ISOLATION, STRUCTURAL ELUCIDATION AND BIOLOGICAL ACTIVITIES OF SECONDARY METABOLITES FROM ENDOPHYTIC FUNGI OF *Picralima nitida*

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APPROVAL

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DEDICATION

To the memory of my late Grand Mother, Madam Agnes Nmor Osama. Her encouragement remains my strength in life.

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ABBREVIATIONS

BHT	BUTYLATED HYDROXYTOLUENE
$CDCl_3$	DEUTERATED CHLOROFORM
CDER	CENTRE FOR DRUG EVALUATION AND RESEARCH
DCM	DICHLOROMETHANE
DNA	DEOXYRIBONUCLEIC ACID
DPPH	1,1-DIPHENYL-2-PICRYL HYDRAZYL
ESI	ELECTRON SPRAY IONISATION
ETOAC	ETHYLACETATE
hCMV	HUMAN CYTOMEGALOVIRUS
HCV	HEPATITIS C VIRUS
HIV-1	HUMAN IMMUNODEFICIENCY VIRUS TYPE-1
HPLC	HIGH PRESSURE LIQUID CHROMATOGRAPHY
HSV-1	HEPERS SIMPLEX VIRUS TYPE -1
IZDs	INHIBITION ZONE DIAMETERS
LC-MS	LIQUID CHROMATOGRAPHY-MASS SPECTROMETRY
MEA	MALT EXTRACT AGAR
MeOD	DEUTERIUATED METHANOL
MeOH	METHANOL
MHA	MULLER HILTON AGAR
MIC	MINIMUM INHIBITION CONCENTRATION
mm	MILLIMETER
MTT	3-(4,5 DIMETHY THIAZOLE 2 YL)-2,5- DIPHENYL TETRAZOLIUM BROMIDE
NDA	NEW DRUG APPLICATIONS
nm	NANOMETER
NMR	NUCLEAR MAGNETIC RESONANCE
NRPS	NON-RIBOSOMAL PEPTIDES
ORAC	OXGYEN RADICAL ABSORBANCE CAPACITY
PKS	POLY KETIDES

PN 3	FUNGUS *CURVULARIA SP*
PN 4	FUNGUS *ENDOMELANONIOPSIS SP*
RNA	RIBONUCLEIC ACID
ROSs	REACTIVE OXYGEN SPECIES
SDA	SABOURAUD DEXTROSE AGAR
US-FDA	UNITED STATES –FOOD AND DRUG ADMINISTRATION
UV	ULTRA VIOLET
VOC	VOLATILE ORGANIC COMPOUNDS
XO	XANTHINE OXIDASE
λ	WAVE LENGTH

ABSTRACT

Fungi are promising source of bioactive compounds whose potentials are important for drug discovery programme. Endophytic fungi have raised the interest of scientists in drug discovery programme due to their immense contribution to the discovery of new bioactive compounds. This study was aimed at isolating bioactive secondary metabolites from endophytic fungi from *Picralima nitida*. Objectives include; isolation, purification and identification of endophytic fungi; fermentation and extraction of secondary metabolites from the endophytic fungi; screening of extracts for biological activities; isolation, structural elucidation and bioassay of compounds from the endophytic fungal extracts. Endophytic fungi were isolated from fresh and healthy leaves of *Picralima nitida*, by culturing the leaves on malt extract agar. The fungi were purified on malt extract agar and subjected to molecular identification. The fungi were fermentation on solid state media and metabolites were extracted using ethyl acetate. The fungal extracts were screened for antimicrobial activity using agar well diffusion method, antioxidant activity using 1,1-diphenyl-2-picryl-2-hydrazyl (DPPH) assay and cytotoxicity using 3-(4,5-dimethylthiazole-2-yl)-2,5-diphenyl tetrazolium bromide assay. The active extracts were subjected to vacuum liquid chromatography and size exclusion separation methods. The resulting fractions were subjected to high performance liquid chromatography (HPLC) coupled to diode array detector. Pure compounds were isolated using semi preparative HPLC and the structures were elucidated using mass spectroscopy and nuclear magnetic resonance spectroscopy. Two endophytic fungi were isolated from *P. nitida* and identified as *Curvalaria sp.* and *Endomelanconiopsis sp.* At a concentration of 10 µg/ml, the fungal crude extracts showed poor cytotoxic activity against mouse lymphoma cell lines (L5178Y), and no anti-tubercular activity against *Mycobacterium tuberculosis.* Results of the DPPH antioxidant assay revealed that at a concentration of 500 µg/ml, the fungal crude extracts and fractions showed varying degrees of antioxidant activities, with higher activities recorded for the fractions. Two fractions of *Endomelanconiopsis sp.* extract PNMR4F7 and PNMR4F15 showed good antioxidant activity with percentage inhibitions of 89.8 and 77.1 respectively. One fraction of *Curvularia sp*. extract, PNMR3AF13, also showed good antioxidant activity with inhibition of 76.9%. The results of the antimicrobial screening revealed that at a concentration of 10 mg/ml, *Curvalaria sp*. and *Endomelanconiopsis sp*. crude extracts showed antibacterial and antifungal activities. Some bioactive compounds were isolated from the two endophytic fungi. These compounds include acropyrone, 4-hydroxyphenylacetic acid and indole-3 acetic acid which were isolated from *Curvalaria sp.*; and orthosporin, tenuazoic acid, methyl 4-methoxybenzoate and methyl 2-hydoxyl-4-methylbenzoate isolated from *Endomelanconiopsis sp*. Results of bioassays carried out on the isolated pure compounds showed that at 500 µg/ml, orthosporin and tenuazonic acid both showed *Aspergillus niger* and *Candida albicans.*This study shows that endophytic fungi associated with *P. nitida* can be a potential source of antifungal agent against *Aspergillus niger* and *Candida albicans*, and antioxidant lead molecules.

CHAPTER ONE
INTRODUCTION

1.1 BACKGROUND

The discovery and development of drugs have a long history that dates back to the early days of human civilization. Most of the drugs used in early days were crude natural products, some of which were used for religious and spiritual healing. Today, natural products are integral part, in one form or another, of several indigenous therapeutic systems including traditional Chinese medicine (TCM) and Ayurveda (Liu and Wang, 2008), as well as African traditional medicine (ATM).

After over a century of research and development in modern pharmaceutical industries, there is still a great need for new drug molecules. Only one third of all diseases can be treated efficiently (Muller *et al*., 2000) and with the increase in the knowledge and better understanding of the mode of action of different remedies, more and more new drugs are being developed through chemical modification.

Most of the renowned drugs used today are of natural origin. Newman and Cragg, 2007 noted that over 60 % of the approved drugs and pre-NDA (New Drug Applications) received at Center for Drug Evaluation and Research (CDER) of the United State Food and Drug Administration and Control (US-FDA) from 1981 to 2006 were developed from natural origin. Especially in the fields of immune suppressants and antibacterial drug discovery, natural products are playing a pivotal role because of the occurrence of great numbers of compounds with these kinds of activities.

Nature itself has constantly provided mankind with a broad and diverse array of pharmacologically active compounds that continue to be utilized as highly effective drugs to combat a legion of diseases (some life threatening) or as lead molecules for the development of novel synthetically derived drugs that are stereochemical models from nature.

The need for new and safe bioactive compounds to provide aid and comfort to mankind in all aspects of life is in the increase in the world today. This is principally due to emerging new diseases, development of different drug resistance by pathogenic and non-pathogenic microorganisms, appearance of life threatening viruses, complications in patients with organ transplantation, etc. (Akansksha and Pavan, 2014).

Worldwide, the threat posed by diseases such as cancers and infection is ever increasing (Strobel, 2003). For example, the emergence and increasing incidence of antibiotic resistant bacteria has resulted in the need for new antibacterial drugs with novel modes of action. Thus, there is a need for new and beneficial compounds that can provide relief against ailments, diseases and healthy environment (Strobel *et al.,* 2004).

There is therefore general call for new antibiotics, chemotherapeutic agents and agrochemicals that are highly effective, with low toxicity, and have a minor environmental impact (Strobel and Diasy, 2003).

In developing countries, tribal people have been using medicinal plants in different ways for the treatment of various diseases, which in turn has resulted in scientific discoveries, with a wealth of literature on plant extracts and their biological activities. But this has a diverse effect on the ecosystem and the use of these plant parts may lead to the extermination of these plant species.

Production of a plant based natural drug is obtainable at a specific developmental stage or under specific environmental condition, stress or nutrient availability. It is estimated that 38,000 pacific yew trees are required to generate 25 kg of taxol to treat 12,000 patients (Sohn and Okos, 1998). Indiscriminate collection and cutting down of medicinal plants from the wild for extraction of products of interest has led to the extinction of certain number of species making them either vulnerable or critically endangered.

The biotechnological approaches involving plant cell and organ cultures and hairy root culture appear to fulfill the ever increasing demand to a certain level.

Different strategies have been used to increase the production of bioactive secondary metabolites in plant cell culture and these include, screening and selection of high producing cell lines, optimization of nutrient media for growth and production, organ culture, culture of immobilized cells, the use of biotic and abiotic elicitors, modulation of cells, feeding of biosynthetic precursors, and scale up in bioreactors (Karuppusamy, 2009; Hussain *et al*., 2012).

Interests in plant parts (leaves, stembark, roots, etc.) as sources of novel therapeutic molecules have greatly diminished over the years owing to low "hit" rates associated with albeit enormous research effort invested in such studies (Atanasov *et al*., 2015).

Considering the limitations and vulnerability of plant species as sources of novel metabolites, microorganisms serve as the ultimate readily renewable, reproducible and inexhaustible source of novel structures bearing pharmaceutical potential. Microorganisms, especially fungi have long been regarded as important source of active metabolites with promising anti-bacterial, antifungal and antiviral activity (Sheela, 2012).

The present work is thus geared towards the bioprospecting of some medicinal plants in Nigeria with the view of generating novel fungi endophytes with potentials of generating novel therapeutic molecules.

1.2 STATEMENT OF PROBLEMS

The effects of most diseases are quite devastating, as most of them lead to death worldwide. There could also be lack of proper medication as some of the treatment agents used today have numerous side effects, creating the continuous need for search for safer medication.

Also, the sustainability of therapeutic drugs in the treatment of infectious diseases requires that replenishment of new drugs and new drug classes occur as existing therapies lose effect.

There is world-wide consensus that the medical need for novel anti-infective drugs is enormous and that we are running out of time. Many achievements of modern medicine especially in the treatment of infectious diseases depend on the availability of efficacious new drugs and the rate these are reaching the market is alarmingly low. The current range of approved drugs and slow pace of chemical testing and regulatory approval will not provide a solution to a looming effect.

Despite the continued need for new antimicrobial agents, this field has been abandoned by the major pharmaceutical companies. The increased size and cost of clinical trials, the new regulatory uncertainty regarding approval criteria, and the low economic return in the market place have driven companies out of new drug molecule research and development (R & D).

The ever-growing gap between the urgent public health need for new drugs and the dwindling potential for the development of new therapeutic agents has resulted in a perilous situation. The reluctance of large pharmaceutical companies to invest in drug development is reflected in the

dwindling number of antibiotics approved for use by US-FDA over the past 30 years (Gollaher and Milner, 2011).

Thus this research seeks to explore the possibility the *Picralima nitida* to produce bioactive active metabolites which could be used in the pharmaceutical industry to produce therapeutic molecules or lead molecule in the synthesis of novel compounds.

1.3 AIM AND OBJECTIVES

- **AIM**

The aim of this study is to isolate, elucidate the structures and evaluate the bioactivities of secondary metabolites from *Curvularia sp*. and *Endomelaconiopsis sp.* isolated from the leaves of *Picralima nitida.*

- **OBJECTIVES**

The objectives of this work include the following to;

1. Isolate, purify and identify endophytic fungi from the leaves of *Picralima nitida.*
2. Screen extracts of fermentation products of endophytic fungi for antibacterial, antifungal, antioxidant and cytotoxic activities.
3. Fractionate bioactive extracts and further screen the various fractions for antibacterial, antifungal, antioxidant and cytotoxic activities.
4. Isolate, purify and characterize the bioactive compounds from endophytic fungal extracts from *Picralima nitida*.

CHAPTER TWO
LITERATURE REVIEW

2.1 SECONDARY METABOLITES AS NATURAL PRODUCTS IN DRUG DEVELOPMENT

Natural products are chemical compounds, derived from living organisms such as terrestrial plants, animals, marine macro-organisms (sponge, corals and algae), and micro-organisms (bacterial, actinomycetes and fungi). Natural products discovery has played major role in the search for new drugs, and is the most potent source for the discovery of novel bioactive molecules (Cragg and Newman, 2013).

The discovery of natural products involves isolation, structural elucidation and establishing the biosynthetic pathways of the secondary metabolites. This also provides the basic chemical framework for deriving semi-synthetic natural products (Suryanarayanan *et al.*, 2009).

The discovery of potent antibiotics against Gram positive bacteria, penicillin from culture of fungus *Penicillium notatum* by Fleming in 1925, led to the search for new drugs from microbial origin. Newman and Cragg (2007) reported many secondary microbial metabolites which showed potent pharmaceutical application against various diseases. These include the therapeutically used ergotamine (**1**), the immunosuppressive peptide cyclosporine A (**2**), peptide antibiotic compounds like the penicillin V (**3**), cephalosporin C (**4**), the polyketide lavastatin used in cholesterol treatment and the antibacterial terpenoid fusidic acid (**5**). Fig. 2.1.

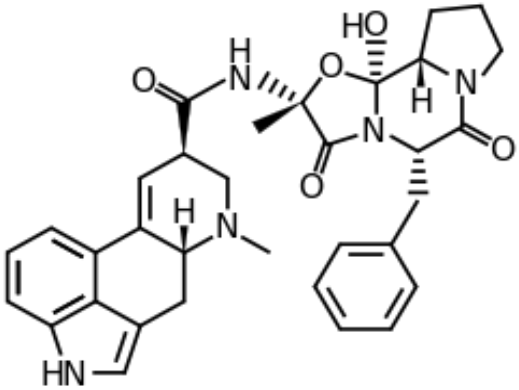
Secondary metabolites are substances which are produced by plants that are not required for normal plants growth and development. They are mostly produced as defense chemicals which

absences do not cause bad effects to the plants. They usually occur in smaller amount than the primary metabolites (Irchariya *et al*, 2015). They are responsible for the medicinal properties of plants to which they belong (Kabera *et al*., 2014). They have a wide range of chemical structures and biological activities. They are not made through metabolic pathways common to all plants instead they are compounds biosynthetically derived from primary metabolites through unique biosynthetic pathways from primary metabolites and intermediates. A secondary metabolite is a natural product, but all natural products are not necessarily secondary metabolites (Bentley, 1999). Secondary metabolites are classified depending on their structural scaffold. These classes include among others polyketides (PKS), non-ribosomal peptides (NRPS), terpenoids, alkaloids and hydrid metabolites of the different classes.

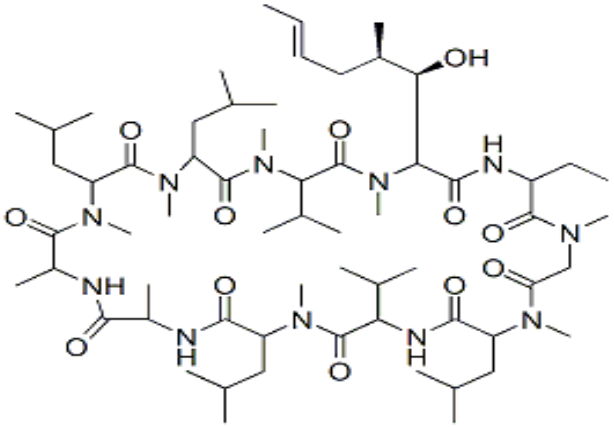
2.2 FUNGI AS SOURCE OF BIOACTIVE METABOLITES

Fungi are among the most important groups of eukaryotic organisms that are well known for producing many novel metabolites which are directly used as drugs, or function as a lead structure for synthetic modification (Mitchell *et al*., 2008; Stadler and Keller, 2008).

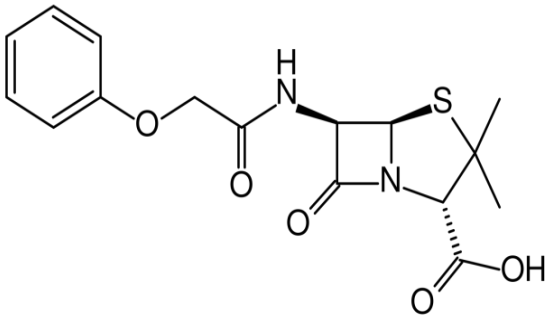
The success of several medicinal drugs from microbial origin such as the antibiotic penicillin (**3**) from *Penicillin sp*., the immunosuppressant cyclosporine (**2**) from *Tolypocladium inflatum* and



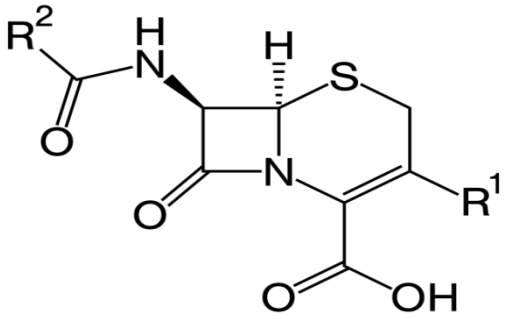
ERGOTAMINE (1)



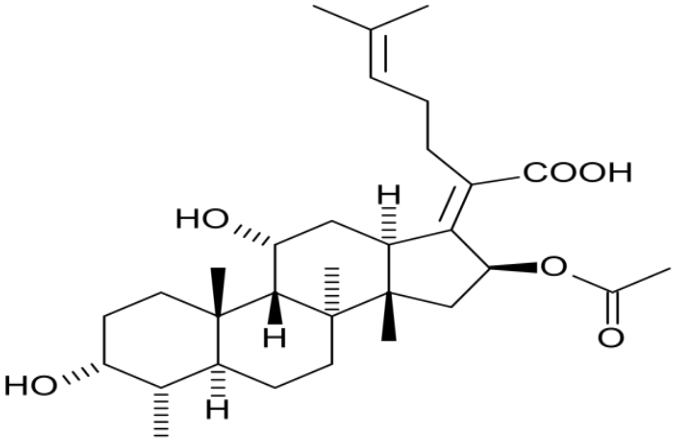
CYCLOSPORINE (2)



PENICILLIN V (3)



CEPHALOSPORIN (4)



FUSIDIC ACID (5)

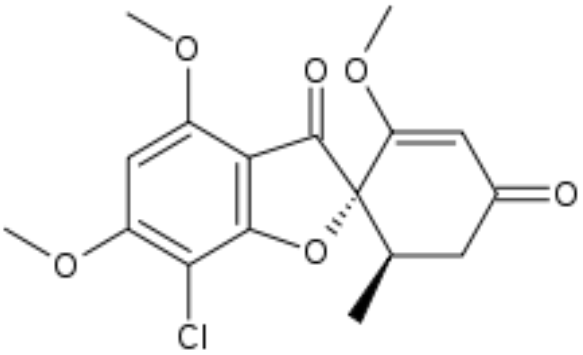
Figure 2.1: Structures of Some Secondary Metabolites (Newman and Cragg, 2007)

Cylindrocarpon lucidum, the antifungal agent griseofulvin (**6**) from the fungus *Penicillium griseofulvum*, the cholesterol biosynthesis inhibitor lovastatin (**7**) from the fungus *Aspergillus terreus*, and others, has shifted the focus of drug discovery from plants to microrganisms.

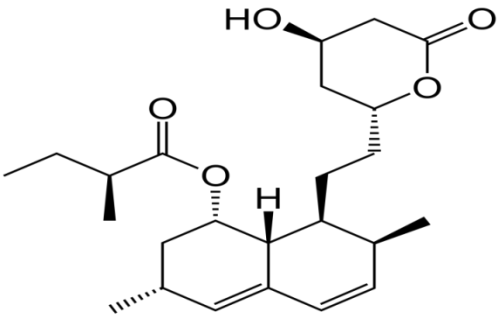
Over 1,500 fungal metabolites have been reported to show anti-tumor and antibiotic activity (Pelaez, 2005), and some of them have been approved as drugs. These include micafungus (**8**), an anti-fungal metabolite from *Coleophoma empetri* (Franttarelli *et al*., 2004), rosuvastatin (**9**) from *Penicillin citrinum*, and *Penicillium brevicompactum*, which is used for the treatment of dyslipidemias (Scott *et al*., 2004), cefditoren pivoxil (**10**), a broad spectrum antibiotic derived from *Cephalosporium sp*. (Darke and Plosker, 2002) among others. Fig 2.2.

It has been estimated that there may be over 5.1 million fungal species, Blackwell, (2011) and only a few taxa have been tested for their biological applications including their ability for drug production and biological control. So, it seems that the discovered percentage of economically valuable fungal metabolites is small.

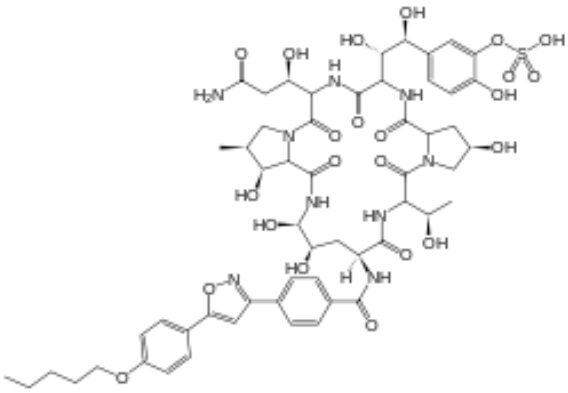
Fungi were usually obtained from the same ecological niche using the same fungal isolation techniques. There, the same fungal strains were re-isolated and this leads to the re-discovery of known compounds as the same taxa produce the same metabolites. Dreyfuss and Chapella (1994) assumed that different environmental factors in nature, including different physical conditions and different biological situations, may change the behaviour of microbes and favour the production of diverse range of secondary metabolites. Therefore, there is need for alternative and unexplored ecological riche. This may lead to novel fungal groups with novel and diverse secondary metabolites. Some of the practically unexplored or practically explored fungal group include: fungi from thermal vents, deep rock sediments, marine environments, desert, fresh water fungi and marine derived fungi (Dreyfuss and Chapella, 1994; Strobel, 2003).



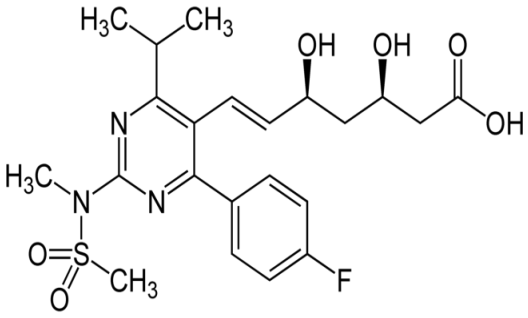
GRISEOFULVIN (6)



LOVASTATIN (7)



MICAFUNGIN (8)



ROSUVASTATIN (9)

CEFDITOREN PIVOXIL (10)

Figure 2.2: **Some bioactive secondary metabolites of Fungi source** (Darkes and Plosker, 2002; Scott *et al*., 2004; Pelaez, 2005)

2.3 ENDOPHYTES

The word endophyte means in the plant' (from greek Endo = within, phyton = plant). "Endophytism" is thus, a unique cost benefit plant – microbe association defined by "location" (not "function") that is transiently symptomless, unobstrusive and established inside the living host plant tissues (Kusari *et al*., 2012). In this association, none of the interacting partners is discernibly harmed and the individual benefits depend on both the interacting partners.

Endophytes are mostly fungi and bacteria. They inhabit the host plants for all or part of their life cycle. They live in the internal plant tissues beneath the epidermal cell layers, living within the intercellular spaces of the tissues and it seems that they may penetrate the living cells (Strobel, 2003). Endophytic fungi are an ecological, polyphyletic group of highly diverse fungi, mostly belonging to ascomycetes and anamorphic fungi (Arnold, 2007).

Since endophytes were first discovered in Darnel (*Lolium temulentum*) (Kusari, 2012), they have been isolated from various organs of different plants. There are near 300,000 plant species on earth and each individual plant may host one or more endophytes and many of them may colonise certain hosts. It has been estimated that there may be as many as five million different endophytic fungal taxa, thus endophytes may be hyperdiverse (Blackwell 2011).

Endophytes produce a plethora of bioactive metabolites that may be involved in the host endophyte relationship (Strobel, 2003), and may serve as potential sources of novel natural products for exploitation in medicine, agriculture and industry (Strobel and Daisy, 2003).

2.3.1 Endophytic Fungi

Endophytic fungi can be divided into 2 major groups; claviciptaceae and non-clavicipitaceae. The clavicipitaceae are generally distributed in grass hosts and several species are systemically transmitted to the next generation vertically by way of the seeds of the host grasses (Kuldau and Beacon, 2008). Examples are *Epichloe sp* and *Neotyphodium sp*. In contrast, the non-clavicipitaceae are composed of a broad range of species in several families, all of which are members of Dikarya (Ascomycota and Bisidiomycota). Non-claviciptaceae are not systemically distributed, but are present in almost all host plant species, including grasses (Sieber, 2007).

Endophytic fungi exist in most plant parts, such as the leaves, stems and roots and are ubiquitously isolated from almost every terrestial and aquatic plant so far studied. Ascomycota in the anamorph state are commonly found endophytes. Endophytic fungi are closely related to pathogens and can sometimes be opportunistic pathogen.

2.3.2 Importance of Endophytic Fungi

The importance of endophytes has been demonstrated over a long period as potential source of pharmaceutical leads, as many of endophytic fungi were reported to produce novel bioactive metabolites such as antimicrobial, anticancer and antiviral agents. The discovery of taxol producing fungi increased the importance of endophytes and shifted natural products research to endophytic fungi. Over the last 20 years, endophytic fungi have been viewed as an outstanding source of secondary metabolites from natural products. A variety of biologically diverse active and chemical structures have been discovered.

2.4. BIOACTIVE COMPOUNDS FROM ENDOPHYTIC FUNGI

2.4.1 ANTICANCER COMPOUNDS

The most fascinating and famous compound in the history of secondary metabolite from endophytic fungi is paclitaxel (**11**) (taxol, one of the brand names). This highly functionalized diterpenoid compound is an active anticancer drug isolated from the bark of a pacific yew tree species, *Taxus brevifolia* (Wani *et al.,* 1971). It is originally used in the treatment of ovarian and breast cancers, but now it is used to treat a number of other human tissue-proliferating disease. The mode of action of paclitaxel is unique in that it prevents the depolymerisation of tubulin during the process of cell division.

A specific cytotoxic quinone dimer, torreyanic acid (**12**), was isolated from the endophytic fungus, *Pestalotiopsis microspora* associated with the tree *Torreya taxifolia*. Torreyanic acid showed 5 – 10 times more potent cytotoxicity against several cancer cell lines that are protein kinase C agonist and caused cell death by apoptosis (Lee *et al*., 1996).

Alkaloids are one of most potent anticancer agents, usually found in endophytic fungi. 22-oxa-12- cytochalasin (**13**) an alkaloid, possessing antitumor activity, was isolated from *Rhinocladiella sp*. an endophytic fungus on *Triptergium wilfordii* (Wagenaar *et al*., 2000).

Cancer is a disease characterized by unregulated cell proliferation, and leads to spread of abnormal cells and uncontrolled tissue growth (American Cancer Society, 2009). Many endophytic fungi have been reported as novel sources of anticancer lead agents to combat this deadly disease.

Three novel cytochalasins alkaloids were identified by Wagenaar *et al*., (2000) to have anticancer activities. They were isolatesd from the endophytic genus *Rhino cladiella*.

Camptothecin, 9-methoxycamptothecin, and 10-hydroxycamptothecin are important precursors for the synthesis of clinically useful anticancer drugs, toprotecan and irinotecan (Uma *et al*., 2008). They were obtained from the endophytic fungus *Fusarium solani* isolated from *Camptotheca acuminate* (Kusari *et al*., 2009 b). Though, it has been reported that camptothecin and its derivatives can be produced by other endophytes (Liu *et al*., 2010b; Shadeta *et al*., 2010).

Lignans are other kinds of anticancer agents from secondary metabolites of endophytic fungi through the Shikimic acid pathway (Gordaliza *et al*., 2009).

Podophyllotoxins, aryl tetralin lignans, were obtained from fungal endophyte *Trametes hirsute* (Puri *et al*., 2006). These ligans produced by the microorganism are biologically active, and exhibit potent antioxidant, anticancer, and radioprotective properties. Derivatives of podophyllotoxins are presently being used as antitumor treatment agent in different types of cancer diseases.

The endophytic fungus, *Penicillium brasilianum* isolated from the root back of *Melia azedarach*, promoted the biosynthesis of phenylpropanoid amides (Fill *et al.*, 2010). The phenylpropanoids have attracted much interest for medicinal applications mainly as anticancer and antioxidant agents (Korkina *et al*., 2003).

Endophytic fungus *Fasarium sp*., isolated from the stems of the mangrove tree *Kandeila candel*, led to the isolation of isoflavone, 5-O-methyl-2-methoxy-3-methylalpinumisoflavone. It inhibits

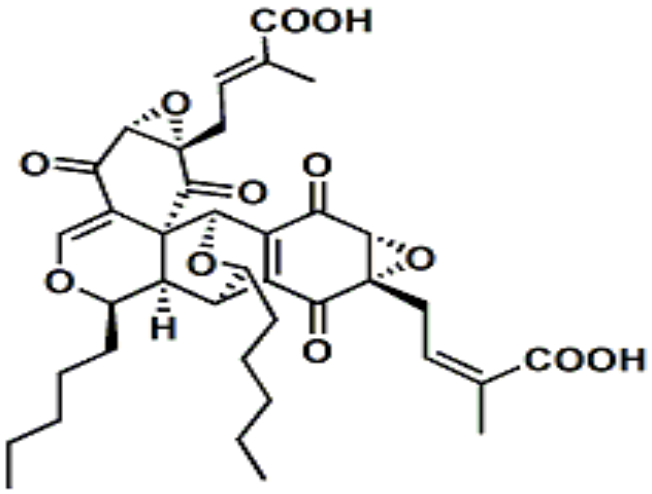
the growth of Hep-2 and HepG2 cancer cell lines with IC_{50} values of 4 and 11 µM respectively (Huang *et al*., 2010).

Eremophilane – type sesquiterpenoids obtained from the endophytic fungus *Xylarioa sp*., from the palm *Licuala spinosa* exhibited moderate cytotoxic activities with $1C_{50}$ values ranging from 3.8 to 21µM against human cancer cell lines (KB, MCF – 7, and NCI – H187 and non maligant Vero cells) (Isaka *et al*. 2010).

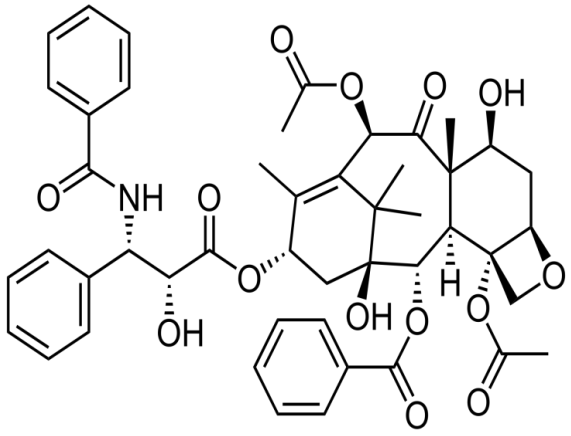
Polyphenols: expansols A and B were obtained from the endophytic fungus *Penicillium expansum*, isolated from the roots of the mangrove plant, *Excoecaria agallocha*. Expansol A exhibited moderate cytotoxic activity against HL-60 cell line with an $1C_{50}$ value of 15.7 µM. Expansols B showed pronounced activity with $1C_{50}$ value 1.9 µM. Figure 2.3 shows the chemical structure of some anticancer agents isolated from endophytic fungi.

2.4.2 ANTIMICROBIAL COMPOUNDS

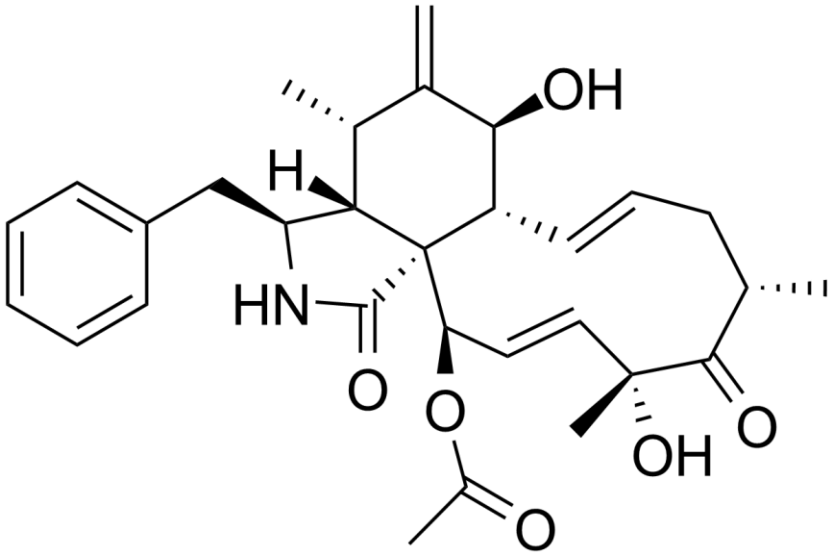
The screening of endophytic crude extracts for antimicrobial activity indicates that they may possess antimicrobial activities against tested pathogens. So far, a large number of metabolites displaying antimicrobial activity have been isolated from endophytic fungi. Li *et al*., (2005) reported that 30 % of tested isolates exhibited anti-fungal activity. Also, anti-microbial activity was demonstrated for 80-92 % of endophytic extracts in other studies (Banu and Kamar, 2009; Hazalin *et al*., 2009, Tong *et al*., 2011).



TORREYANIC ACID (12)



PALITAXEL (TAXOL) (11)



CYTOCHALASIN (13)

Figure 2.3: **Structures of Some Endophytic Secondary Metabolites with Anticancer Activity** (Wani *et al*., 1971; Lee *et al.*, 1996*;* Wagenaar *et al.*, 2000)

Antimicrobial agents with antibacterial activity include periconicins A and B (**14**), phomopsichalasin (**15**), javanicin (**16**). Antimicrobial agents with antifungal activity include, cryptocandin (**17**), ecomycins (**18**), pseudonoycin (**19**), pestalopyrone (**20**) (See Figure 2.4).

Antimicrobial metabolites (antibiotics) are low molecular- weight organic compounds produced by microorganisms that are active at low concentrations against other microorganisms. They are usually not required for growth, produced as an adaptation for specific functions in nature and are the most bioactive natural products isolated from endophytes (Demain 1981; Strobel and Daisy, 2003; Guo *et al*., 2008).

The compounds are classified into several chemical structural groups such as alkaloids, peptides, steroids, terpenoids, phenols, quinines and flavonoids (Yu *et al*., 2010).

Some of the antimicrobial agents from endophytic fungi are not only active against human pathogens but also against plant pathogens, leading to their application in agricultural fields (Strobel and Daisy, 2003). These compounds are also used as food preservatives in the control of food spoilage and food borne diseases, a serious concern in the World food chain (Liu *et al*., 2008).

It is believed that screening for antimicrobial compounds from endophytes is a promising way to overcome the increasing threat of drug resistant microbes of human and plant pathogens (Yu *et al*., 2010).

Crytocin and cryptocandin are antifungal metabolites obtained from the endophytic fungus *Cryptosporiopsis quercina.* Cryptocandin showed high antifungal activity against some important human fungal pathogens such as *Candida abicans* and *Trichophyton spp*. It also

showed high activity against plant pathogenic fungi such as *Solerotinia sclerotiorum* and *Botrytis cinorea*. Cryptocandin and its related compound are used against a number of fungi causing diseases of the skin and nails (Strobel and Daisy, 2003).

Cryptocin has activity against plant pathogens only, especially against *Pyricularia oryzae*, the causal organism of one of the worst plant disease in the world, with MIC of 0.39 µg/ml (Strobel *et al*., 1999b; Li *et al*., 2000).

The endophytic fungus *Pestalotiopsis microspora* produce a number of antifungal agents, ambuic acid, pestaloside, pestalotiopsins A and B. *Pestalotiopsis jester* and *Pestalotiopsis adusta* synthesized jestorone and pestalochlorides A respectively. They both exhibit antifungal activity against a variety of plant pathogenic fungi including *Fusarium culmorum, Gibberella zeae*, and *Verticillium albo-atrum* (Li *et al*., 2003a).

Periconicins A and B isolated from endophytic fungus *Periconia sp*., from host plant *Taxis cuspidate* showed antibacterial activity against human bacteria (Kim *et al*., 2004).

Curvularide B isolated from the endophytic *Curvularia geniculata* showed marked antifungal activity with increase in inhibition zone in the presence of fluconazole against *Candida albicans*. This is an example of the azol drugs which indicates the synergistic effect of both drugs. The minimum inhibitory concentration (MIC) values that produced no visible growth (MIC-0) for fluconazole and curvularide B were 26.1 and 782.8 µM, respectively. While in combination, the MIC values reduced to 3.2 and 48.9 µM, respectively. Curvularide B did not exhibit cytotoxicity towards ten human cancer cell lines even at a concentration of 50 µg/ml, which indicates positive results for using it to improve activity of azol antifungal drugs (Chomcheon *et al*., 2010).

The mangrove derived endophytic fungus *Talaromyces sp*., produced the antimicrobial metabolites – 7-epiaustdiol, stemphyperylenol and secalonic acid A. 7-epiaustdiol show significant inhibition against *Pseudomonas aeruginosa*, a multidrug resistant opportunistic pathogen, with MIC value of 26.48 µM. Stemphyperylenol inhibited *Sarcina ventriculi* with MIC value of 8.86 µM, which is lower than that of Ampicillin 35.81 µM. Secalonic acid A showed high activities against all test organisms. The three compounds also showed moderate to strong cytotoxicity against KB and KBv200 cell lines (Liu *et al*., 2010).

Fumigants are also produced by endophytes. They produce bioactive volatile organic compounds (VOCs). This mixture of gases consists primarily of various alcohols, esters, acids, lipids and ketones. The fungus, as well as the VOCs, has enormous potential for use in the industry, agriculture and medicine.

The endophytic fungi produce a mixture of VOCs that act synergistically to kill a wide variety of plant and human fungi and bacteria. It is also effective against nematodes and certain insects. Artificial mixtures of VOCs mimic the biological effects of the fungal VOCs when tested against a wide range of fungal and bacterial pathogens. Potential applications for "mycofumigation" by this genus are currently exploited for treating various plant diseases, buildings, soil, agricultural produce and human wastes. Another promising option includes its use to replace methyl bromide fumigation as a means to control soil-borne plant diseases (Strobel, 2001; Strobel, 2006a & 2006b).

Yu *et al*. (2010) reported that some antimicrobial compounds were isolated from endophytes which only represent a small part of endophytic species. This suggests that there is a great

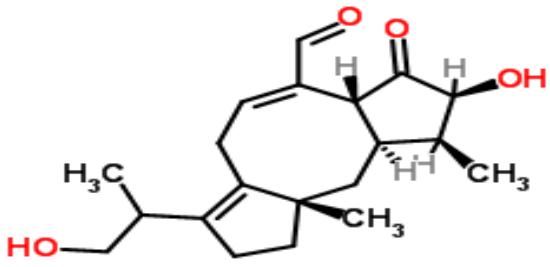
opportunity to utilize endophytes as a new source for production of reliable and novel antimicrobial agents.

They also opined that this could be a promising way to solve the problem of microbial resistance to commonly used drugs and meet the emergency demand of discovering highly effective, low toxicity, and environmentally friendly antibiotics, which may be used as clinically effective antibiotics in future.

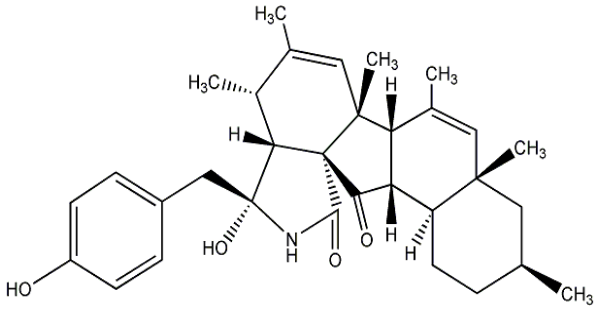
Tuberculosis inhibitors have been found among endophytes. They were found to inhibit *Mycobacterium aurum* and *Mycobacterium tuberculosis*, the causative organisms of tuberculosis. Phomoenamide exhibited *in vitro* antimycobacterial activity against *M. tuberculosis* H37Ra (Rukachaisirikul *et al*., 2008).

Fusaric acid was isolated from *Fusarium sp.,* a mangrove endophytic fungus. Fusaric acid is used in the synthesis of a variety of metal complexes of fusaric acid. Antimycobacterial assays showed that Cadmium (II) and Copper (II) complexes exhibited potent inhibitory activity against *M. bovis* BCG strain with MIC 4 µg/ml and *M. tuberculosis* H37Rv strain with 5 µg/ml (Pan *et al*., 2011).

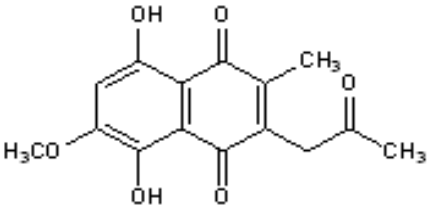
Piperine produced by endophytic fungus *Pericania sp*., showed strong antimycobacterial activity against *M. tuberculosis* and *M. emegmetis* with MIC 1.74 and 2.62 µg/ml, respectively (Verma *et. al*,,2011).



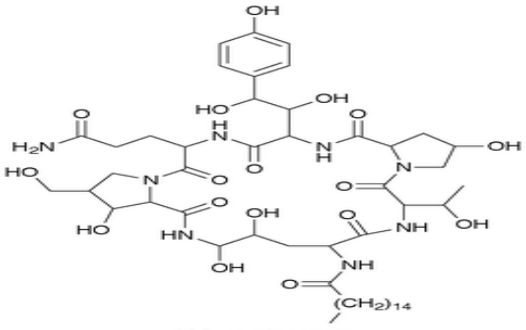
PERICONICINS (14)



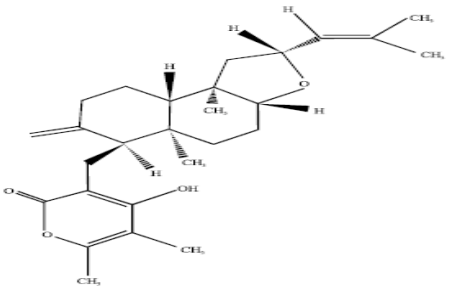
PHOMOPSICHALASIN (15)



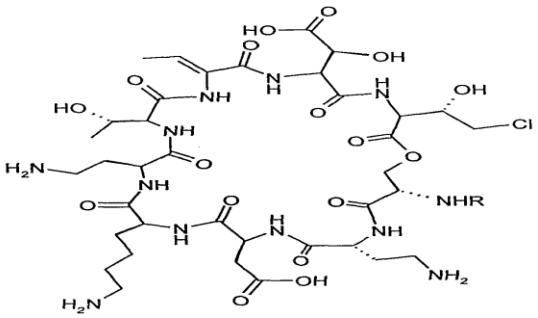
JAVANICIN (16)



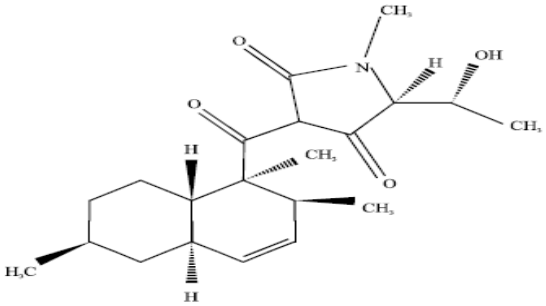
CRYPTOCANDIN (17)



ECOMYCINS (18)



PSEUDOMYCIN (19)



PESTALOPYRONE (20)

FIGURE 2.4: Structure of Some Endophytic Secondary Metabolite with Antimicrobial Activity (Li *et al*., 2005; Hazalin *et al*., 2009; Tong *et al*., 2011)

2.4.3 ANTIOXIDANT COMPOUNDS

The importance of compounds bearing antioxidant activity lies in the fact that they are highly effective against damage caused by reactive oxygen species

(ROSs) and oxygen derived free radicals, which contribute to a variety of pathological effects. Antioxidants have been considered promising therapy for prevention and treatment of ROS-linked diseases such as cancer, cardiovascular disease, atherosclerosis, hypertension, ischemia/reperfusion injury, diabetes mellitus , neurodegenerative diseases (Alzheimer and Parkinson diseases), rheumatoid arthritis and ageing (Valko *et al*., 2007).

Fungal endophytes associated with higher plants appear to be a good source of novel antioxidants. Many antioxidant compounds have been obtained from endophytic fungi. Pestacin (22) and isopestacin (23) (Fig. 2.5) were obtained from *Pestalotiopsis microspora*, isolated from the host plant, *Terminalia merobensis*. Isopestacin antioxidant activity was attributed to its structural similarity to the flavonoids. It is able to scavenge both superoxide and hydroxyl free radical in solutions. The antioxidant activity of pestacin has been demonstrated to be about eleven times higher than that of trolox, a vitamin E derivative. This arises primarily from cleavage of an unusually reactive C-H bond and to a lesser extent, through OH abstraction (Strobel *et al*., 2002; Harper *et al*., 2003).

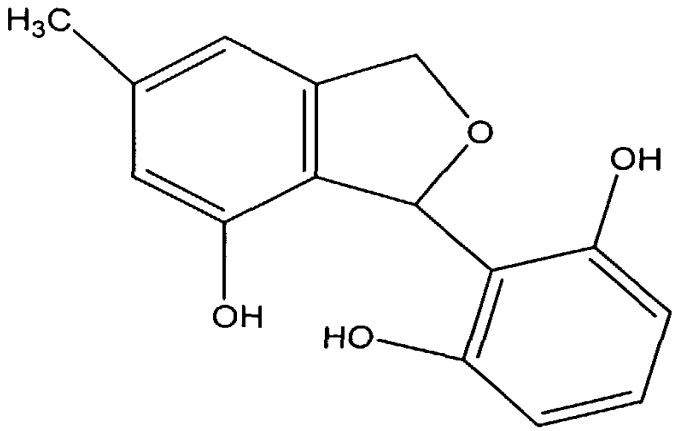
The endophytic fungus *Microsphaeropsis olivacea* isolated from *Pilgerodendron uviferum* produced a phenolic metabolite graphislactone A which showed potent *in vitro* antioxidant and free radical-scavenging activity more than the standards butylated hydroxytoluene (BHT) and ascorbic acid (Song *et al*., 2005). The endophytic fungus *Corynespora cassiicola* produced potent antioxidant compounds corynesidones A and B (Chomcheon *et al*., 2009; Okoye *et al.,*

2013). The oxygen radical absorbance capacity (ORAC) assay of these compounds ranges from 4.3-5.9. Corynesidone B could also scavenge 2, 2-diphenyl-1-picrylhydrazyl (DPPH) free radical with an $1C_{50}$ 22.4 µM, at the same activity as ascorbic acid. Besides antioxidant activity of corynesidone A, it also exhibits aromatase inhibition activity with an $1C_{50}$ value of 5.30µM. This magnitude of activity could be compared to the first generation aromatase inhibition drug, aminoglutethimide. The antioxidant and aromatase inhibition activities of corynesidone A may be useful for cancer chemoprevention, particularly for breast cancer (Chomcheon *et al*., 2009).

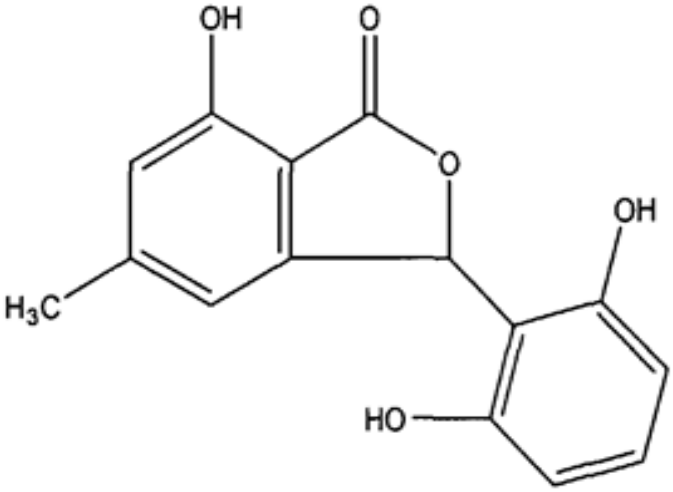
Huang *et al.,* (2007) investigated the antioxidant capacitiers of endophytic fungal cultures of medicinal Chinese plants and its correlation to their total phenolic contents. They suggested that the phenolic content were the major antioxidant constituents of the endophytes (Hujang *et al*., 2008a). Flavonoids were also identified to exhibit strong antioxidant capacity in extracts of endophytic fungi (Liu *et al*., 2007).

2.4.4 ANTIVIRAL COMPOUNDS

Many reports have shown the importance of endophytic fungi in the production of antiviral agents. Prominent among the antiviral agents are cytonic acid A and cytonic acid B (23) (Fig. 2.6). These compounds were isolated from the endophytic fungus *Cytonaema sp*. They are human cytomegalovirus (hCMV) protease inhibitors (Guo *et al*., 2000). There are only very few antiviral compounds that have been isolated from endophytes. These compounds show promising results of activity and could be developed into antiviral drug production.



PESTACIN (21)



ISOPESTACIN (22)

FIGURE 2.5: Structures of Some Endophytic Secondary Metabolites with Antioxidant Activity (Strobel *et al*., 2002; Harper *et al*., 2003).

The structures of Cytonic acid A and B were elucidated by mass spectrometry and NMR techniques as p-tridepside isomer (Guo *et al*., 2000).

Pullularins A-D (*Cyclohexadepsipeptides*) were isolated from *Pullularia sp*. Pullularins A exhibited activities against the herpes simplex virus type – 1 (HSV-1) and also against the malaria parasite *Plasmodium falciparium* the $1C_{50}$ was obtained as 3.3 µg/ml (Isaka *et al*., 2007).

Hunnuiquinone was isolated from endophytic fungi inhabiting the leaves of Oak trees (*Quercus cocciferus*). It was shown to be a potent inhibitor of the HIV-1 protease (Singh *et al*., 2004).

Pestalotheol-C was isolated from the fungal endophyte *Pestalotiopsis theae*. The antiviral compound showed anti-HIV activity (Li *et al*., 2008). Mellisol and 1, 8 –dihydroxynaphthol-1-O-α-glucopyranoside were isolated from endophytic fungus *Xylaria mellisii*. They had activity against herpes simplex virus – type 1 (Hayakhjonwut *et al*., 2005).

Dihydroisocoumarin, (R)-(-)-mellein isolated from endophytic fungi, such as *Pexicula livida*, *Plectophomella sp*., and *Cryptosporiopsis malicoticis*, was reported to have anti-hepatitis C virus (HCV) activity (Florke *et al*., 2006). It was shown to inhibit HCV protease with an $1C_{50}$ value of 35 nM.

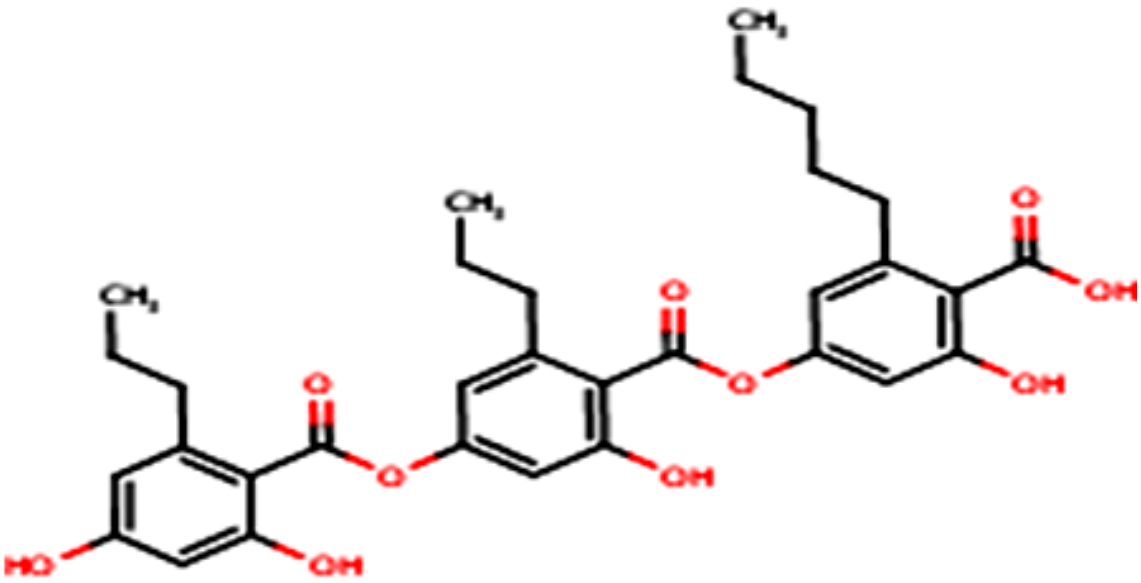
Chloroisosulochrin isolated from *Fagara zanthoxyloides* exhibited an excellent anti- respiratory syncytial viral (RSV) activity with an IC_{50} of 4.22 ± 1.03 µM (Uzor *et al*., 2016).

Sclerotiorin isolated from the endophytic fungus *Penicillium sclerotiorum* was evaluated for its inhibitory effect on human immunodeficiency virus HIV-1 integrase and protease activities.

Arunpanichlert *et al*. (2010) in this investigation reported that (+) – sclerotiorin exhibited anti-HIV 1 integrase and protease activities with $1C_{50}$ values of 45.88 and 198.41 µM, respectively.

However, it was observed that it showed weak anti-fungal activity against *Candida albicans* and *Cryptococcus neoformaris* with MIC values of 202.53 and 101.26 µM, respectively.

Emerimidine A and B were isolated from the culture of the endophytic fungus *Emericella sp*. (Zhang *et al*., 2011). The compounds were found to show moderate inhibition to influenza virus $H_1N_1$with $1C_{50}$ values of 42.07 µg/ml and 62.05 µg/ml respectively. *Phomopsis sp*., an endophyte isolated from *Musa acuminate*, was found to produce hexaketide γ-lactones. Oblongolides Z and 2 – deoxy-4α – hydroxyoblongolides X showed anti-herpes simplex virus type 1 (HSV-1) activity with $1C_{50}$ values of 14 µM and 76 µM, respectively. Chloropupukeanolides A, a chlorinated pupukeananes possessing a unique spiroketal peroxide skeleton was isolated from endophytic *Pestalotiopsis fici*. It was found to inhibit HIV-1 replication *in vitro* in C8166 cells with an $1C_{50}$ value of 6.9 µM. It also showed cytotoxicity against human cancer cell lines Hela, MCF-7 and MDA – MB – 231 with $1C_{50}$ values of 16.9, 15.5 and 15.9 µM, respectively (Liu *et al*., 2010a).



CYTONIC ACID (23)

FIGURE 2.6: Structure of an Endophytic Secondary Metabolite with Antiviral Activity (Guo *et al*., 2000)

2.4.5 OTHER BIOACTIVE AGENTS

Compounds from endophytic fungi have also shown a variety of other biological activities of interest. These may include immunosuppressant, anti-diabetic, anti-malaria, anti-inflammatory as well as insecticidal and anti-nematodal activity.

Nodulisporic acid (24) an indole diterpene, is a novel compound from endophytic fungi, *Nodulisporuim sp*. which exhibits potent insecticidal properties against the larvae of the blowfly (Demain, 2000). Endophytic fungi *Eupenicillium spp*, produced allantryleunone which show insecticidal activity (Fabio *et al*., 2005). 3-Hydroxypropionic acid from endophytic fungus *Phomopsis phaseoli* showed nematicidal activity against the nematodes *Meloidogyne incognita* and *Caenorhaboitis elegans* (Scharz *et al*., 2004).

Phomopsolide A and B (25), produced from endophytic fungus *Phomopsis sp*., act as boring and feeding deterrents of elm bark bettle (Grove, 1985).

Penicinoline (26), a novel pyrrolyl-4-quinolinone alkaloid, isolated from endophytic fungus, *Penicillin sp*. showed strong insecticidal activity against sucking pest *Aphis gossypii* (*Shao et al*., 2010). A number of endophytic fungi could therefore be classified as entomopathogenic and nematophageous fungi. They could be used as biological control and serve as leads to the development of new insecticides or nematocides.

There are reports indicating that endophytic fungi have the potential to produce anti-inflammatory compounds. Phomol (32) was isolated from endophytic fungus *Phomopsis*. It has a very strong anti-inflammatory activity (Weber *et al.*, 2004a). Phenylpropanoids compounds

isolated from endophytic fungi do not only show anti-inflammatory activity but also anticancer, antioxidant, antimicrobial and immunosuppressive activities (Korkina, 2007).

Subglutinol A (27) a noncytotoxic diterpene pyrones isolated from the endophytic fungi *Fusaruim subglutinans*, which along with subglutionol B has shown immunosuppressive activities (Lee *et al*., 1995a). Collutelin A isolated from endophytic fungus *Colletotrichum dematium*, showed strong immunosuppressive activity (Ren *et al*., 2008).

A nonpeptidal fungal metabolite, l-783-281, isolated from *Pseudomassaria sp*. is used as an antidiabetic agent as an insulin mimetic (Zhang *et al*., 1999).

Phenol and mevinic acid isolated from culture of the endophyhtic fungus *Phomopsis sp* were indicated to show strong anti-inflamatory activity. Phenylpropanoids isolated from endophytes have received great interest for medicinal applications because they have multifold activities such as anticancer, antioxidant, antimicrobial, anti-inflammatory and anti-immunosuppressive properties (Korkina, 2007).

Epicoccins M and R, entepicoccin G, and diketopiperazine were isolated from culture of endophytic fungus *Epicoccum nigrum* (Wang *et al*., 2010). The compounds were shown to exhibit potent anti-inflammatory activity. They exhibit the platelet activating factor-induced release of β-glucoronidase from rat polymorphonuclear leukocytes *in vitro* with $1C_{50}$ values of 4.16, 4.95, 3.07 and 1.98 µM, respectively, in comparison to the positive control ginkgolide B which showed $1C_{50}$ value of 2.35 µM value. NF-kB inhibitors 11β – methoxycurvularin, 11α-methoxy-curvularin, 11α – methoxycurcularin, trans-dehydrocurvularin, and 1-chloro-2, 4-dihydroxy-5-methoxy-7-methylanthroquinone have been isolated from culture of endophytic

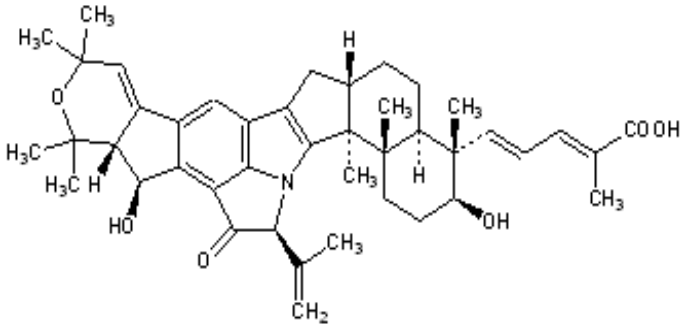
fungus *Penicillin sp*, with $1C_{50}$ values ranging from 1.6 – 10.1 µM for isolated compounds (Aly *et al*., 2011).

Xanthine oxidase (XO) is a key enzyme that catalyses the oxidation of xanthine and hypoxanthine into uric acid, and plays a vital role in hyperuricemia and gout. Allopurinol is known to inhibit XO in the treatment of gout but has unwanted side effects.

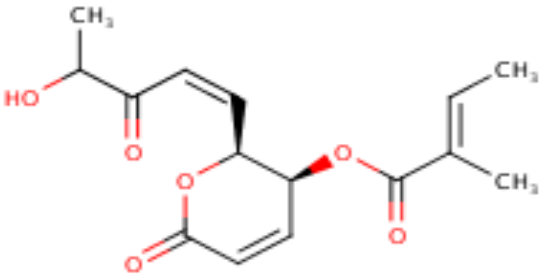
Alternariol which was isolated from the endophytic fungus *Alternaria brassicicola* has been reported to inhibit XO activity with $1C_{50}$ value of 15.5 µM.

Compounds from endophytic fungus *Aspergillus niger*, ritrofusarin B, aurasperone A, also showed strong co-inhibition of XO, cytotoxicity to colon cancer cell lines and inhibition of some microbial pathogens (Sudha *et al*., 2016).

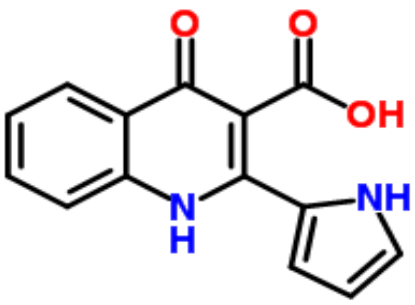
Figure 2.7 shows some secondary metabolites isolated from endophytic fungi that have insecticidal and immunesuppressive activity.



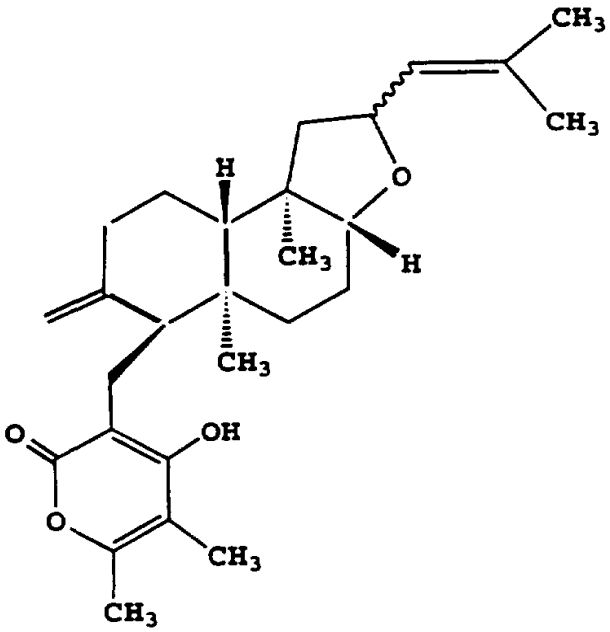
NODULISPORIC ACID (24)



PHOMOPSOLIDE (25)



PENICINOLINE (26)



SUBGLUTINOL (27)

FIGURE 2.7: Structure of Some Endophytic Secondary Metabolites With Bioactive Activity(Grove, 1985; Lee *et al.*, 1995a; Scharz *et al*., 2004; Weber *et al*., 2004a; Reen *et al.*, 2008; Shao *et al*., 2010)

2.5 THE RELATIONSHIP BETWEEN HOST PLANT AND ENDOPHYTES

The relationship between host plants and endophytes seem to be complex. First, the interaction is preceded by a physical encounter between the plant and the fungus. Secondly, several physical and chemical barriers can then be overcome to successfully establish an association. These host endophyte interactions can range from mutualism through commensalism to parasitism, as the manifestation of the interactions are often flexible, depending on the genetic disposition of the two partners, their developmental stage and nutritional status as well as environmental factors (Redman *et al*., 2001; Tan and Zou, 2001; Schulz and Boyle, 2005, Wani *et al*., 2016). The "balanced antagonism" hypothesis, was initially proposed to address how an endophyte avoids activating the host defenses, ensures self-resistance before being incapacitated by the toxic metabolites of the host, and manages to grow within its host without causing visible manifestations of infection or disease (Schulz and Boyle, 2005).

The mutual relationship between the host plant and endophyte benefits the fungal endophytes through provision of shelter, supply of energy, nutrients and protection from environmental stress. On the other hand, fungal endophytes benefit plant growth by producing secondary metabolites and enzymes, which are responsible for the adaptation of plants to abiotic stresses, such as light, drought, climatic and environmental changes and biotic stresses such as insect, nematode and herbivore attack or invading pathogens (Barzet *et al*., 1988; Kogel *et al*., 2006).

Endophytes possess structural similarity with pathogens and both possess many virulence factors, such as production of phytotoxic metabolites and exoenzymes, which are necessary to infect and colonise the host. So, the host plant has to counter by its defense mechanism as a result of non-self-recognition (Kogel *et al*., 2006).

The environmental factors play a major role to destabilize the delicate balance of antagonisms. If the plant defense mechanisms completely counteract the fungal virulence factors, the fungus will perish. Conversely, if the plant succumbs to the virulence of the fungus, a plant pathogen relationship would lead to plant disease (Arnord, 2008). Under certain conditions endophytes may become parasitic and become pathogen causing symptomatic infection.

Hence, parasitism is an exception in plant endophytes interactions, an imbalanced status of a symbiosis when the host is stressed and physiological or ecological conditions favour virulence (Schulz and Boyle, 2005; Kogel *et al*., 2006).

Endophytes of certain plants could be a pathogen of other plants. This depends principally on the balance between pathogenicity and endophytism of the microorganism in the different hosts (Saikkonen *et al.,* 2004; Hallmann *et al*., 2011).

Moricca and Ragozzi (2008) however indicated that the type of interaction between an endophyte and a plant is controlled by the genes of both organisms and modulated by the environment.

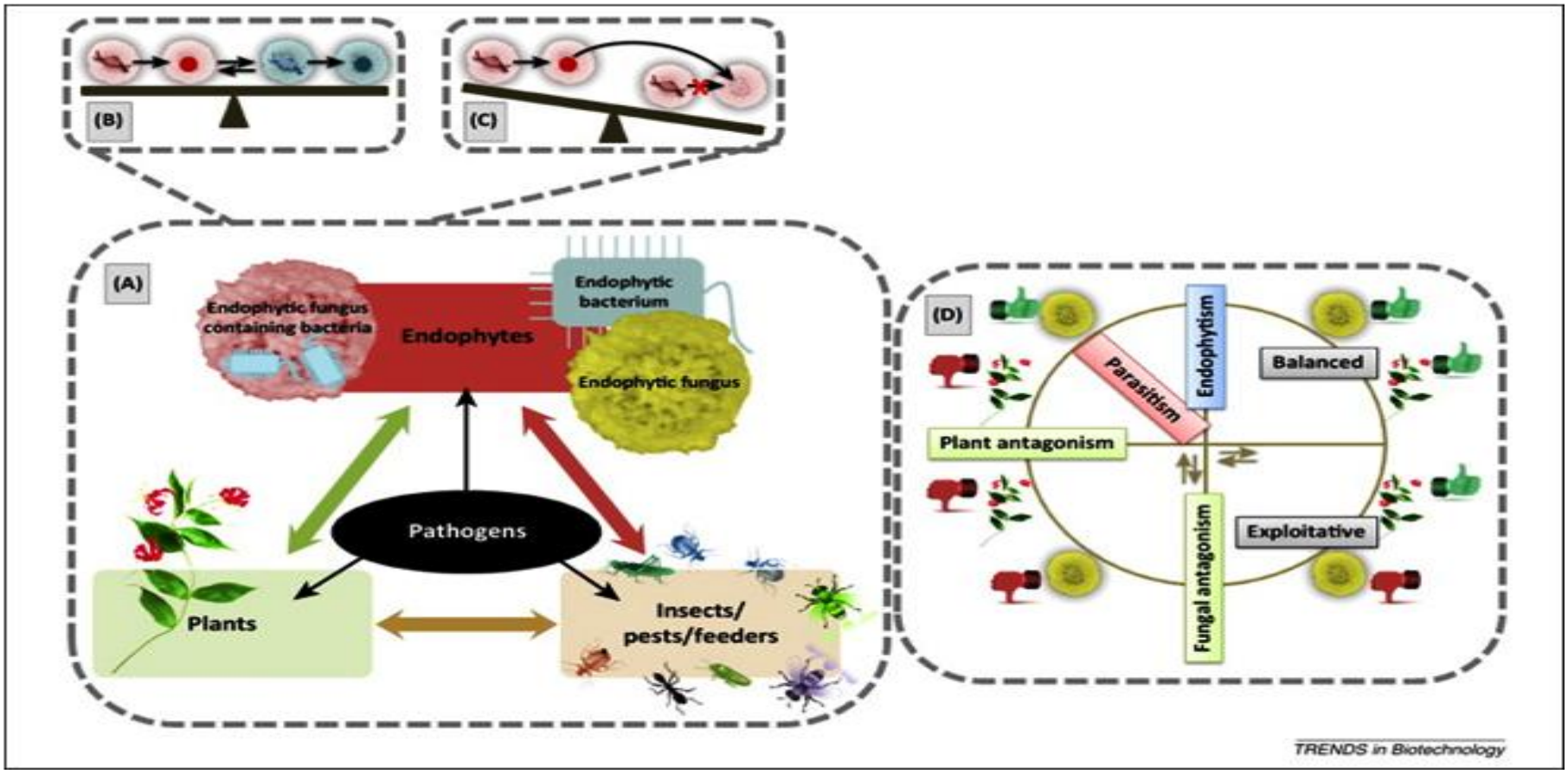


Figure 2.8: Schematic interpretation of the chemical – ecological interaction of plant endophytic fungus (Kusari *et al*., 2012).

2.6 TECHNIQUES FOR ISOLATION AND STRUCTURAL ELUCIDATION OF SECONDARY METABOLITES FROM FUNGAL ENDOPHYTES

2.6.1 CHROMATOGRAPHY

Chromatography is a diverse and important group of methods that enable scientists to separate components of complex mixtures which may be impossible with other techniques.

In all types of chromatographic separations, the sample is transported in a mobile phase, which may be a gas, a liquid or a super critical fluid. The mobile phase is forced through an immiscible stationary phase, which is fixed in place in a column or on a solid surface. The components of the mixture are distributed between the mobile and stationary phases to varying degrees. Some components have higher affinity for the stationary phase than others and as such there is difference in mobility and sample components are separated (Meyer, 2004).

Chromatographic methods can be classified according to the type of mobile phase such as liquid, gas and super critical fluid (Siddiqui *et al*., 2017).

2.6.1.1 LIQUID CHROMATOGRAPHY

This technique implies that the mobile phase used in the chromatographic process is liquid and the stationary phase can be either liquid, solid, bonded phase or ion exchange (Karger *et al*., 1973).

2.6.1.2 VACUUM LIQUID CHROMATOGRAPHY

The vacuum liquid chromatographic technique is essentially a preparative layer chromatographic separation run on a column. It involves step gradients elution and the column is allowed to run after each fraction is collected.

The column is a standard sintered glass filter funnel (25cm in length and 4cm diameter) is suitable. Silica gel 60H is dry packed to the column. The sorbent is allowed to settle by gently tapping under gravity. Sample will be applied in the upper portion of the column. Then vacuum is applied via a vacuum pump. The vacuum is released and solvent of low polarity is poured quickly into the surface of the absorbent and then vacuum is reapplied. The sample in a suitable solvent is applied directly to the top of the column and is drawn gently into the packing by appling the vacuum. The column is developed with appropriate solvent mixtures, starting with solvent of low polarity and gradually increasing the polarity, pulling the column dry between each fraction collected.

For the isolation of natural products (secondary metabolites), different chromatographic techniques are used depending on the nature of the products. The crude extracts of the endophytic fungus that showed positive activities are subjected to vacuum liquid chromatography, to obtain different fractions. A chromatographic system comprises two phases, a stationary phase which absorbs the compound based on their physical properties. For examples, based on polarity, silica gel, normal or reversed phase; based on size, sephadex LH-20, and based on charge, diaion. The mobile phase moves through the stationary phase and thereby eluting the compounds gradually based on the affinity to the solvent (Siddiqui *et al*., 2017).

2.6.2 THIN LAYER CHROMATOGRAPHY (TLC)

Analytical TLC is an inexpensive, fast and qualitative technique for screening of the chemical profile of the crude extraction or fractions. TLC is performed on the pre-coated TLC plates as stationary phase. Aluminum or glass plate TLC on silica gel (60 F_{254}), layer thickness 0.2 mm

are commercially available (Merck). The mobile phase is usually mixture of solvents from non-polar to slightly polar solvents. For example,

n-hexane:ethylacetate (8:2)

n-hexane:ethylacetate (9:1)

dichloromethane:methanol (8:2)

2.6.3 SIZE EXCLUSION CHROMATOGRAPHY

This involves separation based on molecular size of analysed compounds. The stationary phase consists of porous beads (Sephadex LH-20, 0.25-0.1mm mesh size). The mobile phase is usually a mixture of polar and non-polar solvents. For example, methanol: dichloromethane (1:1 V/V). However, the best system of the mobile phase is the one that dissolves all the components to be separated. The mixture flows down the column along with the eluent aided by force of gravity. The components are separated and distributed on the stationary phase according to molecular size. Compounds having larger molecular diameter are excluded from the interior of the bead and thus elute first. The compounds with smaller molecular diameters enter the beads and elute according to their ability to exit from the small size pores where they are trapped (Kjer, 2009).

2.6.4 HIGH PRESSURE LIQUID CHROMATOGRAPHY (HPLC)

High pressure liquid chromatography (HPLC) is high resolution chromatographic technique, also called high performance liquid chromatography. In this technique, the mobile phase is forced through the column containing the stationary phase with the aid of a pump. This results in a fast separation of the components. The high resolution is usually achieved by the used of small particle size of the absorbent material. Mostly used is the reverse phase silica material. Attached

to the HPLC is a detector to monitor the separation of the eluted compounds. This method can be used for semi preparative and analytical purpose (Kjer, 2009).

2.6.4.1 SEMI PREPARATIVE HIGH PRESSURE LIQUID CHROMATOGRAPHY (HPLC)

The semi preparative HPLC is used for the isolation and purification of pure compounds from fractions previously separated using column chromatography. The semi preparative HPLC has a column, the stationary phase, packed with silica beads bonded to octadecylsily (C18-Si-) group of 5 µm in stainless steel tubing with internal diameter of 8 mm and 143 mm length. Other column types are also available. The mobile phase is a mixture of polar solvents including methanol, acetonitrile, nanopure water. The flow rate can be 5 ml per minute or set as required. The composition of the mobile phase usually may be continuously changed from more to less polar condition (gradient elution). The injection volume can be up to 100 µl of the sample applied into the sample loop at each injection. When the injection is injected, the sample loop is suddenly switched into the flow of the mobile phase before it reaches the column.The eluted peaks are detected by an online detector. An online chromate-integrator is connected to the detector to monitor the individual components as they elute from the column after separation. The individual peaks are collected separately in glass tubes (Kjer, 2009).

2.6.4.2 ANALYTICAL HIGH PRESSURE LIQUID CHROMATOGRAPHY (HPLC)

All extracts fractions and pure compounds are monitored by using the analytical HPLC to determine the composition as well as to evaluate the purity of isolated compound. For the routine analytical HPLC detection a solvent system of nanopure water adjusted to pH 2.0 by addition of 0.1% o.phosphoric acid (eluent A) and methanol (eluent B) is used. The standard gradient system

is used: starting with A:B 10:90 to A:B 0:100 in 60 minutes. In some cases, special programs are used. Peaks are detected by UV-VIS diode array detectors. Known substances are identified by comparism of the obtained UV spectra with the internal UV- spectra library using the online software (Kjer, 2009).

2.6.5 STRUCTURAL ELUCIDATION OF THE SECONDARY METABOLITES

The structural elucidation of the isolated compounds follows a standard scheme. Firstly, the data obtained from analytical HPLC is compared with the in-house substance library regarding their retention times at the standard gradient programme and their UV spectra. Comparable hit indicates the class to which the compound belongs. From LC-MS measurement, the mass of the compound and from ^{1}H-NMR measurement substructures could be compiled.

With this information together with the identity of the fungus, a literature search using the latest versions of dictionary of natural products (DNP) (Chapman and Hall, 2005-2009; Antibase, 2002-2007; Antimarin 0512, 2012 and SciFinder) can be performed. However, where these data are insufficient, additional measurements, especially one and two dimensional NMR experiments are necessary to finally identify the secondary metabolites (Kjer, 2009).

2.6.5.1 MASS SPECTROMETRY (MS)

Mass spectrometry (MS) is an analytical technique used to determine the molecular weight, the elemental composition of a molecule and in the structural elucidation of the molecules. It is a very sensitive technique and even from microgram amounts good spectra can be obtained. Mass spectrometry, technically, consists of three parts: ionization source, analyzer and detector. The sample is ionized in the ionization source, and the rising ions are sorted and separated according

to their mass (m) to charge (z) ratio (m/z) in the mass analyser. Both negative and positive charged ions can be detected. The base ion (parent ion) has to be identified giving the molecular weight of the compound. From the fragmentation patterns of the compound, information about substructures can be obtained (Kjer, 2009). Many different methods of ionization are used in mass spectrometry and the selection of the method to be used depends on the type of samples to be analysed.

They include:

Electron Impact (E1)

Electron Spray Ionisation (ES1)

Fast Atom Bombardment (FAB)

Chemical Ionisation (CI)

Atomospheric Pressure Chemical Ionisation (APCI)

Matrix Assisted Laser Desorption Ionisation (MALDI)

Field desorption/Field Ionisation (FD/F1)

Thermo-Spray Ionisation (TSI)

2.6.5.2. ELECTRON SPRAY IONIZATION-MASS SPECTROMETRY (ESI-MS)

ESI-MS is a gentle ionization method where the sample is passed through a high voltage metal capillary. At the end of this capillary, the sample is sprayed by a flow of nitrogen gas at atmospheric pressure to form an aerosol. Together with heating, the nitrogen evaporates the emerging droplets forcing the ions in each droplet together until repulsion causes them to eject the surface. The ions are extracted into the vacuum of the mass analyzer where they are detected.

In addition to the molecular ion peaks $(M+H)^+$or $(M-H)^-$ fragments of these can be detected (Kjer, 2009).

2.6.5.3 LIQUID CHROMATOGRAPHY – MASS SPECTROMETRY (LC/MS)

High pressure liquid chromatography is a powerful method for the separation of complex mixtures, especially when many of the components may have similar polarities. If a mass spectrum of each component can be recorded as it elutes from the LC column, quick characterization of the components is greatly facilated. Typically, the LC-MS system also contains a UV detector so that from each peak found in the chromatogram, a mass spectrum and a UV spectrum can be obtained. Usually, ESI-MS is interfaced with LC to make an effective online LC/MS (Kjer, 2009).

2.6.5.4 NUCLEAR MAGNETIC RESONANCE SPECTROSCOPY (NMR)

Nuclear Magnetic Resonance (NMR) was first observed in 1946. This has led to the development of a technique called NMR Spectroscopy, which is now almost indispensible for structural elucidation. NMR Spectroscopy allows determining chemical and physical properties of the nuclei of atoms having magnetic properties. This technique utilizes the atomic nuclei spinning behaviour of atoms with an odd number of nucleons, e.g. ^{1}H and ^{13}C.

It is nowadays routinely used to study chemical structure by giving not only the constituent of a molecule but also its relative stereochemistry. A NMR spectrum is acquired by varying the magnetic field that is applied to the sample dissolved in a deuterated solvent over a small range while observing resonance signal from the compound. Depending on the electron density around each proton they obtain different shielding and deshielding effects appearing in different parts of

the resulting NMR spectrum and thus providing information about the environment of each proton. The resulting frequency where the nuclei resonate, the so-called chemical shift, is given in ppm and the coupling constants between adjacent nuclei in Hertz (Hz). NMR experiments can be conducted in a one (ID) or two (2D) dimensional manner. 2D NMR spectra can be either measured between two equal (H, H-COSY), correlation spectroscopy) or two different (H, C-COSY) frequency axis. For the H-H 2D experiments the connection between two adjacent proton (COSY), between two protons through space (NOESY, nuclear over-hauser enhancement spectroscopy; ROESY, rotating-frame enhancement spectroscopy) or between all protons in one spin system (TOCSY, total correlation spectroscopy) are given. H-C 2D experiments measure the direct correlation between a proton and a carbon (HMQC, heteronuclear multiple quantum correlation) or the connection of protons over two, three and even four bonds to carbon atoms, so called long range coupling (HMBC, heteronuclear multiple bond correlation). Correlations are shown as cross peaks in the plane between two axes containing the ID NMR shifts (Kjer, 2009).

2.7 *Picralima nitida*

Picralima nitida belong to the family Apocynaceae. The plant is widely distributed in the high deciduous rain forest of West Central Africa from Ivory Coast (Cote de ivore) to West Cameroon and extends to Congo basin and Uganda (Burkill 1985; NNMDA- Nigeria, 2008).

The plant is commonly called Picralima, pile plant or Akuamma (as called in Ghana). It is known as Limeme (Congo), Eban or Obero (Gabon), Bamborutuk or Eban (Cameroon), Erin (Yoruba), Osuigwe (Igbo) (Nkere and Iroegbu, 2005).

Picralima nitida is an understory tree and reaches up to 35 meters in height, crown dense, trunk 5-60 cm diameter; cylindrical, the wood is pale yellow, hard, elastic, fine grained and taking a high polish. The leaves are broad (3-10 cm) and oblong (6-20 cm) with tough tiny lateral nerves. The leaves are pinnate with about 14 to 28 pairs. *Picralima nitida* bears white flowers (about 3 cm long) with ovoid fruits which at maturnity are yellowish in colour (Burkill, 1985). Each fruit contains three flattened seeds embedded in pulps. Figure 2.9 shows the pictures of leaves, flower, fruits and seeds of *Picralima nitida*.

Ethnomedicinally, preparations from different parts of the plant are employed as crude drug or crude herbal extract as remedy for various kinds of human disease (Erhanyi *et al*., 2014). The leaves of *P. nitida* are used as a vermifuge and the leaf-sap is dripped into the ears for the yeartment of otitis (Iwu, 1993). The fruit is used in some West African countries in the treatment of gastrointestinal disorder and dysmenorrhea (Ansa-Asamoah, 1986; Ajanohoun *et al.*, 1996). The seeds are widely used in West Africa especially in Nigeria, Cote d' Ivoire and Ghana as antipyretic, aphrodisiac, for the treatment of malaria, pneumonia and other chest conditions (Nkere and Iroegbu 2005; Kapadia *et al*., 1993). The bark is used as laxatives and purgative, antihelmintic, treatment of veneral diseases, as febrifuges and also to treat hernia (Dalziel, 1937). The root is used as vermifuge, aphrodisiac, for fevers, malaria, pneumonia and gastrointestinal disorder (Oliver, 1960).

2.7.1 TAXONOMIC CLASSIFICATION

Kingdom	Plantae
Family	Apocynaceae
Subfamily	Rauvolfioideae
Genus	Picralima
Species	*Picralima nitida*

2.7.2 PHARMACOLOGICAL PROPERTIES OF *PICRALIMA NITIDA*

2.7.2.1 Antimalarial activity

The *in vitro* antimalarial activity of *P. nitida* extracts has been investigated (Ezeamuzie *et al*., 1994; François *et al*., 1996; Iwu and Klayman, 2002; Bickii *et al*., 2007). The root, stem bark and fruit rind extracts of *P. nitida* were reported to show antiplasmodial activity (Ezeamuzie *et al*., 1994; François *et al*., 1996 Iwu and Klayman, 2002). The seed extract of *P. nitida* demonstrated significant activity against the chloroquine-resistant *Plasmodium falciparum* W2 strain (Bickii *et al*., 2007). The *in vivo* antiplasmodial activity of the ethanol seed extract of *P. nitida* was evaluated in chloroquine-sensitive *Plasmodium berghei* infected mice and the result of this study showed that the ethanol seed extract of *P. nitida* exhibited a significant *in vivo* antiplasmodial activity in both early and established infections (Okokon *et al*., 2007).

FIGURE 2.9: Leaves (A), Flowers (B), Fruits (C), and Seeds (D) of *Picralima nitida.*

Table 2.1: Pharmacological activities of *P. nitida*

Extract of plant part/compound	Pharmacological activity
Seed	Antimicrobial, antileshmanial, larvicidal, analgesic,anti-inflammatory, antioxidant, antimalarial, antiulcer, hypoglycaemic, cytotoxic
Fruit	Antimalarial, antipyretic, anti-inflammatory, antidiarrheal, hypoglycaemic
Leaf	Larvicidal, antimicrobial, hypoglycaemic, antioxidant
Stem bark	Antimalarial, trypanocidal, antimicrobial, antioxidant
Root	Antimalarial, antimicrobial, antioxidant, cytotoxic
Akuammidine	Analgesic
Akuammine	Analgesic
Akuammicine	Analgesic, antidiabetic
Pseudoakuammigine	Analgesic, anti-inflammatory
Coumestan derivatives	Antimicrobial

Adapted from Erharuyi *et al*. (2014)

2.7.2.2 Trypanocidal activity

The trypanocidal activity of hot water extract of *P. nitida* bark in rats was investigated by Wosu and Ibe (1989). Results of their study revealed that the extract (8 mg/kg) showed activity against *Trypanosoma brucei.*

2.7.2.3 Antileishmanial activity

Using a radiorespirometric microtest technique, the antileishmanial activity of chloroform extract of *P. nitida* seeds was evaluated. The result of the study showed that the plant extract recorded activity against *Leishmania donovani* at 50 µg/mL (Iwu *et al*., 1992).

2.7.2.4 Larvicidal activity

The larvicidal activity of leaf and seed extracts of *P. nitida* against fourth instar larvae of *Anopheles gambiae* was evaluated in static bioassay. The results obtained from the studies

showed a concentration and time dependent increase in larvicidal activity of the plant extracts (Ubulom *et al*., 2012; Dibua *et al*., 2013).

2.7.2.5 Antipyretic activity

The antipyretic activity of methanol fruit extract of *P. nitida* was evaluated by Ezeamuzie *et al*. (1994). The result of their study showed that the extract 100 mg/Kg exhibited a mean percentage antipyrexia of 38.7 % on lipopolysaccharide induced pyrexia in rabbits, which was comparable to aspirin (29.0 % at 200 mg/kg) (Ezeamuzie *et al*., 1994).

2.7.2.6 Analgesic activity

The ethanol seed extract of *P. nitida* was reported to increase the mean pain threshold of rats in a dose-dependent manner and also significantly ($P<0.05$) suppressed bradykinin-induced hyperalgesia in rats (Ezeamuzie *et al*., 1994). The opioid analgesic activity of five alkaloids viz akuammidine, akuammine, akuammicine, akuammigine and pseudoakuammigine extracted from the seeds of *P. nitida* were reported by Menzies *et al*. (1998). The result of their study revealed that four of the alkaloids viz akuammidine, akuammine, akuammicine and pseudoakuammigine competitively bound to opioid sites in homogenates of guinea pig brain (Menzies *et al*., 1998).

2.7.2.7 Anti-inflammatory activity

Ezeamuzie *et al.,* (1994) reported that the methanol fruit extract of *P. nitida* showed potent and dose-dependent anti-inflammatory activity. Using the carrageenan-induced rat paw oedema method, Duwiejua *et al*. (2002) investigated an alkaloid from *P. nitida* pseudo-akuammigine for anti-inflammatory activity. The alkaloid significantly ($P<0.05$) reduced the mean maximal and total paw swelling over a 6 h period when administered orally 1 h before induction of oedema in a dose-dependent manner.

2.7.2.8 Antidiarrheal activities

Extracts of *P. nitida* have been reported to show antimicrobial activity against diarrhea-causing bacteria which include *Shigella dysenteriae, Escherichia coli, Staphylococcus aureus, Proteus vulgaris, Enterobacter cloacae, Streptococcus feacalis, Pseudomonas aeruginosa, Proteus mirabilis, Salmonella typhi,* and *Bacillus cereus* (Kouitcheu 2013; Erharuyi *et al*., 2014).

2.7.2.9 Antiulcer activity

Mabeku *et al*. (2008) evaluated the antiulcer activity of the methanol extract, chloroform and methanol fractions of *P. nitida* seeds using the aspirin-pylorus-ligation method in rats. Their study revealed that the extract and fractions of *P. nitida* seeds produced significant ($P<0.05$) reduction of ulcer index, total acidity, pepsin activity and increase in mucoprotective parameter such as phenol red content. The study indicated potent antiulcer activity of *P. nitida* with percentage ulcer inhibition of 56.36%, 40.00% and 56.36% for the methanol extract, chloroform and methanol fractions respectively (Mabeku *et al*., 2008).

2.7.2.10 Cytotoxic activity

The antiproliferative and apoptotic effects of the crude methanol extract and fractions of *P. nitida* root bark were investigated *in-vitro* using human breast cancer cell line (Erhanyi *et al*., 2011). The result indicated a marked reduction in cell proliferation and increased apoptosis in the cancer cells after extract treatment. These effects were highly significant ($P<0.001$) in the chloroform fraction of the extract (Erhanyi *et al*., 2014).

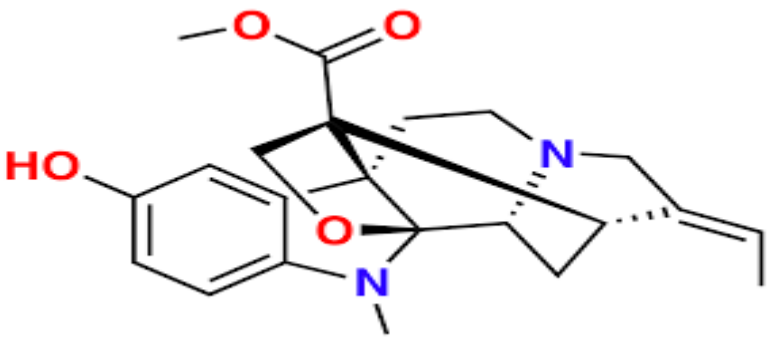
2.7.2.11 Antimicrobial activity

Extracts of *P. nitida* have been reported to show antibacterial activity against a wide range of Gram-positive *B. subtilis, S. aureus* and Gram-negative bacteria such as *E. coli, P. aeruginosa,*

and *Salmonella kintambo* (Iroegbu and Nkere, 2005; Fakeye *et al.*, 2000; Nkere and Iroegbu, 2005; Kouitcheu, 2013; Erharuyi *et al*., 2014). The plants extracts also showed antifungal activities against *Aspergillus flavus* and *C. albicans* (Ubulom *et al.*, 2012).

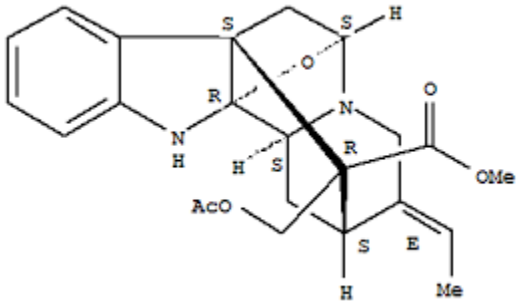
2.7.3 PHYTOCHEMICAL CONSTITUENTS OF *P. nitida*

Phytochemical screening of *P.nitida* has shown that it contains alkaloids, tannins, flavonoids, terpenoids, saponins, steroids, polyphenols and glycosides (Mabeku *et al*., 2008; Obasi *et al*., 2012). Alkaoids are the major constituent especially in the seeds. The first set of alkaloids isolated were indole alkaloids and they include; akuammine, pseudoakuammine, akuammidine, akuammicine, akuammigine, pseudoakuammigine, akuammiline and akuammenine (Erharuyi *et al*., 2014). Thereafter, other alkaloids isolated include, picraphylline, picraline, picralicine, picratidine, picranitine, burnamine, picracalline and pericine (Erharuyi *et al*., 2014). However, some of these alkaloids have been reported to be present in the leave, bark and roots of the plant.



AKUAMMINE

Akuammicine



PICRALINE

FIGURE 2.10: Chemical structure of some alkaloids from *P. nitida*

CHAPTER THREE

MATERIALS AND METHODS

3.1 MATERIALS

3.1.1 ANALYTICAL INSTRUMENTS

A. Analytical HPLC

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

B. Semi preparative HPLC

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

C. LC-MS

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

D. NMR:

^{1}H spectra were recorded at 300º K on Bruker DPX 300, ARX 400, 500 NMR spectrometers. The 1D spectrum was obtained using the standard Bruker software. Solvents for NMR include: acetone, chloroform, dimethoxylsulfoxide, and methanol (Uvasol, Merck).

E. OTHER INSTRUMENTS

Other instruments used in this study include: high resolution ESI-MS (Qtof 2, Micromass), freeze dryer (Lyovac GT2, Steris; pump Trivac D10E, Savant), Analytical balance (MC-1, Sartorius), desiccator (Glaswerk Wertheim), Hot plate and magnetic stirrer (IKA-Combimag RCH, Janke & Kunkel KG), drying oven (ET6130, Heraeus), ultra sonicator (RK 510H, Bandelin Sonorex), rotary evaporator (RV 05-ST, Vaccubrand), membrane vacuum pump (Vaccubrand), UV lamp (Camag, 254 and 366 nm), nitrogen generator (UHPN 3001, Nitrox) (All were made in Germany).

3.1.2 EQUIPMENT/INSTRUMENTS FOR FUNGAL ISOLATION, CULTIVATION AND IDENTIFICATION

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

Culture media used in this study include: fungal isolation and purification medium [Malt Extract Agar (MEA) (Oxoid, UK)], fermentation medium [Rice medium (Rice-100 g + distilled water-110 mL of distilled water)], antimicrobial assay media [Mueller Hinton Agar (MHA) and Sabouraud Dextrose agar (SDA) (Oxoid, UK)].

3.1.4 SOLVENTS

Solvents used in general procedures include methanol, ethyl acetate, dichloromethane and n-hexane, ethanol, sodium hypochlorite (Merck, Germany). They were off analar grade.

HPLC solvents include: methanol and acetonitrile (LiChroSolv HPLC grade, Merck, Germany). Distilled was obtained from Millipore water purifier. De-ionised water was obtained by passing water through nano- and ion-exchange filter cells (Barnstead) to yield nanopure water.

3.1.5 GENERAL LABORATORY CHEMICALS/REAGENTS

Other chemicals/reagents include: acetic acid (Fischer Scientific, UK), formic acid (Fischer Scientiic, UK), chloramphenicol (United State Pharmacoepeia Maryland, USA), Mueller Hinton Agar and Sabouraud Dextrose Agar (Merck, Germany). Chemicals and reagents used were of analytical grade.

3.1.6 TEST MICROORGANISMS

Laboratory strains of *Staphylococcus aureus, Bacillus subtilis, Samonella typhi, Escherichia coli, Aspergilus niger and Candida albicans* were obtained from the Department of Pharmaceutical Microbiology and Biotechnology, Faculty of Pharmaceutical Sciences, Nnamdi Azikiwe University, Awka, Nigeria, were used in the antimicrobial assay of the endophytic fungal extracts.

Mycobacterium tuberculosis from the laboratory of Prof. Dr. R. Kalscheuer at the Institüt für Pharmazeutische Biologie und Biotechnologie, Heinrich-Heine-Universität, Düsseldorf, Germany, was used in the anti-tubercular assay.

3.2 METHODS

3.2.1 COLLECTION OF PLANT MATERIALS

Branches from *Picralima nitida* were collected from the terrestrial forest of Igbo-Ukwu in Aguata Local Government Area, in Anambra State, Nigeria. The plant material was identified by Mrs. A. U. Emezie, a plant technologist in the Department of Pharmacognosy and Traditional Medicine, Faculty of Pharmaceutical Sciences, Nnamdi Azikiwe University, Awka, Anambra State, Nigeria. This was authenticated by a plant taxonomist in the Department of Botany, Nnamdi Azikiwe University, Awka. Sample of the plant was deposited in the herbarium of Department of Pharmacognosy and Traditional Medicine as voucher specimen (PCG474/A/027). Freshly collected healthy leaves from the branches were then used for the fungi isolation.

3.2.2 ISOLATION OF ENDOPHYTIC FUNGI FROM PLANT LEAVES

Fresh leaves of the plant were picked and then rinsed with fresh water under a running tap and was finally rinsed with distilled water. The leaves were cleaved aseptically into small segments (approximately 1 x 1 cm) from the lamina with sterile blades.

Surface sterilization was achieved by immersing the chips in 1 % sodium hypochlorite for 2 min after which the leaves were soaked in 70 % ethanol for 2 min and then rinsed with fresh sterilized distilled water for 5 min. They were then dried under laminar air flow chamber (modified fom Ndakidemi *et al*., 2013).

Using sterilized forceps each leaf was placed on Petri dishes containing Malt Extract Agar (MEA) supplemented with chloramphenicol to suppress bacterial growth. The plates were then incubated at 27 ^{o}C and monitored periodically until mycelia growth were observed from the leaves. Each hyphal tip from distinct colony was transferred onto fresh malt extract plates to obtain pure cultures of each fungal isolate.

3.2.3 IDENTIFICATION OF ENDOPHYTIC FUNGI

Taxonomic identification of all fungal strains was achieved by DNA amplification and sequencing of the fungal internal transcribed spacer (ITS) region. Total fungal genomic DNA was extracted and purified directly from fresh, axenic mycelia using fungal DNA extraction and purification kits. The molecular identification was performed according to a molecular biologic protocol as described by Kjer (2009). Details are presented below.

3.2.3.1 PROTOCOL FOR FUNGAL DNA ISOLATION, PURIFICATION, AMPLIFICATION AND SEQUENCING

Fungal strains were identified based on the analysis of the DNA sequences of the internal transcribed spacer region (ITS) of its ribosomal RNA gene.

A. DNA Extraction/Isolation

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

2. The ZR Bashing Bead™ Lysis Tube was placed on a vortexer and processed (vortexed) at maximum speed (500 revolutions per minute) for 5 min.

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

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

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

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

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

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

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

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

Ultra-pure DNA was then obtained.

B. DNA Amplification (PCR)

DNA amplification by PCR was performed using 'Hot StarTaq Master Mix Taq polymerase and the primer pair ITS1 and ITS4 (Invitrogen) in an iCycler (Bio-Rad) thermocycler.

In 0.2 ml PCR tubes, the following were added

25 µl HotStarTaq Master Mix

1.5 µl primer mix (10 pmol/µl each)

10-100 ng template DNA (usually 1 µl is enough)

Water until 50 µl

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

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

ITS 1 5'-TCCGTAGGTGAACCTGCGG-3'

ITS4 5'-TCCTCCGCTTATTGATATGC-3'

C. PCR Program

Initial denaturation 95^{o}C, 15 min

Denaturation 95^{o}C, 1 min

Annealing 56^{o}C, 1 min

Extension 72^{o}C, 1 min

Final extension 72^{o}C, 10 min.

D. DNA Purification/Gel Electrophoresis

(i) Gel Electrophoresis

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

A volume of 50 µl of the PCR product was mixed with 10 µl of 6X gel loading dye (blue) and loaded in the wells (a volume of 25-30 µl of sample was loaded into the wells). At least 1 or 2 wells was loaded with 10 µl Quick-Load® 100bp DNA Ladder (blue, i.e. already mixed with gel loading dye) (New England Biolabs, Inc. USA).

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

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

(ii) DNA Purification from Agarose Gel

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

(iii) DNA Recovery

1. A volume of three times ADB was added to each volume of agarose excised from the gel (e.g.for 100 µl (mg) of agarose gel slice, 300 µl of ADB was added).
2. This was incubated at 37-55 °C for 5-10 mins until the gel slice is completely dissolved.
3. The melted agarose solution was transferred to a Zymo-Spin™ Column in a Collection Tube.
4. This was centrifuged at 10000rpm for 30-60 sec. The flow-through was then discarded.
5. A volume of 200 µl of DNA Wash Buffer was added to the column and centrifuged for 30 seconds. The flow-through was discarded and the wash step repeated.
6. A volume of ≥ 6 µl of DNA Elution Buffer or water was added directly to the column matrix. The column was then placed into a 1.5 ml tube and centrifuged at 10000rpm after 5 min for 30-60 sec to elute DNA. Ultra-pure DNA was then obtained.

E. DNA Sequencing

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

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

F. BLAST Search

BLAST search of the sequences was then performed under:

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

3.2.4 FERMENTATION AND EXTRACTION OF SECONDARY METABOLITES

Solid state fermentation was carried out in Erlenmeyer flasks containing 100 g of rice and 110 ml of tap water. This was autoclaved at 121 ^{o}C for 1 h and allowed to cool. The flasks was inoculated with 3-5 mm diameter agar blocks containing test fungi and incubated at 25-27 °C for 21 days and extracted with ethyl acetate. The ethyl acetate was evaporated and dried in vacuum evaporator at 40^{0}C to yield crude extract. The extract was stored as stock materials at 4 ^{o}C for further studies. A flow diagram showing the fermentation and extraction of secondary metabolites is presented in Figure 3.1.

3.2.5 ISOLATION OF SECONDARY METABOLITES

For the isolation of natural products (secondary metabolites), different chromatographic techniques was used depending on the nature of the product. The crude extracts of the endophytic fungi that showed positive antimicrobial and antioxidant activities were subjected to vacuum liquid chromatography, to obtain different fractions. A chromatographic system

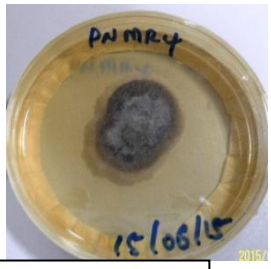
comprises two phases, a stationary phase which absorbs the compound based on their physical properties. For examples, based on polarity, silica gel, normal or reversed phase; based on size, Sephadex LH-20, and based on charge, Diaion. And the mobile phase which moves through the stationary phase and thereby eluting the compounds gradually based on the affinity to the solvent.

3.2.5.1 VACUUM LIQUID CHROMATOGRAPHY (VLC)

About 1.5g each of the crude extracts obtained from the fermentation were subjected to vacuum liquid chromatographic fractionation. The extracts were adsorbed on silica gel in a glass column 4x50 cm packed with silica gel (230-400 mesh size) to a bed height of 15 cm and connected to a vacuum pump. The column was eluted using four solvents in binary combination as shown in Table 3.1.

Each of the fractions were collected in different flasks and concentrated with vacuum rotary evaporator. They were then left to air dry and the weight of each fraction determined.

Rice Medium
100g of rice was put in I000mL Erlenmeyer flask containing 110 ml of distilled water which was then autoclaved and allowed to cool.



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

Fermentation
After inoculation, flasks were incubated at 25-27 °C for 21 days.

Extraction of secondary metabolites using ethyl acetate and filtration

Vacuum drying (40 ^{o}C)

Crude Extracts

Figure 3.1: Flow Diagram for Fermentation and Extraction of Secondary Metabolites.

Table 3.1: Fractions and solvent system used for the fractionation

FRACTION	SOLVENT SYSTEM	RATIO	VOLUME(ML)
F_1	n-hexane/Ethyl acetate	10: 0	100
F_2	n-hexane/Ethyl acetate	9: 1	100
F_3	n-hexane/Ethyl acetate	8:2	100
F_4	n-hexane/Ethyl acetate	7:3	100
F_5	n-hexane/Ethyl acetate	6:4	100
F_6	n-hexane/Ethyl acetate	5:5	100
F_7	n-hexane/Ethyl acetate	4:6	100
F_8	n-hexane/Ethyl acetate	3:7	100
F_9	n-hexane/Ethyl acetate	2:8	100
F_{10}	n-hexane/Ethyl acetate	1:9	100
F_{11}	n-hexane/Ethyl acetate	0: 10	100
F_{12}	Dichloromethane/methanol	10:0	100
F_{13}	Dichloromethane/methanol	8:2	100
F_{14}	Dichloromethane/methanol	6:4	100
F_{15}	Dichloromethane/methanol	5:5	100
F_{16}	Dichloromethane/methanol	4:6	100
F_{17}	Dichloromethane/methanol	0:10	100

3.2.5.2 THIN LAYER CHROMATOGRAPHY (TLC)

Fractions obtained from the Sephadex LH-20 chromatography were monitored by TLC analysis. TLC was performed on the pre-coated TLC plates on Silica gel 60 F_{254}, layer thickness 0.2 mm (Merck). The samples were loaded close to one edge of the plate and the plate "developed" by immersing the loaded edge in a mobile phase in a solvent vapour-saturated development chamber. The mobile phases were a mixture of two solvents: n-hexane:ethylacetate (8:2; 9:1) – for non- polar compounds, dichloromethane:methanol (8:2) – for semi-polar compounds. The band separation on TLC was observed under an ultraviolet light at 254 nm. The different compounds were compared and identified with their specific retraction factor. Fractions with the same R_f values and hence similarity in composition were merged.

3.2.5.3 SIZE EXCLUSION CHROMATOGRAPHY

Bioactive fraction of F5 from *endomelaconiopsis sp*. VLC was subjected to the Sephadex LH-20 gel/size exclusion chromatography.The column was packed with the Sephadex LH-20 gel prepared in the mobile phase, 0.25 – 0.1 mm mesh size, (GE Healthcare) as stationary phase. The mobile phase was a mixture of polar and non-polar solvent. Elution was performed using MeOH:DCM [1:1 (V/V)] as mobile phases. The best system of the mobile phase for each sample was the one that dissolved all components to be separated.

3.2.5.4 SEMI-PREPARATIVE HIGH PRESSURE LIQUID CHROMATOGRAPHY (HPLC)

The semi preparative HPLC was used for the isolation and purification of pure compounds from fractions previously separated using column chromatography. The semi preparative HPLC used

has a column, the stationary phase, packed with silica beads bonded to octadecylsily (C18-Si-) group of 5 µm particle size in stainless steel tubing with internal diameter of 8 mm and 143 mm length. The mobile phase was a mixture of polar solvents including methanol and nanopure water. The flow rate was 5 ml per minute. The composition of the mobile phase was continuously changed from more to less polar condition (gradient elution). The samples were prepared at a concentration of 3 mg per ml. The injection volume was 100 µl of the sample applied into the sample loop at each injection. When the injection was operated, the sample loop was suddenly switched into the flow of the mobile phase before it reaches the column.The eluted peaks were detected by an online UV detector. The wavelength was set at UV absorption maximum of 235 or 254 nm depending on the results obtained from initial analyte HPLC. An online chromate-integrator was connected to the detector to monitor the individual components as they elute from the column after separation.The individual peaks were collected separately in glass tubes.

3.2.5.5 ANALYTICAL HPLC

All extracts, fractions and pure compounds were monitored by using the analytical HPLC to determine the composition as well as to evaluate the purity of isolated compound. Optimal conditions for semi preparative HPLC were determined and known substances identified by comparison of the obtained UV spectra with the internal UV-spectra library using the online software.

For the routine analytical HPLC detection a solvent system of nanopure water adjusted to pH 2.0 by addition of 0.1% formic acid (eluent A) and methanol (Eluent B) was used. The standard gradient system was used as shown in the Table 3.2. The peaks were detected using UV-VIS diode array detector.

3.2.6 STRUCTURAL ELUCIDATION OF THE ISOLATED SECONDARY METABOLITES

The structural elucidation of the isolated compounds followed a standard scheme. Firstly, the data obtained from analytical HPLC were compared with the in-house substance library regarding their retention times at the standard gradient programme and their UV spectra. Comparable hit indicated the class to which the compound belongs. From LCMS measurement the mass of the compound and from ^{1}H-NMR measurement substructures were compiled.

With this information together with the identity of the fungus, a literature search using the dictionary of natural products (DNP, Chapman and Hall, 2005-2009), Antibase, SciFinder, etc was performed to finally identify the secondary metabolites.

3.2.6.1 MASS SPECTROMETRY (MS)

HPLC/ESI-MS was used in the analyses of some of the compounds using a Finnigan LC QDECA mass spectrometer connected to a UV detector. The HPLC was run on a Nucleosil C-18 reversed-phase column. The nanopure water (containing 0.1% formic acid) and methanol were used as the mobile phase. The flow rate was 0.4 ml/min and was according to the standard gradient elution method as shown in Table 3.2. The samples were dissolved in water/Methanol mixtures and injected to the HPLC/ESI-MS set-up. The compounds were measured with ESI and measurements were done at the Institute of Pharmaceutical Biology, HHU, Düsseldorf.

Table 3.2: Standard gradient for Analytical HPLC

Time (min)	Eluent A (%)	Eluent B (%)
0	90	10
5	90	10
35	0	100
45	0	100
46	90	10
60	90	10

3.2.6.2 NUCLEAR MAGNETIC RESONANCE SPECTROSCOPY (NMR)

NMR measurements were done at the Institut für Anorganische Chemie und Makromolekulare Cheie of HHU, Düsseldorf. ^{1}H spectra were recorded on Brucher DPX 300, ARX 500 NMR spectrometers. These were obtained using the standard Bruker software. The samples were dissolved in different solvents, $CDCl_3$ and CD_3OD, the choice of which depended on the solubility of the samples. Residual solvent signals were used as internal standards (reference signals). The observed chemical shift (δ) values were given in ppm and the coupling constants (J) in Hz. All the pure isolates were identified with $^{1}HNMR$.

3.2.7 ANTIMICROBIAL ASSAY

Antimicrobial screening of the crude fungal extracts and fractions was carried out using the agar well diffusion assay method as described by Subbulakshmi *et al.* (2012) (with modification). A concentration of 10 and 1 mg/mL of the fungal extracts/fractions was prepared by dissolving the extracts in dimethyl sulphoxide (DMSO).

Standardized broth cultures of test bacterial isolates (*Staphylococcus aureus, Salmonella typhi, Bacillus subtilis* and *Escherichia coli* and fungal isolates (*Aspergillus niger* and *Candida albicans*) were spread aseptically onto the surface of Mueller Hinton Agar (MHA) and Sabouraud Dextrose Agar (SDA) plates respectively by using sterile cotton swabs. All culture plates were allowed to dry for about 5 min and agar wells were made by using a sterile cork-borer (6 mm in diameter). These wells were respectively filled with 20 µL of the fungal extracts/fractions and controls. The plates were then kept at room temperature for 1 h to allow the agents to diffuse into the agar medium. Ciprofloxacin (5 µg/mL) and miconazole (50 µg/mL) were used as positive controls in the antibacterial and antifungal evaluations respectively; while DMSO was used as the negative control. The MHA plates were then incubated at 37 ^{o}C for 24 h, and the SDA plates were incubated at 25-27 ^{o}C for 2-3 days. The inhibition zones diameters (IZDs) were measured and recorded. The size of the cork borer (6 mm) was deducted from the values recorded for the IZDs to get the actual diameter. This procedure was conducted in triplicate and the mean IZDs calculated and recorded.

Also, the antimicrobial activity of pure compounds isolated from the fungal extracts was determined using the method described above.

3.2.8 CYTOTOXICITY ASSAY

3, (4,5- dimethylthiazolyl-2)-2,5-diphenyltetrazolium bromide (tetrazolium MTT) assay was performed following the method described by Carmichael *et al*. (1987), and % cell viability was deduced by spectrophotometric determination of accumulated formazan derivative in treated cells at 560 nm in comparison to control cells (Ashour *et al*., 2006). L5178Y mouse lymphoma cells were grown in Eagle's minimal essential medium supplement with 10% horse serum in

roller tube culture. The medium contained 100 units/mL penicillin and 100 units/mL streptomycin. The cells were maintained in a humidified atmosphere at 37 °C with 5% CO_2. Of the test samples to be analyzed in the bioassay, stock solutions of crude extracts (10 µg/mL) in ethanol (96% v/v) were prepared. Exponentially growing cells were harvested, counted and diluted appropriately. Of the cell suspension, 50 µL containing 3750 cells were pipetted into 96-well microtiter plates. Subsequently, 50 µL of a solution of the test samples containing the appropriate concentration was added to each well. The test plates were incubated at 37 °C with 5% CO_2 for 72 h.

A solution of MTT was prepared at 5 µg/mL in phosphate buffered saline (PBS; 1.5 mM KH_2PO_4, 6.5 mM Na_2HPO_4, 137 mM NaCl, 2.7 mM KCl; pH 7.4) and from this solution, 20 µL were pipetted into each well. The yellow MTT penetrated the cells and in the presence of mitochondrial dehydrogenases, MTT was transformed to its blue formazan complex. After an incubation period of 3 h 45 min at 37°C in a humidified incubator with 5% CO_2, the medium was centrifuged (15 min at 210 x g) with 200 µL DMSO and the cells were lysed to liberate the formazan product.

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

$$\text{Survival \%} = \frac{100}{\text{Absorbance of treated cells - Absorbance of culture medium}} \times \text{Absorbance of untreated cells - Absorbance of culture medium}$$

All experiments were carried out in triplicate.

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

3.2.9 ANTI-TUBERCULAR ASSAY

Growth inhibition of *Mycobacterium tuberculosis* was tested using a metabolic activity assay employing the resazurin dye reduction method as described by Daletos *et al.*, (2015). *M. tuberculosis* cells were grown aerobically at 37 °C in Middlebrook 7H9 media supplemented with 0.5 % (v/v) glycerol, 0.05 % (v/v) tyloxapol, and 10 % (v/v) ADS enrichment (5 %, w/v, bovine serum albumin fraction V; 2 %, w/v, glucose; 0.85 %, w/v, sodium chloride). Bacteria were pre-cultured until log-phase (OD 600 nm ~1) and then seeded at 1×10^5 cells per well in a total volume of 100 µL in 96-well round-bottom microtiter plates. The test substances were then added to achieve a working concentration of 10 µg/mL for the fungal extracts. The microtiter plates were then incubated at 35°C for 6 days. For viability determination, 10 µL of resazurin solution (100 µg/mL, Sigma-Aldrich) was added per well and incubated for about 8 h. Then cells were fixed by addition of formalin (5%, v/v) for 30 min, and fluorescence was measured using a microplate reader (excitation 540 nm, emission 590 nm). Residual growth was calculated relative to rifampicin-treated (0% growth) and DMSO-treated (100% growth) controls.

3.2.10 ANTIOXIDANT ASSAY

The free radical scavenging activity of all the extracts was evaluated by 1, 1-diphenyl-2 picryl-hydrazyl (DPPH) according to the method by Shen *et al.*, (2010) with modification.

A volume of 50 ml of 0.2 mM solution of DPPH in methanol was prepared by weighing 3.94 mg of DPPH to 50 ml of methanol. Also, 2 ml of 0.2 mM solution of DPPH was added to 2 ml of the sample and quercitin respectively and dissolved in methanol (1 mg/ml, 1000 µg/ml). These final

reaction mixtures result in a 2-fold dilution of both the extract and DPPH concentrations bringing them to a final concentration of 0.1 mM for the DPPH solution and 500 µg/ml for the samples. The mixtures were shaken vigorously and allowed to stand at room temperature for 30 min. Then the absorbance was measured at 517 nm using a UV-VIS spectrophotometer.

Lower absorbance values of reaction mixture indicate higher free radical scavenging activity.

The capability of scavenging the DPPH radical was calculated by using the following formula.

$$\text{DPPH scavenging effect (\% inhibition)} = \frac{A_0 - A_1}{A_0} \times \frac{100}{1}$$

Where, A_0 is the absorbance of the control and A_1 is the absorbance in the presence of all of the extract samples and reference. All the tests were performed in triplicates. The mean value and standard deviation were determined and recorded.

Also, the antioxidant activities of some of the pure compounds isolated from the fungal extracts were determined using the method described above.

3.2.11 BIOASSAY OF PURE COMPOUNDS

3.2.11.1 Antimicrobial Assay

Antimicrobial screening of the pure compounds isolated from the endophytic fungal extracts was carried out using the agar well diffusion assay method as described in section 3.2.7 above. A concentration of 500 µg/mL was prepared for each of the pure compound by dissolving the extracts in Dimethyl sulphoxide (DMSO). The samples were tested against test bacterial isolates (*E. coli, S. aureus, S. typhi and B. subtilis*) and fungal isolates (*Aspergillus niger* and *Candida albicans*). Ciprofloxacin (5 µg/mL) and miconazole (50 µg/mL) were used as positive controls in the antibacterial and antifungal evaluations respectively; while DMSO was used as the negative

control. The inhibition zones diameters (IZDs) produced by the samples and controls against test organisms were measured and recorded. This procedure was conducted in triplicate and the mean IZDs calculated and recorded.

3.2.11.2 Antioxidant Assay

The antioxidant activities of the pure compounds isolated from the endophytic fungal extracts were evaluated using the 1, 1-diphenyl-2-picryl-hydrazyl (DPPH) assay described in section 3.2.10 above. The percentage inhibition of the compounds and positive control (Quercetin) were determined at a concentration of 250 µg/mL from absorbance values recorded from the UV at 517nm. The capability of scavenging the DPPH radical was calculated by using the following formula.

$$\text{DPPH scavenging effect (\% inhibition)} = \left(\frac{\text{Abs of blank } (A_0) - \text{Absorbance of sample } (A_1)}{\text{Abs of blank } (A_0)} \right) \text{X} \quad \frac{100}{1}$$

3.2.12 STATISTICAL ANALYSES

All bioassays carried out in this study were run in triplicates (n=3) and the average calculated and recorded. Results of the preliminary antimicrobial screening of the fungal extracts and fractions were presented as mean ± standard error of mean (SEM) inhibition zone diameters (IZDs).

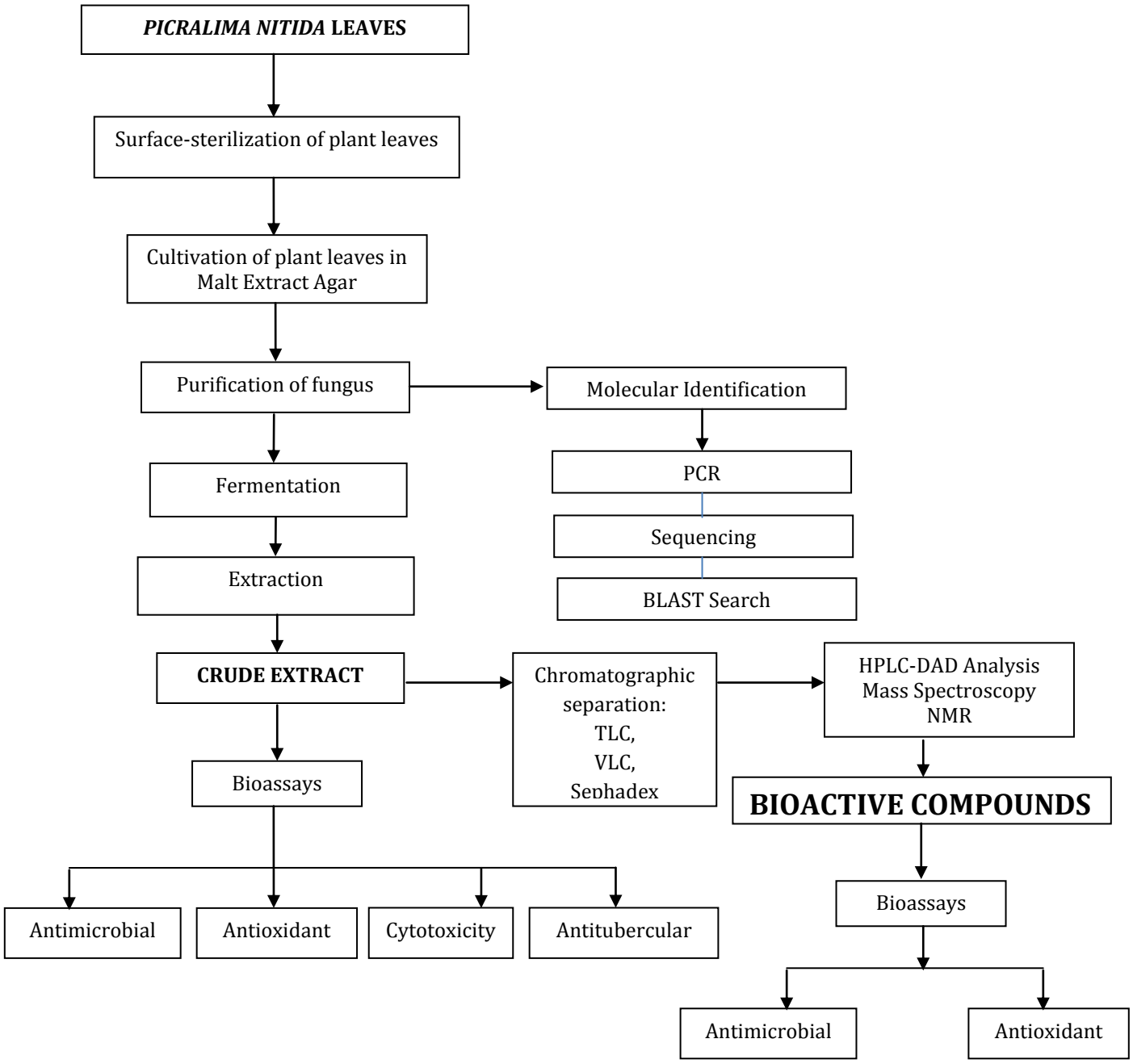


Figure 3.2: Summary of experimentations employed in this study

CHAPTER FOUR

RESULTS AND DISCUSSION

4.1 RESULTS

4.1.1 FUNGAL ISOLATION AND IDENTIFICATION

Two pure endophytic fungal strains that were isolated from *Picralima nitida* were grown in small scale basis on rice and their crude extracts screened for their antimicrobial, antifungal, cytotoxic and antioxidant properties.

Based on these results and in conjunction with the initial HPLC-DAD screening, they were chosen for molecular identification procedures.

Also, following the identification experiments carried out according to the molecular biological protocol of DNA amplification and sequencing of the ITS region (described in section 3.2.3), the resulting fungal DNA sequences were subjected to BLAST searches in the NCBI GenBank database. The DNA sequences and identity of the isolated fungi are presented in Table 4.1 below.

The fungi were identified as *Curvuralia sp*. and *Endomelanconiopsis sp.*

Also, growths of the isolated fungi on Malt Extract Agar are shown in Figures 4.2 and 4.3

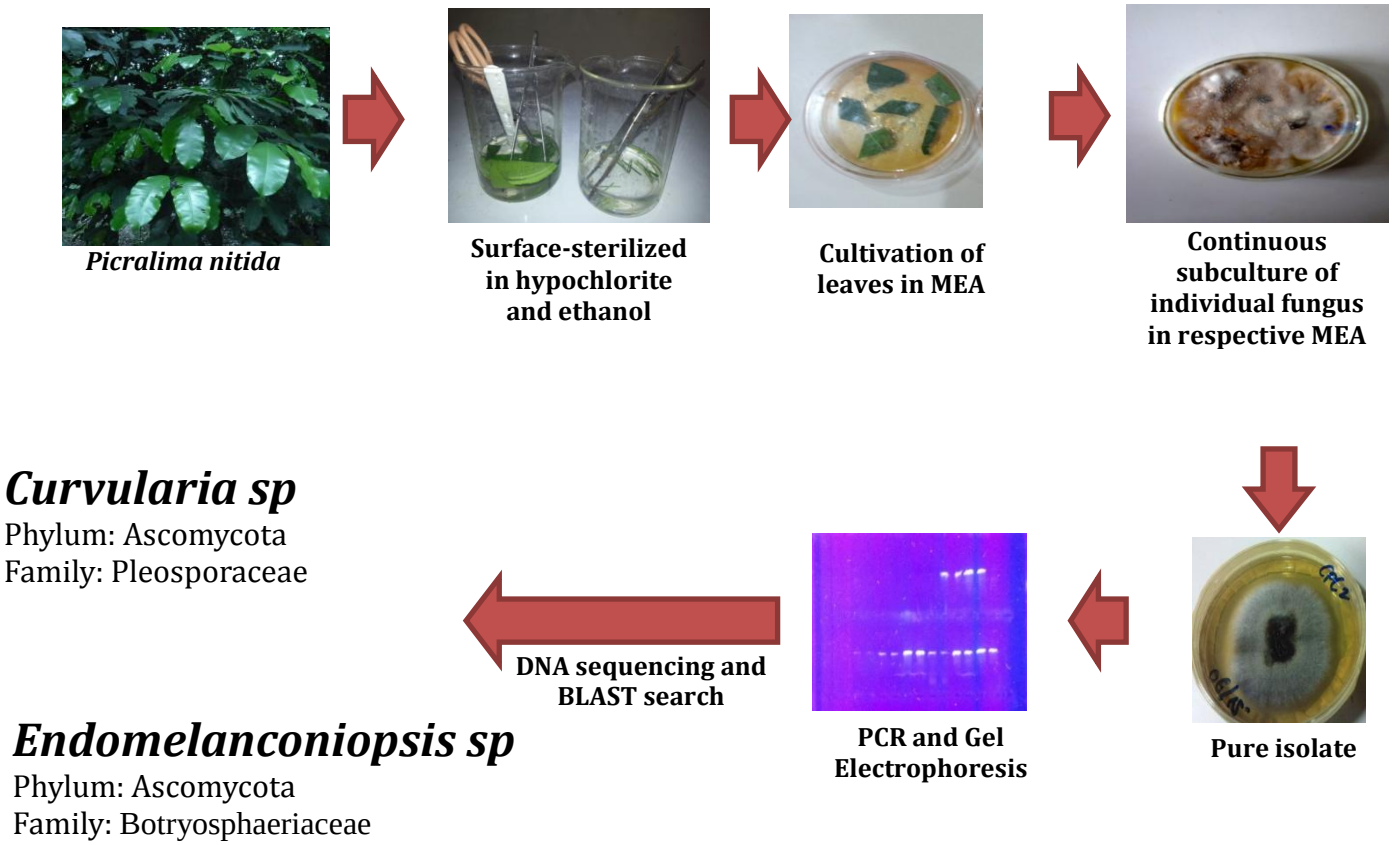


Figure 4.1: Isolation and Identification of Endophytic Fungi from *Picralima nitida*

Figure 4.2: Growth of *Curvularia sp.* in Malt Extract Agar

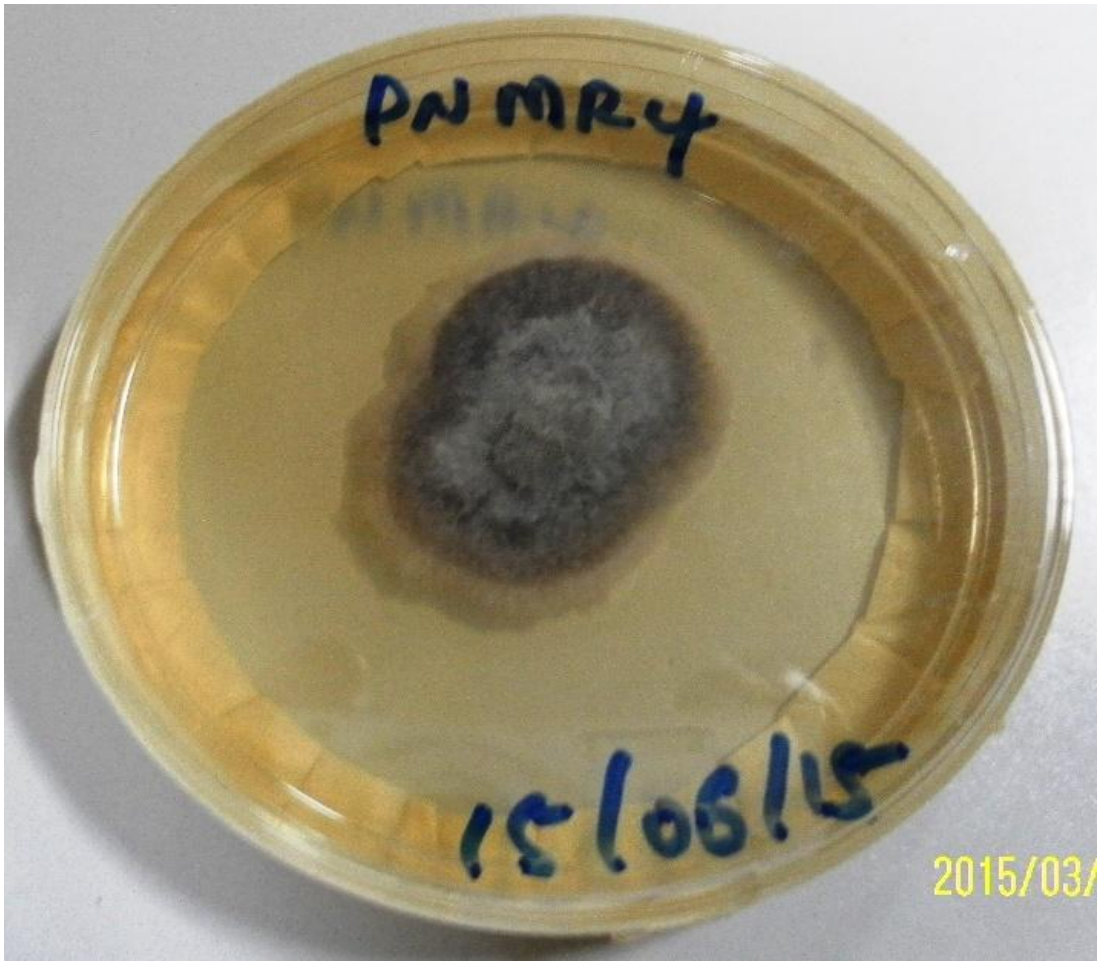


Figure 4.3: Growth of *Endomelanconiopsis sp.* in Malt Extract Agar

Table 4.1: Results of Fungal Identification

Source	Fungal DNA Sequence (FASTA format)	Fungal Name
Picralima nitida	TTCAAACCGGCTGGATTATTTTTCTTCACCCTTGTCTTTTGCGCACTTGTTGTTTCCTGGGCGGGTTCGCTCGCCACCA GGACCACACCATAAACCTTTTTGTTAATGCAATCAGCGTCAGTAAAAAGTAATAATTATTTTACAACTTTCAACAAC GGATCTCTTGGTTCTGGCATCGATGAAGAACGCAGCGAAATGCGATACGTAGTGTGAATTGCAGAATTCAGTGAAT CATCGAATCTTTGAACGCACATTGCGCCCTTTGGTATTCCAAAGGGCATGCCTGTTCGAGCGTCATTTGTACCCTCAA GCTTTGCTTGGTGTTGGGCGTTGTTTGTCTTTGGCCTTGCCCAAAGACTCGCCTTAAAACAATTGGCAGCCGGCCTAC TGGTTTCGCAGCGCAGCACATTTTTGCGCTTGCAATCAGCAAAAGAGGTTGGCCATCCATCAAGACTACATTTATAC GTTTGACCTCGGATCAGGTAGGGATACCCGCTGAACTTAAGCATATCAAT	*Curvularia sp*
	CTCTTCTCTCACACCCTATGTGTACCTACCTCTGTTGCTTTGGCGGGCCGCGGTCCTCCGCGGCCGGCCCCCTAACCG GGGCTGGCCAGCGCCCGCCAGAGGACTACCAAACTCCAGTCAGTAAACGTAGCTGTCTGATCAAAAGTTTAATAAA CTAAAACTTTCAACAACGGATCTCTTGGTTCTGGCATCGATGAAGAACGCAGCGAAATGCGATAAGTAATGTGAAT TGCAGAATTCAGTGAATCATCGAATCTTTGAACGCACATTGCGCCCCTTGGTATTCCGAGGGGCATGCCTGTTCGAG CGTCATTTCACCACTCAAGCTCTGCTTGGTATTGGGCGCCGTCCTTCACCGGACGCGCCTCAGGACCTCGGCGGAGT CTTCCTCTCTGGGTTGGATTAATTGCGTCGCGTTGGGGGTAGTAGGTTTGTTCGTAAGATTAACCTTTTGAATTGTTT CAAGGTGGTCCTCG	*Endomelanconiopsis sp*

After BLAST searches of fungal DNA sequences in the NCBI GenBank database (http://blast.ncbi.nlm.nih.gov/Blast.cgi), the endophytic fungi isolated from *Picralima nitida* were identified as *Curvularia sp and Endomelanconiopsis sp*respectively.

Table 4.2: Yield of Crude Extract and VLC Fractions of *Curvularia sp*.

EXTRACT/FRACTION	SOLVENT RATIO		YIELD (g)
Crude	-		2.6391
F1	Hex: EtAOC	10 : 0	0.0059
F2	Hex: EtAOC	9 : 1	0.0028
F3	Hex: EtAOC	8 : 2	0.0939
F4	Hex: EtAOC	7 : 3	0.0776
F5	Hex: EtAOC	6 : 4	0.0776
F6	Hex: EtAOC	5 : 5	0.0892
F7	Hex: EtAOC	4 : 6	0.0534
F8	Hex: EtAOC	3 : 7	0.0049
F9	Hex: EtAOC	2 : 8	0.0623
F10	Hex: EtAOC	1 : 9	0.0037
F11	Hex: EtAOC	0 : 10	0.0053
F12	DCM:/METH	10 : 0	0.0015
F13	DCM:/METH	8 : 2	0.1140
F14	DCM:/METH	6 : 4	0.0908
F15	DCM:/METH	5 : 5	0.0463
F16	DCM:/METH	4 : 6	0.0101
F17	DCM:/METH	0 : 10	0.0149

Table 4.3: Yield of Crude Extract and VLC Fractions of *Endomelanconiopis sp*.

EXTRACT/FRACTION	SOLVENT RATIO		YIELD (g)
Crude			2.5297
F1	Hex: EtAOC	10 : 0	0.0020
F2	Hex: EtAOC	9 : 1	0.0577
F3	Hex: EtAOC	8 : 2	0.0479
F4	Hex: EtAOC	7 : 3	0.0169
F5	Hex: EtAOC	6 : 4	0.1351
F6	Hex: EtAOC	5 : 5	0.0351
F7	Hex: EtAOC	4 : 6	0.0987
F8	Hex: EtAOC	3 : 7	0.0162
F9	Hex: EtAOC	2 : 8	0.0111
F10	Hex: EtAOC	1 : 9	0.0058
F11	Hex: EtAOC	0 : 10	0.0063
F12	DCM:/METH	10 : 0	0.0019
F13	DCM:/METH	8 : 2	0.1655
F14	DCM:/METH	6 : 4	0.0672
F15	DCM:/METH	5 : 5	0.4356
F16	DCM:/METH	4 : 6	0.0104
F17	DCM:/METH	0 : 10	0.0163

4.1.2 BIOASSAYS OF THE CRUDE EXTRACTS OF *CURVULARIA SP.* AND *ENDOMELANCONIOPSIS SP.*

4.1.2.1 Antimicrobial Assay

The antimicrobial activity of the fungal crude extracts and fractions was determined using the agar well diffusion method.

Results of Antimicrobial Assay of the crude extracts and VLC Fractions of *Curvularia sp* and *Endomelanconiopsis sp* crude extract are presented in Tables 4.4 - 4.7.

4.1.2.2 Cytotoxicity Assay

The cytotoxicity assay was carried out at a concentration of 10 µg/mL, for both *Curvularia sp.* and *Endomelanconiopsis sp.* crude extracts, against mouse lymphoma cell lines (L56178Y).

The results of the cytotoxicity assay are presented in Table 4.8. From the result, it can be seen that *Curvularia sp.* and *Endomelanconiopsis sp.* crude extracts showed no cytotoxic activity at 10 µg/mL.

4.1.2.3 Anti-tubercular Assay

The results of the anti-tubercular assay showed that at a concentration of 10µg/mL, extracts from both *Curvularia sp.* and *Endomelanconiopsis sp.* showed no anti-tubercular activities as shown in Table 4.9.

Table 4.4: Result of Antimicrobial Assay of *Curvularia sp* Crude Extract showing the Mean ±SEM Inhibition Zone Diameters (IZDs) (mm) produced against test organisms

Test Organisms	Mean±SEM IZD (mm) of *Curvularia sp* Crude Extract		Positive control	Negative control
	10 mg/mL	1 mg/mL	Ciprofloxacin (5 µg/mL)	DMSO
E. coli	11±0.58	0±0.00	7±0.33	0±0.00
S.aureus	10±0.00	0±0.00	6±0.00	0±0.00
S. typhi	4±0.00	0±0.00	6±0.33	0±0.00
B. subtilis	20±0.88	0±0.00	8±0.00	0±0.00
			Miconazole (50 µg/mL)	DMSO
A. niger	6±0.33	0±0.00	12±0.33	0±0.00
C. albicans	10±0.33	0±0.00	17±0.33	0±0.00

SEM: Standard Error of Mean

Table 4.5: Result of Antimicrobial Assay of VLC fractions of *Curvularia sp* showing the Mean Inhibition Zone Diameters (IZDs) (mm) produced against test organisms

Test Organisms	IZD (mm) of VLC fractions of *Curvularia sp* Extract (1 mg/mL)																Positive control	Negative control
	F1	F2	F4	F5	F6	F7	F8	F9	F10	F11	F12	F13	F14	F15	F16	F17	Ciprofloxacin (5 µg/mL)	DMSO
E. coli	0±0.00	0±0.00	0±0.00	0±0.00	0±0.00	0±0.00	0±0.00	0±0.00	0±0.00	0±0.00	0±0.00	0±0.00	0±0.00	0±0.00	0±0.00	0±0.00	7±0.33	0±0.00
S. aureus	0±0.00	0±0.00	0±0.00	0±0.00	0±0.00	0±0.00	0±0.00	0±0.00	0±0.00	0±0.00	0±0.00	0±0.00	0±0.00	0±0.00	0±0.00	0±0.00	6±0.00	0±0.00
P. aeruginosa	0±0.00	0±0.00	0±0.00	0±0.00	0±0.00	0±0.00	0±0.00	0±0.00	0±0.00	0±0.00	0±0.00	0±0.00	0±0.00	0±0.00	0±0.00	0±0.00	6±0.33	0±0.00
B. subtilis	0±0.00	0±0.00	0±0.00	0±0.00	0±0.00	0±0.00	0±0.00	0±0.00	0±0.00	0±0.00	0±0.00	0±0.00	0±0.00	0±0.00	0±0.00	0±0.00	8±0.00	0±0.00
																	Miconazole (50 µg/mL)	**DMSO**
A. niger	0±0.00	0±0.00	0±0.00	0±0.00	0±0.00	0±0.00	0±0.00	0±0.00	0±0.00	0±0.00	0±0.00	0±0.00	0±0.00	0±0.00	0±0.00	0±0.00	12±0.33	0±0.00
C. albicans	0±0.00	0±0.00	0±0.00	0±0.00	0±0.00	0±0.00	0±0.00	0±0.00	0±0.00	0±0.00	0±0.00	0±0.00	0±0.00	0±0.00	0±0.00	0±0.00	17±0.33	0±0.00

Table 4.6: Result of Antimicrobial Assay of *Endomelanconiopsis sp* Crude Extract showing the Mean±SEM Inhibition Zone Diameters (IZDs) (mm) produced against test organisms

Test Organisms	Mean±SEM IZD (mm) of *Endomelanconiopsis sp* Crude Extract		Positive control	Negative control
	10 mg/mL	1 mg/mL	Ciprofloxacin (5 µg/mL)	DMSO
E. coli	7±0.33	0±0.00	7±0.33	0±0.00
S.aureus	11±0.33	0±0.00	6±0.00	0±0.00
P.aeruginosa	8±0.00	0±0.00	6±0.33	0±0.00
B. subtilis	10±0.33	0±0.00	8±0.00	0±0.00
			Miconazole (50 µg/mL)	**DMSO**
A. niger	15±0.88	0±0.00	12±0.33	0±0.00
C. albicans	11±0.58	0±0.00	17±0.33	0±0.00

Table 4.7: Result of Antimicrobial Assay of VLC fractions of *Endomelanconiopsis sp* showing the Mean Inhibition Zone Diameters (IZDs) (mm) produced against test organisms

Test Organisms	IZD (mm) of VLC fractions of *Endomelanconiopsis sp* Extract (1 mg/mL)														Positive control	Negative control
	F1	F4	F5	F6	F7	F8	F9	F10	F11	F12	F14	F15	F16	F17	Ciprofloxacin (5 µg/mL)	DMSO
E. coli	0±0.00	0±0.00	0±0.00	0±0.00	0±0.00	0±0.00	0±0.00	0±0.00	0±0.00	0±0.00	0±0.00	2±0.00	0±0.00	0±0.00	7±0.33	0±0.00
S. aureus	0±0.00	0±0.00	0±0.00	0±0.00	0±0.00	0±0.00	0±0.00	0±0.00	0±0.00	0±0.00	0±0.00	0±0.00	0±0.00	0±0.00	6±0.00	0±0.00
P. aeruginosa	0±0.00	0±0.00	0±0.00	0±0.00	0±0.00	0±0.00	0±0.00	0±0.00	0±0.00	0±0.00	0±0.00	0±0.00	0±0.00	0±0.00	6±0.33	0±0.00
B. subtilis	0±0.00	0±0.00	0±0.00	0±0.00	0±0.00	0±0.00	0±0.00	0±0.00	0±0.00	0±0.00	0±0.00	2±0.00	0±0.00	0±0.00	8±0.00	0±0.00
															Miconazole (50 µg/mL)	**DMSO**
A. niger	0±0.00	0±0.00	0±0.00	0±0.00	0±0.00	0±0.00	0±0.00	0±0.00	0±0.00	0±0.00	0±0.00	0±0.00	0±0.00	0±0.00	12±0.33	0±0.00
C. albicans	0±0.00	0±0.00	0±0.00	0±0.00	0±0.00	0±0.00	0±0.00	0±0.00	0±0.00	0±0.00	0±0.00	0±0.00	0±0.00	0±0.00	17±0.33	0±0.00

Table 4.8: Result of CytotoxicAssay of the crude extracts of the endophytes

Fungal Extract	Concentration (µg/mL)	% inhibition
Curvularia sp	10	-0.7
Endomelanconiopsis sp	10	4.4

Table 4.9: Result of Anti-tubercular Assay of the crude extracts of the endophytes

Fungal Extract	Concentration (µg/mL)	*M. tuberculosis*
Curvularia sp	10	-
Endomelanconiopsis sp	10	-

4.1.2.4 Antioxidant Assay

Antioxidant assay was determined using the DPPH free radical scavenging assay. At a concentration of 500 µg/mL, the antioxidant activity of the fungal crude extracts was determined. Fractions of active fungal extracts were also evaluated.

Results of DPPH antioxidant assay of crude extracts and VLC fractions of *Curvularia sp.* and *Endomelanconiopsis sp.* are presented in Figure 4.4 and 4.5 respectively.

4.1.3 *CURVULARIA SP* BIOACTIVE METABOLITES

The detection and isolation of bioactive secondary metabolites in the *Curvularia sp.* crude extract followed the steps outlined in Figure 4.6 below.

Three known fungal metabolites were isolated from the fungus namely; acropyrone (**1**), 4-hydroxyphenylacetic acid (**2**) and indole-3-acetic acid (**3**).

They were isolated from the VLC fraction (DCM:MeOH 80:20) by semiprep-HPLC.

The HPLC chromatograms, UV spectra, LC-MS, NMR data and structures of the compounds isolated from *Curvularia sp.* are shown in Figures 4.7 – 4.18.

The reported bioactivities of the compounds are presented in Table 4.14.

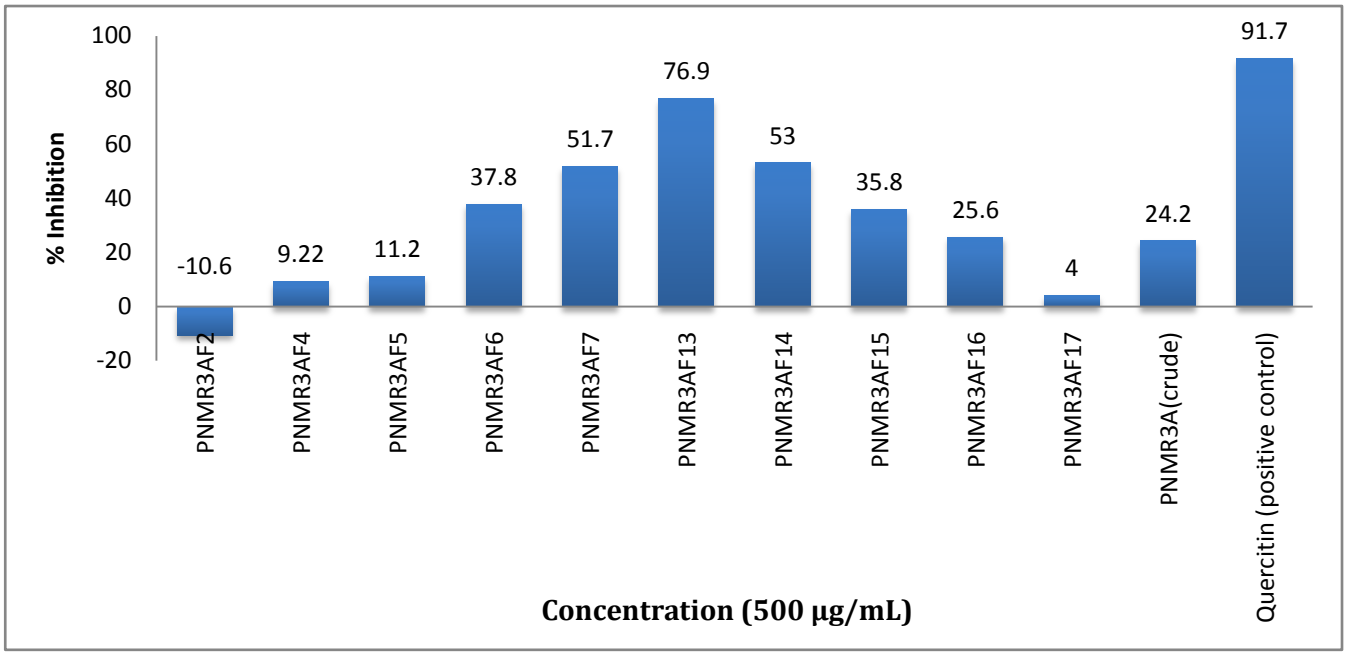


Figure 4.4: Results of DPPH Antioxidant Assay of crude extracts and VLC fractions of *Curvularia sp.* (PNMR3A)

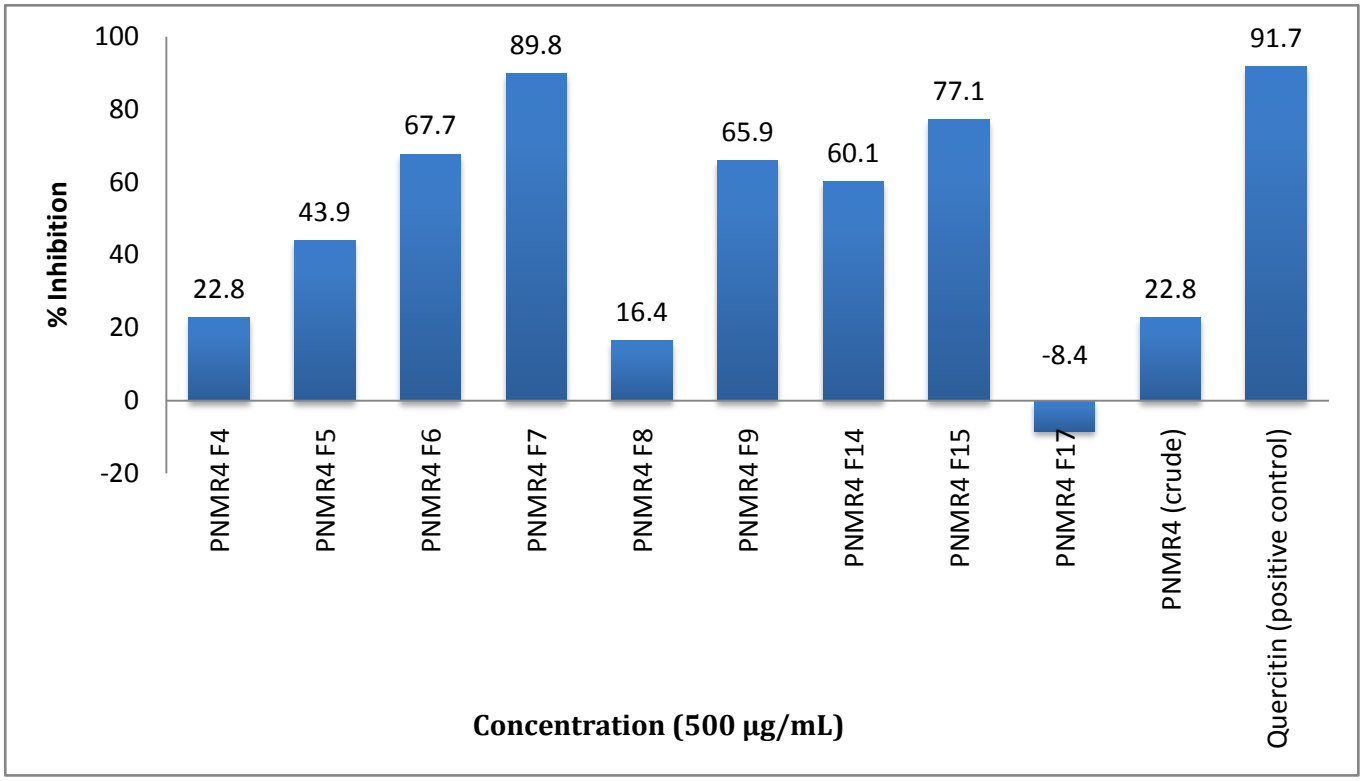


Figure 4.5: Results of DPPH Antioxidant Assay of crude extracts and VLC fractions of *Endomelanconiopsis sp* (PNMR4).

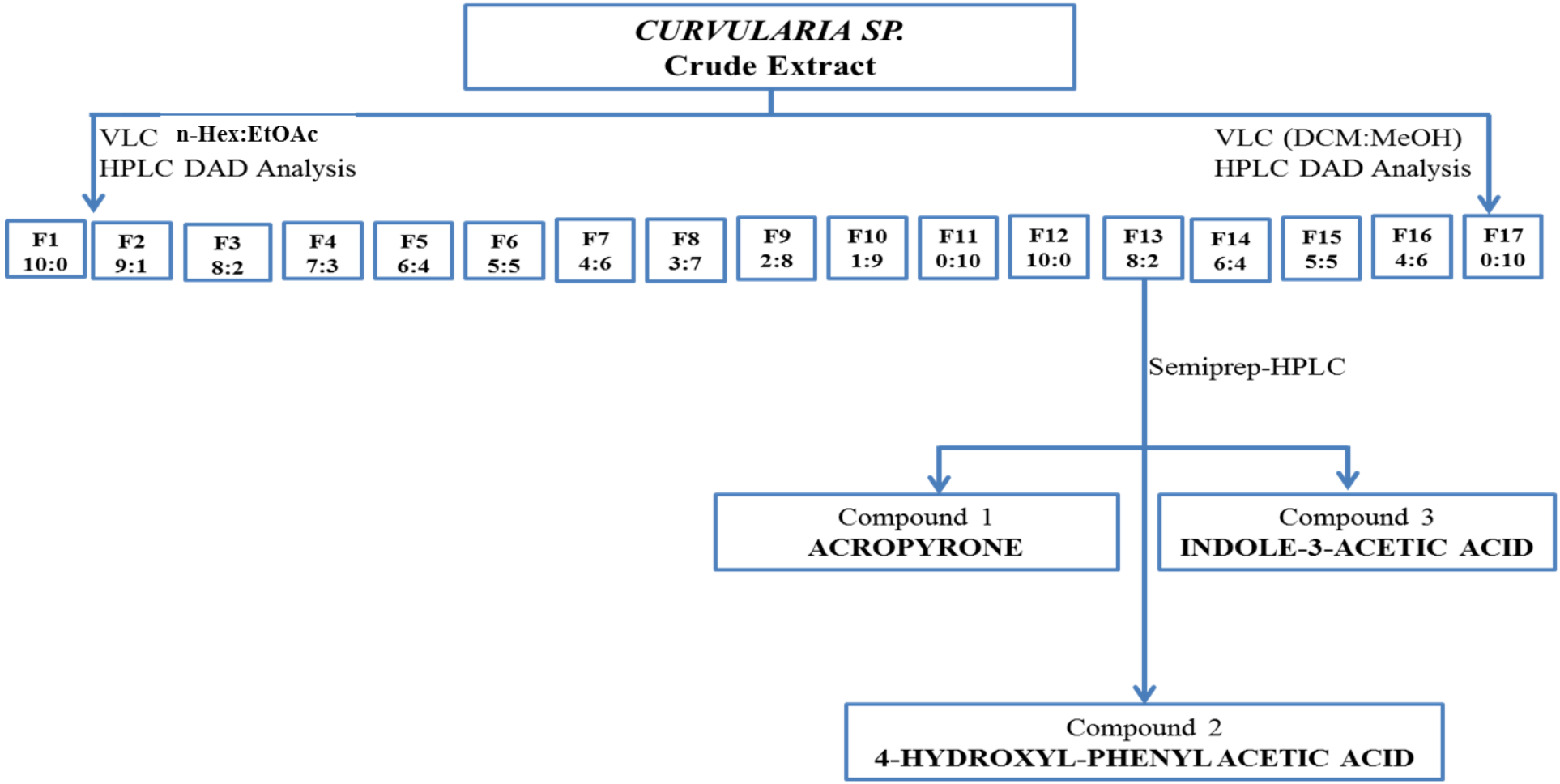


Figure 4.6: Detection and Identification of Compounds from *Curvularia sp.* Crude Extract

COMPOUND 1 (ACROPYRONE)

ACROPYRONE	
SYNONYM(S)	2-(4-methoxy-5-methyl-6-oxopyran-2-yl) but-2-enoic acid
SAMPLE CODE	PN 3-13A-1 (Or PN3-13B-1 or PN3-13C-3)
BIOLOGICAL SOURCE	*Curvularia sp*. (from *Picralima nitida*)
ISOLATED QUANTITY	11.8mg
APPEARANCE	Yellowish Powder
MOLECULAR FORMULA	$C_{11}H_{12}O_5$
MOLECULAR WEIGHT	224 g/mol
ABSORPTION MAXIMUM	UV λmax (Methanol): 237.1 and 341 nm
RETENTION TIME (HPLC)	24.12 min.

Compound **1** was isolated from the VLC fraction (DCM: MeOH 80:20) of ethylacetate crude extract of *Curvularia sp*. as a yellow powder. It exhibited UV maxima at 237.1 nm and 341 nm. Its molecular weight was determined as 224 g/mol based on the ESI-MS quasi peak at m/z 225.1 $(M+H)^+$ upon positive ionization and 223.1 $(M–H)^-$ upon negative ionization. These suggested a molecular formular of $C_{11}H_{12}O_5$.

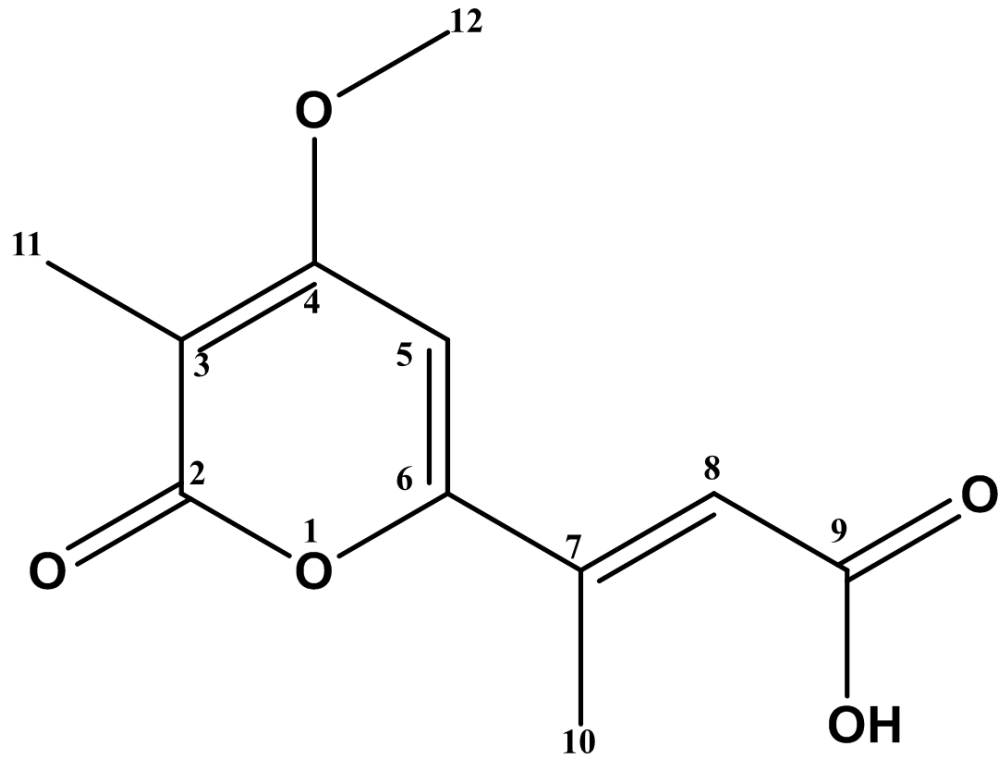
The UV maxima observed is typical of an alpha pyrone which suggest the presence of pyrone as the basic structure.

A look at ^{1}H-NMR showed that there might be three CH_3 groups. One singlet was observed at δ4.04 (H-12) due to the methoxy group; a doublet at δ 2.43 (H-11), which is a methyl group; and a singlet appeared at δ1.95 (H-10), a methyl group. Protons 5 and 8 resonated downfield at δ6.85s (H-5) and δ6.70s (H-8) due in principle to the olefinic nature. The desheilding is due to the anisotropic effect of the olefinic system.

The presence of the carboxyl unit was supported by the fragments 179.0 $[M\text{-}COO^-]^-$ in the negative mode which corresponds to the loss of ion of carboxylate.

Analysis of the MS and NMR data in comparism with those reported in the literature (Lena *et al.*, 2014) led to the elucidation of compound **1** as acropyrone.

ACROPYRONE



Chemical formula: $C_{11}H_{12}O_5$
Molecular mass: 224.21 g·mol^{-1}
UV-max: 237.1, 341 nm

Figure 4.7: The chemical structure of Acropyrone.

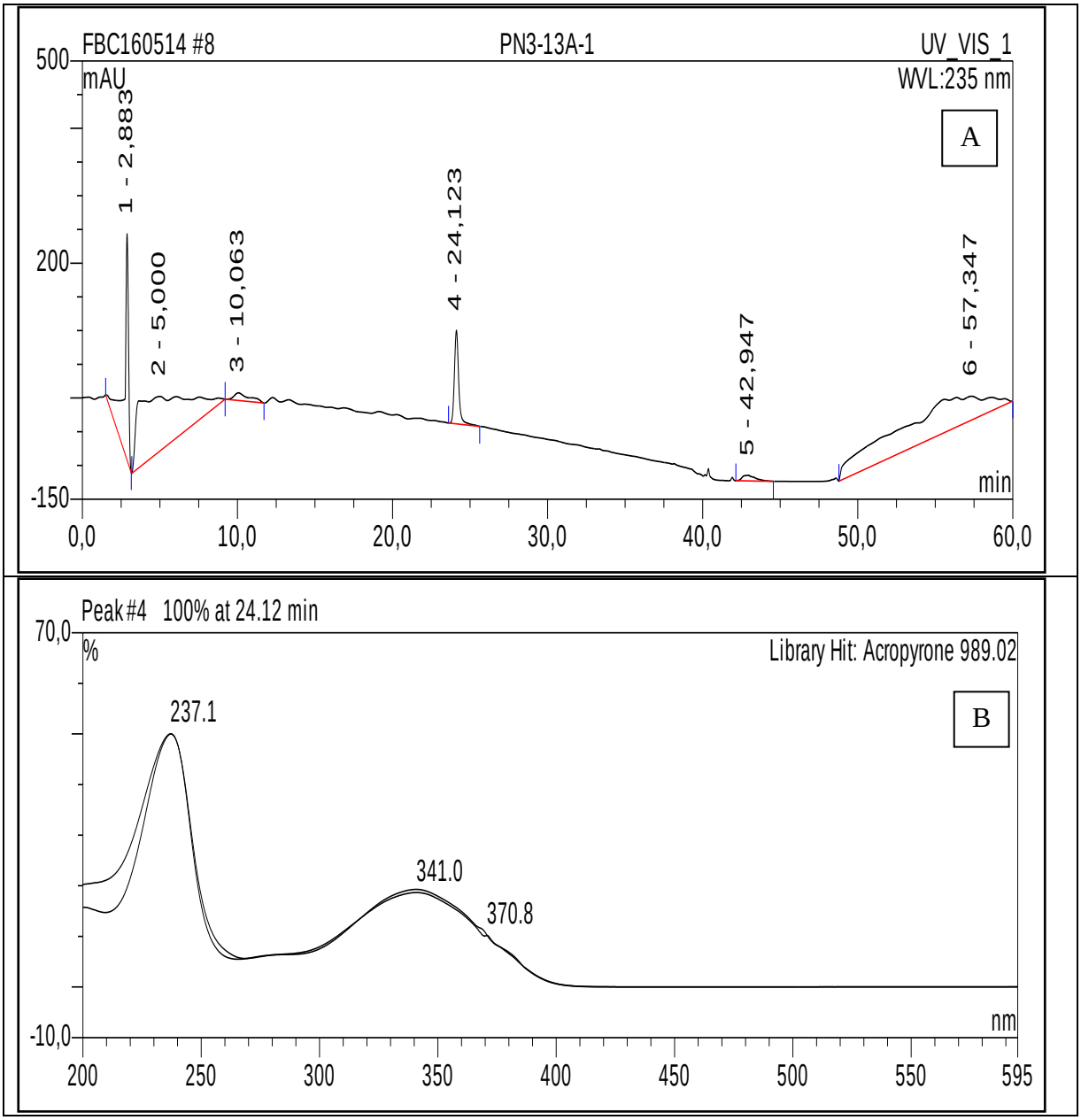


Figure 4.8: Acropyrone: HPLC Chromatogram (A), UV spectrum (B)

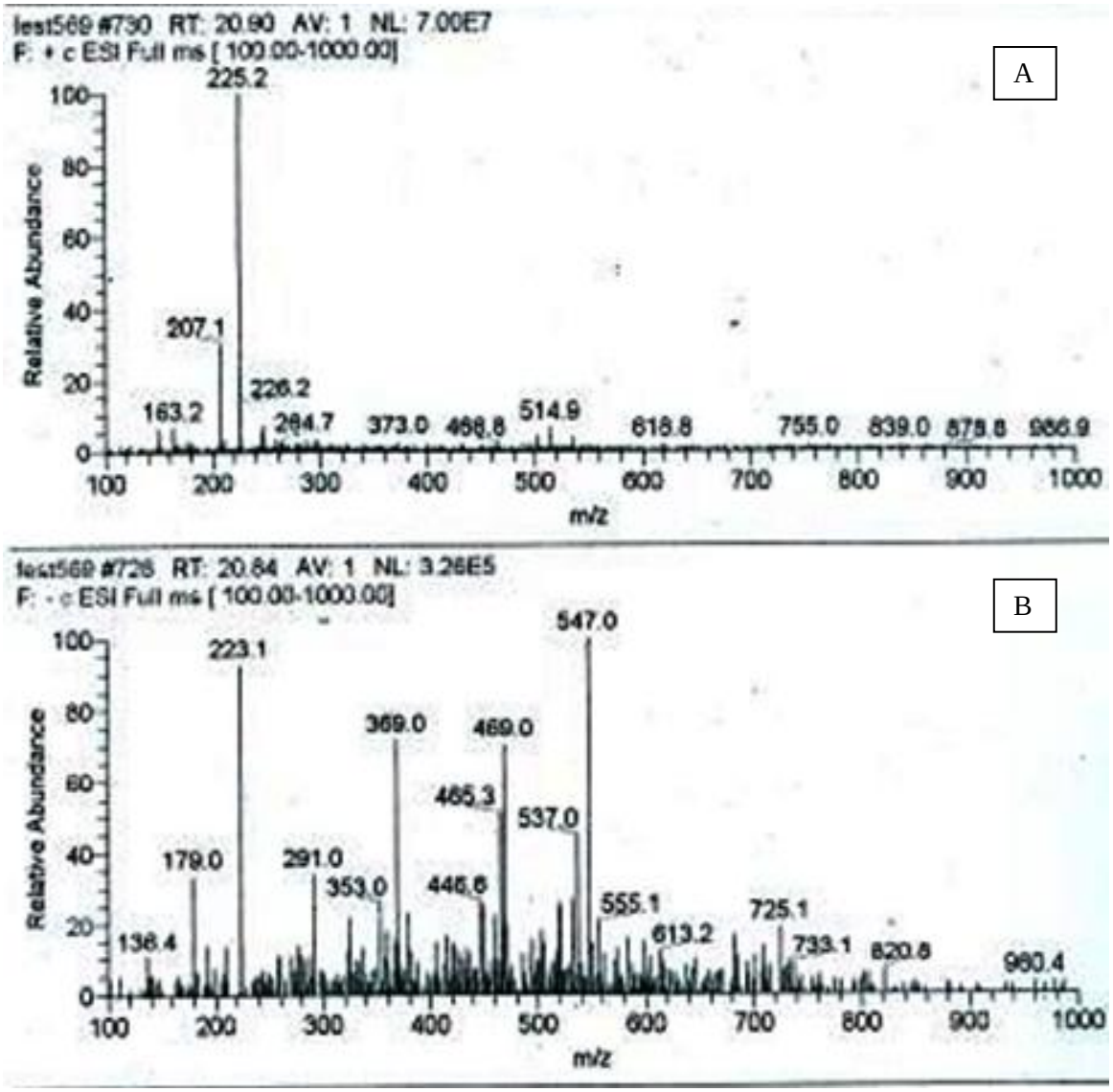


Figure 4.9: Acropyrone: LC-MS data showing the molecular mass [M+H] (A) and [M-H] (B)

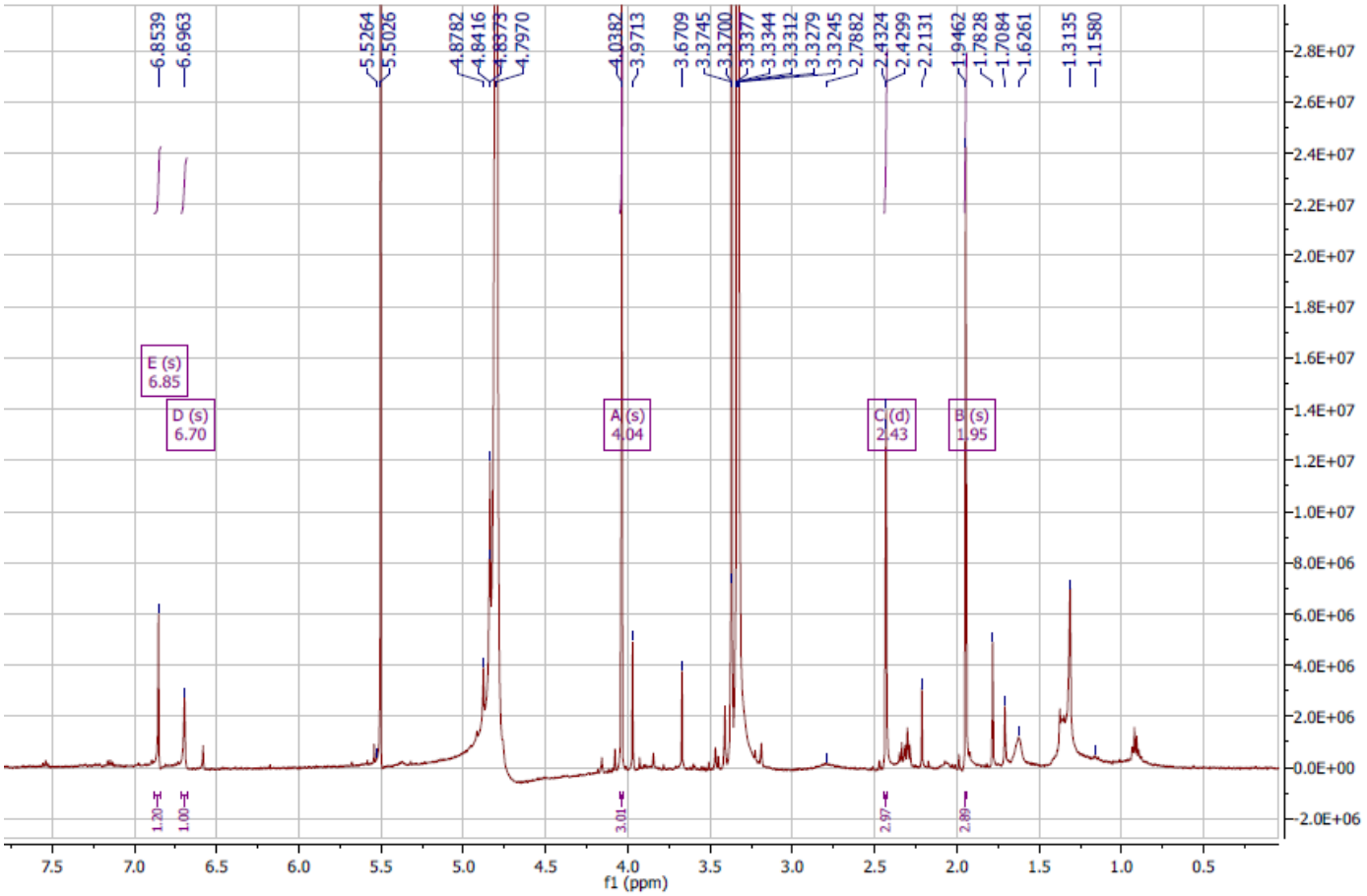


Figure 4.10: ^{1}H-NMR spectrum of Acropyrone: ^{1}H NMR (500 MHz,) δ 6.85 (s, 1H), 6.70 (s, 1H), 4.04 (s, 3H), 2.43 (d, J = 1.2 Hz, 3H), 1.95 (s, 3H).

TABLE 4.10: Comparison of the ^{1}H NMR data of Acropyrone

Carbon No.	δH^{a} (J in Hz)	δH^{b} (J in Hz)
1	-	-
2	-	-
3	-	-
4	-	-
5	6.90 (s)	6.85 (s)
6	-	-
7	-	-
8	6.50 (d, J =1.3)	6.70 (s, J=1.20)
9	-	-
10	1.83 (s)	1.95 (s, J=2.89)
11	2.36 (d, J = 1.3)	2.43 (d, J=2.97)
12	3.98 (s)	4.04 (s)

a. Lema *et al*. (2014) and Hammerschmidt *et al*. (2014) at 600 MHz in $CDCl_3$ (Reference Compound)
b. Derived from ^{1}H spectrum at 500 MHz in MeOD from this study (Isolated Compound)

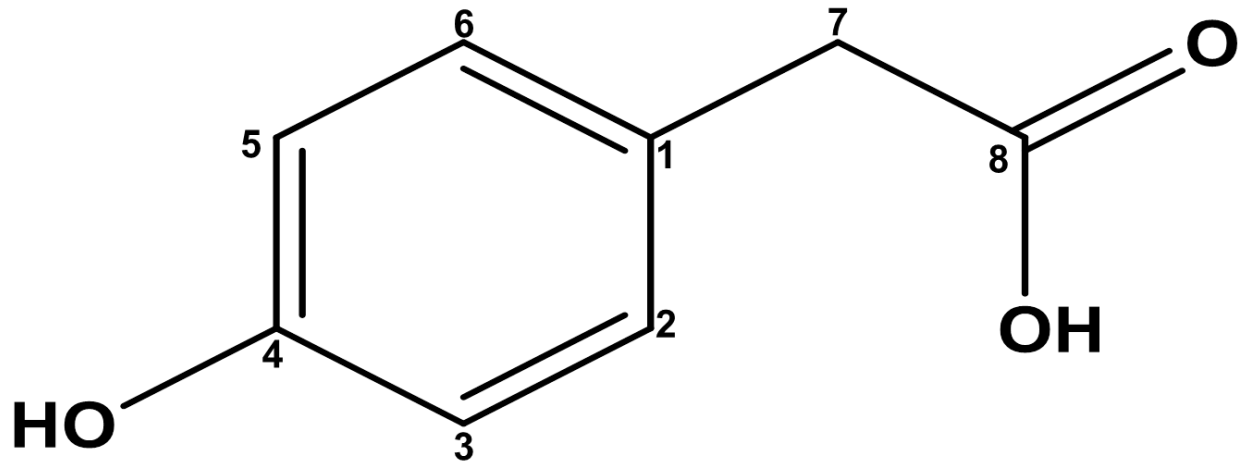
COMPOUND 2 (4-HYDROXYPHENYLACETIC ACID)

4-HYDROXYPHENYL ACETIC ACID	
SYNONYM(S)	*p*-Hydroxyphenylacetic acid; 2-(4-Hydroxyphenyl) acetic acid 2-(4-Hydroxyphenyl) ethanoic acid.
SAMPLE CODE	PN 3-13C-1
BIOLOGICAL SOURCE	*Curvularia sp*. (from *Picralima nitida*)
ISOLATED QUANTITY	8.5 mg
APPEARANCE	Beige Powder
MOLECULAR FORMULA	$C_8H_8O_3$
MOLECULAR WEIGHT	152 g/mol
ABSORPTION MAXIMUM	UV λmax (Methanol): 221 .9 and 275.9nm
RETENTION TIME (HPLC)	13.9 min.

Compound **2** (4- Hydroxyphenylacetic acid) was isolated from the VLC fraction (DCM:MeOH 80:20) of ethylacetate crude extract of *Curvularia sp*. as a beige powder. It exhibited UV maxima at 221.9 and 275.9 nm. This is typical of a simple phenolic compound. Its molecular weight was determined as 152 g/mol based on the molecular ion peak 152 upon positive ionization.The fragment peak at 107 suggest the lost of carboxylic group. This therefore suggests a formular of $C_8H_8O_3$.

A look at the ^{1}H-NMR showed that, there are two pairs of meta-coupled protons at δ 7.11 (m) (H-2 and H-6), 2/6 and δ 6.75 (m) (H-3 and H-5), 3/5. The H-NMR also showed deshielded methylene signal at δ3.50 (s, 2H) (H-7) which is the CH_2 group of the acetic acid. These data appears to correspond with the literature data for p-hydroxyphenylacetic acid (Willeke and Barz, 1982; Abba, 2016). Analysis of the MS and NMR data in comparison with literature (Willeke and Barz, 1982; Abba, 2016) led to the elucidation of compound 2 as 4-hydroxyphenylacetic acid.

4-HYDROXYPHENYL ACETIC ACID



Chemical formula: $C_8H_8O_3$
Molecular mass: 152.15 g·mol^{-1}
UV-max: 221.9, 275.9 nm

Figure 4.11: The chemical structure of 4-Hydroxyphenyl acetic acid.

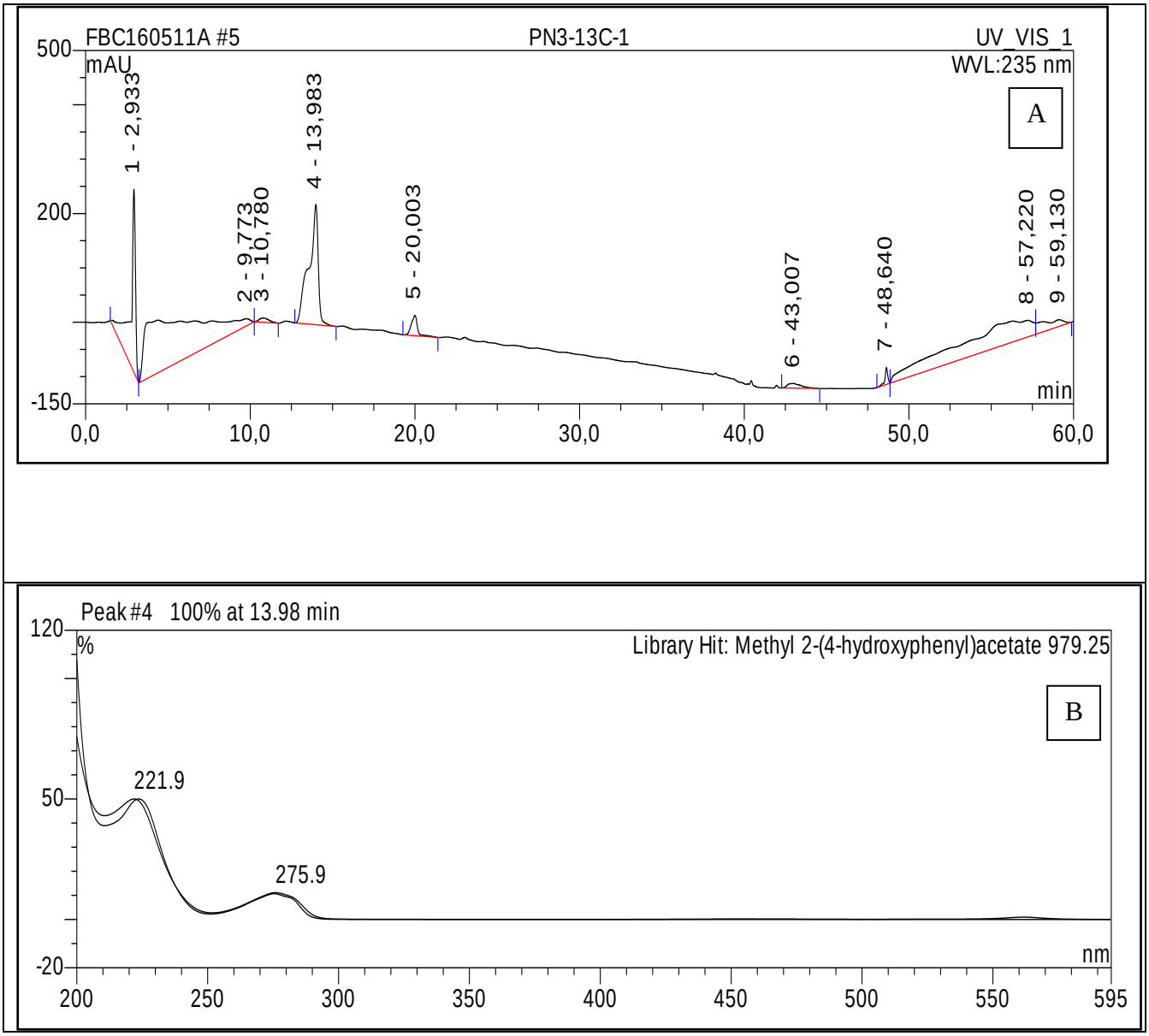


Figure 4.12: 4-Hydroxyphenylacetic acid: HPLC Chromatogram (A), UV spectrum (B)

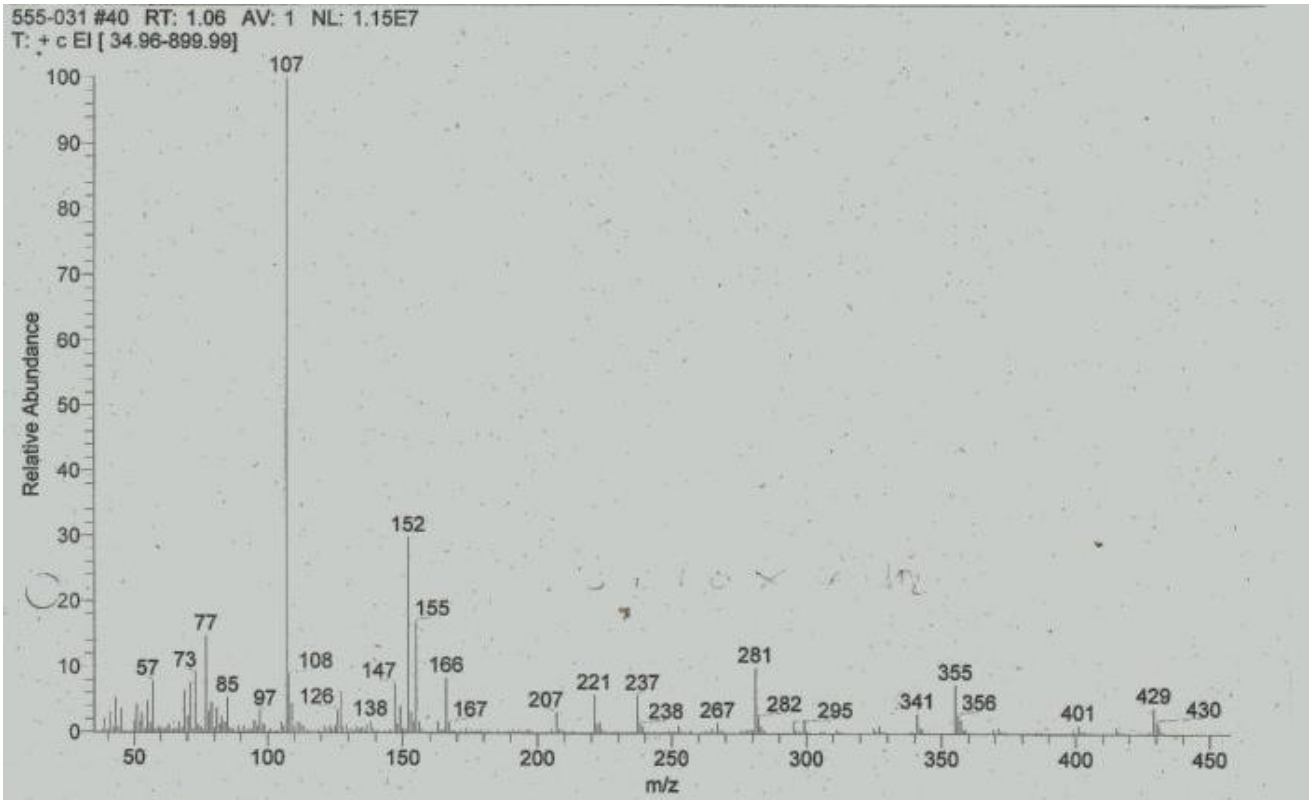


Figure 4.13: 4-Hydroxyphenylacetic acid: MS data showing the molecular mass 152 g/mol

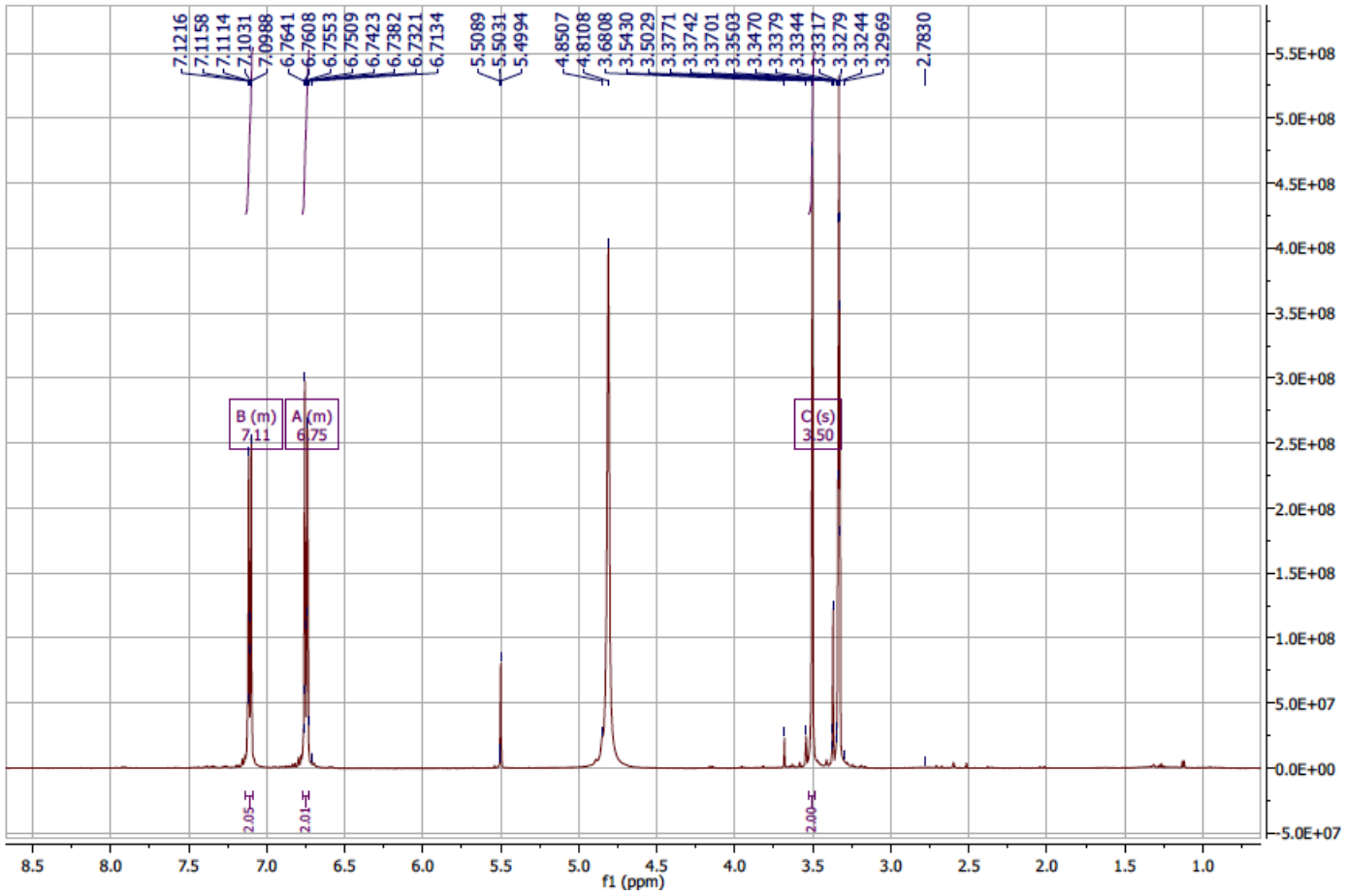


Figure 4.14: ^{1}H-NMR spectrum of 4-Hydroxyphenylacetic acid: ^{1}H NMR (500 MHz,) δ 7.13 – 7.09 (m, 2H), 6.77 – 6.73 (m, 2H), 3.50 (s, 2H).

Table 4.11: Comparison of the ^{1}H-NMR Data of 4-Hydroxyphenylacetic acid

Carbon No.	δH^{a} (J in Hz)	δH^{b} (J in Hz)
1	-	-
2	7.09(m)	7.11(m)
3	6.72 (m)	6.75(m)
4	-	
5	6.72(m)	6.75(m)
6	7.09 (m)	7.11(m)
7	3.47 (m)	3.50(s)
8	-	-

a. Abba (2016) at 300 MHz in MeOD (Reference Compound).

b. Derived from ^{1}H NMR at 500 MHz in MeOD from this study (Isolated Compound).

COMPOUND 3 (INDOLE-3-ACETIC ACID)

INDOLE-3- ACETIC ACID	
SYNONYM(S)	3-Indoleacetic acid; 2-(1H-indole-3-yl) acetic acid; 2-(1H-indole-3-yl) ethanoic acid
SAMPLE CODE	PN 3-13C-2
BIOLOGICAL SOURCE	*Curvularia sp*. (from *Picralima nitida*)
ISOLATED QUANTITY	9.6 mg
APPEARANCE	Light brown
MOLECULAR FORMULA	$C_{10}H_9NO_2$
MOLECULAR WEIGHT	175 g/mol
ABSORPTION MAXIMUM	λ max (Methanol): 217.6 and 279.1 nm
RETENTION TIME	17.5 min.

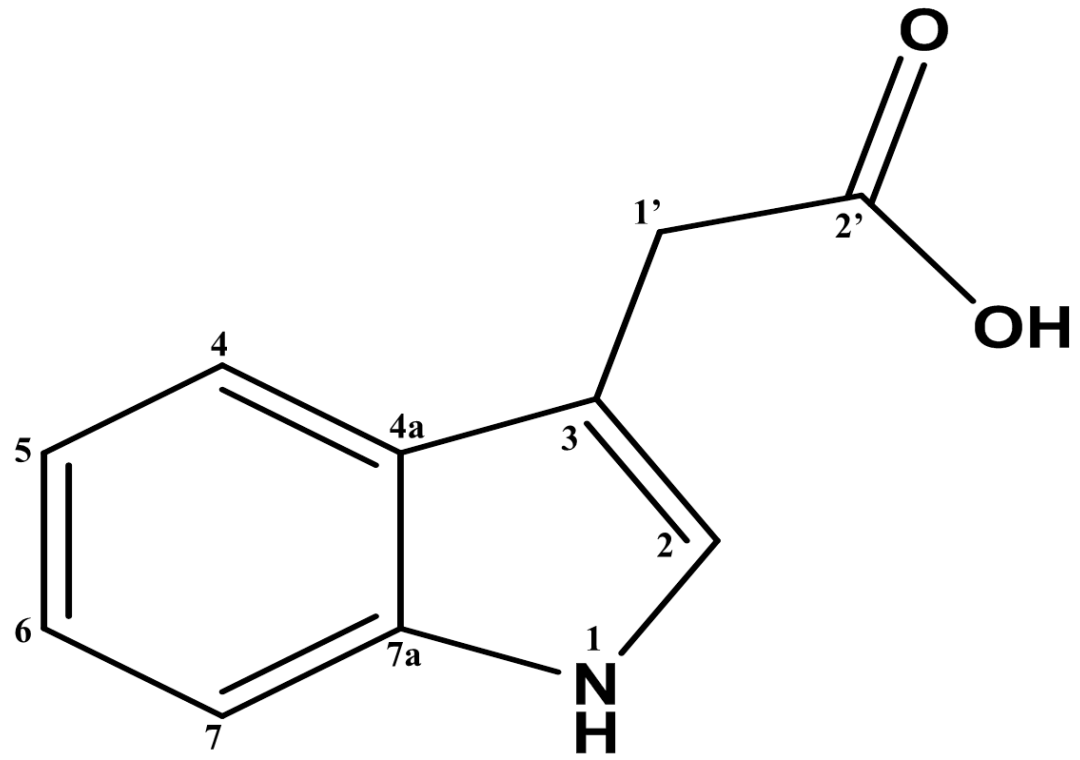
Compound 3 was isolated as a colourless solid from the VLC fraction (DCM:MeOH 80:20) of ethyl acetate crude extract of *Curvularia sp*.

The UV spectrum showed absorption maximum at 217.6 nm and 279.1 nm. This suggested the presence of indole as the basic structure.

The ESI-MS spectrum gave the base peak M/Z = 130.2 indicating the molecular fragment which has lost a carboxylic group $[M\text{-}COOH]^+$. This suggested likely odd number molecular weight given therefore as 175mol/g. This implied the presence of Nitrogen atom in the compound.

The H-NMR spectrum shows four aromatic proton signals at δ 7.58 (d, J=1.03, 1H), 7.05 (m, J=0.89, 1H), 7.15 (m, J=0.99, 1H), 7.38 (d, J=1.01, 1H). The observed multiplicity pattern is typical of ortho-disubstituted benzene ring and the signals are assigned to H-4, H-6, H-5 and H-7 respectively. The deshielded proton signal at δ7.2 (s) was assigned to H-2. The deshielding position was being contributed by both the inductive effect of the nitrogen atom and the anisotropic effect of the olefenic system. The methylene proton signal observed at δ 3.69 (s) was assign to H-1^1A/B. Compound 3 was elucidated as the previously reported indole-3-acetic acid and the data corresponds to that reported in the literature (Von and Zhou, 2012).

INDOLE-3-ACETIC ACID



Chemical formula: $C_{10}H_9NO_2$
Molecular mass: 175.19 $g \cdot mol^{-1}$
UV-max: 217.6, 279.1 nm

Figure 4.15: The chemical structure of Indole-3-acetic acid.

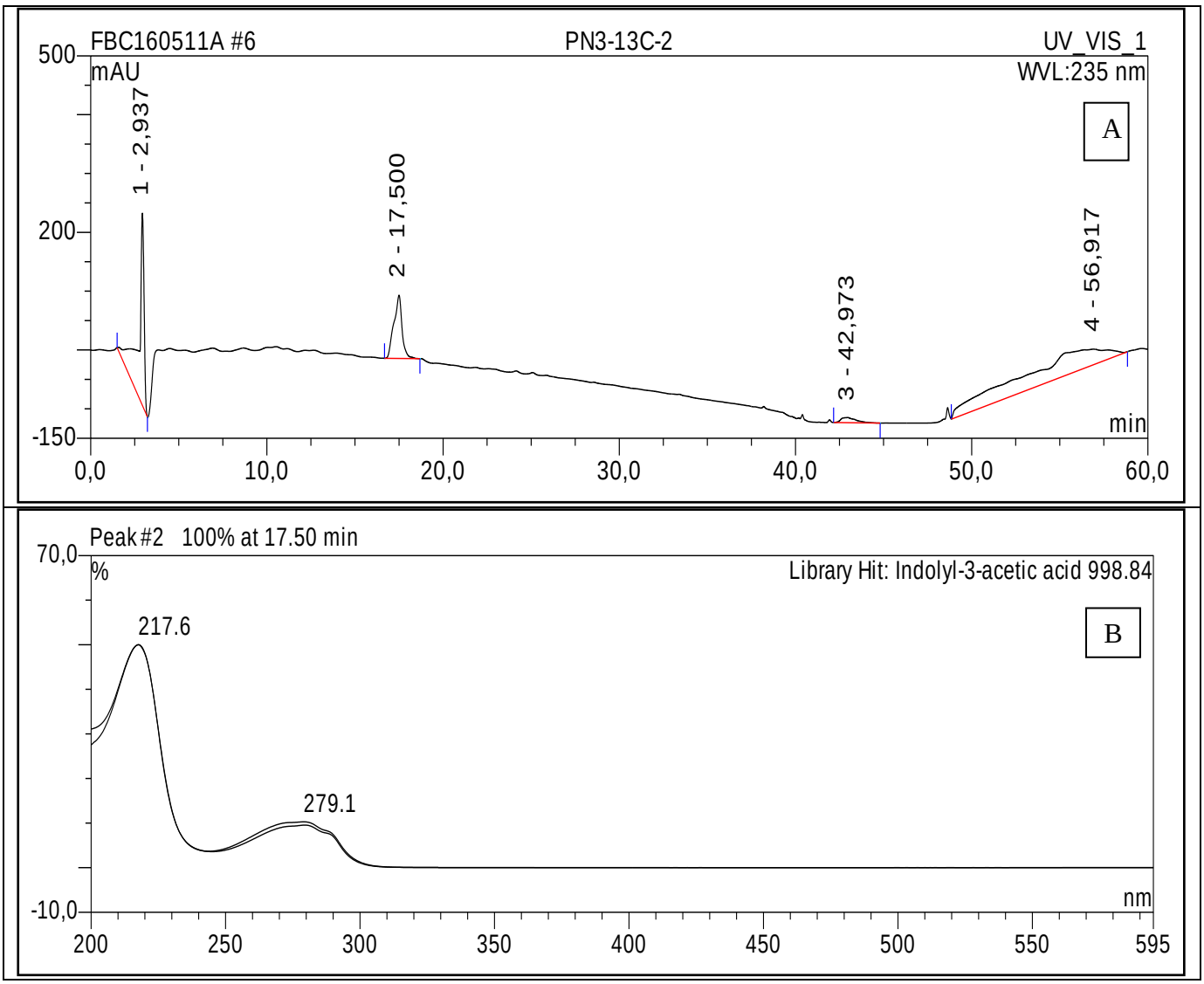


Figure 4.16: Indole-3-acetic acid: HPLC Chromatogram (A), UV spectrum (B)

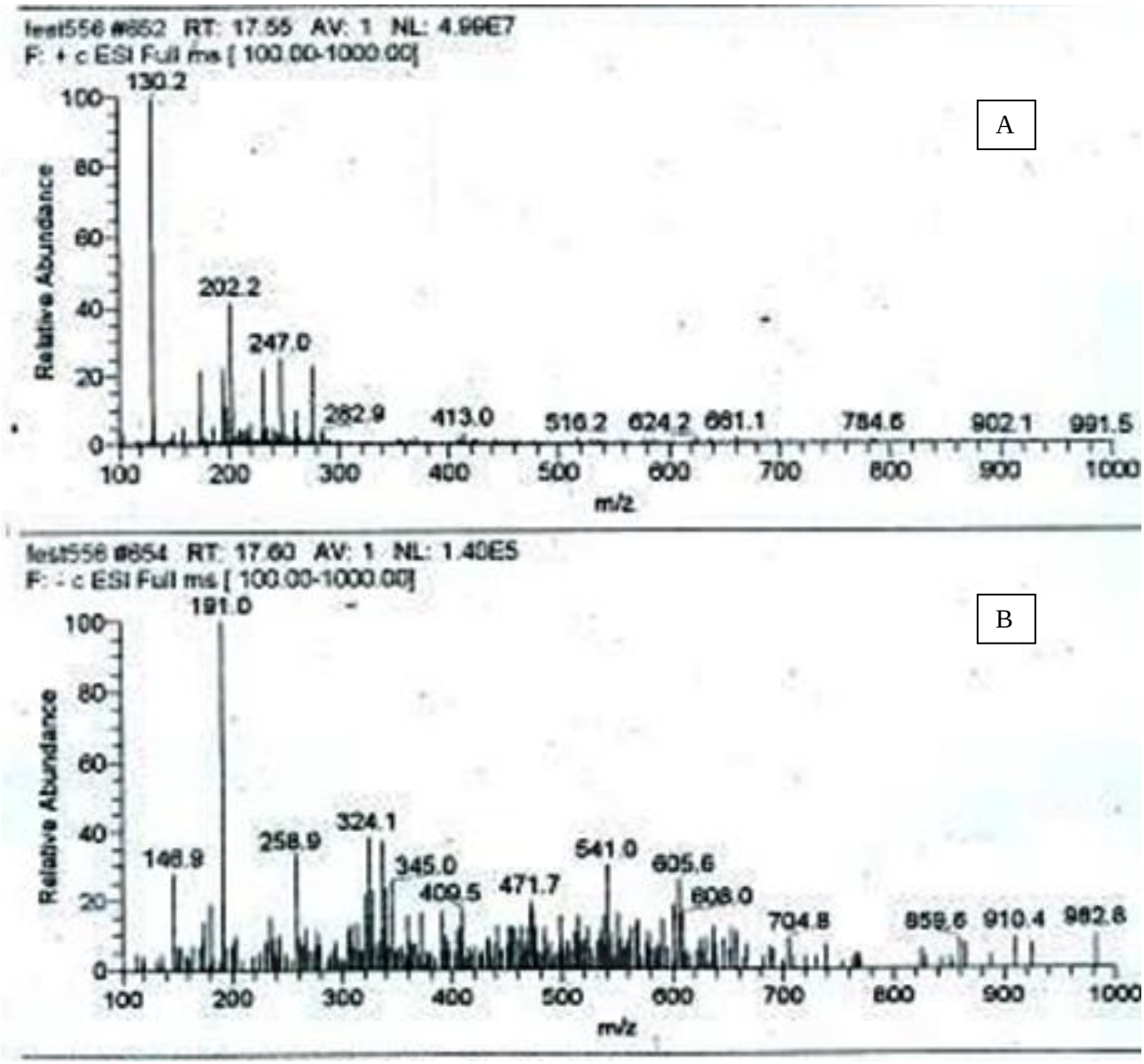


Figure 4.17: Indole -3- acetic acid: LC-MS data showing the positive ionisation (A) and negative ionisation (B)

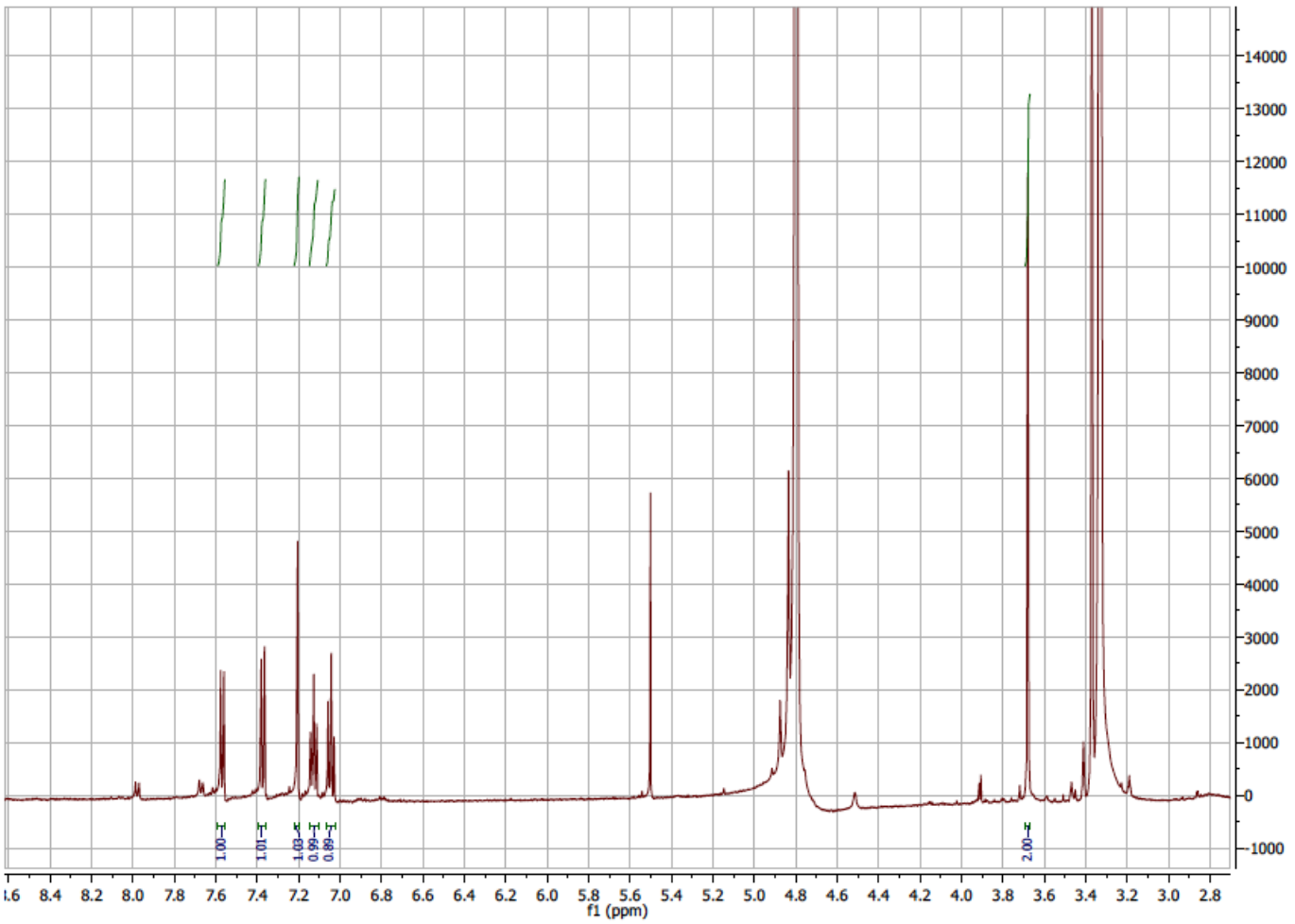


Figure 4.18: ^{1}H-NMR spectrum of Indole-3-acetic acid: ^{1}H NMR at 500 MHz. δ 7.2 (s, 1H), δ 7.58 (d, 1H), δ 7.17 (m, 1H), δ 7.05 (m, 1H), δ 7.38 (d, 1H), δ 3.69 (s, 2H)

Table 4.12: Comparison of ^{1}H-NMR Data of Indole-3-Acetic Acid

Carbon No.	δH^{a} (J in Hz)	δH^{b} (J in Hz)
1	-	-
2	7.12(s)	7.2 (s)
3	-	-
4	7.54(d)	7.58 (d)
5	7.00(ddd)	7.15 (m)
6	7.07 (ddd)	7.05 (m)
7	7.34 (d)	7.38 (d)
8	3.69(s)	3.69 (s)
9.	-	-
10	-	-

a. Von and Zhou (2012) at 300 MHz in $CDCl_3$ (Reference Compound).

b. Derived from ^{1}H spectrum at 500 MHz in MeOD from this study (Isolated Compound).

4.1.4 *ENDOMELANCONIOPSIS SP.* BIOACTIVE METABOLITES

The identification and isolation of bioactive secondary metabolites from *Endomelanconiopsis sp.* extract followed the steps shown in Figure 4.19 below.

Four known fungal metabolites orthosporin (**4**), tenuazonic acid (**5**), methyl 4-methoxybenzoate (**6**) and methyl 2-hydroxy-4-methylbenzoate (**7**) were isolated from the fungus.

Compound 4 was isolated from VLC fraction (n-Hex:EtOAc 40:60) by semiprep-HPLC. Compound 5 was isolated from the VLC fraction (DCM:MeOH 50:50) by semiprep-HPLC. Compounds 6 and 7 were isolated from the VLC fraction (n-Hex:EtOAc 60:40) by sephadex and then semiprep-HPLC.

The HPLC chromatograms, UV spectra, LC-MS, NMR data and structures of the compounds isolated from *Endomelanconiopsis sp* are shown in Figures 4.20 - 4.27.

The reported bioactivities of the compounds are presented in Table 4.14.

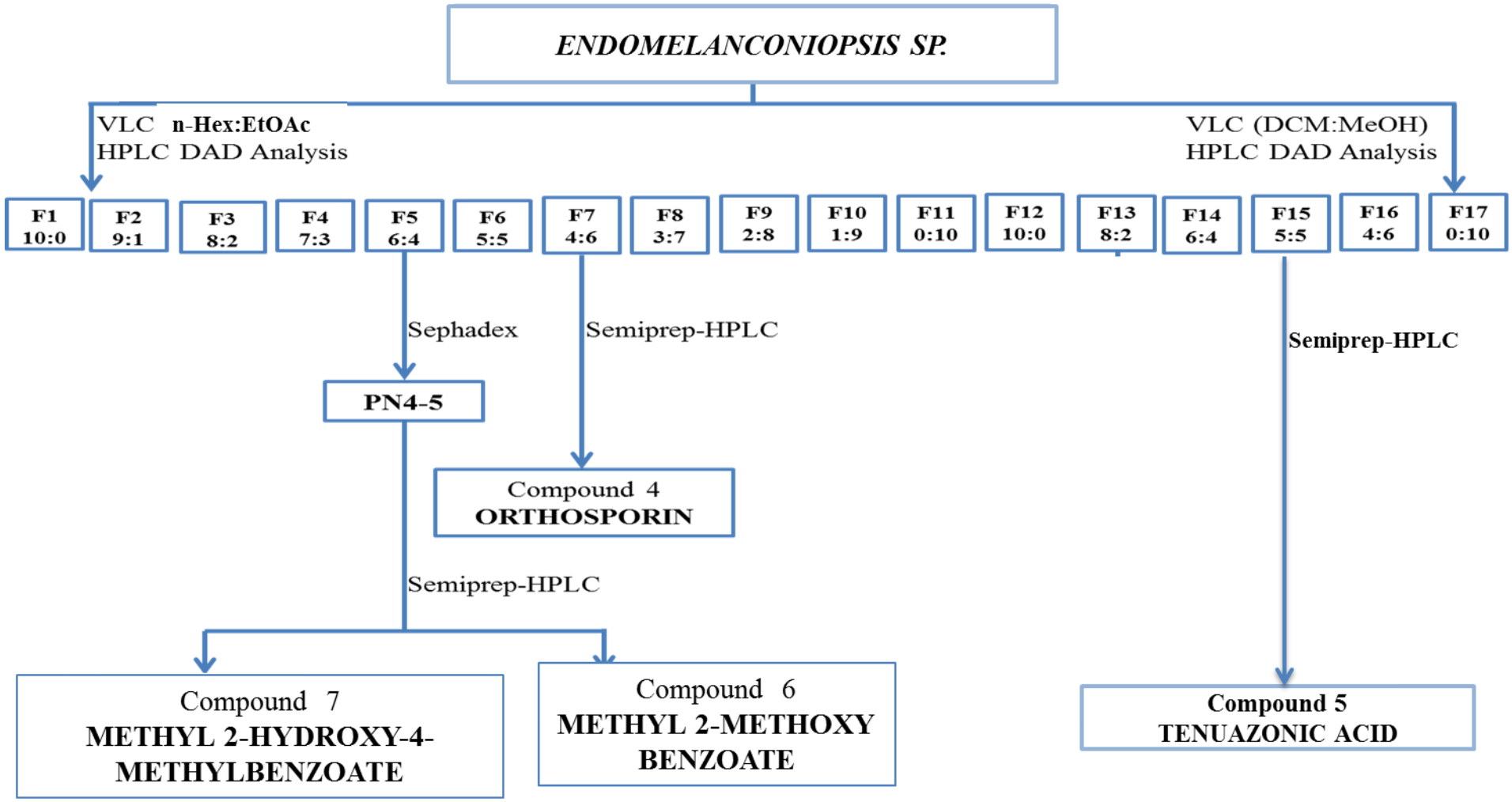


Figure 4.19: Detection and Identification of Compounds from *Endomelanconiopsis sp.* Crude Extract

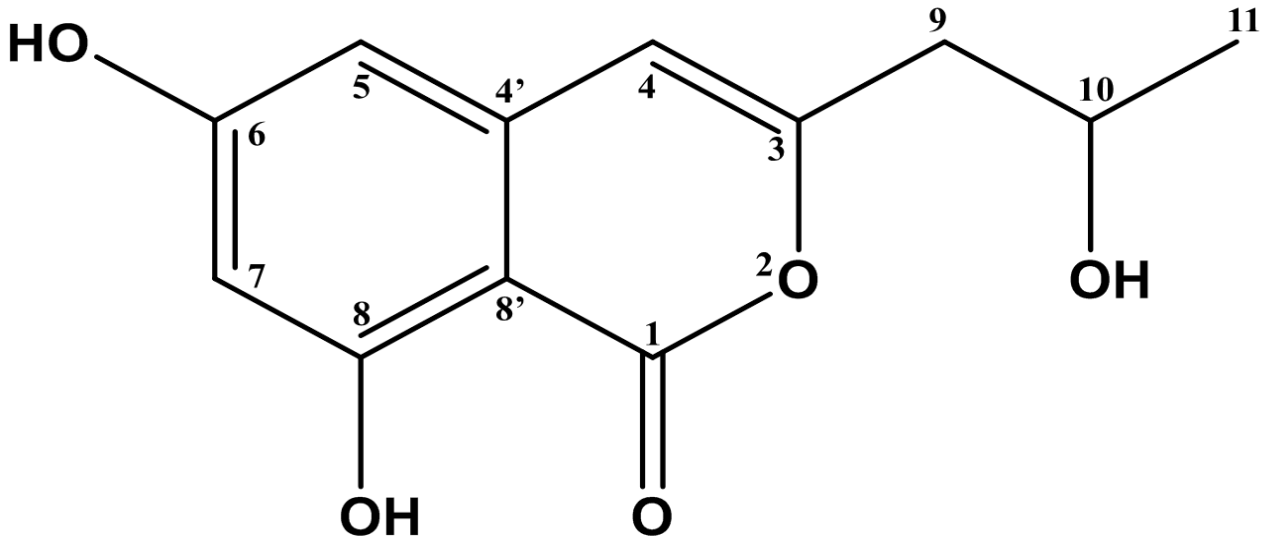
COMPOUND 4 (ORTHOSPORIN)

ORTHOSPORIN	
SYNONYM(S)	3 – (10-Hydropropyl) – 6, 8 dihydroxyl isocoumarin; De-o-methyldiaporthrin; 6, 8-dihydroxy-3-(2-hydroxyl propyl) isocoumarin;
SAMPLE CODE	PN4-7D-1
BIOLOGICAL SOURCE	*Endomelanconiopsis sp* (*Picralima nitida*)
ISOLATED QUANTITY	11.4 mg
APPEARANCE	Yellow solid
MOLECULAR FORMULA	$C_{12}H_{12}O_6$
MOLECULAR WEIGHT	236 g/mol
ABSORPTION MAXIMUM	λ max (Methanol): 243.8, 278 and 326.5 nm
RETENTION TIME (HPLC)	24.8 min.

Compound 4 was isolated from the VLC fraction (n-Hex: EtOAC 40:60) of ethylacetate crude extract of *Endomelanconiopsis sp.* It exhibited UV maxima at 243.8, 278 and 326.5 nm. Its molecular weight was determined as 236 g/mol based on the ESI-MS quasi molecular ion peak at m/z 237 $[M + H]^+$ upon positive ionization and 235 $[M – H]^-$ upon negative ionization. This suggested a molecular formula of $C_{12}H_{12}O_5$.

The H-NMR spectrum shows the presence of aromatic proton signals at δ 6.33 (s, 2H). This signal, which integrated to 2 protons, was assigned to 2 equivalent aromatic protons in H-5 and H-7. The singlet at δ 6.39 (s, 1H) was assigned to H-4, an olefinic signal. The H-NMR also shows the presence of a multiplet signal at δ 4.17 (m, 1H) assigned to the oxymethine proton at H-10, a methyl doublet at δ 1.27 (d, J=6.24, 3H) assigned to Me-11, the presence of a diastropic methylene signals at δ 2.74 (dd, J= 17.7, 4.71, 1H) and δ 2.55 (ddd, J=17.7, 3.4, 1.9. 1H) assigned to H-9A and H-9B respectively. The appearance of methylene at position 9 supports the asymmetric nature of C-10. The presence of OH group at that position was also supported in the fragment m/z 219 (M-18) which resulted from loss of H_2O molecule. Compound 4 was thus identified as the previously reported orthosporin and the data agrees well with literature report (Lee *et al*., 2006).

ORTHOSPORIN



Chemical formula: $C_{12}H_{12}O_5$
Molecular mass: 236.22 g·mol^{-1}
UV-max: 243.8, 278, 326.5 nm.

Figure 4.20: The chemical structure of Orthosporin.

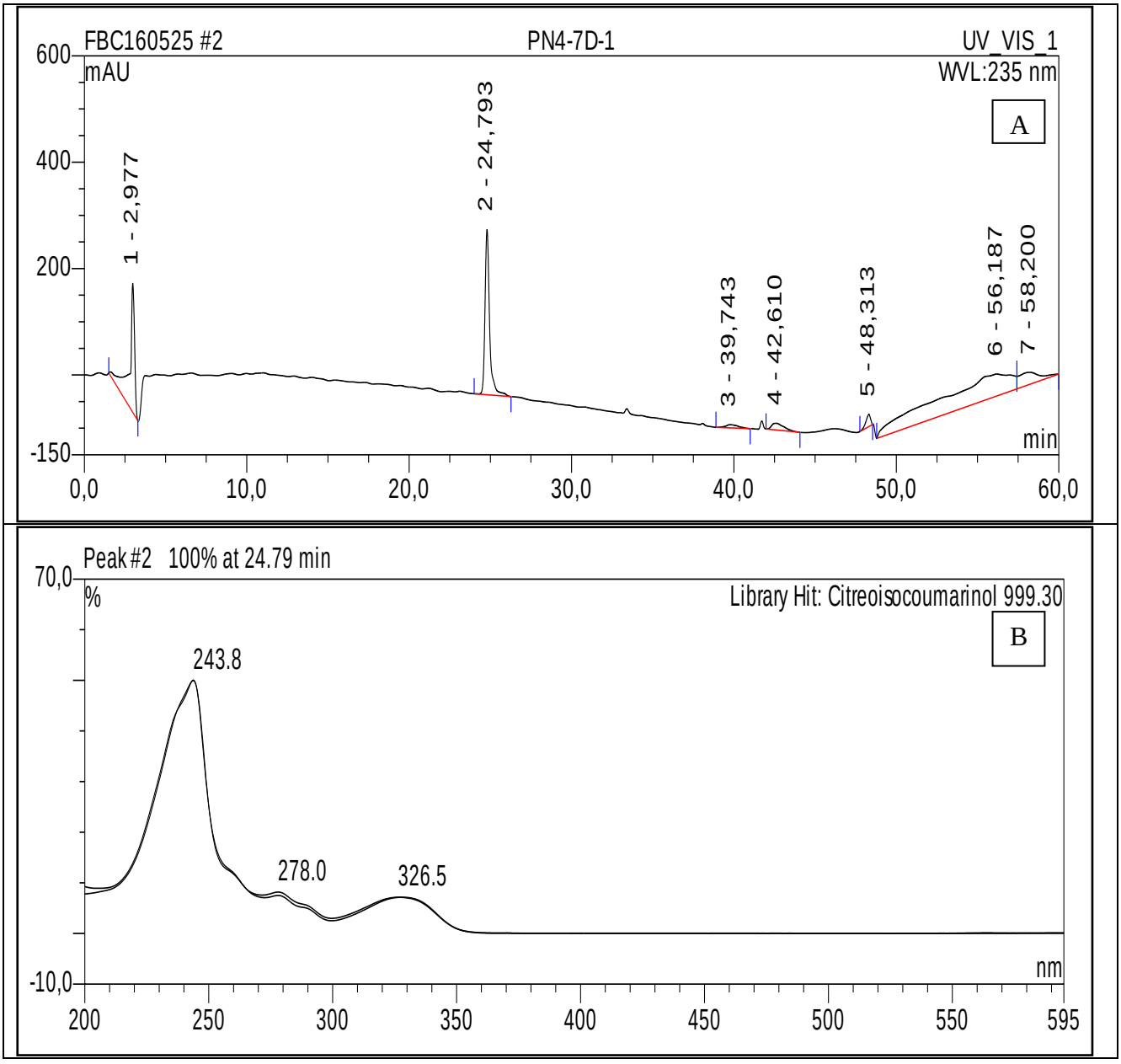


Figure 4.21: Orthosporin: HPLC Chromatogram (A), UV spectrum (B)

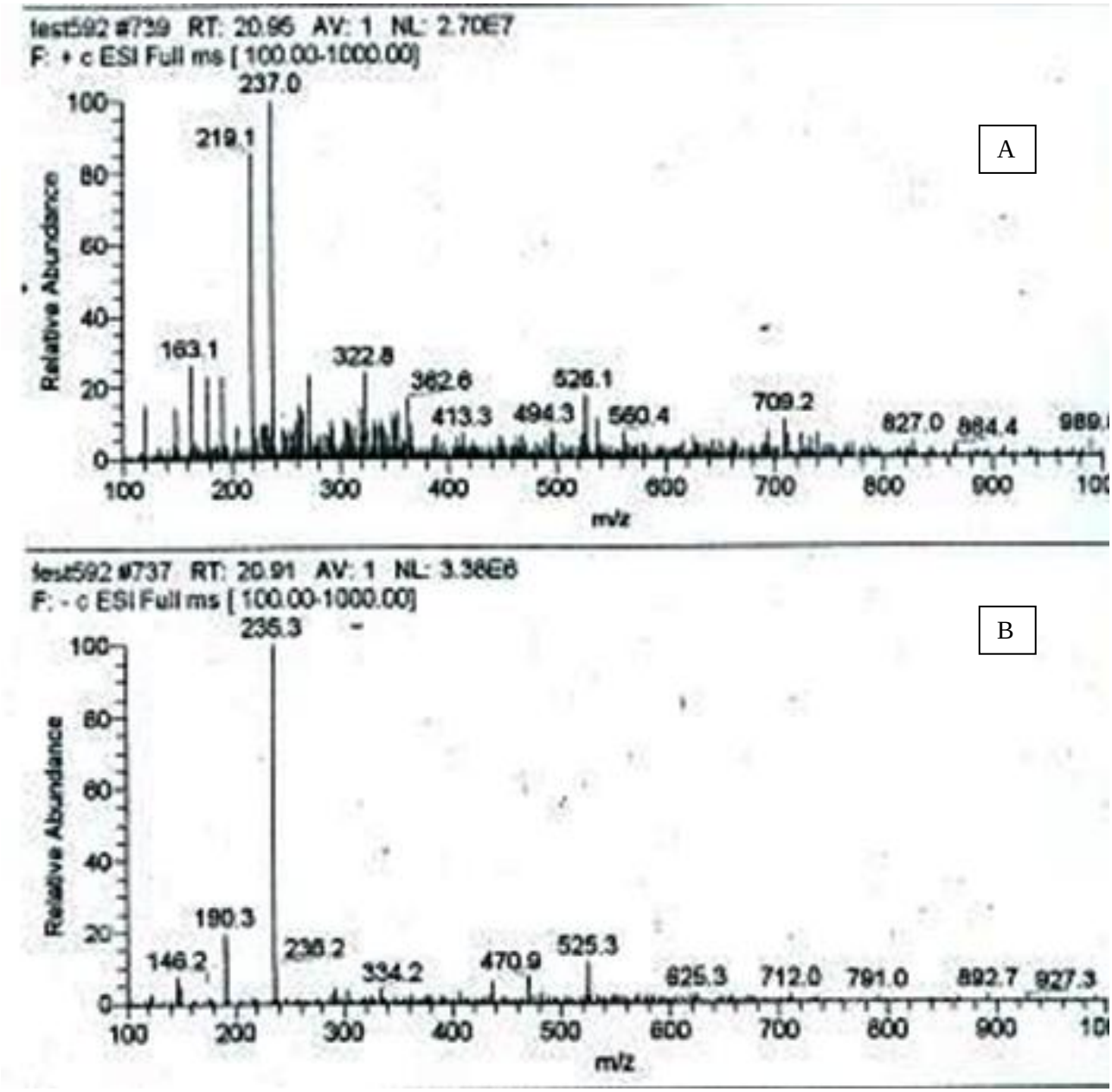


Figure 4.22: Orthosporin: LC-MS data showing the molecular mass [M+H] (A) and [M-H] (B)

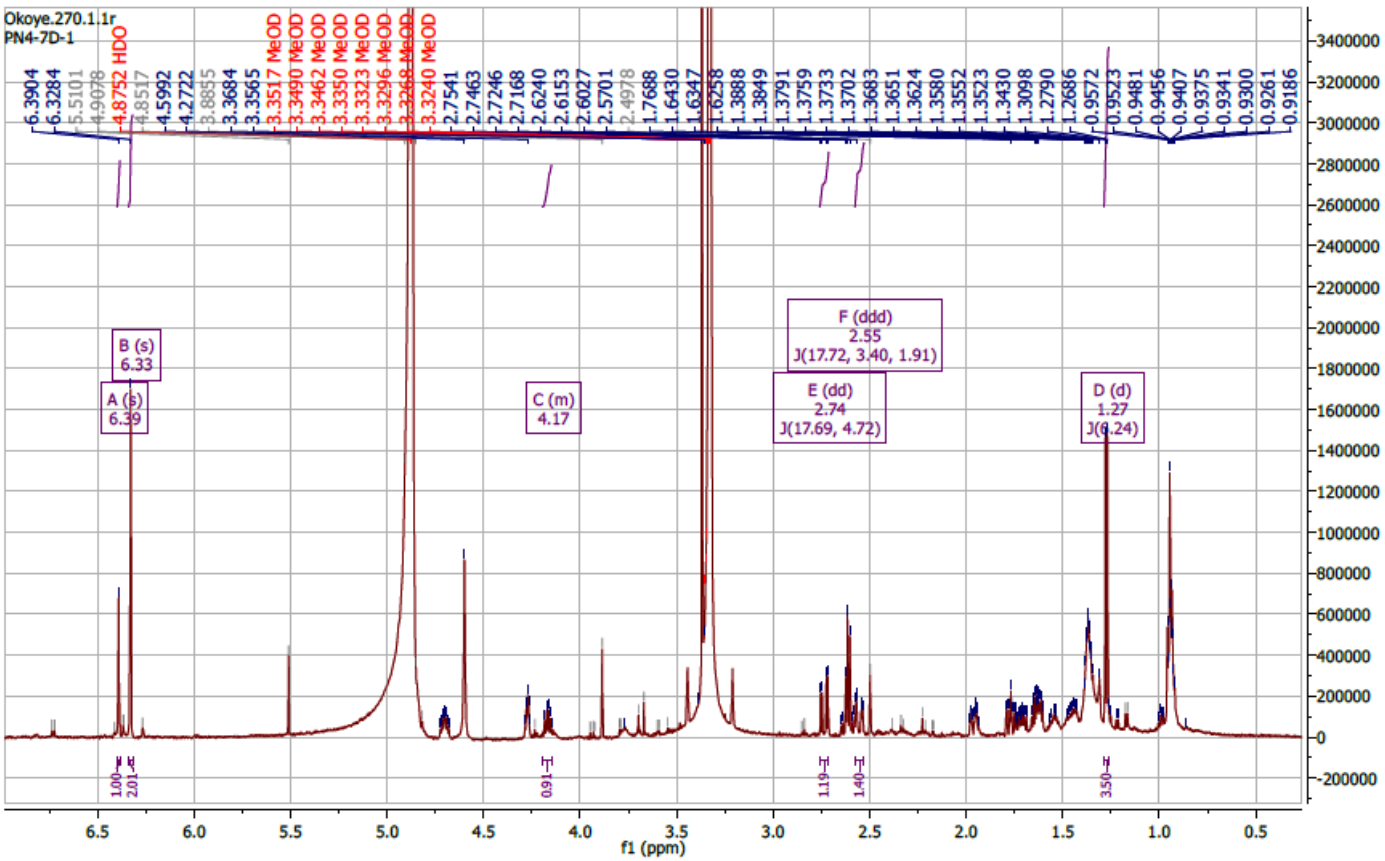


Figure 4.23: ^{1}H-NMR spectrum of Orthosporin: ^{1}H- NMR (500 MHz, Methanol-d4) δ 6.39 (s, 1H), 6.33 (s, 2H), 4.19 – 4.15 (m, 1H), 2.74 (dd, J = 17.7, 4.7 Hz, 1H), 2.55 (ddd, J = 17.7, 3.4, 1.9 Hz, 1H), 1.27 (d, J = 6.2 Hz, 3H).

Table 4.13: Comparison of ^{1}H- NMR Spectrum data of Orthosporin

Carbon No.	δH^{a} (J in Hz)	δH^{b} (J in Hz)
1	-	-
2	-	-
3	-	-
4	6.25 (1H, s)	6.39 (s)
5	6.14 (1H,d, J =1.7)	6.33 (s)
6	-	-
7	6.18 (1H,d, J = 1.7)	6.33(s)
8	-	-
9A	2.60 (1H,d, J = 6.5)	2.55 (ddd)
9B	2.81 (1H, d)	2.74 (dd)
10	4.15 (1H, m)	4.17 (m)
11	1.20 (3H,d, J = 6.2)	1.27 (d, J=6.24)

a. Lee *et al*. (2006) at 600MHz in MeOD (Reference Compound).
b. Derived from ^{1}H spectrum at 500MHz in MeOD from this study (Isolated Compound).

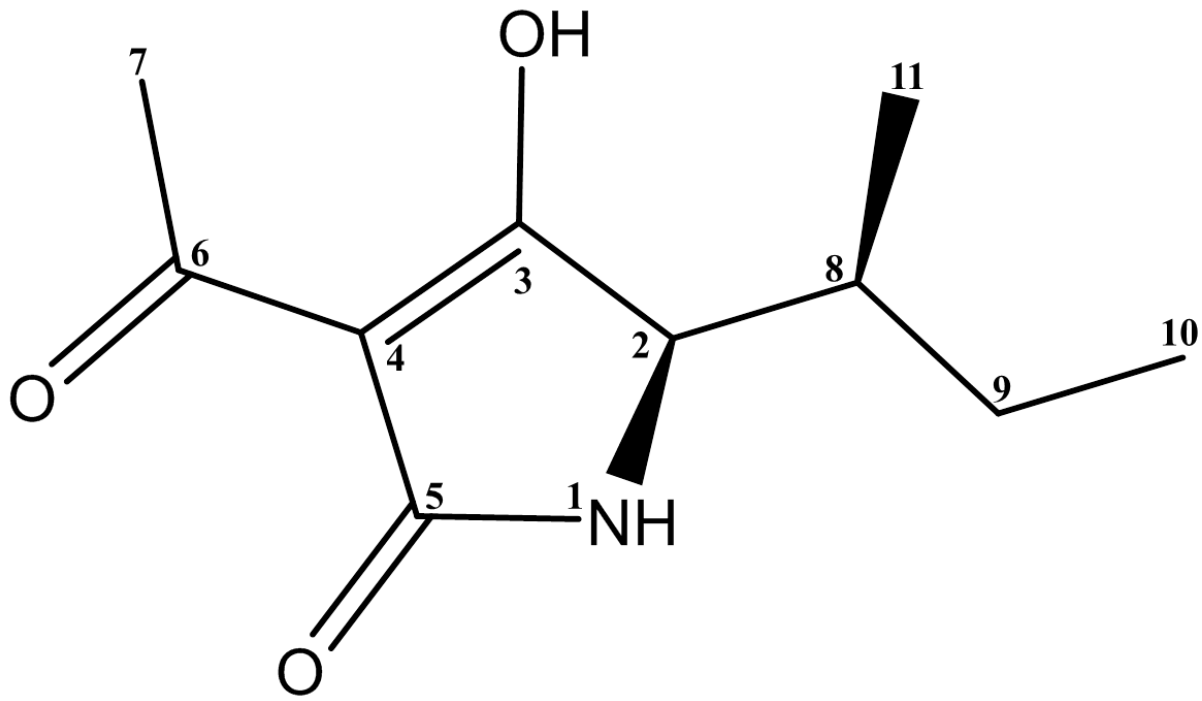
COMPOUND 5 (TENUAZONIC ACID)

TENUAZONIC ACID	
SYNONYM(S)	(S) – 3 –acetyl-5-sec-butyl-4-hydroxyl-1H-Pyrolin-2-one; 4-Acetyl-2-butan-2-yl-3-hydroxyl-1, 2-dihydropyrol-5-one
SAMPLE CODE	PN 4-13B
BIOLOGICAL SOURCE	*Endomelanconiopsis sp* (*Picralima nitida*)
ISOLATED QUANTITY	12.1 mg
APPEARANCE	Viscous brown oil
MOLECULAR FORMULA	$C_{10}H_{15}NO_3$
MOLECULAR WEIGHT	197 g/mol
ABSORPTION MAXIMUM	λmax (Methanol): 217.1 and 276.7 nm.
RETENTION TIME (HPLC)	26.75mins.

Compound 5 was isolated from the VLC fraction (DCM: MeOH 50:50) of ethylacetate crude extract of *Endomelanconiopsis sp*. It exhibited UV maxima at 217.1 and 276.7nm. Its molecular weight was determined as 197g/mo based on the molecular ion peaks at m/z 198.1 upon positive ionization $[M + H]^+$ and 196.3 upon negative ionization $[M - H]^-$. This therefore suggested the presence of an odd number nitrogen atom and the molecular formula $C_{10}H_{15}NO_3$.

The ^{1}H-NMR shows the presence of a deshielded proton signal at δ 3.84 (s) assigned to H-2, the deshieded position being contributed by inductive effect of the adjacent nitrogen atom. There is also the presence of shielded methine proton signal at δ 1.91 (m) assigned to H-8 and diastropic methylene signal at δ 1.34(m) and δ 1.24 (ddd, J=13.4, 8.7, 6.9, IH) assigned to H-9A and H-9B respectively. The methyl doublet at δ 0.99 (d, J=6.8, 3H) was assigned to Me-11, while the methyl triplet at δ 0.90 (t, J=7.4, 3H) was assigned to Me-10. The NMR also shows the presence of a slightly deshielded methyl singlet at δ 2.42 (s) assigned to Me-7, the deshielding being as a result of the anisotropic effect of the carbonyl group. The stereochemistry was deduced as 2-cis, 8-cis as a result of the very low coupling constant between H-2 and H-9 signals. Compound 5 was thus identified as 2 (cis), 8 (cis) tenuazonic acid and the spectra data agree well with literature report Notre *et al.*, (1980).

TENUAZONIC ACID



Chemical formula: $C_{10}H_{15}NO_3$
Molecular mass: 197.23 g·mol^{-1}
UV-max: 217.1, 276.7 nm.

Figure 4.24: The chemical structure of Tenuazonic acid.

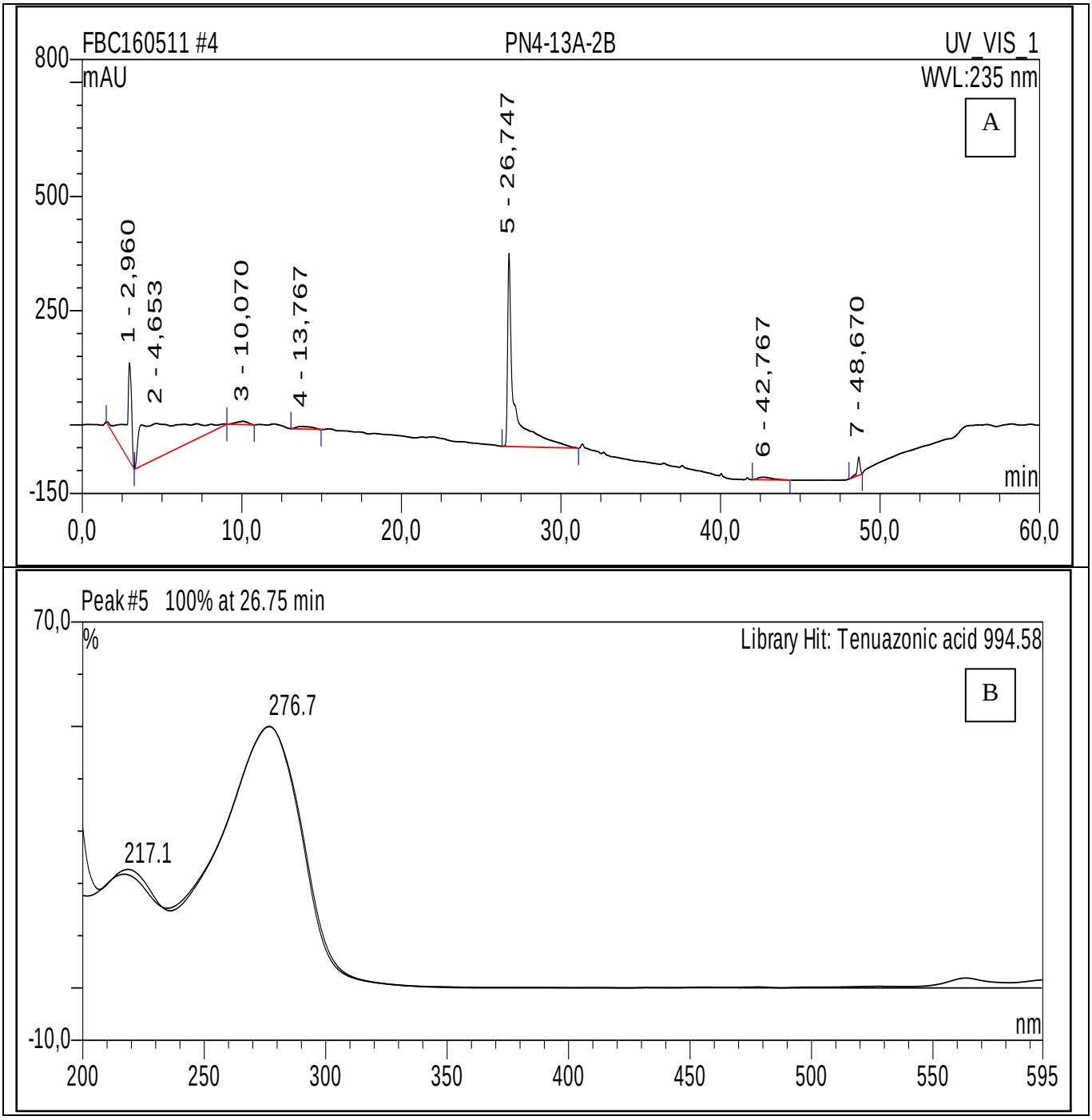


Figure 4.25: Tenuazonic acid: HPLC Chromatogram (A), UV spectrum (B)

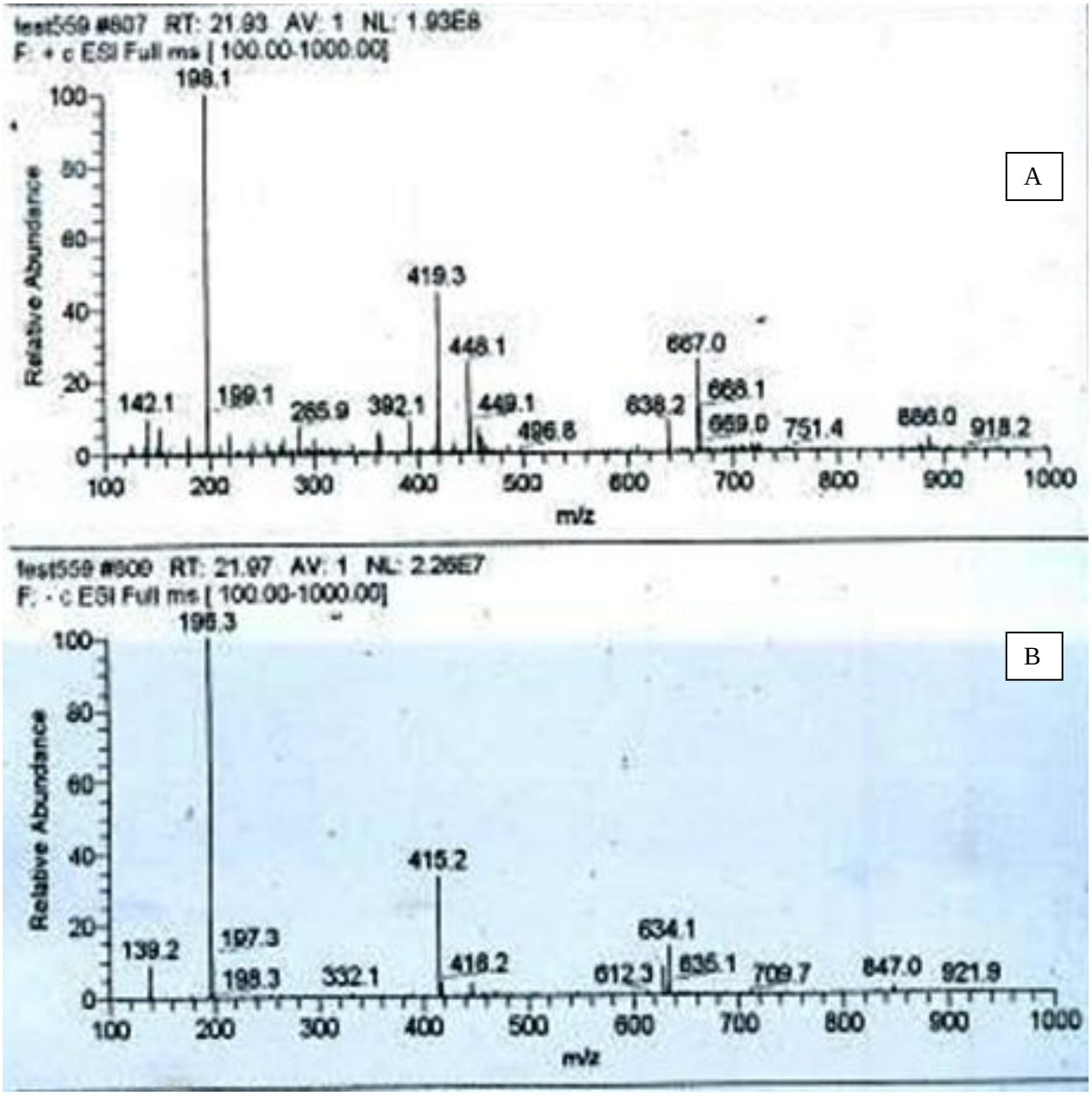


Figure 4.26: Tenuazonic acid: LC-MS data showing the molecular mass [M+H] (A) and [M-H] (B)

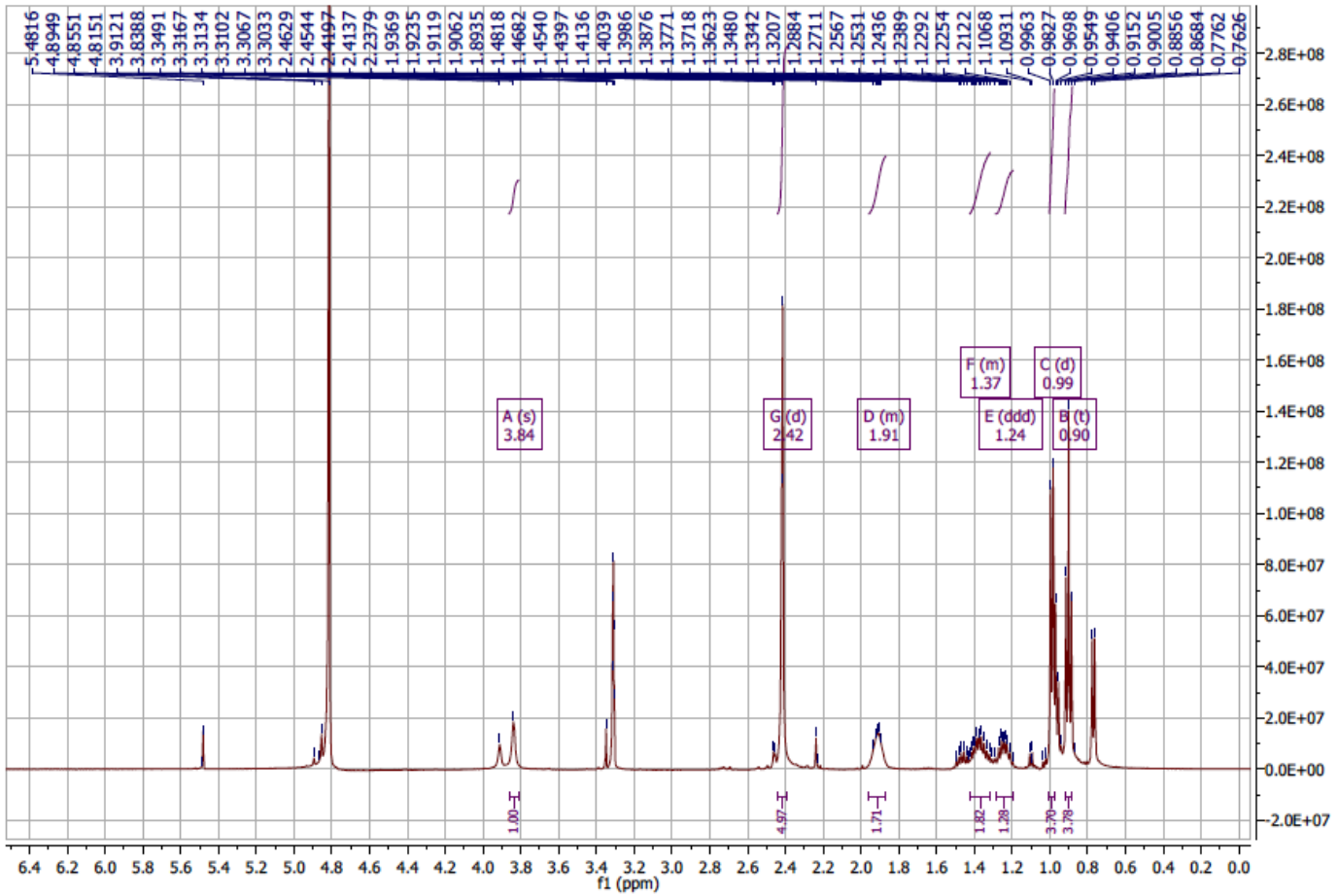


Figure 4.27: ^{1}H-NMR spectrum of Tenuazonic acid: ^{1}H -NMR (500 MHz,) δ 3.84 (s, 1H), 2.42 (d, J = 3.0 Hz, 5H), 1.96 – 1.87 (m, 2H), 1.42 – 1.31 (m, 2H), 1.24 (ddd, J = 13.4, 8.7, 6.9 Hz, 1H), 0.99 (d, J = 6.8 Hz, 4H), 0.90 (t, J = 7.4 Hz, 4H).

Table 4.14: Comparison of ^{1}H data of Tenuazonic acid

Carbon No.	δH^{a} (Jin Hz)	δH^{b} (Jin Hz)
1	-	-
2	-	-
3	-	-
4	-	-
5	2.33 (1H, d, J = 2.8)	2.42 (s)
6	-	-
7	3.83 (1H, s)	3.84 (s)
8	1.77 (1H, m)	1.91 (m)
9A	1.24 (1H, m)	1.37 (m)
9B	1.09 (1H, m)	1.24 (m)
10	0.80 (t, J = 7.2)	0.90 (t)
11	0.90 (d, J = 6.9)	0.99 (d)

a. Notre *et al*. (1980) at 600 MHz in $CDCL_3$ (Reference Compound).

b. Derived from ^{1}H-NMR spectrum at 500 MHz in MeOD in this study (Isolated Compound).

COMPOUND 6 (METHYL 2-METHOXYBENZOATE)

METHYL 2-METHOXYBENZOATE	
SYNONYM(S)	Methyl O-anisate, 2-methoxybenzoic acid methyl ester
SAMPLE CODE	PN4-5FI
BIOLOGICAL SOURCE	*Endomelanconiopsis sp.* (from *Picralima nitida*)
ISOLATED QUANTITY	2.6 mg
APPEARANCE	Colourless liquid
MOLECULAR FORMULA	$C_9H_{10}O_3$
MOLECULAR WEIGHT	166 g/mol
ABSORPTION MAXIMUM	UV λmax (Methanol): 230, 276 and 304 nm
RETENTION TIME	19:22 mins.

Compound 6 was isolated from the VLC fraction (n-Hex, EtOAC 60.40) of Ethylacetate crude extract of *Endomelanconiopsis sp*. as a colourless liquid. It exhibited UV maxima at 230 nm, 276 nm and 304 nm. Its molecular weight was determined as 166 g/mol based on the ESI – MS quasi peak at m/z 167.1 $[M + H]^+$ upon positive ionization and 165.2 $[M – H]^-$ upon negative ionization. This suggested a molecular formular of $C_9H_{10}O_3$.

The UV maxima observed is typical of benzene which suggested the presence of benzene ring.

A look at ^{1}H-NMR showed that two CH_3 group. One singlet was observed at δ 3.92 (H-9) due to the methoxy group. The singlet at δ 3.68 (H-8) is a methyl group. The deshielding was also due to the methoxy group.

The protons 3, 4, 5 and 6 resonated downfield at δ 7.80 (H-3), δ 7.47 (H-6), δ 6.75-7.0 (H-4, H-5), principally due to the olefenic nature associated with benzene ring. The deshielding is due to the anisotropic effect of the olefinic system.

Analysis of the LCMS and NMR data in comparison with those reported in the literature (Bia *et al*, 2012) led to the elucidation of compound 6 as Methyl 2-methoxybenzoate.

METHYL 2-METHOXYBENZOATE

Chemical formula: $C_9H_{10}O_3$

Molecular mass: 166 g·mol−1

UV-max: 227, 276.5, 304.5 nm.

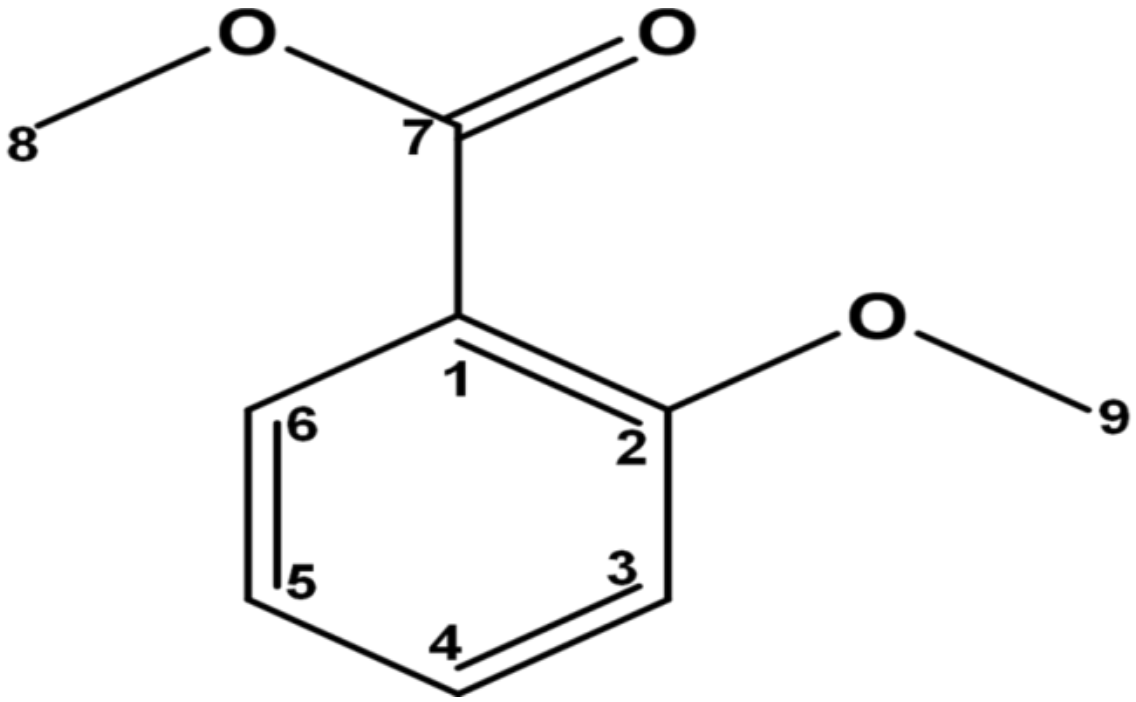


Figure 4.28: The chemical structure of Methyl 2-Methoxybenzoate.

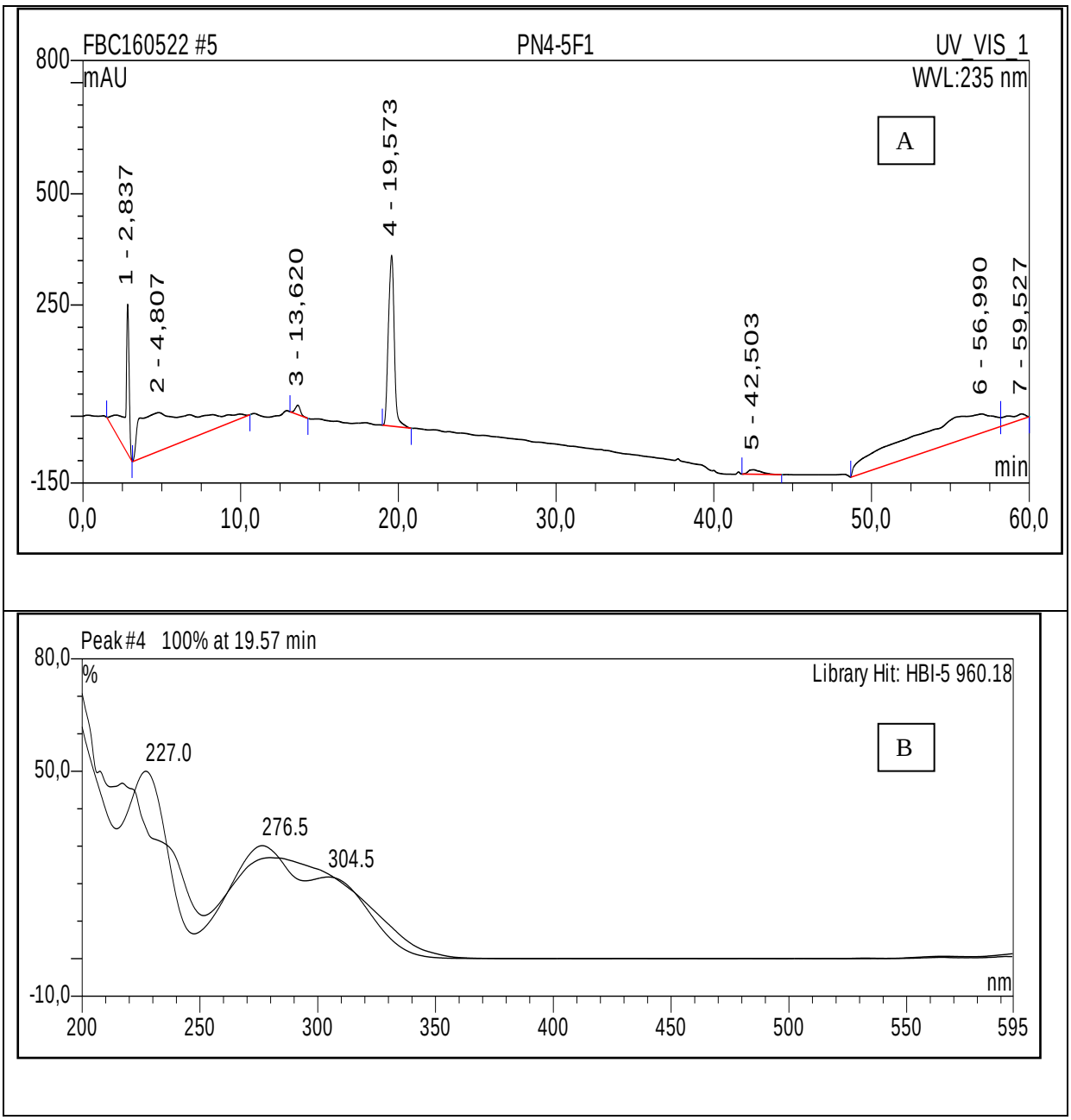


Figure 4.29: Methyl 2-Methoxybenzoate: HPLC Chromatogram (A), UV spectrum (B)

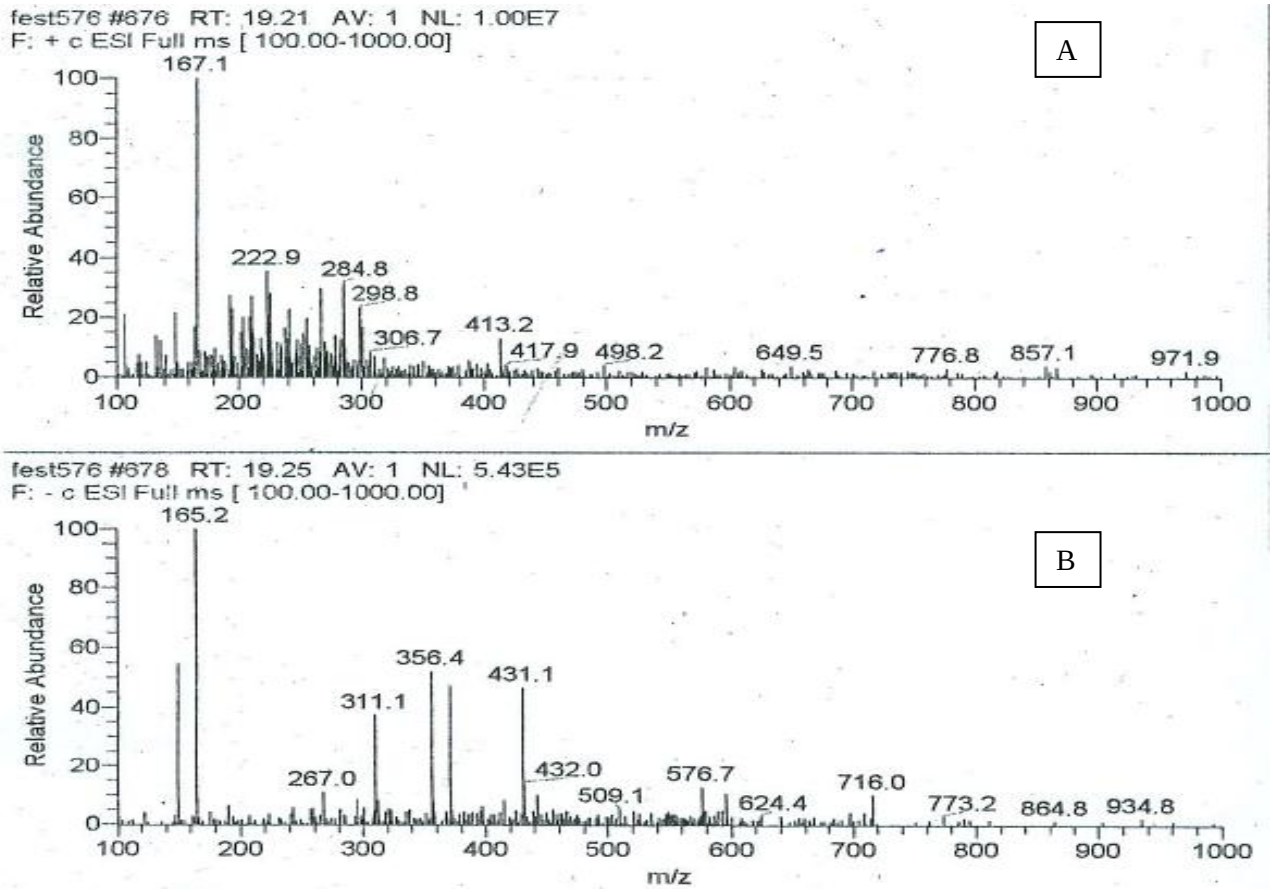


Figure 4.30: Methyl 2-Methoxybenzoate: LC-MS data showing the molecular mass [M+H] (A) and [M-H] (B)

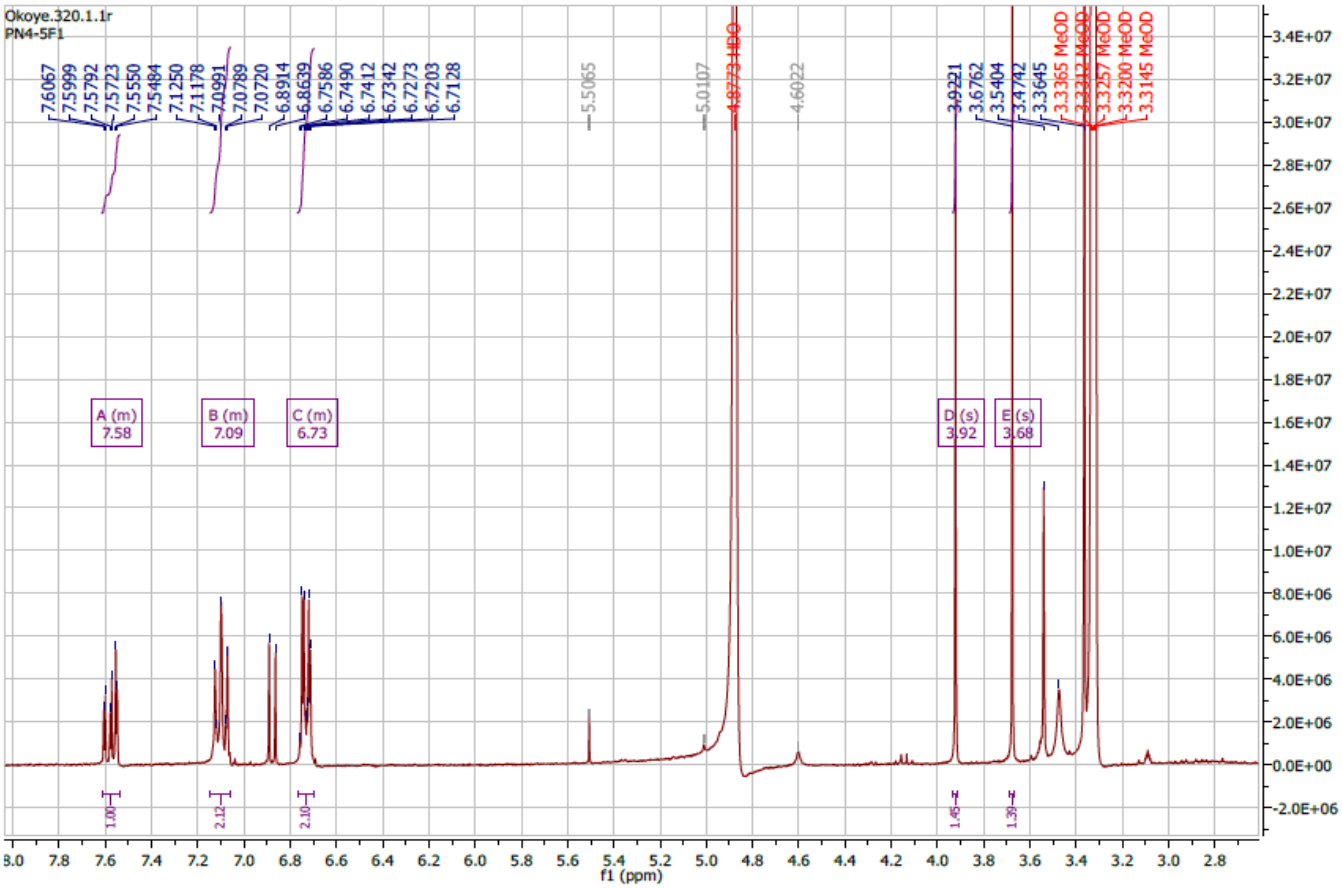


Figure 4.31: ^{1}H-NMR spectrum of Methyl 2-Methoxybenzoate

Table 4.15: Comparison of ^{1}H NMR Data of Methyl 4-methoxybenzoate

Carbon No.	δ H[a](J in Hz)	δ H[b] J in Hz)
1	-	-
2	-	-
3	7.80(dd, J = 80, 2.0Hz)	7.58 (m)
4	6.76 – 7.0(m, 2H)	6.73 (m)
5	6.76 – 7.0(m, 2H)	6.73 (m)
6	7.47 (dd, J = 8.0, 1.6Hz 1H)	7.07 (m)
7	-	-
8	3.91 (s, 3H)	3.68 (s)
9	3.90 (s, 3H)	3.90 (s)

a. Bia X *et al*, (2012) at 500 MHz in $CDCl_3$ (Reference Compound).

b. Derived from ^{1}H spectrum at 500 MHz in MeOD from this study.

COMPOUND 7 (METHYL 2-HYDROXY -4-METHYLBENZOATE)

METHYL 2 - HYDROXY- 4 – METHYLBENZOATE	
SYNONYM(S)	Methyl 4-methylsalicylate, Methyl m-Cresotate
SAMPLE CODE	PN4-5F2
BIOLOGICAL SOURCE	*Endomelanoiopsis sp*. (from *Picralima nitida*)
ISOLATED QUANTITY	2.2 mg
APPEARANCE	Pale yellow powder
MOLECULAR FORMULA	$C_9H_{10}O_3$
MOLECULAR WEIGHT	166 g/mol
ABSORPTION MAXIMUM	UV λmax (Methanol): 234 nm, 280 nm and 314 nm
RETENTION TIME	21:04 mins.

Compound 7 was isolated from the VLC fraction (n-Hexane: EtOAc 60:40) of Ethyl acetate crude extract of *Endomelaniopsis sp*.

The UV spectrum should absorption maximum at 234 nm, 280 nm and 314 nm. This suggested the presence of benzene ring as the basic structure.

The molecular weight was determined as 166 g/mol based on the ESI-MS quasi molecular io peak at m/z 167.2 $[M + H]^+$ upon positive ionization and 165.2 $[M - H]^-$ upon negative ionization. This suggests a molecular formular of $C_9H_{10}O_3$.

The H-NMR spectrum showed the presence of one singlet, a methyl group, observed at δ 3.76. The slight deshielding was due to the presence of methyoxy group. This is assigned to (H-8). The doublet at δ 2.34 is assigned to methyl group at (H-9).

The protons at positions 3, 5 and 6 resonated downfield at δ 6.35(d) (H-3), δ 6.23(m) [H-5, H-6]. This is due to the olefinic nature associated with benzene ring. The deshielding is due to the anisoptropic effect of the olefinic system. Compound 7, based on the MS and NMR data was identified as Methyl 2-hydroxy-4-methylbenzoate.

METHYL 2-HYDROXY -4-METHYLBENZOATE

Chemical formula: $C_9H_{10}O_3$

Molecular mass: 166 $g \cdot mol^{-1}$

UV-max: 231.4, 280.9, 314.2 nm.

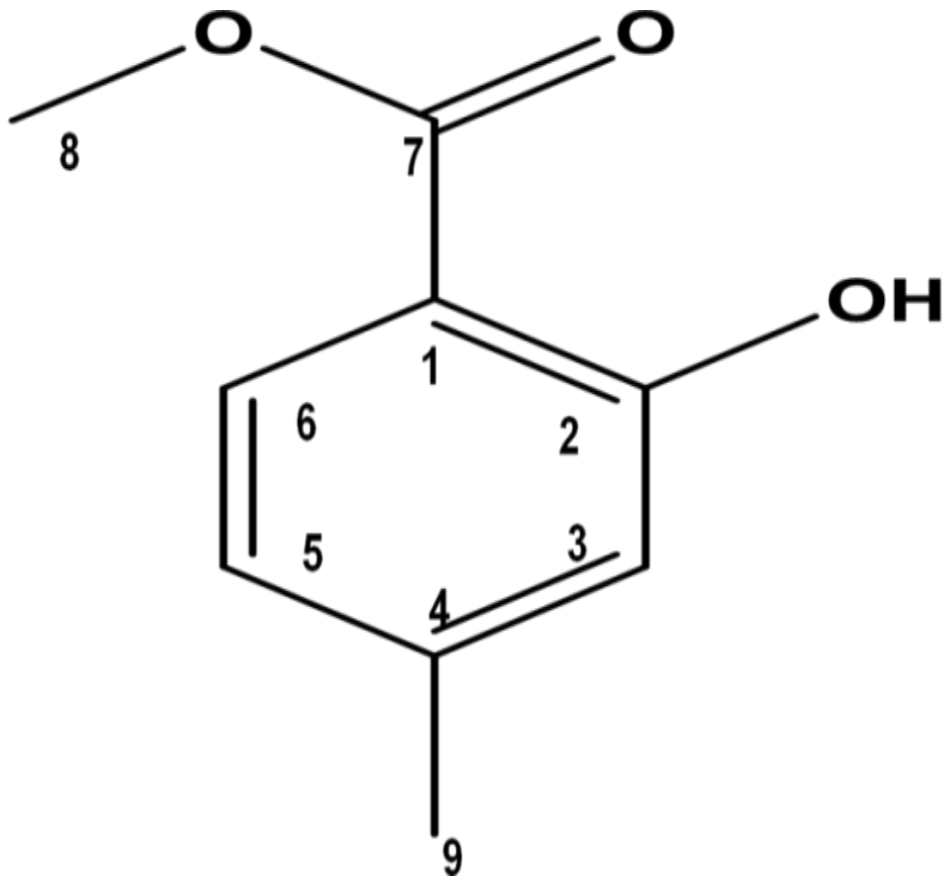


Figure 4.32: The chemical structure of Methyl 2-hydroxy-4-methylbenzoate

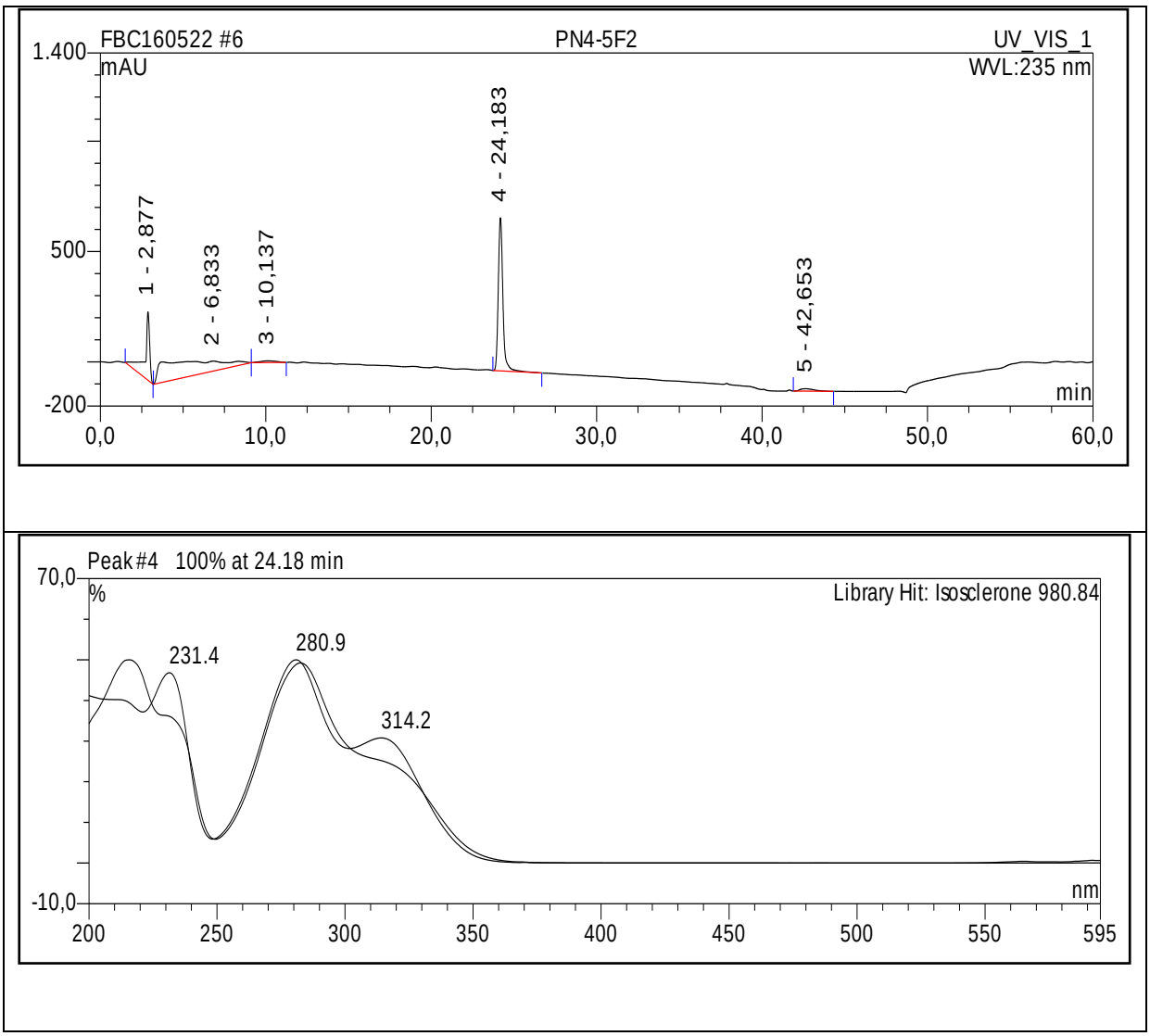


Figure 4.33: Methyl 2-hydroxy-4-methylbenzoate: HPLC Chromatogram (A), UV spectrum (B)

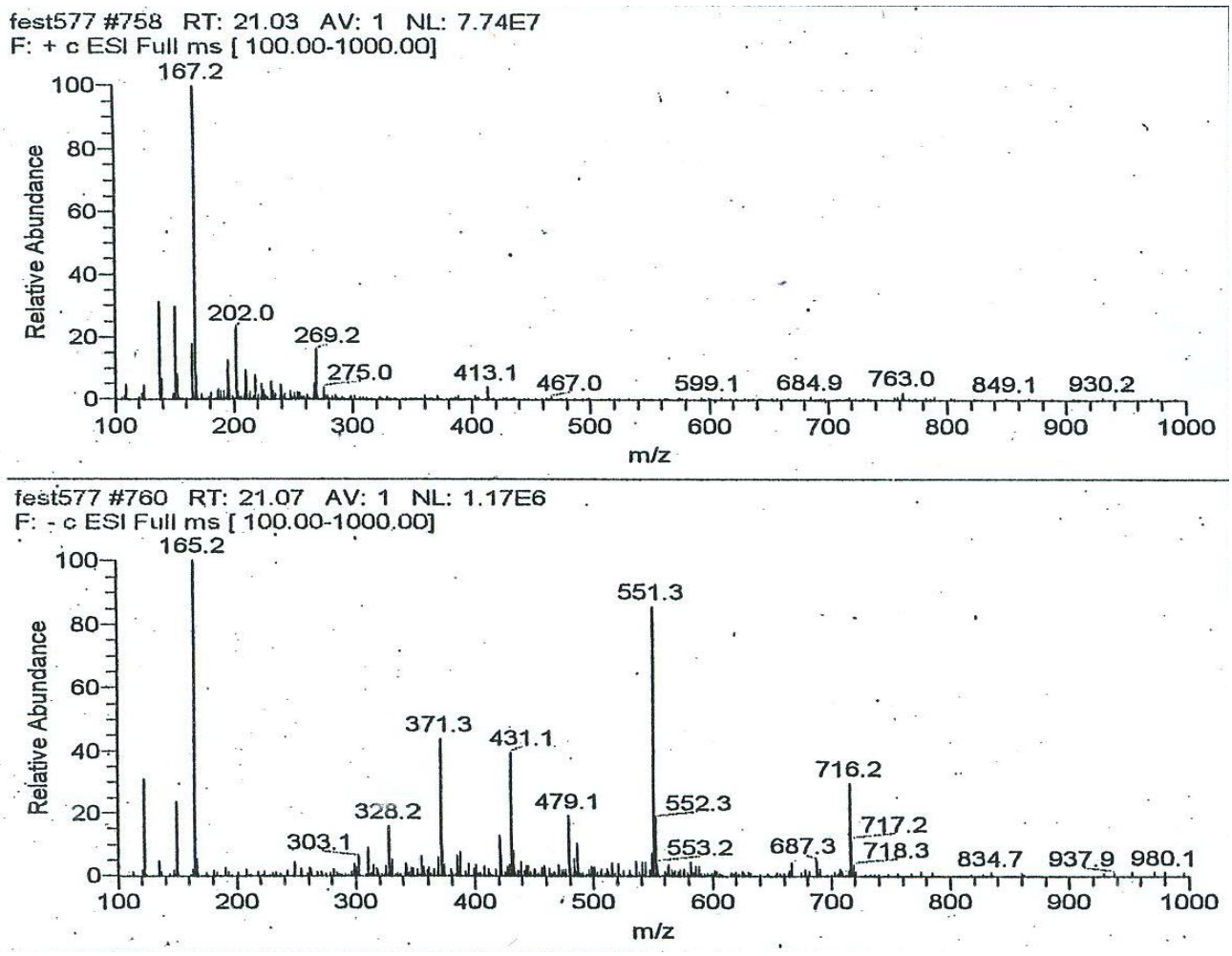


Figure 4.34: Methyl 2-hydroxy-4-methylbenzoate: LC-MS data showing the molecular mass [M+H] (A) and [M-H] (B)

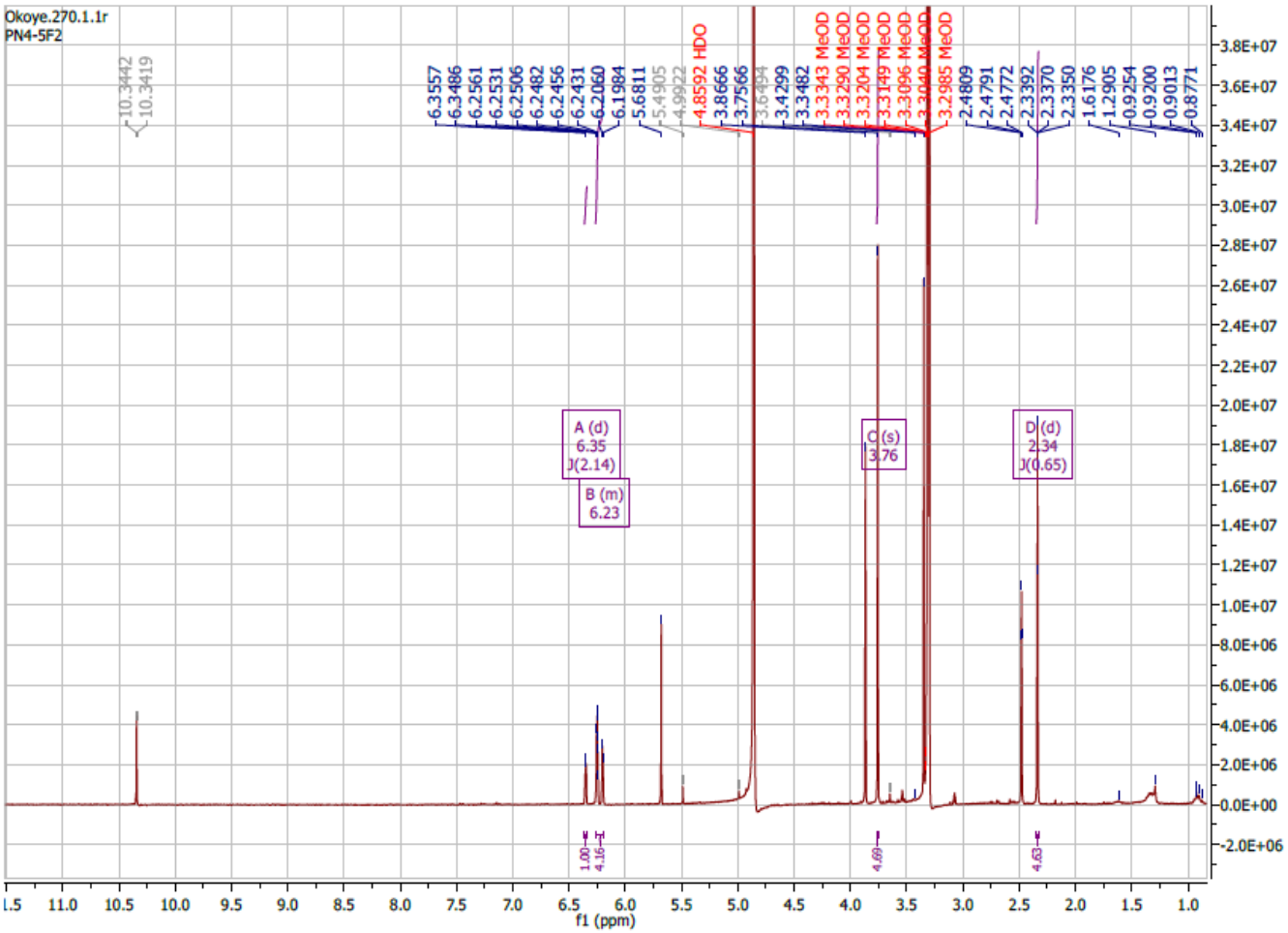


Figure 4.35: ^{1}H-NMR spectrum of Methyl 2-hydroxy-4-methylbenzoate

4.1.5 BIOASSAY OF ISOLATED PURE COMPOUNDS

4.1.5.1 Antimicrobial Assay

The antimicrobial activity of pure compounds isolated from extracts of *Curvularia sp.* and *Endomelanconiopsis sp.* was determined using the agar well diffusion method. Results of the antimicrobial assay are presented in Table 4.16.

4.1.5.2 Antioxidant Assay

The antioxidant activity of pure compounds isolated from extracts of *Curvularia sp* and *Endomelanconiopsis sp.* was determined using the DPPH antioxidant assay method. Results of the antioxidant assay are presented in Figure 4.36.

Table 4.16: Result of Antimicrobial Assay of isolated compounds

	Mean ± SEM Inhibition Zone Diameters (mm)						
Test organisms	**Acropyrone (500 µg/ml)**	**4-hydroxyphenyl acetic acid (500 µg/ml)**	**Indole-3-acetic acid (500 µg/ml)**	**Orthosporin (500 µg/ml)**	**Tenuazonic acid (500 µg/ml)**	**Ciprofloxacin (5 µg/ml)**	**DMSO**
E. coli	0±0.00	0±0.00	0±0.00	0±0.00	0±0.00	5±0.33	0±0.00
S. aureus	0±0.00	0±0.00	0±0.00	0±0.00	0±0.00	8±0.00	0±0.00
S. typhi	0±0.00	0±0.00	0±0.00	0±0.00	0±0.00	7±0.33	0±0.00
B. subtilis	0±0.00	0±0.00	0±0.00	0±0.00	0±0.00	8±0.33	0±0.00
						Miconazole (50 µg/ml)	DMSO
A. niger	0±0.00	0±0.00	0±0.00	3±0.33	2±0.00	12±0.89	0±0.00
C. albicans	0±0.00	0±0.00	0±0.00	0±0.00	3±0.33	14±0.33	0±0.00

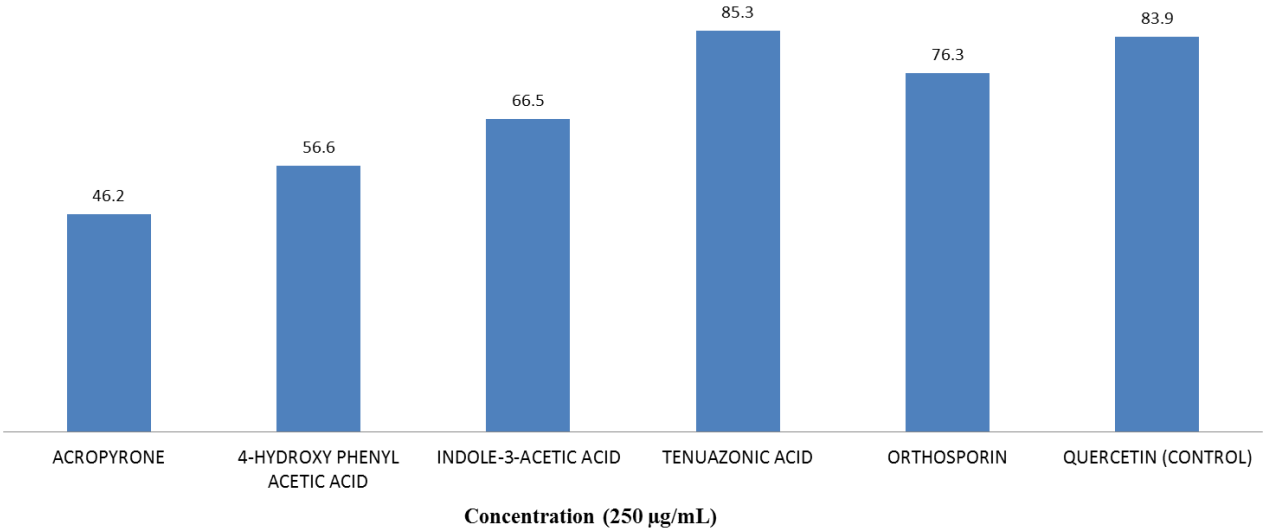


Figure 4.36: Result of Antioxidant Assay of Isolated Pure Compounds

Table 4.17: Reported Bioactivities of Isolated compounds

S/N	Isolated Compounds	Source	Reported Bioactivity	Reference
1	Acropyrone	*Curvularia sp*	Cytotoxic	Ito *et al*., 2016.
2	2-(4-hydroxyphenyl) acetate	*Curvularia sp*	Nematicidal, antimicrobial	Zuo *et al*., 2014; Chapla *et al*., 2014; Ohtani *et al*., 2011
3	Indole-3-acetic acid	*Curvularia sp*	Cytotoxic/Anticancer, antioxidant, anti-inflammatory	Wardman, 2002; Jeong *et al*., 2010; Jones *et al*., 1995.
4	Orthosporin	*Endomelanconiopsis sp*	-	-
5	Tenuazonic acid	*Endomelanconiopsis sp*	Herbicidal, Antitumor/Cytotoxic, Antibacterial, Antiviral,	Devi *et al*., 2010; Gitterman *et al*., 1965; Miller *et al*., 1963.

4.2 DISCUSSION

4.2.1 *CURVULARIA SP*

Curvularia is a genus in the family Pleosporaceae. It is a hyphomycete (mold) fungus which is a facultative pathogen of many plant species and of the soil. Most *Curvularia* are found in tropical regions, though a few are found in temperate zones (Alex, 2013).

Endophytic *Curvularia sp* has been isolated from several plants such as *Ipomoea carnea* (Tayung *et al*., 2012), *Cymbopogon caesius* (Avinash *et al*., 2015), *Lippia sidoides* (de Siqueira *et al*., 2011), *Oryza sativa* (Zakaria *et al*., 2010), etc.

Several compounds have been isolated from endophytic *Curvularia sp*. These include (2'S)-2-(propan-2'-ol)-5-hydroxy-benzopyran-4-one, 2,3-dihydro-2-methyl-benzopyran-4,5-diol, 2-methyl-5-methoxy-benzopyran-4-one and (2R)-2,3-dihydro-2-methyl-5-methoxy-benzopyran-4-one (Teles *et al*., 2005).

In this study, *Curvalaria sp.* crude extract and fractions were tested for antimicrobial, antioxidant, antitubercular and cytotoxic activities.

In the cytotoxicity assay, at a concentration of 10 µg/ml, *Curvalaria sp.* crude extract showed poor cytotoxic activity against mouse lymphoma cell lines (L5178Y), with a percent growth inhibition of -0.7.

At same concentration, the *Curvalaria sp.* crude extracts showed no antitubercular activity against *Mycobacterium tuberculosis.*

Results of the DPPH antioxidant assay revealed that at a concentration of 500 µg/ml, the *Curvularia sp.* crude extract and fractions showed varying degrees of antioxidant activities, with higher activity recorded for the fractions. The fungal crude extracts recorded percent inhibition of 24. One fraction of *Curvularia sp*. extract (PNMR3AF13) showed good

antioxidant activity with percent inhibition of 76.9 which was comparable to the percent inhibition of 91.7 recorded for the positive control, quercetin. The results of the antimicrobial screening revealed that at a concentration of 10 mg/ml, *Curvalaria sp.* crude extract showed antibacterial and antifungal activities against the bacterial and fungal test organisms.

From the bioactive fraction (PNMR3A F13) of *Curvalaria sp.*, three bioactive compounds were isolated. These compounds are acropyrone, 4-hydroxyphenylacetic acid, and indole-3-acetic acid.

4.2.1.1 ACROPYRONE

Acropyrone is an α-pyrone that has been isolated from the fungus like the mangrove-derived endophytic fungus *Acremonium strictum* (Hammerschmidt *et al*., 2014). This acetophenone dimer was also isolated from endophytic fungus *Acronychia pedunculata* (Kauloura *et al.,* 2012).

Acropyrone is an alpha-pyrone (2-pyrone) which possesses a lactone. 2- Pyrones are prevalent in natural products isolated from plants, animals, marine organisms, bacteria, fungi, and insects that exhibit a broad range of biological activities, such as antifungal, antibiotic, cytotoxic, neurotoxic and phytotoxic (McGlacken and Fairlamb, 2005). Moreover, 2-pyrone can serve as versatile building block for the synthesis of key intermediate in synthetic organic chemistry as well as in medicinal chemistry due to the existence of functional groups, such as conjugated diene and the ester group (Lee, 2015).

Kauloura *et al*., 2012, reported that Acropyrone did not exhibit cytotoxic effect against the DU145 postrate and A2058 melanoma human cancer cell lines as well as the NHDF normal cell. However, Hammerschmidt *et al*., (2014), reported cytotoxic activity against human cisplatin sensitive, resistant A2780 cell lines and antibacterial activity.

4.2.1.2 4-HYDROXYPHENYLACETIC ACID

4-Hydroxyphenylacetic acid is an antifungal phenolic acid. It was isolated along with four other phenolic acids from stromata of *E. typhina* (Tan and Zou, 2001). It is a metabolite of the non essential amino acid, tryosine and phenylalanine, in human. It has also been isolated from other fungi such as sponge, *Phycopsis sp.* (Venkateswarlum *et al*., 1995), the endophytic fungus HP-1 of Chinese eaglewood (Zuo *et al.,* 2014), and *Annona muricata*, *Pseudofusicoccum sp*. (Abba, 2016).

It has been shown to have anti-nematicidal activites against root-lesion nematode, *Pratylenchus penetrans*, and the pine wood nematode, *Bursaphelenchus xylophilis* (Ohtani *et al*., 2011). It is also reported to have antibacterial activity against *Staphylococcus aureus* (Zuo *et al*., 2014). A degradation product of *p*-hydroxylphenylacetic acid is 3, 4-dihydrobenzenaldehyde, a precursor of the anti-parkinson drug, levodopa.

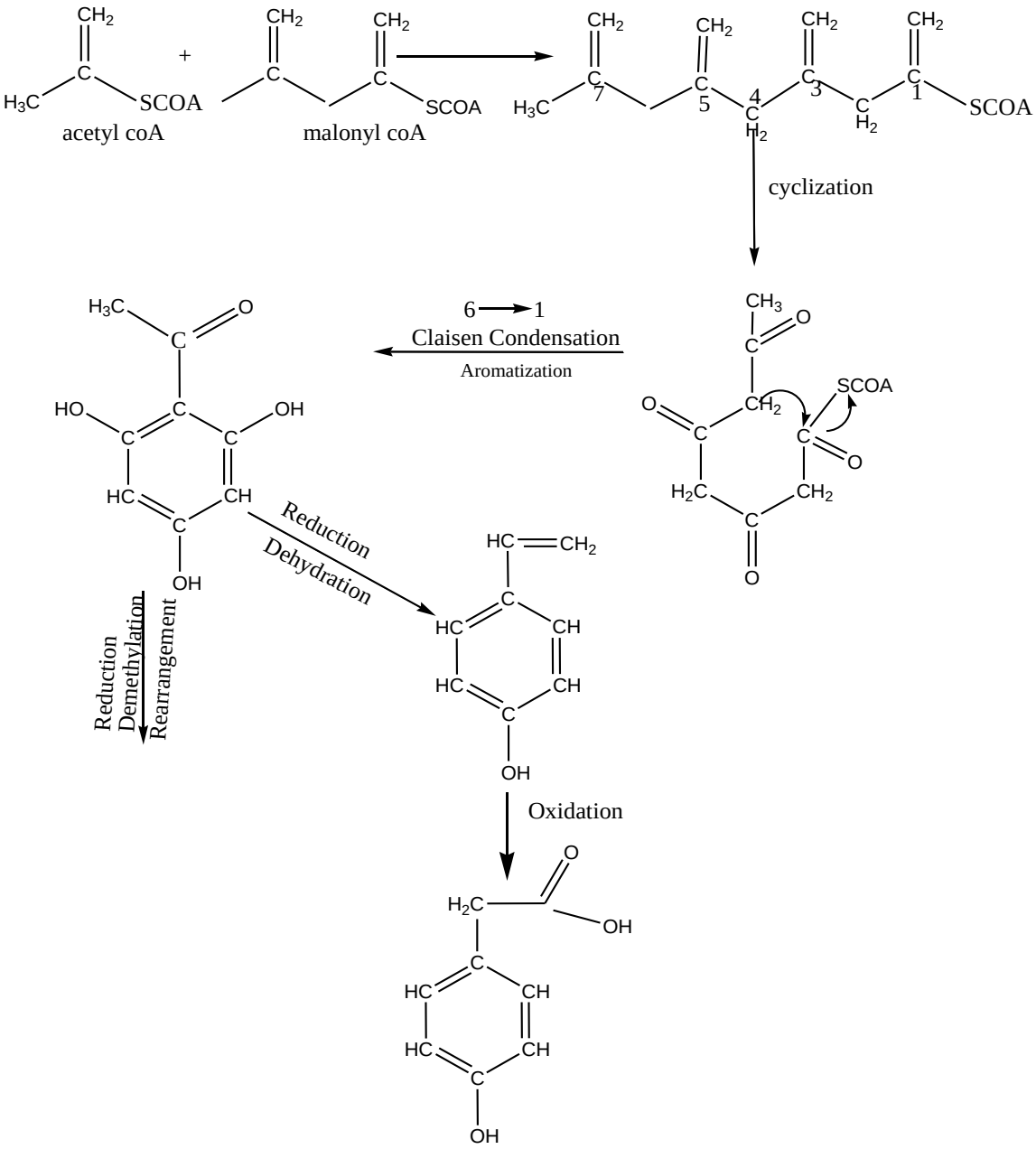


Figure 4.37: Plausible biosynthetic pathway for the simple *p*-Hydroxyl acetic acid

4.2.1.3 INDOLE-3-ACETIC ACID

Plant hormones are undoubtedly among the main secondary metabolities that can influence plant fitness and enhance development when exogenously administered. Many fungal species have been reported to be able to produce compounds such as indole-3-acetic acid (IAA) (Manci *et al.*, 2015).

Indole-3-acetic acid is the most common plant hormone of the auxin class and it regulates various aspects of plant growth and development (Teale, *et al*., 2006; Spaepen *et al*., 2007). Therefore, the term auxin and indole-3-acetic acid are sometimes used interchangeably.

Experimental findings have shown that biosynthetic pathways for IAA may evolve independently in plant and fungi (Manci *et al*., 2015).

4.2.1.4: BIOSYNTHESIS OF INDOLE-3-ACETIC ACID

Fu *et al*., (2015) reviewed the biosynthesis of indole-3-acetic acid in different organisms. They indicated that the various pathways in plants and microorganisms are highly similar, although some intermediates are different. Both tryptophan (Trp)-dependent and Trp-independent indole-3-acetic acid biosynthetic coexist in plants and microbes. The intermediate stages, and genes involved in Trp-independent pathways remain undefined, so information on the biochemical processes involved is limited (Fu *et al*., 2015).

Four Trp-dependent pathways have been proposed: Indole-3-acetamide (IAM), indole-3-pyruvic acid (IPA), tryptamine (TRA), and indole-3-acetaldoxime pathways (Mano and Nemoto, 2012). Although different plant species might use specific strategies or modifications to optimize synthetic pathways, plants would be expected to share evolutionarily conserved core mechanisms for indole-3-acetic acid.

However, only few studies on the indole-3-acetic acid biosynthesis pathway in fungi have been conducted (Basse *et al*., 1996; Robinson *et al*., 1998; Reineke *et al*., 2008). Feeding experiments and *in vitro* assays have confirmed the presence of IAM and IPA pathways in the mycoherbicide, *Colletotrichum gloeosporioides f. sp. aeschynomene*. In addition, these experiments and assays showed that the IAM pathway was the major pathway used by the fungus to produce indole-3-acetic acid in culture (Robinson *et al*., 1998).

Different environmental factors modulate and influence the biosynthesis of indole-3-acetic acid. These include pH and temperature (Spaepen *et al*., 2007). Optimal pH of 6.0 -9.0 and temperature at 28^{o}C have been suggested.

4.2.2 *ENDOMELANCONIOPSIS SP.*

Endomelanconiopsis sp. is a new anamorph genus in the Botryosphaeriaceae, and usually lives inside plant tissues (Rojas *et al.,* 2008; Sun *et al*., 2016). *Endomelanconiopsis* is characterized by eustromatic conidiomata and holoblastically produced, brown, nonapiculate, unicellular conidia, each with a longitudinal germ slit (Rojas *et al.,* 2008). Endophytic *Endomelanconiopsis spp.* have been isolated from several plants which includes *Theobroma cacao* (Rojas *et al.,* 2008), *Heisteria concinna* (Rojas *et al.,* 2008), *Dendropanax arboreus* (Ramos-Garza *et al*., 2016), *Ficus hirta* (Sun *et al*., 2016), etc.

Secondary metabolites isolated from *Endomelanconiopsis sp* include endomeketals A and B, 2, 3-dimethylcyclopent-2-en-1-one and 2-hydroxymethyl-3-methylcyclopent-2-enone (Sun *et al*., 2016).

In this study, the *Endomelanconiopsis sp*. crude extract and fractions were tested for antimicrobial, antioxidant, antitubercular and cytotoxic activities.

In the cytotoxicity assay, at a concentration of 10 µg/ml, *Endomelanconiopsis sp*. crude extract showed poor cytotoxic activity against mouse lymphoma cell lines (L5178Y), with a percentage growth inhibition of 4.4. At same concentration, the *Endomelanconiopsis sp* crude extract showed no antitubercular activity against *Mycobacterium tuberculosis.*

Results of the DPPH antioxidant assay revealed that at a concentration of 500 µg/ml, the *Endomelanconiopsis sp* crude extract and fractions showed varying degrees of antioxidant activities, with higher activity recorded for the fractions. The fungal crude extract recorded percentage inhibition of 22.8. Two fractions of *Endomelanconiopsis sp* extract (PNMR4F7 and PNMR4F15) showed good antioxidant activity with percentage inhibition of 89.8 and 77.1 respectively. These were comparable to the percentage inhibition of 91.7 recorded for the positive control, quercetin.

The results of the antimicrobial screening revealed that at a concentration of 10 mg/ml, *Endomelanconiopsis sp* crude extracts showed antibacterial and antifungal activities against the bacterial and fungal test organisms.

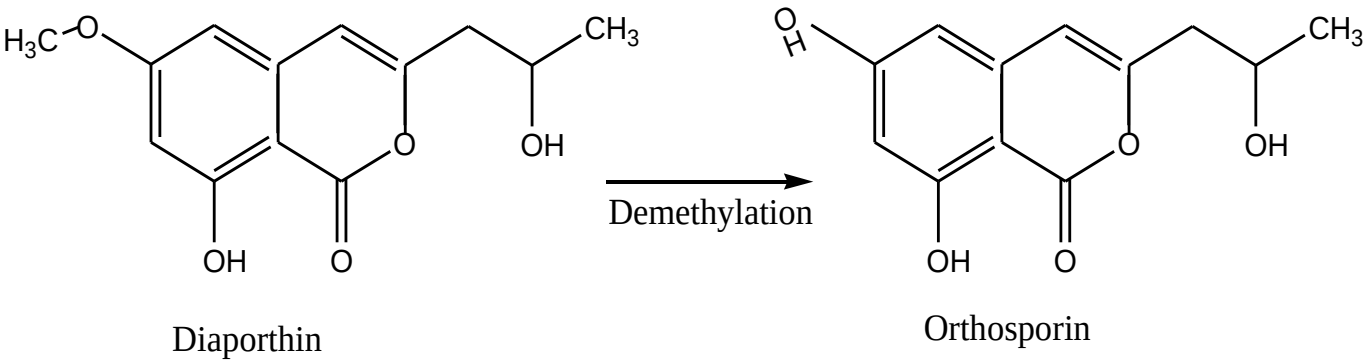
From the fractions of *Endomelanconiopsis sp.* extract, four bioactive compounds were isolated.These are orthosporin, tenuazonic acid, methyl 4-methoxybenzoate and methyl 2-hydroxyl-4-methylbenzoate.

Orthosporin was extracted from PNMR4 F7, tenuazonic was from PNMR4 F15 while methyl 4-methoxybenzoate and methyl 2-hydroxyl-4-methylbenzoate were extracted from PNMR4 F5.

4.2.2.1 ORTHOSPORIN

Orthosporin has been isolated from several fungi such as *Drechslera siccanus* (Hallock *et al* 1988) and from the fruiting body of *Daldinia concentric* (In-Keyoung *et al*. 2006).

Orthosporin was isolated in 1988 by Hallock *et al*, from the pathogenic fungus, *Drechslera siccans* that causes irregular brown spots on leaves of oats and both perennial and Italian ryegrass. It was also isolated from the *Rhynochosporuim orthosporum*, which causes leaf scaled on orchard grass (Ichihara *et al.*, 1989); and from the fruiting body of *Daldinia concetrica* (Lee *et al.*, 2006). Mantle (2001) suggested that the biosynthesis of orthosporin may occur through demethylation of diaporthin a phytotoxins as shown in Figure 4.38. However, this is the first time it is being isolated from this endophytic fungus.



Diaporthin Orthosporin

Figure 4.38: Demethylation of Diaporthin to Orthosporin

4.2.2.2 TENUAZONIC ACID

A look at the ^{1}H – NMR showed that, there might be three methyl groups. One singlet was shifted down field to δ3.84 (H-7) due to the adjacent carbonyl group. A doublet was observed at δ0.99 (H-11) adjacent to a CH group at position H-8, (δ1.91). A triplet appeared at δ0.90 (H-10). This is adjacent to CH_2 group (H-9A, δ1.17 and H-9B, δ1.24). The hydroxyl group was not observed. The proton 5 was shifted to δ2.42 due to the neighboring NH group and appeared as a doublet.

From the mass spectrum, and ^{1}H-NMR, this compound was found to be tenuazonic acid. It showed comparable UV spectra in comparison with the report of Nolte *et al* (1980).

Tenuazonic acid, a tetramic acid, was first reported as an isolated metabolite from *Alternaria tenius* (Rossett *et al* 1957). Since then, it has been reported to have been found in some other fungi including alternaria species and Aspergillus (Hassan, 2007).

Tetramic acids are pyrolidine-2,4-diones acetylated at the 3-position. They constitute an important group of secondary metabolites which exhibit diverse biological activities.

In this study, tenuazonic acid was isolated from the *Endomelanconiopsis sp.* It has been found in several *Alternaria, Aspergillus, Sphacopsidales* strains and *Nigrospora sp*. (Kjer, 2009).

Pyrolidine-2,4-dione is biosynthesized from L-isoleucine and two (2) molecules of acetate, by elimination of 2 molecules of water (Nolte *et al* 1980; Royles 1995). Tenuazonic acid displayed keto-enol tautomerism and these different forms of the tetrameric acids in solution must be considered in the determination of the structure of the substance.

Tenuazonic acid has been described to possess insecticidal, antibacterial, antineoplastic and antiviral activities (Royles 1995). It has however been associated to the cause of several

diseases in crops such as sheet rot disease in rice and tobacco. Furthermore, due to its pronounced toxicities, it is considered unsafe for pharmaceutical use.

4.2.2.2.1 TAUTOMERISM AND BIOSYNTHESIS OF TENUAZONIC ACID

The tautomeric behaviour of 3-acylpyrrolidine-2, 4 diones (e.g. tenuazonic acid involves two sets of rapidly interchanging internal tautomers (a-b) and (c-d), where each set arises through proton transfer along the intramolecular hydrogen bond, together with two pairs of slowly interconverting external tautomers (ab – cd), arising from the rotation of the acyl side chain (Figure 4.39). It was found that the internal tautomerization occurs too rapidly to be detected on the time scale of an NMR experiment, the external tautomerism, however, occurs at a rate which can be measured on the NMR time scale. In non polar solvents (e.g. $CDCI_3$) the interconversion between the external enolic tautomers (ab–cd) was found to be a comparatively slow process, while interconversion between the pairs of internal tautomers (a-b, c–d) was found to be fast. Thus, the two sets of resonances observed in the NMR spectra are attributed to the external tautomer (ab) and (cd). In polar solvent (e.g. CD_3OD) the two external pairs were found to interconvert at a much faster rate and, therefore, the NMR signals of the external tautomers coalesce (Nolte, 1980; Royles, 1995).

From biosynthetic studies on tenuazonic acid established by feeding experiments using ^{14}C-labelled acetate it was concluded that the biosynthesis occurs via cyclization of N-acetoacetyl-L isoleucine to produce tenuazonic acid as shown in Figure 4.40 (Royles, 1995).

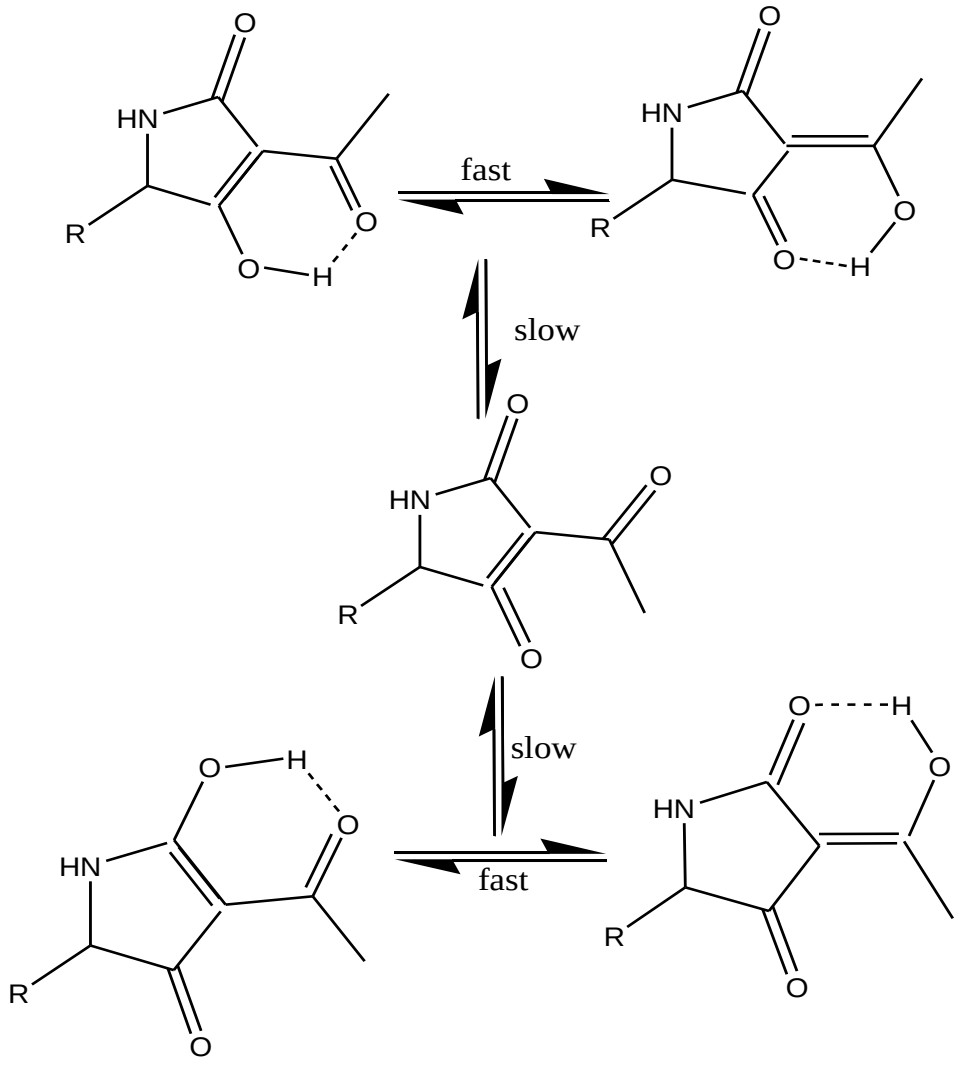


Figure 4.39: Tautomerism of 3-acylpyrrolidine-2, 4-diones (Nolte, 1980)

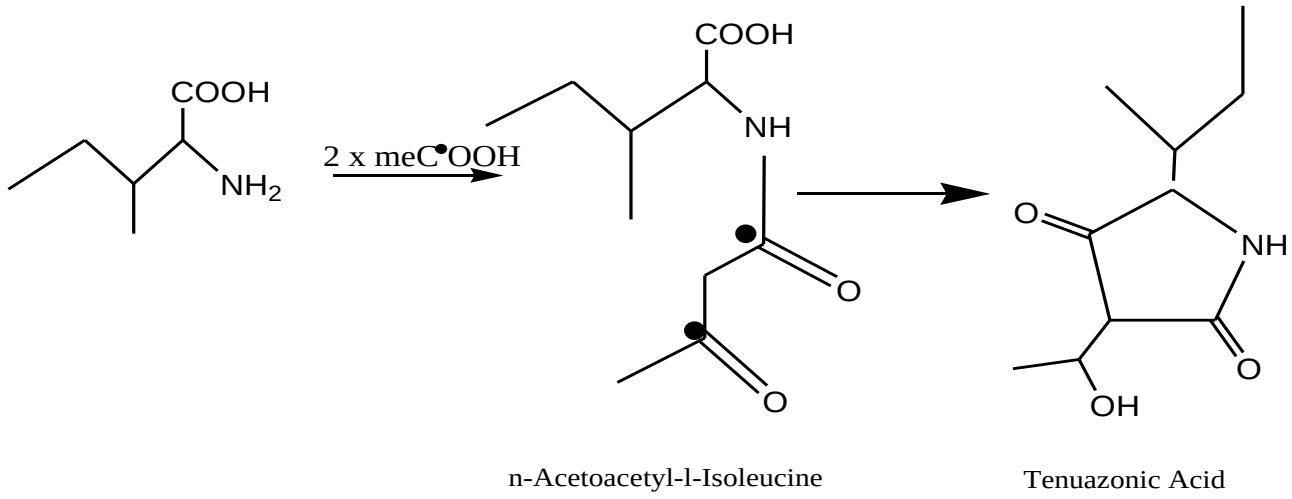


Figure 4.40: Postulated Biosynthesis of Tenuazonic Acid (Royles, 1995)

4.2.2.3 BENZOATE COMPOUNDS

Methyl 4-methoxybenzoate and methyl 2-hydroxy-4-methylbenzoate are natural esters (salicylate) which are useful in the preservation of food, pain control, and fever control (Ameen and Olatunji 2005).

Demethylation of methyl 2-hydroxy-4-methylbenzoate leads to the formation or synthesis of methyl salicylate which is used as a analgestic agent and disinfectant commercially used in toothpaste, mounth wash, perfumes as well as flavouring agent (Finar, 1993).

Various derivatives of benzoate have been attributed to have antifeedants effect on adult pine weevils (Legrands *et al*., 2004). Therefore, further studies on the compound may reveal its potential as a bioinsectide.

CHAPTER FIVE

CONCLUSION AND RECOMMENDATION

5.1 CONCLUSION

Though lots of researches have been carried out in other countries of the world, especially with the advances in new tools used in natural products research, published works on the isolation and characterization of secondary metabolites from fungi associated with Nigerian plants seem to be very scanty.

There are a lot of medicinal plants in Nigeria whose endophytes have not been studied for production of secondary metabolites. Such studies are important as there seem to be a relationship between the host plant and their endophytes.

Isolation of compounds with bioactivities from endophytes instead of harvesting a great quantity of plant material to extract the bioactive molecules will help in preserving our natural flora and biodiversity.

From this work, two endophytic fungi were isolated from a Nigerian terrestrial plant, *Picralima nitida.* This is the first report on the isolation of endophytic fungus from the plant.

Three pure compounds were isolated from the endophytic fungus, *Curvularia sp*. They are acropyrone, indole-3-acetic acid and 4-hydroxylphenyl acetic acid. Their pharmacological properties are already well documented.

From endophytic fungus, *Endomelanconiopsis sp*. were isolated four compounds, orthosporin, tenuazonic acid, methyl 4-methoxybenzoate and methyl 2-hydroxy-4-methylbenzoate. Tenuazonic acid is well known and its pharmacological properties are well known. This research work presented the first report of the isolation of orthosporin from this fungal species and from a Nigerian plant.

From this study, it implies that Nigerian indigenous plants whether of terrestrial, fresh water or marine source, habour lots of endophytes which could be a source or reservoir of compounds with potential pharmacological activities. They could also harbour chemical compound which could be lead molecules in the production of bioactive substances with pharmacological activity.

5.2 RECOMMENDATIONS

1. Large scale fermentation of the endophytic fungi should be adopted to enhance the isolation of large quantities of bioactive compounds of interest. This will aid in more pharmacological investigation on all the isolated compounds and may lead to discovery of new lead compounds.

2. Modulation studies on the isolated endophytic fungus should be encouraged. This may lead to discovery of new lead compounds with new pharmacological activities and less side effect.

3. Government, corporate organizations as well as well-meaning individuals by way of corporate social responsibility should invest in drug discovery programmes especially in the procurement of research equipment and infrastructure to facilitate studies of this nature.

5.3 CONTRIBUTIONS TO KNOWLEDGE

This research work presents the first report of the isolation of acropyrone, 2-(4-hydroxyphenyl) acetate, indole-3-acetic acid, orthosporin, tenuazonic acid, methyl 4-methoxybenzoate and methyl 2-hydroxy-4-methylbenzoate from the endophytic fungal

species used in the study. One of the isolated compounds, orthosporin was isolated for the first time from a Nigerian plant.

This is the first time endophytes from *P. nitida* are evaluated.

This study further revealed the potentials possessed by Nigerian indigenous plants as sources of endophytes that hold key of possibilities to the discovery of novel molecules for both pharmaceutical and agricultural applications.

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APPENDICES

APPENDIX 1: MOLECULAR IDENTIFICATION OF ENDOPHYTIC FUNGI

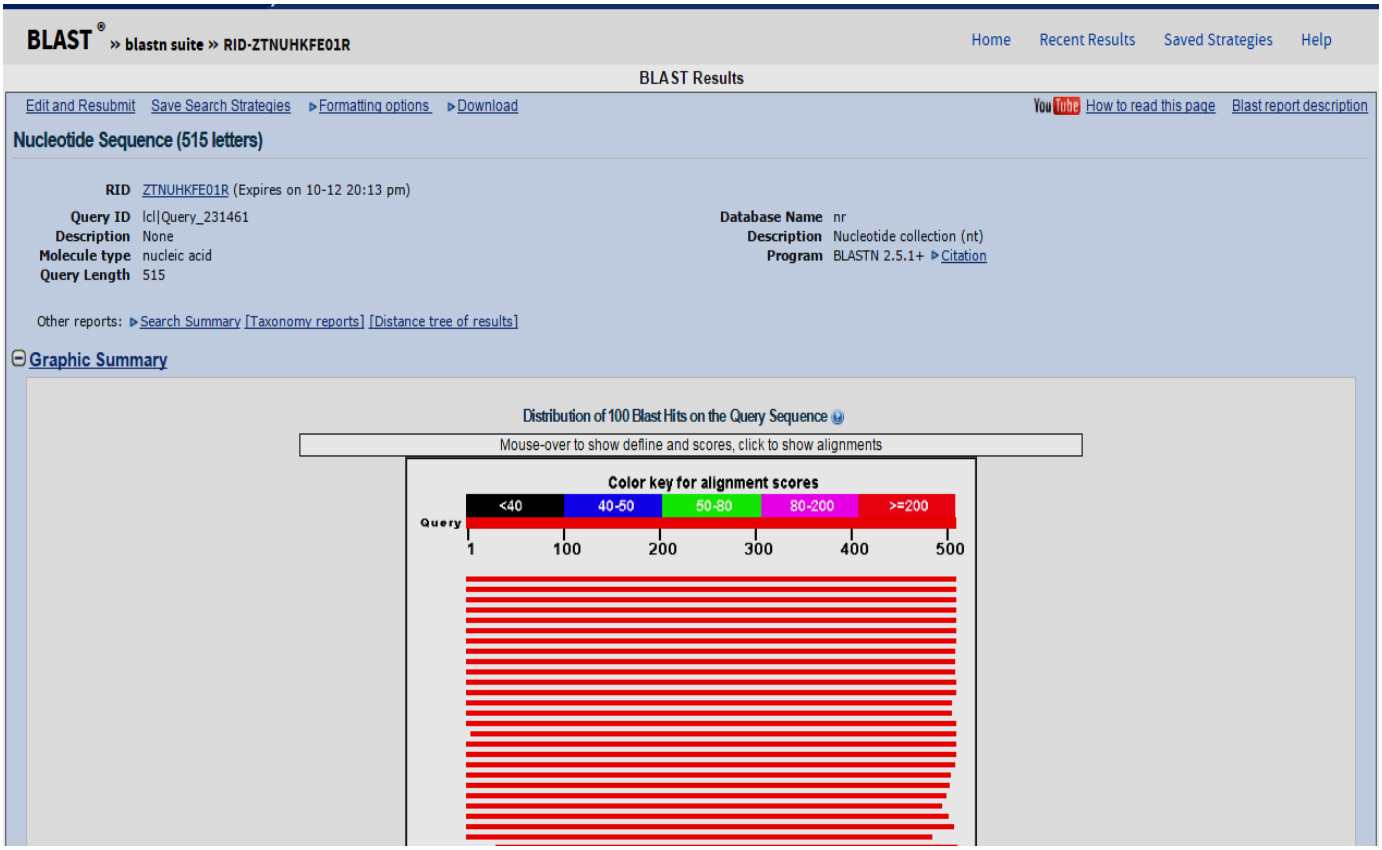
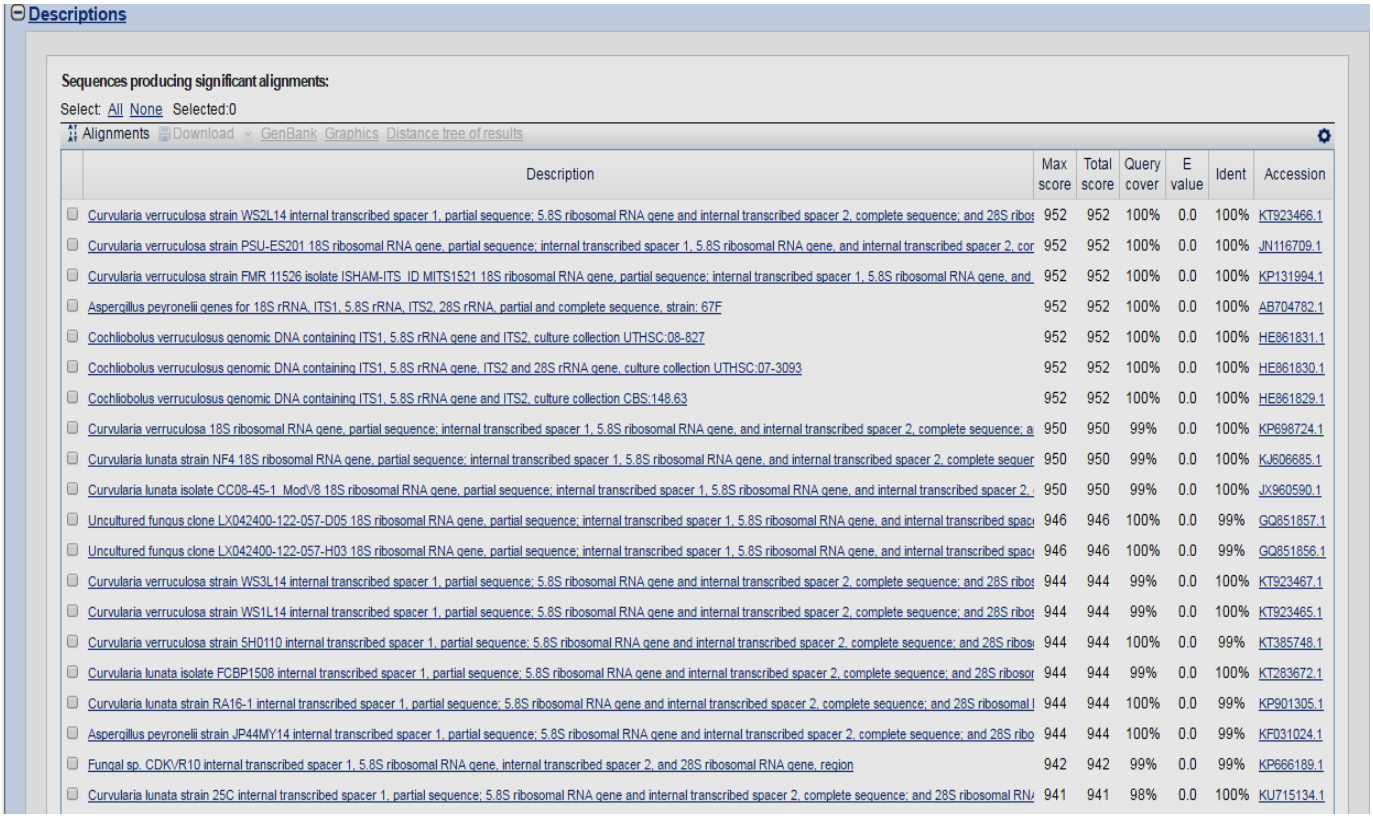


Figure A1: BLAST search result for *Curvularia sp* showing the distribution of BLAST hits on the Fungal DNA sequence



Descriptions

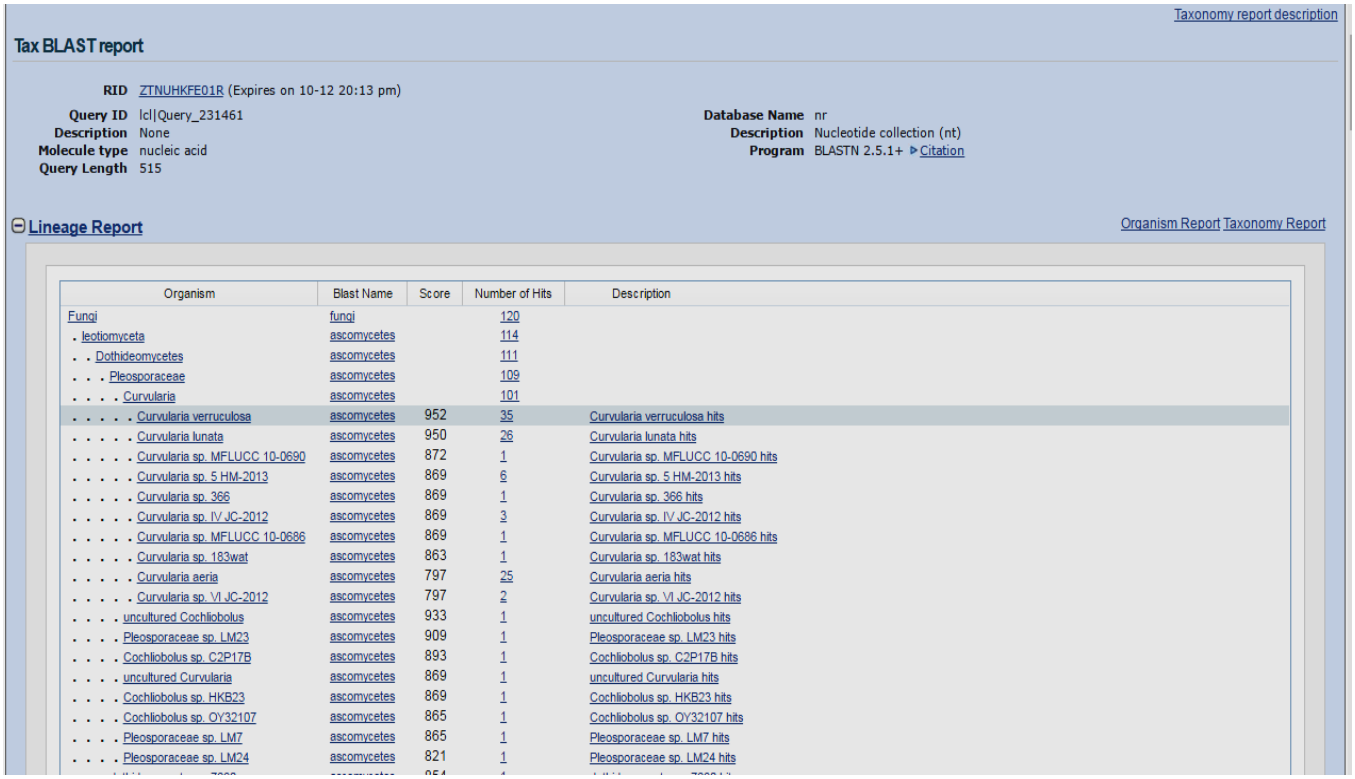
Sequences producing significant alignments:

Select: All None Selected:0

Alignments Download GenBank Graphics Distance tree of results

Description	Max score	Total score	Query cover	E value	Ident	Accession
Curvularia verruculosa strain WS2L14 internal transcribed spacer 1, partial sequence; 5.8S ribosomal RNA gene and internal transcribed spacer 2, complete sequence; and 28S ribos	952	952	100%	0.0	100%	KT923466.1
Curvularia verruculosa strain PSU-ES201 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal transcribed spacer 2, cor	952	952	100%	0.0	100%	JN116709.1
Curvularia verruculosa strain FMR 11526 isolate ISHAM-ITS_ID MITS1521 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene, and	952	952	100%	0.0	100%	KP131994.1
Aspergillus peyronelii genes for 18S rRNA, ITS1, 5.8S rRNA, ITS2, 28S rRNA, partial and complete sequence, strain: 67F	952	952	100%	0.0	100%	AB704782.1
Cochliobolus verruculosus genomic DNA containing ITS1, 5.8S rRNA gene and ITS2, culture collection UTHSC:08-827	952	952	100%	0.0	100%	HE861831.1
Cochliobolus verruculosus genomic DNA containing ITS1, 5.8S rRNA gene, ITS2 and 28S rRNA gene, culture collection UTHSC:07-3093	952	952	100%	0.0	100%	HE861830.1
Cochliobolus verruculosus genomic DNA containing ITS1, 5.8S rRNA gene and ITS2, culture collection CBS:148.63	952	952	100%	0.0	100%	HE861829.1
Curvularia verruculosa 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal transcribed spacer 2, complete sequence; a	950	950	99%	0.0	100%	KP698724.1
Curvularia lunata strain NF4 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal transcribed spacer 2, complete sequer	950	950	99%	0.0	100%	KJ606685.1
Curvularia lunata isolate CC08-45-1_ModV8 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal transcribed spacer 2,	950	950	99%	0.0	100%	JX960590.1
Uncultured fungus clone LX042400-122-057-D05 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal transcribed spac	946	946	100%	0.0	99%	GQ851857.1
Uncultured fungus clone LX042400-122-057-H03 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal transcribed spac	946	946	100%	0.0	99%	GQ851856.1
Curvularia verruculosa strain WS3L14 internal transcribed spacer 1, partial sequence; 5.8S ribosomal RNA gene and internal transcribed spacer 2, complete sequence; and 28S ribos	944	944	99%	0.0	100%	KT923467.1
Curvularia verruculosa strain WS1L14 internal transcribed spacer 1, partial sequence; 5.8S ribosomal RNA gene and internal transcribed spacer 2, complete sequence; and 28S ribos	944	944	99%	0.0	100%	KT923465.1
Curvularia verruculosa strain 5H0110 internal transcribed spacer 1, partial sequence; 5.8S ribosomal RNA gene and internal transcribed spacer 2, complete sequence; and 28S ribos	944	944	100%	0.0	99%	KT385748.1
Curvularia lunata isolate FCBP1508 internal transcribed spacer 1, partial sequence; 5.8S ribosomal RNA gene and internal transcribed spacer 2, complete sequence; and 28S ribosor	944	944	99%	0.0	100%	KT283672.1
Curvularia lunata strain RA16-1 internal transcribed spacer 1, partial sequence; 5.8S ribosomal RNA gene and internal transcribed spacer 2, complete sequence; and 28S ribosomal I	944	944	100%	0.0	99%	KP901305.1
Aspergillus peyronelii strain JP44MY14 internal transcribed spacer 1, partial sequence; 5.8S ribosomal RNA gene and internal transcribed spacer 2, complete sequence; and 28S ribo	944	944	100%	0.0	99%	KF031024.1
Fungal sp. CDKVR10 internal transcribed spacer 1, 5.8S ribosomal RNA gene, internal transcribed spacer 2, and 28S ribosomal RNA gene, region	942	942	99%	0.0	99%	KP666189.1
Curvularia lunata strain 25C internal transcribed spacer 1, partial sequence; 5.8S ribosomal RNA gene and internal transcribed spacer 2, complete sequence; and 28S ribosomal RN	941	941	98%	0.0	100%	KU715134.1

Figure A2: BLAST search result for *Curvularia sp* showing identical sequences with similar alignments



Taxonomy report description

Tax BLAST report

RID ZTNUHKFE01R (Expires on 10-12 20:13 pm)

Query ID lcl|Query_231461
Description None
Molecule type nucleic acid
Query Length 515

Database Name nr
Description Nucleotide collection (nt)
Program BLASTN 2.5.1+ ▷Citation

Lineage Report

Organism Report Taxonomy Report

Organism	Blast Name	Score	Number of Hits	Description
Fungi	fungi		120	
. leotiomyceta	ascomycetes		114	
. . Dothideomycetes	ascomycetes		111	
. . . Pleosporaceae	ascomycetes		109	
. . . . Curvularia	ascomycetes		101	
. . . . . Curvularia verruculosa	ascomycetes	952	35	Curvularia verruculosa hits
. . . . . Curvularia lunata	ascomycetes	950	26	Curvularia lunata hits
. . . . . Curvularia sp. MFLUCC 10-0690	ascomycetes	872	1	Curvularia sp. MFLUCC 10-0690 hits
. . . . . Curvularia sp. 5 HM-2013	ascomycetes	869	6	Curvularia sp. 5 HM-2013 hits
. . . . . Curvularia sp. 366	ascomycetes	869	1	Curvularia sp. 366 hits
. . . . . Curvularia sp. IV JC-2012	ascomycetes	869	3	Curvularia sp. IV JC-2012 hits
. . . . . Curvularia sp. MFLUCC 10-0686	ascomycetes	869	1	Curvularia sp. MFLUCC 10-0686 hits
. . . . . Curvularia sp. 183wat	ascomycetes	863	1	Curvularia sp. 183wat hits
. . . . . Curvularia aeria	ascomycetes	797	25	Curvularia aeria hits
. . . . . Curvularia sp. VI JC-2012	ascomycetes	797	2	Curvularia sp. VI JC-2012 hits
. . . . uncultured Cochliobolus	ascomycetes	933	1	uncultured Cochliobolus hits
. . . . Pleosporaceae sp. LM23	ascomycetes	909	1	Pleosporaceae sp. LM23 hits
. . . . Cochliobolus sp. C2P17B	ascomycetes	893	1	Cochliobolus sp. C2P17B hits
. . . . uncultured Curvularia	ascomycetes	869	1	uncultured Curvularia hits
. . . . Cochliobolus sp. HKB23	ascomycetes	869	1	Cochliobolus sp. HKB23 hits
. . . . Cochliobolus sp. OY32107	ascomycetes	865	1	Cochliobolus sp. OY32107 hits
. . . . Pleosporaceae sp. LM7	ascomycetes	865	1	Pleosporaceae sp. LM7 hits
. . . . Pleosporaceae sp. LM24	ascomycetes	821	1	Pleosporaceae sp. LM24 hits

Figure A3: BLAST search result for *Curvularia sp* showing the taxonomic details of the fungus

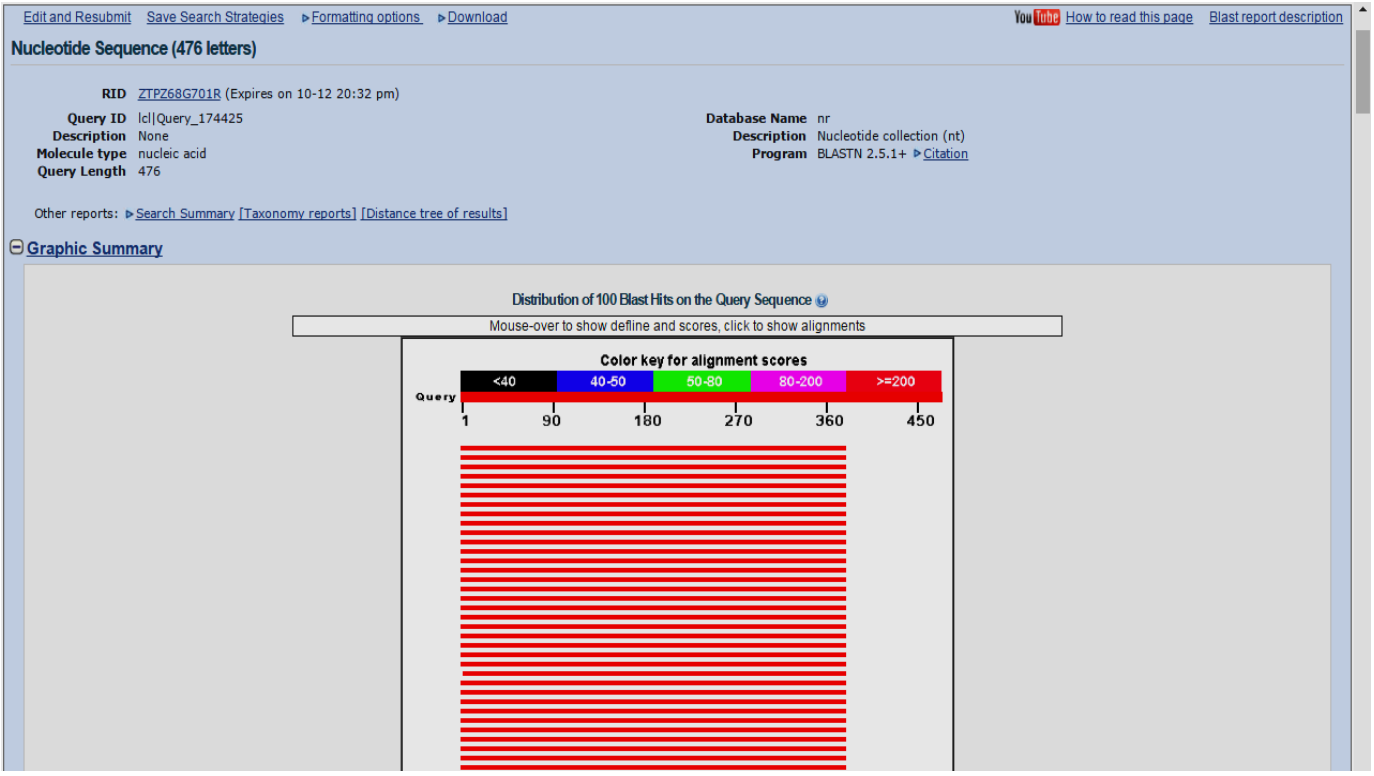
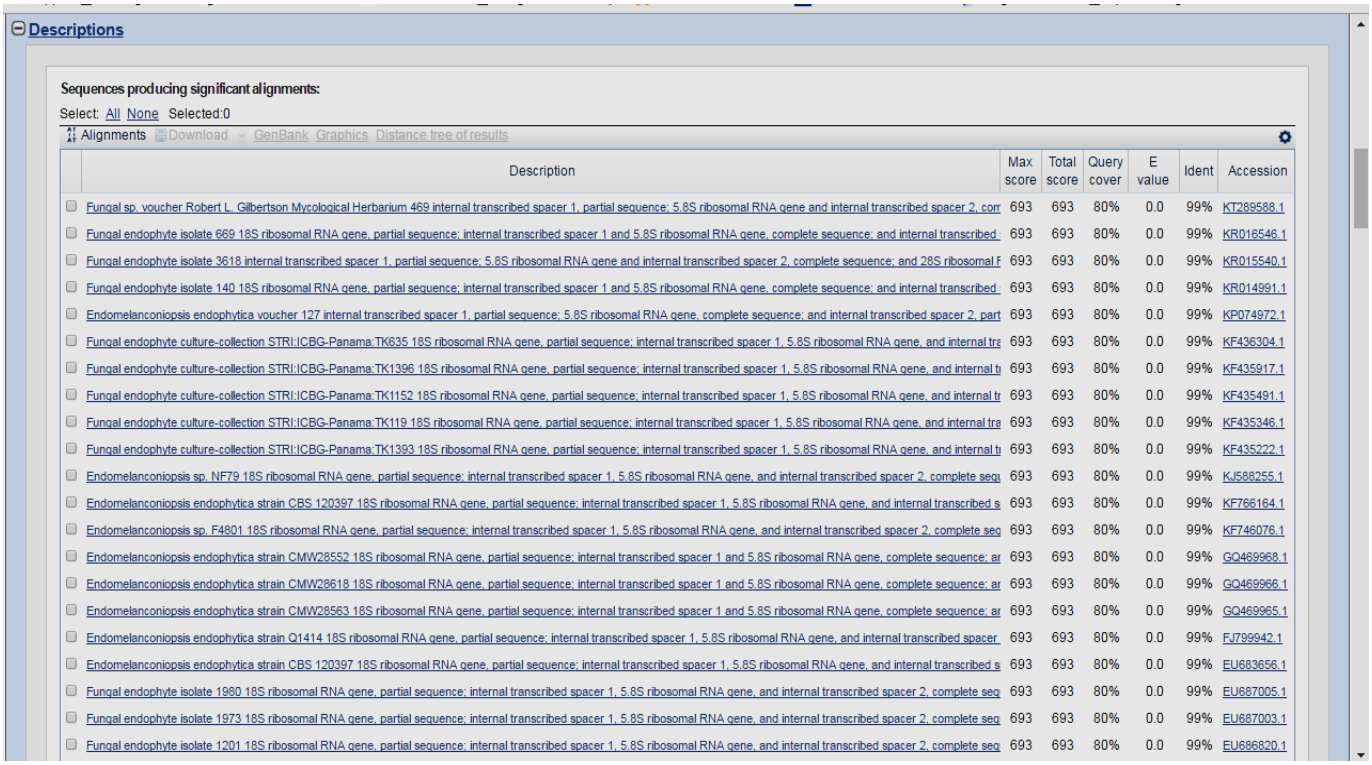


Figure A4: BLAST search result for *Endomelanconiopsis sp* showing the distribution of BLAST hits on the Fungal DNA sequence



Descriptions

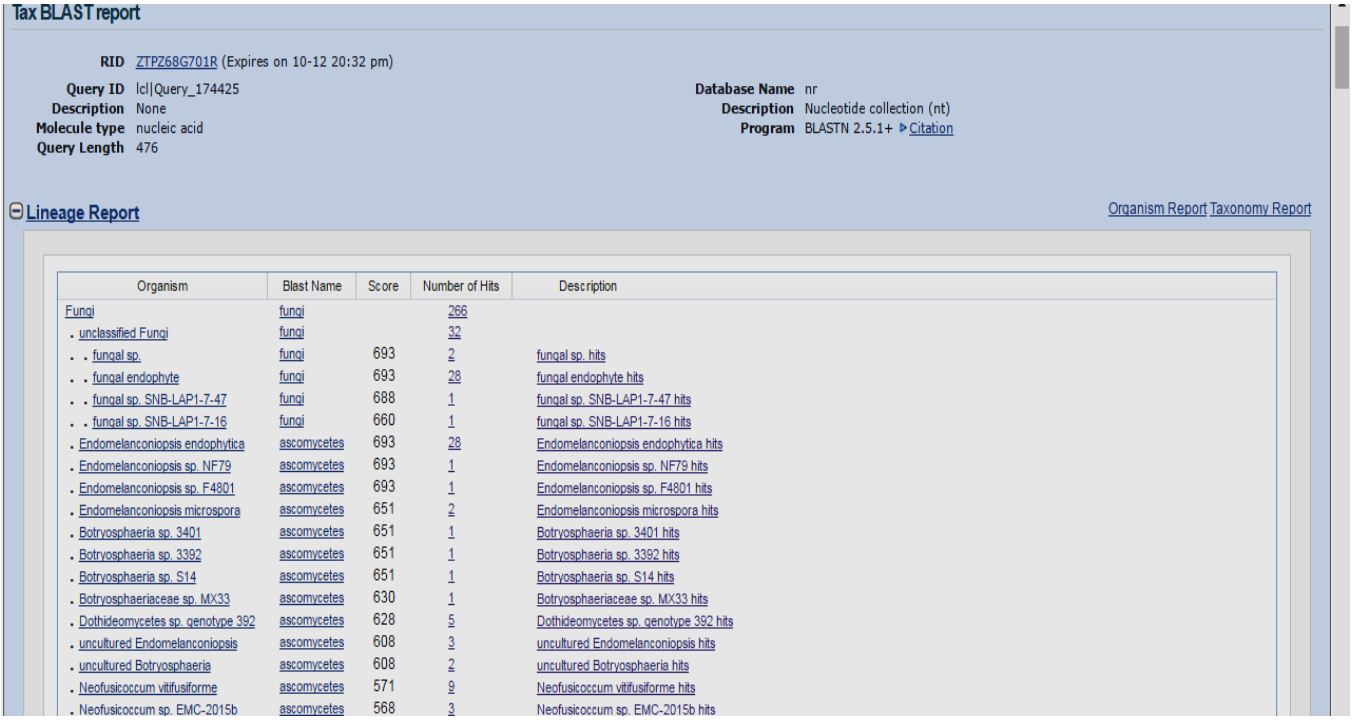
Sequences producing significant alignments:

Select: All None Selected:0

Alignments Download GenBank Graphics Distance tree of results

Description	Max score	Total score	Query cover	E value	Ident	Accession
Fungal sp. voucher Robert L. Gilbertson Mycological Herbarium 469 internal transcribed spacer 1, partial sequence; 5.8S ribosomal RNA gene and internal transcribed spacer 2, cor	693	693	80%	0.0	99%	KT289588.1
Fungal endophyte isolate 669 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1 and 5.8S ribosomal RNA gene, complete sequence; and internal transcribed	693	693	80%	0.0	99%	KR016546.1
Fungal endophyte isolate 3618 internal transcribed spacer 1, partial sequence; 5.8S ribosomal RNA gene and internal transcribed spacer 2, complete sequence; and 28S ribosomal F	693	693	80%	0.0	99%	KR015540.1
Fungal endophyte isolate 140 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1 and 5.8S ribosomal RNA gene, complete sequence; and internal transcribed	693	693	80%	0.0	99%	KR014991.1
Endomelanconiopsis endophytica voucher 127 internal transcribed spacer 1, partial sequence; 5.8S ribosomal RNA gene, complete sequence; and internal transcribed spacer 2, part	693	693	80%	0.0	99%	KP074972.1
Fungal endophyte culture-collection STRI:ICBG-Panama:TK635 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal tra	693	693	80%	0.0	99%	KF436304.1
Fungal endophyte culture-collection STRI:ICBG-Panama:TK1396 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal t	693	693	80%	0.0	99%	KF435917.1
Fungal endophyte culture-collection STRI:ICBG-Panama:TK1152 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal tr	693	693	80%	0.0	99%	KF435491.1
Fungal endophyte culture-collection STRI:ICBG-Panama:TK119 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal tra	693	693	80%	0.0	99%	KF435346.1
Fungal endophyte culture-collection STRI:ICBG-Panama:TK1393 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal t	693	693	80%	0.0	99%	KF435222.1
Endomelanconiopsis sp. NF79 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal transcribed spacer 2, complete sequ	693	693	80%	0.0	99%	KJ588255.1
Endomelanconiopsis endophytica strain CBS 120397 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal transcribed s	693	693	80%	0.0	99%	KF766164.1
Endomelanconiopsis sp. F4801 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal transcribed spacer 2, complete seq	693	693	80%	0.0	99%	KF746076.1
Endomelanconiopsis endophytica strain CMW28552 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1 and 5.8S ribosomal RNA gene, complete sequence; ar	693	693	80%	0.0	99%	GQ469968.1
Endomelanconiopsis endophytica strain CMW28618 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1 and 5.8S ribosomal RNA gene, complete sequence; ar	693	693	80%	0.0	99%	GQ469966.1
Endomelanconiopsis endophytica strain CMW28563 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1 and 5.8S ribosomal RNA gene, complete sequence; ar	693	693	80%	0.0	99%	GQ469965.1
Endomelanconiopsis endophytica strain Q1414 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal transcribed spacer	693	693	80%	0.0	99%	FJ799942.1
Endomelanconiopsis endophytica strain CBS 120397 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal transcribed s	693	693	80%	0.0	99%	EU683656.1
Fungal endophyte isolate 1980 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal transcribed spacer 2, complete seq	693	693	80%	0.0	99%	EU687005.1
Fungal endophyte isolate 1973 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal transcribed spacer 2, complete seq	693	693	80%	0.0	99%	EU687003.1
Fungal endophyte isolate 1201 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal transcribed spacer 2, complete seq	693	693	80%	0.0	99%	EU686820.1

Figure A5: BLAST search result for *Endomelanconiopsis sp* showing identical sequences with similar alignments



Tax BLAST report

RID ZTPZ68G701R (Expires on 10-12 20:32 pm)

Query ID lcl|Query_174425
Description None
Molecule type nucleic acid
Query Length 476

Database Name nr
Description Nucleotide collection (nt)
Program BLASTN 2.5.1+ ▸Citation

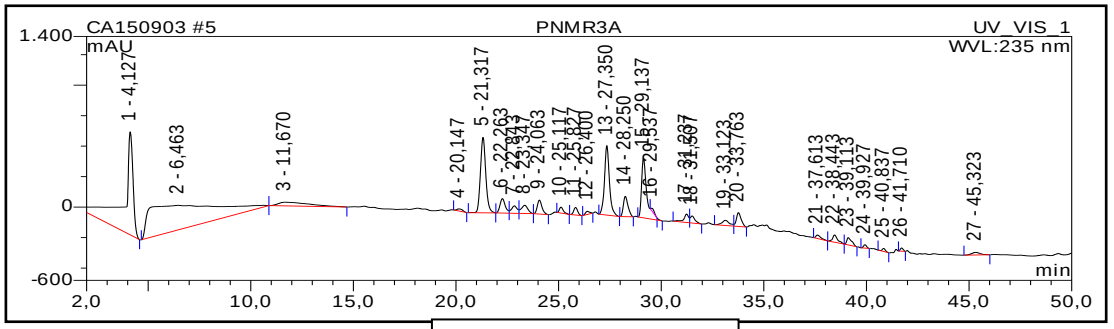
Lineage Report

Organism Report Taxonomy Report

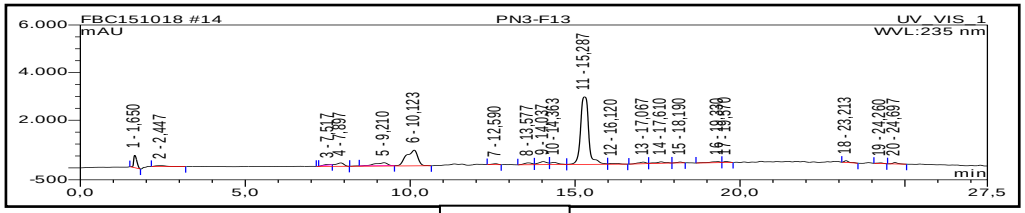
Organism	Blast Name	Score	Number of Hits	Description
Fungi	fungi		266	
. unclassified Fungi	fungi		32	
. . fungal sp.	fungi	693	2	fungal sp. hits
. . fungal endophyte	fungi	693	28	fungal endophyte hits
. . fungal sp. SNB-LAP1-7-47	fungi	688	1	fungal sp. SNB-LAP1-7-47 hits
. . fungal sp. SNB-LAP1-7-16	fungi	660	1	fungal sp. SNB-LAP1-7-16 hits
. Endomelanconiopsis endophytica	ascomycetes	693	28	Endomelanconiopsis endophytica hits
. Endomelanconiopsis sp. NF79	ascomycetes	693	1	Endomelanconiopsis sp. NF79 hits
. Endomelanconiopsis sp. F4801	ascomycetes	693	1	Endomelanconiopsis sp. F4801 hits
. Endomelanconiopsis microspora	ascomycetes	651	2	Endomelanconiopsis microspora hits
. Botryosphaeria sp. 3401	ascomycetes	651	1	Botryosphaeria sp. 3401 hits
. Botryosphaeria sp. 3392	ascomycetes	651	1	Botryosphaeria sp. 3392 hits
. Botryosphaeria sp. S14	ascomycetes	651	1	Botryosphaeria sp. S14 hits
. Botryosphaeriaceae sp. MX33	ascomycetes	630	1	Botryosphaeriaceae sp. MX33 hits
. Dothideomycetes sp. genotype 392	ascomycetes	628	5	Dothideomycetes sp. genotype 392 hits
. uncultured Endomelanconiopsis	ascomycetes	608	3	uncultured Endomelanconiopsis hits
. uncultured Botryosphaeria	ascomycetes	608	2	uncultured Botryosphaeria hits
. Neofusicoccum vitifusiforme	ascomycetes	571	9	Neofusicoccum vitifusiforme hits
. Neofusicoccum sp. EMC-2015b	ascomycetes	568	3	Neofusicoccum sp. EMC-2015b hits

Figure A6: BLAST search result for *Endomelanconiopsis sp* showing the taxonomic details of the fungus

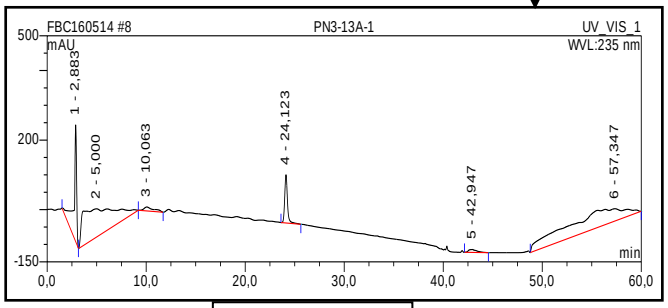
APPENDIX 2: HPLC-GUIDED ISOLATION OF PURE COMPOUNDS FROM CRUDE EXTRACT OF *CURVULARIA SP.*



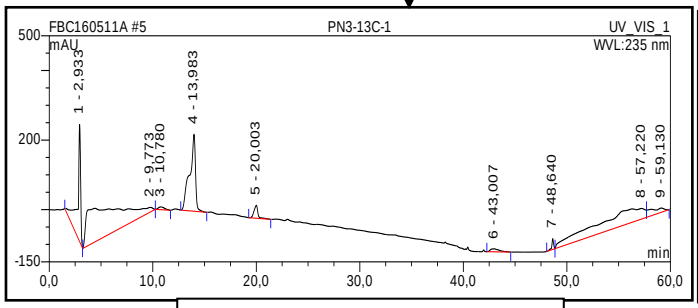
Curvularia sp Crude Extract



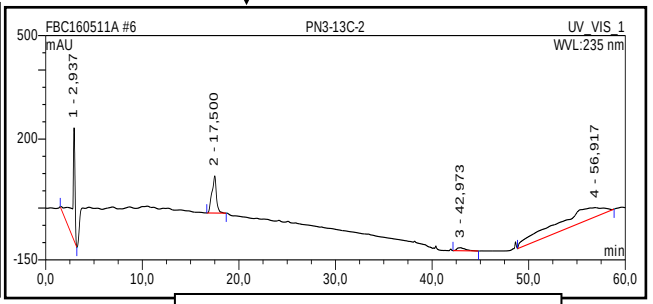
F13



Acropyrone

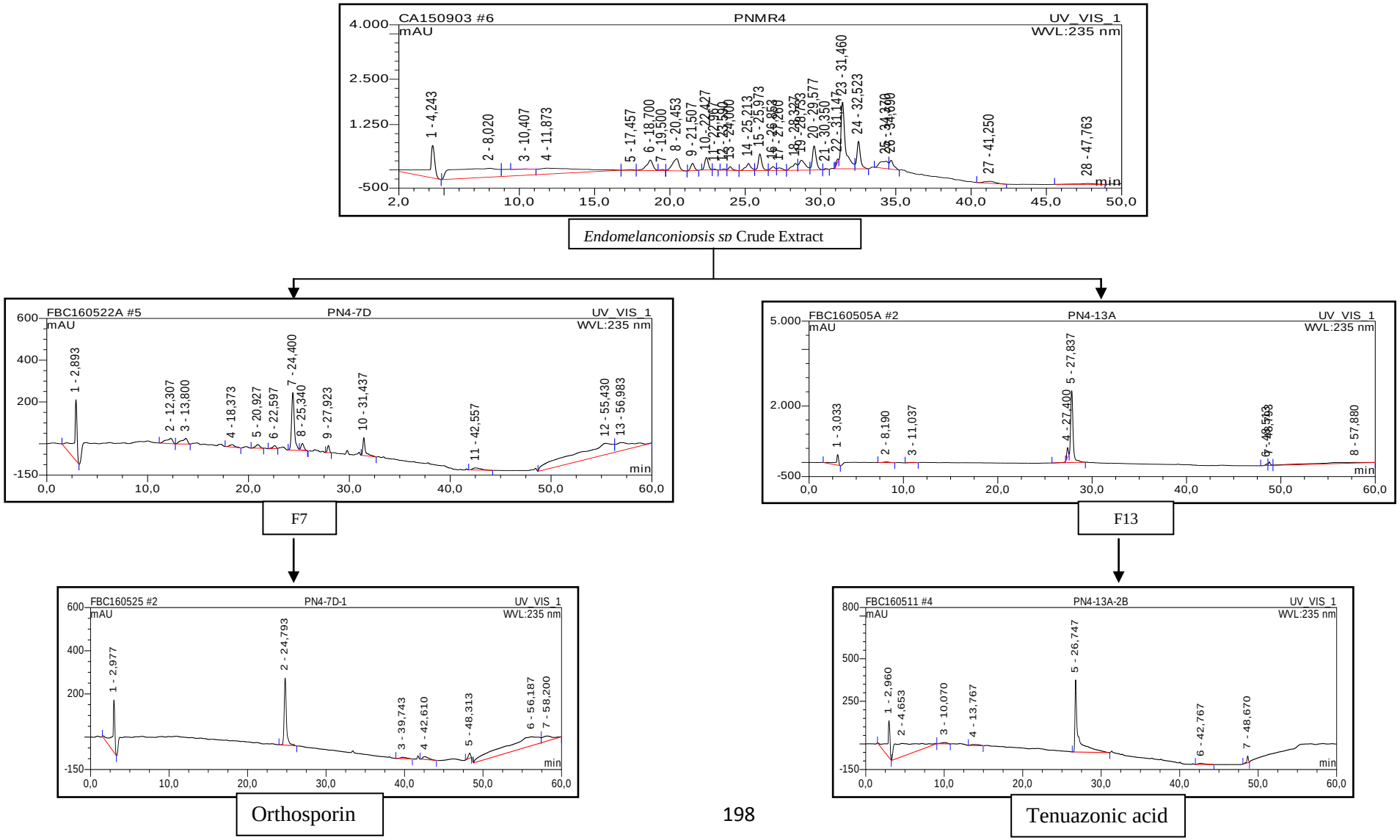


4-Hydroxyphenyl acetic acid



Indole -3- acetic acid

APPENDIX 3: HPLC-GUIDED ISOLATION OF PURE COMPOUNDS FROM CRUDE EXTRACT OF *ENDOMELANCONIOPSIS SP*



APPENDIX 4: ANTIMICROBIAL ASSAY

Table 4.2: Result of Antimicrobial Assay of *Curvularia sp* Crude Extract showing the Mean Inhibition Zone Diameters (IZDs) (mm) produced against test organisms

Test Organisms	IZD (mm) of *Curvularia sp* Crude Extract						Positive control			Negative control		
	10 mg/mL			1 mg/mL			Ciprofloxacin (5 µg/mL)			DMSO		
	A	B	C	A	B	C	A	B	C	A	B	C
E. coli	11	12	10	0	0	0	7	7	8	0	0	0
S. aureus	10	10	10	0	0	0	6	6	6	0	0	0
P. aeruginosa	4	4	4	0	0	0	6	6	5	0	0	0
B. subtilis	20	18	21	0	0	0	8	8	8	0	0	0
							Miconazole (50 µg/mL)			DMSO		
A. niger	6	6	5	0	0	0	12	12	13	0	0	0
C. albicans	10	10	9	0	0	0	17	16	17	0	0	0

Table 4.3: Result of Antimicrobial Assay of VLC fractions of *Curvularia sp* showing the Mean Inhibition Zone Diameters (IZDs) (mm) produced against test organisms

Test Organisms	IZD (mm) of VLC fractions of *Curvularia sp* Extract (1 mg/mL)																																					Positive control			Negative control		
	F1			F4			F5			F6			F7			F8			F9			F10			F11			F12			F14			F15			F16			F17			Ciprofloxacin (5 µg/mL)			DMSO		
	A	B	C	A	B	C	A	B	C	A	B	C	A	B	C	A	B	C	A	B	C	A	B	C	A	B	C	A	B	C	A	B	C	A	B	C	A	B	C	A	B	C	A	B	C
E. coli	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	7	7	8	0	0	0
S. aureus	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	6	6	6	0	0	0
P. aeruginosa	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	6	6	5	0	0	0
B. subtilis	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	8	8	8	0	0	0
																																							Miconazole (50 µg/mL)			DMSO		
A. niger	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	12	12	13	0	0	0
C. albicans	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	17	16	17	0	0	0

Table 4.4: Result of Antimicrobial Assay of *Endomelanconiopsis sp* Crude Extract showing the Mean Inhibition Zone Diameters (IZDs) (mm) produced against test organisms

Test Organisms	IZD (mm) of *Endomelanconiopsis sp* Crude Extract						Positive control			Negative control		
	10 mg/mL			1 mg/mL			Ciprofloxacin (5 µg/mL)			DMSO		
	A	B	C	A	B	C	A	B	C	A	B	C
E. coli	7	8	7	0	0	0	7	7	8	0	0	0
S. aureus	11	11	10	0	0	0	6	6	6	0	0	0
P. aeruginosa	8	8	8	0	0	0	6	6	5	0	0	0
B. subtilis	10	10	9	0	0	0	8	8	8	0	0	0
							Miconazole (50 µg/mL)			DMSO		
A. niger	15	13	16	0	0	0	12	12	13	0	0	0
C. albicans	11	12	10	0	0	0	17	16	17	0	0	0

Table 4.5: Result of Antimicrobial Assay of VLC fractions of *Endomelanconiopsis sp* showing the Mean Inhibition Zone Diameters (IZDs) (mm) produced against test organisms

Test Organisms	IZD (mm) of VLC fractions of *Endomelanconiopsis sp* Extract (1 mg/mL)																																									Positive control			Negative control		
	F1			F4			F5			F6			F7			F8			F9			F10			F11			F12			F14			F15			F16			F17			Ciprofloxacin (5 µg/mL)			DMSO		
	A	B	C	A	B	C	A	B	C	A	B	C	A	B	C	A	B	C	A	B	C	A	B	C	A	B	C	A	B	C	A	B	C	A	B	C	A	B	C	A	B	C	A	B	C	A	B	C
E. coli	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	2	2	2	0	0	0	0	0	0	7	7	8	0	0	0
S. aureus	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	6	6	6	0	0	0
P. aeruginosa	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	6	6	5	0	0	0
B. subtilis	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	2	2	2	0	0	0	0	0	0	8	8	8	0	0	0
																																										Miconazole (50 µg/mL)			DMSO		
A. niger	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	12	12	13	0	0	0
C. albicans	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	17	16	17	0	0	0

APPENDIX 5: DPPH ANTIOXIDANT ASSAY

Antioxidant Assay of Crude Extracts and Fractions of *Endomelanconiopsis sp* and *Curvularia sp*

Sample code	A	B	C	X	% Inhibition
PNMR4 F4	0.902	0.901	0.912	0.905	22.78157
PNMR4 F5	0.661	0.656	0.654	0.657	43.94198
PNMR4 F6	0.38	0.379	0.378	0.379	67.662116
PNMR4 F7	0.114	0.129	0.114	0.119	89.846416
PNMR4 F8	0.982	0.967	0.99	0.979667	16.410666
PNMR4 F9	0.397	0.397	0.404	0.399333	65.927218
PNMR4 F14	0.469	0.467	0.466	0.467333	60.125171
PNMR4 F15	0.282	0.265	0.258	0.268333	77.104949
PNMR4 F17	1.28	1.271	1.26	1.270333	-8.390188
PNMR4 (*Endomelanconiopsis sp*crude extract)	0.897	0.906	0.912	0.905	22.78157
PNMR3AF2	1.302	1.297	1.289	1.296	-10.5802
PNMR3AF4	1.021	1.021	1.15	1.064	9.215017
PNMR3AF5	1.041	1.043	1.037	1.040333	11.234386
PNMR3AF6	0.728	0.73	0.729	0.729	37.798635
PNMR3AF7	0.589	0.573	0.568	0.576667	51.649317
PNMR3AF13	0.266	0.27	0.275	0.270333	76.934044
PNMR3AF14	0.545	0.554	0.555	0.551333	52.957935
PNMR3AF15	0.755	0.749	0.754	0.752667	35.779266
PNMR3AF16	0.882	0.886	0.884	0.884	25.573379
PNMR3AF17	1.131	1.122	1.122	1.125	4.010239
PNMR3A (*Curvularia sp* crude extract)	0.897	0.885	0.884	0.888667	24.175171
Quercitin (positive control)	0.097	0.097	0.098	0.097333	91.695137
Blank(DPPH+MeOH)	1.178	1.167	1.171	1.172	0

Antioxidant Assay of Isolated Pure Compounds

	Sample code	Compound Name	A	B	C	X	% Inhibition
1	PN3-13C-3	Acropyrone	0.305	0.305	0.302	0.304	46.2
2	PN3-13C-1	4-hydroxy phenyl acetic acid	0.247	0.244	0.244	0.245	56.6
3	PN3-13C-2	Indole-3-acetic acid	0.189	0.189	0.189	0.189	66.5
4	PN4-13B	Tenuazonic acid	0.083	0.083	0.082	0.082666667	85.3
5	PN4-7D-1	Orthosporin	0.138	0.132	0.132	0.134	76.3
6	Quercetin	Quercetin	0.091	0.091	0.09	0.090666667	83.9
7	Blank	Blank	0.568	0.564	0.564	0.565333333	0

ccv