Antimicrobial Evaluation of Selected Medicinal Plants Using Molecular Approach

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Table of Contents

List of Tables

List of Figures

Abstract

Resistance to antibiotics is one of the greatest menaces to the success of modern medication. It has lately become more dangerous because we can no longer be certain that any antibiotic chosen will work and because of the emergence of multidrug resistant bacteria. It is more and more clear that antimicrobial resistance is easy to make but hard to miss. Resistance is a major concern with any new agent, and will become even more important in the future if new classes of drugs are established. In pharmaceutical studies, natural products, either as pure compounds or as standardised extracts, provide limitless opportunities for novel drug leads because of the unmatched availability of chemical diversity. Hence, natural products require a powerful and deep assessment of their antibacterial qualities, to help in this unprecedented crisis.

Accordingly, the main objective of this project was concentrating on the development of natural products that can act as an antibacterial agent, with a special reference to the MRSA bacteria, and involved an investigation of the role and mechanism of action of compounds derived from selected medicinal plants, these are *Centella asiatica* (L.), *Imperata cylindrica* (L.), *Morinda citrifolia* (L.), and *Sauropus androgynus* (L.), these plants have previously been implicated as having antibacterial properties. It is a fundamental necessity to uncover and understand the mechanism of action of the tested drug, and its biological pathways in the cell to obtain information for optimisation of lead compounds. During the course of this study, bioassay-guided plant extraction, isolation protocols, and many supporting analytical and biological methodologies were used and developed in order to evaluate the antibacterial activity of the four medicinal plants. Crude extracts were screened against Gram-positive and Gram-negative bacteria, including resistant strains. The isolation procedures were performed using advanced chromatographic techniques including RP-HPLC, guided by the Bioscreen-C results, in order to purify the most active compounds of extracts. These compounds were later identified by employing Mass Spectrometry (MS) and Nuclear Magnetic Resonance (NMR) techniques. Asiatic acid from *Centella asiatica* (L.), Quercetin from *Imperata cylindrica* (L.), and Aucubin from *Morinda citrifolia* (L.), were identified. Aucubin showed to have the highest activity among all other active compounds.

Results suggested that extracts/compounds, from the medicinal plants under investigation, have a potency as an antibacterial agent, with the lowest MIC observed in the compound derived from *Morinda citrifolia* (L.) named as aucubin, which was later subjected to the label-free quantitative proteomics and pathway analysis by using *Staphylococcus aureus* (MSSA) as a drug discovery model. The label free proteomics technique conducted with an aid of several software packages, as an attempt to identify the fundamental principles of the mechanism of action of aucubin. Pathway enrichment analysis suggested that the bacterial central metabolism might be the main target of the aucubin, and the pyruvate metabolism pathway showed the highest enrichment score followed by glycolysis/gluconeogenesis pathway, these results yet to be validated. An antibacterial that have different mechanism of actions to conventional antibiotics are desirable as they will help slow the onset and spread of microbial drug resistance. Overall, current study suggested that the compounds isolated from *Centella asiatica* L., *Imperata cylindrica* L., *Morinda citrifolia* L., and *Sauropus androgynus* L. might have a potency as a cheap natural source of antibiotics.

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You were all a big part in helping me complete my PhD and I thank you.

Dedication

This thesis is dedicated to my precious family.

…For their endless love, support, and encouragement

Declaration

This thesis submitted under the University of Salford regulations for the award of a Doctorate of Philosophy degree by research, and the work was performed under the supervision of Dr. David Pye, at the University of Salford, Manchester, United Kingdom. I hereby declare that this thesis and the work presented are my own work and has not been previously submitted, in whole or in part, to meet requirements for another degree or qualification in this or any other higher education institution. I also certify that to the best of my knowledge, wherever contributions of others are involved, every effort is made to indicate this clearly, and all the sources used have been duly acknowledged and referenced.

Abdullah Ghanim Qaddoori

List of Abbreviations

2D-PAGE	*2-Dimensional Polyacrylamide Gel Electrophoresis*
ABC	*Ammonium Bicarbonate*
ACN	*Acetonitrile*
ADP	*Adenosine Diphosphate*
APS	*Ammonium Persulphate*
ATCC	*American Type Culture Collection*
ATP	*Adenosine Triphosphate*
BSA	*Bovine Serum Albumin*
CC	*Column Chromatography*
CFU	*Colony forming unit*
CHCl3	*Chloroform*
CID	*collision Induced Dissociation*
COSY	*Correlation Spectroscopy*
DAVID	*Database for Annotation, Visualization, and Integrated Discovery*
DDA	*Data Dependent Acquisition*
DIA	*Data Independent Acquisition*
DMSO	*Dimethyl Sulfoxide*
DNA	*Deoxyribonucleic Acid*
DTT	*Dithiothreitol*
EDT	*Electron Transfer Dissociation*
ESI	*Electrospray Ionisation*
EtOH	*Ethanol*
FAME	*Fatty Acid Modifying Enzymes*
FDR	*False Discovery Rate*
FT-ICR	*Fourier Transform Ion Cyclotron Resonance*
FT-IR	*Fourier Transform Infrared*
GC	*Gas Chromatography*
GO	*Gene Ontology*
Hex	*Hexane*
HMBC	*Heteronuclear Multiple-bond Correlation Spectroscopy*
HMQC	*Heteronuclear Multiple-Quantum Correlation*
HPLC	*High-Performance Liquid Chromatography*
HPTLC	*High Performance Thin Layer Chromatography*
IEF	*Isoelectric Focusing*
IPG	*Immobilised pH Gradients*
iTRAQ	*Isotope Tagged Relative and Absolute Quantitation*
KEGG	*Kyoto Encyclopaedia of Genes and Genomes*
LC	*Liquid Chromatography*
m/z	*mass-to-charge*
MALDI	*Matrix-Assisted Laser Desorption Ionisation*
MBC	*Minimum Bactericidal Concentration*

MDR	*Multiple Drug Resistance*
MeOH	*Methanol*
mg	*Milligram*
ml	*Millilitre*
mM	*Millimolar*
MRSA	*Methicillin-Resistant Staphylococcus Aureus*
MS	*Mass Spectrometry*
MS/MS	*Tandem Mass Spectrometry*
MSSA	*Methicillin Sensitive Staphylococcus Aureus*
MW	*Molecular weight*
NCTC	*National Collection of Type Cultures*
NMR	*Nuclear Magnetic Resonance*
NOESY	*Nuclear Overhauser Effect Spectroscopy*
NP-HPLC	*Normal Phase High-Performance Liquid Chromatography*
OD	*Optical Density*
PAGE	*Polyacrylamide Gel Electrophoresis*
PANTHER	*Protein Analysis Through Evolutionary Relationships*
PMS	*Peptide Mass Fingerprinting*
ppm	*Parts Per Million*
PTMs	*Post-Translational Modifications*
qNMR	*Quantitative Nuclear Magnetic Resonance*
RP-HPLC	*Reverse Phase High-Performance Liquid Chromatography*
RNA	*Ribonucleic Acid*
ROESY	*Rotating Frame Nuclear Overhauser Effect Spectroscopy*
SDS	*Sodium Dodecyl Sulfate*
SILAC	*Stable Isotopic Amino Acids in Culture*
TEMED	*Tetramethylethylenediamine*
TFA	*Trifluoroacetic Acid*
TLC	*Thin-Layer Chromatography*
TOCSY	*Total Correlation Spectroscopy*
TOF	*Time of Flight*
tR	*Retention Time*
Tris	*Trisaminomethane*
TSA	*Tryptone Soya Agar*
TSB	*Tryptone Soya Broth*
UHPLC	*Ultra-High-Performance Liquid Chromatography*
UV	*Ultra Violet*
VRSA	*Vancomycin Resistant Staphylococcus Aureus*
WHO	*World Health Organization*
μg	*Microgram*
μl	*Microlitre*

Chapter 1
Introduction

1.1 Project background

Bacteria, are the oldest forms of life on earth, they are remarkably diverse and exist in surprising numbers. Bacteria are group of micro-organisms that are single cells and approximately one micron in transverse diameter. Diseases caused by bacteria comprise some of the most common infections in the world, it is considered one of the threatening issues in medical field, past, present, and probably future (Relman, 2002). Bacteria have been classified according to phenotype, including shape, size, staining properties and biochemical properties. Recently, the classification has been dominated by genotype analysis, especially using conserved molecules such as 16S ribosomal RNA. On the other hand, bacteria can be classified as either pathogenic or nonpathogenic bacteria. Pathogenic bacteria cause bacterial infection, whereas the others do not. Nonpathogenic are commonly called normal flora. Some species of bacteria, such as *Pseudomonas aeruginosa*, are opportunistic pathogens and cause disease mainly in people suffering from immunosuppression (Entenza *et al.*, 2014).

Infections that are induced by bacteria are treated with drugs called antibiotics or antibacterials. Antibiotics can be described as a compound that works to either stop bacteria from growing (bacteriostatic agents) or by killing them entirely (bactericidal agents). The effectiveness of these compounds against the survival of bacteria stems from their ability to block critical bacterial cellular processes (Sommer & Dantas, 2011). The application of modern antibiotics therapy (Post-world war II) has had a deep impact on our societies and changed the features of the medicine and the patient care. Modern procedures in medicine rely on the use of antibiotics to control infections, organ transplants, surgery, care of premature neonates and to allow successful patient rehabilitation (WHO, 2014). The use of antibiotics has removed infectious diseases being the top priority healthcare concern in the western countries over the last few decades. However, infectious diseases remain the major causes of mortality in low-income countries and the third highest cause of mortality worldwide (Thomas & Rima, 2011).

The treatment of bacterial infections has been obstructed by resistance to antibiotics. First observed in Flemings' lab shortly after his discovery of penicillin (1928), the detection of resistance to antibiotics continued throughout the golden age of antibiotic discovery (1950s) where many new drugs and drug classes were discovered to keep up with the increasing rates of resistance (Johnson 2011). Bacterial resistance to antibiotic drugs is one of the most serious

hazards to global public health and the problem knows no boundaries. Drug-resistant microbes of all kinds can move amongst people and animals, from one country to another without notice. The problem is clearly more severe in developing countries where drug availability is limited and resistance is high (Breu *et al.,* 2008). The World Health Organisation (WHO) describes antibiotic resistance as a serious threat that is no longer predictable. It is occurring in every part of the world and has the potential to affect anyone, of any age, in any country (WHO, 2014). The Infectious Diseases Society of America, which represents the infectious disease specialists, who are in the front line of antibiotic use, has called for the delivery of a new antibiotic drugs in the upcoming years, due to the growing threat of antibiotic resistant pathogens which increasingly causes death and weakening disease (Venter, 2014). The new multi-drug pathogenic strains which accumulating large numbers of resistance elements, greatly limiting therapeutic options. Furthermore, the massive financial and logistical burden on health care sectors across the globe (Wright, 2012).

Ultimately, our primary concern regarding resistance is that resistant bacteria are more difficult to get rid of and that complications and deaths resulting from infections caused by them will only increase in time. Very few really new antibiotics have been developed recent years' term, and tradition natural sources of antibiotics such as soil bacteria and fungi may have given up all their arsenal of antibiotics (Carlos, 2010). Hence we need to look elsewhere for new sources of antibiotics, and plant material is an obvious choice. In pharmacognosy studies, natural products, which are mainly derived from plants, have been widely exploited as a resource in drug development. For thousands of years, natural products have played an important role throughout the world in treating and preventing human diseases and once served humankind as the source of all drugs. Higher plants provided most of these therapeutic agents. Until recently, the use of traditional medicine was a common practice in poor or developing countries, where it serves as an alternative medicine because of the lack of the modern health facilities (Calixto *et al.*, 1998).

In terms of the discovery of novel biologically active compounds, including antibacterial agents, what is needed are molecules that are diverse in their structure (and consequently diverse in their functions). Small organic molecules have always been of interest in pharmaceutical and biochemistry science, due to their potential to exert powerful effects on the roles of

macromolecules that make up living systems (Ji, Li, & Zhang, 2009). A collection of such compounds has the potential to provide hits against different biological targets. There are a number of potential sources of small molecules for use in biological screening. Most of the major categories of antibiotics in therapeutic use are natural products or semi-synthetic derivatives. In addition, the natural products have the ability to interact with target proteins and the hit rates in high-throughput screens are usually several times higher for natural products compared to small molecule from traditional synthetic sources (Galloway *et al.*, 2009).

Plants represent an inexhaustible source of novel chemical compounds, which are of potential use in medicine and other applications. Plants consist of many active compounds such as alkaloids, steroids, tannins, glycosides, volatile oils, fixed oils, resins, phenols and flavonoids, which found in their specific parts such as leaves, flowers, bark, seeds, fruits, root, etc. The beneficial medicinal effects of plant materials typically result from the combination of these secondary products. These compounds selectively inhibit the function of biological targets (Gupta, Naraniwal, & Kothari, 2012). Today, natural products (and their derivatives and analogs) still represent over 50 % of all drugs in clinical use, with higher plant-derived natural products representing 25 % of the total. Some relevant examples are galegine, from *Galega officinalis* L., which was the model for the synthesis of metformin and other antidiabetic drugs; papaverine from *Papaver somniferum* L., which formed the basis for verapamil used in the treatment of hypertension. Opium is better known as being the source of painkillers such as morphine and codeine (Cragg & Newman, 2014).

The World Health Organization (WHO) estimates that 80 % of the people in developing countries rely on traditional medicine for their primary health care, and about 85 % of traditional medicine involve the use of plant extracts. This means that about 3.5 to 4 billion people in the world rely on plants as sources of drugs (Chandrawat *et al.*, 2014). The use of plants for medicinal purposes represents the largest use of biodiversity in the world. Many more species of plants are used as medicines, than are used for food. This fact concludes a significant role for natural products in the new drug discovery (Maridass, 2010). Recently, researchers are involved in an intensive screening of plants used in traditional medicine in an attempt to discover new drugs.

Natural products have been a rich source of compounds in antibiotic drug discovery with most antibiotic drugs being derived from a natural product or natural product lead. However, the rapid onset of resistance to most antibiotics diminishes their effectiveness considerably in the last two decades. (Maria, 2012). More reasons to add to the current crisis in antibiotic development is the poor return of investment, which is substantial in drug development. Despite this, smaller pharmaceutical companies are attempting to address the medical need for new antibiotics. In addition, the structural complexity of many natural products has often been understood as an obstacle, since it may impose serious challenges to chemical synthesis and derivatisation during the lead optimisation process (Luzhetskyy *et al.,* 2007). There is an urgent necessitates of a constant supply of new antibiotics for effective treatment of infections.

In the development of new antibiotic agents from natural products, several issues need to be addressed, including the establishment and selection of primary screening assays, which are crucial to ensure a selection of extracts or molecules with relevant pharmacological action are worthy to follow up. The assay must be easy to perform, speedy, uncomplicated, produce quick results, and preferably at a low cost (Monteiro *et al.*, 2012). In order to evaluate the plant constituents for biological activity, the plant material must firstly be subjected to a suitable extraction process. Extraction (as the term is pharmaceutically used) is the procedure used in separation of medicinally active molecules of natural product using selective solvents through standard processes. A wide range of technologies is available for the extraction of active components. The crude extract is screened to obtain an evaluation of the potential biological activities, the crude extracts are sequentially fractioned, and each fraction subjected to a further suitable bio-assay tests (Bobzin, Yang, & Kasten, 2000).

There are various chromatographic techniques available to separate the bio-active components from the crude mixture, such as Column Chromatography (CC), Liquid Chromatography (LC), and Gas Chromatography (GC). Purification of the bio-active compounds from plants by chemical and chromatographic approaches usually followed by structural characterisation using spectroscopic methods including Ultra Violet (UV), Fourier Transform Infrared (FT-IR), Nuclear Magnetic Resonance (NMR), and Mass Spectrometry (MS). NMR and MS are the most powerful and widely used techniques for the structure determination of bio-active compounds (Amsath, 2013).

In the field of drug discovery, it is necessary to distinguish between the cause of the disease and its mechanism at the molecular level. Protein molecules that linked to a given disease are identified first. Alongside the determination of the target, we must understand the mechanism of action of the tested drug and its biological pathways in the cell. Currently, approaches to analyse how a drug works fall into these broad categories. First, includes several strategies that rely on model organisms that are compatible with the genetic manipulations. Second category involves affinity-based methods employed to identify proteins that stick to the drug. (Wacker *et al.,* 2012). Modern drug discovery seeks to identify new small molecules that potently and selectively modulate the functions of target proteins (Monica *et al.,* 2013). Good drugs must have significant effects on a specific biological pathway and minimal effects on all other pathways. Confirmation that a tested compound inhibits any intended target and the identification of mainly secondary effects are among the main challenges in the development of new drugs. Extensive methods that enable researchers to determine which genes or activities are affected by a given drug might improve the efficiency of the drug discovery process by quickly identifying potential protein targets (Marton *et al.,* 1998).

Proteomics may be defined as the genome-scale analysis of the structure, abundance, localisation, modification, and function of proteins present in a cell, organ, or organism at a given time. Currently, proteomics has gained comprehensive attention in the field of drug development and mechanism studies, since proteomic analysis provides a direct reflection of gene expression. Identifying protein targets of bio-active compounds are an efficacious approach to discover unknown protein functions, as well as dissect molecular mechanisms of drug effectiveness. It is really important to systemically characterise the drug and its reactive intermediate (Ji, 2015). Technically, proteomic analysis requires the combination of several technologies for protein separation and processing such as two-dimensional polyacrylamide gel electrophoresis (2D-PAGE), high-performance liquid chromatography (HPLC), and mass spectrometry (MS) which is the primary tool for protein identification and characterisation. There are many types of each technique and the researcher may choose the one useful for the intended work. Bioinformatics used in the analysis of qualitative and quantitative proteomic data, various search engines and software packages available for researchers, i.e. MaxQuant, MultiQuant, Progenesis QI, and much more (Hu, Paul Fawcett, & GU, 2012).

Most proteomic studies involve the high-resolution separation of proteins from a complex protein mixture isolated under certain experimental conditions. Particular proteins of interest are identified using MS data linked to a genome sequence database by specialised softwares. The ultimate goal of proteomics is the complete identification and quantification of a specific proteins, with aim of revealing proteins function and post-translational modifications (PTMs) as part of a complex associated system. It is crucial to include proteomic study in the field of drug discovery to reveal the mechanism of action and functional pathways of the drug under investigation (Cooper & Carucci, 2004). There are a number of ways to perform comparative analysis of proteins using MS techniques within samples, either using labeling techniques or label-free techniques. Labeling of samples for subsequent mass spectrometric analysis allows for direct comparison of individual protein abundance between two different conditions by comparison of the mass shift induced in labeled peptides with their unlabeled counterpart (Alan *et al.,* 2005). Despite the success of labeling techniques in quantification of relative abundances of proteins between two samples, the labeling reagents are expensive, and data analysis of mixed samples is often complicated and time consuming. On the other hand, label-free techniques have the advantage of comparing two samples with different conditions. In addition, label-free approaches can offer the advantage of relatively uncomplicated data analysis and less costly (Tuli & Ressom, 2009). Current study employed the label-free proteomics approach to investigate the antibacterial mode of action of the compounds under investigation in *Staphylococcus aureus* (MSSA) as a drug discovery model.

The noticeable developments in gene and protein sequence databases, provides a means to identify proteins expressed in organisms. Bioinformatics tools, such as Mascot, make it much easier to access these databases to search, match and identify a protein component. UniProt, trEMBL ExPASy, Swiss-Prot and NCBInr are the widely used protein sequence databases (Kambiz, Luc and Sylvia 2010). Identification of the cellular targets for the tested compounds is a crucial step in understanding their true mode of action (Wang *et al.,* 2011).

After a protein is synthesised, it can undergo posttranslational modifications (PTMs), the post-translational modification of proteins plays a critical role in the regulation of a broad range of cellular processes. The analysis of posttranslational modifications is particularly critical for the study of drug discovery, PTMs can occur at any step in the life cycle of a protein and can

influence their structure, stability, function, protein-protein interactions, and localisation within the cell. There are multiple assays available to measure post-translational modifications, including western blots, ELISAs, and mass spectrometry (Freiberg et al., 2004; Brötz-Oesterhelt et al., 2005).

1.2 Aims and objectives of the present study

The main aim of this PhD project was the identification and characterisation of natural products that can act as antibacterial agents. The objective of this research was to investigate the role and mechanism of action of compounds derived from four medicinal plants; *Centella asiatica* L., *Imperata cylindrica* L., *Morinda citrifolia* L., and *Sauropus androgynous* L. These plants have previously been implicated as having antibacterial properties and have previously raised a significant interest of many researchers. In order to evaluate these plants therapeutically, many lab techniques have been used and developed throughout this project. The outline strategies followed in this project, are:

1. Extraction and purification of small molecules from *Centella asiatica* L., *Imperata Cylindrical* L., *Morinda citrifolia* L. *and Sauropus androgynous* L. by using various extraction and purification techniques.
2. *In-vitro* evaluation of the antibacterial activity of these compounds against various known pathogens by utilising convenient biological assays in order to investigate their potential therapeutic activities, and determination of their Minimum Inhibitory Concentration (MICs) and Minimum Bactericidal Concentration (MBCs).
3. Isolation and structure identification of the active compounds comprised in *Centella asiatica* L., *Imperata Cylindrical* L., *Morinda citrifolia* L., *and Sauropus androgynous* L. extracts that are responsible for inhibition of bacterial growth by standard spectroscopy techniques.
4. Elucidation of the mechanism of action of selected compounds shown to have the potent antibacterial activities via liable-free proteomics analysis and MS fingerprinting.
5. Identification of the targets on the bacteria that are bound to the active compounds contained in the medicinal plants under investigation using available software packages.

1.3 Significance of the study

Bacteria, which cause diseases, react to the antibiotics used as treatment by becoming resistant to them, sooner or later, this natural process of adaptation and antimicrobial resistance affect the lifespan of antibiotics. The antimicrobial resistance has increased recently due to unnecessary use and inappropriate use of antibiotics especially. Accordingly, this lead to hyper mutation of many bacterial strains and spread of resistant bacteria (Chauhan *et al.,* 2013). As resistance and virulence increases, the cost and burden on society increases as well, the damaging effects of the antimicrobial resistance are already manifesting themselves across the world. Antimicrobial resistant infections currently claim at least 50,000 lives each year across Europe and the US alone, with many hundreds of thousands more dying in other areas of the world. Latest figures suggest that drug resistant infections could kill an extra 10 million people across the world every year by 2050 if they are not tackled. World Health Organization (WHO) is calling on governments and the pharmaceutical industry to work together in taking comprehensive action against drug-resistant infections by increasing the investment in antibiotic researches to meet global public health needs (Suraj & Sapkal, 2015).

New strategies have been implemented to combat this growing crisis and to help the healthcare sector coping with antimicrobial resistance. Investigation of the antimicrobial activity of the secondary metabolites from natural products represents a promising possible solution. Currently, there is an increasing interest in the isolation, screening and exploitation of antimicrobial activity of natural products of plants as an inexpensive and rich source of secondary metabolites. In this project, different strategies were implemented to evaluate the antibacterial activity of four medicinal plants, *Centella asiatica* L., *Imperata cylindrica* L., *Morinda citrifolia* L., and *Sauropus androgynous* L., *in-vitro* against various strains of pathogenic bacteria.

Although the biological effects of *Centella asiatica* L., *Imperata cylindrica* L., *Morinda citrifolia* L., and *Sauropus androgynous* L. have been studied, there is no comprehensive study available to explain the actual role of active compounds from these plants and the mechanisms of their action. Accordingly, this project conducted a screening of the effects of various extracts of these plants to demonstrate their antibacterial activity. The extracts of interest were further explored to isolate and identify the compounds which were responsible for the observed

activities. The elucidation of possible mechanisms of action of the activity was also studied using LC-MS/MS proteomics approach. Success in combatting antimicrobial resistance requires sustainable commitment and partnership with the private sector, governments, and international aid organisations, no one can do this alone.

Chapter 2
Literature Reviews

2.1 Antibiotics

The word antibiotic is derived from the Greek words anti (against) and bios (life) and means, in principle, a substance, which kills any living organism. However, in medical practice, antibiotics are compounds produced by, or derived from, certain fungi, bacteria, and other organisms, which used to combat bacterial infections by either prevent the bacteria from growing (bacteriostatic agents) or to kill them outright (bactericidal agents) (Sommer & Dantas, 2011). The term antibiotic is used to refer to a drug that cures infections caused by bacteria, while an antimicrobial agent is a general term mostly applied to substances working on bacteria (antibacterial), but can also be applied to agents working on viruses (antivirals), fungi (antifungals) and parasites (antiparasitic agents). Antimicrobials include antibiotics produced by other organisms (e.g. penicillin, tetracycline, and erythromycin), chemically modified antibiotics (e.g. doxycycline) as well as chemically produced substances (e.g. fluoroquinolones) (Davies, J, & Davies, D., 2010).

There has been an ongoing conflict throughout history between humans and the uncountable numbers of microorganisms that cause infection and disease. Around the middle of the twentieth century, there was a major evolution in antibacterial drug development and other means of infection control, which helped turn the tide in favour of humans. This development was dramatically improved when penicillin became available for use in the early 1940s. However, the euphoria of this potential victory over infectious diseases last only for a limited time (Lynne, 2000). The first antibiotic, penicillin, was discovered by Alexander Fleming in 1928 when he observed that a common mold (*Penicillum*) produced a substance that lysed colonies of *Staphylococcus* spp. The first major development after the introduction of penicillin was ampicillin, which offered a broader spectrum of activity than the original penicillin. Ampicillin has been used extensively to treat bacterial infections since 1961. In the following decades, many new antibiotics with novel properties were discovered, including streptomycin, chloramphenicol, and tetracycline. Modification of already known antibiotics has led to several derivatives having different antibacterial activities, pharmacokinetic properties, and resistance characteristics as compared to the older drugs (Sharma *et al.,* 2013).

The effectiveness of these compounds ‘against life’ stems from their ability to block or modify critical bacterial cellular processes. Different antibiotics inhibit certain process in the

pathogen, which are different from that found in the host. For instance, the antibiotics chloramphenicol and tetracycline inhibit the bacterial ribosome (serve as the site of mRNA translation and protein synthesis), but not the structurally-different eukaryotic ribosome, therefore it shows selective toxicity against bacteria only (Olgica *et al.*, 2012).

Almost as soon as antibiotic drugs were rolled out for general use in the world's population, bacteria responded by developing various forms of resistance. As antibacterial usage increased, so did the level and complexity of the resistance mechanisms exhibited by bacterial pathogens (Nikaido, 2009). The struggle to control the infections continues to this day, and surprisingly the number of scientists who are developing new antibiotic agents has begun to fall back, even as bacteria evolve ever more advanced mechanisms of resistance. Nonetheless, recent lobbying of governments, worldwide, has brought a change in attitudes toward antibiotic research and more money is being made available to research centres to support this most urgent experimentation (Tenover, 2006).

The number of new antibiotic agents coming on to the market is falling, for instance, sixteen agents were approved for use between "1983-87" but only seven were approved between "1998-2002" (Bush & Pucci 2011). Recently, only two new classes of antibiotics; daptomycin and oxazolidinones have been utilised to treat Gram-positive infections, whereas, innovation to address Gram-negative bacteria are still struggling. Furthermore, drugs with novel modes of action are even fewer in number. For instance, linezolid, which was approved in 2003, had a novel mechanism of action (protein synthesis inhibitor, which works by blocking the initiation of protein production, and not one of the later steps), while the remainder were solely modified structures of existing agents. (Miller *et al.*, 2014).

A major problem of novel antibiotic development is that many drug companies see it as an unattractive financial risk. This is due to huge production costs for compounds which require relatively small doses and short treatment cycles to be effective and which may not have a long clinical shelf life. Many 'Big Pharma' are focusing their efforts on compounds used in long-term treatment plans, for chronic illnesses, obesity and quality of life drugs, all of which are more probable to yield a larger profit than antibiotics. Still, many governments and international authorities are aware of the situation and measures are being conducted to enhance awareness of prescribing and misuse of antibiotics (Kraus, 2008).

2.1.1 Antibiotic resistance of bacterial pathogens

Antibiotics originally evolved within bacteria almost a billion years ago as organisms competed with each other, long before mankind had discovered antibiotics. It is fair to presume that resistance to antibiotics developed shortly afterwards, as susceptible bacteria developed means to defend themselves against antibiotic-producing bacteria (Bradley, 2014). Since the introduction of antibiotics into clinical use in the mid-1940s, microorganisms have shown a remarkable ability to protect themselves by developing and acquiring antibiotic resistance. By 1942, penicillin resistance was reported after only a few months of limited clinical trials. With the development of resistance to tetracycline, streptomycin, chloramphenicol and erythromycin, other classes of antibiotics began to emerge (Mengiste, Hagos, & Moges, 2014).

Many antibiotics from the days of Alexander Fleming were used for healing diseases. When microorganisms are exposed to antibiotics they adapt some alternative survival routes related to their metabolism, enzyme production and gene transfers. Microbes found adaptation to unfavourable environments and continuous doses of antibiotics. This phenomenon is frequently found throughout the world where organisms come into contact with antibiotics, and the occurrence of the resistance is more likely when patients being exposed to high dosages of antibiotic, such as during hospitalisation (Smitha *et al.,* 2014). Figure (2.1) summaries the timeline of antibiotics deployment and their resistance development.

The overwhelming use of antibiotics has played a significant role in the emergence of antibiotic resistant bacteria. Resistance of pathogenic organisms to approved antibiotics has become one of the medical world's biggest concerns with serious consequences for the treatment of infectious diseases in patients. The main cause of this phenomenon is the increased use or misuse of antibiotics. There has been a terrifying increase of antibiotic resistance in bacteria that cause either community infections or hospital - acquired infections (Nosocomial infections). There is a particular interest in multidrug resistant pathogens (MDR), and one of these in particular is methicillin resistant *staphylococcus aureus* MRSA (Alekshun & Levy, 2007). Hospital infections with methicillin resistant *staphylococcus aureus* raised serious public health concerns recently, since the infection is resistant to numerous antibiotics, including methicillin, amoxicillin, penicillin and oxacillin, thus making it challenging to treat the infection successfully. (Goulet *et al.,* 2009).

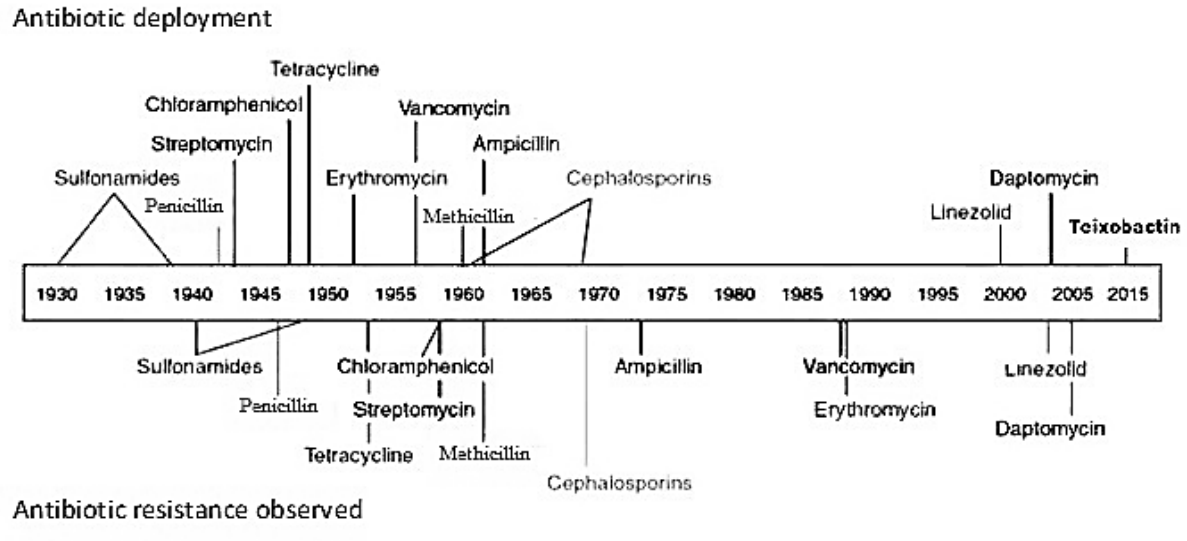


Figure 2.1: Timeline of antibiotics deployment (top) and the development of antibiotic resistance (bottom). Adapted from (Clatworthy *et al.*, 2007)

Pharmaceutical companies have ignored or dropped dramatically investments in the antibiotic research as mentioned earlier, and as a result the race to develop new classes of antibiotics with novel modes of action has been compromised in the last decades (Fox, 2006). In the absence of major new classes of antibiotics, we need to focus our efforts on new sources, such as natural products, as an alternative provider of antimicrobial agents in order to address this problem of mounting resistance. In addition, other strategies have to be considered to minimise the development of resistance, such as judicious antibiotic use, narrow spectrum antibiotics should also be used whenever possible, and antibiotics given for short courses and at appropriate times (Breu, Guggenbichler, & Wollmann, 2008). Accordingly, in search of a better drug, we have concentrated our search on natural product compounds derived from plants. Recently, natural product compounds have attracted attention of medical researchers towards their search for potential antibiotic.

2.1.2 The Basic characteristics of antibiotics

Nowadays, there are about 4000 compounds identified with antibiotic properties. Antibiotics are derived from three main sources: moulds or fungi, bacteria and synthetic or semi-synthetic compounds. They can be used either topically or internally, and the results of their action are either inhibition of the growth of pathogens or their elimination. Therefore, antibiotics can be divided into two classes, bacteriostatic and bactericidal drugs. However, the distinction

is not absolute, and depends on the drug concentration, the bacterial species, and the phase of growth. (Shamnas, Arya, & Deepak, 2013).

Antibiotics can be further classified on their ability to target Gram positive and Gram negative bacteria, these classifications are broad and narrow spectrum antibiotics. Broad spectrum antibiotics are active against both Gram-positive and Gram-negative organisms. Examples include: tetracyclines, phenicols, and fluoroquinolones. Narrow spectrum antibacterials have limited action and are mainly useful against particular species of microorganisms. For instance, glycopeptides and bacitracin are only effective against Gram-positive bacteria, whereas polymixins are usually only effective against Gram negative bacteria. Aminoglycosides and sulfonamides are only effective against aerobic organisms, while nitroimidazoles are generally only effective against anaerobes organisms (Shaikh *et al.*, 2015).

2.1.3 Mechanisms of antibiotic actions

Antibiotics are used to treat bacterial infections by interrupting the physiological mechanisms inside the bacterial cell that allow normal cellular function. Antibiotics agents act selectively on vital bacterial functions with minimal effects, or without affecting host cell mechanisms. Different antimicrobial agents act in different ways, the understanding of these mechanisms as well as the chemical nature of the antimicrobial agents is crucial in the understanding of the ways in which resistance against them develops (Kohanski *et al.*, 2010).

As mentioned earlier, antibiotic agents may be described as either bacteriostatic or bactericidal. Bacteriostatic antibacterial agents only inhibit the growth or multiplication of the bacteria, giving the immune system the time to clear them. Complete elimination of the bacteria in this case therefore is dependent on the efficiency of the immune system. Bactericidal agents kill the bacteria with or without the assistance of the immune system of the host. However, the mechanism of action of antimicrobial agents can be categorised further based on the structure of the bacteria or the functions that are affected by the agents (Salih, Salimon, & Yousif, 2012). Accordingly, antibiotics can be classified into five distinct classes based upon their mechanism of action. Those classes are (1) inhibition of bacterial cell wall biosynthesis, (2) disruption of bacterial cell membrane, (3) inhibition of protein biosynthesis, (4) inhibition of deoxyribonucleic acid (DNA) or ribonucleic acid (RNA) synthesis, and (5) inhibition of folate synthesis (Wright, 2012). These categories can be summarised in table (2.1.).

Mechanism of action	Target	Antibiotic families
Inhibition of cell wall synthesis	**Penicillin binding proteins, D-alanyl-D-alanine, Muropeptide transport**	**β-lactam (Penicillins, Cephalosporins, Carbapenems, Monobactams); Glycopeptides; Cyclic lipopeptides (Daptomycin)**
Inhibition of protein synthesis	**30s and 50s subunits of the ribosome**	**Tetracyclines; Aminoglycosides; Oxazolidononones (Linezolid); Streptogramins (Quinupristin-dalfopristin); Ketolides; Macrolides; Lincosamides**
Inhibition of DNA synthesis	**DNA gyrase, DNA structure integrity**	**Fluoroquinolones (Ciprofoxacin)**
Inhibition of RNA synthesis	**RNA polymerase**	**Rifampin**
Inhibition of folic acid pathway	**Dihydrofolate reductase, Dihydropteroate synthetase**	**Sulfonamides; Trimethoprim**
Disruption of bacterial membrane	**Phospholipid structure**	**Polymyxins (Polymyxin-B, Colistin)**

Table2.1: Summary of mechanisms of action and their targets of selected classes of antibiotic. Adapted from (Brotz-Oesterhelt & Brunner, 2008).

2.1.4 Mechanisms of antibiotic resistance

After the discovery and commercial introduction of antibiotics, it was soon noticed that usually treatable infections were not affected by the treatment with antibiotics and had adapted mechanisms of resistance. Alexander Fleming predicted that too low frequent doses of penicillin would lead to the development of penicillin resistance. Surprisingly, bacterial resistance towards penicillin was actually noticed before it was made widely available to use, after a while, it was finally recognised that there should be some antibiotic control, as the use of penicillin and other

antibiotics were not restricted by any means (Hellen *et al.*, 2015). Antibiotic resistance is a result of the evolutionary pressure that bacteria undergo, resistance occurs for all antibiotics after their clinical administration, and there is a limit to the number of antibiotic substances which fulfil all pharmacokinetics demands. Because of this, much of the antibiotic work done after the 1960s was focused on chemically modifying existing antibiotics to make them more potent to resistant pathogens and to improve pharmacokinetics properties. Recently, researchers have tried to find a new antibiotic candidate from natural products (e.g. medicinal plants) in a hope to meet the health sector demands (Davies & Dorothy, 2010).

There are many methods by which bacteria can become resistant to antibiotics. The current scale of the problem and the number of resistances against drugs across different classes is unprecedented. Resistance can be caused by a variety of mechanisms and it can be summarised as: (i) the presence of an enzyme that inactivates the antibiotic agent, bacteria can produce enzymes that are capable of adding different chemical groups to antibiotics that prevents binding between the antibiotic and its target in the bacterial cell; (ii) the presence of an alternative enzyme for the enzyme that is inhibited by the antibiotic agent, for instance, *Staphylococcus aureus* can acquire the resistance gene *mecA* and produce a new penicillin-binding protein, the new penicillin-binding protein has low affinity to β-lactam antibiotics and results resistant to the drugs, (iii) a mutation in the antibiotic agent's target which results changes in the composition or structure of the target in the bacterium and stop the antibiotic from interacting with the target. Alternatively, the bacteria can add different chemical groups to the target structure, in this way shielding it from the antibiotic; (iv) destroy the antibiotic through enzymes that can inactivate antibiotics. One obvious example is β-lactamase that destroys the active component (the β-lactam ring) of penicillin. Later, bacteria developed extended-spectrum β-lactamases and become a major problem, due to the ability of destroying a wide spectrum of β-lactam antibiotics; (v) reduced uptake of the antibiotic agent, bacteria achieve that by decrease the permeability of the membrane that surrounds the bacterial cell which make it more difficult to pass through; and (vi) efflux pumps, bacteria can produce pumps that are localised in their membrane or cell wall. In some cases, mutations in the bacterial DNA can make the bacteria produce more pumps to reduce the antibiotic concentration inside the bacterial cell (Fluit *et al.*, 2001; Chanda *et al.*, 2010). Figure 2.2 summaries major resistance mechanisms.

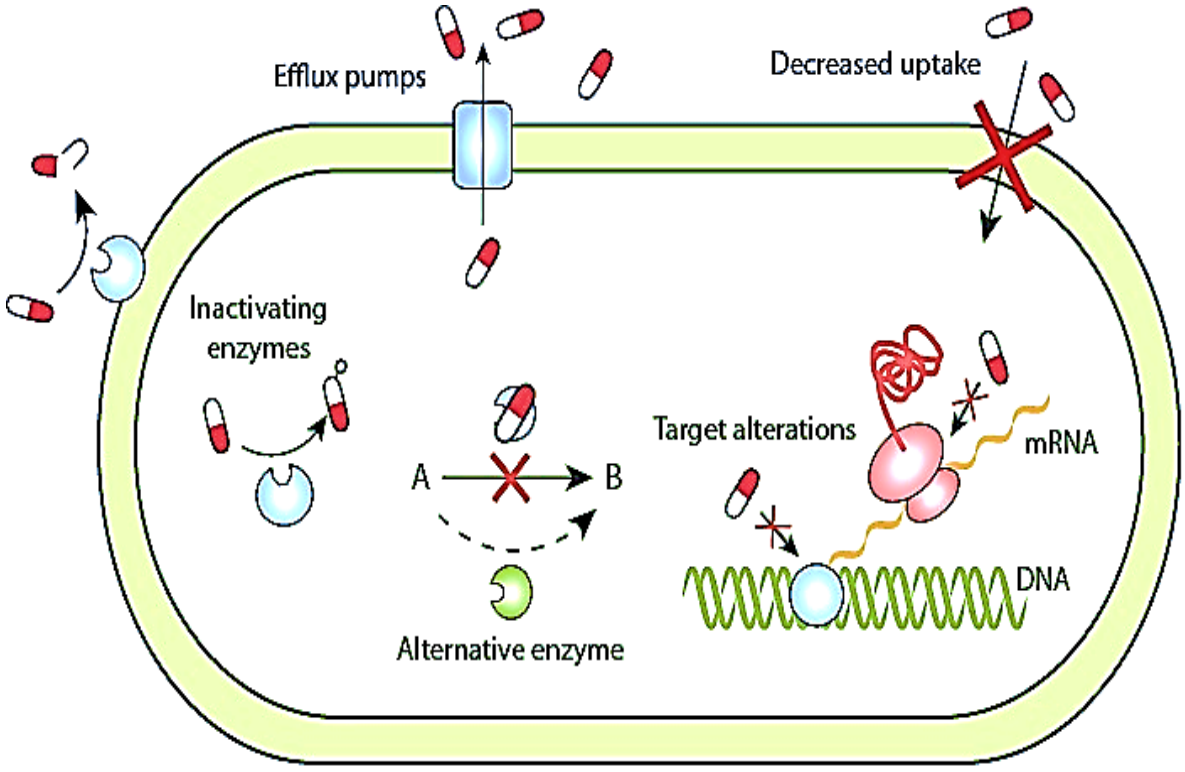


Figure 2.2: Schematic diagram of major antibiotic mechanisms of resistance (Gullberg, 2014).

At least seventeen different classes of antibiotics have been produced to date. Unfortunately, for each one of these classes at least one mechanism of resistance has developed over the years. In fact, in some cases these bacteria have been able to develop simultaneous resistance to two or more antibiotic classes, making the treatment of infections caused by these microorganisms extremely difficult, very costly and in many cases associated with high morbidity and mortality (Alanis, 2005).

Antibiotic resistance can be divided into natural resistance and acquired resistance. Natural resistance means that the bacteria are intrinsically resistant, an example of this can be due to increased efflux activity, a mechanism responsible for moving out toxic substances and antibiotics outside the cell. Acquired resistance refers to bacteria that are usually sensitive to antibiotics, but are liable to develop resistance. Acquired resistance is often caused by mutations in chromosomal genes, or by the acquisition of mobile genetic elements, such as plasmids or transposons, which carry the antibiotic resistance genes. (Katrijn and Arthur 2009).

It is important to have a good understanding of the molecular basis by which development of resistance occurs, because it allows us to develop new approaches to managing the infections caused by these bacteria and to create new strategies for the development of new treatments against these bacteria (Sefton, 2002). In most cases of bacterial resistance, changes in the genetic composition of the susceptible bacteria take place, either via a mutation or by the introduction of new genetic information. The expression of these genetic changes within the cell result in changes to one or more biological mechanism of the affected bacteria and ultimately determines the specific type of resistance that the bacteria develops, resulting in a myriad of possible biological forms of resistance (Figure 2.3). (Dzidic *et al.,* 2008).

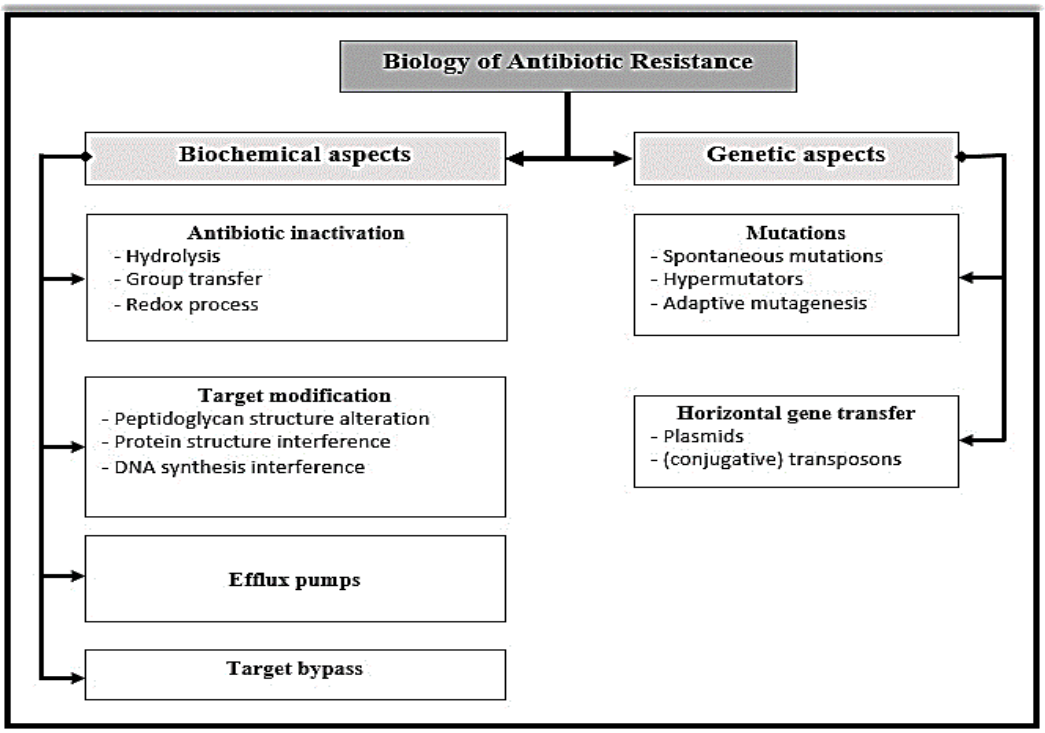


Figure 2.3: Schematic diagram shows biochemical and genetic aspects of antibiotic resistance mechanisms in bacteria. Adapted from (Dzidic *et al.,* 2008).

The resistance among various microbial species (infectious agents; e.g., bacteria, fungi, virus, and parasite) to different antimicrobial drugs has emerged as a cause of public health threat all over the world, at a terrifying pace. One such bacterial pathogen is methicillin-resistant *Staphylococcus aureus* (MRSA), it has for several years been increasingly spreading at inpatient and outpatient health care facilities worldwide. This organism is capable of causing a range of infections from skin and soft tissue infections to more life threatening illnesses such as

pneumonia, bacteremia and surgical site infections (Landecker, 2015). The next section will focus on MRSA, as it comes a huge clinical burden that is causing great public and political concern, specially that the current treatments proved to be inactive.

2.2 Methicillin-resistant *staphylococcus aureus* (MRSA)

Staphylococcus aureus is a Gram-positive bacterium normally found as a commensal organism living on the human skin and mucosa. It is found in up to 30% of the population living harmlessly on the skin. Nevertheless, it is a versatile organism able to acquire entry to the physical structure and it is able to colonise in many situations, and often causing disease (Hardy, 2004). The genus staphylococcus comprises of thirty-two species in which approximately 50 % are indigenous to man, and was first identified in 1880 by Alexander Ogston as the cause of many abscesses. The name is derived from the Greek words staphyle meaning "a bunch of grapes" and kokkos meaning "berry". *Staphylococcus aureus* is a facultative anaerobic, non-motile, non-spore forming cocci that have an average diameter of 1µm. They are arranged in grape-like clusters, in pairs and sometimes in short chains (Figure 2.4) (Stevens, 2006).

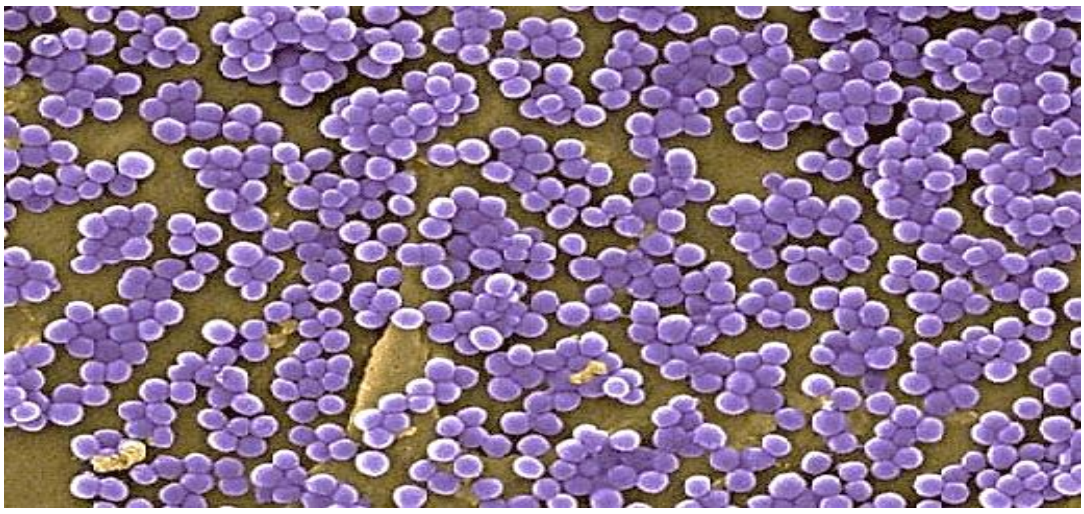


Figure 2.4: Scanning Electron Micrograph of MRSA shows abundant clumps of MRSA. Magnification 4780x. Centers for Disease Control and Prevention (David, 2015).

Of all the species of staphylococci, *Staphylococcus aureus* is by far the most important human pathogen, it causes a wide range of infections related to its ability to produce a variety of cellular and extracellular proteins associated with the virulence of the bacterium. When it finds the way to enter the body and bloodstream, it has become a major cause of nosocomial infection of surgical wounds and infections associated with some medical devices such as

catheters (Livermore, 2000). It also causes toxic shock syndrome (sudden high fever, low blood pressure (hypotension), vomiting or diarrhea, confusion, muscle aches, redness of eyes) by the release of super antigens into the bloodstream of the host. Most of the pathogenic effects of *Staphylococcus aureus* are associated with the amount of potential virulence factors. For instance, adherence, toxins, invasiveness and antibiotic resistance (Spaulding *et al.,* 2013).

Methicillin-resistant *Staphylococcus aureus* MRSA strains emerged soon after the introduction of methicillin into clinical practice, but were generally rare until the 1980. In the late 1970, however, MRSA emerged as a major pathogen of hospital infection worldwide and in addition it had also become a community pathogen. Clinical infections are most common in patients in hospital intensive care units, nursing homes, and other chronic care facilities. The increased frequency of community-associate MRSA infection has been associated with reports of increased morbidity and death rate. Specifically, a longer duration of fever, prolonged hospitalisation, a higher incidence of pulmonary complications along with bone and joint infections (Kazmierczak *et al.,* 2014).

MRSA infections are classified as either hospital-acquired or community-acquired MRSA infections (HA-MRSA or CA-MRSA). HA-MRSA is usually resistant not only to β-lactams, but also to other types of antibiotics. Because many hospitalised patients are weak and their immune systems compromised, to some degree HA-MRSA infections are often quite serious. CA-MRSA however, began to emerge in the 1990s in patients who do not have the risk factors usually associated with HA-MRSA. Such as recent hospitalisation, chronic diseases, kidney dialysis, human immunodeficiency virus infection, and intra-venous drug. CA-MRSA strains are usually resistant to β-lactams but susceptible to other antimicrobials like clindamycin and tetracycline. MRSA infections represent a burden for both patients and health care systems and these problems originate initially in large tertiary care hospitals with patients in burn, post-operative, prolonged hospitalization and intensive care wards (Elliott *et al.,* 2010).

2.2.1 Pathogenesis of Methicillin-resistant *Staphylococcus aureus*

Several factors are required to establish an infection. The pathogenicity of *Staphylococcus aureus* is dependent on production of more than forty different extra-cellular toxins, cell surface proteins and enzymes. Numerous surface proteins, mediate adherence to host tissues including

fibronectin binding protein, fibrinogen binding protein, collagen binding protein and clumping factor. Protein *A*, is an important virulence factor and may play a role in host defence evasion. *Staphylococcus aureus* produces numerous enzymes, such as proteases, lipases, hyaluronidase, and DNase, which enable it to invade and destroy host tissues and spread to other sites. The enzyme coagulase promotes the fibrin mesh development that contributes to abscess formation. Hemolysins and leucocidins cause leucocyte destruction by the formation of pores in the cell membrane, thereby altering their permeability and resulting in cell damage or death (Figure 2.5) (Collins *et al.*, 2010).

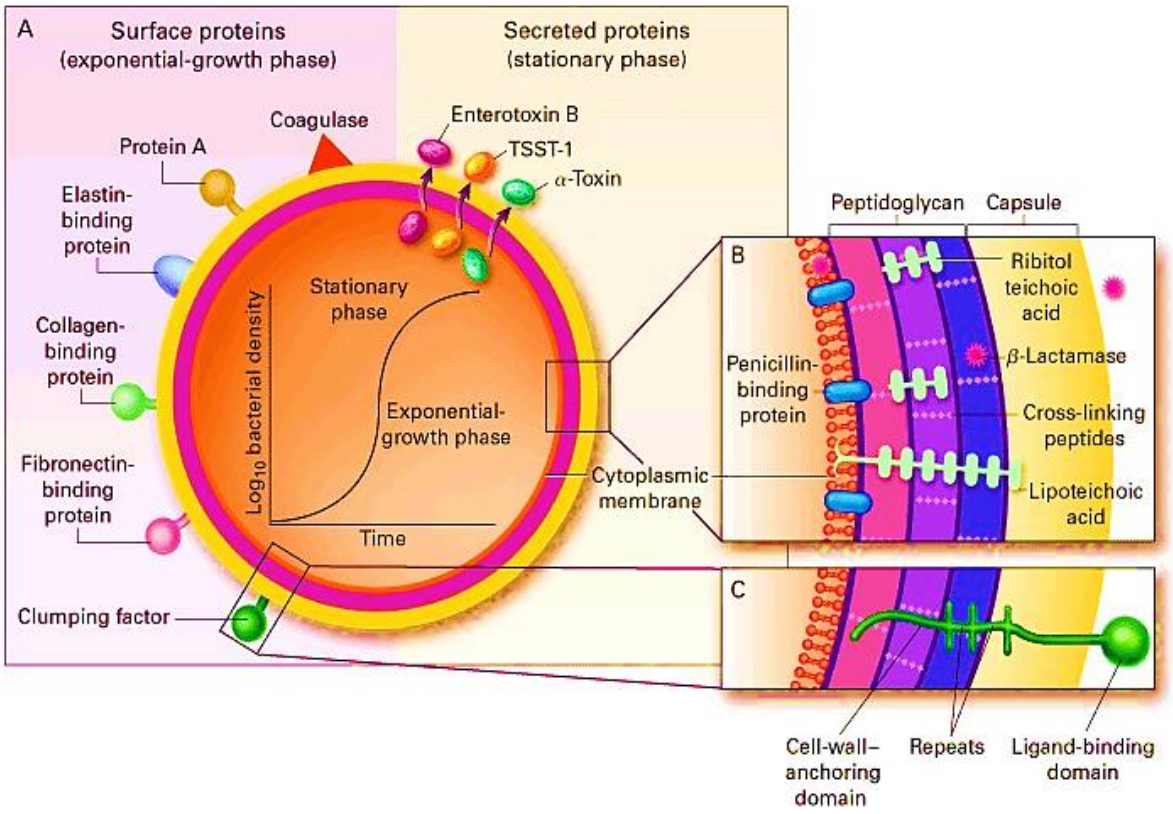


Figure 2.5: Pathogenic factors of *Staphylococcus aureus*, with structural and secreted products both playing roles as virulence factors (Gordon and Lowy, 2008).

Staphylococcus aureus virulence factors include the production of a wide range of extracellular toxins which released by the bacterium either by secretion or cell lysis. When released act upon the host, causing localised damage or systemic infection. *Staph. aureus* is also capable of producing septic shock, it does this by interacting with and activating the host immune system and coagulation pathways. Peptidoglycan, lipoteichoic acid, and α- toxin may

all play a function during the invasion. Other virulence mechanisms include biofilm formation (slime), which allows *Staphylococcus aureus* to resist host defences or certain antibiotics. Moreover, the majority of strains produce extracellular proteins, described as fatty acid modifying enzymes (FAME) that detoxify fatty acids, increasing the invasiveness of *Staphylococcus aureus* (Gordon and Lowy, 2008).

The key determinant of methicillin resistance is the mec*A* gene, which encodes in a novel penicillin-binding protein (PBP2/PBP2a) that reduces the binding of all β-lactam drugs to the cell wall and therefore, mediates cross-resistance to all these compounds. The mec*A* gene is carried on a mobile genetic element assigned the staphylococcal cassette chromosome *mec* (SCCmec), SCCmec is inserted into the chromosome at a unique site (attBscc) located near the *Staphylococcus aureus* origin of replication. SCCmec is composed of the *mec* gene complex, which gives resistance to methicillin, and the *ccr* gene complex, which encodes recombinases responsible for its mobility (Figure 2.6) (Deurenberg & Stobberingh, 2008).

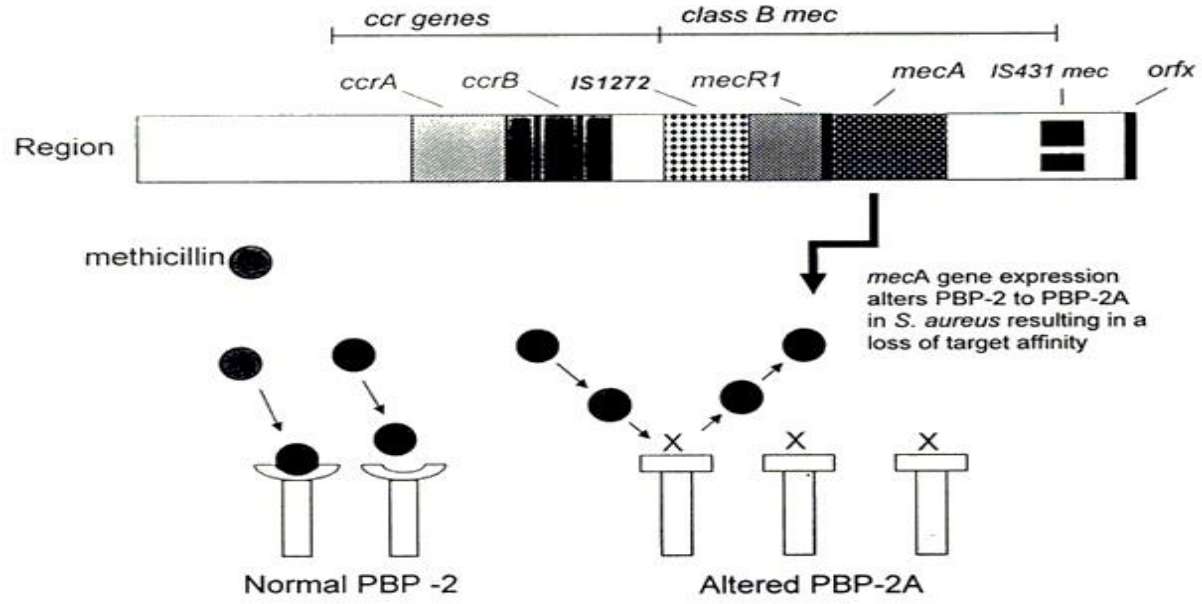


Figure 2.6: Structure of the staphylococcal cassette chromosome *mec* (SCCmec), with the recombinase genes complex upstream of the mec complex (Rybak & La Plante, 2005).

Apart from the inability of an antibiotic to bind to the target site due to structural defect of such site, other mechanisms that may usually play significant roles in the development of resistance in MRSA include efflux phenomenon resulting in continuous pumping of

antibacterial drugs out of the bacterial cell. Others alter membrane proteins, which limit the access of drugs to the cell. Resistance can also occur from high level production of β lactamase. MRSA is produced when *Staphylococcus aureus* acquires a genetic element called SCCmec. SCC is a basic mobile genetic element that serves as the vehicle for gene exchange among staphylococcal species (Luong *et al.,* 2002).

2.3 Proteomics

2.3.1 Origin, definition and significance

The first proteomics studies were reported nearly 40 years ago with the introduction of two dimensional gel electrophoresis (2D-GE) by O'Farrel who attempted to characterise the proteins expressed by *Escherichia coli*. Although many proteins could be separated and visualised, they could not be identified (O'Farrel, 1975). This ground breaking work was followed by attempts to map the proteins of mouse (Klose, 1975), guinea pig (Scheele, 1975) and human (Graves & Haystead, 2002). The term proteomics was first used in 1995, and it was defined as a large-scale characterisation of all proteins that are expressed by an organism at a certain time under a certain condition. Proteomics studies involve characterisation of protein mixtures in order to help understanding biological systems and determine the relationships among proteins, the diverse structural characteristics, functionality and interactions of proteins, and to determine post-translational modified sites (Thelen, 2007).

Currently, there are two definitions of the term proteomics. The first is the more classical definition, restricting proteomics to large scale analysis of proteins only. The second, more inclusive definition, combining protein expression studies with mRNA studies, genomics and studies of the 3D structures of the proteins. However, the goals of proteomics remain the same. The most important goal is to obtain a more global and integrated view of biology by studying all proteins in a cell rather than each one individually (Pandey & Mann 2000).

The field of proteomics covering the analysis of whole proteins. Proteome study would provide the absolute quantitative measurement of every protein, its isoforms, modifications and complexes within a given sample. Typically, there are multiple proteins associated with each gene, the average number of protein formed per gene was predicted to be one or two in bacteria, three in yeast and three or more in human (Daniel, 2002). Comprehensive cellular information cannot be obtained from the study of genes alone. For instance, proteins, not genes, are

responsible for the phenotypes of cells. Furthermore, it is impossible to demonstrate mechanisms of disease, senility, and effects of the environment just by studying the genome. Only through the study of proteins, can protein modifications be characterised and the targets of drugs identified. Thus, the focus of research is now moving to intensify the task of identifying the structure, function, and interactions of the proteins produced by individual genes (Bradshaw & Burlingame, 2005). Many different areas of study are now grouped under the name of proteomics. These include protein expression, protein modifications, protein-protein interaction, protein localisation, and protein function studies (Figure 2.7).

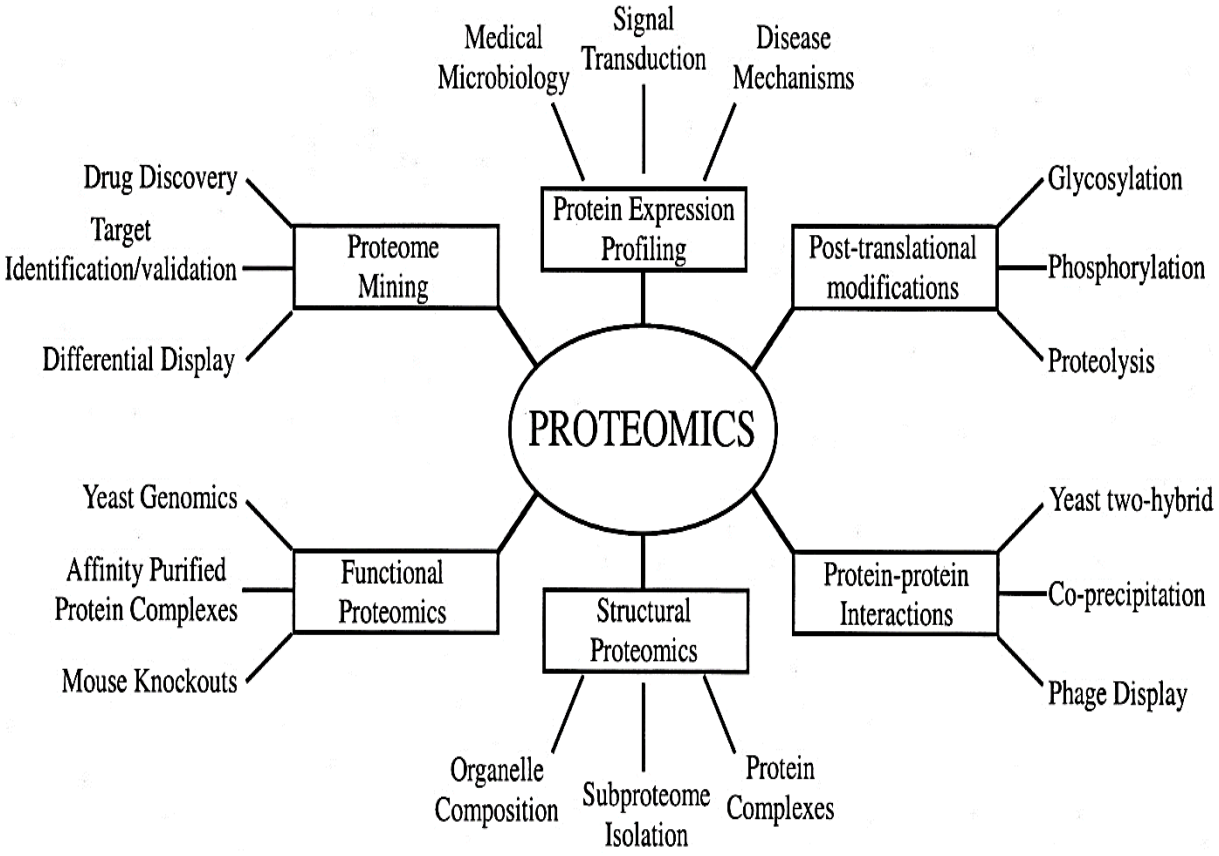


Figure 2.7: Type of proteomics and their applications to biology.

The development of proteomics is a direct consequence of advances made in large-scale nucleotide sequencing and in the sensitivity of protein identification methods. Without this information, proteins could not be identified even with the improvements made in mass spectrometry. Protein identification relies on the presence of databases for the given organism. The majority of DNA and protein sequence information has been accumulated within the last twenty years. One of the first applications of proteomics is to identify the total number of genes

in a given genome. This is necessary because it is difficult to predict gene function accurately from genomic data (Eisenberg *et al.*, 2000).

The other application of proteomics includes providing the means for studying protein expression which cannot be achieved by mRNA analysis alone. Transcriptomic studies have become increasingly popular and includes DNA arrays and serial analysis of gene expression (SAGE). However, many studies have shown that mRNA is not a direct reflection of the protein content of the cell; in fact, there is a very poor correlation between mRNA levels and protein expression levels. The reason for this is that the formation of mRNA is only the first step in a series of events leading to the synthesis of a protein (Figure 2.8). After a protein is synthesised it can undergo posttranslational modifications (PTMs) and it has been estimated that up to 200 different posttranslational modifications may exist. Proteins are also regulated by proteolysis and compartmentalisation (Gygi *et al.*, 2000; Venkateshwar *et al.*, 2008). Proteomics also include the study of protein function. The complete function of genes in a genome will aid the scope of structural genomics, and the ultimate goal is to obtain 3D structures of the products of all genes in a genome to conclude the function of many proteins (David, 2000).

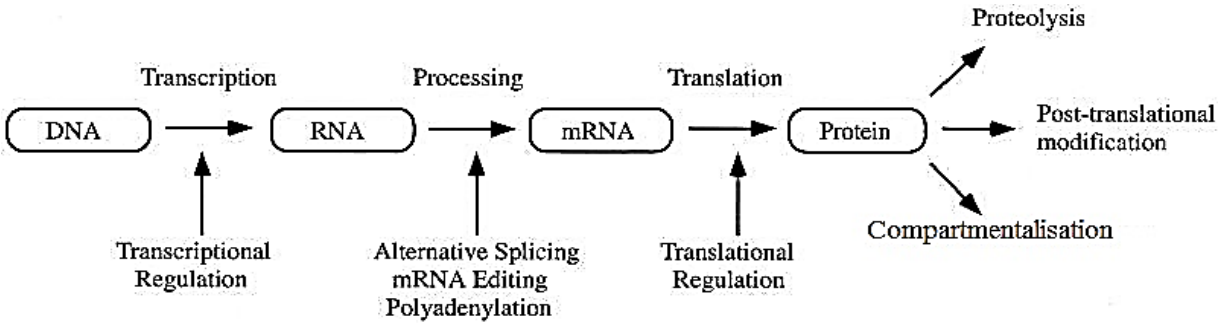


Figure 2.8: Schematic diagram showing the mechanisms of how a single gene can lead to multiple gene products.

Other important applications of proteomics are the characterisation of the posttranslational modifications (PTMs), compartmentalisation of the proteins and protein-protein interactions. Cell growth, life span, and the decision to proceed through the cell cycle are all regulated by signal transduction through protein complexes. Therefore, there are different subjects of proteomics, such as protein expression proteomics, structural proteomics, and functional proteomics. The quantitative studies of protein expression among samples that differ by some

variables is known as expression proteomics. In this way proteins specific for a disease state, cell line or specific growth condition can be identified (Graves & Haystead, 2002).

When the goal of proteomic studies is to map out the structure of a protein complex, or proteins present in a specific organelle, then it known as "cell mapping" or structural proteomics. Structural proteomics attempts to identify all proteins within a complex, determining their location and illustrate the protein-protein interaction. Isolation of specific subcellular organelles or protein complexes by purification can greatly simplify the proteomic analysis. Functional proteomics could include isolation of protein complexes and also the study of their 3D structure which mainly provide important information of protein signaling, disease mechanisms and protein-drug interaction (Au *et al.,* 2007; Csermely *et al.,* 2013). In what is termed comparative proteomics, comparing two protein extracts of the same isolate under two different conditions, or treatment, will result two lists of proteins. The differentially expressed proteins may be related to the condition that induced their expression (e.g. high osmolality) and ultimately allowing the elucidation of which proteins are important or required for the condition (e.g. outer membrane porins) and the characterisation of the physiological response (Kaake, Wang, & Huang, 2010).

2.3.2 Proteomics in Microbiology

Before the universal accessibility of methods for protein identification, two-dimensional polyacrylamide gel electrophoresis 2D-PAGE was widely applied to distinguish between related isolates of various bacterial species, like (Jackson *et al.,* 1985) did with *Neisseria* spp., and (Dunn *et al.,* 1987) did with *Campylobacter* spp., and many more studies in this context. Early application of 2D-PAGE used the method simply as a sensitive technique for the differentiation of closely related microbial isolates on the basis of protein charge and molecular weight. When interpreting the results, it is broadly assumed that co-migrating protein spots are functionally identical proteins with amino acid homology. However, it has to be considered that even a single charged amino acid substitution can alter the position of a protein to what may seem a unique protein spot on a 2D-PAGE. In fact, bacterial strains can sometimes indicate a bigger level of variability when compared on 2D-PAGE in comparison to DNA-DNA hybridisation. (Jungblut, 2001).

The improvements made in the area of mass spectrometry, for identification of electrophoretically separated proteins, allowed the technique of 2D-PAGE to grow further. Nowadays, 2D-PAGE is infrequently utilised as a tool for epidemiological studies, and instead, it is used for elucidating the mechanism of bacterial pathogenicity, antibiotic resistant, physiology and vaccine development (Vesela & Robert, 2010). Many studies have been conducted to analyse the global bacterial proteome. A few examples are the reference maps of the cytosolic proteins created for *Staphylococcus aureus* (Cordwell *et al.,* 2002), *Bacillus licheniformis* (Voigt *et al.,* 2004), *Bacillus subtilis* (Eymann *et al.,* 2004) and many others. The protein expression patterns obtained are typical of growing cells containing glycolytic enzymes, elongation factors and chaperones among the most abundant proteins. Therefore, for several microorganisms, with extensively characterised proteins such as *Escherichia coli*, *Bacillus subtilis* and the *Cyanobacterium synechocystiss*, databases are now available which are freely accessible via the Internet (Cash, 2003).

The publication of the first complete genome sequence of a living organism, the bacterium *Haemophilus influenzae* in 1995 (Fleischmann *et al.,* 1995), opened up a new era in biology. The exponential increase in genome sequence information that followed after this imprint has posed a challenge for bioinformatics and for researchers to design new "global" experiments to attempt to understanding the functions of all of these new genes (Hecker *et al.,* 2003). Today, as the genomes of various bacteria have been sequenced, new technologies for genome wide approaches for analysis of bacterial protein regulation are being explored. These include transcriptional profiling using whole genome DNA microarrays to analyse bacterial gene expression profiles and quantitative protein profiling. Although transcriptional profiling is relatively quick and easy, mRNA abundance is not always a reliable indicator of corresponding protein abundance. In addition, bacterial RNA is relatively unstable and difficult to purify, which might limit the detection of rare transcripts. A large number of genes encoding for proteins with unknown functions have been derived from genome sequencing, among them new global regulators such as alternative sigma factors, (σ factor, is a protein needed only for initiation of RNA synthesis), response regulators or other activators. It appears that even for model organisms, many sections of their cell physiology or molecular biology remain unexplained for the time being (Guina *et al.,* 2003; Zhou *et al.,* 2013).

Quantitative proteomics have become an indispensable analytical tool for microbial research nowadays. Modern microbial proteomics covers a wide range of areas in basic and applied research from in vitro characterisation of individual organisms to unravel the physiological implications of stress, to the characterisation of the protein content of a cells at a given time. With the wide range of techniques available, ranging from classical gel-based procedures to modern MS-based quantitative techniques, including metabolic and chemical labeling, as well as label-free techniques, quantitative proteomics today is highly successful in sophisticated settings of high complexity, such as host-pathogen interactions, mixed microbial communities, and microbial metaproteomics (genes and / or proteins most abundantly expressed in environmental samples) (Andreas *et al.*, 2014).

A proteomics study can be conducted in a top down method where the proteins are studied directly, enabling post translational modifications and splice variants to be determined, key amino acid sequence can also be determined, although specialised MS analysis needed. A bottom up (shotgun) method can also be used where the protein identity is conferred through the study of peptides generated through enzymatic proteolysis of the total proteins from the cell and is the preferred method for complex samples, even though it increases sample complexity through the generation of many peptides for each protein. The optimal methodology depends on the overall complexity of the sample and the information required from the analysis (Wu *et al.*, 2010). Bottom up quantitative proteomics was performed in this research due to the complexity of our sample.

2.3.3 Proteomics as a tool in drug discovery

Drug discovery is a lengthy and highly expensive procedure. To speed up the procedure, a number of biotechnologies, including genomics, proteomics and cellular/organismic methodologies, have been produced and developed over the years (Veenstra, 2007). Proteomics development faces many multidisciplinary challenges, including both the traditional technologies (Biology and Chemistry) and the emerging of high-throughput automation technologies and Bioinformatics. Emergent technologies include two-dimensional ployacrylamide gel electrophoresis 2D-PAGE, mass spectrometry MS, protein arrays, isotope-encoding, two-hybrid systems, bioinformatics technology and activity-based assays. These

technologies are promoting the utility of proteomics in the drug discovery process (Burbanm & Tobal, 2002). Some commonly used technologies are shown in figure (2.9).

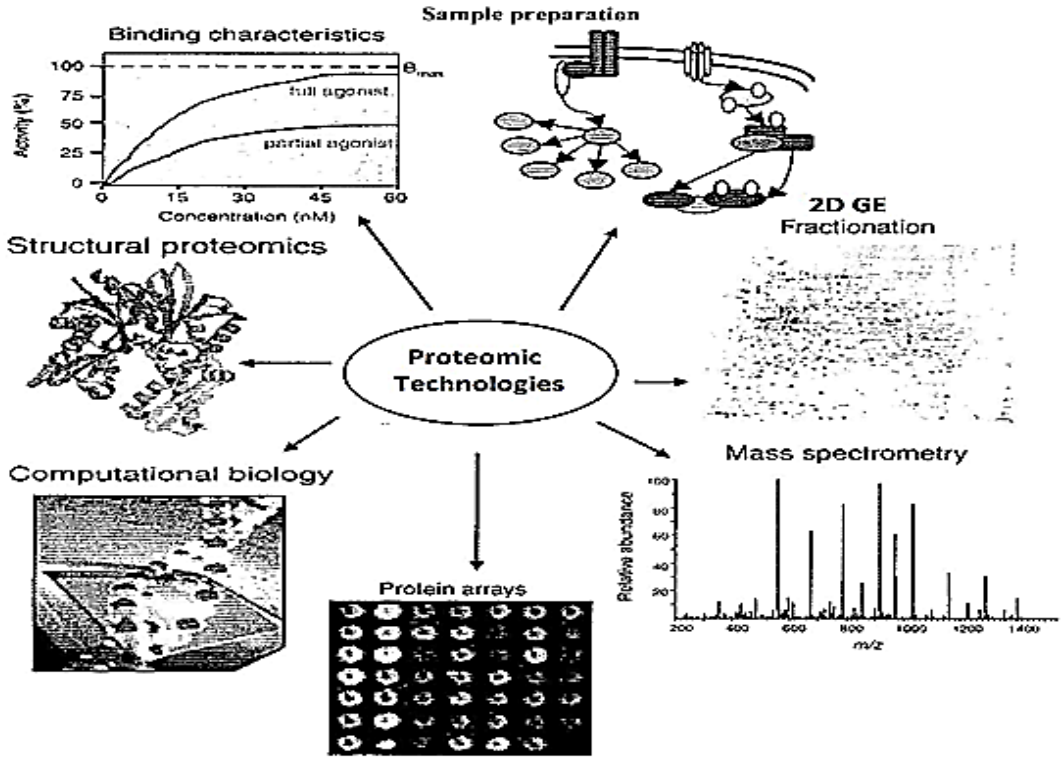


Figure 2.9: A partial view of various proteomic technologies important in drug discovery.

The decisive point of the drug discovery process is getting the best drug for the most appropriate target with minimal side effects to the host. Proteins are the fundamental targets of drug discovery, it represents more than 90 % of druggable targets and will most likely remain the single most important class of molecules targeted by pharmacological agents (Natural or synthesised) as more novel targets are identified. However, the huge excellence of potential drug targets and the hype about target validation has led to a confusion over how to determine the optimal molecular targets for pharmacological intervention. Functional proteomics represent a powerful approach providing valuable information on target druggability (term used in drug discovery to describe a biological target, such as a protein, that is known to or is predicted to bind with high affinity to a drug), and facilitate rapid access to relevant therapeutic leads (Walgren & Thompson, 2004).

The drug discovery process involves many phases, and the proteomics investigation is the important one. Common applications of proteomics in the drug industry include target

identification and validation, identification of efficacy and toxicity biomarkers from readily accessible biological fluids, investigations into mechanisms of drug action or toxicity, exploring protein-protein interactions, and pre-clinical/clinical development. Effectiveness of this procedures relies on timely knowledge of biological cause-and-effect in the course of disease and treatment, which in the end rests on knowledge of protein function and regulation (Jhanker *et al.*, 2012).

Target identification and validation are the first key stages in the drug discovery, which involves identifying proteins whose expression levels or activities change in disease states. Drug target is the specific binding site of a drug in vivo through which the drug exerts its action. The drug target is a biomolecule(s), normally a protein, the biomolecules have special sites that match other molecules, and these molecules could be endogenous or extraneous substances such as drug. The biomolecule structure might change when it binds to small molecules (drug) and the changes in structure normally are reversible (Chen & Du, 2007). Following the change in the biomolecule's structure, various physiological responses occur and induce regulation of the cell, organ, tissue, or body status. The expression, activity, and structure of the biomolecule might change over the duration of the pathological process. This application provides a protein profile of a cell or tissue that can be used to compare a healthy with a diseased state for protein differences in the search for new drug targets. This is one of the most important applications for proteomics (Latterich *et al.*, 2008).

Proteomics technologies may also help identify protein-protein interactions that influence either the diseased state or the proposed therapy. Protein-protein interactions have been identified through the use of the latest proteomics technology, including a method known as stable isotopic amino acids in culture (SILAC). Other methods used to study protein interaction include surface plasmon resonance (SPR) technology, the more recent techniques of reverse transfection and tandem affinity purification. Advances in mass spectrometry have also facilitated the study of protein-protein complexes. Fourier transform ion cyclotron resonance mass spectrometry (FTICR-MS) provides a high mass resolution and accuracy of whole proteins and protein-protein complexes via the circulation of ions in a superconducting magnet (Walgren & Thompson, 2004).

Efficacy biomarkers are used to assess whether target modification has taken place. They are used for the characterisation of disease models and to assess the effects and mechanism of action of lead candidates in animal models. Toxicity biomarkers are used to screen compounds in pre-clinical studies for target organ toxicities as well as later on in development during clinical trials. Complementary approaches such as metabolomics and genomics can be utilised in conjunction with proteomics throughout the drug development process to create more of a unified systems biology approach (Hewick, Lu, & Wang, 2003).

Post-translational processes such as protein modifications or protein degradation remain unaccounted for in genomic analysis. Because both cell function and its biochemical regulation depend on protein activity, and because the correlation between message level and protein activity is low, the measurement of expression has proven to be inadequate. Consequently, the development of drug discovery technologies has begun to shift from genomics to proteomics. The data from recent proteomics studies have demonstrated the potential value that proteomics has to offer in drug development. Proteomics techniques provide precise and fairly rapid methods to screen both target proteins and potential therapeutic compounds (Elangovan, 2013).

2.3.4 Proteomic Analysis Techniques

The rapid growth in the field of proteomics is a direct result of the advances made in the technologies for protein extraction, solubilisation, separation, and the sensitivity of the methods used for protein identification. Attempts to overcome the challenges of heterogeneity, instability and the dynamic range of proteins lead to two main requirements of any techniques apply to proteomic analysis. The first, is the ability to reduce sample complexity to manageable levels, the second is the ability to identify proteins once they are separated. The most popular methods for separating proteins are gels and chromatography columns, and in both cases, the principle of their action is to split a complex mixture of proteins into multiple lower complexity fractions. The most effective tool for protein identification has become the mass spectrometer and bioinformatics (Michael and John, 2000). The ultimate goal of technologies applies in proteomics studies is the characterisation of protein mixtures in order to understand biological systems and determine relationships between proteins, the diverse structural characteristics, functionality and interactions of proteins, and to determine post-translational modified sites.

2.3.4.1 Protein separation using gel based techniques

The modern approach of proteomics consists of two basic elements: the separation of the complex mixture of proteins, and identification of the individual proteins. High resolution separations are the most important feature in separation sciences, in order to be able to analyse complex samples especially in the biological samples. Electrophoretic separations of proteins are no exception to this rule, with the additional difficulty that proteins are very complex substances that have a strong tendency to precipitate (Graves & Haystead, 2002). Two high-performance electrophoretic separations of proteins were available at the very beginning of the 70s, the first one is electrophoresis of proteins in the presence of SDS, as described by (Laemmli, 1970), a technique that instantly became very popular, and the second one is denaturing isoelectric focusing, as described by (Gronow & Griffith, 1971). As these two techniques used completely independent separation parameters (molecular mass and isoelectric point, respectively) it is not surprising that it was soon tried to couple them. The first successful, detailed study, utilising these two different parameters was reported by (O'Faffel, 1975). Subsequently, this technique got its famous name the two-dimensional polyacrylamide gel electrophoresis 2D-PAGE (Rabilloud & Lelong, 2011).

Some complex biological sample may contain too many proteins or peptides for the mass spectrometer to detect them all at once, so separation techniques can be utilised to reduce the complexity of an analyte proteome. By far, the most common technique for the analysis of either a subset of proteins or all resolvable proteins in a cell is gel electrophoresis including either 1D sodium dodecyl sulfate polyacrylamide gel electrophoresis SDS-PAGE or two-dimensional polyacrylamide gel electrophoresis 2D-PAGE. When using SDS-PAGE, the proteins are both solubilised and given a charge by the detergent SDS and further separated according to their molecular weight. Negatively charged proteins traps in the polyacrylamide matrix (gel) as they travel towards a positive electrode (anode), facilitated by an electric field applied across the gel. The ionic detergent SDS is added to the proteins where it binds and denatures the proteins, distributing a negative charge evenly across the whole protein. SDS-PAGE is simple to perform, reproducible and can separate proteins with molecular weights between 10 and 300 kDa. For many applications SDS-PAGE is the method of choice, but due to its limited resolving power it is only suitable for separation of mixtures after some form of purification (John, 2002). Reducing SDS-PAGE is usually performed, which includes the addition of dithiothreitol (DTT)

to the sample, this denatures the proteins further by breaking any disulphide bonds and disrupting any tertiary protein folding and quaternary structure, giving an improved protein separation (Claudia *et al.,* 2012).

When separation of more complex samples is required, such as total cell lysates, 2D-PAGE is the method of choice. 2D-PAGE is an extension step of the 1D separation with the addition of isoelectric focusing (IEF) before SDS-PAGE separation. IEF of proteins is performed along an acrylamide gel strip with an immobilised pH gradient, where proteins are separated based on their charge in a specific buffer and their differing isoelectric points, which is the point on the pH gradient where each protein has no net charge. Proteins migrate along the gel with the electric field to their isoelectric point. This immobilised pH gel is then added to the top of a large polyacrylamide gel and SDS-PAGE is performed (Figure 2.10). The combination of these two methods achieves resolution far exceeding that of SDS-PAGE alone (Rogowska *et al.,* 2013).

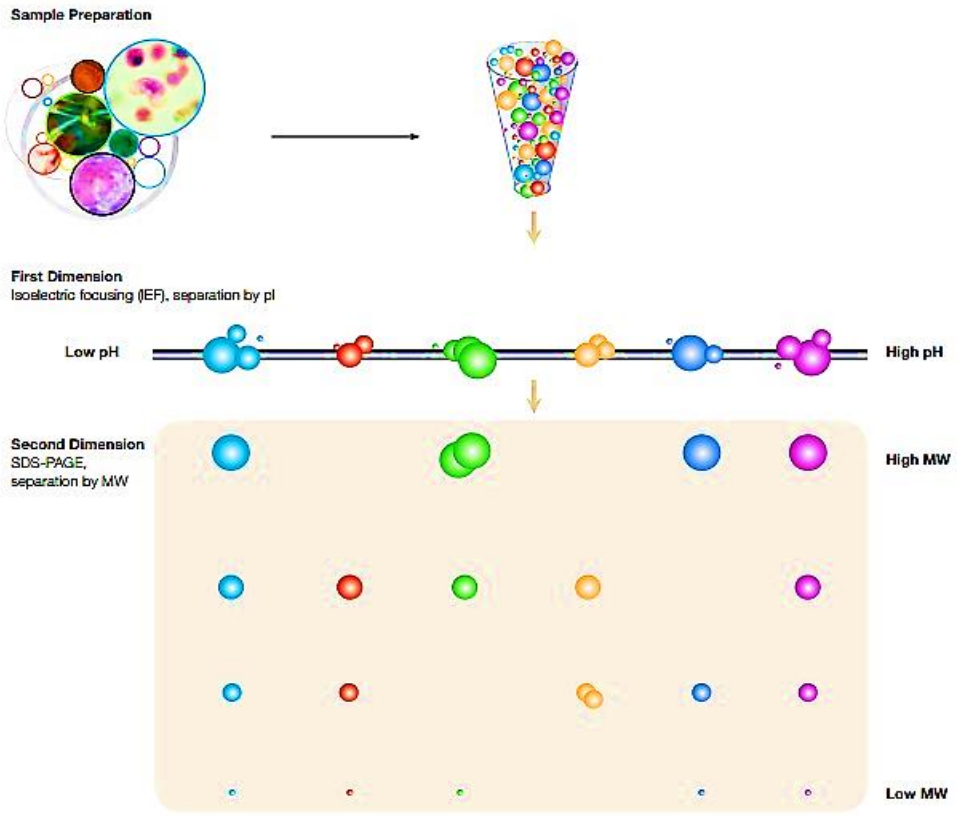


Figure 2.10: 2D Electrophoresis. Protein spot result from two separations: first by pI (IEF) and then by size (SDS-PAGE). (Bio-Rad, 2014).

Isoelectric focusing became more reproducible with the development of immobilised pH gradients (IPG) and Immobiline™, eliminating the possibility of the pH gradient drift and brought superior resolution and reproducibility to first dimension IEF. The primary application of 2D-PAGE is expression the profile of complex protein mixtures extracted from cells, tissues, or other biological samples, when the comparison of the resulting images of two samples is possible, and that gives both quantitative and qualitative information. Another application of 2D-PAGE is monitoring post-translational modifications, which is possible because most of the modifications would alter the charge and the molecular weight of the protein. Currently there are many software packages designed to detect, match and calculate the volumes of spots between gels, this enables quantitative and statistical analyses. For instance, BioNumerics 2D, Delta 2D, ImageMaster 2D, and many others. Protein spots of interest can be excised from the 2D-PAGE, digested, and the peptides subjected to mass spectrometry (MS) to identify the parent protein (Yerlekar & Dudhe, 2014).

With all advantages and features of 2D-PAGE based proteomics, there are still a number of limitations when using this approach. Major shortcomings of 2D-PAGE include the poor representation of basic and membrane proteins (Due to poor solubility in non-ionic lysis buffers and inefficient transfer from the isoelectric focusing strip to the SDS-PAGE gel), limited dynamic range and the potential for "hidden" proteins, 2D-PAGE spots may be made up of more than one protein (two or more may have similar isoelectric point and molecular weights), as well as tendency towards high abundance proteins (isoelectric point of 4-7), and finally the 2D-PAGE is time consuming to run and manually intensive. These disadvantages of 2D-PAGE demonstrate that the choice of method depends largely on the type of sample and its complexity (Wittmann-Liebold, Graack, & Pohl, 2006).

2.3.4.2 Methods of protein quantitation and identification

Although electrophoretic separation of proteins can effectively separate all the component proteins of a proteome, and provide quantitative data, protein identification and function remain unknown. The methods for the identification of proteins present in single gel spots/bands have been improved significantly with the development of mass spectrometry techniques. After performing gel electrophoresis, the protein of interest can be excised from the gel and digested into small peptides by proteases in preparation for MS analysis. Trypsin is the most widely used

protease, which cleaves proteins at the position C-terminal to lysine and arginine residues (Rappsilber & Mann, 2002).

Until recently, the identification of a protein mostly relied on antibody recognition using western blot through transference of proteins from the SDS-PAGE gel, or Edman sequencing (A method of sequencing amino acids in a peptide developed by Pehr Edman). Antibodies are a very useful tool, however, their binding to a given protein is not necessarily proof that it is the product of a specific gene, as cross-reactivity can result in the antibody binding unrelated proteins. Edman sequencing depends on the stepwise cleavage of amino acids from the N-terminus of a protein. Often no peptides that were sufficiently long enough to provide protein-specific identification could be sequenced, or the N-terminus was acetylated or otherwise blocked, preventing the Edman reaction (Smith, 2001).

Nowadays, mass spectrometry has played an important role in proteomics as it is a powerful tool for the structural characterisation of proteins, and it allows the analysis of proteins separated by electrophoretic technique with great accuracy, rapidly and sensitively. MS is an analytical technique that measures an intrinsic property of a molecule based upon the motion of a charged particle in an electric or magnetic field. MS enables protein structural information, such as peptide masses or amino acid sequences, to be obtained. The sample molecules are converted into ions in the gas phase and separated according to their mass to charge (*m/z*) ratio. In order to form a multiple-charge ion, an analyte molecule must have more than one site which can be ionised. In electrospray analyses, these charge loci may be any of the functional groups that normally associate with protonation (positive charge) or deprotonation (negative charge). Amines are excellent proton acceptors and a very common site for positive charges, while carboxylic acids are common site to form a negative ion (Guerrera & Kleiner, 2005).

A typical mass spectrometer is composed of (i) Ionisation source, to produce ions from sample (ii) Mass analyser, to separate the ions based on their mass-to-charge ratio (*m/z*) (iii) A detector system, to detect the ions, (iv) Computer, to record and analyse data. Electrospray ionisation (ESI) and matrix-assisted laser desorption ionisation (MALDI) are the most commonly used ion source techniques. Samples ionised by MALDI are dry and co-crystallised with a matrix, it is primarily used to analyse simple peptide mixtures. Samples ionised by ESI are transferred into gaseous ions at atmospheric pressure and delivered to the mass analyser

through a capillary tube which experiences an electric field. ESI combined with LC-MS is used to analyse more complex samples. Accuracy and precision are two important standards for any mass analyser. Ion trap, time-of-flight (TOF), quadrupole and Fourier transform ion cyclotron resonance (FT-ICR) are the common types of mass analyser. Proteins from gels can be characterised using mass spectrometry via peptide mass fingerprinting (PMF) (Lane, 2005).

The use of peptide mass fingerprinting (PMF) in combination with the use of software algorithms was first described for the identification of proteins in 1993 by a number of research groups (Henzel *et al.,* 1993; Mann *et al.,* 1993; Pappin *et al.,* 1993). The approach utilised peptide masses, in the absence of tandem MS information, to identify a protein from a database containing many thousands of sequences. This approach is rapid and most effective when the sample under investigation was relatively simple, either purified or pre-fractionated. For more complex samples, many protein sequences in the database could lead to the PMF observed by ESI or MALDI, with an incorrect identification. If a protein cannot be identified by its PMF alone, primary sequence information on the peptides is required. This can be obtained by tandem MS. In 1994, McCormack and coworkers published an approach that utilised both peptide mass and tandem MS data, using a computer algorithm to identify proteins from *Escherichia coli* and *Saccharomyces cerevisiae* cell lysates (McCormack *et al.* 1994).

This method couples two stages of MS, it utilises collision-induced dissociation, the process breaks proteins within the peptide backbone and because of this fragmentation, comparisons between the observed fragment sizes and the database of predicted masses is possible. A very similar method to the tandem MS approach is peptide fragmentation fingerprinting (PFF), Instead of utilising collision-induced dissociation, this method uses enzymatic digestion of a single peptide to generate a fragmentation pattern. These fragments are analysed and compared to a database of observed fragments for the particular enzyme in a method similar to the Tandem MS approach. Tandem MS and peptide fragmentation fingerprinting PFF can also be used for protein sequencing (Nilsson *et al.,* 2010).

Mass spectrometry has become a powerful tool in quantitative proteomics, it gives a list of identified proteins and profiling global protein abundances in biological matrices, which can provide important information for the study of cellular response to disturbance and diseases, as well as the drug targets. Quantitative proteomics provides quantitative information of the

analysed sample proteins, as a major advance in proteomics, which allows researchers to quantitatively compare the samples (Rodriguez and Whetton, 2013). Relative and absolute quantification represents two forms of quantitative data. In relative quantification, the protein amount is calculated relatively for the same protein present in a reference sample, whereas absolute quantification determines exactly how many copies or amount of a protein there are per cell. Compared to the classic methods of differential protein gel or blot staining by dyes and fluorophores, mass spectrometry-based approaches enable us to identify and quantify many proteins in parallel, which has been hugely successful in the large-scale proteomics research field (Al Feteisi *et al.*, 2015).

There are two quantitative strategies in proteomics, the top-down protein profiling approach, which often involves the use of 2D-PAGE, followed by MS analysis. Top-down proteomics is capable of identifying and quantitating unique proteoforms through the analysis of intact proteins. this method served as the main proteomics method of choice in the past and has been utilised in many bacterial pathogen studies, e.g. (Kohler *et al.*, 2005; Becher *et al.*, 2009). This approach has some disadvantages including lack of resolution and sensitivity, especially with low abundance proteins, proteins with extremely high /low molecular weights and it is inefficient for the analysis of insoluble proteins. The inherent limitations associated with 2D-PAGE/MS lead to the development of gel-free MS-based approaches, better known as the bottom-up "shotgun" proteomics approach, the method demonstrated better sensitivity and reproducibility over the 2D-PAGE based method, it involves protein digestion of a complex mixture followed by peptide separation and mapping by UHPLC-MS. For the shotgun protein quantification by mass spectrometry, there are two kinds of strategies involved, isotope-labelled and label-free methods (Soufi & Boumediene, 2016).

Label free and isotope labeling are two basic strategies with a different basis. Isotope labeling approaches introduce a differential mass tag to the samples that can be recognised by a mass spectrometer and at the same time provide the basis for quantification. However, this approach has potential limitations such as high cost of reagents, time consuming, complicated sample preparation, incomplete labeling, requirement of larger sample amounts, and sophisticated software required for data analysis. Label free quantification approach, however, represents a strategy which avoids the isotope labeling step limitations. It became a popular

technique very recently. There are two categories under label-free based measurements: peak area (or ion intensity) and the number of peptide sequencing events (spectral counting). These two categories are mostly used to achieve the relative or absolute quantification. Label-free method has become the preferred method of choice for quantification of global protein expressions as an alternative to isotope labelling. The label free technique is simple and relatively cost effective. One of the main advantages of label free quantitation is that there is no limit to the number of samples analysed, whereas isotope labelling methods will always be limited by the number of labels available to the researcher (Bantscheff *et al.*, 2007).

A typical workflow for a label free quantitation experiment is the digestion of protein extracts from different samples, normally using trypsin, so generating complex peptide mixtures. An aliquot of this, for each sample, is analysed with the LC-MS system for the initial data acquisition. Typically, for initial acquisition, only MS^1 data is collected, as a high frequency of MS spectra is required to obtain the required chromatographic resolution for peak detection and integration. Following the label free analysis and identification of features of interest, the sample aliquot is re-analysed, and MS-MS (MS^2) data is selectively acquired in a targeted manner (Griffiths *et al.*, 2014).

The fast development of gene and protein sequence databases provides a means to identify proteins expressed in organisms. Sequencing of bacterial genome is now a standard procedure, by using third-generation DNA sequencing (TGS) (sequencing single DNA molecules without the need to halt between read steps), it is possible to completely sequence a bacterial genome in a few hours. This has enabled MS-based proteomics technologies to rapidly advance in order to study microbial models and their communities in a robust and systematic pattern. Bioinformatics tools make it easier to access these databases to search, match and identify a protein component. There are number of an online database search engines which facilitate the matching and identification, Mascot is a search engine used widely in this field. UniProt, trEMBL and NCBInr are the widely used protein sequence databases (Ribeiro *et al.*, 2012).

2.4 Natural Products
2.4.1 General Introduction

Nature has been a source of therapeutic agents for thousands of years, and an impressive number of modern drugs have been derived from natural sources, many based on their use in traditional medicine (Murugan & Mohan, 2011). Products came from natural origins can be called "natural products". Natural products come from four major sources: (i) An entire organism (e.g., a plant, an animal, or a microorganism) that has not been subjected to any kind of processing or treatment other than a simple process of preservation, (ii) Part of an organism (e.g., leaves or flowers of a plant, or an isolated animal organ), (iii) An extract of an organism or part of an organism, and (iv) Pure compounds isolated from plants, animals, or microorganisms , (e.g., alkaloids, flavonoids, glycosides, steroids, sugars, terpenoids, etc.) Nevertheless, in most cases the term natural products refer to secondary metabolites (small molecules produced by an organism that are not strictly necessary for the living of the organism). Natural products can be from any terrestrial or marine source: plants, animals, or microorganisms. The databases of natural products have recorded more than two hundred thousand compounds from almost all part of the world (Satyajit, Zahid & Gray, 2006).

Research into the chemical and biological properties of natural products over the past two centuries has not only yielded drugs for the treatment of human illnesses, but have provided the motivation for the development of modern synthetic organic chemistry and the emergence of medicinal chemistry as a major field for the discovery of novel and more effective therapeutic agents. Natural products not only served as the main source of medicine for mankind, but played a vital role in the history of medicine, with the plants represented a rich source among them in the ancient time. The structure determination and biological activity screening of natural products, particularly those with a history of medicinal use, taking clues from folklore medicines (Gordon & David, 2005). Plant preparations have a very special characteristic that distinguish them from chemical drugs and any other natural sources, a single plant may contain a considerable number of bio-active compounds and a combination of plants even more. This complexity is one of the most important challenges to phytoscientists attempting to identify a single bio-active phytocompound or chemical group in the enormous molecules that comprises a single crude extract (Mendonca-Filho, 2006).

The natural product drug discovery process in general includes the testing of extracts of plant, marine or microbial origin in adequate in vitro assays target based, followed by bioassay-guided fractionation of the active extracts and isolation and purification of active constituents. Those constituents showing significant *in-vivo* activity in appropriate animal models are considered as lead molecules, which may be chosen as candidates for preclinical development. Initially, such leads may be structurally modified through use of medicinal or combinatorial chemistry techniques to provide agents having prevailing activity or decreased toxicity. The development of natural product-derived drugs poses significant challenges in several areas (Cragg & Newman, 2005). Figure (2.11) summaries the natural product drug discovery process.

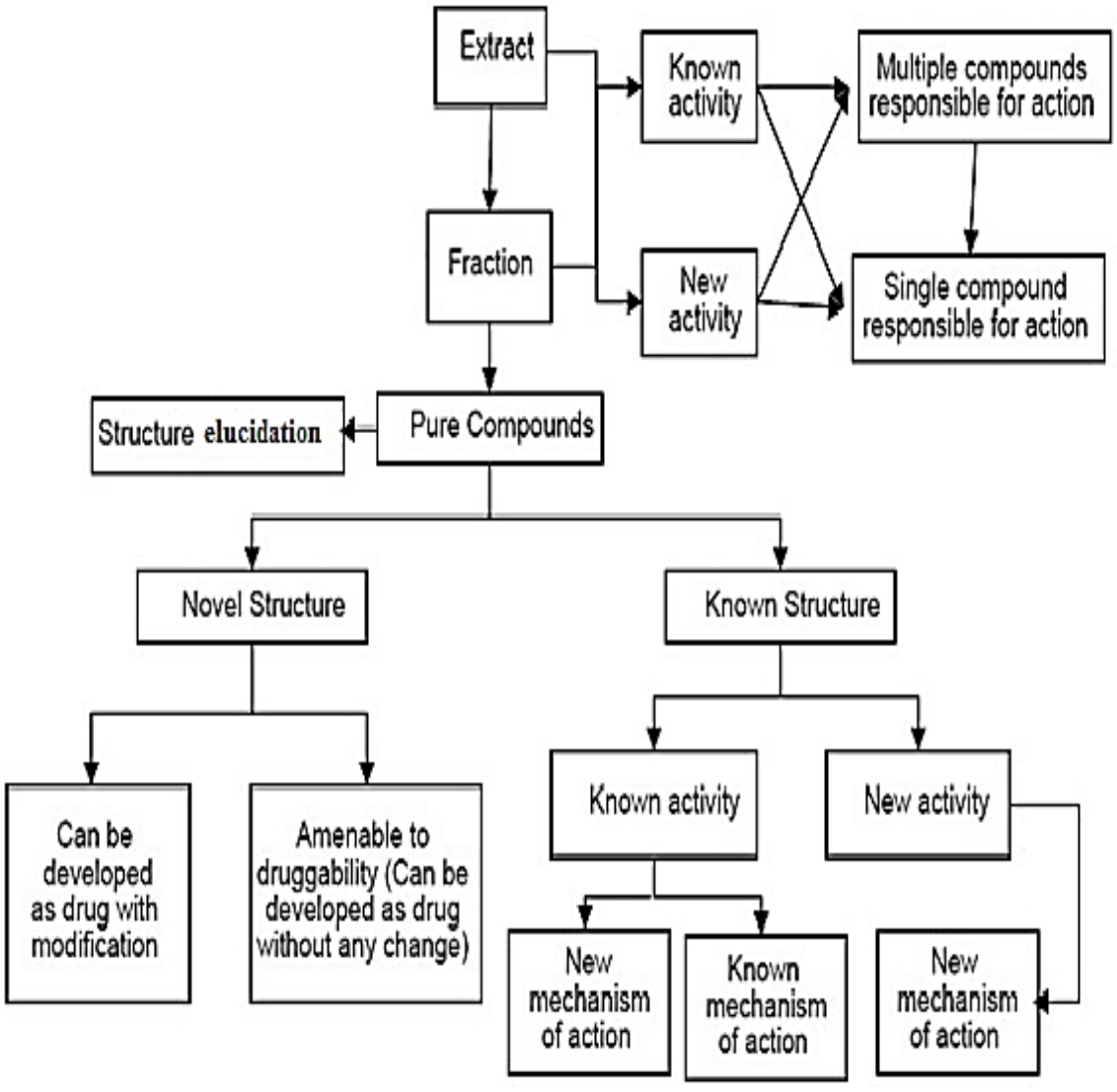


Figure 2.11: Steps of natural products discovery. Modified from (Katiyar *et al.*, 2012).

2.4.2 Importance of Medicinal Plants

There has been an increasing concern, in the health sector, due to the growing resistance of bacteria to the available antibiotics. As result, it is very important to develop new strategies that could discover new therapeutic agents by exploring a new agents derived from plants (Monteiro *et al.*, 2012). Bio-active compounds in plants are compounds produced by them that have pharmacological or toxicological effects in any other organism. Although nutrients can show pharmacological or toxicological effects when consumed at high dosages (e.g. vitamins and minerals), nutrients in plants are generally not included in the term bio-active plant compound. The typical bio-active compounds in plants are produced as secondary metabolites. Thus, a definition of bio-active compounds in plants could be the secondary plant metabolites showing pharmacological or toxicological effects in man and animals (Aksel, 2010).

The use of plants as traditional medicines has been known for a long time by the ancient people from Egypt, India, Africa and Asia. There are large numbers of written herbal manuscripts that explain how the plants were prepared and applied as traditional medicine, although some of the traditional prescriptions have only been transmitted by word of mouth through the generations. One of the ancient scripts from Egypt (2600 BC), which mentioned the uses of plants as the major ingredient of their traditional remedies, another ancient scripts from Chinese scientists (100 BC) and Greek physicians (100 AD) documented the prescription of herbal medications to their patients. In general, parts of the plants used as medicines were only treated with simple galenical preparations to obtain impure crude extracts in the form of liquid, semi-solid or powder prior to oral or external use (Rates, 2001).

Even today, according to the World Health Organization (WHO), about 80% of the population in many third world countries still use traditional medicine (e.g., medicinal plants) for their primary health care, due to poverty and lack of access to modern medicine. About 25% of all prescription drugs in industrialised countries also contain active compounds that are still extracted or derived from higher plants and this situation has continued over the last few decades. Studies on a significant number of novel plant derived substances have entered into western drug markets and clinical plant based research has made great progress in important fields, such as in anti-cancer and anti-malarial therapies (Paulsen, 2010).

The search for useful drug molecules from plants began right after the discovery of morphine in 1804. Since then many thousands of novel structures have been discovered. In spite of this considerable research activity the number of plant-based drugs on the market is still less than two hundred. This is partly because of the poor pharmacological/toxicological profile of many plants (Harvey, 2008). There are presently no scientific methods applicable for the selection of plants that can be anticipated to contain novel biologically active substances. The provision for plant based drug development programs must utilise approaches that are considered to be "nonscientific" to save the time and money spend on these tasks. Historically, scientists claiming to have an interest in drug discovery from natural products have used approaches like, phytochemical screening, followed by bioassay and pharmacological screenings. The last and best approach however is selection based on "folklore" use. A considerable number of drugs based on higher plants came to the attention of pharmaceutical researchers because of their use in traditional medicine (Marderosian & Liberti, 1988).

The WHO considers phytotherapy in its health programs and suggests basic procedures for the validation of drugs from plant origin in developing countries. Eastern countries, such as China and India have well established herbal medicines industries and ten Latin American countries have been investing in research programs in medicinal plants and the standardisation and regulation of phytomedicinal products following European countries, such as France and Germany. Despite all the activities described up to this point, it is still the case that of the estimated five million plant species, only a small percentage has been investigated phytochemically and even a smaller percentage has been properly studied in terms of their pharmacological properties. In most cases, only pharmacological screening or preliminary studies have been carried out (Phillipson, 2001; Bent & Richard2004)

Over the last decade, exploration of active compounds contained in the traditionally-known medicinal plants has become a significant interest in drug development research. Many sophisticated methods are currently available, especially in separation, spectroscopic and bioassay techniques, as a result of the growing demand of natural products research. For instance, GC-MS, LC-MS, LC-FTIR, LC-NMR, LC-NMR-MS and CE-MS have made possible the pre-isolation analyses of crude extracts or fractions from different natural sources. The emphasis on previous strategies has an impact on developing new methods based on

straightforward extraction and elucidation along with the new bioassay screening methods of the bioactive compounds (Sarker, Latif & Gray, 2006). Figure (2.12) gives an example of natural product drug discovery process from plants.

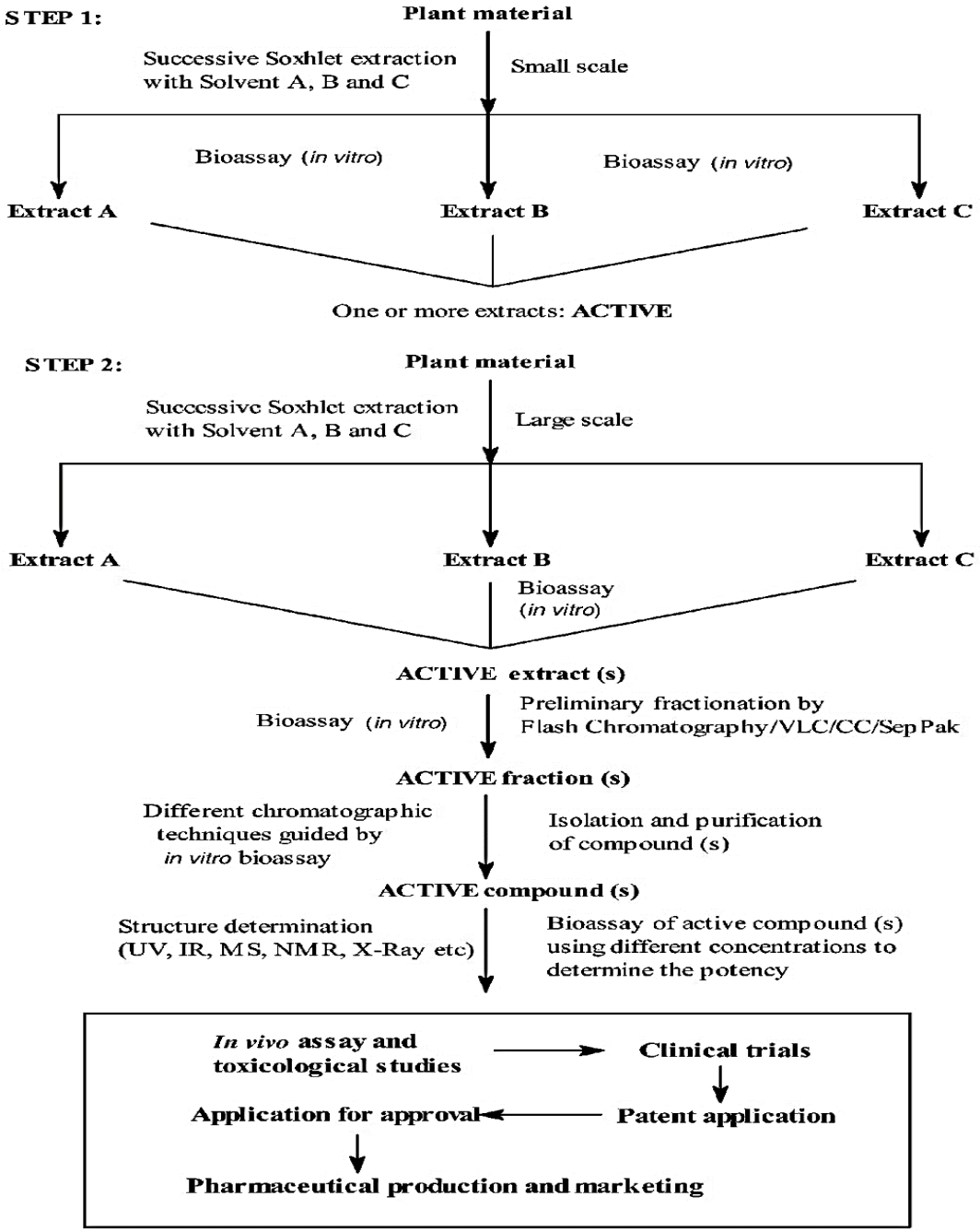


Figure 2.12: An example of natural product drug discovery process (bio-assay guided approach). (Satyajit *et al.*, 2006).

2.4.3 Extraction of plant-derived natural products

The history of the extraction of natural products dates back to the Mesopotamia and Egyptian times, where production of perfumes, or pharmaceutically - active oils and waxes was a major business. Several Sumerian texts, found 400 km south of Baghdad (Iraq) from about 2100 BC, also confirm that a sophisticated pharmaceutical and chemical technology existed at that time. In a papyrus of 1600 BC, beer and wine were used as alcoholic solvents, which give the distinguished advantages of achieving a higher solubility for a solute when producing perfumes. Otherwise up to forty repetitive extraction procedures were necessary to give a high yield. There was no more development until Middle Ages times, when pure ethanol became available as a solvent in about 900 AD (Bart, 2011). Medicinal plants are the richest bio-resource of drugs from traditional systems of medicine, modern medicines, nutraceuticals, food supplements, folk medicines, pharmaceutical intermediates and chemical entities for synthetic drugs. Extraction of the bio-active plant constituents has always been a challenging task for the researchers (Tiwari *et al.*, 2011).

Extraction, as the term is used pharmaceutically, is the separation of medicinal active compounds from plants using selective solvents through standard procedures. The purpose of standardised extraction procedures for crude drugs (medicinal plant parts) is to achieve the therapeutically desired compounds and to eliminate unwanted material by treatment with a selective solvent. The solvent attracts the soluble molecules and leave the insoluble material behind (Das, 2010). There are many protocols available to extract bio-active molecules from medicinal plants such as maceration, infusion, percolation, digestion, hot continuous extraction (Soxhlet), microwave-assisted extraction, boiling, and ultrasonic extraction (sonication). Selection of procedures and solvents for extraction of the particular components of the plants depends on the nature of the desired molecules. Variation in extraction methods usually depends on some factors such as: length of the extraction period, solvent used, Temperature, particle size of the plant tissues, the solvent-to-sample ratio (Mendonça-Filho, 2006)

Successful Identification of biologically active compounds from plant material is significantly dependent on the type of solvent used in the extraction procedure (Table 2.2). Solvent characteristics need to be carefully considered when deciding on the extraction protocol to follow. Solvents have to be easily removed from the extract; therefore, the highly volatile

solvents are preferred. Other factors such as physical and chemical properties of the compounds of interest have to be taken into consideration as they may affect the extraction procedure. Standardisation of extraction procedures contributes significantly to the final quality of the herbal drug (Handa, 2008).

Water	Ethanol	Methanol	Chloroform	Ether	Acetone
Anthocyanins	Tannins	Anthocyanins	Terpenoids	Alkaloids	Phenol
Starches	Polyphenols	Terpenoids	Flavonoids	Terpenoids	Flavonols
Tannins	Polyacetylenes	Saponins		Coumarins	
Saponins	Flavonol	Tannins		Fatty acids	
Terpenoids	Terpenoids	Xanthoxyllines			
Polypeptides	Sterols	Totarol			
Lectins	Alkaloids	Quassinoids			
		Lactones			
		Flavones			
		Phenones			
		Polyphenols			

Table 2.2: Typical solvents used to extract active components from medicinal plants. Adapted from (Handa, 2008).

Standard steps in the extraction of plant material start with size reduction of dried plant material, the purpose of this step is to break the cell wall in order to increase the surface are exposed to the solvent. The next step is the treatment with the desired solvent followed by the filtration of the extract. The final step is the concentration and drying process to remove the solvent and obtain the dried crude extract. This step can be achieved by evaporating the solvent under reduced pressure followed by a freeze dried or the use of a low temperature oven (Tiwari *et al.*, 2011).

2.4.4 Isolation and purification of natural products

Bio-active compounds in plants can be classified according to different criteria. It could be based on clinical function, their pharmacological or toxicological effects, and that relevant to the clinician, pharmacist or toxicologist. It might be a botanical categorisation based on families and genera of the plants producing the bio-active compounds. It could be useful to categories them according to biochemical pathways and chemical classes. The main chemical groups of bio-active compounds in plants are: Flavonoids, Glycosides, Tannins, Resins, Lignans, and Alkaloids (Ramawat, Dass, & Mathur, 2009).

Plant extracts are mixtures of complex molecules that have different physical, chemical and biological properties. It is not easy to obtain pure bio-active compounds with only one isolation procedure; more than one protocol is usually applied to isolate them to homogeneity. The initial step is the separation of the extracted components into various fractions of similar properties, these fractions can be obtained by elution with a particular solvent in various chromatography techniques. These techniques based on separation of substances between a stationary and a mobile phase. The mobile phase moves close to the stationary phase and the mixture to be separated move together with the mobile phase, the isolation occurs due to the different interactions of the mixture with both phases (Czaplicki, 2013).

Chromatography is one of the most important techniques in the isolation and purification of natural products. The older techniques comprised of thin layer chromatography (TLC), column chromatography (CC) and flash chromatography. The latest techniques include high performance thin layer chromatography (HPTLC) and high performance liquid chromatography (HPLC). Selection of isolation protocols of the known molecules can be carried out based on the general features of the molecules itself such as, size, stability, charge and solubility. However, it is more difficult to design an isolation protocol for unknown components. In this case, the nature of the crude extracts is an important factor to be considered. It is also worthwhile carrying out qualitative tests for the presence of various types of compounds, such as, phenolics, steroids, alkaloids, flavonoids, as well as analytical thin-layer chromatography (TLC), or HPLC profiling in order to determine the general profile of the extracts (Sarker, Latif and Gray, 2006). Figure (2.13) Illustrates analytical methodology used for isolation and identification of natural bio-active compounds.

In the most cases, it is not easy to apply a single separation technique to isolate individual compounds from a crude mixture due to its complexity. Hence, the crude extract is initially separated into various detached fractions containing compounds of similar polarities or molecular sizes. For initial fractionation of any crude extract, it is advisable not to generate too many fractions, because it may spread the target compound over several fractions and might avoid detection. Modern preparative, or semi preparative high-performance liquid chromatography (HPLC) can be used for finer fractionation and often guided by an on-line detection technique (Sarker, Latif & Gray, 2006).

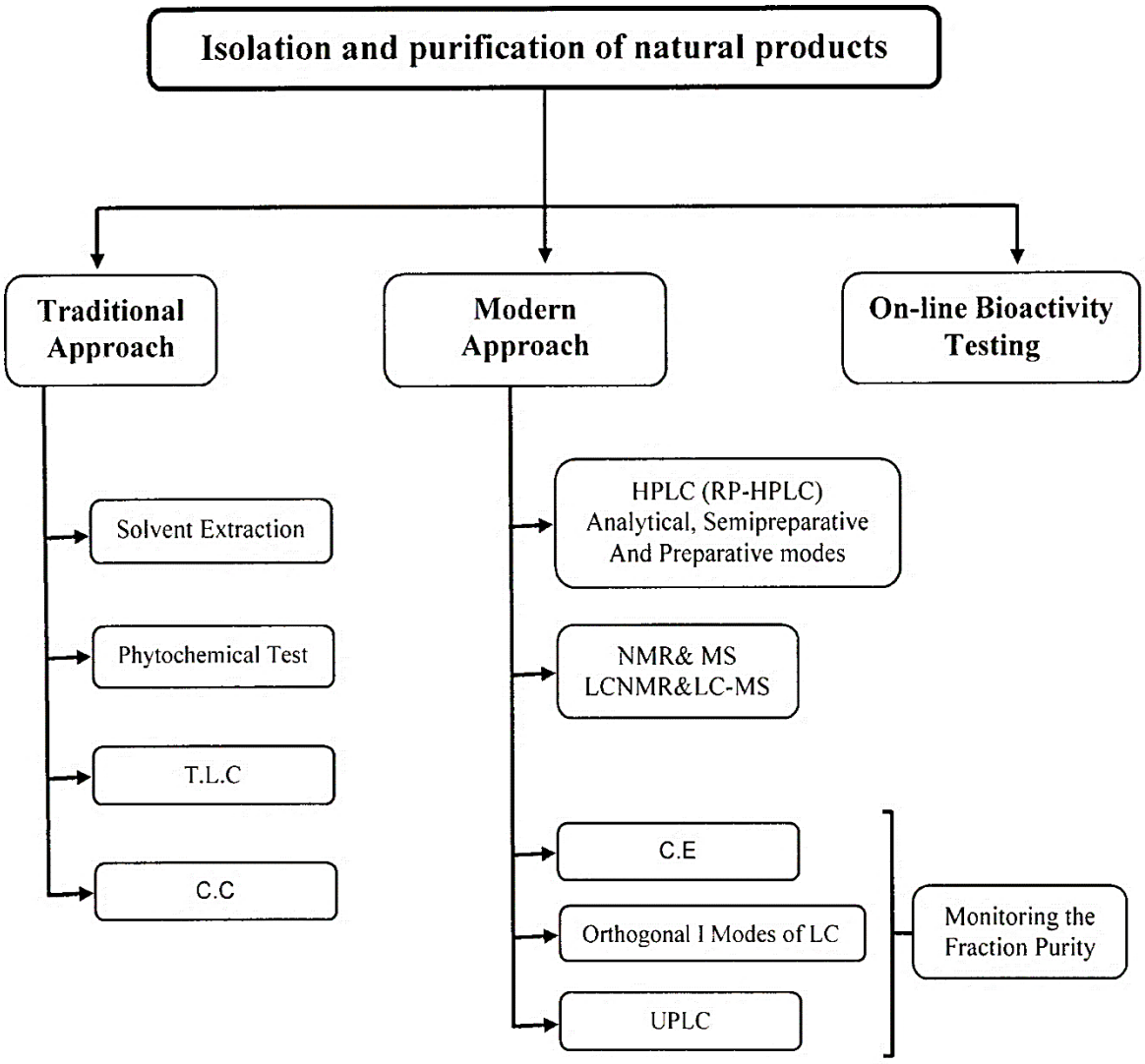


Figure 2.13: Schematic diagram illustrates analytical methodology used for isolation and purification of natural bio-active compounds

2.4.4.1 High-performance liquid chromatography (HPLC)

As mentioned earlier, prior to the 1970's, few reliable chromatographic technologies were commercially available for routine laboratory work. Before that, most chemical separations were carried out using a variety of techniques including open-column chromatography, paper chromatography, and thin-layer chromatography. Later, high pressure liquid chromatography (HPLC) began to emerge in the industries, it was developed in the mid-1970's and quickly improved with the development of column packing materials and the improvement of the on-line detectors. In the late 1970's, new methods including reverse phase liquid chromatography allowed for improved separation between very similar compounds (Charles *et al.*, 2001). HPLC is a chromatographic technique that can separate a mixture of compounds and is used in biochemistry and analytical chemistry to identify, quantify and purify the individual components of the mixture. By the 1980's HPLC was commonly used for the separation of chemical compounds. New techniques improved separation, identification, purification and quantification of HPLC, computers and automation was added, improvements in type of columns such as micro-column, affinity columns, all that lead to improve the reproducibility and reliability, and finally fast HPLC began to emerge (Sailaja *et al.*, 2014).

HPLC system consists of a computer, solvent reservoirs, pumps, injector, column, detector, and fraction collector. The main part of the system is the column where separation occurs. Separation of components occurs as the analytes and mobile phase are pumped through the column (stationary phase) (Kupiec, 2004). Types of HPLC generally depend on the phase system used in the process, these types including; normal phase HPLC (NP-HPLC), this method uses a polar stationary phase and a non-polar mobile phase; Reversed phase HPLC (RP-HPLC) has a non-polar stationary phase and an aqueous, moderately polar mobile phase; Size exclusion chromatography (SEC), also called as gel permeation chromatography mainly separates particles on the basis of size, this technique is widely used for the molecular weight determination of polysaccharides; In ion-exchange chromatography, retention is based on the attraction between solute ions and charged sites bound to the stationary phase. Ions of the same charge are excluded; Bio-affinity chromatography based on specific reversible interaction of proteins with ligands, because of specificity of the interaction, this type can result in very high purification in a single step (Verma, 2014; Malviya *et al.*, 2010).

Among all available techniques, reversed-phase chromatography is the most commonly used separation modes due to its broad application range, especially with the separation of natural products from plant material. The reasons for this include the simplicity, versatility, and range of the reversed-phase method as it is able to handle compounds in a wide range of polarity and molecular mass. The separation mechanism in reverse phase chromatography depends on the hydrophobic binding interaction between the solute molecule in the mobile phase and the immobilised hydrophobic material in the stationary phase (Prathapa *et al.,* 2013).

2.4.5 Structure elucidation of natural products

The final step of natural product extraction is to identify, unequivocally, the molecule that is responsible for a given pharmacological effect. It is possible to determine the chemical composition through advanced spectroscopic techniques including Mass Spectrometry (MS), Nuclear Magnetic Resonance (NMR), MS-MS, and LC-NMR-MS (Bobzin *et al.,* 2000). The purified extracts are subjected to NMR and MS methods to characterise and identify the components. The characterisation and identification of the components requires to know the most appropriate technique for the sample to suggest a reasonable structure. It would also require knowledge of the available literature and databases which the suggested structure can be compared with them (Queiroz *et al.,* 2002).

As a spectroscopic method, nuclear magnetic resonance (NMR) has seen spectacular growth, both as a technique and in its applications. Today's applications of NMR span a wide range of scientific disciplines, from physics to biology to medicine. Since the development of higher resolution NMR spectrometers, NMR spectra have been a major tool for the study of both newly synthesised and natural products isolated from plants, bacteria, etc. The introduction of reliable superconducting magnets combined with newly developed, highly sophisticated pulse techniques provided the chemist with methods suitable to determine the 3-dimensional structure of very large molecules in solution. For the last decade, NMR methods have been introduced for quantitative analysis in order to determine the impurity profile of a drug, to characterise the composition of drug products, and to investigate metabolites of drugs in body fluids (Holzgrabe, Diehl, & Wawer, 1998).

NMR spectroscopy is routinely used by chemists to study the chemical structure of simple molecules using simple one dimensional techniques (1D-NMR). Two-dimensional techniques (2D-NMR) are used to determine the structure of more complicated molecules. (Krystyna *et al.*, 2010). The nuclei of many elemental isotopes have a characteristic spin, the common isotopes used in organic chemistry is ^{1}H and ^{13}C. The NMR behaviour of ^{1}H and ^{13}C nuclei has been exploited by organic chemist since they provide valuable information that can be used to conclude the structure of organic compounds. 1D proton spectra (^{1}H NMR) are far too complex for interpretation when analysing plant products, as most of the signals overlap heavily. By the introduction of additional spectral dimensions these spectra are simplified and extra information is obtained, the invention of 2D NMR was the major leap in NMR spectroscopy. Types of 2D NMR experiments include Correlation Spectroscopy (COSY), Total Correlation Spectroscopy (TOCSY), Nuclear Overhauser Effect Spectroscopy (NOESY), Rotating Frame Nuclear Overhauser Effect Spectroscopy (ROESY), Heteronuclear Single-quantum Correlation Spectroscopy (HSQC) and Heteronuclear Multiple-bond Correlation Spectroscopy (HMBC) (Timothy, 2009).

In phytochemical and small molecules research work, mass spectrometry plays a very important role. Generally, MS provides information related to molecular mass, molecular formula, and fragmentation pattern. While NMR gives useful data to identify the molecular structure of target compounds such as the number and types of protons in the molecule of interest. The protons in a molecule appear as an NMR signal and this provides information about the proton environment and coupling partners. Nonetheless, this information needs an accurate interpretation before the final structure can be concluded that can be achieved through various automated structure elucidation software, which have been developed to simplify the structure interpretation process (Stevenson, 2004; Queiroz *et al.*, 2002).

2.5 Medicinal plants used in the study

2.5.1 *Centella asiatica* (L.)

2.5.1.1 Description of the plant

Centella asiatica L. (Synonym: *Hydrocotyle asiatica* L.), is a plant belonging to Mackinlayaceae family. It grows well in both tropical and sub-tropical countries including India, Sri Lanka, Northern Australia, Indonesia, Iran, Malaysia, China, and other parts of Asia. Being

herbaceous, it contains thin long stems, creeping stolons, green to reddish green in colour, interconnecting one plant to another. It has 1-5 small fan-shaped green leaves per node with long stalks (Figure 2.14). Each plant has 3-6 small flowers which are purple to white-green in colour and arranged in umbels (flower cluster) arising from the axils of the leaves. The plant is a popular herb, it has been exploited for several hundred years by traditional medicine dealers, and it consumed fresh, or processed into tea or juice (Rahman, Sayeed, & Haque, 2012).

Figure 2.14: Aerial parts of *Centella asiatica* (L.) (Harry, 2005)

2.5.1.2 Pharmacological properties of *Centella asiatica* (L.)

There have been a number of studies exploring the pharmacological properties of *Centella asiatica* L. and to exploit its effects. The results from these studies confirmed the medicinal effects of this plant, and determined that this plant is a rich source of potentially active compounds. *C. asiatica* possesses a wide range of pharmacological properties; the plant has a good effect in ulcer prevention, it has also been used for improvement of wound healing, mental disorders, antibacterial, antioxidant and anticancer purposes. The plant is found to improve the power of concentration, general ability and behaviour of mentally retardation in children, and to treat rheumatic disorders. asiaticoside is one of the prime triterpene saponins found in leaves in large amount and is utilised commercially as a wound healing agent due to its potent anti-inflammatory effect and showed the potential use as an anti -gastric ulcers drugs (Seevaratnam *et al.*, 2012). A very recent study carried out by Rishikesh (2013) demonstrated thrombolytic

activity of *C. asiatica* leaves. In addition to above mentioned activities, *C. asiatica* were also claimed to be effectively applied for anti bilharzial, antifertility, immunomodulatory and antagonizing liver fibrosis.

Within antimicrobial studies, *C. asiatica* showed a significant effect as an antibacterial agent. Rahman, Sayeed, & Haque, (2012) reported that the methanolic extract of the plant showed good activity against a wide range of microorganism. Arumugam *et al.*, (2011) have found that the methanol, acetone, chloroform and water extracts of leaf have a significant antibacterial activity against *Bacillus cereus*, *Escherichia coli*, *Staphylococcus aureus* and *Pseudomonas aeruginosa*. However, methanol extracts of leaf and callus showed maximum inhibitory effects. In addition, there are many studies carried out by a number of researchers (College & Naka, 2009; Dash *et al.*, 2011; Vadlapudi, 2012; Udoh *et al.*, 2012) which confirmed the significant antibacterial activity of the aqueous and alcoholic extracts of the plant. However, these studies were completed on crude extracts only. Despite a large number of studies reported over the past decades on the evaluation of biologically active components and their mechanisms of action, the outcome of these studies is still unsatisfactory, more scientific investigation is needed to explore the active compounds responsible for this activity, and their mechanism of actions inside the microorganism.

2.5.1.3 Chemical constituents isolated from *Centella asiatica* (L.)

According to a number of studies exploring *C. asiatica*, it has been reported to contain a vast number of compounds belonging to different chemical classes, chemical constituents of *C.asiatica* have a very important role in medical and pharmaceutical applications (Table 2.3). The triterpenes are a major component of *C.asiatica*, and are composed of many compounds including acetic acid, madecassic acid, asiaticosside, madecassoside, brahmoside, brahmic acid, brahminoside, thankiniside, isothankunisode, centelloside, madasiatic acid, centicacid, and cenellicacid. Among these triterpenes, the most important biologically active compounds are asiatic acid, madecassic acid, asiaticosside and madecassoside. In addition to terpenoids, *C. asiatica* also contains phenolics flavonoid components, such as quercetin, kaempherol, catechin, rutin, apigenin and naringin. It also has volatile oils such as caryophyllene, farnesol and elemene. *C.asiatica* is also a rich source of vitamin C, vitamin B1, vitamin B2, niacin, carotene and vitamin A. (Seevaratinam *et al.*, 2012).

Main groups	Constituents
Amino acids	Alanine and serine (major components), aminobutyrate, aspartate, glutamate, histidine, lysine, threonine, arginine, leucine, iso-leucine, valine, methionine, tyrosine, phenylalanine, proline, cystine, glycine.
Carbohydrates	Glucose, mesoinositol, centellose, pectin, arabinogalactan
Phenols	*Flavonoids:* Kaempferol, kaempferol-3-o-β-d-glucuronide, castilliferol, quercetin, quercetin-3-o-β-d-glucuronide, castillicetin, apigenin, rutin, luteolin, naringin
	Phenylpropanoids: Rosmarinic acid, chlorogenic acid, 3,4-di-*o*-caffeoyl quinic acid 1,5-di-*o*-caffeoyl quinic acid, 3,5-di-*o*-caffeoyl quinic acid, 4,5-di-*o*-caffeoyl quinic acid, isochlorogenic acid *Tannin:* Tannin, phlobatannin
Terpenoids	Triterpenes, asiaticoside, centelloside, madecassoside, brahmoside, brahminoside (saponin glycosides), asiaticentoic acid, centellic acid, centoic acid, madecassic acid, terminolic acid and betulic acid.
Volatile oils and fatty oils	*Various terpenoids:* β-caryophyllene, trans β-farnesene and germacrene D (sesquiterpenes), α-pinene and β-pinene. *Fatty acids:* linoleic acid, linolenic acid, lignocene, oleic acid, palmitic acid, stearic acid.
Vitamins	Ascorbic acid, nicotinic acid, β-carotene
Mineral	Calcium, phosphorus, iron, potassium, magnesium, manganese, zinc, sodium, copper
Other constituents	Hydrocotylin (an alkaloid), vallerine (a bitter principle), phytosterols (e.g. campesterol, sitosterol, stigmasterol), resin. ~14 different polyacetylenes (8-acetoxycentellynol, cadiyenol, dotriacont-8-en-1-oic acid, 11-oxoheneicosanyl cyclohexane).

Table 2.3: Chemical constituents of *C. asiatica.* Modified from (Roy, Barman &Shaik, 2013)

2.5.2 *Imperata cylindrica* (L.)

2.5.2.1 Description of the plant

Imperata cylindrica L. is popular weed worldwide, the common name is cogon grass in English, the plant belongs to the Poaceae (Gramineae) grass family. It is a perennial grass that thrives around areas, it is distributed primarily in Southeast Asia and throughout the tropical and subtropical regions including Africa, Australia, and the Pacific Islands. *Imperata cylindrica* L. vary considerably in appearance, the leaves appear light green, with older leaves becoming orange-brown in colour. The leaves originate directly from ground level and range from one to four feet in length with 1/2 to 3/4 of an inch width (Figure 2.15). (MacDonald, 2004). *Imperata cylindrica* L. has been classified as one of the ten worst weeds in the world. This aggressive, rhizomatous plant is considered a very harmful plant due to its ability to spread and colonise

successfully, which leads it to compete with desirable vegetation and disturb ecosystems. It has also been found that even with its disappearance from certain place; other plants cannot grow where it has previously been grown (Omezine and Skiri-Harzalla, 2009).

Figure 2.15: Aerial parts of *Imperata cylindrica* L. in two different life stages. (John, 2013).

2.5.2.2 Pharmacological properties of *Imperata cylindrica* (L.)

The leaves and rhizome of *I. cylindrica* have traditionally been used for treating a wide range of illnesses, or as herbal supplements for health promotion. It has some wound healing properties and it is used for the treatment of nose bleeds. It is also an important drug in folk medicine for the treatment of urinary stones, retention of urine, diabetes, cardiac disorders, gout, common cough and anaemia. *I.cylindrica* reported to has an antibacterial effect against different bacterial species. It also has some activity in conditions like, arthritis, diarrhea and anthelmintic (used to treat parasitic worms). (Parvathy *et al.*, 2012). Previous studies have clearly revealed that a crude extract of *I. cylindrica* has a wide range of pharmacological effects; one of the most important benefit is the potential antimicrobial activity. A recent study conducted by Balangcod *et al.* (2012) demonstrated good activity of a methanolic extract of *I. cylindrica* against four different pathogenic bacteria, these were *Escherichia coli, Staphylococcus aureus,*

Pseudomonas aeruginosa and Salmonella typhimurium. Another study carried out by Voukeng *et al.,* (2012) showed that the methanol extract of *I. cylindrica* has a significant effect against Gram-negative multi-drug resistant bacteria. A further recent study conducted by Parkavi *et al.,* (2012) revealed a good inhibition effect of aqueous and ethanolic extracts against *Escherichia coli* and *Staphylococcus aureus*.

2.5.2.3 Chemical constituents isolated from *Imperata cylindrica* (L.)

Imperata cylindrica is a rich source of medicinal bio-active compounds. The preliminary phytochemical screening study, which was carried out by Parvathy and his co-worker (2012) confirmed the presence of Alkaloids, Carbohydrates, Flavonoids, Glycosides, Saponins, Steroids and Tannins. It has been suggested that phenolic compounds are the major constituents of *I. cylindrica*. A very recent study about the phenolic compounds from *I. cylindrica* revealed the presence of 7-hydroxy-4-methoxy-5-methylcoumarin, 5,7 dihydroxy-8- methoxyflavone, 4-hydroxybenzaldehyde, 4-hydroxy-cinnamic acid, 3,4-dimethoxyphenyl-O-α-L-rhamnopyranosyl-(1→6)-β-D-glucopyranoside, graminone A, 7-O-β-D-glucopyranosyl- 4-methoxy-5-methylcoumarin, 6-hydroxy-5-methoxyflavone, 5-methoxyflavone, 4-hydroxy-cinnamic acid and impecyloside. The last four compounds were isolated from this plant for the first time in 2013 (Rong-hua *et al.,* 2013).

The presence of jaceidin, quercetagetin-3,5,6,3`-tetramethyl ether, tricin, 3,5-di-omethyl-kaempferol and b-sitosterol-3-O-b-D-glucopyranosy l-6´- tetradecanoate have been confirmed for the first time in the study conducted by Mohamed *et al.,* (2009). In addition, six new compounds, 1-(3, 4, 5-trimethoxyphenyl)-1, 2, 3-propanetriol, arundoin, impecylone, deacetylimpecyloside, seguinoside K 4-methylether and impecylenolide were isolated for the first time from *Imperata cylindrica* by a group of researcher in two separate studies (Liu *et al.,* 2012, Liu *et al.,* 2014).

2.5.3 *Morinda Citrifolia* (L.)

2.5.3.1 Description of the plant

Morinda citrifolia L. belongs to the Rubiaceae family, and commonly known as “noni” which is the Hawaiian and Tahitian island's name for its fruit. *M. citrifolia* is deemed to be the second most important herb plant in the Hawaiian Island. It is also known in different

communities with different names such mengkudu in Indonesia and Malaysia, apatot in Philippines and Indian Mulberry in India. The plant is a small evergreen tree or bush found in the Southeast Asian region and believed to have spread across Australia and Indo-Pacific region. Usually it grows in open coastal regions at sea level and in forest areas, up to about 1300 feet above sea level (Singh *et al.,* 2006). *M. citrifolia* usually less than 10 feet height but occasionally rise up to 20 feet. The leaves are large with a shiny green colour and generally occur in pairs. Leaves are deep veined, short stemmed and 8 inches or longer. The flower heads grow to become mature fruit, 3 to 4 inches in diameter. The fruit is initially green in colour, turns to yellowish-white, normally 4 to 6 inches in length with a surface covered in polygonal-shaped sections (Figure 2.16) (Elizabeth and Murugesan, 2012).

Figure 2.16: Aerial parts of *Morinda citrifolia* (L.) and its fruit (Arthur, 2011).

2.5.3.2 Pharmacological properties of *Morinda Citrifolia* (L.)

Morinda Citrifolia L. has a long history of use as a medicinal plant and is reported to have a broad range of therapeutic effects. Its root, leaves, stem, bark, flowers and fruits have all been used to treat a wide range of illness such as diabetes, diarrhea, hypertension, malaria, pain, and topical infections. The fruits are also eaten as a food in times of famine. There are a number of in vitro studies about the biological activities of crude extracts or fractions of *M. citrifolia*, such as antibacterial, antiviral, antifungal, analgesic, antioxidant and immune enhancing effects (Su *et al.,* 2005).

There are some studies carried out to determine the antimicrobial effect of crude extracts of *M. citrifolia*, Kumar and co-workers, (2010) have evaluated the petroleum ether and alcoholic extracts of *M. citrifolia* leaves for their antimicrobial activities. The alcoholic extract exhibited significant antibacterial and antifungal activity, comparable to the standard drug tetracycline. Rivera and co-workers (2011) have revealed that the *M. citrifolia* fruit juice has a noticeable effect on mycoplasmas clinical isolates and type strain. Previous studies have shown clearly that *M. citrifolia* contains some useful compounds, which may lead to develop a new antibacterial agent, and potentially with a new mechanism of action. More research has to be done to achieve that.

2.5.3.3 Chemical constituents isolated from *Morinda Citrifolia* (L.)

Morinda Citrifolia L. is considered a very rich source of chemical compounds with many biological activities. To date, approximately 200 phytochemicals have been identified and isolated from different parts of the plant. However, chemical composition differs broadly according to the part of the plant used in the studies. *M.citrifolia* contained a broad spectrum of secondary metabolites (Table 2.4), which include acids, alcohols, phenols, flavonoids, anthraquinone, glycosides, carotenoids, esters, iridoids, ketones, lactones, lignans, nucleosides, triterpenoids, sterols and several minor compounds. Anthraquinones considered the major phenolic compounds that have been identified and isolated from different parts of *M. citrifolia* (Singh, 2012).

Researches are trying to explore *M. citrifolia* unknown compounds and evaluate its potential activities. Deng *et al.*, (2007) have revealed for the first time two unknown compounds from this plant, (+)-3,4,3',4'-tetrahydroxy-9,7'α-epoxylignano-7α,9'-lactone and (+)-3,3'-bisdemethyltanegool along with seven known compounds. The structures of the new compounds were determined by spectroscopic techniques. While Takashima *et al.*, (2007) has isolated a new iridoid glycoside, citrifoside, and a new anthraquinone, 1, 5, 15-trimethylmorindol from the leaves of this plant, along with 24 other known compounds. The new isolated anthraquinone in this study has shown a significant cytotoxicity against selected pathogenic microorganisms.

Classes	Compounds	Occurrence
Anthraquinones	Morindine, rubiadine, lucidin-3-prineresal	Roots & fruit
Glycosides	Glycoside of coumarin, flavone and anthraquinones	Fruit
Essential oils	Volatile oil	Ripe fruit
Coumarone	Scopoletin	Fruit
Flavonol	Vomifoliol	Ripe fruit
Monoterpenes	Iridoid	Leaves
Sterol	Stigmpesterol, Stigmasterol, Sitosteryl palmitate, Isofucosteryl palmitate	Cell suspension culture
vitamins	Vitamin C	Dried fruit

Table 2.4: Examples of compounds isolated from *Morinda citrifolia* (L.).

2.5.4 *Sauropus androgynus* (L.)

2.5.4.1 Description of the plant

Sauropus androgynus L., also known as star gooseberry, katuk, or sweet leaf, is one of the most popular leafy vegetables. It is a small shrub belonging to Euphorbiaceous family (Figure 2.19). This plant is spread mostly in tropical regions, such as India, Sri Lanka, Thailand, Laos, Malaysia, and Indonesia, and almost in all countries of Southeast Asia. *S. androgynus* has upright, multiple stems and can grow up to three meter in height. The leaves are simple, 5-6 cm long, alternately arranged and lanceolate or oblong in shape. The upper surface of the leaf is dark green in colour while the lower surface is light green. The flowers are orange to red in colour, unisexual and arranged in axillary clusters. The fruits are rounded capsules with six distinct partitions (Figure 2.17) (Kaushik & Dhiman, 1999).

Figure 2.17: Aerial part of *Sauropus androgynus* (L.) and its fruit (Phuong, 2010)

2.5.4.2 Pharmacological properties of *Sauropus androgynus* (L.)

Sauropus androgynus L. has high nutritional and medicinal value; it is widely used in cooking and as a medicinal herb for curing of illnesses. *S. androgynus* claimed to be effective in the treatment of diabetics, cancer, inflammation, microbial infection, cholesterol and allergy. Different parts of this plant have different benefits, for instance, leaves and roots can be used to relieve fever and urinary problems, whereas the juice of *S. androgynus* leaves can serve as a remedy for earache. The overconsumption of this plant has been reported to cause drowsiness and constipation. The young tips and leaves are a common nutritious vegetable for cooking due to its high content of protein (Wei *et al.,* 2011; Senthamarai & Basker, 2012).

There have been a number of studies carried out by researchers to study the potential antimicrobial activity of *S. androgynus*. A study conducted by Ariharan *et al.,* (2013) showed that the methanol extract of *S. androgynus* leaves exhibited significant antibacterial activity against all bacterial strains in their study. Another experiment conducted by Gayathramma, Pavanik and Raji (2012) to evaluate the antibacterial activity of *S. androgynus* leaves, they tested the petroleum ether, methanol, ethanol, and water extracts on selected bacterial species like *Klebsiella pneumoniae*, *Bacillus cereus*, *Staphylococcus aureus*, *Escherichia coli* and *Pseudomonas aeruginosa*. The *in vitro* antibacterial activity revealed that the methanol and ethanol leaf extracts have a significant effect against pathogenic organisms tested.

2.5.4.3 Chemical constituents isolated from *Sauropus androgynus* (L.)

Sauropus androgynus L. is commonly used in folk medicine in different regions of the world due to its effectiveness in treating some diseases. There are few studies that have documented the chemical properties of this plant. *S. androgynus* claimed to have a high level of vitamin A, especially in freshly picked leaves, as well as high levels of vitamins B and C, protein and minerals. Nutrient content of the leaves is usually higher in more maturing leaves. A lignin diglycoside, (−) - isolariciresinol 3α-O-β- apifuranosyl- (1→2) – O - β-glucopyranoside, and a megastigmane glucoside, sauroposide and papaverine have isolated as well. This plant has also been reported to have resins, steroids, tannins, glycosides, reducing sugar, carbohydrates, saponins, sterols, terpenoids, acidic compounds, cardiac glycosides, phenols, flavonoids and considerable amount of alkaloids (Gayathramma, Pavanik and Raji, 2012). Senthamarai and Basker (2012), isolated 2(1H) naphthalenone,3,5,6,7,8,8a-hexahydro-4, 8a-dimethyl-6-(1-methylethenyl), azulene, pyrene hexadecahydro, squalene and phytol from *S. androgynus* leaves extract.

Chapter 3
Research Methodology

3.1 Materials

3.1.1 Plant Materials

This study used four medicinal plants, which are believed to have important antibacterial activities according to previous studies. There are *Centella asiatica* L., *Imperata cylindrica* L., *Morinda citrifolia* L. and *Sauropus androgynus* L. The dried leaves were obtained as a powder from PT Haldin Pacific Semesta, Jakarta, Indonesia. Product specification for each plant are shown in Appendices.

3.1.2 Chemicals

Unless otherwise stated, all chemicals used for the present study were of molecular biology grade, or spectroscopic grade. All chemicals were purchased from local suppliers, unless otherwise stated, such as Sigma-Aldrich, UK Ltd. (Gillingham, Dorset, UK), Fisher Scientific UK Ltd. (Loughborough, UK), GE Healthcare (Buckinghamshire, UK) and Oxoid Ltd. (Basingstoke, Hampshire, UK).

3.1.3 Equipments

All procedures were performed using the equipment available in the Kidscan laboratories of the Centre for Biochemistry, Drug Design and Cancer Research, University of Salford, unless otherwise stated, such as HPLC, NMR, 1D/2D-PAGE. For the biological screening, the equipment available in the Hygiene and Disinfection Centre, University of Huddersfield, were used, such as the Bioscreen-C Automated Growth Curve Analysis System for routine microbiology work, Whitley Automated Spiral Plater (WASP) for culturing bacteria on plates and Automated Colony Counting Methods (ACCM) for counting bacterial colonies.

3.1.4 Culture media

The following microbiological media were used throughout this study:

- Tryptone Soy Agar (TSA, Oxoid Ltd.), prepared by dissolving 40g in 1L of ddH_2O.
- Tryptone Soy Broth (TSB, Oxoid Ltd.), Prepared by dissolving 30g in 1L of ddH_2O.
- Maximum Recovery Diluent (Peptone Saline Diluent) (MRD, Oxoid Ltd.), prepared by dissolving 9.5g in 1L of ddH_2O.
- DE neutraliser broth (DE, LabM Ltd.), prepared by dissolving 9.5g in 1L of ddH_2O.

All microbiology investigations were carried out via a set of standard microbiological techniques. All cultures were incubated at 37ºC for 24 hours under aerobic conditions. MRD was used as an isotonic diluent for maximum recovery of microorganisms. The low concentration of peptone does not cause multiplication of the organisms within 1-2 hours of dilution of the sample. DE broth was used to neutralise the inhibitory action of our tested compounds and determine the Minimum Bactericidal Concentration (MBC). All media were sterilised by autoclaving (Portable Prestige Medical Autoclave 2100) at 121°C, with a pressure of 15 psi for 20 mins, heat labile components were sterilised via filtration through sterile Millex-GP syringe filter 0.22 μm (Sigma-Aldrich, UK Ltd.).

3.1.5 Bacterial strains

A wide range of bacterial strains were utilised in this study, these strains are outlined in table (3.1). All isolates were preserved on Microbank beads (Prolab Diagnostics Ltd.) in TSB media with 15 % glycerol and stored at -80°C until they were needed. These beads were revived on TSA media under standard conditions. Bacterial strains were kindly provided by Professor Howard Foster (School of Environment & Life Sciences, University of Salford), and Dr. Paul Humphries (Hygiene and Disinfection Centre, School of Applied Science, University of Huddersfield). These organisms are often used as a model for antibacterial susceptibility testing studies.

NO.	Strains	Reference
1	*Escherichia coli*	ATCC 25922
3	*Staphylococcus aureus (MSSA)*	ATCC 6538
4	*Staphylococcus aureus (MRSA)*	NCTC 13142
5	*Pseudomonas aeruginosa*	ATCC 15442
6	*Klebsiella pneumoniae*	NCTC 13438
8	*Enterococcus hirae*	ATCC 10541

Table 3.1: Bacterial strains used throughout this project.

- (NCTC) National Collection of Type Cultures
- (ATCC) American Type Culture Collection

3.2 Methods

3.2.1 Bacterial culture conditions and preparation of inoculums

Fresh cultures were prepared from frozen stocks every two weeks. Bacterial cultures were maintained in an appropriate medium and incubated aerobically at 37°C. A single colony of working microorganisms, grown overnight on TSA media, was inoculated into a 50 ml TSB media in Corning tubes (SANYO MIR-262 Incubator). The liquid cultures were then incubated in a desktop shaking incubator set at 37°C/200 rpm (NB-205L, N-BIOTEK Co., Ltd.) for 24 h.

The optical density (OD) of the culture can be used to measure the concentration of bacterial cells. Accordingly, the concentration of the cells in broth media was measured using plastic, disposable cuvettes at a wavelength of 600 nm with (Biochrom WPA Lightwave II UV/Visible Spectrophotometer). Stock suspensions were prepared by taking cultures from TSA stock plates and dispersing them in a sterile universal tube containing 10 ml of MRD and 5 g of glass beads by vortex mixer. The OD of the suspension was adjusted to be equivalent to a 0.5 McFarland standard at 600 nm in a spectrophotometer. This OD approximated to a 1-1.5 x 10^8 cfu/ml suspension. For monitoring the cells concentration throughout the work, several dilutions were made: 1-1.5 x 10^7, 10^6, 10^5 and 10^4 cfu/ml. Culture dilutions 1 x 10^5 and 10^4 were plated out on TSA Petri dishes via Whitley Automated Spiral Plater (WASP II Spiral Plater, Don Whitley, Scientific Ltd.), figure (3.1/1), the plates incubated overnight at 37ºC and cells count was done via Automatic Colony Counter (aCOLyte3, Synbiosis Ltd.) Figure (3.1/2).

Figure 3.1: (1) Whitley Automated Spiral Plater (WASP II Spiral Plater, Don Whitley, Scientific Ltd.), (2) Automatic Colony Counter (aCOLyte3 Colony Counter, Synbiosis Ltd.).

3.2.2 *In-vitro,* Antibacterial activity assays

The testing of potential antibiotic compounds carried out via two different techniques, the disc diffusion assay and the bioscreening technique. The disc diffusion assay is designed for rapid determination of antibacterial activity and as such is not particularly useful to generate an accurate research data. Nowadays, more sophisticated approaches for evaluating the antibacterial activity are available, on top of them, the revolutionary machine, the Bioscreen-C Automated Growth Curve Analysis System (Growth Curves Ltd., USA), (Figure 3.2).

Figure 3.2: Automated microbial growth curve analysis system (Bioscreen-C systems).

3.2.2.1 Disc diffusion assay

In the disc diffusion assay, Tryptone Soya agar (TSA) plates were incubated with freshly grown bacterial cultures of approximately 1 x 10^4 cfu/ml. Crude extracts were dissolved separately in 10 % DMSO, and all extracts were filtered through 0.22 µm syringe filter (Millex-GP). Then, 50 µl of each plant crude extract was applied to 6 mm sterile filter paper discs (Whatman No.1, Sigma Aldrich, UK) at a concentration of 500, 250, 125 µg/ml, and dried in sterilised conditions to evaporate the residual solvent and were later placed on the TSA plate. Discs with 10 % DMSO only used as a negative control, while chloramphenicol 30 µg/ml discs were used as a positive control for antibacterial activity assay. The experiment was done in triplicate and plates were incubated for 24 h at 37°C. The inhibitory activity of the compounds was determined by comparing the average sizes of inhibition zones (mm), including disc diameter, of the different extracts with those of the controls.

3.2.2.2 MIC Calculation using Bioscreen-C system

The Bioscreen system provides a tightly controlled environment for monitoring the growth of bacteria by measuring turbidity of the liquid growth medium in the 100 well honey comb microtiter plates at specific temperature with or without shaking. These measurements were done by a real-time recording of the optical density (OD) of the cultures under investigation. The measurement interval can be set from minutes to hours. The filter chosen was a wide band filter 420-580 nm. The Bioscreen technology is highly efficient, which can create up to 200 growth curves from 200 samples in one run, a number of modelling approaches have been developed to transform Bioscreen-C data into a key results, such as minimum inhibitory concentration (MIC) and minimum biocidal concentration (MBC) (Dalgaard & Koutsoumanis, 2001; Lambert & Lambert, 2003). The MIC may be defined as the lowest concentration of tested compound, which is required to inhibit the growth of a microbe *in vitro*. Bioscreen-C technology employs a 100 well microtiter plate, with two plates being utilised per run (Figure 3.3), allowing up to 200 samples to run simultaneously. To determine the MIC of our compounds, eight serial dilutions were created vertically (500-3.9 μg/ml), down the plate, using drugs dissolved in 10 % DMSO, TSB media was used throughout this procedure. 50 μl of a 1 x 10^4 cfu/ml suspension of the tested bacteria were added to all wells. The plates were incubated for 24 h at 37°C with a continuous shaking, and optical density measurements were carried out every 60 minutes at a wavelength of 420-580 nm. Data obtained is in the form of an array of OD readings per well per 60-minute intervals. Data was transferred to Microsoft Excel to convert it to growth curves.

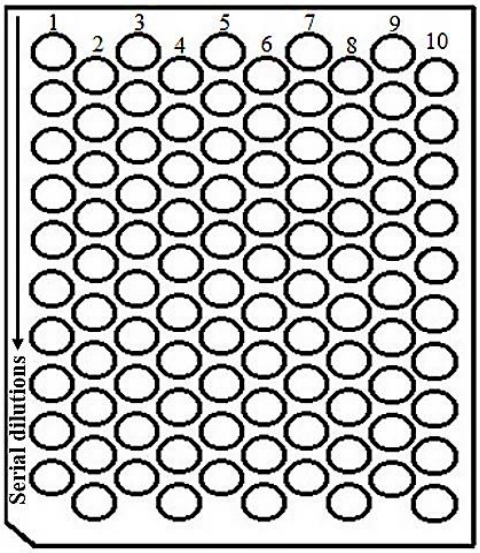


Figure 3.3: Schematic diagram of a 100 well microtiter plate used in Bioscreen-C machine.

The data obtained from the Bioscreen-C machine used to calculate the MIC of our compounds, an adapted approach described by Lambert (2001) was employed. Firstly, the area under the growth curve (Figure 3.4) was determined using the trapezoidal formula rule in Microsoft Excel. Comparison of the test curve with that of the positive and negative controls allows the calculation of a fractional area (*fa*) (Equation 3.1). Once a range of fractional areas have been calculated, data can be plotted against the log concentration (Figure 3.5) to allow analysis via a modification of the Gompertz function in Excel (Equation 3.2). This function was fitted to the data using the solver function in Excel which allowed the MIC values to be calculated with the best fit to the experimental data from the constants M and B (Equation 3.3).

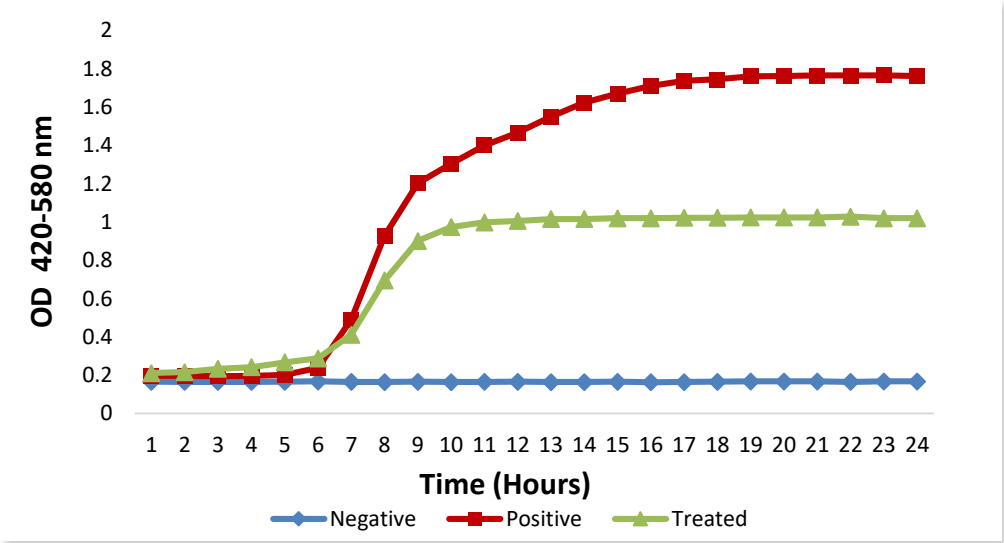


Figure 3.4: A typical Bioscreen growth curve (OD vs. Time).

Equation 3.1: $$fa = \left(\frac{Area_T - Area_{NC}}{Area_{PC} - Area_{NC}}\right)$$

$Area_t$ = Area under the test curve
$Area_{nc}$ = Area under the negative control
$Area_{pc}$ = Area under the positive control

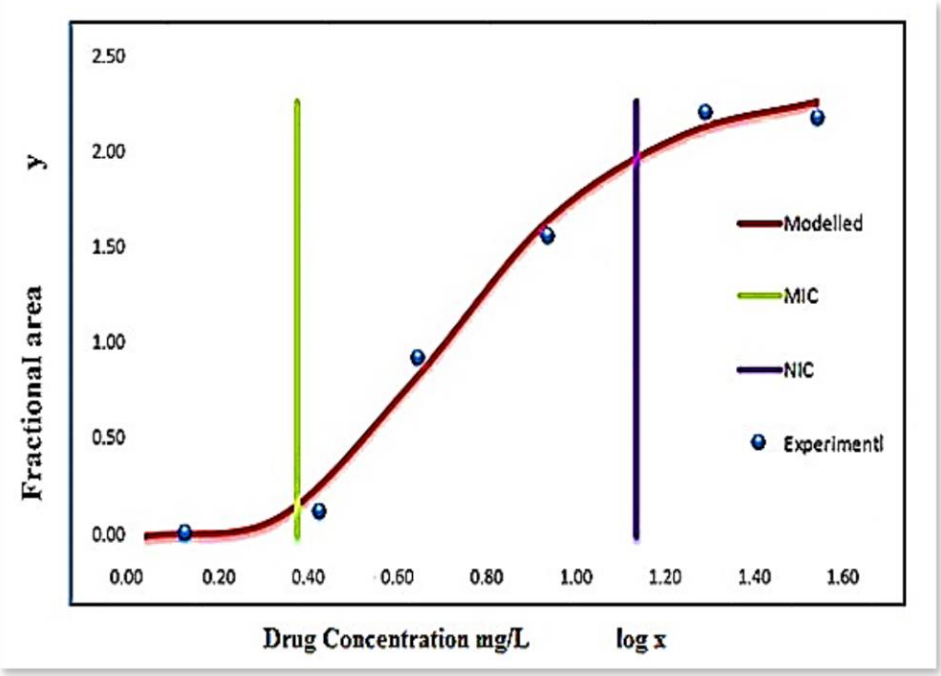


Figure 3.5: A typical profile of fractional area *fa* vs. drug concentration

Equation 3.2: $y = A + Ce^{-e^{B(x-M)}}$

A= Lower asymptote of y

C = distance between upper and lower asymptote

B= Slope parameter

X= Biocides concentration

M= Log Concentration of the inflexion point

Equation 3.3: $MIC = 10^{(M+\frac{1}{B})}$

B= Slope function

M= the log concentration at the inflexion point

3.2.2.3 MBC Calculation of tested molecules

The minimum biocidal concentration (MBC) test determines the lowest concentration at which an antimicrobial agent will kill a particular microorganism. The MBC was determined via a series of steps undertaken after the end of the Bioscreen-C run. To determine the MBC, the dilutions representing the MIC and at least two of the more concentrated tested product were plated on TSA plate and incubated to determine if there were any viable cells. Briefly, 100 µl of the cells suspension were transferred from each well to another Bioscreen-C plate containing 100 µl of DE neutralising broth (LabM Ltd.), after 30 min, 100 µl from each well was removed and plated out on a solidified TSA plate. Inoculums were spread out using disposable L-Shaped sterile spreader. After 24 h incubation under standard conditions, the colonies were counted, and the lowest concentration with no grown cells considered the MBC for that product.

3.2.3 Natural products techniques

3.2.3.1 Plant material extraction

Crude extracts of the dried powder of medicinal plants *Centella asiatica* (L.), *Imperata cylindrica* (L.), *Morinda citrifolia* (L.) and *Sauropus androgynus* (L.) were obtained by maceration and Soxhlet extraction techniques. Successive extractions were carried out using a modified procedure described by Kisangau *et al.,* (2007). Briefly, Soxhlet extraction was carried out by placing plant material in a thimble which was then inserted inside the Soxhlet apparatus and exposed to four different absolute solvents of different polarities (Methanol, Ethanol, Chloroform and Hexane), the ratio of sample to solvent was 1:10 (w/v). The extraction continued until the solvent appeared clear in the upper extraction chamber (Figure 3.6). For maceration, plant material macerated with four different solvents (methanol, ethanol, acetone and chloroform), solvents were changed daily and the collected solvent stored in closed containers in the fridge, this process continued until the solvent appeared clear. Extracts obtained from both procedures were then filtered twice through a Whatman No.1 under vacuum pressure. Extracts were concentrated by vacuum rotary evaporator (IKA RV 10 digital rotary evaporator, Germany), and then extracts freeze dried to completely eradicate solvents. The crude extracts were stored in glass containers and kept in the freezer at -20°C until further analyses.

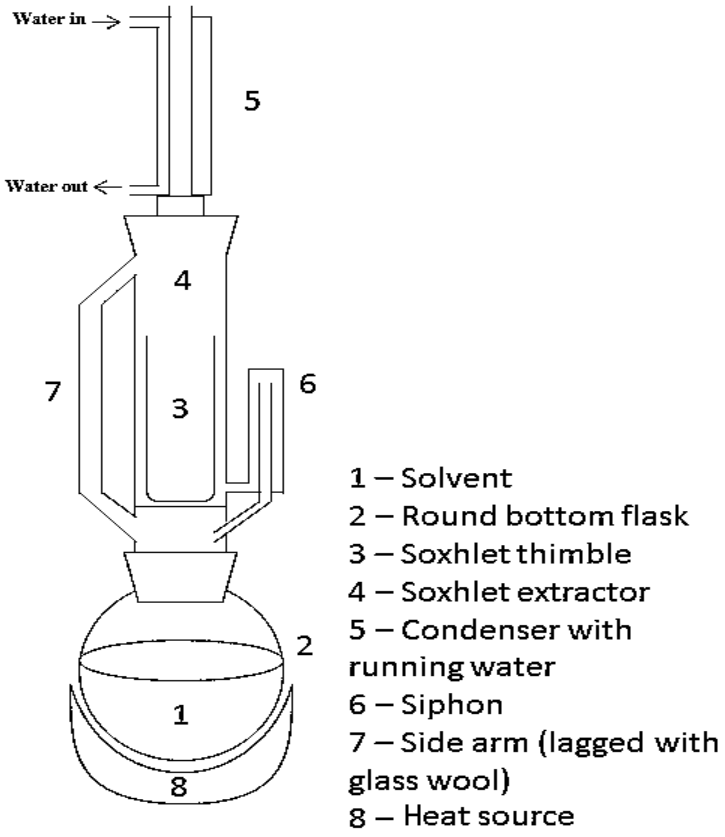


Figure 3.6: Schematic diagram of Soxhlet extraction apparatus

3.2.3.2 Isolation and purification of natural products

Isolation and purification of the active compounds were conducted according to the guidance of the bioassay-guided isolation protocol. Briefly, the active extracts were separated by high performance liquid chromatography (HPLC), followed by bioassay test to determine which fractions were most likely to contain the active substances. Finally, active fractions were analysed by spectrometry techniques to identify their molecular structures. All crude extracts obtained from the previous processes were separated further by (HPLC). High performance liquid chromatography is an efficient technique for the isolation and purification of natural products, due to its ability to separate complex mixtures of compounds in a short time with a high purity. In our study, the reverse phase high performance liquid chromatography (RP-HPLC) was applied. For the preparative reverse phase RP-HPLC analysis, an ACE reverse phase column (5 µm, C18, 100Å, 250 x 21.2 mm, Hichrom Ltd., UK) was used. Analytical RP-

HPLC work was carried out with Kinetex reverse phase column (5µm, C18, 100Å, LC Column 250 x 4.6 mm, Phenomenex, UK).

Analyses were performed using a Gilson 307 HPLC pump with Varian Prostar 320 UV-VIS HPLC Detector. Rheodyne 7725i injection valve was used to deliver the sample to the system, and was fitted with a 20 µl and 500 µl sample loop for analytical and preparative HPLC respectively. The sample concentration was 10 mg/ml for analytical and 100 mg/ml for preparative HPLC. The output signal was monitored and processed using PicoLog data acquisition software (Pico technology Ltd., Cambridge, UK). Both analytical and preparative RP-HPLC methods were optimised for each extract. Analytical HPLC provides a general indication of the compounds in the crude extracts and monitors the purity of the compounds. Whilst preparative HPLC is used to separate large amounts of crude extracts. The peaks eluted from the preparative column were collected manually.

More than one approach was employed in order to develop a mobile phase system for each plant extract, and to achieve that, isocratic solvent systems were used. The mobile phase which gave a consistent good separation, at room temperature conditions (25 ±2ºC), consisted of 15 % (v/v) HPLC grade methanol with 0.1 % (v/v) trifluoroacetic acid (TFA) in water. Solvents were filtered through a 0.45µm filter (Millipore, Bedford, MA, USA) and degassed in an ultrasonic bath prior to use. Samples were dissolved in the mobile phase solvent and filtered with a 0.22µm syringe filter, and then injected to the column. The flow rate was set at 1.0 ml/min for analytical column and 10 ml/min for the preparative column, and 260nm was the best wavelength that showed a good detection.

3.2.3.3 Structure elucidation of active compounds

Structure elucidation and identification is a crucial part of natural products discovery. Spectroscopic methods, such as nuclear magnetic resonance NMR (tell us about the environments of the various elements in a molecule) and mass spectrometry MS (gives the molecular weight and formula, both of the molecule itself and various structural units within it), have become indispensable tools in in this field. Development of computerised techniques has permitted utilisation of these methods for acquisition of spectra and greatly increased the capabilities of these methods (Kind & Fiehn, 2010).

3.2.3.3.1 Nuclear magnetic resonance (NMR) spectroscopy

The measurement of 1D/2D NMR spectra was carried out at Salford Analytical Services centre (SAS), (University of Salford, Manchester, UK). Proton (^{1}H) and carbon (^{13}C) NMR spectra were recorded on Bruker Ultra-shield Avance AMX 400 MHz spectrometer. Samples were dissolved in deuterated DMSO (DMSO-d_6) for NMR measurement. Samples were filtered through a cotton wool plugged into Pasteur pipette and then placed in Wilmad NMR tubes (5 mm diam., precision, 500 MHz, Sigma-Aldrich, UK). More sophisticated NMR tube was used as well (Shigemi 5mm Symmetrical NMR Microtube assembly matched with DMSO-*d6*, Shigemi INC., USA). Solvent peaks were used as an internal standard and all resonance assigned were relative to them. Spectra of pure compounds were processed using TopSpin software (v 3.5) under automation controlled by "iconnmr". Structural assignment was based on spectra resulting from The 1D ^{1}H and ^{13}C NMR experiments. In addition, homo and heteronuclear 2D NMR experiments were also acquired.

3.2.3.3.2 Purity check of the active compounds

Nowadays, softwares specifically created for analysing primarily 1D quantitative NMR spectra (^{1}H and ^{13}C) have emerged, in addition to analysis software provided by the respective NMR instrument manufacturers. For the purity determination of the bio-active compounds isolated in this study, q^{1}H NMR was employed by using Mnova NMR software (v 10.0.2) for windows, (Mestrelab Research, Santiago de Compostela, Spain). This software has been developed in the last few years, and it is a multivendor, multiplatform software for visualisation, processing, analysing and reporting of 1D and 2D-NMR data, it has designed to support the specific needs of researchers (Mestrelab, 2015). qNMR plugin has been used, which can interact with other Mnova plugins to offer the user access to a very advanced functionality with an accuracy, which is needed for the purity check of natural products. The qNMR process was done by using the automatic functions available in the software, (phase and baseline correction), whilst the solvent peaks were used as an internal reference. The raw data is preserved in the background to allow more detailed processing.

3.2.3.3.3 Mass Spectroscopy (MS) of the active compounds

Samples of the active small molecules were sent to the Intertek pharmaceutical services laboratories (Manchester, UK) for accurate mass analysis. Waters ZQ detector integrated with

Waters 2695 Alliance HPLC system (Waters Corp, Milford, MA) was used for the experiment. Prior to the injection into the HPLC system, the sample was dissolved in 50 % (v/v) acetonitrile in water, and the volume injected was 10 µl of approximately 1 mg/ml. The eluent was 9:1 ACN/water at 0.15 ml/min and 2 second scans were acquired over the range 100 to 1500 *m/z* in positive and negative ion modes. Subsequently, samples were introduced into an electrospray (ES) ionization source to ionize the molecules at atmospheric pressure. The ions were separated according to their mass-to-charge ratio. The separated ions were detected and then the signals were amplified. Collected spectra sent to the Waters Empower software for further MS analysis.

3.2.4 Proteomics analysis

3.2.4.1 Bacterial total protein extraction

A starter culture of 10 ml TSB media was inoculated with a single colony from TSA plate and grown overnight under standard conditions. 50 µl of a 1 x 10^4 cfu/ml suspension of the tested bacteria were used to inoculate 10 ml of TSB media, after 8 hours of standard incubation conditions (log phase), the tubes were inoculated with sub-inhibitory dose of our tested compounds for 2.5 h, to give the cells a chance to respond to the stress of the compound and continue to grow (Yun *et al.*, 2011). This allows the proteome profiling to pick up perturbations owing to compound response in the context of relatively normal cellular processes. A higher dose would have triggered massive disruptions in the cellular processes as the cells struggle to survive, which can potentially masked proteome changes directly related to antibiotic response. Protein extraction was done by using Minute^{TM} Bacterial Total Protein Extraction Kit MBTPE, (Invent Biotechnologies, Inc. USA). The MBTPE kit is designed to rapidly extract denatured total proteins from cultured bacterial cells for different assays, such as SDS-PAGE, immunoblotting, ELISA and other applications.

Briefly, the protein extraction filter cartridge, with collection tube, was pre-chilled on ice for 30 min. Bacterial cells were harvested by centrifugation in a 2.0 ml microcentrifuge tube with a table-top microcentrifuge (Microcentrifuge Labnet Spectrafuge, 24D) at 13000 rpm/16000 x g for 2 min. The supernatant was removed and discarded. Then, the cell pellet washed in cold 1X Phosphate-buffered saline (PBS). The supernatant was again removed leaving a small amount of PBS (about equivalent volume of packed cells) in the tube. The cell pellet with the PBS was vortexed briefly to resuspend the cells, and 250 µl of bacterial lysis

buffer A added to the cell suspension then vortexed vigorously for 10 seconds, at room temperature. 25 µl of buffer B (1/10 of buffer A) was then added to the lysate and vortexed vigorously for 10 seconds. The suspensions were transferred into the pre-chilled filter cartridge, which was then centrifuged, in a microcentrifuge at 13,000 rpm/16,000 x g, for 1 min. The clear cell lysate in collection tube was ready to use and the total bacterial protein yielded with this method was about 2.0-2.5 mg/ml.

3.2.4.2 Protein concentration assay

Pierce™ BCA Protein Assay Kit (Thermo Fisher Scientific, UK) was used to determine the protein concentration of bacterial lysates. Briefly, a protein standard curve was created using Bovine Serum Albumin Standard (BSA) in the range of 25-2000 µg/ml. A working dye reagent was prepared by mixing 50 parts of BCA Reagent A (sodium carbonate, sodium bicarbonate, bicinchoninic acid and sodium tartrate in 0.1 M sodium hydroxide) with 1 part of BCA Reagent B (4 % cupric sulfate) (50/1, Reagent A/B). 25 µl of each standard or unknown sample was added into a 96 microtiter plate wells (working range = 20-2000 µg/ml). 200 µl of the working dye reagent was added to each well and the plate agitated thoroughly on a plate shaker for 30 seconds followed by incubation at 37°C for 30 min. Samples and standards were measured in three replicates. Plates were read at 562 nm using a Multiskan™ FC Microplate Reader (Thermo, UK). The absorbance values of each extract was converted into protein concentrations using the gradient of the BSA standard curve (Figure 3.7). All replicates values were averaged and a final concentration was recorded.

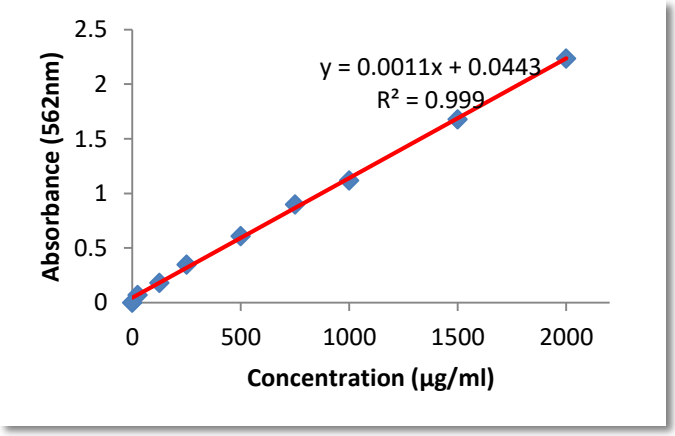


Figure 3.7: Standard curve for Bovine Serum Albumin (BSA) using the microtiter plate Protocol (37°C/30 minute incubation). Diluted BSA standards (25–2,000 µg/mL) were added to Pierce BCA Protein Assay working reagent, and absorbance (562nm) was measured. Data points and standard deviations were calculated from three replicates.

3.2.4.3 Two-dimensional gel electrophoresis (2D-PAGE)

Isoelectric focusing (IEF) (first dimension) was conducted using PROTEAN® IEF System (Bio-Rad, Hertfordshire, UK) with the Bio-Rad Focusing Tray which holds 1-12 Ready IPG strips for flexibility and streamline handling. The second dimension was performed on the Hoefer SE600 Standard Dual Cooled Vertical Unit (GE Healthcare).

3.2.4.3.1 Rehydration

Immobiline Dry Strip gels (IPG strips) with a linear separation range of pH 3-11(11 cm, GE Healthcare) was rehydrated overnight at room temperature in the Immobiline dry strip reswelling tray (Bio-Rad). 125 µl of the rehydration solution (Table 3.2) containing 150 µg protein was applied along the reservoir channel of the reswelling tray, then followed by the application of the IPG strip onto the sample. Strips were prevented from dehydration and oxidation by overlaying with a covering fluid (Immobiline DryStrip Cover Fluid, GE Healthcare).

Components	Final concentration	Volumes
Urea (M.W 60.06)	7 M	8.40 mg
Thiourea (M.W 76.12)	2 M	3.04 mg
CHAPS	2 %	400 mg
DDT	0.4 %	80 mg
IPG Buffer 3-10	0.5 %	100 µl
Bromophenol blue	0.002 %	50 µl of 1 % solution
Milli-Q water		add to final volume 20 ml

Table 3.2: Components of the rehydration solution (recipe for 20 ml final volume)

3.2.4.3.2 Isoelectric focusing (IEF)

The rehydrated IPG strips were placed in the PROTEAN® IEF strip holder. 8 µl of nanopure water was pipetted to the electrode wicks, to act as receptacles for salts and other non-amphoteric constituents of the sample. IPG strips were then covered with 2 ml of covering fluid and proteins were separated on the basis of their isoelectric point (pI). The IEF was carried out at 20°C with the PROTEAN® IEF system. The isoelectric focusing conditions were 150 V for 40 min, 300 V for 1 h, 450 V for 2 h, 750 V for 15 min, 2000 V for 15 min, reaching a total of 8000 V in 2 h (max 0.125 mA and 0.125 W per strip). After focusing, the second dimension was run immediately or the strips were stored at -80°C until required.

3.2.4.3.3 Preparation of SDS- polyacrylamide gels

Prior to preparation of the polyacrylamide gel solutions, the SE600 Standard Dual Cooled Vertical Unit (GE Healthcare) was assembled. The polyacrylamide gels used for the second dimension of 2D-PAGE contained 12 % acrylamide (1 mm thick). To make six gels, 187 ml Milli-Q water was added to 140 ml 40 % acrylamide/bis solution 37.5:1 (Bio-Rad) and 112.5 ml of 1.5 M Tris-HCl (pH 8.8) and stirred. To this mixture, 4.5 ml of 10 % SDS solution was added, with 5 ml of 10 % ammonium persulphate (APS), and 125 µl of N,N,N,N'-tetramethylethane-l,2-diamine (TEMED) to catalyse polymerisation. Each gel was overlaid with 4 ml of a Butanol- Milli-Q water mix solution (10:1) to obtain a uniform gel surface. The overlaid solution was removed after gels were polymerised at the room temperature (≃1 h). Gels were either used immediately or kept in the fridge for short-term storage (1-4 days) after wrapping it with wet paper towels.

3.2.4.3.4 IPG strips Equilibration

Frozen strips were allowed to thaw at room temperature for roughly 30 min. Prior to the separation in the second dimension, the IEF gels (IPGs) were equilibrated twice under mild shaking for 20 min in 10 ml equilibration buffer. First equilibration buffer consisted of 6 M urea, 75 mM Tris-HCl, pH 8.8, 30 % (v/v) glycerol, 2 % (w/v) SDS, 0.5 % (w/v) of DTT to reduce the side chains of cysteine amino acids, and a trace of bromophenol blue. Strips were then equilibrated in 10 ml of the same buffer for 20 minutes with 2.5 % (w/v) iodoacetamide (replacing the DTT) to alkylate the reduced cysteine side chains. This reduction and alkylation

prevented re-oxidation of the reduced proteins during electrophoresis. Excess of equilibration solution was removed by briefly dipping the IPG gels into deionised water.

3.2.4.3.5 Second Dimension gel electrophoresis

SE600 Standard Dual Cooled Vertical Unit (GE Healthcare) was filled with 1 x electrophoresis buffer (Prepared from 10 x electrophoresis buffer: Table 3.3). The casting cassettes (containing the polymerised gels) were washed with deionised water and put into the separation chamber of the apparatus. Equilibrated IPG strips were placed directly on top of 12 % polyacrylamide gel surface. 10 µl of protein marker (Molecular Weight Marker Rainbow, Sigma) dropped on a piece of filter paper (marker pad) and a dried marker pad were also placed onto the acrylamide gel surface next to the strips. Strips and marker pad were covered with an air bubble free heated agarose sealing solution (Table 3.4). Finally, the 2D gels were immersed in 1 x running buffer in the lower buffer chamber while the upper buffer chamber contained 2 x running buffer. Proteins were separated for 30 min at 25°C with 50 W per gel (PowerPac™ HC High-Current Power Supply, Bio-Rad), and afterwards with 200 W per gel until the bromophenol blue dye reached the bottom of the gel (usually 4-5 h).

Components	Amount
Tris	60.4 g
Glycine	288.4 g
SDS	20 g
Milli-Q water	up to 2 L

Table 3.3: Components of (10x) electrophoresis buffer

Components	Amount
Agarose molecular grade	125 g
Electrophoresis buffer (1x)	25 ml
Bromophenol blue	trace

Table 3.4: Components of agarose-sealing solution

3.2.4.3.6 Protein staining and Gel imaging

After proteins were separated, the gels were gently removed from the glass slab, transferred into a suitable container, and washed briefly with distilled water to remove the remaining buffer. Subsequently, gels were fixed in fixing solution (50:40:10, ddH_2O, methanol, acetic acid) for 1 h and then stained overnight with Coomassie Brilliant Blue R-250 stain (Sigma Aldrich). The staining steps were performed under mild agitation on an IKA® KS 260 orbital shaker (IKA®-WERKE, Germany). The gels were rinsed twice with distilled water and de-stained with 10 % (v/v) methanol for one hour before imaging. Gels stained with Coomassie blue were scanned using GS-800 calibrated densitometer (Bio-Rad) and analysed using a Quantity One® image analyser software.

3.2.4.4 Separation of proteins by 1D SDS-PAGE

Protein samples were solubilised using 2 x sample buffer (0.125 M Tris-HCl (pH 6.8), 4 % (w/v) SDS, 20 % (v/v) glycerol, 2 % (v/v) 2-mercaptoethanol, 0.05 % (w/v) bromophenol blue). Samples were heated at 95ºC for 10 min and allowed to cool to room temperature prior to loading. 10 µl of protein marker (Molecular Weight Marker Rainbow, Sigma) was used. Separation was performed using 12 % polyacrylamide resolving gels and 4 % stacking gel. SE600 Standard Dual Cooled Vertical Unit (GE Healthcare) was used at 200 v for approximately 5 hours. Gels were stained with 100 ml of Coomassie Brilliant Blue R-250 (BioRad) overnight, at room temperature, with continuous shaking. Then, gels were destained with destining solution overnight, at room temperature, again with continuous shaking. Finally, gels were scanned using GS-800 calibrated densitometer (Bio-Rad) and Quantity One image analyser software was used. After scanning, gels either used immediately for the Label-free protein quantitation, or stored in the fridge for a short term use.

3.2.4.5 Label-free protein quantitation and proteomics analysis

Label-free quantitative proteomics analysis is a proteomics platform based on the observation that the intensity of the MS spectrum is linearly proportional to the concentration of the ion being detected. Quantitative and qualitative proteomics analysis of the total bacterial protein of *Staphylococcus aureus* (MSSA) was performed in this study according to the protocol described by Liu *et al.* (2014), at the Biological Mass Spectrometry Facility (BioMicS), based in the Faculty of Science, University of Sheffield, UK. The isolated protein samples (section

3.2.4.1) were quantified and the concentrations were adjusted to obtain an equal amount of protein between all samples. After 1D SDS-PAGE being performed, bands were excised into a fresh siliconised Eppendorf tubes, with a fresh clean scalpel blade, inside the fume cabinet. Gel slices then covered with 200 µl of solution 1 (200 mM ammonium bicarbonate (ABC), 40 % acetonitrile (ACN)), and incubated at 37°C for 30 min. Solution then discarded, and the washing step repeated twice with a fresh solution. The last step was drying down gel slices in a vacuum concentrator (Vacufuge® plus Eppendorf, UK) for 30 min.

Subsequently, the samples were reduced using 200 µl of reduction buffer (100 mM dithiothreitol (DTT) and 50 mM ammonium bicarbonate ABC). Then, gel slices incubated at 56°C for 1 h. Later, gel slices were centrifuged in Eppendorf tube centrifuge at 13000 x g for 10 min and the supernatants were discarded. Next step was the alkylation, where 200 µl of alkylation buffer (55 mM iodoacetamide (IAA) and 50 mM ABC) was added to the gel slices and then incubated at 20°C for 30 min in dark. Gel slices later washed with 200 µl of Solution 2 (50 mM ammonium bicarbonate ABC) for 15 min at room temperature. Washing step repeated twice with a fresh solution. The last step was washing gel slices with 200 µl of Solution 3 (50 mM ammonium bicarbonate ABC, 50 % acetonitrile ACN) and incubate them for 15 min at 37°C. Then, gel slices centrifuged at 13000 x g for 10 min, the supernatants then discarded and the gel slices dried down in the vacuum concentrator for 1 h.

Proteolytic digestion was performed after trypsin solution was made, the solution consists of one vial of proteomics grade trypsin (TPCK 20 µg, Sigma, UK), 100 µl of 1 mM HCl, 900 µl of 40 mM ammonium bicarbonate (ABC), and 9 % acetonitrile (ACN). After ensuring the gel slices were covered by fluid at the bottom of the Eppendorf tube, it was Incubated overnight at 37°C. Following the trypsin digestion, gel slices were centrifuged at 13000 x g for 10 min, and the supernatant was recovered to a fresh siliconised Eppendorf tube. Then, gel slices incubated at 37°C for 15 min in 20 µl of 100 % acetonitrile (ACN). Subsequently, 50 µl of 5 % (v/v) formic Acid (FA) was added to the gel slices and incubated again at 37°C for 15 min. The last step was centrifuging the tube at 13000 x g for 10 min and the supernatant then collected. The gel slices were discarded then. The recovered supernatants were placed in a vacuum concentrator and the extracted peptides dried down overnight at 20°C. Tubes were stored at -20°C and it was ready for MS analysis.

3.2.4.6 LC-MS/MS Analysis of digested portions

Nanoscale LC separation was performed using a Dionex Ultimate 3000 uHPLC. Peptide mixtures were first injected onto a pre-column (PepMap 100, C18 2 cm x 75 µm I.D trap column, Thermo Fisher Scientific, UK), where the flow rate was 10 µl/min, and the mobile phase consisted of 0.1 % (v/v) Formic Acid (FA) and 2 % (v/v) Acetonitrile (ACN). Peptides were eluted from the pre-column to the analytical column (EasySpray 15 cm PepMap 100 C18, 2µm particle size, 100Å pore size 75µm I.D, Thermo Fisher Scientific, UK). Sample was separated over a 64 minutes using uHPLC run of increasing acetonitrile (ACN) 2.4-72 % and 0.1 % Formic Acid (FA). The flow rate set to 250 nl/min with a constant temperature of 35°C.

Mass spectrometry analysis of the complex peptide mixtures was performed using electron transfer dissociation (ETD) enabled Thermo Fisher-Scientific Orbitrap Elite, equipped with an Easyspray (ESI) source. Nanospray ionization was carried out at 1.8 KV, with the ion transfer capillary at 250 (C, and S-lens setting of 60 %. MS1 spectra were acquired at a resolving power of 60,000 with an automatic gain control (AGC) target value of 1 x 106 ions by the Orbitrap detector, with a range of 350-2000 m/z. Following MS1 analysis, the top 20 most abundant precursors were selected for MS2 data dependent analysis (DDA) using collision induced dissociation (CID). Charge state screening and monoisotopic precursor selection were enabled with precursor ions of single charge rejected, and a 30 second dynamic exclusion window setting was used after a single occurrence of an ion with a +/- 10 ppm tolerance.

The resulting spectra were searched, for protein identification, using Mascot (Matrix Science, UK) against the Swissprot database (Eubacteria taxonomic filter) and a decoy database. Progenesis QI for proteomics (Nonlinear Dynamics, Water, UK) software package (v 4.1) was used as proteomics data analysis search engine. Proteins required a minimum of two peptides with a 95 % confidence or above in order to be reported. Proteins were investigated to assess their cellular localisation, as well as their biological and molecular functions by the aid of PANTHER classification system (Huaiyu *et al.*,2015). Finally, a comprehensive Gene Ontology (G.O.) and enriched pathways analysis of the proteins functions was conducted by the aid of DAVID pathway analysis tool (Database for Annotation, Visualization and Integrated Discovery, v 6.7) (Huang *et al.,* 2009), given the set of differentially expressed proteins in treated and untreated bacterial groups. The data analysis workflow is summarised in figure (3.8).

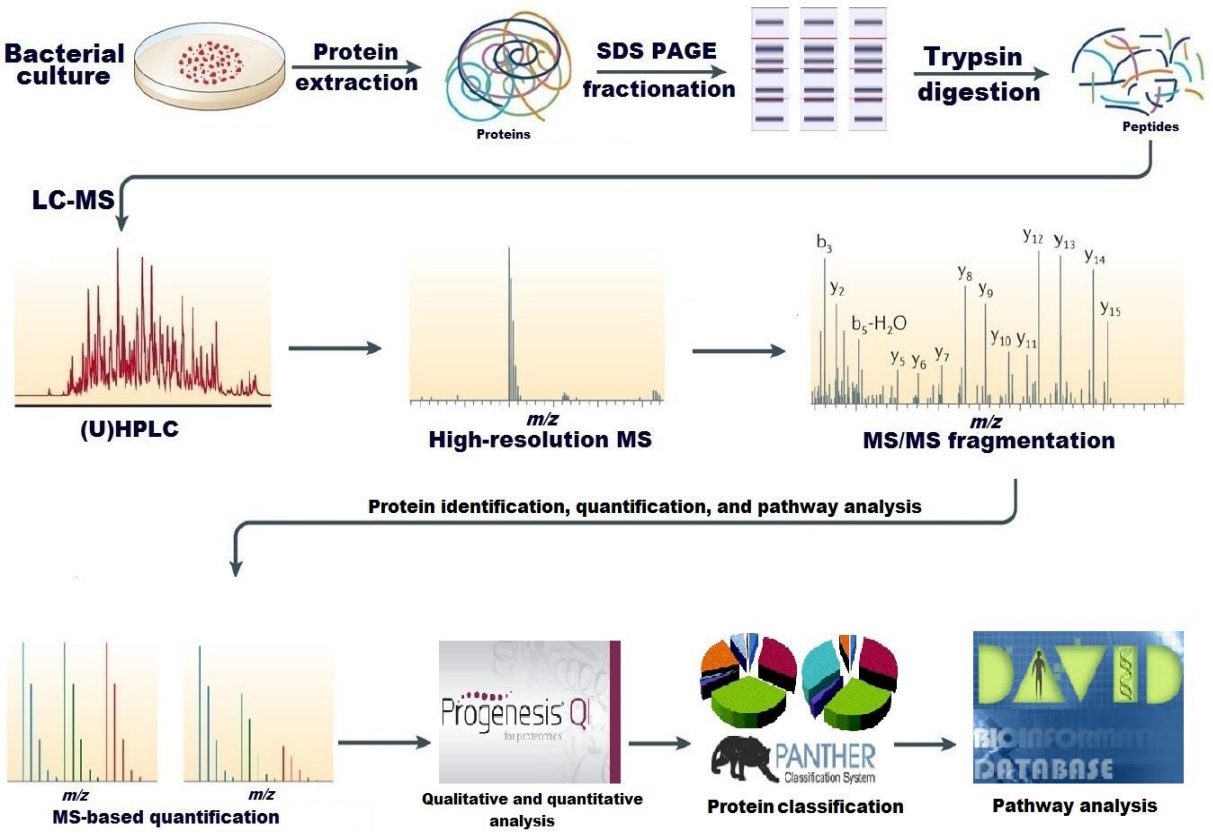


Figure 3.8: Experimental workflow for the sample preparation and data analysis.

3.2.5 statistical analysis

All statistics were performed using Minitab Statistical Software, version 17 (Minitab Inc., USA). Data were expressed as standard error of mean ± (SEM). Statistical analysis was performed by one-way analyses of variance (ANOVA) test with Duncan test. A p-value of <0.05 was considered statistically significant.

Chapter 4

Bioassay Guided Extraction

4.1 Crude extracts

4.1.1 Introduction

A thorough review of the current literature led us to choose the four plant species that were used in this study. However, critically, little is known regarding the actual structure and mechanism of action of the antibacterial compounds derived from these plants. Extraction, as the term is pharmaceutically used, can be defined as the technique used for separation of therapeutically active natural compounds and elimination of unwanted insoluble material by treatment with selective solvents. Screening the crude plant extracts for the desired bioactivity is among the most important operations in medicinal plant research, and extraction is the first crucial step of the process. Several approaches can be employed to extract (or release) the compounds from the biomass. Although water is used as an extractant in many traditional protocols, organic solvents of varying polarities are generally selected in modern methods of extraction to exploit the various solubilities of the plant constituents. This can be referred to as the solvents' ability to increase cell wall permeability, facilitating the efficient extraction of large amounts or polar and medium-to-low polarity constituents. The use of less-polar solvents (hexane and chloroform) is beneficial for the extraction of a group of relatively non-polar substances, i.e., lipids and terpenoids (Seidel, 2006).

There are several methods that have been used to extract potential antibacterial compounds from natural products. The conventional way of testing an antibacterial compound from natural products is to prepare the crude extract, which is thereafter purified, analysed and tested against bacterial strains. Solvent extraction procedures applied to plant natural products including maceration, percolation, Soxhlet apparatus, ultrasound assisted solvent extraction, microwave assisted extraction, and steam distillation. Soxhlet extraction is used widely in the extraction of plant metabolites, this method is adequate for both initial and bulk extraction. The main advantage of Soxhlet extraction is that it is a continuous process, as the solvent (saturated in solubilised metabolites) empties into the flask, the solvent is re-condensed again and extract more material. This makes Soxhlet extraction less time and solvent consuming than other methods. In addition, heating during the process induce the small molecules to be extracted from the biomass by disrupting the cell wall of the plant, diffuse into the cells, solubilise the intracellular metabolites, and diffuse out into the enriched extractant (Sarker *et al.,* 2006).

Many comparative studies have been carried out in order to assess the best method to be followed in the extraction of plant natural products and then use as antibacterial agents. Berka-Zougali *et al.*, (2012), and Kothari *et al.*, (2012) suggested in their comparative studies that the Soxhlet extraction method was the best amongst the other extraction techniques, in terms of high extraction efficiency and extraction of many phytochemical groups compared to other techniques. Soxhlet extracts from different medicinal plants have demonstrated the highest antibacterial activity against Gram positive and Gram negative bacteria. Current study employed two different extraction techniques to compare their efficiency in term of antibacterial activities. Soxhlet and maceration were the methods of choice.

4.1.2 Results

4.1.2.1 Plant material

The starting materials of *Centella asiatica* L., *Imperata cylindrica* L., *Morinda citrifolia* L., and *Sauropus androgynus* L. were obtained from air-dried healthy leaves, after grinding them into a fine powder. It is crucial in herbal medicine to pulverise plant materials to a fine powder in order to increase the surface area of the biomass to be exposed to the extraction solvents. The products were packed in an airtight container to maintain optimal conditions during shipping. The physical characteristics of the products were dry yellowish-brown to pale brown powders with different consistencies, and an approximate 6 % moisture was recorded before packing. Dried plant material can be used as a source for secondary plant components for several reasons. Differences in water content may affect the solubility of subsequent separation, and the secondary metabolic plant components should be relatively stable, especially if it is to be used as an antibacterial agent and that would not be achieved with the presences of water (Ncube *et al.*, 2008).

In compliance with the standard requirement for the production of herbal preparations, plant materials had been previously screened by the manufacturer for the presence of contaminants such as toxic metals and non-metals impurities and biological contaminants, which might affect the purity of the product or serve as hazardous contaminants. All the screening results were within the acceptable limits of herbal medicine products guidelines of the world health organisation (WHO, 2007). Comprehensive information of the plant material is available in Appendix 1.

4.1.2.2 Preliminary Plant extraction

In the preliminary plant extraction, solvents with different polarities were used; methanol, ethanol, chloroform and hexane in both maceration and Soxhlet extraction techniques and the protocol adapted from Kisangau *et al.* (2007) has been followed. These two techniques were used for obtaining the crude extracts of medicinal plants under investigation as described in section (3.2.3.1). The yields of crude extract % (w/w), from medicinal plant materials are illustrated in table (4.1) for Soxhlet extraction, and table (4.2) for maceration.

Solvents	Methanol	Ethanol	Chloroform	Hexane
Source of material	% Of extracts yield out of 50 grams from plant materials			
Cantella asiatica L.	8.7 %	8.1 %	3.4 %	1.1 %
Imperata cylindrica L.	9.3 %	7.2 %	2.7 %	0.9 %
Morinda citrifolia L.	10.1 %	9.8 %	2.4 %	0.7 %
Sauropus androgynus L.	10.2 %	9.6 %	3.1 %	1.3 %

Table 4.1: Total extracts produced by maceration extraction technique (w/w) %, yield = (weight of the extract / weight of plant material) × 100.

Solvents	Methanol	Ethanol	Chloroform	Hexane
Source of material	% Of extracts yield out of 50 grams from plant materials			
Cantella asiatica L.	14.1 %	12.9 %	3.1 %	1.6 %
Imperata cylindrica L.	11.3 %	10.7 %	3.2 %	1.4 %
Morinda citrifolia L.	12.1 %	11.1 %	2.9 %	0.9 %
Sauropus androgynus L.	11.9 %	8.3 %	2.4 %	1.5 %

Table 4.2: Total extracts produced by Soxhlet extraction technique (w/w) %, yield = (weight of the extract / weight of plant material) × 100.

In maceration extraction technique, methanolic extract of *Sauropus androgynus* L. recorded the highest yield amongst all extracts with 5.05 g, while hexane extract of *Morinda citrifolia* L. recorded the lowest yield amongst all extracts with 0.35 g. The highest yield from Soxhlet technique was recorded in methanolic extracts, while the lowest yield recorded in hexane extracts. Methanolic extract of *Cantella asiatica* L. recorded the highest yield amongst all extracts with 7.05 g, whilst hexane extract of *Morinda citrifolia* L. recorded the lowest yield amongst all Soxhlet extracts with 0.45 g. The highest yield in both techniques was recorded in methanolic extracts and the lowest yield was recorded in hexane extracts. The appearance of plant residues left after extraction were relatively similar to the starting materials in colour. The appearance of methanolic and ethanolic extracts were flaky, while the consistency of chloroform and hexane extracts were thicker and tend to be oily.

4.2 Antibacterial screening of crude extracts

4.2.1 Introduction

The first step towards validating the effectiveness of natural products as a potential antibacterial is the *in-vitro* antibacterial activity assay, in which many methods for susceptibility testing of bacteria have been used in the past. However, published susceptibility assessment reviews have shown that the findings could be slightly different if two methods being followed for the same sample (Seidel, 2006). The Minimum Inhibitory Concentration (MIC) test, is an important tool in the field of microbiology, it gives an idea of the microbial activity assessment. The MIC test tells us about the efficiency of the natural products under investigation, it helps to identify the most effective drug candidate from a number of natural product extracts. Thus, the results obtained from the MIC test can be used to monitor the preparation of a new antibacterial agent which has greater efficiency (Sarker *et al.*, 2006).

Disk diffusion assays followed by MIC determination by broth macro/micro dilution is widely used. The agar dilution method has also been proven and accepted to be an equally good technique as compared to broth dilution, and has been recommended as an alternative to broth macro/micro dilution. However, these methods are tedious and time consuming to implement as routine tests in many microbial laboratories. Therefore, this study utilised the Bioscreen-C system to evaluate the antibacterial activities, and to determine the MICs, of tested extracts following the disc diffusion assay. The Bioscreen-C system is an *in-vitro* machine for

quantitative antimicrobial susceptibility and MIC testing, and it has been validated for its high accuracy and ease of performance for routine microbiological use (Weiss *et al.,* 2004).

In this study, four medicinal plants (*Centella asiatica* L., *Imperata cylindrica* L., *Morinda citrifolia* L., and *Sauropus androgynus* L.) that have been reported in many studies for their antibacterial activities were investigated. These plants are widely distributed in tropical areas and have been used as natural remedies in folk medicine. As a result, they have drawn the attention of many scientists who interested in exploring the pharmacological properties of them. This section focuses on the screening of the activities of the crude extracts from these medicinal plants towards two Gram negative bacteria, *Escherichia coli* (ATCC 25922) and *Pseudomonas aeruginosa* (ATCC 15442), and two Gram positive bacteria, *Staphylococcus aureus* (ATCC 6538) and *Enterococcus hirae* (ATCC 10541).

4.2.2 Results

4.2.2.1 Antibacterial activity of crude extracts (Disc Diffusion Assay)

The potential antibacterial activity of the crude extracts was initially determined by the disc diffusion approach, then MICs were calculated from bioscreening results as described in chapter 3(section 3.2.2.1). Results are given in table (4.3) for *Centella asiatica* L., table (4.4) for *Imperata cylindrica* L., table (4.5) for *Morinda citrifolia* L., and table (4.6) for *Sauropus androgynus* L.

Microorganisms		***E. coli***			***S.aureus***			***P. aeruginosa***			***E.hirae***		
Conc. (µg/ml)		**500**	**250**	**125**	**500**	**250**	**125**	**500**	**250**	**125**	**500**	**250**	**125**
Maceration Extraction *Centella asiatica* (L.)	Methanol	**21.3^{b} ±0.57**	**19.6^{c} ±0.57**	**17^{d} ±1**	**22.6^{b} ±0.57**	**20^{c} ±1**	**18.3^{d} ±0.57**	**19^{b} ±0**	**17.6^{c} ±1.15**	**13^{e} ±1**	**21^{b} ±0**	**18^{c} ±0**	**12^{e} ±1**
	Ethanol	**19.6^{b} ±0.57**	**18^{c} ±1**	**13^{d} ±0**	**21.3^{b} ±1.15**	**17.3^{c} ±0.57**	**14^{d} ±0**	**18.3^{b} ±1.52**	**16^{c} ±1**	**14.3^{d} ±0.57**	**19.6^{b} ±0.57**	**15.3^{c} ±1.15**	**12^{e} ±0**
	Chloroform	**14^{b} ±1**	**13^{c} ±1.73**	**11.3^{d} ±0.57**	**16.3^{b} ±1.52**	**14.6^{c} ±0**	**9.6^{d} ±1.15**	**14.3^{b} ±0.57**	**12^{c} ±0**	**9.6^{d} ±0**	**12.6^{b} ±1.15**	**9.6^{c} ±0.57**	**7^{e} ±1.73**
	Hexane	**9.6^{c} ±1.52**	**8.6^{d} ±0.57**	**0^{e} ±0**	**10^{c} ±1**	**8^{d} ±0.57**	**0^{e} ±0**	**9^{c} ±1**	**0^{d} ±0**	**0^{d} ±0**	**10.3^{c} ±0.57**	**7^{d} ±0**	**0^{e} ±0**
Microorganisms		***E. coli***			***S.aureus***			***P. aeruginosa***			***E.hirae***		
Conc. (µg/ml)		**500**	**250**	**125**	**500**	**250**	**125**	**500**	**250**	**125**	**500**	**250**	**125**
Soxhlet Extraction *Centella asiatica* (L.)	Methanol	**23.3^{b} ±0.57**	**21.6^{c} ±0.57**	**16.3^{d} ±1.15**	**24^{a} ±1.73**	**21^{b} ±1**	**19.3^{c} ±1.52**	**22^{b} ±0**	**20.6^{c} ±1.15**	**17^{d} ±1**	**20.6^{b} ±0.57**	**18.6^{c} ±1.15**	**15.3^{d} ±0.57**
	Ethanol	**22.6^{b} ±0.57**	**18^{c} ±1**	**14.3^{d} ±0.57**	**22.6^{b} ±1.15**	**19^{c} ±0**	**15^{d} ±1**	**20^{b} ±0**	**17.3^{c} ±1.53**	**13.6^{d} ±1.15**	**21^{b} ±0**	**18.3^{c} ±0.57**	**13.6^{d} ±0.57**
	Chloroform	**16^{b} ±0**	**14.6^{c} ±1.15**	**12.6^{d} ±0.57**	**18^{b} ±1.73**	**16.6^{c} ±0.57**	**11^{e} ±0**	**14^{b} ±0**	**11.3^{c} ±1.15**	**8.3^{e} ±0.57**	**15^{b} ±1**	**12.3^{c} ±0.57**	**9.3^{e} ±0.57**
	Hexane	**10^{c} ±0**	**7.3^{d} ±0.57**	**0^{e} ±0**	**9.3^{c} ±1.52**	**0^{d} ±0**	**0^{d} ±0**	**9^{c} ±0**	**0^{d} ±0**	**0^{d} ±0**	**12.6^{b} ±0.57**	**9^{c} ±1**	**0^{d} ±0**
-Ve Control		No inhibition			No inhibition			No inhibition			No inhibition		
+Ve Control		27.3±0.57			24.3±0.57			25±0			22±1		

Table 4.3: Antibacterial activity (zone of inhibition) of methanol, ethanol, chloroform, and hexane crude extracts (maceration and Soxhlet) of *Centella asiatica* (L.) leaves against *E. coli, S. aureus, P. aeruginosa,* and *E. hirae* bacteria.

- Negative Control = 10 % DMSO and Positive control = Chloramphenicol 30 µg/ml.
- Values expressed as Mean ± standard error (SEM); n=3 in each group.
- Inhibition zones were measured in mm and disc diameter was included.
- Means in the same raw within groups not followed by the same superscript are significantly different; one-way ANOVA and Duncan's test were conducted ($P<0.05$).

Microorganisms		***E. coli***			***S.aureus***			***P. aeruginosa***			***E.hirae***		
Conc. (µg/ml)		**500**	**250**	**125**	**500**	**250**	**125**	**500**	**250**	**125**	**500**	**250**	**125**
Maceration Extraction *Imperata cylindrica* (L.)	Methanol	**16.3^{b} ±0.57**	**15.3^{c} ±0.57**	**11.6^{d} ±0.57**	**17.6^{b} ±0.57**	**17^{c} ±0**	**13.6^{d} ±1.15**	**17.6^{b} ±1.15**	**16.6^{c} ±0.57**	**15.3^{d} ±0.57**	**13^{b} ±1.73**	**12.6^{c} ±0.57**	**8^{e} ±1**
	Ethanol	**16^{b} ±0.57**	**14.6^{c} ±1.52**	**8.3^{d} ±0**	**16.3^{b} ±1.15**	**13^{c} ±0**	**9.3^{d} ±2.08**	**13.3^{b} ±0.57**	**12^{c} ±1**	**8.6^{d} ±0.57**	**12.6^{b} ±0.57**	**11.3^{c} ±0.57**	**10^{d} ±1**
	Chloroform	**11.6^{c} ±0.57**	**10.3^{d} ±0.57**	**8^{e} ±1.73**	**10.3^{c} ±0.57**	**10^{d} ±0**	**0^{e} ±0**	**11.3^{c} ±0.57**	**9.3^{d} ±1.15**	**7.6^{e} ±0.57**	**0^{b} ±0**	**0^{b} ±00**	**0^{b} ±0**
	Hexane	**8.3^{c} ±1.15**	**0^{d} ±0**	**0^{d} ±0**	**0^{b} ±0**	**0^{b} ±0**	**0^{b} ±0**	**9.6^{d} ±0.57**	**0^{e} ±0**	**0^{e} ±0**	**0^{b} ±0**	**0^{b} ±0**	**0^{b} ±0**
Microorganisms		***E. coli***			***S.aureus***			***P. aeruginosa***			***E.hirae***		
Conc. (µg/ml)		**500**	**250**	**125**	**500**	**250**	**125**	**500**	**250**	**125**	**500**	**250**	**125**
Soxhlet Extraction *Imperata cylindrica* (L.)	Methanol	**18.3^{b} ±0.57**	**15.6^{c} ±0.57**	**13.6^{d} ±0.57**	**19^{b} ±0**	**17.6^{c} ±0.57**	**13^{d} ±1**	**19 ^{b} ±0**	**17^{c} ±1.73**	**14.3^{d} ±0.57**	**15.6^{b} ±1.15**	**13^{c} ±0**	**9.3^{d} ±1.52**
	Ethanol	**16.3^{b} ±0.57**	**13.6^{c} ±0.57**	**10^{e} ±0**	**19.3^{b} ±0.57**	**17^{c} ±1**	**13.6^{d} ±1.15**	**16^{b} ±0**	**13.3^{c} ±0.57**	**9.6^{e} ±1.15**	**13^{b} ±1**	**10^{c} ±1**	**7.3^{e} ±0.57**
	Chloroform	**11.3^{c} ±1.15**	**9.3^{d} ±0.57**	**7.6^{e} ±0.57**	**11 ^{c} ±0**	**9.6^{d} ±0.57**	**0^{f} ±0**	**14^{b} ±0**	**11.6^{c} ±0.57**	**8.6^{e} ±1.52**	**8.6 ^{c} ±0.57**	**0^{d} ±0**	**0^{d} ±0**
	Hexane	**0^{b} ±0**	**0^{b} ±0**	**0^{b} ±0**	**0^{b} ±0**	**0^{b} ±0**	**0^{b} ±0**	**0^{c} ±0**	**0^{c} ±0**	**0^{c} ±0**	**0^{b} ±0**	**0^{b} ±1**	**0^{b} ±0**
-Ve Control		No inhibition			No inhibition			No inhibition			No inhibition		
+Ve Control		27.3±0.57			24.3±0.57			25±0			22±1		

Table 4.4: Antibacterial activity (zone of inhibition) of methanol, ethanol, chloroform, and hexane crude extracts (maceration and Soxhlet) of *Imperata cylindrica* (L.) leaves against *E. coli, S. aureus, P. aeruginosa,* and *E. hirae* bacteria.

- Negative Control = 10 % DMSO and Positive control = Chloramphenicol 30 µg/ml.
- Values expressed as Mean ± standard error (SEM); n=3 in each group.
- Inhibition zones were measured in mm and disc diameter was included.
- Means in the same raw within groups not followed by the same superscript are significantly different; one-way ANOVA and Duncan's test were conducted ($P<0.05$).

Microorganisms		***E. coli***			***S.aureus***			***P. aeruginosa***			***E.hirae***		
Conc. (µg/ml)		**500**	**250**	**125**	**500**	**250**	**125**	**500**	**250**	**125**	**500**	**250**	**125**
Maceration Extraction *Morinda Citrifolia* (L.)	Methanol	**24.6^{b} ±0.57**	**22.3^{c} ±0.57**	**20.6^{d} ±1.52**	**25.3^{a} ±0.57**	**23.6^{b} ±1.15**	**21^{c} ±1**	**21^{b} ±0**	**19^{c} ±1.15**	**14.6^{d} ±0.57**	**20^{b} ±0**	**16.3^{c} ±0.57**	**13^{d} ±1.73**
	Ethanol	**22.6^{b} ±0.57**	**20.3^{c} ±0.57**	**16.6^{d} ±0.57**	**23.3^{b} ±1.15**	**22.3^{c} ±0.57**	**21.6^{d} ±1.15**	**19^{b} ±1**	**16.3^{c} ±0.57**	**13.3^{d} ±0.57**	**18^{b} ±0**	**11.6^{c} ±2.08**	**9.3^{e} ±0.57**
	Chloroform	**17.3^{b} ±0.57**	**15^{c} ±1**	**11^{d} ±1**	**16.3^{b} ±1.15**	**11^{c} ±0**	**0^{e} ±0**	**9.3^{b} ±0.57**	**0^{d} ±0**	**0^{d} ±0**	**16.3^{b} ±0.57**	**13^{c} ±0**	**10.3^{d} ±0.57**
	Hexane	**10.6^{c} ±1.15**	**8.3^{d} ±1.15**	**0^{e} ±0**	**10^{c} ±0**	**7.6^{d} ±0.57**	**0^{e} ±0**	**0^{b} ±0**	**0^{b} ±0**	**0^{b} ±0**	**8.6^{c} ±0.57**	**0^{d} ±0**	**0^{d} ±0**
Microorganisms		***E. coli***			***S.aureus***			***P. aeruginosa***			***E.hirae***		
Conc. (µg/ml)		**500**	**250**	**125**	**500**	**250**	**125**	**500**	**250**	**125**	**500**	**250**	**125**
Soxhlet Extraction *Morinda Citrifolia* (L.)	Methanol	**26^{b} ±0**	**24^{c} ±0**	**21.3^{d} ±0.57**	**26.6^{a} ±0.57**	**25.3^{b} ±1.15**	**23^{c} ±1**	**24.3^{b} ±1.15**	**21.6^{c} ±1.15**	**19.6^{d} ±0.57**	**24^{a} ±1**	**22.6^{b} ±0.57**	**19.3^{c} ±0.57**
	Ethanol	**24.6^{b} ±0.57**	**22^{c} ±1**	**18.6^{d} ±0.57**	**23.6^{b} ±0.57**	**20.6^{c} ±0.57**	**18^{d} ±0**	**22.6^{b} ±1.15**	**22.3^{c} ±0.57**	**19^{d} ±1**	**21.6^{a} ±0.57**	**17^{b} ±0**	**12^{c} ±1**
	Chloroform	**14.3^{b} ±1.15**	**12^{c} ±0.57**	**10^{d} ±0.57**	**16.3^{b} ±1.52**	**13.6^{c} ±0.57**	**10^{d} ±1**	**9.6^{c} ±0.57**	**0^{d} ±0**	**0^{d} ±0**	**12.6^{b} ±1.15**	**8.6^{c} ±0.57**	**0^{d} ±0**
	Hexane	**9^{c} ±1**	**0^{d} ±0**	**0^{d} ±0**	**11^{c} ±1.73**	**8.3^{d} ±2.08**	**0^{e} ±0**	**8.6^{c} ±1.52**	**0^{d} ±0**	**0^{d} ±0**	**7.6^{c} ±0.57**	**0^{d} ±1**	**0^{d} ±0**
-Ve Control		No inhibition			No inhibition			No inhibition			No inhibition		
+Ve Control		27.3±0.57			24.3±0.57			25±0			22±1		

Table 4.5: Antibacterial activity (zone of inhibition) of methanol, ethanol, chloroform, and hexane crude extracts (maceration and Soxhlet) of *Morinda Citrifolia* (L.) leaves against *E. coli*, *S. aureus*, *P. aeruginosa*, and *E. hirae* bacteria.

- Negative Control = 10 % DMSO and Positive control = Chloramphenicol 30 µg/ml.
- Values expressed as Mean ± standard error (SEM); n=3 in each group.
- Inhibition zones were measured in mm and disc diameter was included.
- Means in the same raw within groups not followed by the same superscript are significantly different; one-way ANOVA and Duncan's test were conducted ($P<0.05$).

Microorganisms		***E. coli***			***S.aureus***			***P. aeruginosa***			***E.hirae***		
Conc. (µg/ml)		**500**	**250**	**125**	**500**	**250**	**125**	**500**	**250**	**125**	**500**	**250**	**125**
Maceration Extraction *Sauropus androgynus* (L.)	Methanol	**19.3^{b} ±0.57**	**17.6^{c} ±0.57**	**14.6^{d} ±1.15**	**20^{b} ±0**	**18.3^{c} ±0.57**	**16.6^{d} ±1.15**	**20.6^{b} ±0.57**	**18.6^{c} ±0.57**	**16.3^{d} ±1.52**	**18^{b} ±1.73**	**16.3^{c} ±1**	**13.6^{d} ±0.57**
	Ethanol	**18^{b} ±0**	**16.3^{c} ±0.57**	**14^{d} ±1**	**18.3^{b} ±0.57**	**16^{c} ±0**	**12.6^{d} ±0.57**	**17^{b} ±0.57**	**14^{c} ±0**	**11.6^{d} ±0.57**	**15.6^{b} ±1.15**	**12.6^{c} ±0.57**	**9^{d} ±1**
	Chloroform	**13.6^{b} ±1.73**	**11.6^{c} ±2.08**	**10^{d} ±1.15**	**13.3^{b} ±0.57**	**10.3^{c} ±0.57**	**8^{d} ±1**	**8.3^{c} ±0.57**	**0^{d} ±0**	**0^{d} ±0**	**11.6^{b} ±0.57**	**9.3^{c} ±1.52**	**7.6^{e} ±0.57**
	Hexane	**11.6^{c} ±1.15**	**10^{d} ±0**	**8.3^{e} ±0.57**	**13.3^{b} ±0.57**	**10.6^{c} ±0.57**	**8^{d} ±1**	**0^{b} ±0**	**0^{b} ±0**	**0^{b} ±0**	**8.6^{c} ±0.57**	**0^{d} ±0**	**0^{d} ±0**
Microorganisms		***E. coli***			***E. coli***			***S.aureus***			***P. aeruginosa***		
Conc. (µg/ml)		**500**	**250**	**125**	**500**	**250**	**125**	**500**	**250**	**125**	**500**	**250**	**125**
Soxhlet Extraction *Sauropus androgynus* (L.)	Methanol	**21.3^{b} ±0.57**	**18.6^{c} ±1.15**	**15^{d} ±1**	**22^{b} ±0**	**19.3^{c} ±0.57**	**16.3^{d} ±0.57**	**21 ^{b} ±1**	**19.6^{c} ±0.57**	**17^{d} ±1.73**	**20^{b} ±0**	**18^{c} ±0**	**15.6^{d} ±0.57**
	Ethanol	**19.3^{b} ±0.57**	**17^{c} ±1.52**	**13^{d} ±1**	**19.3^{b} ±0.57**	**15.3^{c} ±0.57**	**12.6^{d} ±0.57**	**22.3^{b} ±0.57**	**20^{c} ±1**	**16.6^{d} ±0.57**	**18.3^{b} ±2.08**	**17^{c} ±0**	**14.3^{d} ±0.57**
	Chloroform	**15^{b} ±0.57**	**12.6^{c} ±0.57**	**9^{d} ±1.73**	**8.6^{c} ±0.57**	**0^{d} ±0**	**0^{d} ±0**	**12^{c} ±0**	**10.3^{d} ±0.57**	**8^{e} ±0**	**10.6 ^{c} ±1.15**	**0^{d} ±0**	**0^{d} ±0**
	Hexane	**10.3^{c} ±1.15**	**7.3^{d} ±0.57**	**0^{e} ±0**	**9.6^{c} ±1.52**	**7^{d} ±1**	**0^{e} ±0**	**11^{c} ±1**	**10^{d} ±0**	**8.6^{e} ±0.57**	**0^{b} ±0**	**0^{b} ±1**	**0^{b} ±0**
-Ve Control		No inhibition			No inhibition			No inhibition			No inhibition		
+Ve Control		27.3±0.57			24.3±0.57			25±0			22±1		

Table 4.6: Antibacterial activity (zone of inhibition) of methanol, ethanol, chloroform, and hexane crude extracts (maceration and Soxhlet) of *Sauropus androgynus* (L.) leaves against *E. coli, S. aureus, P. aeruginosa,* and *E. hirae* bacteria.

- Negative Control = 10 % DMSO and Positive control = Chloramphenicol 30 µg/ml.
- Values expressed as Mean ± standard error (SEM); n=3 in each group.
- Inhibition zones were measured in mm and disc diameter was included.
- Means in the same raw within groups not followed by the same superscript are significantly different; one-way ANOVA and Duncan's test were conducted ($P<0.05$).

The results obtained from the disc diffusion assay showed that there has been a significant increasing effect on bacterial growth inhibition ($P<0.05$), with the increasing concentration of the extracts (Figure 4.1). *Staphylococcus aureus* showed the highest sensitivity in maceration extracts when treated with 500 µg/ml of *Morinda citrifolia* L extracts, and the inhibition zones recorded were, 25.3, 23.3, 16.3, and 10 mm in methanol, ethanol, chloroform, and hexane extracts, respectively. However, the recorded inhibition zones of *Staphylococcus aureus* when treated with 125 µg/ml of the same extract were 21mm in methanolic extract and 21.6 mm in ethanolic extract, while no inhibition zones were recorded in chloroform and hexane extracts. Soxhlet methanolic extracts exhibited stronger activity compering to the maceration methanolic extracts. The highest inhibition zones among Soxhlet methanolic extracts were recorded in *Morinda citrifolia* L., and the inhibition zones were 26, 26.6, 24.3 and 24 mm in *Escherichia coli*, *Staphylococcus aureus*, *Pseudomonas aeruginosa*, and *Enterococcus hirae* respectively. The lowest recorded in *Imperata cylindrica* L. with inhibition zones of 18.3, 19, 19, and 15.6 mm in *Escherichia coli*, *Staphylococcus aureus*, *Pseudomonas aeruginosa*, and *Enterococcus hirae* respectively. The maximum inhibitory zones were recorded in methanolic maceration extracts from *Morinda citrifolia* L., and ranged from 24.6, 25.3, 21, and 20 mm in *Escherichia coli*, *Staphylococcus aureus*, *Pseudomonas aeruginosa*, and *Enterococcus hirae* respectively. The lowest recorded was in *Imperata cylindrica* L. with activity zones ranged from 16.3, 17.6, 17.6, and 13 mm in *Escherichia coli*, *Staphylococcus aureus*, *Pseudomonas aeruginosa*, and *Enterococcus hirae* respectively.

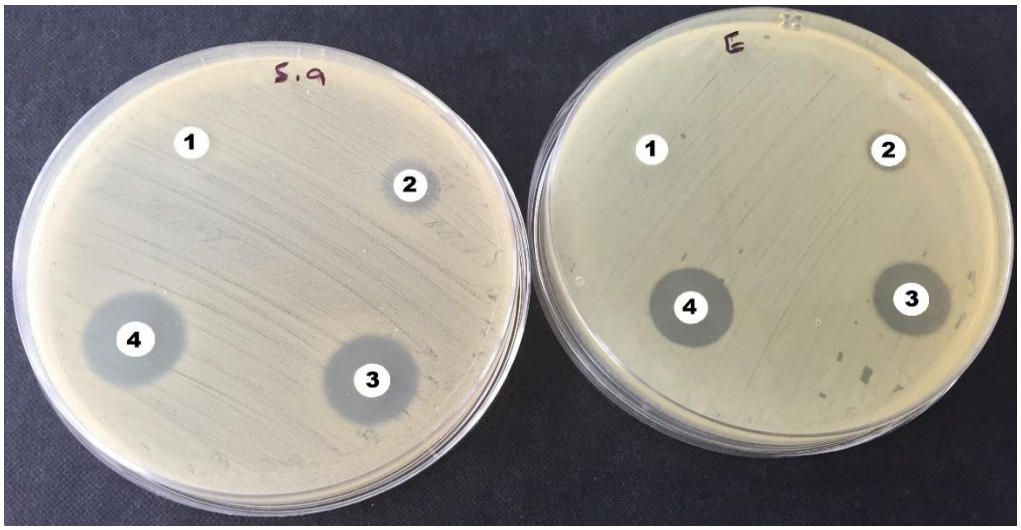


Figure 4.1: Disc diffusion assay of *Imperata cylindrica* L. ethanolic extract in *S. aureus* (left) *and E. coli* (right). 1=10 % DMSO, 2=125 µg/ml, 3=250 µg/ml, 4= 500 µg/ml.

Ethanolic extracts were slightly less active than the methanolic extracts in both extraction techniques (*P<0.05*), however, all tested bacteria were susceptible to ethanolic extracts. The highest activity recorded in Soxhlet ethanolic extracts were in *Morinda citrifolia* L. with activity ranging from 24.6, 23.6, 22.6, and 21.6 mm in *Escherichia coli*, *Staphylococcus aureus*, *Pseudomonas aeruginosa*, and *Enterococcus hirae* respectively, and the lowest were recorded in Imperata cylindrica L. with inhibition zones ranged from 18.3, 19, 19, and 15.6 mm in *Escherichia coli*, *Staphylococcus aureus*, *Pseudomonas aeruginosa*, and *Enterococcus hirae* respectively. The maximum inhibitory zones recorded in maceration ethanolic extracts were in *Morinda citrifolia* L. and ranged from 22.6, 23.3, 19, and 18 mm of *Escherichia coli*, *Staphylococcus aureus*, *Pseudomonas aeruginosa*, and *Enterococcus hirae* respectively, while the lowest recorded in *Imperata cylindrica* L. with activity zones ranged from 17.6, 15.3, 13.3, and 12.6 mm in *Escherichia coli*, *Staphylococcus aureus*, *Pseudomonas aeruginosa*, and *Enterococcus hirae* respectively.

Chloroform extracts were less active than methanolic and ethanolic extracts, and in some cases there were no activity recorded even with the higher concentrations. *Enterococcus hirae* did not record any inhibition zone when tested with maceration chloroform extract of *Imperata cylindrica* L., however, Soxhlet extract of the same plant recorded a very weak activity of 8.6 mm at 500 μg/ml with the same microorganism. The highest inhibition zone recorded was in the chloroform Soxhlet extracts of *Centella asiatica* L. was 18 mm at 500 μg/ml in *Staphylococcus aureus*, while the lowest recorded was 7 mm in the same plant with maceration extract at 500 μg/ml. Chloroform extracts obtained by Soxhlet and maceration techniques of *Centella asiatica* L. demonstrated a varied range of activity against all tested strains even with the lower concentrations, unlike other plant extracts which did not record any activity in some lower doses, especially with maceration extracts.

The least activity recorded was in the hexane extracts of all plants under investigation. In most cases, hexane extracts did not show any activity with lower concentrations, while higher concentrations showed a very weak activity comparing to the positive control at (*P<0.05*). The maximum inhibition zone was recorded in hexane maceration extract of *Sauropus androgynus* L. against *Staphylococcus aureus*, the inhibition zone was 13.3 mm at 500 μg/ml. While the lowest inhibition zone recorded at a concentration of 500 μg/ml was 7.6 mm in hexane Soxhlet

extract of *Morinda citrifolia* L. against *Enterococcus hirae*. Hexane extracts of *Imperata cylindrica* L. obtained by Soxhlet extraction method did not demonstrate any activity against all tested organisms, even with higher doses, however, maceration extract of *Imperata cylindrica* L. showed a very weak activity against Gram negative bacteria *Escherichia coli* and *Pseudomonas aeruginosa* at a concentration of 500 μg/ml, and the inhibition zones were 8.3 mm and 9.6 mm respectively.

4.2.2.2 Minimum inhibitory concentration (Bioscreening assay)

In antibiotic drug discovery, MICs must not be analysed as an isolated data, it should be used for initial screening of natural products as well as for the selection of the material that continue in the following assays and target identification research. The MICs are the starting point in a bioprospecting program. For this reason, the protocols use must be implemented using international standards, because it takes into account the ability of the extract, fraction or compound to penetrate into the microorganism (Bueno, 2012). In order to provide a more developed understanding of the impact of the crude extracts on the growth of bacterial strains under investigation, the Bioscreen-C technology was employed to determine the relevant MICs. As described in section 3.2.2.2, MIC values were calculated from the Bioscreen-C system outputs using an adapted approach described by Lambert, (2001).

The data obtained from the disc diffusion assay of the crude extracts suggested that methanolic and ethanolic Soxhlet extracts had potential antibacterial activity against Gram positive and Gram negative bacteria. Nonetheless, other solvent extracts showed antibacterial activity against tested bacteria to various degrees, but the inhibition zones were more eminent in the methanolic and ethanolic Soxhlet extracts. For this reason, these extracts were tested further on the same bacterial species to determine the minimum inhibitory concentrations (MICs) and the minimum bactericidal concentrations (MBCs). Figure 4.2 (methanolic Soxhlet extracts) and figure 4.3 (ethanolic Soxhlet extracts) represent the MIC values of *Centella asiatica* L., *Imperata cylindrica* L., *Morinda citrifolia* L., and *Sauropus androgynus* L., whilst, figure 4.4 (methanolic Soxhlet extracts) and figure 4.5 (ethanolic Soxhlet extracts) represent the MBC values of the same extracts.

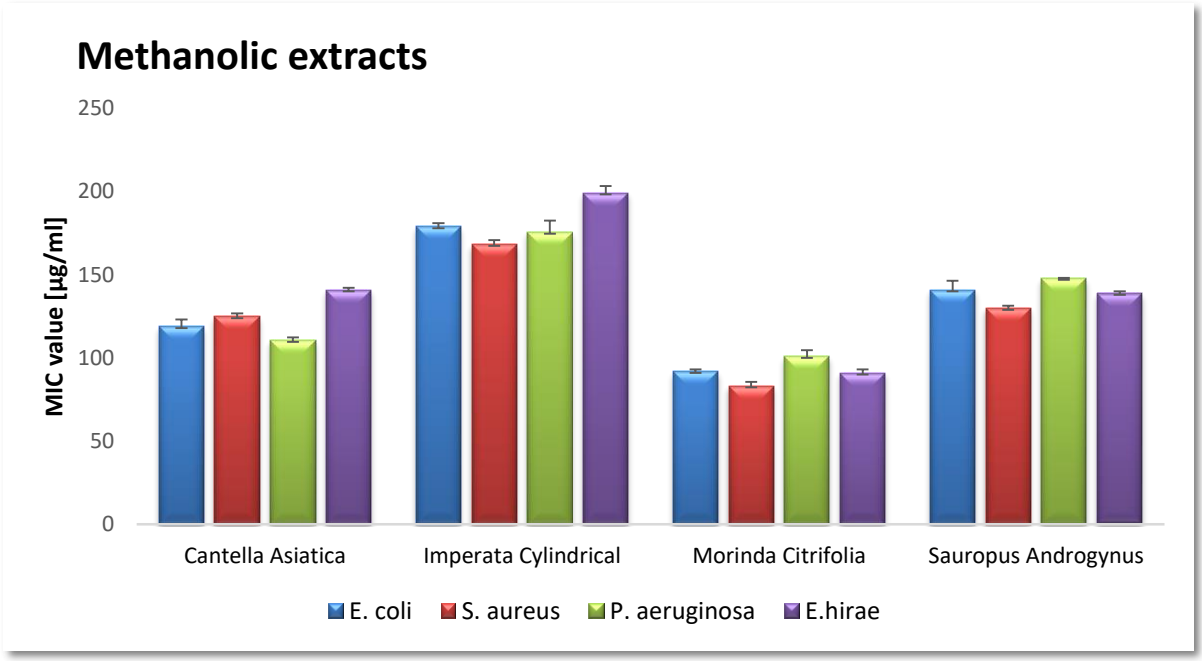


Figure 4.2: Comparative analysis of all bacterial culture MICs of methanolic Soxhlet crude extracts for *Centella asiatica* (L.), *Imperata cylindrica* (L.), *Morinda citrifolia* (L.) and *Sauropus androgynus* (L.). All values represent a mean of three replicate tests and the standard error of the mean (SEM) has been calculated.

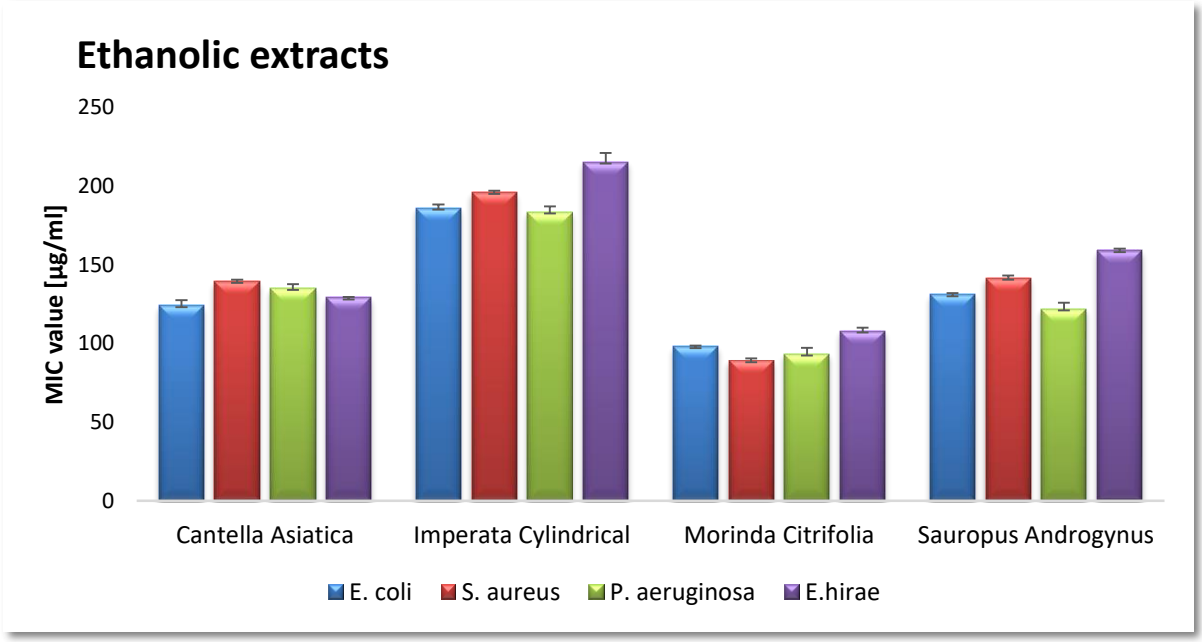


Figure 4.3: Comparative analysis of all bacterial culture MICs of ethanolic Soxhlet crude extracts for *Centella asiatica* L., *Imperata cylindrica* L., *Morinda citrifolia* L., and *Sauropus androgynus* L. All values represent a mean of three replicate tests and the standard error of the mean (SEM) has been calculated.

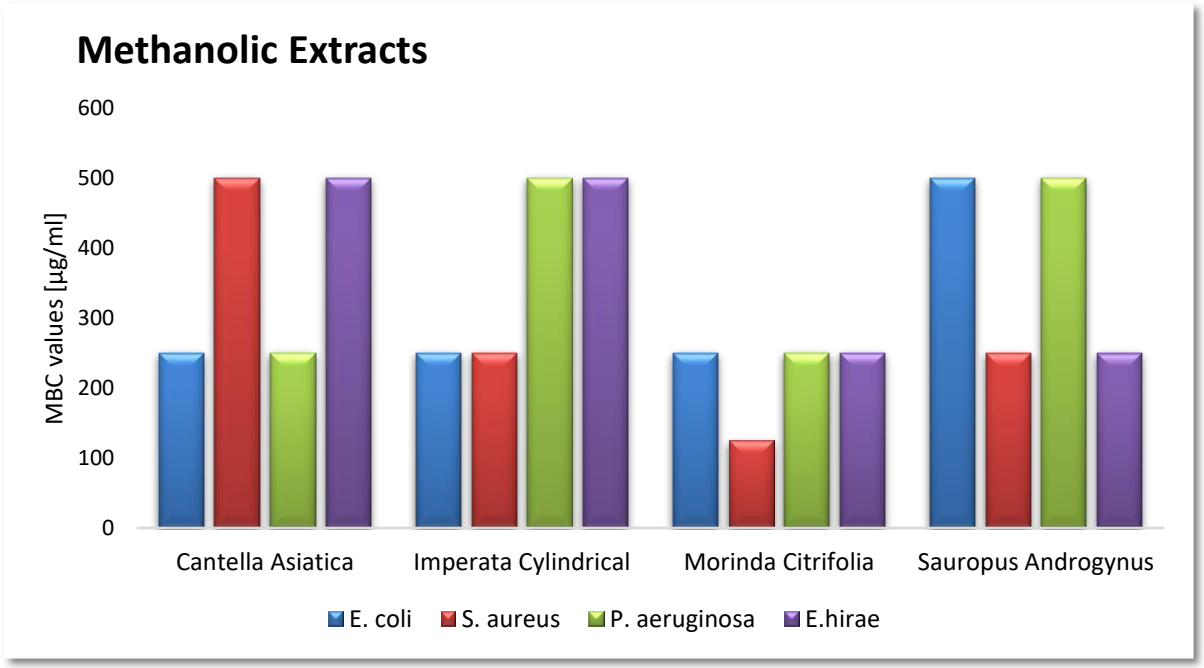


Figure 4.4: MBC values of methanolic Soxhlet crude extracts of *Centella asiatica* L., *Imperata cylindrica* L., *Morinda citrifolia* L., and *Sauropus androgynus* L. Tested bacteria are *Escherichia coli, Staphylococcus aureus, Pseudomonas aeruginosa*, and *Enterococcus hirae.*

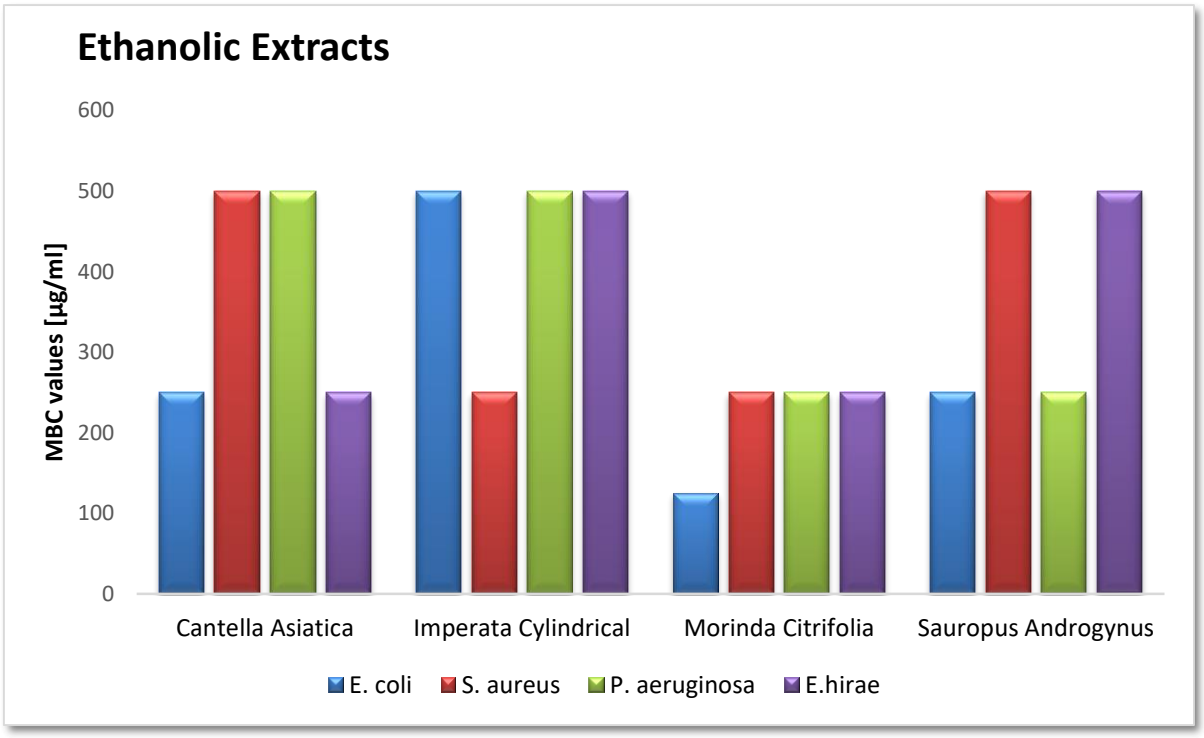


Figure 4.5 MBC values of ethanolic Soxhlet crude extracts of *Centella asiatica* L., *Imperata cylindrica* L., *Morinda citrifolia* L., and *Sauropus androgynus* L. Tested bacteria are *Escherichia coli, Staphylococcus aureus, Pseudomonas aeruginosa*, and *Enterococcus hirae.*

The result of the minimum inhibitory concentration test indicated different levels of MICs depending on the bacterial strain being tested. In methanolic extracts of the four medicinal plants, MICs ranged from 83.3 µg/ml to 199.3 µg/ml, while the range was 89 µg/ml to 215.3 µg/ml for the ethanolic extracts. *Morinda citrifolia* L. recorded the lowest MICs for all tested bacteria with methanolic and ethanolic extracts, the MICs in methanolic extracts were 92, 83.3, 101, and 91µg/ml in *Escherichia coli*, *Staphylococcus aureus*, *Pseudomonas aeruginosa*, and *Enterococcus hirae*, respectively. *Morinda citrifolia* L. ethanolic extract showed MICs of 98, 89, 93.3, and 108 µg/ml in *Escherichia coli*, *Staphylococcus aureus*, *Pseudomonas aeruginosa*, and *Enterococcus hirae* respectively. *Imperata cylindrica* L. showed the weakest MICs among all tested extracts. Methanolic extract of *Imperata cylindrica* L. gave MIC values of 179, 168.3, 175.6, and 199.3µg/ml in *Escherichia coli*, *Staphylococcus aureus*, *Pseudomonas aeruginosa*, and *Enterococcus hirae*, respectively, whilst ethanolic extract recorded 186, 196, 183.6, and 215.3 µg/ml in *Escherichia coli*, *Staphylococcus aureus*, *Pseudomonas aeruginosa*, and *Enterococcus hirae*, respectively. All tested bacteria showed different levels of MICs in *Centella asiatica* L. and *Sauropus androgynus* L. The lowest MIC observed in the methanolic extract of *Centella asiatica* L. was 110.6 µg/ml in *Pseudomonas aeruginosa*, whereas, the ethanolic extract of the same plant recorded the lowest MIC in *Escherichia coli* with a value of 124 µg/ml. On the other hand, *Sauropus androgynus* L. recorded an MIC of 122 µg/ml in the ethanolic extract against *Pseudomonas aeruginosa*, whilst the lowest MIC in the methanolic extract of the same plant was 130 µg/ml with *Staphylococcus aureus.*

The MBC is complementary to the MIC; whereas the MIC test demonstrates the lowest level of antibacterial agent that inhibits growth, the MBC demonstrates the lowest level of antimicrobial agent that results in microbial death. 125 µg/ml, is the lowest MBC value recorded in *Morinda citrifolia* L. against *Staphylococcus aureus* and *Escherichia coli* of, with the methanolic and ethanolic extracts, respectively. Whereas, MBC were ranged between 250 and 500 µg/ml of all tested bacteria in other plant extracts.

Figures 4.6, 4.7, 4.8, and 4.9 represents 1 x and ½ x MIC growth curve, in addition to the standard growth curve of particular strain of all tested bacteria with methanolic Soxhlet crude extract of *Morinda citrifolia* L., which demonstrated the highest activity against tested bacteria.

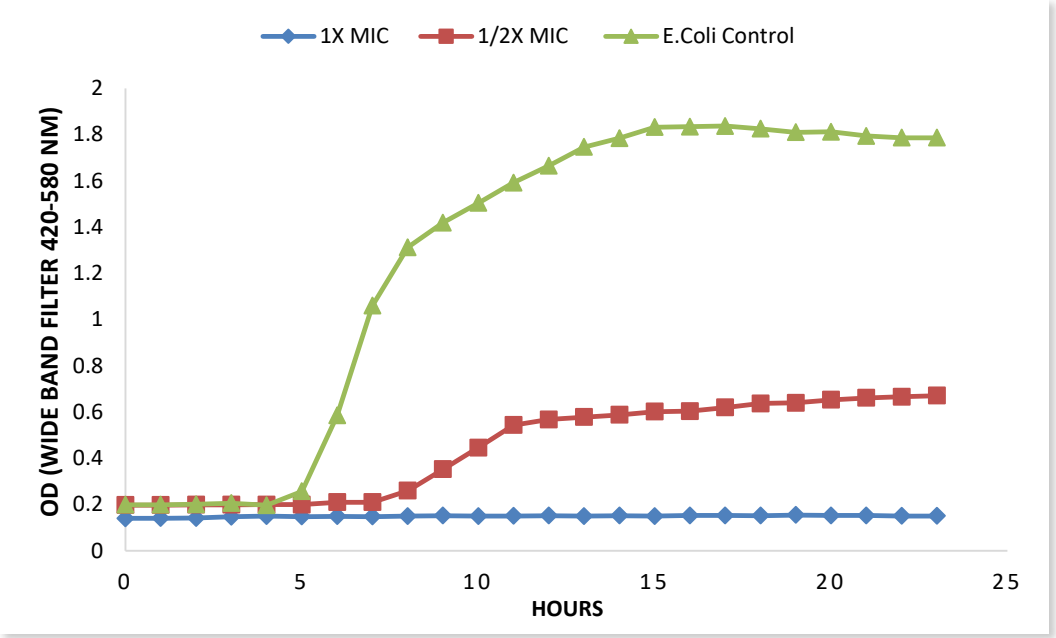


Figure 4.6: Standard *Escherichia coli* growth curve, of 1 x and ½ x MIC, activity of methanolic Soxhlet crude extract of *Morinda citrifolia* L. under standard conditions, 37ºC and 24 h incubation. All values represent a mean of three replicate tests.

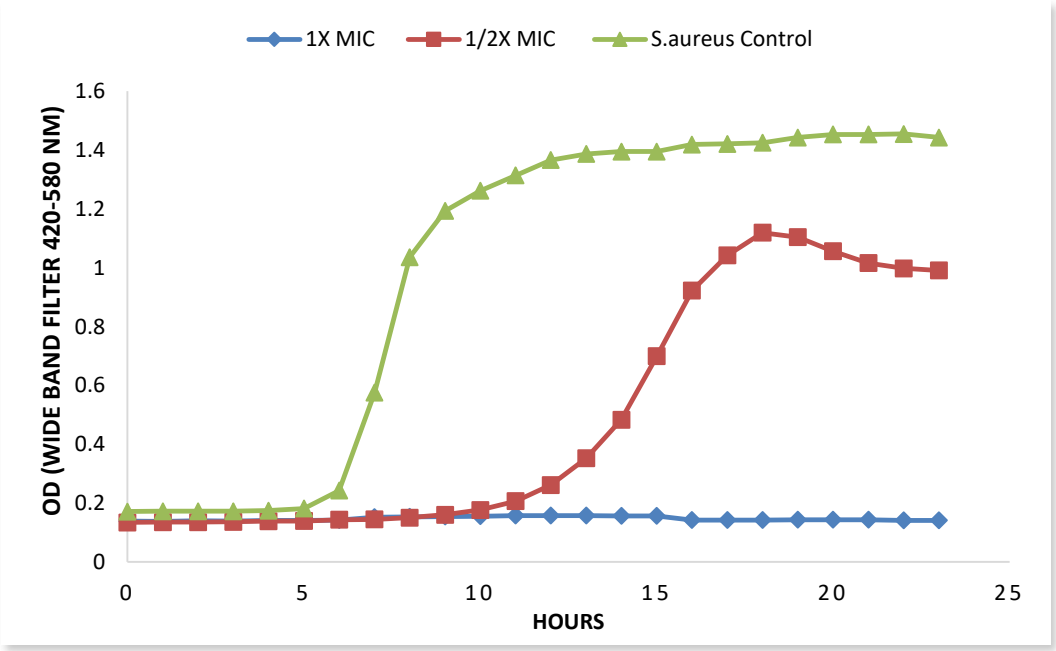


Figure 4.7: Standard *Staphylococcus aureus* growth curve, of 1 x and ½ x MIC, activity of methanolic Soxhlet crude extract of *Morinda citrifolia* L. under standard conditions, 37ºC and 24 ho incubation. All values represent a mean of three replicate tests.

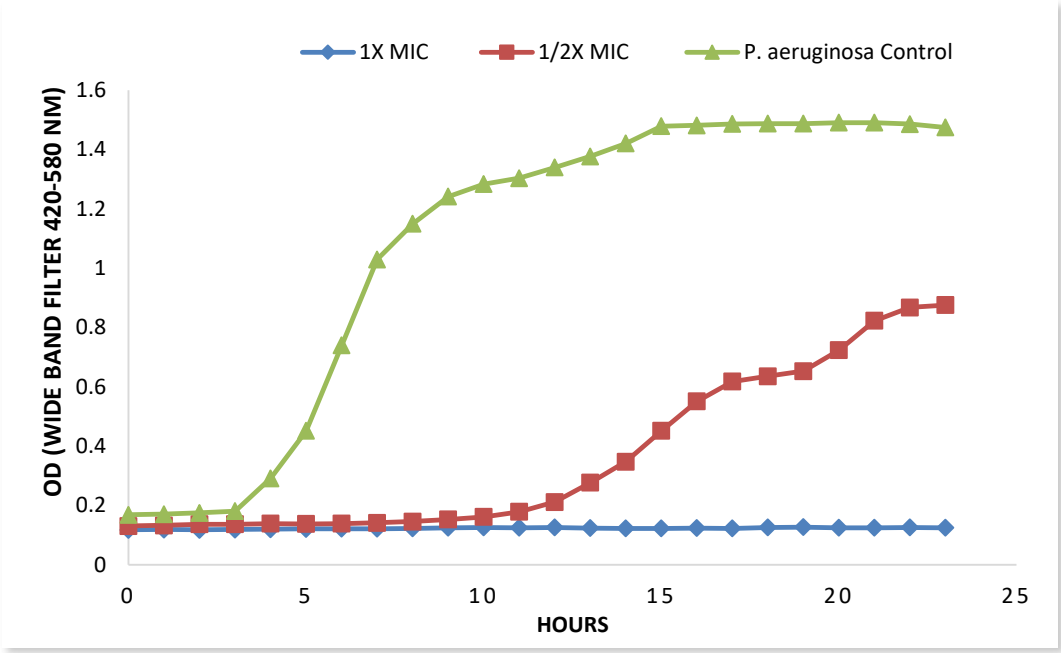


Figure 4.8: Standard *Pseudomonas aeruginosa* growth curve with, of 1 x and ½ x MIC, activity of methanolic Soxhlet crude extract of *Morinda citrifolia* L. under standard conditions, 37ºC and 24 h incubation. All values represent a mean of three replicate tests.

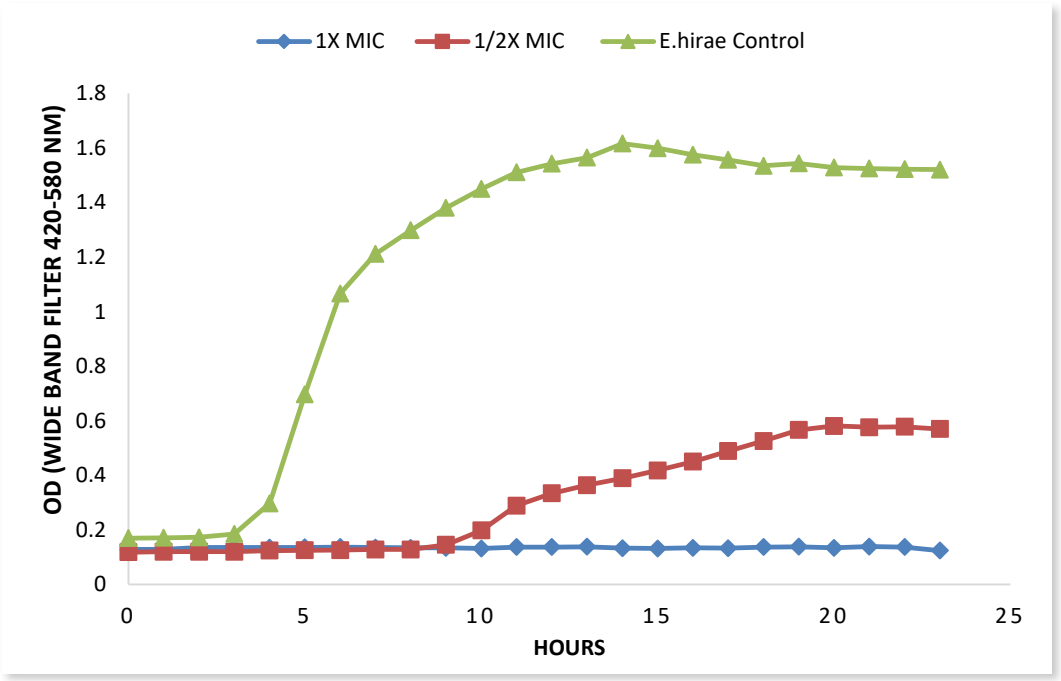


Figure 4.9: Standard *Enterococcus hirae* growth curve with, of 1 x and ½ x MIC, activity of methanolic Soxhlet crude extract of *Morinda citrifolia* L. under standard conditions, 37ºC and 24 h incubation. All values represent a mean of three replicate tests.

4.3 Discussion

Natural resources are now being sought as a source of novel antimicrobial agents since conventional agents are increasingly ineffective due to the acquisition of resistance mechanisms. Complex plant based products contain various secondary metabolites with varying modes of action and complex interactions (Seidel, 2006). The presence of various secondary metabolites with different modes of action, reduces the likelihood that resistance to the products will develop in bacteria. Traditionally, the search for novel antibacterial agents, to tackle bacterial resistance, has focused around products which originate from microbial sources, though most of the antibiotics used clinically are produced by soil microorganisms (Aiyegoro & Okoh, 2009). Plant based antimicrobials have enormous therapeutic potential as they are effective in the treatment of infectious diseases while simultaneously reducing many of the side effects that are often associated with synthetic antimicrobials. The healing power of medicinal plants are in most instances not understood and their mechanism of action remains to be elucidated. With limited access to antibiotics in developing countries and with the upsurge in multi-resistant bacteria around the world, alternative therapies derived from plant extracts may be vital. However, validation of natural products is vital and often a challenging task (Pandey & Kumar, 2013).

The initial stage in studying medicinal plants is the preparation of plant samples to preserve biomolecules in the biomass prior to the extraction. Plant samples such as leaves, barks, roots, fruits and flowers can be extracted from fresh or dried plant material. In most cases, dried sample is preferred as fresh samples are fragile and tend to deteriorate faster than dried samples. for this reason, pulverised dry leaves were used in this study of *Centella asiatica* (L.), *Imperata cylindrica* (L.), *Morinda citrifolia* (L.) and *Sauropus androgynus* (L.). Other pre-preparation of plant materials such as grinding and drying also influences the preservation of bioactive molecules in the final extracts. Lowering the particle size increases surface contact between samples and extraction solvents as powdered samples have a more homogenious and smaller particles, leading to better surface contact with extraction solvents (Azwanida, 2015). Conventional mortar and pestle or electric blenders and mills are commonly used to reduce particle size of the sample. This particular pre-preparation is important, as for efficient extraction to occur, the solvent must make contact with the target analytes. In a study conducted by Sulaiman (2011) and coworkers, they concluded that particle size smaller than 400 µm (0.4 mm) or less is ideal for an efficient extraction.

Extraction is the second crucial step in the preparation of plant potential drug candidates in the field of natural product drug discovery. The extraction procedure determines both the quantity and quality of the crude extracts obtained from each extraction solvent. Modern methods of extraction are effective in advancing the development of potential antibacterial candidate. Considerable efforts have been made by researchers to find an efficient extraction method, for obtaining better quality and high efficiency of extraction of medicinal plants, methods have to be optimised. The need for selection of most appropriate extraction methodology is evident from the fact that when different methods are applied on the same plant material with same solvent, extraction efficiency can vary significantly. In addition, the method selected as the most appropriate one also needs to be standardised so as to achieve an acceptable degree of reproducibility (Jadhav *et al.,* 2009).

In this project, a comparative study between maceration and Soxhlet extraction techniques has been conducted to choose the most efficient method, in term of antibacterial activity and yield of the material, to follow with. Results obtained in this experiment suggested that Soxhlet extraction of four different solvents with different polarities gave a higher yield and bioactivity than that obtained by the maceration method. This result is in line with the work conducted by Selma *et al.* (2004), where they performed a comparative study in order to find a better technique for bioactive molecules extraction. Four extraction techniques were applied using organic and aqueous solvents to obtain various crude extracts: static maceration, dynamic maceration, extraction with the assistance of ultrasonic waves and Soxhlet extraction. Among all other extraction methods, Soxhlet extraction was the most efficient extraction technique, with the highest yield with the most antibacterial activity. However, researchers stated that Soxhlet extraction was time-consuming comparing with other techniques. They argued that its efficiency is due to the employment of a small volume of solvent, which is continually renovating contact with the plant material, promoting more interactions between them. The heat applied in Soxhlet extraction increases solvent's transfer rate for the active site of the plant biomass.

More recent studies conducted by Gothandam *et al.,* (2010); Ismail *et al.,* (2011); Rahman *et al.,* (2013); and Nayak *et al.,* (2015), to compare various extraction techniques for isolation and determination of phytochemical groups with their antibacterial activity in *Sauropus androgynous* L., *Imperata cylindrica* L., *Centella asiatica* L., and *Morinda citrifolia* L

respectively. Supercritical fluid extraction, pressurised fluid extraction, ultrasonic extraction, maceration, and Soxhlet apparatus are the extraction techniques being used for extracting active compounds from the four medicinal. They concluded that the Soxhlet extracts contained more active chemical groups, in the phytochemical assays, than other extraction techniques. Extracts of Soxhlet apparatus has demonstrated the greatest antibacterial activity, among other techniques, against Gram positive and Gram negative bacteria. Soxhlet extraction technique had more yield than other techniques, this finding agreed with the finding in our study, the highest was recorded in *Centella asiatica* L. with 14.1 % out of starting material, while the lowest was recorded in *Imperata cylindrica* L. with 11.3 % out of starting material. However, researchers stated that Soxhlet extraction was more solvent and time-consuming than other methods. Even though other extraction techniques had less active groups, they had some chemical groups which were not found in Soxhlet extraction. As a result, researchers suggested that more than one technique might be employed (to bring more active chemical groups) in the evaluation of the bioactive compounds of particular medicinal plant.

According to Altıok *et al.* (2008), the choice of the solvent is another important factor affecting the efficiency of solid-liquid extraction. They stated that successful determination of biologically active compounds from plant material is largely dependent on the type of solvent used in the extraction procedure. Solvents to be used for medicinal plant extraction should be with a low toxicity, ease of evaporation at low heat, promotion of rapid physiologic absorption of the extract, preservative action and inability to cause the extract to complex or dissociate. Moreover, the choice of solvent is influenced by the intended use of the extract (Eloff, 1998).

Solvents differ in their extraction capabilities depending on their own chemical properties and the solute's chemical structures. Other factors affecting solvent selection are boiling point, density, surface tension, viscosity, stability, flammability, toxicity, compatibility with product, availability, and the cost (Cowan, 1999). Many types of solvents are available for extracting plant materials, including water, methanol, ethanol, acetone, chloroform, hexane, and petroleum ether. The most widely used solvents are water and methanol. Water is a universal solvent and for extracting compounds from medicinal plants. However, organic solvent extracts give more consistent reading of antimicrobial activity than water extracts. Surprisingly, most of the antimicrobial active components have been identified from organic solvent extracts (not water

soluble material) (Ncube *et al.,* 2008). Water-soluble compounds, such as polysaccharides and polypeptides, are generally more effective as inhibitors of pathogen adsorption and have no real impact as antimicrobial agents. Moreover, water soluble flavonoids (mostly anthocyanins) have no antimicrobial significance and water soluble phenolics are only important as antioxidant compounds (Handa, 2008).

The higher impact of the methanolic and ethanolic extracts as compared to the aqueous extract can be attributed to the presence of higher amounts of polyphenols as compared to aqueous extracts. This means that they are more efficient in cell walls degradation which have non-polar character and cause polyphenols to be released from cells. A more useful explanation for the decrease in activity of aqueous extracts can be ascribed to the enzyme polyphenol oxidase, which degrade polyphenols in water extracts, whereas in methanol and ethanol extracts they are inactive. Moreover, water is a better medium for the occurrence of the microorganisms as compared to alcoholic extracts (Tiwari, *et al.,* 2011). Since nearly all of the identified active components from plants are aromatic or saturated organic compounds, they are most often obtained through initial ethanol or methanol extraction. The hydroxyl group in methanol and ethanol helps dissolve both polar molecules and ionic substances, Methanol is more polar than ethanol, but due to its cytotoxic nature, it is unsuitable for extraction in certain kind of studies as it may lead to incorrect results (Mandana *et al.,* 2011).

In our study, polar solvents, like methanol and ethanol, demonstrated stronger antibacterial activities than the less polar solvents, like chloroform and hexane. Many published studies came in line with the results obtained in this study, these studies have evaluated the ability of various solvents to solubilise antibacterial agents from plants. Arumugam *et al.,* (2011) and Romika *et al.,* (2015) examined a variety of solvents for their ability to solubilise antibacterials from *Centella asiatica* L. These studies used acetone, methanol, ethanol, and chloroform for the extraction. Results showed that methanol and ethanol extracts were active against the Gram positive and Gram negative bacteria, even with lower concentrations up to 50 µg/ml. Antibacterial activities of acetone extracts were stronger on Gram negative bacteria than on Gram positive bacteria, while chloroform extract displayed very weak activity on all tested bacteria. Ismail (2011) and others, evaluated the antibacterial activity of *Imperata cylindrica* L., using methanol and chloroform as extraction solvents, they employed Soxhlet technique to

obtain crude extracts. The results suggested that methanolic extract was much more active than chloroform extract against *Escherichia coli, Pseudomonas aeruginosa and Staphylococcus aureus.*

Usha (2010) and coworkers, had subjected *Morinda citrifolia* L. to successive solvent extraction in the increasing order of polarity, from non-polar to highly polar. The solvents used were petroleum ether, benzene, chloroform, ethyl acetate, ethanol, methanol and water. Extracts were tested against *Escherichia coli* and *Staphylococcus aureus*, their results revealed that the maximum activity recorded with the high polar organic solvents. Although water has a very strong polarity index (9), but aqueous extracts had a very weak activity against both tested strains. Sinica *et al.* (2015), evaluated phytochemical, antibacterial and antifungal activities of leaf extract of *Morinda citrifolia* L. using various solvents. The antibacterial activity was tested against gram positive bacteria *Bacillus subtilis*, *Escherichia coli*, *Pseudomonas fluorescence* and *Salmonella typhi* using disc diffusion method. The methanolic extract showed the highest zone of inhibition with more than 20 mm in diameter.

Results by Ariharan and coworkers (2013), come in line with the results of our study. They carried out an antibacterial evaluation of the methanolic, ethanolic, and aqueous extracts of *Sauropus androgynous* L. leaves, against some Gram positive and Gram negative bacterial strains. Agar diffusion assay was used in their study, and Streptomycin was used as a reference antibiotic where all results were compared with it. Methanolic extract of *Sauropus androgynous* L. demonstrated significant antibacterial activity against all tested bacterial strains, while the aqueous and ethanolic extracts showed less activity against *Salmonella typhimurium* and *Klebsiella pneumonia*.

Noticeable attention has been paid to antibacterial activity screening and evaluation methods. Several bioassays such as disk diffusion, well diffusion and broth dilution are well known and commonly used. Others, such as the time-kill test and flow cytofluorometry methods, provide information on the nature of the inhibitory effect (bactericidal or bacteriostatic; time-dependent or concentration-dependent) and the cell damage inflicted to the test microorganism. However, these methods require specified equipment and further evaluation for reproducibility and standardisation (Mayers *et al.,* 2009). Bioscreen-C automated system has been utilised in this study, for its accuracy and ability to handle 200 sample at a time.

Automated systems are not without any limitations, particularly in laboratories with low resource settings. In a study conducted by Felmingham and Brown (2001), they argued that automated systems are considerably more expensive than other non-automated counterparts. They also stated that automated systems are generally broth-based analysis and cannot be used for all clinically important organisms, particularly those species that are more fastidious in nature. From the results given in this study, those disadvantages did not affect the reliability and accuracy of the experiments comparing to other methods.

Samy and coworkers (2011), reported that *in-vitro* results of extracts taken from the leaves of *Centella asiatica* L. demonstrated an antibacterial action. Three solvents were used in their study; hexane, dichloromethane, and methanol, where dichloromethane extracts of *C. asiatica* showed significant and higher activity against various bacterial strains used in the study. While the hexane extract exhibited a weak activity, the methanolic extract had a moderate activity against all tested bacteria. The highest activity recorded with dichloromethane extract in *Bacillus cereus* and *Escherichia coli* with an inhibition zone of 26 mm. Results from our study contradict the results obtained in their study, as the *Centella asiatica* L. methanolic extract showed a significant activity against *Escherichia coli*, *Staphylococcus aureus*, *Pseudomonas aeruginosa*, and *Enterococcus hirae*. The difference in findings between our study and previous studies might be due to different extraction techniques being followed, tested microorganism, and choice of antibacterial test. In our study, the antibacterial susceptibility tests followed all the standards of the Clinical and Laboratory Standards Institute (2007). Other studies conducted by Harun *et al.,* (2011); Thamyris *et al.,* (2014); Paul *et al.,* (2011) have previously reported that *Imperata cylindrica* L., *Morinda citrifolia* L., and *Sauropus androgynus* L. methanolic Soxhlet extracts had a potential antibacterial activity, in particular against *Staphylococcus aureus*, their results agreed with the findings of our study.

Antibacterial compounds may affect bacterial cells in a variety of ways, many of them are poorly understood. At high concentrations, many agents are so destructive. Under certain conditions, some agents may specifically disrupt the cell membrane (Filomena *et al.,* 2013). Many of the cell's essential enzymes possess sulfhydryl (-SH) groups and can only function if they remain free and reduced, hence agents which oxidise or combine with sulfhydryl groups possess a strong inhibitory action. Many agents may act by interfering with one or specific

enzymatic reactions which is called chemical antagonism (Rakesh, 2012). There are few principle modes of action of antibacterial agents, such as the inhibition of the cell wall synthesis, inhibition of protein synthesis, inhibition of DNA replication, and disruption of the cell wall membrane function in intracellular metabolism. Some of the phytochemical groups of medicinal plants, such as flavonoids, appear to make the external structure of the cell wall permeable thus changing the physiology of bacteria increased leading to leakage of the cell contents (Kumar & Pandey, 2013). Most of methanolic extracted compounds in this study acted preferentially on Gram-positive bacteria, in particular *Staphylococcus aureus*, this may be due to the high lipid and low peptidoglycan content in Gram negative bacteria.

4.4 Conclusion

With a dwindling arsenal of effective antibiotics and increasing rates of bacterial resistance, alternative agents are desperately needed. In the present study, according to the presented data, it was found that methanolic Soxhlet crude extracts of *Centella asiatica* L., *Imperata cylindrica* L., *Morinda citrifolia* L., and *Sauropus androgynus* L. demonstrated a significant inhibitory effect on both Gram positive and negative bacteria ($P< 0.05$). Other extracts, which were ethanol, chloroform, and hexane, inhibited most of bacterial strains under investigation with less to that shown by methanolic extracts, and in some cases there was no activity. Quantitative measurements of the effect of the extracts were conducted using various assays. According to the results given in this chapter, methanolic Soxhlet extracts were chosen for further fractionation and purification to revel the compounds responsible for inhibitory effects demonstrated.

Chapter 5

Bioassay Guided Purification

5.1 Separation and purification of methanolic extracts by (HPLC)

5.1.1 Introduction

Methanolic crude extracts of *Centella asiatica* L., *Imperata cylindrica* L., *Morinda citrifolia* L., and *Sauropus androgynus* L. were the most active extracts against tested bacteria. In this study, reverse phase, analytical and preparative, high performance liquid chromatography (RP-HPLC) was employed for fractionation and purification of the crude extracts. Application of HPLC has increased in recent years for the analysis of plant-derived natural products, it has proven to be a very powerful technique in the detection and separation of many compounds. The success of HPLC separations is due in part to the selection of mobile phases (solvent), column type, oven temperature and type of detector. All of these variables are changeable depending on the type of compounds trying to be identified. For plant extracts, the choice of column is of the utmost importance as a crude sample could decrease the lifetime of a column, which should be protected by the addition of a guard column and following the right protocols (Bernal *et al.*, 2011).

When developing a method for HPLC analysis, there are three import overall goals, an adequate resolution of analytes, short running time, and reproducible process that is not affected by small variations throughout the process. The stationary phase should be chosen based on the compounds present in the sample. In this project, reverse-phase (RP) was chosen, this method works best for low molecular mass molecules as well as neutral compounds (Cseke *et al.*, 2006). Isocratic elution was selected in this study where one solvent system at a fixed flow rate was used throughout the entire elution process. According to the study conducted by Friesen *et al.*, (2015), this method is easy to perform, reliable, and sufficient for separations of plant material mixtures. However, for a more complex sample, it may be necessary to use a gradient elution, which uses two or more different solvents of varying elution strengths.

5.2 Results

5.2.1 Optimisation of the solvent system using analytical RP-HPLC

As an initial step in the separation process, analytical RP-HPLC was performed on the absolute methanolic extracts of medicinal plant materials. The aims of this analysis were to evaluate the complexity of the mixture of compounds contained within these samples and to

develop a method with the best separation possible (see chapter 3/section 3.2.3.2). The separations were conducted by injecting an equal amount of sample onto the C-18 analytical HPLC column. Three solvent systems were used in this study for optimisation of the analytical RP-HPLC parameters. The first system was an isocratic elution using a mobile phase of 0.1 % TFA in water: methanol 90:10 (v/v), the second was an isocratic elution run using 0.1 % TFA in water: methanol with a ratio of 85:15 (v/v), and the last solvent system was an isocratic elution using a ratio 80:20 (v/v) of water: methanol containing 0.1 % TFA.

The chromatograms of analytical HPLC crude extract profile of *Centella asiatica* L., *Imperata cylindrica* L., *Morinda citrifolia* L., and *Sauropus androgynus* L. (as shown in figure 5.1, 5.2, 5.3, and 5.4), displayed several major peaks within the first 10 minutes of the separation. The chromatograms showed that the early eluting peaks, ranging from 0 min to 20 minutes, in retention time, were overlapped when the mobile phases of 0.1 % TFA in water: methanol 90:10 and 80:10 (v/v) were used, and no separation was achieved. However, from visual observation, mobile phase of 0.1 % TFA in water: methanol 85:15 (v/v) produced a better separation with sharp and relatively symmetrical peaks. As a result, the 85:15 (v/v) of water: methanol containing 0.1 % TFA isocratic system was chosen for a larger scale preparative HPLC in this study.

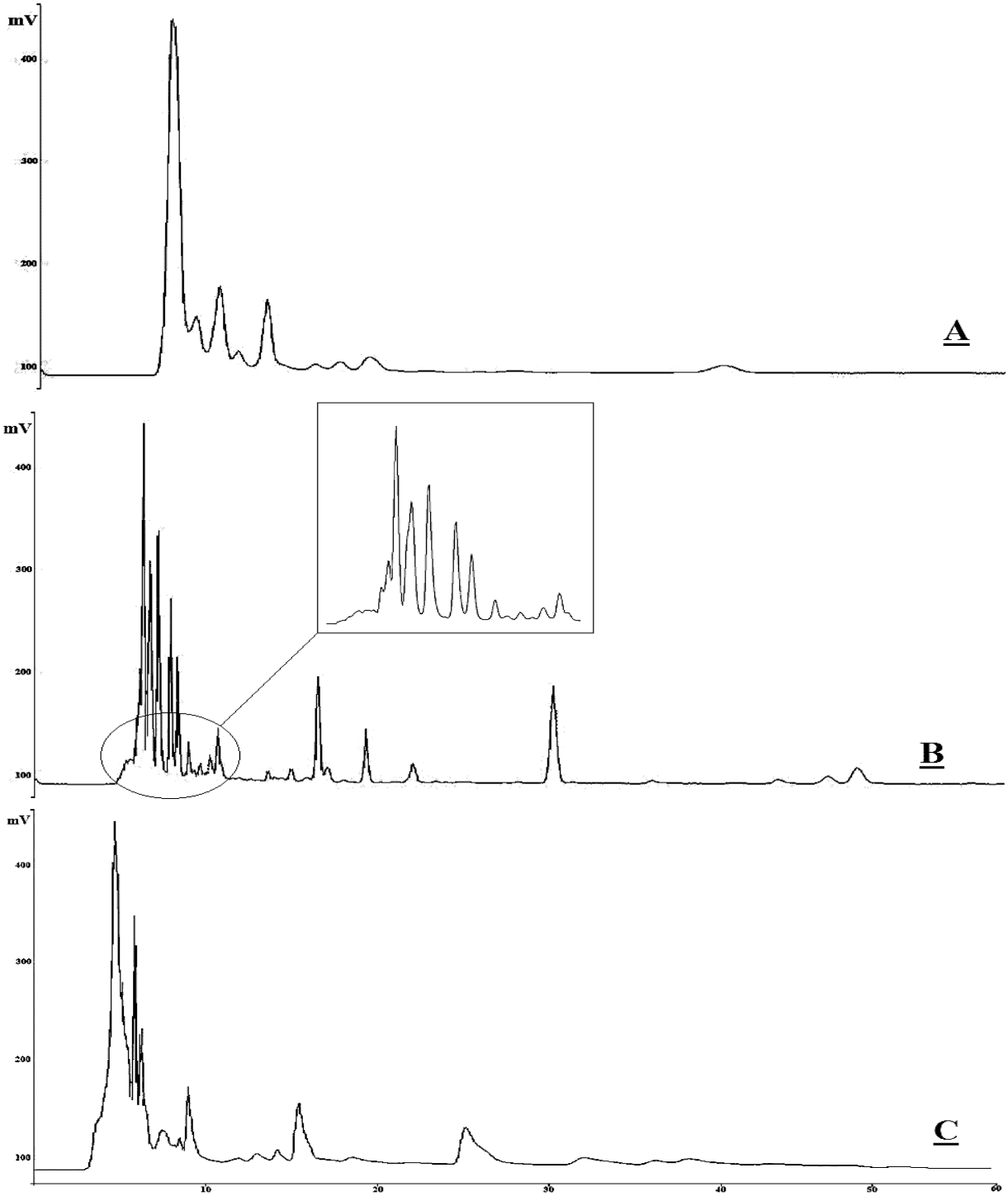


Figure 5.1: Analytical HPLC profiles of *Centella asiatica* L. methanolic extract using different solvent system.

The HPLC column was a reverse-phase C-18 Kinetex analytical column (250 x 4.6 mm and 5 µm particle diameter). The solvent systems were: (A) isocratic mobile phase of 0.1% TFA in water: methanol 90:10 (v/v), (B) isocratic mobile phase of 0.1 % TFA in water: methanol 85:15 (v/v), and (C) isocratic mobile phase of 0.1 % TFA in water: methanol 80:20 (v/v). The injection volume was 20 µl with a sample concentration of 10 mg/ml. The chromatograms were recorded at 260 nm for 60 min.

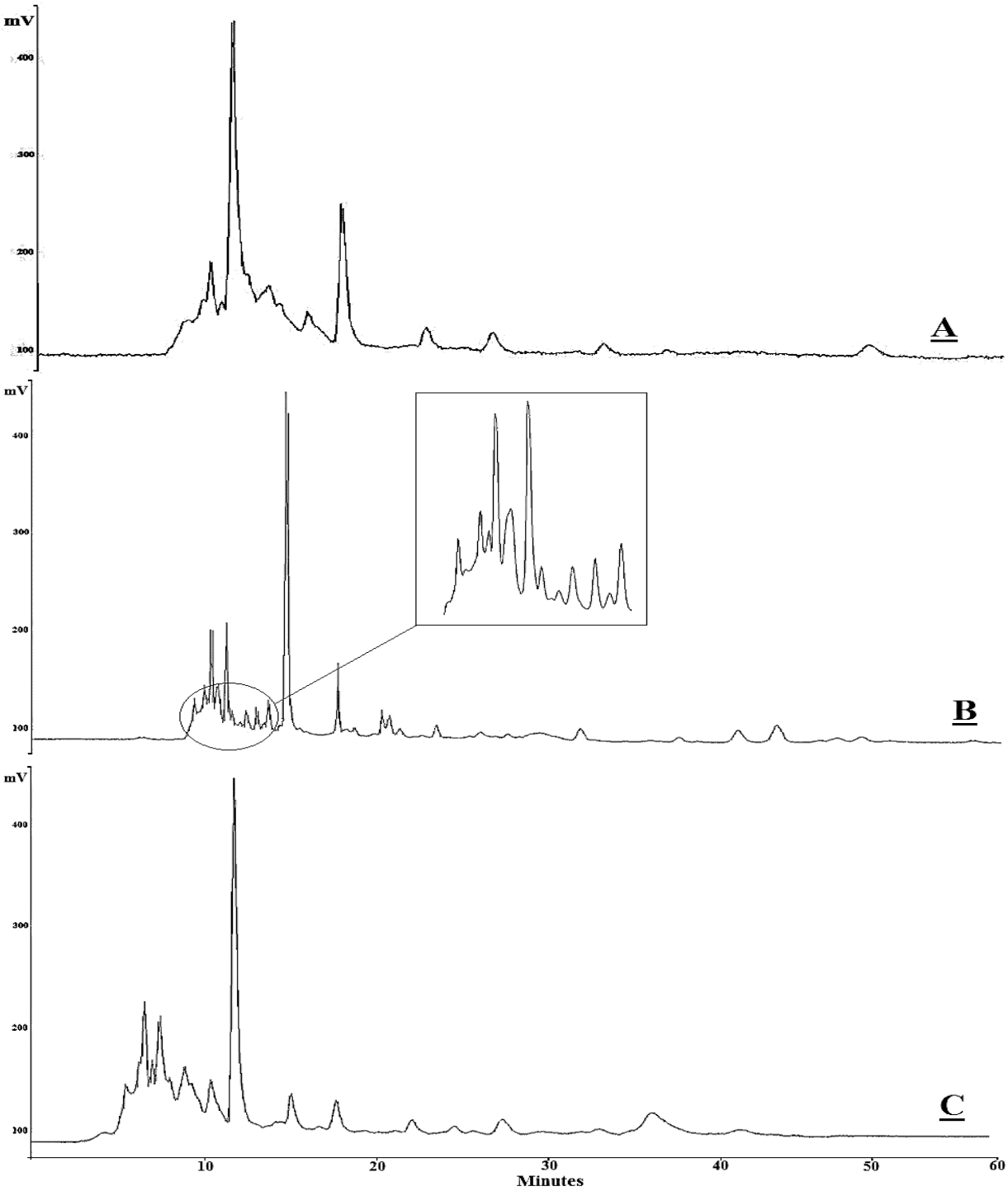


Figure 5.2: Analytical HPLC profiles of *Imperata cylindrica* L. methanolic extract using different solvent system.

The HPLC column was a reverse-phase C-18 Kinetex analytical column (250 x 4.6 mm and 5 µm particle diameter). The solvent systems were: (A) isocratic mobile phase of 0.1% TFA in water: methanol 90:10 (v/v), (B) isocratic mobile phase of 0.1 % TFA in water: methanol 85:15 (v/v), and (C) isocratic mobile phase of 0.1 % TFA in water: methanol 80:20 (v/v). The injection volume was 20 µl with a sample concentration of 10 mg/ml. The chromatograms were recorded at 260 nm for 60 min.

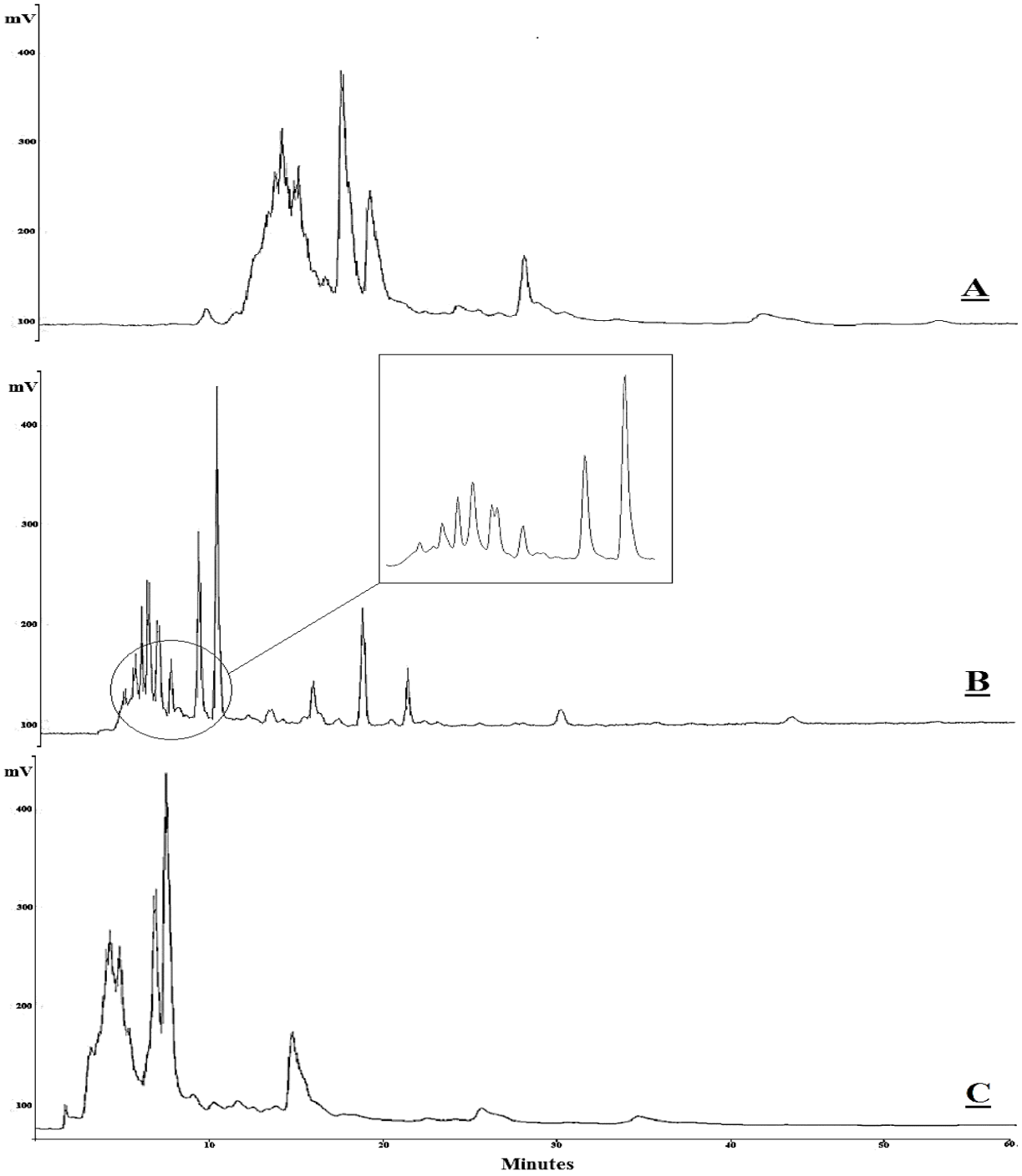


Figure 5.3: Analytical HPLC profiles of *Morinda citrifolia* L. methanolic extract using different solvent system.

The HPLC column was a reverse-phase C-18 Kinetex analytical column (250 x 4.6 mm and 5 µm particle diameter). The solvent systems were: (A) isocratic mobile phase of 0.1% TFA in water: methanol 90:10 (v/v), (B) isocratic mobile phase of 0.1 % TFA in water: methanol 85:15 (v/v), and (C) isocratic mobile phase of 0.1 % TFA in water: methanol 80:20 (v/v). The injection volume was 20 µl with a sample concentration of 10 mg/ml. The chromatograms were recorded at 260 nm for 60 min.

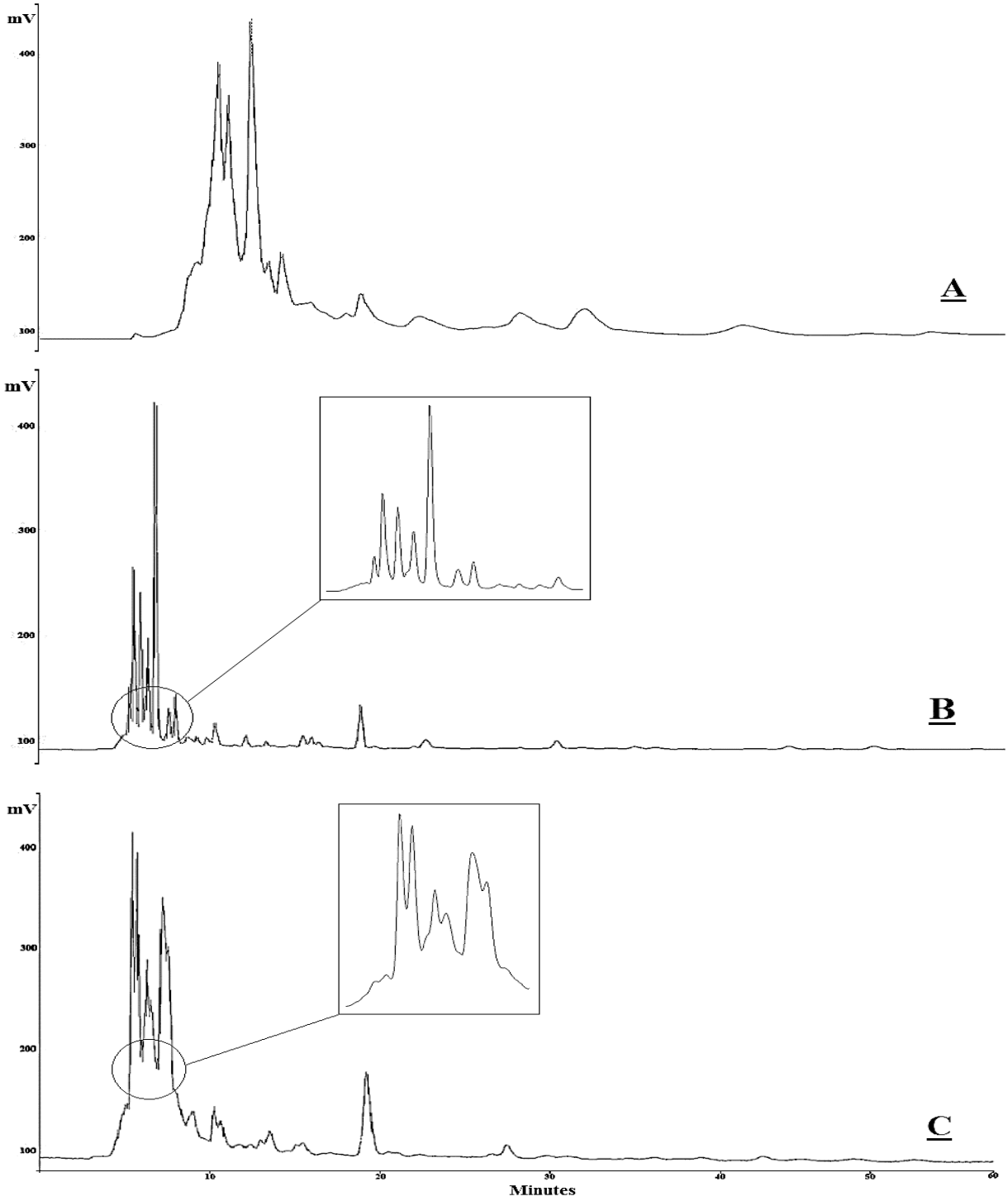


Figure 5.4: Analytical HPLC profiles of *Sauropus androgynus* L. methanolic extract using different solvent system.

The HPLC column was a reverse-phase C-18 Kinetex analytical column (250 x 4.6 mm and 5 µm particle diameter). The solvent systems were: (A) isocratic mobile phase of 0.1% TFA in water: methanol 90:10 (v/v), (B) isocratic mobile phase of 0.1 % TFA in water: methanol 85:15 (v/v), and (C) isocratic mobile phase of 0.1 % TFA in water: methanol 80:20 (v/v). The injection volume was 20 µl with a sample concentration of 10 mg/ml. The chromatograms were recorded at 260 nm for 60 min.

5.2.2 Separation of methanolic extracts using preparative RP-HPLC

According to the analytical HPLC separation results, obtained from previous stage, preparative chromatography was conducted on the methanolic extracts of medicinal plants under investigation. The HPLC elution used was an isocratic mobile phase consisted of 0.1 % TFA in water: methanol 85:15 (v/v). The column used was ACE reverse phase column (C-18, 5µm, 100Å, 250x21.2 mm, Hichrom Ltd, UK) with a flow rate of 10 ml/min for the period of 60 min. Preparative RP-HPLC showed various component elution of methanolic extracts, each peak was collected and pooled together manually according to the retention time. Given that, one or more of that eluted compounds were responsible for the antibacterial activity shown in previous chapter. All fractions were evaporated under reduced pressure and freeze dried. Each fraction was weighed, and the percentage yield was calculated relative to the amount of the material injected into the column.

Ten fractions were collected from *Centella asiatica* L. (figure 5.5), eight of them eluted during first 20 minutes of the run. Fraction 9 produced the highest yield (3.3 %) out of the total injected material, and the retention time was 30.28 min. While fraction 10 recorded the lowest yield of only (1.1 %), with a retention time of 44.50 min. Table (5.1) illustrates the retention time (tR) and the names given to the fractions with the percentage yield of each fraction. *Imperata cylindrica* L. methanolic extract produced twelve fractions (figure 5.6). The highest yield recorded in fraction 1 (4.6 %) with a retention time of 4.47 min, whilst the lowest yield in was recorded in fraction 9 of only (0.7 %) with a retention time of 22.55 min. The yield of this fraction was the lowest recorded amongst all fractions collected in this study. Table (5.2) illustrates the retention time and the names given to the fractions with the percentage yield of each fraction. Ten fractions were collected from *Morinda citrifolia* L. methanolic extract (figure 5.7). The highest yield was recorded in fraction 1 (3.5 %) (table 5.3), with a retention time of 4.40 min, whilst the lowest recorded in fraction 10, (1 %) with a retention time of 46 min. Finally, the preparative RP-HPLC of *Sauropus androgynus* L. methanolic extract produced nine fractions (figure 5.8), fraction 1 showed the highest yield in all collected fractions in this study (5.3 %), with a retention time of 6.17 min. While, fraction 9 produced the lowest yield in this plant (0.9 %) with a retention time of 47.10 min. Table (5.4) shows the retention time and the names given to the fractions with their percentage yield.

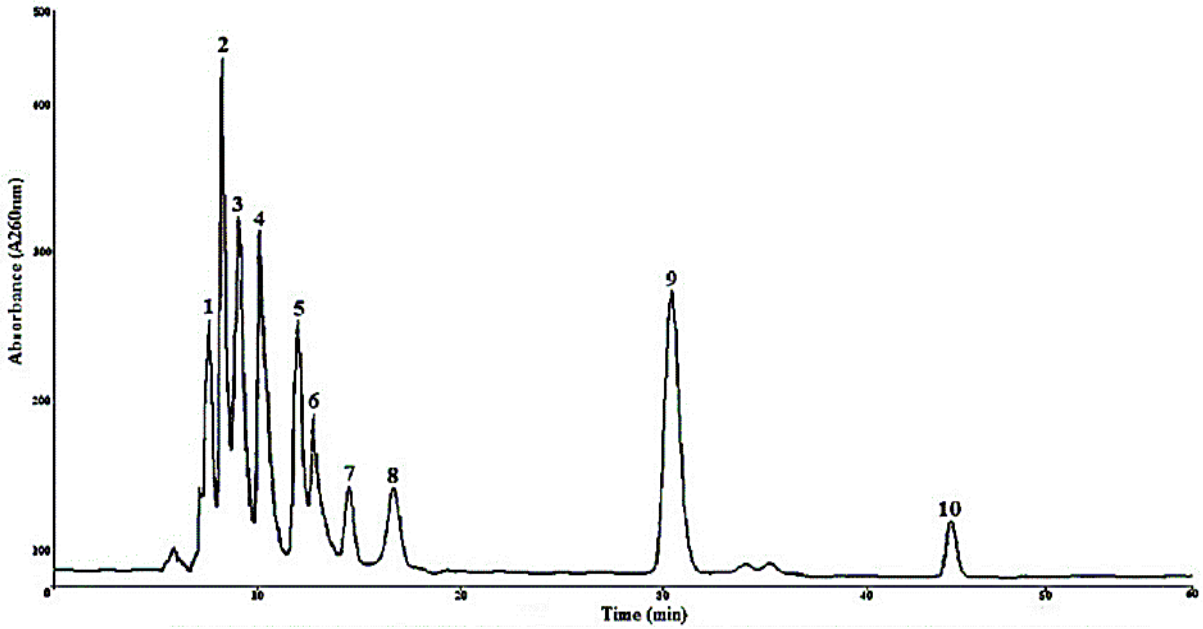


Figure 5.5: Preparative RP-HPLC chromatogram of *Centella asiatica* L.

The preparative HPLC of the Soxhlet methanolic extract was performed using a reverse-phase ACE 5 C18 250 x 21.2 mm column, with 5 µm particle size, the isocratic mobile phase consists of water: methanol 85:15 (v/v) with 0.1 % TFA (v/v), at a flow rate of 10 ml/ min. The chromatogram was run for 60 min. The injected sample volume was 0.5ml, and the concentration was 100 mg/ml. The chromatogram was recorded at 260 nm.

***Centella asiatica* (L.) fractions**			
Number	**Name of the Fraction**	**Retention time (min)**	**Yield % (w/w)**
1	CA1	7.20	2.6 %
2	CA2	8.37	2.0 %
3	CA3	9.26	1.8 %
4	CA4	10.40	2.9 %
5	CA5	12.30	2.2 %
6	CA6	13.40	1.3 %
7	CA7	14.51	1.9 %
8	CA8	16.45	2.3 %
9	CA9	30.28	3.3 %
10	CA10	44.50	1.1 %

Table 5.1: The retention time and weight of the compounds separated from the methanolic extract of *Centella asiatica* L. by preparative RP-HPLC.

- % yield = (weight of the fraction / weight of plant material injected) ×100

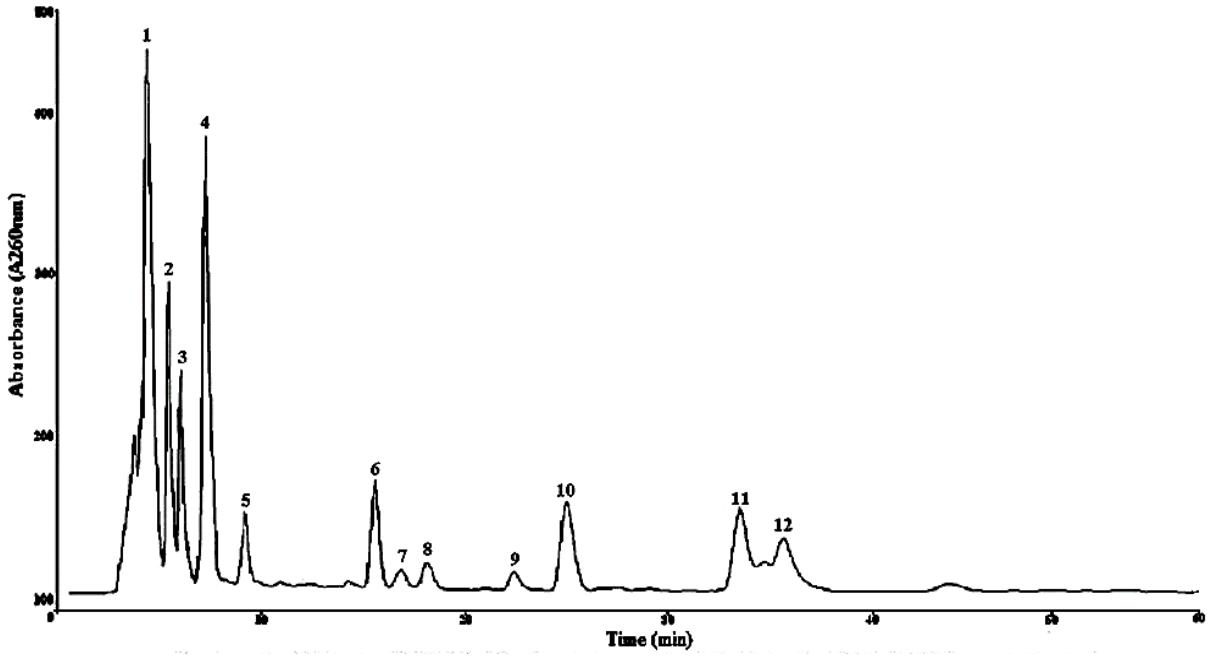


Figure 5.6: Preparative RP-HPLC chromatogram of *Imperata cylindrica* L.

The preparative HPLC of the Soxhlet methanolic extract was performed using a reverse-phase ACE 5 C18 250 x 21.2 mm column, with 5 μm particle size, the isocratic mobile phase consists of water: methanol 85:15 (v/v) with 0.1 % TFA (v/v), at a flow rate of 10 ml/ min. The chromatogram was run for 60 min. The injected sample volume was 0.5ml, and the concentration was 100 mg/ml. The chromatogram was recorded at 260 nm.

***Imperata cylindrica* (L.) fractions**			
Number	**Name of the Fraction**	**Retention time (min)**	**Yield % (w/w)**
1	IC1	4.47	4.6 %
2	IC2	6.12	2.3 %
3	IC3	7.40	1.5 %
4	IC4	8.10	2.9 %
5	IC5	9.25	1.4 %
6	IC6	16.20	1.8 %
7	IC7	17.40	0.8 %
8	IC8	18.45	1.1 %
9	IC9	22.55	0.7 %
10	IC10	24.50	2.1 %
11	IC11	34	1.8 %
12	IC12	36.10	1.2 %

Table 5.2: The retention time and weight of the compounds separated from the methanolic extract of *Imperata cylindrica* L. by preparative RP-HPLC.

- % yield = (weight of the fraction / weight of plant material injected) ×100

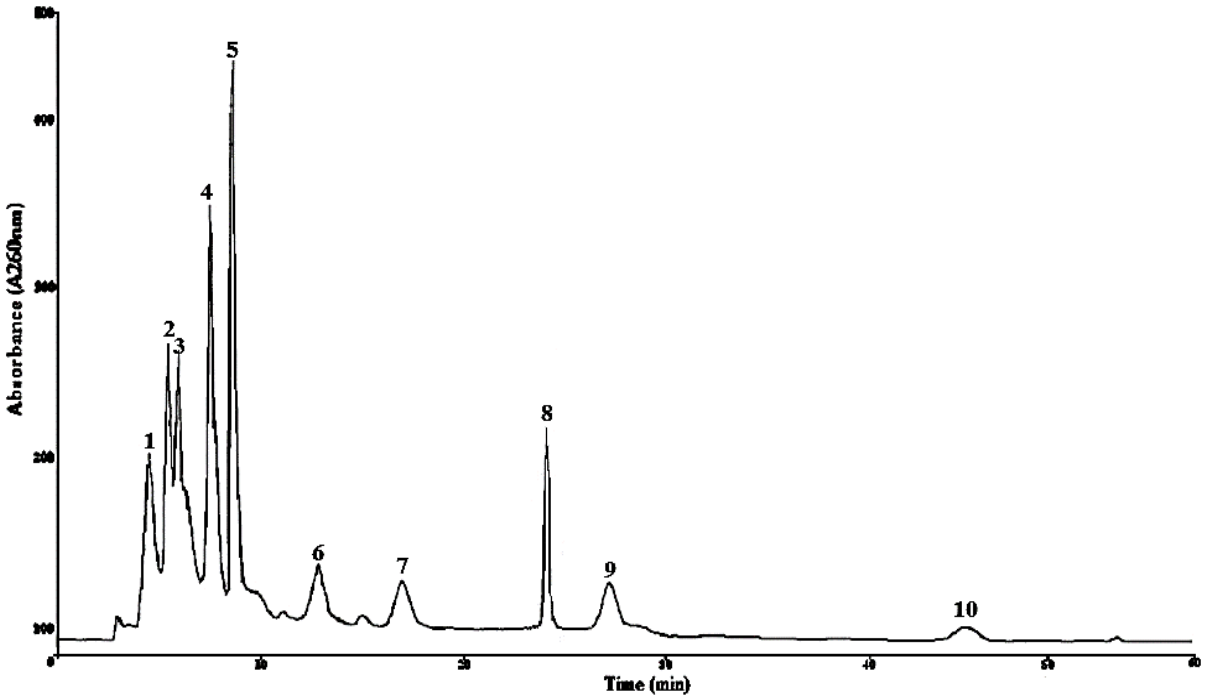


Figure 5.7: Preparative RP-HPLC chromatogram of *Morinda citrifolia* L.

The preparative HPLC of the Soxhlet methanolic extract was performed using a reverse-phase ACE 5 C18 250 x 21.2 mm column, with 5 µm particle size, the isocratic mobile phase consists of water: methanol 85:15 (v/v) with 0.1 % TFA (v/v), at a flow rate of 10 ml/ min. The chromatogram was run for 60 min. The injected sample volume was 0.5ml, and the concentration was 100 mg/ml. The chromatogram was recorded at 260 nm.

***Morinda citrifolia* (L.) fractions**			
Number	**Name of the Fraction**	**Retention time (min)**	**Yield % (w/w)**
1	MC1	4.40	3.5 %
2	MC2	5.25	2.4 %
3	MC3	6.14	1.7 %
4	MC4	7.48	2.2 %
5	MC5	8.35	1.8 %
6	MC6	13.10	1.6 %
7	MC7	16.50	2.4 %
8	MC8	24	1.3 %
9	MC9	26.40	2.1 %
10	MC10	46	1 %

Table 5.3: The retention time and weight of the compounds separated from the methanolic extract of *Morinda citrifolia* L. by preparative RP-HPLC.

- % yield = (weight of the fraction / weight of plant material injected) ×100

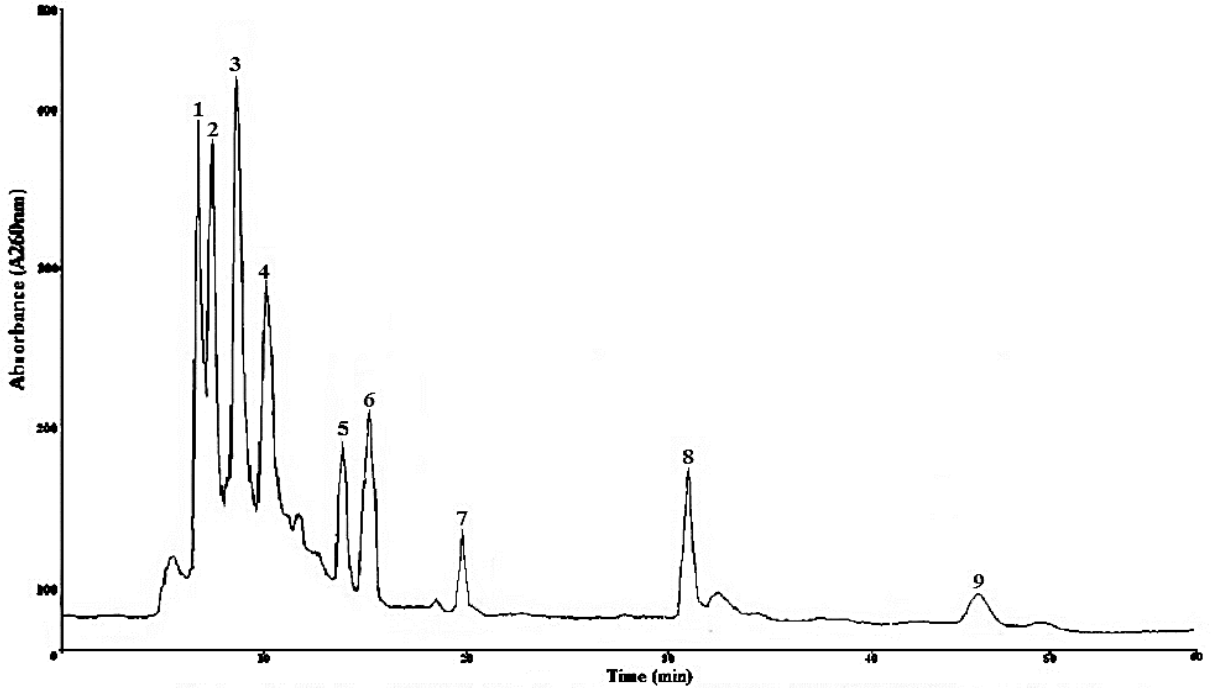


Figure 5.8: Preparative RP-HPLC chromatogram of *Sauropus androgynus* L.

The preparative HPLC of the Soxhlet methanolic extract was performed using a reverse-phase ACE 5 C18 250 x 21.2 mm column, with 5 µm particle size, the isocratic mobile phase consists of water: methanol 85:15 (v/v) with 0.1 % TFA (v/v), at a flow rate of 10 ml/ min. The chromatogram was run for 60 min. The injected sample volume was 0.5ml, and the concentration was 100 mg/ml. The chromatogram was recorded at 260 nm.

***Sauropus androgynus* (L.) fractions**			
Number	**Name of the Fraction**	**Retention time (min)**	**Yield % (w/w)**
1	SA1	6.17	5.3 %
2	SA2	7.10	3.8 %
3	SA3	8.30	3.1 %
4	SA4	10.10	2.0 %
5	SA5	14	1.9 %
6	SA6	15.50	2.4 %
7	SA7	19.40	1.5 %
8	SA8	31.25	1.9 %
9	SA9	47.10	0.9 %

Table 5.4: The retention time and weight of the compounds separated from the methanolic extract of *Sauropus androgynus* L. by preparative RP-HPLC.

- % yield = (weight of the fraction / weight of plant material injected) ×100

5.2.3 Antibacterial testing of HPLC purified fractions

All the HPLC purified fractions from the four medicinal plants under investigation were next subjected to an antibacterial screening to see the most active fractions. Three Gram-negative and three Gram-positive bacterial strains were screened, Gram-negative strains were; *Escherichia coli* (ATCC 25922), *Pseudomonas aeruginosa* (ATCC 15442) and *Klebsiella pneumoniae* (NCTC 13438), whilst, Gram-positive strains were; *Staphylococcus aureus* MSSA (ATCC 6538), *Staphylococcus aureus* MRSA (NCTC 13142), and *Enterococcus hirae* (ATCC 10541). All the data under the growth curve, obtained from the Bioscreen-C system, were processed using Microsoft Excel, according to the approach described earlier (chapter 3, section 3.2.2.2), to calculate the MIC of each fraction.

The results of the antibacterial assay of *Centella asiatica* L. fractions from preparative RP-HPLC revealed that out of 10 fractions (Named as CA1-CA10), only three fractions demonstrated significant inhibitory effects against the tested strains. The fractions that showed antibacterial activity were CA3, CA4 and CA7 (Figure 5.7). The highest inhibitory activity was recorded in fraction CA7. The Gram-positive bacteria were significantly more sensitive than Gram negative bacteria. The MICs of fraction CA7 were 16, 21, 23, 17, 19, and 16 μg/ml respectively in *Escherichia coli*, *Pseudomonas aeruginosa*, *Klebsiella pneumoniae*, *Staphylococcus aureus* (MSSA), *Staphylococcus aureus* (MRSA), and *Enterococcus hirae*. The other two fractions were less active than CA7, *Klebsiella pneumoniae* was the most resistant strain among all three fractions with MICs of 41, 35, and 23 μg/ml respectively in CA3, CA4, and CA7.

The results of antibacterial screening assay of the *Imperata cylindrica* L. chemical compounds revealed that only two fractions (IC6 and IC10) had demonstrated significant antibacterial activity ($P < 0.05$), comparing with the positive control (Figure 5.8). Fraction IC10 was more active than a fraction IC6 with MIC values of 33, 24, 32, 36, 39, and 32 μg/ml respectively in *Escherichia coli*, *Pseudomonas aeruginosa*, *Klebsiella pneumoniae*, *Staphylococcus aureus* (MSSA), *Staphylococcus aureus* (MRSA), and *Enterococcus hirae*. However, *Enterococcus hirae* was more sensitive when exposed to fraction IC6, the MIC value recorded was (29 μg/ml), which is less than the value recorded in a fraction IC10 (32 μg/ml) for the same bacteria under the same conditions.

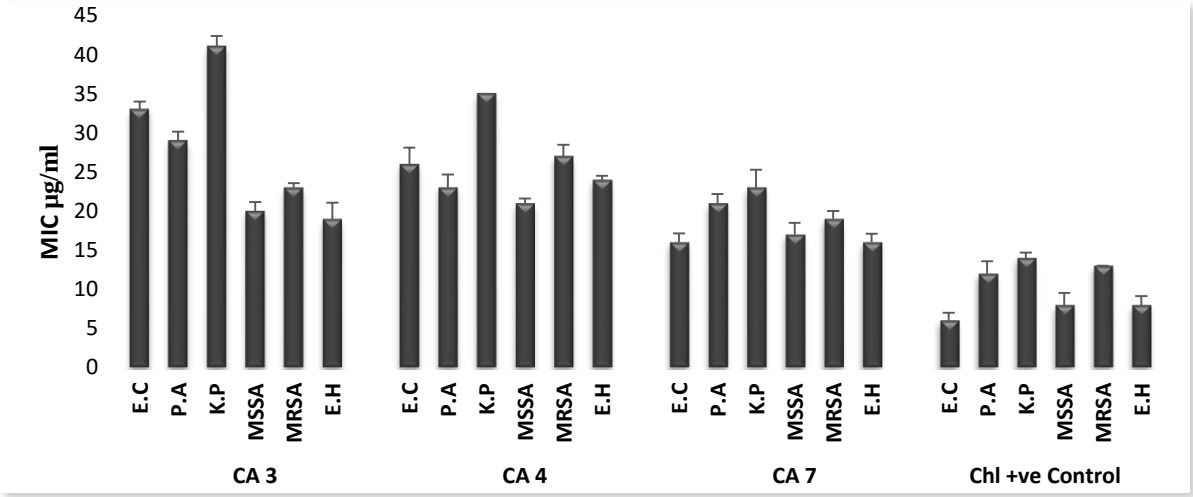


Figure 5.7: MIC values of *Centella asiatica* L. fractions (CA3, CA4 and CA7) against six different bacterial strains, namely; *Escherichia coli* (E.C), *Pseudomonas aeruginosa* (P.A), *Klebsiella pneumoniae* (K.P), *Staphylococcus aureus* (MSSA), *Staphylococcus aureus* (MRSA), and *Enterococcus hirae* (E.H). Chloramphenicol (Chl) used as a positive control.

- Data obtained from the Bioscreen-C system exported to the Microsoft Excel 2013 for MIC calculations under the growth curve.
- The result for each data point represents the average of three replicates, and the standard error of the mean (SEM) has been calculated for each dose.

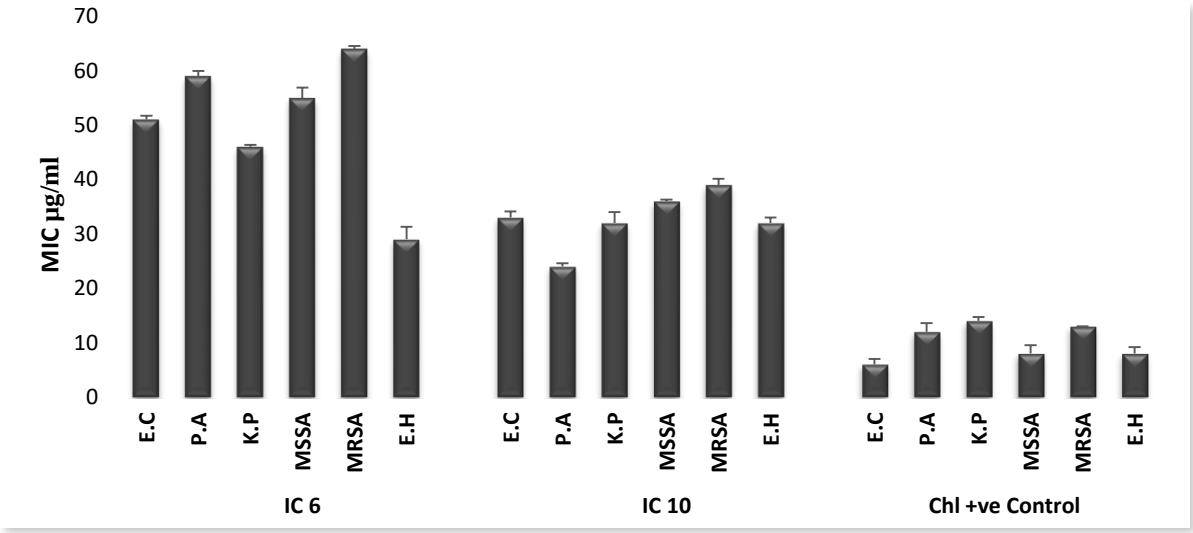


Figure 5.8: MIC values of *Imperata cylindrica* L. fractions (IC6 and IC10) against six different bacterial strains, namely; *Escherichia coli* (E.C), *Pseudomonas aeruginosa* (P.A), *Klebsiella pneumoniae* (K.P), *Staphylococcus aureus* (MSSA), *Staphylococcus aureus* (MRSA), and *Enterococcus hirae* (E.H). Chloramphenicol (Chl) used as a positive control.

- Data obtained from the Bioscreen-C system exported to the Microsoft Excel 2013 for MIC calculations under the growth curve.
- The result for each data point represents the average of three replicates, and the standard error of the mean (SEM) has been calculated for each dose.

Antibacterial activity was observed in three fractions only from *Morinda citrifolia* L., namely; MC4, MC7, and MC8 (figure 5.9). The fraction that demonstrated the highest activity, was MC4. The MIC values for MC4 were 8, 10, 17, 9, 13, and 7 μg/ml respectively in *Escherichia coli, Pseudomonas aeruginosa*, *Klebsiella pneumoniae*, *Staphylococcus aureus* (MSSA), *Staphylococcus aureus* (MRSA), and *Enterococcus hirae*. All bacterial strains tested were sensitive to MC7 and MC8 with variable MIC values. MC7 recorded the highest MIC of 36 μg/ml in *Pseudomonas aeruginosa*, whilst the lowest MIC recorded in *Staphylococcus aureus* (MSSA) was 16 μg/ml. MC8 recorded the lowest MIC in *Staphylococcus aureus* (MSSA) with 22 μg/ml, just like MC7, whilst the highest MIC was recorded in *Pseudomonas aeruginosa* of 41 μg/ml. Moreover, MC4 recorded lower MIC in *Enterococcus hirae* and *Pseudomonas aeruginosa* than the MIC of the positive control, the MICs were 7 μg/ml and 10 μg/ml in MC4 fraction, while the positive control recorded 8 μg/ml and 12 μg/ml respectively in the same strains. Finally, only three fractions demonstrated antibacterial activity in *Sauropus androgynus* L., the fractions were SA2, SA3, and SA7 (figure 5.10). Fraction SA7 showed the highest activity, among other two fractions with MICs of 24, 21, 34, 20, 18, and 20 μg/ml respectively in *Escherichia coli, Pseudomonas aeruginosa, Klebsiella pneumoniae, Staphylococcus aureus* (MSSA), *Staphylococcus aureus* (MRSA), and *Enterococcus hirae*.

By looking closely at figure (5.11), the data clearly showed that fraction MC4 of *Morinda citrifolia* L. was the most active fraction against all tested bacteria comparing to the positive control ($P<0.05$). The second most active fraction was CA7 of *Centella asiatica* L., then followed by fraction SA3 of *Sauropus androgynus* L., and the least active fraction was IC10 of *Imperata cylindrica* L. Now that we have identified active fractions from the four plant extracts, the four active fractions had been chosen for the next step of the project to illustrate their molecular structure by employing advanced spectrometry techniques including NMR and MS. Figure (5.12) showed the MBC values of tested bacterial strains. MC4 fraction of *Morinda citrifolia* L. showed the lowest MBCs of all tested bacteria with a value of 31.25 μg/ml, except in *Klebsiella pneumoniae* where the recorded MBC was 62.5 μg/ml. Figures (5.13 – 5.18) represent 1 X and ½ X MIC of the standard growth curve of a particular strain of all tested bacteria with MC4 fraction of *Morinda citrifolia* L., which was shown the highest activity among all other fractions.

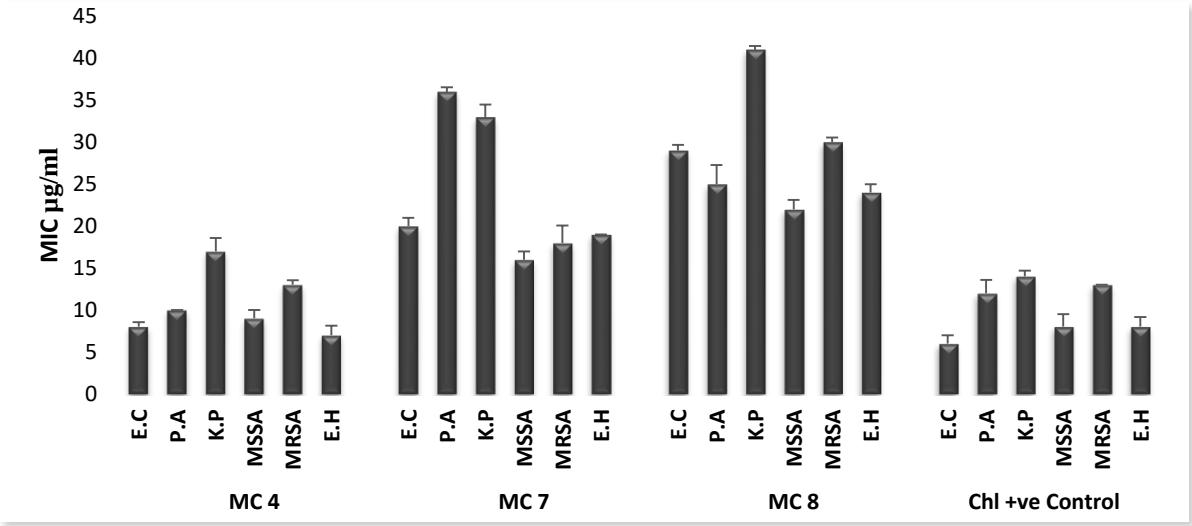


Figure 5.9: MIC values of *Morinda citrifolia* L. fractions (MC4, MC7 and MC8) against six different bacterial strains, namely; *Escherichia coli* (E.C), *Pseudomonas aeruginosa* (P.A), *Klebsiella pneumoniae* (K.P), *Staphylococcus aureus* (MSSA), *Staphylococcus aureus* (MRSA), and *Enterococcus hirae* (E.H). Chloramphenicol (Chl) used as a positive control.

- Data obtained from the Bioscreen-C system exported to Microsoft Excel 2013 for MIC calculations under the growth curve.
- The result for each data point represents the average of three replicates, and the standard error of the mean (SEM) has been calculated for each dose.

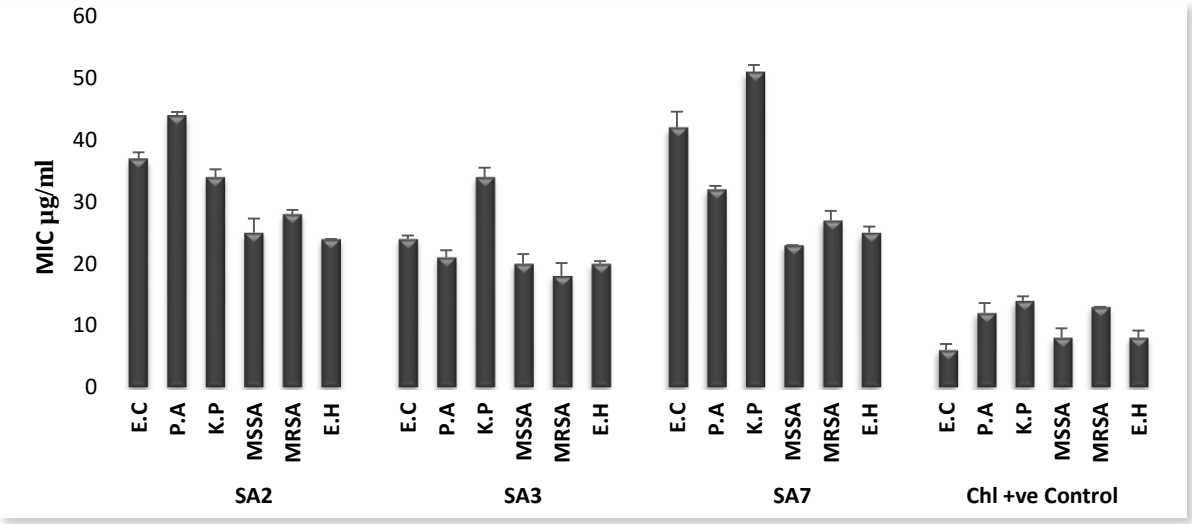


Figure 5.10: MIC values of *Sauropus androgynus* L. fractions (SA2, SA3 and SA7) against six different bacterial strains, namely; *Escherichia coli* (E.C), *Pseudomonas aeruginosa* (P.A), *Klebsiella pneumoniae* (K.P), *Staphylococcus aureus* (MSSA), *Staphylococcus aureus* (MRSA), and *Enterococcus hirae* (E.H). Chloramphenicol (Chl) used as a positive control.

- Data obtained from the Bioscreen-C system exported to Microsoft Excel 2013 for MIC calculations under the growth curve.
- The result for each data point represents the average of three replicates, and the standard error of the mean (SEM) has been calculated for each dose.

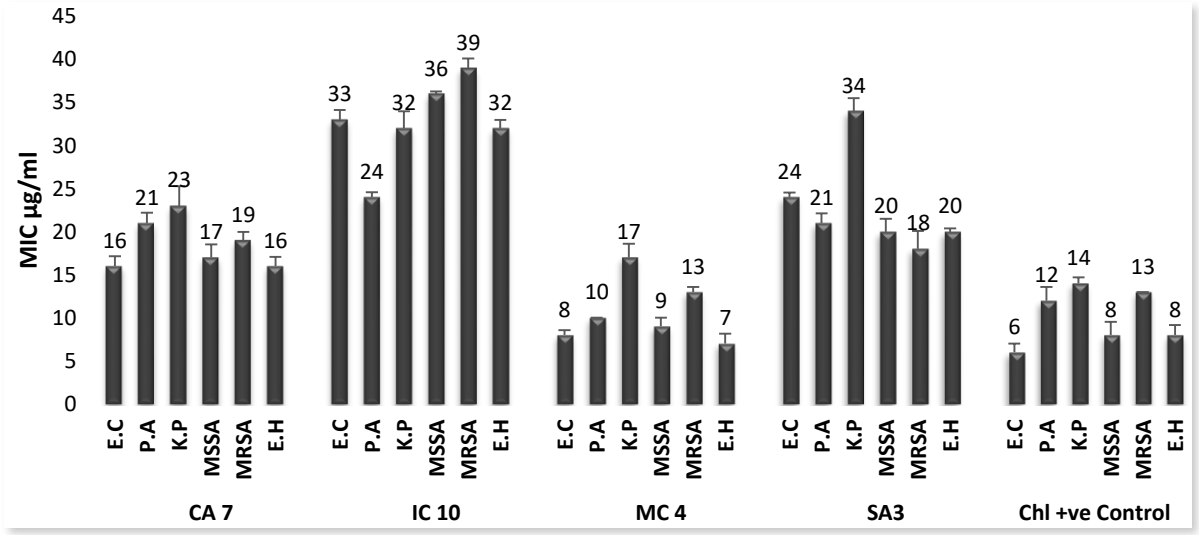


Figure 5.11: MIC values of the most active fractions CA7, IC10, MC4, and SA3, from medicinal plants under investigation, against six different bacterial strains, namely; *Escherichia coli* (E.C), *Pseudomonas aeruginosa* (P.A), *Klebsiella pneumoniae* (K.P), *Staphylococcus aureus* (MSSA), *Staphylococcus aureus* (MRSA), and *Enterococcus hirae* (E.H). Chloramphenicol (Chl) used as a positive control.

- Data obtained from the Bioscreen-C system exported to Microsoft Excel 2013 for MIC calculations under the growth curve.
- The result for each data point represents the average of three replicates, and the standard error of the mean (SEM) has been calculated for each dose.

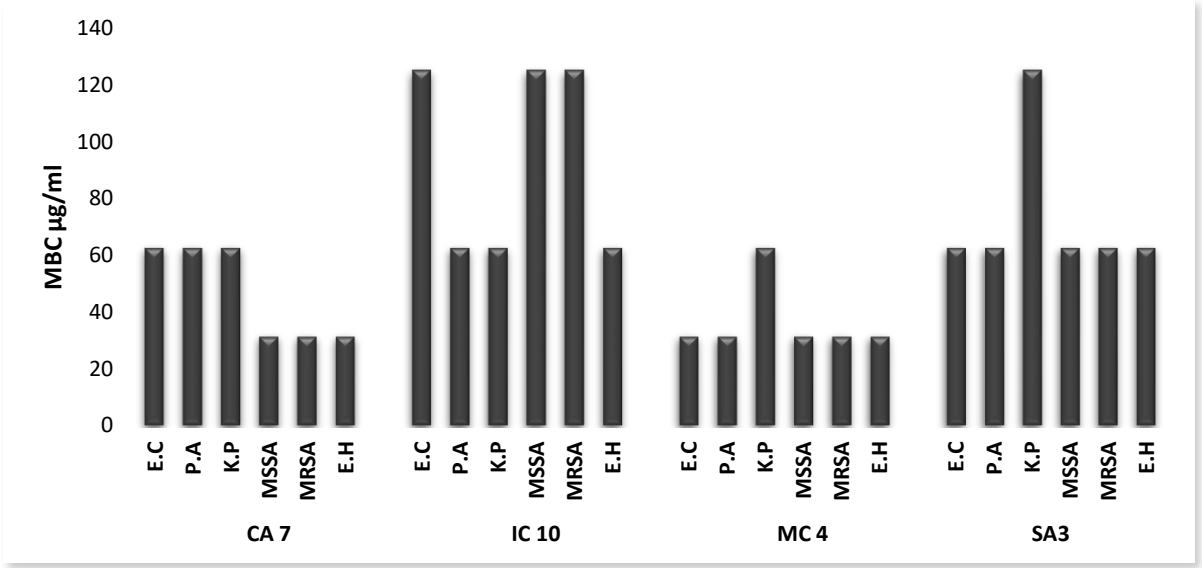


Figure 5.12: MBC values of the most active fractions CA7, IC10, MC4, and SA3, from medicinal plants under investigation, against six different bacterial strains, namely; *Escherichia coli* (E.C), *Pseudomonas aeruginosa* (P.A), *Klebsiella pneumoniae* (K.P), *Staphylococcus aureus* (MSSA), *Staphylococcus aureus* (MRSA), and *Enterococcus hirae* (E.H).

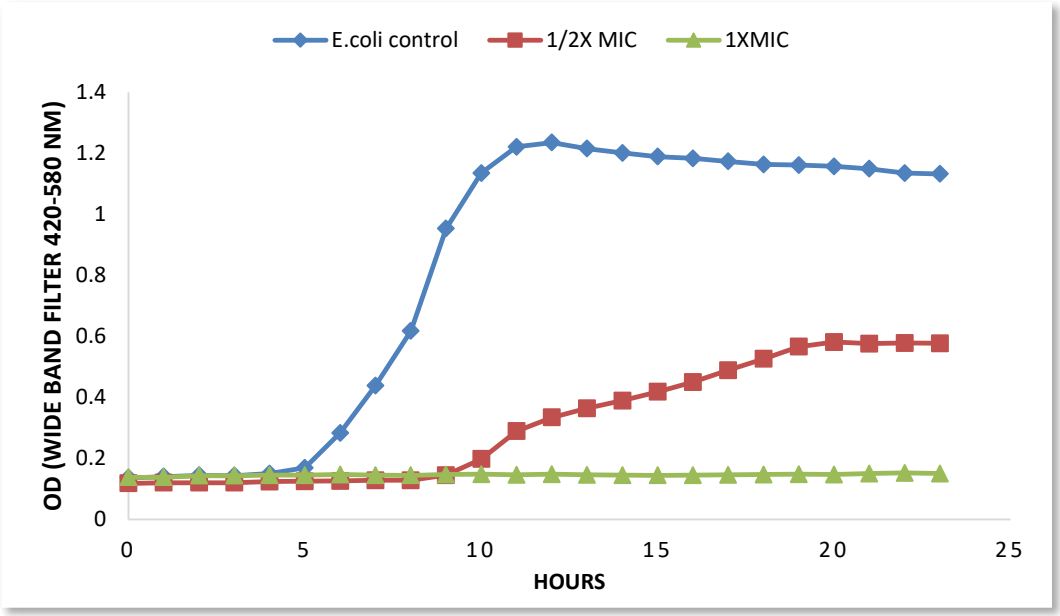


Figure 5.13: Standard *Escherichia coli* growth curve with 1 x and ½ x MIC activity of MC4 fraction of *Morinda citrifolia* L. under standard condition, 37ºC and 24 h incubation. All values represent a mean of three replicate tests.

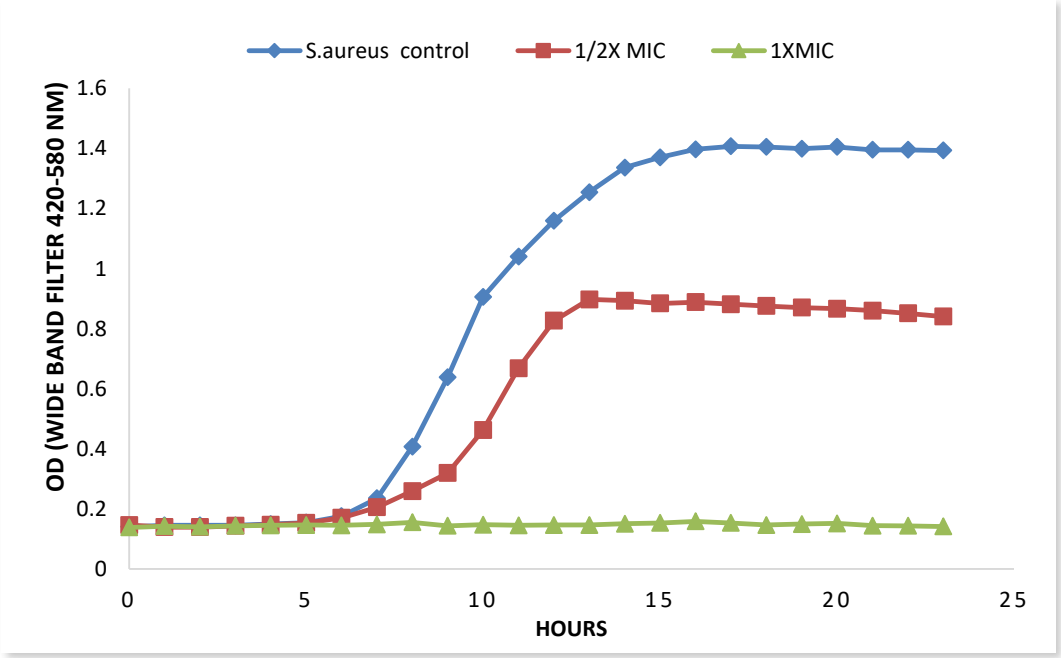


Figure 5.14: Standard *Staphylococcus aureus* growth curve with 1 x and ½ x MIC activity of MC4 fraction of *Morinda citrifolia* L. under standard condition, 37ºC and 24 h incubation. All values represent a mean of three replicate tests.

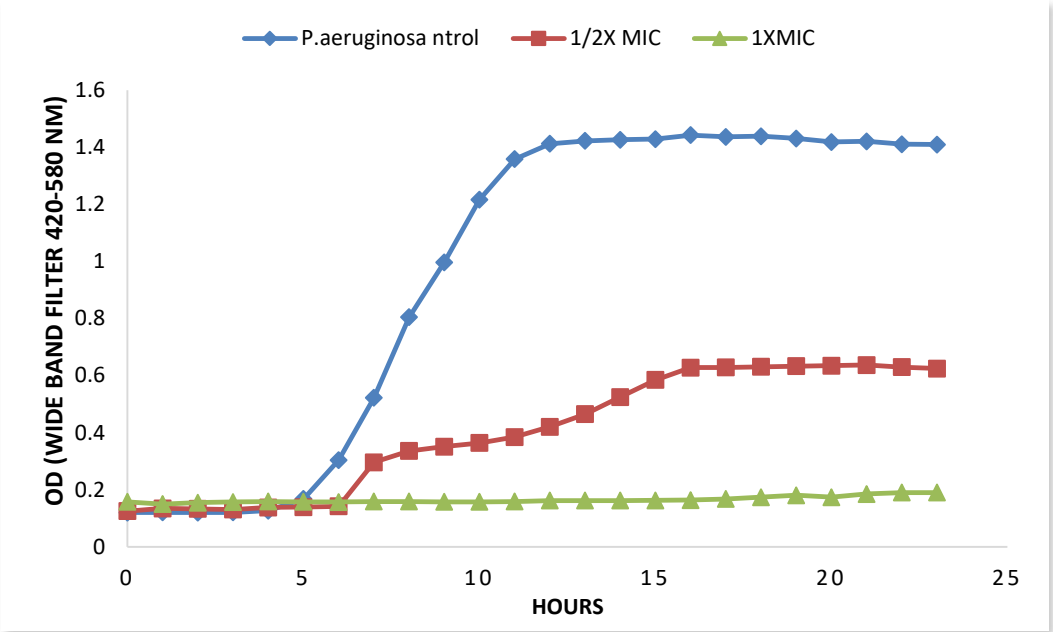


Figure 5.15: Standard *Pseudomonas aeruginosa* growth curve with 1 x and ½ x MIC activity of MC4 fraction of *Morinda citrifolia* L. under standard condition, 37ºC and 24 h incubation. All values represent a mean of three replicate tests.

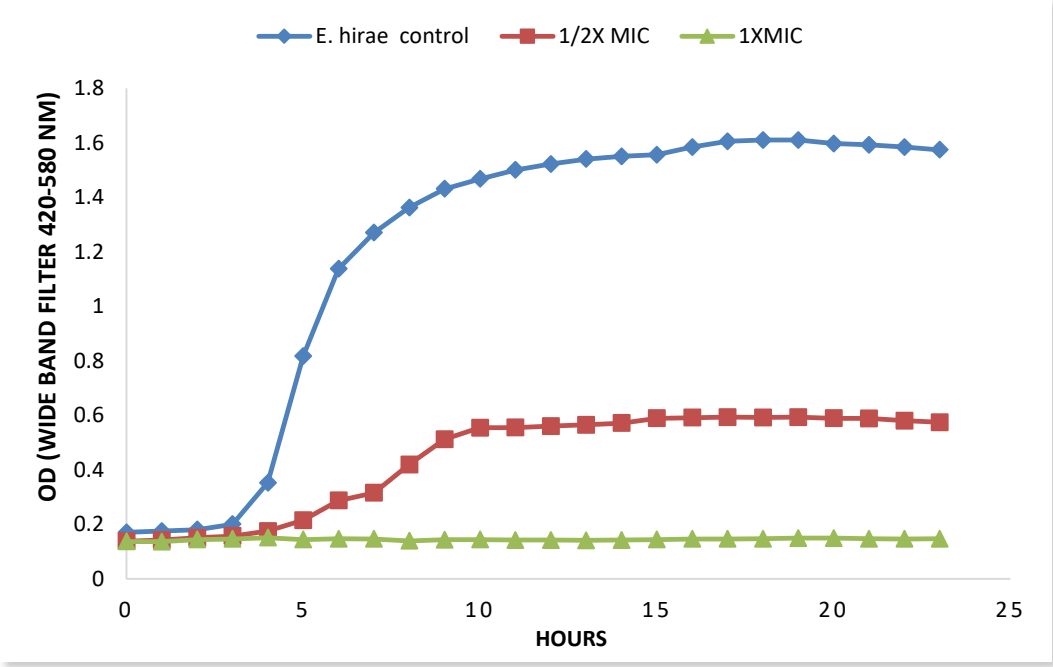


Figure 5.16: Standard *Enterococcus hirae* growth curve with 1 x and ½ x MIC activity of MC4 fraction of *Morinda citrifolia* L. under standard condition, 37ºC and 24 h incubation. All values represent a mean of three replicate tests.

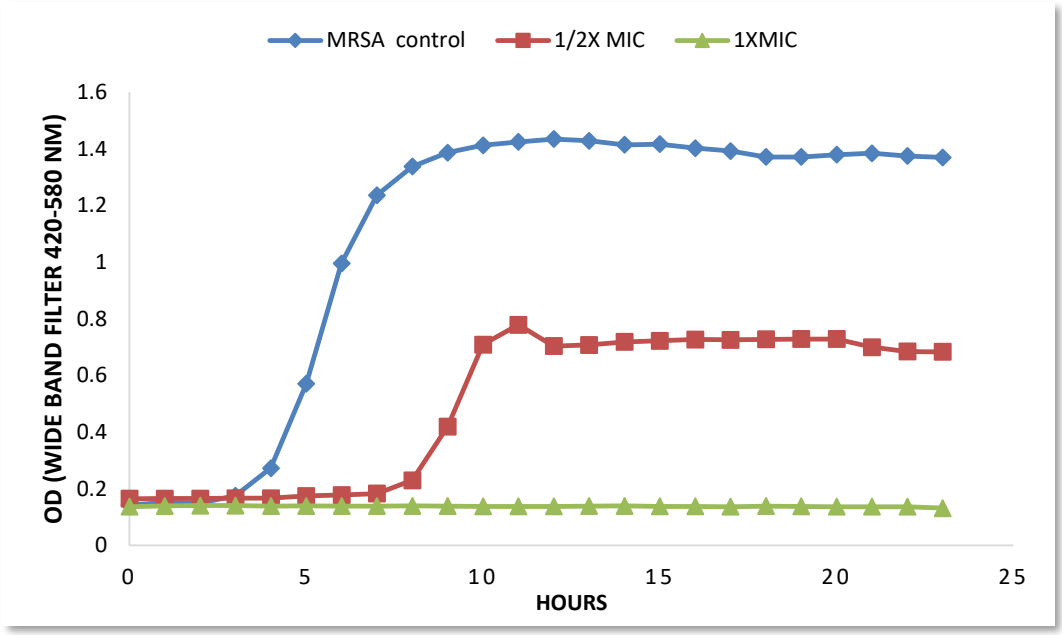


Figure 5.17: Standard *Staphylococcus aureus* (MRSA) growth curve with 1 x and ½ x MIC activity of MC4 fraction of *Morinda citrifolia* L. under standard condition, 37ºC and 24 h incubation. All values represent a mean of three replicate tests.

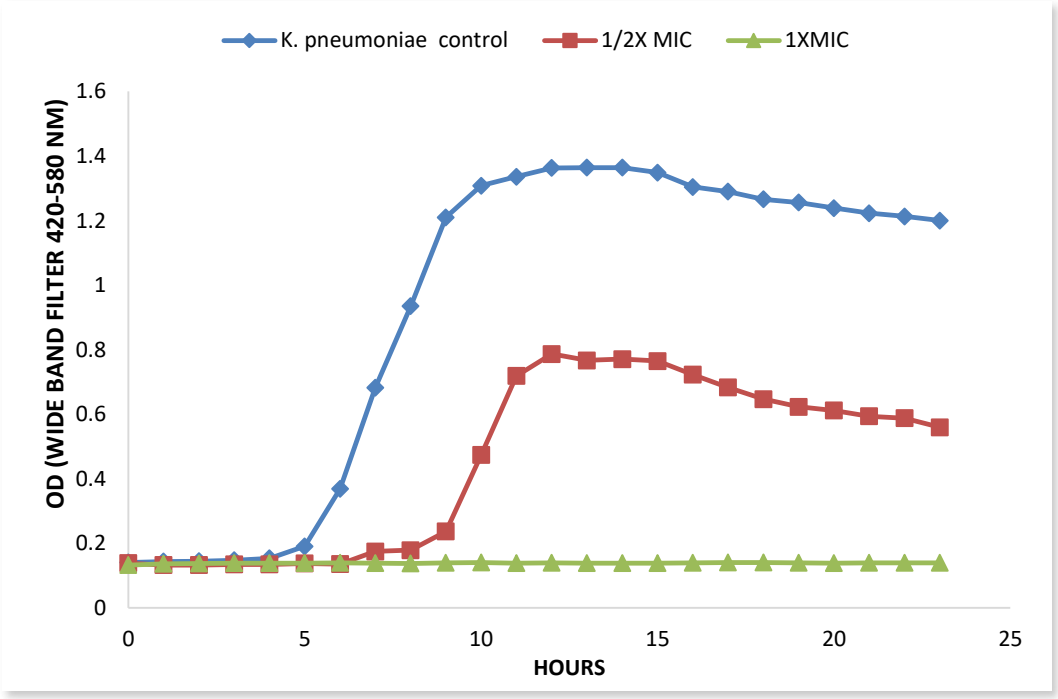


Figure 5.18: Standard *Klebsiella pneumoniae* growth curve with 1 x and ½ x MIC activity of MC4 fraction of *Morinda citrifolia* L. under standard condition, 37ºC and 24 h incubation. All values represent a mean of three replicate tests.

5.3 Discussion

Bioassay-guided isolation integrates the processes of separation of compounds in a mixture, using various analytical methods, with results obtained from biological testing. The process begins by testing a crude extract to confirm its activity, followed by separation of the compounds in the extracts and *in-vitro* biological testing of purified compounds. In some cases, further fractionation will be needed if the high purity level not achieved. Structural identification of the pure compounds, then follows (Jamil *et al.,* 2012). During the last few decades, there are several compounds have been isolated from different plants, where the bioassay-guided isolation has been used, and many of these have been reported to have substantial biological activities, some of which are of interest from the point of view of potential drug development (Khurram *et al.,* 2015; Ejele *et al.,* 2014; Kang *et al.,* 2013). In a study conducted by Unno *et al.,* (2004), they have isolated two bioactive compounds from the aqueous extract of the *Lagerstroemia speciosa* leaves by employing bioassay-guided fractionation approach. These compounds are valoneic acid dilactone and ellagic acid. Another study conducted by Malheiros and coworkers (2005), used using bioassay-guided fractionation to determine the antimicrobial activity of drimane sesquiterpenes from *Drimys brasiliensis*. Militao *et al.,* (2007) isolated six bioactive compounds by using bioassay-guided approach.

High performance liquid chromatography (HPLC) is an extremely robust, versatile, and usually rapid technique. It has the ability to separate, identify, and quantitate the compounds that are present in any sample that can be dissolved in a liquid. Nevertheless, HPLC methods present some limitations, especially in a very complex samples, thus, an initial separation and purification of a complex matrix is crucial prior to instrumental analysis by HPLC (Ignat *et al.,* 2011). RP-HPLC was used extensively in the separation and identification of the bio-active secondary metabolites from medicinal plants, such as, mahanine and mahanimbine alkaloids from *Murraya koenigii* (Pandit *et al.,* 2011), ginkgolide A, ginkgolide B, and bilobalide in *Ginkgo biloba* (Mesbah *et al.,* 2005), some triterpene saponins from *Chenopodium quinoa* (Tiwatt *et al.,* 2008). Analytical and preparative chromatography can be found in many studies dealing with the bio-active compounds from natural products. Analytical HPLC is commonly used in the early stages, prior to the isolation and purification of the active compounds for method development (Gupta *et al.,* 2012). The aim of the preparative HPLC is to produce more

yields of collected fractions for later assays. The use of prep HPLC has become an indispensable tool nowadays in the isolation of most classes of natural products (Zahid and Satyajit, 2012).

Bucar and coworkers (2013) stated that RP-HPLC is superior in separating a relatively-polar substance (such as carbohydrates, amino acids, and alkaloids) and illustrating the profile of bio-active compounds in plant extracts. Moreover, this method is a straightforward technique that usually does not require complex preliminary sample preparation. Mobile phases used in this method need careful preparation, and additives such as formic and acetic acids or trifluoroacetic acid (TFA) may be added to the mobile phase for pH adjustment and optimisation of the separation (Veronika, 2004). In this study, the data obtained from the Bioscreen-C system (section 4.2), provide strong evidence that methanolic extracts were the most active extract, and in turn indicates that the compounds contained in methanolic extracts, which are responsible for the antibacterial activity, might be of polar constituents. Solvent extraction usually follows the rule of like-dissolve-like, where the solubility of solvents of similar polarity can be high.

The most common eluents used in the RP-HPLC analysis of natural products of plant extracts are mixtures of aqueous and water-soluble organic solvent, methanol and acetonitrile are mostly used (Dolan, 2000). Methanol-water mixture is the most commonly used reverse-phase HPLC mobile phase for the isolation of natural compounds. However, there is much published research that utilised a mixture of water-acetonitrile in HPLC-based natural products isolation. Bucar *et al.* (2013) have summarised that about half of natural products isolation employed methanol-water mixture as a solvent system in a survey study of the period between 2008 and 2012. Some selective methods have been published in which tetrahydrofuran has been selected as the only organic solvent in the mobile phase (Nicolino *et al.,* 1997). 2-propanol, *n*-butanol or other organic solvents are used only rarely in the mobile phase and, when used, it is usually for selectivity reasons (Bronner and Beecher 1995). Sticher, (2008) argued that acetonitrile is better to use compared to other alcoholic solvents for RP-HPLC, due to its low UV cut off point, lower viscosity, thus reduces back pressure. However, Gilar and coworkers (2014), argued that acetonitrile is relatively expensive, especially the highly pure solvent. Furthermore, viscosity properties do not affect the separation quality and it causes no problems, neither to the HPLC system, nor to the column. This research group suggested that the use of methanol instead of acetonitrile as an alternative choice with the same separation quality.

Results obtained in this study confirmed that the mobile phase containing methanol has demonstrated a good separation of the natural compounds extracted from medicinal plants. In any case, it cannot be recommended to use mobile phases with less than 10 % organic solvent in water. (Zahid and Satyajit, 2012). The ionic state of the compounds and the stationary phases are critical for producing efficient and reproducible chromatography. 1 % of trifluoroacetic acid (TFA) in water: methanol 85:15 (v/v) has been used in this study. Toru and coworkers (2007), suggested that the isocratic reverse-phase HPLC separation employing a solvent system containing TFA the mobile phase showed a good separation of plant extracts. From the data presented in this chapter, the HPLC chromatograms have provided an evidence for the hypothesis described before, that several compounds were selectively extracted and isolated in the absolute methanolic extract, some of which were responsible for the antibacterial activity.

Studies have reported *Centella asiatica* L. as a rich source of bio-active compounds, the biologically active ingredients are believed to be its phenolic compounds. Several studies have revealed the phenolic derivatives of *Centella asiatica* L., the main technique used was the RP-HPLC (Skalicka-Wozniak & Garrard, 2014; Du *et al.,* 2004). HPLC method was set for quantitative determination of bio-active components in *Centella asiatica* L. extracts by Schaneberg *et al.,* (2003), a gradient mobile phase of water: acetonitrile in 0.1 % TFA has been used. comparative studies have employed RP-HPLC to reveal the variations in secondary metabolite in different geographical areas of *Centella asiatica* L. (Anjana *et al.,* 2010; Devkota *et al.,* 2010). Several biologically active substances have been reported to be present in *Imperata cylindrica* L., a very recent study carried out by Nugroho and coworkers (2015), where they successfully isolated four compounds from a crude methanolic extract of *Imperata cylindrica* L. using analytical RH-HPLC, compounds were, isoeugenin, ferulic acid, p-coumaric acid, and caffeic acid. Jiao *et al.,* (2011) developed a method for the content determination in the extracts from *Imperata cylindrica* L. using RP-HPLC. An isocratic mobile phase was used composed of methanol-water 70:30 (v: v), they concluded that the method was sensitive, reproducible and should be used for the isolation of bio-active components from plant natural products.

Qualitative analytical RP-HPLC analysis of *Morinda citrifolia* L. was recently performed by Serafini and coworkers (2011), they successfully identified some phenolic compounds from *Morinda citrifolia* L., the compounds were characterised by retention times relative to external

standards, and comparison of their ultraviolet spectra with the literature. A selective and validated reversed phase HPLC method for analysing the contents of major anthraquinones in *Morinda citrifolia* L. leaves was developed and reported by (Deng *et al.*, 2009). Among the different options, the methanol: water gradient solvent system offered a good separation with symmetrical peak shapes. Further research undertaken by Singh and coworkers (2012) for identification of bio-active compounds in ten anatomical parts (bark, branches, flowers, leaves, pulp, immature and mature fruit, stem, heartwood and root) of *Morinda citrifolia* L., using reverse phase HPLC. A number of major components were identified in this study, such as terpenoids, alkaloids, anthraquinones, flavones, glycosides and amino acids. The mobile phase used for HPLC analysis consisted of methanol and acetonitrile 90:10 (v/v) at a flow rate of 1.0 ml/min. The bioassay-guided HPLC analysis indicated that different parts of *Morinda citrifolia* L. had various number of bio active contents.

Sauropus androgynus L. is rich in the active chemical compositions. Several studies have been conducted to evaluate and isolate the bio-active compounds from this plant, most of which used HPLC as the main technique for the separation. A study was conducted by Andarwulan and coworkers (2010), to quantify the flavonoid content of *Sauropus androgynus* L., and to evaluate their biological activates. Compounds were isolated and quantified using RP-HPLC as a tool to achieve this. Flavonoids were quantified on the basis of comparison to their standards. Pewarna and coworkers (2015) employed reversed phase HPLC to analyse the carotenoids profile of *Sauropus androgynus* L., this plant was found to be rich in carotenoids with three different compounds isolated, tneoxanthin, violaxanthin and lutein. The group suggested that these targeted phytochemical compounds need to be investigated further due to their potential biological activity.

5.4 Conclusion

In this study, the separation of the methanolic extracts of *Centella asiatica* L., *Imperata cylindrica* L., *Morinda citrifolia* L., and *Sauropus androgynus* L. by preparative RP-HPLC resolved many distinct peaks showed in the chromatograms. Each peak represents either an individual compound, or more than one which eluted at the same time. Ten fractions were collected from *Centella asiatica* L. (CA1-CA10), twelve fractions from *Imperata cylindrical* L. (IC1-IC12), ten fractions from *Morinda citrifolia* L. (MC1-MC10), and finally, nine fractions

were collected from *Sauropus androgynus* L. (SA1-SA9). Each plant had more than one biologically active fraction, however, fractions CA7, IC10, MC4, and SA3 were the most active when compared with the positive control ($P<0.05$). These finding provided a strong evidence that these fractions (Compounds) were responsible for the antibacterial activity seen before. As there is a continuous and an urgent need to discover new antibacterial compounds with diverse chemical structures and novel mechanisms of action for new and re-emerging infectious diseases, any substance that can inhibit pathogens and have little toxicity to host cells are considered as a candidate for developing new antibacterial drug. For that reason, fractions CA7, IC10, MC4, and SA3 were chosen for structure elucidation in the next stage of this project.

Chapter 6
Structure Elucidation of the Active Fractions

6.1 Sample purity analysis

The separation of the methanolic extracts of *Centella asiatica* L., *Imperata cylindrica* L., *Morinda citrifolia* L., and *Sauropus androgynus* L., produced four very active components, which were responsible for the antibacterial activity *in-vitro*. The isolated compounds CA7, IC10, MC4, and SA3, might be the compounds responsible for the activity shown in methanolic extracts of these plants. In any biomedical and natural product studies, a truthful description of chemical constitution requires coverage of both structure and purity aspects (Pauli *et al.,* 2014). Screening for biological activity in pharmaceutical research requires that the compounds being tested are pure, where the purity assessment is particularly critical in discovery studies. The designation of a substance as experimental material (research grade) makes it clear that it differs from material intended for human use (pharmaceutical grade). Purity assessment is perhaps most critical in the case of novel compounds to which a biological activity is ascribed, because trace impurities of a high potency can lead to false conclusions being made. Obtaining the appropriate level of purity is essential for assuring the biological activity of the potential candidates of the active compounds, as well as for a high success rate of the molecular structure elucidation (Guido *et al.,* 2008).

For many years, the chromatographic methods such as HPLC were used extensively for the compound purity checking in the field of pharmaceutical research, however, these techniques require an authentic reference standard for construction of calibration curves, along with a specific method for absolute purity determination. On the other hand, for unknown compounds, it only gives the area under the curve which is not an indicator of absolute purity and depends on many factors, such as the detector response. Furthermore, it does not indicate impurities that are not detected or are retained on the column resulting into a misleading outcomes, or an impurity that has the same retention time as the sample under the conditions employed. The increased availability of high sensitivity analytical techniques, such as NMR, facilitated the means to quantify and determine the purity of compounds of interest. Among many analytical techniques, NMR has been recently used to determine/estimate and report the purity of natural compounds. Over the last few years, quantitative NMR (qNMR) has evolved in the pharmaceutical industry and the use of it is increased (Mahajan & Singh, 2013).

The results of quantitative NMR (qNMR) can be routinely considered as reliable results for purity determination much like other established methods (i.e. HPLC). The qNMR can be described as a fast, easy, accurate, and non-destructive alternate technique for purity check to speed up the whole analytical process and serves the purpose of both identification and purity determination of compounds using a single technique. Another major advantage of this technique, is that it can be used for a very small amount of the sample. Among the major applications of one-dimensional 1D NMR, proton quantitative NMR (q^1H NMR) has become a valuable technique for the analysis of complex mixtures and the determination of the purity without the need for the chromatography assays (Pauli *et al.,* 2014).

6.1.2 Results

All NMR measurements were performed on a Bruker Ultra-shield Avance AMX 400 MHz spectrometer, using 5-mm NMR tubes (Wilmad NMR tubes, 5 mm diam., precision 500 MHz Sigma-Aldrich, UK). Samples were dissolved in a (600 µl) deuterated DMSO-d_6. For purity determination of the bio-active compounds isolated in this study, q^1H NMR used the Mnova NMR software as described in section (3.2.3.3.2).

Figure (6.1) illustrates the proton NMR chromatograms of CA7 analysed using the Mnova software tools, and table (6.1) contains a description of the chemical shift (δ in ppm), intensity, width, area, and classified type of each signal. The purity check analysis of Centella asiatica L. active fraction (CA7) revealed a purity of 97.05 %.

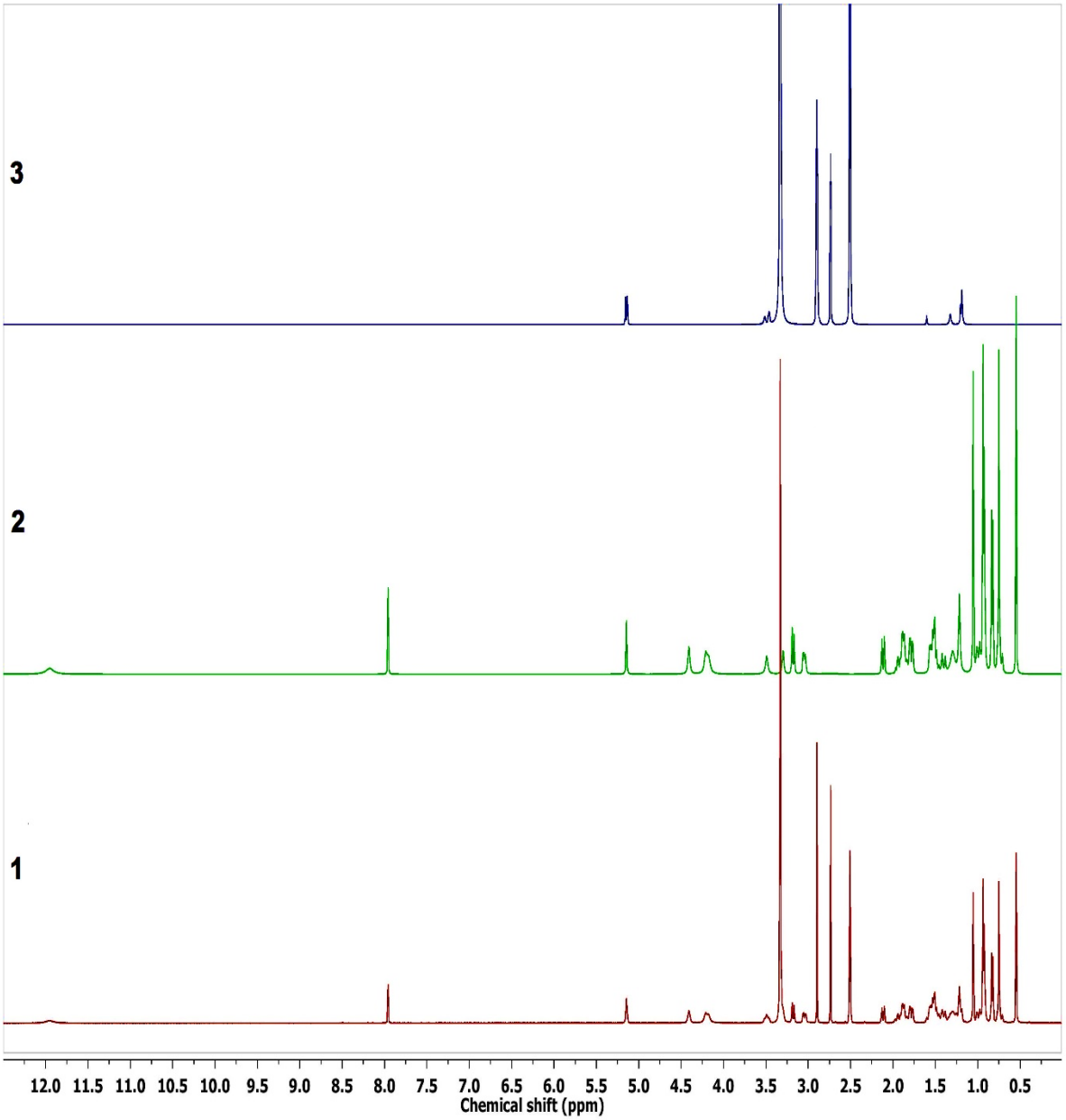


Figure 6.1: Stacked sub-spectra view obtained from the q^{1}H NMR spectrum of the purity check analysis of the active fraction (CA7) in *Centella asiatica* L.

1. Containing all the compound and "solvents+impurities" peaks.
2. Containing only the compound fit peaks.
3. Containing only solvents+impurities peaks.

1D proton NMR spectra acquired by Bruker Ultra-shield Avance AMX 400 MHz spectrometer. The sample was dissolved in a (600 µl) deuterated DMSO-*d6*, and data analysed by using Mnova NMR software (V 10.0.2, Mestrelab Research) with the aid of a qNMR plugin and the purity auto detect feature, to calculate the purity of the fraction. Solvent peaks used as an internal reference and the qNMR process were done automatically. The noise, phasing errors, and baseline flatness were improved by using the appropriate functions available in the software.

	ppm	Hz	Intensity	Width	Area	Type
1	0.07	29.3	6	1.77	117.71	Impurity
2	0.39	155.1	30.3	2.04	810.49	Impurity
3	0.55	218.4	6006	1.9	143947.18	Compound
4	0.59	237.1	24.8	2.95	927.65	Impurity
5	0.71	284	265.7	4.49	14683.16	Compound
6	0.74	295.4	366.1	3.9	16336.48	Compound
7	0.75	300	5077.4	2.65	168111.79	Compound
8	0.77	307.9	289.4	4.21	14658.03	Compound
9	0.78	312.6	23.4	3.25	1072.49	Compound
10	0.82	328.4	2285.4	2.53	71432.97	Compound
11	0.84	334.7	2486.4	2.66	84306.13	Compound
12	0.88	353.5	29.9	2.38	701.72	Impurity
13	0.91	364.4	264.9	2.7	8592.04	Compound
14	0.92	368	1405.5	4.05	70773.33	Compound
15	0.92	370	2535.6	3.17	98475.23	Compound
16	0.94	375.2	4880.3	2.63	157395.78	Compound
17	0.96	383.5	127.2	11.42	14681.75	Compound
18	0.97	386.5	27	1.47	386.61	Impurity
19	0.98	390.7	361.7	7.3	30142.78	Compound
20	1	400.1	43.3	2.29	998.92	Compound
21	1.01	403.9	344	8.15	35820.75	Compound
22	1.05	421.7	4808.3	2.59	152643.03	Compound
23	1.08	431.5	65.8	0.76	620.95	Impurity
24	1.09	436.8	24.7	2.73	951.01	Compound
25	1.11	442.2	17.9	2.79	703.95	Impurity
26	1.15	461.4	24.3	2.93	803.09	Impurity
27	1.18	472.3	38.8	1.89	1035.53	Compound
28	1.19	475.3	368	5.56	23703.16	Compound
29	1.21	482.5	361.7	4.26	19327.55	Compound
30	1.22	486.2	790	4.37	43200.77	Compound
31	1.22	488.7	671.5	5.04	41887.86	Compound
32	1.25	499.4	56	2.73	1752.68	Impurity
33	1.26	502.6	156.8	7.89	15149.42	Impurity
34	1.28	511.7	41.5	3	1547.99	Impurity
35	1.3	518.8	341.3	19.84	84205.67	Compound
36	1.32	528.7	96	6.65	7896.76	Impurity
37	1.33	532.4	64.3	4.1	3364.9	Impurity
38	1.35	538.9	39.1	2.7	1312.91	Impurity
39	1.38	553.3	241.8	5.36	15250.99	Compound
40	1.39	555.5	69.8	2.47	2031.3	Compound
41	1.41	564.2	79.7	3.39	3358.57	Compound
42	1.42	567.4	243.3	5.17	16140.18	Compound
43	1.43	570.6	184.7	3.93	8766.71	Compound
44	1.43	573.9	18	1.32	216.73	Impurity
45	1.46	583.5	126.4	6.13	9406.22	Compound
46	1.49	594.7	256.4	3.26	10264.3	Compound
47	1.51	603.2	737.7	5.59	54278.23	Compound
48	1.52	607	425	4.99	27289.48	Compound
49	1.53	612.3	535.2	4.73	33186.18	Compound
50	1.55	619.8	359.8	8.01	37973.25	Compound
51	1.56	624.4	200.2	3.22	8238.31	Compound
52	1.57	628.1	369.8	5.46	24954.39	Compound
53	1.58	631.7	79.8	2.03	1910.86	Compound
54	1.59	637.5	54.9	2.28	1442.73	Compound
55	1.6	640.7	94.1	2.96	3648.55	Compound
56	1.61	642.8	47.4	7.15	3941.86	Compound
57	1.72	687.1	6.8	1.21	98.95	Impurity
58	1.76	705.2	362	3.58	15251.01	Compound
59	1.77	709.5	429.7	3.4	18375.21	Compound
60	1.79	717.6	349.3	3.01	11691.76	Compound
61	1.8	721.9	502.1	6.23	38154.96	Compound
62	1.83	731.9	25.4	1.89	626.47	Impurity
63	1.83	733.2	89.6	3.53	3924.77	Compound
64	1.84	736.7	68.6	4.67	4520.55	Compound
65	1.86	744.6	79.4	3.32	3264.94	Compound
66	1.87	747.2	539.5	9.55	64813.15	Compound
67	1.88	754	365.6	4.34	21019.58	Compound
68	1.89	757.7	428.7	4.84	26900.05	Compound
69	1.9	762.1	89.3	2.78	2759.03	Compound
70	1.91	765.8	174.7	3.94	8095.23	Compound
71	1.94	775	198.9	4.15	9866.75	Compound
72	1.95	779.2	222.7	4.64	12969.81	Compound
73	1.97	788.2	74.9	2.77	2442.37	Compound
74	1.98	792	80.6	5.76	5314.37	Compound
75	1.99	798.2	30.4	0.8	286.19	Impurity
76	2.09	837	396.9	0.74	3641.35	Compound
77	2.1	841.3	585	3.97	24463.68	Compound
78	2.13	852.6	545.4	3.9	23621.12	Compound
79	2.3	921.8	7.2	0.85	69.45	Impurity
80	2.33	932.1	21.5	1.9	560.95	Solvent
81	2.33	934	25.4	1.14	351.63	Solvent
82	2.34	935.9	19.9	1.4	344.57	Solvent
83	2.5	999.6	1428	1.34	23244.41	Solvent
84	2.5	1001.4	3416.4	1.33	54772.74	Solvent
85	2.51	1003.2	5315.3	1.42	93934.78	Solvent
86	2.51	1005.1	3675.9	1.36	62779.75	Solvent
87	2.52	1007	1508	1.24	21977.99	Solvent
88	2.55	1019	7.1	0.73	72.81	Impurity
89	2.56	1025.2	49.5	1.4	693.54	Impurity
90	2.6	1039.6	5.5	0.97	64.84	Impurity
91	2.67	1069	19.4	1.67	457.28	Solvent
92	2.68	1070.9	27.4	1.27	480.27	Solvent
93	2.68	1072.8	19.1	2.89	754.82	Solvent
94	2.72	1089.1	45.6	1.07	477.56	Impurity
95	2.74	1095.1	8380.2	1.26	116172.84	Compound
96	2.9	1158.9	9893.9	1.15	123191.68	Compound
97	2.91	1162.6	41.6	5.22	2622.08	Impurity
98	2.98	1192.2	11.6	52.36	5516.72	Compound
99	3.02	1210.4	190.2	3.61	7789.84	Compound
100	3.03	1214	255.6	4.71	13776.17	Compound
101	3.05	1221.4	285.4	5.16	17687.99	Compound
102	3.06	1224.7	189.2	3.54	7900.26	Compound
103	3.07	1226.7	122.9	2.31	3698.04	Compound
104	3.17	1267	608.2	3.37	25257.42	Compound
105	3.19	1276.4	727.9	3.31	30084.65	Compound
106	3.29	1316.6	223.6	4.3	10992.16	Compound
107	3.3	1320.4	238.3	4.44	12804.2	Compound
108	3.33	1332.4	24284.5	2.59	783552.5	Solvent
109	3.43	1372.7	7.6	1.58	128.84	Impurity
110	3.46	1384.3	112.5	5	6670.57	Impurity
111	3.47	1388.5	60	3.12	2201.22	Impurity
112	3.49	1396.7	296	13.12	44896.59	Compound
113	3.51	1405.2	53.1	3.88	2485.99	Impurity
114	3.52	1409.2	76.9	4.35	3831.87	Impurity
115	3.6	1440.2	10.1	1.96	248.26	Impurity
116	3.62	1450.4	8.1	1.11	126.76	Impurity
117	3.76	1505.5	6.5	1.07	93.09	Impurity
118	4.03	1611.1	6	2.42	136.92	Impurity
119	4.18	1671.1	236.9	17.21	46636.22	Compound
120	4.21	1684.9	294.2	14.9	50132.45	Compound
121	4.35	1742.1	7.4	1.12	90.75	Impurity
122	4.41	1763.4	323	5.49	20284.74	Compound
123	4.42	1768.3	201.2	7.77	18088.13	Compound
124	4.48	1792.8	5.6	1.68	92.73	Impurity
125	4.61	1846.2	5.3	0.75	49.86	Impurity
126	5.14	2055.1	289.9	2.83	9040.49	Impurity
127	5.15	2058.8	835.8	3.26	34152.94	Compound
128	5.15	2062.3	299	2.56	8894.21	Impurity
129	5.95	2380.7	5.5	0.8	54.13	Impurity
130	6.76	2706	5.5	0.65	47.52	Impurity
131	7.35	2939.4	16.6	1.78	410.56	Impurity
132	7.71	3086.9	9.5	1.79	210.93	Impurity
133	7.96	3184.4	1386.3	2.65	41236.73	Compound
134	8.2	3279.8	9.3	1.54	186.46	Impurity
135	8.5	3400.2	23.4	2.41	702.26	Impurity
136	10.74	4298.9	6	1.3	89.4	Impurity
137	11.95	4783	91.8	44.39	52058.74	Compound
138	12.27	6108.8	5.4	0.58	40.61	Impurity

Table 6.1: The reported list of ^{1}H NMR peaks of the active fraction (CA7) in *Centella asiatica* L. The table includes the following information: chemical shift in ppm and Hz, Intensity (height), Width (in Hz, at half maximum of the peak), and Type (compound, solvent, impurity).

Figure (6.2) illustrates the proton NMR chromatograms of IC10 analysed using the Mnova software tools, and table (6.2) contains a description of the chemical shift (δ in ppm), intensity, width, area, and classified type of each signal. The purity check analysis of *Imperata cylindrica* L. active fraction IC10 revealed a purity of 95.93 %.

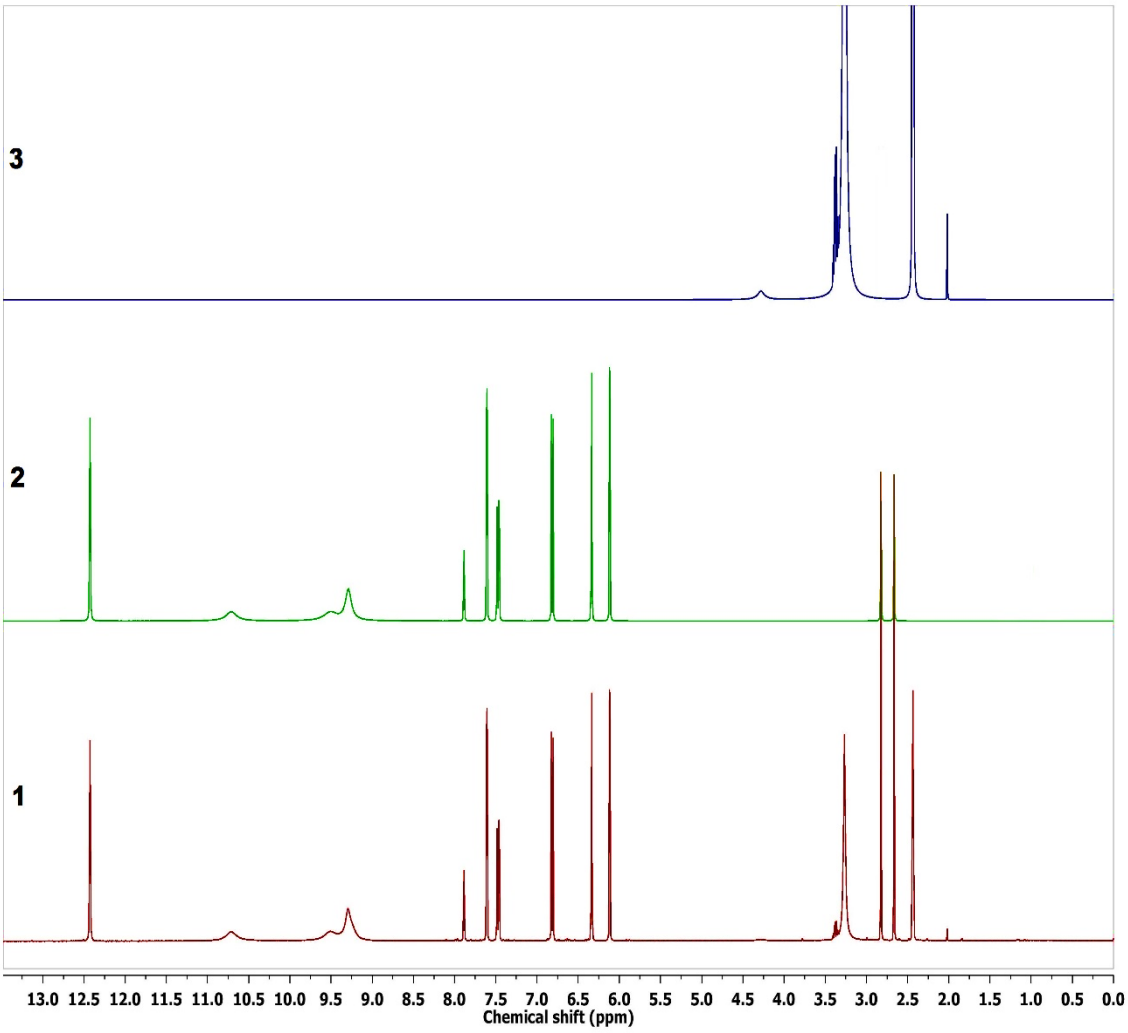


Figure 6.2: Stacked sub-spectra view obtained from the q^{1}H NMR spectrum of the purity check analysis of the active fraction (IC10) in *Imperata cylindrica* L.

1. Containing all the compound and "solvents+impurities" peaks.
2. Containing only the compound fit peaks.
3. Containing only solvents+impurities peaks.

1D proton NMR spectra acquired by Bruker Ultra-shield Avance AMX 400 MHz spectrometer. The sample was dissolved in a (600 µl) deuterated DMSO-*d6*, and data analysed by using Mnova NMR software (V 10.0.2, Mestrelab Research) with the aid of a qNMR plugin and the purity auto detect feature, to calculate the purity of the fraction. Solvent peaks used as an internal reference and the qNMR process were done automatically. The noise, phasing errors, and baseline flatness were improved by using the appropriate functions available in the software.

	ppm	Hz	Intensity	Width	Area	Type
1	0.04	16.4	1	1.93	26.22	CS Reference
2	0.06	22.6	2.7	1.86	60.01	Impurity
3	0.47	189.4	1.2	3.88	65.15	Impurity
4	0.59	235.3	2.3	4.45	130.82	Impurity
5	0.62	246.3	11	3.09	431.01	Impurity
6	0.71	285.3	17.7	10.69	1809.45	Impurity
7	0.77	307.9	28.5	7.94	2735.6	Impurity
8	0.79	315.3	18.7	2.8	623.29	Impurity
9	0.8	320.5	33.5	9.47	3997.68	Impurity
10	0.84	336.3	10.3	3.76	469.7	Impurity
11	0.88	352.3	2.2	1.75	39.84	Impurity
12	0.95	380.2	30.7	4.98	1885.49	Impurity
13	1.02	408.2	9.6	3.14	371.73	Impurity
14	1.04	414.3	8.6	2.7	283.72	Impurity
15	1.06	423.7	1.3	3.26	49.29	Impurity
16	1.1	440	19.5	3.51	808.35	Impurity
17	1.11	444.8	63.4	12.91	10110.64	Impurity
18	1.16	465.2	9.9	19.14	2346.62	Impurity
19	1.19	474.5	14.3	18.21	3650.78	Impurity
20	1.25	501.9	1.8	12.12	263.76	Impurity
21	1.28	514	2.9	18.33	653.7	Impurity
22	1.44	576.9	4.8	33.03	2135.31	Impurity
23	1.46	585.3	2.6	6.24	218.73	Impurity
24	1.53	611.9	2.8	11.76	438.15	Impurity
25	1.59	634.7	1.1	9.22	95.54	Impurity
26	1.69	676.4	6.9	24.6	1822.24	Impurity
27	1.91	764.3	2.5	4.08	143.91	Impurity
28	1.94	776.1	4.3	18.93	954.21	Impurity
29	2.14	856.9	5.1	2.85	177.11	Impurity
30	2.16	864.3	9.8	2.84	332.19	Impurity
31	2.18	871.7	4.4	3.12	149.38	Impurity
32	2.23	891.1	1.3	2.63	48.54	Impurity
33	2.25	900.9	2.4	13.79	417.54	Impurity
34	2.33	934.2	1.8	6.23	127.68	Solvent
35	2.5	999.9	58.4	1.96	1120.51	Solvent
36	2.5	1001.6	159	1.98	3711.62	Solvent
37	2.51	1003.5	281.4	2.3	7941.31	Solvent
38	2.51	1005.4	183.7	2.21	4812.2	Solvent
39	2.52	1007.2	59.3	1.76	1090.36	Solvent
40	2.68	1071.1	2.3	7.84	251.63	Solvent
41	2.96	1185.1	1	5.5	80.5	Impurity
42	3.05	1219.6	1.4	17.97	306.16	Impurity
43	3.12	1247.6	2.4	3.42	106.53	Impurity
44	3.14	1257.1	1.5	2.97	51.62	Impurity
45	3.2	1280.6	3.3	8.51	349.07	Impurity
46	3.28	1312.6	3.2	18.75	817.96	Impurity
47	3.32	1328.4	4.1	4.84	256.46	Impurity
48	3.35	1339.3	2.9	3.41	139.97	Impurity
49	3.56	1426.5	106.4	31.34	42588.31	Solvent
50	3.71	1483.4	1.8	2.79	65.12	Impurity
51	3.74	1495.8	3.5	4.02	159.45	Impurity
52	3.76	1506.3	2.7	5.79	189.91	Impurity
53	3.81	1523.4	2.1	2.32	59.58	Impurity
54	3.84	1535.7	2.6	1.74	54.89	Impurity
55	3.86	1542.8	284.9	2.07	7083.83	Impurity
56	3.99	1595.8	0.9	3.35	27.33	Impurity
57	4.03	1613.9	1.5	1.9	34.26	Impurity
58	4.11	1646.2	2.9	13.54	396.78	Impurity
59	4.28	1711.4	1.3	2.96	53.36	Impurity
60	4.3	1718.9	1.4	2.93	48.97	Impurity
61	4.38	1751.1	0.9	34.06	419.43	Impurity
62	4.43	1773.8	4.7	3.14	154.92	Impurity
63	4.91	1963.2	1.1	1.07	10.8	Impurity
64	4.91	1965.6	0.9	1.12	12.62	Impurity
65	5.05	2022.5	5.1	10.51	554.23	Impurity
66	5.12	2048.3	2.4	33.03	1056.5	Impurity
67	5.24	2098.2	1.4	3.01	59.52	Impurity
68	5.26	2104.4	1.9	5.48	143.6	Impurity
69	5.34	2136	1.8	8.19	137.35	Impurity
70	5.38	2152.9	1.9	2.49	62.35	Impurity
71	5.4	2160.1	2	3.33	83.64	Impurity
72	6	2400.9	10.3	4.77	532.46	Impurity
73	6.18	2472.1	1.4	1.51	19.17	Impurity
74	6.18	2474.3	1.2	1.98	26.1	Impurity
75	6.2	2480.9	2333.9	1.71	46008.92	Compound
76	6.21	2483	2587	1.87	60307.56	Compound
77	6.22	2489.5	5.3	2.49	144.15	Impurity
78	6.4	2560.6	2	1.27	30.27	Impurity
79	6.41	2563	5.7	1.27	81.93	Impurity
80	6.42	2569	2369.5	1.66	45447.37	Compound
81	6.43	2571	2378.6	1.87	52451.97	Compound
82	6.45	2579	33.9	2.05	850.96	Compound
83	6.45	2581.1	26.4	1.74	550.98	Impurity
84	6.48	2593.6	54.7	1.7	1098.1	Impurity
85	6.49	2595.6	53.9	1.83	1197.66	Impurity
86	6.59	2636	1.6	1.77	33.19	Impurity
87	6.63	2651.7	9.2	4.29	472.25	Impurity
88	6.63	2653.4	3.3	1.91	76.27	Impurity
89	6.68	2673.8	3.1	2.15	70.97	Impurity
90	6.7	2681	7.5	2.76	258.03	Compound
91	6.72	2689.5	6.7	3.09	252.7	Compound
92	6.84	2735.3	1.2	2.03	33.03	Impurity
93	6.87	2748.3	3.5	2.02	86.19	Impurity
94	6.9	2760.6	2254.7	1.85	48751.46	Compound
95	6.92	2769.1	2336.3	1.96	55062.63	Compound
96	6.94	2778	65.6	2.05	1674.62	Compound
97	6.95	2779.7	39.5	2.02	971.7	Compound
98	6.95	2782.7	3.3	1.57	59	Compound
99	6.96	2786.5	54.1	1.91	1236.7	Compound
100	7.06	2826	1.2	1.21	15.11	Impurity
101	7.1	2839.5	6.6	3.34	274.52	Compound
102	7.12	2848	8.1	3.33	327.76	Compound
103	7.14	2857.6	1	1	12.55	Compound
104	7.35	2941.5	5.4	5.02	321.36	Compound
105	7.37	2950	5.2	4.23	258.47	Compound
106	7.49	2998.7	1.1	0.9	10.42	Compound
107	7.5	3000.9	1.1	1.12	11.13	Compound
108	7.52	3007.7	8.3	5.38	408.45	Compound
109	7.55	3021.7	1204.3	1.78	25005.12	Compound
110	7.56	3024	1272.1	1.81	27603.66	Compound
111	7.57	3030.2	1068.7	1.91	24709.44	Compound
112	7.58	3032.4	1192.7	1.87	26958.74	Compound
113	7.65	3060.1	3.1	2.17	83.82	Impurity
114	7.69	3076.9	23.8	1.71	473.61	Impurity
115	7.69	3078.9	17.3	1.42	287.65	Impurity
116	7.71	3086.6	2380.3	1.84	50654.93	Compound
117	7.72	3088.8	2274.1	1.87	50404.92	Compound
118	7.74	3095.8	1.7	1.93	36.58	Impurity
119	7.76	3104.2	5.1	4.6	239.86	Impurity
120	7.77	3109.5	51.6	1.88	1135.7	Impurity
121	7.78	3111.6	52.1	2.23	1445.32	Compound
122	7.91	3165.9	5.1	2.61	150.6	Compound
123	7.92	3168	8.7	3.9	403.86	Compound
124	7.93	3175	0.9	1.08	9.55	Compound
125	7.95	3181.9	1	1.71	16.35	Compound
126	8.05	3219.3	46.1	1.97	1107.33	Compound
127	8.05	3221.4	8.5	1.92	188.03	Impurity
128	8.06	3226.1	7.4	1.87	159.81	Compound
129	8.07	3228.2	42.5	2.18	1154.53	Compound
130	8.15	3260.7	1.6	1.03	19.8	Compound
131	8.24	3296.7	1.3	1.45	20.13	Compound
132	8.29	3315.4	2.5	2.12	65.56	Compound
133	9.37	3747.5	253.6	74.43	218301.39	Compound
134	10.7	4281.9	39.3	182.09	86669.71	Compound
135	12.47	4990.9	55.5	3.24	2228.14	Impurity
136	12.5	5002.2	2592	3.3	104311.58	Compound
137	12.62	5050.7	6.9	2.39	198.28	Impurity

Table 6.2: The reported list of ^{1}H NMR peaks of the active fraction (IC10) in *Imperata cylindrica* L. The table includes the following information: chemical shift in ppm and Hz, Intensity (height), Width (in Hz, at half maximum of the peak), and Type (compound, solvent, impurity).

Figure (6.3) illustrates the proton NMR chromatograms of MC4 analysed using the Mnova software tools, and table (6.3) contains a description of the chemical shift (δ in ppm), intensity, width, area, and classified type of each signal. The purity check analysis of *Morinda citrifolia* L. active fraction MC4 revealed a purity of 97.65 %.

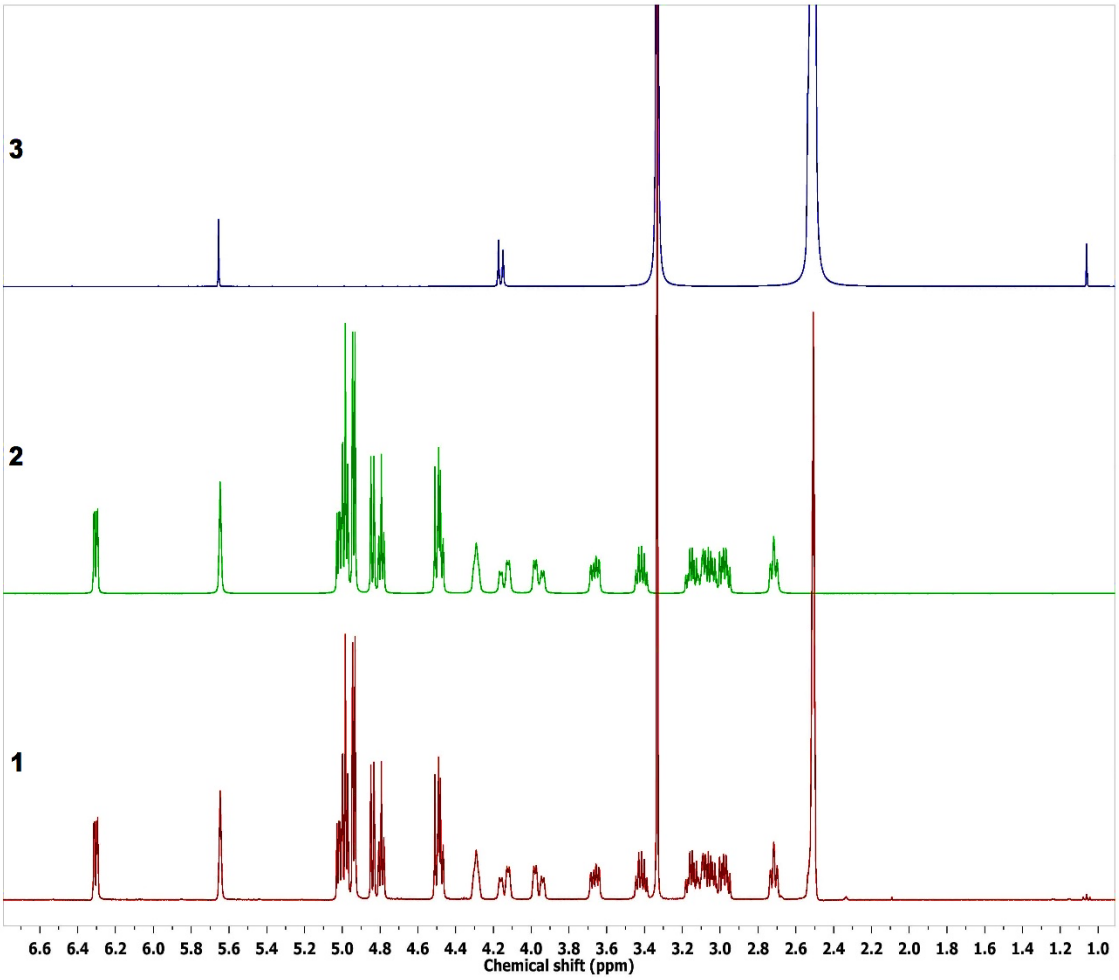


Figure 6.3: Stacked sub-spectra view obtained from the q^{1}H NMR spectrum of the purity check analysis of the active fraction (MC4) in *Morinda citrifolia* L.

1. Containing all the compound and "solvents+impurities" peaks.
2. Containing only the compound fit peaks.
3. Containing only solvents+impurities peaks.

1D proton NMR spectra acquired by Bruker Ultra-shield Avance AMX 400 MHz spectrometer. The sample was dissolved in a (600 µl) deuterated DMSO-*d6*, and data analysed by using Mnova NMR software (V 10.0.2, Mestrelab Research) with the aid of a qNMR plugin and the purity auto detect feature, to calculate the purity of the fraction. Solvent peaks used as an internal reference and the qNMR process were done automatically. The noise, phasing errors, and baseline flatness were improved by using the appropriate functions available in the software.

	ppm	Hz	Intensity	Width	Area	Type
1	0.06	22.6	9.8	1.51	174	Impurity
2	1.04	418	10.8	0.69	72.93	Impurity
3	1.06	425.1	6.3	0.84	63.68	Impurity
4	1.14	454.7	22.5	3.63	850.77	Impurity
5	1.23	490.4	18.8	4.28	1111.95	Impurity
6	1.5	599.4	5.4	0.44	26.37	Impurity
7	1.72	687.3	6	0.53	38.87	Impurity
8	1.8	719.7	6.7	0.5	39.48	Impurity
9	1.86	743.2	6.9	10.34	653.03	Impurity
10	1.95	781.2	5.5	0.42	27.39	Impurity
11	2.08	830.5	204.7	0.77	1902.1	Impurity
12	2.17	867.3	5.2	0.58	37.87	Impurity
13	2.25	898.4	6.7	7.89	743.6	Impurity
14	2.31	925.7	19.1	1.45	342.73	Solvent
15	2.32	927.6	26	1.49	519.73	Solvent
16	2.32	929.4	17.1	1.06	245.57	Solvent
17	2.48	990.7	184.2	1.12	2227.4	Impurity
18	2.48	993.1	1609.1	1.45	27209.74	Solvent
19	2.49	995	3558.9	1.39	57976.47	Solvent
20	2.49	996.8	5562.5	1.4	96956.48	Solvent
21	2.5	998.7	3844.2	1.4	67482.71	Solvent
22	2.5	1000.6	1604.4	1.42	26742.44	Solvent
23	2.51	1002.9	266.6	3.66	8882.88	Solvent
24	2.51	1004.1	206.6	1.72	4457.1	Solvent
25	2.51	1005.4	179.3	2.08	4877.43	Solvent
26	2.52	1007.4	244.8	1.52	4699.03	Impurity
27	2.52	1009.3	246.3	1.36	3815.56	Impurity
28	2.66	1062.7	13.9	0.93	160.72	Solvent
29	2.66	1064.5	22.5	1.42	364.65	Solvent
30	2.66	1066.3	12.5	0.93	140.36	Solvent
31	2.68	1073.3	478.9	3.39	18525.86	Compound
32	2.69	1074.8	98.8	1.76	1992.59	Compound
33	2.7	1080.7	880.3	4.06	39670.67	Compound
34	2.72	1086.6	92.5	2.09	2464.88	Compound
35	2.72	1088.1	385	3.21	14757.03	Compound
36	2.87	1147.5	8.3	26.2	2913.44	Impurity
37	2.93	1173.4	410.7	1.52	7783.17	Compound
38	2.95	1178.5	406.3	1.63	8257.67	Compound
39	2.95	1181.3	404.3	1.48	7060.06	Compound
40	2.95	1182.3	538.1	1.64	11235.78	Compound
41	2.96	1186.3	397.4	1.53	7488.24	Compound
42	2.97	1187.4	551.4	1.68	11498	Compound
43	2.97	1190.2	520.5	1.61	10157.76	Compound
44	2.99	1195.5	646.1	1.95	14858.47	Compound
45	3	1201.4	183.4	1.73	3806.81	Compound
46	3.01	1204.6	274.4	1.63	5320.64	Compound
47	3.01	1205.8	451.5	1.82	10210.23	Compound
48	3.02	1209.7	218.3	1.76	4534.41	Compound
49	3.03	1211	472.8	1.83	10831.54	Compound
50	3.03	1214.3	628.9	1.88	14360.78	Compound
51	3.05	1219.4	735.2	1.76	15863.83	Compound
52	3.06	1224.2	443.6	2.17	11675.11	Compound
53	3.06	1226.1	523.4	2.14	13735.63	Compound
54	3.07	1230.1	535.4	2.05	13604.41	Compound
55	3.08	1232	453	2.19	12337.32	Compound
56	3.08	1233.9	121.2	2.63	4176.27	Compound
57	3.09	1235.7	179.5	1.83	3867.33	Compound
58	3.1	1239.7	224.1	2.04	5406.66	Compound
59	3.1	1241.6	207.6	1.82	4575.56	Compound
60	3.11	1244.6	545.8	1.67	10999.54	Compound
61	3.12	1249.5	509.3	1.78	10696.51	Compound
62	3.13	1253.3	718.5	1.95	16569.02	Compound
63	3.14	1258.3	705.6	1.99	16526.04	Compound
64	3.15	1260.7	2245.8	0.87	24194.97	Compound
65	3.15	1262.2	191.6	1.52	3167.21	Compound
66	3.16	1266	2383.5	0.9	27213.01	Compound
67	3.27	1308.5	31.2	1.39	539.19	Impurity
68	3.32	1326.8	51833	0.92	574481.48	Solvent
69	3.33	1331	117	2.39	3854.7	Compound
70	3.34	1334.6	16	0.81	165.76	Impurity
71	3.37	1349.2	324.9	2.13	8618.47	Compound
72	3.39	1355	622.3	2.16	16261.81	Compound
73	3.4	1360.9	736.3	2.06	18596.87	Compound
74	3.42	1366.8	740.6	2.3	20887.84	Compound
75	3.43	1372.6	349	2.39	10254.35	Compound
76	3.62	1449.6	368.5	2.14	9399.42	Compound
77	3.63	1451.6	400.7	2.14	10377.87	Compound
78	3.64	1455.6	366.3	2.25	9852.75	Compound
79	3.64	1457.5	454.5	2.06	11631.16	Compound
80	3.65	1461.4	343.3	1.85	7664.97	Compound
81	3.66	1463.3	312.2	2.25	8783.49	Compound
82	3.67	1467.4	321.4	2.09	8093.15	Compound
83	3.67	1469.3	313.8	2.29	9019.1	Compound
84	3.72	1489.3	16.2	1.82	288.95	Impurity
85	3.74	1498.1	15.3	1.84	322.68	Impurity
86	3.91	1565.4	55.4	1.55	983.31	Compound
87	3.92	1566.8	121.3	2.39	3699.29	Compound
88	3.92	1568	204	3.24	7560.72	Compound
89	3.93	1572.8	341.4	5.38	23312.49	Compound
90	3.96	1583.1	292.2	3.49	10980.44	Compound
91	3.96	1586.1	268.2	7.39	19041.97	Compound
92	3.97	1589	319.9	3.04	11000.69	Compound
93	3.98	1590.9	81.3	1.47	1345.89	Compound
94	4.07	1627	187.1	0.96	2243.08	Impurity
95	4.08	1632.3	497.2	0.93	5633.81	Compound
96	4.09	1637.5	561.8	1.01	6623.44	Compound
97	4.1	1640.4	388.6	2.99	14469.2	Compound
98	4.11	1642.8	397.4	2.01	10723.08	Compound
99	4.11	1645.8	437.1	3.37	19167.73	Compound
100	4.12	1647.9	137.1	2.37	4035.94	Compound
101	4.13	1654.3	66.5	1.64	1149.19	Impurity
102	4.14	1656.2	226.5	2.25	6102.4	Compound
103	4.15	1658.6	140.6	2.33	4033.47	Compound
104	4.15	1661.4	297.5	3.78	15841.29	Compound
105	4.16	1663.6	89.9	2.22	2483.3	Compound
106	4.25	1701.5	27.2	1.22	377.53	Impurity
107	4.26	1703.3	150.9	1.91	3445.83	Compound
108	4.26	1705.3	245	2.41	7550.44	Compound
109	4.27	1707.3	233.2	3.43	10015.08	Compound
110	4.27	1709.1	301.5	2.86	10708.52	Compound
111	4.28	1710.9	475.6	2.96	17574.4	Compound
112	4.28	1712.7	272	2.69	9275.26	Compound
113	4.28	1714.4	224.7	2.93	8305.88	Compound
114	4.29	1716.2	246.3	2.41	7393.17	Compound
115	4.29	1718	168.5	2.2	4731.42	Compound
116	4.3	1720	18.6	0.77	183.2	Compound
117	4.37	1749.7	6.8	1.11	106.4	Impurity
118	4.45	1780.2	870.5	1.84	19508.58	Compound
119	4.46	1786	1914.8	1.69	39025.61	Compound
120	4.47	1790.4	2112.3	1.41	36524.65	Compound
121	4.48	1791.8	898	1.91	21578.29	Compound
122	4.49	1798.3	2059.8	1.47	37262.65	Compound
123	4.6	1841.7	6.6	1.9	170.71	Impurity
124	4.62	1848.4	5.7	1.4	94.29	Impurity
125	4.65	1862.3	9.2	1.07	92.58	Impurity
126	4.68	1871.5	16.8	1.64	297.65	Impurity
127	4.69	1875.7	13.8	1.29	244.43	Compound
128	4.74	1897.5	8.8	1.51	157.52	Impurity
129	4.76	1905.7	976.3	1.6	19420.24	Compound
130	4.78	1911.2	2358.6	1.31	37803.9	Compound
131	4.79	1916.6	918.1	1.61	18294.28	Compound
132	4.82	1927.2	2290.9	1.38	38043.1	Compound
133	4.83	1934	2272.5	1.38	37782.82	Compound
134	4.86	1945.5	17.3	1.53	324.98	Impurity
135	4.88	1950.7	22.3	2.09	526.47	Impurity
136	4.89	1955.7	13.1	1.66	265.65	Impurity
137	4.92	1966.8	4454.7	1.37	73460.72	Compound
138	4.93	1971.9	4452.6	1.42	77767.56	Compound
139	4.95	1981.8	2117.4	1.39	35982.26	Compound
140	4.97	1986.7	1623.1	1.2	23845.23	Compound
141	4.97	1987.4	3012.1	1.4	52475.81	Compound
142	4.98	1993.3	2333.1	1.23	35188.49	Compound
143	4.99	1995.7	1288.1	1.27	20574.6	Compound
144	5	1999.5	1149.8	1.24	17505.32	Compound
145	5	2001.8	1233.1	1.37	21234.31	Compound
146	5.01	2005.6	1286.1	1.27	20358.16	Compound
147	5.05	2021.1	10.2	1.58	197.16	Impurity
148	5.21	2082.7	5.5	3.08	224.52	Impurity
149	5.42	2170.7	7.8	4.68	403.57	Impurity
150	5.53	2213.8	5.3	0.55	35.71	Impurity
151	5.58	2231.9	8.7	3.08	332.94	Impurity
152	5.62	2249.6	115.2	1.31	1620.69	Compound
153	5.63	2251.3	861.3	1.81	19336.91	Compound
154	5.63	2253	1403.9	1.93	32994.35	Compound
155	5.63	2254.6	868.7	1.72	17850.03	Compound
156	5.64	2256.3	212.5	1.72	4492.84	Compound
157	5.83	2334.5	7.6	5.51	572.76	Impurity
158	6.04	2416.6	5.5	0.5	34.25	Impurity
159	6.28	2512.5	1170.1	1.18	15681.82	Compound
160	6.28	2514.4	1287.9	1.45	23147.02	Compound
161	6.29	2518.5	1153.9	1.38	18711.14	Compound
162	6.3	2520.5	1226	1.38	21633.8	Compound
163	8.5	3399.3	35.3	1.07	480.99	Impurity
164	9.02	3609.3	5.5	0.62	30.8	Impurity
165	10.14	4058.3	5.4	0.49	33.15	Impurity
166	10.2	4082.8	5.5	1.08	76.94	Impurity
167	10.92	4367.6	5.3	0.93	61.05	Impurity
168	13.49	5396.5	5.3	0.55	36.45	Impurity
169	14.79	5917.1	5.3	0.69	45.36	Impurity
170	14.8	5922.9	5.3	0.5	32.89	Impurity
171	15.05	6020.8	5.1	0.58	37.31	Impurity
172	15.35	6142.6	5.3	0.38	22.4	Impurity

Table 6.3: The reported list of ^{1}H NMR peaks of the active fraction (MC4) in *Morinda citrifolia* L. The table includes the following information: chemical shift in ppm and Hz, Intensity (height), Width (in Hz, at half maximum of the peak), and Type (compound, solvent, impurity).

6.2 Molecular structure identification of the isolated compounds

Elucidating the structure of the bio-active compounds isolated from medicinal plants often involves the accumulation of data from numerous sources. A wide range of spectroscopic instrumentation currently form the backbone of modern structure analysis. In the present study, the identification of the molecular structure was possible, as the purity of the isolated compounds was sufficient for spectroscopic analysis. Structure determination of the natural compounds was performed by extensive nuclear magnetic resonance (NMR) spectroscopy. Additional information was gained by mass spectrometry (MS). Only the combined application of these methods allowed a certain determination of the structure. The structure of the compounds was confirmed by comparison with spectral data available in the literature and databases. Structural elucidation started by recording 1D ^{1}H and ^{13}C NMR spectra. Although they are very basic experiments, a lot of useful information about the chemical functions present in a molecule can be gained from the obtained chemical shifts. In addition, extensive of 2D-NMR experiments were performed in this study (COSY, TOCSY, HMQC, HMBC, NOESY, and ROESY). They are superior to their 1D NMR counterparts, both for the information on the connection of nuclei and for the easier assignment of nuclei resonating in crowded regions of the spectra (signal overlapping is much less likely in two dimensions than in one) (Dominique, 2014). Additional ^{13}C_APT experiment were carried out as well. The number of carbons and protons were verified by mass spectrometry.

The identification of the molecular structure of the CA7 compound from *Centella asiatica* L. revealed a known triterpene, and was assigned as (2α, 3β)-2, 3, 23-Trihydroxyurs-12-en-28-oic acid, also known as asiatic acid (Figure 6.4). The spectral details and their assignments on the molecular structure of 1D NMR as follow; ^{1}H-NMR (DMSO-d_6) δ; 0.54 (s, 3H), 0.75 (s, 3H), 0.82 (s, 3H), 0.93 (d, 3H, *J*=6.8), 1.05 (s, 3H), 1.16-1.44 (m, 8H), 1.47-1.62 (m, 8H), 1.73-1.82 (m, 2H), 1.85-1.98 (m, 3H), 2.11 (m, 1H), 3.04 (m, 1H), 3.18 (d, 1H, *J*=8.4), 3.48 (m, 1H),4.19 (bm, 3H), 4.41 (d, 3H, *J*=8.8), 5.14 (m,1H), 11.94 (brs, 1H); ^{13}C-NMR (DMSO-*d6*) δ; 46.93 (C-1), 67.38 (C-2), 75.47 (C-3), 42.45 (C-4), 47.28 (C-5), 17.39 (C-6), 30.74 (C-7), 38.46 (C-8), 52.33 (C-9), 38.37 (C-10), 21. 6 (C-11), 124.46 (C-12), 138.24 (C-13), 41.70 (C-14), 27.45 (C-15), 23.29 (C-16), 46.77 (C-17), 40.34 (C-18), 45.94 (C-19), 30.15 (C-20); 36.29 (C-21), 35.75 (C-22), 63.85 (C-23), 13.9 (C-24), 16.96 (C-25), 17.2 (C-26), 23.76 (C-27), 178.25 (C-28), 32.14 (C-29), 22.93 (C-30).

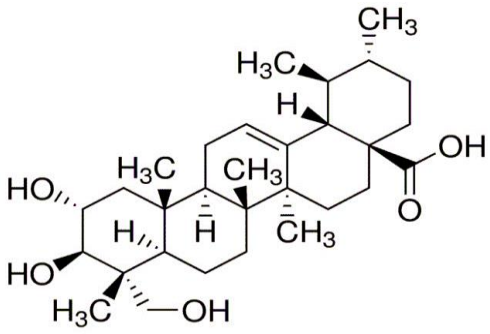


Figure 6.4: Chemical structures of asiatic acid isolated from *Centella asiatica* L.

The assignment of the compound was supported by comparison with the available literature data of asiatic acid (Acebey-Castellon *et al.*, 2011). 2D-NMR spectra of this compound are available in appendices. The MS spectrum (available in the appendices) gave a molecular weight of 488 g/mol, which is appropriate for a molecular formula of $C_{30}H_{48}O_5$. As a final confirmation, commercial asiatic acid was purchased from Sigma-Aldrich and used as a standard in the proton and carbon 1D NMR test. The comparison between the standard asiatic acid and CA7 compound showed very close spectra, results is presented in figures (6.5 and 6.6). Thus, the compound present in CA7 fraction was confirmed as asiatic acid.

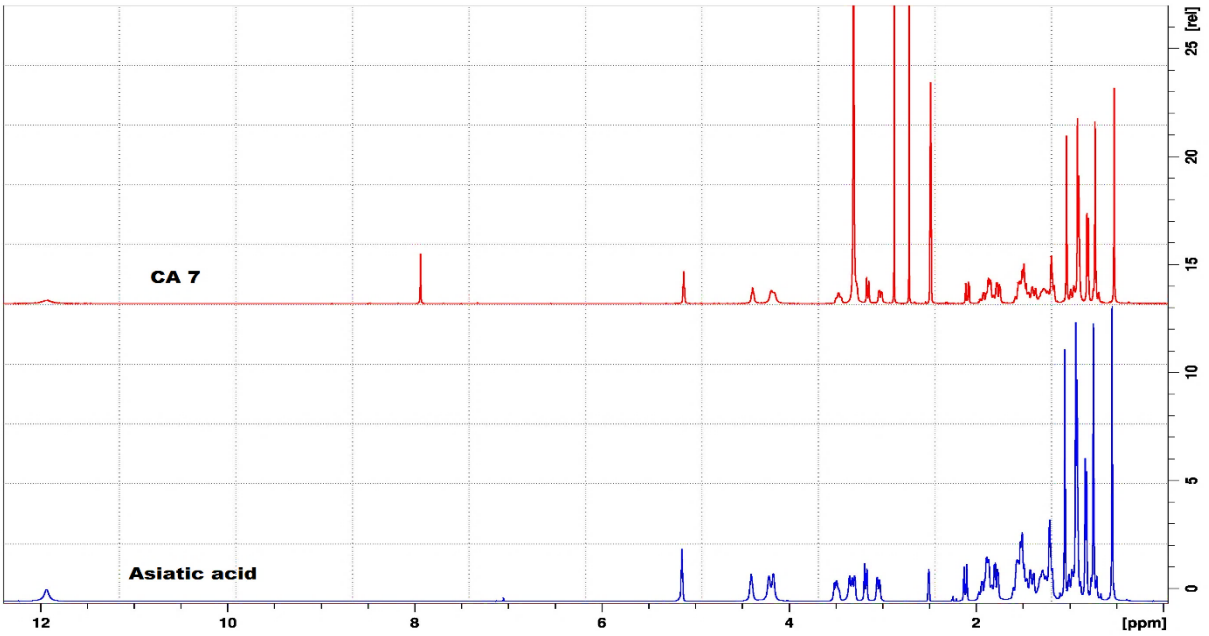


Figure 6.5: ^{1}H NMR spectra of CA7 fraction and standard asiatic acid.

1D proton NMR spectra acquired by Bruker Ultra-shield Avance AMX 400 MHz spectrometer, and the sample was dissolved in a (600 µl) deuterated DMSO-*d6*. The data were analysed by TopSpin NMR software (v 3.5) under automation controlled by "iconnmr". The noise, phasing errors, and baseline flatness were improved by using the appropriate functions available in the software.

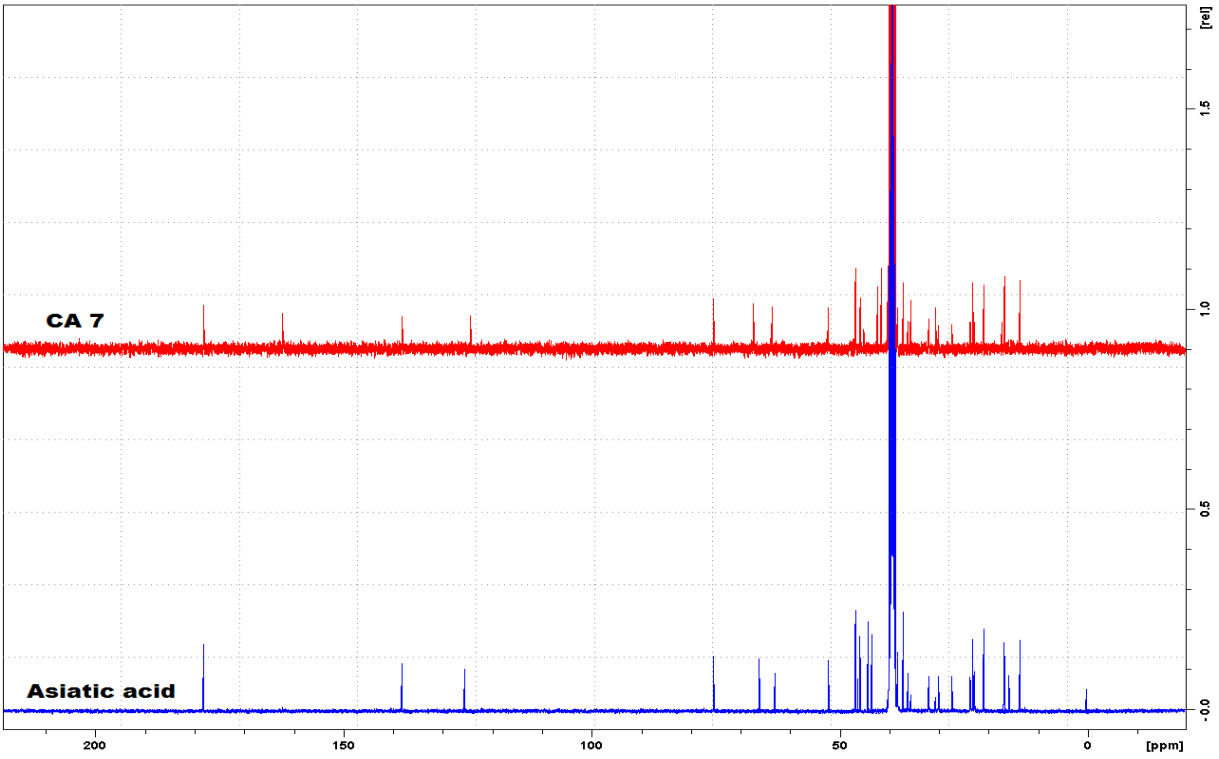


Figure 6.6: ^{13}C NMR spectra of CA7 fraction and standard asiatic acid.

1D carbon NMR spectra acquired by Bruker Ultra-shield Avance AMX 400 MHz spectrometer, and the sample was dissolved in a (600 µl) deuterated DMSO-*d6*. The data were analysed by TopSpin NMR software (v 3.5) under automation controlled by "iconnmr". The noise, phasing errors, and baseline flatness were improved by using the appropriate functions available in the software.

The identification of the molecular structure of IC10 compound from *Imperata cylindrica* L. revealed a known flavonol, and was assigned as 2-(3, 4-dihydroxyphenyl)-3, 5, 7-trihydroxy-4*H*-chromen-4-one, also known as quercetin (Figure 6.7). The spectral details and their assignments on the molecular structure of 1D NMR as follow; ^{1}H-NMR (DMSO-d_6) δ; 6.12 (d, 1H, *J*=2.03), 6.34 (d, 1H, *J*=2.03), 6.83 (d, 1H, *J*=8.47), 7.48 (d, 1H, *J*=6.28), 7.61 (d, 2H, *J*=2.18), 9.29 (s, 1H), 9.51 (s, 1H), 10.71 (s, 1H), 12.43 (s, 1H); ^{13}C-NMR (DMSO-*d6*) δ; 146.73 (C-2), 135.68 (C-3), 175.78 (C-4), 160.66 (C-5), 98.13 (C-6), 136.84 (C-7), 93.29 (C-8), 156.7 (C-9), 102.94 (C-10), 121.89 (C-1'), 115 (C-2'), 145 (C-3'), 147.64 (C-4'), 115.54 (C-5'), 119.90 (C-6').

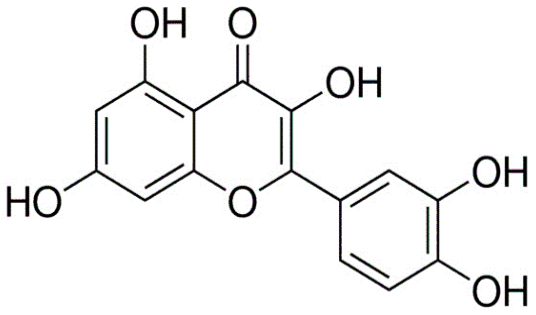


Figure 6.7: Chemical structures of quercetin isolated from *Imperata cylindrica* L.

The assignment of the compound was supported by comparison with the available literature data of quercetin (Materska *et al.,* 2014). 2D-NMR spectra of this compound are available in the appendices. The MS spectrum (available in the appendices) gave a molecular weight of 302 g/mol, which is appropriate for a molecular formula of $C_{15}H_{10}O_7$. As a final confirmation, commercial quercetin was purchased from Sigma-Aldrich and used as a standard in the proton NMR spectral comparison. The comparison showed a very close similarity in 1D NMR spectra, result is presented in figures (6.8 and 6.9). Subsequently, the compound present in IC10 fraction was confirmed as quercetin.

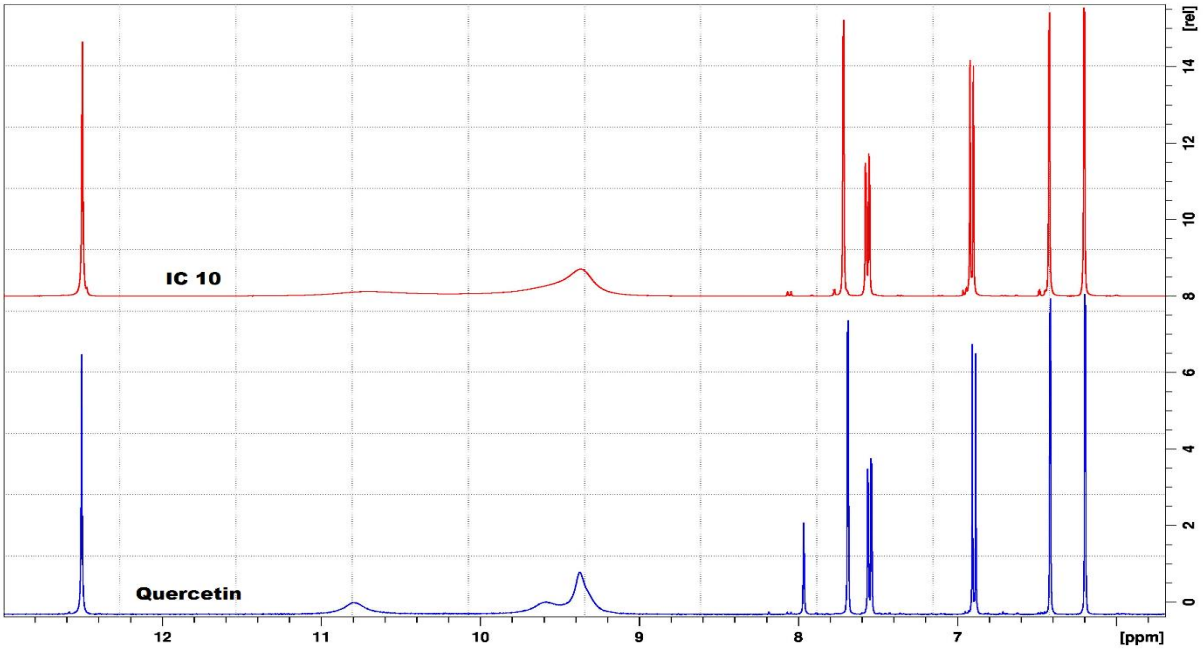


Figure 6.8: ^{1}H NMR spectra of IC10 fraction and standard quercetin.

1D proton NMR spectra acquired by Bruker Ultra-shield Avance AMX 400 MHz spectrometer, and the sample was dissolved in a (600 µl) deuterated DMSO-*d6*. The data were analysed by TopSpin NMR software (v 3.5) under automation controlled by "iconnmr". The noise, phasing errors, and baseline flatness were improved by using the appropriate functions available in the software.

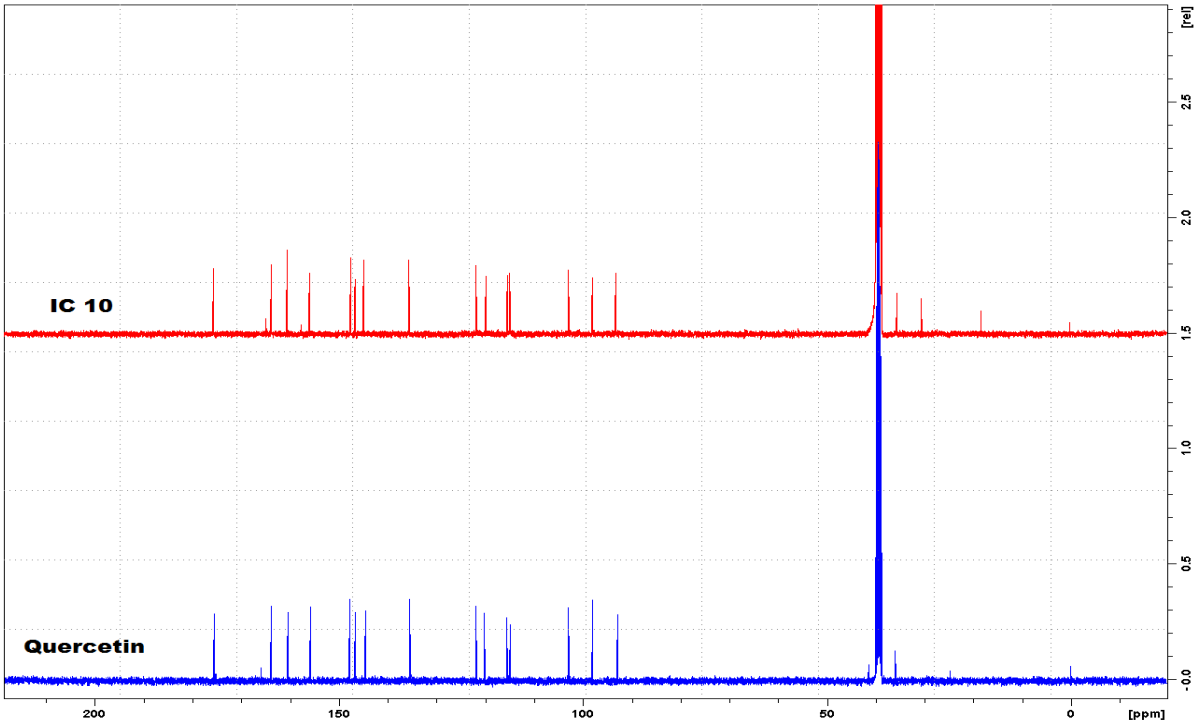


Figure 6.9: ^{13}C NMR spectra of IC10 fraction and standard quercetin.

1D carbon NMR spectra acquired by Bruker Ultra-shield Avance AMX 400 MHz spectrometer, and the sample was dissolved in a (600 µl) deuterated DMSO-*d6*. The data were analysed by TopSpin NMR software (v 3.5) under automation controlled by "iconnmr". The noise, phasing errors, and baseline flatness were improved by using the appropriate functions available in the software.

The identification of the molecular structure of MC4 compound from *Morinda citrifolia* L. revealed a known iridoid glycoside, and was assigned as (1S,4aR,5S,7aS)-5-Hydroxy-7-(hydroxymethyl)-1,4a,5,7a-tetrahydrocyclopenta[c]pyr-1-yl-β-D-glucopyranoside, also known as aucubin (Figure 6.10). The spectral details and their assignments on the molecular structure of 1D NMR as follow; ^{1}H-NMR (DMSO-*d6*) δ; 2.72 (m, 1H), 2.98 (m, 1H), 3.05 (m, 1H), 3.10 (m, 1H), 3.14 (m, 1H), 3.17 (d, 1H, *J*=5.05), 3.42 (m, 1H), 3.66 (m, 1H), 3.96 (m, 1H), 4.13 (m, 1H), 4.16 (m, 1H), 4.29 (m, 1H), 4.48 (s, 1H), 4.50 (d, 1H, *J*=8.08), 4.79 (s, 1H), 4.84 (d, 1H, *J*=6.82), 4.94 (d, 1H, *J*=5.31), 4.97 (s, 1H), 5.01 (s, 1H), 5.4 (dd, 1H, *J*=6.06, 3.79), 5.65 (m, 1H), 6.31 (dd, 1H, *J*=6.06, 2.02); ^{13}C-NMR (DMSO-*d6*) δ; 96.82 (C-1), 139.94 (C-3), 104.91 (C-4),44.54 (C-5), 80.38 (C-6), 129.09 (C-7), 146.04 (C-8), 46.34 (C-9), 60.95 (C-10), 97.94 (C-1'), 73.34 (C-2'), 76.61 (C-3'), 70.02 (C-4'), 77.14 (C-5'), 59.44 (C-6').

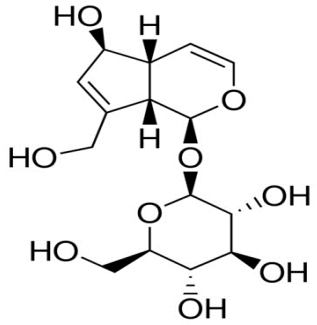


Figure 6.10: Chemical structures of aucubin isolated from *Morinda citrifolia* L.

The assignment of the compound was supported by comparison with the available literature data of aucubin (Gutierrez, Rangel, & Baez, 2008). 2D-NMR spectra of this compound available in the appendices. The MS spectrum (Available in the appendices) gave a molecular weight of 346 g/mol, which is appropriate for a molecular formula of $C_{15}H_{22}O_9$. As a final confirmation, commercial aucubin was purchased from Sigma-Aldrich and used as a standard in the proton NMR test. The comparison between the standard aucubin and MC4 compound showed almost an identical 1D NMR spectra, result is presented in figures (6.11 and 6.12). Therefore, the compound present in MC4 fraction was confirmed as aucubin.

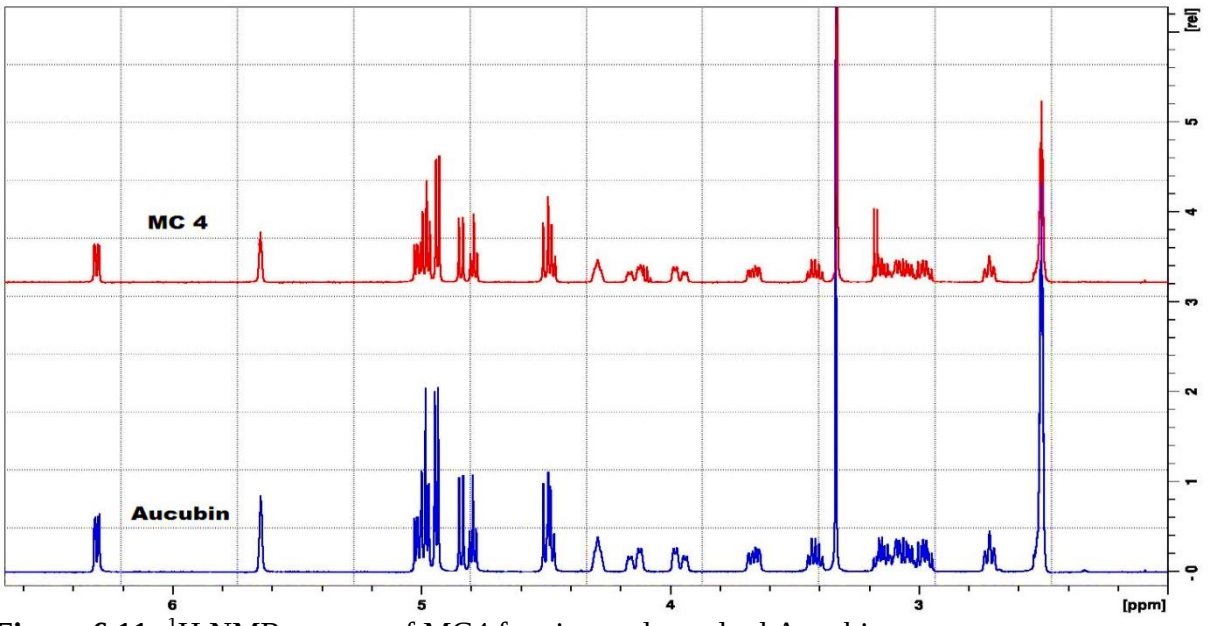


Figure 6.11: ^{1}H NMR spectra of MC4 fraction and standard Aucubin.

1D proton NMR spectra acquired by Bruker Ultra-shield Avance AMX 400 MHz spectrometer, and the sample was dissolved in a (600 µl) deuterated DMSO-*d6*. The data were analysed by TopSpin NMR software (v 3.5) under automation controlled by "iconnmr". The noise, phasing errors, and baseline flatness were improved by using the appropriate functions available in the software.

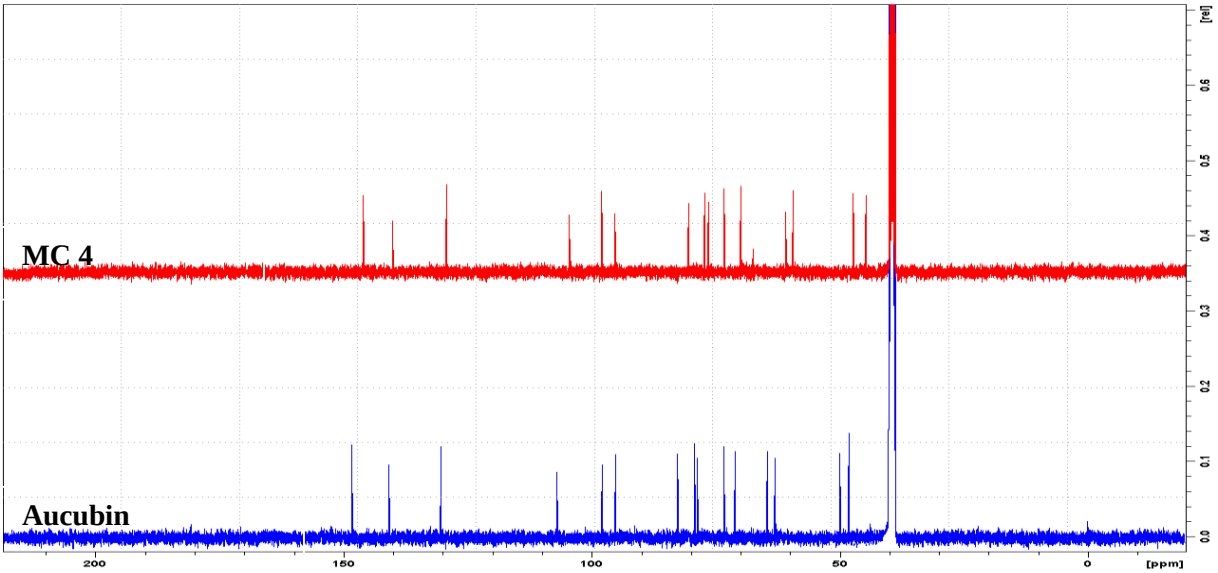


Figure 6.12: ^{13}C NMR spectra of MC4 fraction and standard aucubin.

1D carbon NMR spectra acquired by Bruker Ultra-shield Avance AMX 400 MHz spectrometer, and the sample was dissolved in a (600 µl) deuterated DMSO-*d6*. The data were analysed by TopSpin NMR software (v 3.5) under automation controlled by "iconnmr". The noise, phasing errors, and baseline flatness were improved by using the appropriate functions available in the software.

The identification and the molecular structure of SA3 fraction, which was isolated from *Sauropus androgynus* L., still unknown for the time being. The MS spectrum (available in the appendices) of this compound gave a molecular weight of 354 g/mol. 1D NMR spectra are presented in figures (6.13 and 6.14), whilst 2D NMR spectra available in the appendices.

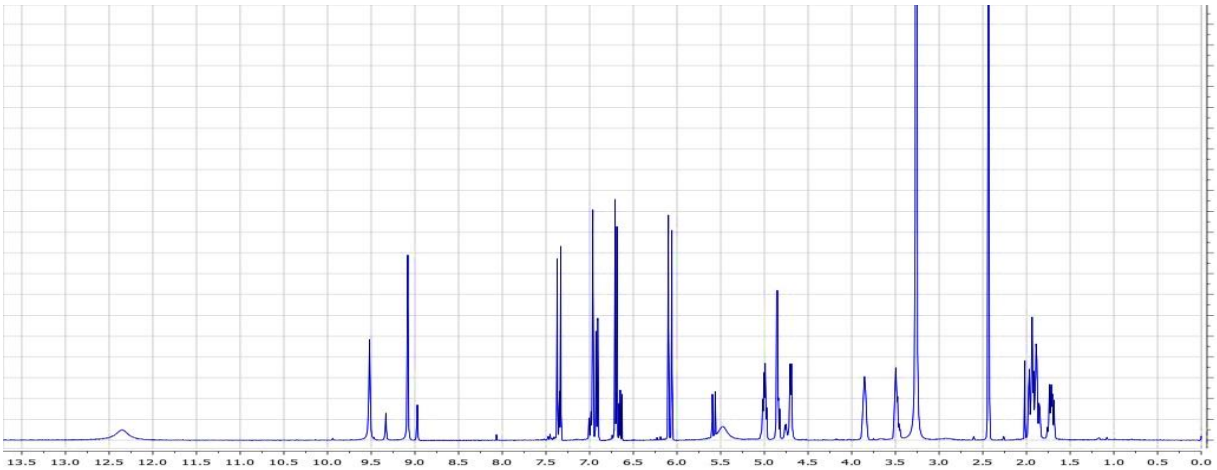


Figure 6.13: ^{1}H NMR spectra of SA3 fraction from *Sauropus androgynus* L.

1D carbon NMR spectra acquired by Bruker Ultra-shield Avance AMX 400 MHz spectrometer, and the sample was dissolved in a (600 µl) deuterated DMSO-*d6*. The data were analysed by TopSpin NMR software (v 3.5) under automation controlled by "iconnmr". The noise, phasing errors, and baseline flatness were improved by using the appropriate functions available in the software.

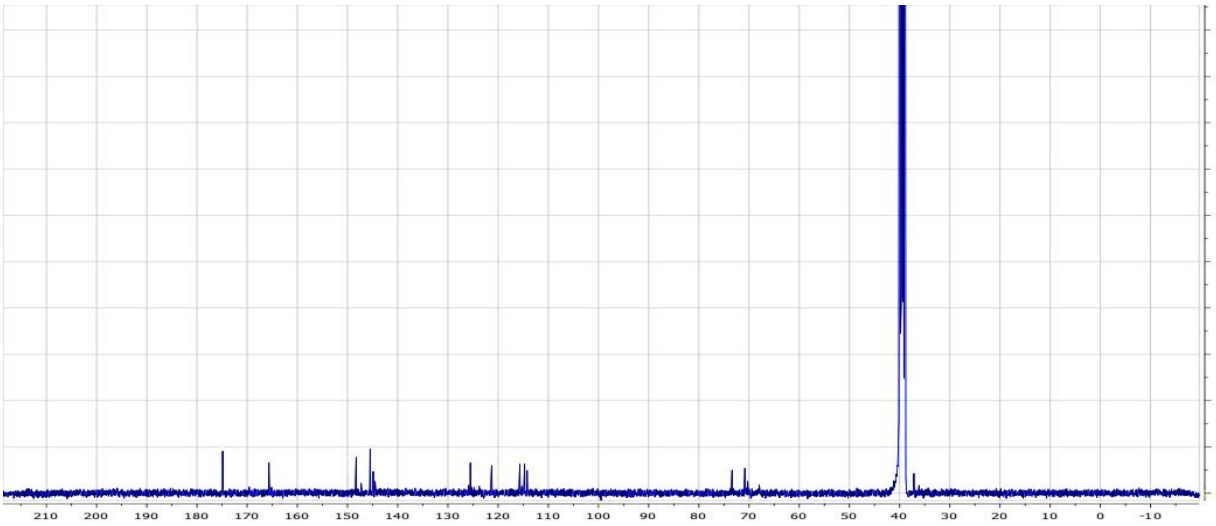


Figure 6.14: ^{13}C NMR spectra of SA3 fraction from *Sauropus androgynus* L.

1D carbon NMR spectra acquired by Bruker Ultra-shield Avance AMX 400 MHz spectrometer, and the sample was dissolved in a (600 µl) deuterated DMSO-*d6*. The data were analysed by TopSpin NMR software (v 3.5) under automation controlled by "iconnmr". The noise, phasing errors, and baseline flatness were improved by using the appropriate functions available in the software.

6.3 Discussion

The biosynthesis and breakdown of proteins, fats, nucleic acids and carbohydrates, which are essential to all living organisms, are known as primary metabolism with the compounds involved in the pathways known as primary metabolites. In contrast to these primary metabolites, the mechanism by which an organism biosynthesises compounds that have a much more limited distribution in nature and are less involved in metabolic pathways. Such compounds, called secondary metabolites, are also important natural products. They are not necessarily produced under all conditions, and in the vast majority of cases the function of these compounds and their benefit to the organism are not fully understood (Paul, 2009). Secondary metabolites are generally not essential for the growth, development, or reproduction of an organism and are produced either as a result of the organism adapting to its surrounding environment, or produced to act as possible defence mechanisms against predators to assist in the survival of the organism. It is this area of secondary metabolism which provides most of the pharmacologically active natural products. It is thus fairly obvious that these metabolites should be focused on when discovering and developing new drug candidates and the antibiotics are not excluded (Dias, Urban, & Roessner, 2012).

The isolation and purification of natural products troublesome and have always been the main obstacle in the overall process of the discovery of medicinal active compounds. The reason for it is the complexity of the naturally occurring secondary metabolites in plants, and often the low concentration of the natural product in plants, this can result in an exhausting and time-consuming separation and purification process. Finally, the yields usually are often very small with relatively low purity levels. That is why researchers in the field of natural products need to make an extra effort when isolating and purifying these compounds. If a natural compound is to be used for biological testing, it is important to know the degree of purity of the material, as the impurities can contribute significantly to any biological activity observed in the bio-screening assay (Olivier & Matthias, 2008). If the material is to be used for *in-vitro* biological activity testing and for chemical characterisation, Charles, (2005) stated that the acceptable level of purity can range from 95 to 99 percent. Such a range of purity will generally be sufficient for the biological activity assays, and for the determination a complex chemical structure via various spectroscopic techniques such as NMR and MS.

When isolating the substances from plants, a purity exceeding 99 % can only be achieved with some considerable effort. Usually, each purification step results in the loss of material, and when working to achieve a very high level of purity, losses can be extreme. If this the case, it may be necessary to take only the very centralised cuts of the peak, by leaving the tail cuts, which can be later subjected to reprocessing. This study employed the later approach in an attempt to achieve a high purity level of the isolated compounds. The purities obtained were 97.05 %, 95.93%, and 97.65 %, respectively for the bio-active fractions CA7, IC10, and MC4 respectively. When purity is less than 90 %, there is a reasonable possibility of the interactions between one or more existing component. In this case, it might not be possible to conclude whether the activity shown is demonstrated by one compound, or resulted from synergism or the antagonistic effect of the containing materials (Satyajit *et al.,* 2006).

In the study of natural products, researchers mainly rely upon the chromatography techniques such as HPLC to determine the quantity of active constituents relative to the impurities, with several examples known, (Jain *et al.,* 2008; Sathyaraj *et al.,* 2011; Holkar *et al.,* 2012). Establishing the purity of samples during the isolation process of the natural products is quite difficult. A large effort goes into developing methods using chromatography techniques,

rather than trying to find a new promising technique, these methods come with many drawbacks as mentioned earlier. It is necessary for monitoring impurities in natural products to be very selective in the analytical methodology (Prabu & Suriyaprakash, 2010). The q^1H NMR is different from other analytical and chromatographic techniques. it considered to be a very useful technique for impurity determination of chemical compounds, and is selective, and reproducible. Impurities with similar chemical structure may easily be identified and quantified by q^1H NMR technique, making it very cost effective alternate compared to other analytical methods that require the acquisition of separate standard of the target analyte and possible impurities, or relies on the calculation of under the peak area for unknown compounds (Bharti & Roy, 2012).

The use of quantitative NMR spectrometry for purity determination has been reported in many studies, (Parmar *et al.*, 2014; Wahl & Holzgrabe, 2014; Simmler *et al.*, 2014; Li *et al.*, 2015; Sahoo *et al.*, 2015). A q^1H NMR method was developed and validated by Yang and coworkers (2015) for Tadalafil (used for pulmonary arterial hypertension) and impurities content determination in different batches of bulk drugs and tablets of various specifications. The method was proved to be accurate, precise, selective, and linear over the assessed concentration range. A unique aspect of the NMR spectrum is the direct proportionality between the peak areas and number of protons responsible for the peak. By comparing the q^1H NMR results with the HPLC approach results, the contents of Tadalafil in the bulk drugs and tablets were almost identical. In our study, One-dimensional and two-dimensional NMR were utilised and examined for structure elucidation of the bio-active natural products isolated in this project, and the molecular weights of them were confirmed by using an external MS service (Intertek Pharmaceutical Services, Manchester, UK).

The structure of the bio-active fraction isolated from the methanolic extract of *Centella asiatica* L. (which has given a virtual name CA7) was identified and assigned as asiatic acid, on the basis of its NMR and MS spectra, and in comparison with data available in the literature (Acebey-Castellon *et al.*, 2011). There are several published studies that have indicated a number of secondary metabolites isolated from *Centella asiatica* L. including asiatic acid. The isolated compounds from these studies have demonstrated a number of biological activities with some related to the antibacterial activity (Taemchuay & Rukkwamsuk, 2009; Jacob &

Shenbagaraman, 2011; Dash *et al.,* 2011; Kalita & Saikia, 2012). The pharmacological activities of *Centella asiatica* L. is thought to be due to a vast number of compounds belonging to different chemical classes. The major chemical class found in this plant is triterpene saponosides (Alqahtani *et al.,* 2015). The most abundant ones are known as asiatic acid, madecassic acid, msiaticoside, madecassoside, and madasiatic acid (Williamson, 2002; Pan *et al.,* 2007; Orhan, 2012). The presence of several flavonoid derivatives such as kaempferol, patuletin, rutin, apigenin, castillicetin, myricetin, and castilliferol has been reported in *Centella asiatica* L. (Kuroda *et al*., 2001; Matsuda *et al*., 2001; Subban *et al*., 2008). More chemical groups have been isolated and identified in *Centella asiatica* L., such as polysaccharides (Wang *et al.,* 2004), polyacetylenes (Govindan *et al.,* 2007), sterols (Srivastava and Shukla, 1996), and phenolic acids (Subban *et al*., 2008; Suntornsuk and Anurukvorakun, 2005).

Asiatic acid (2α, 3β, 23-trihydroxyurs-12-ene-28-oic acid, molecular formula $C_{30}H_{48}O_5$), is one of the important bio-active pentacyclic triterpenoids found in *Centella asiatica* L. Asiatic acid was reported to possess a wide range of biological activities such as antioxidant (Ullah *et al.,* 2009; Lee *et al*., 2003), hepatoprotective (Wenjie *et al*., 2012; Ma Zhang *et al.* 2009), anticancer (Jing et al., 2015; Liu *et al*. 2006; Hsu *et al.,* 2005), anti-inflammatory (Huang *et al.* 2011), neuroprotective (Jew *et al.,* 2000), and anti-Alzheimer (Zhang *et al.,* 2012) properties. However, the biological effects and mechanism of asiatic acid actions in human remains unknown. It has also reported to lower blood glucose level in type 1 diabetic in the rats (Ramachandran & Saravanan, 2013; Liu *et al.,* 2010).

The antibacterial activity of asiatic acid has been reported in some published studies (Djoukeng *et al.,* 2005; Wong *et al.,* 2012; Wojnicz *et al.,* 2013). Garo and coworkers (2007) indicated that asiatic acid could enhance the susceptibility of *Pseudomonas aeruginosa* biofilms to tobramycin. These studies suggested that asiatic acid is a potent antimicrobial agent, however, action modes of asiatic acid against bacterial strains remain unclear. In a study conducted very recently by Liu and coworkers (2015), they have investigated the inhibitory effects of asiatic acid against seven bacterial pathogens. In an attempt to illustrate its mode of action, they have evaluated the influence of this compound on membrane damage, potassium ions (K^+ efflux), and nucleotides release in these bacteria. Their findings suggested that asiatic acid might be a potent antibacterial agent, and its antibacterial action might be explained by the disruption of

the bacterial cell membrane, or enhanced the release of potassium ions and nucleotides from the cells. Bacterial cell membrane integrity and permeability play crucial roles for bacterial survival and growth. Thus, any agent with the ability to destroy the bacterial cell membrane integrity and/or disrupt membrane permeability may cause bacterial cell damage, and even death. The rupture of bacterial cytoplasmic membrane easily impairs bacterial critical functions, which in turn affects bacterial survival and growth. In addition, it promotes the release of intracellular components such as potassium ions and nucleotides, which in turn diminishes bacterial ability to repair and replicate (Da Silva *et al.,* 2014).

The structure of the bio-active fraction IC10 from the methanolic extract of *Imperata cylindrica* L. was identified and assigned as quercetin, on the basis of its NMR and MS spectra, in comparison with the data available in the literature (Materska *et al.,* 2014). *Imperata cylindrica* L. is used medicinally in traditional practices to treat bacterial infections, and helminths. It is also found effective in conditions like arthritis, diarrhoea, dysentery, gonorrhoea, cancer, diuretic, and restorative (Shankar & Aravind, 2015). The major active chemical constituents isolated from *Imperata cylindrica* L. includes carbohydrates, glycosides, flavonoids, and triterpenoids (Asolkar *et al.,* 2005; Parvathy *et al.,* 2012). It has been reported that *Imperata cylindrica* L. has some antibacterial properties (Harun *et al.,* 2011; Parkavi *et al.,* 2012), and plenty of molecules are isolated and characterised from this plant (Wang *et al.,* 1996; Rodriguez *et al.,* 2005; Mohamed *et al.,* 2009). Despite that, a detailed review of the literature showed that not much work has been done in finding out the best bio-active candidate of these molecules. Thus, further research should be done with determination of bioactivity of these new molecules which might yield a potential antibacterial agent.

Quercetin is a polyphenolic molecule belonging to the flavonoid family of compounds, which are found inclusively in the aerial part of plants. Flavonoids are low-molecular-weight polyphenol substances generally composed of a three-ring structure, till now, more than 4000 varieties of flavonoids have been identified (Table 6.4). Available reports tend to show that secondary metabolites of phenolic nature, including flavonoids are responsible for a variety of pharmacological activities (Shashank & Abhay, 2013; Batra & Sharma, 2013).

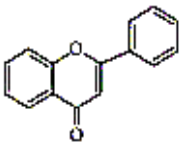
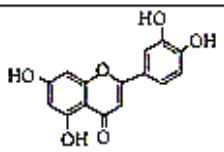
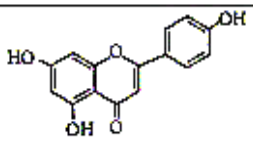
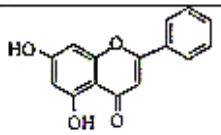
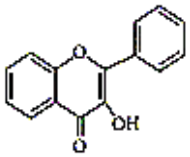
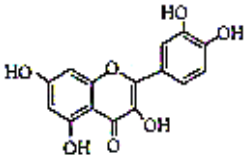
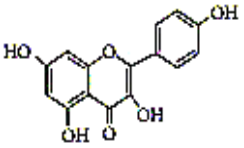
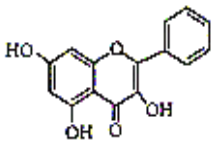
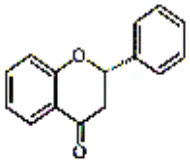
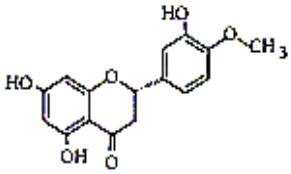
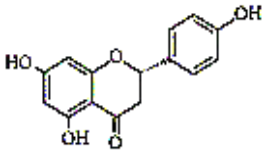
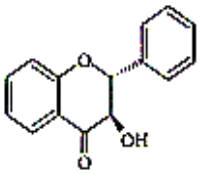
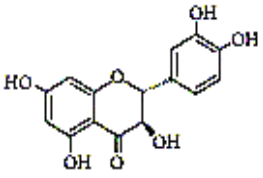
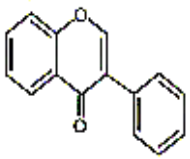
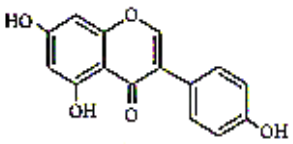
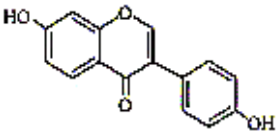
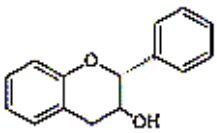
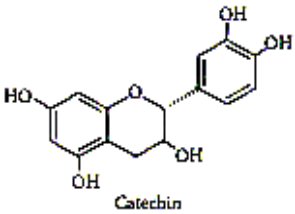
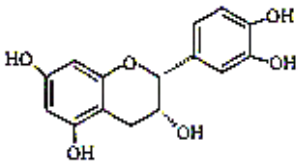
Group of flavanoid	Structure backbone	Examples		
Flavones		 Luteolin	 Apigenin	 Chrysin
Flavonols		 Quercetin	 Kaempferol	 Galangin
Flavanones		 Hesperetin	 Naringenin	
Flavanonol		 Taxifolin		
Isoflavones		 Genistein	 Daidzein	
Flavan-3-ols		 Catechin	 Epicatechin	

Table 6.4: Structure of flavonoids with some examples (Shashank & Abhay, 2013).

Quercetin is one of the most abundant flavonoids which is widely exist in many parts of plants. Interest in quercetin within the scientific community has increased in recent years due to an increase in the popularisation of quercetin's health benefits (Pawlikowska-Pawlega *et al.*, 2007). Many studies have shown that it may has a great potential for acting as anticancer (Feng *et al.*, 2011), antioxidant (Mei *et al.*, 2011; Mihaylova & Schalow, 2013), anti-inflammatory (Choi *et al.*, 2012; Wang et al., 2012), cardiovascular protection (Perez-Vizcaino and Duarte,

2010) and antiviral (Johari *et al.,* 2012). Many recent available studies have confirmed that quercetin has the ability to inhibit many types of bacteria. The results of these studies are in line with the results obtained in this study. Ragunathan (2015) and coworkers analysed the antibacterial properties and possible drug target of quercetin against *Staphylococcus aureus*. Eight protein molecules from *Staphylococcus aureus* belonging to various pathways for cell survivability were chosen. These protein molecules were docked with quercetin to find the most significant protein target. Their proposed mechanism of action for quercetin is the prevention of protein synthesis and folic acid synthesis. However, they stated that these identifications need further *in-vitro* validation before confirmation.

In studies conducted by (Li and Xu, 2008; Woznicka *et al*., 2013; Maalik *et al*., 2014; Walid *et al*., 2015), the quercetin antibacterial activity has been evaluated. The results of these studies suggested that quercetin could be an alternative new antibacterial natural drug candidate to treat bacterial infections. Although these studies demonstrated a significant antibacterial activity of quercetin, the exact mechanism underlying the antibacterial activity for quercetin against tested bacteria remained unclear, and it needs to be subjected to further investigations. Moreno (2006) and coworkers reported that the antibacterial action of quercetin and other phenolic compounds might be related to inactivation of certain cellular enzymes proteins, which are responsible for bacterial membrane formation, by forming irreversible complexes. These changes lead to a changes in membrane permeability. Increased membrane permeability is a major factor in the mechanism of antibacterial action, where compounds may disrupt membranes and cause a loss of cellular integrity and eventual cell death. They stated that further studies need to be done to confirm their results.

The structure of the bio-active fraction isolated from the methanolic extract of *Morinda citrifolia* L. (fraction MC4) was identified and assigned as aucubin, on the basis of its NMR and MS spectra, and in comparison with data available in the literature (Gutierrez, Rangel, & Baez, 2008). *Morinda citrifolia* L. is considered a very rich source of biologically active phytochemicals, and a variety of chemical constituents have been identified in the leaves, bark, stem, flowers and fruits of the plant. Several classes of compounds have been isolated from *Morinda citrifolia* L., including, anthraquinones (Duduku *et al*., 2012), terpenoids (Saludes *et al*., 2002; Takashima *et al*., 2007), iridoids and their glycosides (Sang *et al*., 2003), and flavonol

glycosides (Serafini *et al.*, 2011). Iridoid glycosides are one of the important active chemical group found in *Morinda citrifolia* L. These secondary metabolites have a significant role in the area of natural product chemistry and pharmacology, due to their diverse therapeutic efficacies. Several researches over the past years indicate their wide range of bioactivity including; anithepatotoxic, antiinflammatory, anticancer, antiviral, and antimicrobial (Dinda *et al*., 2007; Debnath *et al*., 2011; Bhakta *et al*., 2013).

Aucubin is one of the iridoid glycosides isolated from *Morinda citrifolia* L. and many more medicinal plants (Barreto *et al*., 2007; Peng *et al*., 2010). Aucubin is an active component and has been reported in many studies, it has shown wide pharmacological activities. Aucubin pharmacological properties encompass a range of therapeutic effects, including; hepatoprotective (Chang, 1998), antioxidant (Hong-Yu *et al*., 2009), antiinflammatory (Maria *et al*., 1994), antispasmodic (Fleer and Verspohl, 2007), antimicrobial (Zheng *et al*., 2012), and promotion of dermal wound healing (Lee *et al*., 1999).

The antibacterial activity of aucubin was reported in a few publications, Zheng (2012) and coworkers evaluated the antibacterial activity of aucubin extracted from *Eucommia ulmoides* leaves, the extracted aucubin could inhibit the growth of *Escherichia coli* and *Staphylococcus aureus*, however, aucubin presented weak inhibitory effect on *Streptococcus pneumonia*. Li (2009) and others, studied the antibacterial activity of the aucubin and 10 more compounds isolated from the leaves of *Scrophularia ningpoensis*. An *in-vitro* study against eight reference strains of bacteria, using the disc-diffusion method and micro-well dilution assay, revealed that aucubin had a good antibacterial activity against gram positive and gram negative bacteria. After a thorough search in the literature, no study has illustrated the aucubin antibacterial activity isolated from *Morinda citrifolia* L. The mechanism of aucubin action is also still unclear with no study available to illustrate it. Most of the published studies evaluated iridoid glucosides from medicinal plants as a complex material, with only few focused on the pure compounds, without any considerable efforts to illustrate the mechanism of its action.

Although the bioactivity of the SA3 fraction, which was isolated from *Sauropus androgynus* L. has been demonstrated in this study, and discussed intensively in the previous chapter, the identification and the molecular structure of this fraction is still unknown for the time being. Due to the scale of this project, and some technical issues we have faced throughout

the study, we could not identify the SA3 fraction. However, it will be identified later, and will be subjected to more biological studies to reveal its antibacterial actions and its potential mechanism of action.

5.4 Conclusion

In this study, aucubin, was isolated from the separation of methanolic extract of *Morinda citrifolia* L., through the bioassay guided extraction, with a purity level of 97.65 %, it showed a significant potency as an antibacterial agent, and recorded the highest activity among other active fraction obtained from medicinal plants used in the present study. For this reason, aucubin has been chosen for the next step of this project to illustrate its mechanism of action. *Staphylococcus aureus* (MSSA) has been employed as a drug discovery model in the mechanistic studies, in the hope of finding a novel drug target to tackle the global problem of the antibiotic resistant in general, and the methicillin-resistant *Staphylococcus aureus* (MRSA) in particular.

Chapter 7

Elucidation of the Aucubin Mechanism of Action

7.1 Introduction

In this study, aucubin was isolated from the methanolic extract of *Morinda citrifolia* L., through the bioassay guided extraction. As a result, aucubin, with a purity level of 97.65 %, has shown a significant potency to act as an antibacterial agent. It had the highest activity (Lowest MIC) amongst all active fractions of tested medicinal plants in this study. For this reason, the aucubin was selected for mechanism of action studies on *Staphylococcus aureus* (MSSA). It is essential to uncover the fundamental basis of the inhibitory mechanism, by which aucubin affects the bacterial growth. Hence, further experiments were conducted, aiming at the elucidation of the mechanism of action of aucubin.

Drug targets are proteins or signal transduction pathways in which an external drug can interfere with normal cell activities. Common applications of proteomics in drug discovery include target identification and investigations into mechanisms of drug action. This study comprehensively analysed the global proteome changes of a susceptible *Staphylococcus aureus* (MSSA) strain, which was affected by the addition of the aucubin to the growth environment (see chapter 3/section 3.2.4). There are several approaches generally used for global proteomics quantitative profiling including gel electrophoresis and label-free LC-MS/MS total bacterial protein analysis. The gel-based separation techniques involve sodium dodecyl sulfate polyacrylamide gel (SDS-PAGE) to separate intact proteins or protein complexes followed by in-gel digestion of proteins into peptides for LC-MS/MS. SDS-PAGE separates denatured proteins depending on the charge and molecular weight. Proteins with low molecular weight migrate faster compared to high molecular weight proteins (John, 2002).

Mass spectrometry (MS) based proteomics has the capability to study total proteins and their interactions in order to find significant new targets for future drug discovery. Current MS-based proteomics technologies have advanced to the point where they are amenable to any biological system. With regards to bacterial organisms, they are particularly attractive models to apply proteomics based approaches due to their smaller proteomes and limited post transactional modifications compared to eukaryotes. This allows for comprehensive proteome coverage (Lee *et al.,* 2015).

Recent improvements in high throughput and automation of LC-MS instruments and especially the development of novel algorithms dealing with LC-MS data have facilitated significant challenges in proteomics studies. Recently, there has been a growing interest towards label-free mass spectrometry based quantification in the field of proteomics. Following the advances in mass spectrometer technology, new techniques for data analysis evolve and new tools for quantification and pathway analysis are being developed. Pathway analysis is a rapidly developing discipline that combines software tools, database models, and computational algorithms, all of which help molecular biologists to convert molecular interaction data into a set of computational models. The models are developed for better prediction of cell behaviour in response to the drug or other outside stimuli (Pan *et al.*, 2014).

7.2 Results

7.2.1 Label-free quantitative proteomics analysis

Label-free quantitative proteomics was employed in this project to study the aucubin responses in *Staphylococcus aureus.* Although 2D-PAGE has some significant disadvantages such as the low load ability, difficulty in separation of hydrophobic proteins, and low reproducibility, the initial aim of the study was to apply the two-dimension gel electrophoresis to quantify only differentially expressed proteins in bacteria, and detect the most abundant proteins in control and treated samples. The reason for this is we tried to stay within the budget limit assigned for this project, as the MS analysis is too expensive for the total protein quantitation. Unfortunately, after many trials with 2D gel electrophoresis, we could not achieve the reproducibility, there was a lot of vertical and some horizontal streaking, even when the 2D cleanup kit was used. An example of the 2D-PAGE gels conducted in this study is available in the appendices. To tackle this problem, total bacterial protein quantification was employed instead. 1D SDS-PAGE was applied for protein separation (Figure 7.1). Bands were excised into a fresh siliconised Eppendorf tube with a fresh, clean scalpel blade, followed by In-Gel trypsin digestion (see chapter 3/section 3.2.4.5).

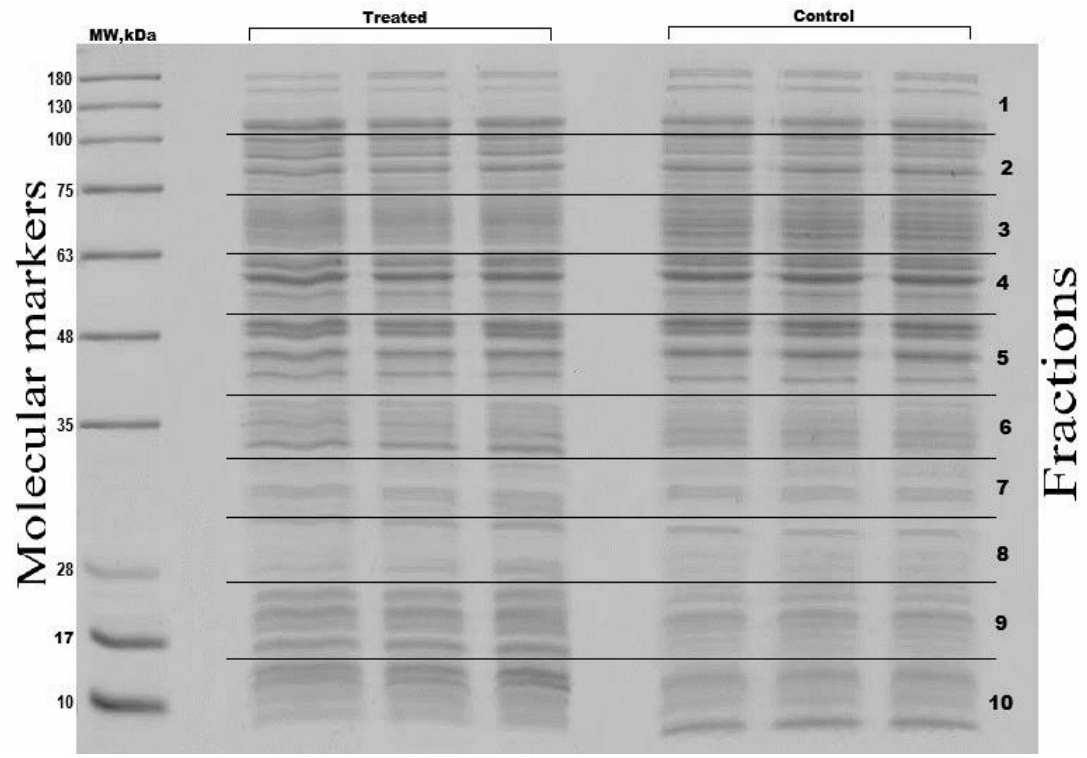


Figure 7.1: Image of 1D-SDS-PAGE separation of *Staphylococcus aureus* (MSSA) total proteins in two conditions; untreated cells, and treated cells with aucubin.

Protein samples were solubilised using 2 x Final Sample Buffer then heated at 95ºC for 10 min and allowed to cool to room temperature prior to loading. 20 µl of protein marker was used. Separation was performed using 12 % polyacrylamide resolving gels and 4 % stacking gel. SE600 Standard Dual Cooled Vertical Unit (GE Healthcare) was used at 50 v for 15min then followed by 200 v. The gel was stained with Coomassie Brilliant Blue R-250 (BioRad) and destined with destining solution. The gel was scanned using GS-800 Calibrated Densitometer (Bio-Rad) and Quantity One image analyser software was used.

Standard shotgun proteomics (bottom-up proteomics) technique were used to analyse the samples. The resulting peptides mixture, after tryptic digestion, were processed on ultra-high-pressure liquid chromatography (uHPLC) coupled with mass spectrometry (MS) system (BioMicS, University of Sheffield, UK), (See chapter 3/section 3.2.4.6). Data-Dependent Acquisition (DDA) approach were applied in this study. The produced MS2 spectra were further analysed using Progenesis QI for proteomics software (v 4.1 with a trial license generously provided by Nonlinear Dynamics, Waters, UK). The raw data files were loaded into the software followed by automatic alignment using the default parameters as recommended by the developer (Di Luca *et al.,* 2015). The software processed the raw data where each sample run was subjected to alignment, which involved aligning the data based on the LC retention time of each sample. This allows for any drift in retention time giving an adjusted retention time for all runs in the analysis. The sample run that yielded most features (i.e. peptide ions) was used as the

reference run, to which retention time of all of the other runs were aligned and peak intensities were normalised. Fractions were recombined at the end of the analysis (Figure 7.2).

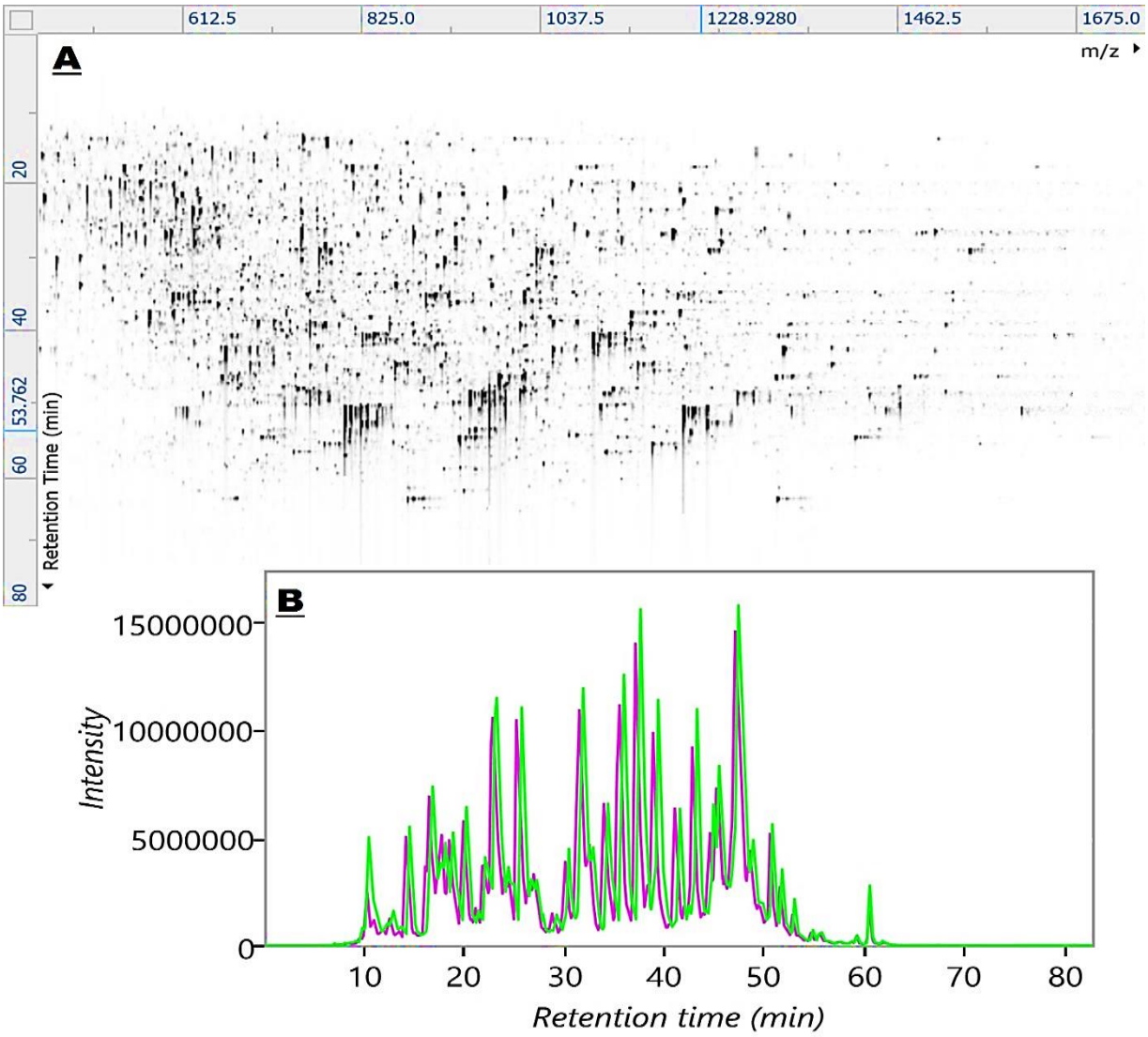


Figure 7.2: Graphical views of the treated sample alignment used in Progenesis QI for proteomics software. (A) Ion intensity map, and (B) Total Ion chromatogram shows the automatic alignment (green) overlaid on the reference chromatogram (magenta).

The resulting spectra were searched with Mascot engine (Matrix Science) against the Swissprot database for peak identification, followed by protein identification and quantification. Two different conditions of total bacterial proteome were analysed: untreated and treated group. Principal component analysis (PCA), was performed on Progenesis QI for proteomics software in order to observe the variation between sample groups (figure 7.3).

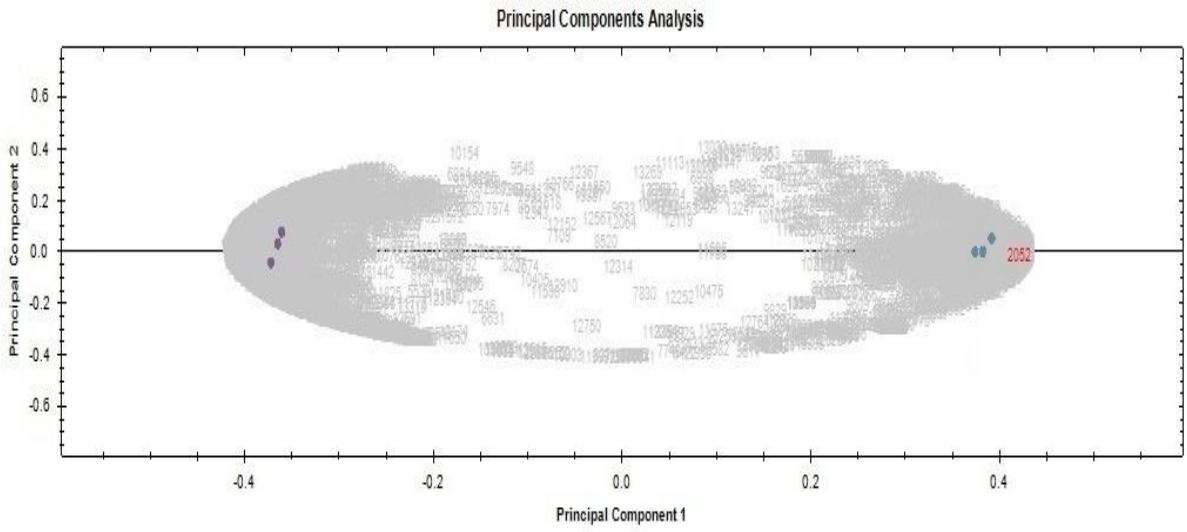


Figure 7.3: Biplot of Principal Component Analysis (PCA) analysis of MSSA proteome data sets. Control group on the left (purple), and aucubin treated group on the right (Blue).

In this study, 827 and 795 quantifiable proteins were identified in *Staphylococcus aureus* (MSSA) in the treated and control group, respectively. Among these proteins, 743 proteins are common in both groups, 84 proteins (10.15 %) are unique for aucubin treated MSSA, and 52 proteins (6.54 %) are unique for control group (Figure 7.4).

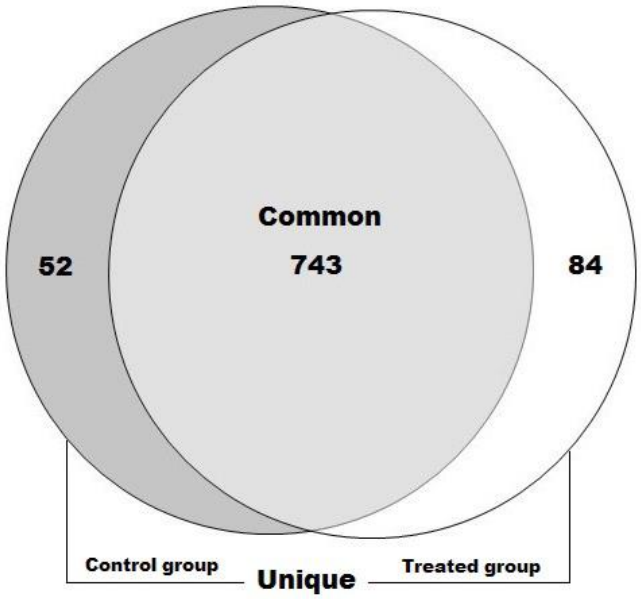


Figure 7.4: Venn diagram of proteome comparison on control sample of *Staphylococcus aureus* (MSSA) and aucubin-treated MSSA.

Aligned runs were analysed to create a data set containing the information about all the signals identified in all sample runs. In order to compare relative protein level between control and treated *Staphylococcus aureus* proteome, the relative abundance of a protein was calculated after normalisation using Progenesis QI software. This was done by calculating the average of the integrated intensity of the most abundant peptides for each protein across all their peptide ions. In this study, all 1622 proteins were used to normalise the samples. Data was filtered according to the *p*-value ≤0. 05 and fold changes higher/lower than ±2 fold in order to rely on proteins that were confidently identified. A cutoff for at least three unique peptides per protein was applied (To ensure only high quality data contributed to the analysis). Using these criteria, results showed only 74 proteins were differentially expressed in aucubin treated MSSA compared with untreated, 47 of which were significantly upregulated and 26 were significantly downregulated (Full list of up/down regulated proteins is available in the appendixes).

Furthermore, in order to obtain a general figure of the distribution of the differentially expressed proteins in the aucubin-treated *Staphylococcus aureus* (MSSA) cells, enrichment gene ontology (GO) analysis was undertaken through PANTHER (Protein Analysis Through Evolutionary Relationships) classification systems (Huaiyu *et al.*,2015), using the list of the 74 differentially expressed proteins. Enrichment Gen ontology is an annotation database, where standardised term are grouped under three main ontologies; cellular component, biological process and molecular function. The ontology terms are assigned to individual proteins by collaboration with numerous databases. The gen ontology annotation database can easily be used to identify overrepresentation of proteins set to obtain initial insights in the sample characterisation (Burge *et al.,* 2012). The analysis classified the differentially expressed proteins into several categories (Figure 7.5).

According to the protein and gene annotation database available in PANTHER classification system, the molecular function classification (Figure 7.5 A) showed that the majority of the identified up/downregulated proteins (59.6 %) were categorised as enzyme-related proteins and shown to have a catalytic activity which are involved in all aspects of cell metabolism, this includes the digestion of the large nutrient molecules (such as proteins, carbohydrates, and fats), the conservation and transformation of chemical energy, and the construction of cellular macromolecules from smaller precursors (Stephen & Hammes, 2003).

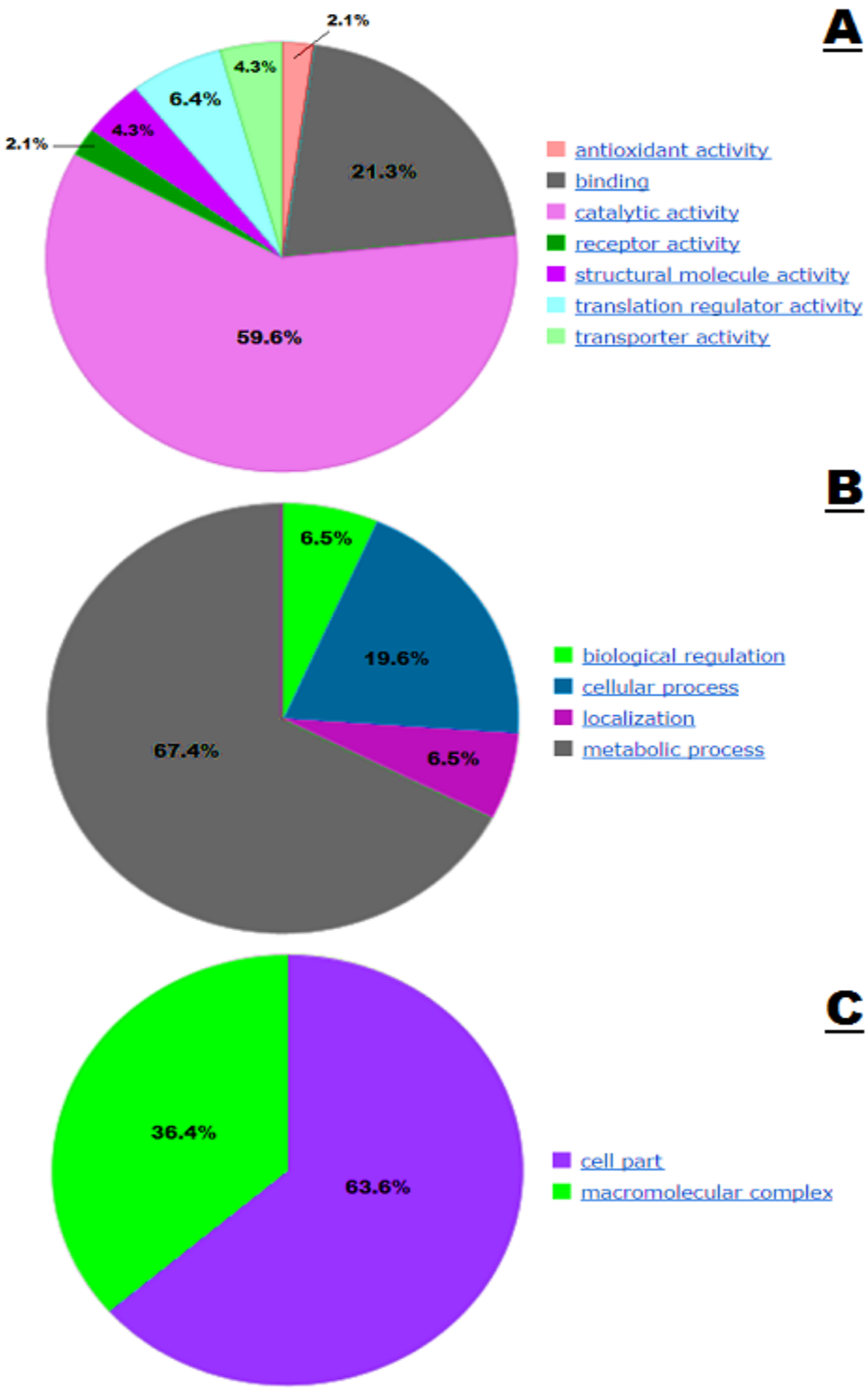


Figure 7.5: Gene ontology (GO) of the differentially expressed proteins in MSSA. 74 proteins were inputted into the PANTHER protein classification software, in order to determine the GO terms for (A) molecular function, (B) biological processes and (C) sub-cellular localisation. Pie charts are representations of the percentage of proteins within each classification.

An example of these enzymes, which were overexpressed in aucubin-treated MSSA are; hydrolase (enzymes catalysing hydrolysis of a variety of bonds, such as esters, glycosides, or peptides), dehydrogenase (enzyme that oxidizes a substrate by transferring hydrogen to an acceptor that is either NAD/NADP or a flavin enzyme.), glycosyltransferase (enzyme that catalyses the transfer of a sugar (monosaccharide) unit from a sugar nucleotide derivative to a sugar or amino acid acceptor), transaminase (A subclass of enzymes that catalyse the transfer of an amino group from a donor (generally an amino acid) to an acceptor (generally 2 keto acid)), and ligase (A class of enzymes that catalyse the formation of a bond between two substrate molecules, coupled with the hydrolysis of a pyrophosphate bond in ATP or a similar energy donor). This high percentage (59.6 %) of catalytic enzymes gives a hint that the aucubin might be involved in one or more of the metabolic process (cycles) in *Staphylococcus aureus* (MSSA) cells pathways. Binding proteins have a total share of (21.3 %), which includes nucleic acid binding proteins such as ribosomal protein, isomerase, and G-protein. Translation regulator activity proteins represent (6.4 %), whilst structural molecules and transporter proteins represent (4.3 %), in each group, of all 74 differentially expressed proteins. Antioxidant activity and receptor activity proteins represent only (2.1 %) in each group.

By looking at the protein's biological process classification results (Figure 7.5 B), the majority of differentially expressed proteins (67.4 %) related to bacterial metabolic process. A total of (19.6 %) is related to cellular processes proteins, an example of that is the aminoacyl-tRNA synthetase enzyme (aaRS), which is an enzyme that attaches the appropriate amino acid onto its tRNA to form an aminoacyl-tRNA. Biological regulation and localisation represent (13 %) of the differentially expressed proteins in each of them. The distribution of cellular component proteins classification (Figure 7.5 C) showed that the differentially expressed proteins fall into only two categories. (63.6 %) classified as macromolecular proteins and (36.4 %) classified as cell part proteins. The general figure for the distribution of total bacterial proteins, in the aucubin-treated *Staphylococcus aureus* (MSSA) and untreated (MSSA) samples, are illustrated in figures (7.6 and 7.7). Enrichment gene ontology (GO) analysis was undertaken through the PANTHER classification systems and revealed that the majority of identified proteins in both groups were involved in the metabolic process of the cells, and mainly localised in the cytoplasm. Other proteins groups were distributed in all other parts of the cells with different functionality.

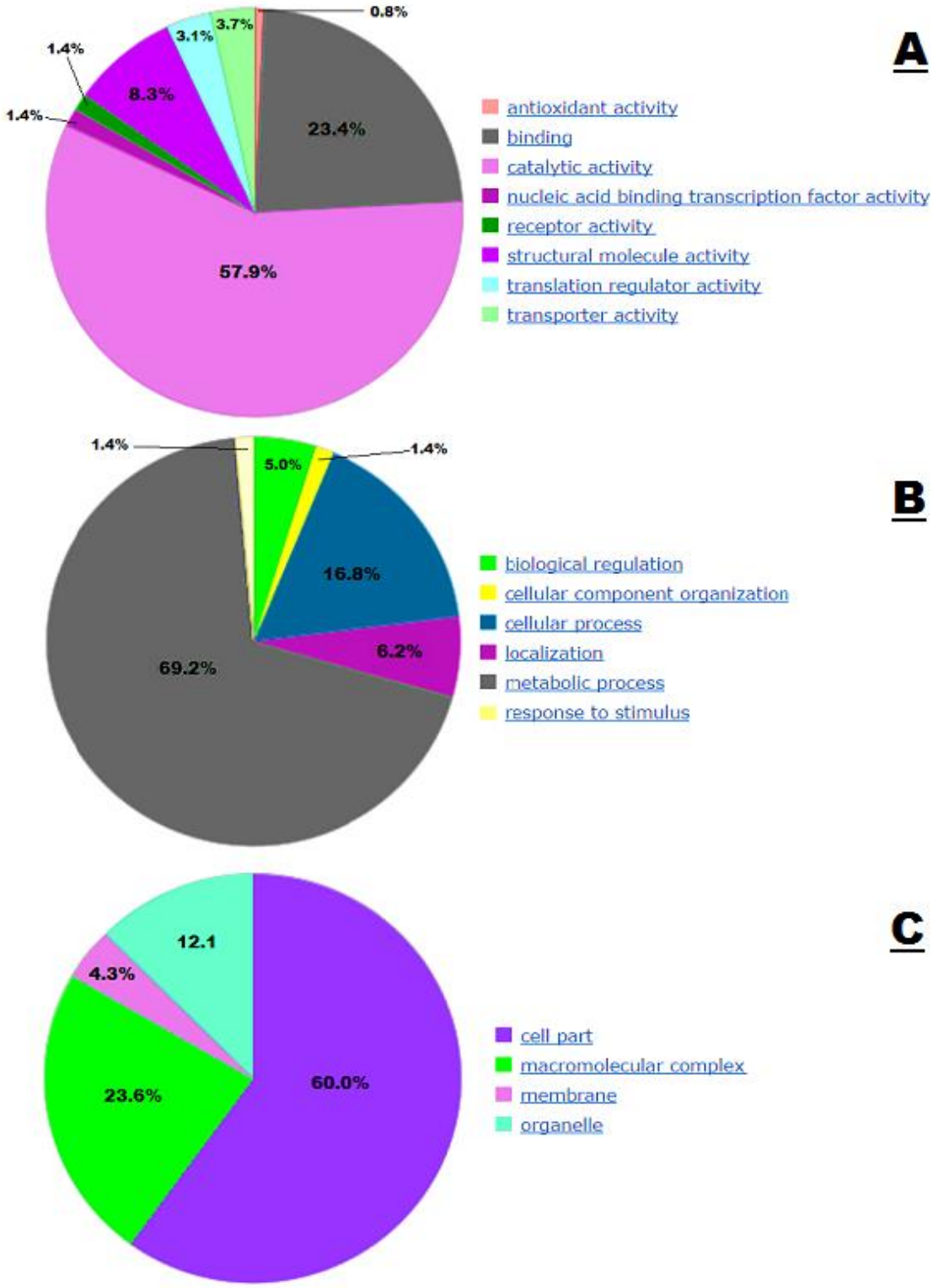


Figure 7.6: Gene ontology (GO) of total MSSA proteome in untreated sample. 795 proteins were inputted into the PANTHER protein classification software, in order to determine the GO terms for (A) molecular function, (B) biological processes and (C) sub-cellular localisation. Pie charts are representations of the percentage of proteins within each classification.

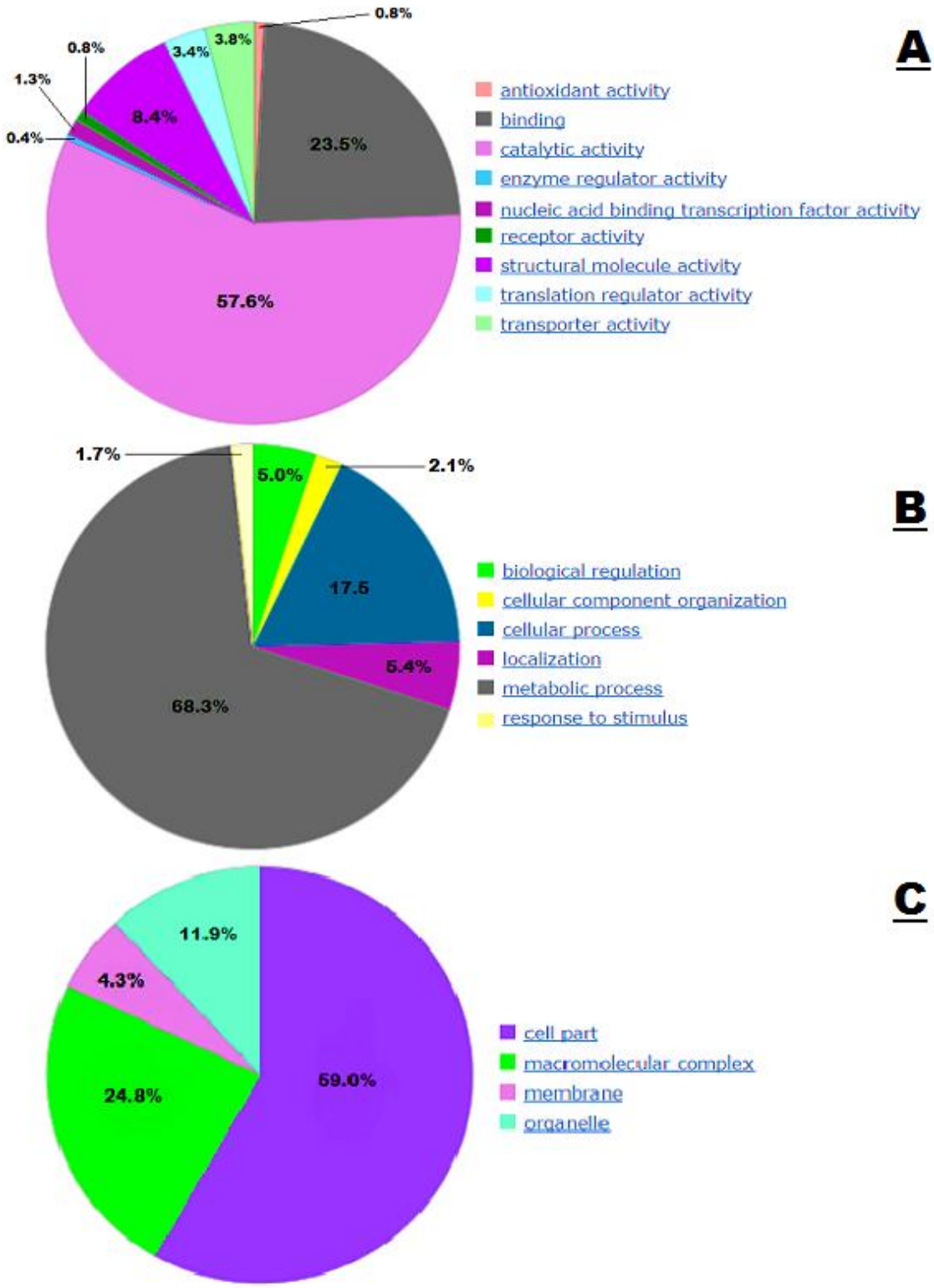


Figure 7.7: Gene ontology (GO) of total MSSA proteome in treated sample. 795 proteins were inputted into the PANTHER protein classification software, in order to determine the GO terms for (A) molecular function, (B) biological processes and (C) sub-cellular localisation. Pie charts are representations of the percentage of proteins within each classification.

7.2.2 Aucubin pathway analysis

Pathway analysis is a rapidly developing discipline that combines software tools, database models, and computational algorithms, all of which help molecular studies to convert molecular interaction data into a set of computational models. The models are developed for better prediction of cell behavior in response to any external stress, such as an exposure to a drug. The development of pathway analysis was triggered by the expansion of high throughput methods in genomic/proteomic researches. Due to these technological advances, the emphasis of molecular biology has shifted from reductionism to system integration (Anton, 2008). Proteomics studies provide the data needed to perform the pathway analysis, by focusing on the generation of information about proteins, such as expression levels, interacting partners, PTMs and activity, all of which contribute to elucidate pathways active inside the cells, and ultimately, to a functional understanding of a biological system of living cells. The first and most important thing in the pathway analysis is to provide tools and infrastructure that facilitate building a pathway model for a giving set of data. These tools must enable adequate data exchange, automatic data integration, communication with central public depositories of pathways, and molecular interaction information for better understanding of any changing in cell functions (Dimitri & Eric, 2009).

In the present study, the experimental data obtained from quantitative experiment of total bacterial proteome analysis, using a label-free proteomics approach, have shown an enriched subset of proteins, whose expressions were increased/decreased in the presence of aucubin in the growth environment. All of the differentially expressed proteins might be involved in the aucubin inhibitory action in *Staphylococcus aureus* (MSSA). A comprehensive Gene Ontology (G.O.) and enriched pathways analysis of the protein functions was performed and predicted by DAVID software (Database for Annotation, Visualization and Integrated Discovery, V. 6.7) (Huang *et al.,* 2009), given the set of differentially expressed proteins in aucubin treated (MSSA). This analysis gives a deeper insight into the mechanism of inhibitory action of aucubin.

DAVID, is a web based software suite designed to categorise complex, high content, genomic and proteomic data sets. It comprises a set of functional annotation tools allowing the user to examine the biological meaning behind large list of genes, with common outputs from

DAVID including identification of overrepresented biological pathways, visualisation of gene lists overlaid onto KEGG (Kyoto Encyclopedia of Genes and Genomes) pathway maps, and identification of interacting proteins. An advantage of DAVID is its intend to address several issues that other tools have not been able to extensively address; (i) to dramatically expand the biological information coverage in the DAVID knowledgebase by comprehensively integrating more than twenty types of major gene/protein identifiers and more than forty well-known functional annotation categories from dozens of public databases; (ii) to address the enriched and redundant relationships among many genes by developing a set of novel algorithms, such as the DAVID Gene Functional Classification Tool and the Functional Annotation Clustering Tool (iii) to dynamically visualise genes from a user's list within the most relevant KEGG pathways with the DAVID Pathway Viewer (Huang *et al.,* 2007).

Initially, data subset of the (74) differentially expressed proteins in aucubin treated MSSA, were obtained from quantitative proteomics analysis with Progenesis QI for proteomics software, out of 827, were subjected to the pathway enrichment analysis by the DAVID pathway enrichment tools. The analysis was performed by uploading the differentially expressed protein list to DAVID gene analysis tools, which automatically searched for the matched protein IDs within the its protein library. If a significant portion (>20 %) of input gene IDs fail to be mapped to an internal DAVID ID, a specially designed module (DAVID Gene ID Conversion Tool) will start up automatically in order to help map such IDs. This feature enables input gene ID to be mapped across the entire database, thus providing comprehensive coverage of gene associated annotation. The search retrieved 70 differentially expressed protein hits.

Subsequently, all identified proteins (up/down regulated) were analysed with enrichment analysis tool by setting up *Staphylococcus aureus* as an analysis background. The principle foundation of the enrichment analysis is that if a biological process is abnormal in a given study, the co-functioning genes should have a higher potential (enriched) to be selected as a relevant group by the high-throughput screening technologies. To decide the degree of enrichment, a certain background must be set up (MSSA in this study) in order to perform the comparison, and therefore decide if the enriched proteins played any important roles in the study (Huang *et al.*, 2009). The resulting pathways list was filtered by using a minimum of 2-fold enrichment and p value ≤ 0.01 for the enrichment of data output (Da-Wei *et al.*, 2009). The significant

enriched pathways predicted by DAVID (given the set of differentially expressed proteins in aucubin-treated MSSA) are summarised in (Table 7.1). These pathways might be the possible pathways involved in aucubin mechanism of action.

Up-regulated Pathways			
Pathway	Protein count	Enrichment	P value
Pyruvate metabolism	6	6.22	≤ 0.01
Glycolysis / Gluconeogenesis	5	4.47	≤ 0.01
Valine, leucine and isoleucine biosynthesis	4	3.6	≤ 0.01
Aminoacyl-tRNA biosynthesis	5	3.1	≤ 0.01
Purine metabolism	4	2.86	≤ 0.01
Citrate cycle (TCA cycle)	3	2.42	≤ 0.01
Propanoate metabolism	3	2.34	≤ 0.01
Down-regulated Pathways			
Pathway	Protein count	Enrichment	P value
Ribosome	4	3.66	≤ 0.01
Pyrimidine metabolism	3	2.52	≤ 0.01

Table 7.1: Pathway enrichment analysis by DAVID of the differentially expressed proteins in aucubin-treated MSSA compared with its untreated control. Fold enrichment of >2 was considered as significant.

In this study, aucubin isolated from *Morinda citrifolia* L. was used as an antibacterial to study its effect on *Staphylococcus aureus* (MSSA). Under this condition, aucubin-treated MSSA has activated some potential pathways to tolerate the drug and allow the cells to continue growing. Proteins that are differentially expressed in MSSA are potentially important as a generic tolerance response to the aucubin. We found that seven up-regulated pathways/proteins were identified by DAVID for these 70 differentially expressed proteins which matched with a DAVID genomic library. The pyruvate metabolism pathway (Figure 7.8) showed the highest enrichment score (6.22) with six abundant enzymes involved in this pathway; acetate kinase [EC 2.7.2.1], dihydrolipoyl dehydrogenase [EC 1.8.1.4], phosphate acetyltransferase [EC 2.3.1.8], probable malate: quinone oxidoreductase 2 [EC 1.1.5.4], pyruvate dehydrogenase E1 component subunit alpha [EC 1.2.4.1], and pyruvate kinase [EC 2.7.1.40], these enzymes are

encoded by the genes; ackA, pdhD, pta, mqo2, pdhA, and pyk, respectively (Zoraghi *et al.*, 2011).

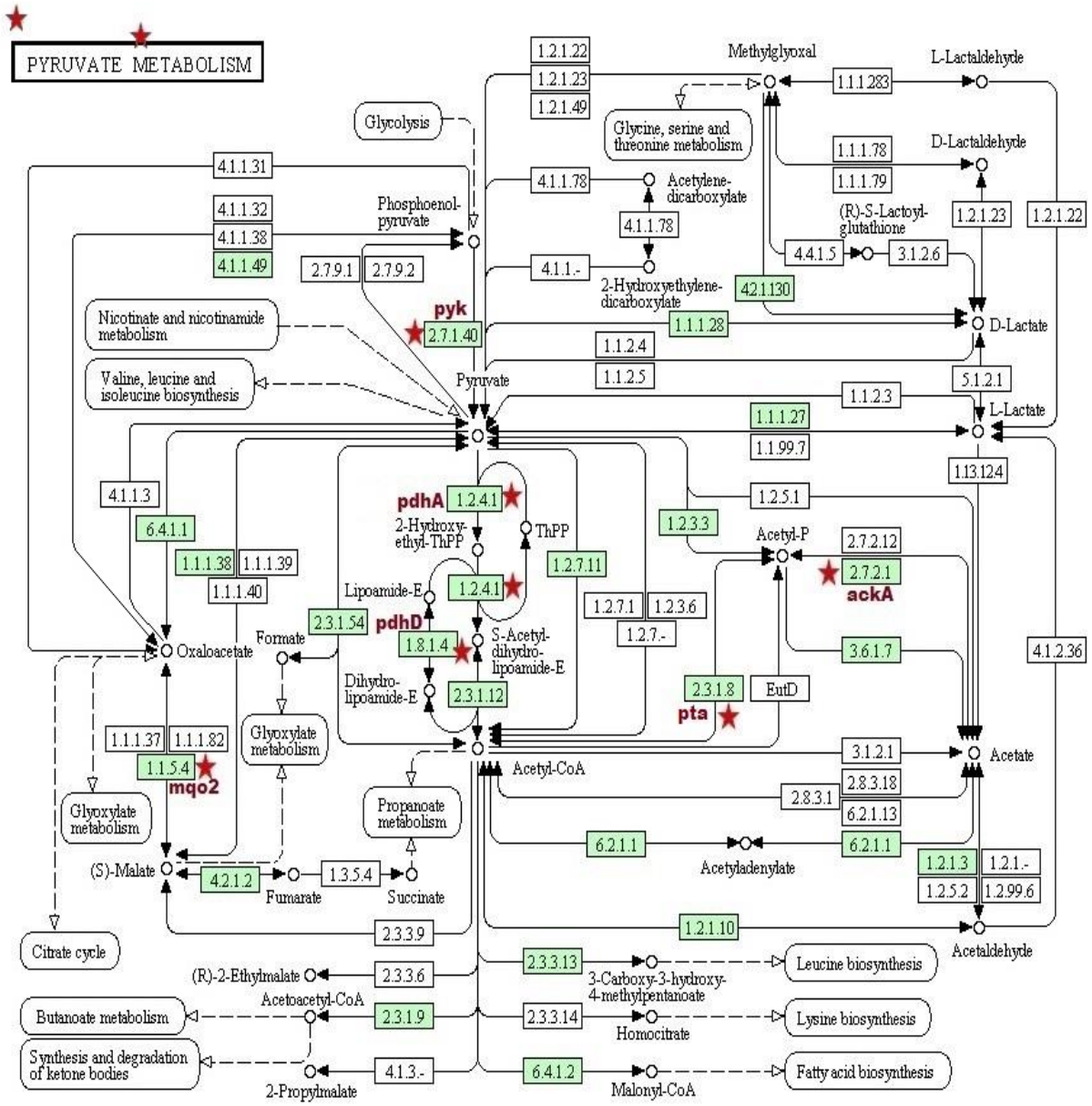


Figure 7.8: Pyruvate metabolism pathway showing the up-regulated genes in aucubin-traded MSSA relative to control MSSA. The underlying KEGG pathway was generated by DAVID and components of the pathway containing enriched genes are indicated by red stars.

The glycolysis/gluconeogenesis pathway (Figure 7.9) showed the second highest enrichment score (4.47) with five abundant enzymes from this pathway involved; enolase [EC 4.2.1.11], L-lactate dehydrogenase1 [EC 1.1.1.27], phosphoglycerate kinase [EC 2.7.2.3],

pyruvate dehydrogenase E1 component subunit beta [EC 1.2.4.1], and dihydrolipoyl dehydrogenase [EC 1.8.1.4], these enzymes are encoded by the genes: eno, IctE, pgk, phdB, and pdhD, respectively.

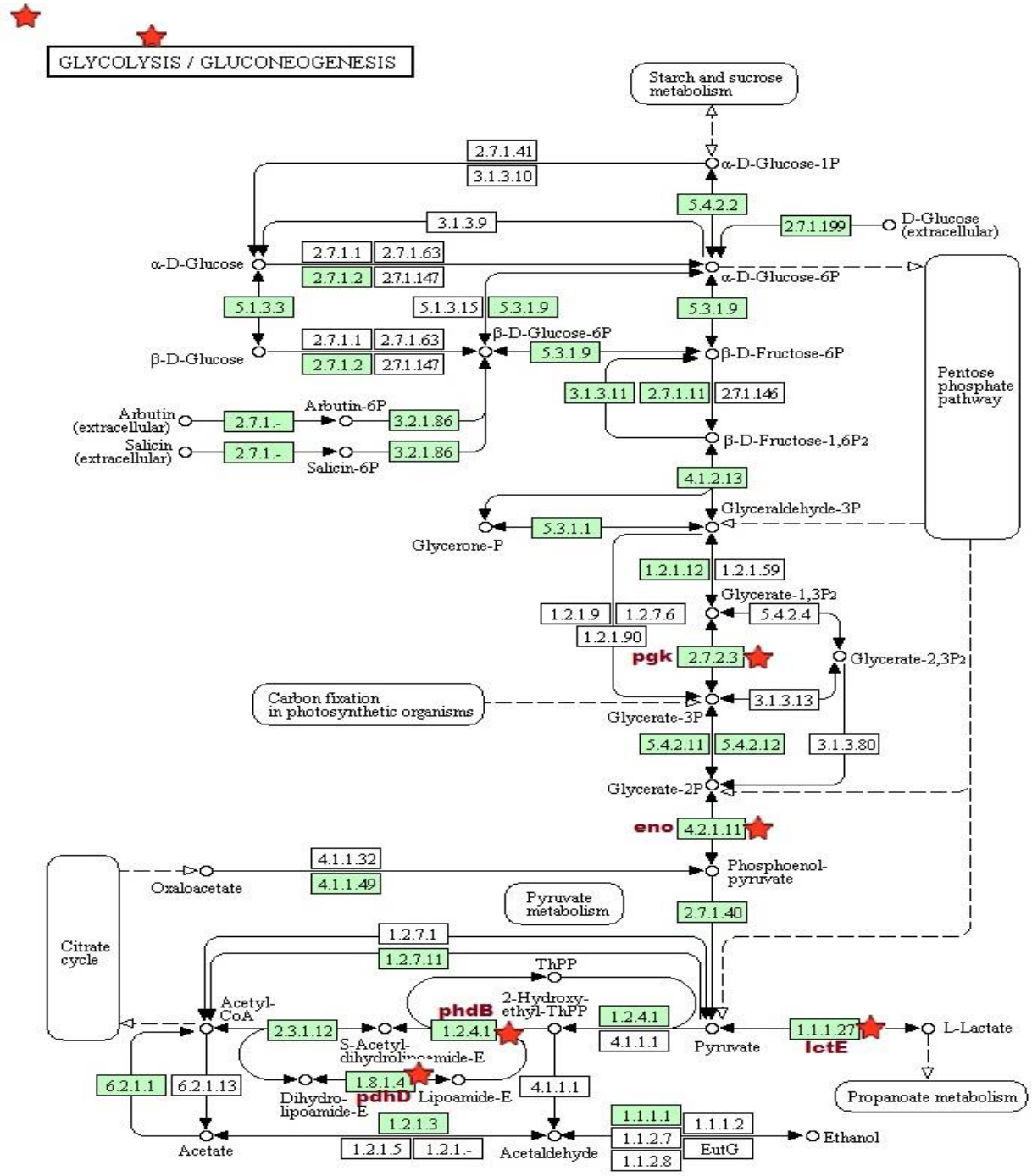


Figure 7.9: Glycolysis/gluconeogenesis metabolism pathway showing the up-regulated genes in aucubin-traded MSSA relative to control MSSA. The underlying KEGG pathway was generated by DAVID and components of the pathway containing enriched genes are indicated by red stars.

In the valine, leucine and isoleucine biosynthesis and purine metabolism pathways, four enzymes were involved. Whilst in the citrate cycle (TCA cycle) and propanoate metabolism pathways, three abundant enzymes were involved. Finally, five abundant enzymes were involved in the aminoacyl-tRNA biosynthesis pathway. Other than these pathways, there were a few efflux pump proteins detected when the stringency of the analysis being reduced. Several studies have suggested that the increase in the efflux pump protein expression is a natural activity response of bacterial cells to transport antibiotics out to the intracellular space to help cells survive the antibiotic treatment (Piddock, 2006). Two down-regulated pathways/ proteins detected in aucubin-treated MSSA, the first one is the ribosome, with an enrichment score of (3.66), four proteins were involved in this pathway; 30S ribosomal protein S11, 50S ribosomal protein L16, 50S ribosomal protein L29, and 50S ribosomal protein L6. These proteins are encoded by the genes: rpsK, rplP, rpmC, and rplF respectively. The second down regulated pathway is the pyrimidine metabolism, three enzymes were involved in this pathway way: bifunctional protein pyrR, carbamoyl-phosphate synthase large chain, and DNA-directed RNA polymerase subunit beta. These enzymes are encoded by the genes: pyrR, carB, and rpoB, respectively. A comprehensive list of enzymes/proteins involved in all pathways is available in the appendices.

7.3 Discussion

Proteins are vitally important components of organisms, which are responsible for executing the biological functions, and play important roles in the biological pathways. The whole set of proteins in the organism is called proteome, and the large-scale study of all the proteins in the organism is called proteomics. Proteomics is involved in different aspects of proteins, including protein identification, quantitation, post-translational modification (PTMs), structure and protein-protein interaction, and others. (Droit, Poirier, & Hunter, 2005). In recent years, label-free quantitative proteomics have become an emerging area, as an alternative to the conventional isotopic labelling approach. The scientific world has witnessed tremendous progress in this field. Remarkable developments in the state of art MS instrumentation, analytical workflows, and data analysis software have made proteomics an important tool for answering biological questions. Recently, proteomics has expanded from mere protein profiling to accurate and high-throughput protein quantification. With the appearance of a powerful MS

fragmentation technology, and the gradual shift from the gel-based to the MS-based proteomics, some entirely new methods has become available for quantitative comparisons (Ying *et al.*, 2015).

Nowadays, proteomic studies no longer focus only on identifying as many proteins as possible in a given sample, but aim for an accurate quantification of them. Especially in clinical proteomics, the investigation of variable protein expression profiles can yield useful information on pathological pathways or biomarkers and drug targets related to a particular disease. Over time, many quantitative proteomic approaches have been established allowing researchers in the field to refer to a comprehensive toolbox of different methodologies (Megger *et al.*, 2013). Due to these technological advances, quantitative proteomic approaches have become a lead solution in medical research, with applications in fields such as cancer research (Gamez-Pozo *et al.*, 2015), clinical research (Zhang *et al.*, 2015), antibiotic research (Liu *et al.*, 2014), malaria (Lindner *et al.*, 2013), and cardiovascular disease (Mirza, 2012). These are but a few of the many examples where proteomic approaches have been used in valuable quantitative proteomic studies.

In this study, shotgun proteomics approach, also known as "bottom-up" proteomics, was employed. This method is currently the most widely applied workflow in proteomics nowadays. It begins with the extraction of the whole set of proteins from the sample of interest. Typically, microgram to milligrams of proteins need to be extracted from the cells or tissues for the protein analysis. Following the protein extraction, both in solution and in gel digestion can be applied. In our workflow, proteins were separated by one-dimensional gel electrophoresis followed by the in gel digestion, and the whole protein gel bands were analysed. The digested peptides were collected and the LC-MS/MS analysis was performed. Gilmore & Washburn (2010), indicated that shotgun proteomics allows global protein identification as well as the ability to systematically profile dynamic proteomes. It also avoids the modest separation efficiency and poor mass spectral sensitivity associated with intact protein analysis, "top-down based proteomics". However, comprehensive proteomic analysis remains challenging specially with mass-limited samples. Shotgun proteomics is a continually changing field to address emerging obstacles (Feist & Hummon, 2015).

Data dependent acquisition (DDA) has been employed in our study, where the MS picked up the most intense peaks (corresponding to more abundant peptide ions) from the MS1 scan to fragment and acquire MS^2 spectra for those peaks. In this way, the instrument is sampling peptide peaks for sequencing, same intense peaks sampling can be avoided by setting the MS to exclude the same peak for MS2 during a certain time period after the peak is fragmented (Zhu, Chen & Subramanian, 2015). On the other hand, Gillet and co-worker (2012), reported that data independent acquisition (DIA) is utilised more typically in targeted proteomics workflows. In this method, a wider *m/z* window in the MS1 scan selected to be fragmented. All the peptide precursors within the mass window will be fragmented together to get a complicated MS^2 spectra, even rare peptides will be fragmented and can potentially be detected. Researcher must be aware when selecting this method as the data analysis is quite challenging.

As mentioned before, this study utilised label-free quantitative proteomics approach, rather than metabolic labeling approach, to study the *Staphylococcus aureus* (MSSA) proteome changes when treated with aucubin as an alternative antibiotic, and to illustrate the mechanism of aucubin action. The two main strategies for label-free quantification are peak area (or ion intensity) and spectral counting approach. The first one relies on the comparison of chromatographic peak intensities across multiple consecutive LC-MS runs. The second estimates relative protein abundance based on the number of relevant peptide fragment MS/MS spectra. Karpievitch and co-workers (2009), stated that spectral counts are easy to collect and do not require peak area integration like spectral peak analysis or label-based methods. It was found that relative protein abundance is mostly correlated with spectral counts when compared to other factors such as sequence coverage and peptide number. Lundgren and co-worker (2010), indicated that spectral counting method is especially suitable for low mass resolution data, for which intensity-based quantification is less reliable due to the difficulty in LC-MS alignment and peak integration. They mentioned another advantage of spectral counting over peak area which lies in the limited software requirements, whereas peak area needs very sophisticated computational algorithms to handle the LC-MS data for feature detection and peak alignment. On the other hand, Zhou, Liotta, & Petricoin (2012), argued that the reliability of final results strongly depends on peptide identification. In addition, the quality of inferred protein abundance depends on software and parameters used for MS/MS acquisition as well as on post-processing

of spectral counts. Moreover, spectral counting methods tend to be less accurate and have smaller dynamic range than intensity-based based approach.

Intensity-based quantitation measures the precursor ion intensities or areas belonging to a particular protein, and compares the relative quantitation based on the intensities or areas. This method will greatly rely on the MS alignment due to the retention time shift between injections. The MS alignment will align the peaks and ascertain the reproducibility of the LC-MS runs (Zhang *et al.,* 2013). There are different ways and programs, which can perform LC-MS alignment. Progenesis QI for proteomics is one of the LC-MS alignment softwares that have been used in this project. This tool aligns different runs to compensate for between-run variation in the LC separation technique. This results in increased reliability and precision of peak picking and peptide abundance measurements. To ensure consistent peak picking and matching across all data files, an aggregate data set was created from the aligned runs. This contains all peak information from all sample files, allowing the detection of a single map of peptide ions. This map is then applied to each sample, giving 100 % matching of peaks with no missing values, enabling users to apply multivariate statistical tools to explore the data and measure differential analysis. The peak picking algorithm handles complex samples and can discern overlapping peptide ions. Then, the survey scan data is used for ion abundance quantification (Oveland *et al.,* 2015).

This study employed label free quantitation based on signal intensity data, to obtain a global view of the altered protein expression in treated MSSA with aucubin. Some literature suggested that signal intensity data is more robust than spectral counting, as spectral counting does not consider retention time, and spectral counting method struggles to assign peptides when they are present in more than one protein (Elliott *et al.,* 2010). On the other hand, missing peptides are common in MS-based proteomic data according to Karpievitch and co-workers (2011), who mentioned that it is common to have 20 % of all attempted intensity measures missing. Abundance measurements are missed if a peptide was identified in some samples but not in others. This can happen because the peptide is present in low abundance, and in some samples the peak intensities are not high enough to be detected or for the corresponding ions to be selected for MS/MS fragmentation, another reason is that peptides whose chemical or

physical structure cause them to get trapped in the LC column, among others. Therefore, a robust LC-MS application is required when using this method (Cappadona *et al.,* 2012).

Proteomics technologies have been successfully applied to investigate differences in the protein expression profiles of cells grown under a different spectrum of growth conditions and with different stress factors including antibiotics treatment. The evaluation of protein profiles in response to multiple stress mechanisms, such as sensitivity to antibiotics or modifications related to antibiotic resistance, could represent a valid and integrating approach for the development of new therapeutic strategies (Sleno & Emili, 2008).

Bandow and co-workers (2003), used proteomics approach to elucidate the complex cellular responses of *Bacillus subtilis* to antimicrobial compounds belonging to classical and emerging antibiotic classes. They established their investigation on two-dimensional gel electrophoresis. A comprehensive database of cytoplasmic proteins with pIs covering a pH range of 4 to 7 were synthesised during treatment with antibiotics or agents known to cause generalised cell damage. They suggested that novel compounds with unknown mechanisms of action may be classified, as each antibiotic showed an individual protein expression profile, and overlaps in the expression of marker proteins reflected similarities in molecular drug mechanisms. They concluded that proteomics technique gives new insights into the bacterial response toward antibiotics and hints at modes of action of novel compounds. Such a method should prove useful in the process of antibiotic drug discovery.

The complete sequencing of the genomes of many pathogenic bacteria has led to an explosion in the knowledge about these organisms on the molecular bases (Monaghan and Barrett, 2006). The first whole bacterial genome to be sequenced was *Haemophilus influenzae* in 1995 (Fleischmann *et al.,* 1995). Since then, hundreds of bacterial genomes have been completely sequenced, yielding a massive amount of new information. Genomics, which is the infrastructure of the proteomics, is used to select potential antibacterial targets and can also be used to provide insights into pathogenesis and antibiotic resistance (Coates & Hu, 2007). An antibacterial target may be the receptor of a ligand in a bacterial molecule, a specific function of a bacterial molecule such as an enzyme, or a metabolic pathway. Target identification is achieved in a variety of ways, but the main principle is that the target should not be shared with the human host, should be present in those bacteria that cause the disease for which a cure is

needed, and if removed or inhibited leads to the death of the bacteria (essentiality) (Bakheet & Doig, 2010).

Recent studies conducted by Shatalin *et al* (2011) and Belenky & Collins (2011), have shown that when bacteria are exposed to an antibiotic, the defences in the bacteria are much more complicated than previously believed, affecting not only proteins directly involved in the target or specific resistance proteins, but also proteins with no apparent relationship to the antibiotic. It is reported that antibiotics can produce more oxidative stress on the cells, and in return, combating this stress is a general bacterial response to the antibiotics. Fajardo and Martinez (2008); Laubacher and Ades (2008), have employed a transcriptomics approach to study the global mRNA changes of bacteria related to antibiotic resistance, they have revealed that antibiotics can trigger a wide-ranging of transcriptional responses, including many metabolic or signal transduction pathways. These results support the idea that bacterial responses to antibiotics involve many different pathways and complicated networks, and should be examined from a global point of view. In this point of view, bacterial cells respond to antibiotic damage by regulating its metabolic pathways globally to repair the damage caused by antibiotic stress. Furthermore, bacteria that survive the antibiotic treatment will develop persistent adaptive responses over time, which eventually lead to resistance (Burchmore, 2014). The knowledge gained from such transcriptomics and proteomics studies can potentially provide new insights into the key players in the antibiotic response of the bacteria, and give a new hope for developing a new antibiotic with a potentially new pathway to cope with the uprising resistance.

In three studies conducted by Bonar *et al.* (2015); Vranakis *et al*. (2014); and Lima *et al*. (2013) they have reviewed the research applied on proteomics to investigate bacterial response to different antibiotics. The vast majority of studies have shown that bacterial responses to antibiotic damage are not limited to a few molecular targets but directly related to the known antibiotic resistance mechanisms, the effect was global inside the cells. No matter what classes of antibiotics were used, large number of proteins involved in various pathways were differentially regulated in the presence of antibiotics. Such studies proved that proteomics can potentially provide a more comprehensive picture of bacterial responses to antibiotics, and the pathways that are responsible for the observed inhibition.

Since the first genome sequence of *Staphylococcus aureus* was published (Makoto *et al.*, 2001), proteomics has been applied to study the mechanism of antibiotic actions, along with the resistance mechanisms. Among the 2600 proteins in *Staphylococcus aureus*, about 1000 show no homology to proteins in other organisms with known functions, and about third of these proteins are likely to be a novel virulence factor (Hecker *et al.*, 2010). Most of the proteomics studies on *Staphylococcus aureus* employed 2D-PAGE proteomics to investigate antibiotics resistance mechanisms, Singh *et al*. (2001) studied the antibiotic effects on *Staphylococcus aureus*, and nine proteins were found up-regulated and five of them were identified. Pieper *et al*. (2006), had combined 2-DE gels with MS and identified 65 overexpressed proteins in the presence of vancomycin. Fischer *et al*. (2011), employed transcriptomics and comparative proteomics to study the daptomycin and vancomycin mechanism of action on *Staphylococcus aureus*. Liu *et al.* (2014), investigated how the *Staphylococcus aureus* respond to antibiotic stress. They have applied a shotgun proteomics with spectral counting-based label-free quantitative proteomics. 81 proteins were shown differentially expressed in the presence of oxacillin, 65 were up-regulated and 16 were down-regulated proteins. The peptidoglycan biosynthesis pathway, pantothenate pathway, and CoA biosynthesis pathway were found to be up-regulated, along with a series of energy metabolism pathways.

Based on the knowledge of the most successful antibiotic classes, the ideal drug target should be an enzyme with a critical function in a biochemical pathway of central importance to the bacterial cell. In the search for novel antibiotics for which there is no pre-existing resistance, an obvious starting point is to identify novel targets. Proteomics approach have been utilised to identify genes coding for potential essential target functions in relevant pathogen species. The ultimate aim is to identify novel and essential targets (not targeted by currently used antibiotics) present in most interesting pathogens, whose inhibition will not risk collateral damage to the human host. However, there are several cautions and fine points to consider before limiting the search for novel drug hits to what might be a very short target list (Hughes & Karlen, 2014).

Novel targets are not always obvious, for instance, tetracyclines, and aminoglycosides, each of which interacts directly with the ribosome to inhibit protein synthesis. However, resistance to any one of these antibiotic classes, at the level of alterations to the structure of the ribosome, does not give resistance against either of the others, because the targets at the

molecular level are different. The ribosome is a large and complex machine, and the actual targets for each antibiotic class are structurally distinct (Laureti, Matic, & Gutierrez, 2013). The essential features required in a novel antibiotic target interaction are that it should inhibit bacterial growth (and preferably be bactericidal) that it should not be affected by existing resistance mechanisms, and that it should be available in a wide range of key bacterial pathogens. Avoiding targets present in human cells also comes with an important caution (Davies & Davies, 2010).

In our study, the ultimate aim was to investigate the aucubin-treated *Staphylococcus aureus* (MSSA) proteome to reveal the cellular responses and the potential pathways of this antibacterial candidate. More than one pathway was detected in the overrepresentation enrichment pathway analysis using DAVID enrichment tool. Among the seven upregulated pathways, the highest enrichment score detected with pyruvate metabolic pathway (Figure 7.10), and six enzymes were involved as a response to the aucubin treatment, glycolysis/gluconeogenesis pathway came second in the enrichment test with five enzymes involved, followed by valine, leucine and isoleucine biosynthesis pathway. Four more pathways were detected with lower enrichment scores. Two downregulated pathways were detected: ribosome with five proteins involved, and pyrimidine metabolism with three proteins involved in this pathway.

These pathways targeting mainly bacterial central metabolism. Researchers in this field are desperately trying to target this route, as an alternate to the conventional antibiotic targets, which they act on by inhibiting a limited spectrum of cellular processes essential during logarithmic growth. Giving the fact that all bacteria must utilise the energy sources in their environment in order to produce ATP (adenosine triphosphate), where ATP is required for all of the biosynthetic processes that bacteria use for their maintenance and reproduction. Bacterial central metabolism pathways could be the target of the next generation of antibiotics to combat the worldwide explosion of drug resistance (Stuart, 2015). In the pyruvate metabolism pathway, which showed to be up regulated in the overrepresentation enrichment pathway analysis, of the aucubin treated cells, the enzymes involved are: acetate kinase, dihydrolipoyl dehydrogenase, phosphate acetyltransferase, malate: quinone oxidoreductase 2, pyruvate dehydrogenase E1

component subunit alpha, and pyruvate kinase. These enzymes encoded by the genes ackA, pdhD, eutD, mqo2, pdhA, and pyk respectively.

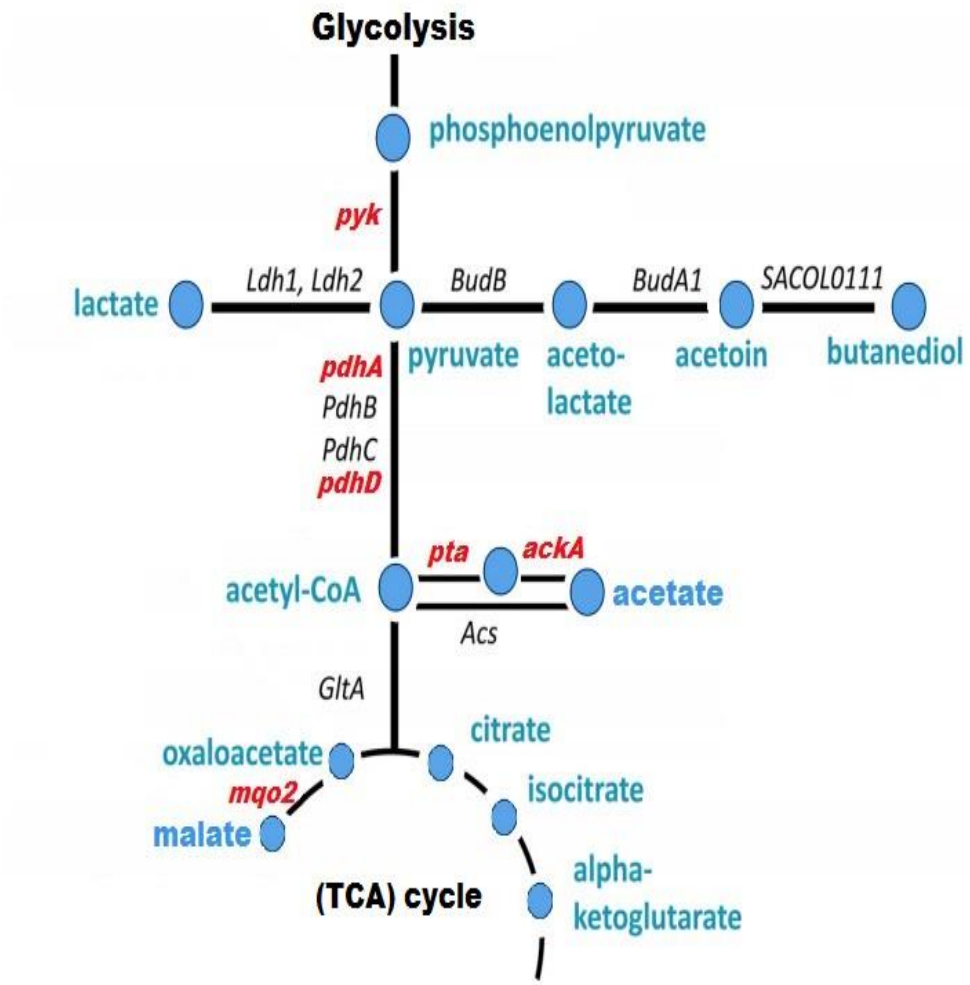


Figure 7.10: Schematic representation of the pyruvate metabolic pathways in *Staphylococcus aureus*. Pyruvate is metabolised to Acetyl-CoA via PDH complex, which is formed by four protein subunits (PdhA, -B, -C, and -D) or reduced to lactate or to butanediol via acetolactate and acetoine. Acetyl-CoA is either funneled into the tricarboxylic acid cycle (TCA), used for various biosynthetic pathways, or hydrolysed to form free acetate.

Six enzymes showed to be upregulated in the overrepresentation enrichment pathway analysis of aucubin-treated *Staphylococcus aureus* (MSSA). The involved enzymes marked in red. (ackA) Acetate kinase, (pdhD) Dihydrolipoyl dehydrogenase, (pta) Phosphate acetyltransferase, (mqo2) Probable malate: quinone oxidoreductase 2, (pdhA) Pyruvate dehydrogenase E1 component subunit alpha, and (pyk) Pyruvate kinase.

Pyruvate kinase (EC 2.7.1.40) catalyzes the final step in glycolysis with the irreversible conversion of phosphoenolpyruvate (PEP) to pyruvate with the concomitant phosphorylation of ADP to ATP. As pyruvate kinase plays a major role in the regulation of glycolysis, its inhibition leads to the interruption of carbohydrate metabolism and energy depletion. Moreover, both the substrate and the product of this reaction feed into a number of biosynthetic pathways, placing pyruvate kinase at a pivotal metabolic intersection. Pyruvate kinase in *Staphylococcus aureus* possesses different kinetic properties and regulatory mechanisms than do human isozymes (Vander *et al.,* 2010). These differences can be attributed to differences in protein sequences and structures. (Zoraghi *et al.,* 2011).

The first step in the oxidation of pyruvate is an oxidative decarboxylation reaction. This reaction is carried out by a very large enzyme complex, the pyruvate dehydrogenase complex (PDHC) (EC 1.2.1.4). The reaction catalyzed by the pyruvate dehydrogenase complex is irreversible, and is tightly regulated. The pyruvate dehydrogenase complex is closely related to the a-ketoglutarate dehydrogenase complex (an important TCA cycle enzyme) and to the branched chain a-ketoacid dehydrogenase complex (an important enzyme in the metabolism of leucine, valine, and isoleucine). The protein complexes of this family contain three main enzymes: E1, E2, and E3. The same E3 gene is used for each of enzyme complexes, while the E1 and E2 genes are specific to the different types of substrate (Vilhelmsson & Miller, 2002). The pyruvate dehydrogenase complex is inhibited by acetyl-CoA, which is the product of the reaction, and can also be inhibited by nicotinamide adenine dinucleotide (NADH). This enzyme can be produced by fatty acid breakdown in another route (Patel & Korotchkina, 2006).

Acetate kinase (EC 2.7.2.1) is another enzyme found to be upregulated in this study which involved in the pyruvate metabolism. It is a conserved enzyme that is widespread in bacteria, it is responsible for the phosphorylation of acetate (Lawrence & Ferry, 2006). Since acetyl coenzyme A is a precursor of fatty acid synthesis, it is acceptable that primary cellular growth depends on the activation of acetate through the process of intracellular phosphorylation. Acetate kinase has different roles in bacteria, bacteria use acetate kinase for energy generation by converting ADP and acetyl phosphate to ATP and acetate (Asgari *et al.*, 2013). This enzyme is present in prokaryotic organisms and some eukaryotic organisms e.g. parasites, but absent in mammals. For that reason, it has been reported as an attractive drug target for the development

of new antibiotic drug (Yadav *et al.,* 2012). The importance of targeting the pyruvate metabolism pathway steams from the fact that it is located at a major junction of assimilatory and dissimilatory reactions which make it vital pathway for the cells to survive.

7.4 Conclusion

In summary, current study suggested that more than one pathway being alerted in the presence of aucubin, whereas the pyruvate metabolism, upregulated, showed the highest enrichment score in the pathway analysis and might be the main route for the drug. Our results come in line with many studies (Liu *et al.,* 2014; Lima *et al.,* 2013), these studies indicated that bacterial responses against antibiotics involved many pathways other than the main drug pathway, as bacterial cells trying to overcome the antibiotic stress which causes some vital routes to be inhibited. This study has suggested several important clues in explaining the mode of action by which aucubin exert its inhibitory activity against *Staphylococcus aureus*, and confirmed the antibacterial activity of aucubin from natural sources. This project is the first to apply label-free quantitative proteomics to study natural antibacterial candidate and illustrate the response in *Staphylococcus aureus*. These new data offer a more complete view of the proteome changes in bacteria in response to the antibiotic. Data obtained from the pathway analysis will be subjected to some validation methods so it can be confirmed.

Chapter 8
General Conclusions and Future Perspectives

8.1 General conclusions

In the field of drug discovery, natural products (secondary metabolites) have been the most successful source of potential drug leads. The use of natural products as medicines has been described throughout history in the form of traditional medicines, remedies, and oils with many of these bio-active natural products still being unidentified. Natural product drug discovery leading to robust and viable lead candidates remains a challenging scientific task, which is the transition from a screening hit to a drug candidate, the task requires expertise and experience. However, the development of new technologies has revolutionised the screening of natural products in discovering new drugs. Applying these technologies compensates for the inherent limitations of natural products and offers a unique opportunity to re-establish natural products as a major source for drug discovery (Dias, Urban, & Roessner, 2012). The present study attempted to describe the utilisation of bio-active compounds, derived from natural resources, as an antibacterial candidate, with a focus on the most active candidate to illustrate its mechanism of action.

Natural products have been a rich source of antibiotic drugs for many decades, but investments in this area have declined over the past three decades. Of all the drugs currently approved as antibacterial agents, a significant percentage of those are either natural products themselves or derived from a natural product scaffold. Although the discovery of antibiotics is one of the biggest medical breakthroughs of the twentieth century, it was immediately followed by the unfortunate emergence of bacterial antibiotic resistance. The rapid rate of bacterial evolution to overcome the antibiotic action, the ability of a single pathogen strain to resist multiple drugs, as well as the stunning frequency of resistance occurring constitute a major challenge to the medical profession, and thus raised retrospective discussions of currently existing antibiotics. In spite of the fact that there are hundreds of antibiotics on the market, it remains a fact that almost all existing antibiotics target only four cellular functions: protein synthesis, nucleic acid synthesis, cell walls synthesis or folate synthesis. Repetitively striking the same cellular sites leads to defensive bacterial gene mutation, which remains the primary cause of the prevalence of antibiotic-resistant bacteria.

The problems with the antibiotic resistant bacterial pathogens that it became a significantly worrying issue and costs thousands of lives if not millions, all around the globe.

The latest figures showed that by 2050, the lack of an effective antibiotics will claim 10 million people each year, and most of them from the poor countries. For this reason, the World Health Organisation (WHO) has called for an immediate action to address this major issue. The current shortage of effective antibiotics is not simply a biological problem (evolution of resistance), or even a social problem (misuse of antibiotics), but is in large part the result of a long-term lack of investment in discovery and development programmes by the large pharmaceutical companies. There are currently very few antibiotics in late-phase clinical trials, and nearly all of these belong to existing classes. The reasons for this investment gap in novel classes are complex, but they are associated with a lack of confidence on the part of the pharmaceutical industry in the relative profitability of antibiotics. However, this may be about to change with the help of public/private partnership programmers, like the recently launched EU Innovative Medicines Initiative (New Drugs 4 Bad Bugs, ND4BB) programme, which represents an unprecedented partnership between industry, academia and biotech organisations to combat antibiotic resistance in Europe, and eventually in the world, by tackling the scientific, regulatory, and business challenges that are hampering the development of new antibiotics.

This study has been conducted as a contribution to addressing the uprising resistance problem, the main focus of this study was on the exploration of *Centella asiatica* L., *Imperata cylindrica* L., *Morinda citrifolia* L., and *Sourpuss androgynous* L. to pursue the search for new antibacterial candidate of natural compound. This was done through a bioassay-guided isolation and purification protocols. Initially, the plants were extracted using Soxhlet and maceration techniques, to obtain soluble containing materials, as the main constituents of the crude extracts. The plant extracts were subjected to the bioscreening test against Gram positive and Gram negative bacteria. Upon the initial screening, further isolation and purification procedures were performed using an advance chromatography technique (HPLC), in order to purify the most-active compounds that represent the final candidates of natural product. Finally, this study has employed further spectroscopy and label free proteomics studies, in an effort to identify the structure of most active components, and illustrate the fundamental principles of the mechanism of action of the most active one among them.

Initial bioscreening tests conducted on the four medicinal plant extracts, the test indicated that these extracts have a potency as antibacterial candidate. Methanolic extracts were proven

to exhibit the highest potency in inhibiting bacterial growth, and the Gram positive strains were the most sensitive ones. Chromatography techniques have applied on methanolic extracts, because it showed the highest activity, and collected fractions underwent the bioscreening test to reveal the most active fractions against tested bacterial strains. The spectroscopy techniques resulted in the identification of asiatic acid from *Centella asiatica* (L., quercetin from *Imperata cylindrica* L., and aucubin from *Morinda citrifolia* L. The active fraction of *Sourpuss androgynous* L. yet to be identified. Aucubin demonstrated the highest activity with lowest MIC against tested strains, for this reason, it has been chosen to illustrate its mode of antibacterial action. Aucubin, is found in a number of medicinal plants and known for its biological activities. However, until recently, only few studies have reported its antibacterial properties, and little progress has been made with regards to the exploration of aucubin fundamental antibacterial activity. Moreover, all the past studies are limited to demonstrate its MIC values only. Accordingly, to the best of our knowledge, current study was the first to discover the antibacterial activity of aucubin isolated from *Morinda citrifolia* L. with an illustration of its potential mechanism of action. Moreover, many other active compounds were isolated and showed a good antibacterial activity. However, due to the time and budget limitation, this study could not elucidate their molecular structure or their mechanism of action at this time.

The investigation of the mechanism of action of aucubin in *Staphylococcus aureus* (MSSA) suggested that the bacterial cells have responded to the drug by activating several pathways to survive the stress. The pathway enrichment test suggested that the aucubin targeted the bacterial central metabolism pathways, and the results yet to be verified. Recently, many studies suggested that the bacterial central metabolism pathways will be the next generation of the antibiotic. Six proteins were involved in the pyruvate metabolism pathway and it might be the main route for the drug, followed by the glycolysis/gluconeogenesis pathway in the enrichment test, with five proteins were involved in this route. Few other pathways have shown to be up/down regulated in the presence of aucubin. Latest studies suggested that it is a normal response from bacterial cells to combat the drug and continue the biological processes. In summary, this study has investigated in scientific approaches the aucubin activity as an antibacterial agent. Moreover, the mechanism of action of aucubin was also studied by applying label free proteomics analysis of global bacterial proteome, which was never been reported before. In fact, this study is one of the very few studies to apply label free proteomics to study

the effects of antibiotics *in-vitro*. Finally, the results produced in this study are an additional contribution into the natural product and antibiotic researches to help finding a solution to the bacterial resistance crisis.

8.2 Future perspectives

The results of the antibacterial evaluation of the four medicinal plants suggested that these plants have the potential to be a good natural source for antibacterial agents. The future work should focus on investigating of other active compounds isolated in this study. Another potential idea for future work could be using other extraction techniques with more extracting solvents in different polarities to retrieve as many active compounds as possible. This study has proved that the bioscreening test using new technologies, such as the Bioscreen-C machine was better than the traditional techniques in term of accuracy, such as disc diffusion assay. Regarding the purity check analysis of the isolated compounds, the qNMR technique has proven to be fast, easy, and reliable method, and might be followed for drug purity checks rather than the traditional chromatographic techniques. With regards to mechanism of action studies, label-free proteomics method was used to illustrate the mechanism of tested drug action on molecular level. The investigation will be continued in the future to identify another active compound from medicinal plants used in this study, and illustrate the mechanism of their actions. Another approaches will be considered, the protein-protein interactions, and the drug target validation, to see the whole picture of the candidate drug.

Validation of the data is a crucial step in the drug discovery process. Most drugs are inhibitors that block the action of a particular target protein. The only way to be completely certain that a protein is contributory in a given disease is to test the potential drug *In-vivo*. Obviously, such clinical trials cannot be used for initial drug development, which means that a potential target must undergo a validation process. Validating proteomics data can be done in a number of ways, western blotting is an accepted standard, and flow cytometry is another technique can be used. RT-PCR and transcript level methods are good for complementing protein level results.

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Appendices

Appendix 1: Product specification of *Centella asiatica* L. plant material

haldin

MATERIAL SAFETY
DATA SHEET

Centella Asiatica

Product Code | 10000069

SECTION 1.		**Product Identification**
Product Name	:	Centella Asiatica
Product Code	:	10000069
SECTION 2.		**Composition**
Product Appearance	:	Pale brown to light brown powder
Description	:	Powdered extract, produced from selected leaves of *Centella asiatica*
Botanical Name	:	*Centella asiatica*
SECTION 3.		**Hazard Identification**
		Non-Hazardous Material
SECTION 4.		**First Aid Procedures**
Inhalation Exposure	:	Remove person to ventilated area
Skin Contact	:	Wash with soap and water
Eye Contact	:	It may irritate if contact with eyes. Flush with large amount of water, seek prompt medical attention if irritation persist.
Other		N/A
SECTION 5.		**Fire Fighting Measures**
Extinguishing Media	:	Water, fog, CO2, foam or dry chemical
Special Fire Fighting Procedures	:	Cool containers exposed to flame with water
Unusual Fire and Explosion Hazard	:	None material is not pyrophoric, does not react with water, not an oxygen donor, material is shock stable
Hazard Combustible or Decomposition products	:	Form carbon monoxide (CO) and or dioxide (CO2) upon burning

MSDS/TSD/025-01

Appendix 2: Product specification of *Imperata cylindrica* L. plant material

haldin

MATERIAL SAFETY
DATA SHEET

Imperata Cylindrical

Product Code | 10000045

SECTION 1.	Product Information
Product Name	| Cogon Grass Powder Extract
Product Code	| 10000045
Product Appearance	| Light brown to brown powder
Description	| Powdered extract, produced from selected roots of *Imperata cylindrica*
Common Name	| Alang Alang (Indonesian) Cogon Grass (English)

SECTION 2.	Fire and Explosion Hazard
Extinguishing Media	| Water, fog, CO_2, foam or dry chemical
Special Fire Fighting Procedures	| Cool containers exposed to flame with water
Unusual Fire and Explosion Hazard	| None, material is not pyrophoric, does not react with water, not an oxygen donor, material is shock stable
Hazardous Combustible or Decomposition Products	| Forms Carbon monoxide (CO) and or dioxide (CO_2) upon burning
Stability	| Stable under normal conditions
Conditions To Avoid	| Excessive Heat
Materials To Avoid	| Strong Oxidizing Agents
Hazardous Polymerization Products	| None

SECTION 3.	Physical and Chemical Properties
Appearance	| Light brown to brown powder
Odor	| Characteristic
Solubility	| Soluble in water
Moisture Content (%)	| NMT 8.0

MSDS/TSD/013-02

Appendix 3: Product specification of *Morinda citrifolia* L. plant material

haldin

MATERIAL SAFETY
DATA SHEET

Morinda Citrifolia

Material Code | 10000109

SECTION 1. Product Identification

Product Name	:	Morinda Citrifolia
Product Code	:	10000109

SECTION 2. Composition

Product Appearance	:	Pale brown powder
Description	:	Natural powdered extract, produced from selected fruits of *Morinda citrifolia*
Botanical Name	:	*Morinda citrifolia*

SECTION 3. Hazard Identification

Non-Hazardous Material

SECTION 4. First Aid Procedures

Inhalation Exposure	:	Remove person to ventilated area
Skin Contact	:	Wash with soap and water
Eye Contact	:	It may irritate if contact with eyes. Flush with large amount of water, seek prompt medical attention if irritation persist.
Other		N/A

SECTION 5. Fire Fighting Measures

Extinguishing Media	:	Water, fog, CO2, foam or dry chemical
Special Fire Fighting Procedures	:	Cool containers exposed to flame with water
Unusual Fire and Explosion Hazard	:	None material is not pyrophoric, does not react with water, not an oxygen donor, material is shock stable
Hazard Combustible or Decomposition products	:	Form carbon monoxide (CO) and or dioxide (CO2) upon burning

MSDS/TSD/050-00

Appendix 4: Product specification of *Sauropus androgynus* L. plant material

haldin

MATERIAL SAFETY
DATA SHEET

Sweet Leaf Bush

Product Code | 10000130

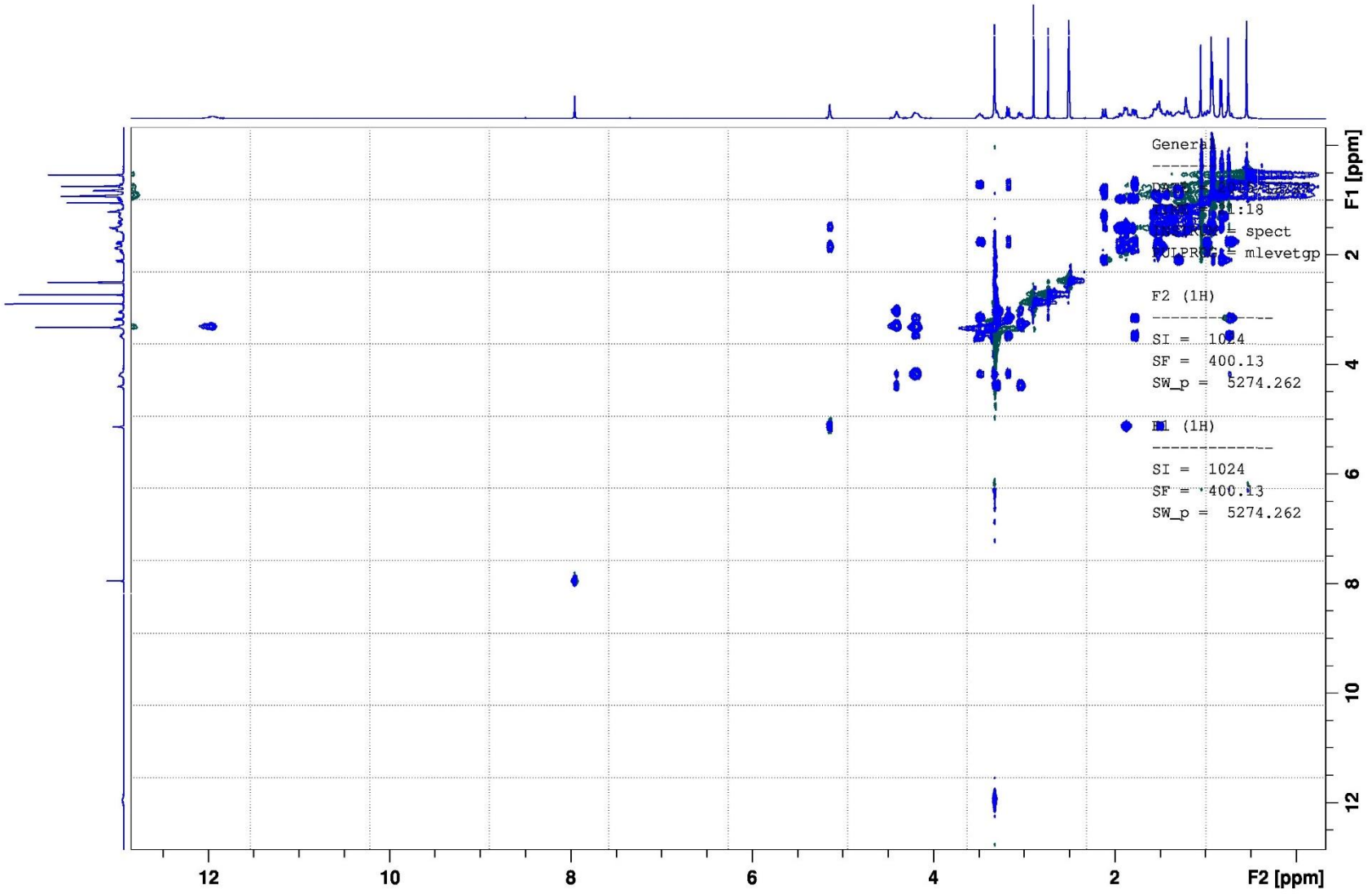
SECTION 1.	Product Information
Product Name	| Sweet Leaf Bush
Product Code	| 10000130
Product Appearance	| Light yellowish powder
Description	| Powdered extract, produced from selected leaves of *Sauropus androgynus*
Common Name	| Katuk (Indonesian) Sweet Leaf Bush (English)

SECTION 2.	Fire and Explosion Hazard
Extinguishing Media	| Water, fog, CO_2, foam or dry chemical
Special Fire Fighting Procedures	| Cool containers exposed to flame with water
Unusual Fire and Explosion Hazard	| None, material is not pyrophoric, does not react with water, not an oxygen donor, material is shock stable
Hazardous Combustible or Decomposition Products	| Forms Carbon monoxide (CO) and or dioxide (CO_2) upon burning
Stability	| Stable under normal conditions
Conditions To Avoid	| Excessive Heat
Materials To Avoid	| Strong Oxidizing Agents
Hazardous Polymerization Products	| None

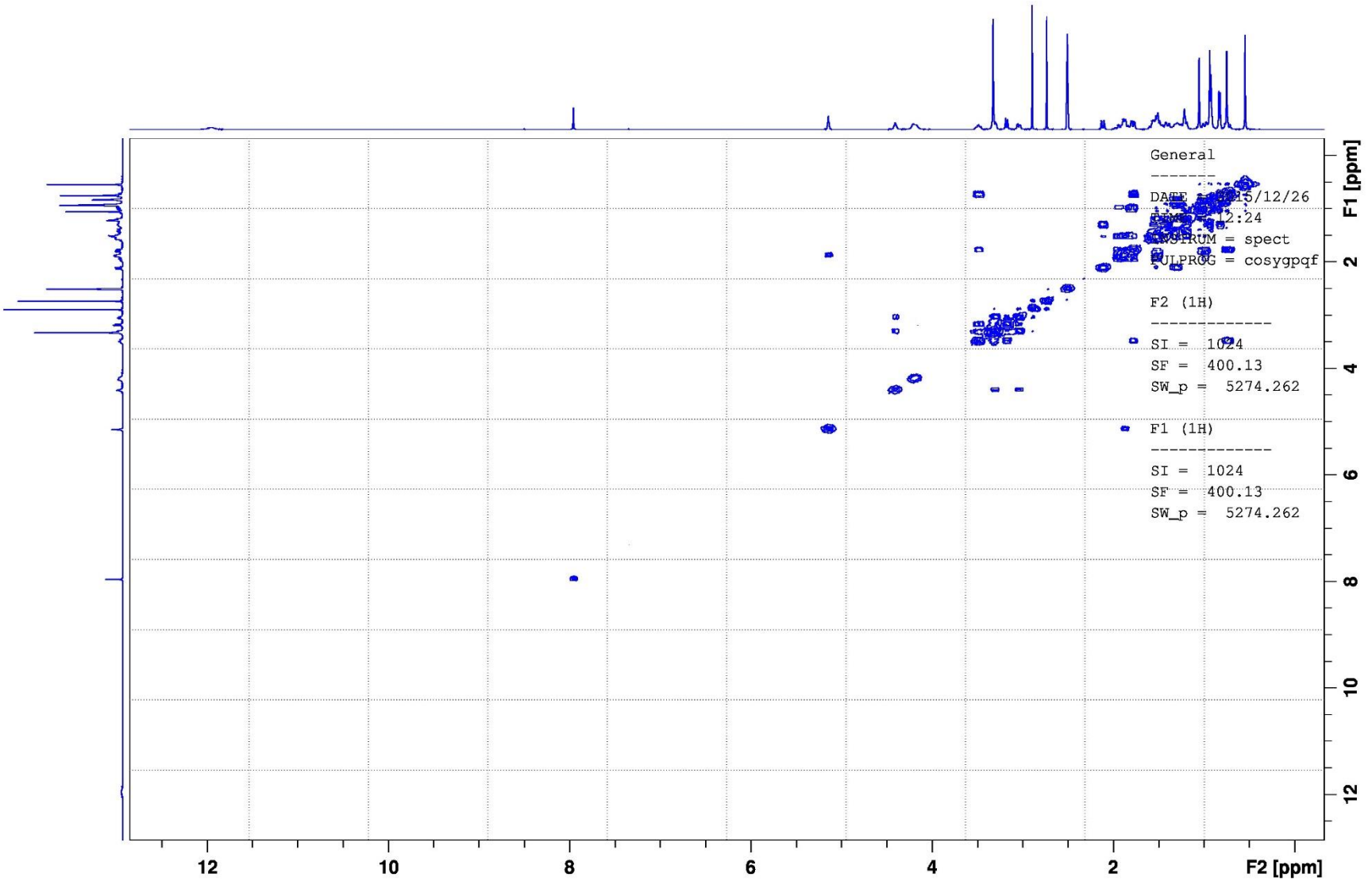
SECTION 3.	Physical and Chemical Properties
Appearance	| Light yellowish powder. Since this is a natural product, color may vary from lot to lot
Odor	| Characteristic
Solubility	| Soluble in water
Moisture Content	| NMT. 8.0 %

MSDS/TSD/063-01

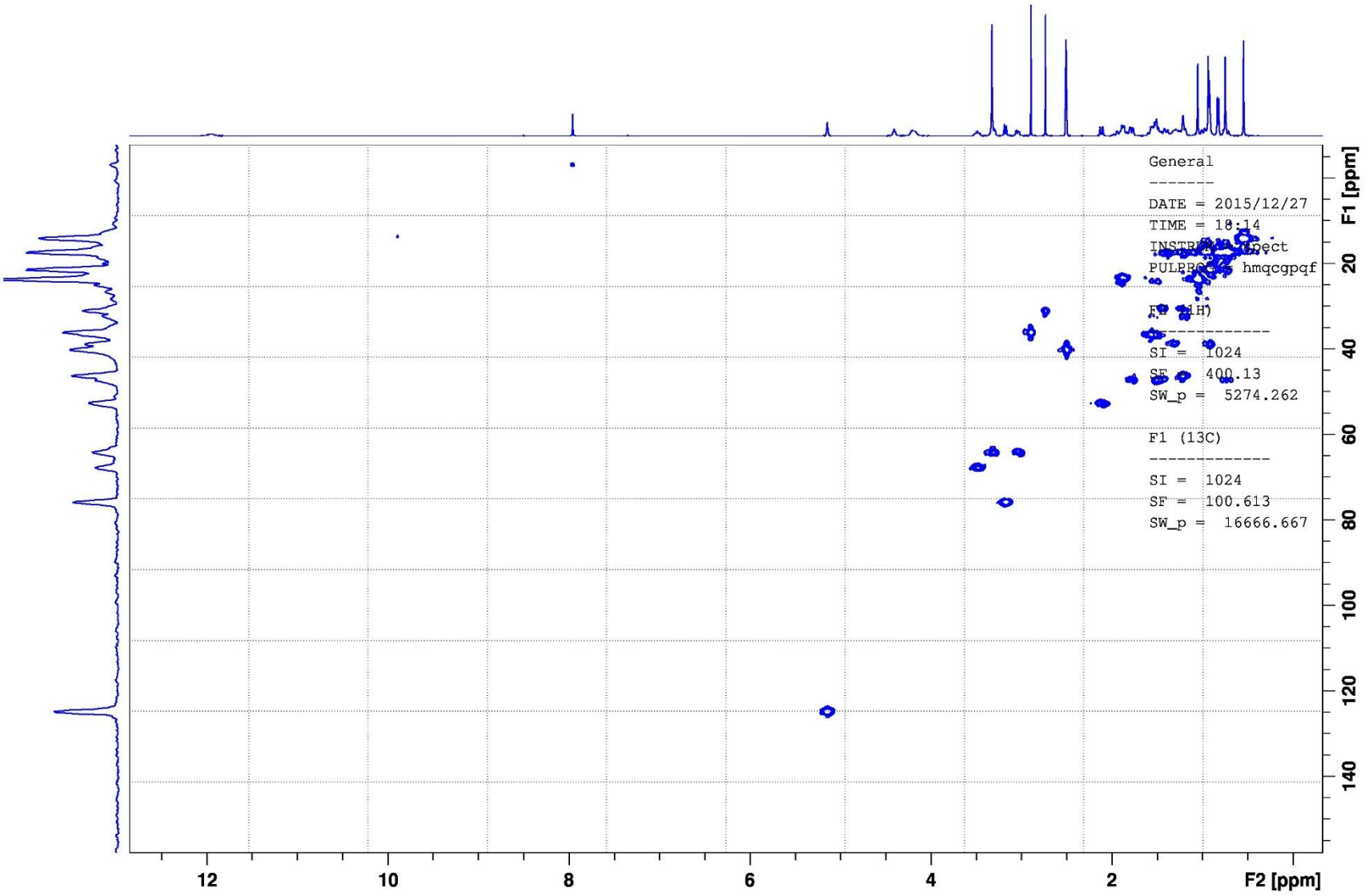
Appendix 5: 2D NMR TOCSY spectrum of CA7 fraction (400 MHz, DMSO- d_6)



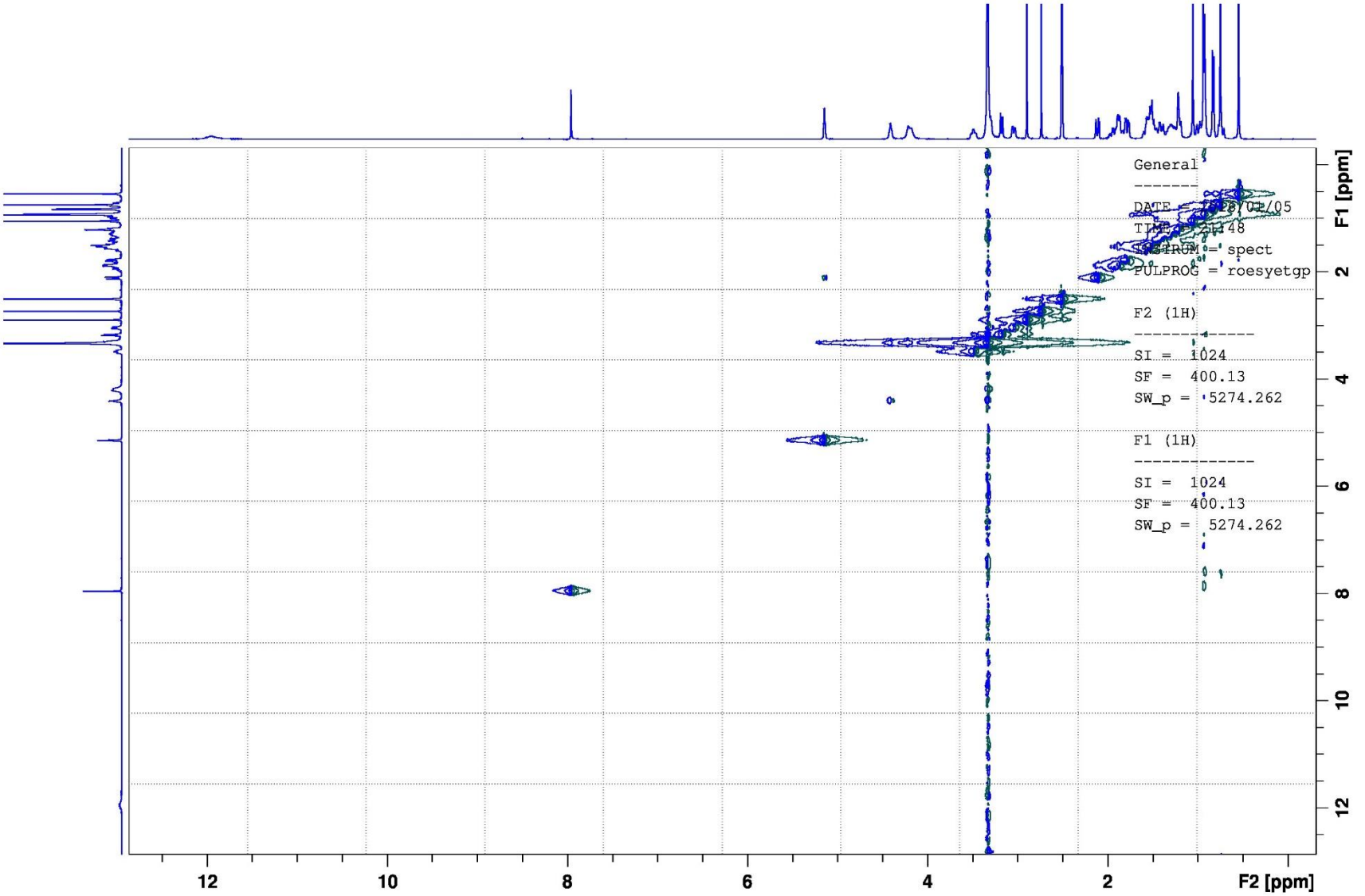
Appendix 6: 2D NMR COSY spectrum of CA7 fraction (400 MHz, DMSO- d_6)



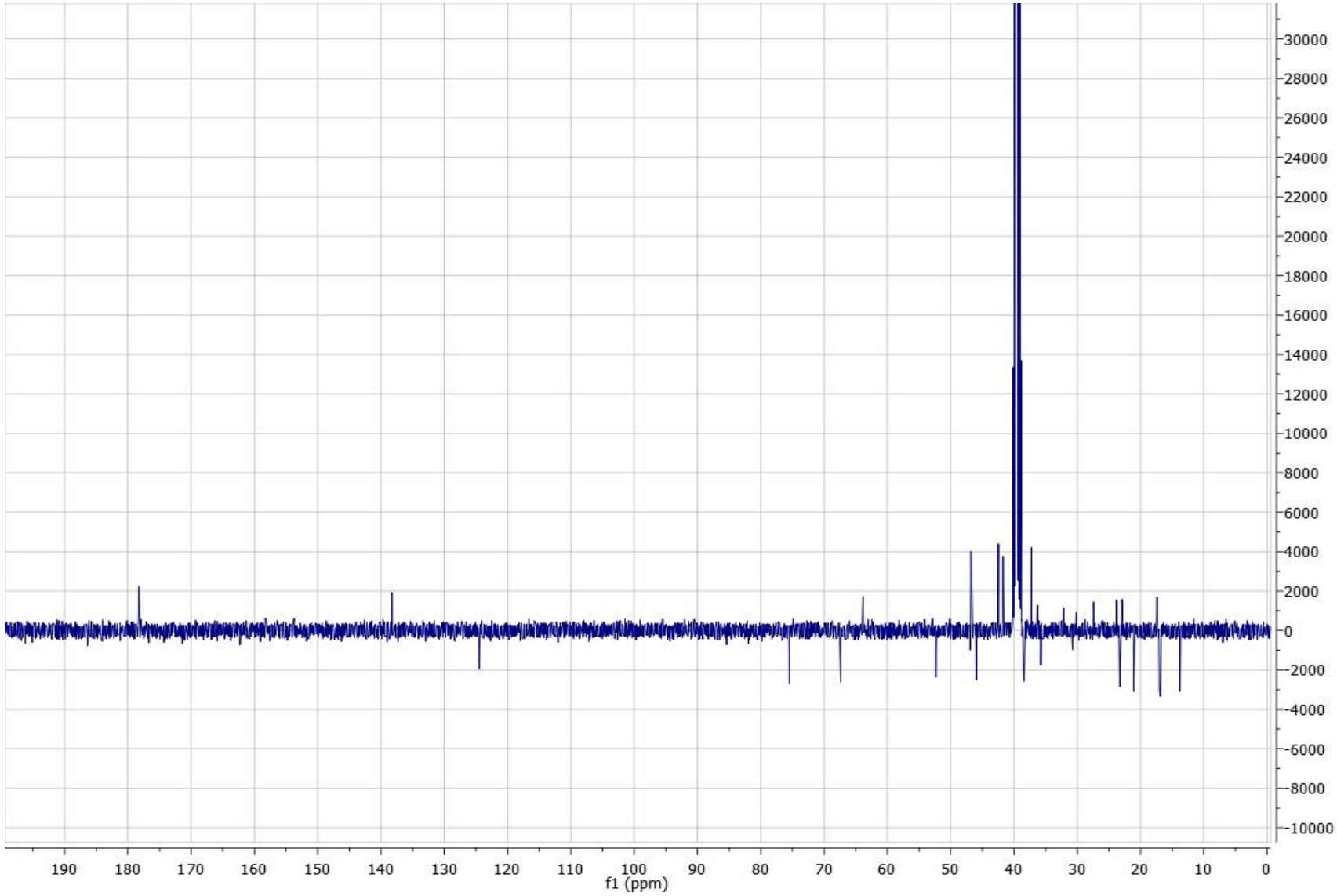
Appendix 7: 2D NMR HMQC spectrum of CA7 fraction (400 MHz, DMSO- d_6)



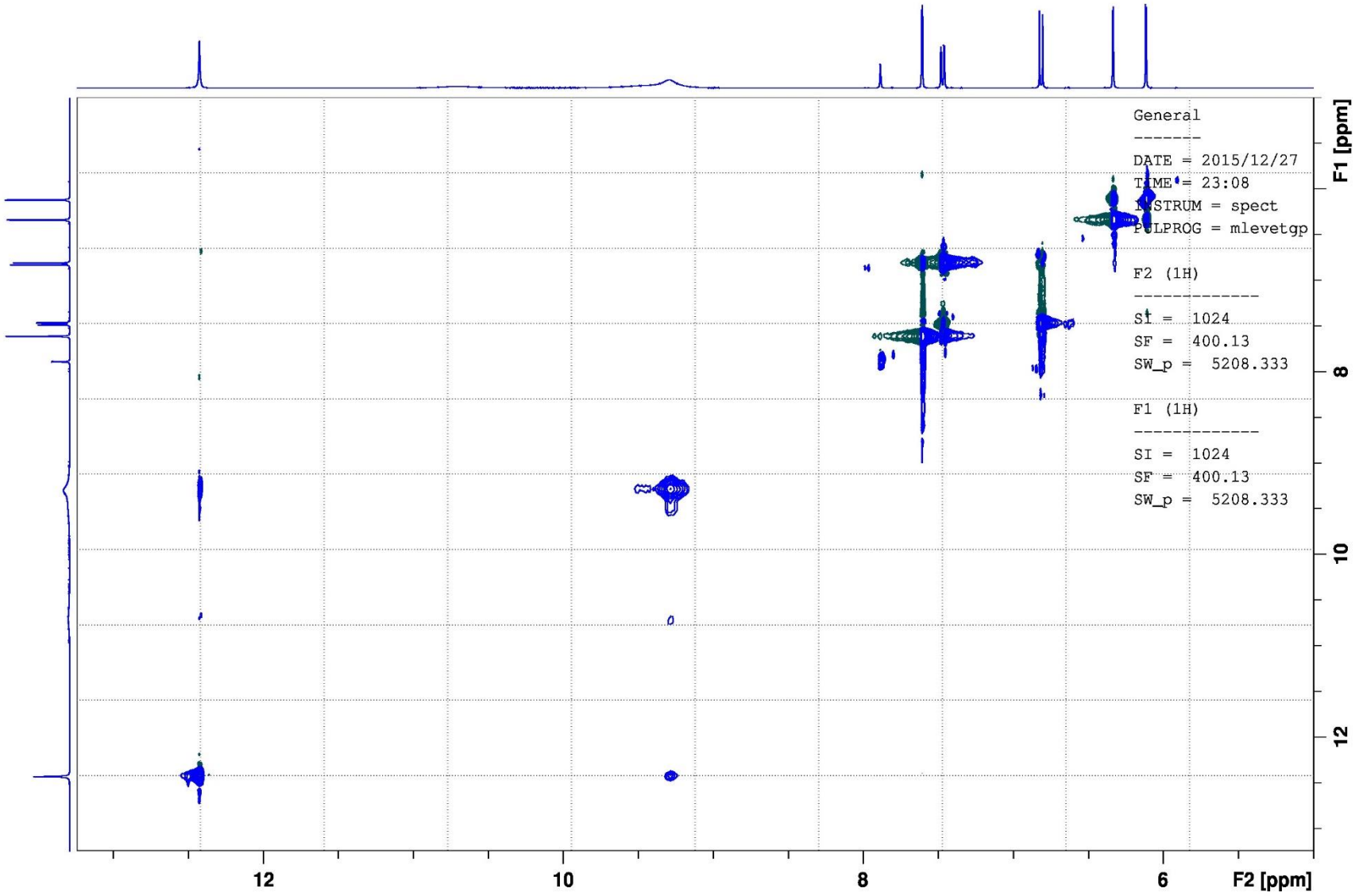
Appendix 8: 2D NMR ROESY spectrum of CA7 fraction (400 MHz, DMSO- d_6)



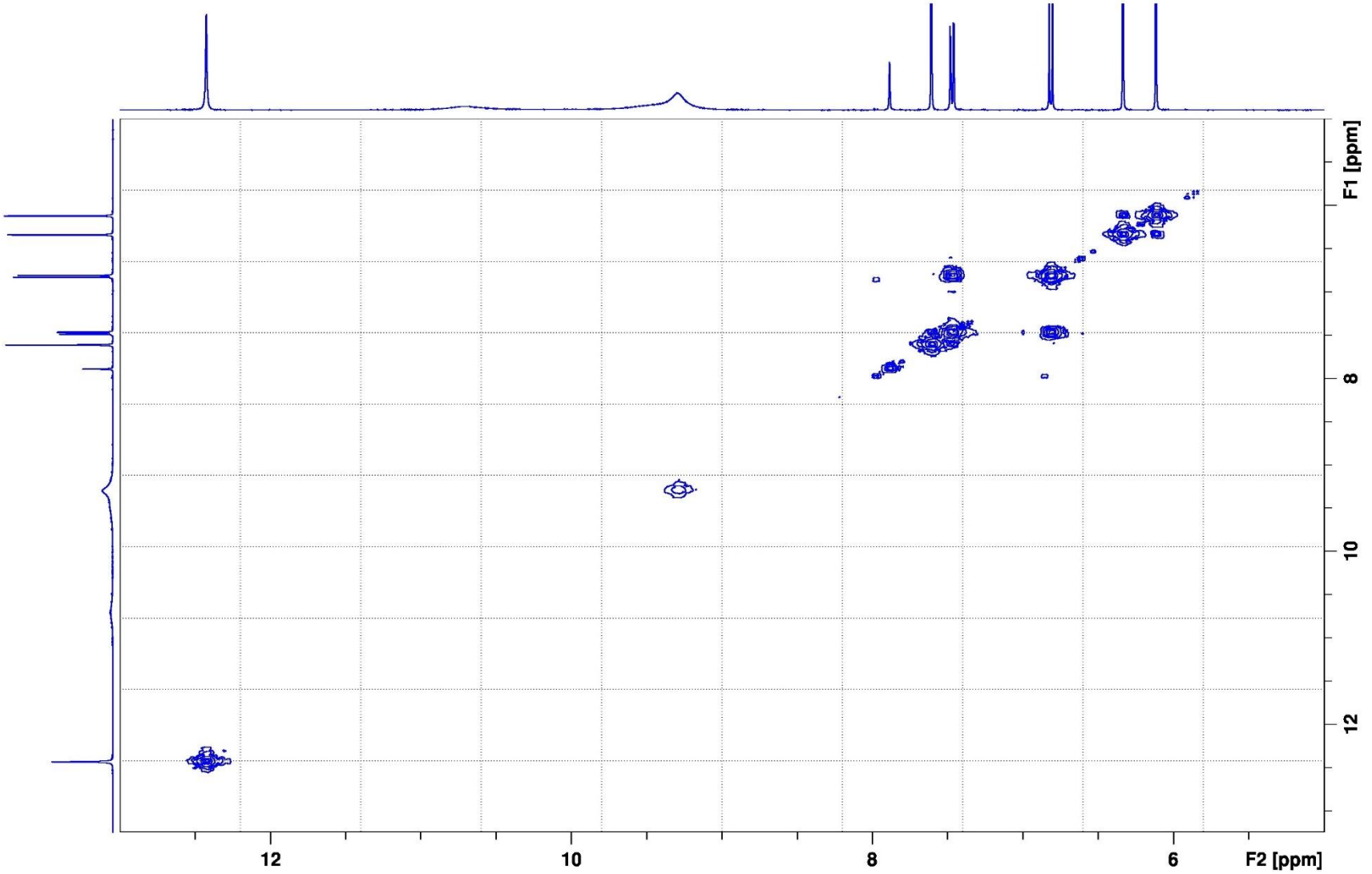
Appendix 9: ^{13}C_APT NMR spectrum of CA7 fraction (100 MHz, DMSO- *d6*)



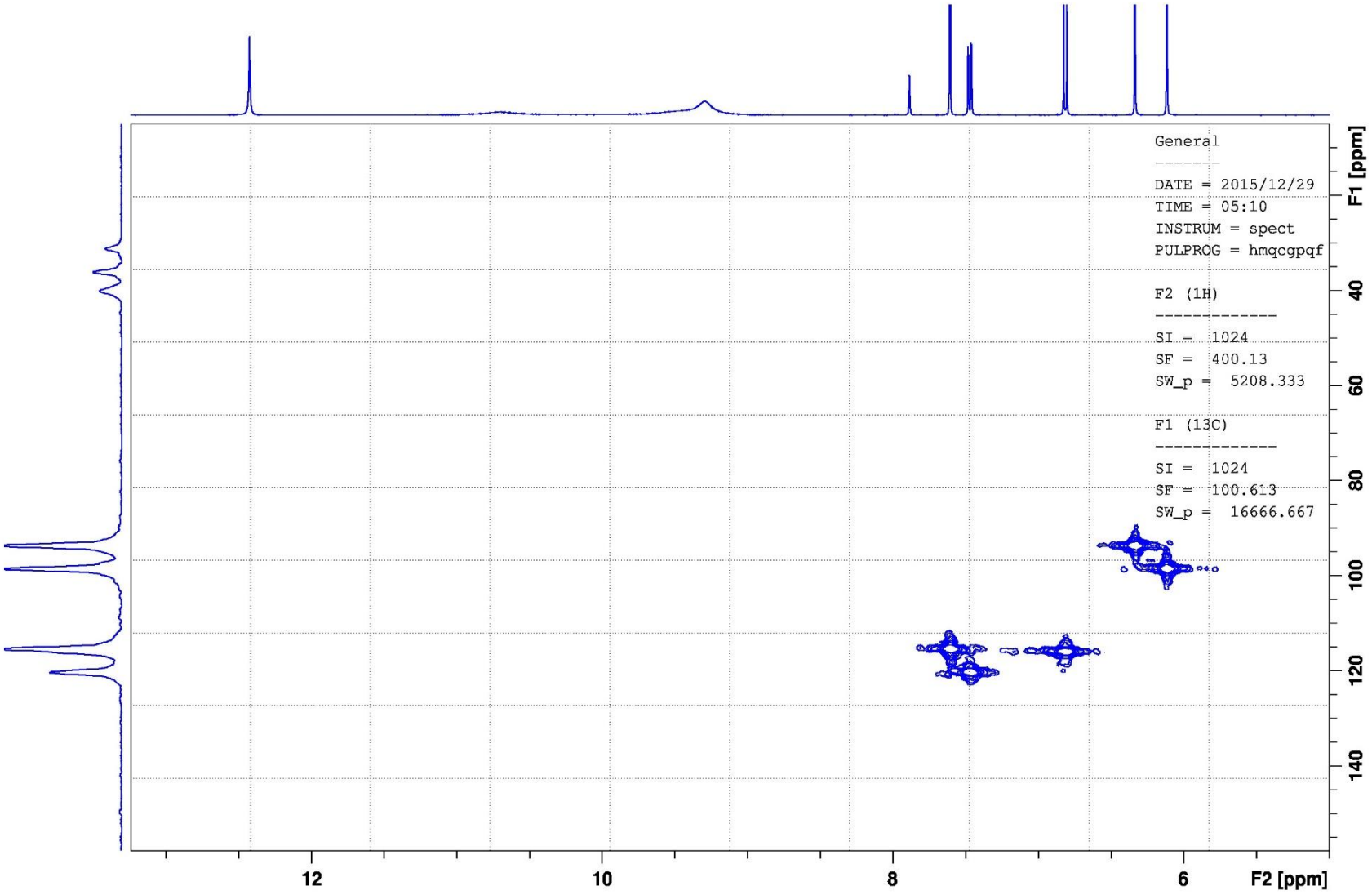
Appendix 10: 2D NMR TOCSY spectrum of IC10 fraction (400 MHz, DMSO- d_6)



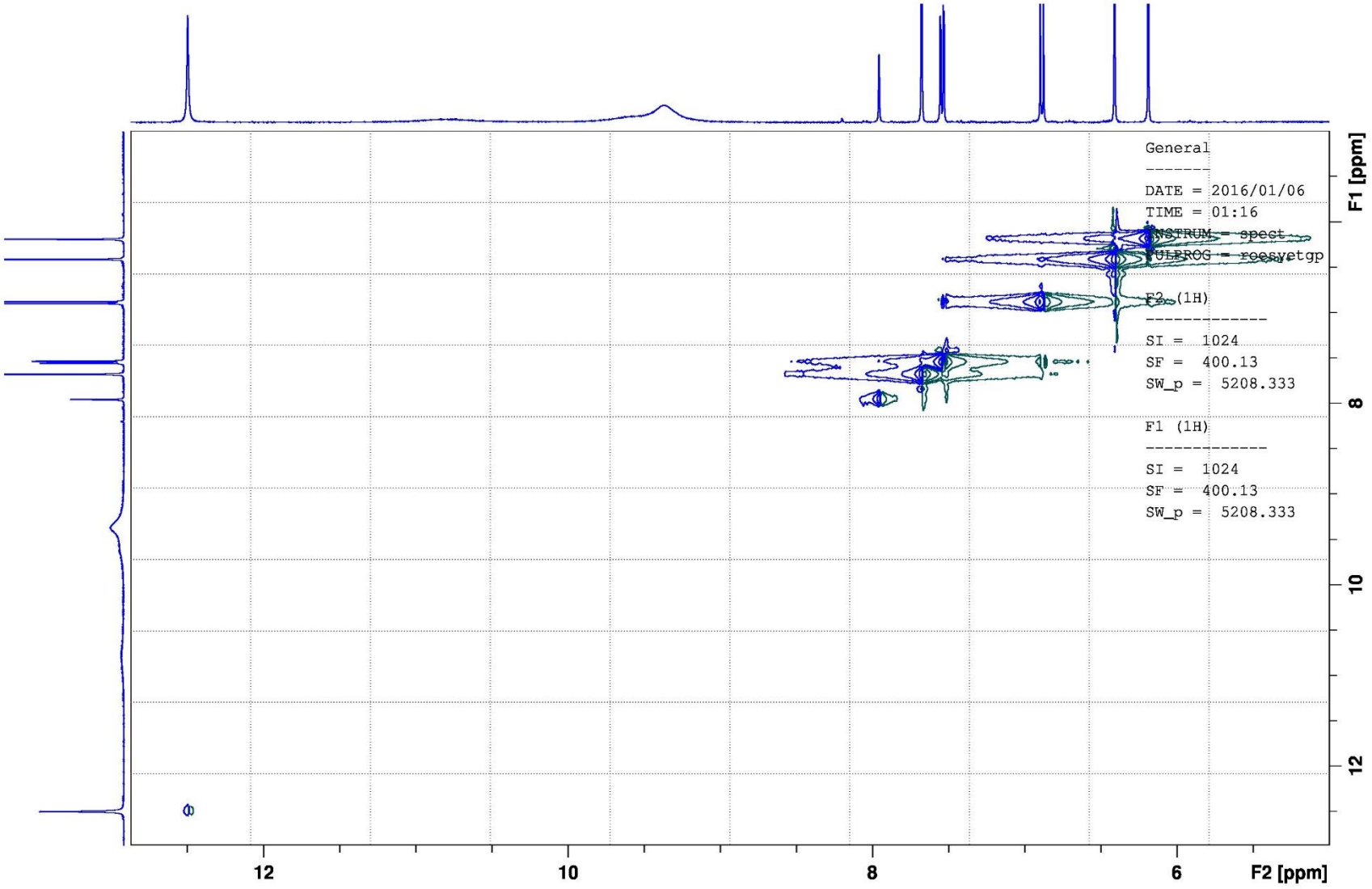
Appendix 11: 2D NMR COSY spectrum of IC10 fraction (400 MHz, DMSO- d_6)



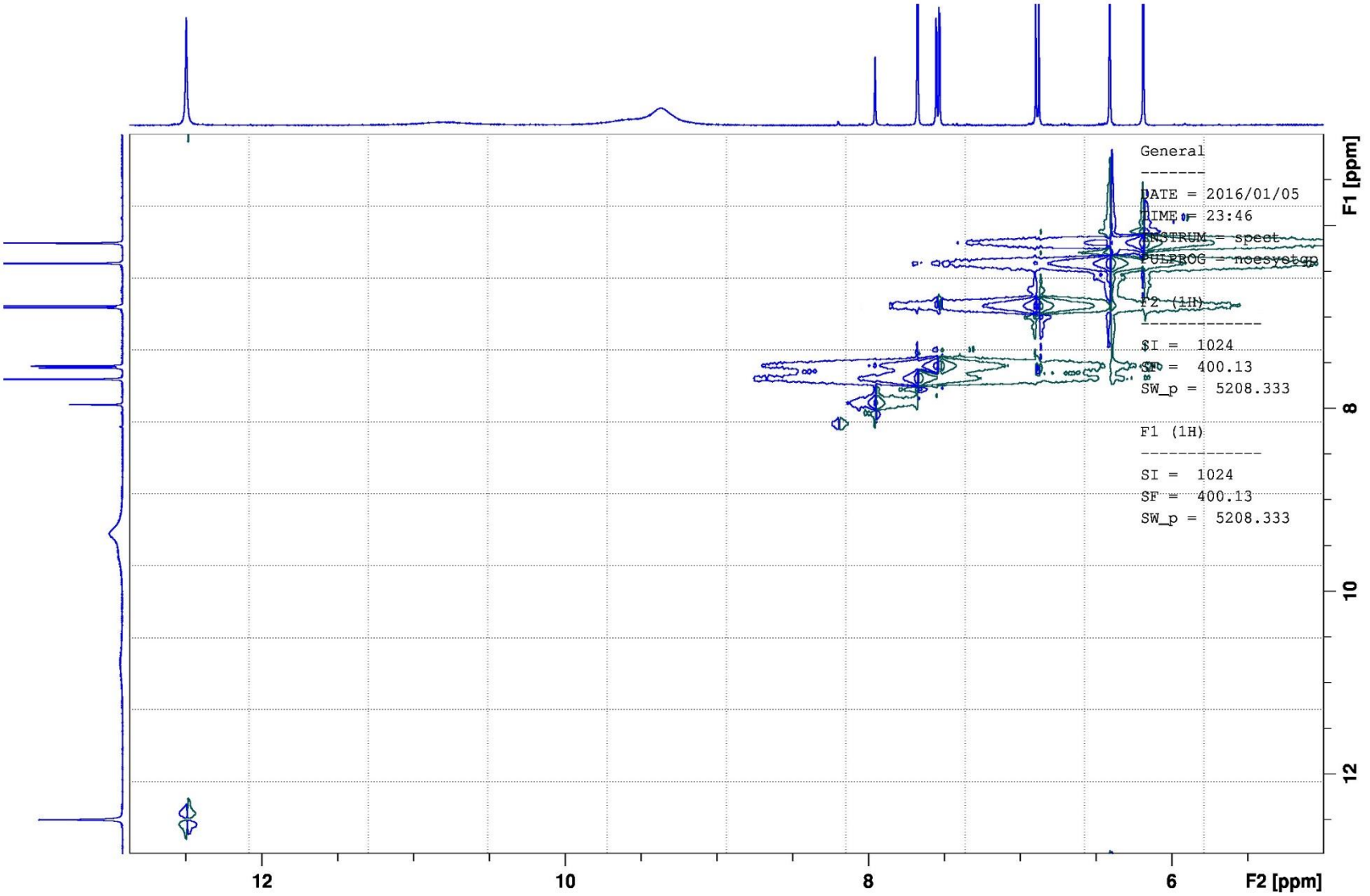
Appendix 12: 2D NMR HMQC spectrum of IC10 fraction (400 MHz, DMSO- d_6)



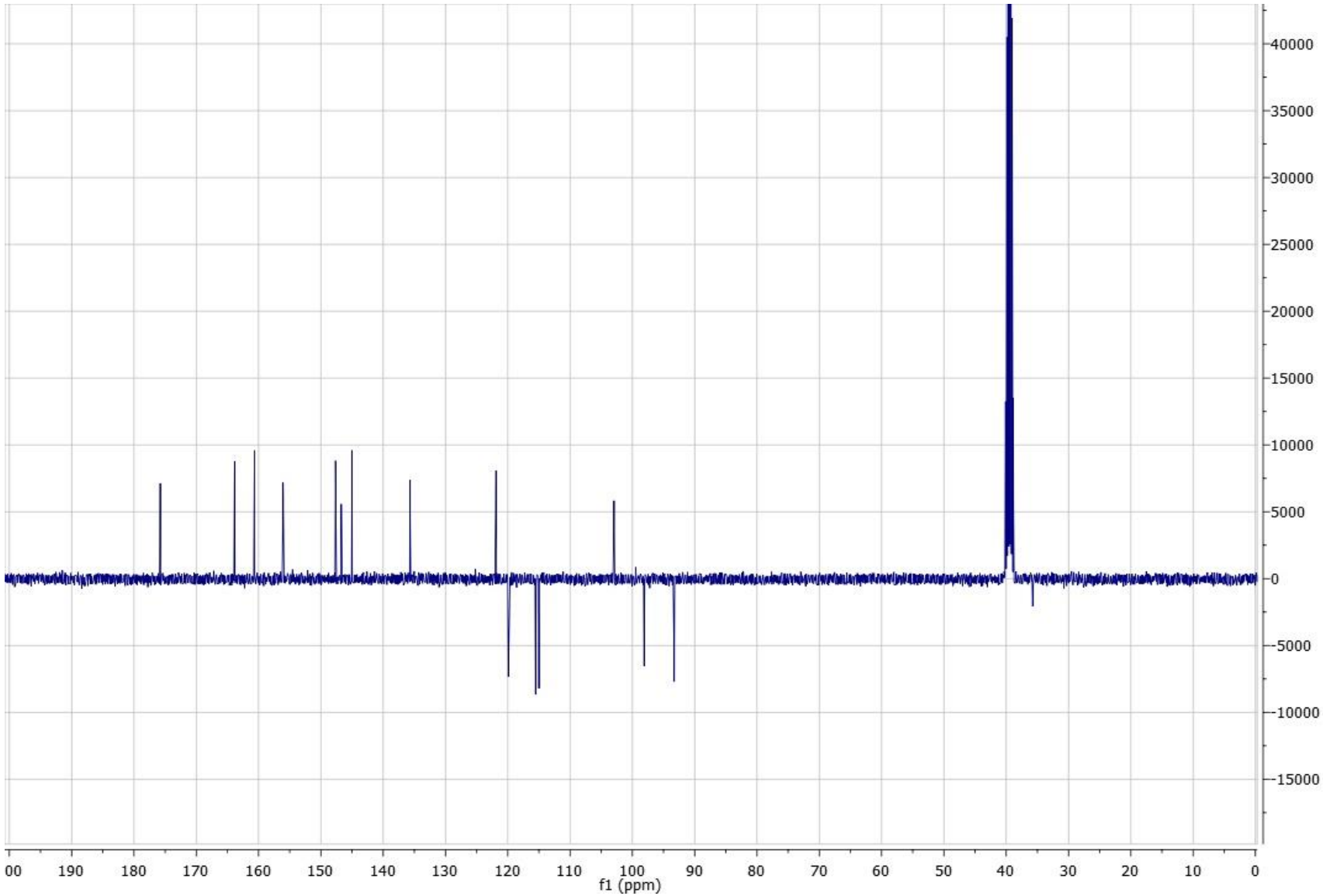
Appendix 13: 2D ROESY NMR spectrum of IC10 fraction (400 MHz, DMSO- *d6*)



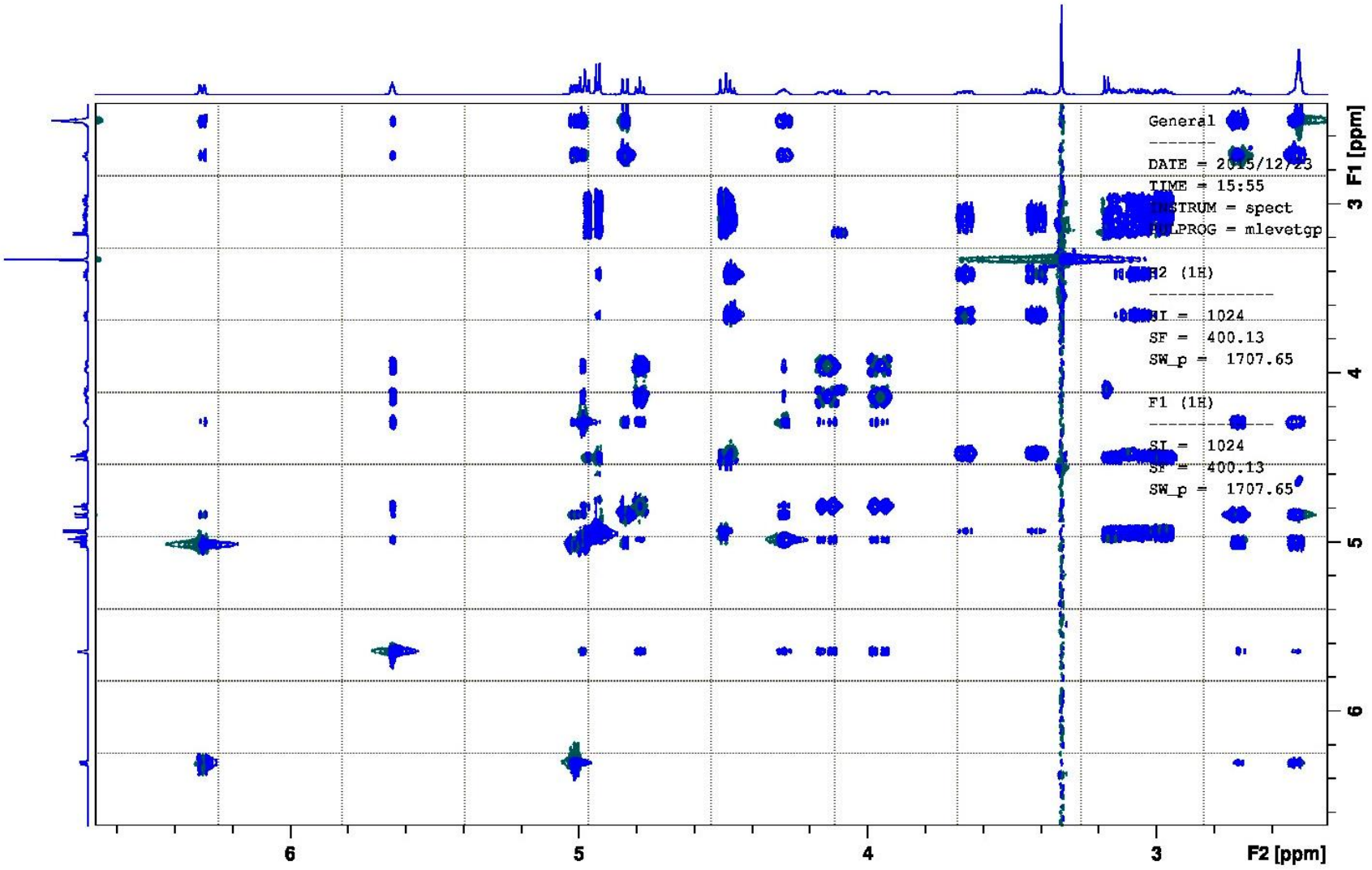
Appendix 14: 2D NOESY NMR spectrum of IC10 fraction (400 MHz, DMSO- d_6)



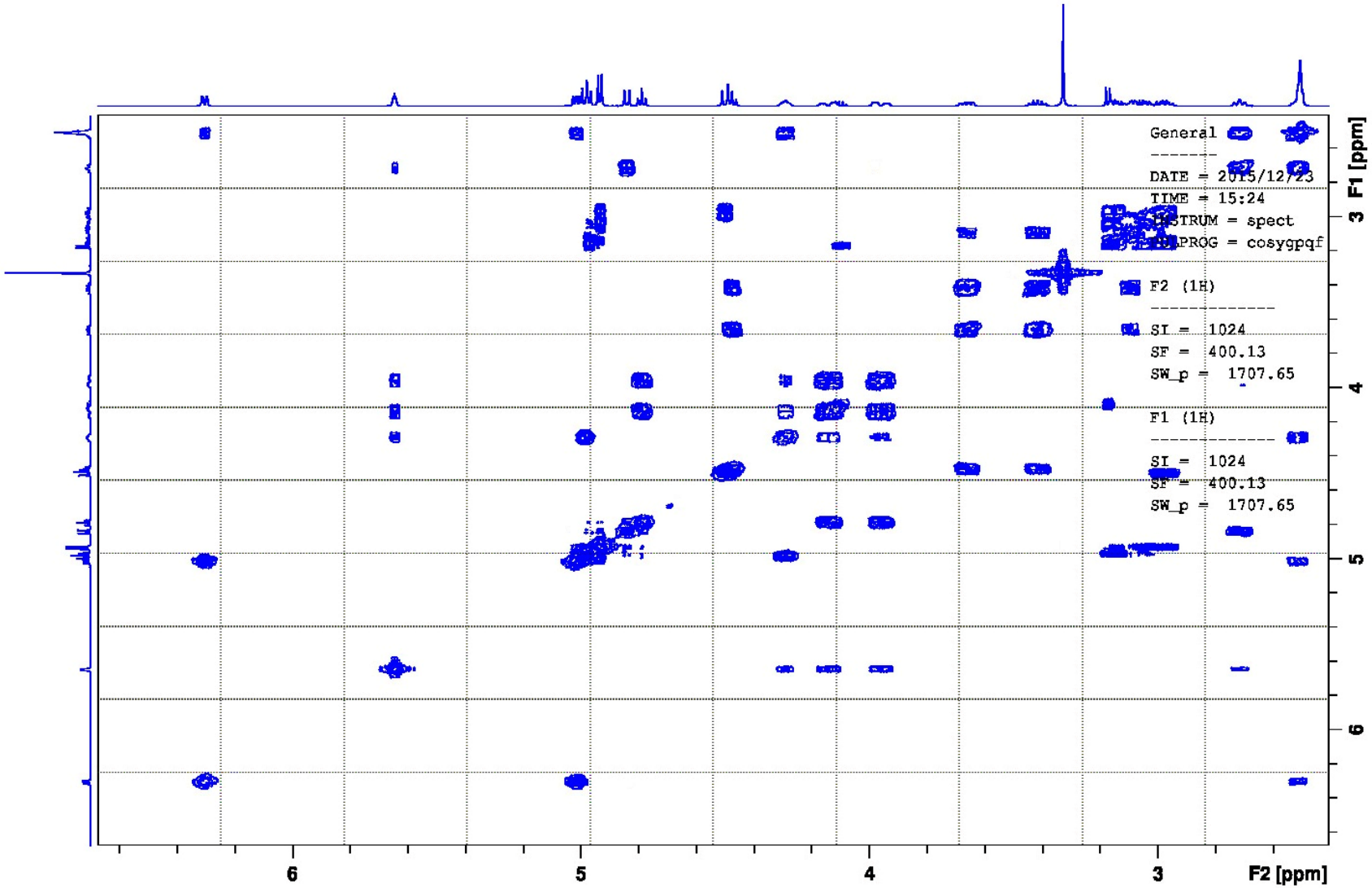
Appendix 15: ^{13}C_APT NMR spectrum of IC10 fraction (100 MHz, DMSO- *d6*)



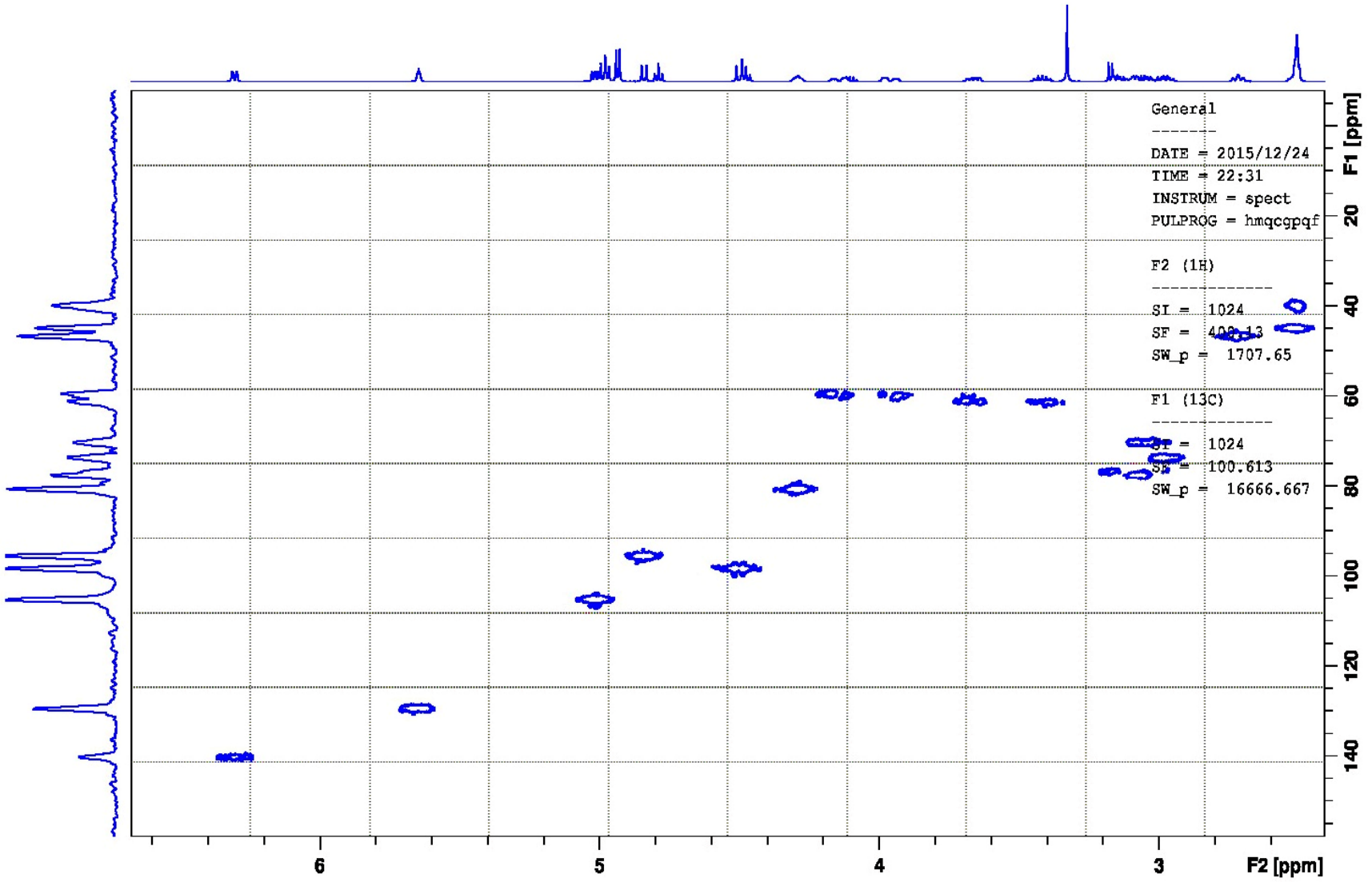
Appendix 16: 2D TOCSY NMR spectrum of MC4 fraction (400 MHz, DMSO- d_6)



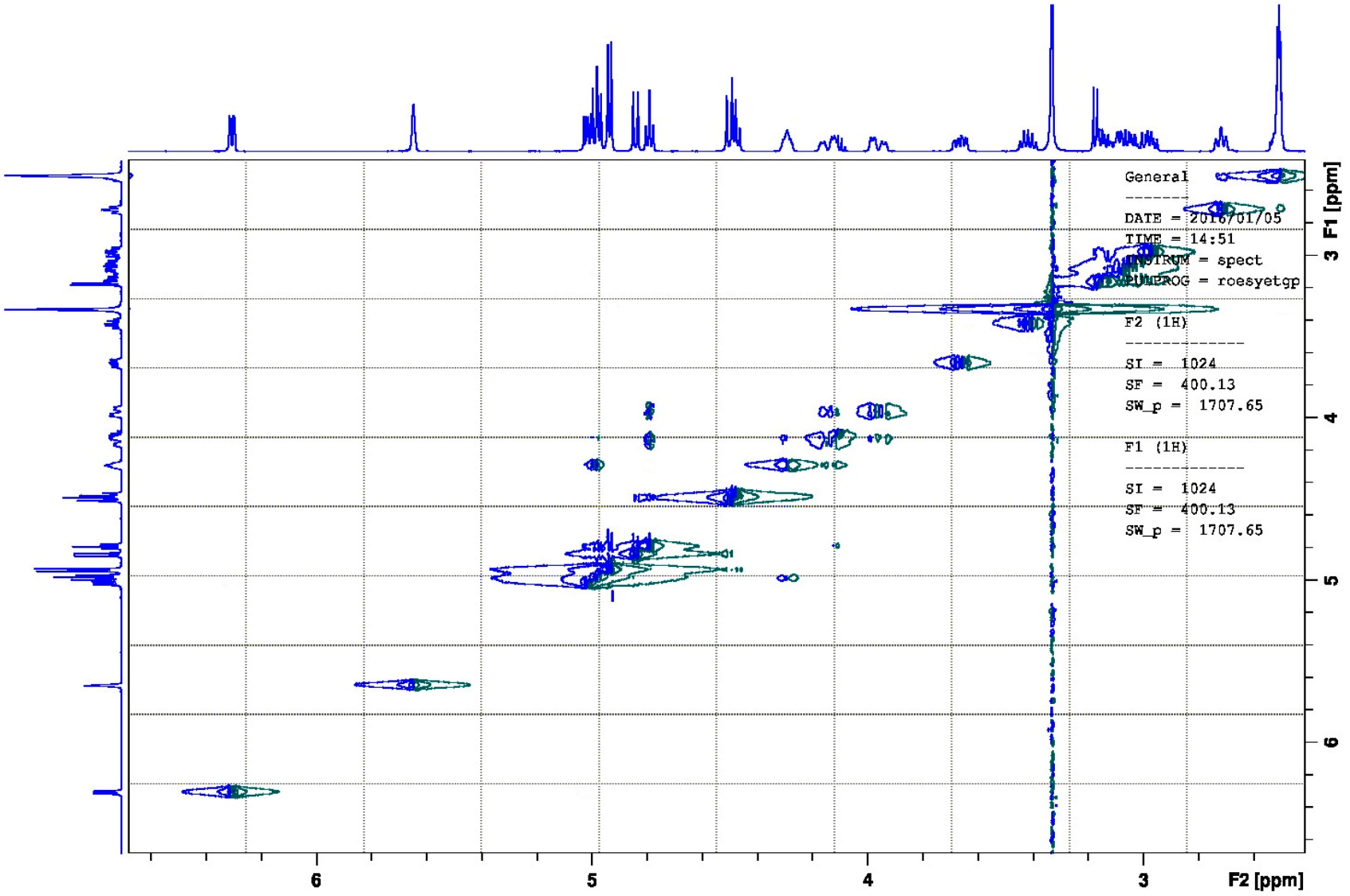
Appendix 17: 2D COSY NMR spectrum of MC4 fraction (400 MHz, DMSO- d_6)



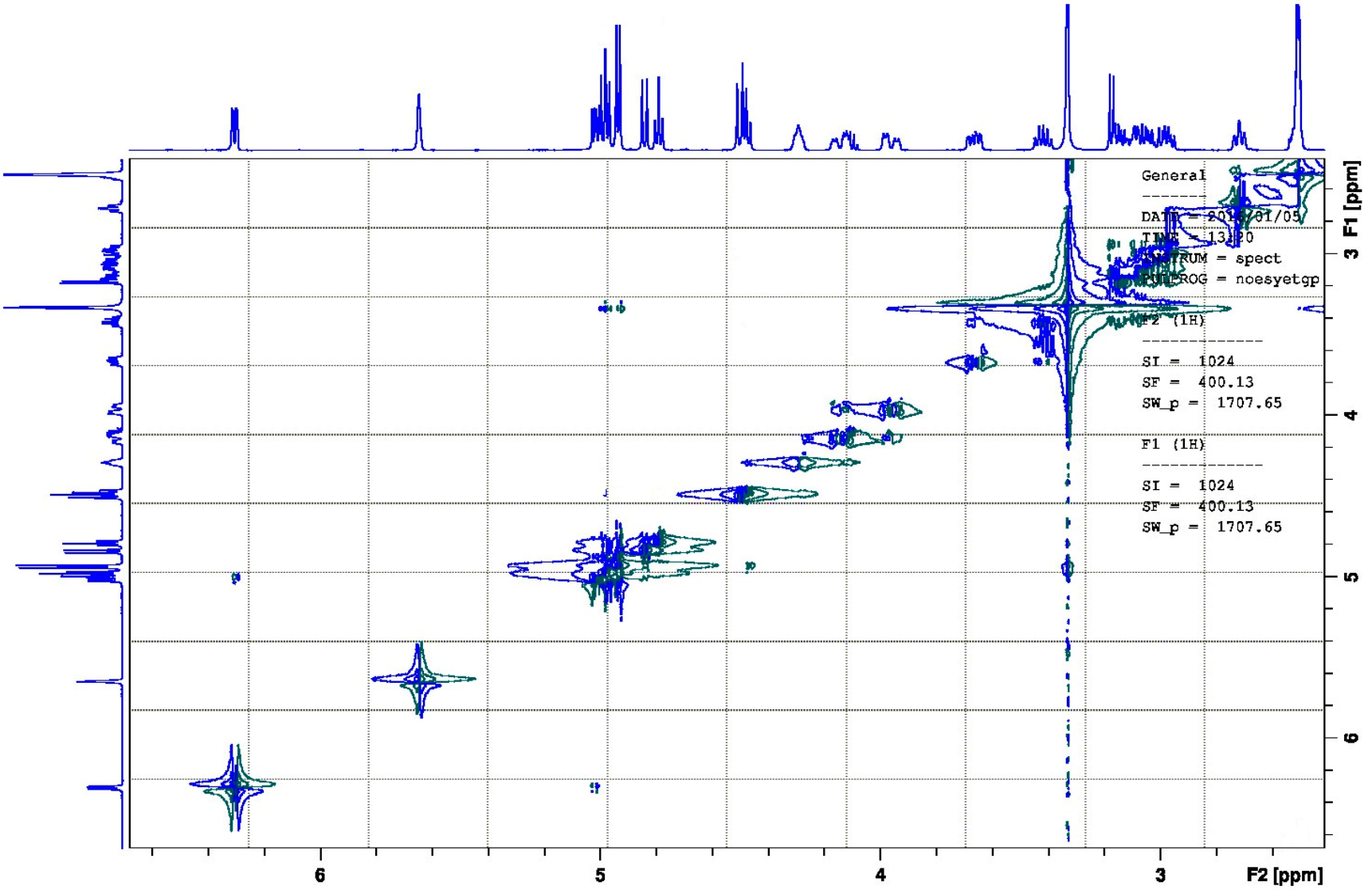
Appendix 18: 2D HMQC NMR spectrum of MC4 fraction (400 MHz, DMSO- d_6)



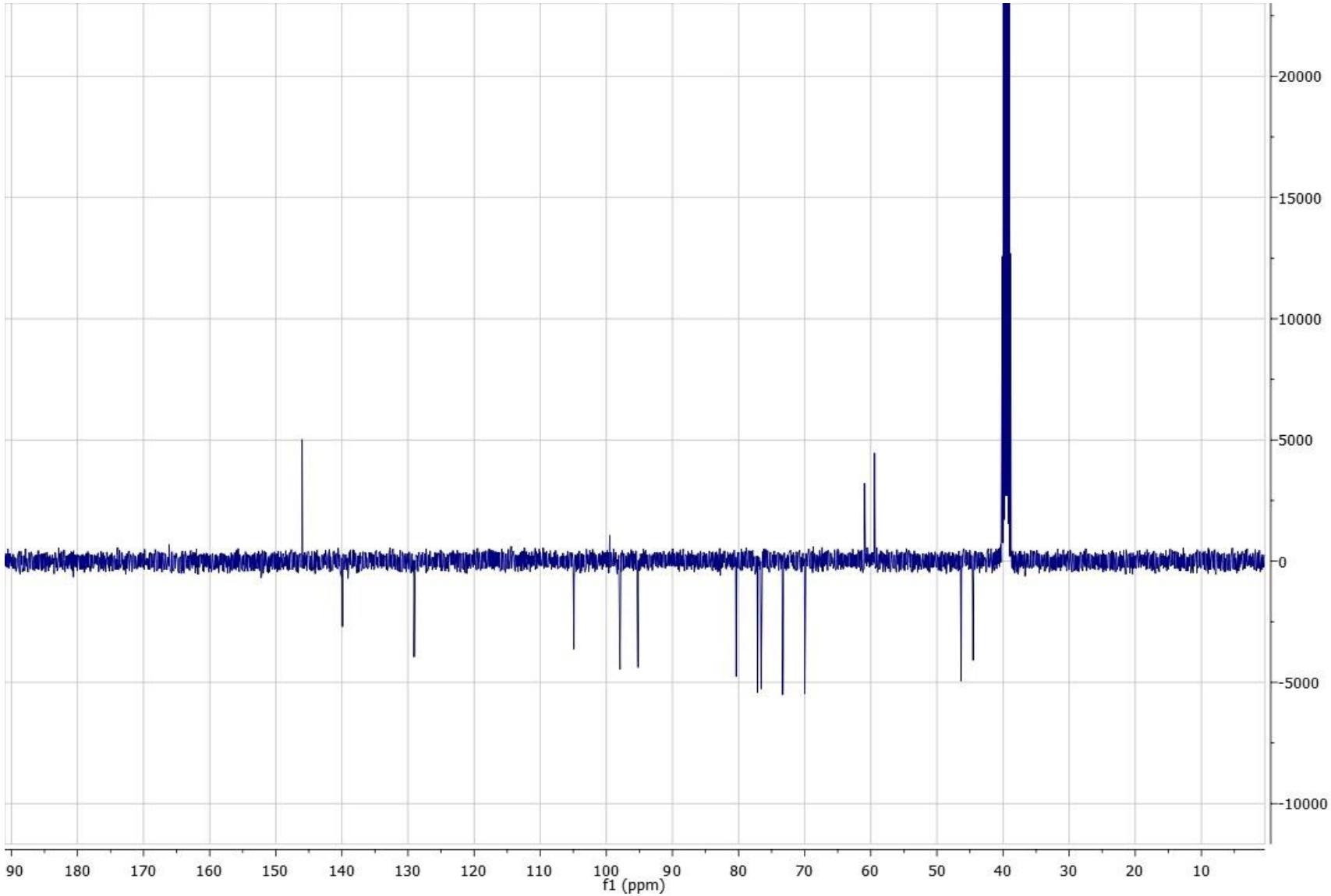
Appendix 19: 2D ROESY NMR spectrum of MC4 fraction (400 MHz, DMSO- d_6)



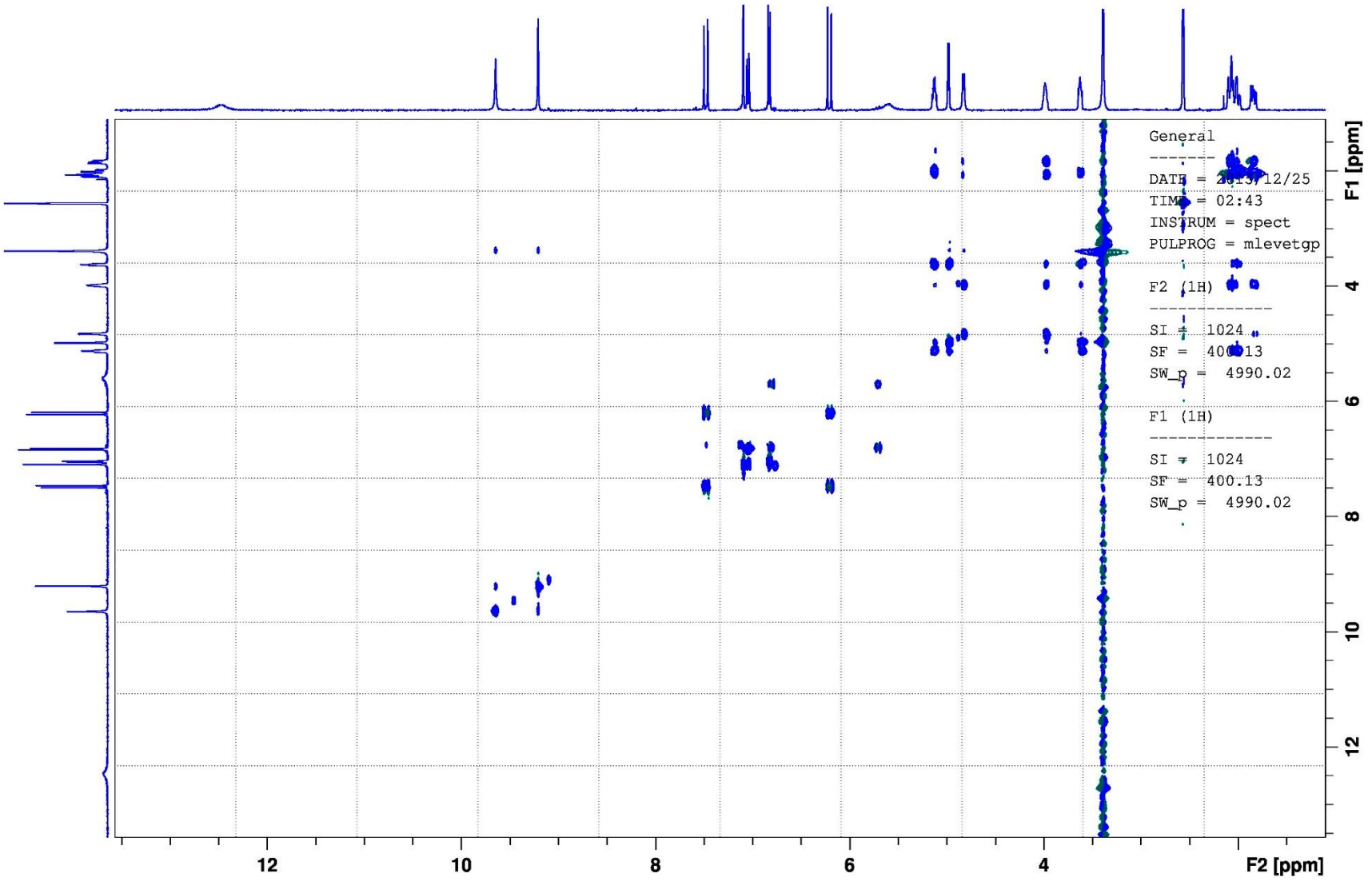
Appendix 20: 2D NOESY NMR spectrum of MC4 fraction (400 MHz, DMSO- *d6*)



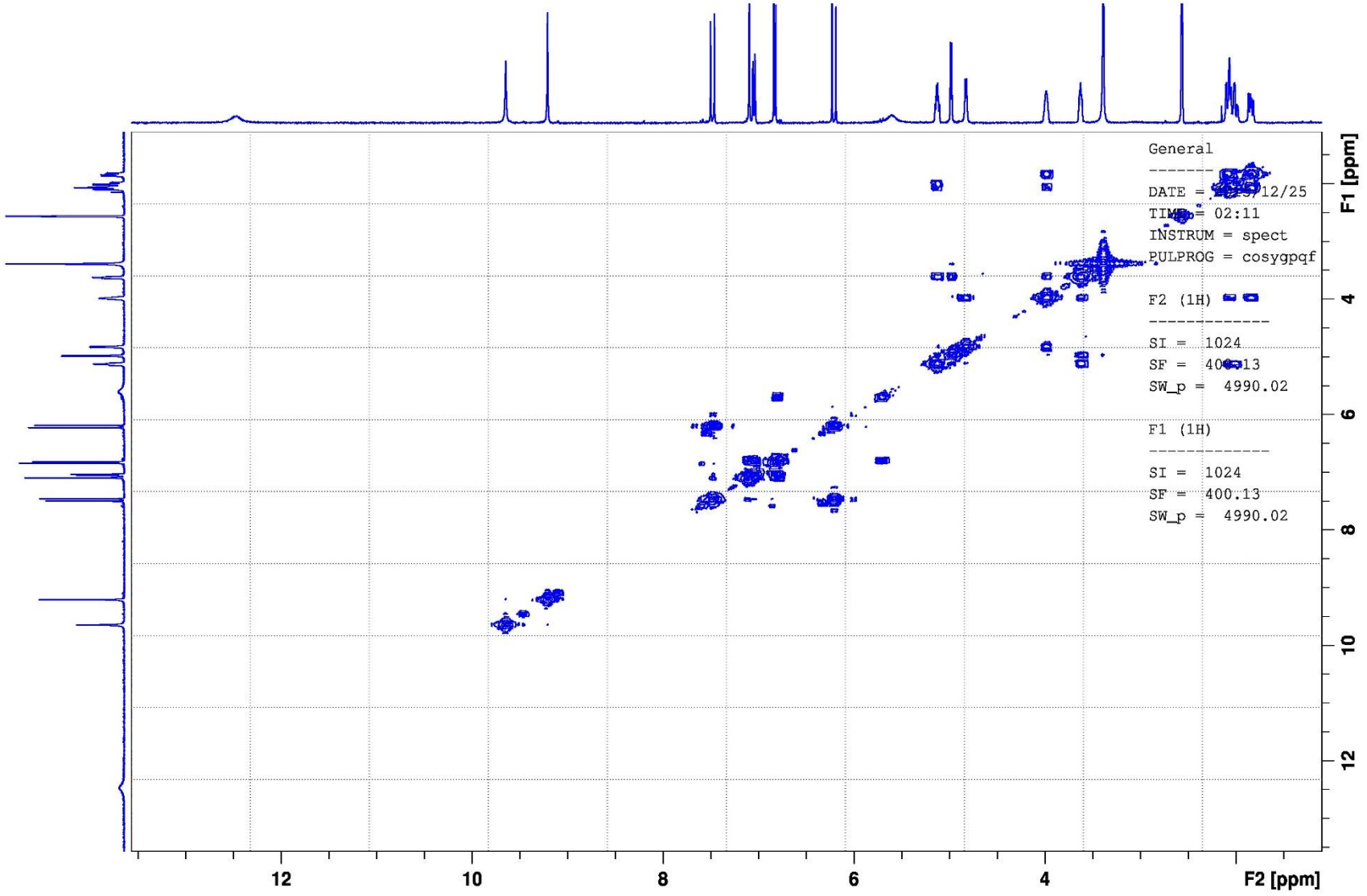
Appendix 21: ^{13}C_APT NMR spectrum of MC4 fraction (100 MHz, DMSO- *d6*)



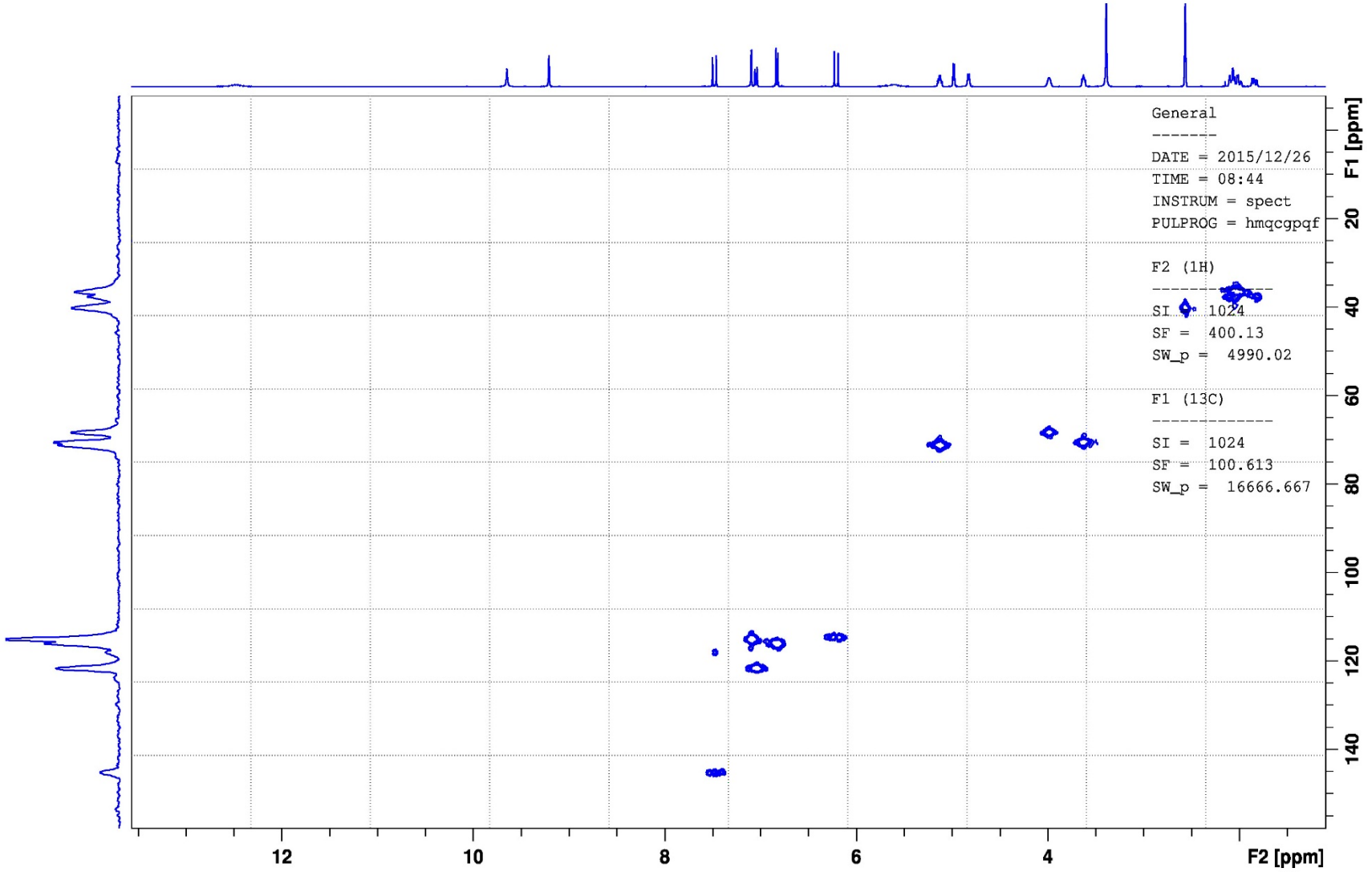
Appendix 22: 2D TOCSY NMR spectrum of SA3 fraction (400 MHz, DMSO- d_6)



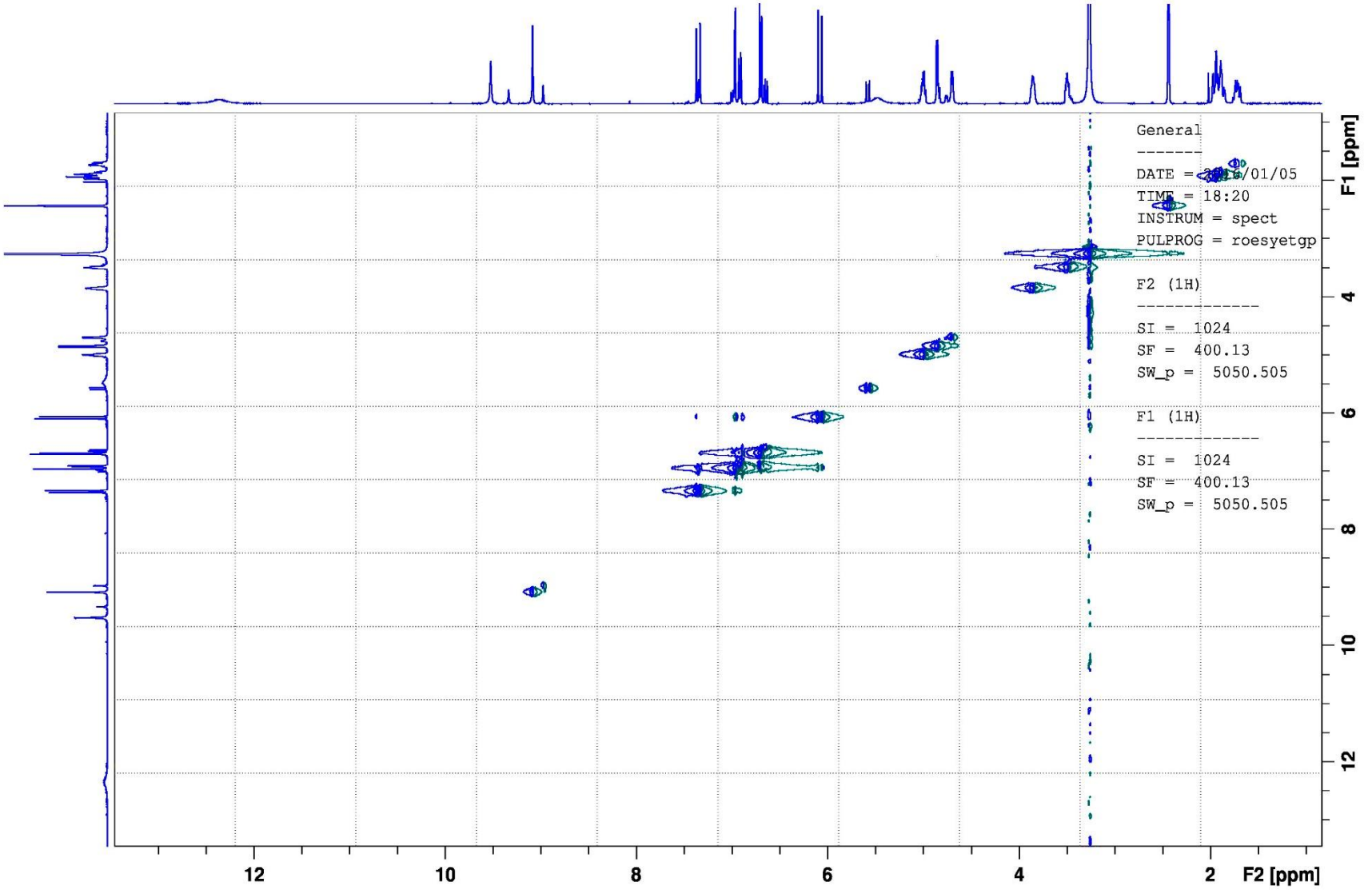
Appendix 23: 2D COSY NMR spectrum of SA3 fraction (400 MHz, DMSO- d_6)



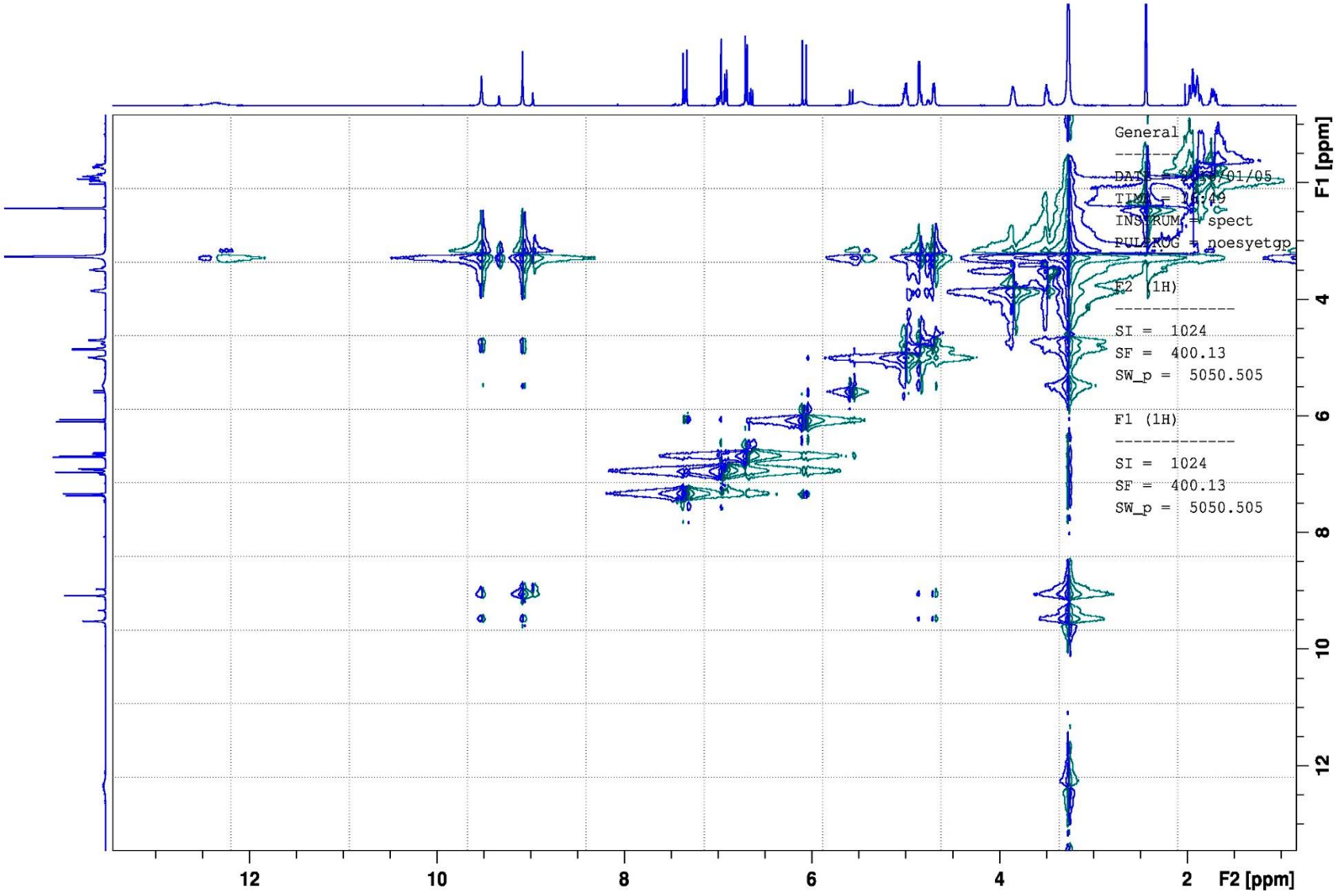
Appendix 24: 2D HMQC NMR spectrum of SA3 fraction (400 MHz, DMSO- d_6)



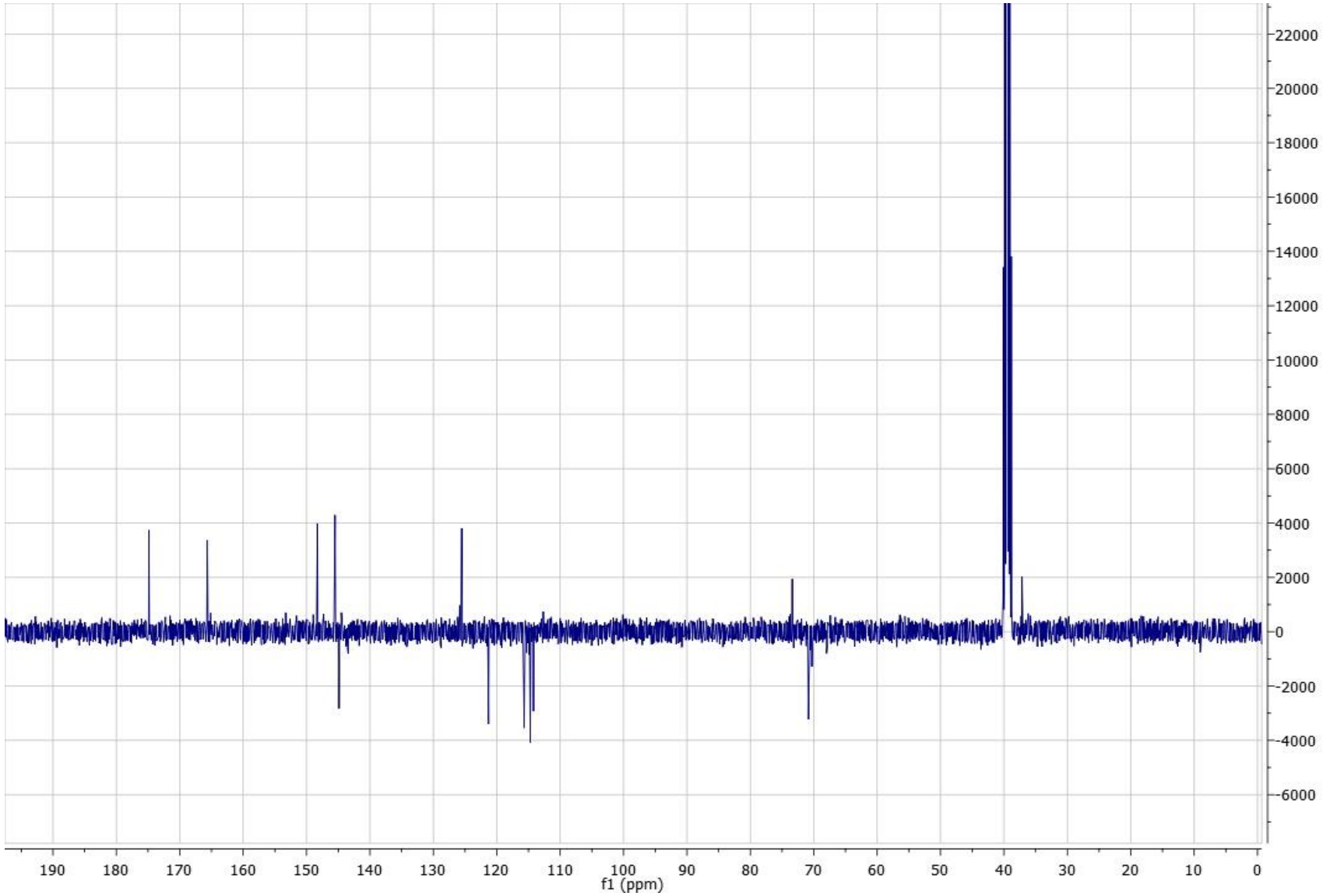
Appendix 25: 2D ROESY NMR spectrum of SA3 fraction (400 MHz, DMSO-d_6)



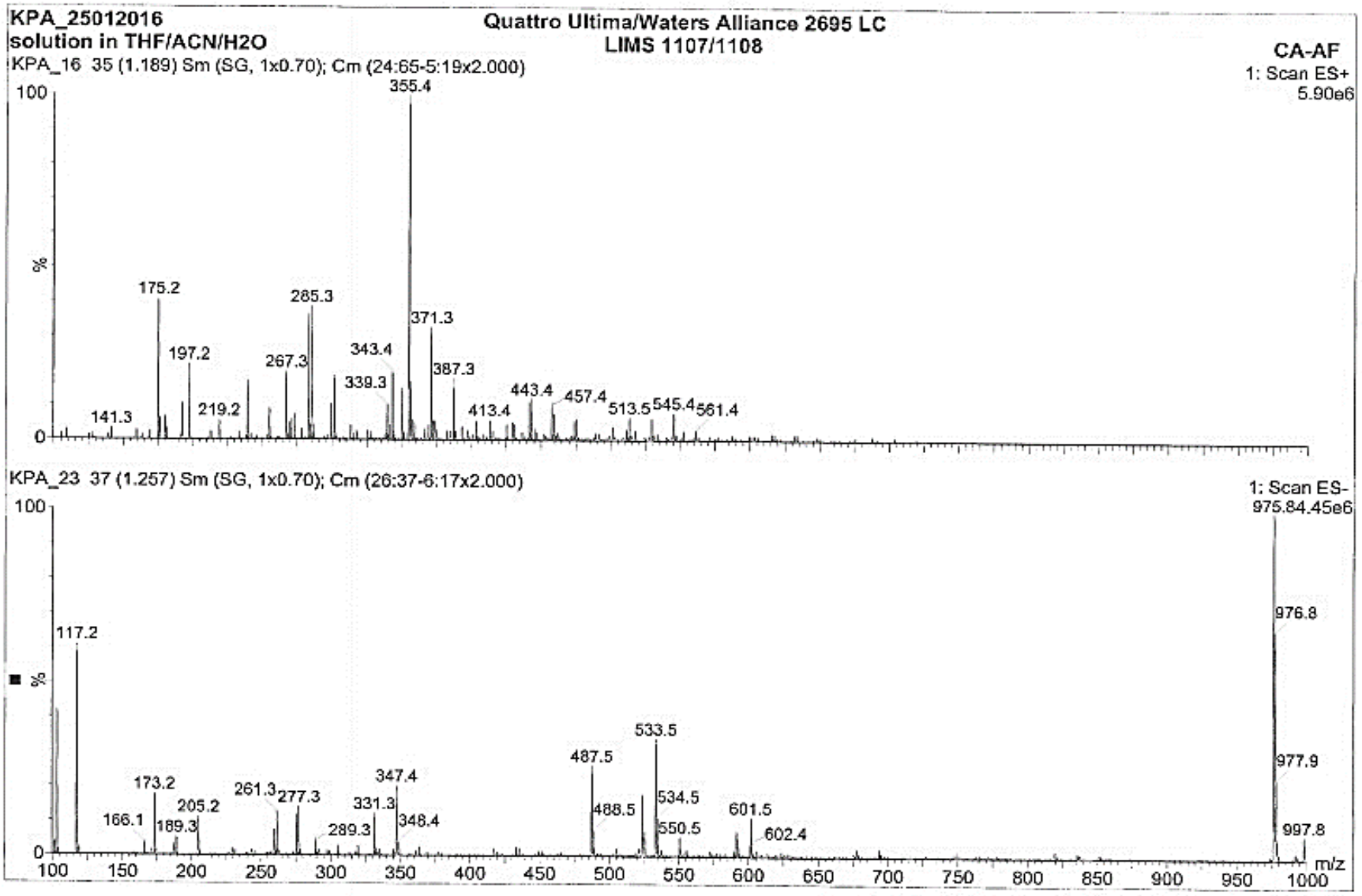
Appendix 26: 2D NOESY NMR spectrum of SA3 fraction (400 MHz, DMSO-*d6*)



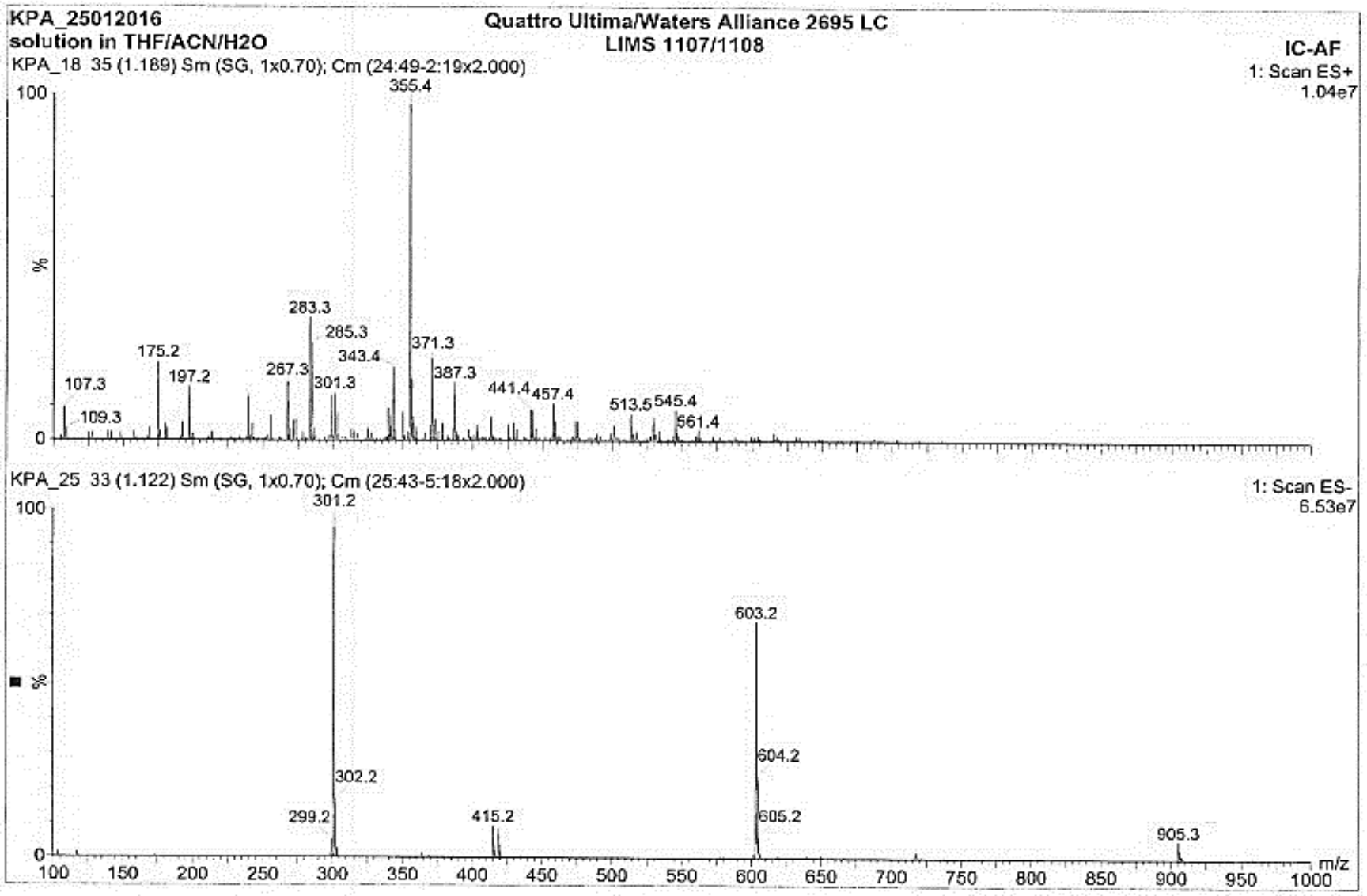
Appendix 27: ^{13}C_APT NMR spectrum of SA3 fraction (100 MHz, DMSO- *d6*)



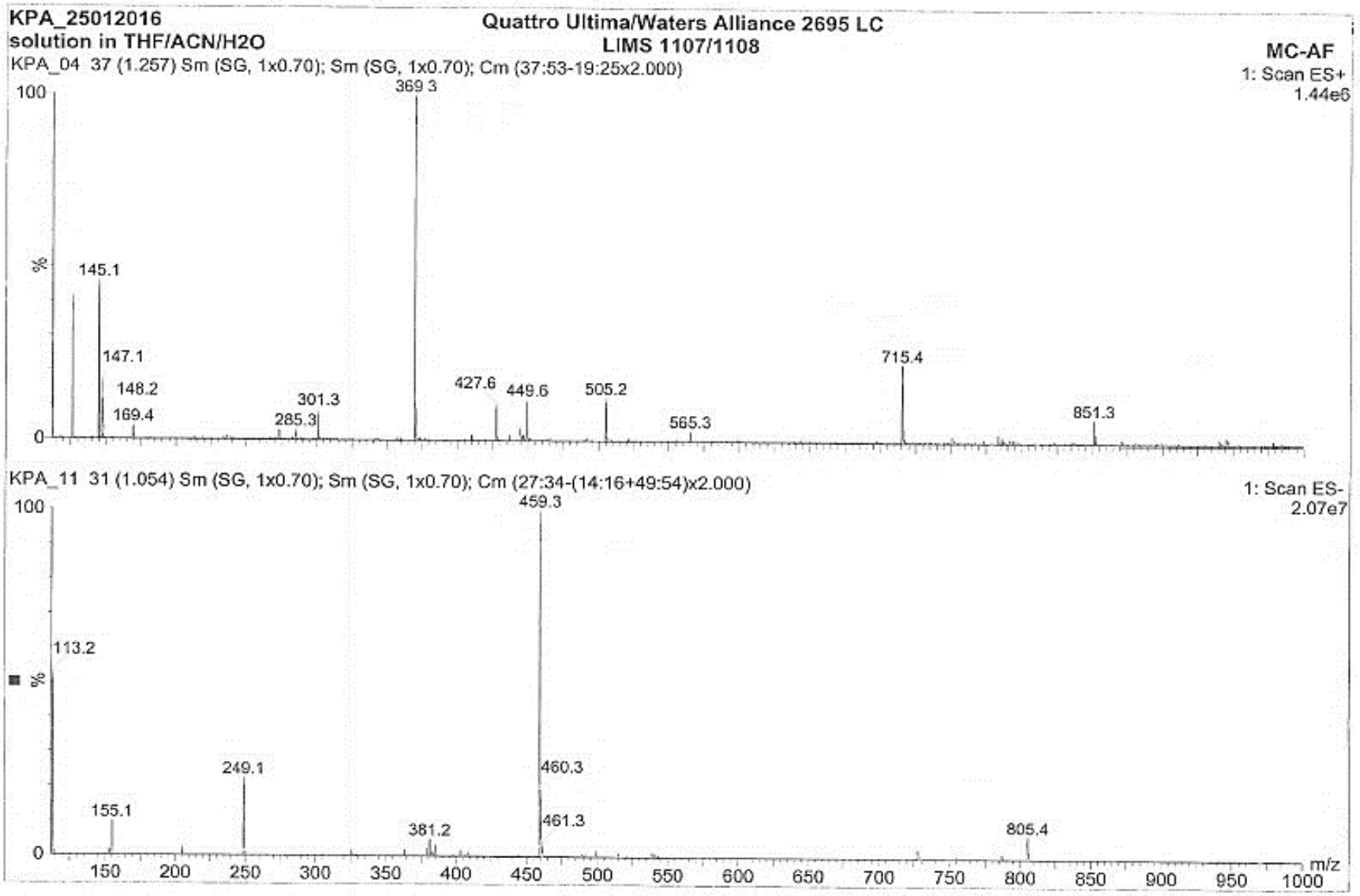
Appendix 28: Mass spectrometry spectra of CA7 fraction



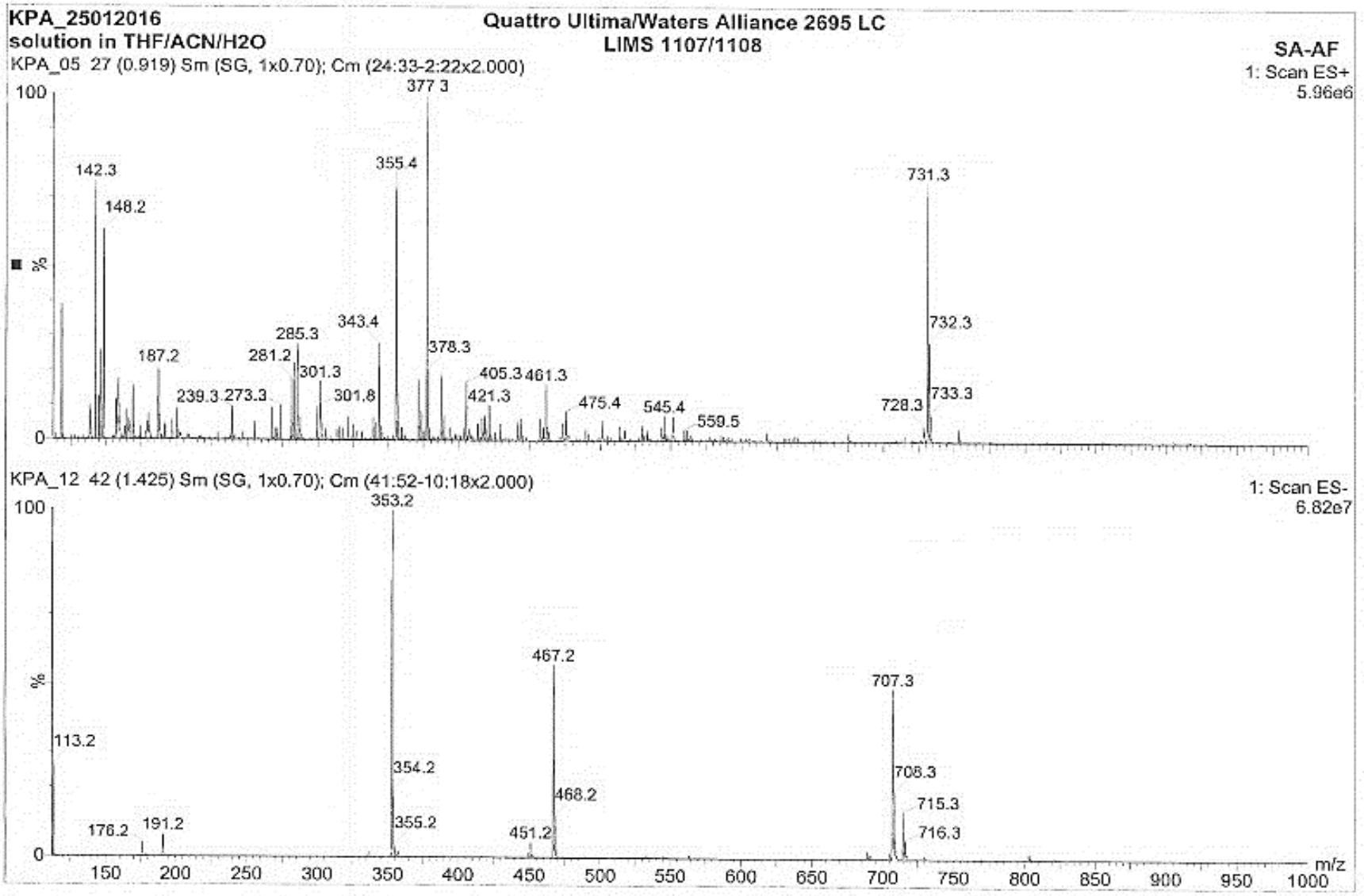
Appendix 29: -Mass spectrometry spectra of IC10 fraction



Appendix 30: -Mass spectrometry spectra of MC4 fraction



Appendix 31: -Mass spectrometry spectra of SA3 fraction



Appendix 32: Differentially expressed proteins involved in the enrichment pathway analysis of aucubin-treated MSSA.

UNIPROT ID	Gene ID	Description	Fold change	calc. pI	Peptides	Coverage	Score	Unique. Peptides	Area	PSMs
A6QHJ8	valS	Valine--tRNA ligase	6.89	4.65	24	67.51	24543.98	24	964900000	718
A7WXP7	ldh1	L-lactate dehydrogenase 1	7.39	4.86	30	85.53	16676.97	30	1180000000	589
A7X1D8	ileS	Isoleucine--tRNA ligase	5.12	4.88	41	72.01	15969.34	41	344500000	468
A7X1Q1	infB	Translation initiation factor IF-2	8.37	5.02	66	73.63	13491.74	66	265900000	455
A7X344	aspS	Aspartate--tRNA ligase	9.96	6.89	69	60.98	11987.61	16	233000000	364
A7X396	clpX	ATP-dependent Clp protease ATP-binding subunit ClpX	4.53	5.05	22	80.36	9617.2	22	164900000	278
A7X4X7	pyrG	CTP synthase	11.03	6.21	55	48.47	8146.42	2	228000000	261
P09616	hly	Alpha-hemolysin	3.72	5.35	40	79.66	7612.16	40	110900000	209
P0A0A1	pdhB	Pyruvate dehydrogenase E1 component subunit beta	6.55	4.82	27	77.87	7237.79	27	200900000	203
P65236	prs	Ribose-phosphate pyrophosphokinase	10.2	5.88	40	89.75	6246.99	39	176900000	209
Q2FF95	groL	60 kDa chaperonin	9.31	5.24	35	46.5	5581.71	35	49320000	169
Q2FJ31	adh	Alcohol dehydrogenase	6.89	5.03	30	56.65	5016.5	30	179200000	145
Q2FVK2	hlgC	Gamma-hemolysin component C	5.11	5.02	21	63.25	4835.7	21	246000000	152
Q2FXB0	lukEv	Leucotoxin LukEv	13.9	5.45	22	90.32	4069.45	22	694300000	128
Q2FXB1	lukDv	Leucotoxin LukDv	4.68	7.56	27	8.87	3929.51	3	209500000	108
Q2FZ89	ftsZ	Cell division protein FtsZ	5.83	7.64	26	4.79	3927.34	2	20950000	107

Q2G296	fhs	Formate--tetrahydrofolate ligase	5.44	5.25	28	58.39	3800.18	28	38970000	123
Q2G2Q0	gyrA	DNA gyrase subunit A	7.52	5.12	18	50.29	3746.27	18	11130000	118
Q2YSB3	tuf	Elongation factor Tu	8.32	4.97	25	28.48	3737.43	25	28890000	125
Q2YSE8	eno	Enolase	4.87	9.44	21	57.14	3721.06	21	13280000	114
Q2YSF1	pgk	Phosphoglycerate kinase	6.61	8.73	23	68.97	3605.02	23	68930000	117
Q2YSX0	adh	Alcohol dehydrogenase	14.86	4.73	17	65.54	3588.08	17	45670000	110
Q2YTC9	ackA	Acetate kinase	7.54	9.35	20	60.31	3516.83	17	30580000	118
Q2YTE3	pyk	Pyruvate kinase	11.8	5.33	21	59.85	3427.57	21	74410000	126
Q2YVL5	guaA	GMP synthase [glutamine-hydrolyzing]	9.85	9.96	24	38.87	3313.38	7	12030000	124
Q2YVL6	guaB	Inosine-5'-monophosphate dehydrogenase	5.92	5.24	24	72.35	3257.92	24	17950000	109
Q2YZ66	arcA	Arginine deiminase	16.14	5.16	29	64.09	3214.32	29	14560000	100
Q49Z50	atpD	ATP synthase subunit beta	4.73	5.03	24	48.75	3200.03	24	12970000	92
Q4L3K8	fusA	Elongation factor G	8.06	4.78	26	44.43	3187.48	25	77330000	102
Q5HDD3	hlgB	Gamma-hemolysin component B	8.75	9.85	27	28.8	3125.88	10	98190000	120
Q5HDD4	hlgC	Gamma-hemolysin component C	5.2	4.6	20	58.57	3089.5	1	17210000	93
Q5HE49	glmS	Glutamine--fructose-6-phosphate aminotransferase	4.88	4.78	14	31.75	3070.63	14	28560000	86
Q5HFJ5	glyQS	Glycine--tRNA ligase	10.47	5.22	27	43.38	3067.59	27	51030000	103
Q5HGH4	tsf	Elongation factor Ts	4.98	5.07	15	54.26	3010.75	15	89230000	89
Q5HGY8	pdhD	Dihydrolipoyl dehydrogenase	6.24	6.55	26	61.45	2987.25	26	10160000	96
Q5HGZ1	pdhA	Pyruvate dehydrogenase E1 component subunit alpha	13.45	6	24	69.25	2944	24	23760000	97
Q5HHP5	gapA1	Glyceraldehyde-3-phosphate dehydrogenase 1	7.32	4.59	21	60.36	2917.31	2	17770000	86
Q5HID2	rpoC	DNA-directed RNA polymerase subunit beta'	7.89	4.77	17	50.52	2908.43	17	10460000	80
Q5HIG2	cysK	Cysteine synthase	6.25	5.12	23	61.99	2862.54	23	82430000	100
Q5HII6	metG	Methionine--tRNA ligase	7.3	5.2	17	28.23	2782.89	17	14260000	81
Q5HMB9	atpD	ATP synthase subunit beta	9.48	5.47	17	26.83	2771.4	17	52720000	79
Q5HQX6	secA1	Protein translocase subunit SecA 1	6.32	9.29	20	61.27	2708.46	18	74030000	91

Q6GBP8	pta	Phosphate acetyltransferase	7.46	5.52	20	74.4	2671.06	20	24290000	70
Q6GH64	tkt	Transketolase	14.3	5.07	20	37.93	2660.44	20	74550000	68
Q7A5C5	SA1402	UPF0365 protein SA1402	6.09	5.14	24	54.1	2635.77	24	74440000	90
Q7A6L4	SA0778	UPF0051 protein SA0778	6.87	5.15	29	33.48	2623.17	29	12280000	86
Q8NUM4	mqo2	Probable malate:quinone oxidoreductase 2	4.82	6.25	15	53.58	2513.79	15	24130000	63
O05204	ahpF	Alkyl hydroperoxide reductase subunit F	-5.63	4.64	18	47.58	2482.6	18	71840000	63
A6QEP0	NWMN_0550	UPF0447 protein NWMN_0550	-7.32	5	12	48.65	2458.27	12	26740000	66
A6TYN9	xpt	Xanthine phosphoribosyltransferase	-4.32	4.98	17	66.41	2451.49	17	97000000	75
A7X4P1	rsbW	Serine-protein kinase rsbW	-6.31	5.11	27	34.7	2364.06	27	33380000	87
A7X432	ligA	DNA ligase	-4.98	4.63	13	52.86	2321.84	13	67900000	63
A7X4L6	rex	Redox-sensing transcriptional repressor rex	-12.37	5.94	13	30.45	2293.12	13	36350000	69
Q2FH00	ald1	Alanine dehydrogenase 2;1	-4.75	5.14	25	74.36	2261.15	25	86020000	72
A7X5F4	rplP	50S ribosomal protein L16	-5.08	5.25	24	54.14	2246.89	24	62090000	78
A6QGY5	ebhB	Extracellular matrix-binding protein ebhB	-3.98	5.22	17	41.77	2217.18	17	29260000	59
Q2FJH7	sle1	N-acetylmuramoyl-L-alanine amidase sle1	-5.61	9.22	13	47.71	2171.85	10	22140000	63
A7X5C5	rpsK	30S ribosomal protein S11	-4.32	8.75	12	46.75	2095.89	12	52520000	68
Q2FFA2	SAUSA300_1975	Uncharacterized leukocidin-like protein 2	-6.05	4.91	20	64.11	2060.08	20	28770000	74
A7X1K9	rimM	Ribosome maturation factor rimM	-9.98	5.3	14	53.27	2057.3	13	51580000	62
A7X1E5	pyrR	Bifunctional protein pyrR	-4.63	5.2	14	42.89	2050.54	14	29630000	43
P66954	tpx	Probable thiol peroxidase	-3.52	5.39	16	36.43	2029.31	16	42390000	67
P65895	purD	Phosphoribosylamine--glycine ligase	-4.56	5.21	19	52.03	2014.48	19	33940000	60
Q2YSH6	secA	Protein translocase subunit secA;A1	-7.12	5.12	16	24.43	2003.2	16	17020000	67
A7X203	sbcC	Nuclease SbcCD subunit C	-5.23	5.48	19	34.85	1956.59	19	21240000	54
Q5HGM9	carB	Carbamoyl-phosphate synthase large chain	-5.3	4.88	16	67.38	1921.44	16	15000000	55
A7X4U4	atpG	ATP synthase gamma chain	-4.98	5.2	18	52.47	1885.57	18	89570000	56
A7X5F2	rpmC	50S ribosomal protein L29	-6.75	4.98	19	33.3	1775.41	19	20450000	63
P08065	sdhA	Succinate dehydrogenase flavoprotein subunit	-3.88	5.48	17	46.14	1753.78	17	66060000	51

Q2YSB9	rpoB	DNA-directed RNA polymerase subunit beta	-12.42	4.87	21	44.86	1730.66	21	40600000	60
A7X5E1	rplF	50S ribosomal protein L6	-4.35	5.88	15	51.98	1698.68	15	13640000	42
Q06AK7	topA	DNA topoisomerase 1	-6.1	5.31	25	58.06	1686.03	25	70210000	68
P0CZ42	SpyM3_0208	Probable ABC transporter ATP-binding protein	-4.36	5.01	18	35.06	1644.55	18	27750000	68

Upregulated
Downregulated

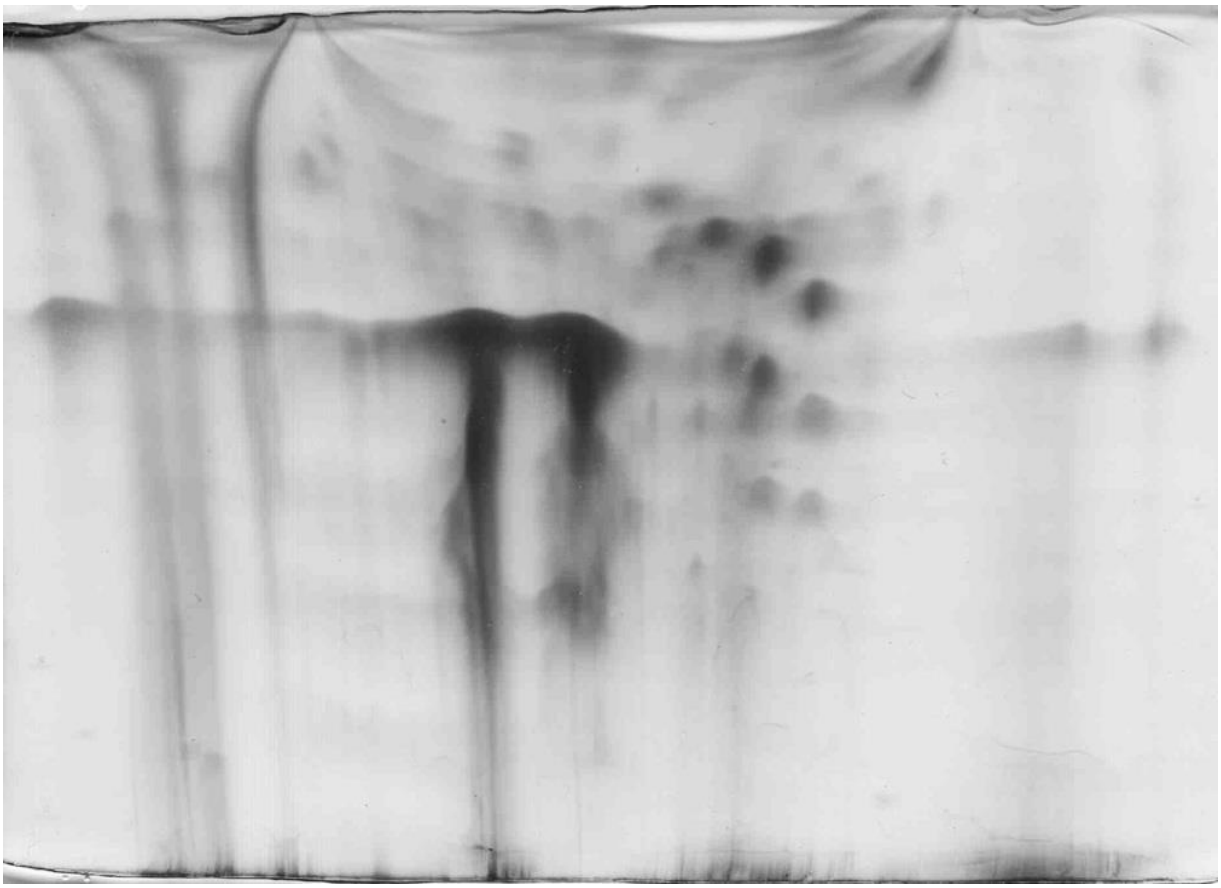
Appendix 33: Up-regulated proteins involved in the pathway enrichment analysis of differentially expressed proteins in aucubin-treated MSSA.

Pyruvate metabolism / Enrichment Score 6.22		
UNIPROT ID	Gene ID	Protein Description
Q2YTC9	ackA	Acetate kinase
Q5HGY8	pdhD	Dihydrolipoyl dehydrogenase
Q6GBP8	pta	Phosphate acetyltransferase
Q8NUM4	mqo2	Probable malate-quinone oxidoreductase 2
Q5HGZ1	pdhA	Pyruvate dehydrogenase E1 component subunit alpha
Q2YTE3	pyk	Pyruvate kinase
Glycolysis-Gluconeogenesis/ Enrichment Score 4.47		
UNIPROT ID	Gene ID	Protein Description
Q2YSE8	eno	Enolase
A0A0D6GGD0	IctE	L-lactate dehydrogenase 1
Q2YSF1	pgk	Phosphoglycerate kinase
Q6GHZ1	phdB	Pyruvate dehydrogenase E1 component subunit beta
Q5HGY8	pdhD	Dihydrolipoyl dehydrogenase
Valine, leucine and isoleucine biosynthesis / Enrichment Score 3.6		
UNIPROT ID	Gene ID	Protein Description
A7X1D8	ileS	Isoleucine--tRNA ligase
Q5HGZ1	pdhA	Pyruvate dehydrogenase E1 component subunit alpha
Q6GHZ1	phdB	Pyruvate dehydrogenase E1 component subunit beta
A6U2D1	valS	Valine--tRNA ligase
Aminoacyl-tRNA biosynthesis / Enrichment Score 3.1		
UNIPROT ID	Gene ID	Protein Description
A7X344	aspS	Aspartate--tRNA ligase
A8Z4A4	glyS	Glycine--tRNA ligase
A7X1D8	ileS	Isoleucine--tRNA ligase
A0A0U1MI40	metS	Methionine--tRNA ligase
A6U2D1	valS	Valine--tRNA ligase
Purine metabolism/ Enrichment Score 2.86		
UNIPROT ID	Gene ID	Protein Description
Q5HID2	rpoC	DNA-directed RNA polymerase subunit beta'
Q2YVL5	guaA	GMP synthase [glutamine-hydrolyzing]
Q2YVL6	guaB	Inosine-5'-monophosphate dehydrogenase
P65236	prs	Ribose-phosphate pyrophosphokinase
Citrate cycle (TCA cycle)/ Enrichment Score 2.42		
UNIPROT ID	Gene ID	Protein Description
Q5HGY8	pdhD	Dihydrolipoyl dehydrogenase
Q5HGZ1	pdhA	Pyruvate dehydrogenase E1 component subunit alpha
Q6GHZ1	phdB	Pyruvate dehydrogenase E1 component subunit beta
Propanoate metabolism / Enrichment Score 2.34		
UNIPROT ID	Gene ID	Protein Description
Q2YTC9	ackA	Acetate kinase
A0A0D6GGD0	IctE	L-lactate dehydrogenase 1
Q6GBP8	pta	Phosphate acetyltransferase

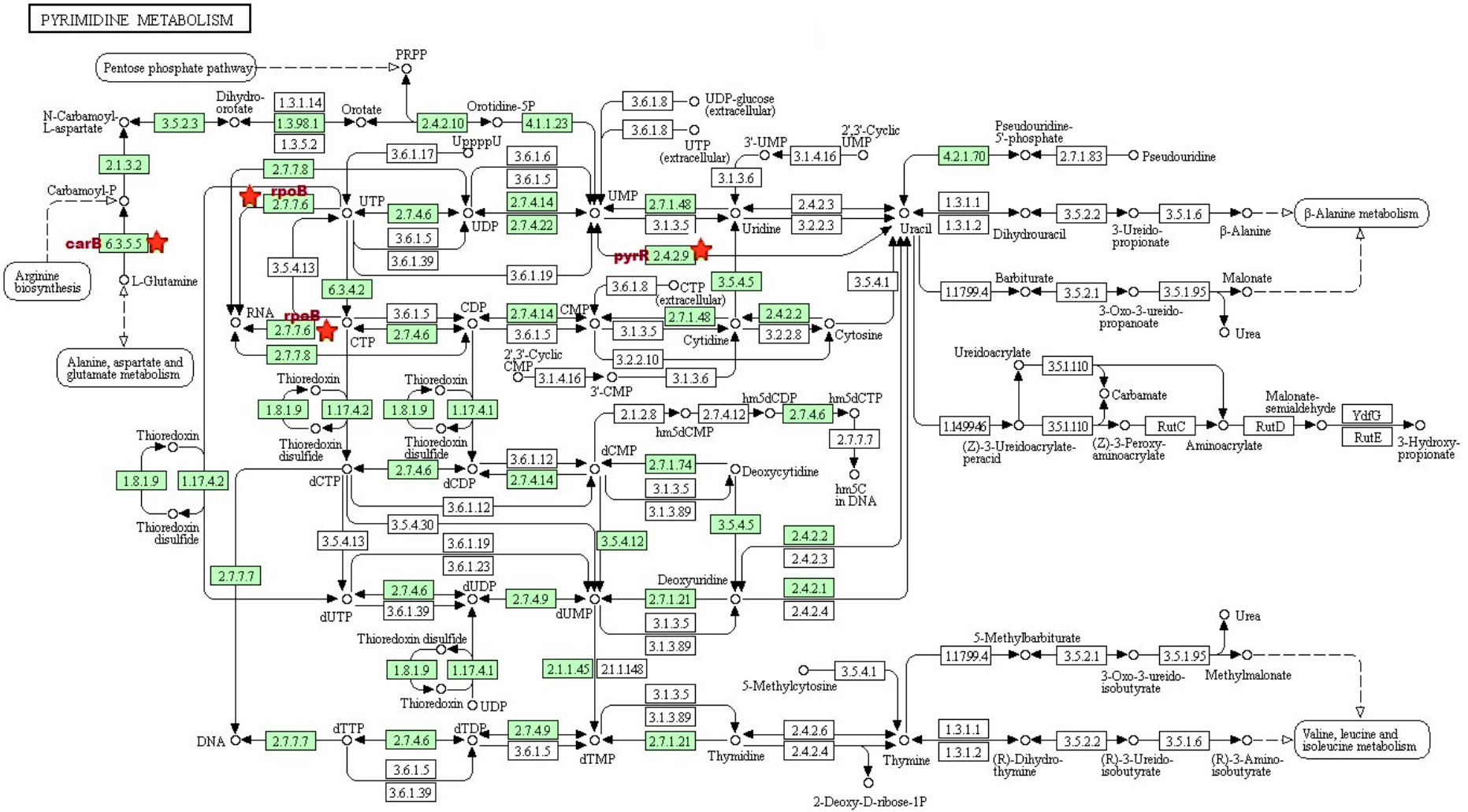
Appendix 34: Down-regulated proteins involved in the pathway enrichment analysis of differentially expressed proteins in aucubin-treated MSSA.

Ribosome / Enrichment Score 3.66		
UNIPROT ID	Gene ID	Protein Description
A7X5C5	rpsK	30S ribosomal protein S11
A7X5F4	rplP	50S ribosomal protein L16
A7X5F2	rpmC	50S ribosomal protein L29
A7X5E1	rplF	50S ribosomal protein L6
Pyrimidine metabolism / Enrichment Score 2.52		
UNIPROT ID	Gene ID	Protein Description
P65945	pyrR	Bifunctional protein pyrR
P63740	carB	Carbamoyl-phosphate synthase large chain
Q2YSB9	rpoB	DNA-directed RNA polymerase subunit beta

Appendix 35: An example of 2D-PAGE profile of total *Staphylococcus aureus* proteins.



Appendix 36: Pyrimidine metabolism pathway showing the down-regulated genes in aucubin-traded MSSA relative to control MSSA.



Appendix 37: ribosome pathway showing the down-regulated genes in aucubin-traded MSSA relative to control MSSA.