**COLLEGE OF BASIC AND APPLIED SCIENCES**

**UNIVERSITY OF GHANA**

# DISCOVERY AND DEVELOPMENT OF NEW CHEMICAL COMPOUNDS WITH ANTIFUNGAL ACTIVITY FROM MARINE ENDOPHYTIC FUNGAL SOURCES

**BY**

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**THIS THESIS IS SUBMITTED TO THE SCHOOL OF GRADUATE STUDIES IN PARTIAL FULFILMENT OF THE REQUIREMENT FOR THE AWARD OF DOCTOR OF PHILOSOPHY DEGREE IN MOLECULAR CELL BIOLOGY OF INFECTIOUS DISEASES**

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# DECLARATION

I Ethel Juliet Blessie hereby declare that this thesis is the outcome of my own research under the supervision of Dr. Patrick Kobina Arthur, Dr. Jonathan P. Adjimani, Dr. Dorcas Osei-Safo and Prof. James Adjaye. This thesis is submitted in fulfilment of the requirements of the PhD research project, and except where duly acknowledged or referenced it is entirely my own work. It has not been submitted, either in whole or in part, for any other award at the University of Ghana or elsewhere.

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# DEDICATION

To my grandmother Madam Mabel Adzo Ansah.

Your constant care and support for me throughout my life till your death in July 2018, keeps urging me to pursue greater heights. I was fortunate to have been nurtured by you.

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# TABLE OF CONTENTS

# LIST OF TABLES

# LIST OF FIGURES

# LIST OF ABBREVIATIONS

| | |
|---|---|
| **5-FU** | 5-Fluorouracil |
| **Acr** | Acrolein |
| **Amp B** | Amphotericin B |
| **Ben** | Benzoic acid |
| **Ca** | *Candida albicans* |
| **CIN** | Chromosome instability |
| **CyH** | Cycloheximide |
| **DNA** | Deoxyribonucleic acid |
| **DNTP** | Deoxyribonucleotide triphosphate |
| **EDTA** | Ethylenediaminetetra acetic acid |
| **erg2 (hom)** | Ergosterol 2 homozygous mutant |
| **erg2 (het)** | Ergosterol 2 heterozygous mutant |
| **eft1 (het)** | Elongation factor 1 heterozygous mutant |
| **eft2 (het)** | Elongation factor 2 heterozygous mutant |
| **FB** | Fraction from *n*-Butanol |
| **FD** | Fraction from dichloromethane |
| **FE** | Fraction from ethyl acetate |
| **FH** | Fraction from hexane |
| **Flu** | Fluconazole |
| **FM50** | Fraction from methanol diluted to 50% |
| **FW** | Fraction from water |
| **g** | Gram |

| | |
|---|---|
| **GFP** | Green fluorescent protein |
| **Gri** | Griseofulvin |
| **HPLC** | High Performance Liquid Chromatography |
| **HRMS** | High-resolution Mass Spectrometry |
| **ITS** | Internal transcribed spacers |
| **MEA** | Malt extract agar |
| **MEF** | Marine endophytic fungi |
| **mg/mL** | milligram/milliliter |
| **MIC** | Minimum inhibitory concentration |
| **mL** | Milliliter |
| **MTT** | 3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyl tetrazolium bromide |
| **NCBI** | The National Center for Biotechnology Information |
| **ng/µL** | Nanogram per microliter |
| **nm** | Nanometer |
| **OD** | Optical density |
| **Para** | Paromomycin |
| **PCR** | Polymerase chain reactions |
| **rDNA** | Ribosomal Deoxyribonucleic acid |
| **SC** | *Saccharomyces cerevisiae* |
| **Set** | Sertraline |
| **TLC** | Thin layer Chromatography |
| **UV** | Ultraviolet light |

| | |
|---|---|
| **yef3 (het)** | Yeast elongation factor 3 heterozygous mutant |
| **YPDA** | Yeast extract, Peptone, Dextrose agar |
| **YPMD** | Yeast extract, Peptone, Malt extract, Dextrose |
| **µL** | Microliter |

# ABSTRACT

Discovery of new drugs have shifted from plants sources to microorganisms due to the enormous diversity of the latter. Marine endophytic fungi (MEF) is underexplored as compared to their terrestrial counterparts for the discovery of anti-infection drugs. This study set out to identify and develop novel antifungal compounds from marine endophytic fungi sources using yeast genomics guided approach. Six priority marine fungal endophytes were fermented and the secondary metabolites produced were extracted using ethyl acetate. Crude extracts and fractions from solvent-solvent fractionation and chromatography were tested against *S. cerevisiae* and *C. albicans*. Majority of the extracts showed satisfactory antifungal activity (zone of inhibition > 11 mm). Sequence analysis of the fungi ITS-rDNA revealed that the isolates belonged to three major genera; *Aspergillus*, *Clonostachys* and *Meyerozyma*. An investigation of the mechanism of action of the extracts from the 6 endophytes indicated that these extracts targeted protein synthesis, ergosterol biosynthesis and RNA translation. The study also confirmed that production of bioactive metabolites by marine fungal endophytes is time-dependent and favours shorter incubation of fungal fermentation cultures. *In vitro* interactions of phenotype modulating compounds with either standard antifungal drugs or fungal extracts resulted in novel synergistic interactions against *Candida albicans* and *Saccharomyces cerevisiae*. Structural elucidation of the compounds in the bioactive fractions using mass spectrometry (ms/ms) identified 22 novel chemical structures which is anticipated to exhibit antifungal activity. Three fractions from MEF 134; 134 FD K1V1 V1, 134 FD K1V5V3 and 134 FD K2V3V5 were active against *S. cerevisiae* but not the pathogenic *C. albicans*. This is an indication of an antitumor cytotoxic compound and not an antifungal. The cytotoxicity effect of the three compounds against HepG2 cells was tested in a dose dependent manner. It was demonstrated that several cancer, metabolism-related pathways and gene

ontologies were regulated by the treatment with the 3 fractions from marine endophytic fungi. The data generated in this study highlights the opportunities of exploring fungal endophytes as useful sources of antifungal compounds for treatment of infections caused by pathogenic fungi.

# CHAPTER ONE

## INTRODUCTION

### 1.1 Background

When it comes to the treatment of human diseases there are many different sources from which compounds for treatment can be derived. One of these sources, which is of great importance for the treatment of disease, is that of fungi. In fact, the first ever antibiotic for the treatment of bacterial infections was isolated from a fungus in 1928 when Alexander Fleming discovered that a compound, now known as penicillin, produced by fungus was able to inhibit the growth and spread of certain types of bacteria. This discovery paved the way for the discovery of other antibiotics, which are the main treatment option for bacterial infections (Tan & Tatsumura, 2015). While antibiotics are an important aspect of modern medicine, there are also many other diseases which are not caused by bacterial pathogens but by fungi. In addition to antibiotics, fungi also produce many other compounds which are routinely used in the fight against human diseases. These compounds include Sordarins, Ergots, Cyclosporine A (Misiek & Hoffmeister, 2007).

Sordarins are produced by fungi and can be used for the treatment of human fungal pathogens. In particular, they are used for the treatment of yeast-like fungal infections (Liang, 2008). Besides infections, fungal compounds also play an essential role in the treatment of metabolic diseases. For example, ergots are alkaloids which act on the central nervous system and function by inhibiting noradrenaline and have been used for the treatment of high blood pressure (Panaccione, 2005). In addition, fungal compounds can also act on the immune system, one important fungal compound in this category is that of cyclosporine A. It is produced by fungi and is an immunosuppressant which is routinely used after organ or bone marrow transplantation (Kück *et*

*al.*, 2014) . Of all the different types of fungi, one group of fungi, the Marine Fungal Endophytes, has recently been recognized as one of the most promising sources for drug discovery (Sarasan *et al.*, 2017).

Endophytic fungi are species that live symbiotically with the host such as algae by entering their cells. Previously the compounds isolated from fungi focused on land-based fungi. Endophytes are distinct from other types of fungi, such as the mycorrhizal fungi in that they live completely within their host plant. Fungal endophytes can be present in both algae and sea-grasses. In general, endophytes invade all tissue and can be present both intracellular and intercellular (Rodriguez *et al.*, 2009). With respect to fungal endophytes, they significantly impact the host in that they provide disease resistance, increased environmental stress tolerance, and increased recycling of nutrients (Sturz & Nowak, 2000). Because of this symbiotic relationship, many endophytes produce unique bioactive metabolites that could be exploited for the treatment of human disease (Flewelling *et al.*, 2013). The vast majority of the research into marine fungal endophytes has focused on *Aspergillus sp.* (Deshmukh *et al.*, 2018).

Therefore, endophytic fungi from algae offer a new potential source of compounds. For over 2000 years various forms of algae have been used in ancient medicine and in cosmetics for their antimicrobial and anti-spoilage properties (Bhadury & Wright, 2004).

Due to the vast number of species of fungal endophytes, there is a potentially large resource of species from which to discover new compounds. Recently there have been several significant studies with regards to bioactive metabolites from marine fungal endophytes. In particular, there are several different classes of bioactive compounds which are of particular interest, these include anticancer compounds, antibacterial compounds, antimycotic compounds, antioxidant compounds, and additional bioactive compounds. Previously, epipolysulfanyl-diozopiperazines,

which are used in the treatment of leukemia have been identified from marine fungal endophytes. Additionally, other anticancer compounds such as noduliprevenone which is a competitive inhibitor of cytochrome P450-1A has been identified (Sarasan *et al.*, 2017).

With regards to antibacterial compounds, research in marine endophytes has focused on searching for novel derivatives of xanthones. Previously, a study identified several new xanthone derivatives which were isolated from *Wardomyces anomalus*. Similarly, novel xanthone compounds have been identified from red algae. Novel macrolides have been isolated from algae such as Asporyzin C and JBIR-03 (indole-diterpene) (Sarasan *et al.*, 2017). With respect to antimycotic compounds, screening from endophytes has identified antimycotics such as Nigerasperone C from fungi which inhabit brown algae (Zhang *et al.*, 2007). While antibiotics and antimycotics are essential for fighting human diseases, antioxidant compounds also are essential for improving human health. In this regard, effective hydroquinone derivatives have been identified from fungi which inhabit brown algae (Abdel-Lateff *et al.*, 2003). Finally, in addition to these compounds, other important therapeutics such as a tyrosine kinase inhibitor isolated from *Chaetomium sp* have been found. As currently there is a demand for novel protein kinase inhibitors, this is a significant development (Nagle *et al.*, 2004).

### 1.2 Significance

Overall, when it comes to the treatment of human diseases researchers are constantly searching for new bioactive compounds. One group of species which hold promise for the discovery of bioactive compounds for therapeutics is that of the marine fungal endophytes. In terms of future directions, while there have been many compounds discovered one of the main concerns is that of commercial production. Therefore more research is necessary in order to develop large-scale identification and large-scale production methods. Based on the previously identified compounds, marine fungal

endophytes are a rich source of bioactive compounds for fighting various human diseases. Thus, increased scientific information on the potential health benefits of these compounds is critical. An expanding body of research suggests the ability of compounds from marine fungal endophytes to inhibit fungal pathogens. Thus, by searching for and identifying more of these compounds, treatment of fungal infections may be improved vastly in immunocompromised people such as people living with AIDS, transplant patients among others.

### 1.3 Hypotheses

① Secondary metabolites produced by marine endophytic fungi will exhibit anti-fungal activity against *Saccharomyces cerevisiae* and *Candida albicans*.

② The secondary metabolites will have new structures within existing classes of compounds and may also belong to new classes of compounds.

③ The antifungal compounds produced by the marine endophytic fungi may have a specific mechanism of action.

④ The new antifungal compounds will exhibit very low toxicity to human cells.

### 1.4 Aim

The aim of the project was to discover and develop potential novel antifungal compounds from marine endophytic fungi sources using a yeast genomics guided approach

### 1.5 Specific research objectives

Five research objectives were proposed to test these hypotheses

① To isolate marine endophytic fungi and to determine the anti-fungal activity against *Saccharomyces cerevisiae* and *Candida albicans*.

② Identify the bioactive metabolites and to elucidate their structures.

③ Determine the specific mechanisms of action of the antifungal compounds produced by the marine endophytic fungi.

④ Determine the cytotoxicity of the new antifungal compounds against human cells.

⑤ Identify the fungal species that produced the bioactive metabolites

# CHAPTER TWO

## 2.1 LITERATURE REVIEW

## CURRENT OPPORTUNITIES AND BOTTLENECKS IN ANTIFUNGAL DRUG DISCOVERY

### 2.2 Introduction

Antifungals are difficult to produce because fungi are eukaryotes hence any substance toxic to fungi may also be toxic to the human host (Dixon *et al.*, 1996). There are currently only three structural classes of compound used to fight systemic fungal infections, namely polyenes, azoles and echinocandins with the other classes only being used externally due to the negative side effects associated with their systemic administration (Vandeputte *et al.*, 2011). The rate of antifungal discovery has been much slower than antibiotic discovery, with the newest class of antifungal agents, echinocandins, having taken 30 years to develop (Butts & Krysan, 2012). Whilst the rate of discovery of antifungal agents is comparatively slow, there is increasing incidence of fungal infections as a result of increasing incidence of immunodeficiency in patients, often related to AIDS, cancer, old age, diabetes and invasive surgical procedures like organ transplants (Vandeputte *et al.*, 2011). The limited number of new antifungal agents being introduced and also the misuse of the ones available have led to increased antifungal resistance and this is a major course of concern (Pfaller, 2012). Antifungal resistance is the means by which a fungal pathogen persists in the presence of an antifungal agent that would normally limit its growth or eradicate it (Kanafani & Perfect, 2008). Treatment of people infected with these resistant pathogens may lead

to death due to untreatable infections (Drgona *et al.*, 2014). Hence, the development of antifungal drugs that are highly effective is urgently needed.

The marine environment has been neglected as a source of novel bioactive compounds with the investigation of its capacities only beginning as recently as in 1970 (Dias *et al.*, 2012). The harsh habitat of the ocean requires organisms which live there to have special adaptations that enhances their survival in those environments. Adaptation to the environment may be mediated by means of expression of genes that confer an advantage to species living in that environment. Studies have shown that marine endophytic fungi are capable of producing secondary metabolites that are potent anticancer, antifungal and antibacterial agents (Rateb & Ebel, 2011).

However, there are many challenges encountered in the search of compounds from basic resources. Strategies such as bioactivity guided fractionation are important steps to consider in order to shorten the duration required for compound discovery while avoiding waste of materials. There is also the need to develop more sensitive and mechanism-specific bioassays to make it possible to detect and isolate compounds that are produced in low amounts by using mutant strains and chemical treated yeast cells. Another important strategy for antifungal drug discovery is the use of scalable fermentation processes which makes it possible to produce large amounts of starting materials to be able to identify low abundance metabolites. This can be coupled to powerful separation schemes that rely on several orthogonal techniques capable of resolving the high level of complexity of fungal metabolites. Since fungi are eukaryotes, the search for new compounds against fungi can be advantageous in terms of finding treatment options for other eukaryotic pathogens and cancer. Understanding how fungal pathogens respond to stressful conditions could provide knowledge about how resistance is built by fungi against drugs over time. In this review,

some strategies for prospecting for natural products, predicting cytotoxicity and mechanism of action at early stages of drug discovery are discussed (Fig 2.1).

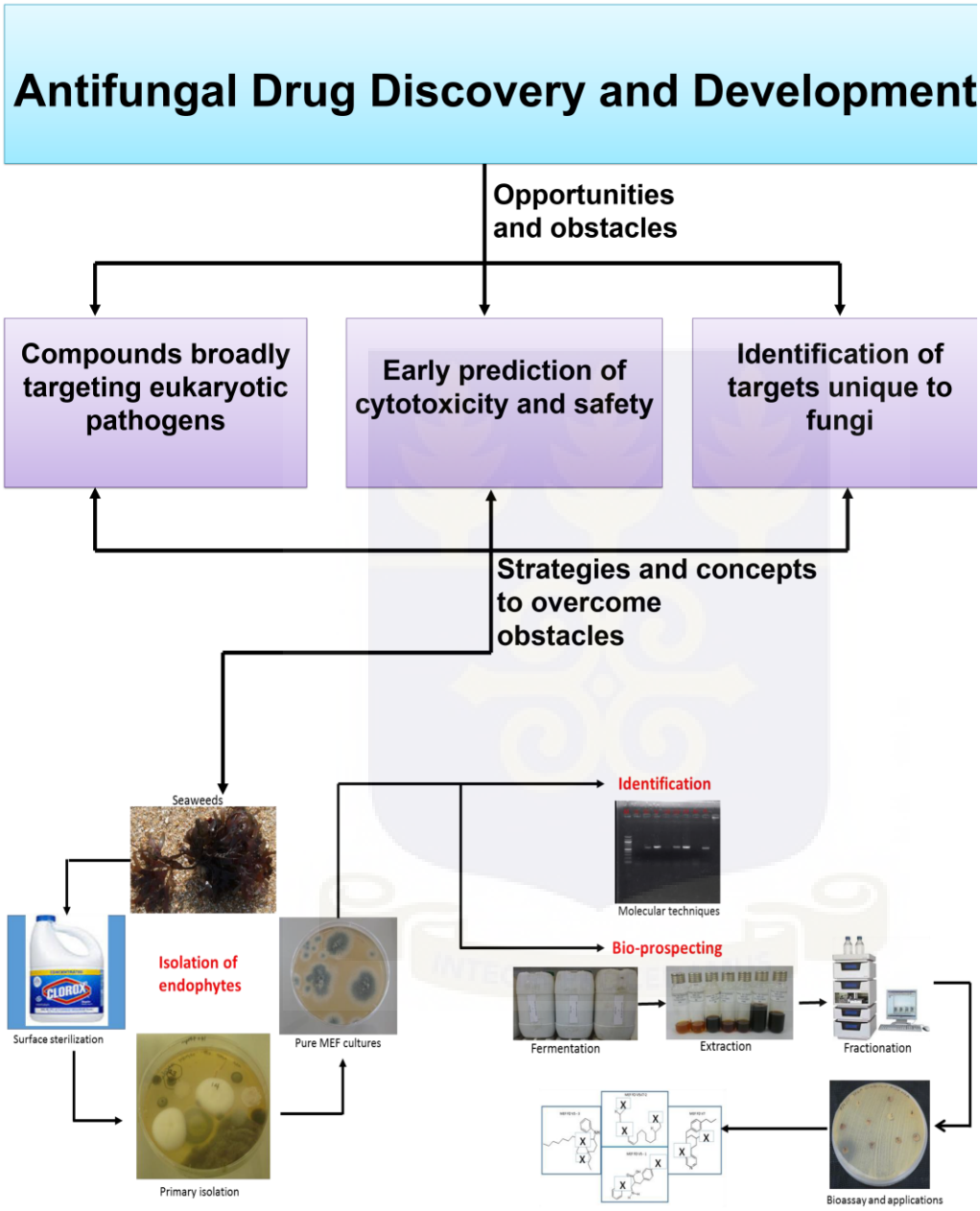


Fig 2.1. A schematic diagram showing bioprospecting techniques from marine fungal endophytes.

## 2.3 Opportunities and obstacles in antifungal drug discovery

### 2.3.1 Selectivity of eukaryotic host targets

Fungi are eukaryotes and numerous possible targets for treatment exist in mammals (Roemer & Krysan, 2014). A number of studies revealed that there are genes in yeast and humans that are responsible for producing similar proteins (Gibbs *et al.*, 1998; R. S. Williams *et al.*, 1997). Functional analysis of RAS1 and RAS2 from *Saccharomyces cerevisiae* show that the genes have similar evolutionary origin to mammalian RAS gene (Kataoka *et al.*, 1984). Sequence comparison protein coding genes of yeast to that of mammal showed that 31% of genes from yeast, capable of producing proteins (open reading frames), were related to mammalian protein sequences (Botstein *et al.*, 1997). Therefore, there are high probabilities of discovering and characterizing novel proteins from yeast that are homologous to that of humans. This observation pose a lot of challenges in terms of antifungal drug discovery due to high level of toxicity to human cells. In this context, *Saccharomyces cerevisiae* which used to identify potential targets for antifungal compounds can also be used as a model organism to screen drugs for many human diseases (Karathia *et al.*, 2011). As humans and yeast have many similar genes, compounds that are toxic to yeast could be potentially toxic to humans due to some conserved genes. Therefore, the task is to identify targets in fungi that are unique and not found in humans for a potential antifungal drug development which require a more stringent screening assay.

### 2.3.2 Broadly targeting eukaryotic pathogens

The current antifungal therapeutic choices for invasive fungal diseases are restricted to these structural types of drugs: echinocandins, azoles and polyene (Pound *et al.*, 2011). All of these drugs target either the ergosterol, ergosterol biosynthesis pathway or cell wall of fungal pathogens. The polyenes were the first class of drugs, of which Amphotericin B is the most frequently used.

Amphotericin B (AmB) binds to ergosterol, a membrane sterol responsible for yeast cell physiology (Gray *et al.*, 2012). Another mechanism of action is membrane permeabilization through channels which enhances drug efficacy (Gray *et al.*, 2012). This drug is very effective and pivotal in treating systemic fungal infections. Despite its proven effectiveness, its use has to be discontinued in some cases because of toxicity to certain organs of the human body (Laniado-Laborín & Cabrales-Vargas, 2009). It has shown that only free AmB can affect the membranes of both mammals and fungi whilst, Conjugated AmB can only infiltrate the membrane of fungi (Kagan *et al.*, 2012).

Azoles are the most widely used antifungal drugs with the most available azoles being fluconazole (Tavallaee & Rad, 2009). Ergosterol is the major controller of membrane fluidity and membrane integrity in fungal cells (H. Yang *et al.*, 2015). For cell integrity to be maintained, the sterols must not have C-4 methyl groups. The major mode of action of azoles is to inhibit 14a-lanosterol demethylase that changes lanosterol to ergosterol which leads to malformation of plasma membrane (Hitchcock *et al.*, 1990). Resistant species are a major problem in most fungal infections involving *Candida albicans* and *Aspergillus fumigatus* (Leonardelli *et al.*, 2016). Fluconazole resistance involves modifications in the following genes; ERG11 and efflux pump genes with the increased expression of efflux pump genes contributing immensely to the fluconazole drug resistance in *C. albicans* (Pourhajibagher *et al.*, 2017).

The new class of antifungal agents is the echinocandin family and Caspofungin is the first compound discovered in this class (Letscher-Bru & Herbrecht, 2003). Other members include Micafungin and Anidulafungin with Caspofungin exerting its antifungal activity by inhibiting synthesis of β (1, 3)-D-glucan (Letscher-Bru & Herbrecht, 2003). There are cases of echinocandin

resistance, particularly resistance of C. *albicans* to Caspofungin has been shown to be connected to alterations in the FKS1 gene (Balashov *et al.*, 2006).

Most of these antifungal agents target a wide-range of fungal pathogens and other eukaryotic pathogen e.g. *Leishmania* sp. However, these other pathogens have also developed ways to overcome the lethal effects of these antifungal agents with resistance to fluconazole linked to the increased expression of efflux pumps (Pourakbari *et al.*, 2017). Hence, there is a need to discover more robust antifungal agents that can overcome the activities of the efflux pumps and also have a better mechanism of action superior to what the present compounds are known for. Similarly, these new compounds must target a broad range of fungal and eukaryotic pathogens.

### 2.3.3 Predicting safety/cytotoxicity

*Saccharomyces cerevisiae* is a useful model for studying eukaryotic biology, its genome has been sequenced and research is ongoing to define the role of genes whose function is unknown (Sherman, 1991). It is now known that about 31% of protein-encoding genes of yeast have high homology to that of mammals (Botstein *et al.*, 1997), therefore, yeast can be a good model and predictor of human cytotoxicity.

The cytotoxicity of allyl alcohol has been investigated using yeast as an eukaryotic model organism. The enzymatic conversion of allyl alcohol into the acrolein (Acr), a very toxic substance *in vivo*, is mediated by histone tails and chromatin modifiers (Golla *et al.*, 2015). In a related study to monitor toxicity and genotoxicity in water samples using a yeast (eukaryotic) based assay, the cells were adapted to display a green fluorescent protein (GFP) as an indicator every time DNA damage was repaired and the general toxicity was measured as a function of yeast cell proliferation (Knight *et al.*, 2004).

Drugs are continuously pulled out of the market due to late discovery of toxicities and it is therefore essential to test novel compounds at early stages of discovery for toxicity (Jaeschke *et al.*, 2002). Currently, drug candidates and toxicity screening rely heavily on human cell-based assays estimated to represent important features of *in vivo* pharmacology, but yeast-based assay can offer cost-effective alternative.

### 2.3.4 Predicting anti-cancer activities

Screening for anticancer agents has progressed from broad phenotypic cell viability assays to target-oriented and pathway-specific cell-based assay. This has led to the approval of many molecularly targeted drugs and cancer chemotherapeutic discovery focuses more on identifying compounds which target many cancer pathways while causing minimal toxicity to non-cancer cells (D. Gao *et al.*, 2014). Humans and yeast have some similar genes including those well known to be associated with cancer (Foury, 1997). The generation of yeast strains with mutations in the genome integrity maintenance genes leads to Chromosome instability (CIN), making yeast suitable for cancer drug screening (Stirling *et al.*, 2011).

There has been modeling of many human cancer-associated DNA polymerase in yeast with the modeling of DNA polymerase ε Variants (Polε-P286R) in yeast revealing an effect that surpass proofreading insufficiency and suggest the involvement of other infidelity factors (Barbari *et al.*, 2018). It was thought that ultra-mutated cancers without mismatch repair mistakes had defects in the DNA polymerase ε (Polε) which resulted in defective proofreading. However, recent findings in yeast show the involvement of other factors (Barbari *et al.*, 2018). *Saccharomyces cerevisiae* is an excellent organism for studying eukaryotic processes such as DNA replication and segregation, stress responses among others as well as the therapeutic target associated with these processes. Genetic and epigenetic modifications in these processes are also common in cancers hence, yeast

can be used as a predictor of anti-cancer activity of novel compounds (Menacho-Marquez & Murguia, 2007). Studying the antifungal activity of a particular compound using yeast as a model can provide the unique opportunity to also discover potential anticancer compounds as shown in this study.

### 2.4 New strategies and concepts to overcome the bottlenecks of antifungal drug discovery

#### 2.4.1 Marine fungal endophytes and their useful metabolites

The discovery of penicillin by Sir Alexander Fleming in 1928 from the fungus *Penicillium notatum*, has resulted in a breakthrough in the treatment of bacterial infections (Drews, 2000; Kjer *et al.*, 2010). This encounter has shifted the attention of drug discovery from plants to microorganisms.

Fungi are found everywhere in the environment including the air, soil, plant tissues and water bodies. They have the ability to colonize different ecological niches and are able to feed on various types of organic and inorganic substances. Fungi are members of the five eukaryotic kingdoms. The kingdom fungi are divided into three sections which are hinged on the type of sexual reproductive structures possessed. These divisions include; Zygomycetes (zygospores), Ascomycetes (ascospores) and Basidiomycetes (basidiospores). Fungi are mostly heterotrophic, filamentous and have cell walls made of chitin (Pusztahelyi, 2018).

Endophytes reside symbiotically within the internal tissues of higher plants without causing any injuries to the plants (J. Liu *et al.*, 2004). Remarkably, endophytes maintain virtually the same virulence factors as pathogens and manufacture many enzymes leading to the colonization of the host (Schulz *et al.*, 2002). The interaction is often without symptoms, for as much as endophytic

virulence and host defense maintain balance. Often, endophytes are not host specific and their relationship with plants are mostly accidental encounters (Joseph & Priya, 2011).

In spite of this, plants are affected by endophytes in various ways as is reported for the fungal endophyte-grass association between ascomycete *Epichloë typhina* and *Dactylis glomerata*. It was observed that *E. typhina* was harmful to this grass orchard even though it is not clear what particular effect the fungi had on the grass (Sampson, 1933). In a related study it was observed that cattle feeding on fescue grass were suffering from a strange disease. It was later recognized that the presence of the endophyte *E. typhina* in the fescue grass produced a vaso-constricting mycotoxin which resulted in the symptoms that were reported (Bacon *et al.*, 1977; Pulsford, 1950). Studies have shown that endophytic fungi, especially root mycorrhizal fungi, facilitate plant nutrient uptake which leads to growth stimulation (Schmidt *et al.*, 2008). Endophytes are also known to enhance growth by producing phyto-hormones. They protect the host from pathogens by competing with the pathogen for the same resources (Lockwood, 1992). Generally endophytes may include; fungi, bacteria and archaebacterial among others (Strobel & Daisy, 2003).

Fungal endophytes have strong effects on stimulation of plant growth and many metabolites are produced as a result of this fungi-plant interaction. These metabolites may be potential sources of new compounds for exploration in medicine and industry (Sturz & Nowak, 2000). However, endophytes are not well studied specifically towards the discovery of drugs effective against human diseases. Due to this, compounds from endophytes are only categorised to few classes of biological activities like anticancer and antifungal properties. Exploration of the capacity of endophytes as potential sources of useful drugs only began in the 1990s after the discovery of taxol, which is an anticancer drug (Strobel *et al.*, 1996).

In the ocean environment, algae presents a huge prospect of therapeutic opportunities and have been studied in old-fashioned medicine for more than 2,000 years in Asia and Africa (Sarasan *et al.*, 2017). Present experimental evidence has attributed the potency of algae to colonization by endophytes. There are two main categories of algae: macroalgae and microalgae. Macroalgae, also known as seaweeds are made up of red, green and brown algae (El Gamal, 2010) and represent the second largest source of marine fungi (Jones, 2000).

Marine endophytic fungi are eukaryotic spore producing organisms that can be derived from mangrove plants, algae, sponges among others (Kjer *et al.*, 2010). The marine environment is multifaceted and contains a wide-ranging variety of fungal diversity (Hong *et al.*, 2015). Many marine fungi are similar to their terrestrial counterparts, for example, *Aspergillus sp*., *Cephalosporium sp*. or *Penicillium sp*. (Manimegalai *et al.*, 2013; Park *et al.*, 2014). Hence marine fungi are not a taxonomically distinct group but rather ecologically and physiologically categorized and adapted. Also these fungi can be obligate or facultative in nature and reproduce in salt or brackish water environment which implies that this study had to use similar conditions for culturing in the laboratory (Hyde *et al.*, 2000). Fungi that are designated endophytes are found throughout the eight phyla of the fungi kingdom and are not limited to any phylum of a group phyla.

Marine endophytes are able to synthesize structurally complex chemicals due to extreme environmental conditions associated with marine life in the form of sharp variations in pH and salinity, reduced oxygen levels, nutrients and pressure (Maria, 2003). These chemicals are known as secondary metabolites and are not directly involved in normal growth, development and reproduction of the organism. They usually have functions which are used as survival tools (pigments) for the organism.

Although distinct from primary metabolites, secondary metabolites are derived from primary metabolites. For example, the primary metabolite acetate which is involved in fatty acid metabolism is also a regular source of carbon for the biosynthesis of secondary metabolites from polyketide and terpene classes (Mann, 1982). Terpenes derived from acetate are synthesized through the mevalonate pathway (Fig 2.2) (Turner, 1971). Acetyl coenzyme A, a primary metabolism intermediate, feeds into the mevalonic acid pathway to form terpenoid secondary metabolites. Phosphoenolpyruvate, an intermediates of the pentose phosphate pathway can be directed through the shikimic acid pathway for the production of phenols and nitrogen containing secondary metabolites (Fig 2.2) (Dias *et al.*, 2012; Taiz & Zeiger, 2010). The shikimate pathway is known to produces natural products that are aromatic in nature. Secondary metabolites are assigned into several classes depending on the functionality of the molecule (Non-ribosomal peptide, Indole alkaloids, Terpenes and Polyketides).

**Table 2.1.** Fungal secondary metabolites and their application.

| Antifungal metabolite | Class | Application |
|---|---|---|
| Aflatoxin B1 | Polyketides | Carcinogenic |
| Cyclosporine | Nonribosomal peptide | Immunosuppressant |
| Ergots | Indole alkaloids | Hypotensive; induce contractions |
| Gibeberellins | Terpenes | Plant growth hormone |
| Penicillins | Nonribosomal peptide | Antibiotic |
| Statins | Polyketides | Hypocholesterolemic |
| Leptosin A | Glycoside | Anticancer |
| Citrinal A | Terpenes | Anticancer |
| Cytochalasin D | alkaloid | Anticancer |
| Asporyzin C | indoloditerpene | Antibacterial |
| Asperamides A | sphingolipid | Antifungal |
| Penicisteroid A | steroid | Antifungal |
| Epicoccone | Polyketides | Antioxidant |

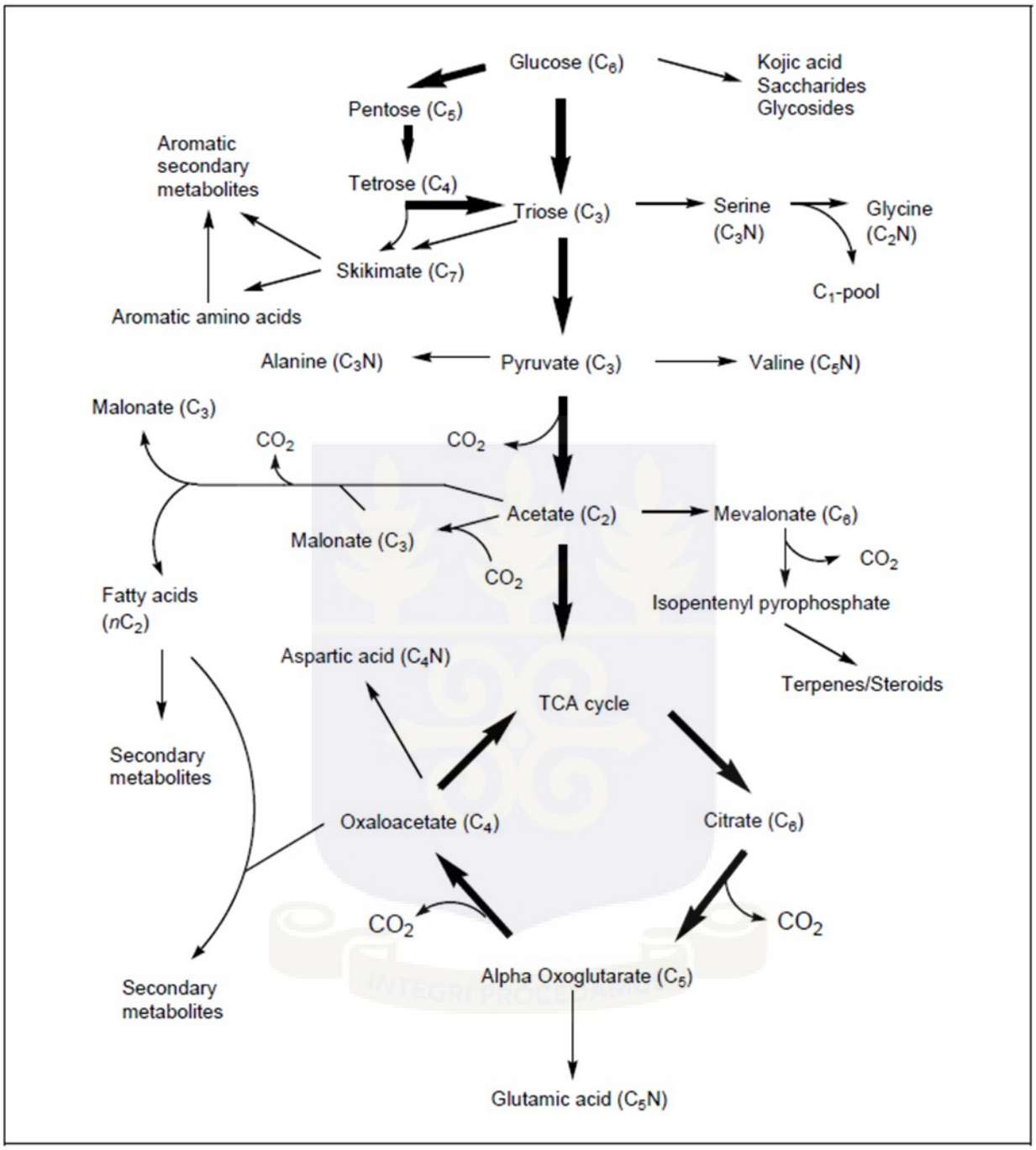


Fig 2.2. A schematic diagram showing the formation of secondary metabolites from primary metabolites (Turner, 1971). The tick black lines represent the main pathways for glucose oxidation.

### 2.4.2 Biopharmaceutical compounds from marine endophytic fungi

Secondary metabolites exhibiting antimicrobial activity are usually small molecular weight compounds and are active against other organisms at very low concentrations. As very limited antifungal drugs are currently available, algal endophytes could be excellent sources of bioactive secondary metabolites which can be used as templates for creating new pharmaceutical compounds. Current studies have shown the effectiveness of these metabolites as antibacterial, antifungal and anticancer among others. This section of the review will describe these compounds in more detail.

Antifungal screening of a brown alga endophytic fungus found Lactones which exhibited fungicidal activity against *Fusarium oxysporum* at an MIC of 100 µg/mL (R.-y. Yang *et al.*, 2006). In addition, a number of new indole alkaloids from marine-derived endophytic fungus *Eurotium cristatum* EN-220 showed antibacterial activity against *Escherichia coli* and *Staphyloccocus aureus* (F.-Y. Du *et al.*, 2012). *Penicillium chrysogenum* QEN-24S obtained from marine red algae, produced metabolites which showed inhibitory activity against a plant fungal pathogen (S.-S. Gao *et al.*, 2010). It was observed that Penicisteroid A also from the same organism displayed potent antifungal and cytotoxic activities.

Many bioactive metabolites exhibiting antifungal activities against *Candida albicans* were isolated from *Xylaria*, among them were multiplolides A and B which had activity against *C. albicans* (Boonphong *et al.*, 2001). Chaetomugilin A and D, which had antifungal activities, were isolated from *C. globosum*. Moreover, 45 endophytic fungal strains were isolated from the sea weed *Benthonella tenella*. Extracts and fractions from 10 of these 45 endophytic fungi were evaluated for cytotoxicity, antifungal and antibacterial activities. It was observed that two out of the ten

selected strains exhibited strong antifungal activity and were less toxic to HL-60 (human leukaemia) cells (Felício *et al.*, 2015).

Helicascolide C from an endophytic *Daldinia eschscholzii* strain showed fungistatic activity against the plant pathogenic fungus *Cladosporium cucumerinum* (Tarman *et al.*, 2012). New xanthone derivatives (yicathin A-C) obtained from endophytic fungi *A. wentii* pt-1 of marine red alga exhibited inhibitory activity against *Escherichia coli*, *Staphylococcus aureus* and *Colletotrichum lagenarium* (R. R. Sun *et al.*, 2013). Chemical investigation of *Leptosphaeria* spp. isolated from brown alga also showed that leptosin-A which is a metabolite produced by this organism was effective against P-388 leukemia cells (König & Wright, 1996).

It has also been observed that metabolites from endophytes are potential sources of antioxidants (X. Liu *et al.*, 2007). Hydroquinone derivatives obtained from *Acremonium* sp., derived from brown alga, exhibited antioxidant activities (Abdel-Lateff *et al.*, 2003). These hydroquinone derivatives exhibited DPPH radical scavenging activity at 25.0 µg/mL and were also able to hinder peroxidation of linolenic acid at 37.0 µg/mL. *Aspergillus ochraceus* produces benzodiazepine which has antioxidant properties (Abdel-Lateff *et al.*, 2003).

### 2.4.3 Media Formulation, Fermentation Parameters, Diversity of Metabolites and Yield

The ability to manipulate fungi genetically or metabolically can be of great importance in the production of varying compounds. In the fermentation process, metabolic manipulation often has an important role in improving secondary metabolite profiles of microorganisms. The quantity and diversity of secondary metabolites produced by a particular fungi is largely dependent on nutritional and environmental factors (Darah *et al.*, 2011). Therefore, in formulating media for culturing fungi, parameters such as aeration, agitation intensity, culture duration and light exposure

must be considered carefully in order to ensure optimal production of desirable secondary metabolites. The liquid Wickerham's (yeast extract, peptone, malt extract and glucose; YPMG) medium is usually ideal for submerged culturing of fungi (Rose, 1975). In the case of marine endophytes, formulating this medium with sea water has been shown to be suitable for endophytic fungal growth. In support of this, work done in our laboratory involving wood decay fungi showed that supplementing the liquid Wickerham's medium with soil extracts vastly improved the yield of secondary metabolites. From literature, there is no documented procedure for determining the duration of culturing a particular fungus for optimal secondary metabolite production. However, studies have shown that longer incubation periods result in a gradual decrease in the production of secondary metabolites (Merlin *et al.*, 2013). In the case of submerged cultures, the speed of agitation was found to be an important factor that improved fungal growth and biomass (Darah *et al.*, 2011). Moreover, high rate of aeration resulted in increased production of mycelial biomass (S. Kim *et al.*, 2003) while excessive exposure of fungal cultures to light inhibited sporulation (Dahlberg & Etten, 1982; Rakoczy, 1963).

Fungi produce diverse secondary metabolites which are of interest due to their pharmaceutical properties. Understanding the genes that affect production of these diverse fungal secondary metabolites and manipulating these genes could result in improved yield. The use of small molecules such as Histone Deacetylase inhibitors could result in constitutive transcription of genes which encode enzymes that are essential to the various fungal secondary metabolite biosynthetic pathways (Hertweck, 2009; Scherlach & Hertweck, 2009; Vervoort *et al.*, 2010). Thus, the use of Histone Deacetylase inhibitors might be instrumental in facilitating discovery of new compounds from fungal cultures (Henrikson *et al.*, 2009; R. B. Williams *et al.*, 2008).

### 2.4.4 Metabolite isolation by activity guided fractionation

Bioactivity-based assays can provide good evidence about biological properties of extracts (Weller, 2012). However, activities of extracts cannot be allocated to a particular chemical compound in the complex mixture. Therefore, it is necessary to tease out the bioactive compound as purification proceeds. Bioactivity guided fractionation is one technique widely used in examining the effect of fractionated extracts on organisms or in biological assays enables early identification or isolation of substances of biological relevance.

There are usually a lot of substances contained in a particular extract, and it is more complex with fungal extracts making it very challenging to obtain the relevant compound(s) of interest. The general idea is to reduce the complexity of the extract and obtain fractions from which could be tested against a particular organism or target. Therefore, it is important to avoid the same compounds in each fractionation step by ensuring that each separation technique produces none-overlapping fractions. Some of the techniques used include solvent-solvent partitioning techniques (Kupchan fractionation), thin layer chromatography (TLC), and the different formats of high performance liquid chromatography (HPLC). A critical approach for successful product isolation effort is the unique combination of the different separation techniques in a particular strategic sequence optimized for the total extract under consideration. Separation schemes that align with certain dominant chemical properties of a complex mixture, such as polarity and size, and the ones that are based on specific interactions (e.g. amide, phenyl and diol solid phase) are promising ideas that are suitable for the complexity of fungal metabolites. Structures of the compounds isolated are elucidated using one or more of the following techniques; Ultra Violet (UV), Infra-Red (IR) spectroscopy, Mass spectrometry and Nuclear Magnetic Resonance (NMR) spectroscopy.

The preferred first fractionation step is the solvent extraction method. This method works by partitioning the crude extracts among different immiscible solvent systems (Kupchan *et al.*, 1973). Its unique attribute is the ability to handle bulk samples from 20 g to more than 500 g. These solvents may include; hexane, water, ethyl acetate, diclomethane and methanol (fig S3.6). These solvents are often selected based on their polarity index. The basic principle of this technique is that polar compounds are soluble in polar solvents and non-polar compounds are soluble in nonpolar solvents. Solvents chosen must be carefully considered to ensure that they are immiscible and offer maximum dissolution of secondary metabolites since most metabolites have different degrees of polarity. The solvent must be inert, nontoxic and not easily flammable (Visht & Chaturvedi, 2012).

**Table 2.2**. Polarity index for some extraction solvents (Snyder, 1974).

| Extraction Solvent | Polarity Index |
|---|---|
| *n*-Hexane | 0 |
| Dichloromethane | 3.4 |
| *n*-Butanol | 3.9 |
| Ethyl acetate | 4.3 |
| Chloroform | 4.3 |
| Ethanol | 5.2 |
| Methanol | 6.6 |
| Water | 9 |

The techniques frequently used after solvent-solvent partitioning are liquid or thin layer chromatography which could be set up in many dynamic formats and sequence as dictated by the data from the previous steps. TLC involves a stationary phase mostly made of silica gel immobilized on glass or aluminum sheet and an organic solvent (Mobile phase). The liquid or

dissolved samples are spotted on the stationary phase to dry. The plate is run in a solvent reservoir and is removed when the solvent front reaches the top edge of the plate. The separated bands on the TLC plates are visualize under visible and UV light. The bands are cut and eluted as partially purified compounds. By using this method, an extract can be divided into its distinct compound or fractions comprising fewer compounds.

Fractions obtained can be purified further by the high performance liquid chromatography (HPLC). This technique employs the uses of high-pressure pumps to ensure good separation. Many compounds have been identified using this technique (Sasidharan *et al.*, 2011). The resolving power of preparative HPLC is suited for isolating pure metabolites which is a requisite for structural elucidation. When a metabolite has been successfully isolated, mass spectrometry along with 1-dimension (1D) and 2-dimension (2D) NMR studies are used to elucidate the structures.

**2.4.5 Bioassays**

Many types of bioactivity assays are used for *in vitro* and *in vivo* testing of fractions and compounds from natural product origin. The filter paper discs diffusion method is the easiest for organisms like bacteria and fungi that can grow on agar plates as long as the appropriate controls are used and established protocols are applied to ensure reproducibility (De Beer & Sherwood, 1945). A fraction or compound of interest is loaded on filter paper discs and the discs are placed on agar plates spread with the microorganisms. The growth of the organism is monitored over a period of time and the diameter of the zones of inhibition are measured and recorded. The 96 well plate can also be used for broth dilution assays in which the optical density of a cell suspension is measured with a plate reader. This method allows for the quantitative comparison of growth with the controls.

There are several examples of the use of bioactivity guided fractionation in analyzing many complex mixtures beyond the search for antimicrobial assays. The activities of extracts and the diverse fractions of the five plant extracts were tested on *in-vitro* thrombolysis and several fractions showed an array of clot lysis activity (Emran *et al.*, 2015). A similar study observed that the butanol fraction had potent antihyperglycemic and anti-inflammatory activities (Michel *et al.*, 2013).

**2.5 Phenotypic screening for bioactive compounds**

Target-based screening leads to the identification of drug candidates for diseases that results from a defect in a single gene. However, human diseases are mostly caused by multiple factors. Target based assays have generally produced many drugs but only a few of these drugs are new (Priest & Erdemli, 2014). Therefore, the whole cell-based assays are very relevant in terms of understanding the exact mode of action of a particular compound targeting a particular disease condition or a pathogen. Another advantage is the possibility of finding new compounds that inhibit new pathways which also has the potential to find complex mechanisms of action. In order to dramatically change the odds of finding new classes of bioactive compounds to combat fungal infections, there is the need to broadly model the growth of the pathogenic cells to go beyond the routine single type media formulation. Moreover, an attempt to explore a more dynamic and diverse cellular states to simulate phenotypic heterogeneity which dramatically impact the cell's response to the drug could be utilized. It is highly likely that drugs that are selected based on their activities across a wide array of phenotypes will show more robust efficacy and stall the development of resistance.

A major challenge of natural product isolation is the unearthing of existing compounds that target already known pathways. In order to eliminate this occurrence, phenotype modifiers can be

employed in whole cell -based assays to create a wide array of physiological conditions in the laboratory. This can be analyzed further to create chemical models for drug screening that is capable of detecting differences in growth inhibitory actives even at the crude extract level. The major advantage of this approach is that crude extracts that look similar and therefore might contain many similar active ingredients can be eliminated quickly and cheaply. The pathogenic organism is usually primed with the known compounds altering the normal physiological state of the organism. The primed organisms are challenged with the new compounds. The screening of new compounds against pathogens in the presence of different known chemical compounds is necessary because it may lead to the development of efficient combination therapies (Nyilasi *et al.*, 2010). Also, this permits easy identification of compounds with new bioactivity, discriminates between compounds and determines the mechanism of action of compounds. This technique also helps to identify compounds with robust activities that would be maintained over an extensive set of simulated physiological conditions. The challenge of genetic variations in humans and also in pathogenic organisms on the efficacy of drug candidates can be overcome with this phenotypic screening due to the wide and dynamic range of physiological and cellular conditions under which the drug discovery campaigns are conducted.

Screening an unknown compound with other known compounds is also a means of finding out whether there can be synergy or antagonism between the two compounds. Sertraline hydrochloride (psychoactive) and fluconazole combinations were examined against two pathogenic fungi and the two drugs led to the conclusion that a potential combination therapy was possible for certain fungal infections (Nayak & Xu, 2010).

## 2.6 Pre-stress treatments and response as a cell culture model for bioassays in antifungal drug discovery

Microorganisms experience stressful conditions in their environment and their ability to overcome these conditions can provide a significant fitness advantage. They experience constant variations in their surroundings, such as the availability of nutrients and temperature (Święciło & Zych-Wężyk, 2013). Exposure of microorganisms to mild stress can make them tolerant to lethal doses of subsequent stress (Berry *et al.*, 2011). *Saccharomyces cerevisiae* mutants developed forbearance to stressful conditions which occurs preceding other cellular experiences (Berry *et al.*, 2011). This is a major mechanism used by most pathogenic organisms to build resistance and a deeper understanding on the molecular mechanisms involved in this type of stress response will enhance our knowledge of drug action on cells and drug resistance. More importantly, this cell culture model can present an advantage in testing for sources of novel bioactive compounds that are distinct from those routinely obtained by using routine cell growth assays. The pre-stress model also has the merit of removing the stress/phenotypic condition before the drug assay and thereby reduce any direct interference of the drug entity.

### 2.6.1 The utility of multidrug resistant cell lines

The screening of active compounds is an important step in the drug development process. There are two major screening strategies employed for the purposes of identifying bioactive compounds. These are phenotypic and target-based screening (Chinen *et al.*, 2017). Phenotypic screening is centered on the changes in the morphological features of an organism after treatment with a particular compound (Ingber *et al.*, 1990). Additionally, phenotypic screens are largely based on cytotoxicity of compounds to a cell (Low *et al.*, 2005). Yeast is a very important tool for chemical screens. However, studies have shown that the cell wall of yeast enables the organism to exhibit

high resistance to many compounds compared to other cell types (Chinen *et al.*, 2017). Hence the engineering of mutant strains with variable levels of sensitivity to compounds is desirable. The deletion of four genes from *S. cerevisiae* resulted in a drug sensitive yeast strain which was used to identify targets for reveromycin (Chinen *et al.*, 2017). Studies in our laboratory has shown that a homozygous double deletion mutant of *S. cerevisiae erg2*, exhibited high levels of resistance to many antifungal compounds and extracts. A similar study was conducted where two strains of multidrug resistance *Mycobacteria smegmatis* were developed and used as tools for routine screening of antibacterial agents in our laboratory (P. Arthur *et al.*, 2019). Therefore the use of these mutant organisms could provide an avenue for *in vitro* screening of new compounds that target drug resistant pathogens.

**2.7 Molecular analysis of stress response networks**

Micro-organisms activate certain genes under the control of regulator proteins in response to stressful conditions. Stress conditions allow for the transcription of many genes which enables cells to survive (Chan *et al.*, 2012). Yeast deletion mutants were classified based on the genes required for adaptation to $H_2O_2$; 4 transcription factors were essential for both acute and chronic responses to $H_2O_2$ (Ng *et al*., 2008). Also, Yap1p and Skn7p acted in synergy for adaptation and shows upregulation of antioxidant activities (Ng *et al.*, 2008). *Saccharomyces cerevisiae* genes associated with heat-shock resistance in two growth phases of the organism, the target of rapamycin and some other pathways were crucial for the conservation of resistance after a sudden shift in temperature from 30ºC to 50ºC (Jarolim *et al.*, 2013).

Ixr1p (transcriptional regulatory factor) and the control of hypoxia in yeast is associated with aerobic suppression of 3 hypoxic genes. Transcriptome analysis of the wild-type strain revealed unique control of genes related to hypoxic, oxidative stress responses, catabolic and anabolic

fluxes in response to oxygen deprivation (Vizoso-Vázquez *et al.*, 2012). It has been shown that upon severe osmotic shock, yeast cells stimulate the high-osmolarity glycerol (HOG) pathway which starts the transcriptional response (Tamás *et al.*, 2000).

### 2.8 Use of the selectivity index of new antifungal compounds against *Candida albicans* versus *Saccharomyces cerevisiae*

Drug discovery and development takes time, therefore, it is important to develop new strategies to obtain compounds that can be effective against a wide range of diseases. Using a very effective model for screening a new compound is important. Screening of compounds against *S. cerevisiae* and pathogenic *Candida albicans* could provide a very important avenue to explore both eukaryotic infections and cancer as models for identifying compounds that are effective against *S. cerevisiae* only. These identified compounds could be targeting pathways important to cancer development and progression (Matuo *et al.*, 2012). Those targeting *Candida albicans* could be important for fungal infections in general. Also, enriched fractions of natural products and compounds that are evaluated using human cells on the basis of their selective activity against *S. cerevisiae* only can be distinguished whether they are anti-cancer or just cytotoxic and unsafe. The basic test is the influence on cell proliferation of the period of 3 to 4 days; anti-cancer compounds will generally permit cells to divide over the initial two days and cell death setting from the 3$^{rd}$ day whereas cytotoxic compounds or fractions will suppress growth from the outset and cell death occurring from day 1 to 4.

### 2.9 Haploid insufficiency profiling of *Saccharomyces cerevisiae*

Haploinsufficiency (HIP) is defined as a dominant phenotype when a diploid organism has lost one copy of a gene which results in a single functional copy of that gene (Deutschbauer *et al.*, 2005). In yeast, the phenomenon has been characterized by gene-by-gene analyses of many

pathways in the cell. In drug discovery, this method is usually employed in the analysis of the possible targets of most compounds; mutant strains that are very susceptible to a drug begin to dwindle from a pool of deletion mutants as time goes on. A haploinsufficiency screen to determine drug mode of action was conducted with yeast (Baetz *et al.*, 2004). It was observed that the drug dihydromotuporamine C acts by inhibiting angiogenesis and metastasis (Baetz *et al.*, 2004). The method is a systematic and unbiased genome-wide screening for drug targets.

### 2.9.1 Hypomorphic fungal cells to increase the sensitivity of bioassays

Hypomorphic cell lines of *Saccharomyces cerevisiae* can also be used to enhance the sensitivity of bioassays during screening of compounds for potential drug candidates that target essential genes. These cell lines are also useful for preliminary screening of extracts or fractions that are anticipated to contain novel antifungal compounds in minute quantities. Previously, hypomorphic cell lines were usually obtained from screening following exposure of wild type cells to chemical mutagens. However, recent approaches are based on either destabilizing a significant pool of the mRNA via disruption of the 3' UTR or addition of several adenosine nucleotides within the open reading frame of mRNA (L. L. Arthur *et al.*, 2017; Breslow *et al.*, 2008). In *Saccharomyces cerevisiae*, several hypomorphic cell lines representing approximately 82% of its essential genes have been generated to facilitate in-depth functional characterization of the genome (Breslow *et al.*, 2008). These plethora of hypomorphic cell lines can be used as a platform for discovery of new antifungal compounds targeting essential genes that are conserved across the various pathogenic fungal species. Calcineurin is a protein phosphatase conserved in yeast (Griffith *et al.*, 1995). It is usually a target for many compounds. The Heat shock protein 90 (Hsp90) supervises most regulatory proteins in yeast (Millson *et al.*, 2007). Mutant cell lines like Calcineurin and

Hsp90 knockouts could aid in the discovery of new compounds by increasing the sensitivity of bioassays.

## 2.10 Benefits from the new strategies and approaches

### 2.10.1 New classes of antifungal compounds

Phenotype modifiers help to mimic physiological conditions in the laboratory setting hence, screening new compounds against cells under varying physiological conditions will enable the identification of new targets. Selecting a compound that maintains its activity against an organism under different physiological and morphological changes induced by the different phenotypic modifiers will allow the discovery of more potent compounds. Also screening new compounds using this method will help to distinguish between the existing classes of antifungals and identify potential new ones even before the product isolation starts.

### 2.10.2 Strategies for suppressing stress responses

As fungal pathogens are continuously exposed to harsh environmental conditions which they need to overcome in order to survive, regulating cell homeostasis is important for survival and this is mostly achieved by coordinated gene expression (Rodrigues-Pousada *et al.*, 2005). A major means by which fungi respond to drug stress is the overexpression of efflux pumps. The out flow of antifungal drugs via the ATP-binding cassette (ABC) transporters establishes a chief cause of antifungal resistance (Schuetzer-Muehlbauer *et al.*, 2003). The inhibition of drug out flow pumps is the best strategy for overcoming the response of fungal pathogen to drug stress. For instance, Schuetzer-Muehlbauer and colleagues used ABC transporter blocker drugs FK506 and Propafenones to reverse CDR-mediated azole resistance in yeast (Schuetzer-Muehlbauer *et al.*,

2003). Another strategy is to use a compound that is not a substrate for the pump and also rapidly fungicidal compounds. Thus, antifungal drugs can be given in combination with proton pump inhibitors. Also, a multi-functional inhibitor which affects the target, efflux activity and efflux pump transcription can be used in overcoming the drug stress response.

### 2.11 Conclusions

Although, terrestrial fungi have been explored when it comes to the treatment of human diseases, marine endophytic fungi have not been studied extensively for potential new sources of compounds. It will be beneficial to focus attention on marine endophytic fungi as potential sources of unique compounds. *Saccharomyces cerevisiae* is a useful model for eukaryotic biology. The entire genome has been sequenced and functional roles have been assigned to about 60% of the genes. *Saccharomyces cerevisiae* is a eukaryotic organism just like humans hence has been used in many cancer disease studies in the search for new targets and understanding disease progression. Discovery of new drugs takes a lot of time hence there is the need to develop new methods to limit the long duration that is associated with the process. Bioactivity-guided fractionation discussed here is one of the best methods of saving time during discovery of novel drug candidates, as fractions that contain interesting and active compounds are identified earlier and those that are less interesting are discarded. Also, phenotypic array is a very useful method for selecting compounds whose activities can be maintained when cells are under different physiological conditions. Responding to stressful conditions leads to the development of drug resistance. Investigating stress response networks will give a fair idea about the drug resistance mechanisms employed by pathogenic fungi and this also helps to identify new targets.

# CHAPTER THREE

## MOLECULER IDENTIFICATION OF MARINE DERIVED ENDOPHYTIC FUNGI AND THE DETERMINATION OF THEIR BIOACTIVE METABOLITE PRODUCTION CAPACITY

### 3.1 Introduction

There is an increased impact of fungal diseases globally and urgency is shown by the effort of different advocacy groups and publications campaigning for critical interventions (Krysan, 2017). The fungal diseases contribute to more than 1.5 million deaths, and this is mainly attributed to the paucity of antifungal drugs. There are unmet clinical needs which call for in-depth research to address the existing need. Despite the increasing cases of fungal diseases, only three antifungal medications have been formulated for the treatment of the life-threating fungal diseases (Victoria Castelli *et al.*, 2017). The three antifungal drugs that have been discovered in the last three decades are echinocandins, azoles, and polyenes. Amphotericin B which was developed in the 1950s is still currently being used in the treatment of most of the diseases even in the immunocompromised individuals with profound side effects.

Although it is undisputed that there is already research which is underway for increasing the spectrum of azoles, there is an undeniable need for novel molecules to be used as antifungal drugs. Currently, the available antifungal medications are only in three classes, and this urgency has been raised by the new multi-drug resistance *C. auris* and *C. glabrata* (Gallo-Ebert *et al.*, 2013). It is unimaginable that patients infected by fungi are treated with drugs that were discovered in the early 1960s with limited efficacies. Agreeably, there is a problem in the development of the antifungal drugs, and this requires to be adequately addressed through the use of strategic new

concepts and approaches. In the case of antibacterial resistance, there have been addition of b-lactamase inhibitors while the gap remains in the fungal diseases. Currently, the resistance can be treated through a combination of adjuvants (Krysan, 2017). More so, other diseases such as malaria, HIV and tuberculosis are treated with a combination of drugs, but this does not dispute the need for a new molecule. The difficult challenge in the discovery of the new molecules for antifungal diseases is that they lead to the development of the same chemical molecules or a close one that inhibits the same process (McCarthy *et al.*, 2017). A new approach should be developed that does not only focus on killing the pathogen but preventing the development of drug resistance. Currently, endophytic fungi have been recognized as a vital source of bioactive compounds needed for drug discovery. Consequently, there is significant progress in exploring the richness of these species, diversity, and bioprospecting active metabolites such as antifungal, anticancer, antioxidant and antibacterial compounds. The range of medicinal usefulness credited to fungal endophytes from various environmental settings have been significant and distinctively for endophytes in macroalgae from the marine environment are noted to have structurally different bioactive secondary metabolites. Notwithstanding the significance of these metabolites, little has been explored to find out the full potential of endophytic fungi. These metabolites are found in increased levels in *Phaeophyceae*, *Rhodophyceae*, and *Chlorophyceae*, in that order respectively (Sarasan *et al.*, 2017).

Marine endophytes (MEF) are promising sources for natural products and this spurs attention on their critical applications. New metabolites have been discovered and new biological functions determined. The functions of these metabolites such as radical scavenging, cytotoxic and enzyme inhibition are used in the classification of the metabolites. A total of 178 fungal metabolites were known by 2012 with the novel ones being 138 (Debbab *et al.*, 2012). Among the various functions

of the marine endophyte metabolites, they have been identified to have antitumor functions. Sixty-four fungi isolated from six seaweed species and two Malaysian marine plants, the extracts were tested against the human cancer cell for their activity in inhibiting tumor growth. Forty-Nine extracts showed the ability to inhibit cancer cell lines specifically from colon and lung. However, there were no effects on the liver and fibroblast cell lines, markedly, only three metabolites were known from these endophytes, and 12 were unknown. These metabolites were analyzed using x-ray crystallographic data (Ariffin *et al.*, 2011).

Marine resources form new leads in the discovery of new medical metabolites, nonetheless, fewer studies are discussing the importance of seaweed endophytic fungi. There was the isolation of forty-five microorganism from *Bostrychia tenella* (Brazilian marine seaweed) which were tested for their cytotoxicity, antibacterial and antifungal effect (Felício *et al.*, 2015). Collectively, these studies show that there are numerous metabolites from marine endophytes vital for biopharmaceutical purposes. These different species isolated and studied illustrate the biological functions of preventing fungal, bacterial infections and tumor. Only a small percentage of these secondary metabolites have been discovered and many remains to be identified. Markedly, much of the research should be conducted to fill the knowledge gap that exists, building on the current knowledge and providing a foundation for future research.

Therefore, the aim of this study was to investigate the antifungal potential of endophytic fungi derived from seaweeds, particularly towards the discovery of new compounds against *Candida albicans*. The objectives of this study were to:

1. Isolate marine endophytic fungi (MEF) and determine the antifungal activity against *Saccharomyces cerevisiae* (Wild type and mutants) and *Candida albicans*.
2. Identify the fungal species that produced the antifungal bioactive metabolites.

## 3.2 Materials and Methods

**3.2.1 Growth media for microorganisms**: Yeast extract, nutrient broth, malt extract broth, peptone, dextrose and agar were purchased from Becton, Dickson and company (BD, USA).

**3.2.2 Standard antimicrobials**: Fluconazole, Amphotericin B and Chloramphenicol were purchased from Sigma Aldrich, Germany.

**3.2.3 DNA extraction and PCR reagents**: Taq polymerase, the DNTPs, ITS primers, the reaction buffer (5X PCR buffer), $MgCl_2$ and Nuclease free water were purchased from Inqaba biotec, South Africa. Agarose gel and 100 base pair (bp) DNA ladder were from Sigma Aldrich, Germany. Benchtop 2UV Transilluminator gel photography system (CA, USA),

**3.2.4 Cell viability testing**: Tetrazolium dye MTT (Thermo Fisher Scientific)

**3.2.5 Solvents**: Isopropanol, *n*-Hexane, *n*-Butanol, Dichloromethane, Ethyl acetate, Methanol, Acetonitrile, Petroleum ether, Ethanol and Anisaldehyde reagent were all purchased from Lab Aid, Ghana. Bleach (Clorox, USA). The solvents were of technical grade.

**3.2.6 Isolation of endophytic fungi from seaweeds**

The healthy and mature sea weeds were collected into sterile bags from several beaches in Ghana. The seaweeds were washed thoroughly under running tap water and successively dipped in 70% alcohol and 5% bleach (Clorox) for 10 seconds followed by immersion in sterile seawater for another 10 s; this procedure has been shown to be sufficient to sterilize marine plant samples (Suryanarayanan *et al.*, 2010). The samples were aseptically cut into segments of about 2 cm and positioned on Malt extract agar (MEA) prepared with sea water (Containing 30 g//L malt extract broth: 15 g/L agar: 5 g/L peptone: 0.2 g/L chloramphenicol). Control plates were made by dabbing

intact pieces of seaweed with and without surface sterilization on the surface of MEA plates. Supplementing the media with antibiotic chloramphenicol was to prevent bacteria growth. The petri-dishes were sealed with parafilm and incubated at 30℃ for 8 days. The segments of seaweeds were screened for the presence of endophytes. Fungus that grew from the seaweed segments were separated on MEA until pure colonies were obtained (Silva *et al.*, 2011). A total of 6 of these fungal strains were selected for analysis based on their biological activities.

### 3.2.7 Endophytic fungal DNA extraction

Mycelia discs from the six selected fungal strains (MEF 11, 65, 75,112,122 and 134) were inoculated into 100 ml broth prepared with sea water (comprising Yeast extract 5g/L, Peptone 5g/L, Malt extract 5g/L and Dextrose 30g/L (YPMD) under shaking at 30℃ for 8 days.

DNA extraction from the isolates were conducted using the Guanidine Hydrochloride technique. To about 0.05 g of harvested mycelia, 650 µL of lysis buffer was added together with some glass beads and vortexed vigorously in a bead beater for 15 minutes. The lysate was incubated for 20 minutes at 65℃ in a water bath and span at 5600 g for 2 minutes. Potassium acetate (150 µL) was added to 400 µL of the supernatant and the resulting mixture was kept at -20 ℃ overnight. The solution obtained was span at 5600 g for 30 minutes. Guanidine hydrochloride (400 µL) was added to 400 µL of the supernatant. The resulting solution was transferred into a spin filter column for centrifugation at 5600 g for 2 minutes. Then the flow through was discarded. The column was then washed with the wash solution and the flow through discarded. Chilled ethanol (70%) was added and the spin filter column was span again with the flow through discarded.

The elution buffer (200 µL) was added to the column and incubated for 10 minutes after which the column was span again to elute the DNA. The 260nm/280nm ratios for the DNA extracted were

found to be between 1.8 and 1.9. Full details of how the buffers and reagents used in this section were prepared can be seen in Table S3.1.

### 3.2.8 Molecular identification of endophytic isolates

The internal transcribed spacers (ITS) of the fungal ribosomal DNA (rDNA) were amplified. The Polymerase chain reactions (PCRs) were conducted in 25 µL reaction mixtures comprising 2.5 µL of fungal DNA template, 12.5 µL Top Taq 2X master mix (comprising; Taq polymerase, the DNTPs and the reaction buffer), 0.5µl each of the following primer pairs ITS1 (51TCCGTAGGTGAACCTGCGG31) and ITS4 (51TCCTCCGCTTA TTGATATGC 31) or ITS2(51GCTGCGTTCTTCATCGATGC31)andITS5(51GGAAGTAAAAGTCGTAACAAGG31) and 9 µL of PCR water.

The amplification was conducted using a thermocycler (Applied Biosystems, USA). The PCR thermal cycling protocol was as follows; initial denaturation was at 94 ℃ for 5 minutes, then 35 cycles of denaturation at 94 ℃ for 45 seconds, followed by annealing at 50 ℃ for 1 minute, extension at 72℃ for 1 minutes 15 seconds. The final extension procedure was at 72 ℃ for 7 minutes.

The products from the PCR reaction (8 µL) together with loading dye (2 µL) were separated on 1.5% agarose gel containing ethidium bromide (Promega, Madison, USA) in Tris –borate EDTA buffer. The gel was run at 100 V for 45 minutes alongside 100 base pair (bp) DNA ladder. The bands on the gel were visualized under UV illumination using the gel imager. The purified ITS amplicons were sequenced by the Sanger method with the primers that were used for amplification by PCR. The sequences obtained were matched with the NCBI database by the BLASTN program. Phylogenetic relationships were determined by maximum likelihood phylogeny.

### 3.2.9 Evaluation of extract yields and secondary metabolite content of fungi

A preliminary study was conducted to determine yield of extracts from the fungi cultures and to obtain qualitative overview of the secondary metabolite content of the fungi. Three of the identified fungi (MEF 65, 112 and 134) were selected for these analyses and were cultured as follows: Each fungi was initially cultured on MEA for 4 days. Mycelia discs obtained from the fungal cultures were inoculated into 400 mL autoclaved YPMD broths and incubated at 30 ℃ for 3 weeks. For each isolate, 10 broth cultures were prepared. After incubation, the cultures were extracted with equal volumes of ethyl acetate for 24 hours. The ethyl acetate portion was collected and concentrated using a rotary evaporator (Rotavapor, Buchi) under vacuum. Quantity of dry crude extracts obtained from each broth was measured (Table S3.2).

Analytical thin layer chromatography (TLCs) of the extracts were performed on silica gel pre-coated TLC plates (60 F254 Aluminum sheets, 0.20 mm layer thickness; Macherey-Nagel). The plates were developed in 50 mL TLC developing solvent combination containing: 35 ml ethyl acetate, 10 ml acetonitrile and 5 ml petroleum ether. Subsequently, the plates were sprayed with anisaldehyde reagent followed by heating at 110 ℃. The bands partitioned on the TLC plate, which is also an indication of the separation of compounds, were detected under UV at 254 and 365 nm (Fig S3.3 to S3.5).

### 3.2.10 Large-scale broth fermentation and extraction of fungal isolates

The 6 identified fungal isolates were fermented as follows; two MEA culture plates for each of the isolates were used to inoculate 2 L sterile YPMD broth prepared with sea water. For each isolate, 10 of these cultures were prepared. The broth cultures were incubated at 30℃ for 4 months with constant agitation of the culture vessels. After the culturing period, the cultures were extracted five times with equal volumes of ethyl acetate at 50℃ for 24 hours. The extracts were further

concentrated by drying using a rotary evaporator (Rotavapor, Buchi) under vacuum. The dried extracts were reconstituted in 5 ml of methanol. The total extract yields are shown in Table 3.2.

### 3.2.11 Fractionation of fungal crude extracts

The dried extracts from the 6 isolates were fractionated by the modified Kupchan's solvent partitioning method (Kupchan *et al.*, 1973). Using 1 L separatory funnels, every 10 g of extract was suspended in 100 ml distilled water and then partitioned with 300 mL dichloromethane. This resulted in the formation of two solvent layers; the aqueous (upper layer) and the organic (lower layer). Subsequently, the dried organic layer was dissolved in 90% methanol (100 mL) and partitioned with n-hexane (FH) (300 mL). Then the methanol layer was adjusted with distilled water to 50% (FM50) and successively extracted with dichloromethane (FD) (540 mL) and ethyl acetate (FME) (540 mL). The aqueous layer (FW) was consecutively partitioned with n-butanol (FB) (300 mL) and ethyl acetate (FE) (300 mL). For each extract, 90% of the total weight was used (Table 3.2). The detailed partitioning scheme is shown in Fig S3.6. In all 7 fractions (FW, FE, FB, FM50, FME, FD and FH) were generated from each extract with the total yield of each fraction indicated in Table 3.3. Analytical TLCs were performed on the fractions. Bands were visualized under UV light to obtain an overview of the identity of each fraction and the qualitative purity of each fraction.

The fractions were tested against *Candida albicans* and *Saccharomyces cerevisiae* by the agar disc diffusion method to identify active fractions. Further isolation of compounds from the active fractions were conducted using preparative thin layer chromatography (TLC) method as follows.

About 90% of the total weight of each active Kupchan fraction was successively run on silica gel TLC plates (60 F254 Aluminum sheets 20 x 20 cm, 0.20 mm layer thickness; Macherey-Nagel).

The plates were developed in 50 ml TLC developing solvent combination containing: 35 ml ethyl acetate, 10 ml acetonitrile and 5 ml petroleum ether (Fig S3.7-14). This yielded five TLC fractions (V1-V5) per each active Kupchan fraction analyzed (Table S3.3). These were also tested for activity. A second round of TLCs (2D) were performed on the active fractions from the first round of TLC analyses (Fig S3.15-22). Bands were cut under UV light and eluted as partially purified compounds.

### 3.2.12 Antifungal activity evaluation

The paper disc diffusion method was used for testing the susceptibility of the organisms to the extracts and fractions from the fungal cultures (Bauer *et al.*, 1966). The organisms used in the sensitivity test were; *Saccharomyces cerevisiae*, *Candida albicans* and a panel of diploid deletion mutants of *Saccharomyces cerevisiae* (Δ*erg*2 (homo), Δ*erg*2 (het), Δ*eft*1 (het), Δ*eft*2 (het) and Δ*yef*3 (het)). The yeast strains were donated by Prof. Martha Cyert from University of Stanford USA. The crude extracts (90 µL) , Kupchan fractions (30 µL & 90 µL) and TLC fractions (30 µL & 120 µL) were pipetted from stock solutions on to sterile Whatman No. 5 filter paper disc (5mm in diameter) to dry.

Initial yeast cultivation was performed on agar plates made from Yeast extract (10g/L), Peptone (20 g/L), Dextrose (20 g/L) and Agar (20g/L) (YPDA) incubated at 30℃ for 12 hours. A loop-full of each organism from these YPDA plates was inoculated into 50 mL nutrient broth (8 g/L) and incubated for 12 hours with shaking at 30℃. The cells were grown to mid-log growth phase. The optical density ($O.D_{600nm}$) of the final cultures were adjusted to 0.7; these were used for the sensitivity tests. Infused discs were positioned on YPDA plates which were previously spread with the various yeast inocula. Afterwards, the plates were incubated at 30 ℃ for 24 hours. The zones of inhibition were measured as the average of two biological replicates and recorded in

millimeter (mm). Discs impregnated with Fluconazole (Flu, 5 µg) and Amphotericin B (Amp B, 10 µg) were used as controls.

After evaluation of the antifungal activity of the TLC fractions, by the disc diffusion method, 59 active TLC fractions were obtained. These active fractions were mainly from the second round (2D) of TLC analyses (Table S3.4 and S3.5). The fractions were validated by the MTT assay method.

### 3.2.13 MTT assay method

The MTT assay was conducted as follows; *Candida albicans* was cultured to mid-log growth phase, as described in the previous section. The final $O.D_{600nm}$ of the cell culture was adjusted to 0.1 for the cell viability test. The active 59 TLC fractions (12 µL) were pipetted from stock solutions prepared at a concentration of 10µg/µl into 96 well plates and the solvent allowed to evaporate under sterile conditions. Subsequently, 200µL of the cell culture was added to the dried fractions in the 96 well plates. The plates were then incubated on a shaker at 30 ℃ for 24 hours.

After the 24-hour incubation period, 20µL of the MTT reagent (50 mg/mL MTT reagent) was added to the cell suspension in each well. The plates were wrapped in foil and incubated at 37℃ for 4 hours. Afterwards, the 96 well plates were span at 2000 rpm at 4℃ for 5 minutes. The media from the cell suspensions in the 96 well plates were aspirated and discarded, leaving the purple insoluble formazan pellets. An aliquot of 200 µL of isopropanol was added to each well to solubilize the purple formazan. The plates were incubated again for 15 minutes.

After incubation, absorbance was read at 590 nm using a microplate reader (Thermo fisher). Each fraction was assayed in triplicate. Untreated cells served as negative control and treatment of cells with 5µg of Fluconazole and 10 µg of Amphotericin B served as positive controls. The average of

the triplicate readings for each sample was determined. The absorbance of the culture medium background was subtracted from the assay reading. This was done to correct the absorbance readings. The absorbance reading was proportional to the number of viable cells present.

## 3.3 Results

### 3.3.1 Identification of endophytic fungi from seaweeds

Spore morphology criteria including; shape and size are generally used to identify fungi. However, the fungi studied here were identified using molecular methods. A total of 143 marine endophytic fungal isolates were obtained from the fungal isolation process. Nevertheless, only six of these 143 isolates were selected for further work based on their good bioactivity. The 6 marine endophytic fungi were cultured in Yeast, Peptone, Malt extract, and Dextrose (YPMD) broth until mycelia were visible (Fig S3.2).

DNA was extracted from the mycelium of each isolate using the guanidine hydrochloride method. The concentrations of the extracted DNA were determined using a nanodrop spectrophotometer (ThermoFisher Scientific). The concentrations obtained were; 19.7 ng/µL, 93.2 ng/µL, 186.2 ng/µL, 280.1 ng/µL, 72.8 ng/µL and 143.7 ng/µL for MEF 11, 65, 75, 112, 122 and 134, respectively. The 260nm/280nm ratios for the extracted DNA were found to be between 1.8 and 1.9.

Further identification and classification of the isolated marine endophytes were conducted on the DNA samples using the polymerase chain reaction. The six marine endophytes were identified by sequence analyses of the Internal Transcribed Spacer (ITS) region. Primers targeting the 18S rDNA were used in the amplification of the ITS region. These primer pairs were; ITS 2 and 5/ ITS

1 and 4 fungal primers. The PCR amplicons were ran on 1.5% agarose gel (Fig S3.1). All the isolates were successfully amplified.

Amplification of the ITS-rDNA of the fungi using the ITS1/ ITS4 primer pair yielded amplicons of about 600 base pair (bp) while the ITS2/ ITS5 primer pair yielded DNA fragments of approximately 300 base pair (Fig S3.1). After gel electrophoresis, the PCR amplicons were sent to Noguchi Memorial Institute for Biomedical Research, University of Ghana, for sequencing by the Sanger method. The sequences obtained were assembled to form Contigs using the SeqManPro software and alignment was performed with Bioedit software. The sequences generated were matched with the NCBI database by the BLASTN program. The organisms with the highest sequence identity and the lowest E-value were selected as identified species. The 6 marine endophytic fungi were found to belong to the genera *Aspergillus*, *Clonostachys* and *Meyerozyma* (Table 3. 1). The most represented genus was *Meyerozyma*.

As shown in the phylogenic tree (Fig 3.1), 3 isolates were closely related to *Meyerozyma sp.* with sequence similarity ranging from 97-99%. Two strains were also found to be related to *Aspergillus sp.* (Fig 3.1). A single isolate was assigned to *Clonostachys* genus.

**Table 3.1.** Identification of the 6 endophyte isolates

| MEF Code | Endophyte isolate | % Sequence Homology | Highest sequence identities |
|---|---|---|---|
| MEF 11 | *Aspergillus fumigatus* | 98 | *Aspergillus fumigatus* Af293 |
| MEF 65 | *Clonostachys wenpingii* | 97 | *Clonostachys wenpingii* HMAS 172156 |
| MEF 75 | *Meyerozyma guilliermondii* | 98 | *Meyerozyma guilliermondii* ATCC 6260 |
| MEF 112 | *Aspergillus fumigatus* | 97 | *Aspergillus fumigatus* Af293 |
| MEF 122 | *Meyerozyma guilliermondii* | 97 | *Meyerozyma guilliermondii* ATCC 6260 |
| MEF 134 | *Meyerozyma guilliermondii* | 99 | *Meyerozyma guilliermondii* CBS 2030 |

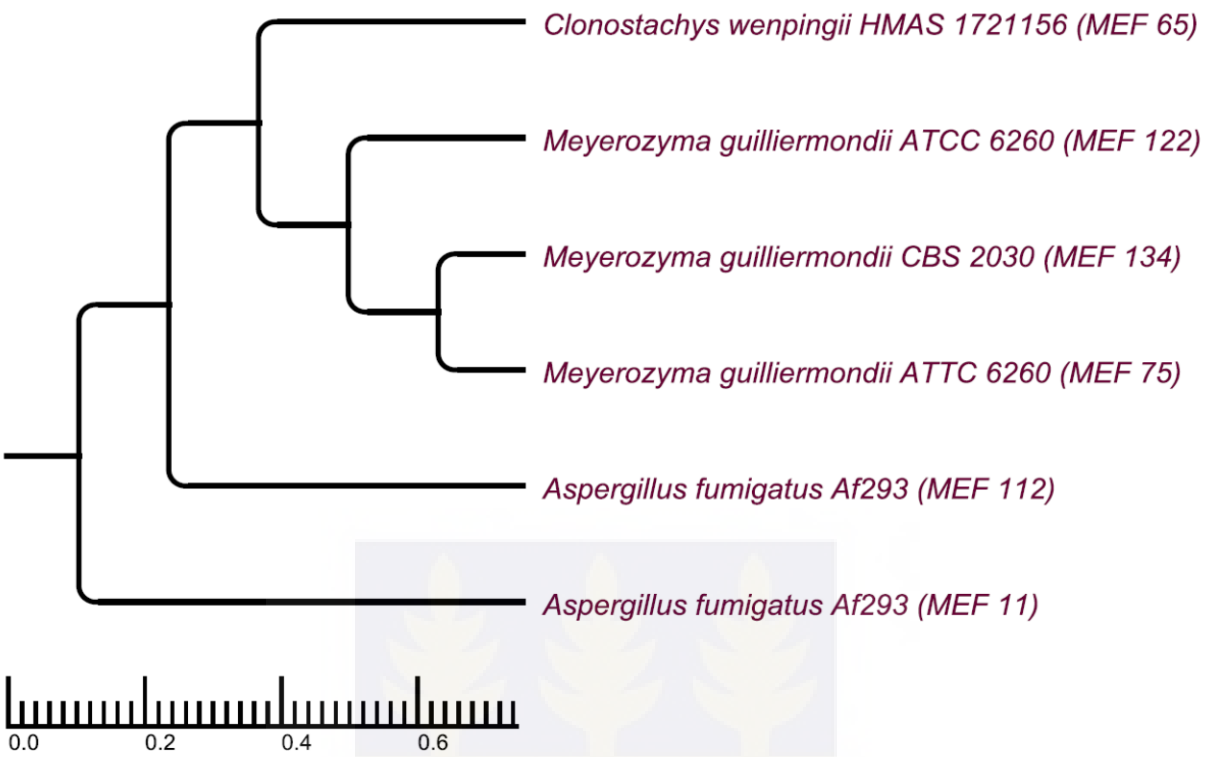


Fig 3.1. Classification of marine endophytes isolated from seaweeds based on a maximum likelihood phylogenetic tree plotted with ITS-rDNA nucleotide sequences. The scale bar represents the distance of the node from the root of the phylogenetic tree.

### 3.3.2 Morphological characteristics of endophytic fungi from seaweeds

Seaweed derived marine endophytic fungi (MEF) were cultured on malt extract agar (MEA 2) prepared with seawater and sub-cultured on fresh MEA2 plates until pure colonies were obtained. Many of the endophytic fungi (99%) were from the leaf-like segments and were morphologically distinct (Fig 3.2).

The fungi formed large multicellular aggregates and could be clearly seen with the naked eye (Fig 3.2). All 6 MEF produced spores and tube-like hyphae which were responsible for the fluffy appearance of some of the colonies (Fig 3.2). An elaborate network of hyphae combined with other structures, formed the mycelia observed for all 6 MEF at day 6 culture periods.

*Aspergillus fumigatus* (MEF 11) appeared green in colour at day 4 but turned dark green by day 6. There was substantial production of green spores by this fungal isolate.

A white fluffy mass was the morphological characteristic of *Clonostachys wenpingii* (MEF 65). This white fluffy aggregate was maintained till day 6. Also, there was production of spores by this isolate. However, the spores did not spread throughout the plates as observed for *Aspergillus fumigatus* (MEF11). *Meyerozyma guilliermondii* (MEF 75) appeared as a green mass and produced a substantial number of broader hyphae. These hyphae quickly intertwined with each other to form the mycelia which covered the entire plate by day 6 of the culture period. *Aspergillus fumigatus* (MEF 112) was brown and fluffy in appearance. It was fast growing and produced many spores. Its numerous hyphae enabled it to penetrate into the substrate and absorb nutrients for the unrelenting growth observed. In contrast, *Meyerozyma guilliermondii* (MEF 122) had a green solid appearance. However, there was not much production of spore or elaborate development of hyphae. Due to this, its growth was slow as compared to *Aspergillus fumigatus* (MEF 112). *Meyerozyma guilliermondii* (MEF 134) displayed a brown color and was fluffy in appearance. The

mycelium produced by this fungus was very large. It was also fast growing and produced numerous brown spores. The 6 marine endophytic fungi grown on MEA 1 (prepared with distilled water) showed the same morphological characteristics as those grown on MEA 2 (Prepared with seawater; Fig 3.2). However, most fungi grown on MEA1 had paler pigments as compared to those grown on MEA 2.

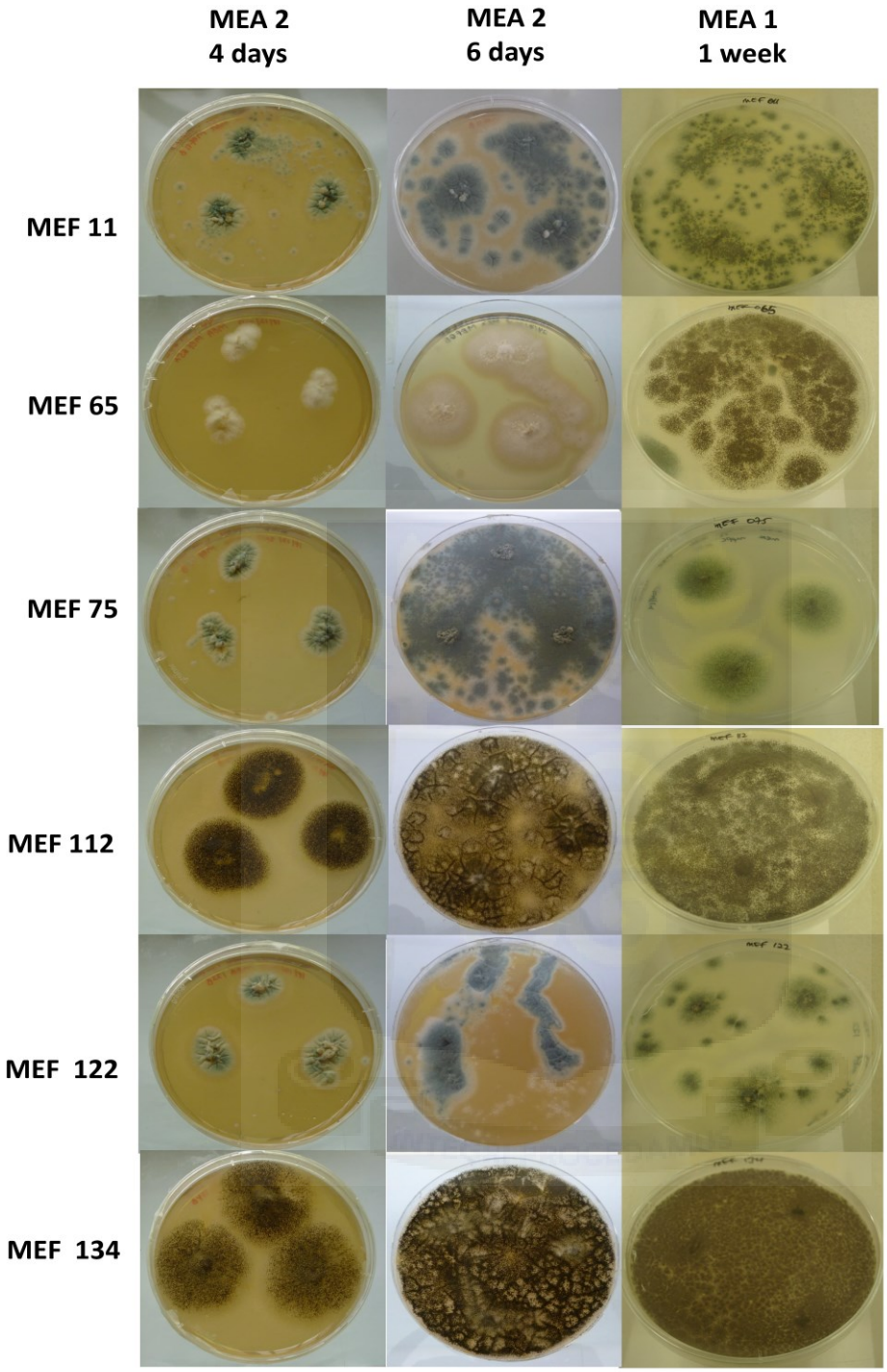


Fig 3. 2. Morphology of the 6 marine endophytic fungi cultured on MEA plates prepared with deionized water (MEA1) and seawater (MEA 2).

### 3.3.3 Extract yield and metabolite content of the various fungal cultures

In order to gain a general overview of the metabolite content and the quantity of extract a particular fungus could potentially produce, this preliminary work was conducted. Ten broth cultures were set up for each of the 3 isolates and metabolites were extracted with ethyl acetate after 3 weeks. The ten sets of cultures for each of the three fungi did not consistently produce the same amount of crude extracts (Table S3.2). However comparing the total extract yield across the 3 fungi, the order of increasing yield was as follows; *Meyerozyma guilliermondii* (MEF 134) < *Clonostachys wenpingii* (MEF 65) < *Aspergillus fumigatus* (MEF 112). The highest extract yield was from the 9th broth of *Aspergillus fumigatus* (MEF 112). In contrast the lowest extract yield was from the 3rd broth of *Meyerozyma guilliermondii* (MEF 134)

Analytical TLC was conducted to qualitatively study the metabolite profile of the extracts obtained (Fig S3.3-S3.5). The band patterns on the TLC plates indicated the separation of potential metabolites contained in the extracts (Fig S3.3). The bands showed many blue emitting compounds across the extracts from the three organisms. For *Clonostachys wenpingii* (MEF 65), there were similar band patterns for extracts from all 10 broth sets, except for extract from broth 4, which had a less prominent fluorescing band close to the solvent front (Fig S3.3). The band pattern observed for extracts from this isolate also confirmed that the same MEF isolate was being propagated.

The profiles of extracts from *Aspergillus fumigatus* (MEF 112) were highly variable. Extracts from broths 1, 2 and 6 had bands that were very faint, which also appeared darker than the fluorescing bands observed close to the solvent front for extracts from the other broths (Fig S 3.4). The separation pattern was not well resolved. The extracts from *Meyerozyma guilliermondii* (MEF 134) produced interesting band patterns on the TLC plates (Fig S3.5). There was a more prominent fluorescing band close to the solvent front for all extracts analyzed. Blue fluorescing compounds

could also be seen close to the origin. Additionally, the band patterns were similar in all the 10 extracts indicating that the same organism was being propagated (Fig S3.5).

### 3.3.4 Determination of antifungal activity of the crude extracts from large scale cultures

The total number of isolates that were found to possess bioactivity were 43 (30%) out of the 143 isolates examined. However only 6 were selected for further studies. The 6 MEF were cultured in Yeast, Peptone, Malt extract, and Dextrose (YPMD) broth prepared with seawater and extracted with ethyl acetate after 4 months of growth. The mass distribution of the extracts obtained from the large scale broth cultures are shown in Table 3.2.

The order of increasing yield was as follows; *Aspergillus fumigatus* (MEF 112) (7.2 g), *Meyerozyma guilliermondii* (MEF 134) (9.6 g), *Clonostachys wenpingii* (MEF 65) (13.2 g), *Meyerozyma guilliermondii* (MEF 122) (16.0 g), *Meyerozyma guilliermondii* (MEF 75) (20.3 g) and *Aspergillus fumigatus* (MEF 11) (26g). Though *Aspergillus fumigatus* (MEF 11) produced the highest extract yield, it was one of the least active when tested against both *Candida albicans* and *Saccharomyces cerevisiae*.

The extracts were tested for antifungal activity against *Saccharomyces cerevisiae* and *Candida albicans* using the disc diffusion method. All crude extracts tested were active against *C. albicans* (Fig 3.3A). However, only the *Clonostachys wenpingii* (MEF65) extract was active against *S. cerevisiae* (Fig. 3.3 B). Extract from *Aspergillus fumigatus* (MEF 112) and *Meyerozyma guilliermondii* (MEF 122) had the highest zone of inhibition against *Candida albicans* (11 mm). This zone of inhibition was comparable to that obtained for Amphotericin B (11mm), which is a standard antifungal compound (Fig 3.3 A). Extracts from *Aspergillus fumigatus* (MEF 11) and *Clonostachys wenpingii* (MEF 65) recorded the lowest zone of inhibition (7 mm) against *C.*

*albicans*. Extracts from *Meyerozyma guilliermondii* (MEF 75) and *Meyerozyma guilliermondii* (MEF 134) had zones of inhibition of 8 mm and 9 mm respectively (Fig 3.3 A).

Only the *Clonostachys wenpingii* (MEF65) extract was active against *S. cerevisiae* (Fig 3.3 B). The zone of inhibition that was recorded for this extract was 7 mm.

**Table 3.2.** Mass of the crude extracts

| Extract code | Mass of extracts (g) |
|---|---|
| MEF 11 | 26.0 |
| MEF 65 | 13.2 |
| MEF 75 | 20.3 |
| MEF 112 | 7.2 |
| MEF 122 | 16.0 |
| MEF 134 | 9.6 |

These extracts were obtained from ten replicates of 2L cultures for each MEF isolate.

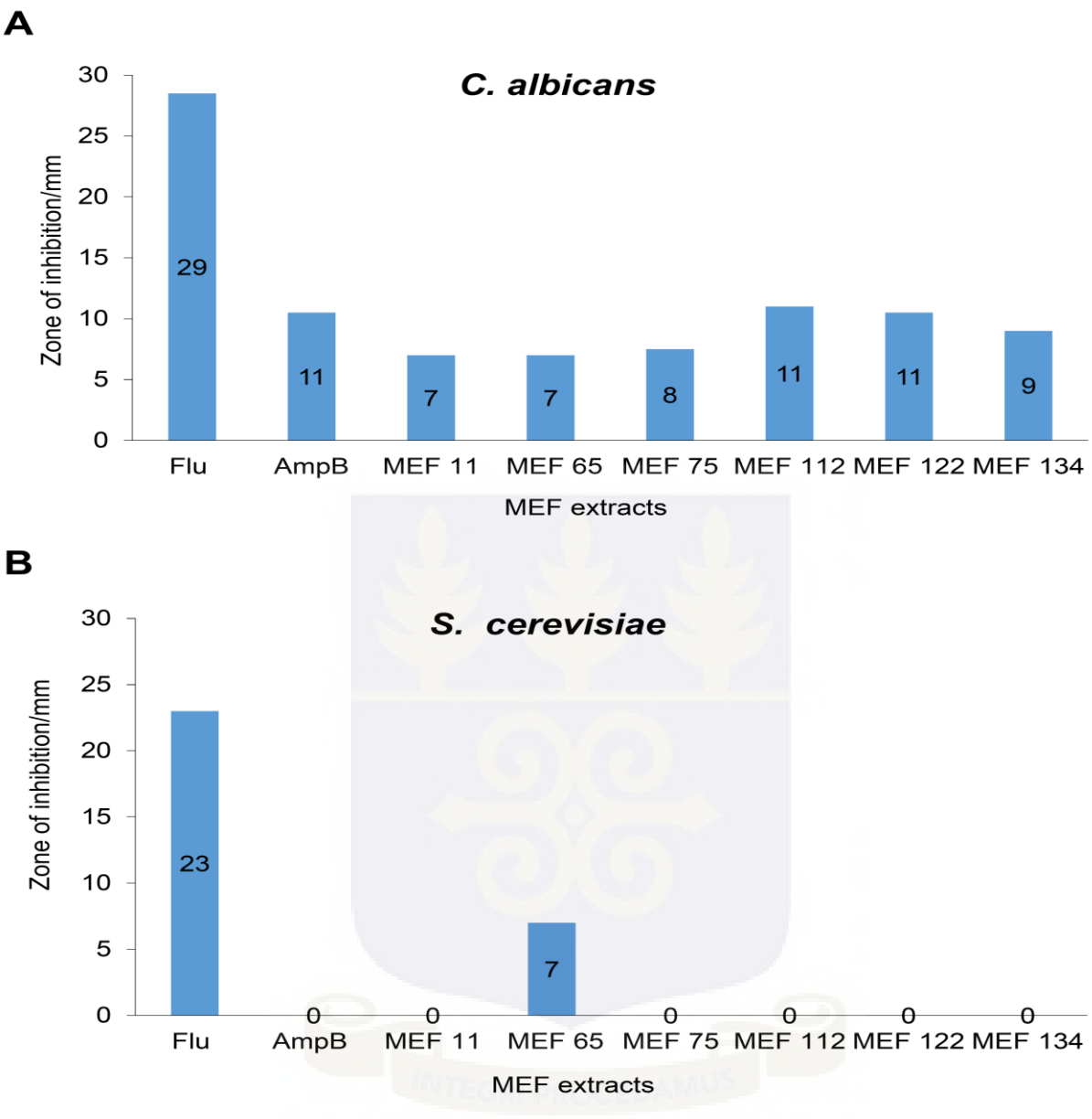


Fig 3.3. Antifungal activity of crude extracts from the six marine endophytic fungal isolates. (A) Antifungal activity of crude extracts on *C. albicans*. (B) Antifungal activity of the crude extract on *S. cerevisiae.*

### 3.3.5 Bioactivity of the crude extracts against the *S. cerevisiae* Mutant strains

After establishing that the 6 MEF produced metabolites with antifungal properties, it was important to have an idea about the potential mechanism of action of the compounds contained in these extracts. To have a general indication of the likely pathways the compound contained in these crude extracts may be targeting, the extracts were tested against a panel of diploid deletion mutants of *Saccharomyces cerevisiae* (Δ*erg*2 (homo), Δ*erg*2 (het), Δ*eft*1 (het), Δ*eft*2 (het) and Δ*yef*3 (het)) (Fig 3.4).

The mutant strains interacted with the extracts in a distinctively different manner, indicating there are different bioactive compounds present. Only 3 strains (Δ*eft*1 (het), Δ*erg* 2 (het) and Δ*yef* 3 (Het)) were sensitive to metabolites produced by *Aspergillus fumigatus* (MEF 11) with the most sensitive being Δ*yef* 3 (16.5 mm) (Fig 3.4). The zones of inhibition produced against Δ*eft*1 (het) and Δ*erg* 2 (het) were 15 mm and 6 mm respectively. These observations indicate that among the 3 sensitive strains, Δ*erg* 2 (het) was not highly affected. Extract from *Clonostachys wenpingii* (MEF 65) was active against Δ*erg*2 (het) and the wildtype *S. cerevisiae* strain, with zones of inhibition of 15 mm and 7 mm respectively. The Δ*erg*2 (het) strain was the most sensitive to this extract.

The most active extract was from *Meyerozyma guilliermondii* (MEF 75); a total of 4 strains (Δ*erg*2 (het), Δ*eft*1 (het), Δ*eft*2 (het) and Δ*yef*3 (het)) were susceptible to this extract. The most sensitive strain was Δ*yef*3 (het) (15 mm) while the least sensitive was Δ*eft*2 (het) (6 mm). The zones of inhibition recorded against the two other strains (Δ*erg*2 (het) and Δ*eft*1 (het)) were 9.5 mm and 14.5 mm, respectively. Extracts from *Aspergillus fumigatus* (MEF112) was active against Δ*yef*3 (het), Δ*eft*1 (het) and Δ*erg*2 (het). The zones of inhibition recorded against these strains were 9 mm, 9.5 mm and 10 mm respectively. Two isolates, *Meyerozyma guilliermondii* (MEF 122) and

*Meyerozyma guilliermondii* (MEF 134) produced extracts which were active against these three strains, (Δ*yef*3 (het), Δ*eft*1 (het) and Δ*erg*2 (het)). However, the extract produced by *Meyerozyma guilliermondii* (MEF 122) was more active (14.5mm, 14.5 mm and 6 mm for Δ*yef*3 (het), Δ*eft*1 (het) and Δ*erg*2 (het), respectively) as compared to that of *Meyerozyma guilliermondii* (MEF 134). The zones of inhibition recorded for the extract from MEF134 against these three strains were Δ*yef*3 (9.5 mm), Δ*eft*1 (11 mm) and Δ*erg*2 (9 mm). None of the extracts were active against Δ*erg*2 (homo) strain. The Δ*erg*2 (homo) strain was resistant to extracts from all the 6 MEF isolates.

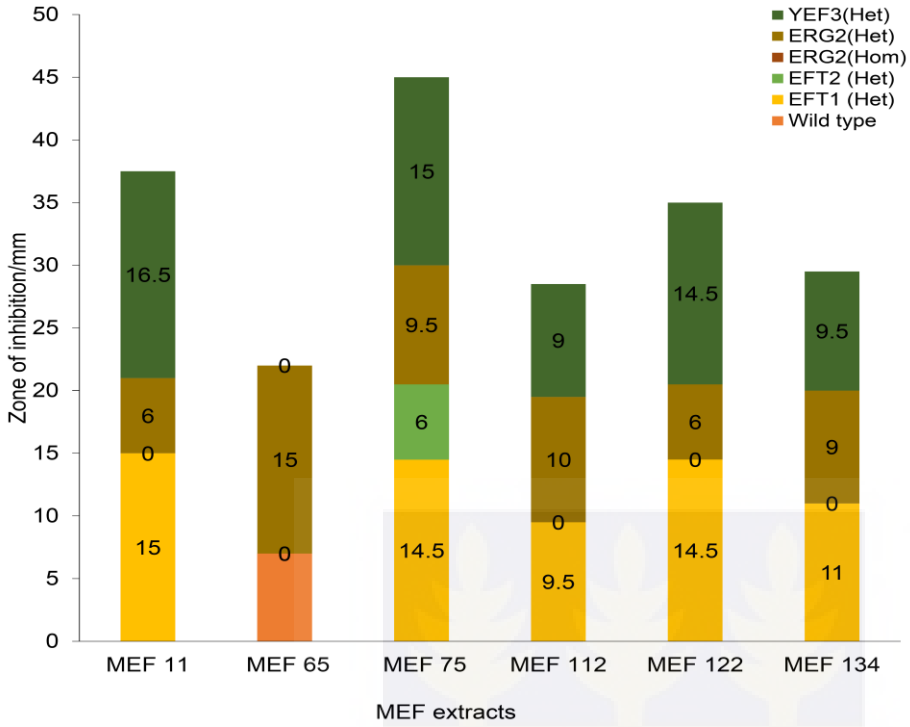


Fig 3.4. Antifungal activity of crude extracts against the *S. cerevisiae* mutant strains. Susceptibility of the *S. cerevisiae* mutant strain and the wild type diploid organism to the 6 fungal extracts was determined by the agar disc diffusion method. The plates were incubated for 24 hours and the zone of inhibition measured in mm.

### 3.3.6 Yield, TLC profiles and antifungal properties of the Kupchan fractions

Crude extracts are complex materials containing numerous compounds some of which have bioactivity of interest. The overall aim is to reduce the complexity of the crude extract and obtain fractions which contain fewer compounds with biological activity. This objective was achieved by utilizing the Kupchan solvent partitioning system (Fig S3.6). This solvent partitioning technique is based on the principle that polar compounds are soluble in polar solvent and non-polar compounds are soluble in non-polar solvents (Visht & Chaturvedi, 2012).

The crude extracts from all 6 MEF were partitioned among 7 solvent systems which led to the generation of 7 (FW, FE, FB, FM50, FME, FD and FH) different fractions for each crude extract that was analyzed. The mass distributions of the Kupchan fractions generated are shown in (Table 3.3). Figure 3.5 shows the percentage mass distribution of the Kupchan fractions separated. For all the 6 crude extracts that were partitioned, high fraction yields were obtained with dichloromethane (FD) and hexane (FH) solvents (Fig 3.5). There were substantial fraction yields obtained when n-Butanol was used in the partitioning process for MEF 112 and MEF 134 (Fig 3.5 D and F). In all cases, more components were resolved with the dichloromethane (FD) and hexane (FH) solvents as compared to all other solvents used.

Analytical TLC profiles were obtained for extracts of the marine endophytes and the Kupchan fractions obtained (Fig 3.6). In comparison with the crude extract from *Aspergillus fumigatus* (MEF 11), which had a very prominent band close to the solvent front, the band pattern for fractions FW, FE, FB and FD revealed extra prominent bands in the mid-section of the TLC plate. Fractions FM50, FME and FH had very faint bands. For *Clonostachys wenpingii* (MEF 65), band patterns of the crude extract and fractions were different, as expected (Fig 3.6). Fainter bands were observed for fraction FW. The profile of fractions FB, FE, FM50, and FME were all similar (even

though it does not imply the presence of the same compounds). Fractions FD and FH also had very similar profiles.

The band profiles of extract and fractions from *Meyerozyma guilliermondii* (MEF 75) were more varied. Similarly, the profiles of extract and fractions from *Aspergillus fumigatus* (MEF112) were distinct with a single observable band at the solvent front of the FD fraction. A similar separation profile was also observed for fractions from *Meyerozyma guilliermondii* (MEF 122) and *Meyerozyma guilliermondii* (MEF 134); a single noticeable band was observed towards the mid-section of the TLC plate for the FD fraction. The bands partitioned on the TLC plates during the analyses of the Kupchan fractions specified the separation of the diverse compounds contained in these fractions. It also gave a general indication of the purity of the fractions.

The Kupchan fractions were tested against *C. albicans* and *S. cerevisiae* to determine their antifungal potentials (Table 3.4 and 3.5). When these fractions were tested against *C. albicans*, a total of 6 fractions were active at the lower concentration (1X). However, this number increased to 18 when the concentration was increased by threefold (3X) (Table 3.4). The highest activity recorded was 16.5 mm and was from the FB fraction of *Clonostachys wenpingii* (MEF 65). The lowest activity was 6 mm and was from the FH fraction of *Aspergillus fumigatus* (MEF 11).

Only 4 fractions showed activity against *S. cerevisiae* at the lower concentration (1X). This number also increased to 13 when the concentration was increased. The highest and lowest zone of inhibition recorded were 20.5 mm and 6 mm, respectively.

**Table 3.3.** Mass of the Kupchan fractions

| Kupchan Fraction | Mass of Kupchan fractions in (g) | | | | | |
|---|---|---|---|---|---|---|
| | MEF 11 | MEF 65 | MEF 75 | MEF 112 | MEF 122 | MEF134 |
| FW | 0.13 | 0.80 | 0.22 | 0.12 | 0.11 | 0.10 |
| FE | 0.43 | 1.21 | 0.33 | 0.19 | 0.10 | 0.10 |
| FB | 0.69 | 1.29 | 1.66 | 1.10 | 0.68 | 2.62 |
| FM50 | 1.45 | 0.17 | 0.40 | 0.15 | 0.18 | 0.16 |
| FME | 0.55 | 0.21 | 0.82 | 0.21 | 0.38 | 0.39 |
| FD | 6.15 | 2.88 | 8.76 | 1.22 | 4.62 | 3.20 |
| FH | 6.99 | 7.67 | 4.41 | 1.87 | 4.75 | 3.87 |
| TOTAL | 16.39 | 14.23 | 16.60 | 4.86 | 10.82 | 10.44 |

FW, FE, FB, FM50, FME, FD, and FH represent the Kupchan fractions from water, ethyl acetate, butanol, 50% methanol, methanol-ethyl acetate, dichloromethane and hexane.

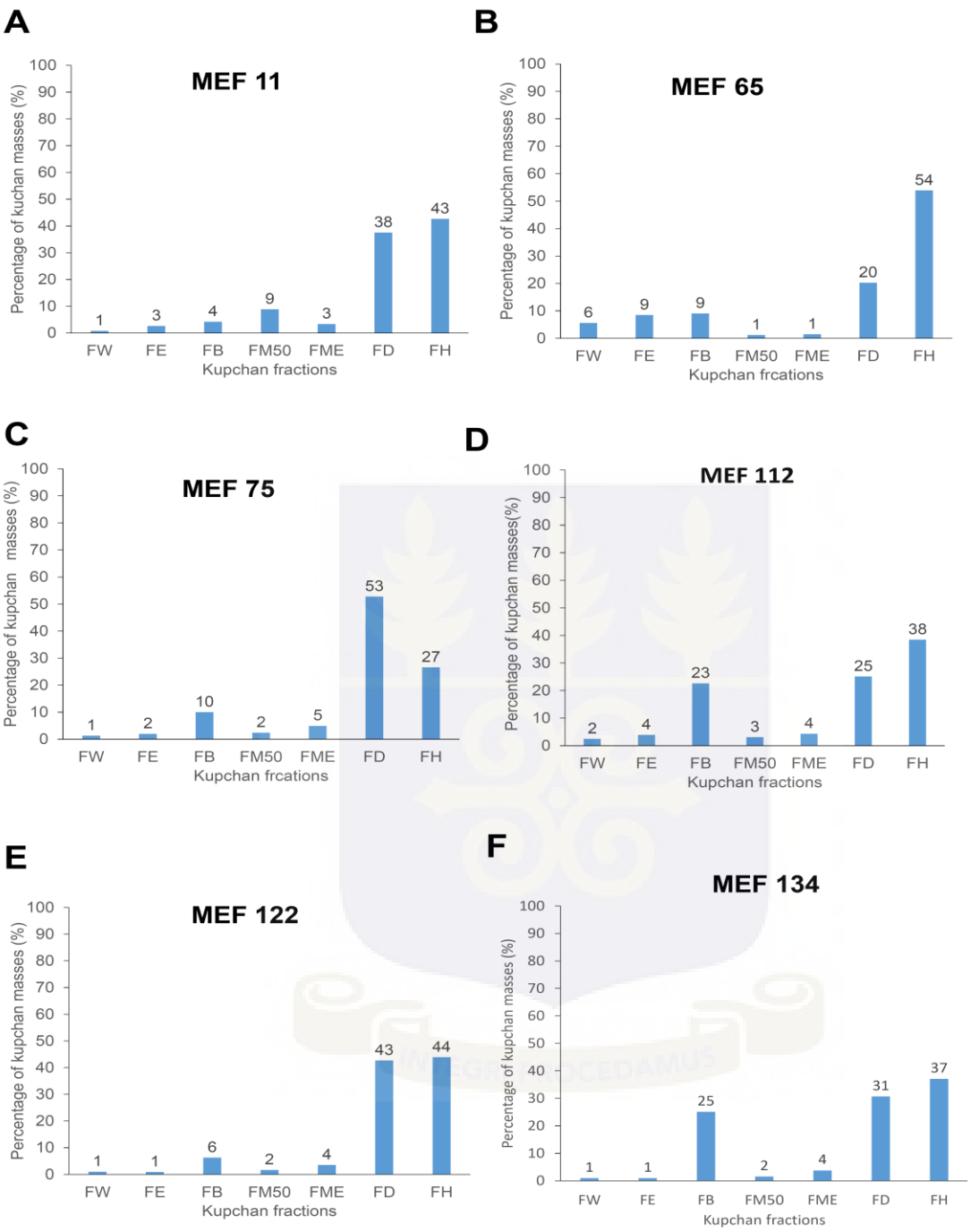


Fig 3.5. Percentage mass distribution of the Kupchan fractions. (A-F) In all cases more products migrated into the Dichloromethane (FD) and Hexane (FH) solvents.

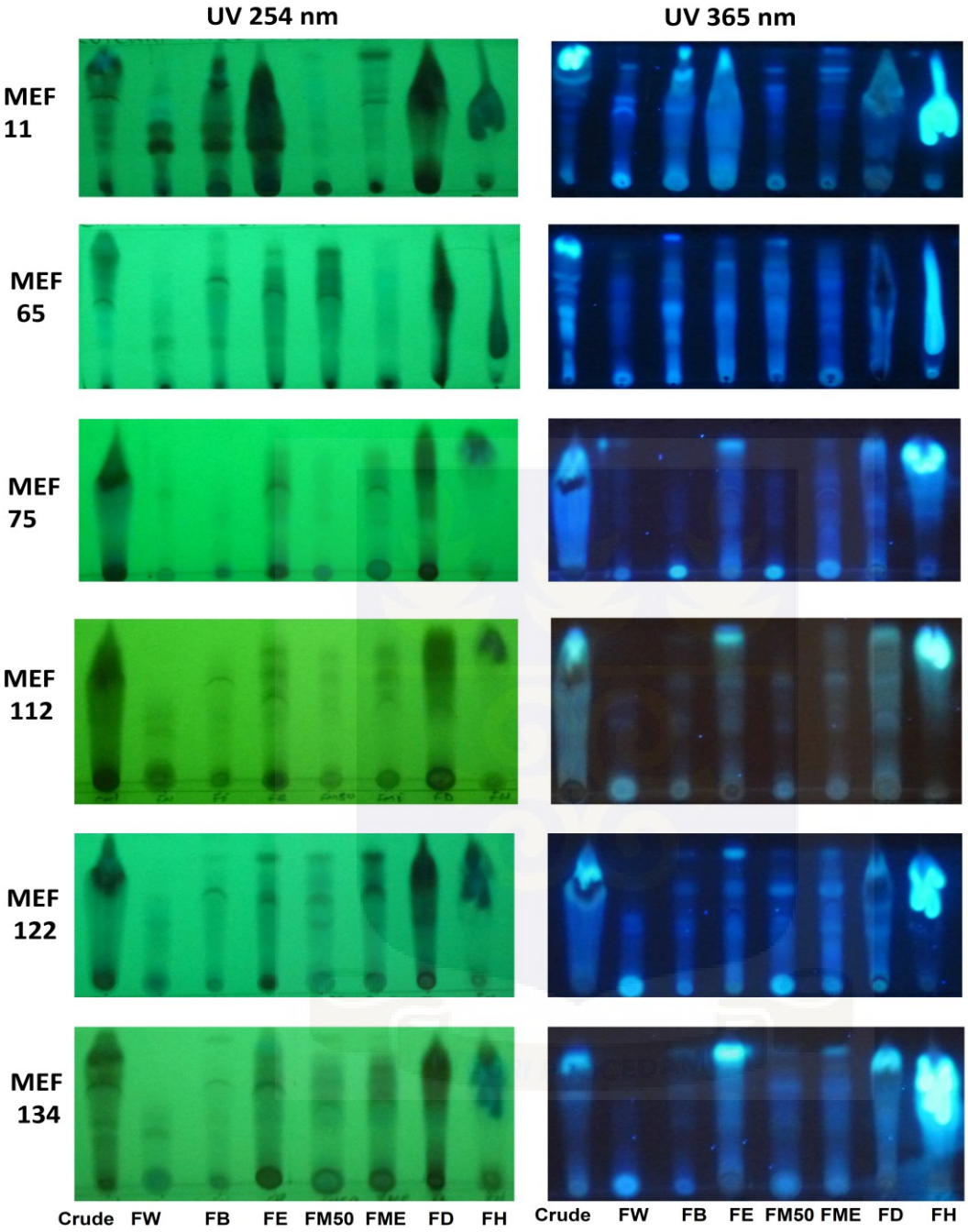


Fig 3.6. Analytical TLC profile of the crude and Kupchan fractions from the 6 isolates viewed under UV light. TLC plates were spotted with 5µl of crude extract and fractions, developed with a solvent system comprising (Ethyl Acetate: Acetonitrile: Petroleum Ether, 7:2:1), and viewed under UV (254nm and 365nm).

**Table 3.4.** Effect of Kupchan fractions from the 6 isolates on *C.albicans*

| | Zone of Inhibition (mm) | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | (MEF 11) | | (MEF 65) | | (MEF 75) | | (MEF 112) | | (MEF 122) | | (MEF 134) | |
| Kupchan Fractions | (1X) | (3X) | (1X) | (3X) | (1X) | (3X) | (1X) | (3X) | (1X) | (3X) | (1X) | (3X) |
| FW | 0 | 0 | 0 | 0 | 0 | 10 | 0 | 0 | 0 | 0 | 0 | 0 |
| FE | 0 | 0 | 6 | 15.5 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| FB | 0 | 0 | 12 | 16.5 | 9.5 | 12 | 0 | 11.5 | 0 | 0 | 0 | 11.5 |
| FM50 | 0 | 0 | 0 | 19 | 0 | 6 | 0 | 0 | 0 | 0 | 0 | 9.5 |
| FME | 0 | 0 | 0 | 0 | 8.5 | 12.5 | 0 | 10.5 | 0 | 0 | 8.5 | 13 |
| FD | 0 | 11 | 9.5 | 13 | 0 | 8.5 | 0 | 10.5 | 0 | 7.5 | 0 | 12 |
| FH | 0 | 6 | 0 | 7 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |

1X= 30 µL – 0.1% unit of total extract and 3x= 90 µL – 0.3% unit of total extract

**Table 3.5.** Effect of Kupchan fractions from the 6 isolates on *S. cerevisiae*

| | Zone of Inhibition (mm) | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | (MEF 11) | | (MEF 65) | | (MEF 75) | | (MEF 112) | | (MEF 122) | | (MEF 134) | |
| Kupchan Fractions | (1X) | (3X) | (1X) | (3X) | (1X) | (3X) | (1X) | (3X) | (1X) | (3X) | (1X) | (3X) |
| FW | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| FE | 0 | 0 | 6 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| FB | 0 | 0 | 0 | 10.5 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 9 |
| FM50 | 0 | 0 | 0 | 15.5 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 10 |
| FME | 0 | 0 | 7 | 0 | 0 | 9.5 | 0 | 0 | 0 | 0 | 0 | 13.5 |
| FD | 0 | 11.5 | 7 | 0 | 0 | 20.5 | 0 | 6 | 0 | 34 | 6 | 10.5 |
| FH | 0 | 11 | 0 | 0 | 0 | 0 | 0 | 7.5 | 0 | 0 | 0 | 0 |

1X= 30 µL – 0.1% unit of total extract and 3x= 90 µL – 0.3% unit of total extract

### 3.3.7 Evaluation of the antifungal properties of the TLC fractions

Some selected active Kupchan fractions were further fractionated by the preparative TLC method in two consecutive rounds, all aimed at further purification of the biologically active components of the extracts. The selected fractions were from *Clonostachys wenpingii* (FE, FB, FD and FME), *Meyerozyma guilliermondii* (MEF 75) (FD, FME and FD) and *Meyerozyma guilliermondii* (MEF 134) (FD) (Table S3.3) extracts.

After the preparative TLC separation the fractions obtained were tested by the disc diffusion method against both *C. albicans* and *S. cerevisiae*. Antifungal activity was only observed against *C. albicans* after analyses of the 1D TLC fractions. The number of active 1D TLC fractions obtained from MEF 65, 75 and 134 were 6, 5, and 5 respectively (Table 3.6). Subsequently, the active fractions were taken through a second round of preparative TLC analyses. The activities of the fractions obtained are shown in Tables S3.4 and S3.5. Further separation of these active fractions resulted in a total of 59 active fractions from the three isolates (Table 3. 6). None of the fractions generated in the TLC separation were active against *S. cerevisiae* (Table 3.7).

**Table 3.6.** Number of active fractions against *C. albicans*

| Fungi | Active fractions | | |
|---|---|---|---|
| | Kupchan | 1D | 2D |
| MEF 11 | 2 | 0 | 0 |
| MEF 65 | 2(5) | 6 | 25 [22] |
| MEF 75 | 2(5) | 5 | 22 [19] |
| MEF 112 | 1(5) | 0 | 0 |
| MEF 122 | 1 | 0 | 0 |
| MEF 134 | 4 | 5 | 12 |

**Table 3.7.** Number of active fractions against *S. cerevisiae*

| Fungi | Active fractions | | |
|---|---|---|---|
| | Kupchan | 1D | 2D |
| MEF 11 | 2 | 0 | 0 |
| MEF 65 | 2 | 0 | 0 |
| MEF 75 | 2 | 0 | 0 |
| MEF 112 | 1 | 0 | 0 |
| MEF 122 | 1 | 0 | 0 |
| MEF 134 | 4 | 0 | 0 |

### 3.3.8 Evaluation of the antifungal properties of the TLC fractions by MTT assay method

Further to this, 59 selected fractions from the second round of TLC analyses were validated by the MTT assay against *C. albicans* (Fig 3.7). Absorbance, which is also proportional to the number of viable cells present, was as high as 11.5 in the presence of Amphotericin B. In contrast, in the presence of fraction MEF134FDV5V9 absorbance reading was as low as 0.1. This 0.1 value was also lower than that recorded for Fluconazole (0.5µg), which is another standard antifungal agent. A total of 57 out of the 59 fractions tested against *C. albicans* had their absorbance values lower than that observed for Amphotericin B (11.5).

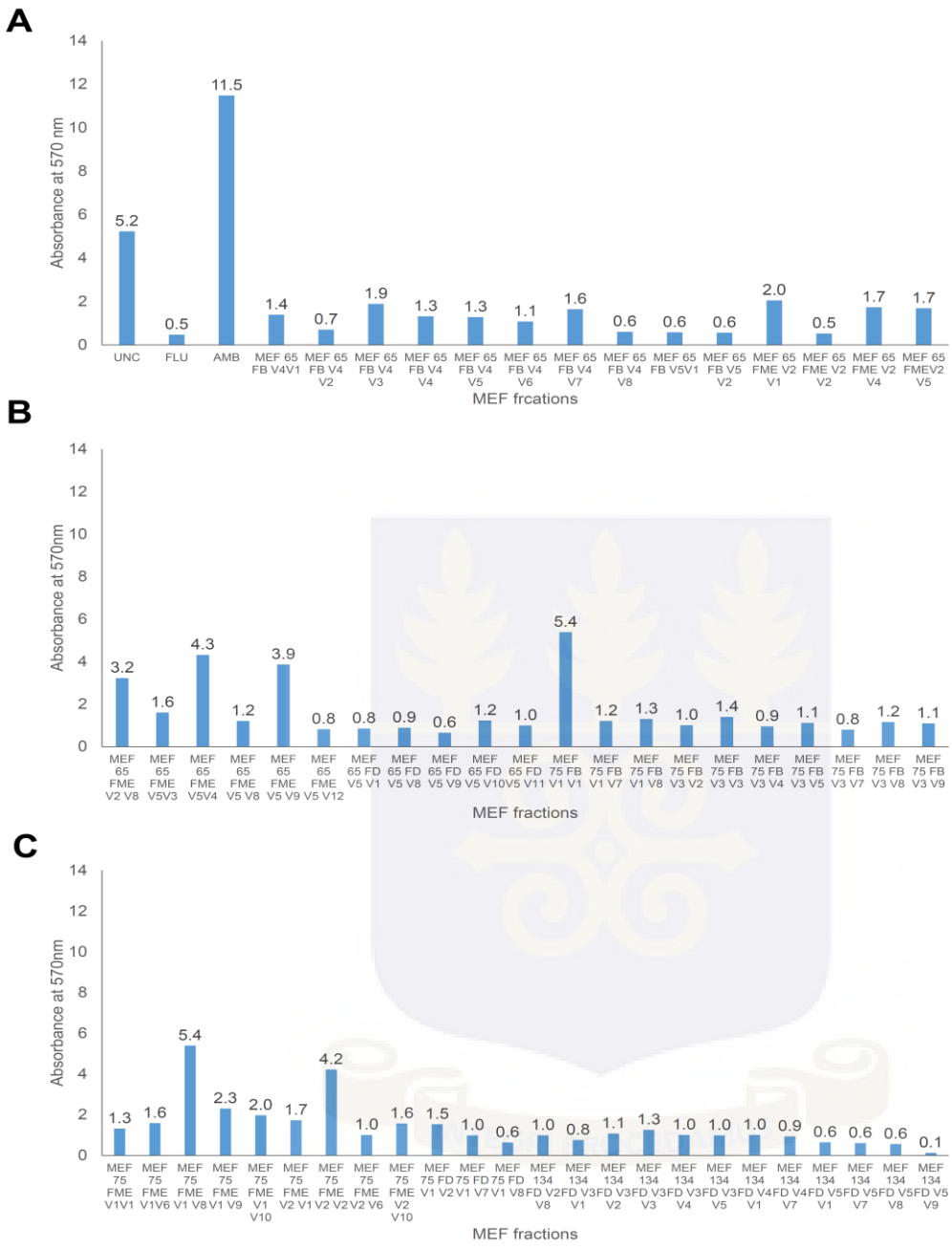


Fig 3.7. Validation of the activities of the 59 active TLC fractions on *C. albicans* by MTT assay. (A) Metabolic activity levels of *C. albicans* after challenging the cells with Fluconazole, amphotericin B and the first 18 fractions (B) Profile after the next set of 21 fractions were tested (c) Profile of the final 24 fractions.

### 3.4 Discussion

In this study six MEFs were shown to produce antifungal metabolites against *C. albicans,* at the level of the Kupchan fractionation; 21 out of 42 fractions for the 6 MEF were active. Out of the 21 active fractions, 8 were fractionated by 1D and 2D prep TLC due to their significant quantities, which ultimately yielded 59 active fractions. It can therefore be projected that the initial 21 active Kupchan fractions are fractionated in a similar manner a total of 154 highly enriched active 2D prep TLC fractions can be obtained which represents a rich resource for the isolation of bioactive compounds. This phase of the work could not be accomplished due to the significantly higher volumes of liquid cultures required to prepare sufficient quantities of pure compounds. Mass spectrometric analysis was employed to determine the structures of compounds in some of the active fractions and the detailed of the promising novel structures are described in chapter 7. Many drugs developed in the 1980s were from natural sources and these drugs have been successful in treating infectious and non-infectious diseases. The sea environment has been ignored as a cradle of structurally and bio-actively unique compounds. Hence, in this study the antifungal potential of endophytic fungi associated with seaweed was explored in an effort to discover new compounds against *Candida albicans*. A large number of endophytic fungi (143) were isolated from the seaweeds. This extraordinary level of endophyte colonization is also supported by earlier findings which indicated that prevalence of endophytes in seaweeds is very high (Venkatachalam *et al.*, 2015).

The ITS sequence analysis of the 6 fungi showed that, these isolates belong to three major genera; *Aspergillus*, *Clonostachys* and *Meyerozyma* (Table 1). Apart from *Clonostachys wenpingii* which has never been isolated from the marine environment, all other fungi identified in this study have been isolated from marine organisms in previous studies (Chen *et al.*, 2015; Zhang *et al.*, 2007).

Chen and colleagues obtained 5 new compounds from *Meyerozyma guilliermondii* isolated in the South China Sea (Chen *et al.*, 2015). Moreover, a new imine derivative was isolated from marine derived *Aspergillus niger* EN-13 (Zhang *et al.*, 2007). Even though some of the identified fungi were from the same genera (Fig 1), their metabolite production and bioactivity patterns were very different. A similar phenomenon was observed by Li and colleagues. Out of the numerous endophytes of the same species isolated from the same host organism, only one of the endophytes produced highly active metabolites in culture (J.-y. Li *et al.*, 1996).

The results from the extraction yields indicated that *Clonostachys wenpingii* (MEF 65), *Aspergillus fumigatus* (MEF112) and *Meyerozyma guilliermondii* (MEF 134) showed different amounts of extractable compounds. It has been shown by the study of different plant samples that the type of sample used has a higher influence on the extraction yield rather than the solvent system used for the extraction (Babbar *et al.*, 2011). This may be the reason for the differences in the total extract yields obtained from the 3 organisms, even though the same amount of starting material was used in each case. From the TLC analyses, there were many blue light emitting compounds (Fig 3.6). The blue light emitting compounds on the TLC plates can easily be synthesized and when explored further can have many applications in light emitting devices (LEDs). Compounds of this nature are desirable because of their thermal and photochemical robustness (li *et al*., 2004). Also these compounds can be used as dyes for many biological application.

Six marine endophyte isolates were screened in this study and they all produced antifungal metabolites against *C. albicans* (Fig 3.3 A). In contrast, only *Clonostachys wenpingii* produced active metabolites against *S. cerevisiae* (Fig 3.3B). These results suggest that an elaborate study of the metabolites of endophytic fungi may reveal a rich collection of antifungal molecules.

Solvent selection for Kupchan fractionation is based on polarity. Generally, secondary metabolites have diverse degrees of solubility, hence careful selection of solvents that will ensure optimum dissolution of secondary metabolites is necessary. The results from the Kupchan fractions analyses showed that the metabolites from the six fungi were mostly less polar because the highest extract yields were from dichloromethane and hexane solvents (Fig 3.5). It is extensively known that polar substance are easier to extract compared to non-polar substances (Razak *et al.*, 2012). Therefore it is interesting to note that in our current study less polar metabolites were extracted. The differences in yield for the other solvents may be due to pH of the extracting solvent, interaction between dissolved components, quantity of metabolite produced, thermostability of metabolites and extraction time.

A higher proportion of the partially purified TLC fractions that were analyzed by the MTT assay against *C. albicans* were more active than Amphotericin B, which is a standard antifungal agent (Fig 3.7). None of these fractions were active against *S. cerevisiae*. Due to genetic similarities between yeast and humans, compounds which are usually toxic to yeast may also be toxic to humans. It is therefore interesting to note that these fractions were only active against *C. albicans* and not S. *cerevisiae*. This further suggests that the compounds contained in these fractions may be targeting specific physiological processes unique to *C. albicans* only. Earlier findings reported that endophyte fungi of *Meyerozyma* and *Aspergillus* species are important sources of antifungal secondary metabolites (Arora & Chandra, 2010; Zhang *et al.*, 2007).

*Saccharomyces cerevisiae* which has been very well characterized genetically provides a means to better understand the mechanism of action of many compounds *in vivo* (Botstein & Fink, 2011). The use of mutant strains, together with the wild type organism, may help to identify drug targets, genes required for protecting drug target trails and to determine the cellular reaction of the

organism to new compounds (Smith *et al.*, 2010). In this study, a yeast chemical genomic assays was performed to predict the potential mechanisms of action of compounds contained in the extracts (Fig 3.4). Three strains (Δ*eft*1 (het), Δ*erg* 2 (het) and Δ*yef* 3 (Het)) were sensitive to metabolites produced by *Aspergillus fumigatus* (MEF 11) (Fig 3.4). Generally, a heterozygous deletion strain which is most sensitive to a particular compound often identifies the compounds target (Giaever *et al.*, 2004). Hence, extracts from *Aspergillus fumigatus* (MEF 11) may contain compounds that inhibit protein synthesis, ergosterol biosynthesis and fungal translational apparatus, respectively (Chakraburtty & Triana-Alonso, 1998; Mukhopadhyay *et al.*, 2002; Shastry *et al.*, 2001). The wildtype *S. cerevisiae* and Δ*erg*2 (het) was susceptible to the extract from *Clonostachys wenpingii* indicating that this extract may contain compounds that inhibit the ergosterol biosynthesis pathway. *Meyerozyma guilliermondii* (MEF 75), *Aspergillus fumigatus* (MEF112) and *Meyerozyma guilliermondii* (MEF 134) all produced metabolites that inhibited similar pathways as described for *Aspergillus fumigatus* (MEF 11).

The Δ*erg*2 (homo) strain was not sensitive to any of the extracts. For Δ*erg*2 (homo), the lack of sensitivity to any of the extracts may imply that there exist an alternative pathway for ergosterol biosynthesis that enables it to maintain the cell membrane integrity. However, we could not confirm this hypothesis from the scope of this study. Previous studies have shown that *erg*3 mutants are able to tolerate azoles via accumulation of 14-methyl-fecosterol, which is a harmless intermediate from ergosterol biosynthesis (Kontoyiannis, 2000). It has been demonstrated that fluconazole bioactivity against yeast cells require the combination of an intact Erg3 protein and mitochondria in order to exhibit its toxicity (Kontoyiannis, 2000). Like *erg*3, *erg*2 is also a gene in the ergosterol biosynthetic pathway. Since Δ*erg*2 (homo) is a homozygous deletion mutant,

there would be no production of the Erg2 protein which may be important for activity of the extracts against this strain.

The findings from our studies suggest that the ability of extracts to exhibit activity in *S. cerevisiae* might be connected to the integrity of membrane lipids, protein synthesis and translation.

A significant benefit of this mutant assay is that it identifies extracts that are permeable to cells and their possible targets. Usually, these targets are important for growth hence may create an avenue for development of novel antifungal agents. In spite of this, yeast assays are not without limitations. Due to the obstruction presented by the yeast cell wall, high amounts of extracts are necessary to produce a biological response. Also, the presence of efflux pumps ensure that compounds are eliminated from the cells almost immediately after uptake.

Our observations have shown that fungi from the genera *Clonostachys*, *Meyerozyma* and *Aspergillus* must be considered comprehensively in the development of novel antifungal compounds. It would be important to carry out studies aimed at the isolation and characterization of the compounds contained in the fractions. These compounds might represent novel antifungal agents against *C. albicans*.

### 3.5 Conclusion

In this study, the antifungal potential of endophytic fungi from seaweed was investigated. The results revealed that all the 6 selected fungi showed promising antifungal activity against *C. albicans*. The extracts might be targeting protein synthesis, ergosterol synthesis and the yeast translational apparatus. Further molecular research needs to be conducted in order to understand the full mechanisms of actions of the compounds contained in these active fractions and extracts.

The findings in this work further gives prominence to endophytic fungi from marine environment as a promising source of unique lead antifungal compounds.

### 3.6 Supplementary Data

**Table S3.1.** Reagents used for DNA extraction

| Lysis Buffer | Component |
|---|---|
| | 1 M Tris pH 8.0 |
| | 0.5 M EDTA |
| | 0.5 M NaCl |
| | SDS |
| | Distilled water |
| **Elution Buffer** | **Component** |
| | 1M Tris pH 8.0 |
| | Distilled water |
| **Wash Solution** | **Component** |
| | 1M Tris pH 8.0 |
| | 0.5 M EDTA |
| | 5.0 M NaCl |
| | Ethanol |
| | Distilled water |
| **Potassium acetate** | **Component** |
| | Potassium acetate |
| | Distilled water |
| **Guanidine Hydrochloride** | **Component** |
| | Guanidine Hydrochloride |
| | Ethanol |
| | Distilled water |

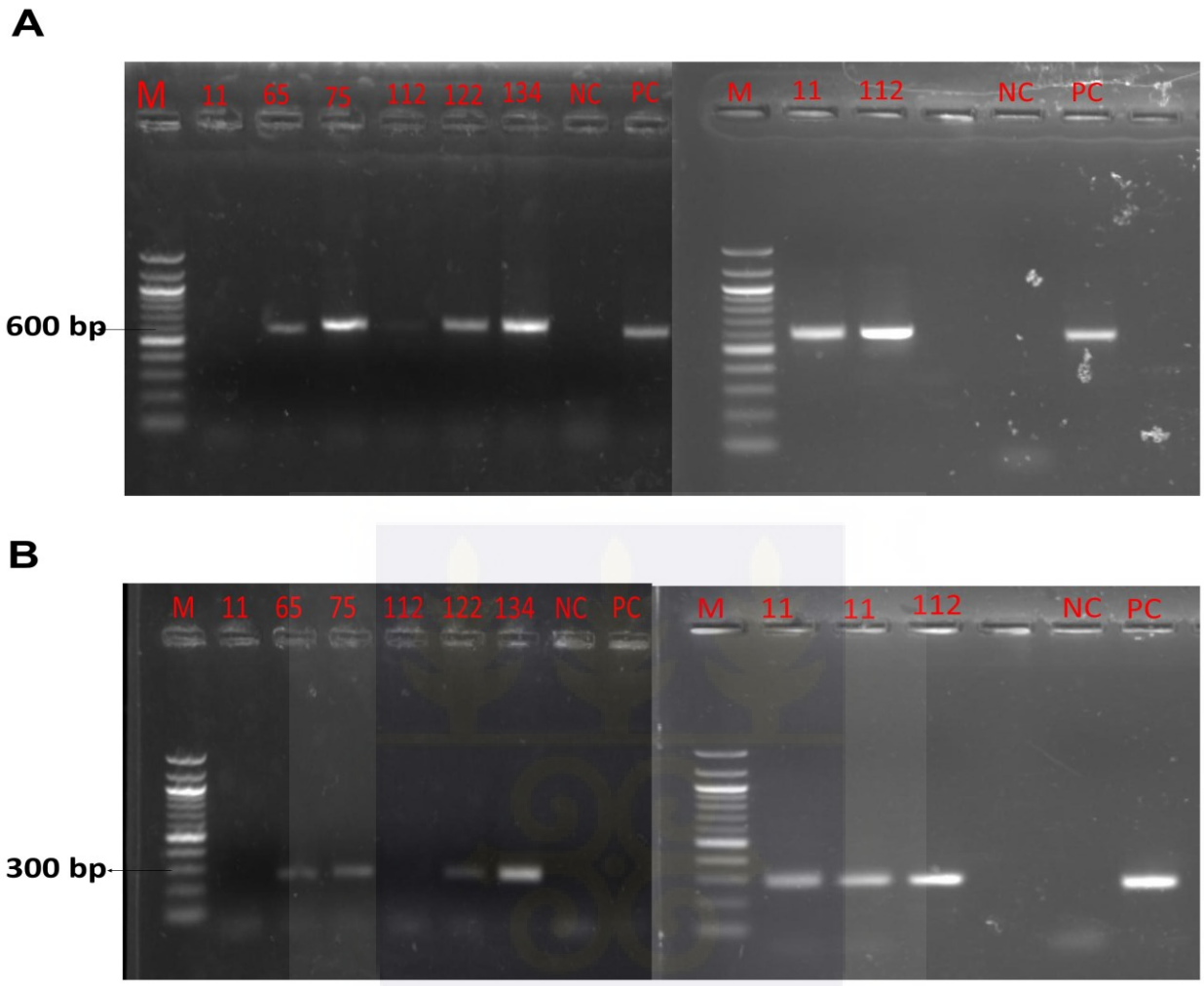


Fig S3.1. PCR products separated on 1.5% agarose gel after the amplification of the ITS region. (A) Using the primer pairs ITS 1 and ITS 4 the PCR amplicons obtained for MEF 11, 65, 75, 112,122 and 134. (B) Using the primer pairs ITS 2 and ITS 5 the PCR amplicons obtained for MEF 11, 65, 75,112, 122 and 134. M: 100-bp DNA ladder, Negative control (NC) and Positive control (PC).

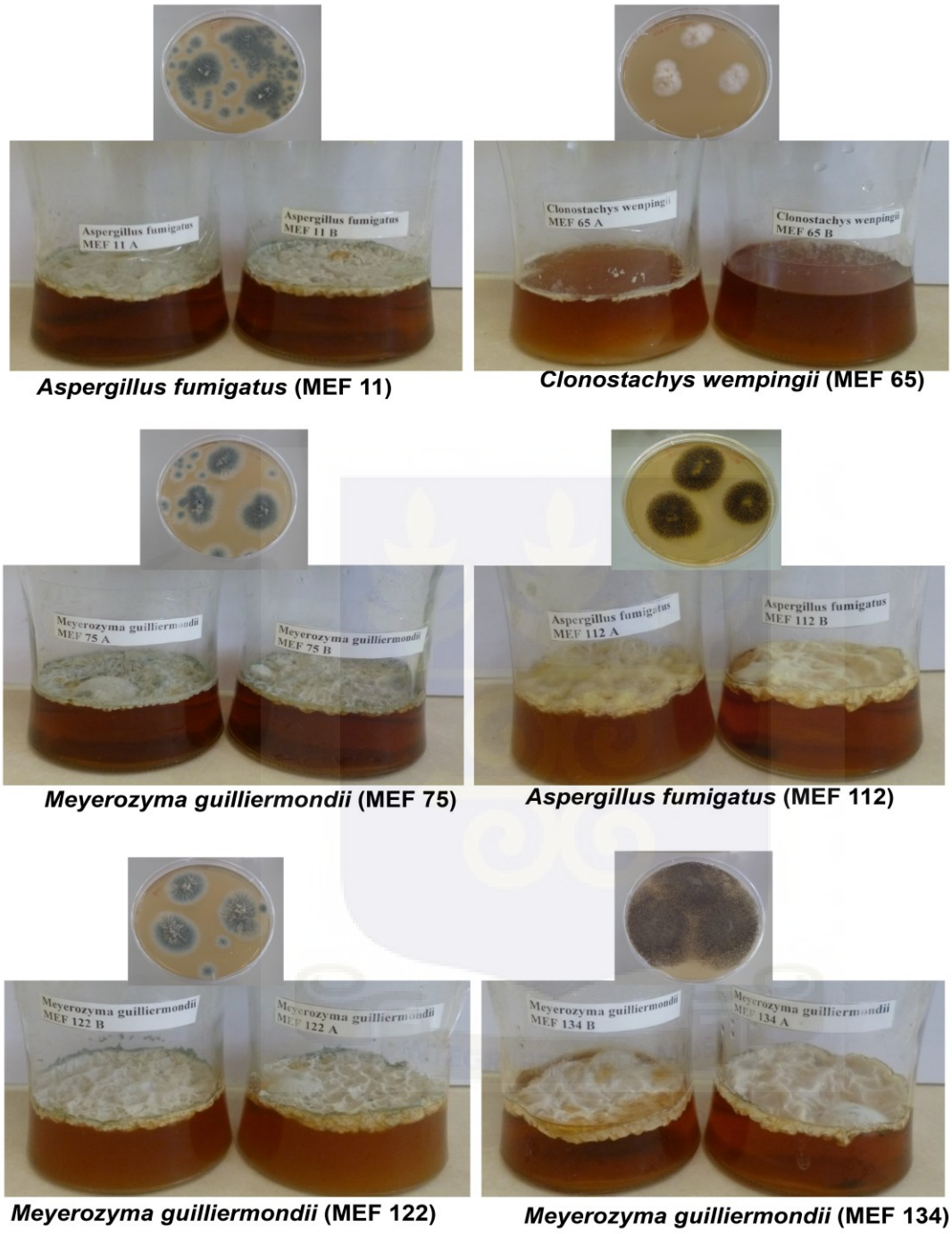


Fig S 3.2. The morphology of the 6 endophytic fungi grown in YPMD broth media and incubated at room temperature with constant agitation.

**Table S3.2.** Evaluation of culture yield (10×0.4 L broths)

| | Masses of extracts obtained in (g) | | |
|---|---|---|---|
| Broth number | MEF 65 | MEF 112 | MEF 134 |
| 1 | 0.25 | 0.66 | 0.45 |
| 2 | 0.23 | 0.69 | 0.22 |
| 3 | 0.29 | 0.36 | 0.1 |
| 4 | 0.72 | 0.32 | 0.33 |
| 5 | 0.14 | 0.55 | 0.4 |
| 6 | 0.22 | 0.15 | 0.4 |
| 7 | 0.51 | 0.31 | 0.13 |
| 8 | 0.45 | 0.28 | 0.29 |
| 9 | 0.23 | 0.72 | 0.23 |
| 10 | 0.35 | 0.33 | 0.43 |

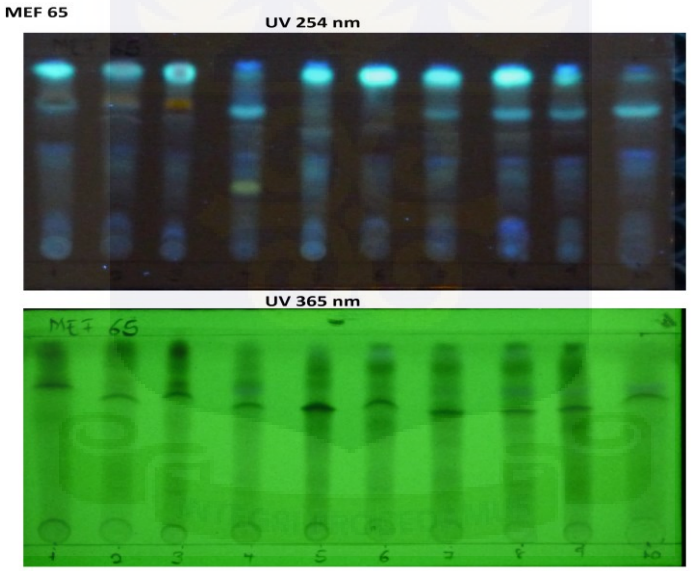


Fig S3.3. TLC profiles of the crude extracts obtained from the 10 broth cultures made with *Clonostachys wenpingii*. The plates were developed in an ethyl acetate-acetonitrile-petroleum ether solvent system (7:2:1) and viewed under UV light.

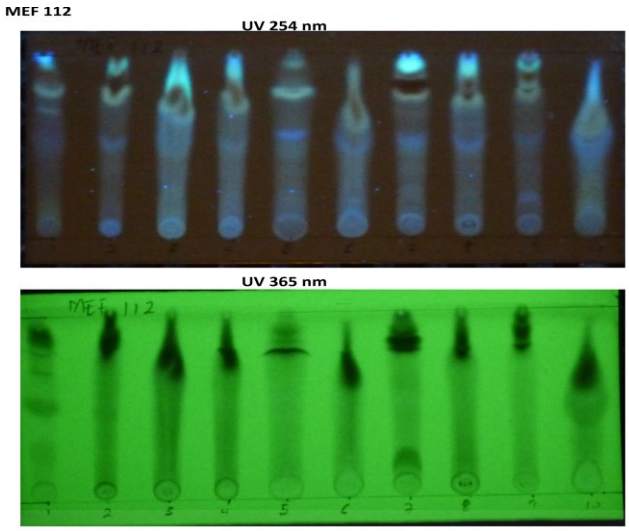


Fig S3.4. TLC profiles of the crude extracts obtained from the 10 broth cultures made with *Aspergillus fumigatus* (MEF 112). The plates were developed in an ethyl acetate-acetonitrile-petroleum ether solvent system (7:2:1) and viewed under UV light.

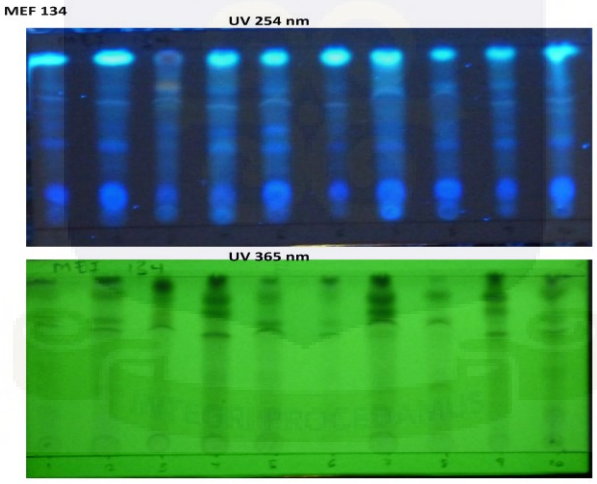


Fig S3.5. TLC profiles of the crude extracts obtained from the 10 broth cultures made with *Meyerozyma guilliermondii* (MEF 134). The plates were developed in an ethyl acetate-acetonitrile-petroleum ether solvent system (7:2:1) and viewed under UV light.

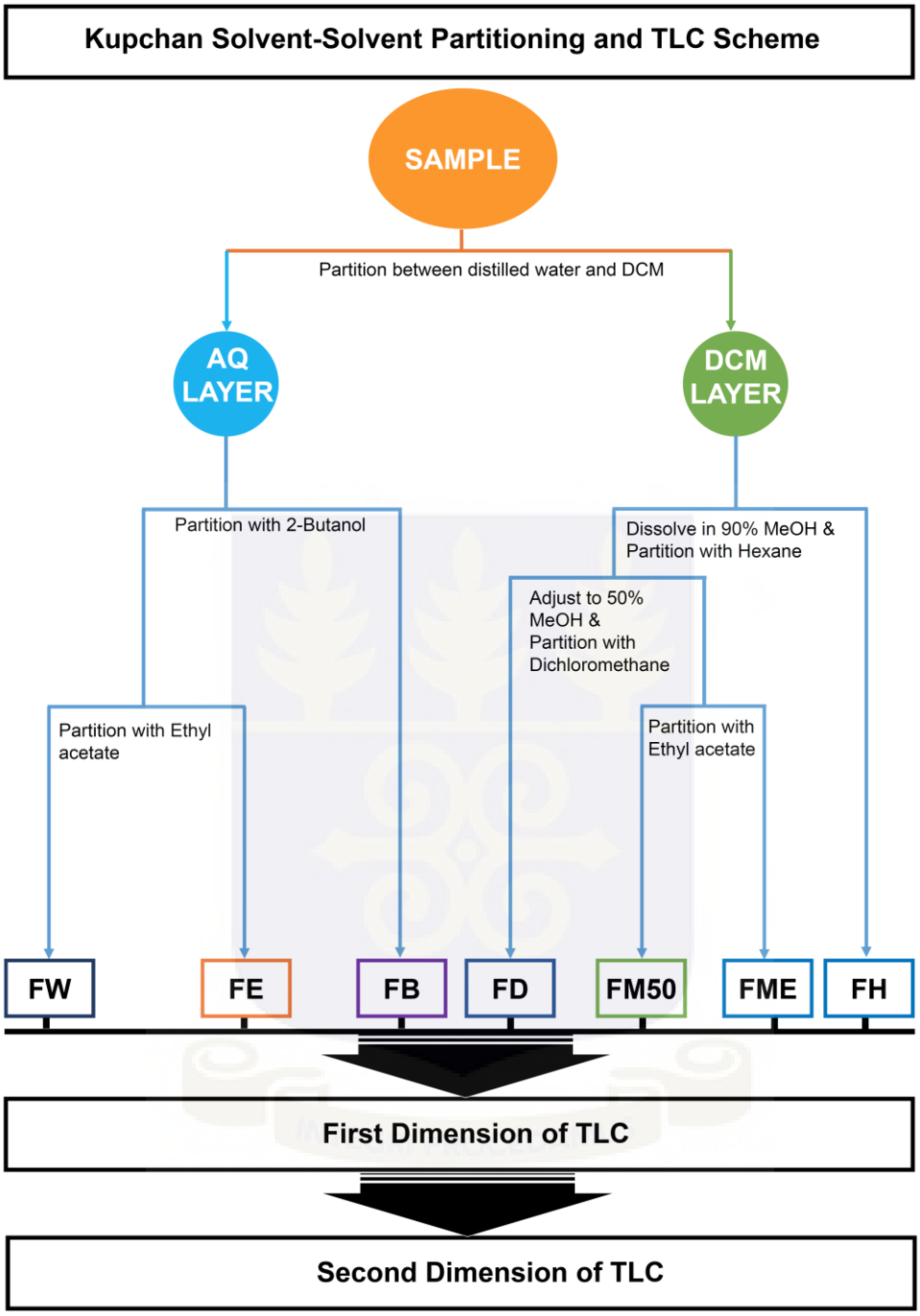


Fig S3.6. Schematic diagram showing the modified Kupchan separation and TLC procedure.

**Table S3.3.** Effect of TLC (1D) fractions on *C. albicans*

| | Zone of inhibition (mm) | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | MEF 65 | | | | MEF 75 | | | MEF 134 |
| TLC Fractions | FE | FB | FME | FD | FB | FME | FD | FD |
| V1 | 0 | 0 | 0 | 0 | 9 | 6 | 12 | 6 |
| V2 | 0 | 6 | 0 | 0 | 0 | 6 | 0 | 6 |
| V3 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 6 |
| V4 | 0 | 8 | 8.5 | 0 | 6 | 0 | 0 | 9 |
| V5 | 0 | 10 | 14.5 | 9 | 0 | 0 | 0 | 7 |

**Table S3.4.** Effect of TLC (2D) fractions on *C. albicans*

| | Zone of Inhibition (mm) | | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | MEF 65 | | | | | | MEF 75 | | | | | | MEF 134 | | | |
| TLC Fraction | FB V2 | FB V4 | FB V5 | FME V2 | FME V5 | FD V5 | FB V1 | FB V3 | FB V4 | FME V1 | FME V2 | FD V1 | FD V2 | FD V3 | FD V4 | FD V5 |
| V1 | 0 | 8.5 | 8.5 | 7.5 | 0 | 8 | 6 | 0 | 0 | 8.5 | 9 | 0 | 0 | 8.5 | 10.5 | 9 |
| V2 | 0 | 8.5 | 8 | 7 | 0 | 0 | 0 | 10 | 0 | 0 | 0 | 6 | 0 | 8 | 0 | 0 |
| V3 | 0 | 0 | 0 | 0 | 9 | 0 | 0 | 8.5 | 0 | 0 | 0 | 0 | 0 | 10 | 0 | 0 |
| V4 | 0 | 9 | 0 | 0 | 7.5 | 0 | 0 | 6.5 | 0 | 0 | 0 | 0 | 0 | 10.5 | 0 | 0 |
| V5 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 7 | 0 | 0 | 0 | 0 | 0 | 11 | 0 | 0 |
| V6 | 0 | 7 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 8.5 | 10 | 0 | 0 | 0 | 0 | 0 |
| V7 | 0 | 9 | 0 | 0 | 0 | 0 | 0 | 8 | 0 | 0 | 0 | 0 | 0 | - | 10 | 0 |
| V8 | 0 | 0 | 0 | 0 | 9 | 9 | 0 | 0 | 0 | 4.5 | 0 | 8 | 9.5 | - | - | 0 |
| V9 | 0 | - | 0 | 0 | 7 | 8 | 0 | 10 | 0 | 8.5 | 0 | 0 | 0 | - | - | 0 |
| V10 | 0 | - | 0 | 0 | 0 | 7.5 | 0 | 0 | 0 | 8.5 | 9 | 0 | 0 | - | - | 0 |
| V11 | 0 | - | 0 | 0 | 0 | 8 | - | - | 0 | 0 | 0 | - | 0 | - | - | 0 |
| V12 | - | - | - | - | 0 | - | - | - | 0 | 0 | - | - | 0 | - | - | - |
| V13 | - | - | - | - | 0 | - | - | - | 0 | - | - | - | 0 | - | - | - |
| V14 | - | - | - | - | 0 | - | - | - | | - | - | - | | - | - | - |

An aliquot of 30uL of the fractions were tested against *C. albicans*. '- 'represents none available TLC fractions.

**Table S3.5.** Effect of TLC (2D) fractions on *C. albicans*

| | Zone of Inhibition (mm) | | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | MEF 65 | | | | | | MEF 75 | | | | | | MEF 134 | | | |
| TLC Fraction | FB V2 | FB V4 | FB V5 | FME V2 | FME V5 | FD V5 | FB V1 | FB V3 | FB V4 | FME V1 | FME V2 | FD V1 | FD V2 | FD V3 | FD V4 | FD V5 |
| V1 | 0 | 8.5 | 6 | 7.5 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| V2 | 0 | 8.5 | 0 | 6.5 | 0 | 0 | 0 | 0 | 0 | 0 | 6 | 0 | 0 | 0 | 0 | 0 |
| V3 | 0 | 7 | 0 | 0 | 9 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 6 | 0 | 0 |
| V4 | 0 | 7.5 | 0 | 6 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| V5 | 0 | 6 | 0 | 6 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| V6 | 0 | 7 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 8 |
| V7 | 0 | 8 | 0 | 0 | 0 | 0 | 6 | 0 | 0 | 0 | 0 | 7.5 | 0 | - | 0 | 6 |
| V8 | 0 | 8.5 | 0 | 6 | 0 | 0 | 11 | 0 | 0 | 0 | 0 | 12 | 7 | - | - | 13 |
| V9 | 0 | - | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | - | - | 0 |
| V10 | 0 | - | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | - | - | 0 |
| V11 | 0 | - | 0 | 0 | 0 | 0 | - | - | 0 | 0 | 0 | - | 0 | - | - | - |
| V12 | 0 | - | - | - | 6 | - | - | - | 0 | 0 | - | - | 0 | - | - | - |
| V13 | - | - | - | - | 0 | - | - | - | 0 | - | - | - | 0 | - | - | - |
| V14 | - | - | - | - | 0 | - | - | - | - | - | - | - | - | - | - | - |

An aliquot of 120uL of the fractions were tested against *C. albicans*. '- 'represents none available TLC fractions.

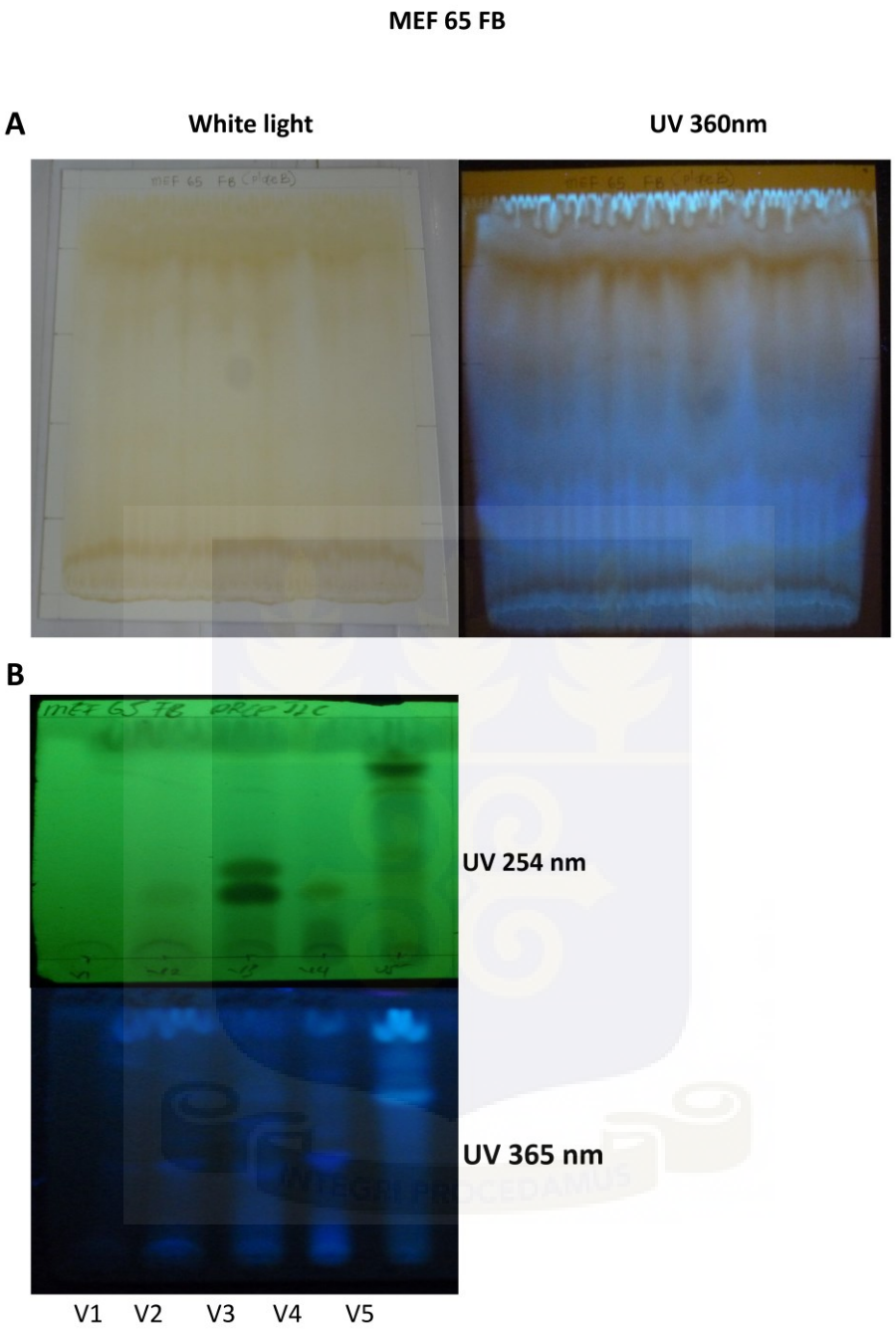


Fig S3.7. Preparative (A) TLC plate and analytical (B) TLC plate of the fractions obtained from MEF 65 FB. (Ethyl Acetate: Acetonitrile: Petroleum Ether, 7:2:1).

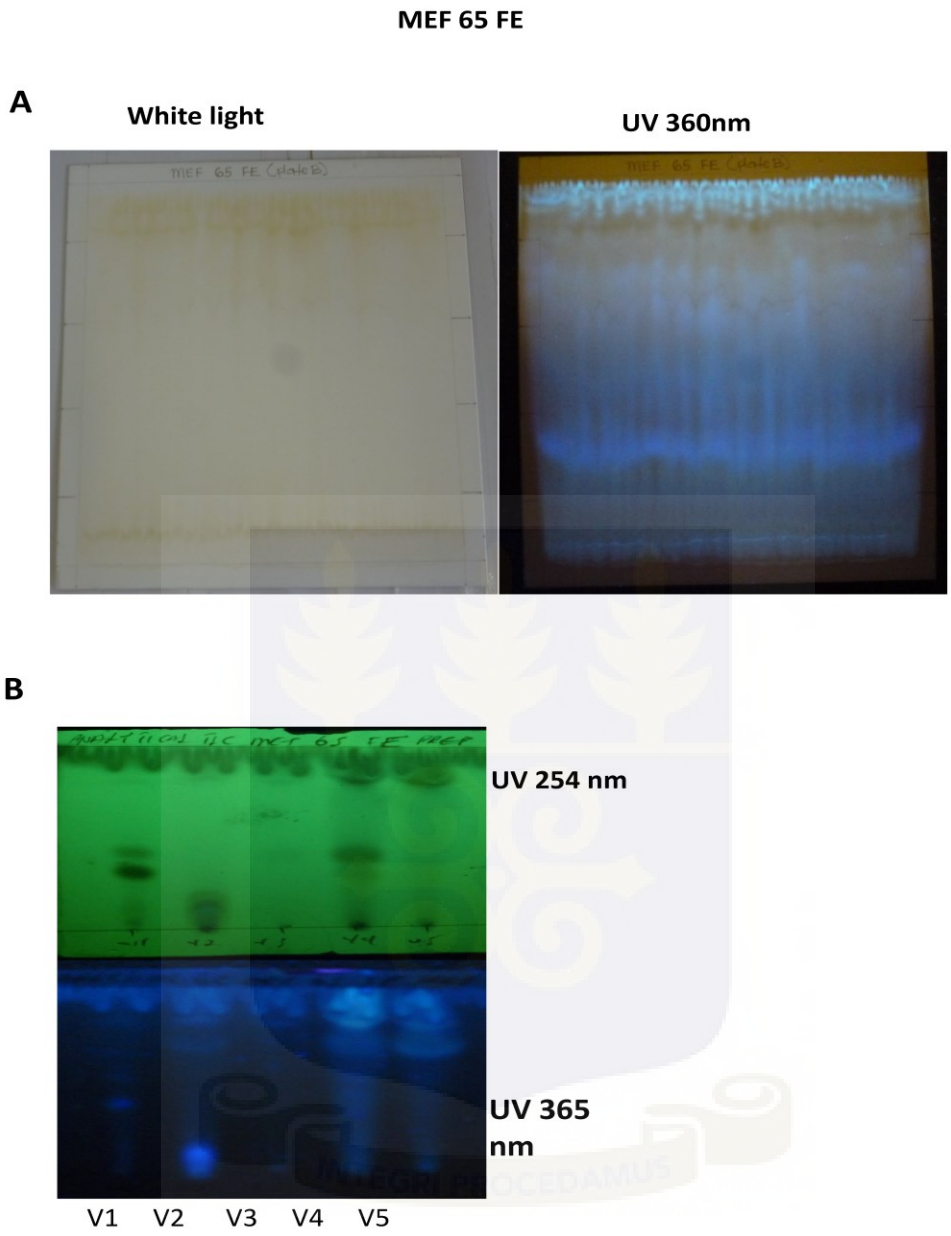


Fig S3.8. Preparative (A) TLC plate and analytical (B) TLC plate of the fractions obtained from MEF 65 FE. (Ethyl Acetate: Acetonitrile: Petroleum Ether, 7:2:1).

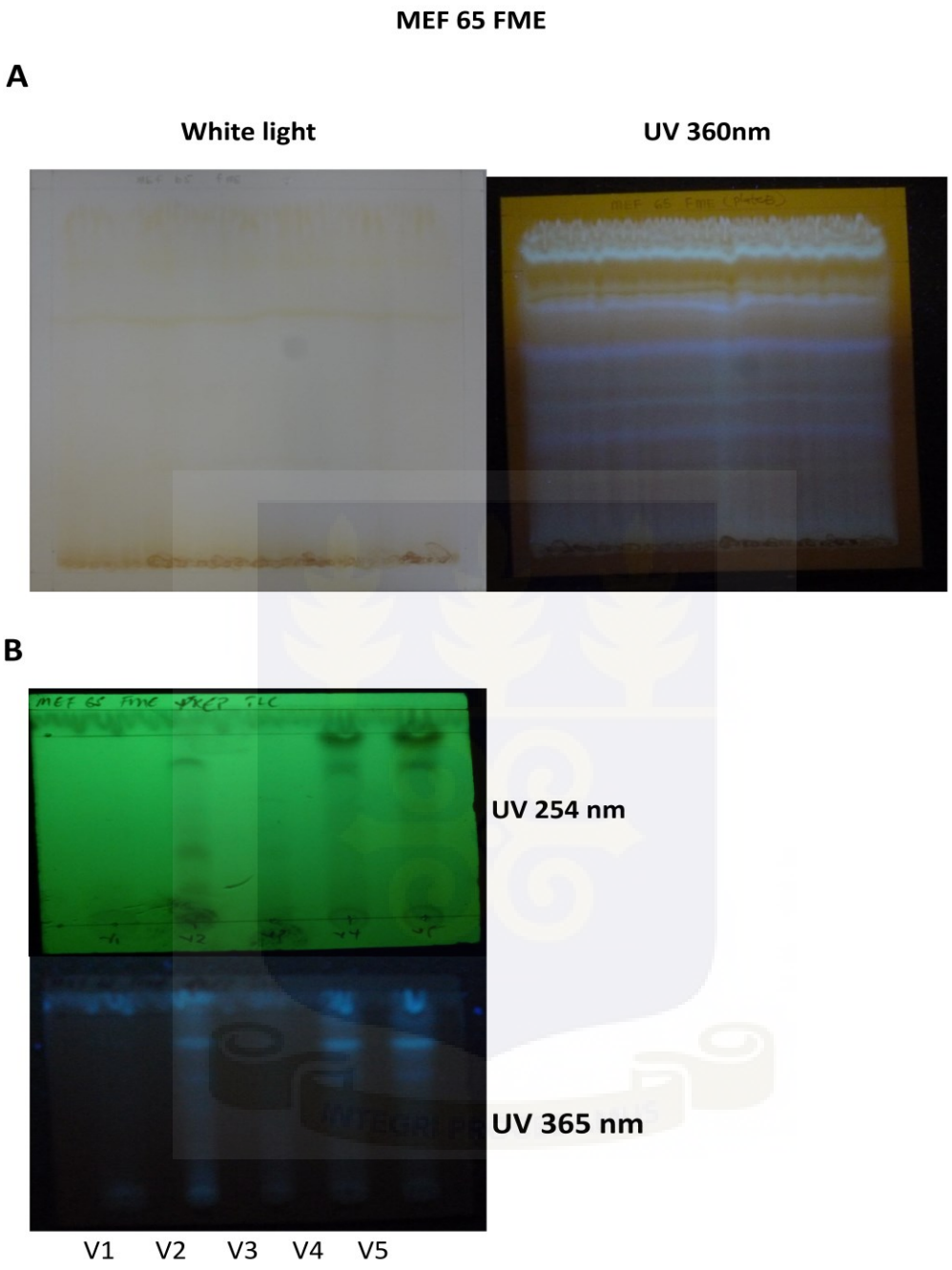


Fig S3.9. Preparative (A) TLC plate and analytical (B) TLC plate of the fractions obtained from MEF 65 FME. (Ethyl Acetate: Acetonitrile: Petroleum Ether, 7:2:1).

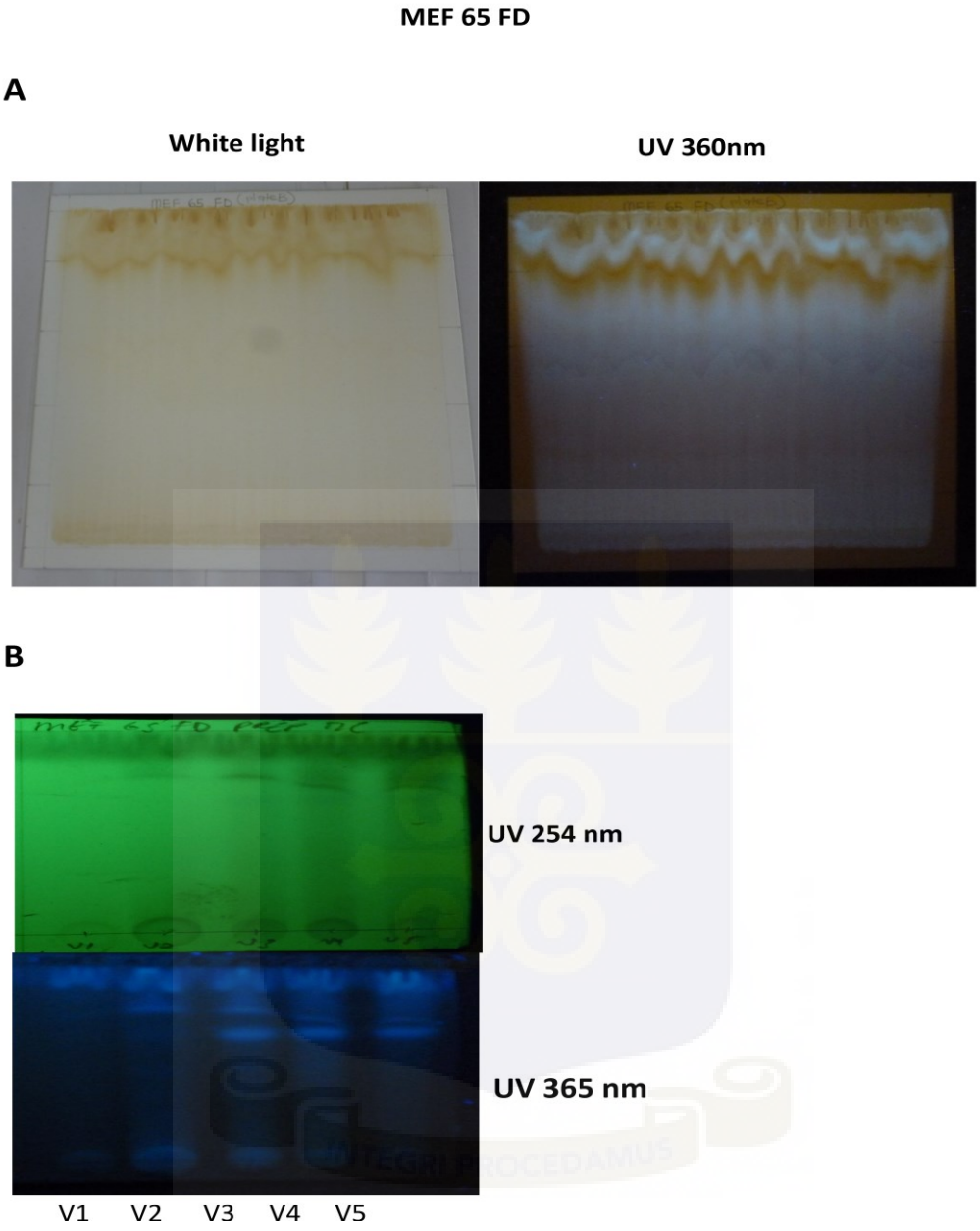


Fig S3.10. Preparative (A) TLC plate and analytical (B) TLC plate of the fractions obtained from MEF 65 FD. (Ethyl Acetate: Acetonitrile: Petroleum Ether, 7:2:1).

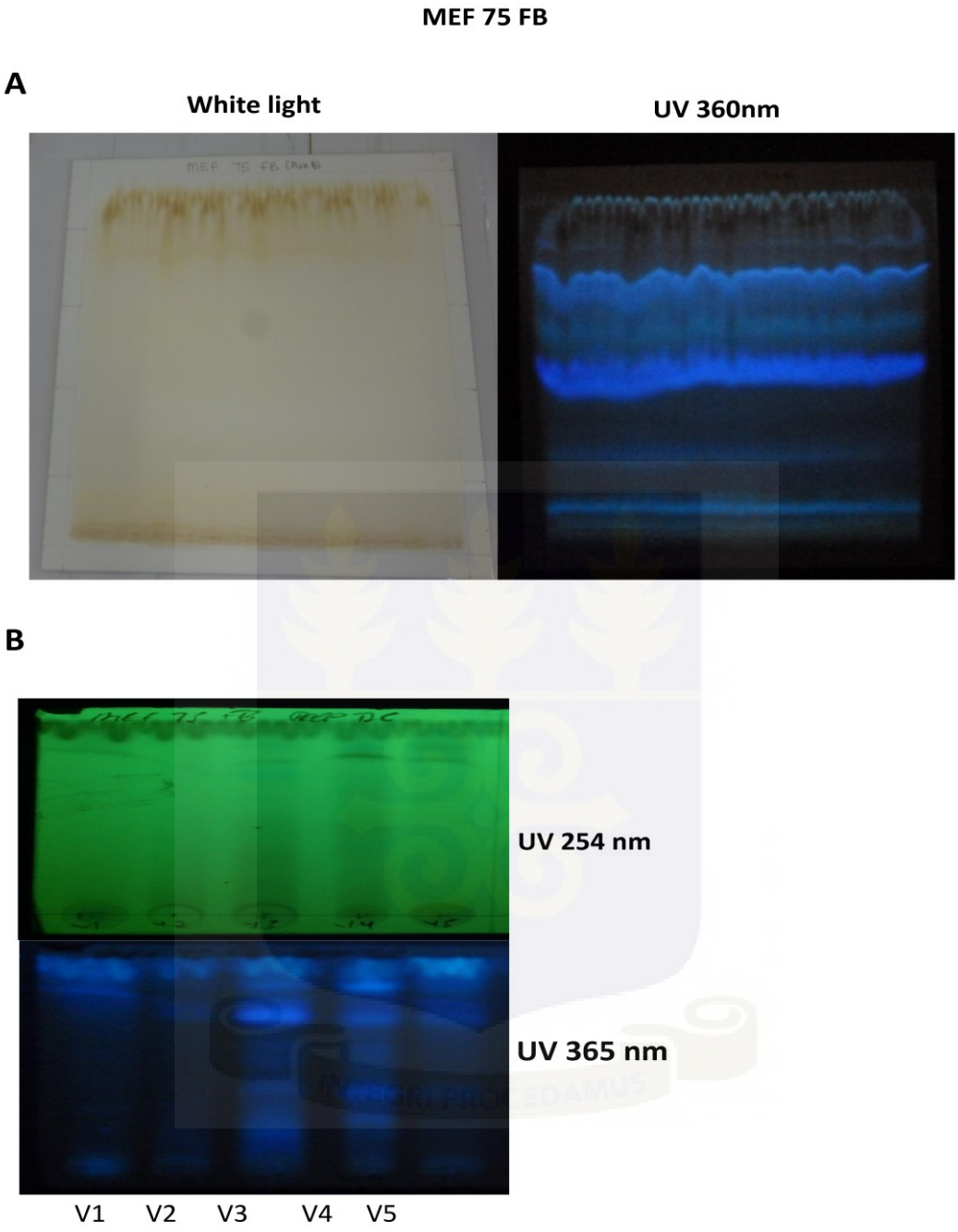


Fig S3.11. Preparative (A) TLC plate and analytical (B) TLC plate of the fractions obtained from MEF 75 FB. (Ethyl Acetate: Acetonitrile: Petroleum Ether, 7:2:1).

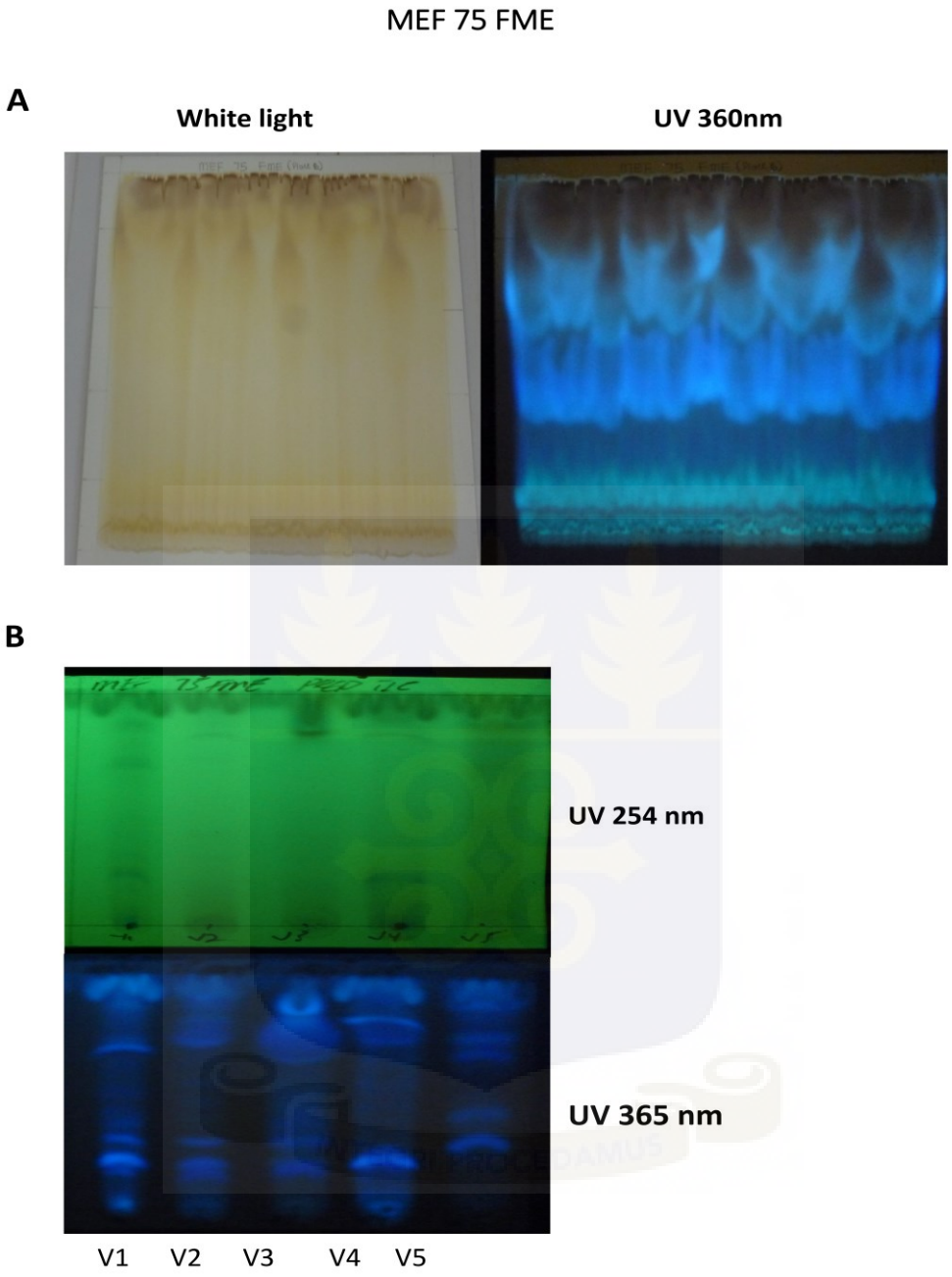


Fig S3.12. Preparative (A) TLC plate and analytical (B) TLC plate of the fractions obtained from MEF 75 FME. (Ethyl Acetate: Acetonitrile: Petroleum Ether, 7:2:1).

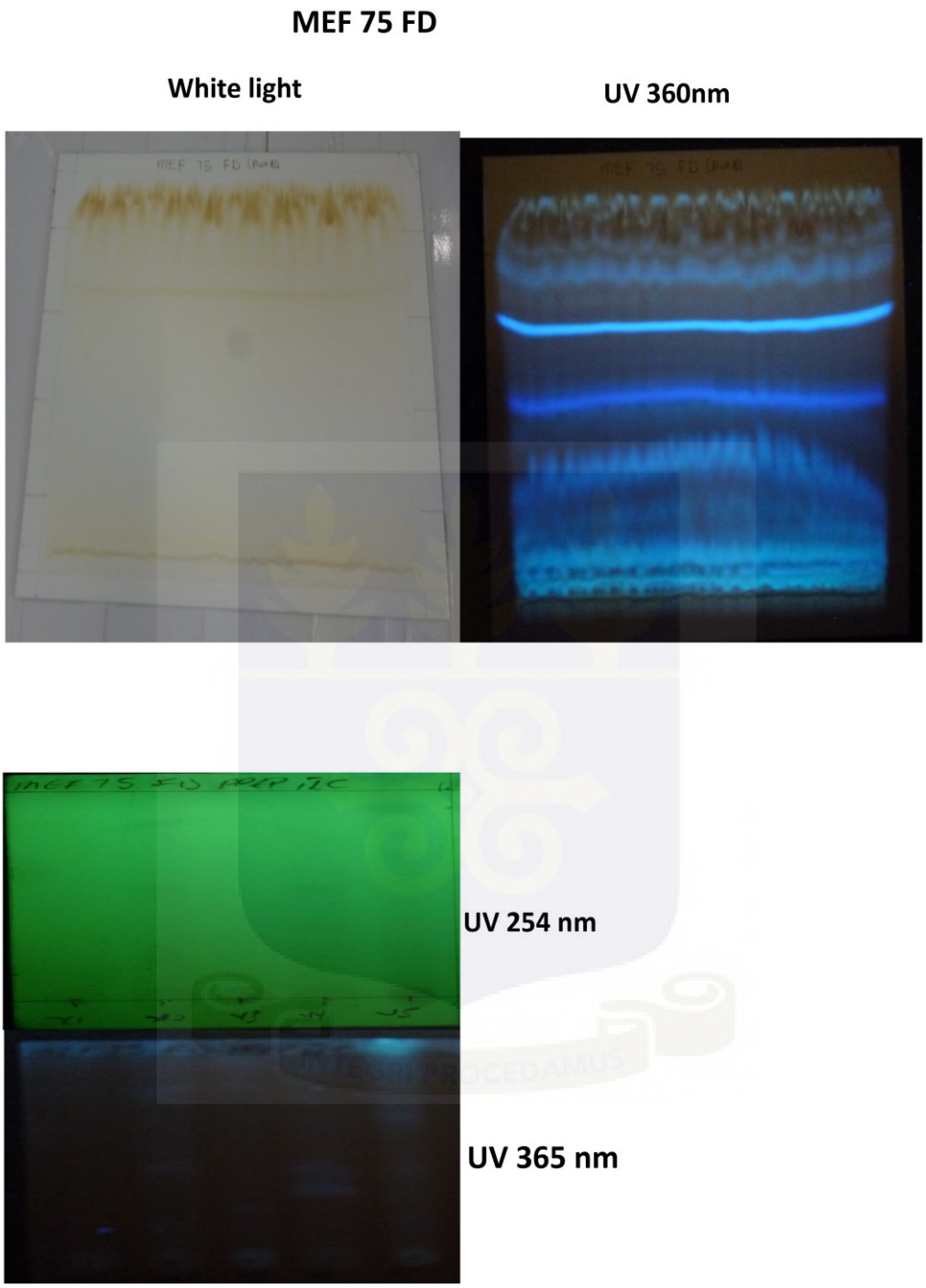


Fig S3.13. Preparative (A) TLC plate and analytical (B) TLC plate of the fractions obtained from MEF 75 FD. (Ethyl Acetate: Acetonitrile: Petroleum Ether, 7:2:1).

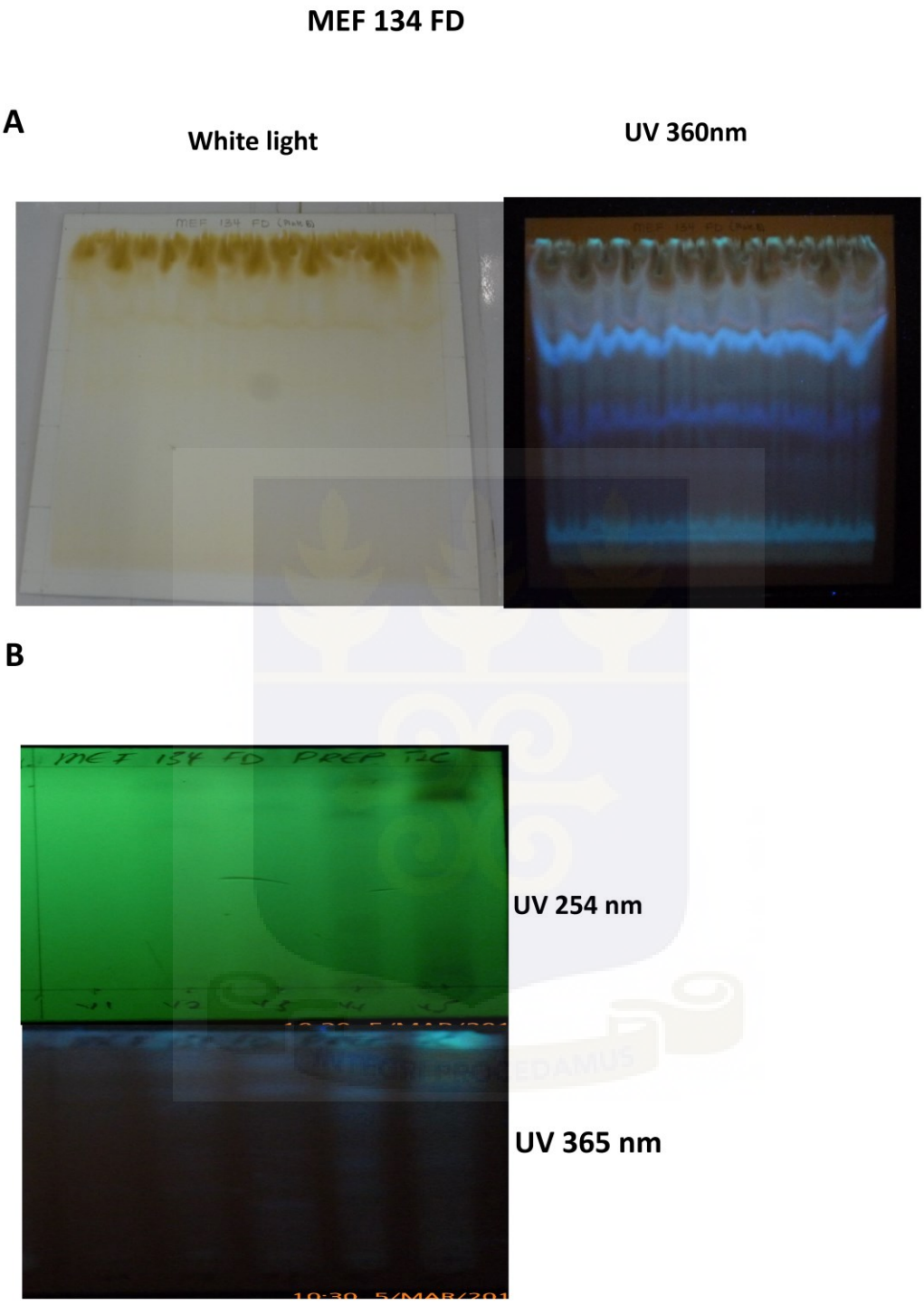


Fig S3.14. Preparative (A) TLC plate and analytical (B) TLC plate of the fractions obtained from MEF 134 FD. (Ethyl Acetate: Acetonitrile: Petroleum Ether, 7:2:1).

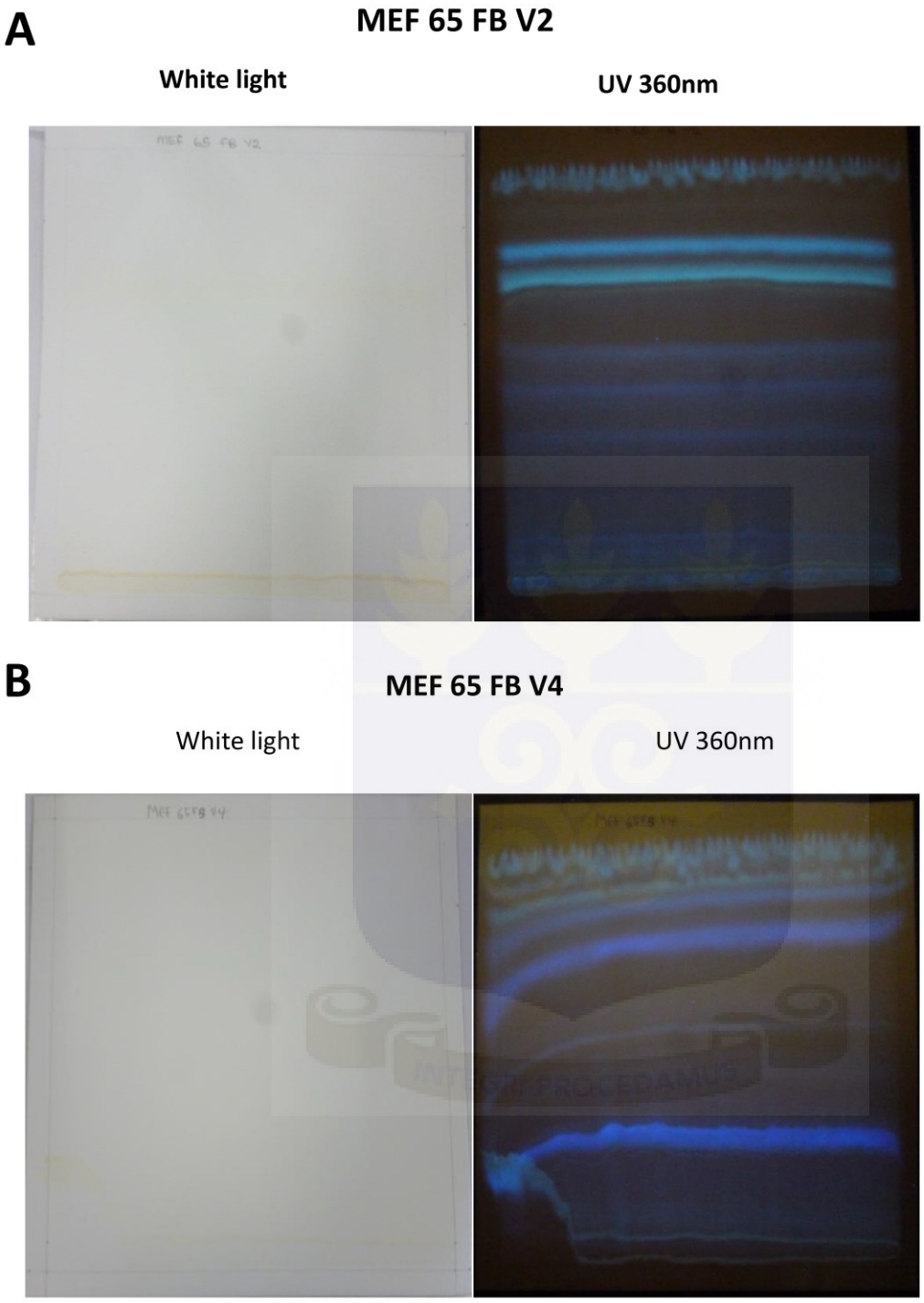


Fig S3.15. Preparative 2D TLC plates of FB V2 and FB V4 1D Prep TLC fraction from MEF 65.

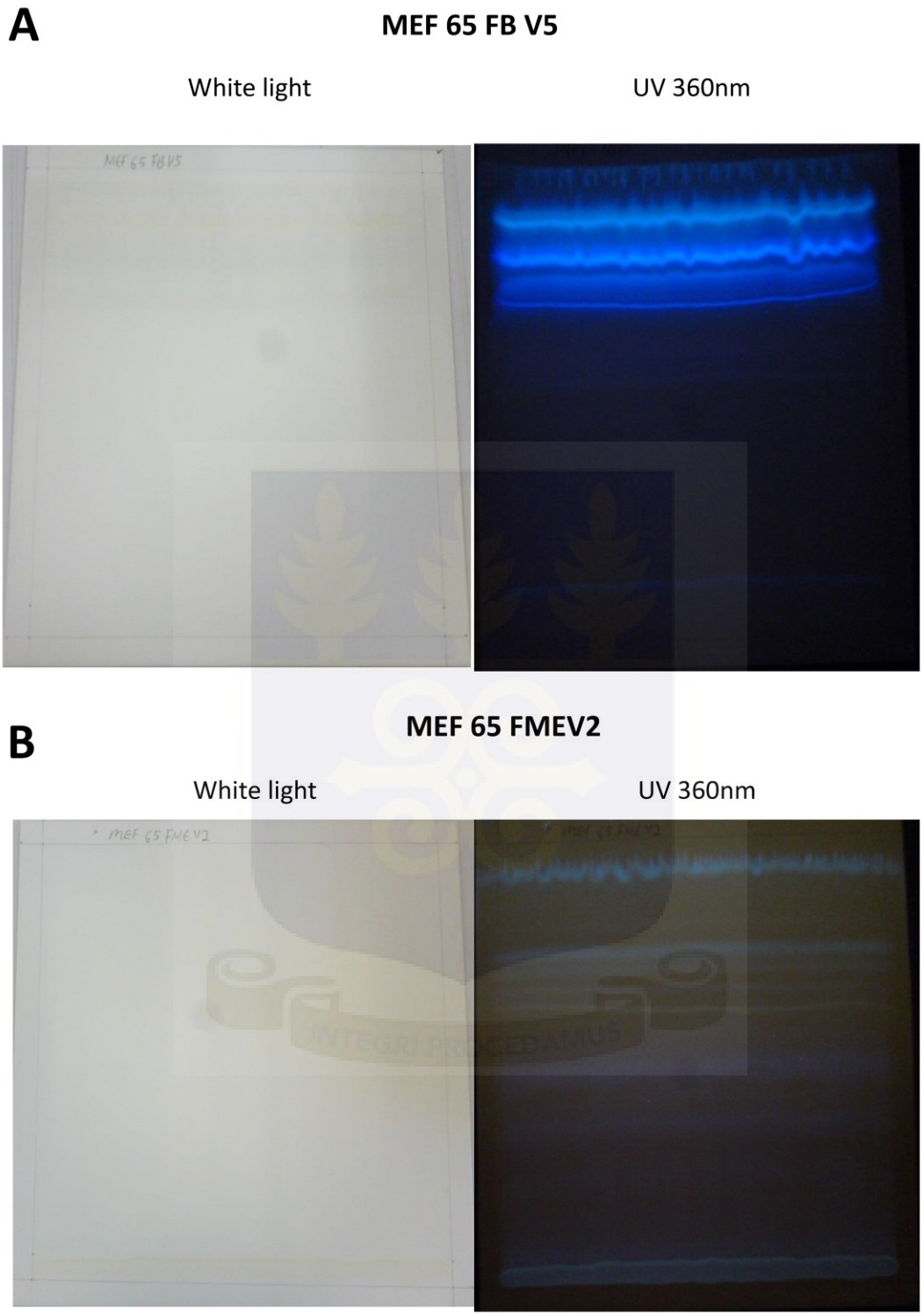


Fig S3.16. Preparative 2D TLC plates of FB V5 and FME V2 1D TLC fraction from MEF 65.

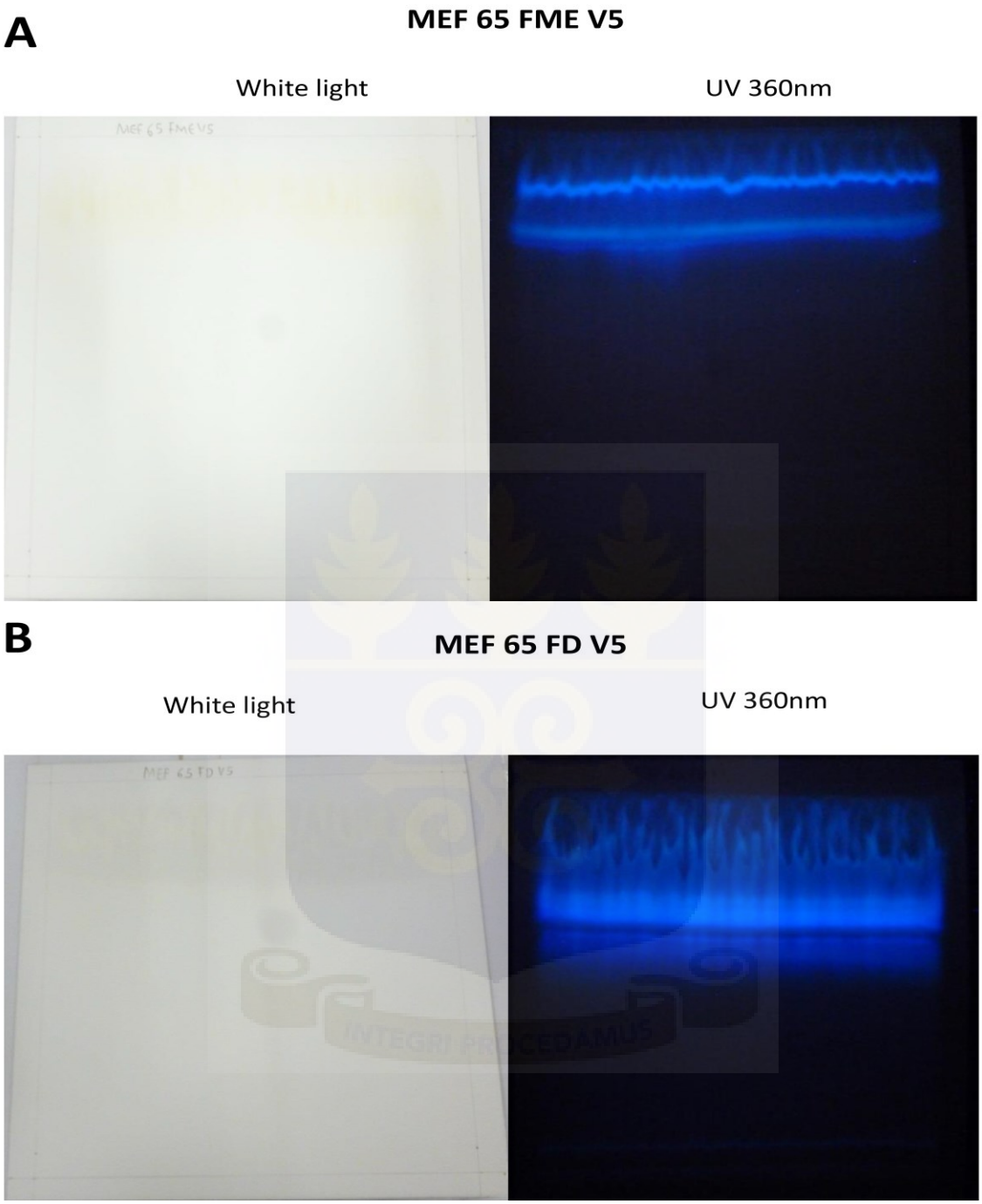


Fig S3.17. Preparative and analytical 2D TLC plates of FME V5 and FD V5 1D TLC fraction from MEF 65.

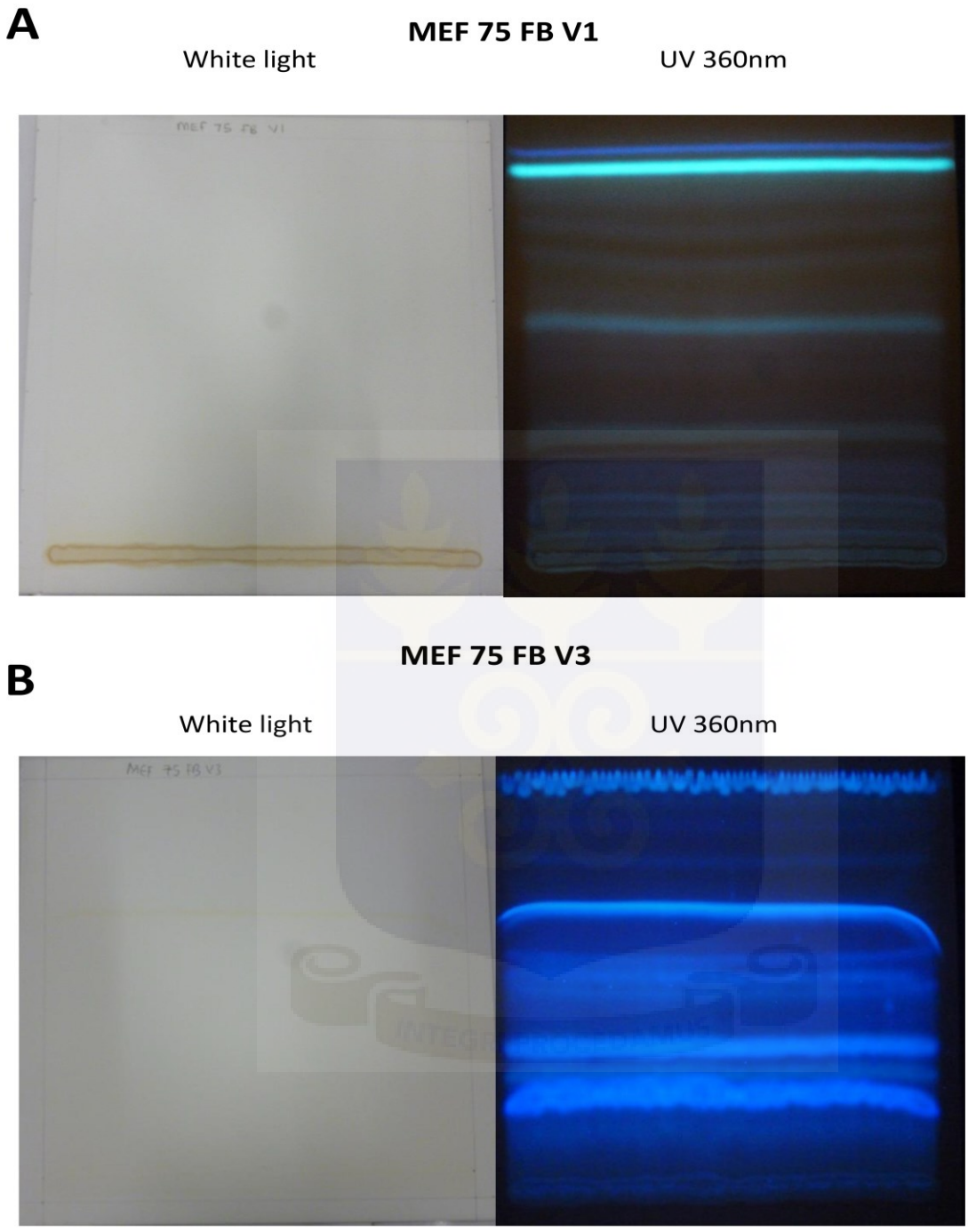


Fig S3.18. Preparative 2D TLC plates of FB V1 and FB V3 1D TLC fraction from MEF 75.

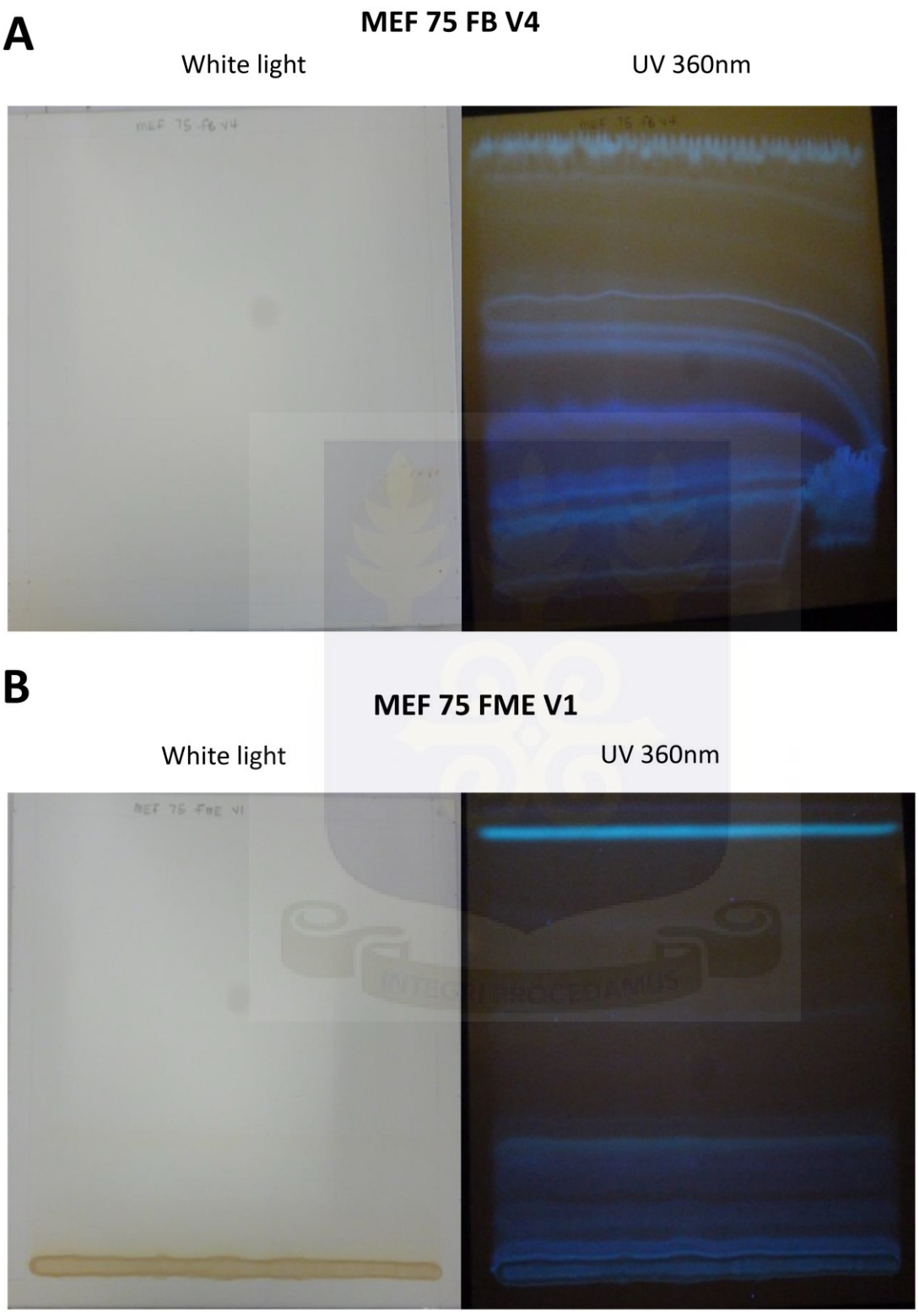


Fig S3.19. Preparative 2D TLC plates of FB V4 and FME V1 1D TLC fraction from MEF 75.

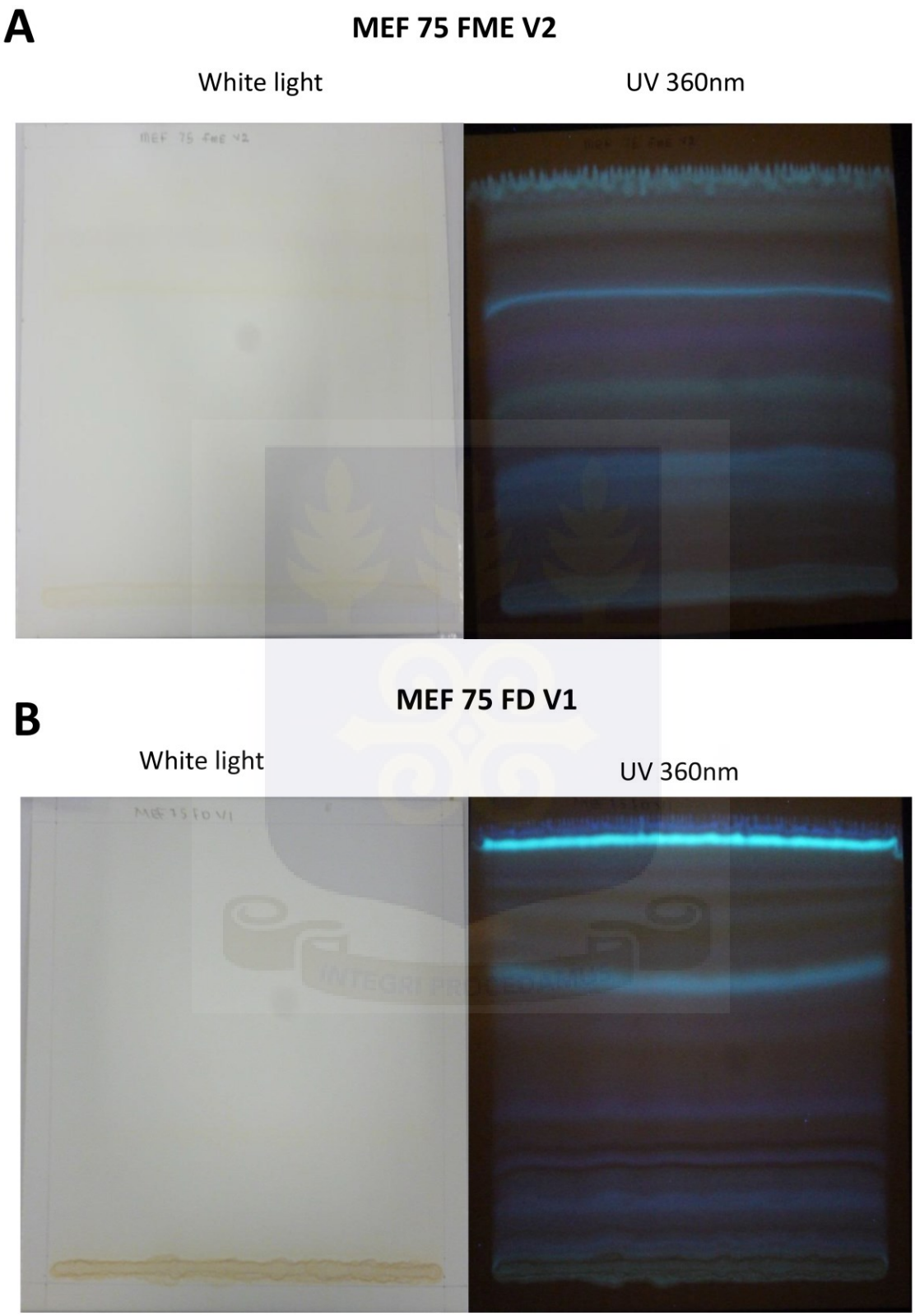


Fig S3.20. Preparative 2D TLC plates of FME V2 and FD V1 1D TLC fraction from MEF 75.

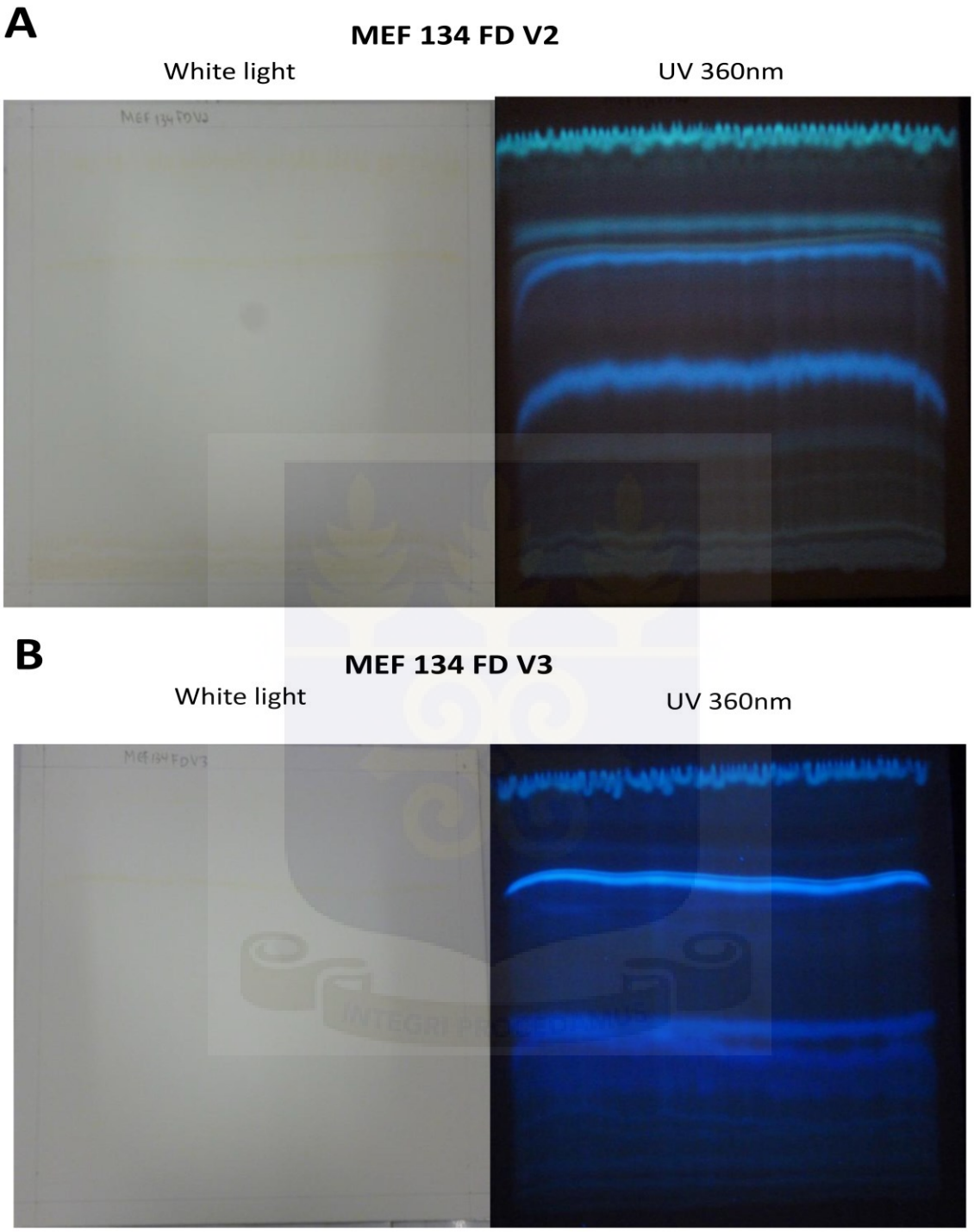


Fig S3.21. Preparative 2D TLC plates of FD V2 and FD V3 1D TLC fraction from MEF 134.

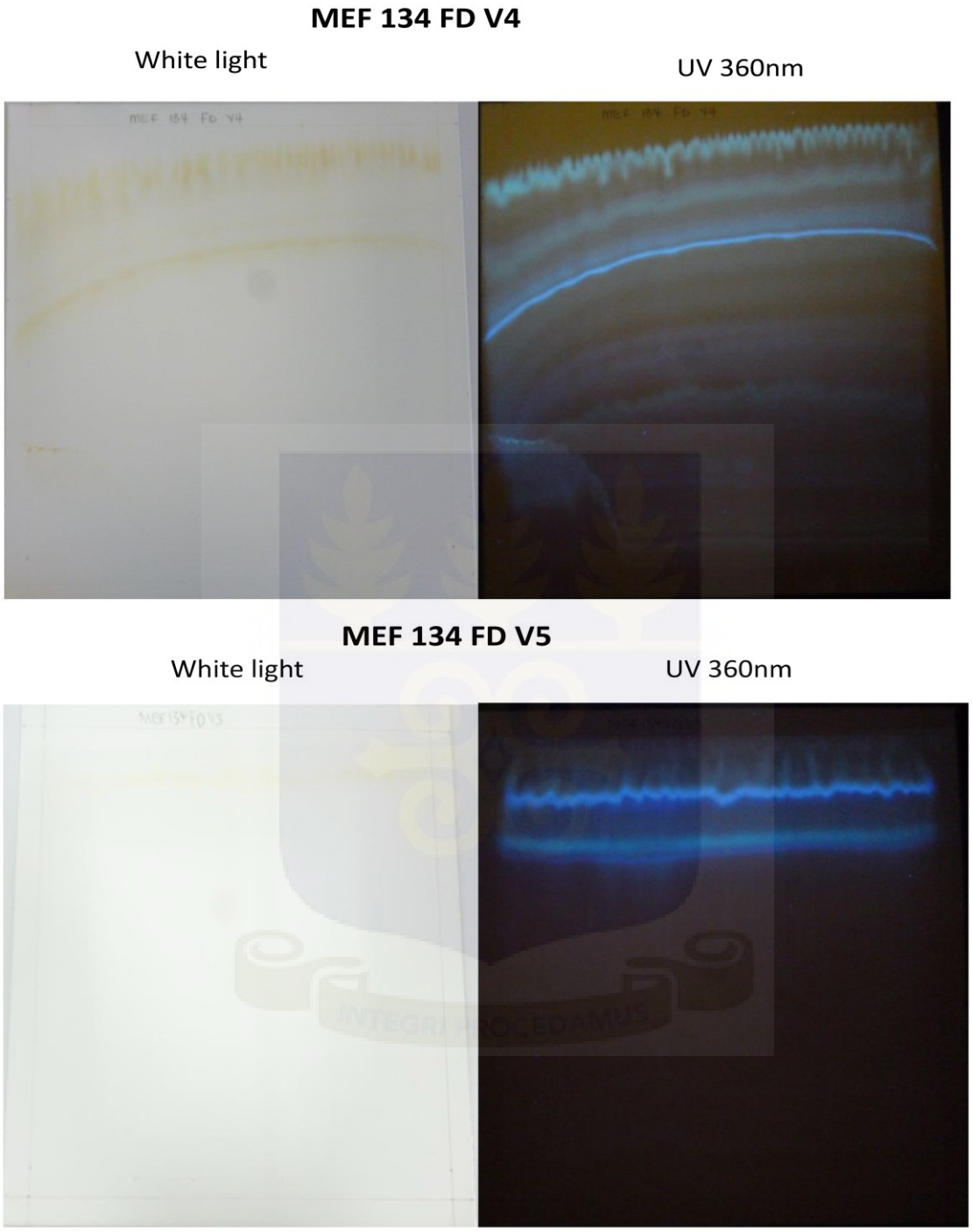


Fig S3.22. Preparative 2D TLC plates of FD V4 and FD V5 1D TLC fraction from MEF 134.

# CHAPTER FOUR

## TIME COURSE ANALYSES TO DETERMINE NUMBER, DIVERSITY, YIELD AND TIME OF PRODUCTION OF BIOACTIVE METABOLITES FOR 3 IDENTIFIED MARINE ENDOPHYTIC FUNGI

### 4.1 Introduction

Fungal cultures are typically incubated for at least four weeks to maximize the recovery of fungi that are growing slowly. Nevertheless, only limited data support this valid reasoning in the context of the optimum time of culture needed for the best results for fungal fermentation. The best period of incubation was found to be two weeks for samples which are suspected to be yeast and moulds. However, it was thought that there was a need for an algorithm to determine the specific incubation time for each fungal culture (Bosshard, 2011).

The fungus *Nigrospora* sp derived from deep sea was grown in potato dextrose broth prepared with sea water and incubated for 15 days at room temperature, the results showed that biomass growth reached its optimum at 10 days while pigment production (secondary metabolite) reached its maximum at 6 days (Arumugam *et al.*, 2015). *Nigrospora sp* was also found to propagate quickly to produce white colonies on potato dextrose agar during incubation within 120 hours, the colonies matured without sporulation and released abundant extracellular red metabolites (Merlin *et al.*, 2013). Growth conditions were optimized to 25-days incubation period and 25ºC temperature for the endophytic fungus from *Pterispellucida* leaf to ensure improved bioactive metabolites production and increased bioactivity against pathogens (Goutam *et al.*, 2014).

Based on the molecular and morphological characteristics of *Fusarium solani*, the optimum incubation time for the production of bioactive secondary metabolite and growth was 9 days (Hasan *et al.*, 2015). *Aspergillus flavus* showed that optimum sporulation time was 12 days while maximum accumulation of aflatoxin B1 occurred after 7 days of incubation (Lahouar *et al.*, 2016). Shaking condition of incubation was found to be optimum for production of antimicrobial metabolites in comparison to stationary culture (Moore *et al.*, 2011).

Overall, these studies have shown that there is currently no established protocol to determine the length of time a particular fungus should be incubated in order to reach optimum production of bioactive metabolites. Since metabolite production by fungus may be based on some stress related conditions, such as nutrient deprivation, earlier research work conducted in our laboratory selected 4 months as the length of incubation time for a particular marine endophytic fungus. This was to afford the organism enough time to deplete the nutrients in the medium and then enter some kind of stressful condition which would trigger secondary metabolite production. However, it is not clear whether optimal levels of bioactivity of the extracts was obtained from these 4-month-old cultures. Out of the 21 active Kupchan fractions obtained from the 4 month culture, 8 were fractionated by TLC (1D and 2D) based on their significant quantities and this yielded a total of 59 active TLC fractions. These observations necessitated our current study as in most of the studies discussed above, different time points were selected to incubate the fungi for metabolite production. There is the need to systematically compare the metabolite production of different fungal species at various time points.

Therefore, the aim of this study was to perform a time course analyses to determine number, diversity, yield and time of production of bioactive metabolites from 3 marine endophytic fungi.

The specific objectives were;

- To culture 3 marine endophytic fungus at different time points in order to determine the optimum time for production of bioactive metabolites.
- To determine number, yield and diversity of the secondary metabolites via bioactivity guided fractionation.
- To conduct an interaction study between the TLC fractions generated and Cyclosporine A.

## 4.2 Materials and Methods

**4.2.1 Chemical agents**; Cyclosporine A, Amphotericin B and Fluconazole were purchased from Sigma Aldrich Germany and Carl Roth, unless otherwise indicated.

**4.2.2 Solvents**: *n*-Butanol, *n*-Hexane, ethyl acetate, dichloromethane, methanol, acetonitrile, petroleum ether and ethanol were all purchased from Lab Aid, Ghana. All the solvents were of technical grade and were all distilled prior to use.

**4.2.3 Growth media for microorganisms:** Yeast extract, nutrient broth, malt extract broth, peptone, dextrose and agar were purchased from Becton, Dickson and company (BD, USA).

### 4.2.4 Fungi isolation and identification

The seaweed-associated fungal strains used for this study were collected from beaches in Ghana. The entire process of isolation and identification has been extensively described in chapter 3. Briefly, the collected fungi were surface sterilized with alcohol and bleach. Afterwards, pieces of the seaweeds were sliced under sterile conditions and then inoculated on the surface of malt extract agar (MEA) plates. The plates were then incubated at 30 ℃ until fungi began to emerge from the plant tissues. Several subcultures of the fungi were carried out onto fresh MEA plates in order to obtain a pure mono-culture of the fungi. The mono-cultures were maintained on malt extract agar

(MEA) plates at 4℃ for future use. To prevent bacterial contaminants, chloramphenicol (0.2 g/L) was added to the MEA.

The internal transcribed spacer (ITS) region is the main fungal identification barcode (Schoch *et al.*, 2012). The genomic identities of the fungi were determined by obtaining and amplifying DNA sequences from the ITS region of the fungal DNA. Genetic matches were identified by performing BLAST searches of the ITS sequences.

Selection of fungi for the metabolite production analysis was based on the biological activities observed in the earlier study. Also, the decision to select a particular fungus for further investigation was centred on the search results from literature on the number of secondary metabolites already isolated from that particular fungus. Those fungi with comparatively fewer reported compounds isolated were selected. Though two of the isolates considered in this study were the same organism (*Meyerozyma guilliermondii* (MEF 75) and *Meyerozyma guilliermondii* (MEF 134)), they were treated as different isolates because they were isolated from different seaweeds.

### 4.2.5 Experimental setup for time course of fungi metabolite production

Three fungal strains were selected and studied for their rate of bioactive metabolite production at 6 weeks, 2 months and 3 months. The three fungi strains were *Clonostachys wenpingii* (MEF 65), *Meyerozyma guilliermondii* (MEF 75) and *Meyerozyma guilliermondii* (MEF 134). The experimental setup for culturing of the fungi at the various time points and the subsequent activity screening tests are illustrated in Fig 4.1.

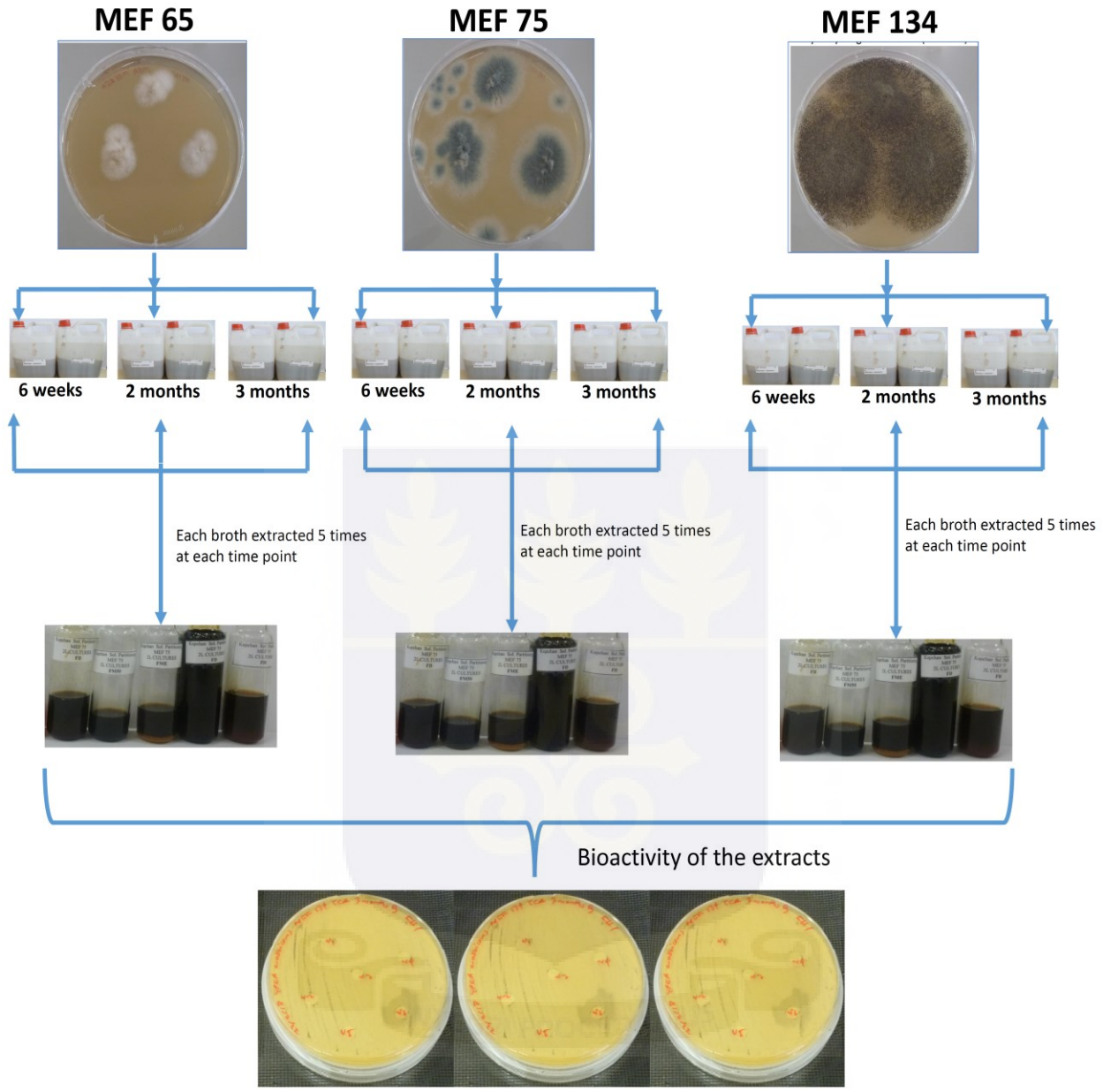


Fig 4.1. Fermentation scheme for time course of production of bioactive metabolites from the three marine endophytic fungi. The 3 fungal isolates were grown in YPMD broth for 6 weeks, 2 months and 3 months period. The extracts from these cultures were tested against *C. albicans* and *S. cerevisiae.*

To obtain the starting inoculum for the study, fungi were initially inoculated from stored MEA slants unto MEA plates. Then mycelia were plugged from the MEA plates and used as starting inoculum for the broth cultures. Mycelia were transferred into 5 L high- density polyethylene culture vessels containing 2 L sterile YPMD broth prepared with seawater. Each broth was inoculated with two fungi culture plates. The broths were made in duplicate (A and B) and at three different time points; 6 weeks, 2 months and 3 months.

Due to the fact that it is challenging to make a particular fungus produce the same metabolites every time, it was decided that the duplicate broths should be kept as separate experiments.

### 4.2.6 Extraction of fungal cultures

Ethyl acetate was used to obtain the crude extracts; equal volume of ethyl acetate (2 L) was added to each fungal broth culture. Subsequently, the broth cultures were shaken thoroughly and allowed to incubate at 50 ℃ for 24 hours. After incubation, the organic phase was collected and filtered under suction through a Büchner funnel lined with a Whatman No. 5 filter paper. The filtrate was collected and concentrated with a rotary evaporator. Each broth culture was extracted 5 times and the extracts obtained were reconstituted in methanol and kept in separate glass vials. The crude extracts (5 vials per broth) were tested against *C. albicans* and *S. cerevsiae* to determine the appropriate incubation period that was optimal for maximum production of bioactive metabolites. Extracting each broth culture 5 times was sufficient to deplete the culture of the bioactive metabolites produced. The masses of the extracts obtained are shown in Table S4.1.

### 4.2.7 Solvent-Solvent extraction

In order to separate the various components of the crude extracts, the extracts in the 5 vials collected per broth were combined and dried. The modified Kupchan's solvent partitioning method

was used and has been extensively described in chapter 3 (Kupchan *et al*., 1974). Using 1 L separation funnels, about 90% of the total mass of each extract was saturated with distilled water (aqueous phase) and extracted with dichloromethane (organic phase). The organic phase was collected in a round bottom flask and dried using a rotatory evaporator. Once the solvent had been removed, the dried fraction was dissolved in 90% methanol and subsequently extracted with *n*-hexane. The 90% methanol layer was diluted with distilled water to 50% then extracted with dichloromethane and ethyl acetate. The aqueous phase was extracted with n-butanol and ethyl acetate. The detailed separation scheme is shown in Fig S3.6. All solvents were dried off and the fractions were reconstituted in methanol.

### 4.2.8 Thin layer chromatography (TLC)

In order to characterize the metabolites produced at the various time points, TLCs were performed on all the fractions generated from the solvent-solvent extraction analyses. Pre-coated silica gel TLC plates were used in the analysis. About 90% of the total mass of the fractions were run on the plates. The spots were allowed to air-dry and developed in a glass tank filled to about 5 mm and saturated with the developing solvent (Ethyl Acetate: Acetonitrile: Petroleum Ether, 7:2:1). The bands were visualized under UV light after which they were cut and eluted as partially purified metabolites (Fig S4.13-32). The TLC fractions obtained were reconstituted in methanol.

### 4.2.9 Bioactivity screening of the crude extracts and the fractions

The crude extracts and fractions were screened for antifungal properties using the disc diffusion assay. Stock solutions of the crude extracts and fractions were prepared to a concentration of 1µg/µL. An aliquot of 30 µL of each crude extract and 120 µL of the fractions (Kupchan and TLC) were loaded unto sterile filter paper discs (5mm). The discs were air dried for complete evaporation

of the solvent. The amount of crude extract and fraction loaded onto each disc was 30 µg and 120 µg respectively.

*Candida albicans* and *Saccharomyces cerevisiae* were streaked on YPDA plates and incubated at 30 ℃ for 12 hours. The isolates were inoculated into 50 mL sterile nutrient broth and incubated in a rotary shaker for 12 hours at 30℃. Subsequently, the 12 hour cultures were used to inoculate fresh 50ml nutrient broth to obtain an $OD_{600}$ of 0.01. The inoculated broths were incubated as described previously. The cultures were maintained till the mid-log growth phase was reached. The optical density of the cultures were adjusted to 0.7 before being used for the antifungal assay.

For the sensitivity tests, the impregnated filter paper discs were placed on the surface of YPDA plates spread with either *Candida albicans* or *Saccharomyces cerevisiae* inocula. Plates were incubated for 12 hours at 30℃. Each plate was observed and growth inhibition recorded. Each extract and fraction was assayed in duplicates.

**4.2.10 Synergistic interaction assay between the TLC fractions and Cyclosporine A**

An interaction study was conducted using cyclosporine A and the TLC fractions. The first step was to determine the minimum inhibitory concentration (MIC) of cyclosporine A against *C.albicans*. Once the MIC was determined, an interaction study was conducted between cyclosporine A and standard antifungal agents. After which the same concept was applied for the interaction study between the TLC fractions and cyclosporine A. The antifungal agents used in this study are shown in the Table 4.1.

The disc diffusion assay was used to determine the MIC of cyclosporine A against *C. albicans.* Solutions of cyclosporine A were prepared in the concentration range from 0.1 to10 µg/µL. These

solutions were pipetted onto filter paper discs to achieve concentrations of 40, 20, 10, 5, 1 and 0.5 µg per disc. The discs were placed on the YPDA plates that were inoculated with *C. albicans*, as described earlier. The plates were incubated at 30℃ for 12 hours and zones of inhibition around each disc (in mm) were measured. There were no activities recorded for all the concentrations tested. Hence the MIC was determined to be >40 µg.

For the interaction study, YPDA plates were modified with cyclosporine A. The amount of cyclosporine A per 15 ml YPDA plate was 500 µg. Discs were infused with the standard antifungal drugs at their effective concentrations as listed in Table 4.1. The discs were placed on the modified agar plates spread with *C. albicans* inoculum prepared at an $OD_{600nm}$ of 0.7. The sensitivity pattern of the antifungals across the modified condition were determined by measuring the zone of inhibition.

The interaction study between the TLC fractions and cyclosporine A was conducted in the same manner as described for the standard antifungal agents. Briefly, YPDA plates were each modified with 500 µg of Cyclosporine A. A stock solution of 10 µg/µL of each TLC fraction was prepared in methanol. Discs were infused with 100 µL of these stock solutions and were air-dried for complete evaporation of the solvent. The modified YPDA plates were spread with cell suspensions of *C. albicans* at an OD600nm of 0.7 and the discs infused with the TLC fractions were placed on the plates. YPDA plates with no modifications were also taken through the same assaying procedure and used as controls. The plates were incubated and the sensitivity patterns were recorded, as describe previously.

**Table 4.1.** List of antifungals and concentrations

| Antifungals | Amount per disc (ug) |
|---|---|
| Amphotericin B (AmpB) | 5 |
| Cycloheximide (CyH) | 20 |
| Paramomycin (Para) | 5 |
| Benzoic acid (Ben) | 40 |
| Fluconazole (Flu) | 1.25 |
| 5-Fluorouracil (5-Fu) | 0.25 |
| Griseofulvin (Gri) | 50 |
| Sertraline (Set) | 100 |

## 4.3 Results

### 4.3.1 Effect of the duration of incubation of MEF fungal cultures on yield and bioactivity of antifungal metabolites.

Three marine endophytic fungal isolates (MEF 65, MEF75 and MEF134) were cultured in YPMD broth in duplicates for 6 weeks, 2 months and 3 months (Fig 4.1). The secondary metabolites produced by these fungal cultures were obtained via five rounds of extraction using ethyl acetate as solvent (Table S 4. 1). MEF65 generated the least yield of crude extracts from the duplicates (Broth A or B) of the fungal cultures. Importantly, the MEF65 cultures that were incubated for 2 or 3 months did not generate higher yield relative to the same fungal culture incubated for 6 weeks, demonstrating that the low yield from the MEF65 cultures was independent on the duration for incubation of the fungus in the YPMD broth. For MEF134, the highest yield was recorded when the fungus was cultured for 2 months in the YPMD broth. Moreover, the data show that the secondary metabolites were not completely recovered from the fungal broth cultures following the first or second rounds of extraction using ethyl acetate, thereby validating the rationale for multiple (five) rounds of recovering these metabolites from the fungal cultures. Collectively, the data shown in Table S4. 1 indicate that yield of secondary metabolites from fungal cultures is not boosted by solely increasing the duration of incubation of these fungal cultures.

The study also investigated the antifungal activity of the crude extracts obtained from the duplicate cultures of MEF65, MEF75 and MEF134 which were incubated for either 6 weeks, 2 months or 3 months. The bioassays were performed using *C. albicans* and *S. cerevisiae* as model organisms and fluconazole and amphotericin B were used as positive controls for this assay. All the crude extracts obtained from the biological duplicate cultures of MEF65 following 3 months of incubation (and five rounds of the extraction procedure) did not exhibit antimicrobial activity

against *C. albicans* and *S. cerevisiae* (Tables 4.2 and 4.3); the same observations were recorded for the MEF134 cultures (Tables 4.6 and 4.7). The crude extracts obtained from the first and second rounds of extractions from the MEF65 cultures, incubated for either 6 weeks or 2 months, exhibited significant antifungal activity against *C. albicans* and *S. cerevisiae* (Tables 4.2 and 4.3). In general, the antifungal activity decreased with successive extractions for the specific fungal cultures whose crude extracts from the five rounds of extractions exhibited bioactivity (Tables 4.6 and 4.7). Hence, it can be inferred that most of the compounds that possessed antifungal activity were usually recovered from the cultures during the initial extraction procedures. The low level of bioactivity detected for the crude extract could be due to proportionally low levels of active compounds, a situation that can improve with fractionation and enrichment.

**Table 4.2.** Bioactivity of extracts from *Clonostachys wenpingii* (MEF 65) grown at different time points

| *C. albicans* | | | | | |
|---|---|---|---|---|---|
| Incubation period | Zone of Inhibition/mm | | | | |
| | Round 1 | Round 2 | Round 3 | Round 4 | Round 5 |
| 6 WEEKS A | 10.5 | 10.5 | 0 | 0 | 0 |
| 6 WEEKS B | 0 | 0 | 0 | 0 | 0 |
| 2 Months A | 9.5 | 7.5 | 0 | 0 | 0 |
| 2 Months B | 9 | 10 | 0 | 0 | 0 |
| 3 Months A | 0 | 0 | 0 | 0 | 0 |
| 3 Months B | 0 | 0 | 0 | 0 | 0 |

Fluconazole: 20 mm, Amphotericin B: 9 mm, 0: no activity observed
Round 1-5: fractions obtained from various rounds of exhaustive extraction conducted per culture

**Table 4.3.** Bioactivity of extracts from *Clonostachys wenpingii* (MEF 65) grown at different time points

| *S. cerevisiae* | | | | | |
|---|---|---|---|---|---|
| Incubation period | Zone of Inhibition/mm | | | | |
| | Round 1 | Round 2 | Round 3 | Round 4 | Round 5 |
| 6 WEEKS A | 10.5 | 13 | 0 | 0 | 0 |
| 6 WEEKS B | 8.5 | 8 | 0 | 0 | 0 |
| 2 Months A | 10 | 10 | 0 | 0 | 0 |
| 2 Months B | 11.5 | 0 | 0 | 0 | 0 |
| 3 Months A | 0 | 0 | 0 | 0 | 0 |
| 3 Months B | 0 | 0 | 0 | 0 | 0 |

Fluconazole: 20 mm, Amphotericin B: 9 mm, 0: no activity observed
Round 1-5: fractions obtained from various rounds of exhaustive extraction conducted per culture

**Table 4.4.** Bioactivity of extracts from *Meyerazyma guilliermondii* (MEF 75) grown at different time points

| *C. albicans* | | | | | |
|---|---|---|---|---|---|
| Incubation period | Zone of Inhibition/mm | | | | |
| | Round 1 | Round 2 | Round 3 | Round 4 | Round 5 |
| 6 WEEKS A | 0 | 0 | 0 | 0 | 0 |
| 6 WEEKS B | 8 | 9 | 8 | 6 | 0 |
| 2 Months A | 6 | 0 | 0 | 0 | 0 |
| 2 Months B | 0 | 0 | 0 | 0 | 0 |
| 3 Months A | 9.5 | 6.5 | 0 | 0 | 0 |
| 3 Months B | 6 | 0 | 0 | 0 | 0 |

Fluconazole: 20 mm, Amphotericin B: 9 mm, 0: no activity observed
Round 1-5: fractions obtained from various rounds of exhaustive extraction conducted per culture

**Table 4.5.** *Bioactivity of extracts from Meyerazyma guilliermondii (MEF 75) grown at different time points*

| *S. cerevisiae* | | | | | |
|---|---|---|---|---|---|
| Incubation period | Zone of Inhibition/mm | | | | |
| | Round 1 | Round 2 | Round 3 | Round 4 | Round 5 |
| 6 WEEKS A | 0 | 0 | 0 | 0 | 0 |
| 6 WEEKS B | 9 | 0 | 0 | 0 | 0 |
| 2 Months A | 6 | 0 | 0 | 0 | 0 |
| 2 Months B | 0 | 0 | 0 | 0 | 0 |
| 3 Months A | 6 | 0 | 0 | 0 | 0 |
| 3 Months B | 12 | 6 | 0 | 0 | 0 |

Fluconazole: 20 mm, Amphotericin B: 9 mm, 0: no activity observed
Round 1-5: fractions obtained from various rounds of exhaustive extraction conducted per culture

**Table 4.6.** Bioactivity of extracts from *Meyerazyma guilliermondii* (MEF134) grown at different time points

| *C. albicans* | | | | | |
|---|---|---|---|---|---|
| Incubation period | Zone of Inhibition/mm | | | | |
| | Round 1 | Round 2 | Round 3 | Round 4 | Round 5 |
| 6 WEEKS A | 7.5 | 8.5 | 6 | 0 | 0 |
| 6 WEEKS B | 12.5 | 12 | 0 | 0 | 0 |
| 2 Months A | 11.5 | 16.5 | 12.5 | 10 | 6 |
| 2 Months B | 16 | 13 | 7.5 | 0 | 0 |
| 3 Months A | 0 | 0 | 0 | 0 | 0 |
| 3 Months B | 0 | 0 | 0 | 0 | 0 |

Fluconazole: 20 mm, Amphotericin B: 9 mm, 0: no activity observed
Round 1-5: fractions obtained from various rounds of exhaustive extraction conducted per culture

**Table 4.7.** Bioactivity of extracts from *Meyerazyma guilliermondii* (MEF134) grown at different time points

| *S. cerevisiae* | | | | | |
|---|---|---|---|---|---|
| Incubation period | Zone of Inhibition/mm | | | | |
| | Round 1 | Round 2 | Round 3 | Round 4 | Round 5 |
| 6 WEEKS A | 6 | 0 | 0 | 0 | 0 |
| 6 WEEKS B | 13.5 | 9.5 | 7 | 0 | 0 |
| 2 Months A | 15 | 10 | 9 | 7.5 | 0 |
| 2 Months B | 12.5 | 10.5 | 9.5 | 0 | 0 |
| 3 Months A | 0 | 0 | 0 | 0 | 0 |
| 3 Months B | 0 | 0 | 0 | 0 | 0 |

Fluconazole: 20 mm, Amphotericin B: 9 mm, 0: no activity observed
Round 1-5: fractions obtained from various rounds of exhaustive extraction conducted per culture

### 4.3.2 Diversity and time of production of antifungal metabolites

An initial screening of the crude extract from the cultures showed the existence of antifungal metabolites. Therefore it was important to investigate the diversity of these metabolites and estimate the particular time of production of the metabolites. Diversity and time of production were determined by testing the fractions obtained (via the solvent-solvent separation of the crude extracts) against *C. albicans* and *S. cerevisiae*. Based on the polarities of the solvents used for the separation procedure, it was expected that each solvent fraction would contain metabolites with similar polarities.

The fractions obtained from MEF65 after separation were highly active against *C. albicans*, though in some instances the fractions from the duplicate broths for some time points did not consistently show activity (Fig 4.2 A). In addition, the FM50 (6weeks broth), FW (2 months broth) and FW (3 month broth) fractions did not show activity against *C. albicans*. The duration of incubation of the MEF cultures at which optimal metabolites were produced against *C. albicans* was 6 weeks. However, the most active metabolites were produced when the cultures were incubated for 3 months. The activity recorded against *S. cerevisiae* were lower and in some cases non-existent particularly for the older cultures (2 and 3 months) (Fig 4.2 B). Also, the best time for metabolite production against *S. cerevisiae* was 6 weeks; most active metabolites were produced in the cultures incubated for 3 months.

The fractions obtained from MEF 75 were highly active against both *C. albicans* and *S. cerevisiae* (Fig 4.3 A and B). In both instances, the data show that the period between 6 weeks and 2 months were adequate for optimal metabolite productions. Moreover, it was observed that the most active fractions were from the cultures incubated for 2 months.

The fractions from MEF134 were highly active against *C. albicans* and *S.cerevisiae* (Fig 4.4 A and B). The fractions that produced most of the activity against *C. albicans* were obtained from the 2- and 3-month old cultures (Fig 4.4 A). The activity observed against *S. cerevisiae* were mostly from the fractions obtained from cultures that were incubated for 2 months (Fig 4.4 B). The antifungal activity of the fractions from the 3-month old MEF134 cultures was relatively low when tested against *S. cerevisiae*.

It can be concluded from this study that the three selected marine endophytic fungi produced diverse metabolites based on the observation that the fractions from the different solvents all yielded active metabolites against both test organisms. It can also be inferred from the study that the optimal time for metabolite production is dependent on the producing organism.

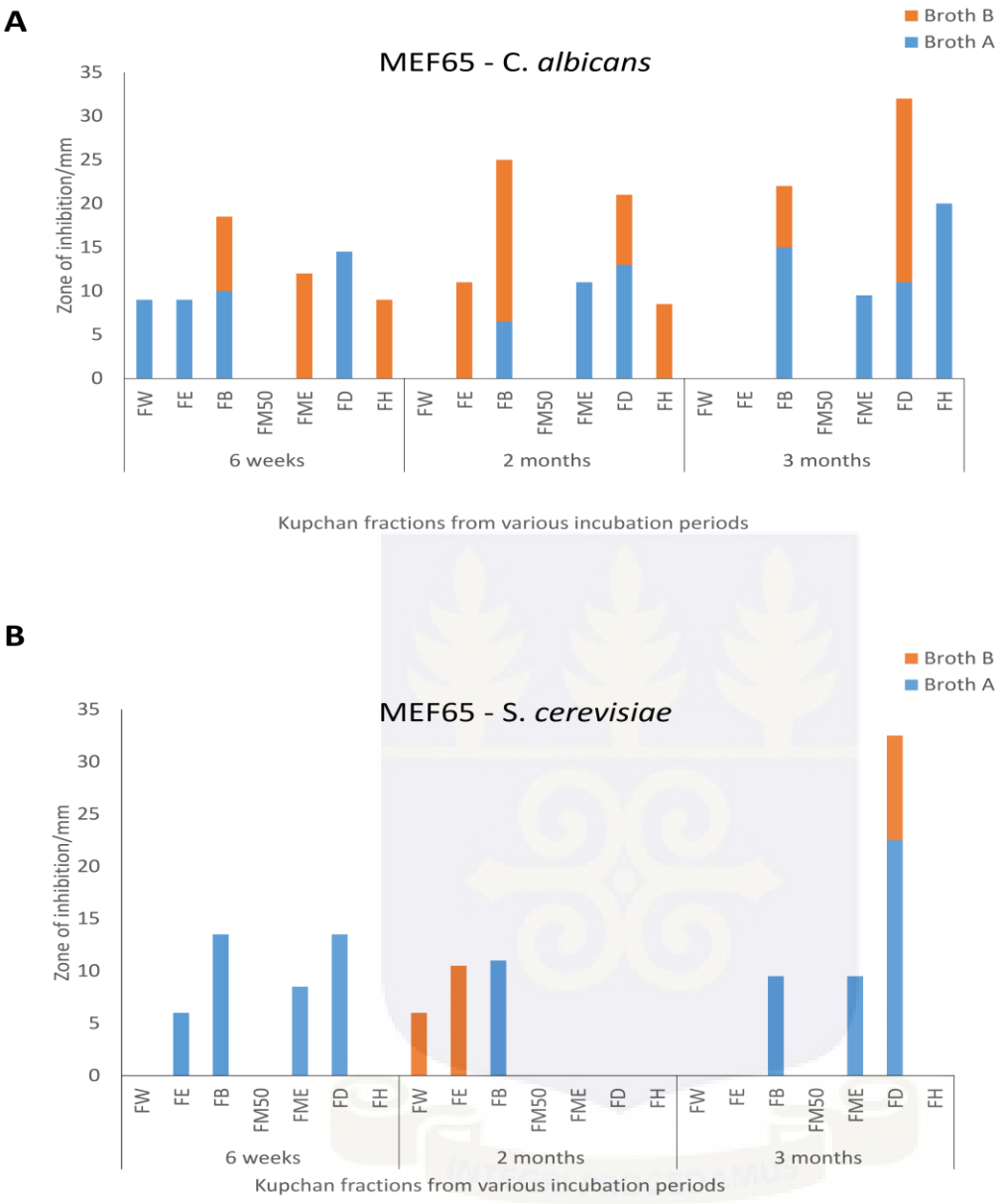


Fig 4.2. Bioactivity of Kupchan fractions obtained from *Clonostachys wenpingii* (MEF65) cultures at different times. Two replicate broths (A and B) were prepared at each time point. The fractions obtained from these broths were tested against (A) *C. albicans* and (B) *S. cerevisiae*. The most active fraction was FD from the 3 months' time point. The blue bars represent fractions from broth A and the orange bars represent fractions from broth B.

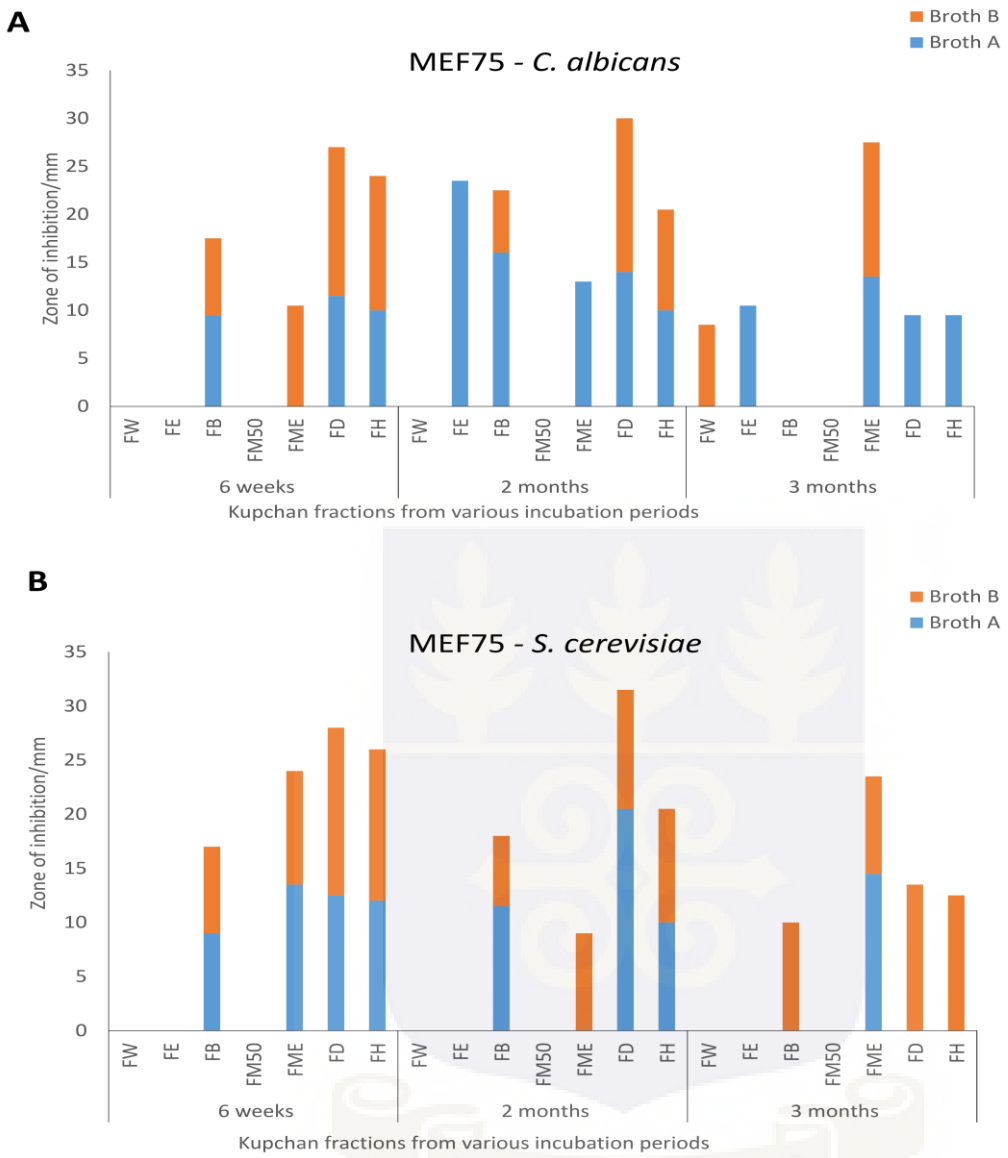


Fig 4.3. Bioactivity of Kupchan fractions obtained from *Meyerozyma guilliermondii* (MEF 75) cultures at different times. Two replicate broths (A and B) were prepared at each time point. The fractions obtained from these broths were tested against (A) C. *albicans* and (B) *S. cerevisiae*. Fractions obtained from this isolate were mostly very active with the most active fraction being FD from the 2 months' time point. Blue bars represent fractions from broth A and orange bars represent fractions from broth B.

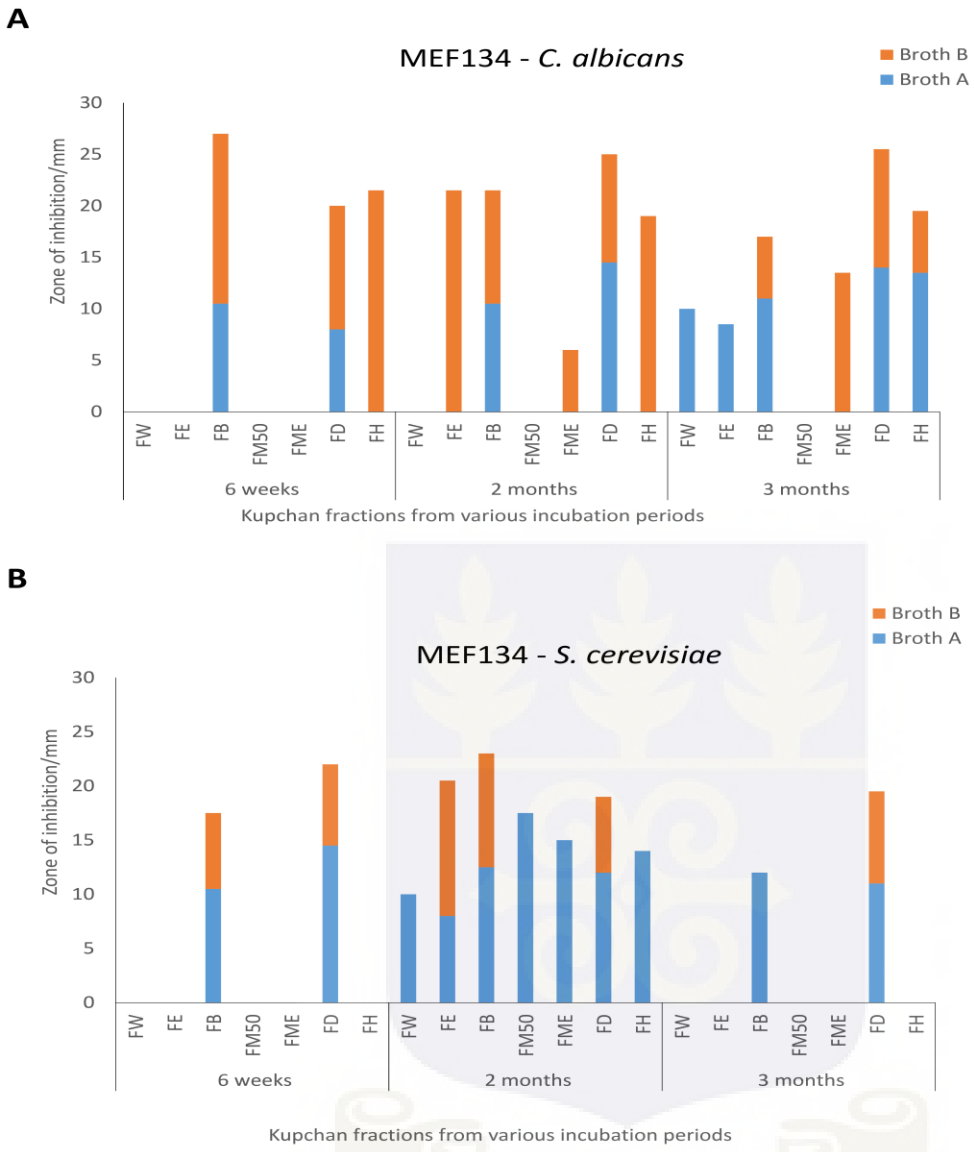


Fig 4.4. Bioactivity of Kupchan fractions obtained from *Meyerozyma guilliermondii* (MEF 134) cultures at different times. Most of the fractions obtained from this isolate were consistently active against both test organisms. The fractions from the two replicate broths (A and B) at each time point were tested against (A) *C. albicans* and (B) *S. cerevisiae*. The blue bars represent fractions from broth A and the orange bars represents fractions from broth B.

### 4.3.3 Antifungal activity of fractions from preparative Thin Layer Chromatography

Fractionation of a crude extract leads to enrichment of the components in the solvent fractions obtained. In some cases, antagonistic components are separated from each other, leading to enhancement of activity of the remaining components. In this study, the individual Kupchan fractions (from the solvent-solvent separation procedure) were further fractionated via preparative Thin Layer Chromatography (TLC). The Kupchan fractions from the MEF134 cultures (incubated at 6 weeks, 2 months and 3 months) were used for the second (TLC) fractionation procedure to generate six TLC fractions (V1-V6) (Fig S4.7-12). The TLC fractions obtained were tested against *C. albicans* and *S. cerevisiae*. A total of 20 TLC fractions, from the MEF134 culture incubated for 6 weeks, were active against *C. albicans* while 33 fractions were active against *S. cerevisiae* (Fig 4. 5). For the MEF134 culture incubated for 2 months, 28 fractions were active against *C. albicans* and 35 fractions were active against *S. cerevisiae* (Fig 4. 5). Furthermore, 34 and 35 TLC fractions were active against *C. albicans* and *S. cerevisiae*, respectively, for the MEF134 cultures incubated for 3 months (Fig 4.5). These observations demonstrate that incubation of the MEF134 culture for 3 months leads to production of the highest number of active antifungal fractions. Even though the Kupchan fractions from the 3-month old MEF134 culture exhibited very low antifungal activity against *S. cerevisiae*, the second TLC fractionation procedure boosted the activity of fractions from that fungal culture. From our data it could be concluded that fractionation aids in eliminating antagonistic compounds, thereby enhancing activity and also showed the production of bioactive metabolites in a time-resolved manner. This allows the selection of time points for the specific Kupchan fraction of peak yield for product isolation. In comparison to the data from the 4 month culture of MEF 134, a total of 5 1D TLC fractions were active against *C. albicans*. However, none of the fractions were active against *S. cerevisiae*.

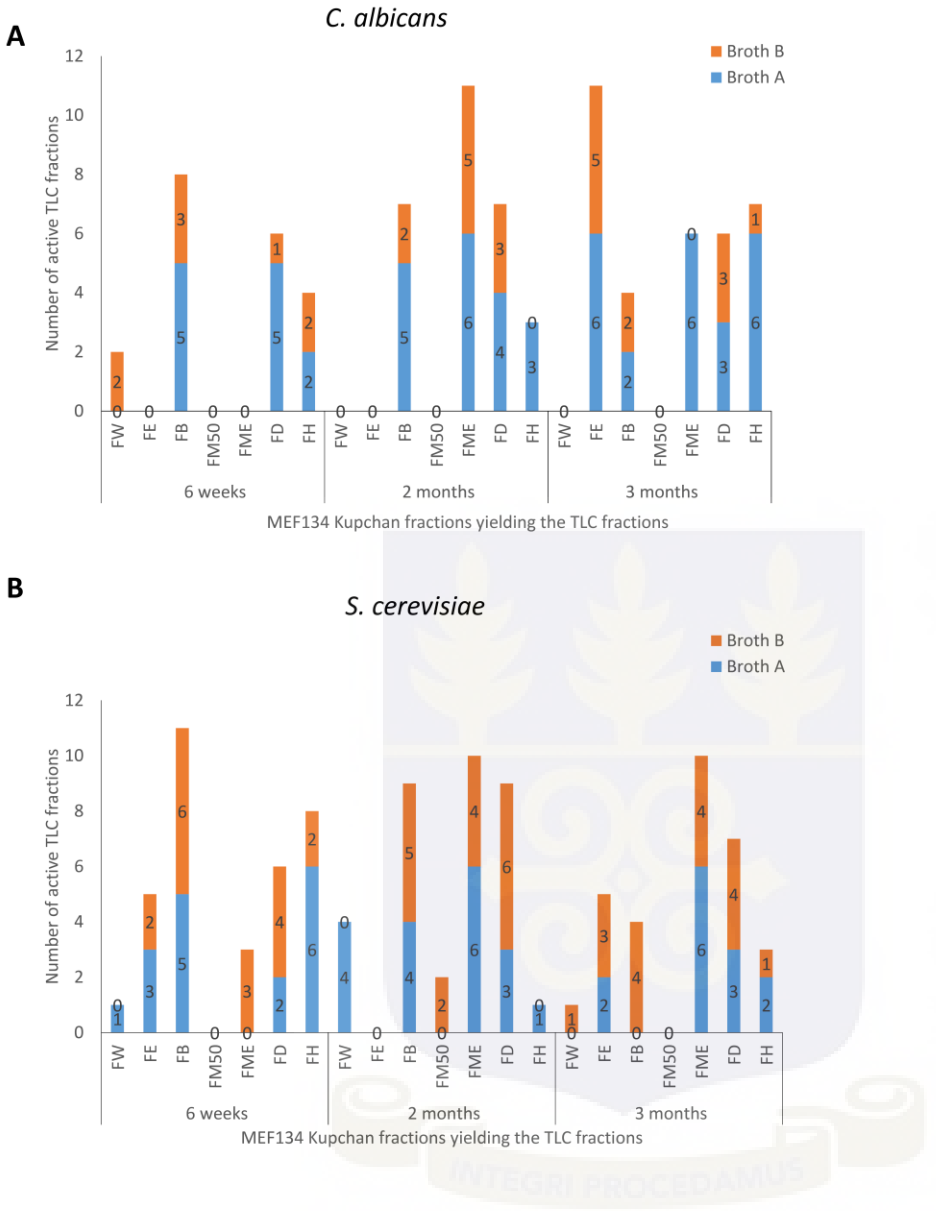


Fig 4.5. The number of TLC sub-fractions obtained from the Kupchan fractions of *Meyerozyma guilliermondii* (MEF 134) tested against (A) *C. albicans* and (B) *S. cerevisiae.* (A) The total number of active TLC fractions obtained against *C. albicans* were 20, 28 and 34 at 6 weeks, 2 months and 3 months respectively. (B) The total number of active TLC obtained against S. cerevisiae at the various incubation times were 33, 35 and 35 for 6 weeks, 2 months and 3 months respectively.

### 4.3.4 Solvent fractions yielding the most active antifungal compounds

Each crude extract contains many compounds, and the separation procedures required for obtaining the compound(s) of interest can be very challenging. In this study, a selection of solvents were used for the separation of compounds in the crude extracts on the basis of differences in polarity of these compounds. The detailed procedure for separation of crude extracts by solvent-solvent fractionation have been extensively described in the methods section.

The butanol and dichloromethane fractions from the MEF 134 extracts produced the most active TLC fractions against *C. albicans* and *S. cerevisae* (Fig 4.5). This showed that the less polar compounds, which constituted these fractions, were very active against *C. albicans*. Moreover, a significant number of active TLC fractions were obtained from the combined methanol/ethyl acetate Kupchan fraction (Fig 4.5). TLC Fractions obtained from the more polar Kupchan fractions, such as water and diluted methanol, yielded the least active metabolites against *C. albicans* (Fig 4.5).

The most active TLC fractions against *S. cerevisiae* were obtained from the butanol and the methanol/ethyl acetate Kupchan fraction (Fig 4.5). In addition, the dichloromethane Kupchan fraction yielded active TLC fractions against *S. cerevisiae* (Fig 4.5). As observed with *C. albicans*, the water and the diluted methanol Kupchan fractions yielded the least active TLC fractions against *S. cerevisiae* (Fig 4.5).

### 4.3.5 Interaction assay between the TLC fractions and cyclosporine A

The extraction and fractionation of crude metabolites from fungal cultures might lead to the rediscovery of already known compounds. To avoid this, there is a need to expand the inventory of starting materials and techniques used in the discovery process in order to increase the

probability of isolating new compounds. It is essential to extend the limit of detection, with respect to antimicrobial activity, for each fraction obtained. A strategy for addressing these limitations is to perform interaction assays between a known antifungal compound and the solvent fractions from the separation procedure (solvent-solvent separation or TLC fractionation). An interaction assay was performed using cyclosporine A and the TLC fractions to determine the effect of the interaction on the antifungal activity of the TLC fractions. This type of interaction assay is important in the identification of the TLC fractions that did not exhibit antifungal activity on their own but showed activity following interaction with cyclosporine A.

As a proof of concept, all the antifungal compounds listed in Table 4.1 were each tested in combination with cyclosporine A. The data from this interaction assay revealed that the bioactivity of these known antifungal compounds against *C. albicans* was boosted in the presence of cyclosporine A (Fig 4.6). Notably, the interaction between sertraline and cyclosporine A resulted in a 60% increase in the antifungal activity relative to the activity of sertraline alone. Moreover, 5-fluorouracil exhibited antifungal activity in the presence of cyclosporine A even though it showed no detectable activity in the absence of cyclosporine A. Following these observations, the effect of the presence of cyclosporine A on the antifungal activity of all the TLC fractions was investigated to determine whether interaction of the TLC fractions with cyclosporine A boosted the antifungal activity of these TLC fractions against *C. albicans*.

Fig 4. 7 revealed that the presence of Cyclosporine A caused antagonistic effects on a significant proportion of the TLC fractions that initially showed antifungal activity in the absence of Cyclosporine A (45% of the active TLC fractions from the 6 weeks fungal cultures and 56% of the active TLC fractions from the 3 months fungal cultures). Synergistic interactions were

generally detected for the TLC fractions from the 2 month fungal cultures; 34 TLC fractions exhibited antifungal activity in the presence of cyclosporine A while 28 TLC fractions showed activity in the control assay.

Out of the number of TLC fractions whose antifungal activity was boosted in the presence of cyclosporine A, some TLC fractions did not exhibit antifungal activity in the absence of cyclosporine A; these category of TLC fractions only showed antifungal activity due to their interaction with cyclosporine A and were obtained from the butanol (Figs. S4. 1 & 4), dichloromethane (Figs. S4.2 & 3), hexane (Figs. S4.3& 4) or water fractions (Fig. S4.4).

In the drug development process, many useful compounds are often ignored because they were not active on their own. Our results show that many compounds could be very useful as a synergistic partner to other known compounds. Hence, more should be done to explore the usefulness of inactive fractions by utilizing interactions assays.

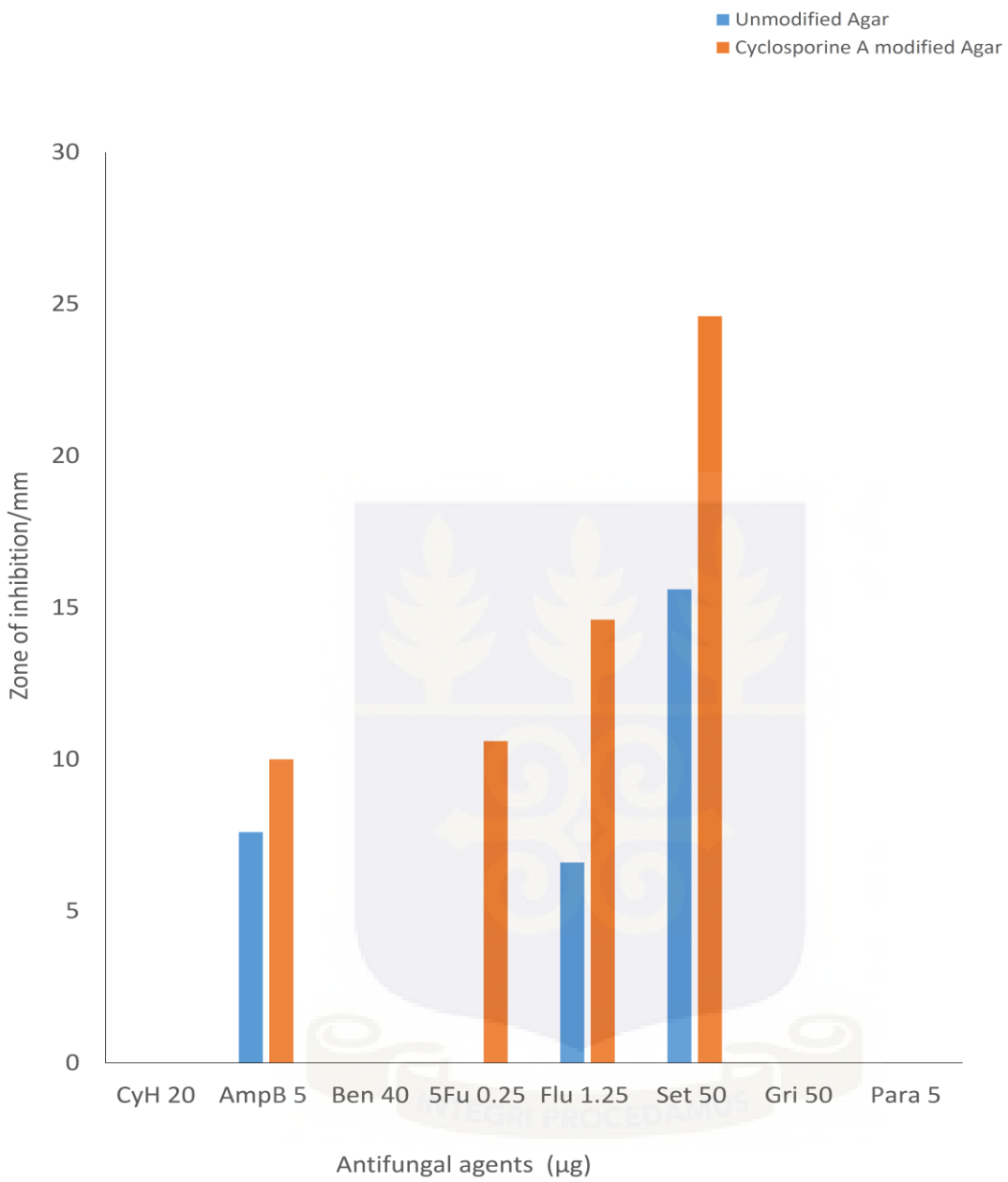


Fig 4.6. The collective effects of standard antifungal compounds combined with cyclosporine A on *C. albicans*. *Candida albicans* was grown on agar plates modified with or without cyclosporine A. The activities of the standard antifungal compounds against *C. albicans* across both conditions are shown in the figure.

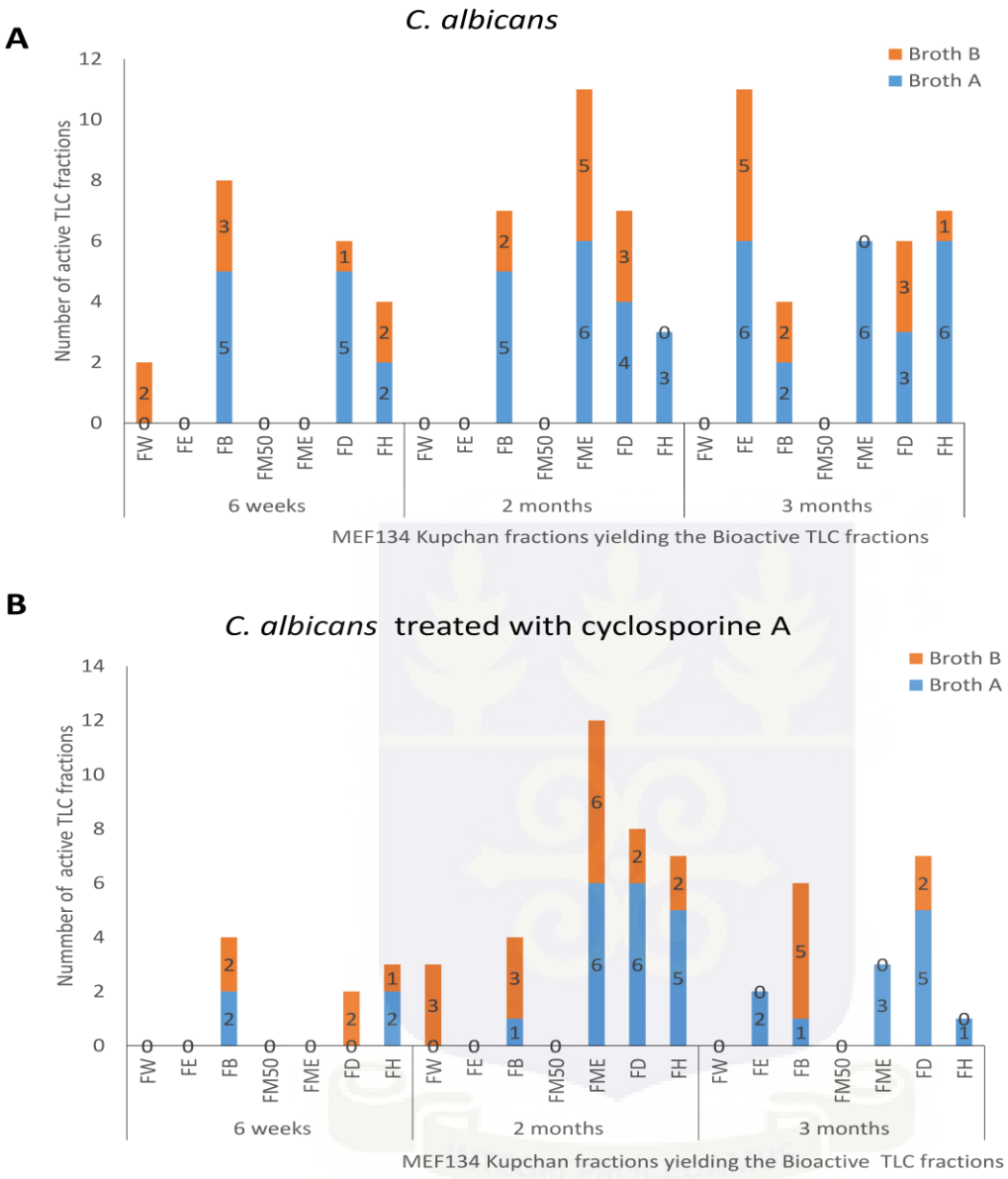


Fig 4.7. The number of active TLC fractions identified after the synergistic interaction of cyclosporine A with the TLC fractions against *C. albicans*. (A)The number of active TLC fractions obtained after interaction with *C. albicans* untreated. This data also served as the control. (B) The number of active TLC fractions obtained after interaction with Cyclosporine A treated C. *albicans* cells. The producing organism was *Meyerozyma guilliermondii* (MEF 134).

### 4.4 Discussion

In this study, three marine endophytic fungal isolates (MEF 65, MEF75 and MEF134) were cultured to ascertain their production of metabolite over time (Fig 4.1, Table S4.1). The data obtained showed that the three isolates presented different yields of extractable soluble compounds (Table S4.1). It has been shown that production of secondary metabolites by microorganisms is usually connected and impacted by the primary metabolic processes (Ludwig-Müller, 2015). It has been asserted that biosynthesis of bioactive molecules is influenced by the concentration and composition of the media and the metabolic activity of the organism (Holkar *et al.*, 2013).

The data presented here suggest that the secondary metabolites were not fully recovered from the fungal broth cultures after the first and second rounds of extraction, and supported the need for multiple (five) rounds of recovering these metabolites. The assessment of the impact of culture media on metabolite yield revealed that the species of fungal isolate was more important than the growth medium used for culturing the fungus (VanderMolen *et al.*, 2013). Studies have shown that media composition has variable effects on the production of fungal secondary metabolites and variation of culture conditions can be used to improve the yields of a specific compound (Bode *et al.*, 2002). Previous studies in the lab of Dr Patrick K. Arthur observed that the YPDM medium is most suitable for culturing of marine endophytic fungi, hence the low yield observed for MEF65 could only be explained by the isolate's metabolic capacity. Overall, the data shown in Table S4.1 indicate that yield of secondary metabolites from fungal cultures is not increased by exclusively increasing the duration of incubation of these cultures.

This study also investigated the production of antifungal agents by the three MEF isolates over the designated time periods. The antifungal activity of the crude extracts obtained from the duplicate

cultures of MEF65, MEF75 and MEF134 demonstrated that the production of these antifungal agents is time dependent. Importantly, the data demonstrates that longer incubation periods do not necessarily favor production of highly active antifungal compounds. Hence, the most suitable time for production of these antifungal agents varies from one organism to the other. Merlin et al. (2013) has also reported a similar observation when they used the endophyte *Fusarium solani*; production of bioactive secondary metabolite was very low after 15 days of culturing the organism (Merlin *et al.*, 2013).

The data form the Kupchan fractionation procedure showed that the fractions obtained from the three selected marine endophytic fungi contained diverse metabolites. Water was more effective in dissolving and extracting the solutes because of its high polarity index (Pin *et al.*, 2010). Water has hydroxyl groups which can form hydrogen bonds with solutes, which makes it effective in extracting solutes. However, in this study, the yields from the water fractions were very low and the corresponding activities were also low. Most of the metabolites were concentrated in the solvent with low polarity indices such butanol and the dichloromethane fractions (Fig 4.5).

Data from the TLC fractionation showed that resolving the components of the crude extracts could help identify compounds whose activities were masked by others. Though the extracts obtained from the 3 months cultures of MEF 134 were not active (Table 4.6 and 4.7), it was observed that after further fractionation by TLC many active fractions were obtained (Fig 4.5 A and B). A similar observation was made with the assessment of the antioxidant properties of crude and fractionated extracts of *Alpinia mutica rhizomeshe.* Their results showed that the ethyl acetate fraction had the highest antioxidant property in comparison to the other extracts (Phang *et al.*, 2011).

Many studies have demonstrated that fluconazole and cyclosporine (Cy) exhibit synergistic activity against *Candida albicans*, *in vitro* (Y. Li *et al.*, 2008; Marchetti *et al.*, 2000). The present study confirmed that, not only does cyclosporine A synergize with fluconazole to elicit fungicidal activity against *C. albicans*, but also synergizes with sertraline and 5-Fluorouracil (Fig 4.5). In *candida albicans*, efflux pumps mediates the extrusion of many molecules and this has been shown to be the major mechanisms of fluconazole resistance *(Sanglard et al., 1997)*. Thus it was thought that membrane-active drugs might increase the efficacy of drugs such as fluconazole by allowing accumulation of the drugs inside of the yeast cell. Our results show that, cyclosporine A exhibited antagonistic effects on a significant proportion of the TLC fractions that initially showed antifungal activity in the absence of Cyclosporine A (Fig 4.7 B). In a few instances, synergistic interactions were detected for the TLC fractions, generally from the 2 month fungal cultures. Unlike the pure compounds, little can be said about this observation because these are still fractions with their largely unknown major constituents. However, the results suggest that there is a potential to synergistically combine some of the compounds from the fractions with cyclosporine A for a more superior activity.

### 4.5 Conclusion

The data shown in this study confirm that production of bioactive metabolites by marine fungal endophytes is time dependent and is favoured by shorter culture incubation periods. The metabolite yield is not entirely dependent on the duration of culture, but also on the metabolism of the fungal isolate. It was observed that the most potent metabolites were in solvents with very low polarity indexes. In addition, activity was distributed throughout the 7 solvents systems used for the solvent-solvent fractionation indicating that there may be diverse metabolites with unique

structures in each fraction obtained. It was also confirmed that cyclosporine A synergizes very well with sertraline and 5-flurouracil.

### 4.6 Recommendation for future works

- The crude fractions from isolates MEF65 and 75 should also be fractionated by TLC and tested in the same manner to enable comparison with the activities observed for MEF 134.
- Isolation of the compounds from the TLC fractions for interaction studies with cyclosporine A to identify any synergistic partners against *C. albicans.*

## 4.7. Supplementary Data

**Table S4.1.** Yield of extracts obtained from the 3 marine endophytic fungi

| | MASS/ g | | |
|---|---|---|---|
| **Samples** | **MEF 65** | **MEF75** | **MEF 134** |
| Broth A 6WKS X1 | 0.008 | 4.091 | 3.541 |
| Broth A 6WKS X2 | 0.007 | 1.781 | 2.507 |
| Broth A 6WKS X3 | 0.008 | 0.657 | 2.111 |
| Broth A 6WKS X4 | 0.006 | 0.657 | 2.739 |
| Broth A 6WKS X5 | 0.006 | 1.246 | 2.173 |
| | | | |
| Broth B 6WKS X1 | 0.007 | 2.851 | 2.885 |
| Broth B 6WKS X2 | 0.004 | 2.329 | 2.332 |
| Broth B 6WKS X3 | 0.008 | 1.298 | 2.415 |
| Broth B 6WKS X4 | 0.007 | 2.589 | 2.525 |
| Broth B 6WKS X5 | 0.004 | 1.554 | 1.78 |
| | | | |
| Broth A 2MTHS X1 | 0.008 | 3.055 | 4.476 |
| Broth A 2MTHS X2 | 0.010 | 1.707 | 3.669 |
| Broth A 2MTHS X3 | 0.009 | 0.577 | 4.064 |
| Broth A 2MTHS X4 | 0.010 | 2.074 | 3.732 |
| Broth A 2MTHS X5 | 0.005 | 1.208 | 2.335 |
| | | | |
| Broth B 2MTHS X1 | 0.013 | 1.779 | 4.346 |
| Broth B 2MTHS X2 | 0.016 | 1.409 | 3.994 |
| Broth B 2MTHS X3 | 0.015 | 0.823 | 3.934 |
| Broth B 2MTHS X4 | 0.013 | 1.614 | 4.368 |
| Broth B 2MTHS X5 | 0.010 | 1.233 | 2.435 |
| | | | |
| Broth A 3MTHS X1 | 0.003 | 3.819 | 1.71 |
| Broth A 3MTHS X2 | 0.005 | 1.325 | 1.54 |
| Broth A 3MTHS X3 | 0.003 | 0.415 | 1.026 |
| Broth A 3MTHS X4 | 0.004 | 1.352 | 1.336 |
| Broth A 3MTHS X5 | 0.002 | 0.58 | 1.111 |
| | | | |
| Broth B 3MTHS X1 | 0.005 | 2.463 | 2.571 |
| Broth B 3MTHS X2 | 0.004 | 1.081 | 1.318 |
| Broth B 3MTHS X3 | 0.004 | 0.96 | 0.635 |
| Broth B 3MTHS X4 | 0.005 | 1.463 | 0.477 |
| Broth B 3MTHS X5 | 0.003 | 0.906 | 0.639 |

X1-X5: Various rounds of extraction

**Table S4.2.** Mass distribution of the kupchan fractions obtained at the 3 time points

| | MASS/g | | | | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | MEF65 6 WEEKS | | MEF 65 2 MONTHS | | MEF 65 3 MONTHS | | MEF 75 6 WEEKS | | MEF 75 2 MONTHS | | MEF 75 3 MONTHS | | MEF 134 6 WEEKS | | MEF 134 2 MONTHS | | MEF 134 3 MONTHS | |
| FRACTIONS | A | B | A | B | A | B | A | B | A | B | A | B | A | B | A | B | A | B |
| FW | 0.44 | 0.19 | 0.05 | 0.59 | 0.04 | 0.01 | 0.08 | 0.04 | 0.36 | 0.21 | 0.34 | 0.07 | 0.27 | 0.07 | 1.11 | 0.71 | 0.04 | 0.11 |
| FE | 0.57 | 0.57 | 0.14 | 0.73 | 0.02 | 0.18 | 0.03 | 0.27 | 1.07 | 0.22 | 0.51 | 0.41 | 0.09 | 0.23 | 0.55 | 1.25 | 1.16 | 0.27 |
| FB | 2.97 | 0.92 | 2.77 | 3.39 | 0.72 | 0.81 | 0.90 | 2.29 | 0.88 | 2.22 | 0.59 | 0.37 | 3.22 | 3.10 | 3.81 | 2.97 | 0.14 | 0.63 |
| FM50 | 0.05 | 0.04 | 0.10 | 0.15 | 0.17 | 0.19 | 0.15 | 0.12 | 0.14 | 0.07 | 0.01 | 0.01 | 0.04 | 0.06 | 0.07 | 0.06 | 0.03 | 0.26 |
| FME | 0.20 | 0.99 | 0.48 | 0.09 | 0.10 | 4.41 | 0.08 | 0.29 | 0.43 | 0.19 | 0.80 | 0.50 | 0.51 | 0.14 | 1.22 | 3.62 | 0.85 | 0.39 |
| FD | 1.03 | 0.63 | 0.45 | 3.81 | 0.20 | 0.74 | 4.05 | 1.36 | 1.18 | 1.10 | 1.27 | 1.28 | 0.54 | 68.55 | 1.16 | 1.83 | 0.73 | 3.32 |
| FH | 0.78 | 1.02 | 0.38 | 3.19 | 0.24 | 0.30 | 1.50 | 0.63 | 1.21 | 1.50 | 1.45 | 0.53 | 0.80 | 0.48 | 0.58 | 0.72 | 0.51 | 1.13 |

**Table S 4.3.** Synergistic effect of cyclosporine A and the TLC fractions against *C. albicans*. (MEF134)

| | Number of active fractions before and after synergistic interaction | | | | | |
|---|---|---|---|---|---|---|
| | 6 Weeks | | 2 Months | | 3 Months | |
| 1DTLC fractions of MEF 134 | Control | Cylosporine A | Control | Cyclosporine A | Control | Cyclosporine A |
| FW A | 0 | 0 | 0 | 0 | 0 | 0 |
| FW B | 2 | 0 | 0 | 3 | 0 | 0 |
| FE A | 0 | 0 | 0 | 0 | 6 | 2 |
| FE B | 0 | 0 | 0 | 0 | 5 | 0 |
| FB A | 5 | 2 | 5 | 1 | 2 | 1 |
| FB B | 3 | 2 | 2 | 3 | 2 | 5 |
| FM50 A | 0 | 0 | 0 | 0 | 0 | 0 |
| FM50 B | 0 | 0 | 0 | 0 | 0 | 0 |
| FME A | 0 | 0 | 6 | 6 | 6 | 3 |
| FME B | 0 | 0 | 5 | 6 | 0 | 0 |
| FD A | 5 | 0 | 4 | 6 | 3 | 5 |
| FD B | 1 | 2 | 3 | 2 | 3 | 2 |
| FH A | 2 | 2 | 3 | 5 | 6 | 1 |
| FH B | 2 | 1 | 0 | 2 | 1 | 0 |
| **Total number of fractions** | **20** | **9** | **28** | **34** | **34** | **19** |

**Table S 4.5.** Mass distribution of the TLC fractions obtained after fractination of the kupchan fraction from the 2 months time period. (MEF134)

| | Mass/g | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | FW | | FB | | FE | | FM50 | | FME | | FH | | FD | |
| Fraction | A | B | A | B | A | B | A | B | A | B | A | B | A | B |
| V1 | 0.01 | 0.00 | 0.13 | 0.71 | 0.03 | 0.18 | -0.01 | 0.01 | 0.19 | 0.16 | 0.28 | 0.18 | 0.52 | 0.54 |
| V2 | 0.01 | 0.07 | 0.03 | 0.39 | 0.04 | 0.20 | -0.02 | 0.00 | 0.20 | 0.14 | -0.15 | 0.14 | 0.35 | 0.31 |
| V3 | 0.02 | 0.20 | 0.01 | 0.29 | 0.03 | 0.09 | -0.01 | 0.00 | 0.10 | 0.07 | 0.03 | 0.04 | 0.13 | 0.19 |
| V4 | 0.02 | 0.05 | 0.01 | 0.14 | 0.00 | 0.09 | 0.00 | -1.00 | 0.06 | 0.02 | 0.13 | 0.10 | 0.22 | 0.13 |
| V5 | 0.01 | 0.09 | 0.00 | 0.14 | 0.00 | 0.44 | -0.01 | 0.00 | 0.03 | -0.07 | 0.05 | 0.08 | 0.09 | 0.14 |
| V6 | 0.00 | 0.05 | 0.00 | 0.16 | 0.01 | 0.05 | -0.01 | 0.01 | 0.01 | 0.01 | 0.39 | 0.62 | 1.14 | -1.33 |

**Table S 4.6.** Mass distribution of the TLC fractions obtained after fractination of the kupchan fraction from the 3 months time period. (MEF134**)**

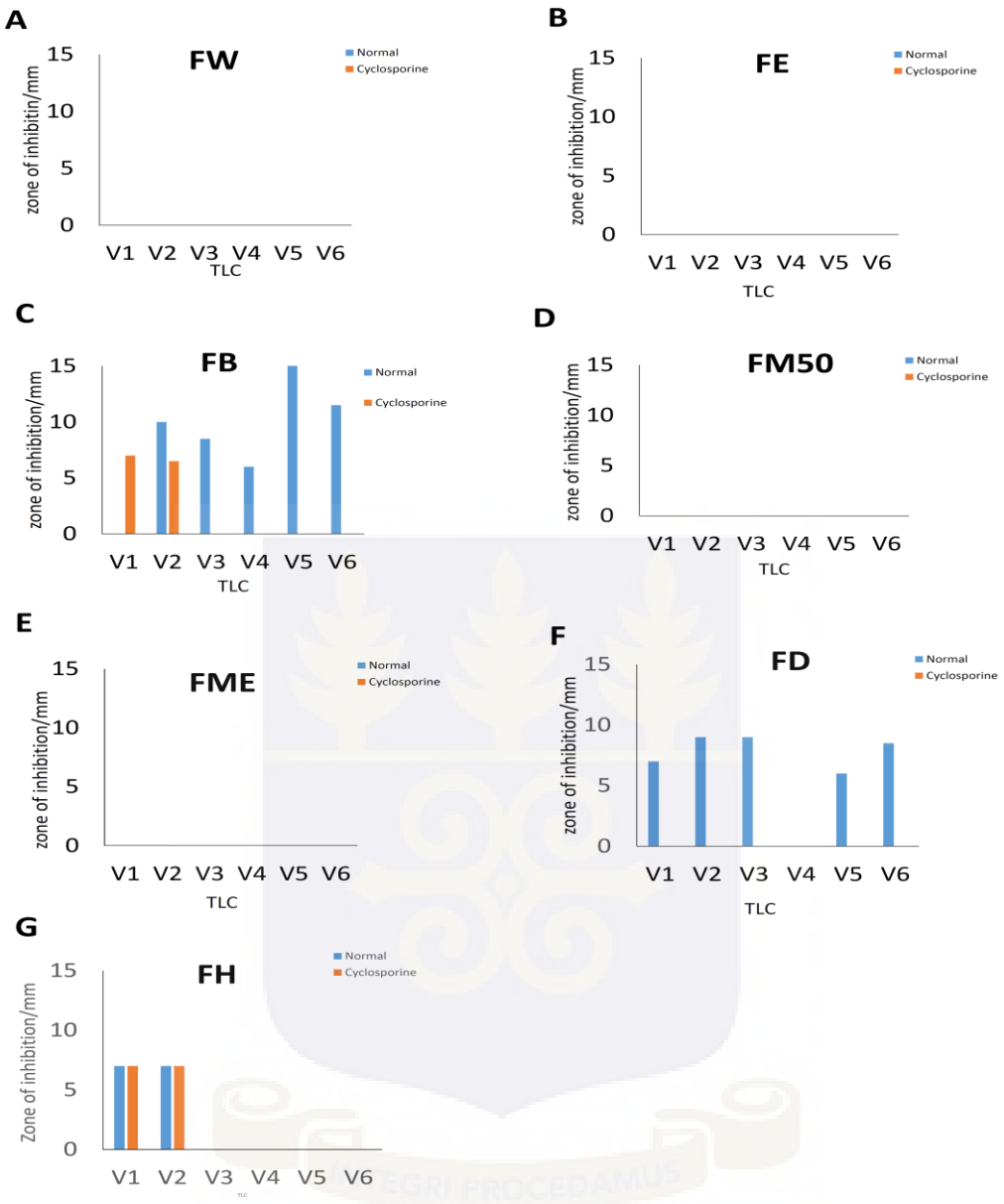


Fig S4.1 A-G. Antifungal activity of the TLC fractions from the MEF134 6 weeks' time point combined with cyclosporine A. The activity of the TLC fractions from MEF134 broth A were examined in combination with cyclosporine A. *Candida albicans* was grown on agar plates with or without cyclosporine A. The plates without cyclosporine A served as the control.

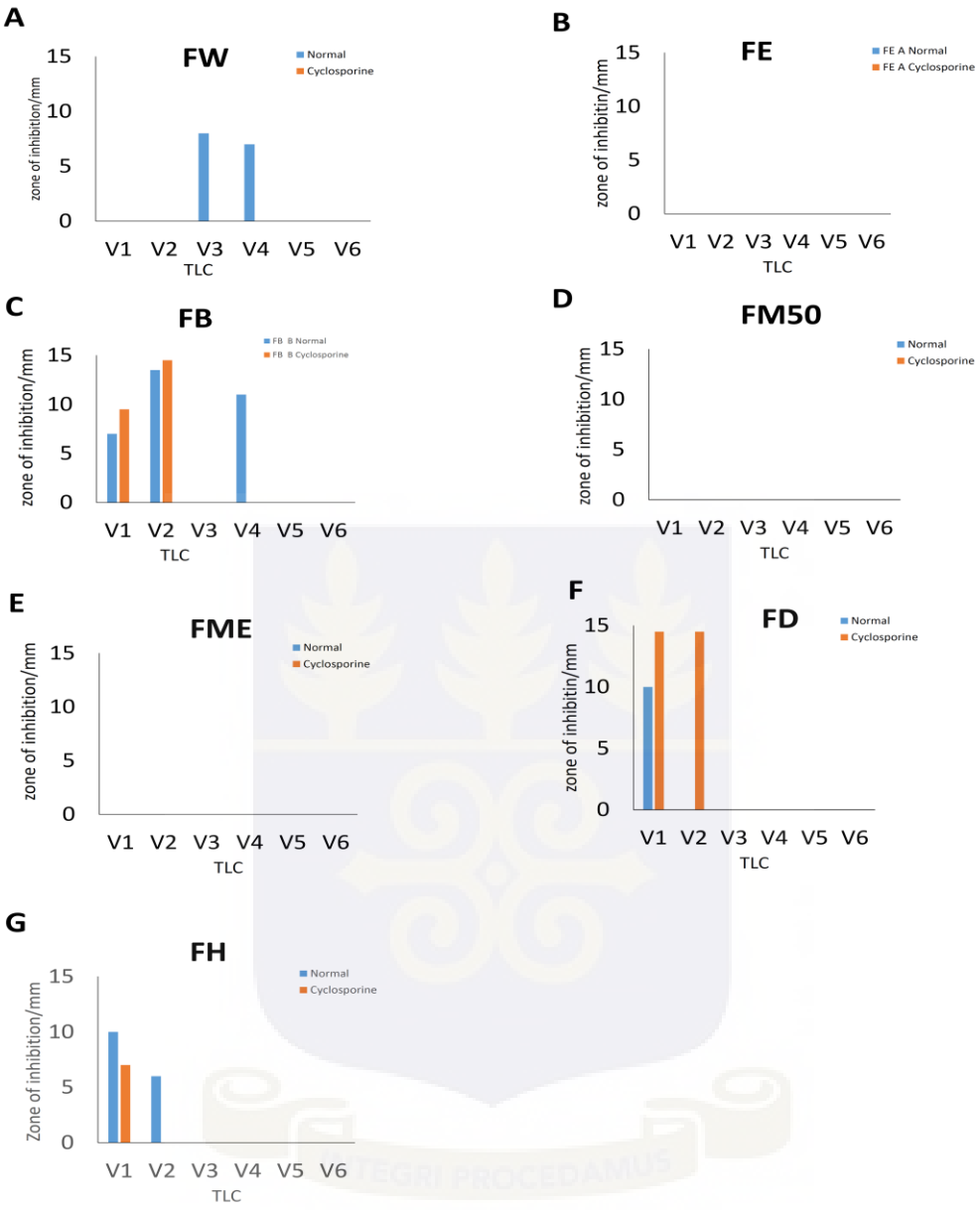


Fig S4.2 A-G. Antifungal activity of the TLC fractions from the MEF134 6 weeks' time point combined with cyclosporine A. The activity of the TLC fractions from MEF134 broth B were examined in combination with cyclosporine A. *Candida albicans* was grown on agar plates with or without Cyclosporine A. The plates without cyclosporine A served as the control.

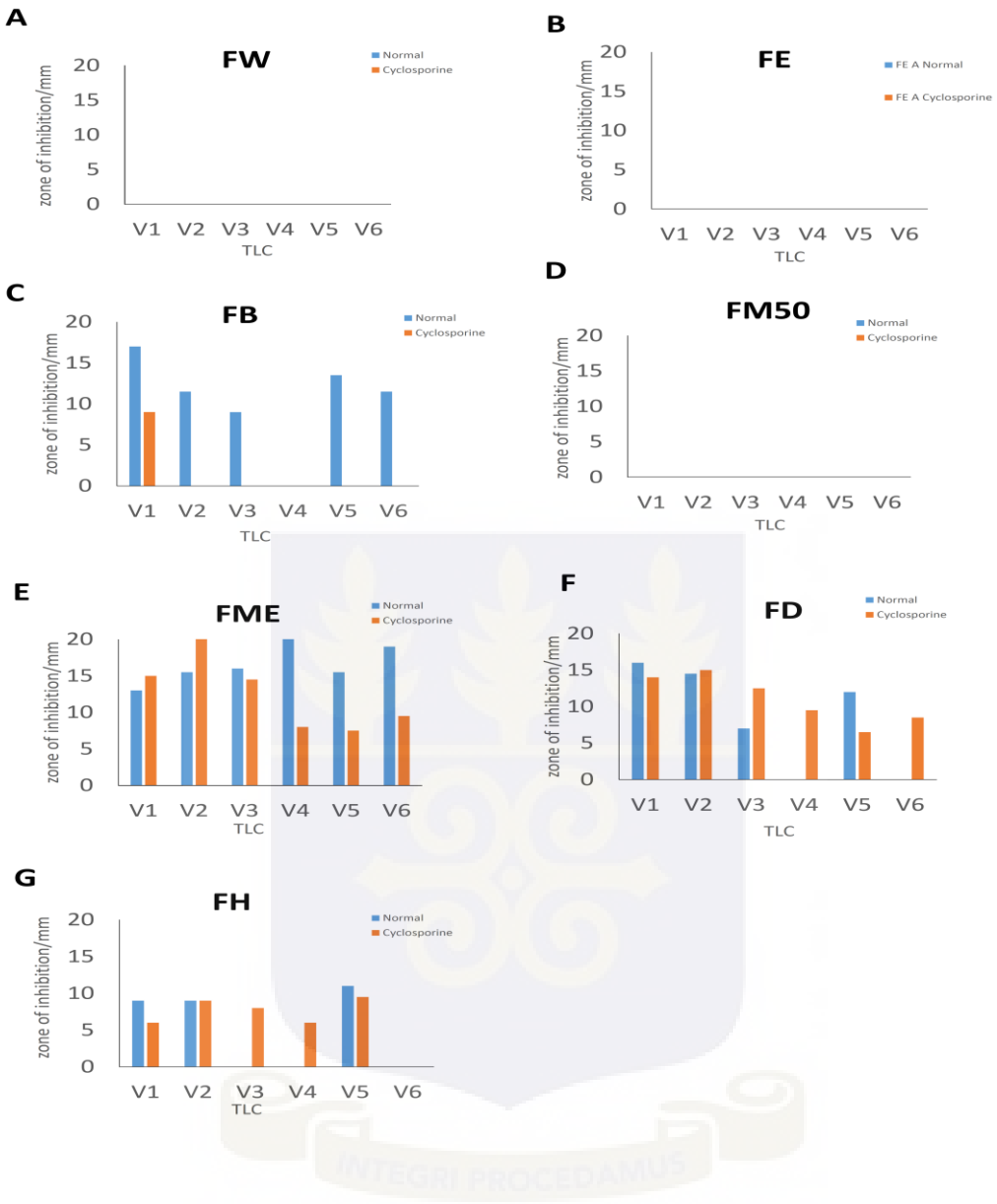


Fig S 4.3 A-G. Antifungal activity of the TLC fractions from the MEF134 2 months' time point combined with cyclosporine A. The activity of the TLC fractions from MEF134 broth A were examined in combination with cyclosporine A. *Candida albicans* was grown on agar plates with or without cyclosporine A. The plates without cyclosporine A served as the control.

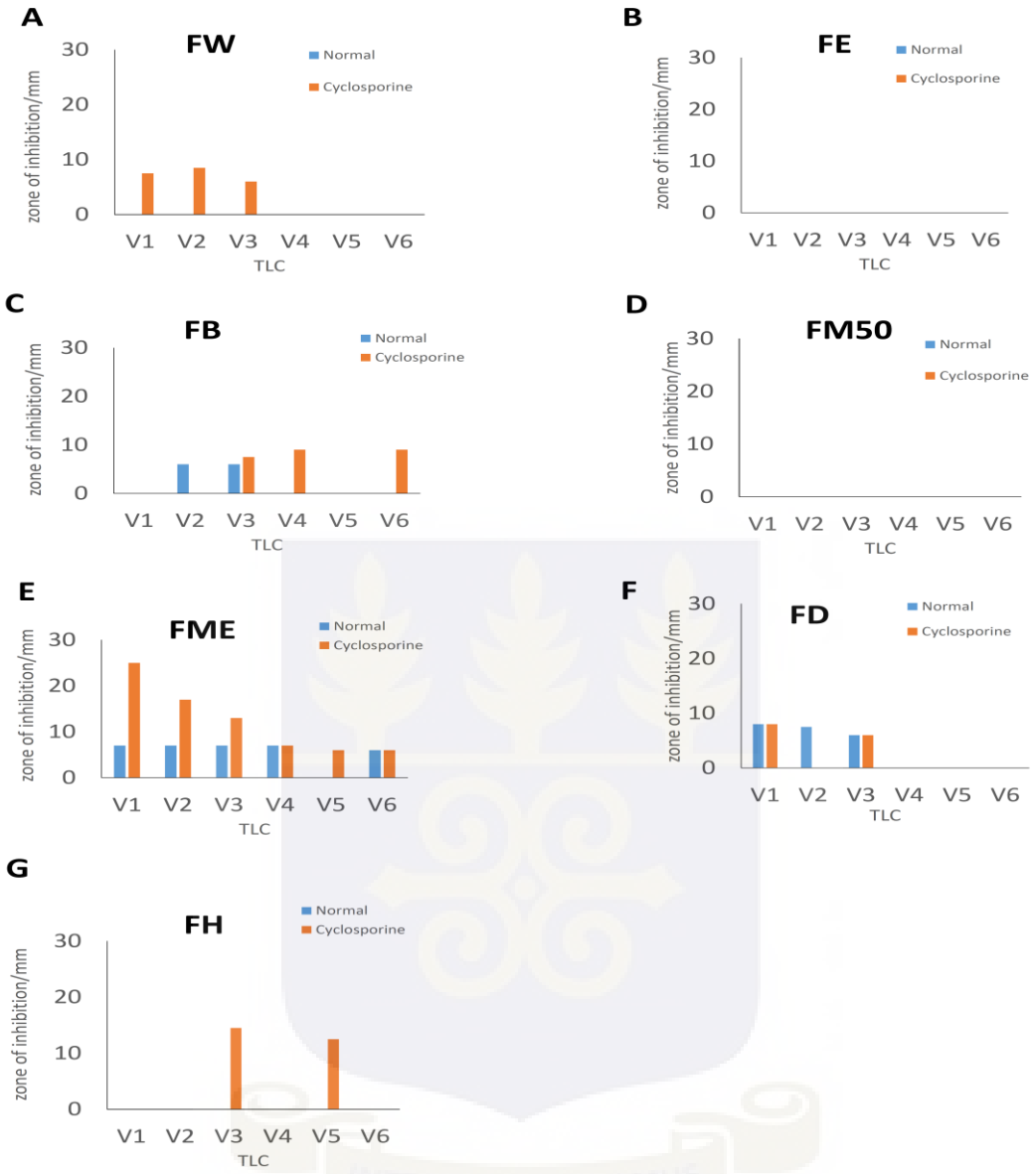


Fig S4.4 A-G. Antifungal activity of the TLC fractions from the MEF134 2 months' time point combined with cyclosporine A. The activity of the TLC fractions from MEF134 broth B were examined in combination with cyclosporine A. *Candida albicans* was grown on agar plates with or without cyclosporine A. The plates without cyclosporine A served as the control.

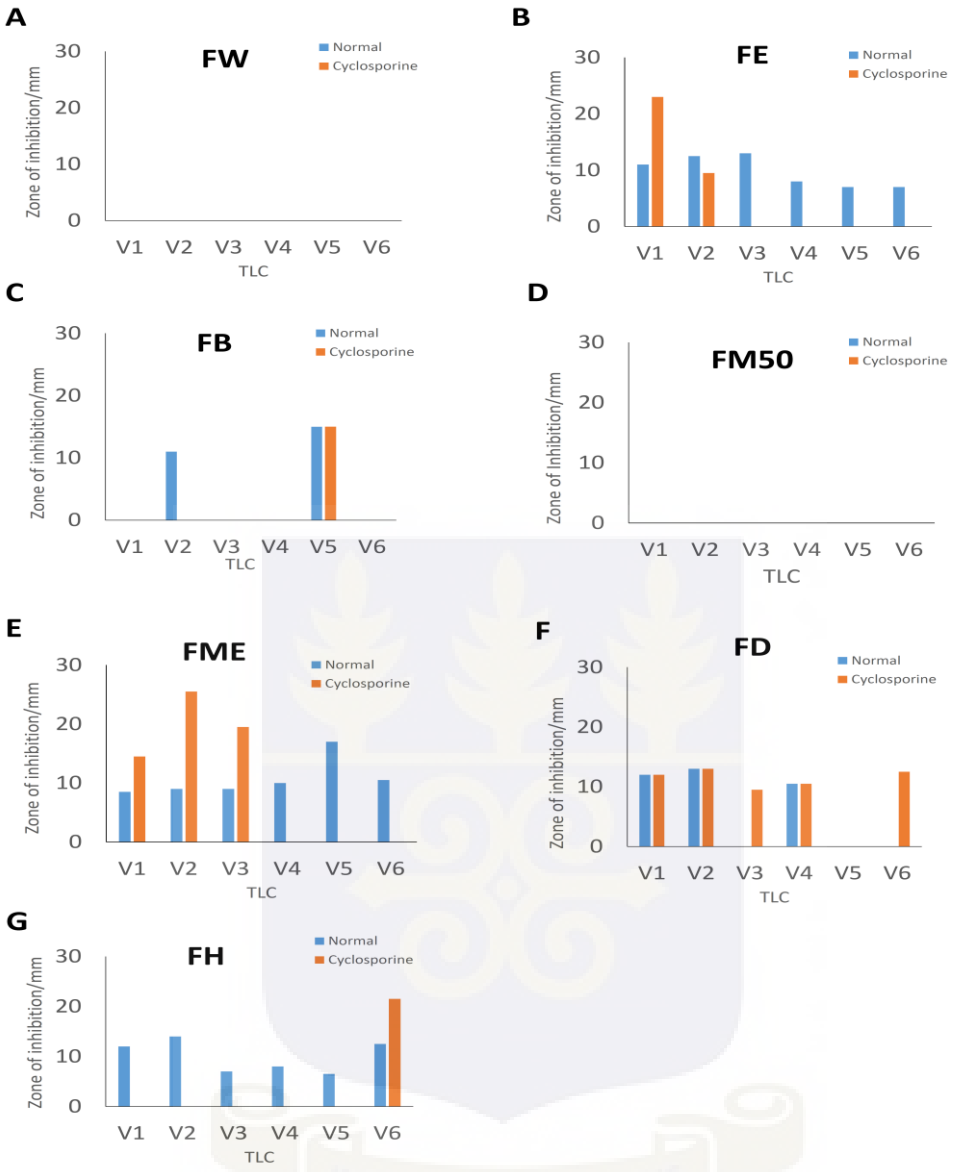


Fig S4.5 A-G. Antifungal activity of the TLC fractions from the MEF134 3 months' time point combined with cyclosporine A. The activity of the TLC fractions from MEF134 broth A were examined in combination with cyclosporine A. *Candida albicans* was grown on agar plates with or without cyclosporine A. The plates without cyclosporine A served as the control.

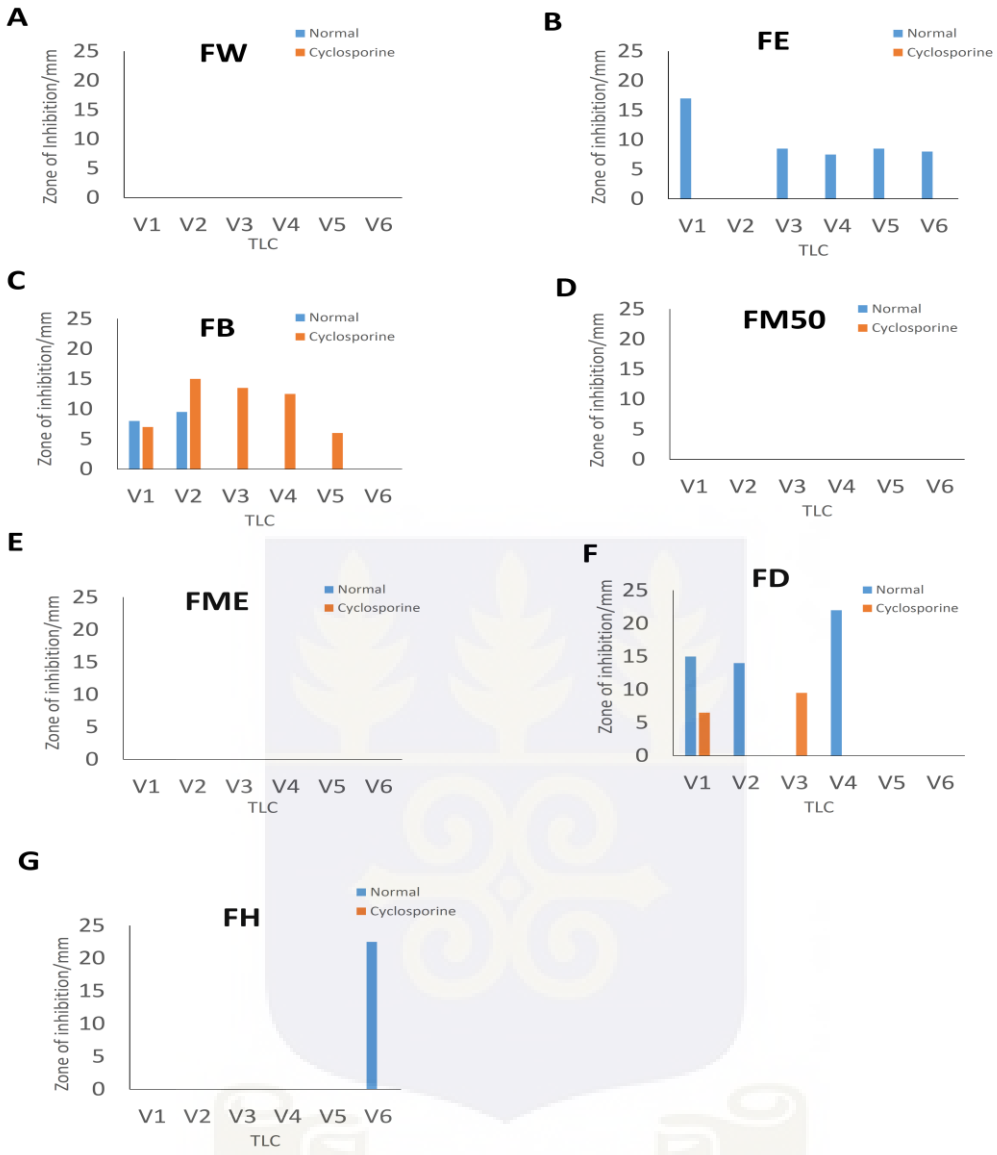


Fig S4.6 A-G. Antifungal activity of the TLC fractions from the MEF134 3 months' time point combined with cyclosporine A. The activity of the TLC fractions from MEF 134 broth B were examined in combination with cyclosporine A. *Candida albicans* was grown on agar plates with or without cyclosporine A. The plates without cyclosporine A served as the control.

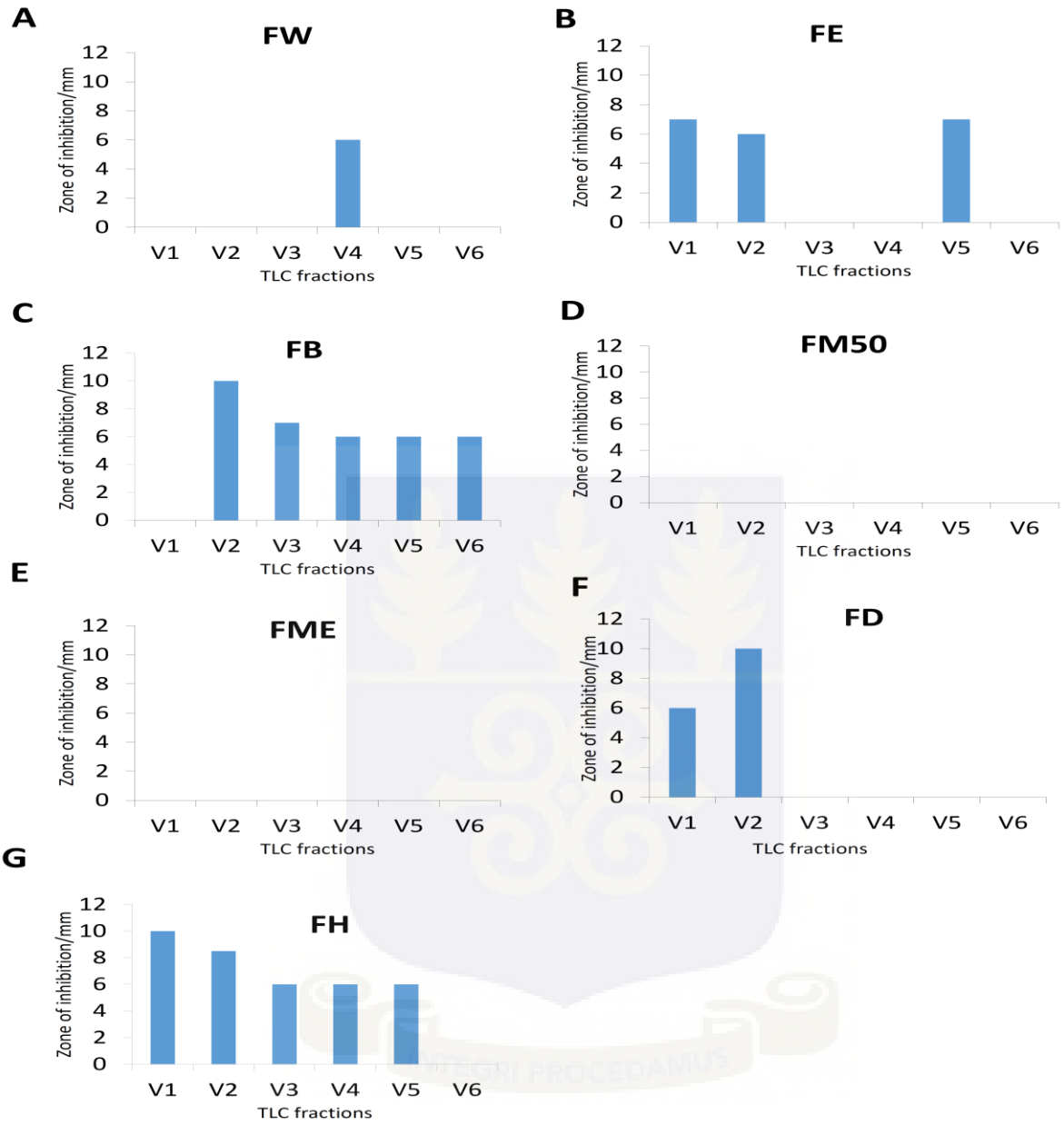


Fig S4.7. Bioassay of TLC fractions (Broth A) from the 6 weeks incubation period against *S. cerevisiae*. The 7 Kupchan fractions (FW, FE, FB, FM50, FME, FD and FH) were separated further by preparative TLC. Fractions from FW, FE, FB, FD and FH fractions showed antifungal activity. The activities of TLC fractions V1-V6 are shown in the figure. *Meyerozyma guilliermondii* (MEF 134) was the producing organisms.

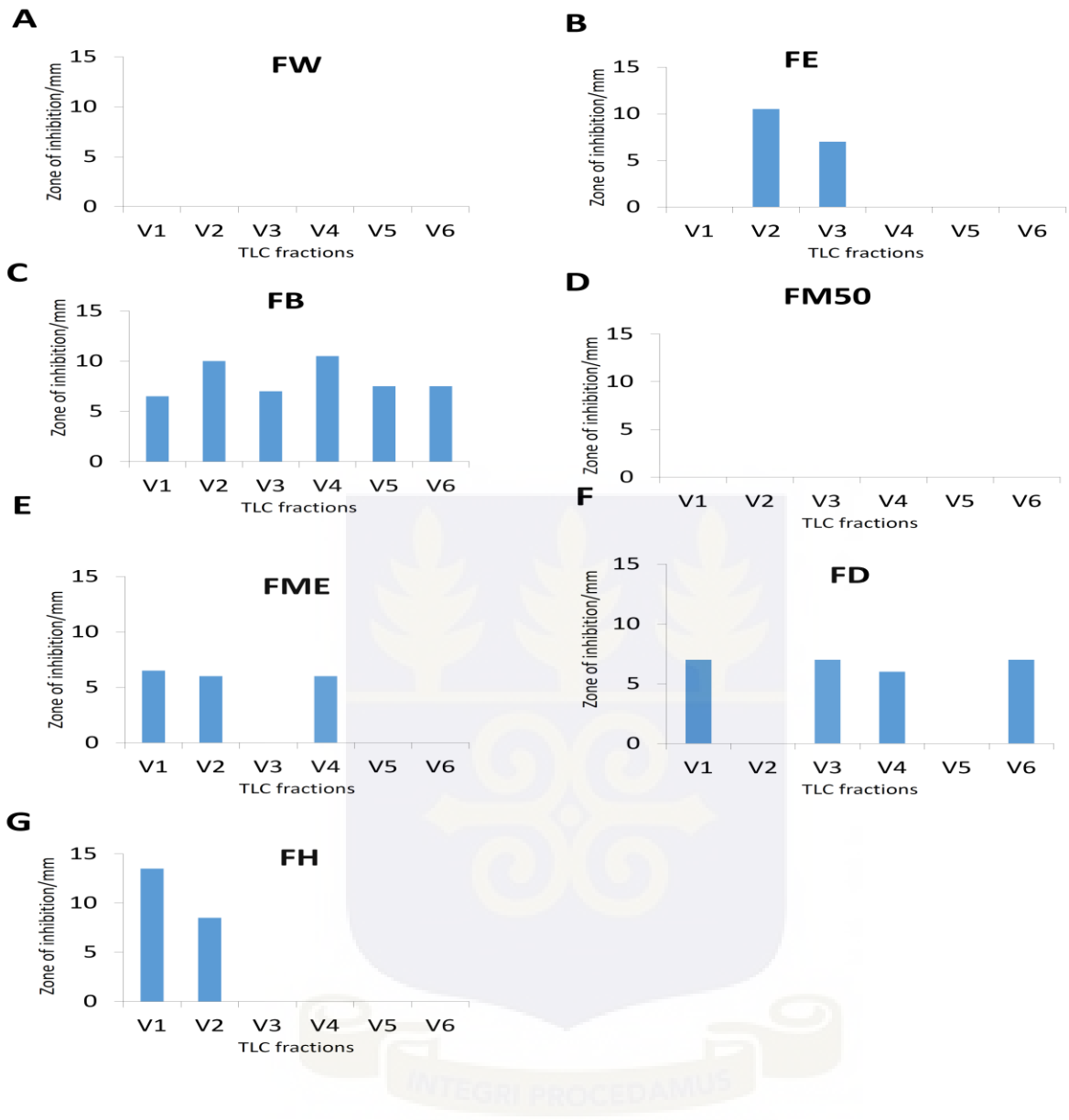


Fig S 4.8. Bioassay of TLC fractions (Broth B) from the 6 weeks incubation period against *S. cerevisiae*. The 7 Kupchan fractions (FW, FE, FB, FM50, FME, FD and FH) were separated further by preparative TLC. Fractions from FE, FB, FME, FD and FH fractions showed antifungal activity. The activities of TLC fractions V1-V6 are shown in the figure. *Meyerozyma guilliermondii* (MEF 134) was the producing organisms.

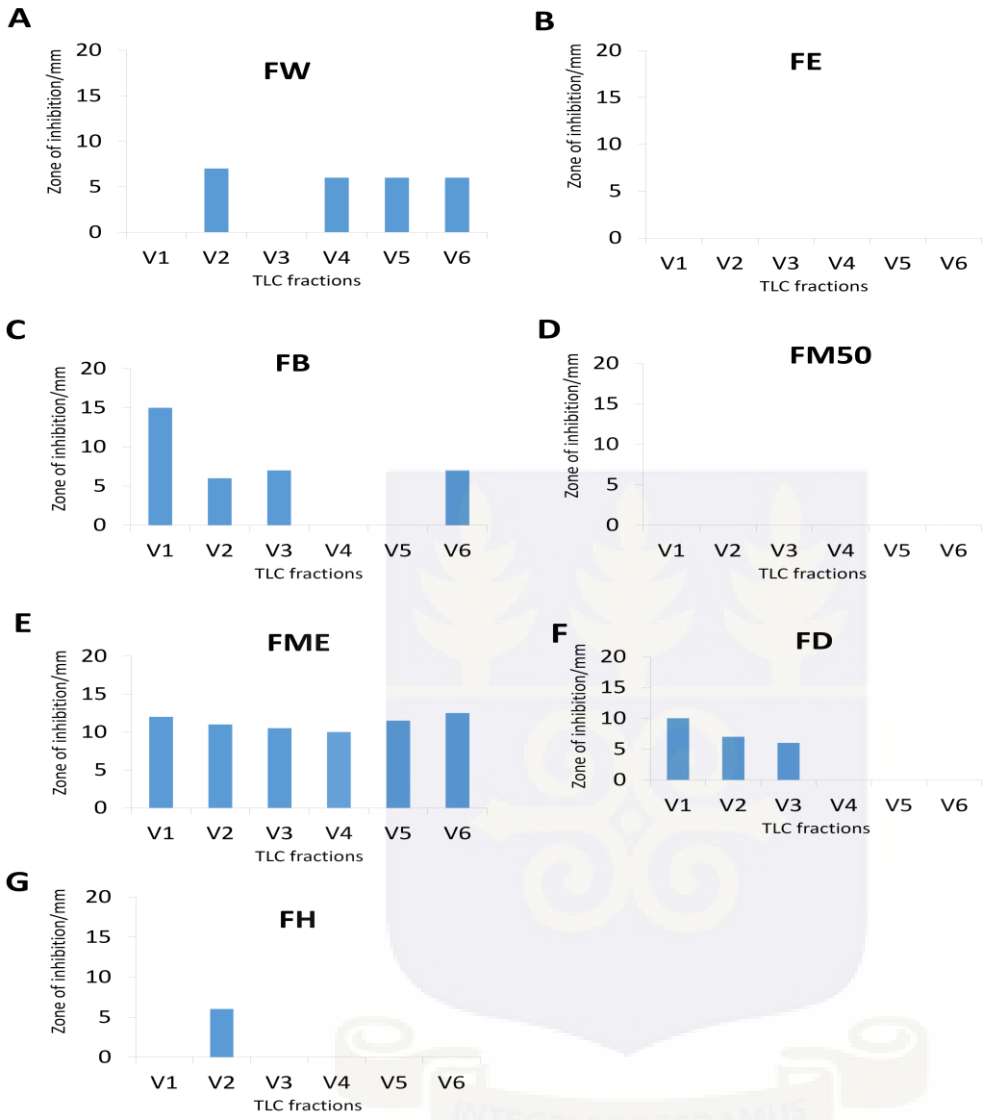


Fig S 4.9. Bioassay of TLC fractions (Broth A) from the 2 months incubation period against *S. cerevisiae.* The 7 Kupchan fractions (FW, FE, FB, FM50, FME, FD and FH) were separated further by preparative TLC. Fractions from FW, FB, FME, FD and FH fractions showed antifungal activity. The activities of TLC fractions V1-V6 are shown in the figure. *Meyerozyma guilliermondii* (MEF 134) was the producing organisms.

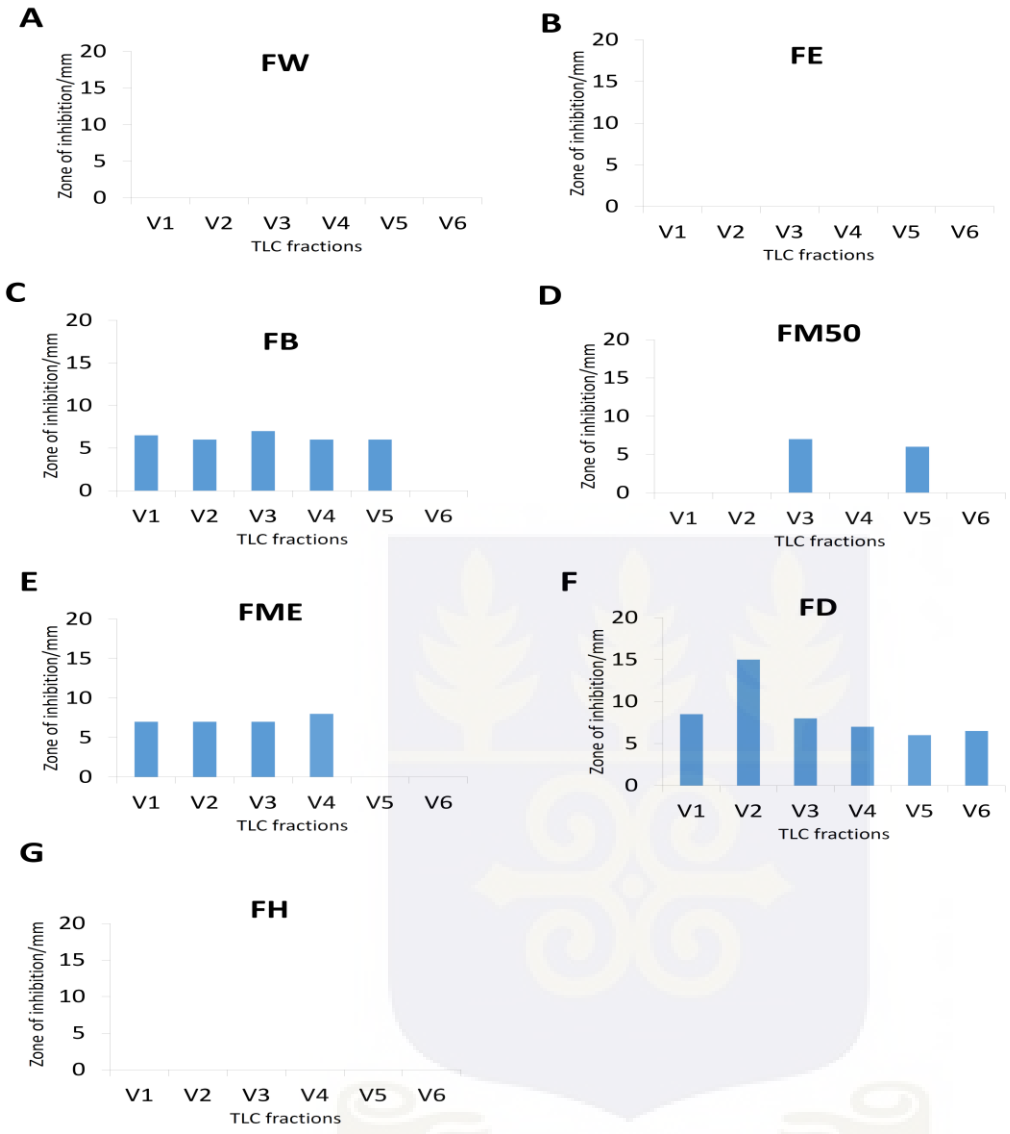


Fig S 4.10. Bioassay of TLC fractions (Broth B) from the 2 months incubation period against *S. cerevisiae*. The 7 Kupchan fractions (FW, FE, FB, FM50, FME, FD and FH) were separated further by preparative TLC. Fractions from FB, FME50 and FD fractions showed antifungal activity. The activities of TLC fractions V1-V6 are shown in the figure. *Meyerozyma guilliermondii* (MEF 134) was the producing organisms.

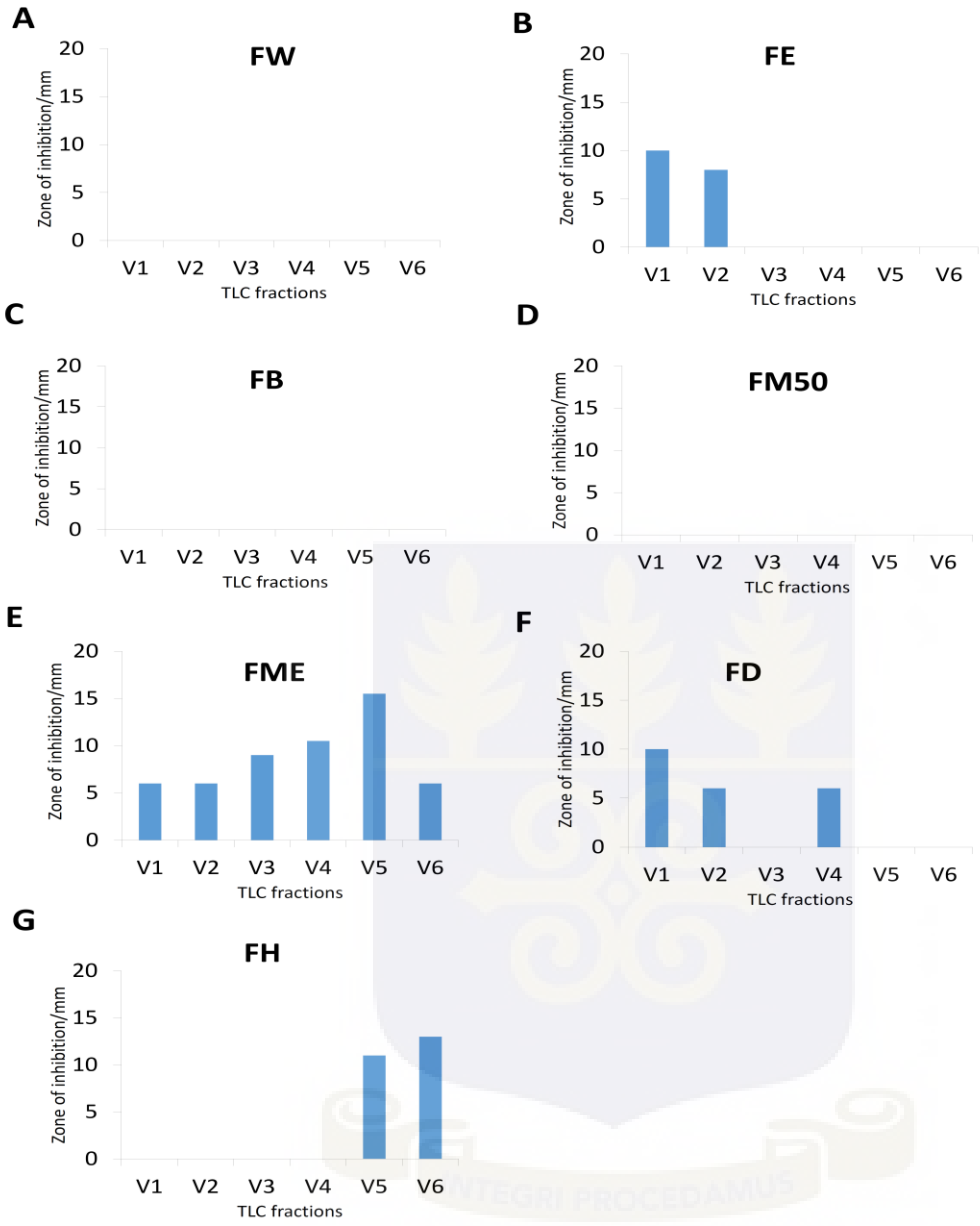


Fig S 4.11. Bioassay of TLC fractions (Broth A) from the 3 months incubation period against *S. cerevisiae*. The 7 Kupchan fractions (FW, FE, FB, FM50, FME, FD and FH) were separated further by preparative TLC. Fractions from FE, FME, FD and FH fractions showed antifungal activity. The activities of TLC fractions V1-V6 are shown in the figure. *Meyerozyma guilliermondii* (MEF 134) was the producing organisms.

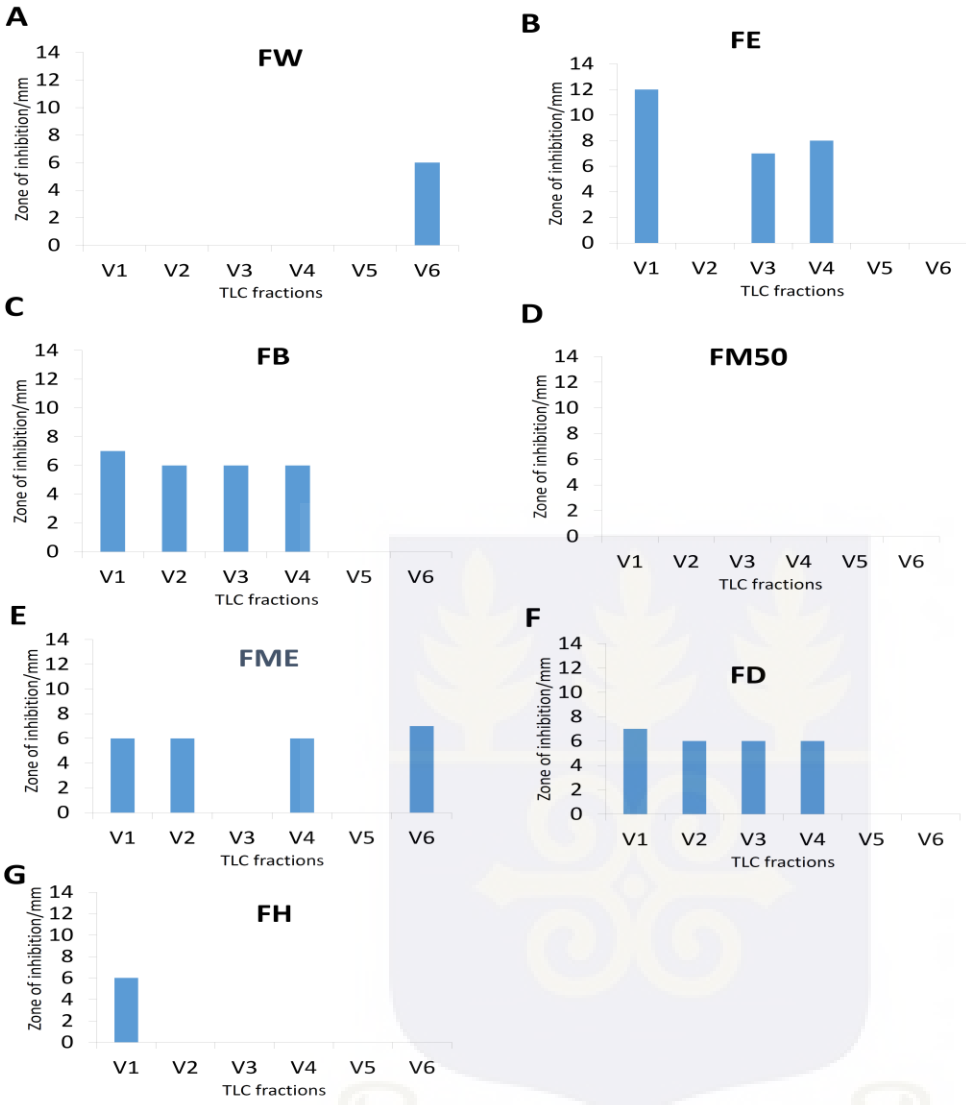


Fig S 4.12. Bioassay of TLC fractions (Broth B) from the 3 months incubation period against *S. cerevisiae*. The 7 Kupchan fractions (FW, FE, FB, FM50, FME, FD and FH) were separated further by preparative TLC. Fractions from FW, FE, FB, FME, FD and FH fractions showed antifungal activity. The activities of TLC fractions V1-V6 are shown in the figure. *Meyerozyma guilliermondii* (MEF 134) was the producing organisms.

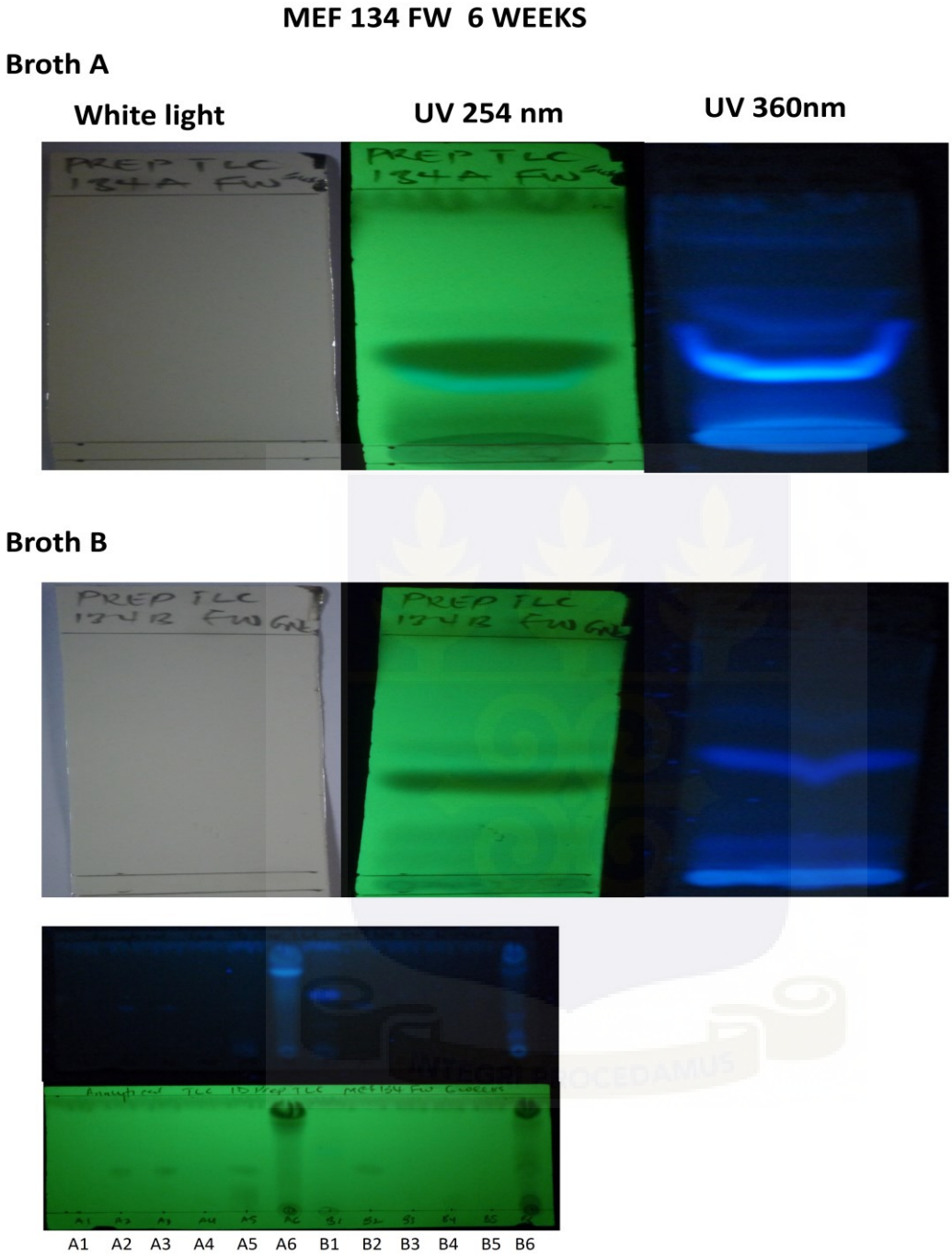


Fig S4.13. Preparative and analytical TLC plates of FW fractions obtained from MEF 134 grown at 6 weeks. The solvent system used as mobile was acetonitrile: ethyl acetate: petroleum ether (2:7:1).

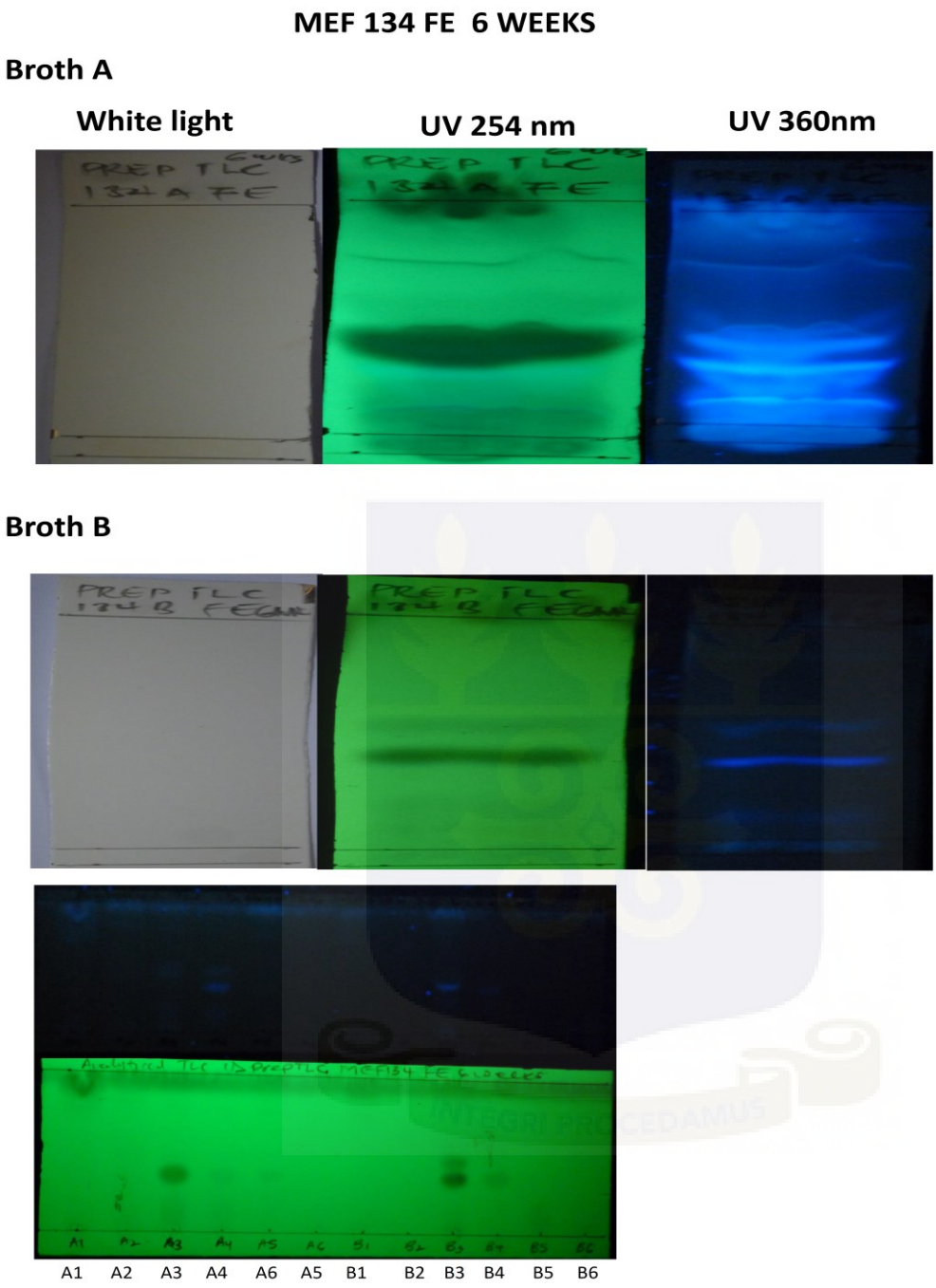


Fig S4.14. Preparative and analytical TLC plates of FE fractions obtained from MEF 134 grown at 6 weeks. The solvent system used as mobile was acetonitrile: ethyl acetate: petroleum ether (2:7:1).

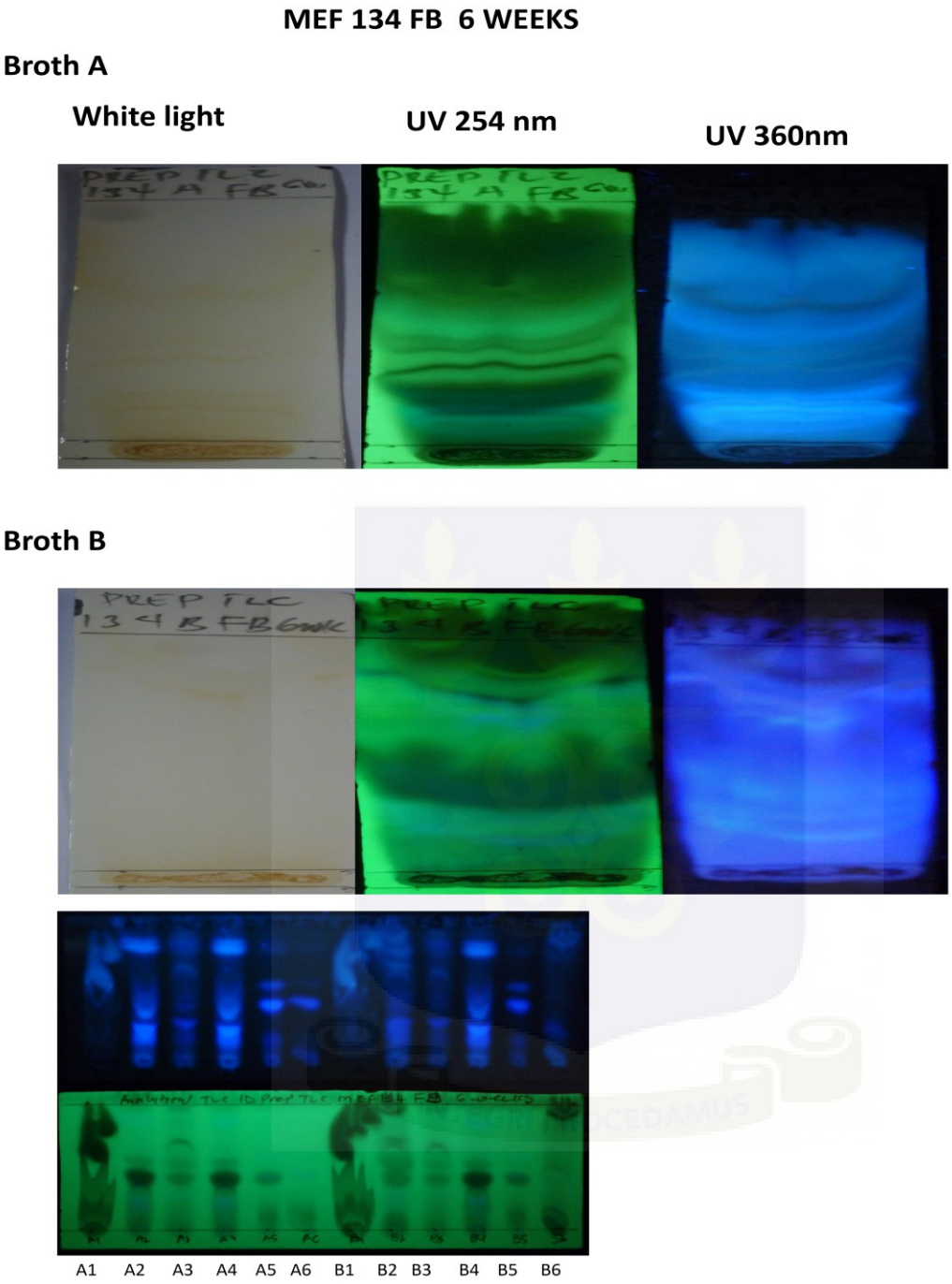


Fig S4.15. Preparative and analytical TLC plates of FB fractions obtained from MEF 134 grown at 6 weeks. The solvent system used as mobile was acetonitrile: ethyl acetate: petroleum ether (2:7:1).

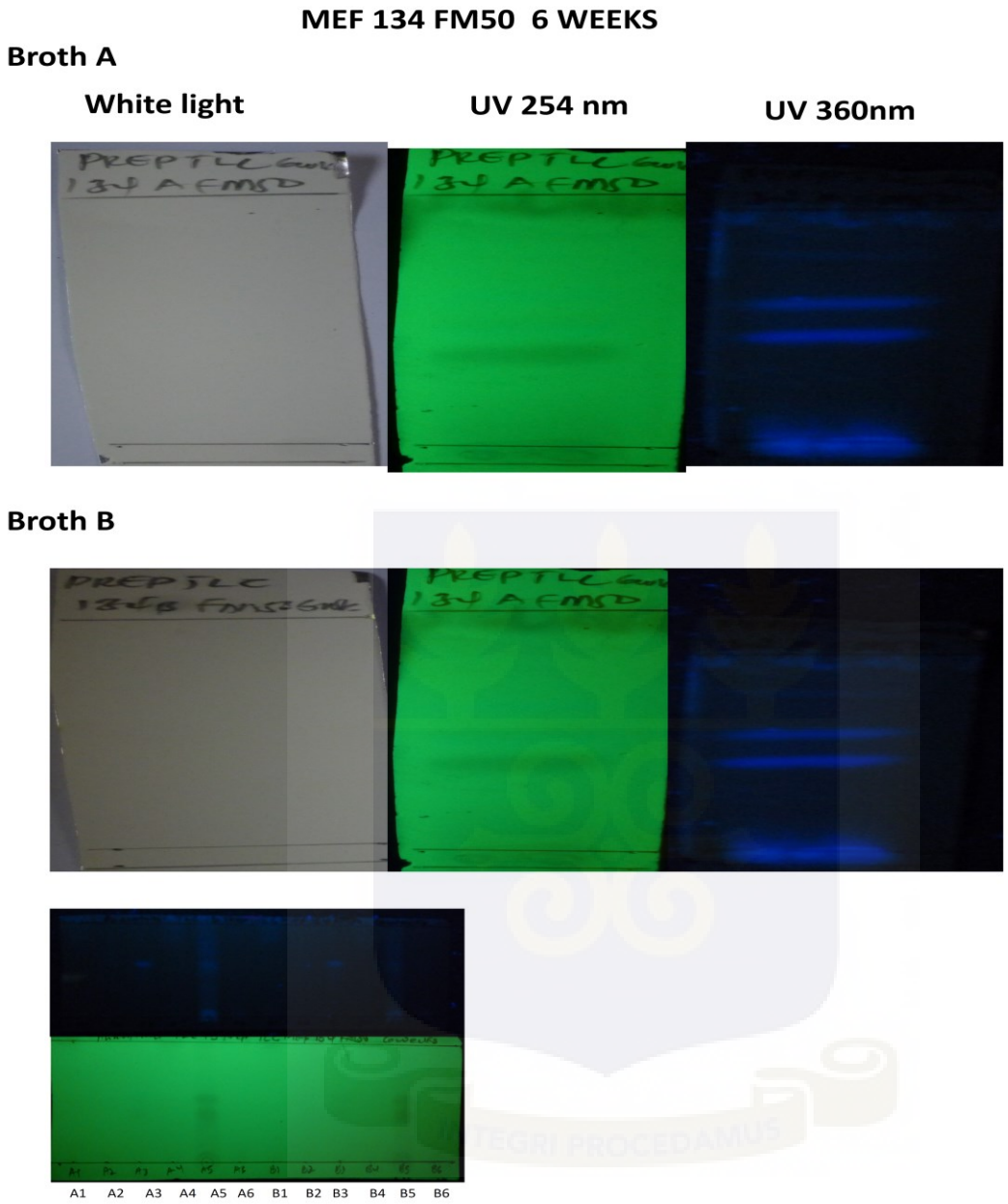


Fig S4.16. Preparative and analytical TLC plates of FM50 fractions obtained from MEF 134 grown at 6 weeks. The solvent system used as mobile was acetonitrile: ethyl acetate: petroleum ether (2:7:1).

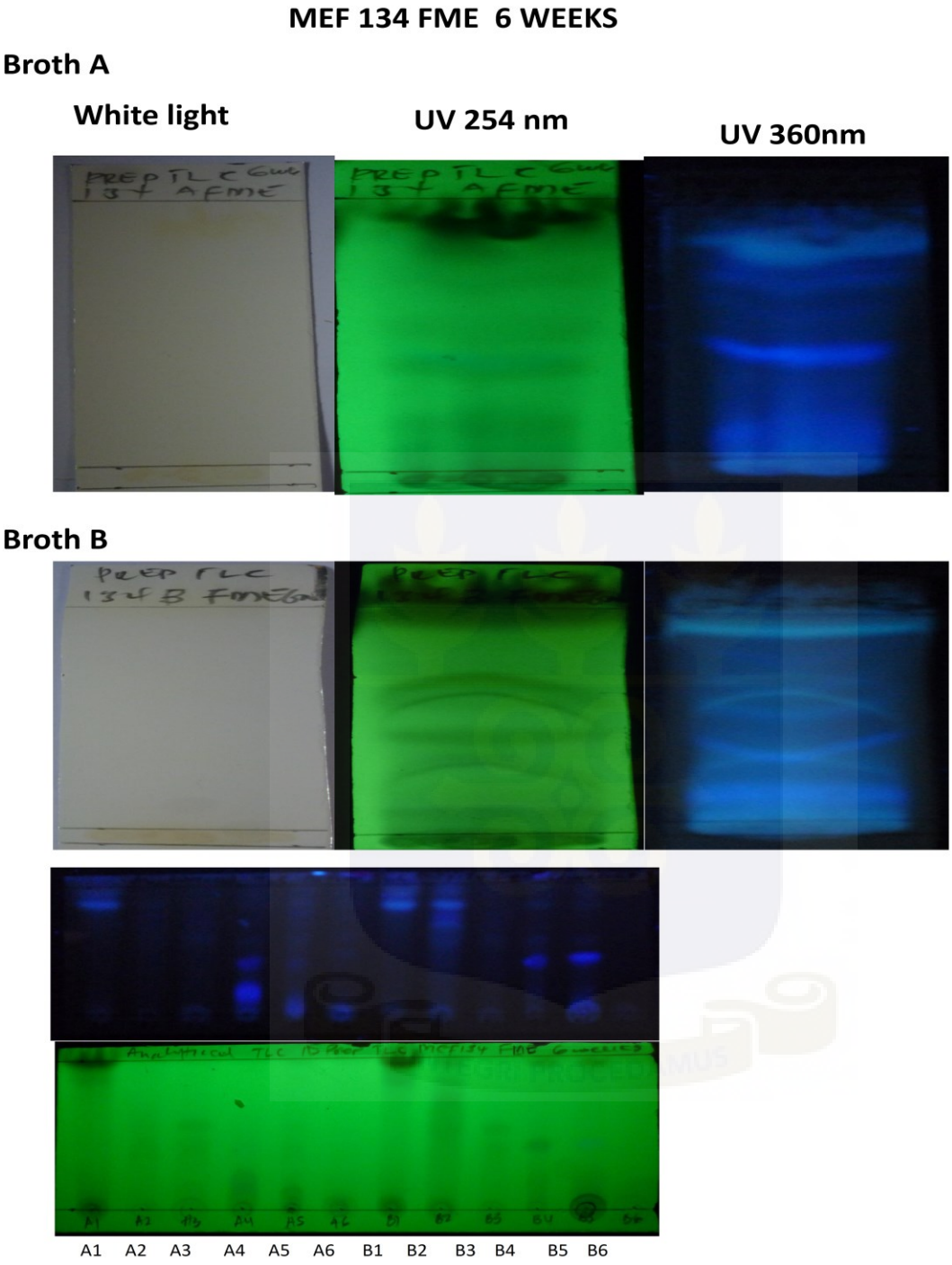


Fig S4.17. Preparative and analytical TLC plates of FME fractions obtained from MEF 134 grown at 6 weeks. The solvent system used as mobile was acetonitrile: ethyl acetate: petroleum ether (2:7:1).

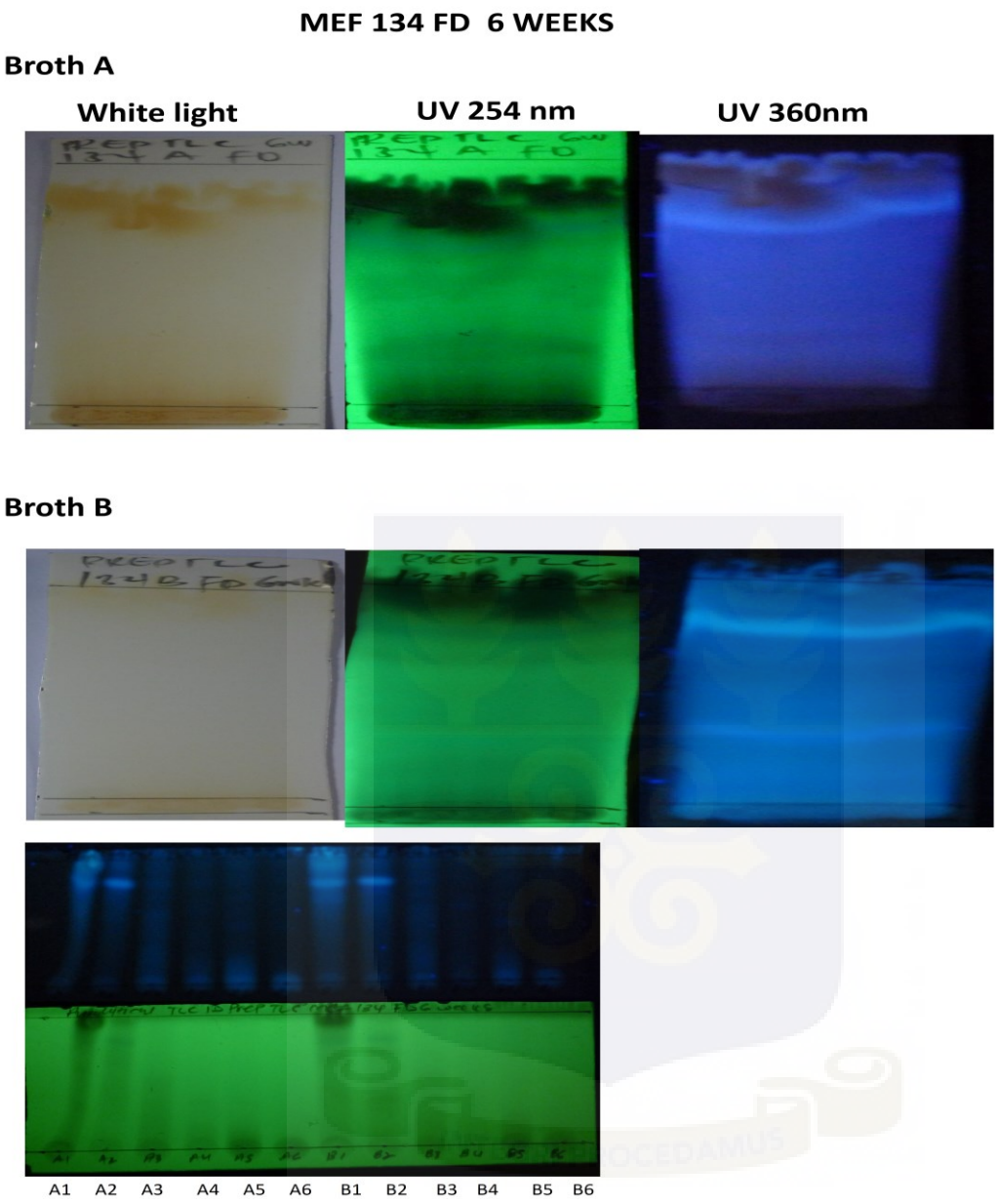


Fig S4.18. Preparative and analytical TLC plates of FD fractions obtained from MEF 134 grown at 6 weeks. The solvent system used as mobile was acetonitrile: ethyl acetate: petroleum ether (2:7:1).

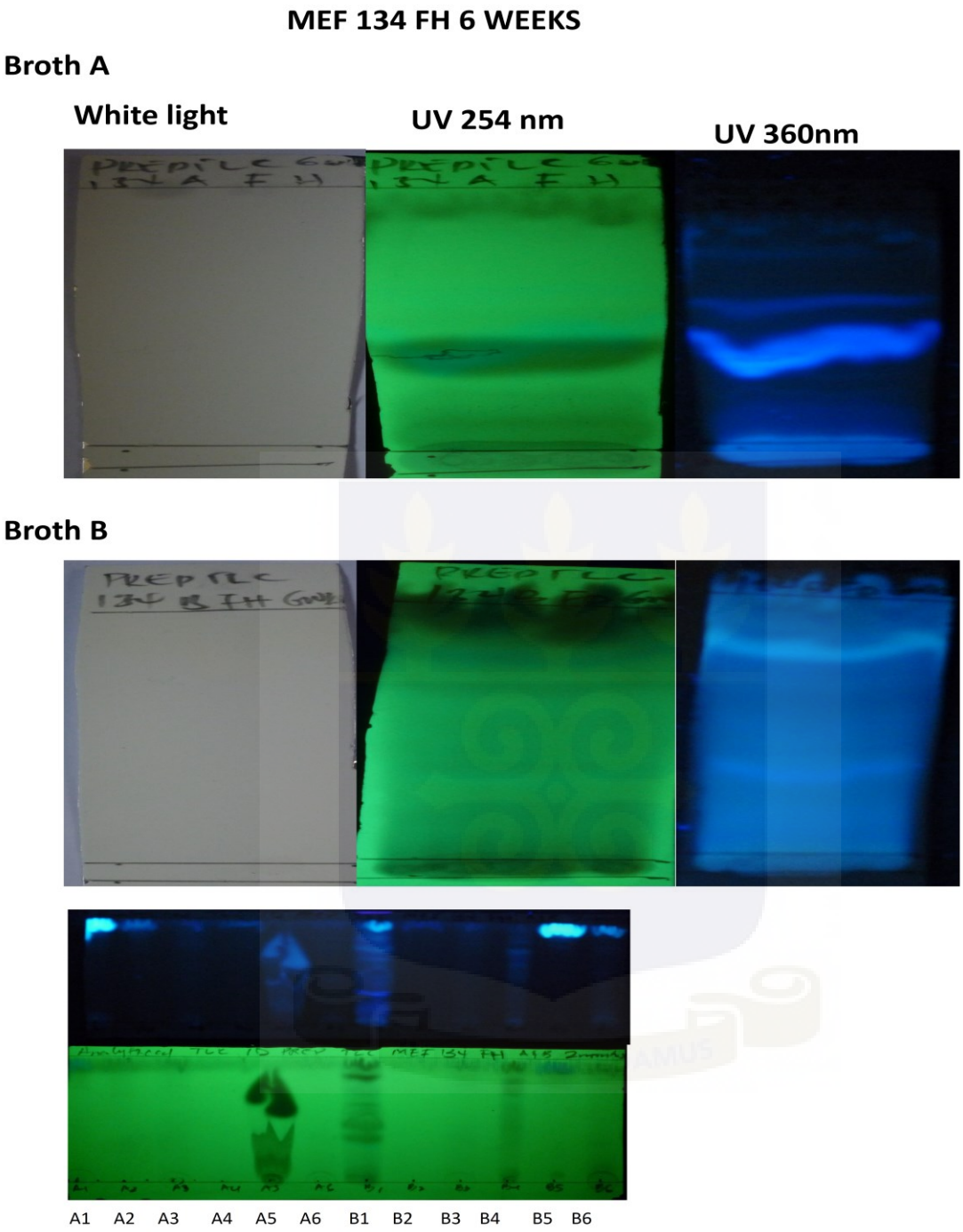


Fig S4.19. Preparative and analytical TLC plates of FH fractions obtained from MEF 134 grown at 6 weeks. The solvent system used as mobile was acetonitrile: ethyl acetate: petroleum ether (2:7:1).

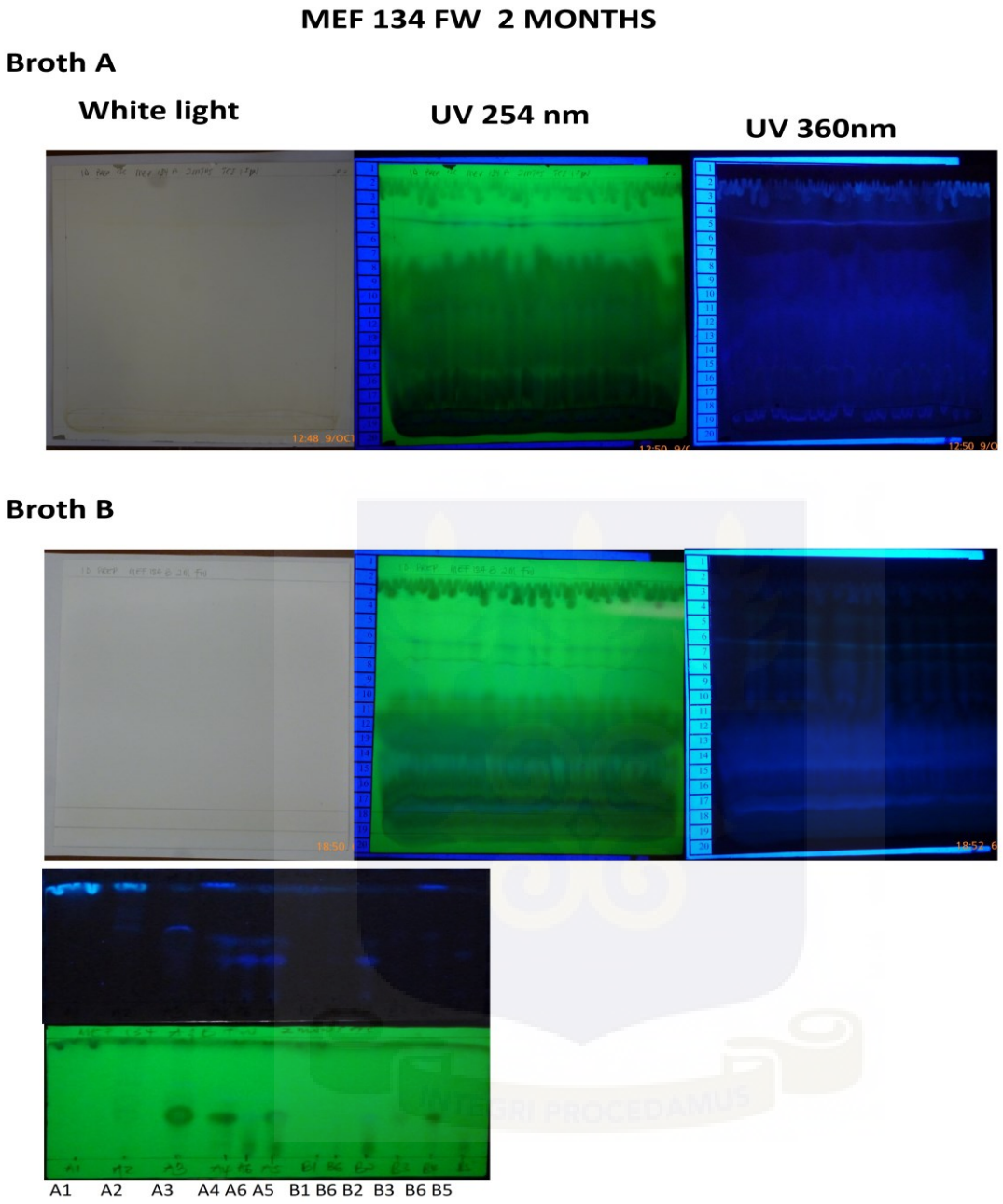


Fig S4.20. Preparative and analytical TLC plates of FW fractions obtained from MEF 134 grown for 2 months. The solvent system used as mobile was acetonitrile: ethyl acetate: petroleum ether (2:7:1).

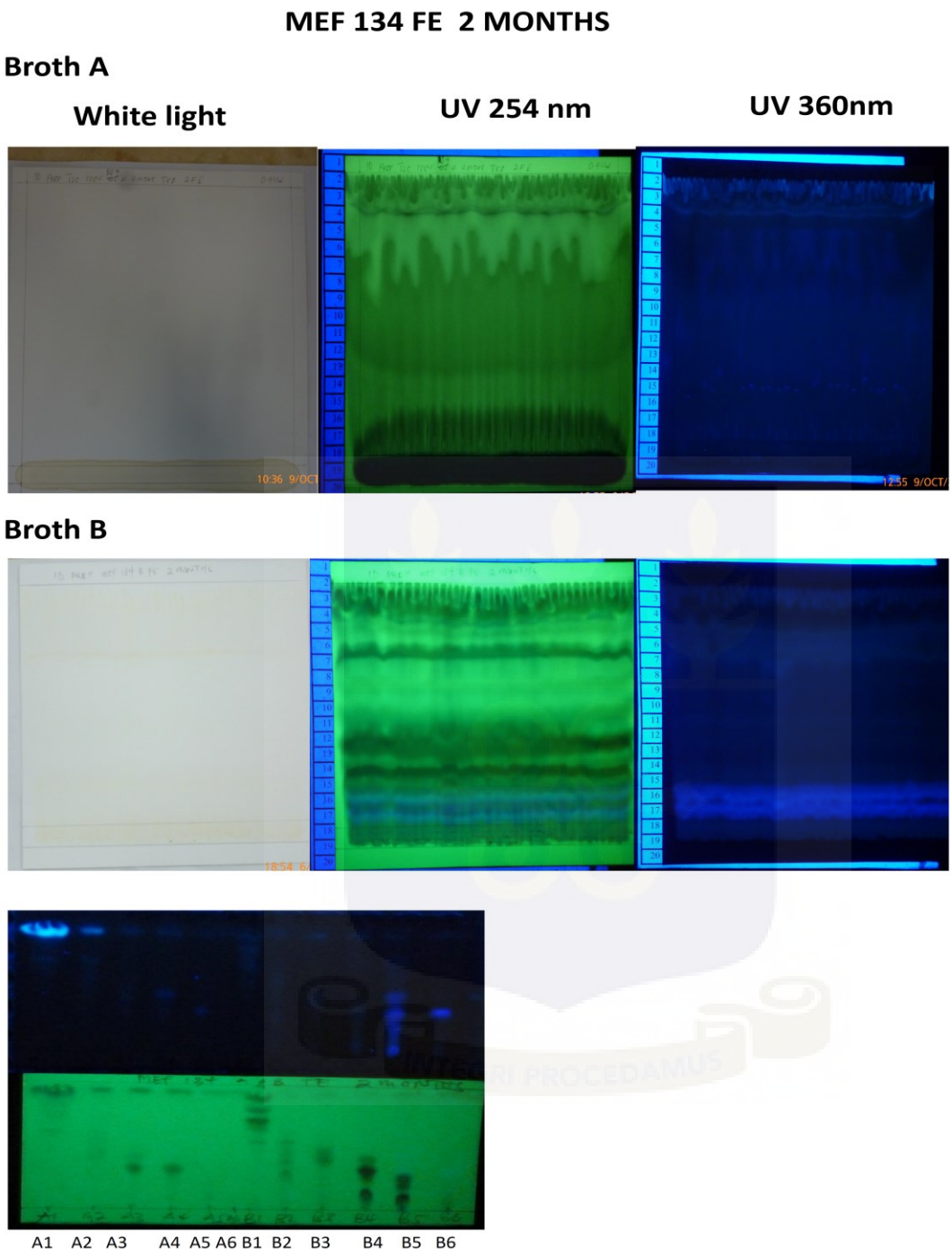


Fig S 4.21. Preparative and analytical TLC plates of FE fractions obtained from MEF 134 grown for 2 months. The solvent system used as mobile was acetonitrile: ethyl acetate: petroleum ether (2:7:1).

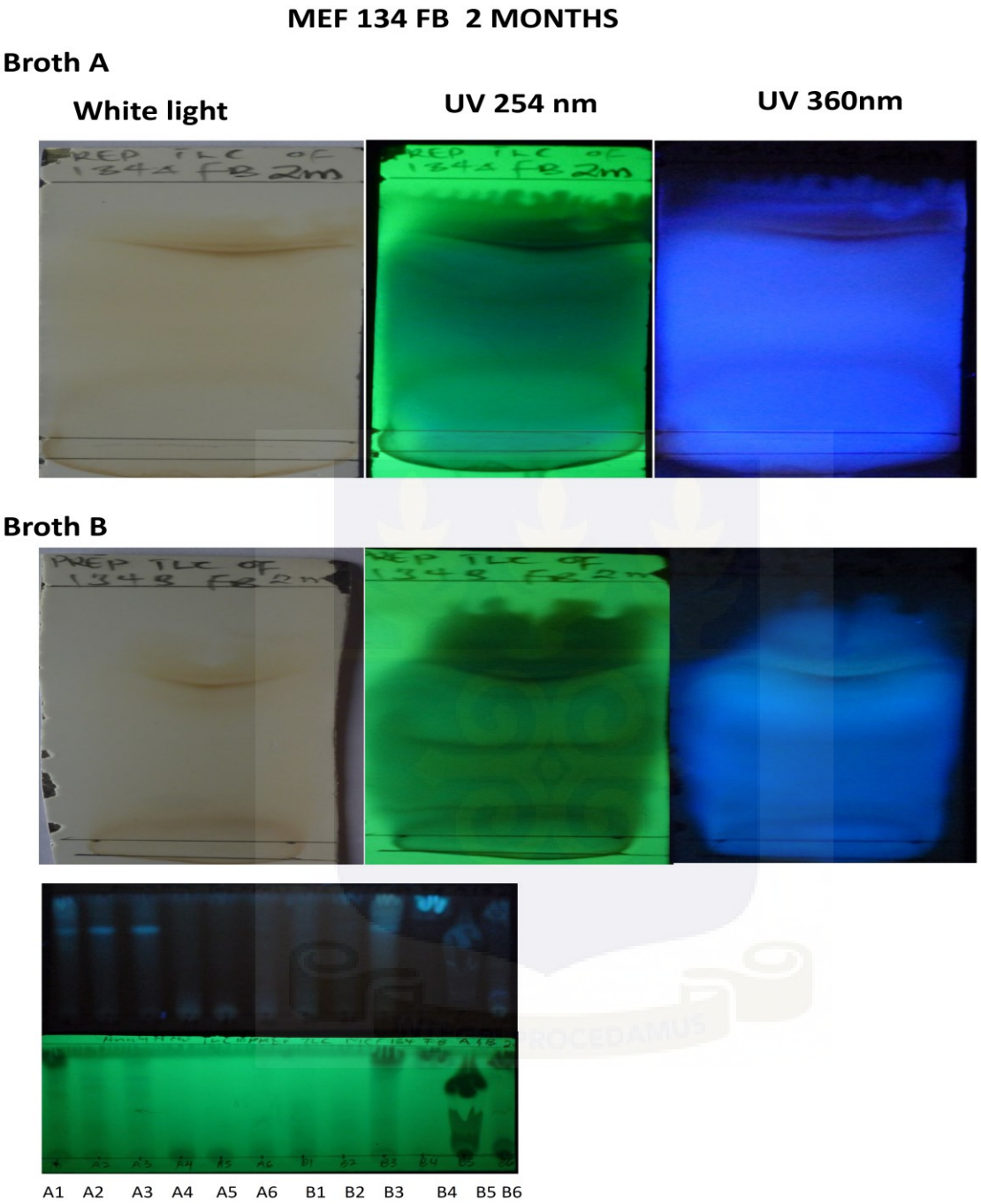


Fig S 4. 22. Preparative and analytical TLC plates of FB fractions obtained from MEF 134 grown for 2 months. The solvent system used as mobile was acetonitrile: ethyl acetate: petroleum ether (2:7:1).

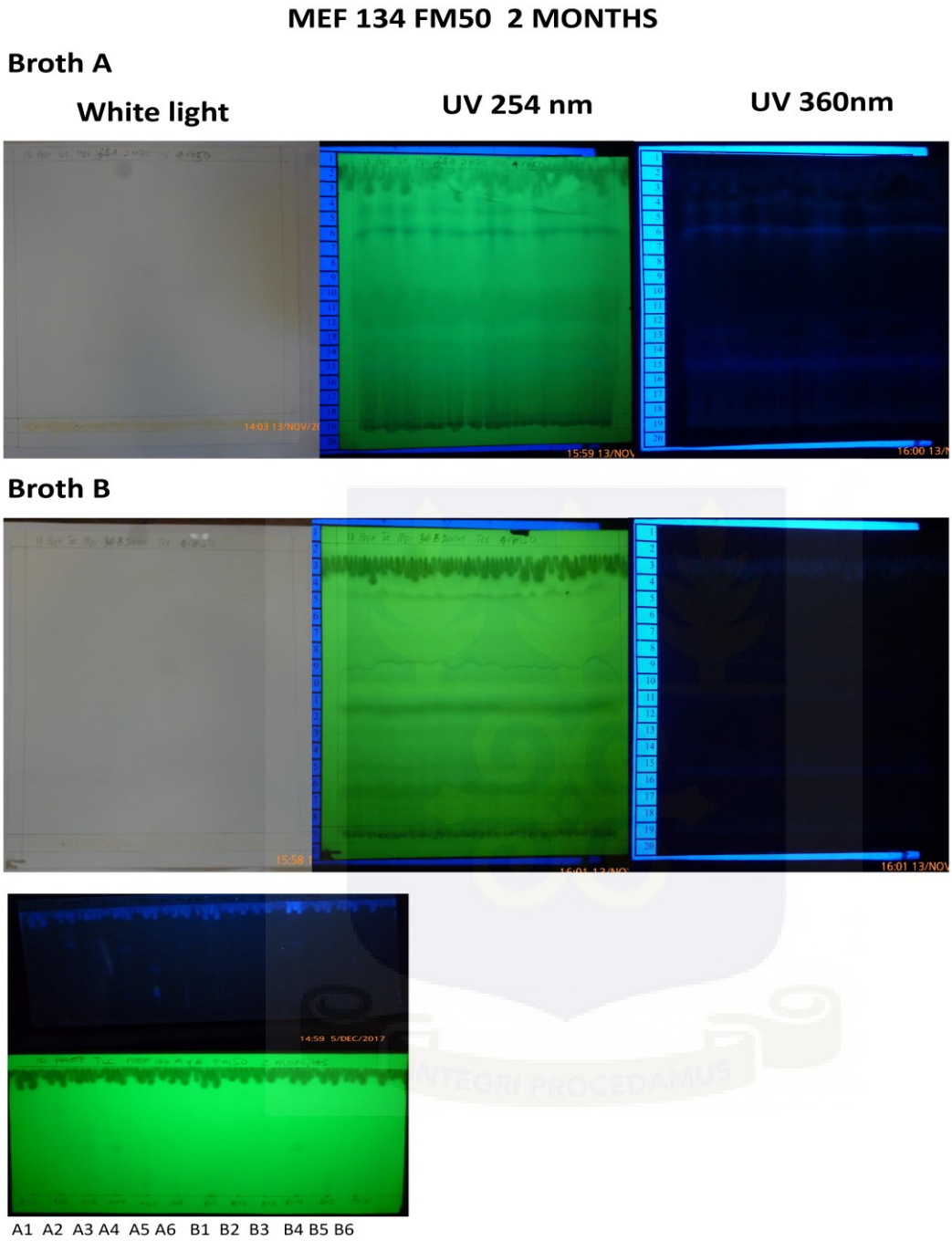


Fig S4. 23. Preparative and analytical TLC plates of FM50 fractions obtained from MEF 134 grown for 2 months. The solvent system used as mobile was acetonitrile: ethyl acetate: petroleum ether (2:7:1).

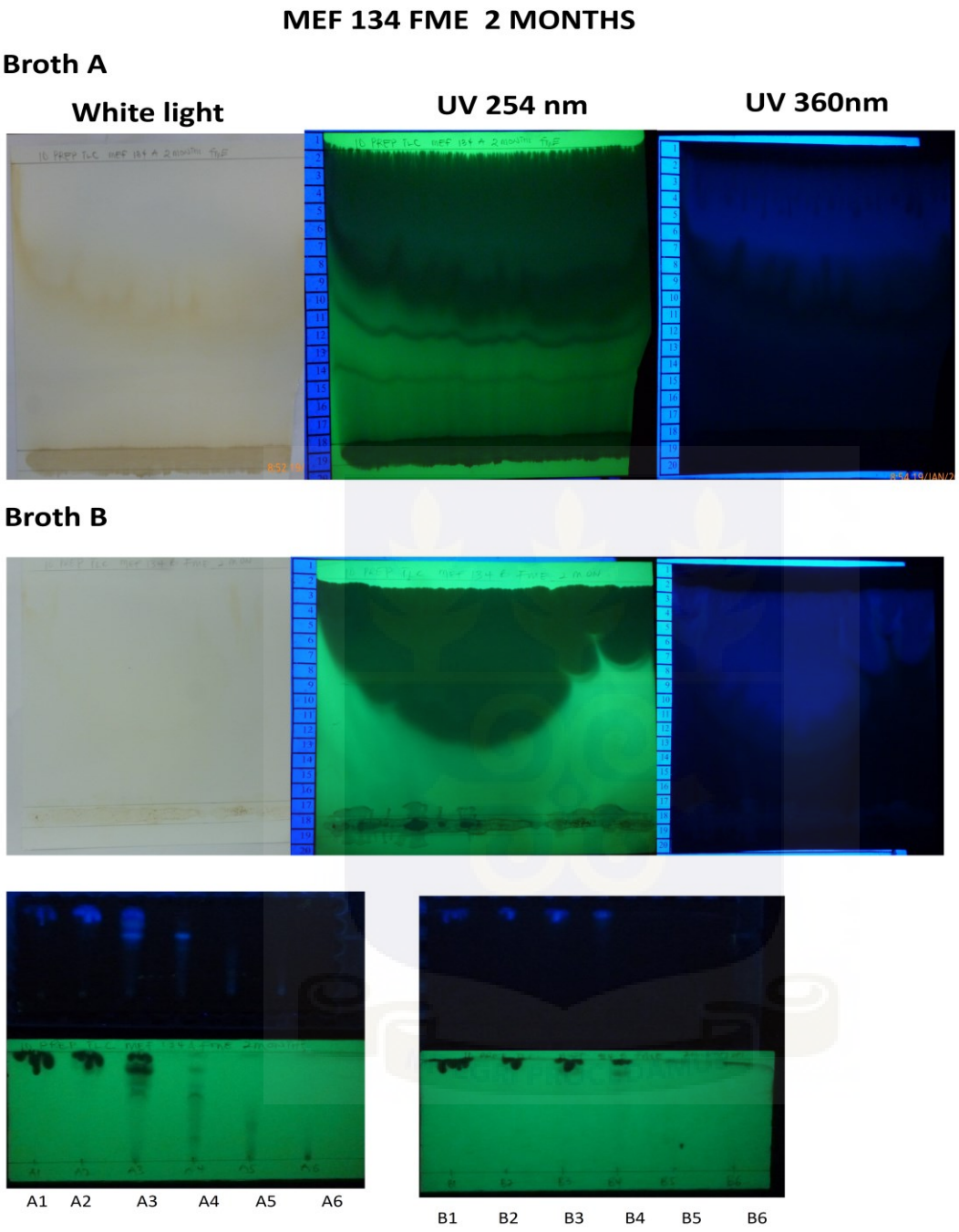


Fig S4.24. Preparative and analytical TLC plates of FME fractions obtained from MEF 134 grown for 2 months. The solvent system used as mobile was acetonitrile: ethyl acetate: petroleum ether (2:7:1).

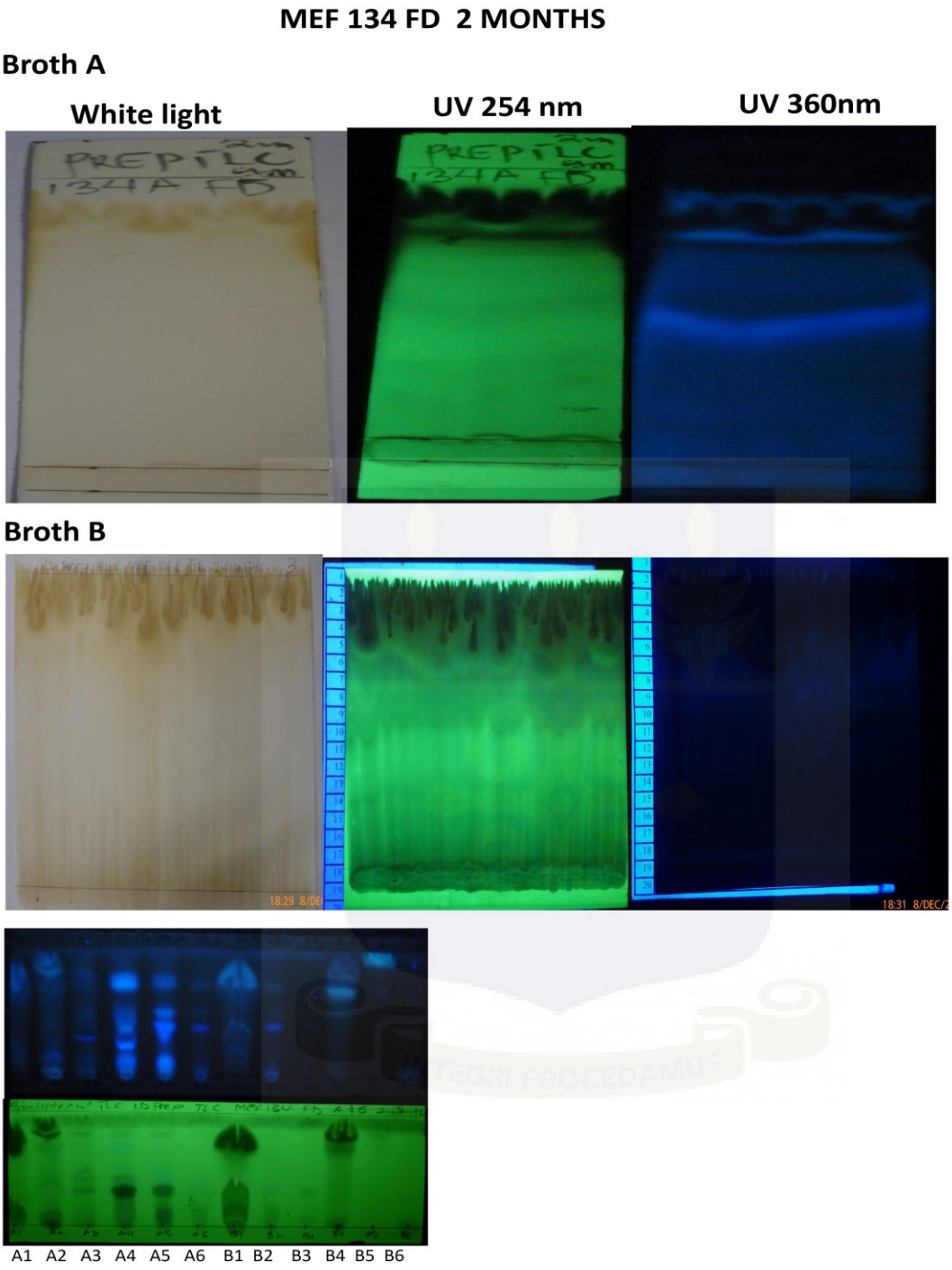


Fig S4.25. Preparative and analytical TLC plates of FD fractions obtained from MEF 134 grown for 2 months. The solvent system used as mobile was acetonitrile: ethyl acetate: petroleum ether (2:7:1).

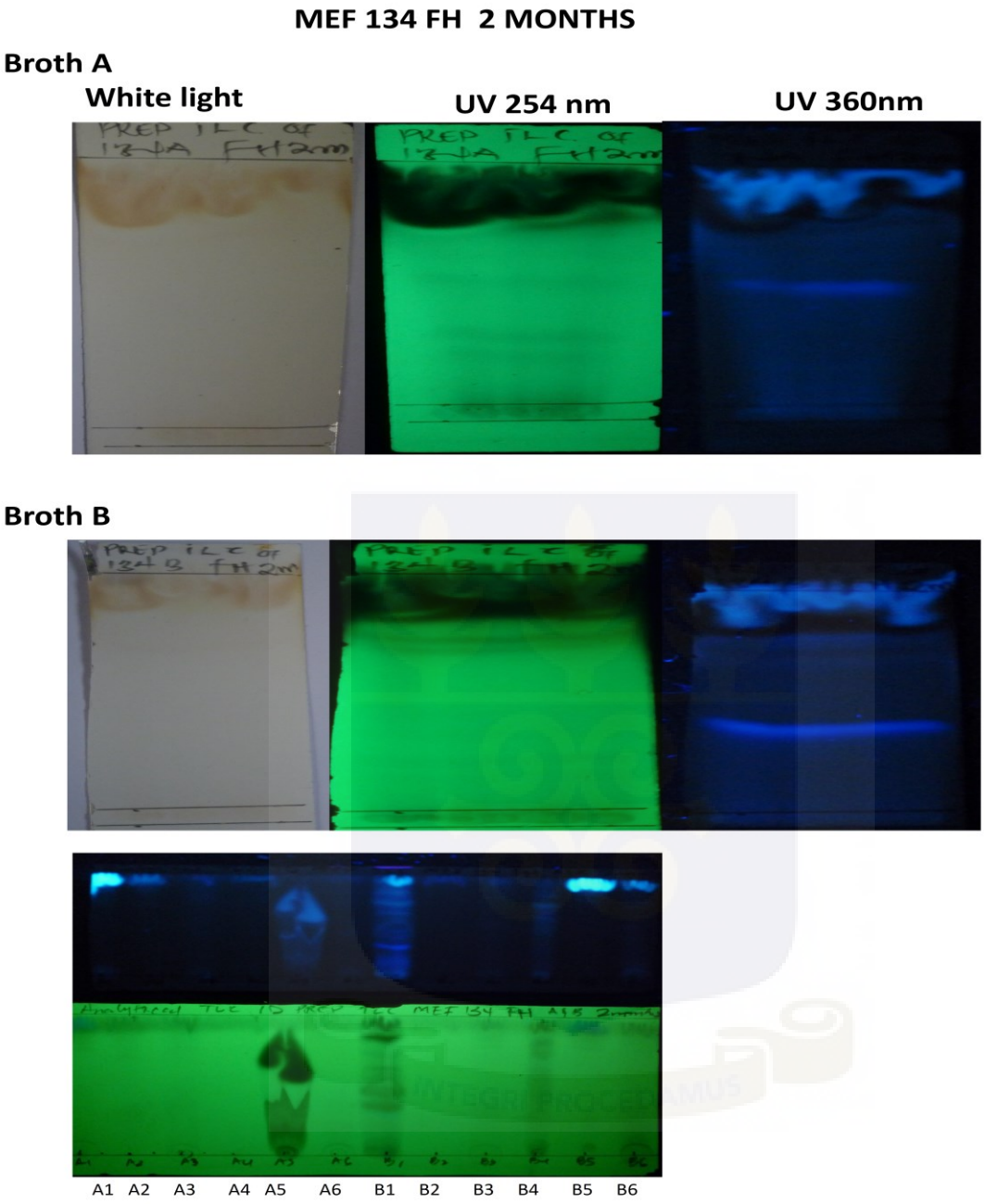


Fig S4.26. Preparative and analytical TLC plates of FH fractions obtained from MEF 134 grown for 2 months. The solvent system used as mobile was acetonitrile: ethyl acetate: petroleum ether (2:7:1).

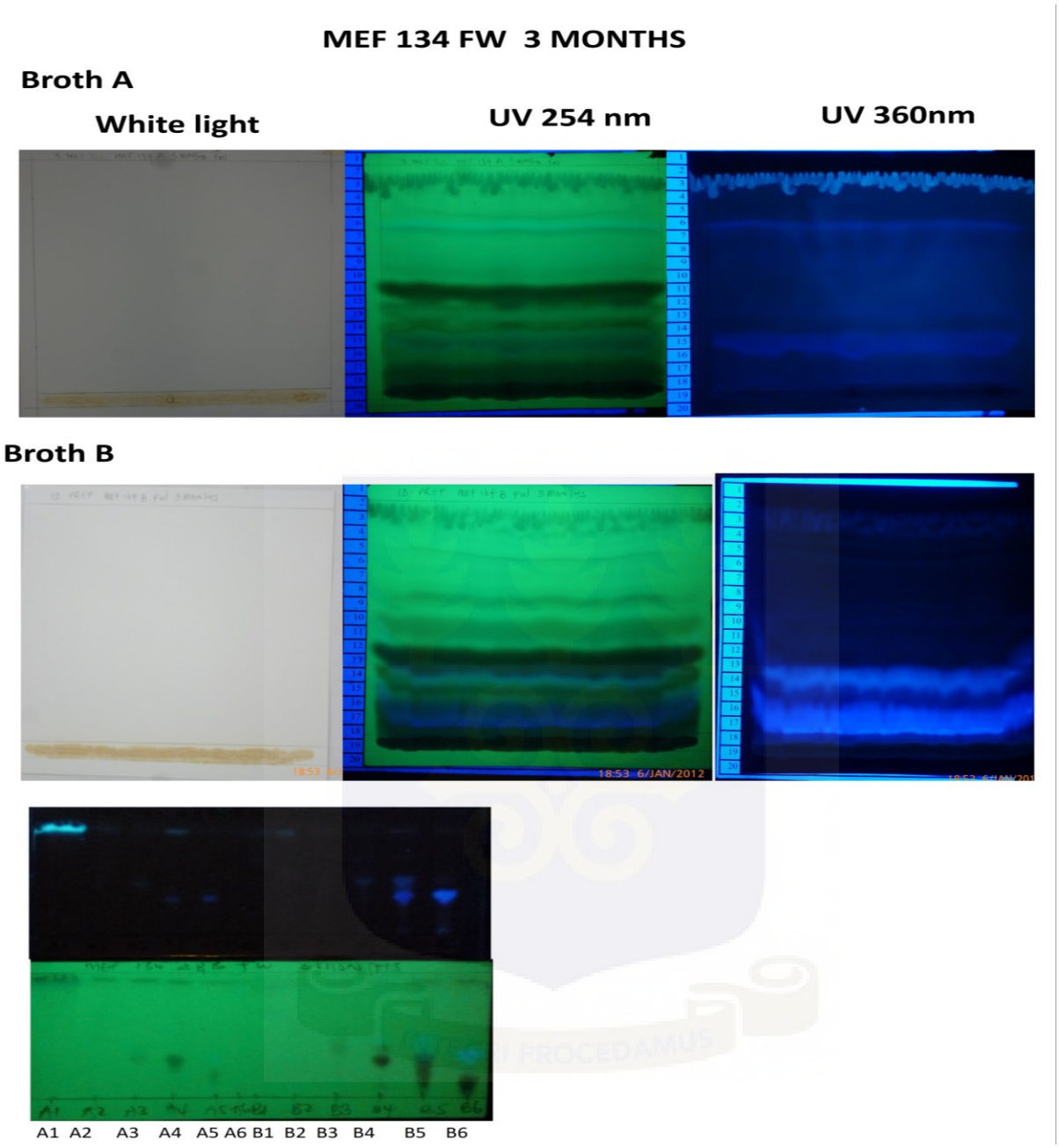


Fig S4.26. Preparative and analytical TLC plates of FW fractions obtained from MEF 134 grown for 3 months. The solvent system used as mobile was acetonitrile: ethyl acetate: petroleum ether (2:7:1).

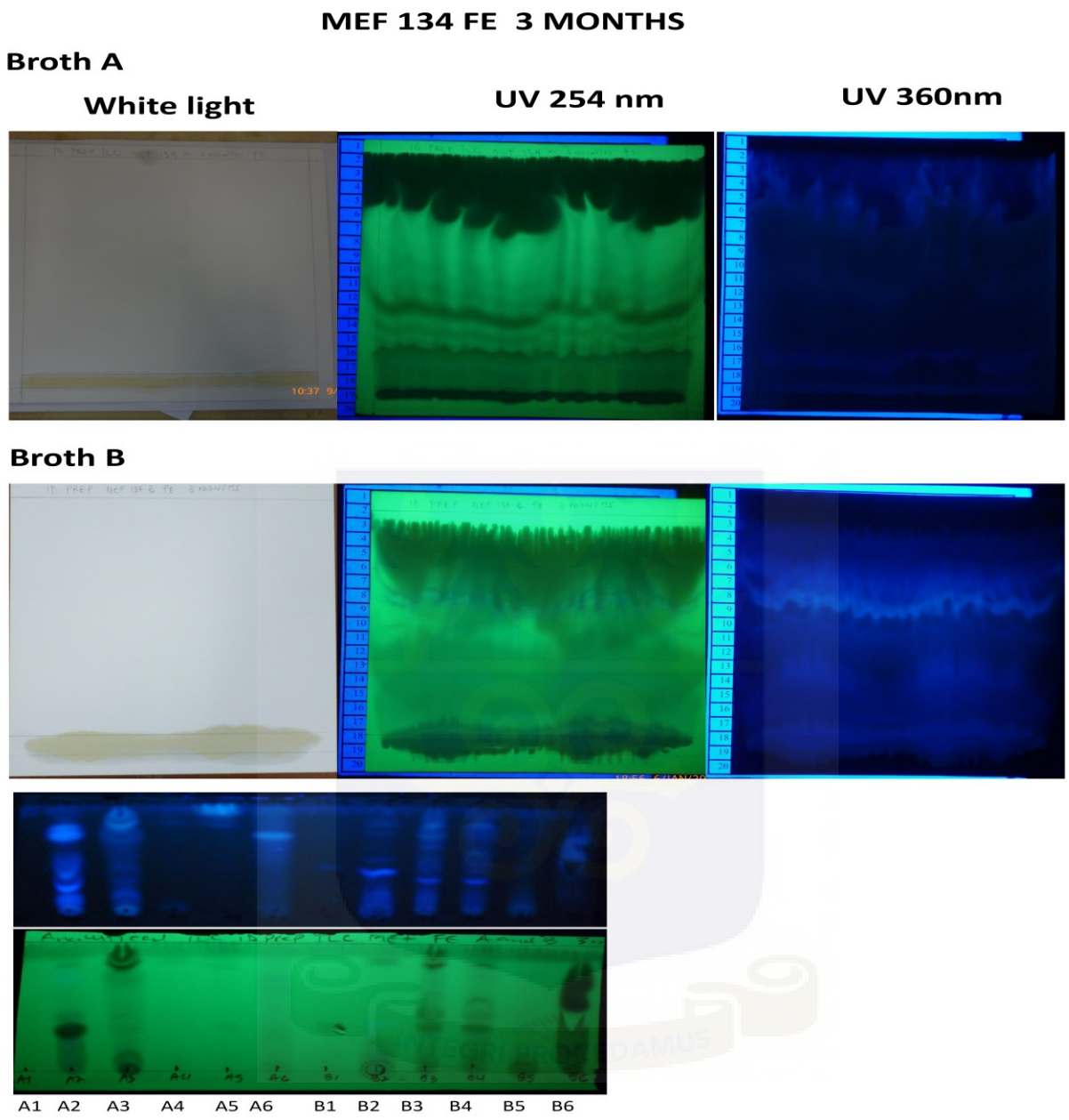


Fig S4.27. Preparative and analytical TLC plates of FE fractions obtained from MEF 134 grown for 3 months. The solvent system used as mobile was acetonitrile: ethyl acetate: petroleum ether (2:7:1).

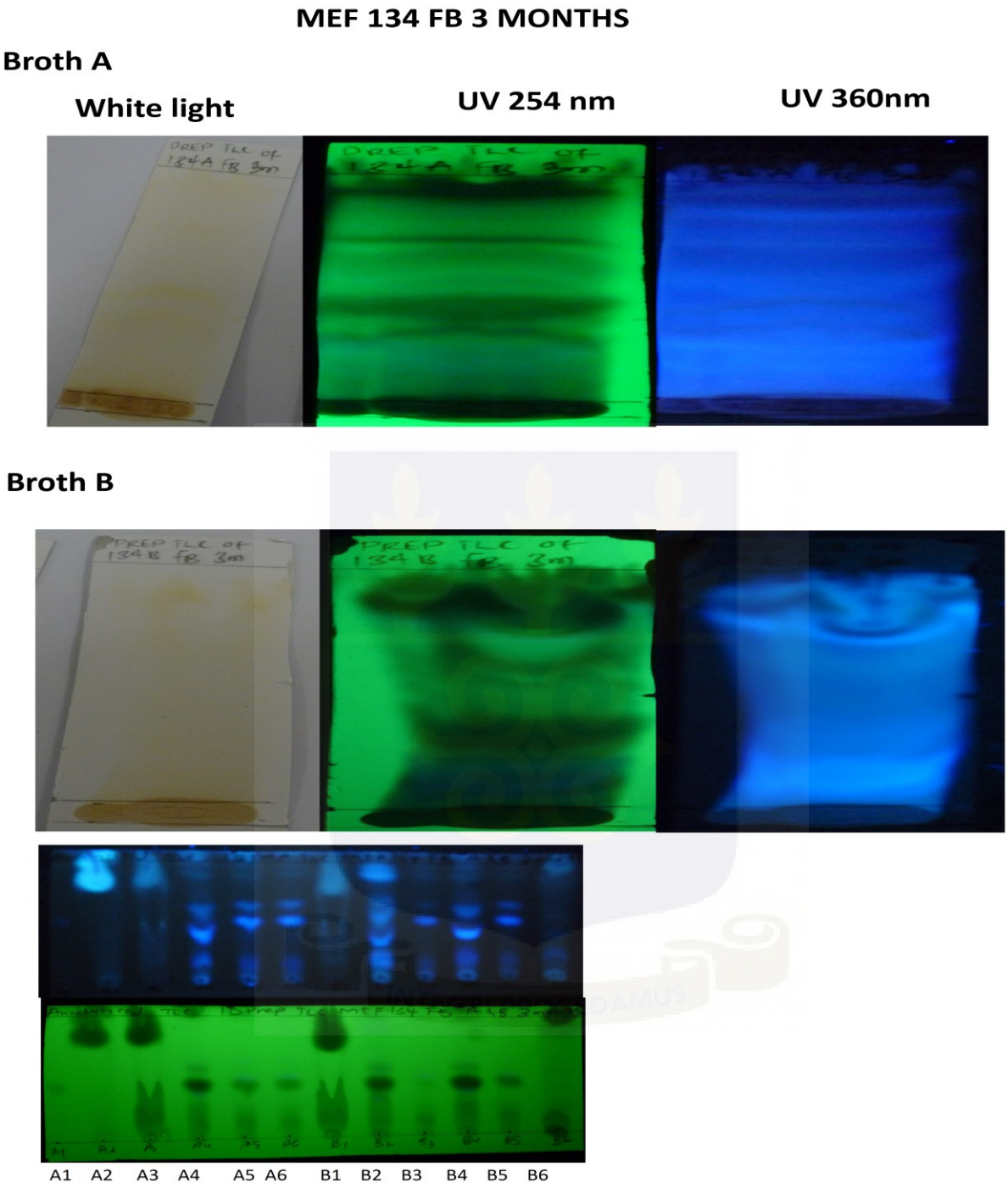


Fig S4.28. Preparative and analytical TLC plates of FB fractions obtained from MEF 134 grown for 3 months. The solvent system used as mobile was acetonitrile: ethyl acetate: petroleum ether (2:7:1).

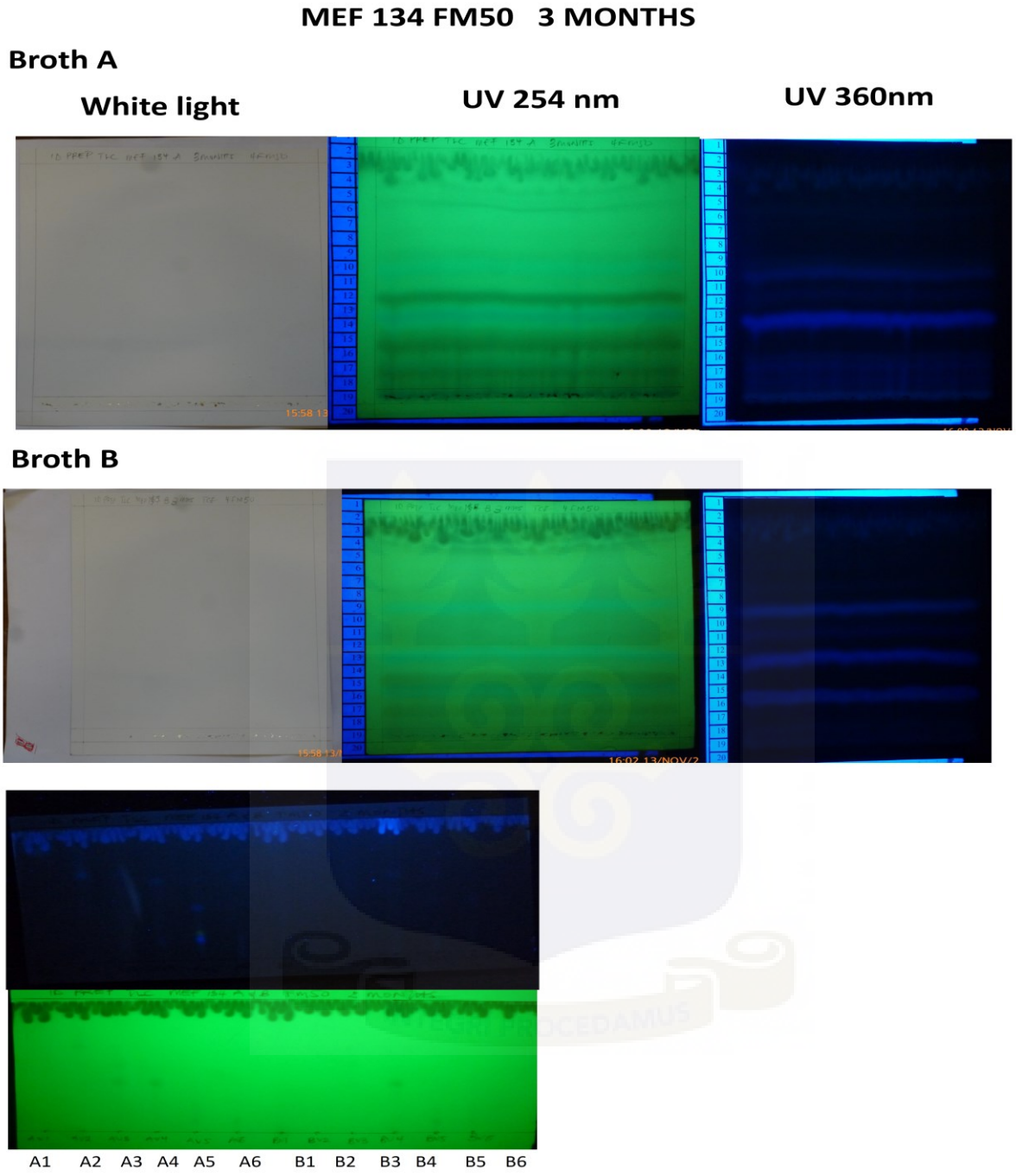


Fig S4.28. Preparative and analytical TLC plates of FM50 fractions obtained from MEF 134 grown for 3 months. The solvent system used as mobile was acetonitrile: ethyl acetate: petroleum ether (2:7:1).

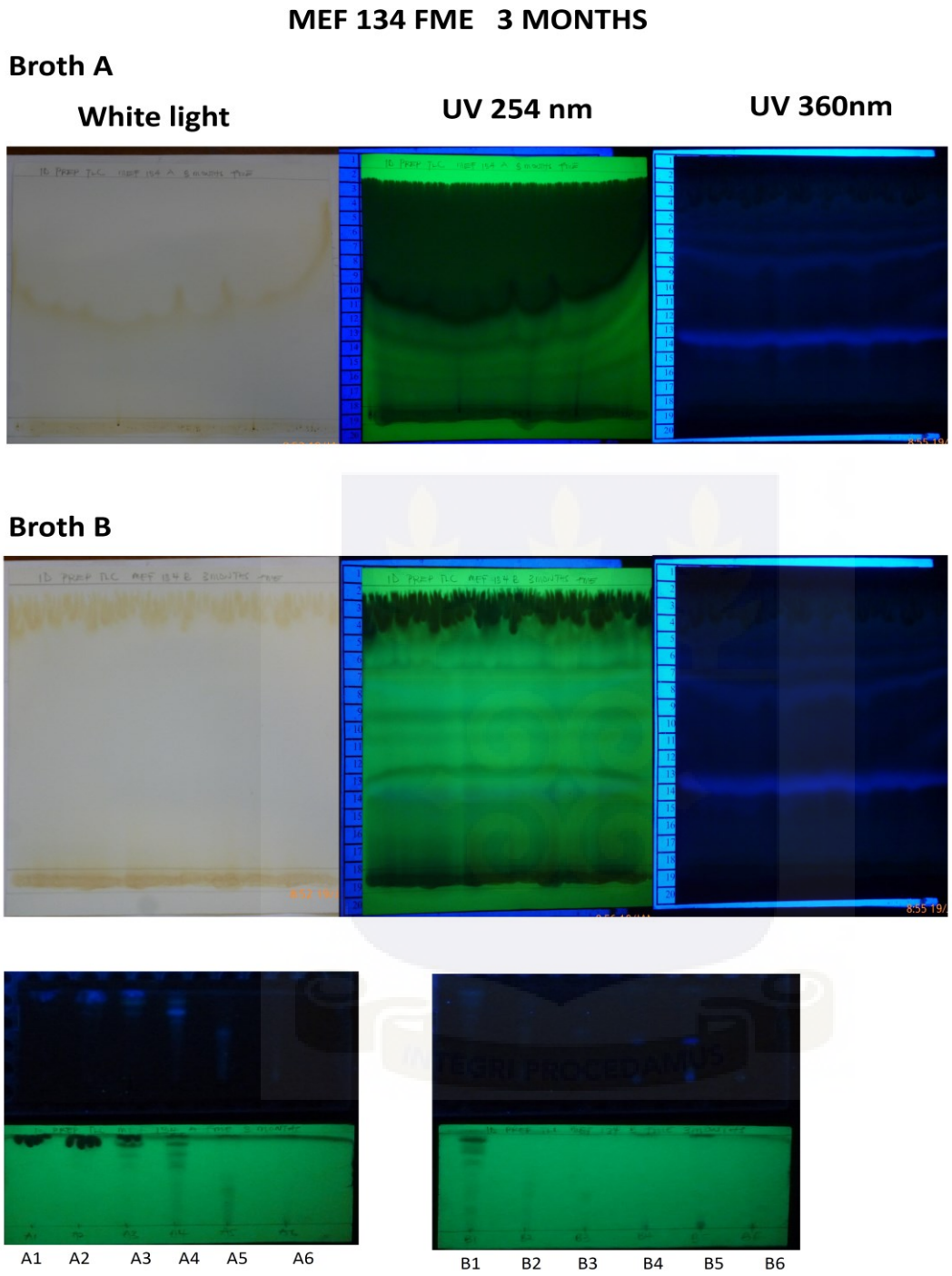


Fig S4.29. Preparative and analytical TLC plates of FME fractions obtained from MEF 134 grown for 3 months. The solvent system used as mobile was acetonitrile: ethyl acetate: petroleum ether (2:7:1).

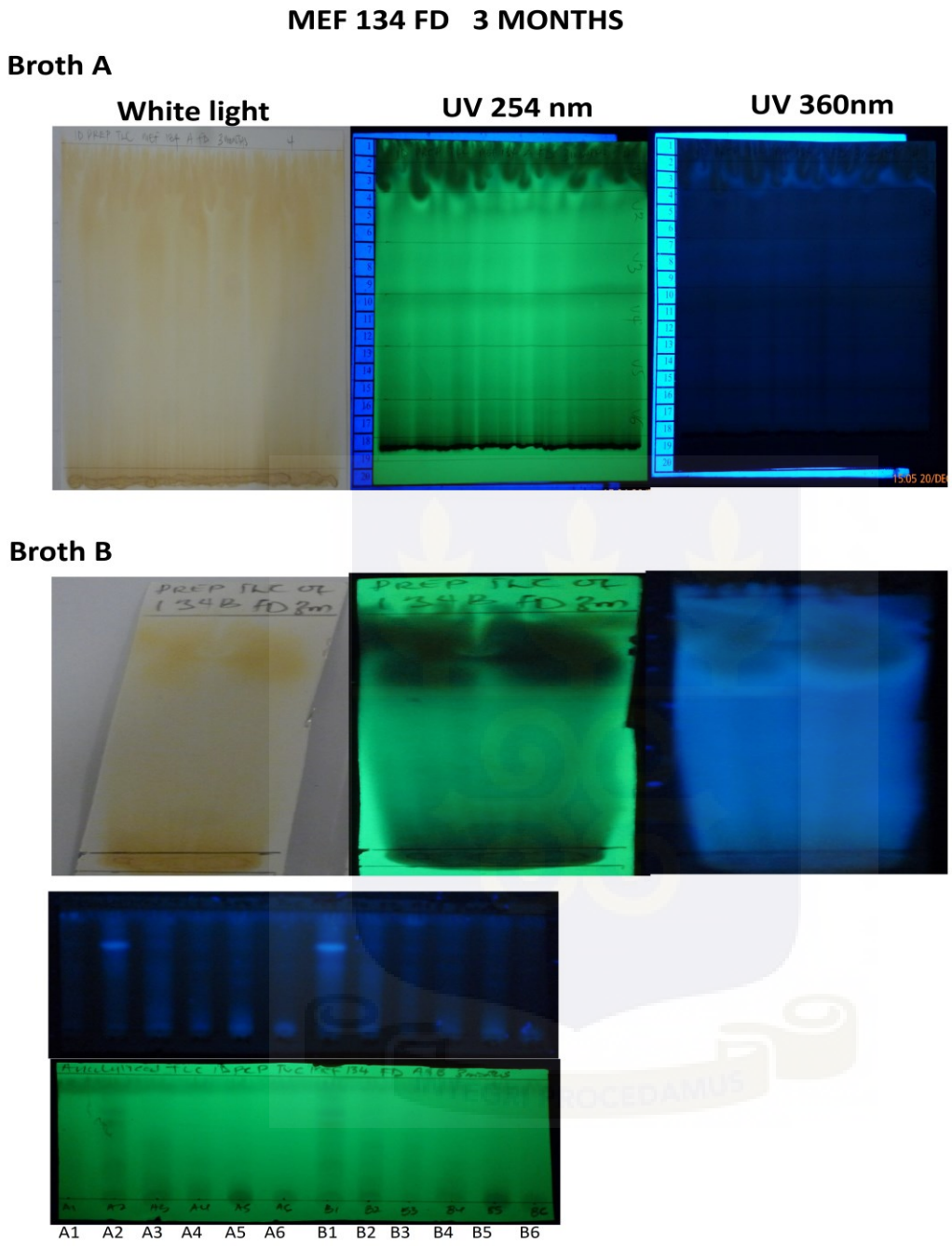


Fig S4.30. Preparative and analytical TLC plates of FD fractions obtained from MEF 134 grown for 3 months. The solvent system used as mobile was acetonitrile: ethyl acetate: petroleum ether (2:7:1).

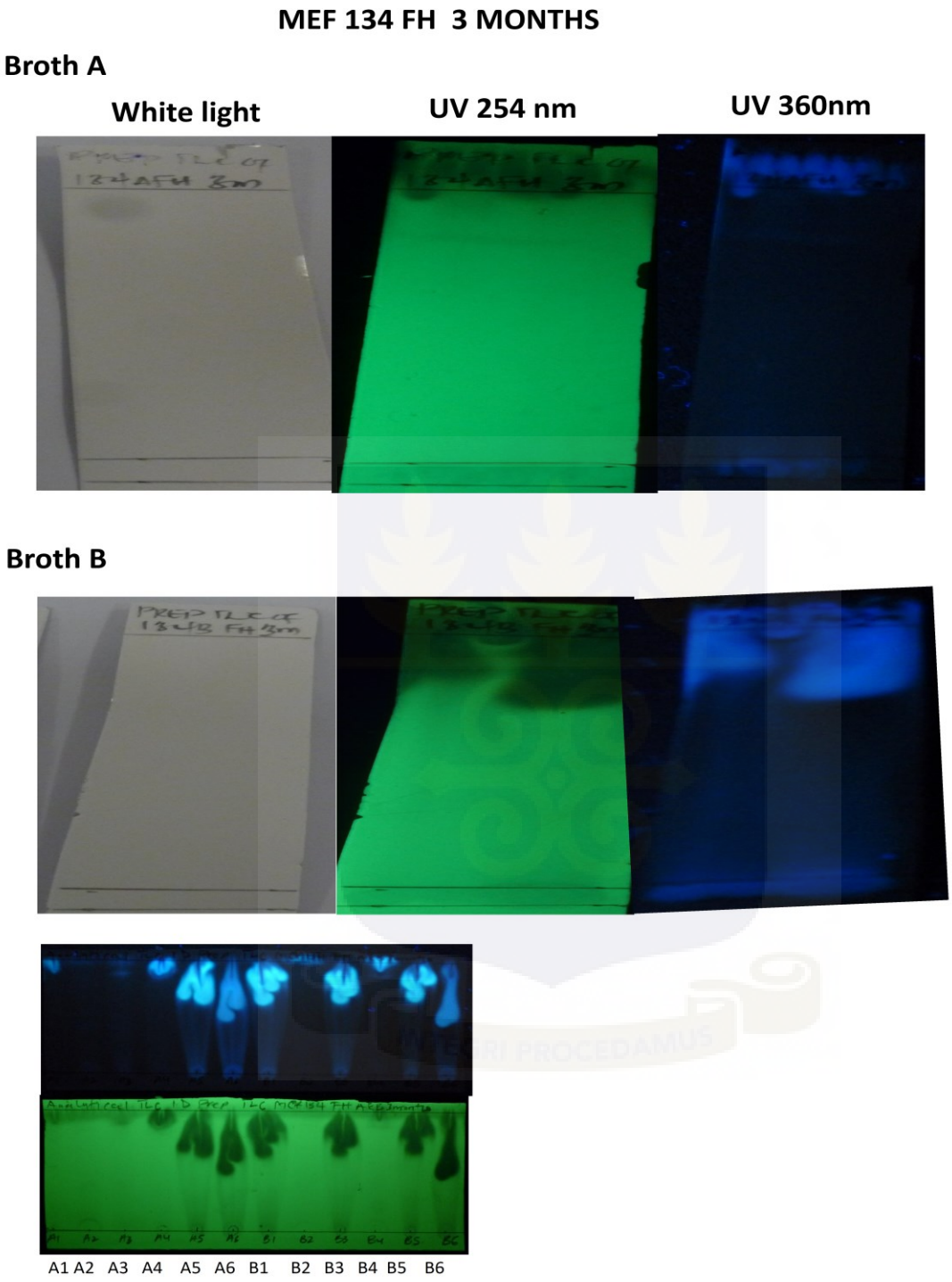


Fig S4.30. Preparative and analytical TLC plates of FH fractions obtained from MEF 134 grown for 3 months. The solvent system used as mobile was acetonitrile: ethyl acetate: petroleum ether (2:7:1).

# CHAPTER FIVE

## *IN VITRO* INTERACTIONS OF PHENOTYPE MODULATING COMPOUNDS, STANDARD ANTIFUNGAL DRUGS AND MARINE FUNGAL EXTRACTS AGAINST *CANDIDA ALBICANS* AND *SACCHAROMYCES CEREVISIAE*

### 5.1 Introduction

Treatment of fungal infections, predominantly caused by *Candida* species, is often hindered by the lack of effective antifungal agents (Walsh & Groll, 1999). There is growing experimental evidence suggesting the possibility of combined application of antifungal and non-antifungal drugs for development of effective antifungal combination chemotherapy against these infections (Afeltra & Verweij, 2003). Administration of two or more compounds in the treatment of fungi infections has many potential advantages the main being synergistic effects that lowers the therapeutic doses. This type of therapy may enhance the efficacy of compounds used, since the compounds may have different mechanisms of action leading to efficient eradication of the pathogens (Nosanchuk, 2006). Also the spectrum of activity of these compounds may be potentially broadened and there by lower the incidence of toxicity often observed in the administration of Amphotericin B (AmpB) due to lowered concentrations of these therapeutic agents administered in combination therapies (Nosanchuk, 2006). Finally, resistance to these compounds may be circumvented since the selected compounds always have multiple mechanisms of action which often overwhelms the pathogens (Nosanchuk, 2006). Azoles are primarily used as antifungal agents, administered orally or topically and can be used to treat clinically important fungi such as *Candida* or *Aspergillus s*pecies. *Candida* species can exist in either the planktonic form or can form biofilms. Of the two forms, biofilms are difficult to treat as they display relatively higher potential for development of drug resistance

(Cui *et al.*, 2015). There are many different types of azoles including fluconazole, miconazole, ketoconazole and itraconazole, with the study focusing mainly on studying interactions of fluconazole. Another drug which is often used to treat fungal infections is amphotericin B and unlike azoles, amphotericin B is typically administered by injection. However, while azoles and amphotericin B may be used alone, they can have synergistic effects when combined with other compounds which is the expected outcome of this study.

Certain azole derivatives can exhibit synergistic effects when administered in combination with amphotericin B, for example, the combination of fluconazole and amphotericin B had synergistic effects on the planktonic growth of *Candida albicans*. However, when the same combination was used on biofilms, the combination did not offer any benefit over using amphotericin B alone (Bachmann *et al.*, 2003). The combination of azoles with amphotericin B can also lead to the occurrence of antagonistic effects between azoles and amphotericin B. In particular, co-incubation of amphotericin B with either miconazole, ketoconazole, itraconazole, or fluconazole led to inhibition of the activity of amphotericin B (Scheven & Schwegler, 1995). Despite these inconsistent observations, both azoles and amphotericin B have displayed synergistic effects against clinically relevant fungi when administered with other compounds.

Combined application of tacrolimus and azoles against clinical isolates of fluconazole-susceptible and resistant strains of *Candida glabrata* displayed different effects depending on the azole. Tacrolimus and itraconazole generated the most effective synergistic effect against only fluconazole-susceptible of *Candida glabrata*. In contrast, all three other azoles (fluconazole, ketoconazole, and itraconazole) displayed synergistic effects with tacrolimus against fluconazole-resistant *Candida glabrata* (Denardi *et al.*, 2015). In a separate study investigating the combination

of tacrolimus and azoles against *Candida albicans,* it was found that strong synergy occurred against azole-resistant strains when analysis was performed using the fractional inhibitory concentration index, the ΔE model or the time-killing test (S. Sun *et al.*, 2008). Another compound which has also shown synergistic effects with azoles is fluoxetine. Fluoxetine with either fluconazole or itraconazole led to synergistic effects against resistant strains of *Candida albicans*, both *in vitro* and *in vivo*. The occurrence of these effects was due to increased attenuation of virulence factors (Gu *et al.*, 2016). Similarly, fluoxetine with fluconazole generated synergistic effects against fluconazole-resistant *Candida* strains (Oliveira *et al.*, 2014).

Fungal infections caused by biofilms are difficult to treat, the biofilms significantly reduce the effectiveness of azoles such as fluconazole. However, using fluconazole with calcineurin inhibitors significantly increased the effectiveness of fluconazole. This was attributed to increased membrane perturbation and alteration of fluconazole from a fungistatic compound to a fungicidal compound (Uppuluri *et al.*, 2008). Similarly, doxycycline with fluconazole produced synergistic effects against *Candida albicans* which was significantly enhanced with addition of calcium channel blockers. Therefore, this observation indicates that calcium signaling may play an important role in mediating the antifungal activity of doxycycline and fluconazole (Y. Gao *et al.*, 2013).

Like azoles, synergistic effects of amphotericin B with other compounds have been reported, especially against biofilms. Amphotericin B with essential oil of *Myrtus communis* led to increased effectiveness against clinical isolates of *Candida albicans*. *Mytrus communis* is also known as the common myrtle plant and is native to the Mediterranean region. While the oil from this plant exhibited antifungal properties alone, with amphotericin B it produced significant synergistic effects (Mahboubi & Bidgoli, 2010). Similarly, significant synergistic effects of amphotericin B

and tyrosol against biofilms of *Candida krusei* and *Candida tropicalis* in individuals who use intrauterine devices have been observed. Using a biofilm model and clinical isolated strains, the combination of amphotericin B and tyrosol produced significant synergistic effects against these biofilms, as biofilms can be difficult to treat, this study further highlights the clinical importance of the synergistic effects between drugs (Shanmughapriya *et al.*, 2014).

Besides *Candida,* another clinically relevant fungi is *Aspergillus* which are typically spread as spores in the air and cause disease under immunocompromised conditions. In individuals with a compromised immune system or lung damage, it can cause serious and sometimes life-threatening conditions. Previous research has shown caspofungin and amphotericin B can produce synergistic effects against *Aspergillus* (Arikan *et al.*, 2002).

Overall, azoles and amphotericin B have been shown to exhibit synergistic effects with other compounds, particularly against drug-resistant fungal strains. However, the mechanisms for these synergistic effects are often not well understood. Therefore, more research is necessary in order to fully understand and potentially enhance these synergistic effects. The aim of this work was to investigate the inhibition of fungal growth by a pairwise combination of compounds leading to the identification of an effective drug combination. Each pair contained a standard antifungal agent and one of the 20 selected phenotype modulating compounds. The knowledge from this combination assay was used in an interaction study between the crude extracts of 6 marine endophytic fungi and the 20 phenotype modulating compounds. Thus the objectives of the study were;

- To determine the phenotypic response of *C. albicans* and *S. cerevisiae* to specific drug-drug interactions using disc diffusion assay (antifungal agents versus phenotype modulating compounds).
- To determine the phenotypic response of *S. cerevisiae* and *C. albicans* to specific drug-fungal extract interactions using disc diffusion assay (fungal extracts versus phenotype modulating compounds).

## 5.2 Materials and Methods

**5.2.1 Phenotype modulating compounds**: the phenotype modulating compounds used in this study were Artesunate, Artemisinin, 5-Fluorouracil (0.0001X and 0.125X), Aspirin, Verapamil, Serine hydroxamate, Diclofenac, Cysteine, Metronidazole, Fludioxinil, Vitamin C, Sertraline, Farnesol, Phenyl ethyl alcohol, Cycloserine, N-acetylglucosamin, Cycloheximide, Lactate and 3-O-Methyl glucose. These were all purchased from Sigma Aldrich and Carl Roth, Germany, unless otherwise indicated.

**5.2.2 Antifungal agents:** the antifungal agents used were provided by the manufacturer (Sigma-Aldrich) as standard powders and included; Fluconazole (Flu), Amphotericin B (Amp B), 5-Fluorouracil (5-FU), Benzoic acid (Ben), Cycloserine (Cys), Paromomycin (Para) and Cycloheximide (CyH). The phenotype modulating compounds and antifungal agents were dissolved in methanol with exception of Vitamin C, Amphotericin B and Paromomycin which were dissolved in distilled water.

**5.2.3 Marine fungal extracts used**; Ethyl acetate extracts of these 6 fungal isolates; (*Aspergillus fumigatus* (MEF11), *Clonostachys wenpingii* (MEF65), *Meyerozyma guilliermondii* (MEF 75), *Aspergillus fumigatus* (MEF 112), *Meyerozyma guilliermondii* (MEF 122), *Meyerozyma*

*guilliermondii* (MEF 134) were also used in an interaction assay. Preparation of these extracts have been previously described in Chapter three.

### 5.2.4 Preparation of fungal cultures

The fungal strains used as test organisms in this study were *Saccharomyces cerevisiae* and *Candida albicans*. The strains were donated to the laboratory by Prof. Martha Cyert from the Stanford University. Cultures of the two fungi were prepared from frozen stocks. They were grown on YPDA (Yeast extract (10g/L), Peptone (20 g/L), Dextrose (20 g/L) and Agar (20g/L)) and then incubated for 12 hours at 30℃. Single colonies of the fungi were suspended in 50 mL nutrient broths (8g/L). The culture suspensions were incubated for 24 hours at 30℃, these were labelled as the starter cultures. After incubation, the optical densities of the fungi cultures were measured with the aid of a spectrophotometer (SANYO SP50) at a wave length of 600 nm ($OD_{600}$). The values obtained from the measurements were used to determine the starting $OD_{600}$ for the seed cultures. The starter cultures were used to inoculate fresh 50 mL nutrient broths to obtain an $OD_{600}$ of 0.01. These new cultures were labelled seed cultures; the organisms were kept in these cultures for an extra 24 hours. Afterwards, the $OD_{600}$ of the seed cultures were adjusted to 0.7 before being used for the interaction assays.

### 5.2.5 Minimum inhibitory concentration (MIC) determination of the phenotype modulating compounds

Stock solutions of each phenotype modulating compound was made in their respective solvents for dilution (methanol and distilled water) at concentrations of 0.1 and 10 µg/µL. These were pipetted unto discs at the following working concentrations per disc; 50, 20, 10, 5, 1 and 0.5 µg/µL. The minimum inhibitory concentrations (MIC) of 13 phenotype modulating compounds were

determined using the disc diffusion method (Table S5.1). Inocula of *C. albicans* and *S. cerevisiae* were adjusted to $OD_{600}$ of 0.7 and were prepared from the seed cultures as described previously. Discs infused with the phenotype modulating compounds were placed on YPDA plates spread with the yeast inocula. The compounds at each concentration were assayed in duplicate. Subsequently, the plates were sealed with parafilm and incubated at 30°C. The diameter of the inhibition zones was measured after 24 hours and recorded in millimeters. The MIC of the remaining 6 phenotype modifying compounds were selected from literature (Table S5.2)

### 5.2.6 Interactions between the antifungal agents and the phenotype modulating compounds

The *in vitro* interactions between the antifungal agents and the phenotype modulating compounds were studied against the two fungal strains. YPDA plates were modified with one-fourth of the MIC of each phenotype modulating compound determined previously. The final working concentrations and the volumes pipetted to modify each plate are indicated in Table S5.1. The antifungal agents at their effective concentrations were infused into sterile filter paper discs (5mm) and placed on the modified YPDA plates spread with the two yeast inocula at $OD_{600}$ of 0.7. The plates were incubated at 30°C and observed after 24 hours. The experiments were conducted in duplicate. Test condition where there was no modification of the YPDA plate was the control. This was to demonstrate the inhibitory effect of the antifungal agents alone.

### 5.2.7 Interactions between the fungal extracts and the phenotype modulating compounds

The interactions between the antifungal agents and the crude extracts of the 6 marine endophytes against *C. albicans* and *S. cerevisiae* were evaluated using the disc diffusion method. A volume of 10 µL of crude extracts from stock solutions (3 µg/µL) were added unto sterile filter paper discs and allowed to dry under sterile conditions. Final extract amount per disc was 30 µg. The crude extract soaked discs were placed on YPDA modified with the phenotype modulating agents and

spread with either *C. albicans* or *S. cerevisiae* as described above. The tests were performed in duplicate. Unmodified YPDA spread with the two yeast inocula having the fungal extracts discs placed on them were used as the test controls. This was to demonstrate the inhibitory activity of the fungal extracts alone.

### 5.2.8 Morphological characterization of *C. albicans* and *S. cerevisiae* in the presence of phenotype modifying compounds

The morphological features of *S. cerevisae* and *C. albicans* were also studied when these cells were grown in media supplemented with the phenotype modifying compounds. Colonies of cells from both organisms were obtained from the agar plates that were modified with these compounds during the disc diffusion assay. These colonies of cells were used for making smears on microscope slides. Gram staining and acid fast staining were used to characterize the effect of these phenotype modifying compounds on the morphology of these organisms. After staining, these cells were viewed with the oil immersion (100x) lens of the Leica light microscope (Germany). Images of the cells were acquired at 100x magnification with a Panasonic Lumix digital camera using the 3X zoom feature.

## 5.3 Results

### 5.3.1 Effects of phenotype modifying compounds on the activities of antifungal compounds against *C. albicans* and *S. cerevisiae*

In this study, 5 antifungal compounds (Fluconazole (5µg/µL), Amphotericin B (10µg/µL), 5-Fluorouracil (1µg/µL), Cycloheximide (20 µg/µL), Paromomycin (10µg/µL)) and 1 compound that does not exhibit antifungal activity (Benzoic acid, (10µg/µL) were used for an interaction assay. For the purpose of this study, these 6 compounds are collectively referred to as antifungal

compounds. The study investigated whether interaction of these 6 antifungal compounds with 20 phenotype-modifying compounds would result in an increase or decrease in the activity of the antifungal compounds.

The first step was to determine the minimum inhibitory concentrations (MICs) of the 20 phenotype modulating compounds. The MIC is the lowest concentration of a compound that will prevent the noticeable growth of an organism after 24 hours of incubation. As a research tool, MICs are used to determine the *in vitro* activity of new bioactive compounds. The MICs of the 20 phenotype modulating compounds were determined against *C. albicans* and *S. cerevisiae* using the disc diffusion assay method. The MICs obtained for the majority of the phenotype modifying compounds were above 20 µg against both *C. albicans* and *S. cerevisiae* (Table S5.1 and S5.2). Lower MIC values were recorded for Serine Hydroxamate and 5-Fluorouracil (Table S5.1).

The 20 phenotype modifying compounds were used for the interaction assay alongside the 6 antifungal compounds; *C. albicans* and *S. cerevisiae* were used as test organisms for this assay. A positive interaction was defined as an increase in zone of inhibition by at least 4 mm or more relative to the control. Conversely, a negative interaction was defined as a reduction in the zone of inhibition by 4 mm, or more, relative to the control. Based on the above definitions, the results showed that there were antagonistic interactions between Amphotericin B and the following phenotype modifying compounds against *C. albicans*; redox inducing compounds (Table 5.1), DNA/RNA synthesis inhibitors (Table 5.2), efflux inhibitor/inducers (Table 5.3), haem interacting compounds (Table 5.4) and kinase inhibitor (Table 5.5). The results also show synergistic interactions between Fluconazole and the following phenotype modifying compounds against *C. albicans*; redox inducing compounds and DNA/RNA synthesis inhibitors (Tables 5.1 and 5.2). Benzoic acid did not exhibit antifungal activity against *C. albicans* on its own nor in combination

with all the phenotype modifying compounds. However, many interesting observations were made from the interaction assay against *S. cerevisiae*. The results showed that Amphotericin B exhibited a strong synergism against *S. cerevisiae* when used in combination with Artemisinin, Artesunate or Fludioxonil (Tables 5.4 and 5.5). In contrast, antagonism against *S. cerevisiae* was detected when Fluconazole was used in combination with Aspirin, Artesunate, Fludioxonil, Cycloheximide and compounds involved in cell wall biosynthesis (Cycloserine and N- acetyl glucosamine) (Tables 5.4, 5.5, 5.6 and 5.7). For most of the interaction assay involving Fluconazole, against *S. cerevisiae*, a decrease in the zones of inhibition from 21 mm to 15 mm was observed.

Sertraline a serotonin inhibitor exhibited a strong synergistic effect against both test organisms when used in combination with Fluconazole, Cycloheximide, 5-Fluorouracil or Paromomycin (Table 5.10). Moreover, positive interactions were observed when lactate (metabolic intermediate) was combined with all the antifungal agents except benzoic acid and paromomycin against *C. albicans* (Table 5.9). In contrast, negative interactions were observed when Lactate was used in combination with many of the antifungal agents against *S. cerevisiae* (Table 5.9). Fluconazole exhibited a strong synergism against *C. albicans* when used in combination with Phenyl Ethyl alcohol and Lactate (Tables 5.8 and 5.9). There was no significant synergism for the interaction assay when Phenyl Ethyl alcohol, 3-methyl glucose or N-acetyl glucosamine was used in combination with an antifungal compound against *S. cerevisiae*.

**Table 5.1.** Effect of growth media modified with two redox compounds on the sensitivity of *C. albicans* and *S. cerevisiae* to standard antifungal agents

| | Redox | | | | | |
|---|---|---|---|---|---|---|
| | Zone of Inhibition/mm | | | | | |
| | *C. albicans* | | | *S. cerevisiae* | | |
| Antifungal (ug) | Control | Cysteine | vitamin C | Contol | Cysteine | vitamin C |
| CyH (20) | 0 | 0 | 0 | 50 | 50 | 50 |
| Amp B (10) | 6 | 0 | 0 | 12 | 12 | 12 |
| Ben (10) | 0 | 0 | 0 | 0 | 0 | 0 |
| Flu (5) | 28 | **32.5** | **32** | 21 | **18** | **15** |
| Para (20) | 0 | 0 | 0 | 0 | 0 | 0 |
| 5-Fu (1) | 0 | 0 | 0 | 8 | 0 | 12 |

**Table 5.2.** Effect of growth media modified with DNA/RNA synthesis inhibitors on the sensitivity of *C. albicans* and *S. cerevisiae* to standard antifungal agents

| | DNA/RNA sysnthesis inhibitors | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | Zone of Inhibition/mm | | | | | | | |
| | *C. albicans* | | | | *S. cerevisiae* | | | |
| Antifungal (u g) | Control | Metroni dazole | 5-Fu (0.001X) | 5-Fu (0.125X) | Control | Metronida zole | 5-Fu (0.001X) | 5-Fu (0.125X) |
| CyH (20) | 0 | 0 | 0 | 0 | 50 | 50 | 50 | 50 |
| Amp B (10) | 6 | 0 | 6 | 7 | 12 | 12 | 12 | 12 |
| Ben (10) | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| Flu (5) | 28 | 28.5 | **33.5** | **29.5** | 21 | 20 | **16.5** | **16.5** |
| Para (20) | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| 5-Fu (1) | 0 | 0 | 0 | 0 | 8 | 10 | 10 | 10 |

**Table 5.3.** Effect of growth media modified with efflux inhibitor/inducers on the sensitivity of *C. albicans* and *S. cerevisiae* to standard antifungal agents

| | Efflux inhibitor/inducers | | | | | |
|---|---|---|---|---|---|---|
| | Zone of Inhibition/mm | | | | | |
| | *C. albicans* | | | *S. cerevisiae* | | |
| Antifungal (ug) | Control | Diclofenac | Verapamil | Contol | Diclofenac | Verapamil |
| CyH (20) | 0 | 0 | 0 | 50 | 51 | 50 |
| Amp B (10) | 6 | 0 | 0 | 12 | 12.75 | 10 |
| Ben (10) | 0 | 0 | 0 | 0 | 0 | 0 |
| Flu (5) | 28 | 28 | 27.5 | 21 | 22.25 | 20 |
| Para (20) | 0 | 0 | 0 | 0 | 0 | 0 |
| 5-Fu (1) | 0 | 0 | 0 | 8 | 8 | 12 |

**Table 5.4.** Effect of growth media modified with Heme interacting compounds on the sensitivity of *C. albicans* and *S. cerevisiae* to standard antifungal agents

| | Heme interacting compounds | | | | | |
|---|---|---|---|---|---|---|
| | Zone of Inhibition/mm | | | | | |
| | *C. albicans* | | | *S. cerevisiae* | | |
| **Antifungal (ug)** | **Control** | **Artemisinin** | **Artesunate** | **Contol** | **Artemisinin** | **Artesunate** |
| CyH (20) | 0 | 0 | 0 | 50 | 50 | 50 |
| Amp B (10) | 6 | 0 | 6 | 12 | **20** | **20** |
| Ben (10) | 0 | 0 | 0 | 0 | 0 | 0 |
| Flu (5) | 28 | **31** | 27.5 | 21 | 21 | **15** |
| Para (20) | 0 | 0 | 0 | 0 | 0 | 0 |
| 5-Fu (1) | 0 | 0 | 0 | 8 | 0 | 0 |

**Table 5.5.** Effect of growth media modified with methyltransferese inducer, quorum-sensing molecule and Hybrid histidine kinase inhibitor on the sensitivity of *C. albicans* and *S. cerevisiae* to standard antifungal agents

| | Methyltransferase inducer, Quorum-sensing molecule and Hybrid histidine kinase inhibitor | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | Zone of Inhibition/mm | | | | | | | |
| | C. albicans | | | | S. cerevisiae | | | |
| Antifungal (ug) | Control | Aspirin | Farnesol | Fludioxonile | Contol | Aspirin | Farnesol | Fludioxonile |
| CyH (20) | 0 | 0 | 0 | 0 | 50 | 50 | **0** | 50 |
| Amp B (10) | 6 | 6 | 0 | 0 | 12 | 12 | **10** | **20** |
| Ben (10) | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| Flu (5) | 28 | 30.5 | **23** | 30.5 | 21 | **15** | **6** | **15** |
| Para (20) | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| 5-Fu (1) | 0 | 0 | 0 | 0 | 8 | **15** | **0** | **0** |

**Table 5.6.** Effect of growth media modified with TRNA stress inducer and protein synthesis inhibitor on the sensitivity of *C. albicans* and *S. cerevisiae* to standard antifungal agents

| | TRNA stress inducer and Protein synthesis inhibitor | | | | | |
|---|---|---|---|---|---|---|
| | Zone of Inhibition/mm | | | | | |
| | *C. albicans* | | | *S. cerevisiae* | | |
| **Antifungal (ug)** | **Control** | **serine hydroxamate** | **Cycloheximide** | **Control** | **serine hydroxamate** | **Cycloheximide** |
| CyH (20) | 0 | 0 | 0 | 50 | 52 | **0** |
| Amp B (10) | 6 | 6 | 8.5 | 12 | 13.5 | **0** |
| Ben (10) | 0 | 0 | 0 | 0 | 0 | 0 |
| Flu (5) | 28 | 28.5 | 28.5 | 21 | 23.5 | **0** |
| Para (20) | 0 | 0 | 0 | 0 | 0 | 0 |
| 5-Fu (1) | 0 | 0 | 0 | 8 | 0 | 0 |

**Table 5.7.** Effect of growth media modified with compounds involved in cell wall biosynthesis on the sensitivity of *C. albicans* and *S. cerevisiae* to standard antifungal agents

| | Cell wall biosynthesis | | | | | |
|---|---|---|---|---|---|---|
| | Zone of Inhibition/mm | | | | | |
| | *C. albicans* | | | *S. cerevisiae* | | |
| **Antifungal (ug)** | **Control** | **Cycloserine** | **N- acetyl glucosamine** | **Contol** | **Cycloserine** | **N- acetyl glucosamine** |
| CyH (20) | 0 | 0 | 0 | 50 | **0** | **0** |
| Amp B (10) | 6 | 6 | 7.5 | 12 | 11 | **9.5** |
| Ben (10) | 0 | 0 | 0 | 0 | 0 | 0 |
| Flu (5) | 9 | **13.5** | **17** | 21 | **13.5** | **11** |
| Para (20) | 0 | 0 | 0 | 0 | 0 | 0 |
| 5-Fu (1) | 0 | 0 | 0 | 8 | 0 | 0 |

**Table 5.8.** Effect of growth media modified with oxidative stress inhibitor on the sensitivity of *C. albicans* and *S. cerevisiae* to standard antifungal agents

| | Oxidative phosphorylation inhibitor | | | |
|---|---|---|---|---|
| | Zone of Inhibition/mm | | | |
| | *C. albicans* | | *S. cerevisiae* | |
| **Antifungal (ug)** | **Control** | **Phenyl ethyl alcohol** | **Contol** | **Phenyl ethyl alcohol** |
| CyH (20) | 0 | 0 | 50 | **0** |
| Amp B (10) | 6 | 6.5 | 12 | 9.5 |
| Ben (10) | 0 | 0 | 0 | 0 |
| Flu (5) | 9 | **19** | 21 | **11.5** |
| Para (20) | 0 | 0 | 0 | 0 |
| 5-Fu (1) | 0 | 0 | 8 | **0** |

**Table 5.9.** Effect of growth media modified with metabolic intermediate on the sensitivity of C albicans and *S. cerevisiae* to standard antifungal agents

| | Metabolic Intermediate | | | | | |
|---|---|---|---|---|---|---|
| | Zone of Inhibition/mm | | | | | |
| | *C. albicans* | | | *S. cerevisiae* | | |
| **Antifungal (ug)** | **Control** | **Lactate** | **3-methyl glucose** | **Contol** | **Lactate** | **3-methyl glucose** |
| CyH (20) | 13.5 | **24** | **0** | 24 | **9.5** | **0** |
| Amp B (10) | 10.5 | **15** | **8** | 6 | 7 | **12** |
| Ben (10) | 0 | 0 | 0 | 0 | **7** | 0 |
| Flu (5) | 28.5 | **40** | **17.5** | 24 | **43** | **14.5** |
| Para (20) | 28.5 | **38.5** | **0** | 25.5 | **8** | **0** |
| 5-Fu (1) | 10.5 | **6** | **0** | 8.5 | 8 | **0** |

**Table 5.10.** Effect of growth media modified with on the sensivity of *C. albicans* and *S. cerevisiae* to standard antifungal agents

| | Serotonin inhibitor | | | |
|---|---|---|---|---|
| | Zone of Inhibition/mm | | | |
| | *C. albicans* | | *S. cerevisiae* | |
| **Antifungal (ug)** | **Control** | **Sertraline** | **Contol** | **Sertraline** |
| CyH (20) | 13.5 | 32 | 24 | 31.5 |
| Amp B (10) | 10.5 | 10.5 | 6 | 9 |
| Ben (10) | 0 | 0 | 0 | 0 |
| Flu (5) | 28.5 | 28.5 | 24 | 29 |
| Para (20) | 28.5 | 33 | 25.5 | 27 |
| 5-Fu (1) | 10.5 | 11 | 8.5 | 23.5 |

### 5.3.2 *In vitro* interactions of the phenotype modulating compounds and the MEF extracts against *C. albicans* and *S. cerevisiae*

A panel of phenotype modifying compounds were tested for their ability to boost the antifungal activity of the extracts from the marine endophytic fungi. In general, the interaction assay revealed that the antifungal activities of the extracts were boosted against *C. albicans*, than against *S. cerevisiae*.

The extract from MEF 11 exhibited the highest antifungal activity against *C. albicans* (Fig. 5.1). The antifungal activity of the MEF 11 extract was maintained in the presence of phenotype modifying compounds, with metronidazole and lactate producing the highest synergistic effect. The extract from MEF 65 did not generate high synergy in the presence of most of the phenotype modifying compounds; the interactions with aspirin, serine hydroxamate or phenyl-ethyl alcohol were the exceptions. Farnesol produced the most antagonistic interaction with all the extracts that were tested. None of the extracts generated antifungal activity against *C. albicans* when tested in combination with farnesol (Fig 5.1). Seven phenotype modifying compounds (aspirin, verapamil, serine hydroxamate, diclofenac, metronidazole, fludioxinil and lactate) boosted the antifungal activity of the extract from MEF 75: Notably, artemisinin and serine hydroxamate were the only phenotype modifying compounds that boosted the antifungal activity of the extract from MEF 112. The antifungal activity of the extract from MEF 122 was only boosted in the presence of lactate. Also, there was no increase in the antifungal activity of the extract from MEF134 against *C. albicans* following interaction with the phenotype modifying compounds. Serine hydroxamate and lactate produced the most synergistic interactions.

The antifungal activities of the MEF extracts that were detected against *S. cerevisiae* were generally low (Fig 5.2). With the exception of the extract from MEF 65, the extracts that were

tested did not exhibit antifungal activity against *S. cerevisiae*, in the absence of interaction with the phenotype modifying compounds. These observations from the interaction assay suggested that the antifungal activity of the MEF extracts against *S. cerevisiae* was boosted in the presence of most of the phenotype modifying compounds. The combination of lactate with the MEF extracts resulted in an increase in the antifungal activity against *S. cerevisiae*. Notably, the interaction of the extract from MEF 65 and lactate led to an increase in the zone of inhibition from 0 mm to 30 mm. The observations from this study demonstrate the potential of boosting antifungal activity of novel and known antifungal compounds via interaction with phenotype modifying compounds.

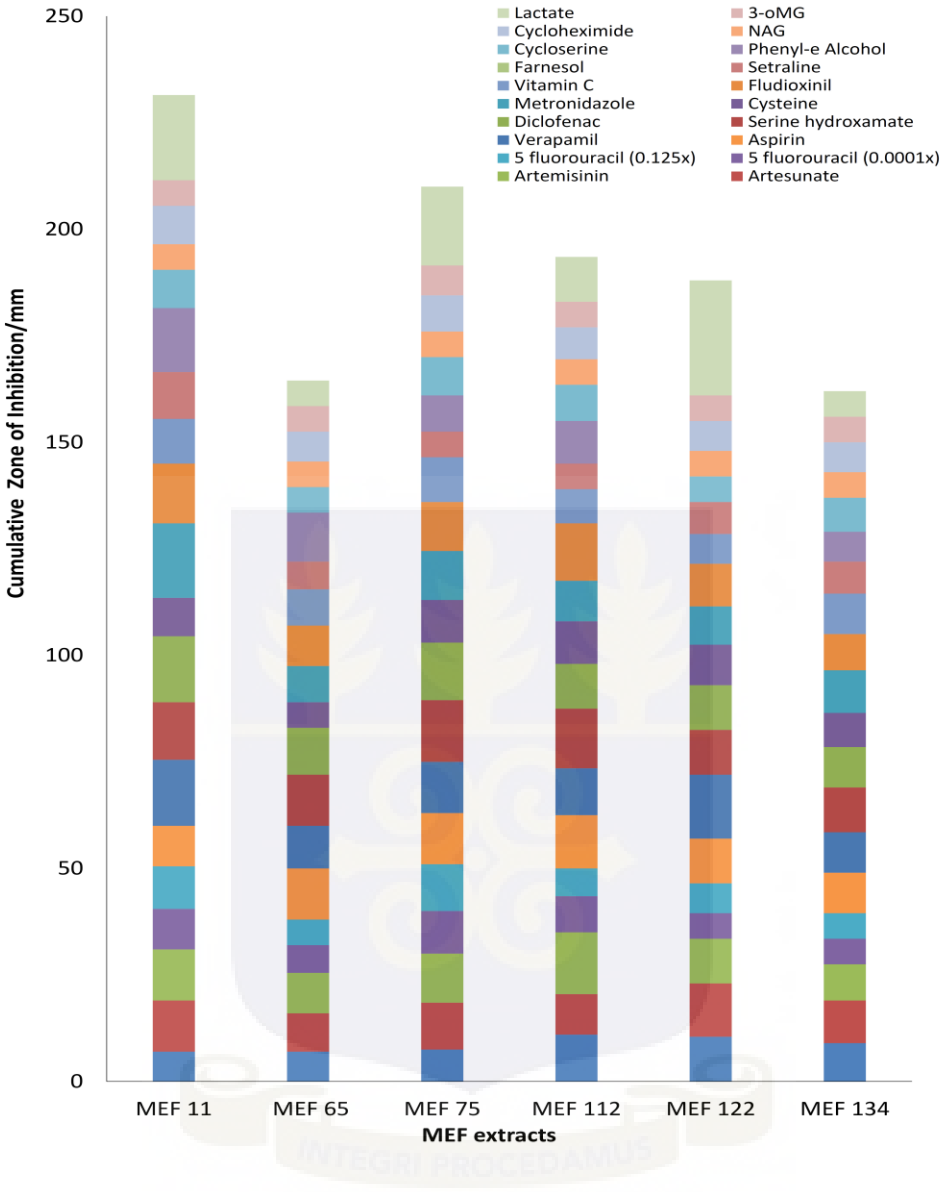


Fig 5.1. In vitro Interactions of phenotype modulating compounds and MEF extracts against *C. albicans*. The figure displays the effectiveness of the interactions of the extracts and the phenotype modulating compounds tested against *C. albicans*. The control for this experiment was unmodified YPDA spread with *C. albicans* having the antifungal discs placed on them.

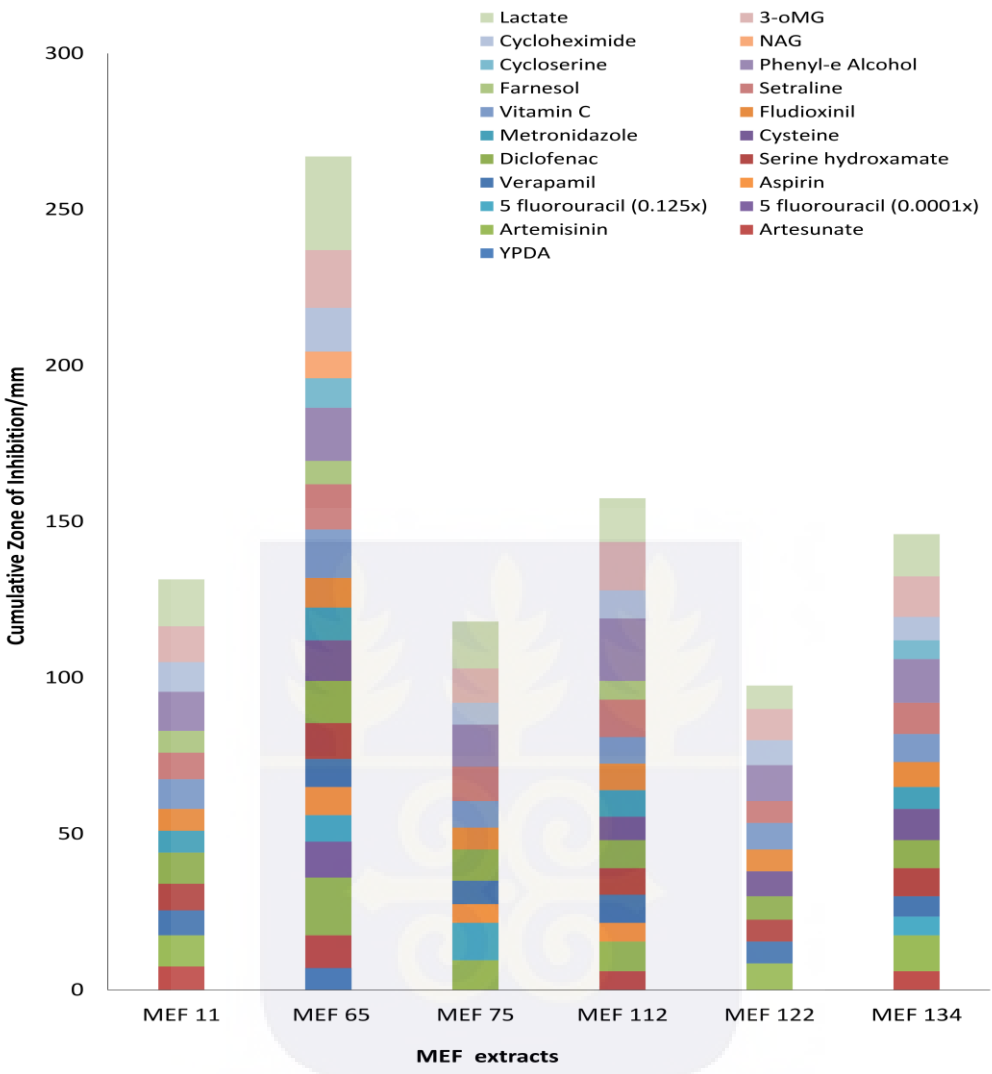


Fig 5.2. In vitro Interactions of phenotype modulating compounds and MEF extracts against *S. cerevisiae*. Effects of phenotype modulating compounds-antifungals combinations on inhibiting *S. cerevisiae* growth were evaluated. The control was unmodified YPDA spread with *S. cerevisiae* having the antifungal discs placed on them.

### 5.3.3 Morphological characterization of *C. albicans* and *S. cerevisiae* in the presence of phenotype modifying compounds

The effect of the 20 phenotype modifying compounds on the morphology of *S. cerevisiae* and *C. albicans* was investigated via light microscopy. The cells used for this assay were obtained from the modified YPDA plates that were generated by the interaction assay.

*C. albicans* cells treated with either N-acetylglucosamine, artemisinin, 5-fluorouracil (0.001x) or cycloheximide were smaller in size as compared to the control cells that were not exposed to any of these compounds (Fig 5.3 and 5.4). Most of these cells also retained the primary carbol fuchsin and crystal violet stain. The farnesol treated cells were very small in size as compared to the control while the lactate treated cells were relatively bigger in size and were elongated rod-like shaped cells. Metronidazole also induced cell elongation in *C. albicans*. Fludioxinil and vitamin C caused the cells to become smaller in size as compared to the control cells. Eleven of the phenotype modifying compounds did not alter the morphology of *C. albicans.* For *S. cerevisiae*, there were no major morphological changes induced by most of the phenotype modifying compounds (Fig 5.5 and 5.6). The exceptions were cycloheximide, farnesol, artemisinin and cycloserine which caused cell elongation and increase in cell population relative to the control cells that were not exposed to these compounds. The phenotype modifying compounds did not affect the shape of the *S. cerevisiae* cells when compared to the control cells. These observations led us to conclude that modification of the YPDA plates with the phenotype modifying compounds did not cause major morphological changes for the *S. cerevisiae* cells.

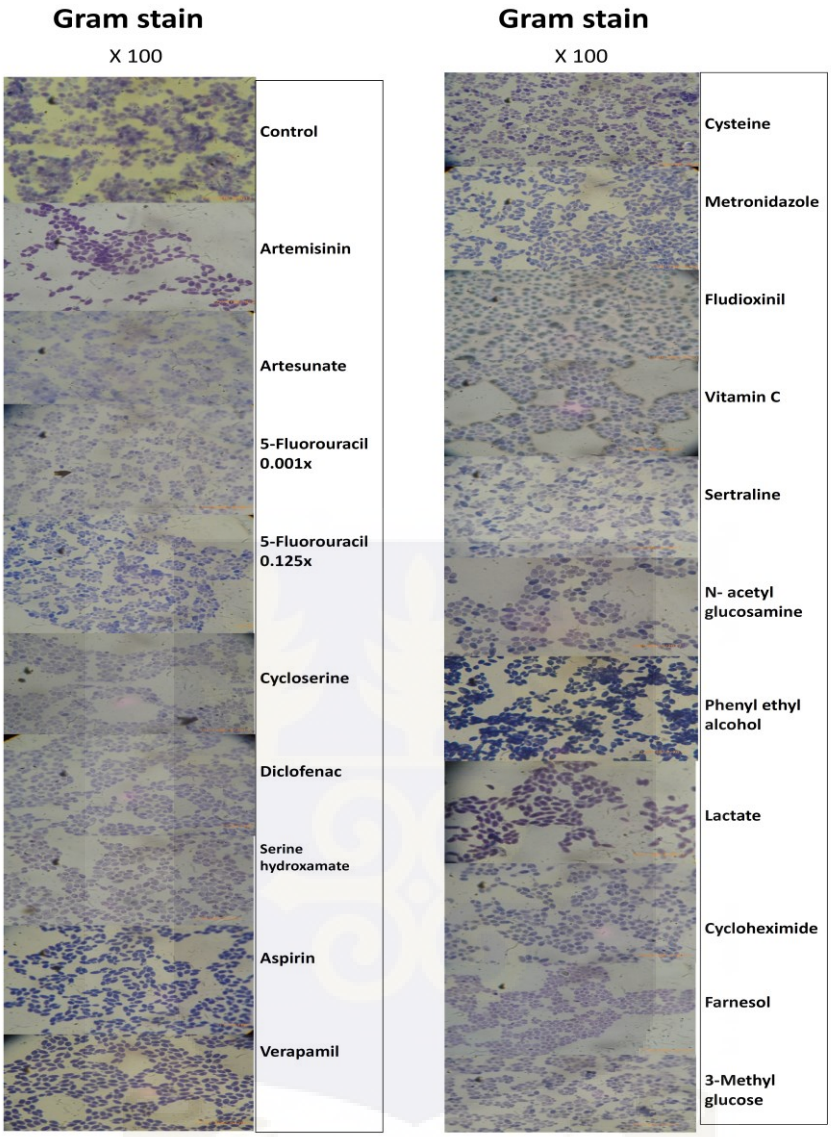


Fig 5.3. Morphological characteristics of *C. albicans* grown on chemically modified plates. *C. albicans* was grown on YPDA plates modified with phenotype modulating compounds. Cells grown on YPDA agar plates without chemical modifications served as the control. The cells were stained with Gram stains and viewed under 100 x magnification of the light microscope. Pictures of the slides were taken with a Panasonic lumix digital cameras using the 3x zoom feature**.**

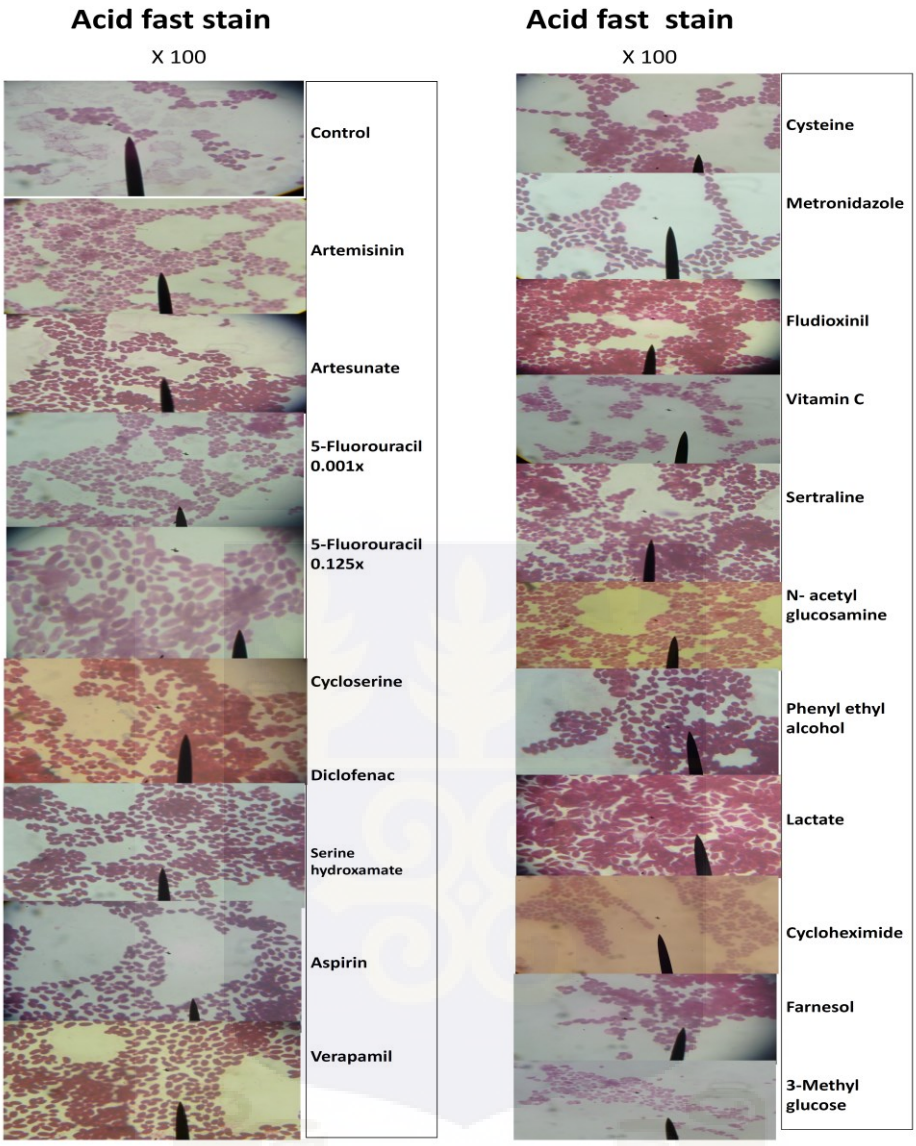


Fig 5.4. Morphological characteristics of *C. albicans* grown on chemically modified plates. *C. albicans* was grown on YPDA plates modified with phenotype modifying compounds. Cells grown on YPDA agar plates without chemical modifications served as the control. The cells were stained with Acid fast stains and viewed under 100 x magnification of the light microscope. Pictures of the slides were taken with a Panasonic lumix digital cameras using the 3x zoom feature.

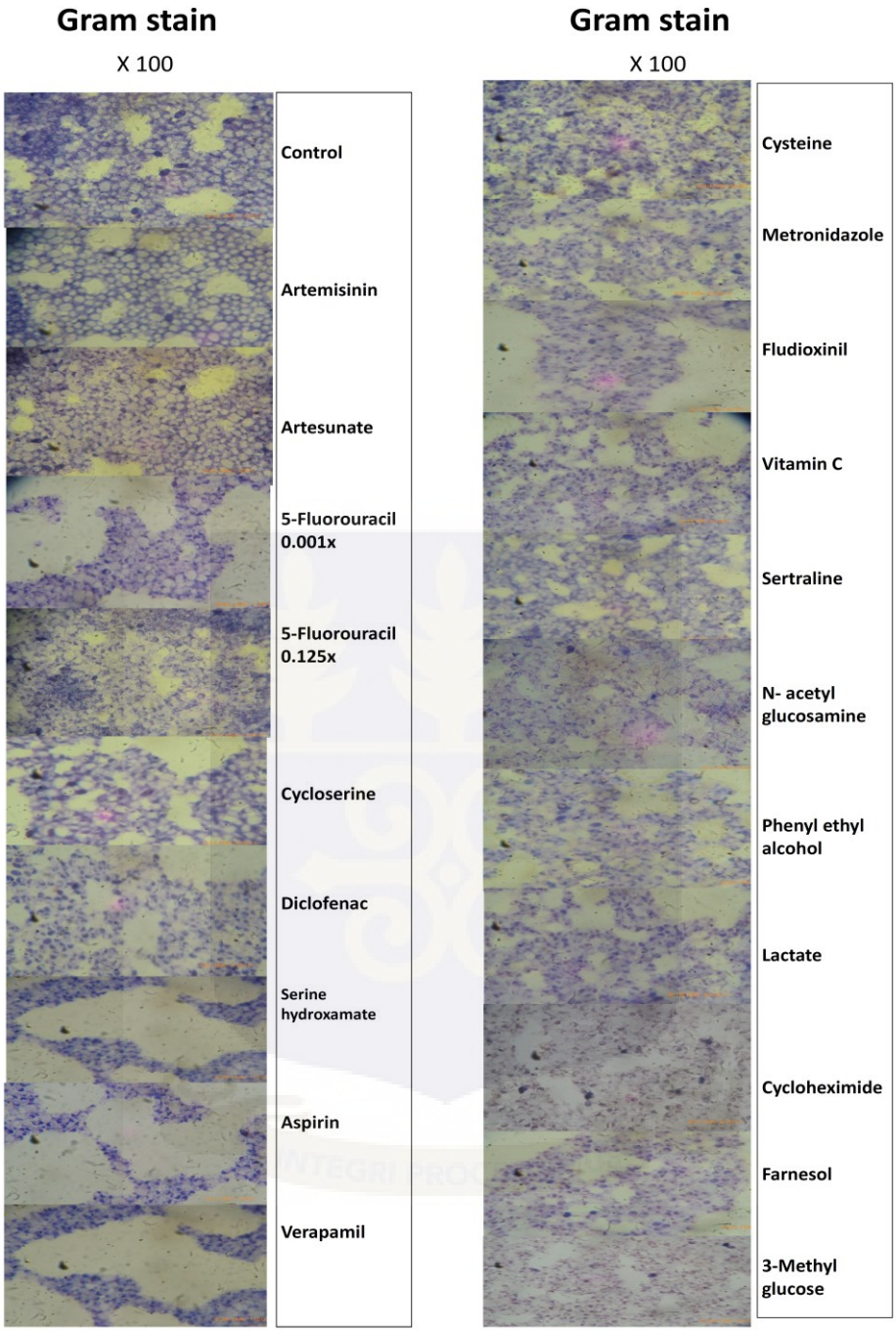


Fig 5.5. Morphological characteristics of *S. cerevisiae* grown on chemically modified plates. *S. cerevisiae* was grown on YPDA plates modified with Phenotype modifying compounds. Cells grown on YPDA agar plates without chemical modifications served as the control. The cells were stained with Gram stain and viewed under 100 x magnification of the light microscope. Pictures of the slides were taken with a Panasonic lumix digital cameras using the 3x zoom feature.

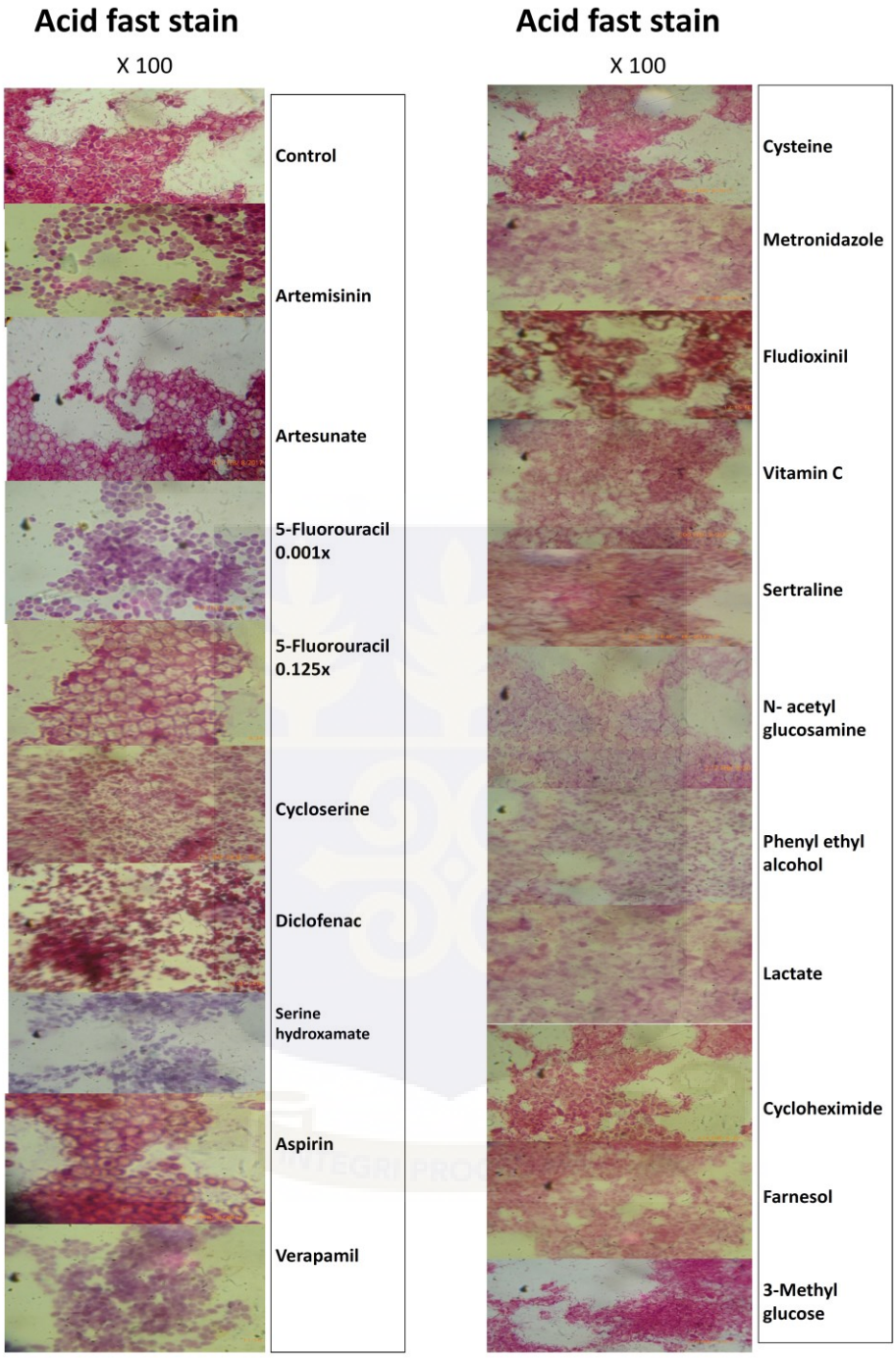


Fig 5.6. Morphological characteristics of *S. cerevisiae* grown on chemically modified plates. *S. cerevisiae* was grown on YPDA plates modified with phenotype modifying compounds.Cells grown on YPDA agar plates without chemical modifications served as the control. The cells were stained with acid fast stains and viewed under 100 x magnification of the light microscope. Pictures of the slides were taken with a Panasonic lumix digital cameras using the 3x zoom feature.

### 5.4 Discussion

Treatment of most fungal infections are generally based on fluconazole and amphotericin B therapy. Though both classes of drugs are very effective, there are many side effects associated with their use. Azoles require longer treatment durations in order to inhibit growth of fungal pathogens (Rex *et al.*, 1993). This phenomenon could increase the probability of selecting for resistant individuals in an infectious fungal population. Amphotericin B can also be very toxic to human cells after prolong usage. Hence exploring ways to boost effectiveness of these drugs at low dosage is very important. This study was conducted to ascertain whether there was a synergistic relationship between 6 standard antifungal agents and 20 phenotype modifying compounds, and whether the observations could be applied to active extracts from marine endophytic fungi. It could also allow for the determination of the mechanism of action in the context of the distinct panel of compounds the antifungal agents interact with in the bioassays. The same method can be used to determine whether bioactive crude extracts have the same kind of activity and predict the differences in the active compounds ahead of product isolation.

The results from the MIC study of the 20 phenotype modifying compounds demonstrated that, individually, the 20 compounds had weak or no *in vitro* antimicrobial activities against the two-model organism used in the assays. Although, most of these compounds are widely used in the treatment of other infections.

In the interaction study involving *C. albicans*, the highest synergistic interactions were observed when fluconazole was combined with either cysteine, vitamin C or 5-flurouracil (5FU). Fluconazole inhibits lanosterol 14a-demethylase during synthesis of ergosterol (Scheven & Scheven, 1996). It has also been demonstrated that 5FU causes inhibition of thymidylate synthase, thus interrupting DNA synthesis (Kesavan & Joyee, 2005). Inhibition of these biochemical

processes underlie the high antifungal activity that was observed when Fluconazole was administered in combination with 5-Flurouracil. The synergism observed between vitamin C and fluconazole could be attributed to the ability of vitamin C to inhibit the response of *C. albicans* to stressful conditions. Vitamin C (ascorbic acid) is also known to inhibit morphogenesis of *Candida albicans* (Van Hauwenhuyse *et al.*, 2014). The ability of *Candida albicans* to exhibits different morphotypes enables it to respond robustly against environmental stress and display virulent phenotypes (Tyc *et al.*, 2014). Hence, the enhanced antifungal activity of fluconazole could be due to suppression of stress response by vitamin C against *C. albicans*. The interaction observed between cysteine and fluconazole could not be clearly explained. However, it has been shown that variation in the biosynthesis of cysteine affects the sensitivity of yeast to various pharmacological compounds (H. S. Kim & Fay, 2007). The synergistic antifungal activities observed for fluconazole and either of the three phenotype modifying compounds highlight the potential of these compound combinations as candidates for combination therapy against fungal infections. Notably, there was a strong antagonistic interactions between Amphotericin B and 7 of the phenotype modifying compounds against *C. albicans*. However, there were no interactions observed between the other antifungal agents and the compounds studied.

The study also demonstrated a strong synergistic effect by amphotericin B in the presence of either artemisinin, artesunate or fludioxonil against *S. cerevisiae* (Tables 5.4 and 5.5). Fludioxonil is an antifungal agent used for the treatment of infected crops and is known to inhibit transport-associated phosphorylation of glucose (Furukawa *et al.*, 2012). Artemisinin and artesunate are known to produce free radicals which cause apoptosis of their target cells (Sen *et al.*, 2010). The combination of any of these three compounds with amphotericin B could present a very fatal outcome for *C. albicans* or *S. cerevisiae*. The study also demonstrated a strong antagonism

between fluconazole and 5 phenotype modifying compounds. Among the antagonistic compounds is fludioxonil, which has been shown to inhibit fluconazole activity (Buschart *et al.*, 2012). There was a positive interaction among 5-flurouracil, aspirin and verapamil. Aspirin can disrupt biofilm formation in yeast and that prostaglandin production may be a virulence factor in yeast biofilm-associated infection (Alem & Douglas, 2004). Aspirin which is a prostaglandin inhibitor can produce the observed activity. Verapamil is a calcium channel blocker and has been shown to be able to inhibit expression and transport of yeast Hwp1. This affects hyphal development, adhesion and colonization by yeast (Yu *et al.*, 2014). The interesting activities of these compounds make them good candidates for the development of combination therapy using one of the known antifungal compounds.

Sertraline and lactate synergized the activity of most of the antifungal agents (Table 3 and 4). Sertraline is an antidepressant which inhibits protein synthesis in fungi (Lin *et al.*, 2010; Zhai *et al.*, 2012). It has been shown that carbon source for fungal growth influences cell wall design and drug resistance (Ene *et al.*, 2012). The cell wall of *C. albicans* grown in the presence of lactate was observed to be thinner with β-glucan and chitin components reduced drastically (Ene *et al.*, 2012). Therefore the presence of lactate could affect enzymes involved in cell wall synthesis leading to the inhibition of ergosterol synthesis hence the observed synergistic activity. Fluconazole synergized well with the many of the compounds used in the interaction assay. The most prominent interaction was between fluconazole and cycloheximide. Cycloheximide is a protein synthesis inhibitor hence the pronounced activity observed.

From the interaction assays using pure compounds, extracts from marine endophytes were used as a proof of concept to determine whether compounds contained in these extracts could potentially form synergistic partners with the phenotype modifying compounds and the outcome could be

analyzed to understand the potential mechanisms of action of the active components. The assay was also used to determine the limit of detection of the bioactive compounds contained in the extracts. Generally, the extracts were more active against *C. albicans* than *S. cerevisiae*. Notably, farnesol produced the most antagonistic interaction with all extracts tested against *C. albicans*. While lactate boosted the activity of most of the extracts against both organisms. Though there were many instances where activity of extracts was enhanced particularly against *S. cerevisiae*, more investigations need to be conducted in order to explain the possible mechanisms underlying the occurrences. Extracts which maintained their activities across many phenotype conditions were identified as the most promising for further product isolation and characterization. These extracts could contain compounds with potent activities which could be maintained under all conditions during their use in the treatment of fungal infections.

Generally, yeast displays a wide range of morphological phenotypes, the capability to shift between yeast and hyphal growth forms contributes to virulence (Jacobsen *et al.*, 2012). In this study, the morphology of the two yeast cells were studied in the absence and presence of the chemical phenotype modifiers. It was observed that compounds such as farnesol, N-acetyl glucosamine, artemisinin and cycloheximide induced frequent budding in *C. albicans*. N-Acetyl glucosamine has previously been shown to induce both apoptosis and necrosis in *C. albicans* (H. Du *et al.*, 2015). Cycloheximide is a known protein synthesis inhibitor while artemisinin causes DNA damage to its target cells (Gopalakrishnan & Kumar, 2014; Schneider-Poetsch *et al.*, 2010). The number of buds per field of view could indicate rapid cell division, which could be a mechanism that is adapted by these cells to cope with the impact of the compounds. This could also explain why cells grown under these condition were highly sensitive to the standard antifungal compounds. Accumulation of farnesol has been shown to affect biofilm formation and dimorphism

(Nickerson *et al.*, 2006). This could explain why the cells that were grown in the presence of farnesol were smaller and more spherical in shape.

### 5.5 Conclusion

In conclusion, this study confirmed that the combination of some phenotype modifying compounds with antifungal agents could be developed as effective combinational therapy for treatment of fungal infections involving *C. albicans*. Many of the combinations resulted in synergistic interactions which have not yet been reported elsewhere. However, antagonistic interactions were also observed for a few of the compound combinations. The results of this study provide critical information that will facilitate the selection of compounds that could form potent therapeutic partners with the compounds which will be isolated from the extracts.

### 5.6 Recommendations for future works

- The active compounds from the extracts should be tested in combination with the most potent phenotype modifying compounds in order to identify potent pairs of compounds effective against the selected pathogens.
- The mechanism of action of the compounds in the pairs identified should be properly elucidated.

## 5.7 Supplementary Data

**Table S 5.1.** Concentrations of phenotype modulating compounds and the various concentrations used in modifying the plates

| Compound | MIC (µg) | (1/4 MIC (µg) | Working Concentration (µg/µL) | Volume Spread on plate (µL) |
|---|---|---|---|---|
| Serine Hydroxamate | 0.5 | 0.125 | 0.125 | 100 |
| Diclofenac | 10 | 2.5 | 2.5 | 100 |
| Artemisinin | 25 | 6.25 | 6.25 | 100 |
| Aspirin | 40 | 10 | 10 | 100 |
| Metronidazole | 30 | 7.5 | 7.5 | 100 |
| Verapamil | 40 | 10 | 10 | 100 |
| Cysteine | 25 | 6.25 | 6.25 | 100 |
| Artesunate | 50 | 12.5 | 12.5 | 125 |
| Fludioxonil | 10 | 2.5 | 2.5 | 100 |
| Vitamin C | 50 | 12.5 | 12.5 | 125 |
| 5-Fluorouracil 0.001X | 0.001 | 0.00025 | 0.00025 | 100 |
| 5-Fluorouracil 0.125X | 0.125 | 0.03125 | 0.03125 | 100 |
| Sertraline | 25 | 6.25 | 6.25 | 80 |
| Lactate | 0.50% | 0.13% | 0.13% | 100 |

**Table S5.2.** Phenotype modulating compounds used in modifying the plates

| Compound | (1/4 MIC (ug) |
|---|---|
| Farnesol | 5uM |
| Phenyl ethyl alcohol | 0.15mg/ml |
| N-acetyl glucosamine | 10nM |
| Cycloserine | 1mM |
| 3-O-methyl glucose | 10mM |
| Cycloheximide | 0.25mg/ml |

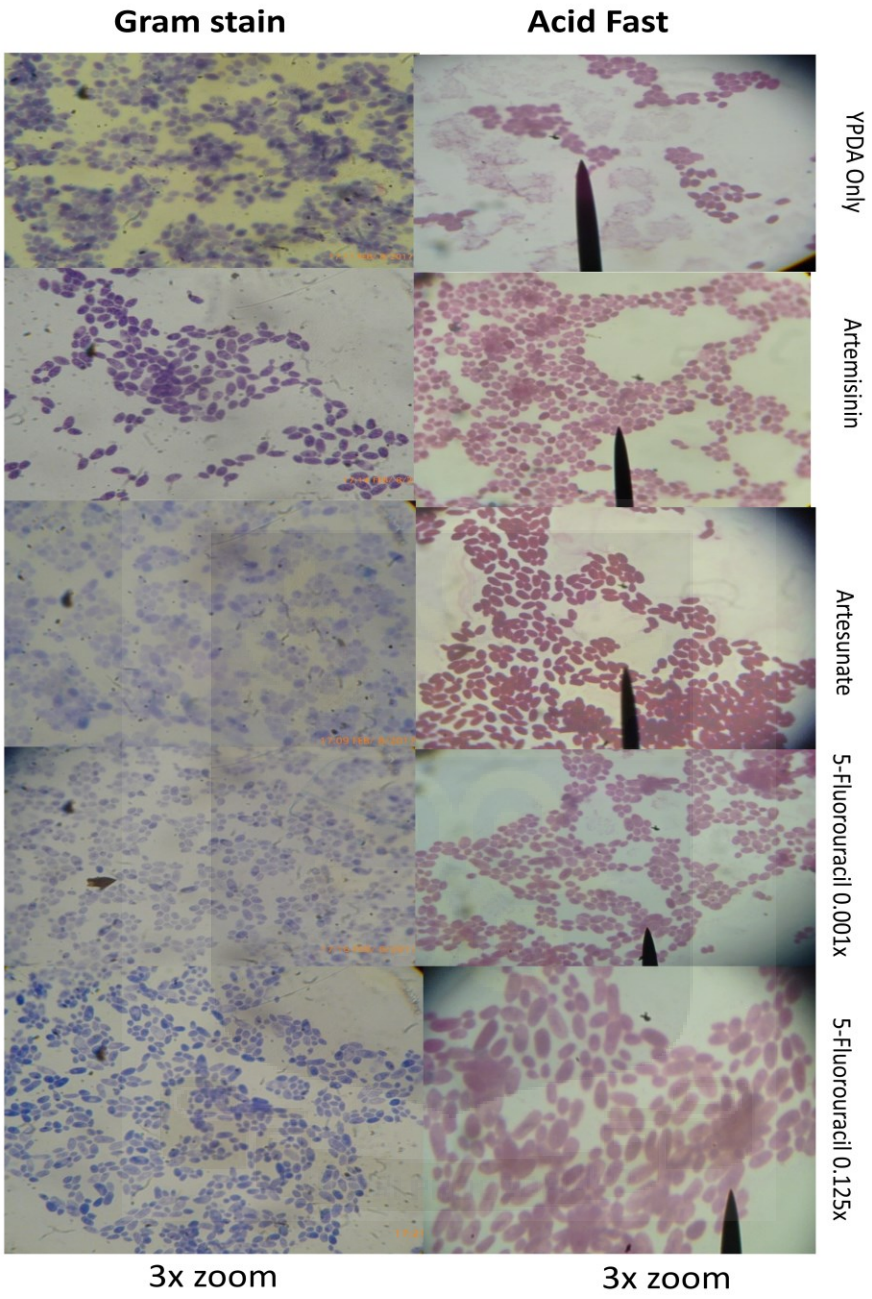


Fig S5.1. Morphological characteristics of fungi grown on chemically modified plates. *C. albicans* was grown on YPDA plates modified with Artenisinin, Artesunate , 5- Fluorouracil (at 2 different concentrations) and the control plate with no chemical modification (YPDA only). The cells were stained with Gram or acid fast stains and viewed under 100 x magnification of the light microscope. Pictures of the slides were taken with a Panasonic lumix digital cameras using the 3x zoom feature.

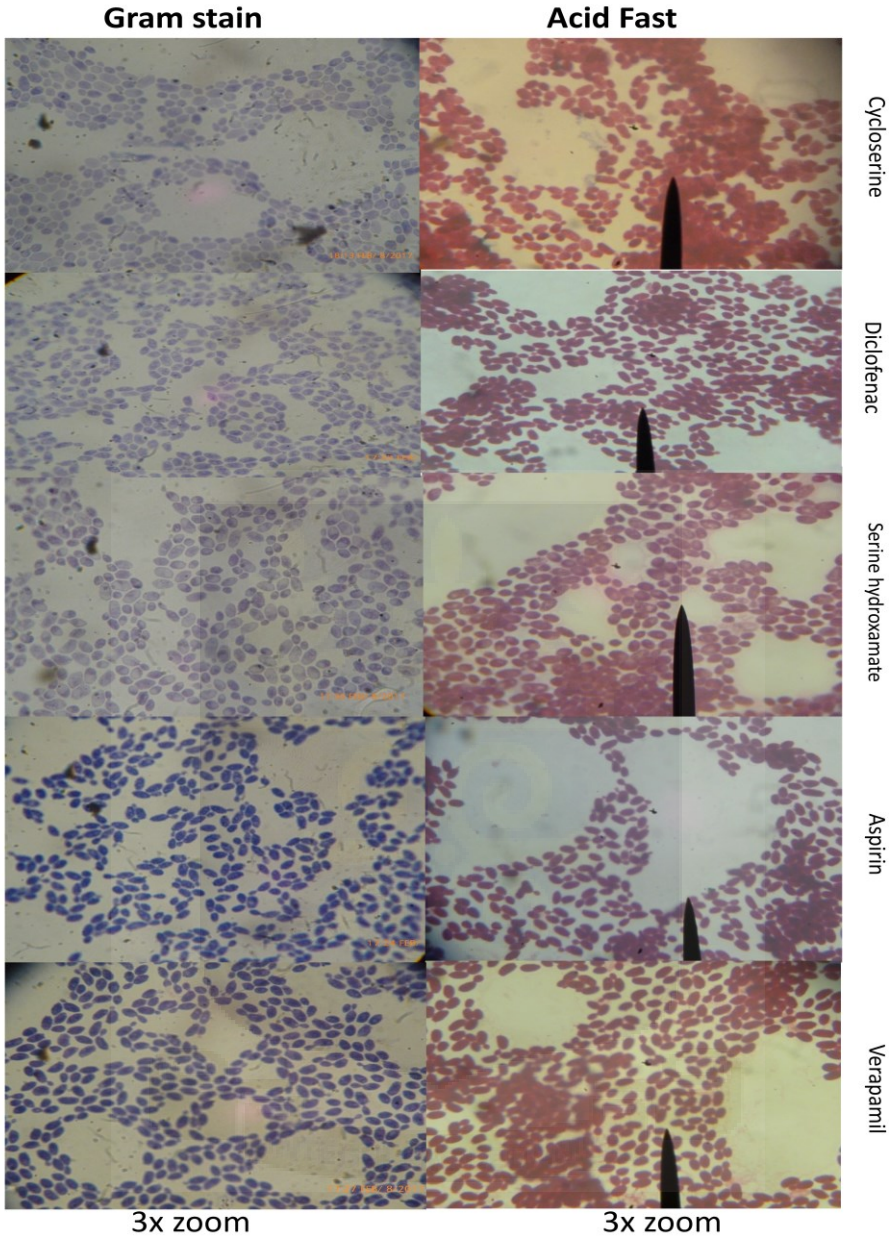


Fig S5.2. Morphological characteristics of fungi grown on chemically modified plates. *C. albicans* was grown on YPDA plates modified with Cycloserine, Diclofenac, Serine hydroxamate, Aspirin and Verapamil. The cells were stained with Gram or acid fast stains and viewed under 100 x magnification of the light microscope. Pictures of the slides were taken with a Panasonic lumix digital cameras using the 3x zoom feature.

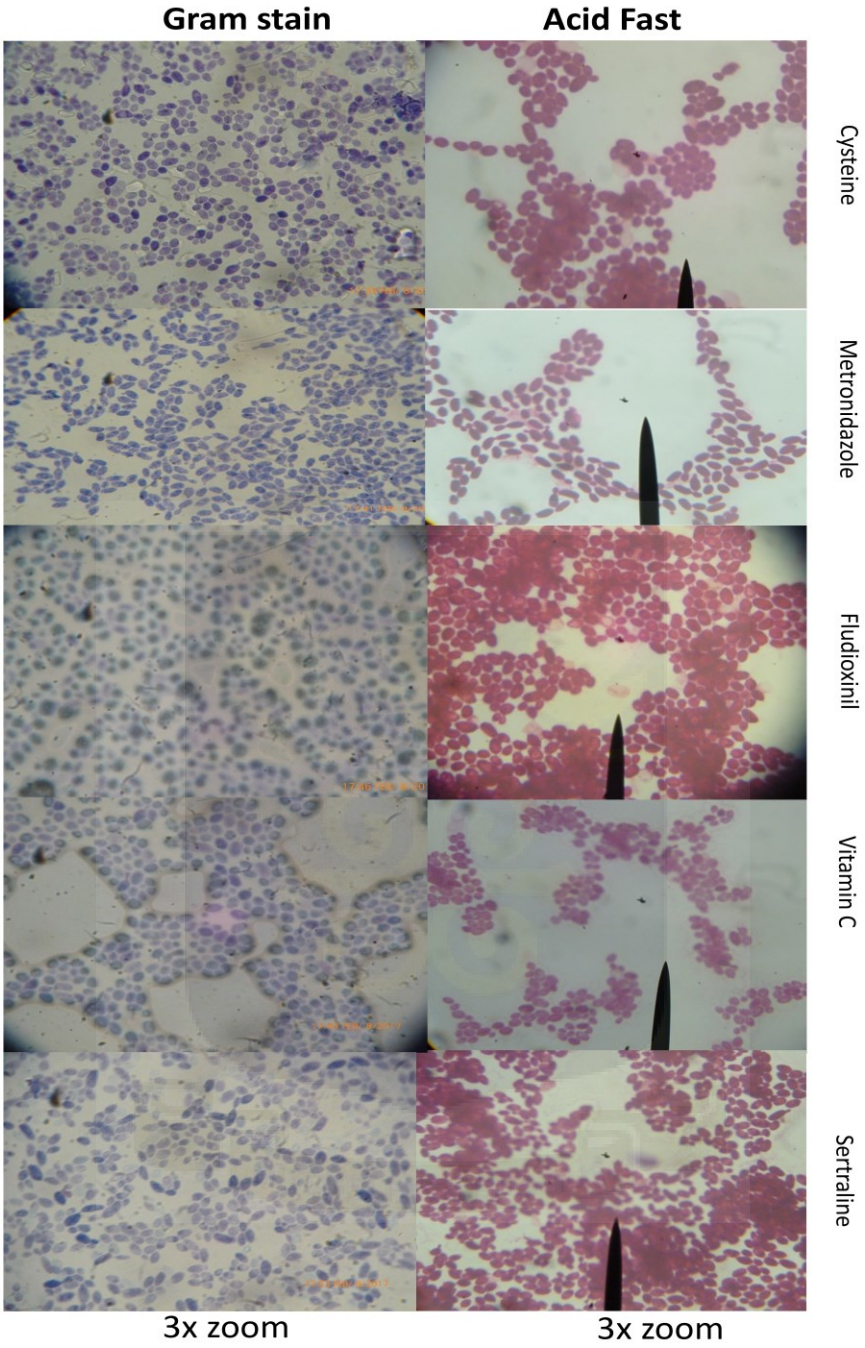


Fig S5.3. Morphological characteristics of fungi grown on chemically modified plates. *C. albicans* was grown on YPDA plates modified with Cysteine, Metronidazole, Fludioxinil, Vitamin C and Sertraline. The cells were stained with Gram or acid fast stains and viewed under 100 x magnification of the light microscope. Pictures of the slides were taken with a Panasonic lumix digital cameras using the 3x zoom feature.

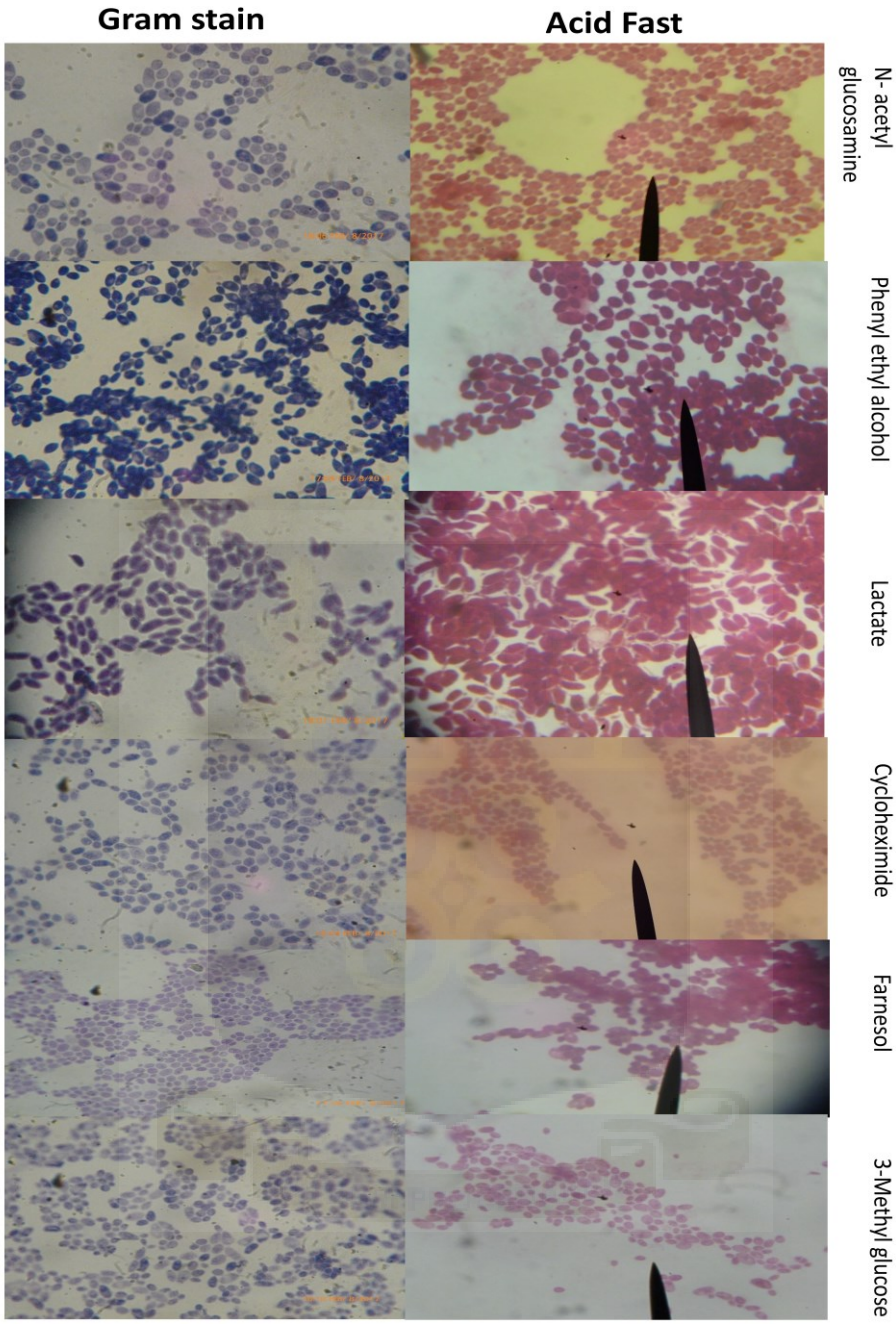


Fig S5.4. Morphological characteristics of fungi grown on chemically modified plates. *C. albicans* was grown on YPDA plates modified with N-acetyl glucosamine, Phenyl ethyl alcohol, Lactate, Cyclohecimide, Farnesol and 3- methyl glucose. The cells were stained with Gram or acid fast stains and viewed under 100 x magnification of the light microscope. Pictures of the slides were taken with a Panasonic lumix digital cameras using the 3x zoom feature.

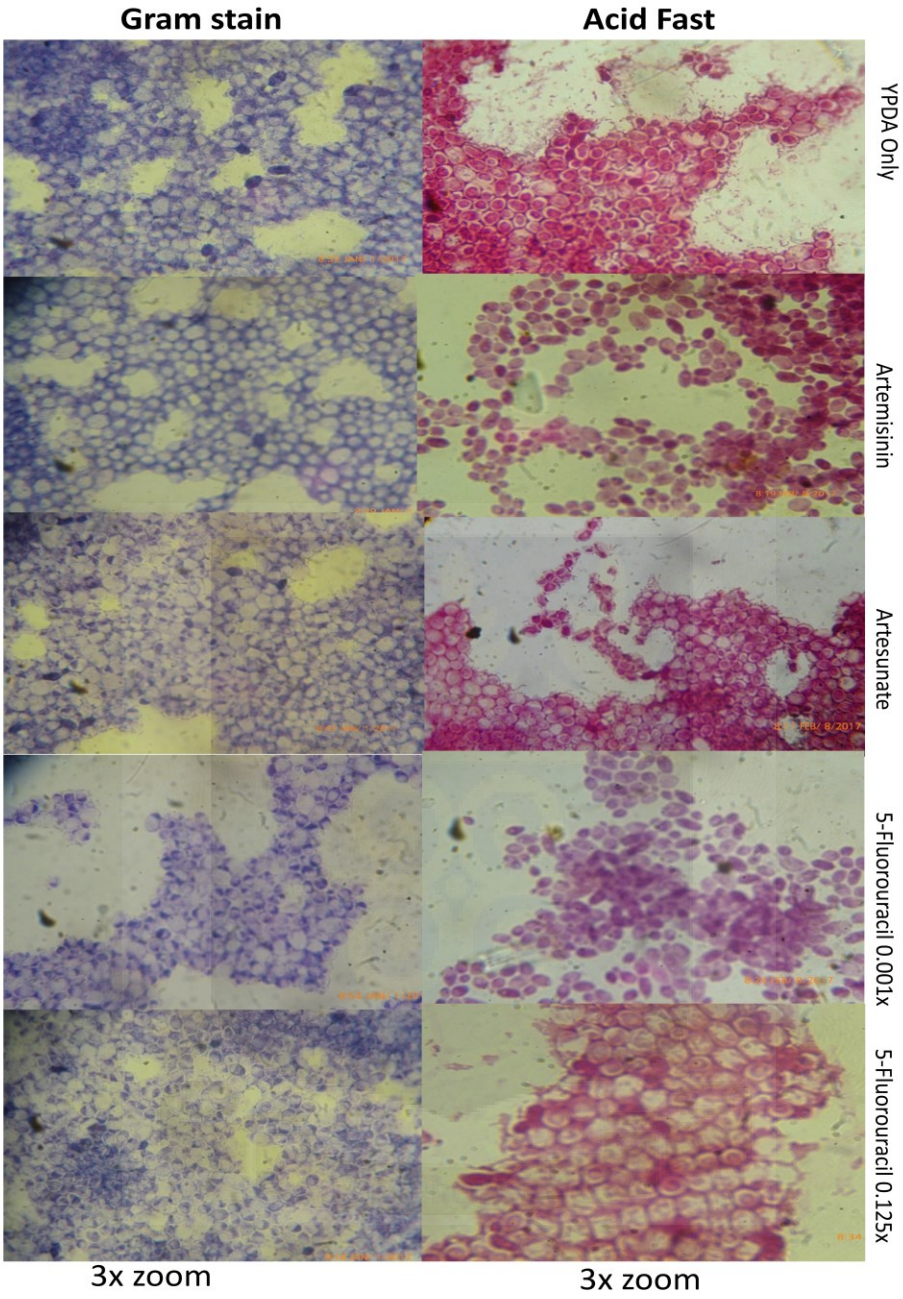


Fig S5.5. Morphological characteristics of fungi grown on chemically modified plates. *S. cerevisiae* was grown on YPDA plates modified with Artenisinin, Artesunate , 5- Fluorouracil (at 2 different concentrations) and the control plate with no chemical modification (YPDA only). The cells were stained with Gram or acid fast stains and viewed under 100 x magnification of the light microscope. Pictures of the slides were taken with a Panasonic lumix digital cameras using the 3x zoom feature.

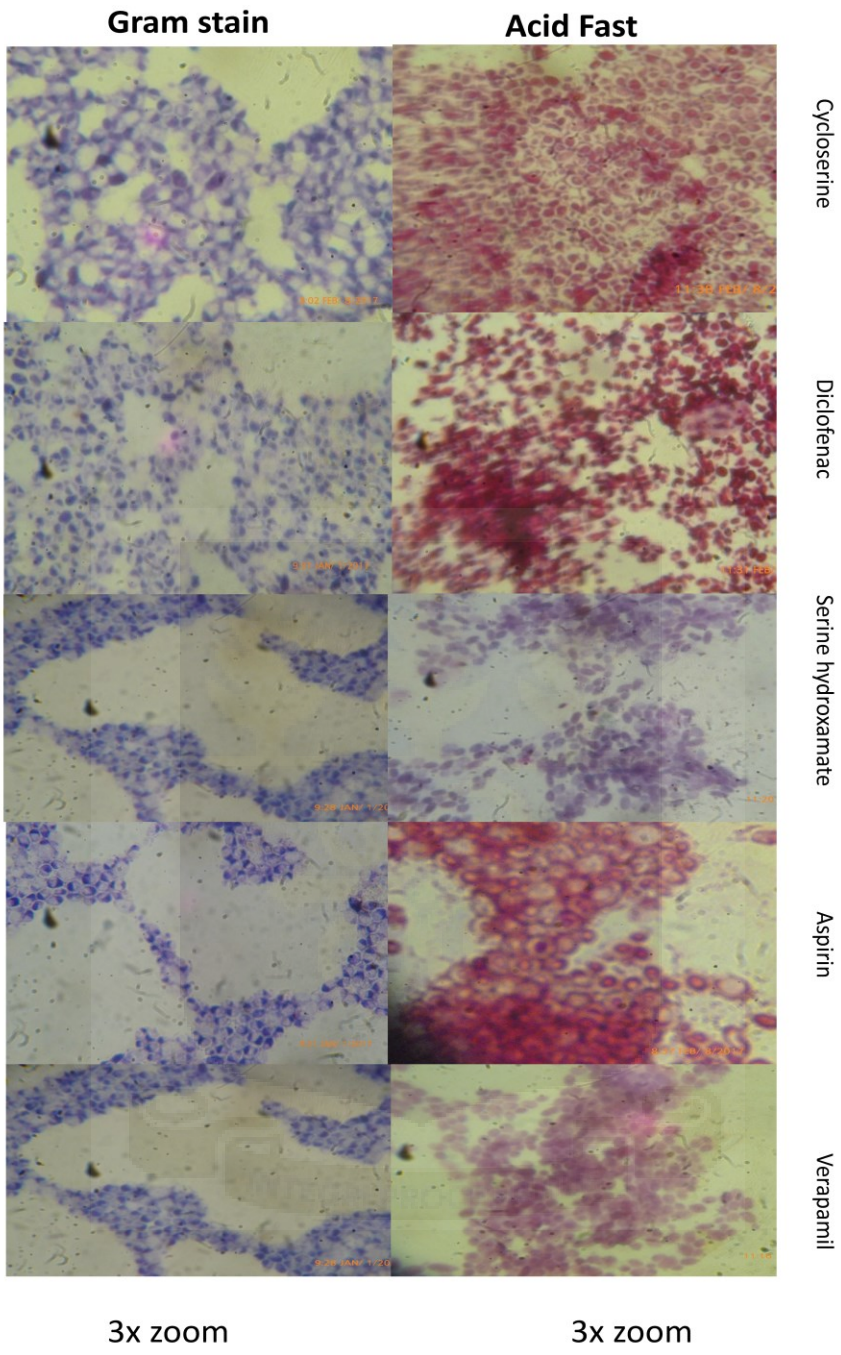


Fig S5.6. Morphological characteristics of fungi grown on chemically modified plates. *S. cerevisiae* was grown on YPDA plates modified with Cycloserine, Diclofenac, Serine hydroxamate, Aspirin and Verapamil. The cells were stained with Gram or acid fast stains and viewed under 100 x magnification of the light microscope. Pictures of the slides were taken with a Panasonic lumix digital cameras using the 3x zoom feature.

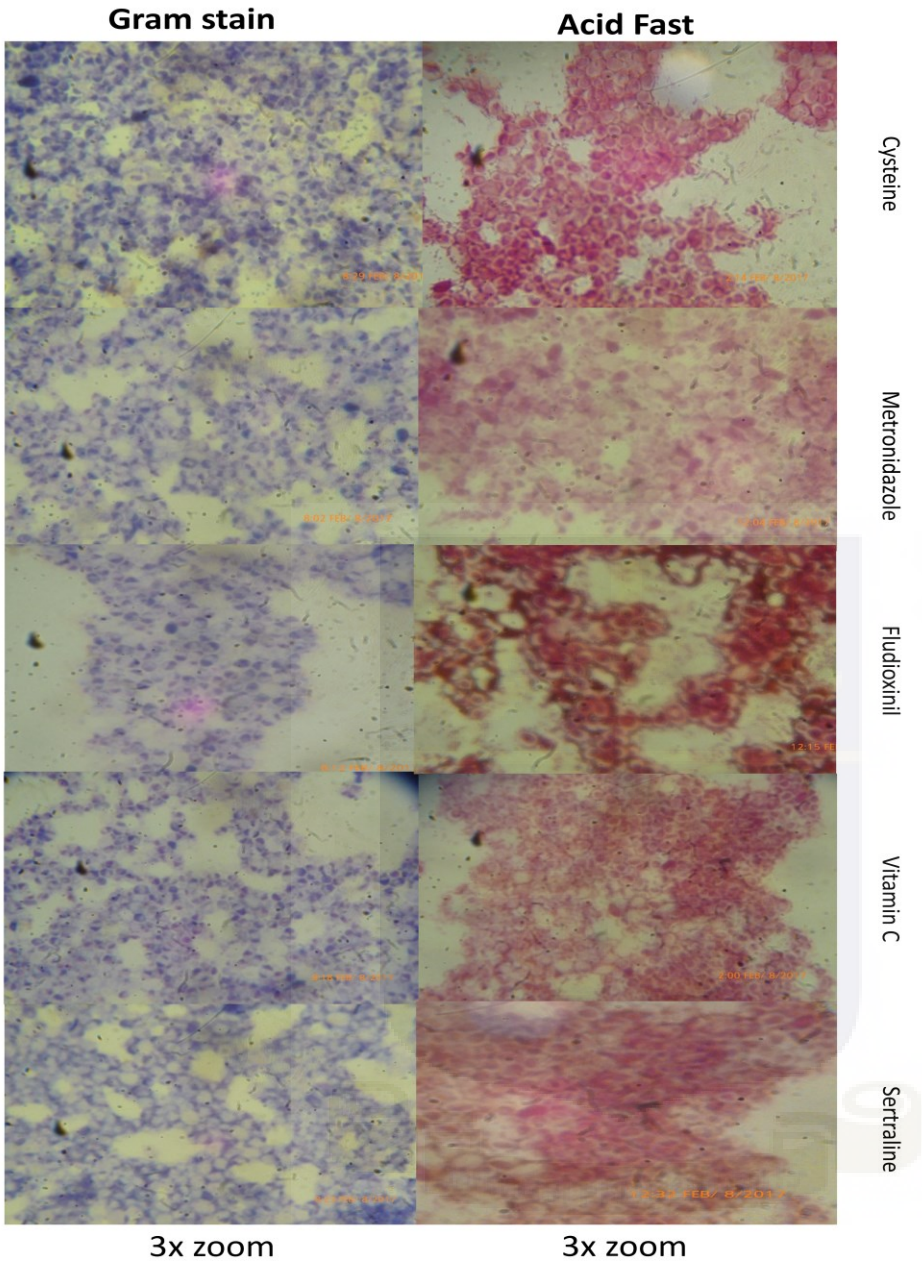


Fig S5.7. Morphological characteristics of fungi grown on chemically modified plates. *S. cerevisiae* was grown on YPDA plates modified with Cysteine, Metronidazole, Fludioxinil, Vitamin C and Sertraline. The cells were stained with Gram or acid fast stains and viewed under 100 x magnification of the light microscope. Pictures of the slides were taken with a Panasonic lumix digital cameras using the 3x zoom feature.

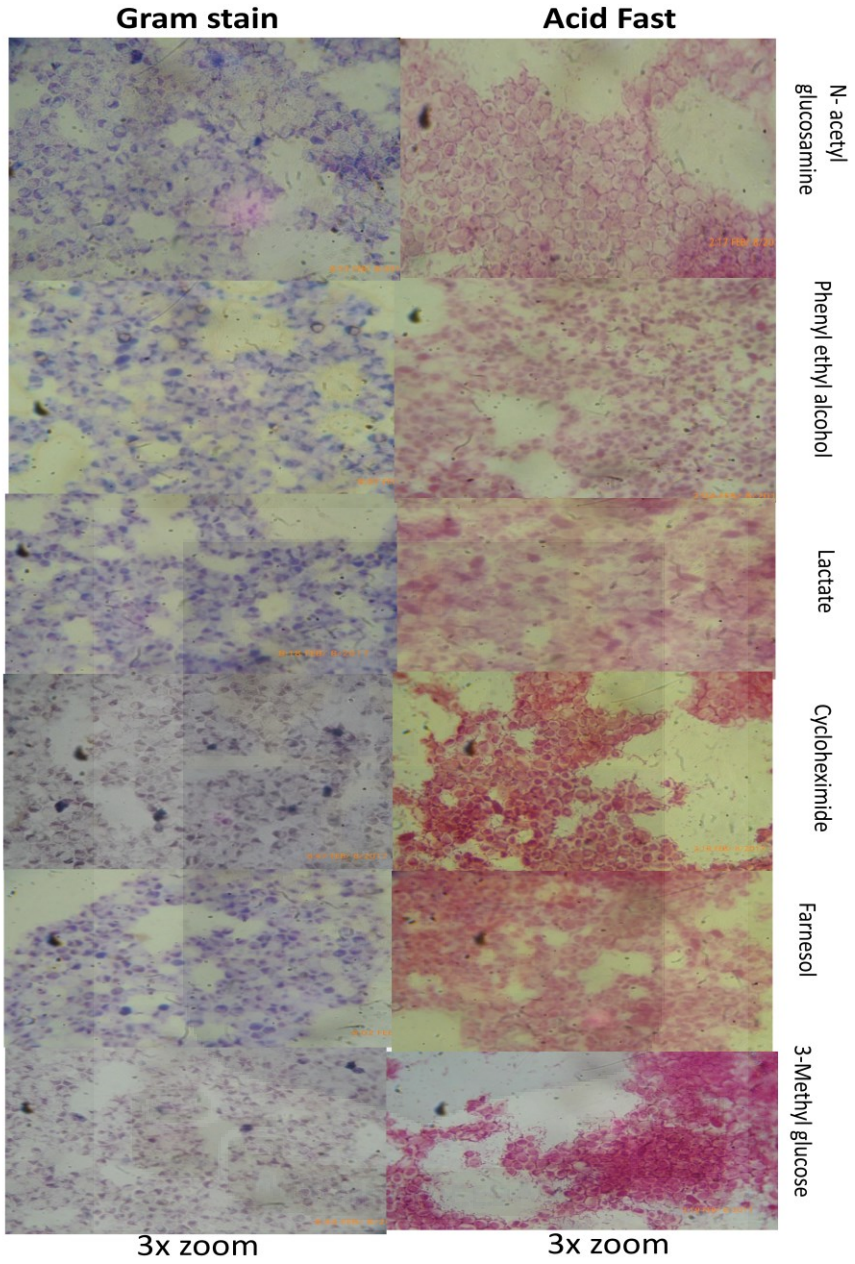


Fig S5.8. Morphological characteristics of fungi grown on chemically modified plates. *S. cerevisiae* was grown on YPDA plates modified with N-acetyl glucosamine, Phenyl ethyl alcohol, Lactate, Cyclohecimide, Farnesol and 3- methyl glucose. The cells were stained with Gram or acid fast stains and viewed under 100 x magnification of the light microscope. Pictures of the slides were taken with a Panasonic lumix digital cameras using the 3x zoom feature.

Article

# CHAPTER SIX

## TRANSCRIPTOMIC ANALYSIS OF MARINE ENDOPHYTIC FUNGI EXTRACT IDENTIFIES HIGHLY ENRICHED ANTI-FUNGAL FRACTIONS TARGETING CANCER PATHWAYS IN HEPG2 CELL LINES

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**Abstract:** Marine endophytic fungi (MEF) from mangrove plants, algae, sponges among others are a good source of structurally unique and biologically active secondary metabolites which suggests therapeutic potential. However, their use in the treatment of human diseases has not yet been explored as compared to their terrestrial counterparts. On the other hand, anti-fungal resistance continues to increase and complicate patient management. In this study we derived partially purified antifungal fractions of extracts from marine endophytic fungal sources and tested their anticancer effects by applying them to the human liver cancer cell line HepG2. The highly enriched fractions were also analyzed with high performance liquid chromatography-high resolution mass spectrometry (HPLC-HRMS) and their effect on the HepG2 cells was assessed on the transcriptome level and with a proliferation assay. We demonstrated that the fractions could reduce proliferation in HepG2 cells. The detailed transcriptome analysis revealed regulation of several cancer- and metabolism-related pathways and gene ontologies. For instance, those down-regulated included, cell cycle, p53 signaling, DNA replication, sphingolipid metabolism and drug metabolism – cytochrome. The upregulated pathways included HIF-1 signaling, focal adhesion, necroptosis and transcriptional mis-regulation of cancer. Furthermore, we could construct a protein interaction network based on 26 proteins distinguishing the three investigated treatments from the control cells. This network is composed of central functional components associated with metabolism and cancer such as TNF, MAPK, TRIM21 and one component containing APP.

**Keywords:** Marine endophytic fungi; seaweed; anti-cancer extract; anti-fungal resistance; HepG2; proliferation

## 6.1. Introduction

The majority of neglected tropical diseases are caused by fungal pathogens as well as helminths and protozoan. Drug discovery effort that uses fungal cells as target has the potential of finding compounds that targets many pathogenic fungi in addition to the other eukaryotic fungi since they share cellular targets. Marine endophytic fungi (MEF) which can be derived from mangrove plants, algae, sponges among others have not been explored as compared to their terrestrial counterparts in the treatment of human diseases [1]. They are interesting because they have been proven to be a good source of structurally unique and biologically active secondary metabolites [1]. On the other hand, anti-fungal resistance continues to increase and complicate patient management [2]. Although new antifungal agents

have been introduced to combat this problem, the development of resistance to anti-fungal drugs has increased, particularly in patients who require long-term treatment of patients with severe immunosuppression [2]. Hence, there is a global demand for new antifungal agents that eliminates fungal pathogens from a host with minimal toxicity to the host. However, such ideal antifungals are difficult to develop because fungi are eukaryotes hence any substance toxic to fungi is also most likely to be toxic to the host [3]. Therefore, the desirable antifungal agent should be able to differentially target the fungal pathogen.

Screening for anticancer agents has progressed from general experimental procedures to target-oriented screens. This has led to the approval of many molecularly targeted drugs. Cancer chemotherapeutics discovery focuses on identifying compounds which target many cancer pathways while causing minimal toxicity to non-cancer cells [4]. Humans and yeast have similar genes including those known to be involved in cell proliferation and cancer [5]. *Saccharomyces cerevisiae* is used as a model for investigating many processes of humans. These processes include cell cycle progression, DNA replication and segregation, maintenance of genomic integrity and stress responses. In cancer, these processes are affected by genetic and epigenetic alterations. Hence, yeast can be used as a predictor of anti-cancer activity of novel compounds [6].

In this project we identified antifungal fractions from marine endophytic fungi sources with many potential novel compounds, tested their capability of reducing proliferation in the established human liver cancer cell line HepG2 and analyzed the effect of the treatment on the transcriptome level. The preliminary evidence presented in this study will inform future effort to scale up the fermentation of this fungal isolate to purify the potential novel compounds for detailed characterization.

## 6.2. Results

### 6.2.1 Antifungal activity of the MEF 134 crude extract and FD

Several rounds of preliminary screening of 143 MEF isolates was carried out to identify the most active extract as that from MEF 134 isolate (manuscript #1 in preparation). This was based on the evaluation using the phenotypic array bioassay (manuscript #2 in preparation). The crude extract (MEF 134) showed antifungal activity against *Candida albicans* the pathogenic fungi with a maximum zone of inhibition of 9 mm (Table 6.1 A). However, it was not active against *Saccharomyces cerevisiae* the nonpathogenic fungi (Table 6.1). The entire separation work has been summarized in Fig 6.1 to streamline the workflow on which the bioassay data was generated. Two rounds of Kupchan fractionation were conducted with 7.74 g of extracts as starting material for the first round and 18.3 g for second round (Fig 6.2). An amount of 2.58 g of the Dichloromethane fraction (MEF 134 FD) from the round 1 sample was run on silica gel (TLC).

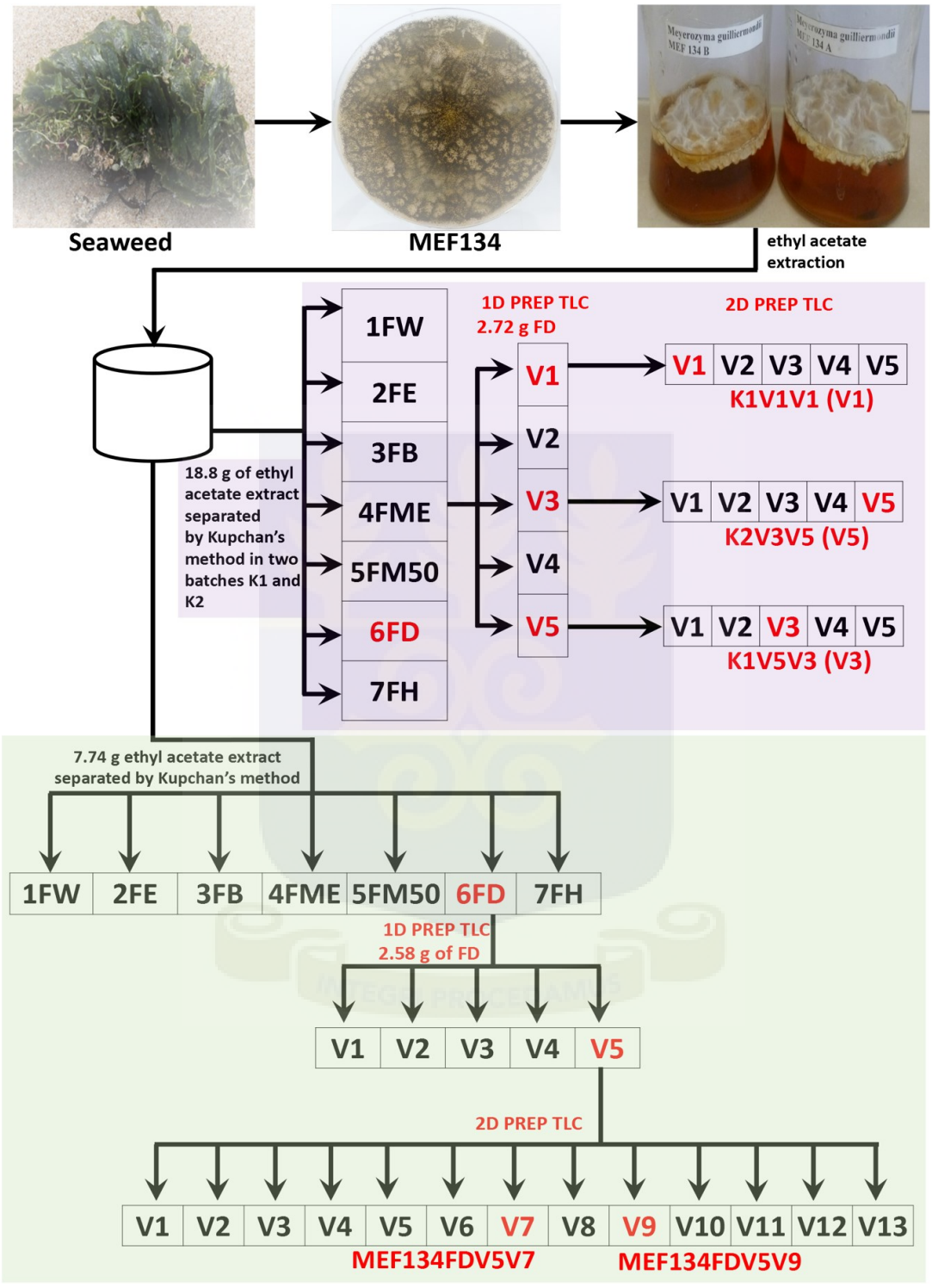



Figure 6.1. Schematic of the purification process. MEF 134 isolate from seaweed was cultured in 20 L of broth. The crude extract was separated by Kupchan and two rounds prep-TLC fractionation. Samples marked in red were selected based their antifungal activities and further analysed using transcriptomics and mass spectromtery.

All the dichloromethane (DCM) fractions obtained from the Kupchan fractionation analysis were active against both *Candida albicans* and *Saccharomyces cerevisiae* (Table 6.1). The 1D TLC fraction FD K1V1 was only active against *Saccharomyces cerevisiae* while FD K1V5 was active against *Candida albicans* (Table 6.1). However, FD K2V3 was active against both organisms recording a zone of 12.5 and 10 mm respectively against *Candida albicans* and *Saccharomyces cerevisiae* (Table 6.1).

**Table 6.1. Antifungal activity of MEF 134 Crude extract and DCM fractions (1 TLC).**

Antifungal activity of MEF 134 Crude extract and DCM fractions ( 1 TLC)

| MEF 134 Samples | Zone of Inhibition in mm (Disc size 5 mm) | |
|---|---|---|
| | *Candida albicans* | *Saccharomyces cerevisiae* |
| Crude extract | 9 | 0 |
| FD-K1 | 12 | 10.5 |
| FD-K2 | 8 | 6.5 |
| FD K1V1 | 0 | 11.5 |
| FD K2V3 | 12.5 | 10 |
| FD K1V5 | 18.5 | - |

Fluconazole (5µg) = 23 mm

The 1-dimension TLC fractions were selected for further TLC fractionation. None of the 2D TLC fractions from FDK1V1, FDK1V5 and FDK2V3 were active against *Candida albicans* however, they were all active against *Saccharomyces cerevisiae* (Table 6.2). Two other fractions FD V5V7 and 134 FD V5V9 were active against *Candida albicans* (Table 6.2). Partially purified fractions FDV5V7 and FDV5V9 were obtained from the TLCs conducted (Fig. 6.2a).

The second round of Kupchan fractionation was conducted with 18.3 g of extract as new starting material obtained repeating the extraction of the matured culture. Here 2.7 g of the dichloromethane fraction obtained was again separated on the preparative plates made of aluminum oxide. It turned out to be difficult to elute the fractions from the aluminum oxide stationary phase. Hence 12 different solvent systems were employed to perform the elutions since it takes considerable amount of time and resources to obtain the extract. The active fractions obtained (Fig 6.2b) were analyzed with an antifungal bioassay and High-performance liquid chromatography (HPLC) together with High resolution mass spectrometry (HRMS) (Table 6.2 and Figure 6.3).

**Table 6.2. Antifungal activity of the DCM fractions (FD) of MEF 134 (2 TLC).**

Antifungal activity of the DCM fractions (FD) of MEF 134 (2 TLC)

| MEF 134 Fractions | Zone of Inhibition in mm (Disc size 5 mm) | |
|---|---|---|
| | *Candida albicans* | *Saccharomyces Cerevisiae* |
| FD V5V7 | 8 | - |
| FD V5 V9 | 13 | - |
| FD K1V1V1 | 0 | 10.5 |
| FD K1V5V3 | 0 | 8.5 |
| FD K2V3V5 | 0 | 11 |

Fluconazole (5µg) = 23 mm

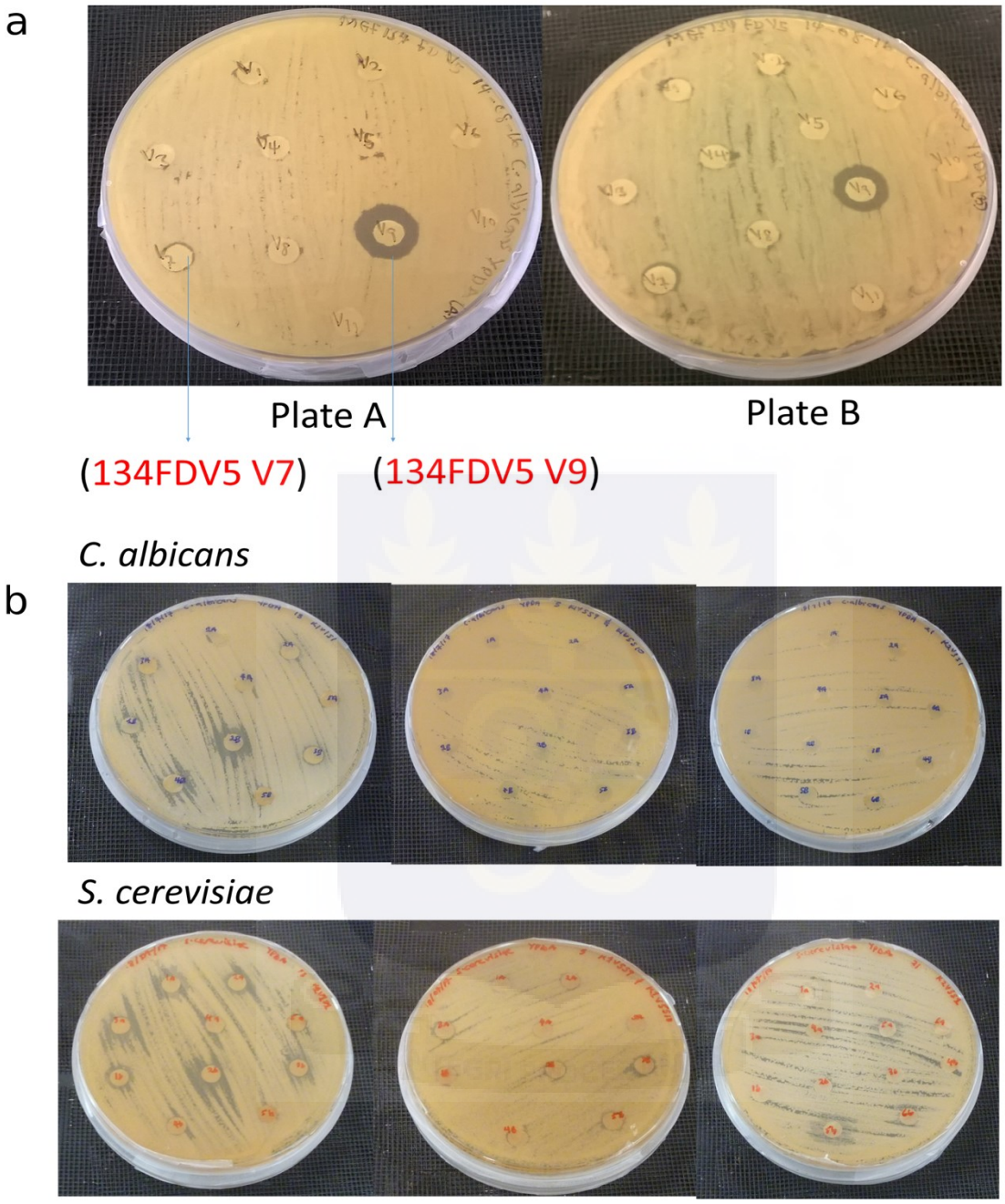



Figure 6.2. Representative photo showing the inhibition of growth of *C. albicans* and *S. cerevisiae* by the antifungal fractions from MEF 134. (a) Growth inhibition of C. albicans by FDV5V7 and FDV5V9 fractions. (b) Plate cultures showing the antifungal activities of FDK1V1V1, FDK1V5V3 and FDK2V3V5 against S. Cerevisiae and C. albicans.

### 6.2.2 HPLC- HRMS analysis of MEF 134 dichloromethane fraction and the 2 DTLC fractions

For the first selected fraction, FD K1V1V1, there were 11 major peaks detected. However, 5 of these major peaks were important. These peaks were important because of their interesting fragmentation patterns. The fragmentation pattern of the masses is complex (Data not shown). Also, FD K1V5V3, had 6 major peaks. Three of these major peaks were interesting also because of fragmentation pattern. For FD K2V3V5, there were 11 major peaks detected (Fig 6.3 c). Approximately 17 major peaks were detected after the analysis of the MEF134 FD samples (Fig 6.3 d).

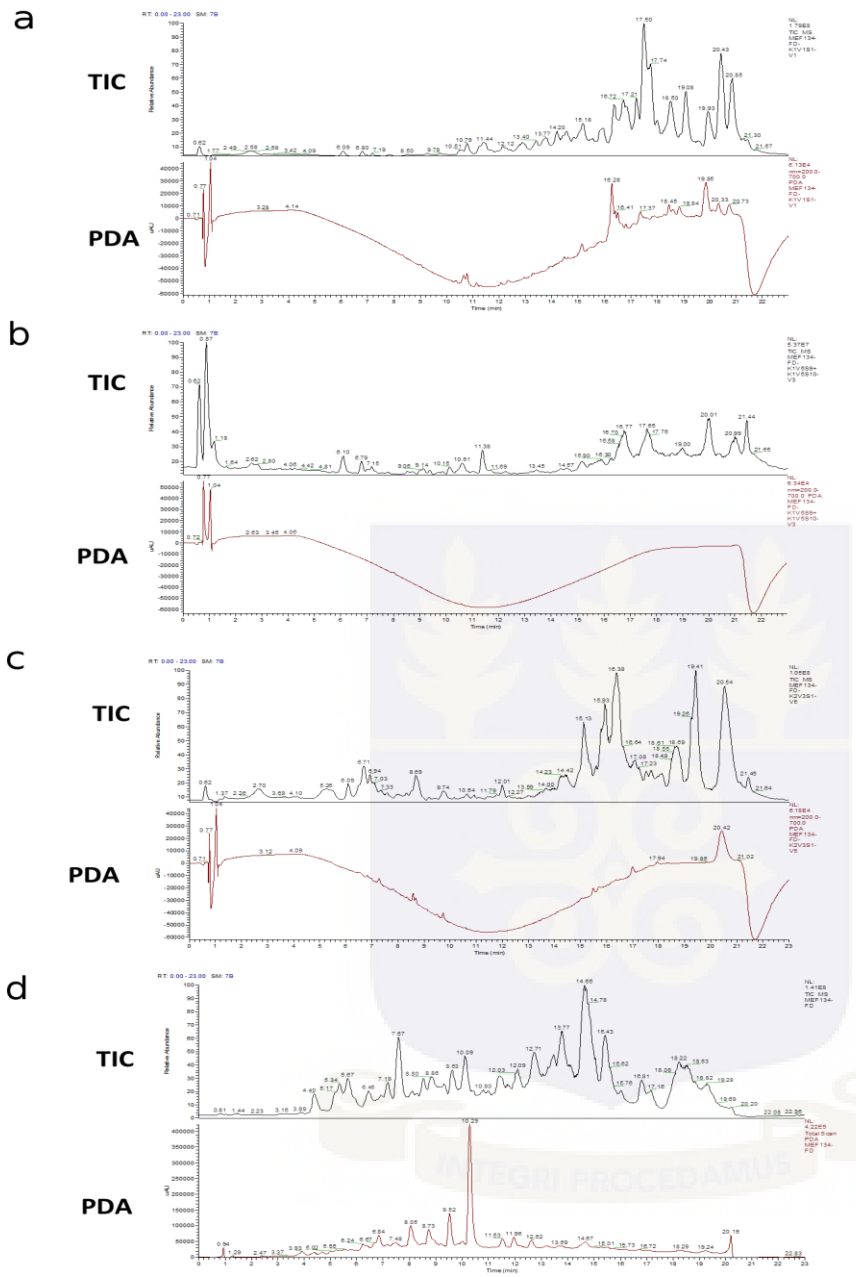



Figure 6.3. HPCL-HRMS full scan analysis of the MEF 134 Dichloromethane fraction and the 2 DTLC fractions of MEF 134 FD. (a) The profile of the FD K1V1V1 fraction. (b) The profile of the FD K1V5V3 fraction. (c) The profile of the FD K2V3V5 fraction. (d) The profile of the MEF 134 FD fraction.

### 6.2.3 Proliferation of HepG2 cells after treatment with the various fractions

Proliferation of HepG2 cells after treatment with FD K1V1V1 (V1), FD K1V5V3 (V3) and FD K2V3V5 (V5) (Fig 6.4a). All three fractions reduced HepG2 cell proliferation. However, the reduction in cell proliferation was to some extent in a dose dependent manner for fractions V1 and V3 (Fig 6.4b & c). Reduction in cell proliferation was in a clear dose dependent manner for V5 (Fig 6.4d).

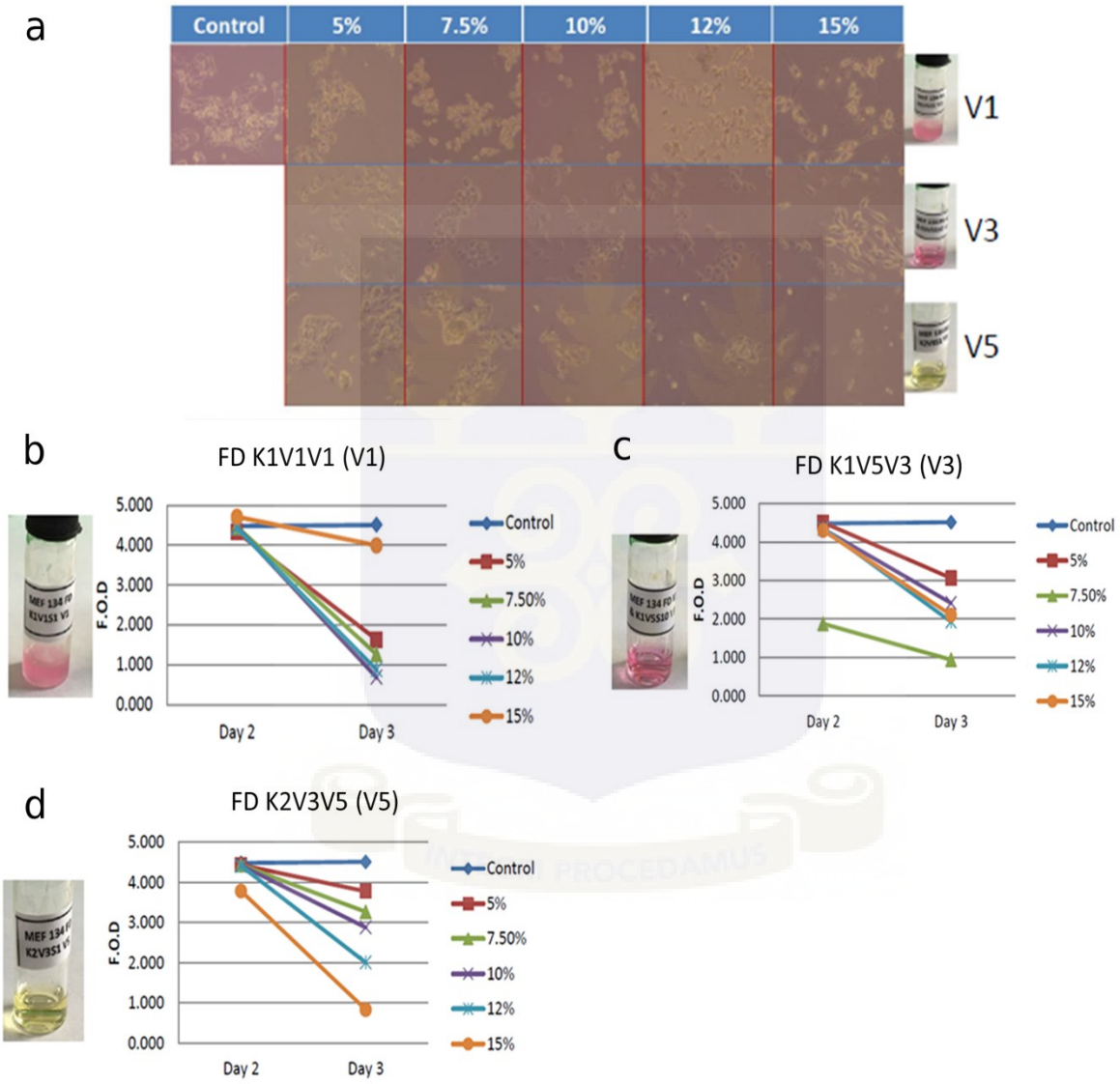



Figure 6.4. Inhibition of HepG2 cell proliferation by the three active fractions from FD K1V1V1 (V1), FD K1V5V3 (V3) and FD K2V3V5 (V5). All three had antifungal activity against S. cerevisiae only.

### 6.2.4 Cell viability after treatment with the three fractions

HepG2 cells were treated with 2% of every fraction in order to assess their respective influences on the cell viability and proliferation capacity. To this end, the expression of 4 key markers, namely P53, KI67, Caspase3, and CDKN1B was analyzed by qRT-PCR (Fig 6.5). Expression of all 4 genes was reduced in every condition compared to untreated cells.

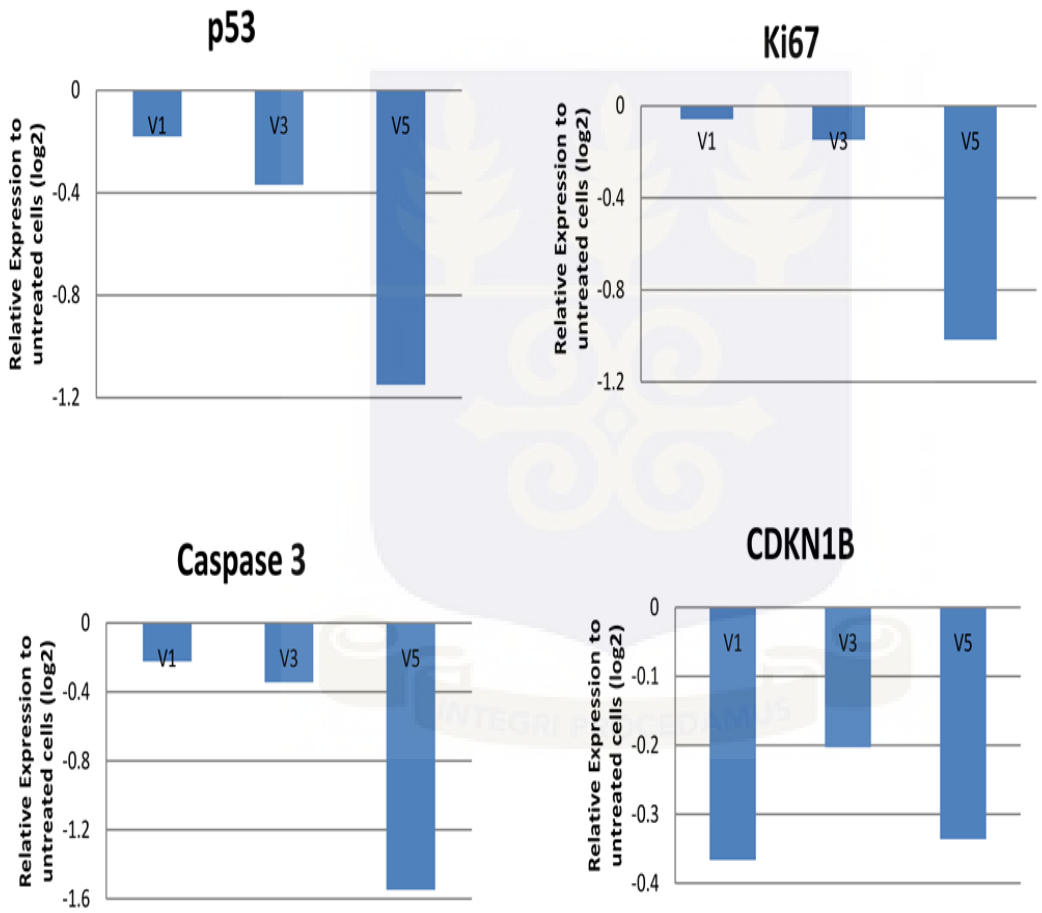
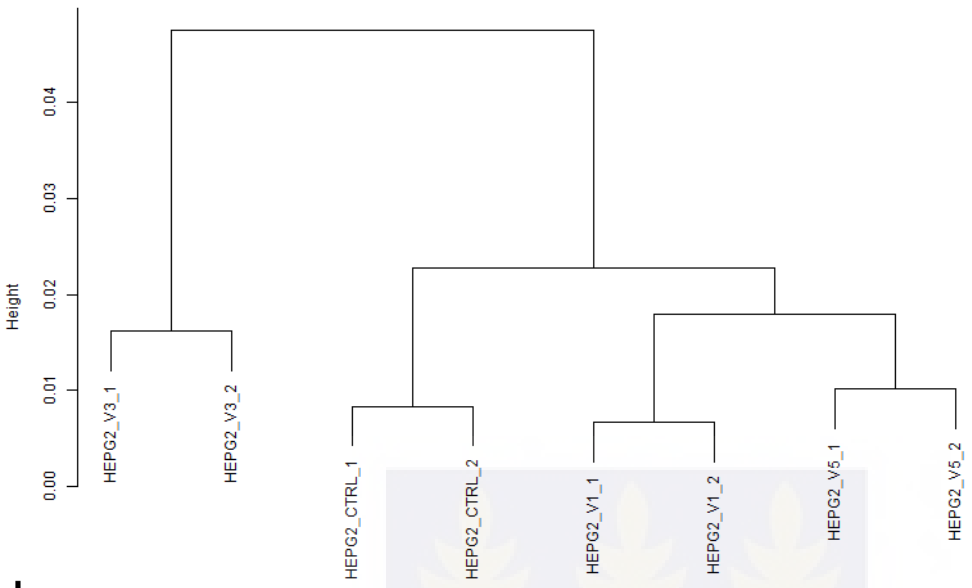



Figure 6.5. qRT-PCR of cell viability markers. HepG2 cells were treated with 2% of every fraction and qRT-PCR was performed for the indicated genes. Gene expression was normalized to RPL37A and fold change was calculated respective to untreated controls.

### 6.2.5 Transcriptome analysis

Cluster analysis of RMA-normalized microarray data showed that duplicates cluster together - as expected (Fig 6.6a). HepG2 cells treated with V1 and V5 come together in a cluster and thus have the highest similarities between experiments. The V1-V5-cluster is then further extended by HepG2 controls demonstrating higher similarity of V1 and V5 with control than with V3 treated HepG2 cells. HepG2 cells treated with V3 cluster separately suggesting that V3 treatment has the greatest effect on the whole transcriptome level. This is reflected by the Pearson correlation table (Fig 6.6b) where the V3 treatment has the lowest correlation to all other samples. Furthermore, the V1 treatment has over all the highest correlation with V5 treatment although there is intra-sample variability and there is one V1 sample having a slightly higher correlation to control than to V5.

a




b

| sample | HEPG2_CTRL_1 | HEPG2_CTRL_2 | HEPG2_V1_1 | HEPG2_V1_2 | HEPG2_V3_1 | HEPG2_V3_2 | HEPG2_V5_1 | HEPG2_V5_2 |
|---|---|---|---|---|---|---|---|---|
| HEPG2_CTRL_1 | 1 | 0.996042795 | 0.99357682 | 0.99158639 | 0.98786236 | 0.98077523 | 0.99066198 | 0.99469222 |
| HEPG2_CTRL_2 | 0.996042795 | 1 | 0.99337992 | 0.99350966 | 0.99146503 | 0.98577988 | 0.99559328 | 0.99536071 |
| HEPG2_V1_1 | 0.993576817 | 0.993379922 | 1 | 0.99686531 | 0.98850559 | 0.98385013 | 0.9921684 | 0.99524881 |
| HEPG2_V1_2 | 0.991586394 | 0.993509658 | 0.99686531 | 1 | 0.99262217 | 0.989201 | 0.99402391 | 0.99525778 |
| HEPG2_V3_1 | 0.987862355 | 0.991465029 | 0.98850559 | 0.99262217 | 1 | 0.99394583 | 0.99175371 | 0.99088234 |
| HEPG2_V3_2 | 0.980775231 | 0.985779883 | 0.98385013 | 0.989201 | 0.99394583 | 1 | 0.98765441 | 0.98652732 |
| HEPG2_V5_1 | 0.990661985 | 0.995593278 | 0.9921684 | 0.99402391 | 0.99175371 | 0.98765441 | 1 | 0.9950695 |
| HEPG2_V5_2 | 0.994692216 | 0.995360713 | 0.99524881 | 0.99525778 | 0.99088234 | 0.98652732 | 0.9950695 | 1 |

Figure 6.6. Treatment of HepG2 cells with V3 induced the highest transcriptional differences compared to V1 and V5. (a) RMA-normalized microarray data was subjected to a cluster analysis using complete linkage as agglomeration method and Pearson correlation as similarity measure. The dendrogram shows that duplicates cluster together - as expected - and that V1 and V5 fall together in a cluster which is further extended than HepG2 controls. HepG2 treated with V3 clusters separately. (b) The Pearson correlation table reflects the dendrogram structure showing that V3 has the lowest correlation to all other samples. However, all correlation coefficients are close to the possible maximum of 1 demonstrating a high overall similarity of the samples.

The Venn diagram analysis of genes expressed at a detection p value threshold of $p < 0.05$ showed that in all comparisons by far the most genes were expressed in common and that fewer genes were expressed in the treatment cases than in the control (Fig. 6.7, Tables S6.1-S6.4). From the pairwise

comparisons with control (Fig. 6.7a-c) the comparison HepG2 treated with V1 vs. HepG2 control (Fig. 6.7a, Table S6.1) resulted in the most genes expressed in common (14453) and the comparison HepG2 treated with V3 vs. HepG2 control (Fig. 6.7b, Table 6.S2) resulted in the fewest genes expressed in common (14146). This may further confirm that the V3 treatment has the biggest effect on the transcriptome (Fig. 6.7b, Table 6.S2). Moreover, expressed genes in all three treatments and control were compared in a Venn diagram (Fig. 6.7d, Table 6.S4). In this comparison again most (13699) genes were expressed in common in V1, V3, V5 and control. From the subsets expressed exclusively in only one experiment most genes (285) were in the control followed by 123 genes in the V3 treatment.

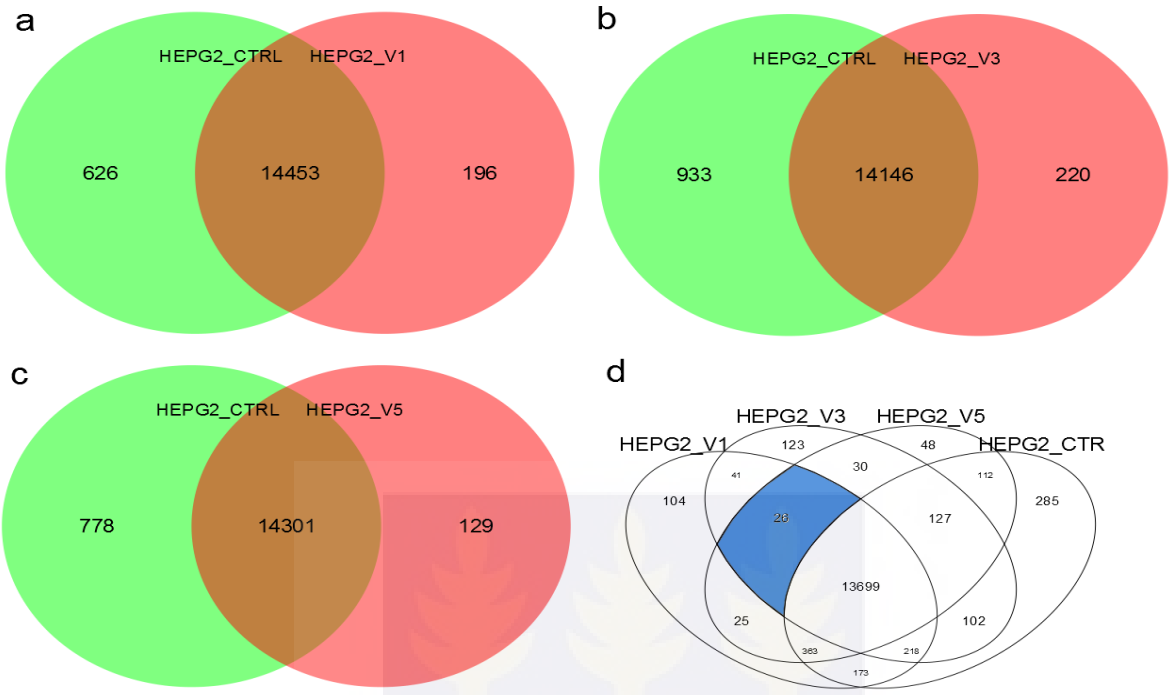



Figure 6.7. Treatments of HepG2 cells with V1, V3 and V5 result in regulated distinct treatment-specific genes and a common treatment signature. Expressed genes were detected using a detection p value threshold of $p < 0.05$. In all comparisons by far the most genes were expressed in common. Fewer genes were expressed in the treatment cases than in the control. (a) In the comparison HepG2 treated with V1 vs. HepG2 control 196 genes were expressed in the V1 treatment, 626 genes in the control and 14453 in common. (b) In the comparison HepG2 treated with V3 vs. HepG2 control 220 genes were expressed in the V3 treatment, 933 genes in the control and 14146 in common. (c) In the comparison HepG2 treated with V5 vs. HepG2 control 129 genes were expressed in the V5 treatment, 778 genes in the control and 14301 in common. (d) In the comparison of all HepG2 treatments (V1, V3, V5) with HepG2 control 104 genes were exclusively expressed in the V1 treatment, 123 genes in the V3 treatment, 48 genes in the V5 treatment and 285 genes in the control. 13699 genes were expressed in common in V1, V3, V5 and control. The common treatment signature consists of 26 genes expressed in common in V1, V3 and V5 but not in control (marked blue).

### 6.2.6 Overrepresented pathways and gene ontologies (GOs)

Overrepresentation analysis for KEGG pathways in the up- and down-regulated genes (ratio < 0.75; ratio > 1.33; p-value < 0.05) from the intersection sets of the pairwise Venn diagrams demonstrated that treatments of HepG2 cells with V1, V3 and V5 have impact on many cancer- and metabolism-related pathways (Fig. 6.8a-c, Tables S6.5-S6.10).

### 6.2.7 Genes and associated pathways regulated by treatment with V1

In V1 treatment cancer-related pathways such as *p53* and *chemical carcinogenesis* and metabolic pathways such as *cytochrome P450 metabolism* emerge in the down-regulated genes while metabolic pathways such as *Arginine, Retinol* and *Insulin resistance* emerge in the up-regulated genes (Fig. 6.8a, Tables S6.5-S6.6). Visual inspection of the pathway charts showed that nearly the whole *Steroid Biosynthesis* pathway (Fig S6.1) is down-regulated. Interestingly, in *Drug metabolism – cytochrome P450* (Fig S6.2), in the cyclophosphamide subsystem ALDH3B, ADH4 and GSTA1 are down-regulated.

### 6.2.8 Genes and associated pathways regulated by treatment with V3

In V3 treatment cancer-related pathways such as *cell cycle*, *Mismatch repair* and *Viral carcinogenesis* emerge in the down-regulated genes and also in the up-regulated genes cancer-related pathways such as *MAPK-signaling* and *Transcriptional misregulation in cancer* come up (Fig. 6.8b, Tables S6.7-S6.8). The chart of the *cell cycle* pathway (Fig. S6.4) shows down-regulation of most genes involved in this pathway thus suggesting reduced cell cycle activity. In all subsystems (*Hepatitis B/C, Epstein-Barr virus, human Papillomavirus, human T*

*Lymphotrophic virus Type I* and *Kaposi's sarcoma-associated Herpesvirus*) of the *viral carcinogenesis* pathway (Fig. S6.5) genes are significantly down-regulated.

### 6.2.9 Genes and associated pathways regulated by treatment with V5

In V5 treatment Cancer-related pathways such as *cell cycle* and various other cancers emerge in the up-regulated genes while various metabolic pathways including *cytochrome P450 metabolism* emerge in the down-regulated genes (Fig. 6.8c). Interestingly, contrary to V3 treatment in V5 treatment up-regulated genes are significantly overrepresented in the *cell cycle* pathway. Regarding this in detail in the *cell cycle* pathway chart (Fig. S6.6, Tables S6.9-S6.10) demonstrates that fewer and other genes than in V3 are up-regulated, particularly *TGFB1* and the cyclin-dependent kinase inhibitors *CDKN1A* and *CDKN2C*.

### 6.2.10 Common Gene ontologies over-represented in all treatments

Furthermore, we were interested in the set of genes expressed in common in all three treatments but not in the control in order to find commonality of all treatments distinguishing them from the control. The corresponding set of 26 genes expressed in common in all treatments (V1, V3 and V5) but not in the HepG2 control revealed overrepresentation of gene ontologies annotated with cancer-associated *MAPK/ERK-signaling* (Fig. 6.8d, Table S6.11).

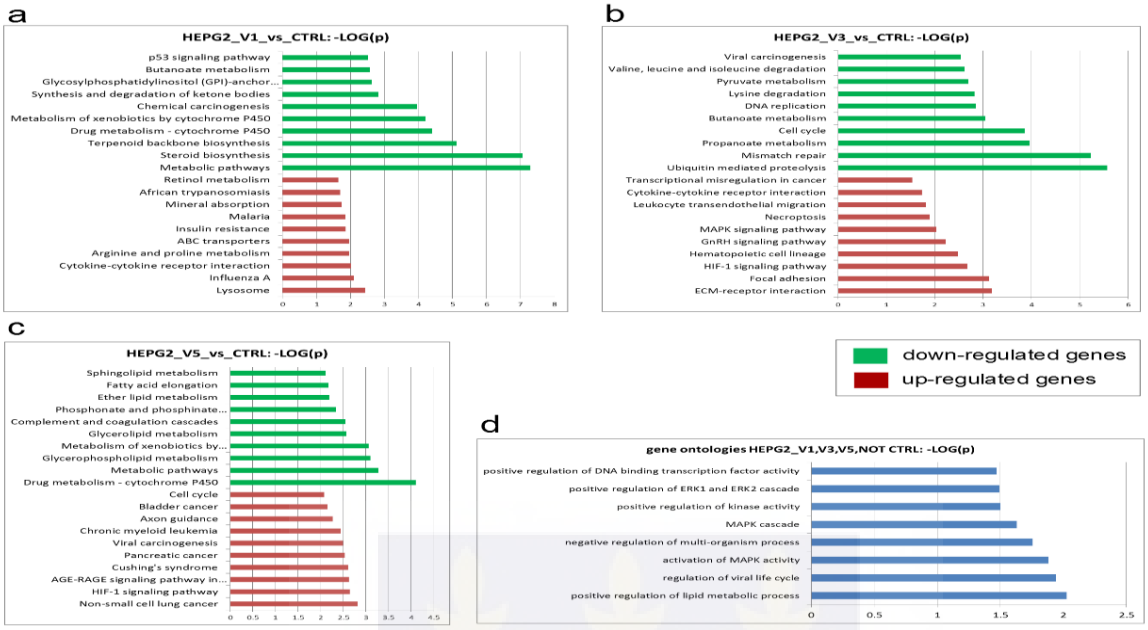



Figure 6.8. Treatments of HepG2 cells with V1, V3 and V5 impact cancer-related pathways. Up- and Down-regulated genes (ratio < 0.75; ratio > 1.33; p-value < 0.05) from the intersection sets of the pairwise Venn diagrams were analyzed for overrepresentation of KEGG pathways and gene ontologies with the hypergeometric test. The bar charts show the negative logarithm (base 10) of the p-value - higher values corresponding to higher significance. In (a-c) green bars indicate overrepresentation of down-regulated genes and red bars of up-regulated genes in pathways. (a) In V1 treatment cancer-related pathways such as p53 and chemical carcinogenesis and metabolic pathways such as cytochrome P450 metabolism emerge in the down-regulated genes while metabolic pathways such as Arginine, Retinol and Insulin resistance emerge in the up-regulated genes. (b) In V3 treatment cancer-related pathways such as cell cycle, Mismatch repair and Viral carcinogenesis emerge in the down-regulated genes and also in the up-regulated genes cancer-related pathways such as MAPK-signaling and Transcriptional misregulation in cancer come up. (c) In V5 treatment Cancer-related pathways such as cell cycle and various other cancers emerge in the up-regulated genes while various metabolic pathways including cytochrome P450 metabolism emerge in the down-regulated genes. (d) The set of 26 genes expressed in common in all treatments (V1, V3 and V5) but not in the HepG2 control revealed over-representation of gene ontologies related to cancer-associated MAPK/ERK-signaling.

### 6.2.11 Analysis of protein interaction networks activated by the treatments

The set of 26 genes expressed in common in all treatments (V1, V3 and V5) but not in the HepG2 control was further analyzed for protein interaction networks based on the Biogrid database. Most of the genes from the 26-gene set (Fig 6.9a, green nodes) had interactions with other proteins as reported in the Biogrid database (Fig 6.9a, red nodes). Within the network we looked for communities with similar network features via community clustering (Fig 6.9b, Fig. S6.1) and found several communities including those characterized by tumor necrosis factor (TNF, red), mitogen-activated protein kinase 4 (MAPK4, skyblue), tripartite motif containing 21 (TRIM21, yellow), amyloid beta precursor protein (APP, green).

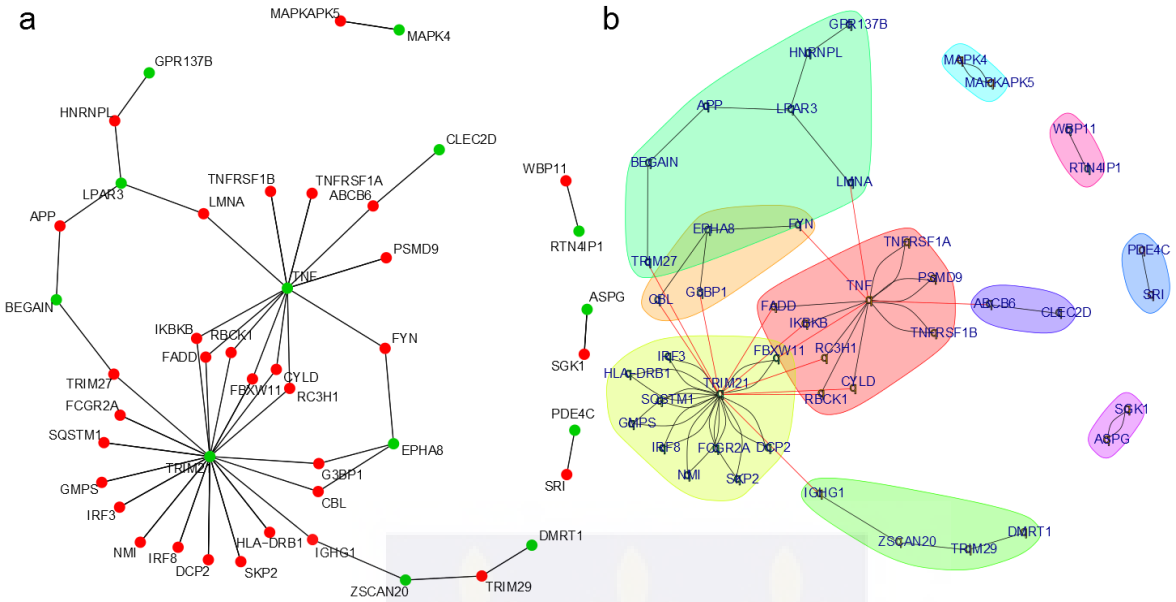



Figure 6.9. TNF, TRIM21 and MAPK are hubs of protein interaction networks of HepG2 cells treated with V1, V3 and V5. Based on the Biogrid database protein interaction networks have been constructed from the set of 26 genes expressed in common in all treatments (V1, V3 and V5) but not in the HepG2 control. (a) Most of the genes from the 26-gene set can be connected to a network with interactions reported in the Biogrid database. Genes from the original 26-gene-set are colored green, genes added as Biogrid interactions are colored red. (b) Community clustering of the network via edge-betweenness reveals several communities including those characterized by TNF (red), MAPK4 (skyblue), TRIM21 (yellow), APP (green).

## 6.3. Discussion

In this study we extracted partially purified antifungal fractions from marine endophytic fungal sources. The crude extract MEF134 which was found as the most active of 143 extracts in several rounds of preliminary screening showed antifungal activity against the pathogenic *Candida albicans* but not against *Saccharomyces cerevisiae*. However, activity against *Candida albicans* or *Saccharomyces cerevisiae* or both fungi could be observed after two rounds of Kupchan fractionation and TLC runs of DCM fractions.

These fractions were analyzed with HPLC-HRMS and complex patterns including multiple peaks in mass spectra were detected. For FD K1V1V1 11 major peaks, for FD K1V5V3 6 major peaks and for FD K2V3V5 11 major peaks were detected. However, the fragmentation patterns of the masses were complex. These three fractions have been evaluated using the highly sensitive microarray-based analysis of gene expression. This allows for the determination of the prospects of obtaining important chemical structural scaffold that can serve as new leads to the development of new anticancer chemotherapy. Obtaining milligram quantities for natural products is extremely resource intensive and an approach like this provides the key evidence for making future commitments.

### 6.3.1 Proliferation and viability of HepG2 after treatment with the fractions

Anticancer effects could be demonstrated by reduced proliferation in a proliferation assay of the human liver cancer cell line HepG2 after application of the fractions. Expression of the 4 viability markers *P53*, *KI67*, Caspase3, and *CDKN1B* as analyzed by qRT-PCR was reduced in every condition compared to untreated cells. The reduction of *KI67*, which is a marker for proliferation [7], correlates well with the proliferation assay. The concomitant reduction of Caspase 3 indicates

that these observations are not related to increased apoptosis [8]. Interestingly, also *p53* and *CDKN1B*, which both negatively regulate cell cycle progression [9],[10], are down-regulated. This is probably due to complex interaction mechanisms and reflects stress reactions. Fraction V5 has the strongest negative effect on all genes except *CDKN1B*, which indicates that is has the highest impact on overall cell survival.

### 6.3.2 Transcriptome analysis

Pathway analyses based upon Venn diagram dissections of the genes expressed exclusively in the V1, V3 or V5 treated HepG2 cells but not in the controls revealed many overrepresented cancer-related pathways described in sections 6.3.2.1-6.3.2.3. From the genes expressed in common in all treatments but not in the untreated HepG2 cells a protein interaction network could be constructed (section 6.3.2.4).

#### 6.3.2.1 Pathways regulated by treatment with V1

The down-regulation of ALDH3B in V1 treatment in the pathway *Drug metabolism – cytochrome P450* (Fig S6.2)would support the central effect of the chemotherapeutic agent cyclophosphamide depending on its metabolite phosphoramide mustard which is only produced in cells that have low levels of ALDH [11]. Phosphoramide mustard induces apoptosis by building DNA crosslinks between and within DNA strands at guanine N-7 positions. In the *p53 signaling pathway* down-regulated genes are in the cancer-suppressing sub-paths leading to cell cycle arrest, inhibition of angiogenesis and metastasis and DNA repair and damage prevention. However, also the p53 negative feedback sub-path is down-regulated which finally may lead to p53 up-regulation again.

#### 6.3.2.2 Pathways regulated by treatment with V3

The down-regulation of most genes involved in the cell cycle pathway (Fig. S6.4) in V3 treatment is in line with our finding of down-regulated proliferation upon V3 treatment. The significantly down-regulated genes in all subsystems of the *viral carcinogenesis* pathway (Fig. S6.5) indicate the inhibitory effect of the V3 treatment on many viral induced cancer hallmarks, e.g. down-regulated proliferation by SKP2 in the Epstein-Barr virus.

#### 6.3.2.3 Pathways regulated by treatment with V5

In V5 treatment the up-regulation of genes from the *cell cycle* pathway, particularly *TGFB1*, *CDKN1A* and *CDKN2C* (Fig. S6.6, Tables S6.9-S6.10), is in line with reports that in most transformed cells the usual association of the cyclin-CDK complexes with *CDKN1A* (alias *p21*) is absent [12]. More general, Cyclin-dependent kinase inhibitors are considered to function often as tumor-suppressors and to lead to cell cycle arrest [13]. This again would confirm our observation of reduced proliferation upon treatment with the fractions.

#### 6.3.2.4 Analysis of protein interaction networks activated by the treatments

The protein interaction network derived from the set of 26 genes expressed in common in all treatments (V1, V3 and V5) but not in the HepG2 control revealed communities (Fig 6.9b, Fig. S6.1) with similar network features including those characterized by tumor necrosis factor (TNF, red), mitogen-activated protein kinase 4 (MAPK4, skyblue), tripartite motif containing 21 (TRIM21, yellow), amyloid beta precursor protein (APP, green). The protein interactions of brain enriched guanylate kinase associated (BEGAIN) and lysophosphatidic acid receptor 3 (LPAR3) with APP retrieved from Biogrid have been reported as results from high-throughput in vitro

experiments [14]. The role of aberrant expression of LPARs in cancer has already been established [15] .Although APP is mostly associated with Alzheimer's disease many recent publications have described its impact on cancer [16], [17], [18]. Tumor-suppressing activity of TRIM21 has been reported although there may be variability between different cancer types and treatment conditions: Ding et al. find it to be a tumor-suppressor in hepatocellular carcinoma [19] and also Espinosa et al. suggest TRIM21 (alias RO52) to be a tumor suppressor [20] while Nguyen et al. describe that TRIM21 down-regulates the tumor-suppressor PAR4 in pancreatic cancer in the presence of cisplatin [21]. The relevance of TNF in cancer is unquestionable however there are therapeutic effects of its inflammatory response on the one and tumor advancing properties on the other hand [22]. Also for MAPK4 (alias ERK4) anti- and pro-ontogenic properties have been reported thus extending established knowledge about the major role typical MAP kinase pathways ERK1/2-MEK1/2 play in cancer [23]. Furthermore, MAPK-signaling is downstream of TNF [22] thus emphasizing the importance of these components of the network.

## 6.4. Materials and Methods

### 6.4.1 Large-scale fermentation and preparation of crude extracts of MEF 134

Pure isolates of MEF 134 were introduced into 20 liter culture vessels containing 10 liter Yeast, Peptone, Malt, Dextrose (YPMD) (Ratio: 5g/l;5g/l;5g/l;30g/l respectively) broth prepared with filtered seawater and grown at room temperature approximately 30oC for 4 months [24] . The extraction was performed as previously described by [24]. The matured fungi cultures were extracted with ethyl acetate (1:1, V/V). The extracts were dried by rotary evaporation (Buchi) at 450C under reduced pressure and the residues were reconstituted in methanol.

### 6.4.2 Fractionation and Structural characterization of bioactive fractions

The crude extract of MEF134 was fractionated by modified Kupchan fractionation method and preparative thin Layer Chromatography (TLC). The modified Kupchan fractionation method was performed by partitioning the crude extracts among seven different solvent systems [25]. These solvents included; hexane (FH), water (FW), ethyl acetate (FE), dichloromethane (FD), Butanol (FB), methanol (FM50%) and ethyl acetate & methanol (FME). A total of 7 different fractions were obtained. The next step was a preparative TLC in 50 ml TLC solvent combination containing: 35ml ethyl acetate, 10 ml acetonitrile and 5 ml petroleum ether. Dry and developed plates were visualized under UV light at high and low wavelengths. The bands were cut and eluted as the pure fractions. The bands obtained from the silica gel pre-coated plates were eluted with methanol.

### 6.4.3 Determination of the antifungal activity of the MEF 134 crude extract and Dichloromethane fractions

Antifungal activity of the crude extract and the dichloromethane fractions (FD) were tested by the agar plate disc-diffusion method. A volume of 30 µl and 120 µl from stock solution of crude extract and FD were added on to sterile filter paper disc which were 5 mm in diameter respectively. The final amount of the crude extract and FD in each disc were 0.05 g and 0.2 g respectively and the discs were left dry. All other fractions were tested by this method at 0.01 g/disc. A disc-diffusion assay was carried out in which the impregnated filter paper discs were laid on to plates freshly inoculated with mid-log phase *Saccharomyces cerevisiae* and *Candida albicans*. Zones of inhibition were measured in mm, 12 hours after incubation of inoculated plates at 300c.

### 6.4.4 Determination of cytotoxicity of the MEF 134 FD

Hepato-carcinoma (HepG2) cells (ATCC®HB-8065TM) were cultured in DMEM low glucose 10 % FCS, 1% Penicillin/Streptomycin (P/S) (all Gibco) at 37°C and 5% CO2 in a humidified atmosphere. Cells were split with trypsin (Gibco) when they reached confluency. Monolayers were treated with FD K1V1 V1, FD K1V5V3 and FD K2V3V5 at varying concentrations. Cell proliferation was determined by the resazurin metabolic assay [26]. After treatment for 2 or 3 days with the indicated concentrations of the three fractions, cells were incubated for 4 h with fresh medium containing 10% of a resazurin solution consisting of 0.1 mg/mL resazurin (Sigma-Aldrich) in phosphate buffer saline (PBS) (Gibco). Resazurin reduction was measured with a spectrophotometer (Bio-tek instruments) at 570 and 600 nm. A final resazurin value (F.O.D.) was calculated as the difference between the O.D. 570/O.D. 600 nm of the treated ample and that of the negative control (resazurin media incubated for 4 h in the absence of cells).

### 6.4.5 RNA isolation and quantitative real time PCR (qRT-PCR)

Cells were lysed in 300-500 µl Trizol and RNA was isolated with the Direct-zol™ RNA Isolation Kit (Zymo Research) according to the user's manual including the optional on-column DNase digestion. For cDNA synthesis the TaqMan Reverse Transcription (RT) Kit (Applied Biosystems) was used. Real time PCR was performed in technical triplicates of biological duplicates with Power Sybr Green Master Mix (life technologies) on a VIIA7 (life technologies). Mean Ct values were calculated and normalized to RPL37A as a housekeeping gene. Fold change was calculated relative to the untreated controls. Results are depicted as mean values (log2).

### 6.4.6 Transcriptome analysis

Untreated HepG2 samples and samples treated with FD K1V1V1 (V1), FD K1V5V3 (V3) and FD K2V3V5 (V5) were hybridized in duplicates on the Affymetrix Human Clariom S Array (Affymetrix, Thermo Fisher Scientific) at the BMFZ (Biomedizinisches Forschungszentrum) core facility of the Heinrich-Heine Universität, Düsseldorf. Data analysis of the Affymetrix raw data was performed in the R/Bioconductor [27] environment using the package oligo [28]. The obtained data were background-corrected and normalized by employing the Robust Multi-array Average (RMA) method from the package oligo. The heatmap.2 function from the gplots package was employed to generate hierarchical clustering and heatmaps using Pearson correlation as similarity measure and color scaling per rows containing genes [29]. Venn diagrams were drawn based on gene expression employing package VennDiagram [30]. A gene was considered expressed when its detection p value was less than 0.05. The detection p value was calculated as described in the supplementary methods in Graffmann *et al.* [31].

### 6.4.7 Gene ontology and pathway analysis

Based on the transcriptome analysis over-represented gene ontology terms and KEGG (Kyoto Encyclopedia of Genes and Genomes) pathways [32] were determined. The GOstats package [33] was used for over-representation analysis of GO terms and the hypergeometric test provided by the R base package was used for over-representation analysis of KEGG pathways. KEGG pathway annotation had been downloaded from the KEGG database in March 2018. Visualization of significant genes in KEGG pathway charts was achieved with the R/Bioconductor pathView package [34].

### 6.4.8 Construction of protein interaction networks

Based on the venn diagram analysis the set of 26 genes expressed in common in all treatments (V1, V3 and V5) but not in the HepG2 control a protein interaction network was constructed. Interactions annotated with the taxonomy id 9606 (Homo sapiens) were filtered from the Biogrid database version 3.4.161 [35]. From this dataset all protein interactions containing at least one protein coded by the above-mentioned set of 26 genes were extracted. As this network was already too complex it was reduced by adding only the n=30 interacting proteins with the most interactions to proteins coded by genes from the original set. These interactions were plotted employing the R package network [36] marking proteins from the original set in green. An in-betweenness clustering analysis was performed via the method cluster_edge_betweenness() from the R package igraph [37] in order to identify communities of related proteins within the network.

## 6.5. Conclusions

In this study we extracted novel antifungal fractions from seaweed and demonstrated that they could reduce proliferation in the human liver cancer cell line HepG2. Employing detailed transcriptome analysis, we detected several cancer- and metabolism-related pathways and gene ontologies regulated by the treatment with the novel antifungal fractions from marine endophytic fungi and could construct a protein interaction network distinguishing the fraction-treated HepG2 cells from the untreated control HepG2 cells. Major functional components of this network (TNF, MAPK, TRIM21 and APP) are associated with metabolism and cancer. The future directions for this study are to synthesize the prominent compounds detected from the sub-fractions and retest their antifungal and anti-proliferative activities on numerous cancer cell lines and primary tumor cells. The producing organism will be re-cultured in larger volumes to obtain milligram quantities of the active molecules for exhaustive chemical and functional characterization.

**Supplementary Materials:** The following are available online at www.mdpi.com/xxx/s1, Table S6.1: Venn diagram HepG2 V1 vs. HepG2 control sets, Table S6.2: Venn diagram HepG2 V3 vs. HepG2 control sets, Table S6.3: Venn diagram HepG2 V5 vs. HepG2 control sets, Table S6.4: Venn diagram HepG2 V1, HepG2 V3, HepG2 V5 and HepG2 control sets, Table S6.5: Overrepresented KEGG pathways in the down-regulated genes between HepG2 V1 vs. HepG2 control sets, Table S6.6: Overrepresented KEGG pathways in the up-regulated genes between HepG2 V1 vs. HepG2 control sets, Table S6.7: Overrepresented KEGG pathways in the down-regulated genes between HepG2 V3 vs. HepG2 control sets, Table S6.8: Overrepresented KEGG pathways in the up-regulated genes between HepG2 V3 vs. HepG2 control sets, Table S6.9: Overrepresented KEGG pathways in the down-regulated genes between HepG2 V5 vs. HepG2 control sets, Table S6.10: Overrepresented KEGG pathways in the up-regulated genes between HepG2 V5 vs. HepG2 control sets, Table S6.11: Overrepresented gene ontologies in the subset of 26 genes expressed in HepG2 V1, V3 and V5 but not in HepG2 control from the venn diagram in Fig 6.7D, Figure S6.1: KEGG pathway chart of pathway Steroid Biosynthesis in genes down-regulated in HepG2 cells treated with V1 vs. control, Figure S2: KEGG pathway chart of pathway Drug metabolism – cytochrome P450 in genes down-regulated in HepG2 cells treated with V1 vs. control, Figure S6.3: KEGG pathway chart of pathway p53 signaling pathway in genes down-regulated in HepG2 cells treated with V1 vs. control, Figure S6.4: KEGG pathway chart of pathway cell cycle in genes down-regulated in HepG2 cells treated with V3 vs. control, Figure S6.5: KEGG pathway chart of pathway viral carcinogenesis in genes up-regulated in HepG2 cells treated with V3 vs. control, Figure S6.6 Fig: KEGG pathway chart of pathway cell cycle in genes up-regulated in HepG2 cells treated with V5 vs. control, Figure S6.7: Dendrogram of community clustering of protein interaction networks of HepG2 cells treated with V1, V3 and V5.

**Author Contributions:** Conceptualization, J.A. and P.A.; methodology, W.W., J.A. and P.A:.; software, W.W.; validation, A.N. and N.G.; formal analysis, W.W.; investigation, E.B., B.A., A.N. and N.G.; resources, E.B., P.A. and B.A.; data curation, W.W.; writing—original draft preparation, E.B. and W.W., J.A., B.A., A.N., N.G.and P.A; writing—review and editing, W.W., J.A. and P.A .; visualization, W.W.; supervision, P.A. and J.A.; funding acquisition, P.A. and J.A. .

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## 6.6 Supplementary Data

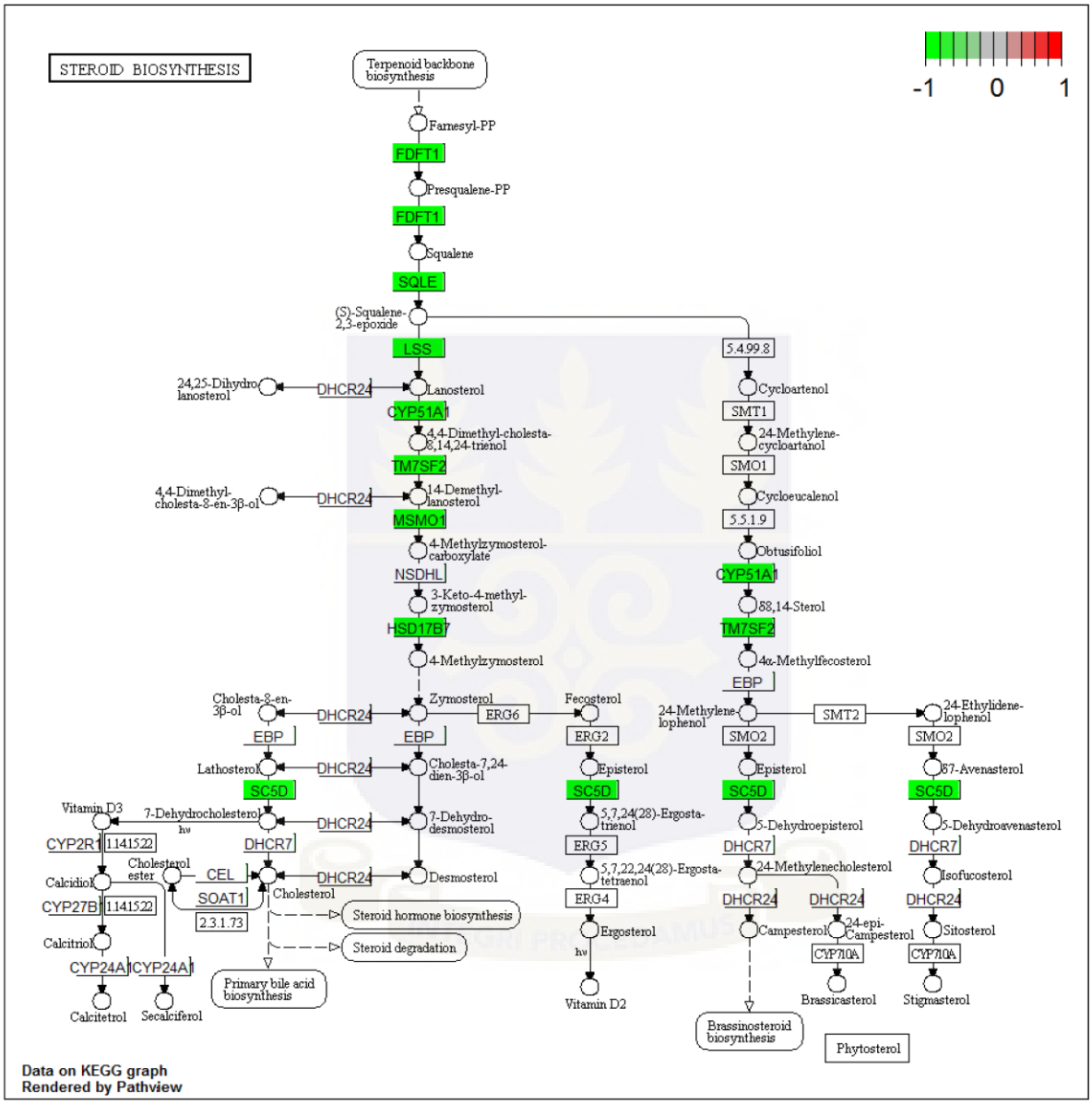



Fig S6.1. KEGG pathway chart of pathway Steroid Biosynthesis in genes down-regulated in HepG2 cells treated with V1 vs. control.

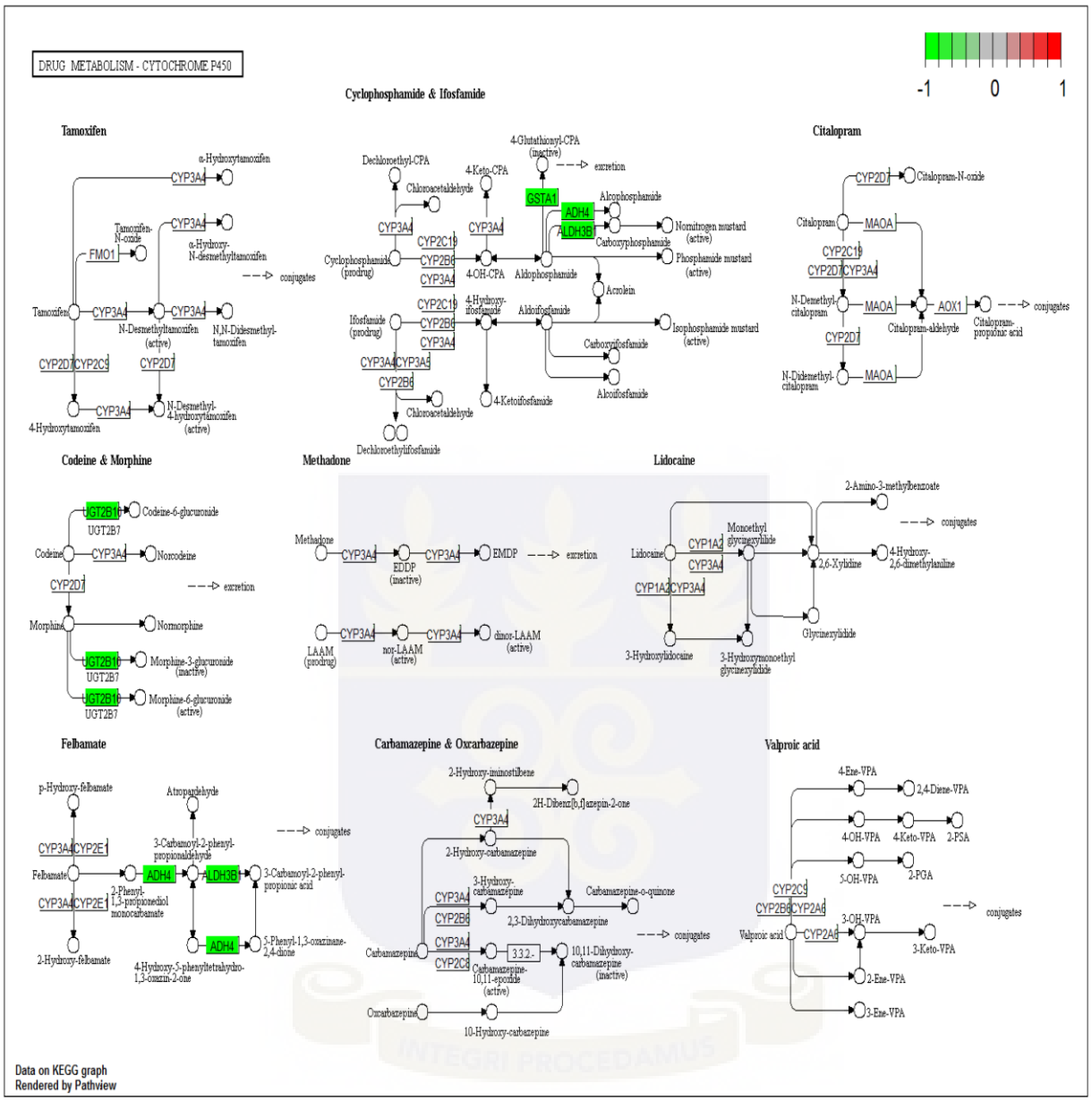



Fig S6.2. KEGG pathway chart of pathway Drug metabolism – cytochrome P450 in genes down-regulated in HepG2 cells treated with V1 vs. control.

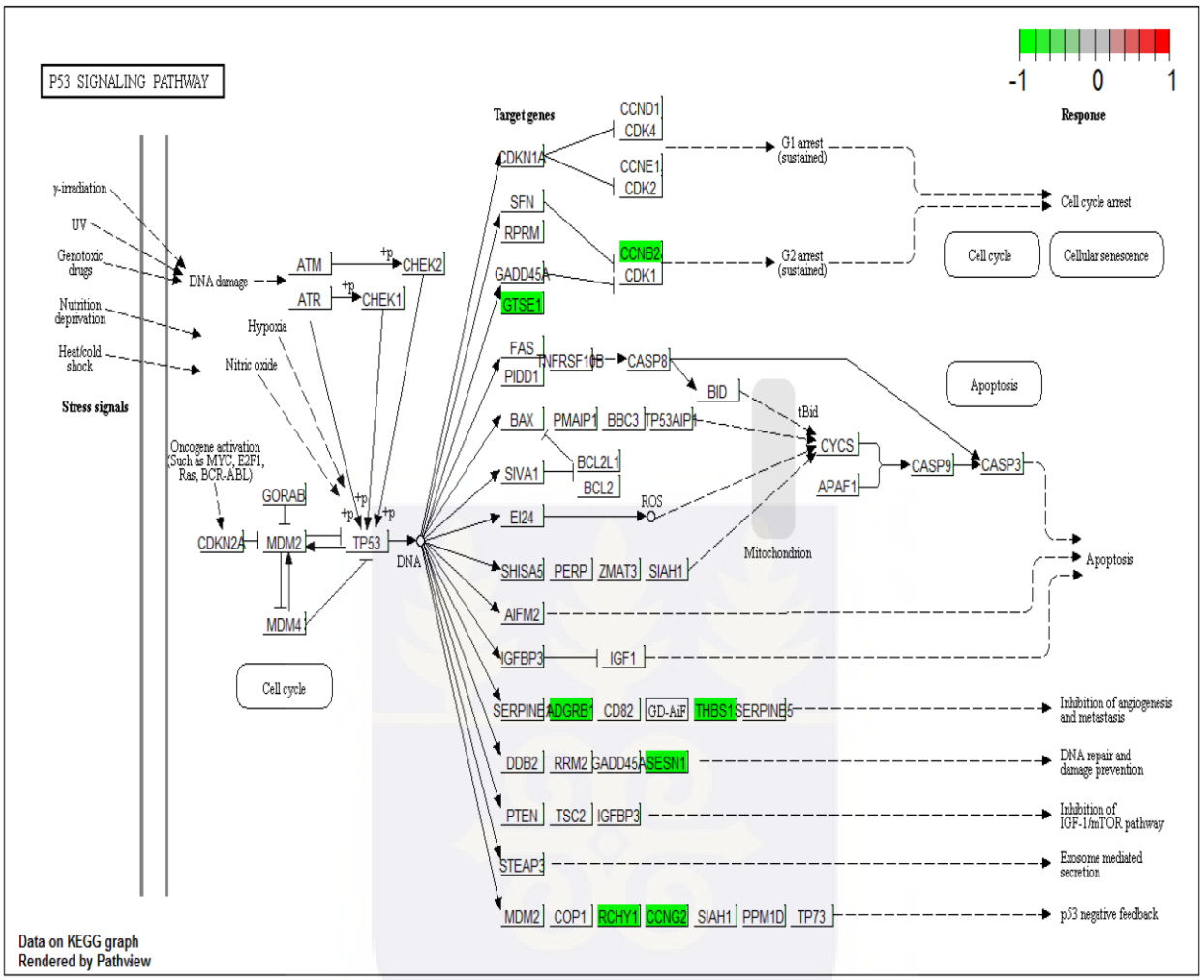



Fig S6.3. KEGG pathway chart of pathway p53 signaling pathway in genes down-regulated in HepG2 cells treated with V1 vs. control.

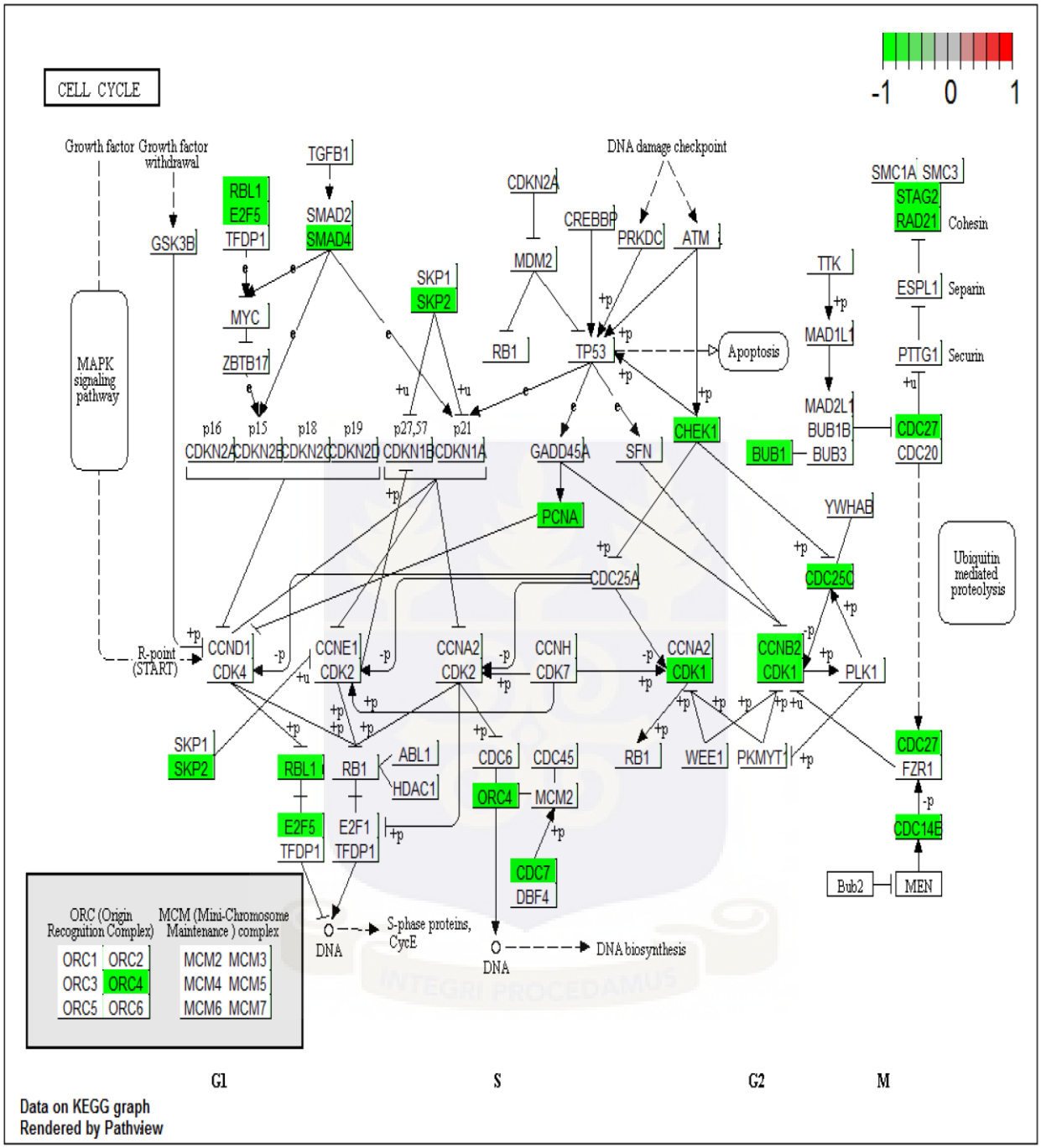



Fig S6.4. KEGG pathway chart of pathway cell cycle in genes down-regulated in HepG2 cells treated with V3 vs. control.

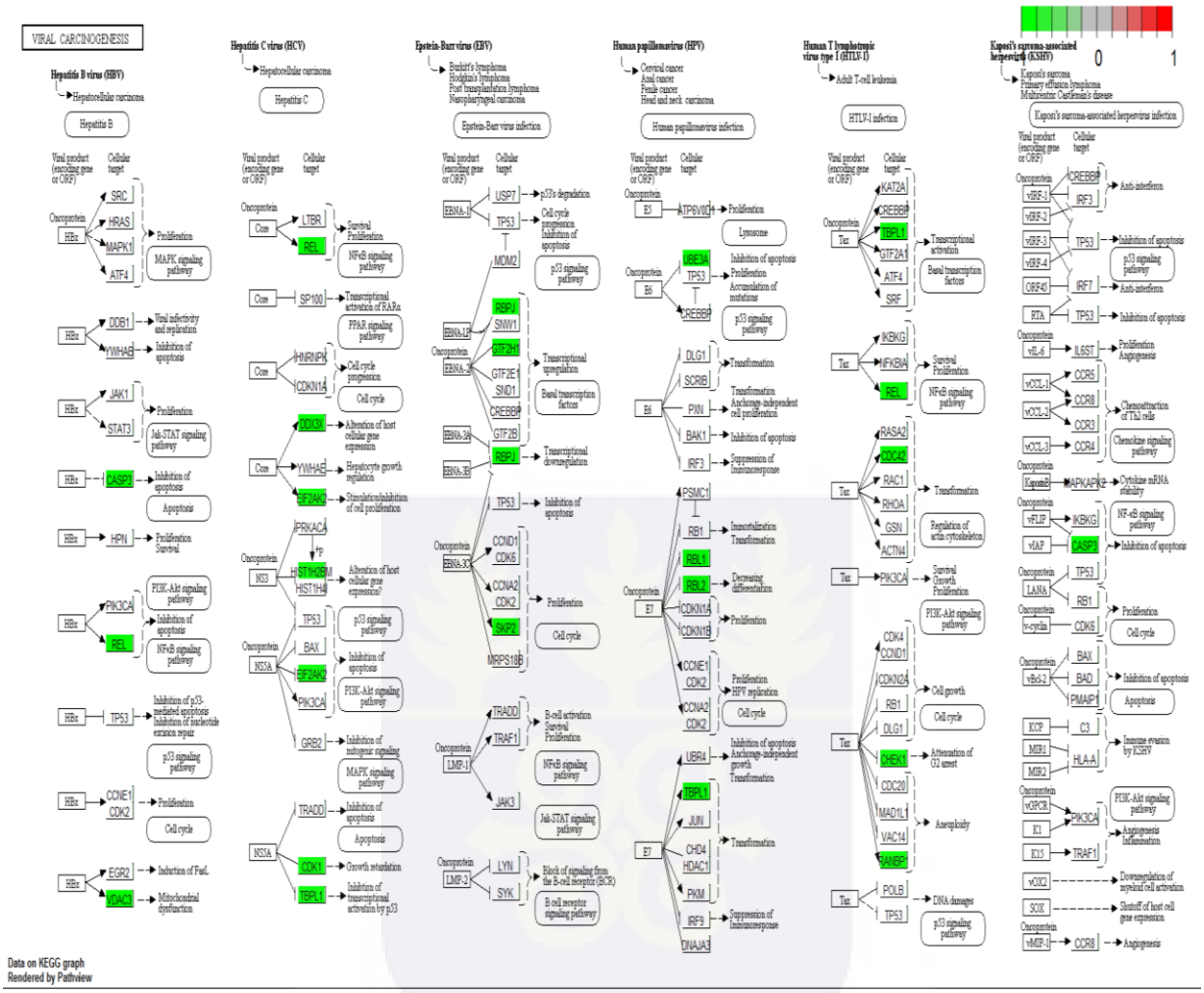



Fig S6.5. KEGG pathway chart of pathway viral carcinogenesis in genes down-regulated in HepG2 cells treated with V3 vs. control.

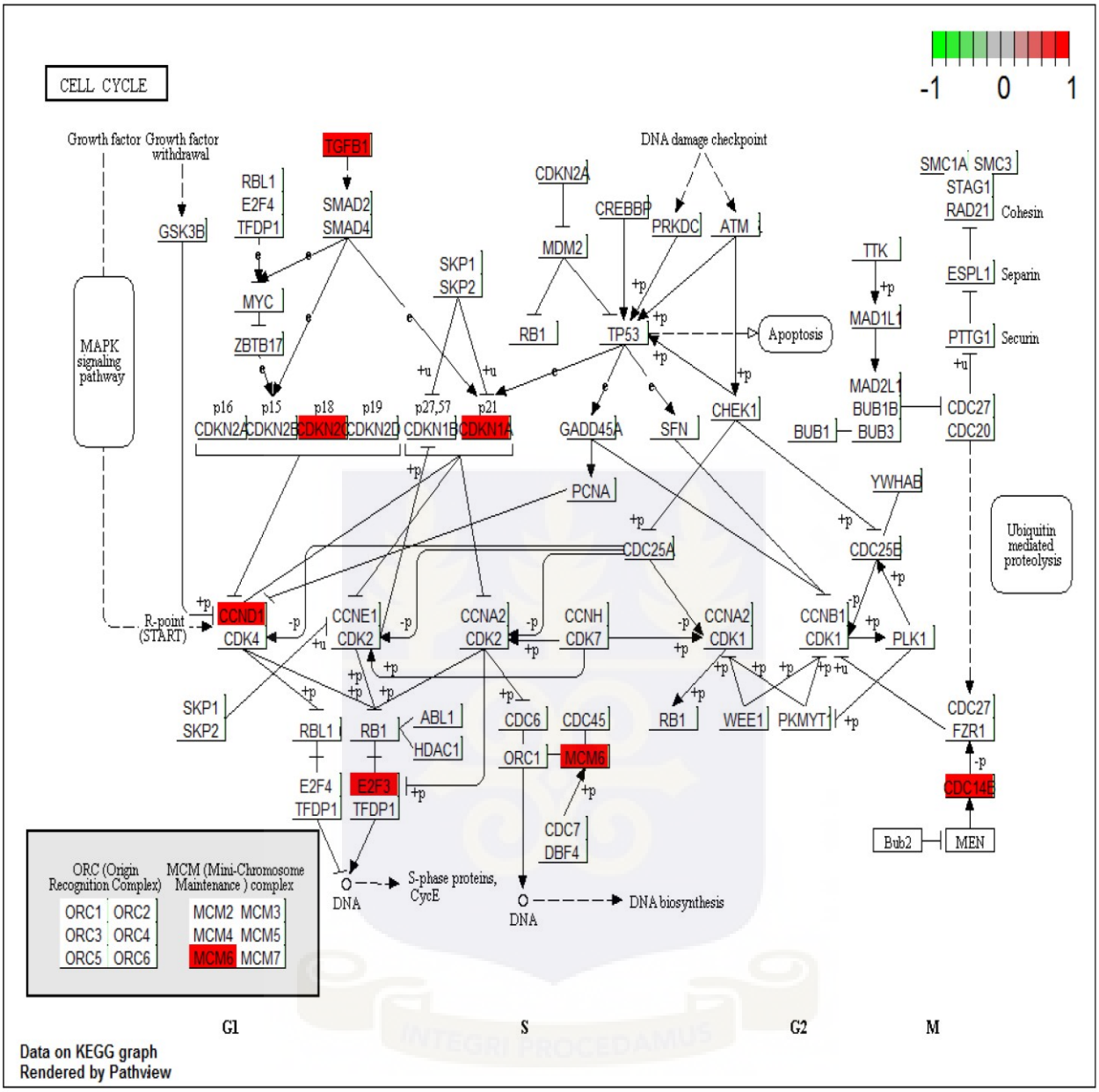



Fig S6.6. KEGG pathway chart of pathway cell cycle in genes up-regulated in HepG2 cells treated with V5 vs. control.

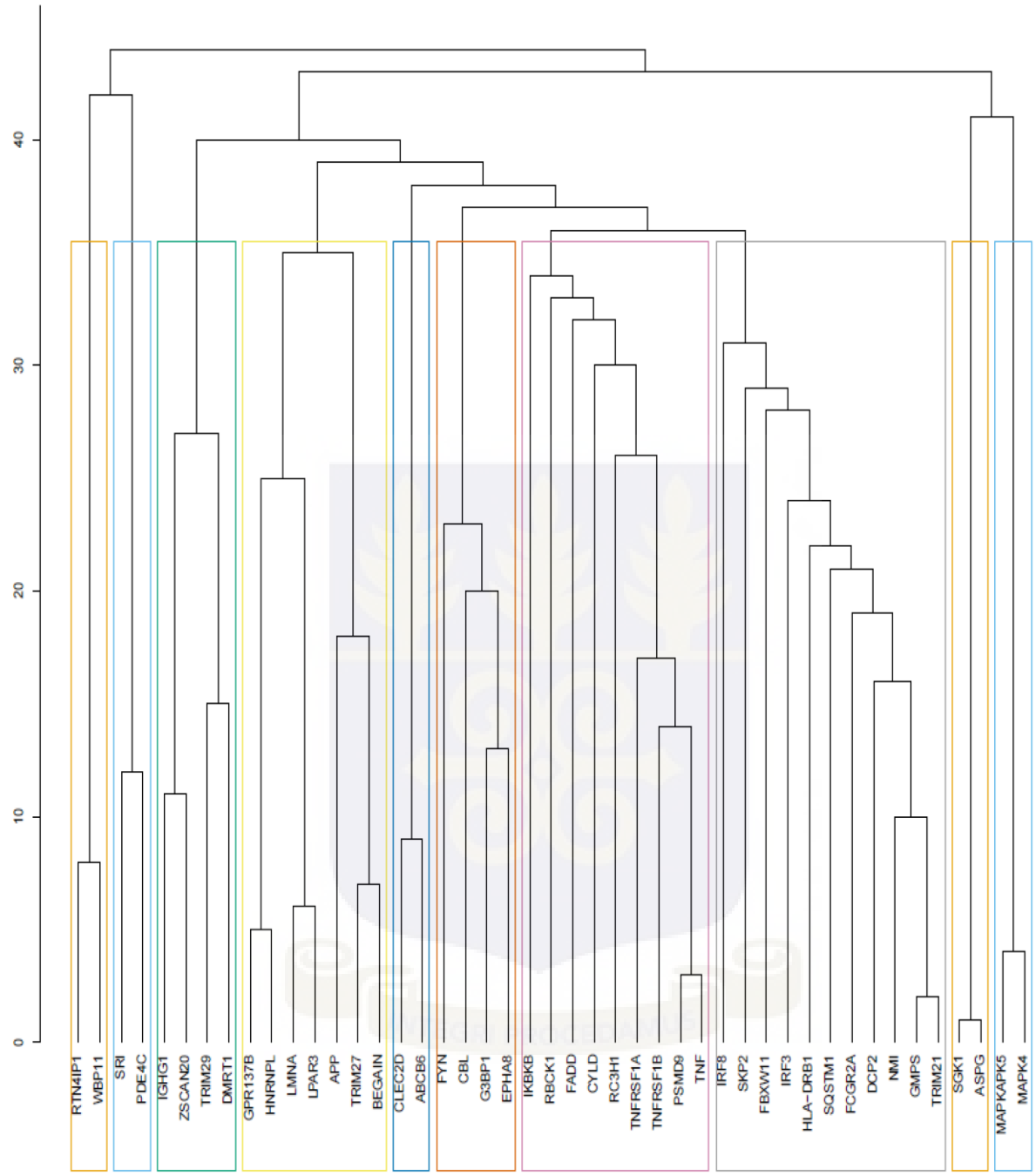



Fig S6.7. Dendrogram of community clustering of protein interaction networks of HepG2 cells treated with V1, V3 and V5. Based on the Biogrid database protein interaction networks have been constructed from the set of 26 genes expressed in common in all treatments (V1, V3 and V5) but not in the HepG2 control. TNF, TRIM21 and MAPK are focuses determining dedicated clusters of the protein interaction networks.

# CHAPTER SEVEN

## STRUCTURAL ELUCIDATION OF COMPOUNDS ISOLATED FROM BIOACTIVE FRACTIONS OF 5 MARINE ENDOPHYTIC FUNGI

### 7.1 Introduction

The widespread use of standard antifungal drugs have hastened the emergence of drug resistant pathogenic fungi (Vandeputte *et al.*, 2011), hence the need for development of novel antifungals (Fisher *et al.*, 2018). To strengthen the antifungal armamentarium, a number of approaches have been used in the search for novel antifungal agents for the treatment of fungal infections. Natural products offer a limitless source of unique compounds and a reservoir for new potential drugs. Other groups of antifungal agents are even derived from compounds obtained from natural sources (J. W.-H. Li & Vederas, 2009). Natural products offer wide structural diversity and can serve as templates and scaffolds for semi-synthetic and whole synthetic modification (Chin *et al.*, 2006). It has been reported that about 40% of the chemical scaffolds of published natural products are distinct and have never been chemically synthesized, thus making natural products a compelling driving force for pharmaceutical companies and drug discovery investigators to target (Shen *et al.*, 2003).

Modifications in the biosynthetic pathways of microorganism as a result of exposure to environmental changes and other unnatural situations enable them to adapt to these extreme conditions (Sarker *et al.*, 2006). The accumulation of remarkable physiological and functional heterogeneity as a survival strategy to adaptation to various selective environmental pressures, make microbial life forms in marine and terrestrial ecosystems potential sources of bioactive compounds (Lozada & Dionisi, 2015). Since less than 10% of the world's biodiversity has been

evaluated for potential of biological activities, a lot more functional natural lead compounds await discovery (Cragg & Newman, 2005). A significant number of recent reviews have highlighted the important contributions of natural products to drug discovery and development (Butler, 2005, 2008). Marine-derived endophytic fungi which colonize internal tissues of their hosts have emerged as alternative sources of promising biologically active agents, including alkaloids, terpenoids, polyketides, lipids, glycosides, isoprenoids, and hybrids of natural and synthetic compounds (Debbab *et al.*, 2012; Kjer *et al.*, 2010). A number of studies have showed that secondary metabolites from marine derived endophytic fungi are potential novel antimicrobial agents (Flewelling *et al.*, 2013; Manivannan *et al.*, 2011). From the body of literature, little is known of the diversity of compounds from marine-derived endophytes. The aim of this study was to identify, characterize and elucidate the structures of bioactive metabolites from 9 2D preparative TLC fractions of 5 marine fungal endophytes. The objectives of the study were;

- To further purify the bioactive fractions using high performance liquid chromatography.
- To analyze the component of the fractions using electrospray ionization (ESI) - mass spectrometry.
- To elucidate the structures of the identified compounds via analysis of their mass spectra.

## 7.2 Materials and Methods

**7.2.1 Solvents for HPLC analyses:** acetonitrile, methanol and deionized water

**7.2.2 Fractions used for the HPLC and mass spectrometry analyses:** MEF 134 FDV5V9, MEF 134 FDV5V7, MEF 134 FD K1V1V1, MEF 134 FD K1V5V3, MEF 134 FD K2V3V5, MEF 11 FH V1V1, MEF 65 FH V3V3, MEF 75 FH V1V1 and MEF 112 FH V1V1

**7.2.3 Analytical HPLC analyses of the TLC 9 tlc fractions**

After conducting a series of thin layer chromatographic separations, the 9 fractions selected from TLC separations were characterized by analytical HPLC (Thermo Scientific). Prior to the analytical HPLC studies, it was necessary to confirm the quality of samples. The mobile phase system was evaluted to determine the most appropriate combination of solvent system; either methanol and deionized water, or acetonitril and deionized water, with or without the addition of 0.1% formic acid. Addition of formic acid was to improve the shape of the peaks from the chromatography. The HPLC system was equiped with photo diode array detector (PDA) for the detection of the eluted peaks. Chromatographic separation of the fractions was conducted using Phenomenex Gemini C18 column (3 µm, 0.3 × 150mm) (Torrance, CA) with water/ acetonitrile gradient solvent system with 4.0 µL/min flow rate. The run time was often adjusted per fraction in order to establish improved resolution of peaks (Kusari & Spiteller, 2012).

**7.2.4 High resolution mass spectrometry (HRMS) evaluation of the TLC 9 fractions**

ESI-MS measurements were conducted by Prof. Spiteller at TU Dortmund, Institute of Environmental Research.

Separation of compounds by mass spectrometry is based on mass to charge ratio (m/z) of the atoms. This technique is important in obtaining chemical and structural information of molecules. Each of the 9 fractions were analyzed on an HPLC system equipped with photo diode array detector coupled to a high resolution tandem mass spectrometer (LC-MS) (Thermo Scientific) using a LTQ-Orbitrap, operating in positive and negative electrospray ionization mode (Kusari & Spiteller, 2012). The mass spectrometer together with the HPLC were used to scan the 9 fractions first to detect the total ion currrent of each fraction before the spectroscopy analysis was conducted.The mass spectrometry analyses were conducted in tandem (MS1 and MS2). Subsequently, the ESI-mass spectra of the compounds were also acquired. In electrospray ionization mass spectrometry, vaporised samples are often blasted with a beam of electrons. This reaction results in intense fragmentation, giving rise to unique fragmentation patterns which can be used to characterize compounds. This method has been described in detail in work done by Ivanova and colleagues (Ivanova *et al.*, 2001). Multiple reaction monitoring modes were employed in analysing the fractions. The most intense peaks in the spectra also known as the base peaks were selected from the first round of mass spectrometry (MS1) analyses. These were further analysed in the subsequent mass spectrometry (MS2) reaction.

### 7.2.5 Structure elucidation of compounds identified from the 9 TLC fractions

The structure elucidation workflow was design with the help of Mr. Bismarck Kyei Amaniampong a graduate intern at the West African Center for Cell Biology of Infectious Pathogens.

The data from a mass spectrometer is usually a plot of relative abundance of each ion against mass-to-charge ratio (m/z). The tallest peak in a mass spectrum is called the base peak. All other peaks are reported comparative to the base peak. The parent molecule is the higest molecular weight peak in a spectrum and this parent molecule often looses one electron ($M^+$). Compounds are often

stabilised by double bonds, aromatic rings among others. Energetically unstable ions can be dissociated from the parent compound during fragmentation. The fragmetation patterns of a compound provides structural information and follow predictable chemical pathways. Functional groups on a compound control the way a compound may resist fragmentation. Some chemical groups that are often lost during fragmentation include; OH, $CH_3$, CN and $OCH_3$. Generally, mass spectra aids to identify the structure of a molecular ion by examining the fragments of the orginal molecules. Based on the fragmentation patterns obtained from the mass spectrometry (MS2) analyses, the structures of the compounds were solved. Chemdraw (PerkinElmer) software was used to predict the mass spectra of the tentative structures and compared with that obtained from the mass spectrometer analysis. The structures were matched with SciFinder® and ChemSpider (Royal society of chemistry) chemistry databases to determine whether the structures were patented or novel.

## 7.3 Results

### 7.3.1 HPLC-HRMS full scan of the 9 TLC fractions

The criteria for selecting the 9 TLC fractions for HPLC-HRMS analyses was based on the activity profiles of the fractions against *C. albicans* and *S. cerevisiae* (Table 7.1; Figs S7.13-S.7.63). The methods used to generate these fractions have been extensively discussed in chapters 1 and 6. Even though two out of the nine selected fractions (MEF 65 FH V3V3 and MEF 75 FH V1V1) did not show activity against both organisms, they were included in this analysis because their TLC patterns showed only single bands. When a mass spectrometer is used together with a chromatographic equipment, the data received from the analyses are often displayed as the total ion current (TIC) chromatogram. The following description elaborates more on the total ion current (TIC) chromatogram of the 9 fractions. For the fraction MEF 134 FDV5V7, there were 11

major components detected from the HPLC-HRMS scan (Fig S7.4 a). The TIC chromatogram from MEF 134 FDV5V9 showed 8 major components; components 1 and 8 were the most dominant (Fig S 7.5a). There were 6 major peaks detected from MEF 134 FD K1V5V3 (Fig S7.6a). These peaks were not prominent as compared to those observed in figure 1; there were also impurities from the 19$^{th}$ to the 22$^{nd}$ minute (Fig S7.6a). The TIC chromatogram from the analysis of MEF 134 FD K1V1V1 revealed 11 prominent peaks, these peaks were detected from the 11$^{th}$ minute to the 21$^{st}$ minute. The peak that projected the most was peak 7 which appeared at the 18$^{th}$ minute (Fig S7.7 a). There were 11 peaks detected for MEF 134 FD K2V3V5 (Fig S7.8a). These peaks were detected from the 15$^{th}$ minute and the most prominent peaks were 10 and 11. The TIC chromatogram for MEF 11 FH V1V1 showed 11 peaks (Fig S7.9a). These peaks occurred from the 2$^{nd}$ minute of analysis to about 19$^{th}$ minutes. The chromatogram for MEF 65 FH V3V3 showed 7 peaks and peak number one was the most prominent (Fig S7.10 a). Though 10 peaks were detected for MEF 75 FH V1V1, there were many impurities recorded from time point 17 (Fig S7.11 a). The HPLC-HRMS full scan of MEF 112 FH V1V1 revealed 7 peaks (Fig S7.12a).

**Table 7.1.** Activity profile of the 9 fractions use the mass the spectrometry analyses

| | | Zone of inhibition/mm | |
|---|---|---|---|
| **Number** | **Fractions used for Spectrometry analyses** | ***C. albicans*** | ***S. cerevisiae*** |
| 1 | MEF 134 FDV5V9 | 13 | 0 |
| 2 | MEF 134 FDV5V7 | 8 | 0 |
| 3 | MEF 134 FD K1V1V1 | 0 | 10.5 |
| 4 | MEF 134 FD K1V5V3 | 0 | 8.5 |
| 5 | MEF 134 FD K2V3V5 | 0 | 11 |
| 6 | MEF 11 FH V1V1 | 0 | 0 |
| 7 | MEF 65 FH V3V3 | 0 | 0 |
| 8 | MEF 75 FH V1V1 | 0 | 9 |
| 9 | MEF 112 FH V1V1 | 0 | 8 |

Fluconazole: 22 mm, Amphotericin B: 10 mm, 0: no activity observed

### 7.3.2 Major Peaks Selected for MS2 Analysis

The mass spectrometry analyses were conducted in tandem. Some of the major peaks identified in the TIC were selected for mass spectrometry analyses (Tables 7.2 and 7.3).

Peaks 1, 2, 3 and 5 were selected from the TIC of MEF 134 FD V5V7 for the MS1 analysis (Table 7.2). The spectra obtained are shown in Fig S7.4b and c. From these spectra, base peaks were selected for the MS2 analysis. The structures of the compounds were elucidated using the MS2 spectra; these spectra are not shown here because they could be used to deduce the structures of novel compounds that were identified from this study. For the chromatogram data from MEF 134 FDV5V9, only peak one was selected for MS1 and the fragmentation pattern of that compound is shown in Fig S7.5b. The base peak selected for MS2 analysis from the spectrum was m/z 354. A total of 6 peaks were anaysed via MS1 from the chromatogram of MEF 134 FD K2V3V5 (Fig S 7. 8b and c). The base peaks selected from those spectra ranged between m/z 277 and 486. Also, 8 peaks were studied from the TIC obtained from MEF 134 FD K1V1V1 (Fig S7.7b and c). From the spectra obtained, the base peaks were selected and fragmentated in the second round of mass spectrometry analyses as decribed for others. The selected base peaks ranged from m/z 211 to 453. A total of 3 peaks were chosen and studied for MEF 134 FD K1V5V3 (Fig S7.6a and c). The base peaks from the spectra for MS2 were in the range of m/z 267 to 453. Peaks 1, 3, 4 and 7 were studied from the TIC of MEF 11 FH V1V1 (Fig S7.9 b and c). The range of these peaks were from m/z 227 to 453. A total of three peaks (1, 4 and 5) were analyzed from the TIC of MEF 65 FH V3V3. The peaks selected were in the range of m/z 165 and 309 (FigS7.10 b and c). From the TIC of MEF 75 FH V1V1(Fig S7.11b and c) and MEF 112 FH V1V1 (Fig S7.12 band c),only one peak each was selected for analyses. The mass spectra information from the MS2 analyses together

with the fragmentation patterns of the selected peaks were used to elucidate the structures of the compounds identified.

**Table 7.2.** Structure characteristics of the compounds identified from MEF 134 by Mass spectrometry

| Entry | MS/MS Spectra | Masses selected for MS2 fragmentation | Classification of structures obtained | |
|---|---|---|---|---|
| | | | Novel | Patented |
| 1 | MEF 134 FD V5V7-1 | 227 | | √ |
| 2 | MEF 134 FD V5V7-2 | 340 | √ | |
| 3 | MEF 134 FD V5V7-3 | 453 | √ | |
| 4 | MEF 134 FD V5V7-5 | 354 | √ | |
| 5 | MEF 134 FD V5V9-1 | 354 | | √ |
| 6 | MEF 134 FD K2V3-V5-1 | 227 | √ | |
| 7 | MEF 134 FD K2V3-V5-4 | 200 | | √ |
| 8 | MEF 134 FD K2V3-V5-5 | 254 | √ | |
| 9 | MEF 134 FD K2V3-V5-6 | 468 | √ | |
| 10 | MEF 134 FD K2V3-V5-8 | 425 | √ | |
| 11 | MEF 134 FD K2V3-V5-10 | 300 | | √ |
| 12 | MEF 134 FD K1V1-V1-1 | 211 | √ | |
| 13 | MEF 134 FD K1V1-V1-2 | 213 | √ | |
| 14 | MEF 134 FD K1V1-V1-3 | 453 | | √ |
| 15 | MEF 134 FD K1V1-V1-4 | 149 | √ | |
| 16 | MEF 134 FD K1V1-V1-5 | 293 | √ | |
| 17 | MEF 134 FD K1V1-V1-6 | 309 | √ | |
| 18 | MEF 134 FD K1V1-V1-7 | 237 | √ | |
| 19 | MEF 134 FD K1V1-V1-9 | 407 | √ | |
| 20 | MEF 134 FD K1V5V3-2 | 453 | √ | |
| 21 | MEF 134 FD K1V5-V3-3 | 228 | √ | |
| 22 | MEF 134 FD K1V5-V3-4 | 267 | | √ |

The numbers indicated in red were the peaks selected for MS fragmentation after the TIC scan for that particular fraction

**Table 7.3. Structure characteristics of the compounds identified from the hexane fractions of MEF 11, 65 and 75 by Mass spectrometry analyses**

| | | | Classification of structures obtained | |
|---|---|---|---|---|
| Entry | MS/MS Spectra | Masses selected for MS2 fragmentation | Novel | Patented |
| 23 | MEF 11 FH V1V1-1 | 227 | | √ |
| 24 | MEF 11 FH V1V1-3 | 453 | √ | |
| 25 | MEF 11 FH V1V1 - 4 | 301 | | √ |
| 26 | MEF 11 FH V1V1-7 | 385 | √ | |
| 27 | MEF 65 FH V3V3-1 | 165 | √ | |
| 28 | MEF 65 FH V3V3-4 | 297 | √ | |
| 29 | MEF 65 FH V3V3 -5 | 309 | √ | |
| 30 | MEF 75 FH V1V1-2 | 340 | √ | |
| 31 | MEF 112 FH V1V1 - 7 | 237 | | √ |

The numbers indicated in red were the peaks selected for MS fragmentation after the TIC scan for that particular fraction

### 7.3.3 Putative Structures of Compounds Identified in the Fractions

The spectrum from the mass spectrometry analyses indicated the exact masses of the elements and hence, can be used to characterize and identify compounds with high accuracy and precision. The spectrum generated from analyses of the samples were used to elucidate the tentative structures of the compounds in each of the 9 TLC fractions (Fig 7.1). The structures were elucidated by identifying molecular ion peaks from the MS2 spectra that were generated and applying the rule of 13 to these spectra. The rule of 13 was applied to every MS2 data selected for structural characterization. This rule is based on the assumption that each compound being investigated contains both Carbon and Hydrogen atoms. Thus, the masses of the molecular ions were divided by 13 and then modified to obtain a molecular formula for their corresponding alkane ($C_nH_{2n+2}$ ). Prominent peaks were labelled and differences in peaks were related to appropriate molecules. Peaks corresponding to fragments of the compounds were labelled. The difference between each peak corresponds to the loss of small molecule like $H_2O$ and $NH_3$. The difference between the peaks, both long range and short range, were determined in order to relate these differences to the loss of specific molecules or fragments. For example, a difference of 18 implies loss of $H_2O$ while a difference of 17 may imply loss of $NH_3$.

Elemental substitutions were also used to incorporate relevant information, such as the degree of unsaturation, to the alkane formula obtained from application of the rule of 13. This procedure was used to determine the number of double bonds, triple bonds or rings that might be present in the compounds.With knowledge of the degree of unsaturation and molecular formula, the tentative structures of the compounds were determined using computational chemistry. The software, Chemsketch, was used to create images of the chemical structures that were solved from the MS2 data. The spectrum of the tentative structures were generated by Chemdraw and compared to the

ones obtained from the mass spectrometry. The novelty of the various compounds obtained from the 9 TLC fraction were ascertained using Chemistry database platforms such as SciFinder® and ChemSpider (Royal society of chemistry). Altogether, 31 compounds were identified from the 9 TLC fractions (Figs. 7.2 to 7.7), 9 of the compounds were already known and had been patented while 22 of the compounds were novel (Table 2 and 3). The spectra from MS2 fragmentation of the compounds are not shown in this thesis for the purposes of confidentiality of the novel compounds that are represented by most of these spectra.

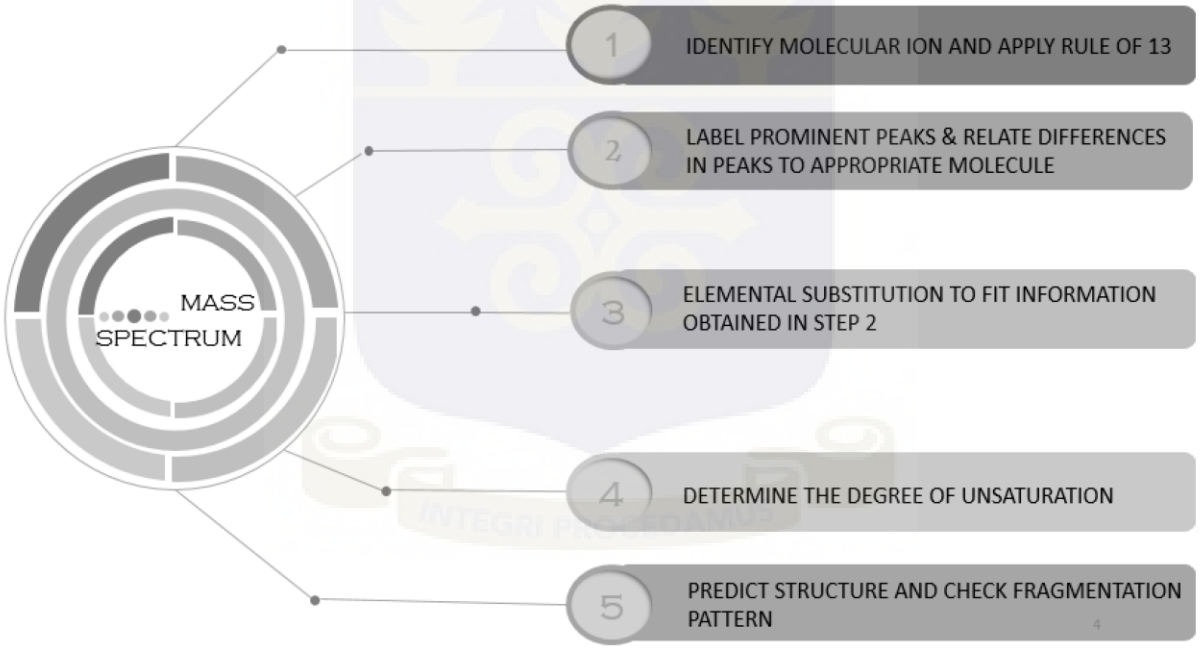



Fig 7.1. A diagram showing the structure elucidation technique used to predict the structures of the 31 compounds.

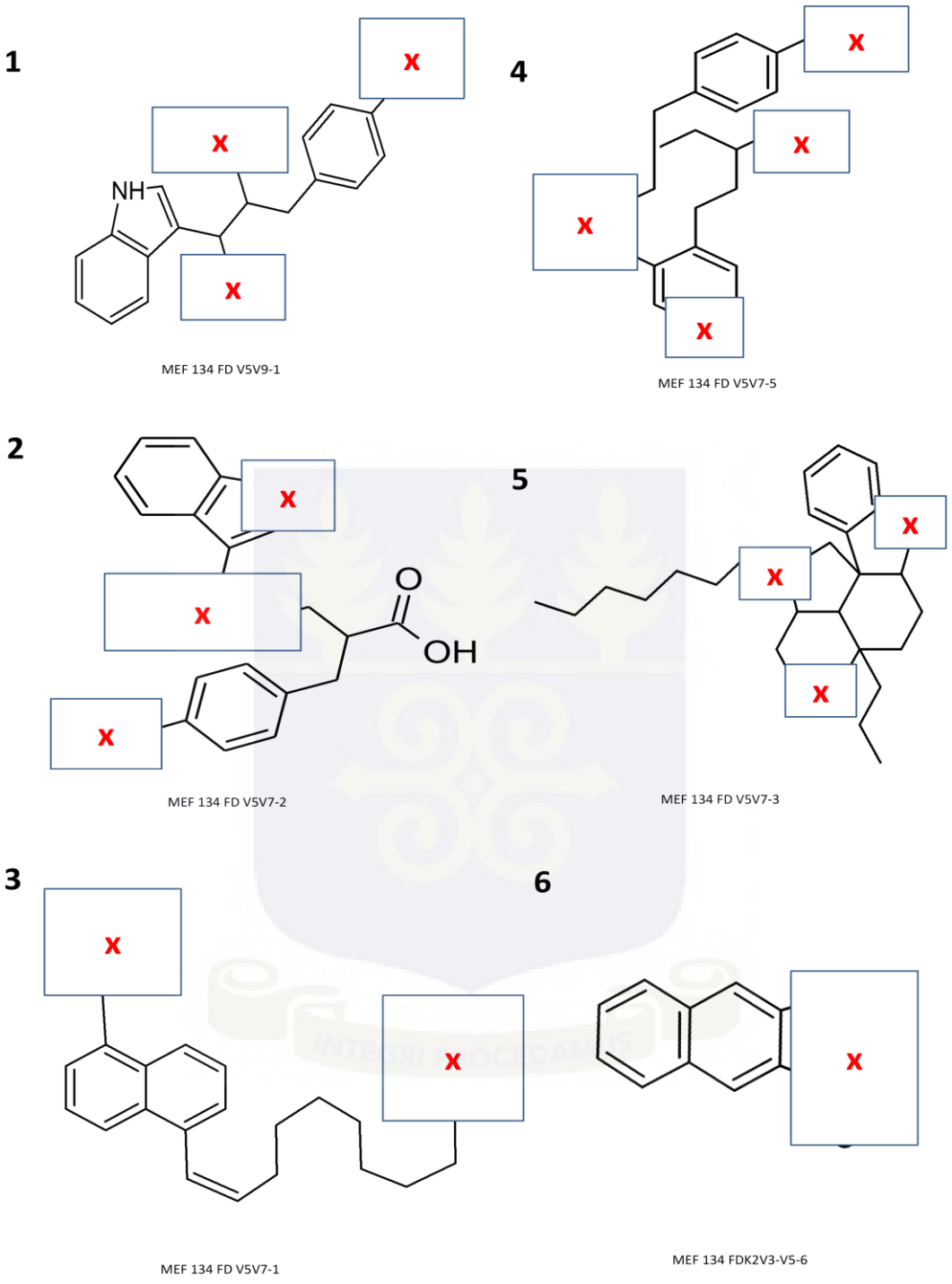



Fig 7.2. The putative structures of the compounds identified from the fractions.

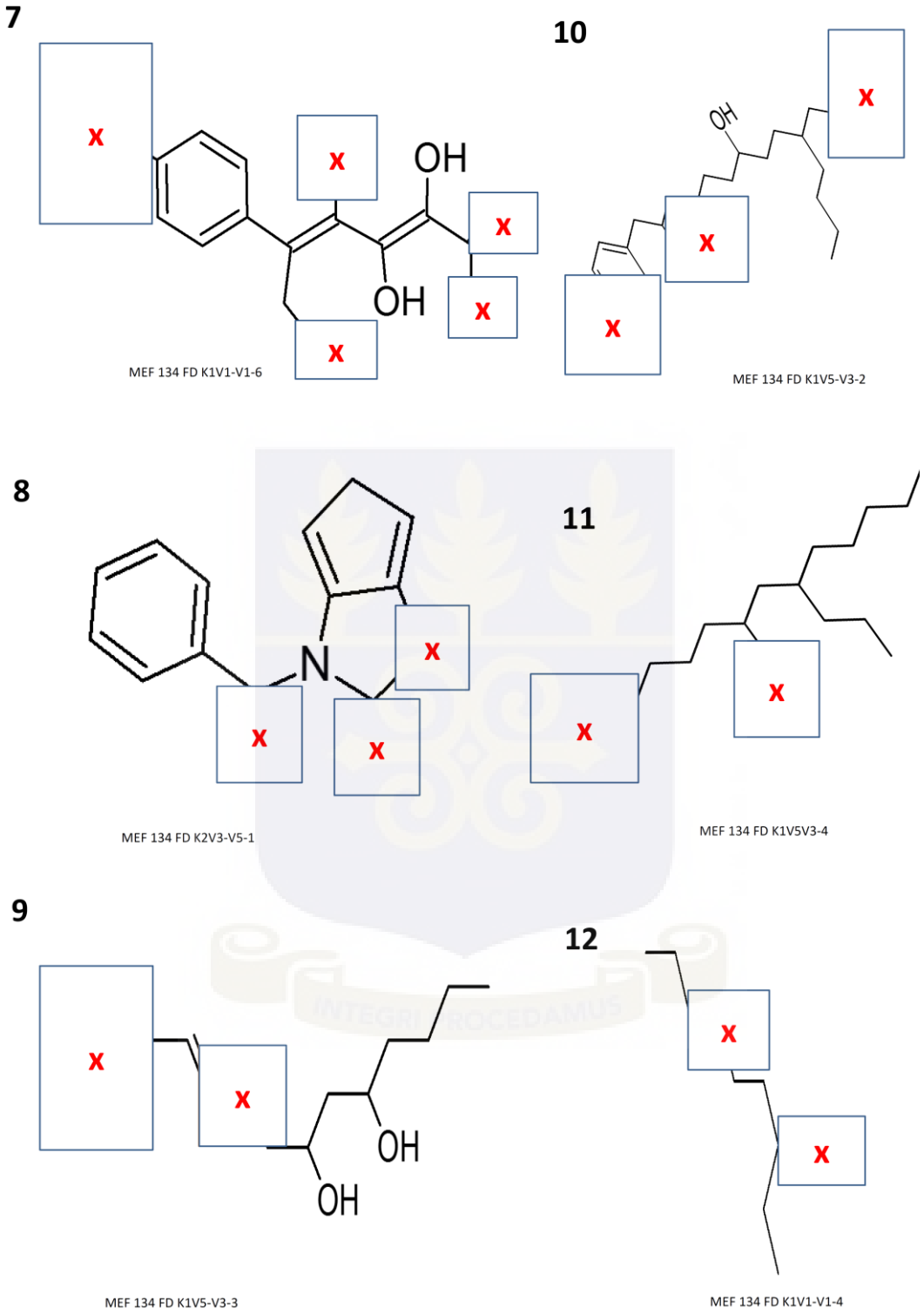



Fig 7.3. The putative structures of the compounds identified from the fractions.

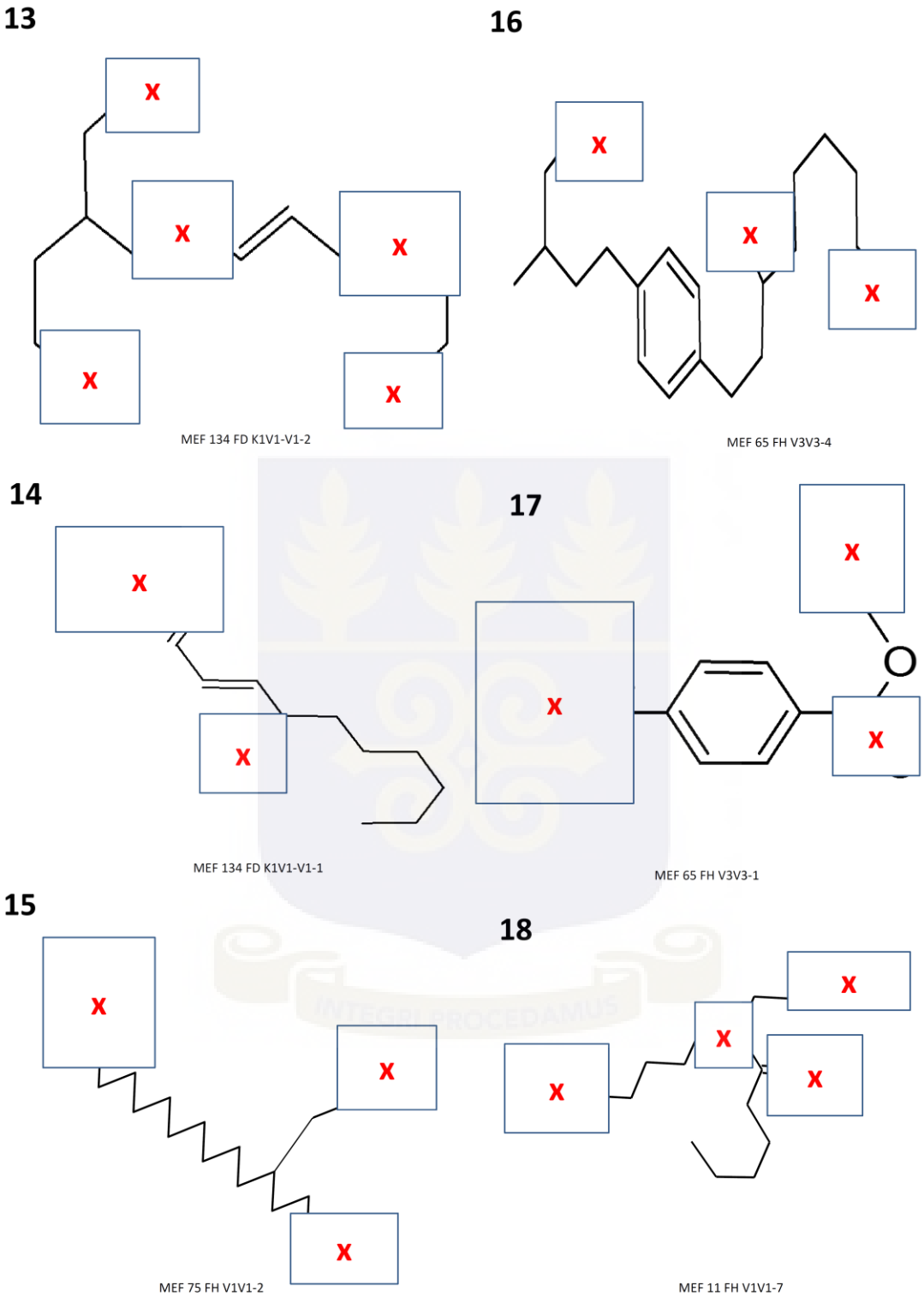



Fig 7.4. The putative structures of the compounds identified from the fractions.

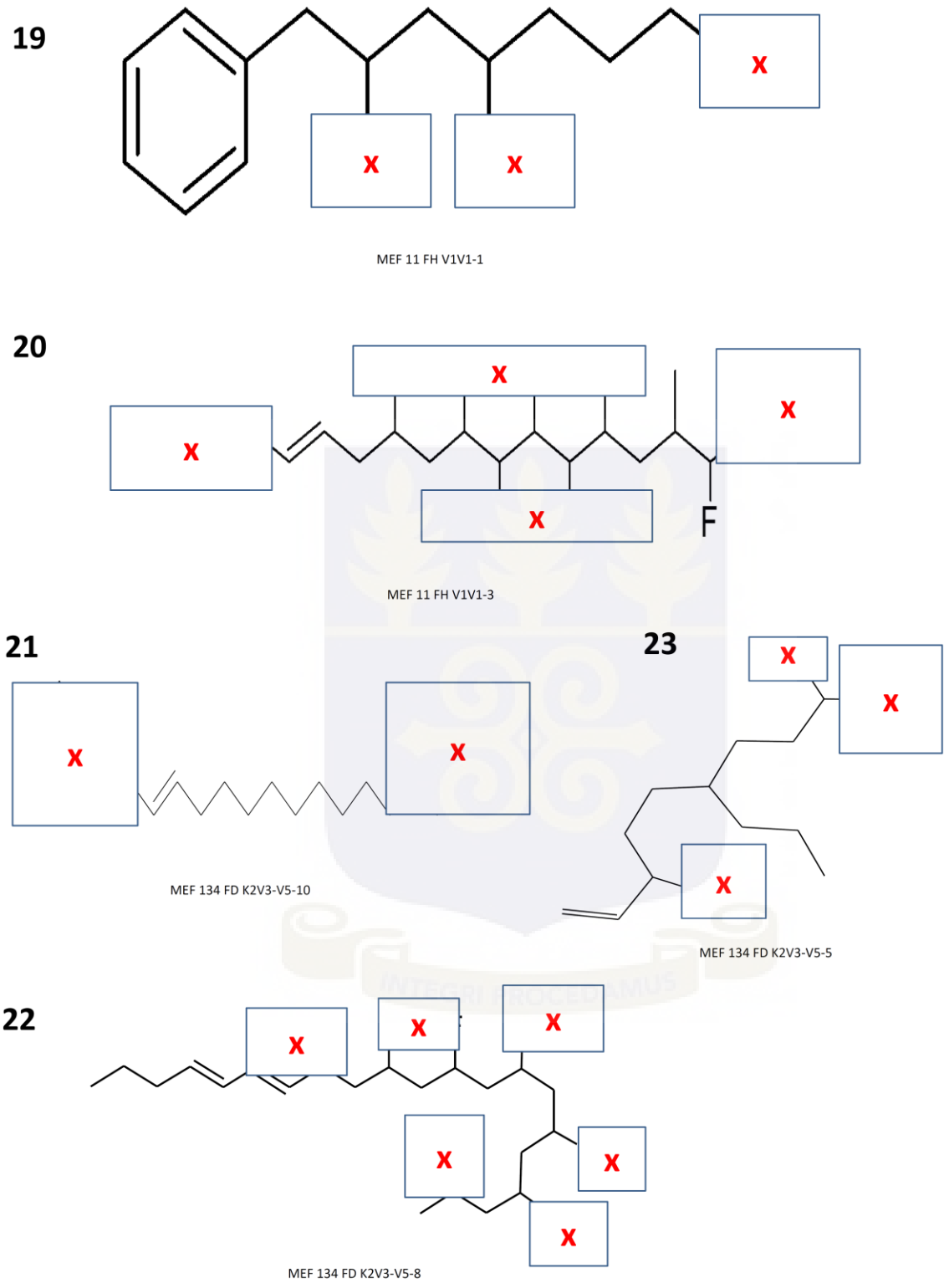



Fig 7.5. The putative structures of the compounds identified from the fractions.

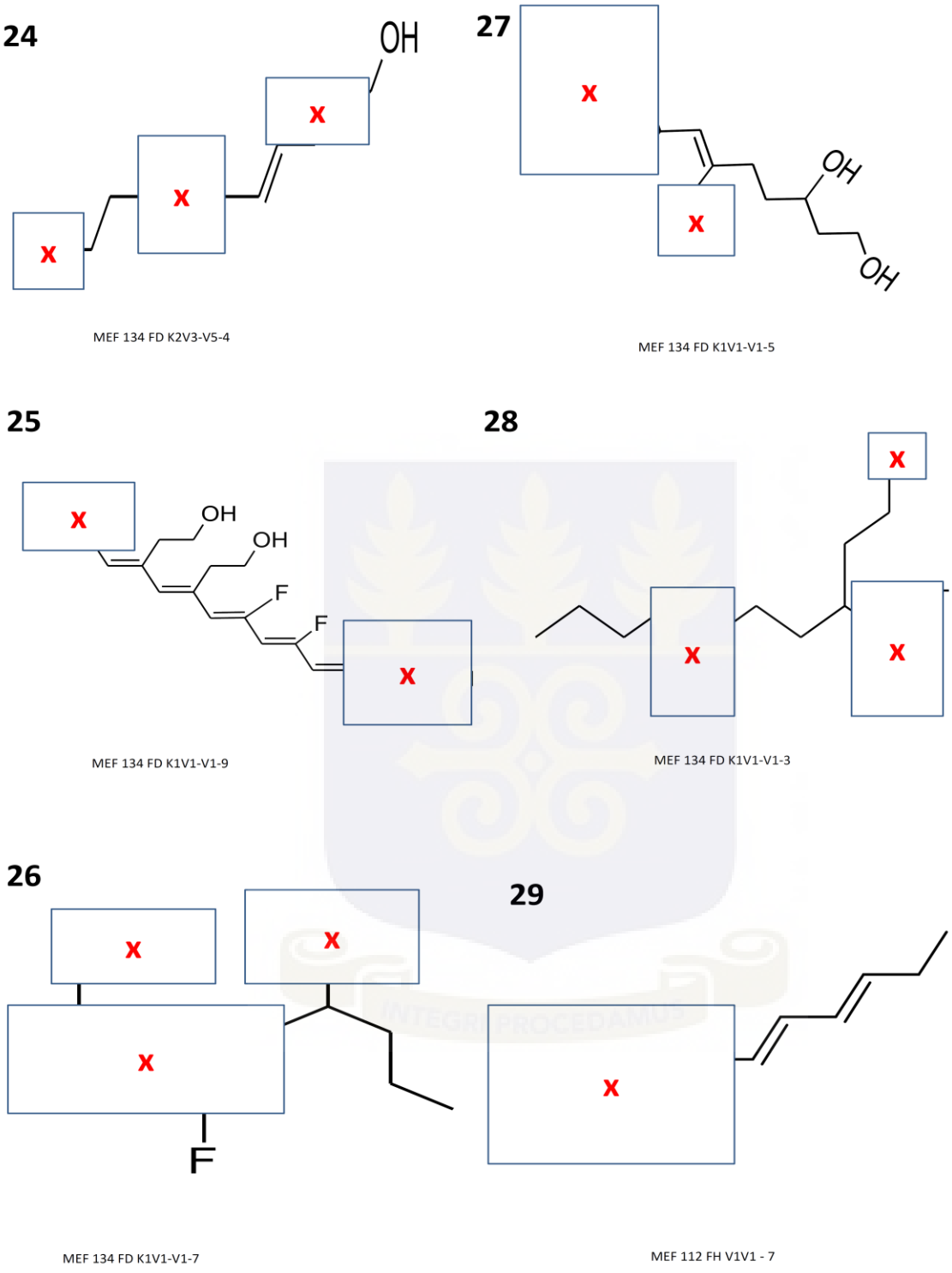
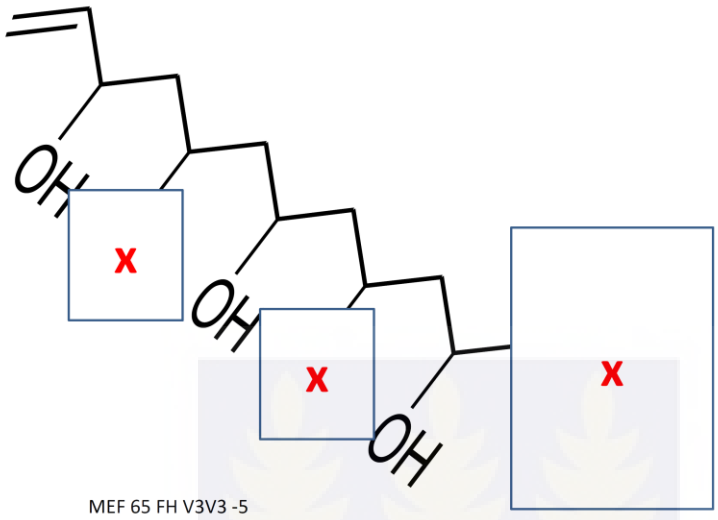


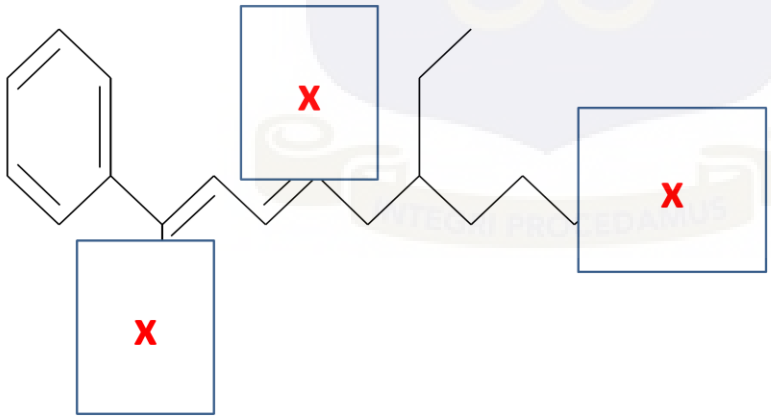

Fig 7.6. The putative structures of the compounds identified from the fractions.

**30**




MEF 65 FH V3V3 -5

**31**




MEF 11 FH V1V1 - 4

Fig 7.7. The putative structures of the compounds identified from the fractions.

### 7.4 Discussion

In this study, HPLC analysis was coupled to mass spectrometry and were used to analyze 9 TLC fractions which exhibited antifungal activity against *C. albicans* or *S. cerevisae*. These analyses were performed to identify the components of the fractions that contributed to the antifungal activities. Altogether, 31 compounds were identified from fractions of 5 marine fungal endophytes. Of these compounds, 22 were novel when they were analyzed using the available chemical databases such as SciFinder. The remaining 9 compounds were already known and were present in these database. Previous studies have assinged biological activities to a few of the known compounds.

Natural product research often involve the use of mass spectrometry as part of the discovery workflow. When used appropriately, mass spectrometry might provide relevant information for in-depth characterization of new compounds even in the absence of nuclear magnetic resonnance spectroscopy. Several structural information can be generated from a single mass spectrum using very small quantities of samples. HPLC/ESI-FTMS/MS profiling is a potent quantitative analyzation method for studying the constituent of complex extracts.

A bioactive molecule consist of an assembly of functional groups and these functional groups are a collection of atoms that confer specific chemical & physical properties to the bioactive molecule. Generally, functional groups of a molecule confer characteristics such as chemical reactivity, chemical stability, solubility and susceptibility towards metabolism. Functional groups could be acidic, basic, hydrophilic or lipophilic (Pop *et al.*, 2004; Sung & Topp, 1995). A hydrophilic compound is easily soluble in water while lipophilic compound can readily cross cellular membranes through passive diffusion. The structure of one of the compounds from fraction MEF 134 FD V5V9 was successfully elucidated in this study. This compound is known as 3-amino-3-

(1*H*-indol-3-yl)-2-(4-nitrobenzyl) propanoic acid and has been patented (Fig 7.2; Compound 1). The compound has a desirable functional group which is acidic and provides opportunity for easy ionization. Moreover, its mechanism of action *in vivo* may include formation of hydrogen bond with receptors and transport proteins. Though no biological studies have been conducted with this particular compound, the parent compound, propanoic acid has been used to inhibit growth of bacteria and mold in the food industry for many years. The efficacy of many organic acids against fungi, of which propanoic acid was one of the organic acids tested , only 0.1 to 0.2% was required to cause total inhibition of mold growth (Higgins & Brinkhaus, 1999). Since 3-amino-3-(1*H*-indol-3-yl)-2-(4-nitrobenzyl) was one of the most abundant components of the MEF 134 FD V5V9, the antifungal activity observed against *C. albican*s could be as a result of this compound. Hence, it is not surprising that the MEF 134 FD V5V9 fraction exhibited antifungal activity. A total of 4 compounds were obtained from MEF 11 FH V1V1 (Table 7.3), among these compounds, two are already known; 7-fluoro-1-phenylheptane-2,4-diol was one of the known compounds (Fig 7.5, compound 19). Heptane-based compounds have been shown to exhibit biological activities including anti-inflamatory and antimicrobial activities (Thennarasu & Perumal, 2002). It has also been shown that fluorine-containing compounds exhibit adequate anticancer activity (Song *et al.*, 2005).

All the three compounds identified from MEF 65 FH V3V3 were novel. Compound 17 (Fig 7.4) is a benzoic acid derivative. There is a growing interest in benzoic acid derivatives because of their desirable biological activities. For instant *p*-aminobenzoic acid is a precursor of folic acid (Basset *et al.*, 2004). Also *p*-aminobenzoic acid often forms the basis in the design of most new drugs and has been shown to exhibit very strong antibacterial activity (Michael *et al.*, 1995). The amino group on compound 3 (Fig 7.2) could be a contributing factor to the antifungal activity of the

fraction from which it was obtained. Amino group-containing compounds have been shown to display biological activity against fungal isolates (Vilipić *et al.*, 2015).

## 7.5 Conclusion

This study has provided insight into the compounds contained in the 9 TLC fractions obtained from 5 marine fungal endophytes. Many of the structures obtained were novel and could serve as precursor compounds in the design of new drugs or could be used as drugs on their own following in-depth characterization. Many interesting functional groups such as alkanoic acids, flourine, amino and hydroxyl groups formed part of the structures obtained from these fractions. It was evident from the study that all the 9 fractions studied contained multiple compounds. Hence the activity of the 9 fractions would not be attributed to any individual compound identified in these fractions. However, the high amounts of these compounds in some of the fractions might provide a clue about the probability of the identified compounds being the main bioactive constituent of those fractions. The biological activities of the known compounds (such as derivatives of propanoic acids or benzoic acids) further confirmed that this study is on the right path to discovery of novel compounds with potent antifungal and anticancer activities. Benzoic acid and its derivatives are already important components of many drugs while propanoic acid is an already known antifungal compound. Currently, there is limited data found in literature on the biological activities of the known compounds that were identified from this study, indicating that further work needs to be done on these already known compounds. In this study, the fractions were only profiled to identify possible chemical components because the starting material of the fractions were not sufficient for purification and isolation of the bioactive compounds in the fractions. Nonetheless, the information provided by this study is vital for subsequent compound isolation from marine fungal endophytes.

## 7.6 Recommendation for Future Works

- There is a need to chemically synthesize the novel structures in order to have enough material to validate their antifungal activites.

- It is important to study the mechanism of action of all the synthesized compounds to know the relevant pathways being targeted.

- It is also important to determine the toxicity profile of these novel compounds.

## 7.7 Supplementary Data

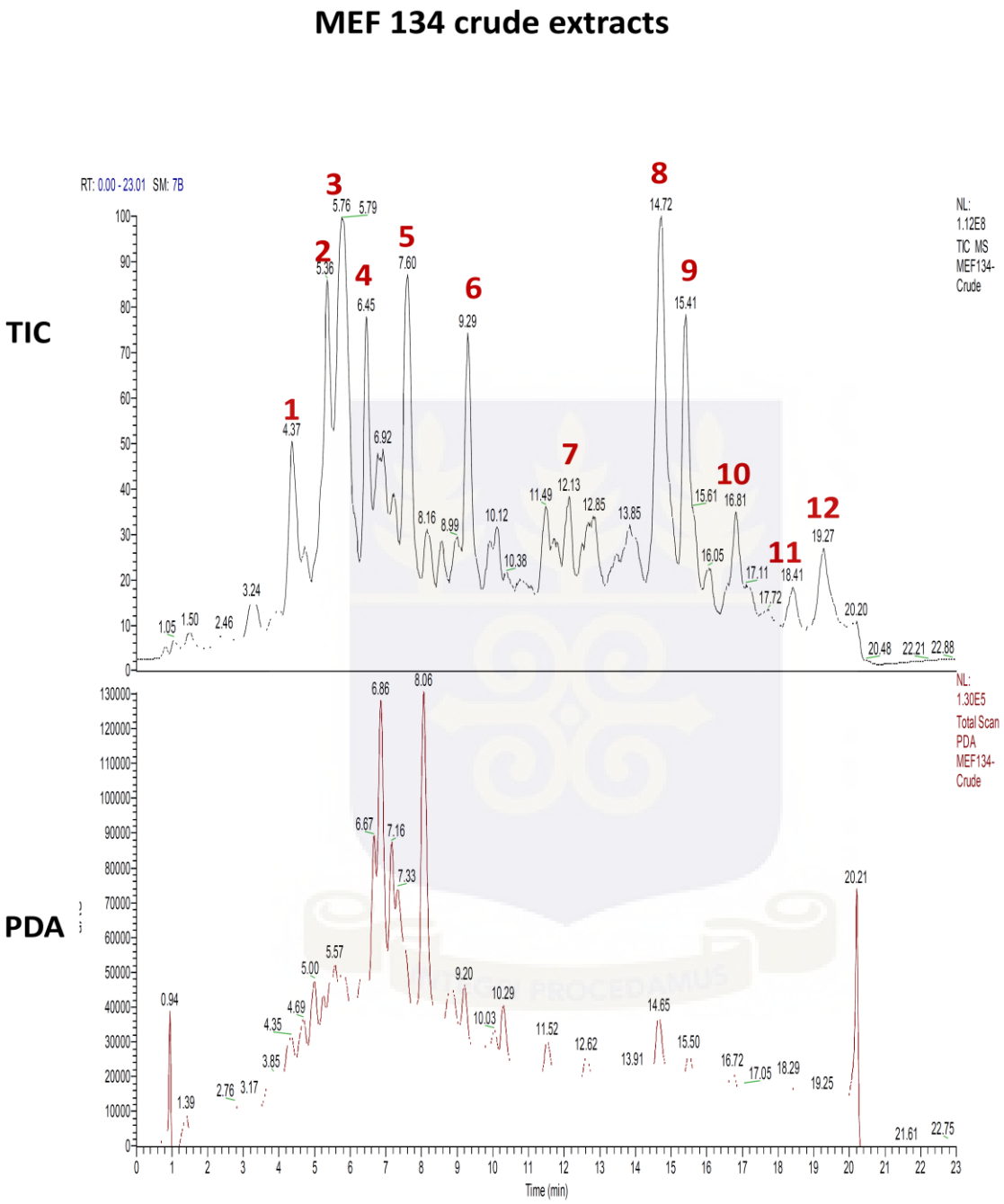



Fig S7.1a.Total ion current chromatogram of MEF 134 crude extracts after scanning by HPLC-HRMS

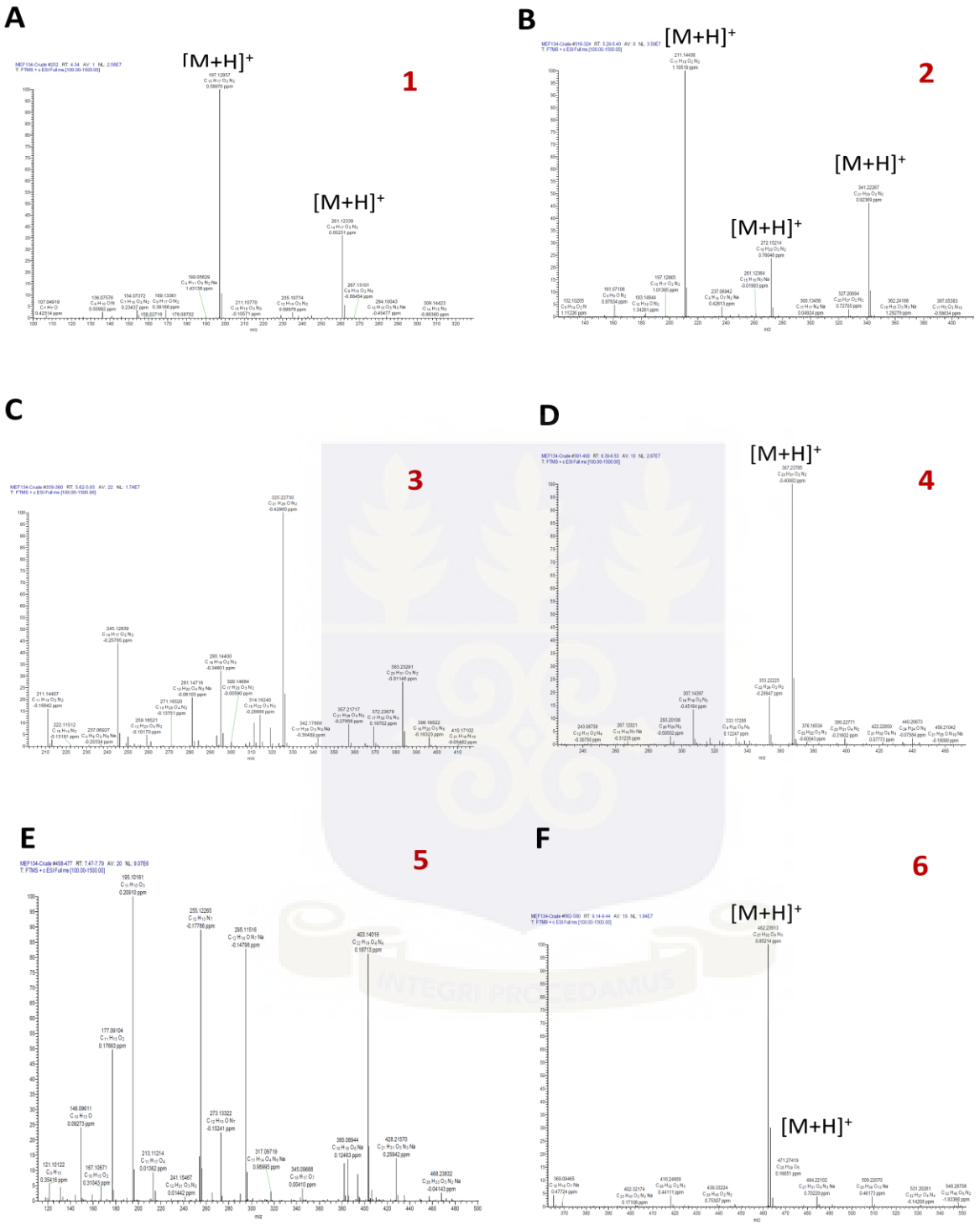



Fig S7.1 b. Mass spectra of peaks 1, 2, 3, 4, 5 and 6 detected in the TIC of the crude extract

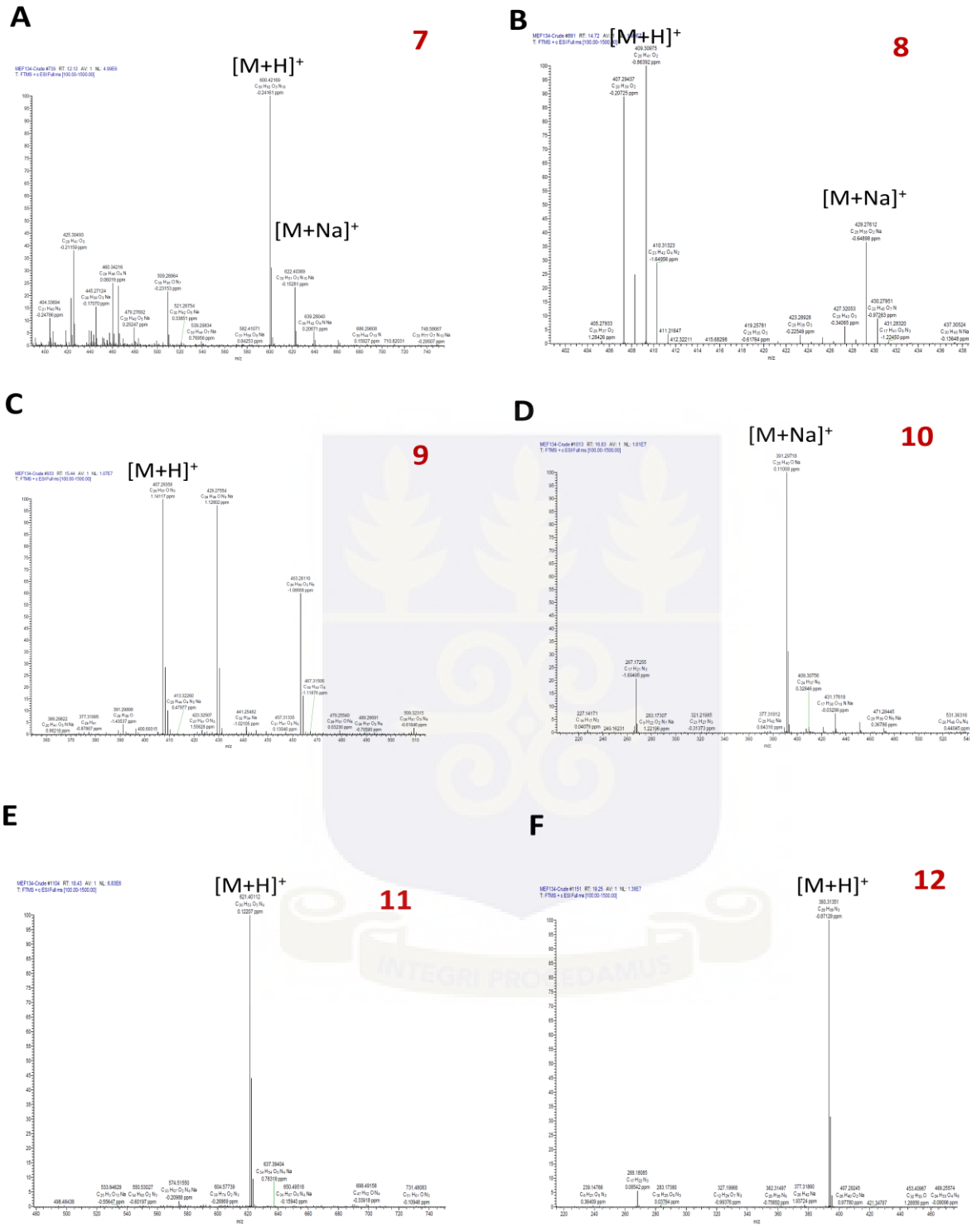



Fig S7.1c. Mass spectra of peaks 7, 8, 9, 10, 11 and 12 detected in the TIC of the crude extract

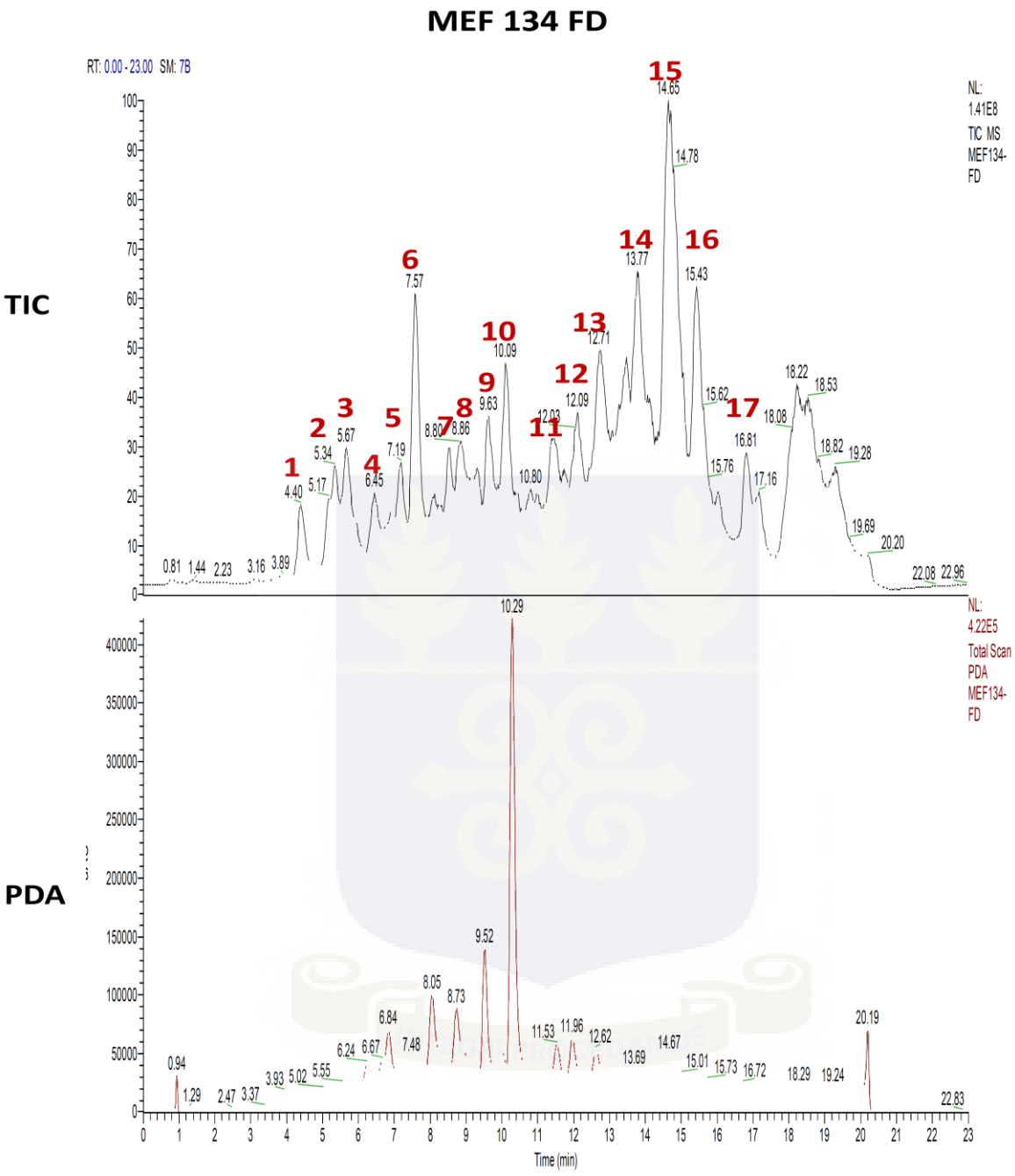



Fig S7.2a. Total ion current chromatogram of MEF 134 FD after scanning by HPLC-HRMS.

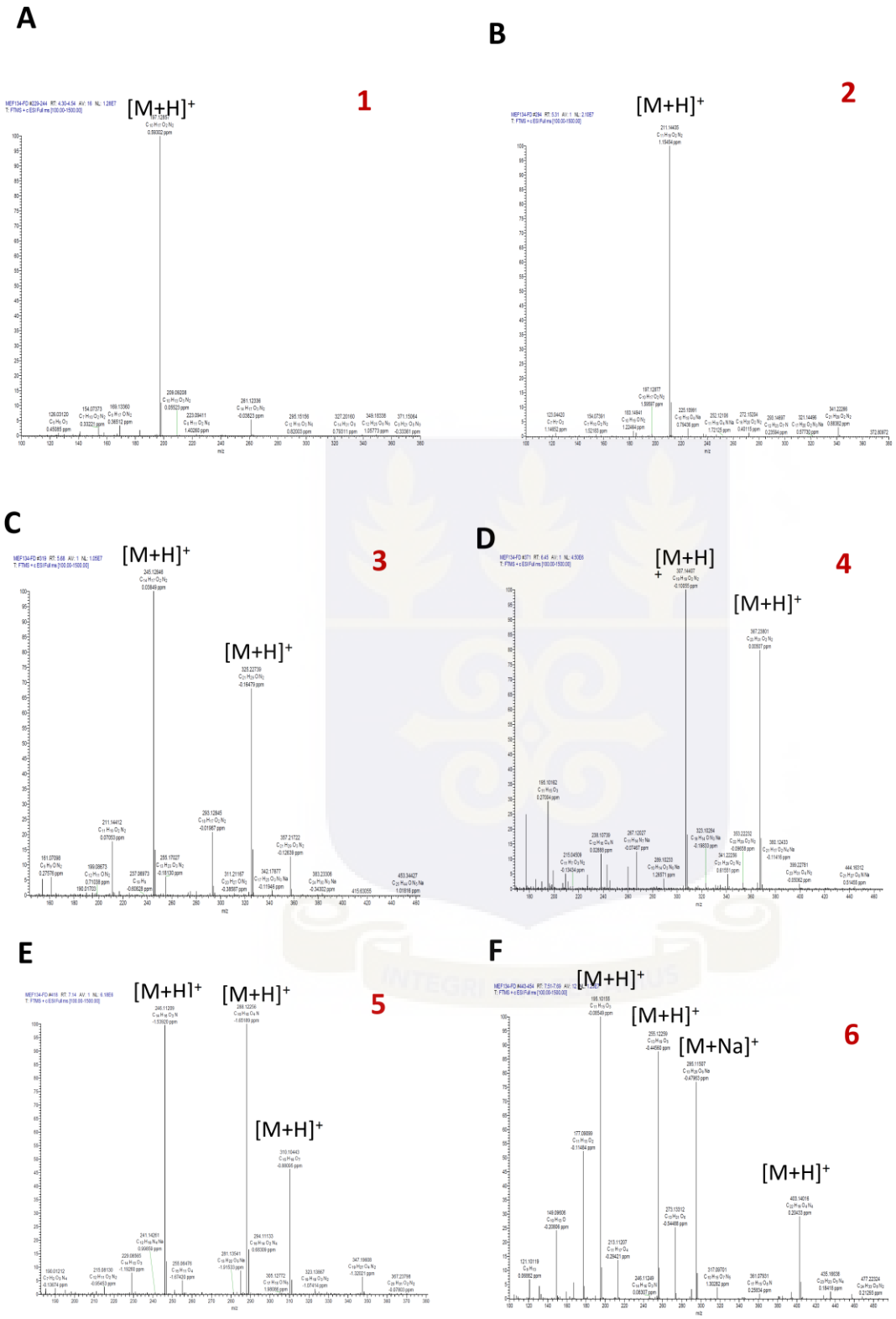



Fig S7.2b. Mass spectra of peaks 1, 2, 3, 4, 5 and 6 detected in the TIC of MEF 134 FD

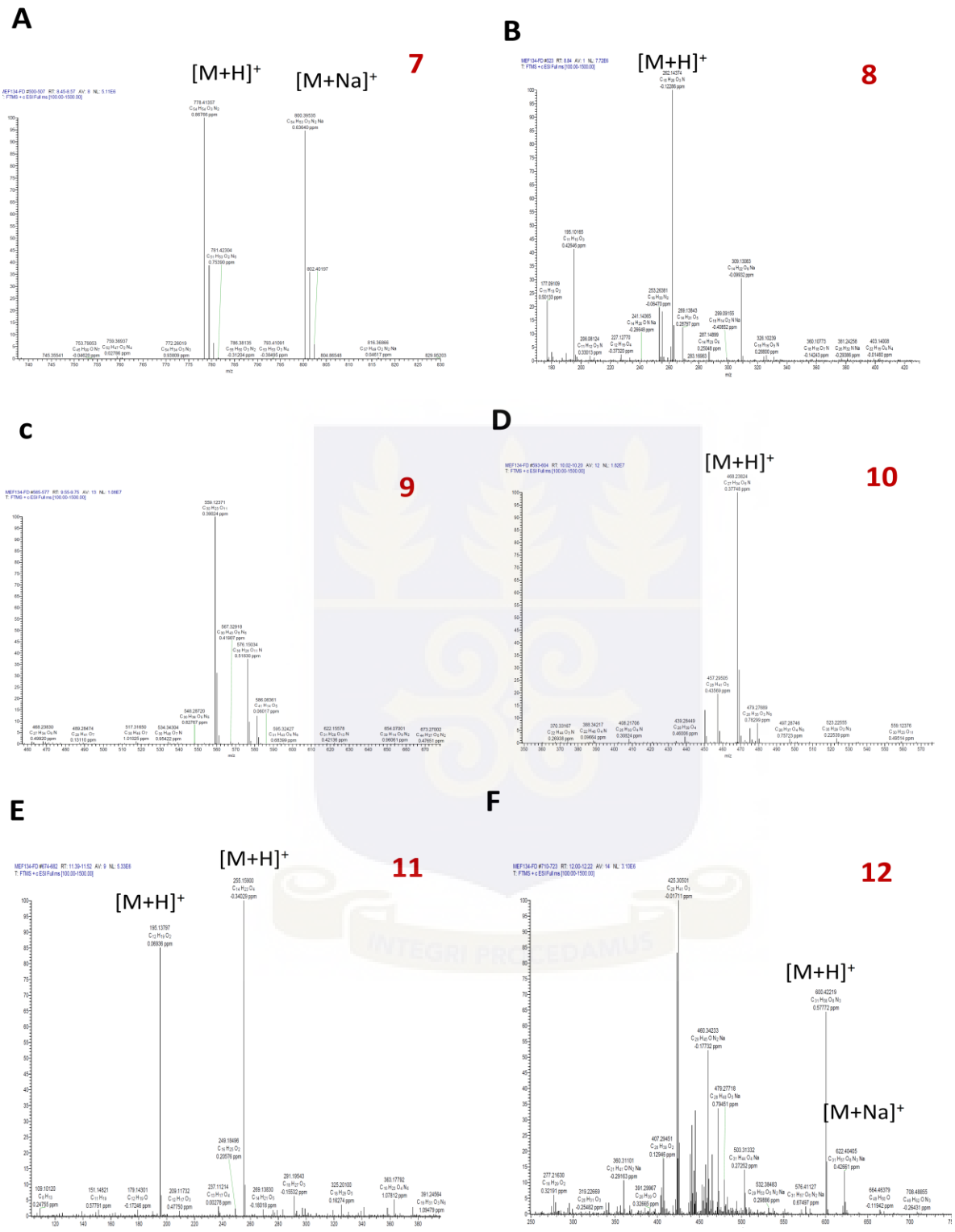



Fig S7.2c. Mass spectra of peaks 7,8,9,10,11 and 12 detected in the TIC of MEF 134 FD

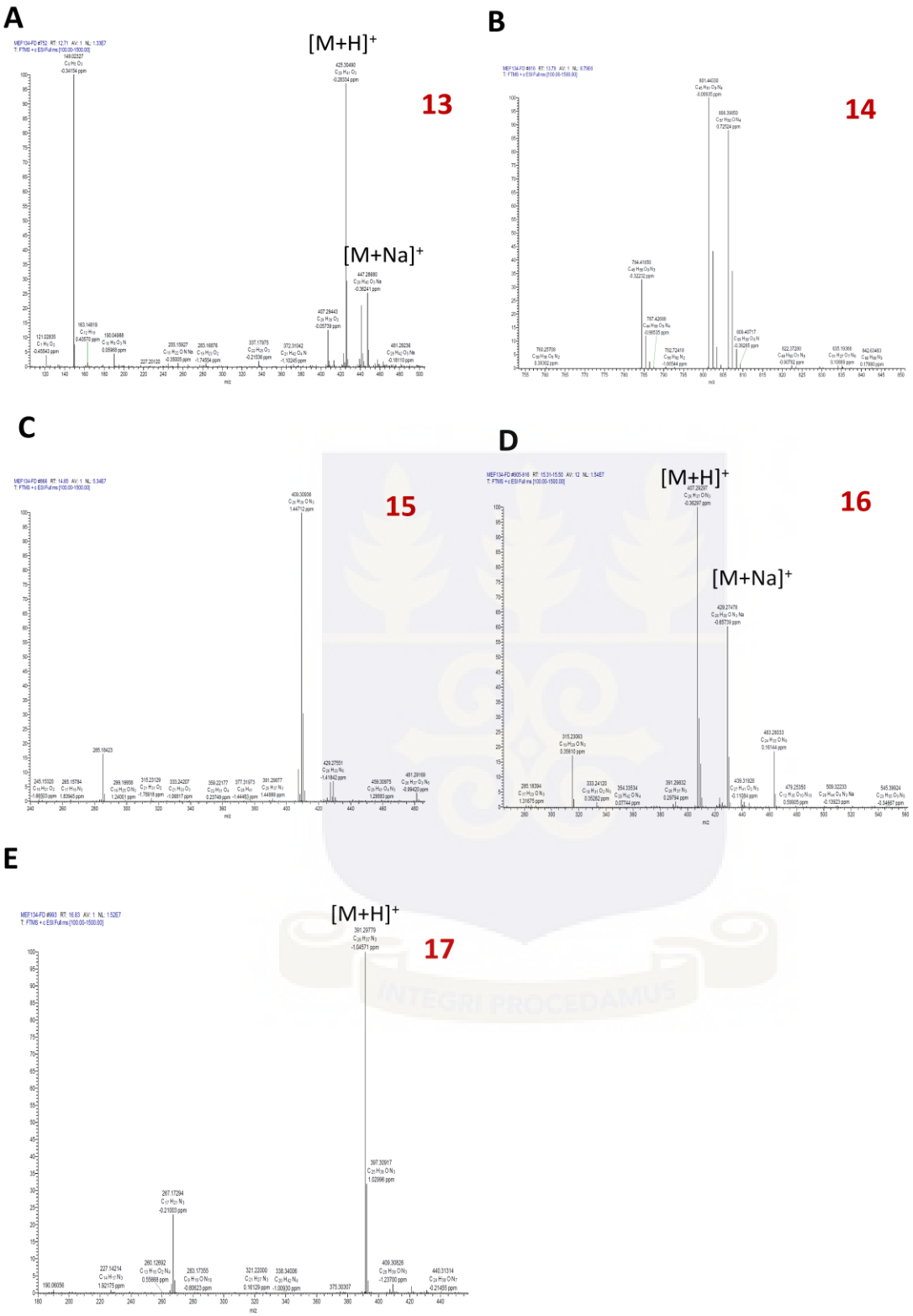



Fig S7.2d. Mass spectra of peaks 13, 14, 15, 16 and 17 detected in the TIC of MEF 134 FD

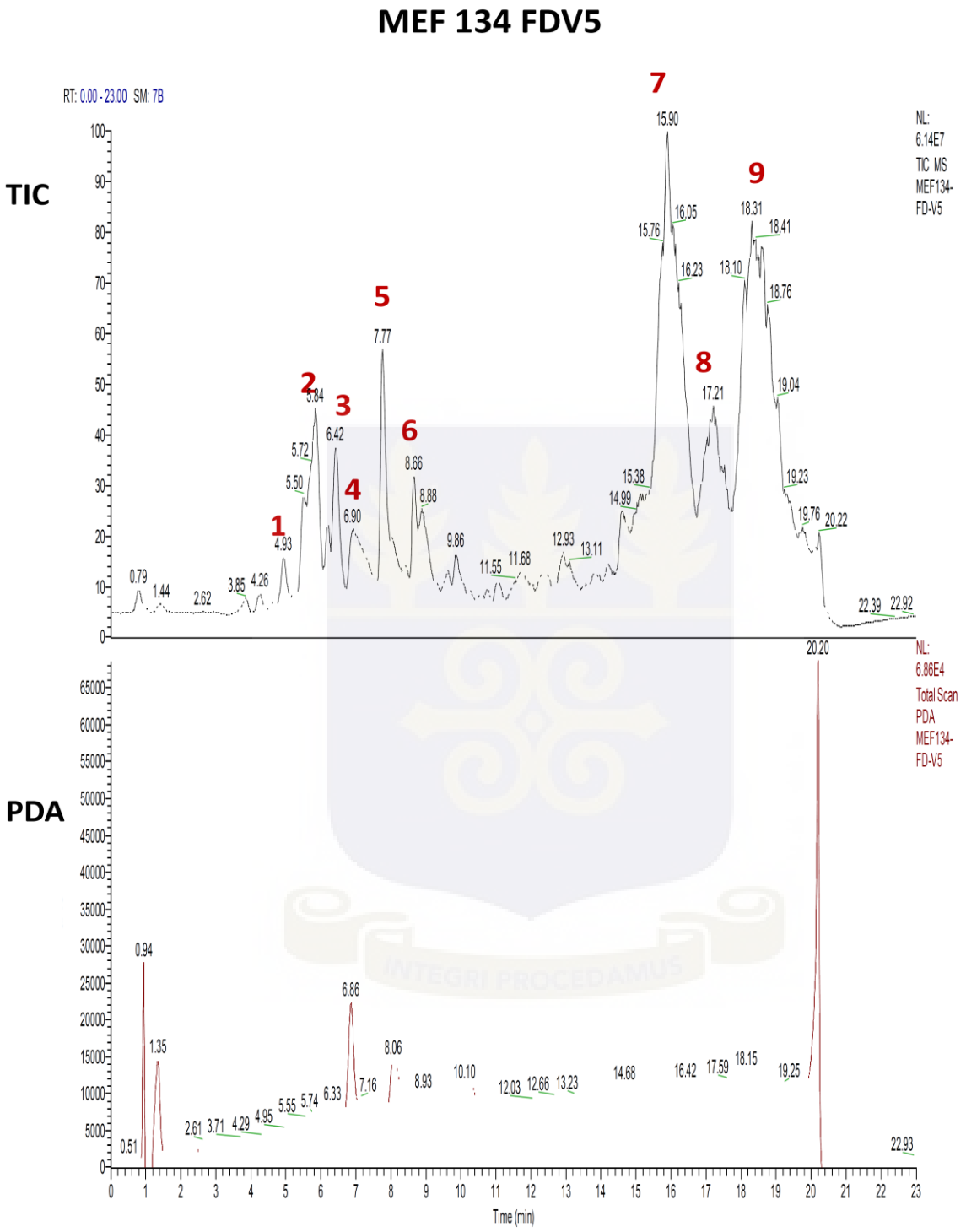



Fig S7.3 a. Total ion current chromatogram of MEF 134 FDV5 after scanning by HPLC-HRMS.

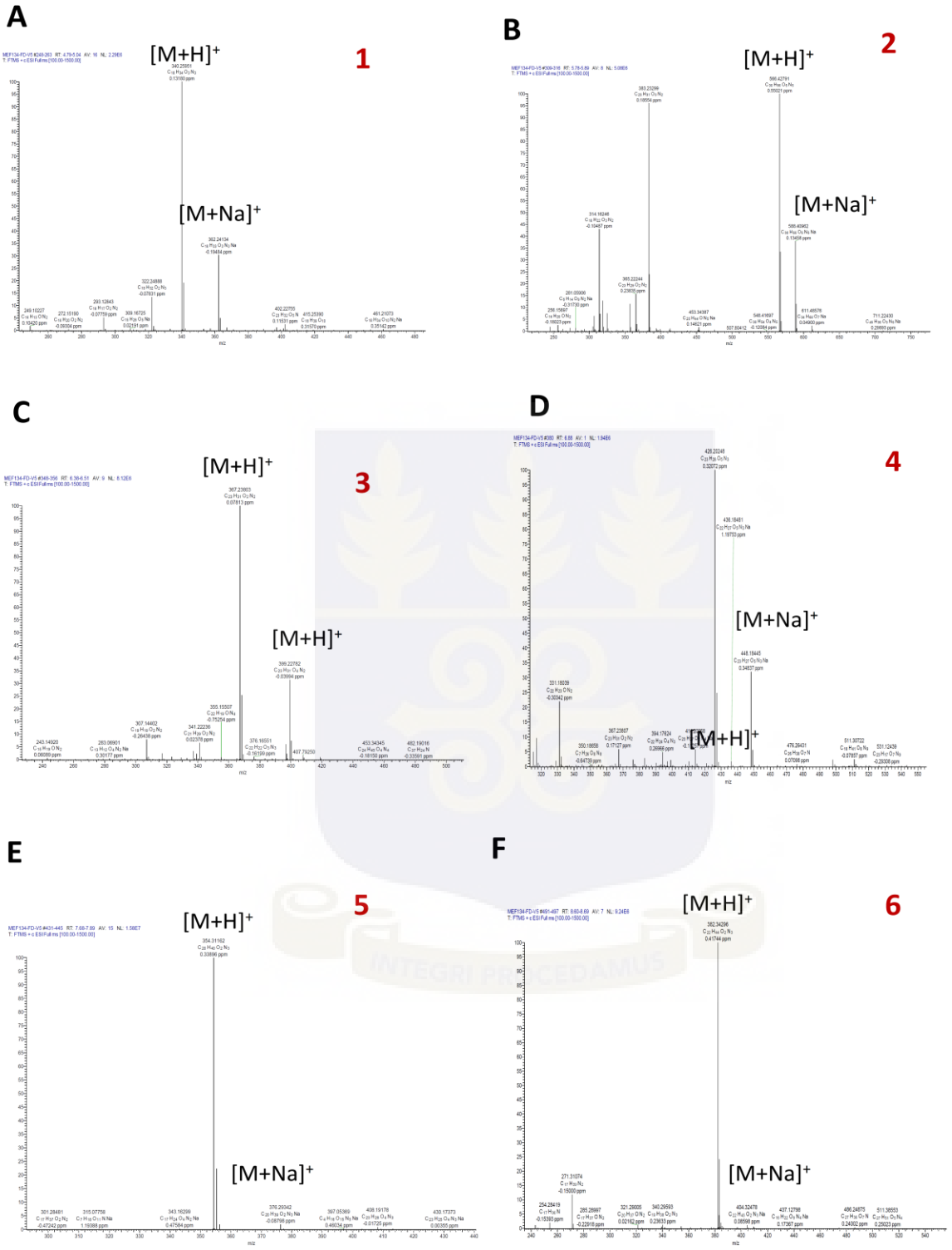



Fig S7.3 b. Mass spectra of peaks 1, 2, 3, 4, 5 and 6 detected in the TIC of MEF 134 FDV5

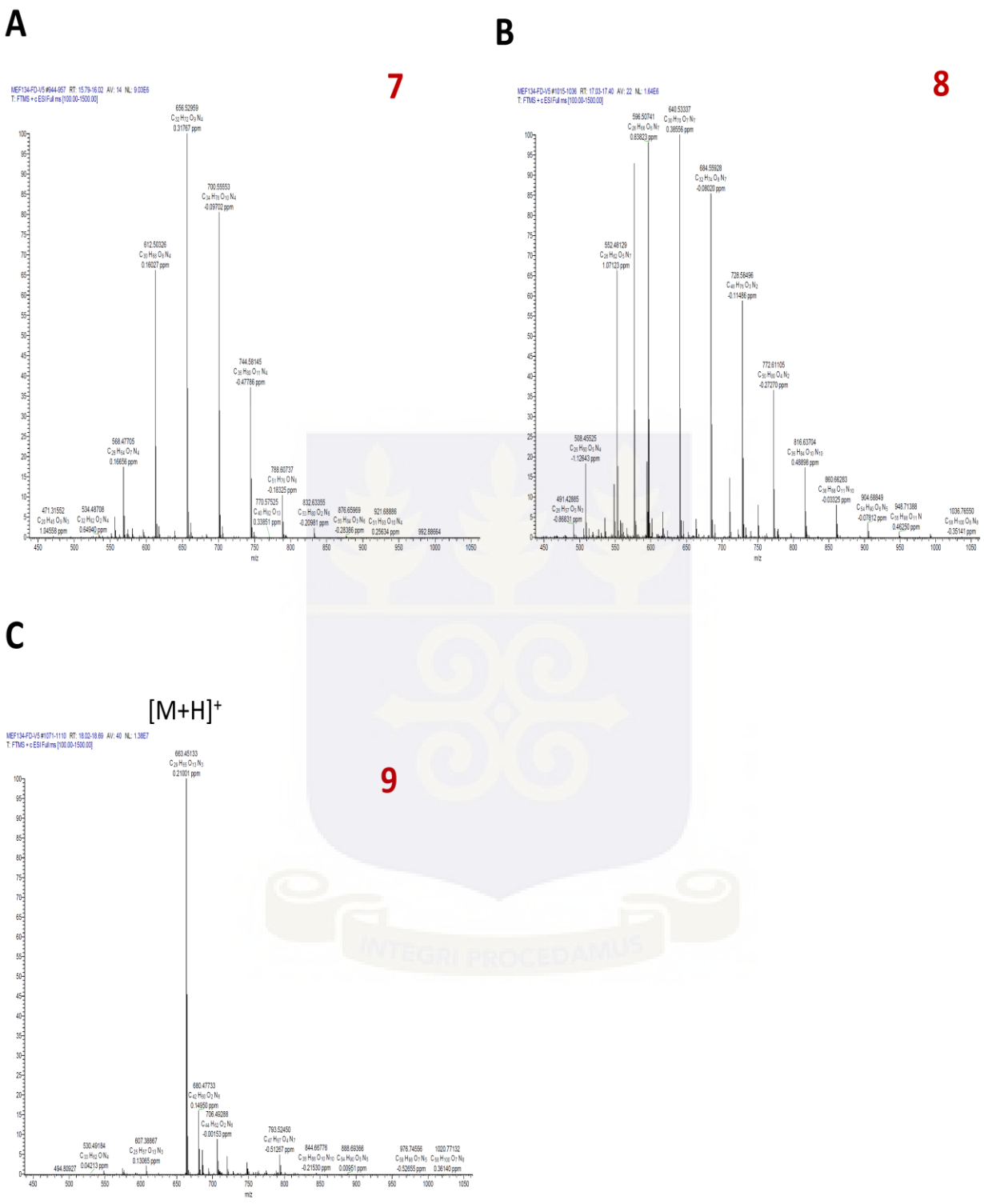



Fig S7.3 c. Mass spectra of peaks 7, 8 and 9 detected in the TIC of MEF 134 FDV5

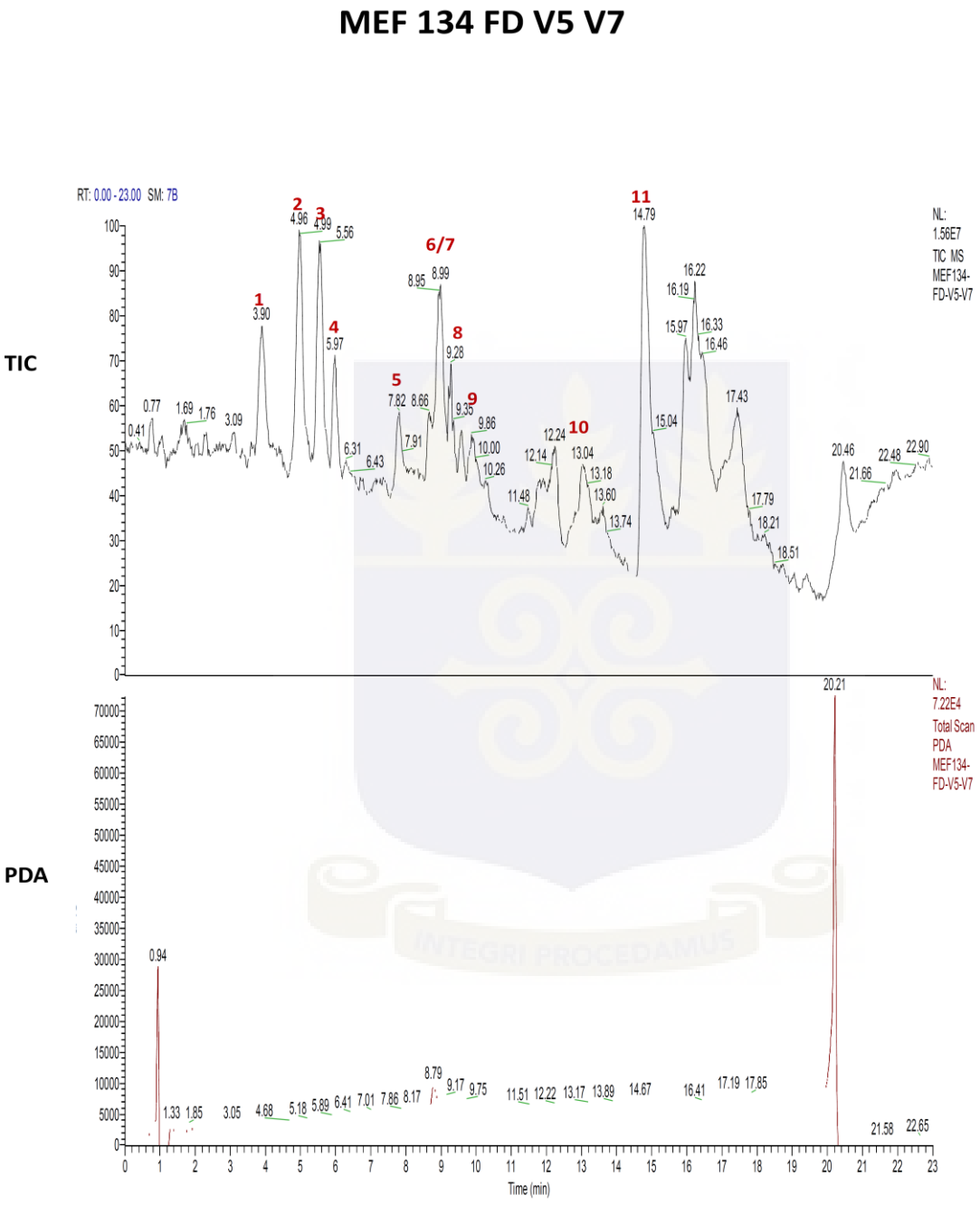



Fig S7.4a. Total ion current chromatogram of MEF 134 FD V5 V7 fractions after scanning by HPLC-HRMS.From the chromatogram of MEF134 FDV5V7, 11 major peaks were detected.

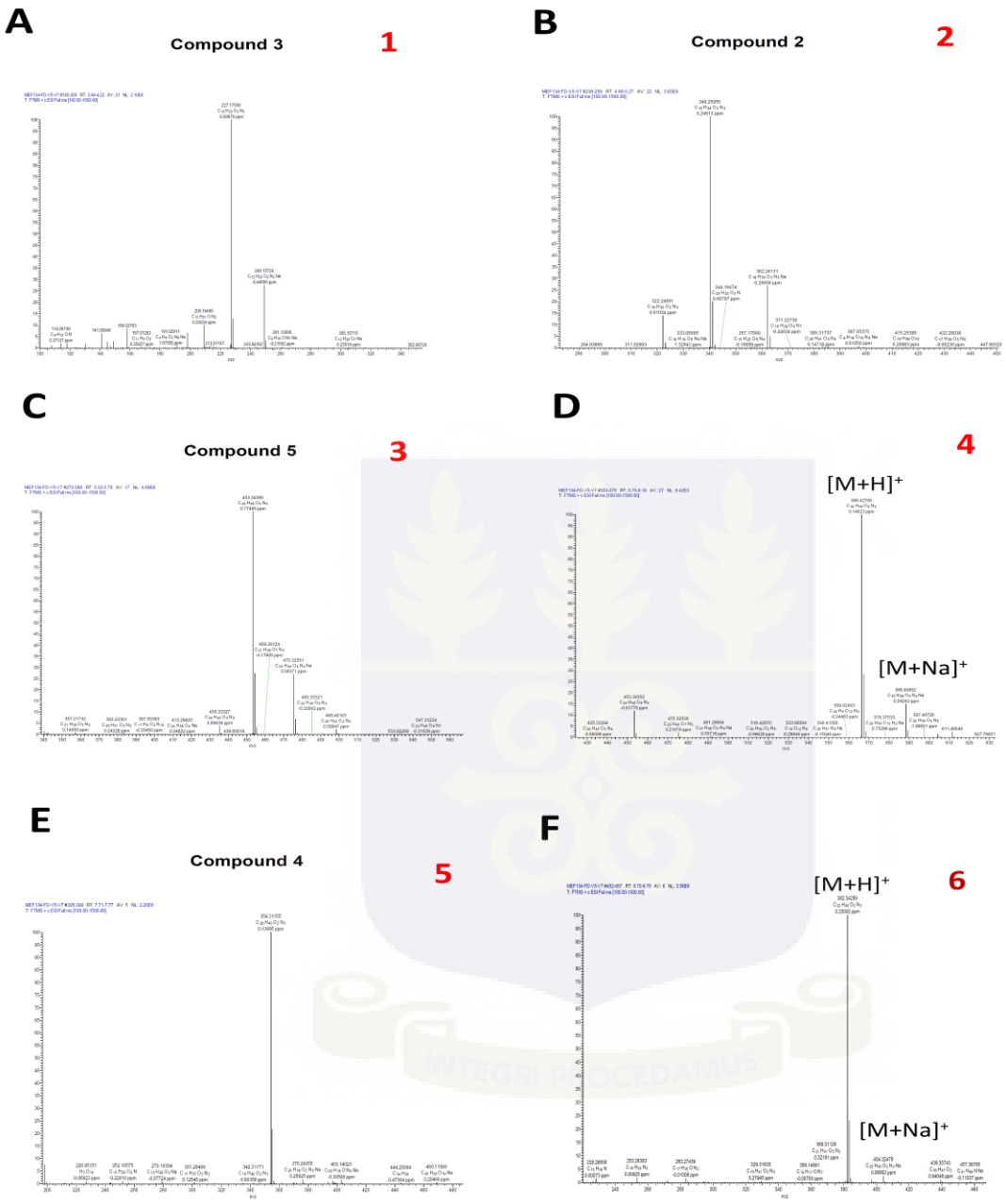



Fig S7.4b. Electrospray ionization-HRMS spectra of TLC fractions from MEF 134 FD V5 V7

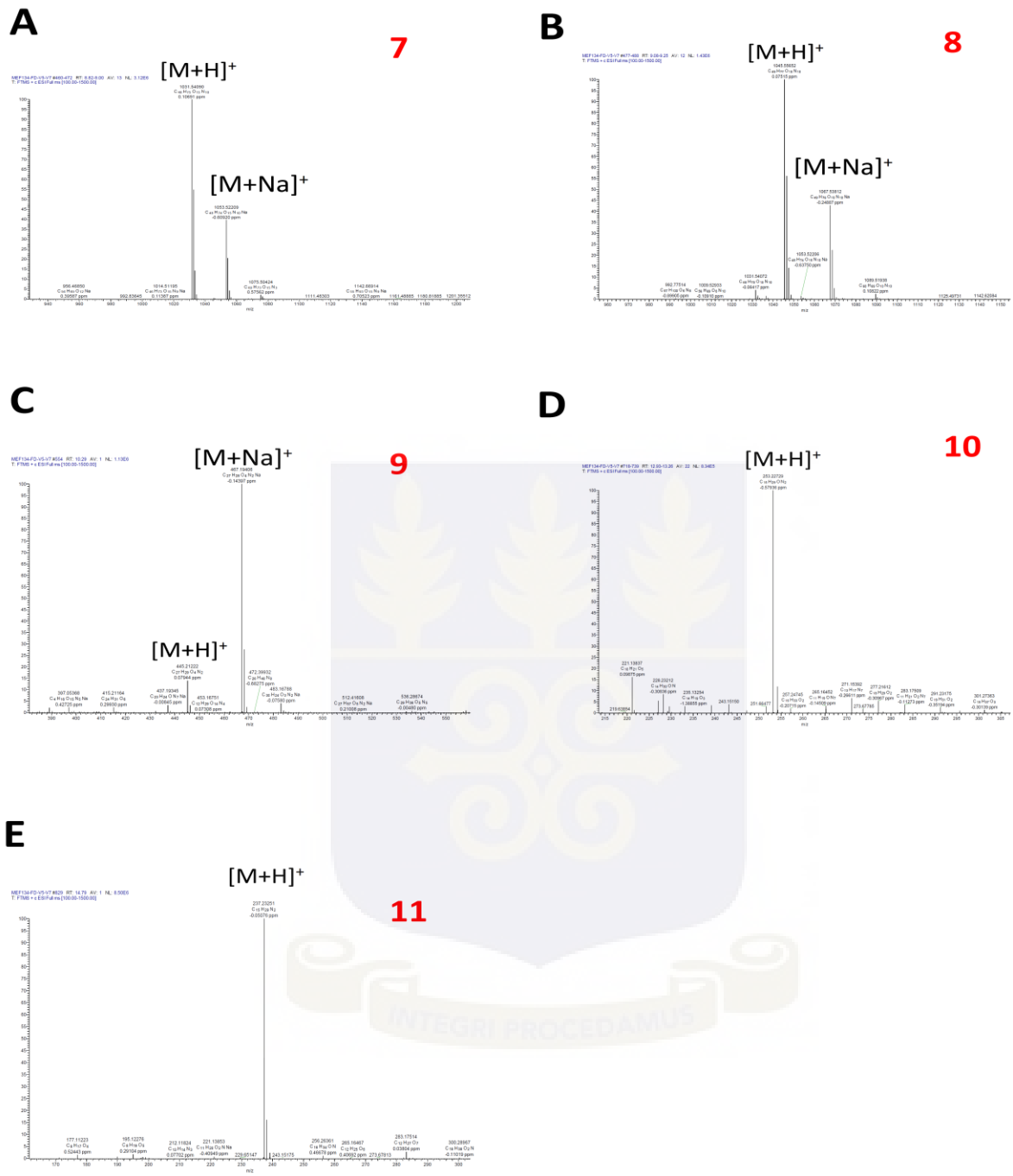



Fig S7.4c. Electrospray ionization-HRMS spectra of TLC fractions from MEF 134 FD V5 V7.

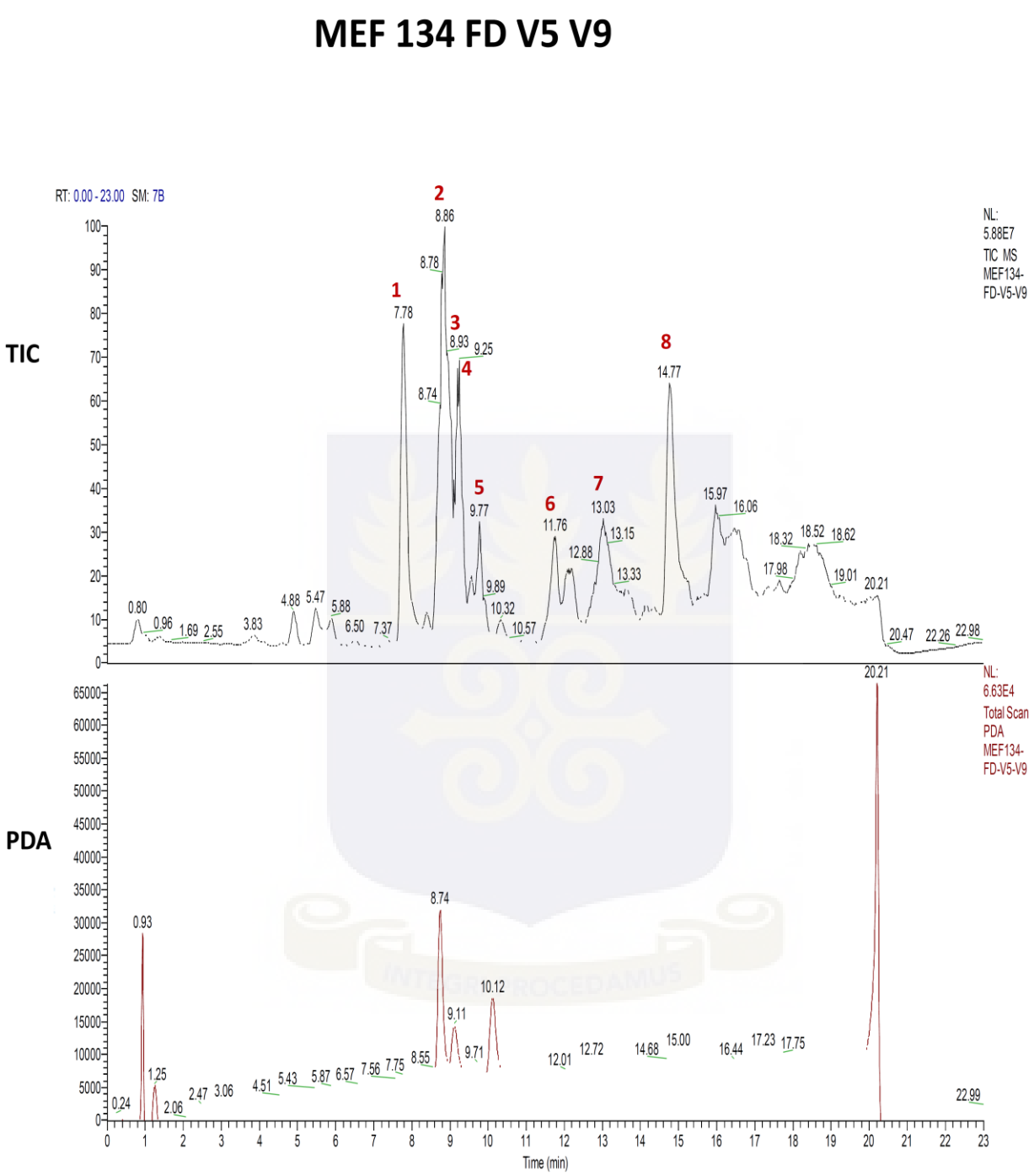



Fig S7.5a. Total ion current chromatogram of MEF 134 FD V5 V9 fractions after scanning by HPLC-HRMS.The chromatogram of MEF 134 FDV5V9 showed 8 major peaks

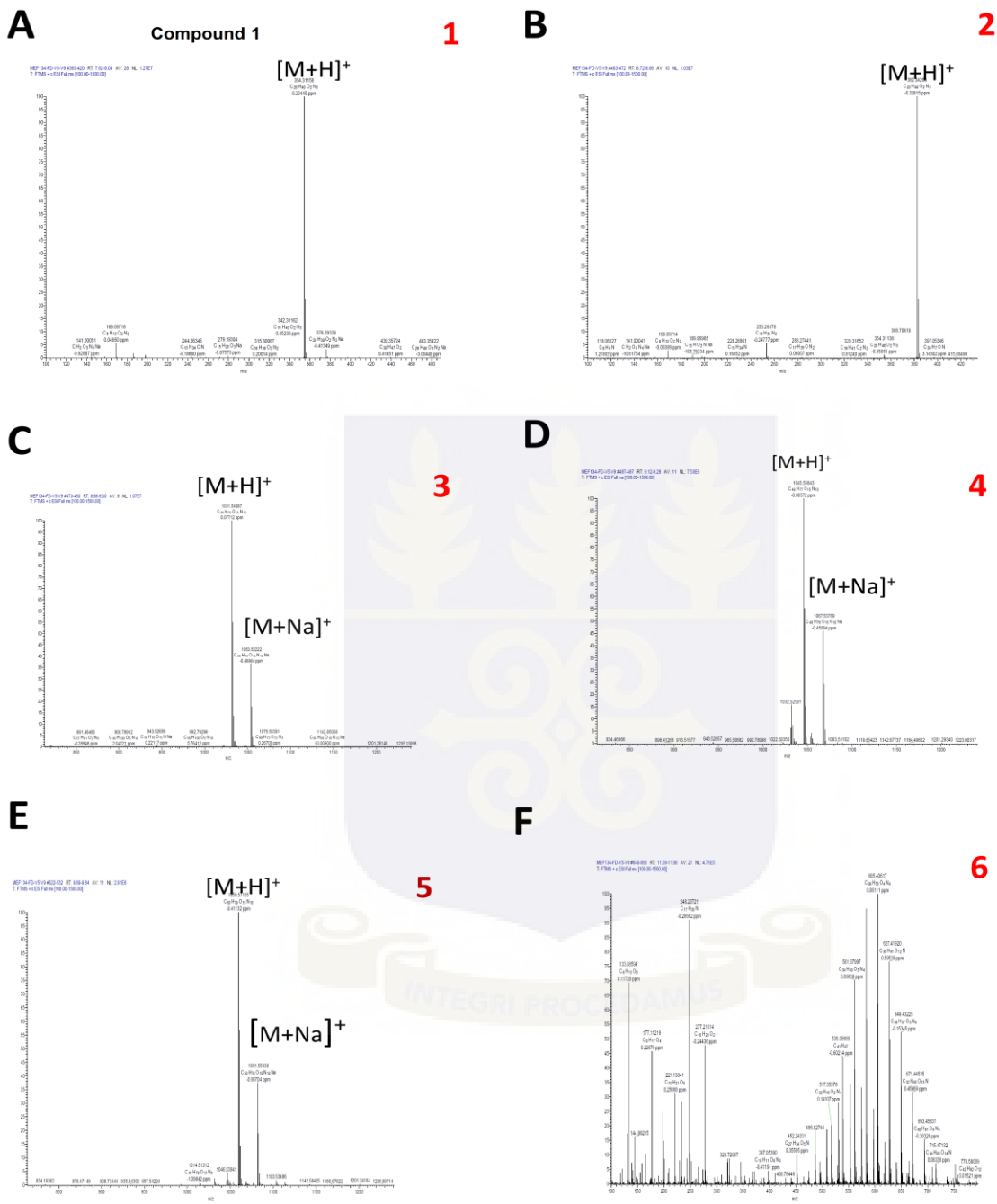



Fig S7.5b. Electrospray ionization-HRMS spectra of TLC fractions from MEF 134 FD V5 V9.

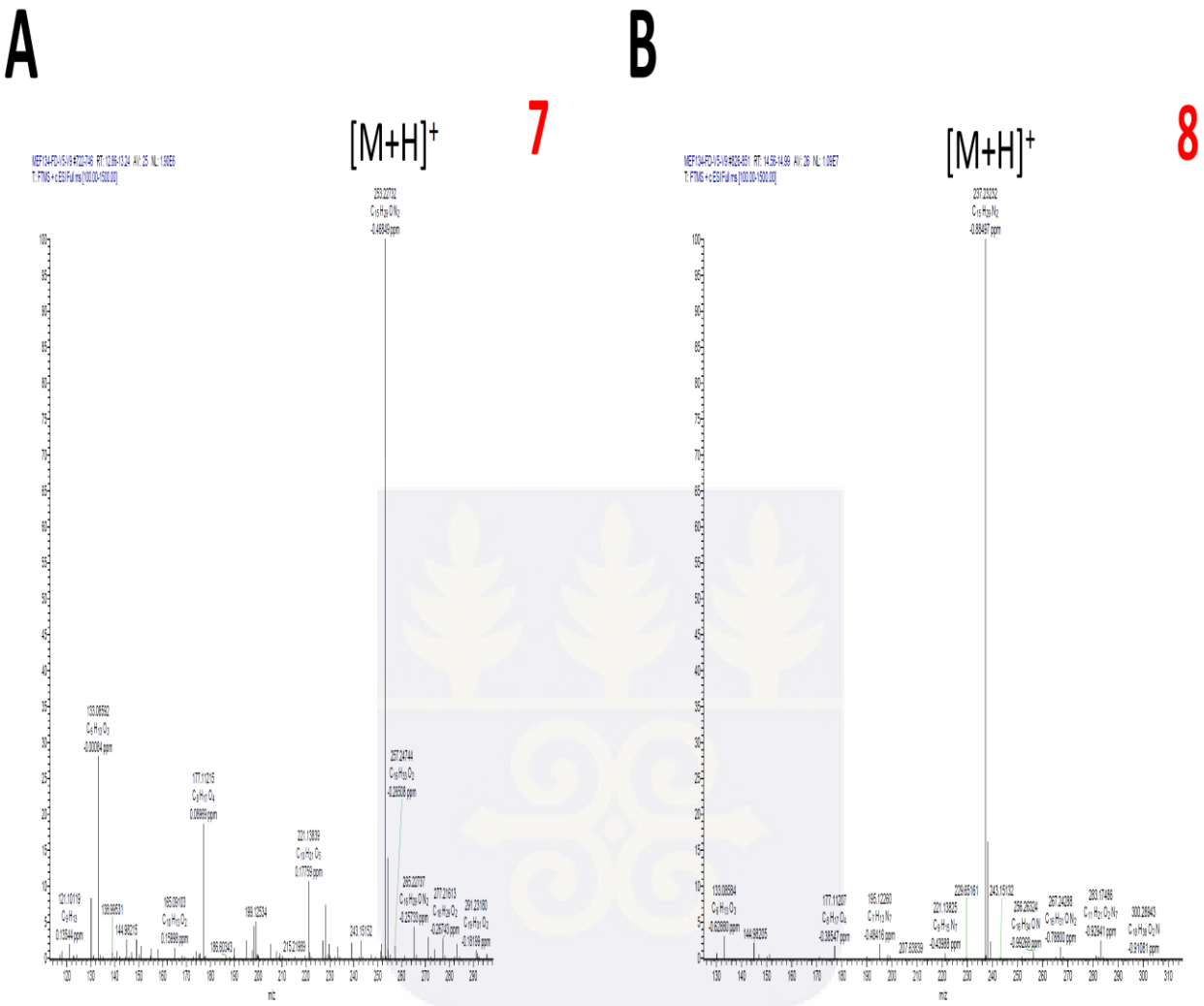



Fig S7.5c. Electrospray ionization-HRMS spectra of TLC fractions from MEF 134 FD V5 V9.

# MEF 134 FD K1V5-V3

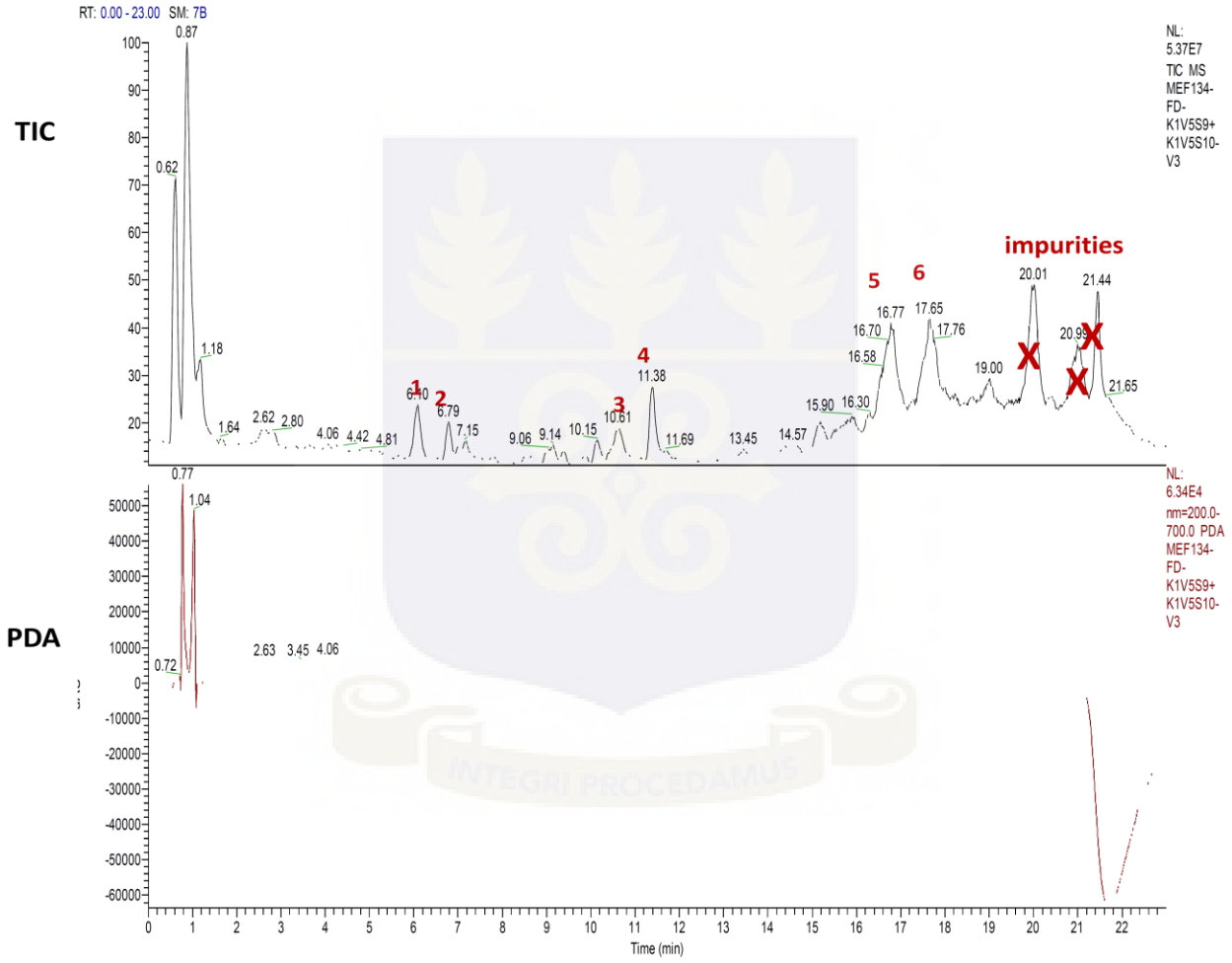



Fig S7.6a. Total ion current chromatogram of MEF 134 FD K1V5-V3 fractions after scanning by HPLC-HRMS.From the chromatogram of MEF 134 FD K1V5-V3, 6 major peaks were detected.

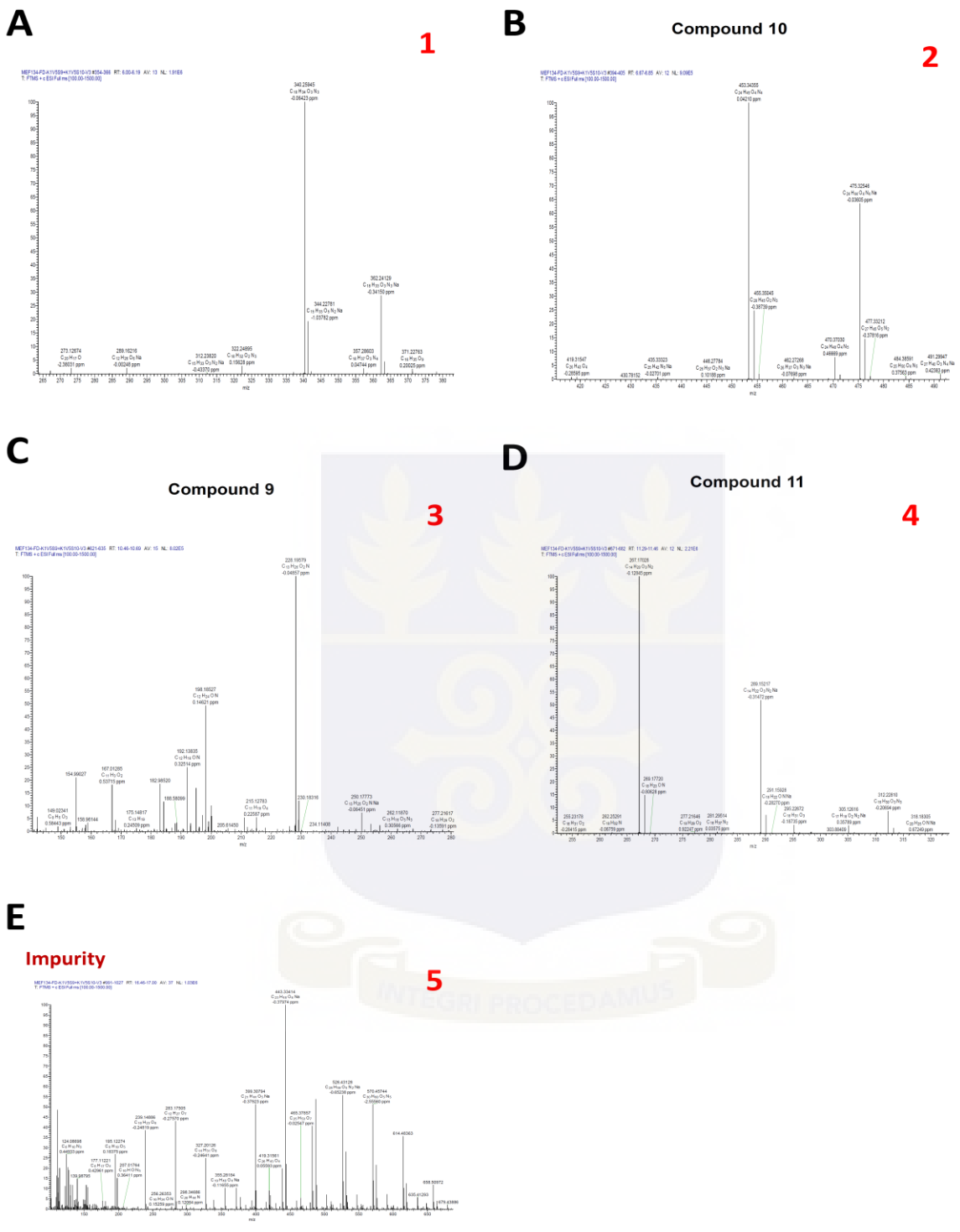



Fig S7.6b. Electrospray ionization-HRMS spectra of TLC fractions from MEF 134 FD K1V5-V3

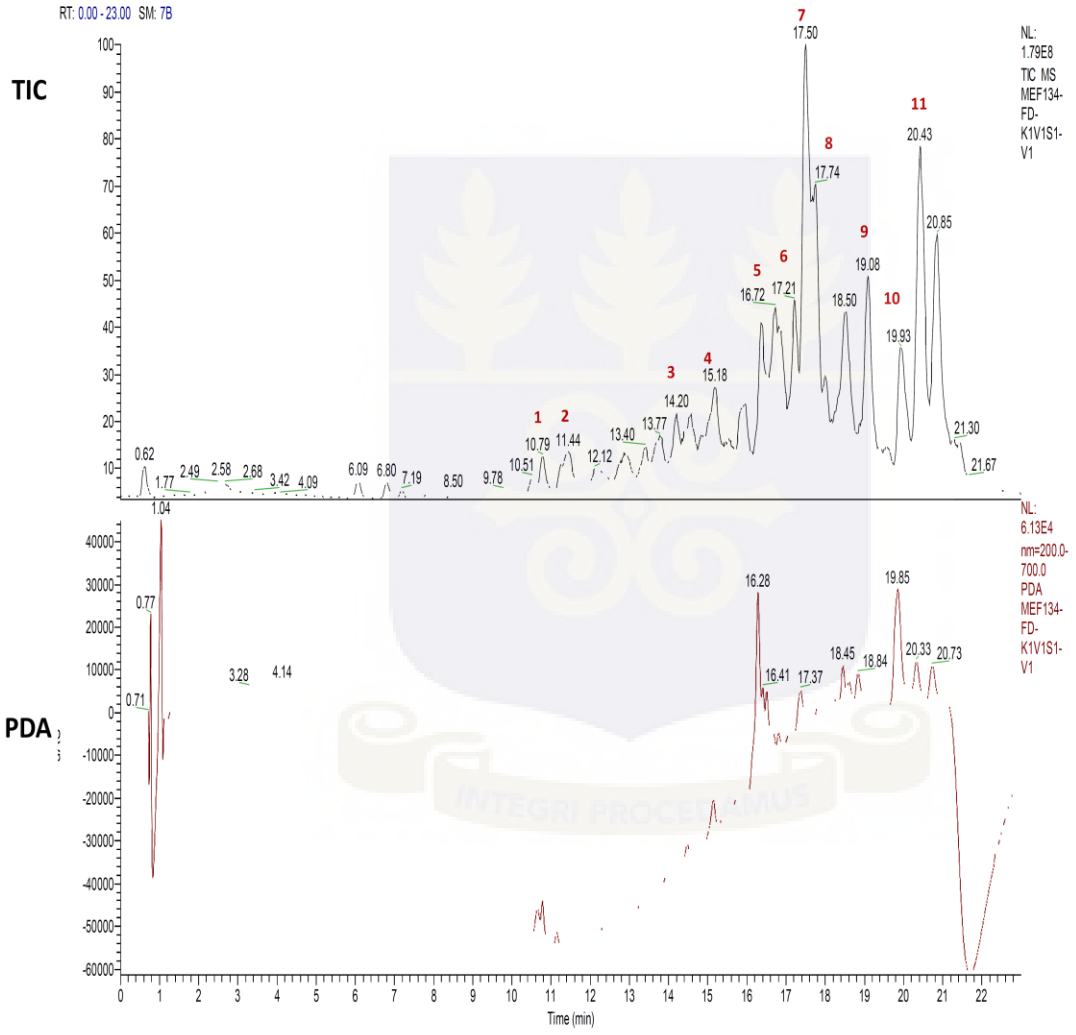



Fig S7.7a. Total ion current chromatogram of MEF 134 FD K1V1-V1 fractions after scanning by HPLC-HRMS. The chromatogram of MEF 134 FD K1V1-V1 showed 11 major peaks.

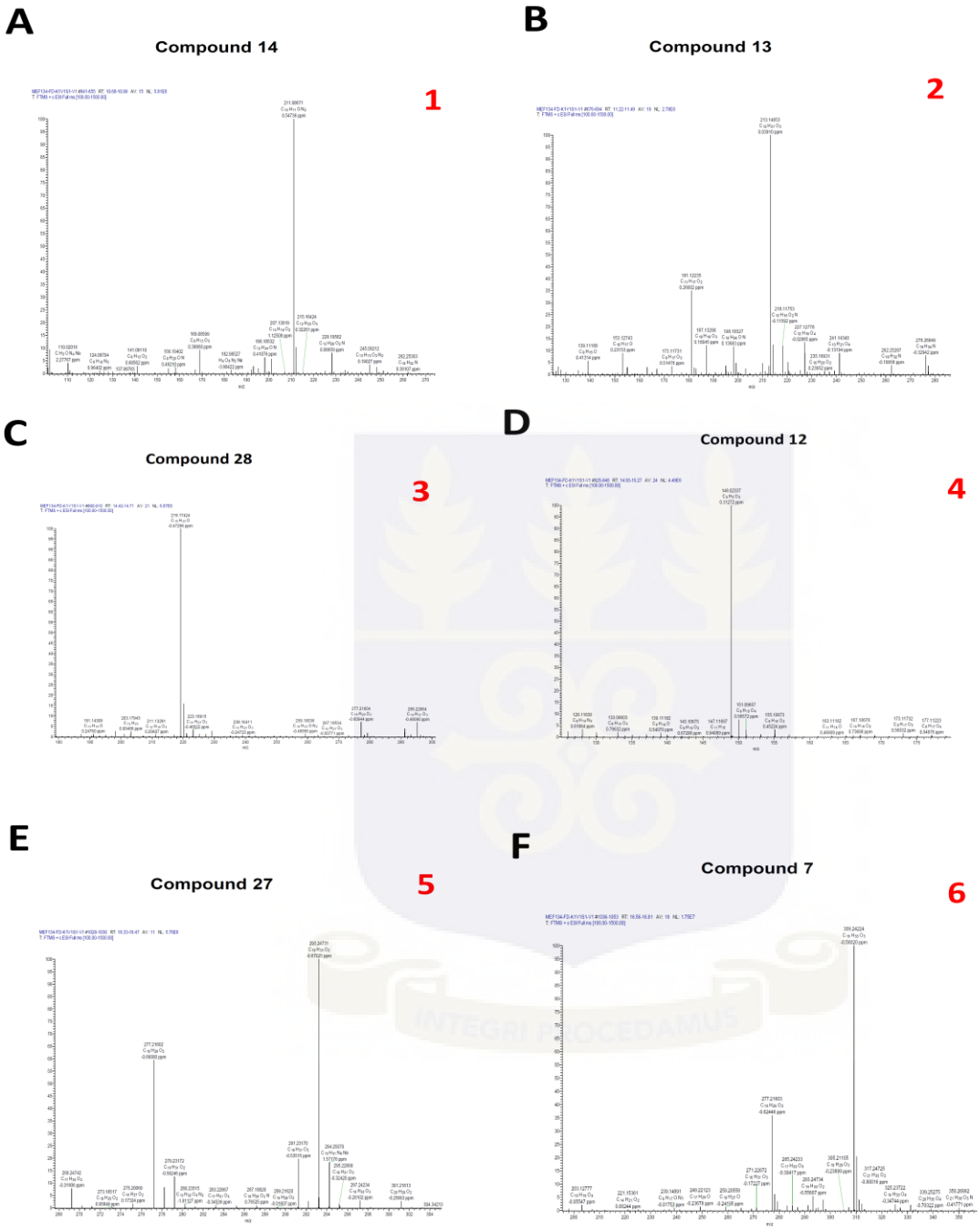



Fig S7.7b. Electrospray ionization-HRMS spectra of TLC fractions from MEF 134 FD K1V1-V1.

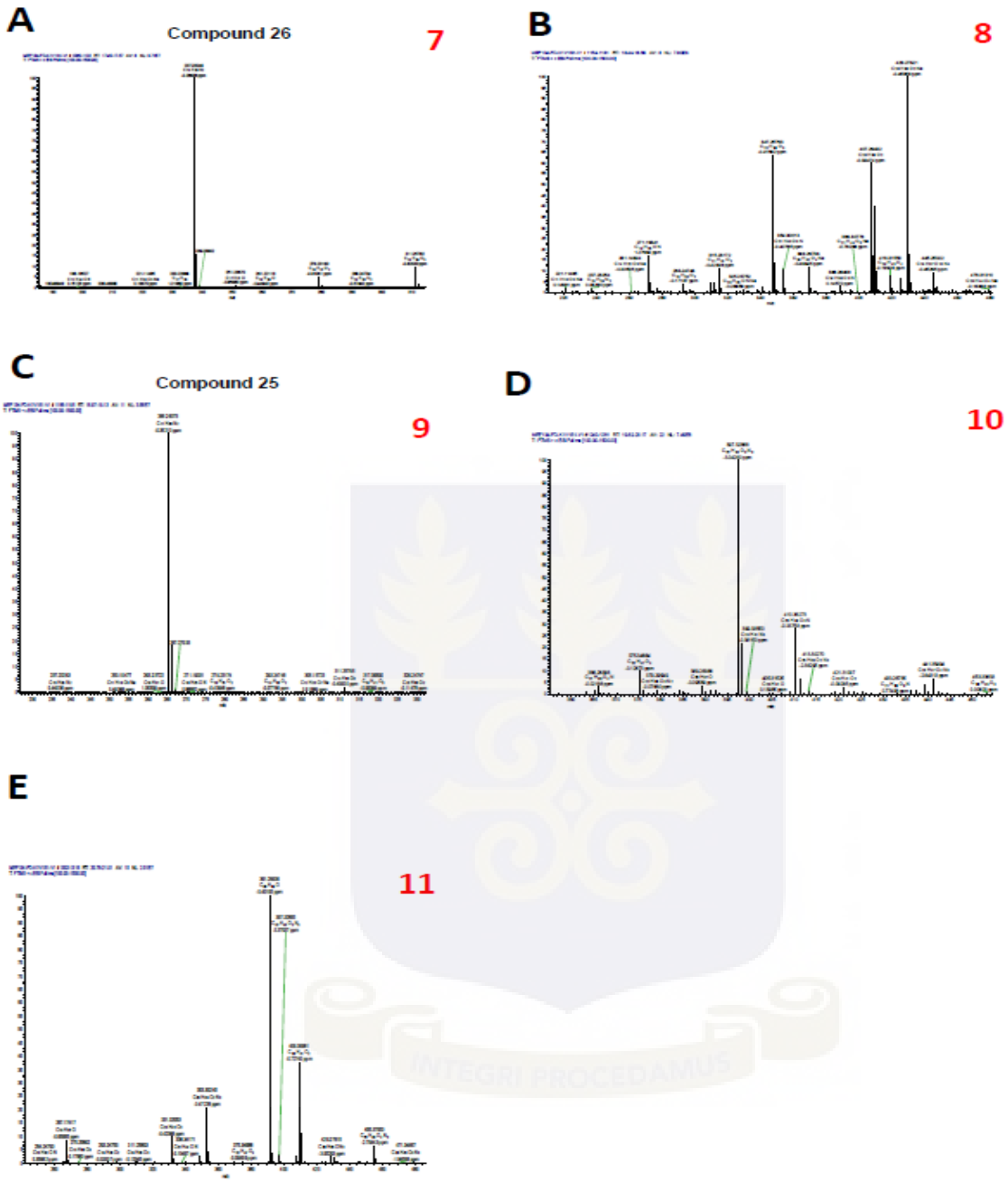



Fig S7.7c. Electrospray ionization-HRMS spectra of TLC fractions from MEF 134 FD K1V1-V1.

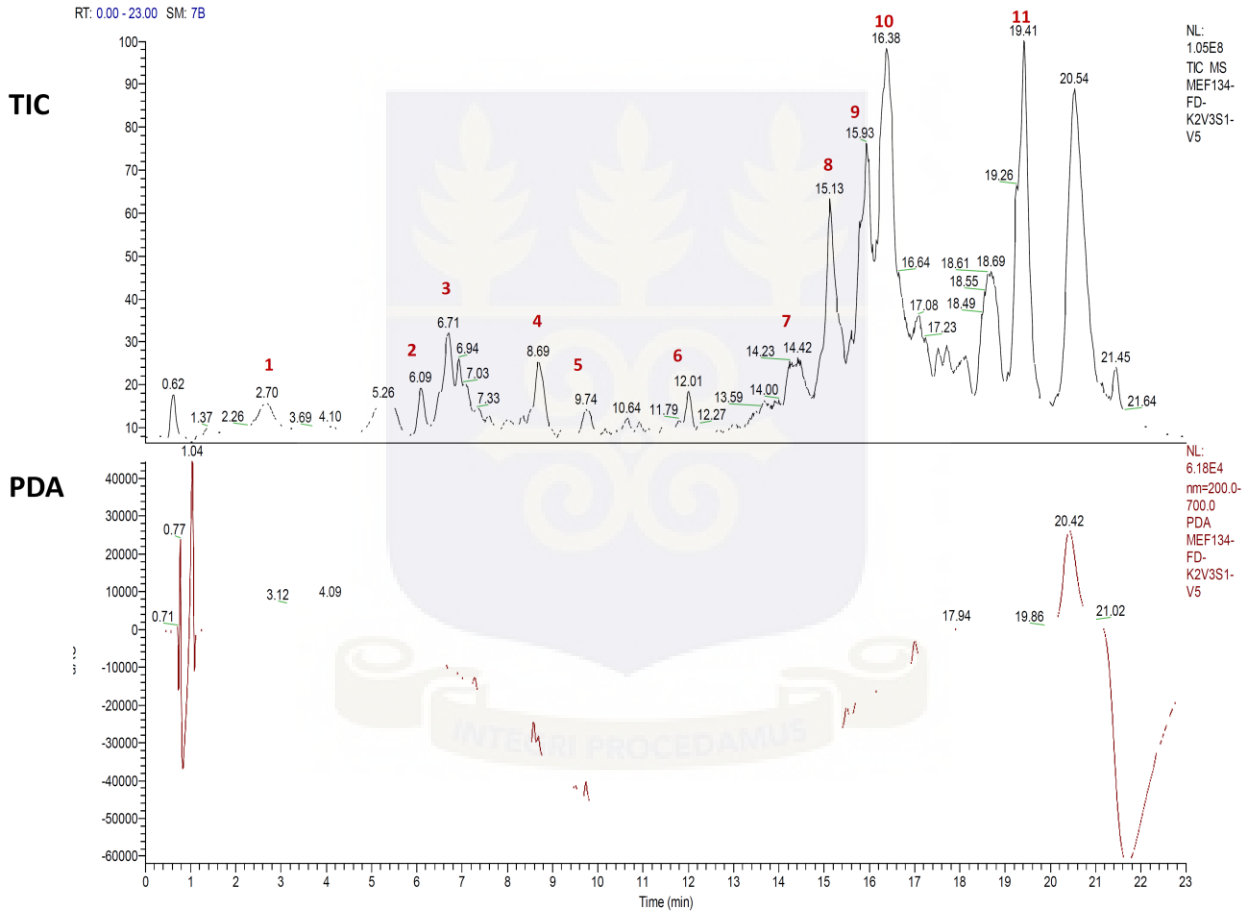



Fig S7.8a. Total ion current chromatogram of MEF 134 FD K2V3-V5 fractions after scanning by HPLC-HRMS. From the chromatogram of MEF 134 FD K2V3-V5, 11 major peaks were detected.

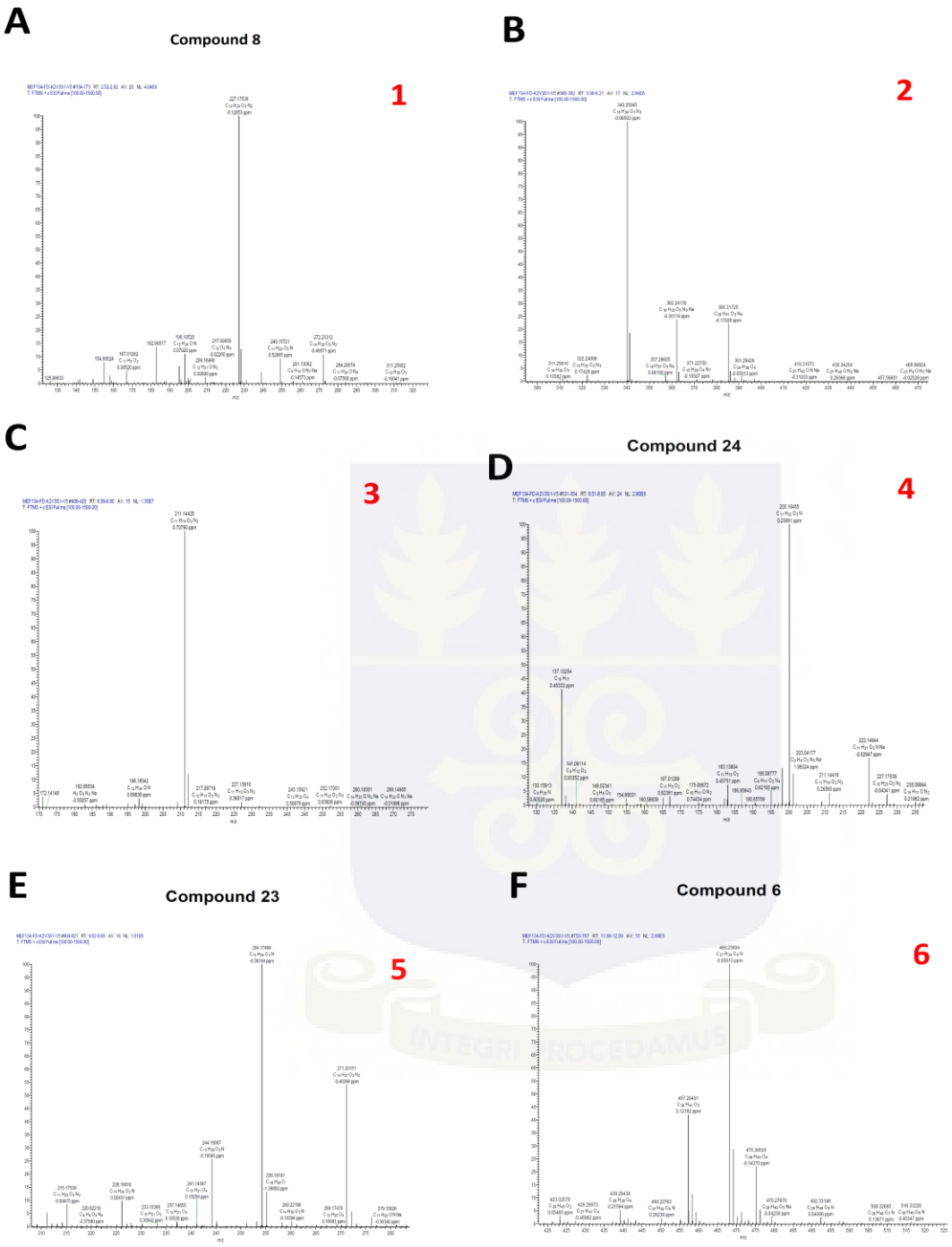



Fig S7.8b. Electrospray ionization-HRMS spectra of TLC fractions from MEF 134 FD K2V3-V5.

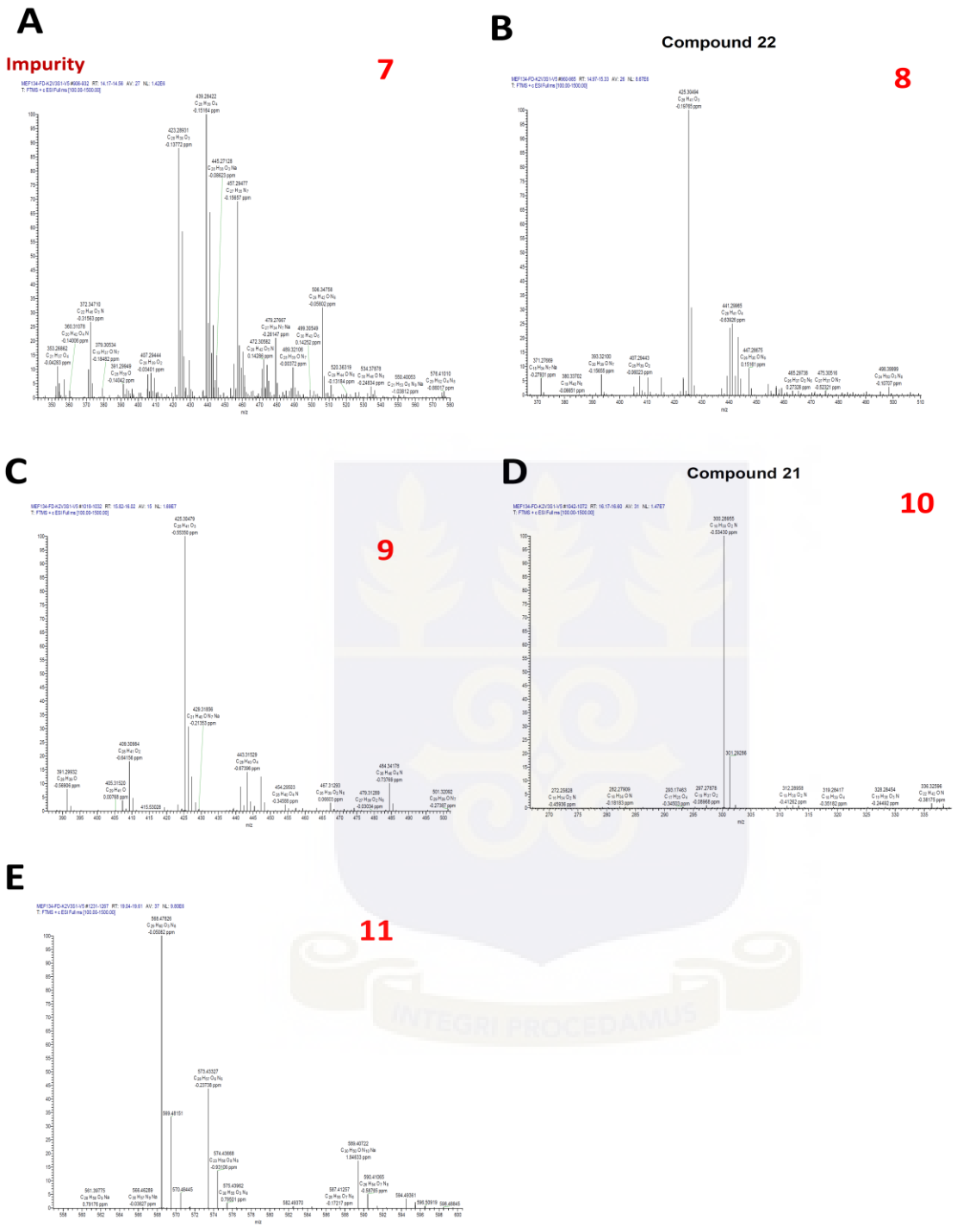



Fig S7.8c. Electrospray ionization-HRMS spectra of TLC fractions from MEF 134 FD K2V3-V5.

# MEF 11 FH V1V1

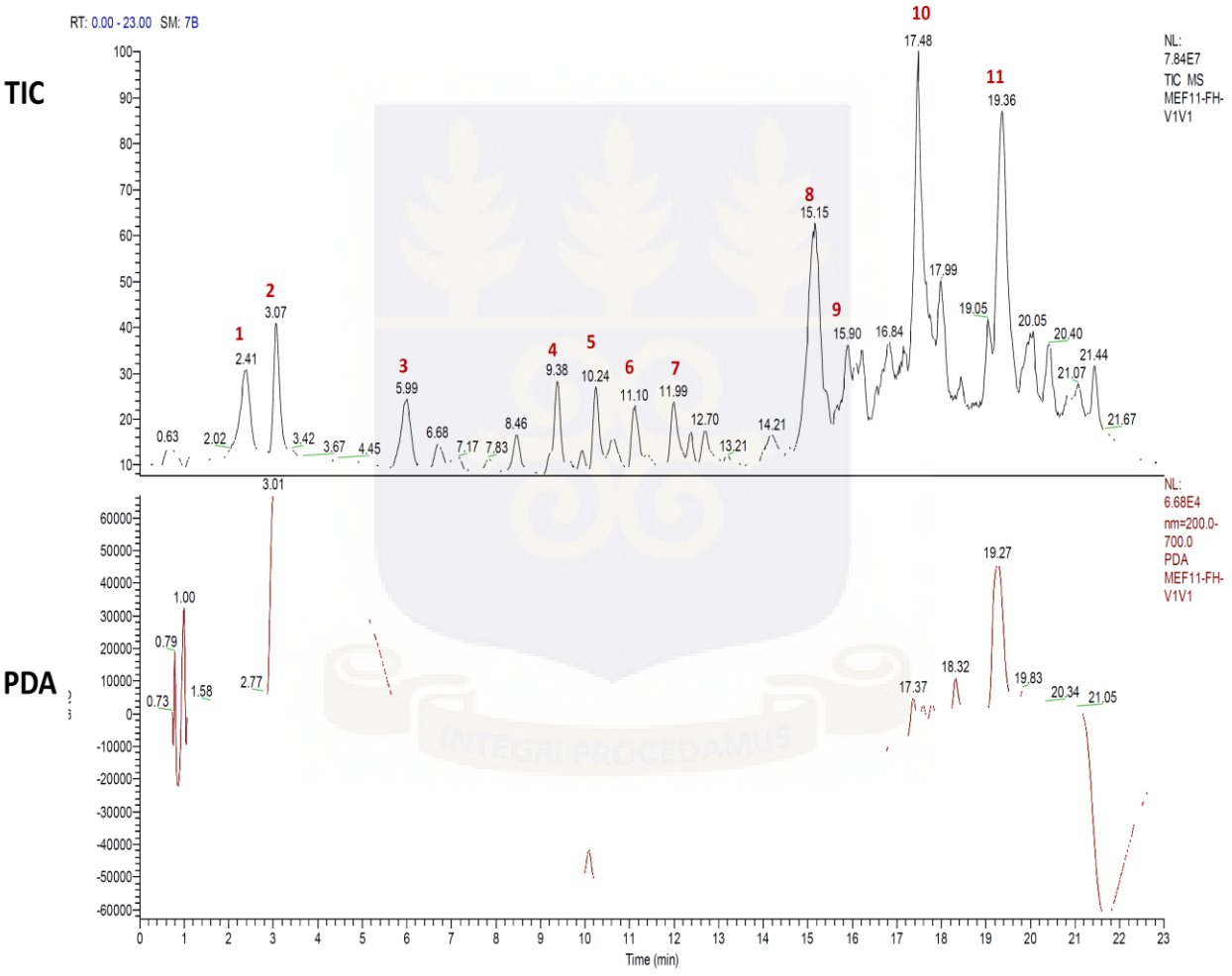



Fig S7.9a. Total ion current chromatogram of MEF 11 FH V1V1 fractions after scanning by HPLC-HRMS. The chromatogram of MEF 11 FH V1V1 showed 11 major peaks.

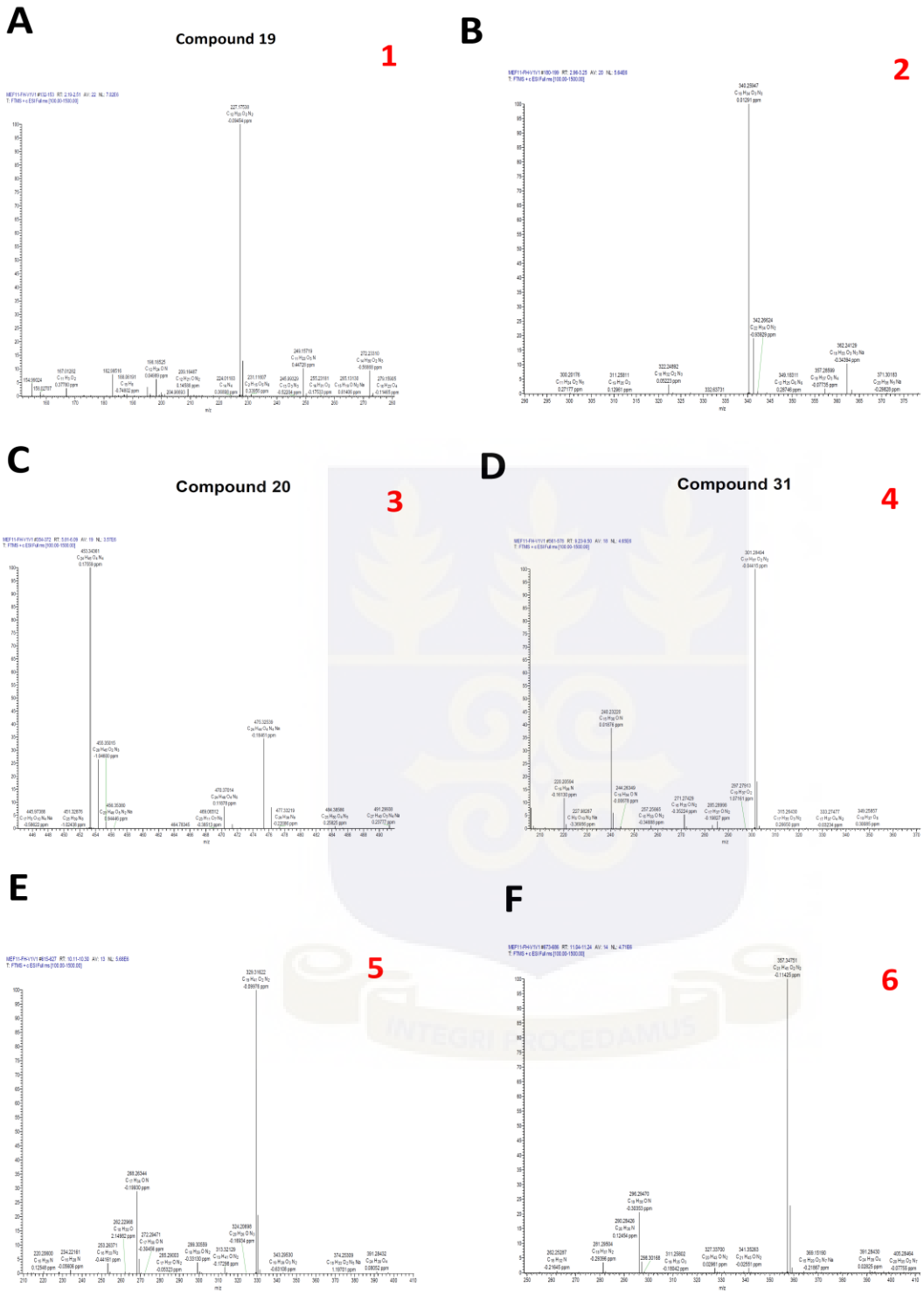



Fig S7.9b. Electrospray ionization-HRMS spectra of TLC fractions from MEF 11FH V1V1.

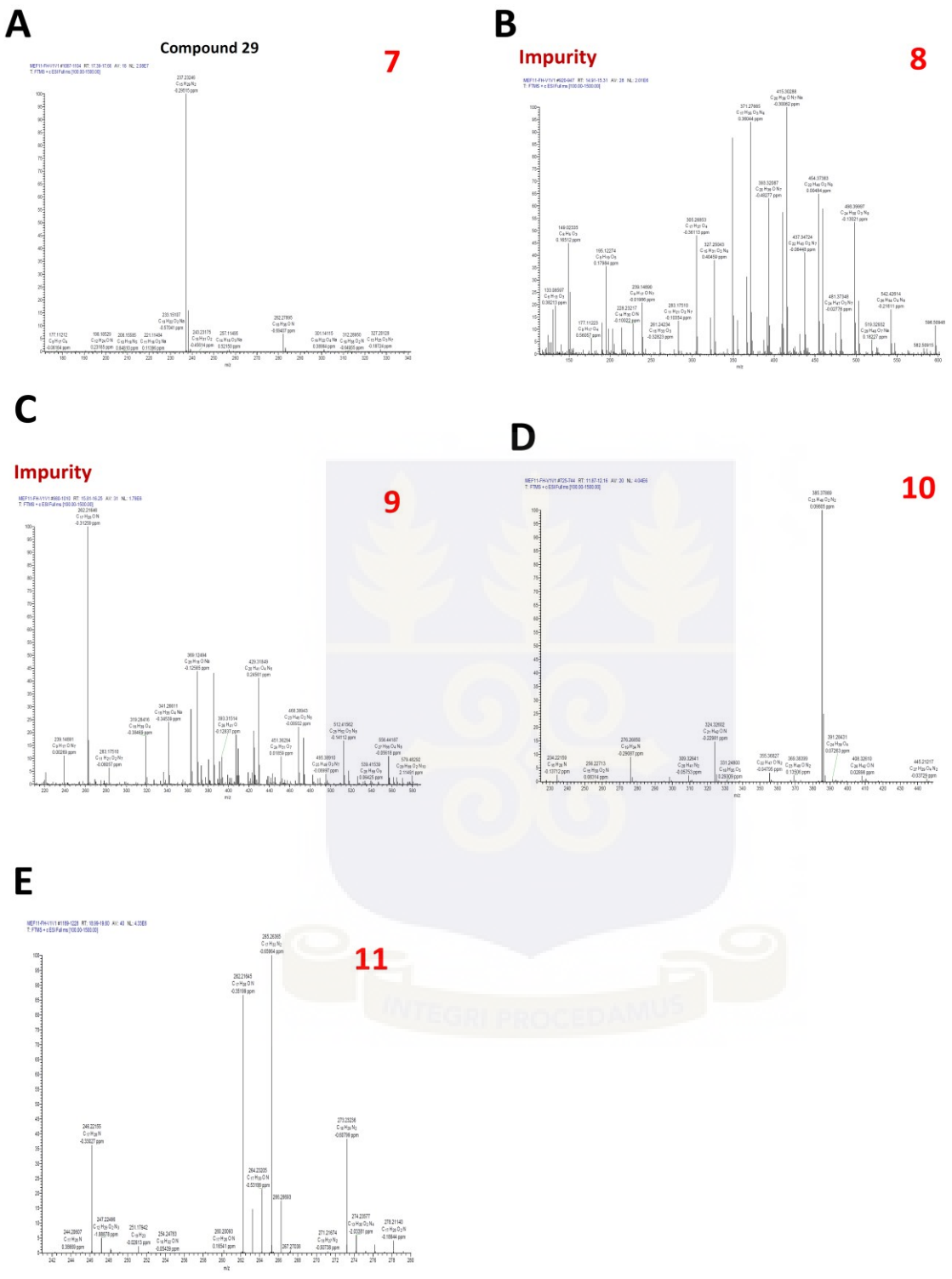



Fig S7.9c. Electrospray ionization-HRMS spectra of TLC fractions from MEF 11FH V1V.

# MEF 65 FH V3V3

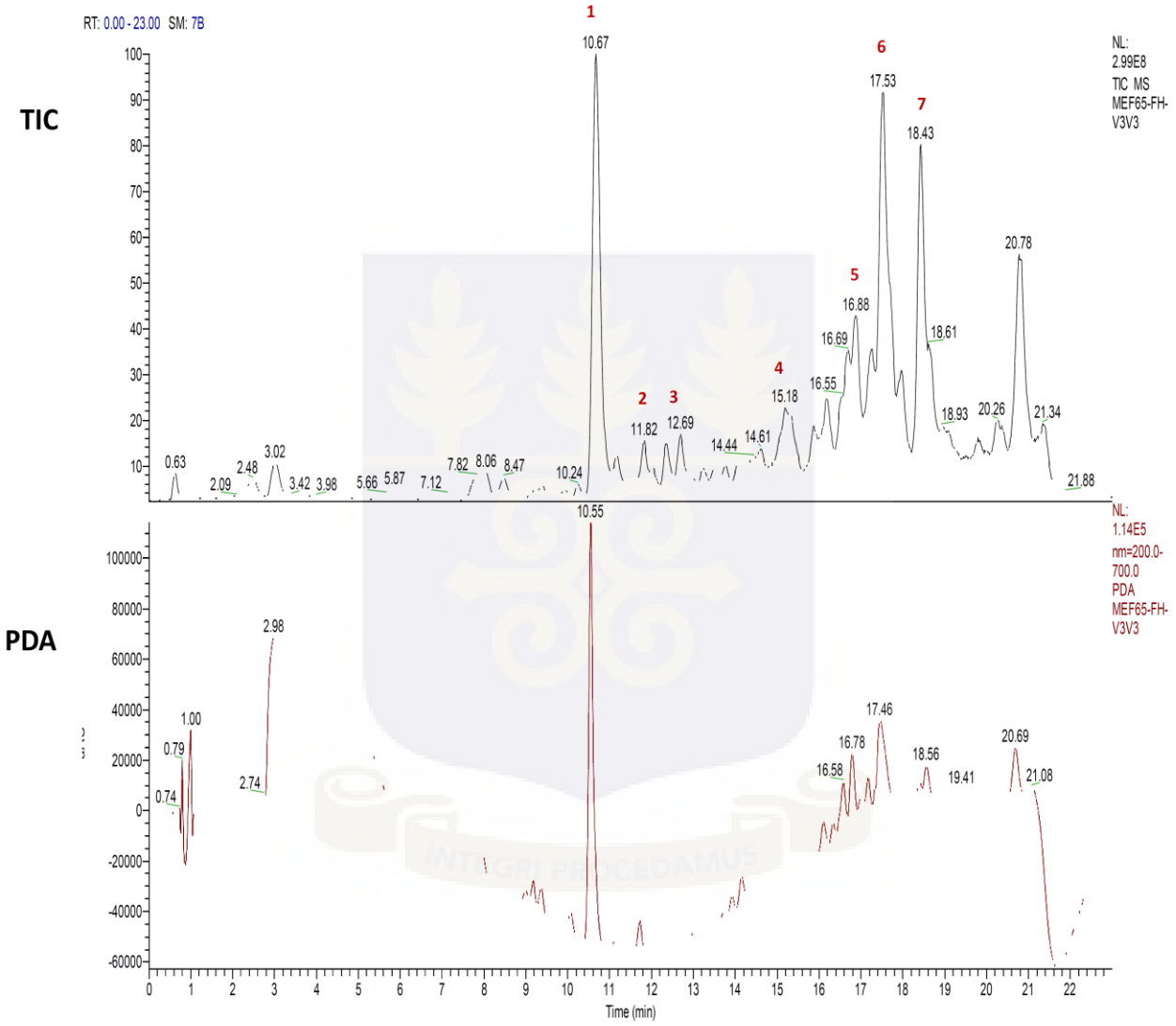



Fig S7.10a. Total ion current chromatogram of MEF 65 FH V3V3 fractions after scanning by HPLC-HRMS.

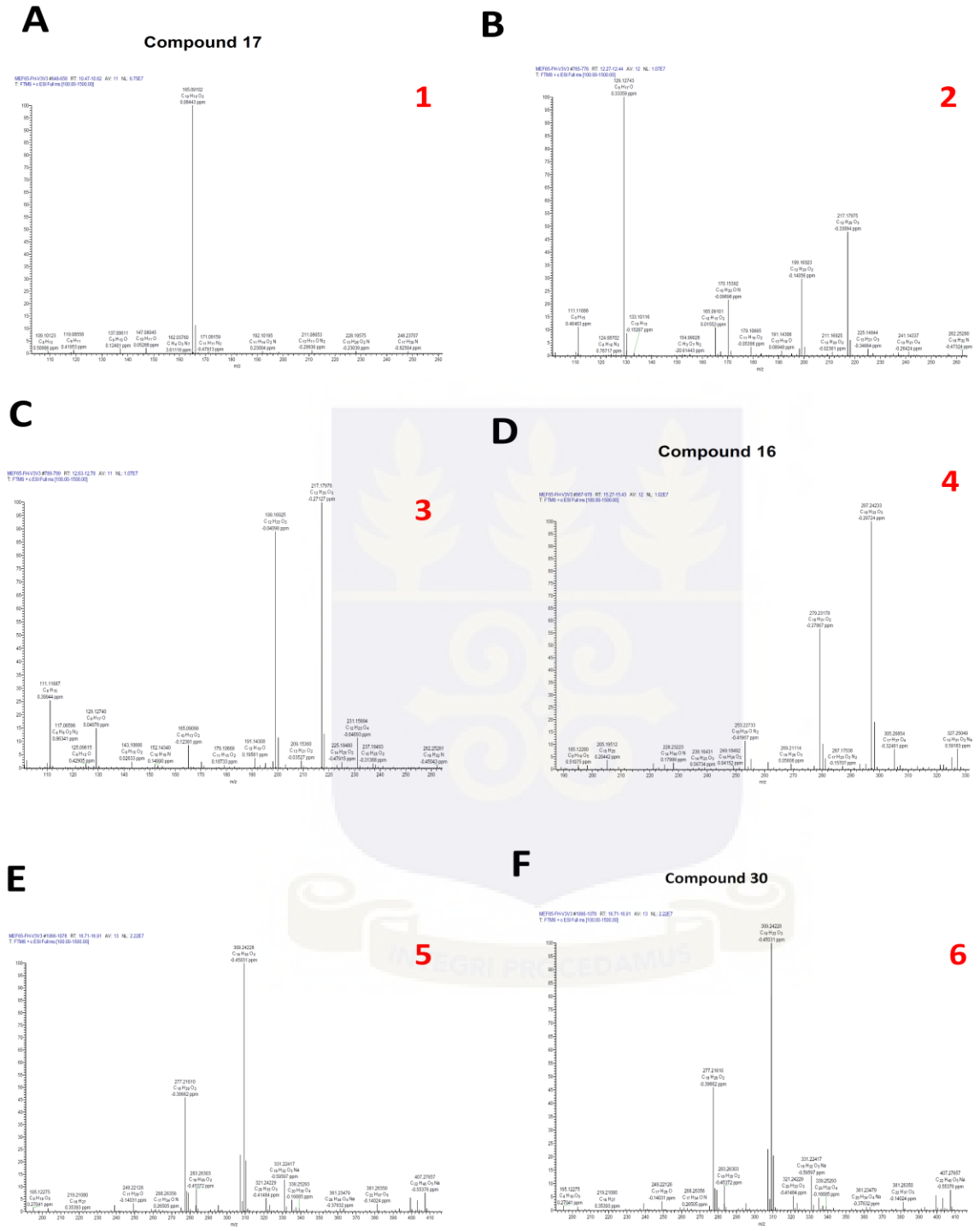



Fig S7.10b. Electrospray ionization-HRMS spectra of TLC fractions from MEF 65 FH V3V3

A

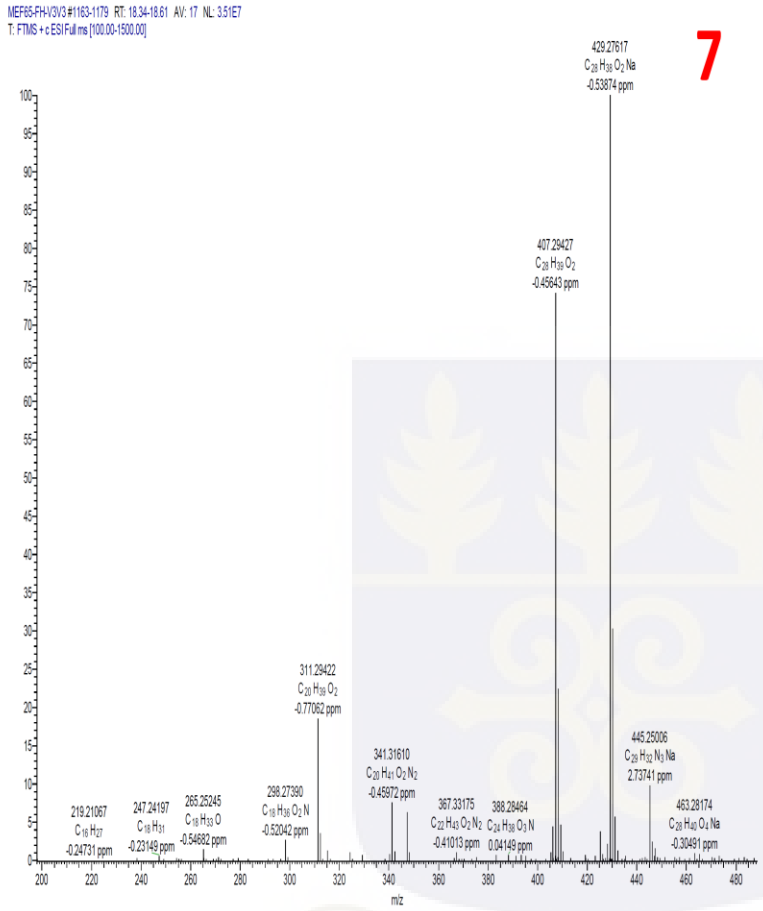



Fig S7.10c. Electrospray ionization-HRMS spectra of TLC fractions from MEF 65 FH V3V3.

# MEF 75 FH V1V1

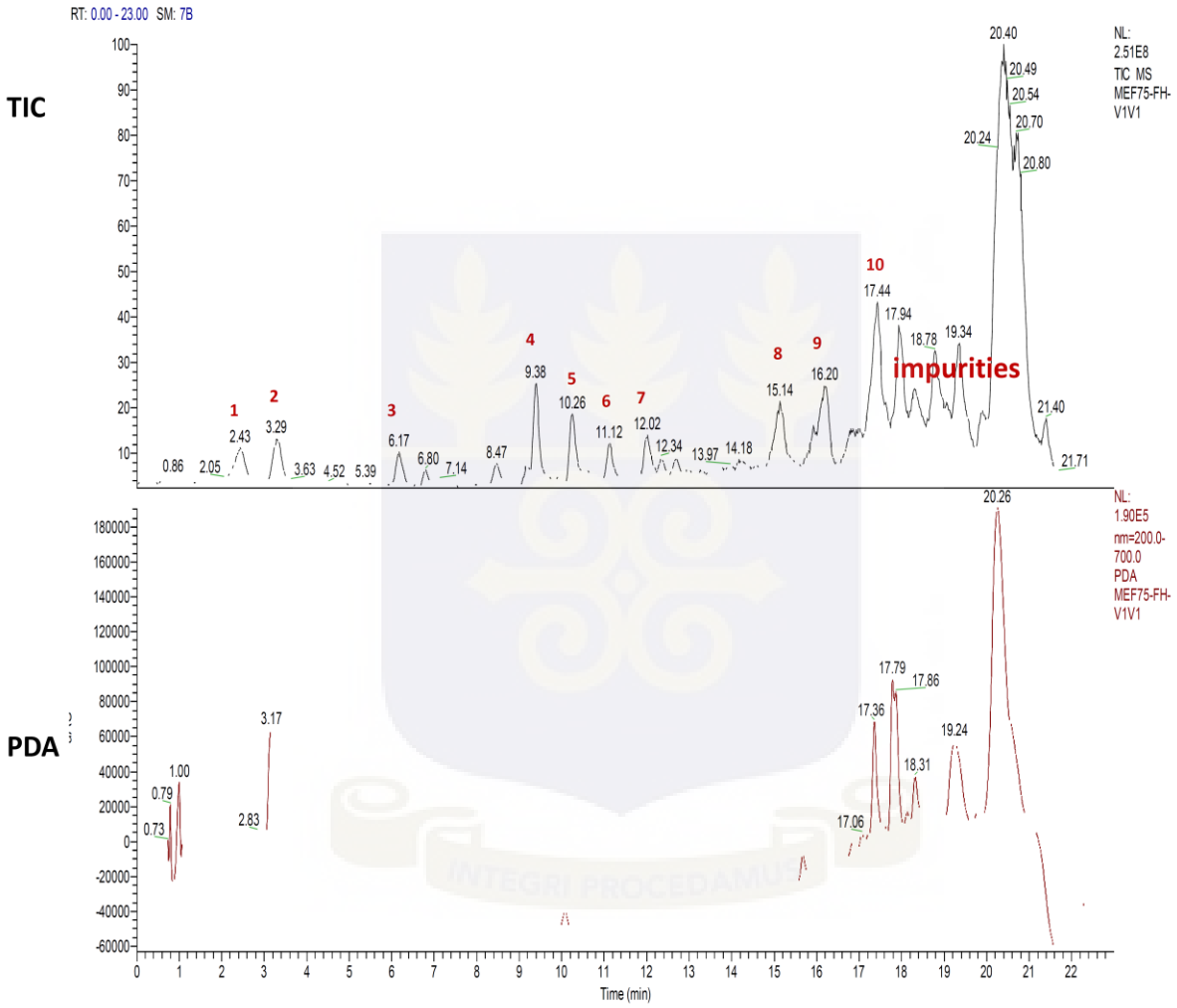



Fig S7.11a. Total ion current chromatogram of MEF 75 FH V1V1 fractions after scanning by HPLC-HRMS. The chromatogram of MEF 75 FH V1V1 showed 10 major peaks.

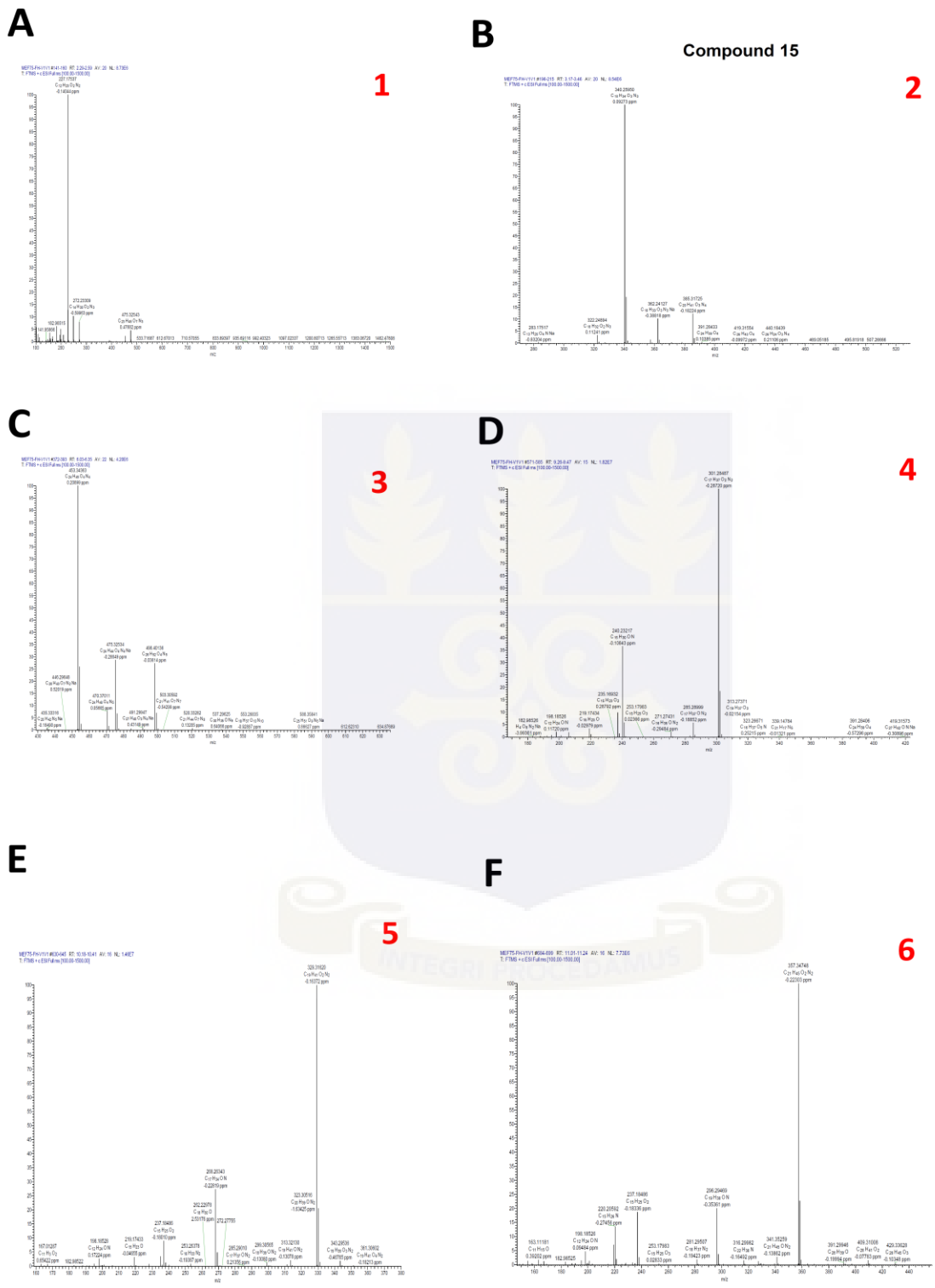



Fig S7.11b. Electrospray ionization-HRMS spectra of TLC fractions from MEF75 FH V1V1.

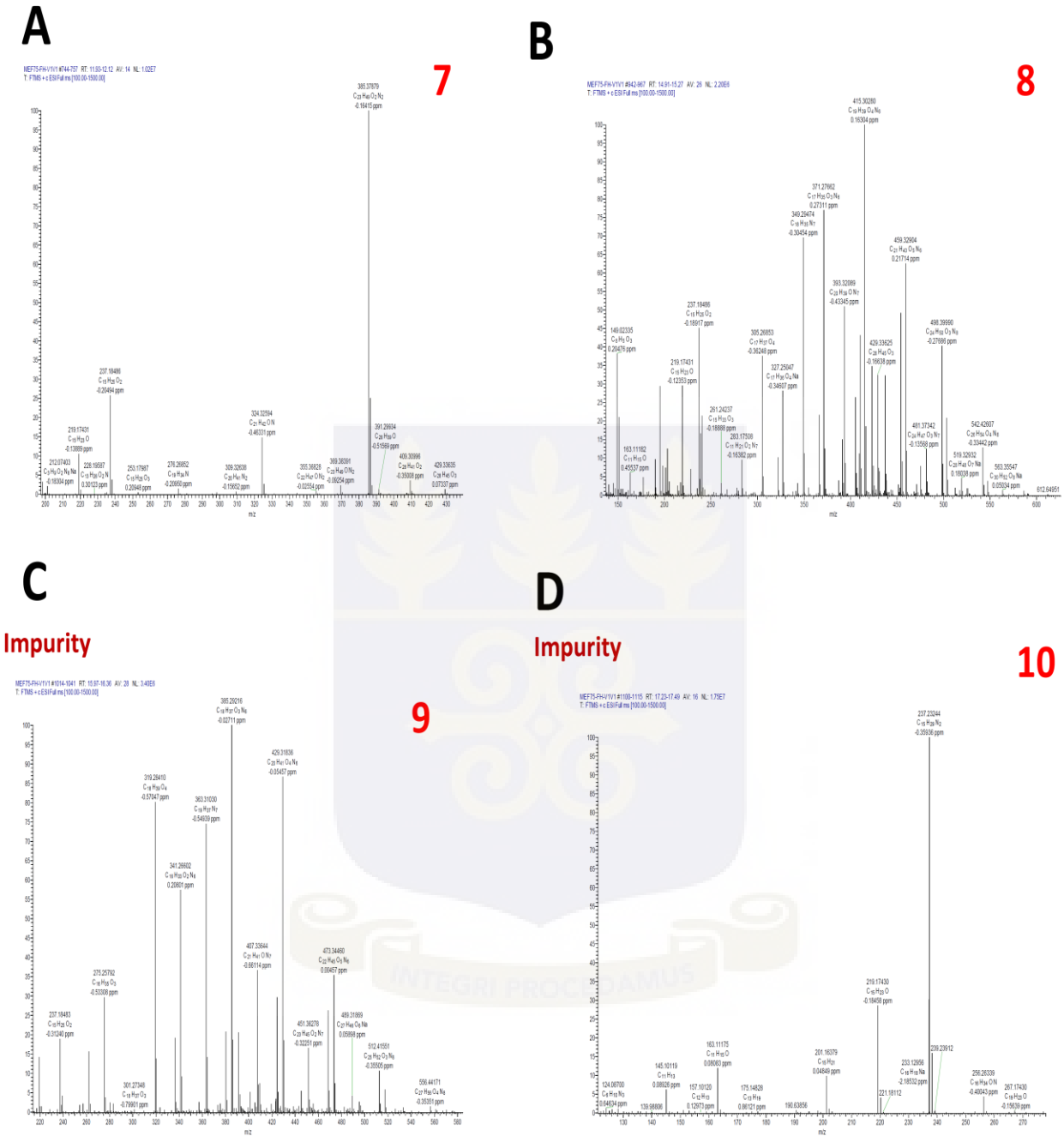



Fig S7.11c. Electrospray ionization-HRMS spectra of TLC fractions from MEF75 FH V1V1.

# MEF 112 FH V1V1

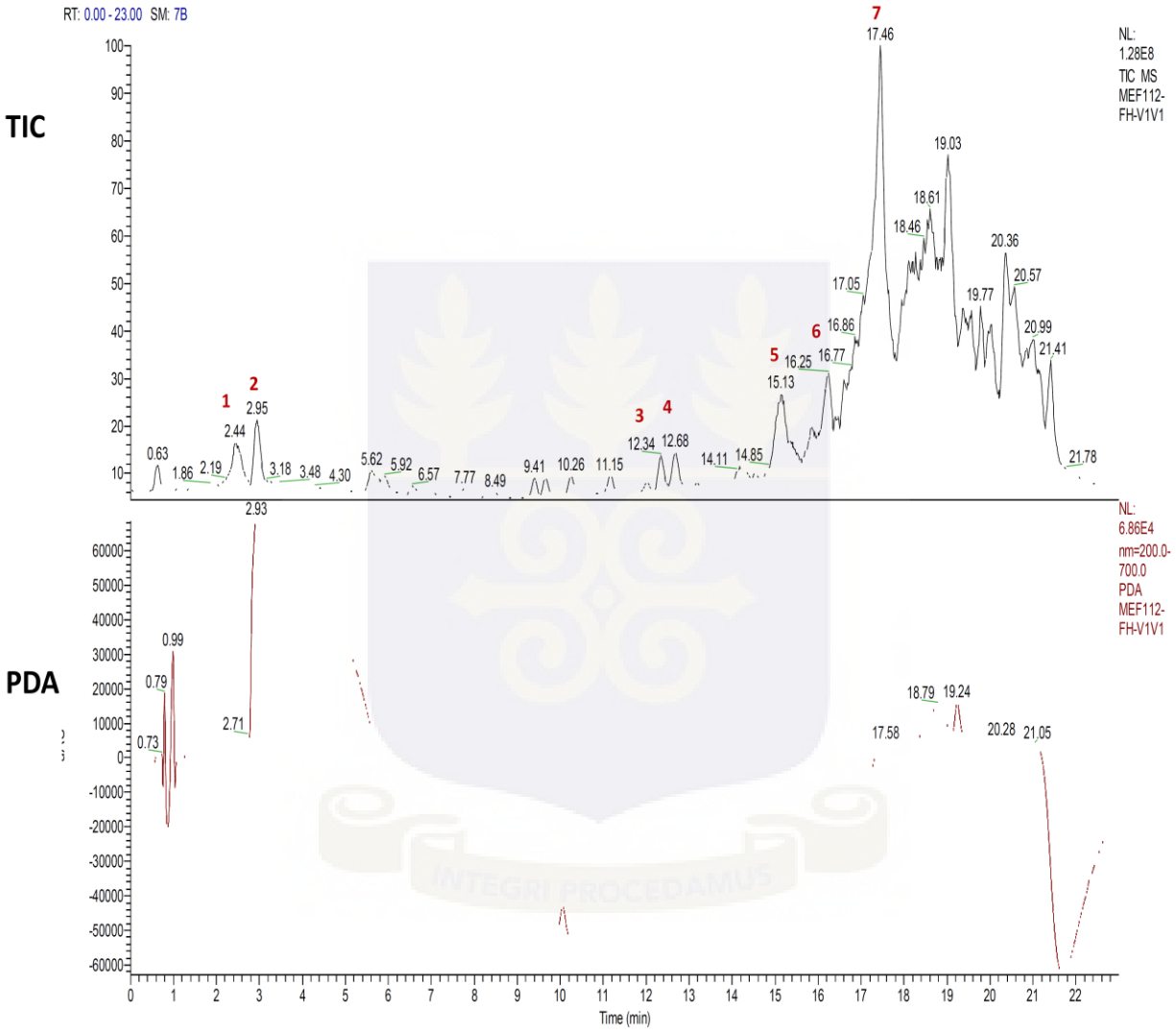



Fig S7.12a. Total ion current chromatogram of MEF 112 FH V1V1 fractions after scanning by HPLC-HRMS.A total of 7 major peaks were detected.

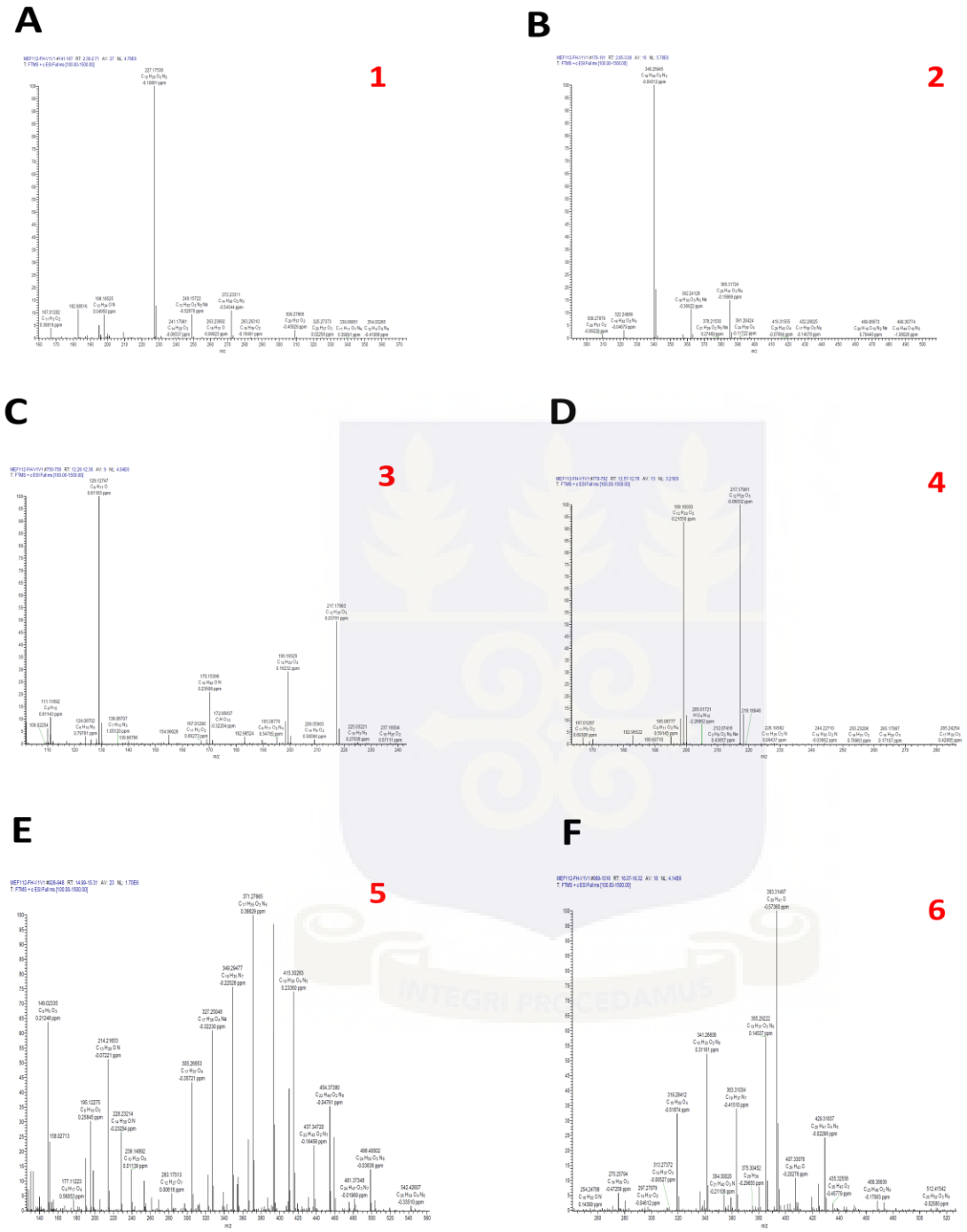



Fig S7.12b. Electrospray ionization-HRMS spectra of TLC fractions from MEF 112 FH V1V1.

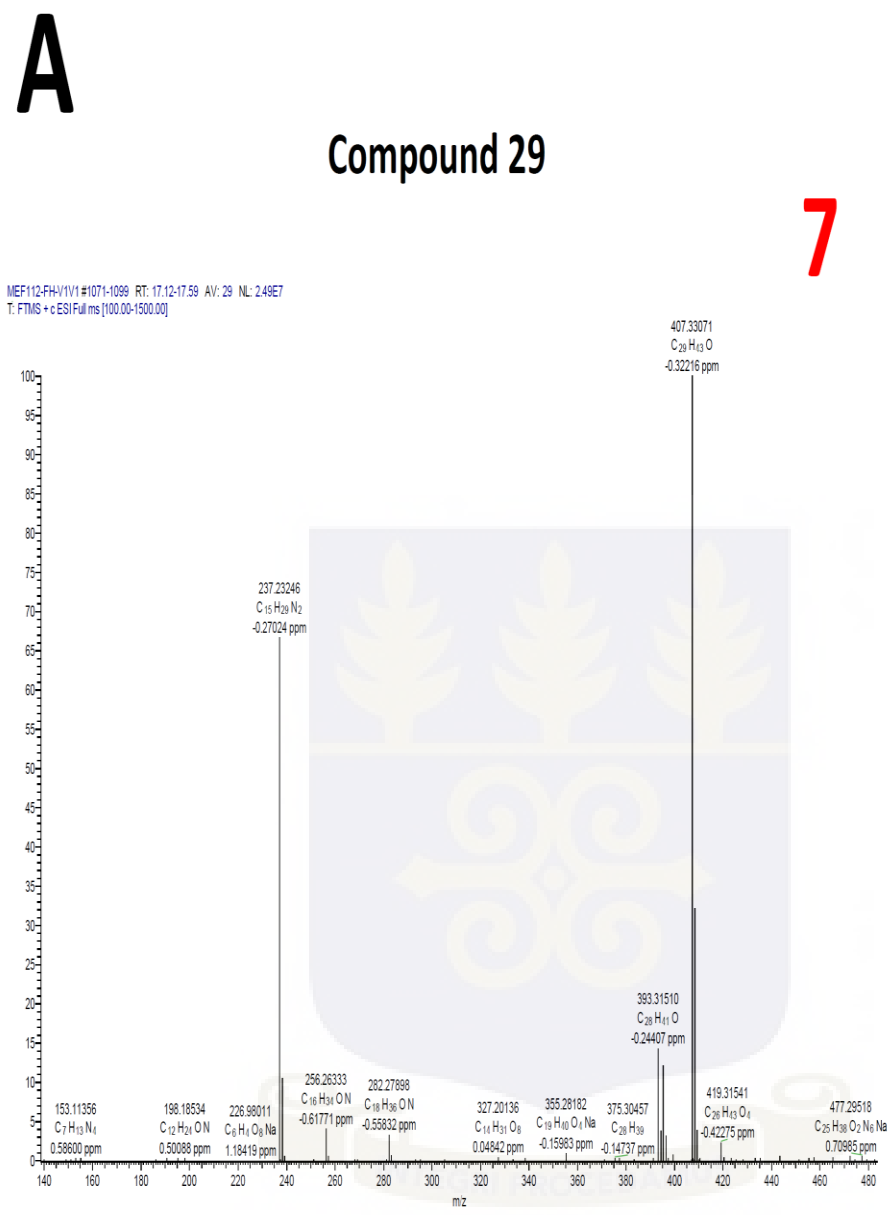



Fig S7.12c. Electrospray ionization-HRMS spectra of TLC fractions from MEF 112 FH V1V1.

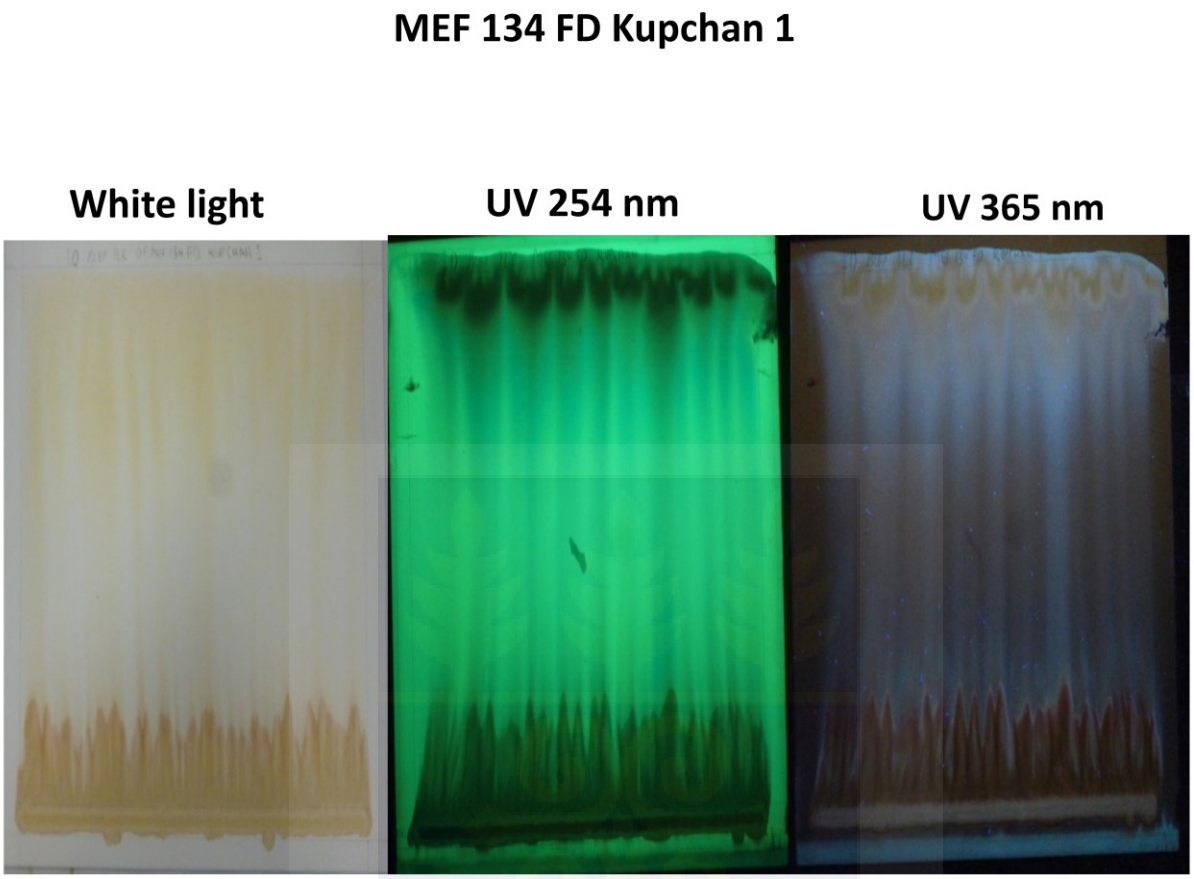



Fig S7.13. Preparative TLC (1D) of MEF 134 FD Kupchan. The solvent system used as mobile phase Acetonitrile: ethyl acetate: ether (2:7:1).

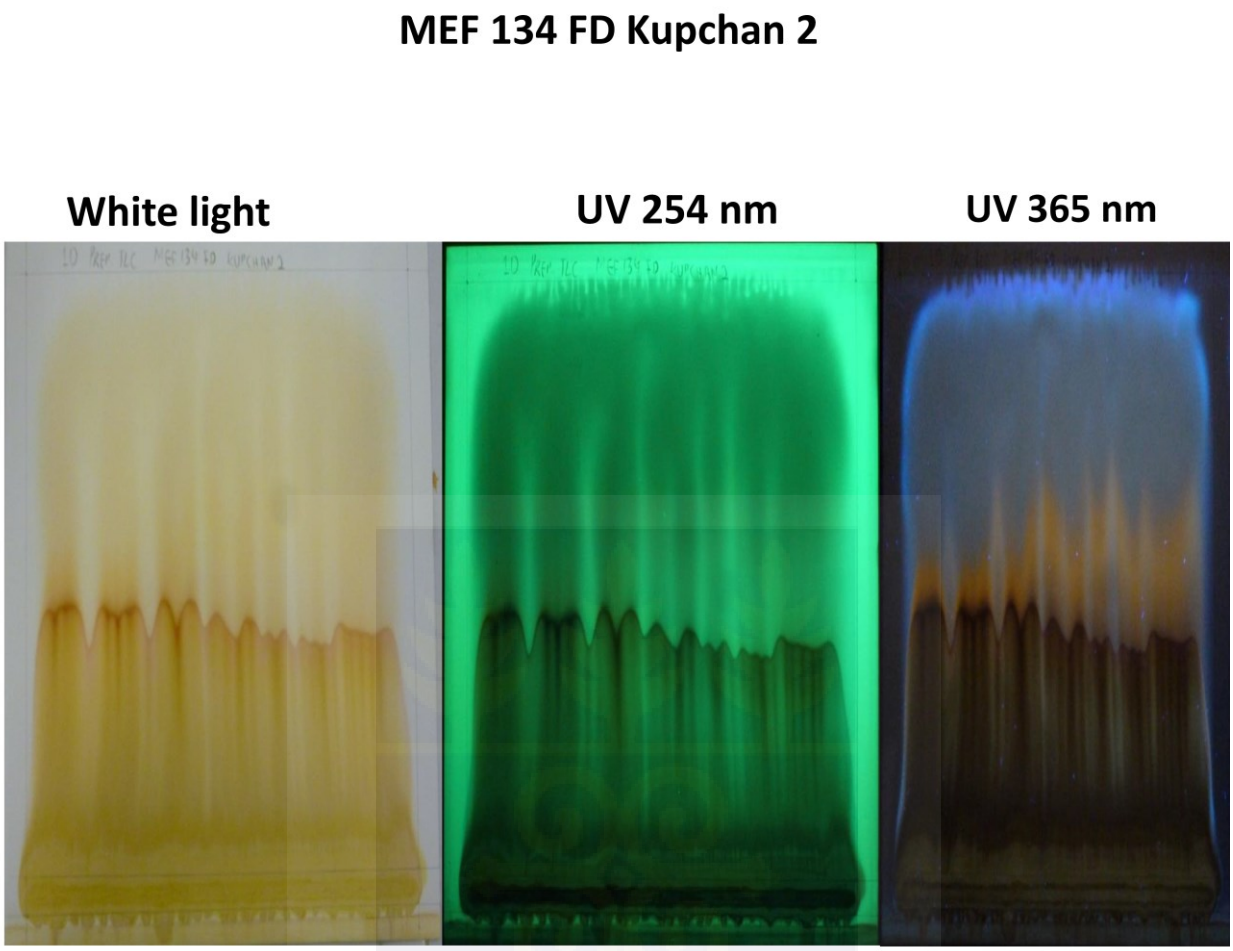



Fig S7.14. Preparative TLC (1D) of MEF 134 FD Kupchan 2.

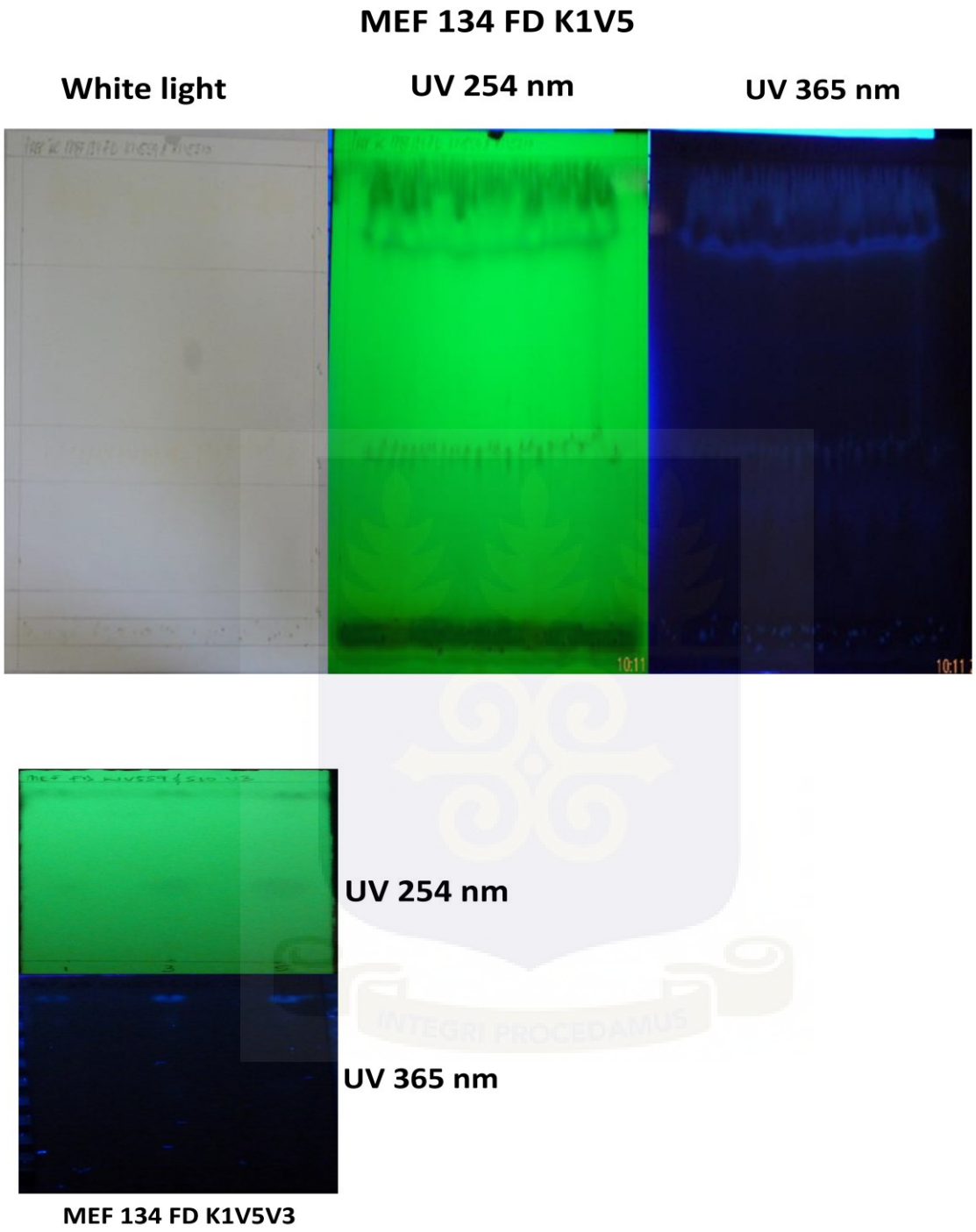



Fig S7.15. Preparative TLC (2D) of MEF 134 FD K1V5 from which fraction MEF 134 FD K1V5V3 was obtained.

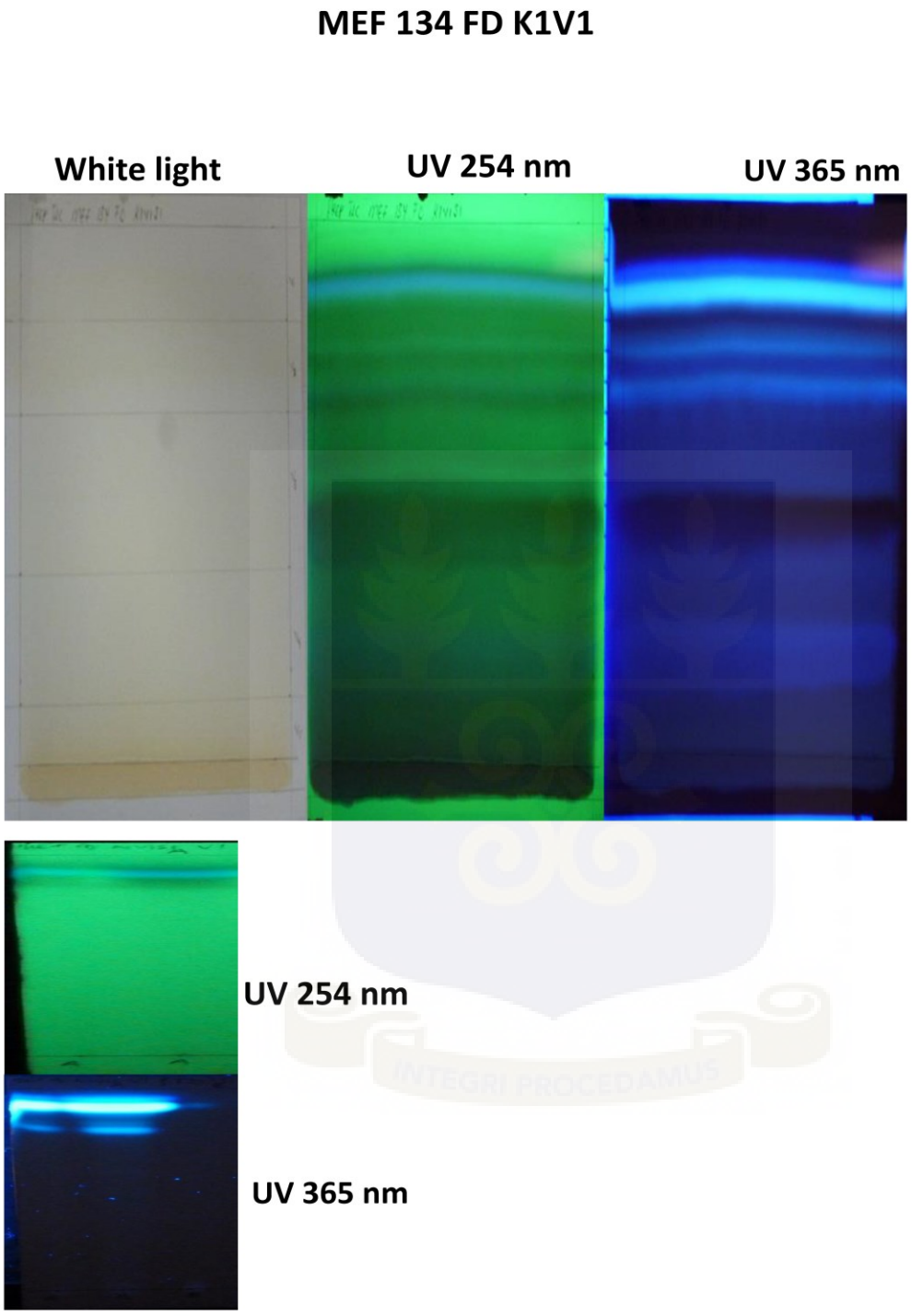



Fig S7.16. Preparative TLC (2D) of MEF 134 FD K1V1 from which fraction MEF 134 FD K1V1V1 was obtained.

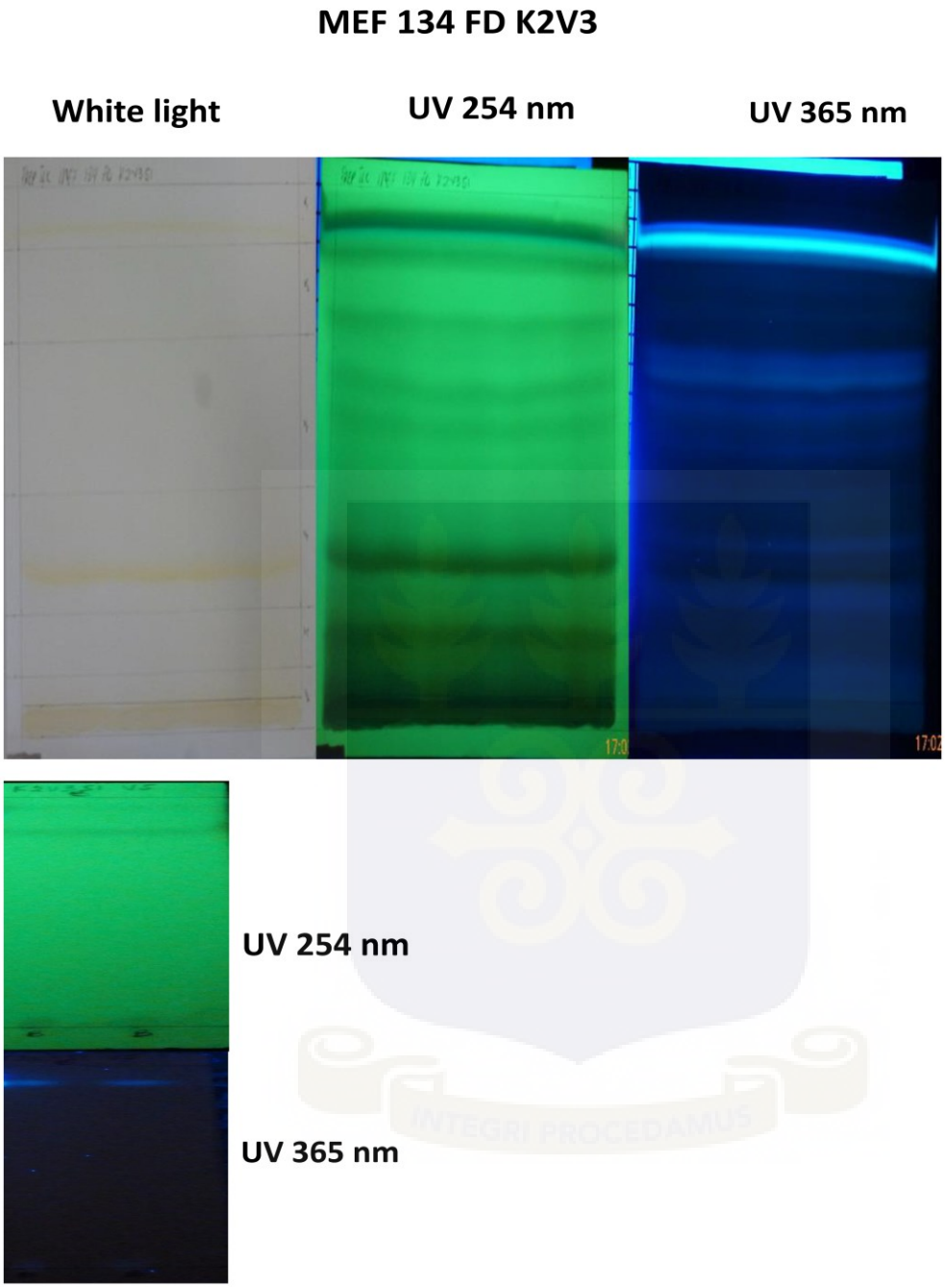



Fig S7.17. Preparative TLC (2D) of MEF 134 FD K2V3 from which fraction MEF 134 FD K2V3V5 was obtained.

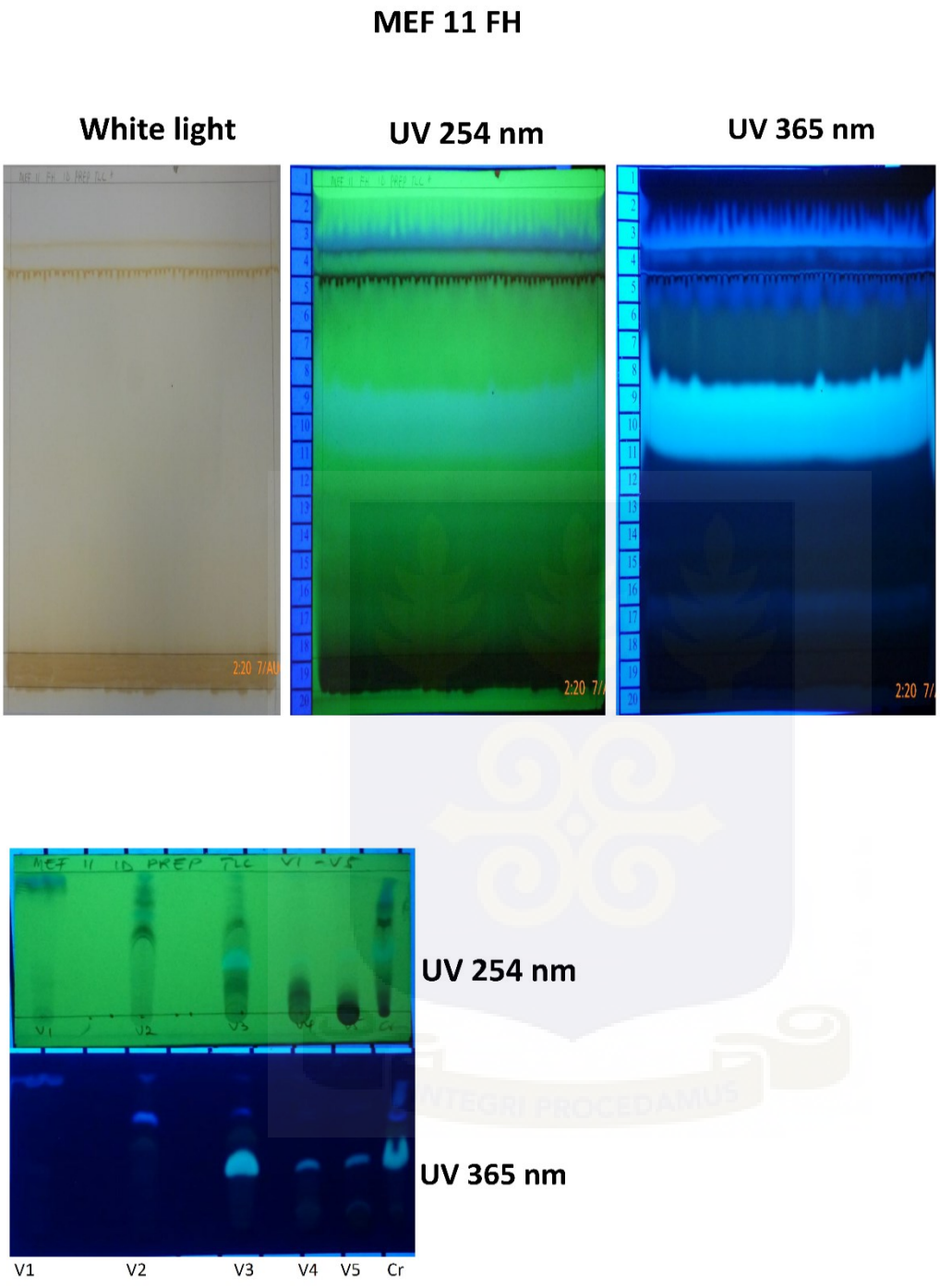



Fig S7.18. Preparative TLC (1D) of MEF 11 FH.

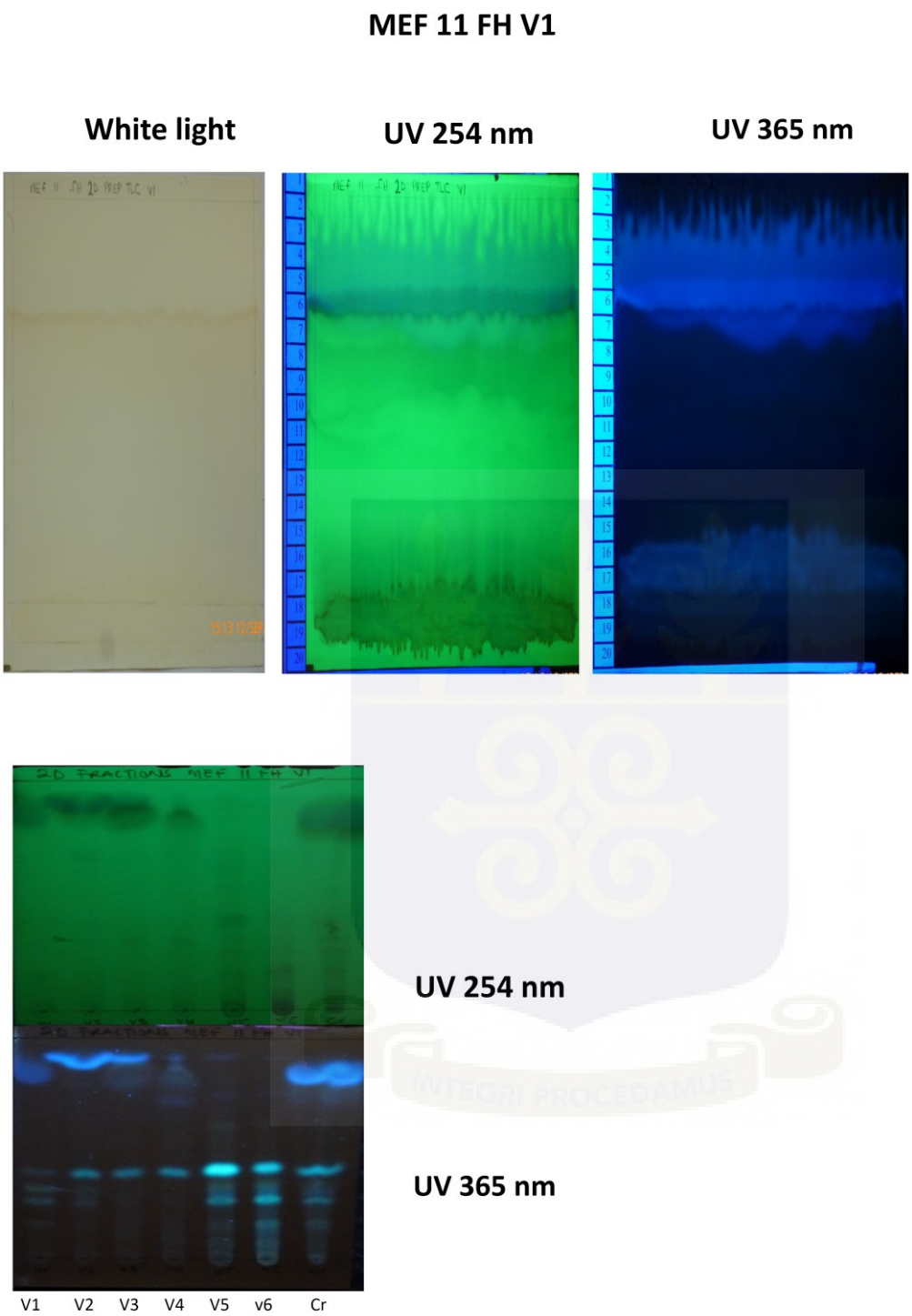



Fig S7.19. Preparative TLC (2D) of MEF 11 FH V1.

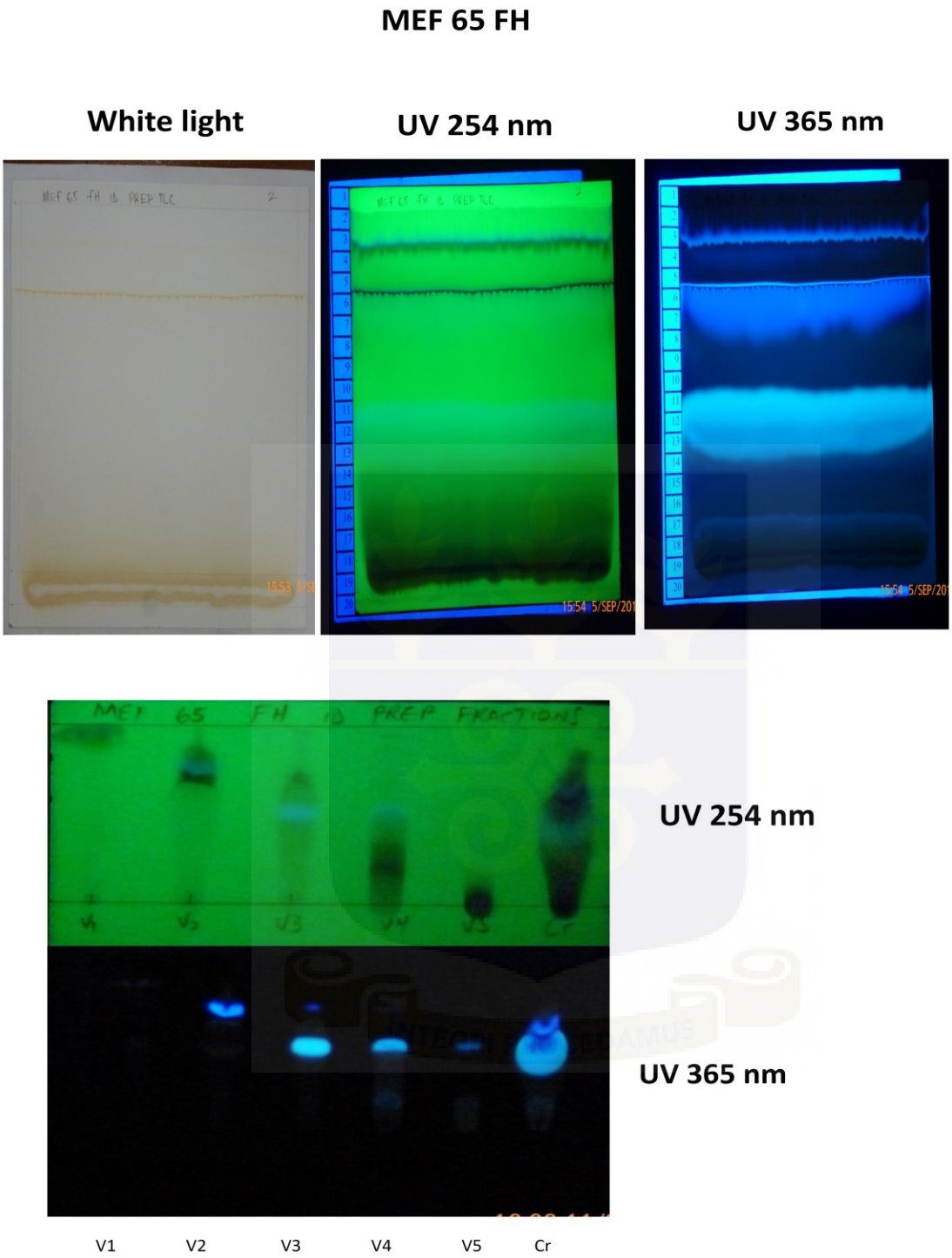



Fig S7.20. Preparative TLC (1D) of MEF 65 FH.

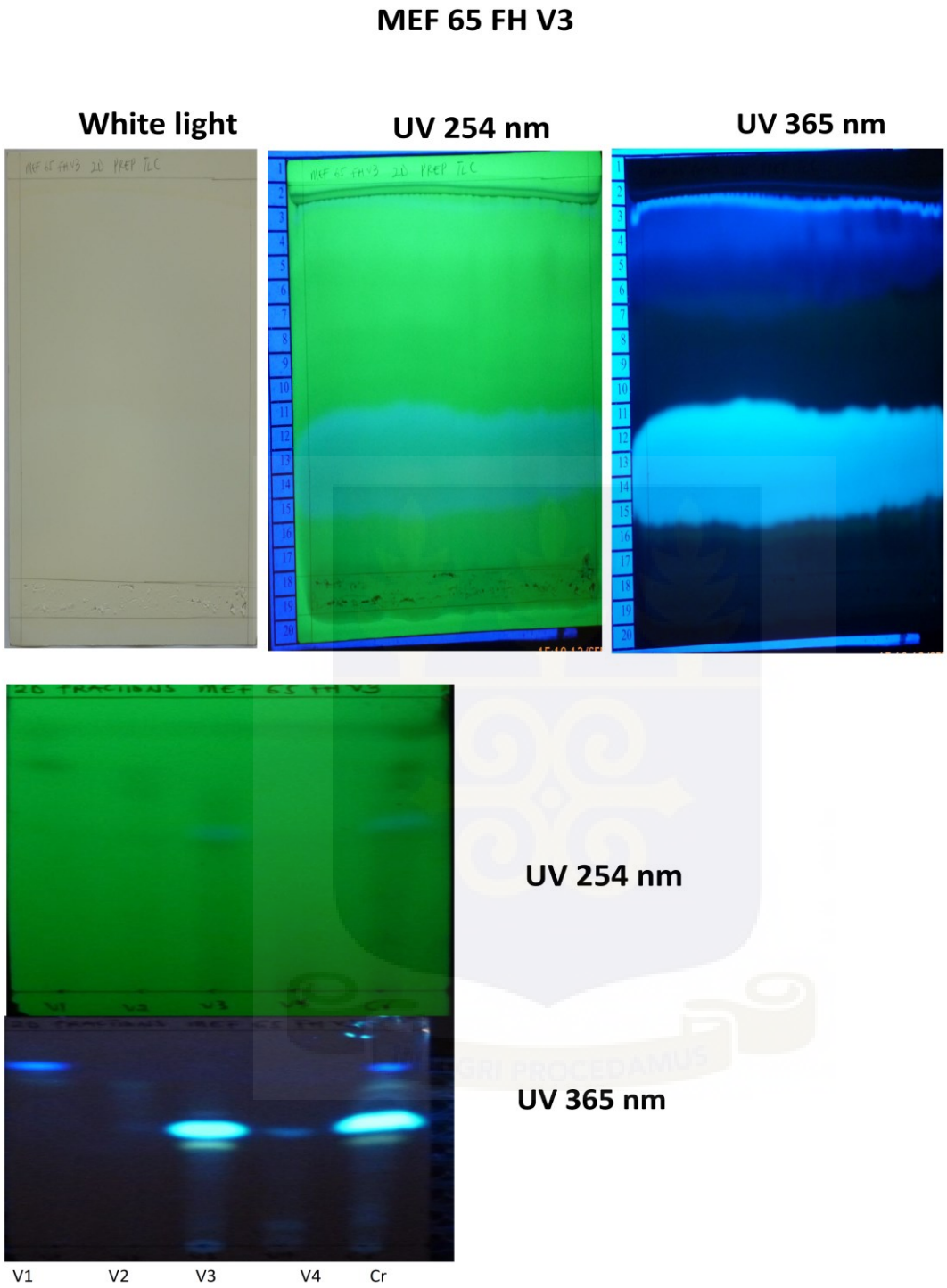



Fig S7.21. Preparative TLC (2D) of MEF 65 FH V3.

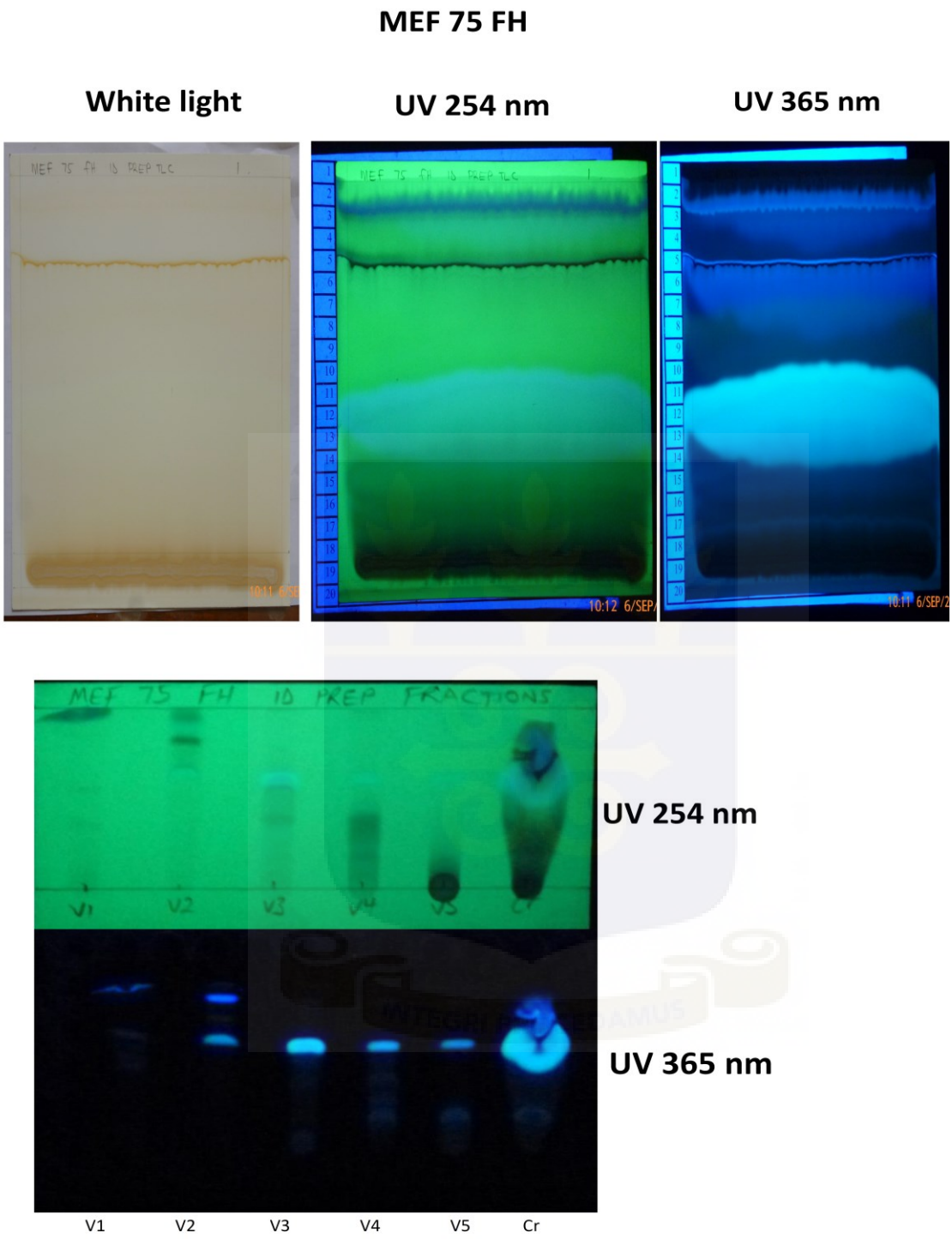



Fig S7.22. Preparative TLC (1D) of MEF 75 FH.

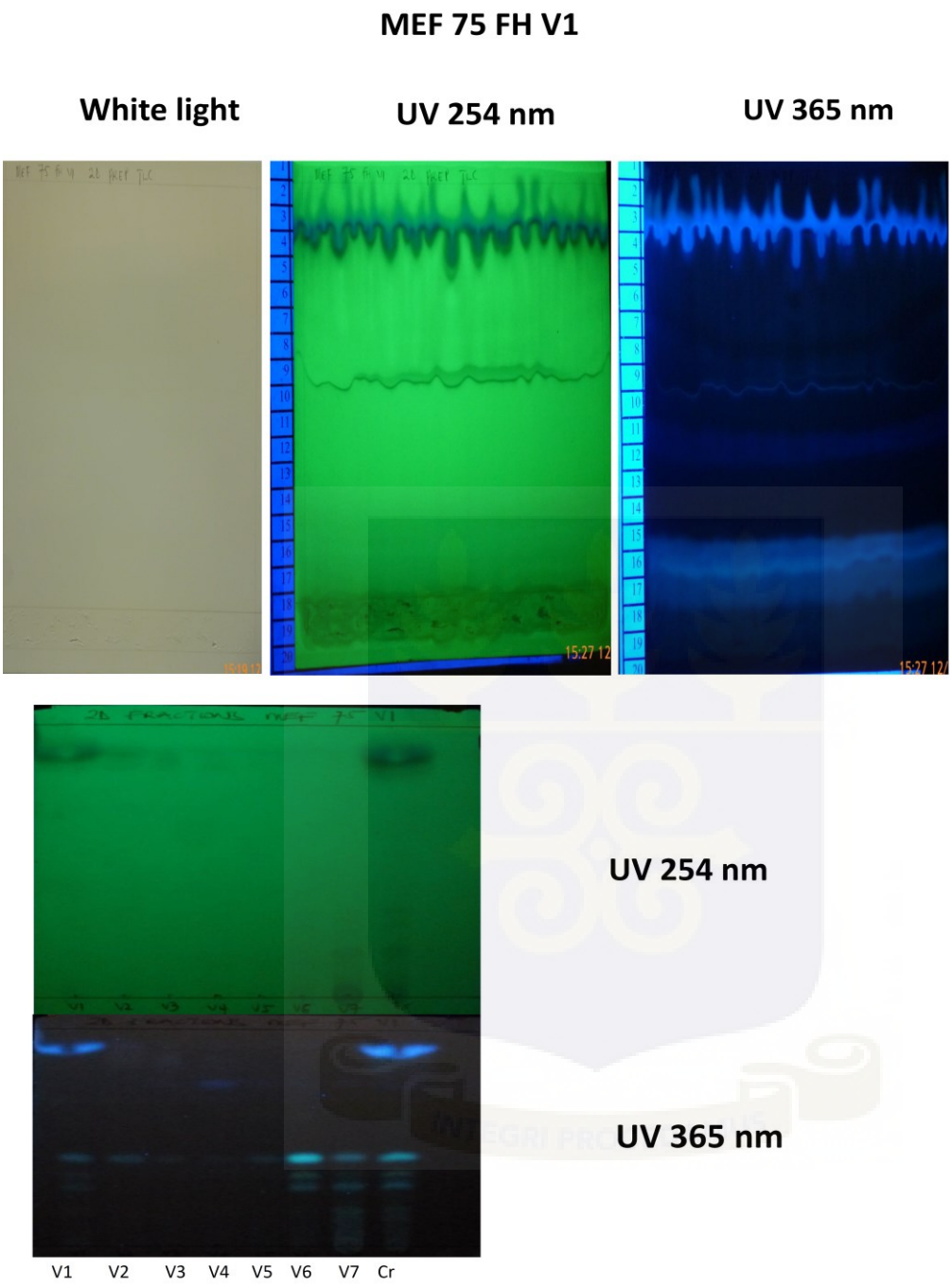



Fig S7.23. Preparative TLC (2D) of MEF 75 FHV1.

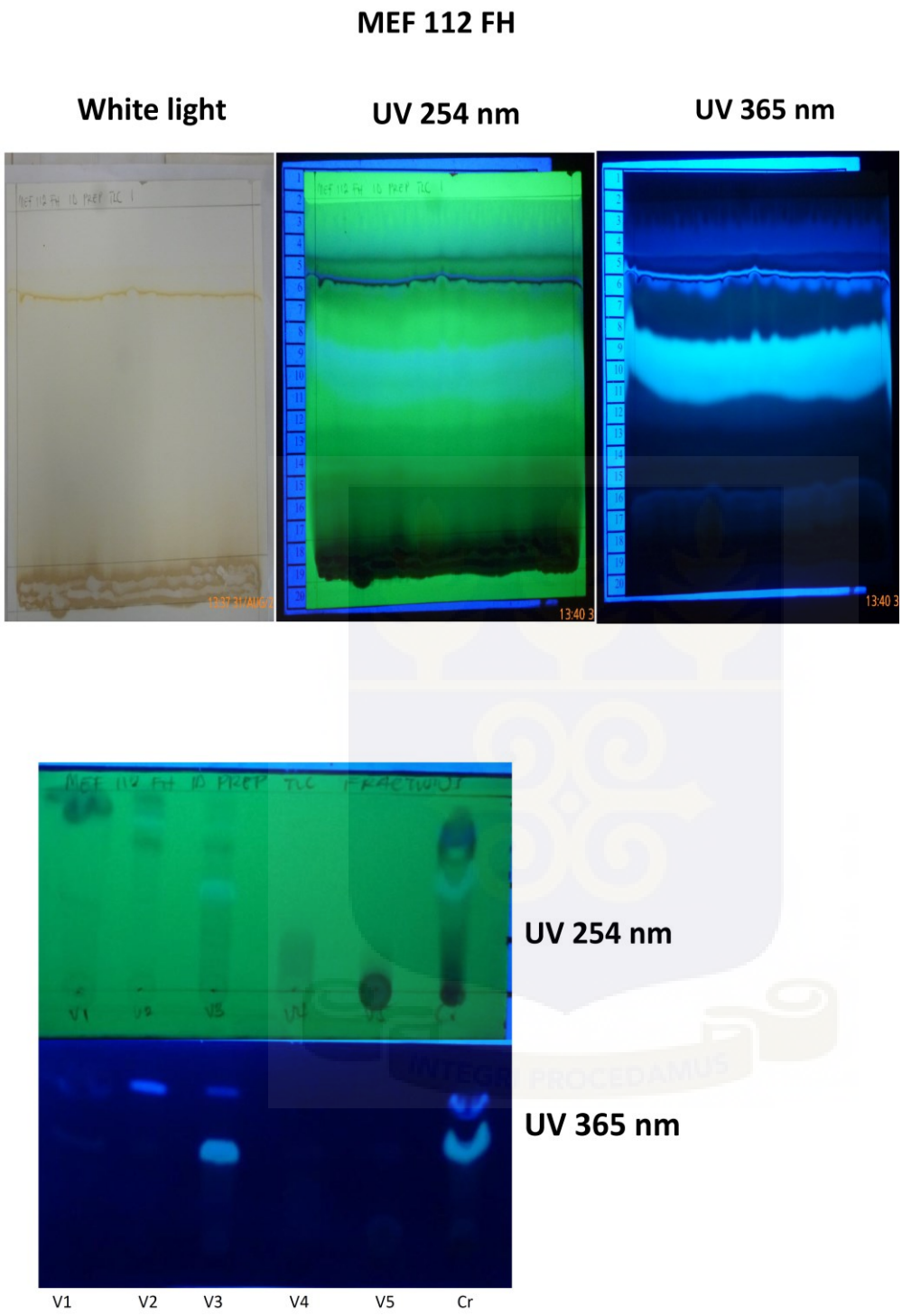



Fig S7.24. Preparative TLC (1D) of MEF 112 FH.

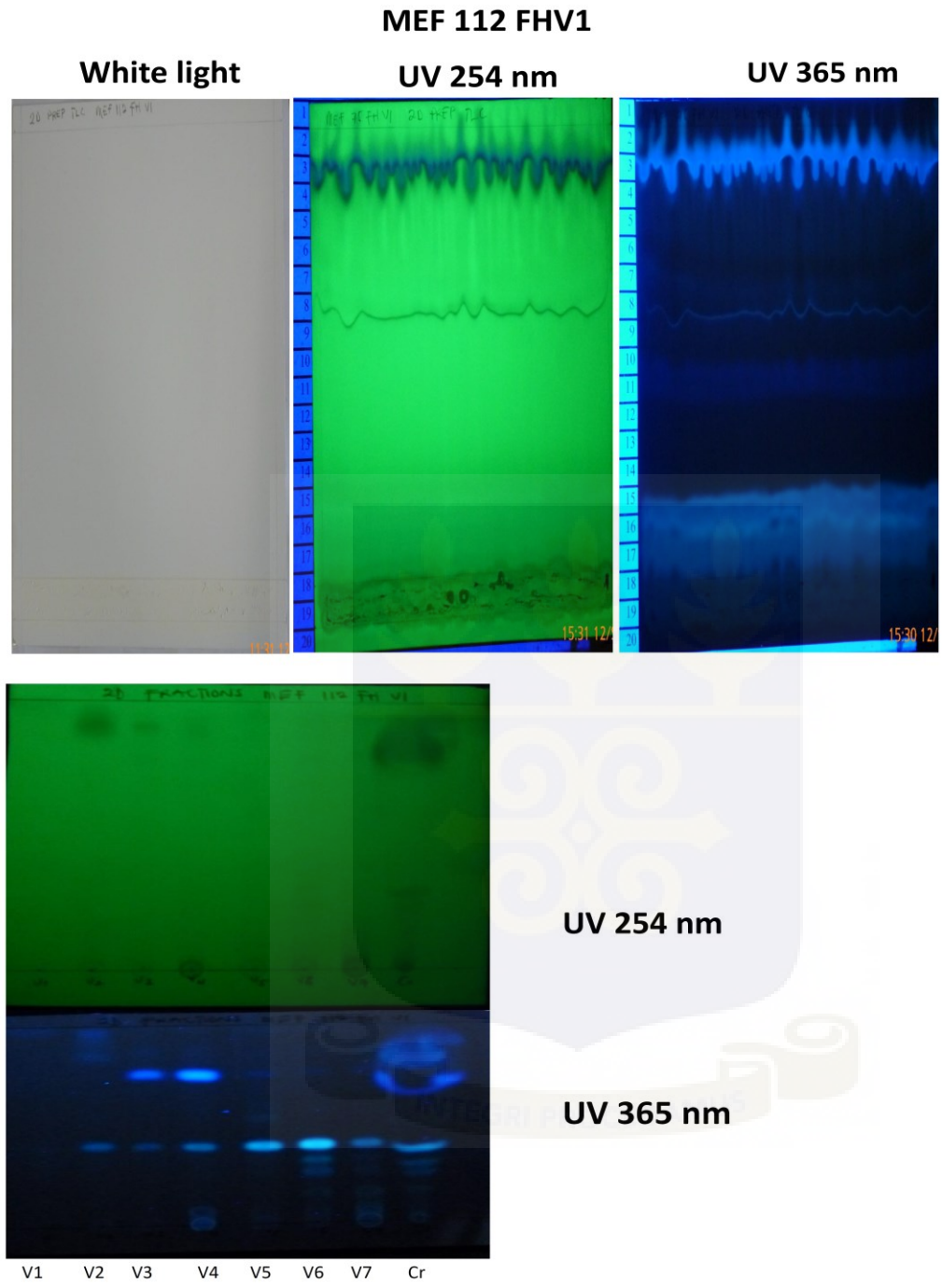



Fig S7. 25. Preparative TLC (2D) of MEF 112 FH V1.

# CHAPTER EIGHT

## GENERAL DISCUSSION AND CONCLUSIONS

### 8.1 General discussion

Marine fungal endophytes, and the bioactive metabolites they produce, have not been widely explored as potential sources of antimicrobial chemotherapy. Current reports indicate that fungal endophytes have been recognized as a promising source of bioactive compounds for drug discovery (Sarasan *et al.*, 2017). Globally, lung diseases caused by infectious fungal pathogens affect an estimated 10 million people, resulting in over 1 million deaths annually (Denning, 2015). Fungal infections are not restricted to the lungs alone; fungi are opportunistic pathogens that affect every part of the human body when the body's natural defense is compromised (de Pauw, 2011). Data on the global burden of fungal diseases affecting the human race is limited. However, fungal related deaths in developing countries remain significantly high because of the huge population of immune compromised people, such as people infected with HIV AIDS (Armstrong-James *et al.*, 2014). Additional measures, such as the introduction of more effective drugs, remain paramount in addressing the therapeutic needs of the population. This measure can be facilitated by searching for new drug candidates in areas that are not commonly explored. Indeed, some progress has been made in discovering drugs particularly from fungal sources. For instance, the isolation of taxol from *Pestalotiopsis microspore*, which is an endophytic fungus, has revolutionized cancer chemotherapy (Strobel *et al.*, 1996).

Due to the continuous pressures imposed on organisms by the marine environment, it is thought that marine fungal endophytes may be a good source of structurally diverse bioactive metabolites (Sarasan *et al.*, 2017). Several studies on discovery and isolation of bioactive metabolites from

marine fungal endophytes have been conducted with many unique bioactive compounds revealed. These compounds include Nigerasperone C, Asporyzin C and Multiplolides A and B (Boonphong *et al.*, 2001; Sarasan *et al.*, 2017; Zhang *et al.*, 2007). These studies give prominence to endophytic fungi from marine environment as promising sources of unique bioactive compounds. This study was performed to further explore marine fungal endophytes as potential sources of antifungal compounds, with emphasis on isolation techniques, molecular identification and bioprospecting (Fig 8.1). The objectives of the study were to: (1) isolate marine endophytic fungi and to determine the antifungal activity of their extracts against *Saccharomyces cerevisiae* and *Candida albicans*; (2) identify the bioactive metabolites and to elucidate their structures; (3) determine the specific mechanisms of action of the antifungal compounds produced by the marine endophytic fungi; (4) determine the cytotoxicity of the new antifungal compounds against human cells; (5) identify the fungal species that produced the bioactive metabolites.

Isolation of marine fungal endophytes from seaweed was conducted with great understanding of the method and rationale governing the process. The seaweeds that were harvested were processed immediately after sampling. In order to eliminate epiphytic organisms, different surface sterilization techniques were employed. Seaweeds are delicate in nature and they deteriorate easily when manipulated excessively. Hence several disinfection techniques were explored until successful surface sterilization of these seaweeds was achieved. The optimal disinfection protocol involved sterilization of the exterior tissues of the seaweeds with 70% isopropanol followed by immersion in 5% bleach. This technique has been shown to be sufficient to sterilize marine plant samples (Suryanarayanan *et al.*, 2010). A total of 143 fungal endophytes were obtained; a selection of these endophyte isolates were prioritized for in-depth studies of their ability to produce bioactive compounds. The selection criteria was based on bioactivity and the amount of secondary

metabolites produced by these fungal isolates during a preliminary antimicrobial screening assay; isolates that have previously been reported to produce bioactive compounds were also taken into consideration during selection of the fungi.

Thus, endophytic fungi with good bioactivity profiles and relatively fewer isolated compounds were prioritized for this study. Overall, 6 endophytic fungi were prioritized for in-depth investigation of the content of the secondary metabolites they produce (Table 3.1). Usually, fungal isolates are identified to the genus level using morphological characteristics such as the pattern of their colony during growth, size and pigmentation (Mailafia *et al.*, 2017). Nevertheless, species located within a genus can vary widely in their biological activities (Hong *et al.*, 2015). Hence high-resolution DNA-based information is necessary for taxonomic identification. Molecular techniques were used to identify the fungal endophytes. The targets used for molecular identification of fungal isolates to the genus and specie level may include one or a combination of the following; 18S rRNA gene, mitochondrial DNA and the internal transcribed spacer (ITS) regions (Alwakeel, 2013). In the present study, the region situated between the 18S and 28S rRNA genes, often referred to as the internal transcribed spacer (ITS) region, was sequenced and used to identify the fungal isolates, as reported previously (Pfaller & McGinnis, 2009). The copy number of the internal transcribed spacer region is approximately 100 per genome, making it highly sensitive. In instances where sufficient variability exist in this spacer region, fungal identification to species level can be achieved (Hinrikson *et al.*, 2005). The 6 marine endophytic fungi that were prioritized for this study were found to belong to the genera *Aspergillus*, *Clonostachys* and *Meyerozyma*. The most represented genus was *Meyerozyma* (Fig 2.1).

The test organisms that were used in this study were *C. albicans* and *S. cerevisiae*. The genome of *S. cerevisiae* has been sequenced and is well characterized (Goffeau *et al.*, 1996). Several gene

function analyses have been performed in *S. cerevisiae* to identify the function of all the protein-encoding genes in the genome (Gibney *et al.*, 2013; Guan *et al.*, 2007; Winzeler *et al.*, 1999). Based on these criteria, *S. cerevisiae* was chosen as a test organism for identification of the targets of the bioactive metabolites obtained from the 6 marine fungal endophytes. *C. albicans* is an opportunistic yeast pathogen and almost 70% of its protein coding genes have not been characterized(http://www.candidagenome.org/cache/C_albicans_SC5314_genomeSnapshot.html ). Even though the main aim of this study was to identify bioactive metabolites against *C. albicans*, it was necessary to also include *S. cerevisiae* as a test organism because of the limited data on the function of the genes found in *C. albicans*.

This study demonstrated that the extracts from all the 6 selected endophytic fungi showed promising antifungal activity against *Candida albicans*. The bioactivity of the extracts against *Saccharomyces cerevisiae* was generally low, which might indicate that these extracts would be less toxic to human cell lines. Solvent-solvent fractionation of the extracts exhibiting bioactivity against *C. albicans* generated fractions that contain antifungal compounds. These solvent fractions can be analyzed in-depth using high performance liquid chromatography (HPLC) to obtain the individual bioactive compounds in the pure form. In order to understand the possible mechanism of action, the extracts from the 6 endophytic fungi were screened against a library of deletion mutants of diploid *S. cerevisiae*. From this screening assay, it was concluded that the extracts might target protein synthesis, ergosterol biosynthesis and the yeast translational apparatus.

A major hindrance to the production of high quantities of bioactive metabolites from fungal cultures is the inability to determine the particular time at which these metabolites are produced. For many studies, the duration for culturing of fungi was chosen without any strong scientific evidence. It was important therefore in this study to systematically determine the duration for

production of metabolites from 3 out of the 6 selected endophytic fungi. The data obtained indicated that the duration for production of bioactive metabolites from the endophytic fungi is dependent on time and was favored by shorter incubation periods of the fungal cultures. Moreover, the data showed that the yield of metabolites is not entirely dependent on the duration of incubation of the cultures but also on the metabolism of the fungal isolate. It was observed from TLC analysis of solvent extract fractions that the endophytic fungi produced a broad spectrum of metabolites. The most bioactive metabolites were found in solvents with very low polarity indexes. Overall 59 active fractions were detected in the fairly crude fractions and it was projected that there could be as many as 154 in total when all the active solvent-solvent fractions are fully fractionated. All the 59 active fraction were validated in liquid cultures to show that there was activity irrespective of the culture format.

The production of compounds from marine fungal isolates which could have superior activity over the commercially available antifungal compounds was investigated. In order to ensure that the relevant extracts were studied in-depth, a phenotypic screening assay was developed in our laboratory to reduce redundancy among the selected extracts. The phenotypic screening assay was also used to identify compounds that could form synergistic partners with the bioactive compounds contained in the extracts and to generate an activity map for all the standard antifungal compounds assayed in the presence of the phenotype modifying compounds against both test organisms. Phenotypic screening is usually included in the drug discovery workflow to identify molecules that modify the phenotype of cells (Kotz, 2012). In this study, 20 phenotype-modifying compounds were screened together with either standard antifungal compounds or the extracts using *C. albicans* and *S. cerevisiae* as test organisms. Several new synergistic interactions between the antifungal compounds and the phenotype modifying compounds were detected; these observations have not

been reported in literature. During the interaction assay against *S. cerevisiae*, it was observed that even though the extracts were not active on their own, they became active in the presence of the phenotype modifying compounds. Additionally, the extent of those activities formed the basis for determining how different the extracts were from each other, it is very important to avoid redundancy in the selection for the crude extracts for product isolation. The phenotypic assay was instrumental in extending the limit of detection of bioactivity of the extracts against *S. cerevisiae*.

For a lead compound to become a clinical drug candidate, the compound must meet high safety standards. In the drug discovery and development process, testing toxicity of new compounds at the early stages of the investigation is vital. Effort was made in this project to evaluate the cytotoxicity of the bioactive solvent fractions of interest. It was observed that three dichloromethane fractions from MEF 134 were active only against *S. cerevisiae*. These fractions were tested for their ability to reduce proliferation in the established human liver cancer cell line HepG2. This cell line is usually used in toxicity testing of compounds. The findings indicated that all 3 fractions reduced HepG2 cell proliferation. Studies have shown that humans and yeast have similar genes including those known to be involved in cell proliferation and cancer (Foury, 1997). *S. cerevisiae* is a model organism for investigating processes such as cell cycle progression, DNA replication and segregation, maintenance of genomic integrity and stress responses. In cancer disease progression, these processes are affected by genetic and epigenetic alterations (Menacho-Marquez & Murguia, 2007). Since our data showed that these fractions were only active against *S. cerevisiae* and reduced HepG2 cell proliferation, it was hypothesized that the fractions could affect certain pathways and genes relevant in cancer development. Therefore, the effect of the 3 fractions on proliferation of HepG2 cells at the transcriptome level was also analyzed. It was demonstrated that several cancer and metabolism-related pathways and gene ontologies were

regulated by the treatment with the 3 fractions from marine endophytic fungi. Furthermore, a protein interaction network was constructed to distinguish the fraction-treated HepG2 cells from the untreated control HepG2 cell. It was observed that major functional components of this network were associated with metabolism and cancer. It was concluded that the prominent compounds detected from these fractions should be synthesized and validated.

The metabolite content of 9 selected fractions was investigated using HPLC and mass spectroscopy. The structures of 31 compounds were elucidated with 22 of these structures identified to be novel while 9 structures were already known compounds. Many of the novel structures obtained could serve as precursor compounds in the design of new drugs or could be used as drugs on their own following in-depth characterization.

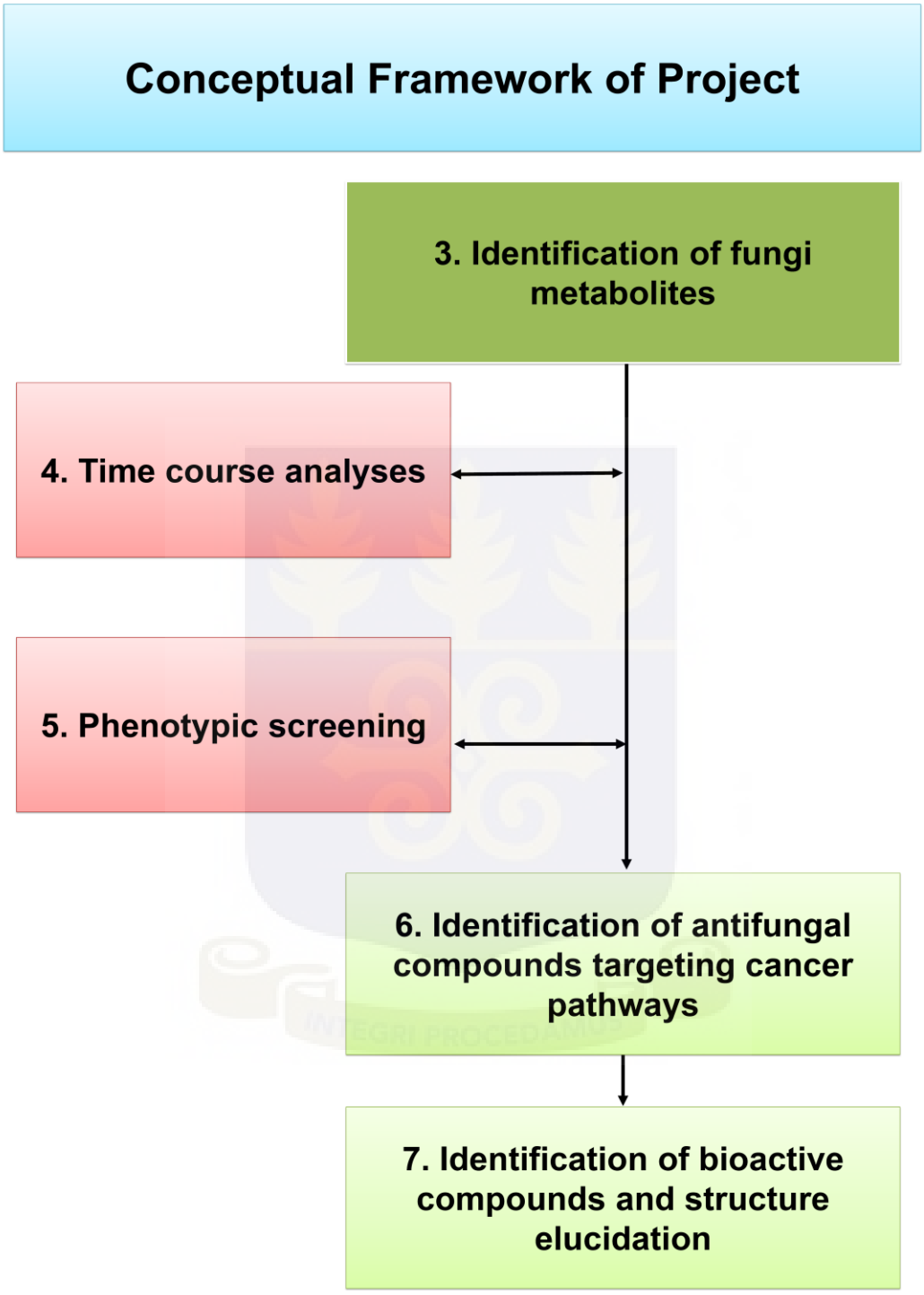



Fig 8.1. Conceptual frame work of the antifungal drug discovery project.

## 8.2 Conclusions

This study reports on the potential of obtaining antifungal compounds from endophytic fungi which were isolated from seaweeds along the beaches in Ghana. A total of 143 morphologically distinct marine fungal endophytes were obtained from these seaweeds. In this study, 6 out of the 143 marine fungal isolates were analyzed in-depth and were found to produce compounds which were effective against *C. albicans* and *S. cerevisiae*. Taxonomic identification of the 6 marine fungal isolates via amplification of the DNA sequences from the internal transcribed spacer (ITS) region of the ribosomal RNA revealed that these fungal isolates were from three major genera; *Aspergillus, Clonostachys* and *Meyerozyma*. Future studies would be aimed at identifying the genus and species of the remaining fungal isolates in order to identify the predominant fungal species at the beaches from which these fungi were obtained.

Production and yield of bioactive metabolites from these marine fungal endophytes was dependent on time and the metabolism of the fungus. Diverse classes of metabolites were produced by these fungi and several novel synergistic interactions were observed between the known antifungal compounds and the phenotype modifying compounds. Cytotoxicity assay was used to demonstrate that the solvent fractions from the extracts reduce HepG2 Cell proliferation. This solvent fractions targeted major functional components of the protein network which were associated with metabolism and cancer. Future studies would be aimed at synthesizing the prominent compounds detected from these fractions and retesting their antifungal and anti-HepG2 activities. One of the main observations from this study was the identification of 31 compounds from the 9 fractions that were analyzed. These compounds could be the active components in the fractions that exhibited antifungal activities; this hypothesis would be tested using pure forms of these compounds (chemically-synthetized or isolation from the fractions via HPLC). The data generated in this study

highlights the opportunities of exploring fungal endophytes as useful sources of antifungal compounds for treatment of infections caused by pathogenic fungi

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