ISOLATION AND CHARACTERISATION OF BIOACTIVE COMPOUNDS FROM *VITEX PINNATA* AND ASSOCIATED FUNGAL ENDOPHYTES

A THESIS PRESENTED FOR THE DEGREE OF DOCTOR OF PHILOSOPHY IN THE FACULTY OF SCIENCE THE UNIVERSITY OF STRATHCLYDE

BY

NURKHALIDA KAMAL

BSc. (Honours)

Strathclyde Institute of Pharmacy and Biomedical Sciences

University of Strathclyde

161 Cathedral Street

Glasgow

G4 0RE

United Kingdom

April 2015

This thesis is the result of the author's original research. It has been composed by the author and has not been previously submitted for examination which has led to the award of a degree.

Signed:

Date:

Acknowledgement

First and foremost, I would like to express my gratitude to Allah SWT for blessing me and giving me strength to complete this study.

I am greatly indebted to my first supervisor Dr. RuAngelie Edrada-Ebel for her constantly patience in guiding and shaping me to be a creative and better researcher during the course of my PhD study. Her encouragement, supervision and advises are invaluable and I am grateful to have a supervisor like her. My appreciation also goes to my second supervisor Prof. Alexander I. Gray for his assistance, suggestion and ideas.

My sincerely thanks also to Ms. Carol Clements for the antimicrobial assays, Dr. Alex Thong Zhang for his assistance in NMR and MS instruments, Dr. Roth Tate for his help on molecular DNA isolation procedure, Dr John Igoli for his assistance on soxhlet extraction and Dr. Marc Schumacher for the NF-κB inhibition assay. I also would like acknowledge all MSc and MPharm students who involved in the project including Sirisha, Safwan and Farah.

I would also like to thank to Malaysian Government and Universiti Sultan Zainal Abidin Malaysia for their generosity to fund my PhD study in SIPBS.

My appreciation also goes to all my labmates: Dr. Chiara Viegelmann, Dr. Enitome Bafor, Dr. Ahmed F. Tawfike, Noorwini Mazlan, Weqas Alotaibi, Dr. Lynsey McIntyre, Cheng Cheng, Dr. Tantima Kumlung, Dr. Daniela de Paula, Najwa, Hazniza, Yusnaini, Nashwa, Kirsty, Yahia, Kirsty, Bella, Bish and Hanan for their friendship, support and kindness.

My heartfelt thanks dedicated to my beloved husband Abdul Muhaimin Ismail and my daughter Zara Maryam for their faith, sacrifices, support and endless love during the study. To my parent, parent in-laws, siblings and all my families and friends, thank you for your du'a and prayers.

Last but not least, I wish to express my best regards and gratitude to all of those who supported me in any respect in making this study a success.

Table of Contents

Abstract

Drug discovery from natural sources including plants, marine organisms and endophytes is still pivotal in pharmaceutical research due to their ability of producing diverse and unique secondary metabolites. Discovery of secondary metabolites from leaves of *Vitex pinnata*, a medicinal plant from Malaysia and its associated endophytic fungi, *Lasiodiplodia theobromae*, *Nigrospora* sp and *Pestalotiopsis olivacea* afforded nineteen compounds and several of these compounds exhibited good anticancer, antitrypanosomal and anti-mycobacterial activities. Secondary metabolites isolated from leaves of *V. pinnata* previously reported to exhibit good anti-inflammatory activity and interestingly the leaves were used by local Malay community to treat cuts and wounds. In recent years new strategy using metabolomics in natural products is trending among scientists and found to be efficient, intelligent and robust. Implements of metabolomics including hyphenated HR-LCMS, NMR and integrated with *in-house* database, AntiMarin were utilised to exploit endophytic fungal natural products from *V. pinnata.* Preliminary exploration of secondary metabolites production of all three endophytic fungi was simply achieved by applying metabolomics strategy for medium-scale fermentation. Metabolomics also was used as decision-making strategy in mining active metabolomes of endophytic fungus, *Lasiodiplodia theobromae* against *Trypanosoma brucei brucei.* Dereplication approach using metabolomics for identification of compounds from *Pestalotiopsis olivacea* was easily accomplished.

Abbreviations

AChE	Acetylcholinesterase
BCRP	Breast cancer resistance protein
BLAST	Basic Local Alignment Search Tool
CCl_4	Carbon tetrachloride
CHI	Chalcone isomerase
CoA	Coenzyme A
COX-1	Cyclooxygenase-1
COX-2	Cyclooxygenase-2
DCM	Dichloromethane
DEPT	Distortionless Enhancement by Polarization Transfer
DMSO-*d6*	Deuterated dimethyl sulfoxide
DNA	Deoxyribonucleic acid
DNP	Dictionary of Natural Products
DPPH	2,2-diphenylpicrylhydrazyl
ELSD	Evaporative light scattering detector
Endothelin	ET
ESI-MS	Electrospray ionisation mass spectrometry
EtOAc	Ethyl acetate
FDA	The Food and Drug Administration
FNS	Flavone synthase
GCMS	Gas Chromatography Mass Spectrometry
H_2O_2	Hydrogen peroxide
HCV	Hepatitis C virus
HIV	Human immunodeficiency virus
HMGB1	High-mobility group protein B1
HMBC	Heteronuclear Multiple-Bond Correlation
HMDB	The Human Metabolome Database
HMQC	Heteronuclear Multiple-Quantum Correlation

HPLC	High-performance liquid chromatography
HRESI-MS	High Resolution Electrospray Ionisation Mass Spectrometry
HR-LCMS	High Resolution Liquid Chromatography mass spectrometry
HSV-1	Herpes simplex type 1
HTS	High-Throughput screenings
LC-NMR	Liquid Chromatography Nuclear Magnetic Resonance
MDCK	Madin-Darby canine kidney
MeOH	Methanol
MIC	Minimum inhibitory concentration
MPLC	Medium Pressure Liquid Chromatography
MRSA	meticillin-resistant *Staphylococcus aureus*
MS	Mass Spectrometry
NF-κB	Nuclear factor-κB
NIST	The National Institute of Standards and Technology
NMR	Nuclear Magnetic Resonance
NO	Nitric oxide
NTDs	The neglected tropical diseases
$^1O_2$	Singlet oxygen
$O_2\bullet^-$	Superoxide anion
$OH^\bullet$	Hydroxyl radicals
OPLS-DA	Orthogonal Partial Least Square-Discriminant Analysis
PCA	Principal Component Analysis
PLS-DA	Partial Least Square-Discriminant Analysis
QR1	Quinone oxireductase type1
ROS	Reactive oxygen species
RT	Retention Time
SA	*Staphylococcus aureus*

SIDR	Strathclyde Institute for Drug research
SIPBS	Strathclyde Institute of Pharmacy and Biomedical Sciences
TBE	Tris/Borate/EDTA
TNF-α	Tumour necrosis factor alpha
UK	United Kingdom
USD	United States Dollar
UV	Ultra Violet

CHAPTER 1

1 Introduction

1.1 Natural products

Any substance produced by living organisms can be defined as a natural product, of which there are three major groups (Hanson, 2003). The first group consists of small molecular weight primary metabolites found in every cellular system. These include sugars, lipids, nucleic acids and amino acids. Primary metabolites play an essential role in metabolism and reproduction. Second are the high molecular weight compounds that are important in cellular structures, such as lignin, proteins and celluloses. The last group are the secondary metabolites. Their role depends on the interaction of the organism with its environment and other organisms either through symbiosis or commensalism. Secondary metabolites may serve as attractants or play a defensive mechanism to protect the living organisms which make them interesting compounds to study. Most of the secondary metabolites have their own biological effects and may contribute important activity for pharmaceutical application (Hanson, 2003; Sarker and Nahar, 2012). Since ancient times, plants have been well-known sources of secondary metabolites as they were used as medicines to treat various ailments. More than 270,000 secondary metabolites are recorded in the Dictionary of Natural Products (DNP), the most comprehensive database containing natural products information to date (Press, 2015). Aside from plants, other sources of these natural products are lichens, marine organisms and endophytes. Endophytes, either from terrestrial or marine origin, encompass a great chemical diversity with more yet to be discovered (Newman and Cragg, 2012).

1.1.1 History of Natural Products

A long time ago before modern doctors and pharmacists began prescribing capsules and tablets to their patients, ancient doctors used aromatic spices and medicinal plants to treat their patients. Ancient medicine employed gargles, snuffs, poultices, infusions, pills, ointments, potions, remedies and oils. The earliest record was written on clay tablets in cuneiform and described that Mesopotamian medical doctors (2600 BC) were

using oils from cypress (*Cupressus sempervirens*), poppy juice (*Papaver somniferum*) and myrrh (*Commiphora* species) to treat coughs, cold and inflammation. To date, these oils are still being used for the same purpose and to some extent are also utilised against parasitic infection, inflammation, as diagnostic tool in liver cancer patients and to treat iodine deficiency disorder (Benmiloud *et al.*, 1994; Borchardt, 2002; Heinrich, 2004; Newman *et al.*, 2000; Takayasu *et al.*, 1987). Other ancient civilisations such as those from Egypt, China, India, Greece and Muslim Arabia were also widely using medicinal and herbal plants for treating ailments. The most popular history of Egyptian medicine was recorded as early as 1500 BC in the "Eber Papyrus". In this document, more than 700 drugs were listed and the majority of them were of plant origin. For the Chinese, their Materia Medica is the encyclopaedia for traditional Chinese medicine. The first written record from 1100 BC is Wu Shi Er Bing Fang, which documented 52 drug prescriptions. Later in about 100 BC, the Shennong Herbal recorded 365 drugs. In 659 AD, the Tang herbal was made available with 850 drugs prescribed. In India, the Ayurvedic system such as Chakara recorded 341 drugs while the Sushruta and Samhitas chronicled 516 drugs. For the western world, the history of herbal medicines started during the time of the Greek emperors. Dioscorides (100 AD) was a physician who kept and catalogued the uses of medicinal herbs. Theophrastus, a Greek philosopher and natural scientist (~300 BC), used medicinal herbs to treat sick people. The breakthrough of the medicinal world was when Muslim Arabs privately opened the first pharmacies in the 8th century. Ibnu Sina, or known as 'Avicenna' by western people, was a Muslim Persian physician, pharmacist, philosopher and poet. He made great contributions in pharmacy, science, and medicine through his book, *Canon Medicinae* (Cragg and Newman, 2005).

1.1.2 Trends in natural products

The idea of single substance drugs from medicinal plants started in the United Kingdom (UK) in 1618 when the London Pharmacopoeia was first published. In the early 19th century, scientists began to isolate pure compounds from medicinal plants. Morphine **1.1** was the first bioactive compound isolated in 1816 by Serturner, a German pharmacist, and in 1826 morphine was commercialised as a drug in the market. It was isolated from the medicinal plant *Papaver somniferum*, which was used in ancient

Mesapotamia as an analgesic plant while the Arabs described this plant as an addictive agent (Buss *et al.*, 2003; DerMarderosian and Beutler, 2002). This work was followed by the production of acetylsalicylic acid, known as aspirin **1.2**, by Bayer in 1899. Aspirin is a semi-synthetic pure drug based from salicylic acid derived from the willow tree (Weissmann, 1991). Later, in 1820, quinine **1.3** was isolated from the bark of Cinchona species by the French pharmacists Caventou and Pelletier. This bark was used traditionally by indigenous groups in the Amazon to treat fever. Many active compounds such as morphine **1.1**, atrophine **1.4** and colchine **1.5** were isolated from plants in the early 1800s by scientists and all of these plants had been used by local communities to cure various types of diseases (Dias *et al.*, 2012).

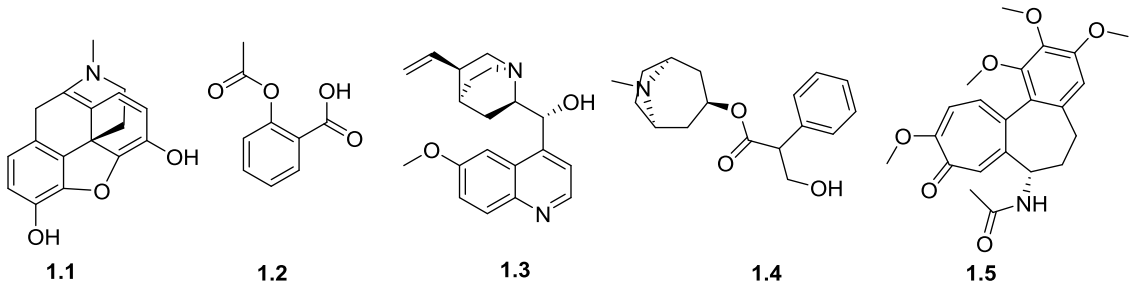


Fig 1.1: Natural products from plants

The isolation of secondary metabolites was overwhelmingly growing over the centuries. One of the most important discoveries occurred in 1929 when Fleming isolated penicillin **1.6** from *Penicillium notatum* (Fleming, 1929). The discovery was very essential and proved that microorganisms have the ability to produce remarkable secondary metabolites. However, it was not until the 1940s that penicillin was extensively studied by Chain and Florey. As a drug, penicillin created a new era in medicine and pharmacy known as the 'golden age of antibiotics'. Many antibiotics were discovered during this era and a number of them are still being produced commercially, such as streptomycin **1.7**, chloramphenicol **1.8**, chlorotetracycline **1.9**, cephalosporin C **1.10**, erythromycin **1.11**, and vancomycin **1.12** (Newman *et al.*, 2000; Wainwright and Wainwright, 1990). The height of natural products research was between the 70s and 80s when many huge pharmaceutical companies invested in natural products research. This was influenced by the idea of bioassay-guided isolation and mechanism-based screening studies.

However, in recent times, the trend has shown that attention of pharmaceutical companies to natural product research is fading away due to several reasons. Natural products research schemes for finding promising target drugs are extremely slow and not economical, making them less popular among pharmaceutical companies. Lead compounds isolated from nature give very small yields which has made further studies such as preclinical and clinical trials not feasible. High-Throughput screenings (HTS) that could assay thousands of compounds in a day caught the attention of pharmaceutical companies. However, the complexity of natural product extract libraries made it difficult to apply HTS to them. The companies therefore preferred to use HTS of synthetic libraries against defined molecular targets. In addition, the introduction of combinatorial chemistry offered more drug-type and simple chemical compounds, seducing drug companies to change their drug discovery programs from natural products to the synthetic route. Surprisingly, despite the fact that natural products research and development is considered to be less competitive to pursue by pharmaceutical companies, the latest update by Agnolet *et al.* (2010) showed that more than 50% of drugs approved by the Food and Drug Administration (FDA) are from natural products and their derivatives as well as natural products inspired through synthesis or by combinatorial chemistry. This update clearly reinforces the fact that compounds from natural products are still essential and important in drug discovery pipelines and cannot be neglected. Chemical structures of natural products are unique and diverse. Thus, to ensure that the most promising drugs can be discovered and produced, a holistic approach, which includes the combination of natural products research with high-throughput screening, should be applied to the drug discovery pipelines.

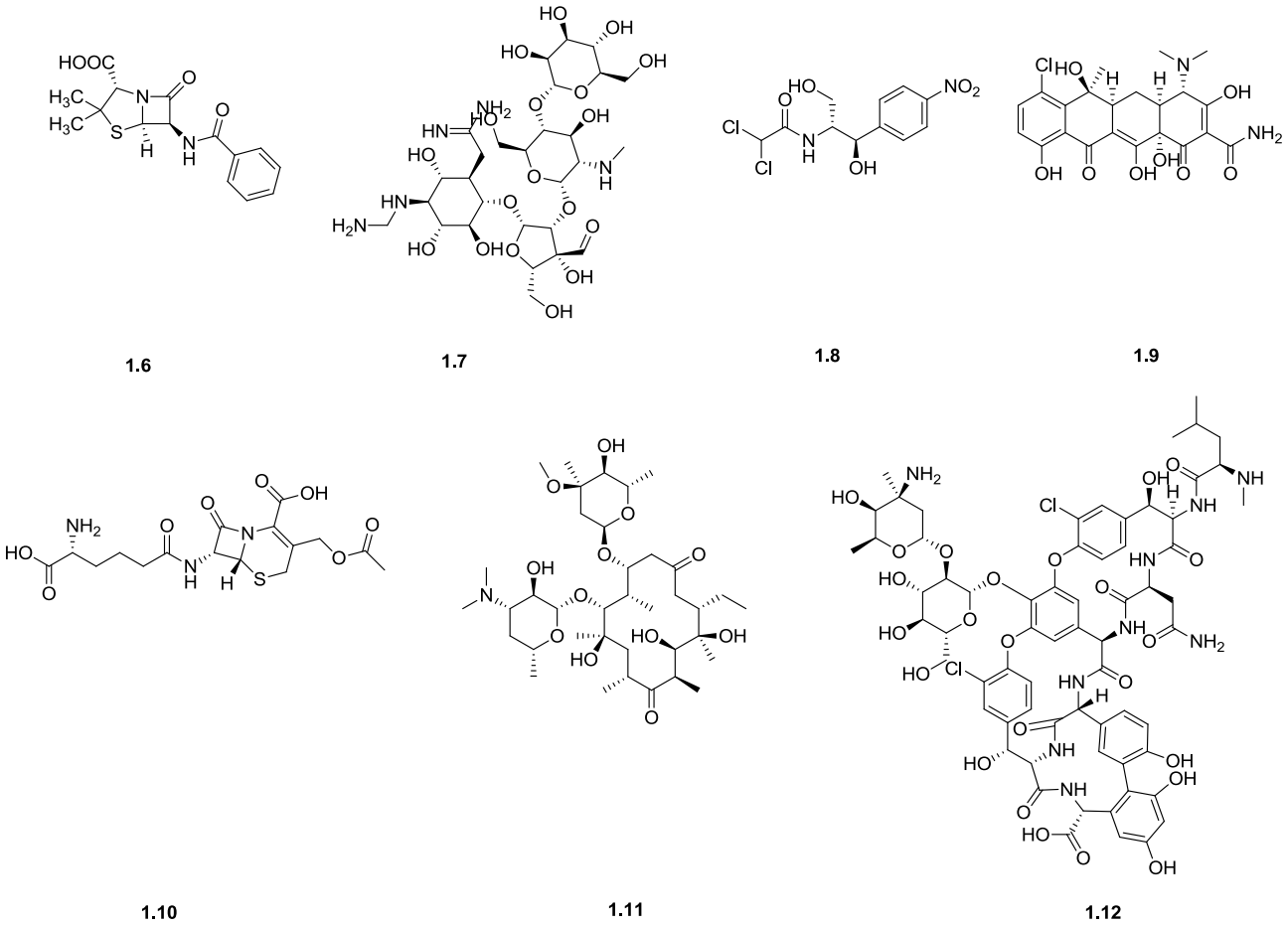
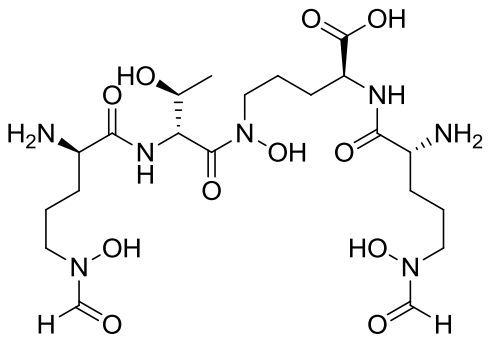


Fig 1.2: Isolated natural products during the golden age of antibiotics research

1.1.3 New frontiers in natural products

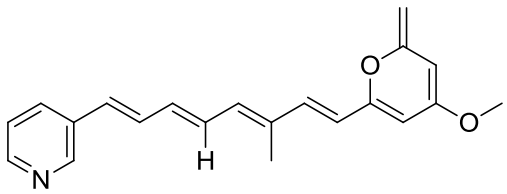
The employment of genome mining in particular to discover new secondary metabolites is rising very fast with more and more research groups around the world showing their interest in this area (Challis, 2008; Corre and Challis, 2007; Gross *et al.*, 2007; Peric-Concha and Long, 2003; Van Lanen and Shen, 2006). Challis and co-workers established their research strategy by manipulating cryptic genes in *Streptomyces* sp to find new target compounds as well as to study their biosynthetic pathways. Their work to find new metabolic products from cryptic (also known as orphan) genes from *Streptomyces coelicolor* A3 yielded the new peptide coelichelin **1.13** (Lautru *et al.*, 2005). Through this approach, they also isolated a bioactive 51-membered macrolide complex from *Streptomyces ambofacient* by the activation of an orphan polyketide synthase (Laureti *et al.*, 2011). This tremendous finding suggested that by activating silent genes, more new secondary metabolites can be produced by microorganisms. This methodology by Challis best answered why most research institutes that cultured their microbes in the early 1990s always yielded the same types of compounds, as many cryptic genes had not been expressed in the optimum incubating or fermentation conditions.



1.13

Fig 1.3: The new peptide coelichelin isolated from *S. coelicolor* based on cryptic genome mining

Another promising tool which has been developed to promote the re-emergence of the importance of natural products in current drug discovery is metabolomics. Metabolomics is defined as a global study of all chemical entities including primary and secondary metabolites that present in living organisms (cells or tissues) under certain conditions (Moco *et al.,* 2007; Verpoorte *et al.*, 2007). Metabolomics, or metabolome mining, in natural products research has been used for dereplication studies of both known and new compounds in crude plant, marine or microbial extracts, in differentiating biologically active from non-active fractions, optimising the production of bioactive secondary metabolites, as well as in developing cultivation processes for large-scale fermentation and understanding their biosynthetic pathways. Normally, MS and NMR are used as metabolomics tools to assess the presence of interesting secondary metabolites in target organisms (Agnolet *et al.*, 2010; Gray *et al.*, 2012; Peric-Concha and Long, 2003; Wolfender *et al.*, 2010; Yuliana *et al.*, 2011). With commercial and free internet-available databases, compounds can be dereplicated, and therefore the occurrence of interesting new compounds can be determined and subjected to further isolation work (Kamal *et al.*, 2011). Metabolomics along with statistical "gizmos" are employed as decision-making tools to select the most interesting plant or marine species or microbial strains (Hou *et al.*, 2012) which produced novel and diverse chemical compounds. For example new and rare class of polyenepyrone **1.14** together with another 20 compounds was successfully identified and isolated through the metabolomics route from a marine microbe.



1.14

Fig 1.4: A new rare class polyenepyrone isolated from a marine microbe using the metabolomics approach.

1.1.4 Natural products from plants in drug discovery

Plants play a vital role in drug discovery by producing various types of bioactive compounds which have later been approved by FDA as drugs. Plant natural products have been used successfully as precursors and drug leads in the production of more potent drugs and also as pharmacological probes (Salim *et al.*, 2008). The most ground-breaking drug discovery was taxol, marketed under the trade name Paclitaxel® **1.15**, an anticancer drug isolated from the bark of *Taxus brevifolia* Nutt. (Taxaceae), and was worth about 1.6 billion USD in 2000. Taxol binds tubulin without causing any depolymerisation or interference with the tubulin assembly (Horwitz, 2004). Another success story is podophyllotoxin **1.16**, a resin isolated from *Podophyllum peltatum* L. The synthesised derivative called etoposide **1.17** has been utilised to treat small-cell lung cancer and refractory testicular cancer. Epotoside acts as topoisomerase II inhibitor which binds tubulin and unravels deoxyribonucleic acid (DNA), causing the strands to break during the G2 phase of the cell cycle and leads to cell death (Baldwin and Osheroff, 2005). Another plant drug lead is camptothecin **1.18,** a quinoline alkaloid isolated from the bark and stem of *Camptotheca acuminate* Decne. (Nyssaceae). It was used in Traditional Chinese Medicine to treat cancer (Efferth *et al.*, 2007). Camptothecin is a topoisomerase I inhibitor (Liu *et al.*, 2000). During the clinical trials, it was found to have poor solubility and high adverse drug reactions, thereupon analogues were synthesised. Two analogues, topotecan **1.19** and irinotecan **1.20** were approved for cancer therapy. A number of new plant natural products-inspired drugs have been introduced in the U.S market to treat other various types of diseases (Balunas and Kinghorn, 2005). Galantamine **1.21**, isolated from *Galanthus woronowii* Losinsk by Russian researchers, has been developed as a drug to treat Alzheimer's disease. It has a dual mechanism of action by inhibiting acetylcholinesterase (AChE) as well as binding to and modulating the nicotinic acetylcholine receptor which slows down the process of neurological degeneration (Aly *et al.*, 2011). Another example is arteether **1.22,** a promising drug to combat acute malaria. It is actually a derivative of artemisinin **1.23,** a sesquiterpene lactone from *Artemisia annua* L (van Agtmael *et al.*, 1999).

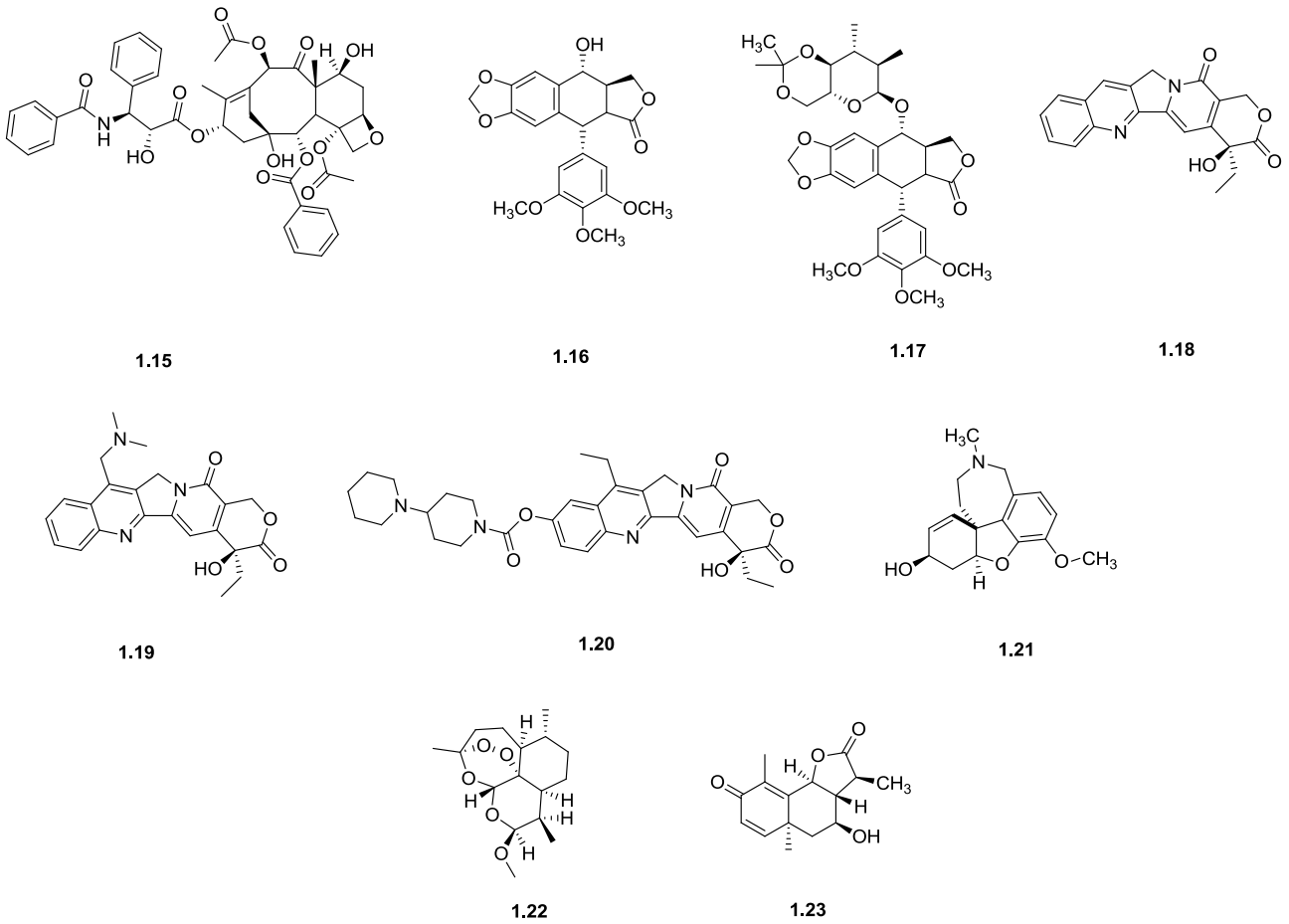


Fig 1.5: FDA-approved drugs isolated or inspired from plant natural products

1.2 Metabolomics approach in natural products research

1.2.1 Introduction

Historically, the word metabolome was first coined in 1998 (Oliver *et al.*, 1998). In metabolomics research, hyphenated analytical techniques such as High Resolution Liquid Chromatography Mass Spectrometry (HR-LCMS), Gas Chromatography Mass Spectrometry (GCMS) and Liquid Chromatography Nuclear Magnetic Resonance (LC-NMR) (Dunn *et al.*, 2005) are integrated with available databases such as The Human Metabolome Database (HMDB), METLIN, The Golm Metabolome Database, Fiehn GC-MS Database, AntiMarin, Dictionary of Natural Products (DNP) and MassBank (Blunt, 2013; Horai *et al.*, 2010; Hummel *et al.*, 2007; Kind *et al.*, 2009; Smith *et al.*, 2005; Wishart *et al.*, 2007). Together with innovative chemometric tools like MZmine, MZmatch, and MetaboLab, data are statistically analysed utilising multivariate software such as SIMCA-P, for easy pattern recognition (Ludwig and Günther, 2011; Pluskal *et al.*, 2010; Scheltema *et al.*, 2011; Umetrics). Therefore, a combination of these techniques and methodologies makes metabolomics an efficient and robust tool for drug discovery. Since metabolomics joined other 'OMICS' predecessors such as genomics, trancriptomics, and proteomics, attention on metabolomics has grown tremendously as indicated by the exponential increase in the number of publications in the field between the years 2000 to 2015 as recorded in Pubmed (Fig. 1.6).

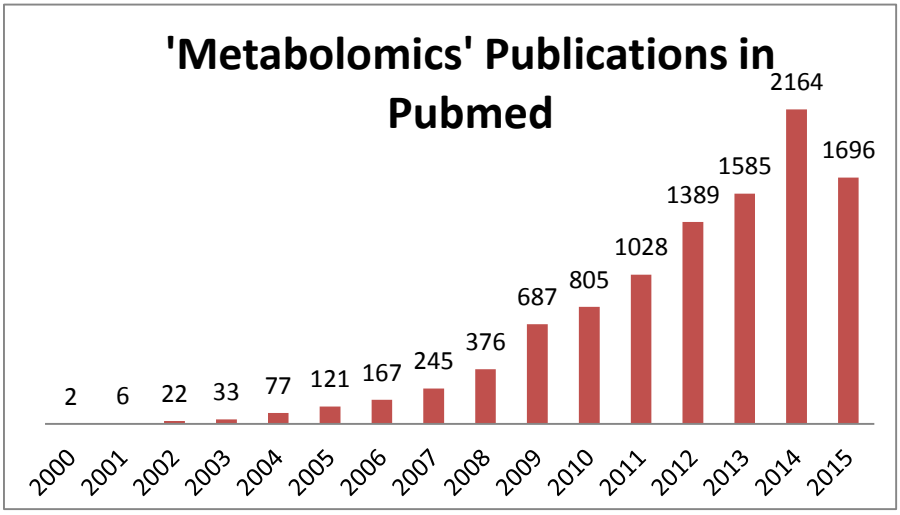


Fig. 1.6: Number of metabolomics publications recorded in Pubmed according to year

Metabolomics can be divided into two types: targeted or untargeted (shown in Fig. 1.7). Targeted metabolomics is designed to study a specific pathway and extensively scrutinizes the role of a target metabolite as a biomarker (Griffiths *et al.*, 2010). On the other hand, an untargeted approach measures simultaneously as many metabolites as possible in a biological sample without preference to any specific pathway, enzyme, or biomarker. Metabolomics as an analytical platform has been adopted in a vast variety of research fields, including ecology (Lankadurai *et al.*, 2013), biomedical and systems biology (Weckwerth, 2003), toxicology (Ramirez *et al.*, 2013), nutrition and food science (Wishart, 2008), herbal products (Sun *et al.*, 2012a), and recently in natural products research (Robinette *et al.*, 2011; Tawfike *et al.*, 2013).

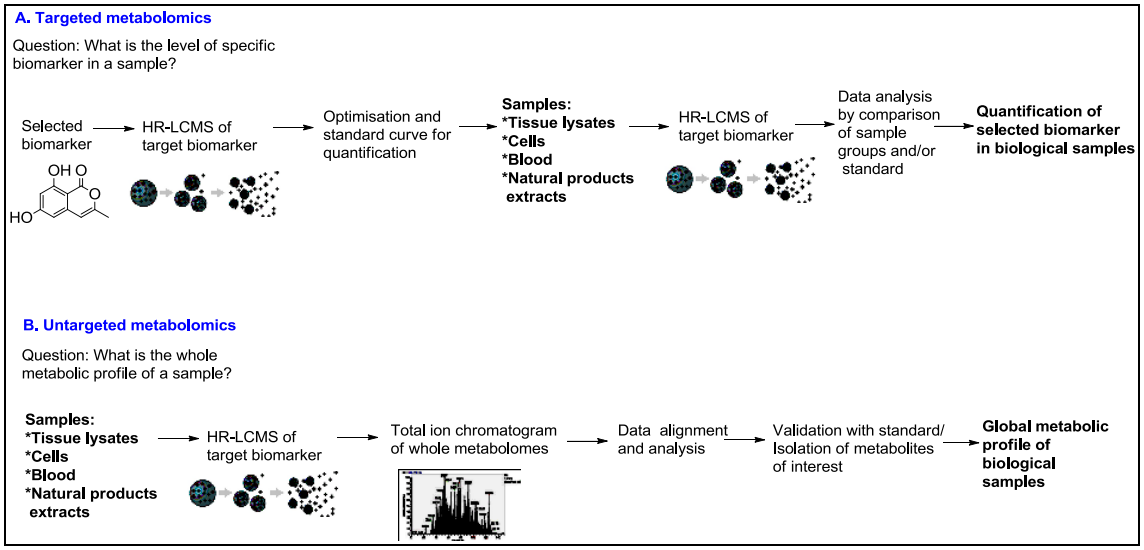


Fig. 1.7: Metabolomic methods workflow for a) targeted metabolomics b) untargeted metabolomics (Figure was modified from Patti *et al.* (2012) published in *Nature Reviews Molecular Cell Biology* **13**, 263-269 (April 2012)

1.2.2 Spectral analysis tool

In natural products metabolomics research, the spectral analytical tool is one of the crucial components to measure metabolomes in any biological samples. MS coupled with gas chromatography (GC), liquid chromatography (LC), capillary electrophoresis/ time of flight (CE-TOF) and/or matrix-assisted laser desorption/ionization-TOF (MALDI-TOF) are among the selection of high-sensitivity hyphenated techniques that could cover a comprehensive range of metabolites (Dunn *et al.*, 2005; Yang *et al.*, 2011). GC-MS-based metabolomics is mostly used to identify volatile metabolites isolated from various biological sources including pheromones, toxins, terpenes and essential oil components. One study utilised this technique to identify known and unknown metabolites from *Arabidopsis thaliana* crude leaf extracts (Fiehn *et al.*, 2000). It has also been used to classify 322 different compounds from 94 samples of ripe tomato (*Lycopersicon esculentum* Mill.) (Tikunov *et al.*, 2005). CE-TOFMS is an analytical technique used in metabolomics that is particularly suitable for polar and charged metabolites including amino acids, amines, organic acids and nucleic acids (primary metabolites) (Ramautar *et al.*, 2009). CE-TOFMS metabolomic profiling

mainly has been used as a tool in the quality control of herbal medicines since most polar and charged compounds are the major components in the water extracts (Iino *et al.*, 2012; Lao *et al.*, 2009). Several comprehensive reviews about CE-MS-based metabolomics have been published (Barbas *et al.*, 2011; Ramautar *et al.*, 2009; Ramautar *et al.*, 2013; Ramautar *et al.*, 2011). For the past 17 years MALDI-TOF-imaging mass spectrometry (IMS) technology has been applied to molecular pathology (Caprioli *et al.*, 1997; Seeley and Caprioli, 2011) to localise and detect proteins, peptides (Chaurand *et al.*, 1999; Stoeckli *et al.*, 2001) and lipids (Puolitaival *et al.*, 2008) as biomarkers of diseases directly from tissue. Recent developments in MALDI-TOF-imaging MS metabolomics has allowed researchers to visualise the spatial distribution of metabolomes produced by two microbial samples as individual and interacting colonies like between *Streptomyces coelicolor* and *Bacillus subtilis* (Yang *et al.*, 2009).

In natural products metabolomics, the most frequently used technique is high-resolution LCMS. This is due to a number of advantages, including high sensitivity, the ability to cover a broad range of chemical classes, the fact that there is no need for chemical derivatisation prior to sample preparation as in gas chromatography, and it is also beneficial for elucidating unknowns by multiple MS fragmentation (MS^n) (Shulaev, 2006). LCMS-PCA based metabolomics has been used as a tool to prioritize microbial strain selection in finding novel compounds (Hou *et al.*, 2012) and bioactive secondary metabolites (Klitgaard *et al.*, 2014; Macintyre *et al.*, 2014; Nielsen *et al.*, 2011; Samat *et al.*, 2014). A recent publication on UPLC-MS-based metabolomics exhibited its application in optimising culture conditions to study the effect on biosynthetic pathways in the production of secondary metabolites (Rao *et al.*, 2013).

Another common detection technique used in metabolomics is NMR. The application of ^{1}H NMR based-metabolomics has been described as a promising tool in chemical fingerprint analysis (Pauli *et al.*, 2004). Efficient identification of plant-derived compounds was easily achieved (Exarchou *et al.*, 2005) with a hyphenated liquid chromatography-NMR (LC-NMR) system along with the development of cryogenic and capillary probes. Choi and co-workers effectively used ^{1}H-NMR based metabolomics associated with multivariate analysis to differentiate chemical compounds in varieties of

Cannabis sativa cultivars without any pre-fractionation step (Choi *et al.*, 2004). ^{1}H-NMR based metabolomics has also been applied to determine the presence of bioactive molecules (Abdelmohsen *et al.*, 2014; Ali *et al.*, 2013).

1.2.3 Databases available for dereplication in natural products metabolomics

Numerous commercially available databases may be used in metabolomics studies. For natural products metabolomics in particular, several established databases are often used as references for dereplication studies. The Dictionary of Natural Products (Press, 2015) and The Dictionary of Marine Natural Products (Press, 2014) are comprised of more than 260 000 and 48 000 known metabolites, respectively. AntiMarin (Blunt, 2013) is a recent database that covers more than 58 000 compounds. It resulted from a merger of AntiBase (Laatsch, 2012), a database containing secondary metabolites from terrestrial and marine microorganisms, and MarinLit (Blunt and Munro, 2005), a database of marine natural products literature. Klitgaard *et al.* (2014) reported using a combination of databases dedicated for fungal natural products which consisted of approximately 1500 reference standards (Nielsen *et al.*, 2011), 500 identified compounds (Frisvad *et al.*, 2008; Månsson *et al.*, 2010; Nielsen *et al.*, 2009), and also a commercially-available database, AntiBase (Laatsch, 2012), to support a dereplication strategy in their research.

1.2.4 Multivariate analysis tool

Multivariate data analysis refers to the simultaneous analysis of data sets containing more than one independent variable which takes into account all variables involved (Abdi, 2003). For example, in LCMS-based metabolomics, variables encompass retention time range, a mass range and mass tolerance. Therefore by applying multivariate rather than univariate data analysis in metabolomics, data loss is minimal. The multivariate data analysis can be either unsupervised or supervised. Unsupervised analysis is used if the groupings of the samples are unknown, while the supervised analysis is used if the groups of samples are known, such as in forensic applications where samples can be grouped into authentic and unauthentic samples of seized drugs

(J. Anzanello *et al.*, 2014), and in the natural products drug discovery field where active secondary metabolites can be traced by comparing active fractions against inactive fractions (Harvey *et al.*, 2015). The summary of multivariate analysis methods and their primary uses is showed in Table 1.1.

Table 1.1: Summary of approaches used in multivariate analysis (adapted from (Wiklund, 2008))

Overview	**Classification**	**Discrimination**	**Regression**
PCA	**SIMCA**	**PLSDA** **OPLSDA**	**O2-PLS**
Trends	Pattern recognition	Discriminating between groups	Comparing blocks of omics data
Outliers	Diagnostics	Biomarker candidates	Metabolomics vs proteomic vs genomic
Quality control	Health/disease	Comparing studies or instrumentation	Correlation spectroscopy
Biological diversity	Disease progression		
Patient monitoring	Toxicity mechanisms		

One of the multivariate techniques is called as principal component analysis (PCA) (Hotelling, 1933). PCA is a type of exploratory data analysis where the data sets are initially analysed to get a general idea of class separation, trends and outliers (Bouhifd *et al.*, 2013). PCA data can be pictured by graphic scatter plots. The basic idea of PCA is to reduce the dimensions (Jolliffe, 2005) of a set of variables (principal components) to deduce a linear relationship between the variables (Everitt, 2013). Two matrices, known as scores and loadings, are formed based on the conversion of the original data set by PCA. The principal component loading gives information on which variables are the greatest contributors in the transformation to the new variables. The relationship among measured variables is also shown in the loading plot. The scores plot provides a

summary of all points (such as chromatograms or spectrums) and shows the correlation between each point. Therefore, the points those are close to each other share similar profiles. The correlation between score and loading plot corresponds to direction in the loading plot. Hence by examining the loading plot, any point found to be clustered on the score plot can be interpreted (Smolinska, 2012). For example, active fractions against *T. brucei brucei* can cluster together in the loading plot; therefore a series of masses in the score plot can then be analysed to predict the potential hits responsible for the antitrypanosomal activity.

For the supervised methods, examples of multivariate techniques are partial least squares – discriminant analysis (PLS-DA) or orthogonal projection of latent structures – discriminant analysis or Orthogonal PLS-DA (OPLS-DA). Both of these methods are types of supervised multivariate analysis. PLS is a method that involves an additional 'bogus' y- variable to discriminate two classes such as healthy *versus* diseased states to target biomarkers for diagnosis and treatment, or bioactive *versus* inactive natural products to pinpoint secondary metabolites responsible for certain bioactivity. OPLS- is an extension of supervised PLS regression method with an additional feature called orthogonal signal correction filter (OSC) (Wold *et al.*, 1998) which permits systemic variation in X that is orthogonal or uncorrelated to Y to be identified.

1.2.5 Metabolomics as a tool in the dereplication study of fungal endophytes

When pharmaceutical companies found that natural products isolation was not as attractive as total synthesis, combinatorial chemistry or genomic biomarkers, new tactics were urgently needed. One of the major obstacles in natural products is that it is time-consuming; the dereplication strategy was therefore introduced. The dereplication strategy is the rapid identification of known compounds based on available *in-house* or commercial databases; therefore, undesirable extracts or fractions can be eliminated at an early stage while a specific target can be prioritized to find new bioactive compounds. Without investing any huge isolation effort and time, the bioactive compounds can be identified easily by applying high resolution NMR and MS on crude organic fungal extracts (Frisvad *et al.*, 2008; van der Sar, 2006). Recently, a robust,

extensive dereplication strategy was described using a 15 min gradient run on an ultrahigh-performance liquid chromatography–diode array-MS (UHPLC–DAD-MS) hyphenated system along with an *in-house* 7000 entry fungal database to identify new nitrogen-containing biomarkers from extracts of *Aspergillus carbonarius* and *Penicillium melanoconidium* (Klitgaard *et al.*, 2014).

1.3 Secondary metabolites isolated from *Vitex sp.*

Vitex pinnata is a woody plant which can be found in primary, secondary forests and savannahs. This species is under the genus *Vitex* with the family Lamiaceae. It can grow up to 25 metre tall. The leaves are palmately compound with 3 to 5 foliolate and each leaflet is spear shaped. The flowers are purple-bluish and fruits are globose and black. The flowering season is from November to May while fruiting is from November to August. Normally, fruits are eaten by birds (de Kok, 2008). It can be found in Southeast Asia like in Malaysia, Indonesia, Thailand, Cambodia and India. Interestingly, in the Philippines, *V. pinnata* can only be found in the islands of Palawan, Culion and Tawi Tawi (de Kok, 2008). *V. pinnata* is used as traditional medicine among local in Malaysia. The young leaves of *V. pinnata* are used for antipyretic treatment and bark is used to treat gastric ulcer (Corner, 1951). In 1966, Burkill (Burkill, 1966) reported that bark decoction is used for stomach ache and post-childbirth medicine while leaves are used for fever and wound poultices. Later publication by Ong (1999) (Ong and Nordiana, 1999) reported that in Machang, Kelantan, Malaysia, leaves of *V. pinnata* were applied on cuts and wounds.

There are approximately 270 species of *Vitex* trees and shrubs identified. Most of the species can be found in tropical and sub-tropical regions with a few of species found in temperate areas. Various types of compounds have been isolated from *Vitex,* mainly iridoids, flavonoids, diterpenoids and phytosteroids. In addition, many of these secondary metabolites have been found to have interesting bioactivities.

1.3.1 Iridoids

Iridoids are cyclopentano[*c*]pyran monoterpenoids which have been isolated from both terrestrial and marine organisms. Those mainly isolated from plants are glycosidic in nature. In terms of chemotaxonomy and biogenesis, iridoids are structurally linked to both terpenes and alkaloids (Bruneton, 1995). A previous study on these compounds described their diverse bioactivities, such as cardiovascular, anti-inflammatory, antispasmodic, anticancer, antiviral and antihepatotoxic effects (Ghisalberti, 1998). *V. negundo* is the most widely studied species, yielding five new iridoids. Two

mussaenosidic acid derivatives named 2′-p-hydroxybenzoyl mussaenosidic acid (negundoside) **1.24** and 6′-p-hydroxybenzoyl mussaenosidic acid **1.25** were the first iridiods isolated from the genus *Vitex* by Sehgal and co-workers in 1982 and 1983, respectively (Sehgal *et al.*, 1982; Sehgal *et al.*, 1983). Negundoside showed protective effects against carbon tetrachloride (CCl_4)-induced toxicity in HuH-7 cells and oxidative stress (Tasduq *et al.*, 2008). Another iridoid, nishindaside **1.26**, was isolated from *V. negundo* for the first time (Dutta *et al.*, 1983). In 1994, lagundinin **1.27** was discovered from this same species (Dayrit and Lagurin, 1994). In 2009, carboxylic acid derivatives **1.28** were isolated from *V. negundo* (Sharma *et al.*, 2009). Most iridoids were discovered after 2000 with more advanced isolation methods and techniques. From the Brazilian *V. cymosa*, tarumal **1.29** was isolated (Santos *et al.*, 2001). *V. peduncularis* yielded pedunculariside **1.30** which was shown to inhibit COX-2 with IC_{50} values of 0.15 +/- 0.21 mg/ml. However, it only showed low inhibition of COX-1 and no cytotoxicity against Vero African green monkey kidney cell lines was reported (Suksamrarn *et al.*, 2002a). Later in 2003, three iridoids, namely agnucastoside A **1.31**, agnucastoside B **1.32** and agnucastoside C **1.33**, were described from *V. agnus-castus*. However all three compounds showed no activity in both antimicrobial and anticancer assays (Kuruüzüm-Uz *et al.*, 2003). In the following year, another six iridoids, 6‘-O-trans-feruloylnegundoside **1.34**, 6‘-O-trans-caffeoylnegundoside **135**, 2‘-O-p-hydroxybenzoyl-6‘-O-trans-caffeoylgardoside **1.36**, 2‘-O-p-hydroxybenzoyl-6‘-O-trans-caffeoyl-8-epiloganic acid **1.37**, 2‘-O-p-hydroxybenzoyl gardoside **1.38**, and 2‘-O-p-hydroxybenzoyl-8-epiloganic acid **1.39** were isolated from the ethyl acetate extract of *V. altissima.* Compounds **1.35-1.37** showed promising antioxidant activity in both DPPH-radical-scavenging and the superoxide free-radical-scavenging assays (Sridhar *et al.*, 2004). Finally, in 2009, a new iridoid metabolite, pinnatoside **1.40** was described from *V. pinnata* as one of its minor compounds (Ata *et al.*, 2009).

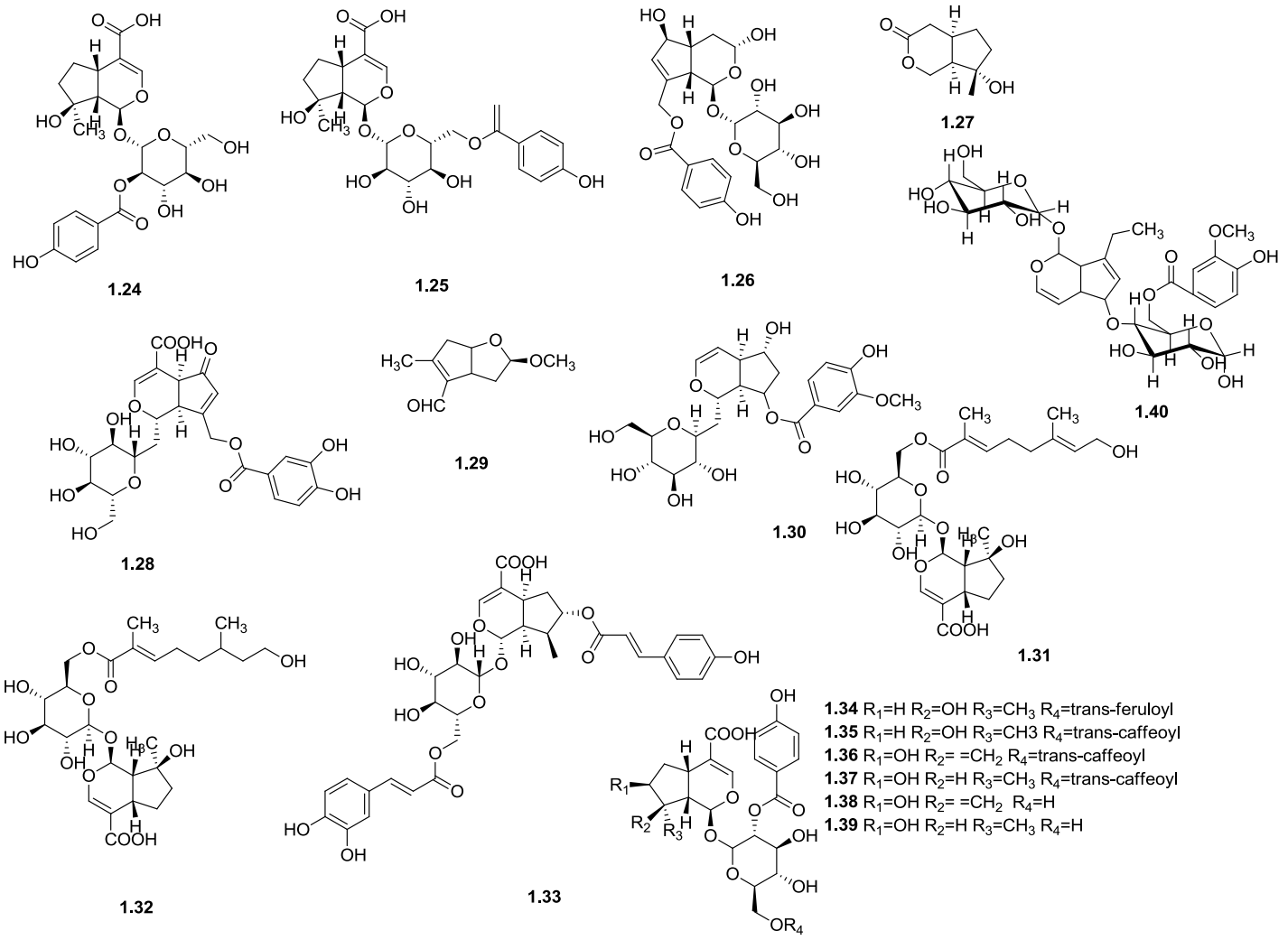
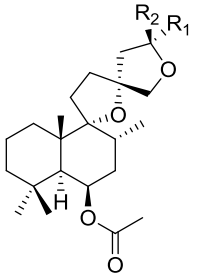


Fig. 1.8: Iridoid derivatives previously isolated from the genus *Vitex*

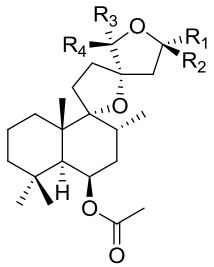
1.3.2 Diterpenoids

A diterpene is a type of terpene that has twenty carbons and biosynthetically originates from geranylgeraniol pyrophosphate. It can be found in many living organisms such as plants, fungi, marine organisms and insects. Paclitaxel® (taxol) is a diterpene isolated from plants and fungi and was approved by the FDA in 1998 to treat lung, ovarian, breast, head and neck cancer (Zhi-Da, 2011). Another example is andrographolide, a labdane-type diterpene isolated from the herbaceous plant *Andrographis paniculata*, which can be widely found in Asian countries like China, Thailand, Malaysia and India. These secondary metabolites are potentially active against cancer and inflammation (Lim *et al.*, 2012). In *Vitex* species, labdane-type diterpenes are most typical. Interestingly, all labdane diterpenes have been isolated from fruits of *V. rotundifolia*, *V. agnus-castus* and *V. trifolia* ``and not from other plant part of this genus. In addition, abietane-, nor- and halimane-type diterpenes have also been found in *Vitex*. The group of Masateru Ono from Japan has dedicated their research to finding new labdane diterpenes from the fruits of *V. rotundifolia* and *V. agnus-castus*. Since 1999, five journal papers have been published by this group, reporting 27 labdane-type diterpenes. In 1999, eight labdane-type diterpenes **1.41-1.48** were isolated from *V.rotundifolia*. Compound **1.48** was subjected to antioxidant assay but did not exhibit any bioactivity (Ono *et al.*, 1999). Three years later, another ten labdane-type diterpene congeners **1.49-1.58** were isolated from the same species. Unfortunately no bioactivies were demonstrated. Compounds **1.51-1.58** could have been artefacts resulting from the reaction with aldehyde during isolation work (Ono *et al.*, 2001). Later in 2008, 2009, and 2011, a further eight labdane diterpenes, viteagnusin C, D, E, F, G, H, J, and viteagnuside I **1.59-1.66** and one labdane diterpene glucoside, viteagnuside A **1.67** were isolated from the fruit of *V. agnus castus* (Ono *et al.*, 2011; Ono *et al.*, 2009; Ono *et al.*, 2008). The labdane diterpene 6β,7β-diacetoxy-13-hydroxy-labda-8,14-diene **1.68** was isolated from the hexane extract of the fruit of *V. agnus castus* and showed strong affinity to the dopamine-D2-receptor with IC_{50} value of 15µg/mL (Hoberg *et al.*, 1999). In 2002, a novel nitrogen-containing labdane diterpene, vitexlactam A **1.69**, was isolated from the fruits of *V. agnus castus* (Li *et al.*, 2002). And more recently, another two labdane diterpene alkaloids, named as vitexlactam B and C **1.70-1.71**, were isolated

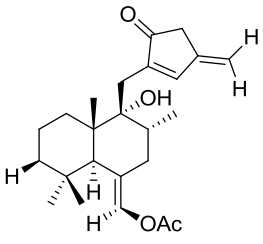
along with compound **1.68**. Their cancer chemoprevention effect was tested and only compound **1.71** showed moderate result on NADP(H); quinone oxireductase type1 (QR1) induction activity (Li *et al.*). Another two labdane diterpenes, vitetrifolin H and vitetrifolin I **1.72-1.73**, were isolated from the fruits of *Vitex trifolia* L. and both compounds inhibited HeLa cell proliferation with IC_{50} between 4-28 μM. Moreover, compound **1.73** was found to induce G_0/G_1 phase arrest and apoptosis of HeLa cells (Wu *et al.*, 2009a). Later in 2011, viteagnusin I **1.74** was described; however no activities were shown on opioid receptor assays (DOR and MOR) (Chen *et al.*, 2011). The labdane diterpene negundol **1.75** isolated from *V. negundo* was found to be a mixture of two diastereoisomers **1.75a and 1.75b** and showed antifungal activity with MIC_{80} values in the range of 16–64 μg/mL (Zheng *et al.*, 2012). Recently in 2013, two new labdane diterpenoids, 6α,7α-diacetoxy-13-hydroxy-8(9),14-labdadien **1.76** and 9-hydroxy-13(14)-labden-15,16-olide **1.77** were found in the leaf extract of *V. trifolia*. Compound **1.76** was found to be active against *Mycobacterium tuberculosis* with MIC of 100μg/mL (Tiwari *et al.*, 2013). The norlabdane diterpene vitrifolin A **1.78** was also isolated from *V. trifolia* Linn. *var. simplicifolia* and was shown to have moderate inhibitory effect against NO production in lipopolysaccaride-activated mouse macrophages (Zhang *et al.*, 2013). Seven labdane diterpenes, vitextrifolin A-G **1.79-1.85**, were recently described from the fruits of *V. trifolia*; however, they were found to be inactive against four human cancer cell lines (A549, HCT116, HL60, and ZR-75-30) (Zheng *et al.*, 2013).



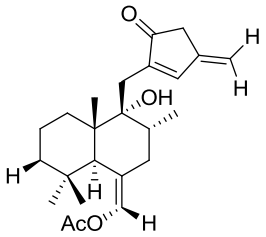
1.41 R_1=OMe R_2=H
1.42 R_1=H R_2=OMe



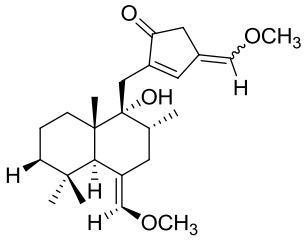
1.43 R_1=OMe R_2=H R_3=H R_4=H
1.44 R_1=H R_2=OMe R_3=H R_4=H
1.45 R_1=OMe R_2=H R_3=OMe R_4=H
1.46 R_1=H R_2=OMe R_3=OMe R_4=H
1.47 R_1=OMe R_2=H R_3=H R_4=OMe
1.48 R_1=H R_2=OMe R_3=H R_4=OMe



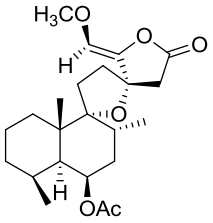
1.49



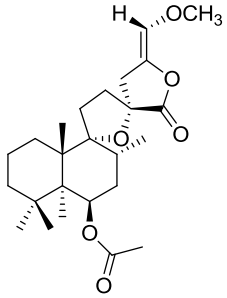
1.50



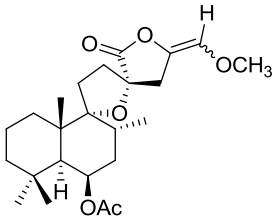
1.51



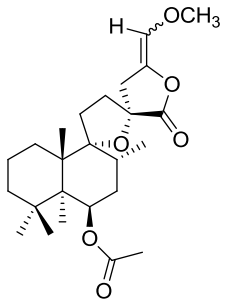
1.52



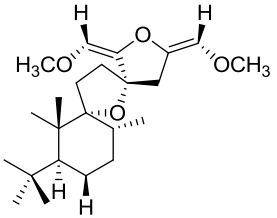
1.53



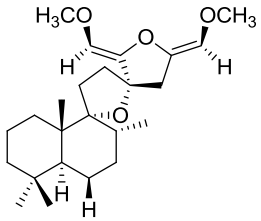
1.54



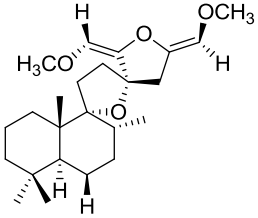
1.55



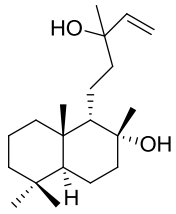
1.56



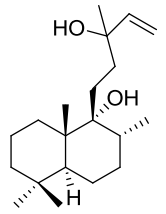
1.57



1.58



1.59



1.60

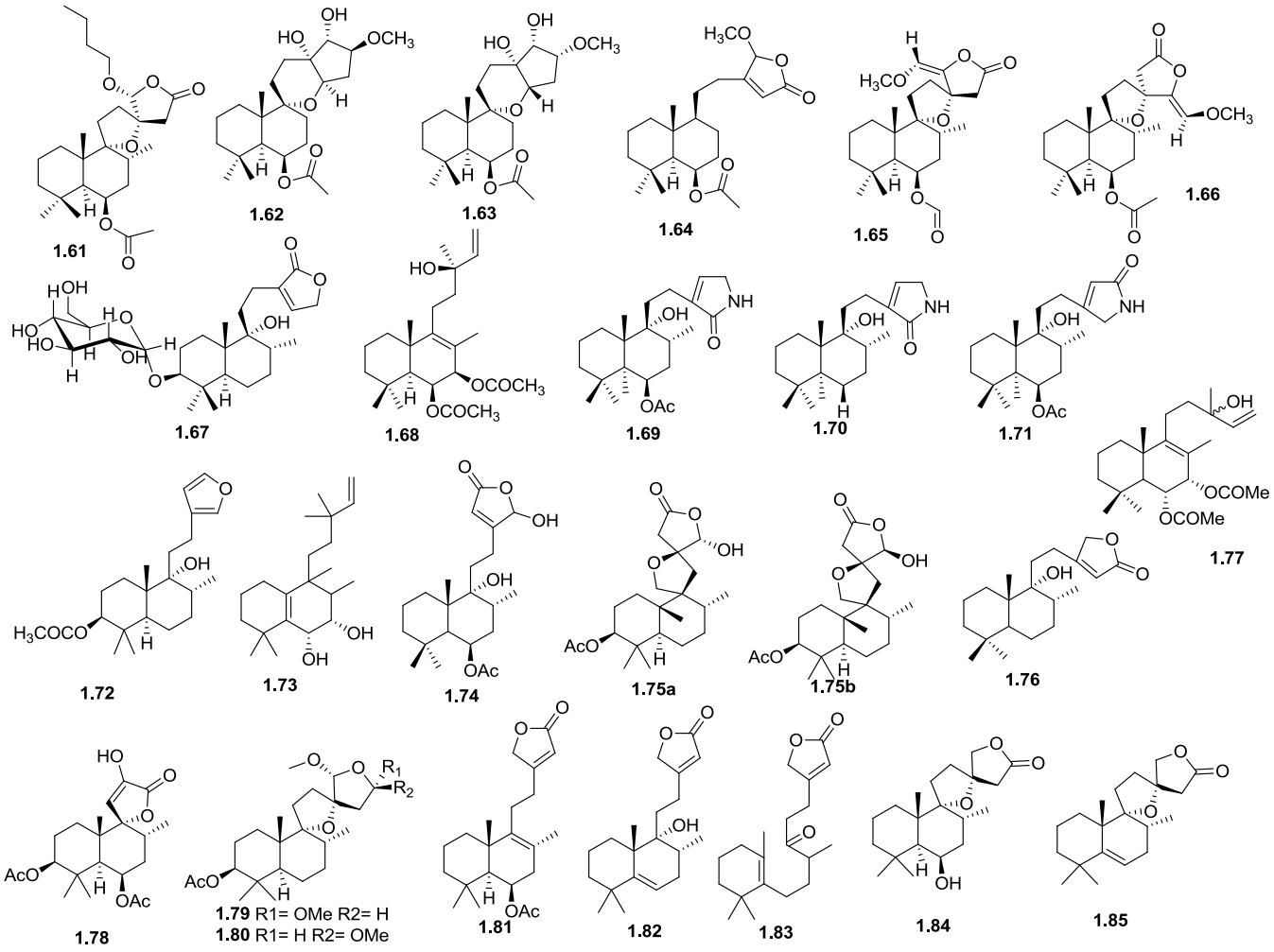


Fig. 1.9: Diterpenoids derivatives previously isolated from *Vitex*

1.3.3 Ecdysteroids

Ecdysteroids are hormones produced by insects that were initially believed to control moulting and the metamorphosis process. However, today their functions are known to be wider than first described, and it was found that this hormone is produced at all development stages of an insect commencing from the newly-laid eggs, embryonic stage, metamorphosis, reproduction and diapause (Dinan, 2001). Plants also contain ecdysteroids (phytoecdysteroids) in large amounts as defence chemicals against phytophagous insects (Horn, 1983). In *Vitex* species, ecdysteroids are one of the common compounds that have been reported and a review was published suggesting that ecdysteroids can act as chemotaxonomic markers in this genus (Sena Filho *et al.*, 2008). Various ecdysteroids have been discovered from *Vitex*, such as pinnatasterone **1.86** isolated from *V. pinnata* and showed weak activity against *Musca domestica* larvae (Suksamrarn and Sommechai, 1993). Several ecdysteroids were isolated from *V. canescens*, including canescensterone **1.87**, 24-*epi*-abutasterone **1.88**, (24R)-11α,20,24-trihydroxyecdysone **1.89** and 11α,20,26-trihydroxyecdysone **1.90** (Suksamrarn *et al.*, 2000; Suksamrarn *et al.*, 1997; Suksamrarn *et al.*, 1995). Other ecdysteroids are 26-hydroxypinnatasterone **1.91**, isolated from *V. cymosa* (dos Santos *et al.*, 2001), 24-*epi*-pinnatasterone **1.92** and scabrasterone **1.93** discovered from *V. scabra* which displayed very weak moulting activity in *Musca* bioassay (Suksamrarn *et al.*, 2002b). More recently, *V. doniana* yielded 21-hydroxyshidasterone **1.94**, 11*β*-hydroxy-20-deoxyshidasterone **1.95**, and 2,3-acetonide-24-hydroxyecdysone **1.96** which showed anti-inflammatory activity in a rat paw oedema development assay at 100mg/kg+ dose (Ochieng *et al.*, 2013).

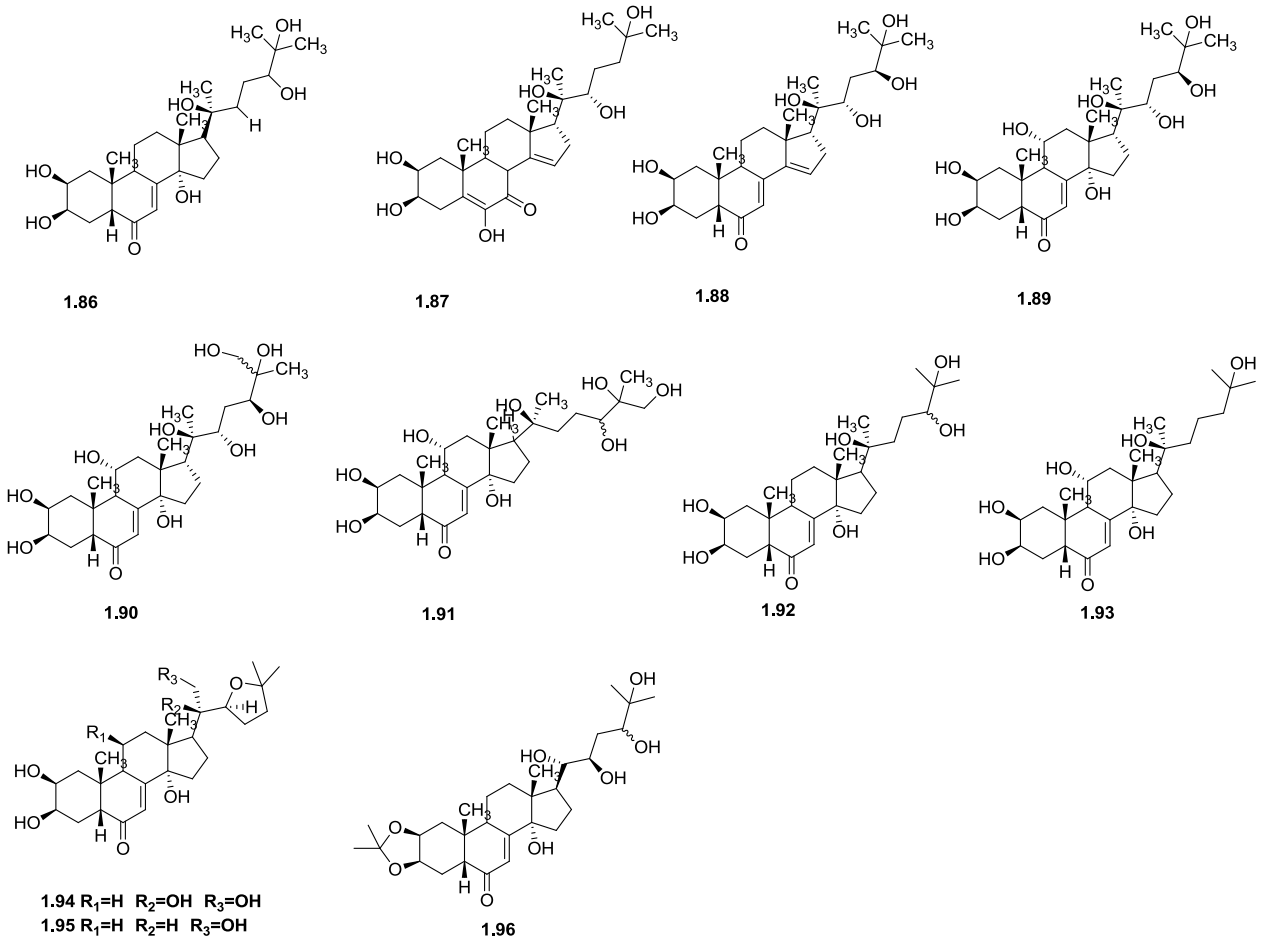


Fig. 1.10: Ecdysteroids derivatives previously isolated from *Vitex*

1.3.4 Flavonoids

Flavonoids are a group of pigment compounds that are found to be abundant in the plant kingdom (Havsteen, 1983). These compounds are responsible for normal growth, development and defence in plants (V. Cody, 1986). Flavonoid biosynthesis is via the shikimic acid and acylpolymalonate pathways (Samuelsson, 1993). In the genus *Vitex*, a small number of flavonoids have been reported. Four flavonoid glucosides isolated from *V. agnus-castus* exhibited cytotoxicity against P388 lymphocytic leukemic cells. This includes luteolin 6-C-(4″-methyl-6″-O-trans-caffeoylglucoside) **1.97**, luteolin 6-C-(6″-O-trans-caffeoylglucoside) **1.98**, luteolin 6-C-(2″-O-trans-caffeoylglucoside) **1.99**, and luteolin 7-O-(6″-p-benzoylglucoside) **1.100** with IC_{50} of 7.6, 14, 56 and 70 μg/mL, respectively (Hirobe *et al.*, 1997). Vitegnoside **1.101** was found in *V. negundo* and exhibited antifungal activity against *T. mentagrophytes* and *C. neoformans* with MIC values of 6.25 μg/mL (Sathiamoorthy *et al.*, 2007).

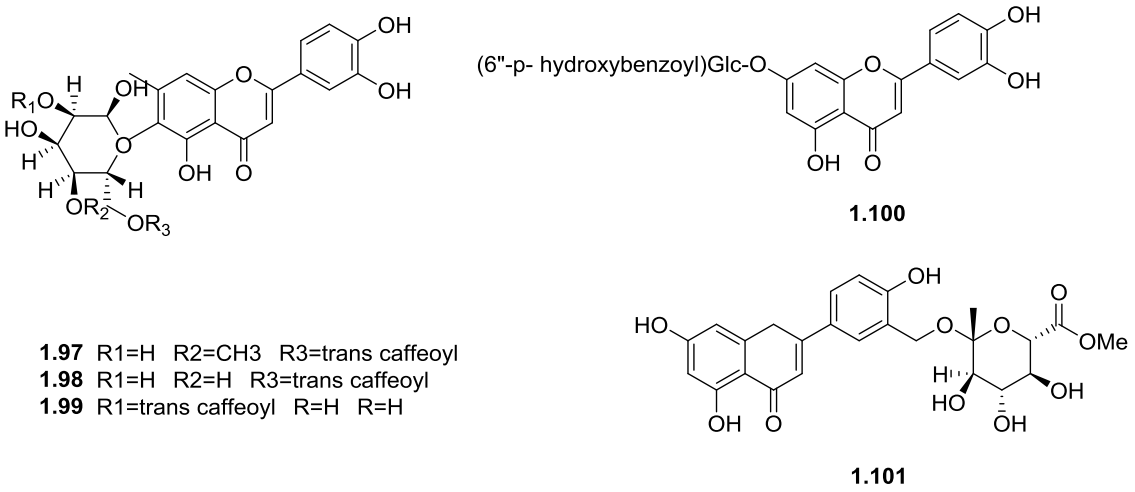


Fig. 1.11: Flavonoid derivatives previously isolated from *Vitex*

1.3.5 Previous pharmacological studies on *V. pinnata*

Extracts of *V. pinnata* showed broad activities when tested against various bioassays. Bark extracts of *V. pinnata* were shown to have antifungal activity against *Candida albicans* (Ata *et al.*, 2009). An antimelanogenesis assay was also carried out with the

root extract of *V. pinnata.* This extract possessed DPPH radical-scavenging activity of more than 70% when it was tested at 100 μg/ml (Arung *et al.*, 2009). Photocytotoxicity activity was detected with the leaf extract of *V. pinnata* at 20µg/ml. The extract was incubated with a promyelocytic leukimia cell line, HL60, which was then irradiated with 9.6 J/cm^2 of broad spectrum light. The result showed the reduction of the viability of HL60 cell line by more than 50% (Ong *et al.*, 2009).

1.4 Endophytic fungi and their bioactive natural products

Since the discovery of penicillin **1.6** from *P. notatum* by Fleming in 1929 (Fleming, 1929), many studies were embarked upon to find bioactive secondary metabolites from fungi. Many compounds with interesting bioactivities have been isolated from fungi and some of these compounds were approved as drugs by the FDA, such as erythromycin **1.11** and vancomycin **1.12**. Retapamulin **1.102** is an analogue of the fungal metabolite pleuromutilin **1.103** and was shown to treat skin infections such as impetigo (Jacobs, 2007). Pleuromutilin **1.103** binds to 50S bacterial ribosomes, inhibiting bacterial protein synthesis (Schlünzen *et al.*, 2004). It was first isolated from *Clitopilus passeckerianus* and it was used as an antibiotic to treat swine flu in animals in the 1950s (Sneader, 1996; Springer *et al.*, 2003). Pneuomocandin **1.104**, another fungal metabolite, was isolated from *Zalerion arboricola* in 1992 by a group of researchers from Merck Laboratory (Schwartz *et al.*, 1992). Caspofungin **1.105**, the semi-synthetic analogue of pneumocandin **1.104**, has been administered to treat fungal infections by inhibiting fungal β-1,3 glucan synthesis which disrupts the fungal cell wall (Saravolatz *et al.*, 2003). In 2006, anidulafungin **1.106**, another FDA approved antifungal drug, was introduced to the market. Anidulafungin is a semi synthetic analogue of echinocandin B **1.107** found in *Aspergillus rugulovalvus*. It has a similar mechanism of action to caspofungin (Denning, 2003; Krause *et al.*, 2004). Recently CKD-732 **1.108** was developed from fumagillin **1.109** and has successfully reached clinical trials as an angiogenesis inhibitor. Fumagillin was previously isolated from *Aspergillus fumigatus* in 1951 as an antibiotic (McCowen *et al.*, 1951) but it was not until 1990 that fumagillin derivatives were synthesised and found to be promising angiogenesis inhibitors (Ingber *et al.*, 1990).

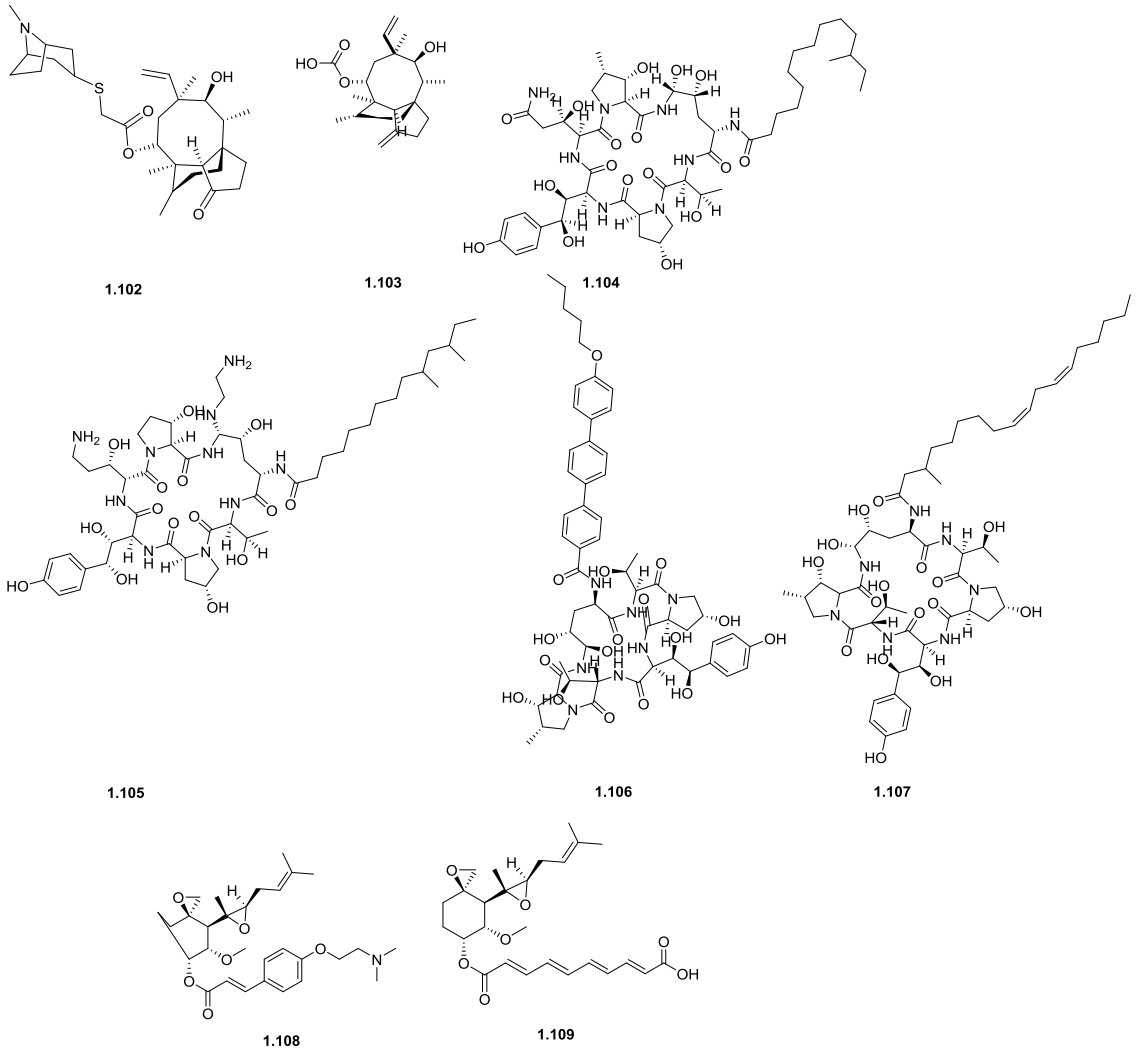
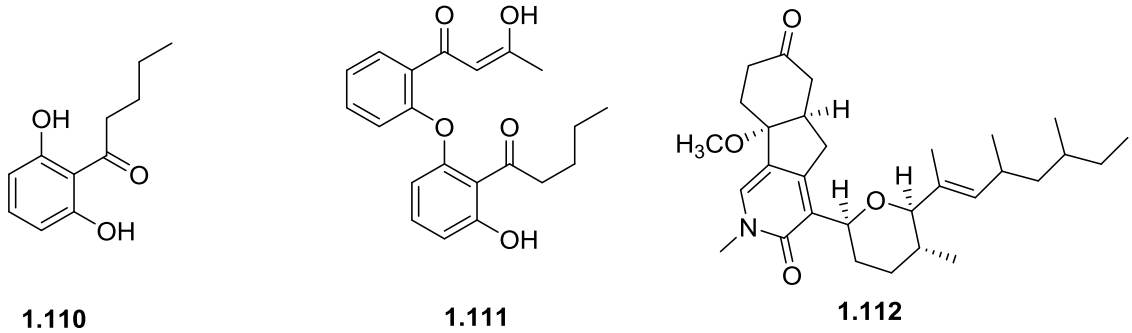


Fig 1.12: FDA-approved drugs isolated or inspired from endophytic fungi natural products

1.4.1 Bioactive compounds from terrestrial endophytic fungi as antimicrobial agents

The multidrug resistance of human pathogenic bacteria against existing antibiotics is increasing every year. This problem is very serious, especially in hospitals. Five bacterial species, namely *Enterococcus faecium*, *Staphylococcus aureus, Klebsiella pneumoniae*, *Acinetobacter baumanii*, *Pseudomonas aeruginosa*, and *Enterobacter* species were identified as the main cause of the majority of hospital infections (Rice, 2008). The discovery of new alternatives to the currently available antibiotics is hence of particular urgency.

Two new secondary metabolites identified as 1-(2,6-dihydroxyphenyl)pentan-1-one **1.110** and (Z)-1-(2-(2-butyryl-3-hydroxyphenoxy)-6-hydroxyphenyl)-3-hydroxybut-2-en-1-one **1.111** were isolated from the endophytic fungus *Cryptosporiopsis* sp. derived from its host plant *Clidemia hirta.* It was found that 1-(2,6-dihydroxyphenyl)pentan-1-one **1.110** exhibited antimicrobial activity against *Bacillus cereus*, *Escherichia coli* and *S. aureus* with IC_{50} values from 18 to 30 μg/mL while (Z)-1-(2-(2-butyryl-3-hydroxyphenoxy)-6-hydroxyphenyl)-3-hydroxybut-2-en-1-one **1.111** was active against *Pseudomonas fluorescens* with an IC_{50} value of 6 μg/mL (Zilla *et al.*, 2013). Furthermore, one new alkaloid, namely Fusapyridon A **1.112**, isolated from an endophytic fungus, *Fusarium* sp. YG-45 derived from the stem of *Maackia chinensis*, showed activity against *P. aeruginosa* and *S. aureus*, with MIC values of 6.25 and 50 μg/mL (Tsuchinari *et al.*, 2007).

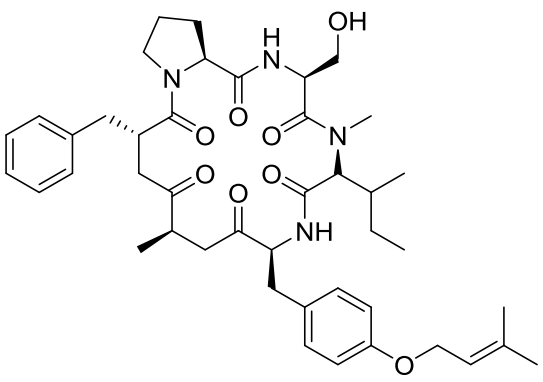


1.110 **1.111** **1.112**

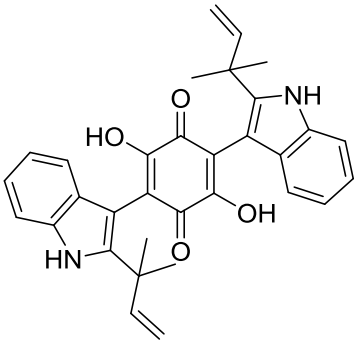
Fig 1.13: Antimicrobial secondary metabolites produced by terrestrial endophytic fungi

1.4.2 Bioactive compounds from terrestrial endophytic fungi as antiviral agents

Several viral diseases, especially human immunodeficiency virus (HIV) and hepatitis C virus (HCV), are still hard to combat by vaccination. Finding new, effective antiviral drugs, including those from nature, is therefore still a priority in order to ensure that these viral diseases are curable. Pullularin A **1.113** was reported to exhibit anti-HSV-1 (herpes simplex type 1) activity (IC_{50} 3.3 μg/mL). This compound was found in the extract of *Pullularia* sp. isolated from a leaf of *Culophyllum* sp. (Guttiferae) in Hala–Bala Wildlife Sanctuary, Narathiwat Province, Thailand (Isaka *et al.*, 2007). In addition to this, a new quinone named as hinnuliquinone **1.114** was isolated from an unidentified endophytic fungus derived from the leaves of its host plant, *Quercus coccifera*, collected in Ontígola, Aranjuez (Madrid, Spain). This compound displayed anti-HIV activity by the inhibition of wild-type HIV-1 protease activity with an IC_{50} of 2.5 μM. Further bioassays of hinnuliquinone against a highly resistant mutant enzyme A44 showed an IC_{50} value of 1.8 μM, revealing the potential of this compound as a promising candidate against drug-resistant strains of HIV-1 (Singh *et al.*, 2004).



1.113



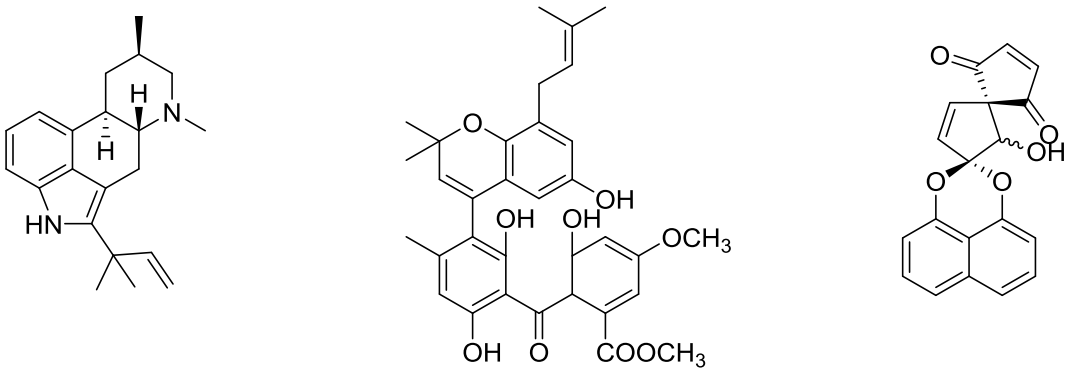
1.114

Fig 1.14: Antiviral agents derived from terrestrial endophytic fungi

1.4.3 Bioactive compounds from terrestrial endophytic fungi as anticancer agents

Cancer is the most common disease causing death worldwide. It has been estimated that by 2030 approximately a total number of 23.6 million new cancer cases will be reported. In 2012, the death toll caused by cancer was 17.5 million people worldwide (UK, 2015). Natural products such as those from higher plants, microorganisms and marine organisms offer unique and diverse bioactive secondary metabolites. Published data by Newman *et al* showed that out of 79 new anticancer agents, 50 were related to natural products, *i.e* natural product derivatives and natural product-inspired total synthesis, while only 25 were from total synthesis (Newman and Cragg, 2012). For this reason, mining and developing new anticancer drugs from natural products is still crucial and essential.

A new alkaloid determined as 9-deacetoxyfumigaclavine C **1.115** (shown in Fig 1.15), isolated from *Aspergillus fumigatus*, a fungal endophyte of *Cynodon dactylon* displayed promising cytotoxicity against human leukaemia cells (K562) with an IC_{50} value of 3.1 μM. The concentration used by this compound is about the same as the concentration of a current drug for leukemic treatment, doxorubicin hydrochloride (1.2 μM) (Ge *et al.*, 2009). Additionally, a new isoprenylated chromone derivative named as pestaloficiol L **1.116,** which can be seen in Fig. 1.15**,** isolated from *Pestalotiopsis fici* derived from *Camellia sinensis,* showed potent cytotoxicity with IC_{50} values of 8.7 and 17.4 μM against HeLa and MCF7 cells lines, respectively (Liu *et al.*, 2009). Furthermore, one novel spirobisnaphthalene identified as spiropreussione A **1.117** (illustrated in Fig. 1.15) was purified from *Preussia* spp, an endophytic fungus of *Aquilaria sinensis*. It exhibited *in vitro* cytotoxicity against the A2780 human ovarian carcinoma cell line and the BEL-7404 human liver carcinoma cell line, with IC_{50} values of 2.4 and 3.0 μM, respectively (Chen *et al.*, 2009).



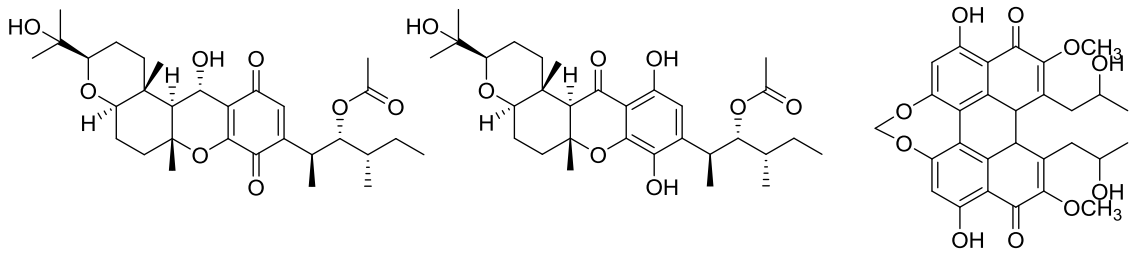
1.115 **1.116** **1.117**

Fig 1.15: Bioactive compounds from terrestrial endophytic fungi as anticancer agents

1.4.4 Bioactive compounds from terrestrial endophytic fungi as drugs against neglected tropical diseases

The neglected tropical diseases (NTDs) form a group of chronic infectious diseases caused by parasites, bacteria, viruses and fungi, and are endemic in underprivileged populations in developing countries of Africa, Asia and America (Hotez *et al.*, 2007). The NTDs lead to poverty resulting from long-term disability, long-term illness, deficient childhood growth and development, various problems in pregnancy and diminished worker productivity. Approximately more than 500,000 deaths annually are caused by the NTDs (Hotez *et al.*, 2006; Hotez *et al.*, 2009). In consequence, it is necessary to find new drugs to fight the NTDs, which in the end will improve the quality of life of affected populations or individuals.

Two quinone compounds known as cochlioquinone A **1.118** and isocochlioquinone A **1.119** (shown in Fig 1.16) were isolated from *Cochliobolus* sp., the fungal endophyte of *Piptadenia adiantoides*. Both compounds exhibited potent activity against *L. amazonensis* with EC50 values of 1.7 μM and 4.1 μM, respectively (Campos *et al.*, 2008). In addition, one known compound, cercosporin **1.120**, was purified from the new endophytic fungus, *Mycosphaerella* sp. nov. strain F2140 isolated from *Psychotria horizontalis*. It displayed potent activity against *L. donovani* and *T. cruzi* with IC_{50} values of 0.46 and 1.08 μM, respectively (Moreno *et al.*, 2011).



1.118 1.119 1.120

Fig 1.16: Bioactive compounds from terrestrial endophytic fungi as drugs for neglected tropical diseases

1.5 Secondary metabolites previously isolated from *Lasiodiplodia theobromae, Nigrospora* spp and *Pestalotiopsis* spp

1.5.1 Known secondary metabolites isolated from *Lasiodiplodia theobromae*

Lasiodiplodia theobromae is a common endophytic fungus that is also known to be an opportunistic pathogen on more 500 tree species in the tropics and subtropics (Burgess *et al.*, 2006; E., 1976). *L. theobromae* is the anamorph of *Botryosphaeria rhodina*, where it causes shoot blight and dieback in trees and shrubs (Mohali *et al.*, 2005). Previous isolation studies showed that this fungus produced a number of metabolites (shown in Fig. 1.17) such as lasiodiplodin **1.121**, de-O-methyl-lasiodiplodin **1.122**, *cis*-4-hydroxymellein **1.123**, indole-3-carboxylic acid **1.124**, 3-formylindole **1.125**, ethyl hydrogen fumarate **1.126**, (-)-mellein **1.127** (Aldridge *et al.*, 1971), jasmonic acid **1.128**, theobroxide **1.129** (Nakamori *et al.*, 1994), 5-oxolasiodiplodin **1.130**, 5-hydroxylasiodiplodins **1.131a** and **1.131b** (Matsuura *et al.*, 1998), (3R), (4S)-4-hydroxylasiodiplodin **1.132**, (3R), (6R)-6-hydroxy-de-O-methyllasiodiplodin **1.133**, (3R), (5R)-5-hydroxy-de-O-methyllasiodiplodin **1.134** (Yang *et al.*, 2000), (3S,4R)-3-carboxy-2-methylene-heptan-4-olide **1.135** (He *et al.*, 2004), (4S,5S)-4,5-dihydroxy-2-methyl-cyclohex-2-enone **1.136,** (3aS,4R,5S,7aR)-4,5-dihydroxy-7-methyl-3a,4,5,7a-tetrahydrobenzo[1,3]dioxol-2-one **1.137** (Takei *et al.*, 2008) and 4,5-dihydroxy-3-methyl-cyclohex-2-enone **1.138** (Kitaoka *et al.*, 2009).

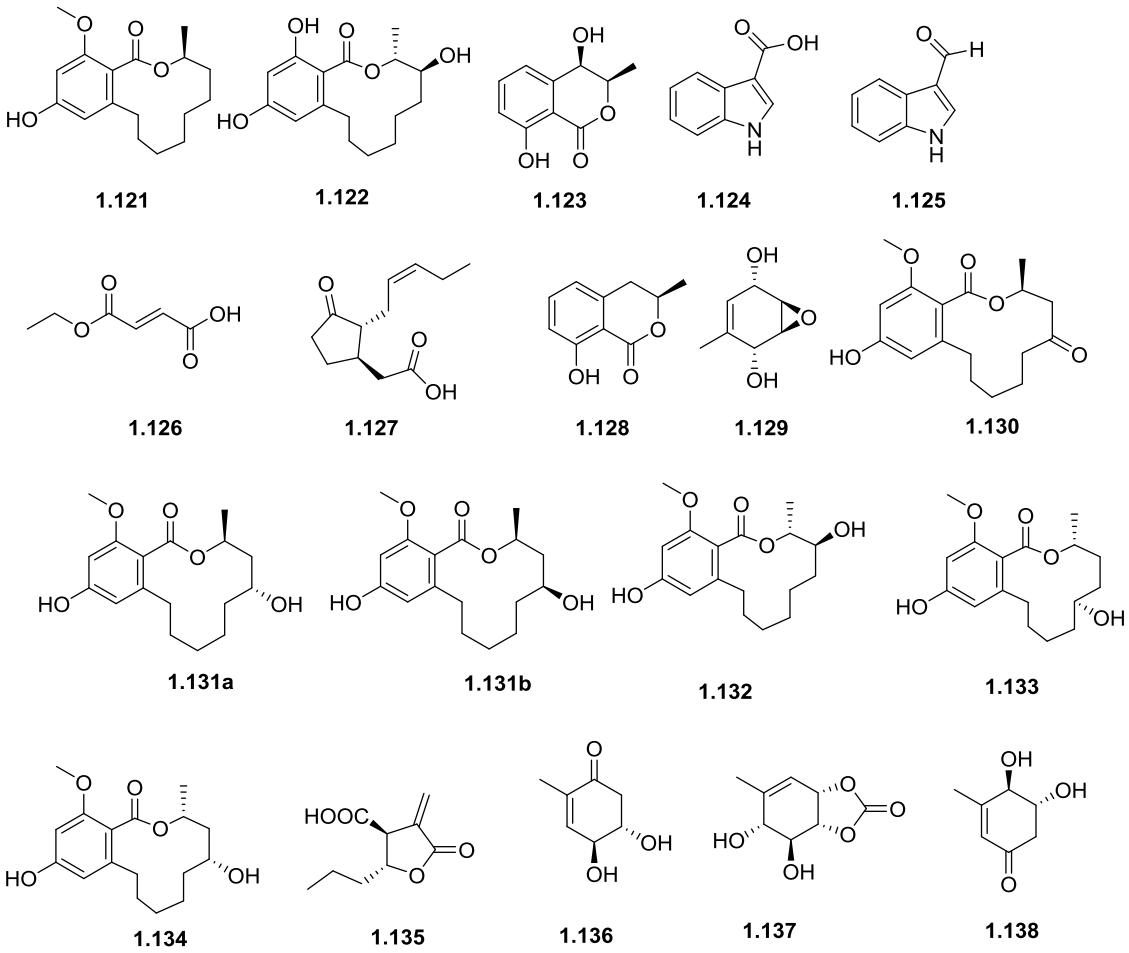


Fig. 1.17: Secondary metabolites previously isolated from *L. theobromae*

1.5.2 Known secondary metabolites isolated from *Nigrospora* spp

Nigrospora spp are endophytic and saprophytic fungi found in terrestrial and marine environments (Ding *et al.*, 2011; dui al Banerjee, 2011; Kuthubutheen, 1981; Li and Wang, 2009). Species such as *Nigrospora oryzae* have been reported to cause minute leaf and grain spot in rice crops (Mew and Gonzales, 2002), and even though it does not create any problematic disease against its host, yet it still can affect the quality of the seed, germination, plant strength and root growth (Sempere and Santamarina, 2006). Another common species in this genus is *Nigrospora sphaerica*. A recent publication reported that it can cause diseases on root of palm dates (Al-Sadi *et al.*, 2012). Despite their role as saprophytes during part of their life cycle, this species continuously produces innumerable secondary metabolites such as lactones, coumarins, quinone and semiquinone derivatives, epoxydons, pyrones, furanones, phenolic compounds,

nitrogen-containing compounds, terpenes and steroids. In this section, previous studies on the chemical investigation of *Nigrospora* genus will be included together with their bioactivities.

1.5.2.1 Polyketide derivatives

1.5.2.1.1 Lactones

Phomalactone **1.139** (Fukushima *et al.*, 1998) is a common fungal metabolite isolated from *Nigrospora* species (Evans Jr *et al.*, 1969; Fukushima *et al.*, 1998; Trisuwan *et al.*, 2009; Wu *et al.*, 2009b). Two other lactones have also been isolated from a culture broth of *N. sacchari* and identified as musacin D **1.140** (Fukushima *et al.*, 1998) and E **1.141** (Fukushima *et al.*, 1998)**.** Among these three metabolites, phomalactone showed the highest phytotoxicity effect in a leaf puncture assay and strong herbicidal effects in an electrolyte leakage assay (Fukushima *et al.*, 1998). Another lactone derivative, 5-(S)-[1-(1(S)-hydroxybut-2-enyl)]-dihydrofuran-2-one (Fukushima *et al.*, 1998) **1.142**, was also isolated from marine-derived *Nigrospora* sp. PSU-F18 along with musacin F **1.143** (Trisuwan *et al.*, 2009).

Other chemical studies on *N. sphaerica* led to the isolation of two known lactone derivatives, 4-(hydroxymethyl)-3,5-dimethyl dihydrofuran-2(3H)-one **1.144** (Rukachaisirikul *et al.*, 2009) and 3-(1-hydroxyethyl)-4-methyl dihydrofuran-2(3H)-one **1.145** (Choi *et al.*, 2009). Both compounds exhibited anticancer activity against two leukemic cancer cell lines, HL60 and chronic K562 with IC_{50} values of 0.2, 0.4 μg/mL and 0.49, 0.01 μg/mL, respectively (Metwaly *et al.*, 2014).

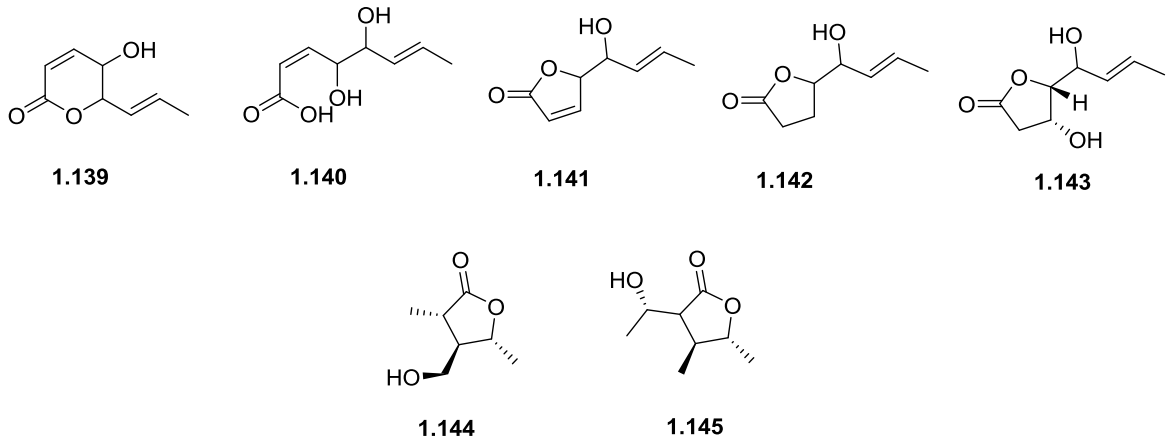


Fig. 1.18: Lactone derivatives previously found in *Nigrospora* spp

1.5.2.1.2 Coumarins

One known coumarin analogue, 3-isochromanone **1.146** (Rukachaisirikul *et al.*, 2010), has been obtained from *Nigrospora* sp. PSU-F12 isolated from a gorgonian sea fan (*Annella* sp.). Two common fungal endophytic metabolites, mellein **1.147** (Nishikawa, 1933) and 8-dihydroramulosin **1.148** (Stierle *et al.*, 1998), have both been isolated from potato dextrose broth cultures of *Nigrospora* sp. LLGLM003.

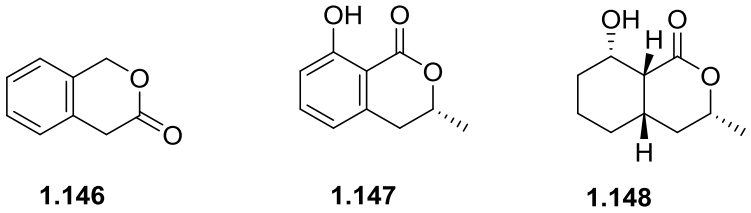


Fig. 1.19: Coumarin derivatives previously found in *Nigrospora* spp

1.5.2.1.3 Pyrones

Chemical investigation of *Nigrospora* sp. PSU-F5 afforded a new pyrone, nigrosporapyrone **1.159** (Trisuwan *et al.*, 2008), together with two known ones, **1.150** and **1.151** (Lee *et al.*, 1995). However, due to the insufficient amounts isolated, the antibacterial activities of all three pyrones against *Staphylococcus aureus* ATCC 25923 and methicillin-resistant *S. aureus* were not evaluated (Trisuwan *et al.*, 2008).

Four new pyrones analogues, nigrosporapyrones A–D **1.152-1.155** (Trisuwan *et al.*, 2009), and one known analogue, solanopyrone A **1.156** (Alam *et al.*, 1989), have been isolated from the broth of marine-derived *Nigrospora* sp. PSU-F18. Nigrosporapyrone C **1.154** exhibited good antibacterial activity against *S. aureus* ATCC 25923 and methicillin-resistant *S. aureus* with MIC values of 128μg/mL against both strains. In another study, a known pyrone derivative, aplysiopsene D **1.157** (Ciavatta *et al.*, 2009), was found from *Nigrospora* sp. PSU-F12 (Rukachaisirikul *et al.*, 2010).

Two new solanopyrone derivatives, solanapyrones N and O **1.158-1.159,** and one known derivative, solanopyrone C **1.160** (Alam *et al.*, 1989; Ichihara *et al.*, 1983), were isolated from a terrestrial endophytic fungus, *Nigrospora* sp. YB-141 derived from *Azadirachta indica* (Wu *et al.*, 2009b). The antifungal assay revealed that compound **1.158** was active against *Penicillium islandicum* with an MIC value of 31.25 μg/mL, which was equivalent with the MIC value of the positive control, nystatin (Wu *et al.*, 2009b).

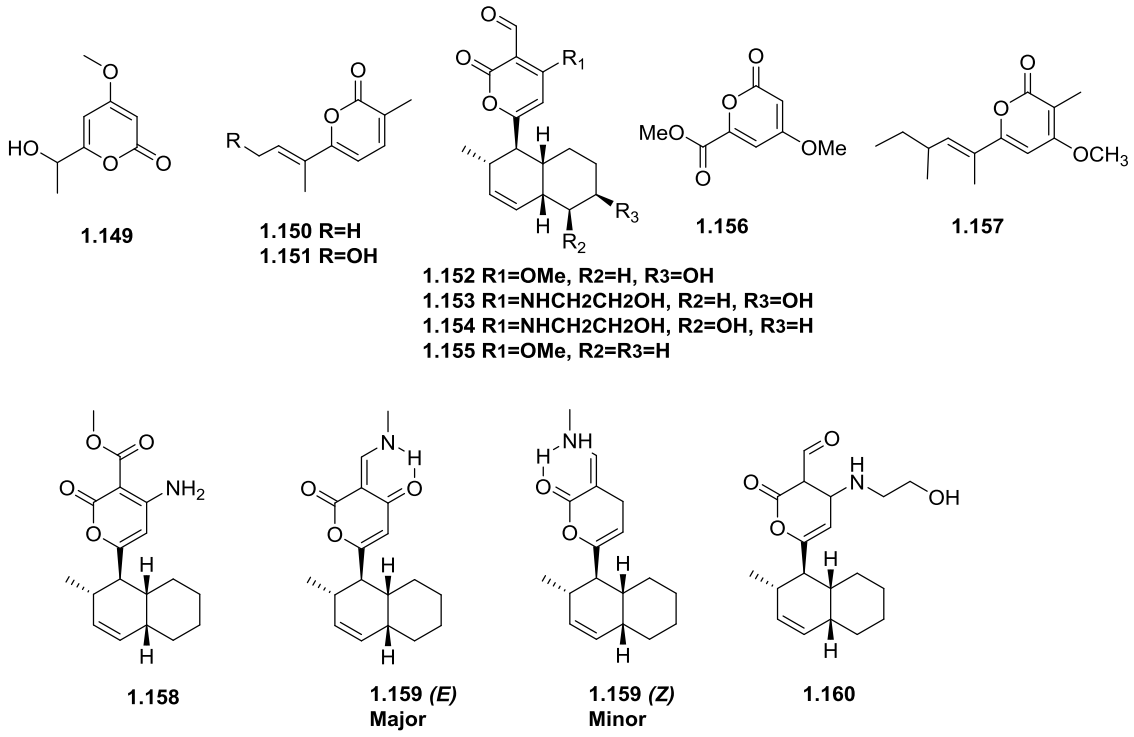


Fig. 1.20: Pyrone derivatives previously found in *Nigrospora* spp

1.5.2.1.4 Quinone and semiquinone derivatives

1.5.2.1.4.1 Epoxydons

Three new epoxydon analogues, nigrospoxydons A-C **1.161-1.163**, along with four known metabolites, (+)-epoxydon **1.164** (Mehta and Islam, 2004), 5(+)-deoxyabscisic acid **1.165**, 6(+)-abscisic acid **1.166** (Ferreres *et al.*, 1996), and (+)-phaseic acid **1.167** (Todoroki *et al.*, 2000) were isolated from the marine-derived fungus *Nigrospora* sp. PSU-F5. Evaluation of their antibacterial activity showed that compound **1.161** was active against *Staphylococcus aureus* (SA) and meticillin-resistant *Staphylococcus aureus* (MRSA) with MIC values of 64 μg/mL and >128 μg/mL, respectively, while compound **1.164** displayed an MIC value of 128 μg/mL against both strains (Trisuwan *et al.*, 2008).

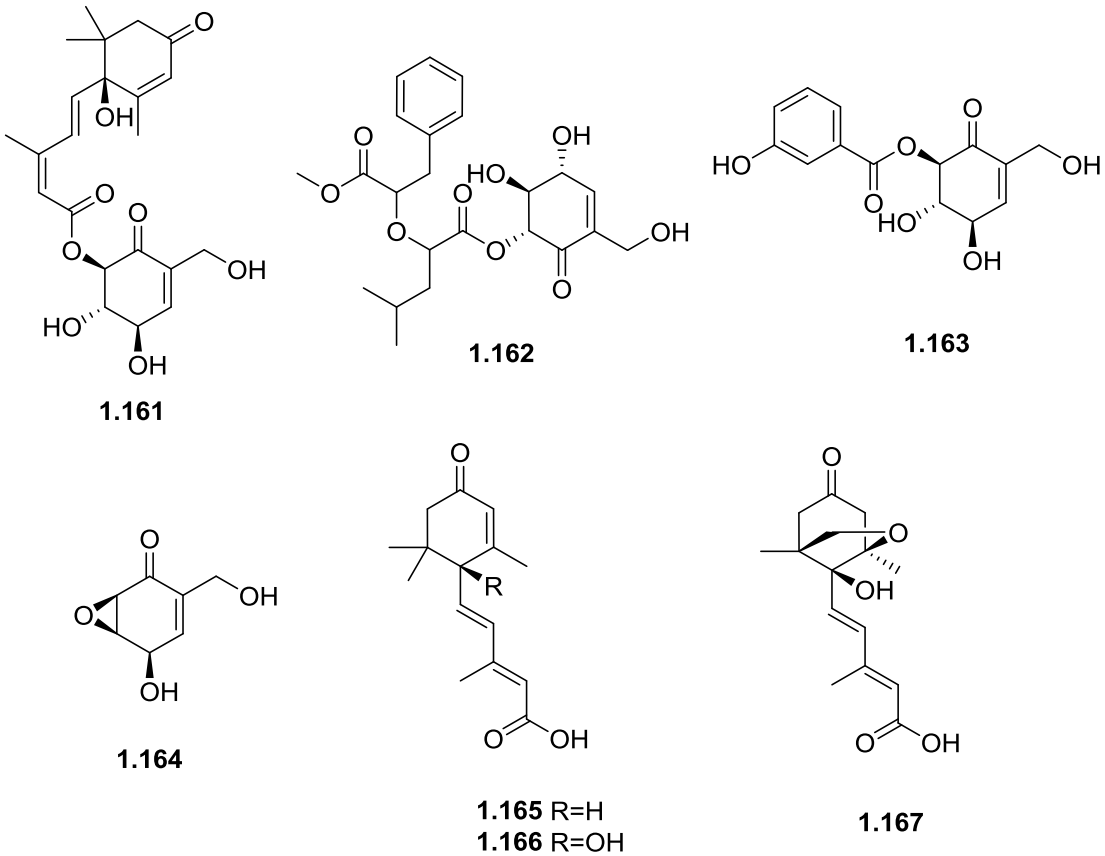


Fig. 1.21: Epoxydon derivatives previously found in *Nigrospora* spp

1.5.2.1.4.2 Anthraquinone

The first anthraquinone derivative isolated from *N.* oryzae was rhodosporin (bostrycin) **1.168** (Furuya and Shirasaka, 1969). Another isolation work from *N. oryzae* yielded two new compounds identified as nigrosporin A and B **1.169-1.170**. These two compounds inhibited the growth of *Bacillus sublitis* at 100 and 200 ppm with inhibition zones of 11 and 14 mm, respectively, using the disc diffusion assay (Tanaka *et al.,* 1997). A series of anthraquinone metabolites was isolated from a marine-derived *Nigrospora* sp., obtained from an unidentified sea anemone in the South China Sea. Among the anthraquinones isolated were two new compounds, 4a-epi-9α-methoxydihydrodeoxybostrycin and 10-deoxybostrycin **1.171-1.172** (Yang *et al.,* 2012a), and seven known anthraquinone derivatives: nigrosporin B **1.170** (Tanaka *et al.*, 1997), 9α-hydroxydihydrodesoxybostrycin **1.173** (Shang *et al.*, 2012; Sommart *et al.*, 2008), 9α-hydroxyhalorosellinia A **1.174** (Shang *et al.*, 2012; Sommart *et al.*, 2008), 4-deoxybostrycin **1.175** (Charudattan and Rao, 1982; Xia *et al.*, 2011), bostrycin **1.168** (Charudattan and Rao, 1982; Furuya and Shirasaka, 1969), 3,5,8-trihydroxy-7-methoxy-2-methylanthracene-9,10-dione **1.176** (Xia *et al.,* 2007) and austrocortirubin **1.177** (Xia *et al.,* 2007). Of all the metabolites, nigrosporin B **1.170** exhibited the greatest antibacterial activity against *B. subtilis* and *B. cereus* with MIC values of 312 nM against both organisms. Its activity against *B. subtilis* was therefore similar to the positive control, ciprofloxacin, which also had an MIC value of 312 nM, whereas its activity against *B. cereus* was greater than that of ciprofloxacin (MIC: 1250 nM). Compounds **1.170** and **1.175** were tested for anti-mycobacterial activity using the Kirby-Bauer disk diffusion susceptibility test, and both compounds displayed inhibition zone sizes of 30 mm and 27 mm, respectively, against *M. bovis* BCG. A further microarray study revealed that the expression of several genes of *M. tuberculosis* H37Rv4 were affected by 4-deoxybostrycin **1.175**. 4-deoxybostrycin **1.175** might therefore be a promising anti-TB drug candidate for future development (Wang *et al.*, 2013).

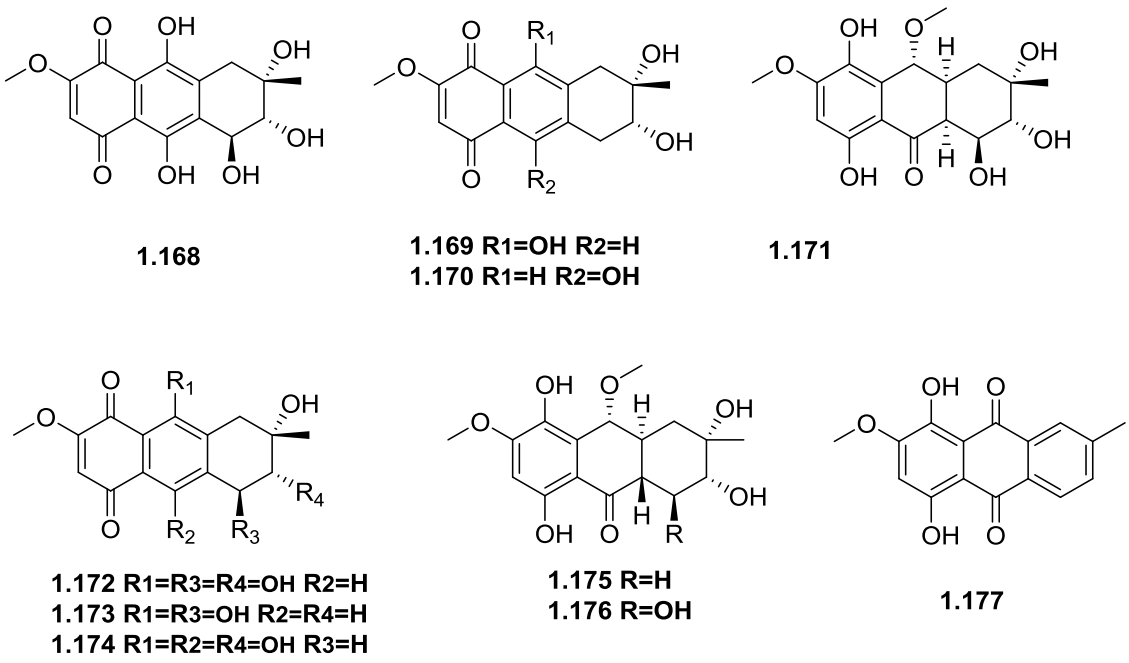


Fig. 1.22: Anthraquinone derivatives previously found in *Nigrospora* spp

1.5.2.1.4.3 Other quinones

One new quinone, 2,3-didehydro-19a-hydroxy-14-epicochlioquinone B **1.178** (Shang *et al.*, 2012), along with three known xanthones analogues, 3,8-dihydroxy-6-methoxy-1-methylxanthone **1.179** (Kingston *et al.*, 1976), 3,6,8-trihydroxy-1-methylxanthone **1.180** (Harris *et al.*, 1976) and griseophenone **1.181** (Rhodes *et al.*, 1961), were obtained from *Nigrospora* sp. MA75, an endophytic fungus isolated from the marine semi-mangrove plant *Pongamia pinnata*. Compound **1.178** showed promising antibacterial activity against various bacteria including MRSA, *E. coli*, *P. aeruginosa*, *P. fluorescens,* and S. *epidermidis* with MIC values of 8, 4, 4, 0.5, and 0.5 μg/mL, respectively (Shang *et al.*, 2012).

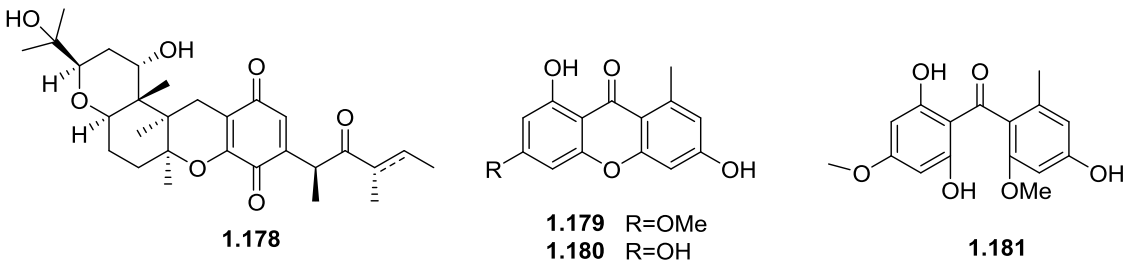


Fig. 1.23: Other quinones derivatives previously found in *Nigrospora* spp

1.5.2.1.4.4 Furanones

Five new 2(5H)-furanone-type derivatives, pestalafuranones F–J **1.182-1.186**, along with two known compounds, pestalafuranones A **1.187** (Liu *et al.*, 2012) and B **1.188** (Liu *et al.*, 2012), were isolated from modified PDA broth cultures of *Nigrospora* sp. BM2 from the leaf of *Saccharum arundinaceum* Retz., collected from Yichang Hubei Province, China (Zhang *et al.*, 2014). In this study, Zhang and co-workers used the OSMAC (one strain many compounds) approach to activate cryptic genes in *Nigrospora* sp. BM2. This strategy demonstrated that *Nigrospora* sp is capable of producing new metabolites **1.182-1.186** as a result of its adaptation to a modified environment (Zhang *et al.*, 2014).

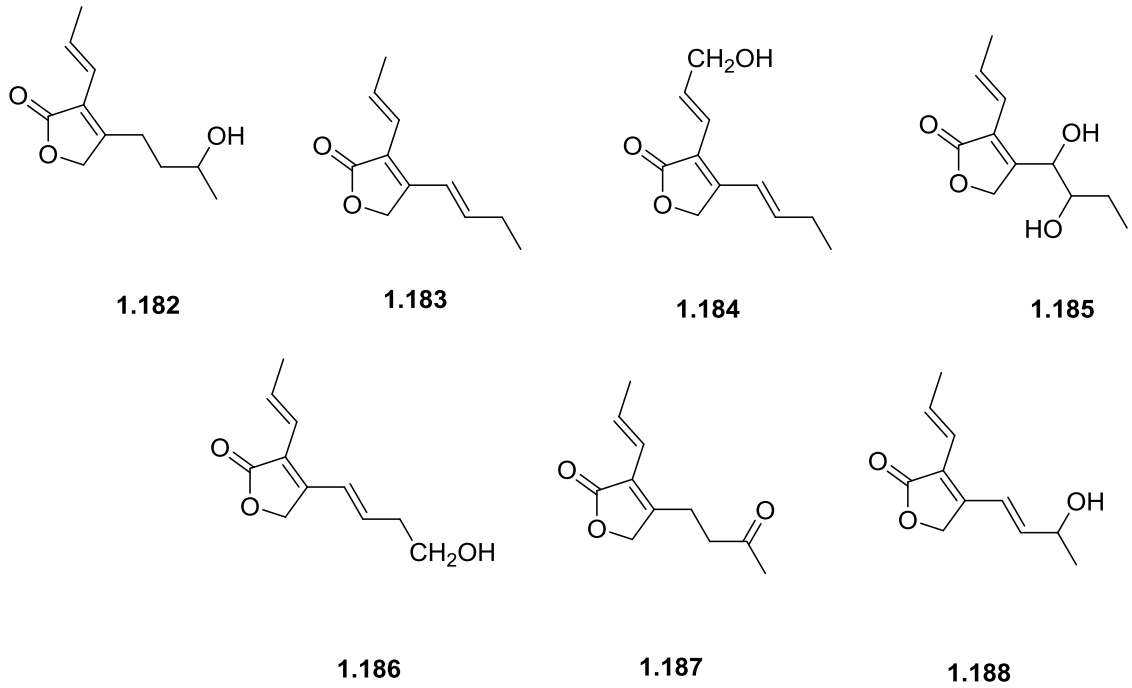


Fig. 1.24: Furanones derivatives previously found in *Nigrospora* spp

1.5.2.1.5 Other polyketides

Two new polyketide cyclohexene derivatives, nigrosporanenes A **1.189** and B **1.190**, were obtained from the broth fermentation of the marine-derived endophytic fungus *Nigrospora* sp. PSU-F11. Compound **1.189** displayed cytotoxicity against MCF-7 and Vero cells with respective IC_{50} values of 9.37 and 5.42 μg/mL. In addition, weak radical

scavenging activity was observed for both **1.189** and **1.190** with IC_{50} values of 0.34 and 0.24 mg/mL, respectively (Rukachaisirikul *et al.*, 2010).

Griseofulvin **1.191** (Oxford *et al.*, 1939) and dechlorogriseofulvin **1.192** (MacMillan, 1953), analogues of benzofurans, were successfully isolated from *Nigrospora* sp. LLGLM003, an endophytic fungus derived from the root of *Moringa oleifera* Lam (Zhao *et al.*, 2012). Only compound **1.191**, an antifungal antibiotic currently used to treat fungal infection in humans (Gupta *et al.*, 1997), showed strong antifungal activity against the plant pathogenic fungi *Botrytis cinerea* and *Colletotrichum orbiculare* with EC_{50} values of 0.20 and 0.49 μg/mL, respectively (Zhao *et al.*, 2012).

Two new griseofulvin derivatives, 6-O-desmethyldechlorogriseofulvin **1.193** and 6'-hydroxygriseofulvin **1.194**, along with two known metabolites **1.191** (Oxford *et al.*, 1939) and **1.192** (MacMillan, 1953) were obtained from extracts of the marine-derived fungus *Nigrospora* sp. MA75 by applying the OSMAC approach. Interestingly, the dereplication analysis using HPLC found that compounds **1.193** and **1.194** were only produced in the rice culture medium while compound **1.191** and **1.192** were produced in all media (Shang *et al.*, 2012).

Isolation work on the extract of an endolichenic fungus, *N. sphaerica*, yielded two new heptaketides, (+)-(2S,3S,4aS)-altenuene **1.195a** (He *et al.*, 2012) and (−)-(2S,3S,4aR)-isoaltenuene **1.196a** (He *et al.*, 2012) along with five known mycotoxin derivatives identified as (−)-(2R,3R,4aR)-altenuene **1.195b** (Jiao *et al.*, 2006b), (+)-(2R,3R,4aS)-isoaltenuene **1.196b** (Jiao *et al.*, 2006b), alternariol **1.197** (Gu, 2009), alternariol-9-methyl ether **1.198** (Gu, 2009), and 4-hydroxyalternariol-9-methyl ether **1.199** (Gu, 2009). All compounds were tested for antiviral activity against HSV and only compounds **1.197** and **1.198** were active with IC_{50} values of 13.5 and 21.3 μM and selective index values of 26.5 and 17.1, respectively (He *et al.*, 2012).

A new isochromene derivative, nigrosphaerin A **1.200**, was isolated from the terrestrial endophytic fungus *N. sphaerica* derived from *Vinca rosea* (Metwaly *et al.*, 2014). One new macrolide analogue, nigrosporolide **1.201**, was isolated from *N. sphaerica* (strain UK181RRC) and displayed plant growth inhibitor activity on etiolated wheat coleoptiles (Harwooda *et al.*, 1995).

Nigrospins B and C **1.202-1.203**, two new citrinins and one known compound 1-acetonyl-7-carboxyl-6,8-dihydroxy-3,4,5-trimethylisochroman **1.204** (Xu *et al.*, 2006) were obtained from the marine-derived fungus *N. oryzae* SCSGAF 0111 isolated from the gorgonian *Verrucella umbraculum* (Dong *et al.*, 2014). All three compounds exhibited weak antifungal activity against *Aspergillus versicolor* with inhibition zones of 8 cm at 50 µg/paper disc in comparison with thiram as a positive control against *Aspergillus sydowii* and *A. versicolor* with an inhibition zone of 8 cm at 5 µg/paper disc.

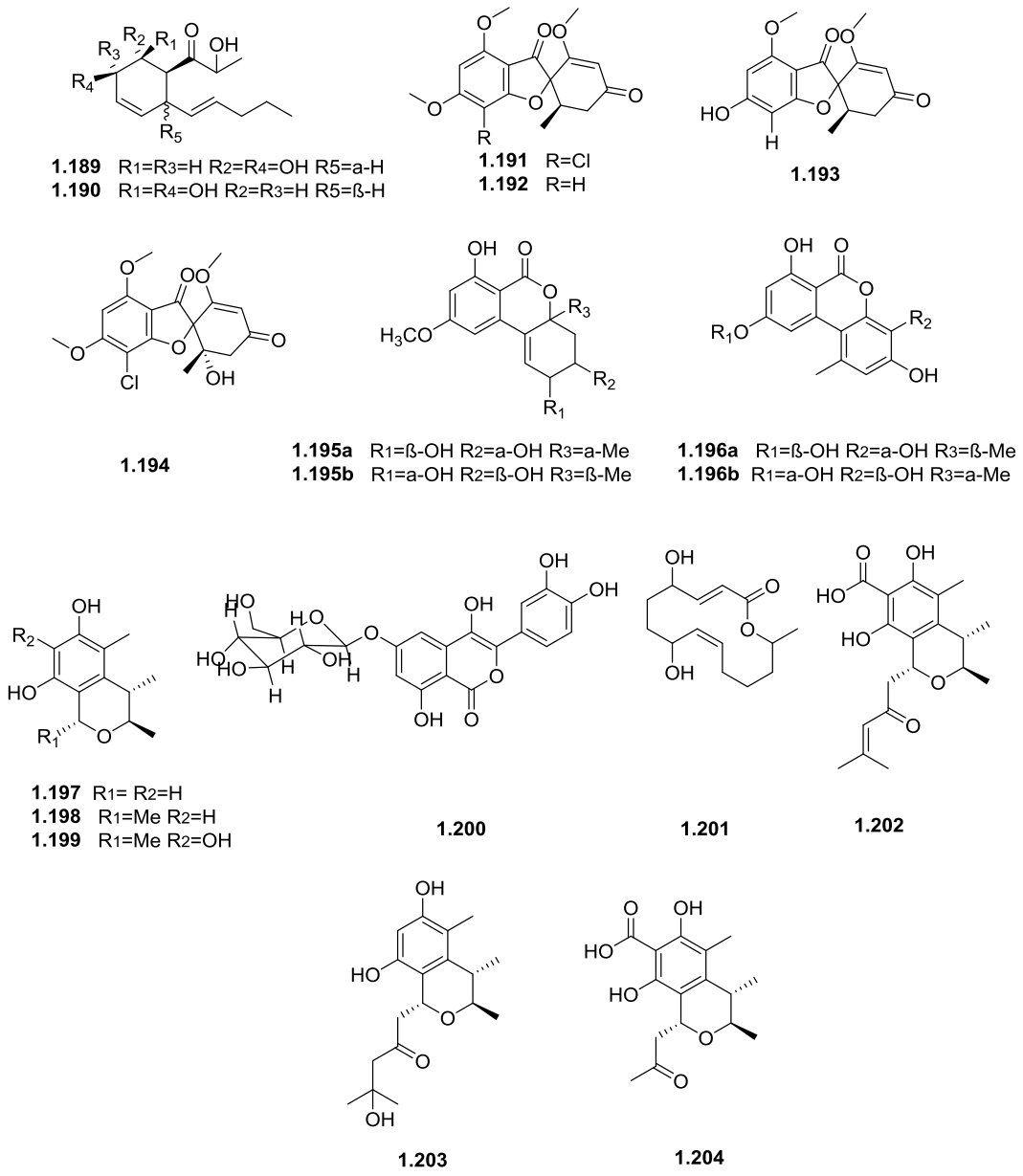
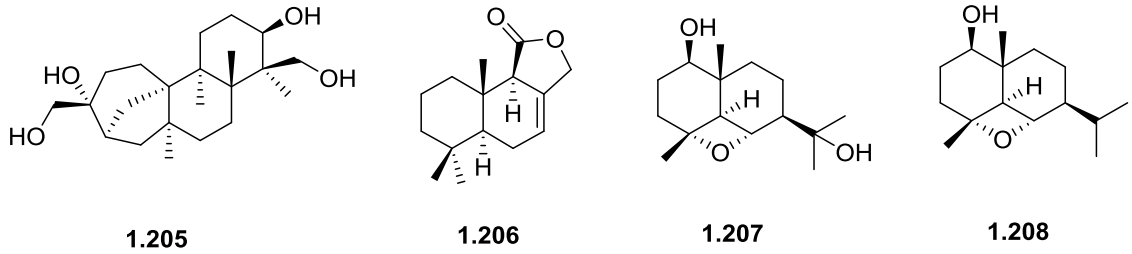


Fig. 1.25: Other polyketide derivatives previously found in *Nigrospora* spp

1.5.2.2 Terpenoids

Aphidicolin **1.205** (Brundret *et al.*, 1972), a known antibiotic diterpene derivative, was isolated from the endophytic fungus *N. sphaerica* (Starratt and Loschiavo, 1974). One known drimane sesquiterpenoid, (-)-drimenin **1.206** (Akita *et al.*, 2000), was found in the broth of a marine-derived fungus *Nigrospora* sp. PSU-F12 collected near the Similan Islands, Southern Thailand. (-)-drimenin **1.206** was inactive in several bioassays, including cytotoxity assays against MCF-7 and Vero cell lines, antioxidant assays using DPPH, and antibacterial assays against SA and MRSA (Rukachaisirikul *et al.*, 2010). 11-Hydroxycapitulatin B **1.207**, a new metabolite belonging to the group of eudesmane sesquiterpene derivatives together with the sesquiterpene capitulatin B **1.208** (Li *et al.*, 2005), were obtained from the endophytic fungus *N. oryzae* A8 isolated from *Aquilaria sinensis* (Li *et al.*, 2014a).



1.205 **1.206** **1.207** **1.208**

Fig. 1.26: Terpenoids derivatives previously found in *Nigrospora* spp

1.5.2.3 Steroids

Seven known steroid derivatives were obtained from the solid rice extract of *N. sphaerica.* These were identified as ergosta-6,22-diene-3β,5α,8α-triol **1.209** (Cateni *et al.*, 2007), ergosta-7,22-diene-3β-ol **1.210** (Qing-fang *et al.*, 2010), ergosta-4,6,8(14),22-tetraene-3β-ol **1.211** (Pang and Sterner, 1993), ergosta-4,6,8(14),22-tetraene-3one **1.212** (Lee *et al.*, 2005), ergosta-5(6),7,22-triene-3β-ol **1.213** (Li *et al.*, 2007), ergosta- 7,9(14), 22-triene-3β-ol **1.214** (Li *et al.*, 2008), and ergosta-7,22-epidioxy-3β-ol **1.215** (Cateni *et al.*, 2007). Compounds **1.212** and **1.214** exhibited antileukemic activity against acute HL60 cells with IC_{50} values of 0.03 and 0.39μg/mL and against chronic K562 cells with IC_{50} values of 0.35μg/mL for both compounds, respectively. The antileishmanial assay revealed that compounds **1.210**, **1.211** and

1.213 showed adequate activity with IC_{50} values of 30.2, 26.4 and 36.4 μg/mL, respectively. Compound **1.214** also exhibited good antifungal activity against *Cryptococcus neoformans* with an IC_{50} value of 14.81 μg/mL (Metwaly *et al.*, 2014).

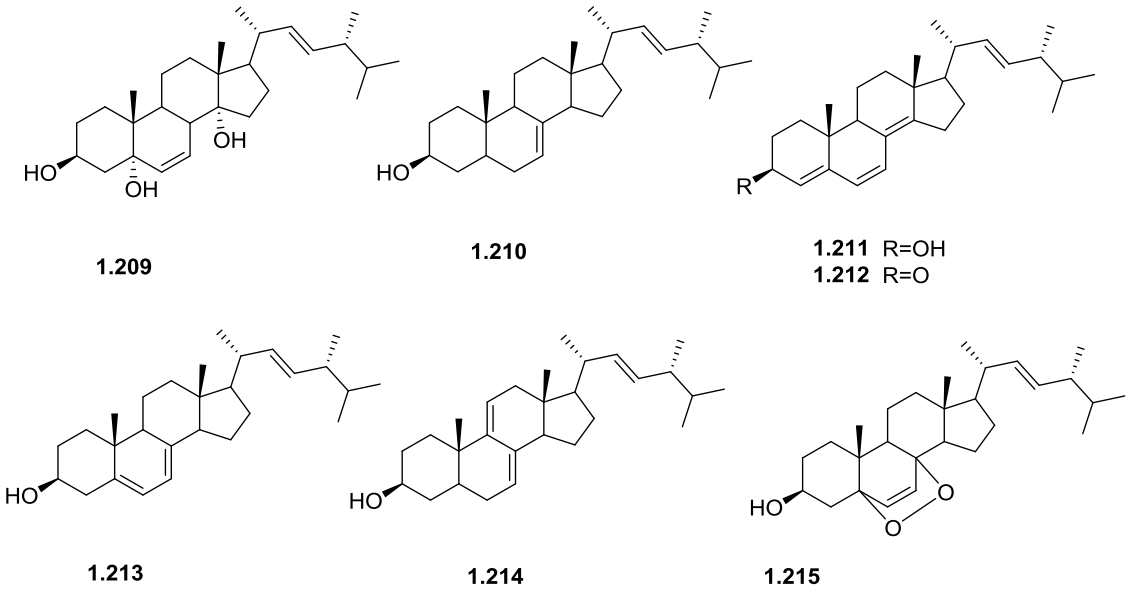


Fig. 1.27: Steroid derivatives previously found in *Nigrospora* spp

1.5.2.4 Aromatic compounds

Three phenolic compounds, 3,4-dihydro-3,4,8-trihydroxy-1[2H]-naphthalenone **1.216** (Borgschulte *et al.*, 1991), clavatol **1.217** (Astudillo *et al.*, 2000) and 3-hydroxymethylphenol **1.218** (Alfaro *et al.*, 2003), were reported from the potato dextrose broth of the marine-derived fungus *Nigrospora* sp. PSU-F5 isolated from a sea fan *Anella* sp collected near Similan Island, Thailand. Tyrosol **1.219** (Chen *et al.*, 2004; Rasser *et al.*, 2000) was isolated from the broth of the marine-derived fungi *Nigrospora* sp. PSU-F18 (Trisuwan *et al.*, 2009) and PSU-F11 (Rukachaisirikul *et al.*, 2010). Furthermore, the same paper reported that 4-hydroxybenzoic acid **1.220** (Pyo *et al.*, 2002) was obtained from *Nigrospora* sp. PSU-F12 (Rukachaisirikul *et al.*, 2010), another fungal strain which they worked on. Compounds **1.220** (Chen *et al.*, 2004; Rasser *et al.*, 2000) and 1-(4-hydroxyphenyl)ethan-1-one **1.221** (Li, 2008) were also isolated from the terrestrial endophytic fungus, *N. sphaerica* isolated from it host plant *Vinca rosea* (Metwaly *et al.*, 2014). Tyrosol **1.219** is a high value-added antioxidant metabolite which can be found in olive oil. Its function as a neutraceutical in the

prevention of cancer and cardiovascular diseases is evolving remarkably (Capasso *et al.*, 1995; de la Puerta *et al.*, 1999; Deiana *et al.*, 1999; Konstantinidou *et al.*, 2010; Lozano-Sánchez *et al.*, 2010; Waterman and Lockwood, 2007). One known phenolic compound, 5′-methoxy-6-methyl-biphenyl-3,4,3′-triol **1.222**, was isolated from *N. sphaerica*, an endolichenic fungus derived from *Parmelinella wallichiana* (Taylor) Elix & Hale collected from Zixi Mountains of Yunnan Province of China (He *et al.*, 2012).

Two new phenolic compounds, 1-(5-oxotetrahydrofuran-2-yl) ethyl 2-phenylacetate **1.223** and 3-hydroxybutan-2-yl 2-hydroxy-3-phenylpropanoate **1.224**, together with three known compounds, harzialactone A **1.225** (Amagata *et al.*, 1998), benzeneethanol 4-hydroxy-1-acetate **1.226** (Procopiou *et al.*, 1998), and 1,4-dioxane-2,5-dione-3,6-bis(penylmethyl)-homopolymer **1.227** were obtained from the endophytic fungus *N. sphaerica*. 4-Hydroxyphenethyl 2-hydroxypropanoate **1.228** (Lu *et al.*, 2012), a known aromatic compound, was isolated from *Nigrospora* sp. derived from *Scyphiphora hydrophyllacea* (Chen *et al.*, 2012).

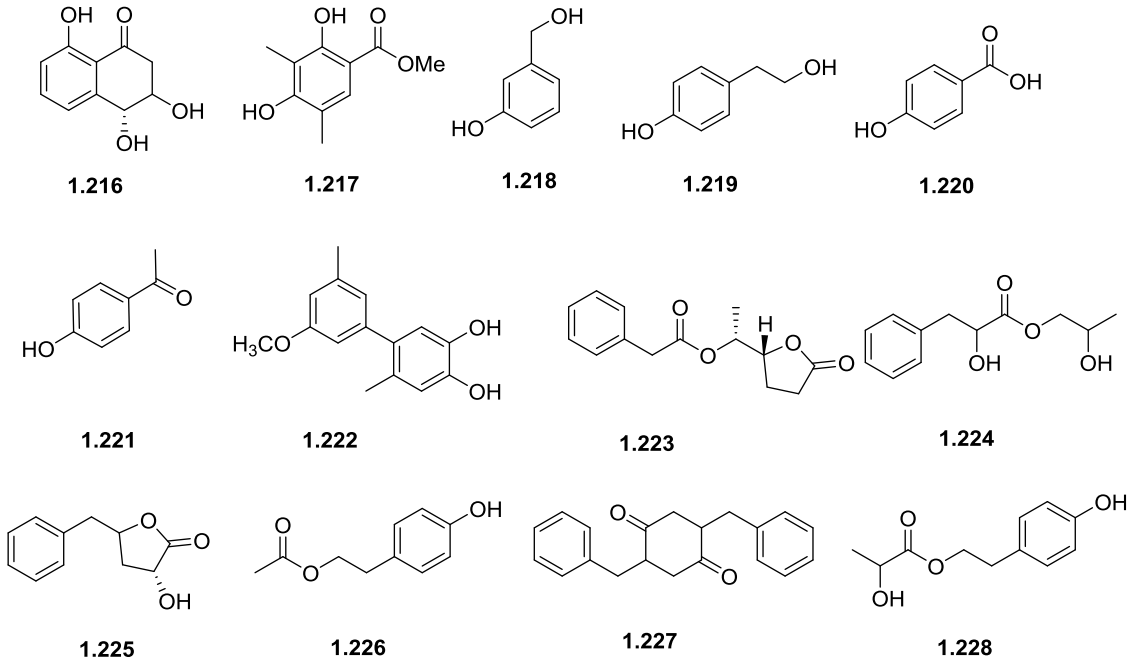


Fig. 1.28: Aromatic compounds previously found in *Nigrospora* spp

1.5.2.5 Nitrogen-containing compounds

One known diketopiperazine derivative Sch 54796 **1.229** (Chu *et al.*, 1993) was isolated from a marine-derived endophytic fungus, *Nigrospora* sp. PSU-F11 (Rukachaisirikul *et al.*, 2010). Two other diketopiperazine analogues, 3-benzylhexahydropyrrolo[1,2-a]pyrazine-1,4-dione **1.230** (Fdhila *et al.*, 2003) and 3-methylhexahydropyrrolo[1,2-a]pyrazine-1,4-dione **1.231** (Hendea *et al.*, 2006) were obtained from the terrestrial endophytic fungus *N. sphaerica* isolated from fresh and healthy leaves of *Vinca rosea* (Metwaly *et al.*, 2014).

Two new alkaloids, nigrospine and nigrospin A **1.232-1.233**, along with one known alkaloid, 2-(4-hydroxybenzyl) quinazolin-4(3H)-one **1.234** (Ma *et al.*, 2010a), were obtained from the marine-derived fungus *N. oryzae* SCSGAF 0111 (Dong *et al.*, 2014).

Two new nitrogen-containing compounds, methyl 5-acetamido-6-(4-hydroxyphenyl)-4-oxohexanoate **1.235** and uridine-5'α-hydroxypropanoate **1.236**, together with three known metabolites, methyl pyroglutamate **1.237** (Yang *et al.*, 2003), cyclo (Pro-Val) **1.238** (Jayatilake *et al.*, 1996), and cyclo (Phe-Hyp) **1.239** were isolated from the terrestrial endophytic fungus *Nigrospora* sp. isolated from *Scyphiphora hydrophyllacea*. The two new compounds were inactive for cytotoxicity activity against HL-60 cell line with IC_{50} values over 100 μM for both compounds (Chen *et al.*, 2012).

Two known nucleoside analogues, uridine **1.240** (Mantsch and Smith, 1973) and adenosine **1.241** (Ciuffreda *et al.*, 2007), were isolated from the solid rice culture extract of *N. sphaerica* obtained from fresh and healthy leaves of *Vinca rosea*.

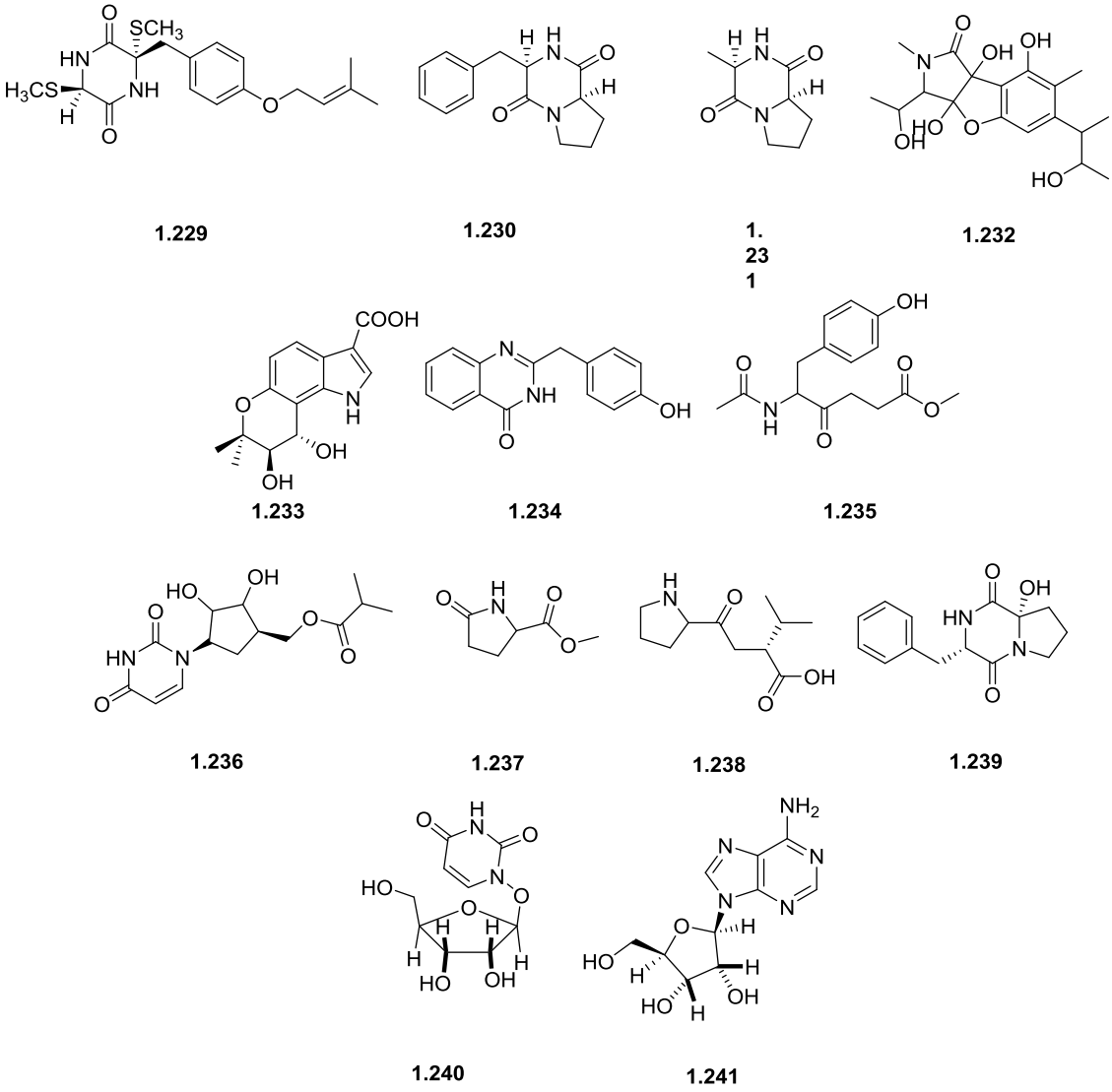
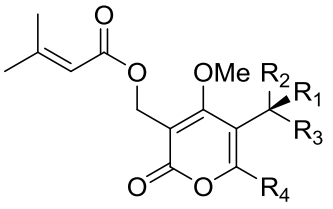


Fig. 1.29: Nitrogen-containing compounds previously found in *Nigrospora* spp

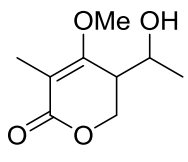
1.5.3 Known secondary metabolites isolated from *Pestalotiopsis* spp

Pestalotiopsis species are continuously fascinating scientists by producing highly diverse and unique biochemical metabolites. In recent years, three reviews reported more than 100 metabolites produced by these species (Xu *et al.*, 2010b; Xu *et al.*, 2014; Yang *et al.*, 2012b). In this section, I will include several recent findings on secondary metabolites isolated from *Pestalotiopsis* spp.

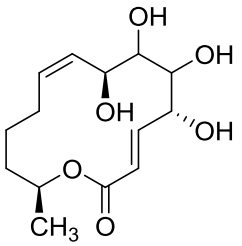
Five compounds named as pestalotiopyrones A–C **1.242-1.244** and pestalotioprolides A-B **1.245-1.246** and two known compounds, seiricuprolide **1.247** (Ballio *et al.*, 1988) and 20-hydroxy-30,40- didehydropenicillide **1.248** (Kawamura *et al.*, 2000) were isolated from *Pestalotiopsis* spp. PSU-MA92 and PSU-MA119 derived from mangrove plant *Rhizophora apiculata* and *Rhizophora mucronata*, respectively (Rukachaisirikul *et al.*, 2012). Due to insufficient yields, only pestalotiopyrone B was sent for antibacterial assays against *Staphylococcus aureus* ATCC25923 and a methicillin *S. aureus* clinical isolate, as well as for antifungal assays against *Candida albicans* NCPF3153, *Cryptococcus neoformans* ATCC90113 and a *Microsporum gypseum* clinical isolate. However this compound was inactive in all bioassays (Rukachaisirikul *et al.*, 2012).



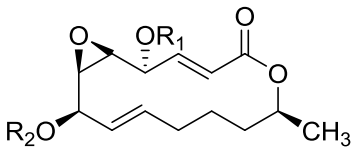
1.242 R1=OH, R2=R4= H, R3 = CH3
1.243 R1=R2=H R3=OH, R4=CH3



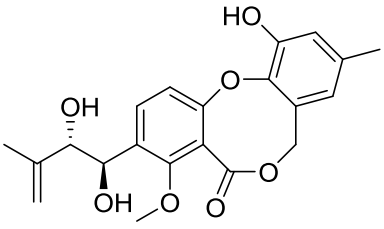
1.244



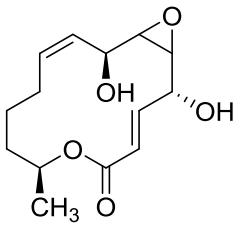
1.245



1.246 R1=R2=H

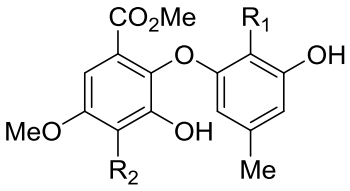


1.247

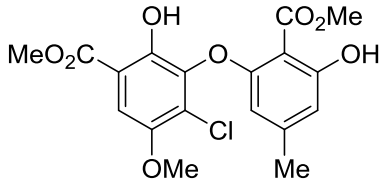


1.248

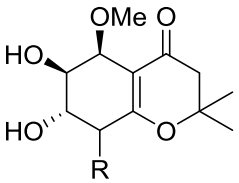
Nine secondary metabolites including four new diphenyl ethers, pestalotethers A-D **1.249-1.252**, three new chromones, pestalochromones A-C **1.253-1.255**, one new xanthone, pestaloxanthone **1.256**, and one new butenolide, pestalolide **1.257**, were isolated from the mangrove-derived fungus *Pestalotiopsis* sp. PSU-MA69. All compounds were tested against *Candida albicans* and *Cryptococcus neoformans* but only pestalolide **1.257** exhibited weak antifungal activity (Klaiklay *et al.*, 2012).



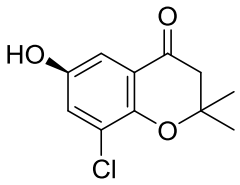
1.249 R1=CO2Me R2= Cl
1.250 R1=H R2=Cl
1.252 R1=CO2Me R2= H



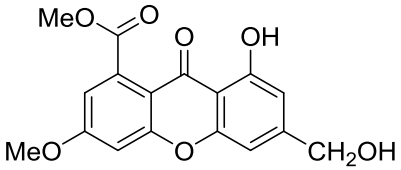
1.251



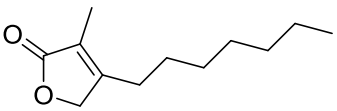
1.253 R=ß-Cl
1.254 R=a-Cl



1.255

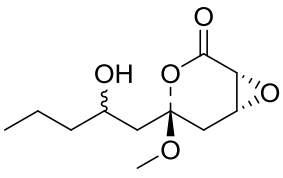


1.256

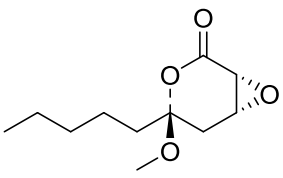


1.257

Isolation work on the fermentation broth of *Pestalotiopsis karstenii* derived from stems of *Camellia sasanqua* led to the identification of two oxysporone derivatives, pestalrone A **1.258** and pestalrone B **1.259**. Only pestalrone B was active against HeLa, HepG2 and U-251 with IC_{50} values of 12.6, 31.7 and 5.4 μg/mL, respectively (Luo *et al.*, 2012).



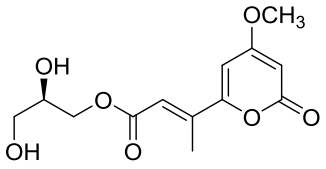
1.258



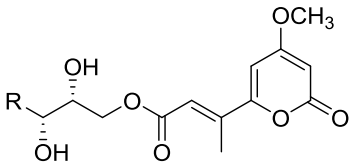
1.259

Four a-pyrone derivatives, pestalotiopyrones I–L **1.260-1.263** and one new hydroxypestalotin diastereomer **1.264** were isolated from the mangrove-derived

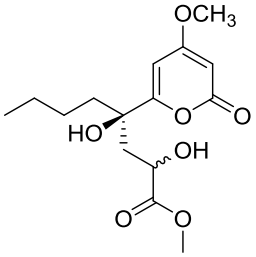
endophytic fungus *Pestalotiopsis virgatula*. However these compounds were inactive in antimicrobial and cytotoxicity assays (Rönsberg *et al.*, 2013).



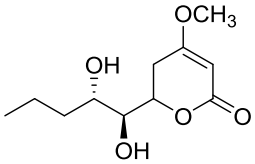
1.260



1.261 R=CH2OH
1.262 R=CH2OCOCH3

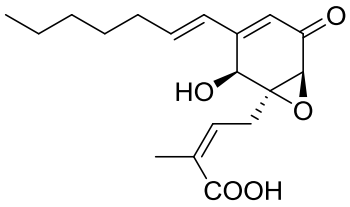


1.263

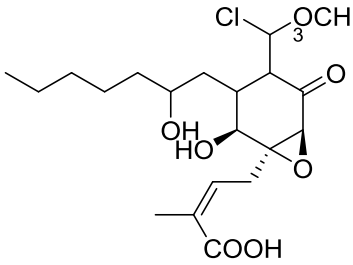


1.264

Phytochemical investigation on a *Cronartium ribicola*-associated fungus, *Pestalotiopsis* sp. cr013 led to the isolation of two ambuic acids **1.265-1.266**. Only compound **1.266** exhibited weak cytotoxicity activities against various cancer lines, respectively (Xie *et al.*, 2014).



1.265



1.266

1.6 Aim of Study

Generally, there is a paucity of phytochemical studies on *V. pinnata* but no study has been conducted yet on endophytic fungi isolated from *V. pinnata*.

Therefore the first aim of this study was the isolation of bioactive secondary metabolites from the leaves of *V. pinnata* by employing efficient, robust and reproducible MPLC methods while characterization and structure elucidation of bioactive compounds were achieved by applying high resolution NMR and MS. All

isolated compounds of sufficient yields were submitted for various available bioassays such as antineoplastic, antitrypanosome and anti-mycobacterium activities.

The second aim was the purification of endophytic fungal strains from the leaves of *V. pinnata*, as well as the isolation and characterization of major compounds from extracts of endophytic fungi and their bioactivities against *T. brucei brucei* and *M. marinum*.

The third aim was the utilisation of metabolomics in the preliminary exploration study for the optimal condition for medium-scale fermentation of *V. pinnata*-associated fungi. A dereplication study also was applied to pinpoint and isolate known and potential new compounds in comparison with available *in-house* databases. Finally metabolomics was used in the mining of active metabolites responsible for antitrypanosomal activity against *T. brucei brucei*.

CHAPTER 2

2 Experimental Parameters

2.1 Experimental Parameters for Isolation of Secondary Metabolites from the leaves of *Vitex pinnata*

2.1.1 Plant materials

Leaf parts of *V. pinnata* were collected from Kuala Terengganu, Malaysia in September 2009. The plant was identified by Dr. Nashriyah Mat from Faculty of Bioresources and Food Industry, Universiti Sultan Zainal Abidin and voucher specimen was deposited (collection number VP 01).

2.1.2 Extraction and Isolation

The ground leaves of *V. pinnata* (1 kg) were extracted in a Soxhlet apparatus by utilising three different solvents based on their polarity. The extraction was started with *n*-hexane followed by ethyl acetate (EtOAc) then methanol (MeOH) with solvent volumes of 7.5 L each for 72 hours. Later the extracts were concentrated *in vacuo* using a rotary evaporator (BUCHI Labortechnik AG, Switzerland) at 40ºC. The yields were 6.33 g, 6.5 g and 5.2 g for the *n*-hexane, EtOAc and MeOH extracts, respectively. All dried extracts were stored at -20ºC freezer.

Fractionation of the *n*-hexane extract (6.33g) was accomplished by using Medium Pressure Liquid Chromatography (MPLC). Linear gradient elution was employed with hexane (A) and EtOAc (B) as the solvents at a flow rate of 100 mL/min. A pre-packed VersaPak silica cartridge (particle size 20–45 μm, diameter and length 40 x 150 mm) from Supelco was used in this run. 100% A was run for 5 min followed by 100% A to 100% B for 20 min then 100% B for the last 5 min. The run time was 30 min in total. For the first and last 5 min of the run, the 100 mL fractions were collected and followed by 50 mL volume fractions for 20 min, resulting in 50 fractions. Fractions with similar TLC profiles were pooled together yielding 28 fractions.

Fraction 12 contained a mixture of two compounds assigned as *β*-sitosterol **3.1** and stigmasterol **3.2** (15 mg). Fraction 13 was further subjected to MPLC over a pre-packed VersaPak silica cartridge (particle size 20–45 μm, diameter and length 23 x 53 mm) utilising an isocratic gradient system with 80% hexane and 20% EtOAc for 30 min at a flow rate of 20 mL/min. Fraction 13 afforded six sub-fractions and gave one pure compound named as 5-hydroxy-3, 7, 4'-trimethoxyflavone **3.3** (20 mg). Fraction 16 afforded one major compound 5-hydroxy-7, 4'-dimethoxyflavone **3.4** (4 mg). Fraction 19 yielded the major compound retusin **3.5** (130 mg) by crystallisation after washing with mixtures of hexane and MeOH (see Fig. 2.1).

2.1.3 Identification and structural elucidation of isolated compounds

One and two dimensional NMR experiments were measured in Strathclyde Institute of Pharmacy and Biomedical Sciences (SIPBS) by using 400 (^{1}H) and 100 (^{13}C) MHz on an AS-400 JEOL NMR instrument. All isolated samples were dissolved in 650 μL of deuterated chloroform ($CDCl_3$).

In LCMS analysis, all samples were dissolved in MeOH (HPLC-grade) to give a final concentration of 1 mg/mL. The analysis was carried out using the Finnigan Exactive Orbitrap instrument in both positive and negative ionisation (ThermoFisher Corporation, Hemel Hempstead, UK). A reversed phase silica C-18 HPLC column, 75.0 x 3.0 mm^2 (Hichrom Limited, UK) with particle size of 5 μm, and pore size 100 ˚A was used. The approximate pressure was at 37 bar while the temperature was maintained at 22 °C. The mobile phase consisted of purified water (A) and acetonitrile (B) with 0.1 % formic acid in each solvent. The samples were eluted using a linear gradient of 90% A and 10% B to 100% B for 30 min which changed to isocratic mode for 5 min before decreasing back to 10% of B for 1 min. Then the column was re-equilibrated with 10% of B for 9 min before the next sample injection. The flow rate used in this method was 300 μL /min and the injection volume was 10 μL.

In GCMS analysis, sample (1 μl) was injected into the the Gas Chromatography Mass Spectrometry (GCMS) (Focus GC-DSQ2) system from Thermo Fisher Scientific (Bremen, Germany) using with 30 m long, 0.25 mm i.d., and 0.25 μm film thickness InertCap 1 MS capillary column from GL Sciences (Japan). The oven temperature was

set at 80 ^{o}C for 1 minute and the temperature was increased at a rate of 15 ^{o}C /min until it reached to 200 ^{o}C and was maintained for 15 min. Then the temperature was again increased at a rate of 5 ^{o}C /min until the final temperature of 320 ^{o}C (held for 10 min). The base temperature of the SSL was 250 ^{o}C. The mode was splitless. The split flow was on at 15 mL/min. The splitless time was 1 minute. The carrier method was set to constant flow. The initial value was 1.50 mL/min and the initial time was 1 minute. The MS transfer line was maintained at a temperature of 320 ^{o}C. The source temperature of the DSQ II mass spectrometer was set to 250 ^{o}C. The mass range used was 50.0-800.0.

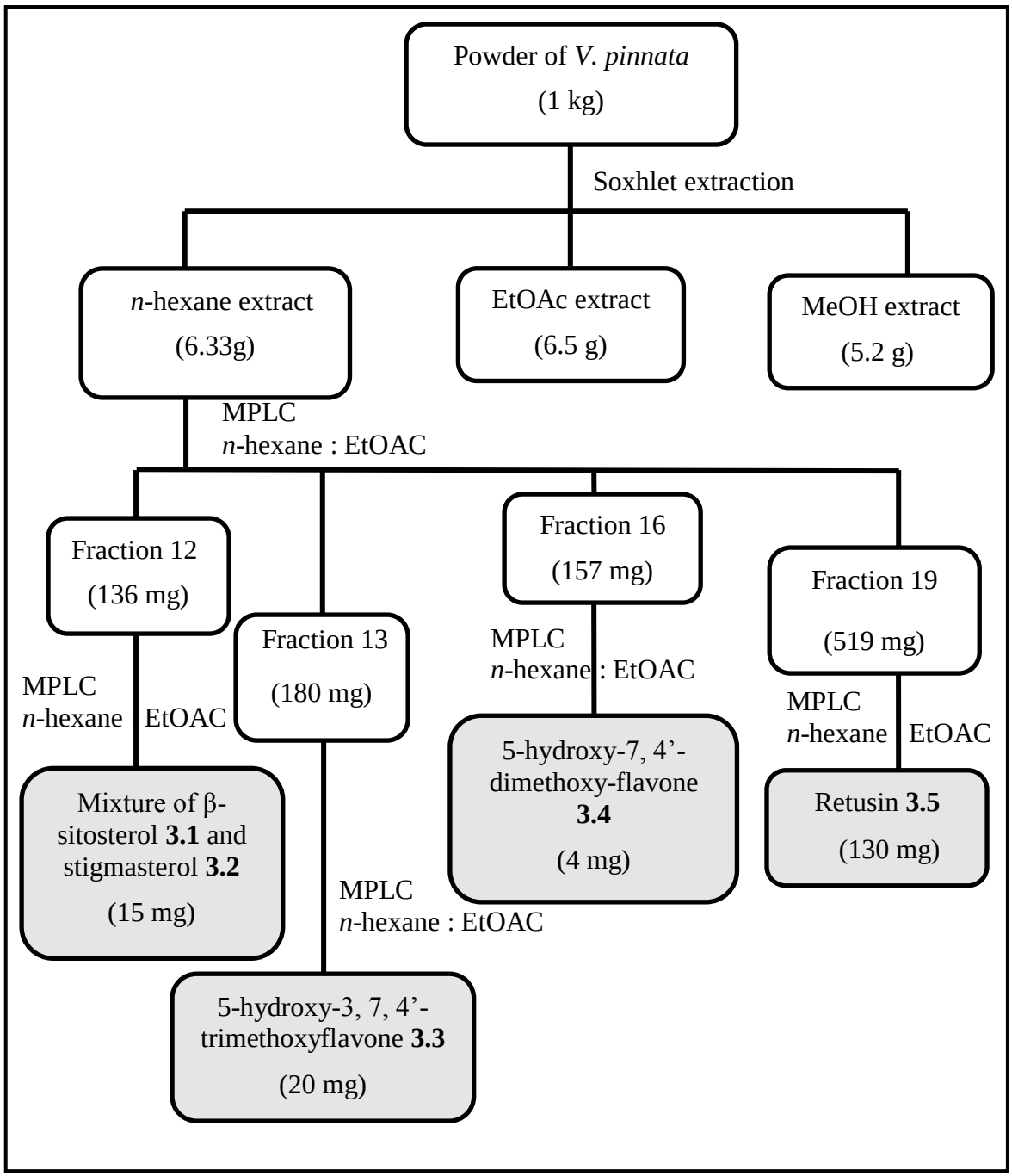


Fig 2.1: Separation procedure of *V. pinnata* extract

2.2 Experimental Procedures for Isolation of Secondary Metabolites from Endophytic Fungi Derived from *V. pinnata*

2.2.1 Chemicals and reagents

2.2.1.1 Chemicals for culture media

Agar	Oxoid, Basingstoke, UK
Chloramphenicol	Sigma-Aldrich, Dorset, UK
Glucose	Alfa Aesar, Heysham, UK
Malt extract	Oxoid, Basingstoke, UK
NaCl	Merck, Hoddesdon, UK
Peptone	Fisher, Loughborough, UK
Rice	Aldi, Glasgow, UK
Yeast extract	Oxoid, Basingstoke, UK

2.2.1.2 Chemicals for agarose gel electrophoresis

Agarose	Sigma-Aldrich, Dorset, UK
Ethidium bromide	Sigma-Aldrich, Dorset, UK
TBE-buffer	Sigma-Aldrich, Dorset, UK
Standards	Sigma-Aldrich, Dorset, UK

2.2.2 Fungal materials

Fresh leaves and stems of *V. pinnata* were collected from Kuala Terengganu, Malaysia in April 2011. The plant was identified by Dr. Nashriyah Mat from from Faculty of

Bioresources and Food Industry, Universiti Sultan Zainal Abidin. Samples were kept in zip lock bags and stored at 4°C until the isolation of endophytic fungi was performed four days later. The surfaces of the leaves and stems were sterilised with 70% iso-propanol for two minutes, rinsed in sterile water. Small tissue samples from inside the leaves and stems were cut aseptically and pressed onto agar plates (composition of isolation medium: 15 g/L malt extract, 15 g/L agar, and 0.2 g/L chloramphenicol in distilled water, pH 7.4-7.8, adjusted with 10% NaOH or 36.5% HCl). Chloramphenicol was added to inhibit bacterial growth. Plates were left for few days until fungal growth were observed. Reinoculation onto new malt agar plates was repeated several times until pure strains were observed.

2.2.3 Microbiology Equipment

The laminar flow cabinet (BioMAT2) was procured from Medical Air Technology, Manchester, UK. The incubator (Incu-160S) used for agar plates was from SciQuip Ltd., Shropshire, UK. The homogenizer (IKA T18 Basic Ultra-Turrax) and handheld homogenizer (Ultra-Turrax T8) bought from IKA Labortechnik, Germany.

2.2.4 Media

2.2.4.1 Composition of malt agar (MA) medium

MA medium was used to prepare fresh fungal seeding for liquid and solid cultures.

Agar	15.0 g
Malt extract	7.5 g
Distilled water	to 1000 mL
pH	7.4 - 7.8 (adjusted with NaOH/HCl)

Chloramphenicol (0.2 g) or streptomycin (0.1 g) was added to the medium to suppress bacterial growth.

2.2.4.2 Composition of Wickerham medium for liquid cultures

Yeast extract	3.0 g
Malt extract	3.0 g
Peptone	5.0 g
Glucose	10.0 g
Distilled water	to 1000 mL
pH	7.2 - 7.4 (adjusted with NaOH/HCl)

2.2.4.3 Composition of rice medium for solid cultures

Rice	100 g
Distilled water	100 mL

Water was added to the rice and kept overnight before autoclaving.

2.2.5 Methodologies

2.2.5.1 Identification of fungal strains

Three strains were isolated from the malt extract agar plates and were identified by using DNA amplification and sequencing of the internal transcribed spacer (ITS) region. Out of the three strains, one could not be identified at the species level due to the lack of similar sequences in GenBank. All voucher strains were deposited at Natural Product Metabolomics Laboratory in SIPBS.

2.2.5.2 DNA extraction

DNA isolation and purification from the fungal endophytes was achieved using the REDExtract-N-Amp™ Plant polymerase chain reaction (PCR) Kit (Sigma-Aldrich, Dorset, UK), which contains Dilution solution, Extraction solution, and REDExtract-N-Amp PCR ReadyMix Kit. First, a small piece of fungal mycelium, approximately 0.5 cm^2, was cut and transferred into an Eppendorf tube. 100 µL of Extraction Solution was subsequently added to the Eppendorf tube and the tube was vortexed. The sample was then incubated for 10 min at 95^0C. Later 100 µL of dilution solution was added to the sample and these were mixed thoroughly using a centrifuge. The diluted sample was stored at 2-8^0C until it was ready for PCR amplification.

2.2.5.3 DNA amplification

The isolated DNA was amplified by Polymerase Chain Reaction (PCR). The PCR was done using the REDExtract-N-Amp PCR ReadyMix Kit (Sigma-Aldrich, Dorset, UK). Two primers were selected: ITS 1 (with base sequences TCCGTAGGTGAACCTGCGG) as the forward primer and ITS 4 (with base sequences TCCTCCGCTTATTGATATGC) as the reverse primer (Life Technologies, Paisley, UK). The following reagents were added to a thin-walled PCR micro-centrifuge tube: 10 µL REDExtract-N-Amp PCR ReadyMix, 4 µL of RNA-free water, 1 µL of ITS 1 and ITS 4 primers and 4 µL of fungal DNA (10-20 ng) with a total volume of 20 µL.

The mixture was applied to the thermal cycler (Perkin Elmer, Seer Green, UK using the Genofund PCR cycle as below (Table 2.1):

Table 2.1: PCR Cycle step using thermal cycler

Step	Temperature (°C)	Time (mins)	Cycles
Initial denaturation	95	3	1
Denaturation	95	1	
Annealing	56	1	35
Extension	72	1	
Final Extension	72	10	1
Hold	4	Indefinitely	

2.2.5.4 Purification of PCR products and DNA sequencing

The PCR product was purified on 1% agarose gel using gel electrophoresis at 60 V for 45 minutes in Tris/Borate/EDTA (TBE) buffer. 1% of ethidium bromide was added as staining agent. Then, the stained DNA fragment was sliced from the agarose gel. The PCR product was then purified using a GenElute Gel Extraction Kit (Sigma Aldrich, Dorset, UK) which consisted of a Column Preparation Solution, Gel Solubilization Solution, Wash Solution Concentrate G, Elution Solution (10 mM Tris-HCl, pH 9.0), GenElute Binding Column G and Collection Tubes. The sliced gel was mixed with Gel Solubilization Solution, incubated at 50-60^{0}C for 10 min and vortexed for 2-3 minutes. The mixture was mixed with isopropanol and then centrifuged. The solubilised gel solution mixture was loaded into the binding column and centrifuged for 1 min. The flow-through liquid was discarded. 700 µL of Wash Solution was then added to the binding column and this was centrifuged for 1 min. The filtrate was removed from the column. 700 µL Elution Solution was added to the binding column onto which the PCR product was incorporated and incubated for 1 min at 37°C in order to elute the product. The binding column was then centrifuged and the flow-through liquid was collected. This contained the PCR product that had dissolved in the elution solution. The purified PCR product was then submitted to Dr. Rothwelle Tate for sequencing and the base sequence was compared with publicly available databases such as GenBank with the help of the Basic Local Alignment Search Tool (BLAST)

(http://blast.ncbi.nlm.nih.gov/Blast.cgi). The identified fungi with accession data is presented in Table 2.2. (Please see Appendix III for the sequence results of each strain).

Table 2.2: Identification of isolated fungi using GenBank

No. of samples	Sample ID	Fungal strains	Accession Data
1	S3	*Lasiodiplodia theobromae*	KC960898
2	P13	*Nigrospora* sp.	JQ246358
3	S7	*Pestalotiopsis olivacea*	KF312153

2.2.5.5 Cultivation

For small scale cultivation (during optimisation) fungal strains were grown in a 500 mL Erlenmeyer flasks of solid rice medium and liquid Wickerham medium while for medium scale fermentation, the fungal strains were grown in either 20 flasks of solid rice medium or 20 flasks of Wickerham liquid medium in 2 L flasks.

2.2.5.6 Extraction of fungi grown on solid rice medium

EtOAc was added (500 ml x 3 times) into rice culture for extraction prior to homogenisation with an IKA T18 Basic Ultra-Turrax and left overnight. The total extract was filtered, collected and then was dried under *vacuo* using a rotovap. Then the extract was partitioned with a 1: 1 volume of *n*-hexane and aqueous MeOH (10% water and 90% MeOH) to remove fatty acids. The part of the sample that was soluble in aqueous MeOH was collected for further isolation work. Isolation and purification of secondary metabolites from rice culture and liquid culture extracts were established by using various types of chromatography such as MPLC and conventional column chromatography.

2.2.5.7 Extraction of fungi grown on Wickerham liquid medium

For samples that were grown in Wickerham liquid culture, EtOAc (200 ml x 3 times) was poured inside the Erlenmeyer flasks to separate mycelia from culture media. The sample was homogenised using a T18 Basic Ultra-Turrax (IKA, Germany) at maximum speed and then was kept overnight. The supernatant was filtered under vacuum using a Buchner funnel. The supernatant was partitioned with EtOAc using a separating funnel. The material that was soluble in ethyl acetate was collected and dried using a rotary evaporator. Isolation and purification of secondary metabolites from rice and liquid culture extracts was accomplished by using various types of chromatography such as MPLC, high-performance liquid chromatography (HPLC) and conventional column chromatography.

2.2.6 Isolation and purification of secondary metabolites from fungal endophytes

2.2.6.1 Secondary metabolites isolated from rice culture (30 days) of *Lasiodiplodia theobromae*

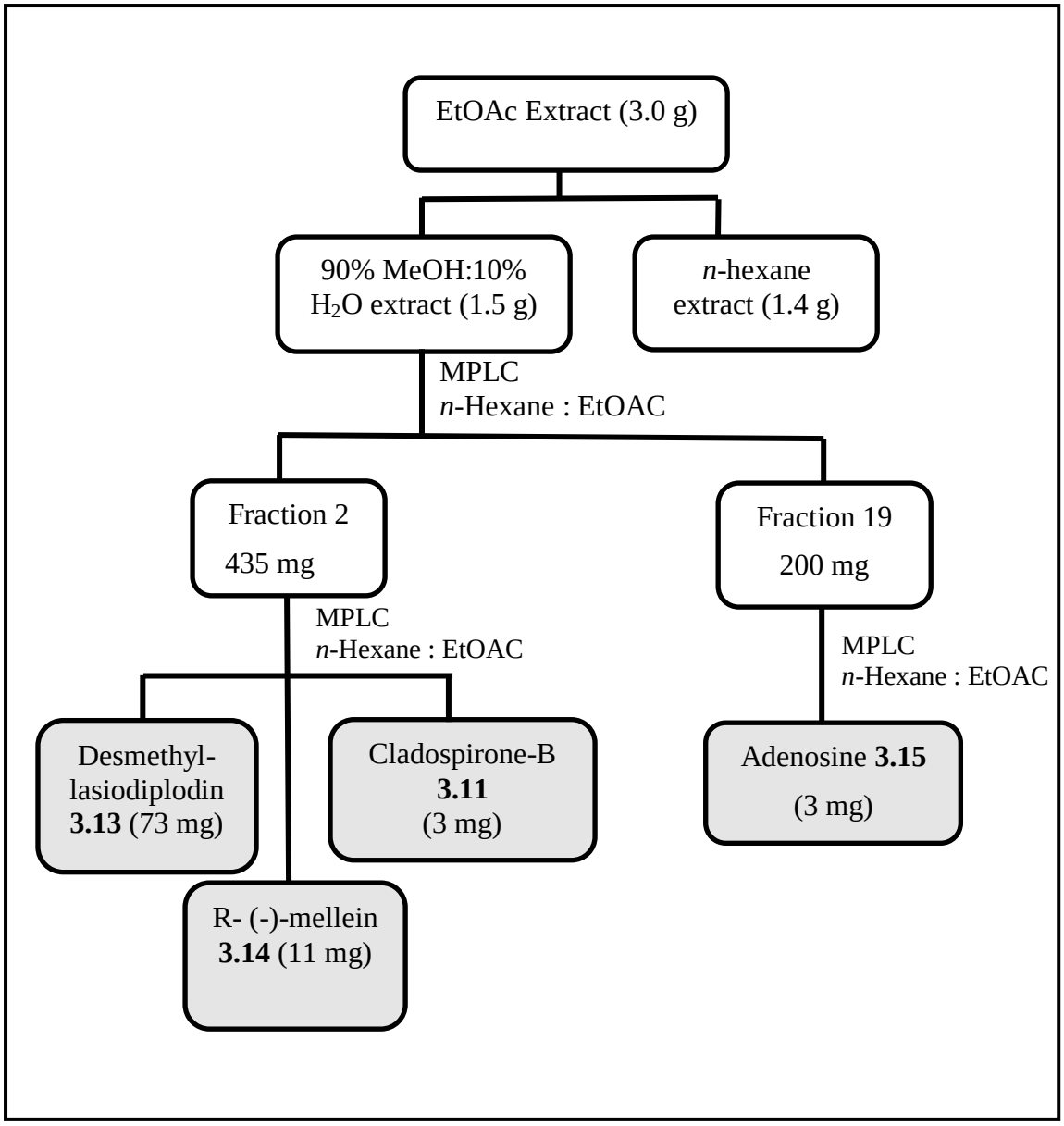


Fig 2.2: Separation procedure of *L. theobromae* extract (30 days in rice culture)

2.2.6.2 Secondary metabolites isolated from seven days rice culture of *Nigrospora* sp.

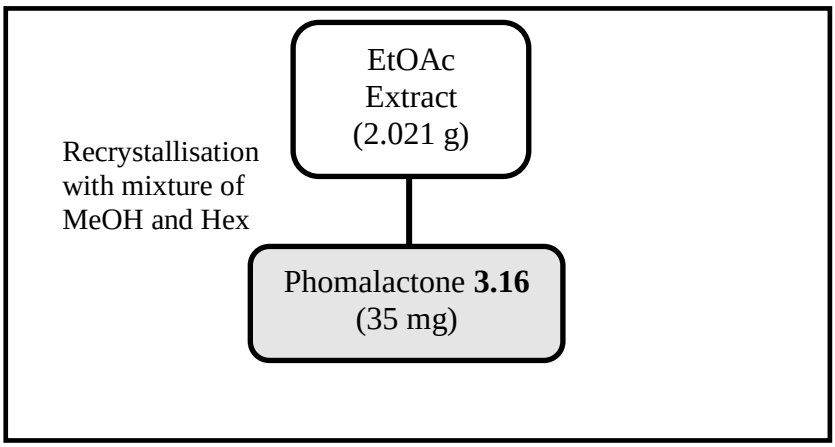


Fig 2.3: Separation procedure of *Nigrospora* sp extract (seven days in rice culture)

2.2.6.3 Secondary metabolites isolated from rice culture (thirty days) of *Nigrospora* sp.

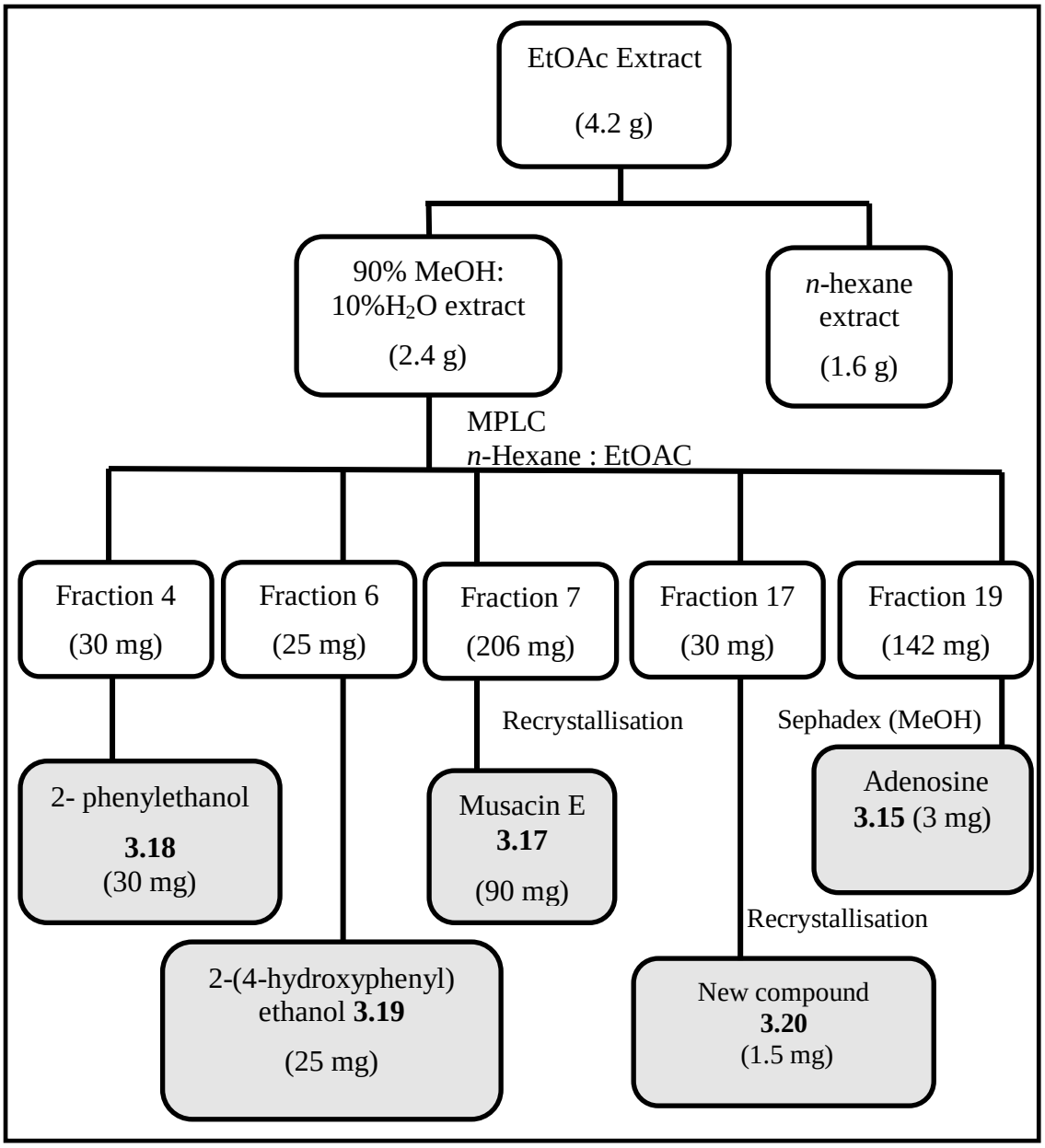


Fig 2.4: Separation procedure of *Nigrospora* sp. extract

2.2.6.4 Secondary metabolites isolated from rice culture (fifteen days) of *Pestalotiopsis olivacea*

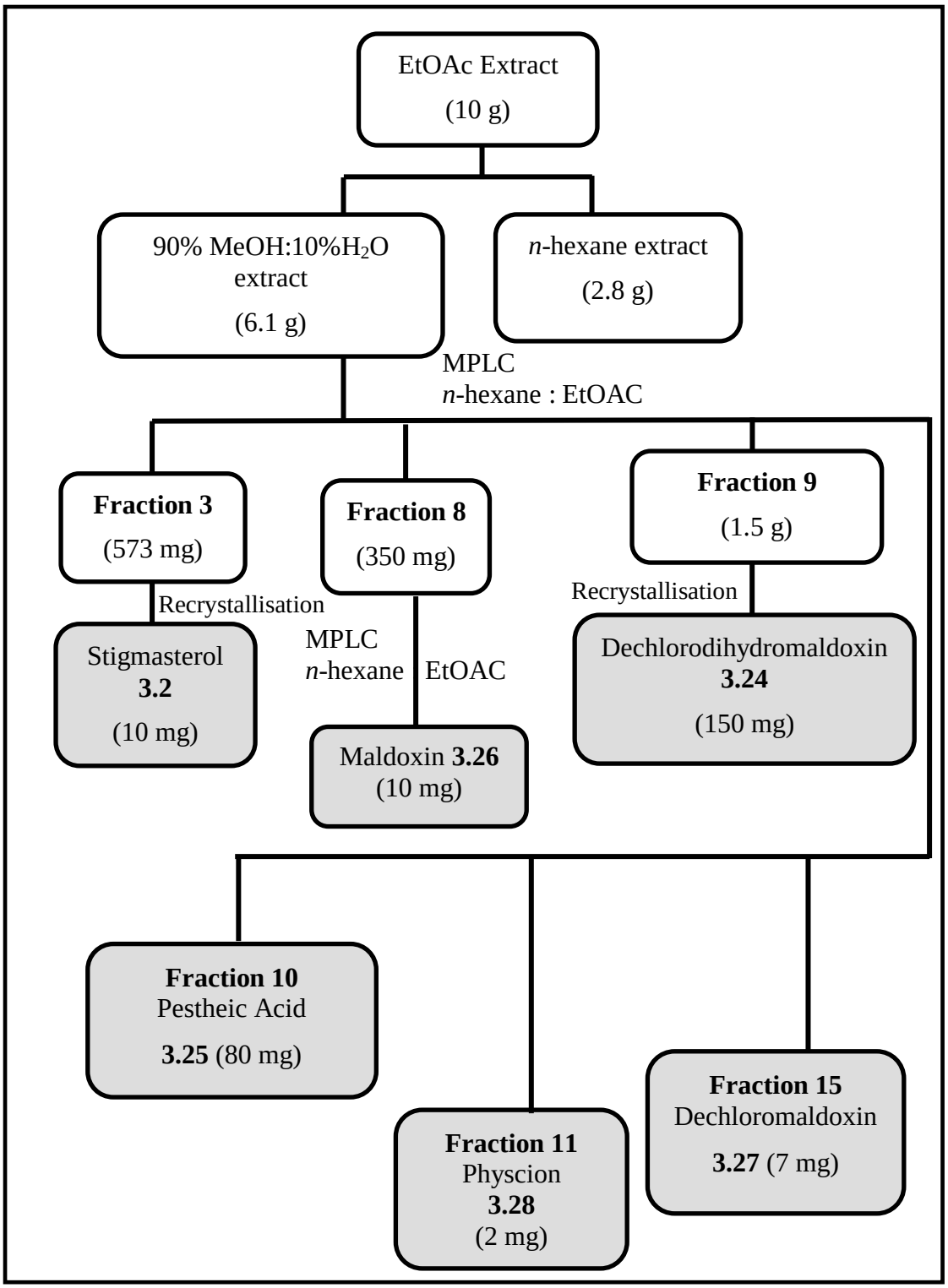


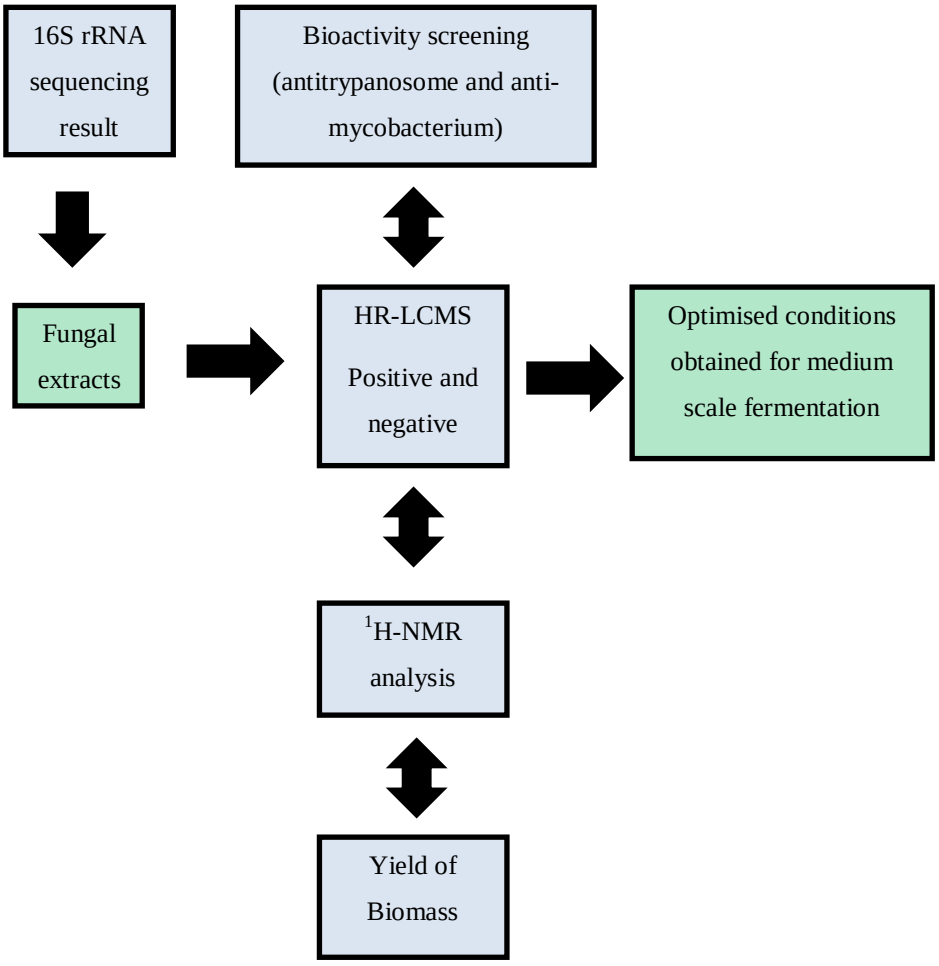
Fig 2.5: Separation procedure of *P. olivacea* extract

2.3 Experimental Procedures for metabolomics

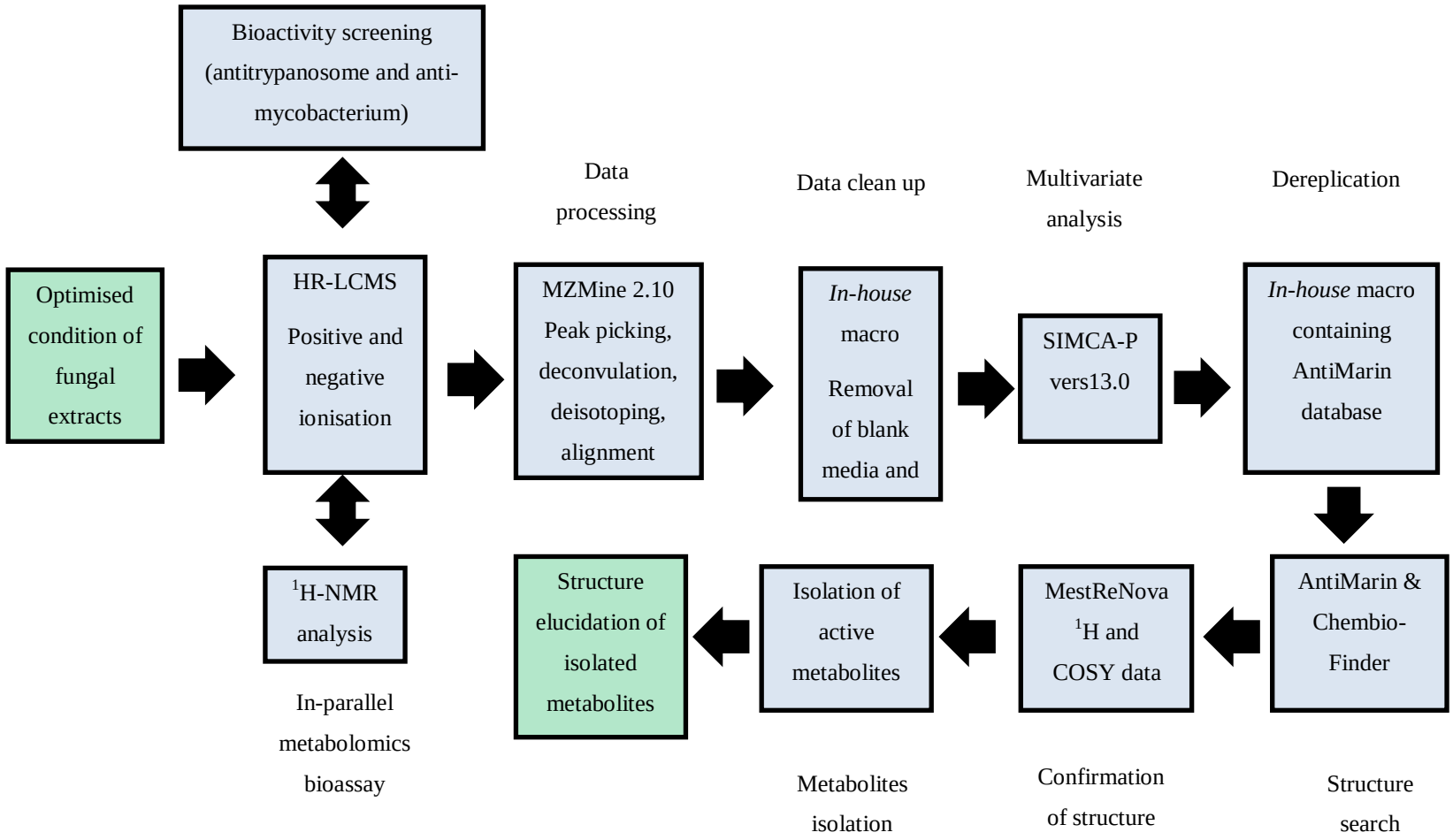
2.3.1 Metabolomics workflow

A pre-defined metabolomics workflow was designed depending on the aim of the study. Several steps were involved and illustrated as below:

2.3.1.1 Metabolomics-bioassay guided screening as a strategy to determine the optimal condition of fungal culture for medium-scale fermentation



2.3.1.2 Metabolomics as decision-making tools in mining active metabolites of the endophytic fungus, *Lasiodiplodia theobromae* against *Trypanosoma brucei brucei* / Metabolomics as a tool in dereplication study of *Pestalotiopsis olivacea*



2.3.2 Data Analysis Using MZmine 2.10 (Macintyre *et al.*, 2014)

The LC-MS raw data were sliced into positive and negative ionisation modes using the MassConvert tool file slicer from ProteoWizard. The sliced MS data were imported and processed using MZmine 2.10 (Pluskal *et al.*, 2010). The chromatograms were first cropped to 0.0-38.0 minutes. For peak detection, centroid mass detector was selected with the noise level set to $1.0E^5$ and the MS level set to 1. The chromatogram builder was set to a minimum time span of 0.2 min, minimum height of $1.0E^5$ and *m/z* tolerance of 0.001 *m/z* or 5.0 ppm. The algorithm used for chromatogram deconvolution was the local minimum search. The chromatographic threshold was set to 90.0%. The search minimum in Retention Time (RT) range was 0.4 minutes, minimum relative height was 5.0%, minimum absolute height was $3.0E^5$, minimum ratio of peak top/edge was 2 and the peak duration range was 0.3-5.0 min. For isotopes isotopic peaks grouper was used with the *m/z* tolerance at 0.001 *m/z* or 5.0 ppm, RT tolerance at 0.2 absolute (minutes), the maximum charge at 2, and the representative isotope used was the most intense. RT normalization was performed using the RT normalizer with *m/z* tolerance of 0.001 *m/z* or 5.0 ppm while the RT tolerance and the minimum standard intensity were set to 5% (relative) and $5.0E^4$ respectively. The peak lists were all aligned using the join aligner (*m/z* tolerance 0.001 *m/z* or 5.0 ppm, weight for *m/z*: 20, RT tolerance: 5.0% relative, weight for RT: 20). The aligned peak list was gap-filled using the peak finder function (intensity tolerance: 1%, *m/z* tolerance: 0.001 *m/z* or 5.0 ppm, RT tolerance: 0.5 min). An adduct search was performed with the RT tolerance set at 0.2 absolute (min), the *m/z* tolerance at 0.001 *m/z* or 5.0 ppm and the maximum relative adduct peak height at 30%. The adducts searched were Na, K, NH_4 and ACN+H. A complex search was also performed using $[M+H]^+$ for the positive ionisation mode and $[M-H]^-$ for the negative ionisation mode. The RT tolerance was set at 0.2 absolute (min), *m/z* tolerance was kept at 0.001 *m/z* or 5.0 ppm, and the maximum complex peak height was set at 50%. Data was then exported as CSV file for further clean up.

2.4 Materials and Equipment

2.4.1 Reagents

All HPLC-grade organic solvents including acetone, acetonitrile, dichloromethane, ethyl acetate, hexane and methanol for isolation work were purchased from Fisher Scientific, UK. Celite® 545AW-Reagent Grade and Diaion® HP20 were obtained from Supelco, USA. Sephadex® LH-20 was bought from Fluka Chemie AG Buchs, Switzerland. Silica gel 60 0.035-0.070 mm (220-440 mesh) was purchased from Alfa Aesar, Heysham, England. The deuterated solvents used for NMR were deuterated dimethyl sulfoxide (DMSO-*d6*), deuterated chloroform ($CDCl_3$) deuterated methanol (MeOH-*d6*) from Euriso-Top, France or from Sigma-Aldrich Co., Missouri, USA.

2.4.2 Equipment

2.4.2.1 General Equipment

The analytical mill, IKA A11 Basic was bought from IKA, Germany. The two rotary evaporators, R-110 and R-3, were purchased from BÜCHI, Switzerland. The UV lamp model UVGL-55 Handheld Ultra Violet (UV) Lamp was bought from UVP, Cambridge, UK. The heat gun HL 2010 E Type 3482 was produced by Steinel, USA. The freeze dryer, Christ Alpha 2-4 was purchased from Martin Christ Gefriertrocknungsanlagen GmbH, Germany. The Ultrawave sonicator was from Scientific Laboratory Supplies, Ltd, Coatbridge, UK. The optical rotations of the compounds were measured on a 341 Polarimeter from PerkinElmer, Inc., USA.

2.4.2.2 Medium Pressure Liquid Chromatography (MPLC) Equipment

MPLC is one of the numerous preparative column chromatography techniques with the addition of pre-packed columns and the application of medium air pressure (between 5-20 bar) to the system. It is a quick, robust and reproducible technique which is widely used to separate a variety of organic compounds. Normally, the columns contain pre-packed dry silica gel (for normal phase) and modified silica gel phase such as RP-18,

RP-8 or RP-4 (for reversed phase). Before loading the sample (solid or liquid), the column will be conditioned with 100% non-polar solvent of the selected solvent system for a normal phase. Once the sample is loaded on top of the column, the mobile phase will be eluted through the column with a pump at low to medium pressure, facilitating the separation of the sample. The fractions will be collected either manually or with a fraction collector. Two MPLC instruments were used in this research work. The BÜCHI MPLC instrument consisted of C-601 pump modules and the C-615 pump manager (BÜCHI, Switzerland) which allowed binary solvent gradients with flow rates from 2.5 to 250 mL/min. The column stand and pre-packed cartridge columns (particle size 20–45 μm, diameter and length 23 x 53 mm, 23 x 110 mm and 40 x 150 mm) were from VersaFlash/Supelco, Sigma-Aldrich, Germany. The fraction collector was bought from Spectrum Lab. The Reveleris® Flash Forward system (Grace Davison Discovery Sciences, Illinois US) was also used for isolation and purification work. This system allowed a solvent gradient with four solvents able to be used in one single run. This MPLC had a flow rate range of 4 to 200 mL/min and the flow rate was automatically adjusted if the pressure was too high. This system also came with two detectors, a UV detector (wavelength range: 200-500 nm) and an evaporative light scattering detector (ELSD). This enabled better sensitivity of peak detections and was not limited to UV-active compounds. The built-in fraction collector and trays were also included in this system.

2.4.2.3 Spectroscopic Equipment

2.4.2.3.1 Liquid Chromatography – Mass Spectrometry (LCMS)

The LCMS Finnigan Exactive Orbitrap instrument in both positive and negative ionisation switch mode was used for analysis (ThermoFisher Corporation, Hemel Hempstead, UK). Accela 600 HPLC pump with Accela autosampler and UV/Vis detector (Thermo Scientific, Bremen, Germany) was coupled to the MS instrument. A low-resolution mass spectrometry system, Finnigan LCQ-Deca coupled to an HPLC (series 1100) from Hewlett Packard also was used in some of the experiment.

2.4.2.3.2 Gas Chromatography- Mass Spectrometry (GCMS)

The capillary electron impact GCMS was carried out by using a Focus GC coupled to a DSQ II (Thermo Scientific, Germany). The column, InertCap 1MS (ID: 0.25 mm, length: 30 m, df: 0.25 μm) was brought from GL Sciences Inc., Japan.

2.4.2.3.3 Nuclear Magnetic Resonance (NMR)

The NMR machine JNM-LA400 instrument (JEOL, Japan) and the magnet NMR AS400 model EUR0034 (Oxford Instruments, England) was used in this study. The NMR has a Pulse-Field Gradient "Autotune"TM probe 40TH5AT/FG broadband high sensitivity probe for 5mm tubes and FG coils, 2H lock channel which can operate at various temperatures. Another NMR machine used was AVANCE-III 600 instrument with a 14.1 T Bruker UltraShield magnet from the Department of Pure and Applied Chemistry. It has a 24 position autosampler, 3 channel console, is DQD and Waveform-equipped and can use either a BBO-z-ATMA-[31P-183W/1H] probe or a TBI-z-[1H, 13C, 31P-15N] probe. DMSO and $CDCl_3$ Shigemi® tubes and Wilmad® NMR capillary tubes were purchased from Sigma-Aldrich Inc., USA.

2.5 General Methods

2.5.1 Chromatography Methods

Chromatography is a technique used to separate mixtures of two or more molecules in which the molecules are distributed between two phases: the stationary and the mobile phase.

2.5.1.1 Thin layer chromatography (TLC)

Thin layer chromatography is a separation technique used to identify various types of compounds with the aid of spray reagents for visualisation and through observation under a UV lamp. It also can be used to monitor the purity of compounds. This method is very easy, quick and requires small quantities of compounds. TLC was performed on

pre-coated TLC plates with normal silica gel 60 F254 and reverse phase TLC silica gel 60 RP-18 F254S (layer thickness 0.2 mm, Merck, Germany). The fractions were dissolved in suitable solvents depending on whether the normal or reversed phase TLC plates were used. In general, n-hexane, DCM, EtOAc, Acetone and MeOH were used for the normal phase solvent systems while H_2O and MeOH were used for reversed phase. The length of the chromatogram was generally 5 cm. The band separation on the plates was detected under the UVGL-55 Handheld UV Lamp (UVP, Cambridge, UK) at 254 and 365 nm, after which the TLC plates were sprayed with anisaldehyde/H_2SO_4 reagent and subsequently heated at 110 °C. This spray reagent was used to see many chemical compounds such as essential oil components, steroids, terpenes, sugars, phenolic compounds, and sapogenins.

2.5.1.2 Column chromatography

Selected extracts/fractions of plant and fungal endophytes were subjected to column chromatography to purify desired compounds. The optimum solvent systems were determined using TLC. The following separation systems of column chromatography were used:

I. Normal phase chromatography utilises polar material such as silica gel, which has silanol groups at the surface as stationary phase. The less polar compounds will elute faster because they have minimal interaction with the silanol groups (Braithwaite and Smith, 1999). In a gradient method, the mobile phase will be started with a non-polar organic solvent such as n-hexane or dichloromethane (DCM) with gradually increasing amounts of a polar solvent (e.g. Acetone, EtOAc or MeOH).

II Sephadex® LH-20 is a beaded, hydroxypropylated cross-linked dextran. It has both hydrophilic and lipophilic characteristics. It is suitable for gel filtration of natural compounds such as steroids, terpenoids, lipids and low-molecular weight peptides. In this PhD project Sephadex® LH-20 was used to eliminate lipids which were found to be abundant in extracts of solid rice fungal cultures. Sephadex® LH-20 was suspended in methanol and poured into the column in one continuous manner. A glass rod was held against the column wall to prevent air bubbles. The column was left to settle overnight. The sample was then slowly introduced to the top of the column using a pipette. After

the sample was adsorbed onto the column, a larger volume of mobile phase was added into the column. Fractions were collected during elution, and after all the bands eluted the column was washed and kept in MeOH.

2.5.2 Spray reagents

Spray reagents were used to monitor the types of compounds found in every fraction and also to observe the purity of the isolated compound spotted on the TLC plate. The spray reagent was always covered with aluminium foil to avoid light and kept refrigerated until use. In this study Anisaldehyde/H_2SO_4 spray reagent was used.

Anisaldehyde/H_2SO_4 Spray Reagent

Anisaldehyde	0.5 mL
Methanol	85 mL
Glacial acetic acid	10 mL
Conc. H_2SO_4	5 mL (added slowly)

2.5.3 Analysis and elucidation methods

2.5.3.1 Liquid Chromatography Mass Spectrometry (LCMS)

The following gradient method was used for low-resolution LCMS ((HP 1100-Finnigan LCQ Deca):

- Column: ACE 5 C18 75x3.0 mm (Hichrom Ltd., UK)
- Mobile Phase: 0.1% formic acid in water (A)

  0.1% formic acid in acetonitrile (B)

- Injection volume: 10 μL
- Flow rate: 400 μL/min
- Gradient: 0-30 min 10%-100% B
  30-35 min 100% B
  35-36 min 100%-10% B
  36-40 min 10% B (equilibration)

For high-resolution LCMS Finnigan Exactive Orbitrap ((ThermoFisher Corporation, Hemel Hempstead, UK), the following gradient method was used:

- Column: ACE 5 C18 75x3.0 mm (Hichrom Ltd, UK)
- Mobile Phase: 0.1% formic acid in water (A)
  0.1% formic acid in acetonitrile (B)
- Injection volume: 10 μL
- Flow rate: 300 μL/min
- Gradient: 0-30 min 10-100% B
  30-35 min 100% B
  35-36 min 100%-10% B
  36-40 min 10% B (equilibration)

2.5.3.2 Nuclear Magnetic Resonance Spectroscopy

One and two dimensional ^{1}H and ^{13}C NMR spectra were recorded at 400 MHz on an AS-400 JEOL NMR spectrometer. Samples were reconstituted in suitable deuterated solvents (600 μL). Shigemi® tubes or Wilmad® NMR capillary tubes were used for

samples with low quantities, allowing the samples to be dissolved in just 180 µL of deuterated solvents. Samples were then processed with MNova 2.9.

2.5.3.3 Optical rotation

Absolute stereochemistry can be determined for molecules that possess at least one chiral centre because these molecules interact with linear polarised light. In this case enantiomers will be differentiated between - or + form. For example, the (+) isomer can be confirmed when the orientation of linearly polarized light is clockwise whereas the (-) isomer is counter-clockwise.

The specific optical rotation at the wavelength of the sodium D-line, 589 nm at 20°C can be calculated as

$$[\alpha]_D^{20} = \frac{100 * \alpha}{l * c}$$

where α = the measured angle of rotation in degrees °

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

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

2.5.4 Biological activities

2.5.4.1 Antitrypanosomal assay

An Alamar Blue assay was used to determine drug sensitivity against African trypanosomes *in vitro*. The tests were carried out by Mrs. Carol Clements from the Strathclyde Institute for Drug research (SIDR).

2.5.4.2 Antibacterial assay

Alamar Blue Assay (resazurin–reduction test) was carried out against *Mycobacterium marinum*. The tests were carried out by Mrs. Carol Clements from the SIDR.

2.5.4.3 NF-κB inhibition assay

The NF-κB inhibition assay on chronic myelogenous leukemia, K562 cell lines was performed by Dr. Marc Schumacher from the Laboratoire de Biologie Moléculaire et Cellulaire du Cancer, Luxembourg.

CHAPTER 3

3 Results

3.1 Bioactive Secondary Metabolites from the leaves of *Vitex pinnata*

3.1.1 Extraction, fractionation and isolation of compounds from *V. pinnata*

The obtained extracts from soxhlet extraction were labelled as hexane, EtOAc and MeOH extracts. The ^{1}H NMR data of each was compared and showed that only the hexane and EtOAc extracts contained peaks at aromatic region (6-8.5 ppm) which belong to phenolic compound congeners and also very crowded peaks were observed at 0.5-2.5 ppm which possibly related to terpene or steroid type of compounds while MeOH extract contained only sugars as major compounds therefore further fractionation was done on hexane and EtOAc extracts.

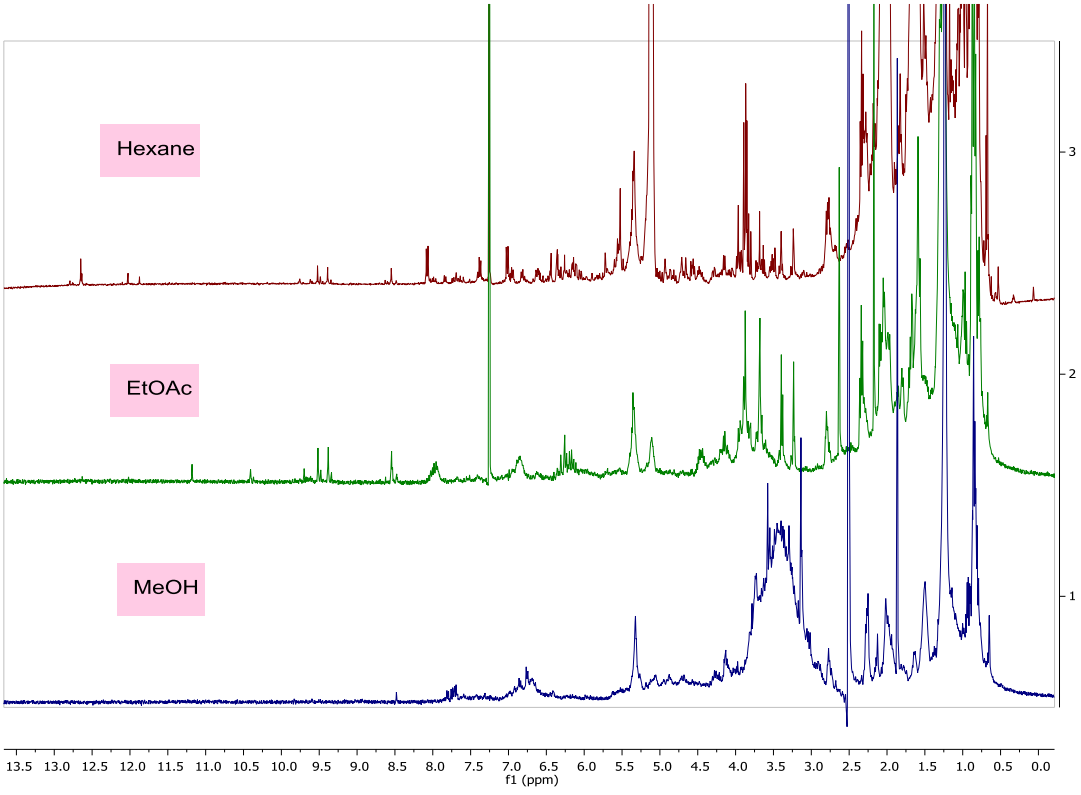


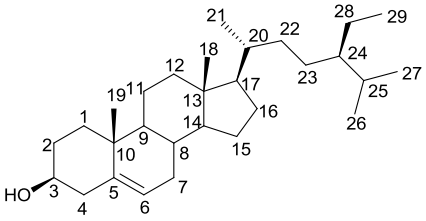
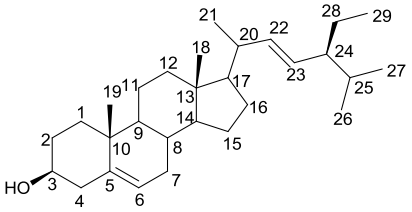
Fig. 3.1: The ^{1}H NMR of extracts derived from *V. pinnata*

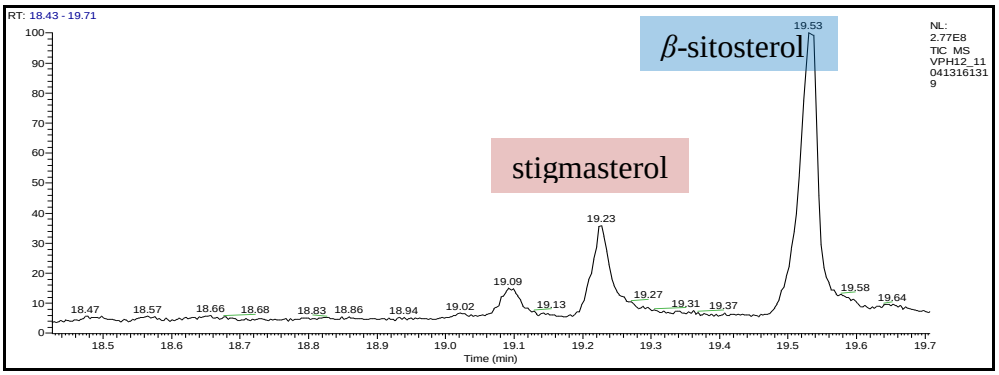
All extractions were done by using MPLC. MPLC is a hybrid technique of conventional column chromatography and flash chromatography but with higher resolution and

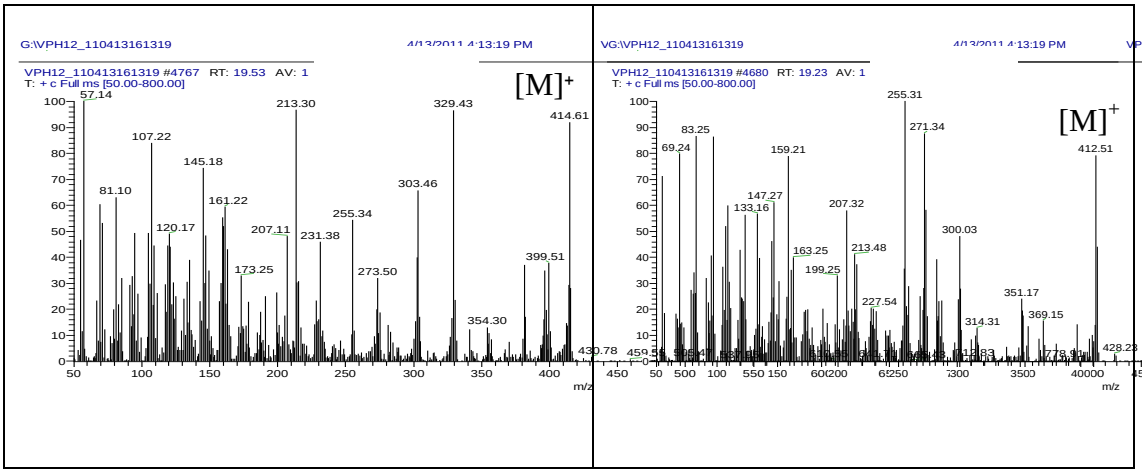
shorter separation times (K. Hostettman, 1989). Based on fractionation of *V. pinnata* extracts using MPLC, it was found to be fast, robust and reproducible. As a result, time and money are saved in comparison to the conventional open columns that require more time and huge quantity of solvents. From the isolation work of the hexane extract, two inseparable steroids and four flavonoid congeners were isolated. The compounds were elucidated as *β*-sitosterol **3.1** and stigmasterol **3.2**, 5-hydroxy-3, 7, 4'-trimethoxyflavone **3.3**, 5-hydroxy-7,4'-dimethoxy-flavone **3.4** and 5-hydroxy-3,3',4',7-tetramethoxyflavone **3.5**, respectively. Fractionation of the EtOAc extract was also accomplished however most of the isolated compounds were chlorophyll type of compounds such as pheophytin a while fractions contain phenolic compounds were too small in yields to undergo further purification work.

3.1.2 Secondary metabolites isolated from *V. pinnata*

3.1.2.1 Mixture of *β*-sitosterol and stigmasterol (known compounds)

***β*-sitosterol (3.1)**	**Stigmasterol (3.2)**
Source: Leaves of *V. pinnata* Sample amount: 15 mg Physical description: White powder Molecular formula: $C_{29}H_{50}O$ Molecular weight: 414.7067 g/mol	Source: Leaves of *V. pinnata* Sample amount: 15 mg Physical description: White powder Molecular formula: $C_{29}H_{48}O$ Molecular weight: 412.6908 g/mol
	





The mixture of steroids **3.1** and **3.2** was observed as a white powder. From the GCMS result, it showed two peaks at 19:23 and 19:53 minutes with the molecular ion peaks at *m/z* 412.51 $[M]^+$ and 414.51 $[M]^+$, respectively. Online GCMS NIST library database suggested this fraction is a mixture of two compounds. GCMS spectrum at *m/z* 414.51 suggested the compound is *β*-sitosterol **3.1,** with the molecular formula $C_{29}H_{50}O$ while at *m/z* 412.51 is stigmasterol **3.2** with the molecular formula $C_{29}H_{48}O$. The HMQC spectrum (Fig. 3.4) was done to confirm the identity of the individual compounds in each of the mixture which were elucidated as *β*-sitosterol and stigmasterol by observing the specific signals at positions C-22 and C-23. In the HMQC spectrum of stigmasterol the chemical shifts at positions C-22 and C-23 are 138.0 and 129.2 ppm, respectively which suggested an alkene group while in *β*-sitosterol, chemical shift signals at C-22 and C-23 were at 34.0 and 26.1 ppm, respectively for an alkyl group (see Fig. 3.4). Based on comparison to ^{13}C NMR data of **3.1** and **3.2** to the previous literatures (Alam *et al.*, 1996; Mahato and Kundu, 1994) supported that compound **3.1** and **3.2** is a mixture of *β*-sitosterol and stigmasterol.

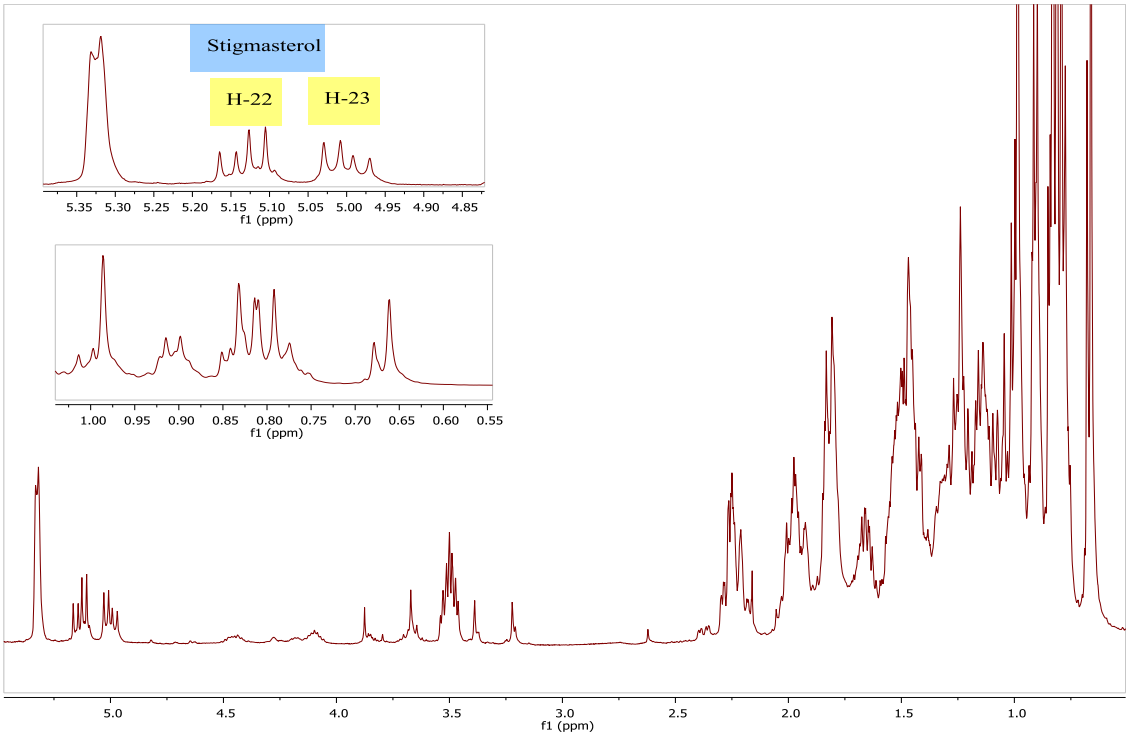


Fig. 3.2: ^{1}H NMR of a mixture of *β*-sitosterol and stigmasterol

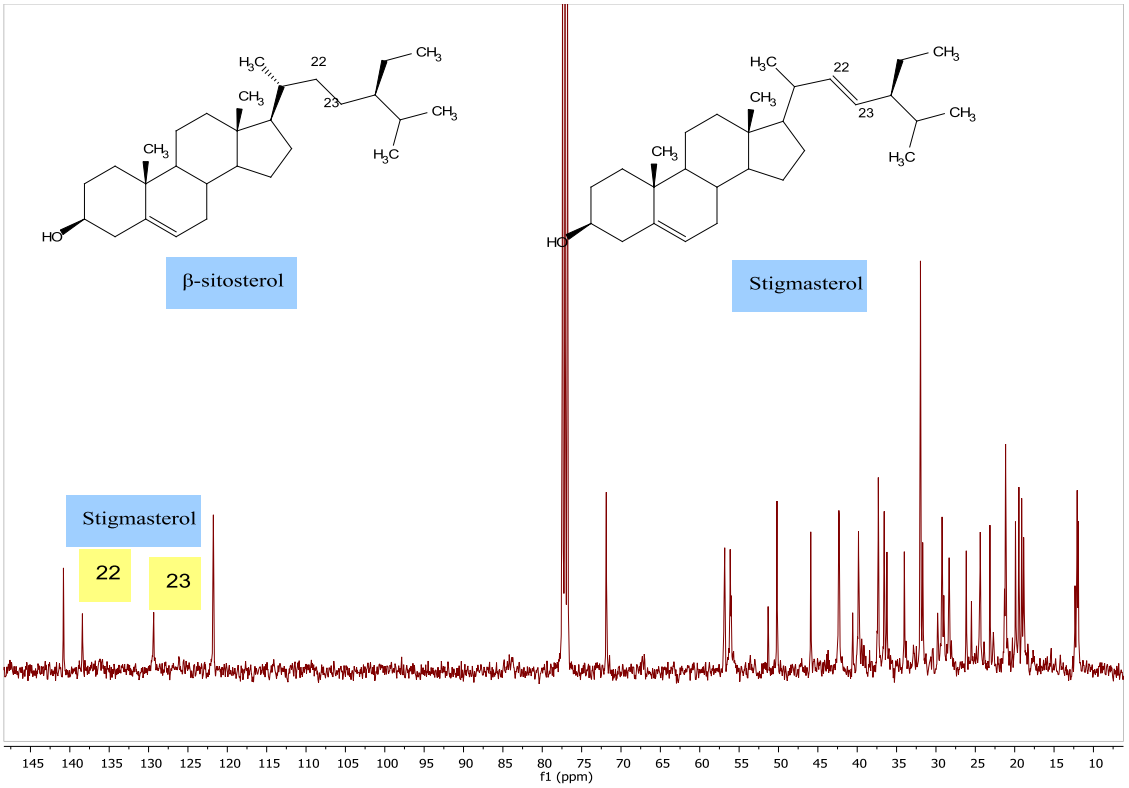


Fig. 3.3: ^{13}C NMR of a mixture of *β*-sitosterol and stigmasterol. The carbon peak at δ_C 138.0 and 129.2 belong to stigmasterol which corresponded to the double bond at positions C-22 and C-23.

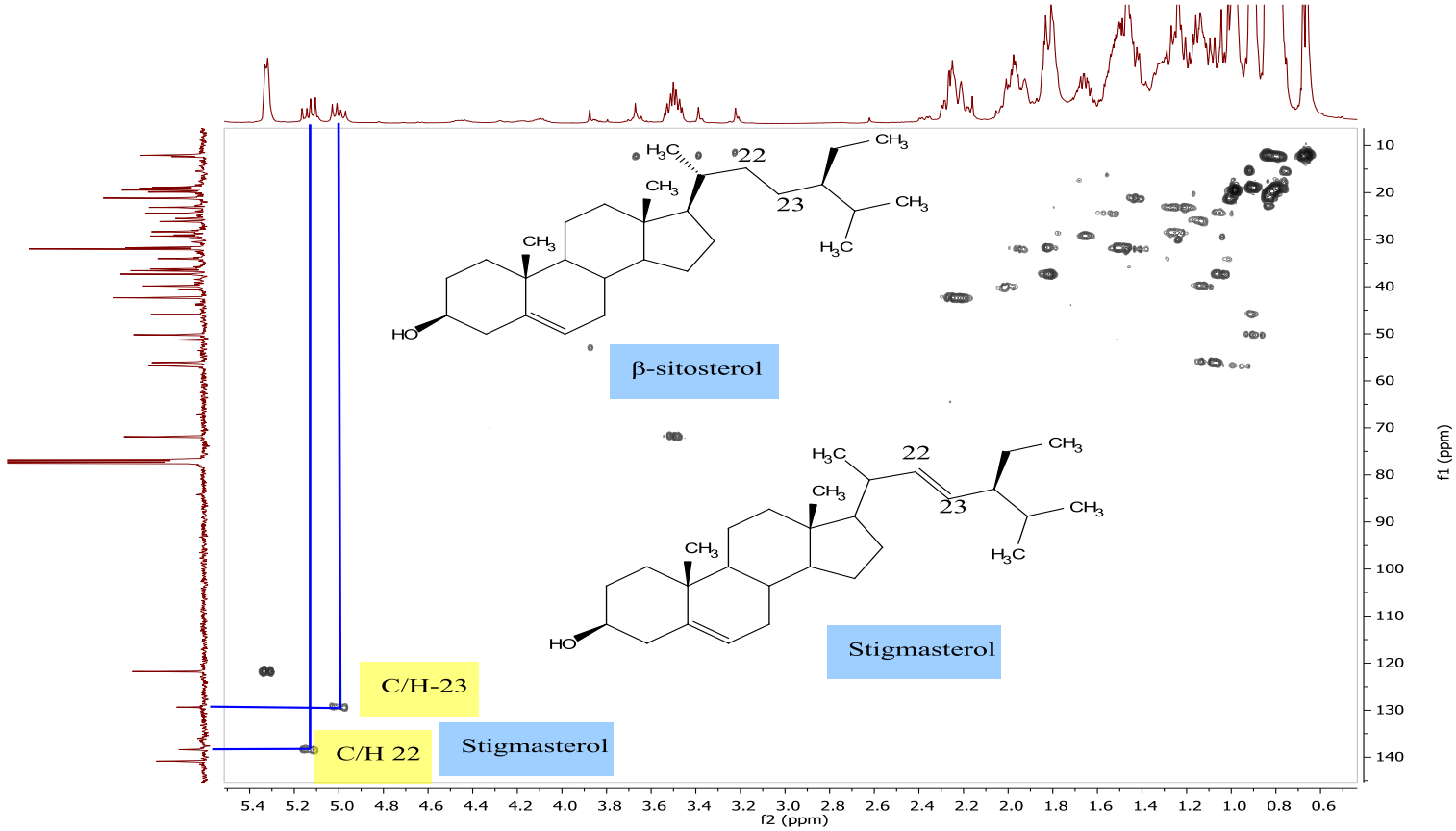
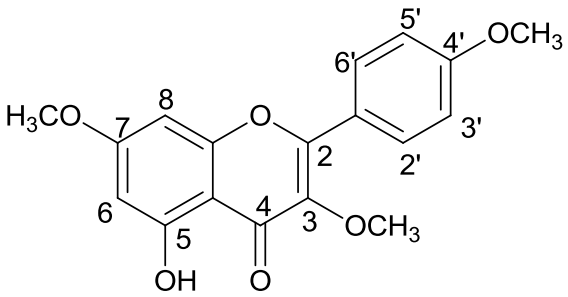
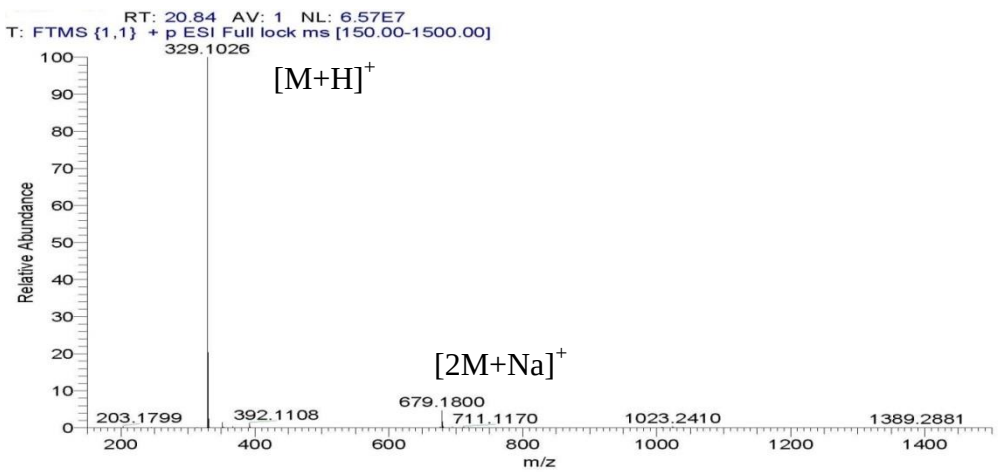


Fig. 3.4: The HMQC spectrum showed C-H direct correlation at positions 22 and 23 which belong to stigmasterol

Table 3.1: ^{13}C NMR data of *β*-sitosterol and stigmasterol at 400 MHz in Chloroform-*d*

Carbon	***β*-sitosterol**	**Literature (Alam *et al.*, 1996)**	**Stigmasterol**	**Literature (Mahato and Kundu, 1994)**
Position	δ_C	δ_C	δ_C	δ_C
1	37.4	37.3	37.1	37.3
2	31.9	31.9	31.6	31.7
3	71.8	71.9	71.8	71.9
4	42.3	42.3	41.5	42.3
5	140.3	140.8	140.3	140.8
6	121.7	121.8	121.7	121.8
7	31.7	31.7	31.7	32.1
8	32.0	32.0	32.0	32.0
9	50.1	50.2	50.1	50.2
10	36.5	36.6	36.7	36.6
11	21.1	21.2	20.8	21.2
12	39.6	39.8	39.6	39.8
13	42.3	42.4	42.3	42.4
14	56.7	56.8	56.7	56.8
15	24.3	24.4	24.3	24.4
16	28.6	28.3	28.6	28.3
17	55.9	56.1	55.9	56.1
18	11.6	11.9	11.6	11.9
19	19.4	19.5	19.2	19.1
20	36.2	36.2	40.1	40.6
21	18.8	18.9	20.5	20.0
22	34.0	34.0	138.0	138.4
23	26.1	26.2	129.2	129.2
24	45.8	46.0	51.1	51.3
25	29.2	29.2	32.0	34.0
26	19.8	19.9	19.0	18.9
27	19.0	19.1	21.2	21.3
28	23.1	23.1	25.4	25.5
29	12.0	12.1	12.0	12.1

3.1.2.2 5-hydroxy-3, 7, 4’-trimethoxyflavone (known compound)

5-hydroxy-3, 7, 4’-trimethoxyflavone (3.3)
Synonym(s): 3,7,4'-trimethylkaempferol Source: Leaves of *V. pinnata* Sample amount: 20 mg Physical description: White needles Molecular formula: $C_{18}H_{16}O_6$ Molecular weight: 328.316 g/mol



Compound **3.3** was isolated from hexane extract of leaves of *V. pinnata* after running through MPLC. ESI-MS peak in the positive mode was found at *m/z* 329.1026 $[M+H]^+$ (base peak) which revealed a molecular weight of 328.32 g/mol and a molecular formula of $C_{18}H_{16}O_6$. Consequently, compound **3.3** had 11 degree of unsaturation. The ^{1}H NMR spectrum of **3.3** displayed a singlet signal at δ 12.65 due to the strongly

hydrogen bonded phenolic hydroxyl moiety. The presence of two doublet signals at δ_H 6.44 (d, *J*=2) and δ 6.35 (d, *J*=2) were characteristic of two *meta*-related H-6 and H-8 as in a 5,7disubstituted A-ring flavonoid. In the B ring system, two sets of symmetric proton doublet signals at δ 8.07 (2H, d, *J*=9) and 7.02 (2H, d, *J*=9) were detected for an AA' BB' system which indicated that C-4' was substituted. In addition, the ^{1}H NMR spectrum of this compound exhibited three singlets at δ_H 3.89, 3.87 and 3.85 showing the existence of three methoxyl groups. By comparing the data with previous literature (Rossi *et al.*, 1997), the compound **3.3** was identified as 5-hydroxy-3, 7, 4'-trimethoxyflavone previously isolated from *Aniba* species.

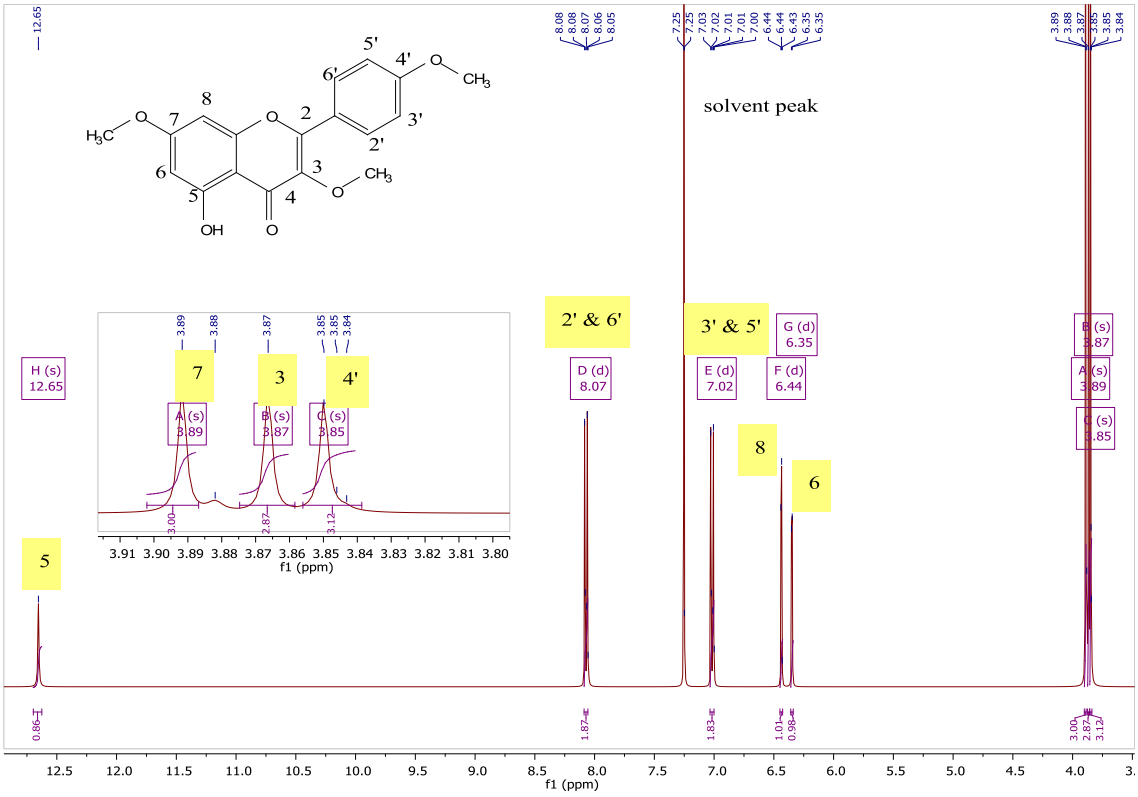
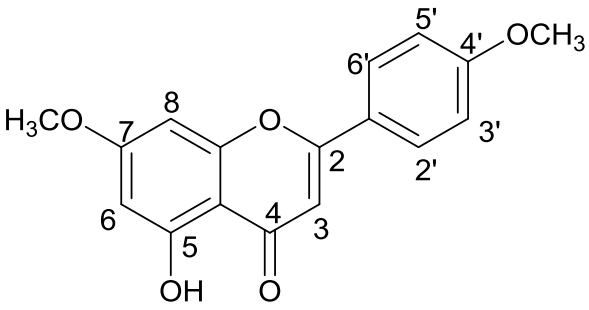
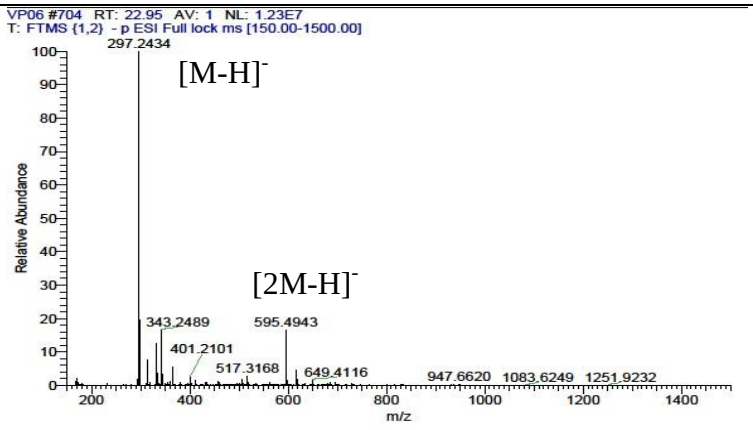


Fig. 3.5: The ^{1}H NMR spectrum of 5-hydroxy-3, 7, 4'-trimethoxyflavone at 400 MHz recorded in Chloroform-*d*

Table 3.2: ^{1}H NMR data of 5-hydroxy-3, 7, 4'-trimethoxyflavone at 400 MHz in Chloroform-*d*

Position	5-hydroxy-3, 7, 4'-trimethoxyflavone in Chloroform-*d*	Literature (Rossi *et al.*, 1997) in Chloroform-*d*
	δ_H (*J* Hz)	δ_H (*J* Hz)
6	6.35, d (*J*=2)	6.33, d (*J*=2)
8	6.44, d (*J*=2)	6.43, d (*J*=2)
2' and 6'	8.07, d (*J*=9)	8.07, d (*J*=9)
3' and 5'	7.02, d (*J*=9)	7.0, d (*J*=9)
3-OCH_3	3.87,s	3.84, s
7-OCH_3	3.89, s	3.84, s
4'-OCH_3	3.85, s	3.84, s
5-OH	12.65, s	12.6, s

3.1.2.3 5-hydroxy-7, 4'-dimethoxyflavone, (known compound)

5-hydroxy-7, 4'-dimethoxyflavone (3.4)
Synonym(s): Apigenin 7, 4'-dimethyl ether Source: Leaves of *V. pinnata* Sample amount: 4 mg Physical description: White needles Molecular formula: $C_{17}H_{14}O_5$ Molecular weight: 298.29 g/mol



Compound **3.4** was obtained as white needles. The ESI-MS spectrum showed a molecular ion at *m/z* 297.2434 $[M-H]^-$ suggested a molecular formula of $C_{19}H_{18}O_7$. The ^1H NMR spectrum showed of two methoxy signals at δ_H 3.88 and 3.89. One downfield singlet at δ_H 12.80 assigned to a phenolic hydroxyl group. The ^1H NMR spectrum of compound **3.4** was found to be similar to spectrum compound **3.3** except that compound **3.4** showed additional singlet peak at δ_H 6.58 suggested its position at C-3. In the A ring, AB system was assigned to two doublet protons (H-6 and H-8) at δ_H 6.37

and 6.48 with typical *meta* coupling constant of *J*= 2.2 Hz. In the B ring system, two sets of symmetric proton signals δ 7.84 (2H, d, *J*=9) and 7.01 (2H, d, *J*=9) were detected for an AA' BB' system which indicated that C-4' was substituted. The structure of **3.4** was identified as 5-hydroxy-7, 4'-dimethoxyflavone based on comparison to the previous published data (Kolak *et al.*, 2009).

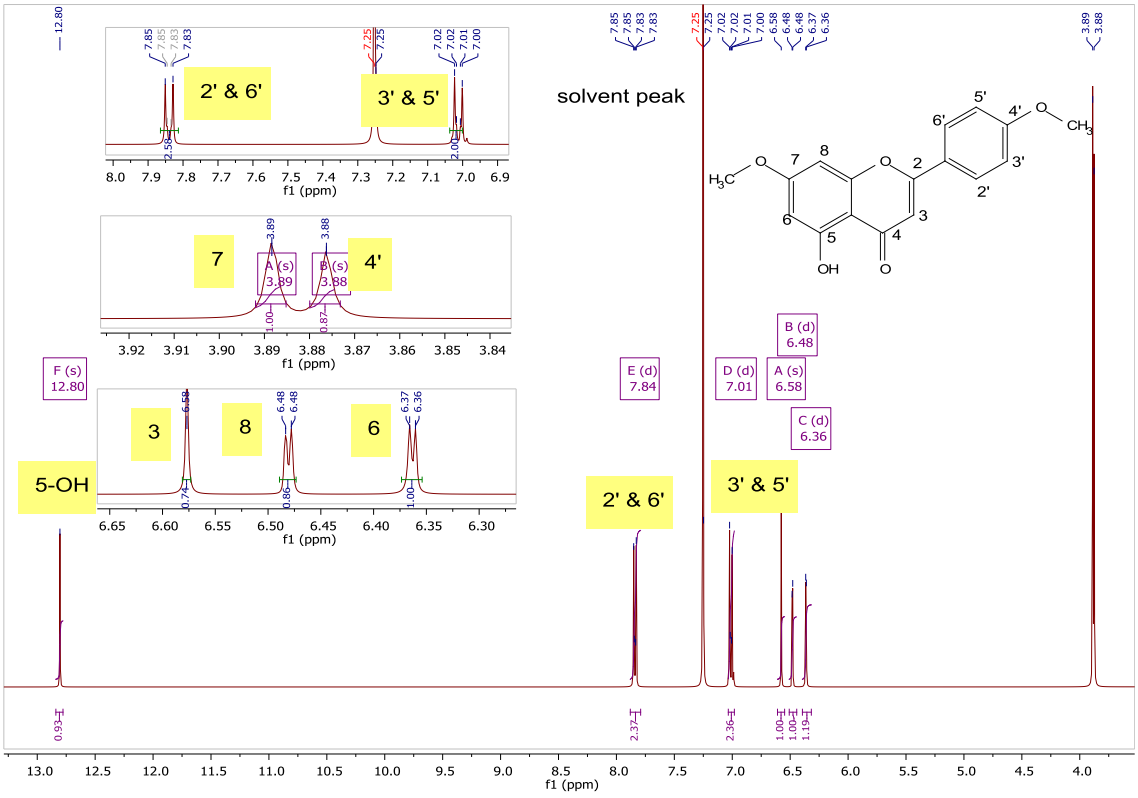
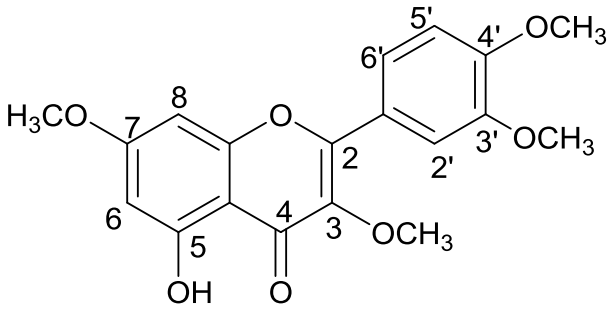
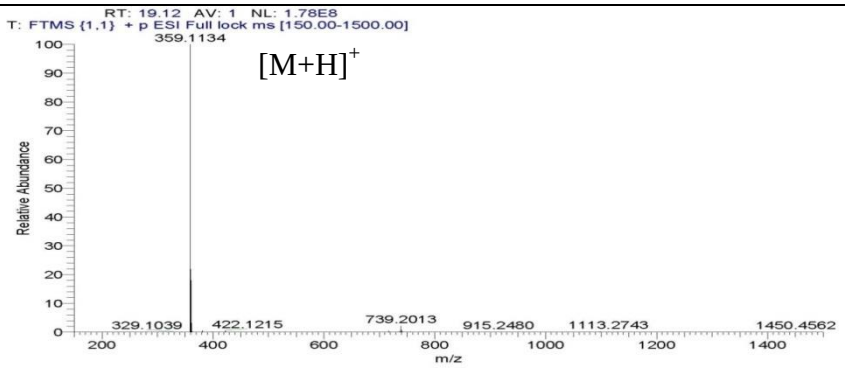


Fig. 3.6: The ^{1}H NMR spectrum of 5-hydroxy-7, 4'-dimethoxyflavone at 400 MHz recorded in Chloroform-*d*

Table 3.3: ^{1}H NMR data of 5-hydroxy-7, 4'-dimethoxyflavone at 400 MHz in Chloroform-*d*

Position	5-hydroxy-3, 7, 4'-trimethoxyflavone in Chloroform-*d*	Literature(Kolak *et al.*, 2009) in Chloroform-*d*
	δ_H (*J* Hz)	δ_H (*J* Hz)
3	6.58, s	6.58, s
6	6.36, d (*J*=2.2)	6.37, d (*J*=2.4)
8	6.48, d (*J*=2.2)	6.48, d (*J*=2.4)
2' and 6'	7.84, d (*J*=9.0)	7.85, d (*J*=9.0)
3' and 5'	7.01, d (*J*=9.0)	7.02, d (*J*=9.0)
7-OCH_3	3.89, s	3.84, s
4'-OCH_3	3.88, s	3.84, s
5-OH	12.80, s	12.80, s

3.1.2.4 5-hydroxy-3, 3', 4', 7-tetramethoxyflavone, (known compound)

5-hydroxy-3, 3', 4', 7-tetramethoxyflavone (3.5)
Synonym(s): Retusin Source: Leaves of*V. pinnata* Sample amount: 130 mg Physical description: White needles Molecular formula: $C_{19}H_{18}O_7$ Molecular weight: 358.34 g/mol



Compound **3.5** was obtained as white crystalline needles. A molecular formula of $C_{19}H_{18}O_7$ was deduced by ESI-MS with molecular ion peak at *m/z* 359.1134 $[M+H]^+$ (base peak) with 11 degrees of unsaturation. The ^1H NMR spectrum showed four methoxy signals at δ_H 3.86, 3.88, 3.96 and 3.97. One downfield singlet at δ_H 12.64 was assigned to a phenolic hydroxyl group. In the A ring, AB system was assigned to two doublet protons (H-6 and H-8) at δ_H 6.36 and 6.45 with a typical *meta* coupling constant of *J*= 2.2 Hz. The remaining three aromatic protons for the B ring showed an ABX

system at δ_H 7.73 (dd, *J* = 8.9, 2 Hz), 7.69 (d, *J* = 2 Hz) and 6.99 (d, *J* = 8.9 Hz). The structure of **3.5** was identified as retusin based on comparison to the previous published data (Li *et al.*, 2006) which was previously isolated from *Distemonanthus benthamianus* Baillon (Malan and Roux, 1979).

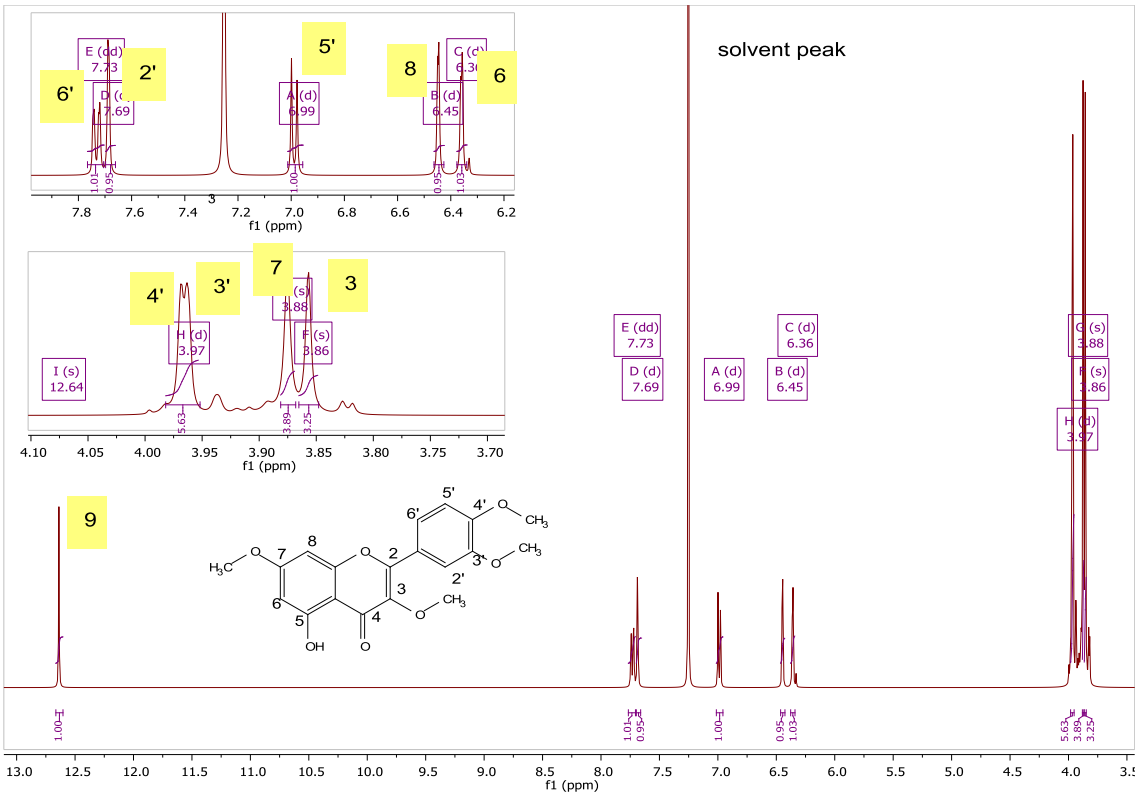


Fig. 3.7: The ^{1}H NMR spectrum of retusin at 400 MHz recorded in Chloroform-*d*

Table 3.4: ^{1}H NMR data of retusin at 400 MHz in Chloroform-*d*

Position	Retusin in in Chloroform-*d*	Literature (Li *et al.*, 2006) in DMSO-d_6
	δ_H (*J* Hz)	δ_H (*J* Hz)
6	6.36, d (*J*=2.2)	6.39, d (*J*=2.2)
8	6.45, d (*J*=2.2)	6.80. d (*J*=2.2)
2’	7.69, d (*J*=2.0)	7.67, d (*J*=2.0)
5’	6.99, d (*J*=8.9)	7.17, d (*J*=9.0)
6’	7.73, dd (*J*=8.9, 2.0)	7.74, dd (*J*=9.0, 2.0)
3- OCH_3	3.86, s	3.83, s
7- OCH_3	3.88, s	3.87, s
3’- OCH_3	3.96, s	3.87, s
4’- OCH_3	3.97, s	3.88, s
9-OH	12.64, s	12.62, s

3.1.3 Biological activities

3.1.3.1 Anti trypanosomal assay

The isolated compounds were evaluated for their antitrypanosomal activities against *T. brucei brucei.* Compounds **3.1/3.2, 3.3, 3.4 and 3.5** showed antitrypanosomal activity with MIC values of 6.25µg/mL, 19.0, 21.0 and 17.0µM, respectively.

3.1.3.2 Antibacterial assay

No activity was observed in all isolated compounds against *M. marinum.*

3.1.3.3 NF-κB inhibition assay

All compounds except **3.4** were sent for NF-κB inhibition assay on K562 human chronic leukemia cells. Compounds **3.1/3.2, 3.3 and 3.5** were active on NF-κB inhibition activity with IC_{50} of 2.3µg/mL, 52.1 and 10.0µM, respectively.

Table 3.5: Summary of bioactivities of isolated compounds from *V. pinnata*

Compounds	Antitrypanosomal activity against *T. brucei brucei*, MIC (μM)	Antibacterial activity against *M.marinum* MIC (μM)	Inhibition of NF-κB activity (IC_{50}) (μM)
3.1/3.2	6.25μg/mL	>100	2.3μg/mL
3.3	19.0	>100	52.1
3.4	21.0	>100	Not tested
3.5	17.0	>100	10.0
Suramin	0.1		
Gentamycin		13.5	

Compounds considered active on NF-κB inhibition assay when IC_{50} <10 μM

3.2 *Lasiodiplodia theobromae*

3.2.1 Metabolomics -bioassay guided isolation as decision-making strategy in mining antitrypanosomal active metabolites from the endophytic fungus *L. theobromae*

3.2.1.1 Metabolomics-bioassay guided screening as a approach to prioritize the most optimal condition of culture of *L. theobromae* for medium-scale fermentation

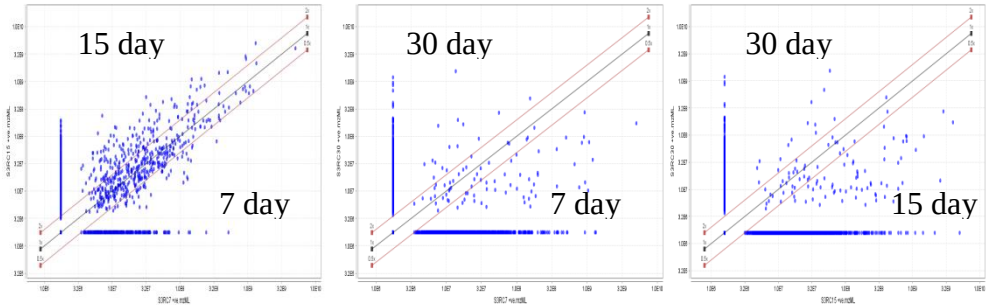
In search of the best condition for scaling up the endophyte *L. theobromae* culture obtained from the leaves of *V. pinnata*, HR-LCMS and NMR-based metabolomics along with the bioassay data were utilised. Fungal extracts were grown in solid rice culture and liquid Wickerham cultures for seven, fifteen and thirty days then extracts were submitted for antitrypanosomal assay. Three different incubation times were chosen based on the fungal life-cycle (Webster and Weber, 2007), which in this case the first seven days represent the germination phase, the fifteen days culture for the hyphal growth stage and thirty days culture covers the sporing phase of *L. theobromae*. The production of secondary metabolites was monitored at each of the growth phases parallel to the bioassay result; all extracts were monitored by HR-LCMS and NMR. Based on the bioassay results (Table 3.6), the 30-days rice culture extract exhibited the strongest activity against *T. brucei brucei* with MIC of less than 25µg/mL. The HR-LCMS raw data was processed using MZMine 2.10 (Pluskal *et al.*, 2010). Metabolite production and distribution between cultures were analysed through a scatter plot (Fig. 3.10). Based on the MS data, occurrences of the metabolites on the 7th and 15th days were almost similar while a decrease in metabolite production was observed on the 30th day. In addition, the total ion chromatogram revealed a different data set to those of the 7th and 15th -day extracts (Fig. 3.8). In addition to that, the ^{1}H NMR data revealed the similar finding with MS data (Fig. 3.9). Therefore, the 30-days rice culture condition was chosen for scale-up and further isolation work.

Table 3.6: Antitrypanosomal and anti-mycobacterium activity of *L. theobromae* extracts derived from *V. pinnata* in different types of media and incubation periods. MIC was only determined for the bioactive extracts

Sample	*T. brucei brucei* 20ug/ml % D control	*T. brucei brucei* MIC (µg/ml)	*M. marinum* ATCC BAA 535 100µg/ml % D control	*M. marinum* ATCC BAA 535 MIC (µg/ml)
LT-LC-7	112.3	Not tested	112.4	Not tested
LT-LC-15	106.3	Not tested	112.9	Not tested
LT-LC30	126.0	Not tested	102.4	Not tested
LT-RC-7	103.9	Not tested	113.3	Not tested
LT-RC-15	94.3	Not tested	99.0	Not tested
LT-RC30	**1.4**	**25**	100.9	Not tested

*LT- *L. theobromae*

A



B

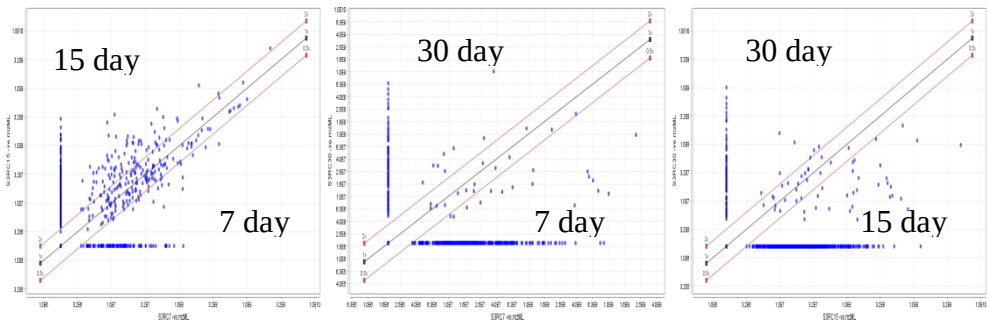


Fig. 3.8: Scatter plot of the mass spectral data of *L. theobromae* extracts at different incubation periods in A) positive ionisation and B) negative ionisation mode

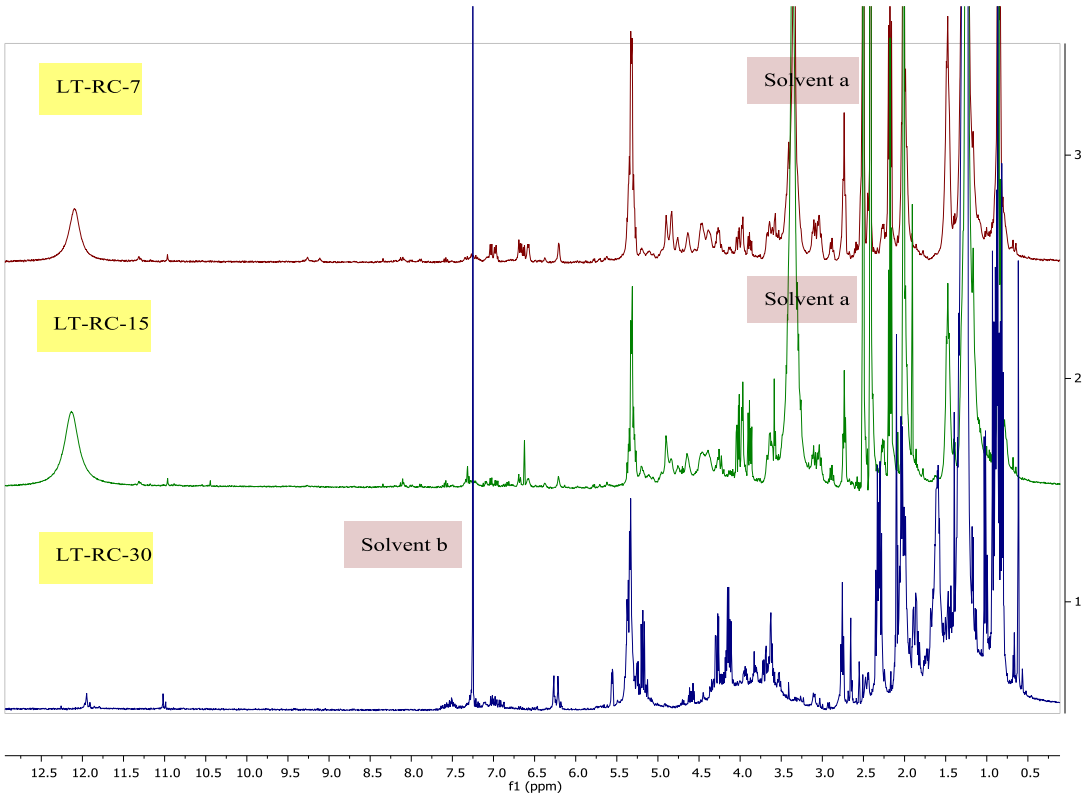


Fig. 3.9: The ^{1}H NMR data of *L. theobromae* extracts obtained from solid rice culture at three different incubation periods (a in DMSO-*d6*; b in Chloroform-*d*). Rice culture extracts at 30-days can only be fully dissolved in chloroform indicating the compounds occurring in this extract are semi non-polar.

3.2.1.2 The antitrypanosomal activity and ^{1}H NMR comparison on *L. theobromae* fractions

The rice culture extract of *L. theobromae* was fractionated yielding 19 fractions (LT-1 until LT-19) and these were submitted for the antitrypanosomal assay and ^{1}H NMR experiment. In the bioassay screening, LT-1 was excluded because it contains only fatty acids as indicated by its ^{1}H NMR data (see Fig. 3.11). The non-polar fractions LT-2 to LT-8 exhibited strong bioactivity except for LT-4 which showed only moderate activity against *T. brucei brucei* (Fig. 3.10). The ^{1}H NMR spectrum data of 19 fractions (Fig. 3.11) were analysed and unique chemical fingerprints of the active fractions were detected. Among these active fractions, two distinctive sub-groups, LT-2 to LT-4 and LT-6 to LT-8, clustered together as they share similar spectral data. Fraction LT-5 was

a mixture of both groups. ^{1}H NMR spectrum of fractions LT-2 to LT-5 displayed a pair of *meta*-coupled aromatic proton at δ_H 6.23 and 6.28 (J= 2.7 Hz) and a multiplet peak at δ_H 5.15 which could probably be an olefinic or oxygenated methine. The upfield shift of the *meta*-coupled aromatic protons at the 6 ppm region suggested the presence of an electron withdrawing group such as hydroxyl or halogen substituent. In fractions LT-5 to LT-8 two *meta*- coupled doublets at δ_H 6.18 and 6.25 (J=2.6 Hz) were observed (see red arrow in Fig. 3.11). The proton signals between 4.2- 4.8 ppm revealed the presence of oxygenated methines while proton signals between 6.6 to 7.7 ppm indicated the presence of aromatic compounds in fractions LT-6 to LT-8.

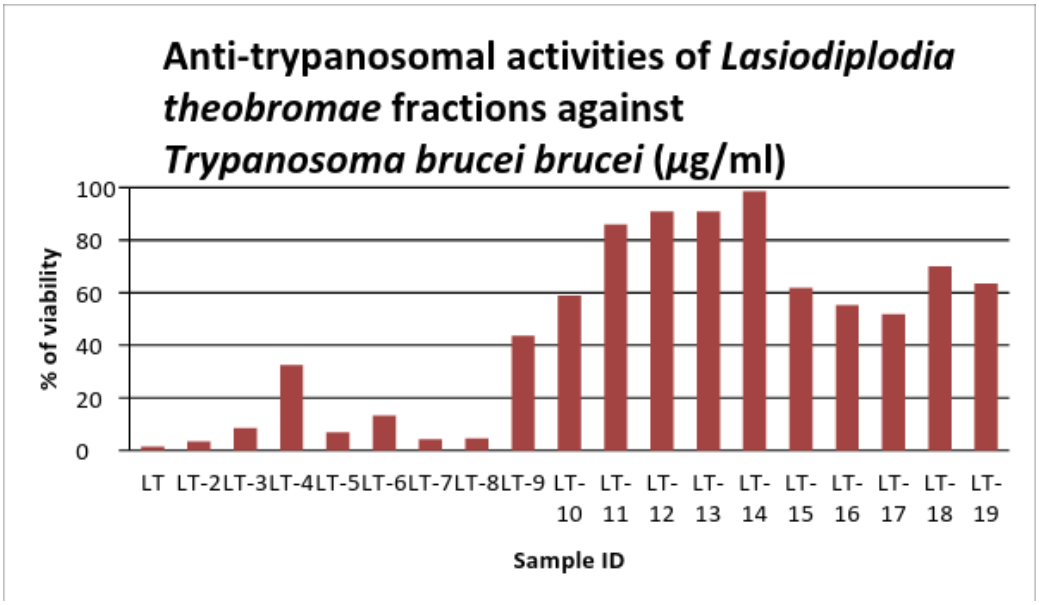


Fig. 3.10: Antitrypanosomal activity of *L. theobromae* fractions against *T. brucei brucei*. LT: *L. theobromae* extract as positive control; LT-2 to LT-19: *L. theobromae* fractions

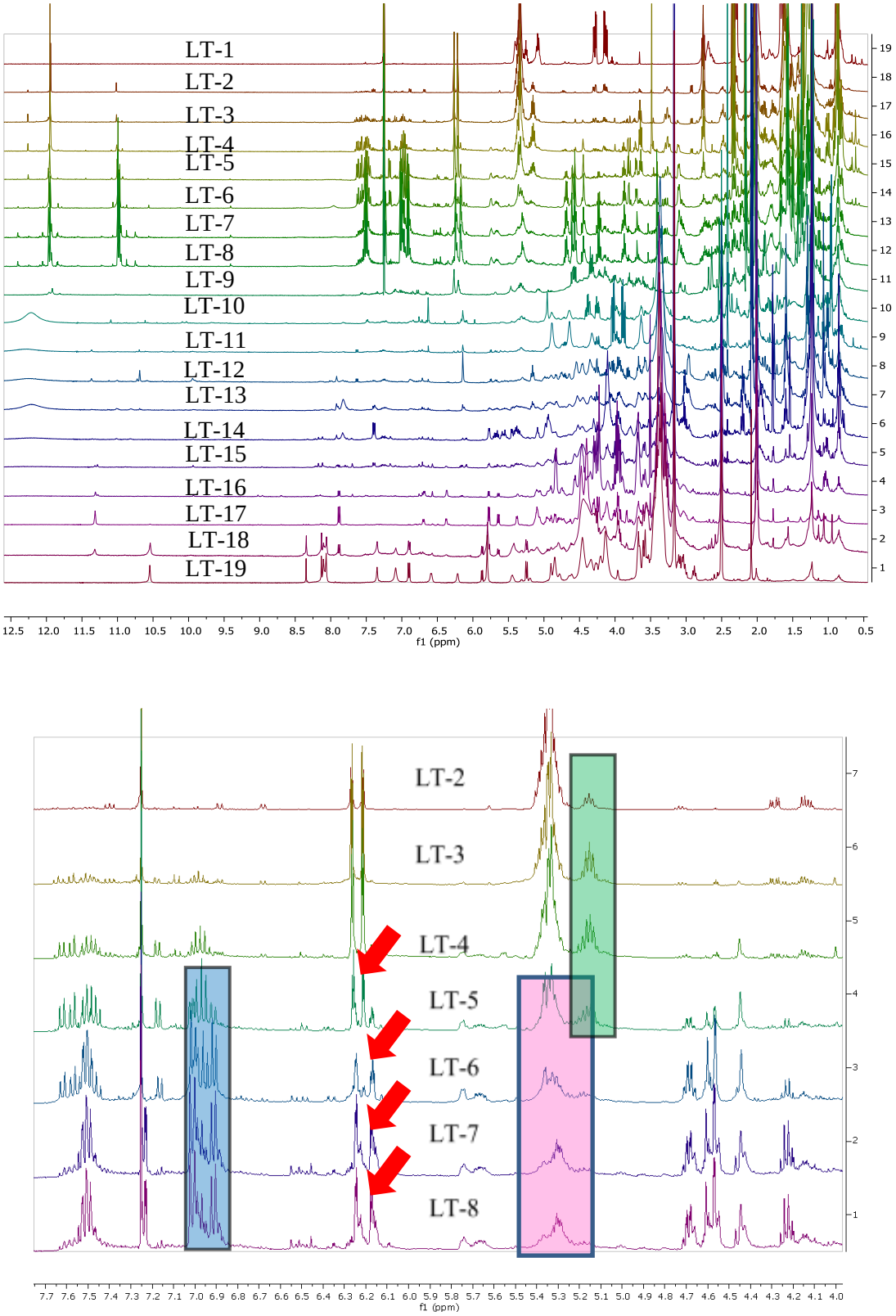
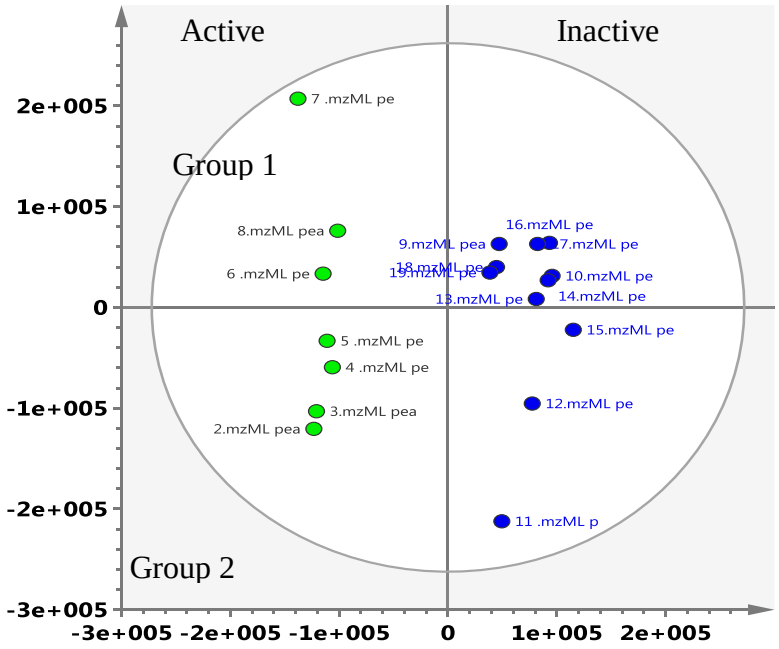


Fig. 3.11: Above: The ^{1}H NMR spectrums of 19 fractions; Below: The expansion of the ^{1}H NMR data for active antitrypanosome fractions which showed several unique chemical finger prints found only in these fractions. Red arrow in fractions LT-5 to LT-8 indicate the presence of two *meta*- coupled doublets at δ_H 6.18 and 6.25 (J=2.6 Hz)

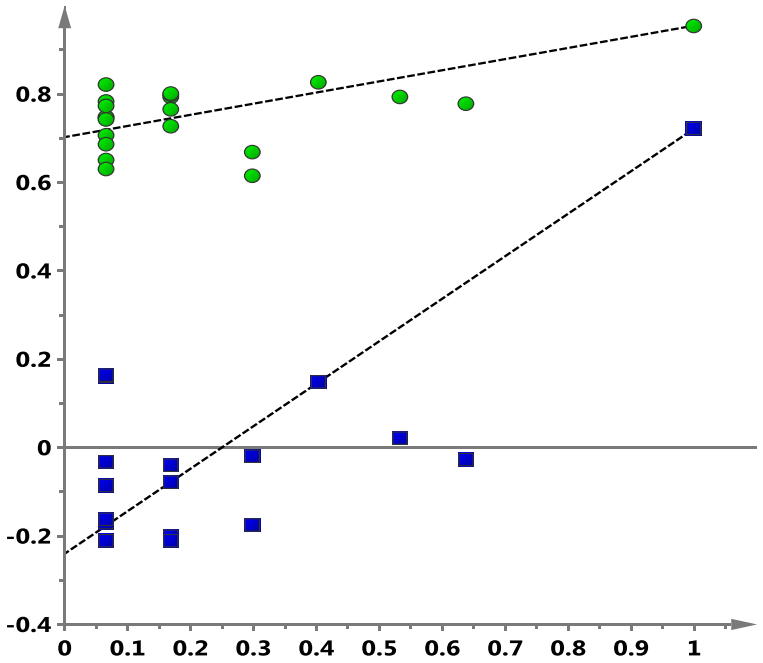
3.2.1.3 Chemometrics analyis of HR-LCMS data

Multivariate data analysis with supervised method was used to analyse the similarity of the data sets between samples. In this study, the distribution difference of the type of metabolites between active *vs* inactive fractions of *L. theobromae* against *T. brucei brucei* was analysed by subjecting the data to Partial Least Square-Discriminant Analysis (PLS-DA) and Orthogonal Partial Least Square-Discriminant Analysis (OPLS-DA). Furthermore, the results of the analysis led to the prediction of the compounds that contribute towards the antitrypanosomal activity. For the PLS and OPLS-DA models (Fig. 3.12a), the MS-based metabolomics data set was assigned as the X independent variable while the fractions' antitrypanosomal response was the Y dependent variable. To validate the model of PLS-DA, a permutation test (Fig. 3.12b) was performed to calculate the goodness of fit (R2) and prediction of the model (Q2). The model's R2 was 0.9 and Q2 was 0.7. This indicated a well fitted model exhibiting good prediction. OPLS-DA was employed to eliminate the structured noise obtained from the X-variable which can cause system variation (Ali *et al.*, 2013). For the OPLS-DA score plot (Fig. 3.12a), the active fractions were grouped together versus the inactive ones. Under the active group, fractions LT-2 to LT-5 (Group 1) and LT-6 to LT-8 (Group 2) clustered together indicating a shared set of metabolites while fractions LT-9 to LT-19 (Group 2) were observed as outliers of the group. The generated S-plot (Fig. 3.12d) determined the "end point" compounds which suggested the unique metabolites responsible towards the bioactivity against *T. brucei brucei* and discriminates them from the inactive fractions of *L. theobromae*. Eight metabolites were identified from Antibase and MarinLit as shown in Table 3.7. The end point compounds were targeted for bioassay-guided isolation work with three active compounds in Group 1 and remaining five active compounds in Group 2.

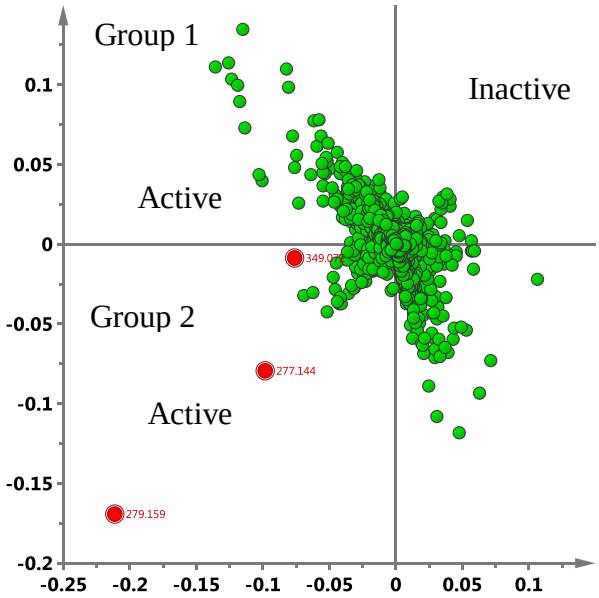
A


Active
Inactive
Group 1
Group 2
7 .mzML pe
8.mzML pea
6 .mzML pe
5 .mzML pe
4 .mzML pe
3.mzML pea
2.mzML pea
16.mzML pe
9.mzML pea
17.mzML pe
10.mzML pe
13.mzML pe
14.mzML pe
15.mzML pe
12.mzML pe
11 .mzML p
2e+005
1e+005
0
-1e+005
-2e+005
-3e+005
-3e+005 -2e+005 -1e+005 0 1e+005 2e+005

B


0.8
0.6
0.4
0.2
0
-0.2
-0.4
0 0.1 0.2 0.3 0.4 0.5 0.6 0.7 0.8 0.9 1

C



D

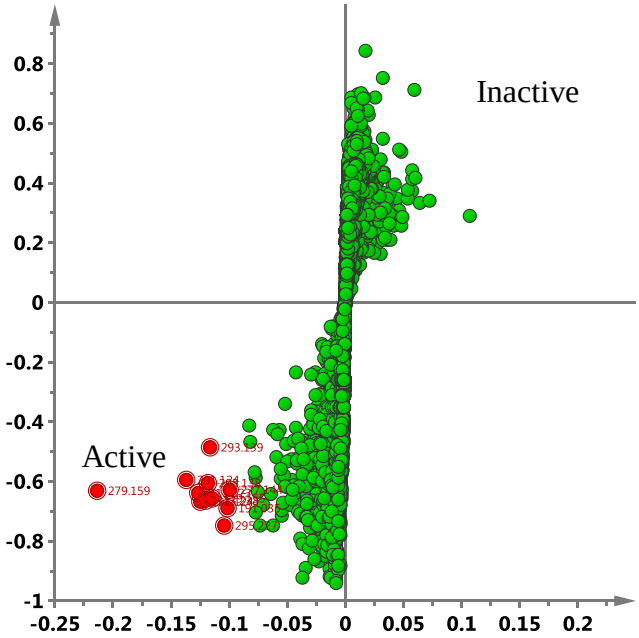


Fig. 3.12: Chemometrics analysis data of *L. theobromae* fractions in correlation to their antitrypanosomal activity data. A) In score scatter plot of OPLS-DA, the samples were grouped based on their bioactivity. B) Permutation test result of the PLS-DA model. C) The loading scatter plot of OPLS-DA showed feature ion peaks of active metabolomes D) S-plot generated from the OPLS-DA model predicted metabolites responsible for the activity.

Table 3.7: List of unique metabolites of *L. theobromae* active fractions obtained from S-plot "end-point" data shown on Figure 3d. (P=positive mode; N=negative mode)

Ionisation Mode	MS *m/z*	Rt (min)	Chemical Formula	Putative ID	Active Fractions	
					Group 1	Group 2
N	191.035	11.3197	$C_{10}H_8O_4$	6,8-Dihydroxy-3-methylisocoumarin(**3.6**)		√
N	291.124	13.1707	$C_{16}H_{20}O_5$	6-Oxo-de-*O*-methyllasiodiplodin (**3.7**)		√
P	293.139	13.7128	$C_{16}H_{20}O_5$	6-Oxo-de-*O*-methyllasiodiplodin (**3.7**)		√
N	395.077	13.6028	$C_{21}H_{16}O_8$	Preussomerin-C (**3.8**)		√
N	363.051	14.6074	$C_{20}H_{12}O_7$	Preussomerin-H (**3.9**)		√
N	333.077	17.6053	$C_{20}H_{14}O_5$	Palmarumycin CP17 (**3.10**)	√	
N	349.072	13.7125	$C_{20}H_{14}O_6$	Cladospirone-B (**3.11**)	√	
P	321.170	19.1427	$C_{18}H_{24}O_5$	Phomopsin B (**3.12**)		√
N	319.155	19.1605	$C_{18}H_{24}O_5$	Phomopsin B (**3.12**)		√
N	277.144	21.789	$C_{16}H_{22}O_4$	Desmethyl-lasiodiplodin (**3.13**)	√	
P	279.159	21.7967	$C_{16}H_{22}O_4$	Desmethyl-lasiodiplodin (**3.13**)	√	
N	555.296	21.7948		Complex of 277.144	√	

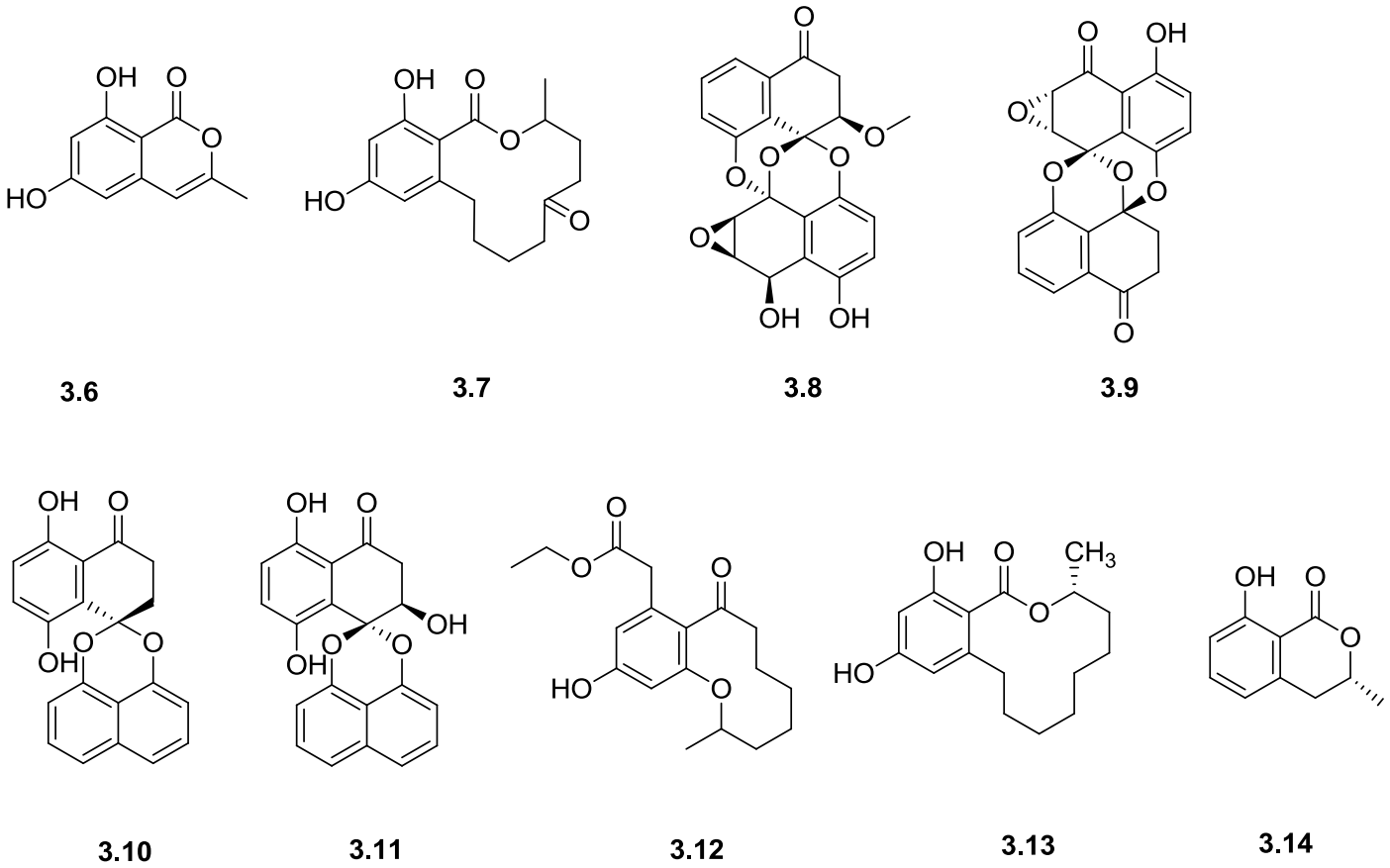


Fig. 3.13: Chemical structures of unique metabolites (**3.6-3.13**) predicted from the S-plot and further secondary metabolites (**3.11, 3.13,** and **3.14**) isolated from an active antitrypanosomal fractions of *L. theobromae* (Group 1).

3.2.1.4 Confirmation of the structure of the metabolites through ^{1}H-^{1}H COSY correlation

In Group 1, the active metabolites were further identified from the dereplication step as palmarumycin CP17 **3.10**, cladospirone-B **3.11** and desmethyl-lasiodiplodin **3.13**. Further analyses of the ^{1}H-^{1}H COSY NMR spectrum of fraction LT-3 revealed the partial correlation of the predicted active metabolites. For example, characteristic correlations for the major component, desmethyl-lasiodiplodin **3.13** were exhibited by the *meta*-coupled aromatic protons at δ_H 6.23 and 6.28 while for the aliphatic system, cross peaks were observed between the methyl doublet at δ_H 1.35 and the oxygenated methine proton at δ_H 5.15 which further correlated to the rest of the alkyl chain (see Fig. 3.14). A similar approach was used for 6-oxo-de-O-methyllasiodiplodin **3.7** (Fig. 3.15a). On the other hand, COSY correlations for preussomerin-C's ABC spin system were also revealed in the aromatic region (Fig. 3.15b). These COSY correlations confirm the dereplication results obtained from the HRMS data.

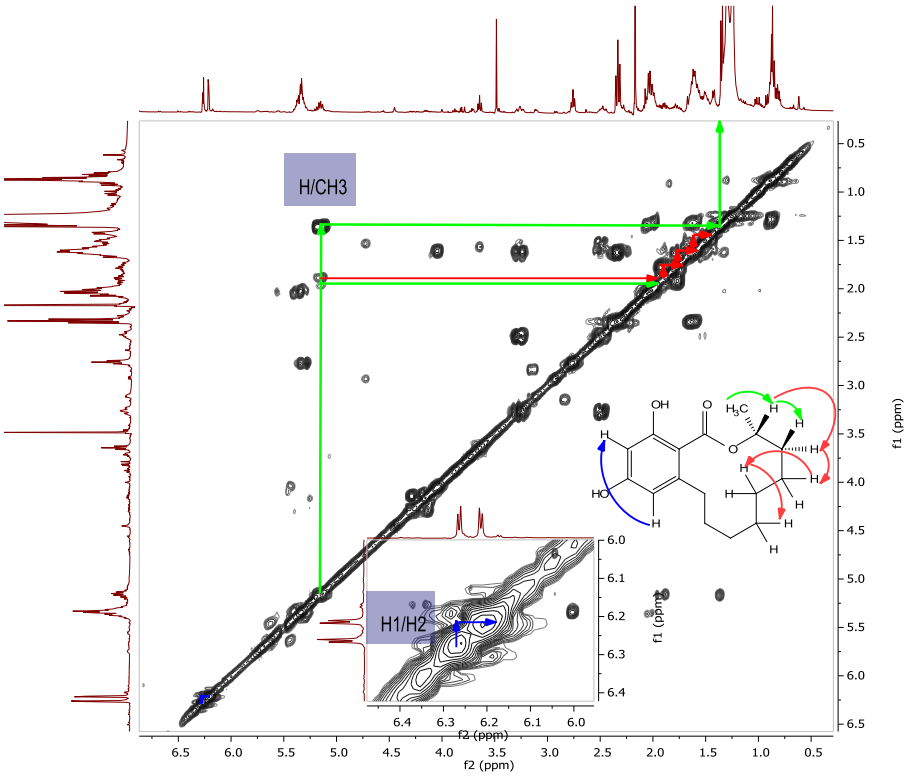


Fig. 3.14: ^{1}H-^{1}H COSY spectrum of fraction LT-3 showed correlations depicting a desmethyl-lasiodiplodin substructure.

A

B

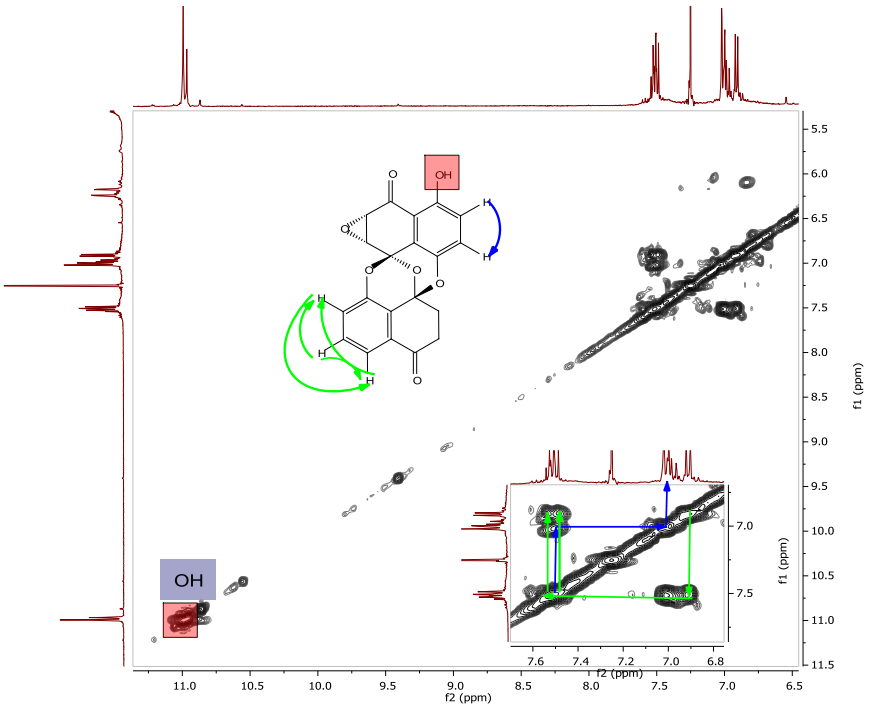


Fig. 3.15: The ^{1}H-^{1}H COSY spectrum of fraction LT-7 show partial correlation for substructures of a) 6-oxo-de-O-methyllasiodiplodin and b) preussomerin-C.

3.2.1.5 Isolation and identification of bioactive metabolites

Isolation and purification of the bioactive compounds was done by high-throughput MPLC. Three known compounds were isolated and elucidated based on their NMR and MS data as cladospirone-B **3.11** (Bode *et al.*, 2000), desmethyl-lasiodiplodin **3.13** (Aldridge *et al.*, 1971) and R-(-)-mellein **3.14** (Schulz *et al.*, 1995). Cladospirone-B and desmethyl-lasiodiplodin were among the three target "end point" compounds for Group 1 which were earlier predicted from the S-plot of the OPLS-DA model. Meanwhile R-(-)-mellein is a close analogue of 6,8-dihydroxy-3-methylisocoumarin **3.6**. All isolated compounds were tested against *T. brucei brucei* (Table 3.8). Cladospirone-B **3.11** and desmethyl-lasiodiplodin **3.13** were found active with MICs of 22.5 and 17.8 µM, respectively. The positions of the three metabolites on the S-plot were again checked. R-(-)-mellein **3.14** was located in the middle of the S-plot suggesting a weaker antitrypanosomal bioactivity (Fig. 3.16). Among the predicted bioactive metabolites is palmarumycin CP17 **3.10.** However, due to a lower yield, palmarumycin CP17 **3.10** was not isolated from this sample.

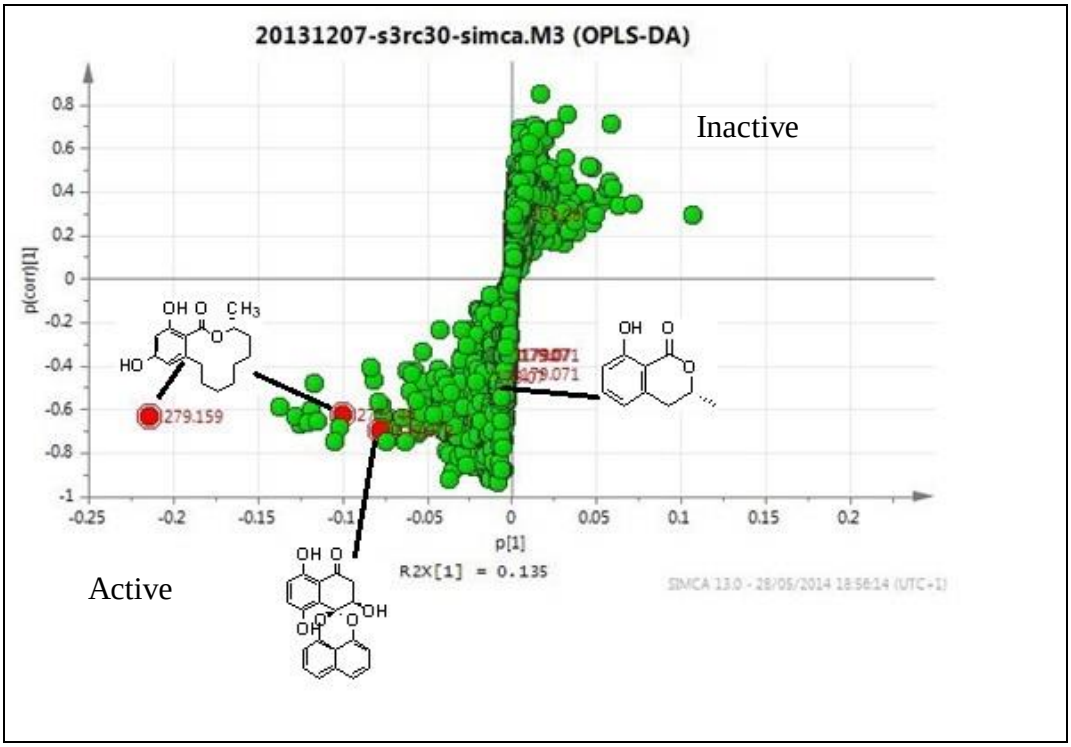


Fig. 3.16: Three isolated compounds from *L. theobromae* labelled in the S-plot. R-(-)-mellein was in the middle of the plot, suggesting less antitrypanosomal activity for this compound.

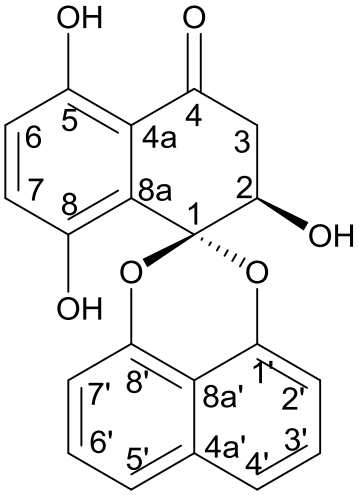
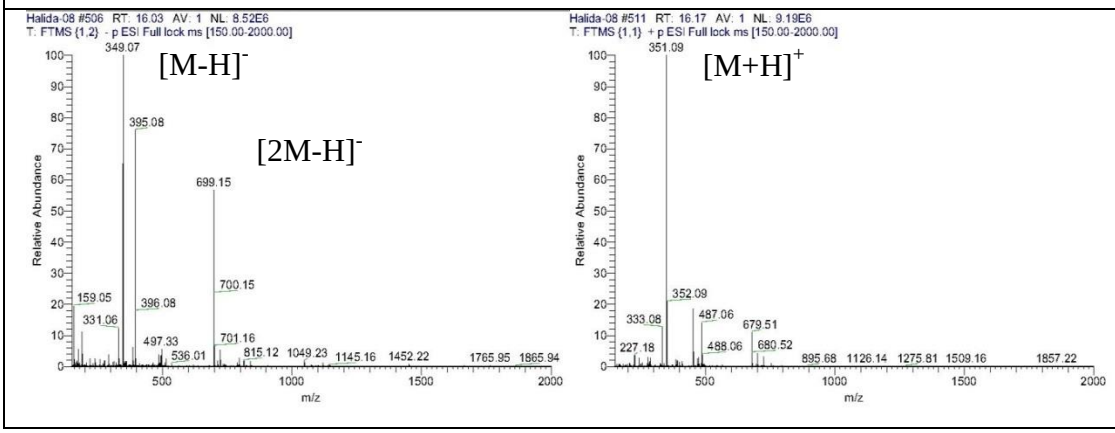
Table 3.8: Antitrypanosomal and anti-mycobacterial activities of isolated compounds obtained from *L. theobromae* fermented in solid rice culture for thirty days.

Compound	**Antitrypanosomal activity** ***(T. brucei brucei)***	**Anti-mycobacterial activity** **(*M. marinum*)**
	MIC (μM)	**MIC (μM)**
Cladospirone-B **3.11**	17.8	>100
Desmethyl-lasiodiplodin **3.13**	22.5	43.1
R-(-)- mellein **3.14**	>100	>100
Adenosine **3.15**	>100	>100
Suramin	0.1	
Gentamycin		13.5

In fraction 19, adenosine **3.15** also was isolated. This fraction was selected for purification based on the complexity of ^{1}H NMR data suggesting the purity of the major compound occurring in the respective fraction, thus only requiring a purification step.

3.2.2 Secondary metabolites isolated from *L. theobromae*

3.2.2.1 Cladospirone-B (known compound)

Cladospirone-B (3.11)
Synonym(s): 2,5,8-trihydroxy-2H-spiro[naphthalene-1,2'-naphtho[1,8-de][1,3]dioxin]-4(3H)-one Source: *L. theobromae* (isolated from *V. pinnata)* Sample amount: 3.0 mg Physical description: White amorphous solid Molecular formula: $C_{20}H_{14}O_6$ Molecular weight: 350.322 g/mol Optical rotation: $[\alpha]_D^{20}$ = -276 ($CHCl_3$)



Compound **3.11** was isolated as a white powder amounting to 3.0 mg (0.1% yield). An analysis of the HRESI-MS gave a molecular ion at *m/z* 351.0866 (base peak) in positive mode, while in the negative mode the molecular ion was found at *m/z* 349.0720 (base peak), indicating a molecular weight of 350.322 g/mol. The ^{1}H NMR spectrum measured in DMSO-*d6* showed the presence of an ABX spin system in the aliphatic region. An oxygenated methine multiplet at δ_H 4.27 (H-2) showed correlations with two separate methylene doublet of doublet signals belonging to the geminal protons of H-3 (δ_{H3a}= 2.67, dd, $J_{3a, 3b}$ = 16, $J_{3a, 2}$ = 4 Hz; δ_{H3b}= 3.21, dd, $J_{3b, 3a}$ = 16, $J_{3b, 2}$ = 3 Hz). In the aromatic region, two doublets at δ_H 7.03 (*J*= 9 Hz) and 7.25 (*J*= 9 Hz), denoting a 1,2,3,4-tetrasubstituted aromatic ring, were observed (Fig. 3.17). The signals for the 1,8-dihydroxynaphthalene moiety were overlapping in the ^{1}H NMR and thus was confirmed by analysis of the 2D COSY and *J*-resolved NMR spectra [(δ_H = 7.48, dd, *J*=8.7, 7.5 Hz, H-3') and (δ_H = 7.05, dd, *J*=7.5 and 1.2 Hz, H-7')] (Fig. 3.18, 3.19). To support this, the chemical shifts of the carbons were extracted from the HMBC spectrum (Fig. 3.20), and these confirmed the presence of the oxygenated carbons of the naphthalene moiety at δ_C 148.6 (C-1') and 149.5 (C-8'), respectively. Furthermore, three hydroxyl singlets were found at δ_H 5.75, 9.15 and 11.97 (OH-2, OH-8, and OH-5, respectively). These correlated in the HMBC with signals at δ_C 66.3, 150.5 and 154.8 which corresponded to the phenolic carbons C-2, C-5 and C-8, respectively (Fig. 3.20). The structure was confirmed through the comparison of the ^{1}H, ^{13}C NMR and mass spectral data with those of published data, and the compound was thus identified as cladospirone-B which was previously isolated from *Sphaeropsidales* sp F24'707 (Bode *et al.*, 2000).

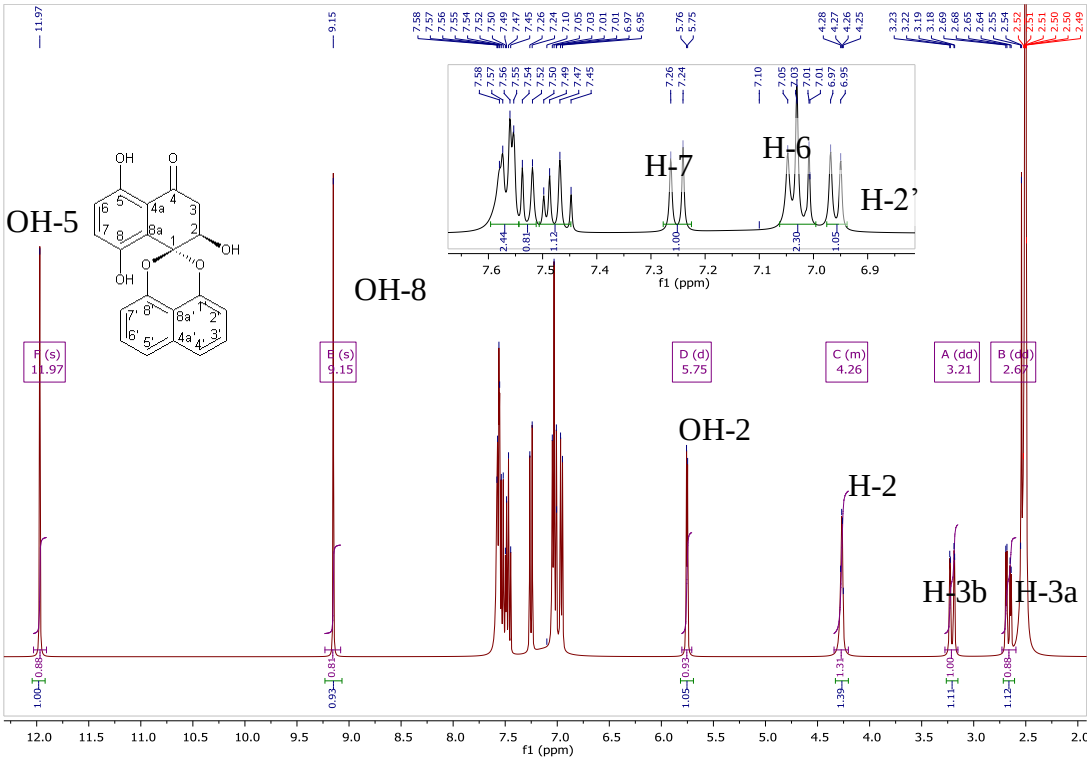


Fig. 3.17: The ^{1}H NMR spectrum of cladospirone-B revealed the proton integration and multiplicity of each peak. The expansion in the upper right corner shows the peaks in the aromatic region.

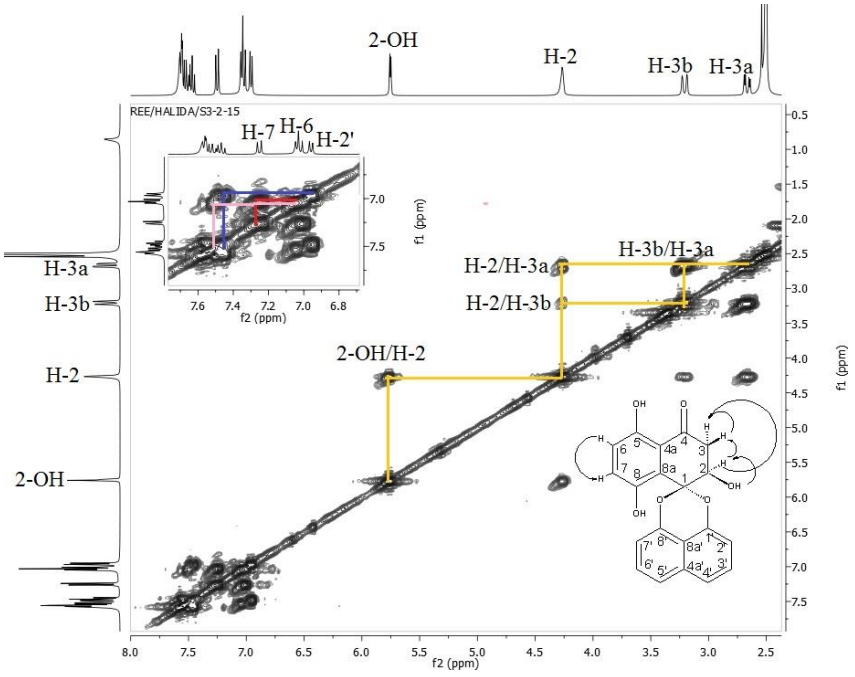


Fig. 3.18: The ^{1}H-^{1}H COSY correlation of cladospirone-B. The overlapping peak correlations were observed in the region at 7.45- 7.65 ppm.

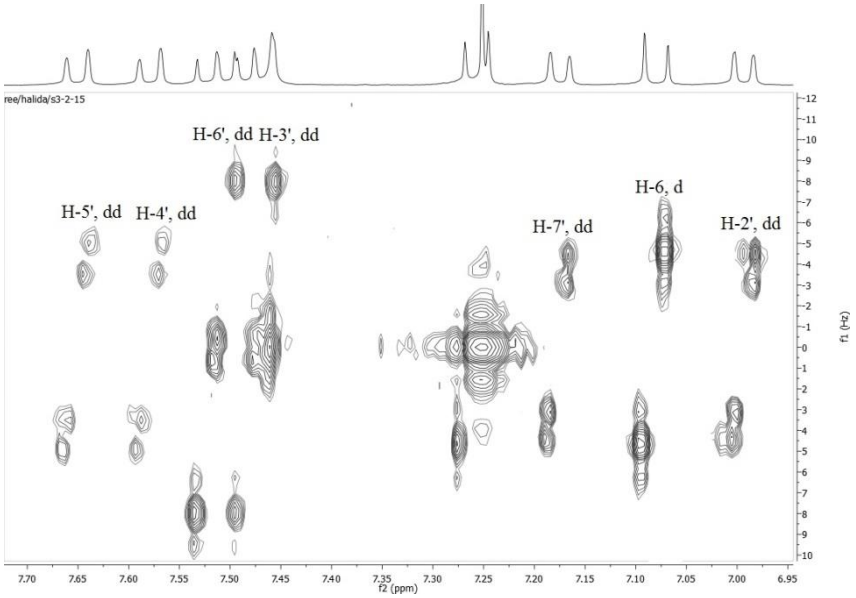
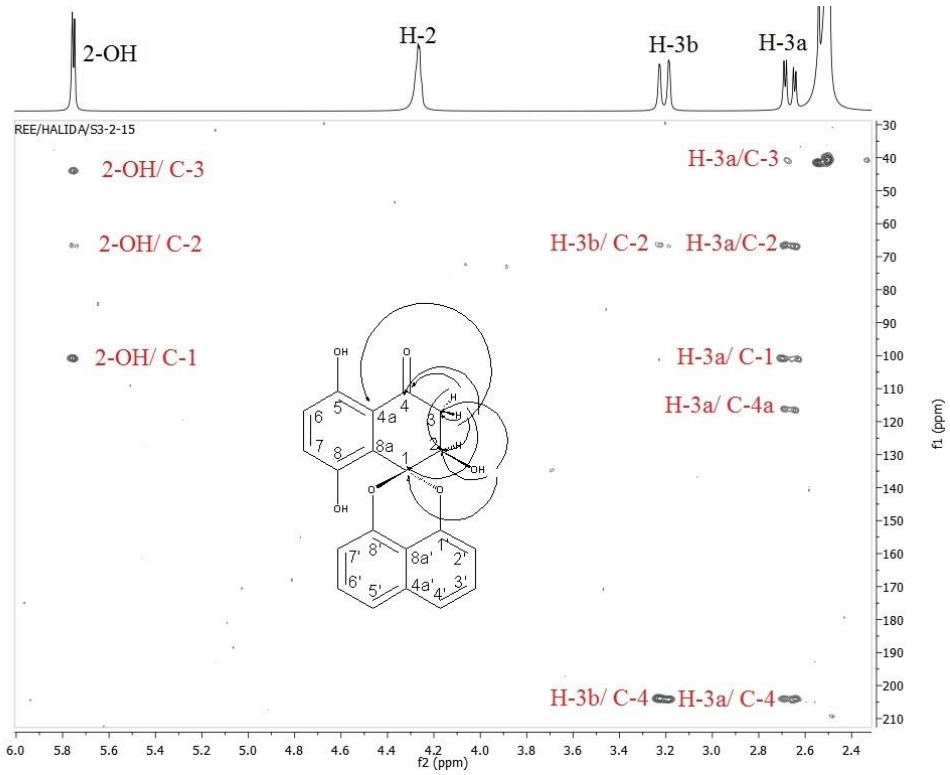


Fig. 3.19: The expanded *J*-resolved spectrum of cladospirone-B (measured in Chloroform-*d*) showed the multiplicity of the overlapping peaks at 7.45- 7.65 ppm.



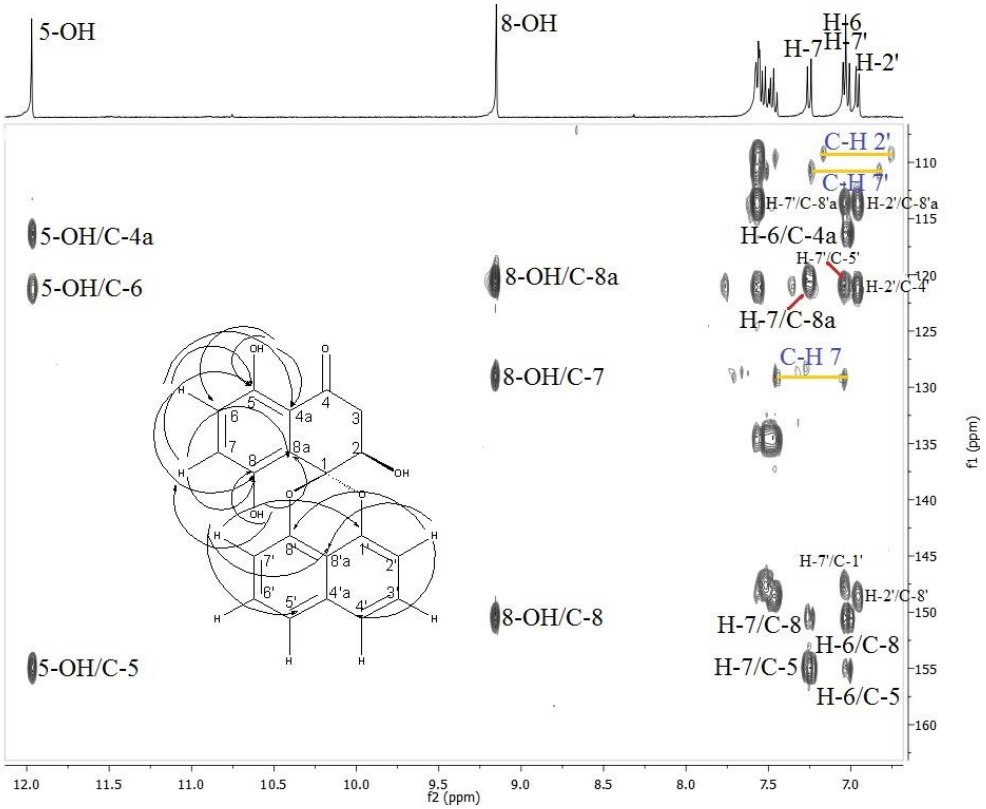
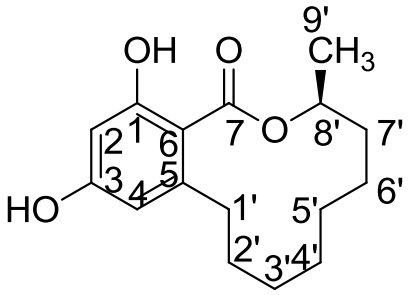
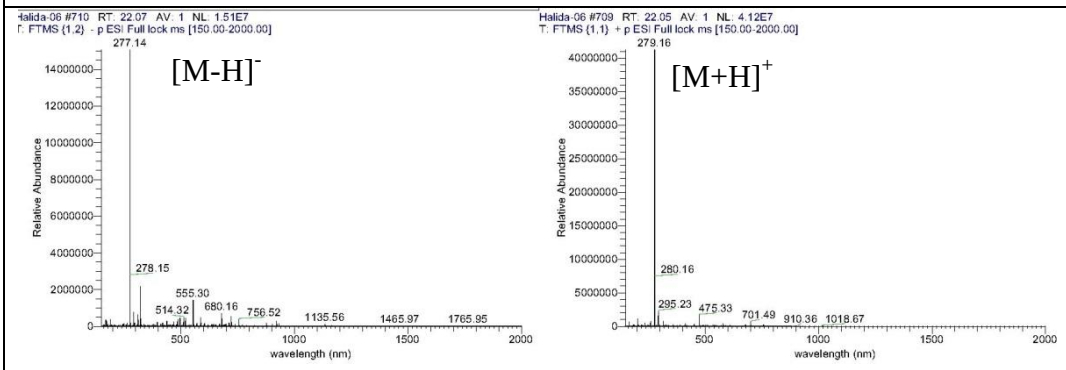


Fig. 3.20: Expansions of the upfield (top) and downfield (bottom) regions of the ^{1}H-^{13}C HMBC spectrum showing the correlations within cladospirone-B

Table 3.9: The ^{1}H and ^{13}C NMR data of cladospirone-B at 400 MHz in Chloroform-*d*[#] and DMSO-*d6**

	Cladospirone-B in Chloroform-*d*[#] and DMSO-*d6**				Literature (Bode *et al.*, 2000) in Acetone-*d6*	
	δ_H (*J* Hz)[#]	δ_H (*J* Hz)*	δ_C*	HMBC*	δ_H (*J* Hz)	δ_C
1			100.9			102.8
2	4.55, t (*J*=3.2)	4.26, m	66.3		4.54, m	66.4
2-OH		5.75, d (*J*=4.0)		1, 2, $3H_a$	5.03, br.	
$3H_a$	2.85, dd (*J*=17.4, 3.5)	2.67, dd (*J*=16.0, 4.0)	43.9	1, 2, 4, 4a	2.74, dd (*J*=17.0, 4.0)	43.0
$3H_b$	3.13, dd (*J*=17.4, 2.9)	3.21, dd (*J*=16.0, 3.0)		2, 3	3.26, dd (*J*=17.0, 3.0*)*	
4			204.0			202.1
4a			116.6			114.5
5-OH	12.25, s	11.97, s	154.8	4a, 5, 6	12.27, s	156.8
6	7.08, d (*J*= 8.5)	7.03, d (*J*=9.0)	121.3	4a, 5, 8,	7.05, d (*J*=9.0)	121.8
7	7.28, d (*J*=8.5)	7.25, d (*J*=9.0)	129.0	5, 8, 8a	7.27, d (*J*=9.0)	129.4
8-OH		9.16, s	150.5	7, 8, 8a	8.08, br.	150.1
8a			120.7			119.3
1’			148.6			146.3
2’	6.99, dd (*J*=7.5, 1.2)	6.99 d (*J*=7.5)	109.8	1’, 4’, 8’a	7.03, dd (*J*=7.5, 1.0)	110.0
3’	7.48, dd (*J*=8.7, 7.5)	7.48, dd (overlap)	128.6	1’, 2’, 3’	7.51, dd (*J*=8.5, 7.5)	128.4
4’	7.58, dd (*J*=8.7, 1.5)	7.57, dd (overlap)	121.1	2’, 4’, 4’a, 8’a	7.61, dd (*J*=8.5, 1.0)	121.7
4’a			134.1			134.9
5’	7.65, dd (*J*=8.7, 1.5)	7.57, dd (overlap)	121.1		7.65, dd (*J*=8.5, 1.0)	121.9
6’	7.51, dd (*J*=8.7, 7.5)	7.53, dd(overlap)	128.6		7.55, dd (*J*=8.5, 7.5)	128.4
7’	7.18, dd (*J*=7.5, 1.2)	7.05, d (*J*=7.8)	110.9	1’, 4’, 8’a	7.16, dd (*J*=7.5, 1.0)	110.8
8’			149.5			148.3
8’a			113.3			

3.2.2.2 Desmethyl-lasiodiplodin

Desmethyl-lasiodiplodin (3.13)
Synonym(s): Des-O-methyllasiodiplodin Source: *Lasiodiplodia theobromae* (isolated from *V. pinnata*) Sample amount: 73 mg Physical description: White amorphous solid Molecular formula: $C_{16}H_{22}O_4$ Molecular weight: 278.343 g/mol Optical rotation: [$^{\alpha}$]D20 = +7.0 ($CHCl_3$)



Compound **3.13** (73 mg) was isolated as an amorphous solid from the methanol extract of *L. theobromae* obtained by a partition process between n-hexane and 90% aqueous methanol. It was found that **3.13** was the major compound in *L. theobromae* with a total yield of 2.4% from 3.0 g of EtOAc extract. The High Resolution (HR) ESI-MS gave the mass ion peaks of 279.1589 $[M+H]^+$ (base peak) and 277.1443 $[M-H]^-$ (base peak) which confirms that the M^+ of compound **3.13** is 278.19 g/mol. From the HRESI-MS data it was suggested that the molecular formula of **3.13** is $C_{16}H_{22}O_4$ with six degrees of

unsaturation. The ^{1}H NMR spectrum showed signals for one pair of *meta*-coupled aromatic protons at δ_H 6.23 (H-4) and 6.28 (H-2). The presence of one oxygenated methine at δ_H 5.15 (H-8') and a methyl doublet at δ_H 1.35 (H-9') were also observed (Fig. 3.21).

From the ^{13}C NMR spectrum, sixteen signals were deduced (Fig. 3.22). In addition to seven methylene signals, one methyl and three methylenes were observed in the Distortionless Enhancement by Polarization Transfer (DEPT) spectrum. Five quaternary carbons were also defined, including an ester carbonyl carbon (δ_C=172.08) and two oxygenated carbons in aromatic system (δ_C=160.68 and δ_C=165.26) (Fig. 3.22). Due to the busy overlapping signals in the aliphatic region of the ^{1}H NMR spectrum, 2D NMR experiments such as Correlation spectroscopy (COSY), Heteronuclear Multiple-Bond Correlation spectroscopy (HMBC) and Homonuclear Multiple Quantum Correlation spectroscopy (HMQC) were applied to elucidate the structure. The COSY spectrum indicated that H-9' is adjacent to H-8', which further correlated to the aliphatic chain of seven methylene units (Fig. 3.23). It was supported by the DEPT spectrum which exhibited the occurrence of seven methylene signals. The HMBC spectrum confirmed the position of H-4 (δ_H = 6.23) by having a ^{3}J correlation with the carbon at position C-1' (δ_C = 33.6) (Fig. 3.25). A comparison of the ^{1}H and ^{13}C NMR (Table 3.10) and mass spectral data with those of desmethyl-lasiodiplodin previously isolated from *Euphorbia fidjiana* (Cambie *et al.*, 1991) confirmed that the two are identical.

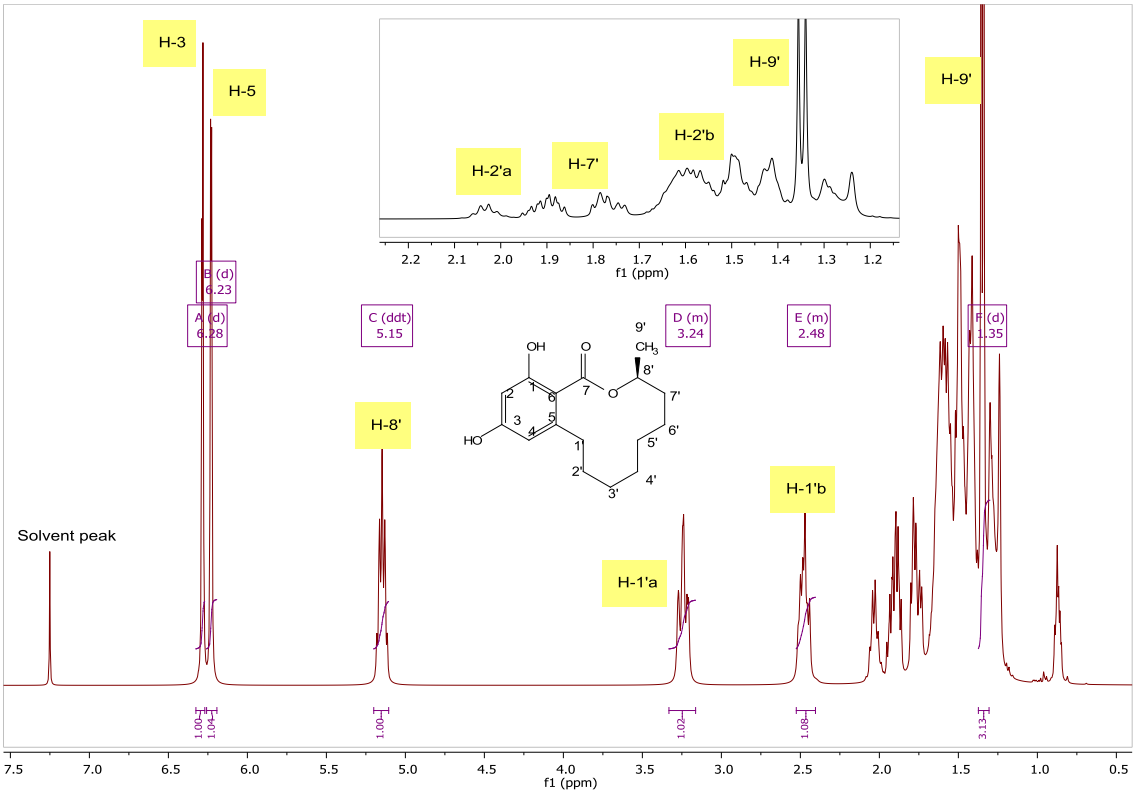


Fig. 3.21: ^{1}H NMR of desmethyl-lasiodiplodin. The chemical shifts, multiplicity and integration of some peaks are shown. The proton peaks at chemical shift δ_H 6.23 and δ_H 6.28 indicate the presence of aromatic protons.

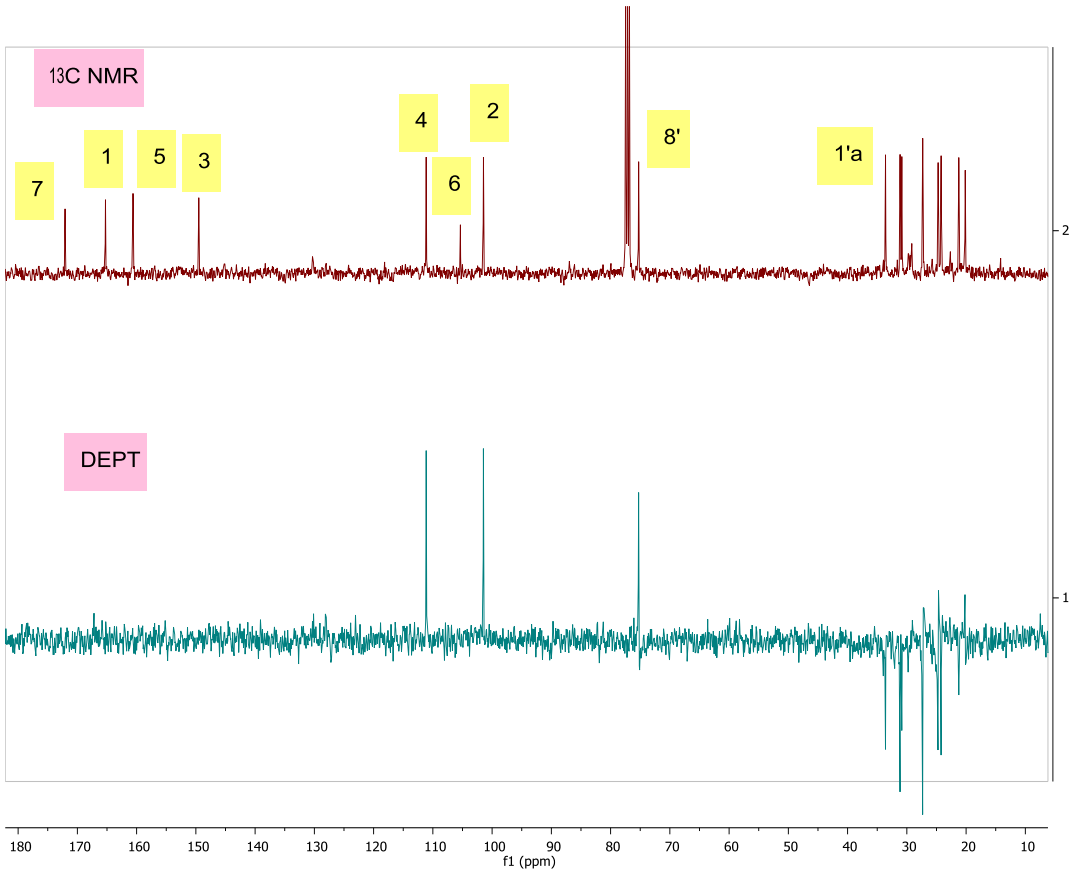


Fig. 3.22: ^{13}C and DEPT NMR of desmethyl-lasiodiplodin. Five quaternary carbons were also defined in ^{13}C NMR spectrum.

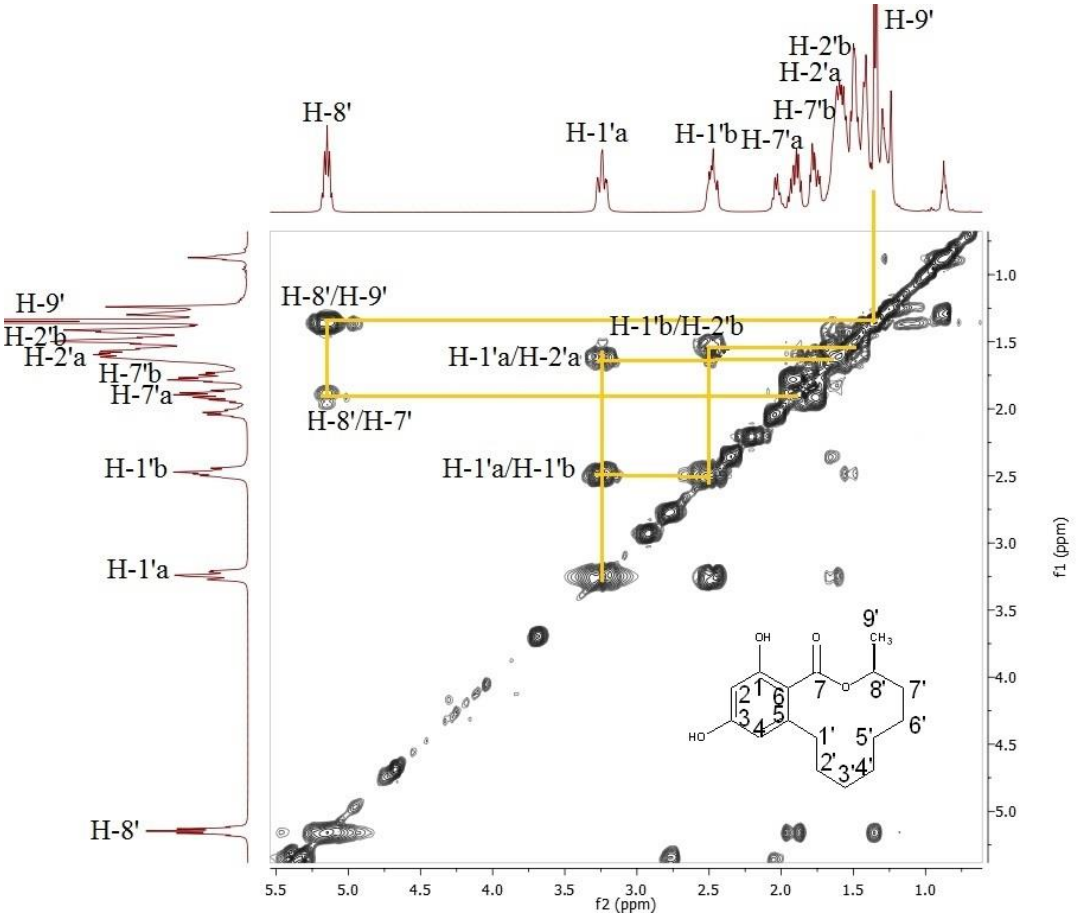


Fig. 3.23: The ^{1}H-^{1}H COSY spectrum (expanded) of desmethyl-lasiodiplodin

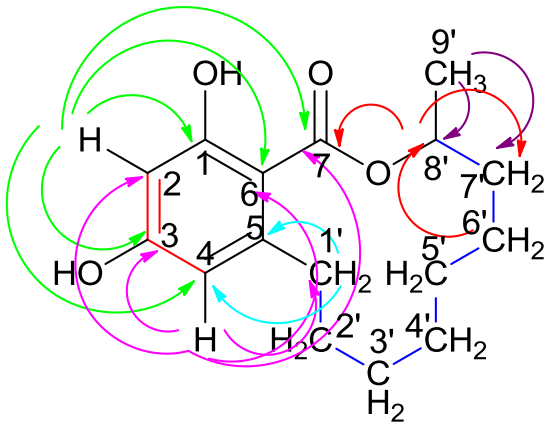


Fig. 3.24: The ^{1}H-^{1}H COSY correlations of compound desmethyl-lasiodiplodin are depicted by the bold lines and the ^{1}H-^{13}C HMBC correlations are indicated by the colourful arrows.

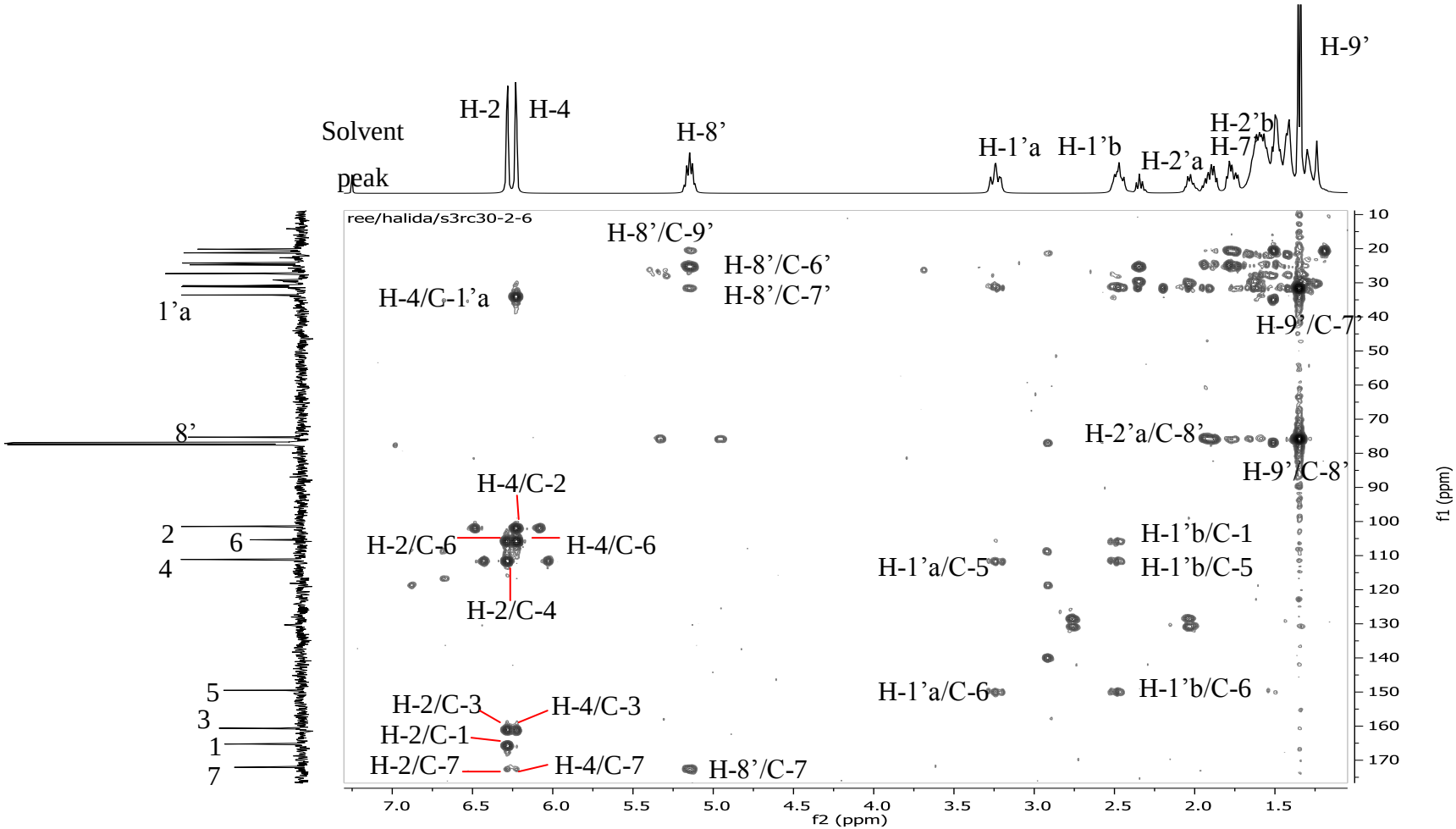
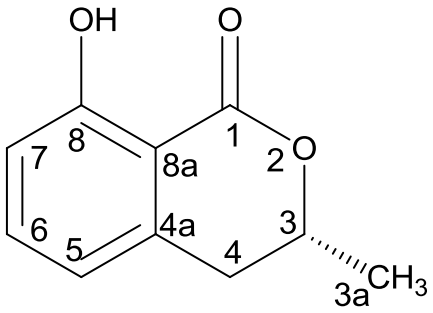
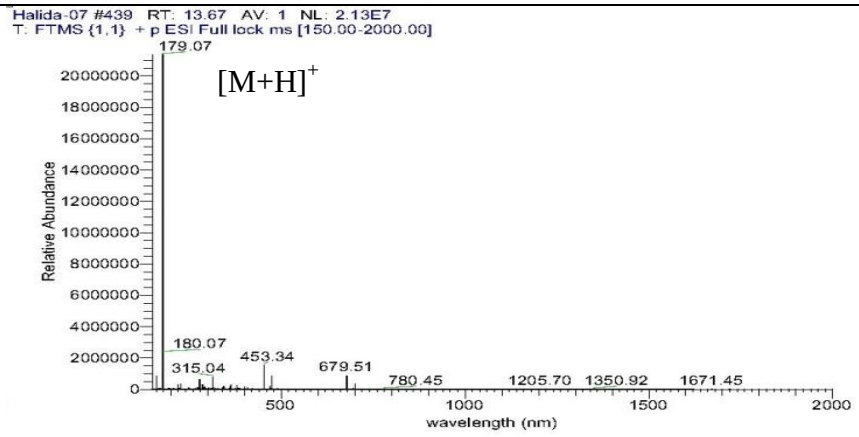


Fig. 3.25: The ^{1}H-^{13}C HMBC NMR spectrum of compound desmethyl-lasiodiplodin

Table 3.10: The ^{1}H, ^{13}C and HMBC NMR data of desmethyl-lasiodiplodin at 400 MHz in Chloroform-*d*

Position	Desmethyl-lasiodiplodin in Chloroform-*d*			Literature (Cambie *et al.*, 1991) in D_2O	
	δ_H (*J* Hz)	δ_C	HMBC	δ_H (J Hz)	δ_C
1-OH		160.6			
2	6.28, d (*J*=2.7)	101.5	1, 5, 2, 4, 7	6.27, d	101.3
3-OH		165.3			159.9
4	6.23, d (*J*=2.7)	111.1	1, 1'a, 2, 3, 7	6.22, d	110.6
5		149.5			149.4
6		105.4			
7		172.1			172.0
1'a	3.27, m	33.6	1, 5, 6', 7'	3.30, m	33.5
1'b	2.48, m		5, 6', 7'	2.50, m	
2'a	1.93, m				
2'b	1.61, m				
3'					
4'					
5'					
6'					
7'	1.75, m	21.2		1.79, m	21.1
	1.88, m			1.92, m	
8'	5.15, ddt (*J*=10.6, 6.5, 3.2)	75.3	6', 7, 7', 9	5.17, m	75.1
9'	1.35, d (*J*=6.2)	20.2	7', 8'	1.36, d	20.1

3.2.2.3 R-(-)-mellein (known compound)

R-(-)-mellein (3.14)
Synonym(s): (-)-3,4-Dihydro-8-hydroxy-3-methylisocoumarin Source: *L. theobromae* (isolated from *V. pinnata)* Sample amount: 11.0 mg Physical description: White amorphous solid Molecular formula: $C_9H_{10}O_3$ Molecular weight: 178.185 g/mol Optical rotation: $[\alpha]_D^{20}$ = -119 ($CHCl_3$)



Compound **3.14,** a white amorphous solid, was purified from the non-polar fraction of the methanol extract of *L. theobromae*. The total yield was 11.0 mg (0.36%). The HRESI-MS in the positive mode showed a molecular ion peak at *m/z* 179.0705 $[M+H]^+$ (base peak), providing the molecular formula $C_9H_{10}O_3$. The ^1H NMR spectrum showed signals for three aromatic protons corresponding to a 1, 2, 3-trisubstituted benzene [δ_H

7.39 (H-6), 6.88 (H-7) and 6.68 (H-5)]. The singlet at δ 11.01 was assigned to the hydroxyl group attached to position C-8 of the benzene ring (Fig. 3.26). The TOCSY spectrum showed another spin system with three signals correlating to each other. These included an oxygenated methine multiplet at δ_H 4.73 (H-3), a methyl doublet at δ_H 1.52 (H-3a) and a methylene doublet at δ_H 2.93 (H-4) (Fig. 3.27) (see Appendix I for COSY spectrum). Six C-H direct signals and four quaternary carbons were found in the HMBC spectrum, including an ester carbonyl carbon at δ_C 170.3 and one aromatic hydroxylated carbon at δ_C 162.8 (Fig. 3.29). Comparison of the ^{1}H NMR data of **3.14** (Table 3.11) with that of R-(-)-mellein, together with the HRESI-MS and the optical rotation (compound **3.14**: $[\alpha]_D^{20}$ = -119.0, R-(-)-mellein: $[\alpha]_D^{25}$ = -102.000) (Schulz *et al.*, 1995) determined that they are the same compound. This compound was previously isolated from endophytic *Pezicula* species (Schulz *et al.*, 1995).

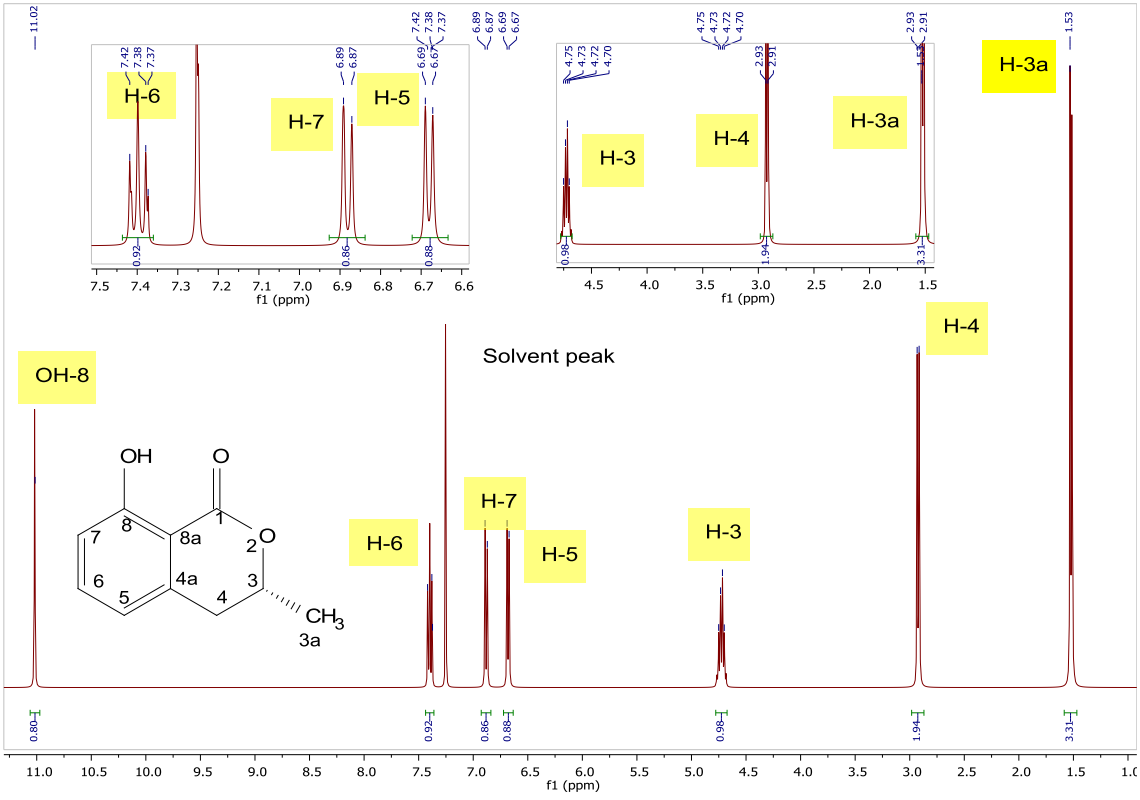


Fig. 3.26: The ^{1}H NMR spectrum of R-(-)-mellein revealed the proton integration and multiplicity of each peak

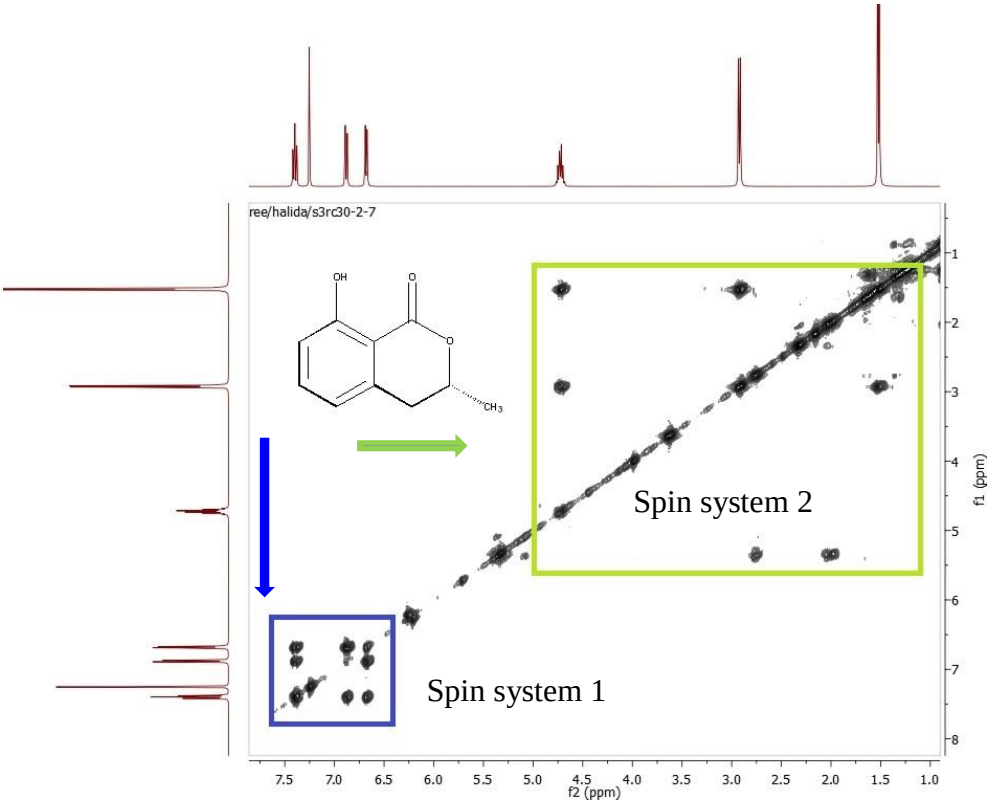


Fig. 3.27: The TOCSY correlation data of R-(-)-mellein showed that it has two spin systems

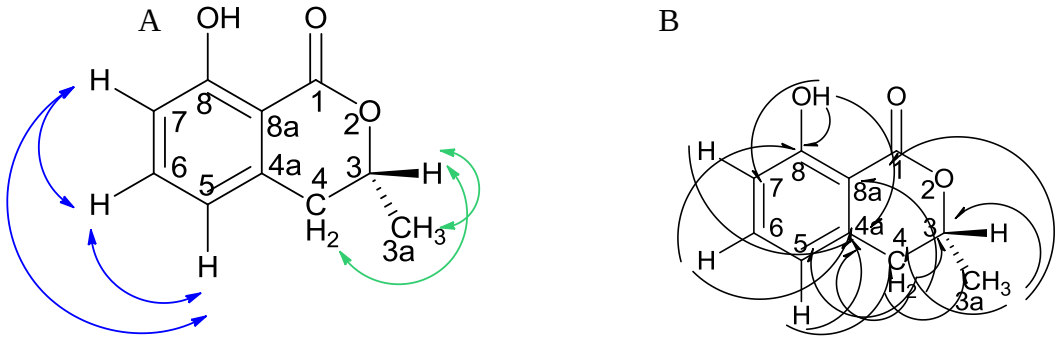


Fig. 3.28: A) The ^{1}H-^{1}H COSY correlation and B) The ^{1}H-^{13}C HMBC correlation for R-(-)-mellein

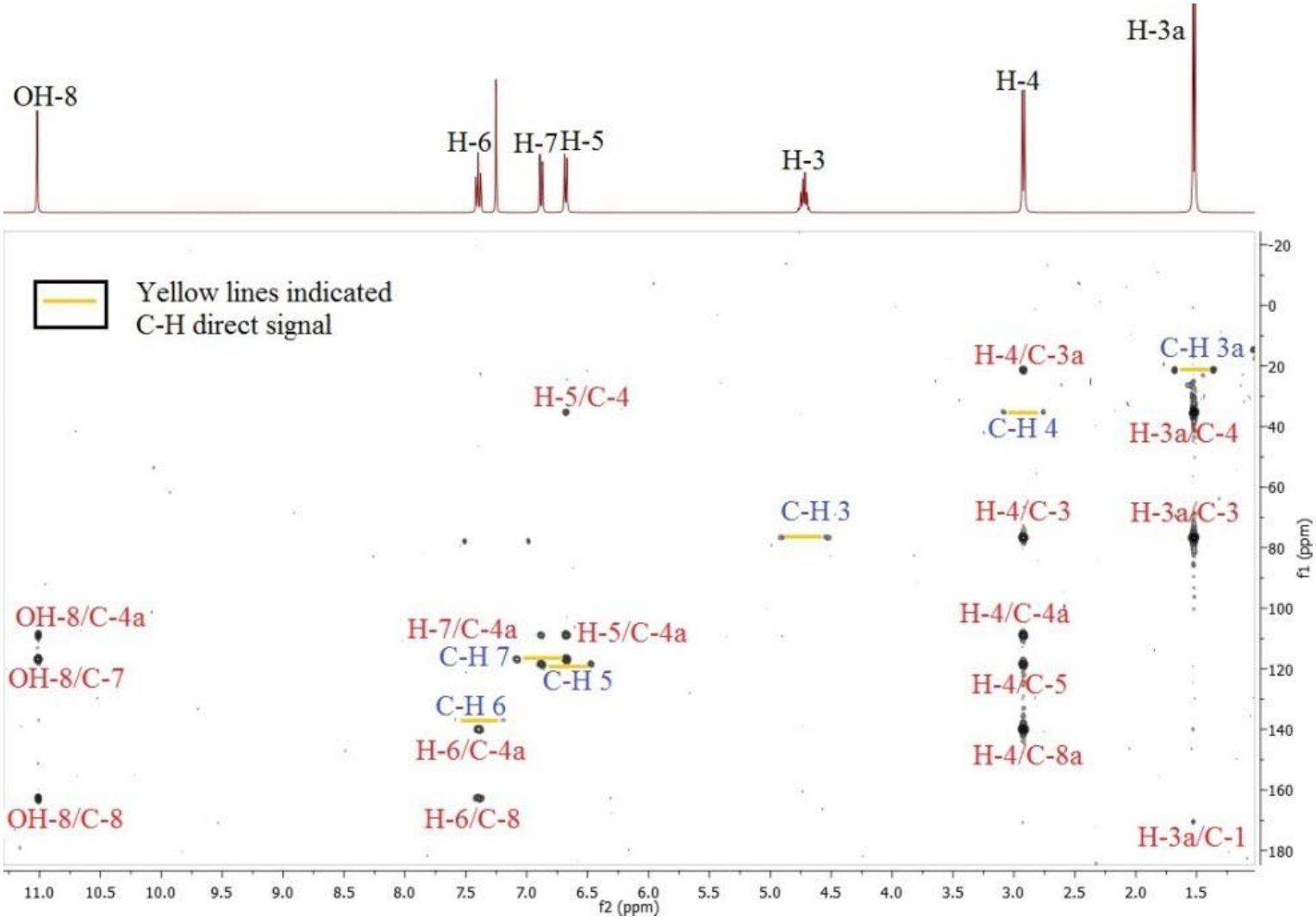
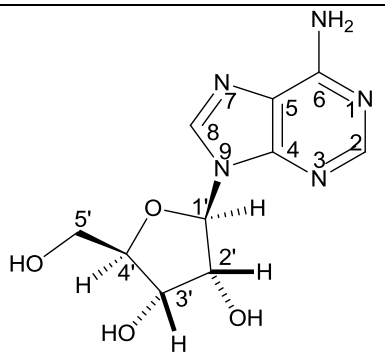
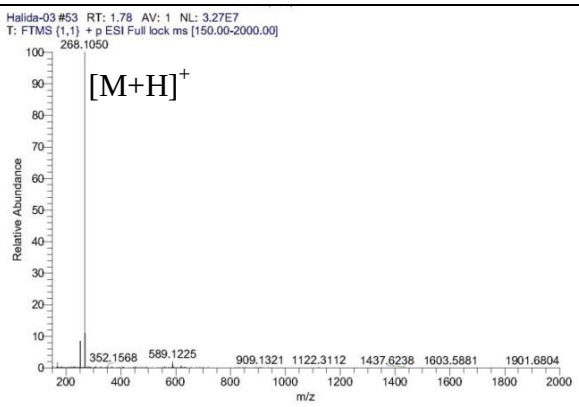


Fig. 3.29: The HMBC correlations for R-(-)-mellein. Yellow lines indicate direct C-H coupling

Table 3.11: ^{1}H, ^{13}C and HMBC NMR data of R-(-)-mellein at 400 MHz in Chloroform-*d*

Position	R-(-)-mellein in Chloroform-*d*			Literature (Schulz *et al.*, 1995) in Chloroform-*d*	
	δ_H (*J* Hz)	δ_C	HMBC	δ_H (*J* Hz)	δ_C
1		170.3			169.96
3	4.73, m	77.9		4.73, m	76.11
4	2.93, d (*J*=7.3)	35.2	3, 3a, 4a, 5, 8a	2.93, d (*J*=7.3)	34.56
5	6.68, dd (*J*=7.4, 1.0)	118.5	4, 4a	6.69, dd (*J*=7.4, 0.8)	117.92
6	7.39, dd (*J*=7.4, 8.4)	136.9	4a, 8	7.41, dd (*J*=8.0)	136.13
7	6.88, dd (*J*=8.4, 1.1)	116.9	4a	6.89, d (*J*=8.1)	116.18
8-OH	11.01, s	162.8	4a, 7, 8	11.04, s	162.15
4a		108.8			108.29
8a		139.9			139.41
3a	1.52, d (*J*=6.3)	21.36	1, 3, 4	1.53, d (*J*=6.3)	20.75

3.2.2.4 Adenosine (known compound)

Adenosine (3.15)
Synonym(s): Adenine ribonucleoside; 9-*β*-D-ribofuranosyl adenine Source: *L. theobromae* (isolated from *V. pinnata)* Sample amount: 3 mg Physical description: White powder Molecular formula: $C_{10}H_{13}N_5O_4$ Molecular weight: 267.241 g/mol



Compound **3.15** was obtained as a white powder with a molecular ion peak at *m/z* 268.1060 $[M+H]^+$ in the positive mode by HR-ESIMS, indicating that the molecular formula of compound **3.15** is $C_{10}H_{13}N_5O_4$ with seven degree of unsaturation. The odd-numbered molecular weight of 267 denoted the presence of at least one nitrogen in the structure of **3.15** according to the nitrogen rule; this agreed with the determined molecular formula. The ^{1}H NMR spectrum (400MHz, DMSO-*d6*) showed one broad

singlet at δ_H 7.35 that was assigned to two protons of the amine group attached to the aromatic ring (Fig. 3.30). In addition, two singlet peaks at δ_H 8.35 and 8.13 were directly coupled with carbon peaks at δ_C 140.86 and 153.3, respectively, as determined by HMQC. These were characteristic resonances corresponding to an adenine moiety (Fig. 3.31). Furthermore, the ^{1}H NMR spectrum showed a doublet signal at δ_H 5.87 for the anomeric proton of a ribofuranose unit, and a coupling constant of 6.2 Hz for the anomeric proton indicated its *β*-configuration (Fig. 3.30). In the HMQC spectrum (Fig. 3.31), δ_C 87.9 (C-1') and δ_C 85.9 (C-4') correlated to the proton resonance at δ_H 5.87 and δ_H 3.96, respectively. The proton signals at δ_H 4.14 and 4.61 were assigned to H-2' and H-3', respectively (Fig. 3.30). Proton signals at δ_H = 3.55 and 3.67 were assigned to CH_2-5' with a geminal coupling constant of 12 Hz. This was confirmed in the HMQC spectrum where both protons correlated to one carbon signal at δ_C 61.6. The exchangeable signals at δ_H 5.44, 5.18, and 5.43 correlated with the hydroxylated methine carbons for positions 2', 3' and 5' (Fig. 3.30). The exchangeable proton signals were confirmed by the absence of HMBC correlations (Fig. 3.32). Based on mass spectral, 1D and 2D NMR data, and in comparison to the published data, it was confirmed that **3.15** is adenosine (Ciuffreda *et al.*, 2007).

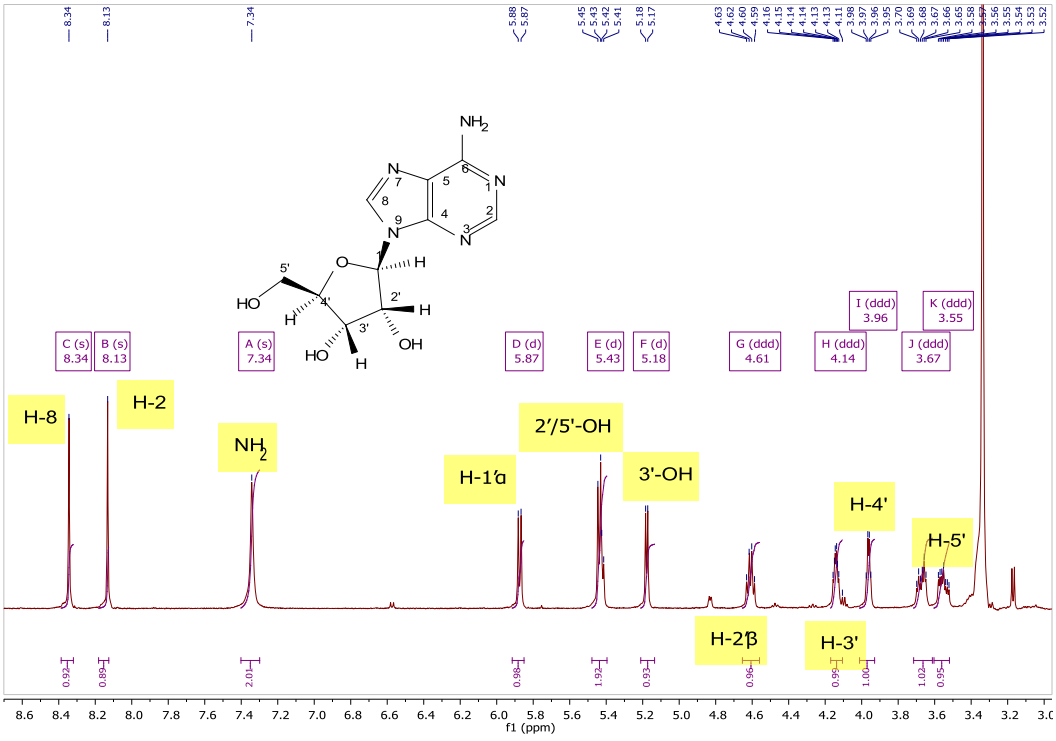


Fig. 3.30: The ^{1}H NMR spectrum of adenosine revealed the proton integration and multiplicity of each peak.

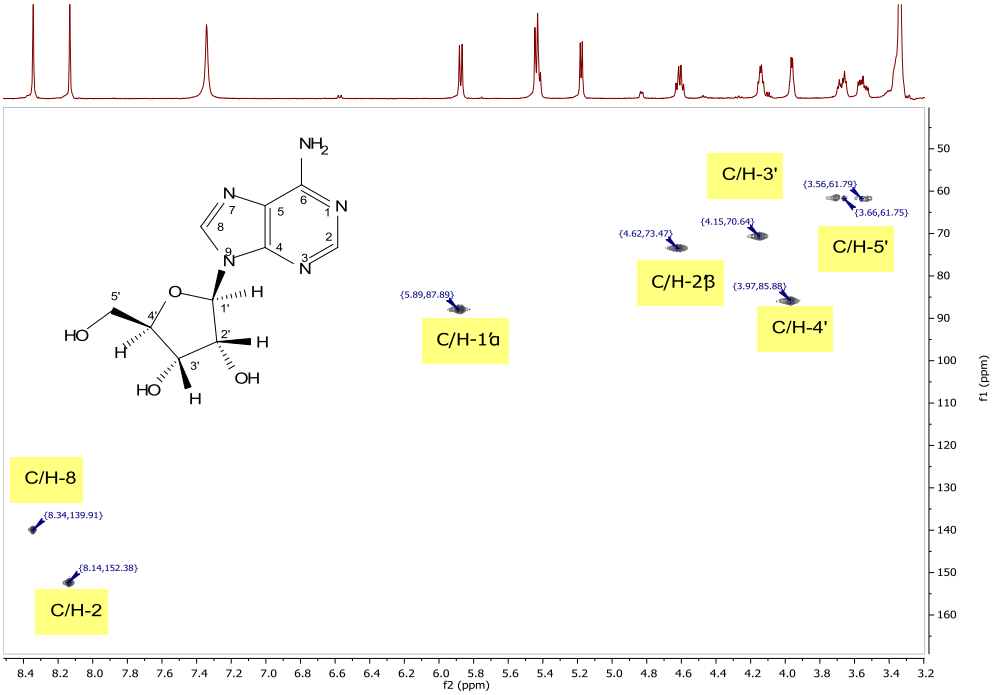


Fig. 3.31: The HMQC correlations of adenosine measured at 400 MHz in DMSO-*d6*.

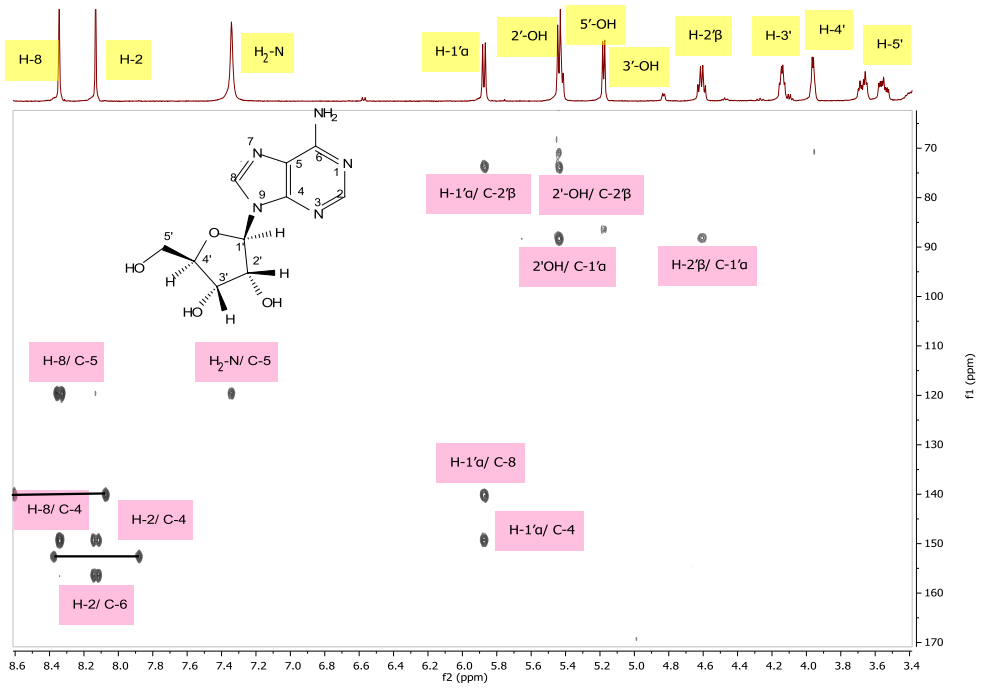


Fig. 3.32: The HMBC correlations of adenosine measured at 400 MHz in DMSO-*d6*.

Table 3.12: ^{1}H and ^{13}C NMR data of adenosine at 400 MHz in DMSO-*d6*

Position	Adenosine in DMSO-*d6*		Literature(Ciuffreda *et al*., 2007) in DMSO-*d6*	
	δ_H (*J* Hz)	δ_C	δ_H (*J* Hz)	δ_C
2	8.13, s	152.4	8.13, s	152.4
4		149.0		149.0
5		119.3		119.3
6		156.1		156.1
8	8.34, s	139.9	8.34, s	139.9
NH_2	7.34, bs		7.33, bs	
1'α	5.87, d (*J*=6.2)	87.9	5.87, d (*J*=6.2)	87.9
2'β	4.61, ddd (*J*=5.1, 6.2, 6.1)	73.4	4.61, ddd (*J*=5.1, 6.2, 6.3)	73.4
3'	4.14, ddd (*J*=3.0, 4.6, 5.1)	70.6	4.14, ddd (*J*=3.0, 4.6, 5.1)	70.6
4'	3.96, ddd (*J*=3.0, 3.6, 3.7)	85.9	3.96, ddd (*J*=3.0, 3.6, 3.7)	85.9
5'a	3.67, ddd (*J*=3.6, 4.4, 11.8)	61.6	3.67, ddd (*J*=3.6, 4.4, 12.1)	61.6
5'b	3.55, ddd (*J*=11.8, 7.2, 3.6)		3.55, ddd (*J*=3.7, 7.2, 12.1)	
2'-OH	5.43, d (*J*=6.1)		5.43, d (*J*=6.3)	
3'OH	5.17, d (*J*=4.6)		5.17, d (*J*=4.6)	
5'OH	5.41, d (overlap)		5.41, dd (*J*=4.4, 7.2)	

3.3 *Nigrospora* sp

3.3.1 Metabolomics-bioassay guided screening as a approach to prioritize the most optimal condition of culture of *Nigrospora* sp for medium-scale fermentation

Same strategy on *L. theobromae* (Chapter 3.2.2.1) was applied on the exploratory study to search the most optimal cultivation of *Nigrospora* sp. Bioassays on extract of *Nigrospora* sp revealed this extract didn't show any strong bioactivities against *T. brucei brucei* and *M. marinum* (Table 3.13). Therefore the decision making step was focused on the biomass yield (Table 3.14), scatter plot of MS data (Fig. 3.33) and ^{1}H NMR data (Fig. 3.34). ^{1}H NMR data of liquid medium showed the culture in thirty days produced interesting compounds with more peak signals appearing in aromatic region in comparison to other incubation periods (Fig. 3.34a). In terms of extract yield of liquid culture, there is no significant difference between incubation periods (Table 3.14). Thus, the best incubation time for *Nigrospora* sp in liquid culture is in thirty days.

On the other hand, the ^{1}H NMR spectrum of the solid rice medium showed strong interesting peak signals at 5.5-6.5 ppm region indicating the presence olefinic functional groups especially in seven and thirty days (Fig. 3.34b). However, based on the MS scatter plot (Fig. 3.33b), comparison between the solid rice culture in seven and thirty days showed that more ion peaks were detected in thirty days. Comparison of the MS scatter plot between the fifteen and thirty days showed that these two incubation periods shared similar type of compounds but the total weight of extracts in thirty days 30% more than that of the fifteen days. Therefore the best incubation period for the rice culture was thirty days.

To choose between liquid and solid rice medium, both cultivated in thirty days, as the most ideal condition for medium scale fermentation, a MS scatter plot comparison was done. The MS scatter plot deduced that more ion peaks were detected from the rice culture medium than from the liquid medium. In addition to that, the total biomass yield from the rice culture is higher than from the liquid culture. Therefore based on all these

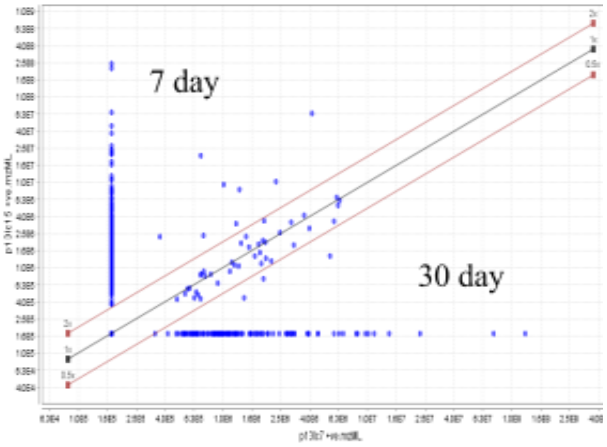
data, *Nigrospora* sp culture was optimized in the thirty days rice culture for scale up fermentation.

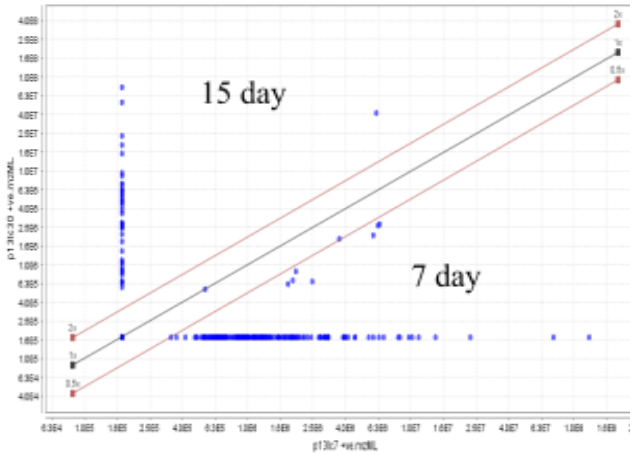
Table 3.13: Antitrypanosomal and anti-mycobacterium activity of *Nigrospora* sp extracts derived from *V. pinnata* in different types of media and incubation periods. MIC was only detected for the bioactive extracts.

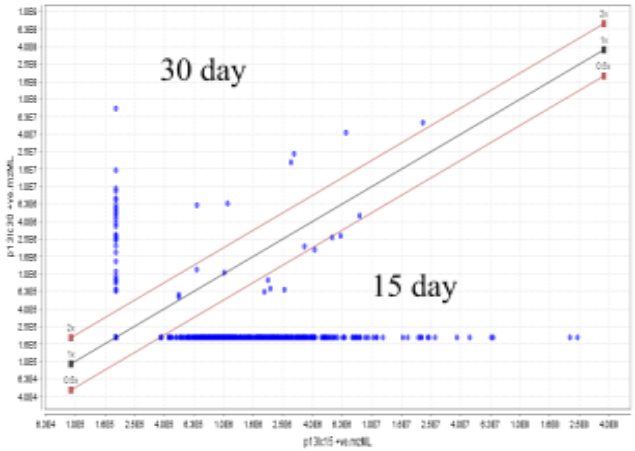
Sample	*T. brucei brucei* 20ug/ml % D control	*T. brucei brucei* MIC (µg/ml)	*M. marinum* ATCC BAA 535 100µg/ml % D control	*M. marinum* ATCC BAA 535 MIC (µg/ml)
N-LC-7	44.6	Not tested	43.3	Not tested
N-LC-15	50.1	Not tested	64.3	Not tested
N-LC30	67.4	Not tested	45.2	Not tested
N-RC-7	96.9	Not tested	26.2	Not tested
N-RC-15	91.2	Not tested	16.2	Not tested
N-RC30	90.5	Not tested	14.2	Not tested

*N-*Nigrospor*a sp

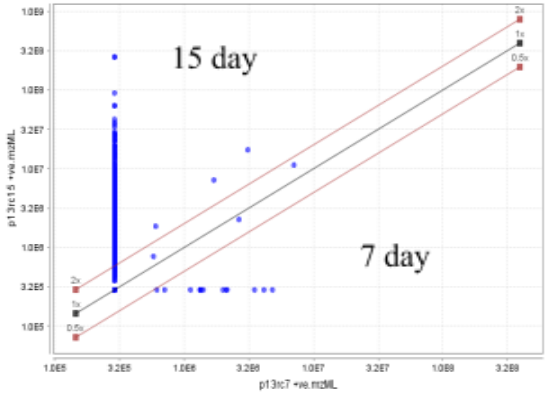
A1

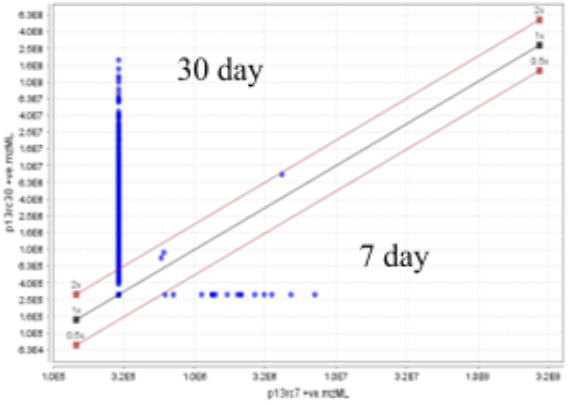

7 day
30 day

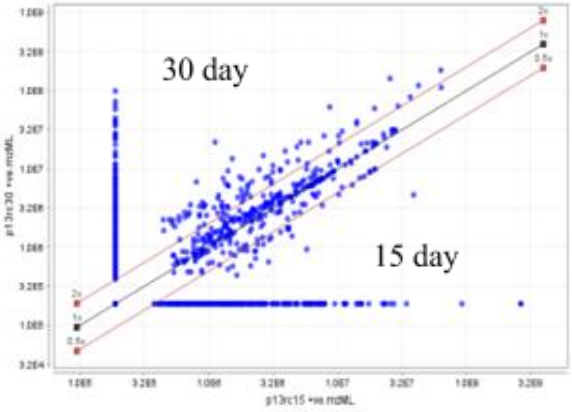

15 day
7 day


30 day
15 day

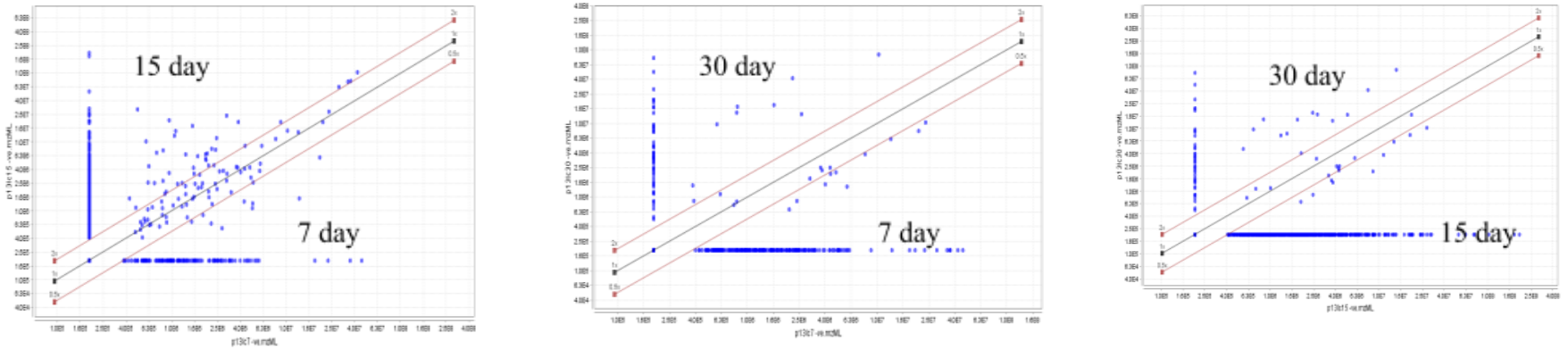
B1


15 day
7 day


30 day
7 day


30 day
15 day

A2



B2

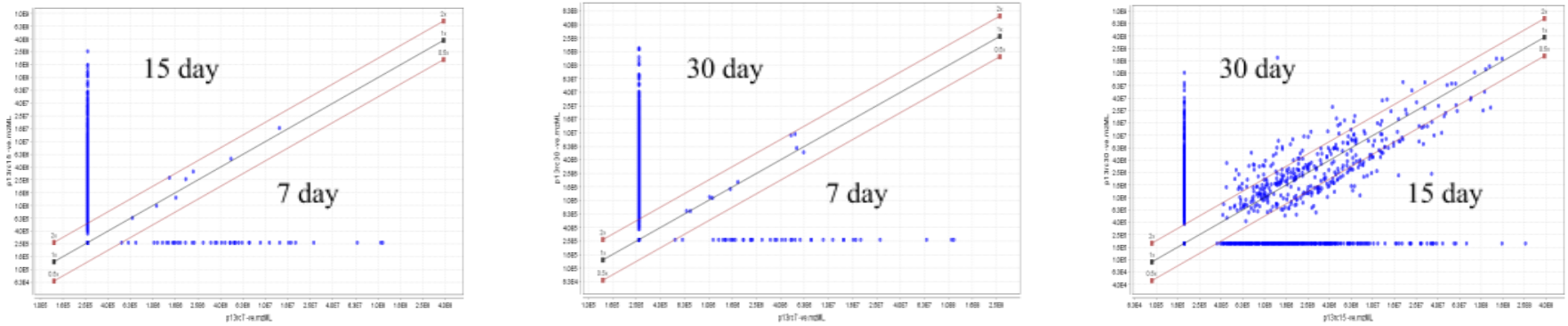
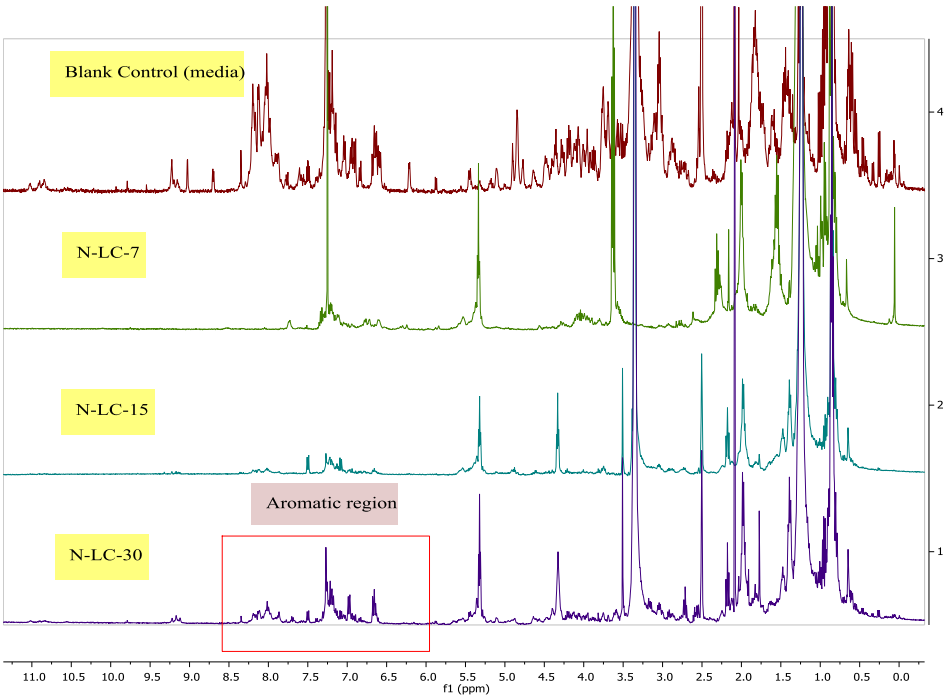


Fig. 3.33: MS scatter plot of *Nigrospora* sp extracts obtained from A1) Wickerham liquid culture (positive ionization); A2) Wickerham liquid culture (negative ionization); B1) solid rice culture (positive ionization); and B2) solid rice culture (negative ionization)

A



B

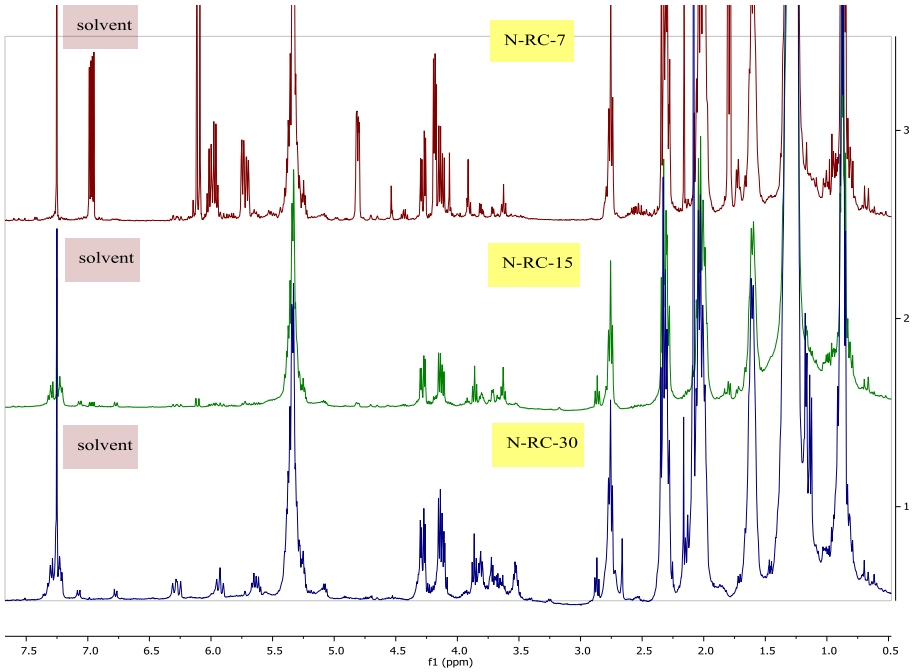
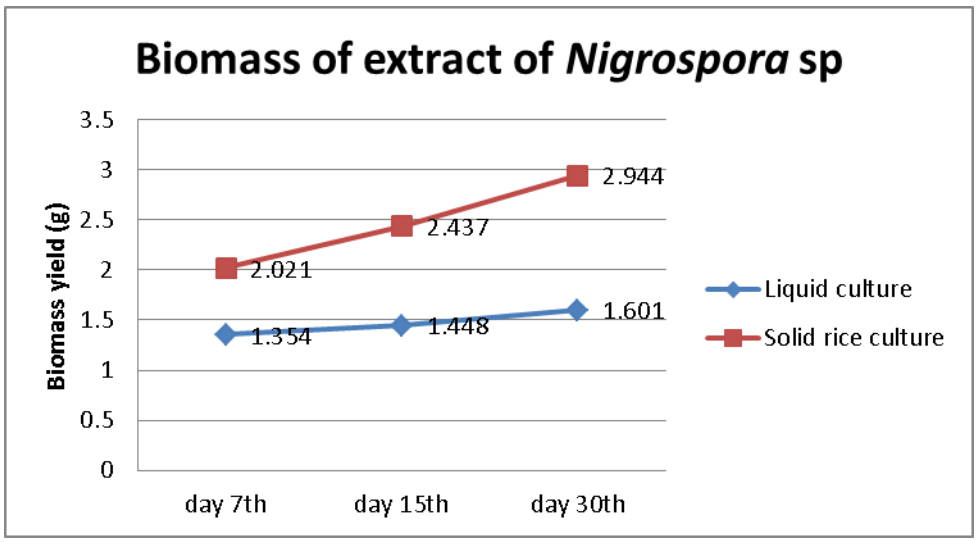


Fig. 3.34: The ^{1}H NMR spectrum of *Nigrospora* sp extracts obtained from A) liquid Wickerham and B) solid rice culture medium

Table 3.14: The total biomass yield (g) of *Nigropsora* sp extracts obtained from the Wickerham liquid and solid rice cultures grown in 500 mL flasks at different incubation periods



3.3.2 Secondary metabolites isolated from *Nigrospora* sp and their bioactivities

The endophytic fungus, *Nigrospora* sp, was isolated from the petiole of a healthy leaf of *V. pinnata*. Based on the metabolomic analysis of the optimization of the fermentation process, *Nigrospora* sp was fermented in rice culture medium for seven and thirty days and later was extracted with ethyl acetate. The NMR and LCMS spectral data revealed that the extract of *Nigrospora* sp in rice culture for seven days produced phomalactone **3.16** as the major compound. In contrast, the extract of *Nigrospora* sp cultured in thirty days produced musacin E **3.17** as the major compound, together with 2- phenyl ethanol **3.18**, tyrosol **3.19**, nigrosporone *β*-glucoside **3.20 (new)** and adenosine **3.15**.

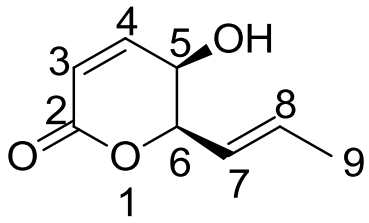
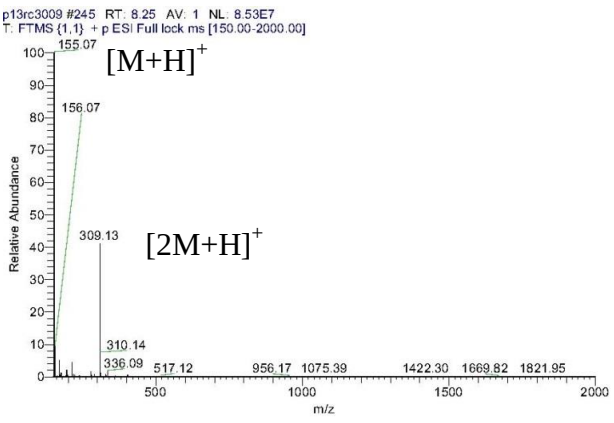
3.3.2.1 Bioactivities of secondary metabolites isolated from *Nigrospora* sp

Table 3.15: Antitrypanosomal and anti-mycobacterial activities of isolated compounds obtained from *Nigrospora* sp. fermented in solid rice culture for seven and thirty days.

Compound	**Antitrypanosomal activity** ***(T. brucei brucei)***	**Anti-mycobacterial activity** **(*M. marinum*)**
	MIC (μM)	**MIC (μM)**
Phomalactone **3.16**	40.5	80.0
Musacin E **3.17**	80.0	80.0
2- phenyl ethanol **3.18**	> 100	> 100
Tyrosol **3.19**	> 100	> 100
Nigrosporone *β*-glucoside **3.20**	48.5	> 100
Suramin	0.1	
Gentamycin		13.5

Of the five isolated compounds, phomalactone, nigrosporone *β*-glucoside and musacin E showed moderate and weak activities against *T. brucei brucei*, respectively, whereas no activity was detected for the remaining metabolites. For anti-mycobacterial activity, most compounds were inactive except phomalactone and musacin E which exhibited weak activity against *M. marinum*.

3.3.2.2 Phomalactone (known compound)

Phomalactone (3.16)
Synonym(s): (5R,6R)-5-hydroxy-6-((E)-prop-1-en-1-yl)-5,6-dihydro-2H-pyran-2-one Source: *Nigrospora* sp (isolated from *V. pinnata)* Sample amount: 35 mg Physical description: Colourless oil Molecular formula: $C_8H_{10}O_3$ Molecular weight: 154.163 g/mol Optical rotation: $[\alpha]D^{20}$ = +72.5 (MeOH)



Compound **3.16** was isolated as colourless oil with a total yield of 30 mg. The HR-LCMS spectral data showed a molecular ion peak in the positive mode at 155.0706 $[M+H]^+$ (base peak), giving a molecular formula of $C_8H_{10}O_3$. The ^1H NMR spectrum showed the presence of one methyl doublet signal at δ_H 1.80 and four olefinic proton signals, suggesting that two double bonds were present in the molecule (Fig. 3.35). In

the COSY spectrum (Fig. 3.36), a ^{1}H-^{1}H correlation was observed between the methyl doublet peak at δ_H 1.80 (H-9) and an olefinic multiplet at δ_H 5.99 (H-8), and the correlation continued with another olefinic doublet of doublet of quartet at δ_H 5.71 (H-7). The signal at H-7 then connected to a doublet of doublet at δ_H 4.81 (H-6). This proton was attached to a carbonyl group, which explained why the chemical shift was more downfield. An olefinic signal at δ_H 6.97 (H-4) showed a dual correlation to another olefinic proton signal at δ_H 6.12 (H-3) and also to a methine doublet of doublet signal at δ_H 4.18 (H-5) which also coupled with H-6. The carbon and DEPT NMR spectra (Fig. 3.37) displayed eight carbon signals, including one methyl, six methines, and one quaternary carbonyl. In addition HMBC experiments were conducted to determine long-range C-H correlations, respectively. Direct C-H correlation was deduced from the HMQC data (Appendix II). These correlations can be seen in Fig. 3.38 and Fig. 3.39. Based on ^{1}H and ^{13}C NMR, mass spectral data, and the comparison of these with literature, compound **3.16** was confirmed to be phomalactone previously isolated from entomopathogenic fungus *Hirsutella thompsonii* var.synnematosa (Fukushima *et al.*, 1998; Krasnoff and Gupta, 1994).

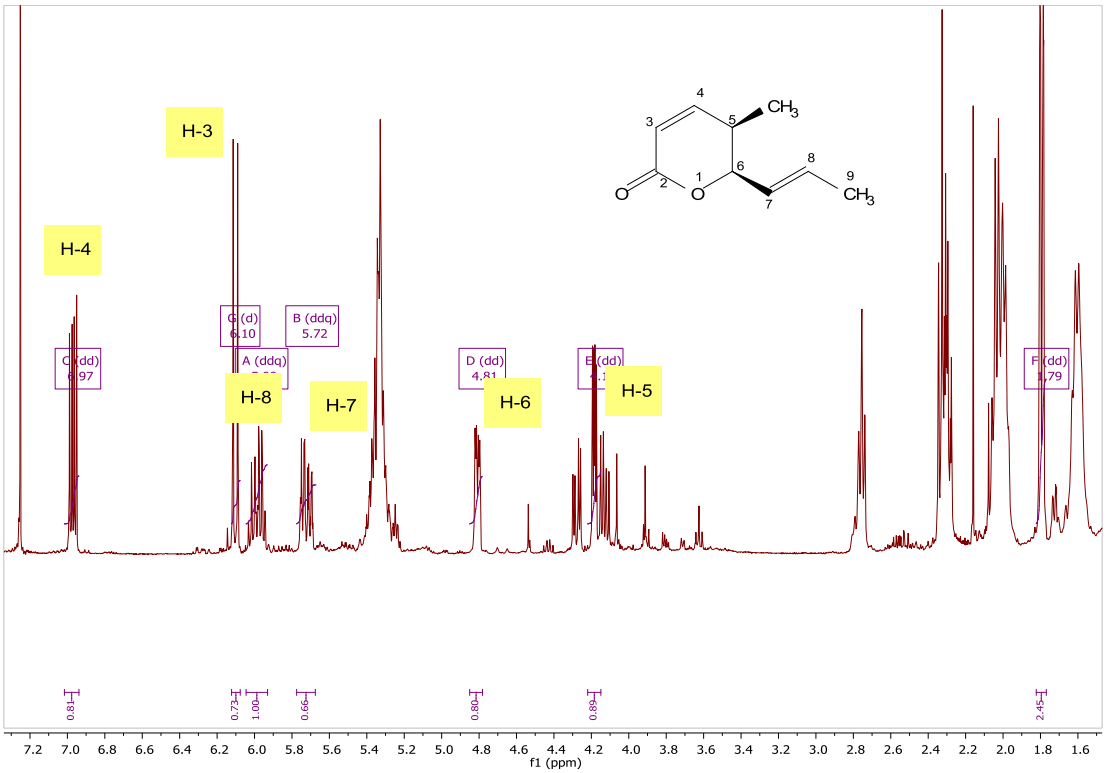


Fig. 3.35: ^{1}H NMR of phomalactone in the semi-pure extract, which contained fatty acids as impurities

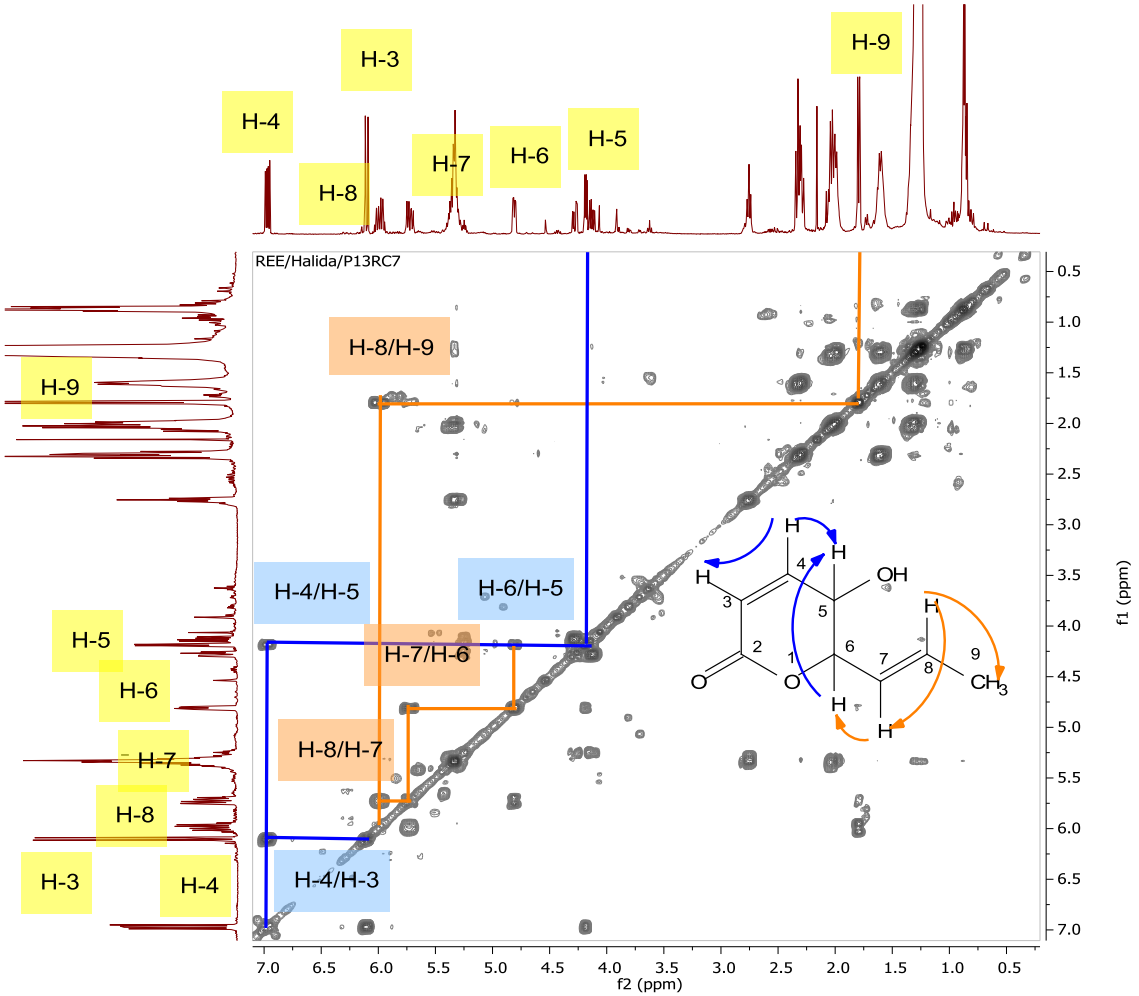


Fig. 3.36: ^{1}H-^{1}H COSY spectrum of phomalactone.

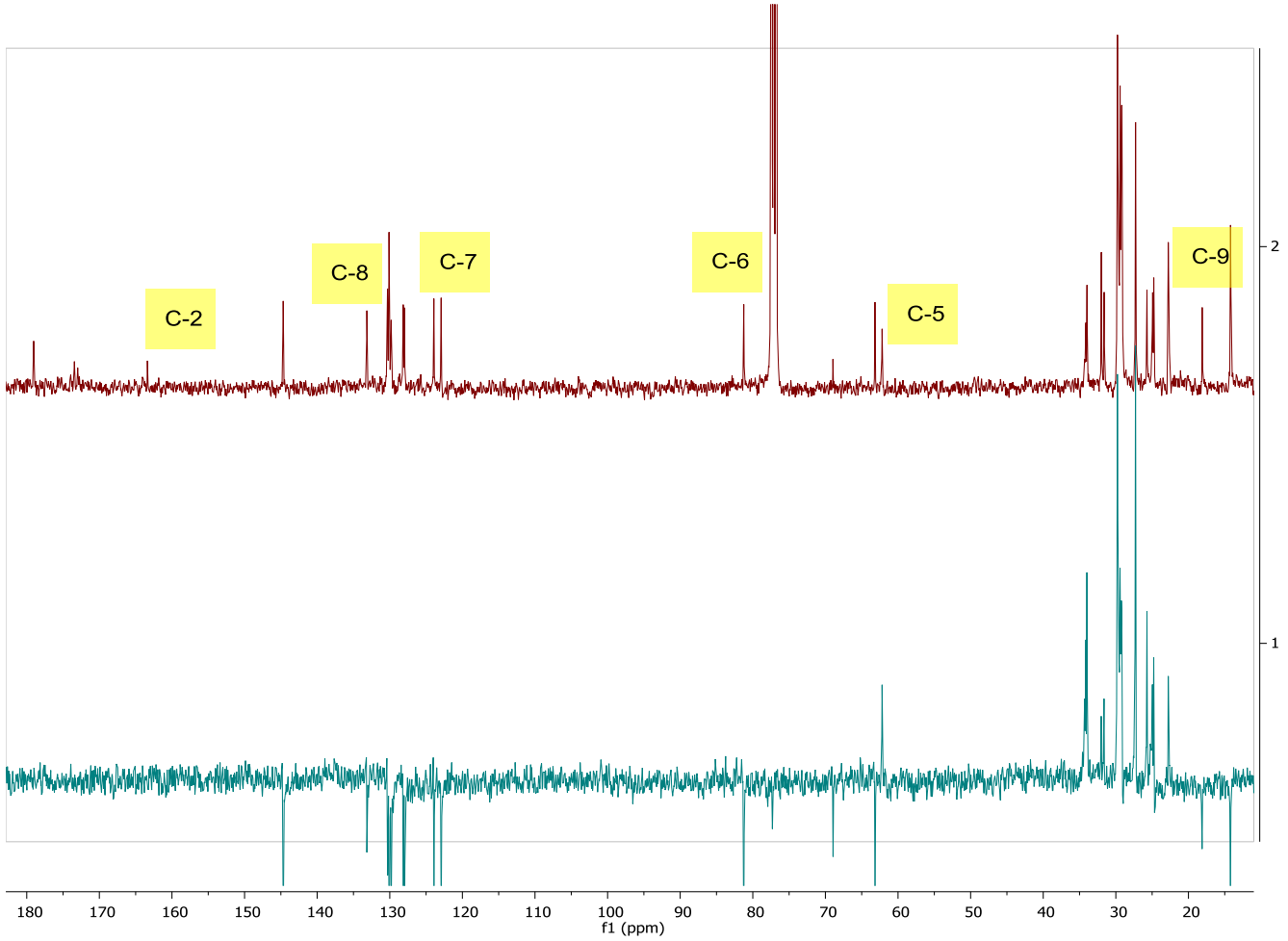


Fig. 3.37: ^{13}C and DEPT NMR spectrum of phomalactone

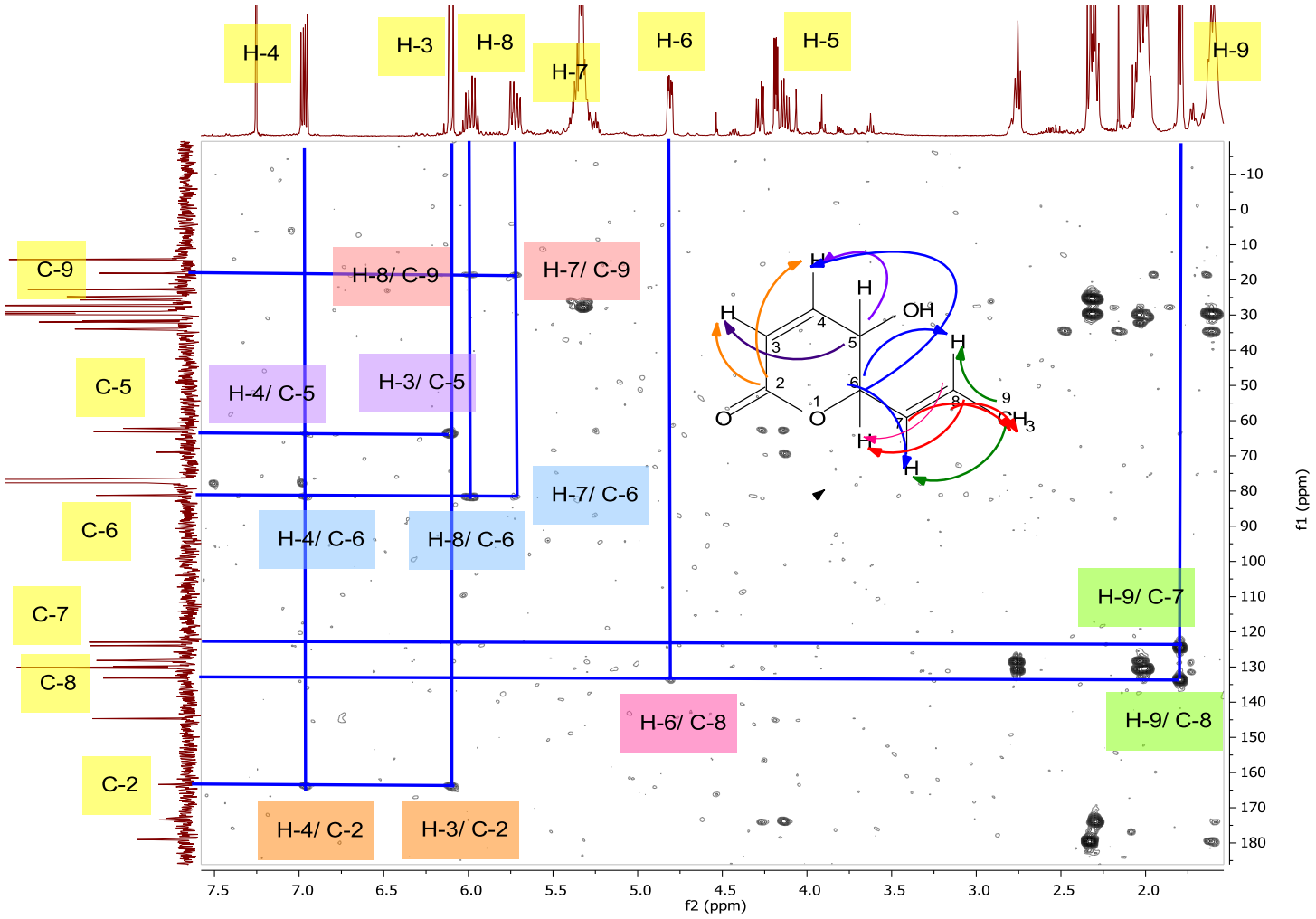


Fig. 3.38: HMBC spectrum of phomalactone

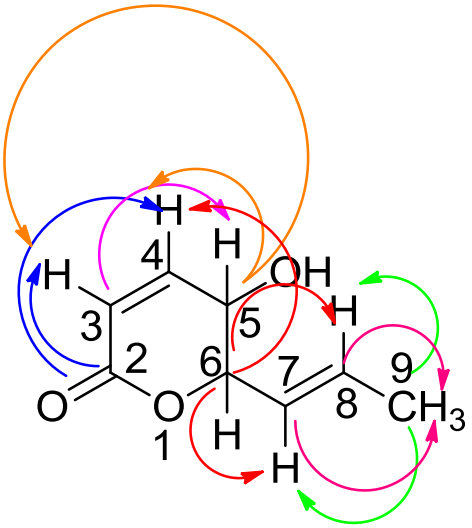
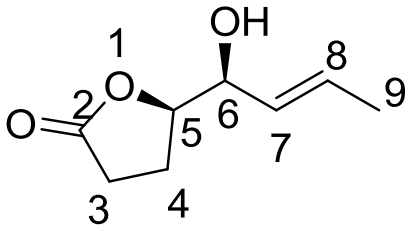
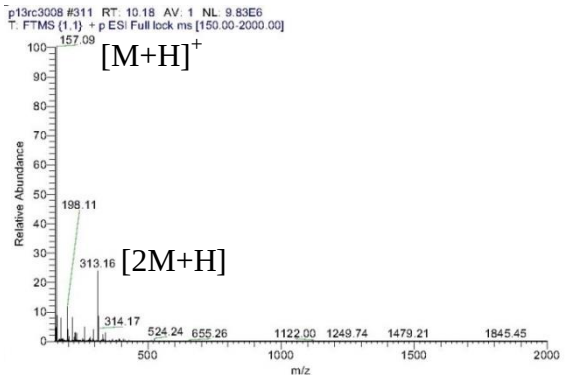


Fig. 3.39: The HMBC correlations of phomalactone

Table 3.16: ^{1}H, ^{13}C and HMBC NMR data of phomalactone at 400 MHz in Chloroform-*d6*

Position	Phomalactone in Chloroform-*d*			Literature (Fukushima *et al.*, 1998) in Chloroform-*d*	
	δ_H (*J* Hz)	δ_C	HMBC	δ_H (*J* Hz)	δ_C
2		163.9			163.6
3	6.10, d (*J*=9.7)	123.2	2, 5	6.13, dd (*J* =0.4, 9.7)	122.7
4	6.97, dd (*J* =5.3, 9.7)	144.9	2, 5, 6	6.99, dd (*J* =5.3, 9.7)	144.9
5	4.18, dd (*J* =3.1, 5.3)	63.5	2, 6	4.20 ,ddd (*J* =3.1, 5.3, 8.0)	63.2
5-OH				2.14, d (*J* =8.0)	
6	4.81, dd (*J* =3.0, 7.1)	81.3	8	4.83, ddquint (*J* =3.1, 7.0, 8.0)	81.4
7	5.72, ddq (*J* =1.7, 7.1, 15.9)	124.4	6, 9	5.73, ddq (*J* =1.4 , 7.0, 15.3)	124.0
8	5.99, ddq (*J* =1.5, 6.5, 15.9)	133.5	6, 9	6.01, ddq (*J* =1.1 , 6.5, 15.3)	133.1
9	1.79, dd (*J* =1.6, 6.5)	18.5	7, 8	1.82, ddd (*J* =0.8, 1.6, 6.5)	16.1

3.3.2.3 Musacin E (known compound)

Musacin E (3.17)
Synonym: 5-[I-(l-hydroxybut-2-enyl)]-dihydrofuran-2-one Source: *Nigrospora* sp (isolated from *V. pinnata*) Sample amount: 90 mg Physical description: Colourless oil Molecular formula: $C_8H_{12}O_3$ Molecular weight: 156.163 g/mol Optical rotation: $[\alpha]D^{20}$ = +38.2 (MeOH)



Compound **3.17** was isolated as oil from *Nigrospora* sp extract cultured in rice medium for thirty days. The total amount of compound **3.17** was 90 mg. The HR-LCMS spectral data showed a molecular ion in positive mode at 157.0861 $[M+H]^+$ (base peak), indicating the molecular formula of $C_8H_{12}O_3$ with two degrees of unsaturation. The ^1H NMR spectrum data (Fig. 3.40) revealed the presence of one methyl doublet at δ_H 1.68 (H-9), two olefinic methines at δ_H 5.48 (H-7) and δ_H 5.78 (H-8), and two oxygenated methines at δ_H 4.40 (H-5) and δ_H 4.05 (H-6). From the information given it was deduced

that compound **3.17** is a phomalactone derivative. The difference observed in compound **3.17** was the presence of two methylene signals observed at δ_H 2.49 and δ_H 2.16, and their geminal protons at δH 2.57 and 2.04, respectively, instead of the two olefinic methines in phomalactone **3.16**. Further analysis of the ^{1}H-^{1}H COSY (Fig. 3.41) and ^{1}H-^{13}C HMBC (Fig. 3.42) supported this argument. In the COSY spectrum (Fig. 3.41), the proton at δ_H 2.49 (H-3) showed a correlation at δ_H 2.16 (H-4a) and δ_H 2.04 (H-4b). In the HMBC spectrum (Fig. 3.42), long range ^{1}H-^{13}C correlations were also found between H-3 and C-2, C-4a and C-5, showing that two methylenes were present. The difference also was observed in the HMBC where the chemical shifts of C-5 and C-6 were δ_C 82.7 and δ_C 75.0, respectively, while those of C-5 and C-6 of phomalactone were δ_C 63.5 and δ_C 81.3. This revealed that C-5 was more downfield, suggesting that C-5 is attached to a hydroxyl group. Based on the ^{1}H and ^{13}C NMR and mass spectral data, and in comparison with literature, compound **3.17** was determined to be musacin E, previously isolated from *Nigrospora sacchari* (Fukushima *et al.*, 1998).

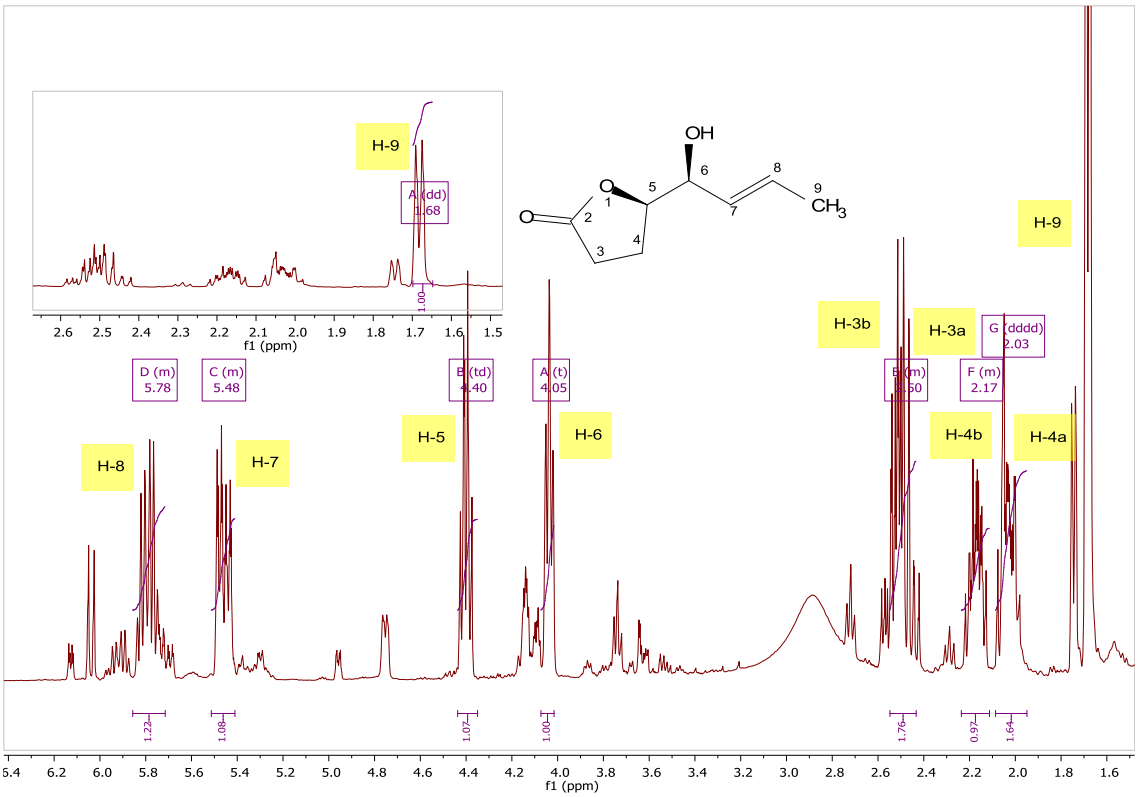


Fig. 3.40: ^{1}H NMR spectrum of musacin E

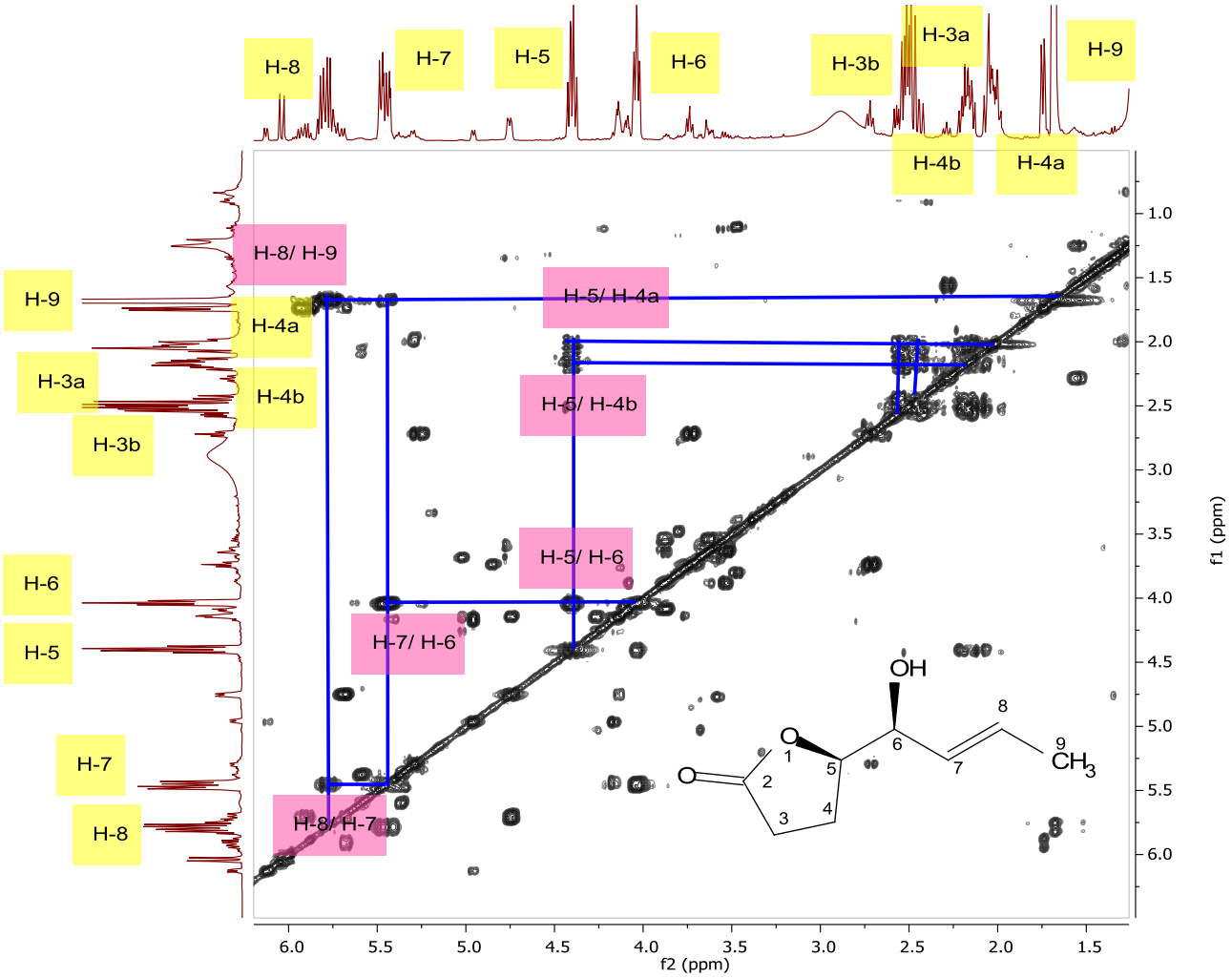


Fig. 3.41: COSY spectrum of musacin E

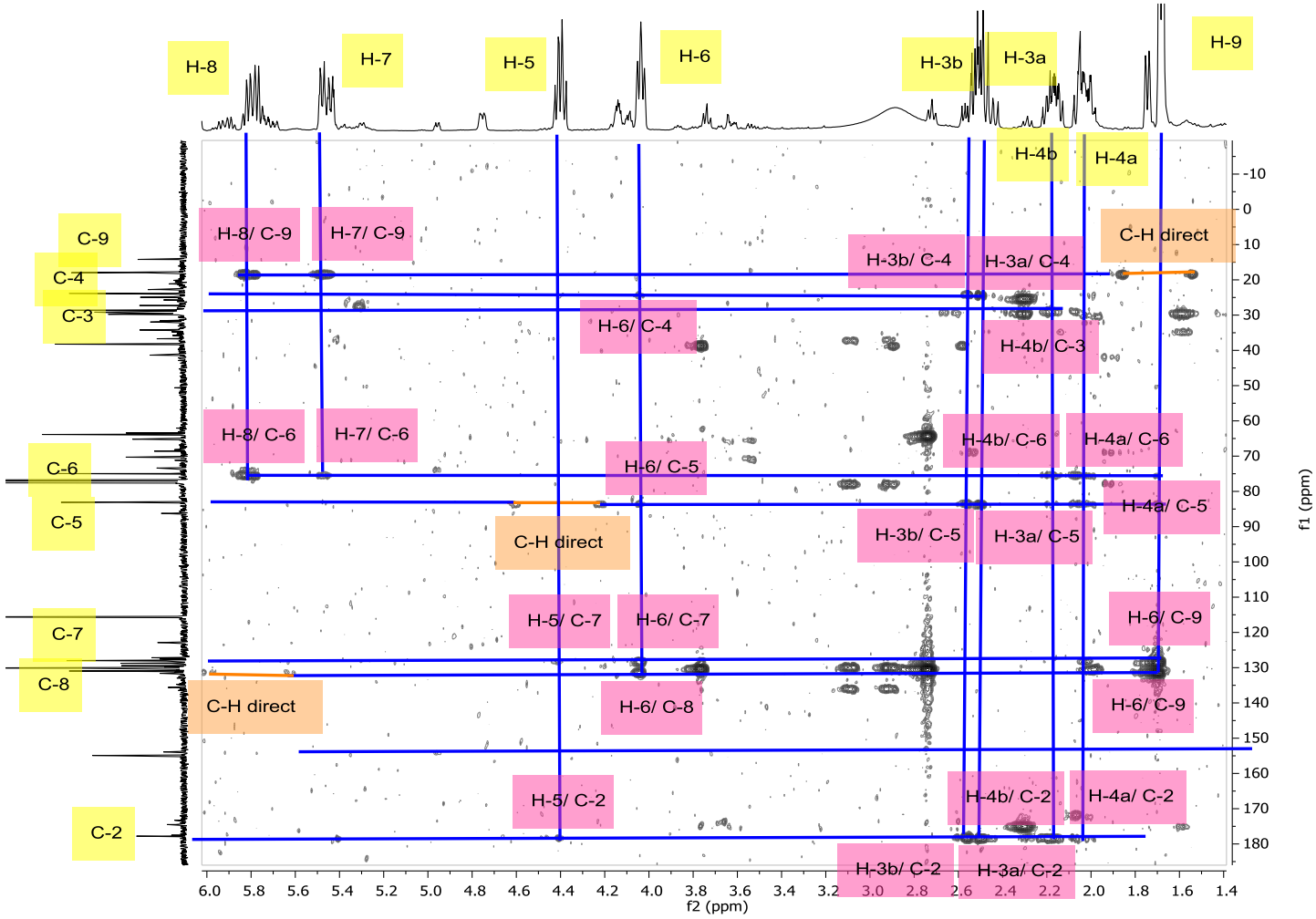


Fig. 3.42: HMBC spectrum of musacin E

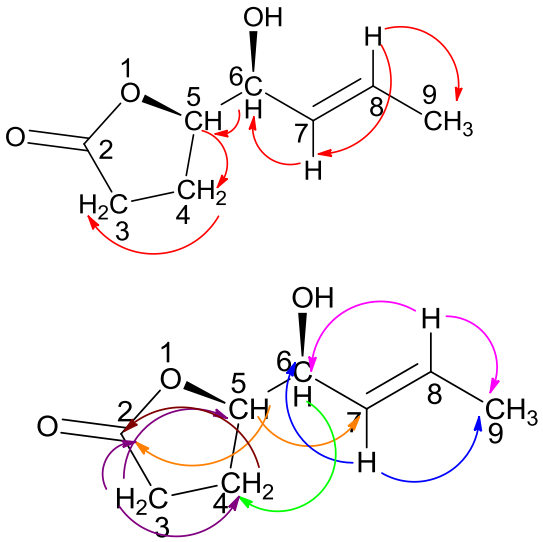
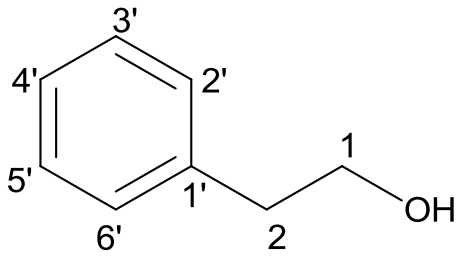


Fig. 3.43: COSY (red arrows) and HMBC (colourful arrows) correlation of musacin E

Table 3.17: ^{1}H and ^{13}C NMR data of musacin E at 400 MHz in Chloroform-*d*

Position	Musacin E in Chloroform-*d*			Literature(Fukushima *et al.*, 1998) Chloroform-*d*	
	δ_H (*J* Hz)	δ_C	HMBC	δ_H (*J* Hz)	δ_C
2		177.0			177.1
3a	2.48, m	28.5	2, 4a, 5	2.52, ddd (*J*=8.4. 9.4, 17.9)	28.6
3b	2.57, m		2, 4a, 5	2.61, ddd (*J*=6.0. 9.9, 17.9)	
4a	2.17, dddd, (*J*=14.1, 8.4, 6.0, 3.3)	23.8	2, 3, 6	2.23, dddd (*J*=6.0, 7.2. 9.4, 13.1)	23.9
4b	2.03, m		2, 5, 6,	2.08, dddd (*J*=7.2, 8.4, 9.9, 13.1)	
5	4.40, dd (*J*=5.5, 7.3)	82.7	2, 7	4.44, dd (*J*=5.5,7.2)	82.8
6	4.05, t (*J*=6.5)	75.0	4a, 5, 7, 8	4.09, t (*J*=6.0)	75.1
6-OH					
7	5.48, m	127.9	6, 9	5.52, ddq (*J*=0.9, 15.4, 6.5)	128.0
8	5.78, m	131.2	6, 9	5.86, ddq (*J*=0.9, 15.4, 6.5)	131.3
9	1.68, dd (*J*=6.6, 1.8)	18.0	7, 8	1.74, dd (*J*=1.7, 6.5)	18.0

3.3.2.4 2-phenylethanol (known compound)

2-phenylethanol (3.18)
Synonym(s): Phenethyl alcohol, Benzenethanol Source: *Nigrospora* sp (isolated from *V. pinnata)* Sample amount: 30 mg Physical description: colourless oil Molecular formula: $C_8H_{10}O$ Molecular weight: 122.16 g/mol


Compound **3.18** was isolated as colourless oil and was found to have a floral smell. The structure was confirmed by the ^{1}H NMR spectrum (Fig. 3.44), which showed overlapping peaks between δ_H 7.22-7.28, representing five aromatic proton signals. This indicated that this aromatic molecule had a mono-substituted spin system. The ^{1}H NMR data also showed the presence of two triplet methylene protons at δ_H 3.80 (H-1') and δ_H 2.81 (H-2') (Fig. 3.44) which were found to be correlating to each other in the ^{1}H-^{1}H COSY spectrum (Fig. 3.45). Based on the ^{1}H and COSY NMR and comparison with literature, compound **3.18** was identified as 2-phenylethanol (Velasco B *et al.*, 2010).

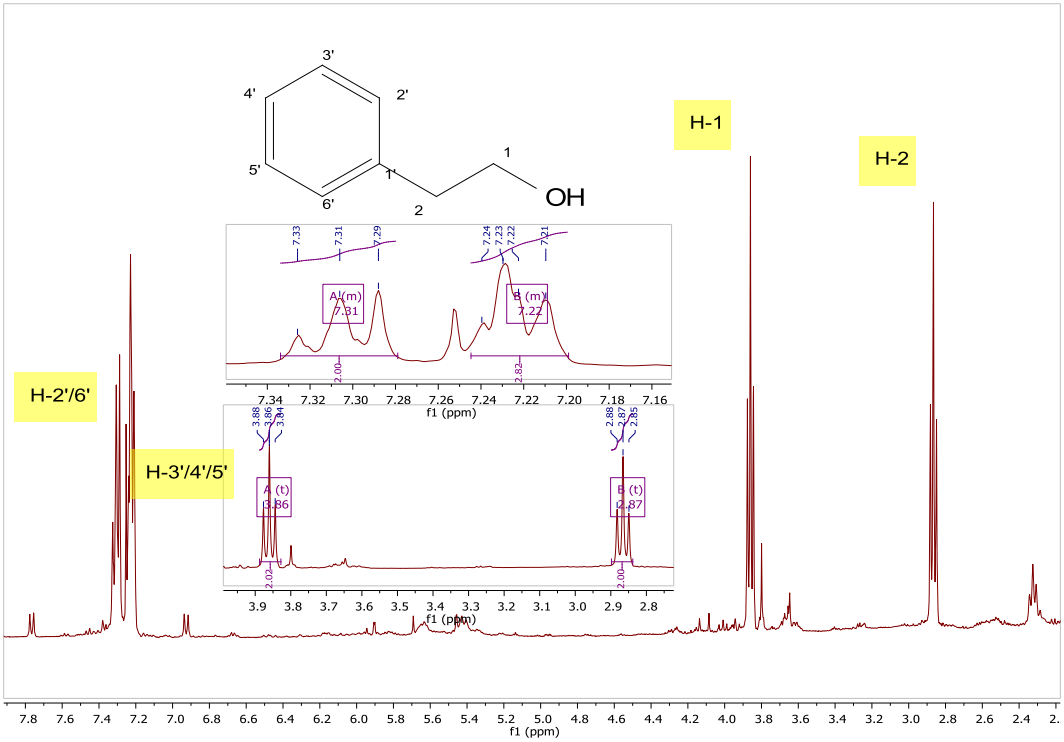


Fig. 3.44: ^{1}H NMR of 2-phenylethanol

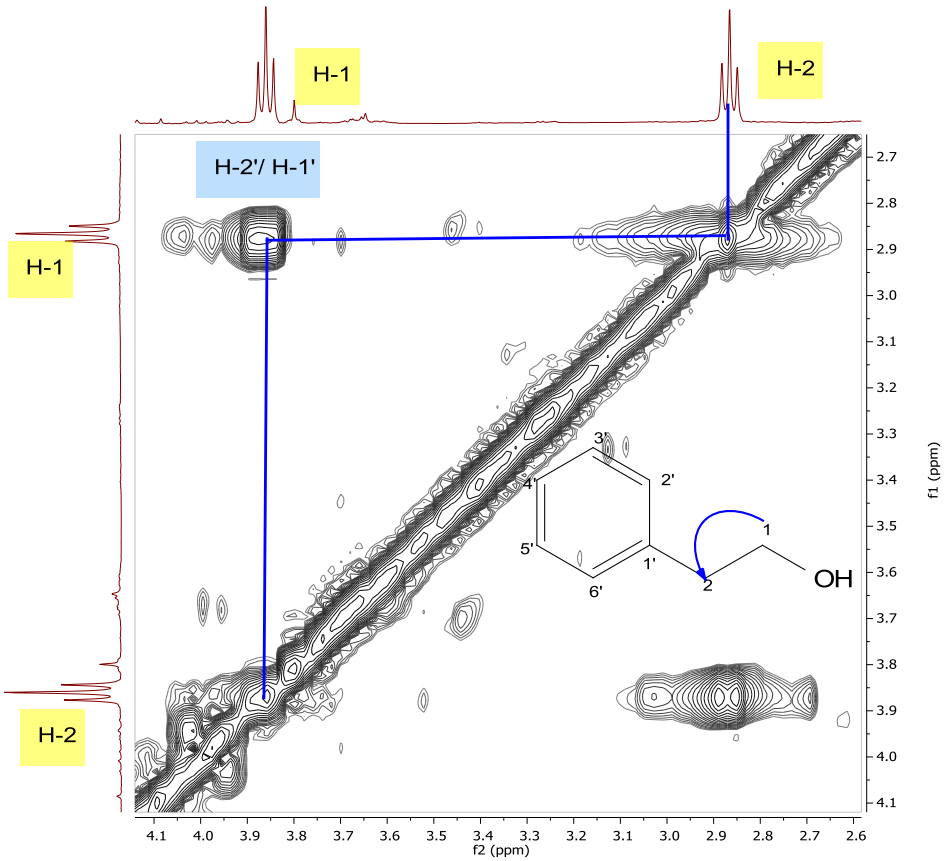
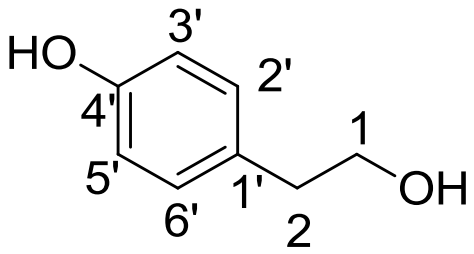


Fig. 3.45: COSY spectrum of 2-phenylethanol

Table 3.18: ^{1}H NMR data of 2-phenylethanol at 400 MHz in Chloroform-*d*

Position	2-phenylethanol in Chloroform-*d*			Literature(Velasco B *et al.*, 2010) in Chloroform-*d*	
	δ_H (*J* Hz)	COSY	δ_C	δ_H (*J* Hz)	δ_C
1	3.86, t (*J*=6.6)	2		3.69, t (*J*=7.0)	59.4
2	2.87, t (*J*=6.6)	1		2.76, t (*J*=7.0)	35.2
1’					
2’	7.29-7.33, m		129.2	7.07-7.27, m	125.0
3’	7.21-7.24, m		127.4	7.07-7.27, m	124.5
4’	7.21-7.24, m			7.07-7.27, m	
5’	7.21-7.24, m		127.4	7.07-7.27, m	124.5
6’	7.29-7.33, m		129.2	7.07-7.27, m	125.0

3.3.2.5 2-(4’-hydroxyphenyl) ethanol (known compound)

2-(4’-hydroxyphenyl) ethanol (3.19)
Synonym(s): Tyrosol Source: *Nigrospora* sp (isolated from *V. pinnata)* Sample amount: 25 mg Physical description: colourless oil Molecular formula: $C_8H_{10}O_2$ Molecular weight: 138.16 g/mol


Compound **3.19** was isolated as colourless oil. The aromatic region showed two *ortho*-doublet peaks at δ_H 7.02 (H-2’ and H-6’) and δ_H 6.75 (H-3’ and H-5’), denoting an AA’BB’ spin system (Fig. 3.46). The ^{1}H NMR data also showed the presence of two triplet methylene protons at δ_H 3.77 (H-1) and δ_H 2.73 (H-2) which were similar to 2-phenylethanol. The COSY spectrum showed *ortho* correlation between H2’/H6’ and H3’/H5’ (Fig. 3.47). Based on the ^{1}H and COSY NMR and mass spectral data and comparison with literature, compound **3.19** was identified as 2-(4’-hydroxyphenyl) ethanol (Chen *et al.*, 2004).

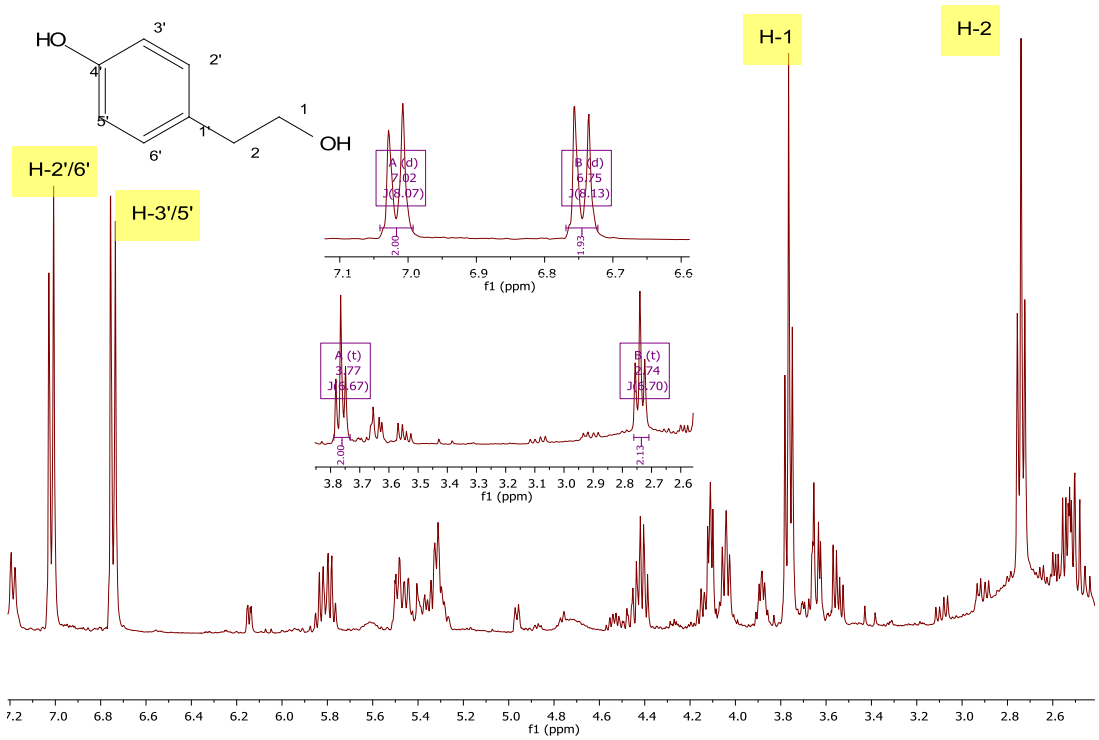


Fig. 3.46: ^{1}H NMR of 2-(4'-hydroxyphenyl) ethanol

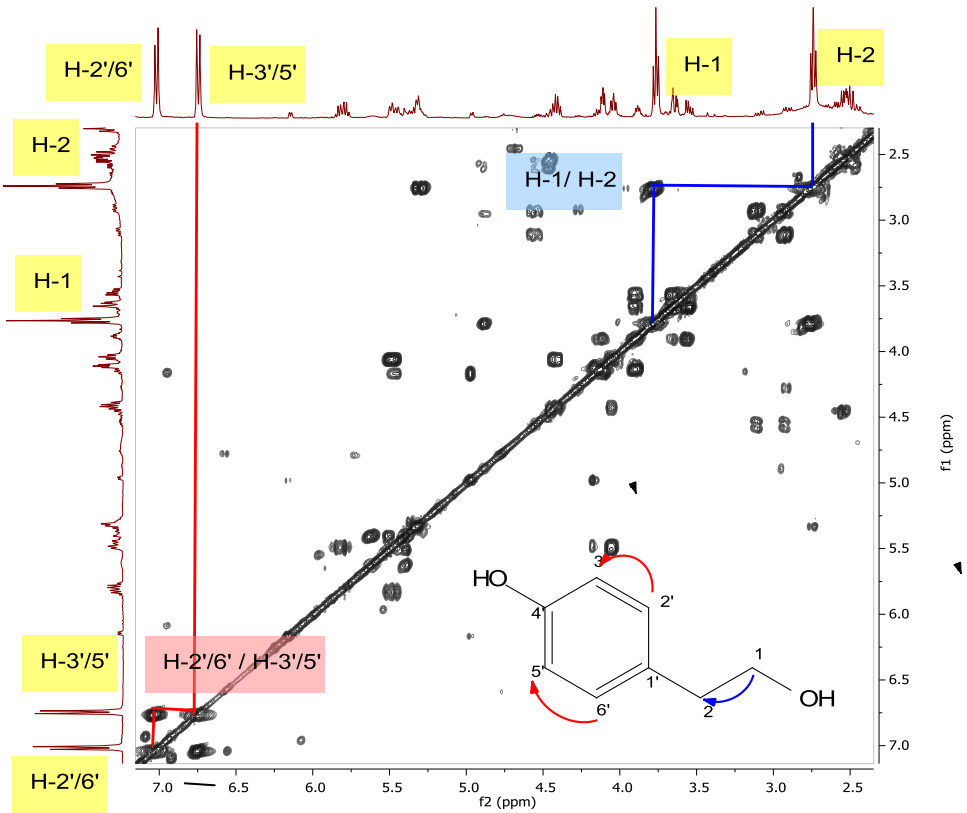
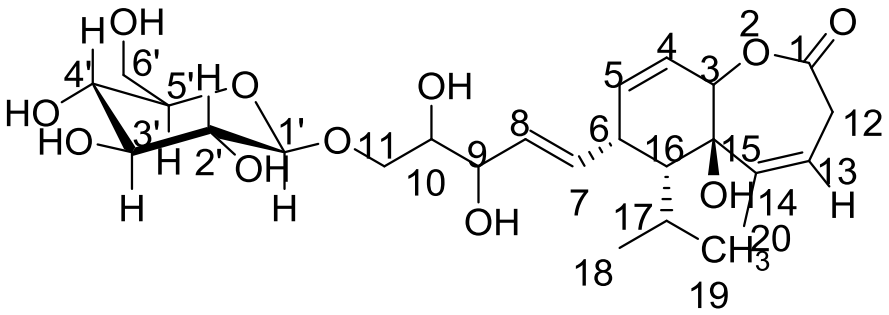
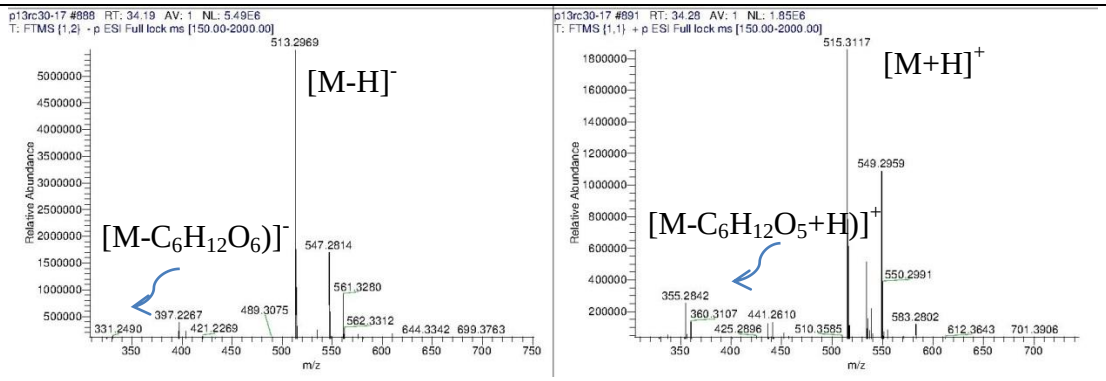


Fig. 3.47: COSY NMR of 2-(4'-hydroxyphenyl) ethanol

Table 3.19: ^{1}H data of 2-(4'-hydroxyphenyl) ethanol at 400 MHz in Chloroform-*d*

Position	2-(4'-hydroxyphenyl) ethanol in Chloroform-*d*		Literature(Chen *et al.*, 2004) in Chloroform-*d*
	δ_H (*J* Hz)	COSY	δ_H (*J* Hz)
2', 6'	7.02, d (*J*=8.5)	3', 5'	6.98, d (*J*= 8.5)
1'			
3', 5'	6.75, d (*J*=8.5)	2', 6'	6.65, d (*J*=8.5)
4'			
1	3.79, t (*J*=6.6)	2'	3.85, t, (*J*=6.0)
2	2.81, t (*J*=6.6)	1	2.80, t, (*J*=6.0)

3.3.2.6 Nigrosporone *β*-glucoside (New)

Nigrosporone *β*-glucoside (3.20)
Synonym: 7-((1E,4E)-6,7-dihydroxy-8*β*–glucosyl)oxy)octa-1,4-dien-1-yl)-6-hydroxy-6-isobutyl-5-methyl-6,7-dihydrooxepin-2(3H)-one Source: *Nigrospora* sp (isolated from *V. pinnata*) Sample amount: 1.5 mg Physical description: White powder Molecular formula: $C_{25}H_{38}O_{11}$ Molecular weight: 514.5785 g/mol



Compound **3.20** was isolated as white powder with a total yield of 1.5 mg. The HR-LCMS spectral data showed the molecular ion peak in the positive mode at 515.3117 $[M+H]^+$ (base peak), and 513.2969$[M-H]^-$ (base peak) which confirms that the M^+ of compound **3.20** is 514.5785 g/mol, compatible with the molecular composition of $C_{25}H_{38}O_{11}$. Fragmentation ions observed at *m/z* 331.25 $[M-C_6H_{12}O_6)]^-$ and 355.28 $[M-C_6H_{12}O_5+H)]^+$ reveals the loss of a hexose unit. The ^1H NMR spectrum (Fig. 3.48)

showed one methyl singlet at δ_H 1.60 (H-20), two methyl doublets at δ_H 0.89 (H-18) and δ_H 0.90 (H-19), three methine multiplets at δ_H 1.30 (H-17), 1.97 (H-16) and 2.06 (H-6) and additional signals for sugar moiety (δ_H 4.26 - 3.19). The presence five olefinic proton signals were detected (δ_H 5.82, 5.50, 5.71, 5.45 and 5.13) suggesting that three non-conjugated double bonds occurred in the molecule. Moreover analysis of the ^{1}H-^{1}H COSY (Fig. 3.49) revealed the presence three spin systems including a continuous correlation between H-3 to H-4, H-4 to H-5, H-5 to H-6, H-6 to H-7, H-7 to H-8, H8 to H-9, H-9 to H-10, H-10 to H-11, H-6 to H-16, H-16 to H-17 and H-17 to H-18 and H-19 (green and red lines), H-12 to H-13 and H-13 to H-14 (pink line) and sugar correlation (cyan line) as shown in Fig. 3.53. The coupling constant of anomeric proton, δ_H 4.26 (H-1') is large with J=7.8 Hz indicated β-glycosidic linkages at which the anomeric proton is axially oriented. In the HMBC data (Fig. 3.51), anomeric proton at δ_H 4.26 (H-1') correlated with C-11 at δ_C 68.8 (^3J correlation). The HMQC data (Fig. 3.50) provides C-H direct correlation and was established as shown in Table 3.16. The presence of a lactone unit as in R-CO-O-R was confirmed by the HMBC data δ_C 174.6 (C1), δ_C 73.2 (C3) and δ_H 4.42 (H3). The NMR correlation data of the lactone ring was similar to (E)-ethyl 4-methyloct-3-enoate from previous data (Didier *et al.*, 2014). The chemical shift for C-15 was deduced based on the long range H-C correlation between proton at δ_H 5.13 (H-13) and carbon at δ_C 81.5 (C-15) as found in the HMBC spectrum. The chemical shift for C-15 went downfield suggesting an OH bound carbon. The ^{1}H-^{1}H COSY (Fig. 3.49) showed correlation between H-16 (δ_H 1.97) and H-6 (δ_H 2.06) proposing these protons are located adjacent to each other to form a 7-membered cyclic ring. This suggestion was confirmed by the HMBC data revealed the long range 4-J correlation between proton at position H-18 (δ_H 0.90) and carbon at C-16 (δ_C 32.5). Based on the NMR and MS data, compound **3.20** was deduced as nigrosporone β-glucoside.

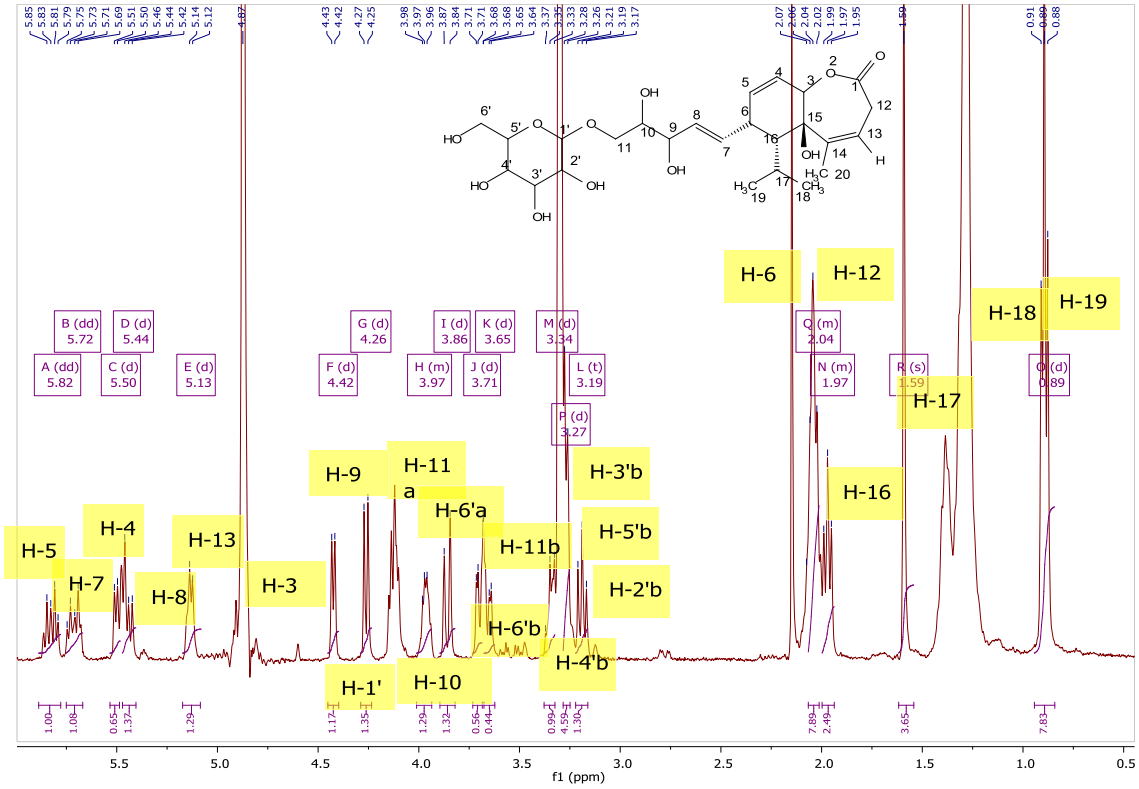
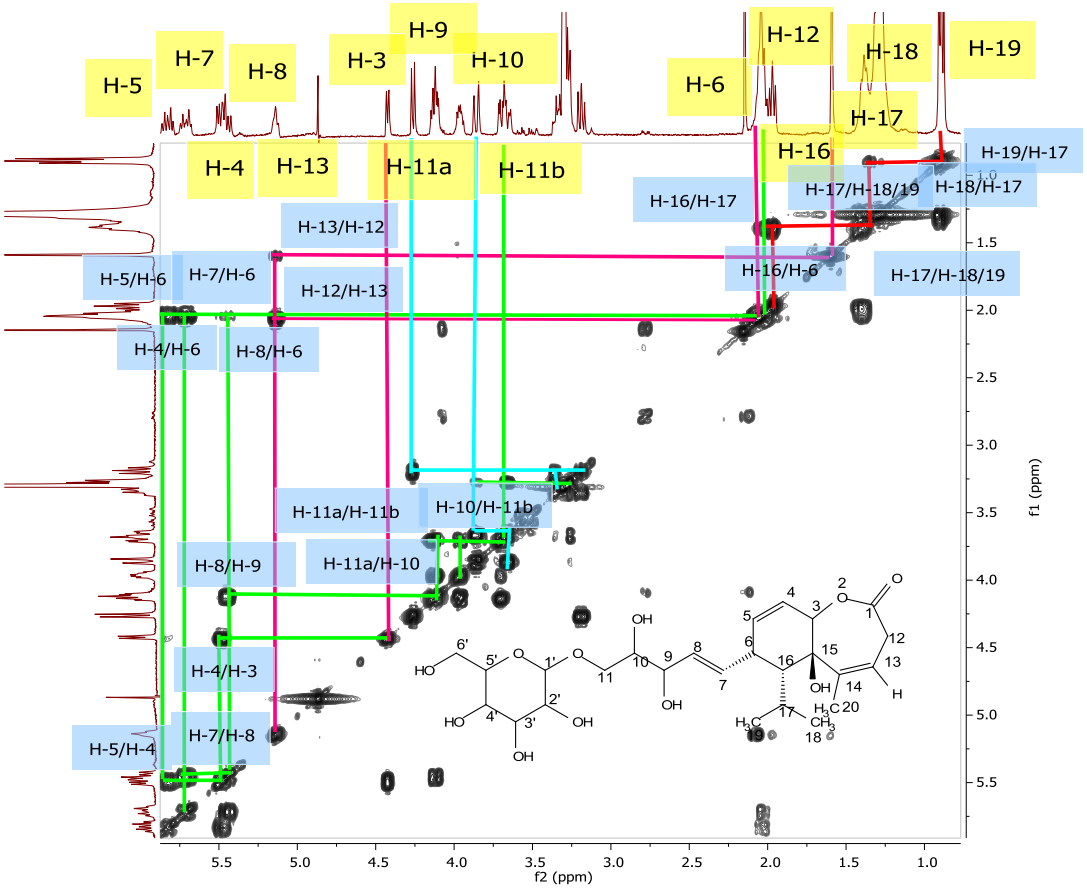


Fig. 3.48: The ^{1}H NMR spectrum of nigrosporone *β*-glucoside revealed the proton integration and multiplicity of each peak



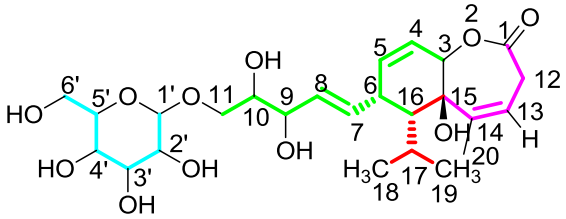


Fig. 3.49: The ^{1}H -^{1}H COSY of nigrosporone *β*-glucoside. Different line colours represent different COSY correlation found in the spectrum data.

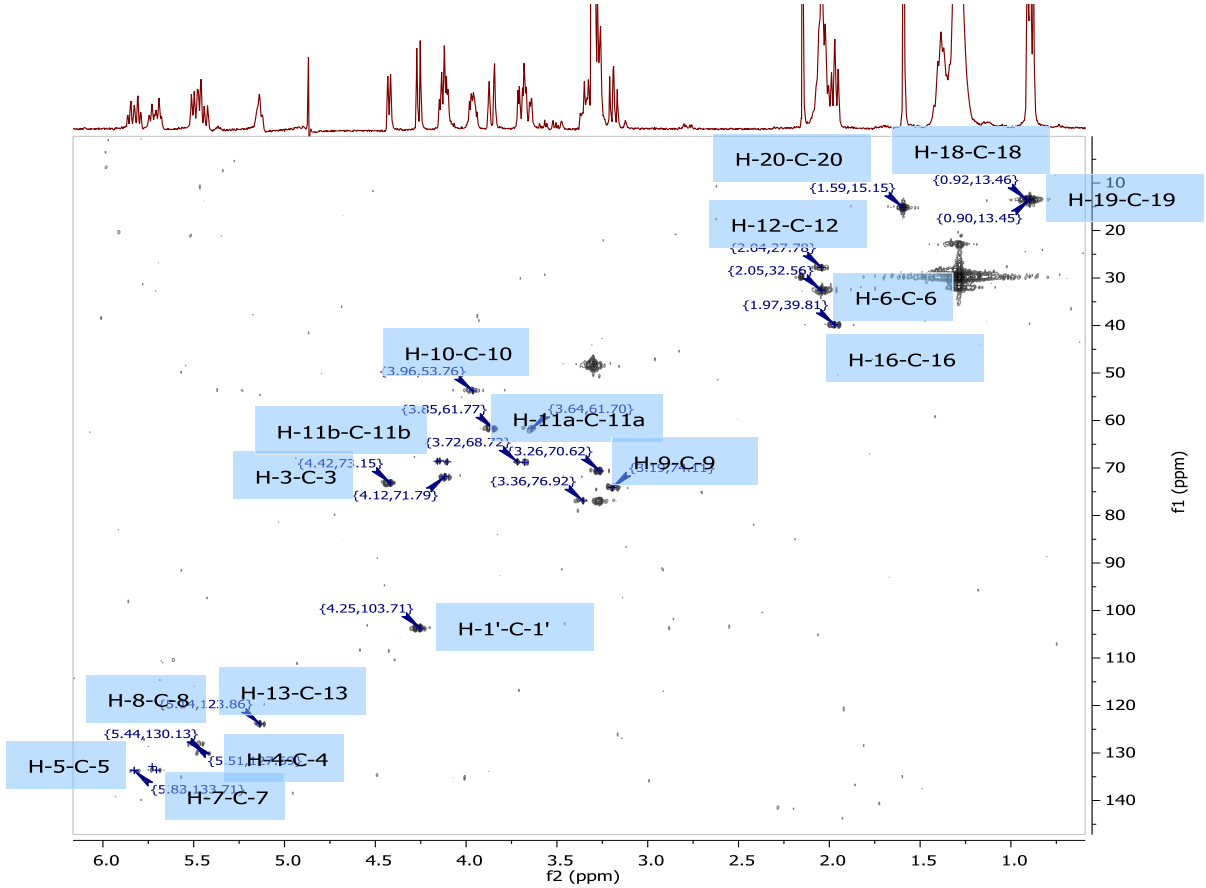


Fig. 3.50: HMQC of nigrosporone *β*-glucoside

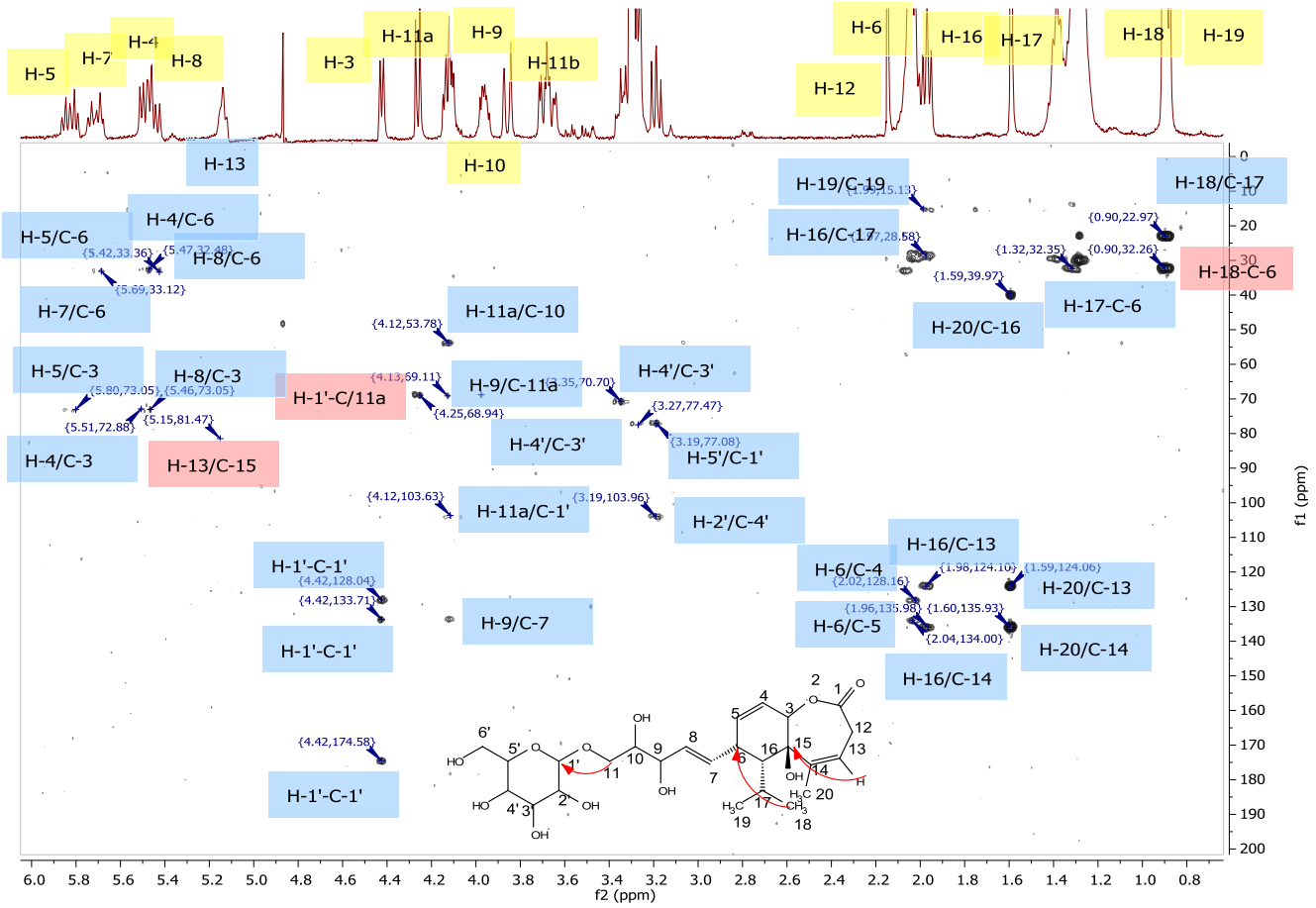


Fig. 3.51a: HMBC NMR of nigrosporone *β*-glucoside. Red boxes and red arrows correspond to 3-*J* and 4-*J* correlation of the compounds.

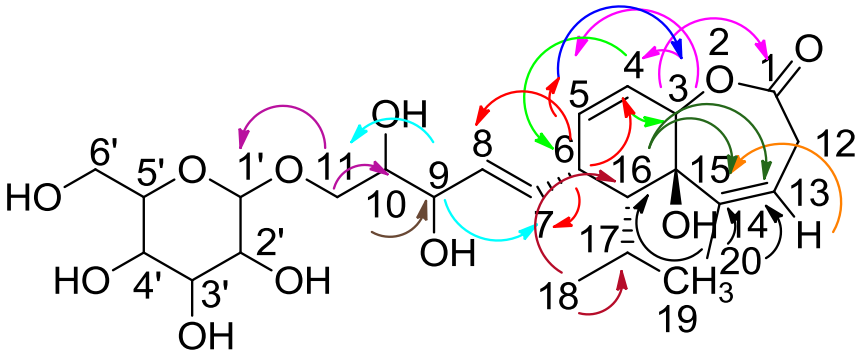


Fig. 3.51b: The HMBC correlations of nigrosporone *β*-glucoside.

Table 3.20: The NMR data of nigrosporone *β*-glucoside at 400 MHz in methanol–*d6.*

Position	P13RC30-17 in CD_3OD		
	δ_H (*J*, Hz)	δ_C	HMBC
1		174.6	
2			
3	4.42, d (*J*=6.1)	73.2	4, 5, 1
4	5.50, dd (*J*=14.0, 6.1)	128.3	3, 6
5	5.82, dt (*J*=14.0, 6.1)	133.6	3
6	2.06, m	32.5	4, 5, 7, 8
7	5.71, dt (*J*=15.3, 7.0)	132.8	6
8	5.45, dd (*J*=15.3, 7.0)	129.5	6
9	4.12, m	71.8	7, 11a
10	3.97, m	53.8	9
11a	4.11, m	68.8	1', 10
11b	3.71, dd (*J*= 2.0, 8.0)		
12	2.04, m	28.5	
13	5.13, m	123.9	15
14		136.0	
15		81.5	
16	1.97, m	39.4	13, 14
17	1.30, m	22.9	
18	0.90, d (*J*=6.6)	13.5	17, 6
19	0.90, d (*J*=6.6)		
20	1.60, s	15.2	13, 14, 16
1'	4.26, d (*J*=8.0)	103.7	
2'	3.19, t (*J*=8.0)	74.1	
3'	3.27, t (*J*=8.0)	70.6	
4'	3.34, t (*J*=8.0)	76.9	
5'	3.19, overlap	74.1	
6'b	3.65, dd (*J*=11.8, 4.0)	61.4	
6'a	3.86, d (*J*=11.8)		

3.4 *Pestalotiopsis olivacea*

3.4.1 Metabolomics and bioassay-guided screening of *P. olivacea* for medium-scale fermentation

For *P. olivacea*, the rice culture extracts in all three incubation periods exhibited strong anti-mycobacterial activity against *M. marinum* while only weak bioactivity was detected for extracts obtained from the liquid culture (Table 3.21). Hence, the rice culture extracts were chosen for further isolation work. The ^{1}H NMR spectrum of the seven, fifteen and thirty-days rice culture extracts showed the production two major compounds known as dechlorodihydromaldoxin and pestheic acid (Fig. 3.52). ^{1}H NMR and MS data (Fig. 3.52 and Fig. 3.53) of the extracts from all incubation periods showed very similar chromatograms and spectrums suggesting all these extracts produced comparable secondary metabolome. However, the fifteen days rice culture condition was chosen for scale-up of *P. olivacea* to optimise the yield as well as to save time when compared to a 30-days culture condition.

Table 3.21: Antitrypanosomal and anti-mycobacterium activity of *P. olivacea* extracts derived from *V. pinnata* in different types of media and incubation periods. MIC was only detected for the bioactive extracts.

Sample	*T. brucei brucei* 20ug/ml % D control	*T. brucei brucei* MIC (µg/ml)	*M. marinum* ATCC BAA 535 100µg/ml % D control	*M. marinum* ATCC BAA 535 MIC (µg/ml)
PO-LC-7	104.0	Not tested	125.6	Not tested
PO-LC-15	102.5	Not tested	116.6	Not tested
PO-LC30	104.8	Not tested	116.1	Not tested
PO-RC-7	98.6	Not tested	**5.3**	100
PO-RC-15	100.7	Not tested	**2.4**	50
PO-RC30	101.5	Not tested	**1.4**	50

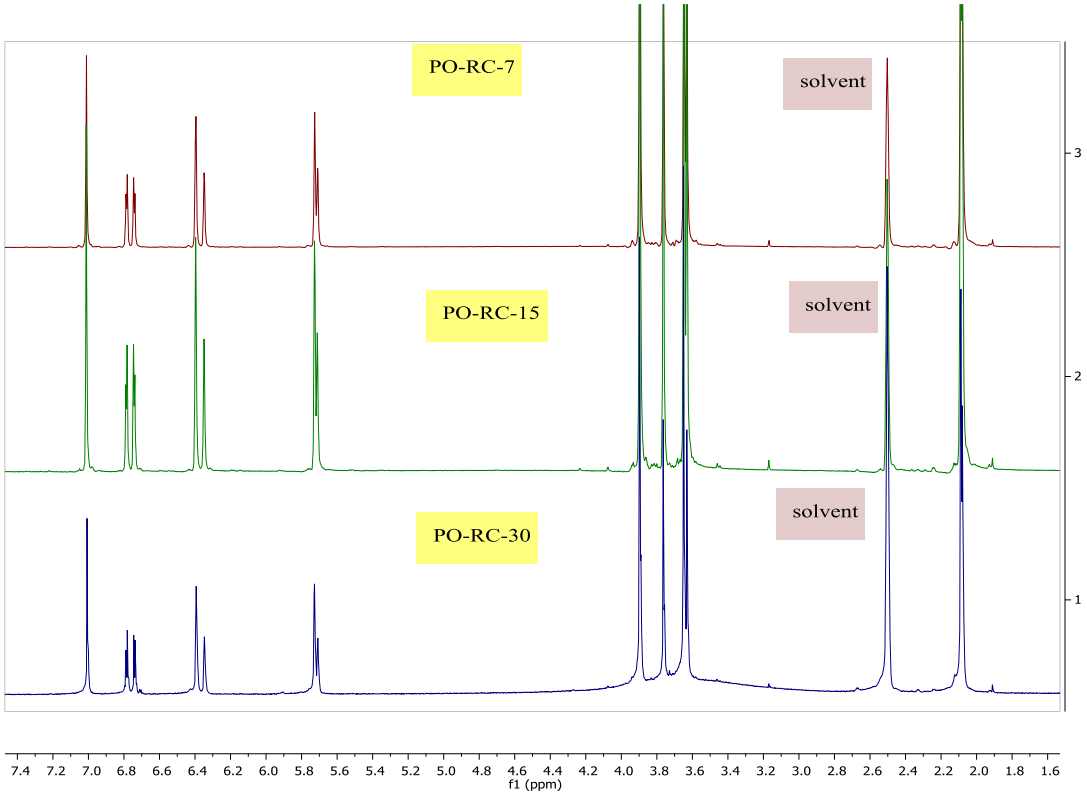
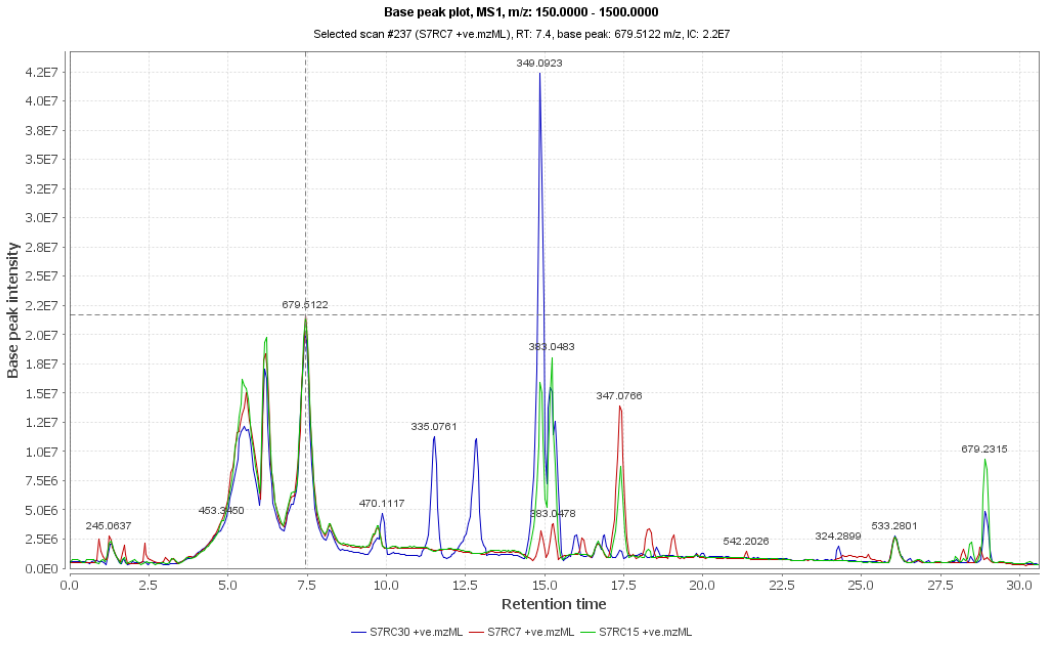


Fig. 3.52: The ^{1}H NMR data of *P. olivacea* extracts in solid rice medium after three different incubation durations

A



B

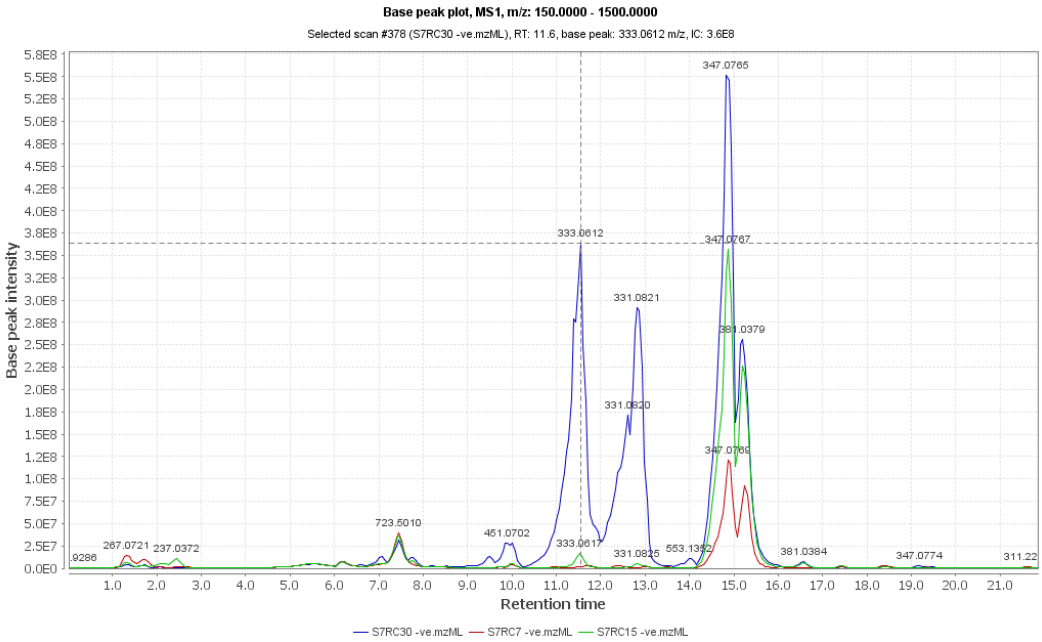


Fig. 3.53: The Total Ion Chromatogram (TIC) of *P. olivacea* extracts A) positive mode B) negative mode in solid rice medium after three different incubation durations

3.4.2 Dereplication of *P. olivacea*

3.4.2.1 The ^{1}H NMR data of the *P. olivacea* extract

To get an overview of the types of compounds found in the extract of *P. olivacea* cultured for 15 day on the rice medium, ^{1}H NMR and MS experiments were performed. The ^{1}H NMR data (Fig. 3.54) revealed that *P. olivacea* produced one or two major abundant compounds which perhaps masked all other promising minor compounds. Therefore, to acquire an overview of the secondary compounds produced by *P. olivacea*, HRLCMS was used due to its high sensitivity, allowing the identification of ion peaks at low concentrations.

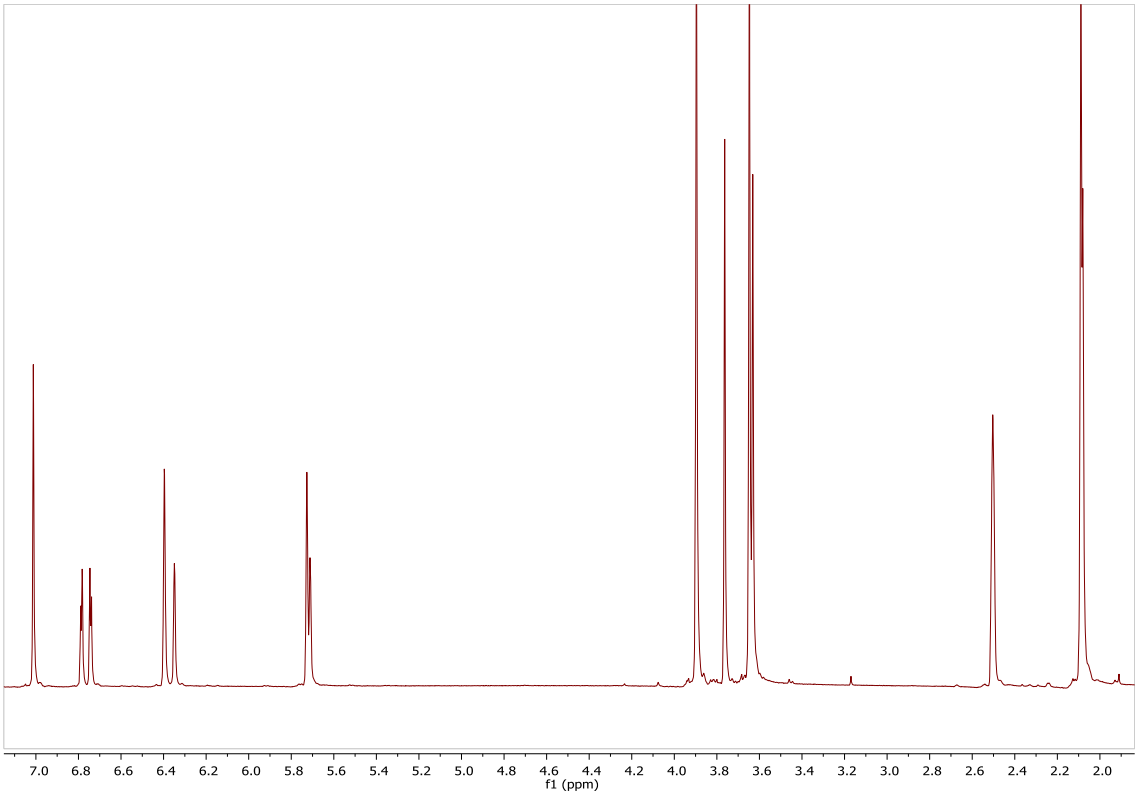


Fig. 3.54: The ^{1}H NMR spectrum of the extract of *P. olivacea* cultured in rice medium for 15 days showed that the spectrum is very clean, suggesting major compounds are abundant in the extract

3.4.2.2 HR-LCMS metabolite profiling of the *P. olivacea* extract

Metabolite profiling of the *P. olivacea* extract was accomplished by applying HR-LCMS (Thermo Exactive). The raw data was processed using MZMine 2.10 (Pluskal *et al.*, 2010) and the secondary metabolites were then identified with databases such as AntiMarin, a database dedicated for microbial secondary metabolites (Blunt, 2013). Most of the known compounds putatively identified in *P. olivacea* extract were previously isolated from *Pestalotiopsis* sp. as well as other endophytic fungi, as shown in Table 3.22. Table 3.22 reveals that the ionisation peaks found in the sample are mostly $[M+H]^+$, $[2M+H]^+$, $[2M+Na]^+$ and $[2M+NH_4]^+$ in positive mode and $[M-H]^-$ and $[2M-H]^-$ in negative mode. The list of identified metabolites showed the presence of pyrones and also inter-related metabolites including diphenyl ethers and spirocyclohexadienones. Two known pyrone peaks, Pestalofuranone E **3.21** and Pestalofuranone D **3.22**, which were both previously isolated from *Pestalotiopsis*

besseyi (Liu *et al.*, 2012), were detected in the MS chromatograms. Diphenyl ethers and spirocyclohexadienones were also found in the sample including dechlorodihydromaldoxin **3.26**, dihydromaldoxin; pestheic acid **3.27** and maldoxin **3.29**.

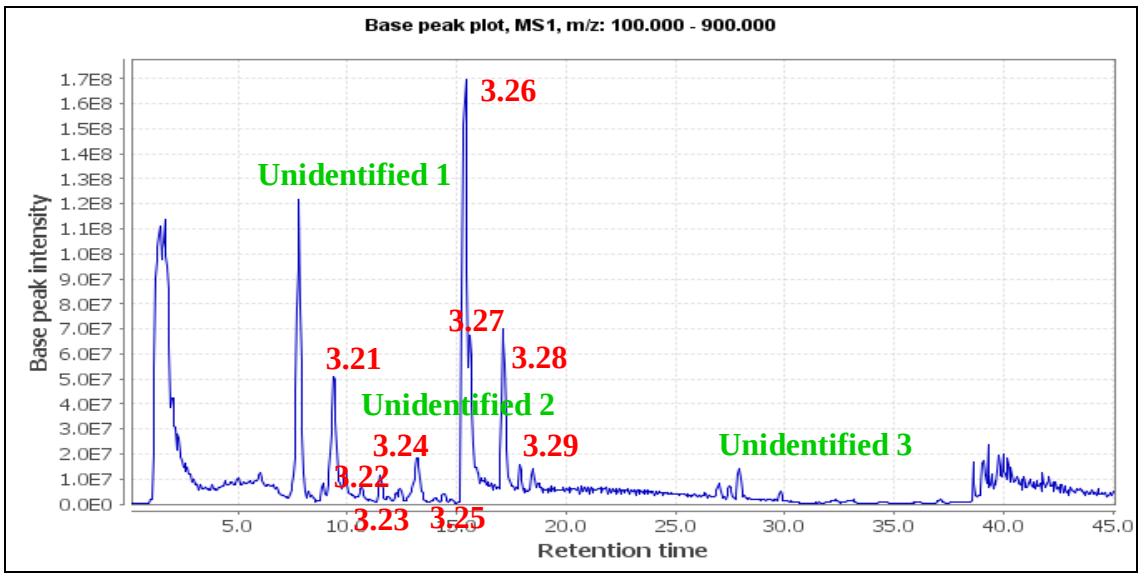


Fig. 3.55: The MS chromatogram of the *P. olivacea* extract in positive mode generated with the MZmine 2.10 software that was used in the dereplication study

The dereplication of known metabolites was easily achieved with MS without any isolation work needing to be done. For example, chloroisosulochrin **3.25** was easily identified by observing the isotopic distribution of the base peak at *m/z* 367.058 $[M+H]^+$ and 369.058 $[M+H+2]^+$ in the MS spectrum (Fig. 3.56a), which clearly showed the presence of one Cl due to the isotope pattern ratio of 70:30 ^{35}Cl: ^{37}Cl. The fragmentation pattern at *m/z* 331.0813 $[M+H-Cl]^+$ corresponded to the loss of Cl. Its derivative, isosulochrin **3.26**, also was easily identified in positive mode at 333.097 $[M+H]^+$ (base peak) with a retention time of 13.30 min (Fig. 3.56b). The fragmentation pattern at 209.045 displayed the loss of $C_7H_8O_2$ from its parent ion. Both compounds **3.25** and **3.26** were isolated previously from *Pestalotiopsis thea* (Shimada *et al.*, 2001). Barcelonic acid **3.23** was recognized in the MS data after careful selection from the hits list generated from the AntiMarin database. Of the nine hits listed, eight hits were previously found in fungi and one was isolated from lichen. However, barcelonic was selected based on its similar structure to dechlorodihydromaldoxin **3.26** which one of major metabolites produced by *P. olivacea* (see Table 3.22).

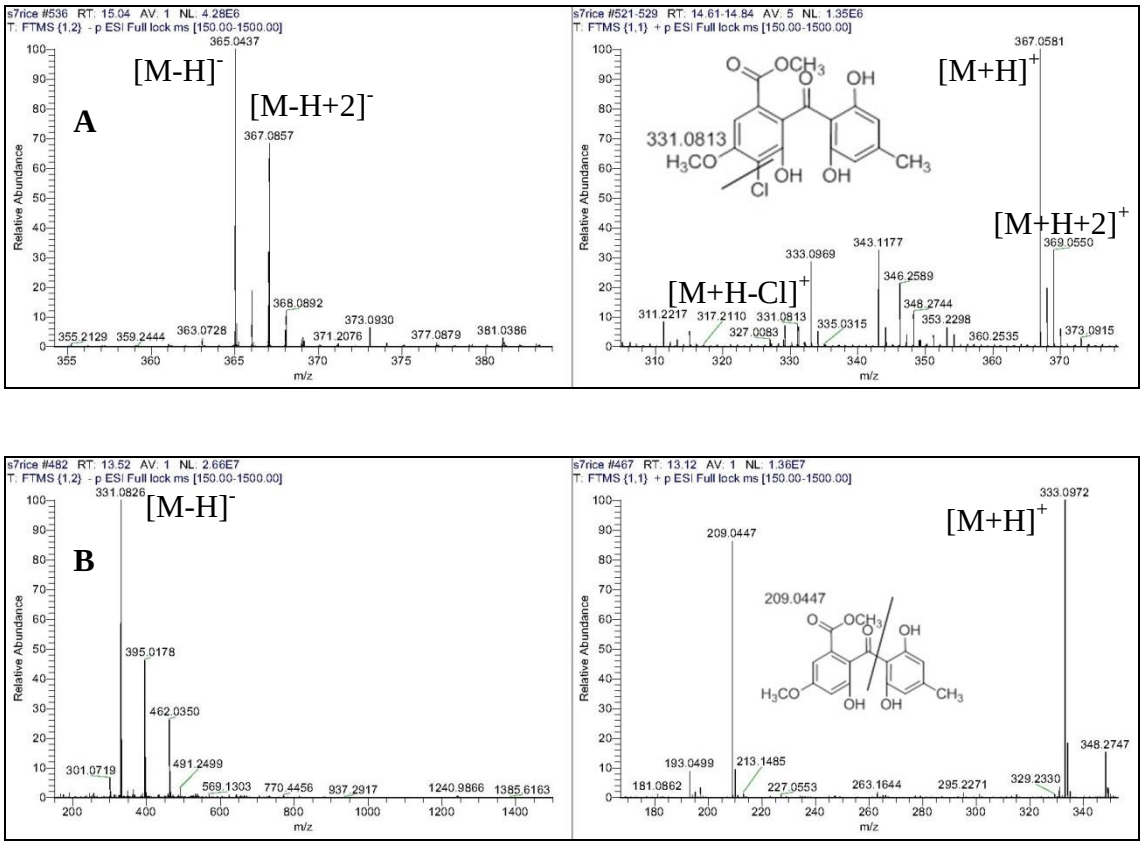


Fig. 3.56: MS/HRMS spectra and fragmentation pattern of A) chloroisosulochrin and B) isosulochrin.

Three unidentified peaks were detected in the *P. olivacea* extract as shown in the chromatogram in Fig. 3.60, revealing the potential of finding new secondary metabolites in this extract. Based on the MS data, the predicted molecular formula for **Unidentified 1** is $C_{14}H_{20}O_2N$. After searching the AntiMarin database for secondary metabolites from the *Pestalotiopsis* genus, the chemical structure of **Unidentified 1** was proposed to be a derivative of Pestalactam C **3.31** which was previously isolated from *Pestalotiopsis* sp. (Davis *et al.*, 2010). The proposed structure of **Unidentified 1** is (*E*)-4-methyl-7-(2-methylhex-1-en-1-yl)-1H-azepine-2,5-dione **3.32**.

Table 3.22: List of known compounds putatively identified by using HR-LCMS data set processed in MZmine 2.10 and cross-checked with available database, AntiMarin

$[M+H]^+$; $[M-H]^-$	**$[2M+H]^+$; $[2M+Na]^+$; $[2M+NH4]^+$; $[2M-H]^-$**	**MS^2**	**Rt (min)**	**Chemical Formula**	**Name**	**Source**
197.118 $[M+H]^+$	393.227 $[2M+H]^+$	179.107 -$[OH]^-$	9.25	$C_{11}H_{16}O_3$	Pestalofuranone E **(3.21)**	*Pestalotiopsis besseyi(Liu et al., 2012)*
195.102 $[M+H]^+$	389.196 $[2M+H]^+$		9.80	$C_{11}H_{14}O_3$	Pestalofuranone D **(3.22)**	*Pestalotiopsis besseyi (Liu et al., 2012)*
335.076 $[M+H]^+$ 333.062 $[M-H]^-$	686.175 $[2M+NH_4]^+$	317.066 -$[OH]^-$	11.90 11.85	$C_{16}H_{14}O_8$	Barceloneic acid-B **(3.23)**	*Phoma* sp.,*(Jayasuriya et al., 1995) Penicillium albocoremium(Overy et al., 2005)*
333.097 $[M+H]^+$ 331.082 $[M-H]^-$	663.365 $[2M+H]^+$ 661.171 $[2M-H]^-$	209.045 -$[C_7H_8O_2]$	13.30 13.24	$C_{17}H_{16}O_7$	Isosulochrin **(3.24)**	*Pestalotiopsis theae,(Shimada et al., 2001) Pestalotiopsis guepinii(Oliveira et al., 2011)*
367.058 $[M+H]^+$ 365.043 $[M-H]^-$			14.70 14.79	$C_{17}H_{15}ClO_7$	Chloroisosulochrin **(3.25)**	*Pestalotiopsis theae*(Shimada *et al.*, 2001)
349.091 $[M+H]^+$ 347.077 $[M-H]^-$	697.180 $[2M+H]^+$	331.081 -$[OH]^-$	15.40	$C_{17}H_{16}O_8$	Dechlorodihydromaldoxin **(3.26)**	*Pestalotiopsis* sp.,(Ogawa *et al.*, 1995) *Xylaria* sp.(Adeboya *et al.*, 1996)
383.053 $[M+H]^+$ 381.039 $[M-H]^-$	782.120 $[2M+Na]^+$	365.040 -$[OH]^-$	15.60 15.66	$C_{17}H_{15}ClO_8$	Dihydromaldoxin; Pestheic Acid **(3.27)**	*Pestalotiopsis* sp.*(Ogawa et al., 1995), Pestalotiopsis theae,*(Shimada *et al.*, 2001) *Xylaria* sp.,(Adeboya *et al.*, 1996) *Pestalotiopsis* sp.,*(Klaiklay et al., 2012)*
347.077 $[M+H]^+$ 345.061$[M-H]^-$	710.173 $[2M+NH_4]^+$		17.10 16.99	$C_{17}H_{14}O_8$	Dechloromaldoxin **(3.28)**	*Pestalotiopsis fici(Liu et al., 2013b)*
381.038 $[M+H]^+$ 379.022$[M-H]^-$	778.098 $[2M+NH_4]^+$		18.30 21.93	$C_{17}H_{13}ClO_8$	Maldoxin **(3.29)**	*Xylaria* sp.(Adeboya *et al.*, 1996)

Table 3.23: List of peaks from the HR-LCMS data set processed in MZmine 2.10

Peak	**$[M+H]^+$; $[M+NH_4]^+$; $[M-H]^-$; $[M]^+$**	**$[2M+H]^+$; $[2M+Na]^+$; $[2M+NH4]^+$; $[2M-H]^-$; $[2M]^+$**	**Rt (min)**
Unidentified 1	234.149 $[M+H]^+$ 253.0715 $[M+Na-2H]^-$		7.83
Unidentified 2	470.117 $[M+NH_4]^+$ 453.0851$[M+H]^+$ 451.0704 $[M-H]^-$	922.190 $[2M+NH_4]^+$ 903.147 $[2M-H]^-$	10.11 10.14
Unidentified 3	696.2580 $[M+NH_4]^+$ 677.2152 $[M-H]^-$	1374.4835 $[2M+H]^+$ 1355.4362 $[2M-H]^-$	27.89 27.17

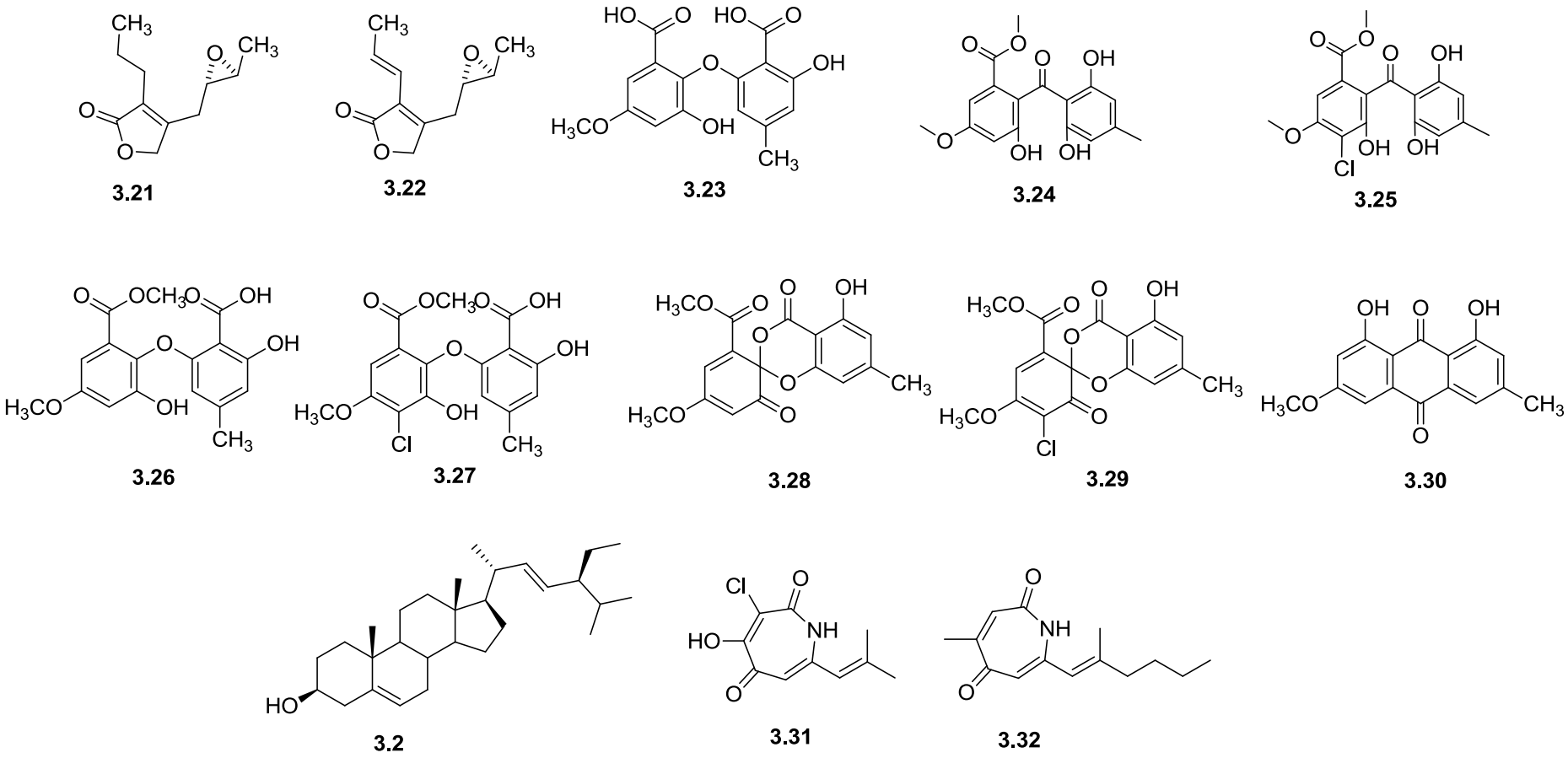


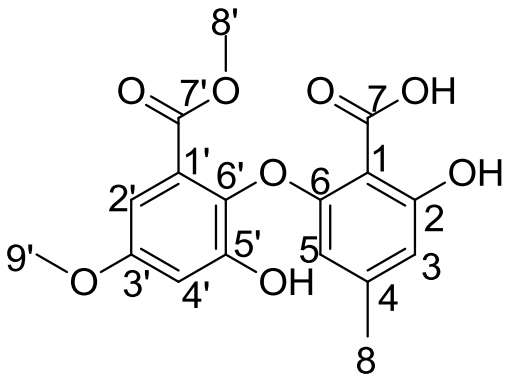
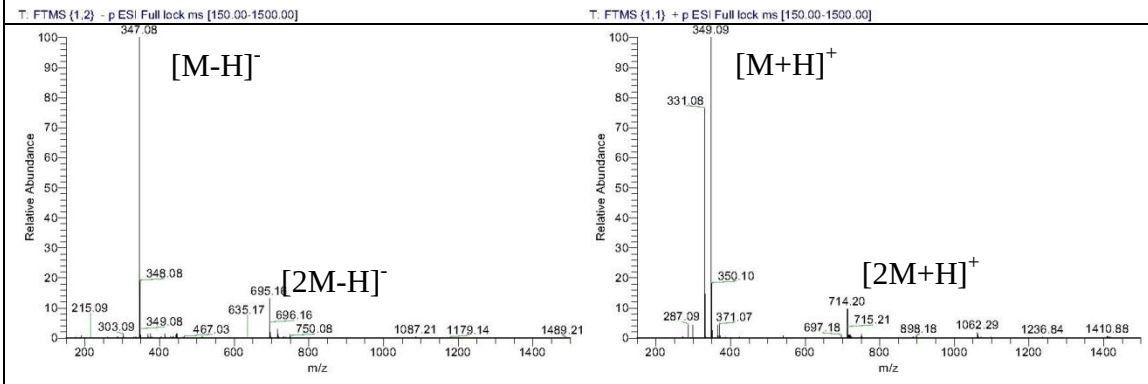
Fig. 3.57: List of known metabolites putatively identified in the extract of *P. olivacea* based on HR-LCMS data processed with MZmine 2.10 and cross-checked with the available database, AntiMarin **(3.21-3.29). 3.26-3.30** and **3.2** were isolated from *P. olivacea*

3.4.3 Isolation and identification of unidentified peaks in *P. olivacea*

The chemical investigation of *P. olivacea* resulted in six known compounds. Dechlorodihydromaldoxin **3.26** and pestheic acid **3.27** were identified as the major compounds while further purification yielded maldoxin **3.28**, dechloromaldoxin **3.29**, physcion **3.30** and stigmasterol **3.2**. Four isolated compounds **(3.26, 3.27, 3.28 and 3.29)** were putatively identified from the MS data integrated with the AntiMarin database. The database-generated hits therefore demonstrated that the dereplication was reliable and produced true positive results. However, the other five putatively identified compounds **(3.21, 3.22, 3.32, unidentified 2 and unidentified 3)** were not isolated due to the limited amount in the samples. Furthermore, physcion **3.30** was identified in one fraction even though it was not detected in the MS data of the crude *P. olivacea* extract. The concentration of physcion was relatively higher in the fraction as opposed to the crude extract, and as such it was only identified in the fraction. Unfortunately, none of the unidentified peaks were isolated in this study, again due to the small amount of the fractions, precluding further purification. It is therefore important to highlight that in future work the volume of the fermentation of the fungi should be increased from 20 L to 40 L to ensure that the extract yield is sufficient for further isolation work.

3.4.4 Secondary metabolites isolated from *P. olivacea*

3.4.4.1 Dechlorodihydromaldoxin (known compound)

Dechlorodihydromaldoxin (3.26)
Synonym(s): RES-1214-1 Source: *P. olivacea* (isolated from *V. pinnata)* Sample amount: 150 mg Physical description: Yellow crystal needle Molecular formula: $C_{17}H_{16}O_8$ Molecular weight: 348.30 g/mol



Compound **3.26** was isolated as yellow crystal needles with a total amount of 150 mg. This compound was found to have a molecular formula of $C_{17}H_{16}O_8$, determined by HRLCMS ($[M+H]^+$). The ^1H NMR data (Fig. 3.58) showed the presence of one methyl singlet at δ_H 2.08 and two methoxy signals at δ_H 3.63 and δ_H 3.76. In the aromatic

regions, two pairs of *meta* doublet signals from two different spin systems were detected at δ_H 5.81 (H-5) and δ_H 6.43 (H-3) and also at δ_H 6.76 (H-4') and δ_H 6.98 (H-2'), which was confirmed by the ^{1}H-^{1}H COSY experiment. The HMBC experiment (Fig. 3.59) showed ^{1}H-^{13}C long-range correlation of the *meta-coupled* proton in the A ring, H-3, to C-1, C-2, C-5 and C-8 along with H-5 to C-1, C-3, C-6 and C-8. Apart from that, in the B ring, the HMBC spectrum revealed the correlation of another *meta*-coupled proton, H-2', to C-1', C-3', C-4', C-5', C-6' and C-7' as well as H-4' to C-2', C-3', C-6', and C-5'. The ^{1}H, two dimensional NMR and mass spectral data were found to be identical to the published data of dechlorodihydromaldoxin (RES-1214-1) which was previously isolated from *Pestalotiopsis* sp (Ogawa *et al.*, 1995).

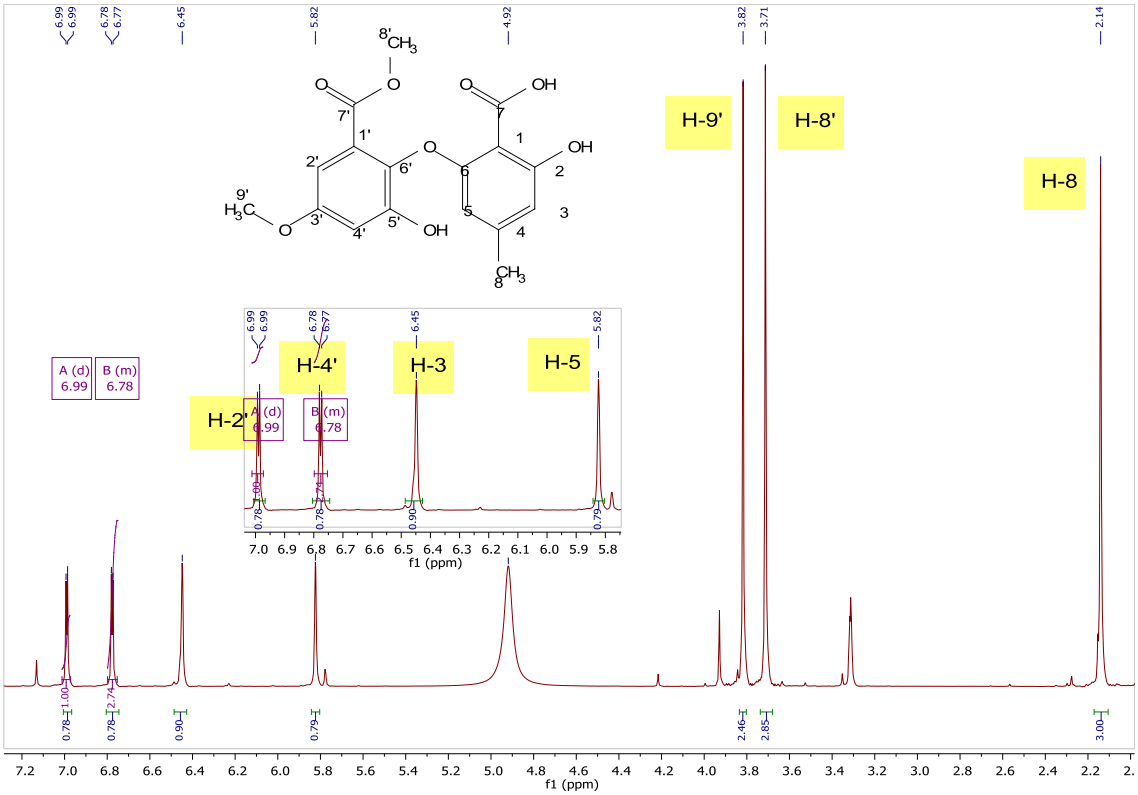


Fig. 3.58: ^{1}H NMR of dechlorodihydromaldoxin

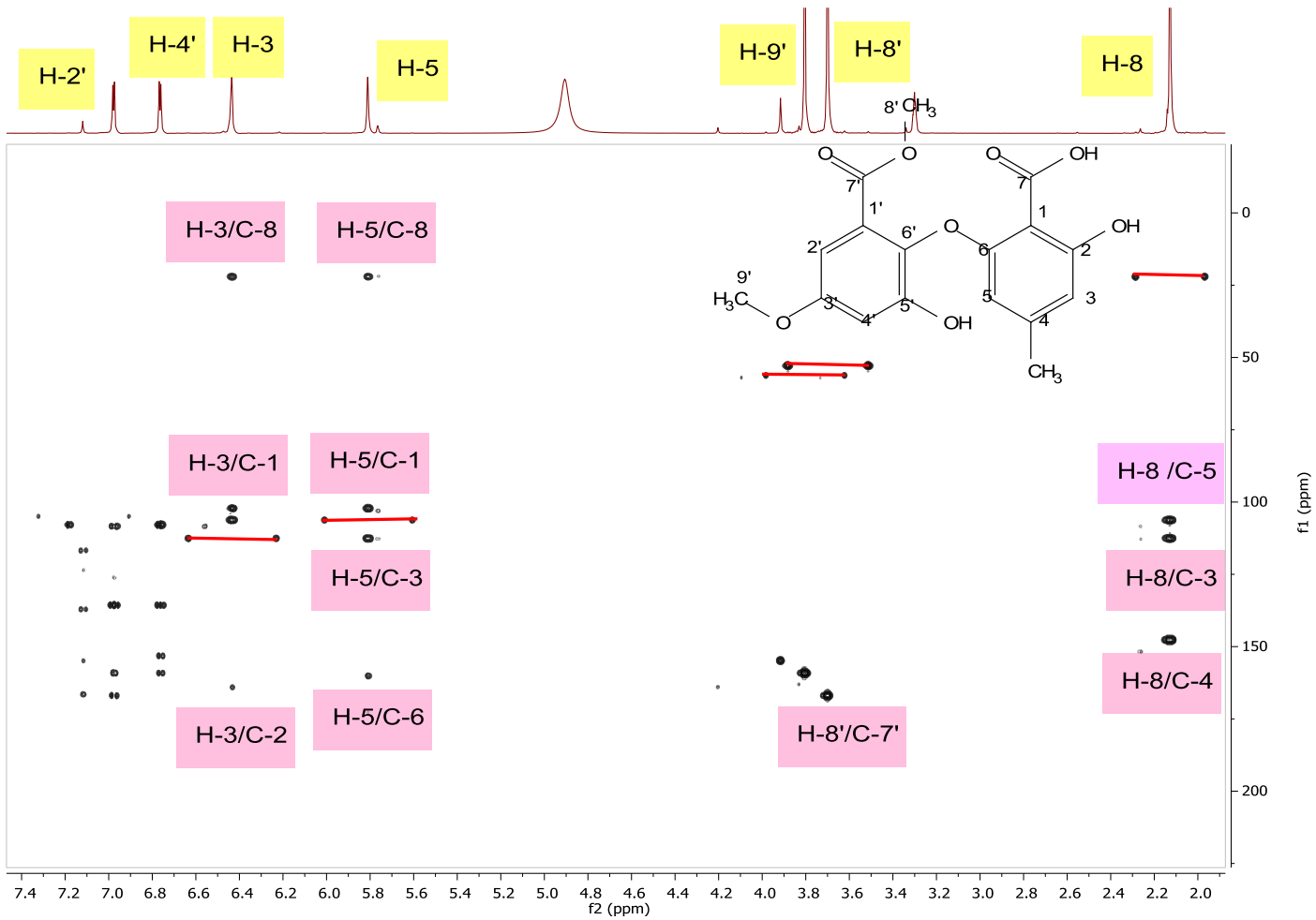
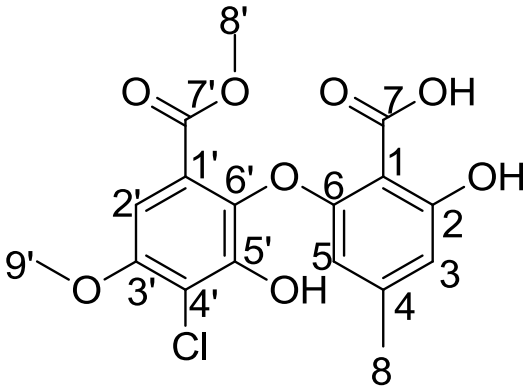
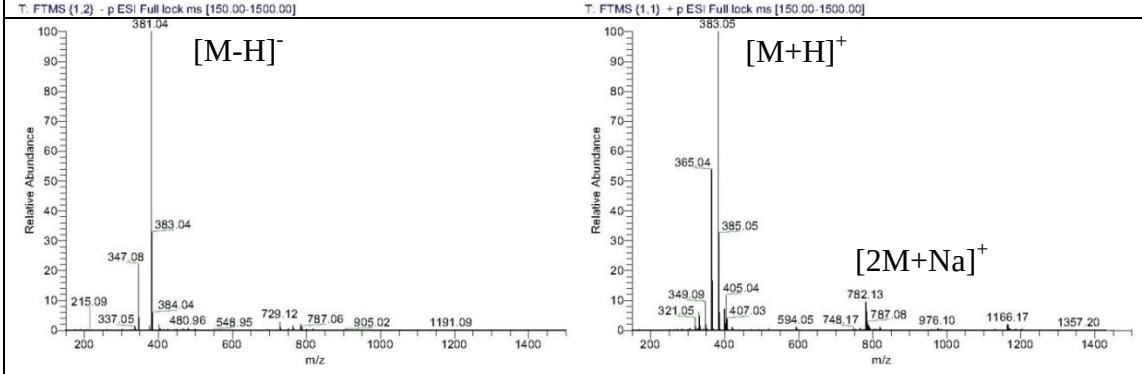


Fig. 3.59: HMBC of dechlorodihydromaldoxin

Table 3.24: ^{1}H and ^{13}C NMR data of dechlorodihydromaldoxin at 400 MHz in CD_3OD

Position	Dechlorodihydromaldoxin in CD_3OD			Literature(Ogawa *et al.*, 1995) in CD_3OD	
	δ_H (*J*, Hz)	δ_C	HMBC	δ_H (J Hz)	δ_C
1		102.3			102.3
2		164.1			164.1
3	6.45, d (*J*=0.8)	112.6	1, 2, 5, 8	6.45, d (*J*=0.8)	112.6
4		147.7			147.7
5	5.82, d (*J*=0.8)	106.3	1, 3, 6, 8	5.82, d (*J*=0.8)	106.3
6		160.2			160.2
7		172.7			172.7
8	2.13 (s)	22.1	3, 4, 5	2.13 (s)	22.1
1’		126.2			126.2
2’	6.99, d (*J*=3.1)	108.0	1’, 3’, 4’, 5’, 6’, 7’	6.99, d (*J*=3.0)	108.0
3’		159.2			159.2
4’	6.78, d (*J*=3.0)	108.4	2’, 3’, 5’, 6’	6.78, d (*J*=3.0)	108.4
5’		153.2			153.2
6’		135.7			135.7
7’		167.0			167.0
8’	3.71 (s)	52.9	7’	3.71 (s)	52.9
9’	3.82 (s)	56.3	3’	3.82 (s)	56.3

3.4.4.2 Pestheic Acid (known compound)

Pestheic Acid (3.27)
Synonym(s): dihydromaldoxin, RES-1214-2 Source: *P. olivacea* (isolated from *V. pinnata)* Sample amount: 80 mg Physical description: Yellow crystal needles Molecular formula: $C_{17}H_{15}ClO_8$ Molecular weight: 382.75 g/mol
   

Compound **3.27** was isolated as yellow crystal needles (80 mg). The HR-LCMS data showed $[M+H]^+$ at *m/z* 383.0537 (base peak) and $[M-H]^-$ at *m/z* 381.0386, denoting a molecular weight of 382.75 g/mol, which had an increase of 34 mass units compared to dechlorodihydromaldoxin. The data also showed that the base peak had an intensity ratio of 3:1 which indicated the presence of a Cl and this, together with the difference of 34 mass units from dechlorodihydromaldoxin, finally confirmed that the molecular

formula of this compound is $C_{17}H_{15}ClO_8$. The ^1H NMR spectrum (Fig. 3.60) showed the presence of one methyl singlet at δ_H 2.09, two methoxy signals at δ_H 3.65 and δ_H 3.90, a pair of *meta*-coupled aromatic protons at δ_H 5.73 (H-5) and δ_H 6.40 (H-3), and one singlet aromatic proton at δ_H 7.01 (H-2'). The HMBC spectrum (Fig. 3.61) of this compound was found to have close similarity with dechlorodihydromaldoxin, suggested a similar structure. Based on mass spectral and 1D and 2D NMR data in comparison to the published data, it was confirmed that **3.27** is pestheic acid, previously isolated from *Pestalotiopsis* sp (Ogawa *et al.*, 1995).

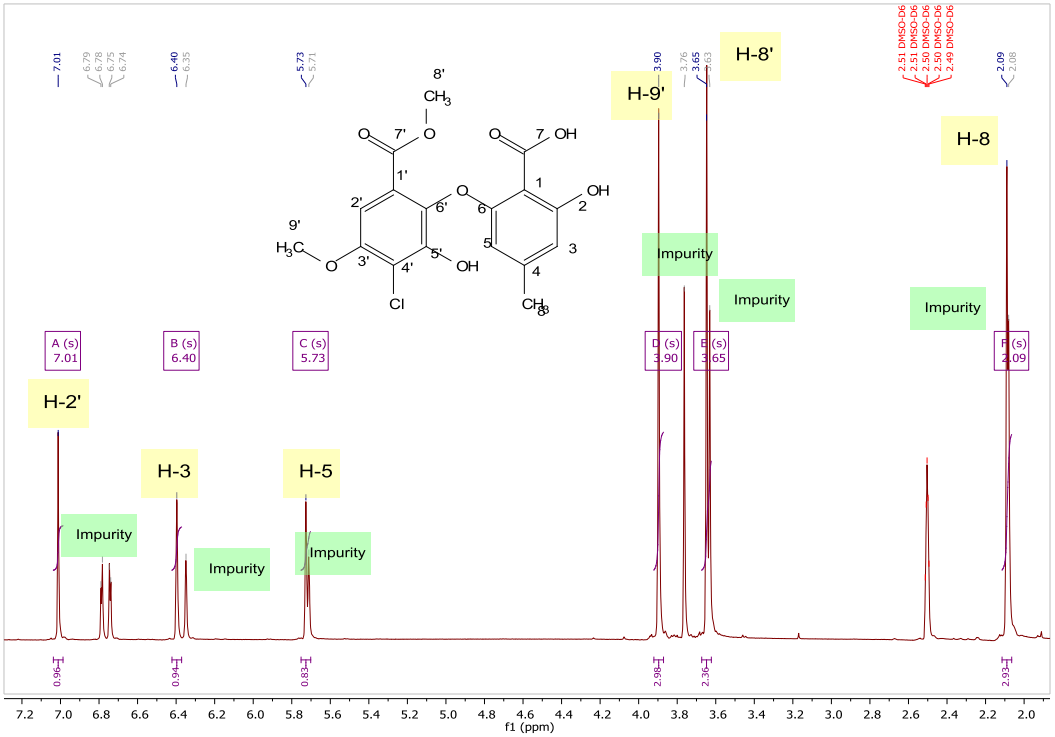


Fig. 3.60: ^1H NMR of pestheic acid

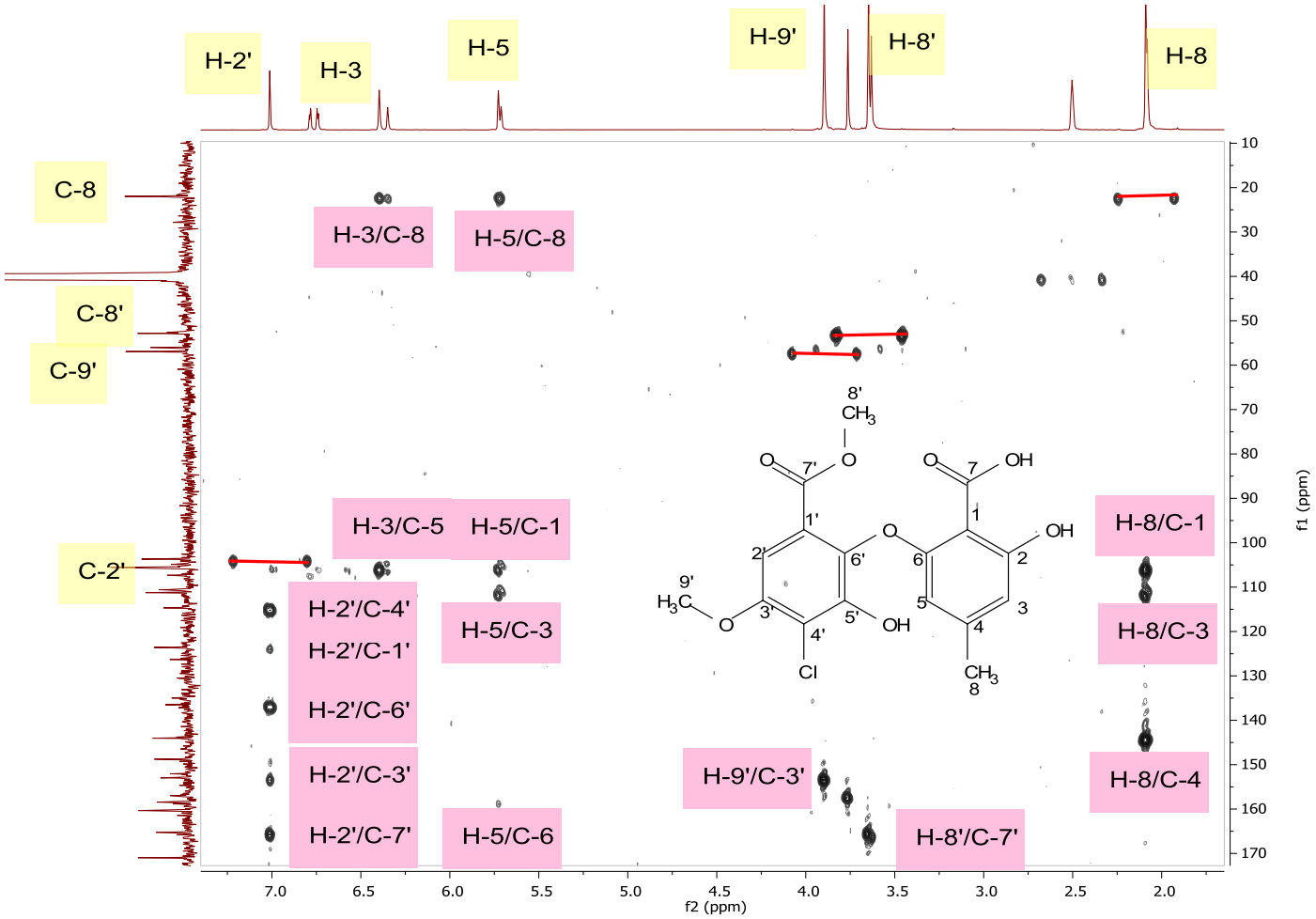
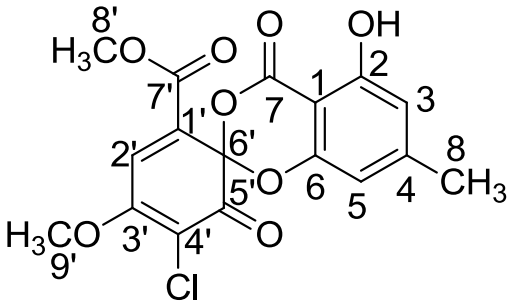
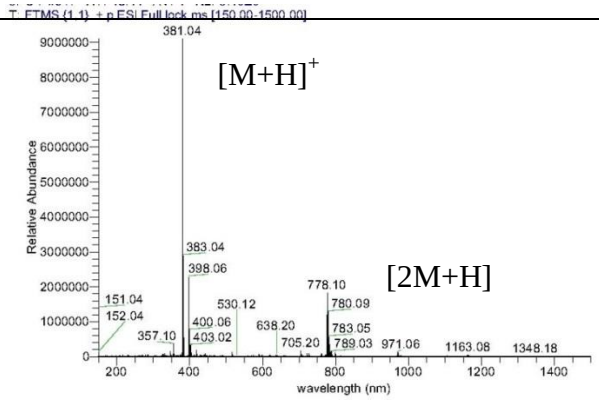


Fig. 3.61: HMBC of pestheic acid

Table 3.25: ^{1}H and ^{13}C NMR data of pestheic acid at 400 MHz in DMSO-*d6*

Position	Pestheic acid in DMSO-*d6*			Literature(Ogawa *et al.*, 1995) in DMSO-*d6*	
	δ_H (*J* Hz)	δ_C	HMBC	δ_H (J Hz)	δ_C
1		105.0			105.0
2		159.8			159.8
3	6.40, d (*J*=0.8)	110.7	5, 8	6.39, d (*J*=0.7)	110.7
4		143.4			143.4
5	5.73, d (*J*=0.8)	105.2	1, 3, 8	5.73, d (*J*=0.7)	105.2
6		157.8			157.8
7		170.4			170.4
8	2.09 (s)	21.3		2.09 (s)	21.3
1’		123.0			123.0
2’	7.01 (s)	103.1	1’, 3’, 4’, 6’, 7’	7.01 (s)	103.1
3’		152.4			152.4
4’		114.1			114.1
5’		148.2			148.2
6’		136.0			136.0
7’		164.6			164.6
8’	3.65 (s)	52.2	7’	3.65 (s)	52.2
9’	3.90 (s)	56.4		3.89 (s)	56.4

3.4.4.3 Maldoxin (known compound)

Maldoxin (3.28)
Synonym(s): Methyl 5'-chloro-5-hydroxy-4'-methoxy-7-methyl-4,6'-dioxo-4H-spiro[benzo[d][1,3]dioxine-2,1'-cyclohexa[2,4]diene]-2'-carboxylate Source: *P. olivacea* (isolated from *V. pinnata)* Sample amount: 10 mg Physical description: Yellow powder Molecular formula: $C_{17}H_{13}ClO_8$ Molecular weight: 380.73 g/mol



Compound **3.28** was isolated as a yellow powder (10 mg). The molecular weight of 380.73 g/mol was deduced from the positive and negative molecular ion peaks at *m/z* 381.0387 $[M+H]^+$ (base peak) in HR-LCMS. A Cl atom was detected in the compound as the base peak had an intensity ratio of 3:1. The ^1H NMR spectrum (Fig. 3.62) showed one methyl singlet at δ_H 2.26, two methoxy peaks at δ_H 3.84 (H-8') and δ_H 4.16 (H-9'), one hydroxyl singlet at δ_H 9.85, a pair of *meta*-coupled aromatic protons at δ_H 6.15 (H-5) and δ_H 6.46 (H-3) and one aromatic proton singlet at δ_H 7.61 (H-2'). The ^1H NMR spectrum of compound **3.28** was found to be similar to pestheic acid; however,

the mass of compound **3.37** is two units less than that of pestheic acid. In the HMBC spectrum, the chemical shift of compound **3.37** at position C-1 (δc 96.8) and C-6' (δc 94.2) were relatively upfield because of the de-shielding effect of C-7 and C-5'. The HMBC experiment (Fig. 3.63) showed correlations of the *meta*-coupled proton in the A ring, H-3, to C-1, C-2, C-5, C-7 and C-8 as well as H-5 to C-1, C-3, C-7 and C-8. Also, in the B ring, the proton singlet H-2' showed a correlation to C-2', C-4', C-6', and C-7'. The NMR and mass spectral data obtained were found to be identical with published data for maldoxin, which was previously isolated from *Xylaria* sp (Adeboya *et al.*, 1996) and was synthesised by Yu and Snider in 2011(Yu and Snider, 2011a).

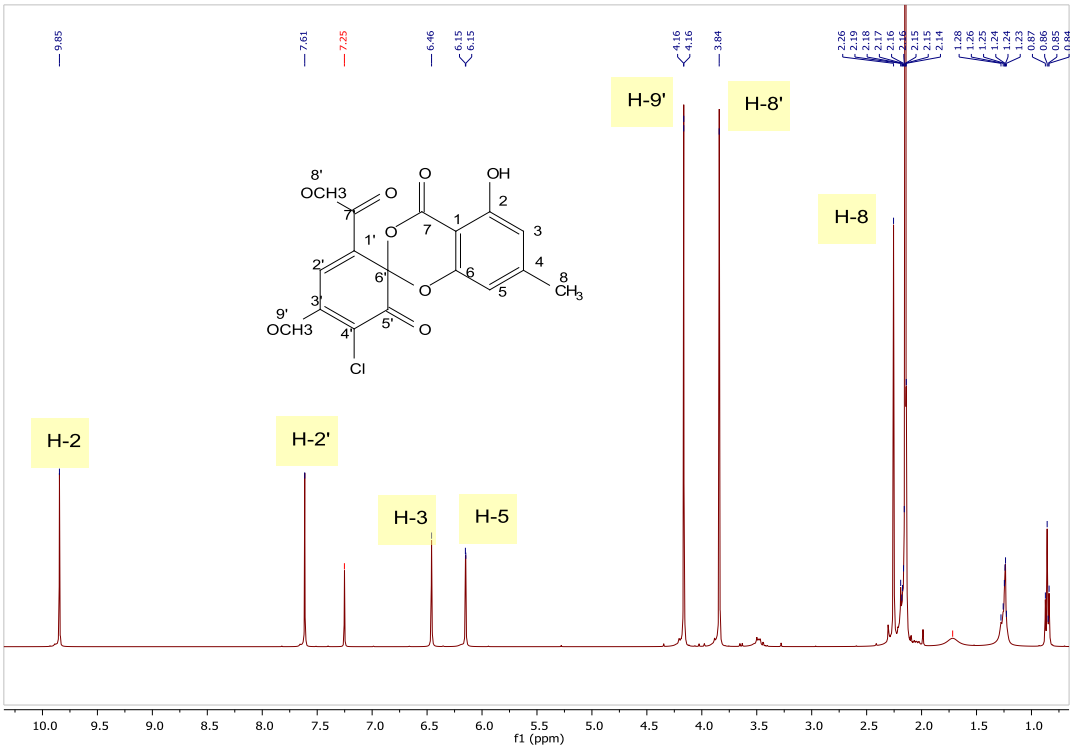


Fig. 3.62: ^{1}H NMR of maldoxin

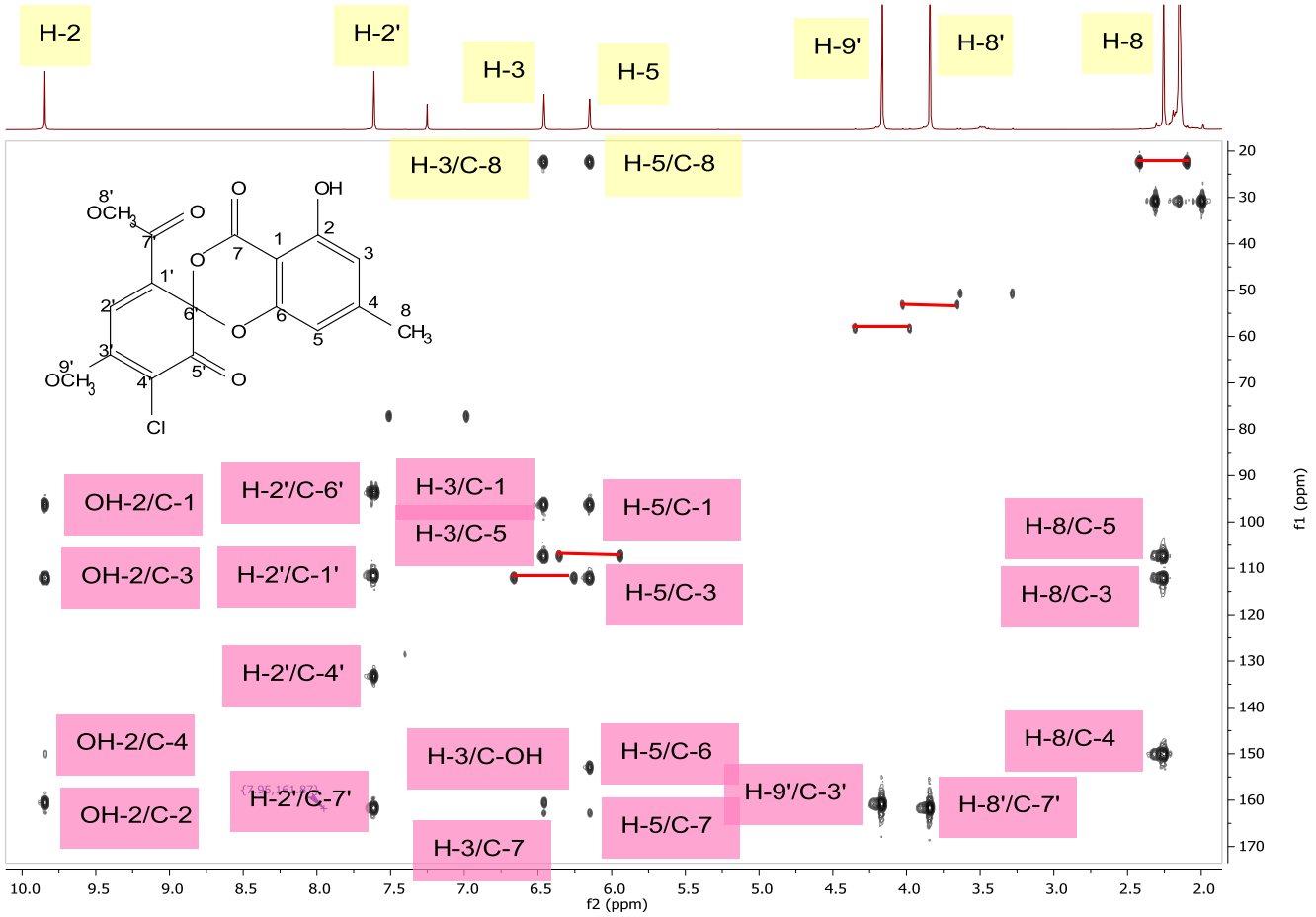
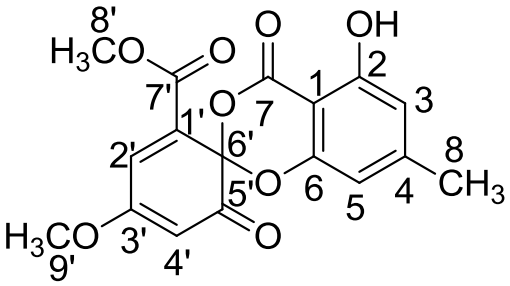
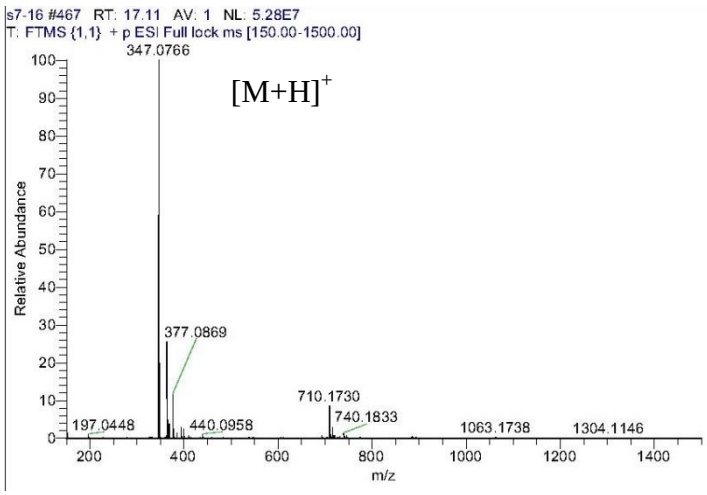


Fig. 3.63: HMBC of maldoxin

Table 3.26: ^{1}H and ^{13}C NMR data of maldoxin at 400 MHz in Chloroform-*d*

Position	Maldoxin in Chloroform-*d*			Literature(Adeboya *et al.*, 1996) in Chloroform-*d*	
	δ_H (*J* Hz)	δ_C	HMBC	δ_H (J Hz)	δ_C
1		96.4			96.4
2		160.7	1, 3, 4, 7		160.7
OH-2	9.85 (s)			9.87 (s)	
3	6.46, d (*J*=1.4)	112.1	1, 5, 2-OH, 7,8	6.48, d (*J*=1.1)	111.7
4		112.5			112.3
5	6.15, d (*J*=0.8)	107.5	1, 3, 6, 7, 8	6.17, d (*J*=1.1)	107.5
6		153.1			153.1
7		162.9			162.9
8	2.26 (s)	22.5	3, 4, 5	2.28 (s)	22.5
1’		133.4			133.4
2’	7.61 (s)		1’, 6’, 7’	7.63 (s)	128.6
3’		161.0			161.0
4’		150.1			150.2
5’					183.1
6’		93.8			93.8
7’		161.8			161.8
8’	3.84 (s)	53.2	7’	3.86 (s)	53.2
9’	4.16 (s)	58.3	3’	4.18 (s)	58.3

3.4.4.4 Dechloromaldoxin (known compound)

Dechloromaldoxin (3.29)
Synonym(s): Methyl 5-hydroxy-4'-methoxy-7-methyl-4,6'-dioxo-4H-spiro[benzo[d][1,3]dioxine-2,1'-cyclohexa[2,4]diene]-2'-carboxylate Source: *P. olivacea* (isolated from *V. pinnata)* Sample amount: 7 mg Physical description: Yellow powder Molecular formula: $C_{17}H_{14}O_8$ Molecular weight: 346.29 g/mol



Compound **3.29** was isolated as a yellow powder (7.0 mg). The HR-LCMS exhibited a strong peak at 347.0760 $[M+H]^+$, suggesting the molecular formula of $C_{17}H_{14}O_8$. The ^1H NMR data (Fig. 3.64) showed a spectrum similar to that of maldoxin, suggesting a close relationship between the two structures. The similarities included proton signals at δ_H 2.25, two methoxy peaks at δ_H 3.82 and δ_H 3.86, one singlet hydroxyl at δ_H 9.94, a

pair of *meta*-coupled aromatic protons in the A ring at δ_H 6.15 (H-5) and δ_H 6.45 (H-3). The difference was that compound **3.29** contained one additional pair of aromatic *meta*-coupled protons in the B ring at δ_H 5.51 (H-4') and δ_H 7.23 (H-2'), replacing the Cl atom at H-4' position. Further structural investigation confirmed this by the HMBC experiment (Fig. 3.65) which showed correlations of OH-2 to C-1, C-2, C-3, and C-4, and of the methyl proton H-8 to C-1, C-3, C-4 and C-5. In addition to that, a *meta*-coupled proton in the A ring H-3, correlated with C-1, C-2, C-5, C-6, C-7 and C-8. H-5 also correlated with C-1, C-2, C-3, C-6, C-7 and C-8. In the B ring, a *meta*-coupled proton, H-2', showed correlations to C-1', C-3', C-4', C-6', and C-7'. H-4' correlated with C-2', C-3', C-5' and C-6. The structure was further identified as dechloromaldoxin based on comparison of the NMR and mass spectral data with published data. Dechloromaldoxin was previously isolated from *Xylaria* sp (Adeboya *et al.*, 1996) and was synthesised in 2011(Yu and Snider, 2011a).

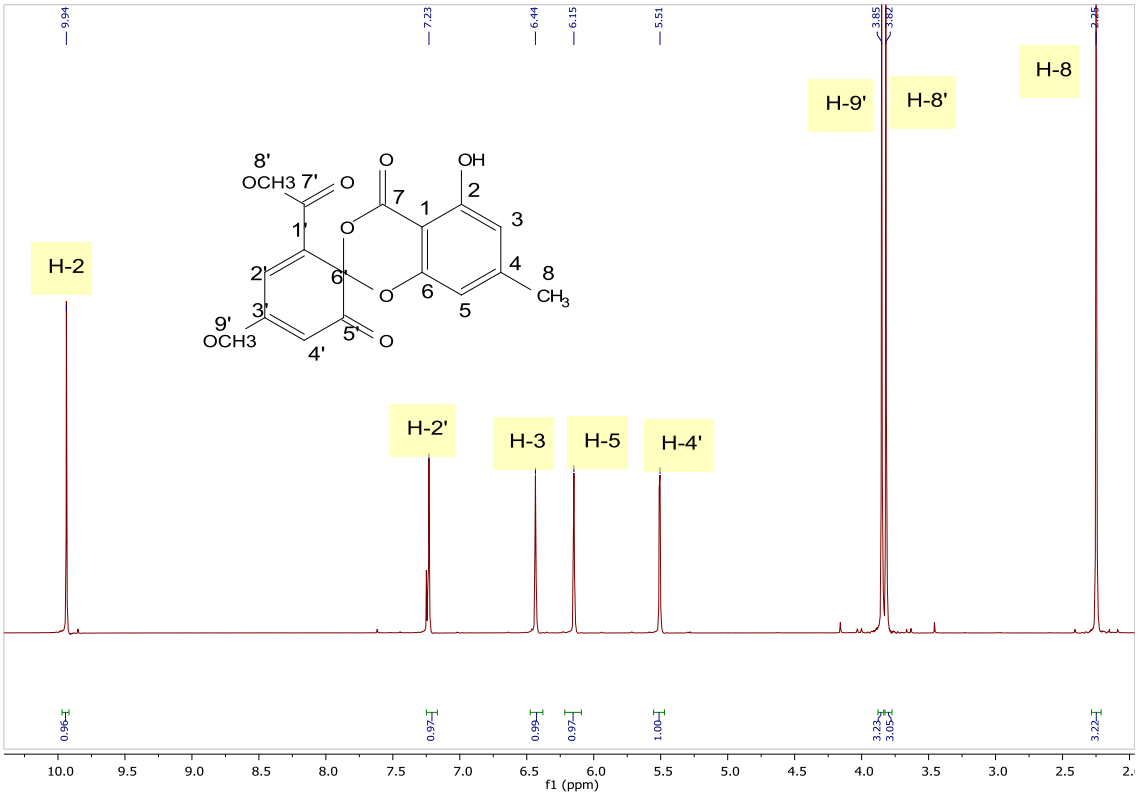


Fig. 3.64: ^{1}H NMR of dechloromaldoxin

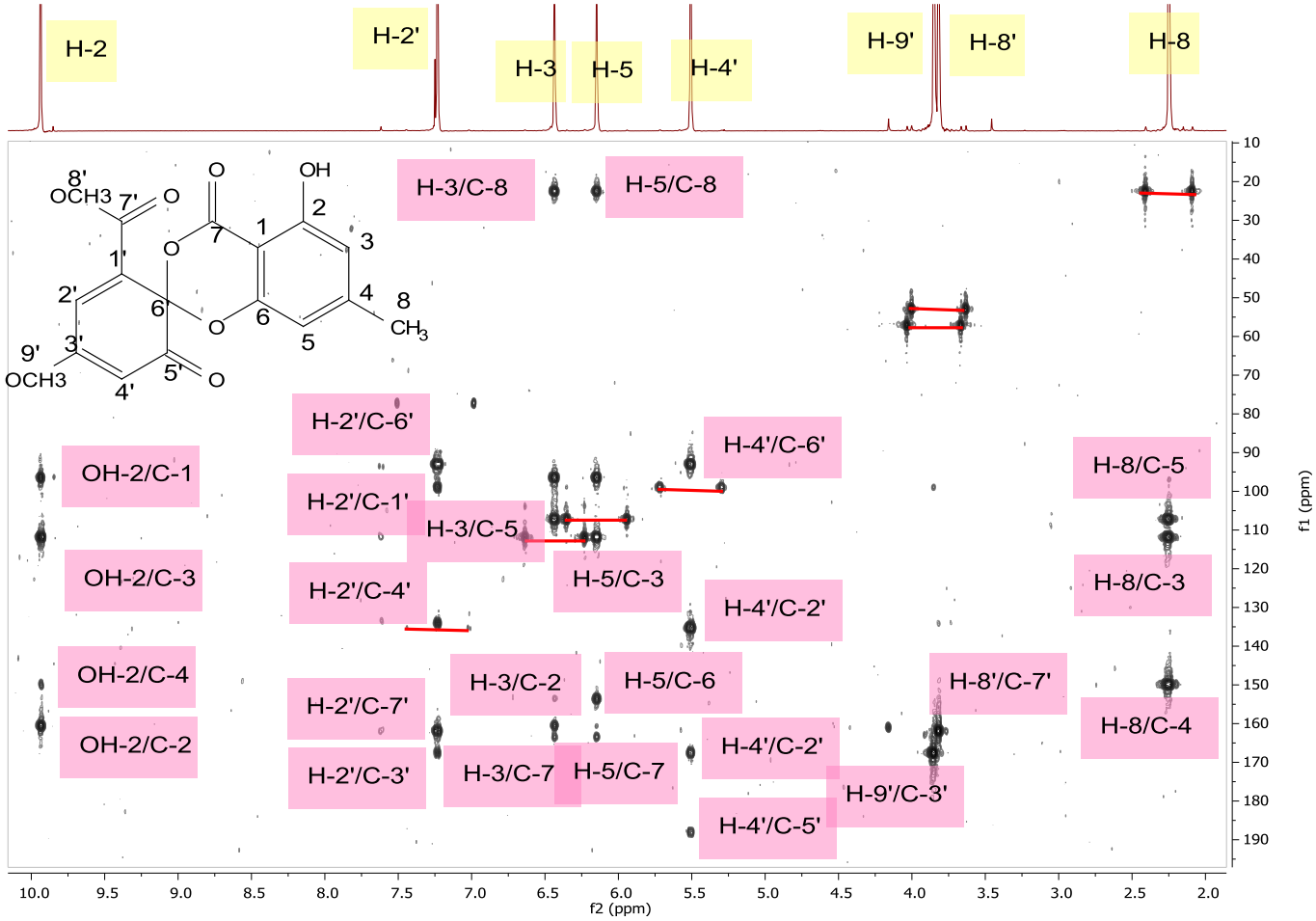
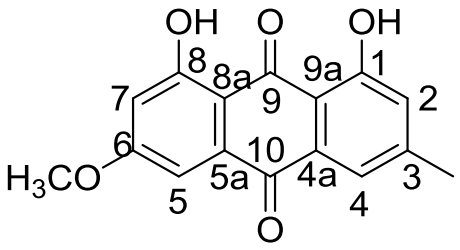
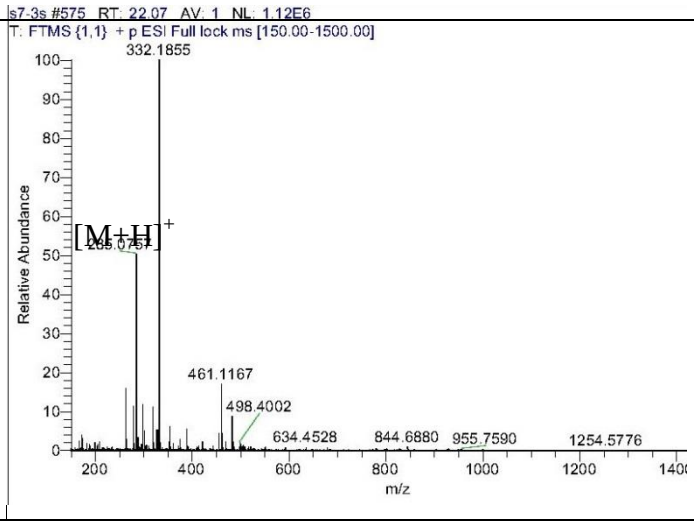


Fig. 3.65: HMBC of dechloromaldoxin

Table 3.27: ^{1}H and ^{13}C NMR data of dechloromaldoxin at 400 MHz in Chloroform-*d*

Position	Dechloromaldoxin in Chloroform-*d*			Literature (Yu and Snider, 2011a) in Chloroform-*d*	
	δ_H (*J* Hz)	δ_C	HMBC	δ_H (J Hz)	δ_C
1		96.8			96.4
2	9.94		1, 2, 3, 4	9.96 (s)	
OH-2		160.9			160.5
3	6.44 (s)	112.0	1,2,5, 6, 7,8	6.46 (s)	111.8
4		150.5			149.9
5	6.15 (s)	108.0	1, 2, 3, 6, 7, 8	6.17 (s)	107.2
6		153.5			153.6
7		163.9			163.3
8	2.26 (s)	22.9	1, 3, 4, 5	2.28 (s)	22.4
1’		134.9			134.0
2’	7.23, d (*J*=2.5)	136.1	1’, 3’, 4’, 6’, 7’	7.25, d (*J*=1.8)	135.3
3’		168.0			167.5
4’	5.51, d (*J*=2.5)	99.3	2’, 3’, 5’, 6’	5.53, d (*J*=1.8)	98.9
5’		188.1			188.1
6’		99.3			93.7
7’		162.2			161.9
8’	3.82 (s)	53.5	7’	3.84 (s)	52.8
9’	3.86 (s)	56.5	3’, 4’	3.88 (s)	57.1

3.4.4.5 Physcion (known compound)

Physcion (3.30)
Synonym(s): 6-methylemodin, 1,8-dihydroxy-3-methoxy-6-methylanthracene-9,10-dione, parietin Source: *P. olivacea* (isolated from *V. pinnata)* Sample amount: 2 mg Physical description: Yellow powder Molecular formula: $C_{16}H_{12}O_5$ Molecular weight: 284.26 g/mol



Compound **3.30** was obtained as a yellow powder (2.0 mg). The molecular formula was deduced as $C_{16}H_{12}O_5$ based on the HR-LCMS peak in the positive mode at 285.0757 $[M+H]^+$. The ^1H NMR data (Fig. 3.66) showed one methyl singlet at δ_H 2.45, one methoxy peak at δ_H 3.93 (OCH_3-6), two hydroxyl singlets at δ_H 12.10 (OH-1) and 12.30 (OH-8), and two pairs of *meta*-coupled aromatic protons in the A ring at δ_H 7.07 (H-2) and δ_H 7.62 (H-4) and in the B ring at δ_H 7.35 (H-5) and δ_H 6.67 (H-7), respectively. In the HMBC spectrum (Fig. 3.67) it was seen that protons at positions H-4 and H-5 were

associating with carbon signals at δ_C 182.7 (C-9) and 182.2 (C-10), respectively, indicating the presence of a *para* benzoquinone in the structure. The ^{13}C NMR, however, was not executed due to the limited amount of material. With the aid of mass spectral, ^{1}H and HMBC data, the structure of the compound was constructed and it was found to be similar to physcion which was previously reported to be in *Penicillium* sp., (Wang *et al.*, 2008) marine alga-derived fungus *Aspergillus wentii* EN-48 (Li *et al.*, 2014b), marine sponge-associated fungus *Eurotium cristatum*, (Gomes *et al.*, 2012) lichen *Canderalia concolor*, (Salinas *et al.*, 2012) and rhubarb (Danielsen *et al.*, 1992).

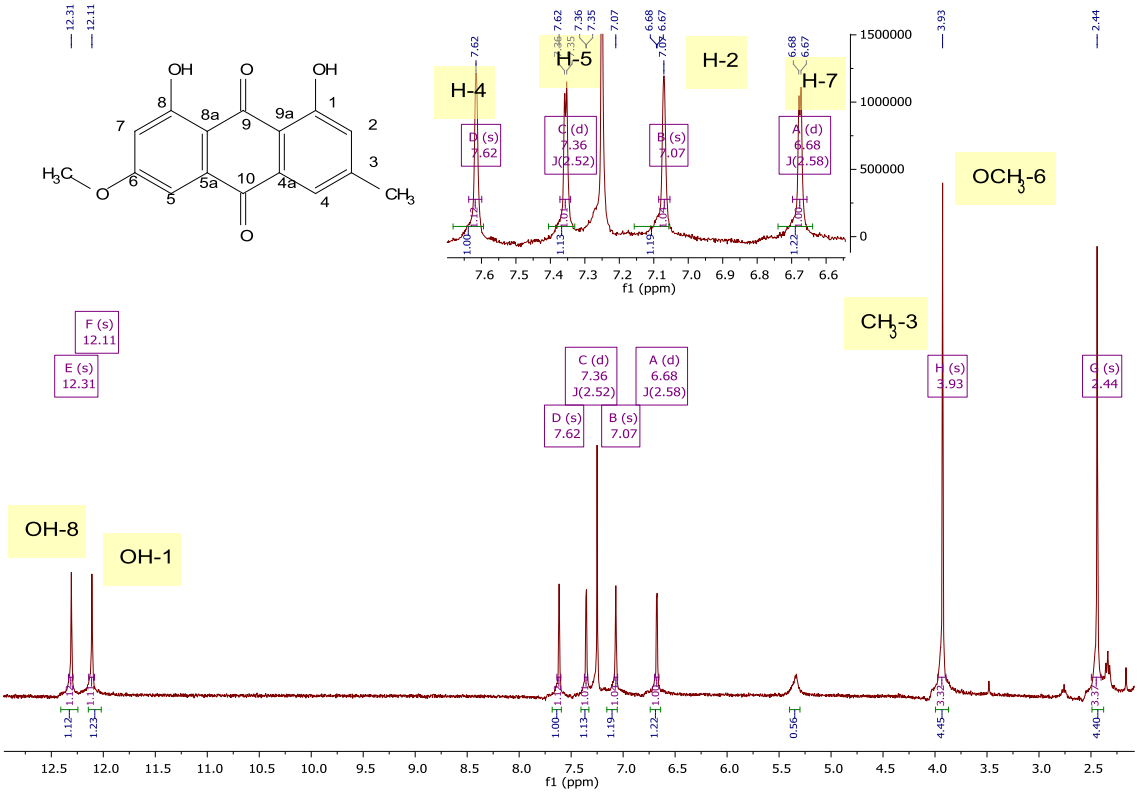
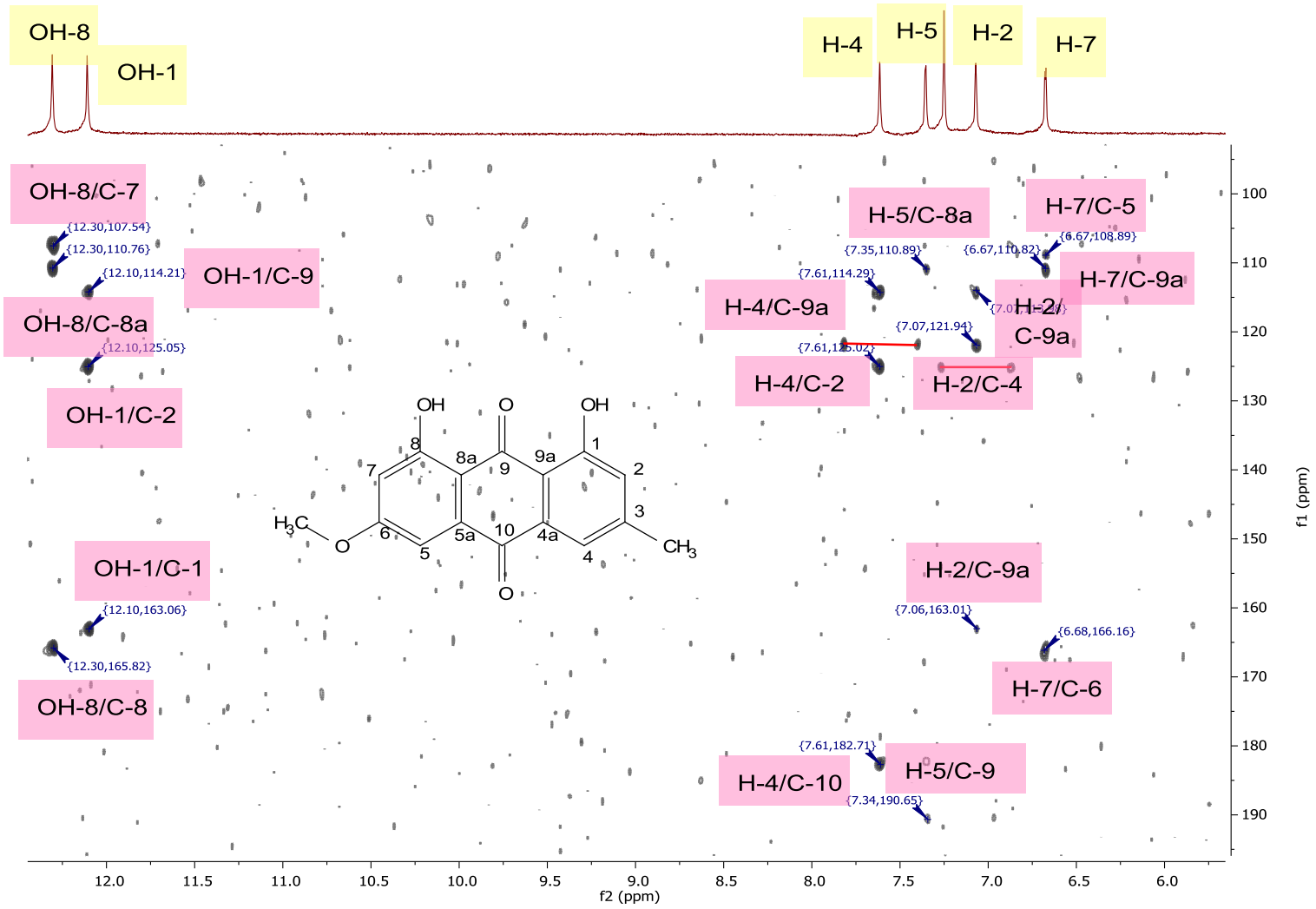


Fig. 3.66: ^{1}H NMR of physcion


OH-8
OH-1
H-4
H-5
H-2
H-7
OH-8/C-7
{12.30,107.54}
{12.30,110.76}
{12.10,114.21}
OH-1/C-9
OH-8/C-8a
{12.10,125.05}
OH-1/C-2
OH-1/C-1
{12.10,163.06}
{12.30,165.82}
OH-8/C-8
H-5/C-8a
{7.35,110.89}
H-7/C-5
{6.67,108.89}
{6.67,110.82}
H-7/C-9a
{7.61,114.29}
H-4/C-9a
H-2/C-9a
{7.07,121.94}
{7.61,125.02}
H-4/C-2
H-2/C-4
H-2/C-9a
{7.06,163.01}
{6.68,166.16}
H-7/C-6
{7.61,182.71}
H-4/C-10
H-5/C-9
{7.34,190.65}
OH
O
OH
8
8a
9
9a
1
2
7
3
H3C
O
6
5
5a
10
4a
4
CH3
O
12.0
11.5
11.0
10.5
10.0
9.5
9.0
8.5
8.0
7.5
7.0
6.5
6.0
f2 (ppm)
100
110
120
130
140
150
160
170
180
190
f1 (ppm)

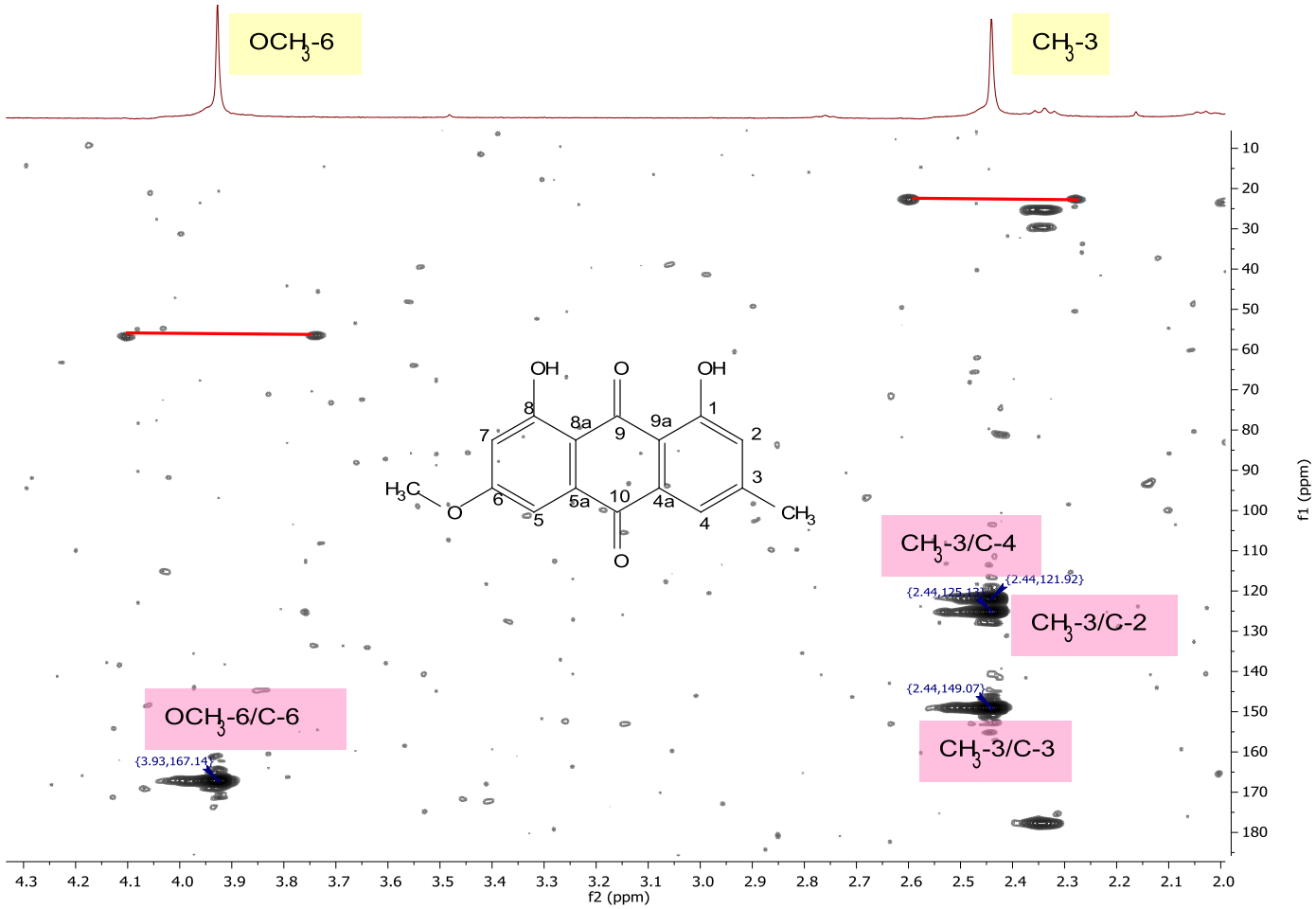


Fig. 3.67: HMBC NMR spectrum of physcion (upper and below)

Table 3.28: ^{1}H and ^{13}C NMR data of physcion at 400 MHz in Chloroform-*d*

Position	Physcion in $CDCl_3$			Literature (Danielsen *et al.*, 1992) in $CDCl_3$	
	δ_H (*J* Hz)	δ_C	HMBC	δ_H (J Hz)	δ_C
1		163.0			162.5
OH-1	12.10, s		1, 2, 9a	12.10, s	
2	7.07, s	125.0	1, 4, 9a	7.07, s	124.5
3		149.1			148.4
CH_3-3	2.45, s	22.5	2, 3, 4	2.44, s	22.2
4	7.62, s	122.0	2, 9a, 10	7.62, d (*J*=0.7)	121.3
4a					133.3
5	7.35, d (*J*=2.5)	108.9	8a, 9	7.36, d (*J*=2.5)	108.2
5a					135.3
6		167.1			166.7
OCH_3-6	3.93, s		6	3.93, s	
7	6.67, d (*J*=2.5)	107.5	5, 6, 8a	6.68, d (*J*=2.5)	106.8
8		165.8			165.2
OH-8	12.30, s	56.9	7, 8, 8a	12.30, s	56.1
8a		110.8			110.3
9		190.7			190.8
9a		114.3			113.7
10		182.7			182.1

3.4.5 Bioactivities of isolated compounds

Table 3.29: Antitrypanosomal and anti-mycobacterial activities of isolated compounds obtained from *Pestalotiopsis olivacea* fermented in solid rice culture for thirty days.

Compound	**Antitrypanosomal activity** ***(T. brucei brucei)***	**Anti-mycobacterial activity** **(*M. marinum*)**
	MIC (µM)	**MIC (µM)**
Dechlorodihydromaldoxin **3.26**	> 100	> 100
Pestheic Acid **3.27**	> 100	> 100
Maldoxin **3.28**	> 100	78.8
Dechloromaldoxin **3.29**	> 100	**14.4**
Physcion **3.30**	> 100	> 100
Stigmasterol **3.2**	**21.1**	> 100
Suramin	0.1	
Gentamycin		13.5

From the bioactivity results (Table 3.29) only stigmasterol **3.2** was active against *T. brucei brucei* with MIC value of 21.1 µM while dechloromaldoxin 3.29 exhibited strong anti-mycobacterial activity against *M. marinum* with MIC value of 14.4 µM, respectively.

CHAPTER 4

4 Discussion

4.1 Bioactive Secondary Metabolites from the leaves of *Vitex pinnata*

4.1.1 Flavones isolated from *V. pinnata*

The vibrant pigments such as red, blue, and purple found in plants have sparked scientists' interest to study about it and later they discovered that flavonoids are the compounds that play role in pigmentation. Fascinatingly these compounds also were found to have numerous applications in pharmaceutical and dietary functions especially as antioxidant, antianxiety, antimicrobial and anti-inflammatory agents (Hollman and Katan, 1999; Kitamura, 2006; Ross and Kasum, 2002; Viola *et al.*, 1995). Flavonoids are a group of phenylbenzopyran molecules which have a C_6-C_3-C_6 carbon framework. These groups may be divided into three classes including the flavonoids (2-phenylbenzopyrans) **4.1**, isoflavonoids (3-benzopyrans) **4.2**, and the neoflavonoids (4-benzopyrans) **4.3**.

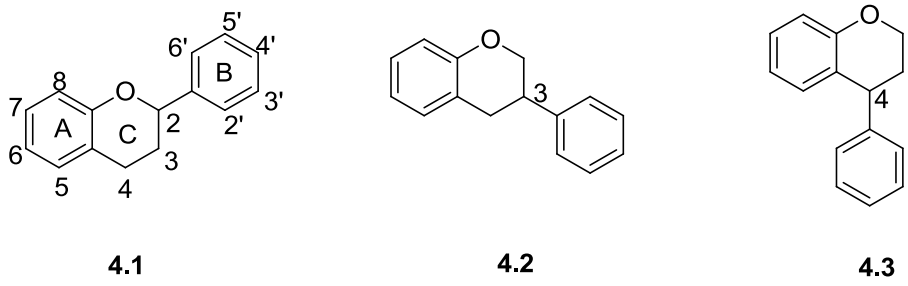


Fig. 4.1: Classes of flavonoids

The flavonoids (2-phenylbenzopyrans) **4.1** can be divided into the following groups such as flavan **4.4**, flavanone **4.5**, flavone **4.6**, flavonol **4.7**, dihydroflavonol **4.8**, flavan-3-ol **4.9**, flavan-4-ol **4.10** and flavan-3, 4-diol **4.11**.

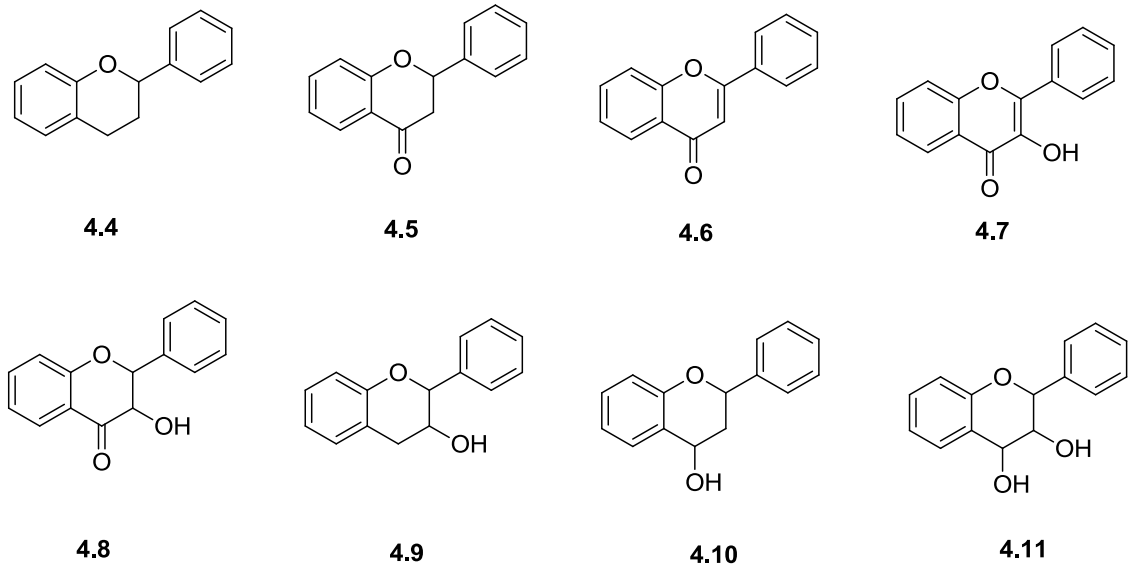
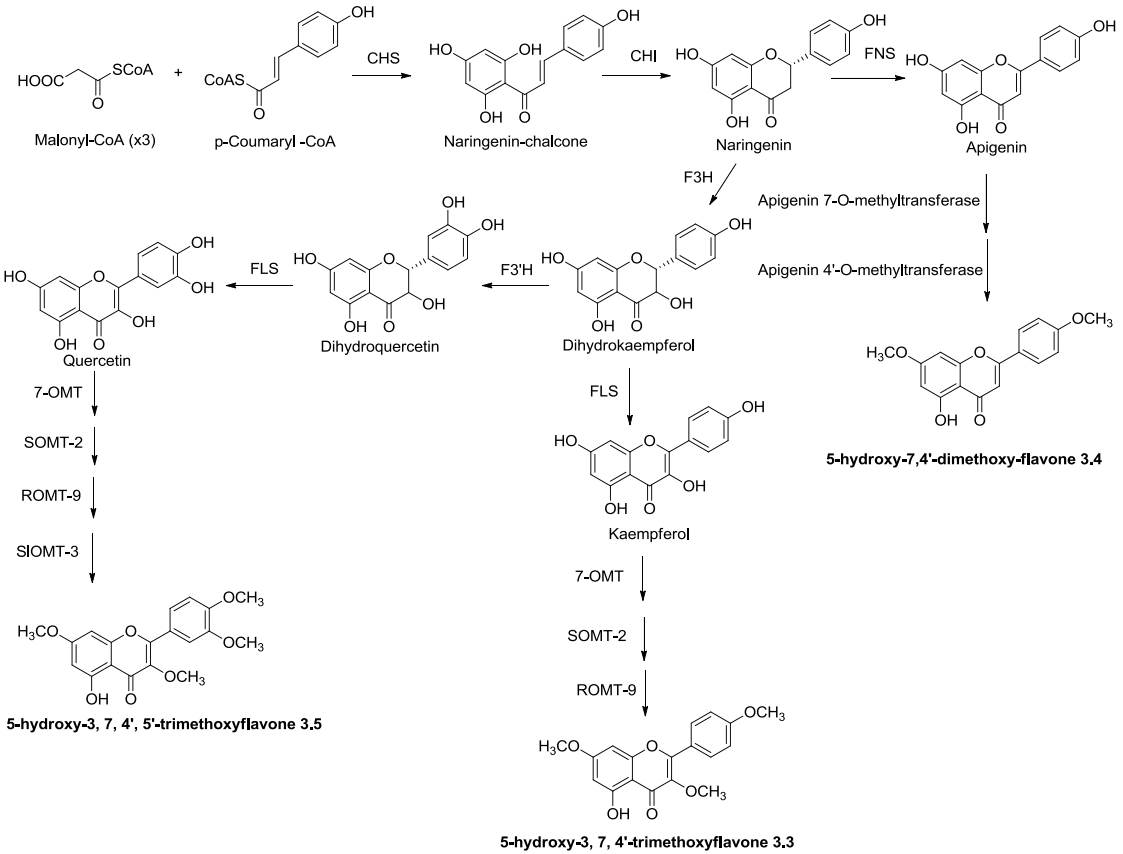


Fig. 4.2: Type of flavonoids (2-phenylbenzopyrans)

In this study, three flavone types of compounds were isolated from *V. pinnata* which are 5-hydroxy-3,7,4'-trimethoxyflavone **3.3**, 5-hydroxy-7,4'-dimethoxyflavone **3.4** and retusin **3.5** respectively. The biosynthesis of these three flavones are proposed (see Scheme 4.1) from the reaction between *p*-coumaryl and three molecules of malonyl-coenzyme A (CoA; via the fatty acid pathway) to produce naringenin-chalcone compound. Then chalcone isomerase (CHI) was catalysed and naringenin was produced as a key substrate of these three flavones. In next step flavone synthase (FNS) was involved to produce apigenin. Finally apigenin 7-O-methyltransferase and apigenin 4'-O-methyltransferase were catalysed for methylation and therefore 5-hydroxy-7,4'-dimethoxy-flavone was formed (Fig. 2.10). The other two flavone biosynthesis were also proposed in Scheme 4.1 (Berim and Gang, 2013; Cho *et al.*, 2012; Jung *et al.*, 2000; Kim *et al.*, 2006).



Scheme 4.1: Schematic diagram of proposed biosynthesis of flavones 2, 3 and 4. Enzyme abbreviations: CHS, chalcone synthase; CHI, chalcone isomerase; FNS, flavone synthase; F3H, flavanone 3-hydroxylase; F3'H, flavonoid 3'-hydroxylase; FLS, flavonol synthase; 7-OMT, 3-methylquercetin 7-O-methyltransferase; SOMT-2, soybean O-methyltransferase-2; ROMT-9, rice O-methyltrasnferase-9; SIOM based on Berim and Gang (2013); Cho *et al.* (2012); Jung *et al.* (2000); Kim *et al.* (2006)

4.1.2 Role of isolated flavones from *V. pinnata* as anticancer, anti-inflammatory and antimicrobial agents

Reactive oxygen species (ROS) including superoxide anion ($O_2^{\bullet -}$), hydroxyl radicals ($OH^{\bullet}$), singlet oxygen ($^1O_2$) and hydrogen peroxide (H_2O_2) play an essential role in the pathogenesis of numerous diseases such as inflammation, diabetes, cancer, cardiovascular diseases, degenerative diseases, anaemia and ischemia (Aruoma, 1998). The activated macrophages which responsible to release pro-inflammatory cytokines such as TNF-α, HMGB1 and interleukin-1 are believed to have an important role in eliciting the local inflammatory response (Baumann and Gauldie, 1994). Moreover the over production of these pro-inflammatory cytokines stimulated by ROS are understood to cause severe injuries which cause cell death (Wang *et al.*, 2004). TNF-α is known to activate transcription factors such as NF-κB and the genes induced by transcription factors including IL-6, IL-8 are responsible toward acute inflammatory response (Barnes, 1997). The association of inflammation and cancer has been well recognised in many types of cancer and inflammation has been regarded as the 'seventh hallmark of cancer' (Mantovani et al, 2008; Mantovani, 2009). Accumulating evidence has shown that TNF-α is a key mediator of inflammation and cancer (Sethi et al, 2008; Balkwill, 2009). The phenolics and flavonoids exhibited outstanding anti-inflammatory and anticancer activities (Chahar *et al.*, 2011; Talhouk *et al.*, 2007; Zhang *et al.*, 2011a) and these properties were extensively studied in plenty animal models (Chahar *et al.*, 2011; Talhouk *et al.*, 2007). In my study, TNF-α -induced NF-κB activity on K562 human chronic leukemia cells were examined and it was found that compound **3.3** and **3.5** were significantly inhibit TNF-α -induced NF-κB activation with IC_{50} of 52.1 and 10.0μM respectively. Previous study showed retusin **3.5** inhibited breast cancer resistance protein (BCRP) expression in MDCK BCRP and MCF-7 MX cell lines by using Hoechst 33342 assay with IC_{50} of 0.39 ± 0.14 and 0.90 ± 0.32 μM respectively (Pick *et al.*, 2011). It was reported by other study that BCRP is down regulated by selectively inhibiting the PI3K/AKT/NF-kB signalling pathway which play an important role in HER2-mediated chemo resistance of MCF7 cells (Zhang *et al.*, 2011b).

As we know plant antioxidant agents play a crucial role in preventing the generation of free radicals which can cause diseases related to oxidative stress including

inflammation (Akinmoladun *et al.*, 2010). Even though antioxidant activity was not examined in this study, however previous studies showed that compound **3.3** exhibited good antioxidant activity. Previous study showed that compound **3.3** isolated from leafy vegetable known as Cat's whiskers was an active antioxidant agent with IC_{50} of 0.085 mg/mL respectively (Kolak *et al.*, 2009). Recent study also revealed that this compound have strong effect on carrageenan-induced acute inflammation by reducing the swelling of hind paw in rats from 1 to 3 h upon carrageenan injection. Additionally the production of serum nitric oxide (NO) was found to be decreased. This compound also was tested on patients with rheumatoid arthritis. It was found that compound **3.3** inhibited the hydroxyl and NO radicals in the isolated peripheral blood mononuclear lymphocytes of patients. This further suggested that compound **3.3** diminished acute inflammation by NO inhibition and protecting the oxidative DNA (Bala *et al.*, 2013). Traditionally, the Malay community used leaves of *V. pinnata* to treat fever and wounds (Burkill, 1966) and based from this evidence, this suggested the anti-inflammatory response in treating fever and wounds were possibly a bioactivity of these two compounds.

Tumor necrosis factor-α (TNF-α) is a group of cytokines that involved systemic inflammatory reaction. Previous report describes the level of TNF-α was increased in the sera of bacterial, viral, and parasitic-infected patients including malaria and leishmaniasis (Barral-Netto *et al.*, 1991; Scuderi *et al.*, 1986; Shaffer *et al.*, 1991). The level of TNF-α in serum of *T. b. gambiense*-infected patient also corresponding with the disease severity (Okomo-Assoumou *et al.*, 1995). Other finding also reported the level of TNF-α was high in patient with late-stage of *T. b. gambiense* infection and was declined dramatically after treatment with melarsoprol (Rhind *et al.*, 1997). Interestingly, we found that compounds **3.3** and **3.5** which actively inhibited TNF-α -induced NF-κB activation also exhibited good antitrypanosomal activity with MIC of 19.0 and 17.0 μM, respectively. Previous study reported that compound **3.5** was inactive against *T. b. rhodesiense* and *T. cruzi* (IC_{50} >80 μM) however showed adequate antileismanial activity against *Leishmania donovani* with IC_{50} value of 21.77 μM (Tasdemir *et al.*, 2006)

In conclusion, this finding reported that compounds **3.3**, **3.4** and **3.5** were isolated efficiently for the first time from *V. pinnata* by using MPLC. It is also described their

promising cytotoxicity activity on K562 human chronic leukemia cells. In this thesis, I reported a good antitrypanosomal activity of these compounds against *T. brucei brucei* for the first time. In addition, Malaysian *V. pinnata* did not yield any diterpenes, iridiods, and ecdysteroids as earlier reported for the genus *Vitex*.

4.2 Bioactive secondary metabolites from *L. theobromae*

4.2.1 Metabolomics and bioassay-guided isolation as decision-making tools in mining bioactive metabolomes

Bioassay-guided screening and isolation is widely used in natural products research. However a common problem of this method is the generation of false positive hits due to the complex chemical composition of natural extracts (Keserű and Makara, 2006). One of many approaches to improve the bioassay-guided isolation method is by adopting a metabolomics approach (LCMS and/or NMR) in the strategy. Yuliana *et al.* (2011) published a review in utilising metabolomics in natural products research to identify and pinpoint the bioactive compounds prior to a labour-intensive isolation work. A research group in Leiden university successfully applied NMR-metabolomics to identify active metabolites on the adenosine A1 binding receptor from plant extracts (Ali *et al.*, 2013; Yuliana *et al.*, 2013). Other groups showed the application of HR-LCMS and quantitative NMR metabolomics with zebrafish bioassay screening of plant extracts which proved to be an efficient and robust strategy to isolate anti-angiogenesis and anti-inflammatory metabolites (Bohni *et al.*, 2013). In our group, other colleagues were effectively using the metabolomics approach to isolate active anti-trypanosomal compounds known as Actinosporins A and B from a marine sponge associated-*Actinokineospora* sp. EG49 (Abdelmohsen *et al.*, 2014).

4.2.2 Related bioactivities of isolated compounds and their known derivatives

In this study, two known active antitrypanosome compounds were isolated from fraction two and identified as cladospirone-B **3.11**, desmethyl-lasiodiplodin **3.13**, together with other compounds identified as R-(-)-mellein **3.14** and adenosine **3.15**. Palmarumycin CP17 **3.10** was also predicted as one of active metabolites however due to low yield, palmarumycin CP17 was not isolated in this study. Palmarumycin CP_{17} was previously reported as a promising drug lead against *Leishmania donovani* with IC_{50} of 1.34 uM. Palmarumycin CP17 was inactive against *Plasmodium falciparum* and

Trypanosome cruzi at a concentration of 10 μg/mL indicating its selectivity against *Leishmania* parasites (Martínez-Luis *et al.*, 2008). In other fraction adenosine **3.15**, was also isolated by applying robust and reproducible MPLC techniques.

Spirobisnaphthalenes (also known as bisnaphthospiroketals) are secondary metabolites found in fungi such as *Decaisnella thyridioides* (Jiao *et al.*, 2006a), *Edenia gomezpompae* (Macías-Rubalcava *et al.*, 2008), *Preussia* sp (Chen *et al.*, 2009) and *Rhytidhysteron* sp. AS21B (Pudhom *et al.*, 2014). It consists of two naphthalene units (1,8-dihydroxynaphthalene, DHN) connected through a spiroketal linkage with either two or three oxygens (Cai *et al.*, 2010). This type of compound can be divided into three subgroups, which are palmarumycin **4.12**, preussomerin **4.13,** and spiroxin **4.14** (Fig. 3.32). The biosynthetic studies of these types of compounds were established elsewhere (Bode and Zeeck, 2000; Cai *et al.*, 2010). Cladospirone-B **3.11** is a derivative of spirobisnaphthalene, which was isolated for the first time from *L. theobromae* in this study. Previously it had been isolated from *Sphaeropsidales* sp F-24-707 (Bode *et al.*, 2000). The biosynthetic pathway of cladospirone-B **3.11** was depicted *via* the intermediary of DHN **4.15** followed by the epoxidation of palmarumycin CP1 **4.16**, which produced palmarumycin C2 **4.17** that was later oxidized to form palmarumycin C3 **4.18**, which becomes cladospirone-B **3.11** via the reductive opening of the epoxide (see Scheme 4.2).

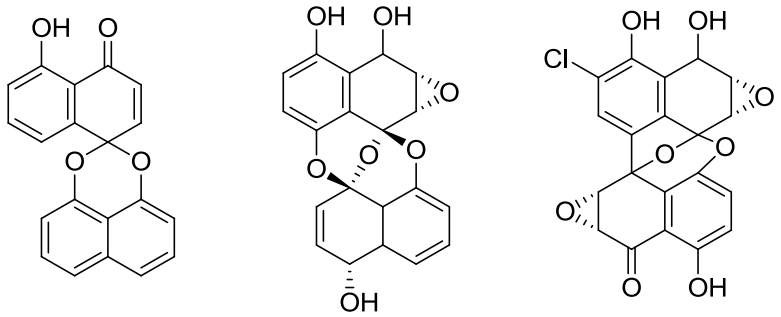
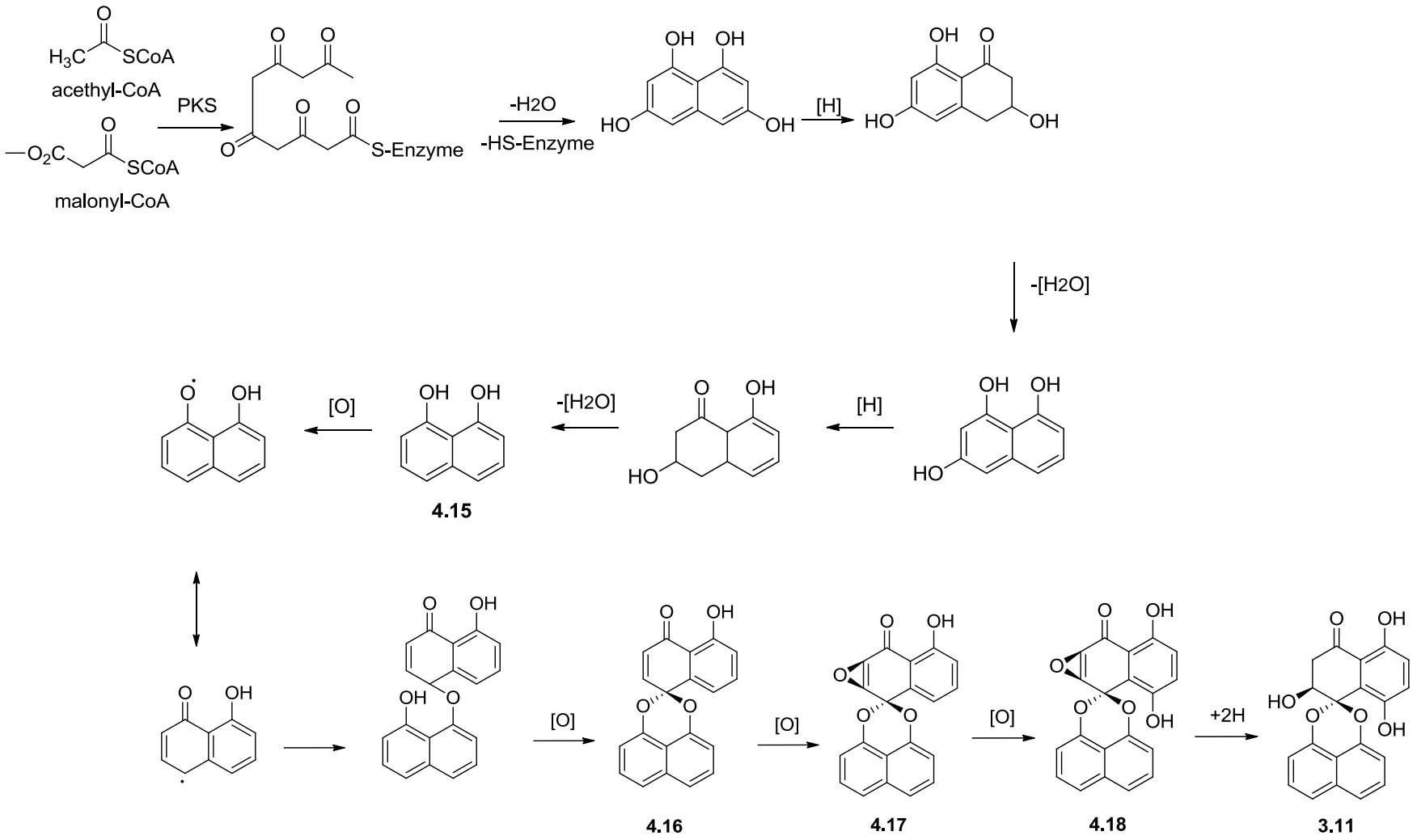


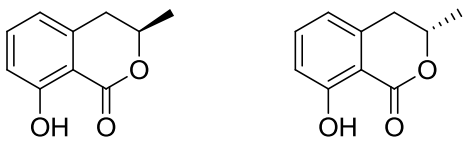
Fig. 4.3: Spirobisnaphthalene types of metabolites



Scheme 4.2: Postulated biosynthetic pathway of cladospirone-B based on Bode and Zeeck (2000); Cai *et al.* (2010)

Desmethyl-lasiodiplodin **3.13**, was a major secondary metabolite found in fraction 2, having a percentage yield of 4.87%. Interestingly, several previous studies have reported the isolation of compound **3.13** from *L. theobromae*, including isolation of this compound for the first time by Aldridge *et al.* (1971). Other studies reported strong activity of compound **3.13** in potato micro-tuber induction at a concentration of 10^{-4} M (Yang *et al.*, 2000) and the strong anticancer activity of compound **3.13** against MCF-7 breast cancer cell lines (Hazalin *et al.*, 2013). Another fungal species identified as *Syncephalastrum racemosum* also was found to produce desmethyl-lasiodiplodin, which exhibited cytotoxicity activity against KB, BC1, and NCI-H187 cell lines was observed with IC_{50} values of 12.67, 9.65, and 11.07 μg/mL, respectively (Buayairaksa *et al.*, 2011). This compound was isolated as well from higher plants including *Euphorbia fidjiana, Kirkia acuminata* Oliver, *Osbeckia opipara* and *Cerbera manghas* (Cambie *et al.*, 1991; Mulholland *et al.*, 2003; Wang *et al.*, 2009; Zhou *et al.*, 2013). A recent study reported that desmethyl-lasiodiplodin found in the marine plant, *Cerbera manghas* displayed antagonistic activity against mineralocorticoid receptors in mice models (Zhou *et al.*, 2013).

Mellein is a ubiquitous isocoumarin derivative that is produced by terrestrial and mangrove fungi (Aldridge *et al.*, 1971; Cabras *et al.*, 2006; Evidente *et al.*, 2012; Klemke *et al.*, 2004; Ramos *et al.*, 2013; Rukachaisirikul *et al.*, 2009; Shao *et al.*, 2009; Sommart *et al.*, 2008). It was discovered for the first time from *Aspergillus melleus* Yukawa (Nishikawa, 1933). Mellein is formed naturally in the R-(-) and S-(+)-configurations. The absolute configuration of the R-(-) form was first established by Arakawa e al., in 1969 (Arakawa *et al.*, 1969). Its enantiomer, S-(+)-mellein, which is also known as ochracin, was found in several fungi including *Aspergillus ochraceus*, *Fusarium larvarum* and *Helicascus kanaloanus* (Claydon *et al.*, 1979; Poch and Gloer, 1989; Yabuta and Sumiki, 1933).



S-(+)-mellein R-(-)-mellein

Fig. 4.4: Enantiomers of mellein found naturally in endophytic fungi

Based on the optical rotation result done in $CHCl_3$ ($[\alpha]_D^{20}$ = -119.0), the mellein isolated from this study was identified as R-(-)-mellein **3.14**, which was also isolated previously from *L. theobromae* (Aldridge *et al.*, 1971). Based on recent study, mellein was proposed to be synthesis by a partially reducing iterative polyketide synthase derived from reaction between acetyl-CoA and malonyl-CoA (Sun *et al.*, 2012b). R-(-)-mellein **3.14** is a known phytotoxin produced by *Botryosphaeria obtusa* derived from apple trees which causes black rot and frogeye leaf spot (Venkatasubbaiah *et al.*, 1991). This compound exhibited various types of biological activities including fungicidal, antibacterial and algicidal activity in agar diffusion tests (Höller *et al.*, 1998; Krohn *et al.*, 1997). It also inhibited HCV protease with an IC_{50} value of 35 mM; however, it was found to be inactive against HIV-1 reverse transcriptase (Dai *et al.*, 2000).

Adenosine **3.18** is a nucleoside found in many fungi (Ma *et al.*, 2010b; Metwaly *et al.*, 2014; Talontsi *et al.*, 2012; Yuan *et al.*, 2014) including *Cordyceps sinensis*, a popular traditional Chinese medicine used to treat numerous diseases such as asthma, hyperglycemia, hyperlipidemia, respiratory disease, renal failure and arrhythmias. It is also used to restore sexual function (Zhu *et al.*, 1998). This compound is a chemical marker and contributes to the biological activities of *C. sinensis* (Hsu *et al.*, 2002). For example, adenosine displayed a promising therapeutic effect against epilepsy (Boison, 2005). Adenosine is hypothesized to play role in fungal reproduction, morphogenesis, nutrition and dimorphism (Griffin, 1996; Pall, 1981). This is the first report of the isolation of adenosine from *L. theobromae*. It also can be postulated that this compound is a common metabolite in endophytic fungi, hence more and more fungi are reported to produce adenosine.

4.3 Bioactive secondary metabolites from *Nigrospora* sp

4.3.1 Metabolomics- and bioassay-guided screening tools to optimise the production of bioactive secondary metabolites in *Nigrospora* sp for medium-scale fermentation

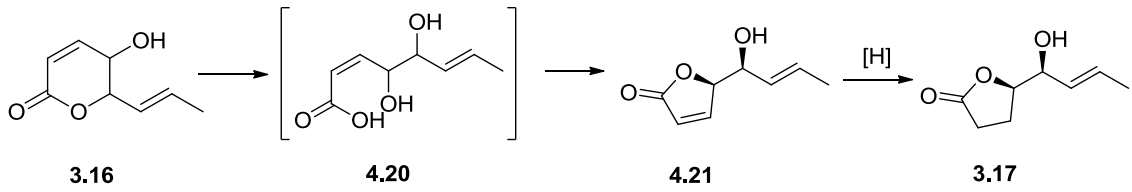
Microorganisms including fungi and bacteria show enormous potential to produce various types of metabolites when affected by environmental stress (Bills *et al.*, 2008; Fiedurek *et al.*, 1996; Mohanty and Prakash, 2009; Shang *et al.*, 2012). One Strain Many Compounds (OSMAC) is a method of manipulating several cultivation parameters such as media composition, incubation time and addition of enzyme inhibitors to optimise the production of bioactive secondary metabolites (Bode *et al.*, 2002). Medium compositions such as carbon source, nitrogen source and other growth factors affect the production of various metabolites (Singh, 2003).

In this particular study, media composition and incubation time were the two parameters that were manipulated. Based on the MS metabolomics- and bioassay-guided scheme implemented on *Nigrospora* sp, the optimum cultivation condition occurred with the solid rice medium. Previously, VanderMolen *et al.* (2013) found that rice media is the ideal medium for fungal metabolite screening program while a liquid media showed to be less productive. Another study by Hormazabal *et al.* (2005) revealed the best condition to grow fungus *Microsphaeropsis olivacea* was also in solid rice media where most of major secondary metabolites were produced in abundance than when grown in potato/glucose and yeast/malt/glucose liquid media . However, these findings should not be used to generalise the preferred medium to grow endophytic fungi. The production of sclerotiorin by *Penicillium sclerotiorum* was optimised in a liquid medium which contained dextrose, peptone, and mineral salts along with a sodium chloride supplement. While on the other hand, lower yields of sclerotiorin was produced in solid rice medium (Lucas *et al.*, 2010). In a more recent study by Shang *et al.* (2012), the marine-derived fungus *Nigrospora* sp. MA75 yielded certain compounds in liquid culture containing 3.5% NaCl which were not produced in solid rice medium and vice versa. Findings from other colleagues in our group also showed that optimization of the production of bioactive secondary metabolites in

endophytic fungi (terrestrial and marine) varied in media preferences (Abdelmohsen *et al.*, 2014; Mazlan *et al.*, 2013; Tawfike *et al.*, 2013).

In term of incubation time of endophytic fungal culture, most research groups grow the fungi between 7-14 days (Isaka *et al.*, 2007; Liu *et al.*, 2012). It is worth to take note that in this study, the bioactive compounds against *T. brucei brucei* were clearly produced beyond 14 days of incubation. Hence to assist this study application of a metabolomics approach simplified the decision-making step.

Phomalactone **3.16** and musacin E **3.17** were previously isolated from an unidentified species of *Nigrospora* (Evans Jr *et al.*, 1969). In my study these two chemically related compounds were detected and identified as major compounds at different incubation periods. Optimum production of phomalactone was detected on the 7th day while muscacin E was produced at its highest yield on the 30th day (see Fig. 3.38b). Trisuwan *et al.* (2009) isolated both compounds from *Nigrospora* sp PSU-F18 in a potato dextrose broth for 4 weeks at room temperature. Interestingly the total % yield of phomalactone and musacin E isolated were 0.2% (8.3 mg) and 9.3% (240.9 mg,) respectively which suggested that the major bio-product of *Nigrospora* sp PSU-F18 cultivated in 4 weeks was musacin E which is similar with my findings. Wu *et al.* (2009b) also reported similar finding by isolating 6.0% of phomalactone (1.5 g) and 8.8% of musacin E (2.2 g) from *Nigrospora* sp. YB-141 culture which was cultivated in potato dextrose broth (shaken) for only 6 days. Perhaps by shaking the conical flasks in potato dextrose broth is the factor affected the production of musacin E to be faster. Based on the optimisation on *Nigrospora* sp. culture, this finding indicates that phomalactone might be biosynthetically transformed to compound musacin E over the course of the fermentation with the cleavage between the C-O bonds of the lactone ring to form intermediate **4.20** and later compound **4.21** (musacin D) in the rice culture of *Nigrospora* sp (Fukushima *et al.*, 1998) as shown in Scheme 4.3.



3.16 **4.20** **4.21** **3.17**

Scheme 4.3: Putative biosynthesis of phomalactone, musacin D and E (Fukushima *et al.*, 1998)

Thus based on our finding, we illustrated that the metabolomics approach in de-replication potentially could be used to study the production of target compounds in plants or microbes.

For the past few decades, chemical investigations on the genus *Nigrospora* have led to the isolation of numerous new interesting compounds. However this genus is still under-utilised in terms of isolation work compared to other endophytic fungi like *Aspergillus, Fusarium, Penicillium* and *Pestalotiopsis* (Larsen and Frisvad, 1995; Nelson *et al.*, 1993; Nielsen *et al.*, 2009; Xu *et al.*, 2010a; Yang *et al.*, 2012b). Exhaustive isolation work should therefore be done in order to tap new and exciting metabolites produced by this genus. For example, manipulation of the OSMAC technique by adding NaCl to the media afforded five new 2(5H)-furanone-type derivatives, pestalafuranones F–J **1.185-1.189** (Zhang *et al.*, 2014) and also one new quinone deduced as 2,3-didehydro-19ahydroxy-14-epicochlioquinone B **1.179** (Shang *et al.*, 2012)**.** This technique was used to activate the cryptic gene of *Nigrospora* sp. Hence metabolomics could be applied in the future for dereplication studies of orphan compounds and also for the optimization of secondary metabolites, especially unidentified compounds.

4.1.3 Related bioactivities of isolated compounds

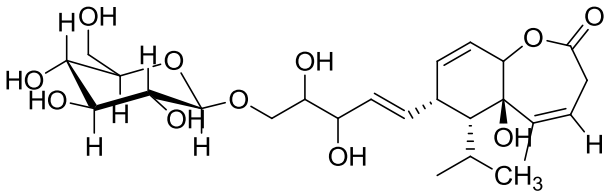
In this study, six compounds were isolated from *Nigrospora* sp including one new compound identified as nigrosporone *β*-glucoside **3.20** and five known compounds named as phomalactone **3.16**, musacin E **3.17**, 2-phenylethanol **3.18**, tyrosol **3.19** and adenosine **3.15**. Phomalactone was reported to inhibit the growth of cotton bollworm *Helicoverpa armigera* larvae after 48 h incubation (Wu *et al.*, 2012). Musacin E was inactive against various bioassays including antibacterial, antifungal, antiprotozoal,

herbicidal and insecticidal activities (Burkhardt *et al.*, 1996). Recent publication suggested the occurrence of compound phomalactone, musacin E and musacin D **4.21** in fungi play an important role to inhibit other microbial competitors to colonize in the host (Lai *et al.*, 2014).

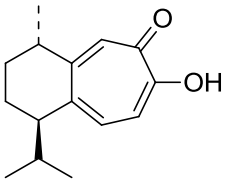
2-Phenylethanol **3.18** (Velasco B *et al.*, 2010) is highly desirable essential oil that is giving a rose-like aroma in foods and drinks (Etschmann and Schrader, 2006). Compound **3.18** is usually produced in yeast as intermediate in the production of amino acid L-phenylalanine. Previously this particular compound was detected in nine strains of *Aspergillus niger* (Lomascolo *et al.*, 2001). 2-Phenylethanol was reported to possess bacteriostatic and antifungicidal activities, hence was added in the preparation of antiseptic creams and deodorants (Chaudhari *et al.*, 2000). To the best of my knowledge this is the first report of the isolation of 2-phenylethanol from *Nigrospora* sp.

Tyrosol **3.19** (Chen *et al.*, 2004) is a high value-added antioxidant metabolite which can be found in olive oil(Bu *et al.*, 2007). Its function as a neutraceutical in the prevention of cancer and cardiovascular diseases is evolving remarkably (Capasso *et al.*, 1995; de la Puerta *et al.*, 1999; Deiana *et al.*, 1999; Konstantinidou *et al.*, 2010; Lozano-Sánchez *et al.*, 2010; Waterman and Lockwood, 2007). This compound also showed antifungal activities against *Lagenidium callinectes* (Gil-Turnes and Fenical, 1992) and *Gibberella pulicaris* (Slininger *et al.*, 2004). It also was detected to act as autoregulatory molecule and have significant effect on growth and morphogenesis in *Candida albicans* (Chen *et al.*, 2004).

In this study new compound named nigrosporone *β*-glucoside **3.20** was successfully isolated from *Nigrospora* sp. Based on the chemical structure the compound might derived from sesquiterpene type of compound. Similar congener identified as scodopin **4.22** was also isolated from the fruits of Scorodocarpus borneensis, respectively (Wiart *et al.*, 2001)



3.20



4.22

895

In this study, unfortunately none of isolated compounds exhibited strong anti-trypanosomal and anti-mycobacterial activities. However finding from previous study suggested the anthraquinone derivative 10-deoxybostrycin **1.173** isolated from genus of *Nigrospora* exhibited promising anti-mycobacterial activity against *M. tuberculosis* (Yang *et al.*, 2012a). Therefore more effort could be focused on the discovery of new exciting anthraquinone derivatives from *Nigrospora* sp. To aid this strategy, utilisation of metabolomics strategy plays an important role in the initial stage of this project. Compounds **3.16, 3.17** and **3.18** had previously been isolated from the *Nigrospora* genus, whereas compounds **3.19** and **3.20 (new)** were isolated from this genus for the first time in this study.

4.4 Bioactive secondary metabolites from *Pestalotiopsis olivacea*

4.4.1 Related bioactivities of isolated compounds and their putative biosynthesis

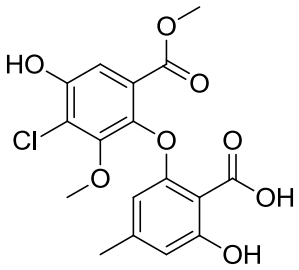
In this study, four inter-related diphenyl ethers and spirocyclohexadienones derivatives (dechlorodihydromaldoxin **3.26**, dihydromaldoxin (pestheic acid) **3.27**, maldoxin **3.28**, dechloromaldoxin **3.29**), one quinone named physcion **3.30** and one steroid known as stigmasterol **3.2** were successfully isolated from *P. olivacea*. The bioactivity results showed that only dechloromaldoxin **3.29** exhibited very good anti-mycobacterial activities with MIC value of 14.4 µM, while stigmasterol **3.2** was active against *T. brucei brucei* with MIC value of 21.1 µM, respectively. Dechlorodihydromaldoxin **3.27** and dihydromaldoxin **3.27** were isolated previously from *Pestalotiopsis* sp and demonstrated endothelin (ET) antagonist activities which specifically inhibited ET type A receptor with the IC_{50} values of 1.5 µM and 20 µM, respectively (Ogawa *et al.*, 1995). Adeboya *et al*. conducted an isolation study from *Xylaria* sp. collected in Malaysia and obtained five inter-related congeners, including dechlorodihydromaldoxin **3.26**, dihydromaldoxin **3.27**, isodihydromaldoxin **4.23**, maldoxin **3.29** and maldoxone **4.24**. He and co-workers also discussed the biosynthetic pathways of these related compounds (Adeboya *et al.*, 1996). Dechlorodihydromaldoxin **3.35**, dechloromaldoxin **3.38** dihydromaldoxin **3.36**, maldoxin **3.29** and maldoxone **4.24** have also been successfully synthesized from isosulochrin **3.24** and chloiroosulochrin **3.25** by the biomimetic sequence reported in a previous study (Yu and Snider, 2011a). This study also proposed that the biosynthetic precursor of isosulochrin **3.24** and chloroisosulochrin **3.25** might originate from physcion **3.30** and fragilin **4.25** (Yu and Snider, 2011a). Another study reported that maldoxin **3.26** and isopropenylallene **4.26** can undergo a Diels-Alder reaction to yield compounds related to chloropestolide A **4.27,** chloropupukeanolide D **4.28,** chlorochloropupukeananin **4.29** and chloropupukeanolide C **4.30** as shown in Scheme 4.5 (Yu and Snider, 2011b) . Dechloromaldoxin **3.28** was isolated naturally for the first time from *P. fici* (Liu *et al.*, 2013a). Dihydromaldoxin or pestheic acid **3.27** occurs in several endophytic fungi, including *Pestalotiopsis* genus (Klaiklay *et al.*, 2012; Liu *et al.*, 2008; Ogawa *et al.*, 1995; Shimada *et al.*, 2001), *Xylaria* sp., (Adeboya *et al.*, 1996) and *Steganospora* sp.

(Schreiber *et al.*, 2012). It was reported that dihydromaldoxin ; pestheic acid **3.27** acts as an inhibitor of CXCL10 expression in MonoMac6 (MM6) cells with IC_{50} values of 41µM (Schreiber *et al.*, 2012). Physcion **3.30** is an anthraquinone congener and has previously been isolated from several endophytic fungi including *Penicillium* sp., (Wang *et al.*, 2008) *Aspergillus fumigatus* CY018, (Liu *et al.*, 2004), *Pleospora* sp., (Ge *et al.*, 2005) *Eurotium cristatum* (Gomes *et al.*, 2012) and from the lichen *Candelaria concolor* (Salinas *et al.*, 2012). Physcion **3.30** has been reported to exhibit antifungal, antineoplastic and antioxidant activities (Agarwal *et al.*, 2000; Kuo *et al.*, 1997; Li *et al.*, 2014b).

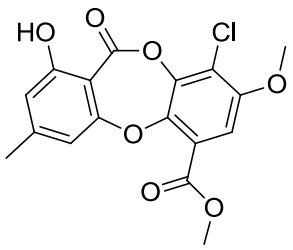
In this study, physcion **3.30** was obtained for the first time from *Pestalotiopsis* species, which supports previous claims by Yu and Snider who postulated that physcion **3.30** is a precursor in the biosynthesis of dechloromaldoxin **3.28** (Yu and Snider, 2011a) (Scheme 4.4). They also proposed fragilin **4.25** as a biosynthetic precursor of maldoxin **3.29**. However, in this study fragilin **4.25** was not isolated. The occurrence of these five metabolites in *P. olivaceae* supports that chlorination and methylation routes are taking place in the biosynthesis of maldoxin **3.29** and dechloromaldoxin **3.28**. The biosynthesis of dechloromaldoxin **3.28** postulated that the oxidative cleavage of physcion **3.30** will yield isosulochrin **3.24** which then will convert to dechlorodihydromaldoxin **3.26** by oxidation and finally will give dechloromaldoxin **3.28** by an oxidation step. A similar oxidation of fragilin **4.25** will give chloroisosulochrin **3.25**, which then might be oxidatively cyclized to afford dihydromaldoxin **3.27** which is later oxidized to have maldoxin **3.28** as a final product. Although isosulochrin **3.24** and chloroisosulochrin **3.25** were not isolated, nonetheless the dereplication study of secondary metabolites from this species using HRLCMS incorporated with the AntiMarin database confirmed the presence of isosulochrin **3.24** and chloroisosulochrin **3.25** in the extract of *P. olivacea*

This is the first report on the secondary metabolites of *P. olivacea* derived from *V. pinnata*. Interestingly, five biosynthetically-related metabolites were isolated from this species. These are dechlorodihydromaldoxin **3.26**, dihydromaldoxin (pestheic acid) **3.27**, maldoxin **3.29**, dechloromaldoxin **3.28** and physcion **3.30**. Physcion **3.30** was isolated for the first time from *Pestalotiopsis* species. This finding suggests that the involvement of physcion **3.30** in the biosynthesis of dechloromaldoxin as postulated by

a previous study. A dereplication study of *P. olivacea* confirmed the occurrence of isosulochrin **3.24** and chloroisosulochrin **3.25** which are also involved in the biosynthesis of dechloromaldoxin **3.28** and maldoxin **3.29**. However, the presence of fragilin **4.25** in *P. olivacea* could not be detected. This might be due to the full conversion of fragilin **4.25** to dihydromaldoxin **3.27** or poor ionization of the fragilin **4.25** in the mass spectrometer. Of the compounds isolated, only dechloromaldoxin **3.29** exhibited very good anti-mycobacterial activities with MIC value of 14.4 µM, while stigmasterol **3.2** was active against *T. brucei brucei* with MIC value of 21.1 µM, respectively.

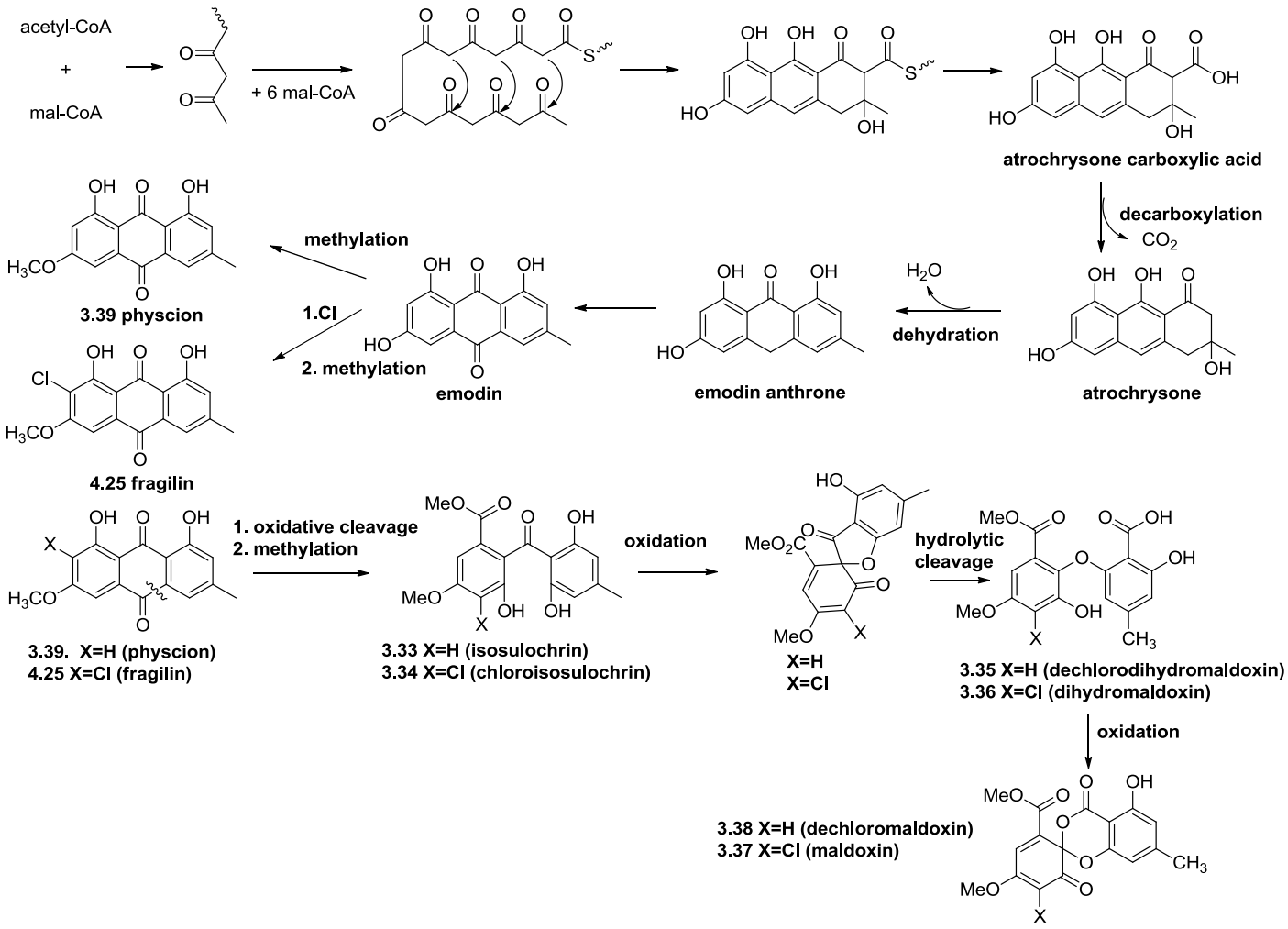


4.23

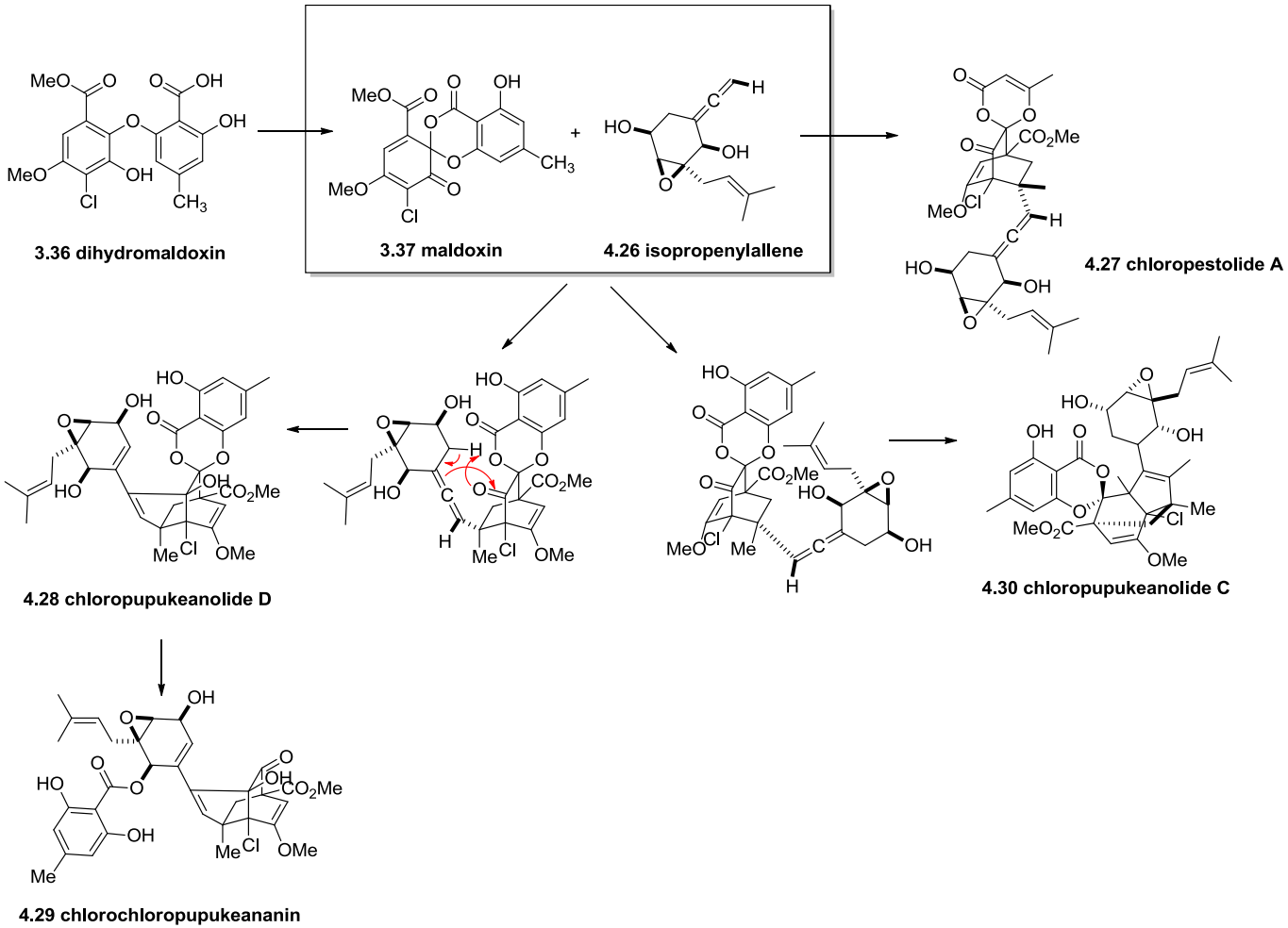


4.24

Fig. 4.5: Other related metabolites previously reported from *Pestalotiopsis* sp



Scheme 4.4: Postulated biosynthesis of dechloromaldoxin and maldoxin (Yu and Snider, 2011a)



Scheme 4.5: Putative biogenesis of chloropestolide A, chloropupukeanolide C, chloropupukeanolide D and chloropupukeananin (Yu and Snider, 2011b)

CHAPTER 5

5 Conclusions and future recommendation

5.1 Discovery bioactive metabolites from leaves of *V. pinnata*

V. pinnata is one of traditional medicinal plants used by local Malay in Peninsula and East Malaysia to treat various diseases. The leaves were applied by indigenous people to treat fever, cuts and wounds. Phytochemical study of the leaves of *V. pinnata* employing fast and reproducible MPLC afforded mixture of steroids: *β*-sitosterol **3.1** and stigmasterol **3.2** and three flavones: 5-hydroxy-3,7,4'-trimethoxyflavone **3.3**, 5-hydroxy-7,4'-dimethoxy-flavone **3.4** and 5-hydroxy-3,3',4',7-tetramethoxyflavone **3.5**, respectively. Compounds **3.3**, **3.4** and **3.5** were isolated efficiently for the first time from *V. pinnata* by using MPLC. Compounds **3.3** and **3.5** were cytotoxic against NF-κB inhibition assay with IC_{50} values of 52.1 and 10.0μM, respectively. All compounds also exhibited good antitrypanosomal activity with MIC values of 6.25 μg/mL, 19.0, 21.0 and 17.0μM, respectively. However no activity was observed in the anti-mycobacterial assay. Among all isolated secondary metabolites, compound **3.5** showed the strongest activities in both assays. Previous studies also described their promising anticancer (Zhang *et al.*, 2011a), antioxidant (Kolak *et al.*, 2009) and anti-inflammatory (Bala *et al.*, 2013) activities which perhaps describes the usage of this medicinal plant by local traditional medicine practise in Peninsula Malaysia to treat fever, cuts and wounds (Ong and Nordiana, 1999). In this thesis, a good antitrypanosomal activity of these compounds isolated from *V. pinnata* against *T. brucei brucei* was reported for the first time.

5.2 Metabolomics as decision-making tools in mining antitrypanosomal active metabolites from the endophytic fungus *L. theobromae*

With the application of HR-LCMS and NMR-based metabolomics in-parallel to the antitrypanosomal activity on extracts of *L. theobromae* (different duration time of incubation and types of media), production of bioactive secondary metabolites in medium-scale fermentation was optimised at 30 days in rice culture media. Analysis of

^{1}H NMR between active *versus* inactive fractions identified several unique chemical fingerprints belonging to the active fractions. Furthermore, by integrating HR-LCMS data with chemometric analysis, such as PLS-DA, OPLS-DA, S-plots along with the bioactivity results of the fractions of *L. theobromae*, the antitrypanosomal compounds were easily discerned and predicted. With available databases such as Antibase and MarinLit coupled to MZmine by *in-house* algorithms optimized in our laboratory, the bioactive metabolites were readily identified prior to isolation work. 1D and 2D ^{1}H NMR data were utilized to verify the presence of active metabolites in the samples. Fractionation was performed on one of the active fractions and three known compounds were isolated, namely cladospirone-B **3.11**, desmethyl-lasiodiplodin **3.13**, and R-(-)-mellein **3.14.** Cladospirone-B **3.11** and desmethyl-lasiodiplodin **3.13** were among the predicted bioactive compounds generated by S-plot, and interestingly these compounds exhibited good activity against *T. brucei brucei* with MICs of 17.8 µM and 22.5 µM, respectively. Therefore, HR-LCMS and NMR-based metabolomics proved to be a powerful decision-making tool in mining active metabolites from *L. theobromae* against *T. brucei brucei*.

5.3 Secondary metabolites isolated from *Nigrospora* sp

The most ideal condition for medium-scale fermentation of *Nigrospora* sp was easily achieved by applying HR-LCMS and NMR-based metabolomics on 30 days rice culture. Nevertheless analysis of the ^{1}H NMR of *Nigrospora* sp extract from the seventh day revealed the presence of phomalactone **3.16** as a major compound which simply can be elucidated with further 1D and 2D NMR experiment data. Meanwhile isolation work on *Nigrospora* sp extract from the 30 days culture afforded five known compounds identified as musacin E **3.17** as the major compound, together with 2-phenyl ethanol **3.18**, tyrosol **3.19**, adenosine **3.15** and a new lactone derivative, nigrosporone *β*-glucoside **3.20**, respectively. The putative biosynthetic study suggested production of phomalactone in early fermentation was converted to musacin E in later stage incubation. Hence this finding created the idea of utilising metabolomics in biosynthetic study of secondary metabolites in endophytic fungi for future work.

5.4 Dereplication of *P. olivacea*

The metabolomics study on analysing the optimum condition for medium-scale fermentation of *P. olivacea* was accomplished on the 15th day rice culture. ^{1}H NMR data revealed two major abundant compounds known as dechlorodihydromaldoxin **3.26** and pestheic acid **3.27** which masked the occurrence of other metabolites. Dereplication step on the MS data of this extract with AntiMarin database, revealed putatively the production of various secondary metabolites previously isolated from other *Pestalotiopsis* spp. Potential new compounds also were detected in this step. Isolation work on the extract afforded five known compounds, four of which were already putatively identified during dereplication analysis (dechlorodihydromaldoxin **3.26**, pestheic acid **3.27**, maldoxin **3.28** and dechloromaldoxin **3.29**) and physcion **3.30**. This is the first report the isolation of inter-related compounds in one single species. The putative biosynthetic pathway of the production of this inter-related compounds also were constructed.

5.5 Future recommendation

Detection of fungal metabolites in the host plant fractions by using strategy of metabolomics can be done in the future work to study the biosynthetic relationship of fungal endophytes with their host plants. Also mode of action of anti-inflammatory, antitrypanosomal and anti-mycobacterial effects of isolated compounds can be carried out to support this initial finding.

For secondary metabolites derived from fungal endophytes, the major limitation of study is the yield. Therefore suggestion for future study is to increase the volume of medium-scale fermentation from 20 L to 60 L to obtain more yields. Application of hyphenated NMR for example cryoprobe NMR may be utilized to analyse pure compound at less than 1 mg. Previous chemical study of genus *Nigrospora* reported anthraquinone derivative exhibited promising anti-mycobacterial activity against *M. tuberculosis*. Therefore future isolation of anti-mycobacterial study could focus more on isolation of anthraquinone congeners by using strategy of metabolomics including HPLC UV-PDA spectrum profile, HR-LCMS, NMR and integrated with any available

in-house and commercial databases to guide the isolation work. Biosynthetic study of inter-related metabolomes from *P. olivacea* could be further analysed in the future by using suitable heterologous host for example *Escherichia coli* and biosynthesis will be monitored by HR-LCMS and NMR.

Manipulations of different solid mediums such as oat could be used in the future study as these sources are abundant and cheaper in the UK and European region compared to rice, which is the main source in Asia to observe the production of different and similar biomarkers in each medium. Additionally co-cultivation between 'fungi *versus* fungi' or 'fungi *versus* bacteria' could be explored to activate the orphan genes which then will produce new diverse metabolomes.

References

Abdelmohsen U, Cheng C, Viegelmann C, Zhang T, Grkovic T, Ahmed S, Quinn R, Hentschel U, Edrada-Ebel R. 2014. Dereplication Strategies for Targeted Isolation of New Antitrypanosomal Actinosporins A and B from a Marine Sponge Associated-*Actinokineospora* sp. EG49. *Marine Drugs* **12**(3):1220-1244.

Abdi H. 2003. Multivariate analysis. *Encyclopedia for research methods for the social sciences. Thousand Oaks: Sage*:699-702.

Adeboya MO, Edwards RL, Lassoe T, Maitland DJ, Shields L, Whalley AJS. 1996. Metabolites of the higher fungi. Part 29. Maldoxin, maldoxone, dihydromaldoxin, isodihydromaldoxin and dechlorodihydromaldoxin. A spirocyclohexadienone, a depsidone and three diphenyl ethers: keys in the depsidone biosynthetic pathway from a member of the fungus genus *Xylaria*. *Journal of the Chemical Society, Perkin Transactions 1*(12):1419-1425.

Agarwal SK, Singh SS, Verma S, Kumar S. 2000. Antifungal activity of anthraquinone derivatives from *Rheum emodi*. *Journal of Ethnopharmacology* **72**(1–2):43-46.

Agnolet S, Jaroszewski J, Verpoorte R, Staerk D. 2010. ^{1}H NMR-based metabolomics combined with HPLC-PDA-MS-SPE-NMR for investigation of standardized *Ginkgo biloba* preparations. *Metabolomics* **6**(2):292-302.

Akinmoladun AC, Obuotor EM, Farombi EO. 2010. Evaluation of antioxidant and free radical scavenging capacities of some Nigerian indigenous medicinal plants. *Journal of medicinal food* **13**(2):444-451.

Akita H, Nozawa M, Mitsuda A, Ohsawa H. 2000. A convenient synthesis of (+)-albicanol based on enzymatic function: total syntheses of (+)-albicanyl acetate, (−)-albicanyl 3,4-dihydroxycinnamate, (−)-drimenol, (−)-drimenin and (−)-ambrox. *Tetrahedron: Asymmetry* **11**(6):1375-1388.

Al-Sadi AM, Al-Jabri AH, Al-Mazroui SS, Al-Mahmooli IH. 2012. Characterization and pathogenicity of fungi and oomycetes associated with root diseases of date palms in Oman. *Crop Protection* **37**(0):1-6.

Alam MS, Chopra N, Ali M, Niwa M. 1996. Oleanen and stigmasterol derivatives from *Ambroma augusta*. *Phytochemistry* **41**(4):1197-1200.

Alam SS, Bilton JN, Slawin AMZ, Williams DJ, Sheppard RN, Strange RN. 1989. Chickpea blight: Production of the phytotoxins solanapyrones A and C by *Ascochyta rabiei*. *Phytochemistry* **28**(10):2627-2630.

Aldridge D, Galt S, Giles D, Turner W. 1971. Metabolites of *Lasiodiplodia theobromae*. *Journal of the Chemical Society C: Organic*:1623-1627.

Alfaro C, Urios A, González MC, Moya P, Blanco M. 2003. Screening for metabolites from *Penicillium novae-zeelandiae* displaying radical-scavenging activity and oxidative mutagenicity: isolation of gentisyl alcohol. *Mutation Research/Genetic Toxicology and Environmental Mutagenesis* **539**(1–2):187-194.

Ali K, Iqbal M, Yuliana N, Lee Y-J, Park S, Han S, Lee J-W, Lee H-S, Verpoorte R, Choi Y. 2013. Identification of bioactive metabolites against adenosine A1 receptor using NMR-based metabolomics. *Metabolomics* **9**(4):778-785.

Aly AH, Debbab A, Proksch P. 2011. Fungal endophytes: unique plant inhabitants with great promises. *Applied microbiology and biotechnology* **90**(6):1829-45.

Amagata T, Usami Y, Minoura K, Ito T, Numata A. 1998. Cytotoxic substances produced by a fungal strain from a sponge: physico-chemical properties and structures. *The Journal of antibiotics* **51**(1):33-40.

Arakawa H, Torimoto N, Masui Y. 1969. Die absolute Konfiguration der optisch aktiven, nat rlichen Dihydroisocumarine, II Bestimmung der absoluten Konfiguration von Agrimonolid und Mellein. *Justus Liebigs Annalen der Chemie* **728**(1):152-157.

Arung ET, Kusuma IW, Christy EO, Shimizu K, Kondo R. 2009. Evaluation of medicinal plants from Central Kalimantan for antimelanogenesis. *Journal of natural medicines* **63**(4):473-480.

Aruoma O. 1998. Free radicals, oxidative stress, and antioxidants in human health and disease. *J Amer Oil Chem Soc* **75**(2):199-212.

Astudillo L, Schmeda-Hirschmann G, Soto R, Sandoval C, Afonso C, Gonzalez MJ, Kijjoa A. 2000. Acetophenone derivatives from Chilean isolate of *Trichoderma pseudokoningii* Rifai. *World Journal of Microbiology and Biotechnology* **16**(6):585-587.

Ata A, Mbong N, Iverson CD, Samarasekera R. 2009. Minor chemical constituents of *Vitex pinnata*. *Natural product communications* **4**(1):1.

Bala A, Chetia P, Dolai N, Khandelwal B, Haldar PK. 2013. Cat's whiskers flavonoid attenuated oxidative DNA damage and acute inflammation: its importance in lymphocytes of patients with rheumatoid arthritis. *Inflammopharmacology*:1-7.

Baldwin EL, Osheroff N. 2005. Etoposide, Topoisomerase II and Cancer. *Current Medicinal Chemistry -Anti-Cancer Agents* **5**(4):363-372.

Ballio A, Evidente A, Graniti A, Randazzo G, Sparapano L. 1988. Seiricuprolide, a new phytotoxic macrolide from a strain of *Seiridium cupressi* infecting cypress. *Phytochemistry* **27**(10):3117-3121.

Balunas MJ, Kinghorn AD. 2005. Drug discovery from medicinal plants. *Life Sciences* **78**(5):431-441.

Barbas C, Moraes EP, Villaseñor A. 2011. Capillary electrophoresis as a metabolomics tool for non-targeted fingerprinting of biological samples. *Journal of Pharmaceutical and Biomedical Analysis* **55**(4):823-831.

Barnes PJ. 1997. Nuclear factor-κB. *The International Journal of Biochemistry & Cell Biology* **29**(6):867-870.

Barral-Netto M, Badaró R, Barral A, Almeida RP, Santos SB, Badaró F, Pedral-Sampaio D, Carvalho EM, Falcoff E, Falcoff R. 1991. Tumor Necrosis Factor (Cachectin) in Human Visceral Leishmaniasis. *Journal of Infectious Diseases* **163**(4):853-857.

Baumann H, Gauldie J. 1994. The acute phase response. *Immunology Today* **15**(2):74-80.

Benmiloud M, Chaouki ML, Gutekunst R, Teichert H, Wood WG, Dunn JT. 1994. Oral iodized oil for correcting iodine deficiency: optimal dosing and outcome indicator selection. *The Journal of clinical endocrinology and metabolism* **79**(1):20-24.

Berim A, Gang DR. 2013. The Roles of a Flavone-6-Hydroxylase and 7-O-Demethylation in the Flavone Biosynthetic Network of Sweet Basil. *Journal of Biological Chemistry* **288**(3):1795-1805.

Bills G, Platas G, Fillola A, Jimenez M, Collado J, Vicente F, Martin J, Gonzalez A, Bur-Zimmermann J, Tormo J. 2008. Enhancement of antibiotic and secondary metabolite detection from filamentous fungi by growth on nutritional arrays. *Journal of applied microbiology* **104**(6):1644-1658.

Blunt J, Munro M. 2005. MarinLit database. *Department of Chemistry, University of Canterbury*.

Blunt JW, Munro, M. H. G. & Laatsch, H. 2013. AntiMarin Database. Göttingen, Germany: University of Canterbury, Christchurch, New Zealand and University of Göttingen.

Bode HB, Zeeck A. 2000. UV mutagenesis and enzyme inhibitors as tools to elucidate the late biosynthesis of the spirobisnaphthalenes. *Phytochemistry* **55**(4):311-316.

Bode HB, Bethe B, Höfs R, Zeeck A. 2002. Big Effects from Small Changes: Possible Ways to Explore Nature's Chemical Diversity. *ChemBioChem* **3**(7):619-627.

Bode HB, Walker M, Zeeck A. 2000. Cladospirones B to I from *Sphaeropsidales* sp. F-24′ 707 by Variation of Culture Conditions. *European Journal of Organic Chemistry* **2000**(18):3185-3193.

Bohni N, Cordero-Maldonado ML, Maes J, Siverio-Mota D, Marcourt L, Munck S, Kamuhabwa AR, Moshi MJ, Esguerra CV, de Witte PA. 2013. Integration of Microfractionation, qNMR and Zebrafish Screening for the In Vivo Bioassay-Guided Isolation and Quantitative Bioactivity Analysis of Natural Products. *PLoS ONE* **8**(5):e64006.

Boison D. 2005. Adenosine and epilepsy: from therapeutic rationale to new therapeutic strategies. *The neuroscientist* **11**(1):25-36.

Borchardt JK. 2002. The beginnings of drug therapy: Ancient mesopotamian medicine. *Drug News Perspect* **15**(3):187.

Borgschulte K, Rebuffat S, Trowitzsch-Kienast W, Schomburg D, Pinon J, Bodo B. 1991. Isolation and structure elucidation of hymatoxins B - E and other phytotoxins from *Hypoxylon mammatum* fungal pathogen of leuce poplars. *Tetrahedron* **47**(39):8351-8360.

Bouhifd M, Hartung T, Hogberg HT, Kleensang A, Zhao L. 2013. Review: toxicometabolomics. *Journal of Applied Toxicology* **33**(12):1365-1383.

Braithwaite A, Smith FJ. 1999. Planar chromatography. Chromatographic Methods: Springer Netherlands. p 44-116.

Brundret K, Dalziel W, Hesp B, Jarvis J, Neidle S. 1972. X-Ray crystallographic determination of the structure of the antibiotic aphidicolin: a tetracyclic diterpenoid containing a new ring system. *J. Chem. Soc., Chem. Commun.*(18):1027-1028.

Bruneton J. 1995. Pharmacognosy, Phytochemistry, Medicinal Plants. Intercept, U.K.

Bu Y, Rho S, Kim J, Kim MY, Lee DH, Kim SY, Choi H, Kim H. 2007. Neuroprotective effect of tyrosol on transient focal cerebral ischemia in rats. *Neuroscience Letters* **414**(3):218-221.

Buayairaksa M, Kanokmedhakul S, Kanokmedhakul K, Moosophon P, Hahnvajanawong C, Soytong K. 2011. Cytotoxic lasiodiplodin derivatives from the fungus *Syncephalastrum racemosum*. *Archives of Pharmacal Research* **34**(12):2037-2041.

Burgess TI, Barber PA, Mohali S, Pegg G, de Beer W, Wingfield MJ. 2006. Three new *Lasiodiplodia* spp. from the tropics, recognized based on DNA sequence comparisons and morphology. *Mycologia* **98**(3):423-435.

Burkhardt K, Fiedler HP, Grabley S, Thiericke R, Zeeck A. 1996. New cineromycins and musacins obtained by metabolite pattern analysis of *Streptomyces griseoviridis* (FH-S 1832). I. Taxonomy, fermentation, isolation and biological activity. *The Journal of antibiotics* **49**(5):432-437.

Burkill IH. 1966. A Dictionary of the Economic Products of the Malay Peninsula. Ministry of Agriculture Co-operatives: Kuala Lumpur 2280.

Buss AD, Cox B, Waigh RD. 2003. Natural Products as Leads for New Pharmaceuticals. Burger's Medicinal Chemistry and Drug Discovery: John Wiley & Sons, Inc.

Cabras A, Mannoni M, Serra S, Andolfi A, Fiore M, Evidente A. 2006. Occurrence, isolation and biological activity of phytotoxic metabolites produced in vitro by *Sphaeropsis sapinea*, pathogenic fungus of *Pinus radiata*. *European Journal of Plant Pathology* **115**(2):187-193.

Cai Y-S, Guo Y-W, Krohn K. 2010. Structure, bioactivities, biosynthetic relationships and chemical synthesis of the spirodioxynaphthalenes. *Natural Product Reports* **27**(12):1840-1870.

Cambie RC, Lal AR, Rutledge PS, Woodgate PD. 1991. Ent-14[S],16β,17-trihydroxyatisan-3-one and further constituents from *Euphorbia fidjiana*. *Phytochemistry* **30**(1):287-292.

Campos FF, Rosa LH, Cota BB, Caligiorne RB, Rabello ALT, Alves TMA, Rosa CA, Zani CL. 2008. Leishmanicidal metabolites from *Cochliobolus* sp., an endophytic fungus isolated from *Piptadenia adiantoide*s (fabaceae). *PLoS neglected tropical diseases* **2**(12):e348.

Capasso R, Evidente A, Schivo L, Orru G, Marcialis M, Cristinzio G. 1995. Antibacterial polyphenols from olive oil mill waste waters. *Journal of Applied Bacteriology* **79**(4):393-398.

Caprioli RM, Farmer TB, Gile J. 1997. Molecular imaging of biological samples: localization of peptides and proteins using MALDI-TOF MS. *Analytical chemistry* **69**(23):4751-4760.

Cateni F, Doljak B, Zacchigna M, Anderluh M, Piltaver A, Scialino G, Banfi E. 2007. New biologically active epidioxysterols from *Stereum hirsutum*. *Bioorganic & medicinal chemistry letters* **17**(22):6330-6334.

Chahar MK, Sharma N, Dobhal MP, Joshi YC. 2011. Flavonoids: a versatile source of anticancer drugs. *Pharmacognosy reviews* **5**(9):1.

Challis GL. 2008. Mining microbial genomes for new natural products and biosynthetic pathways. *Microbiology* **154**(6):1555-1569.

Charudattan R, Rao K. 1982. Bostrycin and 4-deoxybostrycin: two nonspecific phytotoxins produced by *Alternaria eichhorniae*. *Applied and environmental microbiology* **43**(4):846-849.

Chaudhari RV, Telkar MM, Rode CV. 2000. Process for the preparation of 2-phenyl ethanol. Google Patents.

Chaurand P, Stoeckli M, Caprioli RM. 1999. Direct profiling of proteins in biological tissue sections by MALDI mass spectrometry. *Analytical chemistry* **71**(23):5263-5270.

Chen G, Tian L, Wu H-H, Bai J, Lu X, Xu Y, Pei Y-H. 2012. Secondary metabolites from fungus Nigrospora sp. *Journal of Asian natural products research* **14**(8):759-763.

Chen H, Fujita M, Feng Q, Clardy J, Fink GR. 2004. Tyrosol is a quorum-sensing molecule in Candida albicans. *Proceedings of the National academy of Sciences of the United States of America* **101**(14):5048-5052.

Chen S-N, Friesen JB, Webster D, Nikolic D, van Breemen RB, Wang ZJ, Fong HHS, Farnsworth NR, Pauli GF. 2011. Phytoconstituents from Vitex agnus-castus fruits. *Fitoterapia* **82**(4):528-533.

Chen X, Shi Q, Lin G, Guo S, Yang J. 2009. Spirobisnaphthalene Analogues from the Endophytic Fungus Preussia sp. *Journal of Natural Products* **72**(9):1712-1715.

Cho M-H, Park H, Park J-H, Lee S-W, Bhoo S, Hahn T-R. 2012. Characterization of regiospecific flavonoid 3′/5′-O-methyltransferase from tomato and its application in flavonoid biotransformation. *J Korean Soc Appl Biol Chem* **55**(6):749-755.

Choi J-h, Horikawa M, Okumura H, Kodani S, Nagai K, Hashizume D, Koshino H, Kawagishi H. 2009. Endoplasmic reticulum (ER) stress protecting compounds from the mushroom Mycoleptodonoides aitchisonii. *Tetrahedron* **65**(1):221-224.

Choi YH, Kim HK, Hazekamp A, Erkelens C, Lefeber AWM, Verpoorte R. 2004. Metabolomic Differentiation of Cannabis sativa Cultivars Using 1H NMR Spectroscopy and Principal Component Analysis. *Journal of Natural Products* **67**(6):953-957.

Chu M, Mierzwa R, Truumees I, Gentile F, Patel M, Gullo V, Chan T-M, Puar MS. 1993. Two novel diketopiperazines isolated from the fungus Tolypocladium sp. *Tetrahedron Letters* **34**(47):7537-7540.

Ciavatta ML, Manzo E, Nuzzo G, Villani G, Cimino G, Cervera JL, Malaquias MAE, Gavagnin M. 2009. Aplysiopsenes: an additional example of marine polyketides with a mixed acetate/propionate pathway. *Tetrahedron Letters* **50**(5):527-529.

Ciuffreda P, Casati S, Manzocchi A. 2007. Complete 1H and 13C NMR spectral assignment of α-and β-adenosine, 2′-deoxyadenosine and their acetate derivatives. *Magnetic Resonance in Chemistry* **45**(9):781-784.

Claydon N, Grove JF, Pople M. 1979. Insecticidal secondary metabolic products from the entomogenous fungus Fusarium larvarum. *Journal of Invertebrate Pathology* **33**(3):364-367.

Corner EJH. 1951. Wayside trees of Malaya. . Singapore Govern: Singapore.

Corre C, Challis GL. 2007. Heavy Tools for Genome Mining. *Chemistry & Biology* **14**(1):7-9.

Cragg GM, Newman DJ. 2005. Biodiversity: A continuing source of novel drug leads. *Pure and applied chemistry* **77**(1):7-24.

Dai J-R, Carté BK, Sidebottom PJ, Sek Yew AL, Ng S-B, Huang Y, Butler MS. 2000. Circumdatin G, a New Alkaloid from the Fungus Aspergillus ochraceus. *Journal of Natural Products* **64**(1):125-126.

Danielsen K, Aksnes DW, Francis GW. 1992. NMR study of some anthraquinones from rhubarb. *Magnetic Resonance in Chemistry* **30**(4):359-360.

Davis RA, Carroll AR, Andrews KT, Boyle GM, Tran TL, Healy PC, Kalaitzis JA, Shivas RG. 2010. Pestalactams A–C: novel caprolactams from the endophytic fungus Pestalotiopsis sp. *Organic & Biomolecular Chemistry* **8**(8):1785-1790.

Dayrit F, Lagurin L. 1994. Identification of four iridoids in the pharmacologically-active fraction of Vitex negundo, L. *Philippine Journal of science* **123**.

de Kok R. 2008. The genus *Vitex* (Labiatae) in the flora Malesiana region, excluding New Guinea. *Kew Bulletin* **63**(1):17-40.

de la Puerta R, Gutierrez VR, Hoult JRS. 1999. Inhibition of leukocyte 5-lipoxygenase by phenolics from virgin olive oil. *Biochemical pharmacology* **57**(4):445-449.

Deiana M, Aruoma OI, Bianchi MdLP, Spencer JP, Kaur H, Halliwell B, Aeschbach R, Banni S, Dessi MA, Corongiu FP. 1999. Inhibition of peroxynitrite dependent

DNA base modification and tyrosine nitration by the extra virgin olive oil-derived antioxidant hydroxytyrosol. *Free Radical Biology and Medicine* **26**(5):762-769.

Denning DW. 2003. Echinocandin antifungal drugs. *The Lancet* **362**(9390):1142-1151.

DerMarderosian A, Beutler JA. 2002. The review of natural products: the most complete source of natural product information. Facts and Comparisons.

Dias DA, Urban S, Roessner U. 2012. A Historical Overview of Natural Products in Drug Discovery. *Metabolites* **2**(2):303-336.

Didier D, Delaye P-O, Simaan M, Island B, Eppe G, Eijsberg H, Kleiner A, Knochel P, Marek I. 2014. Modulable and Highly Diastereoselective Carbometalations of Cyclopropenes. *Chemistry – A European Journal* **20**(4):1038-1048.

Dinan L. 2001. Phytoecdysteroids: biological aspects. *Phytochemistry* **57**(3):325-339.

Ding B, Yin Y, Zhang F, Li Z. 2011. Recovery and Phylogenetic Diversity of Culturable Fungi Associated with Marine Sponges Clathrina luteoculcitella and Holoxea sp. in the South China Sea. *Mar Biotechnol* **13**(4):713-721.

Dong J-J, Bao J, Zhang X-Y, Xu X-Y, Nong X-H, Qi S-H. 2014. Alkaloids and citrinins from marine-derived fungus Nigrospora oryzae SCSGAF 0111. *Tetrahedron Letters* **55**(16):2749-2753.

dos Santos TC, Delle Monache F, Leitão SG. 2001. Ecdysteroids from two Brazilian Vitex species. *Fitoterapia* **72**(3):215-220.

dui al Banerjee D. 2011. Endophytic fungal diversity in tropical and subtropical plants. *Research Journal of Microbiology* **6**(1):54-62.

Dunn WB, Bailey NJ, Johnson HE. 2005. Measuring the metabolome: current analytical technologies. *Analyst* **130**(5):606-625.

Dutta PK, Chowdhury US, Chakravarty AK, Achari B, Pakrashi SC. 1983. Studies on indian medicinal plants-part LXXV: Nishindaside, a novel iridoid glycoside from vitex negundo. *Tetrahedron* **39**(19):3067-3072.

E. P. 1976. Botryodiplodia theobromae. CMI Description of Pathogenic Fungi and Bacteria. Kew N, editor: Surrey, UK: Commonwealth Mycological Institute.

Efferth T, Fu Y-j, Zu Y-g, Schwarz G, Konkimalla VSB, Wink M. 2007. Molecular target-guided tumor therapy with natural products derived from traditional Chinese medicine. *Current medicinal chemistry* **14**(19):2024-2032.

Etschmann MMW, Schrader J. 2006. An aqueous–organic two-phase bioprocess for efficient production of the natural aroma chemicals 2-phenylethanol and 2-phenylethylacetate with yeast. *Applied microbiology and biotechnology* **71**(4):440-443.

Evans Jr RH, Ellestad GA, Kunstmann MP. 1969. Two new metabolites from an unidentified nigrospora species. *Tetrahedron Letters* **10**(22):1791-1794.

Everitt BS. 2013. Applied multivariate data analysis. John Wiley & Sons, Ltd.

Evidente A, Masi M, Linaldeddu BT, Franceschini A, Scanu B, Cimmino A, Andolfi A, Motta A, Maddau L. 2012. Afritoxinones A and B, dihydrofuropyran-2-ones produced by Diplodia africana the causal agent of branch dieback on Juniperus phoenicea. *Phytochemistry* **77**(0):245-250.

Exarchou V, Krucker M, van Beek TA, Vervoort J, Gerothanassis IP, Albert K. 2005. LC–NMR coupling technology: recent advancements and applications in natural products analysis. *Magnetic Resonance in Chemistry* **43**(9):681-687.

Fdhila F, Vázquez V, Sánchez JL, Riguera R. 2003. dd-Diketopiperazines: Antibiotics Active against Vibrio a nguillarum Isolated from Marine Bacteria Associated with Cultures of Pecten m aximus. *Journal of Natural Products* **66**(10):1299-1301.

Ferreres F, Andrade P, Tomás-Barberán FA. 1996. Natural Occurrence of Abscisic Acid in Heather Honey and Floral Nectar. *Journal of agricultural and food chemistry* **44**(8):2053-2056.

Fiedurek J, Gromada A, Jamroz J. 1996. Effect of medium components and metabolic inhibitors on β-galactosidase production and secretion by Penicillium notatum 1. *Journal of basic microbiology* **36**(1):27-32.

Fiehn O, Kopka J, Trethewey RN, Willmitzer L. 2000. Identification of uncommon plant metabolites based on calculation of elemental compositions using gas chromatography and quadrupole mass spectrometry. *Analytical chemistry* **72**(15):3573-3580.

Fleming A. 1929. On the antibacterial action of cultures of a penicillium, with special reference to their use in the isolation of B. influenzae. *British journal of experimental pathology* **10**(3):226.

Frisvad JC, Rank C, Nielsen KF, Larsen TO. 2008. Metabolomics of Aspergillus fumigatus. *Medical Mycology* **47**(S1):S53-S71.

Fukushima T, Tanaka M, Gohbara M, Fujimori T. 1998. Phytotoxicity of three lactones from Nigrospora sacchari. *Phytochemistry* **48**(4):625-630.

Furuya K, Shirasaka M. 1969. Antibiotics from fungi. IV. Production of rhodosporin (bostrycin) by Nigrospora oryzae. *Sankyo Kenkyusho Nempo* **21**:165-168.

Ge HM, Song YC, Shan CY, Ye YH, Tan RX. 2005. New and Cytotoxic Anthraquinones from Pleospora sp. IFB-E006, an Endophytic Fungus in Imperata cylindrical. *Planta Med* **71**(11):1063-1065.

Ge HM, Yu ZG, Zhang J, Wu JH, Tan RX. 2009. Bioactive alkaloids from endophytic Aspergillus fumigatus. *Journal of Natural Products* **72**(4):753-755.

Ghisalberti E. 1998. Biological and pharmacological activity of naturally occurring iridoids and secoiridoids. *Phytomedicine* **5**(2):147-163.

Gil-Turnes MS, Fenical W. 1992. Embryos of Homarus americanus are protected by epibiotic bacteria. *The Biological Bulletin* **182**(1):105-108.

Gomes NM, Dethoup T, Singburaudom N, Gales L, Silva AMS, Kijjoa A. 2012. Eurocristatine, a new diketopiperazine dimer from the marine sponge-associated fungus Eurotium cristatum. *Phytochemistry Letters* **5**(4):717-720.

Gray AI, Igoli JO, Edrada-Ebel R. 2012. Natural Products Isolation in Modern Drug Discovery Programs #. T Natural Products Isolation. p 515-534.

Griffin DH. 1996. Fungal physiology. John Wiley & Sons.

Griffiths WJ, Koal T, Wang Y, Kohl M, Enot DP, Deigner HP. 2010. Targeted metabolomics for biomarker discovery. *Angewandte Chemie International Edition* **49**(32):5426-5445.

Gross H, Stockwell VO, Henkels MD, Nowak-Thompson B, Loper JE, Gerwick WH. 2007. The Genomisotopic Approach: A Systematic Method to Isolate Products of Orphan Biosynthetic Gene Clusters. *Chemistry & Biology* **14**(1):53-63.

Gu W. 2009. Bioactive metabolites from Alternaria brassicicola ML-P08, an endophytic fungus residing in Malus halliana. *World Journal of Microbiology and Biotechnology* **25**(9):1677-1683.

Gupta AK, Sibbald RG, Lynde CW, Hull PR, Prussick R, Shear NH, De Doncker P, Daniel III CR, Elewski BE. 1997. Onychomycosis in children: prevalence and treatment strategies. *Journal of the American Academy of Dermatology* **36**(3):395-402.

Hanson JR. 2003. Natural products: the secondary metabolites. Royal Society of Chemistry.

Harris CM, Roberson JS, Harris TM. 1976. Biosynthesis of griseofulvin. *Journal of the American Chemical Society* **98**(17):5380-5386.

Harvey AL, Edrada-Ebel R, Quinn RJ. 2015. The re-emergence of natural products for drug discovery in the genomics era. *Nat Rev Drug Discov* **14**(2):111-129.

Harwooda JS, Cutler HG, Jacyno JM. 1995. Nigrosporolide, a Plant Growth-Inhibiting Macrolide from the Mould Nigrospora sphaerica. *Natural Product Letters* **6**(3):181-185.

Havsteen B. 1983. *Biochem. Pharmacol* **32**:141-148.

Hazalin NAM, Lim SM, Cole AL, Majeed ABA, Ramasamy K. 2013. Apoptosis induced by desmethyl-lasiodiplodin is associated with upregulation of apoptotic genes and downregulation of monocyte chemotactic protein-3. *Anti-cancer drugs*.

He G, Matsuura H, Yoshihara T. 2004. Isolation of an α-methylene-γ-butyrolactone derivative, a toxin from the plant pathogen Lasiodiplodia theobromae. *Phytochemistry* **65**(20):2803-2807.

He J-W, Chen G-D, Gao H, Yang F, Li X-X, Peng T, Guo L-D, Yao X-S. 2012. Heptaketides with antiviral activity from three endolichenic fungal strains Nigrospora sp., Alternaria sp. and Phialophora sp. *Fitoterapia* **83**(6):1087-1091.

Heinrich M, Barnes, J., Gibbons, S., and Williamson, E.M., editor. 2004. Fundamentals of Pharmacognosy and Phytotherapy. Churchill Livingstone: Elsevier Science Ltd.

Hendea D, Laschat S, Baro A, Frey W. 2006. Diastereoselective Alkylation of a Proline-Derived Bicyclic Lactim Ether. *Helvetica Chimica Acta* **89**(9):1894-1909.

Hirobe C, Qiao Z-S, Takeya K, Itokawa H. 1997. Cytotoxic flavonoids from Vitex agnus-castus. *Phytochemistry* **46**(3):521-524.

Hoberg E, Orjala J, Meier B, Sticher O. 1999. Diterpenoids from the fruits of *Vitex agnus-castus*. *Phytochemistry* **52**(8):1555-1558.

Höller U, König GM, Wright AD. 1998. Three New Metabolites from Marine-Derived Fungi of the Genera Coniothyrium and Microsphaeropsis. *Journal of Natural Products* **62**(1):114-118.

Hollman PCH, Katan M. 1999. Dietary flavonoids: intake, health effects and bioavailability. *Food and Chemical Toxicology* **37**(9):937-942.

Horai H, Arita M, Kanaya S, Nihei Y, Ikeda T, Suwa K, Ojima Y, Tanaka K, Tanaka S, Aoshima K. 2010. MassBank: a public repository for sharing mass spectral data for life sciences. *Journal of mass spectrometry* **45**(7):703-714.

Hormazabal E, Schmeda-Hirschmann G, Astudillo L, Rodriguez J, Theoduloz C. 2005. Metabolites from Microsphaeropsis olivacea, an endophytic fungus of Pilgerodendron uviferum. *Zeitschrift fur Naturforschung C-Journal of Biosciences* **60**(1-2):11-21.

Horn Ba. 1983. Distribution and role of insect hormones in plants. In: Bergamasco R, Horn, D.H.S, editor. Endocrinology of Insects. New York: A.R. Liss Inc. p 627-654.

Horwitz SB. 2004. Personal Recollections on the Early Development of Taxol⊥. *Journal of Natural Products* **67**(2):136-138.

Hotelling H. 1933. Analysis of a complex of statistical variables into principal components. *Journal of educational psychology* **24**(6):417.

Hotez P, Ottesen E, Fenwick A, Molyneux D. 2006. The neglected tropical diseases: the ancient afflictions of stigma and poverty and the prospects for their control and elimination. Hot Topics in Infection and Immunity in Children III: Springer. p 23-33.

Hotez PJ, Fenwick A, Savioli L, Molyneux DH. 2009. Rescuing the bottom billion through control of neglected tropical diseases. *The Lancet* **373**(9674):1570-1575.

Hotez PJ, Molyneux DH, Fenwick A, Kumaresan J, Sachs SE, Sachs JD, Savioli L. 2007. Control of Neglected Tropical Diseases. *New England Journal of Medicine* **357**(10):1018-1027.

Hou Y, Braun DR, Michel CR, Klassen JL, Adnani N, Wyche TP, Bugni TS. 2012. Microbial Strain Prioritization Using Metabolomics Tools for the Discovery of Natural Products. *Analytical chemistry* **84**(10):4277-4283.

Hsu T-H, Shiao L-H, Hsieh C, Chang D-M. 2002. A comparison of the chemical composition and bioactive ingredients of the Chinese medicinal mushroom DongChongXiaCao, its counterfeit and mimic, and fermented mycelium of Cordyceps sinensis. *Food Chemistry* **78**(4):463-469.

Hummel J, Selbig J, Walther D, Kopka J. 2007. The Golm Metabolome Database: a database for GC-MS based metabolite profiling. In: Nielsen J, Jewett M, editors. Metabolomics: Springer Berlin Heidelberg. p 75-95.

Ichihara A, Tazaki H, Sakamura S. 1983. Solanapyrones A, B and C, phytotoxic metabolites from the fungus Alternaria solani. *Tetrahedron Letters* **24**(48):5373-5376.

Iino K, Sugimoto M, Soga T, Tomita M. 2012. Profiling of the charged metabolites of traditional herbal medicines using capillary electrophoresis time-of-flight mass spectrometry. *Metabolomics* **8**(1):99-108.

Ingber D, Fujita T, Kishimoto S, Sudo K, Kanamaru T, Brem H, Folkman J. 1990. Synthetic analogues of fumagillin that inhibit angiogenesis and suppress tumour growth. *Nature* **348**(6301):555-557.

Isaka M, Berkaew P, Intereya K, Komwijit S, Sathitkunanon T. 2007. Antiplasmodial and antiviral cyclohexadepsipeptides from the endophytic fungus Pullularia sp. BCC 8613. *Tetrahedron* **63**(29):6855-6860.

J. Anzanello M, S. Ortiz R, Limberger R, Mariotti K. 2014. Performance of some supervised and unsupervised multivariate techniques for grouping authentic and unauthentic Viagra and Cialis. *Egyptian Journal of Forensic Sciences* **4**(3):83-89.

Jacobs MR. 2007. Retapamulin: a semisynthetic pleuromutilin compound for topical treatment of skin infections in adults and children. *Future microbiology* **2**(6):591-600.

Jayasuriya H, Ball RG, Zink DL, Smith JL, Goetz MA, Jenkins RG, Nallin-Omstead M, Silverman KC, Bills GF, Lingham RB. 1995. Barceloneic acid A, a new farnesyl-protein transferase inhibitor from a Phoma species. *Journal of Natural Products* **58**(7):986-991.

Jayatilake GS, Thornton MP, Leonard AC, Grimwade JE, Baker BJ. 1996. Metabolites from an Antarctic Sponge-Associated Bacterium, Pseudomonas aeruginosa. *Journal of Natural Products* **59**(3):293-296.

Jiao P, Swenson DC, Gloer JB, Campbell J, Shearer CA. 2006a. Decaspirones AE, bioactive spirodioxynaphthalenes from the freshwater aquatic fungus Decaisnella thyridioides. *Journal of Natural Products* **69**(12):1667-1671.

Jiao P, Gloer JB, Campbell J, Shearer CA. 2006b. Altenuene Derivatives from an Unidentified Freshwater Fungus in the Family Tubeufiaceae. *Journal of Natural Products* **69**(4):612-615.

Jolliffe I. 2005. Principal component analysis. Wiley Online Library.

Jung W, Yu O, Lau S-MC, O'Keefe DP, Odell J, Fader G, McGonigle B. 2000. Identification and expression of isoflavone synthase, the key enzyme for biosynthesis of isoflavones in legumes. *Nature biotechnology* **18**(2):208-212.

K. Hostettman MHaAM. 1989. Preparative chromatography techniques: Applications in natural product isolation. *Plant Growth Regul* **8**(4):375-376.

Kamal N, Clements C, Gray AI, Edrada Ebel R. 2011. Discovery of bioactive metabolites from the leaves of Vitex pinnata using high-throughput flash chromatography. *Planta Med* **77**(12):PG68.

Kawamura H, Kaneko T, Koshino H, Esumi Y, Uzawa J, Sugawara F. 2000. Penicillides from Penicillium sp. isolated from Taxus cuspidata. *Natural Product Letters* **14**(6):477-484.

Keserű GM, Makara GM. 2006. Hit discovery and hit-to-lead approaches. *Drug Discovery Today* **11**(15–16):741-748.

Kim BG, Kim H, Kim JH, Lim Y, Ahn J. 2006. Synthesis of ermanin, 5, 7-dihydroxy-3, 4'-dimethoxyflavone from kaempferol, 3, 5, 7, 4'-tetrahydroxyflavone with two O-methyltransferases expressed in E. coli. *Bulletin-Korean Chemical Society* **27**(3):357.

Kind T, Wohlgemuth G, Lee DY, Lu Y, Palazoglu M, Shahbaz S, Fiehn O. 2009. FiehnLib: Mass Spectral and Retention Index Libraries for Metabolomics Based on Quadrupole and Time-of-Flight Gas Chromatography/Mass Spectrometry. *Analytical chemistry* **81**(24):10038-10048.

Kingston DG, Chen PN, Vercellotti JR. 1976. Metabolites of Aspergillus versicolor: 6, 8-di-O-methylnidurufin, griseofulvin, dechlorogriseofluvin, and 3, 8-dihydroxy-6-methoxy-1-methylxanthone. *Phytochemistry* **15**(6):1037-1039.

Kitamura S. 2006. Transport of flavonoids: from cytosolic synthesis to vacuolar accumulation. The science of flavonoids: Springer. p 123-146.

Kitaoka N, Nabeta K, Matsuura H. 2009. Isolation and Structural Elucidation of a New Cyclohexenone Compound from <I>Lasiodiplodia theobromae</I>. *Bioscience, biotechnology, and biochemistry* **73**(8):1890-1892.

Klaiklay S, Rukachaisirikul V, Tadpetch K, Sukpondma Y, Phongpaichit S, Buatong J, Sakayaroj J. 2012. Chlorinated chromone and diphenyl ether derivatives from the mangrove-derived fungus Pestalotiopsis sp. PSU-MA69. *Tetrahedron* **68**(10):2299-2305.

Klemke C, Kehraus S, Wright AD, König GM. 2004. New Secondary Metabolites from the Marine Endophytic Fungus Apiospora montagnei†. *Journal of Natural Products* **67**(6):1058-1063.

Klitgaard A, Iversen A, Andersen M, Larsen T, Frisvad J, Nielsen K. 2014. Aggressive dereplication using UHPLC–DAD–QTOF: screening extracts for up to 3000 fungal secondary metabolites. *Analytical and Bioanalytical Chemistry*:1-11.

Kolak U, Hacibekıroğlu I, Öztürk M, Özgökçe F, Topçu G, Ulubelen A. 2009. Antioxidant and anticholinesterase constituents of *Salvia poculata*. *Turkish Journal of Chemistry* **33**(6).

Konstantinidou V, Covas M-I, Muñoz-Aguayo D, Khymenets O, de la Torre R, Saez G, del Carmen Tormos M, Toledo E, Marti A, Ruiz-Gutiérrez V. 2010. In vivo nutrigenomic effects of virgin olive oil polyphenols within the frame of the Mediterranean diet: a randomized controlled trial. *The FASEB Journal* **24**(7):2546-2557.

Krasnoff S, Gupta S. 1994. Identification of the antibiotic phomalactone from the entomopathogenic fungus *Hirsutella thompsonii* var.synnematosa. *J Chem Ecol* **20**(2):293-302.

Krause DS, Simjee A, Van Rensburg C, Viljoen J, Walsh TJ, Goldstein BP, Wible M, Henkel T. 2004. A randomized, double-blind trial of anidulafungin versus fluconazole for the treatment of esophageal candidiasis. *Clinical infectious diseases* **39**(6):770-775.

Krohn K, Bahramsari R, Flörke U, Ludewig K, Kliche-Spory C, Michel A, Aust H-J, Draeger S, Schulz B, Antus S. 1997. Dihydroisocoumarins from fungi: Isolation, structure elucidation, circular dichroism and biological activity. *Phytochemistry* **45**(2):313-320.

Kuo Y-C, Sun C-M, Ou J-C, Tsai W-J. 1997. A tumor cell growth inhibitor from polygonum hypoleucum Ohwi. *Life Sciences* **61**(23):2335-2344.

Kuruüzüm-Uz A, Ströch K, Demirezer LÖ, Zeeck A. 2003. Glucosides from Vitex agnus-castus. *Phytochemistry* **63**(8):959-964.

Kuthubutheen A. 1981. Fungi associated with the aerial parts of Malaysian mangrove plants. *Mycopathologia* **76**(1):33-43.

Laatsch H. 2012. Antibase Version 4.0—The Natural Compound Identifier. Wiley-VCH Verlag GmbH & Co. KGaA: Weinheim, Germany.

Lai Y, Liu K, Zhang X, Zhang X, Li K, Wang N, Shu C, Wu Y, Wang C, Bushley KE *et al.*,. 2014. Comparative Genomics and Transcriptomics Analyses Reveal

Divergent Lifestyle Features of Nematode Endoparasitic Fungus Hirsutella minnesotensis. *Genome Biology and Evolution* **6**(11):3077-3093.

Lankadurai BP, Nagato EG, Simpson MJ. 2013. Environmental metabolomics: an emerging approach to study organism responses to environmental stressors. *Environmental Reviews* **21**(3):180-205.

Lao YM, Jiang JG, Yan L. 2009. Application of metabonomic analytical techniques in the modernization and toxicology research of traditional Chinese medicine. *British journal of pharmacology* **157**(7):1128-1141.

Larsen TO, Frisvad JC. 1995. Characterization of volatile metabolites from 47 Penicillium taxa. *Mycological Research* **99**(10):1153-1166.

Laureti L, Song L, Huang S, Corre C, Leblond P, Challis GL, Aigle B. 2011. Identification of a bioactive 51-membered macrolide complex by activation of a silent polyketide synthase in Streptomyces ambofaciens. *Proceedings of the National Academy of Sciences* **108**(15):6258-6263.

Lautru S, Deeth RJ, Bailey LM, Challis GL. 2005. Discovery of a new peptide natural product by Streptomyces coelicolor genome mining. *Nat Chem Biol* **1**(5):265-269.

Lee JC, Yang X, Schwartz M, Strobel G, Clardy J. 1995. The relationship between an endangered North American tree and an endophytic fungus. *Chemistry & Biology* **2**(11):721-727.

Lee WY, Park Y, Ahn J, Park S, Lee H. 2005. Cytotoxic activity of ergosta-4, 6, 8 (14), 22-tetraen-3-one from the sclerotia of *Polyporus umbellatus*. *Bulletin-Korean Chemical Society* **26**(9):1464.

Li D-l, Li X-m, Cui C-m, Wang B-g. 2008. Chemical constituents of Hypocreales sp., an endophytic fungus derived from red alga Symphyocladia latiscula. *Marine Sciences* **11**:010.

Li D, Chen Y, Pan Q, Tao M, Zhang W. 2014a. A New Eudesmane Sesquiterpene from Nigrospora oryzae, an Endophytic Fungus of Aquilaria sinensis. *Records of Natural Products* **8**(4).

Li N, Chen J-J, Zhou J. 2005. Note: Capitulatin B, A new eudesmane derivative from Curculigo capitulata, and revised assignment of 13C NMR data of 6α,15α-epoxy-1β,4β-dihydroxyeudesmane. *Journal of Asian natural products research* **7**(3):279-282.

Li P, Zuo, T. T., & Wang, X. Q. 2008. Chemical constituents of scutellaria barbata D. don (II). *Chin J Med Chem* **18**(5):374-376.

Li Q, Wang G. 2009. Diversity of fungal isolates from three Hawaiian marine sponges. *Microbiological Research* **164**(2):233-241.

Li S-H, Zhang H-J, Qiu S-X, Niu X-M, Santarsiero BD, Mesecar AD, Fong HHS, Farnsworth NR, Sun H-D. 2002. Vitexlactam A, a novel labdane diterpene lactam from the fruits of Vitex agnus-castus. *Tetrahedron Letters* **43**(29):5131-5134.

Li S, Qiu S, Yao P, Sun H, Fong HH, Zhang H. Compounds from the Fruits of the Popular European Medicinal Plant Vitex agnus-castus in Chemoprevention via NADP (H): Quinone Oxidoreductase Type 1 Induction.

Li S, Lo C-Y, Ho C-T. 2006. Hydroxylated polymethoxyflavones and methylated flavonoids in sweet orange (*Citrus sinensis*) peel. *Journal of agricultural and food chemistry* **54**(12):4176-4185.

Li X, Li X-M, Xu G-M, Li C-S, Wang B-G. 2014b. Antioxidant metabolites from marine alga-derived fungus Aspergillus wentii EN-48. *Phytochemistry Letters* **7**(0):120-123.

Li X, Sun G, Zheng Y, Lin W, Sattler I. 2007. Separation and structures of two steroids from mangrove endophyte Penicillium. *Tianran Chanwu Yanjiu Yu Kaifa* **19**:420-422.

Lim JCW, Chan TK, Ng DS, Sagineedu SR, Stanslas J, Wong W. 2012. Andrographolide and its analogues: versatile bioactive molecules for combating inflammation and cancer. *Clinical and Experimental Pharmacology and Physiology* **39**(3):300-310.

Liu H, Liu S, Guo L, Zhang Y, Cui L, Ding G. 2012. New Furanones from the Plant Endophytic Fungus Pestalotiopsis besseyi. *Molecules* **17**(12):14015-14021.

Liu JY, Song YC, Zhang Z, Wang L, Guo ZJ, Zou WX, Tan RX. 2004. Aspergillus fumigatus CY018, an endophytic fungus in Cynodon dactylon as a versatile producer of new and bioactive metabolites. *Journal of Biotechnology* **114**(3):279-287.

Liu L, Li Y, Li L, Cao Y, Guo L, Liu G, Che Y. 2013a. Spiroketals of Pestalotiopsis fici Provide Evidence for a Biosynthetic Hypothesis Involving Diversified Diels–Alder Reaction Cascades. *The Journal of organic chemistry* **78**(7):2992-3000.

Liu L, Liu S, Niu S, Guo L, Chen X, Che Y. 2009. Isoprenylated chromone derivatives from the plant endophytic fungus Pestalotiopsis fici. *Journal of Natural Products* **72**(8):1482-1486.

Liu L, Liu S, Jiang L, Chen X, Guo L, Che Y. 2008. Chloropupukeananin, the First Chlorinated Pupukeanane Derivative, and Its Precursors from Pestalotiopsis fici. *Organic letters* **10**(7):1397-1400.

Liu LF, Desai SD, LI TK, Mao Y, Sun M, SIM SP. 2000. Mechanism of action of camptothecin. *Annals of the New York Academy of Sciences* **922**(1):1-10.

Liu S, Liu X, Guo L, Che Y, Liu L. 2013b. 2H-Pyran-2-one and 2H-Furan-2-one Derivatives from the Plant Endophytic Fungus Pestalotiopsis fici. *Chemistry & Biodiversity* **10**(11):2007-2013.

Lomascolo A, Lesage-Meessen L, Haon M, Navarro D, Antona C, Faulds C, Marcel A. 2001. Evaluation of the potential of Aspergillus niger species for the bioconversion of L-phenylalanine into 2-phenylethanol. *World Journal of Microbiology and Biotechnology* **17**(1):99-102.

Lozano-Sánchez Js, Segura-Carretero A, Menendez JA, Oliveras-Ferraros C, Cerretani L, Fernández-Gutiérrez A. 2010. Prediction of extra virgin olive oil varieties through their phenolic profile. Potential cytotoxic activity against human breast cancer cells. *Journal of agricultural and food chemistry* **58**(18):9942-9955.

Lu X, Chen G, Hua H, Dai H, Mei W, Xu Y, Pei Y. 2012. Aromatic compounds from endophytic fungus Colletotrichum sp. L10 of Cephalotaxus hainanensis Li. *Fitoterapia* **83**(4):737-741.

Lucas E, Machado Y, Ferreira A, Dolabella L, Takahashi J. 2010. Improved production of pharmacologically-active sclerotiorin by Penicillium sclerotiorum. *Tropical Journal of Pharmaceutical Research* **9**(4).

Ludwig C, Günther UL. 2011. MetaboLab-advanced NMR data processing and analysis for metabolomics. *BMC Bioinformatics* **12**(1):366.

Luo DQ, Zhang L, Shi BZ, Song XM. 2012. Two New Oxysporone Derivatives from the Fermentation Broth of the Endophytic Plant Fungus Pestalotiopsis karstenii Isolated from Stems of Camellia sasanqua. *Molecules* **17**(7):8554-8560.

Ma C, Li Y, Niu S, Zhang H, Liu X, Che Y. 2010a. N-Hydroxypyridones, Phenylhydrazones, and a Quinazolinone from Isaria farinosa. *Journal of Natural Products* **74**(1):32-37.

Ma Y-T, Qiao L-R, Shi W-Q, Zhang A-L, Gao J-M. 2010b. Metabolites produced by an endophyte Alternaria alternata isolated from Maytenus hookeri. *Chemistry of Natural Compounds* **46**(3):504-506.

Macías-Rubalcava ML, Hernández-Bautista BE, Jiménez-Estrada M, González MC, Glenn AE, Hanlin RT, Hernández-Ortega S, Saucedo-García A, Muria-

González JM, Anaya AL. 2008. Naphthoquinone spiroketal with allelochemical activity from the newly discovered endophytic fungus Edenia gomezpompae. *Phytochemistry* **69**(5):1185-1196.

Macintyre L, Zhang T, Viegelmann C, Martinez IJ, Cheng C, Dowdells C, Abdelmohsen UR, Gernert C, Hentschel U, Edrada-Ebel R. 2014. Metabolomic Tools for Secondary Metabolite Discovery from Marine Microbial Symbionts. *Marine Drugs* **12**(6):3416-3448.

MacMillan J. 1953. 351. Griseofulvin. Part VII. Dechlorogriseofulvin. *J. Chem. Soc.*:1697-1702.

Mahato SB, Kundu AP. 1994. 13C NMR Spectra of pentacyclic triterpenoids--a compilation and some salient features. *Phytochemistry* **37**(6):1517-1575.

Malan E, Roux DG. 1979. Flavonoids from Distemonanthus benthamianus Baillon. Methoxylated flavones and inter-relationships of benthamianin, a [2]benzopyrano[4,3-b][1]benzopyran. *Journal of the Chemical Society, Perkin Transactions 1*(0):2696-2703.

Månsson M, Phipps RK, Gram L, Munro MH, Larsen TO, Nielsen KF. 2010. Explorative solid-phase extraction (E-SPE) for accelerated microbial natural product discovery, dereplication, and purification. *Journal of Natural Products* **73**(6):1126-1132.

Mantsch H, Smith IC. 1973. A Study of Solvent Effects on the 13C Nuclear Magnetic Resonance Spectra of Cholesterol, Pyridine, And Uridine. *Canadian Journal of Chemistry* **51**(9):1384-1391.

Martínez-Luis S, Della-Togna G, Coley PD, Kursar TA, Gerwick WH, Cubilla-Rios L. 2008. Antileishmanial Constituents of the Panamanian Endophytic Fungus *Edenia* sp. *Journal of Natural Products* **71**(12):2011-2014.

Matsuura H, Nakamori K, Omer EA, Hatakeyama C, Yoshihara T, Ichihara A. 1998. Three lasiodiplodins from lasiodiplodia theobromae ifo 31059. *Phytochemistry* **49**(2):579-584.

Mazlan N, Tate R, Clements C, Edrada-Ebel R. 2013. Metabolomics studies of endophytic metabolites from Malaysian mangrove plant in the search for new potential antibiotics. *Planta Med* **79**(13):PA22.

McCowen MC, Callender ME, Lawlis JF. 1951. Fumagillin (H-3), a new antibiotic with amebicidal properties. *Science* **113**(2930):202-203.

Mehta G, Islam K. 2004. Enantioselective total synthesis of epoxyquinone natural products (−)-phyllostine,(+)-epoxydon,(+)-epiepoxydon and (−)-

panepophenanthrin: access to versatile chiral building blocks through enzymatic kinetic resolution. *Tetrahedron Letters* **45**(41):7683-7687.

Metwaly AM, Kadry HA, El-Hela AA, Mohammad A-EI, Ma G, Cutler SJ, Ross SA. 2014. Nigrosphaerin A a new isochromene derivative from the endophytic fungus Nigrospora sphaerica. *Phytochemistry Letters* **7**(0):1-5.

Mew T-W, Gonzales P. 2002. A handbook of rice seedborne fungi. Int. Rice Res. Inst.

Moco S, Vervoort J, Bino RJ, De Vos RCH, Bino R. 2007. Metabolomics technologies and metabolite identification. *TrAC Trends in Analytical Chemistry* **26**(9):855-866.

Mohali S, Burgess T, Wingfield M. 2005. Diversity and host association of the tropical tree endophyte Lasiodiplodia theobromae revealed using simple sequence repeat markers. *Forest Pathology* **35**(6):385-396.

Mohanty SS, Prakash S. 2009. Effects of culture media on larvicidal property of secondary metabolites of mosquito pathogenic fungus *Chrysosporium lobatum* (Moniliales: Moniliaceae). *Acta tropica* **109**(1):50-54.

Moreno E, Varughese T, Spadafora C, Arnold AE, Coley PD, Kursar TA, Gerwick WH, Cubilla-Rios L. 2011. Chemical constituents of the new endophytic fungus Mycosphaerella sp. nov. and their anti-parasitic activity. *Natural product communications* **6**(6):835.

Mulholland D, Cheplogoi P, Crouch N. 2003. Secondary metabolites from *Kirkia acuminata* and *Kirkia wilmsii* (Kirkiaceae). *Biochemical systematics and ecology* **31**(7):793-797.

Nakamori K, Matsuura H, Yoshihara T, Ichihara A, Koda Y. 1994. Potato micro-tuber inducing substances from Lasiodiplodia theobromae. *Phytochemistry* **35**(4):835-839.

Nelson PE, Desjardins AE, Plattner RD. 1993. Fumonisins, mycotoxins produced by Fusarium species: biology, chemistry, and significance. *Annual review of phytopathology* **31**(1):233-252.

Newman DJ, Cragg GM. 2012. Natural Products As Sources of New Drugs over the 30 Years from 1981 to 2010. *Journal of Natural Products* **75**(3):311-335.

Newman DJ, Cragg GM, Snader KM. 2000. The influence of natural products upon drug discovery. *Natural Product Reports* **17**(3):215-234.

Nielsen KF, Mogensen JM, Johansen M, Larsen TO, Frisvad JC. 2009. Review of secondary metabolites and mycotoxins from the Aspergillus niger group. *Analytical and bioanalytical chemistry* **395**(5):1225-1242.

Nielsen KF, Månsson M, Rank C, Frisvad JC, Larsen TO. 2011. Dereplication of Microbial Natural Products by LC-DAD-TOFMS. *Journal of Natural Products* **74**(11):2338-2348.

Nishikawa H. 1933. Biochemistry of Filamentous Fungi. II. A Metabolic Product of Aspergillus melleus Yukawa. Part I.

Ochieng CO, Ishola IO, Opiyo SA, Manguro LAO, Owuor PO, Wong K-C. 2013. Phytoecdysteroids from the Stem Bark of Vitex doniana and Their Anti-Inflammatory Effects. *Planta Med* **79**(01):52-59.

Ogawa T, Ando K, Aotani Y, Shinoda K, Tanaka T, Tsukuda E, Yoshida M, Matsuda Y. 1995. RES-1214-1 and-2, novel non-peptidic endothelin type A receptor antagonists produced by Pestalotiopsis sp. *The Journal of antibiotics* **48**(12):1401.

Okomo-Assoumou MC, Daulouede S, Lemesre J-L, N'Zila-Mouanda A, Vincendeau P. 1995. Correlation of High Serum Levels of Tumor Necrosis Factor-α with Disease Severity in Human African trypanosomiasis. *The American Journal of Tropical Medicine and Hygiene* **53**(5):539-543.

Oliveira MN, Santos LS, Guilhon G, Santos AS, Ferreira IC, Lopes-Junior ML, Arruda MSP, Marinho AM, Silva MNd, Rodrigues-Filho E. 2011. Novel anthraquinone derivatives produced by Pestalotiopsis guepinii, an endophytic of the medicinal plant Virola michelii (Myristicaceae). *Journal of the Brazilian Chemical Society* **22**(5):993-996.

Oliver SG, Winson MK, Kell DB, Baganz F. 1998. Systematic functional analysis of the yeast genome. *Trends in Biotechnology* **16**(9):373-378.

Ong CY, Ling SK, Ali RM, Chee CF, Samah ZA, Ho ASH, Teo SH, Lee HB. 2009. Systematic analysis of in vitro photo-cytotoxic activity in extracts from terrestrial plants in Peninsula Malaysia for photodynamic therapy. *Journal of Photochemistry and Photobiology B: Biology* **96**(3):216-222.

Ong H, Nordiana M. 1999. Malay ethno-medico botany in Machang, Kelantan, Malaysia. *Fitoterapia* **70**(5):502-513.

Ono M, Yamamoto M, Masuoka C, Ito Y, Yamashita M, Nohara T. 1999. Diterpenes from the Fruits of Vitex rotundifolia. *Journal of Natural Products* **62**(11):1532-1537.

Ono M, Eguchi K, Konoshita M, Furusawa C, Sakamoto J, Yasuda S, Ikeda T, Okawa M, Kinjo J, Yoshimitsu H *et al.*,. 2011. A New Diterpenoid Glucoside and Two New Diterpenoids from the Fruit of *Vitex agnus-castus*. *Chemical and Pharmaceutical Bulletin* **59**(3):392-396.

Ono M, Nagasawa Y, Ikeda T, Tsuchihashi R, Okawa M, Kinjo J, Yoshimitsu H, Nohara T. 2009. Three New Diterpenoids from the Fruit of *Vitex agnus-castus*. *Chemical and Pharmaceutical Bulletin* **57**(10):1132-1135.

Ono M, Yamasaki T, Konoshita M, Ikeda T, Okawa M, Kinjo J, Yoshimitsu H, Nohara T. 2008. Five New Diterpenoids, Viteagnusins A--E, from the Fruit of *Vitex agnus-castus*. *Chemical and Pharmaceutical Bulletin* **56**(11):1621-1624.

Ono M, Yamamoto M, Yanaka T, Ito Y, Nohara T. 2001. Ten New Labdane-Type Diterpenes from the Fruit of Vitex rotundifolia. *Chemical and Pharmaceutical Bulletin* **49**(1):82-86.

Overy DP, Larsen TO, Dalsgaard PW, Frydenvang K, Phipps R, Munro MH, Christophersen C. 2005. Andrastin A and barceloneic acid metabolites, protein farnesyl transferase inhibitors from Penicillium albocoremium: chemotaxonomic significance and pathological implications. *Mycological Research* **109**(11):1243-1249.

Oxford AE, Raistrick H, Simonart P. 1939. Studies in the biochemistry of micro-organisms: Griseofulvin, C17H17O6Cl, a metabolic product of Penicillium griseo-fulvum Dierckx. *Biochemical Journal* **33**(2):240.

Pall ML. 1981. Adenosine 3', 5'-phosphate in fungi. *Microbiological Reviews* **45**(3):462.

Pang Z, Sterner O. 1993. The Isolation of Ergosta-4, 6, 8 (14), 22-tetraen-3β-ol from Injured Fruit Bodies of Marasmius oreades. *Natural Product Letters* **3**(3):193-196.

Patti GJ, Yanes O, Siuzdak G. 2012. Innovation: Metabolomics: the apogee of the omics trilogy. *Nat Rev Mol Cell Biol* **13**(4):263-269.

Pauli GF, Jaki BU, Lankin DC. 2004. Quantitative 1H NMR: Development and Potential of a Method for Natural Products Analysis§. *Journal of Natural Products* **68**(1):133-149.

Peric-Concha N, Long PF. 2003. Mining the microbial metabolome: a new frontier for natural product lead discovery. *Drug Discovery Today* **8**(23):1078-1084.

Pick A, Müller H, Mayer R, Haenisch B, Pajeva IK, Weigt M, Bönisch H, Müller CE, Wiese M. 2011. Structure–activity relationships of flavonoids as inhibitors of

breast cancer resistance protein (BCRP). *Bioorganic & Medicinal Chemistry* **19**(6):2090-2102.

Pluskal T, Castillo S, Villar-Briones A, Orešič M. 2010. MZmine 2: modular framework for processing, visualizing, and analyzing mass spectrometry-based molecular profile data. *BMC Bioinformatics* **11**(1):395.

Poch GK, Gloer JB. 1989. Helicascolides A and B: new lactones from the marine fungus Helicascus kanaloanus. *Journal of Natural Products* **52**(2):257-260.

Press C. Dictionary of Natural Products. http://www.chemnetbase.com/ (accessed 12 August).

Press C. The Dictionary of Marine Natural Products. http://www.chemnetbase.com/ (accessed 27/06).

Procopiou PA, Baugh SPD, Flack SS, Inglis GGA. 1998. An Extremely Powerful Acylation Reaction of Alcohols with Acid Anhydrides Catalyzed by Trimethylsilyl Trifluoromethanesulfonate†. *The Journal of organic chemistry* **63**(7):2342-2347.

Pudhom K, Teerawatananond T, Chookpaiboon S. 2014. Spirobisnaphthalenes from the Mangrove-Derived Fungus Rhytidhysteron sp. AS21B. *Marine Drugs* **12**(3):1271-1280.

Puolitaival SM, Burnum KE, Cornett DS, Caprioli RM. 2008. Solvent-free matrix dry-coating for MALDI imaging of phospholipids. *Journal of the American Society for Mass Spectrometry* **19**(6):882-886.

Pyo MK, Lee Y, Yun-Choi HS. 2002. Anti-platelet effect of the constituents isolated from the barks and fruits ofMagnolia obovata. *Archives of Pharmacal Research* **25**(3):325-328.

Qing-fang G, Yu-mei Z, Ning-hua T, Zuo-hong C. 2010. Chemical Constituents of Three Poisonous Mushrooms. *Natural Product Research & Development* **22**(2).

Ramautar R, Somsen GW, de Jong GJ. 2009. CE-MS in metabolomics. *Electrophoresis* **30**(1):276-291.

Ramautar R, Somsen GW, de Jong GJ. 2013. CE-MS for metabolomics: Developments and applications in the period 2010–2012. *Electrophoresis* **34**(1):86-98.

Ramautar R, Mayboroda OA, Somsen GW, de Jong GJ. 2011. CE-MS for metabolomics: Developments and applications in the period 2008–2010. *Electrophoresis* **32**(1):52-65.

Ramirez T, Daneshian M, Kamp H, Bois FY, Clench MR, Coen M, Donley B, Fischer SM, Ekman DR, Fabian E. 2013. Metabolomics in toxicology and preclinical research. *Altex* **30**(2):209.

Ramos HP, Simão MR, de Souza JM, Magalhães LG, Rodrigues V, Ambrósio SR, Said S. 2013. Evaluation of dihydroisocoumarins produced by the endophytic fungus Arthrinium state of Apiospora montagnei against Schistosoma mansoni. *Natural Product Research* **27**(23):2240-2243.

Rao M, Feng L, Ruan L, Ge M, Sheng X. 2013. UPLC-MS-Based Metabolomic Study of Streptomyces Strain HCCB10043 Under Different pH Conditions Reveals Important Pathways Affecting the Biosynthesis of A21978C Compounds. *Analytical Letters* **46**(15):2305-2318.

Rasser F, Anke T, Sterner O. 2000. Secondary metabolites from a Gloeophyllum species. *Phytochemistry* **54**(5):511-516.

Rhind SG, Sabiston BH, Shek PN, Buguet A, Muanga G, Stanghellini A, Dumas M, Radomski MW. 1997. Effect of Melarsoprol Treatment on Circulating IL-10 and TNF-α Levels in Human African Trypanosomiasis. *Clinical Immunology and Immunopathology* **83**(2):185-189.

Rhodes A, Boothroyd B, McGonagle MP, Somerfield G. 1961. Biosynthesis of griseofulvin: the methylated benzophenone intermediates. *Biochemical Journal* **81**(1):28.

Rice LB. 2008. Federal funding for the study of antimicrobial resistance in nosocomial pathogens: no ESKAPE. *Journal of Infectious Diseases* **197**(8):1079-1081.

Robinette SL, Brüschweiler R, Schroeder FC, Edison AS. 2011. NMR in Metabolomics and Natural Products Research: Two Sides of the Same Coin. *Accounts of Chemical Research* **45**(2):288-297.

Rönsberg D, Debbab A, Mándi A, Wray V, Dai H, Kurtán T, Proksch P, Aly AH. 2013. Secondary metabolites from the endophytic fungus Pestalotiopsis virgatula isolated from the mangrove plant Sonneratia caseolaris. *Tetrahedron Letters* **54**(25):3256-3259.

Ross JA, Kasum CM. 2002. Dietary flavonoids: bioavailability, metabolic effects, and safety. *Annual review of Nutrition* **22**(1):19-34.

Rossi MH, Yoshida M, Soares Maia JG. 1997. Neolignans, styrylpyrones and flavonoids from an *Aniba* species. 1263-1269 p.

Rukachaisirikul V, Arunpanichlert J, Sukpondma Y, Phongpaichit S, Sakayaroj J. 2009. Metabolites from the endophytic fungi Botryosphaeria rhodina PSU-M35 and PSU-M114. *Tetrahedron* **65**(51):10590-10595.

Rukachaisirikul V, Khamthong N, Sukpondma Y, Phongpaichit S, Hutadilok-Towatana N, Graidist P, Sakayaroj J, Kirtikara K. 2010. Cyclohexene, diketopiperazine, lactone and phenol derivatives from the sea fan-derived fungi *Nigrospora* sp. PSU-F11 and PSU-F12. *Archives of Pharmacal Research* **33**(3):375-380.

Rukachaisirikul V, Rodglin A, Phongpaichit S, Buatong J, Sakayaroj J. 2012. α-Pyrone and seiricuprolide derivatives from the mangrove-derived fungi Pestalotiopsis spp. PSU-MA92 and PSU-MA119. *Phytochemistry Letters* **5**(1):13-17.

Salim AA, Chin Y-W, Kinghorn AD. 2008. Drug discovery from plants. Bioactive molecules and Medicinal plants: Springer. p 1-24.

Salinas J, Mollinedo P, Vila J. 2012. Calycin and physcion from the lichen *Candelaria concolor*. *Revista Boliviana de Química* **29**:94.

Samat N, Tan PJ, Shaari K, Abas F, Lee HB. 2014. Prioritization of Natural Extracts by LC–MS-PCA for the Identification of New Photosensitizers for Photodynamic Therapy. *Analytical chemistry*.

Samuelsson G. 1993. Drugs of Natural Origin. Textbook of Pharmacognosy. Swedish Pharmaceutical Press Editor

Santos TCd, Schripsema J, Monache FD, Leitão SG. 2001. Iridoids from Vitex cymosa. *Journal of the Brazilian Chemical Society* **12**:763-766.

Saravolatz LD, Deresinski SC, Stevens DA. 2003. Caspofungin. *Clinical infectious diseases* **36**(11):1445-1457.

Sarker S, Nahar L. 2012. An Introduction to Natural Products Isolation. In: Sarker SD, Nahar L, editors. Natural Products Isolation: Humana Press. p 1-25.

Sathiamoorthy B, Gupta P, Kumar M, Chaturvedi AK, Shukla PK, Maurya R. 2007. New antifungal flavonoid glycoside from Vitex negundo. *Bioorganic & medicinal chemistry letters* **17**(1):239-242.

Scheltema RA, Jankevics A, Jansen RC, Swertz MA, Breitling R. 2011. PeakML/mzMatch: A File Format, Java Library, R Library, and Tool-Chain for Mass Spectrometry Data Analysis. *Analytical chemistry* **83**(7):2786-2793.

Schlünzen F, Pyetan E, Fucini P, Yonath A, Harms JM. 2004. Inhibition of peptide bond formation by pleuromutilins: the structure of the 50S ribosomal subunit

from Deinococcus radiodurans in complex with tiamulin. *Molecular microbiology* **54**(5):1287-1294.

Schreiber D, Jung M, Sandjo LP, Liermann JC, Opatz T, Erkel G. 2012. 3[prime]-Demethyldihydromaldoxin and dihydromaldoxin, two anti-inflammtory diaryl ethers from a Steganospora species. *J Antibiot* **65**(9):473-477.

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

Schwartz RE, Sesin DF, Joshua H, Wilson KE, Kempf AJ, Goklen KA, Kuehner D, Gailliot P, Gleason C, White R. 1992. Pneumocandins from Zalerion arboricola. I. Discovery and isolation. *The Journal of antibiotics* **45**(12):1853.

Scuderi P, Lam K, Ryan K, Petersen E, Sterling K, Finley P, Ray CG, Slymen D, Salmon S. 1986. Raised serum levels of tumour necrosis factor in parasitic infections. *The Lancet* **328**(8520):1364-1365.

Seeley EH, Caprioli RM. 2011. MALDI imaging mass spectrometry of human tissue: method challenges and clinical perspectives. *Trends in Biotechnology* **29**(3):136-143.

Sehgal CK, Taneja SC, Dhar KL, Atal CK. 1982. 2′-p-Hydroxybenzoyl mussaenosidic acid, a new iridoid glucoside from Vitex negundo. *Phytochemistry* **21**(2):363-366.

Sehgal CK, Taneja SC, Dhar KL, Atal CK. 1983. 6′-p-hydroxybenzoylmussaenosidic acid-an iridoid glucoside from Vitex negundo. *Phytochemistry* **22**(4):1036-1038.

Sempere F, Santamarina MP. 2006. Microscopic and macroscopic study of the interaction betweenAlternaria alternata (Fr.) Keissler andNigrospora oryzae (Berk. & Broome) Petch. *Annals of microbiology* **56**(2):101-107.

Sena Filho JG, Duringer J, Maia GLA, Tavares JF, Xavier HS, Sobral da Silva M, da-Cunha EVL, Barbosa-Filho JM. 2008. Ecdysteroids from Vitex Species: Distribution and Compilation of Their 13C-NMR Spectral Data. *Chemistry & Biodiversity* **5**(5):707-713.

Shaffer N, Grau GE, Hedberg K, Davachi F, Lyamba B, Hightower AW, Breman JG, Nguyen-Dinh P. 1991. Tumor Necrosis Factor and Severe Malaria. *Journal of Infectious Diseases* **163**(1):96-101.

Shang Z, Li X-M, Li C-S, Wang B-G. 2012. Diverse Secondary Metabolites Produced by Marine-Derived Fungus Nigrospora sp. MA75 on Various Culture Media. *Chemistry & Biodiversity* **9**(7):1338-1348.

Shao C, Wang C, Li C, She Z, Gu Y, Lin Y. 2009. Microsphaeropsisin A, a new sesquiterpenoid isolated from the mangrove endophytic fungus (No. DZ39). *Natural Product Research* **23**(17):1579-1583.

Sharma RL, Prabhakar A, Dhar KL, Sachar A. 2009. A new iridoid glycoside from Vitex negundo Linn (Verbenacea). *Natural Product Research* **23**(13):1201-1209.

Shimada A, Takahashi I, Kawano T, Kimura Y. 2001. Chloroisosulochrin, chloroisosulochrin dehydrate, and pestheic acid, plant growth regulators, produced by Pestalotiopsis theae. *Zeitschrift für Naturforschung B* **56**(8):797-803.

Shulaev V. 2006. Metabolomics technology and bioinformatics. *Briefings in Bioinformatics* **7**(2):128-139.

Singh CJ. 2003. Optimization of an extracellular protease of Chrysosporium keratinophilum and its potential in bioremediation of keratinic wastes. *Mycopathologia* **156**(3):151-156.

Singh SB, Ondeyka JG, Tsipouras N, Ruby C, Sardana V, Schulman M, Sanchez M, Pelaez F, Stahlhut MW, Munshi S *et al.*,. 2004. Hinnuliquinone, a C2-symmetric dimeric non-peptide fungal metabolite inhibitor of HIV-1 protease. *Biochemical and Biophysical Research Communications* **324**(1):108-113.

Slininger PJ, Burkhead KD, Schisler DA. 2004. Antifungal and sprout regulatory bioactivities of phenylacetic acid, indole-3-acetic acid, and tyrosol isolated from the potato dry rot suppressive bacterium Enterobacter cloacae S11:T:07. *Journal of Industrial Microbiology and Biotechnology* **31**(11):517-524.

Smith CA, O'Maille G, Want EJ, Qin C, Trauger SA, Brandon TR, Custodio DE, Abagyan R, Siuzdak G. 2005. METLIN: a metabolite mass spectral database. *Therapeutic drug monitoring* **27**(6):747-751.

Smolinska A. 2012. Chemometrics and NMR spectroscopy for metabolomics analysis of neurological disorders [PhD]: Radboud University Nijmegen.

Sneader W. 1996. Drug prototypes and their exploitation. Wiley Chichester, UK.

Sommart U, Rukachaisirikul V, Sukpondma Y, Phongpaichit S, Sakayaroj J, Kirtikara K. 2008. Hydronaphthalenones and a dihydroramulosin from the endophytic fungus PSU-N24. *Chemical and Pharmaceutical Bulletin* **56**(12):1687-1690.

Springer DM, Sorenson ME, Huang S, Connolly TP, Bronson JJ, Matson JA, Hanson RL, Brzozowski DB, LaPorte TL, Patel RN. 2003. Synthesis and activity of a C-

8 keto pleuromutilin derivative. *Bioorganic & medicinal chemistry letters* **13**(10):1751-1753.

Sridhar C, Subbaraju GV, Venkateswarlu Y, Venugopal RT. 2004. New Acylated Iridoid Glucosides from Vitex altissima. *Journal of Natural Products* **67**(12):2012-2016.

Starratt AN, Loschiavo SR. 1974. The production of aphidicolin by Nigrospora sphaerica. *Canadian journal of microbiology* **20**(3):416-7.

Stierle DB, Stierle AA, Kunz A. 1998. Dihydroramulosin from Botrytis sp. *Journal of Natural Products* **61**(10):1277-1278.

Stoeckli M, Chaurand P, Hallahan DE, Caprioli RM. 2001. Imaging mass spectrometry: a new technology for the analysis of protein expression in mammalian tissues. *Nature medicine* **7**(4):493-496.

Suksamrarn A, Kumpun S, Kirtikara K, Yingyongnarongkul B, Suksamrarn S. 2002a. Iridoids with anti-inflammatory activity from Vitex peduncularis. *Planta medica* **68**(01):72-73.

Suksamrarn A, Kumpun S, Yingyongnarongkul B-e. 2002b. Ecdysteroids of Vitex scabra Stem Bark. *Journal of Natural Products* **65**(11):1690-1692.

Suksamrarn A, Promrangsan N, Jintasirikul A. 2000. Highly oxygenated ecdysteroids from Vitex canescens root bark. *Phytochemistry* **53**(8):921-924.

Suksamrarn A, Promrangsan N, Chitkul B, Homvisasevongsa S, Sirikate A. 1997. Ecdysteroids of the root bark of Vitex canescens. *Phytochemistry* **45**(6):1149-1152.

Suksamrarn A, Sommechai C, Charulpong P, Chitkul B. 1995. Ecdysteroids from Vitex canescens. *Phytochemistry* **38**(2):473-476.

Suksamrarn A, Sommechai C. 1993. Ecdysteroids from Vitex pinnata. *Phytochemistry* **32**(2):303-306.

Sun H, Zhang A, Wang X. 2012a. Potential Role of Metabolomic Approaches for Chinese Medicine Syndromes and Herbal Medicine. *Phytotherapy Research* **26**(10):1466-1471.

Sun H, Ho CL, Ding F, Soehano I, Liu X-W, Liang Z-X. 2012b. Synthesis of (R)-Mellein by a Partially Reducing Iterative Polyketide Synthase. *Journal of the American Chemical Society* **134**(29):11924-11927.

Takayasu K, Shima Y, Muramatsu Y, Moriyama N, Yamada T, Makuuchi M, Hasegawa H, Hirohashi S. 1987. Hepatocellular carcinoma: treatment with intraarterial iodized oil with and without chemotherapeutic agents. *Radiology* **163**(2):345-351.

Takei R, Takahashi K, Matsuura H, Nabeta K. 2008. New Potato Micro-Tuber-Inducing Cyclohexene Compounds Related to Theobroxide from Lasiodiplodia theobromae. *Bioscience, biotechnology, and biochemistry* **72**(8):2069-2073.

Talhouk R, Karam C, Fostok S, El-Jouni W, Barbour E. 2007. Anti-inflammatory bioactivities in plant extracts. *Journal of medicinal food* **10**(1):1-10.

Talontsi FM, Kenla TJN, Dittrich B, Douanla-Meli C, Laatsch H. 2012. Paeciloside A, a New Antimicrobial and Cytotoxic Polyketide from< i> Paecilomyces</i> sp. Strain CAFT156. *Planta medica* **78**(10):1020-1023.

Tanaka M, Fukushima T, Tsujino Y, Fujimori T. 1997. Nigrosporins A and B, new phytotoxic and antibacterial metabolites produced by a fungus Nigrospora oryzae. *Bioscience biotechnology and biochemistry* **61**:1848-1852.

Tasdemir D, Kaiser M, Brun R, Yardley V, Schmidt TJ, Tosun F, Rüedi P. 2006. Antitrypanosomal and antileishmanial activities of flavonoids and their analogues: in vitro, in vivo, structure-activity relationship, and quantitative structure-activity relationship studies. *Antimicrobial agents and chemotherapy* **50**(4):1352-1364.

Tasduq SA, Kaiser PJ, Gupta BD, Gupta VK, Johri RK. 2008. Negundoside, an iridiod glycoside from leaves of Vitex negundo, protects human liver cells against calcium-mediated toxicity induced by carbon tetrachloride. *World journal of gastroenterology: WJG* **14**(23):3693.

Tawfike A, Viegelmann C, Edrada-Ebel R. 2013. Metabolomics and Dereplication Strategies in Natural Products. In: Roessner U, Dias DA, editors. Metabolomics Tools for Natural Product Discovery: Humana Press. p 227-244.

Tikunov Y, Lommen A, de Vos CR, Verhoeven HA, Bino RJ, Hall RD, Bovy AG. 2005. A novel approach for nontargeted data analysis for metabolomics. Large-scale profiling of tomato fruit volatiles. *Plant Physiology* **139**(3):1125-1137.

Tiwari N, Thakur J, Saikia D, Gupta MM. 2013. Antitubercular diterpenoids from Vitex trifolia. *Phytomedicine* **20**(7):605-610.

Todoroki Y, Hirai N, Ohigashi H. 2000. Analysis of Isomerization Process of 8′-Hydroxyabscisic Acid and its 3′-Fluorinated Analog in Aqueous Solutions. *Tetrahedron* **56**(12):1649-1653.

Trisuwan K, Rukachaisirikul V, Sukpondma Y, Preedanon S, Phongpaichit S, Sakayaroj J. 2009. Pyrone derivatives from the marine-derived fungus Nigrospora sp. PSU-F18. *Phytochemistry* **70**(4):554-557.

Trisuwan K, Rukachaisirikul V, Sukpondma Y, Preedanon S, Phongpaichit S, Rungjindamai N, Sakayaroj J. 2008. Epoxydons and a pyrone from the marine-derived fungus Nigrospora sp. PSU-F5. *J Nat Prod* **71**(8):1323-6.

Tsuchinari M, Shimanuki K, Hiramatsu F, Murayama T, Koseki T, Shiono Y. 2007. Fusapyridons A and B, novel pyridone alkaloids from an endophytic fungus, Fusarium sp. YG-45. *ZEITSCHRIFT FUR NATURFORSCHUNG B* **62**(9):1203.

UK CR. Worldwide cancer Key Facts. http://publications.cancerresearchuk.org/downloads/Product/CS_KF_WORLDWIDE.pdf.

Umetrics. Simca. http://www.umetrics.com/products/simca (accessed 23/06).

V. Cody EM, J.B. Harborne. 1986. Plant Flavonoids in Biology and Medicine: Biochemical, Pharmacological and Structure-Activity Relationship, Alan. Liss. Inc: New York.

van Agtmael MA, Eggelte TA, van Boxtel CJ. 1999. Artemisinin drugs in the treatment of malaria: from medicinal herb to registered medication. *Trends in pharmacological sciences* **20**(5):199.

van der Sar S. 2006. Studies in the chemistry of fungal natural products.

Van Lanen SG, Shen B. 2006. Microbial genomics for the improvement of natural product discovery. *Current opinion in microbiology* **9**(3):252-260.

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

Velasco B R, Gil G JH, Garcia P CM, Durango R DL. 2010. Production of 2-phenylethanol in the biotransformation of cinnamyl alcohol by the plant pathogenic fungus Colletotrichum acutatum. *Vitae* **17**(3):272-280.

Venkatasubbaiah P, Sutton T, Chilton W. 1991. Effect of phytotoxins produced by Botryosphaeria obtusa, the cause of black rot of apple fruit and frogeye leaf spot. *Phytopathology* **81**(3):243-247.

Verpoorte R, Choi Y, Kim H. 2007. NMR-based metabolomics at work in phytochemistry. *Phytochemistry reviews* **6**(1):3-14.

Viola H, Wasowski C, De Stein ML, Wolfman C, Silveira R, Dajas F, Medina J, Paladini A. 1995. Apigenin, a component of Matricaria recutita flowers, is a central benzodiazepine receptors-ligand with anxiolytic effects. *Planta medica* **61**(03):213-216.

Wainwright M, Wainwright J. 1990. Miracle cure: The story of antibiotics. Blackwell.

Wang C, Wang J, Huang Y, Chen H, Li Y, Zhong L, Chen Y, Chen S, Wang J, Kang J. 2013. Anti-mycobacterial activity of marine fungus-derived 4-deoxybostrycin and nigrosporin. *Molecules* **18**(2):1728-1740.

Wang C, Schuller Levis GB, Lee EB, Levis WR, Lee DW, Kim BS, Park SY, Park E. 2004. Platycodin D and D3 isolated from the root of Platycodon grandiflorum modulate the production of nitric oxide and secretion of TNF-α in activated RAW 264.7 cells. *International Immunopharmacology* **4**(8):1039-1049.

Wang FW, Hou ZM, Wang CR, Li P, Shi DH. 2008. Bioactive metabolites from Penicillium sp., an endophytic fungus residing in Hopea hainanensis. *World Journal of Microbiology and Biotechnology* **24**(10):2143-2147.

Wang H, Wang Y, Shi Y, Li X, Long C. 2009. Chemical constituents in roots of *Osbeckia opipara*. *China journal of Chinese materia medica* **34**(4):414-418.

Waterman E, Lockwood B. 2007. Active components and clinical applications of olive oil. *Alternative medicine review: a journal of clinical therapeutic* **12**(4):331-342.

Webster J, Weber R. 2007. Introduction to Fungi. Cambridge University Press.

Weckwerth W. 2003. METABOLOMICS IN SYSTEMS BIOLOGY. *Annual Review of Plant Biology* **54**(1):669-689.

Weissmann G. 1991. Aspirin. *Scientific American* **264**:84-90.

Wiart C, Martin M-T, Awang K, Hue N, Serani L, Laprévote O, Païs M, Rhamani M. 2001. Sesquiterpenes and alkaloids from Scorodocarpus borneensis. *Phytochemistry* **58**(4):653-656.

Wiklund S. 2008. Multivariate Data Analysis for Omics. *Umetrics, Umeå, Sweden*.

Wishart DS. 2008. Metabolomics: applications to food science and nutrition research. *Trends in Food Science & Technology* **19**(9):482-493.

Wishart DS, Tzur D, Knox C, Eisner R, Guo AC, Young N, Cheng D, Jewell K, Arndt D, Sawhney S. 2007. HMDB: the human metabolome database. *Nucleic acids research* **35**(suppl 1):D521-D526.

Wold S, Antti H, Lindgren F, Öhman J. 1998. Orthogonal signal correction of near-infrared spectra. *Chemometrics and Intelligent Laboratory Systems* **44**(1–2):175-185.

Wolfender J-L, Marti G, Ferreira Queiroz E. 2010. Advances in Techniques for Profiling Crude Extracts and for the Rapid Identificationof Natural Products: Dereplication, Quality Control and Metabolomics. *Current Organic Chemistry* **14**(16):1808-1832.

Wu H-Y, Wang Y-L, Tan J-L, Zhu C-Y, Li D-X, Huang R, Zhang K-Q, Niu X-M. 2012. Regulation of the Growth of Cotton Bollworms by Metabolites from an Entomopathogenic Fungus Paecilomyces cateniobliquus. *Journal of agricultural and food chemistry* **60**(22):5604-5608.

Wu J, Zhou T, Zhang S-w, Zhang X-h, Xuan L-j. 2009a. Cytotoxic Terpenoids from the Fruits of Vitex trifolia L. *Planta medica* **75**(04):367-370.

Wu SH, Chen YW, Shao SC, Wang LD, Yu Y, Li ZY, Yang LY, Li SL, Huang R. 2009b. Two new solanapyrone analogues from the endophytic fungus Nigrospora sp. YB-141 of Azadirachta indica. *Chem Biodivers* **6**(1):79-85.

Xia X-K, Huang H-R, She Z-G, Shao C-L, Liu F, Cai X-L, Vrijmoed LLP, Lin Y-C. 2007. 1H and 13C NMR assignments for five anthraquinones from the mangrove endophytic fungus Halorosellinia sp. (No. 1403). *Magnetic Resonance in Chemistry* **45**(11):1006-1009.

Xia X, Li Q, Li J, Shao C, Zhang J, Zhang Y, Liu X, Lin Y, Liu C, She Z. 2011. Two new derivatives of griseofulvin from the mangrove endophytic fungus Nigrospora sp. (strain No. 1403) from Kandelia candel (L.) Druce. *Planta Med* **77**(15):1735-8.

Xie J, Li J, Yang Y-H, Chen Y-H, Zhao P-J. 2014. Two new ambuic acid analogs from Pestalotiopsis sp. cr013. *Phytochemistry Letters* **10**(0):291-294.

Xu J, Yang X, Lin Q. 2010a. Chemistry and biology of Pestalotiopsis-derived natural products. *Fungal Diversity*:1-32.

Xu J, Ebada S, Proksch P. 2010b. Pestalotiopsis a highly creative genus: chemistry and bioactivity of secondary metabolites. *Fungal Diversity* **44**(1):15-31.

Xu J, Yang X, Lin Q. 2014. Chemistry and biology of *Pestalotiopsis*-derived natural products. *Fungal Diversity* **66**(1):37-68.

Xu L, Xue J, Xu H, Liu X, Ma W, Wei X. 2006. Three new isochromans from the mycelial culture of a Cylindrocarpon fungus. *Heterocycles* **68**(9):1955-1959.

Yabuta T, Sumiki Y. 1933. Metabolite from Aspergillus ochraceus. *Journal of Agricultural Chemical Society of Japan* **9**:1264.

Yang K-L, Wei M-Y, Shao C-L, Fu X-M, Guo Z-Y, Xu R-F, Zheng C-J, She Z-G, Lin Y-C, Wang C-Y. 2012a. Antibacterial Anthraquinone Derivatives from a Sea Anemone-Derived Fungus Nigrospora sp. *Journal of Natural Products* **75**(5):935-941.

Yang Q, Asai M, Matsuura H, Yoshihara T. 2000. Potato micro-tuber inducing hydroxylasiodiplodins from Lasiodiplodia theobromae. *Phytochemistry* **54**(5):489-494.

Yang X-L, Zhang J-Z, Luo D-Q. 2012b. The taxonomy, biology and chemistry of the fungal Pestalotiopsis genus. *Natural Product Reports* **29**(6):622-641.

Yang Y-b, Tan N-h, Zhang F, Lu Y-q, He M, Zhou J. 2003. Cyclopeptides and Amides from Pseudostellaria heterophylla (Caryophyllaceae). *Helvetica Chimica Acta* **86**(10):3376-3379.

Yang Y-L, Xu Y, Straight P, Dorrestein PC. 2009. Translating metabolic exchange with imaging mass spectrometry. *Nature chemical biology* **5**(12):885-887.

Yang YL, Xu Y, Kersten RD, Liu WT, Meehan MJ, Moore BS, Bandeira N, Dorrestein PC. 2011. Connecting chemotypes and phenotypes of cultured marine microbial assemblages by imaging mass spectrometry. *Angewandte Chemie International Edition* **50**(26):5839-5842.

Yu M, Snider BB. 2011a. Syntheses of Chloroisosulochrin and Isosulochrin and Biomimetic Elaboration to Maldoxin, Maldoxone, Dihydromaldoxin, and Dechlorodihydromaldoxin. *Organic letters* **13**(16):4224-4227.

Yu M, Snider BB. 2011b. Diels–Alder reaction of maldoxin with an isopropenylallene. *Tetrahedron* **67**(49):9473-9478.

Yuan Y, Tian J-M, Xiao J, Shao Q, Gao J-M. 2014. Bioactive metabolites isolated from Penicillium sp. YY-20, the endophytic fungus from Ginkgo biloba. *Natural Product Research* **28**(4):278-281.

Yuliana ND, Budijanto S, Verpoorte R, Choi YH. 2013. NMR metabolomics for identification of adenosine A1 receptor binding compounds from Boesenbergia rotunda rhizomes extract. *Journal of Ethnopharmacology* **150**(1):95-99.

Yuliana ND, Khatib A, Choi YH, Verpoorte R. 2011. Metabolomics for bioactivity assessment of natural products. *Phytotherapy Research* **25**(2):157-169.

Zhang H, Deng Z, Guo Z, Tu X, Wang J, Zou K. 2014. Pestalafuranones F–J, Five New Furanone Analogues from the Endophytic Fungus Nigrospora sp. BM-2. *Molecules* **19**(1):819-825.

Zhang L, Ravipati AS, Koyyalamudi SR, Jeong SC, Reddy N, Smith PT, Bartlett J, Shanmugam K, Münch G, Wu MJ. 2011a. Antioxidant and Anti-inflammatory Activities of Selected Medicinal Plants Containing Phenolic and Flavonoid Compounds. *Journal of agricultural and food chemistry* **59**(23):12361-12367.

Zhang T, Zhang CX, Xie WD, Row KH. 2013. Vitrifolin A: A Norlabdane Diterpenoid from the Fruits of Vitex trifolia Linn. var. simplicifolia Cham. *Journal of the Chinese Chemical Society*:n/a-n/a.

Zhang W, Ding W, Chen Y, Feng M, Ouyang Y, Yu Y, He Z. 2011b. Up-regulation of breast cancer resistance protein plays a role in HER2-mediated chemoresistance through PI3K/Akt and nuclear factor-kappa B signaling pathways in MCF7 breast cancer cells. *Acta biochimica et biophysica Sinica*:gmr050.

Zhao J, Zhang Y, Wang L, Wang J, Zhang C. 2012. Bioactive secondary metabolites from Nigrospora sp. LLGLM003, an endophytic fungus of the medicinal plant Moringa oleifera Lam. *World Journal of Microbiology and Biotechnology* **28**(5):2107-2112.

Zheng C-J, Lan X-P, Wang Y, Huang B-K, Han T, Zhang Q-Y, Qin L-P. 2012. A new labdane diterpene from Vitex negundo. *Pharmaceutical Biology* **50**(6):687-690.

Zheng C-J, Zhu J-Y, Yu W, Ma X-Q, Rahman K, Qin L-P. 2013. Labdane-Type Diterpenoids from the Fruits of Vitex trifolia. *Journal of Natural Products* **76**(2):287-291.

Zhi-Da M. 2011. Diterpenes. Introduction to Natural Products Chemistry: CRC Press. p 101-123.

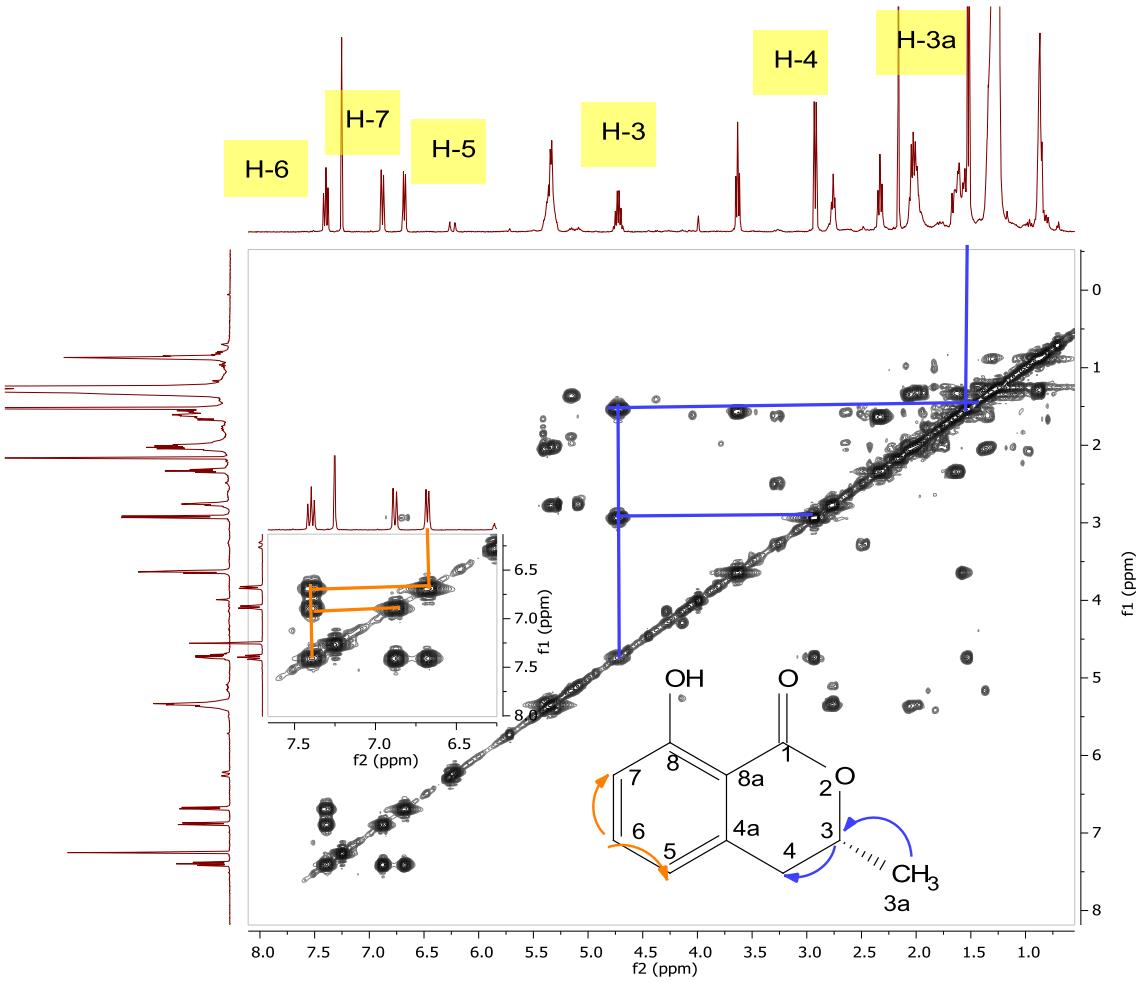
Zhou R, Lin Z-h, Jiang C-s, Gong J-x, Chen L-l, Guo Y-w, Shen X. 2013. Marine natural product des-O-methyllasiodiplodin effectively lowers the blood glucose level in db/db mice via ameliorating inflammation. *Acta Pharmacol Sin* **34**(10):1325-1336.

Zhu J-S, Halpern GM, Jones K. 1998. The scientific rediscovery of an ancient Chinese herbal medicine: Cordyceps sinensis Part I. *The Journal of alternative and complementary medicine* **4**(3):289-303.

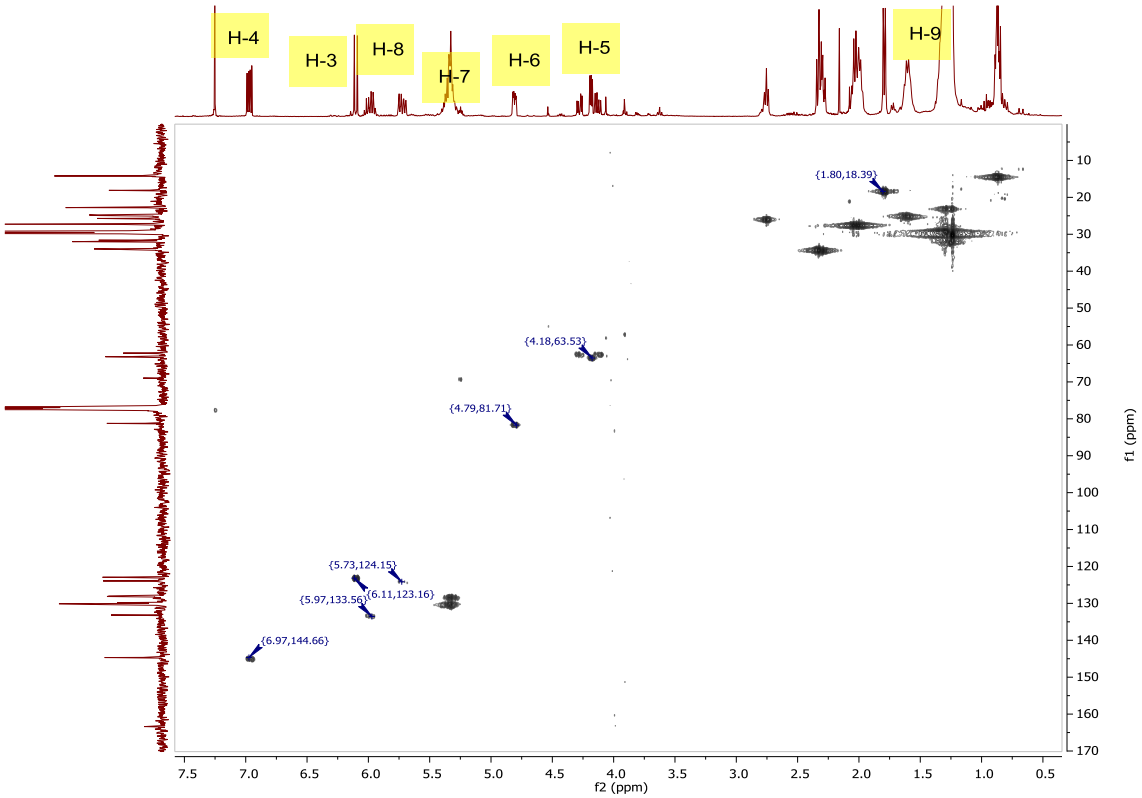
Zilla MK, Qadri M, Pathania AS, Strobel GA, Nalli Y, Kumar S, Guru SK, Bhushan S, Singh SK, Vishwakarma RA *et al.*,. 2013. Bioactive metabolites from an endophytic Cryptosporiopsis sp. inhabiting Clidemia hirta. *Phytochemistry* **95**(0):291-297.

Appendices

Appendix I: The COSY NMR of R-(-)-mellein

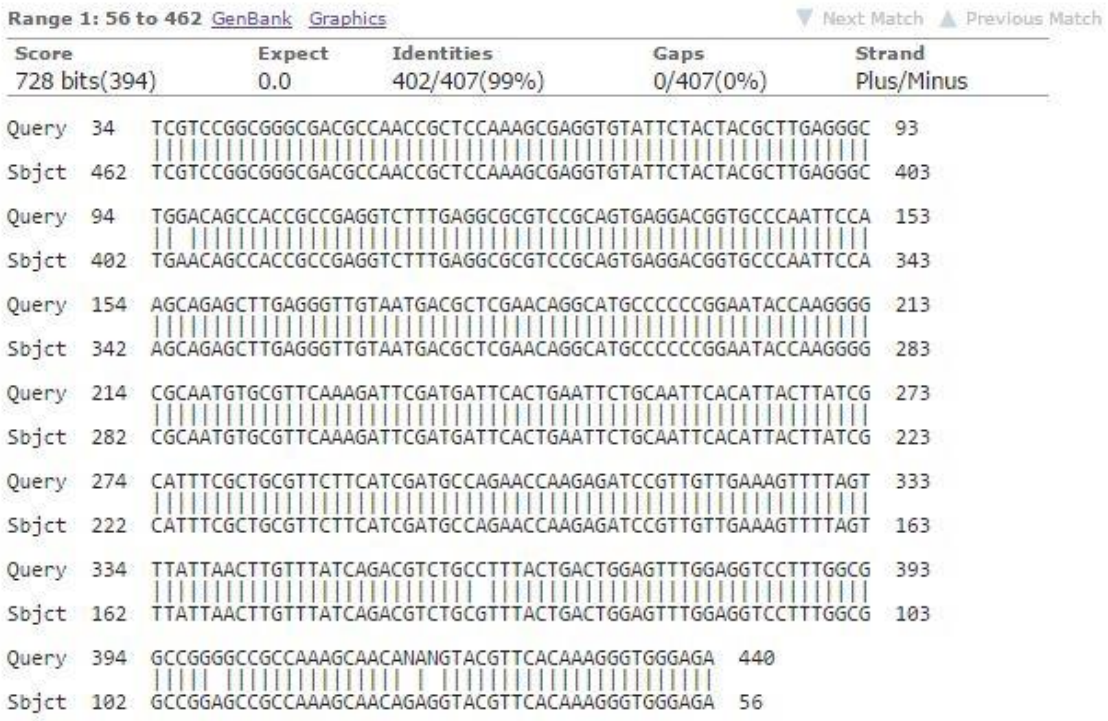


Appendix II: The HMQC spectrum of phomalactone



Appendix III: The sequence result for A) *Lasiodiplodia theobromae* B) *Nigrospora* sp and C) *Pestalotiopsis olivacea*

A

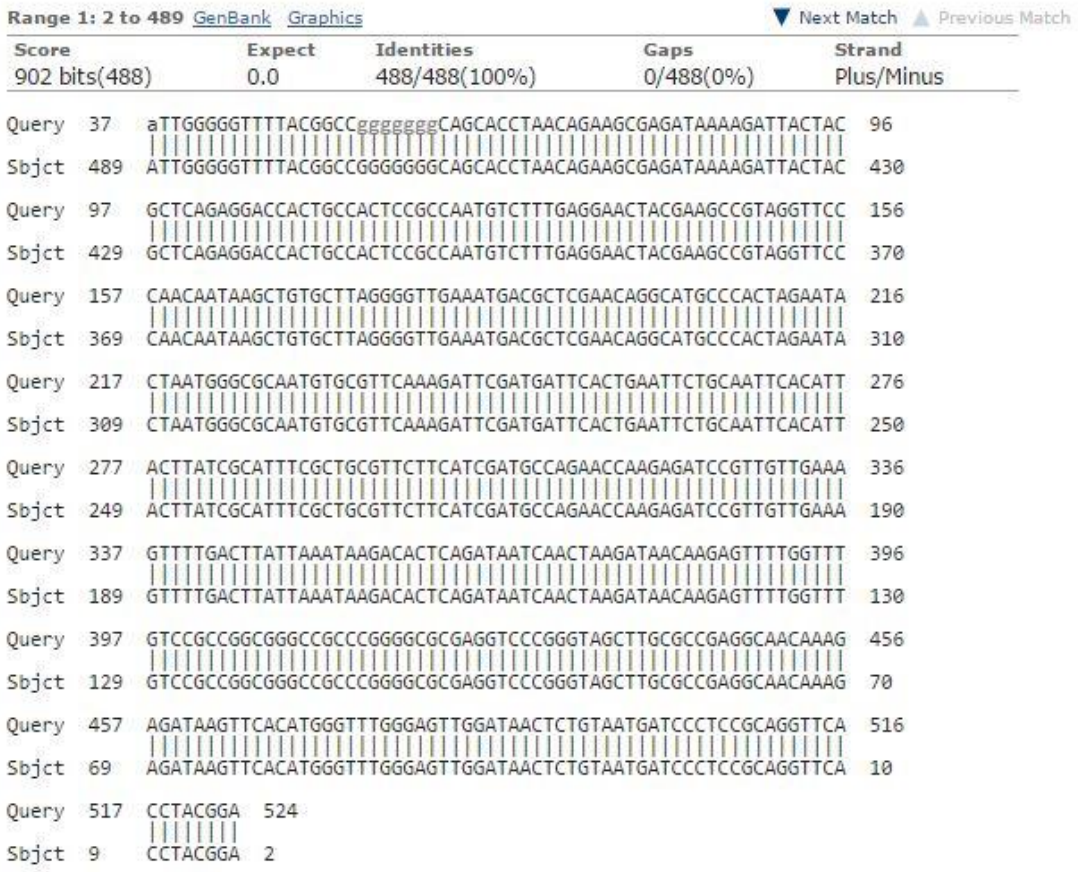


Range 1: 56 to 462 GenBank Graphics ▼ Next Match ▲ Previous Match

Score	Expect	Identities	Gaps	Strand
728 bits(394)	0.0	402/407(99%)	0/407(0%)	Plus/Minus

```
Query  34   TCGTCCGGCGGGCGACGCCAACCGCTCCAAAGCGAGGTGTATTCTACTACGCTTGAGGGC  93
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  462  TCGTCCGGCGGGCGACGCCAACCGCTCCAAAGCGAGGTGTATTCTACTACGCTTGAGGGC  403

Query  94   TGGACAGCCACCGCCGAGGTCTTTGAGGCGCGTCCGCAGTGAGGACGGTGCCCAATTCCA  153
            || ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  402  TGAACAGCCACCGCCGAGGTCTTTGAGGCGCGTCCGCAGTGAGGACGGTGCCCAATTCCA  343

Query  154  AGCAGAGCTTGAGGGTTGTAATGACGCTCGAACAGGCATGCCCCCCGGAATACCAAGGGG  213
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  342  AGCAGAGCTTGAGGGTTGTAATGACGCTCGAACAGGCATGCCCCCCGGAATACCAAGGGG  283

Query  214  CGCAATGTGCGTTCAAAGATTCGATGATTCACTGAATTCTGCAATTCACATTACTTATCG  273
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  282  CGCAATGTGCGTTCAAAGATTCGATGATTCACTGAATTCTGCAATTCACATTACTTATCG  223

Query  274  CATTTCGCTGCGTTCTTCATCGATGCCAGAACCAAGAGATCCGTTGTTGAAAGTTTTAGT  333
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  222  CATTTCGCTGCGTTCTTCATCGATGCCAGAACCAAGAGATCCGTTGTTGAAAGTTTTAGT  163

Query  334  TTATTAACTTGTTTATCAGACGTCTGCCTTTACTGACTGGAGTTTGGAGGTCCTTTGGCG  393
            |||||||||||||||||||||||||| ||||||||||||||||||||||||||||||||||
Sbjct  162  TTATTAACTTGTTTATCAGACGTCTGCGTTTACTGACTGGAGTTTGGAGGTCCTTTGGCG  103

Query  394  GCCGGGGCCGCCAAAGCAACANANGTACGTTCACAAAGGGTGGGAGA  440
            ||||| ||||||||||||||| | ||||||||||||||||||||||
Sbjct  102  GCCGGAGCCGCCAAAGCAACAGAGGTACGTTCACAAAGGGTGGGAGA  56
```

B

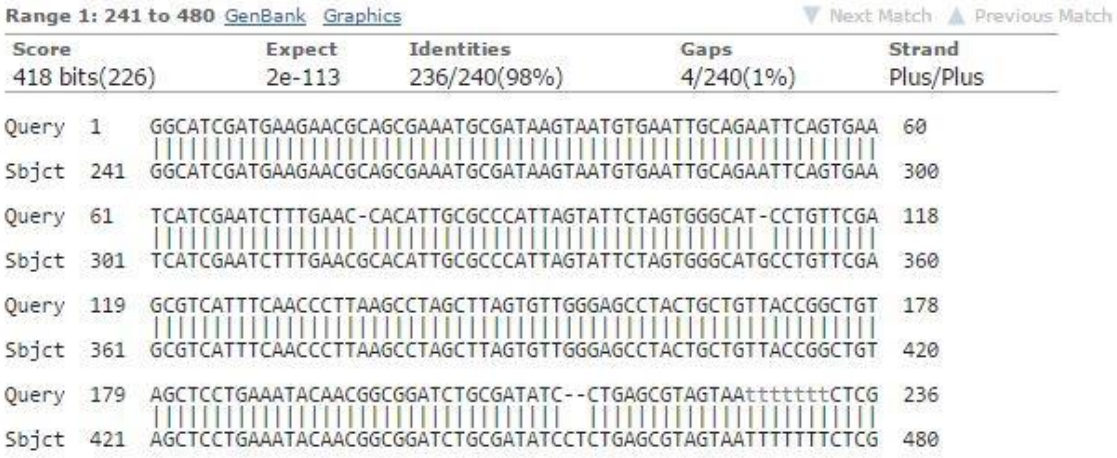


Range 1: 2 to 489 GenBank Graphics Next Match Previous Match

Score	Expect	Identities	Gaps	Strand
902 bits(488)	0.0	488/488(100%)	0/488(0%)	Plus/Minus

```
Query  37   aTTGGGGGTTTTACGGCCgggggggCAGCACCTAACAGAAGCGAGATAAAAGATTACTAC  96
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  489  ATTGGGGGTTTTACGGCCGGGGGGGCAGCACCTAACAGAAGCGAGATAAAAGATTACTAC  430

Query  97   GCTCAGAGGACCACTGCCACTCCGCCAATGTCTTTGAGGAACTACGAAGCCGTAGGTTCC  156
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  429  GCTCAGAGGACCACTGCCACTCCGCCAATGTCTTTGAGGAACTACGAAGCCGTAGGTTCC  370

Query  157  CAACAATAAGCTGTGCTTAGGGGTTGAAATGACGCTCGAACAGGCATGCCCACTAGAATA  216
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  369  CAACAATAAGCTGTGCTTAGGGGTTGAAATGACGCTCGAACAGGCATGCCCACTAGAATA  310

Query  217  CTAATGGGCGCAATGTGCGTTCAAAGATTCGATGATTCACTGAATTCTGCAATTCACATT  276
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  309  CTAATGGGCGCAATGTGCGTTCAAAGATTCGATGATTCACTGAATTCTGCAATTCACATT  250

Query  277  ACTTATCGCATTTCGCTGCGTTCTTCATCGATGCCAGAACCAAGAGATCCGTTGTTGAAA  336
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  249  ACTTATCGCATTTCGCTGCGTTCTTCATCGATGCCAGAACCAAGAGATCCGTTGTTGAAA  190

Query  337  GTTTTGACTTATTAAATAAGACACTCAGATAATCAACTAAGATAACAAGAGTTTTGGTTT  396
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  189  GTTTTGACTTATTAAATAAGACACTCAGATAATCAACTAAGATAACAAGAGTTTTGGTTT  130

Query  397  GTCCGCCGGCGGGCCGCCCGGGGCGCGAGGTCCCGGGTAGCTTGCGCCGAGGCAACAAAG  456
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  129  GTCCGCCGGCGGGCCGCCCGGGGCGCGAGGTCCCGGGTAGCTTGCGCCGAGGCAACAAAG  70

Query  457  AGATAAGTTCACATGGGTTTGGGAGTTGGATAACTCTGTAATGATCCCTCCGCAGGTTCA  516
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  69   AGATAAGTTCACATGGGTTTGGGAGTTGGATAACTCTGTAATGATCCCTCCGCAGGTTCA  10

Query  517  CCTACGGA  524
            ||||||||
Sbjct  9    CCTACGGA  2
```

C



Range 1: 241 to 480 GenBank Graphics Next Match Previous Match

Score	Expect	Identities	Gaps	Strand
418 bits(226)	2e-113	236/240(98%)	4/240(1%)	Plus/Plus

```
Query  1    GGCATCGATGAAGAACGCAGCGAAATGCGATAAGTAATGTGAATTGCAGAATTCAGTGAA  60
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  241  GGCATCGATGAAGAACGCAGCGAAATGCGATAAGTAATGTGAATTGCAGAATTCAGTGAA  300

Query  61   TCATCGAATCTTTGAAC-CACATTGCGCCCATTAGTATTCTAGTGGGCAT-CCTGTTCGA  118
            |||||||||||||||| |||||||||||||||||||||||||||||| |||||||||
Sbjct  301  TCATCGAATCTTTGAACGCACATTGCGCCCATTAGTATTCTAGTGGGCATGCCTGTTCGA  360

Query  119  GCGTCATTTCAACCCTTAAGCCTAGCTTAGTGTTGGGAGCCTACTGCTGTTACCGGCTGT  178
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  361  GCGTCATTTCAACCCTTAAGCCTAGCTTAGTGTTGGGAGCCTACTGCTGTTACCGGCTGT  420

Query  179  AGCTCCTGAAATACAACGGCGGATCTGCGATATC--CTGAGCGTAGTAAttttttCTCG  236
            ||||||||||||||||||||||||||||||||||  ||||||||||||||||||||||||
Sbjct  421  AGCTCCTGAAATACAACGGCGGATCTGCGATATCCTCTGAGCGTAGTAATTTTTTTCTCG  480
```