CHAPTER ONE

INTRODUCTION

1.1 BACKGROUND OF STUDY

Microbial endophytes are microorganisms which grow intercellularly and asymptomatically within living tissues establishing mutual relationship with the host plants (Petrini, 1991). In recent years, researchers have begun to realize that plants may serve as a repository of untold numbers of organisms known as endophytes (Bacon and White, 2000; Strobel, 2002).

Endophytes, which occupy a unique biotope with global estimation up to one million species, are a great choice in the study of natural products to assist in solving not only plant diseases, but also human and animal health problems (Gimenez *et al*., 2007). Since the endophytes can be found in nearly all living plant species, a scientific basis in plant selection is necessary for the study of endophytes in order to isolate microorganisms with pharmaceutical potentials (Tong *et al*., 2011 and Zhao *et al*., 2011).

Most endophytes are capable of synthesizing bioactive compounds that may provide plants with a defense against pathogens, and some of these compounds have proven useful for novel drug discovery (Guo *et al*., 2008; Yan *et al*., 2011).

Approximately 4,000 secondary metabolites of fungal origin have been described to possess biological activities (Dreyfuss and Chapela, 1994). The number of secondary metabolites produced by fungal endophytes is larger than that of any other endophytic microorganisms (Zhang *et al*., 2006).

Since the chemical constituents from medicinal plants are complex, more and more endophytic fungi with novel metabolites of pharmaceutical importance have been isolated from medicinal plants, and a series of new and useful compounds have also been obtained

(Huang *et al*., 2007; Guo *et al*., 2008; Kusari *et al*., 2009a). No doubt, exploiting a variety of new natural products from endophytic fungi of medicinal plants has become a hot spot of new drug research.

World health problems caused by viruses, parasites, and drug resistant bacteria and fungi have become increasingly alarming. Cancer also, has become an increasing global health problem due to its high rate of morbidity and mortality.

The process of drug discovery and development seeks to make available, medications that are safe and effective in improving the length and quality of life. Though there are antimicrobial, antioxidant and anticancer products of plant and other natural origins in clinical testing at the moment, the search for novel bioactive compounds is of utmost importance as diseases, including cancer, are constantly developing resistance to existing drugs.

Many pathogenic microorganisms have developed resistance due to the misuse or long-term use of the same class of antibiotics. An intensive search for newer and more effective antibiotics to deal with these problems is now underway. The isolation of novel secondary metabolites from the endophytes is a progressive field in research (Huang *et al*., 2008).

Endophytes are believed to carry out a resistance mechanism to overcome pathogenic invasion by producing secondary metabolites (Tan and Zou, 2001). So far, studies reported a large number of antimicrobial compounds isolated from endophytes, belonging to several structural classes like alkaloids, peptides, steroids, terpenoids, phenols, quinines and flavonoids (Yu *et al.,* 2010). The discovery of novel antimicrobial metabolites from endophytes is an important alternative to overcome the increasing levels of drug resistance by pathogens, the insufficient number of effective antibiotics against diverse bacterial species and few new antimicrobial agents in development, probably due to relatively unfavourable returns on investment (Yu *et al*., 2010).

Another interesting application of bioactive compounds from endophytic fungi is in the inhibition of viruses. Two novel human cytomegalovirus protease inhibitors, cytonic acids A and B have been isolated from the solid-state fermentation of the endophytic fungus *Cytonaema sp.* (Guo *et al.,* 2000). An endophytic fungus *Pestalotiopsis theae* of an unidentified tree on Jianfeng Mountain, China was capable of producing Pestalotheol C with anti-HIV properties (Li *et al*., 2008b). Although it is apparent that the potential for the discovery for compounds, from endophytes, having antiviral activity is in its infancy, the fact, however, that some compounds have been found is promising. The main limitation in antiviral compound discovery is probably related to the absence of appropriate antiviral screening systems in most compound discovery programs (Strobel and Daisy, 2003).

The burden of cancer in Nigeria is unknown; mainly because of lack of statistics or under-reporting (Abdulkareem, 2009). Conventional cancer chemotherapy has the limitation of multidrug resistance (MDR) caused by overexpression of integral membrane transporters, such as P-gp, which can efflux intracellular anticancer drugs thus decreasing drug accumulation. MDR cells are resistant to cytotoxic effects of various structurally and mechanistically unrelated chemotherapeutic agents (Smyth *et al*., 1998; Ruefli *et al*., 2002; Shi *et al*., 2007). Developing new anticancer drugs that are efficient against MDR cells is a feasible strategy to overcome MDR (Jian-ye *et al*., 2010).

The anticancer properties of several secondary metabolites from endophytes have been investigated recently (Joseph and Priya, 2011). The diterpenoid, Taxol (also known as paclitaxel) has generated more attention and interest than any other new drug since its discovery, possibly due to its unique mode of action compared to other anticancer agents (Gangadevi and Muthumary, 2008; Firakova *et al.,* 2007). This compound interferes with the multiplication of cancer cells, reducing or interrupting their growth and spreading. FDA has

approved Taxol for the treatment of advanced breast cancer and refractory ovarian cancer (Cremasco *et al.*, 2009).

Fungi are promising sources of novel bioactive compounds that are important for drug discovery programs. Recent studies on the molecular biology of fungi have demonstrated that the genetic potential of these microbes, especially in the area of producing a far greater chemical diversity of compounds than is currently known, has been vastly underestimated in the past. For endophytic fungi for example, according to Ul-Hassan *et al*. (2012), the plant-microbe interaction may influence some of their otherwise cryptic biosynthetic pathways, resulting in the production of new molecules and overproduction of other secondary metabolites.

It has been recognized that under standard laboratory conditions most fungal biosynthetic gene clusters are either silent or expressed at very low levels. Those cryptic pathways may be triggered or stimulated by molecular, chemical or environmental signals.

Laboratory-based methods have been applied to express these latent pathways to produce new chemical entities; these include media engineering and optimization of growth conditions (Knappe *et al*., 2008), co-culture (Schroeckh *et al*., 2009), genetic manipulation (Bergmann *et al*., 2007) and epigenetic modulation (Williams *et al*., 2008; Henrikson *et al*., 2009).

Thus, this research seeks to identify and exploit fungi with ability of producing bioactive metabolites with antioxidant, antibacterial, antifungal, antiparasitic, antiviral, and anticancer potentials; and to manipulate these fungi and their environment to facilitate the increased production of specific bioactive metabolite of interest.

1.2 STATEMENT OF PROBLEM(S)

The effects of cancer are quite devastating being one of the leading causes of death worldwide.Also, world health problems caused by viruses, parasites, and drug resistant bacteria and fungi have become increasingly alarming.

Many pathogenic microorganisms have developed resistance due to the misuse or long-term use of the same class of antibiotics. There is also lack of safe medication for cancer as most treatment agents used today have numerous side effects. This creates an urgent need for an intensive search for newer,safer, more effective and less expensive drugs to deal with these problems.

Fungi are well known for producing many novel bioactive molecules, butthe prospect for the commercial production of novel drug compounds by fungi is bedeviled by some challenges. Most of the bioprospecting programs on detection and isolation of bioactive compounds have been based on single culture condition to screen biological activities of many fungi.The reduction of secondary metabolite production on repeated subculturing under axenic monoculture conditions is one of the key challenges that need to be addressed in order to establish, restore, and sustain the *in vitro* biosynthetic potential of fungi. This classical approach of drug discovery has occasionally led to the rediscovery of known secondary metabolites, mostly overlooking the repertoire of ‘‘cryptic’’ natural products that are not produced under standard *in vitro* conditions. There is thepossibility that the fungus might actually express only a subset of their biosynthetic genes under *in vitro* standard laboratory conditions such that only a minor portion of their actual biosynthetic potential is harnessed. Recent whole-genome sequencing strategies have revealed that the number of genes encoding the biosynthetic enzymes in various fungi undoubtedly is greater than the known secondary metabolites of these microorganisms (Scherlach and Hertweck, 2009; Winter *et al*., 2011).

It is therefore obvious that the large reservoir of ‘‘cryptic’’ natural metabolites of fungi is yet to be exploited. Thus, this research seeks to identify and exploit fungi with ability of producing bioactive metabolites with therapeutic potentials; and to manipulate these fungi and their environment to facilitate the possibility of increased production of specific bioactive metabolites of interest, as well as synthesis of novel compounds.

1.3 OBJECTIVES OF STUDY

1. To modulate a lichen-associated fungus for improved biosynthesis of secondary metabolites.
2. To isolate, identify and characterize endophytic fungi from *Carica papaya*.
3. To screen extracts of fermentation products of the endophytic fungi for antimicrobial, antioxidant and cytotoxic activities.
4. To isolate and purify bioactive compounds from endophytic fungal extracts.

CHAPTER TWO

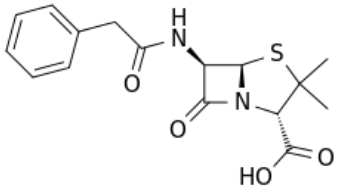
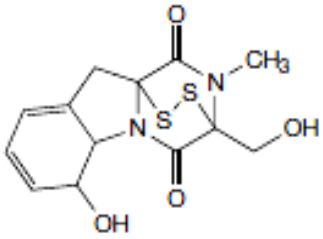
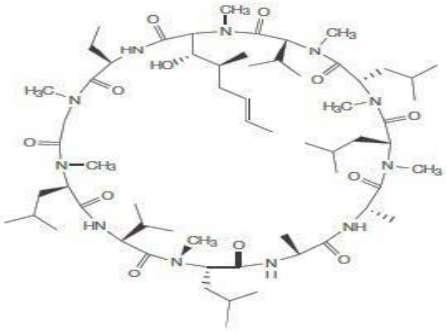
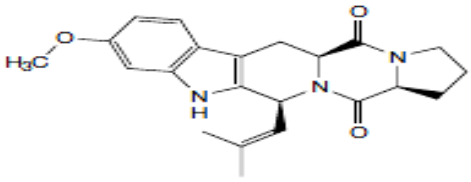
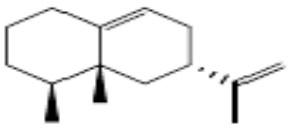
LITERATURE REVIEW

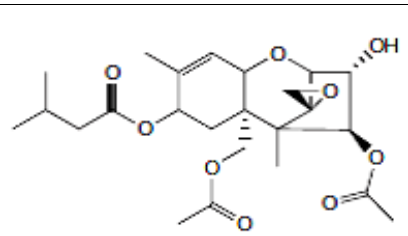
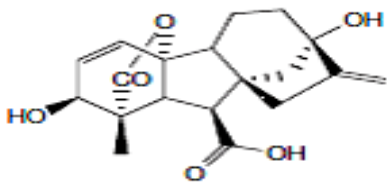
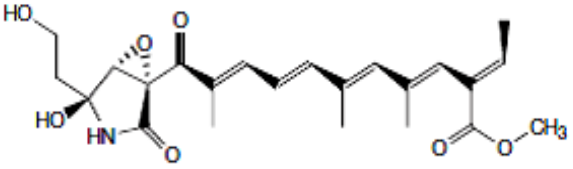
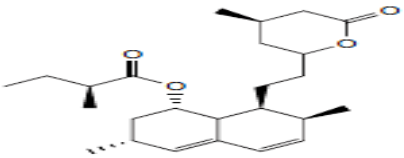
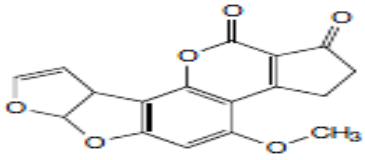
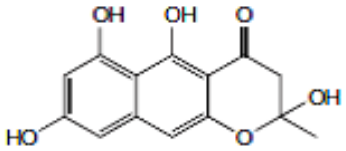
2.1 FUNGI AS SOURCE OF BIOACTIVE COMPOUNDS

Fungi are among the most important groups of eukaryotic organisms that are being explored for their metabolites for clinical applications. Existing drugs of fungal origin include β-lactam antibiotics, griseofulvin, cyclosporine A, taxol, ergot alkaloids, and lovastatin(Suryanarayanan *et al.*, 2009). More new natural products of varied chemical structures are continually being reported from fungi (Ola *et al*., 2013; Marmann *et al*., 2014). The heterotrophic and absorptive mode of nutrition of fungi reflects their ability to exploit a wide variety of substrates and habitats. Only about 7% of the estimated 1.5 million species of fungi are known, and only very few of these have been cultivated and screened for drug production. It is therefore logical to postulate that we have only discovered a small percentage of economically important metabolites of fungal origin(Suryanarayanan *et al*., 2009).

The main groups of fungal secondary metabolites are shown in Table 2.1. Some fungal secondary metabolites of medical importance are presented Table 2.2.

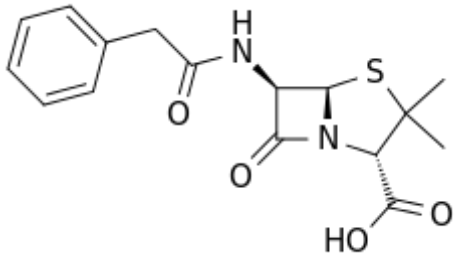
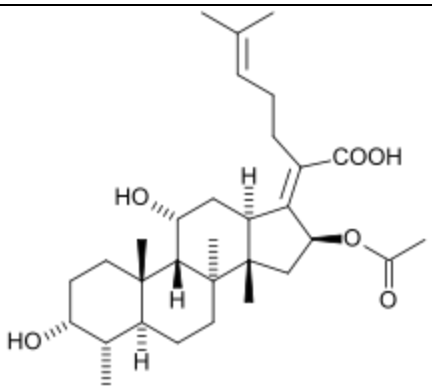
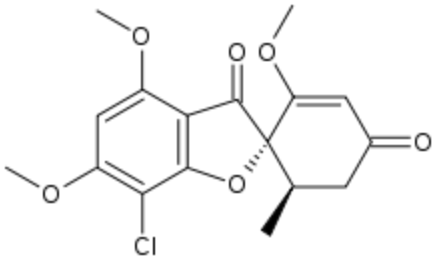
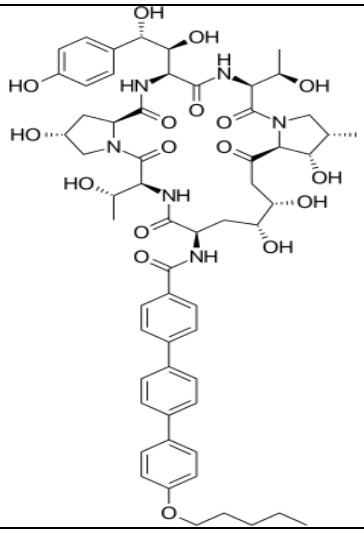
Table 2.1: The Main Groups of Fungal Secondary Metabolites.

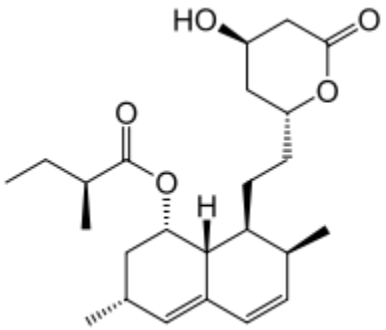
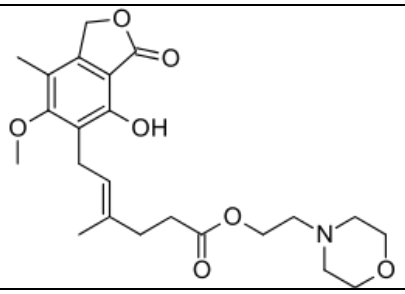
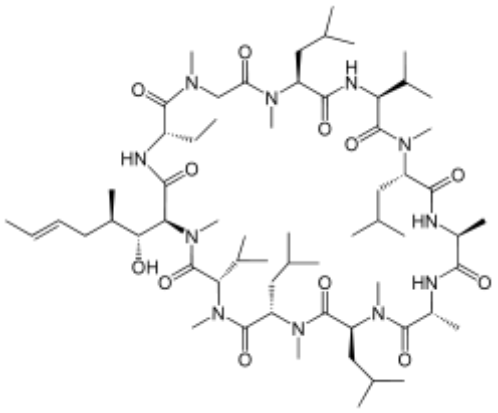
Classes	Compounds	Structures
Peptides	Penicillin G	
	Gliotoxin	
	Cyclosporine	
Alkaloids	Ergopeptides	
	Fumitremorgen C	
Terpenes	Aristolochene	

	Trichothecene T2 toxin	
	Gibberellin GA3	
Polyketides	Fusarin C	
	Lovastatin	
	Aflatoxin B1	
	WA	

Keller *et al*., (2005)

Table 2.2: Some Fungal Metabolites of Medical Importance

Fungal Metabolites	Structure	Fungal Producer	Medical use
Penicillin G		*Penicillium sp.*	Antibacterial agent (Toku-e, 2011)
Fusidic Acid		*Fusidium coccineum, Mucor ramannianus*	Antibacterial agent (Leclercq *et al*., 2000).
Griseofulvin		*Penicillium patulum*	antifungal drug (Goldman, 960).
Anidulafungin		*Aspergillus nidulans, A. rugulosus*	Antifungal agent (Pfaller *et al*., 2005; Krause *et al*., 2004)

Lovastatin		*Aspergillus terreusm Pleurotus ostreatus*	Treatment of dyslipidemia and the prevention of cardiovascular disease (ASHP, 2016)
Mycophenolic Acid		*Penicillium brevicompactum*	Immunosuppressant, antiviral, antifungal, antibacterial, and antipsoriatic agent (Silverman-Kitchin *et al*., 1997)
Cyclosporine		*Tolypocladium inflatum*	Immunosuppressant agent (Cantrell and Smith, 1984)

Fungi are well known for producing many novel chemicals that are directly used as drugs or function as lead structures for synthetic modifications. Although mycophenolic acid, the first fungal secondary metabolite was obtained from *Penicillium glaucoma* as early as 1896, it was the tremendous success of penicillin as an antibiotic in the early 1940s that shifted the focus of natural product-based drug sources from plants to microorganisms (Suryanarayanan *et al*., 2009).

From 1993-2002, about 1500 metabolites exhibiting antitumor or antibiotic activity have been reported from fungi (Pelaez, 2005). Some of the approved drugs of fungal origin are micafungin, an anti-fungal metabolite from *Coleophoma empetri* (Frattarelli *et al*., 2004), mycophenolate, a product of *Penicillium brevicompactum* used for preventing renal transplant rejection (Curran and Keating, 2005), rosuvastatin from *Penicillium citrinum* and *P. brevicompactum* used for treating dyslipidemias (Scott *et al*., 2004) and cefditoren pivoxil, a broad spectrum antibiotic derived from *Cephalosporium sp*. (Darkes and Plosker, 2002). Derivatives of fumagillin, an antibiotic produced by *Aspergillus fumigatus* (Chun *et al*., 2005), and illudin-S, a sesquiterpenoid from *Omphalotus illudens* (McMorris *et al*., 1996) exhibit anti-cancer activities. Fungal metabolites find important applications in agriculture as well (Anke and Thines, 2007).

It is pertinent to mention that, among the microfungi, only certain genera such as *Aspergillus* and *Penicillium* have been rigorously screened for bioactive compounds; of the 6450 bioactive metabolites from microfungi, more than 30% are obtained from these two genera (Berdy, 2005).

According to Schulz *et al*. (2002), the search for new fungal metabolites which previously has concentrated mainly on the random screening of isolates, can be optimized by consideration of the following:

(1) that the secondary metabolites a fungus synthesizes may correspond with its respective ecological niche, e.g. the mycotoxins of plant pathogens and

(2) that metabolic interactions may enhance the synthesis of secondary metabolites.

Thus, the fungi screened should originate from biotopes from which fungi have not been previously isolated for biochemical purposes and they should have metabolic interactions with their environment. This method of intelligent screening is a strategy for exploiting the untapped potential for secondary metabolites that fungi offer (Schulz *et al*., 2002).

2.2 ENDOPHYTIC FUNGI AS SOURCE OF BIOACTIVE COMPOUNDS

The fungal kingdom includes many species with unique and unusual biochemical pathways. The products of these pathways include important pharmaceuticals such as penicillin, cyclosporin and statins; potent poisons, including aflatoxins and trichothecenes; and some metabolites that are both toxic and pharmaceutically useful, such as the ergot alkaloids. All of these natural products, along with many other low-molecular-weight fungal metabolites, are classified together as secondary metabolites (Keller *et al*., 2005).

Endophytic fungi are a very diverse polyphyletic group of microorganisms.They can thrive asymptomatically in the tissues of plants, including stems, leaves, and/or roots (Kusari *et al*., 2012a).Many endophytes possessthe potential and have been reported to synthesize various bioactive metabolites that may directly or indirectly be used as therapeutic agents against numerous diseases (Strobel *et al*., 2004; Staniek *et al*., 2008; Aly *et al*., 2010; Kharwar *et al*., 2011; Kusari and Spiteller, 2012; Okoye *et al*., 2013). Structural diversity of metabolites isolated from endophytic fungi is shown in Figure 2.1.

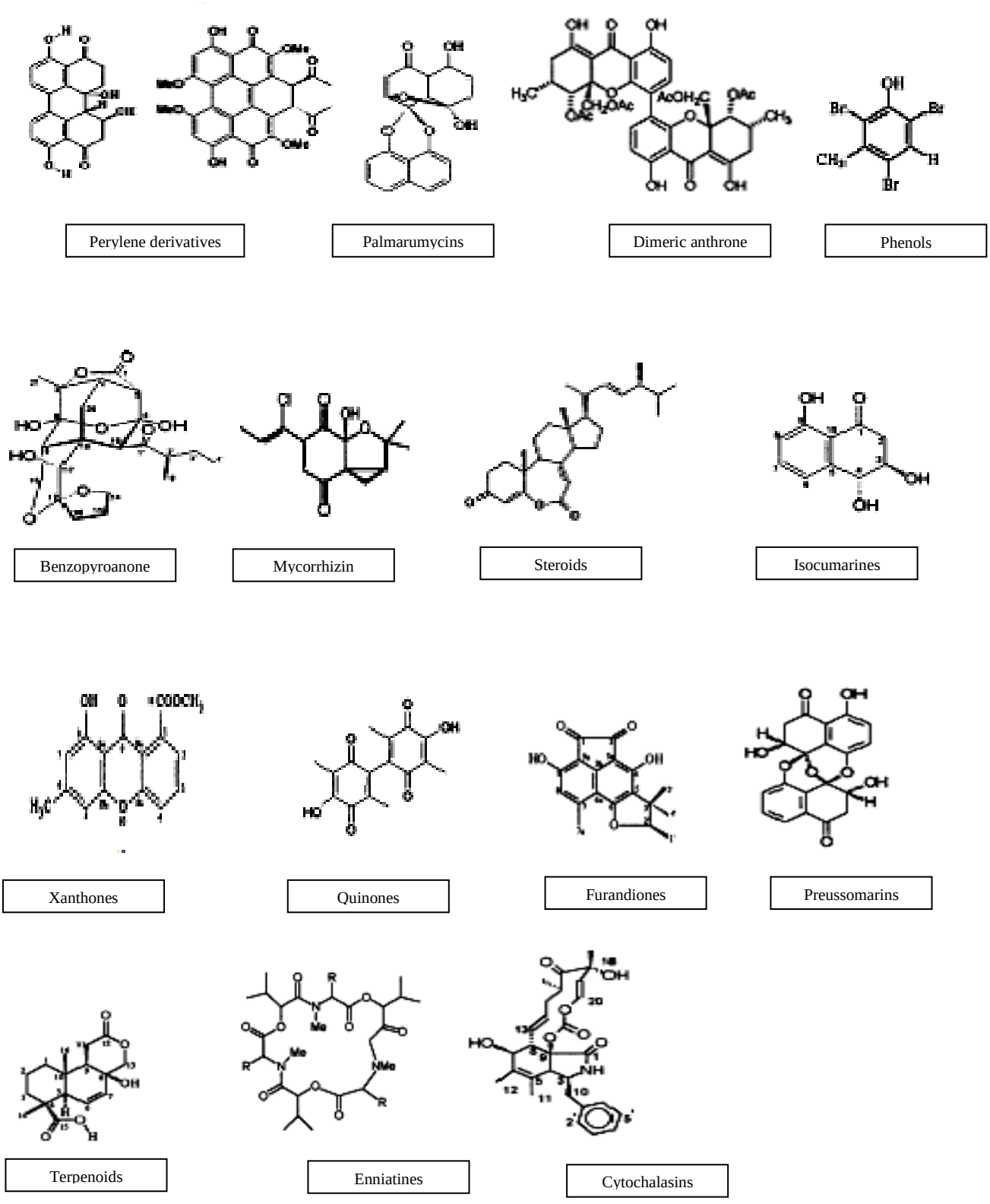


Figure2.1: Structural diversity of metabolites isolated from endophytic fungi (Schulz *et al.*, 2002).

Some endophytes have been reported to produce host plant secondary metabolites with therapeutic value or potential, some examples include paclitaxel (also known as Taxol) (Stierle *et al.,* 1993), podophyllotoxin (Eyberger *et al.,* 2006; Puri *et al.,* 2006), deoxypodophyllotoxin (Kusari *et al.,* 2009a), camptothecin and structural analogs (Puri *et al.,* 2005; Kusari *et al.,* 2009c; Shweta *et al.*, 2010), hypericin and emodin (Kusari *et al.,* 2008, 2009b), and azadirachtin (Kusari *et al.,* 2012b). The production of bioactive compounds by endophytes, especially those exclusive to their host plants, is important from an ecological perspective, as well as from a biochemical and molecular standpoint (Kusari *et al*., 2012a).

Exciting possibilities exist for exploiting endophytic fungi for the production of a plethora of known and novel biologically active secondary metabolites. For example, using controlled fermentation conditions by altering the accessible culture and process parameters (such as media type and composition, aeration, O_2, CO_2, pH, temperature, agitation, sampling, and harvest points), the compounds produced by fungal endophytes might be optimized. This could lead to a cost-effective, environmentally friendly, continuous, and reproducible yield compliant to commercial scale-up. In the case of endophytes capable of producing host plant compounds, such production (under optimized fermentation conditions) would then be independent of the variable quantities produced by plants influenced by environmental conditions (Kusari *et al*., 2012a).

2.3 CURRENT CHALLENGES OF EXPLOITING ENDOPHYTIC FUNGI

The prospect for the commercial production of compounds by endophytic fungi is bedeviled by some challenges, as the practicality still remains unproven. The reduction of secondary metabolite production on repeated subculturing under axenic monoculture conditions is one of the key challenges that need to be addressed in order to establish, restore, and sustain the *in vitro* biosynthetic potential of endophytes (Kusari and Spiteller, 2011). This problem is

intensified by the fact that almost all efforts to obtain natural products from endophytes have so far been made by the ‘‘classical’’ approach, under axenic monoculture conditions (Winter *et al*., 2011). This has occasionally led to the rediscovery of known secondary metabolites, mostly overlooking the repertoire of ‘‘cryptic’’ natural products that are not produced under standard *in vitro* conditions (Scherlach and Hertweck, 2009; Walsh and Fischbach, 2010).

The terms “orphan” and “silent” biosynthetic gene clusters which were suggested by Gross (Gross, 2007), are used to refer to clusters for which the corresponding metabolite is still undiscovered (orphan gene clusters) and clusters in which the genes are not expressed or expressed at very low levels (Silent gene clusters).

It seems that when these microorganisms are cultured under standard laboratory conditions, most biosynthetic gene clusters are either silent or expressed at very low levels. It is then certain that the chemical or environmental signals necessary for triggering these pathways is absent (Chiang *et al*., 2011).

Also, bioprospecting endophytes capable of producing desired bioactive secondary metabolites traditionally involves screening of a plethora of different endophytes isolated from a single host plant in order to identify the ‘‘competent’’ endophyte with the desired trait (Scherlach and Hertweck, 2009). When employing the classical approach, often, only a few or even none of the endophytes is capable of possessing the desired potentials (Kusari and Spiteller, 2011). The rest so-called ‘‘incompetent’’ endophytes are discarded without further investigation leading to the loss of the entire suite of natural products that they might produce under suitable conditions mimicking their natural habitat. However, recent whole-genome sequencing strategies have revealed that the number of genes encoding the biosynthetic enzymes in various fungi and bacteria undoubtedly is greater than the known secondary metabolites of these microorganisms (Scherlach and Hertweck, 2009; Winter *et al*., 2011).

Therefore, it is compelling that the discarded endophytes might actually express only a subset of their biosynthetic genes under *in vitro* standard laboratory conditions such that only a minor portion of their actual biosynthetic potential is harnessed. The large reservoir of ‘‘cryptic’’ natural metabolites is, thus, yet to be exploited. It is even possible that they produce the desired target compounds in quantities below the limit of detection, sometimes coupled with a large ‘‘metabolic background’’ and discrete culture conditions. Hence, it is necessary to understand and unravel the chemical ecological interaction of endophytes to fully exploit their inexhaustible potential of natural product biosynthesis (Kusari*et al*., 2012a).

Several strategies exist that try to overcome these limitations by modulating the organism or manipulating its cultural conditions. These include among others, the ‘one-strain-many-compounds’ (OSMAC) approach where promising strains are cultured in a variety of media and under different culture regimes in order to maximize the diversity of compounds produced (Bode *et al*., 2002), in addition to epigenetic modifications. In the latter case microorganisms are treated with epigenetic modifiers such as histone deacetylase inhibitors or DNA methyl transferase inhibitors aiming at a modulation of histones or of the DNA thereby initiating the transcription of silent genes which in turn may lead to the accumulation of new compounds (Cichewicz, 2010; Henrikson *et al*., 2009; Fisch *et al*., 2009; Asai*et al*., 2011).

A third option tries to mimic the natural ecological situation, where microbes always co-exist within complex microbial communities. Competition for limited resources and antagonism are characteristics of these micro-habitats, which favor various defense mechanisms that rely mainly on the production of bioactive secondary metabolites (Ola *et al*., 2013). It is often assumed that antibiotic production by bacteria and fungi can be interpreted within these ecological frames that will select for chemically defended microbes (Wiener, 1996; Anke,

1995; Slattery *et al*., 2001) even though other authors question this hypothesis (Linares *et al*., 2006; Fajardo and Martínez, 2008; Liu *et al*., 2013). Co-cultivation of two or more different microbes tries to mimic this setting in a laboratory scale. Competition among these microbes is deliberately provoked in the hope that biosynthetic genes that remain silent under luxurious culture conditions are activated and transcribed under stress conditions(Marmann *et al*., 2014).

2.4 MODULATION OF FUNGI AND MANIPULATION OF CULTURE CONDITIONS

New insights into the molecular biology of bacteria and fungi have demonstrated that the genetic potential of these microbes, in terms of producing a far greater chemical diversity of compounds than is currently known, has been vastly underestimated in the past (Knight*et al*., 2003; Brakhage*et al*., 2011). Many microbial biosynthetic genes are apparently not transcribed under standard laboratory conditions but remain silent. As a consequence, only a fraction of the real biosynthetic diversity of microbes is obtained in terms of produced compounds, which leads to the currently experienced bottle neck in drug discovery from microbial sources (Marmann *et al*., 2014).

To find novel compounds with promising bioactivities,many high-cost methods such as high-throughput screeningof different biological sources have been employed(Grabley and Thiericke 1999; Maier *et al*. 1999). Alternatively,an effective screening process can be achievedthrough systematic manipulation of culture conditions for asmall number of promising organisms. In fact, cultureconditions have a major impact on the growth of microbesand the production of microbial products. As far as cultureconditions are concerned, there is usually a dilemmabetween achieving maximal growth rates and maximalantibiotic yields because conditions that allow fast cellgrowth could be unfavorable to metabolite

production(Audhya and Russell 1974; Frisvad and Samson 1991;Chisti and Moo-Young 1993).

The yield of bioactivecompounds can sometimes be substantially increased by application of the one-strain-many-compounds (OSMAC) approach, where new compounds can be elicited by cultivating microorganisms in different fermentation conditions. Parameters that have been manipulated include media composition, aeration rate, type of culture vessel, and the addition of enzyme inhibitors (Bode *et al*., 2002). While very useful, this OSMAC approach can be laborious and there is no certainty that conditions can be found to stimulate the synthesis of all the interesting and useful compounds that organisms can potentially produce. Application ofenvironmental cues i.e. physical (temperature, salinity, pHvalue, and light) and chemical (media components,precursors, and inhibitors) factors for the growth of microbes(Calvo *et al*. 2002; Llorens *et al*. 2004) have also been explored in increasing the yield of bioactive compounds.On the other hand, co-cultivation i.e. the competitive cultivation betweendifferent microbial taxahas been suggested as a new tool toboost the discovery of novel compounds (Mearns-Spragg*et al*. 1997).

The growing number of sequenced microbial genomes has revealed a remarkably large number of natural product biosynthetic clusters for which the products are still unknown. These cryptic clusters are a potential treasure house of medically useful compounds. The recent development of new methodologies has made it possible to begin to unlock this treasure house, to discover new natural products and determine their biosynthesis pathways (Chiang *et al*., 2011).

An alternative strategy takes advantage of the fact that many secondary metabolite biosynthetic gene clusters contain one or more genes that encode transcription factors that transcribe all the genes of the cluster. In organisms amenable to molecular genetic

manipulation, the promoter of the transcription factor can be replaced by an inducible promoter. The synthesis of the transcription factor is, thus, removed from natural controls and put under the control of the researcher. Induction leads to high levels of the transcription factor and this, in turn, can lead to all of the genes of the cluster being turned on and the product produced (Bergmann *et al*., 2007; Chiang *et al*., 2009). This approach has the advantage that the product is immediately ascribable to the target cluster and it offers the potential for production of large amounts of the product. Some transcription factors are regulated post-translationally (Shimizu *et al*., 2003), however and it is possible that this strategy will not work easily for many of the clusters that contain transcription factor genes. In addition, many clusters do not contain transcription factor genes and other approaches must be used.In addition to cluster-specific transcription factors, there are more general regulators of secondary metabolite gene expression. One example is LaeA, a nuclear protein that controls secondary metabolite gene expression. Using microarray analysis of strains over expressing *laeA* and strains carrying a deletion of *laeA,* the biosynthetic gene cluster of terrequinone A in *A. nidulans* was discovered (Bok *et al*., 2006).

While the identification of silent gene clusters has already expanded our consideration of the biosynthetic ability of microorganisms, the development of multiple approaches to stimulate the production of unknown secondary metabolites remains a key challenge in harnessing the value of these cryptic systems (Reen *et al*., 2015) (Figure 2.2).

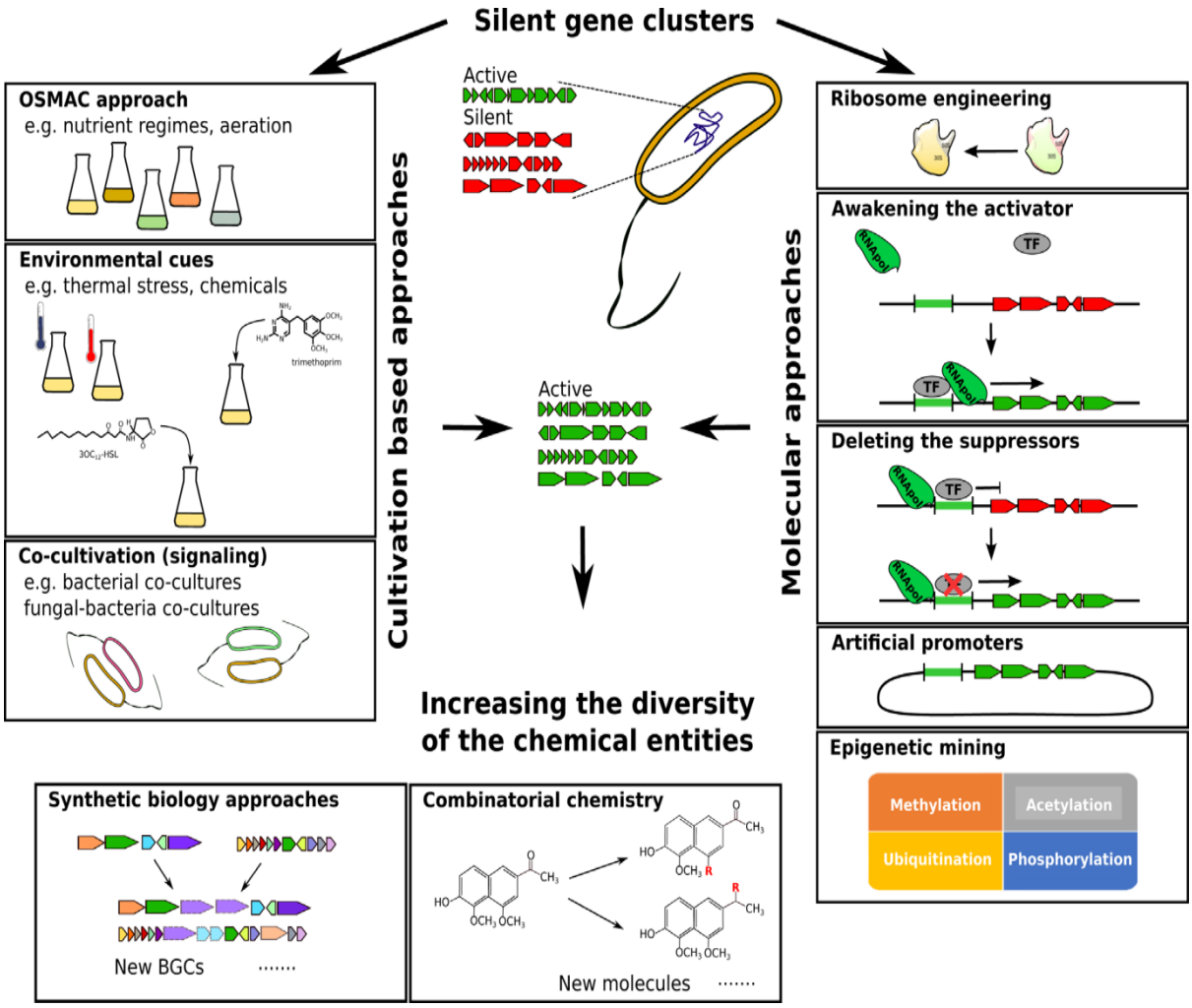


Figure 2.2: classical culture based and molecular biology based approaches for the elicitation of natural products from silent biosynthetic gene clusters (BGCs).
The integration of classical culture based and molecular biology based approaches for the elicitation of natural products from silent BGCs. A major challenge to the biodiscovery community, the cooperation of biological and chemical expertise is driving integrative technology developments that will unlock the silent potential of the cryptic gene clusters. Synthetic biology and combinatorial chemistry approaches further underpin the potential diversity of natural product that can be achieved (Reen *et al*., 2015).

2.4.1 ENVIRONMENTAL CUESAND CO-CULTIVATION

2.4.1.1 Stimulating Secondary Metabolite Production through Changing Culture Conditions: The "OSMAC" Approach

Microbial physiology is influenced by cultivation environment, media and conditions (Egli, 2015). It follows, therefore, that the production of secondary metabolites and bioactive compounds will be influenced by metabolic factors, themselves influenced by the prevailing environment. Broadly considered a form of strain improvement, manipulation of culture conditions has been used for decades as a mechanism of improving outputs from living organisms. Small changes in the composition of the growth media can induce not only variation in the amount of specific compounds, but also the production of a completely different pattern of molecules (Bode *et al*., 2002; Romano *et al*., 2014).

Zeeck and co-workers coined the term "OSMAC" (one strain many compounds), which simply summarized the ability of single strains to produce different compounds when growing under different conditions (Bode *et al*., 2002).

Screening of a panel of extracts from the marine fungus *Spicaria elegans* grown under 10 different culture conditions revealed a dramatic shift in the profile of secondary metabolites produced (Lin *et al*., 2009). This included the isolation of the novel spicochalasin A, five new aspochalasins M–Q, and two known aspochalasins. Subjecting the marine-derived fungus Ascotricha sp. ZJ-M-5 to the OSMAC approach yielded three new caryophyllene derivatives and the known 1,3,6-trihydroxy-8-methylxanthone in Czapek Dox broth with or without Mg^{2+}(Wang *et al*., 2014). Examples from other ecosystems are provided by the detailed work of Martin and co-workers, which repetitively showed how the phosphate concentration greatly influences the production of secondary metabolites in streptomycetes species (Martin, 2004). They showed how cross-talk between global nutritional regulators has a great effect on both primary and secondary metabolites (Martin *et al*., 2011).

Similarly, alsothe carbon sources have been shown to greatly affect secondary metabolites production in a wide range of microorganisms (Sánchez *et al*., 2010). Other easily accessible cultivation parameters can be modified, e.g., temperature, salinity, aeration, shape of the flasks, and these strategies led Zeeck and colleagues (Bode *et al*., 2002) to show that *A. ochraceus*, known to synthesize only aspinonene, was able to produce 15 additional metabolites when grown under different cultivation conditions. Similarly, applying the OSMAC approach three new compounds belonging to the rare class of 22-membered macrolacton polyketides were isolated from the bacterium *Streptomyces sp*. C34 (Rateb *et al*., 2011). The power of this approach was further reinforced by the work from Paranagama and colleagues (Paranagama *et al*., 2007), who were able to characterize six new secondary metabolites produced by the plant-associated fungus *Paraphaeosphaeria quadriseptata*, simply by changing the water used for the media preparation from tap water to distilled water.

Notwithstanding these advances, there are limitations to the use of this approach, highlighted by the strain-specific variation observed in the quantity of metabolite production as well as the seemingly capricious behavior of fungi to alter metabolite profiles when re-cultured (Williams *et al*., 2008).

2.4.1.2Challenging Microorganisms with External Cues

In addition to the cultivation parameters, external cues have been used to deviate cellular metabolism towards secondary metabolite production. For example, Christian and co-workers were able to isolate three new secondary metabolites, chaetoglobosin-510, -540, and -542, from cultures of the marine derivate fungus *Phomopsis asparagi* challenged with the F-actin inhibitor jasplakinolide (Christian *et al*., 2005). Another example is the production of the

potent antibiotic jadomycin by the bacterium *Streptomyces vanazuelae*. When cultured under physiologically favorable conditions in the absence of cellular stress, low amounts of jadomycin are produced. However, when cells are subjected to heat- or ethanol-shock, production of the compound is greatly increased (Doull *et al*., 1994). Similarly, heat- or ethanol-stressed *S. hygroscopicus* cells produce a significantly higher amount of the antibiotic validamycin, with concentrations up to 13 g/L harvested (Wei *et al*., 2012; Zhou *et al*., 2012). Another interesting line of research concerns the use of antibiotics, or molecules strictly related to them, to elicit the production of secondary metabolites (Reen *et al*., 2015). Studies on *Burkholderia thailandensis* showed that sub-lethal concentrations of trimethoprim served as a global activator of secondary metabolism by inducing at least five biosynthetic pathways, when present at sub-inhibitory concentrations (Seyedsayamdost, 2014).

Similarly, a series of molecules related to the synthetic antibiotic triclosan, were able to enhance the yield of secondary metabolites produced by *S. coelicolor* (Craney *et al*., 2012). In a broader sense, these cues or chemicals have been shown to alter the secondary metabolite production in a wide range of streptomycetes strains, leading also to the identification of several novel compounds (Yoon and Nodwell, 2014). With the successful chemical elicitation of other novel secondary metabolites including lunalides A and B, oxylipins, cladochromes F and G, nygerone A, sphaerolone, dihydrosphaerolone, mutolide and pestalone (Pettit, 2011), it has been clearly shownthatstress greatly influence the production of secondary metabolites, suggesting easy approaches to assess the biosynthetic ability in less explored microorganisms.

2.4.1.3 Co-cultivation

It is generally accepted that one of the roles of secondary metabolites is to provide a biological advantage for the producer in response to its environment. In some cases

secondary metabolites may protect against non-biological agents such as ultraviolet radiation, but in many cases, they will be used to compete against other organisms. Use of secondary metabolites to compete with other organisms implies that there must be some sort of sensing mechanism to control production of the metabolites. It follows that by harnessing the interplay between the producer and the environment, it might be possible to stimulate silent clusters to produce secondary metabolites (Chiang *et al*., 2011).

Microorganisms live in complex communities in dynamic and constantly changing environments. In order to increase their fitness, microbes need to adapt to both the different environmental conditions and the presence of different competitive species. Communication between cells and between species has long been known to be central to the activation or suppression of key cellular metabolites, whereby individual cells can monitor group behavior and tailor transcriptional and translational activities accordingly. Based on this assumption, the interplay between strains of the same or different species has been used to enhance the production of known compounds and to discover new bioactive natural products (Netzker *et al*., 2015; Cueto *et al*., 2001; Onaka *et al*., 2011; Schroeckh *et al*., 2009; Marmann *et al*., 2014; Traxler *et al*., 2013).

Among marine microorganisms, the co-cultivation approach has been shown to represent a promising strategy for the discovery of new bioactive compounds. For example, a new benzophenone antibiotic, called pestalone, that exhibits potent antibacterial activity against drug-resistant bacteria, was isolated from a mixed fermentation of a deuteromycete (*Pestalotia* sp.) and an unidentified antibiotic-resistant marine bacterium (Cueto *et al*., 2001). Another successful example comes from the expression of the biosynthetic pathway for the production of four different diterpenoids, libertellenones A–D, which show varying levels of cytotoxicity against the HCT-116 human adenocarcinoma cell line. Libertellenones'

production was obtained by culturing the marine fungus Libertella sp. with a marine alpha-proteobacterium (Oh *et al*., 2005).

Similarly, the co-cultivation of two mangrove endophytic fungi led to the discovery of marinamides A and B (Zhu and Lin, 2006), while Angell and co-workers showed that mixed bacterial culture isolated from ocean floor sediments produced blue pigment with antibiotic activity (Angell *et al*., 2006). The pigment was characterized as the phenazine derivative pyocyanin, and it was produced by a *Pseudomonas aeruginosa* strain only when co-cultivated with a strain of *Enterobacter* sp. Also, thecoral bacterium *Bacillus amyloliquefaciens* GA40 stimulated production of lipopeptide antifungal metabolites when grown in the presence of *A. fumigatus* and *A. niger*(Moree *et al*., 2013).

The strength of this approach has further been shown in one of the first systematic studies conducted to investigate the activation of cryptic or silent biosynthetic gene clusters (BGCs) in the model fungus *A.nidulans* during co-cultivation with different actinomycetes (Schroeckh *et al*., 2009). Schroeck *et al*. employed a microarray - based approach to monitor the selective activation of silent fungal biosynthetic genes *via* bacterial-fungal interaction (Schroeckh *et al*., 2009). Using a collection of 58 actinomycetes, Schroeck *et al*. discovered that when *A. nidulans* is co-cultured with *Streptomyces rapamycinicus*, two secondary metabolite genes clusters are induced. One of the gene clusters produced the aromatic polyketides orsellinic acid, lecanoric acid and the cathepsin K inhibitors F-9775A/F-9775B. Interestingly, dialysis experiments and electron microscopy indicated that a physical contact of the *S. rapamycinicus* and *A. nidulans* is required to elicit expression of the silent PKS gene. This study provides evidence that physical interaction among microorganisms can lead to the induction of silent biosynthesis genes. It also demonstrates the potential of using mixed-cultivation of microorganisms for novel secondary metabolite discovery.

Onaka and colleagues conducted an interesting study on the effect of mycolic-acid containing bacteria on the production of secondary metabolites by actinomycetes strains (Onaka *et al*., 2011). They could show that *Tsukamurella pulmonis* enhances the production of known metabolites in 54.5% of the tested actinomycetes strains and triggers the production of unknown compounds in 36.6% on the strains.

Although applicable only to cultivable microorganisms, the co-cultivation approaches represent a solid and promising strategy for the discovery of new bioactive metabolites (Reen *et al*., 2015). In this respect, the recent effort in the isolation of endophytic fungi,as well as fungi from other environments, for the discovery of unknown secondary metabolites offers an invaluable platform to explore multiple cultivation conditions, external cues, and co-cultivation possibilities, capitalizing on the vast potentials possessed by these organisms.

2.4.1.4 Microbial Signaling and Cryptic Clusters

Another class of compounds that have been shown to elicit activation of BGCs is the quorum sensing class of signaling molecules. A key control mechanism for cellular physiology, virulence and antibiotic production, quorum sensing molecules are widespread among microbial organisms. The nature and chemical composition of these signal molecules can be quite diverse, with the paradigm class of signal for Gram negative organisms being the acyl-homoserine lactone (AHL) class (Miller and Bassler, 2001). This signal based mechanism for control of cellular behavior has already been shown to coordinate antibiotic biosynthesis within producing bacteria, as seen with pyrrolnitrin production in a rhizospheric biocontrol strain of *Serratia plymuthica*(Liu *et al*., 2007), and with production of a polyketide antibiotic in*Burkholderia thailandensis*(Duerkop *et al*., 2009). The quorum sensing controlled Evr transcriptional regulator was shown to activate a conserved cryptic pigment biosynthetic cluster and a novel phenomycin-like locus in the plant pathogen *Pectobacterium*

carotovorum(Williamson *et al*., 2010). Other classes of quorum sensing molecules have also been implicated in the production of natural products, and in many cases these tend to be more species specific than the broad spectrum AHL class. An example of this would be phenazine production in *P. aeruginosa,* which is controlled by the *Pseudomonas* Quinolone Signal (PQS) QS system (Recinos *et al*., 2012). A core element of the synergism between organisms is the role of chemical messages or signal molecules in altering gene expression and secondary metabolite production of competing organisms (Reen *et al*., 2015). A major factor in shaping microbial populations, signal molecules produced by one organism have been shown to modulate phenotypes in species from other families or even kingdoms, in a form of communication or microbial diplomacy (Dworkin, 2014; Lowery *et al*., 2008; Reen *et al*., 2011; Williams, 2007). From an ecological perspective, the machinations of this interaction can be complex, and the terminology surrounding the actual role of these compounds has triggered some debate (Stacy *et al*., 2012, Diggle, 2010).

Notwithstanding this, in the context of natural product discovery, the ability of these signals to activate silent BGCs has significant potential (Netzker *et al*., 2015). Both PQS and its biological precursor HHQ have been shown to elicit phenotypic changes in Gram positive and fungal organisms, with staphyloxanthin production in *Staphylococcus aureus* being enhanced in the presence of the latter compound (Reen *et al*., 2011).

Netzker and colleagues described the interaction between the filamentous fungi*A. nidulans* and *A. fumigatus* with the soil bacterium *S. rapamycinicus* at the molecular level,highlighting the involvement of inter-kingdom communication in the activation of silent BGCs (Netzker *et al*., 2015). Indeed, antibiotics themselves are being recognized as a class of signal or cue involved in inter-kingdom communication with the "weapons or signal" debate receiving a lot of attention (Linares *et al*., 2006). Antibiotics have been widely reported as elicitors of

biosynthetic products, although the degree to which they can activate a broad spectrum of silent BGCs remains to be ascertained (Reen *et al*., 2015).

2.4.2 SEMI-SYNTHETIC AND MOLECULAR ACTIVATION OF SILENT BGCS

2.4.2.1 Epigenetic Mining

Within the broad grouping of microorganisms, but particularly in the higher order organisms such as fungi, phosphorylation, acetylation, methylation, ubiquitination, ADP ribosylation, sumoylation, and glycosylation are known to regulate gene expression (Cichewicz, 2012; Chiang *et al*., 2011). Although this aspect of BGC control is relatively uncharacterized, there is evidence that modulating epigenetic control can enhance or activate production of natural products in fungal organisms (Reen *et al*., 2015).

The Keller group demonstrated the importance of histone acetylation on the regulation of natural products in *Aspergillus* (Shwab *et al*., 2007). Disruption of the *hdaA* gene encoding an *A. nidulans* histone deacetylase (HDAC) led to the increased production of sterigmatocystin and penicillin. The COMPASS (complex associated with Set 1) complex has been shown to be responsible for the methylation of lysine 4 on histone 3 (H3K4) in yeast. Deletion of the *cclA* gene in *A. nidulans*, encoding one of the eight COMPASS complex proteins, led to the activation of at least two silent gene clusters involved in the biosynthesis of emodin and the related compounds, monodictyphenone, and F9775A/F9775B (Bok *et al*., 2009). Besides the acetylation and methylation of histones, deletion of the single sumoylation gene *sumO* in *A. nidulans* caused a dramatic increase in asperthecin and a decrease of austinol/ dehydroaustinol and sterigmatocystin production (Szewczyk *et al*., 2008).

Although the precise mechanism by which epigenetic regulation affects secondary metabolite production still awaits elucidation, the examples above confirm the applicability of using epigenetic modifiers for modulating secondary metabolite production. Moreover, the Cichewicz group was able to obtain novel fungal natural products using a similar concept by culturing a fungus in the presence of DNA methyltransferase (DNMT) or HDAC inhibitors (Williams *et al*., 2008). This chemical epigenetic mining does not require genetic manipulation, and it thus can be applied to many cultivable fungal strains (Chiang *et al*., 2011).

Epigenetics may also play a role in triggering the activation of silent fungal BGCs in response to co-cultivation with bacteria. As already discussed, contact with competing microbial organisms can elicit activation of secondary metabolite production, with interspecies and inter-kingdom communication receiving a lot of attention in the fields of ecology and microbial pathogenesis (Reen *et al*., 2011; Williams, 2007). *S. rapamycinicus* triggered modification of fungal histones, eliciting production of the archetypal polyketide orsellinic acid and its derivatives in *A. nidulans*, following co-cultivation (Nutzmann *et al*., 2010). Deletion analysis of 36 of 40 acetyltransferases, including histone acetyltransferases (HATs) of *A. nidulans*, implicated the Saga/Ada complex which was also shown to play a major role in induction of other BGCs, such as sterigmatocystin, terrequinone, and penicillin (Nutzmann *et al*., 2010).

2.4.2.2 Ribosome and Polymerase Engineering

The regulation of BGC expression is likely to be complex in light of the metabolic burden placed on the cell following production. However, at the simplest level, one of the limitations to overexpression of antibiotics and natural products under culture conditions is the transcriptional and translational requirement on cells. Removing the perception of the burden

has the effect of increasing production, while also changing the profile of compounds produced. Different from other "pathway specific" approaches, this generalized activation has been attributed to mutation at Lys-88 to either Glu or Arg in the ribosomal protein S12 of Streptomyces species, which enhances protein synthesis in stationary growth phase (Reen *et al*., 2015).

Great efforts have been committed to improving the yield of antibiotics in microorganisms, primarily to meet commercial requirements and demands (Chiang *et al*., 2011). The Ochi group developed a method, termed "ribosome engineering", to increase antibiotic production by targeting ribosomal protein S12 or RNA polymerase (RNAP), with the idea that bacterial gene expression may be increased dramatically by altering transcription and translation machineries (Ochi *et al*., 2004).

Since many antibiotics, such as streptomycin, target the ribosome, ribosome mutants that confer antibiotic resistance could be obtained by simply selecting for mutants on streptomycin-containing plates. Similarly, RNAP mutants could be obtained by growing on plates containing rifampicin, which binds to RNAP and inhibits RNA synthesis. Remarkably, these mutants not only had increased antibiotic production but were found to produce novel antibiotics (Hosaka *et al*., 2009).

After screening of 1,068 actinomycetes isolated from soil, 6% of non-*Streptomyces* actinomycetes species and 43% of *Streptomyces* species that did not produce antibacterials were activated to produce them. A mechanistic study indicated that the effect observed was due to a mutation at Lys-88 to either Glu or Arg in the ribosomal protein S12, which enhances protein synthesis in stationary growth phase. In addition, a mutation at His-437 to either Asp or Leu in the RNAP β-subunit increased its promoter binding affinity (Chiang *et al*., 2011; Reen *et al*., 2015).

Similar to co-cultivation and epigenetic mining approaches, ribosome engineering triggers the activation of one or several but not all biosynthesis clusters in the organism. However, these approaches, unlike those that activate pathway-specific activators, do not trigger specific biosynthetic gene clusters (Chiang *et al*., 2011).

2.4.2.3Awakening the Activator

A recurring theme from the genomics based discovery of BGCs is the co-occurrence of genesencoding transcriptional regulatory proteins suggesting a programmable activation or repression of BGC expression. Many secondary metabolite BGCs contain one or more genes that encode transcription factors that potentially transcribe all the genes of the cluster. Of the BGCs characterized to date, a co-occurrence of the LysR-Type Transcriptional Regulator (LTTR) family with BGCs has been described for a diverse spectrum of bacterial organisms (Colombo *et al*., 2001; Rodriguez *et al*., 2008; Waldron *et al*., 2001). Known to control the expression ofmetabolic and virulence related functions in a broad spectrum of microbial species, these regulators have dual domains typically consisting of an N-terminal DNA binding domain and a C-terminal co-inducer or ligand binding domain (Maddocks and Oyston, 2008). Capable of binding to DNA in the absence of a signal, they nonetheless remain inactive and expression is not achieved. Upon interaction with a co-inducer ligand or substrate, a conformational change ensues, with the formation of tetrameric structures and subsequent activation of expression at target promoters. The capacity to interact with promoters in the absence of signal is an interesting feature as it typically retains the promoter in the off-state, as is the case with silent BGCs. Therefore, identifying an activating signal has the potential to change the regulator to the on-state leading to activation of the BGC and production of the bioactive compound. In some cases, LysR proteins may act as repressors of expression whereby deactivation of the protein is required for elicitation of biosynthesis.

ThnI has been shown to be required for synthesis of the β-lactam antibiotic thienamycin in *Streptomyces cattleya*(Rodriguez *et al*., 2008), while another LysR protein designated ORF-L16 is encoded within the spinomycin biosynthetic cluster of *Saccharopolyspora spinosa*(Waldron *et al*., 2001). The oxazolomycin BGC of *S. albus*encodes the OzmR LysR transcriptional regulator (Zhao *et al*., 2010). As with many of the LysR proteins identified to date, the role of this protein in the synthesis of oxazolomycin A, a peptide-polyketide hybrid compound containing a unique spiro-linked β-lactone/γ-lactam, a 5-substituted oxazole ring, is unknown. However, the co-occurrence of LysR proteins within bioactive gene clusters strongly suggests a role in their transcriptional regulation. Intriguingly, LTTRs were found to be most abundant among the Actinobacteria, Proteobacteria and Firmicutes, the three phyla that account for the majority of natural product biosynthetic potential (Cimermancic *et al*., 2014). A recent molecular phylogenetic analysis of LTTRproteins from the Proteobacterial pathogen *P. aeruginosa* revealed clustering of the LTTR repertoire into nine independent clades (Reen *et al*., 2013). Importantly, clustering of either the DNA binding domain or the co-inducer binding domain resulted in conservation of the clades indicating that some degree of structural similarity may exist within the activating signal. Extending such an approach within the genomes of BGCs-rich organisms may yield clues on the activating signals that activate the LTTR regulated module, providing hints on the experimental approaches to follow to express the BGCs.

Several other classes of transcriptional regulator have also been described in association with BGCs. Reports have described the co-occurrence of *Streptomyces* antibiotic regulatory protein (SARP) family proteins with BGCs. These include the aur1 polyketide gene cluster involved in biosynthesis of the angucycline-like antibiotic auricin in *S. aureofaciens*(Novakova *et al*., 2011), ThnU which is required for cephamycin C biosynthesis in *S. cattleya*(Rodriguez *et al*., 2008), as well as several other *Streptomyces sp*. related

clusters (Aigle *et al*., 2005; Arias *et al*., 1999; Rehakova *et al*., 2013). The TetR and AraC families are also represented, with many regulatory systems co-occurring within the large BGCs (Novakova *et al*., 2010). In addition to the pathway specific activation of BGCs, some global regulators of secondary metabolite production have been identified. The cyclic AMP receptor protein (Crp), which is known to regulate catabolite repression in *Escherichia coli*, has recently been shown to be a global regulator for antibiotic production in *Streptomyces*(Gao *et al*., 2012).

Identifying the signal or conditions which elicit activation of transcriptional regulators can be difficult. Where activation remains elusive, an alternative strategy has been pursued in organisms amenable to molecular genetic manipulation, whereby the promoter of the transcription factor can be replaced by an inducible promoter. This has the effect of artificially driving expression of the transcriptional regulator, which should in turn result in production of the associated natural product (Chiang *et al*., 2011, Bergmann *et al*., 2011; Chiang *et al*., 2009).

According to Reen *et al*. (2015), there are obvious limitations to this approach where

(a) post-transcriptional or post-translational regulation occurs;

(b) activation of the transcriptional regulator requires physical interaction with a co-inducing molecule (as with LysR proteins); or

(c) not all biosynthetic genes are under the control of a common regulator (as with many large BGCs in which several transcriptional regulators from different classes are encoded). Notwithstanding this, the use of inducible promoters has potential for application in this field.

2.4.2.4 The Mutation Approach: Deleting the Suppressors

While positive regulation by transcriptional proteins is the dominant phenotype, repression is also afeature of many, whether intrinsic or state dependent. In this case, deletion or deactivation of these suppressor proteins has the potential to free silent BGCs from their locked in state and result in production of the natural compounds. Members of the LysR and TetR families of transcriptional regulator are known to possess suppressive activity and are one potential target for this approach. Null mutation of the hexA LysR from *Photorhabdus luminescens* led to a dramatic increase in the biosynthesis of small molecules (Kontnik *et al*., 2010). Bth_II1681 encodes for a LysR-type TF associated with the previously characterized thailandamide biosynthetic cluster of *Burkholderia thailandensis*(Biggins *et al*., 2011, Nguyen *et al*., 2008), with mutations in this regulator resulting in the production of a novel thailandamide lactone variant (Biggins *et al*., 2011, Ishida et la., 2010). An example TetR repression was shown by the null mutation of repressors within two silent gene clusters in *Streptomyces sp.*PGA64 and *S. ambofaciens*, which induced expression of the BGCs and resulted in the detectable production of the corresponding natural products—novel angucyclinone and already described kinamycins, respectively (Bunet *et al*., 2011, Metsa-Ketela *et al*., 2004). In another study, deleting a presumed pathway-specific regulatory gene (scbR2) that encodes a member of the c-butyrolactone receptor family of proteins and which lies in the cpk gene cluster of *S. coelicolor* A3(2) led to production of novel antibacterial activity (abCPK) and a yellow-pigmented secondary metabolite (yCPK) (Gottelt *et al*., 2010). In some cases, repression of biosynthetic clusters involves both transcriptional and post-translational control, as seen with production of the cryptic orsellinic acid gene cluster in *A. nidulans*. Genetic assessment of mvlA mutants revealed the role of both itself and VeA (but not the VeA partner LaeA) in the suppression of the cryptic ors gene cluster producing orsellinic acid and its F9775 derivatives (Bok *et al*., 2013). Adding to the complexity of this

regulatory system was the involvement of histone 3 acetylation, suggesting the involvement of epigenetic control (Bok *et al*., 2013).

In a broader sense, deletion of cellular systems also has potential for activation of silent BGCs. An example of this is where protein destabilization has been targeted for deletion resulting in recovery of novel bioactive compounds from *A. nidulans*. Specifically, deleting the conserved eukaryotic csnE/CSN5 deneddylase subunit of the COP9 signalosome results in the activation of a previously silenced gene cluster comprising a polyketide synthase gene producing the antibiotic 2,4-dihydroxy-3-methyl-6-(2-oxopropyl)benzaldehyde (DHMBA)(Gerke *et al*., 2012). The highly conserved nature of this system among eukaryotic organisms underpins its potential as a broad spectrum approach for the activation of silent BGCs.

2.4.2.5 Artificial Promoters

While attention has focused on the manipulation of transcriptional activation of BGCs, another approach has been the insertion of inducible artificial promoters to drive expression of the silent genes. This generally takes advantage of the advances in molecular cloning technologies where suitable plasmid systems are generated in which structural biosynthetic genes can be cloned downstream of strong promoters, thus overcoming the cryptic native transcription network (Reen *et al*., 2015). Decoupling pathway expression from the complexity of native regulation circumvents the need for culture specific conditions and in some cases the laborious search for appropriate activating signals. In addition, production of the respective natural products can be controlled under defined culture conditions, ensuring downstream processing and stabilization prior to isolation (Reen *et al*., 2015). This has received a lot of attention in fungal systems where the transcriptional control of large clusters can be manifestly complex (Wasil *et al*., 2013). Described as refactoring, already this

approach has met with some success, as with activation of the silent spectinabilin and taromycin A pathways from *S. orinoci* and *Saccharomonospora* sp. CNQ-490, respectively (Yamanaka *et al*., 2014, Shao *et al*., 2013). In the latter case, transformation-associated recombination (TAR) cloning was exploited to express a 67-kb nonribosomal peptide synthetase BGC from the marine actinomycete *Saccharomonospora* sp., producing the dichlorinated lipopeptide antibiotic taromycin A in the model expression host *S. coelicolor*(Yamanaka *et al*., 2014). In another study, Luo and colleagues used a similar strategy to activate a cryptic BGC SGR810-815 from *S. griseus*, resulting in the production of three novel polycyclic tetramate macrolactams (Luo *et al*., 2013). In what the authors of each study described as a "plug and play scaffold", the respective host strains were engineered using a specific set of heterologous promoters that were functional in a heterologous host under the target culturing condition. More recently, a single genomic capture and expression vector for antibiotic production in *Bacillus subtilis* has been reported (Li *et al.*, 2015), while the pathway for production of alterochromide lipopeptides by *Pseudoalteromonas piscicida* JCM 20779 was heterologously expressed in *E. coli* utilizing native and *E. coli*-based T7 promoter sequences (Ross *et al*., 2015). Heterologous expression of genes from the proposed aspyridone biosynthetic cluster from *A. nidulans* in the host *A. oryzae* led to the production of eight different compounds in addition to aspyridone A 1, one of the previously observed products (Wasil *et al*., 2013). Surprisingly, the previously accepted final product of the pathway, aspyridone B 2, was not detected (Wasil *et al*., 2013). Similarly, expression of silent gene clusters has also been achieved in Streptomyces using the strongly constitutive promoter ermE*p (Baltz, 2010), while introduction of the transcriptional activator of balhimycin biosynthesis, the bbr gene from *Amycolatopsis balhimycina* (bbrAba) into *A. japonicum*also resulted in the production of an antibiotic-active compound (Spohn *et al*., 2014). More

recently, a new high-performance heterologous fungal expression system was described in *Aspergillus terreus*(Gressler, 2015).

Based on regulatory elements from the terrein gene cluster, the system was found to be particularlysuitable for high level expression of polyketides in heterologous hosts. However, despite some successes, challenges abound and molecular-based methods utilizing heterologous expression systems continue to be limited by problems such as locating and cloning genes, difficulties with gene transformation and inactivation, and host incompatibilities (Williams *et al*., 2008).

CHAPTER THREE
MATERIALS AND METHODS

3.1 MATERIALS

3.1.1 ANALYTICAL INSTRUMENTS

Details of analytical instruments and chemicals used in this study are presented as follows:

A. Analytical HPLC

Analytical HPLC components include: pump (P580A LPG, Dionex), autosampler (ASI-100, Dionex), photodiode array detector (UVD 340S, Dionex), column oven (STH 585, Dionex), column (Eurosphere 100-C18; 5 μM; 125 x 4 mm; with integrated pre-column), software (Chromeleon 6.30)(All made in Germany).

B. Semi preparative HPLC

Semi preparative HPLC components include: pump (L-7100, Merck/Hitachi), photodiode array detector (UV-L7400, Merck/Hitachi), column (Eurosphere 100-C18; 10 μm; 300 x 8 mm; Knauer), pre-column (Eurosphere 100-C18; 10 μm; 30 x 8 mm; Knauer), printer (Chromato-Integrator D-2000, Merck/Hitachi) (All made in Germany).

C. LC-MS

LC-MS components include: HPLC System (Agilent 1100 series, pump, photodiode array UV-detector, autosampler and injector; Agilent), MS spectrometer (Finnigan LCQDeca, Thermoquest), column (Eurosphere 100-C18; 5 μM; 227 x 2 mm; Knauer), vacuum pump (Edwards 30, BOC), software (Xcalibur, version 1.3) (All made in Germany).

D. NMR

^{1}H and ^{13}C spectra were recorded at 300º K on Bruker DPX 300, ARX 400, 500 or AVANCE DMX 600 NMR spectrometers(All made in Germany). All 1D and 2D spectra were obtained using the standard Bruker software.

3.1.2 EQUIPMENT/INSTRUMENTS FOR FUNGAL ISOLATION, CULTIVATION AND IDENTIFICATION

The equipment used in the isolation, cultivation and identification of the endophytic fungi include: laminar air flow cabinet (Herasafe HS15, Heraeus, Germany), autoclave (Varioklav, H&P, Germany), pH meter (InoLab, Germany), pH electrode (Sen Tix 21, WTW, Germany), microcentrifuge (Biofuge pico, Heraeus), PCR machine (iCycler, Bio-Rad, Germany), UV transluminator (Syngene GVM 20, Germany), mixer mill (MixerMill MM30, Germany), power supply for electrophoresis (PowerPac 300, Bio-Rad, Germany), ZR fungal/bacterial DNA MiniPrep™ Kit (Zymo Research Corp, USA), Hot Star Taq Master Mix Kit (Qiagen, Germany), agarose gel (Biozym LE Agarose, Biozym Scientific GmbH), Zymoclean™ gel DNA recovery kit (Zymo Research Corp, USA).

3.1.3 GROWTH/ISOLATION/PURIFICATION/FERMENTATION/STORAGE/ ANTIMICROBIAL ASSAY MEDIA

Medium for Isolation of Fungal Strains from Plants/Lichens

- Bacto agar (Galke, Germany) 15.0 g
- Malt extract (Merck, Germany) 15.0 g
- Chloramphenicol (Sigma, Germany) 0.2 g
- Dissolve in 1 L distilled (pH 7.4-7.8).

Medium for Purification and Short-term Storage of Fungal Strains

- Bacto agar (Galke, Germany) 15.0 g
- Malt extract (Merck, Germany) 15.0 g
- Dissolve in 1 L distilled (pH 7.4-7.8).

M**edium for Long-term Storage**

- Malt Extract (Merck, Germany) 20.0 g
- Yeast Extract (Sigma, Germany) 0.1 g
- Glycerin (Roth, Germany) 50 mL
- Artificial sea salt (Sera, Germany) 10.0 g
- Bacto Agar (Galke, Germany) 13.0 g
- Dissolve in 950 ml distilled (pH 7.4-7.6).

Yeast Malt (YM) medium

- Agar-20 g
- Glucose-10 g
- Yeast extract-3 g
- Peptone-5 g
- Malt-3 g
- Dissolve in 1 L distilled water (pH 7.4-7.6).

Rice medium (Co-cultivation)

- Rice-50 g
- Distilled water-60 mL of distilled water

Rice medium (Media Engineering and Optimization of Growth Conditions)

- Rice-100 g
- Distilled water-110 mL of distilled water

Rice YM medium

- Rice-50 g
- YM medium-60 mL of water

Antimicrobial assay Media

- Mueller Hinton Agar (Oxoid, UK)
- Sabouraud Dextrose Agar (Oxoid, UK)

3.1.4 OTHER INSTRUMENTS

These include high resolution ESI-MS (Qtof 2, Micromass), polarimeter (241 MC, Perkin-Elmer), freeze dryer (Lyovac GT2, Steris; pump Trivac D10E, Savant), Speedvac (SPD 111V with cooling trap RVT 400, Savant), balance (BL1500, Sartorius), analytical balance (MC-1, Sartorius), desiccator (Glaswerk Wertheim), hot plate and magnetic stirrer (IKA-Combimag RCH, Janke & Kunkel KG), drying oven (ET6130, Heraeus), ultra sonicator (RK 510H,

Bandelin Sonorex), rotary evaporator (RV 05-ST, Vaccubrand), membrane vacuum pump (Vaccubrand), UV lamp (Camag, 254 and 366 nm), nitrogen generator (UHPN 3001, Nitrox), Ultra Turrax® (T18 basic, IKA), vacuum pump (4EKF56CX-4,Greiffenberger Antriebstechnik)(All made in Germany).

3.1.5 SOLVENTS

A. General solvents

Solvents used in general procedures include methanol, ethyl acetate, dichloromethane,acetone and n-hexane (Merck, Germany).

B. HPLC solvents

HPLC solvents include methanol and acetonitrile (LiChroSolv HPLC grade, Merck, Germany). Distilled and heavy metal free water was obtained by passing through nano- and ion-exchange filter cells (Barnstead) to yield nanopure water.

C. Solvents for optical rotation

Solvents for optical rotation are: chloroform and methanol (Spectroscopic grade, Sigma).

D. Solvents for NMR

Solvents for NMR include: Acetone-$_{d6}$, Chloroform-$_{d}$, DMSO-$_{d6}$, and Methanol-$_{d4}$ (Uvasol, Merck, Germany).

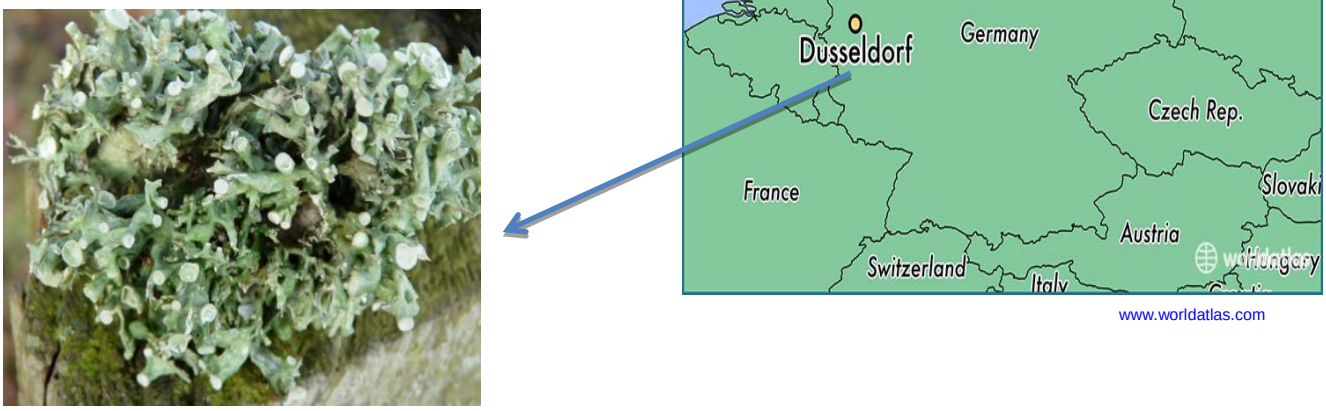
3.1.6 GENERAL LABORATORY CHEMICALS/REAGENTS

Other chemicals/reagents include: acetic acid, formic acid, hydrochloric acid, potassium hydroxide, sodium hydroxide, sulphuric acid, chloramphenicol, ammonium chloride, peptone, yeast extract, corn steep liquor, rhamnose, glucose, sucrose, sodium chloride, sodium iodide, sodium bromide, etc.

3.2 METHODS

3.2.1 COLLECTION OF SAMPLES

The lichen (*Cladonia sp.*) was collected from Düsseldorf, Germany in the month of February, 2015. It was identified by Prof. Dr. Peter Proksch of the Institut für Pharmazeutische Biologie und Biotechnologie, Heinrich-Heine-Universität, Düsseldorf, Germany. Fresh leaves of *Carica papaya* (Caricaceae) were collected in the month of June, 2015 from Agulu, a town near Awkain Anambra State, Nigeria. The plant material was authenticated by a plant taxonomist and a voucher specimen (PCG474/A/023) deposited in the herbarium of the Department of Pharmacognosy and Traditional Medicine, Faculty of Pharmaceutical Sciences, Nnamdi Azikiwe University, Anambra State, Nigeria.



http://www.peterorchard.me.uk/nature-notes/lichen-cladonia-fimbriata

Cladonia sp.

Figure 3.1: Location for collection of lichen (*Cladonia* sp.)

Carica papaya

www.worldatlas.com

Figure 3.2: Location for Collection of Plant leaves (*Carica papaya*)

3.2.2 ISOLATION AND IDENTIFICATION OF FUNGI

3.2.2.1 Isolation

For the endophytic fungi, harvested healthy leaves of *C. papaya*were washed in running tap water and processed as follows: about 1-2 mm segments were cut from the lamina and surface-sterilized by washing in 2% sodium hypochlorite for 2 min and then in 70% ethanol for 2 min. For the lichen-associated fungus, about 1-2 mm segments were cut from the lichen and surface-sterilized by washing only in 70% ethanol for 2 min.

The plant/lichen segments were selected and placed on Petri dishes containing malt extract agar (MEA) supplemented with chloramphenicol. The plates were incubated on laboratory benches at room temperature with ambient light. Periodically, fungal growths from the leaf/lichen segments were monitored and hyphal tips from distinct colonies emerging from leaf segments were sub-cultured on fresh MEA plates to obtain pure colonies.

3.2.2.2 Identification

Taxonomic identification of all fungal strains was achieved by DNA amplification and sequencing of the fungal ITS region. Total fungal genomic DNA was extracted and purified directly from fresh, axenic mycelia using fungal DNA extraction and purification kits. DNA amplification by PCR was performed using Hot StarTaq Master Mix Taq polymerase and the primer pair ITS1 and ITS4 in a thermocycler. The PCR products were visualized on a 1%

agarose gel, and amplified DNAs were recovered from the gel using appropriate kits. Fungal DNAs were sequenced and resulting sequences were subjected to BLAST searches of the NCBI GenBank database for identification.Details of the molecular identification of the fungal isolates are described below.

A. DNA Extraction/Isolation

1. A weight of ~200 mg of the fungal mycelium was added to a ZR Bashing Bead™ Lysis Tube. A volume of 750 µl Lysis Solution was added to the tube.

2. The ZR Bashing Bead™ Lysis Tube was placed on a vortexer and processed (vortexed) at maximum speed for 5 min.

3. The ZR Bashing Bead™ Lysis Tube was centrifuged in a microcentrifuge at 10,000 x *G* for 1 min.

4. A volume of ~ 400 µl of the supernatant was transferred to a Zymo-Spin™ IV Spin Filter in a collection tube and centrifuged at 7,000 rpm (~7,000 x *G*) for 1 min.

5. A volume of 1,200 µl of fungal/bacterial DNA binding buffer was added to the filtrate in the collection tube from Step 4.

6. A volume of 800 µl of the mixture from Step 5 was transferred to a Zymo-Spin™ IIC column in a collection tube and centrifuged at 10,000 x *G* for 1 min.

7. The flow through from the collection tube was discarded and Step 6 repeated.

8. A volume of 200 µl DNA pre-wash buffer was added to the Zymo-Spin™ IIC column in a new collection tube and centrifuged at 10,000 x *G* for 1 min.

9. A volume of 500 µl fungal/bacterial DNA wash buffer was added to the Zymo-Spin™ IIC column and centrifuged at 10,000 x *G* for 1 min.

10. The Zymo-Spin™ IIC column was transferred to a clean 1.5 ml microcentrifuge tube and 30 µl (25 µl minimum) of DNA elution buffer was added directly to the column matrix. After 5min, the tube was centrifuged at 10,000 x *G* for 30 sec to elute the DNA.

Ultra-pure DNA was then obtained.

B. DNA Amplification (PCR)

DNA amplification by PCR was performed using 'Hot StarTaq Master Mix Taq polymerase and the primer pair ITS 1 and ITS 4 (Invitrogen) in an iCycler (Bio-Rad) thermocycler.

In 0.2 ml PCR tubes, the following were added

- 25 µl HotStarTaq Master Mix
- 1.5 µl primer mix (10 pmol/µl each)
- 10-100 ng template DNA (usually 1 µl is enough)
- Water until 50 µl

The negative control, which contained all of the above listed except fungal DNA, was also prepared.

Sequence for ITS 1 and ITS 4 primers are as shown below:

ITS 1 5'-TCCGTAGGTGAACCTGCGG-3'

ITS4 5'-TCCTCCGCTTATTGATATGC-3'

PCR Program

1. Initial denaturation 95^{o}C, 15 min
2. Denaturation 95^{o}C, 1 min
3. Annealing 56^{o}C, 1 min
4. Extension 72^{o}C, 1 min
5. Final extension 72^{o}C, 10 min.

C. DNA Purification/Gel Electrophoresis

Gel Electrophoresis

1. One (1) % Agarose gel was prepared and 1/10 TBE buffer was added enough to cover the gel in the electrophoresis machine.

2. A volume of 50 µl of the PCR product was mixed with 10 µl of 6X gel loading dye (blue) and loaded in the wells (a volume of 25-30 µl of sample was loaded into the wells).

3. At least 1 or 2 wells was loaded with 10 µl Quick-Load® 100bp DNA Ladder (blue, i.e. already mixed with gel loading dye) (New England Biolabs, Inc. USA).

4. The electrophoresis gel was then run at 75 V for 45 min.

After electrophoresis, the gel was transferred into the UV-transilluminator to confirm that the PCR has been successfully carried out and the PCR products had the right size of about 550 bp by comparing them with the DNA ladder.

DNA Purification from Agarose Gel

The DNA fragment was then excised from the agarose gel with a clean, sharp scalpel. The size of the gel slice was minimized by removing extra agarose and the gel fragment was put in a sterile 1.5 or 2 ml microcentrifuge tube.

D. DNA Recovery

1. A volume of x3 of ADB was added to each volume of agarose excised from the gel (e.g.for 100 µl (mg) of agarose gel slice, 300 µl of ADB was added).

2. This was incubated at 37-55 °C for 5-10 mins until the gel slice was completely dissolved.

3. The melted agarose solution was transferred to a Zymo-Spin™ Column in a Collection Tube.

4. This was centrifuged for 30-60 sec. The flow-through was then discarded.

5. A volume of 200 µl of DNA Wash Buffer was added to the column and centrifuged for 30 seconds. The flow-through was discarded and the wash step repeated.

6. A volume of ≥ 6 µl of DNA Elution Buffer or water was added directly to the column matrix. The column was then placed into a 1.5 ml tube and centrifuged after 5 min for 30-60 sec to elute DNA.

Ultra-pure DNA was then obtained.

E. DNA Sequencing

The DNA concentration was confirmed using the NanoDrop and the DNA samples were prepared according to the sequencing company's instructions.

The PCR products/DNA was then submitted to GATC Biotech AG, Köln, Germany for direct sequencing with the ITS 1 primer. i.e. 2.5 µl ITS1 + 7.5 µl purified PCR product (final concentration of DNA was ≥ 20 ng/µl in each case).

F. Blast Search

Blast search of the sequences was then performed under:

http://blast.ncbi.nlm.nih.gov/Blast.cgi?PROGRAM=blastn&BLAST_PROGRAMS=megaBlast&PAGE_TYPE=BlastSearch&SHOW_DEFAULTS=on&LINK_LOC=blasthome

3.2.3 MODULATION OF *APIOSPORA MONTAGNEI* FOR IMPROVED BIOSYNTHESIS OF SECONDARY METABOLITES

3.2.3.1 MEDIA ENGINEERING AND OPTIMIZATION OF GROWTH CONDITIONS OF *APIOSPORA MONTAGNEI*

These experiments, except the evaluation of the effects of temperature, were carried out as described by Zhao *et al*. (2013), but with modification. *A. montagnei* was cultured in rice

media which were constituted with different chemical agents that acted as sources for carbon, nitrogen and salts respectively. Also, the fungus was cultured under different temperature conditions. The effects of the manipulations of the cultural conditions on the metabolic profile of the fungus were monitored. The parameters studied in this experiment are presented in Table 3.1.

Table 3.1: Parameters Studied in the Media Engineering and Optimization of Growth Conditions

Temperature (^{o}C)	**Salts (5%)**	**Carbon sources (4%)**	**Nitrogen sources (1%)**
22	Nacl	Glucose	Peptone
28	NaI	Sucrose	Yeast Extract
	NaBr	Rhamnose	Corn Steep Liquor
			Ammonium chloride

A. Effects of Carbon Sources

A. montagnei was inoculated into flasks of rice medium (50 g of rice + 60 mL of water) each supplemented with 40 g/L of various carbon sources such as of glucose, sucrose, and rhamnose respectively, and were incubated at 22 ^{o}C for 14 days under stationary condition.

B. Effects of Nitrogen Sources

A. montagnei was inoculated into flasks of rice medium (50 g of rice + 60 mL of water) each supplemented with 10 g/L of various nitrogen sources such as of peptone, yeast extract, corn steep liquor, and ammonium chloride respectively, and were incubated at 22 ^{o}C for 14 days under stationary condition.

C. Effects of Temperature

A. montagnei was inoculated into an Erlenmeyer flask containing rice medium (50 g of rice + 60 mL of water) and incubated in various ranges of temperature (22 and 28 ^{o}C) for 14 days under stationary condition.

D. Effects of Salts

The effect of sodium salts on growth and bioactive metabolite production of *A. montagnei* was studied by incubating the fungus in flasks of rice medium (50 g of rice + 60 mL of water)

each supplemented with various concentrations of NaCl, NaBr, and NaI (5% and 10%. i.e. 50 and 100 g/L respectively).

E. Extraction of Secondary Metabolites

After fermentation, the fungal secondary metabolites were extracted using 300 mL of ethyl acetate for all of the experiments carried out in the media engineering and optimization of growth conditions. The extracts were analysed using HPLC and the resulting chromatograms (metabolic profiles) were compared.

3.2.3.2 FUNGI-BACTERIA CO-CULTIVATION

A. montagnei was co-cultured with *Bacillussubtilis* and *Streptomyceslividans* respectively. Both bacteria are known to produce bioactive compounds. Rice medium was used in the co-culture of *A. montagnei* and *B. subtilis*, while Rice YM medium was used in the co-culture of *A. montagnei* and *S. lividans*. The fermentation media were inoculated with both microorganisms, incubated at 22°C for 21 days and then extracted with ethyl acetate. Crude extracts from the fermentation of the various fungi-bacteria co-cultures were subjected to HPLC analysis and the resulting chromatograms (metabolic profiles) were compared. The procedure for the co-cultivation experiment is presented as follows:

A. CO-CULTIVATION OF *B. SUBTILIS* AND *A. MONTAGNEI*

- **Rice medium preparation**

A volume of 60 mL of distilled water was added to 50 g of rice in a 1000 mL Erlenmeyer flask. This was autoclaved at 121 °C for 15 min. The medium was allowed to cool before use.

- **Preparing *Bacillus subtilis* to be injected into the rice medium**

B. subtilis was streaked on Luria-Bertani (LB)agar and incubated overnight at 37 °C. A loopful of the bacterial growth was then transferred to an LB medium which was incubated overnight at 37 °C with shaking.

The broth was diluted to OD600 of 0.05 with an appropriated amount of LB broth.

The bacteria was allowed to grow at 37 °C with shaking to mid-exponential growth phase (OD600 of 0.2 – 0.4) A volume of 10 mL of the bacterial solution was then transferred into the rice medium. This was then incubated at 37 °C for 3-4 days until the bacterial growth in the rice medium is observable.

After 3-4 days, portions of the fungus colony growth on malt agar were transferred into the rice medium containing the bacteria. The flasks were then incubated at 22^{o}C for 14 days before the culture can be harvested. Positive control flasks for the bacteria were prepared which contained only the bacterial isolate. Positive control flasks for the fungus which contained only the fungal isolate were also prepared.

- **Extraction of metabolites and HPLC analysis**

1. The fermentation/growth of the fungus, bacterium, and co-culture was stopped by adding 300 mL of ethyl acetate into each flask.

2. The extracts were properly mixed or agitated using a mechanical shaker for the same duration (8 h).

3. The extracts were concentrated using the rotary evaporator.

4. After drying, 50 mL of HPLC methanol was added to each extract in a round bottom evaporation flask and mixed to be homogenous.

5. A volume of 500 µL of each extract was transferred into clean HPLC vials and sent to the Dionex for HPLC analysis.

B. CO-CULTIVATION OF *S. LIVIDANS* AND *A. MONTAGNEI*

- **Modified rice medium preparation**

A volume of 60 ml of YeastMalt (YM)broth was added to 50 g of rice in the 1L Erlenmeyer flask. This was autoclaved at 121 °C for 15 min. The medium was allowed to cool before use.

- **Preparing *Streptomyces lividans* to be injected into the rice medium**

1. A colony of *S. lividans*from the stock culture was streaked onto a YM agar plate and then incubated at 30 °C for 24 h.

2. The growing *S. lividans*was transferred into the fresh 50 ml YM medium and incubated for ≥ 5 h until mid-exponential growth phase is reached (OD600 of 0.2 - 0.4).

3. A volume of 10 mL of the bacterial solution was transferred into the modified rice medium (Rice YM medium). This was then incubated at 30°C for about 3-4 days until substantial bacterial growth in the rice medium has been observed.

5. After about 3-4 days, some pieces of the fungal growth on malt agar were transferred aseptically into the Rice YM medium containing bacteria. The co-culture was incubated at 22^{o}C for 14 days before the culture was then extracted.

6. Duplicate flasks were prepared, as well as the positive control flasks for the bacteria and fungus.

- **Extraction of metabolites and HPLC analysis**

Extraction of metabolites and HPLC Analysis of samples were carried out as described previously.

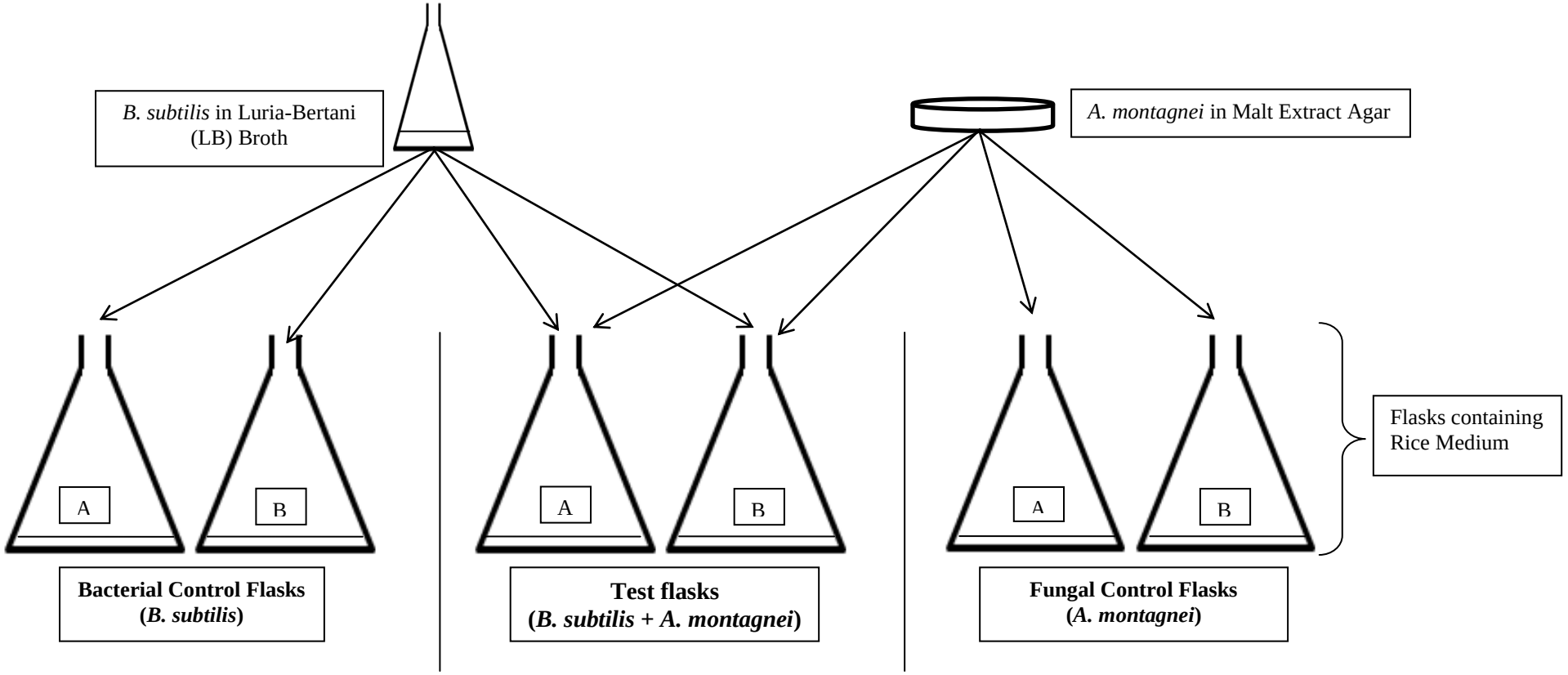


Figure 3.3: Co-cultivation experiment on *A. montagnei* with *B. subtilis*. Bacterial control and test flasks were inoculated with the bacterial isolate and incubated at 37^{o}C for 4 days. After which the test flasks containing *B. subtilis* were then inoculated with the fungus, *A. montagnei*. Also, the fungal control flasks were inoculated with the fungal isolate. All flasks, bacterial control, test and fungal control flasks were then incubated at 22^{o}C for 14 days. The experiment was conducted using duplicate flasks (A and B) for each treatment.

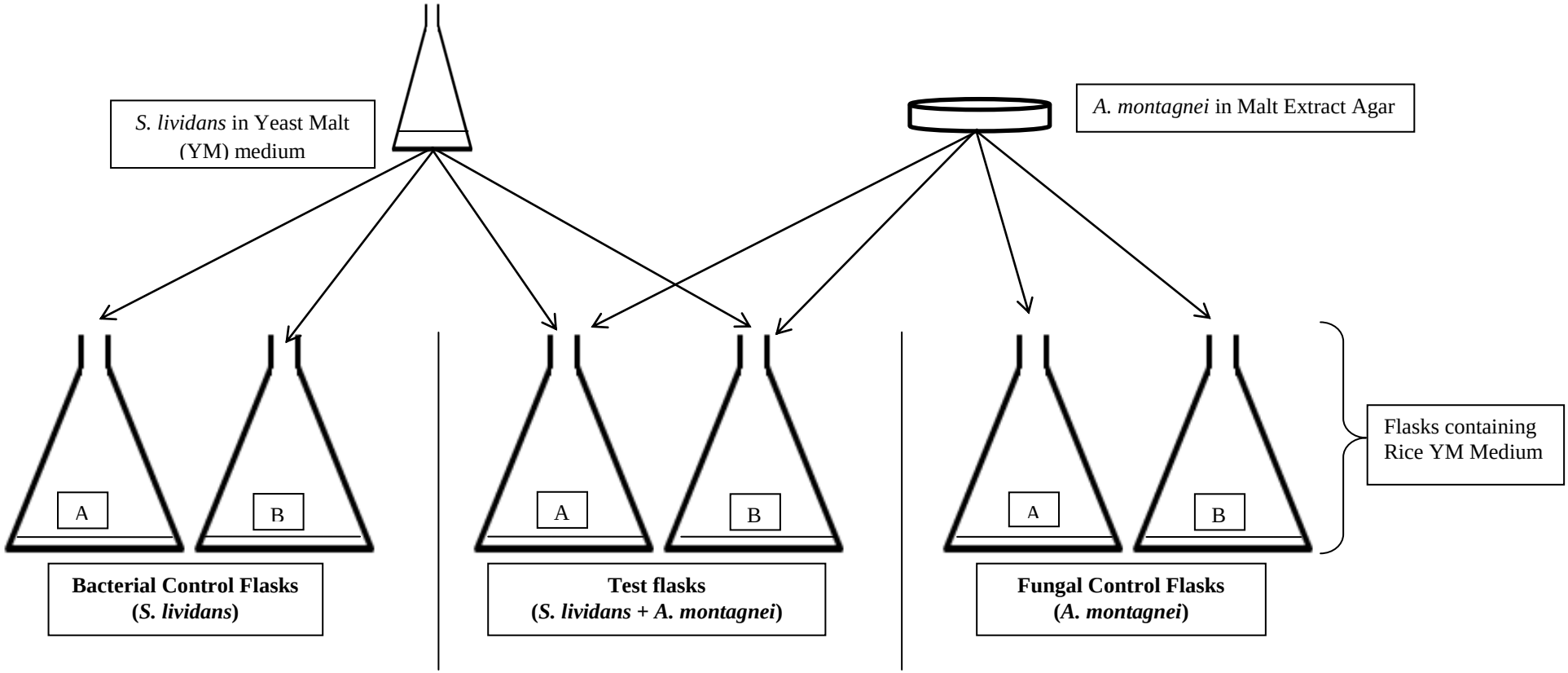


Figure 3.4: Co-cultivation experiment on *A. montagnei* with *S. lividans*. Bacterial control and test flasks were inoculated with the bacterial isolate and incubated at 30^{o}C for 4 days. After which the test flasks containing *S. lividans* were then inoculated with the fungus, *A. montagnei*. Also, the fungal control flasks were inoculated with the fungal isolate. All flasks, bacterial control, test and fungal control flasks were then incubated at 22^{o}C for 14 days. The experiment was conducted using duplicate flasks (A and B) for each treatment.

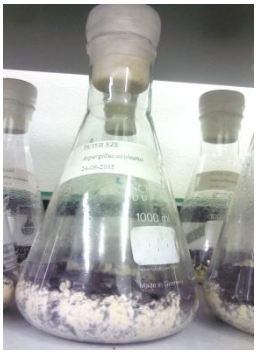
3.2.4 FERMENTATION AND EXTRACTION OF CRUDE SECONDARY METABOLITES FROM *A. ACULEATUS* AND *F. EQUISETI*

Solid state fermentation was carried out in Erlenmeyer flasks containing Rice Medium (i.e. 100 g of rice +110 mL of distilled water, which was autoclaved at 121^{o}C at 15 psi for 1 h). The flasks were inoculated with 3 mm diameter agar blocks containing test fungi and incubated at 22°C for 14-21 days and extracted with ethyl acetate. The organic phase was then vacuum-concentrated using the rotary evaporator at 40^{o}C to obtain the extracts.

A diagrammatic presentation of the fermentation and extraction of crude secondary metabolites from *A. aculeatus* and *F. equiseti*is shown in Figure 3.5.

Preparation of Rice Medium: 100g of rice was put in 1L Erlenmeyer flask containing 110 ml of distilled water which was then autoclaved and allowed to cool.

Inoculation of Rice Medium: Portions of fungal growth in agar were aseptically transferred into sterile Rice Medium.

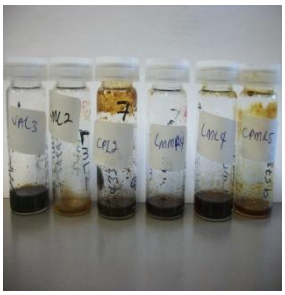


Fermentation: After inoculation, flasks were incubated at 22°C for 14-21 days.

Extraction of Secondary Metabolites: After the completion of fermentation, the secondary metabolites were extracted with ethyl acetate.

Filtration

Vacuum drying (40 ^{o}C)



Crude Extracts

Figure 3.5: Fermentation and Extraction of Secondary Metabolites

3.2.5 CHROMATOGRAPHIC SEPARATION (FRACTIONATION) OF CRUDE EXTRACTS

The crude extracts were subjected to vacuum liquid chromatography and size exclusion chromatography to yield several fractions. The chromatographic separation/fractionation of the crude fungal extracts was carried out as reported by Kjer (2009).

3.2.5.1 Vacuum liquid chromatography (VLC)

Vacuum liquid chromatography was used for initial isolation procedure for the large crude extract. The apparatus consists of a 30 cm sintered glass VLC column with an inner diameter of 15cm attached to a vacuum pump. Silica gel 200-400 mesh size was packed to a hard cake up to a height of 1.5 cm. The sample used was covered onto a small amount of silica gel using volatile solvents. The resulting sample mixture was then packed onto the top of the column. Step gradient elution with a non-polar solvent (n-hexane and dichloromethane) and increasing the amounts of a polar solvent (ethyl acetate or methanol) gave successive fractions. The flow was produced by vacuum and the column was allowed to run dry after each fraction was collected. Fractions were collected in round bottom flasks.

3.2.5.2 Size Exclusion Chromatography

The column was packed with Sephadex LH-20 (0.25 – 0.1 mm mesh size), as stationary phase and the mobile phase was a mixture of polar and non-polar solvents. The components were separated and distributed on the stationary phase according to their molecular size. Since the stationary phase consists of porous beads, the large molecules were excluded from the beads and eluted first. The small molecules were eluted later because they entered the porous beads and stayed inside until they found the certain size of pores to exit from the beads. Elution was performed using methanol or methanol : dichloromethane [1:1 (V/V)] as mobile phases.

3.2.5.3 High Pressure Liquid Chromatography (HPLC)

- **Analytical HPLC**

All extracts, fractions and pure compounds were monitored by analytical HPLC to determine the composition of the fractions, the purity of the isolated substances and the optimum conditions for semi preparative HPLC. Additionally, known substances could be identified by comparison of the obtained UV spectra with the internal UV-spectra library using the online software. For the routine analytical HPLC detection, a solvent system of nanopure water (adjusted to pH 2.0 by addition of phosphoric acid) (eluent A) and methanol (eluent B) with a flow rate of 1 mL/min employing the standard gradient system, was used.

- **Semi preparative HPLC**

This technique was used for isolation and purification of compounds from fractions pre-purified using column chromatographic separation. The most appropriate solvent systems were determined by analytical HPLC before running the preparative HPLC separation. The mobile phase consisted of methanol and nanopure water. Each injection consisted of 1–3 mg of the fraction dissolved in 100 μL of the solvent system. The solvent system was pumped through the column at a rate of 5 mL/min. The eluted peaks were detected by the online UV detector and collected separately in clean test tubes.

3.2.6 ISOLATION OF BIOACTIVE COMPOUNDS AND STRUCTURAL ELUCIDATION

Isolation of bioactive compounds and structural elucidation from fractions of the fungal extracts was carried out as reported by Kjer (2009).

These fractions were subjected to semi-preparative HPLC and HPLC-DAD analyses to isolate pure compounds. The structural elucidation of the isolated compounds then followed a standard scheme. Firstly, the data obtained from analytical HPLC were compared with the

in-house substance library regarding their retention times at the standard gradient program and their UV spectra. Comparable hits indicated the class to which the compound belongs. From the LC-MS measurements the mass of the compoundscould be calculated; and from the ^{1}H-NMR measurements, substructures of the compounds could be compiled. With these information, together with the identity of the fungus, a literature search using the latest versions of Dictionary of Natural Products (DNP, Chapman and Hall, 2005-2009), Antibase (2002-2007) and SciFinder was performed. In cases where these data were insufficient, additional measurements (especially 1 and 2 dimensional NMR experiments) were carried out to finally identify the secondary metabolite.

3.2.6.1 Liquid Chromatography Mass Spectrometry (LC-MS)

Liquid Chromatography Mass Spectrometry (LC-MS)was carried out to determine the molecular weights of the compounds in the fractions/extracts and the isolated pure compounds. This procedure revealed the negative and positive charged ions of the compounds. Itwas from the molecular ions (parent ions) of the compounds, thatthe molecular weights of the compound were calculated. From the fragmentation patterns of the compounds, information about their substructures was attained.

The solvent system of 0.1% formic acid (eluent A) and acetonitrile (eluent B) with a flow rate of 0.4 mL/min employing the standard gradient system was used in the LC-MS analysis. Detection was achieved by an ion trap mass filter together with a UV photodiode array detector.

3.2.6.2 Nuclear Magnetic Resonance Spectroscopy (NMR)

NMR measurements were carried out to acertain the substructures of the isolated compounds. ^{1}H and ^{13}C spectra were recorded at 300ºK using the Bruker DPX 300, ARX 500 or AVANCE DMX 600 NMR spectrometers. All 1 and 2 dimensional NMR spectra were

obtained using the standard Bruker software. The samples were dissolved in different solvents (i.e. $CDCl_3$, and CD_3OD), the choice of which was dependent on the solubility of the samples. Residual solvent signals were used as internal standards (reference signal). The observed chemical shift (δ) values were given in ppm and the coupling constants (*J*) in Hz.

3.2.6.3 Optical Activity

The optical activity of the isolated compounds was determined to differentiate enantiomers of the compounds. Enantiomers were differentiated as the D (+) isomer (which rotates the orientation of linearly polarized light clockwise),or as the L (-) isomer (which rotates the orientation of linearly polarized light counter-clockwise). This measurement allowed the determination of the absolute stereochemistry of the isolated compound.

The optical activity of the compounds was measured at the wavelength of 589 nm and at a temperature of 20°C.The specific optical rotation of the compounds was calculated using the formula:

$$[\alpha]_D^{20} = \frac{100*\alpha}{l*c} \quad \text{.....Equation 1}$$

Where α = the measured angle of rotation in degrees °

l = the length in dm of the polarimeter tube (typically = 1)

c = concentration of the substance in g/100 mL.

Pure substances were dissolved in appropriate spectroscopic grade solvent. The optical rotation of known compounds was compared to literature data.

3.2.7 TESTING OF EXTRACTS FOR BIOACTIVITY

Each crude extract from the fermentation of the various endophytic fungal isolates was tested for activity through antibacterial, anti-tuberculosis, antioxidant and cytotoxicity bioassays.

3.2.7.1 Antimicrobial Assay

- **Preliminary Antimicrobial Screening**

Preliminary antimicrobial screening of the crude fungal extracts was carried out using the agar well diffusion assay method as described by Subbulakshmi*et al.* (2012),with modification. A concentration of 1 mg/mL of the fungal extracts was prepared by dissolving the extracts in Dimethyl sulphoxide (DMSO). Standardized broth cultures of test bacterial isolates (*Staphylococcus aureus, Pseudomonas aeruginosa, Klebsiella pneumoniae, Bacillus subtilis* and *Salmonella typhi*) and fungal isolates (*Aspergillus niger* and *Candida albicans*) were spread aseptically onto the surface of Mueller Hinton Agar (MHA) and Sabouraud Dextrose Agar (SDA) plates respectively using sterile cotton swabs. All culture plates were allowed to dry for about 5 min and agar wells were made using a sterile cork-borer (6 mm in diameter). These wells were respectively filled with 20 µL of the fungal extracts and controls. The plates were then kept at room temperature (25-27 ^{o}C) for 1 hour to allow the agents to diffuse into the agar medium and incubated accordingly. Ciprofloxacin (5µg/mL) and miconazole (50µg/mL) were used as positive controls in the antibacterial and antifungal evaluations respectively; while DMSO was used as the negative control. The MHA plates were then incubated at 37^{o}C for 24 h, and the SDA plates were incubated at 25-27^{o}C for 2-3 days. The inhibition zone diameters (IZD) were measured and recorded. The size of the cork borer (6mm) was deducted from the values recorded for the IZD to get the actual diameter. This procedure was conducted in triplicate and the mean IZD calculated and recorded.

- **Determination of Minimum Inhibitory Concentrations (MICs)**

The Minimum Inhibitory Concentrations (MICs) of the fungal extracts were determined against the test organisms on which the extracts showed activity in the preliminary antimicrobial screening. The MICs of the fungal extracts were determined using the agar dilution method described by Russell and Furr (1972). A stock solution of 5 mg/ml was prepared for each fungal extract and these were further diluted in a 2-fold serial dilution to obtain the following concentrations: 2.5, 1.25, 0.625, and 0.3125 mg/mL. Agar plates were prepared by pouring 4 mL of molten double strength MHA and SDA, for bacterial and fungal isolates respectively, into sterile Petri dishes containing 1mL of the various dilutions of the extract to give the final plate concentrations of 1, 0.5, 0.25, 0.125 and 0.0625 mg/mL.

The test isolates which were grown overnight in broth were adjusted to McFarland 0.5 standard and streaked onto the surface of the agar plates containing dilutions of the extract. The MHA plates were then incubated at 37^{o}C for 24 hours and the SDA plates at 25-27^{o}C for 2-3days, after which all the plates were observed for growth. The minimum dilution (concentration) of the extracts completely inhibiting the growth of each organism was taken as the MIC. This procedure was conducted in triplicate.

3.2.7.2 Anti-tubercular Assay

Growth inhibition of *Mycobacterium tuberculosis* was tested using a metabolic activity assay employing the resazurin dye reduction method as described by Daletos *et al.,* (2015). *M. tuberculosis* cells were grown aerobically at 37 °C in Middlebrook 7H9 media supplemented with 0.5% (v/v) glycerol, 0.05% (v/v) Tyloxapol, and 10% (v/v) ADS enrichment (5%, w/v, bovine serum albumin fraction V; 2%, w/v, glucose; 0.85%, w/v, sodium chloride). Bacteria were pre-cultured until log-phase (OD 600 nm ~1) and then seeded at 1×10^5 cells per well in a total volume of 100 µL in 96-well round-bottom microtiter plates. The test substances were then added to achieve a working concentration of 10µg/mL for the fungal extracts. The

microtiter plates were then incubated at 35°C for 6 days. For viability determination, 10 µL of resazurin solution (100 µg/mL, Sigma-Aldrich) was added per well and incubated for about 8 h. The cells were fixed by addition of formalin (5%, v/v) for 30 min, and fluorescence was measured using a microplate reader (excitation 540 nm, emission 590 nm). Residual growth was calculated relative to rifampicin-treated (0% growth) and DMSO-treated (100% growth) controls.

3.2.7.3 Antioxidant Assay (DPPH Free Radical Assay)

The free radical scavenging activity of all the extracts was evaluated by 1, 1-diphenyl-2-picryl-hydrazyl (DPPH) according to the previously reported method by Shen *et al*. (2010) with modification. Here, the percentage inhibition of the samples and positive control were determined at a concentration of 500 µg/mL from absorbance values recorded from the UV at 517nm.

A volume of 50 mL of 0.2mM solution of DPPH in methanol was prepared (final concentration of 0.1 mM to be obtained in reaction mixture). A weight of 3.94 mg of DPPH was added to 50 mL of methanol (0.2mM DPPH solution). A volume of 2 mL of 0.2mM solution of DPPH was then added to 2 mL of the samples dissolved in methanol (1mg/mL, 1000µg/mL). These final reaction mixtures resulted in a 2-fold dilution of both the extract and DPPH concentrations, bringing them to final concentrations of 0.1 mM for the DPPH solution and 500 µg/mL for the samples.

The mixtures were shaken vigorously and allowed to stand at room temperature for 30 minutes. Then the absorbance was measured at 517 nm using a UV-VIS spectrophotometer. Lower absorbance values of reaction mixture indicate higher free radical scavenging activity. The capability of scavenging the DPPH radical was calculated using the following formula.

DPPH scavenging effect (% inhibition) =

$$\frac{\text{Absorbance of blank } (A_0) - \text{Absorbance of sample } (A_1)}{\text{Absorbance of blank } (A_0)} \times \frac{100}{1} \quad \text{..... Equation 2}$$

3.2.7.4 Cytotoxicity Assay (MTT/Cell Proliferation Assay)

MTT assay was performed following the method described by Carmichael *et al*. (1987), and % cell viability was determined by spectrophotometric determination of accumulated formazan derivative in treated cells at 560 nm in comparison to control cells (Ashour *et al*. 2006).

L5178Y mouse lymphoma cells were grown in Eagle's minimal essential medium supplement with 10% horse serum in roller tube culture. The medium contained 100 units/mL penicillin and 100 units/mL streptomycin. The cells were maintained in a humidified atmosphere at 37°C with 5% CO_2. Of the test samples to be analyzed in the bioassay, stock solutions of crude extracts (10 µg/mL) in ethanol (96% v/v) were prepared. Exponentially growing cells were harvested, counted and diluted appropriately. Of the cell suspension, 50 µL containing 3750 cells were pipetted into 96- well microtiter plates. Subsequently, 50 µL of a solution of the test samples containing the appropriate concentration was added to each well.

The test plates were incubated at 37°C with 5% CO_2 for 72 h. A solution of MTT was prepared at a concentration of 5 µg/mL in phosphate buffered saline (PBS; 1.5 mM KH_2PO_4; 6.5 mM Na_2HPO_4; 137 mM NaCl; 2.7 mM KCl; pH 7.4), and from this solution, 20 µL was pipetted into each well.

The yellow MTT penetrated the cells and in the presence of mitochondrial dehydrogenases MTT was transformed to its blue formazan complex.

After an incubation period of 3 h 45 min at 37°C in a humidified incubator with 5% CO_2, the medium was centrifuged (15 min at 210 x g) with 200 µL DMSO and the cells were lysed to liberate the formazan product.

After thorough mixing, the absorbance was measured at 520 nm. The color intensity could be correlated with the number of healthy living cells and cell survival was calculated using the formula:

$$\text{Survival \%} = \frac{100}{1} \times \frac{\text{Absorbance of untreated cells - Absorbance of culture medium}}{\text{Absorbance of treated cells - Absorbance of culture medium}} \quad \text{..... Equation 3}$$

All experiments were carried out in triplicate.

As negative controls, media with 0.1% (v/v) ethanol were included in all experiments.

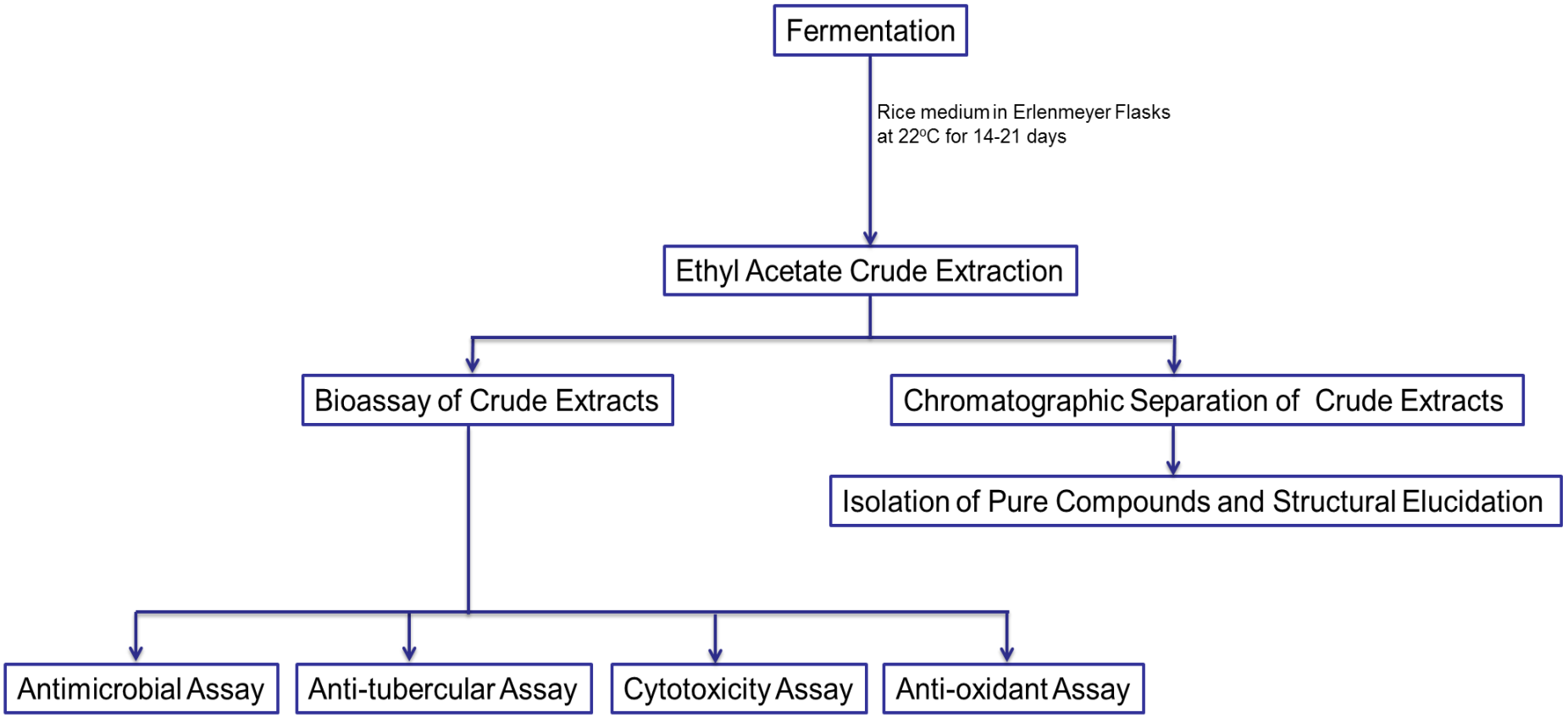


Figure 3.6: Methods for Fermentation/Extraction/Isolation and Bioassay of Compounds from Fungal Secondary Metabolites

3.2.8 STATISTICAL ANALYSES

The Chromeleon 6.30 software of the Dionex was used to create overlays of the HPLC chromatograms of the treatments and controls for comparison of the expression of certain compounds as well as the degree of expression of the compounds.

All modulation experiments (co-cultivation, manipulation of cultural conditions and media engineering) were conducted using duplicate flasks for each treatment and controls. Antimicrobial assay of the crude fungal extracts was carried out in triplicate Petri dishes and results were presented as mean ± standard error of mean (SEM) inhibition zone diameters (IZD). Using Microsoft Excel version 2010, the mean peak areas (%) of detected compounds (which indicate the degree of quantitative expression of the compounds) were calculated and presented graphically for comparisons.

CHAPTER FOUR

RESULTS AND DISCUSSION

4.1 RESULTS

4.1.1 RESULTS OF FUNGAL ISOLATION AND IDENTIFICATION

Following the isolation procedures (described in section 3.2.2), one fungus was isolated from the lichen-*Cladonia sp.,* and two endophytic fungi were isolated from the leaves of the Nigerian plant-*Carica papaya.*

Also, following the identification experiments carried out according to the molecular biological protocol of DNA amplification and sequencing of the ITS region (described in section 3.2.2), the fungus isolated from the lichen was identified as *Athrinium arundinis* (recent name- *Apiospora montagnei*); while, the two endophytic fungi isolated from *Carica papaya* were identified as *Aspergillus aculeatus* and *Fusarium equiseti* respectively. The DNA sequences of the identified fungi were submitted to GenBank, with their accession numbers presented in Table 4.1.

Flowcharts of the isolation and identification of the different fungi are presented in Figures 4.1 and 4.3. Also, growths of the isolated fungi on Malt Extract Agar are shown in Figures 4.2, 4.4 and 4.5.

The three fungal strains,with codes - HuiqinChen/CL-2-1 (for *Apiospora montagnei*), PeterEze/CPMR4 (for *Aspergillus aculeatus*) and PeterEze/CPL1 (for *F. equseti*),were deposited in Prof. Proksch's laboratory, at the Institut für Pharmazeutische Biologie und Biotechnologie, Heinrich-Heine-Universität, Düsseldorf, Germany.

Table 4.1: Fungal Identification

Source	Fungal DNA Sequence (FASTA format)	Fungal Name	GenBank Accession Number
Cladonia sp.	GGCGTAAGCTCGGTTGGAGGCACCTGCAGCTACCCTGTAGTTGCGGACTGCCAACTCCAGCCGCGGCCCG CCGGCGGTACACTAAACTCTGTTTTATTTTATATTCTGAGCGTCTTATTTTAATAAGTTAAAACTTTCAACA ACGGATCTCTTGGTTCTGGCATCGATGAAGAACGCAGCGAAATGCGATAAGTAATGTGAATTGCAGAATT CAGTGAATCATCGAATCTTTGAACGCACATTGCGCCCATCAGTATTCTGGTGGGCATGCCTGTTCGAGCGT CATTTCAACCCTTAAGCCTAGCTTAGTGTTGGGAATCTGCTGTACTGCAGTTCCTTAAAGACAGTGGCGGA GCGGCGGTAGTCCTCTGAGCGTAGTAATTTATTTCTCGCTTTTGTCAGGCTCTGTCCTCCCGCCATAAAACC CCC	*Apiospora montagnei* (*Arthrinium arundinis*)	KX137848
Carica papaya	CCCGTGCTTACCGTACCCTGTTGCTTCGGCGGGCCCGCCTTCGGGCGGCCCGGGGCCTGCCCCCGGGACCG CGCCCGCCGGAGACCCCAATGGAACACTGTCTGAAAGCGTGCAGTCTGAGTCGATTGATACCAATCAGTC AAAACTTTCAACAATGGATCTCTTGGTTCCGGCATCGATGAAGAACGCAGCGAAATGCGATAACTAATGT GAATTGCAGAATTCAGTGAATCATCGAGTCTTTGAACGCACATTGCGCCCCCTGGTATTCCGGGGGGCAT GCCTGTCCGAGCGTCATTTCTCCCCTCCAGCCCCGCTGGTTGTTGGGCCGCGCCCCCCCGGGGGCGGGCCT CGAGAGAAACGGCGGCACCGTCCGGTCCTCGAGCGTATGGGGCTCTGTCACCCGCTCTATGGGCCCGGCC GGGGCTTGCCTCGACCCCCAATCTTCTCAGATTGACCTCGGATCAGGTAGGGATACCCGCTGAACTTAAGC ATATCAATA	*Aspergillus aculeatus*	KX137846
	CCCTAAACTCTGTTTTTAGTGGAACTTCTGAGTAAAACAAACAAATAAATCAAAACTTTCAACAACGGAT CTCTTGGTTCTGGCATCGATGAAGAACGCAGCAAAATGCGATAAGTAATGTGAATTGCAGAATTCAGTGA ATCATCGAATCTTTGAACGCACATTGCGCCCGCCAGTATTCTGGCGGGCATGCCTGTTCGAGCGTCATTTC AACCCTCAAGCTCAGCTTGGTGTTGGGACTCGCGGTAACCCGCGTTCCCCAAATCGATTGGCGGTCACGTC GAGCTTCCATAGCGTAGTAATCATACACCTCGTTACTGGTAATCGTCGCGGCCACGCCGTAAAACCCCAA CTTCTGAATGTTGACCTCGGATCAGGTAGGAATACCCGCTGAACTTAAGCATAT	*Fusarium equiseti*	KX137847

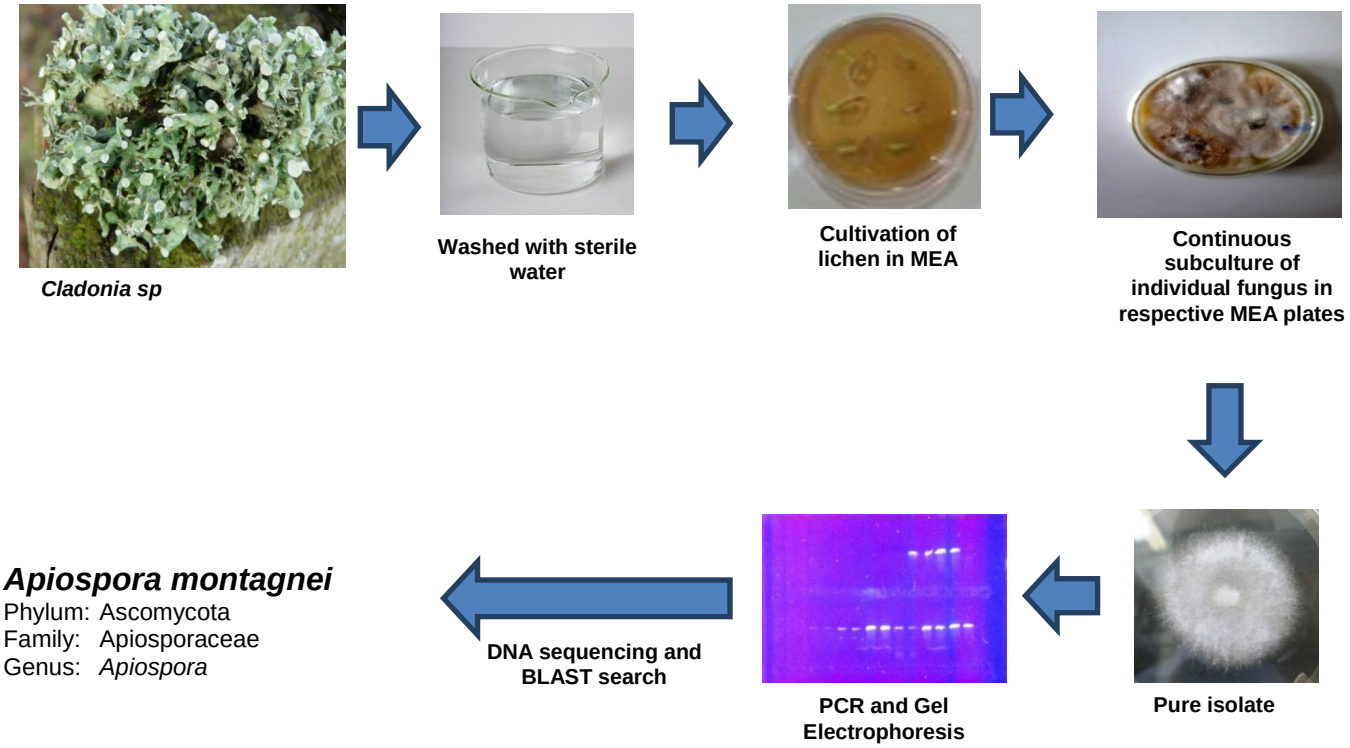


Figure 4.1: Isolation and Identification of fungus from *Cladonia sp*

Young culture Old culture

Figure 4.2: Growth of *Apiospora montagnei* (also known with its old name as *Arthrinium arundinis*) in Malt Extract Agar.

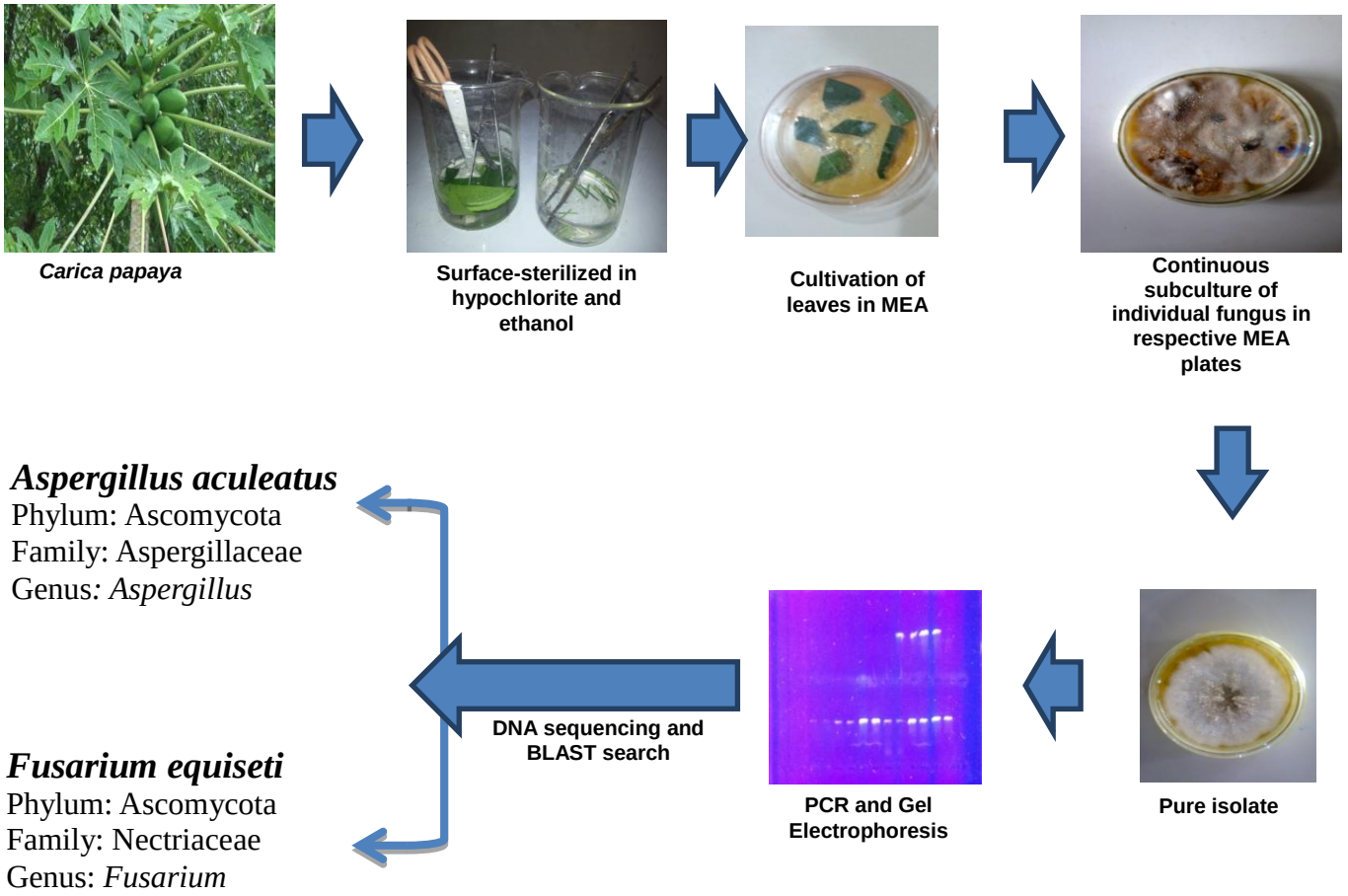
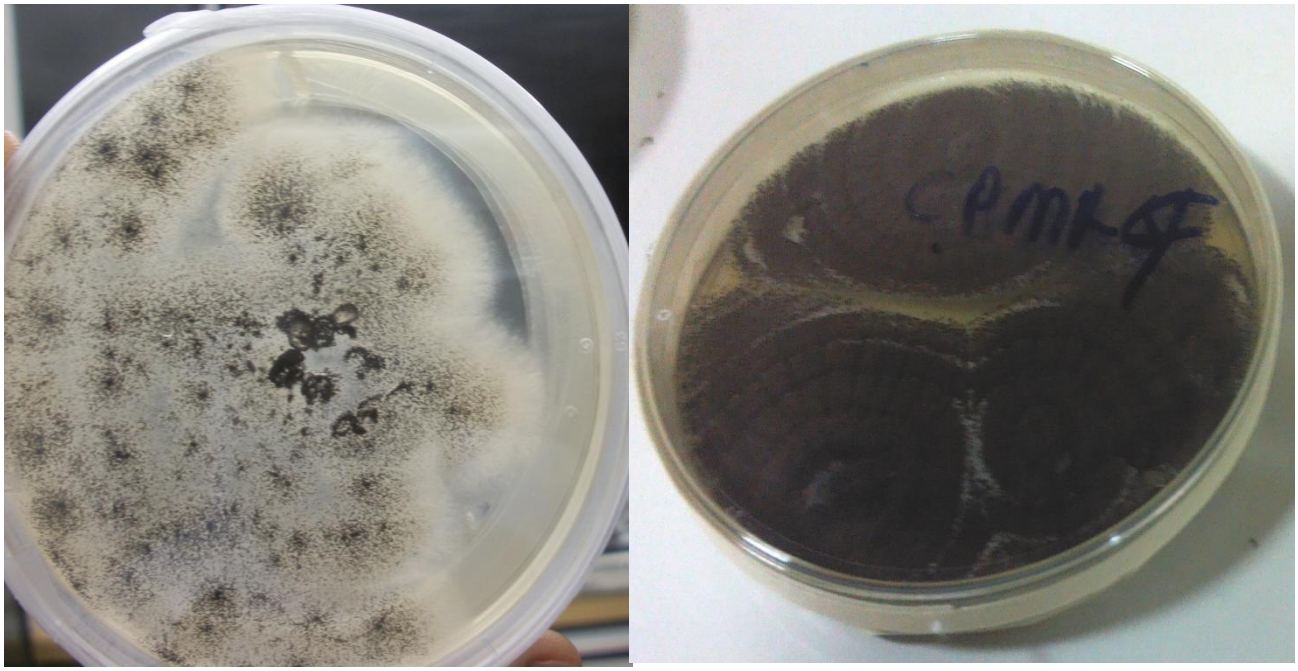
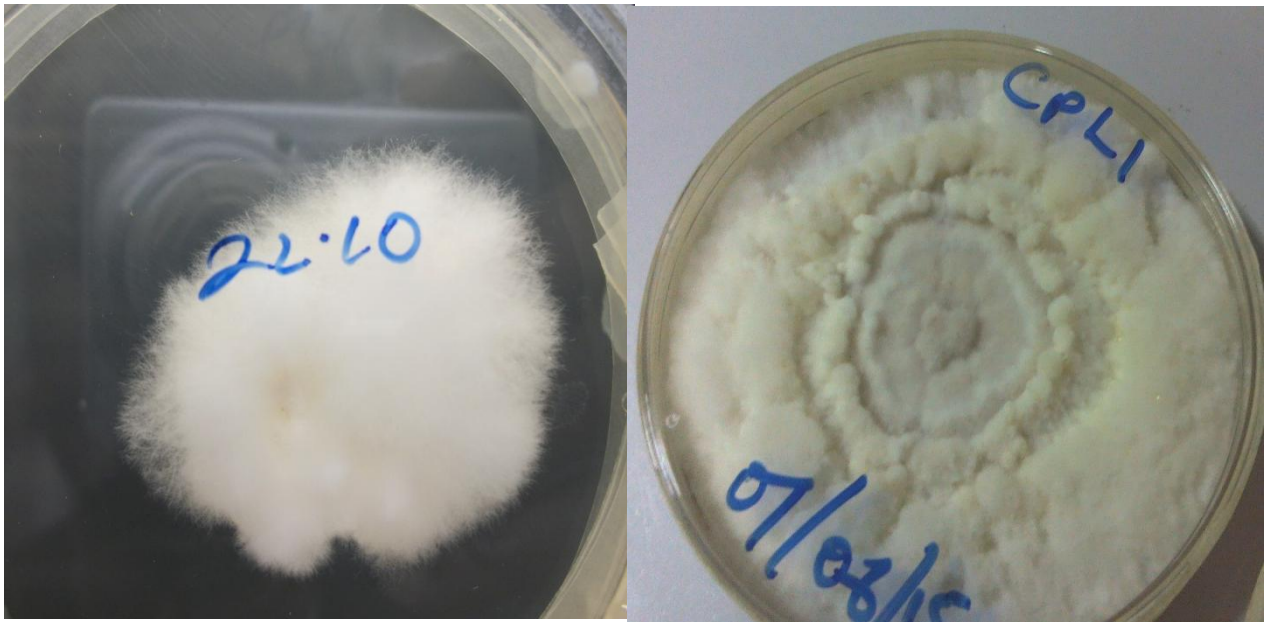


Figure 4.3: Isolation and Identification of Two Fungi from Leaves of *Carica papaya*



Young Culture Old Culture

Figure 4.4: Growth of *Aspergillus aculeatus* in Malt Extract Agar



Young Culture Old Culture

Figure 4.5: Growth of *Fusarium equiseti* in Malt Extract Agar

4.1.2 RESULTS OF MODULATION EXPERIMENTS ON*APIOSPORA MONTAGNEI*

The already established metabolic profile of *A. montagnei* (i.e. compounds expressed and isolated from the fungus) is shown in Figure 4.6, while the reported bioactivities of the compounds are presented in Table 4.2.

A. montagnei with its established metabolic profile was subjected to several modulation experiments with the aim of stimulating the increased production of the bioactive secondary metabolites. It was also expected that the experiments will trigger the expression of silent genes i.e. the expression of compounds which were originally not expressed by the fungus. These modulation experiments include: co-cultivation experiments of *A. montagnei* with *Bacillus subtilis* and *Streptomyces lividans* respectively; manipulation of cultural conditions (which involved culturing the fungus at different temperatures); and media engineering (where the fermentation medium of the fungus was supplemented with various sodium salts, carbon, and nitrogen sources).

After each experiment, the HPLC chromatogram of each treatment was compared with each other as well as with the control (untreated). The Chromeleon 6.30 software of the Dionex was used to create an overlay of the HPLC chromatograms at the same detection. By observing the overlay of the chromatograms, a direct comparison was done on the treatments in relation to the presence or absence of peaks of detected compounds. Also using the same software, the peak areas (%) of detected compounds were calculated and these values were compared for the treatments and controls. The peak areas (%) of detected compounds show the quantity/degree of expression of the compounds.

Decarboxycitrinone

Decarboxyhydroxycitrinone

O-Methylmellein

Myrocin A

Libertellenone G

Norlichexanthone

8-hydroxy-3-methyl-9-oxo-9*h*-xanthene-1-carboxylic acid methyl ether

Arthrinin A

Arthrinin B

6,8-Dihydroxy-4 hydroxymethyl -3,5-dimethylisochromen-1-one

Acremonone G

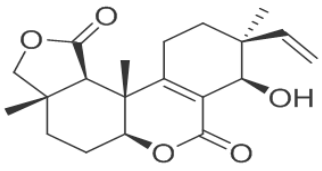
Anomalin A

Anomalin B

Apiosporamide

N-Hydroxyapiosporamide

Didymellamide B



CL2-1E-SE5-S4
(Uncharacterized compound)

CL2-1G-SE3-S4
(Uncharacterized compound)

Figure 4.6: Established metabolic profile of *A. montagnei* (i.e. compounds expressed and isolated from the fungus)

Table 4.2: Compounds Isolated from *A. montagnei* and their Reported Bioactivities

	Compounds	Reported Biological Activities	References
1	Decarboxycitrinone	Antifungal	Whyte *et al*., 1996
2	8-hydroxy-3-methyl-9-oxo-9*H*-xanthene-1-carboxylic acid methyl ether	-	-
3	Libertellenone G	Antibacterial activity	Lu *et al*., 2014
4	Arthrinin A	-	-
5	Arthrinin B	-	-
6	Norlichexanthone	Cytotoxic/Antiproliferative	Ebada *et al*., 2011
7	Myrocin A	Cytotoxic/Antiproliferative	Ebada *et al*., 2011; Tsukada *et al*., 2011;
8	Anomalin A	Cytotoxic/Antiproliferative; Antioxidant	Ebada *et al*., 2011; Abdel-Lateff *et al*., 2003.
9	Anomalin B	-	
10	*O*-methylmellein	Antifungal	Glauser *et al*., 2009
11	Acremonone G	-	-
12	6,8-dihydroxy-4-hydroxymethyl-3,5-dimethylisochromen-1-one	-	-
13	Decarboxyhydroxycitrinone	Cytotoxic/Antiproliferative	Tsukada *et al*., 2011
14	Didymellamide B	Antifungal	Xu *et al*., 2015
15	*N*-hydroxyapiosporamide	Cytotoxic; Antifungal; Antibacterial	Wang 2015
16	Apiosporamide	Antifungal	Alfatafta *et al*., 1994

4.1.2.1 FUNGI-BACTERIA CO-CULTIVATION EXPERIMENTS

In the co-cultivation experiments, *A. montagnei* was co-fermented with *Bacillus subtilis* and *Streptomyces lividans* in Rice medium and Rice YM medium respectively. Fermentation flasks showing co-cultivation experiments are presented in Figures 4.7 (*A. montagnei* + *B. subtilis*) and 4.10 (*A. montagnei* + *S. lividans*).

The overlay of the HPLC chromatograms/profiles of the treatments and controls in the co-cultivation experiments are presented for comparisons in Figures 4.8 (*A. montagnei* + *B. subtilis*) and 4.11 (*S. lividans* + *A. montagnei*). These chromatogram overlays show and compare the presence or absence of peaks of detected compounds for the treatments and controls.

Graphs showing the peak areas (%) of compounds detected by the HPLC analysis of the treatments and controls are presented in Figures 4.9 (*B. subtilis* + *A. montagnei*) and 4.12 (*S. lividans* + *A. montagnei*). These graphs reveal and compare the quantity/degree of expression of the compounds detected for the treatments and control.

The stimulation/expression of compounds/secondary metabolites in the co-cultivation experiments are presented in Tables 4.3(*A. montagnei* + *B. subtilis*) and 4.4 (*A. montagnei* + *S. lividans*).

A. CO-CULTIVATION EXPERIMENT OF *A. MONTAGNEI* + *B. SUBTILIS*

It can be observed in Figure 4.8, that the co-cultivation of *A. montagnei* and *B. subtilis* stimulated the up-regulation of fungal genes expressing anomalin B, norlichexanthone, and N-hydroxy-apiosporamide. Co-cultivation of *A. montagnei* and *B. subtilis* also triggered the expression of silent fungal genes expressing anomalin A and apiosporamide.

It can also be observed in Figure 4.9 that the co-cultivation of *A. montagnei* and *B. subtilis* stimulated the up-regulation of fungal genes expressing anomalin B, norlichexanthone, and N-hydroxy-apiosporamide. Co-cultivation of *A. montagnei* and *B. subtilis* also triggered the expression of silent fungal genes expressing anomalin A and apiosporamide.

It can be observed in Table 4.3 that the co-cultivation of *A. montagnei* and *B. subtilis* triggered the expression of silent fungal genes expressing anomalin A and apiosporamide. These compounds were initially expressed by earliest cultures of the fungus, but after periods of storage and successive sub-culturing, the fungus ceased to express the compounds (i.e. the genes expressing the compounds became silent or turned off). Co-culturing of the fungus with *B. subtilis*, however, turned on the silent genes resulting in the re-expression of these compounds.

B. subtilis ***B. subtilis*** + ***A. montagnei*** ***A. montagnei***

Figure 4.7: Flasks showing co-cultivation experiment of *A. montagnei* + *B. subtilis*

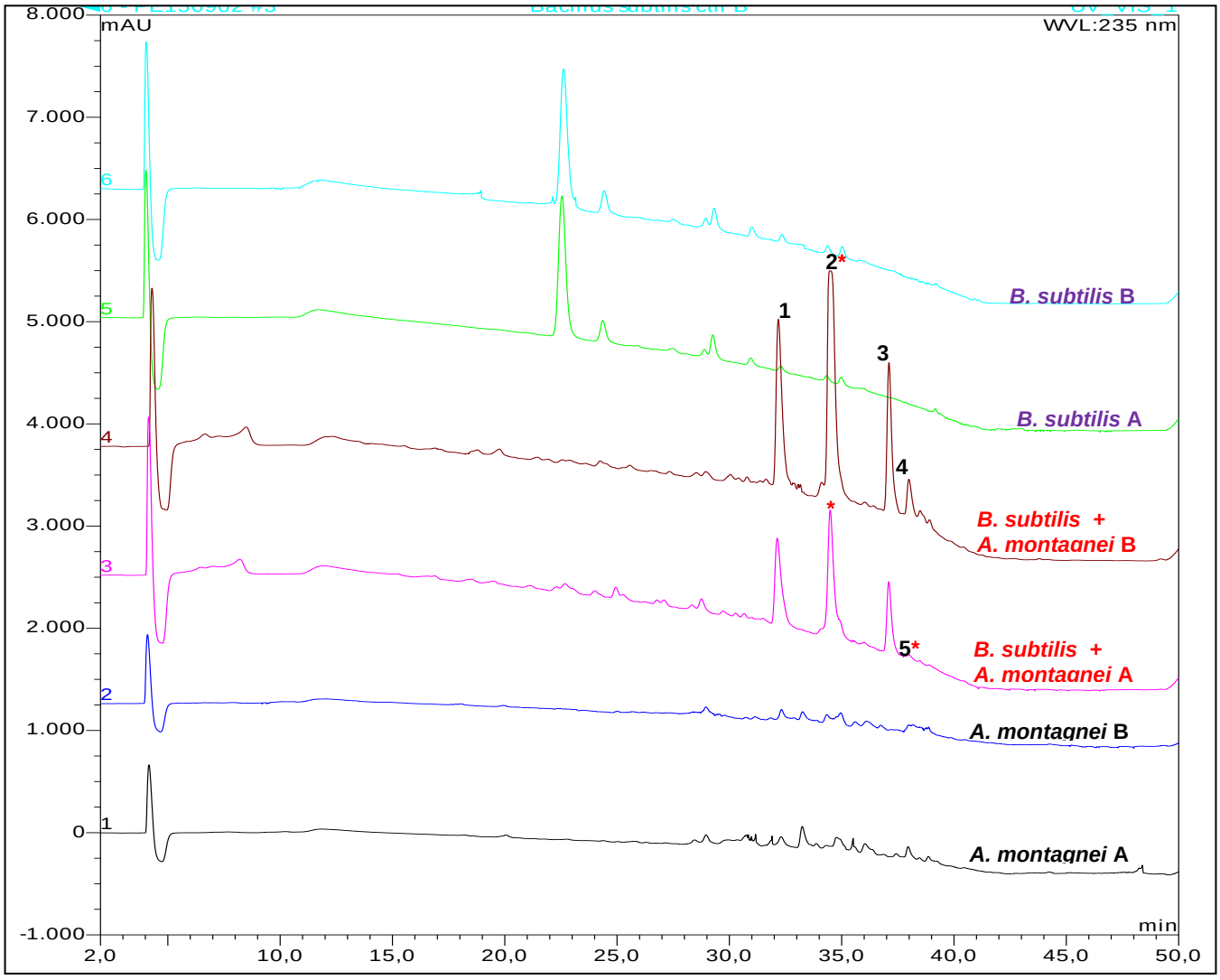


Figure 4.8:Comparison of HPLC Profilesof *B. subtilis*, *A. montagnei* and *B. subtilis* + *A. montagnei*(*B. subtilis* + *A. montagnei*Co-cultivation Experiment)

1-Anomalin B
2- Anomalin A
3- Norlichexanthone
4- N-hydroxy-apiosporamide
5- Apiosporamide

- Up-regulated gene products (1, 3, and 4)
- Turned-on genes (2 and 5)
- *-undetected in fungal controls, but present in chromatogram of earlier fungal fermentation extract.

NB: The experiment was conducted using duplicate flasks (A and B) for each treatment.

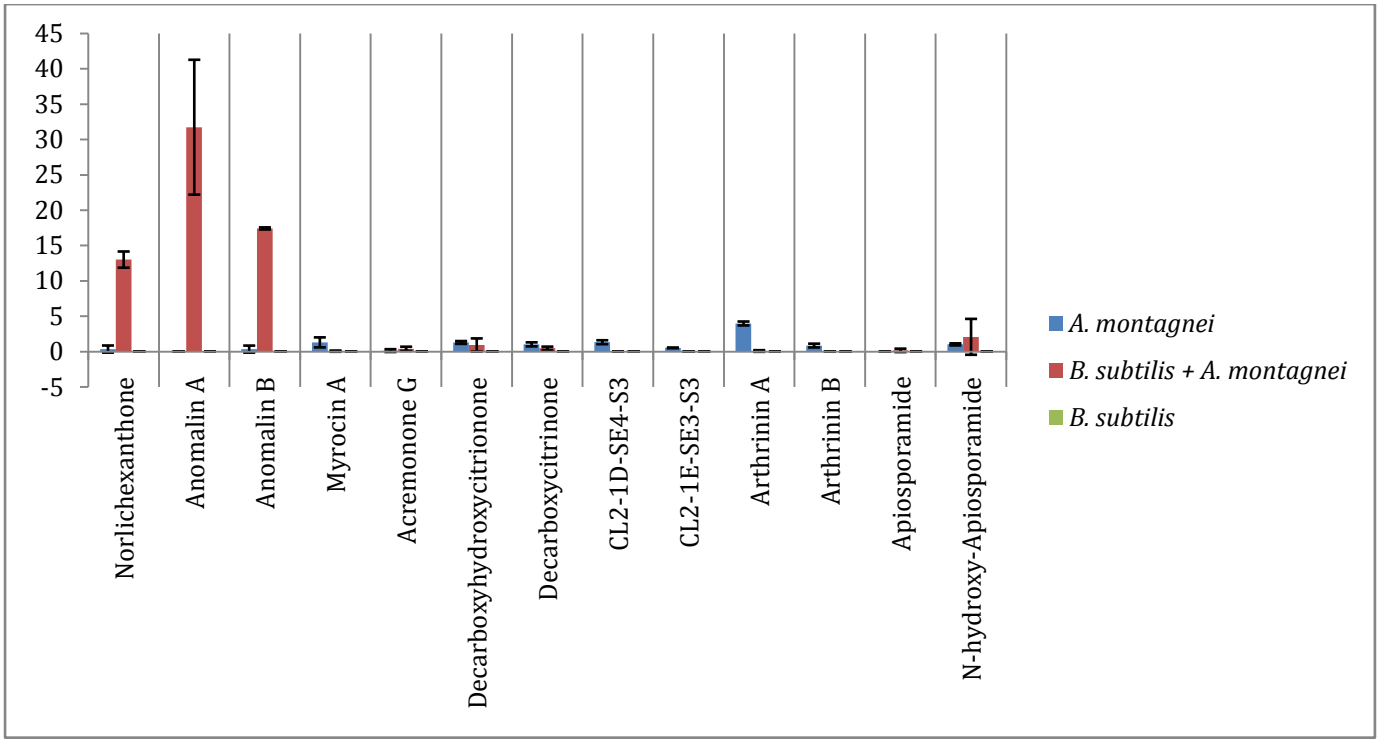


Figure 4.9: Comparison of Mean Peak Areas (%) of Detected Compounds (*B. subtilis* + *A. montagnei*Co-cultivation Experiment)

Table 4.3: Compounds Stimulated by Co-cultivation of fungus with *B. subtilis*

Identified compounds	***A. montagnei*** **(Earliest culture)**	***A. montagnei* A**	***A. montagnei*B**	***B. subtilis*** **+** ***A. montagnei* A**	***B. subtilis*** **+** ***A. montagnei* B**	***B. subtilis* A**	***B. subtilis* B**
Norlichexanthone	Detected	-	Detected	Detected	Detected	-	-
Anomalin A	Detected	-	-	Detected	Detected	-	-
Anomalin B	Detected	Detected	-	Detected	Detected	-	-
Myrocin A	Detected	Detected	Detected	Detected	-	-	-
Acremonone G	Detected	Detected	-	Detected	Detected	-	-
Decarboxyhydroxycitrinone	-	Detected	Detected	Detected	Detected	-	-
Decarboxycitrinone	Detected	Detected	Detected	Detected	Detected	-	-
8-hydroxy-3-methyl-9-oxo-9H-xanthene-1-carboxylic acid methyl ether	-	Detected	Detected	-	-	-	-
Libertellenone G	-	Detected	Detected	-	-	-	-
Arthrinin A	Detected	Detected	Detected	Detected	-	-	-
Arthrinin B	-	Detected	Detected	-	-	-	-
Apiosporamide	Detected	-	-	-	Detected	-	-
N-hydroxy-Apiosporamide	Detected	Detected	Detected	Detected	Detected	-	-

B. CO-CULTIVATION EXPERIMENT OF *A. MONTAGNEI* + *S. LIVIDANS*

From Figure 4.11, it can be seen that co-cultivation of *A. montagnei* and *S. lividans* triggered the production of a new compound by the fungus.

It can be observed in Figure 4.12, that the expression of bioactive metabolites was suppressed or down-regulated by co-culturing *A. montangnei* with *S. lividans.* This co-cultivation tuned off/suppressed the genes expressing some compounds (norlichexanthone and decarboxycitrinone) which were originally expressed by the fungus.

It can also be observed in Table 4.4 that the expression of bioactive metabolites was suppressed or down-regulated by co-culturing *A. montangnei* with *S. lividans.* This co-cultivation tuned off/suppressed the genes expressing some compounds (norlichexanthone and decarboxycitrinone) which were originally expressed by the fungus i.e. *A. montagnei* (earliest culture).

S. lividans ***S. lividans*** + ***A. montagnei*** ***A. montagnei***

Figure 4.10: Flasks showing co-cultivation experiment of *A. montagnei* + *S. lividans*

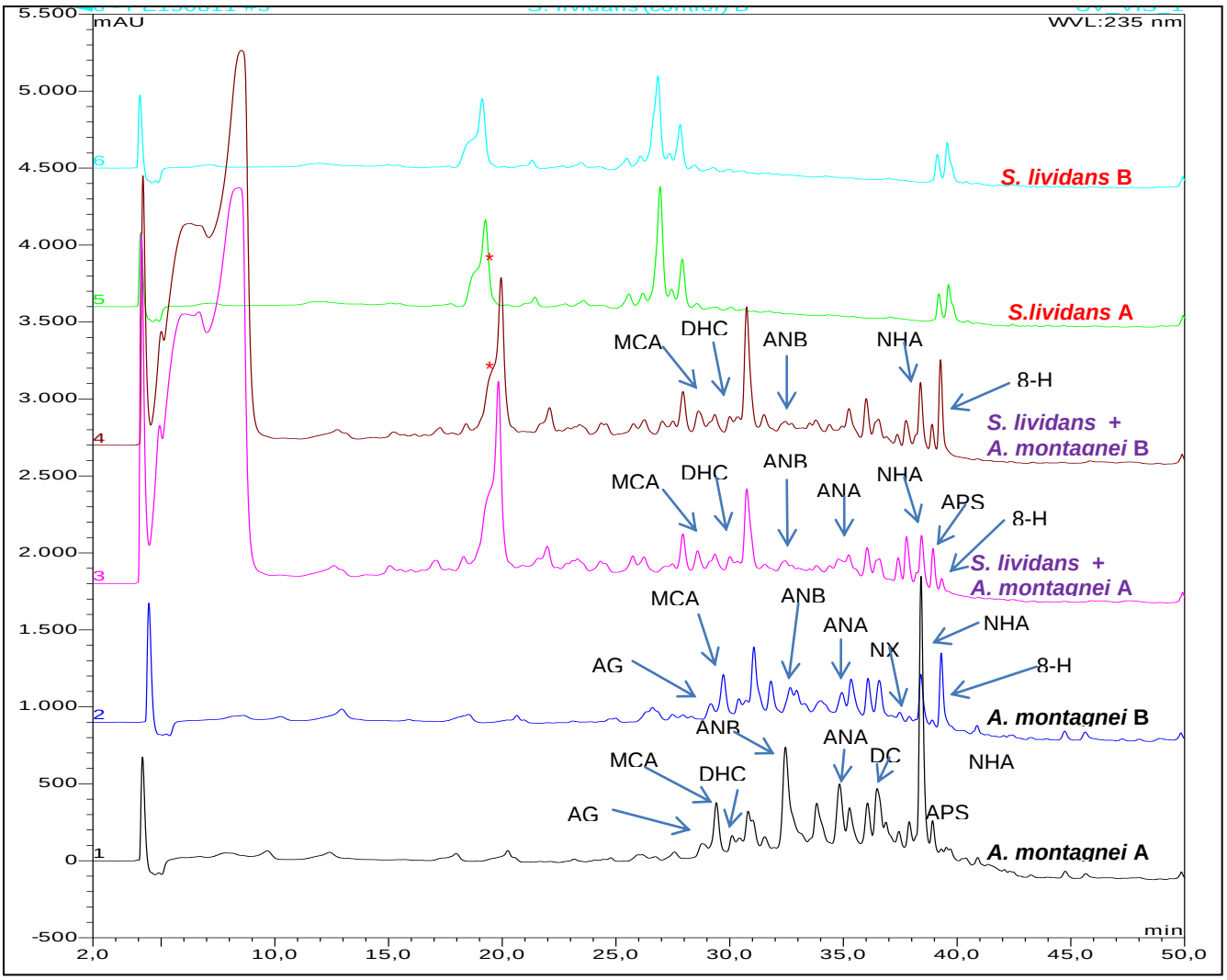


Figure 4.11: Comparison of HPLC Profilesof *S. lividans*, *A. montagnei* and *S. lividans* + *A. montagnei* (*S. lividans* + *A. montagnei* Co-cultivation Experiment)

- APS: Apiosporamide
- AG: Acremonone G
- ANA: Anomalin A
- ANB: Anomalin B
- DC: Decarboxycitrinone
- DHC: Decarboxyhydroxycitrinone
- NHAPS: N-Hydroxy-apiosporamide
- NX: Norlichexanthone
- MCA: Myrocin A
- 8-H: 8-hydroxy-3-methyl-9-oxo-9H-xanthene-1-carboxylic acid methyl ether
- *: New compound detected

NB: The experiment was conducted using duplicate flasks (A and B) for each treatment.

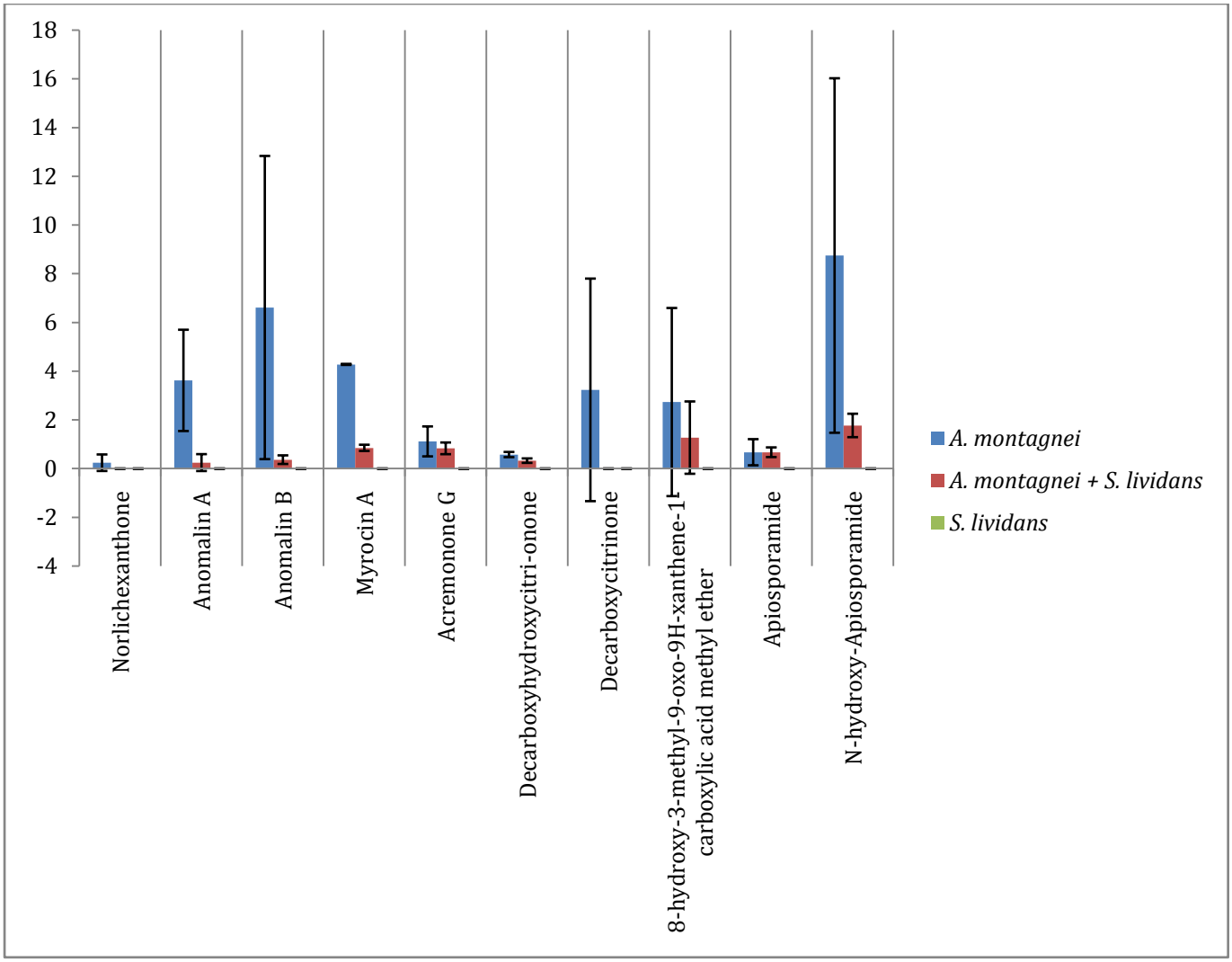


Figure 4.12: Comparison of Mean Peak Areas (%) of Detected Compounds (*S. lividans* + *A. montagnei*Co-cultivation Experiment)

Table 4.4: Compounds Stimulated by Co-cultivation of fungus with *S. lividans*

Compounds	*A. montagnei* (Earliest culture)	*A. montagnei* A (Rice YM Medium)	*A. montagnei*B (Rice YM Medium)	*A. montagnei* + *S. lividans* A	*A. montagnei* + *S. lividans* B	*S. lividans* A	*S. lividans* B
Norlichexanthone	Detected	-	Detected	-	-	-	-
Anomalin A	Detected	Detected	Detected	Detected	-	-	-
Anomalin B	Detected	Detected	Detected	Detected	Detected	-	-
Myrocin A	Detected	Detected	Detected	Detected	Detected	-	-
Acremonone G	Detected	Detected	Detected	Detected	Detected	-	-
Decarboxyhydroxycitrinone	-	Detected	Detected	Detected	Detected	-	-
Decarboxycitrinone	Detected	Detected	-	-	-	-	-
8-hydroxy-3-methyl-9-oxo-9H-xanthene-1-carboxylic acid methyl ether	-	-	Detected	Detected	Detected	-	-
Apiosporamide	Detected	Detected	Detected	Detected	Detected	-	-
N-hydroxy-Apiosporamide	Detected	Detected	Detected	Detected	Detected	-	-

4.1.2.2 MANIPULATION OF CULTURAL CONDITIONS AND MEDIA ENGINEERING

Physical characteristics of flasks during and after completion of fermentation in the experiments for the manipulation of cultural conditions and media engineering are presented in Table 4.5.

In the experiment for the manipulation of cultural conditions of *A. montagnei*, the fungus was fermented in Rice Medium at different temperatures of 22^{o}C and 28^{o}C. The overlay of the HPLC chromatograms/profiles of the treatments and controls in this experiment are presented for comparisons in Figure 4.13, where the presence or absence of peaks of detected compounds resulting from the effects of cultivating the fungus at different temperatures are shown and compared. Also, graphs showing the peak areas (%) of compounds detected by the HPLC analysis of the treatments and controls in this experiment are presented in Figure 4.14. The graph reveals and also compares of the quantity/degree of expression of the compounds detected for the treatments and controls which were cultivated at different temperatures. The stimulation/expression of compounds/secondary metabolites as a result of the effects of cultivating the fungus at different temperatures are presented in Table 4.6.

In the media engineering experiments on *A. montagnei*, the fungus was fermented in several flasks containing Rice Medium supplemented with sodium salts (5% of NaCl, NaI, and NaBr respectively); carbon sources (4% of glucose, sucrose, and rhamnose respectively); nitrogen sources (1% of peptone, yeast extract, corn steep liquor and ammonium chloride respectively).

The overlay of the HPLC chromatograms/profiles of the treatments and controls in the media engineering experiments are presented for comparisons in Figures 4.15(effects of sodium salts), 4.17 (effects of carbon sources), and 4.19 (effects of nitrogen sources). These

chromatogram overlays show and compare the presence or absence of peaks of detected compounds for the treatments and controls.

Also, graphs showing the peak areas (%) of compounds detected by the HPLC analysis of the treatments and controls in the media engineering experiments are presented in Figures 4.16 (effects of sodium salts), 4.18 (effects of carbon sources), and 4.20(effects of nitrogen sources). These graphs reveal and also compare the quantity/degree of expression of the compounds detected for the treatments and control.

The stimulation of the expression of compounds/secondary metabolites by *A. montagnei*as a result of supplementing the fungal medium with different chemical agents are presented in Tables 4.7(effects of sodium salts), 4.8(effects of carbon sources), and 4.9 (effects of nitrogen sources).

Table 4.5: Physical Characteristics of *A. montagnei* Fermentation Flasks during and at the Completion of Fermentation

Parameters studied		Physical observation	
		Coloration	Growth Rate
Sodium Salts(5%)	NaCl	Grey	Slow when compared to controls
	NaI	Dark brown	Growth rate was inhibited and was slowest compared to other sodium salts and controls
	NaBr	Light pink	Slower when compared to controls
Temperature	22^{o}C	Grey	Slower compared to growth at 28^{o}C
	28^{o}C	Grey	Faster compared to growth at 22^{o}C
Carbon sources (4%)	Glucose	Grey	Similar to sucrose and controls, but faster than rhamnose
	Sucrose	Grey	Similar to glucose and controls, but faster than rhamnose
	Rhamnose	Pink	Growth rate was inhibited and was slowest compared to other carbon sources and controls
Nitrogen sources (1%)	Peptone	Grey	Growth rate was almost same as the controls
	Yeast Extract	Grey	Growth rate was almost same as the controls
	Corn Steep Liquor	Grey	Growth rate was almost same as the controls
	Ammonium chloride	Red	Growth rate was almost same as the controls
Controls	-	Grey	-

A. EFFECTS OF TEMPERATURES

It can be observed from Figure 4.13 that the cultivation of *A. montagnei* at 22 ^{o}C resulted to the expression of certain compounds (decarboxyhydroxycitrinone, 8-hydroxy-3-methyl-9-oxo-9H-xanthene-1-carboxylic acid methyl ether). These compounds were however not expressed when the fungus was cultivated at 28 ^{o}C. Also, 28 ^{o}C, the fungus expressed some compounds (anomalin A, anomalin B and norlichexanthone) which were not expressed when it was cultivated at 22 ^{o}C.

It can also be observed from Figure 4.14 that the cultivation of *A. montagnei* at 22 ^{o}C resulted to the expression of certain compounds (decarboxyhydroxycitrinone, 8-hydroxy-3-methyl-9-oxo-9H-xanthene-1-carboxylic acid methyl ether, and CL2-1E-SE5-S4). These compounds were however not expressed when the fungus was cultivated at 28 ^{o}C. Also, at 28 ^{o}C, the fungus expressed some compounds (anomalin A, anomalin B and norlichexanthone) which were not expressed when it was cultivated at 22 ^{o}C.

Table 4.6 shows that cultivation of the fungus at 28 ^{o}C induced the expression of the fungal silent genes which codes for the expression of the three compounds: anomalin A, anomalin B, and norlichexanthone. It can also be seen that at these two temperatures, other compounds which originally were expressed by the fungus (i.e. earliest cultures) where not synthesized (the genes expressing these compounds were silent/turned off). These compounds include, myrocin A, acremonone G, decarboxycitrinone, apiosporamide, and N-hydroxy-apiosporamide.

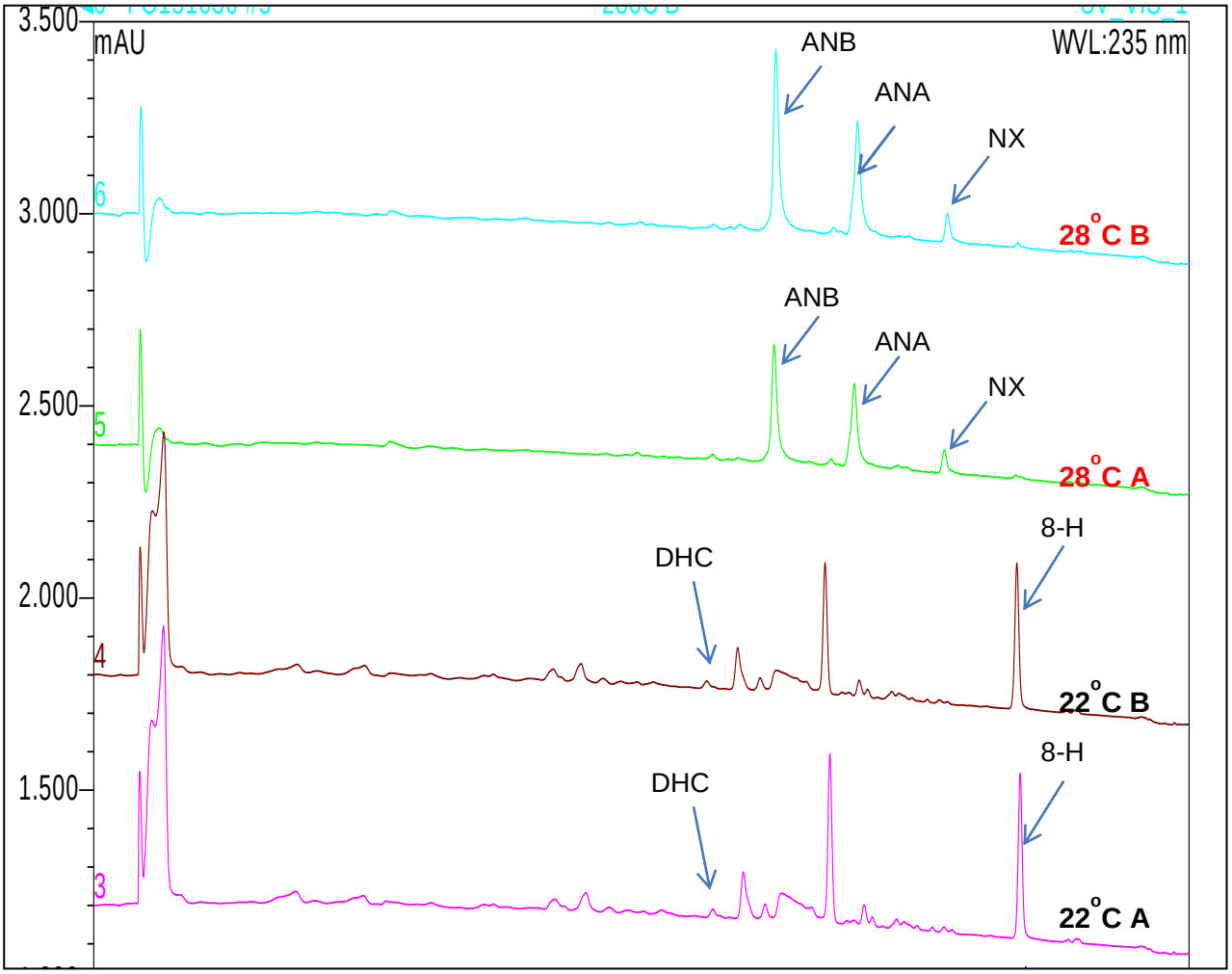


Figure 4.13: Comparison of HPLC Profiles of *A. montagnei* (Effects of Temperatures)

- ANA: Anomalin A
- ANB: Anomalin B
- DHC:Decarboxyhydroxycitrinone
- NX: Norlichexanthone
- 8-H: 8-hydroxy-3-methyl-9-oxo-9H-xanthene-1-carboxylic acid methyl ether

NB: The experiment was conducted using duplicate flasks (A and B) for each treatment.

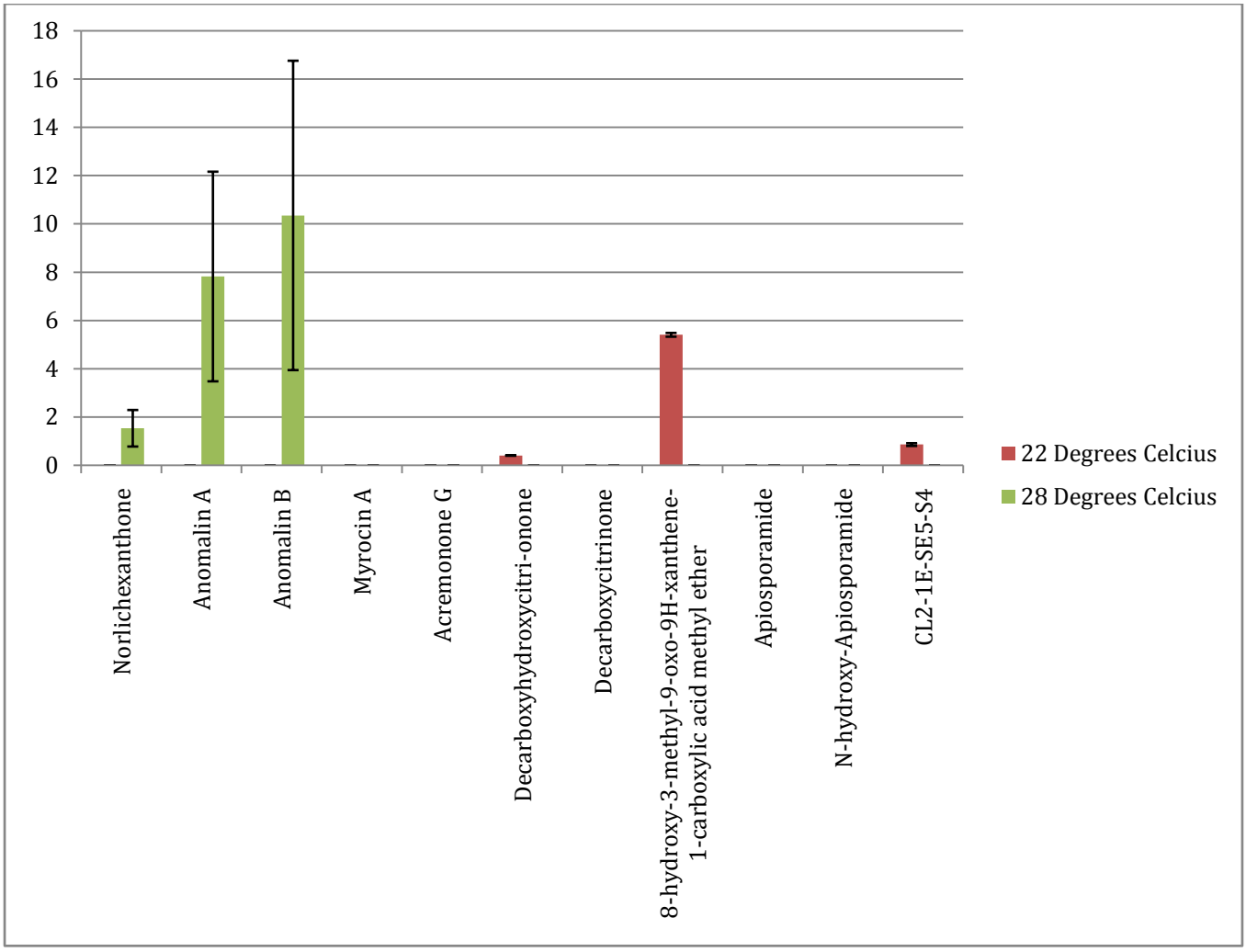


Figure 4.14: Comparison of Mean Peak Areas (%) of Detected Compounds (Effects of Temperatures)

Table 4.6: Compounds Stimulated by Cultivation of *A. montagnei* at Different Temperatures

Compounds	*A. montagnei* (Earliest culture)	22°C A	22°C B	28°CA	28°C B
Norlichexanthone	Detected	-	-	Detected	Detected
Anomalin A	Detected	-	-	Detected	Detected
Anomalin B	Detected	-	-	Detected	Detected
Myrocin A	Detected	-	-	-	-
Acremonone G	Detected	-	-	-	-
Decarboxyhydroxycitrinone	-	Detected	Detected	-	-
Decarboxycitrinone	Detected	-	-	-	-
8-hydroxy-3-methyl-9-oxo-9H-xanthene-1-carboxylic acid methyl ether	-	Detected	Detected	-	-
Apiosporamide	Detected	-	-	-	-
N-hydroxy-Apiosporamide	Detected	-	-	-	-
CL2-1E-SE5-S4	-	Detected	Detected	-	-

B. EFFECTS OF SODIUM SALTS

It can be observed in Figure 4.15 that the addition of different sodium salts (5% w/v) resulted in the expression of silent fungal genes. Of all the sodium salts, only NaCl induced the production of a new compound.

It can be observed in Figure 4.16 that NaCl induced the expression of silent genes for the following compounds: norlichexanthone, anomalin A, anomalin B, myrocin A, decarboxyhydroxycitrinone, 8-hydroxy-3-methyl-9-oxo-9H-xanthene-1-carboxylic acid methyl ether, apiosporamide, and N-hydroxy-Apiosporamide; NaBr induced the expression of silent gene for 8-hydroxy-3-methyl-9-oxo-9H-xanthene-1-carboxylic acid methyl ether; and NaI induced the expression of silent genes for norlichexanthone and apiosporamide.

It can also be seen in Table 4.7 that NaCl induced the expression of silent genes for the following compounds: norlichexanthone, anomalin A, anomalin B, myrocin A, decarboxyhydroxycitrinone, 8-hydroxy-3-methyl-9-oxo-9H-xanthene-1-carboxylic acid methyl ether, apiosporamide, and N-hydroxy-apiosporamide; NaBr induced the expression of silent gene for 8-hydroxy-3-methyl-9-oxo-9H-xanthene-1-carboxylic acid methyl ether; and NaI induced the expression of silent genes for norlichexanthone and apiosporamide.

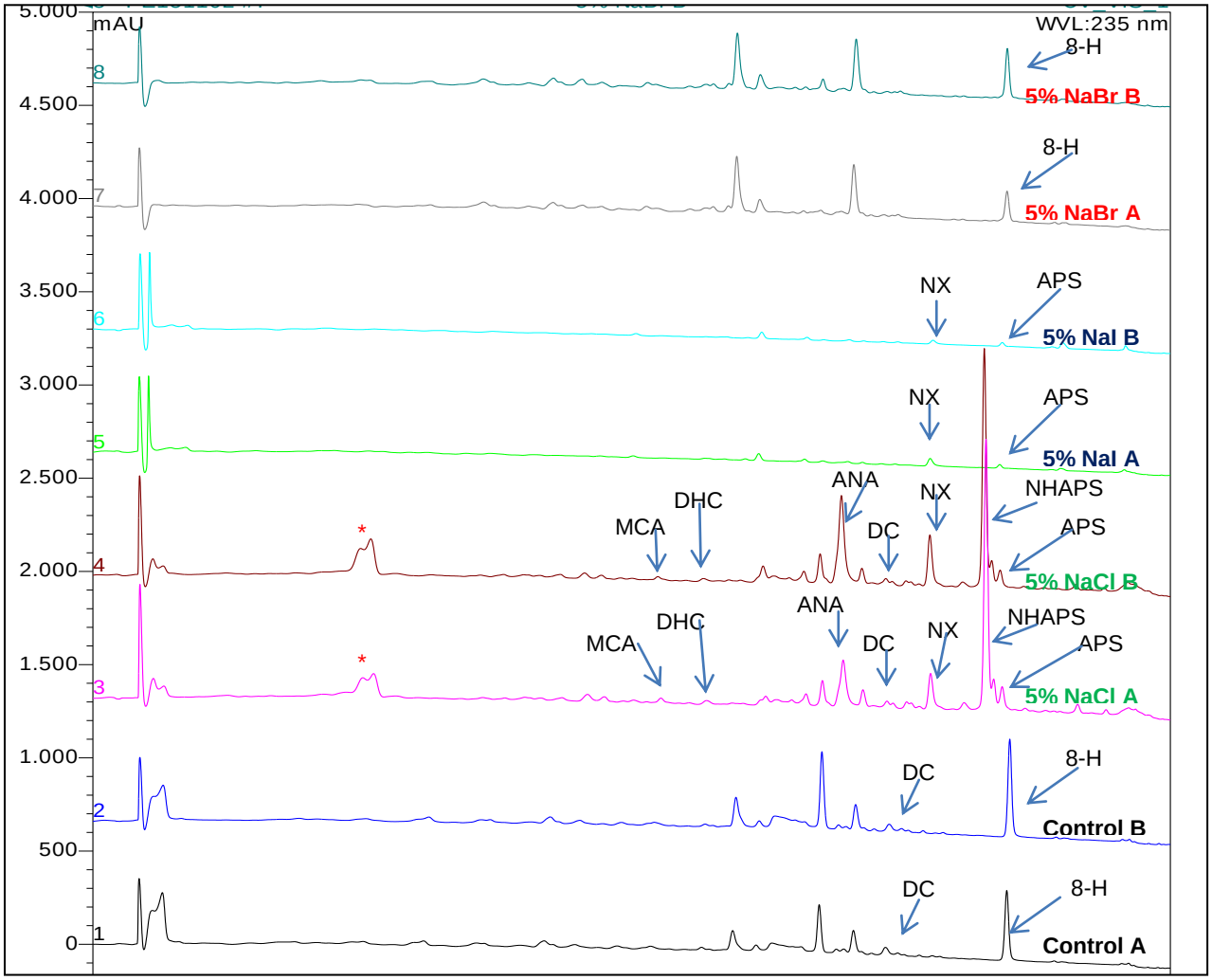


Figure 4.15: Comparison of HPLC Profilesof *A. montagnei* (Effects of Sodium Salts)

- APS:Apiosporamide
- ANA: Anomalin A
- ANB: Anomalin B
- DC: Decarboxycitrinone
- DHC: Decarboxyhydroxycitrinone
- NHAPS: N-Hydroxy-apiosporamide
- NX: Norlichexanthone
- MCA: Myrocin A
- 8-H: 8-hydroxy-3-methyl-9-oxo-9H-xanthene-1-carboxylic acid methyl ether
- * : New compound detected
- NB: The experiment was conducted using duplicate flasks (A and B) for each treatment.

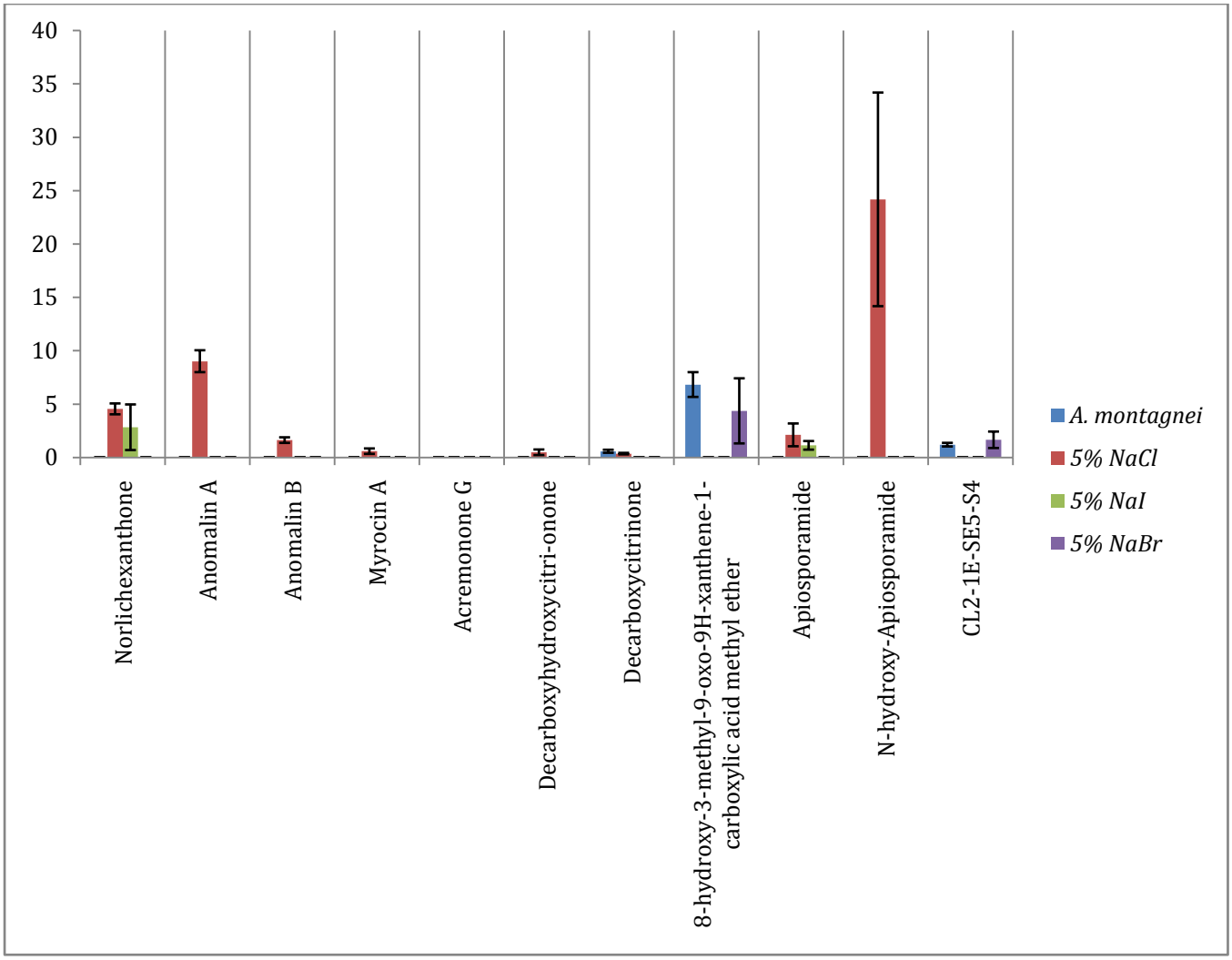


Figure 4.16: Comparison of Mean Peak Areas (%) of Detected Compounds (Effects of Sodium Salts)

Table 4.7: Compounds Stimulated by Cultivation of *A. montagnei*in Medium Containing Different Sodium Salts

Compounds	*A. montagnei* (Earliest culture)	*A. montagnei* A	*A. montagnei* B	5%NaCl A	5%NaCl B	5%NaIA	5%NaI B	5%NaBrA	5%NaBr B
Norlichexanthone	Detected	-	-	Detected	Detected	Detected	Detected	-	-
Anomalin A	Detected	-	-	Detected	Detected	-	-	-	-
Anomalin B	Detected	-	-	Detected	Detected	-	-	-	-
Myrocin A	Detected	-	-	Detected	Detected	-	-	-	-
Acremonone G	Detected	-	-			-	-	-	-
Decarboxyhydroxycitrinone	-	-	-	Detected	Detected	-	-	-	-
Decarboxycitrinone	Detected	Detected	Detected	Detected	Detected	-	-	-	-
8-hydroxy-3-methyl-9-oxo-9H-xanthene-1-carboxylic acid methyl ether	-	Detected	Detected	-	-	-	-	Detected	Detected
Apiosporamide	Detected	-	-	Detected	Detected	Detected	Detected	-	-
N-hydroxy-Apiosporamide	Detected	-	-	Detected	Detected	-	-	-	-
CL2-1E-SE5-S4	-	Detected	Detected	-	-	-	-	Detected	Detected

C. EFFECTS OF CARBON SOURCES

It can be observed in Figure 4.17 that the addition of the various carbon sources (4% w/v) had no much effect on the metabolism of the fungus, as the HPLC profile of the fungus supplemented with these carbon sources were similar to that of the fungus control. Also, none of the carbon sources induced the production of new compounds.

In Figure 4.18it can be seen that three carbon sources stimulated the up-regulation of the fungal gene products. Addition of glucose and sucrose resulted in the up-regulation of genes expressing two compounds, decarboxycitrinone and 8-hydroxy-3-methyl-9-oxo-9H-xanthene-1-carboxylic acid methyl ether. Of all the three carbon sources, the addition of sucrose resulted to the highest degree of up-regulation of gene expression for decarboxycitrinone, and especially 8-hydroxy-3-methyl-9-oxo-9H-xanthene-1-carboxylic acid methyl ether (which recorded the highest percent mean peak area). Also, sucrose triggered the expression of silent fungal genes coding for norlichexanthone and decarboxyhydroxycitrinone; and rhamnose triggered the expression of silent fungal genes coding for norlichexanthone, anomalin A, anomalin B, and decarboxyhydroxycitrinone.

It can also be seen in Table 4.8 that the addition of sucrose triggered the expression of silent fungal genes coding for norlichexanthone and decarboxyhydroxycitrinone; and rhamnose triggered the expression of silent fungal genes coding for norlichexanthone, anomalin A, anomalin B, and decarboxyhydroxycitrinone.

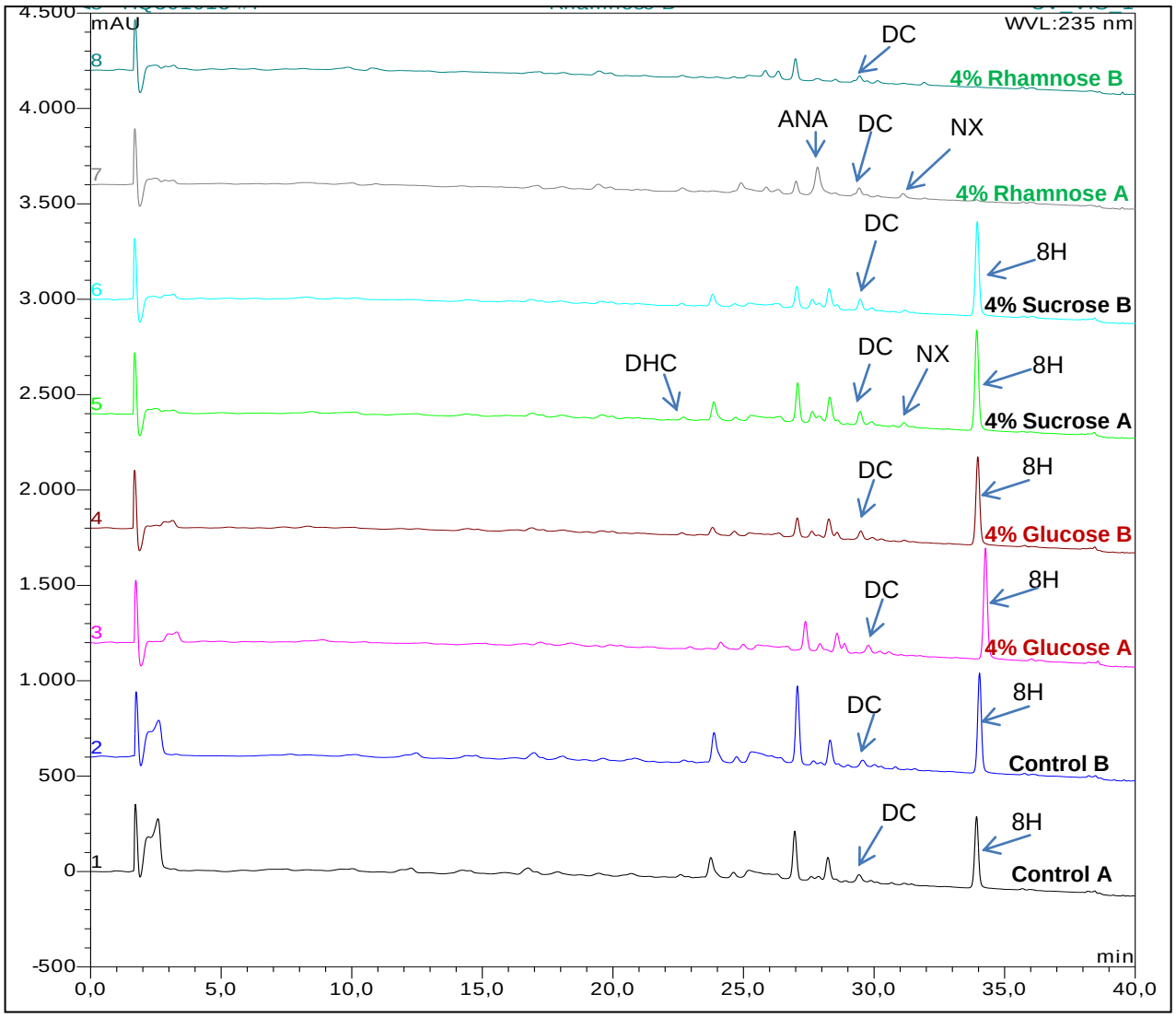


Figure 4.17: Comparison of HPLC Profiles of *A. montagnei* (Effects of Carbon Sources)

- APS:Apiosporamide
- ANA: Anomalin A
- ANB: Anomalin B
- DC: Decarboxycitrinone
- DHC: Decarboxyhydroxycitrinone
- NHAPS: N-Hydroxy-apiosporamide
- NX: Norlichexanthone
- MCA: Myrocin A
- 8-H: 8-hydroxy-3-methyl-9-oxo-9H-xanthene-1-carboxylic acid methyl ether

NB: The experiment was conducted using duplicate flasks (A and B) for each treatment.

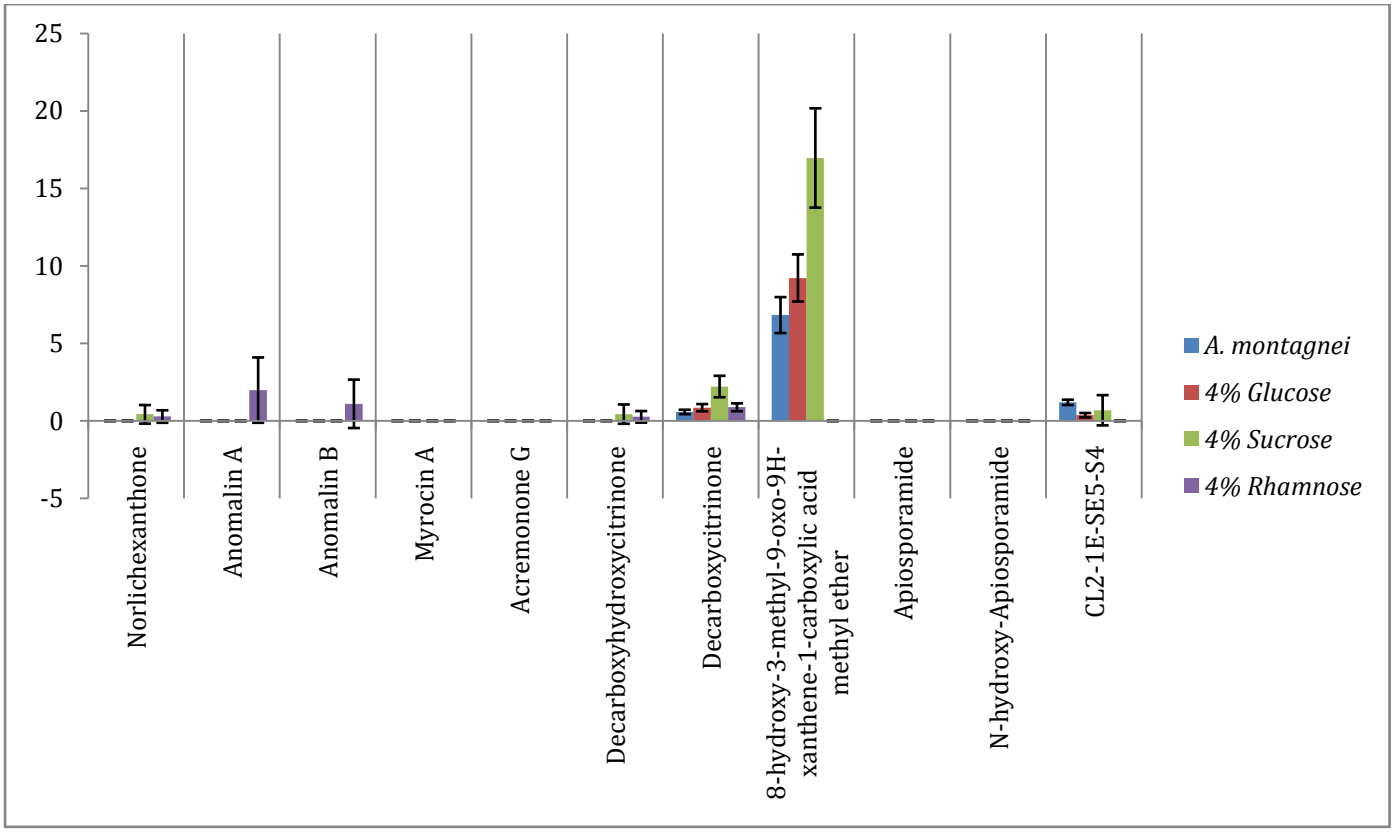


Figure 4.18: Comparison of Mean Peak Areas (%) of Detected Compounds (Effects of Carbon Sources)

Table 4.8: Compounds Stimulated by Cultivation of *A. montagnei*in Medium Containing Different Carbon Sources

Compounds	*A. montagnei* (Earliest culture)	*A. montagnei* A	*A. montagnei* B	4% Glucose A	4% Glucose B	4% Sucrose A	4% Sucrose B	4% Rhamnose A	4% Rhamnose B
Norlichexanthone	Detected	-	-	-	-	Detected	-	Detected	-
Anomalin A	Detected	-	-	-	-	-	-	Detected	Detected
Anomalin B	Detected	-	-	-	-	-	-	Detected	-
Myrocin A	Detected	-	-	-	-	-	-	-	-
Acremonone G	Detected	-	-	-	-	-	-	-	-
Decarboxyhydroxycitrinone	-	-	-	-	-	Detected	-	Detected	-
Decarboxycitrinone	Detected	Detected	Detected	Detected	Detected	Detected	Detected	Detected	Detected
8-hydroxy-3-methyl-9-oxo-9H-xanthene-1-carboxylic acid methyl ether	-	Detected	Detected	Detected	Detected	Detected	Detected	-	-
Apiosporamide	Detected	-	-	-	-	-	-	-	-
N-hydroxy-Apiosporamide	Detected	-	-	-	-	-	-	-	-
CL2-1E-SE5-S4	-	Detected	Detected	Detected	Detected	Detected	-	-	-

D. EFFECTS OF NITROGEN SOURCES

It can be observed from Figure 4.19 that the addition of the some of the nitrogen sources triggered the expression of silent fungal genes. Corn steep liquor (CSL) and yeast extract (YE) both stimulated the expression of silent fungal genes for similar compounds (norlichexanthone, anomalin A, and decarboxyhydroxycitrinone). Addition of NH_4Cl induced the production of new compounds. Peptone had no significant effect on the metabolic profile of the fungus, as it presented similar HPLC profile with the fungus control.

It can also be observed from Figure 4.20 that the addition of CSL and YE both stimulated the expression of silent fungal genes for similar compounds (norlichexanthone, anomalin A, and decarboxyhydroxycitrinone). However, YE had more effect on the yield or degree of expression of the three compounds (with higher percent mean peak areas). Addition of NH_4Cl induced the up-regulated gene expression for one only compound (CL2-1E-SE5-S4). Addition of peptone did not stimulate the expression of the fungal silent genes, or the up-regulation of expressed compounds, nor did it induce the production of new compounds.

Table 4.9 shows that the addition of CSL and YE both stimulated the expression of silent fungal genes for similar compounds (norlichexanthone, anomalin A, and decarboxyhydroxycitrinone). However, YE had more effect on the yield or degree of expression of the three compounds (with higher percent mean peak areas). NH_4Cl did not stimulate the expression of silent fungal genes. Also, the addition of peptone had no significant effect as it did not stimulate the expression of the fungal silent genes, or the up-regulation of expressed compounds, nor did it induce the production of new compounds.

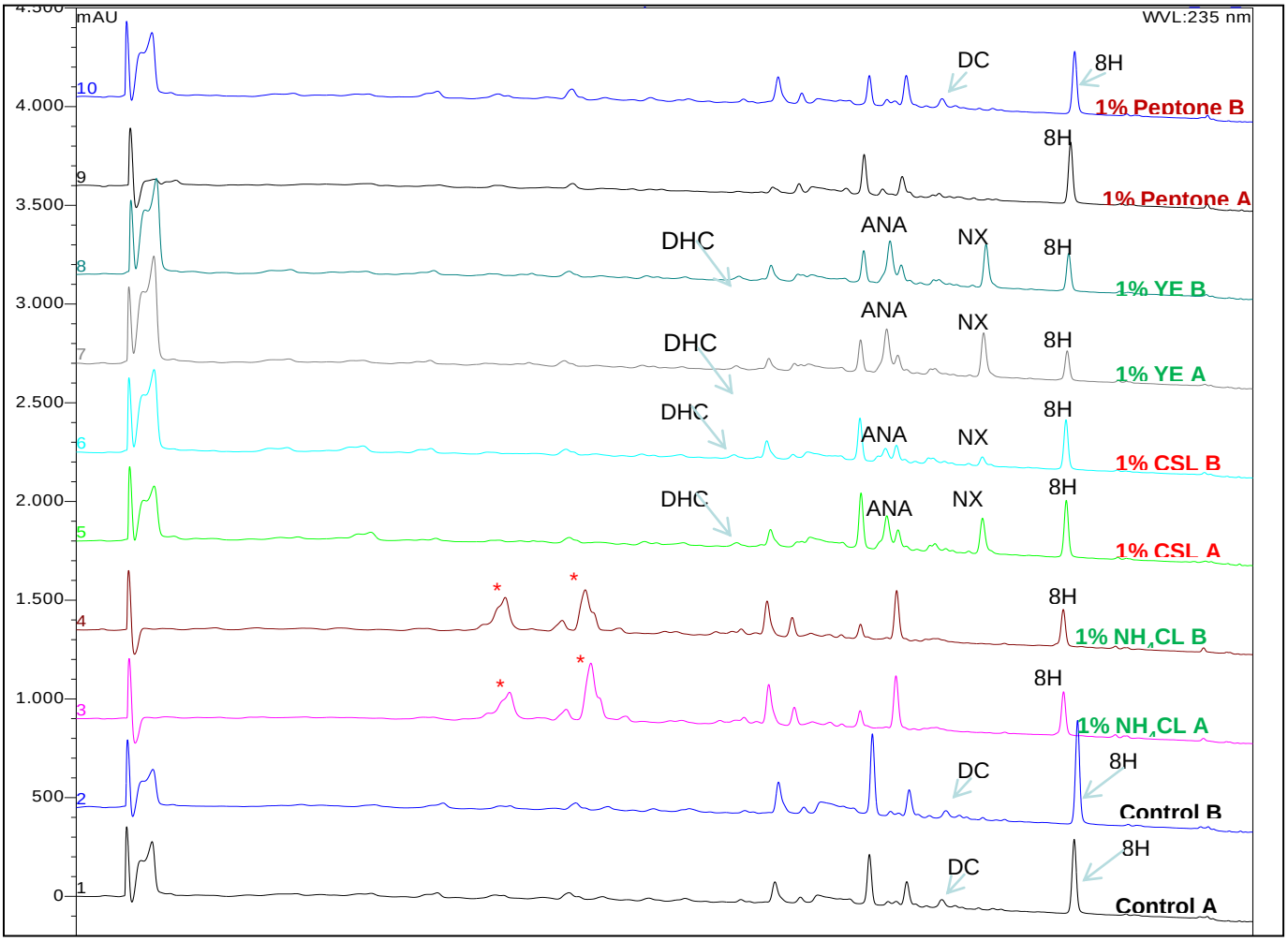


Figure 4.19: Comparison of HPLC Profiles of *A. montagnei* (Effects of Nitrogen Sources)

- ANA: Anomalin A
- ANB: Anomalin B
- DC: Decarboxycitrinone
- DHC:Decarboxyhydroxycitrinone
- NX: Norlichexanthone
- 8-H: 8-hydroxy-3-methyl-9-oxo-9H-xanthene-1-carboxylic acid methyl ether
- YE: Yeast Extract
- CSL: Corn Steep Liquor
- NH_4Cl: Ammonium Chloride
- *: New compound detected

NB: The experiment was conducted using duplicate flasks (A and B) for each treatment.

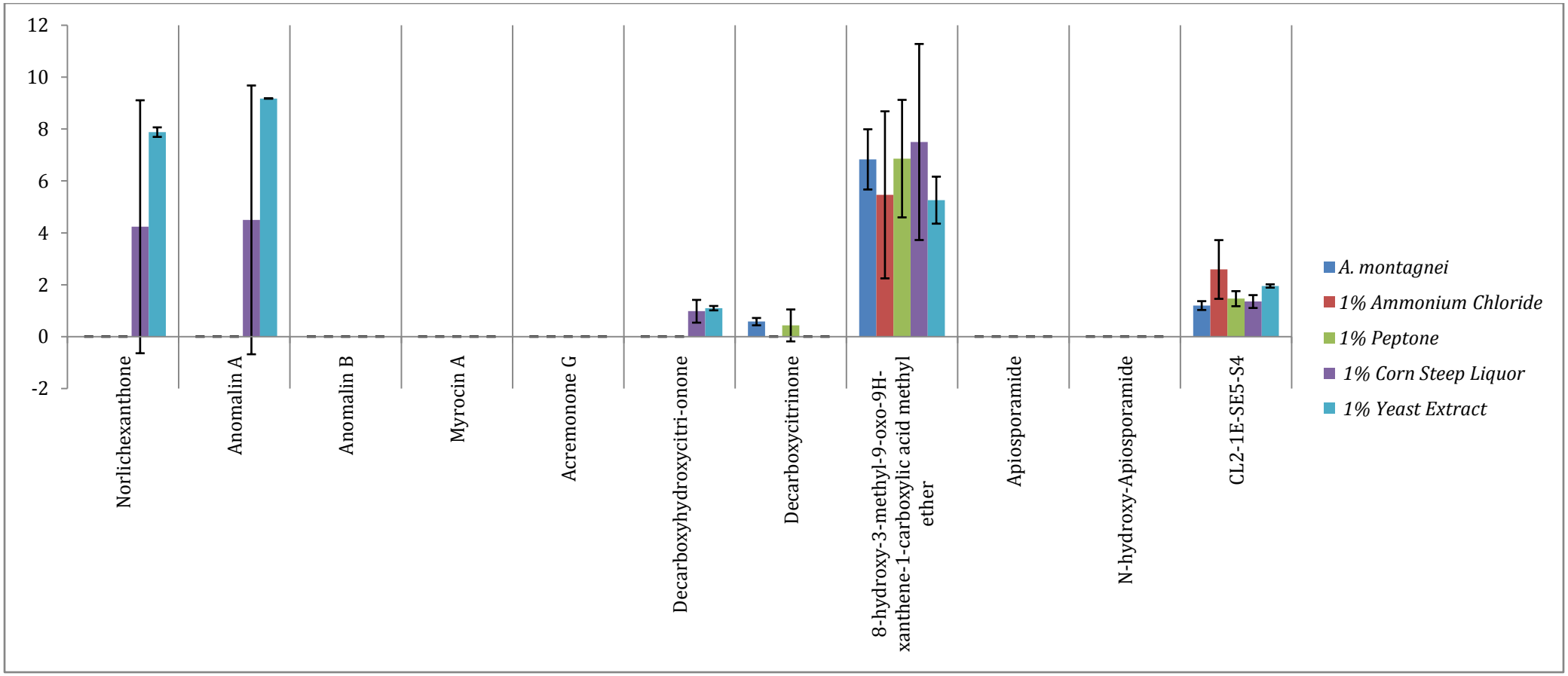


Figure 4.20: Comparison of Mean Peak Areas (%) of Detected Compounds (Effects of Nitrogen Sources)

Table 4.9: Compounds Stimulated by Cultivation of *A. montagnei* in Medium Containing Different Nitrogen Sources

Compounds	*A. montagnei* (Earliest culture)	*A. montagnei* A	*A. montagnei* B	1% NH_4Cl A	1% NH_4Cl B	1% Peptone A	1% Peptone B	1% CSL A	1% CSL B	1% YE A	1% YE B
Norlichexanthone	Detected	-	-	-	-	-	-	Detected	Detected	Detected	Detected
Anomalin A	Detected	-	-	-	-	-	-	Detected	Detected	Detected	Detected
Anomalin B	Detected	-	-	-	-	-	-	-	-	-	-
Myrocin A	Detected	-	-	-	-	-	-	-	-	-	-
Acremonone G	Detected	-	-	-	-	-	-	-	-	-	-
Decarboxyhydroxycitrinone	-	-	-	-	-	-	-	Detected	Detected	Detected	Detected
Decarboxycitrinone	Detected	Detected	Detected	-	-	-	Detected	-	-	-	-
8-hydroxy-3-methyl-9-oxo-9H-xanthene-1-carboxylic acid methyl ether	-	Detected	Detected	Detected	Detected	Detected	Detected	Detected	Detected	Detected	Detected
Apiosporamide	Detected	-	-	-	-	-	-	-	-	-	-
N-hydroxy-Apiosporamide	Detected	-	-	-	-	-	-	-	-	-	-
CL2-1E-SE5-S4	-	Detected	Detected	Detected	Detected	Detected	Detected	Detected	Detected	Detected	Detected

4.1.3 ISOLATION OF BIOACTIVE COMPOUNDS FROM *ASPERGILLUS ACULEATUS*

The isolation of bioactive secondary metabolites from *A. aculeatus* followed the steps shown in Figure 4.21. The reported bioactivities of the compounds are presented in Table 4.10.

The structures, HPLC chromatograms, UV spectra, LC-MS data and other data on the compounds isolated from *A. aculeatus*, as well as their ^{1}H-NMR spectra are shown in Figures 4.22 – 4.30.

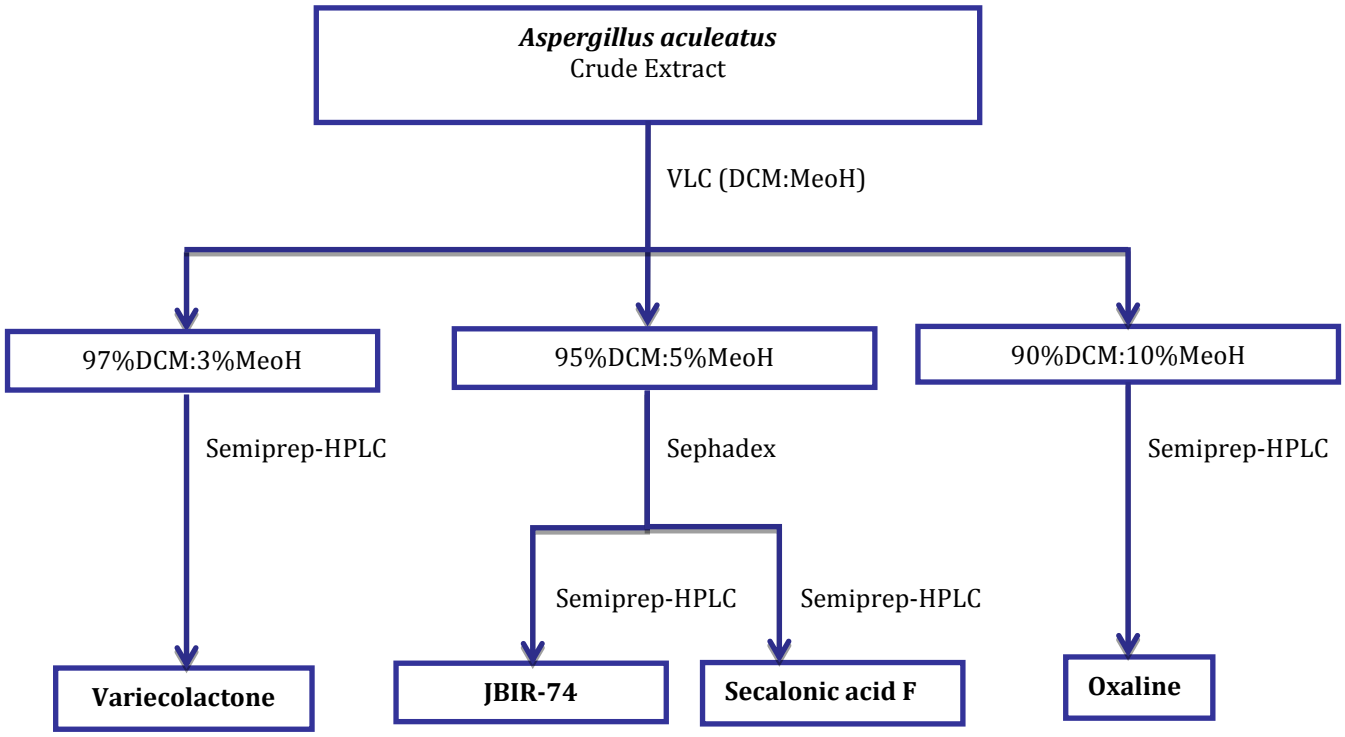


Figure 4.21: Isolation of Compounds from *A. aculeatus* Crude Extract

4.1.3.1 ISOLATED COMPOUNDS FROM *A. ACULEATUS*

A. SECALONIC ACID F

A

Secalonic acid F

Molecular formula: $C_{32}H_{30}0_{14}$
Molecular weight: 638 g/mol
Appearance: Yellowish powder
Absorption maximum: UV λ_{max} (methanol): 336 nm
Quantity isolated: 8 mg

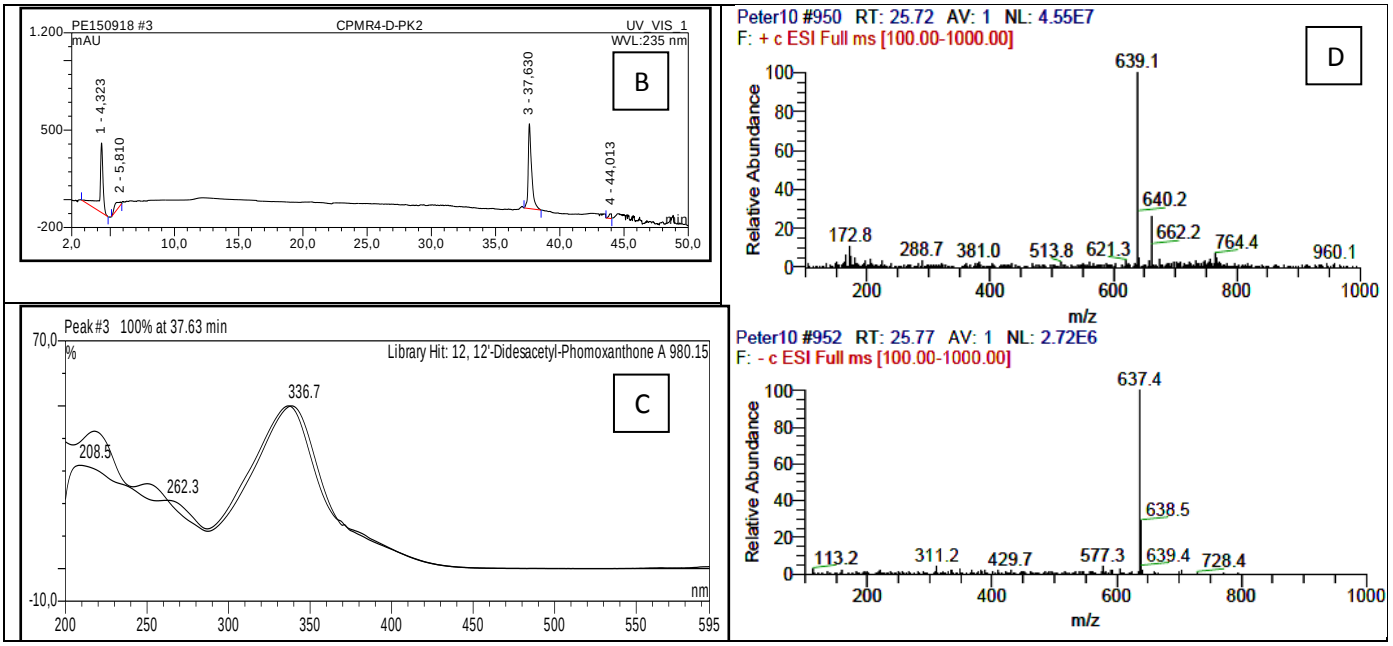


Figure 4.22: Secalonic Acid F: Structure (A), HPLC Chromatogram (B), UV spectrum(C) and LC-MS Data (D)

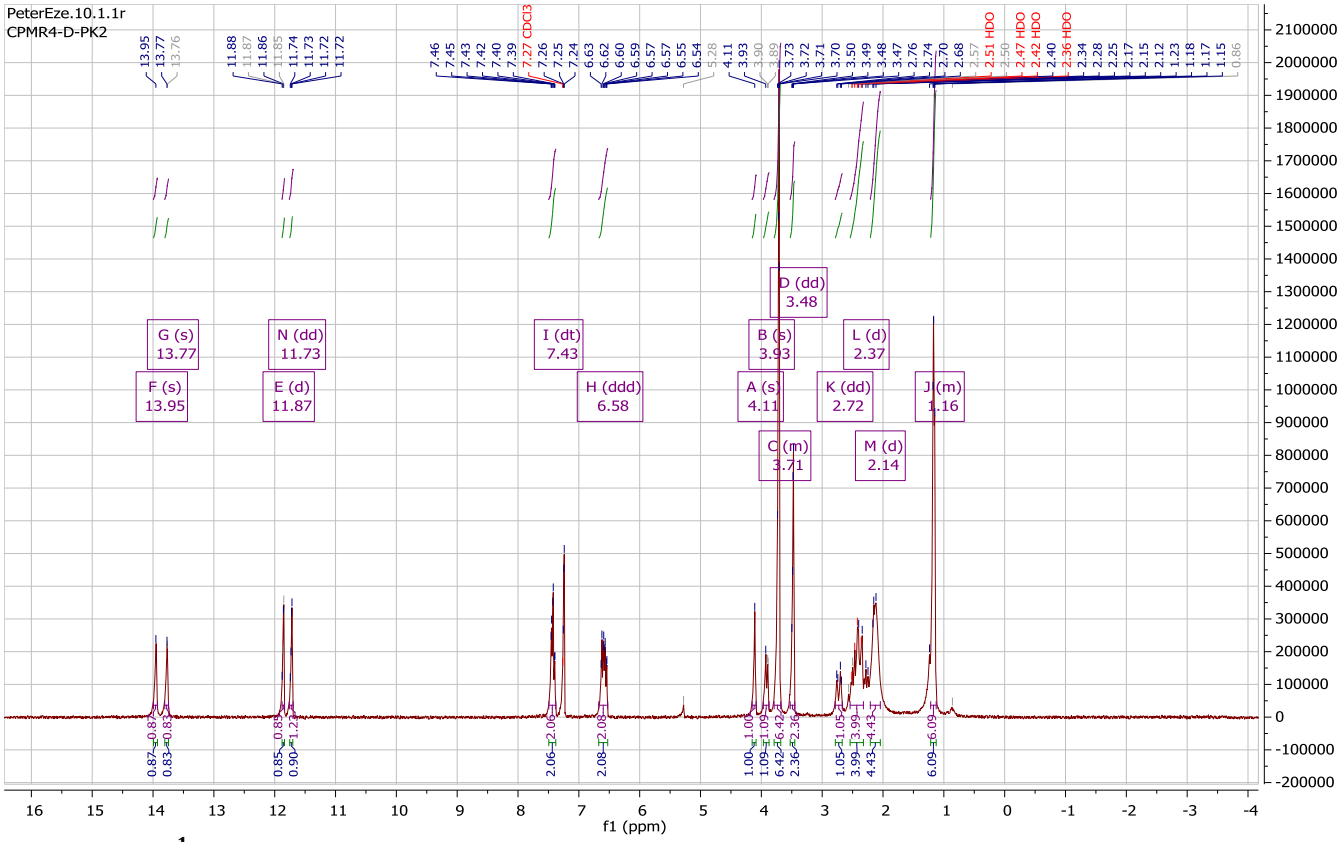


Figure 4.23: ^{1}H-NMR Spectra (300Hz, Chloroform-d)of Secalonic acid F

^{1}H NMR (300 MHz, Chloroform-d) δ 13.95 (s, 1H), 13.77 (s, 1H), 11.87 (d, J = 6.0 Hz, 1H), 11.73 (dd, J = 5.4, 3.0 Hz, 1H), 7.43 (dt, J = 10.7, 5.3 Hz, 2H), 6.58 (ddd, J = 15.6, 9.2, 3.0 Hz, 2H), 4.11 (s, 1H), 3.93 (s, 1H), 3.79 – 3.68 (m, 6H), 3.48 (dd, J = 5.3, 2.8 Hz, 2H), 2.72 (dd, J = 18.1, 5.3 Hz, 1H), 2.37 (d, J = 17.5 Hz, 4H), 2.14 (d, J = 15.4 Hz, 4H), 1.22 – 1.13 (m, 6H).

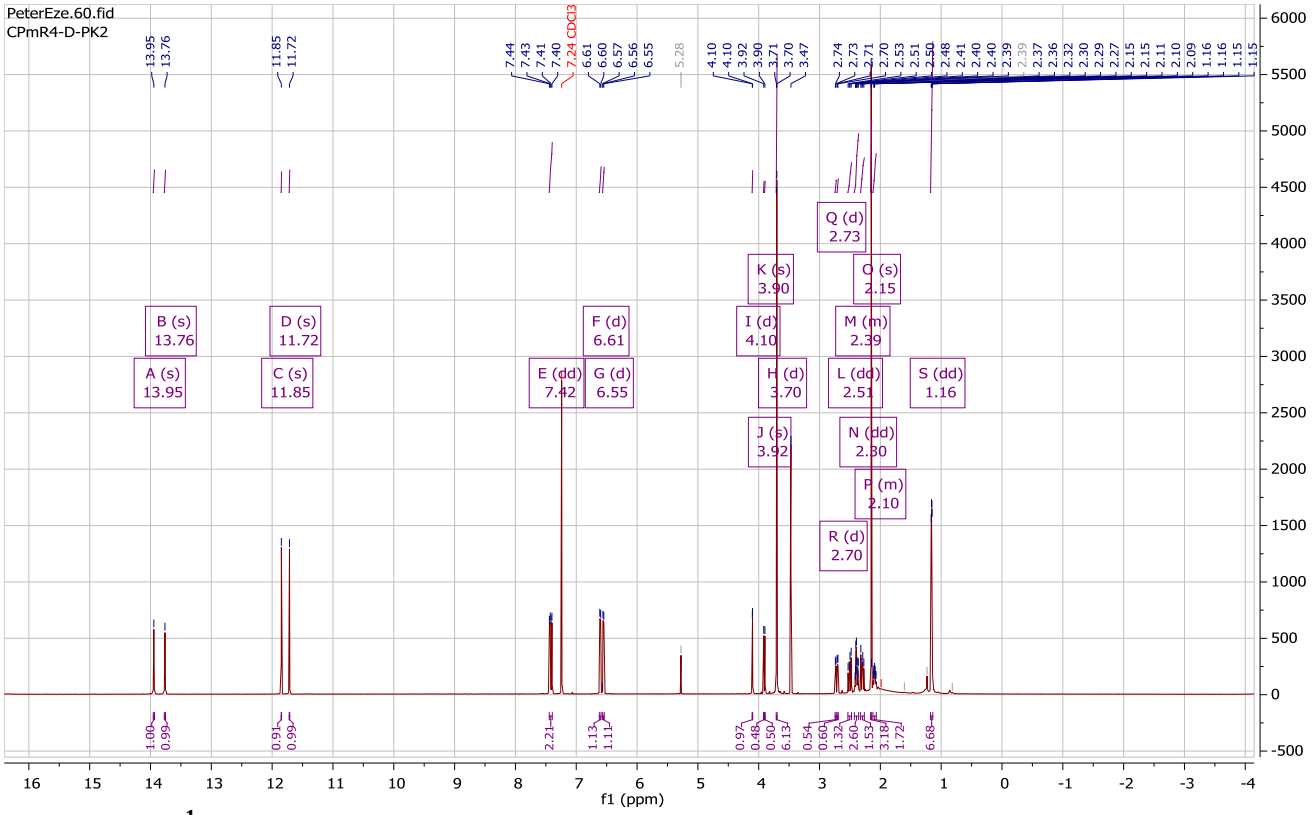


Figure 4.24: ^{1}H-NMR Spectra (600Hz, Chloroform-d) of Secalonic acid F

^{1}H NMR (600 MHz, Chloroform-d) δ 13.95 (s, 1H), 13.76 (s, 1H), 11.85 (s, 1H), 11.72 (s, 1H), 7.42 (dd, J = 16.5, 8.4 Hz, 2H), 6.61 (d, J = 8.5 Hz, 1H), 6.55 (d, J = 8.4 Hz, 1H), 4.10 (d, J = 1.7 Hz, 1H), 3.92 (s, 0H), 3.90 (s, 0H), 3.70 (d, J = 3.4 Hz, 6H), 2.73 (d, J = 6.3 Hz, 1H), 2.70 (d, J = 6.3 Hz, 1H), 2.51 (dd, J = 19.0, 11.3 Hz, 1H), 2.43 – 2.36 (m, 3H), 2.30 (dd, J = 19.2, 10.6 Hz, 2H), 2.15 (s, 3H), 2.12 – 2.07 (m, 2H), 1.16 (dd, J = 6.7, 3.0 Hz, 7H).

B. JBIR-74

A

JBIR-74

Molecular formula: $C_{12}H_{16}N_4O_2$
Molecular weight: 234 g/mol
Appearance: White powder
Absorption maximum: UV λ_{max} (methanol): 231, 298 nm
Quantity isolated: 0.6 mg

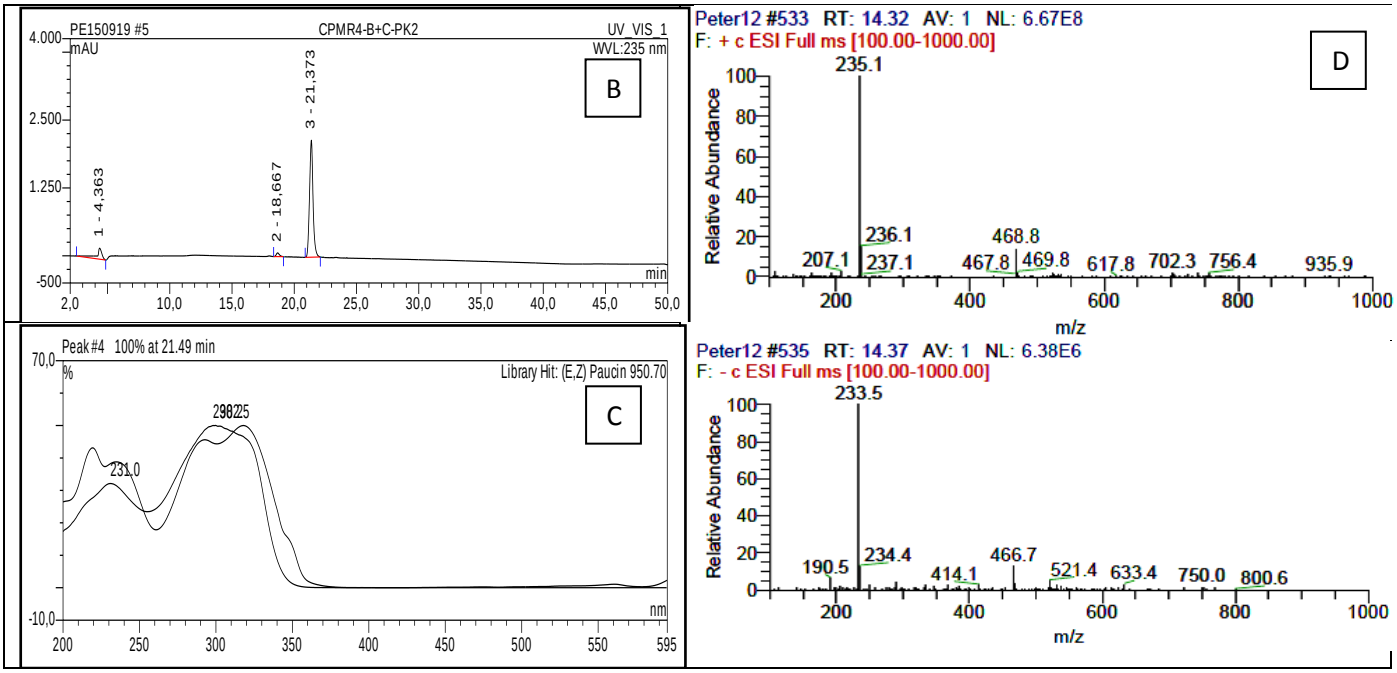


Figure 4.25: JBIR-74: Structure (A), HPLC Chromatogram (B), UV spectrum(C) and LC-MS Data (D)

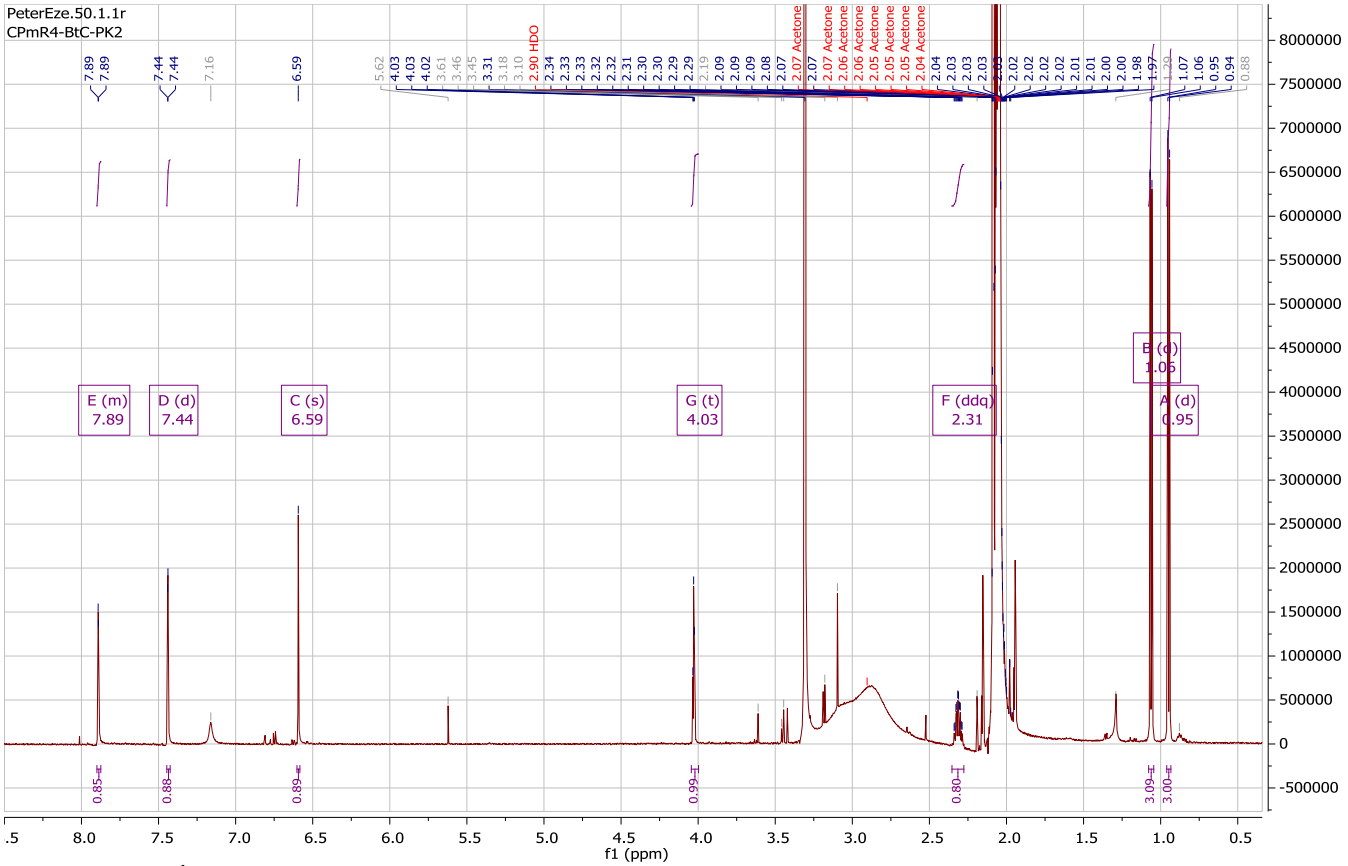


Figure 4.26: ^{1}H-NMR Spectra (600Hz, Acetone-d6) of JBIR-74

^{1}H NMR (600 MHz, Acetone-d6) δ 7.90 – 7.87 (m, 1H), 7.44 (d, J = 1.2 Hz, 1H), 6.59 (s, 1H), 4.03 (t, J = 3.0 Hz, 1H), 2.31 (ddq, J = 10.1, 6.8, 3.5, 3.1 Hz, 1H), 1.06 (d, J = 7.0 Hz, 3H), 0.95 (d, J = 6.8 Hz, 3H).

C. VARIECOLACTONE

A

Variecolactone

Molecular formula: $C_{25}H_{36}O_3$
Molecular weight: 384 g/mol
Appearance: Yellowish powder
Absorption maximum: UV λ_{max} (methanol): 232 nm
Quantity isolated: 4.0 mg

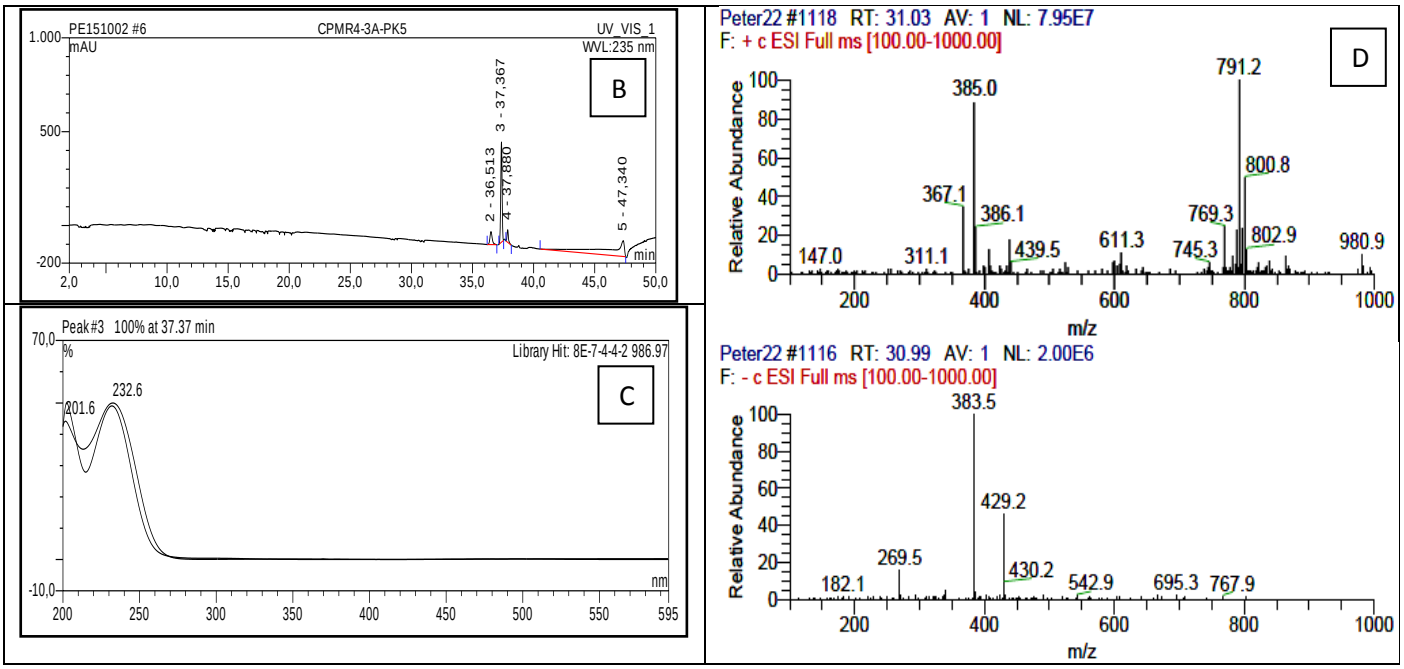


Figure 4.27: Variecolactone: Structure (A), HPLC Chromatogram (B), UV spectrum(C) and LC-MS Data (D)

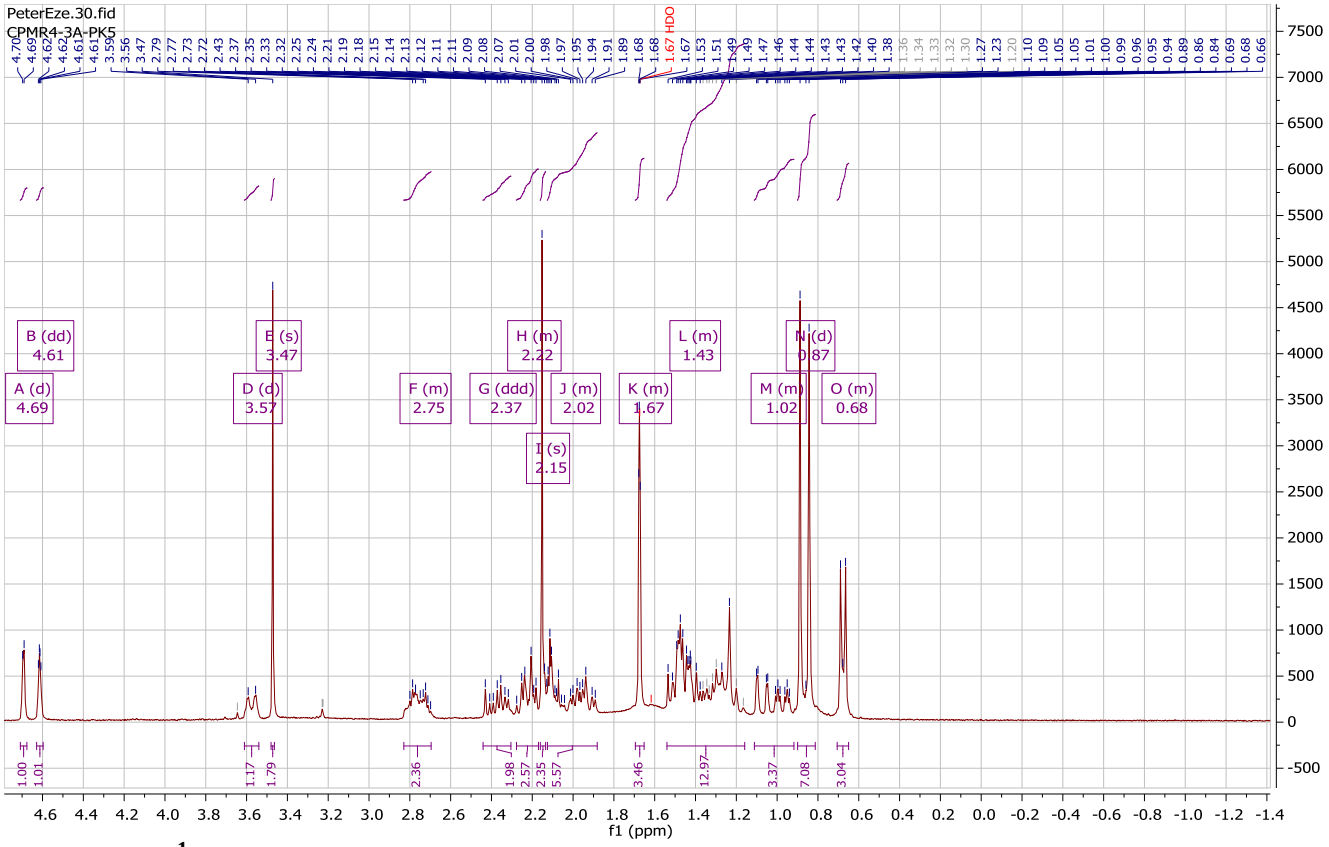
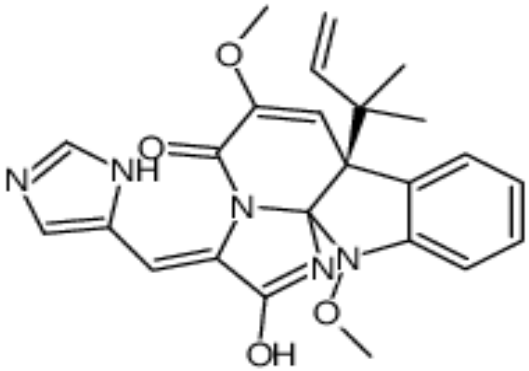


Figure 4.28: ^{1}H-NMR Spectra (300Hz, Chloroform-d) of Variecolactone

^{1}H NMR (300 MHz, Chloroform-d) δ 6.98 (q, J = 3.8 Hz, 1H), 4.69 (d, J = 2.2 Hz, 1H), 4.61 (dd, J = 2.3, 1.4 Hz, 1H), 3.57 (d, J = 10.9 Hz, 1H), 3.47 (s, 2H), 2.83 – 2.70 (m, 2H), 2.37 (ddd, J = 16.3, 11.4, 5.7 Hz, 2H), 2.28 – 2.17 (m, 3H), 2.15 (s, 2H), 2.13 – 1.88 (m, 6H), 1.69 – 1.65 (m, 3H), 1.54 – 1.16 (m, 13H), 1.11 – 0.92 (m, 3H), 0.87 (d, J = 13.3 Hz, 7H), 0.71 – 0.65 (m, 3H).

D OXALINE



A

Oxaline

Molecular formula: $C_{24}H_{25}N_5O_4$
Molecular weight: 447 g/mol
Appearance: Brownish powder
Absorption maximum: UV λ_{max} (methanol): 227, 327 nm
Quantity isolated: 0.6 mg

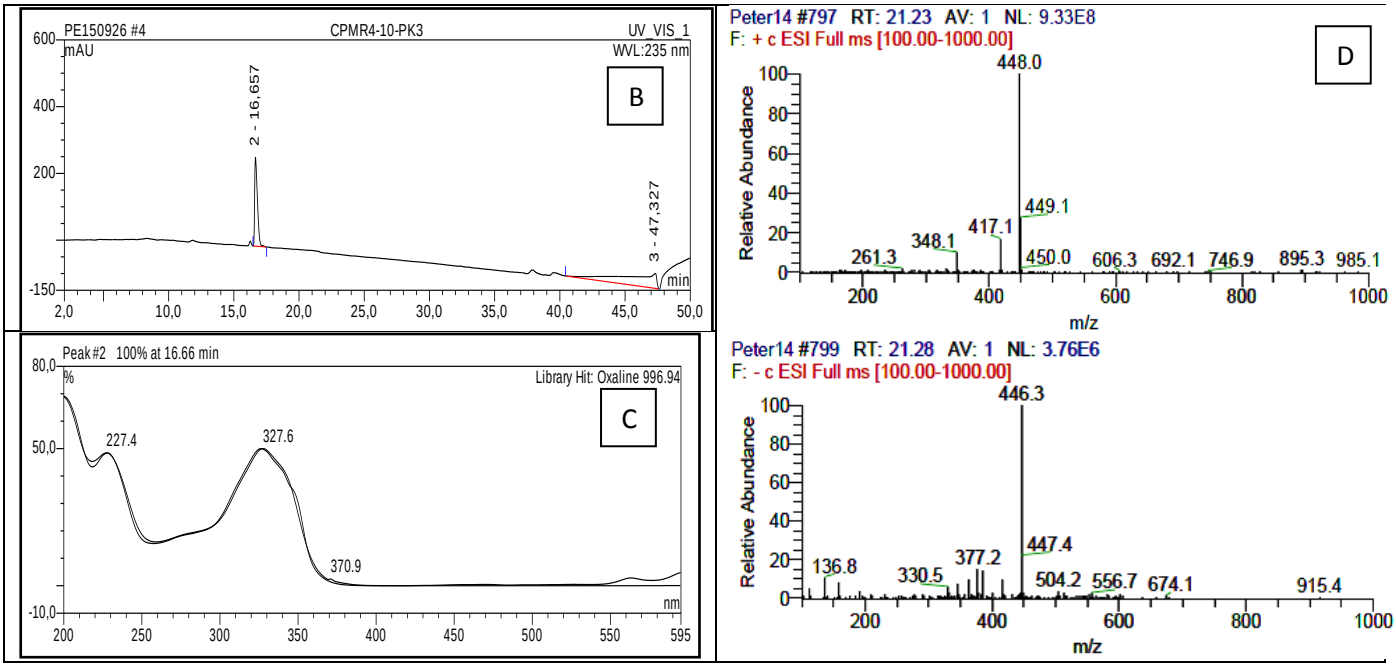


Figure 4.29: Oxaline: Structure (A), HPLC Chromatogram (B), UV spectrum(C) and LC-MS Data (D)

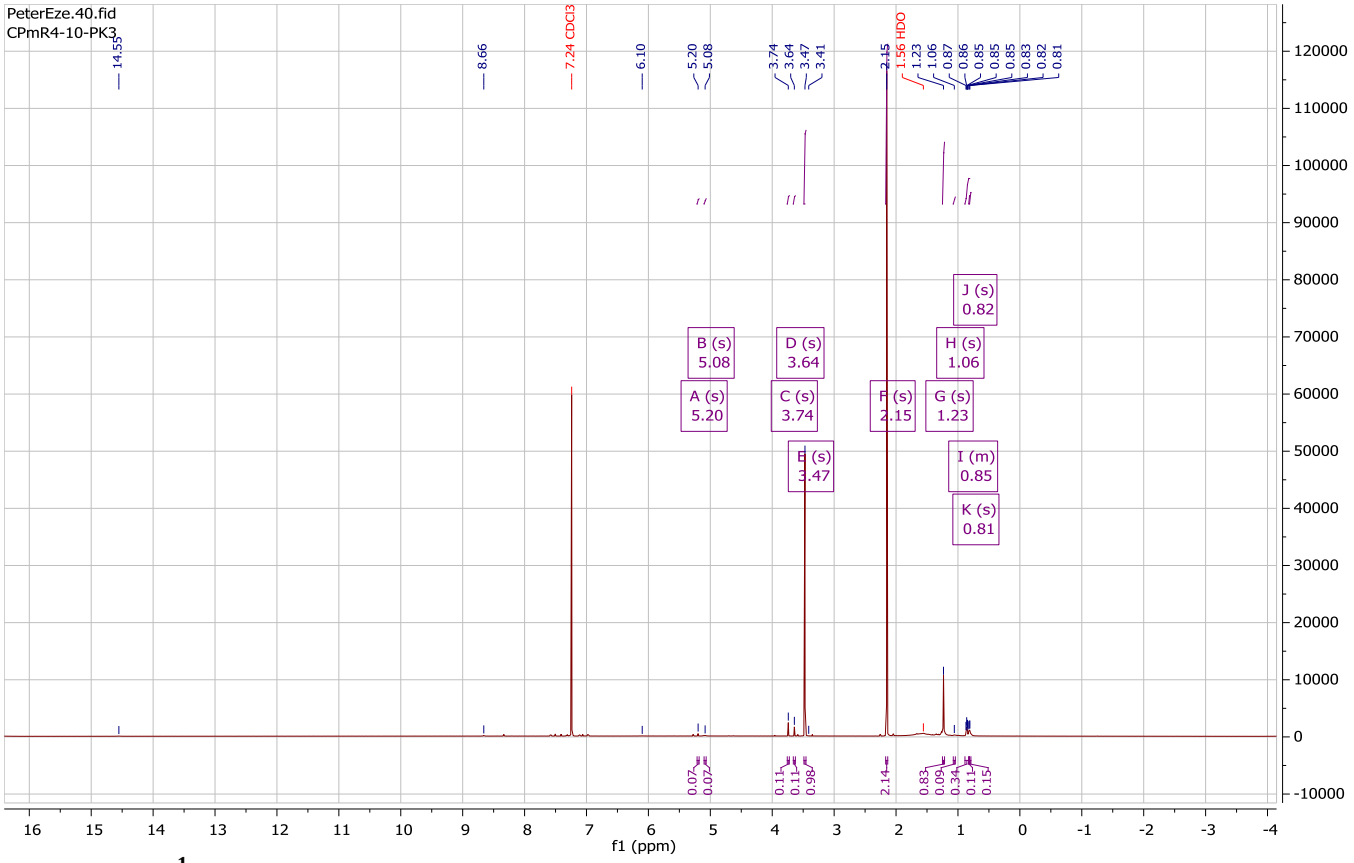


Figure 4.30: ^{1}H-NMR Spectra (600Hz, Chloroform-d) of Oxaline

^{1}H NMR (600 MHz, Chloroform-d) δ 5.20 (s, 0H), 5.08 (s, 0H), 3.74 (s, 0H), 3.64 (s, 0H), 3.47 (s, 1H), 2.15 (s, 2H), 1.23 (s, 1H), 1.06 (s, 0H), 0.89 – 0.83 (m, 0H), 0.82 (s, 0H), 0.81 (s, 0H).

4.1.4 RESULTS OF ISOLATION OF BIOACTIVE COMPOUNDS FROM *F. EQUISETI*

The isolation of bioactive secondary metabolites from *F. equiseti* followed the steps outlined in Figure 4.31. The reported bioactivities of the compounds are presented in Table 4.10.

The structures, HPLC chromatograms, UV spectra, LC-MS data and other data on the compounds isolated from *F. equiseti*, as well as their ^{1}H-NMR Spectra of the compounds are presented in Figures 4.32-4.35.

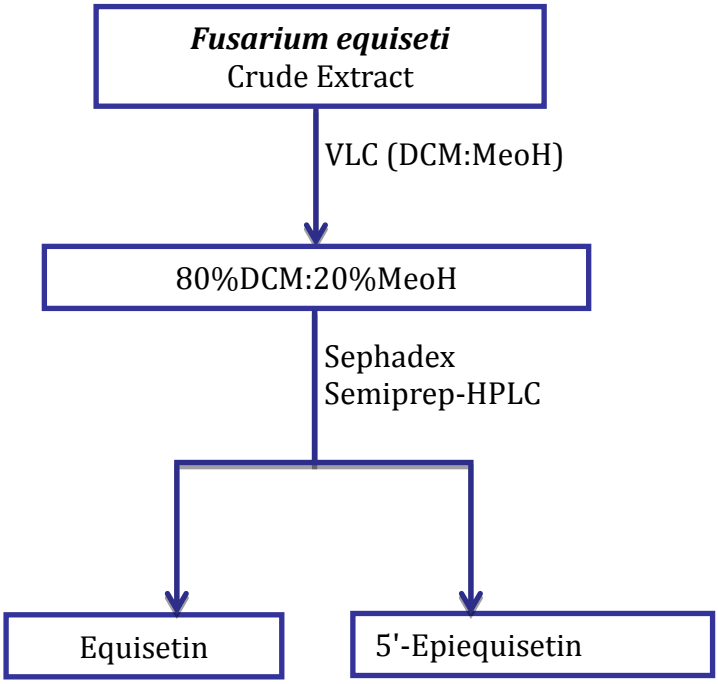


Figure 4.31: Isolation of Compounds from *F. equiseti* Crude Extract

4.1.4.1 COMPOUNDS ISOLATED FROM *F. EQUISETI*

A. EQUISETIN

A

Equisetin

Molecular formula: $C_{22}H_{31}NO_4$
Molecular weight: 373 g/mol
Appearance: Pale/Slightly pinkish
Absorption maximum: UV λ_{max} (methanol): 234, 295 nm
Quantity isolated: 4.4 mg

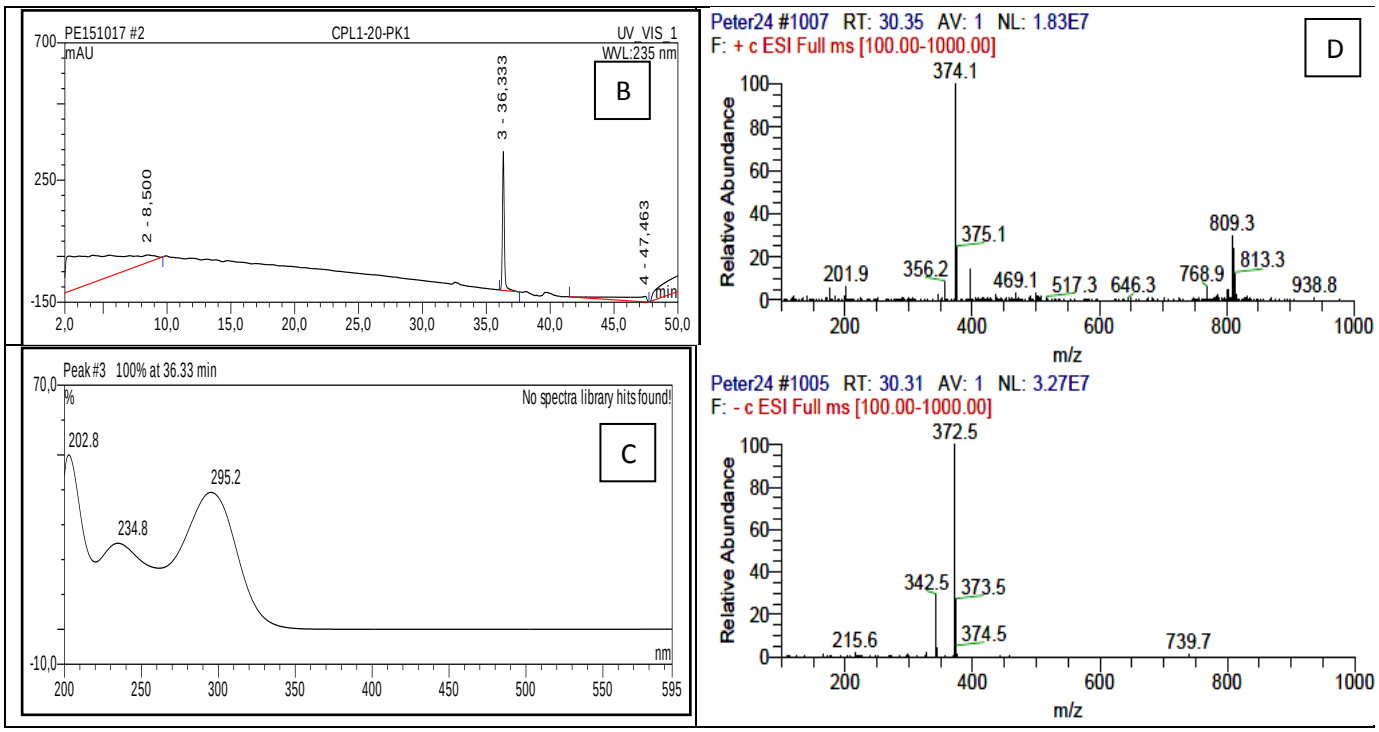


Figure 4.32: Equisetin: Structure (A), HPLC Chromatogram (B), UV spectrum(C) and LC-MS Data (D)

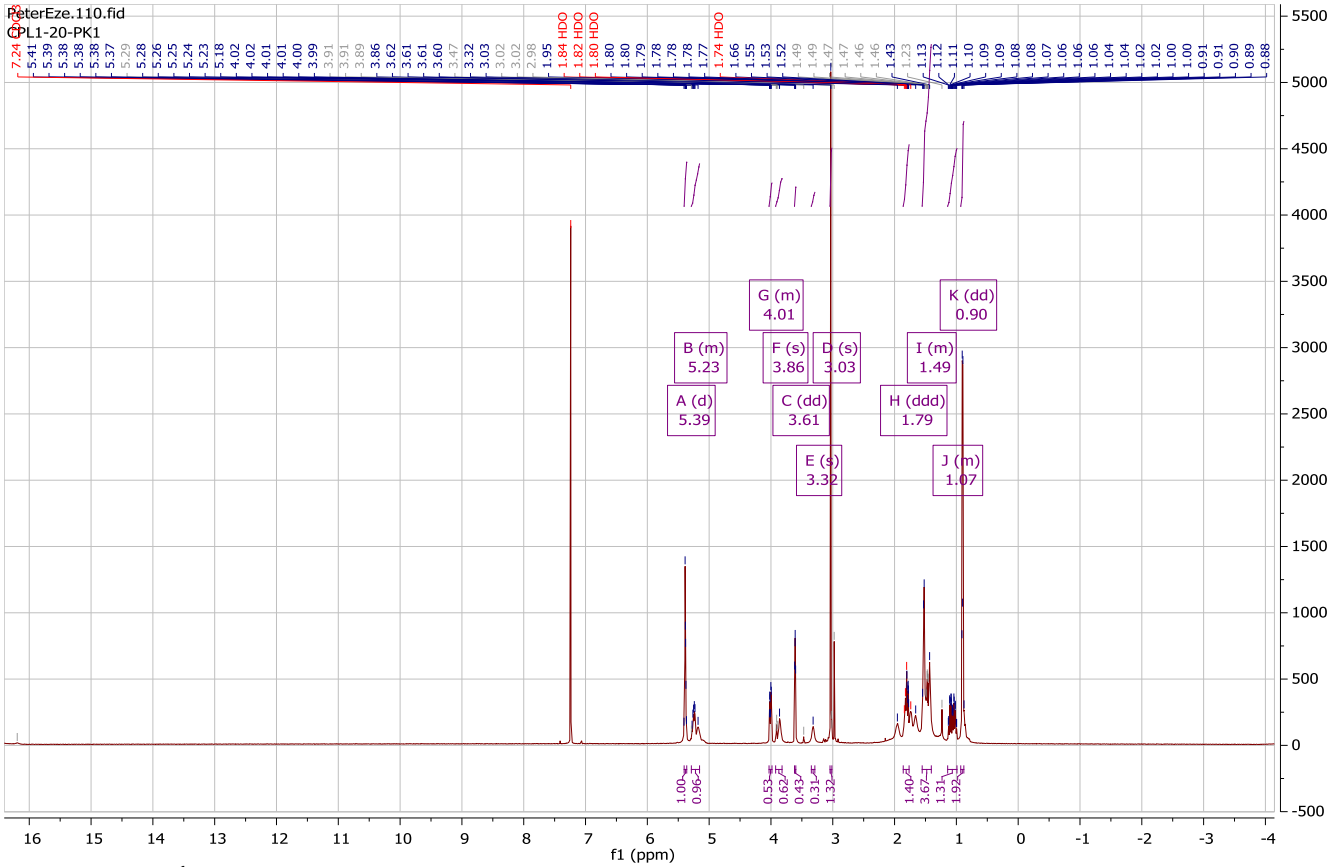


Figure 4.33: ^{1}H-NMR Spectra (600Hz, Chloroform-d) of Equisetin

^{1}H NMR (600 MHz, Chloroform-d) δ 5.39 (d, J = 4.5 Hz, 1H), 5.29 – 5.16 (m, 1H), 4.03 – 3.98 (m, 1H), 3.86 (s, 1H), 3.61 (dd, J = 5.0, 4.0 Hz, 0H), 3.32 (s, 0H), 3.03 (s, 1H), 1.79 (ddd, J = 8.8, 4.3, 2.1 Hz, 1H), 1.55 – 1.41 (m, 4H), 1.14 – 0.99 (m, 1H), 0.90 (dd, J = 6.5, 2.8 Hz, 2H).

B. 5'-EPIEQUISETIN

A

5'-Epiequisetin

Molecular formula: $C_{22}H_{31}NO_4$
Molecular weight: 373 g/mol
Appearance: Pale/Slightly pinkish
Absorption maximum: UV λ_{max} (methanol): 234, 295 nm
Quantity isolated: 2.0 mg

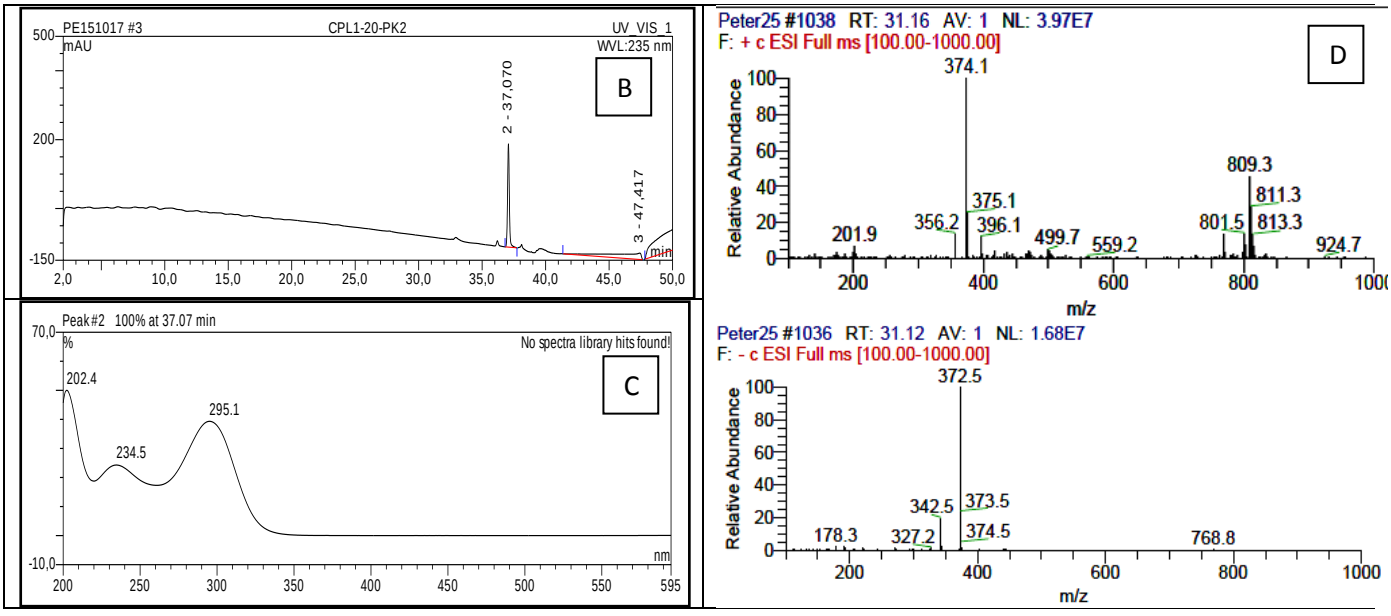


Figure 4.34: 5'-Epiequisetin: Structure (A), HPLC Chromatogram (B), UV spectrum(C) and LC-MS Data (D)

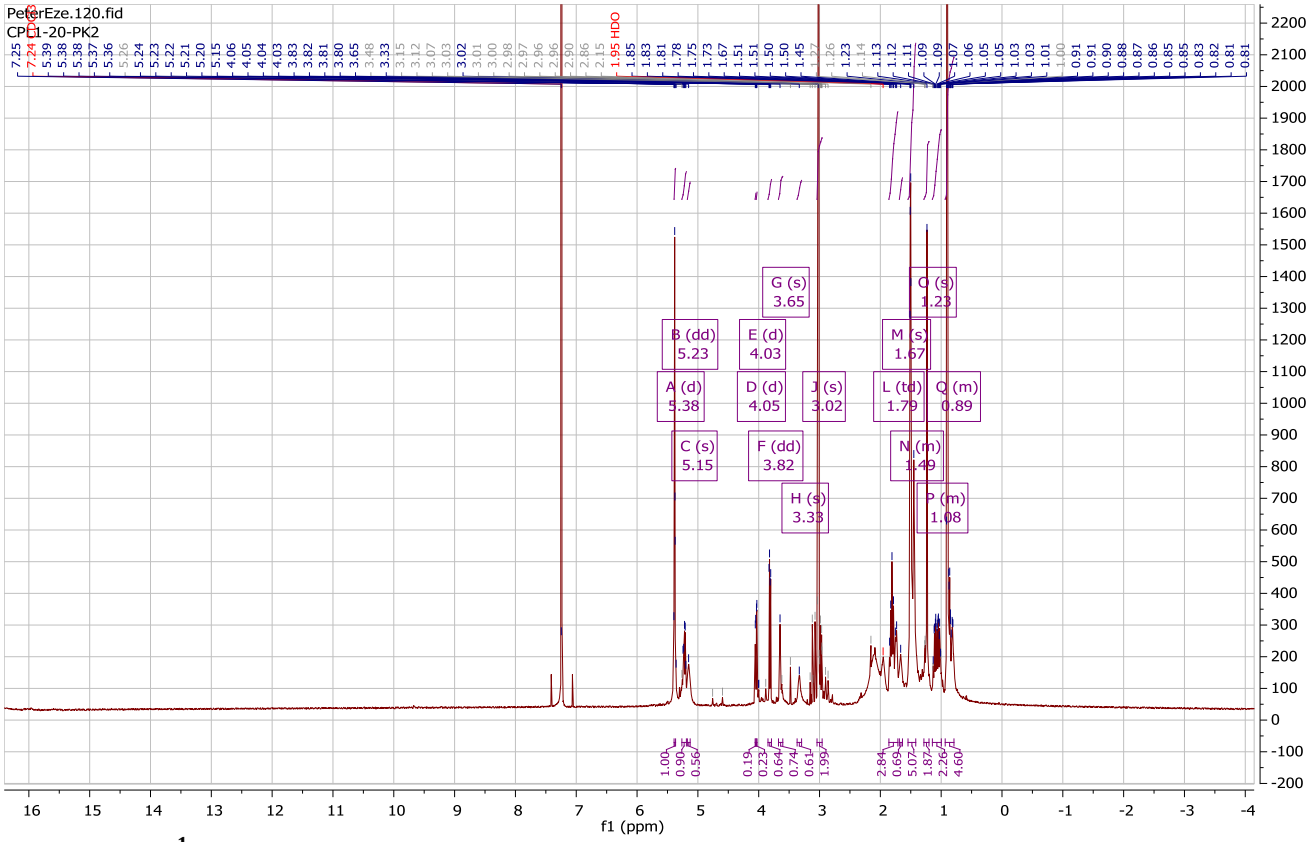


Figure 4.35: ^{1}H-NMR Spectra (600Hz, Chloroform-d) of 5'-Epiequisetin

^{1}H NMR (600 MHz, Chloroform-d) δ 5.39 (d, J = 4.5 Hz, 1H), 5.29 – 5.16 (m, 1H), 4.03 – 3.98 (m, 1H), 3.86 (s, 1H), 3.61 (dd, J = 5.0, 4.0 Hz, 0H), 3.32 (s, 0H), 3.03 (s, 1H), 1.79 (ddd, J = 8.8, 4.3, 2.1 Hz, 1H), 1.55 – 1.41 (m, 4H), 1.14 – 0.99 (m, 1H), 0.90 (dd, J = 6.5, 2.8 Hz, 2H).

Table 4.10: Reported Bioactivities of Isolated Compounds

Isolated compounds	**Source**	**Reported Bioactivity**	**References**
Secalonic acid F	*A. aculeatus*	Antimicrobial	Andersen *et al*., 1977
JBIR-74	*A. aculeatus*	-	-
Variecolactone	*A. aculeatus*	Immunomodulatory	Takahashi *et al*., (1999)
Oxaline	*A. aculeatus*	Anticancer α-glucosidase inhibition	Koizumi *et al*., 2004 Yawei *et al*., 2015
Equisetin	*F. equiseti*	Antibiotic and cytotoxic activity; Inhibitor of mitochondrial ATPases and HIV-1 integrase	Tziveleka *et al*, 2003; Hazuda *et al*., 1999; Burke *et al*., 2005; Singh, 2011
5'-Epiequisetin	*F. equiseti*	Inhibitor of HIV-1 integrase	Singh, 2011

4.1.5 BIOASSAYS ON THE CRUDE EXTRACTS OF *A. ACULEATUS* AND *F. EQUISETI*

Results of cytotoxicity, antioxidant, antimicrobial and anti-tubercular assays on the crude extracts of *A. aculeatus* and *F. equiseti* are presented in Tables 4.11 – 4.15.

In the cytotoxicity assay, at a concentration of 10µg/mL, extracts of *A. aculeatus* and *F. equiseti* were tested against mouse lymphoma cell lines (L56178Y). The extract of *A. aculeatus* showed an excellent cytotoxic activity with a growth inhibition of 106.4%. The extract of *F. equiseti* displayed poor inhibitory activity against the cancer cell line with an inhibition of -15.3% (Table 4.11).In the DPPH antioxidant assay, at a concentration of 500µg/mL, the extract of *A. aculeatus* showed an excellent antioxidant activity with an inhibition of 75.84%. The extract of *F. equiseti* displayed poor DPPH scavenging activity with an inhibition of 13.83% (Table 4.12).

The results of the preliminary antimicrobial screening revealed that a concentration of 1 mg/ml, crude extract from *A. aculeatus* showed antibacterial activity against the two Gram positive bacteria *S. aureus* and *B. subtilis*, with inhibition zone diameters (IZD) of ~ 7 and 4 mm respectively. *F. equiseti*'s extract showed antibacterial activity only against *B. subtilis* with an IZD of 3 mm. No antimicrobial activity was recorded against the Gram negative strains and fungal test isolates for both fungal crude extracts (Table 4.13).

The Minimum Inhibitory Concentrations (MICs) of the fungal extracts were determined against the test organisms on which the extracts showed activity in the preliminary antimicrobial screening. At the concentrations analyzed (1-0.0625 mg/mL), the MICs of the fungal extracts on the test organisms ranged from 0.125 - 0.25 mg/ml. *A. aculeatus* crude extract recorded MICs of 0.125 and 0.25 mg/mL against the two Gram positive bacteria *S. aureus* and *B. subtilis* respectively. *F. equiseti'*s recorded an MIC of 0.25 mg/mL against *B.*

subtilis. No MIC values were recorded against the Gram negative strains and fungal test isolates for both fungal extracts (Table 4.14).

The results of the anti-tubercular assay showed that at a concentration of 10µg/mL, extracts from both *A. aculeatus* and *F. equiseti*showed no anti-tubercular activities (Table 4.15).

Table 4.11: Cytotoxicity Assay on Mouse Lymphoma Cell Lines (L5178Y)

Fungal Extract	Concentration(µg/mL)	Growth inhibition (%)
A. aculeatus	10	106.40
F. equiseti	10	-15.30
Control	-	0

Table 4.12: DPPH Antioxidant Assay

Fungal Extract	Concentration(µg/mL)	% Inhibition
A. aculeatus	500	75.84
F. equiseti	500	13.83
Control (Quercetin)	500	93.93

Table 4.13: Preliminary Antimicrobial Screening of Fungal Extracts

Test Organisms	**Inhibition Zone Diameters (mm)**			
	A. aculeatus (1 mg/mL)	***F. equiseti*** (1 mg/mL)	**Positive control[**Ciprofloxacin (5 µg/mL)]	**Negative control** DMSO
Staphylococcus aureus	6.67±0.33	0±0.00	6±0.00	0±0.00
Bacillus subtilis	3.67±0.33	3±0.00	9.67±0.33	0±0.00
Salmonella typhi	0±0.00	0±0.00	3.67±0.33	0±0.00
Pseudomonas aeruginosa	0±0.00	0±0.00	8±0.00	0±0.00
Klebsiella pneumoniae	0±0.00	0±0.00	4.67±0.33	0±0.00
			Miconazole (50 µg/mL)	**DMSO**
Aspergillus niger	0±0.00	0±0.00	14.67±0.67	0±0.00
Candida albicans	0±0.00	0±0.00	7.67±0.33	0±0.00

Table 4.14: Minimum Inhibitory Concentrations (MICs) of Fungal Extracts against Test Organisms

Test Organisms	MICs (mg/mL)	
	A. aculeatus	***F. equiseti***
Staphylococcus aureus	0.125	-
Bacillus subtilis	0.25	0.25
Salmonella typhi	-	-
Pseudomonas aeruginosa	-	-
Klebsiella pneumoniae	-	-
Aspergillus niger	-	-
Candida albicans	-	-

Table 4.15: Anti-tubercular Assay

Fungal Extract	Concentration (µg/mL)	*M. tuberculosis*
A. aculeatus	10	-
F. equiseti	10	-

4.2 DISCUSSION

The ultimate aim of bioprospecting for novel compounds is to isolate compounds which are safe and efficacious for human use. Efficient screening mechanisms are crucial for targeting potential bioactive compounds (Alvin *et al.*, 2014). With the help of various exciting new tools and experimental techniques introduced in recent years, natural product research, especially in the area of exploiting micro- and macro-organisms of both terrestrial and marine origins for novel bioactive compounds, has now become very promising.

Natural products, especially those from microorganisms, continue to play a major role in drug discovery and development. Fungi remain a promising source of novel bioactive compounds that are important for drug discovery programs. Most of the bioprospecting programs on detection and isolation of bioactive compounds have been based on single culture condition to screen biological activities of many fungi. Nevertheless, microorganisms are able to synthesize a great variety of secondary metabolites according to their environment and the available nutritional resources (Bode, 2002).

The findings of this research confirmthat using one single fungus cultivated under different culture conditions, as well as the co-cultivation (mixed fermentation) of the fungus with other unrelated microbes, have a great potential in the discovery of novel natural products by fungi.

4.2.1 BIOACTIVE COMPOUNDS FROM *APIOSPORA MONTAGNEI*

In this study, several compounds were expressed by the lichen-associated fungus *A. montagnei* (teleomorph), also known as *Arthrinium arundinis* (anarmoph). The compounds include: decarboxycitrinone, 8-hydroxy-3-methyl-9-oxo-9H-xanthene-1-carboxylic acid methyl ether, libertellenone G, arthrinin A, arthrinin B, norlichexanthone, myrocin A, anomalin A, anomalin B, O-methylmellein, acremonone G, 6,8-dihydroxy-4-hydroxymethyl-

3,5-dimethylisochromen-1-one, decarboxyhydroxycitrinone, didymellamide B, N-hydroxyapiosporamide, apiosporamide. The compounds have been reported to possess biological activities that include antimicrobial and anticancer activities (Table 4.2).

Several compounds, including some of those reported in this study have been reported to be expressed by this fungus regardless of its ecological niche.

Wang *et al*. (2015) reported the isolation of arthpyrones A, B, C and N-hydroxyapiosporamide from a sponge-derived *A. montagnei*; Klemke *et al*. (2004) reported the isolation of myrocin A, apiosporic acid, monomethyl ester of 9-hydroxyhexylitaconic acid, (-)-hexylitaconic acid, (+)-epiepoxydon, (+)-epoxydon monoacetate, R-mellein, R-8-methoxymellein, 5-hydroxymethylfuran-2-carboxylic acid, and anomalin A from a marine algae-derived *A. montagnei*. Arundifungin, an antifungal agent was reported to be isolated from *A. montagnei* (*Athrinium arundinis*) as well as from other *Apiospora* species (Cabello *et al*., 2001).

From other species of the genus *Apiospora* (*Arthrinium*), several compounds have been isolated, including some of those expressed by the fungus used in this study.

Qian-Cutrone *et al*. (1994) isolated arthrinone, norlichexanthone, and terpestacin from a typed strain of *Arthriniumsp*.

Bloor (2008) reported the isolation of arthrinic acid from *A. phaeospermum*. Tsukada *et al*. (2011) reported the isolation of myrocin D, libertellenone E, and libertellenone F, decarboxyhydroxycitrinone, myrocin A, libertellenone C, and cytochalasin E from *A. sacchari.*

Ebada *et al*. (2011) reported the isolation of 10 secondary metabolites (arthrinins A–D, myrocin A and D, norlichexanthone, anomalin A, decarboxycitrinone, and 2,5-dimethyl-7-

hydroxychromone) from *Arthrinium sp*., associated with the Mediterranean sponge *Geodia cydonium.*

Tsukamoto *et al*. (2006) reported the isolation of an anticancer compound (-)-hexylitaconic acid from a marine-derived *Arthrinium* sp. The compound [(-)-hexylitaconic acid], which was not isolated from the lichen-associated fungal strain used in this study, was also isolated by Klemke *et al*. (2004) from a specie of *Arthrinium* associated with the marine environment.

Other compounds isolated from *Apiospora* species include: arthrichitin (Vijayakumar *et al*., 1996), terpestacin (Oka *et al*., 1993), CAF-603 (Ondeyka *et al*., 1995), apiosporamide (Alfatafta *et al*., 1994), and the cyclopeptides TMC-95 A–D (Kohno *et al*., 2000).

In addition tothe biological activities reportedly possessed by compounds isolated from fermentation products of *Apiospora* species (Ebada *et al*., 2011; Wang *et al*., 2015; Tsukada *et al*., 2011), crude extracts/secondary metabolites of *A. montgnei*, as well as species in the genus *Apiospora* (*Arthrinium*), have been confirmed to possess either antimicrobial, antioxidant, or cellulolytic/cytotoxic activities (Ramos *et al*., 2010; Hong *et al*., 2015).

The quality of expressing bioactive byproducts and the stability possessed by fungi in the genus *Apiospora* (*Arthrinium*) in expressing certain compounds (this stability increases the possibility of isolating same or similar bioactive compounds from the fungus regardless of the ecological niche from where it is associated or isolated from) makes the fungus *A. montagnei* an ideal candidate for modulation experiments.

In this study, the aim of the modulation experiments on *A. montagnei* was to facilitate/stimulate its production of the desired bioactive metabolites, as well as to unlock the possibility of the fungus to express compounds which it hitherto does not express (i.e. turning on of silent genes).

There have been indications that an effective drug/bioactive natural products discovery process can be achieved through systematic manipulation of culture conditions of promising organisms, the application of the one strain-many compounds (OSMAC) approach-where new compounds can be elicited by cultivating microorganisms in different fermentation conditions, and co-cultivation which is the competitive cultivation between different microbial taxa. All these processes are known to result in increased yields of previously described metabolites, production of previously undetected metabolites, production of analogues of known metabolites resulting from combined pathways and importantly, induction of previously unexpressed pathways for bioactive constituents (Pettit, 2009; Reen *et al*., 2015; Ola *et al*., 2013; Chiang *et al*., 2009; Chiang *et al*., 2011).

In this study, parameters that were manipulated included the alteration of media composition also known as media engineering (where different sodium salts, carbon, and nitrogen sources were added to the fermentation medium), and application of environmental cues/manipulation of cultural conditions (in this case, the fungus was cultivated at different temperature conditions) Also, co-cultivation of the fungus with *B. subtilis* and *S. lividans* respectively was carried out.

4.2.2 MODULATION OF *A. MONTAGNEI*

Several bioactive compounds which were undetected or those detected at very low levels in the initial crude extract of *A. montagnei* were up-regulated after the modulations experiments on the fungus. These compounds include: anomalin B, anomalin A, norlichexanthone, N-hydroxy-apiosporamide, apiosporamide, decarboxycitrinone, decarboxyhydroxycitrinone, myrocin A, and 8-hydroxy-3-methyl-9-oxo-9H-xanthene-1-carboxylic acid methyl ether.

The effects of the different parameters studied in the modulation experiments are presented in the following headings.

A. Co-cultivation

It can be observed in Figures 4.8 and 4.9 that the co-cultivation of *A. montagnei* and *B. subtilis* stimulated the up-regulation of fungal genes expressing anomalin B, norlichexanthone, and N-hydroxy-apiosporamide. Co-cultivation of *A. montagnei* and *B. subtilis* also triggered the expression of silent fungal genes expressing anomalin A and apiosporamide. These compounds were initially expressed by earliest cultures of the fungus (see Table 4.3 and Figure 4.6), but after periods of storage and successive subculturing, the fungus ceased to express the compounds (i.e. the genes expressing the compounds became silent or turned off). Co-culturing of the fungus with *B. subtilis*, however, turned on the silent genes resulting in the re-expression of the compounds.

On the other hand, the expression of bioactive metabolites was suppressed or down-regulated by co-culturing*A. montagnei*with *S. lividans* (Figures 4.11 and 4.12). This co-cultivation resulted in the switching off/suppression of the genes expressing some compounds (norlichexanthone and decarboxycitrinone) which were originally expressed by the fungus (Table 4.4 and Figure 4.6). However, co-cultivation of *A. montagnei* and *S. lividans* triggered the production of a new compound by the fungus (Figure 4.11).

B. Temperature

Results of the modulation experiments on*A. montagnei* showed that when cultivated at 22^{o}C, the fungus expressed certain compounds (decarboxyhydroxycitrinone, 8-hydroxy-3-methyl-9-oxo-9H-xanthene-1-carboxylic acid methyl ether, and CL2-1E-SE5-S4). These compounds were however not expressed when the fungus was cultivated at 28^{o}C (Figures 4.13 and 4.14). Also, at 28 ^{o}C, the fungus expressed some compounds (anomalin A, anomalin B and norlichexanthone) which were not expressed when it was cultivated at 22^{o}C.

At these two temperatures, other compounds which originally were expressed by the fungus (i.e. earliest cultures) were not synthesized (the genes expressing these compounds were turned off/ became silent).

The cultivation of the fungus at 28^{o}C,however,induced the expression of the fungal silent genes which codes for the expression of the three compounds: anomalin A, anomalin B, and norlichexanthone (Table 4.6). Also, at this temperature, the fungus recorded faster growth than when cultured at 22^{o}C (Table 4.5).

C. Sodium salts

Expression of silent fungal genes was also observed after the addition of different sodium salts (5% w/v) (Figures 4.15 and 4.16, Table 4.7). Sodium chloride (NaCl) induced the expression of silent genes for the following compounds: norlichexanthone, anomalin A, anomalin B, myrocin A, decarboxyhydroxycitrinone, 8-hydroxy-3-methyl-9-oxo-9H-xanthene-1-carboxylic acid methyl ether, apiosporamide, and N-hydroxy-Apiosporamide; sodium bromide (NaBr) induced the expression of silent gene for 8-hydroxy-3-methyl-9-oxo-9H-xanthene-1-carboxylic acid methyl ether; and sodium iodide (NaI) induced the expression of silent genes for norlichexanthone and apiosporamide (Table 4.7) . Of all the sodium salts, only NaCl induced the production of a new compound (Figure 4.15). During the fermentation, it was observed that all sodium salts had varying degrees of inhibitory activity on the fungal growth. This was obvious for NaI which almost totally inhibited the growth of the fungal mycelium. The inhibitory activities of these salts and the resultant stress on the fungus may be responsible for triggering the expression of the silent fungal genes (Table 4.5).

D. Carbon sources

Addition of 4%w/v of carbon sources (glucose, sucrose and rhamnose) had no much effect on the metabolism of the fungus, as the HPLC profiles of the fungus supplemented with these

carbon sources were similar to those of the fungus control. Also, none of the carbon sources induced the production of new compounds (Figure 4.17). However, the three carbon sources stimulated the up-regulation of the fungal gene products (Figure 4.18). Also, sucrose and rhamnose both triggered the expression of silent fungal genes (Table 4.8). Glucose which had no effect on the turning-on of silent fungal genes, however resulted in the up-regulation of genes expressing two compounds, decarboxycitrinone and 8-hydroxy-3-methyl-9-oxo-9H-xanthene-1-carboxylic acid methyl ether (Figure 4.18). Of all the three carbon sources, the addition of sucrose resulted to the highest degree of up-regulation of gene expression for decarboxycitrinone, and especially 8-hydroxy-3-methyl-9-oxo-9H-xanthene-1-carboxylic acid methyl ether (which recorded the highest percent mean peak area) (Figure 4.18). Also, the addition of sucrose triggered the expression of silent genes for norlichexanthone and decarboxyhydroxycitrinone. Rhamnose, when compared with the other carbon sources, triggered the expression of silent genes for more compounds (norlichexanthone, anomalin A, anomalin B, and decarboxyhydroxycitrinone) (Table 4.8). This may be attributable to the fact that rhamnose which inhibited the growth rate of the fungus (Table 4.5), may have as a result of the induced stress, triggered the turning-on of fungal genes which were hitherto silent.

E. Nitrogen sources

The fungal cultivation medium was also supplemented with nitrogen sources at a concentration of 1% w/v. The nitrogen sources were ammonium chloride (NH_4Cl), peptone, corn steep liquor (CSL) and yeast extract (YE). These nitrogen sources had measurable effects on the metabolic profile of the fungus. At different degrees, they induced the up-regulation of gene products, as well as, triggered the expression of silent fungal genes (Figures 4.19 and 4.20). The addition of NH_4Cl did not stimulate the expression of silent fungal genes (Table 4.9), but however induced the production of new compounds (Figure 4.19) and the up-regulationof gene expression for one compound (CL2-1E-SE5-S4) (Figure

4.20). CSL and YE both stimulated the expression of silent fungal genes for similar compounds (norlichexanthone, anomalin A, and decarboxyhydroxycitrinone) (Table 4.9). However, YE had more effect on the yield or degree of expression of the three compounds (with higher percent mean peak areas) (Figure 4.20). Peptone had no observableeffect on the metabolic profile of the fungus, as it presented similar HPLC profile with the fungus control (Figure 4.19). It did not stimulate the expression of the fungal silent genes, or the up-regulation of expressed compounds, nor did it induce the production of new compounds (Figure 4.20 and Table 4.9).

Earlier reports convincingly demonstrate that the profiles of fungal secondary metabolites varied under different culture conditions (Wang et al. 1998). Experiments to stimulate the expression of certain metabolites by *A. montagnei*, as well as by species in the genus *Apiospora* (*Arthrinum*), have been reported (Ramos and Said, 2011; Miao *et al*., 2006). The variations observed by *Apiospora sp.* in these reports are in agreement with the the results of this study.

Ramos and Said (2011) investigated the effect of culture conditions on the antibacterial, antifungal, cytotoxic, and antiparasitic activities of bioactive secondary metabolites produced by an endophytic *A. montagnei*. They observed that highest levels of bioactivities were shown by extracts from cultures cultivated at 30°C. Best antimicrobial activity was observed in extracts from cultures grown in medium containing 3.1% (w/v) sucrose and 0.1% (w/v) sodium nitrate at pH 4.0 after 9 days of incubation. Strong cytotoxic activity against tumor cell lines was observed in extracts of cultures incubated at 25°C and 30°C, and in extracts obtained after 27 days of incubation.

Miao *et al.* (2006) investigated the effects of culture conditions and competitive cultivation (co-cultivation) with bacteria on the mycelial growth, metabolite profile, and antibacterial

activity of a marine-derived *A. saccharicola.*They observed that the fungus grew faster at 30°C, at pH 6.5 and in freshwater medium, but exhibited higher antibacterial activity at 25°C, at pH 4.5, 5.5, and 7.5, and in 34 ppt seawater medium. The fungus also grew faster in a high-nitrogen medium that contained 0.5% peptone and/or 0.5% yeast extract, while exhibiting higher bioactivity in a high-carbon medium that contained 2% glucose. It was also observed that the fungal growth was inhibited when it was co-cultured with six bacterial species, particularly the bacterium *Pseudoalteromonas piscida*, but the addition of a cell free culture broth of this bacterium significantly increased the bioactivity of the fungus. Although the bioactivity varied among different treatments in all experiments in their study, the HPLC profiles of the ethyl acetate extracts of these treatments were similar.

Ramos and Said (2011) and Miao *et al*. (2006) indicate that the change in bioactivity among different treatments might be attributed to different amounts of the major bioactive compounds produced by the fungus or the production of new compounds.

The biosynthesis of secondary metabolites in fungi is known to be regulated in response to nutrient availability, or as a result of changes in the environment, and its stage of development (Sanchez and Demain, 2002). The components of culture media may affect secondary metabolite production in fungi (Bode *et al.* 2002). Many carbon and nitrogen substrates can inhibit secondary metabolite production e.g. glucose and ammonium at high concentrations have been generally regarded as repressors of secondary metabolism (Miao *et al.*, 2006). Also, the competition that results from the co-cultivation of different microbes has potential of inducing secondary metabolite synthesis.

Studies using one single fungus cultivated under different culture conditions are not only suitable to produce different compounds, but also provide conditions to guide the production

of a specific compound (Ramos and Said, 2011) and increase the yields of desired metabolites.

4.2.3 BIOACTIVE COMPOUNDS ISOLATED FROM *ASPERGILLUS ACULEATUS*AND *FUSARIUM EQUISETI*

In the second part of this study, the crude ethyl acetate extracts from solid state fermentation of *Aspergillus aculeatus* and *Fusarium equiseti* were tested for antimicrobial, anti-tubercular, antioxidant and cytotoxic activities. The crude extracts showing positive activity were then subjected to several chromatographic procedures for the isolation and characterization of the fungal secondary metabolites. Bioactive compounds were isolated from the two endophytic fungi. Secalonic acid F, variecolactone, oxaline and JBIR-74 were isolated from *A. aculeatus,* while equisetin and 5' epiequisetin were isolated from *F. equiseti*.

A. ASPERGILLUS ACULEATUS

The genus *Aspergillus* (Moniliaceae), with over 180 species, is known to be a rich source of alkaloids, terpenoids, xanthones, steroids, and polyketides, some of which show antimicrobial, antifouling, antifeedant, phytotoxic, or other interesting bioactivities (Gao *et al*., 2015).

A. aculeatus is a filamentous fungus belonging to *Aspergillus* section *Nigri* - the black aspergilli. At least 145 metabolites have been characterized from the black aspergilli, many of which are biologically active (Petersen *et al*., 2014). The black aspergilli can be divided into different clades. *A. aculeatus* belongs to the uniseriate black aspergilli and is closely related to *A. aculeatinus, A. uvarum, A. japonicus, A. fijiensis, A. trinidadensis, A. floridensis, A. brunneoviolaceus* and *A. violaceofuscus* (Varga *et al*., 2011; Jurjević *et al*., 2012). These fungi differ from the other black aspergilli in their morphology, physiological behavior and in

the production of secondary metabolites. The fungi belonging to this group can produce secondary metabolites, which can be both polyketide (PK) and nonribosomal peptide (NRP) derived from mixed biosynthetic origin (Nielsen *et al*., 2009).

A. aculeatus has been reported to generate a variety of bioactive natural products, such as aculeacins A–G (antimicrobial agents) (Mizuno *et al*., 1977; Satoi, *et al*., 1977); CJ-15,183 (squalene synthase inhibitor and antifungal agent) (Watanabe *et al*., 2001); aspergillusol A (α-glucosidase inhibitor) (Ingavat *et al*., 2009); secalonic acids D and F (antimicrobial agents) (Andersen *et al*., 1977;Gao *et al*., 2015); asperparaline A (Ingavat *et al*., 2011); cytotoxic aculeatusquinones A–D (Chen *et al*., 2013); okaramines H and I (Hayashi *et al*., 1999); chrysophanol, emodin, succinic acid and 1,5-dimethyl citrate (Wu *et al*., 2009); and asperaculane A and B, aculenes A–D (Gao *et al*., 2015).

In this study, four bioactive metabolites, which include compounds with antimicrobial, immunonomodulatory and cytotoxic activities, were isolated from *A. aculeatus*(Table 4.10 and Figure 4.21).

Secalonic acid F was isolated from *A. culeatus* and it wasthe main compound present in the fungal crude extract, having the highest yield of 8 mg (Figure 4.22). This compound has been reported to show antimicrobial activity (Andersen *et al*., 1977). The structure of secalonic acid F, its HPLC chromatogram, UV-spectrum, LC-MS data, and ^{1}H-NMR spectra are shown in Figures 4.22 - 4.24.

Secalonic acids are members of the ergochrome group of secondary metabolites. Seven different kinds (A, B, C, D, E, F, and G) have been isolated from fungi and lichens (Kurobane *et al*., 1979).

Secalonic acid F (together with Secalonic acid D) was first reported to be isolated from *A. aculeatus* by Andersen *et al*. (1977). Other secalonic acids are known metabolites of *A.*

ochraceus (secalonic acid A), *Claviceps purpurea* (secalonic acids, A, B, C) and *Phoma terrestris* (secalonic acids, A, E), *Penicillium oralicum* (secalonic acid D) (Andersen *et al*., 1977).

The compound JBIR-74 was also isolated from *A. aculeatus* in this study (Figure 4.25). Takagi *et al*. (2010) first reported the isolation of this compound from *Aspergillus sp.* associated with a marine sponge. JBIR-74 was also isolated by Yawei *et al.*, (2015) from aciduric strain of *Aspergillus sp.* from a mangrove soil. There is no report on the biological activity of JBIR-74, although it has been tested for both antimicrobial and cytotoxic activities by Takagi *et al*. (2010) and showed no activity.

Variecolactone which was isolated from *A. aculeatus* in this study is a fungal sesterterpene (Figure 4.27). It has been reported to possess immunomodulatory property (Takahashi *et al*., 1999). Literature survey revealed that variecolactone has only been isolated from *Emericella purpurea* (Takahashi *et al*., 1999; Hosoe *et al*., 2006), and from *Emericella aurantio-brunnea* (Fujimoto *et al*., 2000). This study however, records the first report on the isolation of variecolactone from an *Aspergillus* species.

The fourth compound isolated from *A. aculeatus* in this study is the alkaloid, oxaline (Figure 4.29). The compound has been reported to have been isolated from aciduric strain of *Aspergillus sp.* from a mangrove soil (Yawei *et al*., 2015),and from *Penicillium sp.* (Koizumi *et al*., 2004). Oxaline has been reported to show anticancer activity (Koizumi *et al*., 2004).

At a concentration of 10 μg/mL, the crude ethyl acetate extract of *A. aculeatus* showed excellent cytotoxic activity against mouse lymphoma cell lines (L5178Y) with a growthinhibition of 106.4% (Table 4.11). At 500 μg/mL, the extract also showed good antioxidant activity in the DPPH antioxidant assay with an inhibition of 75.84%, which is quite comparable with that recorded for the positive control, quercetin (93.93%) (Table 4.12).

At a concentration of 1 mg/mL, crude extract from *A. aculeatus* showed antibacterial activity against the two Gram positive bacteria *S. aureus* and *B. subtilis,* with IZD of ~ 7 and 4 mm respectively. There was no antimicrobial activity recorded against the Gram negative isolates (*S. typhi, P. aeruginosa* and *K. pneumoniae*) and the fungal test isolates (*A. niger* and *C. albicans*) (Table 4.13). The results of the determination of the MICs of the fungal extracts against test organisms showed that *A. aculeatus* crude extract recorded MICs of 0.125 and 0.25 mg/mL against the two Gram positive bacteria *S. aureus* and *B. subtilis* respectively (Table 4.14). Crude extracts of *A. aculeatus* have been reported to possess antimicrobial activity (Katoch *et al*., 2014). Also, crude extracts from other species of *Aspergillus* (Prabavathy and Nachiyar, 2012; El-Zayat, 2008) have shown antimicrobial properties.

In this study, the crude ethyl acetate extract of *A. aculeatus*at 10 µg/mL recorded no anti-tubercular activity (Table 4.15).

While some black aspergilli are important in the biotechnological industry for production of enzymes and organic acids (Goldberg *et al*., 2006; Pel *et al*., 2007), *A. aculeatus* is used to produce important industrial enzymes such as cellulases (Bhat, 2000), xylanases (Fujimoto *et al*., 1995; Polizeli *et al*., 2005) and proteases (Olutiola and Nwaogwugwu, 1982), which are used commercially in the food and feed industries. Considering the potentials *A. aculeatus* possess for industrial applications, as well as its role in food and feed contamination, it is of great importance tostudyand exploit the metabolites produced by this fungus (Petersen *et al*., 2014).

B. FUSARIUM EQUISETI

Many studies have been carried out on metabolites production from several *Fusarium* species especially *F. oxysporum* and *F. solani* (Waskiewicz *et al*., 2010; Tatum *et al*., 1989; Savard *et al*., 1997; Hernandes *et al*., 2012).

Crude extracts of endophytic *Fusarium* species have been confirmed to show both antibacterial and antifungal activities (Devraju and Sreedharamurthy, 2011).

In this study, at a concentration of 1 mg/mL, *F. equiseti*'s extract showed antibacterial activity only against *B. subtilis* with an IZD of 3 mm. There was no antimicrobial activity recorded against the Gram negative isolates and the fungal test isolates (Table 4.13).The results of the determination of the MICs of the fungal extracts against test organisms showed that *F. equiseti'*s recorded an MIC of 0.25 mg/mL against *B. subtilis*(Table 4.14).

In this study, the crude ethyl acetate extract of *A. aculeatus*at 10 µg/mL recorded no anti-tubercular activity (Table 4.15).

Also in the study, *F. equiseti* crude extract showed no cytotoxic activity against mouse lymphoma cell lines (L5178Y) when analyzed at a concentration of 10 µg/mL (Table 4.11). A poor antioxidant activity was recorded for the extract at 500 µg/mL (Table 4.12).

Among the endophytes from medicinal plants, many studies have shown that *Fusariumsp*. is the most common species and a potent source of bioactive compounds (Katoch *et al*., 2014; Devraju and Sreedharamurthy, 2011). Antimicrobial compounds such as the penta-ketide (CR377: 2-methylbutyraldehyde-substituted-α-pyrone) (Sean and Jon 2000), beauvericin (also showed cytotoxic activity) (Wang *et al*. 2011), subglutinol A and B (Lee *et al*. 1995), and two antifungal agents-Fusapyrone and Deoxyfusapyrone (Altomare *et al*., 2000) have been isolated from *Fusarium* sp.

Fusarium equiseti (Nectriaceae) (teleomorph: *Gibberella intricans*) which is a toxigenic species, is a soil inhabitant that can infect seeds, roots, tubers, and fruit of several crop plants from diverse climatic regions. It has been implicated as a causal agent of disease in diverse plant species (Goswami *et al*., 2008; Marín *et al*., 2012).

F. equiseti is capable of synthesizing a vast range of phytotoxic and cytotoxic secondary metabolites (Langseth *et al*., 1998; Burmeister *et al.*, 1974). In this present study, we isolated two bioactive metabolites from *F. equiseti*, namely: equisetin and 5'-epiequisetin (see Table 4.10 and Figure 4.31).

Equisetin is an N-methylserine-derived acyl tetramic acid produced by a number of *Fusarium* species which demonstrates antibiotic and cytotoxic activities (Vesonder *et al*., 1979; Desjardins and Proctor, 2007; Patham *et al*., 2009). The structure of equisetin, its HPLC chromatogram, UV-spectrum, LC-MS data and ^{1}H-NMR spectra are shown in Figures 4.32 - 4.33.

Equisetin inhibits mitochondrial ATPase activity (Singh *et al*., 1998; König *et al*., 1993). It also inhibits HIV-1 integrase (Singh *et al*., 1998; Burke *et al*., 2005; Tziveleka *et al*., 2003; Singh, 2011). Equisetin has been shown to have strong antibiotic activity against certain Gram-positive bacteria and mycobacterium (Burmeister *et al*., 1974). It has been previously isolated from *F. equiseti* (Wheeler *et al*., 1999; Vesonder *et al*., 1979; Burmeister *et al.*, 1974).

5'-Epiequisetin is a phytotoxic isomer of equisetin. Its structure, HPLC chromatogram, UV-spectrum, LC-MS data and ^{1}H-NMR spectra are shown in Figures 4.34 – 4.35. 5'-Epiequisetin is also reported to have been isolated from *F. equiseti* (Wheeler *et al*., 1999). The compound is reported to inhibit HIV-1 integrase (Singh, 2011).

Burmeister *et al.* (1974) evaluated equisetin isolated from *F. equiseti* for its antimicrobial property and observed that the compound, in comparison to standard antibiotics used as positive controls (streptomycin, penicillin, and isoniazid), strongly inhibited some Gram-positive bacteria and some mycobacteria with MICs ranging from 0.5-8.0 μg/mL.

Anticancer compounds have also been isolated from *F. equiseti*. These include Diacetoxyscirpenol (Brian *et al.,* 1961; Dosik*et al.,* 1978) and Fusarochromanone and its derivatives (Xie *et al.,* 1991; Mahdavian *et al.,* 2014). Other reported compoundsfrom *F. equiseti* are 4-acetylnivalenol, nivalenol, scirpentriol zearalenone, beauvericin, fusarochromanone, equisetine and butenolide (Thrane, 1989; Pillai *et al.,* 2015).

Industrially, *F. equiseti* has been reported to have important applications for use as a biocontrol agent of plant pathogens and as a plant growth promoting fungus (Horinouchi *et al.,* 2007, and 2008, 2010; Motlagh, 2011). Also, an *F. equiseti*-dreived protease showed excellent performance in stain removal and good compatibility with several commercial laundry detergent formulations (Juntunen *et al.,* 2015).

CHAPTER FIVE

CONCLUSION AND RECOMMENDATION

5.1 CONCLUSION

With various exciting new tools introduced in recent years, natural product research has now entered a new promising age (Chiang *et al.,* 2011). Co-cultivation (mixed fermentation) of unrelated microbes, as well as the cultivation of an organism under different culture conditions, is an interesting strategy for the discovery of additional silent natural products (Chiang *et al.,* 2011; Ramos and Siad, 2011; Pettit, 2009).

Fungi, which are well known for producing many novel chemicals that are directly used as drugs or function as lead structures for synthetic modifications, are among the most important groups of eukaryotic organisms that are being explored for generation of novel therapeutic molecules.

In the hope of achieving an effective drug/bioactive natural products discovery process through systematic manipulation of culture conditions, the application of the one-strain-many-compounds (OSMAC) approach, and co-cultivation, and with the promising potentials of endophytic fungi in the area of novel drug discovery; this research was carried out to exploit fungi for their ability to produce unique bioactive metabolites, and to manipulate these fungi and their environment to facilitate the increased production of specific bioactive metabolites of interest.

In this study, co-cultivation of the lichen-associated fungus *A. montagnei* and the soil bacteria *B. subtilis* triggered the expression of some of the silent fungal genes. Also, co-cultivation of the fungusand *S. lividans* induced the production of a new compound. The expression of silent fungal genes was also observed when the fungus was cultivated at 28^{o}C, and also after

the addition of various sodium salts, nitrogen, and carbon sources. Sodium chloride (5%) and ammonium chloride (1%) both induced the production of new compounds by the fungus.

Bioactive compounds were isolated from two endophytes-*Aspergillus aculeatus* and *Fusarium equiseti* associated with the leaves of *Carica papaya*, a medicinal plantfrom South-Eastern Nigeria. These include four compounds from *A. aculeatus*: secalonic acid F (antimicrobial), JBIR-74, variecolactone (immunomodulatory activity), oxaline (anticancer activity), and two compounds from *F. equiseti*: equisetin (anticancer, antibacterial and antiviral activities) and 5'-epiequisetin (antiviral activity).

Results of this study indicate that manipulation of the external conditions/environment of a single fungus (co-cultivation, media engineering, and manipulation of cultural conditions), can trigger some desirable changes at the genetic level and this can result in increased yields of previously described metabolites, expression of previously undetected metabolites, analogues of known metabolites resulting from combined pathways and, importantly, induction of previously unexpressed pathways for bioactive constituents. Also, this study confirms endophytic fungi associated with Nigerian indigenous plants as a potential source of bioactive compounds for pharmaceutical and industrial applications.

This reports the isolation of bioactive compounds that are either active against bacteria, fungi, viruses, or cancerous cells. These agents can be synthesized on a larger scale using same biosystems and be developed for use in the treatment of microbial infections and cancers. The findings from this research will also facilitate further studies to gain a better understanding of active metabolite production in fungi, especially in the optimization of culture conditions for mass cultivation and the biotechnological mass production of active compounds in the future.

5.2 RECOMMENDATIONS

Experimental techniques for discovery of the products of cryptic biosynthetic gene clusters continue to be developed and refined. One important challenge that lies ahead is the development of general methods for activating the expression of silent cryptic biosynthetic gene clusters (Challis, 2008).It is clear that co-cultivation as an experimental tool, which aims at a diversified production of bioactive compounds that could be leads for biomedical research, is promising but nevertheless still in its infancy (Marmann *et al.,* 2014). To successfully explore the potentials of microorganisms, especially fungi, in the discovery of novel bioactive molecules, it is therefore recommended that:

- It is important that the underlying molecular mechanisms driving microbial co-cultivations be studied and better understood. This knowledge could be applied to induce silent secondary metabolite biosynthetic gene clustersunder laboratory conditions. This ecologically-driven approach, if adopted by more reserchers, will enhance the chemical diversity of microbes beyond the boundaries that can be reached by routine axenic cultivation.
- It is also important to studythe prevailing circumstancesthat cause these cryptic genes to be silent under laboratory fermentation conditions. Understanding the underlying mechanism(s) that result in the silencing of cryptic genes would help to fully untilize the microbial gene clusters for secondary metabolism.
- The co-cultivation approach can also be complemented by the emerging innovative biotechnological platforms which ecompasses genomics, proteomics, metabolomics, secretomics, transcriptomics, bioinformatics, etc.
- The application of ribosome engineering to activate the silent biosynthetic pathways in fungi is another point of interest. This may result in the efficient exploitation of novel secondary metabolites of these microbial groups.

- It is important that collaborations between mycologists, geneticists, biochemists and chemists be established. This is essential to facilitate the discovery of novel natural products and the genes involved in their biosynthesis.

According to Strobel and Daisy (2003), endophytes which are a poorly investigated group of microorganisms, represent an abundant and dependable source of bioactive and chemically novel compounds. These compounds can be exploited for pharmaceutical, agricultural, and industrial applications. To facilitate the discovery of novel drug compounds and other important metabolites, it is important that the mechanisms through which endophytes exist and respond to their surrounding be better understood. This would aid the researcher to be more predictive about which higher plants to seek, study, and spend time isolating microfloral components.

- A global initiative involving fungal taxonomists, ecologists, and natural product chemists should be established in order to develop systematic and rapid screens for endophytic fungi. This can be achieved by designing strategic bioassays that would indicate the production of novel bioactive compounds.
- It is also recommended to optimize fermentation conditions of endophytes that have been found to show bioactivity in order to enhance the yield of active substances synthesized by endophytes.
- It is hoped that the findings from this research will facilitate further studies to gain a better understanding of bioactive metabolites production in fungi, especially in the optimization of culture conditions for mass cultivation and the biotechnological mass production of active compounds in the future.

5.3 CONTRIBUTIONS TO KNOWLEDGE

- The various techniques used in the modulation experiments of this study (co-cultivation, media engineering, and manipulation of cultural conditions), can be applied by researchers to successfully explore the potentials of microorganisms, especially fungi, in the discovery of novel bioactive molecules.
- This studyreports the isolation of bioactive agents (Secalonic acid F, variecolactone, oxaline, JBIR-74,equisetin and 5' epiequisetin) from two endophytic fungi associated with *Carica papaya*. These isolated compounds which are either active against bacteria, fungi, viruses, and cancerous cells may be developed as activeprinciples of drugs against microbial infections or cancer; or as lead compounds which could be chemically manipulated into effective anticancer, antioxidant or antimicrobial drugs.
- This study also reveals the potentials possessed by Nigerian indigenous plants as source of endophytes that hold keys of possibilities to the discovery of novel molecules for both pharmaceutical and industrial applications.
- The fungistudiedin this research work can be applied inthe large scale synthesis of any of the reported bioactive compounds.

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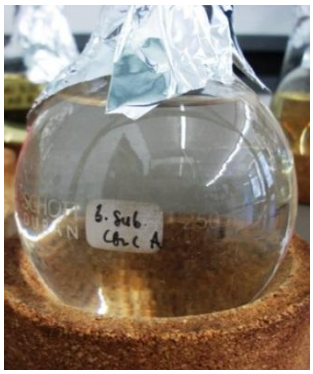
APPENDICES

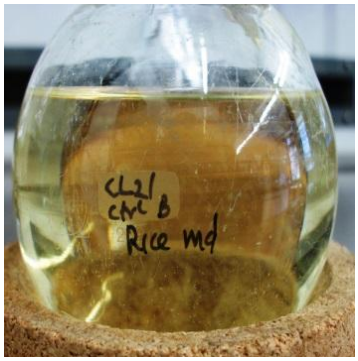
APPENDIX 1: IMAGES FOR MODULATION EXPERIMENTS ON *APIOSPORA MONTAGNEI*

A. CO-CULTIVATION



B. subtilis

B. subtilis + A. montagnei

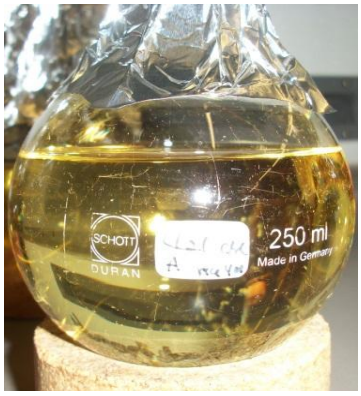


A. montagnei

Figure A1: Ethyl acetate extract of secondary metabolites of *B. subtilis*,*B. subtilis* +*A. montagnei* and *A. montagnei*

S. lividans

S. lividans + *A. montagnei*



A. montagnei

Figure A2: Ethyl acetate extract of secondary metabolites of *S. lividans*,*S. lividans* + *A. montagnei* and *A. montagnei*

B. MEDIA ENGINEERING AND MANIPULATION OF CULTURAL CONDITIONS

Figure A3: Erlenmeyer flasks showing fermentation/cultivation of *A. montagnei* at 22°C and 28°C

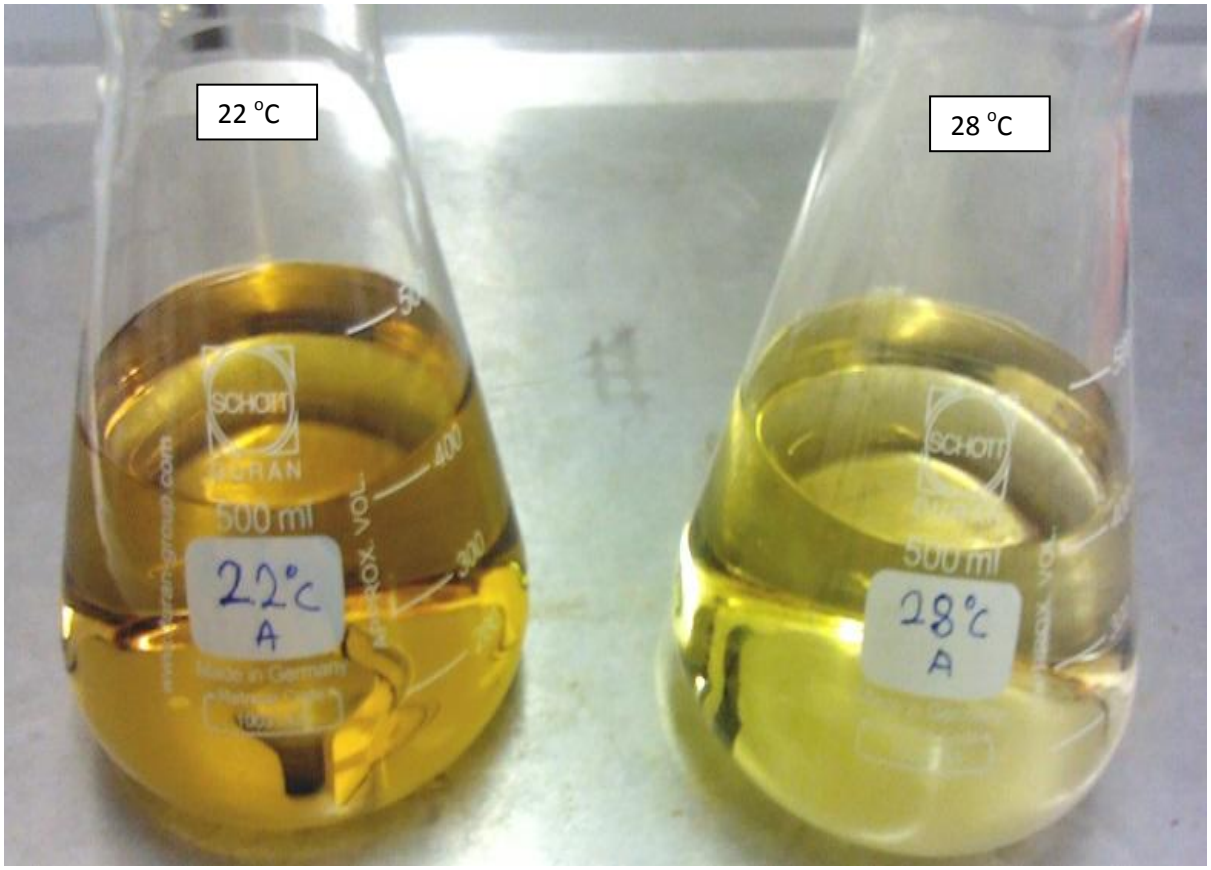
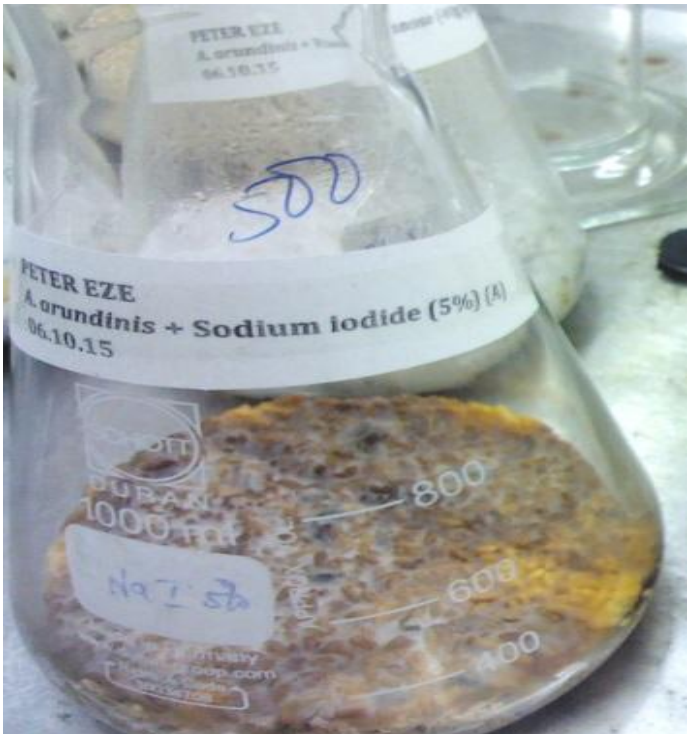


Figure A4: Ethyl acetate extract of secondary metabolites of *A. montagnei* cultivated at 22°C and 28°C



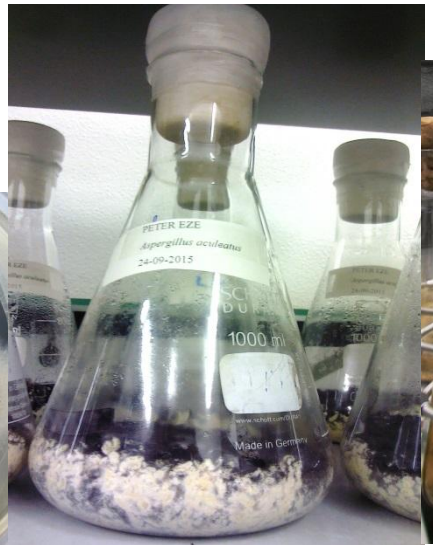
Flask showing cultivation of *A. montagnei* in Sodium Iodide (5% w/v)

Flasks showing cultivation of *A. montagnei* in Rhamnose (4% w/v)

Figure A5: Fungal Mycelia Growth Inhibition by Some of the Treatments

APPENDIX 2: IMAGES OF THE FERMENTATION AND EXTRACTION OF SECONDARY METABOLITES FROM *ASPERGILLUS ACULEATUS* AND *FUSARIUM EQUISETI*

***A. aculeatus* in Petri plate**



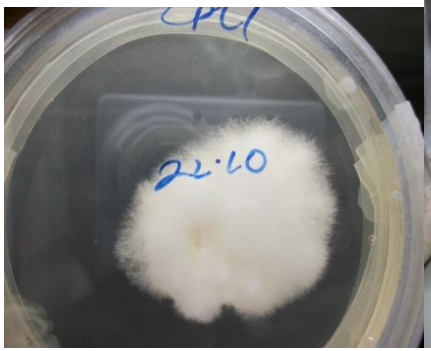
Cultivation of *A. aculeatus* on Rice Medium (Fermentation)

Extraction of secondary metabolites using ethyl acetate and mixed using the mechanical shaker

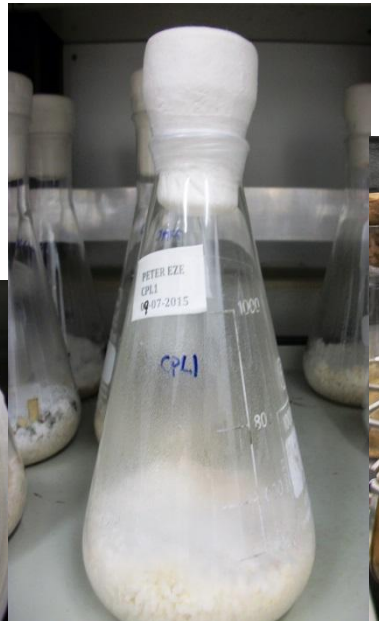
Ethyl acetate extract of secondary metabolites of*A. aculeatus*

Dried crude extract of*A. aculeatus*

Figure A6: Fermentation and Extraction of Secondary Metabolites from *Aspergillus aculeatus*



***F. equiseti* in Petri plate**



Cultivation of*F. equiseti*on Rice Medium (Fermentation)

Extraction of secondary metabolites using ethyl acetate and mixed using the mechanical shaker

Ethyl acetate extract of secondary metabolites of*F. equiseti*

Dried crude extract of*F. equiseti*

Figure A7: Fermentation and Extraction of Secondary Metabolites from *Fusarium equiseti*

APPENDIX 3: HPLC-GUIDED ISOLATION OF BIOACTIVE COMPOUNDS FROM *ASPERGILLUS ACULEATUS* AND *FUSARIUM EQUISETI*

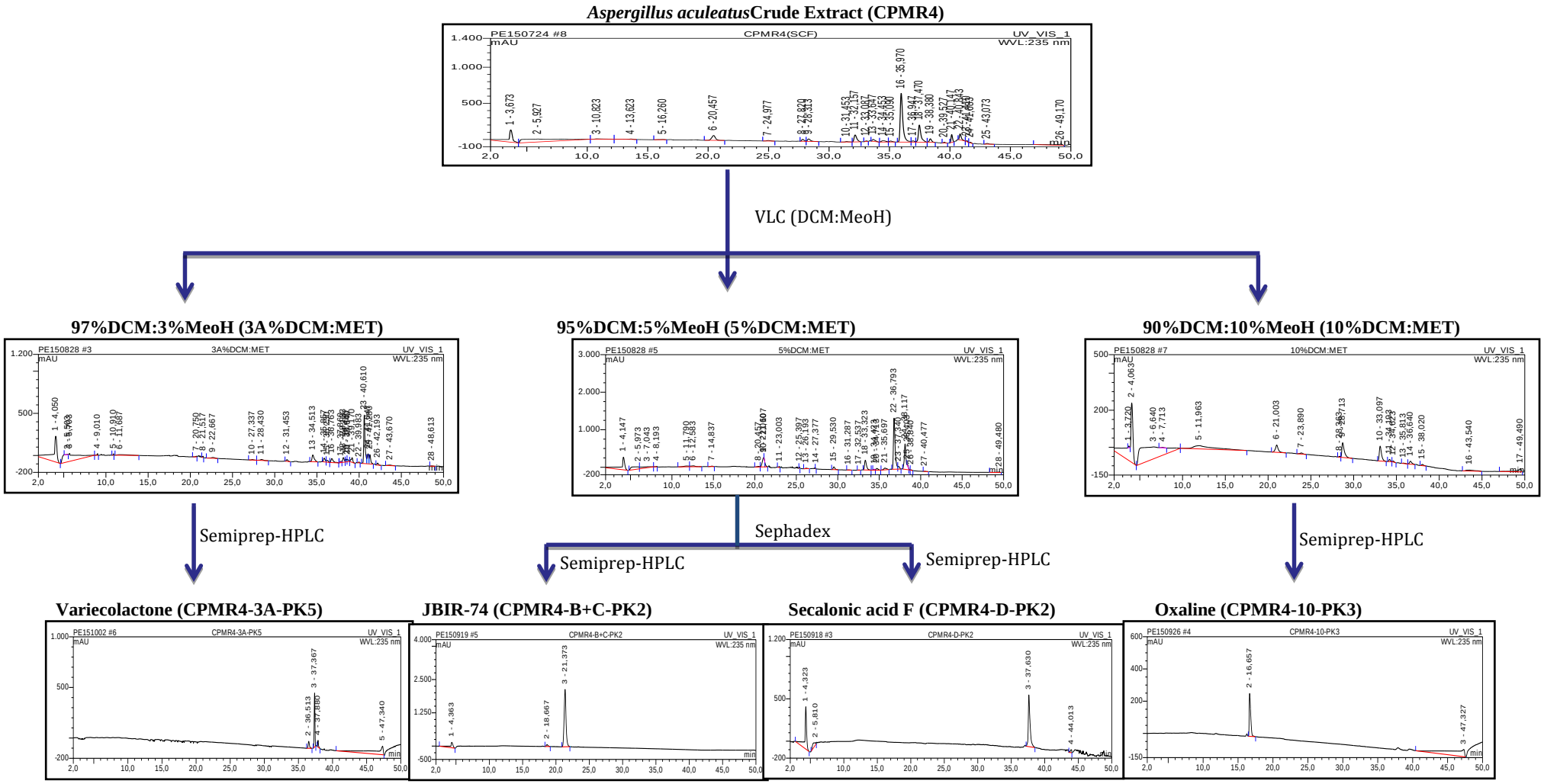


Figure A8: HPLC-guided Isolation of Compounds from *A. aculeatus* Crude Extract

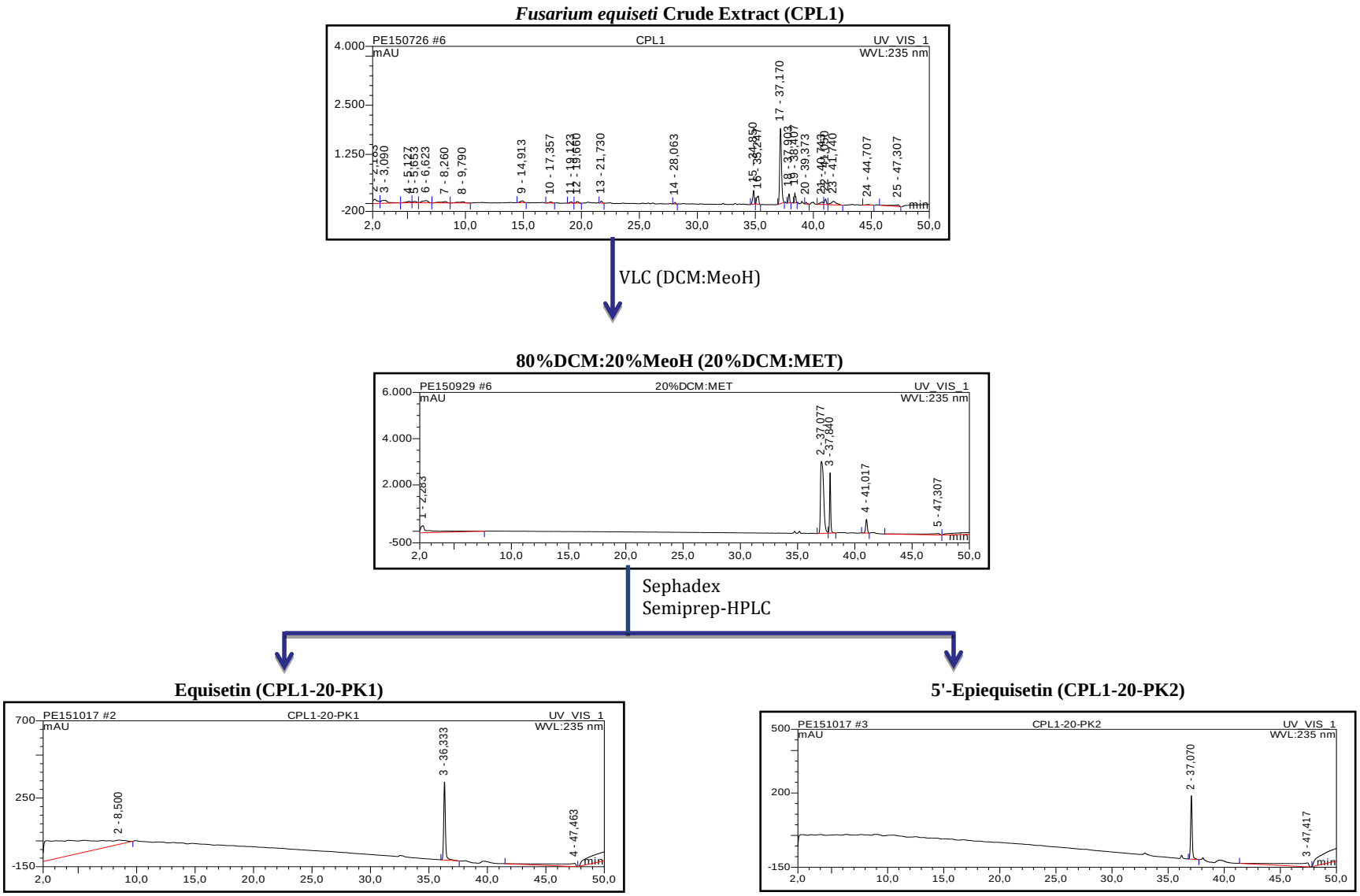


Figure A9: HPLC-guided Isolation of Compounds from *F. equisetin* Crude Extract

APPENDIX 4: ^{1}H-NMR COSY, ^{1}H-NMR HMBC,^{1}H-NMR HSQCAND^{13}C-NMR SPECTRA OF VARIECOLACTONE

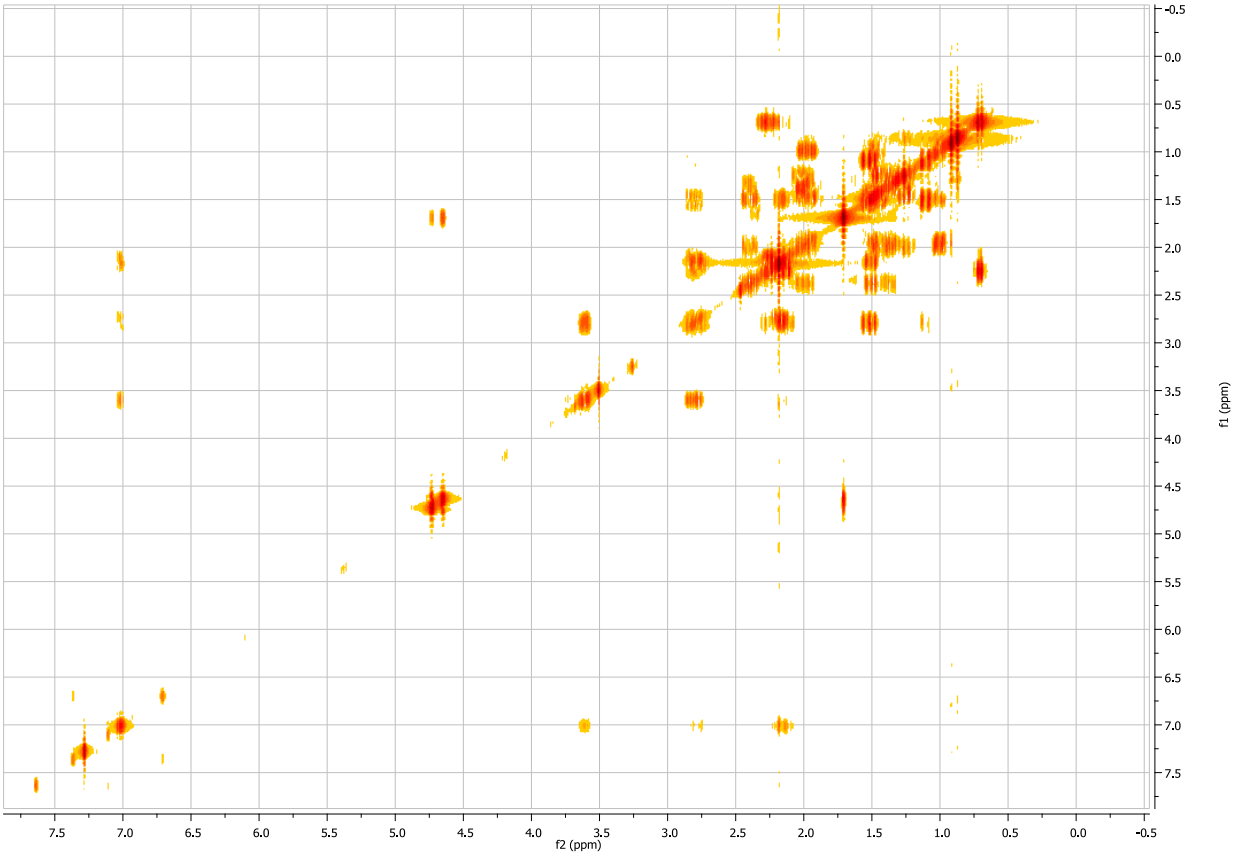


Figure A10: ^{1}H-NMR COSY Spectra (300Hz) of Variecolactone

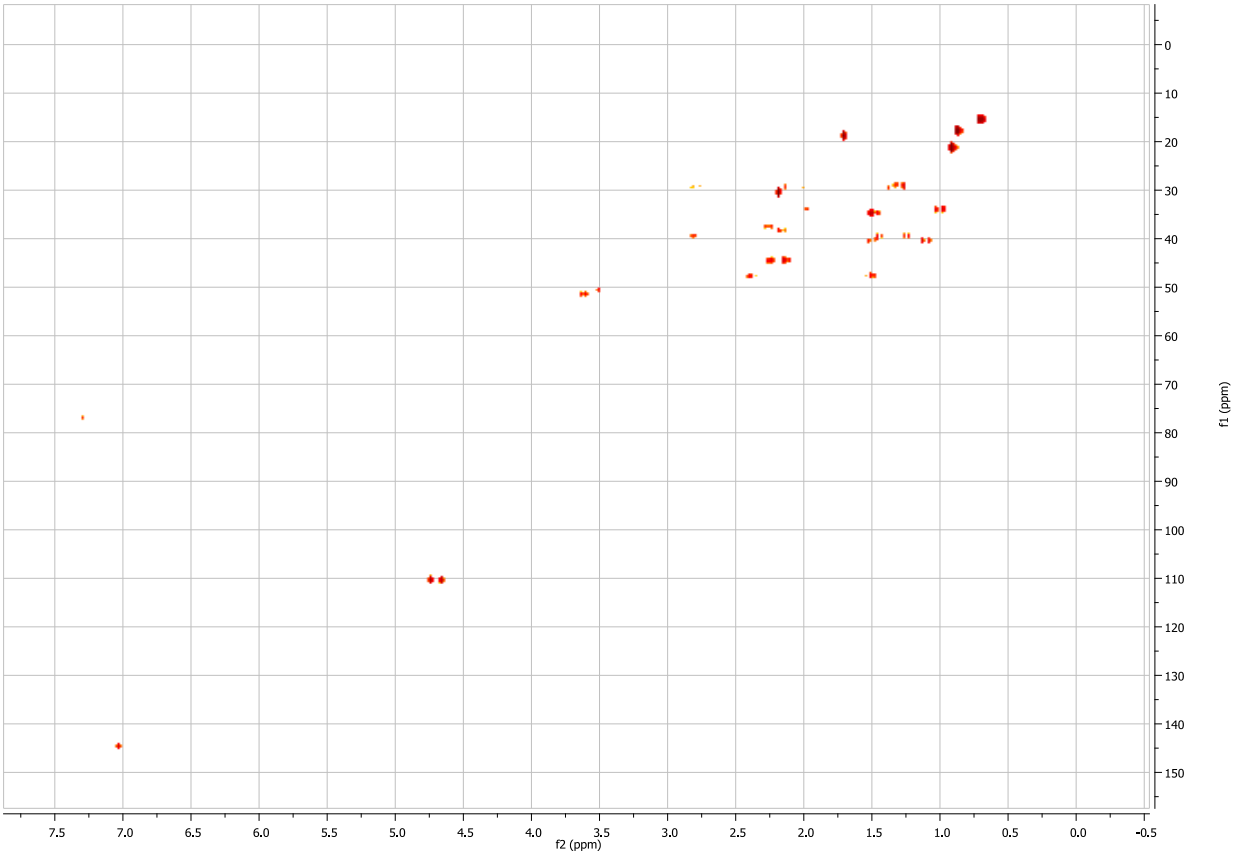


Figure A11: ^{1}H-NMR HMBC Spectra (300Hz) of Variecolactone

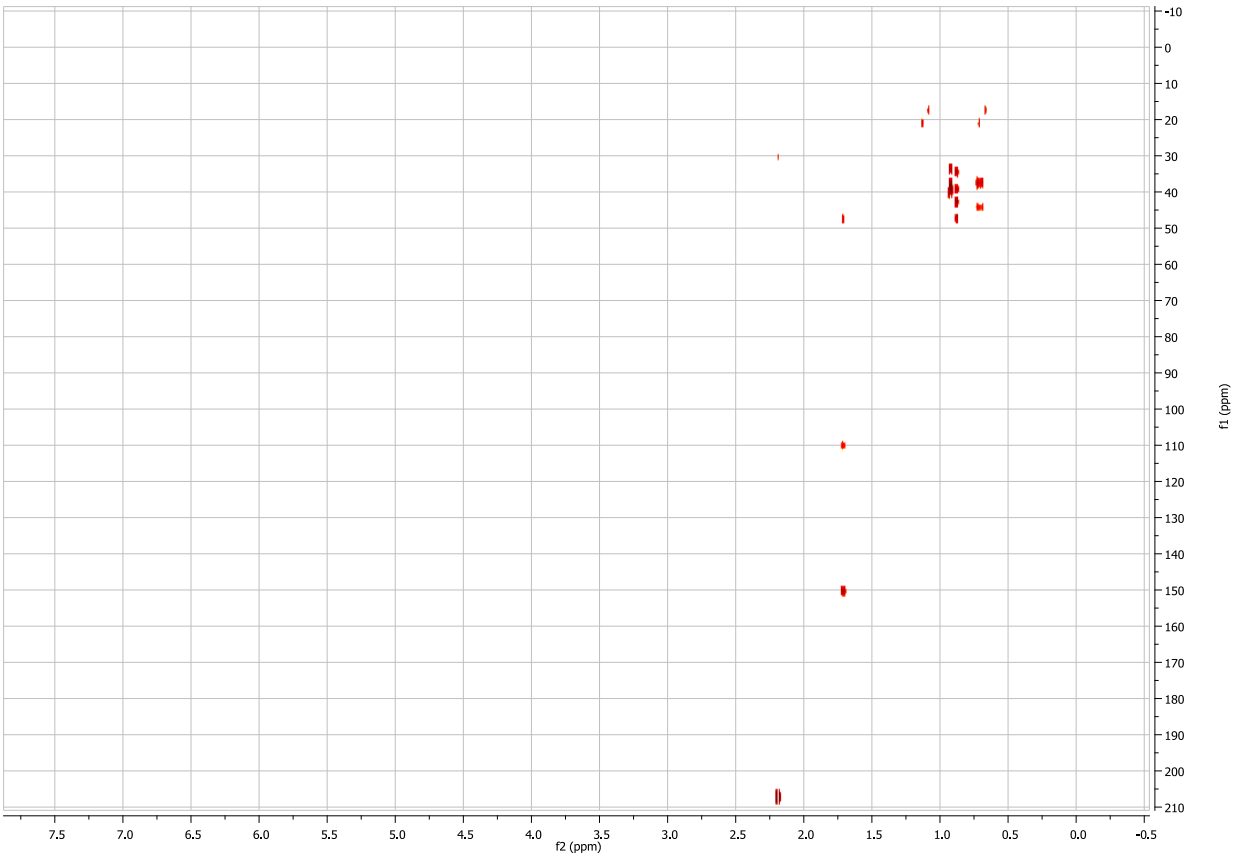


Figure A12: ^{1}H-NMR HSQC Spectra (300Hz) of Variecolactone

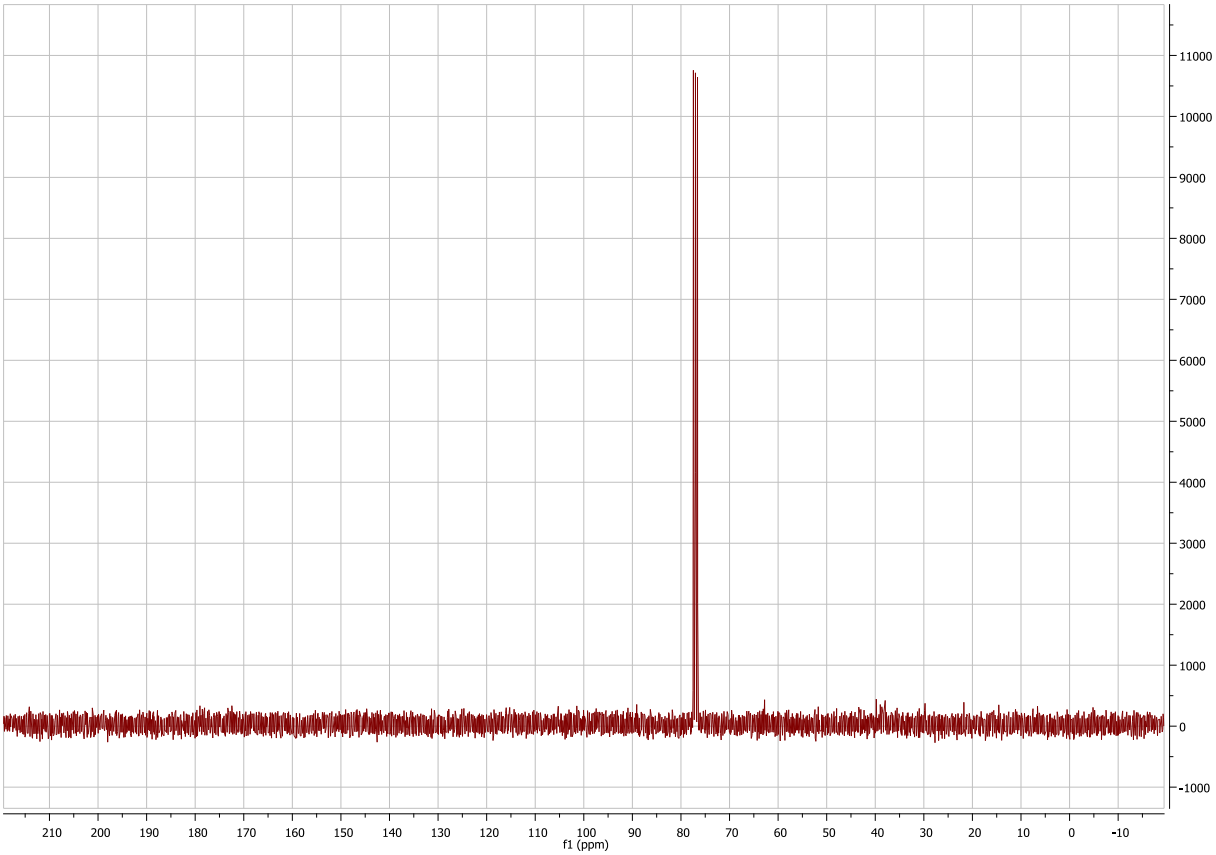


Figure A13: ^{13}C-NMR Spectra (300Hz) of Variecolactone