CHARACTERIZATION AND ANTIMICROBIAL ACTIVITY OF TROPICAL BASIDIOMYCETES FUNGI AND THEIR SECONDARY METABOLITES

SUM WINNIE CHEMUTAI

A thesis submitted to the Graduate School in partial fulfilment for the requirements of Master of Science Degree in Biochemistry of Egerton University

EGERTON UNIVERSITY

OCTOBER, 2018

DECLARATION AND RECOMMENDATION

DECLARATION

This thesis is my original work and has not been submitted for examination in any institution.

Signature…… Date……

Sum Winnie Chemutai

SM14/14192/15

RECOMMENDATION

This thesis has been submitted for examination with our approval as university supervisors.

Signature…… Date……

Prof. J. Matasyoh

Chemistry Department

Egerton University

Signature…… Date…….

Dr. S. Indieka

Department of Biochemistry and Molecular Biology

Egerton University

COPYRIGHT

DEDICATION

To my family and friends for their unconditional love, support, encouragement and prayers throughout my study.

ACKNOWLEDGEMENT

I thank the Almighty God for His love, blessings and protection throughout my entire research period. I'm also thankful towards Egerton University, especially the departments of Chemistry and Biochemistry for their support during my studies. I sincerely thank my supervisors Prof. Josphat. C. Matasyoh and Dr. Stephen A. Indieka for providing me with excellent supervision during my research work. Their guidance, commitment and scientific knowledge drove me towards the innovative exploration of the field and aided in my timely completion of the work. I appreciate their encouraging discussions and positive criticisms. My heartfelt gratitude also goes to the fraternity of Helmholtz Zentrum Für Infektionforschung (HZI), Braunschweig, Germany for allowing me to use their facilities to achieve the objectives of my study. In a special way my heartfelt gratitude goes to Prof. Dr. Marc Stadler of the Microbial Drugs department, HZI and Ms. Clara Chepkirui (PhD student, HZI) for their guidance throughout my laboratory work at the institute. I sincerely thank the Kenya Wildlife Service (KWS) and the local community around Mt Elgon National Park for their assistance during the collection samples for this study. I also owe my sincere gratitude to my loving husband Eng. Joel K. Choge, for his love, care and encouragement and to my parents Mr. Joseph L. Sum and Mrs. Rose C. Sum for their endless support in my life. I remain indebted to them forever.

ABSTRACT

Microbial infections remain to be a major global public health challenge. In Kenya, these infections cause significant morbidity and mortality. Their devastating effects are majorly attributed to antimicrobial drug resistance. Nonetheless, the biodiversity and ecology of tropical Basidiomycetes fungi is an untapped source of potential bioactive compounds. Therefore, the current study aimed to isolate and test crude extracts and antimicrobial compounds from Basidiomycetes against selected microbial pathogens. The fungal basidiocarps were collected from Mt. Elgon National Park forest and the spores cultured in Yeast Malt Agar media (YMG), amended with antibiotics. Subsequent sub-culturing led to the establishment of pure cultures of 12 fungal strains. Their identity was determined using phenotypic- and ITS rDNA molecular-based techniques. They belonged to genera; *Inonotus*, *Fomitiporia*, *Ganoderma*, *Perenniporia*, *Favolaschia*, *Hexagonia*, *Skeletocutis*, *Polyporus*, *Antrodia* and *Echinochaete*. Mycelial cultures were thereafter fermented and metabolites extracted using ethyl acetate. However, *Fomitiporia* sp (KE/16-163) was further subjected to large scale cultivation and the crude extracts fractionated and purified using HPLC (high performance liquid chromatography), owing to it's fast growth rate and initial antagonism. Notably, five previously undescribed pregnenolone-type steroids (assigned trivial names Aethiopinolones A-E) were successfully characterized from the fungus using 1-D and 2-D NMR (nuclear magnetic resonance) and HREIMS (high resolution electron impact mass spectroscopy) data. Antimicrobial assays of fungal crude extracts and pure compounds were performed against *Bacillus subtilis*, *Escherichia coli*, *Mucor plumbeus* and *Candida albicans*. 9 of the 12 tested fungal strains displayed considerable antimicrobial activity. Notably, *Echinochaete* sp (KE/16-198) and *Favolaschia* sp (KE/16-152) demonstrated high activities against *B. subtilis* and *C. tenuis* at minimum inhibitory concentration (MIC) values of 4.69 µg/ml and <2.34 µg/ml respectively. Further, the cytotoxicity of the novel compounds obtained was determined using a panel of seven mammalian cell lines (mouse fibroblast cells, HeLa cells, epidermoid carcinoma cells, human alveolar adenocarcinoma basal epithelial cells, prostate cancer cells, ovarian cancer cells and breast cancer cells**)**. Interestingly, compounds **1**, **3**, **4** and **5** exhibited moderate to high (8-19µg/ml) cytotoxic effects against most cancer cell lines whereas, compound **2** was non-cytotoxic. Thus, the results reveal that Basidiomycetes fungi are a reservoir of fungal metabolites which could be exploited as sources of natural products of pharmaceutical importance.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF APPENDICES

LIST OF ABBREVIATIONS AND ACRONYMS

BLAST	Basic Local Alignment Search Tool
CLSI	Clinical and Laboratory Standards Institute
COSY	Correlation Spectroscopy
DEPT	Distortionless Enhancent of Polarization Transfer
DMSO	Dimethyl Sulfoxide
DNP	Dictionary of Natural Products
HMBC	Heteronuclear Multiple Bond Correlation
HMW	High Molecular Weight
HPLC	High Performance Liquid Chromatography
HREIMS	High Resolution Electron Impact Mass Spectroscopy
HSQC	Heteronuclear single quantum coherence
ITS	Internal Transcribed Spacer Regions
LMW	Low Molecular Weight
MIC	Minimum Inhibitory Concentration
MUCL	Mycothèque de l'Université Catholique de Louvain
NCI	National Cancer Institute
NMR	Nuclear Magnetic Resonance
PCR	Polymerase Chain Reaction
ROESY	Rotating-frame Overhauser Effect Spectroscopy

CHAPTER ONE
INTRODUCTION

1.1 Background Information

Infectious diseases cause an enormous economic and social burden worldwide. In the developing countries, they have been shown to be responsible for one in every two deaths in children and young adults (WHO, 2014). Millions of these victims are mainly affected by diseases attributed to fungal and bacterial pathogens (Danishuddin *et al.,* 2012). These microbial threats continue to emerge, reemerge, and persist with some microbes causing new diseases in the populace, whereas others are pathogens that were previously known but are now infecting new or larger population groups and spreading into new geographic areas.

Antimicrobial drug resistance by microbes is a paramount public health threat of the twenty first century (Ling *et al.,* 2015). Therefore, with the presence of antimicrobial drug resistance, there may come a corresponding increase in mortality and morbidity from untreatable diseases, and increased risk of global spread of drug-resistant pathogens. This consequently may lead to a rise in the total health care costs associated with the need for multidrug therapy, longer and more frequent hospital stays, as well as increased costs of research and development of alternative drugs (Ventola, 2015). For instance, major efforts to control each of the three major global infectious diseases (AIDS, TB, and malaria) have been undermined by the rise of antimicrobial drug resistance (WHO, 2014).

Initially, the problem of microbial resistance to drug compounds was solved by the discovery of new classes of drugs, such as the aminoglycosides, macrolides, and glycopeptides, as well as by the chemical modification of the previously existing drugs (Davies, 2014). However, unfortunately there has been no assurance that the developed antimicrobial drugs have kept up pace with the ability of microbial pathogens to develop resistance. Therefore, unless the antimicrobial drug resistance problems are detected early as they emerge and counter measures immediately implemented, the dangers posed by even previously treatable and manageable diseases will still linger, as microbial diseases continue to persist and re-emerge (Ventola, 2015).

Nevertheless, the exploration of natural sources for novel bioactive agents, may be lead to development of antimicrobial drug compounds to counter the existing drug resistance (Gurnani *et al.,* 2014; Cragg and Newman, 2013). The kingdom fungi is the second most

diverse group of organisms on earth, and it has been postulated that fungal diversity (up to 3 to 5 million species) exceeds the diversity of terrestrial plants by a greater magnitude (Blackwell, 2011; Dai, 2010). However, only a small fraction of all fungal species have so far been described (about 100,000) and a smaller number explored for the production of pharmacologically important metabolites. This is in spite of some of the major successful drugs and agrochemical fungicides on the market having been developed from fungal secondary metabolites (De Silva *et al.,* 2013). A number of antibiotics (penicillins, cephalosporins and fusidic acid), anti-fungal agents (griseofulvin, strobilurins and echinocandins), cholesterol-lowering statin derivatives (mevinolin, lovastatin and simvastatin) and immunosuppressive drugs (cyclosporin) among others have been obtained from fungal metabolites (Kozlovskii *et al.,* 2013; Li and Vederas, 2009; Smith and Ryan, 2009).

Traditionally, mushroom forming fungi mostly from phylum Basidiomycota and some Ascomycota have been used as remedies for various diseases (De Silva *et al.,* 2012; Petrova *et al.,* 2008; Sullivan *et al.,* 2006), owing to their prolific production of secondary metabolites (Wasser, 2011; Kawagishi, 2010). However, macro fungi of the phylum Basidiomycota, are less intensively investigated targets despite the fact that they contain compounds with potential antimicrobial activities (Alves *et al.,* 2012). According to various literatures, novel compounds of different biogenetic origins isolated from Basidiomycota have been shown to have antibacterial and antifungal properties among other pharmacological activities (De Silva *et al.,* 2012; Poucheret *et al.,* 2006). Hence, these mushrooms make a vast yet untapped source of new antimicrobials. Thus, this study aimed to explore tropical fungi of phylum Basidiomycota as a potential source of novel and potent antimicrobial compounds.

1.2 Statement of the Problem

Antimicrobial drug compounds pipeline is drying up with insufficient attention in terms of medical research. In the past 25 years there has been stagnation in the development of new antimicrobial drugs due to high costs associated with developing antimicrobials drug compounds. Hence, for sound commercial reasons pharmaceutical companies are investing in drugs that are used to manage chronic infections, since these drugs might be taken for years or even decades, hence providing profits. The antimicrobial drug compounds currently in the market are few and expensive, hence they are neither affordable nor accessible to the poor.

In addition, some synthetic antimicrobial drug compounds have shown significant toxicity to patients hence causing more adverse effects. Sadly, the situation has been further compromised since the major efforts to combat microbial pathogens, have been seriously undermined by emergence of antimicrobial drug resistance. In most cases, pathogenic microbes are resistant to at least one drug used in its management. Hence, there is need for the discovery and development of cheaper and effective antimicrobial compounds.

1.3 Objectives

1.3.1 General objective

To isolate and characterize Basidiomycetes fungi and their compounds and determine their antimicrobial activities against selected human microbial pathogens.

1.3.2 Specific objectives

i. To identify and characterize selected tropical fungi belonging to phylum Basidiomycota collected from Mt. Elgon National Park forest.
ii. To evaluate the antimicrobial activities of crude extracts and pure secondary metabolites from isolated pure cultures of Basidiomycota fungi against selected human microbial pathogens.
iii. To determine the structures of pure bioactive secondary metabolites using NMR and establish their cytotoxic effects to mammalian cell lines.

1.4 Null Hypotheses

i. Molecular and phenotypic characterization techniques will not reveal different species of Basidiomycetes.
ii. Basidiomycetes fungi will not display significant antagonistic activity against major human microbial pathogens.
iii. The isolated secondary metabolites will not show significant toxicity to human cell lines.

1.5 Justification

Bacterial and fungal pathogens are major causes of devastating human diseases. Antimicrobial drug resistance and the presence of few and expensive antimicrobial drug compounds is a fact of life that we must accept and confront. Currently, the increased incidences of bacterial and fungal infections in most patients, also summons the need for

more effective antimicrobials to replace many of the existing agents, which are not optimal against emerging infections, exhibit host toxicity and might induce the development of resistance. Strikingly, many antimicrobial drug compounds in the market, were developed from naturally occurring products obtained majorly from fungi. The fungi accumulate antibacterial and antifungal secondary metabolites in order to survive the hostility of their natural environment. Thus, they have been shown to be a dominant but untapped source of novel antimicrobial drug compounds. It isn't therefore surprising that antimicrobial drug compounds with strong activities could be isolated from Basidiomycetes fungal species to benefit humans. Furthermore, the secondary metabolites isolated may offer an alternative to microbial diseases' management without the negative impact of synthetic drugs. Therefore, this study aimed to identify and isolate bioactive compounds from Basidiomycetous tropical fungi as lead compounds for development of antibiotic and antifungal drugs.

CHAPTER TWO
LITERATURE REVIEW

2.1 Human Microbial Pathogens

Human beings, animals and the environment is heavily colonized with various microbes majorly bacterial and fungal. Some of these microbes can transit from the colonization state and cause severe life-threatening disease state (Pordeus *et al*., 2008). Microbial pathogenicity usually occurs as a result of many different attributes: microbial attachment, local proliferation, tissue damage, invasion and dissemination to other body parts (Wassenaar and Gaastra, 2001). This depends on the microbial degree of virulence which involves the combined activity of all genes, whose products are involved in interactions with the host leading to pathological damage (Allen and Torres, 2008; Lynch and Wiener-Kronish, 2008). Highly pathogenic microbial species, cause significant mortality and morbidity, whereas the less pathogenic ones primarily cause morbidity. In addition, opportunistic pathogens may or may not cause disease, depending on the natural equilibrium between pathogen and its host (Packey and Sartor, 2009).

2.1.1 Bacterial pathogens

Bacteria such as gram-positive coagulase-negative staphylococci, *Staphylococcus aureus*, and *Enterococcus* sp are extremely important pathogens in the hospital environment (Rice, 2006). This is because, for most nosocomial bloodstream infections, coagulase-negative staphylococci cause 31.9% of the infections, followed by *S. aureus* in 15.7%, enterococci species in 11.1%, and viridans streptococci in 1% (Randazzo *et al* 2016; Wisplinghoff *et al.,* 2004).

Pseudomonas aeruginosa, a versatile Gram-negative bacterium grows in various habitats, as well as on the tissues of plants and animals. It also forms biofilms on wet surfaces and has emerged as a major opportunistic human pathogen resistant to antibiotics and disinfectants used to eliminate bacteria in the environment (Schluter *et al.,* 2015; Petrova and Sauer, 2010). *P. aeruginosa* is the major source of bacteremia in victims of burns, in urinary-tract infections in catheterized patients and in hospital-acquired pneumonia by patients on respirators. Furthermore, it's also responsible for the gross morbidity and mortality in cystic fibrosis (CF) patients (Musk and Hergenrother, 2008).

Escherichia coli is a commonly found normal microflora in the human gastrointestinal tract (Gill *et al.,* 2006). However, some *E. coli* strains can cause serious

illness in humans resulting in major losses of lives annually (Smith *et al.,* 2007). In Kenya, *E. coli* strains and group B *Streptococci* were shown to be the leading causes of bacteremia among children according to a study by Lowe *et al.* (2005). Diarrheagenic *E. coli* strains have been shown to cause major outbreaks in the United States. A significant proportion of *E. coli* isolates also have been shown to cause disease outside the intestinal tract; they are known as extra-intestinal pathogenic *E. coli* (ExPEC) (Russo and Johnson, 2006). ExPEC isolates cause a range of diseases in humans; urinary tract infections and neonatal meningitis. The diarrheagenic pathogenic variants (pathovars) of *E. coli* are also diverse and five distinct clinical groups of diarrheagenic isolates have been identified: enterohemorrhagic *E. coli* (EHEC), enteroaggregative *E. coli* (EAEC), enteropathogenic *E. coli* (EPEC), enteroinvasive *E. coli* (EIEC) and enterotoxigenic *E. coli* (ETEC) (Rasko *et al.,* 2008).

Some bacterial pathogens such as EHEC, *Toxoplasma gondii* and *Salmonella* sp are also known to be frequent among domestic animals hence likely to be introduced into the food chain causing human disease (Schlundt *et al*., 2004). Bacterial pathogens that can be transmitted by vectors, enhance human exposure to such agents (Randolph, 2008). Some bacteria that cause human disease can also be transmitted via various modes such as aerosol, contact and behavioral (i.e. sexual) mechanisms among others. Nasopharyngeal bacterial pathogens (*Streptococcus pneumoniae, Haemophilus influenzae,* and *Moraxella catarrhalis*) for instance, can spread via an airborne route (Mink and Yeh, 2009).

2.1.2 Fungal pathogens

Fungal pathogens have an enormous impact on the life of plants and animals. A recent report has shown an extraordinary and frightening impact of fungal pathogens on various species extinctions, ecosystem disturbances, and food security (Ishii and Holloman, 2015; Fischer *et al.,* 2012). Despite this gross impact, the effects of fungal infections on human health is not widely recognized and deaths that result from these infections are more often overlooked. More than 90% of all reported fungal-related deaths world-wide result from species that belong to one of four genera: *Cryptococcus*, *Candida*, *Aspergillus*, and *Pneumocystis* (Alastruey-Izquierdo *et al.,* 2015).

Most people worldwide will always suffer from superficial fungal infections in their lifetime, which is generally easy to cure. However, millions of individuals worldwide will contract invasive life-threatening infections that are much harder to diagnose and treat (Brown *et al.,* 2012). Superficial fungal infections of the skin and nails are the most common

fungal diseases in humans. These infections are caused by dermatophytes and they affect approximately 25% (or ~1.7 billion) of the general worldwide human population (Havlickova *et al.,* 2008). Dermatophytes give rise to well-known conditions such as athlete's foot, which occurs in 1 out of 5 adults, ringworm of the scalp, usually common in young children and thought to affect 200 million individuals worldwide, and nail infections that affects ~10% of the general population worldwide (Thomas *et al.,* 2010). Infact, a study in an urban slum (Mathare) in Kenya, revealed that Tinea capitis infections rates were about 81.3% in children. *Trichophyton*, *Epidermophyton* and *Microsporum* sp were shown to be the major etiological agents (Moto et al., 2015). Nevertheless, superficial infections are mostly caused by various species of genus *Candida*. This genus is ranked second as the major cause of fungal infections worldwide and the fourth most common cause of nosocomial (hospital-acquired) bloodstream infections (Wisplinghoff *et al.,* 2004). It has been shown that more than a dozen Candida species can cause disease. However, in almost all patient groups and disease manifestations, *Candida albicans* dominates in terms of incidence (Pfaller and Diekema, 2007).

In contrary, invasive fungal infections have an incidence that is much lower compared to superficial infections. However, they are of greater concern since they are associated with unacceptably high mortality rates (Brown *et al.,* 2012). Virtually not all fungi are pathogenic and their infections are majorly opportunistic (de Pauw, 2011). However, the occurrence of systemic fungal infections has shown dramatic increase during the past 2 decades. This is probably due to the immunosuppressed status caused by aggressive chemotherapy, transplantation, HIV infection, and both solid and hematologic malignancies (de Pauw, 2011). HIV/AIDS adds nearly 10 million cases of oral thrush and 2 million cases of esophageal fungal infections annually in the world regions where limited healthcare facilities are available (Lertvannavit and Suankratay, 2013).

2.2 Economic Importance of Bacteria and Fungi

The relative importance of human diseases is conventionally assessed by cause-specific mortality, morbidity, and economic impact. Drug resistance has led to a corresponding increase in mortality and morbidity from microbial diseases. This has led to the spread of superbugs and healthcare associated costs due to the need for multidrug therapy and longer and more frequent hospital stays (Ventola, 2015; Davies and Davies, 2010).

2.2.1 Economic importance of bacterial pathogens

In Kenya, diarrheagenic *E. coli* (DEC) was detected in 171 (55.9%) out of 306 children in a study by Iijima *et al.* (2017). The prevalence was notably higher than reports from developed countries due to consumption of contaminated food and water. A similar study in Europe estimated the total costs attributable to excess hospital stays for MRSA (Methicillin-resistant *Staphylococcus aureus*) and third generation cephalosporin-resistant *E. coli* (G3CREC) blood stream infections (BSIs) to be €44.0 million and €18.1 million, respectively. Based on prevailing trends, the number of BSIs caused by G3CREC is likely to rapidly increase, outnumbering the number of MRSA BSIs in the near future (De Kraker *et al.,* 2011). MRSA is another major cause of healthcare- and community-associated infections worldwide. According to a study, prevalence of health-care-associated MRSA infection (pooled prevalence in high-quality studies, 15.5 per 100 patients [95% CI 12.6–18.9]) was much higher than proportions reported from Europe and the USA whereas pooled overall health-care-associated infection density in adult intensive-care units was 47.9 per 1000 patient-days (95% CI 36.7–59.1), at least three times as high as densities reported from the USA (Allegranzi *et al.,* 2011). In the healthcare setting alone, MRSA infections are estimated to affect more than 150,000 patients annually in the European Union (EU), resulting in attributable extra in-hospital costs of €380 million for EU healthcare systems (Köck *et al.,* 2010).

Multi-drug resistant *Pseudonomas aeruginosa* (MDRPA) have also shown an increased disease burden with hospitalization cost of US$54,081 compared with a mean cost of US$22,116 for patients with susceptible *P. aeruginosa* infections (Paramythiotou *et al.,* 2004). A separate study reported an overall mortality rate of 67% in patients with MDRPA bacteremia compared with 23% in those with susceptible *P. aeruginosa* bacteremia (OR 15.1, p=0.001) (Obritsch *et al.,* 2005).

2.2.2 Economic importance of fungal pathogens

Fungal pathogens are eukaryotic hence they pose a great challenge due their close evolutionary relationship with their human hosts (Baldauf *et al.,* 2000). Fungi are renowned for causing life-threatening disease in immunocompromised individuals, but can also pose a threat also to healthy humans (Fraser *et al.,* 2005). Studies show that the frequency of fungal infections, has increased in recent years. For instance, in the United States, bloodstream infections caused by fungi increased by 207% between 1979 and 2000 (Martin *et al.,* 2003),

along with the number of individuals who have become immunocompromised owing to the treatment of malignancies, organ transplantation and autoimmune disorders (Lertvannavit and Suankratay, 2013). Most deaths attributed to fungal pathogens, are caused by *Candida albicans* and *Aspergillus fumigatus* (Pfaller and Diekema, 2007).

Fungal infections are notoriously difficult to treat leading to high mortality rates, and costs to the health-care system. In Kenya, the burden of fungal disease is high due to tuberculosis and HIV infection. According to a recent study, 7% of Kenyans suffer from significant fungal infections of which 82% are recurrent vaginitis and tinea capitis (Guto *et al.,* 2016). Similarly, in a study done in Cameroon in 2016, it was estimated that 1.2 million people were affected by a serious fungal infection. The situation was also found to be further compromised by the HIV epidermic which is a huge problem in the country of a gross domestic produce (GDP) of $1,033 per capita (Mandengue and Denning, 2018). Furthermore, the projected average incidence of fungal infections was 306 per million US population, with candidiasis accounting for 75% of cases. The estimated total direct cost was US$2.6 billion and the average per-patient attributable cost was $31,200. The most commonly reported comorbid diagnoses with fungal infections i.e. HIV/AIDS, neoplasms, transplants, accounted for only 45% of all infections (Wilson *et al.*, 2002).

Another study using a different methodology, found that invasive fungal infections attributed to a higher mortality, longer hospital stays, and higher costs associated with hospitalization (approximately $1.89 billion was the annual hospital costs in the United States) (Menzin *et al.,* 2009). Furthermore, length of stay (LOS) in the hospital has been shown to increase significantly among candidemia patients. One study estimated a mean LOS increase of 21.1 days for children and 10.1 days for adults while the mean increase in hospital charges attributable to candidemia in hospitalized adults was $40,000 (Zaoutis *et al.,* 2005). Also, a different study estimated the average major costs associated with candidemia at approximately $35,000 for Medicare patients and $45,000 for privately insured patients (Gagne *et al.,* 2006).

2.3 Antimicrobial Drug Compounds

Antimicrobial drug compounds are used in the management of microbial infections. The success of antimicrobial drugs was remarkable and generated a misconception in the late 1960s and early 1970s, that microbial diseases had been conquered. However, 40 years later, these diseases were the second-leading cause of death worldwide (WHO, 2002). This

situation has worsened due to the emergence of multidrug-resistant bacteria, with few or no treatment options for the infections caused (WHO, 2014). Furthermore, recent genetic engineering of pathogens for bioterrorism could also render them resistant to currently available antimicrobials (Wagar, 2016; Lederberg *et al.,* 2003). Therefore, a large market of antimicrobial drugs, is needed to aid in the control of these infections.

Antibiotics are chemical agents that can kill bacteria (bacteriocidal) or nullify their growth (bacteriostatic) (Hubbard and Walsh, 2003). Most antibiotics used as antibacterials, are natural products elaborated by a given species of microorganism (bacteria or fungi) as a chemical weapon, to kill off other microbes in the neighboring microenvironment for survival due to crowding (Walsh, 2000). The management of microbial infections using antimicrobials in ancient Egypt, Greece, and China are well-documented (Walsh, 2015; Sengupta *et al.*, 2013). In the past 60–70 years, most antibiotics were discovered by screening of soil samples for natural products that can kill known bacterial pathogens (Lewis, 2013). These included penicillins and cephalosporins obtained from fungi and several antibiotics from different strains of filamentous bacterium *Streptomyces*, such as streptomycin, erythromycin, tetracycline and vancomycin. Semisynthetic modifications produced second- and third generation b-lactams of both the penicillin and cephalosporin classes, whereas total synthesis created the second-generation erythromycins; clarithromycin and azithromycin. By the end of the year 1999, only the fluoroquinolones such as, ciprofloxacins were totally synthetic significant class of antibiotics (Walsh, 2000). Currently, there is global concern over antimicrobial drug resistance to these drug compounds with an estimated 700, 000 deaths yearly in the world (Tadesse *et al.,* 2017). In fact, multi-drug resistant Tuberculosis (MDR-TB) kills ten times more people annually than the last tragic Ebola epidermic (Weyer *et al.,* 2017), with no existing single-drug formulation to curb the disease.

Antifungals are fungicidal or fungistatic compounds used in treatment and prevention of mycoses. They are grouped into five groups based on their site of action: (a) azoles, which inhibit the synthesis of egosterol which is the major fungal sterol; (b) polyenes, which bind to the fungal membrane sterol thus resulting to the formation of aqueous pores through which essential cytoplasmic materials leak out; (c) allylamines, which block ergosterol biosynthesis leading to accumulation of squalene that is toxic to cells; (d) candins, which are inhibitors of fungal cellwall synthesis via inhibition of the synthesis of β-1,3-glucan the major cellwall structural polymer; and (e) flucytosine, that inhibits the synthesis of macromolecules (Balkis

et al., 2002). Recently, the increased incidences of fungal infections particularly in patients with impaired immune function has summoned the need for more effective antifungals to replace many of the existing agents, which are not very effective due to their toxic effects and exhibit high propensity to induce development of resistance by microbes (Roemer and Krysan, 2014).

2.4 Antimicrobial Drug Resistance

The modern era of the use of antibiotics started with the discovery of penicillin by Sir Alexander Fleming in 1928. Since then, antibiotics have transformed modern medicine and saved millions of lives (Gould and Bal, 2013). Antibiotics were the major class of drugs prescribed to treat serious infections in the 1940s (Chambers and DeLeo, 2009) and penicillin was successful in controlling bacterial infections among World War II soldiers (Sengupta *et al.,* 2013; Walsh, 2015).

However, despite this major breakthrough, shortly thereafter, penicillin resistance became a substantial clinical problem. By 1950s, many of the advances made in the prior decade were threatened (Spellberg and Gilbert, 2014) where, gonococcal and staphylococcal strains' resistance towards penicillin was first noted (Ventola, 2015). To overcome this, new beta-lactam antibiotics were discovered, developed, and deployed, restoring confidence (Sengupta *et al.,* 2013; Spellberg and Gilbert, 2014). However, later in 1970's MRSA emerged, where the first case was identified in the United Kingdom in 1962 and in the United States in 1968 (Walsh, 2015). Vancomycin was then introduced into clinical practice in 1972 for the treatment of methicillin resistance in both *S. aureus* and coagulase-negative staphylococci (Chambers and DeLeo, 2009). Since it had been so difficult to induce vancomycin resistance, it was believed unlikely to occur in a clinical setting. However, cases of vancomycin resistance were reported in coagulase-negative staphylococci in 1979 and 1983 (Sengupta *et al.,* 2013). The aminoglycoside-resistant *Pseudomonas aeruginosa* was then noted after widespread use of gentamicin, whereas ceftazidime-resistant and ciprofloxacin-resistant *P. aeruginosa* remained a concern (Sundar *et al.,* 2005). Furthermore, rapid increase in antibiotic resistance among respiratory pathogens has also been seen recently with resistance to macrolides, trimethoprim-sulfamethoxazole, doxycycline and second and third-generation cephalosporins being on the rise (Sundar *et al.,* 2005).

Resistance to antibiotics can either be active or passive. Active resistance is where there is a specific evolutionary pressure to adapt a counterattack mechanism against an

antibiotic or class of antibiotics (Morar and Wright, 2010; D'Costa and Wright, 2009; Wright, 2005). Passive resistance ensues as a result of general adaptive processes which are not necessarily linked to any given class of antibiotics. An important example is the non-specific barrier at the outer membrane of Gram-negative bacteria (Wright, 2005). Bacteria usually achieve active drug resistance via three major mechanisms: (a) efflux of antibiotics from the bacterial cell membrane-associated pumping proteins (Kumar and Schweizer, 2005); (b) modification of the antibiotic targets through mutation of key binding elements such as ribosomal RNA or by reprogramming of some biosynthetic pathways (D'Costa and Wright, 2009) and (c) via the synthesis of modifying enzymes that target selectively and destroy the activity of antibiotics (Morar and Wright, 2010). The pressure imparted on the bacteria to mutate and become resistant develops due to inappropriate and excessive use of antibiotics in treating infections, the widespread use of low-dose antibiotics in animal husbandry and the use of antimicrobials or antiseptic agents in agriculture and domestic purposes (Marshall and Levy, 2011).

In the recent past, dramatic increase in the incidence of fungal infections has been seen most probably as a result of the alterations in immune status associated with the AIDS epidemic, cancer chemotherapy, and organ and bone marrow transplantation (Kaplan and White, 2016). Although extremely rare some years ago, antifungal drug resistance is quickly becoming a major problem in certain populations, especially those infected with HIV, in whom drug resistance of the agent causing oropharyngeal candidiasis is a major problem. For instance, 33% of late-stage AIDS patients in one study had drug-resistant strains of *Candida albicans* in their oral cavities (Nucci and Colombo, 2002). Recently, studies have also shown antifungal drug resistance by non-albicans *Candida* species, such as fluconazole resistant *Candida parapsilosis* (Grossman *et al.*, 2015). This has exacerbated the need for the development of next generation of antifungal agents.

Several molecular mechanisms enable fungi to survive exposure to antifungal drugs. Increased efflux of a drug from a cell is a ubiquitous resistance mechanism in bacterial, cancerous and also fungal cells. Constitutive upregulation of a multidrug transporter of the major facilitator class has been shown to confer resistance to the azole fluconazole in species of *Candida* and *Aspergillus*. The constitutive upregulation of multidrug transporters of the ATP-binding cassette (ABC) family confers resistance to multiple azoles, as well as other drugs (Chamilos and Kontoyiannis, 2005). Another common mechanism by which cells acquire resistance is via the alteration or amplification of the drug target. For instance,

overexpression of erg11 has been associated with azole resistance in *Candida* and *Aspergillus* species (Chamilos and Kontoyiannis, 2005). Also, mutation of Fks1 targeted by echinocandins, is a common mechanism of resistance to echinocandins by various fungal pathogens (Perlin, 2007). The final category of resistance mechanism involves cellular alterations that minimize the toxicity of the target drug. Specific examples in this category, involve alterations in the ergosterol biosynthetic pathway associated with azole resistance (Cowen, 2008).

Therefore, despite the usefulness of most antimicrobial drugs being compromised by their severe host toxicity or diminished efficacy in killing pathogens, this situation has been worsened by antimicrobial drug resistance exhibited by nearly all the antimicrobial drug compounds that have been already developed. However, under these circumstances all is not bad news since recent advances made in the discovery of bioactive compounds from fungi could facilitate in the return to the world of susceptible microbes. Notably, the Basidiomycetes fungi contain an untapped source of such bioactive natural products with potent antimicrobial properties (Poucheret *et al.,* 2006).

2.5 Bioactivities of Basidiomycetous Tropical Fungi

Fungi contain a huge diversity of biomolecules with nutritional (Kalac, 2009) and/or medicinal properties (Poucheret *et al.,* 2006; Lindequist *et al.,* 2005; Borchers *et al.,* 2004). It has been estimated that there are about 140,000 species of mushroom-forming fungi on the earth, where only 22,000 are known and only a small percentage (5%) have been investigated. Hence, there is need to explore more about their properties and potential applications in medicine (Lindequist *et al.,* 2005). The phylum Basidiomycota contains at least 30,000 different species worldwide and includes all the agarics (mushrooms and toadstools), puffballs and stinkhorns, bracket fungi, chanterelles, club and coral fungi, as well as the plant-parasitic rusts and smuts (Roberts and Evans, 2011). Fungi comprising the phylum Basidiomycota, includes three classes; Hymenomycetes, Ustilaginomycetes, and Urediniomycetes. Hymenomycetes includes orders Agaricales, Aphyllophorales, Auriculariales, Dacrymycetales, Ceratobasidiales, and Tuslasnellales. Among Hymenomycetes, mainly the orders Agaricales, Aphyllophorales, and Auriculariales contain the mushrooms of pharmaceutical importance. The rest of the orders, that is, Dacrymycetales, Ceratobasidiales, and Tuslasnellales, contain parasitic and disease causing forms (Rahi and Malik, 2016). Ustilaginomycetes includes orders Ustilaginales and Exobasidiales which

include smut causing forms, while Urediniomycetes include orders Uredinales, Sporidiales, Septobasidiales and Eocronatirum that are responsible for causing rusts in various crop plants (Alexopoulos, 1996).

The fruiting bodies, mycelia, and spores of various Basidiomycetes tropical fungi accumulate bioactive metabolites which form a major source of medicines and nutraceuticals (Rahi and Malik, 2016). These metabolites have exhibited various pharmacological and biological activities; immunomodulatory, cardiovascular, liver protective, antifibrotic, anti-inflammatory, antidiabetic, antiviral, antioxidant, antitumor, and antimicrobial activities (Alves *et al.,* 2012; Poucheret *et al.,* 2006; Lindequist *et al.,* 2005).

2.5.1 Antimicrobial activity of Basidiomycetes

In the recent past, there has been a renewed interest in discovery of antibacterial compounds since many pathogenic bacterial species have acquired antibiotic resistance mechanisms with limited treatment options (Boucher *et al.,* 2009). Several studies also, have shown that some antibiotic drugs can lead to drug induced hepatoxicity which would be of more severity in hepatitis or HIV patients (Andrade and Tulkens, 2011). This renewed interest on the exploration of natural antimicrobial compounds from Basidiomycetes has resulted in numerous extraction and testing of the extracts with considerable positive outcomes (Alves *et al.,* 2012; Ochoa-Zarzosa *et al.,* 2011; Ramesh and Pattar, 2010).

Basidiomycota are a rich source of natural antimicrobials. For instance, many extracted secondary metabolites of the mycelia have been known to combat bacteria (Deyrup *et al.,* 2007) and fungi (Gilardoni *et al.,* 2007). Several studies on the antimicrobial potential of *Ganoderma* species are well documented. According to a study, the antibacterial potentials of *Ganoderma lucidum*, *G. praelongum* and *G. resinaceum* were evaluated against 30 strains of clinical isolates of methicillin-resistant and methicillin-sensitive *Staphylococcus aureus*, where ethyl acetate extract of *G. praelongum* exhibited the maximum activity (35.67± 0.62 µm) and minimum inhibitory concentration (MIC) of 0.390–6.25 mg/mL (Ameri *et al.,* 2011). In addition, *Ganoderma orbiforme* BCC 22324, have also exhibited significant antimycobacterial activity with MIC of 1.3 µg/ml (Isaka *et al.,* 2013). According to a study, Hymenochaetaceae family genera such as *Inonotus* (*Inonotus obliquus* and *Inonotus dryadeus*) and *Phellinus* possess antimicrobial properties (Alves *et al.,* 2012).

In fungal infections, *Laetiporus sulphureus* ethanolic extract has been demonstrated to be a promising target against *Candida albicans* (IZD = 21 ± 1mm). It showed higher activity against the fungus compared to the positive control, nystatin (IZD=19 mm) (Turkoglu *et al.,* 2007). Furthermore, the fruit bodies of *Polyporus alveolaris* have been shown to contain various bioactive components with antifungal properties (Wang *et al.,* 2004).

2.6 Bioactive Secondary Metabolites Isolated from Tropical Fungi

Medicinal mushrooms have shown therapeutic benefits, since they consist of a number of biologically active compounds (Lee and Hong, 2011; Petrova *et al.,* 2008; Chen and Seviour 2007). Bioactive compounds consist of the high molecular weight (HMW) compounds polysaccharides, proteins and lipids as well as a variety of complex low molecular weight (LMW) metabolites. The LMW compounds include the terpenoids, alkaloids, polyketides and metabolites derived from non-ribosomal peptide synthesis (NRPS) (Gallo *et al.,* 2013; Lau *et al.,* 2012; Erkel and Anke, 2008). Although this research study focused mainly on LMW bioactive metabolites, there are a variety of novel HMW compounds that have also shown promising bioactivities in Basidiomycetes (Silva *et al.,* 2012).

Secondary metabolites are intermediates of primary metabolism and are classified according to five main metabolic pathways; (1) amino acid-derived pathways, including NRPS, (2) the shikimic acid pathway, giving rise to aromatic compounds, (3) the acetate–malonate pathway, that leads to formation of olyacetylenes or polyketides, (4) the mevalonic acid pathway, resulting in the biosynthesis of terpenoids and (5) the polysaccharides and peptidopolysaccharides pathways (Silva *et al.,* 2012; De Silva *et al.,* 2012; Erkel and Anke, 2008).

2.6.1 Antimicrobial secondary metabolites isolated from Basidiomycota

Basidiomycetes' secondary metabolites are essential for their survival in their natural habitats. According to a study, an antimicrobial compound biformin, isolated from a polypore is a polyacetylenic carbinol active against a wide variety of bacteria and fungi (Zjawiony, 2004). Similarly, Applanoxidic acid A isolated from *Ganoderma annulare* showed some antifungal activity against *Trichophyton mentagrophytes* (Smania *et al.,* 2003). Besides, steroids isolated from *Ganoderma applanatum* also proved to be active against a number of Gram-positive and Gram-negative bacteria (Lindequist *et al.,* 2005). Furthermore, ganomycin

A and ganomycin B, isolated from *G. pfeifferi* have shown moderate growth inhibition of several bacterial strains, particularly Gram-positive strains such as *B. subtilis*, *S. aureus*, and *Micrococcus flavus* (Mothana *et al.,* 2000). *Ganoderma applanatum*, provides various sterols and a novel lanostanoid that tested active against Gram-positive bacteria. In addition, Chepkirui *et al.* (2016) characterized antifungal compounds (calocerins) from *Favolaschia calocera* fungus.

Nevertheless, available literature clearly indicates that most research activities have mainly focused on the screening of antimicrobial properties of fungal crude extracts. However, there is still a gap in the identification of the individual compounds responsible for these properties since only a few low-molecular weight compounds including some peptides and proteins have been described (De Silva *et al.,* 2013). Thus, this calls for further exploration of the fungal kingdom for novel bioactive compounds.

2.7 Methods of Fungal Strain Identification

The correct taxonomic identification of fungi is a significant first line step owing to their probable application in pharmaceutical drugs development. The combined use of morphological data with molecular markers such as β-tubulin and internal transcribed spacer (ITS) regions has proven to be successful as illustrated by some studies (Shenoy *et al.,* 2007), as compared to the use of a single approach.

2.7.1 Traditional methods for fungal identification

Fungal identification is essential in their basic (taxonomy, ecology) and applied (bioprospecting, genomics) applications in scientific research. The use of traditional methods to assess fungal diversity relies solely on culture-growth properties and morphological microscopic identification. The use of morphological characters is important in taxonomic studies of fungi at the familial and ordinal level (Toledo *et al.,* 2013). However, most Basidiomycetous fungi obtained from decaying plant material usually consists of vegetative (non-reproducing) hyphae that cannot be identified using conventional microscopy. In addition, in traditional microscopic methods, the fungi are usually examined for the presence of sporulating structures or conidia, which might be absent due to presence of a non-sporulating mycelium (Nikolcheva *et al.,* 2003). Hence due to these obvious shortcomings, fungal detection by molecular approaches come in handy.

2.7.2 Molecular methods for fungal identification

The molecular identification techniques employ the characterization of nucleic acids, which are present in all stages of the fungal life cycle. These methods have proved useful in circumventing the problems associated with the microscopy- and culture-based techniques in fungal identification. These methods are faster, reliable and not influenced by environmental conditions. In addition, phenotypically similar species can be easily distinguished while viable non-culturable fungi can also be detected (Shenoy *et al.,* 2007). Hence, in this study current study molecular techniques employed involved the amplification of ITS (Internal Transcribed Spacer Region) rDNA using ITS1-F and ITS4 primers which are specific for Basidiomycetes fungi (Gardes and Bruns, 1993).

Internal Transcribed Spacer (ITS) region refers to the spacer DNA situated between the ribosomal RNA (rRNA) large sub-unit and small sub-unit genes on the fungal chromosome, or its corresponding transcribed region on the polycistronic rRNA precursor transcript. This DNA barcoding maker is known to evolve very fast, hence it is useful for lower level (species) phylogeny determination. ITS1 and ITS2 are the two ITS regions in eukaryotes. ITS1, located between 18S and 5.8S rRNA genes is amplified by ITS1 and ITS4 primers whereas, ITS2 located between 5.8S and 26S rRNA genes can be amplified using plant-excluding primers ITS1-F and ITS4-B specific for fungi and Basidiomycetes respectively (White *et al.,* 1990).

2.8 Characterization of Secondary Metabolites

The first task following the isolation of a compound from nature is its identification. There are a number of analytical techniques that can be employed. This involve the analysis of physical properties (optical rotation, physical appearance, odor, taste, boiling point and melting point), as compared to a known compound (Braude and Nachod, 2013). However, such elemental analysis methods are relatively crude in determining the identity of unknown compounds.

Among the many techniques, mass spectroscopy (MS) provides a means of measuring the molecular mass and chemical formula of a compound (Courant *et al.,* 2014). Infrared spectroscopy (IR), which measures the absorption of infrared radiations by organic compounds provides information on the functional groups present in a compound (Alpert *et al.,* 2012). However, MS and IR are not sufficient in structure elucidation since they cannot be applied in obtaining structures of novel compounds (Sarker and Nahor, 2012).

Nuclear magnetic resonance (NMR) spectroscopy applies the principle of magnetic spin and nuclei alignment when atoms are placed inside a large magnetic field. The technique analyses moleculescontaining NMR-active nuclei (Diehl, 2008). The power of NMR in structure elucidation cannot be matched by any other method. NMR determines both the basic skeleton and the stereochemistry of a compound, hence giving its comprehensive structural information. Furthermore, NMR is non-destructive and the interpretation of the 1D (one-dimensional) and 2D (two-dimensional) NMR spectra using computational tools aid in successful elucidation of a complete structure of a known or novel/unknown molecule (Holzgrabe, 2017). Therefore, NMR spectroscopy remains to be an indispensable tool in structure elucidation of organic molecules (Silverstein *et al.,* 2014).

2.9 Evaluation of the Toxic Effects of Secondary Metabolites

The recent research findings reveal that extracts from fungi (mushrooms), contain mycotoxins that may be genotoxic, cytotoxic, mutagenic and even carcinogenic (Eisold and Mostrom, 2011). Such adverse effects are usually as a result of interaction between the toxicants and cells (Barb and Neamtu, 2012). Therefore, this necessitates further investigation on the safety of preparations obtained from natural products by toxicological screening (VanderMolen *et al.,* 2017; Cos *et al.,* 2006).

Various *in-vitro* and *in-vivo* methods of metabolite toxicity determination exist (Schuhendler *et al.,* 2014; Astashkina *et al.,* 2012). Cancer cell lines are frequently used in toxicological studies (Wilding and Bodmer, 2014). This is because the drug-induced cytotoxic mechanisms have been shown to occur mostly at the cellular level. Such adverse effects result from disruption of intracellular calcium homeostasis, activation of apoptosis, oxidative stress, inhibition of mitochondrial functions, inhibition of enzyme actions and formation of reactive metabolites (Stephens *et al.,* 2014; Jaeschke *et al.,* 2012).

CHAPTER THREE
MATERIALS AND METHODS

3.1 Sampling Site

The fungal samples were collected from Mt. Elgon national park forest: a tropical rainforest in western Kenya located at 1.1635° N, 34.5930° E. Sampling was undertaken randomly within the forest at sites with little human interference or modification. The fruiting bodies/mushrooms were collected from dead logs and woods: this was achieved by scouting for newly formed mushrooms on decaying tree trunks or logs on the forest floor. Samples were photographed, then carefully dug out and placed in sterile sample khaki bags in triplicates. The sampled mushrooms were thereafter placed in a cool sample box and transported to Egerton University biotechnology laboratory within 24 hours.

3.2 Preparation of Pure Fungal Cultures

For each of the mushroom collected, two pieces of the inner tissues of the pileus was cut out using a sterile surgical blade and a piece stuck on Yeast-Malt-Medium (YMG) media containing, glucose (4 g/l), yeast extract (4 g/l), malt extract (10 g/l), agar (20 g/l), streptomycin sulphate (1.5 mg/ml) and pH 6.3, with gills facing the media. The growth of the fungal hyphae on the media was propagated at room temperature in the dark. Monitoring followed by sub-culturing into fresh antibiotic-free YMG media, was carried out to establish pure cultures. The petri-plates inoculated with hyphae of pure cultures were subsequently incubated at 24^{o} C in a dark room until full-plate growth was achieved.

3.3 Morphological Characterization

The forest collected samples and the pure cultures prepared were further examined to ascertain the strains collected using morphological characteristics in the laboratory. This was achieved using both macroscopic and microscopic fungal characters. Macroscopic characters included the observation of the shapes and color of the pileus and the stipe, the manner of attachment of the gills to the top of the stalk, as well as the observation of cultural characteristics of pure cultures isolated on YMG media, such as growth rate, hyphal branching and mycelial color. Microscopic features observed were color, shape, ornamentation and size of the spores and the pore type. This was achieved using a stereomicroscope by placing a thin dissected gill from the basidium flat on the slide. It was afterwards mounted using water and a cover slip and observed using 40× objective lens. The

results obtained were in addition compared with mycology handbook (Piepenbring, 2015), with the assistance of a mycologist to identify the fungi phenotypically.

3.4 Molecular Characterization

Various molecular biology techniques were considered for the isolation of genomic DNA from the fungal mycelial samples. The steps involved the extraction, amplification, purification, sequencing and analysis of the rDNA ITS consensus sequences.

3.4.1 DNA extraction

Total genomic DNA was extracted from the fungal mycelia using EZ-10 Spin Column Fungal Genomic DNA Mini-Preps kit (BIO BASIC INC.), following manufacturer's instructions. The digestion buffer (180 µl) and Proteinase K (20 µl) were placed in sterile 1.5 ml screw-cap microfuge tubes, before addition of fungal mycelia (~60 mg) for each strain. Mycelia were obtained from actively growing pure fungal isolates on petri-plates and placed in the microfuge tubes using sterile forceps. Thereafter, 6-10 (1.4 mm) Precellys ceramic beads were added into the microfuge tubes and the mycelia homogenized using PeQlab Precellys 24 homogenizer under program 5 (6.0 m/s for 2×40 secs).

The homogenized samples were centrifuged at 10,000 rpm for 30 secs using an Eppendorf Centrifuge 5430R at 24° C to remove the foam produced during homogenization. The samples were then incubated at 56° C for 30 min in Kleinfeld Labortechnik MBT 250 incubator. Universal PF buffer (100µl) was then added to the samples and vortexed for 5 secs, followed by incubation at -20° C for 5 min. The samples were then centrifuged at 14,000 rpm for 5 min to form a clear sample lysate. Prior to DNA isolation from the lysate, the EZ-10 spin columns binding conditions were adjusted using 200µl Universal BD binding buffer and 200 µl 99% ethanol. Clear lysate from the samples were then pipetted into the adjusted EZ- spin columns and centrifuged for 1 min at 12,000 rpm. Washing steps then followed, where 500 µl Universal PW solution was added into the spin columns and centrifuged at 12,000 rpm for 1min, followed by addition of 500 µl Universal Wash solution and centrifugation at 12,000 rpm for 1 min. The washing step was done twice and the flow through after each centrifugation step was discarded. The EZ-10 columns were then centrifuged twice at 12,000 rpm for 2 min to remove left over washing buffer in the spin columns and for drying. To elute the DNA from the EZ-10 columns, 50 µl warm (60° C) TE buffer (10 mM Tris-HCL, 0.5 mM EDTA, pH 9.0) was added at the center of each column membrane and incubated for 1 min at room temperature. The DNA from the column was then

eluted into sterile 1.5 ml microfuge tube via centrifugation at 12,000 rpm for 1 min. All the centrifugation steps were performed at room temperature and the eluted DNA was stored at -4° C.

3.4.2 PCR-amplification of fungal internal transcribed spacer (ITS) region

The ITS spacer rDNA sequences were targeted for the identification of pure fungi cultures prepared from the forest collected samples. The targeted ITS1 region is located between 18S and 5.8S whereas, ITS2 is located between 5.8S and 26S of rRNA genes (Fig. 1). Amplification of the ITS region was conducted using a 25 µl PCR reaction mix consisting of 2 µl fungi genomic DNA and 23 µl PCR master mix (12.5 µl JumpStart Taq Ready Mix, 9.5 µl water, 0.5 µl 10pmol ITS1-F forward primer (5^1-CTTGGTCATTTAGAGGAAGTAA-3^1) and 0.5 µl 10 pmol ITS4 (5^1-TCCTCCGCTTATTGATATGC-3^1) reverse primer. The PCR was conducted using Eppendorf Mastercycler under the following program cycle; initial denaturation at 94° C for 5 min was done, followed by 34 cycles of denaturation, annealing and elongation for 2 minutes, at 94° C, 52° C and 72° C respectively. A final elongation at 72° C for 10 min was then performed. The negative control reaction tubes consisted of all the PCR components except fungal genomic DNA. To confirm successful PCR amplification and the quality of ITS rDNA amplicon, 3 µl aliquots of PCR products were mixed with 2µl Midori green loading dye and resolved on 0.8% agarose gel. Gel electrophoresis was conducted at 100 volts for 30 min in 1×TAE buffer and viewed under non-hazardous Nippon Genetics White light LED trans-illuminator.

The amplified ITS fragments were then purified from the remainder 22 µl PCR products, using EZ-10 Spin Column PCR product purification kit (BIO BASIC INC.) This was achieved by adding 110 µl of Binding Buffer I into the 22 µl PCR product, vortexed for 1 sec, pipetted into an EZ-10 Spin Column, incubated for 2 minutes at room temperature and then centrifuged at 10,000 rpm for 30 sec. The flow through was then discarded and the ITS fragments in the column washed with 500 µl Universal Wash solution. This washing step was done twice followed by centrifugation at 10,000 rpm for 30 sec after each washing, and finally drying by spinning the columns at 10,000 rpm for 1 min. To elute the ITS DNA amplicons, 20 µl of warm (60° C) elution buffer was pippeted at the center of the column membrane, followed by incubation for 2 min at room temperature. Transfer of the ITS amplicons into 1.5 ml microfuge tubes was done by centrifugation at 1000 rpm for 30 sec.

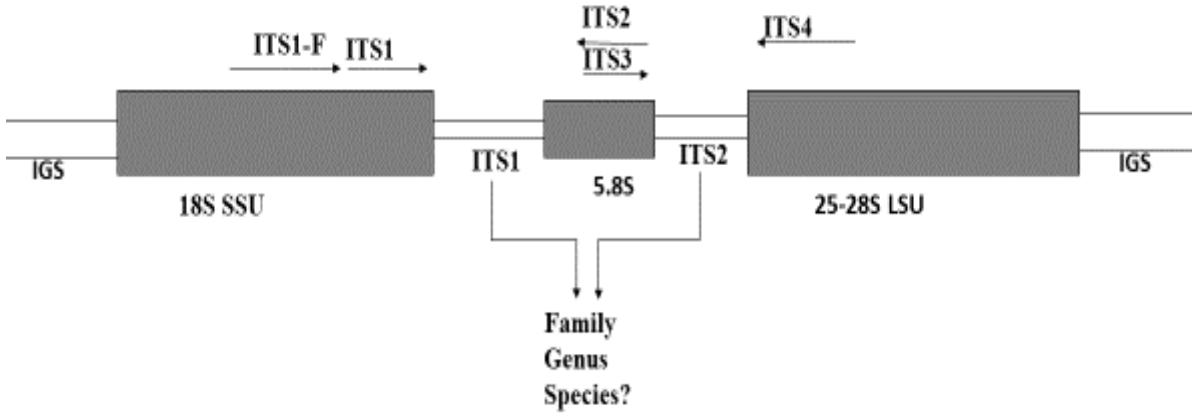


Figure 1: Location of the amplified ITS1 and ITS2 sequences on the fungal chromosome. Amplification primers for the region included; ITS1-F; (CTTGGTCATTTAGAGGAAGTAA), ITS1; (TCCGTAGGTGAACCTGCGG), ITS2; (GCTGCGTTCTTCATCGATGC), ITS3; (GCATCGATGAAGAACGCAGC), ITS4; (TCCTCCGCTTATTGATATGC) and ITS5; (GGAAGTAAAAGTCGTAACAAGG). The sequence direction is from 5^1 to 3^1. The arrows indicate the direction of amplification. 1TS1 is located between 18S SSU-Small-Subunit and 5.8rRNA coding sequences whereas ITS2 is located between 25S LSU- Large-Subunit and 5.8S rRNA coding sequence. The primers used in the amplification of the regions were ITS1-F and ITS4.

3.4.3 Sequencing of ITS rDNA PCR amplicons

The cleaned PCR- amplified ITS rDNA fragments (18-30 ng in 12 µl TE elution buffer) were submitted for sequencing using ITS4 reverse primer (5^1-TCCTCCGCTTATTGATATGC-3^1) based on White *et al.* (1990) and ITS1-F forward primer (5^1-CTTGGTCATTTAGAGGAAGTAA-3^1) based on studies by Gardes, and Bruns (1993). Sequencing was conducted using Applied Biosystems 3730xl DNA Analyzer at Helmholtz Centre for Infection Research, Braunshweig, Germany. The sequence reads obtained were then assembled using Geneious R7 software.

3.4.4 Phylogenetic analysis based on amplified ITS regions

The assembled sequences for each PCR-amplified ITS fragment were subjected to nucleotide BLAST (Basic Local Alignment Search Tool) search on the National Centre for Biotechnology Information (NCBI) database (https://blast.ncbi.nlm.nih.gov/genbank/) to determine the identity of the fungal strains. The BLASTN return sequences that matched the

ITS query, with a coverage of ≥80% and sequence similarity of ≥97-100% were considered a minimum threshold for species identification. Multiple sequence alignment of the ITS sequences was then done using Clustal W version 2.0 (Larkin *et al.*, 2007). The sequences were then edited using Jalview version 2 (Waterhouse *et al.*, 2009) and a phylogenetic tree based on neighbor joining method with 1000 bootstrap replications, constructed using Geneious Tree Builder plugin implemented in Geneious software version 11.0.4 (Kearse *et al.*, 2012). The generated trees were then visualized with iTOL (Interactive Tree of Life) version 3.6.1 online tool (http://itol.embl.de/) (Letunic and Bork, 2016).

3.5 Media Preparation, Fermentation and Antimicrobial Assays

For the cultivation of the fungi strains, both solid and liquid state fermentation media were used. Three types of liquid media namely, YMG (4 g/l glucose, 4 g/l yeast extract, 10 g/l malt extract and pH 6.3), ZM/2 (Sugar-Malt-Medium) (5 g/l molasses, 5 g/l oatmeal, 4 g/l saccharose, 4 g/l mannitol, 1.5 g/l glucose, 1.5 g/l calcium carbonate, 0.5 g Edamin, 0.5 g ammonium sulphate and pH 7.2) and Q6/2 (Cotton Seed Flour-Medium) (2.5 g/l glucose, 10 g/l glycerin, 5 g cotton seed flour and pH 7.2) were used. The liquid media was autoclaved at 121 ºC and 15 pa for 30 minutes. For solid state fermentation, rice media was prepared by weighing 90 g of rice into 500 ml Erlenmeyer flasks containing in 90ml of distilled water per flask and autoclaved twice at 120 ºC for 40 minute. In the culturing of bacterial and fungal isolates, EBS (0.5% casein peptone, 0.5% glucose, 0.1% meat extract, 0.1% yeast extract, 50 mM (11.9g/l) HEPES, pH 7.0) and YMG (glucose 4 g/l, yeast extract 4 g/l, malt extract 10 g/l and pH 6.3) liquid media was used respectively. The pH of media prepared was adjusted using 10% KOH (potassium hydroxide) and/or 10% acetic acid.

3.5.1 Pre-screening

The purified fungi strains were pre-screened for antagonistic activity against microbial pathogens, following the procedure laid out by Clinical and Laboratory Standards Institute (CLSI) with slight modifications (CLSI, 2018). EBS and YMG media each supplemented with 20 g/l agar were prepared and inoculated with known concentrations of overnight grown cultures of microbial test pathogens using the pour plate technique.

Prior to inoculation, the tests microbial cells were counted in a Bürker Neubauer chamber of 0.100 mm depth and 0.0025 mm^2 area, under a light microscope. The chamber was cleaned with 70% ethanol, loaded with 10 µl of the sample and the cells counted. The microbial cell concentrations were calculated and a working microbial cell concentration of

10^5 CFUs/ml used for inoculation of 200 ml EBS and 300 ml YMG warm media with test microbial pathogens *B. subtilis* strain DSM10 and *M. plumbeus* strain MUCL 49355, respectively. The media was then allowed to cool and solidify on petriplates. Fungal plugs (7 mm) were then cut out from fully grown pure cultures using a cork borer. A maximum of four fungal plugs were placed 4 cm apart per plate facing upside down on the media containing inoculated microbial pathogens and labeled. Ciprofloxacin and nystatin antibiotic discs (6 mm) were placed at the center as positive controls for *B. subtilis* and *M. plumbeus* plates, respectively. These plates were then incubated at room temperature and checked for inhibition after 24 hrs for *B. subtilis* plates and 48 hrs for *M. plumbeus* plates. The inhibition zones were then measured with a ruler in mm and images taken.

3.5.2 Small-scale fermentation of fungal isolates

The 12 purified fungi strains were cultivated in various media for secondary metabolite production. Liquid state fermentations were performed based on previous studies by Stadler *et al.* (2003) using YMG, ZM/2 and Q6/2 media. Five mycelial fungal plugs were cut using 7 mm cork borer from a fully grown fungal petri-plates and transferred into 500 ml Erlenmeyer flasks containing 200 ml of media. These were then propagated in a rotary shaker at 23.8° C and 140 rpm in the dark. Glucose exhaustion was tested using sterile glucose strips every two days and the fungal metabolites harvested 3 days after glucose depletion.

3.5.3 Large-scale fermentation of fungal isolates

Besides liquid state fermentation, *Fomitiporia* sp fungal strain was further subjected to large-scale solid state fermentation according to the method by Ye *et al.* (2006) with slight modification, for the production of metabolites. Five 7 mm mycelial plugs were placed into each of the 21, 500 ml Erlenmeyer flasks containing 90 g sterile rice media. The fungal cultures were then incubated in the dark at 17 ± 2^0 C for 28 days after which the fungal metabolites were extracted. The species was also subjected to large-scale liquid fermentation, where five, 7mm hyphal plugs were inoculated into 10, 1000 ml Erlenmeyer flasks consisting of 500 ml YMG media. The cultures were then propagated at 23.8^0 C on a shaker (140 rpm) for 31 days after which fermentation was aborted and metabolites extracted.

3.5.4 Extraction of fungal crude extracts

For liquid state fermented fungi strains, the mycelia and supernatant portions were separated by filtration using Whatman® Grade 1 filter paper for small-scale fermentation.

Vacuum filtration was used for large-scale fermentation. The pH of the supernatant was determined and the mycelia weighed. The supernatant portion from small-scale fermentation was extracted with an equal volume of ethyl acetate and filtered through anhydrous sodium sulphate. The supernatant ethyl acetate extract was then dried using a rotary evaporator (Heidolph Hei-VAP). Adsorbent resin (Amberlite XAD^{TM} -16N), was used to extract the supernatant portion obtained from the large-scale fermented strains where, 100 g of the resin was added to 5 L of the supernatant, then incubated overnight and the resins eluted 4 times with 500 ml acetone. Acetone solvent was evaporated and the aqueous phase partitioned with equal volume of ethyl acetate, followed by filtration through anhydrous sodium sulphate and drying to obtain a solid extract. The mycelial portion was extracted using 4 × 500 ml acetone in an ultrasonic bath for 30 min at 40° C. Subsequently, it was filtered, and the residue discarded. The solvent was then evaporated to yield the aqueous phase, which was partitioned with ethyl acetate in a ratio of 1:1 and evaporated to yield the ethyl acetate mycelial extract. The mycelial and the supernatant ethyl acetate extracts were then dissolved using methanol and transferred into vials which were subsequently dried in nitrogen damper and the weights of the extracts determined.

The fungal strains fermented on solid rice media were extracted after 28 days of culture. This was achieved by addition of 150 ml methanol to each of the 21, 500 ml Erlenmeyer flasks and allowed to stand overnight. The cultures were then diced into smaller pieces with a spatula, followed by repeated extraction in an ultrasonic bath at 40° C for 30 min and filtration until an exhausted residue was yielded. The residue was then discarded and the filtrate concentrated in a rotary evaporator to remove the methanol solvent. An equal volume of water was added to the resulting aqueous phase which was then partitioned with an equal amount of ethyl acetate in a separating funnel. The aqueous phase was discarded and the organic phase filtered after drying with anhydrous sodium sulphate. The ethyl acetate solvent was removed with a rotary evaporator and the weight of the crude extract determined.

3.5.5 Purification of the fungal crude extracts

200 µl of 1 mg/ml methanol fungal crude extracts were prepared and analyzed in an HPLC-MS (Agilent Technologies (Waldbronn, Germany) HP 1100 coupled to MS (Micromass, Manchester, UK) to establish the purity and the mass of metabolites present in the crude fungal extracts. The data from the HPLC-MS was interpreted using Compass Data

Analysis version 4.2 software. The molecular masses of the metabolites present were compared to those in the DNP (dictionary of natural products) to ascertain their identity.

Reverse phase preparative HPLC using Gilson HPLC (PLC 2020, Gilson, Middleton, USA) was then used in the purification of the fungal extracts. The stationary phase consisted of silica gel-packed VP Nucleodur 100-5C 18 ec column (250 × 40 mm, 7 µm: Macherey-Nagel). The chromatogram obtained from the crude extract analysis on Agilent HPLC-MS was used to determine the gradient for the preparative HPLC run. The mobile phase composition used for the gradients was acetonitrile + 0.05% (Tetrafluoroacetic acid) TFA (solvent B) and deionized water (Milli-Q, Millipore, Schwalbach, Germany) + 0.05% TFA (solvent A). The reverse phase preparative HPLC was run under Trilution ® LC software version 2.1 and the UV detection carried out at 210, 254 and 350 nm wavelengths. The fractions obtained were evaporated in a rotary evaporator and the purity of the fractions at a concentration of 1 mg/ml ascertained in the HPLC-MS. The pure compounds were then stored at -4° C for NMR spectroscopic analysis and bio-assays.

3.5.6 Determination of the minimum inhibitory concentration (MIC)

The MIC for the crude extracts and pure compounds was determined using the serial dilution assay method as described by Halecker *et al.* (2014) and Okanya *et al.* (2011). The test microbial pathogens used for the assay were fungal *M. plumbeus* (filamentous fungi) MUCL 49355 and *C. tenuis* (yeast) MUCL 29982. The bacterial strains used were *B. subtilis* (Gram-positive) DSM10 and *E. coli* (Gram-negative) DSM498. The serial dilution assays were conducted using 96-well microtiter plates and liquid EBS and YMG media for bacterial and fungal test organisms, respectively.

The population of overnight-grown 5 ml cultures of pathogenic microorganisms was determined using the Bürker Neubauer counting chamber method and a working microbial cell concentration of 10^5 CFUs/ml prepared. Methanol was then used to dissolve the pure compounds and crude extracts to a starting concentration of 1.5 mg/ml and 4.5 mg/ml, respectively. A multichannel pipette was then used to pipette 150 µl aliquots of microbial pathogen-media mixture into all wells of the 96-well microtiter plates. An additional 130 µl pathogen-media mixture was added to the first row (A1-A12) of the assay plate. Then, 20 µl of the test samples (crude extracts and pure compounds) dissolved in methanol were loaded to the wells of the first rows in separate plates, with the last two wells loaded with 20 µl of the negative (methanol) and positive controls. The positive controls used were 1mg/ml

nystatin for fungal test organisms and 1mg/ml ciprofloxacin for bacterial pathogens. The contents of the first row (A) were then mixed using a multichannel pipette and 150 µl transferred to the adjacent row (B). This 1:1 serial dilution was performed in subsequent rows (C-H) and 150 µl discarded after the last row (H). The 96 well microtiter plate was then incubated at 30° C in a microplate-vibrating shaker (Heidolph Titramax 1000) at 450 rpm. The plates were checked for inhibition after 24 hours for bacteria and 48 hours for fungal test organisms. The lowest concentration of fungal pure and crude extracts that inhibited the growth of the test microorganisms was recorded as the MIC.

3.6 NMR Spectroscopy

NMR Spectroscopy was used to characterize the secondary metabolites. Deuterated solvents (500 µl) were used to dissolve 1-2 mg of purified compounds, which were then placed in an NMR tube and analyzed with Brucker Ascend 700 MHz and Bruker Advance 500 MHz NMR spectrometers. The NMR spectra obtained were ^{1}H, ^{13}C, COSY, ROESY, DEPT, HSQC and HMBC spectra. ^{13}C (Carbon-13) NMR revealed the number of carbon signals present in a compound whereas, DEPT (Distortionless Enhancement of Polarization Transfer) identified the methyl (CH_3), methylene (CH_2) and methine (CH) carbons. ^{1}H NMR spectra showed the number of protons, their splitting patterns and chemical shifts whereas, ^{1}H-^{13}C HSQC (Heteronuclear Single Quantum Coherence) spectra, indicated the protons attached onto adjacent carbons. COSY (Correlation spectroscopy) spectra, identified the spin-spin coupling interactions in the ^{1}H-^{1}H correlations whereas, ROESY (Rotating-frame Overhauser Effect spectroscopy) revealed correlation of protons close to each other in space even if not bonded. The proton-carbon connectivity between protons and carbons 2 and/or 3 bonds away, was determined on the ^{1}H-^{13}C HMBC (Heteronuclear Multiple Bond Correlation) spectra. The structures for various fungal compounds were then solved using ACD/Spectrus processor 2017.1 structure elucidation software. HR-EI-MS mass spectra of the compounds obtained, were then recorded with Agilent 1200 series HPLC-UV system.

3.7 Cytotoxicity Assays

In vitro cytotoxicity (IC50) of pure compounds was determined against a panel of mammalian cell lines. These were; mouse fibroblast (L929), HeLa (KB3.1), epidermoid carcinoma cells A431, breast cancer cells MCF-7, prostate cancer cells PC-3, ovarian cancer cells (SKOV-3) and adenocarcinomic human alveolar basal epithelial cells A549. The cell lines L929, KB3.1 and A549 were grown in DMEM (Gibco), MCF-7 and A431 were

cultured in RPMI (Lonza), and SKOV-3 and PC-3 were cultured in F12-K (Gibco) media. All these cultures were supplemented with 10% fetal bovine serum (Gibco) and incubated under 10% CO_2 at 37° C for 24 hours for the cells to attain confluence. They were then harvested by trypsinization, split and pooled into 50 ml vials.

The cells were then diluted to 50,000 cells/ml and 120 µl of the suspension (6,000 cells/well) transferred into a 96-well cell culture plate. A dilution plate was then prepared and 100 µl media + 50 µl of test compound (1mg/ml) added to column 1. 100 µl of media only was placed in the remaining columns 2-12. A threefold serial dilution was then performed by transferring 50 µl from column 1 to 12. The experiment was done in duplicates.

60 µl was then taken from the dilution plate and added to cell culture plate to make up 180 µl (cell suspension + diluted compound) per well. Hence, the cells were exposed to decreasing concentrations of the fungal compounds ranging from 111.111 µg/ml-0.00067 µg/ml. The cytotoxicity tests were then performed in accordance with the MTT (3-(4, 5-dimethylthiazol-2- yl)-2, 5 diphenyltetrazolium bromide) method (Mosmann, 1983) using 96-well microtitre plates with slight modification. 20 µl of MTT solution (5mg/ml) was first added to every well in the cell culture plate after 5 days of incubation. These were thereafter incubated at 37° C for 2-4 hours. The plates were centrifuged at 3,000 rpm for 5 minutes and the supernatant discarded. 100 µl of PBS was then added and the mixture centrifuged. The supernatant was discarded and 100 µl isopropanol added stabilize the MTT crystals. The plates were finally placed on a rotary shaker (450-700 rpm) for 2 minutes after which absorbance was recorded at 595 nm using a Victor ELISA plate reader. The percentage growth inhibition at each concentration was obtained from Ms Excel, 2003 using the formula; % Growth Inhibition = 100 – (Mean OD of Individual Test Group/ Mean OD of Control Group) X 100 (Patel and Patel, 2011).

The concentration at which the growth of 50% of the cells was inhibited (IC_{50}) compared to the control was obtained from the dose response curves generated by the graphic program. The compounds were considered cytotoxic if their IC_{50} < 20 µg/ml, and non-cytotoxic if otherwise (Geran *et al.,* 1972), according to the guidelines set by the National Cancer Institute (NCI). The positive control used was Epothilone B (Sigma-Aldrich) and the negative control was methanol.

CHAPTER FOUR
RESULTS

4.1 Morphological Characteristics of Fungi

The Basiodiomycetes fungi samples collected from Mt Elgon forest had fruiting bodies with varied shapes, sizes and colors (Fig. 2A-D). Most of the fungi samples collected had either brown or white sporocarps (Fig. 2 A, C & D), however one of the most notable sample collected, namely strain KE/16-176 had a distinct maroon sporocarp (Fig. 2B).

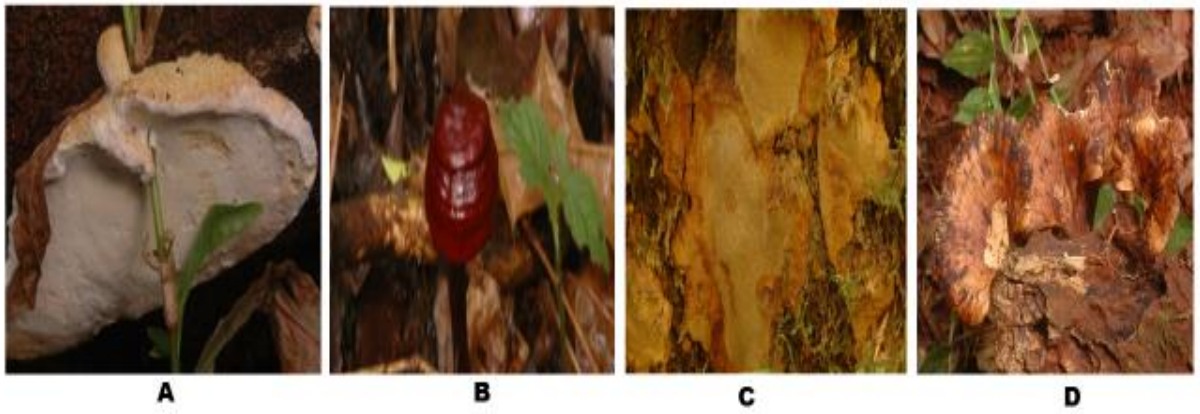


Figure 2. Morphological characteristics of Basiodiomycetes fruiting bodies collected from dead wood in Mt Elgon forest: Initial field identification and phenotypic characterization was based on sporocarp shape and colour following field manual by Piepenbring (2015). (**A)** *Tyromyces* sp; **(B)** *Ganoderma* sp; **(C)** *Fomitiporia aethiopica* and **(D)** *Echinonaete brachypora*

Based on their morphological characteristics, the 14 collected samples were categorized into various species (Table 1). Similarities and differences were observed both in the morphological and microscopic characteristics with regards to the sporocarp colour, shapes of the spores isolated and pores on the sporocarps (Table 1).

Table 1: Morphological characteristics of the Basidiomycetes fungi strains collected from Mt Elgon forest.

Fungal Strain Code	Putative Species	Phenotypic Characteristics
KE/16-152	*Favolaschia* sp	Contains very large prominent pores, cream spores and a brown basidiocarp,
KE/16-101	*Inonotus* sp	The fruiting body is brown, pileate with white basidiospores
KE/16-103	*Perenniporia* sp	Has a bracket-shaped brown cap, round pores and white hyaline basidiospores
KE/16-163	*Fomitiporia aethiopica*	Has yellow thick cap, and pores that produce cream spores
KE/16-165	*Antrodia* sp	Consists of a resupinate white sporocarp, exposed hymenium with round pores that produce white basidiospores
KE/16-176	*Ganoderma* sp	The fruiting body is maroon and bracket-shaped with white basidiospores
KE/16-193	*Polyporus* sp	The basidiome is cream-colored with a ciliate margin, pores are hexagonal and the spores are cylindrical and white in color
KE/16-138	*Hexagonia* sp	Has a brown sporocarp, hexagonal pores, and cream basidiospores. The hyphae are yellowish-brown in color
KE/16-189, KE/16-115 and KE/16-117	*Tyromyces* sp	The basidiome is pileate and brown in color whereas, the pore is white with yellow basidiospores
KE/16-153	*Phellinus* sp	Consists of a brown cap and hyphae and cream spores
KE/16-198	*Echinochaete* sp	Contains a brown basidiome, angular-shaped pores and cylindrical white spores
KE/16-177	*Abortiporus* sp	Contains a brown cap and white spores

After the culturing of spores (on YMG media) obtained from the field-collected sporocarps and successive sub-culturing of their mycelia, axenic cultures for 12 distinct fungi

were successfully obtained (Table 2). This represented about 86% (12 out of 14) of the total number of individual fungi strains collected from the forest. The 12 axenic strains differed in their growth rates, mycelial growth patterns and colors on semi-solid YMG media. Generally, mycelial growths varied from white, cream and brown, or a combination of the colors (Fig. 3A-L). Interestingly, KE/16-153 had a distinct brown mycelial color when growing on YMG media (Fig. 3K).

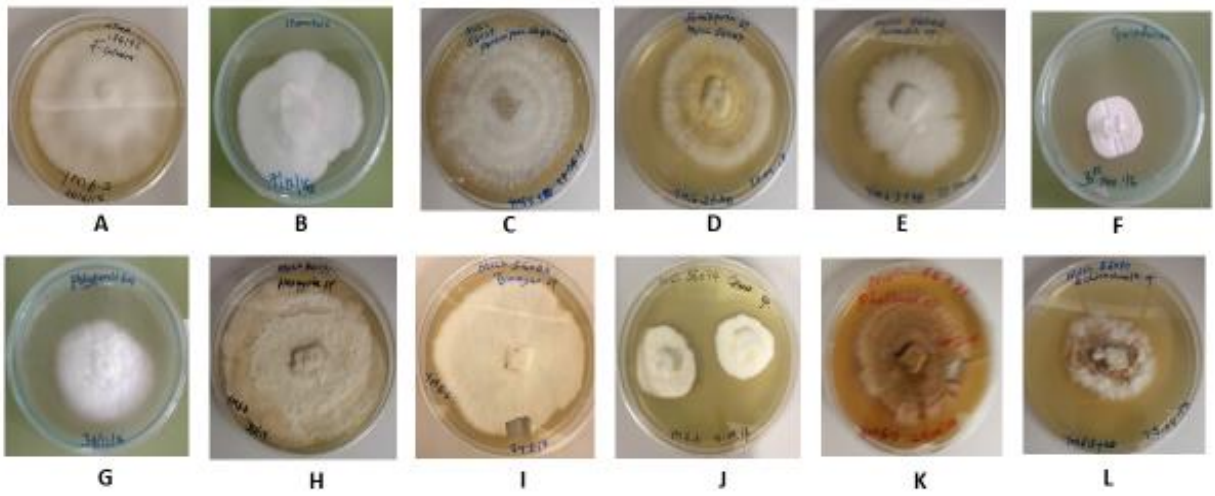


Figure 3: Morphological characteristics of 12 axenic fungi cultures grown on semi-solid YMG media. **(A)** KE/16-152 (*Favolaschia calocera*); **(B)** KE/16-101 (*Inonotus* sp); **(C)** KE/16-103 (*Perenniporia* sp); **(D)** KE/16-163 (*Fomitiporia aethiopica*); **(E)** KE/16-165 (*Antrodia* sp); **(F)** KE/16-176 (*Ganoderma* sp); **(G)** KE/16-193 (*Polyporus* sp); **(H)** KE/16-138 (*Hexagonia* sp); **(I)** KE/16-115 (*Tyromyces* sp); **(J)** KE/16-189 (*Tyromyces* sp); **(K)** KE/16-153 (*Phellinus* sp) and **(L*)** KE/16-198 (*Echinochaete brachypora*).

It was also observed that each fungal isolate varied in the period it took to attain full-growth on semi-solid (15-70 days) and liquid (9-62 days) YMG media (Table 2). Faster growth rate was realized in liquid media compared with semi-solid media. Also, media supplementation with antibiotics (streptomycin sulphate (1.5 mg/ml)) on primary cultures to obtain pure ones led to a slower growth of cultures as compared with those without. In addition, fungal cultures showed a rapid growth in the dark and at a temperature of 24° C compared with those propagated on a clean bench at room temperature (22° C).

Table 2: Growth period (days) of 12 axenic fungi isolates grown on semi-solid and liquid YMG media. A total of 12 out of 14 Basidiomycetes fungi samples collected from Mt Elgon forest were successfully cultured.

Fungal Strain Code	**YMG (liquid media) Growth(days)**	**YMG (semi-solid media) Growth (days)**
KE/16-152	28	34
KE/16-101	13	19
KE/16-103	16	22
KE/16-163	35	42
KE/16-165	18	24
KE/16-176	14	22
KE/16-193	9	15
KE/16-138	15	20
KE/16-115	53	60
KE/16-189	62	70
KE/16-153	14	20
KE/16-198	20	26
*KE/16-117	-	-
*KE/16-177	-	-

*****Samples that were not successfully cultured to obtain pure axenic cultures

4.2 ITS Amplification and Sequencing of the Axenic Fungal Isolates

All the 12 axenic fungi isolates subjected to PCR amplification using genomic DNA and ITS-1F and ITS4 primers, produced the expected amplicons of 500-700 bp (Fig. 4 A and B).

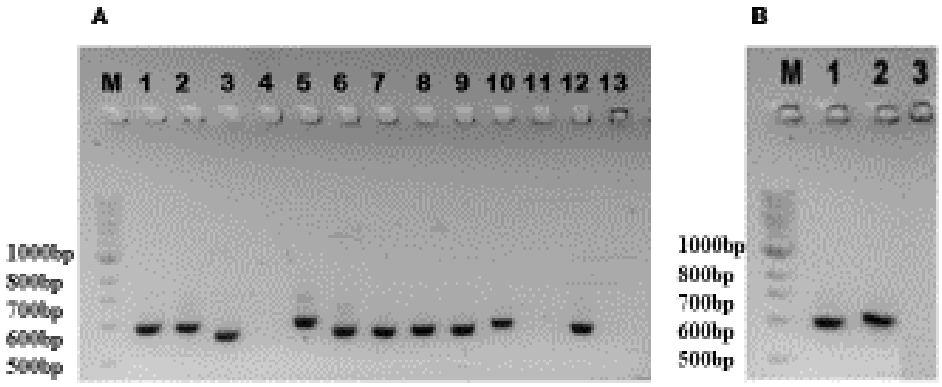


Figure 4: PCR amplification of rDNA fragments using ITS-1F and ITS4 primers pairs flanking ITS1 and ITS2 regions. The amplicons resolved on 0.8% Agarose gel at 100V in TAE buffer. Lane M-1 Kb DNA ladder; **(A)** Lane (L)1-KE/16-152; L2- KE/16-101; L3-KE/16-103; L5- KE/16-163; L6-KE/16-165; L7-KE/16-176; L8- KE/16-193; L9-KE/16-138; L10-KE/16-115; L12-KE/16-189; L13-Negativecontrol; L4 and L11-no amplification; **(B)** Lane L1- KE/16-153 and L2- KE/16-198; L3-Negative control.

The PCR amplicons (rDNA ITS) were sequenced and their sizes ranged between 527 and 688 nucleotides (Appendix 1). Multiple sequence alignment of the amplicon sequences using CLUSTAL/W revealed great variation of nucleotide residues and little conservation of the rDNA ITS regions, among the 12 fungi isolates (Appendix 2). BLASTN analysis of the 12 sequences revealed that all the strains were from phylum/division Basidiomycota and class Agaricomycetes (Table 3). The identities of the 12 strains were based on the best BLASTN matches with sequence similarity of ≥ 97-100%, query coverage ≥ 80% and Expect values (e-values) of zero (Appendix 1). However, the best matches for strains KE/16-101 (*Inonotus pachyphloeus*) and KE/16-198 (*Echinochaete brachypora*), had lower query coverages of 76% and 74%, respectively. Generally, the BLAST searches revealed that the fungi collected from Mt Elgon were of the genera; *Skeletocutis*, *Perenniporia*, *Echinochaete*, *Ganoderma*, *Hexagonia*, *Fomitiporia*, *Antrodia*, *Favolaschia*, *Inonotus* and *Polyporus.* Remarkably, the strains that was phenotypically identified as *Phellinus* and *Tyromyces* gave high sequence similarities to *Inonotus* (97%) and *Skeletocutis nivea* (96%), respectively (Table 3).

Table 3: Identified Basidiomycetes fungal isolates based on top hits of BLASTN search on NCBI database using sequenced ITS rDNA PCR amplicons.

NCBI Corresponding Species	Fungal Strain Code	MUCL Depository Number	E-value	GenBank Accession Number
Favolaschia calocera	KE/16-152	56041	0.0	DQ026249.1
Inonotus pachyphloeus	KE/16-101	56025	0.0	KP030785.1
Perenniporia sp	KE/16-103	56027	0.0	JQ673013.1
Fomitiporia sp	KE/16-163	56047	0.0	GU461958.1
Antrodia sp	KE/16-165	56048	0.0	KC543176.1
Ganoderma sp	KE/16-176	56055	0.0	JQ520185.1
Polyporus arcularius	KE/16-193	56064	0.0	KR673445.1
Hexagonia sp	KE/16-138	56071	0.0	KKY948738.1
Skeletocutis nivea	KE/16-115	56031	0.0	KJ140619.1
Skeletocutis nivea	KE/16-189	56074	0.0	KJ140619.1
Inonotus sp	KE/16-153	56075	0.0	JF895464.2
Echinochaete brachypora	KE/16-198	56080	0.0	AB462320.1

MUCL-Mycothèque de l'Université Catholique de Louvain; E-value-Expect value

4.3 Phylogenetic Analysis of the Fungal Isolates

The evolutionary history of the Basidiomycetes was inferred using Neighbor Joining (NJ) method. The analyses involved 70 sequences (12 study sequences and 58 sequences obtained from GenBank). The resultant trees confirmed that three taxonomic orders were represented by the fungi strains collected from Mt. Elgon forest. These were; Hymenochaetales, Polyporales and Agaricales (Fig. 5). The tree also resolved into various fungal families including; *Hymenochaetaceae* (*Inonotus* sp and *Fomitiporia aethiopica*), *Polyporaceae* (*Polyporus arcularius, Hexagonia* sp, *Ganoderma* sp, *Skeletocutis nivea*), *Mycenaceae* (*Favolaschia calocera*) and *Fomitopsidaceae* (*Antrodia* sp). *Favolaschia calocera* was however observed to be an outgroup taxon (Fig. 5). The NJ method used was shown to give a well resolved tree, due to the high bootstrap values obtained (>50%).

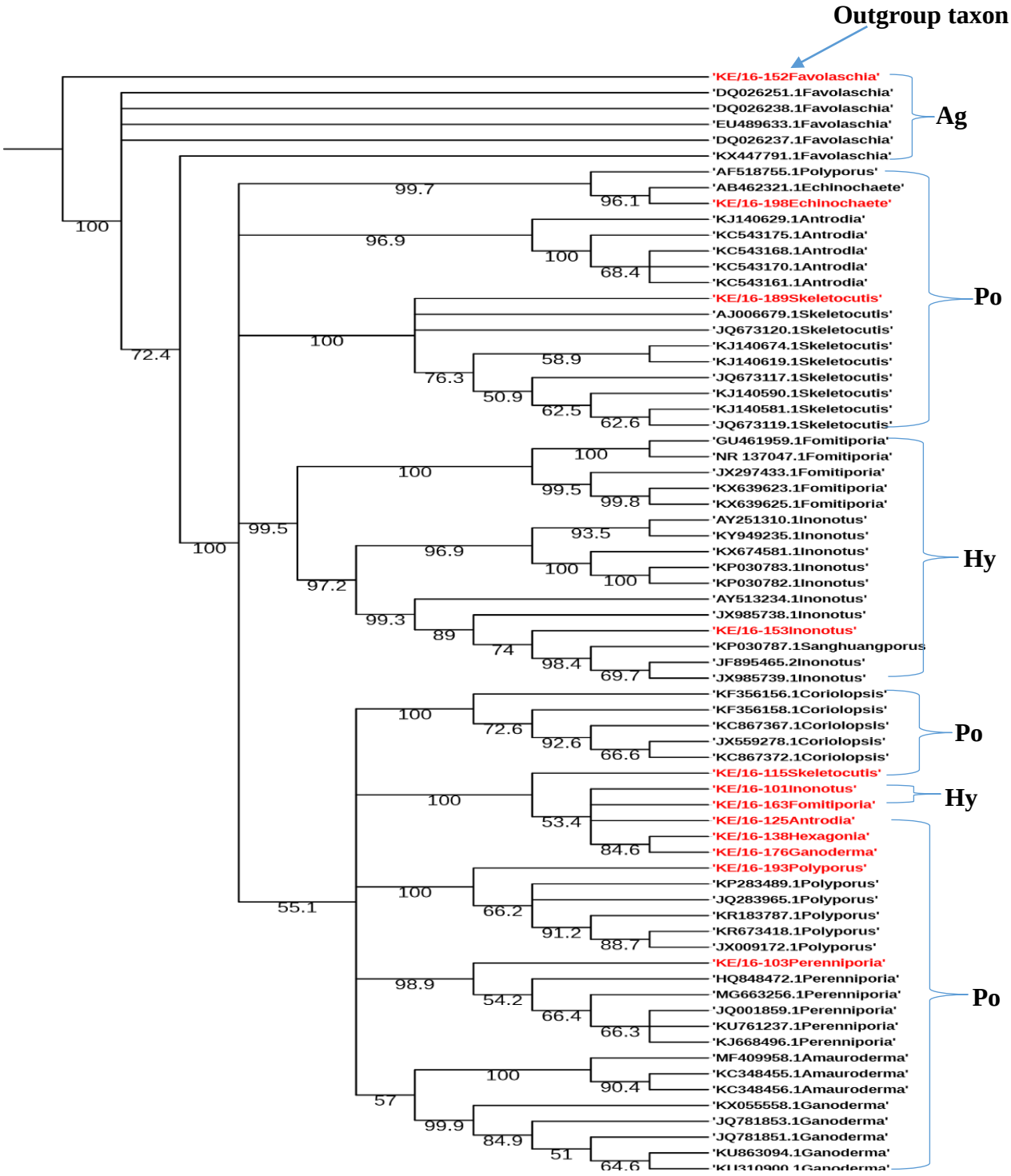


Figure 5: An evolutionary tree obtained from the analyses of the rDNA ITS sequences of the fungal strains under study (red) and their closest relatives obtained from GenBank (black) using iTOL online program. The trees were calculated with Geneious Tree Builder (implemented in Geneious 11.0.4), using Neighbor-Joining approach followed by 1000 bootstrap replications. Orders: Po; Polyporales, Ag-Agaricales and Hy-Hymenochaetales.

4.4 Pre-Screening via Dual Culture Assay

Out of the 12 cultured fungi strains collected from Mount Elgon forest, only four namely KE/16-163 (*Fomitiporia* sp), KE/16-138 (*Hexagonia* sp), KE/16-189 (*Skeletocutis* sp) and KE/16-115 (*Skeletocutis* sp) had positive antagonism against *B. subtilis* (DSM10) test organism: demonstrated by growth inhibition of bacterial lawn around inoculated fungi agar plugs (Fig. 6A). The other 8 strains did not constrain growth of *B. subtilis*, since there was no bacterial growth inhibition around the inoculated fungi plugs (Fig. 6B). Unlike for *B. subtilis*, all the cultured fungi strains did not have any antagonistic activity against fungal test organism, *M. plumbeus* (MUCL49355).

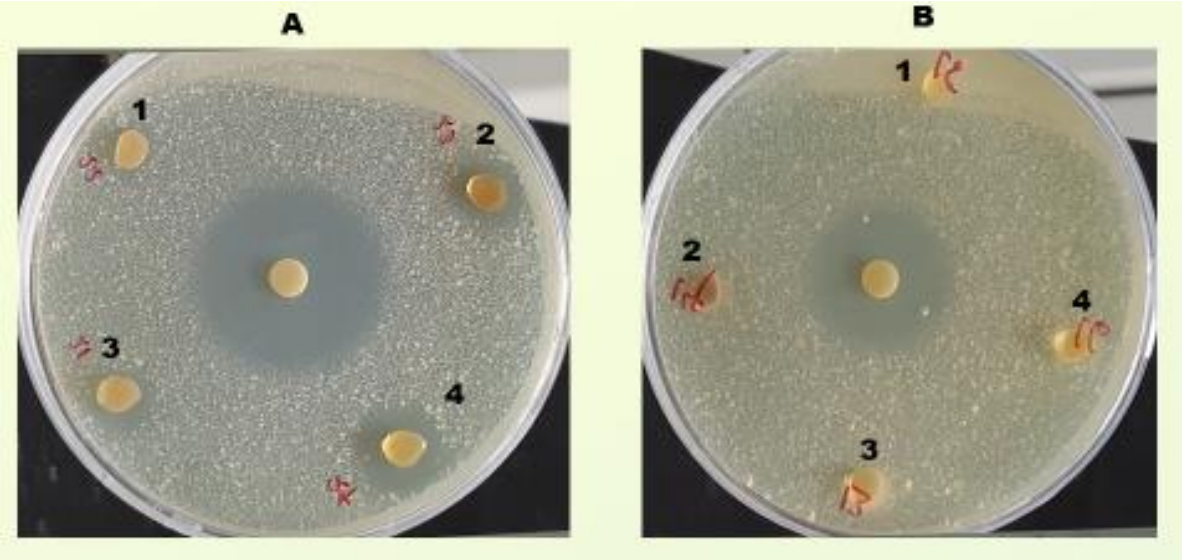


Figure 6: Preliminary screening for antimicrobial activities of cultured Basidiomycetes fungi species collected from Mt. Elgon forest against *B. subtilis* using dual culture assay. (A). Inhibition of *B. subtilis* lawn around agar plugs of; **1**-KE/16-163 (*Fomitiporia* sp), **2**-KE/16-138 (*Hexagonia* sp), **3**-KE/16-189 (*Skeletocutis* sp), **4**-KE/16-115 (*Skeletocutis* sp) ; (B) Agar plugs for; **1**-KE/16-101 (*Inonotus pachyphloeus*); **2**-KE/16-153 (*Inonotus* sp); **3**-KE/16-198 (*Echinochaete brachypora*); **4**-KE/16-103 (*Perenniporia* sp) among others failed to inhibit growth of *B. subtilis*. The centre of each of the petri plates contains a positive control treatment consisting of a disc impregnated with Ciprofloxacin at 1 mg/disc (Oxoid™).

The strains KE/16-115 and KE/16-189 (*Skeletocutis* sp) produced inhibition zones of 13.0±2.6 mm and 15.0±3.0 mm. Similarly, strains KE/16-163 (*Fomitiporia* sp) and KE/16-138 (*Hexagonia* sp) produced inhibition zones of 12.0±1.0 mm and 10.0± 4.6 mm (Table 4). The positive controls ciprofloxacin and nystatin discs at 1mg/ml used against bacterial and

fungal test pathogens produced inhibition zones of 31.0±1.5 and 20.0±2.1mm, respectively (Table 4).

Table 4: Preliminary screening for antimicrobial activities of cultured fungi strain from Mt Elgon forest using dual culture assay on semi solid YMG media against *B. subtilis* and *M. plumbeus* test microorganisms.

Fungal Strain Code	**Identity**	**Test organism (diameter in mm)**	
		B. subtilis	***M. plumbeus***
KE/16-152	*Favolaschia calocera*	-	-
KE/16-101	*Inonotus pachyphloeus*	-	-
KE/16-103	*Perenniporia abyssinica*	-	-
KE/16-163	*Fomitiporia aethiopica*	12.0±1.0	-
KE/16-165	*Antrodia* sp	-	-
KE/16-176	*Ganoderma* sp	-	-
KE/16-193	*Polyporus arcularius*	-	-
KE/16-138	*Hexagonia* sp	10.0±4.6	-
KE/16-115	*Skeletocutis* sp	15.0±3.0	-
KE/16-189	*Skeletocutis* sp	13.0±2.6	-
KE/16-153	*Inonotus* sp	-	-
KE/16-198	*Echinochaete brachypora*	-	-
Ciprofloxacin disc		31.0±1.5	N/A
Nystatin disc		N/A	20.0±2.1

(-)- No activity

4.5 MIC Determination using Fungal Crude Extracts

In the second screening assay, antimicrobial activities of secondary metabolites present in the mycelium and fermentation medium (here in referred to as supernatant) was observed in 9 out of the 12 strains unlike in the dual culture assay reported in section 4.3.1.

The crude extracts obtained from 3 fungal strains i.e KE/16-176 (*Ganoderma* sp), KE/16-103 (*Perenniporia* sp) and KE/16-153 (*Inonotus* sp) did not show any antimicrobial activities against all the 4 selected test microorganisms; bacterial (*B. subtilis* and *E. coli*) and fungal (*M. plumbeus* and *C. tenuis*). (Appendix 3).

4.5.1 MIC assay against *B. subtilis* test pathogen

Irrespective of the source (mycelium or supernatant), secondary metabolites extracted from genera *Inonotus* (KE/16-101), *Echinochaete* (KE/16-198), *Fomitiporia* (KE/16-163), *Antrodia* (KE/16-165), *Hexagonia* (KE/16-138) and *Skeletocutis* (KE/16-189 and KE/16-115) fermented in Q6½, YMG and rice media were active against *B. subtilis* (Fig. 7A-F). In contrast, secondary metabolites extracted from mycelium and supernatant from these fungi strains fermented using ZM ½ medium did not have activity against *B. subtilis* (Appendix 3). Strikingly, of all the extracts tested against *B. subtilis*, mycelial extracts from KE/16-189 and KE/16-198 strains fermented in Q6½ medium demonstrated the highest inhibitory activities at low concentrations of 4.69 µg/ml (Fig. 7A) and 9.38 µg/ml (Fig. 7B), respectively. Similarly, supernatant extracts from these strains also demonstrated inhibitory activities against *B. subtilis* at relatively lower concentrations of 9.38 µg/ml for KE/16-189 and 18.75 µg/ml for KE/16-198. In addition, extracts from the strain KE/16-165 strain fermented in Q6½ medium demonstrated antimicrobial activity at 37.5 µg/ml (mycelial extract) and 300 µg/ml (supernatant extracts) against *B. subtilis* (Fig. 7C). Interestingly, for the strain KE/16-101, only the mycelial extracts inhibited growth of *B. subtilis* at higher concentration of 300 µg/ml (Fig. 7C).

Generally, mycelial extracts from fungi strains fermented in YMG medium were more active against *B. subtilis* than from supernatant. The mycelial extracts obtained from strain KE/16-189, showed activity at MIC of 75 µg/ml, whereas those obtained from KE/16-115 and KE/16-138, as well as KE/16-163 and KE/16-198 were active at 150 µg/ml, and 300 µg/ml respectively against the *B. subtilis*. However, only the YMG supernatant extracts from strain KE/16-138 demonstrated minimum inhibitory activity against the *B. subtilis* at 37.5 µg/ml (Figure 7D). Also, the mycelial extracts obtained from KE/16-163 fermented on rice media, demonstrated activity at a high concentration of 300 µg/ml against *B. subtilis* (Fig. 7E). The positive control (1 mg/ml ciprofloxacin) gave an MIC value of <2.34 µg/ml in all the tests that were performed against *B. subtilis* (Fig 7A-F).

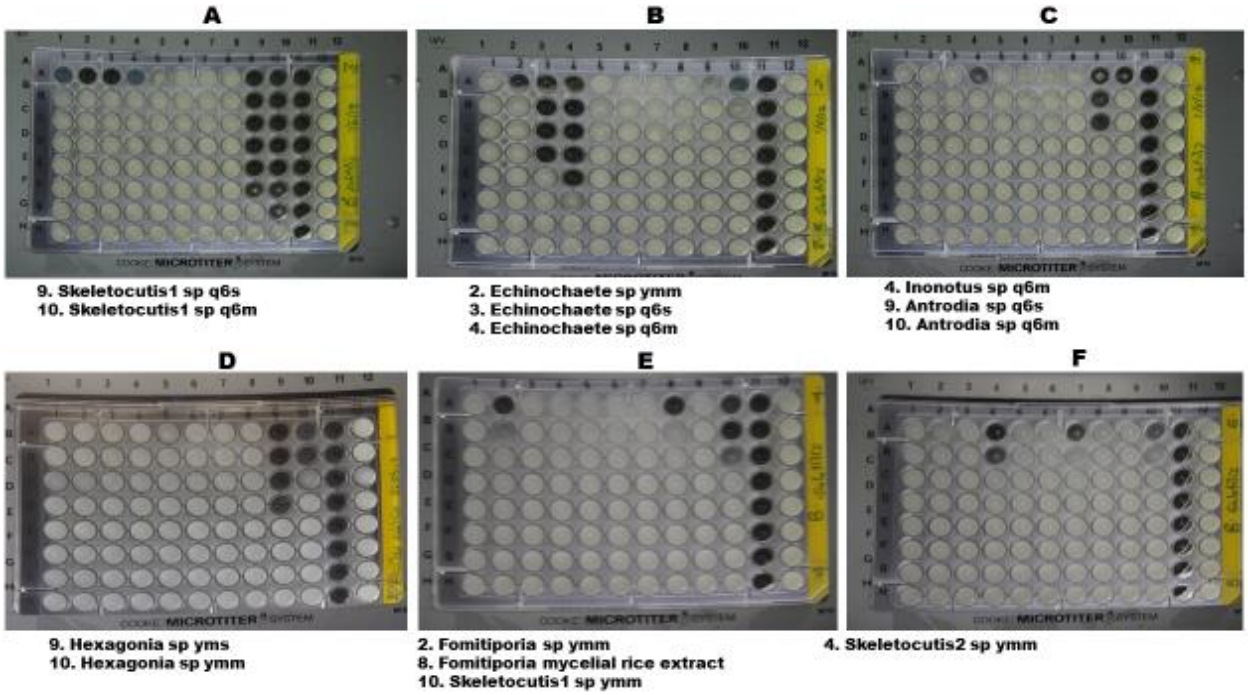


Figure 7: Minimum inhibitory concentration (MIC) assay of fungal extracts against *Bacillus subtilis* test pathogen. (**A**) C9-10, KE/16-189 (*Skeletocutis nivea*); (**B**) C2-4, KE/16-198 (*Echinochaete* sp); (**C**) C4, KE/16-101 (*Inonotus* sp); C9-10, KE/16-165 (*Antrodia* sp); (**D**) C9-10, KE/16-138 (*Hexagonia* sp); (**E**) C2 &C8, KE/16-163 (*Fomitiporia* sp); C10, KE/16-189 (*Skeletocutis* sp); (**F**) C4, KE/16-115(*Skeletocutis* sp); C11, Positive control; C12, Negative control. The letter and number before the fungi strain refers to Column number on the microtiter plate e.g. C4 refers to column 4. Underneath images the numerical value refers to column No., then fungi strain and lower case letters refer to source of the secondary metabolite (supernatant or mycelium): Q6S refers to Q6½ supernatant extracts; Q6M refer to Q6½ mycelial extracts; YMS refer to YMG supernatant extracts; YMM refer to YMG mycelial extracts.

4.5.2 MIC assay against *E.coli* test pathogen

Similar to *B. subtilis* assay, secondary metabolites extracted from fungi fermented in YMG and Q6½ media showed activity against *E. coli*. However, unlike the former where the mycelial and supernatant extracts were active, only the supernatant extracts obtained from KE/16-138 (Fig. 8A) and KE/16-101 (Fig. 8B) demonstrated inhibitions at 300 µg/ml. The positive control (ciprofloxacin) gave an MIC value of <2.34 µg/ml in all the tests that were performed against *E. coli*.

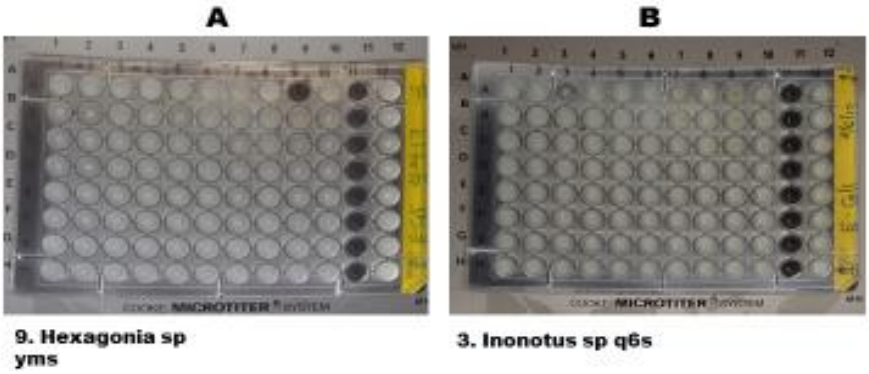


Figure 8: Minimum inhibitory concentration (MIC) assay of fungal extracts against *E. coli* test pathogen. (**A**) C9-10, KE/16/138 (*Hexagonia* sp); (**B**) C3-4, KE/16-101 (*Inonotus* sp). C11, Positive control; C12, Negative control. The letter and number before the fungi strain refers to Column number on the microtiter plate e.g. C8-9 refers to column 8 to 9. Underneath images the numerical value refers to column No., then fungi strain and lower case letters refer to source of the secondary metabolite (supernatant or mycelium): Q6S refers to Q6½ supernatant extracts; YMS refers to YMG supernatant extracts.

4.5.3 MIC Assay against *M. plumbeus* and *C. tenuis* test pathogens

M. plumbeus and *C. tenuis* growth was inhibited by mycelium and supernatant extracted secondary metabolites fermented using YMG, Q6 ½ and ZM ½ media. The mycelium and supernatant extracts from strain KE/16-152 fermented in YMG media demonstrated very low MIC values of <2.34 µg/ml and 9.38 µg/ml against *C. tenuis* (Fig. 9A), unlike for *M. plumbeus* where MIC values of 150 µg/ml and 75 µg/ml were obtained (Fig. 9B). The ZM ½ supernatant extracts from strain KE/16-101 also demonstrated a remarkable low MIC value of 37.5 µg/ml against *C. tenuis*, whereas YMG mycelial extracts showed activity against *C. tenuis* at 150 µg/ml (Fig. 9C). In addition, YMG mycelial and supernatant extracts from strain KE/16-193 (*Polyporus* sp) and Q6 ½ mycelial extract from strain KE/16-189 demonstrated mild activities at 300 µg/ml against *C. tenuis* and *M. plumbeus*, respectively. The positive control, Nystatin at 1 mg/ml produced MIC values of <2.34 µg/ml for *C. tenuis* (Fig. 9A and C) and 18.75 µg/ml for *M. plumbeus* (Fig. 9B).

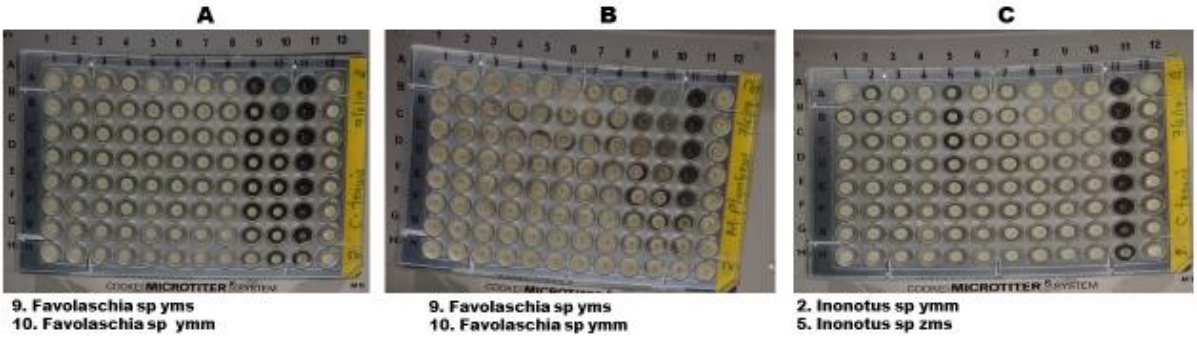


Figure 9: Minimum inhibitory concentration (MIC) assay for fungal extracts from fermented axenic cultures against *C. tenuis* and *M. plumbeus*. (**A**) C9-10, KE/16-152 (*Favolaschia* sp); (**B**) C9-10, KE/16/152 (*Favolaschia* sp); (**C**) C2-6, KE/16/101 (*Inonotus* sp); C11, Positive control; C12, Negative control. The letter and number before the fungi strain refers to Column number on the microtiter plate e.g. C9-10 refers to column 9 to 10. Underneath images the numerical value refers to column No., then fungi strain and lower case letters refer to source of the secondary metabolite (supernatant or mycelium): ZMS refers to ZM½ mycelial extracts; YMS refers to YMG supernatant extract; YMM refers to YMG mycelial extract.

4.6 Characterization of Strain KE/16-163 Secondary Metabolites

The strain KE/16-163 (*Fomitiporia aethiopica*) was selected from the 12 fungi strains for further characterization of secondary metabolites. Selection of strain KE/16-163 was based on three factors, namely definitive taxonomic identification, antimicrobial activity demonstrated in the dual culture assay and fast growth rate of KE/16-163 axenic culture. Large scale production of strain KE/16-163 secondary metabolites from hyphae was undertaken using solid state fermentation with rice medium. The initial analysis of crude secondary metabolites using HPLC-MS generated peaks and subsequent DNP database (Buckingham, 2017) search, suggested presence of new compounds. The 1-D and 2-D NMR spectroscopy and analysis of HREIMS (High Resolution Electron Impact Mass Spectroscopy) data led to the successful characterization of 5 previously undescribed pregnenolone-type steroid compounds, which were assigned trivial names; aethiopinolones A to E.

4.6.1 Compound 1 (Aethiopinolone A)

The compound was isolated as a yellow oil from the ethyl acetate extract, with molecular formula C_{21} H_{30} O_5 and 7 degrees of unsaturation corresponding to the mass ion at 363.2166 *m/z* $[M+H]^+$, according to the data obtained from HREIMS (Fig. 10).

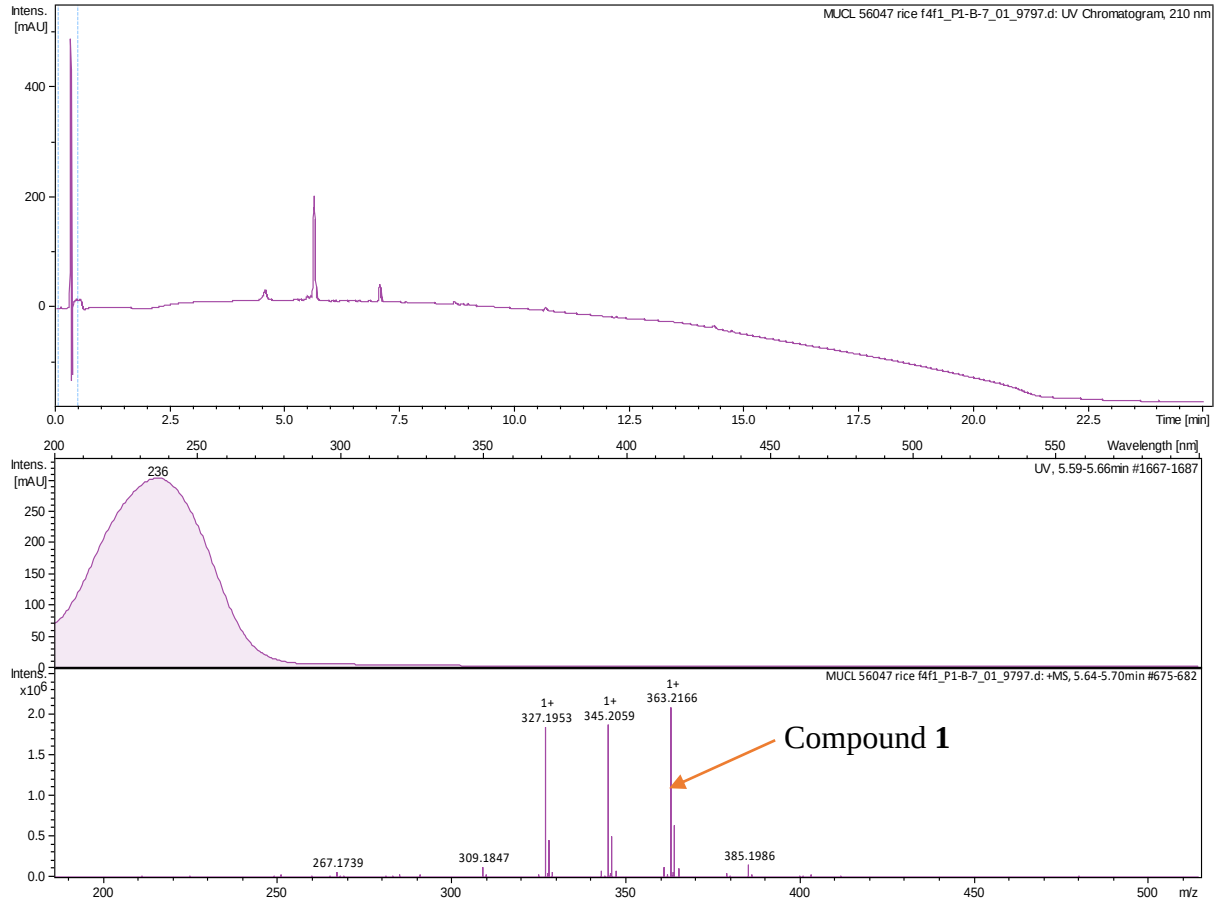


Figure 10: The Mass spectra of compound **1**

^{13}C NMR spectroscopy showed presence of 21 carbon signals, which the DEPT spectrum revealed to be methyl (3), methylene (6), methine (6) and quaternary (6) carbons. ^1H NMR spectrum revealed three methyl singlets resonating at δ 0.58 (H_3-18), δ 0.93 (H_3-19) and δ 2.16 (H_3-21). Other multiplicities (doublets (d), doublet of doublets (dd), doublet of doublet of doublets (ddd), doublet of triplets (dt), triplet of doublets (td), triplet of triplet of triplets (ttt) and multiplets (m) were also observed (Table 5 and Appendices 4-9). Furthermore, ^1H NMR data was supported by HSQC NMR spectrum that confirmed the protons directly attached to carbon atoms. HMBC correlations were also revealed between H_3-18 to C-12, C-13, C-14 and C-17; H_3-19 to C-1, C-5, C-9 and C-10; H_3- 21 to C-17 and C-20 and also H-14 and H-5 correlations to C-7, C-8, C-9, C-13, C-15, C-18 and C-3, C-4, C-6, C-9, C-10 and C-19, respectively. Among other HMBC correlations, these suggested a

pregnenolone-type triterpenoid structure (Fig. 11). The HMBC correlations were supported by COSY correlations that were observed between H_2-2 to H_2-1 and H-3; H_2-4 to H-3 and H-5; H_2-11 to H_2-12; H-14 to H_2-15 and H-16 to H_2-15 and H-17. Also, a network of ROESY correlations were observed between H_3-18 to H_3-19/ H_2-12β/ H-15β/ H-16/ H_3-21 and H_3-19 to H_3-18/ H_2-1β/ H_2-11β, thus suggesting that the protons were on the same side of the plane. Furthermore, H-3 was established to have an α orientation based on it's ROESY correlations to H_2 -1α/ H_2-4α/ H_2-2α and coupling constants of 4.52 Hz and 11.3 Hz. In contrast, H-16 was shown to have a β orientation due to the cross-peaks that were observed between it and H_3-18/ H_2-15β, as well as its small coupling constant of 3.0 Hz.

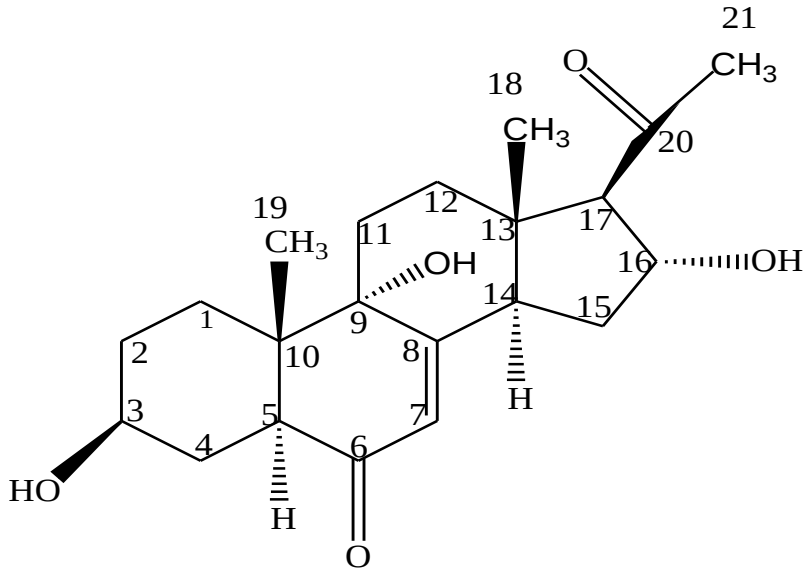


Figure 11: Structure of Compound **1**

Table 5: NMR data for Compound **1** in acetone-d_6

Carbon Position	^{13}C (δ_C)	DEPT	^{1}H/HSQC (δ_H (J in Hz))	HMBC	COSY	ROESY
1.	30.5	CH_2	1.48, dt, (J=13.21, 3.55) 1.29, dd, (J=13.21, 3.55)	19, 2, 10, 5, 3	2	19, 12a, ,16
2.	31.5	CH_2	2.05, 1.79, m	10, 3	1,3	18, 1b, 4a, 11a,2
3.	70.3	CH	3.5, ttt, (J=4.58, 11.29)	5, 2,4,1	2,4	1a, 4b, 2
4.	31.5	CH_2	1.32, 2.12, m	3, 5	5, 3	-
5.	47.2	CH	2.90, dd, (J=12.21,3.81)	19, 4, 10, 3, 9,6	4	1a, 2
6.	199.3	C	-	-	-	-
7.	124.2	CH	5.48, d, (J=2.29)	5, 14	-	-
8.	160.8	C	-	-	-	-
9.	74.2	C	-	-	-	-
10.	42.7	C	-	-	-	-
11.	28.5	CH_2	1.84,dd,s (J=11.40, 6.45) 1.99, dd, (J=11.40, 6.45)	12, 13, 9, 8	12	-
12.	35.5	CH_2	1.97, 2.07	18, 13, 9	11	-
13.	46.8	C	-	-	-	-
14.	50.1	CH	3.12(ddd), (J=12.8,6.67,2.29)	18, 15, 13, 7, 8	15	15a,12b,17
15.	35.1	CH_2	1.61(ddd), (J=1.29,6.67,12.48) 2.00 (m)	13, 14, 16, 17	14	16
16.	71.6	CH	4.74 (m)	14, 20, 17	15,17	15b
17.	74.3	CH	2.71 (d), (J=5.95)	18, 12, 13, 16, 20	16	-
18.	14.8	CH_3	0.58 (s)	12,13,14, 17	-	12b, 14
19.	17.19	CH_3	0.93 (s)	1,10,5,9	-	-
20.	207.6	C	-	-	-	-
21.	31.9	CH_3	2.16 (s)	20,17	-	-

a-alpha; b-beta; (s)-singlet; (d)-doublet (dd)-doublet of doublets; (ddd)-doublet of doublet of doublets; (dt)-doublet of triplets; (td)-triplet of doublets; (ttt)-triplet of triplet of triplets; (m)-multiplets

4.6.2 Compound 2 (Aethiopinolone B)

Compound **2**, a white solid was obtained from ethyl acetate extracts of the fungal strain (KE/16-163). It had a molecular formula of C_{21} H_{30} O_5 and 7 degrees of unsaturation. The mass spectrum revealed correspondence to the mass ion at 363.2162 *m/z* $[M+H]^+$, as deduced from HREIMS (Fig. 12).

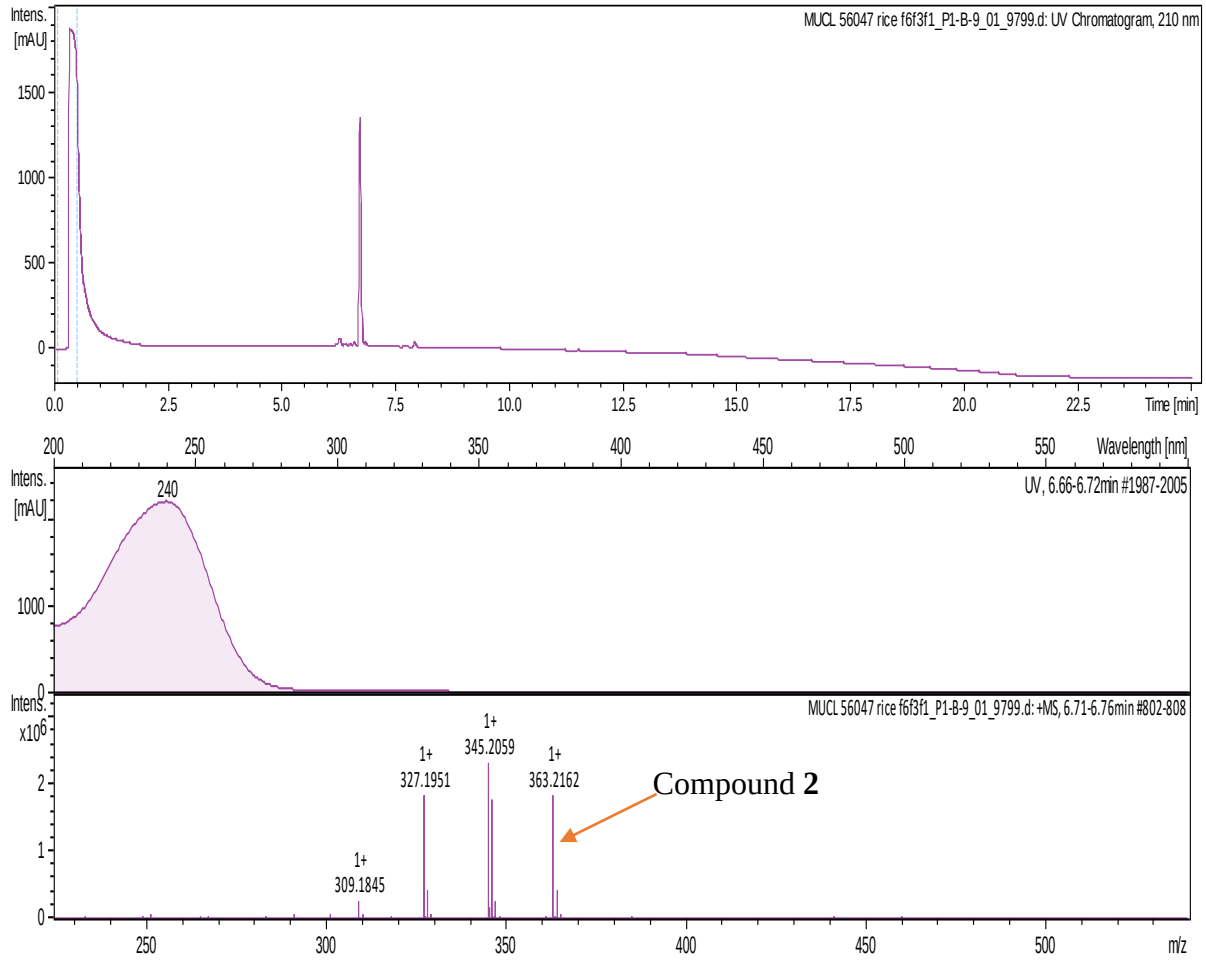


Figure 12: The mass spectrum of compound **2**

1-D and 2-D NMR spectra (Appendices 10-15), suggested that the compound was similar to compound **1** in it's planar structure. ^{13}C NMR data of compound **2** revealed the presence of 21 carbon signals whereas the DEPT NMR spectroscopy revealed 3 methyl, 6 methylene, 6 methine and 6 quaternary carbons (Appendix 11). The ^{1}H NMR data showed that the methyl groups were singlets resonating at δ 2.15 (C-21), δ 0.80 (C-19) and δ 0.46 (C-18). The other ^{1}H signals also gave d, dd, ddd, dt, td, and m multiplicities (Table 5). The HMBC and COSY correlations observed, were similar to those of compound **1**, resulting in the pregnenolone-type triterpenoid (Fig. 13). However, compound **2** differed from compound **1** in the stereochemistry at C-3, where C-3 was slightly shielded (δ 63.1) (Table 6) as

compared to the C-3 of compound **1** which resonated at δ 70.3. Furthermore, ROESY correlations of H-3 to H_3- 19 and OH-3 to H-5 pointed out that H-3 had a β orientation.

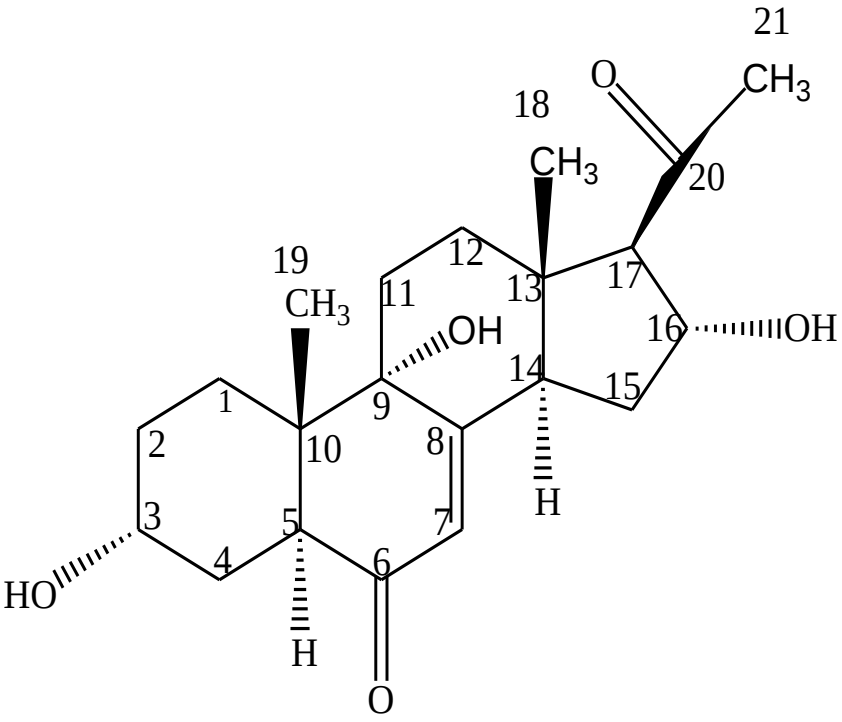


Figure 13: The structure of compound **2**

Table 6: The NMR data for Compound **2** in DMSO-d_6

Carbon Position	^{13}C (δ_C)	DEPT	^{1}H/HSQC (δ_H (J in Hz))	HMBC	COSY	ROESY
1.	24.2	CH_2	1.01 (dt) (J=12.66,3.20) 2.26,(td) (J=13.58,4.12)	19, 2, 9, 5, 3	2	19, 12a, ,16
2.	27.4	CH_2	1.51 (m) 1.43(m)	10, 3	1,3	18, 1b, 4a, 11a,2
3.	63.1	CH	3.91 (m)	1	2,4	1a, 4b, 2, 5,19
4.	27.87	CH_2	1.40 (m) 1.80(m)	2	5, 3	-
5.	41.39	CH	3.17, dd, (J=12.05,3.97)	19, 4, 10, 3, 9,6	4	1a, 2,3
6.	200.7	C	-	-	-	-
7.	122.6	CH	5.39, d, (J=1.98)	5, 14	-	-
8.	160.4	C	-	-	-	-
9.	72.6	C	-	-	-	-
10.	41.9	C	-	-	-	-
11.	26.8	CH_2	1.69,dd, (J=13.58,3.97) 1.79, dd, (J=13.58,4.43)	13, 9	12	-
12.	34.0	CH_2	1.83 (m) 1.94 (m)	13, 9, 14, 11	11	-
13.	45.6	C	-	-	-	-
14.	48.8	CH	3.01 (ddd), (J=12.36,6.56,1.98)	18, 15, 13, 7, 8	15	15a,12b,17
15.	33.8	CH_2	1.41 (ddd), (J=1.25,6.87,12.43) 1.88 (m)	13, 14, 16, 17	14	-
16.	69.9	CH	4.55 (m)	14, 20, 17	15,17	15b
17.	73.0	CH	2.63 (d), (J=6.10)	18, 12, 13, 16, 20	16	-
18.	14.16	CH_3	0.46 (s)	12,13,14, 17	-	12b, 14
19.	15.8	CH_3	0.80 (s)	1,10,5,9	-	3
20.	207.7	C	-	-	-	-
21.	31.6	CH_3	2.15 (s)	20,17	-	-

a-alpha; b-beta; (s)-singlet; (d)-doublet (dd)-doublet of doublets; (ddd)-doublet of doublet of doublets; (dt)-doublet of triplets; (td)-triplet of doublets; (ttt)-triplet of triplet of triplets; (m)-multiplets

4.6.3 Compound 3 (Aethiopinolone C)

Aethiopinolone **3** was isolated as a yellow oil with molecular formula $C_{21}H_{28}O_4$ and 8 degrees of unsaturation according to HREIMS data (Fig. 14). In addition, the compound corresponded to the molecular ion at 345.2060 *m/z* $[M+H]^+$.

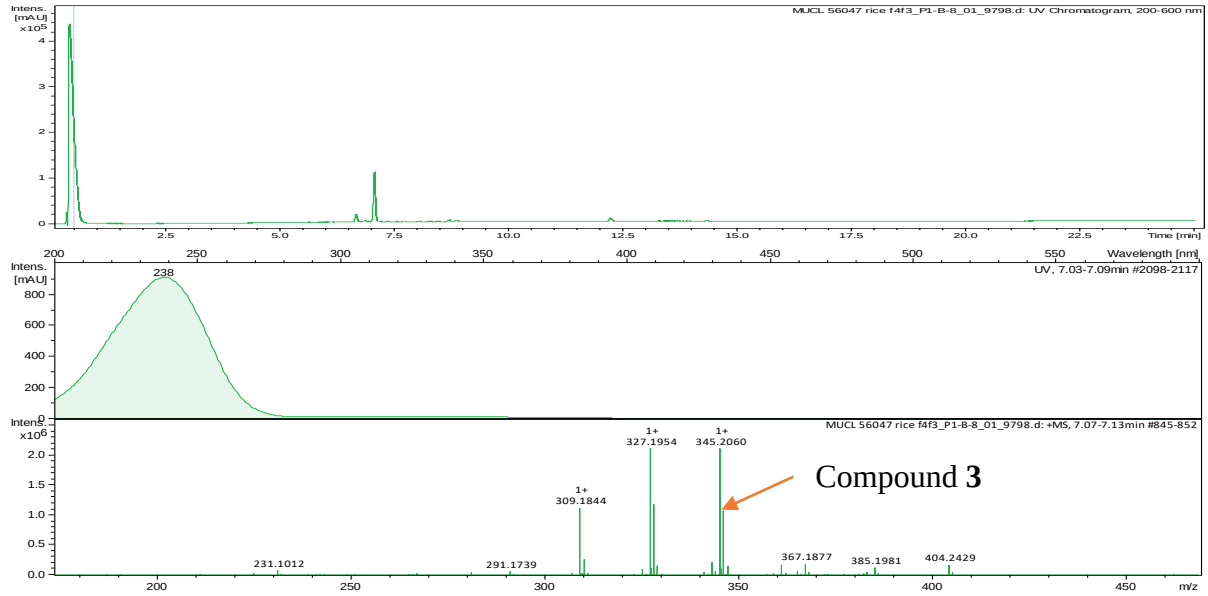


Figure 14: The mass spectrum of compound **3**

The compound was similar to compound **1** having 21 carbon atoms according to ^{13}C NMR data. However, new additional carbon signals were visualized resonating at δ 144.3 and δ 155.1 (Fig. 15). The analysis of the ^{1}H NMR showed that the oxygenated methine proton (H-16) and a methine proton (H-17), resonating at δ 4.74 and δ 2.71 respectively, were absent as compared to their presence in compound **1**. Instead, a doublet of doublet at H-16 resonating at δ 6.91 was revealed. The HMBC NMR data revealed correlation of H-16 to C-13, C-14, C-17 and a COSY correlation of H-16 to H_2-15. Nevertheless, other correlations (Table 7 and Appendices 16-21), implied a similar planar structure between the compounds **1** and **3**. Furthermore, ROESY correlation patterns of compound **3** were shown to be similar to those of compound **1**.

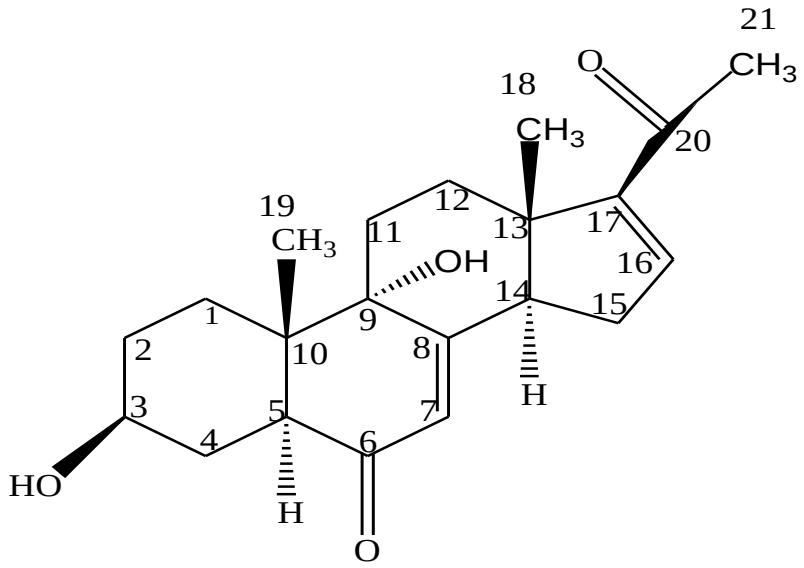


Figure 15: The structure of compound **3**

Table 7: The NMR data for compound **3** in Acetone-d_6

Carbon Position	^{13}C (δ_C)	DEPT	^{1}H/HSQC (δ_H (J in Hz))	HMBC	COSY
1.	30.4	CH_2	1.29,m; 1.49,m	19	-
2.	30.4	CH_2	1.33,m; 1.77,m	3,10	5
3.	70.3	CH	3.47,tt (J=4.4,11.3)	1,2,4,5	5,4
4.	31.5	CH_2	2.10,m; 2.13,m	3,5	3,5
5.	47.3	CH	2.91,dd (J=12.2, 3.8)	19	3,4,2
6.	199.4	C	-	-	-
7.	123.4	CH	5.60,d (J=2.1)	9	-
8.	160.2	C	-	-	-
9.	74.4	C	-	-	-
10.	42.8	C	-	-	-
11.	31.3	CH_2	1.77,m; 2.15,m	13,19	8
12.	32.1	CH_2	1.81,m; 2.30,m	18	11,13
13.	46.8	C	-	-	-
14.	52.9	CH	3.11,ddd (J=11.6,6.6,2.1)	18,15,13,7,8	15
15.	31.3	CH_2	2.39,m; 2.47,m	13,14,16,17	14,16
16.	144.3	CH	6.91,dd; (J=1.9,3.4)	14,17,20	15
17.	155.1	C	-	-	-
18.	16.4	CH_3	0.88s	12,17	-
19.	17.2	CH_3	0.99s	1,5,9,10	-
20.	196.3	C	-	-	-
21.	27.1	CH_3	2.25s	20,17	-

(s)-singlet; (d)-doublet (dd)-doublet of doublets; (ddd)-doublet of doublet of doublets; (dt)-doublet of triplets; (td)-triplet of doublets; (ttt)-triplet of triplet of triplets; (m)-multiplets

4.6.4 Compound 4 (Aethiopinolone D)

Aethiopinolone D was obtained as a yellow oil with a molecular formula of C_{21} H_{28} O_4 and eight degrees of unsaturation. Thus, the compound corresponded to the molecular ion at 344.44 *m/z* $[M+H]^+$ as deduced from HREIMS (Fig. 16).

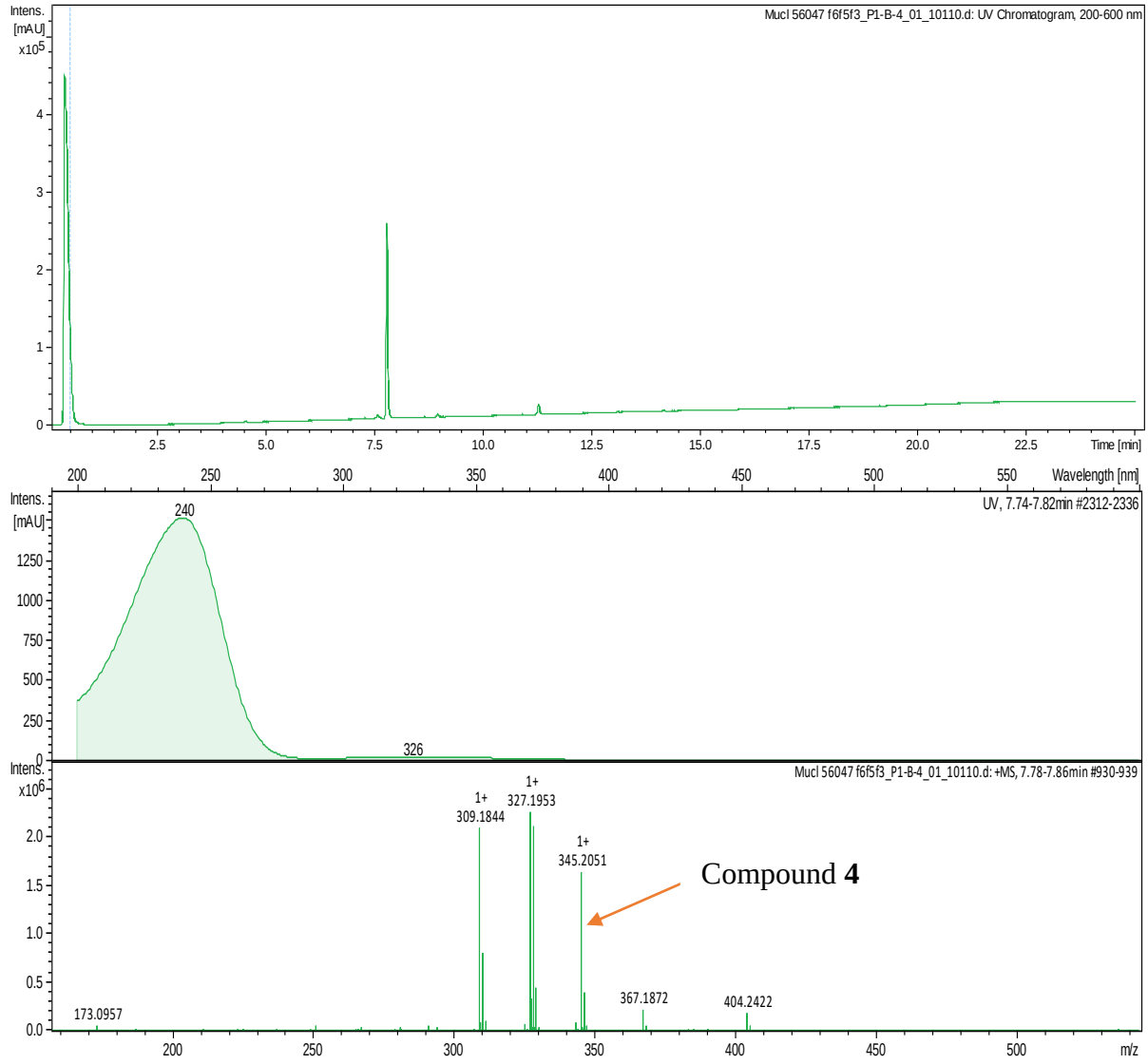


Figure 16: Mass spectrum of compound **4**

The compound was similar to compound **3**, with the only difference in the stereochemistry at C-3, for the two compounds (Fig. 17). In addition, the data obtained from both 1-dimensional (1D) and 2-dimensional (2D) NMR (Appendices 22-27), indicated an existing analogy between the compounds (Table 8). The shielding effect that was observed in the ^{13}C NMR of compound **2** was also visualized. C-3 of compound 4 was slightly shielded (δ 65.0) as compared to C-3 of compound 3 (δ 70.3). Thus, this indicated the same

stereochemistry at position C-3 for the molecules. Also, similar ROESY correlations between compounds **2** and **4** were recorded.

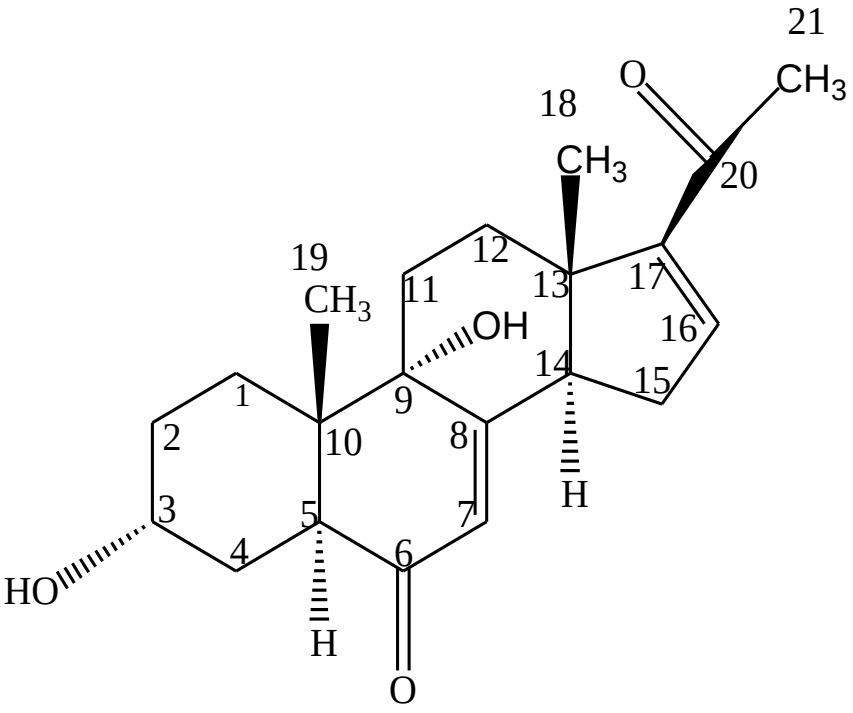


Figure 17: The structure of compound **4**

Table 8: NMR data for compound **4** in Acetone-d_6

Carbon Postion	^{13}C (δ_C)	DEPT	^{1}H/HSQC (δ_H (J in Hz))	HMBC	COSY
1.	25.3	CH_2	1.16 (dt)(J=12.91,3.80) 2.47(dq), (J=12.91,2.80)	19, 2, 9, 3	2
2.	28.7	CH_2	1.59 (dq), (J=2.58,12.48) 1.65 (m), (J=12.48,2.58)	10, 3,1,4	1,3
3.	65.0	CH	4.05 (p), (J=5.81)	1, 2, 5	2,4
4.	29.2	CH_2	1.54 (m), (J=2.80,2.58,11.83) 1.97(m)	2, 5, 3, 6	5, 3
5.	42.7	CH	3.32, dd, (J=12.26,4.09)	19, 4, 10, 3, 9,6	4
6.	201.1	C	-	-	-
7.	123.5	CH	5.58, d, (J=1.15)	5, 14, 9	-
8.	160.0	C	-	-	-
9.	74.5	C	-	-	-
10.	43.4	C	-	-	-
11.	29.4	CH_2	1.84,dd,(J=4.95,1.94) 2.13, dd, (J=1.94,5.59)	12, 13, 9,810	12
12.	32.1	CH_2	1.79 (dd),(J=4.95,12.91) 2.32,m	13, 9, 14, 11	11
13.	49.0	C	-	-	-
14.	52.7	CH	3.13 (ddd), (J=11.62,2.15)	18, 15, 13, 7, 8, 12	15
15.	31.3	CH_2	2.41 (ddd), 2.47 (m)	13, 14, 16, 17	14
16.	144.3	CH	6.91 (dd),(J=3.23,1.94)	15, 14, 20, 17,13	15
17.	155.2	C	-	-	-
18.	16.4	CH_3	0.88 (s)	12,13,14, 17	-
19.	16.5	CH_3	0.99 (s)	1,10,5,9	-
20.	196.3	C	-	-	-
21.	27.2	CH_3	2.26 (s)	20,17	-

a-alpha; b-beta; (s)-singlet; (d)-doublet (dd)-doublet of doublets; (ddd)-doublet of doublet of doublets; (dt)-doublet of triplets; (td)-triplet of doublets; (ttt)-triplet of triplet of triplets; (m)-multiplets

4.6.5 Compound 5 (Aethiopinolone E)

Compound **5** was isolated as a yellow oil having the molecular formula C_{21} H_{26} O_4 and nine degrees of unsaturation as obtained from HREIMS data. Furthermore, the molecule corresponded to a molecular ion at 343.1901 *m/z* $[M+H]^+$ (Fig. 18).

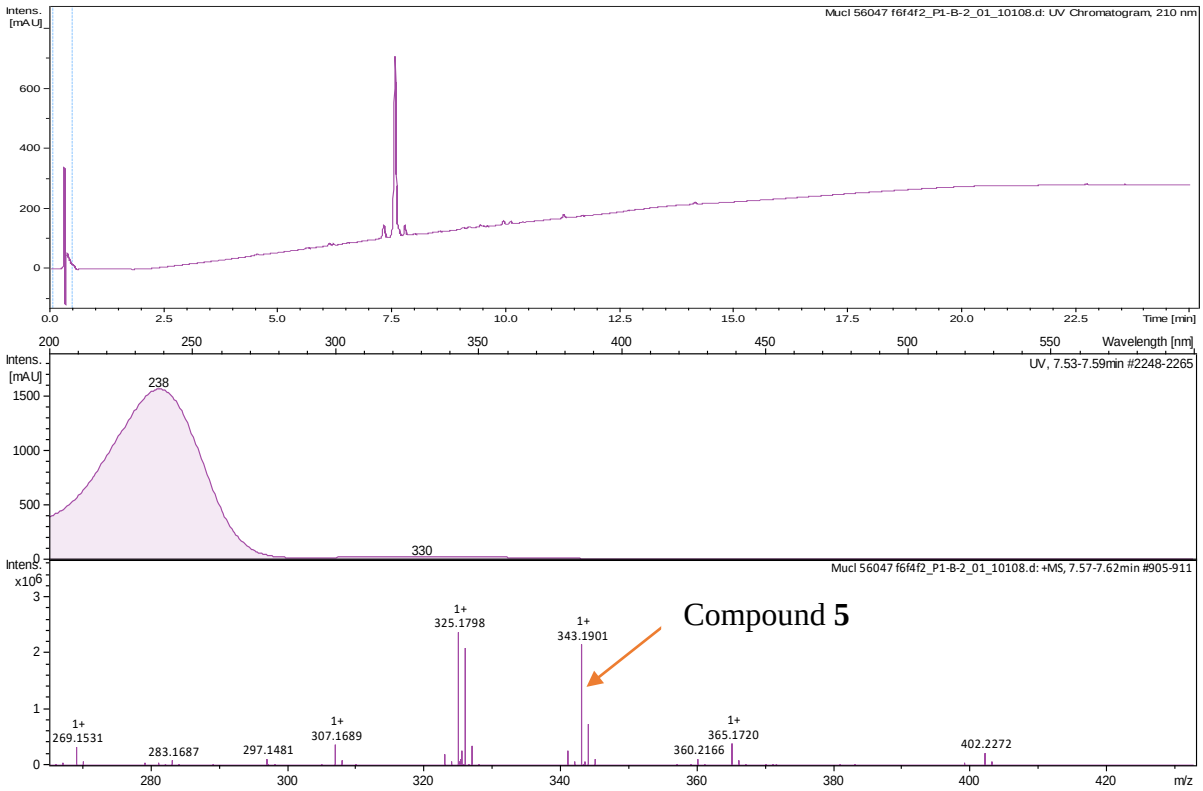


Figure 18: Mass spectrum of compound of **5**

The overall analysis of the data obtained from 1D and 2D NMR (Appendices 28-33) suggested a similarity of the compound's planar structure to compound **3**. However, a difference was noted where a keto group was at C-3 position (Fig. 22). The additional keto carbonyl (C-3) previously absent in compound **3**, was resonating at δ 210.1. Also, the oxygenated methine signal resonating at δ 70.3 that was observed in compound **3** was missing (Table 9).

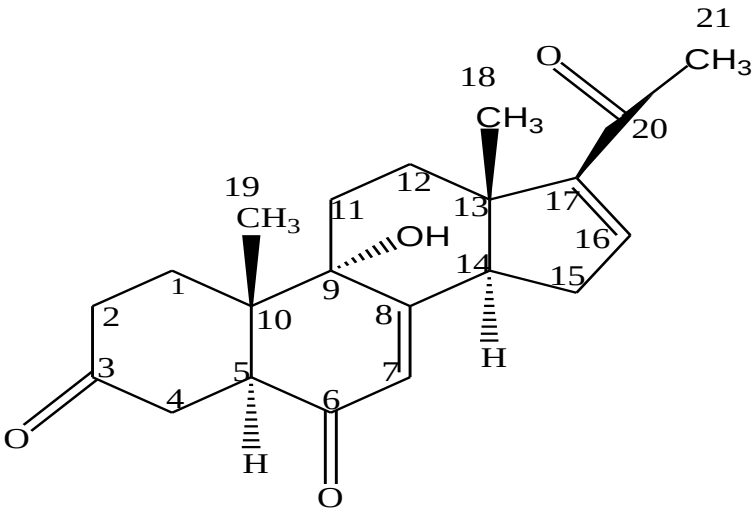


Figure 19: Structure of compound **5**

Table 9: NMR data for compound **5** in Acetone-d_6

Carbon Position	^{13}C (δ_C)	DEPT	^{1}H/HSQC (δ_H (J in Hz))	HMBC	COSY
1.	32.2	CH_2	1.82,m; 1.91,m	2,3,19,5	2
2.	37.5	CH_2	2.26,m; 2.35,m	3,10	1
3.	210.1	C	-	-	-
4.	37.6	CH_2	2.39,m; 2.51,m	5	5
5.	49.0	CH	3.30,dd (J=12.7,4.9)	19,3,10,6	4
6.	198.4	C	-	-	-
7.	123.1	CH	5.68,d (J=2.2)	9,5,14	-
8.	160.8	C	-	-	-
9.	74.6	C	-	-	-
10.	43.1	C	-	-	-
11.	29.6	CH_2	1.92,m; 2.24,m	13,18,12,9	12
12.	32.1	CH_2	1.82,m; 2.35,m	18,13,9	11
13.	49.0	C	-	-	-
14.	52.7	CH	3.14,ddd (J=11.6, 2.2,6.2)	16,7,18,8	15
15.	31.3	CH_2	2.42,m; 2.51,m	16,13,14,17	14,16
16.	144.3	CH	6.92,dd (J=3.4,1.9)	15,13,14,20	15
17.	155.1	C	-	-	-
18.	16.4	CH_3	0.91s	12,13,14,17	-
19.	16.5	CH_3	1.24s	9,1,5,10	-
20.	196.3	C	-	-	-
21.	27.1	CH_3	2.26s	20,17	-

(s)-singlet; (d)-doublet (dd)-doublet of doublets; (ddd)-doublet of doublet of doublets; (dt)-doublet of triplets; (td)-triplet of doublets; (ttt)-triplet of triplet of triplets; (m)-multiplets

4.7 MIC Screening of the Isolated Pure Compounds

The five pregnenolone-type steroids compounds from KE/16-163, were subjected to MIC assays to ascertain the compounds responsible for the previously visualized activity of the strain's crude extracts against *B. subtilis*. Nevertheless, the pure compounds obtained from the fractionation of the strain's mycelial extracts (obtained following fermentation in rice), showed mild (compounds **2** and **3**) to no activity (compounds **1**, **4** and **5**) against the test pathogen (Fig. 20). Ciprofloxacin control used against *B. subtilis* in the assay gave an MIC value of <2.34 µg/ml.

Figure 20: Minimum inhibitory concentration (MIC) assay of 5 pure fungal compounds isolated from KE/16-163 (*Fomitiporia aethiopica*) against *B. subtilis* test pathogen. (**A**) Column (C) 1-compound **1**; C2- compound **2**; C3- compound **3**; C4- compound **4**; C5-compound **5**; C9, Positive control; C10, Negative control. The letter and number before the fungi strain refers to Column number on the microtiter plate e.g. C4 refers to column 4.

4.8 Cytotoxicity Assays

Cytotoxicity assay of the 5 pure compounds isolated from strain KE/16-163 reported in section 4.5 against 7 mammalian cell lines, revealed IC_{50} in the range of 8-70 µg/ml. Generally, selective cytotoxic activities of <20 µg/ml were exhibited by the compounds **1**, **3**, **4** and **5** against 3 cell lines; breast cancer cells (MCF-7), epidermoid carcinoma cells (A431) and HeLa cells (KB3.1). In contrast, compound **2** was non-cytotoxic to all the 7 tested cell lines. Furthermore, all the five compounds were not cytotoxic against 4 cell lines: mouse fibroblast cells (L929), human alveolar basal epithelial cells (A539), prostate cancer cells (PC-3) and ovarian cancer cells (SKOV-3) (Table 10).

Compounds **1**, **3**, **4** and **5** demonstrated considerable cytotoxic effects against MCF-7 cells. Notably, compound **1** was the most cytotoxic compound with IC_{50} at 8 µg/ml against MCF-7. In addition, compounds **3**, **4** and **5** had cytotoxic effects against the MCF-7 cell line at 18 µg/ml, 17 µg/ml and 16 µg/ml respectively. Interestingly, compounds **1** and **5** also revealed cytotoxic effects against HeLa cells (KB3.1) at 19 µg/ml and epidermoid carcinoma cells (A431) at 14 µg/ml respectively. The positive control (Epothilon B) used in the assay, exhibited IC_{50} between 0.0004-0.005 µg/ml (Table 10). The absorbance values/optical densities (ODs) of the compounds and the controls used (at 595 nm) are presented in appendix 34.

Table 10: Cytotoxic effects of the pure pregnenolone-type steroid compounds **1-5** isolated from fungi strain KE/16-163 (*Fomitiporia aethiopica*) against seven mammalian cell lines.

Cell lines	**Cytotoxicity IC_{50} (µg/mL)**					
	1	**2**	**3**	**4**	**5**	**Epothilon B**
L929	28	-	40	45	40	0.0014
KB3.1	19*	-	35	39	33	0.00022
A431	20	-	27	21	14*	0.0006
A549	22	-	70	52	43	0.005
PC-3	26	-	45	40	39	0.0002
SKOV-3	nt	-	38	36	34	0.0014
MCF-7	8*	-	18*	17*	16*	0.0004

- Not active; nt-not tested; data with an asterix IC_{50} values (<20 µg/ml); data without an asterix non-cytotoxic IC_{50} values (≥20 µg/ml); L929-mouse fibroblast cells; KB3.1-HeLa cells; A431-epidermoid carcinoma cells; A549-human alveolar basal epithelial cells; PC-3-prostate cancer cells; SKOV-3-ovarian cancer cells and MCF-7-breast cancer cells.

CHAPTER FIVE
DISCUSSIONS

5.1 Fungal Identification and Characterization

Precise identification and characterization of fungal species is essential in establishing their biology, evolutionary relatedness and their current and possible application in antimicrobial drug discovery. However, the use of a single approach in correctly identifying fungi remains questionable (Ko *et al.,* 2011). Therefore in the study herein, a combination of morphological and molecular based methods were used to identify fungi samples collected from Mt Elgon forest. Although there are shortcomings in the use of morphological based approach, it still remains an important first line step in the identification and characterization of fungi species. Therefore, morphological characteristics were relied on for purposes of identification of fungi strain collected during sampling in Mt Elgon forest and also in identification of cultured fungi in plates. There were fungi samples that possessed distinct morphological characteristics that facilitated their identification. For instance, strain KE/16-176 and KE/16-163 had distinct maroon (Fig. 2B) and thick yellow caps (Fig. 2C), respectively. Furthermore, strain KE/16-193 and KE/16-165 could be easily identified based on their cream ciliate and white resupinate basidiomes, respectively. Also, strains KE/16-138, KE/16-152, KE/16-103 and KE/16-198 were distinguished based on their unique pore shape and spore colors when observed under a microscope.

Cultural characteristics also enhanced the identification of the strains KE/16-153 and KE/16-101 which had a distinct brown and white cottony mycelial growth. However, there were strains which could not easily be distinguished using the obvious morphological characteristics. For example, strains KE/16-115 and KE/16-189, could not be distinguished conclusively due to their similarities in cap and pore colors and shapes as well as their cultural characteristics (Fig. 2A and Fig. 3I and J). A previous study by Toledo *et al.* (2013) also demonstrated that morphological-based approach of identification could be unreliable when used solely due to similarities in the phenotypic and cultural characteristics among fungal strains. This clearly demonstrate the challenges of using morphological or cultural characteristic for precise identification of two or more fungi strains that share almost similar features. Despite shortcoming of morphological characterization, a total of 14 fungi were identified. The data obtained morphologically was hence considered preliminary until their confirmation using molecular methods was performed.

In order to confirm the morphologically identified fungi samples and ascertain the identity of those that were not identified, a second level identification was necessary. Thus molecular markers were used to complement morphological identification strategy to precisely determine the fungi samples, particularly those that were successfully cultured on media in petri-plates. Molecular characterization was conducted using a technique based on sequencing the ITS1-5.8S-ITS2 rDNA region using primers ITS1-F and ITS4 primers that flank the aforementioned rDNA region. The ITS rDNA reliability and effectiveness in Basidiomycetes' identification had earlier been demonstrated by various studies (Schoch *et al.,* 2012; Pryce *et al.,* 2003). Therefore, the successful amplification of the respective ITS region and yielding readable fungal DNA sequences clearly demonstrated the efficiency of ITS1-F and ITS4 primers in identification of fungi belonging to the Basidiomycetes group and in line with earlier reports by Blaalid *et al.* (2013). In addition, other studies have also reported that ITS1 and ITS5 primer pairs are also biased towards the amplification of Basidiomycetes, while pairing primers ITS2 or ITS3 with ITS4 primer have preferentially amplified Ascomycetes (Bellemain *et al*., 2010).

Comparing the sequenced ITS rDNA fragments using CLUSTAL/W confirmed that the 12 axenic fungi cultures were indeed different strains, since there was no full sequence similarity albeit a few short sequences. On the other hand, BLAST search using ITS rDNA sequences aided in the successful identification of 5 out of 12 fungal strains (KE/16-103 (*Perenniporia* sp), KE/16-176 (*Ganoderma* sp), KE/16-138 (*Hexagonia* sp), KE/16-165 (*Antrodia* sp) and KE/16-163 (*Fomitiporia* sp) up to the genus level, and thus supported the morphological-based identification of these strains. Similarly, it helped to reveal the identity of only 4 out of the 12 strains to the species level (KE/16-198 (*Echinochaete brachypora*), KE/16-193 (*Polyporus arcularius*) and KE/16-152 (*Favolaschia calocera*) KE/16-101 (*Inonotus pachyphloeus*)) as previously noted in morphological-based approach. However, there were discordant identifications between morphological- and sequence-based identifications for the strains KE/16-153, KE/16-189 and KE/16-115 which had been morphologically identified as *Phellinus* and *Tyromyces* fungi respectively (Table 1). The BLAST search for the strain KE/16-153 gave 97% sequence similarity to *Inonotus* sp whereas, KE/16-115 and KE/16-189 had 98% sequence similarities to *Skeletocutis nivea* (Appendix 1). According to some studies, fungi of genera *Phellinus* and *Inonotus* have a very close relationship. Hence, this has resulted to some strains being moved from genus *Phellinus* to *Inonotus* and vice versa (Wagner and Fischer, 2002). Also, phylogenetic studies based on

ITS sequences of genera *Tyromyces* and *Skeletocutis* by Yao *et al.* (1999), indicated a very strong link between the two genera. According to the study, *Tyromyces chioneus* was shown to be embedded within *Skeletocutis* clade, revealing their close relationship. Thus, the use of BLAST search tool in identification of fungi could have exhibited drawbacks for the 3 fungal strains, due to databases limited taxonomic scope for the available rDNA ITS sequences for these two genera. It has also been reported that the fungal databases contain inaccurately identified sequences (Ko *et al.*, 2011). Furthermore, there are countless documented examples of failure of BLAST hits reliability in fungi identification, hence leading to wrong conclusions (Christen, 2008). Therefore, such drawbacks may explain the resultant discordant identifications of the 3 fungi strains.

Generally, the fungi strains identified using both the molecular- and morphological-based approaches in this study belonged to the phylum Basidiomycota. Phylogenetic analysis revealed that they shared common taxa; Hymenochaetales (KE/16-101, KE/16-153 and KE/16-163), Polyporales (KE/16-165, KE/16-138, KE/16-198, KE/16-176, KE/16-193, KE/16-115, KE/16-189 and KE/16-103) and Agaricales (KE/16-152) (Fig. 5). Interestingly, similar taxa had been reported previously in Eastern Africa (Decock *et al.,* 2012; Decock *et al.,* 2005; Wagner and Fischer, 2002; Ryverden and Johansen, 1980). Despite the fact that the strains KE/16-115 and KE/16-189 had similar morphological characteristics to *Tyromyces* sp and BLAST hits to *Skeletocutis nivea*, phylogenetic analyses showed that they were different species (Fig. 5). According to various literature, *Skeletocutis nivea* has been only reported in South America, Europe and Asia (Robledo and Rajchenberg, 2007) whereas *Tyromyces* sp has only been reported in the western parts of Africa (Markson *et al.,* 2017).

Similar to morphological approach, the use of the rDNA ITS region in identification of fungal strains is not also devoid of short-comings as demonstrated in the present study. This can also be attributed to high variability among the ITS sequences. According to Lindahl *et al.* (2013), the ITS region is useful in separation of species. For instance, in this study KE/16-115 and KE/16-189 belonging to the same genus, and non-distinguishable due to their similar morphological characteristics did not cluster together on the phylogram. Hence, they were shown to be two distinct species of the same genus. However, the rDNA ITS region is too variable to cater for evolutionary relatedness at higher taxonomic ranks such as orders and families (Lindahl *et al.,* 2013). Therefore, this probably explains why, it was difficult to attain perfect alignment of sequences at the family level of taxonomy for some strains; i.e. *Inonotus* sp and *Fomitiporia* sp which belong to *Hymenochaetaceae* family, as well as

Antrodia sp (*Fomitopsidaceae*), which clustered on the phylogram with fungi of family *Polyporaceae* (Fig. 5). Nonetheless, studies have shown that the use of nuclear large subunit (LSU) rDNA primers could provide a better alternative due to its best resolution at higher levels of taxonomy compared to ITS (Porter and Golding, 2012).

Although a definitive taxonomic identification method for KE/16-115, KE/16-189 and KE/16-153 Basidiomycetes fungi in the study reported herein was not achieved, the data obtained suggests that there exists a great diversity among these fungi in the undisturbed sections of tropical forests such as Mt Elgon. Notably, the results obtained in the study show that in order to approach an effective identification, a combined use of morphological, cultural and molecular characters is essential. Whilst, morphological data and best BLAST matches of ITS sequences were considered to conclusively identify the 12 cultured fungi, the ITS sequences deposited in GenBank cannot be completely relied on to arrive at the identities of fungi strains. Thus, according to Liu *et al.* (2012), Bayesian classifier method which was not used in this study could provide an alternative to BLAST search due to its reviewed accuracy and speed than the latter. Although, ITS rDNA molecular marker formed a significant part of the phylogenetic analysis of the fungi by enhancing characterization and assessment of fungal genetic diversity, additional taxonomic identification markers such as LSU, β-tubulin gene and elongation factor 1α gene (EF-1α) could also be used in addition to the ITS rDNA gene to enhance fungal identification to the species level.

5.2 Antimicrobial Activities of Crude Extracts and Pure Seconday Metabolites

Due to their diverse nature and their potential as a reservoir of novel bioactive compounds with antimicrobial propensities, many studies on tropical fungi have been skewed towards isolating and identification of these novel compounds (Blackwell, 2011; Dai, 2010). In the current study, fungi strains that demonstrated activity against the bacteria were from the genera *Hexagonia* (KE/16-138), *Tyromyces/Skeletocutis* (KE/16-115 and KE/16-189), *Echinochaete* (KE/16-198), *Inonotus* (KE/16-101), *Antrodia* (KE/16-165) and *Fomitiporia* (KE/16-163) (Fig. 7A-F and Fig. 8A and B). On the other hand, the fungal test organisms were inhibited by the extracts from genera *Favolaschia* (KE/16-152), *Inonotus* (KE/16-101) and *Polyporus* (KE/16-193) (Fig. 9A-C). The antagonism visualized might be due to the production of bioactive secondary metabolites in various media as demonstrated by VanderMolen et *al.* (2013). Furthermore, the variation of culture media contents enhanced the diversity and quantity of bioactive fungal compounds as demonstrated by previous studies

(Pu *et al.,* 2013; Xu *et al.,* 2008). Therefore, this undoubtedly led to the diverse antimicrobial activities of the fungi as seen in the study.

5.2.1 Antagonism against bacterial test pathogens

The strains KE/16-115 (*Tyromyces/Skeletocutis* sp) and KE/16-198 (*Echinochaete* sp) were good options to inhibit Gram positive bacteria (*B. subtilis*), exhibited by their low MIC values of 4.69 µg/ml and 18.75 µg/ml respectively in Q6½ mycelial extracts as compared to the ciprofloxacin positive control (2.34 µg/ml) (Fig. 7A and B). Nevertheless, there has been no exhaustive research previously performed on the bioactivities of the strain KE/16-198, despite its taxonomic description by Ryvarden and Johansen (1980). Also, the bioactivities of *Skeletocutis nivea* metabolites have not been reported so far. However, those from genus *Tyromyces* have only been reported for their antioxidant activity (Kim *et al.,* 2012).

The ethyl acetate extracts of KE/16-138 (*Hexagonia* sp) in YMG media were also highly active against *B. subtilis* (Fig. 7D) which is in agreement with a study conducted by Rosa *et al.* (2003), who reported a high growth inhibition of *B. cereus* by *Hexagonia hynoides*. Interestingly, in the present study, the supernatant extracts were more active compared to the mycelial extracts, suggesting that the metabolites secreted into the growth media during fermentation contained active compounds. In fact, only the supernatant extracts of *Hexagonia* sp inhibited *E. coli* (Fig. 8A). A study done by Al-Fatimi *et al.* (2013), showed that *Hexagonia velutina* had a considerable higher antifungal activity against *Trichophyton mentagrophytes* than nystatin reference antibiotic. Contrarily, in the present study, the strain KE/16-138 did not demonstrate any antifungal activity. Although the metabolites responsible for the antimicrobial activity of the fungal genus remains unprecedented, lanostane triterpenoids characterized from *Hexagonia tenuis* have been previously attributed to anti-trypanosomal activity of the strain (Umeyama *et al.,* 2014).

The ethyl acetate extracts of *Antrodia* sp in Q6½ media also demonstrated a considerable activity against *B. subtilis* in the present study (Fig. 7C). According to Ryvarden and Johansen (1980), *Antrodia daedaleiformis* was the first species from the genus to be reported in East Africa. However, up-to-date extensive published works have only been performed on *Antrodia camphorata* which is predominantly used as medicine in Asia (Geethangili and Tzeng, 2011). In fact, similar to the current study, the ethyl acetate as well as ethanol and chloroform extracts of *A. camphorata* exhibited low MIC values against *Streptococcus mutans* and *Porphyromonas gingivalis* bacteria (Lien *et al.,* 2014). The

activities of the fungus have been attributed to terpenoid compounds predominant in the fruiting bodies and mycelial cultures (Geethangili and Tzeng, 2011).

KE/16-163 (*Fomitiporia aethiopica*) was first reported from Ethiopian highlands and described by Decock and Castillo (2005). In this study, antagonistic activities were exhibited by the strain's rice and YMG mycelial extracts against *B. subtilis* (Fig. 7E). However, the five novel pure compounds isolated from the strain's rice grown mycelial extracts were devoid of any significant activity against *B. subtilis*. Lack of activity for the pure compounds against *B. subtilis* unlike crude extract suggests synergistic mode of action. However, studies have reported the association of some species of the genus with Esca disease of grapevine (Graniti *et al.,* 2006; Fischer *et al.,* 2005).

In spite of the fact that antimicrobial activities have not been reported so far on KE/16-101 (*Inonotus pachyphloeus*), the fungus exhibited moderate antimicrobial activity on *E. coli* (Fig. 8B). Reports on related species namely *Inonotus hispidus* and *Inonotus obliquus*, demonstrated their antimicrobial potential (Glamočlija *et al.,* 2015; Suay *et al.,* 2000). In fact, *Inonotus obliquus* ethanolic and aqueous extracts exhibited lower antibacterial activity against *Pseudomonas aeruginosa*, as compared to the control antibiotics (Glamočlija *et al.,* 2015). However, fungal metabolites responsible for the bacterial antagonism by the strain have not been studied.

5.2.2 Antagonism against fungal test pathogens

Fungal strain KE/16-152 (*Favolaschia calocera*) secondary metabolites demonstrated the highest antifungal activities against both filamentous fungi (<2.34 µg/ml) and and yeast cells (9.38 µg/ml) (Fig. 9A and B). Similar results were reported from a previous study by Chepkirui *et al.* (2016). The fungus' strong antifungal activity was attributed to four oxostrobilurins derivatives obtained from the YMG mycelial extracts. However, in the current study, both the supernatant and mycelial extracts of YMG media showed activity.

The metabolites extracted from strains KE/16-193 (*Polyporus arcularius*) and KE/16-101 (*Inonotus pachyphloeus*) showed moderate inhibitory effects against *C. tenuis* (Appendix 2). In a similar way, the crude extracts isolated from genus *Polyporus*, have been used in treatment of various fungal ailments (Zhao, 2013). Although, no antagonism was visualized against the bacteria by strain KE/16-193, methanolic extracts of a related strain *Polyporus squamous* demonstrated excellent antagonism against *P. aeruginosa* without toxicity to hepatocytes according to a study by Fernandes *et al.* (2016). Generally, the activities of the

Polyporus fungal genus have been majorly attributed to steroids, anthraquinones, polysaccharides and nucleosides. The strain KE/16-101 has not been studied extensively however, related species *Inonotus hispidus* was reported to contain phenolic compounds hispolon and hispidin, which could be responsible for its high activity against *Aspergillus fumigatus* (human isolate) (Suay *et al.,* 2000). Furthermore, the activity of *I. hispidus* was higher than ketoconazole antifungal against *Trichoderma viridae* and *Penicillin oochrochloron* according to Glamočlija *et al.* (2015).

5.2.3 Non-antagonistic strains

It was pointed out that KE/16-176 (*Ganoderma* sp), KE/16-103 (*Perenniporia abyssinica*) and KE/16-153 (*Phellinus*/ *Inonotus* sp), were not susceptible to any of the microbial test pathogens used. However, according to reports from previous studies, *Ganoderma lucidium* has been reported as the most famous traditionally used medicinal mushroom (Alves *et al.,* 2012). Its various extracts have been found effective in a similar level to gentamycin sulphate against bacterial pathogens, with the acetone extract being the most active. Nevertheless, moderate inhibitions against *B. subtilis* and *S. aureus* have been reported for any other extract. However, highest activities against both Gram positive and Gram negative bacteria especially *Micrococcus luteus* were obtained with the aqueous extracts (Quereshi *et al.,* 2010; Gao *et al.*, 2005). The ethyl acetate extracts of *Phellinus* sp inhibited growth of *Bacillus cereus*, in a study. However, methanol extracts had moderate activities against *B. subtilis* and *S. aureus* (Rosa *et al.,* 2003; Sheena *et al.,* 2003). *Perenniporia abyssinica* has been described taxonomically by studies done by Decock and Bitew (2012) however, there are no documented bioactivities of the strain. Therefore, absence of any significant antimicrobial activity by the fungus was also noted in this study.

Generally, manipulation of the environment and nutrient composition of the media used in the study, had substantial effects on diversity, quality and quantity of the metabolites produced. Hence, different antimicrobial activities were exhibited by the fungi on various tested microbial pathogens. Similarly, previous research had also shown that optimization of cultural conditions enhance synthesis of a wide range of fungal compounds (VanderMolen *et al.,* 2013; Shih *et al.,* 2007). Therefore, varied media contents due to diverse nutrient preferences by fungal strains, enhanced metabolite output and success of natural product discovery (Bashir *et al.,* 2012), as seen in this study. Furthermore, the results obtained from this work reveal that secondary metabolites present in Basidiomycetes have the potential to

be exploited as leads for antimicrobial drug development to keep up pace with the evolution of 'superbugs'.

5.3 The Isolated Secondary Metabolites

Five novel pregnenolone-type steroid compounds were isolated from the mycelial cultures of the strain KE/16-163 (*Fomitiporia aethiopica*) in rice media. The steroid compounds are the first to be isolated from this particular genus. Nonetheless, steroids and terpenoids had been reported to occur predominantly in Basidiomycetes (Hu *et al.,* 2015; Wang *et al.,* 2015; Yin *et al.,* 2015). Notably, pregnane-type streroids have been isolated from *Phellinus igniarius* and *Phaeosphaeria spartinae* (Yin *et al.,* 2015; Elsebai *et al.,* 2013). However, pregnenolone-type compounds had earlier been unprecedented in fungal metabolism.

5.3.1 Antimicrobial activity and cytotoxic effects of the isolated secondary metabolites

There were no significant antimicrobial activities shown by the pregnenolone-type steroid compounds isolated from the fungus. Probably, the antibacterial activity shown by the YMG and rice media crude extracts against the bacterial test pathogen (*B. subtilis*) was due to synergistic activity of compounds as reported earlier by studies (Savage *et al.,* 2002). Nevertheless, 17 chalconyl derivatives of pregnenolones synthesized in a research study, had antimicrobial activity against all the tested microbial strains (Banday *et al.,* 2011). Thus, this suggests that molecular modifications can be employed to maximize the antimicrobial properties of the compounds.

Studies on the cytotoxic effects of the fungal compounds are also significant in deducing the toxicological risks associated with their use and in their consideration as leads in drug discovery. This is because most biologically active metabolites previously isolated from fungi are mycotoxins (Alassane-Kpembi *et al.,* 2013). In the present study, pure compounds **1**, **3**, **4** and **5** isolated from ethyl acetate extracts of KE/16-163, showed a significant selective cytotoxicity against breast cancer (MCF-7), HeLa (KB3.1) and epidermoid carcinoma (A431) cell lines. Compound **2** was the only metabolite considered non-cytotoxic to all cell lines, since IC_{50} could not be obtained at the highest concentration (111 µg/ml) of the compound (Table 9). The results are therefore in agreement with a previous study on the synthesis and cytotoxicity of various benzylidine pregnenolones and their oximes (Banday *et al.,* 2014). According to the study, the synthesized analogs exhibited significant potency against MCF-7 (breast cancer cells) and HCT-15 (colorectal

adenocarcinoma cells). Furthermore, alkynylaryl pregnenolone derivatives that were previously designed and synthesized exhibited high potency with IC_{50} of 2.18 µg/ml in A431 (epidermoid carcinoma) cells (Szalóki *et al.*, 2014).

Therefore, the selective toxicity of the pregnenolone-type steroids isolated in this study against MCF-7, KB3.1 and A431 cancer cell lines is vital. This is because the previous studies stated, clearly show that synthesized pregnenolone type compounds have anticancer properties. Thus, this sparks an interesting discussion with regards to their probable application in the development of novel fungal-based anticancer drugs.

CHAPTER SIX
CONCLUSIONS AND RECOMMENDATIONS

6.1 CONCLUSIONS

Based on the results obtained in this study, the conclusions made include:

i. Coupled use of morphological-, cultural- and molecular-based methods were efficient in the characterization of most Basidiomycetes except for three strains (KE/16-115, KE/16-189 and KE/16-153), which were not conclusively identified.
ii. The crude extracts of 75% of the fungi obtained from Mt. Elgon National Park forest possessed antibacterial and antifungal activities against *B. subtilis*, *E. coli*, *M. plumbeus* and *C. tenuis*. The activities were enhanced by variation of culture media contents.
iii. The pure compounds isolated from KE/16-163 (*Fomitiporia aethiopica*) in this study had mild antimicrobial activities. This may imply that the antimicrobial activity initially observed in crude extracts and its absence in the purified compounds may be due to synergistic mode of action by the compounds.
iv. The novel compounds obtained from *Fomitiporia aethiopica*, possessed cytotoxic effects against breast cancer cells, epidermoid carcinoma cells and HeLa cells. Thus, their selective toxicity imply that they may be applied in cancer treatment.

6.2 RECOMMENDATIONS

i. Further studies should be conducted on alternative identification methods such as the use of LSU, β-tubulin gene and elongation factor 1α gene (EF-1α) in addition to the ITS rDNA to aid in the identification of all Basidiomycetes fungi to the species level.
ii. Large scale fermentation, fractionation, purification and toxicological studies of the crude and pure compounds of the remaining bioactive fungal strains should be carried out to establish the antimicrobial compounds present.
iii. Although the antimicrobial activity of the pure compounds isolated from KE/16-163 were mild, the structures obtained may be modified to increase their potency and stability.
iv. Further studies are needed to evaluate the potency of the novel cytotoxic pregnenolone-type steroids isolated, in the development of cancer drugs.

REFERENCES

Alassane-Kpembi, I., Kolf-Clauw, M., Gauthier, T., Abrami, R., Abiola, F. A., Oswald, I. P. and Puel, O. (2013). New insights into mycotoxin mixtures: The toxicity of low doses of Type B trichothecenes on intestinal epithelial cells is synergistic. *Toxicology and Applied Pharmacology*, **272**: 191-198.

Alastruey-Izquierdo, A., Melhem, M. S., Bonfietti, L. X. and Rodriguez-Tudela, J. L. (2015). Susceptibility test for fungi: clinical and laboratorial correlations in medical mycology. *Revista do Instituto de Medicina Tropical de São Paulo*, **57**: 57-64.

Alexopoulos, C. J., Mims, C. W. and Blackwell, M. (1996). *Introductory mycology* (4th ed). New York, NY: John Wiley and Sons.

Al-Fatimi, M., Schröder, G., Kreisel, H. and Lindequist, U. (2013). Biological activities of selected Basidiomycetes from Yemen. *Die Pharmazie-An International Journal of Pharmaceutical Sciences*, **68**: 221-226.

Allegranzi, B., Nejad, S. B., Combescure, C., Graafmans, W., Attar, H., Donaldson, L. and Pittet, D. (2011). Burden of endemic health-care-associated infection in developing countries: systematic review and meta-analysis. *The Lancet*, **377**: 228-241.

Allen, C. A. and Torres, A. G. (2008). Host-microbe communication within the GI tract. In *GI Microbiota and Regulation of the Immune System* (pp. 93-101). New York, NY: Springer.

Alpert, N. L., Keiser, W. E. and Szymanski, H. A. (2012). *IR: Theory and practice of infrared spectroscopy* (2nd ed). New York, NY; Plenum Press.

Alves, M. J., Ferreira, I. C., Dias, J., Teixeira, V., Martins, A. and Pintado, M. (2012). A review on antimicrobial activity of mushroom (Basidiomycetes) extracts and isolated compounds. *Planta Medica*, **16**: 1707-1718.

Ameri, A., Vaidya, J. G. and Deokule, S. S. (2011). *In vitro* evaluation of anti-staphylococcal activity of *Ganoderma lucidum*, *Ganoderma praelongum* and *Ganoderma resinaceum* from Pune, India. *African Journal of Microbiology Research*, **3**: 328-333.

Andrade, R. J. and Tulkens, P. M. (2011). Hepatic safety of antibiotics used in primary care. *Journal of Antimicrobial Chemotherapy*, **66**: 1431-1446.

Arimi, S. M., Koroti, E., Kang'ethe, E. K., Omore, A. O. and McDermott, J. J. (2005). Risk of infection with Brucella abortus and Escherichia coli O157: H7 associated with marketing of unpasteurized milk in Kenya. *Acta Tropica*, **96**: 1-8.

Astashkina, A., Mann, B. and Grainger, D. W. (2012). A critical evaluation of *in vitro* cell culture models for high-throughput drug screening and toxicity. *Pharmacology & therapeutics*, **1**: 82-106.

Baldauf, S. L., Roger, A. J., Wenk-Siefert, I. and Doolittle, W. F. (2000). A kingdom-level phylogeny of eukaryotes based on combined protein data. *Science*, **290**: 972-977.

Balkis, M. M., Leidich, S. D., Mukherjee, P. K., and Ghannoum, M. A. (2002). Mechanisms of fungal resistance. *Drugs*, **62**: 1025-1040.

Banday, A. H., Akram, S. M. and Shameem, S. A. (2014). Benzylidine pregnenolones and their oximes as potential anticancer agents: synthesis and biological evaluation. *Steroids*, **84**: 64-69.

Banday, A. H., Zargar, M. I., and Ganaie, B. A. (2011). Synthesis and antimicrobial studies of chalconyl pregnenolones. *Steroids*, **76**: 1358-1362.

Barb, I. V. and Neamţu, M. (2012). ACUTE RENAL FAILURE FROM INTOXICATION BY MUSHROOMS. *Acta Medica Transilvanica*, **17**.208-210.

Bashir, Z. A., Ahmad, A., Nor, S. M. and Usup, G. (2012). Factors affecting bioactivity of secondary metabolites produced by Streptomyces sp. PT1 using Plackett-Burman design. *Advances in Environmental Biology*, **6**: 3043-3052.

Bellemain, E., Carlsen, T., Brochmann, C., Coissac, E., Taberlet, P. and Kauserud, H. (2010). ITS as an environmental DNA barcode for fungi: an in silico approach reveals potential PCR biases. *BMC Microbiology*, **10**: 189-197.

Blaalid, R., Kumar, S., Nilsson, R. H., Abarenkov, K., Kirk, P. M. and Kauserud, H. (2013). ITS1 versus ITS2 as DNA metabarcodes for fungi. *Molecular Ecology Resources*, **13**: 218-224.

Blackwell, M. (2011). The Fungi: 1, 2, 3… 5.1 million species? *American Journal of Botany*, **98***:* 426-438.

Borchers, A. T., Keen, C. L. and Gershwin, M. E. (2004). Mushrooms, tumors, and immunity: an update. *Experimental Biology and Medicine*, **229**: 393-406.

Boucher, H. W., Talbot, G. H., Bradley, J. S., Edwards, J. E., Gilbert, D., Rice, L. B., Scheld, M., Spellberg, B. and Bartlett, J. (2009). Bad bugs, no drugs: no ESKAPE! An update from the Infectious Diseases Society of America. *Clinical Infectious Diseases*, **48**: 1-12.

Braude, E. A. and Nachod, F. C. (Eds.). (2013). *Determination of organic structures by physical methods*. New York, NY: Academic Press Inc.

Brown, G. D., Denning, D. W., Gow, N. A., Levitz, S. M., Netea, M. G. and White, T. C. (2012). Hidden killers: human fungal infections. *Science Translational Medicine*, **4**: 1-9.

Buckingham, J. (2017). *Dictionary of Natural Products on DVD; Chapman & Hall, Chemical Database*. Boca Raton, Florida: CRC Press.

Chambers, H. F. and DeLeo, F. R. (2009). Waves of resistance: *Staphylococcus aureus* in the antibiotic era. *Nature Reviews Microbiology*, **7**: 629-641.

Chamilos, G. and Kontoyiannis, D. P. (2005). Update on antifungal drug resistance mechanisms of *Aspergillus fumigatus*. *Drug Resistance Updates*, **8**: 344-358.

Chen, J. and Seviour, R. (2007). Medicinal importance of fungal β-(1→ 3), (1→ 6)-glucans. *Mycological research*, **111**: 635-652.

Chepkirui, C., Richter, C., Matasyoh, J. C. and Stadler, M. (2016). Monochlorinated calocerins AD and 9-oxostrobilurin derivatives from the basidiomycete *Favolaschia calocera*. *Phytochemistry*, **132**: 95-101.

Christen, R. (2008). Global sequencing: a review of current molecular data and new methods available to assess microbial diversity. *Microbes and Environments*, **23**: 253-268.

Clinical and Laboratory Standards Institute (2018). *Methods for Antimicrobial Susceptibity Testing of Anaerobic Bacteria* (9th ed). Wayne, PA: Clinical and Laboratory Standards Institute.

Cos, P., Vlietinck, A. J., Berghe, D. V. and Maes, L. (2006). Anti-infective potential of natural products: how to develop a stronger *in vitro* 'proof-of-concept'. *Journal of Ethnopharmacology*, **106**: 290-302.

Courant, F., Antignac, J. P., Dervilly-Pinel, G. and Le Bizec, B. (2014). Basics of mass spectrometry based metabolomics. *Proteomics*, **14**: 2369-2388.

Cowen, L. E. (2008). The evolution of fungal drug resistance: modulating the trajectory from genotype to phenotype. *Nature Reviews Microbiology*, **6**: 187-198.

Cragg, G. M. and Newman, D. J. (2013). Natural products: a continuing source of novel drug leads. *Biochimica et Biophysica Acta (BBA)-General Subjects*, **1830**: 3670-3695.

Dai, Y. C. (2010). Hymenochaetaceae (Basidiomycota) in China. *Fungal Diversity*, **45**: 131-343.

Danishuddin, M., Kaushal, L., Baig, M. H. and Khan, A. U. (2012). AMDD: Antimicrobial drug database. *Genomics, Proteomics & Bioinformatics*, **10**: 360-363.

Davies J. (2014). The Origin and Evolution of Antibiotics. In F. Marinelli and O. Genilloud (Eds.), *Antimicrobials* (pp. 3-10). Berlin, Heidelberg: Springer.

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

D'Costa V. and Wright G. D. (2009). Biochemical Logic of Antibiotic Inactivation and Modification. In D. L Mayers. (Ed.), *Antimicrobial Drug Resistance* (pp. 81-95). New York, NY: Humana Press.

De Kraker, M. E., Davey, P. G., Grundmann, H. and BURDEN Study Group. (2011). Mortality and hospital stay associated with resistant *Staphylococcus aureus* and *Escherichia coli* bacteremia: estimating the burden of antibiotic resistance in Europe. *PLoS Med*, **8**: e1001104.

de Pauw, B. E. (2011). What are fungal infections? *Mediterranean journal of hematology and infectious diseases*, **3**: 1-7.

De Silva, D. D., Rapior, S., Fons, F., Bahkali, A. H. and Hyde, K. D. (2012). Medicinal mushrooms in supportive cancer therapies: an approach to anti-cancer effects and putative mechanisms of action. *Fungal Diversity*, **55**: 1-35.

De Silva, D. D., Rapior, S., Sudarman, E., Stadler, M., Xu, J., Alias, S. A. and Hyde, K. D. (2013). Bioactive metabolites from macrofungi: ethnopharmacology, biological activities and chemistry. *Fungal Diversity*, **62**: 1-40.

Decock, C. and Bitew, A. (2012). Studies in *Perenniporia* (Basidiomycota). African taxa VI. A new species and a new record of *Perenniporia* from the Ethiopian Afromontane forests. *Plant Ecology and Evolution*, **145**: 272-278.

Decock, C., Bitew, A. and Castillo, G. (2005). *Fomitiporia tenuis* and *Fomitiporia aethiopica* (Basidiomycetes, Hymenochaetales), two undescribed species from the Ethiopian highlands: taxonomy and phylogeny. *Mycologia*, **97**: 121-129.

Deyrup, S. T., Gloer, J. B., O'Donnell, K. and Wicklow, D. T. (2007). Kolokosides AD: Triterpenoid Glycosides from a Hawaiian Isolate of *Xylaria* sp. *Journal of natural products*, **70**: 378-382.

Diehl, B. (2008). Principles in NMR spectroscopy. In U. Holzgrabe, I. Wawer and B. Diehl (Eds.), *NMR Spectroscopy in Pharmaceutical Analysis* (pp. 1-41). Amsterdam: Elsevier.

Eisold, J. and Mostrom, M. (2011). Mycotoxins and mushrooms. *Small Animal Toxicology Essentials*, **23**: 189-206.

Elsebai, M. F., Kehraus, S. and König, G. M. (2013). Caught between triterpene-and steroid-metabolism: 4α-carboxylic pregnane-derivative from the marine alga-derived fungus *Phaeosphaeria spartinae*. *Steroids*, **78**: 880-883.

Erkel, G. and Anke, T. (2008). Products from basidiomycetes. *Biotechnology Set, Second Edition*, **7**: 489-533.

Fernandes, Â., Petrović, J., Stojković, D., Barros, L., Glamočlija, J., Soković, M., Martins, A. and Ferreira, I. C. (2016). *Polyporus squamosus* (Huds.) Fr from different origins: Chemical characterization, screening of the bioactive properties and specific antimicrobial effects against *Pseudomonas aeruginosa*. *LWT-Food Science and Technology*, **69**: 91-97.

Fischer, M., Edwards, J., Cunnington, J. H. and Pascoe, I. G. (2005). Basidiomycetous pathogens on grapevine: a new species from Australia-*Fomitiporia australiensis*. *Mycotaxon*, **92**: 85-96.

Fisher, M. C., Henk, D. A., Briggs, C. J., Brownstein, J. S., Madoff, L. C., McCraw, S. L. and Gurr, S. J. (2012). Emerging fungal threats to animal, plant and ecosystem health. *Nature*, **484**: 186-194.

Fraser, J. A., Giles, S. S., Wenink, E. C., Geunes-Boyer, S.G., Wright, J. R., Diezmann, S., Allen, A., Stajich, J. E., Dietrich, F. S., Perfect, J. R., and Heitman, J. (2005). Same-sex mating and the origin of the Vancouver Island *Cryptococcus gattii* outbreak. *Nature*, **437**: 1360-1364.

Gagne, J. J., Breitbart, R. E., Maio, V., Horn, D. L., Hartmann, C. W., Swanson, R. and Goldfarb, N. I. (2006). Costs associated with candidemia in a hospital setting. *Pharmacology and Therapeutics*, **31**: 586-619

Gallo, A., Ferrara, M. and Perrone, G. (2013). Phylogenetic study of polyketide synthases and nonribosomal peptide synthetases involved in the biosynthesis of mycotoxins. *Toxins*, **5**: 717-742.

Gao, Y., Tang, W., Gao, H., Chan, E., Lan, J., Li, X. and Zhou, S. (2005). Antimicrobial activity of the medicinal mushroom *Ganoderma*. *Food Reviews International*, **21**: 211-229.

Gardes, M. and Bruns, T. D. (1993). ITS primers with enhanced specificity for basidiomycetes-application to the identification of mycorrhizae and rusts. *Molecular Ecology*, **2**: 113-118.

Geethangili, M. and Tzeng, Y. M. (2011). Review of pharmacological effects of Antrodia camphorata and its bioactive compounds. *Evidence-Based Complementary and Alternative Medicine*, **2011**: 1-17.

Geran, R. I. (1972). Protocols for screening chemical agents and natural products against animal tumors and other biological systems. *Cancer Chemotherapy Reports*, **3**: 51-61.

Gilardoni, G., Clericuzio, M., Tosi, S., Zanoni, G. and Vidari, G. (2007). Antifungal acylcyclopentenediones from fruiting bodies of Hygrophorus chrysodon. *Journal of Natural Products*, **70**: 137-139.

Gill, S. R., Pop, M., DeBoy, R. T., Eckburg, P. B., Turnbaugh, P. J., Samuel, B. S., Gordon, J. I., Relman, D. A., Fraser-Liggett, C. M. and Nelson, K. E. (2006). Metagenomic analysis of the human distal gut microbiome. *Science*, **312**: 1355-1359.

Glamočlija, J., Ćirić, A., Nikolić, M., Fernandes, Â., Barros, L., Calhelha, R. C., Ferreira, I. C., Soković, M. and Van Griensven, L. J. (2015). Chemical characterization and biological activity of Chaga (*Inonotus obliquus*), a medicinal "mushroom". *Journal of Ethnopharmacology*, **162**: 323-332.

Gould, I. M. and Bal, A. M. (2013). New antibiotic agents in the pipeline and how they can help overcome microbial resistance. *Virulence*, **4**: 185-191.

Graniti, A., Giovanni, B., Ciccarone, C. and Sparapano, L. (2006). Infection of Grapevines by Some Fungi Associated with Esca. II. Interaction among «*Phaeoacremonium chlamydosporum*, *P. aleophilum*» and «*Fomitiporia punctata*». *Phytopathologia Mediterranea*, **39**: 53-58.

Grossman, N. T., Pham, C. D., Cleveland, A. A. and Lockhart, S. R. (2015). Molecular mechanisms of fluconazole resistance in *Candida parapsilosis* isolates from a US surveillance system. *Antimicrobial Agents and Chemotherapy*, **59**: 1030-1037.

Gurnani, N., Mehta, D., Gupta, M. and Mehta, B. K. (2014). Natural products: source of potential drugs. *African Journal of Basic and Applied Sciences*, **6**: 171-186.

Guto, J. A., Bii, C. C. and Denning, D. W. (2016). Estimated burden of fungal infections in Kenya. *The Journal of Infection in Developing Countries*, **10**: 777-784.

Halecker, S., Surup, F., Kuhnert, E., Mohr, K. I., Brock, N. L., Dickschat, J. S., Junker, C., Schulz, B. and Stadler, M. (2014). Hymenosetin, a 3-decalinoyltetramic acid antibiotic from cultures of the ash dieback pathogen, *Hymenoscyphus pseudoalbidus*. *Phytochemistry*, **100**: 86-91.

Havlickova, B., Czaika, V. A. and Friedrich, M. (2008). Epidemiological trends in skin mycoses worldwide. *Mycoses*, **51**: 2-15.

Holzgrabe, U. (2017). *NMR Spectroscopy in Pharmaceutical Analysis*. I. Wawer and D. Bernd (Eds.). Amsterdam: Elsevier.

Hu, D. B., Zhang, S., He, J. B., Dong, Z. J., Li, Z. H., Feng, T. and Liu, J. K. (2015). Brasilane sesquiterpenoids and alkane derivatives from cultures of the Basidiomycete *Coltricia sideroides*. *Fitoterapia*, **104**: 50-54.

Hubbard, B. K. and Walsh, C. T. (2003). Vancomycin assembly: nature's way. *Angewandte Chemie International Edition*, **42**: 730-765.

Iijima, Y., Oundo, J. O., Hibino, T., Saidi, S. M., Hinenoya, A., Osawa, K., Shirakawa, T., Osawa, R. and Yamasaki, S. (2017). High prevalence of diarrheagenic Escherichia coli among children with diarrhea in Kenya. *Japanese journal of infectious diseases*, **70**: 80-83.

Isaka, M., Chinthanom, P., Kongthong, S., Srichomthong, K. and Choeyklin, R. (2013). Lanostane triterpenes from cultures of the Basidiomycete *Ganoderma orbiforme* BCC 22324. *Phytochemistry*, **87**: 133-139.

Ishii, H. and Holloman, D. W. (2015). *Fungicide Resistance in Plant Pathogens*. Tokyo: Springer, **10**: 978-4.

Jaeschke, H., McGill, M. R. and Ramachandran, A. (2012). Oxidant stress, mitochondria, and cell death mechanisms in drug-induced liver injury: lessons learned from acetaminophen hepatotoxicity. *Drug Metabolism Reviews*, **44**: 88-106.

Kalač, P. (2009). Chemical composition and nutritional value of European species of wild growing mushrooms: A review. *Food Chemistry*, **113**: 9-16.

Kaplan, R. and White, D. A. (2016). Pulmonary Infections in Immunosuppressed Patients. In L. Claude (Ed.), *Respiratory Infections* (pp. 159-185). Boca Raton, Florida: CRS Press.

Kawagishi, H. (2010). Biologically functional molecules from mushrooms. In S. C. Mandal, V. Mandal and T. Konishi (Eds.), *Natural Products and Drug Discovery: An Integrated Approach* (pp. 309-326). Amsterdam: Elsevier.

Kearse, M., Moir, R., Wilson, A., Stones-Havas, S., Cheung, M., Sturrock, S., Buxton, S., Cooper, A., Markowitz, S., Duran, C. and Thierer, T. (2012). Geneious Basic: an integrated and extendable desktop software platform for the organization and analysis of sequence data. *Bioinformatics*, **28**: 1647-1649.

Kim, S. E., Lee, I. K., Jung, Y. A., Yeom, J. H., Ki, D. W., Lee, M. S., Song, J. G., Jin, Y. J., Seok, S. J. and Yun, B. S. (2012). Mushrooms collected from Deogyu Mountain, Muju, Korea and their antioxidant activity. *Mycobiology*, **40**: 134-137.

Ko, T. W. K., Stephenson, S. L., Bahkali, A. H. and Hyde, K. D. (2011). From morphology to molecular biology: can we use sequence data to identify fungal endophytes? *Fungal Diversity*, **50**: 113-120.

Köck, R., Becker, K., Cookson, B., van Gemert-Pinen, J. E., Harbarth, S., Kluytmans, J., Mielke, M., Peters, G., Skov, R.L., Struelens, M. J., Tacconelli, E., Navarro Torné, A., Witte, W. and Friedrich, A.W. (2010). Methicillin-resistant *Staphylococcus aureus* (MRSA): burden of disease and control challenges in Europe. *Eurosurveillance*, **15**: 19688-19697.

Kozlovskii, A. G., Zhelifonova, V. P. and Antipova, T. V. (2013). Fungi of the genus Penicillium as producers of physiologically active compounds (Review). *Applied Biochemistry and Microbiology*, **49**: 1-10.

Kumar, A. and Schweizer, H. P. (2005). Bacterial resistance to antibiotics: active efflux and reduced uptake. *Advanced Drug Delivery Reviews*, **57**: 1486-1513.

Larkin, M. A., Blackshields, G., Brown, N. P., Chenna, R., McGettigan, P. A., McWilliam, H., Valentin, F., Wallace, I. M., Wilm, A., Lopez, R. and Thompson, J.D. (2007). Clustal W and Clustal X version 2.0. *Bioinformatics*, **23**: 2947-2948.

Lau, C. C., Abdullah, N., Shuib, A. S., and Aminudin, N. (2012). Proteomic analysis of antihypertensive proteins in edible mushrooms. *Journal of Agricultural and Food Chemistry*, **60**: 12341-12348.

Lederberg, J., Hamburg, M. A. and Smolinski, M. S. (Eds.). (2003). *Microbial Threats to Health: emergence, detection, and response*. Washington, U. S. A: National Academies Press.

Lee, J. S. and Hong, E. K. (2011). Immunostimulating activity of the polysaccharides isolated from *Cordyceps militaris*. *International Immunopharmacology*, **11**: 1226-1233.

Lertvannavit, O. and Suankratay, C. (2013). Systemic Trichosporonosis: a mini review. *Journal of Infectious Diseases and Antimicrobial Agents*, **30**: 57-60.

Letunic, I. and Bork, P. (2016). Interactive tree of life (iTOL) v3: an online tool for the display and annotation of phylogenetic and other trees. *Nucleic acids research*, **44**: W242-W245.

Lewis, K. (2013). Platforms for antibiotic discovery. *Nature Reviews Drug Discovery*, **12**: 371-387.

Li, J. W. H. and Vederas, J. C. (2009). Drug discovery and natural products: end of an era or an endless frontier? *Science*, **325**: 161-165.

Lien, H. M., Tseng, C. J., Huang, C. L., Lin, Y. T., Chen, C. C. and Lai, Y. Y. (2014). Antimicrobial activity of *Antrodia camphorata* extracts against oral bacteria. *PloS One*, **9**: e105286.

Lindahl, B. D., Nilsson, R. H., Tedersoo, L., Abarenkov, K., Carlsen, T., Kjøller, R., Kõljalg, U., Pennanen, T., Rosendahl, S., Stenlid, J. and Kauserud, H. (2013). Fungal community analysis by high-throughput sequencing of amplified markers–a user's guide. *New Phytologist*, **199**: 288-299.

Lindahl, B. D., Nilsson, R. H., Tedersoo, L., Abarenkov, K., Carlsen, T., Kjøller, R., Kõljalg, U., Pennanen, T., Rosendahl, S., Stenlid, J. and Kauserud, H. (2015). A new antibiotic kills pathogens without detectable resistance. *Nature*, **517**: 455-459.

Lindequist, U., Niedermeyer, T. H. and Jülich, W. D. (2005). The pharmacological potential of mushrooms. *Evidence-Based Complementary and Alternative Medicine*, **2**: 285-299.

Liu, K. L., Porras-Alfaro, A., Kuske, C. R., Eichorst, S. A. and Xie, G. (2012). Accurate, rapid taxonomic classification of fungal large-subunit rRNA genes. *Applied and Environmental Microbiology*, **78**: 1523-1533.

Lynch, S. V. and Wiener-Kronish, J. P. (2008). Novel strategies to combat bacterial virulence. *Current Opinion in Critical Care*, **14**: 593-599.

Mandengue, C. E. and Denning, D. W. (2018). The Burden of Serious Fungal Infections in Cameroon. *Journal of Fungi*, **4**: 44-52.

Markson, A. A., Akwaji, P.I. and Umana, E. J. (2017). Mushroom Biodiversity of Cross River National Park (Oban Hills Division), Nigeria. *World Scientific News*, **65**: 59-80.

Marshall, B. M. and Levy, S. B. (2011). Food animals and antimicrobials: impacts on human health. *Clinical Microbiology Reviews*, **24**: 718-733.

Martin, G. S., Mannino, D. M., Eaton, S. and Moss, M. (2003). The epidemiology of sepsis in the United States from 1979 through 2000. *New England Journal of Medicine*, **348**: 1546-1554.

Menzin, J., Meyers, J. L., Friedman, M., Perfect, J. R., Langston, A. A., Danna, R. P. and Papadopoulos, G. (2009). Mortality, length of hospitalization, and costs associated

with invasive fungal infections in high-risk patients. *American Journal of Health-System Pharmacy*, **66**: 1711-1717.

Mink, C. M. and Yeh, S. (2009). Infections in child-care facilities and schools. *Pediatrics in Review/American Academy of Pediatrics*, **30**: 259-268.

Morar, M. and Wright, G. D. (2010). The genomic enzymology of antibiotic resistance. *Annual Review of Genetics*, **44**: 25-51.

Mosmann, T. (1983). Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. *Journal of Immunological Methods*, **65**: 55-63.

Mothana, R. A., Jansen, R., Jülich, W. D. and Lindequist, U. (2000). Ganomycins A and B, new antimicrobial farnesyl hydroquinones from the basidiomycete *Ganoderma pfeifferi*. *Journal of Natural Products*, **63**: 416-418.

Moto, J. N., Maingi, J. M. and Nyamache, A. K. (2015). Prevalence of Tinea capitis in school going children from Mathare, informal settlement in Nairobi, Kenya. *BMC Research Notes*, **8**: 274-277.

Musk, D. J. and Hergenrother, P. J. (2008). Chelated iron sources are inhibitors of *Pseudomonas aeruginosa* biofilms and distribute efficiently in an *in vitro* model of drug delivery to the human lung. *Journal of Applied Microbiology*, **105**: 380-388.

Nikolcheva, L.G., Cockshutt, M.A. and Barlocher, F. (2003). Determining Diversity of Freshwater Fungi on Decaying Leaves: Comparison of Traditional and Molecular Approaches. *Applied and Environmental Microbiology*, **69**: 2548-2554.

Nucci, M. and Colombo, A. L. (2002). Emergence of resistant *Candida* in neutropenic patients. *Brazilian Journal of Infectious Diseases*, **6**: 124-128.

Obritsch, M. D., Fish, D. N., MacLaren, R. and Jung, R. (2005). Nosocomial infections due to multidrug-resistant *Pseudomonas aeruginosa*: epidemiology and treatment options. *Pharmacotherapy: The Journal of Human Pharmacology and Drug Therapy*, **25**: 1353-1364.

Ochoa-Zarzosa, A., Vázquez-Garcidueñas, M. S., Robinson-Fuentes, V. A. and Vázquez-Marrufo, G. (2011). Antibacterial and cytotoxic activity from basidiocarp extracts of the edible mushroom *Lactarius indigo* (Schw.) Fr. (Russulaceae). *African Journal of Pharmacy and Pharmacology*, **5**: 281-288.

Okanya, P. W., Mohr, K. I., Gerth, K., Jansen, R. and Müller, R. (2011). Marinoquinolines A− F, Pyrroloquinolines from *Ohtaekwangia kribbensis* (Bacteroidetes). *Journal of Natural Products*, **74**: 603-608.

Oldenburg, K. R., Vo, K. T., Ruhland, B., Schatz, P. J. and Yuan, Z. (1996). A dual culture assay for detection of antimicrobial activity. *Journal of Biomolecular Screening*, **1**: 123-130.

Packey, C. D. and Sartor, R. B. (2009). Commensal bacteria, traditional and opportunistic pathogens, dysbiosis and bacterial killing in inflammatory bowel diseases. *Current Opinion in Infectious Diseases*, **22**: 292-301.

Paramythiotou, E., Lucet, J. C., Timsit, J. F., Vanjak, D., Paugam-Burtz, C., Trouillet, J. L. and Andremont, A. (2004). Acquisition of multidrug-resistant *Pseudomonas aeruginosa* in patients in intensive care units: role of antibiotics with antipseudomonal activity. *Clinical Infectious Diseases*, **38**: 670-677.

Patel, M. R. and Patel, S. K. (2011). Cytotoxic activity of methanolic extract of *Artocarpusheterophyllus* against A549, Hela and MCF-7 cell lines. *Journal of Applied Pharmaceutical Science*, **01**: 167-171.

Perlin, D. S. (2007). Resistance to echinocandin-class antifungal drugs. *Drug Resistance Updates*, **10**: 121-130.

Petrova, O. E. and Sauer, K. (2010). The novel two-component regulatory system BfiSR regulates biofilm development by controlling the small RNA rsmZ through CafA. *Journal of Bacteriology*, **192**: 5275-5288.

Petrova, R. D., Reznick, A. Z., Wasser, S. P., Denchev, C. M., Nevo, E. and Mahajna, J. (2008). Fungal metabolites modulating NF-kappa B activity: an approach to cancer therapy and chemoprevention (Review). *Oncology Reports*, **19**: 299-308.

Pfaller, M. A. and Diekema, D. J. (2007). Epidemiology of invasive candidiasis: a persistent public health problem. *Clinical Microbiology Reviews*, **20**: 133-163.

Piepenbring, M. (2015). *Introduction to Mycology in the Tropics*. St. Paul, Minnesota, U. S. A: The American Phytopathological Society.

Pordeus, V., Szyper-Kravitz, M., Levy, R. A., Vaz, N. M. and Shoenfeld, Y. (2008). Infections and autoimmunity: a panorama. *Clinical Reviews in Allergy & Immunology*, **34**: 283-299.

Porter, T. M. and Golding, G. B. (2012). Factors that affect large subunit ribosomal DNA amplicon sequencing studies of fungal communities: classification method, primer choice, and error. *PLoS One*, **7**: e35749.

Poucheret, P., Fons, F. and Rapior, S. (2006). Biological and pharmacological activity of higher fungi: 20-year retrospective analysis. *Cryptogamie Mycologie*, **27**: 311-333.

Pryce, T. M., Palladino, S., Kay, I. D. and Coombs, G. W. (2003). Rapid identification of fungi by sequencing the ITS1 and ITS2 regions using an automated capillary electrophoresis system. *Medical Mycology*, **41**: 369-381.

Pu, X., Qu, X., Chen, F., Bao, J., Zhang, G. and Luo, Y. (2013). Camptothecin-producing endophytic fungus *Trichoderma atroviride* LY357: isolation, identification, and fermentation conditions optimization for camptothecin production. *Applied Microbiology and Biotechnology*, **97**: 9365-9375.

Quereshi, S., Pandey, A. K. and Sandhu, S. S. (2010). Evaluation of antibacterial activity of different Ganoderma lucidum extracts. *People's Journal of Scientific Research*, **3**: 9-13.

Rahi, D. K. and Malik, D. (2016). Diversity of Mushrooms and Their Metabolites of Nutraceutical and Therapeutic Significance. *Journal of Mycology*, **2016**: 1-19.

Ramesh, C. H. and Pattar, M. G. (2010). Antimicrobial properties, antioxidant activity and bioactive compounds from six wild edible mushrooms of western ghats of Karnataka, India. *Pharmacognosy Research*, **2**: 107-112.

Randazzo, A., Simon, M., Goffinet, P., Classen, J. F., Hougardy, N., Pierre, P., Kinzinger, P., Mauel, E. and Goffinet, J. S. (2016). Optimal turnaround time for direct identification of microorganisms by mass spectrometry in blood culture. *Journal of Microbiological Methods*, **130**: 1-5.

Randolph, S. E. (2008). Tick-borne encephalitis virus, ticks and humans: short-term and long-term dynamics. *Current Opinion in Infectious Diseases*, **21**: 462-467.

Rasko, D. A., Rosovitz, M. J., Myers, G.S., Mongodin, E.F., Fricke, W.F., Gajer, P., Crabtree, J., Sebaihia, M., Thomson, N. R., Chaudhuri, R. and Henderson, I. R. (2008). The pangenome structure of *Escherichia coli*: comparative genomic analysis of *E. coli* commensal and pathogenic isolates. *Journal of Bacteriology*, **190**: 6881-6893.

Rice, L. B. (2006). Antimicrobial resistance in gram-positive bacteria. *American Journal of Infection control*, **34**: S11-S19.

Roberts, P., and Evans, S. (2011). *The Book of Fungi*: A Life-Size Guide to Six Hundred Species from around the World. London, UK: University of Chicago Press.

Robledo, G. L. and Rajchenberg, M. (2007). South American polypores: first annotated checklist from Argentinean Yungas. *Mycotaxon*, **100**: 5-9.

Roemer, T. and Krysan, D. J. (2014). Antifungal drug development: challenges, unmet clinical needs, and new approaches. *Cold Spring Harbor Perspectives in Medicine*, **4**: a019703.

Rosa, L. H., Machado, K. M. G., Jacob, C. C., Capelari, M., Rosa, C. A. and Zani, C. L. (2003). Screening of Brazilian basidiomycetes for antimicrobial activity. *Memórias do Instituto Oswaldo Cruz*, **98**: 967-974.

Russo, T. A. and Johnson, J. R. (2006). Extraintestinal isolates of *Escherichia coli*: identification and prospects for vaccine development. *Expert Review of Vaccines*, **5**: 45-54.

Ryvarden, L. and Johansen, I. (1980). *A preliminary polypore flora of East Africa*. Oslo, Norway: Fungiflora.

Sarker, S. D. and Nahar, L. (2012). An introduction to natural products isolation. *Methods in Molecular Biology*, **864**: 1-25.

Savage, P. B., Li, C., Taotafa, U., Ding, B. and Guan, Q. (2002). Antibacterial properties of cationic steroid antibiotics. *FEMS Microbiology Letters*, **217**: 1-7.

Schlundt, J., Toyofuku, H., Jansen, J. and Herbst, S. A. (2004). Emerging food-borne zoonoses. *Revue Scientifique et Technique-Office International des Epizooties*, **23**: 513-534.

Schluter, J., Nadell, C. D., Bassler, B. L. and Foster, K. R. (2015). Adhesion as a weapon in microbial competition. *The ISME Journal*, **9**: 139-149.

Schoch, C. L., Seifert, K. A., Huhndorf, S., Robert, V., Spouge, J. L., Levesque, C.A., Chen, W., Bolchacova, E., Voigt, K., Crous, P. W. and Miller, A. N. (2012). Nuclear ribosomal internal transcribed spacer (ITS) region as a universal DNA barcode marker for Fungi. *Proceedings of the National Academy of Sciences*, **109**: 6241-6246.

Sengupta, S., Chattopadhyay, M. K. and Grossart, H. P. (2013). The multifaceted roles of antibiotics and antibiotic resistance in nature. *Frontiers in Microbiology*, **4**: 47-59.

Sheena, N., Ajith, T. A., Mathew, A. and Janardhanan, K. K. (2003). Antibacterial activity of three macrofungi, *Ganoderma lucidum*, *Navesporus floccosa* and *Phellinus rimosus* occurring in South India. *Pharmaceutical biology*, **41**: 564-567.

Shenoy, B. D., Jeewon, R., Lam, W. H., Bhat, D. J., Than, P. P., Taylor, P.W. and Hyde, K. D. (2007). Morpho-molecular characterization and epitypification of *Colletotricum capsici* (*Glomerulaceae*, Sordariomycetes), the causative agent of arnthracnose in chilli. *Fungal Diversity*, **27**: 197-211.

Shih, L., Tsai, K. L. and Hsieh, C. (2007). Effects of culture conditions on the mycelial growth and bioactive metabolite production in submerged culture of *Cordyceps militaris*. *Biochemical Engineering Journal*, **33**: 193-201.

Shuhendler, A. J., Pu, K., Cui, L., Uetrecht, J. P. and Rao, J. (2014). Real-time imaging of oxidative and nitrosative stress in the liver of live animals for drug-toxicity testing. *Nature Biotechnology*, **32**: 373.

Silva, S., Martins, S., Karmali, A. and Rosa, E. (2012). Production, purification and characterisation of polysaccharides from *Pleurotus ostreatus* with antitumour activity. *Journal of the Science of Food and Agriculture*, **92**: 1826-1832.

Silverstein, R. M., Webster, F. X., Kiemle, D. J. and Bryce, D. L. (2014). *Spectrometric identification of organic compounds* (8th ed). New York, U. S. A: John wiley & sons Inc.

Smania, E. F. A., Delle Monache, F., Smania, A., Yunes, R. A. and Cuneo, R. S. (2003). Antifungal activity of sterols and triterpenes isolated from *Ganoderma annulare*. *Fitoterapia*, **74**: 375-377.

Smith, D. and Ryan, M. J. (2009). *Fungal Sources for New Drug Discovery*. New York, NY: McGraw-Hill.

Smith, J. L., Fratamico, P. M. and Gunther, N. W. (2007). Extraintestinal pathogenic *Escherichia coli*. *Foodborne Pathogens and Disease*, **4**: 134-163.

Sparapano, L., Bruno, G., Ciccarone, C. and Graniti, A. (2000). Infection of grapevines by some fungi associated with esca. II. Interaction among *Phaeoacremonium chlamydosporum*, *P. aleophilum* and *Fomitiporia punctata*. *Phytopathologia Mediterranea*, **39**: 53-58.

Spellberg, B. and Gilbert, D. N. (2014). The future of antibiotics and resistance: a tribute to a career of leadership by John Bartlett. *Clinical Infectious Diseases*, **59**: S71-S75.

Stadler, M., Tichy, H. V., Katsiou, E. and Hellwig, V. (2003). Chemotaxonomy of Pochonia and other conidial fungi with Verticillium-like anamorphs. *Mycological Progress*, **2**: 95-122.

Stephens, C., Andrade, R. J. and Lucena, M. I. (2014). Mechanisms of drug-induced liver injury. *Current Opinion in Allergy and Clinical Immunology*, **14**: 286-292.

Suay, I., Arenal, F., Asensio, F. J., Basilio, A., Cabello, M. A., Díez, M. T., García, J. B., Del Val, A. G., Gorrochategui, J., Hernández, P. and Peláez, F. (2000). Screening of basidiomycetes for antimicrobial activities. *Antonie van Leeuwenhoek*, **78**: 129-140.

Sullivan, R., Smith, J. E. and Rowan, N. J. (2006). Medicinal mushrooms and cancer therapy: translating a traditional practice into Western medicine. *Perspectives in Biology and Medicine*, **49**: 159-170.

Sundar, S., Rai, M., Chakravarty, J. and Agrawal, S. (2005). Prevention of Antibiotic resistance/Antibiotic Misuse-Abuse. *MeDicine*, **141**: 696-699.

Szalóki, G., Pantzou, A., Prousis, K. C., Mavrofrydi, O., Papazafiri, P. and Calogeropoulou, T. (2014). Design and synthesis of 21-alkynylaryl pregnenolone derivatives and evaluation of their anticancer activity. *Bioorganic & Medicinal Chemistry*, **22**: 6980-6988.

Tadesse, B. T., Ashley, E. A., Ongarello, S., Havumaki, J., Wijegoonewardena, M., González, I. J. and Dittrich, S. (2017). Antimicrobial resistance in Africa: a systematic review. *BMC Infectious Diseases*, **17**: 616-632.

Thomas, J., Jacobson, G. A., Narkowicz, C. K., Peterson, G. M., Burnet, H. and Sharpe, C. (2010). Toenail onychomycosis: an important global disease burden. *Journal of Clinical Pharmacy and Therapeutics*, **35**: 497-519.

Toledo, A. V., Simurro, M. E. and Balatti, P. A. (2013). Morphological and molecular characterization of a fungus, *Hirsutella* sp., isolated from planthoppers and psocids in Argentina. *Journal of Insect Science*, **13**: 1-11.

Turkoglu, A., Duru, M. E., Mercan, N., Kivrak, I. and Gezer, K. (2007). Antioxidant and antimicrobial activities of *Laetiporus sulphureus* (Bull.) Murrill. *Food Chemistry*, **101**: 267-273.

Umeyama, A., Ohta, C., Shino, Y., Okada, M., Nakamura, Y., Hamagaki, T., Imagawa, H., Tanaka, M., Ishiyama, A., Iwatsuki, M. and Otoguro, K. (2014). Three lanostane triterpenoids with antitrypanosomal activity from the fruiting body of *Hexagonia tenuis*. *Tetrahedron*, **70**: 8312-8315.

VanderMolen, K. M., Little, J. G., Sica, V. P., El-Elimat, T., Raja, H. A., Oberlies, N. H., Baker, T. R. and Mahony, C. (2017). Safety assessment of mushrooms in dietary

supplements by combing analytical data with in silico toxicology evaluation. *Food and Chemical Toxicology*, **103**: 133-147.

VanderMolen, K. M., Raja, H. A., El-Elimat, T. and Oberlies, N. H. (2013). Evaluation of culture media for the production of secondary metabolites in a natural products screening program. *AMB Express*, **3**: 71-89.

Ventola, C. L. (2015). The antibiotic resistance crisis: part 1: causes and threats. *Pharmacy and Therapeutics*, **40**: 277-283.

Wagar, E. (2016). Bioterrorism and the role of the clinical microbiology laboratory. *Clinical Microbiology Reviews*, **29**: 175-189.

Wagner, T. and Fischer, M. (2002). Proceedings towards a natural classification of the worldwide taxa *Phellinus* sl and *Inonotus* sl, and phylogenetic relationships of allied genera. *Mycologia*, **94**: 998-1016.

Walsh, C. (2000). Molecular mechanisms that confer antibacterial drug resistance. *Nature*, **406**: 775-781.

Walsh, F. (Ed.). (2015). *The multiple roles of antibiotics and antibiotic resistance in nature*. Lausanne, Switzerland: Frontiers Media SA.

Wang, H., Ng, T. B. and Liu, Q. (2004). Alveolarin, a novel antifungal polypeptide from the wild mushroom *Polyporus alveolaris*. *Peptides*, **25**: 693-696.

Wang, S. R., Zhang, L., Chen, H. P., Li, Z. H., Dong, Z. J., Wei, K. and Liu, J. K. (2015). Four new spiroaxane sesquiterpenes and one new rosenonolactone derivative from cultures of Basidiomycete *Trametes versicolor*. *Fitoterapia*, **105**: 127-131.

Wassenaar, T. M. and Gaastra, W. (2001). Bacterial virulence: can we draw the line? *FEMS Microbiology Letters*, **201**: 1-7.

Wasser, S. P. (2011). Current findings, future trends, and unsolved problems in studies of medicinal mushrooms. *Applied Microbiology and Biotechnology*, **89**: 1323-1332.

Waterhouse, A. M., Procter, J. B., Martin, D. M., Clamp, M. and Barton, G. J. (2009). Jalview Version 2—a multiple sequence alignment editor and analysis workbench. *Bioinformatics*, **25**: 1189-1191.

Weyer, K., Dennis Falzon, D., Jaramillo, E., Zignol, M., Mirzayev, F. and Raviglione, M. (2017). Drug-resistant tuberculosis: what is the situation, what are the needs to roll it back. *AMR control*, **20**: 60-67.

White, T. J., Bruns, T., Lee, S. and Taylor, J. W. (1990). Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. *PCR Proto-cols: A Guide to Methods and Applications*, **18**: 315-322.

Wilding, J. L. and Bodmer, W. F. (2014). Cancer cell lines for drug discovery and development. *Cancer Research*, **74**: 2377-2384.

Wilson, L. S., Reyes, C. M., Stolpman, M., Speckman, J., Allen, K. and Beney, J. (2002). The direct cost and incidence of systemic fungal infections. *Value in Health*, **5**: 26-34.

Wisplinghoff, H., Bischoff, T., Tallent, S. M., Seifert, H., Wenzel, R. P. and Edmond, M. B. (2004). Nosocomial bloodstream infections in US hospitals: analysis of 24,179 cases from a prospective nationwide surveillance study. *Clinical Infectious Diseases*, **39**: 309-317.

World Health Organization. (2002). *The World Health Report 2002: Reducing Risks, Promoting Healthy Life*. Avenue Appia, Geneva: World Health Organization.

World Health Organization. (2014). *Antimicrobial Resistance: Global Report on Surveillance*. Avenue Appia, Geneva: World Health Organization.

Wright, G. D. (2005). Bacterial resistance to antibiotics: enzymatic degradation and modification. *Advanced Drug Delivery Reviews*, **57**: 1451-1470.

Xu, P., Ding, Z. Y., Qian, Z., Zhao, C. X. and Zhang, K. C. (2008). Improved production of mycelial biomass and ganoderic acid by submerged culture of *Ganoderma lucidum* SB97 using complex media. *Enzyme and Microbial Technology*, **42**: 325-331.

Yao, Y. J., Pegler, D. N. and Chase, M. W. (1999). Application of ITS (nrDNA) sequences in the phylogenetic study of *Tyromyces* sl. *Mycological Research*, **103**: 219-229.

Ye, S.D., Ying, S.H., Chen, C. and Feng, M.G. (2006). New solid-state fermentation chamber for bulk production of aerial conidia of fungal biocontrol agents on rice. *Biotechnology Letters*, **28**: 799–804.

Yin, R. H., Zhao, Z. Z., Ji, X., Dong, Z. J., Li, Z. H., Feng, T. and Liu, J. K. (2015). Steroids and sesquiterpenes from cultures of the fungus *Phellinus igniarius*. *Natural Products and Bioprospecting*, **5**: 17-22.

Zaoutis, T. E., Argon, J., Chu, J., Berlin, J. A., Walsh, T. J. and Feudtner, C. (2005). The epidemiology and attributable outcomes of candidemia in adults and children hospitalized in the United States: a propensity analysis. *Clinical Infectious Diseases*, **41**: 1232-1239.

Zhao, Y. Y. (2013). Traditional uses, phytochemistry, pharmacology, pharmacokinetics and quality control of *Polyporus umbellatus* (Pers.) Fries: A review. *Journal of Ethnopharmacology*, **149**: 35-48.

Zjawiony, J. K. (2004). Biologically Active Compounds from Aphyllophorales (Polypore) Fungi. *Journal of Natural Products*, **67**: 300-310.

APPENDICES

Appendix 1: The rDNA ITS sequences obtained following the sequencing of the purified amplicons and their NCBI corresponding species. The NCBI BLAST database was queried to obtain the best BLASTN search hits (query coverage, identity, e-values and accession numbers).

No.	Strain Code	rDNA ITS Sequences	Species	Query Coverage	Identity	Accession No.
1.	KE/16-152	GGTGAACCTGCGGAAGGATCATTATTGAATACGATTGGTACTGATGCTGGCTCTTAACAGGGCATGTGCTCGTGCCGTCTATTTATCTTCTCTTGTGCACATTTTGTAGTCAGTGAATTGGAAACTATGCGTGCTTTCATTAGTACGGTCTGGAGGCTGATTAAACCCTGCTTCTGTTCCTCTGCGCACTCTTTACTGAGTTGCGGTCTGGGAGTTGTTAACCCTTCTCCTGCTTCACTGACTATGTTTTCATATACCTTATAAAGTCATAGAATGTCATTTAACTTGATTGCGCTCGTCGTAGTCGTTAAACCTATACAACTTTCAGCAACGGATCTCTTGGCTCTCCTATCGATGAAGAACGCAGCGAAATGCGATAAGTAATGTGAATTGCAGAATTCAGTGAATCATCGAATCTTTGAACGCACCTTGCGCCCTTTGGTATTCCGAAGGGCATGCCTGTTTGAGTGTCATTAAATTATCAACCTTAGCTTGCTTTATTGCGAGCTTAAGGCTTGGATGTGAGGGCTGCTGGCTTCCTTCAGTGGATGGTCTGCTCCCTTTAAATGCATTAGTGGGATTCTCTTTGTGGACCGTCACTTGGTGTGATAATTATCTACGCCGCTTGACTTGCAGAGATTGAGACCTGCTCATAACGTCCAT	*Favolaschia calocera*	97%	99%	DQ026249.1

2.	KE/16-101	GTGATTTGAGGTCAAAGTGTCAAGAAGTCCGGTGAAGG GATCCTTGTCCAACTTAAGGACGATTAGAGGCAGACCCG TTAGGCAAGCGTTCTGGTGAAGTGATAATTATCACACCG TAAACGCAGACCAAAGTCCAGCCAATGTATTTGAGAGG AGCCGACCAAACCAAAGGCAACACAACACGGTTACCTG GGGCCAGCAAAAACCTCCAAGTCCAAACCGCCACCCCTT TCCGTGAAGAAAGGACAAGTGGTTTGAGATTAACATGAC ACTCAAACAGGCATGCCCCTCGGAATACCAAGGGGCGC AAGGTGCGTTCAAAGATTCGATGATTCACTGAATTCTGC AATTCACATTACTTATCGCATTTCGCTGCGTTCTTCATCG ATGCGAGAGCCAAGAGATCCGTTGTTGAAAGTTGTATTT CATTATCGCTCACAACGAGCATTACCATTCACATAACAA TCAAAGTGTGTTTGTAATATTAGCCAAAAGCCTTCATAA CCTTCCTTTTCTCTAATAAGGCCCTTTTCTGCCGGTTAGC ATACTTCAAAGCCCGTTCATTACAGAGACTACTCCCTTTG GTTCTAGGGGCCCGGGGTAAAAGGATTACCAAAAATTG GGCCTCTTTCCACGTCAGCACAGACCTCTCGCTTTCAAA ACTCGATAATGATCCTTCCGCAGGTTC	*Inonotus pachyphloeus*	76%	93%	KPO30785.1
3.	KE/16-103	CTGCGGAAGGATCATTATCGAGTTTTGACTGGGTTGTAG CTGGCCTTCCGAGGCATGTGCACGCCCTGCTCATCCACT CTACACCTGTGCACTTACTGTGGGTTTCAGATGTTATTAG CGGGGCCTTTACGGGTCTCGTGAAAGCGTCTGTGCCTGC GTTTATTACAAACTCTTACAAGTAACAGAATGTGTATTG CGATGTAACGCATCTATATACAACTTTCAGCAACGGATC TCTTGGCTCTCGCATCGATGAAGAACGCAGCGAAATGCG ATAAGTAATGTGAATTGCAGAATTCAGTGAATCATCGAA TCTTTGAACGCACCTTGCGCTCCTTGGTATTCCGAGGAGC ATGCCTGTTTGAGTGTCATGAAATCTTCAACCTATAAGC CTTTGCGGGTTTTTATTAGGCTTGGACTTGGAGGCTTGTC GGCCCAGCGGTCGGCTCCTCTTAAATGCATTAGCTTGAT TCCTTGTGGATCGGCTCTCGGCGTGATAATTGTCTACGCC	*Perenniporia abyssinica*	100%	98%	JQ673013.1

		GCGACCGTGAAGCGTTTAGCAAGCTTCTAATCGTC				
4.	KE/16-163	TTGAGGCAAGGGTCAAAAATGGTTTAAGGTAACAGAGT ACCTGTCTGACACATAGGCAGACTATTGGAAGCAGACAG TCTAAGTAAGCACTGGTGAATATAGATAGAAAACTATTA CACCAAACAATGCGAACTACAGTCCAGCTAATGCATTTG AGAGGAGCCGATACAGACAGTACCAGCATACATATTGC CTCCAAGTCCAAGCCCCTTCTTCAATTAAGAAAAAGAGG ATTGAGAATTACATGACACTCAAACAGGCATGCCCCTCG GAATACCAAGGGGCGCAAGGTGCGTTCAAAGATTCGAT GATTCACTGAATTCTGCAATTCACATTACTTATCGCATTT CGCTGCGTTCTTCATCGATGCGAGAGCCAAGAGATCCGT TGTTGAAAGTTGTATATTTGTATTTCGCTCACAGGAGCAT TACACATTCACAGGAACAAGAAAATGTTTGTATAGGTAA AAGTCAAAGTGTTCATAGTAAGTAAAGCCAAGATCATTA CTACTGCCAGAAGGGGTACCC	*Fomitiporia tenuis*	100%	98%	GU461958.1
5.	KE/16-165	CTGATCTGAGGTCAAAGGTCAAGATGAATTGTCCTTTAG CAGGAGATTAAGAAGCTGACACCCATACAACATGCTTCA CAGAACAGTGTAAACAAATTATCACACTGAAGCTGATTC ACAAAAGGTTTCAAGCTAATGCATTCAAGAGGAGCTGA ACACAGTAGTATCCAGCACACTCCAAATCCAAGCTCCAT TCACAGAAATGAATAGAGTTGAGAATTCCATGACACTCA AACAGGCATGCTCCTCGGAATACCAAGGAGCGCAAGGT GCGTTCAAAGATTCGATGATTCACTGAATTCTGCAATTC ACATTACTTATCGCATTTCGCTGCGTTCTTCATCGATGCG AGAGCCAAGAGATCCGTTGCTGAAAGTTGTATATAGATG CGTTACACGCAATAGACATTCTTTAAACTGGTTGTGTGT GGGTAAAAACATAGGAAAGACCACAGAGCAAAATCAAT GAAGACTTCACTCCAAGAGCCTAATCTACAGTGTGTGCA CAGGGGTGAGAGAGGATAATGATCAGGGTGTGCACAAT GCCGCAGCCAGCAACAACCCCTTTCAAGATTCATTAATG ATCCTTCCGCAGGTTCAC	*Antrodia* sp	100%	98%	KC543175.1

6.	KE/16-176	CTGATTTGAGGTCAGAGGTCATAAAGCTGTCTTCAAGTA AGACGGTTAGAAGCTCGCCAAACGCTTCACGGTCGCGAT GTAGACATTATCACACCGAGAGCCGATCCGCAAGGAAC CAAGCTAATGCATTTAAGAGGAGCCGACCGACAAAGGG CCGACAAGCCTCCAAGTCCAAGCCTACAAACCCCAAAA AGCTTGTAGGTTGAAGATTTCATGACACTCAAACAGGCA TGCTCCTCGGAATACCAAGGAGCGCAAGGTGCGTTCAAA GATTCGATGATTCACTGAATTCTGCAATTCACATTACTTA TCGCATTTCGCTGCGTTCTTCATCGATGCGAGAGCCAAG AGATCCGTTGCTGAAAGTTGTATATAGATGCGTTACATC GCAATACACATTCTAATACTTTATAGTGTTTGTGATAAAC GCAGGCACAGACGCGCTTCATGAGCCCCGCAAGGAGCA CGCTTCACGGTCTGAAACCCACAGTAAGTGCACAGGTGT AGAGTGGATGAGCAGGGCGTGCACATGCCTCGGAAGGC CAGCTACAACCCAGTCAAAACTCGATAATGATCCTTCCG CAGGTTCA	*Ganoderma* sp	100%	97%	JQ520185.1
7.	KE/16-193	CCTGCGGAAGGATCATTATCGAGTTCTGAAACGGGTTGT AGCTGGCCTTCCGAGGCATGTGCACGCCCTGCTCATCCA CTCTACACCTGTGCACTTACTGTGGGTTTCAGGAGCTTCG AAGCGAGGGTTTAACCGCTCTCGCCGAGTTGTTACTGGG CCTACGTTTATCACAAACTCTTAAAAGTATCAGAATGTA AACGCGTCTAACGCATCTATATACAACTTTCAGCAACGG ATCTCTTGGCTCTCGCATCGATGAAGAACGCAGCGAAAT GCGATAAGTAATGTGAATTGCAGAATTCAGTGAATCATC GAATCTTTGAACGCACCTTGCGCTCCTTGGTATTCCGAG GAGCATGCCTGTTTGAGTGTCATGAAATTCTCAACCTAA CAAGTTCTTAACGGGGCTTGCGTAGGCTTGGACTTGGAG GCTTGTCGGCTCTTAGCAGTCGGCTCCTCTCAAATGCATT AGCTTGGTTCCTTGCGGATCGGCTCACGGTGTGATAATT ATCTGCGCTGCGACCGTTGAAGCGTTTAATGGCCAGCTT CTAAT	*Polyporus arcularius*	100%	99%	KR673445.1

8.	KE/16-138	ATTGAGCGAGGTCATAATAAGCTGTCTCATGCGAGACGG TTAGAAGCTCGCCAAACGCTTCACGGTCGCGGCGTAGAC AATTATCACACCGAGAGCCGATCCGCAAGGAATCAAGCT AATACGTTTAAGAGGAGCCGACCGATATGAAACCGGCC GACAAAAGCCTCCAAGTCCAATCCTAACGAAGCCCGCA AAGACTTTGTAGGTTGAGAATTTCATGACACTCAAACAG GCATGCTCCTCGGAATACCAAGGAGCGCAAGGTGCGTTC AAAGATTCGATGATTCACTGAATTCTGCAATTCACATTA CTTATCGCATTTCGCTGCGTTCTTCATCGATGCGAGAGCC AAGAGATCCGTTGCTGAAAGTTGTATATAGATGCGTTAC ATCGCGATACACATTCTGATACTTTGTGGTTTGTAGTAAA ACGCAGGCCCGATCAACGACCGCAACCCGTGAAGGCGC GACCGTCTCCCGAAACCCACAGTAAGTGCACAGGTGTAG AGTGGATGAGCAGGGCGTGCACATGCCTCGGAAGGCCA GCTACAACCCGTTTCAAAACTCGTTAAT	*Hexagonia* sp	98%	92%	KF356157.1
9.	KE/16-115	GAGGTCGAGTCAAAGATTATTACTCTGTCTTAAAAGACA ACTAGAAGCGGAATTCCATACATGTGCTTAGACAGCTAC AGCGTAGACAATTATCACACTGAAGCTAGACCTGAGCAA AGATTTCCAGCTAATATATTCAAGAGGAGCAGATTTATT ACTAAACCTGCAAAGAGACCTCCAAATCCAAAGCACCA ACATCATCAAAAAATGAAGAGGGCTTTGAGAATACCAT GACACTCAAACGGGCATGCCCTTCGGAATACCAAAGGG CGCAAGTTGCGTTCAAAGATTCGATGATTCACTGAATTC TGCAATTCACATTACTTATCGCATTTCGCTGCGTTCTTCA TCGATGCGAGAGCCAAGAGATCCGTTGCTAAAAGTTATA TATAATGCGTTATTTAAGCGCAAGAGACATTCATGATAC AGCGTGTGTGAATGAAACATAGGAAGGCGTCAACAACT AGAGAGGAACCTAAGTTCTTCTCCTGTATCAACCATCCT ACAATATGTGCACAGGTGTTAAAGATGAGTTGGATTTGA GCGAAGCGTGCACATGCCCCGAAAGGCCAGCTACAACTT CTTTCAAAGACTCGATAA	*Tyromyces* sp	53%	98%	AB509841.1

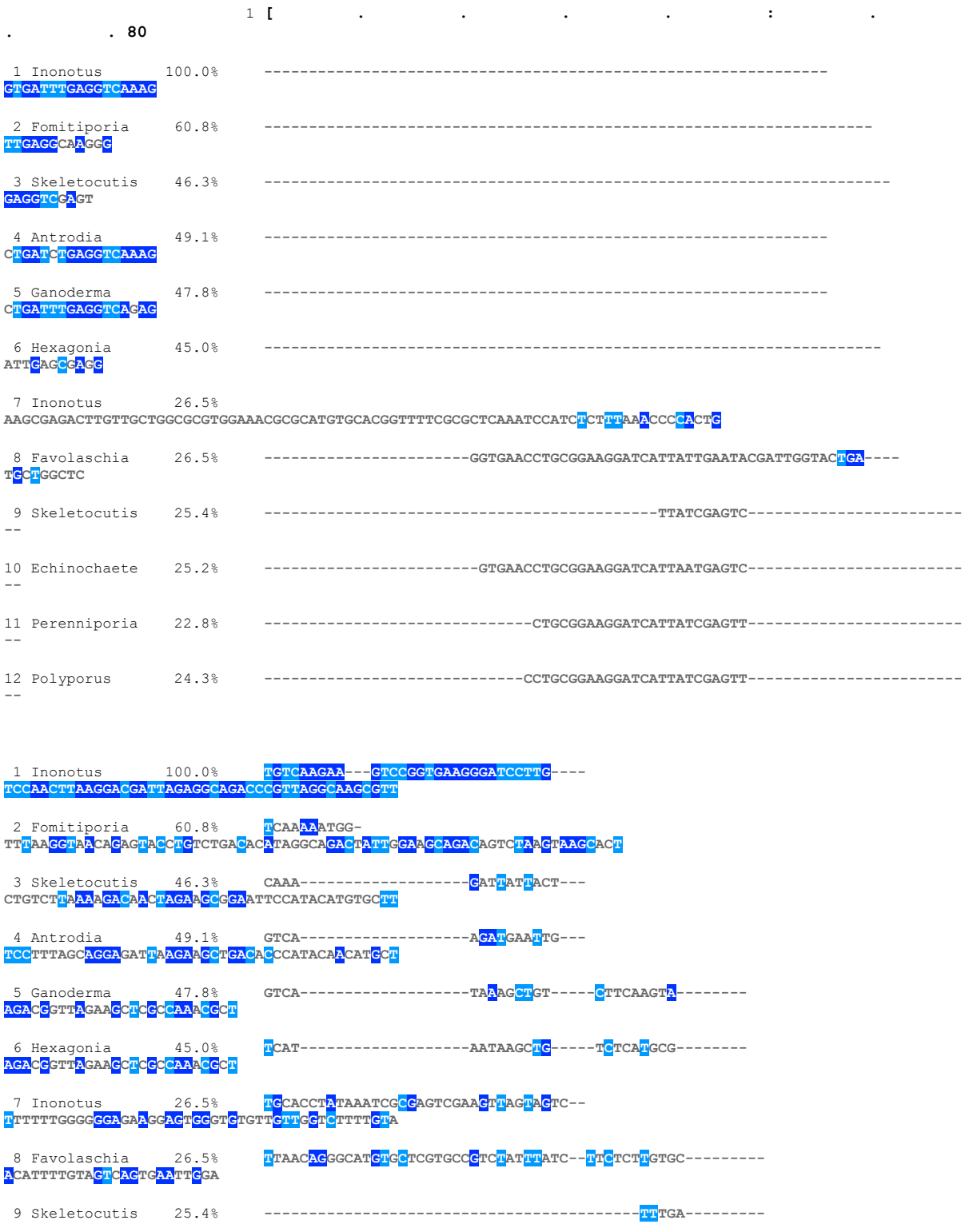
10.	KE/16-189	TTATCGAGTCTTTGAAGAAGTTGTAGCTGGCCTTTCGGG GCATGTGCACGCTTCGCTCAAATCCAACTCATCTTTAAC ACCTGTGCACATATTGTAGGATGGTTGATACAGGAGAAG AACTTAGGTTCCTCTCTAGTTGTTGACGCCTTCCTATGTT TCATTCACACATGCTGTATCATGAATGTCTCTTGCGCTTA AATAACGCATTATATATAACTTTTAGCAACGGATCTCTT GGCTCTCGCATCGATGAAGAACGCAGCGAAATGCGATA AGTAATGTGAATTGCAGAATTCAGTGAATCATCGAATCT TTGAACGCAACTTGCGCCCTTTGGTATTCCGAAGGGCAT GCCCGTTTGAGTGTCATGGTATTCTCAAAGCCCTCTTCAT TTTTTGATGATGTTGGTGCTTTGGATTTGGAGGTCTCTTT GCAGGTTTAGTAATAAATCTGCTCCTCTTGAATATATTAG CTGGAAATCTTTGCTCAGGTCTAGCTTCAGTGTGATAATT GTCTACGCTGTAGCTGTCTAAGCACATGTATGGAATTCC GCTTCTAGTTGTCTT	*Tyromyces* sp	53%	98%	AB509841.1
11.	KE/16-153	AAGCGAGACTTGTTGCTGGCGCGTGGAAACGCGCATGTG CACGGTTTTCGCGCTCAAATCCATCTCTTTAAACCCCACT GTGCACCTATAAATCGCGAGTCGAAGTTAGTAGTCTTTT TTGGGGGGAGAAGGAGTGGGTGTGTTGTTGGTCTTTTGT AAGWAAATCAGTAGAAAGGTGAAATCGGKTGAGCTTAC TTACCCGGTAGTAATCTTTTGAACGTCGAAAGCAAAAGT GAAAACRATCTTCTTCCTATTCCTCCGTTCGGGCGAAGG CTTTGGCTTGTGTGTGTTATTACACAAACACCTTTAATTG TCTTTGTGAATGTATTTGCTCCTTGTGGGCGAAAATAAAT ACAACTTTCAACAACGGATCTCTTGGCTCTCGCATCGAT GAAGAACGCAGCGAAATGCGATAAGTAATGTGAATTGC AGAATTCAGTGAATCATCGAATCTTTGAACGCACCCTGC GCCCCCTGGTATTCCGAGGGGCAYGCCTGTTTGAGTGTC ATGTTAATCTCAAACCGCTAGTCTYTCTTAATTGAAGGG CTCTGAGGTTTGGACT	*Inonotus* sp	100%	97%	JF895464.1
12.	KE/16-	GTGAACCTGCGGAAGGATCATTAATGAGTCTTGATGCGG	*Echinochaete*	74%	93%	AB462320.1

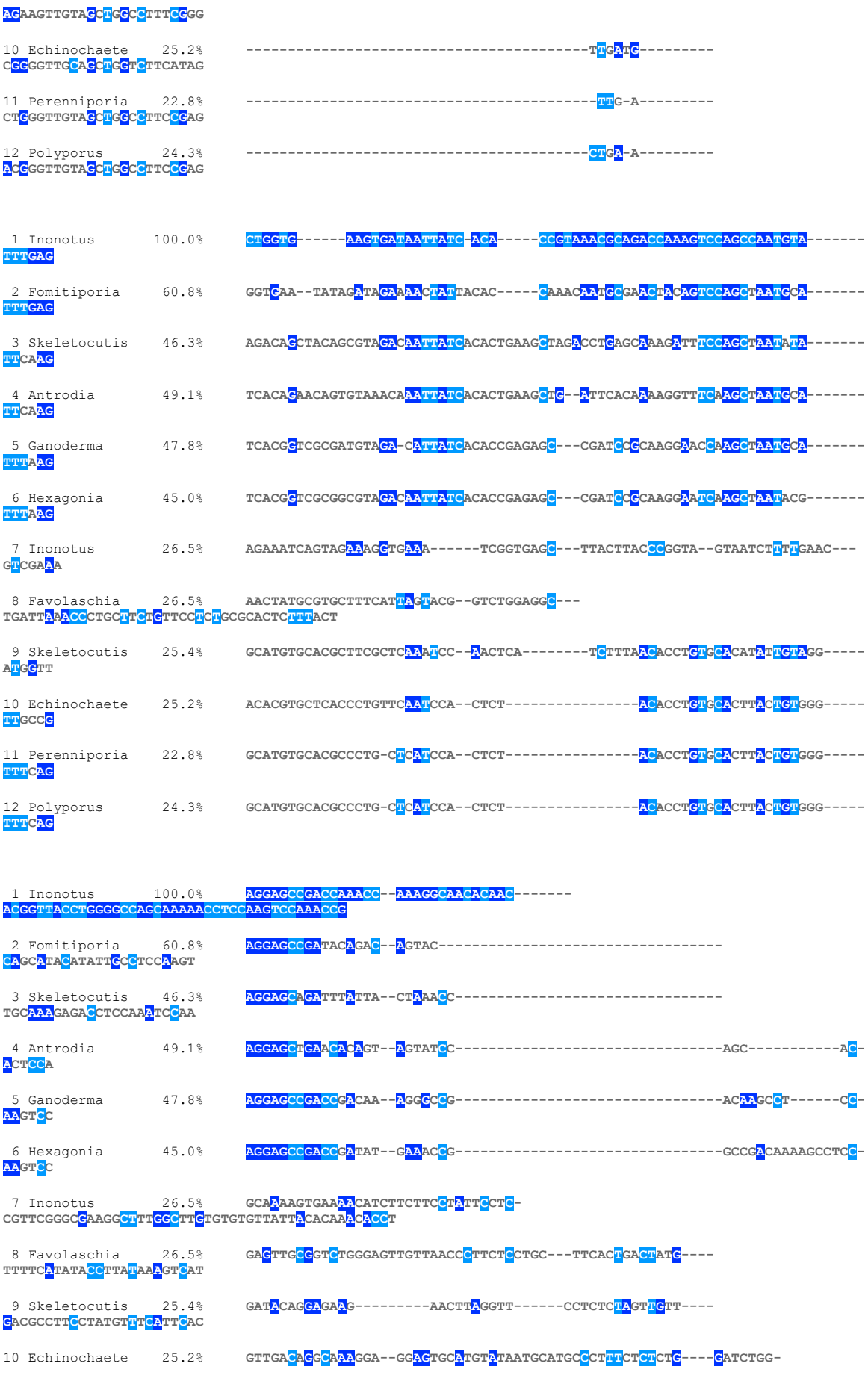
	198	GGTTGCAGCTGGTCTTCATAGACACGTGCTCACCCTGTTC AATCCACTCTACACCTGTGCACTTACTGTGGGTTGCCGGT TGACAGGCAAAGGAGGAGTGCATGTATAATGCATGCCCT TTCTCTCTGGATCTGGCCTCACGTTTTTATTACACACAAG TATTAGAATGTGTACTGCGATGTTGTAACGCATTTATATA CAACTTTCAGCAACGGATCTCTTGGCTCTCGCATCGATG AAGAACGCAGCGAAATGCGATAAGTAATGTGAATTGCA GAATTCAGTGAATCATCGAATCTTTGAACGCACCTTGCG CTCCTTGGTATTCCGAGGAGCACGCCTGTTTGAGTGTCAT GAAATTCTCAACCTTATATGCCCTTGTTATGGGGGTCTGT AAAGTTGGACTTGGAGGTGTATTGTCAGCTTGCCCTCTG CGAGTTGGCTCCTCTCAAATGCATTAGCTTTAGTTCCTTT GTGGATCGGCTTTCGGTGTGATAGTTGTGTCTACGCCGT GACCGTGAAGCGTTTATGGCATAAGCTTATAAACCCCAA CGCCTCTTTCCCATTCTTTGGGACAGCGTTCTCTTGACAA TCTGCTCAA	*brachypora*			

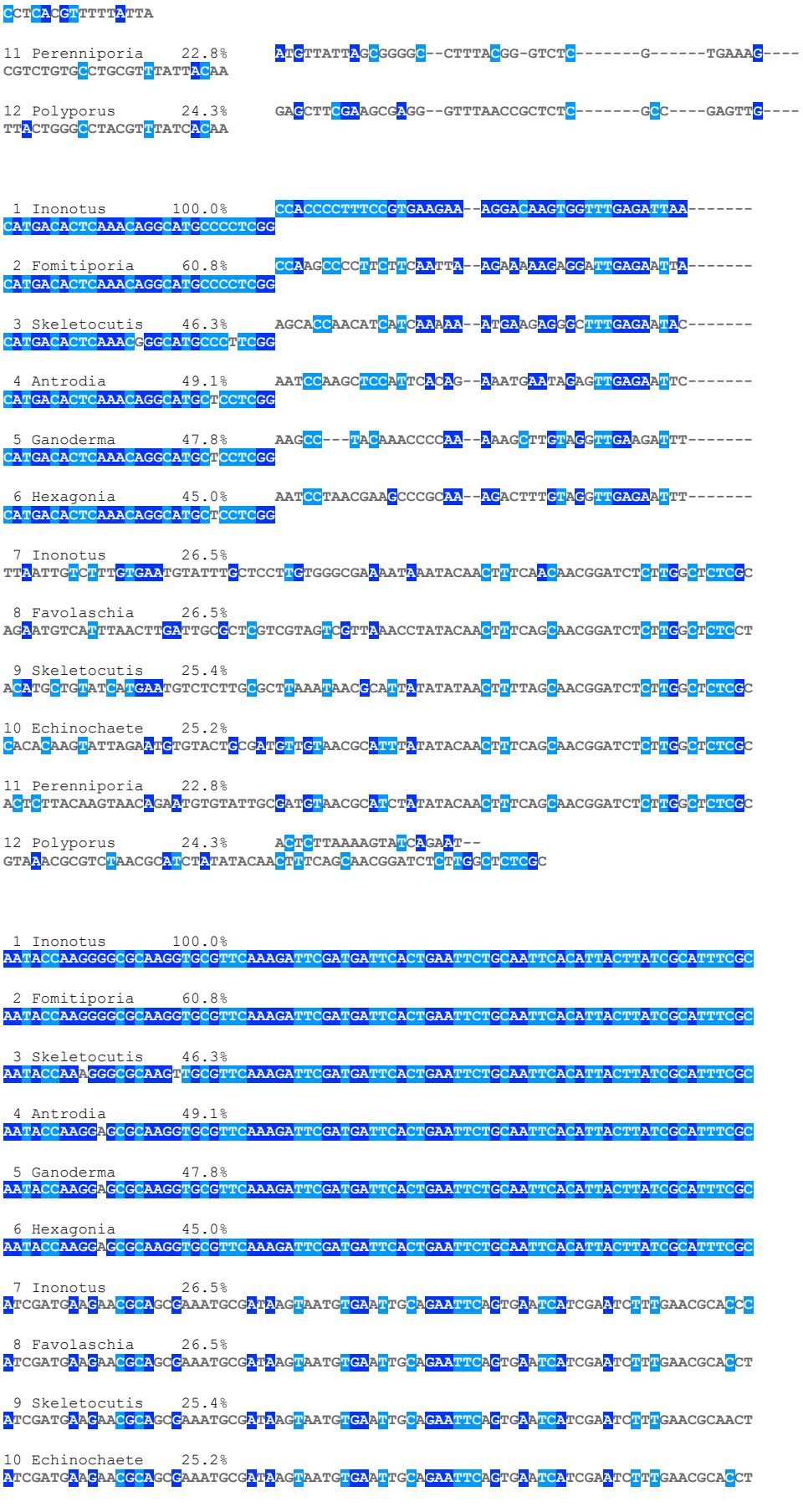
Appendix 2: CLUSTAL W Multiple Sequence Alignment Results of the rDNA ITS Sequences of the 12 Fungal Samples used in the Study.
Reference sequence (1): Inonotus

Identities normalised by aligned length.

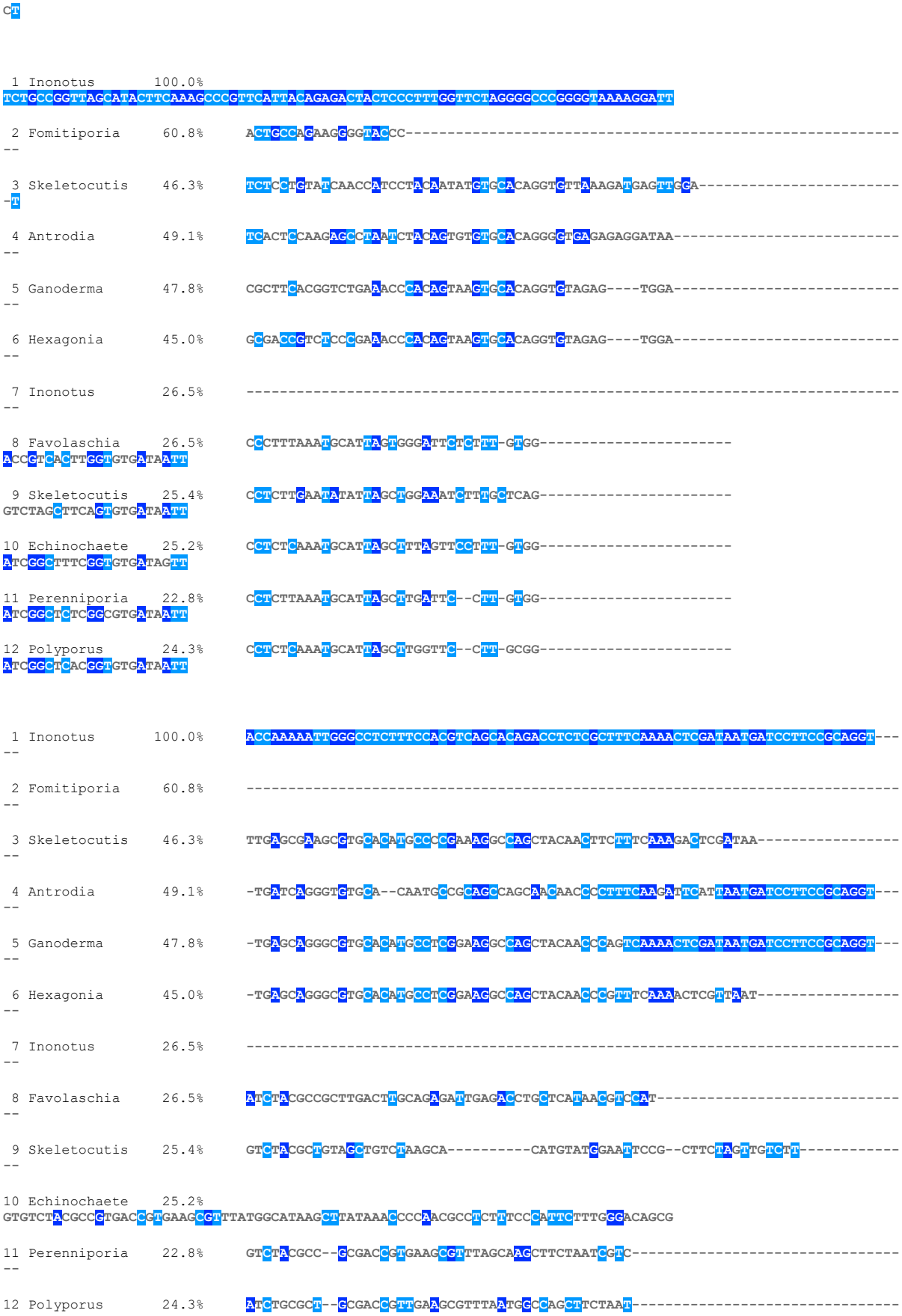
Colored by: identity + property




10 Echinochaete 25.2%
11 Perenniporia 22.8%
12 Polyporus 24.3%
1 Inonotus 100.0%
2 Fomitiporia 60.8%
3 Skeletocutis 46.3%
4 Antrodia 49.1%
5 Ganoderma 47.8%
6 Hexagonia 45.0%
7 Inonotus 26.5%
8 Favolaschia 26.5%
9 Skeletocutis 25.4%
10 Echinochaete 25.2%
11 Perenniporia 22.8%
12 Polyporus 24.3%
1 Inonotus 100.0%
2 Fomitiporia 60.8%
3 Skeletocutis 46.3%
4 Antrodia 49.1%
5 Ganoderma 47.8%
6 Hexagonia 45.0%
7 Inonotus 26.5%
8 Favolaschia 26.5%
9 Skeletocutis 25.4%
10 Echinochaete 25.2%


CCTCACGTTTTTATTA
11 Perenniporia 22.8%
ATGTTATTAGCGGGGC--CTTTACGG-GTCTC-------G------TGAAAG----
CGTCTGTGCCTGCGTTTATTACAA
12 Polyporus 24.3%
GAGCTTCGAAGCGAGG--GTTTAACCGCTCTC-------GCC----GAGTTG----
TTACTGGGCCTACGTTTATCAGAA
1 Inonotus 100.0%
CCACCCCTTTCCGTGAAGAA--AGGACAAGTGGTTTGAGATTAA-------
CATGACACTCAAACAGGCATGCCCCTCGG
2 Fomitiporia 60.8%
CCAAGCCCCTTCTTCAATTA--AGAAAAAGAGGATTGAGAATTA-------
CATGACACTCAAACAGGCATGCCCCTCGG
3 Skeletocutis 46.3%
AGCACCAACATCATCAAAAA--ATGAAGAGGGCTTTGAGAATAC-------
CATGACACTCAAACGGGCATGCCCTTCGG
4 Antrodia 49.1%
AATCCAAGCTCCATTCACAG--AAATGAATAGAGTTGAGAATTC-------
CATGACACTCAAACAGGCATGGTCCTCGG
5 Ganoderma 47.8%
AAGCC---TACAAACCCCAA--AAAGCTTGTAGGTTGAAGATTT-------
CATGACACTCAAACAGGCATGGTCCTCGG
6 Hexagonia 45.0%
AATCCTAACGAAGCCCGCAA--AGACTTTGTAGGTTGAGAATTT-------
CATGACACTCAAACAGGCATGGTCCTCGG
7 Inonotus 26.5%
TTAATTGTCTTTGTGAATGTATTTGCTCCTTGTGGGCGAAAATAAATACAACTTTCAACAACGGATCTCTTGGCTCTCGC
8 Favolaschia 26.5%
AGAATGTCATTTAACTTGATTGCGCTGTCGTAGTCGTTAAACCTATACAACTTTCAGCAACGGATCTCTTGGCTCTCCT
9 Skeletocutis 25.4%
ACATGCTGTATGATGAATGTCTCTTGGGCTTAAATAACGCATTATATATAACTTTTAGCAACGGATCTCTTGGCTCTCGC
10 Echinochaete 25.2%
CACACAAGTATTAGAATGTGTACTGCGATGTTGTAACGCATTTATATACAACTTTCAGCAACGGATCTCTTGGCTCTCGC
11 Perenniporia 22.8%
ACTCTTACAAGTAACAGAATGTGTATTGCGATGTAACGCATCTATATACAACTTTCAGCAACGGATCTCTTGGCTCTCGC
12 Polyporus 24.3%
ACTCTTAAAAGTATCAGAAT--
GTAAACGCGTCTAACGCATCTATATACAACTTTCAGCAACGGATCTCTTGGCTCTCGC
1 Inonotus 100.0%
AATACCAAGGGCGCAAGGTGCGTTCAAAGATTCGATGATTCACTGAATTCTGCAATTCACATTACTTATCGCATTTCGC
2 Fomitiporia 60.8%
AATACCAAGGGCGCAAGGTGCGTTCAAAGATTCGATGATTCACTGAATTCTGCAATTCACATTACTTATCGCATTTCGC
3 Skeletocutis 46.3%
AATACCAAAGGGCGCAAGTTGCGTTCAAAGATTCGATGATTCACTGAATTCTGCAATTCACATTACTTATCGCATTTCGC
4 Antrodia 49.1%
AATACCAAGGAGCGCAAGGTGCGTTCAAAGATTCGATGATTCACTGAATTCTGCAATTCACATTACTTATCGCATTTCGC
5 Ganoderma 47.8%
AATACCAAGGAGCGCAAGGTGCGTTCAAAGATTCGATGATTCACTGAATTCTGCAATTCACATTACTTATCGCATTTCGC
6 Hexagonia 45.0%
AATACCAAGGAGCGCAAGGTGCGTTCAAAGATTCGATGATTCACTGAATTCTGCAATTCACATTACTTATCGCATTTCGC
7 Inonotus 26.5%
ATCGATGAAGAACGCAGCGAAATGCGATAAGTAATGTGAATTGCAGAATTCAGTGAATCATCGAATCTTTGAACGCACCC
8 Favolaschia 26.5%
ATCGATGAAGAACGCAGCGAAATGCGATAAGTAATGTGAATTGCAGAATTCAGTGAATCATCGAATCTTTGAACGCACCT
9 Skeletocutis 25.4%
ATCGATGAAGAACGCAGCGAAATGCGATAAGTAATGTGAATTGCAGAATTCAGTGAATCATCGAATCTTTGAACGCAACT
10 Echinochaete 25.2%
ATCGATGAAGAACGCAGCGAAATGCGATAAGTAATGTGAATTGCAGAATTCAGTGAATCATCGAATCTTTGAACGCACCT
11 Perenniporia 22.8%

```
ATCGATGAAGAACGCAGCGAAATGCGATAAGTAATGTGAATTGCAGAATTCAGTGAATCATCGAATCTTTGAACGCACCT

12 Polyporus        24.3%
ATCGATGAAGAACGCAGCGAAATGCGATAAGTAATGTGAATTGCAGAATTCAGTGAATCATCGAATCTTTGAACGCACCT


 1 Inonotus        100.0%
TGCGTTCTTCATCGATGCGAGAGCCAAGAGATCCGTTGTTGAAAGTTGTATTTCATTATCGCTCACAACGAGCATTACCA

 2 Fomitiporia      60.8%
TGCGTTCTTCATCGATGCGAGAGCCAAGAGATCCGTTGTTGAAAGTTGTATATTTGTATTTCGCTCACAGGAGCATTACA

 3 Skeletocutis     46.3%
TGCGTTCTTCATCGATGCGAGAGCCAAGAGATCCGTTGCTAAAAGTTATATTATAATGCGTTATTTAAGCGCAAGAGACAT

 4 Antrodia         49.1%
TGCGTTCTTCATCGATGCGAGAGCCAAGAGATCCGTTGCTGAAAGTTGTATATTAGATGCGTTACACGCAATAGACATTCT

 5 Ganoderma        47.8%
TGCGTTCTTCATCGATGCGAGAGCCAAGAGATCCGTTGCTGAAAGTTGTATATTAGATGCGTTACATCGCAATACACATTC

 6 Hexagonia        45.0%
TGCGTTCTTCATCGATGCGAGAGCCAAGAGATCCGTTGCTGAAAGTTGTATATTAGATGCGTTACATCGCGATACACATTC

 7 Inonotus         26.5%      TGCGCCCCCTGGTATTCCGAGGGGCAGCCTGTTT--------
GAGTGTCATGTTAATCTCAAACGCCTAGTCTTCTTTA--

 8 Favolaschia      26.5%      TGCGCCCTTTGGTATTCCGAAGGGCATGCCTGTT-------
TGAGTGTCATTAAATTATCAACCTTAGCTTGCTTTAT--

 9 Skeletocutis     25.4%      TGCGCCCTTTGGTATTCCGAAGGGCATGCCCGTT-------
TGAGTGTCATGGTATTCTCAAAGCCTCTTCATTTTT--

10 Echinochaete     25.2%      TGCGCTCCTTGGTATTCCGAGGAGCACGCCTGTT-------
TGAGTGTCATGAAATTCTCAACCTTATATGCGCTTGT--

11 Perenniporia     22.8%      TGCGCTCCTTGGTATTCCGAGGAGCATGCCTGTT-------TGAGTGTCATGAAATCTTCAACCTATAAGCCTT--
TG--

12 Polyporus        24.3%      TGCGCTCCTTGGTATTCCGAGGAGCATGCCTGTT-------
TGAGTGTCATGAAATTCTCAACCTAACAAGTTCTTAA--


 1 Inonotus        100.0%      TTCACATAACAATCAAAGTGTGTTTGTAATA--
TTAGCCAAAAGCCTTCATAACCTTCCTTTTCTCTAATAAGGCCCTTT

 2 Fomitiporia      60.8%      CATTCACAGGAACAAGAAAATGTTTGTATAGGTAAAAGTCAAAGTGTTCATAGTAAGTAAAGCCAAGATCATT----
ACT

 3 Skeletocutis     46.3%      TCATGATACAGCGTGTGTGAATGAAACATAGGAAGGCGTCAACAACTA------GAGAGGAACCTAAGTTC-------
-T

 4 Antrodia         49.1%      TTAAACTGGTTGTGTGTGGTAAAAACATAGGAAAGACCACAGAGCAA------AATCAA-TG-AAGAC---------
-T

 5 Ganoderma        47.8%      TAATACTTTATAGTGTTTGTGATAAACGCAGGCACAGACGCGCTTCAT------GAGCCC-CGCAAGGAGC-------
-A

 6 Hexagonia        45.0%      TGATACTTTGTGGTTTGTAGTAAAACGCAGGCCCGATCAA-----CGA------CCGCAA-CCCGTGAAGG-------
-C

 7 Skeletocutis     26.5%      ---ATTGAAGGGCTCTGAGGTTTGGACT-----------------------------------------------
--

 8 Favolaschia      26.5%      ---TGCG-AGCTTAAGGC---TTGGATGTGAGGGCTGCTGGCTTCCTT--------CAG---TGGATGGTCTG-----
CT

 9 Tyromyces        25.4%      ---TGAT-GATGTTGGTC-CTTTGGATTTGGAGGTCTCTTTGCAGGTT--------TAGT-A--ATAAATCTC-----
CT

10 Echinochaete     25.2%      ---TATG-GGGGTCTGTAAAGTTGGACTTGGAGGTGTATTGTCAGCTT--------GCCC-TCTGCGAGTTGC-----
CT

11 Perenniporia     22.8%      ---CGGG-TTT-TTATTAGGCTTGGACTTGGAGGCTTGTCGGC-------------------CCAGCGGTCGC-----
CT

12 Polyporus        24.3%      ---CGGG-GCT-TGCGTAGGCTTGGACTTGGAGGCTTGTCGGC----------------T-CTTAGCAGTCGC-----
```


CT
1 Inonotus 100.0%
TCTGCCGGTTAGCATACTTCAAAGCCCGTTCATTACAGAGACTACTCCCTTTGGTTCTAGGGGCCCGGGGTAAAAGGATT
2 Fomitiporia 60.8% ACTGCCAGAAGGGGTACCC----------
--
3 Skeletocutis 46.3% TCTCCTGTATCAACCATCCTACAATATGTGCACAGGTGTTAAAGATGAGTTGCA----------
-T
4 Antrodia 49.1% TCACTCCAAGAGCCTAATCTACAGTGTGTGCACAGGGGTGAGAGAGGATAA----------
--
5 Ganoderma 47.8% CGCTTCACGGTCTGAAACCCACAGTAAGTGCACAGGTGTAGAG----TGGA----------
--
6 Hexagonia 45.0% GCGACCGTCTCCCGAAACCCACAGTAAGTGCACAGGTGTAGAG----TGGA----------
--
7 Inonotus 26.5% ----------
--
8 Favolaschia 26.5% CCCTTTAAATGCATTAGTGGGATTCTCTTT-GTGG----------
ACCGTCACTTGGTGTGATAATT
9 Skeletocutis 25.4% CCTCTTGAATATATTAGCTGGAAATCTTTGCTCAG----------
GTCTAGGTTCAGTGTGATAATT
10 Echinochaete 25.2% CCTCTCAAATGCATTAGCTTTAGTTCCTTT-GTGG----------
ATCGGCTTTCGGTGTGATAGTT
11 Perenniporia 22.8% CCTCTTAAATGCATTAGCTTGATTC--CTT-GTGG----------
ATCGGCTCTCGGCGTGATAATT
12 Polyporus 24.3% CCTCTCAAATGCATTAGCTTGGTTC--CTT-GCGG----------
ATCGGCTCACGGTGTGATAATT
1 Inonotus 100.0% ACCAAAAATTGGGCCTCTTTCCACGTCAGCACAGACCTCTCGCTTTCAAAACTCGATAATGATCCTTCCGCAGGT---
--
2 Fomitiporia 60.8% ----------
--
3 Skeletocutis 46.3% TTGAGCGAAGCGTGCACATGCCCCGAAAGGCCAGCTACAACTTCTTTCAAAGACTCGATAA----------
--
4 Antrodia 49.1% -TGATCAGGGTGTGCA--CAATGCCGCAGCCAGCAACAACCCTTTCAAGATTCATTAATGATCCTTCCGCAGGT---
--
5 Ganoderma 47.8% -TGAGCAGGGCGTGCACATGCCTCGGAAGGCCAGCTACAACCCAGTCAAAACTCGATAATGATCCTTCCGCAGGT---
--
6 Hexagonia 45.0% -TGAGCAGGGCGTGCACATGCCTCGGAAGGCCAGCTACAACCCGTTTCAAAACTCGTTAAT----------
--
7 Inonotus 26.5% ----------
--
8 Favolaschia 26.5% ATGTACGCCGCTTGACTTGCAGAGATTGAGACCTGCTCATAACGTCCAT----------
--
9 Skeletocutis 25.4% GTGTACGCTGTAGCTGTCTAAGCA----------CATGTATGGAATTCCG--CTTCTAGTTGTCTT----------
--
10 Echinochaete 25.2%
GTGTCTACGCCGTGACCGTGAAGCGTTTATGGCATAAGCTTATAAACCCCAACGCCTCTTTCCCATTCTTTGGGACAGCG
11 Perenniporia 22.8% GTGTACGCC--GCGACCGTGAAGCGTTTAGCAAGCTTCTAATCGTC----------
--
12 Polyporus 24.3% ATGTGCGCT--GCGACCGTTGAAGCGTTTAATGCCCAGCTTCTAAT----------
--

1	Inonotus	100.0%	-TC----------
2	Fomitiporia	60.8%	----------
3	Skeletocutis	46.3%	----------
4	Antrodia	49.1%	-TCAC----------
5	Ganoderma	47.8%	-TCA----------
6	Hexagonia	45.0%	----------
7	Inonotus	26.5%	----------
8	Favolaschia	26.5%	----------
9	Skeletocutis	25.4%	----------
10	Echinochaete	25.2%	TTCTCTTGACAATCTGCTCAA
11	Perenniporia	22.8%	----------
12	Polyporus	24.3%	----------

Appendix 3: Mininimum inhibitory concentration (MIC (µg/ml) results of crude extracts against *B. subtilis*, *E. coli*, *C.tenuis* and *M. plumbeus* microbial test pathogens. The extracts were obtained following fermentation of fungal mycelia in YMG, ZM ½ and Q6 ½ media.

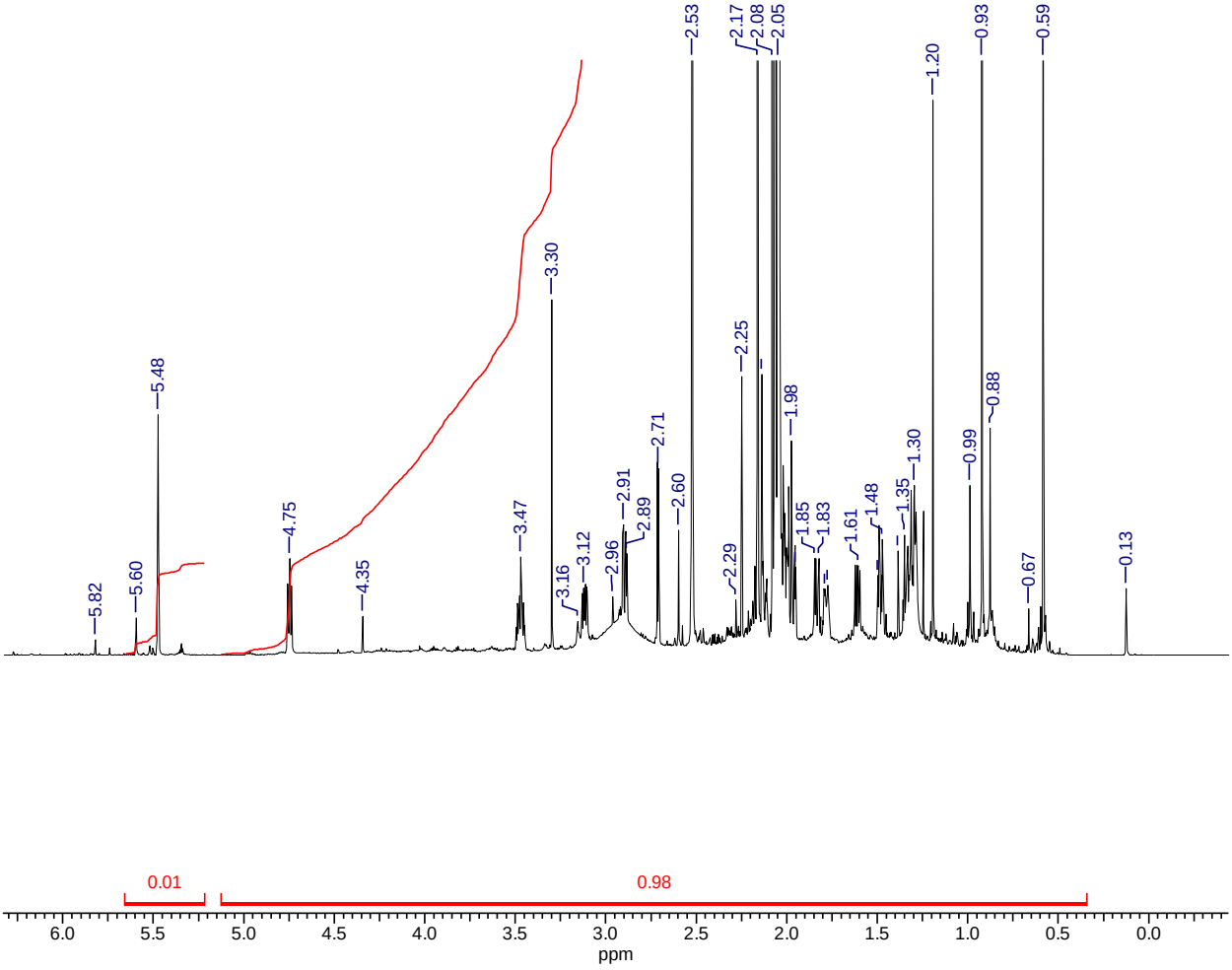
Fungal Strain Code	**Identity**	**Medium**	**Extracts**	***E. coli***	***B. subtilis***	***C. tenuis***	***M. plumbeus***
KE/16-152	*Favolaschia calocera*	**YMG**	**S**	**-**	**-**	**4.69**	**75**
			M	**-**	**-**	**<2.34**	**37.5**
KE/16-101	*Inonotus pachyphloeus*	**YMG**	**S**	**-**	**-**	**-**	**-**
			M	**-**	**-**	**150**	**-**
		Q6 1/2	**S**	**300**	**-**	**-**	**-**
			M	**-**	**300**	**-**	**-**
		ZM 1/2	**S**	**-**	**-**	**37.5**	**-**
			M	**-**	**-**	**-**	**-**
KE/16-103	*Perenniporia* sp	**YMG**	**S**	**-**	**-**	**-**	**-**
			M	**-**	**-**	**-**	**-**
		Q6 1/2	**S**	**-**	**-**	**-**	**-**
			M	**-**	**-**	**-**	**-**
		ZM 1/2	**S**	**-**	**-**	**-**	**-**
			M	**-**	**-**	**-**	**-**
KE/16-163	*Fomitiporia* sp	**YMG**	**S**	**-**	**-**	**-**	**-**
			M	**-**	**300**	**-**	**-**
		RICE	**S**	**-**	**-**	**-**	**-**
			M	**-**	**300**	**-**	**-**
KE/16-165	*Antrodia* sp	**YMG**	**S**	**-**	**-**	**-**	**-**
			M	**-**	**-**	**-**	**-**
		Q6 1/2	**S**	**-**	**75**	**-**	**-**
			M	**-**	**300**	**-**	**-**
		ZM 1/2	**S**	**-**	**-**	**-**	**-**

			M	**-**	**-**	**-**	**-**
KE/16-176	*Ganoderma* sp	**YMG**	**S**	**-**	**-**	**-**	**-**
			M	**-**	**-**	**-**	**-**
		Q6 1/2	**S**	**-**	**-**	**-**	**-**
			M	**-**	**-**	**-**	**-**
		ZM 1/2	**S**	**-**	**-**	**-**	**-**
			M	**-**	**-**	**-**	**-**
KE/16-193	*Polyporus arcularius*	**YMG**	**S**	**-**	**-**	**-**	**-**
			M	**-**	**-**	**300**	**-**
		Q6 1/2	**S**	**-**	**-**	**-**	**-**
			M	**-**	**-**	**-**	**-**
		ZM 1/2	**S**	**-**	**-**	**-**	**-**
			M	**-**	**-**	**-**	**-**
KE/16-138	*Hexagonia* sp	**YMG**	**S**	**300**	**37.5**	**-**	**-**
			M	**-**	**150**	**-**	**-**
		Q6 1/2	**S**	**-**	**-**	**-**	**-**
			M	**-**	**-**	**-**	**-**
		ZM 1/2	**S**	**-**	**-**	**-**	**-**
			M	**-**	**-**	**-**	**-**
KE/16-115	*Skeletocutis nivea*	**YMG**	**S**	**-**	**-**	**-**	**-**
			M	**-**	**150**	**-**	**-**
		Q6 1/2	**S**	**-**	**-**	**-**	**-**
			M	**-**	**-**	**-**	**-**
		ZM 1/2	**S**	**-**	**-**	**-**	**-**
			M	**-**	**-**	**-**	**-**
KE/16-189	*Skeletocutis nivea*	**YMG**	**S**	**-**	**-**	**-**	**-**
			M	**-**	**75**	**-**	**-**

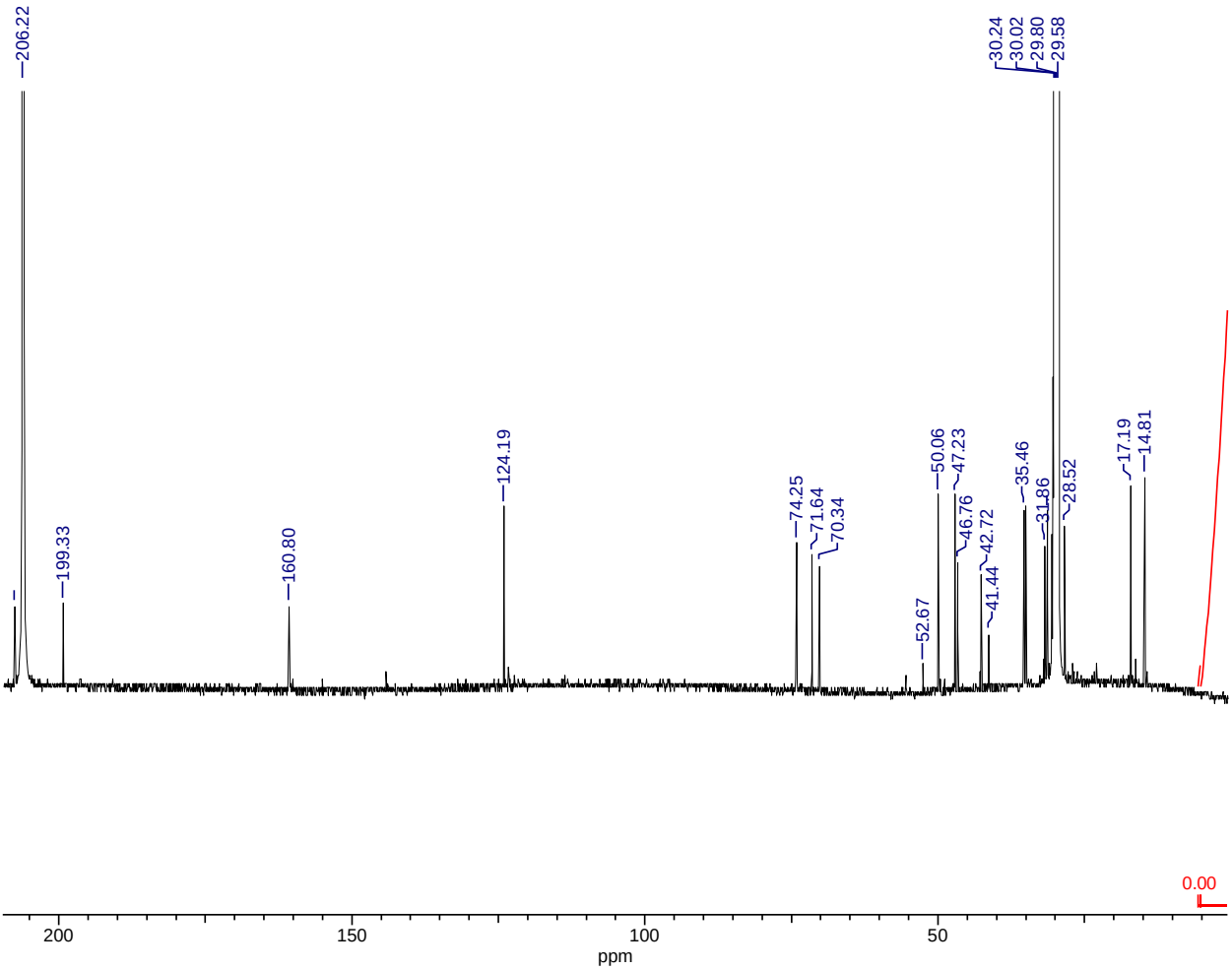
		Q6 1/2	**S**	**-**	**9.38**	**-**	**-**
			M	**-**	**4.69**	**-**	**300**
		ZM 1/2	**S**	**-**	**-**	**-**	**-**
			M	**-**	**-**	**-**	**-**
KE/16-153	*Inonotus* sp	**YMG**	**S**	**-**	**-**	**-**	**-**
			M	**-**	**-**	**-**	**-**
		Q6 1/2	**S**	**-**	**-**	**-**	**-**
			M	**-**	**-**	**-**	**-**
		ZM 1/2	**S**	**-**	**-**	**-**	**-**
			M	**-**	**-**	**-**	**-**
KE/16-198	*Echinochaete brachypora*	**YMG**	**S**	**-**	**-**	**-**	**-**
			M	**-**	**300**	**-**	**-**
		Q61/2	**S**	**-**	**37.5**	**-**	**-**
			M	**-**	**18.75**	**-**	**-**
		ZM1/2	**S**	**-**	**-**	**-**	**-**
			M	**-**	**-**	**-**	**-**

- Not active; S-supernant extracts; M-mycelial extracts; Positive controls-1mg/ml ciprofloxacin (bacteria) and 1mg/ml nystatin (fungi)

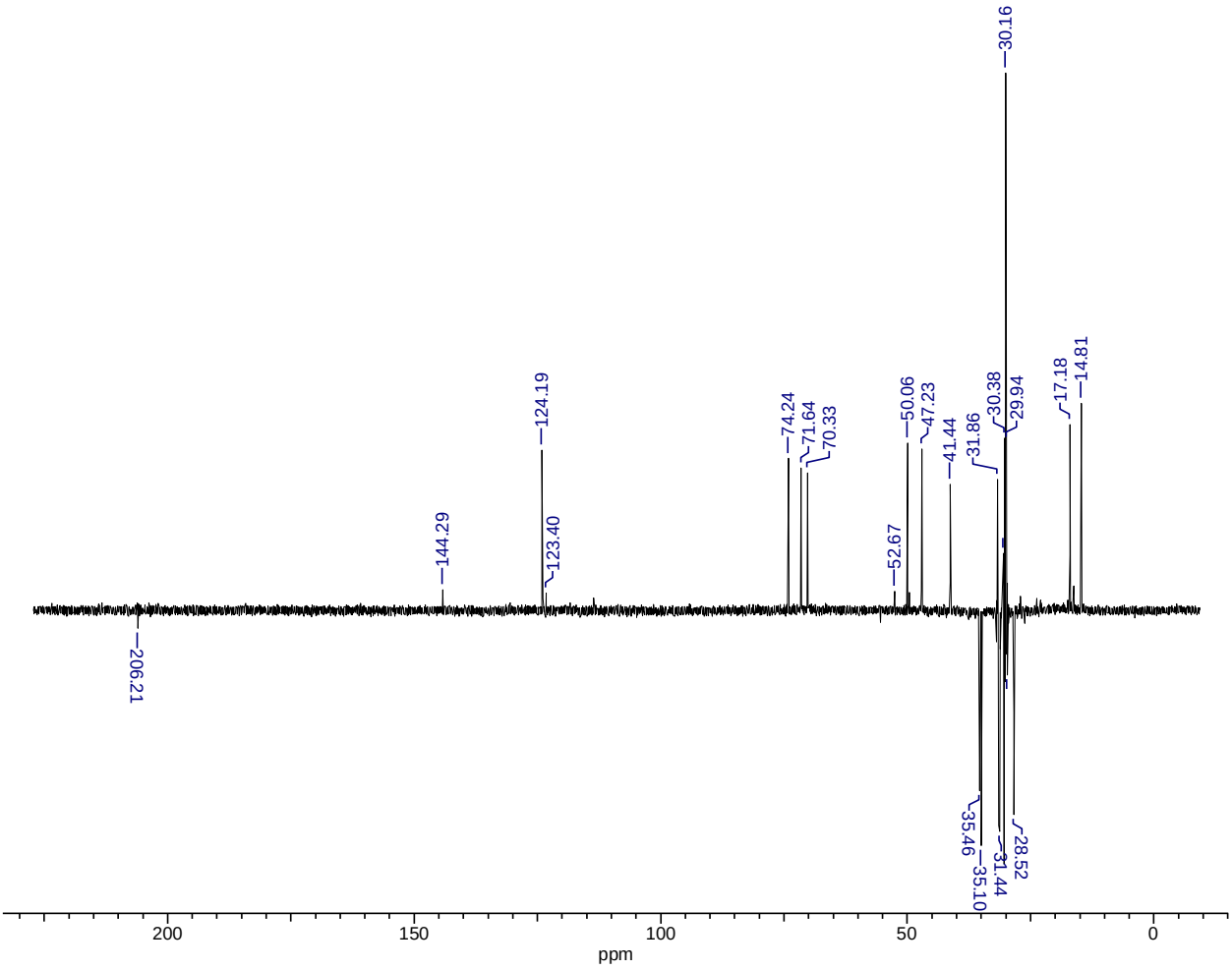
Appendix 4: ^{1}H NMR spectrum of compound **1**



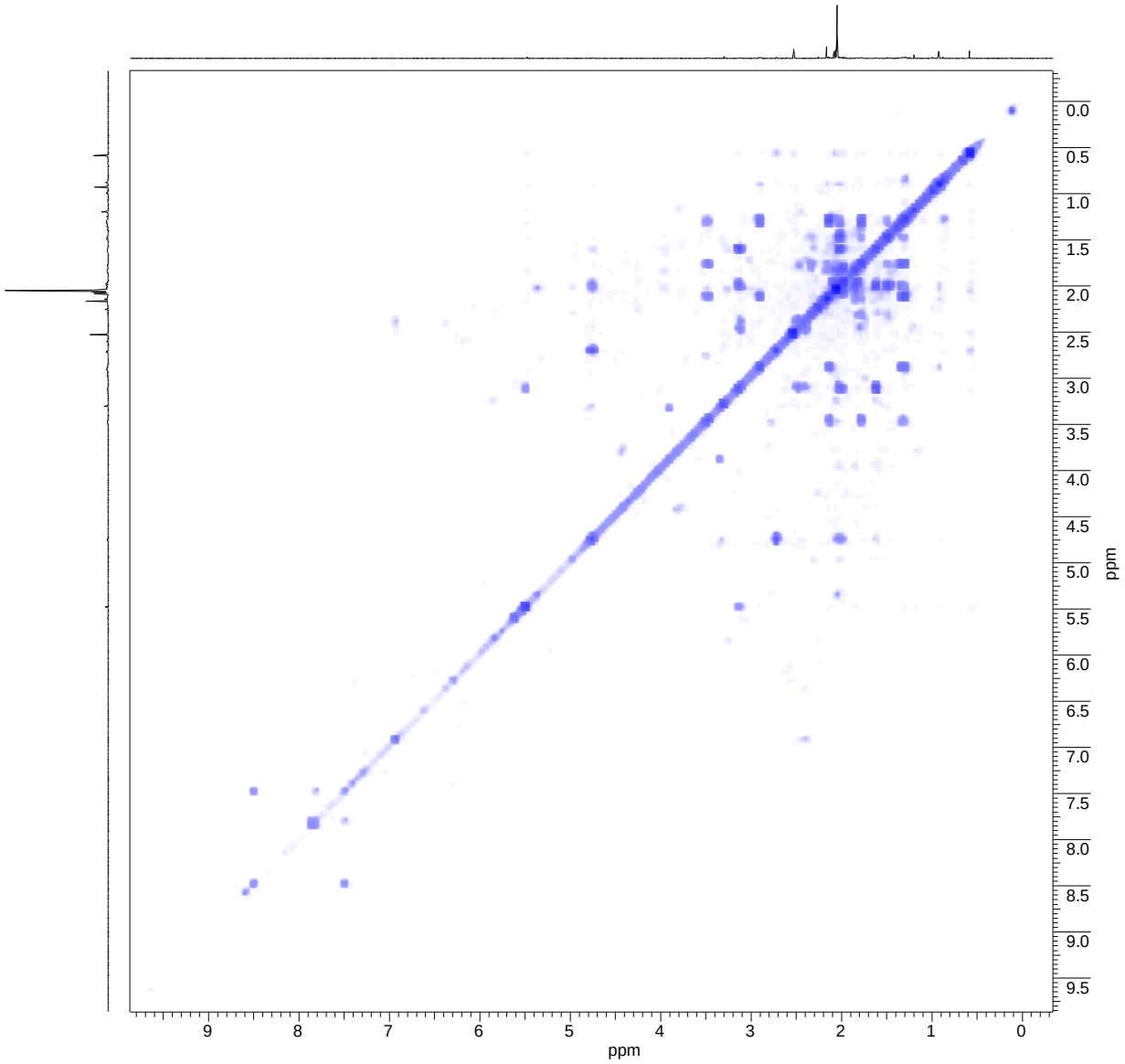
Appendix 5: ^{13}C NMR spectrum of compound **1**



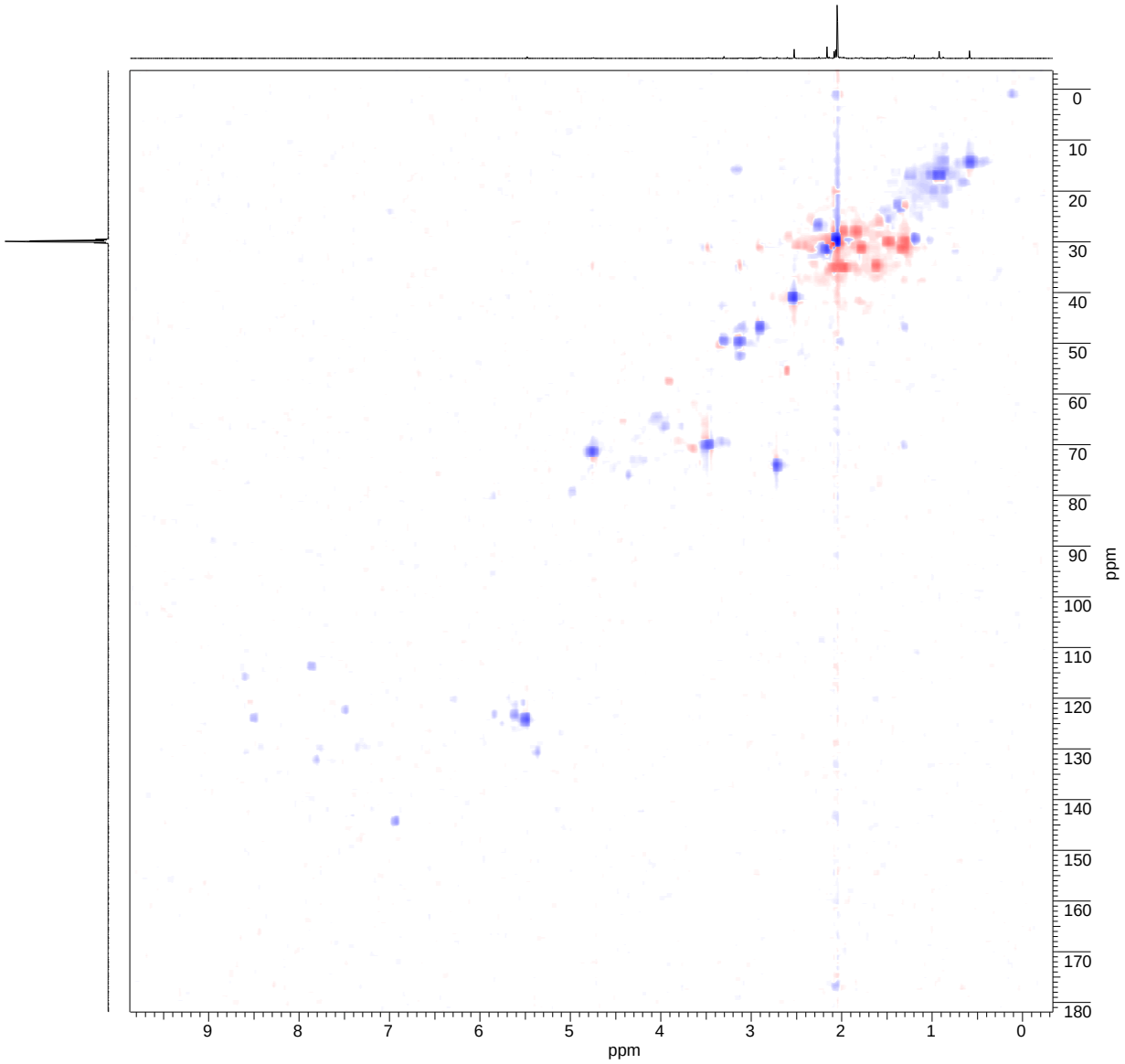
Appendix 6: DEPT NMR spectrum of compound **1**



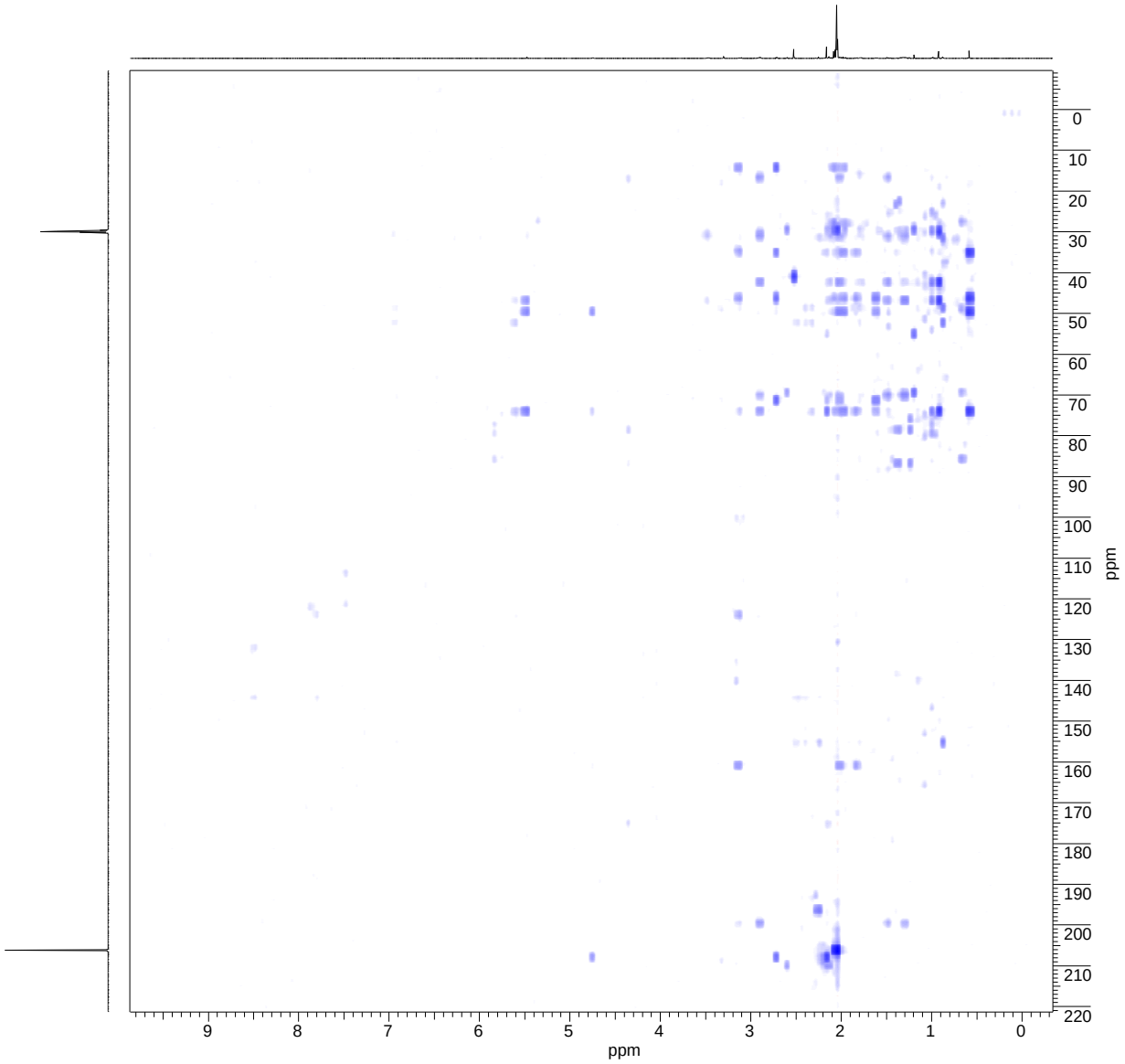
Appendix 7: COSY NMR spectrum of compound **1**



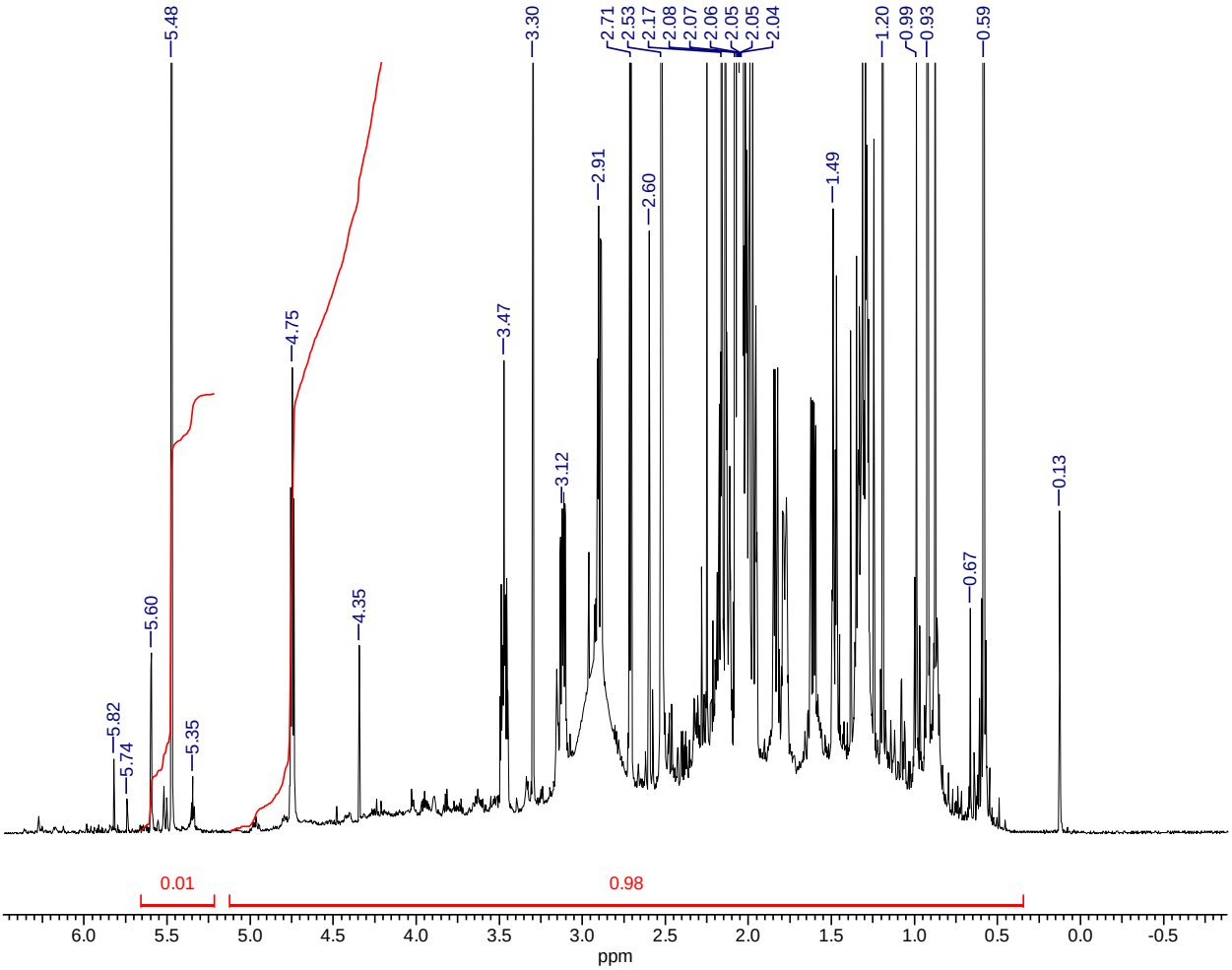
Appendix 8: HSQC NMR spectrum of compound **1**



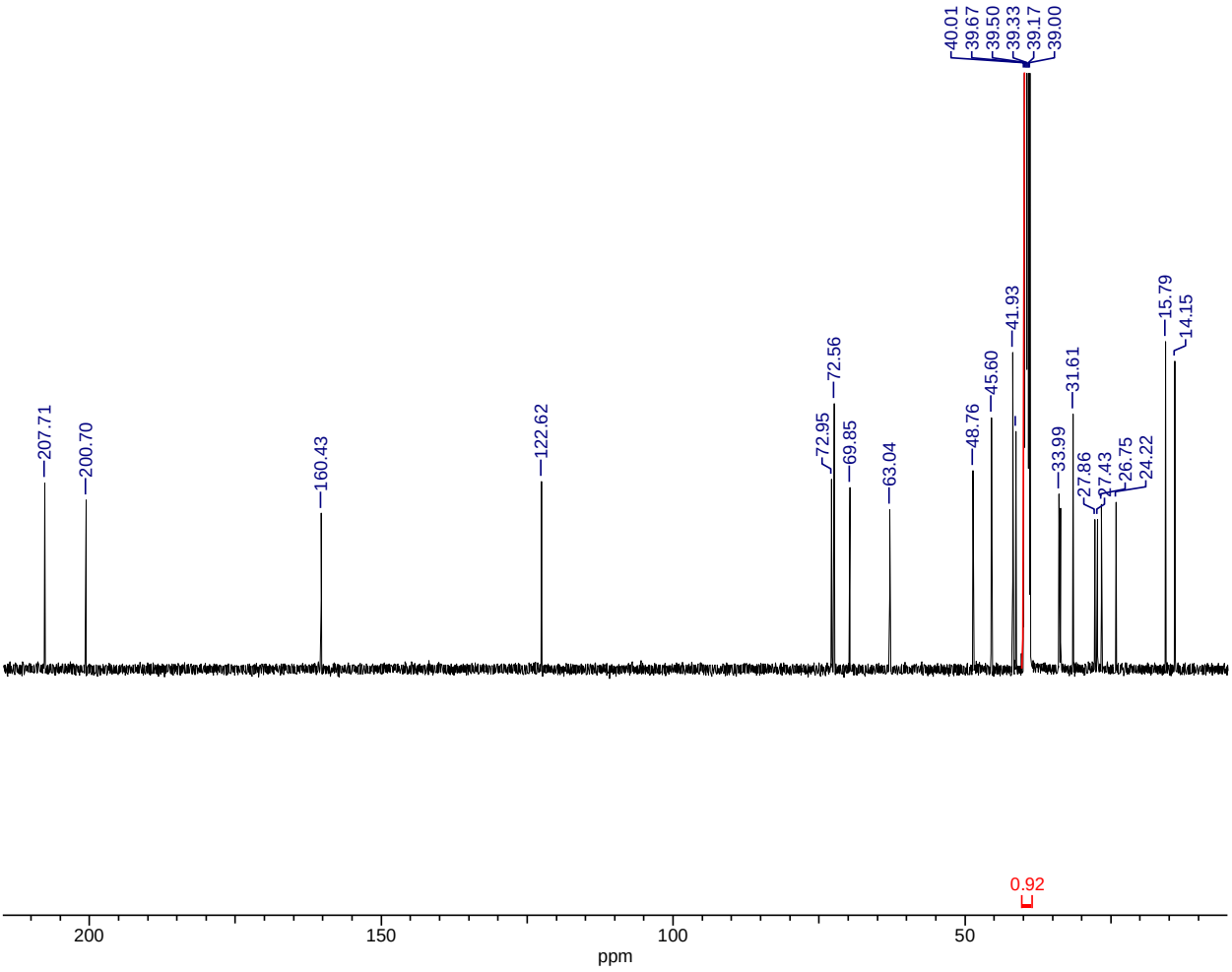
Appendix 9: HMBC NMR spectrum of compound **1**



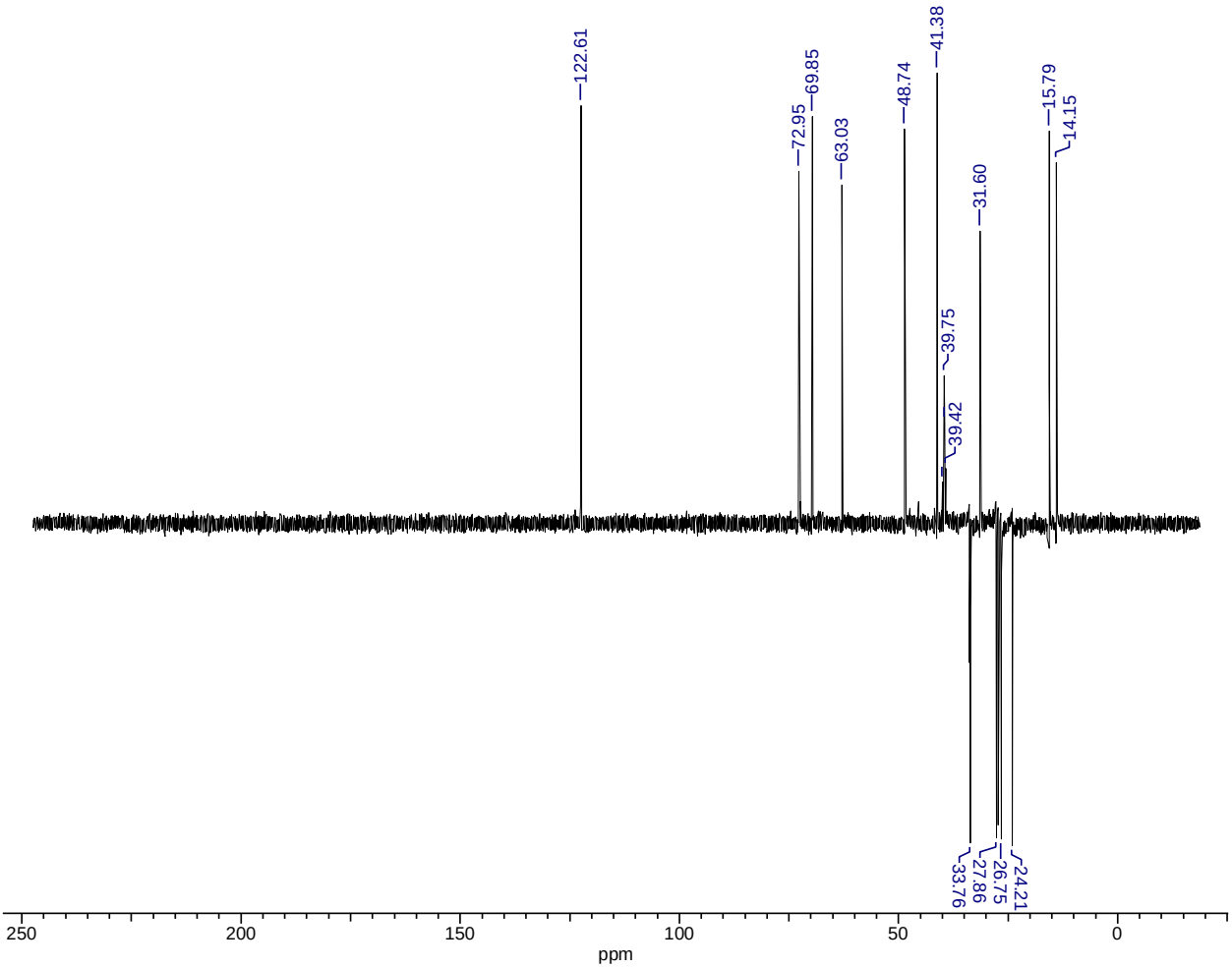
Appendix 10: ^{1}H NMR spectrum of compound **2**



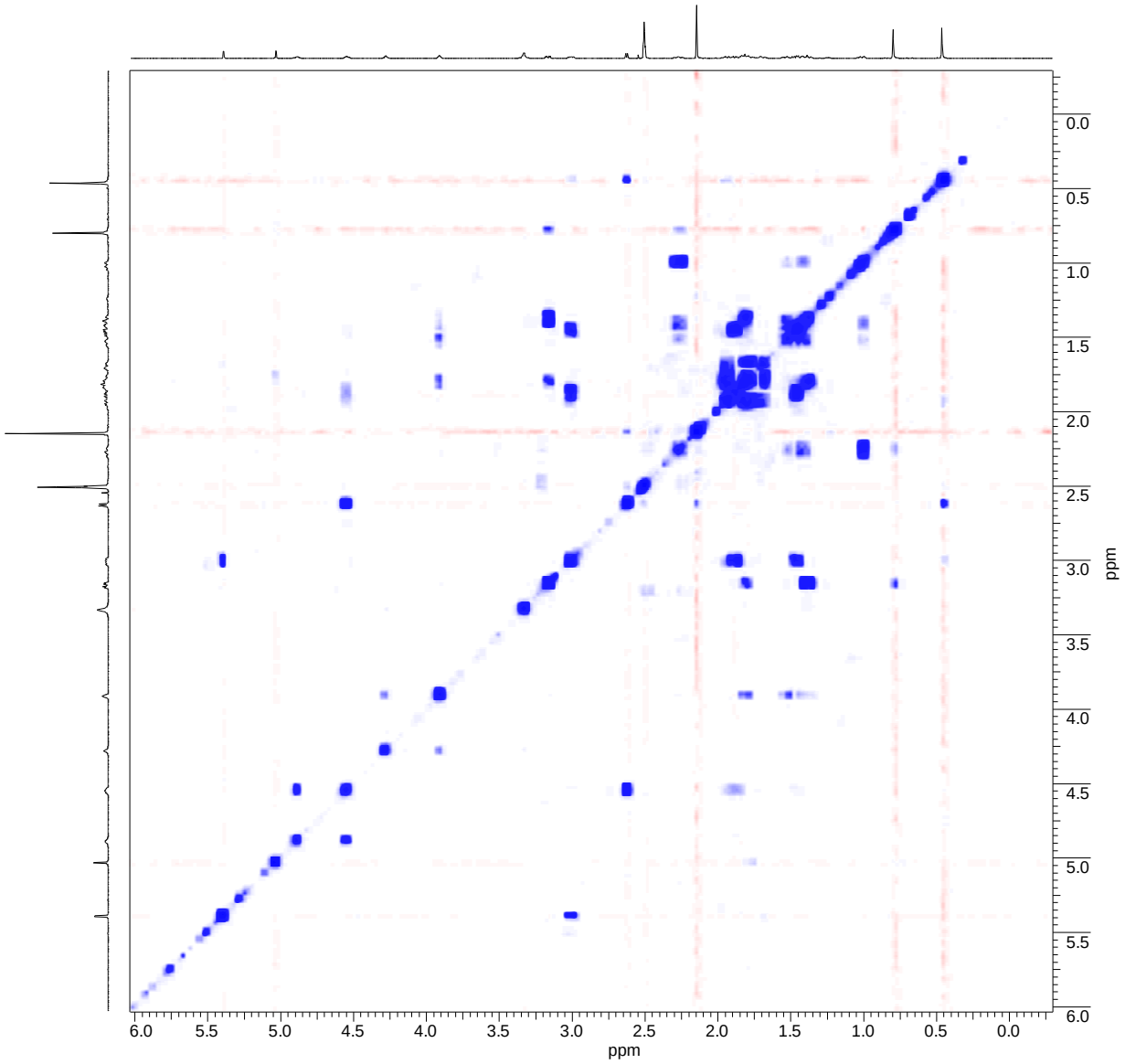
Appendix 11: ^{13}C NMR spectrum of compound **2**



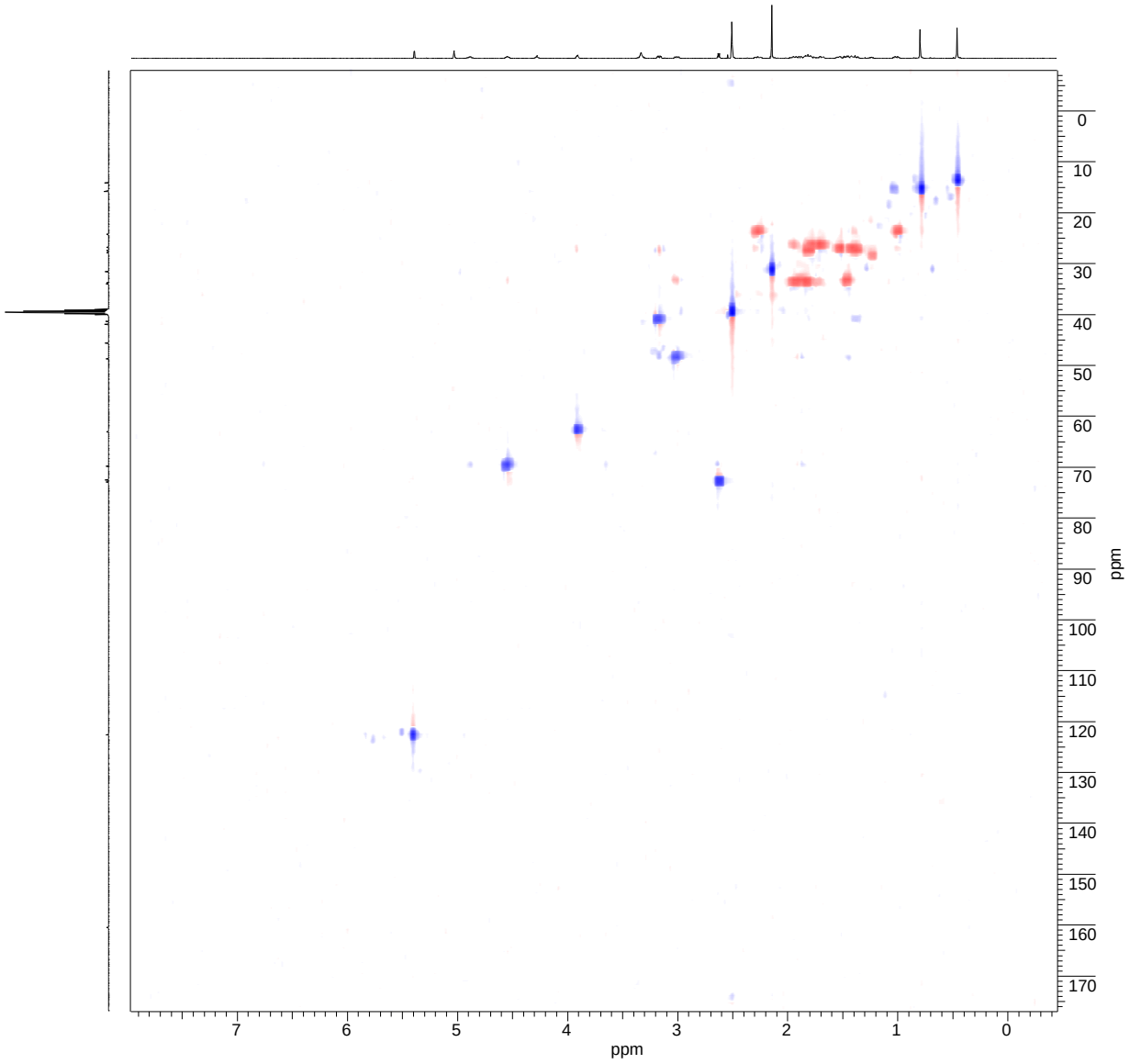
Appendix 12: DEPT NMR spectrum of compound **2**



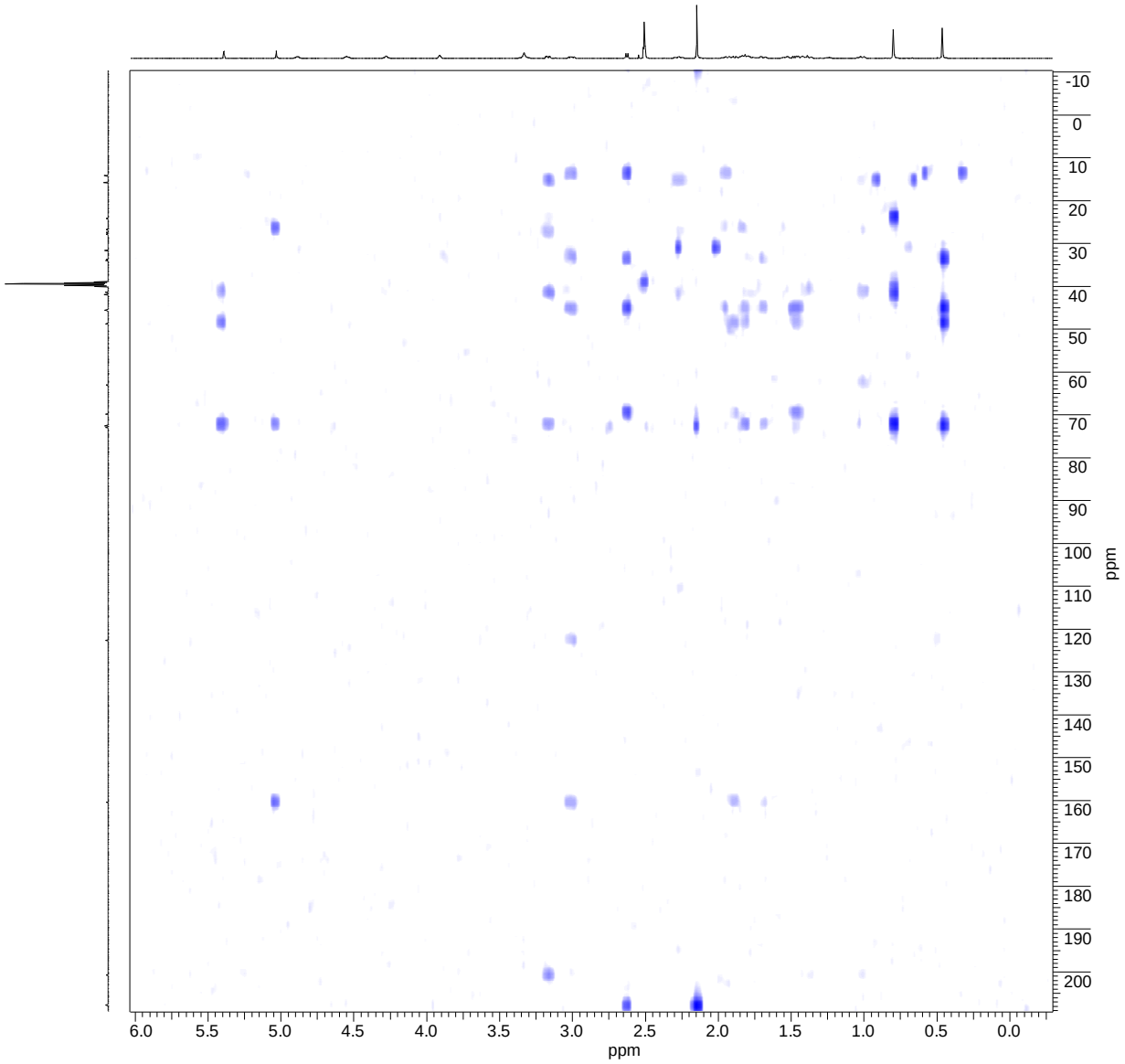
Appendix 13: COSY NMR spectrum of compound **2**



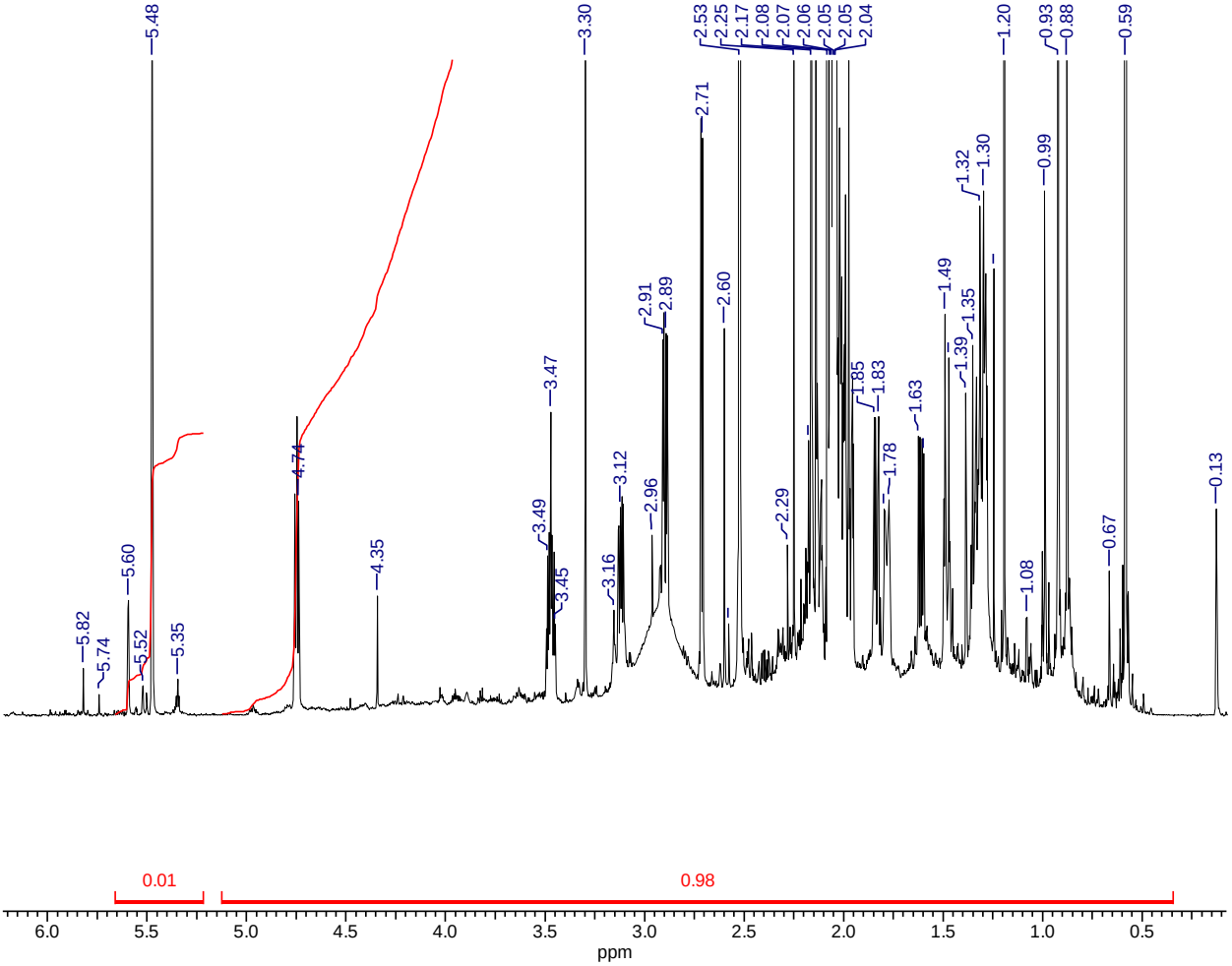
Appendix 14: HSQC NMR spectrum of compound **2**



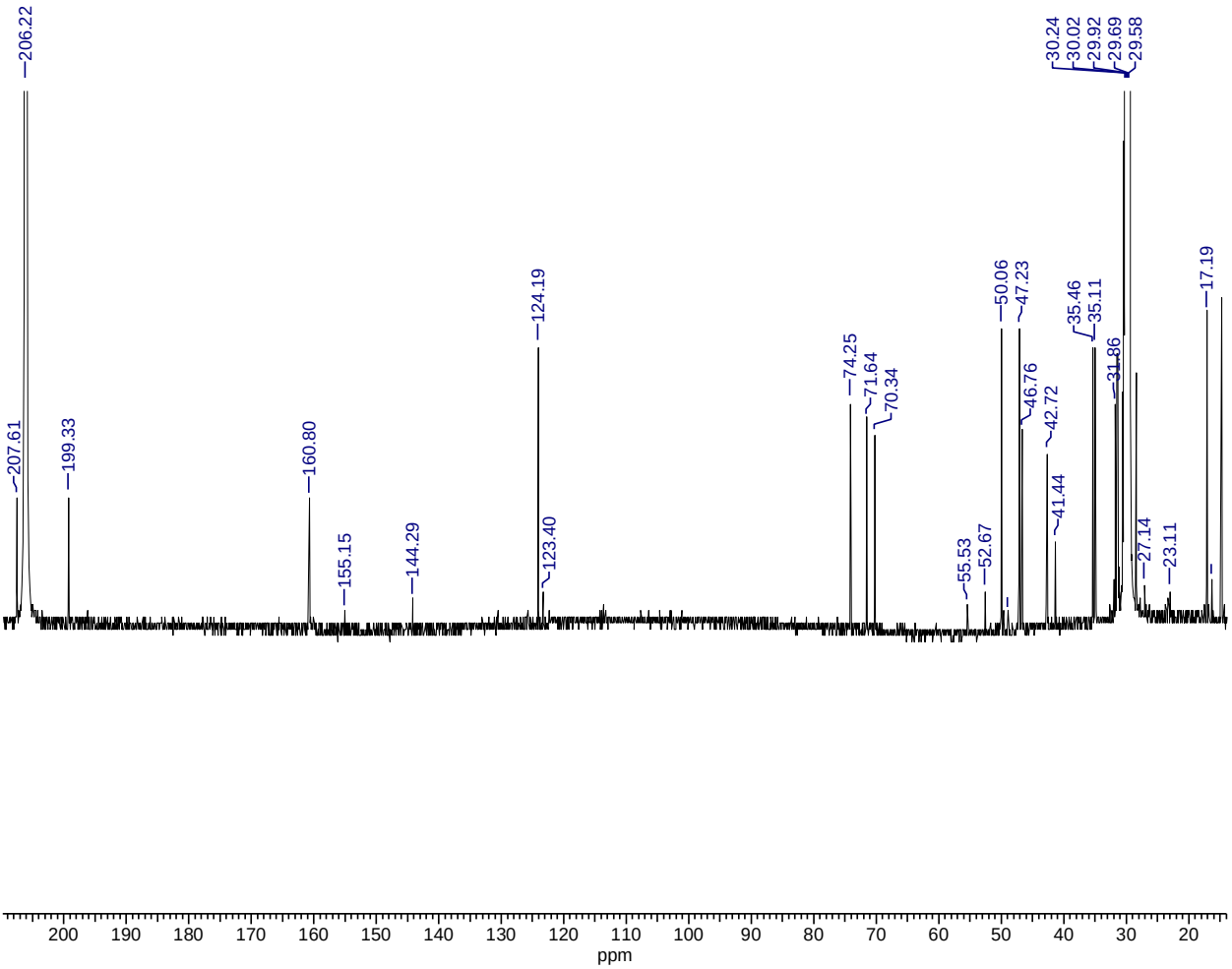
Appendix 15: HMBC NMR spectrum of compound **2**



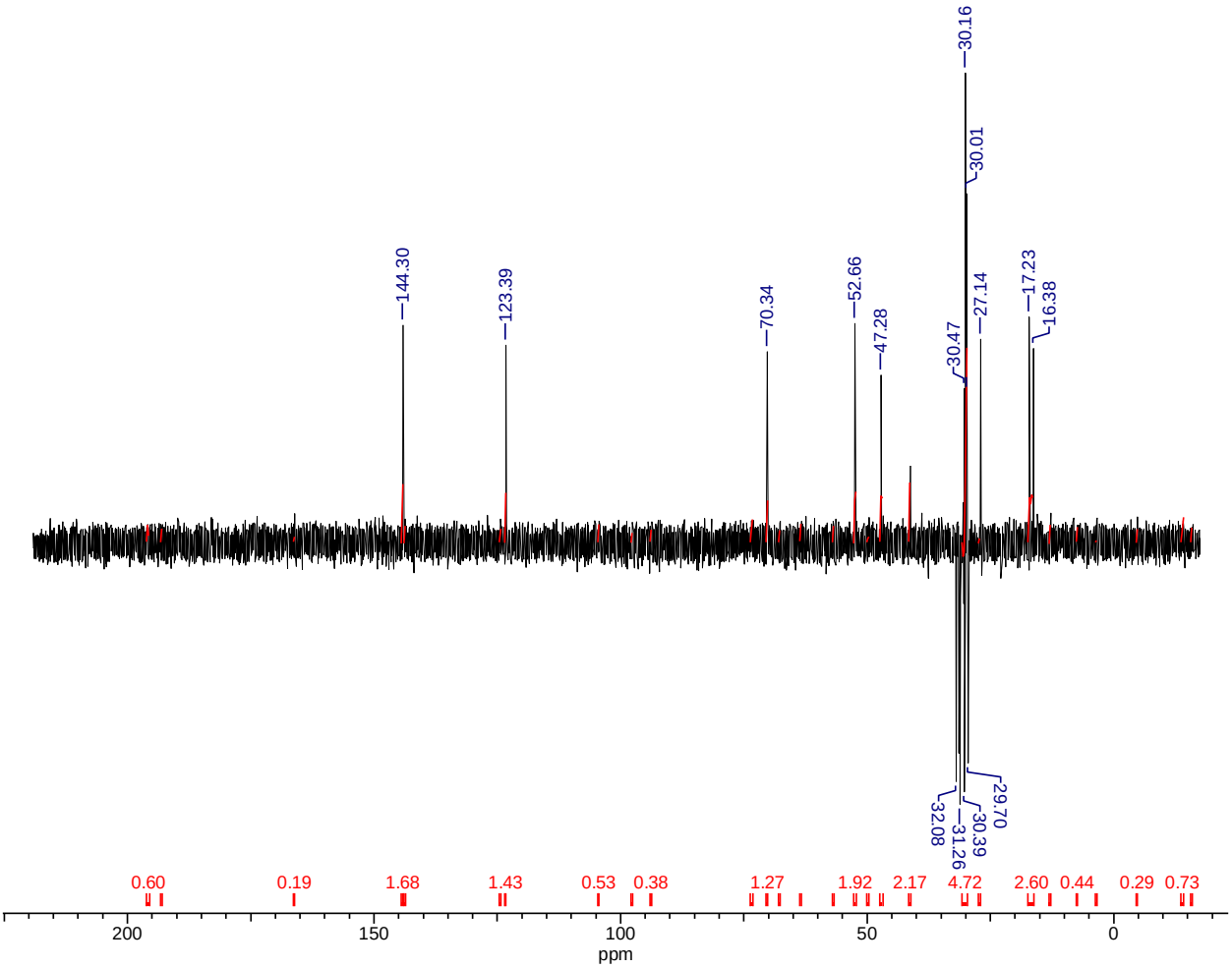
Appendix 16: ^{1}H NMR spectrum of compound **3**



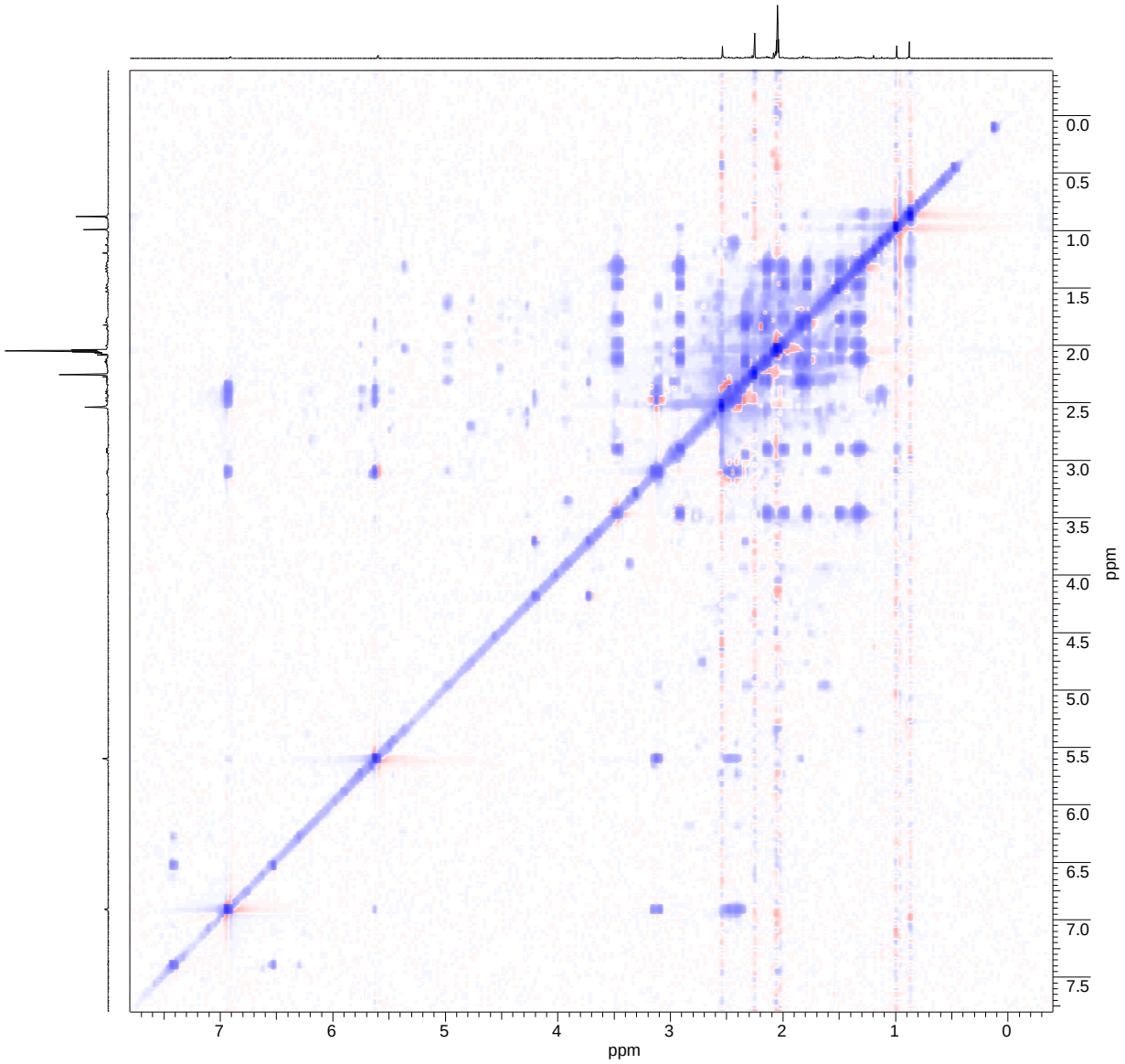
Appendix 17: ^{13}C NMR spectrum of compound **3**



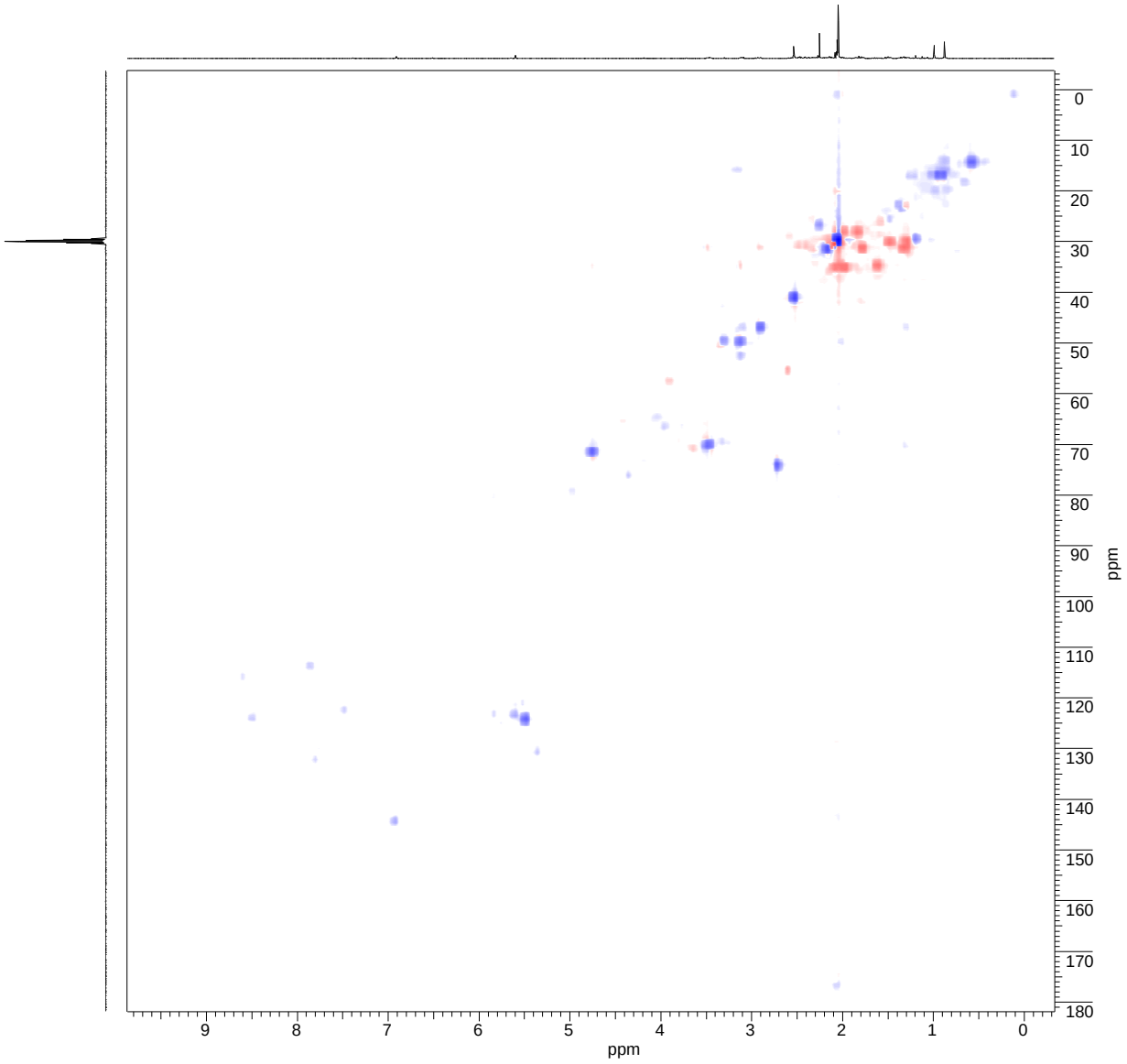
Appendix 18: DEPT NMR spectrum of compound **3**



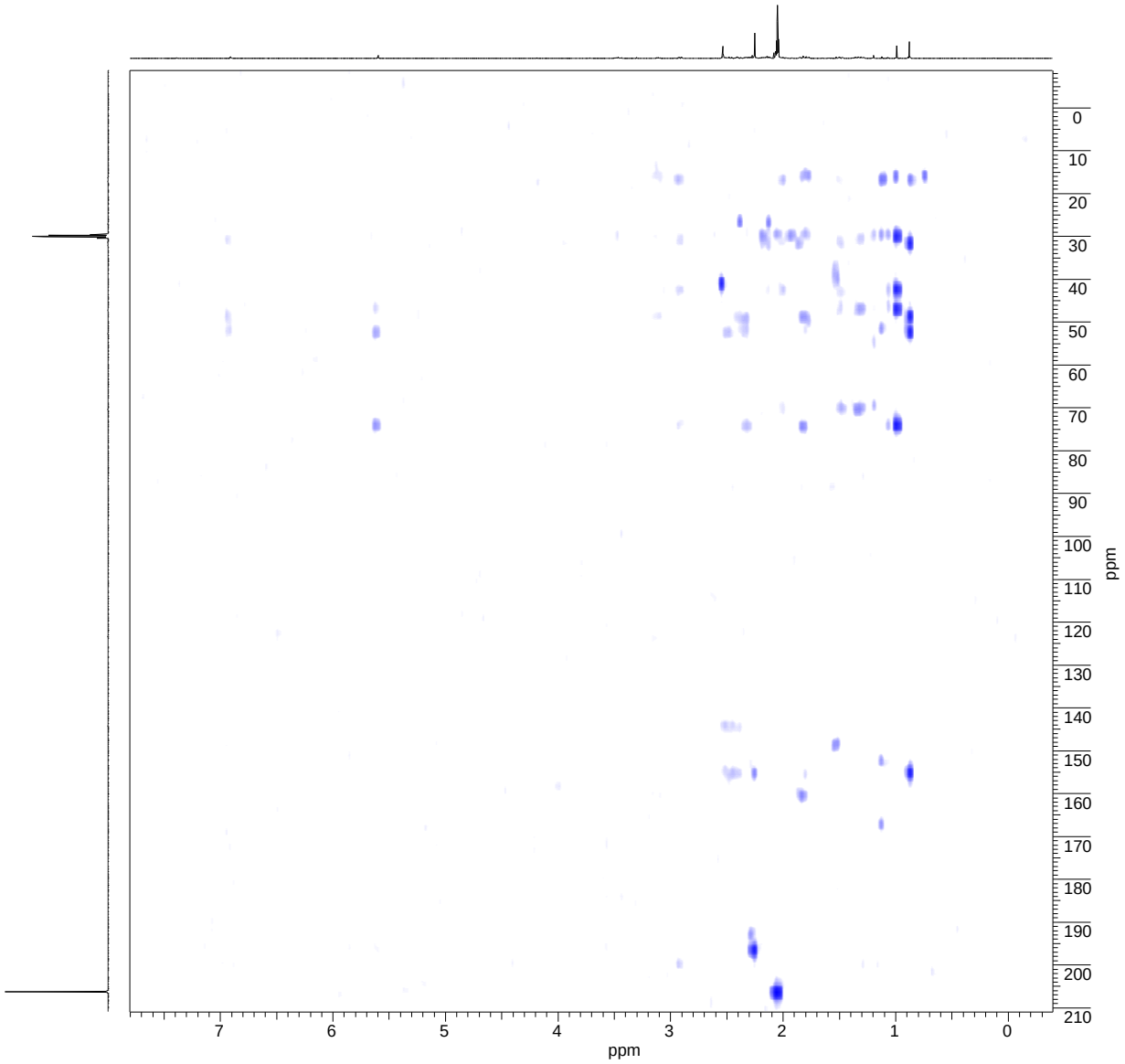
Appendix 19: COSY NMR spectrum of compound **3**



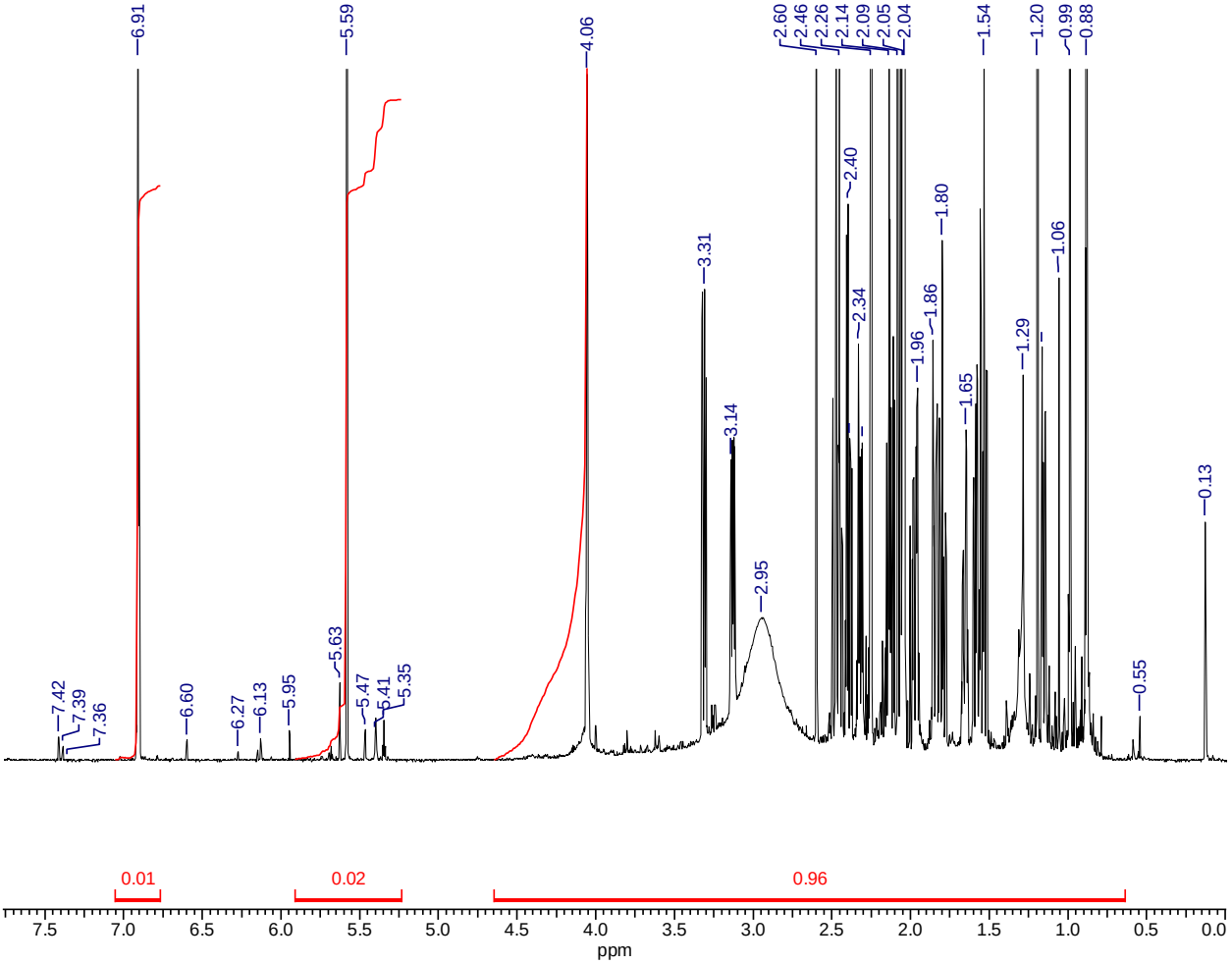
Appendix 20: HSQC NMR spectrum of compound **3**



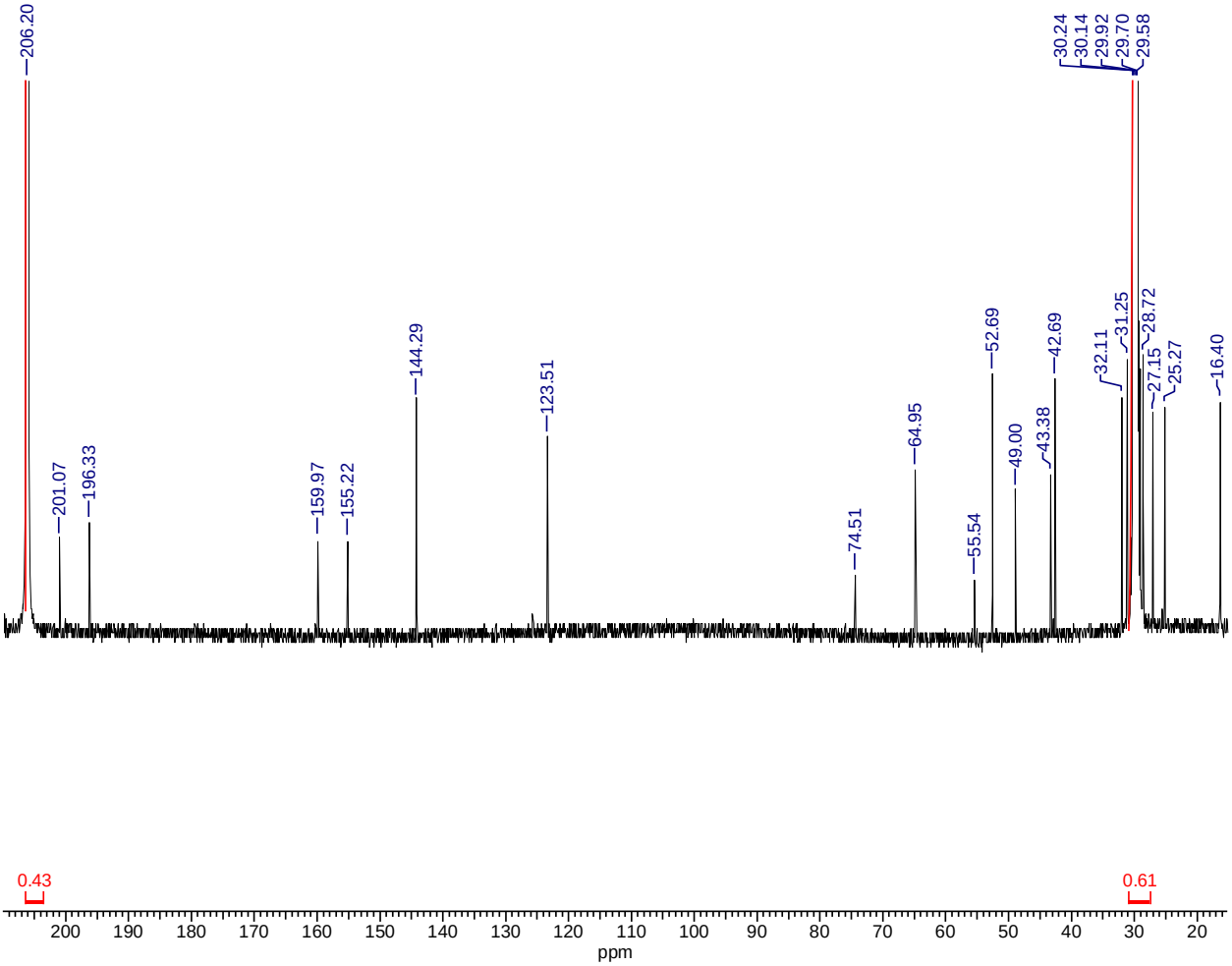
Appendix 21: HMBC NMR spectrum of compound **3**



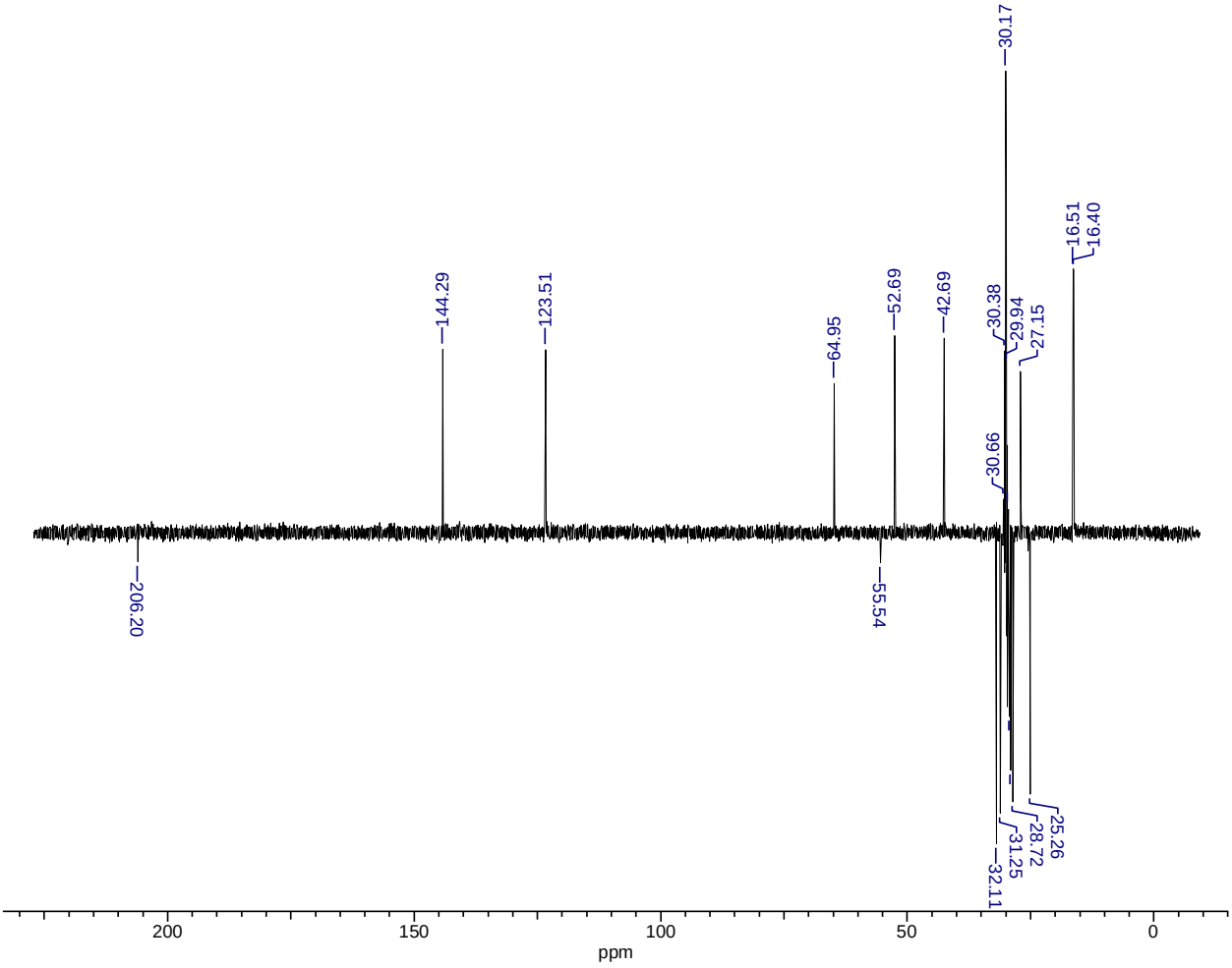
Appendix 22: ^{1}H NMR spectrum of compound **4**



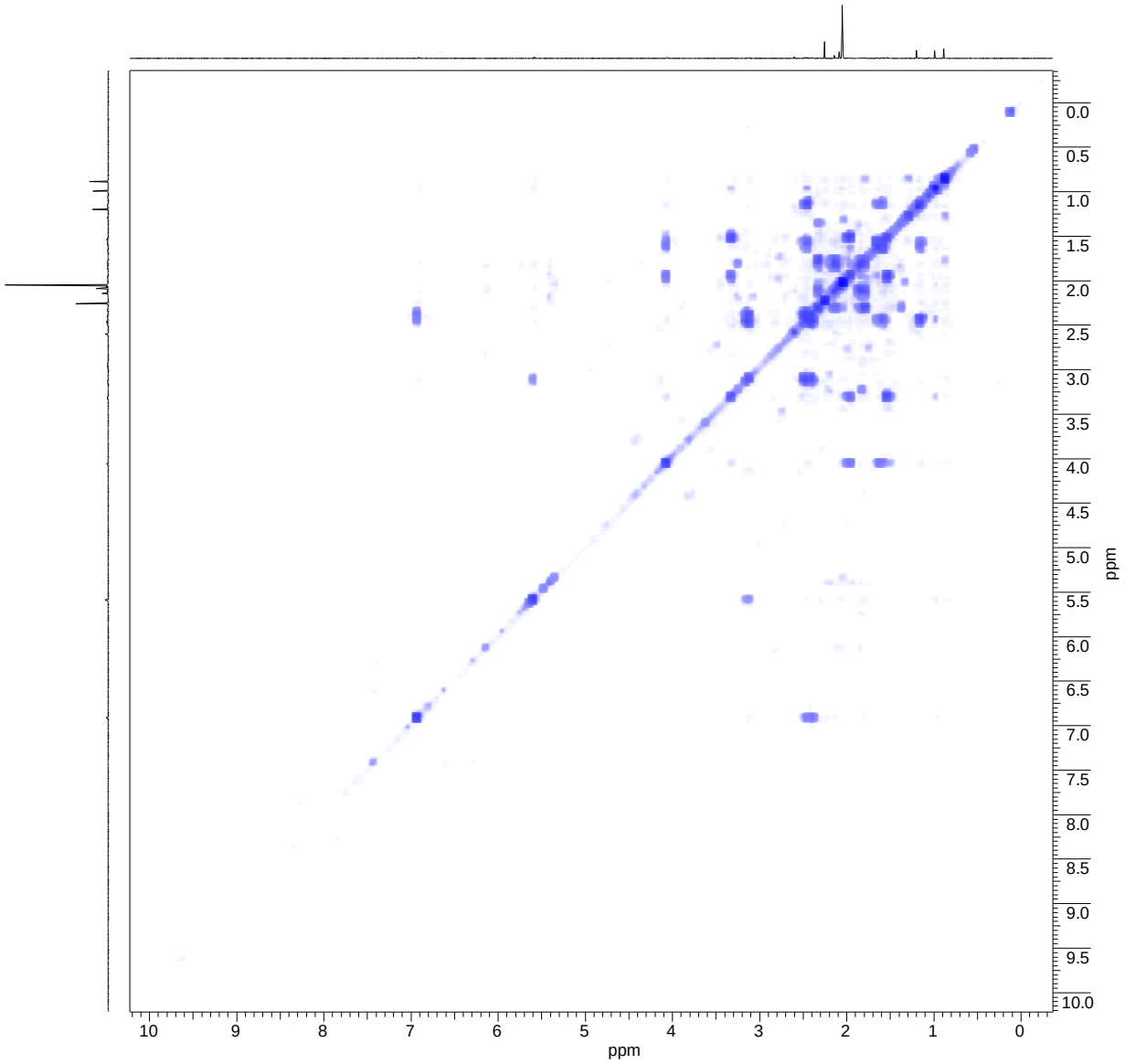
Appendix 23: ^{13}C NMR spectrum of compound **4**



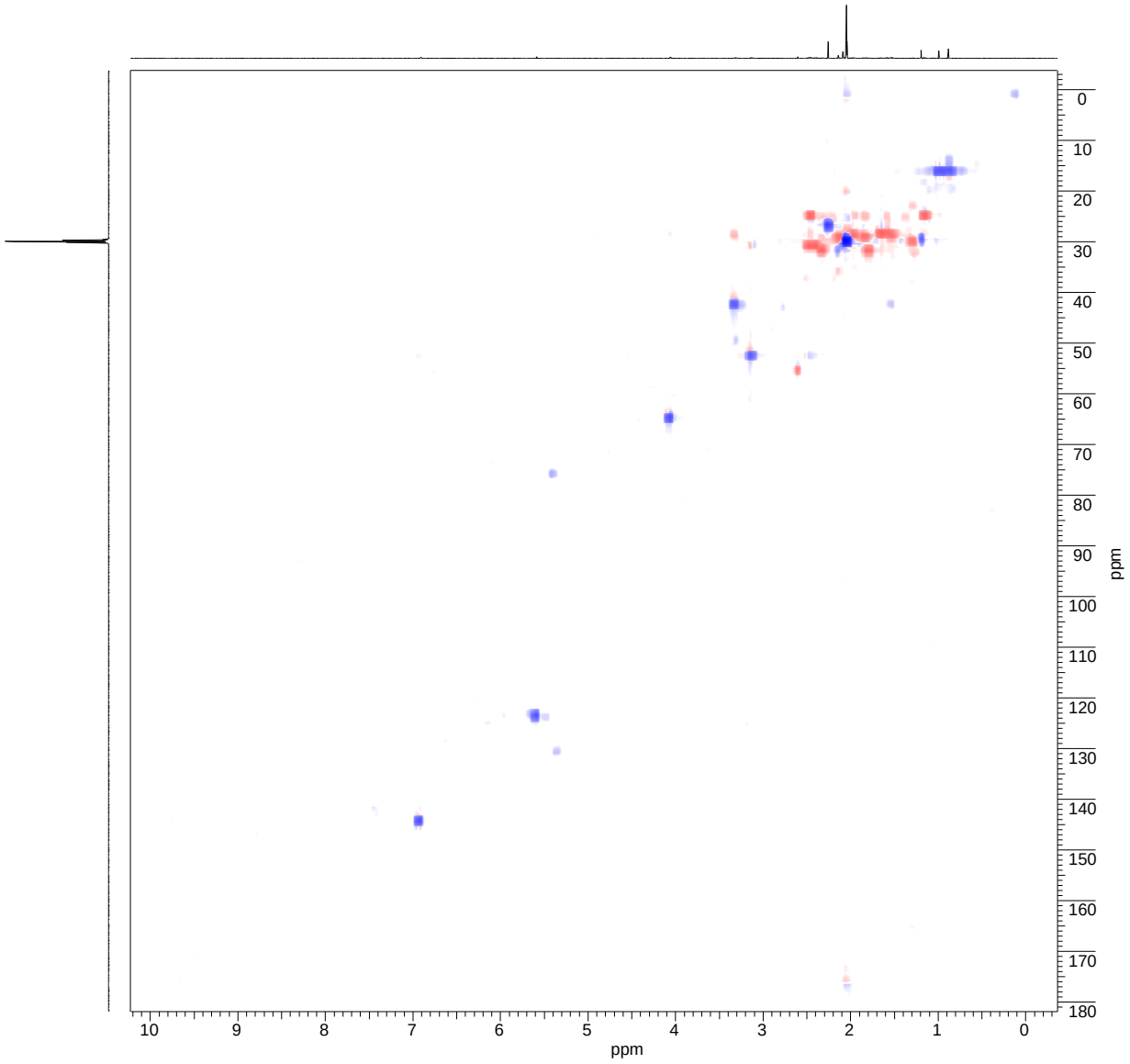
Appendix 24: DEPT NMR spectrum of compound **4**



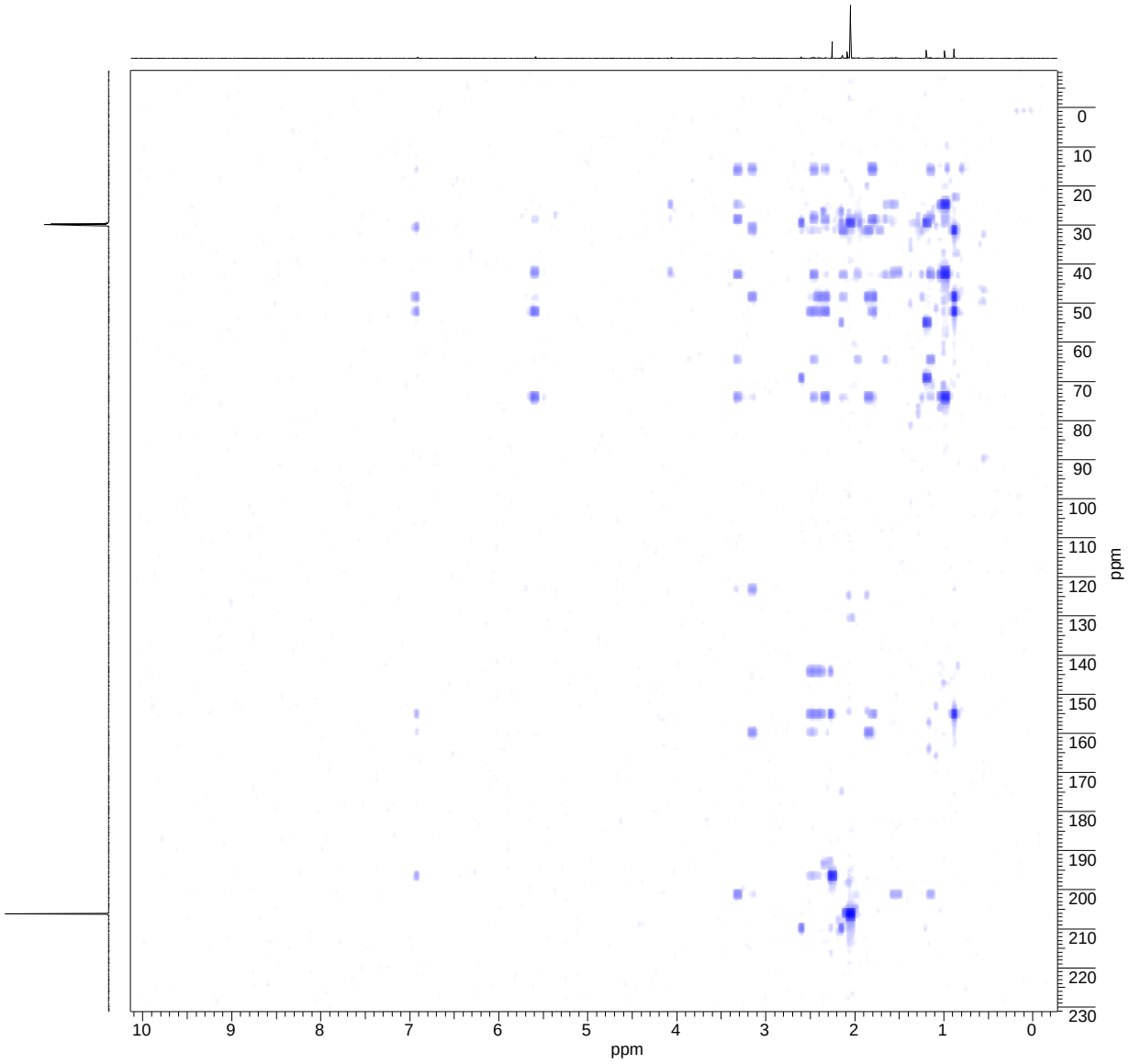
Appendix 25: COSY NMR spectrum of compound **4**



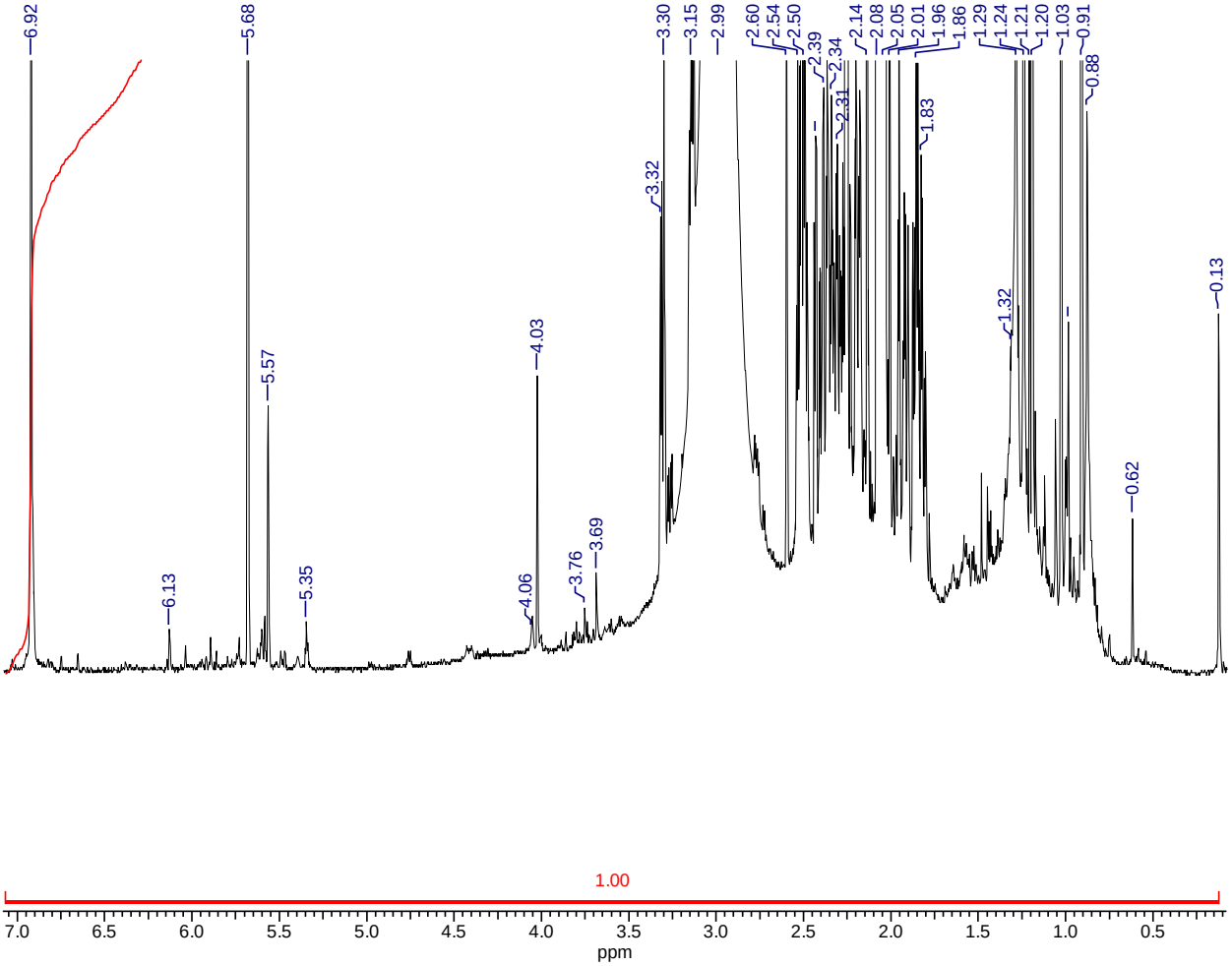
Appendix 26: HSQC NMR spectrum of compound **4**



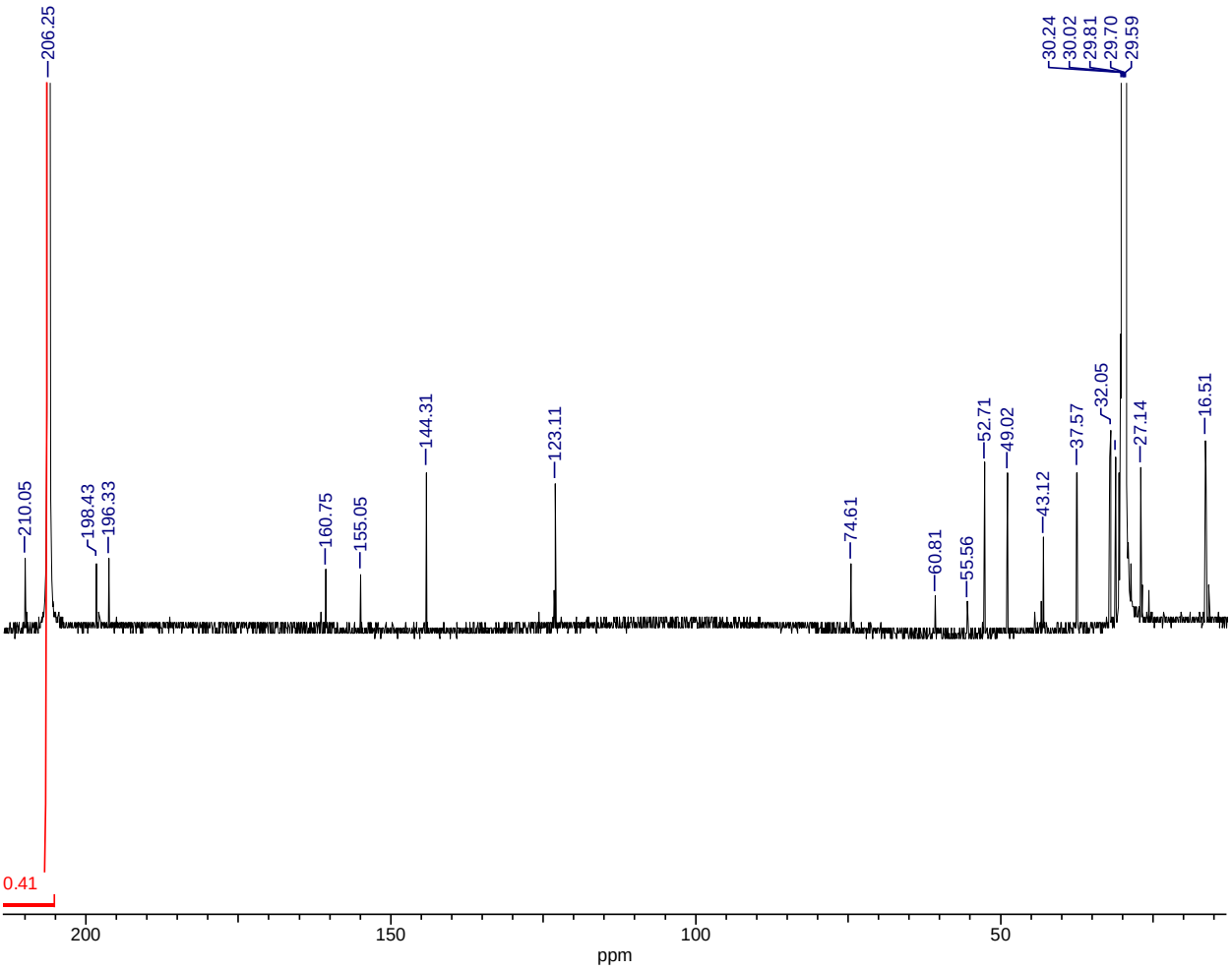
Appendix 27: HMBC NMR spectrum of compound **4**



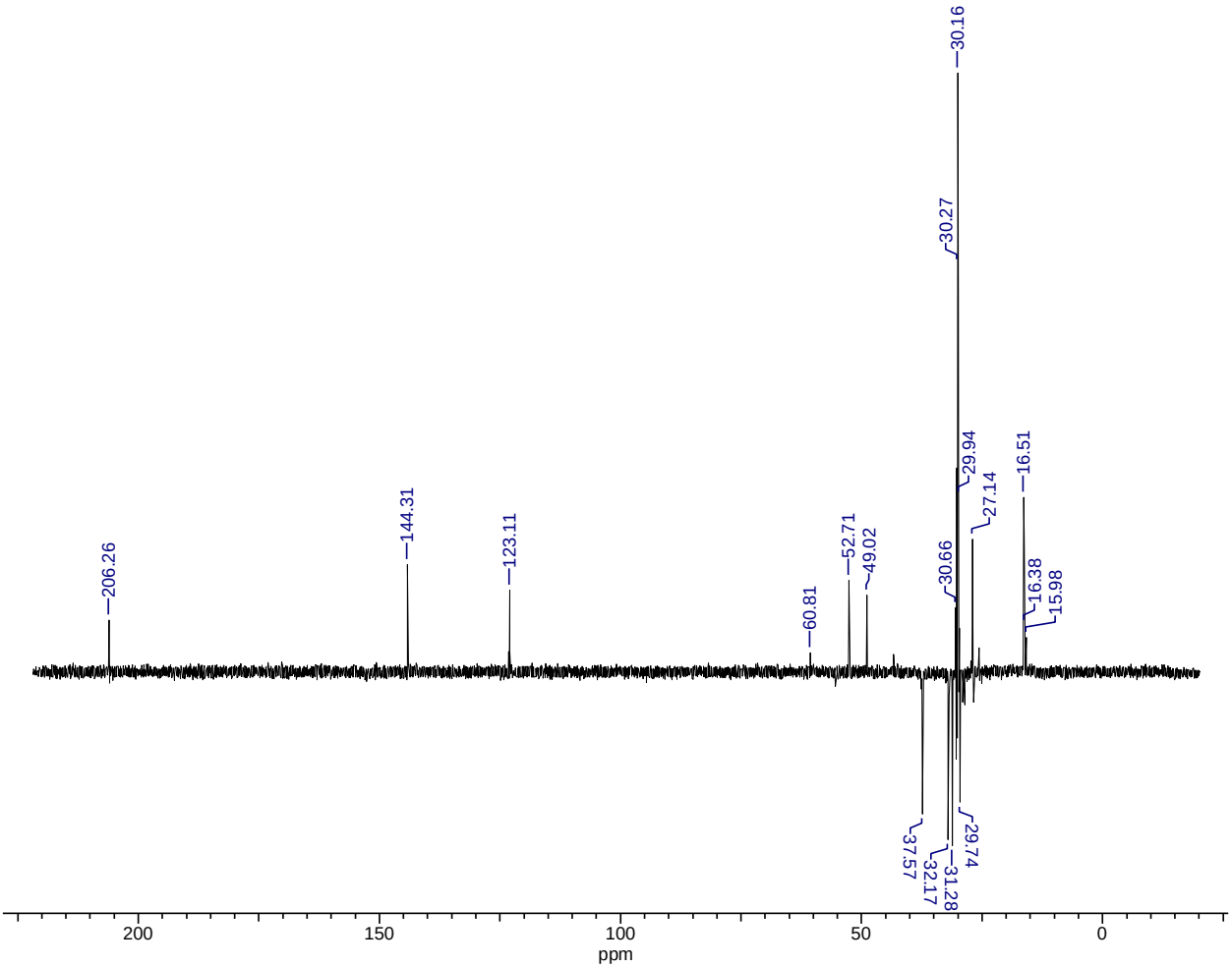
Appendix 28: ^{1}H NMR spectrum of compound **5**



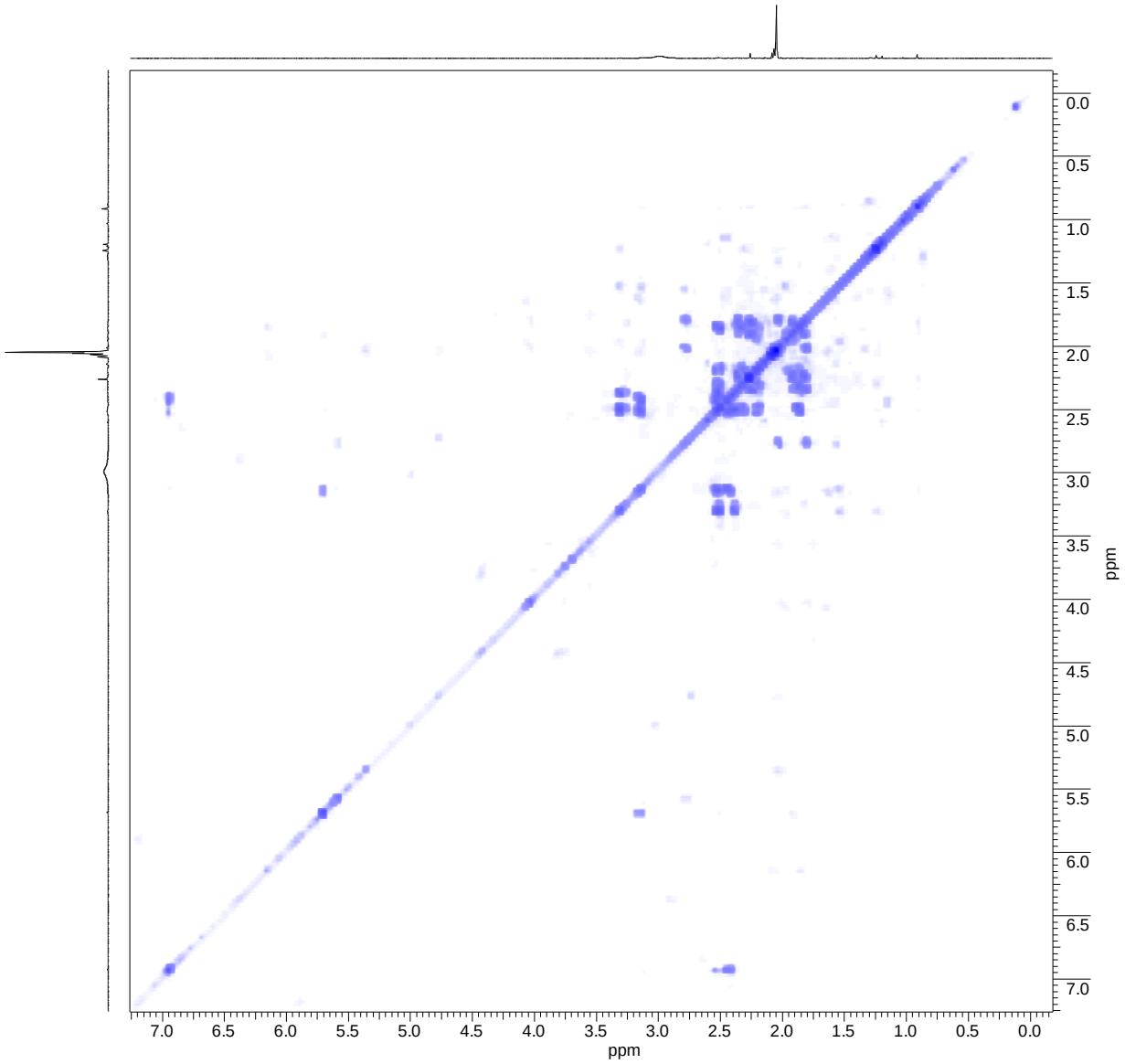
Appendix 29: ^{13}C NMR spectrum of compound **5**



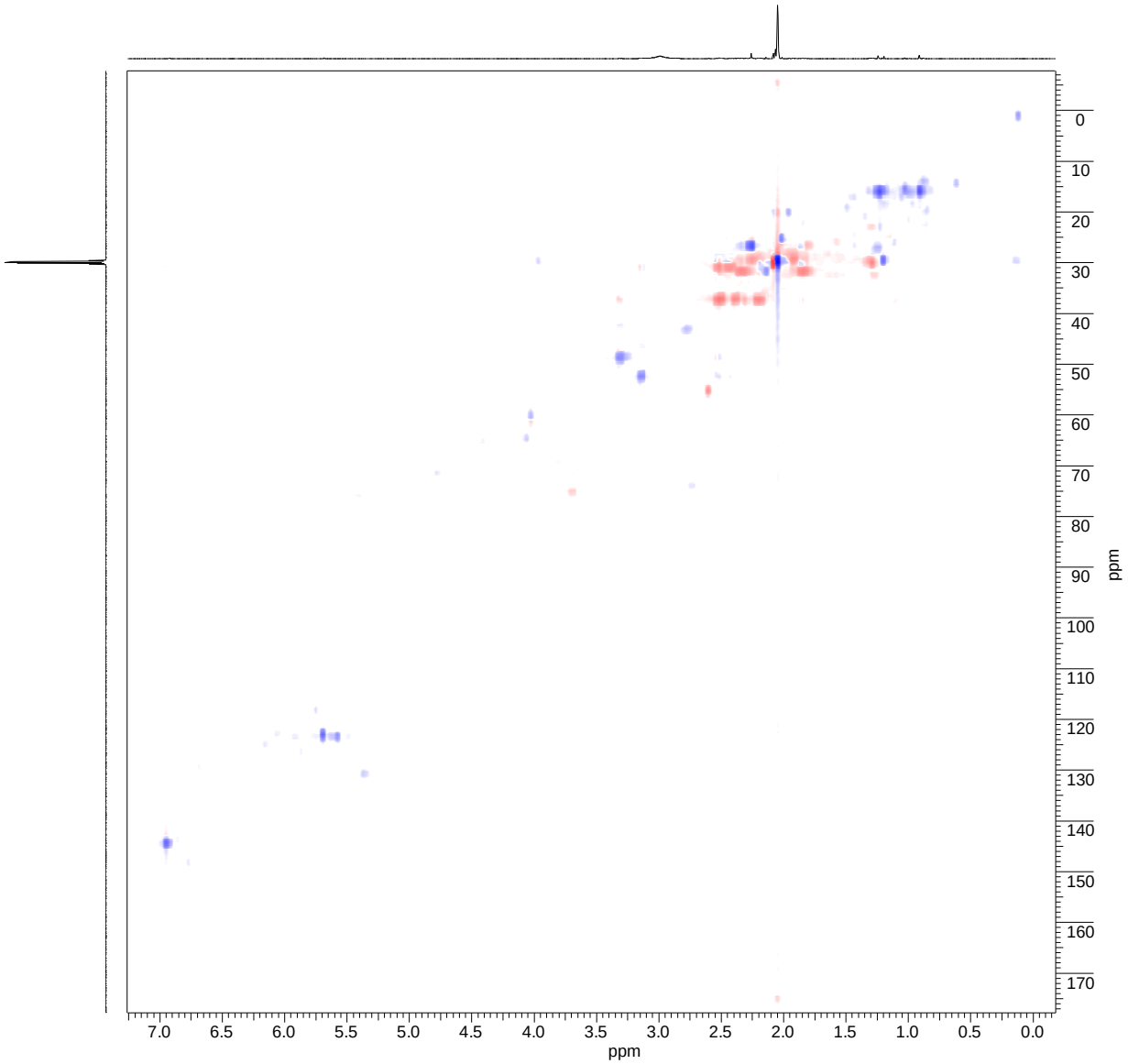
Appendix 30: DEPT NMR spectrum of compound **5**



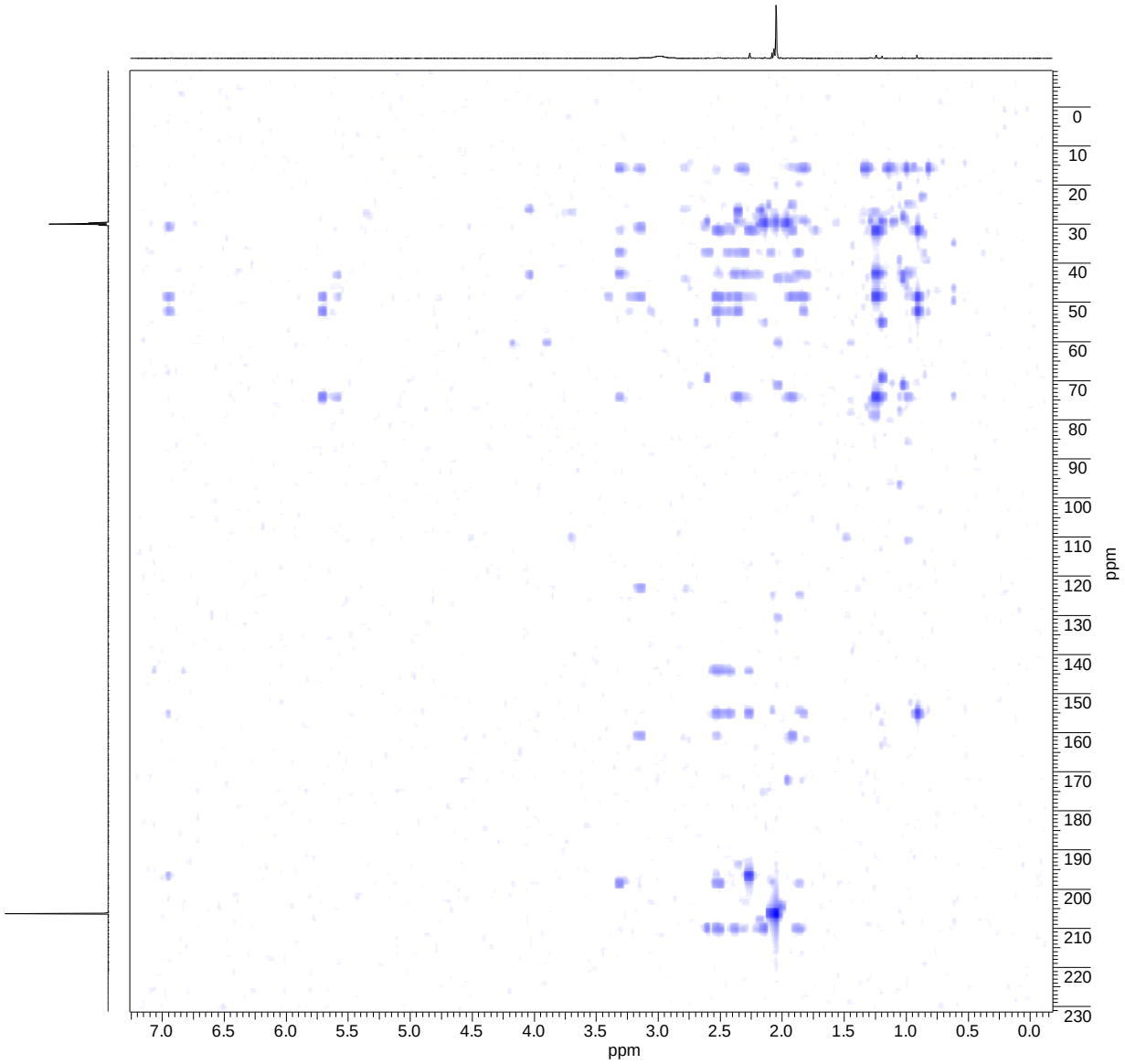
Appendix 31: COSY NMR spectrum of compound **5**



Appendix 32: HSQC NMR spectrum of compound **5**



Appendix 33: HMBC NMR spectrum of compound **5**



Appendix 34: Optical densities (ODs) of the cytotoxic compounds as obtained from the ELISA plate reader. These were converted in relative activities using MS-Excel software

◇	1	2	3	4	5	6	7	8	9	10	11	12
A	0.0490	0.0464	0.0476	0.0501	0.0524	0.0499	0.0571	0.0437	0.0530	0.0513	0.0517	0.0523
B	0.3425	0.3202	0.3947	0.4095	0.4091	0.4186	0.3901	0.3868	0.3776	0.3760	0.3564	0.3471
C	0.3819	0.3019	0.3704	0.4016	0.4037	0.4102	0.3751	0.3660	0.3780	0.3641	0.3680	0.3639
D	0.3832	0.0723	0.1554	0.2746	0.3817	0.4031	0.3724	0.3754	0.3691	0.3928	0.3667	0.3482
E	0.3870	0.0733	0.1432	0.2766	0.3431	0.3564	0.3726	0.3847	0.3730	0.3868	0.3768	0.3574
F	0.3845	0.2980	0.2980	0.2980	0.3429	0.3396	0.4027	0.3735	0.3556	0.3819	0.3757	0.3582
G	0.3844	0.2845	0.2845	0.2845	0.3321	0.3665	0.3568	0.3764	0.3825	0.3466	0.3630	0.4107
H	0.0482	0.0526	0.0564	0.0503	0.0529	0.0483	0.0545	0.0525	0.0545	0.0511	0.0483	0.0465

a). OD values of compound **1** against breast cancer cells (MCF-7) (F and G-Methanol, D and E- Compound **1**).

◇	1	2	3	4	5	6	7	8	9	10	11	12
A	0.0448	0.0473	0.0501	0.0516	0.0509	0.0466	0.0495	0.0487	0.0500	0.0479	0.0493	0.0505
B	1.3726	1.3702	1.6028	1.4006	1.6089	1.5684	1.5780	1.4823	1.3644	1.5765	1.0350	1.3541
C	1.3041	1.4018	1.5922	1.5673	1.5488	1.2961	1.2805	1.2543	1.1170	1.4126	1.1927	1.2156
D	1.4783	0.0514	1.2877	1.6521	1.5706	1.5702	1.7372	1.4621	1.4414	1.4229	1.4937	1.7316
E	1.4553	0.0560	1.1597	1.4813	1.3896	1.4202	1.3615	1.4579	1.3073	1.3172	1.4490	1.1932
F	1.4918	1.5945	1.5945	1.6254	1.5651	1.3120	1.1845	1.2321	1.2648	1.3876	1.1539	1.2436
G	1.3246	1.7029	1.7029	1.7455	1.7571	1.5646	1.3735	1.5960	1.2007	1.2406	1.0523	1.3633
H	0.0472	0.0455	0.0482	0.0501	0.0530	0.0510	0.0524	0.0508	0.0537	0.0530	0.0506	0.0437

b). OD values of compound **1** against HeLa cells KB3.1 (F and G-Methanol, D and E-Compound **1**).

◇	1	2	3	4	5	6	7	8	9	10	11	12
A	0.0434	0.0453	0.0559	0.0497	0.0545	0.0443	0.0526	0.0609	0.0487	0.0464	0.0470	0.0470
B	0.4764	0.0964	0.1532	0.3519	0.3746	0.4070	0.4468	0.4956	0.4577	0.4727	0.4772	0.4930
C	0.4786	0.1103	0.1652	0.3674	0.3767	0.4069	0.4133	0.4481	0.4531	0.4682	0.4683	0.4563
D	0.4509	0.0923	0.1559	0.3486	0.4030	0.4286	0.4651	0.4762	0.4659	0.4630	0.4523	0.4594
E	0.3888	0.0866	0.1563	0.3264	0.3854	0.4348	0.4472	0.4542	0.4537	0.4621	0.4810	0.4357
F	0.3753	0.3143	0.3014	0.3605	0.4031	0.4166	0.4341	0.4658	0.4430	0.4519	0.4559	0.3966
G	0.2923	0.2913	0.3109	0.3572	0.3632	0.3808	0.4074	0.4207	0.4186	0.4635	0.3874	0.3361
H	0.0524	0.0519	0.0523	0.0517	0.0509	0.0521	0.0512	0.0562	0.0604	0.0595	0.0601	0.0563

c). OD values of compound **3** and **4** against breast cancer cells (MCF-7) (F and G-Methanol, B and C-Compound **3**, D and E- Compound **4**).

◇	1	2	3	4	5	6	7	8	9	10	11	12
A	0.0444	0.0487	0.0463	0.0523	0.0513	0.0550	0.0498	0.0507	0.0525	0.0509	0.0504	0.0491
B	0.4993	0.0793	0.1676	0.4379	0.4721	0.5184	0.5320	0.5362	0.5360	0.5172	0.5990	0.5751
C	0.5189	0.0769	0.1477	0.4111	0.4732	0.5014	0.5245	0.5342	0.5348	0.5456	0.5404	0.5580
D	0.4932	0.3868	0.4090	0.4296	0.4751	0.5075	0.5154	0.5338	0.5837	0.5599	0.5391	0.5580
E	0.5284	0.3888	0.4137	0.4051	0.4560	0.4864	0.5033	0.5419	0.5589	0.5370	0.5480	0.5392
F	0.4903	0.3558	0.3719	0.4422	0.4928	0.5129	0.5406	0.5912	0.5591	0.5669	0.5439	0.5580
G	0.5393	0.3822	0.4029	0.4487	0.4890	0.5357	0.5194	0.5542	0.5407	0.5563	0.5169	0.4898
H	0.0488	0.0503	0.0526	0.0509	0.0509	0.0616	0.0528	0.0513	0.0522	0.0542	0.0489	0.0468

d). OD values of compound **5** against breast cancer cells (MCF-7) (F and G-Methanol, B and C-Compound **5**).

◇	1	2	3	4	5	6	7	8	9	10	11	12
A	0.0526	0.0530	0.0557	0.0534	0.0576	0.0549	0.0551	0.0633	0.0654	0.0687	0.0536	0.0549
B	1.0496	0.0771	0.3030	0.8174	0.9522	0.9995	0.9783	1.0354	1.0945	0.9771	1.0820	1.1529
C	1.0255	0.0754	0.3201	0.8493	0.9367	0.9627	1.0458	1.0171	1.0471	1.0736	1.0579	1.1249
D	1.0441	0.8492	0.9630	0.9653	1.0417	0.9831	0.9554	1.0117	1.0234	1.0111	1.0483	1.1238
E	1.1404	0.8440	0.9334	0.9645	0.9269	1.0470	0.9852	0.9903	0.9616	1.0688	1.0488	1.0120
F	1.0994	0.9257	1.0286	0.9569	1.0289	1.0663	1.0478	1.0120	1.0147	1.0256	1.0169	1.1093
G	1.2253	1.1197	1.2665	1.1604	1.1837	1.2849	1.2573	1.3092	1.3237	1.3748	1.3869	1.2886
H	0.0475	0.0494	0.0537	0.0526	0.0523	0.0531	0.0553	0.0575	0.0611	0.0597	0.0538	0.0439

e). OD values of compound **5** against epidermoid carcinoma cells (A431) (F and G-Methanol, B and C-Compound **5**).