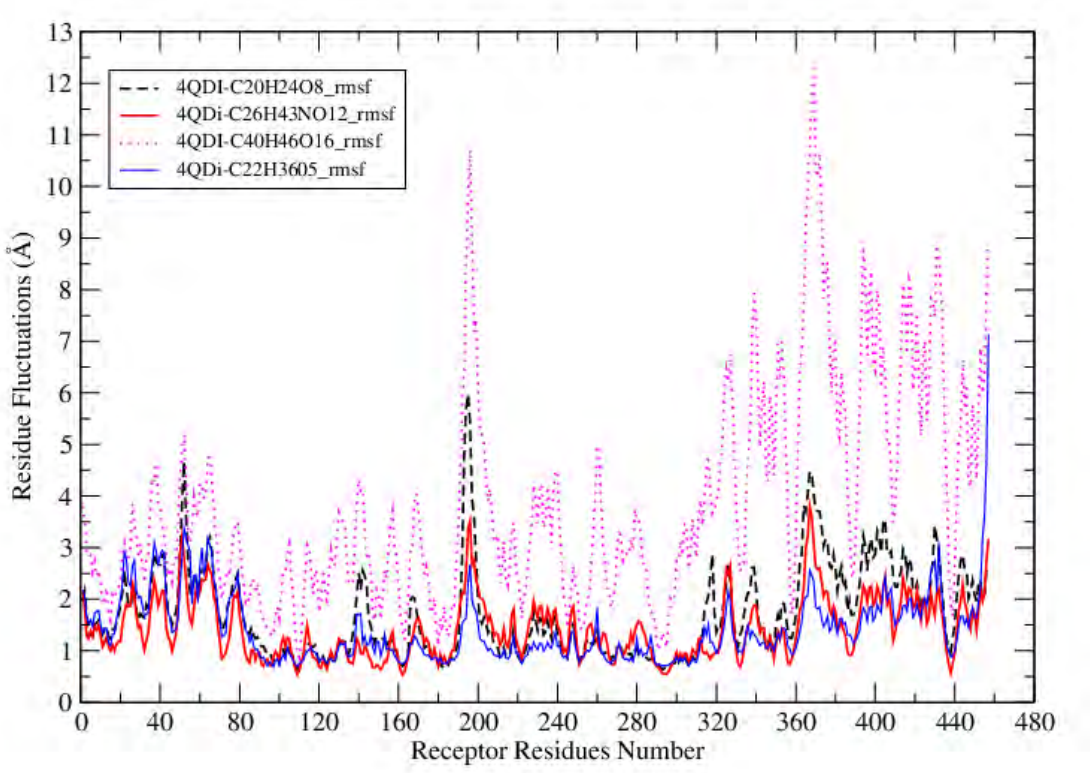
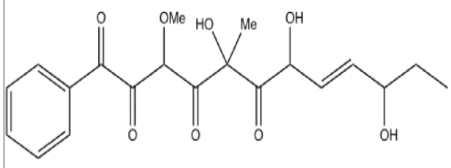
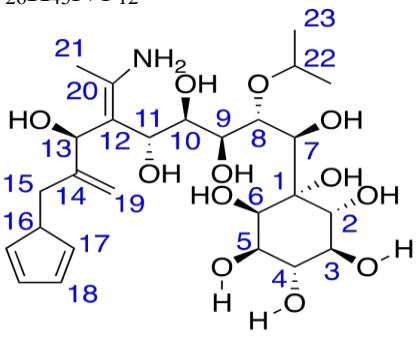


Figure 6.12. RMSF plots for MurF enzyme in the presence of compounds

6.3.9 Estimation of Binding Free Energy

Binding free energy is now widely used in drug designing protocols to assist in selection of high affinity binders and to validation docked posed as real binding mode of the compounds. To this objective, the most employed MMPBSA method was applied on 100 snapshots of the simulation trajectories. All the compounds revealed robust gas phase energy binding to the MurF target with high contribution of both electrostatic and van der Waals. The net binding energy of the complexes in MMPBSA and its complementary MMGBSA are given in Table 6.2 and Table 6.3, respectively. All systems favor strong intermolecular affinity and equilibration to the stable state. This implies that the used compounds can be used as potential leads for future structural optimization to product highly potent antibacterial derivatives.

Table 6.2. Binding free energies of the MurF-compounds complexes estimated by MMGBSA method.

Compound	Energy Component	Average	Std. Dev.	Std. Err. of Mean
$C_{20}H_{24}O_8$ 	VDWAALS	-1.8710	5.9527	0.5953
	EEL	-125.8065	48.1133	4.8113
	EGB	127.0861	50.9262	5.0926
	ESURF	-0.3016	1.0041	0.1004
	DELTA G gas	-127.6775	53.9612	5.3961
	DELTA G solv	126.7845	49.9384	4.9938
	DELTA TOTAL	-0.8930	4.1522	0.4152
$C_{26}H_{43}NO_{12}$ 	VDWAALS	-20.5150	3.0116	0.3012
	EEL	-331.0635	8.2722	0.8272
	EGB	341.7118	7.9159	0.7916
	ESURF	-2.5539	0.3144	0.0314
	DELTA G gas	-351.5785	8.1337	0.8134
	DELTA G solv	339.1579	7.9352	0.7935

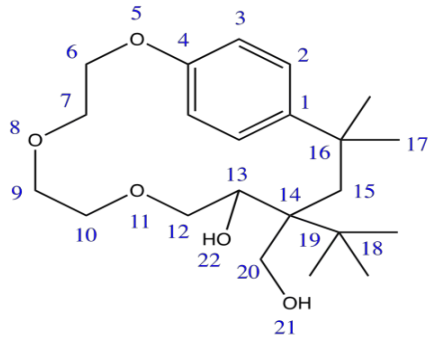
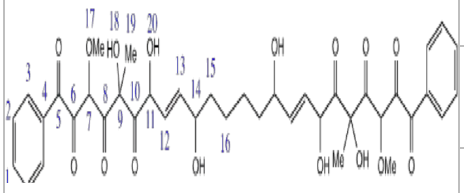
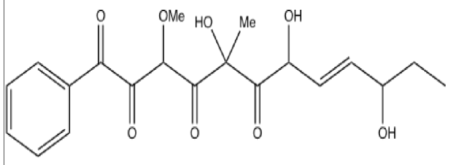
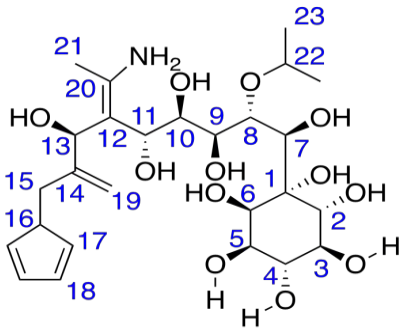
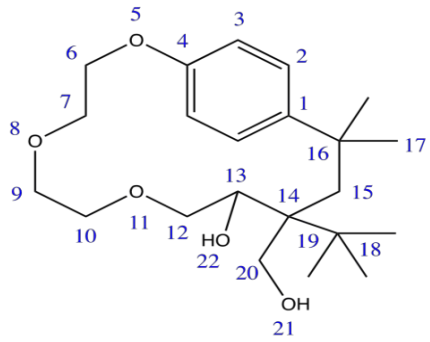
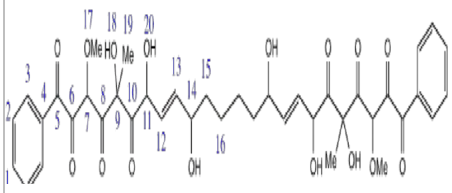
	DELTA TOTAL	-12.4206	2.3095	0.2310
$C_{22}H_{36}O_5$ 	VDWAALS	-5.6793	4.8142	0.4814
	EEL	-315.0517	36.9085	3.6909
	EGB	319.8281	39.7423	3.9742
	ESURF	-0.6632	0.6922	0.0692
	DELTA G gas	-320.7310	40.5815	4.0582
	DELTA G solv	319.1650	39.2196	3.9220
	DELTA TOTAL	-1.5661	1.9188	0.1919
$C_{40}H_{46}O_{16}$ 	VDWAALS	-22.9955	2.4328	0.2433
	EEL	-325.1041	9.1287	0.9129
	EGB	336.9941	9.2152	0.9215
	ESURF	-3.0929	0.3306	0.0331
	DELTA G gas	-348.0996	9.4062	0.9406
	DELTA G solv	333.9012	9.2440	0.9244
	DELTA TOTAL	-14.1984	2.1738	0.2174

Table 6 3. Binding free energies of the MurF-compounds complexes estimated by MMPBSA method.

Compound	Energy Component	Average	Std. Dev.	Std. Err. of Mean
$C_{20}H_{24}O_8$ 	VDWAALS	-1.8710	5.9527	0.5953
	EEL	-125.8065	48.1133	4.8113
	EPB	124.7503	49.9723	4.9972
	ENPOLAR	-0.2830	0.7492	0.0749
	EDISPER	0.0000	0.0000	0.0000
	DELTA G gas	-127.6775	53.9612	5.3961
	DELTA G solv	124.4673	49.2336	4.9234
	DELTA TOTAL	-3.2102	4.8235	0.4823
$C_{26}H_{43}NO_{12}$ 	VDWAALS	-20.5150	3.0116	0.3012
	EEL	-331.0635	8.2722	0.8272
	EPB	336.8998	8.7247	0.8725
	ENPOLAR	-2.2637	0.2033	0.0203
	EDISPER	0.0000	0.0000	0.0000

	DELTA G gas	-351.5785	8.1337	0.8134
	DELTA G solv	334.6361	8.7125	0.8713
	DELTA TOTAL	-16.9424	2.2996	0.2300
$C_{22}H_{36}O_5$ 	VDWAALS	-5.6793	4.8142	0.4814
	EEL	-315.0517	36.9085	3.6909
	EPB	315.2828	39.4424	3.9442
	ENPOLAR	-0.7011	0.5854	0.0585
	EDISPER	0.0000	0.0000	0.0000
	DELTA G gas	-320.7310	40.5815	4.0582
	DELTA G solv	314.5817	38.9423	3.8942
	DELTA TOTAL	-6.1494	2.6879	0.2688
$C_{40}H_{46}O_{16}$ 	VDWAALS	-22.9955	2.4328	0.2433
	EEL	-325.1041	9.1287	0.9129
	EPB	333.2040	9.3581	0.9358
	ENPOLAR	-3.0231	0.2374	0.0237
	EDISPER	0.0000	0.0000	0.0000

	DELTA G gas	-348.0996	9.4062	0.9406
	DELTA G solv	330.1809	9.3441	0.9344
	DELTA TOTAL	17.9187	2.4792	0.2479

6.4 CONCLUSIONS

As resistance to antibiotics by bacterial pathogens is growing concern at present, the need of new potent compounds with broad spectrum antibacterial activity is of high time. This section of the dissertation particularly used the applications of computer aided drug designing to support the potential of the compounds discovered in this work as potent antibacterials. A random screening was performed to check the binding affinity of reported compounds to antibacterial targets. It was unraveled that MurF enzyme of bacterial cell wall machinery might be the target to which these compounds bind with good affinity. It was found that the affinity is the attribute of both hydrophobic and hydrophilic interactions and binding is seen both at the ATP and UDP binding substrate of the MurF. Dynamically all the compounds form stable complexes and variations observed in the structure of MurF though the structural changes are mere adjustments to properly hold the compounds at the docked site. This was validated further by binding energy calculation which affirms the predictions done by docking based simulation studies. In a nutshell, it can be concluded that the set compounds reported in this study might use as leads and need to subject to further in vitro and in vivo experimentations to disclose their real affinity.

CHAPTER 7

7 SUMMARY AND FUTURE PROSPECTS

7.1 SUMMARY

As resistance to antibiotics by bacterial pathogens is growing concern at present, the need of new potent compounds with broad spectrum antibacterial activity is of high time. Endophytic fungi from medicinal plants especially the fungal isolates used in this study are potential source of many secondary metabolites with therapeutic and other biotechnological importance.

Screening of endophytic fungal isolates were performed for production of potential antibacterial compounds in the presence of antibiotics in culture medium. Different strategies were applied where fungal strains were grown in the presence of antibiotic in Sabouraud dextrose broth media and in other cases mycelium collected and dispersed in antibiotic solution in water for any transformation of antibiotic. In the third strategy enzymes or total protein was extracted from the cell free broth of fungal cultures and were used for antibiotic transformation. Antibacterial activities were recorded from the ethyl acetate extracts of every fungal isolate against standard test bacterial strains. Strategy one in which the fungal isolates were grown in the SDB supplemented with antibiotics showed better antibacterial activity after incubation period as compared to other two strategies.

The screening of seven endophytic fungal isolates of *Taxus fauna* was done in the presence of 5 different antibiotics and results showed that three fungal isolates encoded *Epicoccum* sp. NFW1, *Chaetomium* sp. NFW8 and *Fusarium oxysporum* NFW16 showed enhanced antibacterial activity in the presence of clarithromycin and moxifloxacin. *Epicoccum* species NFW1, NFW7 and *Fusarium oxysporum* NFW16 can completely diminish the antibacterial activity of cephradine, ciprofloxacin and cefixime antibiotics.

Further high throughput screening was done for the selected three fungal strains using dereplication studies. The results showed that the fungal isolates are great source of known bioactive metabolites as well as many unknown metabolites. The metabolic profile changed greatly when the medium was supplemented with antibiotics. Several metabolites were induced by the antibiotics in medium some of them are known bioactive metabolites together with many unidentified metabolites. The study suggested

that these endophytic fungal isolates showed enhanced production of bioactive metabolites when they were challenged with antibiotic in liquid medium.

The metabolic profile was changed in terms of bioactivity and HPLC chromatogram. The distinct peaks in HPLC chromatogram of the extracts obtained from fungal isolates grown in the presence of antibiotics were collected and based on their antibacterial activity, purity and unique ^{1}H-NMR they were collected for 1D, 2D NMR and HRMS to elucidate their structure. Five unique compounds were characterized, and all these compounds were produced only when the fungal isolates were challenged with antibiotics. Two novel compounds with moderate antibacterial activity having chemical formula **$C_{40}H_{46}O_{16}$** (8E,16E)-5,7,10,15,18,20-hexahydroxy-3,22-dimethoxy-5,20-dimethyl-1,24-diphenyltetracosa-8,16-dien-1,2,4,6,19,21,23,24-octaone) and **$C_{26}H_{43}NO_{12}$** (1R,2R,3R,4S,5S,6R)-1-((1R,2S,3S,4R,5R,7R,Z)-6-(1-aminoethylidene)-8-(cyclopenta-2,4-dien-1-ylmethyl)-1,3,4,5,7-pentahydroxy-2 isopropoxynon-8-en-1-yl)cyclohexane-1,2,3,4,5,6-hexaol) were isolated from *Epicoccum* sp. NFW1 grown in the presence of clarithromycin and moxifloxacin, respectively. Two other metabolites were isolated from *Chaetomium* sp. NFW8 having chemical formula **$C_{22}H_{36}O_5$** 11-(tert-butyl)-11-(hydroxymethyl)-13,13-dimethyl-2,5,8-trioxa-1(1,4)-benzenacyclotridecaphan-10-ol) a novel compound produced when clarithromycin is present in medium and a compound first time isolated from any type of fungi having chemical formula and **$C_{12}H_{14}O_4$** **(**4-N-butylphthalic acid) was produced when moxifloxacin was supplemented in the medium. A novel compound with antibacterial activities against both gram positive and gram-negative bacteria having chemical formula **$C_{10}H_{11}NO_2$** (S)-1,2,3,6-tetrahydrocyclopenta[d]azepine-6-carboxylic acid) was isolated from *Fusarium oxysporum* NFW16 when grown in the presence of clarithromycin.

In-Silico studies include a random screening to check the binding affinity of reported compounds to antibacterial targets. It was unraveled that MurF enzyme of bacterial cell wall machinery might be the target to which these compounds bind with good affinity. It was found that the affinity is the attribute of both hydrophobic and hydrophilic interactions and binding is seen both at the ATP and UDP binding substrate of the MurF. Dynamically all the compounds form stable complexes and variations observed in the structure of MurF though the structural changes are mere adjustments to properly

hold the compounds at the docked site. This was validated further by binding energy calculation which affirms the predictions done by docking based simulation studies.

This study conclude that the endophytic fungal isolates of *Taxus fauna* are important source of many known and novel bioactive metabolites, and these isolates can completely degrade antibiotics like ciprofloxacin cefixime and cephradine in shake flask experiments.

7.2 FUTURE PROSPECTS

Metabolomics and dereplication studies unrevealed that a number of known and novel bioactive metabolites can be achieved from these endophytic fungal isolates. These metabolites can further be produced under specified conditions in large scale for their potential bioactivities including, antibacterial, antifungal, antiviral, anti-parasitic and anti-cancerous activities as many of the structures identifies in dereplication has potential for all kinds of activities.

Novel compounds are discovered in the present study further, their structure activity relationship (SAR) could be carried out by both *in-silico* and *ex-silico* studies. *Ex-silico* methods which combine *in-silico* production with experimental verification and will add up to the scientific repertoire of these biochemical moieties.

Pharmacokinetics optimization (ADME) and toxicity evaluation of the isolated novel compounds could be further elaborated.

Microbial natural products are widely used in human and veterinary medicine, agriculture, and manufacturing, leading to many microbe-host and microbe-microbe interactions. Natural products when connect to the genes that encode them enable discovery of new molecules. The identification of cryptic genes and many biosynthetic gene clusters (BGCs): sets of physically clustered genes that encode the biosynthetic enzymes for the novel metabolites for these fungal isolates will give further insight to these processes.

Some antibiotics were transformed or degraded to inactive form by the endophytic fungal isolates which can be further studied to degrade antibiotics from contaminated environments especially pharmaceutical industry wastewaters.

8 REFERENCES

Abbasi, A., Sarker, S. and Chiang, R.H., 2016. Big data research in information systems: Toward an inclusive research agenda. *Journal of the Association for Information Systems*, *17*(2), p.3.

Abdalla, M.A., Aro, A.O., Gado, D., Passari, A.K., Mishra, V.K., Singh, B.P. and McGaw, L.J., 2020. Isolation of endophytic fungi from South African plants, and screening for their antimicrobial and extracellular enzymatic activities and presence of type I polyketide synthases. *South African Journal of Botany*, *134*, pp.336-342.

Abd-Elsalam, K.A. and Hashim, A.F., 2013. Hidden fungi as microbial and nano-factories for anticancer agents. *Fungal Genomics & Biology*.

Aboobaker, Z., Viljoen, A., Chen, W., Crous, P.W., Maharaj, V.J. and van Vuuren, S., 2019. Endophytic fungi isolated from Pelargonium sidoides DC: Antimicrobial interaction and isolation of a bioactive compound. *South African Journal of Botany*, *122*, pp.535-542.

Ahmad, S., Raza, S., Uddin, R. and Azam, S.S., 2017. Binding mode analysis, dynamic simulation and binding free energy calculations of the MurF ligase from Acinetobacter baumannii. *Journal of Molecular Graphics and Modelling*, *77*, pp.72-85.

Aksenov, A.A., da Silva, R., Knight, R., Lopes, N.P. and Dorrestein, P.C., 2017. Global chemical analysis of biology by mass spectrometry. *Nature Reviews Chemistry*, *1*(7), pp.1-20.

Alekshun, M.N. and Levy, S.B., 2004. The Escherichia coli mar locus-Antibiotic resistance and more. *ASM News-American Society for Microbiology*, *70*(10), pp.451-456.

Al-Fakih, A.A. and Almaqtri, W.Q.A., 2019. Overview on antibacterial metabolites from terrestrial Aspergillus spp. *Mycology*, *10*(4), pp.191-209.

Alvin, A., Miller, K.I. and Neilan, B.A., 2014. Exploring the potential of endophytes from medicinal plants as sources of antimycobacterial compounds. *Microbiological research*, *169*(7-8), pp.483-495.

Aly, A.H., Debbab, A. and Proksch, P., 2011. Fungal endophytes: unique plant inhabitants with great promises. *Applied Microbiology and Biotechnology*, *90*(6), pp.1829-1845.

Aly, A.H., Debbab, A., Kjer, J. and Proksch, P., 2010. Fungal endophytes from higher plants: a prolific source of phytochemicals and other bioactive natural products. *Fungal diversity*, *41*(1), pp.1-16.

Aly, A.H., Edrada-Ebel, R., Indriani, I.D., Wray, V., M.ll er, W.E., Totzke, F., Zirrgiebel, U., Schächtele, C., Kubbutat, M.H., Lin, W.H. and Proksch, P., 2008. Cytotoxic metabolites from the fungal endophyte Alternaria sp. and their subsequent detection in its host plant Polygonum senegalense. *Journal of natural products*, *71*(6), pp.972-980.

Arnold, A.E. and Lutzoni, F., 2007. Diversity and host range of foliar fungal endophytes: are tropical leaves biodiversity hotspots?. *Ecology*, *88*(3), pp.541-549.

Ashutosh, K. and Absar, A., 2013. Biotransformation of vinblastine to vincristine by the endophytic fungus Fusarium oxysporum isolated from Catharanthus roseus. *Biocatalysis and Biotransformation*, *31*(2), pp.89-93.

Aslam, B., Wang, W., Arshad, M.I., Khurshid, M., Muzammil, S., Rasool, M.H., Nisar, M.A., Alvi, R.F., Aslam, M.A., Qamar, M.U. and Salamat, M.K.F., 2018. Antibiotic resistance: a rundown of a global crisis. *Infection and drug resistance*, *11*, p.1645.

Bacon, C.W. and White, J.F., 2000. Physiological adaptations in the evolution of endophytism in the Clavicipitaceae. *Microbial Endophytes*, pp.237-261.

Bala, M. and Singh, M., 2013. Nondestructive estimation of total phenol and crude fiber content in intact seeds of rapeseed–mustard using FTNIR. *Industrial crops and products*, *42*, pp.357-362.

Balunas, M.J. and Kinghorn, A.D., 2005. Drug discovery from medicinal plants. *Life sciences*, *78*(5), pp.431-441.

Barthélemy, M., Elie, N., Pellissier, L., Wolfender, J.L., Stien, D., Touboul, D. and Eparvier, V., 2019. Structural identification of antibacterial lipids from amazonian palm tree endophytes through the molecular network approach. *International journal of molecular sciences*, *20*(8), p.2006.

Bashyal, B.P., Wijeratne, E.K., Faeth, S.H. and Gunatilaka, A.L., 2005. Globosumones A− C, cytotoxic orsellinic acid esters from the Sonoran desert endophytic fungus Chaetomium globosum. *Journal of natural products*, *68*(5), pp.724-728.

Berdy, J., 2005. Bioactive microbial metabolites. *The Journal of Antibiotics*, *58*(1), pp.1-26.

Bertrand, J.A., Auger, G., Martin, L., Fanchon, E., Blanot, D., Le Beller, D., van Heijenoort, J. and Dideberg, O., 1999. Determination of the MurD mechanism through crystallographic analysis of enzyme complexes. *Journal of molecular biology*, *289*(3), pp.579-590.

Bhardwaj, A. and Agrawal, P., 2014. A review fungal endophytes: as a store house of bioactive compound. *World J. Pharm. Pharm. Sci*, *3*, pp.228-237.

Bills, G.F. and Stadler, M., 2014. Editorial comment–Discovery, distribution and biosynthesis of fungal secondary metabolites.

Bischoff, J.F. and White, J.F., 2005. Evolutionary development of the Clavicipitaceae. *Mycology Series*, *23*, p.505.

Blair, J.M., Webber, M.A., Baylay, A.J., Ogbolu, D.O. and Piddock, L.J., 2015. Molecular mechanisms of antibiotic resistance. *Nature reviews microbiology*, *13*(1), pp.42-51.

Bode, H.B., Bethe, B., Höfs, R. and Zeeck, A., 2002. Big effects from small changes: possible ways to explore nature's chemical diversity. *ChemBioChem*, *3*(7), pp.619-627.

Bugni, T.S. and Ireland, C.M., 2004. Marine-derived fungi: a chemically and biologically diverse group of microorganisms. *Natural Product Reports*, *21*(1), pp.143-163.

Cai, X., Shan, T., Li, P., Huang, Y., Xu, L., Zhou, L., Wang, M. and Jiang, W., 2009. Spirobisnaphthalenes from the endophytic fungus Dzf12 of Dioscorea zingiberensis and their antimicrobial activities. *Natural Product Communications*, *4*(11), p.1934578X0900401105.

Cha, S.S., An, Y.J., Jeong, C.S., Yu, J.H. and Chung, K.M., 2014. ATP-binding mode including a carbamoylated lysine and two Mg2+ ions, and substrate-binding mode in Acinetobacter baumannii MurF. *Biochemical and biophysical research communications*, *450*(2), pp.1045-1050.

Chatterjee, S., Ghosh, R. and Mandal, N.C., 2019. Production of bioactive compounds with bactericidal and antioxidant potential by endophytic fungus Alternaria alternata AE1 isolated from Azadirachta indica A. Juss. *PLoS One*, *14*(4), p.e0214744.

Chen, X., Sang, X., Li, S., Zhang, S. and Bai, L., 2010. Studies on a chlorogenic acid-producing endophytic fungi isolated from Eucommia ulmoides Oliver. *Journal of Industrial Microbiology and Biotechnology*, *37*(5), pp.447-454.

CHEN, X., YU, H., GU, H., LI, K. and YAN, S., 2007. Control of Tobacco Black Root Rot by Applying Trichoderma harzianum and the Interactions Between Trichoderma harzianum and Superior Strains of Soil Microbe. *Guizhou Agricultural Sciences*, *5*.

Chokpaiboon, S., Sommit, D., Teerawatananond, T., Muangsin, N., Bunyapaiboonsri, T. and Pudhom, K., 2010. Cytotoxic nor-chamigrane and chamigrane endoperoxides from a basidiomycetous fungus. *Journal of natural products*, *73*(5), pp.1005-1007.

Chomcheon, P., Wiyakrutta, S., Aree, T., Sriubolmas, N., Ngamrojanavanich, N., Mahidol, C., Ruchirawat, S. and Kittakoop, P., 2010. Curvularides A–E: antifungal hybrid peptide–polyketides from the endophytic fungus Curvularia geniculata. *Chemistry–A European Journal*, *16*(36), pp.11178-11185.

Chomcheon, P., Wiyakrutta, S., Sriubolmas, N., Ngamrojanavanich, N., Kengtong, S., Mahidol, C., Ruchirawat, S. and Kittakoop, P., 2009. Aromatase inhibitory, radical scavenging, and antioxidant activities of depsidones and diaryl ethers from the endophytic fungus Corynespora cassiicola L36. *Phytochemistry*, *70*(3), pp.407-413.

Chopra, I., O'Neill, A.J. and Miller, K., 2003. The role of mutators in the emergence of antibiotic-resistant bacteria. *Drug Resistance Updates*, *6*(3), pp.137-145.

Cota, B.B., Rosa, L.H., Caligiorne, R.B., Rabello, A.L.T., Almeida Alves, T.M., Rosa, C.A. and Zani, C.L., 2008. Altenusin, a biphenyl isolated from the endophytic fungus Alternaria sp., inhibits trypanothione reductase from Trypanosoma cruzi. *FEMS Microbiology letters*, *285*(2), pp.177-182.

Craney, A., Ahmed, S. and Nodwell, J., 2013. Towards a new science of secondary metabolism. *The Journal of antibiotics*, *66*(7), pp.387-400.

Cueto, M., Jensen, P.R., Kauffman, C., Fenical, W., Lobkovsky, E. and Clardy, J., 2001. Pestalone, a new antibiotic produced by a marine fungus in response to bacterial challenge. *Journal of Natural Products*, *64*(11), pp.1444-1446.

Cuperlovic-Culf, M. and Culf, A.S., 2016. Applied metabolomics in drug discovery. *Expert opinion on drug discovery*, *11*(8), pp.759-770.

Dai, J., Krohn, K., Draeger, S. and Schulz, B., 2009. New Naphthalene-Chroman Coupling Products from the Endophytic Fungus, Nodulisporium sp. from Erica arborea.

Dai, J., Krohn, K., Flörke, U., Draeger, S., Schulz, B., Kiss-Szikszai, A., Antus, S., Kurtán, T. and van Ree, T., 2006. Metabolites from the endophytic fungus Nodulisporium sp. from Juniperus cedre.

Dallakyan, S. and Olson, A.J., 2015. Small-molecule library screening by docking with PyRx. In *Chemical biology* (pp. 243-250). Humana Press, New York, NY.

Davies, J. and Davies, D., 2010. Origins and evolution of antibiotic resistance. *Microbiology and Molecular Biology Reviews*, *74*(3), pp.417-433.

Davis, E.C. and Shaw, A.J., 2008. Biogeographic and phylogenetic patterns in diversity of liverwort-associated endophytes. *American Journal of Botany*, *95*(8), pp.914-924.

de Bary, A., 1866. *Morphologie und physiologie der pilze, flechten und myxomyceten*. Engelmann.

de Siqueira, V.M., Conti, R., de Araújo, J.M. and Souza-Motta, C.M., 2011. Endophytic fungi from the medicinal plant Lippia sidoides Cham. and their antimicrobial activity. *Symbiosis*, *53*(2), pp.89-95.

Debbab, A., Aly, A.H., Edrada-Ebel, R., Müller, W.E., Mosaddak, M., Hakikj, A., Ebel, R. and Proksch, P., 2009. Bioactive secondary metabolites from the endophytic fungus Chaetomium sp. isolated from Salvia officinalis growing in Morocco. *Biotechnology, Agronomy, Society and Environment (BASE)*, *13*(2), pp.229-234.

Debbab, A., Aly, A.H., Edrada-Ebel, R., Wray, V., Müller, W.E., Totzke, F., Zirrgiebel, U., Schachtele, C., Kubbutat, M.H., Lin, W.H. and Mosaddak, M., 2009. Bioactive metabolites from the endophytic fungus Stemphylium globuliferum isolated from Mentha pulegium. *Journal of natural products*, *72*(4), pp.626-631.

Deshmukh, S.K., 2018. Translating endophytic fungal research towards pharmaceutical applications. *Kavaka*, *50*, pp.1-13.

Ding, G., Song, Y.C., Chen, J.R., Xu, C., Ge, H.M., Wang, X.T. and Tan, R.X., 2006. Chaetoglobosin U, a cytochalasan alkaloid from endophytic Chaetomium g lobosum IFB-E019. *Journal of Natural Products*, *69*(2), pp.302-304.

Ding, Z., Tao, T., Wang, L., Zhao, Y., Huang, H., Zhang, D., Liu, M., Wang, Z. and Han, J., 2019. Bioprospecting of novel and bioactive metabolites from endophytic fungi Isolated from rubber tree *Ficus elastica* leaves. *J. Microbiol. Biotechnol*, *29*(5), pp.731-738.

dos Reis, C.M., da Rosa, B.V., da Rosa, G.P., do Carmo, G., Morandini, L.M.B., Ugalde, G.A., Kuhn, K.R., Morel, A.F., Jahn, S.L. and Kuhn, R.C., 2019. Antifungal and antibacterial activity of extracts produced from *Diaporthe schini*. *Journal of Biotechnology*, *294*, pp.30-37.

Durrant, J.D. and McCammon, J.A., 2011. Molecular dynamics simulations and drug discovery. *BMC Biology*, *9*(1), pp.1-9.

Dzoyem, J.P., Melong, R., Tsamo, A.T., Maffo, T., Kapche, D.G., Ngadjui, B.T., McGaw, L.J. and Eloff, J.N., 2017. Cytotoxicity, antioxidant and antibacterial activity

of four compounds produced by an endophytic fungus *Epicoccum nigrum* associated with Entada abyssinica. *Revista Brasileira de Farmacognosia*, *27*(2), pp.251-253.

Holanda, F.H., Birolli, W.G., Morais, E.D.S., Sena, I.S., Ferreira, A.M., Faustino, S.M.M., Solon, L.G.D.S., Porto, A.L. and Ferreira, I.M., 2019. Study of biodegradation of chloramphenicol by endophytic fungi isolated from *Bertholletia excelsa* (Brazil nuts). *Biocatalysis and Agricultural Biotechnology*, *20*, p.101200.

Lohning, A., M Levonis, S., Williams-Noonan, B. and S Schweiker, S., 2017. A practical guide to molecular docking and homology modelling for medicinal chemists. *Current Topics in Medicinal Chemistry*, *17*(18), pp.2023-2040.

Ebrahim, W., Özkaya, F.C. and Ebada, S.S., 2020. Antifungal metabolites from endophytic fungus Fusarium verticillioides strain WF18. *South African Journal of Botany*, *133*, pp.40-44.

Ekins, S., Mestres, J. and Testa, B., 2007. In silico pharmacology for drug discovery: methods for virtual ligand screening and profiling. *British journal of Pharmacology*, *152*(1), pp.9-20.

El Amrani, M., Lai, D., Debbab, A., Aly, A.H., Siems, K., Seidel, C., Schnekenburger, M., Gaigneaux, A., Diederich, M., Feger, D. and Lin, W., 2014. Protein kinase and HDAC inhibitors from the endophytic fungus *Epicoccum nigrum*. *Journal of Natural Products*, *77*(1), pp.49-56.

El-Bialy, H.A. and El-Bastawisy, H.S., 2020. Elicitors stimulate paclitaxel production by endophytic fungi isolated from ecologically altered *Taxus baccata*. *Journal of Radiation Research and Applied Sciences*, *13*(1), pp.79-87.

Faeth, S.H. and Hamilton, C.E., 2006. Does an asexual endophyte symbiont alter life stage and long-term survival in a perennial host grass? *Microbial Ecology*, *52*(4), pp.748-755.

Fan, B., Dewapriya, P., Li, F., Blümel, M. and Tasdemir, D., 2020. Pyrenosetins A–C, new decalinoylspirotetramic acid derivatives isolated by bioactivity-based molecular networking from the seaweed-derived fungus *Pyrenochaetopsis* sp. FVE-001. *Marine Drugs*, *18*(1), p.47.

Fatima, N., Kondratyuk, T.P., Park, E.J., Marler, L.E., Jadoon, M., Qazi, M.A., Mehboob Mirza, H., Khan, I., Atiq, N., Chang, L.C. and Ahmed, S., 2016. Endophytic fungi associated with Taxus fuana (West Himalayan Yew) of Pakistan: potential bio-resources for cancer chemopreventive agents. *Pharmaceutical Biology*, *54*(11), pp.2547-2554.

Ferri, M., Ranucci, E., Romagnoli, P. and Giaccone, V., 2017. Antimicrobial resistance: a global emerging threat to public health systems. *Critical Reviews in Food Science and Nutrition*, *57*(13), pp.2857-2876.

Firáková, S., Šturdíková, M. and Múčková, M., 2007. Bioactive secondary metabolites produced by microorganisms associated with plants. *Biologia*, *62*(3), pp.251-257.

Founou, R.C., Founou, L.L. and Essack, S.Y., 2017. Clinical and economic impact of antibiotic resistance in developing countries: a systematic review and meta-analysis. *PloS one*, *12*(12), p.e0189621.

Gao, S.S., Li, X.M., Zhang, Y., Li, C.S. and Wang, B.G., 2012. Conidiogenones H and I, two new diterpenes of cyclopiane class from a marine-derived endophytic fungus Penicillium chrysogenum QEN-24S. *Chemistry & biodiversity*, *8*(9), pp.1748-1753.

Garg, N., Kapono, C.A., Lim, Y.W., Koyama, N., Vermeij, M.J., Conrad, D., Rohwer, F. and Dorrestein, P.C., 2015. Mass spectral similarity for untargeted metabolomics data analysis of complex mixtures. *International Journal of Mass Spectrometry*, *377*, pp.719-727.

Genheden, S. and Ryde, U., 2015. The MM/PBSA and MM/GBSA methods to estimate ligand-binding affinities. *Expert Opinion on Drug Discovery*, *10*(5), pp.449-461.

George, T.K., Joy, A., Divya, K. and Jisha, M.S., 2019. In vitro and in silico docking studies of antibacterial compounds derived from endophytic Penicillium setosum. *Microbial Pathogenesis*, *131*, pp.87-97.

Gouda, S., Das, G., Sen, S.K., Shin, H.S. and Patra, J.K., 2016. Endophytes: a treasure house of bioactive compounds of medicinal importance. *Frontiers in Microbiology*, *7*, p.1538.

Goutam, J., Kharwar, R.N., Tiwari, V.K., Mishra, A. and Singh, S., 2016. Isolation and identification of antibacterial compounds isolated from endophytic fungus *Emericella qaudrilineata*. *Natural Products Chemistry & Research*.

Greco, C., Keller, N.P. and Rokas, A., 2019. Unearthing fungal chemodiversity and prospects for drug discovery. *Current Opinion in Microbiology*, *51*, pp.22-29.

Green, J.R., 2019. Fungal endophytes as source to combat bacterial infections.

Gu, W., Ge, H.M., Song, Y.C., Ding, H., Zhu, H.L., Zhao, X.A. and Tan, R.X., 2007. Cytotoxic benzo [j] fluoranthene metabolites from Hypoxylon truncatum IFB-18, an endophyte of Artemisia annua. *Journal of natural products*, *70*(1), pp.114-117.

Gunalan, G., Rathinamala, R. and Kumar, A.R., 2020. Gas chromatography-mass spectroscopy fingerprinting and in silico molecular docking analysis of secondary metabolites from Anethum graveolens L. seeds for anti-inflammatory activity. *Drug Invention Today*, *13*(5).

Guo, B., Dai, J.R., Ng, S., Huang, Y., Leong, C., Ong, W. and Carté, B.K., 2000. Cytonic acids A and B: novel tridepside inhibitors of hCMV protease from the endophytic fungus Cytonaema species. *Journal of Natural Products*, *63*(5), pp.602-604.

Guo, H., Sun, B., Gao, H., Chen, X., Liu, S., Yao, X., Liu, X. and Che, Y., 2009. Diketopiperazines from the Cordyceps-colonizing fungus *Epicoccum nigrum*. *Journal of natural products*, *72*(12), pp.2115-2119.

Han, X., Tang, X., Luo, X., Sun, C., Liu, K., Zhang, Y., Li, P. and Li, G., 2020. Isolation and Identification of Three New Sterigmatocystin Derivatives from the Fungus Aspergillus versicolor Guided by Molecular Networking Approach. *Chemistry & Biodiversity*.

Haq, F.U., Abro, A., Raza, S., Liedl, K.R. and Azam, S.S., 2017. Molecular dynamics simulation studies of novel β-lactamase inhibitor. *Journal of Molecular Graphics and Modelling*, *74*, pp.143-152.

Harper, J.K., Arif, A.M., Ford, E.J., Strobel, G.A., Porco Jr, J.A., Tomer, D.P., Oneill, K.L., Heider, E.M. and Grant, D.M., 2003. Pestacin: a 1, 3-dihydro isobenzofuran from *Pestalotiopsis microspora* possessing antioxidant and antimycotic activities. *Tetrahedron*, *59*(14), pp.2471-2476.

Harwoko, H., Daletos, G., Stuhldreier, F., Lee, J., Wesselborg, S., Feldbrügge, M., Müller, W.E., Kalscheuer, R., Ancheeva, E. and Proksch, P., 2019. Dithiodiketopiperazine derivatives from endophytic fungi *Trichoderma harzianum* and Epicoccum nigrum. *Natural product research*, pp.1-9.

Harwoko, H., Lee, J., Hartmann, R., Mándi, A., Kurtán, T., Müller, W.E., Feldbrügge, M., Kalscheuer, R., Ancheeva, E., Daletos, G. and Frank, M., 2020. Azacoccones FH, new flavipin-derived alkaloids from an endophytic fungus *Epicoccum nigrum* MK214079. *Fitoterapia*, *146*, p.104698.

Hata, K. and Sone, K., 2008. Isolation of endophytes from leaves of Neolitsea sericea in broadleaf and conifer stands. *Mycoscience*, *49*(4), pp.229-232.

Hatakeyama, T., Koseki, T., Murayama, T. and Shiono, Y., 2010. Eremophilane sesquiterpenes from the endophyte Microdiplodia sp. KS 75-1 and revision of the stereochemistries of phomadecalins C and D. *Phytochemistry Letters*, *3*(3), pp.148-151.

Heinig, U.H., 2012. *Studies on the evolution of complex natural products biosynthetic pathways on basis of Taxol biosynthesis in plants and endophytic fungi* (Doctoral dissertation, Hochschulbibliothek der Rheinisch-Westfälischen Technischen Hochschule Aachen)

Herre, E.A., Mejía, L.C., Kyllo, D.A., Rojas, E., Maynard, Z., Butler, A. and Van Bael, S.A., 2007. Ecological implications of anti-pathogen effects of tropical fungal endophytes and mycorrhizae. *Ecology*, *88*(3), pp.550-558.

Hiroyuki, K., Satoshi, T., Shun-ichi, T., Yoshihara, T., Sakamura, S., Shimanuki, T., Sato, T. and Tajimi, A., 1992. New fungitoxic sesquiterpenoids, chokols AG, from stromata of Epichloe typhina and the absolute configuration of chokol E. *Agricultural and biological chemistry*, *53*(3), pp.789-796.

Horgan, K.A. and Murphy, R.A., 2011. Pharmaceutical and chemical commodities from fungi. *Biology and Applications*, p.147.

Hormazabal, E., Astudillo, L., Schmeda-Hirschmann, G., Rodríguez, J. and Theoduloz, C., 2005. Metabolites from Microsphaeropsis olivacea, an endophytic fungus of Pilgerodendron uviferum. *Zeitschrift für Naturforschung C*, *60*(1-2), pp.11-21.

Hou, Y., Braun, D.R., Michel, C.R., Klassen, J.L., Adnani, N., Wyche, T.P. and Bugni, T.S., 2012. Microbial strain prioritization using metabolomics tools for the discovery of natural products. *Analytical chemistry*, *84*(10), pp.4277-4283.

Huang, W.Y., Cai, Y.Z., Hyde, K.D., Corke, H. and Sun, M., 2007. Endophytic fungi from *Nerium oleander L* (Apocynaceae): main constituents and antioxidant activity. *World Journal of Microbiology and Biotechnology*, *23*(9), pp.1253-1263.

Huang, X.Z., Zhu, Y., Guan, X.L., Tian, K., Guo, J.M., Wang, H.B. and Fu, G.M., 2012. A novel antioxidant isobenzofuranone derivative from fungus Cephalosporium sp. AL031. *Molecules*, *17*(4), pp.4219-4224.

Hussain, H., Akhtar, N., Draeger, S., Schulz, B., Pescitelli, G., Salvadori, P., Antus, S., Kurtán, T. and Krohn, K., 2009. New bioactive 2, 3-epoxycyclohexenes and isocoumarins from the endophytic fungus Phomopsis sp. from Laurus azorica. *Eur. J. Org. Chem*, *2009*, pp.749-756.

Hussain, H., Kliche-Spory, C., Al-Harrasi, A., Al-Rawahi, A., Abbas, G., Green, I.R., Schulz, B., Krohn, K. and Shah, A., 2014. Antimicrobial constituents from three endophytic fungi. *Asian Pacific Journal of Tropical Medicine*, *7*, pp.S224-S227.

Jalgaonwala, R.E., Mohite, B.V. and Mahajan, R.T., 2011. A review: natural products from plant associated endophytic fungi. *Journal of Microbiology and Biotechnology Research*, *1*(2), pp.21-32.

Jansen, N., Ohlendorf, B., Erhard, A., Bruhn, T., Bringmann, G. and Imhoff, J.F., 2013. Helicusin E, isochromophilone X and isochromophilone XI: new chloroazaphilones produced by the fungus *Bartalinia robillardoides* strain LF550. *Marine Drugs*, *11*(3), pp.800-816.

Jiang, C.X., Li, J., Zhang, J.M., Jin, X.J., Yu, B., Fang, J.G. and Wu, Q.X., 2019. Isolation, identification, and activity evaluation of chemical constituents from soil fungus *Fusarium avenaceum* SF-1502 and endophytic fungus *Fusarium proliferatum* AF-04. *Journal of Agricultural and Food Chemistry*, *67*(7), pp.1839-1846.

Jin, X., Ma, H., Wang, F., Jiang, J., Cheng, L., Hu, S. and Zhang, G., 2020. Generation of indole derivatives by an endophytic fungus Chaetomium sp. through feeding 1, 2-dimethylindole. *Natural Product Research*, pp.1-9.

Jin, Z., Gao, L., Zhang, L., Liu, T., Yu, F., Zhang, Z., Guo, Q. and Wang, B., 2017. Antimicrobial activity of saponins produced by two novel endophytic fungi from Panax notoginseng. *Natural product research*, *31*(22), pp.2700-2703.

Joseph, B. and Priya, R.M., 2011. Bioactive Compounds from Endophytes and their Potential in. *American Journal of Biochemistry and Molecular Biology*, *1*(3), pp.291-309.

Karplus, M. and McCammon, J.A., 2002. Molecular dynamics simulations of biomolecules. *Nature Structural Biology*, *9*(9), pp.646-652.

Kaul, S., Ahmed, M., Zargar, K., Sharma, P. and Dhar, M.K., 2013. Prospecting endophytic fungal assemblage of *Digitalis lanata Ehrh*. (foxglove) as a novel source of digoxin: a cardiac glycoside. *3 Biotechnology*, *3*(4), pp.335-340.

Kharwar, R.N., Mishra, A., Gond, S.K., Stierle, A. and Stierle, D., 2011. Anticancer compounds derived from fungal endophytes: their importance and future challenges. *Natural product reports*, *28*(7), pp.1208-1228.

Kharwar, R.N., Verma, S.K., Mishra, A., Gond, S.K., Sharma, V.K., Afreen, T. and Kumar, A., 2011. Assessment of diversity, distribution and antibacterial activity of endophytic fungi isolated from a medicinal plant Adenocalymma alliaceum Miers. *Symbiosis*, *55*(1), pp.39-46.

Kharwar, R.N., Verma, V.C., Kumar, A., Gond, S.K., Harper, J.K., Hess, W.M., Lobkovosky, E., Ma, C., Ren, Y. and Strobel, G.A., 2008. Javanicin, an antibacterial naphthaquinone from an endophytic fungus of neem, Chloridium sp. *Current microbiology*, *58*(3), pp.233-238.

Kiani, Y.S., Ranaghan, K.E., Jabeen, I. and Mulholland, A.J., 2019. Molecular Dynamics Simulation Framework to Probe the Binding Hypothesis of CYP3A4 Inhibitors. *International Journal of Molecular Sciences*, *20*(18), p.4468.

Kjer, J., Wray, V., Edrada-Ebel, R., Ebel, R., Pretsch, A., Lin, W. and Proksch, P., 2009. Xanalteric acids I and II and related phenolic compounds from an endophytic Alternaria sp. isolated from the mangrove plant Sonneratia alba. *Journal of natural products*, *72*(11), pp.2053-2057.

Kohanski, M.A., Dwyer, D.J. and Collins, J.J., 2010. How antibiotics kill bacteria: from targets to networks. *Nature Reviews Microbiology*, *8*(6), pp.423-435

Kontnik, R. and Clardy, J., 2008. Codinaeopsin, an antimalarial fungal polyketide. *Organic letters*, *10*(18), pp.4149-4151.

Krohn, K., Michel, A., Römer, E., Flörke, U., Aust, H.J., Draeger, S., Schulz, B. and Wray, V., 1995. Biologically active metabolites from fungi 61); phomosines AC three new biaryl ethers from Phomopsis sp. *Natural Product Letters*, *6*(4), pp.309-314.

Kumar, A., Patil, D., Rajamohanan, P.R. and Ahmad, A., 2013. Isolation, purification and characterization of vinblastine and vincristine from endophytic fungus *Fusarium oxysporum* isolated from *Catharanthus roseus*. *PloS one*, *8*(9), p.e71805.

Lee, J.C., Strobel, G.A., Lobkovsky, E. and Clardy, J., 1996. Torreyanic acid: a selectively cytotoxic quinone dimer from the endophytic fungus Pestalotiopsis microspora. *The Journal of Organic Chemistry*, *61*(10), pp.3232-3233.

Levy, S.B. and Marshall, B., 2004. Antibacterial resistance worldwide: causes, challenges and responses. *Nature Medicine*, *10*(12), pp.S122-S129.

Li, C., Sarotti, A.M., Yang, B., Turkson, J. and Cao, S., 2017. A new N-methoxypyridone from the co-cultivation of Hawaiian endophytic fungi *Camporesia sambuci* FT1061 and *Epicoccum sorghinum* FT1062. *Molecules*, *22*(7), p.1166.

Li, E., Jiang, L., Guo, L., Zhang, H. and Che, Y., 2008. Pestalachlorides A–C, antifungal metabolites from the plant endophytic fungus Pestalotiopsis adusta. *Bioorganic & medicinal chemistry*, *16*(17), pp.7894-7899.

Li, H., Xiao, J., Gao, Y.Q., Tang, J.J., Zhang, A.L. and Gao, J.M., 2014. Chaetoglobosins from Chaetomium globosum, an endophytic fungus in Ginkgo biloba, and their phytotoxic and cytotoxic activities. *Journal of agricultural and food chemistry*, *62*(17), pp.3734-3741.

Li, H.T., Tang, L.H., Liu, T., Yang, R.N., Yang, Y.B., Zhou, H. and Ding, Z.T., 2020. Protoilludane-type sesquiterpenoids from *Armillaria sp.* by co-culture with the endophytic fungus *Epicoccum sp*. associated with *Gastrodia elata*. *Bioorganic Chemistry*, *95*, p.103503.

Li, J., Wu, G., Qin, C., Chen, W., Chen, G. and Wen, L., 2019. Structure characterization and otoprotective effects of a new endophytic exopolysaccharide from Saffron. *Molecules*, *24*(4), p.749.

Li, J.Y., Strobel, G., Harper, J., Lobkovsky, E. and Clardy, J., 2000. Cryptocin, a potent tetramic acid antimycotic from the endophytic fungus Cryptosporiopsis cf. q uercina. *Organic Letters*, *2*(6), pp.767-770.

Liang, H., Xing, Y., Chen, J., Zhang, D., Guo, S. and Wang, C., 2012. Antimicrobial activities of endophytic fungi isolated from Ophiopogon japonicus (Liliaceae). *BMC Complementary and Alternative Medicine*, *12*(1), p.238.

Lim, W., Park, J., Lee, Y.H., Hong, J. and Lee, Y., 2015. Subglutinol A, an immunosuppressive α-pyrone diterpenoid from Fusarium subglutinans, acts as an estrogen receptor antagonist. *Biochemical and biophysical research communications*, *461*(3), pp.507-512.

Liu, L., Li, Y., Liu, S., Zheng, Z., Chen, X., Zhang, H., Guo, L. and Che, Y., 2009. Chloropestolide A, an antitumor metabolite with an unprecedented spiroketal skeleton from Pestalotiopsis fici. *Organic letters*, *11*(13), pp.2836-2839.

Liu, S., Dai, H., Orfali, R.S., Lin, W., Liu, Z. and Proksch, P., 2016. New fusaric acid derivatives from the endophytic fungus *Fusarium oxysporum* and their phytotoxicity to barley leaves. *Journal of Agricultural and Food Chemistry*, *64*(16), pp.3127-3132.

Longenecker, K.L., Stamper, G.F., Hajduk, P.J., Fry, E.H., Jakob, C.G., Harlan, J.E., Edalji, R., Bartley, D.M., Walter, K.A., Solomon, L.R. and Holzman, T.F., 2005.

Structure of MurF from Streptococcus pneumoniae co-crystallized with a small molecule inhibitor exhibits interdomain closure. *Protein science*, *14*(12), pp.3039-3047.

Lösgen, S., Magull, J., Schulz, B., Draeger, S. and Zeeck, A., 2008. Isofusidienols: Novel Chromone-3-oxepines Produced by the Endophytic Fungus Chalara sp.EurJOC p 698-703

Mai, P.Y., Levasseur, M., Buisson, D., Touboul, D. and Eparvier, V., 2020. Identification of antimicrobial compounds from *Sandwithia guyanensis*-associated endophyte using molecular network approach. *Plants*, *9*(1), p.47.

Majumder, A. and Jha, S., 2009. Biotechnological approaches for the production of potential anticancer leads podophyllotoxin and paclitaxel: an overview. *Journal of Biological Sciences*, *1*(1), pp.46-69.

Mallouk, S., Mohamed, N.S.E.D. and Debbab, A., 2020. Cytotoxic Hydroperoxycochliodinol Derivative from Endophytic *Chaetomium* sp. Isolated from *Salvia officinalis*. *Chemistry of Natural Compounds*, *56*(4), pp.701-705.

Mandyam, K., Fox, C. and Jumpponen, A., 2012. Septate endophyte colonization and host responses of grasses and forbs native to a tallgrass prairie. *Mycorrhiza*, *22*(2), pp.109-119.

Manganyi, M.C., Regnier, T., Tchatchouang, C.D.K., Bezuidenhout, C.C. and Ateba, C.N., 2019. Antibacterial activity of endophytic fungi isolated from *Sceletium tortuosum L*. (Kougoed). *Annals of Microbiology*, *69*(6), pp.659-663.

Marcellano, J.P., Collanto, A.S. and Fuentes, R.G., 2017. Antibacterial activity of endophytic fungi isolated from the bark of *Cinnamomum mercadoi*. *Pharmacognosy Journal*, *9*(3).

Martínez-Luis, S., Della-Togna, G., Coley, P.D., Kursar, T.A., Gerwick, W.H. and Cubilla-Rios, L., 2008. Antileishmanial constituents of the Panamanian endophytic fungus Edenia sp. *Journal of natural products*, *71*(12), pp.2011-2014.

Mei, J., Chen, X., Liu, J., Yi, Y., Zhang, Y. and Ying, G., 2019. A Biotransformation Process for Production of Genistein from Sophoricoside by a Strain of *Rhizopus oryza*. *Scientific Reports*, *9*(1), pp.1-5.

Meng, X.Y., Zhang, H.X., Mezei, M. and Cui, M., 2011. Molecular docking: a powerful approach for structure-based drug discovery. *Current Computer-aided Drug Design*, *7*(2), pp.146-157.

Merrouche, R., Yekkour, A., Coppel, Y., Bouras, N., Zitouni, A., Lebrihi, A., Mathieu, F. and Sabaou, N., 2019. A new dithiolopyrrolone antibiotic triggered by a long fermentation of *Saccharothrix algeriensis* NRRL B-24137 in sorbic acid-amended medium. *Letters in Applied Microbiology*, *69*(4), pp.294-301.

Miller III, B.R., McGee Jr, T.D., Swails, J.M., Homeyer, N., Gohlke, H. and Roitberg, A.E., 2012. MMPBSA. py: an efficient program for end-state free energy calculations. *Journal of chemical theory and computation*, *8*(9), pp.3314-3321.

Mohimani, H., Gurevich, A., Mikheenko, A., Garg, N., Nothias, L.F., Ninomiya, A., Takada, K., Dorrestein, P.C. and Pevzner, P.A., 2017. Dereplication of peptidic natural products through database search of mass spectra. *Nature Chemical Biology*, *13*(1), pp.30-37.

Monserrat-Martinez, A., Gambin, Y. and Sierecki, E., 2019. Thinking outside the bug: molecular targets and strategies to overcome antibiotic resistance. *International Journal of Molecular Sciences*, *20*(6), p.1255.

Nair, D.N. and Padmavathy, S., 2014. Impact of endophytic microorganisms on plants, environment and humans. Sci World Journal 2014: 250693.

Navidinia, M., 2016. The clinical importance of emerging ESKAPE pathogens in nosocomial infections.

Ng, T.B., Cheung, R.C.F., Wong, J.H., Bekhit, A.A. and Bekhit, A.E.D., 2015. Antibacterial products of marine organisms. *Applied Microbiology and Biotechnology*, *99*(10), pp.4145-4173.

Ngashangva, N., Indira Devi, S. and Kalita, M.C., 2019. Screening of Endophytes from Traditionally Used Medicinal Plants of Manipur for Their Antimicrobial Activity: An Impact towards Future Drug Discovery. *Inernationalt Journal of Scientific Research in Biological Sciences Vol*, *6*, p.5.

Nielsen, K.F. and Larsen, T.O., 2015. The importance of mass spectrometric dereplication in fungal secondary metabolite analysis. *Frontiers in Microbiology*, *6*, p.71.

Nobeli, I., Laskowski, R.A., Valdar, W.S. and Thornton, J.M., 2001. On the molecular discrimination between adenine and guanine by proteins. *Nucleic Acids Research*, *29*(21), pp.4294-4309.

Ola, A.R., Tawo, B.D., Belli, H.L.L., Proksch, P., Tommy, D. and Hakim, E.H., 2018. A new antibacterial polyketide from the endophytic fungi *Aspergillus fumigatiaffinis*. *Natural Product Communications*, *13*(12), p.1934578X1801301202.

Oppong-Danquah, E., Parrot, D., Blümel, M., Labes, A. and Tasdemir, D., 2018. Molecular networking-based metabolome and bioactivity analyses of marine-adapted fungi co-cultivated with phytopathogens. *Frontiers in Microbiology*, *9*, p.2072.

Owen, N.L. and Hundley, N., 2004. Endophytes—the chemical synthesizers inside plants. *Science Progress*, *87*(2), pp.79-99.

Pal, P.P., Shaik, A.B. and Begum, A.S., 2020. Prospective Leads from Endophytic Fungi for Anti-Inflammatory Drug Discovery. *Planta Medica*.

Pansanit, A. and Pripdeevech, P., 2018. Antibacterial secondary metabolites from an endophytic fungus, Arthrinium sp. MFLUCC16-1053 isolated from Zingiber cassumunar. *Mycology*, *9*(4), pp.264-272.

Parrot, D., Oppong-Danquah, E., Fan, B. and Tasdemir, D., 2017. Molecular Networking for Identification of Enhanced Chemical Inventories of Baltic Marine Fungi. *Planta Medica International Open*, *4*(S 01), pp.Tu-SL.

Parthasarathy, R., Chandrika, M., Rao, H.Y., Kamalraj, S., Jayabaskaran, C. and Pugazhendhi, A., 2020. Molecular profiling of marine endophytic fungi from green algae: Assessment of antibacterial and anticancer activities. *Process Biochemistry*.

Perrone, G., Susca, A., Cozzi, G., Ehrlich, K., Varga, J., Frisvad, J.C., Meijer, M., Noonim, P., Mahakarnchanakul, W. and Samson, R.A., 2007. Biodiversity of *Aspergillus* species in some important agricultural products. *Studies in Mycology*, *59*, pp.53-66.

Pervaiz, I., Ahmad, S., Khaliq, F.H., Arshad, A., Imran, M., Khan, B.A., Ullah, A., Ali, U., Iqbal, K., Usman, M. and Bibi, H., 2014. Fungal biotransformation of ezetimibe. *Biotechnology & Biotechnological Equipment*, *28*(5), pp.934-940.

Petrini, O., 1991. Fungal endophytes of tree leaves. In *Microbial ecology of leaves* (pp. 179-197). Springer, New York, NY.

Hallmann, J., Quadt-Hallmann, A., Mahaffee, W.F. and Kloepper, J.W., 1997. Endophytic bacteria in agricultural crops. *Can J Microbiol*, *43*(10), pp.895-914.

Praptiwi, M.R., Wulansari, D., Fathoni, A. and Agusta, A., 2018. Antibacterial and antioxidant activities of endophytic fungi extracts of medicinal plants from Central Sulawesi. *Journal Applied Pharmaceutical Science*, *8*(8), pp.69-74.

Prasad, S. and Roy, I., 2018. Converting enzymes into tools of industrial importance. *RecentPpatents on Biotechnology*, *12*(1), pp.33-56.

Puri, S.C., Verma, V., Amna, T., Qazi, G.N. and Spiteller, M., 2005. An endophytic fungus from Nothapodytes f oetida that produces Camptothecin. *Journal of natural products*, *68*(12), pp.1717-1719.

Qader, M.M., Hamed, A.A., Soldatou, S., Abdelraof, M., Elawady, M.E., Hassane, A.S., Belbahri, L., Ebel, R. and Rateb, M.E., 2021. Antimicrobial and Antibiofilm Activities of the Fungal Metabolites Isolated from the Marine Endophytes Epicoccum nigrum M13 and Alternaria alternata 13A. *Marine drugs*, *19*(4), p.232.

Qi, J., Wang, D., Yin, X., Zhang, Q. and Gao, J.M., 2020. New Metabolite with Inhibitory Activity Against α-Glucosidase and α-Amylase from Endophytic

Chaetomium globosum. *Natural Product Communications*, *15*(7), p.1934578X20941338.

Qi, X., Li, X., Zhao, J., He, N., Li, Y., Zhang, T., Wang, S., Yu, L. and Xie, Y., 2019. GKK1032C, a new alkaloid compound from the endophytic fungus *Penicillium sp*. CPCC 400817 with activity against methicillin-resistant *S. aureus*. *The Journal of Antibiotics*, *72*(4), pp.237-240.

Ratnaweera, P.B., Walgama, R.C., Jayasundera, K.U., Herath, S.D., Abira, S., Williams, D.E., Andersen, R.J. and de Silva, E.D., 2018. Antibacterial activities of endophytic fungi isolated from six Sri Lankan plants of the family Cyperaceae. ||| *Bangladesh Journal of Pharmacology*|||, *13*(3), pp.264-272.

Rayner, M.C., 1915. Obligate symbiosis in *Calluna vulgaris*. *Annals of Botany*, *29*(113), pp.97-133.

Rehman, S., Shawl, A.S., Kour, A., Andrabi, R., Sudan, P., Sultan, P., Verma, V. and Qazi, G.N., 2008. An endophytic Neurospora sp. from Nothapodytes foetida producing camptothecin. *Applied biochemistry and microbiology*, *44*(2), pp.203-209.

Reygaert, W.C., 2018. An overview of the antimicrobial resistance mechanisms of bacteria. *AIMS microbiology*, *4*(3), p.482.

Rice, L.B., 2008. Federal funding for the study of antimicrobial resistance in nosocomial pathogens: no ESKAPE.

Richter, M.F. and Hergenrother, P.J., 2017. Reaction: broad-spectrum antibiotics, a call for chemists. *Chem*, *3*(1), pp.10-13.

Rodriguez, R.J., White Jr, J.F., Arnold, A.E. and Redman, A.R.A., 2009. Fungal endophytes: diversity and functional roles. *New Phytologist*, *182*(2), pp.314-330.

Roy, P. and Adhikari, S., 2018. Search for in-silico applications in drug discovery and applications of different disciplines in it: a survey. *International Journal of Advanced Research in Computer Science*, *9*(1).

Rukachaisirikul, V., Sommart, U., Phongpaichit, S., Sakayaroj, J. and Kirtikara, K., 2008. Metabolites from the endophytic fungus *Phomopsis* sp. PSU-D15. *Phytochemistry*, *69*(3), pp.783-787.

Rusch, M., Spielmeyer, A., Meißner, J., Kietzmann, M., Zorn, H. and Hamscher, G., 2017. Efficient reduction of antibacterial activity and cytotoxicity of fluoroquinolones by fungal-mediated N-oxidation. *Journal of Agricultural and Food Chemistry*, *65*(15), pp.3118-3126.

Saha, P., Talukdar, A.D., Choudhury, M.D. and Nath, D., 2019. Bioprospecting for Fungal-Endophyte-Derived Natural Products for Drug Discovery. In *Advances in Endophytic Fungal Research* (pp. 35-49). Springer, Cham.

Sahihi, M., 2016. In-Silico Study on the Interaction of Saffron Ligands and Beta-Lactoglobulin by Molecular Dynamics and Molecular Docking Approach. *Journal of Macromolecular Science, Part B*, *55*(1), pp.73-84.

Saikkonen, K., Saari, S. and Helander, M., 2010. Defensive mutualism between plants and endophytic fungi. *Fungal Diversity*, *41*(1), pp.101-113.

Salehi, M., Moieni, A., Safaie, N. and Farhadi, S., 2019. Elicitors derived from endophytic fungi *Chaetomium globosum* and *Paraconiothyrium brasiliense* enhance paclitaxel production in Corylus avellana cell suspension culture. *Plant Cell, Tissue and Organ Culture (PCTOC)*, *136*(1), pp.161-171.

Santos, I.P.D., Silva, L.C.N.D., Silva, M.V.D., Araújo, J.M.D., Cavalcanti, M.D.S. and Lima, V.L.D.M., 2015. Antibacterial activity of endophytic fungi from leaves of Indigofera suffruticosa Miller (Fabaceae). *Frontiers in Microbiology*, *6*, p.350.

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

Schulz, B., Sucker, J., Aust, H.J., Krohn, K., Ludewig, K., Jones, P.G. and Döring, D., 1995. Biologically active secondary metabolites of endophytic Pezicula species. *Mycological Research*, *99*(8), pp.1007-1015.

Selim, K.A., El-Beih, A.A., AbdEl-Rahman, T.M. and El-Diwany, A.I., 2011. Biodiversity and antimicrobial activity of endophytes associated with Egyptian medicinal plants. *Mycosphere*, *2*(6), pp.669-678.

Selim, K.A., El-Beih, A.A., Abdel-Rahman, T.M. and El-Diwany, A.I., 2016. High expression level of antioxidants and pharmaceutical bioactivities of endophytic fungus Chaetomium globosum JN711454. *Preparative biochemistry & biotechnology*, *46*(2), pp.131-140.

Sharma, D., Pramanik, A. and Agrawal, P.K., 2016. Evaluation of bioactive secondary metabolites from endophytic fungus Pestalotiopsis neglecta BAB-5510 isolated from leaves of Cupressus torulosa D. Don. *3 Biotech*, *6*(2), p.210.

Siddiqui, I.N., Zahoor, A., Hussain, H., Ahmed, I., Ahmad, V.U., Padula, D., Draeger, S., Schulz, B., Meier, K., Steinert, M. and Kurtán, T., 2011. Diversonol and blennolide derivatives from the endophytic fungus Microdiplodia sp.: absolute configuration of diversonol. *Journal of natural products*, *74*(3), pp.365-373.

Silva, G.H., de Oliveira, C.M., Teles, H.L., Pauletti, P.M., Castro-Gamboa, I., Silva, D.H., Bolzani, V.S., Young, M.C., Costa-Neto, C.M., Pfenning, L.H. and Berlinck, R.G., 2010. Sesquiterpenes from Xylaria sp., an endophytic fungus associated with Piper aduncum (Piperaceae). *Phytochemistry Letters*, *3*(3), pp.164-167.

Silva, G.H., Teles, H.L., Zanardi, L.M., Young, M.C.M., Eberlin, M.N., Hadad, R., Pfenning, L.H., Costa-Neto, C.M., Castro-Gamboa, I., da Silva Bolzani, V. and Araújo, Â.R., 2006. Cadinane sesquiterpenoids of Phomopsis cassiae, an endophytic fungus associated with Cassia spectabilis (Leguminosae). *Phytochemistry*, *67*(17), pp.1964-1969.

Singh, M.P., Janso, J.E., Luckman, S.W., Brady, S.F., Clardy, J., Greenstein, M. and Maiese, W.M., 2000. Biological activity of guanacastepene, a novel diterpenoid antibiotic produced by an unidentified fungus CR115. *The Journal of antibiotics*, *53*(3), pp.256-261.

Singh, V., Praveen, V., Tripathi, D., Haque, S., Somvanshi, P., Katti, S.B. and Tripathi, C.K.M., 2015. Isolation, characterization and antifungal docking studies of wortmannin isolated from *Penicillium radicum*. *Scientific reports*, *5*(1), pp.1-13.

Somjaipeng, S., Medina, A. and Magan, N., 2016. Environmental stress and elicitors enhance taxol production by endophytic strains of *Paraconiothyrium variabile* and *Epicoccum nigrum*. *Enzyme and Microbial Technology*, *90*, pp.69-75.

Song, C., Ding, G., Wu, G., Yang, J., Zhang, M., Wang, H., Wei, D., Qin, J. and Guo, L., 2020. Identification of a Unique *Azaphilone* Produced by *Chaetomium globosum* Isolated from *Polygonatum sibiricum*. *Chemistry & Biodiversity*, *17*(3), p.e1900744.

Song, Y.C., Huang, W.Y., Sun, C., Wang, F.W. and Tan, R.X., 2005. Characterization of graphislactone A as the antioxidant and free radical-scavenging substance from the culture of Cephalosporium sp. IFB-E001, an endophytic fungus in Trachelospermum jasminoides. *Biological and Pharmaceutical Bulletin*, *28*(3), pp.506-509.

Sordon, S., Popłoński, J., Tronina, T. and Huszcza, E., 2019. Regioselective O-glycosylation of flavonoids by fungi *Beauveria bassiana, Absidia coerulea* and *Absidia glauca*. *Bioorganic Chemistry*, *93*, p.102750.

Specian, V., Sarragiotto, M.H., Pamphile, J.A. and Clemente, E., 2012. Chemical characterization of bioactive compounds from the endophytic fungus *Diaporthe helianthi* isolated from *Luehea divaricata. Brazilian Journal of Microbiology*, *43*(3), pp.1174-1182.

Stępniewska, Z. and Kuźniar, A., 2013. Endophytic microorganisms—promising applications in bioremediation of greenhouse gases. *Applied Microbiology and Biotechnology*, *97*(22), pp.9589-9596.

Strobel, G. and Daisy, B., 2003. Bioprospecting for microbial endophytes and their natural products. Microbiology Molecular Biol Rev67: 491–502.

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

Tacconelli, E., Carrara, E., Savoldi, A., Harbarth, S., Mendelson, M., Monnet, D.L., Pulcini, C., Kahlmeter, G., Kluytmans, J., Carmeli, Y. and Ouellette, M., 2018. Discovery, research, and development of new antibiotics: the WHO priority list of antibiotic-resistant bacteria and tuberculosis. *The Lancet Infectious Diseases*, *18*(3), pp.318-327.

Talontsi, F.M., Dittrich, B., Schüffler, A., Sun, H. and Laatsch, H., 2013. Epicoccolides: Antimicrobial and antifungal polyketides from an endophytic fungus Epicoccum sp associated with Theobroma cacao. *European Journal of Organic Chemistry*, (15), pp.3174-3180.

Tantapakul, C., Promgool, T., Kanokmedhakul, K., Soytong, K., Song, J., Hadsadee, S., Jungsuttiwong, S. and Kanokmedhakul, S., 2020. Bioactive xanthoquinodins and epipolythiodioxopiperazines from *Chaetomium globosum* 7s-1, an endophytic fungus isolated from Rhapis cochinchinensis (Lour.) Mart. *Natural Product Research*, *34*(4), pp.494-502.

Tapfuma, K.I., Sebola, T.E., Uche-Okereafor, N., Koopman, J., Hussan, R., Makatini, M.M., Mekuto, L. and Mavumengwana, V., 2020. Anticancer activity and metabolite profiling data of *Penicillium janthinellum* KTMT5. *Data in Brief*, *28*, p.104959.

Taufiq, M.M.J. and Darah, I., 2019. Antibacterial activity of an endophytic fungus *Lasiodiplodia pseudotheobromae* IBRL OS-64 residing in leaves of a medicinal herb, *Ocimum sanctum* Linn. *Journal of Applied Biology & Biotechnology Vol*, *7*(02), pp.35-41.

Tejesvi, M.V. and Pirttilä, A.M., 2018. Endophytic fungi, occurrence, and metabolites. In *Physiology and Genetics* (pp. 213-230). Springer, Cham.

Tianpanich, K., Prachya, S., Wiyakrutta, S., Mahidol, C., Ruchirawat, S. and Kittakoop, P., 2011. Radical scavenging and antioxidant activities of isocoumarins and a phthalide from the endophytic fungus Colletotrichum sp. *Journal of natural products*, *74*(1), pp.79-81.

Trosset, J.Y. and Cavé, C., 2019. In silico drug–target profiling. In *Target Identification and Validation in Drug Discovery* (pp. 89-103). Humana Press, New York, NY.

Tyers, M. and Wright, G.D., 2019. Drug combinations: a strategy to extend the life of antibiotics in the 21st century. *Nature Reviews Microbiology*, *17*(3), pp.141-155.

Uzma, F., Mohan, C.D., Siddaiah, C.N. and Chowdappa, S., 2019. Endophytic fungi: promising source of novel bioactive compounds. In *Advances in Endophytic Fungal Research* (pp. 243-265). Springer, Cham.

Van Heijenoort, J., 1994. Biosynthesis of the bacterial peptidoglycan unit. In *New Comprehensive Biochemistry* (Vol. 27, pp. 39-54). Elsevier.

VanderMolen, K.M., Raja, H.A., El-Elimat, T. and Oberlies, N.H., 2013. Evaluation of culture media for the production of secondary metabolites in a natural products screening program. *Amb Express*, *3*(1), p.71.

Vasundhara, M., Kumar, A. and Reddy, M.S., 2016. Molecular approaches to screen bioactive compounds from endophytic fungi. *Frontiers in Microbiology*, *7*, p.1774.

Venisetty, R.K., Keshetty, S. and Ciddi, V., 2011. Biotransformation of silibinin (silybin) using fungal organisms. *Indian Journal of Pharmaceutical Education and Research*, *45*, pp.384-391.

Vidyavathi, M., Sarika, B. and Varalakshmi, S., 2014. Fungal Biotransformation of Fenofibrate. *metabolism*, *5*(6), p.7.

Wang, B., Li, H., Zhu, L., Tan, F., Li, Y., Zhang, L., Ding, Z. and Shi, G., 2017. High-efficient production of citric acid by Aspergillus niger from high concentration of substrate based on the staged-addition glucoamylase strategy. *Bioprocess and biosystems engineering*, *40*(6), pp.891-899.

Wang, F.W., Jiao, R.H., Cheng, A.B., Tan, S.H. and Song, Y.C., 2007. Antimicrobial potentials of endophytic fungi residing in Quercus variabilis and brefeldin A obtained from Cladosporium sp. *World Journal of Microbiology and Biotechnology*, *23*(1), pp.79-83.

Wang, J., Vasaikar, S., Shi, Z., Greer, M. and Zhang, B., 2017. WebGestalt 2017: a more comprehensive, powerful, flexible and interactive gene set enrichment analysis toolkit. *Nucleic acids research*, *45*(W1), pp.W130-W137.

Wang, J.M., Ding, G.Z., Fang, L., Dai, J.G., Yu, S.S., Wang, Y.H., Chen, X.G., Ma, S.G., Qu, J., Xu, S. and Du, D., 2010. Thiodiketopiperazines produced by the endophytic fungus *Epicoccum nigrum*. *Journal of Natural Products*, *73*(7), pp.1240-1249.

Wang, L., Li, Y., Yu, P., Xie, Z., Luo, Y. and Lin, Y., 2010. Biodegradation of phenol at high concentration by a novel fungal strain Paecilomyces variotii JH6. *Journal of Hazardous Materials*, *183*(1-3), pp.366-371.

Wang, M., Carver, J.J., Phelan, V.V., Sanchez, L.M., Garg, N., Peng, Y., Nguyen, D.D., Watrous, J., Kapono, C.A., Luzzatto-Knaan, T. and Porto, C., 2016. Sharing and community curation of mass spectrometry data with Global Natural Products Social Molecular Networking. *Nature biotechnology*, *34*(8), pp.828-837.

Wang, M., Carver, J.J., Phelan, V.V., Sanchez, L.M., Garg, N., Peng, Y., Nguyen, D.D., Watrous, J., Kapono, C.A., Luzzatto-Knaan, T. and Porto, C., 2016. Sharing and community curation of mass spectrometry data with Global Natural Products Social Molecular Networking. *Nature Biotechnology*, *34*(8), pp.828-837.

Wang, Q.X., Li, S.F., Zhao, F., Dai, H.Q., Bao, L., Ding, R., Gao, H., Zhang, L.X., Wen, H.A. and Liu, H.W., 2011. Chemical constituents from endophytic fungus *Fusarium oxysporum*. *Fitoterapia*, *82*(5), pp.777-781.

Wang, S., Li, X.M., Teuscher, F., Li, D.L., Diesel, A., Ebel, R., Proksch, P. and Wang, B.G., 2006. Chaetopyranin, a benzaldehyde derivative, and other related metabolites from Chaetomium globosum, an endophytic fungus derived from the marine red alga Polysiphonia urceolata. *Journal of Natural Products*, *69*(11), pp.1622-1625.

Wang, S., Li, X.M., Teuscher, F., Li, D.L., Diesel, A., Ebel, R., Proksch, P. and Wang, B.G., 2006. Chaetopyranin, a benzaldehyde derivative, and other related metabolites from Chaetomium globosum, an endophytic fungus derived from the marine red alga Polysiphonia urceolata. *Journal of Natural Products*, *69*(11), pp.1622-1625.

Wang, X., Li, P., Li, Z., Yin, J., He, M., Xue, W., Chen, Z. and Song, B., 2013. Synthesis and bioactivity evaluation of novel arylimines containing a 3-aminoethyl-2-

[(p-trifluoromethoxy) anilino]-4 (3 H)-quinazolinone moiety. *Journal of agricultural and food chemistry*, *61*(40), pp.9575-9582.

Wang, Y., Xiang, L., Huang, Y., Yi, X. and He, X., 2019. Microbial transformation of laxogenin by the fungus Syncephalastrum *racemosum*. *Tetrahedron*, *75*(10), pp.1440-1449.

Wang, Z., Xue, R., Cui, J., Wang, J., Fan, W., Zhang, H. and Zhan, X., 2019. Antibacterial activity of a polysaccharide produced from *Chaetomium globosum* CGMCC 6882. *International Journal of Biological Macromolecules*, *125*, pp.376-382.

Wangun, H.V.K. and Hertweck, C., 2007. Epicoccarines A, B and epipyridone: tetramic acids and pyridone alkaloids from an *Epicoccum* sp. associated with the tree fungus *Pholiota squarrosa*. *Organic & Biomolecular Chemistry*, *5*(11), pp.1702-1705.

Wani, Z.A., Ashraf, N., Mohiuddin, T. and Riyaz-Ul-Hassan, S., 2015. Plant-endophyte symbiosis, an ecological perspective. *Applied Microbiology and Biotechnology*, *99*(7), pp.2955-2965.

Watrous, J., Roach, P., Alexandrov, T., Heath, B.S., Yang, J.Y., Kersten, R.D., van der Voort, M., Pogliano, K., Gross, H., Raaijmakers, J.M. and Moore, B.S., 2012. Mass spectral molecular networking of living microbial colonies. *Proceedings of the National Academy of Sciences*, *109*(26), pp.E1743-E1752.

WHO News Report on AMR 29 April 2019 Joint News Release New York.

Wicklow, D.T. and Pearson, T.C., 2005. Detection and removal of single mycotoxin contaminated maize grains following harvest. *Microorganisms, Mycotoxins, and Other Biological Contaminants*, *1*, pp.109-119.

Wilson, D., 1995. Endophyte: the evolution of a term, and clarification of its use and definition. *Oikos*, pp.274-276.

Wingfield, P.T. ed., 2016. Protein precipitation using ammonium sulfate. *Current Protocols in Protein Science*, *84*(1), pp.A-3F.

Wright, A.D., Osterhage, C. and König, G.M., 2003. Epicoccamide, a novel secondary metabolite from a jellyfish-derived culture of Epicoccum purpurascens. *Organic & biomolecular chemistry*, *1*(3), pp.507-510.

Xiao, W.J., Chen, H.Q., Wang, H., Cai, C.H., Mei, W.L. and Dai, H.F., 2018. New secondary metabolites from the endophytic fungus *Fusarium* sp. HP-2 isolated from "Qi-Nan" agarwood. *Fitoterapia*, *130*, pp.180-183.

Xie, J., Wu, Y.Y., Zhang, T.Y., Zhang, M.Y., Peng, F., Lin, B. and Zhang, Y.X., 2018. New antimicrobial compounds produced by endophytic *Penicillium janthinellum* isolated from *Panax notoginseng* as potential inhibitors of FtsZ. *Fitoterapia*, *131*, pp.35-43.

Xu, W., Li, G., Huang, X. and Luo, Z., 2015. Fungal diversity study in the deep sea sediments of three oceans by culture-dependent approach. *Journal of Applied Oceanography*, *34*, pp.103-110.

Yan, J., Qi, N., Wang, S., Gadhave, K. and Yang, S., 2014. Characterization of secondary metabolites of an endophytic fungus from Curcuma wenyujin. *Current microbiology*, *69*(5), pp.740-744.

Yan, W., Cao, L.L., Zhang, Y.Y., Zhao, R., Zhao, S.S., Khan, B. and Ye, Y.H., 2018. New metabolites from endophytic fungus *Chaetomium globosum* CDW7. *Molecules*, *23*(11), p.2873.

Yan, Z., Huang, C., Guo, H., Zheng, S., He, J., Lin, J. and Long, Y., 2020. Isobenzofuranone monomer and dimer derivatives from the mangrove endophytic fungus Epicoccum nigrum SCNU-F0002 possess α-glucosidase inhibitory and antioxidant activity. *Bioorganic chemistry*, *94*, p.103407.

Yan, Z., Li, J., Ye, G., Chen, T., Li, M., Liang, Y. and Long, Y., 2020. Fused multicyclic polyketides with a two-spiro-carbon skeleton from mangrove-derived endophytic fungus *Epicoccum nigrum* SCNU-F0002. *RSC Advances*, *10*(48), pp.28560-28566.

Yan, Z., Wen, S., Ding, M., Guo, H., Huang, C., Zhu, X., Huang, J., She, Z. and Long, Y., 2019. The purification, characterization, and biological activity of new polyketides

from mangrove-derived endophytic fungus *Epicoccum nigrum* SCNU-F0002. *Marine Drugs*, *17*(7), p.414.

Yang, J.Y., Sanchez, L.M., Rath, C.M., Liu, X., Boudreau, P.D., Bruns, N., Glukhov, E., Wodtke, A., De Felicio, R., Fenner, A. and Wong, W.R., 2013. Molecular networking as a dereplication strategy. *Journal of Natural Products*, *76*(9), pp.1686-1699.

Yang, S.Q., Li, X.M., Xu, G.M., Li, X., An, C.Y. and Wang, B.G., 2018. Antibacterial anthraquinone derivatives isolated from a mangrove-derived endophytic fungus *Aspergillus nidulans* by ethanol stress strategy. *The Journal of Antibiotics*, *71*(9), pp.778-784.

Ye, Y., Xiao, Y., Ma, L., Li, H., Xie, Z., Wang, M., Ma, H., Tang, H. and Liu, J., 2013. Flavipin in Chaetomium globosum CDW7, an endophytic fungus from Ginkgo biloba, contributes to antioxidant activity. *Applied microbiology and biotechnology*, *97*(16), pp.7131-7139.

Yu, J.H. and Keller, N., 2005. Regulation of secondary metabolism in filamentous fungi. *Annu. Rev. Phytopathol.*, *43*, pp.437-458.

Zhang, P., Yuan, X.L., Du, Y.M., Zhang, H.B., Shen, G.M., Zhang, Z.F., Liang, Y.J., Zhao, D.L. and Xu, K., 2019. Angularly prenylated indole alkaloids with antimicrobial and insecticidal activities from an endophytic fungus *Fusarium sambucinum* TE-6L. *Journal of Agricultural and Food Chemistry*, *67*(43), pp.11994-12001.

Zhang, P.L., Wang, G., Xu, F.Q., Liu, J.S., Wang, J.T., Zhang, R., Liu, H.T., Hu, J.M., Wang, G.K. and Wu, P.Y., 2019. Aspergilolide, a steroid lactone produced by an endophytic fungus *Aspergillus* sp. MBL1612 isolated from *Paeonia ostii*. *Natural Product Research*, *33*(15), pp.2133-2138.

Zhao, J., Mou, Y., Shan, T., Li, Y., Zhou, L., Wang, M. and Wang, J., 2010. Antimicrobial metabolites from the endophytic fungus Pichia guilliermondii isolated from Paris polyphylla var. yunnanensis. *Molecules*, *15*(11), pp.7961-7970.

Zhao, P., Yang, M., Zhu, G., Zhao, B., Wang, H., Liu, H., Wang, X., Qi, J., Yin, X., Yu, L. and Meng, Y., 2021. Mollicellins SU, three new depsidones from Chaetomium

brasiliense SD-596 with anti-MRSA activities. *The Journal of Antibiotics*, *74*(5), pp.317-323.

Zhu, X., Zhong, Y., Xie, Z., Wu, M., Hu, Z., Ding, W. and Li, C., 2018. Fusarihexins A and B: Novel cyclic hexadepsipeptides from the mangrove endophytic fungus *Fusarium* sp. R5 with antifungal activities. *Planta Medica*, *84*(18), pp.1355-1362.

Zou, W.X., Meng, J.C., Lu, H., Chen, G.X., Shi, G.X., Zhang, T.Y. and Tan, R.X., 2000. Metabolites of Colletotrichum gloeosporioides, an endophytic fungus in Artemisia mongolica. *Journal of Natural Products*, *63*(11), pp.1529-1530.