



Faculty of Science, Engineering & Technology
Department of Chemistry & Biotechnology

Investigation of antimicrobial activity and phytochemical characterisation of plant extracts

Submitted in total fulfillment of the requirements for the degree of

Doctor of Philosophy in Chemistry

By

Savithri Galappathie

Department of Chemistry and Biotechnology
Faculty of Science, Engineering and Technology
Swinburne University of Technology

October 2018

Abstract

Traditional medicine based on plant extracts and herbal remedies has been increasingly accepted in Western countries with the modernisation of traditional medicine being a hot area of research in recent years. During 1980-2014, there were 1562 new pharmaceutical drugs developed to treat diseases with about 49% being derived from medicinal plants. There are around 500,000 plant species worldwide but only one percent of these have been used as therapeutic agent.

The Sarawak Biodiversity Centre (SBC), Kuching, Malaysia, has provided chemical plant extracts from their collection of Southeast Asian plants that were screened for their antibacterial and antifungal properties. The results of this study so far have demonstrated that many of the Sarawak traditional medicinal plants have effects on microorganisms causative agents for diarrhoea, stomachache, fever, headaches, cough and inflammation. Out of fifty Asian plant extract that were screened, six were found to exhibit broad spectrum activity against several strains of bacteria and fungi and there was significant effect against both Gram-positive and Gram-negative bacteria. The use of these plant species by indigenous groups in Malaysia for the treatment of these illnesses can therefore be substantiated by our findings. In addition to this research, a study was undertaken to discover the basis of the healing abilities reported for an Australian bush medicine.

One native Australian and one Bornean plant were the subject of further characterisation using an approach developed for bioguided fractionation. Solid Phase Extraction tubes were used to divide the complex mixture of the plant extract into smaller fractions that were then tested for bioactivity. This process was then repeated for the active fractions and instrumental analysis of these secondary fractions was used for identification.

Eremophila longifolia leaf extract produced using the conventional immersion technique exhibited better antimicrobial activity compared to Accelerated Solvent Extraction, acetonitrile was found to be the most suitable extraction solvent and optimal activity was found with extracts produced using the conventional immersion technique at 21°C and 5 days

There were two novel natural compounds isolated using bioguided fractionation from the leaves of the Australian plant, *Eremophila longifolia*, and they were chemically identified as neryl ferulate and neryl *p*-coumarate. Although the bioactivity was weak, their discovery has further advanced knowledge of an important indigenous medicine.

Asian plant *Baccaurea lanceolata* fruit extracts showed the most antimicrobial activity against Gram-positive and Gram-negative bacterial strains, the active compound was isolated, and it was chemically identified as tartaric acid.

The overall goal of this project was to identify the compounds responsible for observed bioactivity.

*This thesis dedicated to my late father, Mr. Ranjith Perera
Gunaratne, his sister Mrs. Mangala Fernando and my primary
school science teacher Mrs. Jayantha Fernando, especially
because of you, I love science.*

Acknowledgements

Firstly, I would like to express my sincere gratitude to all my supervisors Dr. Peter Mahon, Professor Enzo Palombo and Dr François Malherbe for the continuous support towards my research project. Special thanks to Peter, who gave me the necessary advice for all chromatographic analysis, separation studies and coached me to think accordingly. His words were “Savi, do the experiments systematically and it has to be consistent”. Tremendous thanks to Professor Danny Meyer for her expert advice for statistical pathways and analysis. The Sarawak Biodiversity Center is the primary provider of the Bornean plant extracts for this study and my humble appreciation to the staff who were involved in the collection of the plant from the different geological locations in the forest. To the University of Queensland, I am immensely grateful to Dr. Mark Butler and his colleagues for providing necessary instrumentation analysis to finalise this research. Also, a big thank you to senior LC-MS application specialist Mr. Alex Chen, at Thermo Fisher Sciences Australia, for providing unrestricted assistance of analysis for all my samples. I thank fellow colleagues, Mr. Ngan Nguyen and Yeannette Lizama, always with a smile on their face, Dr. Rebecca Phillips and Dr. Shanthi Joseph providing microbial advice and the cultures. Also, big thank you to Dr. Avinash Karpe, Dr. Rohan Shah, Dr. Snehal Jadhav and Dr. Yulia Alekseeva for their precious support to survive in difficult situations.

Finally, I would love to thank my beloved rebellion gang, my mum Nikulas, husband Suppriya and kids, Amanda and her partner Julian, Tanya, Ruwini and her family, my physics and chemistry teacher/cousin Lalani Kadigamuwa for her support in secondary school and my beloved friend Pjrena, for their unconditional love and support including the encouragement to finish.

I love you guys with all of my heart

Declaration

To the best of my knowledge, this PhD thesis does not contain any published materials including tables, figures and appendices, in whole, or in part, for the award of any other academic degree or diploma by another individual. Except where otherwise indicated, this work is my own oeuvre.

Savithri Galappathie

2018

List of publications

Journal publications

- **Savithri Galappathie**, Enzo A. Palombo, Tiong Chia Yeo, Diana Lim Siok Ley, Chu Lee Tu, François M. Malherbe and Peter J. Mahon, Comparative antimicrobial activity of Southeast Asian plants used in Bornean folkloric medicine. *Journal of Herbal Medicine*, 2014, 4 (2), 96-105
- **Savithri Galappathie**, David J. Edwards, Alysha G. Elliott, Matthew A. Cooper, Enzo A. Palombo, Mark S. Butler and Peter J. Mahon, Antibacterial Nerol Cinnamates from the Australian Plant *Eremophila longifolia*. *Journal of Natural Products*, 2017, 80 (4), 1178–1181

Table of Contents

Abstract	iii
Acknowledgements	vi
Declaration.....	vii
List of publications.....	viii
Table of Contents.....	ix
List of Figures.....	xiv
List of Tables	xvi
List of Abbreviations and Acronyms	xvii
Chapter 1	1
1.1 Aims of the thesis	2
1.2 Outcomes of the work undertaken in the throughout the study	3
1.3 Thesis Overview	5
Chapter 2	7
2.1 Plant medicines with antimicrobial activity	8
2.1.1 Ethnomedicinal significance of plants.....	8
2.1.2 Plants used for antibacterial medicines	9
2.1.3 Plants used for antifungal medicines.....	11
2.1.4 Medicinal plants for the treatment of neurological and psychiatric disorders.....	11
2.1.5 Plants for malaria therapy.....	12
2.1.6 Plant medicine and acupuncture interrelated treatments	13
2.1.7 Plant medicine for cancer therapy.....	13
2.2 Australian native plant collected from Byrock in New South Wales, Australia.....	20
2.2.1 Description, distribution, ethnomedicinal and phytochemical identities of.....	20
2.3 Southeast Asian plant collection from Sarawak, Province of Malaysia	26
2.3.1 Description, distribution, ethnomedicinal and phytochemical identities of	
<i>Baccaurea lanceolata</i> (Miq.) Müll.Arg	26
2.3.2 Description, distribution, ethnomedicinal and phytochemical identities of	
<i>Fibraurea tinctoria</i> Lour	29

2.3.3 Description, distribution, ethnomedicinal and phytochemical identities of <i>Goniothalamus tapisoides</i> Mat Salleh	32
2.3.4 Description, distribution, ethnomedicinal and phytochemical identities of <i>Goniothalamus velutinus</i> Airy Shaw	34
2.3.5 Description, distribution, ethnomedicinal and phytochemical identities of <i>Polyalthia hookeriana</i> King	36
2.3.6 Description, distribution, ethnomedicinal and phytochemical identities of <i>Pyrenaria serrata</i> Bl. var <i>masocarpa</i> (Korth) H. Keng	37
2.4 Isolation and characterisation of bioactive compounds from plant materials.....	38
2.4.1 Extraction methods.....	39
2.4.2 Clean up and isolation methods	42
2.4.3 Separation methods	44
2.4.4 Identification by spectroscopy methods	46
2.5 Conclusions	48
Chapter 3	50
3.1 Introduction	51
3.2 Plants materials used in this study.....	51
3.3 Methods used in this study.....	52
3.3.1 Preparation of Australian crude plant extract	52
3.3.2 Preparation of the Southeast Asian plant extracts.....	52
3.3.3 Bacterial strains and media	53
3.3.4 Bioassays	55
3.3.5 Separation techniques.....	56
3.3.6 Analysis techniques - Separation and identification	60
3.4 Method development	64
3.4.1 Fractionation based on Solid Phase Extraction cartridges.....	64
3.4.2 Development of fractionation methods	65
3.4.3 Results and discussion.....	67

3.5 Conclusions	75
Chapter 4	76
4.1 Introduction	77
4.2 Plant collection	77
4.2.1 Sample preparation.....	77
4.3 Extraction of leaves	77
4.3.1 Different extraction techniques	77
4.4 Extraction efficiency analysis	80
4.4.1 Assessment of antimicrobial activity	80
4.4.2 HPLC analysis.....	82
4.4.3 GC-MS analysis	82
4.5 Results and Discussion	82
4.5.1 Solvent optimisation.....	82
4.5.2 Maximising extraction yield using different extraction techniques	86
4.6 Conclusions	103
Chapter 5	104
5.1 Introduction	105
5.2 Extraction, isolation and characterisation	105
5.2.1 Fractionation of the <i>E.longifolia</i> plant extract	105
5.2.2 GC-MS analysis	106
5.2.3 NMR Analysis.....	106
5.3 Antimicrobial and antifungal assays.....	106
5.3.1 Assessment of biological inhibitory activity of separated fractions	106
5.4 Results and discussion	109
5.4.1 Primary fractionation.....	109
5.4.2 GC-MS analysis	111
5.4.3 Further separation, identification and characterisations of microbially active compounds.....	114
5.4.4 Thermal degradation experiments.....	121
5.5 Conclusion.....	123

Chapter 6	124
Screening of ethnomedicinal Southeast Asian plants for antimicrobial and antifungal activities	
124	
6.1 Introduction	125
6.2 Material and methods.....	126
6.2.1 Preparation of extracts.....	126
6.2.2 Bacterial and fungal isolates and media used	126
6.2.3 Antibacterial and antifungal assays.....	126
6.3 Results and discussion	126
6.3.1 Disc diffusion assay.....	126
6.3.2 MIC and MBC/MFC determination for the selected extracts.....	133
6.4 Conclusions	137
Chapter 7	138
7.1 Introduction	139
7.2 Experimental	139
7.2.1 Preparation of crude extract.....	139
7.2.2 Primary fractionation by Solid Phase Extraction.....	140
7.2.3 Secondary fractionation by Solid Phase Extraction	140
7.2.4 Antimicrobial susceptibility tests.....	140
7.2.5 Chromatographic analysis	141
7.3 Statistical Analysis.....	142
7.4 Results and discussion	142
7.4.1 Primary fractionation.....	142
7.4.2 Secondary fractionation.....	144
7.4.3 Compound identification and verification	146
7.5 The presence of tartaric acid in other plants.	152
7.6 Conclusions	153
Chapter 8	154
8.1 Summary of conclusions and further directions.....	155
8.2 Potential for future work.....	157
References	160

Appendix	173
----------------	-----

List of Figures

Figure 1: Chemical structure of Quinine.....	13
Figure 2: <i>Catharanthus roseus</i> G Don.....	15
Figure 3: Chemical structure of Vinblastine (VBL)	16
Figure 4: Pacific Yew (<i>Taxus brevifolia</i>) bark	17
Figure 5: Chemical structure of Paclitaxel (Taxol).....	17
Figure 6: Chemical structure of Camptothecin.....	18
Figure 7: <i>Camptotheca acuminata</i>	18
Figure 8: Chemical structure of Combretastatin A	19
Figure 9: <i>E. longifolia</i> - Weeping emu bush	21
Figure 10: Branches with leaves and flowers of <i>E. longifolia</i>	21
Figure 11: Distribution of <i>E. longifolia</i> in Australia.....	22
Figure 12: Chemical structure of compounds isolated from <i>E. longifolia</i> ^[102]	25
Figure 13: <i>B. lanceolata</i>	27
Figure 14: <i>F. tinctoria</i> leaves and fruit	30
Figure 15: <i>F. tinctoria</i> vine	30
Figure 16: <i>G. tapisoides</i> K. Mat-Salleh, from Lambir Hills National Park	32
Figure 17: Chemical structures of the compounds identified from <i>G. tapisoides</i> ^[119]	34
Figure 18: <i>G. velutinus</i> Airy Shaw	35
Figure 19: <i>P. hookeriana</i> branch	36
Figure 20: <i>P. serrata</i> branch with fruits	38
Figure 21: Soxhlet extraction ^[141]	41
Figure 22: Supercritical fluid extraction (SFE) apparatus ^[142]	42
Figure 23: Solid Phase Extraction method	43
Figure 24: Standard compounds used to characterise the micro-scale fractionation.....	60
Figure 25: Analytical HPLC chromatogram of the standard mixture of compounds. Peaks: 1. Uracil, 2. Phenol, 3. Acetophenone, 4. Anisole, 5. Toluene, 6. Biphenyl and 7. Pyrene	67
Figure 26: HPLC chromatogram of SPE eluted fractions from the standard test mixture.....	68
Figure 27: Components isolated in each fraction for the two series of eluting solvents using SPE.....	69
Figure 28: SPE results for the standard test mixture.....	70
Figure 29: Secondary fractionation of the standard test mixture with 40% acetonitrile/Milli-Q water.....	72
Figure 30: The analytical HPLC chromatogram of crude plant extract from <i>E. longifolia</i>	73
Figure 31: Analytical HPLC chromatogram of SPE eluted <i>E. longifolia</i> fractions.....	74
Figure 32: Flow chart of extractions and microbial assessment.....	78
Figure 33: HPLC chromatograms with CH ₃ CN for <i>E. longifolia</i> leaves extraction at (A to C) different temperatures for ASE and (D) the conventional extraction method.....	88
Figure 34: HPLC chromatograms with EtOH for <i>E. longifolia</i> leaves extraction at (A to C) different temperatures for ASE and (D) the conventional extraction method.....	89
Figure 35: GC-MS chromatograms with CH ₃ CN for <i>E. longifolia</i> leaves extraction at (A to C) different temperatures for ASE and (D) the conventional extraction method.....	90
Figure 36: GC-MS chromatograms with EtOH for <i>E. longifolia</i> leaves extraction at (A to C) different temperatures for ASE and (D) the conventional extraction method.....	91

Figure 37: Examples for antibacterial activity using DDA for different organisms (<i>S. enterica</i> and <i>S. aureus</i>) and a range of extracts. SA refers to the CH ₃ CN extracts and AA are the EtOH extracts.....	92
Figure 38: HPLC chromatograms from the conventional extraction of <i>E. longifolia</i> leaves using CH ₃ CN with various times represented in A to F	99
Figure 39: GC-MS chromatograms from the conventional extraction of <i>E. longifolia</i> leaves using CH ₃ CN with various times represented in A to F	100
Figure 40: Bioactivity against <i>B. cereus</i> from the Series 1 fractions in DDA	110
Figure 41: Bioactivity against <i>B. cereus</i> from the Series 2 fractions in DDA	110
Figure 42: GC-MS chromatogram of an active fraction of <i>E. longifolia</i> against <i>B. cereus</i> ..	112
Figure 43: GC-MS chromatogram of non-active fraction of <i>E. longifolia</i> against <i>B. cereus</i> . In this figure, red star indicates 2-methoxy-4-vinyl phenol	112
Figure 44: Chemical structure of 2-methoxy-4-vinyl phenol.....	112
Figure 45: GC-MS chromatogram of 2-methoxy-4-vinyl phenol	113
Figure 46: Disc diffusion assay of 2-methoxy-4-vinylphenol against <i>B. cereus</i>	113
Figure 47: Structures of (1) neryl ferulate and (2) neryl p-cinnamate.	117
Figure 48: Proposed autoxidation mechanism of neryl ferulate (1)	118
Figure 49: HPLC chromatograms of the thermal degradation experiment for an active fraction from <i>E. longifolia</i>	122
Figure 50: Chemical structure of (1) neryl ferulate and with the thermal degradation product 2-methoxy-4 vinyl phenol circled in green	122
Figure 51: (A) Disc diffusion assay for <i>C. albicans</i> clinical isolate (SC5314) (B) Disk diffusion assay for <i>C. albicans</i> laboratory isolate (DAY286)	133
Figure 52: Primary SPE fractionation and inhibitory activity against <i>B. cereus</i>	142
Figure 53: Disc diffusion assay results showing antimicrobial activity of the Milli-Q water fraction with various bacterial cultures	143
Figure 54: Antimicrobial activity of secondary fractions (Fractions 1 to 7) of <i>B. lanceolata</i> against <i>S. aureus</i> . Fraction 8 is the positive control (Tetracycline) and Fraction 9 is the negative control (Milli-Q water) in disc diffusion assay.....	144
Figure 55: Relative activities of secondary fractions (Fraction 1-7) of <i>B. lanceolata</i> against <i>S. aureus</i> . The white bars represent relative MIC and those in grey represent MBC.....	145
Figure 56: CFU values against <i>E. coli</i> for active Fractions 1 to 5 with 6 being the negative control.....	146
Figure 57: Direct GC-MS analysis of the Milli-Q water fractions (1 to 6) with dimethyl tartrate eluting at 15.6 minutes	147
Figure 58: Derivatised GC-MS analysis of the Milli-Q water fractions (1 to 6).....	148
Figure 59: Chemical structures of the compounds with Spearman's rank correlation coefficients greater than 0.716. (1) Dimethyl 2, 3-bis[(trimethylsilyl)oxy]succinate, (2) Arabino-hexos-2-ulose, 3, 4, 5, 6-tetrakis-o-(trimethylsilyl)-, bis(dimethyl acetal) and (3) Bis(trimethylsilyl) 2,3-bis[(trimethylsilyl)oxy]succinate.....	150

List of Tables

Table 1: The most frequently occurring types of infections caused by the test pathogens.....	54
Table 2: Solvent mixtures of acetonitrile and Milli-Q water (% v/v) used for fractionation in both series	58
Table 3: Mass of standard compounds used in the test mixture	66
Table 4: Minimum inhibitory, bactericidal and fungal concentrations for <i>E. longifolia</i> leaves extracted using different solvents and hot water decoction extraction against various organisms.....	84
Table 5: Yield extraction efficiency for different extraction methods.....	87
Table 6: Antibacterial activity for different organism from various extracts obtained using DDA	93
Table 7: Minimum inhibitory, bactericidal, and fungicidal concentrations of <i>E. longifolia</i> leaf extracts using various extraction conditions.....	95
Table 8: Minimum inhibitory and bactericidal concentrations of <i>E. longifolia</i> leaf extracts using various extraction conditions.....	97
Table 9: Minimum inhibitory, bactericidal and fungicidal concentrations of <i>E. longifolia</i> leaf extracts using different times with conventional CH ₃ CN extraction.....	102
Table 10: DDA bioactivity against <i>B. cereus</i> of CH ₃ CN/H ₂ O from Series 1 and 2 fractions	111
Table 11: MIC of the crude extract and active fractions against a panel of Gram-positive and Gram-negative bacteria.	116
Table 12: Chemical details for the structures in Figure 47	117
Table 13: NMR spectroscopic data (600 MHz, CDCl ₃) for (1) neryl ferulate and (2) neryl p-cinnamate.....	119
Table 14: Biological activity of (1) Neryl Ferulate and (2) Neryl p-Cinnamate against a panel of bacteria, fungi and cell lines	121
Table 15: Summary of DDA results for the Sarawak extracts	128
Table 16: DDA antimicrobial screening of selected plant extracts	132
Table 17: MIC and MBC/MFC values for selected plant extracts	134
Table 18: Spearman's rank correlation coefficients for chromatographic peak area versus MBC for each fraction.....	149
Table 19: Compounds identified in the most active fractions from the secondary fractionation stage using derivatised GC-MS analysis	150

List of Abbreviations and Acronyms

ASE	Accelerated solvent extractor
BHIA	Brain heart infusion agar
CFU	Colony forming units
CH ₃ CN	Acetonitrile
CMRNG	Chromosomally-mediated resistant <i>Neisseria gonorrhoeae</i>
DDA	Disc diffusion assay
DMSO	Dimethyl sulfoxide
EtOH	Ethanol
GC-MS	Gas chromatography–mass spectrometry
HPLC	High performance liquid chromatography
HRMS	High-resolution mass spectrometry
IC ₅₀	50% inhibitory concentration
LC-MS	Liquid chromatography–mass spectrometry
LLE	Liquid liquid extraction
MBC	Minimum bactericidal concentration
MDR	Multi-drug resistant
MeOH	Methanol
MFC	Minimum fungal inhibitory concentration
MHA	Mueller-Hinton agar
MIC	Minimum inhibitory concentration
MRO	Multi-resistant organisms
NA	Nutrient agar
NB	Nutrient broth
NMR	Nuclear magnetic resonance spectroscopy
PDA	Potato dextrose agar
PDB	Potato dextrose broth
SBC	Sarawak Biodiversity Centre

SPE	Solid phase extraction
WHO	World Health Organisation

Chapter 1

Introduction

1.1 Aims of the thesis

Bacterial infections are among the most dominant illnesses in the world. The alarming spread of drug resistant bacteria and limited access to anti-infective drugs in some populations emphasise the importance of discovering new antibacterial and antifungal compounds. The richest sources of natural compounds to defend against such bacterial pathogens are plants and herbal extracts that have had their side effects empirically tested all the way through their history of human use as bush medicines.

One aim of this research was thus to identify the antimicrobial active plants from a series of plant extracts provided by the Sarawak Biodiversity Centre, in Malaysia. These plants were collected as part of an intensive ethnomedicinal knowledge documentation program of plants used by the local indigenous peoples. Based on the documented evidence of medicinal benefits, fifty plant extracts were provided for the further antibacterial and antifungal analysis. The extracts were tested against four Gram-negative bacteria: *Escherichia coli*; *Pseudomonas aeruginosa*; *Salmonella enterica* serovar Typhimurium and *Moraxella catarrhalis*, three Gram-positive bacteria: *Bacillus cereus*; *Staphylococcus aureus* and *Streptococcus pyogenes* and one fungus *Candida albicans*.

A further aim of this research was to identify the bioactive compounds from the active plants. Among all the plants that were tested, *Baccaurea lanceolata* was selected for further analysis of the bioactive compounds detected in the preliminary analysis conducted on the crude extracts. The crude extract separation was performed using bioguided primary and secondary fractionation based on the solid phase extraction (SPE) format. This method is useful to separate the complex mixtures and concentrate them accordingly. The active compounds were identified using Gas Chromatography–Mass Spectrometry (GC-MS) and Liquid

Chromatography–Mass Spectrometry (LC-MS) with the resulting data statistically analysed based on correlation methods.

The presented research also included a study of the Australian native plant, *Eremophila longifolia* F. Muell, as this plant is widely used in Aboriginal communities as a bush medicine. The plant is commonly known as Emu bush, Berrigan emu bush, Weeping emu bush, Native plum, Juniper tree and Dog wood. In this case, plant material was available and a detailed extraction study was performed, which included optimising the extraction solvents and procedure. Ethanol, hot water and acetonitrile were used as extraction solvents and the optimization was based on bioguided inhibitory assays using bacterial cultures. Different extraction methods and soaking times were also used as variables. After applying the optimised extraction method, the extract was processed using primary and secondary fractionation. The unknown bioactive compounds were identified using GC-MS, LC-MS and Nuclear Magnetic Resonance (NMR) spectroscopic analysis.

1.2 Outcomes of the work undertaken throughout this study

- Identification of plants that can inhibit microbial activity associated with infective disease. From the plants that were collected from Sarawak, Malaysia, six plant extracts out of fifty were identified after thorough bacterial and fungal screening. Of the six active extracts, one was chosen for further analysis.
- The Australian plant, *Eremophila longifolia*, and *Baccaurea lanceolata* from Malaysia were subjected to further chemical analysis. Each plant extract has undergone extensive bioguided SPE fractionation.
- The active compound discovery for *B. lanceolata* was completed based on the Spearman statistical correlation approach. Initially, bioactive fractions were identified

and those fractions were then analysed using GC-MS and LC-MS to determine composition of the mixtures. The entire collection of data was subjected to statistical analysis to identify the compound that is responsible for the bioactivity.

- *E. longifolia* active compound isolation and chemical structure elucidation was performed in collaboration with the Institute of Molecular Biology at the University of Queensland. The chemical identification was completed using a range of instrumental analysis techniques including LC-MS and NMR.

1.3 Thesis Overview

Chapter 1 introduces the primary aims of the study

Chapter 2 offers an inclusive literature review on traditional medicine and its healing abilities. It describes how plant-based remedies can fulfill the demands of Western medicine and how effective they might be for some known diseases. Subsequently, in this chapter, the plants, *Baccaurea lanceolata* (Miq.) Müll. Arg., *Fibraurea tinctoria* Lour, *Goniothalamus tapisoides* Mat Salleh, *Goniothalamus velutinus* Airy Shaw, *Polyalthia hookeriana* King and *Pyrenaria serrata* Bl. var *masocarpa* (Korth) H. Keng used in this research are introduced and more comprehensively detailed. An overview of bioactive compounds produced by the plants has been provided.

Chapter 3 discusses the materials and methods used in this study. A fractionation method was developed using Solid Phase Extraction for a standard mixture of compounds and separation was completed according to the log *P* values for each compound. It was then adapted to actual plant extracts and was demonstrated to be suitable for the predictive separation of complex mixtures.

Chapter 4 introduces the Australian plant *E. longifolia*, the extraction of the bioactive compounds and testing for their antibacterial and antifungal activities.

Chapter 5 discusses the separation, isolation and characterisation of bioactive compounds in *E. longifolia* that are responsible for inhibiting the growth of bacteria.

Chapter 6 introduces the Southeast Asian plants and describes their antibacterial and antifungal inhibitory assays. Out of fifty plant extracts tested, six plants were discovered that had antibacterial ability.

Chapter 7 focuses on primary and secondary SPE fractionation applied to the Asian plant *B. lanceolata* with subsequent mass spectrometry analysis followed by statistical analysis.

Chapter 8 concludes the thesis with a brief summary of research outcomes and suggests further work.

Chapter 2

Literature review

2.1 Plant medicines with antimicrobial activity

2.1.1 Ethnomedicinal significance of plants

Plants have been used as the main source of medicinal remedies for more than 11,000 years and 70% of the worldwide population still relies on some form of traditional herbal medicine [1-2]. According to the World Health Organisation (WHO) statistics for developing countries, more than 80% of people rely on traditional medicine as their main source of medicine for infectious diseases [3]. More recently, during the World Wars of the 20th century most of the world's population depended on herbal medicine due to the disrupted distribution of pharmaceutical drugs and lack of other medicines [4]. As an example, garlic (*Allium sativum*) was used as an antiseptic to prevent numerous diseases [5].

Furthermore, plant-based (phytotherapeutic) medicinal treatments can help the body to heal itself. Most plants are used as diuretics and diaphoretics, antibacterial agents, as blood and bowel cleansers, and as a kidney stimulator [6]. Additionally, these plants contain a range of secondary metabolites that can help to defend against many bacterial and fungal infections. This also establishes the importance of plant-derived medicines for use in modern times as well [7].

Herbs have been widely employed in traditional medicine, which includes Chinese and Ayurvedic medicine [8], and some of these herbal medicines are used in different parts of the world along with more recently developed pharmaceutical medicines. The practice of plant medicine is widespread in developing countries especially in rural areas and is a great deal more accessible and affordable than expensive modern pharmaceutical drugs. It has been revealed that 80% of the population in African and Asian countries are still using herbal medicine as their main medicinal source for common diseases such as indigestions related

diseases including diarrhea , eye infections and skin infections ^[9]. Even in the modern times, herbal medications are more economical compared to pharmaceutical medicines and more readily accepted on the basis that they are “natural”. Nevertheless, the effectiveness of herbal plants can change depending upon the seasons or the month collected, part of the plant used, geographical location, altitude and the type of the soil where they grow. Therefore, extensive indigenous knowledge that has evolved over many generations is an important factor when employing medical treatments based on plants.

2.1.2 Plants used for antibacterial medicines

Bacterial infections are among the most detrimental global health conditions. According to the WHO, the major cause of death in the developing countries from infectious diseases is due to bacterial infections. The proportion of deaths was counted as 50% and this figure is comparatively high compared to other subjective death rates ^[10]. These deaths are related to 1415 species of microbial agents, including numerous viruses, bacteria, fungi, protozoa and helminths ^[11]. The alarming spread of drug resistant bacteria and limited access to many drugs to treat bacterial infections in some populations emphasise the importance to discover new antimicrobial medicines ^[12]. The richest source of natural compounds to defend against pathogens comes from plants and it is generally accepted that plant-derived drugs are less toxic, safer and more environmentally friendly. Also, they are generally more cost effective than synthetically manufactured drugs ^[13-14] .

After the discovery of penicillin by Fleming in the early 1930’s ^[15], antibiotics almost entirely controlled the spread and destruction of bacterial infections globally with death rates from bacterial related infectious diseases dropping significantly ^[16]. With the development of many other new antibiotics and the subsequent increase in the production and usage of new

antibiotics, some bacteria have evolved to evade the antimicrobial action by adapting diverse mechanisms against antibiotics ^[17]. It is estimated that pharmaceutical companies worldwide, manufacture about 100,000 tons of antibiotics annually to cure the bacterial and fungal related infections among the world's population ^[18]. Particularly in developing nations, most of the antibiotic agents are not regulated as prescription medication and they are readily available as over-the-counter medicines. The ease of accessing antibiotics has become the core factor for the rise of antibiotic resilient bacteria, which has been growing continuously in the past decades and has become a global health issue in recent years ^[19].

Nevertheless, a cumulative consequence of multiple genetic mutations has given bacteria the capacity to become resistant and even proliferate in the presence of antibiotics initially used for the handling and curing of pathogenic diseases ^[20]. For example, *Staphylococcus aureus* ('golden staph') and Chromosomally-Mediated Resistant *Neisseria gonorrhoeae* (CMRNG causes gonorrhoea) are, at present, virtually resistant to benzylpenicillin (penicillin G) ^[21]. Previously, these bacterial infections were readily cured by using generic penicillin ^[22]. Certain bacterial species are no longer susceptible to antibiotics and are thus identified as multi-drug resistant organisms (MROs). Most of these bacteria have developed resistance against more frequently used antibiotics like trimethoprim ^[23], amoxicillin ^[24], methicillin ^[25], penicillin ^[26], tetracycline ^[27], erythromycin and vancomycin ^[28]. Multi-drug resistant *Staphylococcus aureus* (MDR-SA), vancomycin resistant *Enterococcus* (VRE) ^[29] and multi drug resistant *Mycobacterium tuberculosis* (MDR-TB) ^[30] are the most important examples of MROs. Subsequently, the MROs are also resistant to most commercially available disinfectants and they can be particularly problematic in the development of hospital-acquired infections ^[31]. Furthermore, they can be a major cause of the secondary infections for hospitalised patients with immune deficiency disorders ^[32].

The WHO is concerned that increasingly, multi resistant bacteria and related infections are elevating rapidly due to insufficient availability of newly synthesised antibiotics. In order to overcome the problem of antimicrobial drug resistance, attention has recently turned to natural products as a potential source of new antimicrobial drugs ^[33].

2.1.3 Plants used for antifungal medicines

According to many recent studies, plant extracts can be applied to prevent skin diseases caused by fungi ^[34]. During the period of 1995-2012, there were 31 plant species identified as effective for skin disorders ranging from itching to skin cancer. *Allium sativum* (garlic) showed significant activity against *Malassezia furfur* (25 strains) which are liable for causing several types of skin infections, including seborrheic dermatitis, tinea versicolor and pityrosporum folliculitis ^[35]. It is also active against two different types of *Candida albicans* ^[23] and *Candida* sp.^[17], which are mostly responsible for oral candidiasis, oropharyngeal candidiasis or thrush and, when it affects the genital area of females it is known as vulvovaginal yeast infection ^[36-37]. Ethanol, ethyl acetate and aqueous extracts of Neem leaves showed inhibitory activity against different *Aspergillus* species that cause serious lung infections for immunocompromised patients ^[38], *C. albicans* and *Microsporum gypseum* ^[39] can be responsible for fungal keratitis ^[40]. The leaves of *Cassia fistula* Linn have been identified as a potential antipathogenic agent for *Aspergillus niger*, *Aspergillus clavatus* and *C. albicans* ^[41].

2.1.4 Medicinal plants for the treatment of neurological and psychiatric disorders

A recent report has revealed some native Iranian plants exhibited an influence on neurological and psychiatric disorders. According to the study, there are as many as 10 different plant families involved with the Family Astreaceae plant species showing the greatest effectiveness with the seed decoction being used as a tranquilizer ^[42].

Controversial arguments related to the medicinal use of marijuana continues all around the world including Australia. A recent Colombian clinical review suggested that marijuana could be useful to handle patients with chronic pain and other psychiatric problems. A further study revealed it could be used as an alternative medication for patients with chronic and neuropathic pain, and spasticity sufferers due to multiple sclerosis. In the United States, the US Food and Drug Administration approved administering medical marijuana to cancer patients after chemotherapy treatments. This decision was granted on the basis of high-quality evidence that proved cannabis can be used as an alternative medicine to treat nausea and regurgitation ^[43]. Moreover, an extensive review indicated that medicinal marijuana can be beneficial especially in psychological disorders like Alzheimer's disease, epilepsy, seizures and post-traumatic stress disorders (PTSD) ^[44].

2.1.5 Plants for malaria therapy

Malaria is one of the most detrimental diseases in developing countries and in recent years there were reports of malaria epidemics in 106 countries. In 2010, as many as 265 million humans suffered from this primeval disease with a significant number of deaths being recorded ^[45]. Effective antimalarial drugs containing amino quinoline and quinine are the best available in the market to treat this mosquito related infection and associated side effects that include arm and leg cramps related to vascular spasms, internal haemorrhoids ^[46] and irregular varicose veins ^[47]. Quinine (Figure 1) is a cinchona alkaloid obtained from the bark of *Cinchona calisaya* originally from South America ^[48] including regions of Brazil, Peru, Bolivia and Costa Rica ^[49]. In the 1600s, native Peruvian used the bark extract from the trees as herbal medicine to cure fever and then in the 1930s, European governments planted cinchona plant species in their tropical colonies to meet the medicinal requirements but because of the high demand,

more recently synthetic quinine has been used as an antimalarial drug. In 1944, a New York Times headline stated that finding quinine was the greatest scientific accomplishment of the century ^[50].

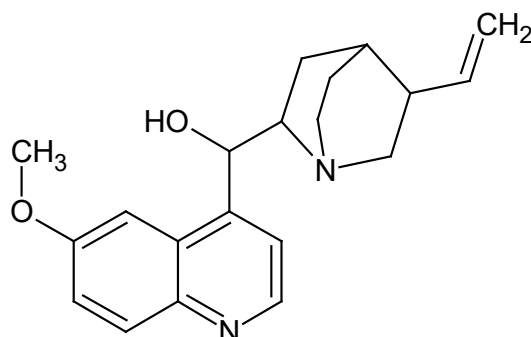


Figure 1: Chemical structure of Quinine

2.1.6 Plant medicine and acupuncture interrelated treatments

Acupuncture and ancient Chinese herbal formulation mixtures showed promising improved results for patients affected with seasonal allergic rhinitis (AR) ^[51]. Additionally, in research based on the Korean herbal medicine, moxibustion, and acupuncture, it was suggested that this complex mixture of the treatments illustrated effectiveness on female infertility ^[52]. Obesity ^[53], breast cancer ^[54] and insomnia-related depression ^[55] were also treated by acupuncture-based herbal medicine. According to published research in 2015, it was revealed that this herbal concept can be useful for female patients with bacterial vaginosis ^[56].

2.1.7 Plant medicine for cancer therapy

The Cancer Council of Australia stated that there are 130,470 new cancer patients diagnosed in every year and estimate that will this figure could reach up to 150,000 in 2020 ^[57]. Furthermore, according to the American Cancer Society, cancer kills approximately 3.5 million individuals annually ^[58]. Malignant neoplastic disease is the major health related problem in the modern world and it can affect any part of the physical structure of the human body ^[59]

with the most common cancers being skin, prostate, breast, bowel and lung ^[60]. Cancer can be caused by many different factors that include cigarette smoking, lifestyle factors like diet and activity, and specific virus infections ^[61]. Some 15-20% of human cancers occur due to long term untreated virus infections, which include human Papilloma virus (HR-HPV) that is responsible for oropharyngeal cancers ^[62] and cervical carcinoma ^[63]. Herpes-simplex virus, also known as Kaposi's sarcoma, (HSV-2) is a cofactor that can be conjugated with HR-HPV and increase the risk of cervical cancer for females ^[64].

Cancer patient often require chemotherapy depending on the aggressiveness of the cancer. Common side effects of chemotherapy include headache, skin rashes, nausea or vomiting, somnolence and abdominal pain. These side effects can result in high blood sugar and type 2 diabetes, as well. Some cancers adapt self-defense mechanisms to become resistant to chemotherapy related drugs ^[65]. This is the most vulnerable situation facing the new era of cancer treatments and therefore there is increasing demand for new cancer related drugs. Consequently, many cancer sufferers seek different solutions for this fatal illness based on alternative or complementary medicines and it has been reported that more than 60% of cancer patients use vitamin and herbal-based therapies ^[66].

Natural products play a major role in the development of novel drugs that target anticancer and antiinfectious agents. Herbal medicine or alternative medication can be very useful with patients with cancer and there have been reports of significant improvement of cancer related agonising pain and pain regularity ^[67]. Past literature has described some poisonous Chinese herbal medicines that are also used as cancer remedies. Evidence suggested that the combination of chemotherapeutic agents and these herbs can reduce the systemic toxicity

effects from conventional Western medicines and can help to maintain high immunity levels based on the theory the author called "fighting fire with fire" [68].

Recently, several plant-based compounds have been successfully employed in the treatment of malignant neoplastic disease. The fruit extract of a plant from Indonesia, *Phaleria macrocarpa* (Scheff), showed antioxidant properties and after the phytochemical isolation and identification, it was confirmed that the gallic acid was the active compound. It resulted in significant inhibitory cell proliferation activity against cancer cell lines (esophageal cancer cell TE-2) compared to non-cancerous (CHEK-1) cell lines [69].

In the 1950s, Vinca alkaloid was discovered by Canadian scientists, Robert Noble and Charles Beer and was derived from *Catharanthus roseus* G. Don (Figure 2), a plant from Madagascar [70]. Vinca alkaloids are the oldest group of the plant alkaloids to be used as chemotherapeutic agents to treat cancer. *Catharanthus roseus* G Don is also well-known to treat diabetes, high blood sugar and is used as a disinfectant.



Figure 2: *Catharanthus roseus* G Don

(Sourced from https://commons.wikimedia.org/wiki/File:Catharanthus_roseus%28L.%29G.Don._._Flickr_-_lalithamba.jpg)

There are four major Vinca alkaloids used as medicine: Vinblastine (VBL) (Figure 3), vinorelbine (VRL), vincristine (VCR) and vindesine (VDS). All these alkaloids are approved in the United States of America to treat patients with cancer related infections. Both VBL and VCR are chemicals initially extracted from plants with VRL and VDS being synthetically developed as vinca alkaloids molecules. Essentially, VBL and VCR are used for treating most of cancers including Hodgkin lymphoma, non-Hodgkin's lymphoma, breast, lung, brain, neck and bladder cancers ^[71].

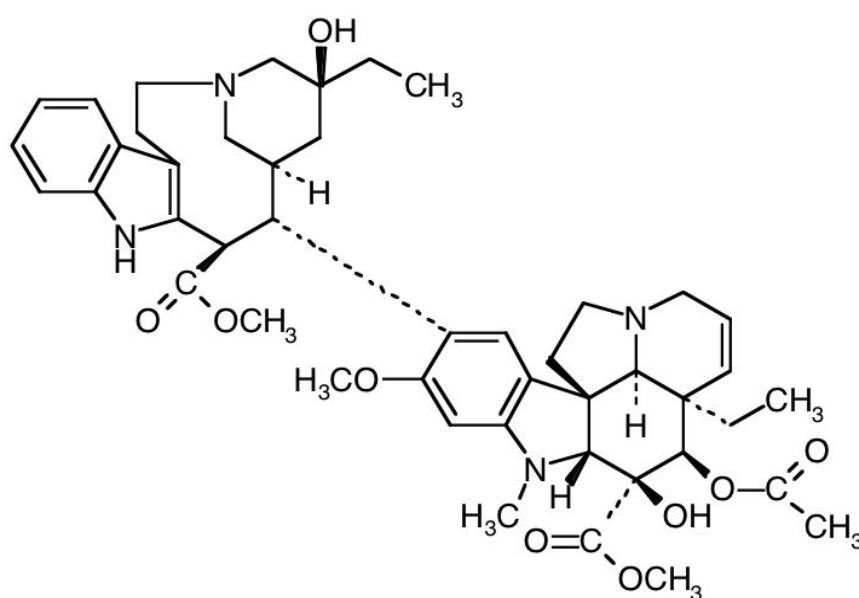


Figure 3: Chemical structure of Vinblastine (VBL)

The discovery of Paclitaxel (Taxol) from the bark of *Taxus brevifolia* Nutt (Taxaceae), Pacific yew (Figure 4) is another example of using natural products for new drug discovery ^[72]. This conifer tree is native to the Pacific North West of North America ^[73].



Figure 4: Pacific Yew (*Taxus brevifolia*) bark

(Sourced from: http://arcadianabe.blogspot.com.au/2011_12_01_archive.html)

Paclitaxel (Figure 5) is a lifesaving compound discovered in 1960's by two American scientists, Mansukh Wani and Monroe Wall. Taxol successfully treats cancer by inhibiting the division of cancer cells and it is used to treat breast cancer, ovarian cancer, non-small lung cancer, pancreatic cancer and AIDS related Kaposi's sarcoma [74].

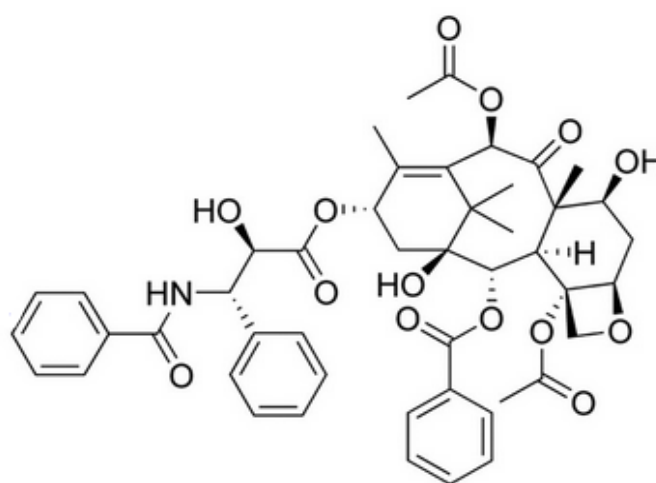


Figure 5: Chemical structure of Paclitaxel (Taxol)

Camptothecin (Figure 6) is a pentacyclic quinolone alkaloid derived from *Camptotheca acuminata* (Figure 7) ^[75]. Topotecan is a chemotherapeutic agent that is semi-synthesised from camptothecin that can be used to treat patients with ovarian, lung cancer and numerous other cancers. This became the first oral topoisomerase I inhibitor and it is trading pharmaceutically as Hycamtin ^[76-77] .

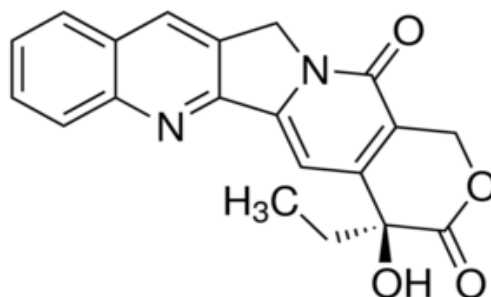


Figure 6: Chemical structure of Camptothecin



Figure 7: *Camptotheca acuminata*

(Sourced from: https://commons.wikimedia.org/wiki/File:Camptotheca_acuminata_-_Chengdu_Botanical_Garden_-_Chengdu,_China_-_DSC03562.JPG)

Combretastatins (Figure 8) are natural phenols that are derived from South African native plants, *Combretum caffrum* Kuntze (Combretaceae) ^[78]. This molecule is mainly deposited in the bark of the tree and exhibits some effect on patients suffering from colon, lung and leukaemia cancers. The non-polar and water-insoluble properties of this molecule have limited its use as a drug and so Combretastatin A-4, which is a more polar molecule, was developed ^[79]. This compound inhibits the development of blood cells within the tumor and mainly disrupts the mitosis of the malignant neoplastic disease cells. Aside from all those positives, this molecule is regarded as the most cytotoxic molecule derived from plants ^[66].

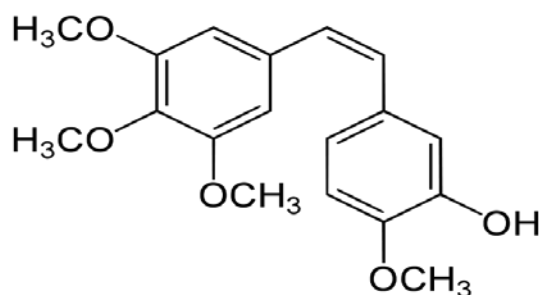


Figure 8: Chemical structure of Combretastatin A

Globally, there is a significant number of plant species that could be used for medicinal purposes, however, phytochemical analysis has only been completed on relatively few plant species ^[80]. In recent years, the exploration of plants has rapidly developed due to the use of modern phytochemical techniques and it has been found that there are vast quantities of chemicals that are associated with biosynthesis and metabolism pathways. Researchers have developed techniques that are needed to separate, purify and perform phytochemical analysis on the plant's secondary metabolites but this has limitations due to the variety and structural complexity of these compounds. A survey of the literature indicates that these secondary metabolites are comprised of hundreds of unknown chemical structures. Furthermore, these previous studies have found that there is a significant number of therapeutic drugs also derived

from plants ^[81] and some of the naturally occurring herbal products have similar active ingredients as synthetically manufactured pharmaceutical drugs.

Often, the natural molecules have issues due to low water solubility and exhibit high cytotoxicity effects on mammalian cells ^[66]. This requires pharmaceutical companies to derivatise and modified these molecules to be compatible for human use without reducing their bioactivity. Further research is required into the discovery of natural products to develop more effective drugs to cure cancer related infections and microbial infectious diseases.

2.2 Australian native plant collected from Byrock in New South Wales, Australia

2.2.1 Description, distribution, ethnomedicinal and phytochemical identities of

***Eremophila longifolia* F. Muell**

Eremophila is a significantly large genus and contains over 260 plant species from the family Scrophulariaceae. This genus is commonly known in Australian indigenous communities as Emu bush, Poverty bush and Fuchsia bush ^[81]. It is endemic to the Australian Continent ^[82] and is scattered across all the states of Australia. It is primarily located in semi-arid, arid or desert areas and it is absent from the wetter coastal belts along eastern Australia and southwestern Western Australia ^[83]. Plants are usually found as small to medium shrubs although a few species may be large bushes or small trees ^[84]. The name *Eremophila* is derived from Greek and translates as “lonely places, desert” and “to love” ^[85].

2.2.1.1 Description of *E. longifolia*

E. longifolia is a desert solitude loving plant with long leaves and brightly coloured flowers (Figures 9 and 10) ^[86]. Indigenous Australian people called it “berrigan” and is commonly

known as the weeping emu bush ^[87]. This plant is not considered as endangered based on its conservation status ^[86].



Figure 9: *E. longifolia* - Weeping emu bush

(Sourced from: http://upload.wikimedia.org/wikipedia/commons/3/33/E.longifolia_shrub.jpg)



Figure 10: Branches with leaves and flowers of *E. longifolia*

(Sourced from: <http://upload.wikimedia.org/wikipedia/commons/3/3d/E.longifolia.jpg>)

This plant is an evergreen with foliage all year round and is a rounded shrub or small tree that grows to a height of about 4-8 m but is often much smaller ^[86]. It is not unusual to see this tree grow in small clusters, as shown in Figure 9, as normally it surrounds the mother plant which

may have been grown from seeds or from root suckers ^[88]. The leaves of this plant are linear shaped and up to 200 mm long and 4-7 mm wide. The leaf tip has a hooked or bent point and is greyish green (dull green) in colour, it is mostly covered with fine hairs (this is an adaptation to dry weather) ^[89]. Tree branches have droopy foliage ^[90] and the flowers arise in the leaf axils. As displayed in Figure 10, the flowers are about 25-30 mm long with a tubular shape, have short stalks of 4-11 mm in length and the colour can vary from pinkish to brick red with spots on the inside ^[91].

2.2.1.2 Distribution of *E. longifolia*

E. longifolia is native to Australia and is predominantly distributed in semi-arid regions of all the mainland states except for the wetter coastal areas in eastern and southwestern Australia (Figure 11). This species rarely grows in the Northern Territory ^[90]. Even though it may grow in dry climates, it has also been effectively cultivated in warm temperate areas. It also can be seen in open, sunny areas with decent water drainage and can be tolerant of at least moderate frost ^[92].

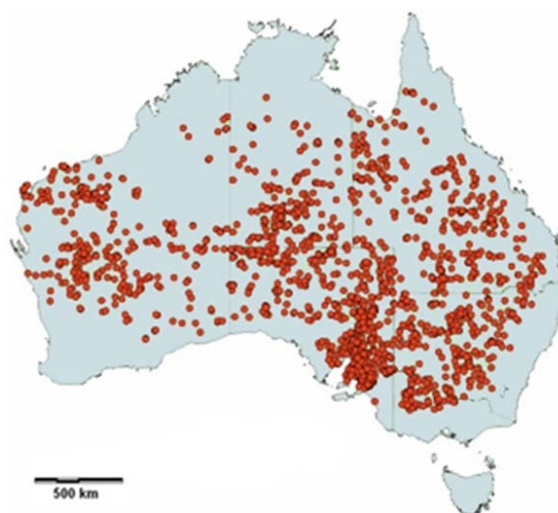


Figure 11: Distribution of *E. longifolia* in Australia

(Sourced from: http://www.florabank.org.au/lucid/key/species%20navigator/media/html/Eremophila_longifolia.htm)

2.2.1.3 Ethnomedicinal significance of *E. longifolia*

Australian indigenous communities have an extensive amount of understanding and knowledge of the edible and medicinal properties of many different plant species, which are established in the outback of Australia, and *E. longifolia* is considered as a prominent medicinal plant ^[93]. Traditional knowledge is playing a significant role in the sourcing, harvesting and preparation of these plants for edible or medicinal purposes.

The knowledge of medicinal plants has been passed from generation to generation but this pattern is being disrupted due to modernisation. A recent study revealed that only elders know how to use these medicinal plants appropriately and this knowledge is still relied on for herbal treatment tailored to the local habitat where they live ^[94]. The research revealed that most of the bush medicines are derived from common plants that could be easily accessed, which is convenient for a nomadic lifestyle as it reduces the size of any medicine kit that needs to be carried.

The plant can be prepared as medicine in many different ways. Leafy branches can be smoked over a fire with the steam that is produced being inhaled. As an aromatherapy, sprigs of aromatic plant leaves are crushed and this can then be inhaled, inserted into the nasal septum or put in the pillow that the patient would sleep on. In addition, an infusion can be prepared by using pounded bark and leaves that are soaked in water for a few days; this was then taken orally or used for bathing. Sometimes, an ointment would be prepared by using finely crushed leaves, seeds, fruit pulp mixed with animal fat for external treatments as a paste to be rubbed over the skin ^[95]. These preparations were frequently used as medicine to cure common colds, fever, sores, wounds, headaches, scabies and common infections as listed in Table A1 of the Appendix ^[96].

2.2.1.4 Phytochemistry of *Eremophila*

Past studies had shown very interesting results by identifying a diverse range of rare secondary metabolites from this genus. One study screened 72 species of *Eremophila duttonii* species against Gram positive and Gram negative bacteria where the results were quite encouraging with an MIC value of 62 µg/mL for *Streptococcus* species. An extract of *E. virens* showed activity against 68 clinical isolates of mMRSA at the MIC value of 31 µg/mL [97].

According to the other experimental results reported, some *Eremophila* species were responsible for producing a diverse class of diterpenoids called serrulatane, which have demonstrated antibacterial activity against pathogenic bacteria. In 1994, some researchers identified that these serrulatane structures were related to the diterpenoid quinone antibiotic biflorin isolated from the extract of *E. duttonii* [93].

Some recent bioguided isolation and identifications were reported for *E. sturtii* extracts that possess antimicrobial and antiinflammatory properties and two novel structures were elucidated. They belonged to a serrulantic acid group and were identified as 3, 8-dihydroxyserrulatic acid and serrulatic acid, and the two known compounds are β-sitosterol and Sesamin [96].

Table A2 in the Appendix indicates that most of the *Eremophila* species produce different resins and the desert-adapted *Eremophila* species are known to especially generate diterpenes, of which the most common skeletal structure is the bicarbocyclic serrulatane [98]. A number of serrulatanes have been identified as antibacterial with activity against Gram-positive and Gram-negative bacteria [82, 99-102].

Members of the *Eremophila* genus produce a limited number of flavonoids that have been isolated and identified. In addition, recent studies describe a series of flavonoids, *i.e.*, luteolin, dinatin, tricetin, 3,6-dimethoxyapigenin, jaceidin, and cirsimaritin, as well as a cembrene diterpene, (3*Z*, 7*E*, 11*Z*)-15-hydroxycembra-3,7,11-trien-19-oic acid, that were identified for the first time from *E. lucida* ^[103].

A previous study disclosed that a combination of eugenol methyl ether and safrole were found in *E. longifolia* from the distillation of pounded leaves ^[104]. Additionally, the methanolic extract from this plant isolated significant amounts of geniposidic acid and a small quantity of mannitol and verbascoside (Figure 12).

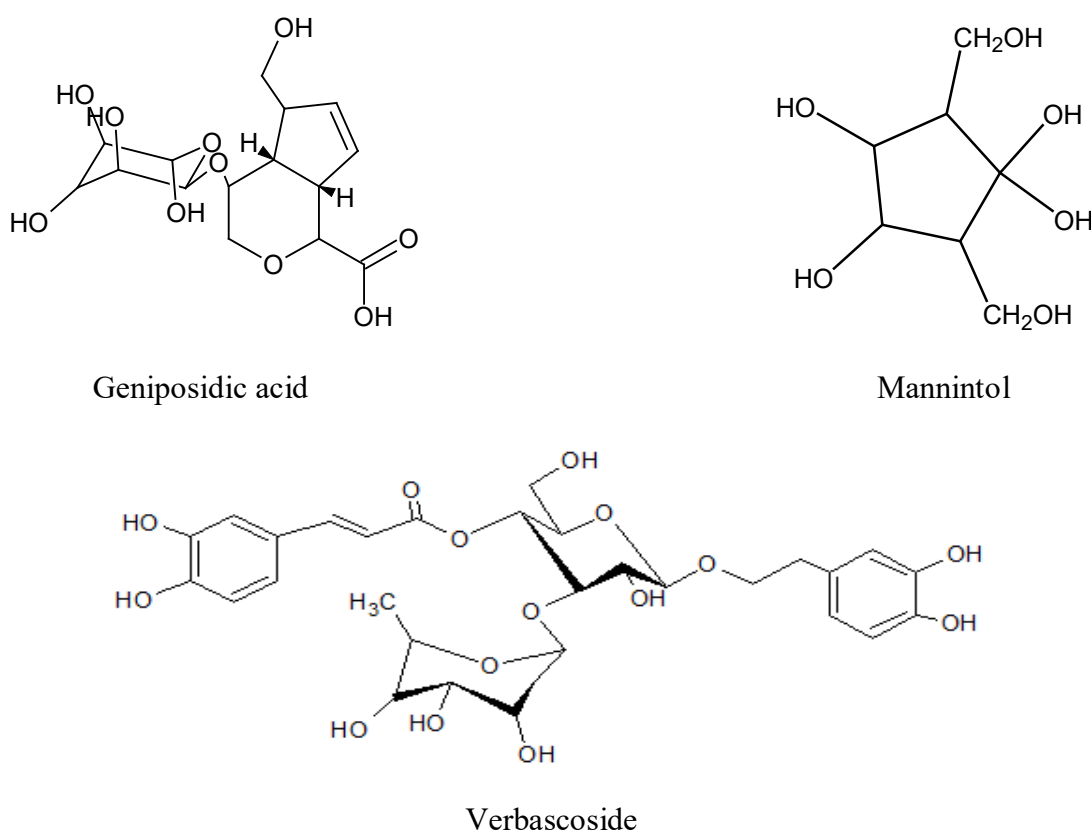


Figure 12: Chemical structure of compounds isolated from *E. longifolia* ^[105]

Appendix Table 2B lists the volatile oils identified in *E. longifolia* in a past study completed in 2009. For this study, the plant samples were collected in different geographical locations and during different seasons throughout the year ^[94]. However, since this report, there has been no further research of the medicinal aspects associated with *E. longifolia* and therefore there are limited data on the biological and phytochemical properties.

In summary, *Eremophila* species are known to have a wide range of ethnomedical properties and the main secondary metabolites are divided into different classes of compounds. The phenolic compounds are mainly classified as flavonoids, lignins and phenylpropanoids, and enriched with sesqui- and di-terpenes. These phenolic and terpenoids compounds are the chemicals most responsible for the pharmacological properties of *Eremophila* extracts and they are liable for observed cardiotonic, antiinflammatory, antimicrobial and cytotoxic activities ^[85].

2.3 Southeast Asian plant collection from Sarawak, Province of Malaysia

The fifty plant species that were initially analysed in this research were collected between 2005 and 2007 from various locations in Sarawak, Malaysia. They are categorised according to their traditional role in healing practices and include treatments for sore throat, fever, conjunctivitis, eye-related diseases, diarrhoea, dysentery, enteritis, vaginomycosis, furunculosis, burns and much more.

2.3.1 Description, distribution, ethnomedicinal and phytochemical identities of

***Baccaurea lanceolata* (Miq.) Müll.Arg**

2.3.1.1 Description of *B. lanceolata*

This plant species belongs to the genus of *Baccaurea* (flowering plants), the family of Phyllanthaceae and is also placed in the family of Euphorbiaceae ^[106]. According to the

literature, diverse Malaysian tribal groups name this plant differently and these names are listed in Table A3 of the Appendix.

Plants of this species can grow up to 21 m tall with bear's thick skin and white fruits that grow off the trunk. The pulp is translucent white with a sour flavour ^[107] (Figure 13).

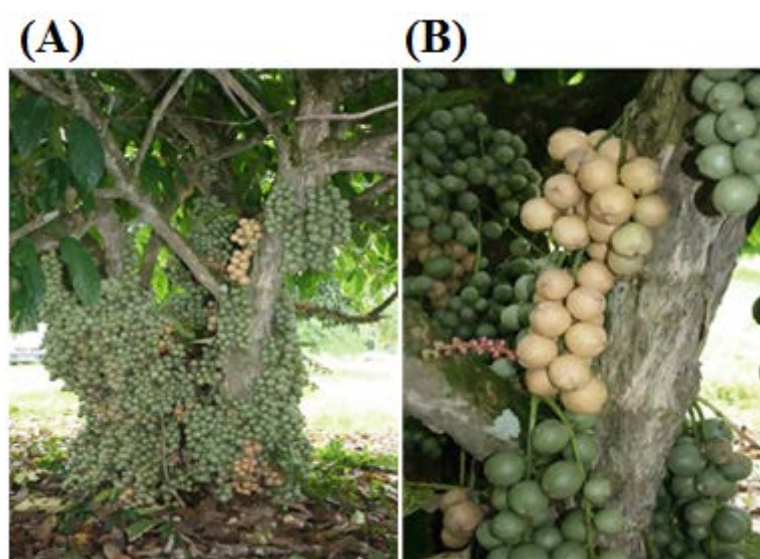


Figure 13: *B. lanceolata*

(A) Branched tree with large leaves and fruits; (B) Magnified image of (A) ^[108]

2.3.1.2 Distribution of *B. lanceolata*

Baccaurea lanceolata is commonly found in the Philippines, Thailand, Malaysian Peninsula, Sumatra, and Borneo (Sarawak, Brunei, Sabah, western, central, southern and eastern Kalimantan) ^[108].

2.3.1.3 Ethnomedicinal significance of *B. lanceolata*

According to the ethnomedicinal knowledge of the seniors from numerous ethnic groups in Borneo, Malaysia, this plant was commonly used against stomachache. It is prepared by

pounding leaves and fruits that were then mixed with water to be converted into a paste and applied to the painful areas of the abdomen ^[106].

The majority of the ethnomedical therapies that used this plant were dependent on the ethnic groups living in rural regions of Malaysia, for example:

- Kelabit: used to cure, alleviate stomachache; prepared using pounded leaves, which are added to a minimal amount of water to dissolve and consumed as a decoction.
- Bidayuh: a cataplasm of the fruit applied as a cream on the inflamed parts of the body.
- Penan: to avoid drunkenness; pounded bark mixed with water and drunk as decoction before consuming any alcohol.
- Iban: to protect against charms; during Gawai Dayak (festival in Sarawak, Malaysia) fruit is eaten before paying house visits ^[109].

According to the book published by Paul P. K. Chai, it has been mentioned that the Penan tribal groups utilised squeezed sap (soft inner bark) from *Baccaurea baccariana* Pax ex Hoffm and applied to the eye to cure eye diseases. Apart from the above-mentioned ethnic groups, some other communities also used the inner bark juice of *Baccaurea macrophylla* Muell.-Arg for sore eyes and related infections ^[110]. The fruit pericarp and seed can be edible as it is but, if it is very sour, then it can be mixed with salt or sugar to overcome the sourness of the fruit. Sometimes the fruit is mixed with chicken rice and consumed as a meal. Other research has revealed that the Murut community in Sabah, Malaysia, also eats the aril of the fruit after it has ripened and the mesocarp is eaten after being converted into a pickle ^[98, 106].

2.3.1.4 Phytochemistry of *B. lanceolata*

Studies have discovered that some specific volatile flavonoids were isolated from *Baccaurea motleyana* (Muell. Arg.) Muell. Arg. (Rumba). After distillation of the dichloromethane extract

and analysis by GC-MS, they identified some sesquiterpene hydrocarbons and also high levels of methyl 2-hydroxy-3-methylbutanoate, methyl 2-hydroxy-3-methylpentanoate and methyl 2-hydroxy-4-pentanoate ^[111]. Additionally, three unknown and four known vanilloid derivative compounds were isolated from the stem of *Baccaurea ramiflora* (same genus). These compounds were confirmed by NMR, with the identified compounds being 4'-O-(6-O-vanilloyl)- β -D-glucopyranosyl tachioside D, 6'-O-vanilloyl picraquassioside D and 6'-O-vanilloylicarisode B ^[112]. Another study revealed there are no significant toxicological effects in rats when utilising 1200 mg/kg of *Baccaurea angulate* (Belimbing Dayak) plant extract as a decoction ^[113].

In a qualitative phytochemical survey carried out in 1989, it was discovered that an extract from the leaf of this plant species had constituents that contain alkaloids ^[114]. However, since this report, there has been no further research of the medicinal activities associated with *B. lanceolata* and, therefore, there are limited data on other biological and phytochemical properties.

2.3.2 Description, distribution, ethnomedicinal and phytochemical identities of *Fibraurea tinctoria* Lour

2.3.2.1 Description of *F. tinctoria*

This species belongs to the genus *Fibraurea* and the family Menispermaceae ^[115]. According to the literature, diverse Malaysian tribal groups have different names for this plant as listed in Table A4 (Appendix) and no common/English name is available.

F. tinctoria is a large, woody, climbing vine, that grows up to 40 m high with a stem diameter of up to 5 cm ^[116] and yellow coloured wood ^[117] (Figures 14 and 15).



Figure 14: *F. tinctoria* leaves and fruit



Figure 15: *F. tinctoria* vine

(Sourced from: <https://www.flickr.com/photos/adaduitokla/6129211373/sizes/m/in/pool-680530@N20/>)

2.3.2.2 Distribution of *F. tinctoria*

This species is widespread in northeastern India, the Nicobar Islands, Burma, Thailand, Vietnam, eastern and southern China, Malaysia (Malaysian Peninsula, Sarawak and Sabah), Brunei, Indonesia and the Philippines. Mostly, this plant species inhabits native forests and is rarely cultivated in gardens or homes in Southeast Asia ^[118].

2.3.2.3 Ethnomedicinal significance of *F. tinctoria*

Ethnomedicinal properties attributed to this plant are diverse depending on the ethnic group living in the region where the plant is found:

- Iban: boil slices of roots to make as a decoction to treat sexually transmitted diseases like gonorrhoea and to avoid drunkenness or morning sickness during pregnancy; a decoction of the stem is consumed as a remedy for various sicknesses and especially for food poisoning and paralysis; a cataplasm of the stem or bark can be applied to snake bites related infections ^[109].
- Penan: fresh juice from the stem is taken to cure poisonous bites from snakes, centipedes and scorpions.
- Penan Totoh: decoctions from boiled roots are consumed as a tea to cure stomachaches.
- Kayan: roots are boiled until they become concentrated and taken as a tea to cure urine infections and mitigate diarrhoea ^[109].

F. tinctoria stem and roots are very popular among these ethnic groups as a dye-producing agent (yellow dye) ^[116].

2.3.2.4 Phytochemistry of *F. tinctoria*

Some alkaloids have been isolated from extracts of the root bark and stem of *Fibraurea chloroleuca* Mier and are known as magnoflorine, pseudocolumbamine, dehydrocorydalmine and palmatrubine. In addition, further alkaloids were documented as barbarians and berberrubine ^[119]. Phytotherapy research produced some interesting results regarding the inhibition of cytochrome P3A4 with secondary metabolites from *F. tinctoria* (CH₃OH extract) exhibiting an IC₅₀ of 5.1 µg/mL ^[120]. It was also found that the non-polar portion of the CH₃OH extract had an inhibitory effect, with an IC₅₀ value of 3.4 µg/mL.

Another study revealed *F. tinctoria* Lour as having an IC₅₀ value ranging between from 0.4 to 8.6 µg/mL for selectivity towards *Plasmodium falciparum* in association to their cytotoxic effects compared to the human cell lines ^[14]. Phytochemical studies on this plant resulted in the isolation of several chemical constituents, including proto-berberine alkaloids and furanodi terpenoids ^[116]. Further analysis revealed that berberrubine, fibleucin, fibraurin, 6-hydroxyfibraurin, palmatrubine, pseudojatrorrhizine, carboxyfibleucin, chasmatine, colombamine, jatrorrhizine, magnoflorine and palmatine were also present in this plant ^[121].

2.3.3 Description, distribution, ethnomedicinal and phytochemical identities of

Goniothalamus tapisoides Mat Salleh

2.3.3.1 Description of *G. tapisoides*

This species belongs to the genus *Goniothalamus* and the family Annonaceae and is commonly known as Selada or Semukau ^[122]. *G. tapisoides* is a small tree of about 5 m in height ^[123] (Figure 16).



Figure 16: *G. tapisoides* K. Mat-Salleh, from Lambir Hills National Park

(Sourced from: <https://www.flickr.com/photos/kmatsalleh/2558119374/>)

2.3.3.2 Distribution of *G. tapisoides*

This plant species is predominately distributed throughout the island of Borneo, Northeast India, Myanmar, Thailand, Vietnam, Sumatra, Malaysian Peninsular, Singapore, Java, Northeast Celebes and the Philippines ^[124].

2.3.3.3 Ethnomedicinal significance of *G. tapisoides*

Various ethnic groups have pounded the leaf and bark, which was then rubbed into the infected area of wounds due to snake and insect bites ^[125].

2.3.3.4 Phytochemistry of *G. tapisoides*

Previous phytochemical studies on this species resulted in the isolation of various essential oils. However, the essential oil from the leaf and root of *G. tapisoides* is comprised almost entirely of the monoterpenoid 1, 8-cineole ^[123]. In 2012, the researchers published data about 11 more compounds isolated from the stem and bark of this species. They were classified as (1) goniomicin A, (2) goniomicin B, (3) goniomicin C, (4) goniomicin D, (5) tapisoidin, (6) goniothalamine, (7) 9-deoxygoniopypyrone, (8) pterodondiol, (9) liriodenine, (10) benzamide and (11) cinnamic acid as shown in Figure 17. Goniothalamine has displayed some mild cytotoxic effects against colon cancer cells (HT-29) and goniomicin B exhibited specific antioxidant properties ^[122].

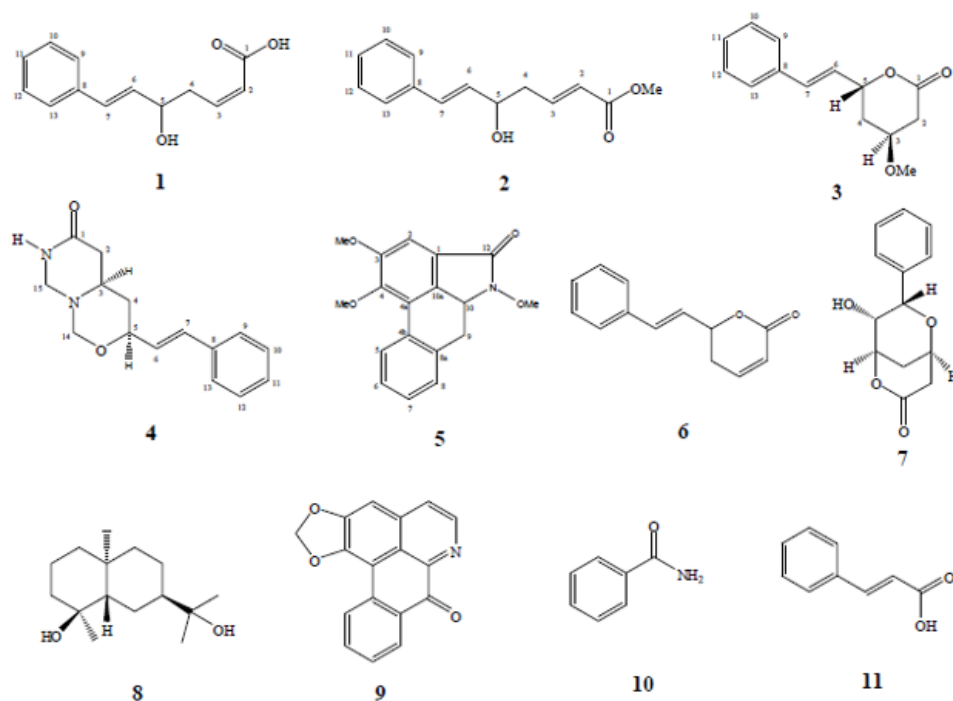


Figure 17: Chemical structures of the compounds identified from *G. tapisoides* ^[122]

2.3.4 Description, distribution, ethnomedicinal and phytochemical identities of *Goniiothalamus velutinus* Airy Shaw

2.3.4.1 Description of *G. velutinus*

This species belongs to the genus *Goniiothalamus* and the family Annonaceae ^[124]. Commonly, it is known as Lim panas (Iban), Kayo ujan panas (Kenyah) and Lakum ^[109]. *G. velutinus* is a small tree and can reach up to 3 m in height with a stem that is 3 cm in diameter ^[81] (Figure 18).



Figure 18: *G. velutinus* Airy Shaw

(Sourced from: Borneo Post, 2014) Photo from Sarawak Biodiversity Centre)

2.3.4.2 Distribution *G. velutinus*

G. velutinus is distributed throughout Southeast Asia and Malaysia ^[126].

2.3.4.3 Ethnomedicinal identities of *G. velutinus*

Various ethnic groups used the twigs as a mosquito repellent. The Iban ethnic group use a decoction from boiled shoots that is consumed as a treatment to alleviate fever and the pounded stem is applied to remedy snake bite related infections ^[81]. The Penan ethnic group use hanging branches of the plant on each corner of the house as protection from spirits ^[109].

2.3.4.4 Phytochemistry of *G. velutinus*

Research conducted in late 1992 showed this species to be rich in phenanthrene lactams (cytotoxic alkaloids) such as velutinam (10-amino-8-hydroxy-3, 4-dimethoxyphenanthrene-1-carboxylic acid lactam) and aristolactam-BII ^[127]. A more recent study has shown that alkaloids such as goniopedaline, aristololactam AII and goniotalactam are produced by *G. velutinus*

^[128]. In 2010, further phytochemical characterisation was completed with another three alkaloids being identified (pinocembrine, goniothalamine, and naringenin) ^[126]. It was also discovered in this study that goniothalamine can have a significant inhibitory effect (98%) against the measles virus ($LC_{50}=0.1 \mu\text{g/mL}$).

2.3.5 Description, distribution, ethnomedicinal and phytochemical identities of *Polyalthia hookeriana* King

2.3.5.1 Description of *P. hookeriana*

This species belongs to the genus *Polyalthia* and the family Annonaceae ^[124] and it is a small tree that grows between 15 to 25 m high with hanging branches, dark green leaves and yellowish red colour flowers ^[129] (Figure 19).



Figure 19: *P. hookeriana* branch

(Sourced from: http://plantsofasia.com/index/polyalthia_hookeriana/0-894)

2.3.5.2 Distribution of *P. hookeriana*

P. hookeriana is native to Malaysia and Singapore, it is found mainly in the lowland and sub montane forests ^[130].

2.3.5.3 Ethnomedicinal significance of *P. hookeriana*

Leaf and stem of this plant are used to cure sore eye-related infections ^[109].

2.3.5.4 Phytochemistry of *P. hookeriana*

There is no known scientifically chemical characterisation or identification available on this plant.

2.3.6 Description, distribution, ethnomedicinal and phytochemical identities of *Pyrenaria serrata* Bl. var *masocarpa* (Korth) H. Keng

2.3.6.1 Description of *P. serrata*.

The genus *Pyrenaria* belongs to the family Theaceae and the plant is commonly known as Kayuh Lilui ^[124]. Species within the genus are distinguished as shrubs or small trees (Figure 20).

2.3.6.2 Distribution of *P. serrata*.

The plant commonly grows in most areas of India, Asia, America, a few places in Africa ^[112] and is native to Java and Sumatra islands.

2.3.6.3 Ethnomedicinal significance of *P. serrata*.

Raw fruits are consumed as a remedy for coughing.

2.3.6.4 Phytochemical identities of *P. serrata*.

There is no known chemical analysis data available for this plant.



Figure 20: *P. serrata* branch with fruits

2.4 Isolation and characterisation of bioactive compounds from plant materials.

Plants have been the most fertile source for the discovery of novel compounds with potential application in drug discovery and development. Application of plant-based natural products in medicine has been practiced for many millennia. Natural products have been used against a multitude of pathogens and it is, yet still true that almost half of the novel therapeutic drugs approved by the US Food and Drug Administration since 1994 were plant-based natural products ^[131].

Despite all the benefits of natural plant-based extracts, large pharmaceutical companies have decreased their screening processes and it is mainly because of inadequate provision of plant materials ^[132]. The isolation and characterisation of plant based natural products have been the most challenging tasks in drug innovation.

Physiological and biochemical research often requires the production of extracts from plant tissues that are purified and then chemically separated before further analysis. However, for this purpose, the amount of starting material can be restricted and, therefore, purification and separation needs to be undertaken carefully. According to research published in 1966, more

than 50 years ago, amino acids extracted from plant leaves were obtained in two separate steps. The plant extract purification was performed using liquid-liquid extraction and the separation was performed with thin-layer high-voltage electrophoresis. The amino acid identification was then confirmed by ninhydrin or autoradiography ^[133]. However, these days there are many novel and advanced techniques used which include extraction, separation, identification and characterisation ^[134] and these techniques will be discussed in later chapters.

2.4.1 Extraction methods

Extraction is the first step for novel drug discovery with the mining of essential compounds from a complex sample depending upon the solvent used for the extraction. There are a number of extraction methods where the choice depends on the form of the sample and the nature of the targeted natural products.

2.4.1.1 Conventional liquid immersion extraction method

This is a form of solid-liquid extraction and this process is the most commonly used technique. The solvent selection is based on the compound of interest where it is usual to match the polarity of the solvent with the polarity of the target molecules. In this method, a homogenised solid sample is immersed into the selected solvent for a given time at room temperature ^[135]. The main disadvantage of this extraction method is that it is extremely time consuming compared to other methods which will be discussed later.

2.4.1.2 Accelerated Solvent extraction (ASE)

An automated form of solid-liquid extraction is the Accelerated Solvent Extraction technique and is useful for extracting semi volatile organic compounds ^[136]. The temperature and pressure are the two parameters that can be adjusted with the temperature range usually 50°C to 200°C

and the pressure ramped between 10 MPa to 15 MPa. Once again the choice of solvent dictates the compounds that are extracted but the range of polarities and efficiencies are much greater than the conventional immersion method. The ASE extraction method is very fast and extraction process can be completed within 15 to 20 minutes ^[137]. The disadvantage of this process is the potential degradation of thermolabile compounds at higher temperatures. However, the high efficiency of this extraction method can also extract unwanted compounds as well, which can impact on separation and further isolation.

2.4.1.3 Liquid-liquid extraction (LLE)

Most commonly, this technique is used to extract polyphenols and non-complex phenolic compounds from aqueous plant samples, such as juices or decoctions. This process is based on the relative solubilities of a solute in two different immiscible solvents, most commonly water (polar) and an organic solvent (non polar). Dichloromethane, diethyl ether, chloroform and ethyl acetate are the main organic solvents utilised in this operation. Researchers have widely used this technique because of its simplicity and its efficiency ^[138-139]. The main disadvantages of this method are due to it being labor intensive and time consuming. It also needs relatively large sample volumes and large amounts of ultra-pure, often toxic, organic solvents. Extraction efficiency can be influenced due to the formation of emulsions between the layers, particularly if there is a large amount of fine plant material present ^[140-141].

2.4.1.4 Soxhlet extraction

This technique is a continuous form of solid-liquid extraction with acetonitrile and water commonly used as solvents where the reflux process time is about 12 to 48 hours. The apparatus is shown in Figure 21. The disadvantages for this method is the processing time and large

solvent consumption ^[142]. Also, this extraction technique requires a significant amount sample of up to 30 g ^[143].

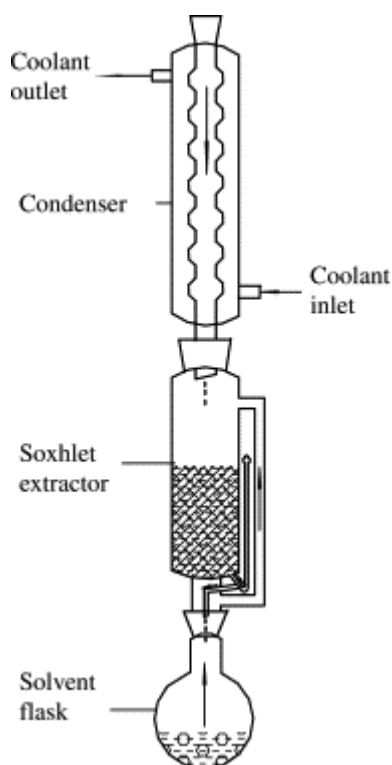


Figure 21: Soxhlet extraction ^[144]

2.4.1.5 Supercritical fluid extraction (SFE)

SFE (Figure 22) has been discussed as the most effective and efficient technique to extract the desirable compound from plants. The extraction process separates one compound from another in a combination of supercritical fluids and it provides a relatively clean extract. Carbon dioxide is commonly utilised as a supercritical fluid. The critical set temperature of the extraction is normally 31°C and required pressure is 74 bar. The normal processing time for the experiment is 10 to 60 minutes ^[145]. This process can be employed especially for thermolabile compound extraction.

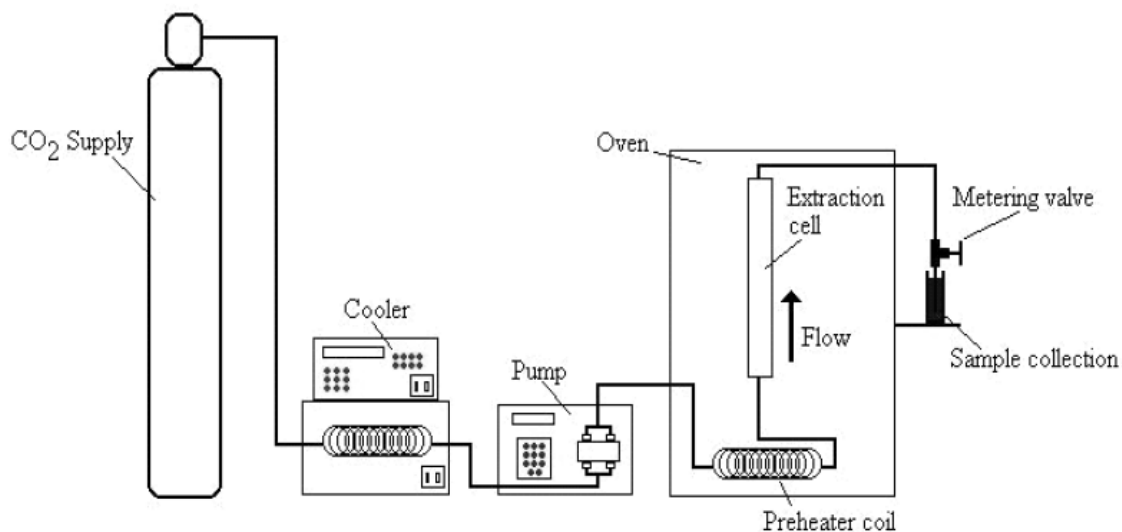


Figure 22: Supercritical fluid extraction (SFE) apparatus ^[145]

2.4.1.6 Microwave assisted extraction (MAE)

The microwave assisted extraction technique is super-fast and is easy to use. The range of the microwave frequencies is between 0.3 to 300 GHz and the adjustable temperature range between 50°C to 200°C, which can be adjusted to the method requirement ^[146]. This technique is especially useful for polar compound extraction as microwave radiation is best absorbed by polar molecules to create heat, which is then transferred throughout the sample matrix ^[147].

2.4.2 Clean up and isolation methods

2.4.2.1 Solid phase extraction (SFE)

Solid phase extraction (Figure 23) is an extensively used sample preparation method that has developed in recent decades. According to literature, it has been reported that most scientists used the SPE technique as the preliminary purifying step when processing complex sample mixtures and it is a useful technique for prompt and selective sample preparation ^[148]. It assists to purify the analytes from the sample solution by sorption on to a solid sorbent and enables the isolation of selected analytes from the non-adsorbed matrix. An advantage is if a smaller

volume is used to elute the analytes compared to the sample volume as an increase in concentration can be achieved, this is also known as preconcentration. This technique is mainly focused on three aspects of the sample preparation that includes preconcentration, sample clean up and matrix exchange. SPE is the most common extraction method used in many research areas such as environmental, clinical, food and industrial chemistry ^[149].

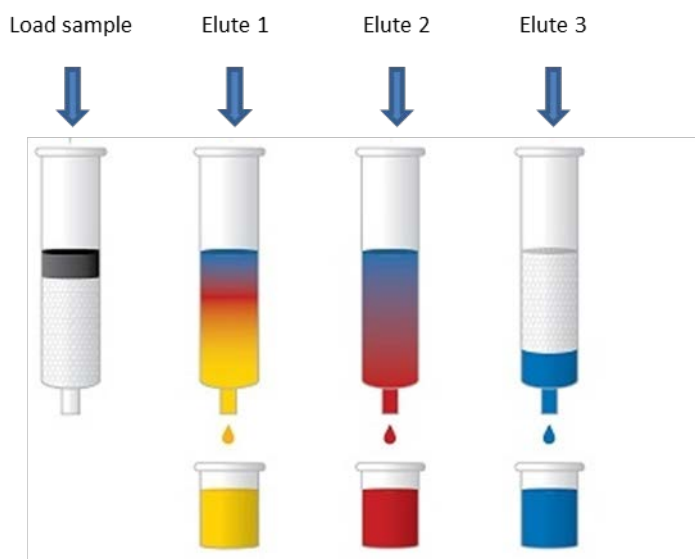


Figure 23: Solid Phase Extraction method

(Sourced from: http://www.waters.com/waters/en_AU/Solid-Phase-Extraction-SPE-Guide/nav.htm?cid=134721476&locale=en_AU)

The extraction process is predominantly dependent on the retention index of each target solute in the sample mixture and the characteristics of the solid particles that are used as the stationary phase of the cartridge. Retention of the solutes in the stationary phase depends on partitioning between the mobile phase and the stationary phase ^[150]. Selection of the type of SPE extraction cartridge mainly depends on the physical and chemical properties of the analyte, with the main criteria based on the functional groups present in the analytes and the interactions with the chosen sorbent ^[150].

The extraction efficiency is strongly influenced by the flow rate of the eluent, the sample loading capacity of the separation cartridge and reproducibility of the separation of the solutes

of the sample mixture ^[151]. Traditional SPE sorbents are available as large particles of silica, polymeric and resin materials packed in syringes or cartridges. The wide range of sorbents provides various applications for various classes of drugs, including ionic and highly polar compounds which are difficult to extract by traditional LLE methods ^[152].

It is considered as digital chromatography as the short format ensures that solutes are either adsorbed or eluted in a solvent that matches the polarity and the migration through the stationary phase is minimal ^[153]. It has replaced liquid-liquid extraction as the most common extraction method in many research disciplines such as environmental, clinical, food and industrial chemical analysis ^[154]. The SPE technique avoids the emulsion problems often encountered in LLE methods and achieves significant simultaneous preconcentration and purification ^[155].

2.4.3 Separation methods

2.4.3.1 Thin layer chromatography (TLC)

Thin layer chromatography is one of the oldest methods utilised beginning in the early 1960s. Silica is used as a stationary phase combined with 2-(diphenylboryl-oxy)ethylamine and poly ethylene glycol or AlCl_3 . Ultraviolet light is used for detection and the selected wavelength range is between 350 - 365nm or 250 - 260nm ^[147, 156]. In biochemistry, including nucleic acid analysis, the TLC separation technique is used extensively. The method involves spotting test compounds onto a TLC plate where the plate is developed with a suitable solvent or mixed solvent and visualization of the migrated spots possible due to their color, the quenching of fluorescence or the use of chemical developing reagents.. Retention factors (R_f values) are calculated from migration distances and individual standards are used for comparisons with the unknowns ^[157].

2.4.3.2 Gas chromatography (GC)

GC is a very commonly used analysis technique in analytical chemistry for volatile analyte separation in complex mixtures. The separation occurs according to chemical and physical properties of the analytes and their interaction with the stationary phase immobilized on the inside of a long tube or column. Each analyte elutes out of the column at different times and this is called the retention time. The retention time is unique for specific analytes under a specific set of conditions and can be used to identify the analytes when compared with a standard. Inert gases such as hydrogen, helium and nitrogen are used as the carrier gas with the choice depending on the type of detector that is employed.

2.4.3.3 High Performance Liquid Chromatography (HPLC)

HPLC is used as a separation and identification technique for high boiling point, non volatile compounds. The technique is well established and is highly reliable for the separation of complex mixtures. It is acceptable for pharmaceutical quality control and is widely used as an analytical tool. The separating column must have sufficiently high sample capacity in addition to high efficiency and this has led to the development of a wide range of columns sizes that suit different applications. Furthermore, the column packing or stationary phase has a number of surface chemistries that can be selected for a particular set of analytes. The nature of the stationary phase influences the selection of solvents or mobile phase that are used as the partitioning of the analyte is dependent on this combination. As the majority of analytes are relatively non polar organic molecules then the most appropriate mode is reversed-phase chromatography (also named as RPC, reverse-phase chromatography or hydrophobic chromatography) where the stationary phase is more non polar than the mobile phase. Therefore, the main column in use consists of high purity silica with a bonded phase of

monomeric C18 offers, which offers classical reversed-phase selectivity. The hydrophobic stationary phase can retain most organic analytes as a result of hydrophobic interactions ^[158]. It is also compatible with water as a mobile phase in combination with less polar solvents such as CH₃CN and MeOH. The composition of the mixed mobile phase can be adjusted at highly controlled rates to improve chromatographic separation performance.

2.4.4 Identification by spectroscopy methods

2.4.4.1 Mass Spectroscopy

2.4.4.1.1 Gas chromatography–mass spectrometry (GC-MS)

GC-MS is a hyphenated analytical technique that combines with GC with mass spectrometry to separate and characterise unknown analytes in complex mixtures that includes drug detection in blood samples, explosive/fire investigations and environmental sample analysis. The main disadvantage of GC-MS is that thermal degradation of analytes can occur due to the high temperatures that are required to transform liquid samples into a gaseous state in the injection port. It is also difficult to analyse polar compounds due to their high boiling points but this can be overcome with chemical derivatisation of the polar functional groups. The main advantage of GC-MS is the availability of mass spectral reference libraries due to the instrument independence of the molecular fragmentation during the ionisation process.

2.4.4.1.2 Liquid chromatography–mass spectrometry (LC-MS)

LC-MS is also a hyphenated combination of physical analyte separation with mass spectroscopy capabilities and is an ideal merger of separation and detection. Unlike GC-MS, the ionisation process for LC-MS is softer and molecular ions are more favored due to less fragmentation. Therefore, the LC-MS combination provides molecular mass and structural information, when multi stage MS-MS is employed, with accurate quantitative analysis of the analyte. Recent improvements have resulted in highly accurate mass detection with molecular

masses able to be determined to ppm levels using high resolution instruments. This technique can be used to analyse organic, inorganic and biochemical compounds found in environmental and biological samples. It therefore finds use in cosmetic, agrochemical, pharmaceutical, food processing and biotechnology industries to analyse samples.

2.4.4.2 Nuclear magnetic resonance spectroscopy (NMR)

NMR is a prominent spectroscopic technique used in research to obtain physical, chemical, electronic and structural information for a molecule due to chemical shifts as a result of interactions with a large external magnetic field.

2.4.4.2.1 Proton nuclear magnetic resonance (proton NMR, hydrogen-1 NMR, or ^1H NMR)

The ^1H NMR spectra records chemical shifts and coupling constants including quantitative relationship information about inter molecular and intra molecular resonances of the protons within a molecule ^[159].

2.4.4.2.2 Carbon-13 nuclear magnetic resonance (^{13}C)

The ^{13}C technique is an application of NMR that responds to the carbon-13 isotope, which has a natural abundance of approximately 1%. The sensitivity is much less than proton NMR but this can be overcome by averaging multiple scans, which results in longer experiment times. From ^{13}C , the number of carbons in a molecule can be identified.

2.4.4.2.3 Homonuclear correlation spectroscopy (COSY)

COSY is the most the popular form of two dimensional NMR spectroscopy and detects the coupling of spins between the H atoms in a molecule. It displays with both axes consisting of proton NMR spectra with the cross peak intensity indicating the extent of coupling.

2.4.4.2.4 Heteronuclear single-quantum correlation spectroscopy (HSQC)

The HSQC technique identifies single bond correlation between protons and carbon. The X axis normally represents the protons and Y-axis corresponds to the carbon atoms.

2.4.4.2.5 Heteronuclear multiple-bond correlation spectroscopy (HMBC)

HMBC also characterises the bonding correlations between carbon and proton. It can show correlations over two, three and, in a conjugated molecule, four bonds within a molecule. Correlation over one-bond is automatically suppressed by this method but is detected using HSQC.

2.5 Conclusions

This study focuses on the antimicrobial activity of bioactive compounds of selected plant or herbal extracts that are effective as medicines and have usually originated in indigenous communities with their side effects being continuously monitored over many centuries based on experiential knowledge. Unfortunately, this important indigenous knowledge is slowly being lost due to habitat changes (civilization), industrialisation (e.g. Palm oil plantations in Malaysia) and the deforestation of their native lands ^[160]. Specifically, the experiments in this research are aimed at identifying the source of the antimicrobial and antifungal activities of these selected extracts based on the knowledge of their usage by the indigenous people of Australia and the Malaysian State of Sarawak in Borneo. In selected cases, these plants will be

further tested to obtain chemical identification for the compounds that are responsible for inhibition of the observed bioactivity.

Chapter 3

Materials, methods and method development

3.1 Introduction

This chapter primarily describes the techniques applied to obtain crude plant extracts from one Australian species *E. longifolia* F. Muell and fifty Southeast Asian species provided by the SBC. It then presents information about six of the most noteworthy extracts interacting with pathogens of Gram negative (four species) and Gram positive (three species) bacterial groups, together with the fungal pathogen *C. albicans*. These were selected for assessing antibacterial and antifungal inhibitory activities of the plant extracts. It continues by explaining general aspects of the techniques used to perform the relevant assessments of the antibacterial and antifungal activities. This includes Disc Diffusion Assay (DDA), along with Minimum Bacterial Inhibitory Concentration (MIC), Minimum Fungal Inhibitory Concentration (MFC), Minimum Bactericidal Concentration (MBC) and Minimum Fungicidal Concentration (MFC) assays.

The chapter concludes with a brief description of extraction (Solid Phase Extraction, SPE), separation (High Performance Liquid Chromatography, HPLC), and identification (Liquid Chromatography–Mass Spectrometry, LC-MS; Gas Chromatography–Mass Spectrometry, GC-MS; and Nuclear Magnetic Resonance spectroscopy, NMR) techniques that were used to isolate, identify and characterise the bioactive plant compounds responsible for the inhibition of the tested pathogens.

3.2 Plants materials used in this study

The plants collected for this study were from two geographically distant locations with different climates. *E. longifolia* F. Muell was collected from near Burke in NSW, Australia, and botanically authenticated by Dr. Robert J. Chinnock (State Herbarium, Adelaide, South Australia with voucher specimen number AD99732199); Canopus BioPharma Inc. (Byrock,

New South Wales, Australia) supplied the plant material. For a brief discussion of this plant, see Section 2.2.1.

In addition, the Sarawak Biodiversity Centre (SBC) in Sarawak, Malaysia, collected fifty ethnomedicinally important plant species; the information about these plants was recorded for the Traditional Knowledge Documentation program carried out by the SBC in Sarawak Malaysia. For further information on all plants presented here, see Section 2.3

3.3 Methods used in this study

3.3.1 Preparation of Australian crude plant extract

E. longifolia leaves were separated and dried for 7 days at 60 °C in an incubator to reduce the moisture content and then the leaves were converted into a very fine powder using a commercial grinder. A mass of 10.25 g of powdered leaves was placed in 50 mL of CH₃CN at 25 °C for 5 days with occasional stirring. The extract was then filtered through a 0.45 µm nylon filter (Grace Part number N14745) and placed in the Speed Vac (RVC-2-18, CHRIST) at 40 °C until all the solvent had evaporated, which was typically a few hours. The dried plant extract was kept in a -20 °C freezer until required for further experimental analysis.

3.3.2 Preparation of the Southeast Asian plant extracts

A selection of 50 plant extracts were obtained from the SBC of which some have been described in Section 2.3. The plants extract were prepared by the SBC using a method where the plant material (i.e. leaves, fruits and/or stems) were dried for 3 days at 45 °C in an incubator to minimise the moisture content and then were converted into a very fine powder using a commercial grinder. Then 6 g of each powder sample was soaked in 60 mL of a dichloromethane-methanol mixture (1:1, v/v) overnight in an orbital shaker. The extracts were

then filtered through a 0.45 µm nylon filter with the filtrate placed in a rotary evaporator at 40 °C until all the solvent had evaporated. The concentrated plant extracts were then re-suspended in dimethyl sulphoxide (DMSO, Merck) at a concentration of 400 µg/mL prior to the evaluation of *in vitro* antimicrobial and antifungal activities (see Section 3.3.4) and the remaining extracts were stored in a -20 °C freezer until required for further analysis.

3.3.3 Bacterial strains and media

Four Gram negative bacteria (*Escherichia coli*, *Pseudomonas aeruginosa*, *Salmonella enterica* serovar Typhimurium and *Moraxella catarrhalis*), three Gram positive bacteria (*Bacillus cereus*, *Staphylococcus aureus* and *Streptococcus pyogenes*) and one fungus (*Candida albicans*) were used to test the plant extracts. Cultures were collected from the microbiology laboratory at Swinburne University of Technology (Melbourne, Australia) and used for the screening of extracts from the fifty plant species from Sarawak, Malaysia. From this, six plant species showed significant activity against above mentioned bacteria and fungi. All microorganisms were selected with respect to their abilities to cause various types of infections in the susceptible hosts (Table 1) ^[161-164].

All bacteria were grown on brain heart infusion (BHI) agar (Oxoid) slants. Overnight cultures were prepared by inoculating a loopful of the agar slant cultures into 2 mL of nutrient broth (NA) (Oxoid). All bacterial cultures were incubated at 37 °C overnight under aerobic conditions, with the exception of *M. catarrhalis*, which was incubated at 30 °C for 48 hrs. The fungus was cultured in potato dextrose broth (PDB) medium (Difco) and incubated at 30 °C for 48 hrs.

Table 1: The most frequently occurring types of infections caused by the test pathogens

Micro organisms	Group	Species	Types of infections									
			BI	BSI	CNS i	GI	LTI	OI	OM	RTI	SSSI	UTI
Bacteria	GNB	<i>E. coli</i> (ATCC 25922)		+		+						+
		<i>M. catarrhalis</i> (ATCC 23246)			+				+	+		
		<i>P. aeruginosa</i> (ATCC 10145)								+	+	+
		<i>S. enterica</i> (ATCC 13311)				+						
	GPB	<i>B. cereus</i> (ATCC 11778)				+						
		<i>S. aureus</i> (ATCC 12600)	+	+						+		
		<i>S. pyogenes</i> (ACM 178)					+			+	+	
Fungi		<i>C. albicans</i> (FRR5580, DAY286) and (SC 5314)		+				+				+

Gram-positive bacteria (GPB); Gram negative bacteria (GNB); bone infection (BI); blood-stream infection (BSI); central nervous system infection (CNSi); gastrointestinal infection (GI); life-threatening infection (LTI); opportunistic infection (OI); middle ear infection, i.e. otitis media (OM); respiratory tract infections (RTI); skin and skin structure infection (SSSI) and urinary tract infection (UTI).

3.3.4 Bioassays

3.3.4.1 Disc diffusion assay

The disc diffusion assay involved inoculating a bacterial or fungal broth suspension (adjusted to match a 0.5 McFarland turbidity standard) onto the surface of Mueller-Hinton agar (MHA) (Oxoid) plates using sterile cotton-tipped swabs. The plates were dried for 2-3 min and then pre-sterilised discs (6 mm in diameter) were placed on the agar surface. Subsequently, 5 μ L of the selected plant extracts (400 μ g/mL) were added individually onto discs that were pre-wetted with 5 μ L of DMSO. Generally, the seeded plates were incubated at 37 °C for 24 hrs. The exceptions being *M. catarrhalis* where nutrient agar (Oxoid) and *C. albicans* where potato dextrose agar (PDA) (Difco) plates were used; these were incubated at 30 °C for 48 hrs. At the end of the incubation period, the diameters of the zones of inhibition were measured.

All assays were performed in triplicate and the mean zones of inhibition were recorded. Commercially available tetracycline (30 μ g) antibiotic discs were used as the antimicrobial positive control and freshly prepared standard amphotericin B (1.4 μ g) was utilised as the antifungal positive control. DMSO wetted discs were used as the negative control for all of these susceptibility testing assays. Pure acetonitrile and methanol that were used as extracting solvents showed no activity when included as solvent blanks.

3.3.4.2 Determination of minimum inhibitory concentration (MIC)

This method was performed by using the Mueller Hinton broth dilution method with doubling serial dilution (50, 25, 12.5, 6.25 and 3.13 μ g/mL using 100 μ g/mL as the starting concentrations) of plant extracts arrayed in triplicates into adjacent wells of 96-well microtitre plates to which freshly prepared bacterial and fungal inocula were added ^[165]. The plates were placed in a POLARSTAR Omega plate reader (Lab Tech) with the incubation temperature set

at 27 °C. The analysis was programmed for 200 cycles where absorbance readings (wavelength 600 nm) were measured in 53 time increments with linear shaking for 5 min before each reading for all the bacteria except *M. catarrhalis* where the number of cycles was 150 and *C. albicans* with 600 cycles. After the incubation period, the breakthrough concentration was established [166]. Pure acetonitrile and methanol that were used as extracting solvents showed no activity when included as solvent blanks.

3.3.4.3 Determination of minimum bactericidal and fungicidal concentrations (MBC/MFC)

The minimum bactericidal and fungicidal concentration is a plant extract that resulted in the killing of more than 99.9% of the microorganisms being tested [167]. The minimum bactericidal concentration (MBC) and minimum fungicidal concentration (MFC) of the plant extracts were determined by taking a loopful of the culture media from samples used in the MIC assays and streak inoculating MHA (to determine MBC) and PDA (to determine MFC). All plates were incubated at 37 °C for 24 hrs (bacteria) or 30 °C for 48 hrs (fungi). All the MBC/MFC and MIC assays were performed in triplicate. DMSO was used as a negative control, with tetracycline and amphotericin B used as positive controls for the antimicrobial and antifungal susceptibility assays, respectively. Pure acetonitrile and methanol that were used as extracting solvents showed no activity when included as solvent blanks.

3.3.5 Separation techniques

3.3.5.1 Solid Phase Extraction (SPE)

SPE has developed due to the need to simplify sample preparation by removing potentially troublesome interferents and produce a less complex sample for analysis. It is a small format

chromatography and, in this thesis, it was used to fractionate plant extracts by eluting with a mixed solvent that has the polarity altered by using different proportions of two solvents.

3.3.5.1.1 Primary fractionation

Commercially available SPE Giga cartridges (SPE column Phenomenex-Strata C18-e, 2 g/12 mL) were used as a preliminary separation column. The SPE separation was developed by using two different series (Table 2) of eluents to generate fractions. Prior to separation, the SPE cartridges were preconditioned with 20 mL of 100% acetonitrile (CH_3CN) followed by 20 mL of Milli-Q water. Fractionation was conducted using a series of different solvent mixtures listed in Table 2 and the volume of solvent mixture used for each fractionation was 20 mL.

Table 2: Solvent mixtures of acetonitrile and Milli-Q water (% v/v) used for fractionation in both series

	Series 1 % CH_3CN	Series 2 % CH_3CN
Fraction 1	0	0
Fraction 2	10	18
Fraction 3	25	33
Fraction 4	40	48
Fraction 5	55	63
Fraction 6	70	78
Fraction 7	85	100
Fraction 8	100	-

The total flow rate was 1 mL/min. Collection tubes were positioned beneath the SPE cartridge to collect the eluent that passed through the cartridge. Identification was performed using HPLC by comparing the retention time for each component ^[168].

A standard mixture was used to correlate the solvent composition of the eluting solvent for each fraction with the standard compound's log P values. It would be expected that relatively

polar compounds would be eluted in the earlier fractions and be isolated from the non-polar compounds. The extent of the isolation during the fractionation procedure would be revealed by the proportions of each compound in each fraction, which was measured by HPLC.

3.3.5.1.2 Secondary fractionation

Based on the results from the primary fractionation for the plant extracts, it was observed that further fractionation would be necessary and this was developed with the following strategy. For a particular fraction identified from the primary fractionation, instead of eluting with a 20 mL aliquot of the appropriate solvent, the elution solvent was added and collected in 1 mL increments. These fractions were also examined using HPLC.

3.3.5.2 Log P values

The polarity of a molecule can be described by the partition coefficient between water and octanol ^[169]. The log P value of a compound is a measure of the polarity that influences many biological processes, separation techniques, as well as drug absorption and distribution in the human body ^[170]. The log P value can be measured but the development of various algorithms that evaluate a molecular structure enables the log P to be estimated ^[171]. For all the compounds in the standard test mixture, the log P values were calculated by using ACD (Advance Chemistry Development) software (Figure 24). It follows that the greater the value for log P , the more non-polar or hydrophobic the molecule.

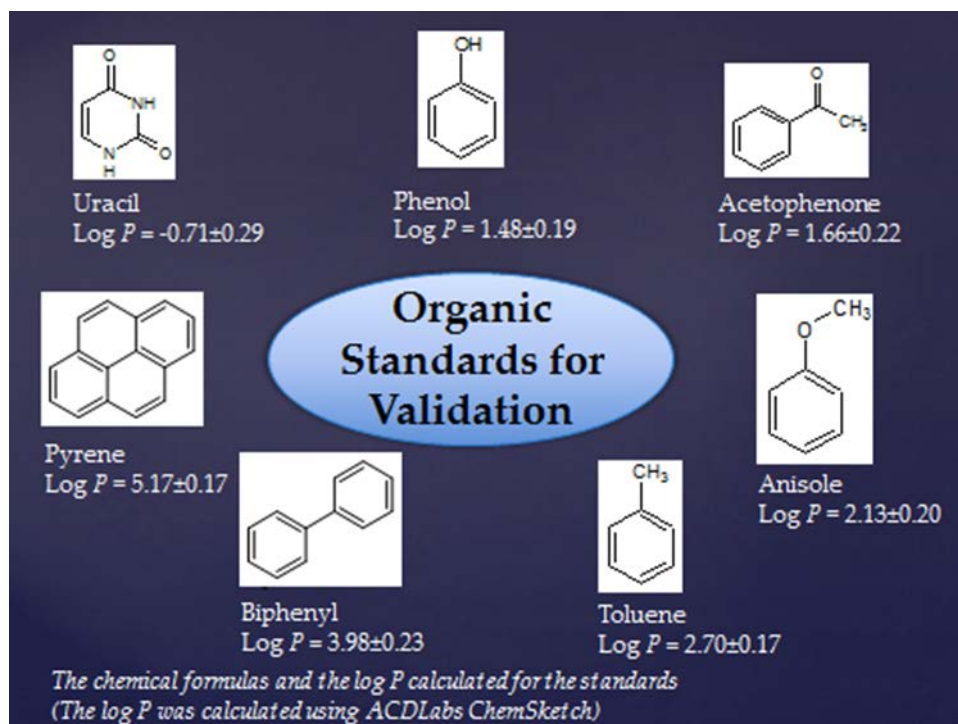


Figure 24: Standard compounds used to characterise the micro-scale fractionation

3.3.6 Analysis techniques - Separation and identification

3.3.6.1 Gas Chromatography-Mass Spectrometry analysis (GC-MS)

The GC-MS analyses were carried out on a Shimadzu GC Chromatograph GC 2010 Plus fitted with Zebron-ZB semi volatile GC capillary column (Phenomenex Australia Pty Ltd) with fused silica (low polarity cross bond silarylene phase, 30 m x 0.25 mm, i.d. 0.50 μ m) interfaced with a Shimadzu mass selective detector QP2010 (Shimadzu, Japan) operated by GC-MS Solution (version 2.61) software. The column oven temperature program used in this analysis was as follows: initially 70 °C for 2 min, followed by an increase of temperature to 300 °C (at a heating rate of 7.5 °C min⁻¹) that was then held for 4.50 min. The injector temperature was set at 230 °C with a split ratio of 75:1 and ultra-high purity helium was used as the carrier gas. The interface and ion source temperatures were maintained at 220 °C and 200 °C, respectively. The mass scan range was 50-600 amu. For non-derivatised samples, the solvent delay time was 2.5 min and the solvent delay time was 7.5 min to eliminate detector saturation from the solvents

in the derivatisation process. A sample volume of 1 μL was injected into the system and identification of the components was based on a comparison of the mass spectra with the Wiley Edition 8.0 Mass Spectral Database (Shimadzu, Japan). The identification of each compound was based on a combination of retention time and spectral matching (minimum of 80% in this case) using the Mass Spectral Database.

3.3.6.1.1 Derivatisation for the polar metabolites

Derivatisation in gas chromatographic analysis was necessary due to the high polarity of some of the compounds in the fractions and a trimethylsilyl reagent was used as the derivatisation agent to form trimethylsilyl adducts. Briefly, dried samples (30 ± 0.2 mg) of various fractions were solubilized in 100 μL of N, O-Bis (trimethylsilyl) trifluoroacetamide (BSTFA) containing 1% of trimethylchlorosilane (TMCS) followed by the addition of 200 μL pyridine (Sigma Aldrich) and homogenised. The samples were then placed in a Microwave reactor (CEM Discover) and 300 W power was applied for 1.50 min for the derivatisation reaction to occur. The samples were subjected to centrifugation and the supernatant was collected. Supernatant (in pyridine) was used for further GC-MS analysis.

3.3.6.2 Analytical High Performance Liquid Chromatography

Analyses were carried out using a Shimadzu HPLC consisting of a Prominence Modular High Performance Liquid Chromatograph coupled to an UV–Vis Photo-Diode Array detector (SPD-M20A). The analytical data were evaluated using the Lab Solution software data processing system (Shimadzu Corporation, Kyoto, Japan). The separation was achieved on a reversed phase chromatography column (Adsorbosphere XL C18, 5 μm , 150 mm x 2.1 mm) with an oven temperature of 35 $^{\circ}\text{C}$. The binary mobile phase consisted of (Reservoir A) 90:10 Milli-Q water with 1% glacial acetic acid and CH_3CN that was combined with a solvent that was

(Reservoir B) 10:90 of the same components. The following gradient run was employed: 0-15 min, 0%-100% B; 15-20 min, 100% B and 20-35 min, 0% B; an equilibration time of 15 min was part of the program. The flow rate was 1.0 mL/min and the injection volume was 10 μ L with the monitoring wavelength being 254 nm.

3.3.6.3 Liquid Chromatography-Mass Spectroscopy (LC-MS)

3.3.6.3.1 Analysis of *E. longifolia* extract

Initial collection of the bioactive compounds was achieved using a Gilson PLC 2020 personal purification system. The purity was analysed by using a Shimadzu Liquid chromatography–mass spectrometer (LC-MS) 2020 with a UV-vis detector (λ =200-400 nm). A Zorbax Eclipse XDB phenyl column (3.0 mm \times 100 mm, 3.5 μ m HPLC column was employed with flow rate of 1 mL/min and oven temperature of 40 °C. The eluents were 0.05% formic acid in water and 0.05% formic acid in CH₃CN. Further analysis was performed to obtain accurate masses using High-resolution mass spectrometry (HRMS) that was performed on a Bruker Micro TOF mass spectrometer using (-)ESI calibrated to sodium formate. This was undertaken at the Institute of Molecular Biosciences at the University of Queensland.

3.3.6.3.2 For Southeast Asian plants

3.3.6.3.2.1. Primary analysis

Analyses were carried out using a Shimadzu instrument consisting of a Prominence Modular Ultra High Performance Liquid Chromatograph (UPLC) coupled with an UV–Vis Photo-Diode Array detector (SPD-M20A). UPLC was used for analysis because of the limited volumes of the samples and the analytical data were evaluated using the Lab Solution software data processing system (Shimadzu Corporation, Kyoto, Japan). The separation was achieved on a reversed phase chromatography column (Vision HT C18, 1.5 μ m, 50 mm x 2 mm) with an

oven temperature of 35 °C. The binary mobile phase consisted of (Reservoir A) Milli-Q water with CH₃CN as the second solvent (Reservoir B). The following gradient run was employed: 0-15 min, 0%-100% B and an equilibration time of 10 min was used before the next injection. The flow rate was 0.2 mL/min and the injection volume was 2 µL with the monitoring wavelength being 254 nm. For further MS/MS analysis was performed using Varian MS-500 mass spectrometer (Varian Inc., Palo Alto, CA, USA) system equipped with an ion trap mass analyser having a mass range from 20 to 2000 amu was connected to the UPLC. Electrospray ionisation (ESI) was operated in negative and positive modes with the ion spray voltage set at -5000 V and 5000 V, respectively.

3.3.6.3.2.2. Secondary Analysis

Secondary analysis was performed by using a Thermofisher Ultra High Performance Liquid Chromatography system (UPLC) coupled with benchtop Q-Exactive HF Hybrid Quadrupole Orbitrap Mass Spectrometer (Thermofisher Scientific USA). Analytical data were evaluated using the Compound Discoverer software data processing system. The separation was achieved on a reversed phase chromatography column (Thermo Hypersil Gold C18, 1.9 µm, 50 mm x 2.1 mm) with an oven temperature of 40 °C. The binary mobile phase consisted of (Reservoir A) Milli-Q water with 0.1% formic acid and 100% CH₃CN with 0.1% formic acid as the second solvent (Reservoir B). The following gradient run was employed: 0-10 min., 2%-95% B, 10-12 min 95% B solvent and equilibration with 2% B time during 12-15 min. The flow rate was 0.2 mL/min. and the injection volume was 2 µL with the monitoring wavelength being 254 nm.

QE HF MS/MS parameters were Scan mode: Full MS/top 3 DDMS2, polarity: Positive and Negative switching, MS resolution Full mas 60,000 and DDMS2 12,000, Scan mass range m/z 120-1200, Collision energy Stepped 17, 35 and 52, Dynamic exclusion 10 sec and Apex trigger

2-5 sec. QE HF Ion source parameters consist of Sheath gas 45, Aux gas 10, Sweep gas 0, Spray voltage positive 3500 V and negative 3000 V, Capillary temperature 320 °C and Vaporizer temperature 350 °C.

3.3.6.4 Nuclear Magnetic Resonance (NMR)

A Bruker Avance-600M Hz NMR spectrometer was used to analyse the structure of the microbial active compounds from the plant *E. longifolia*. It was equipped with a Triple Resonance Probe (TXI cryoprobe) using standard pulse sequences with the resonances referenced with respect to Tetramethylsilane (TMS) (δ_H 0.00 and δ_C ppm). Samples were analysed by 1H (600 MHz) and ^{13}C (150 MHz) with $CDCl_3$ used as the solvent.

3.3.6.5 Statistical Analysis

All testing was completed in triplicate and the data are presented as mean \pm standard deviation (mean \pm SD). Statistical analysis was performed using an online statistical package MetaboAnalyst 3.0 (TMIC, Edmonton, Canada). Various correlation statistical analysis methods were used for evaluation of the data. Alternatively, the IBM SPSS Statistical package V25 was used for some of the earlier statistical analyses (Statistical Package for the Social Sciences in the USA).

3.4 Method development

3.4.1 Fractionation based on Solid Phase Extraction cartridges

SPE has been extensively used in sample preparation where routine use of this technique is as a preliminary purifying technique for complex sample mixtures prior to any analysis ^[148]. An advantage is that not only does this technique purify the target analyte from the sample mixture but also results in a more concentrated target due to volume differences between the original

sample and the eluting solvent. In the process of method development for the complex mixtures of compounds in the plant extracts, the SPE extraction technique became the main focus. This was after considering several separation techniques and it was selected as the most flexible, fast and cost-effective technique ^[172] to use as a primary and secondary fractionation platform.

3.4.2 Development of fractionation methods

3.4.2.1 Primary Solid Phase Extraction

The SPE method for the isolation/separation, purification and concentration of the plant extracts sample before further HPLC analyses was initially characterised using a set of test standards. The standards used were: Uracil (Sigma U0750-25 g), Phenol (Merck 6010188.05), Acetophenone (BDH-27030), Anisole (SigmaA4405-1 L), Toluene (Merck 10284), Biphenyl (Sigma/Aldrich B34656-25g) and Pyrene (Aldrich 18,551.5). All the standard compounds and their log *P* values are presented in Figure 24.

Uracil and phenol were dissolved in 100 mL of Milli-Q water and the remainder of the compounds were dissolved in HPLC gradient grade CH₃CN. The standard mixture was prepared by using 1 mL volumes of each standard listed in Table 3 and was diluted to 20 mL with Milli-Q water. The mass of each standard was adjusted so that the HPLC peak heights at an absorbance of 254 nm were approximately constant with the total mass being 100 mg.

Table 3: Mass of standard compounds used in the test mixture

Compound	Concentration (mg/mL)
Uracil	0.01
Phenol	0.38
Acetophenone	0.02
Anisole	0.20
Toluene	0.40
Biphenyl	0.01
Pyrene	0.02

Disposable syringe cartridge columns were used as the stationary phase and the standard test mixture was extracted using different proportions of CH₃CN and Milli-Q water used as the mobile phase to generate fractions. Elution occurred upon the addition of an appropriate organic solvent to disrupt the interactions between the sorbent and the solutes.

3.4.2.2 HPLC analysis

The standard test mixture and the collected fractions after SPE separation were analysed using HPLC as described in Section 3.3.6.2. The intention of using this standard mixture was to correlate the solvent composition of the eluting solvent for each fraction with the standard compound's log *P* values. It would be expected that relatively polar compounds would be eluted in the early fractions and to be isolated from the non-polar compounds. The extent of the isolation during the fractionation procedure would be revealed by the proportions of each compound in each fraction as measured by HPLC. The characterization of this fractionation procedure using standards would enable reasonable estimates of the log *P* values of the compounds in the plant extract based on the comparison of HPLC retention times ^[173].

3.4.3 Results and discussion

3.4.3.1 Primary Solid Phase Extraction of the standard test mixture

The fractions were analysed using analytical HPLC by comparison of the retention time for each individual compounds with the standard mixture before fractionation (Figure 25). All of the standards were eluted between 0.4 to 12 minutes, which corresponds to the time when the gradient was active. The most polar compound, uracil ($\log P = -0.77$), was eluted at 0.410 min and the least polar compound, pyrene ($\log P = 4.88$), eluted at 11.366 min.

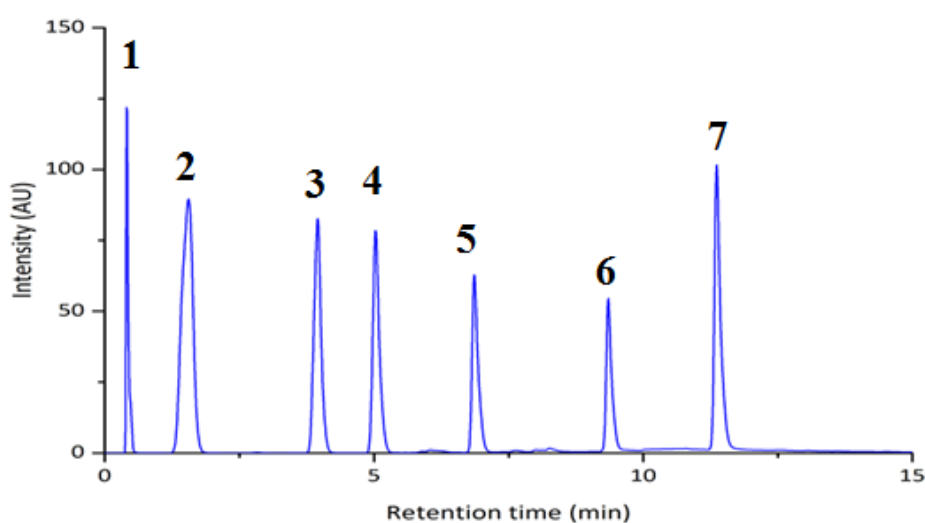


Figure 25: Analytical HPLC chromatogram of the standard mixture of compounds. Peaks: 1. Uracil, 2. Phenol, 3. Acetophenone, 4. Anisole, 5. Toluene, 6. Biphenyl and 7. Pyrene

Figures 26-27 demonstrate how effectively the SPE fractionation technique was able to fractionate the complex standard test mixture with the fractionation performed using two alternate series of CH_3CN and Milli-Q water concentration combinations as listed in Table 3. Uracil eluted in the first fraction as it is normally not retained on the reversed phase column or the SPE cartridge and is often used as an unretained void volume marker for C18 and C8 columns ^[174]. The low $\log P$ value could be easily correlated to the very polar compounds in the plant extracts and the retention of phenol demonstrates the practical lower limit of polarity where the reversed phase separation mechanism is applicable.

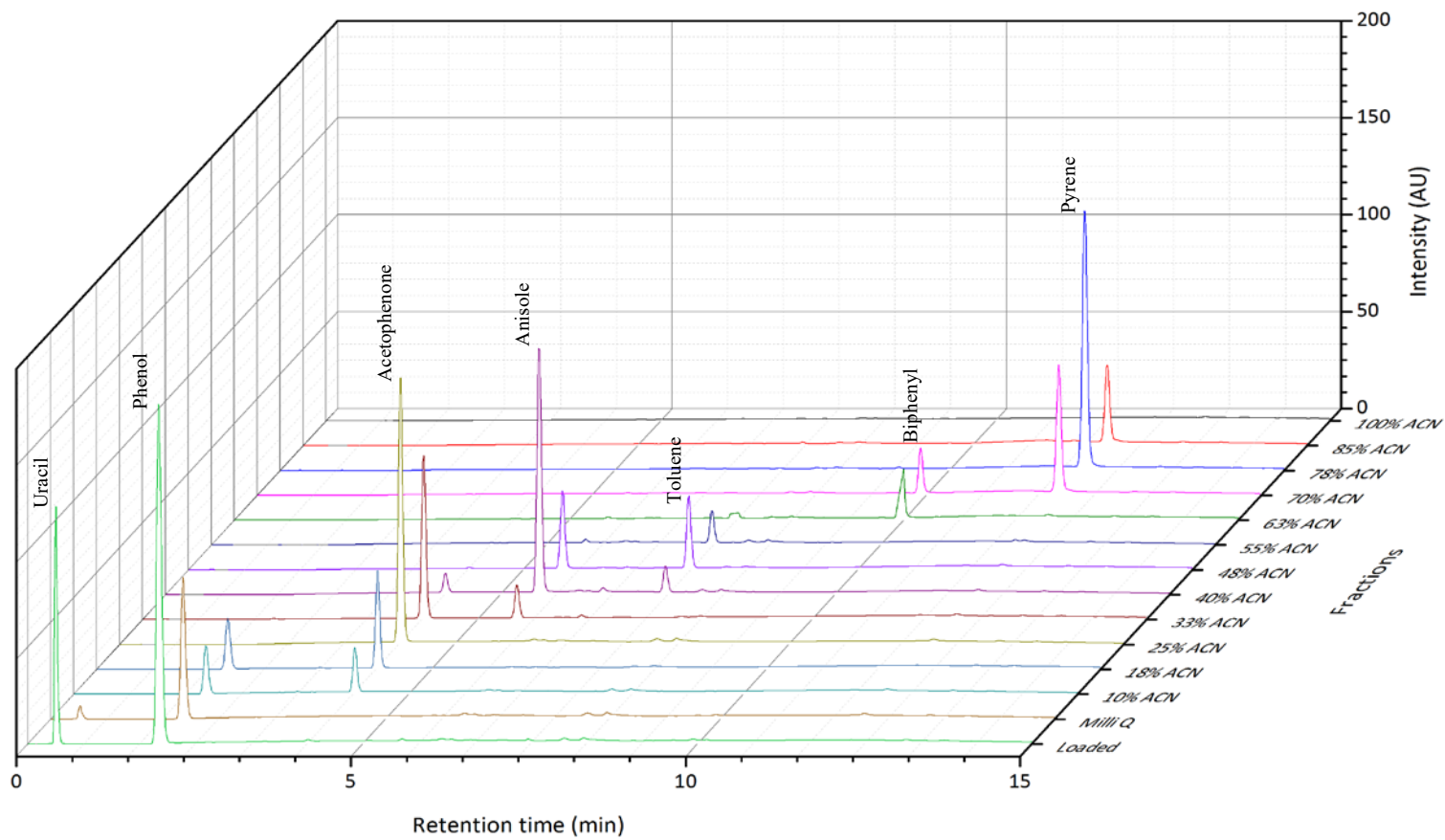


Figure 26: HPLC chromatogram of SPE eluted fractions from the standard test mixture

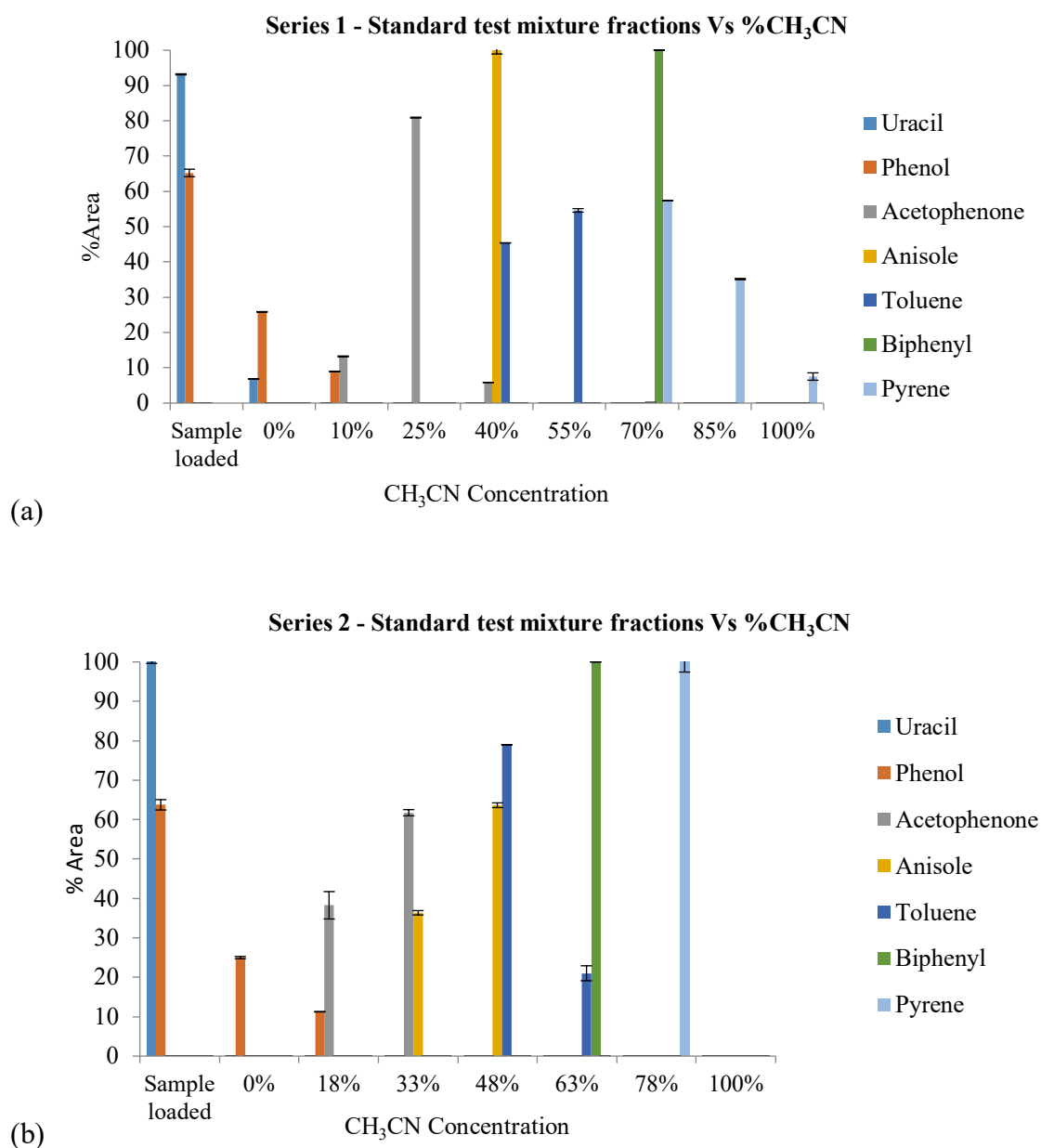


Figure 27: Components isolated in each fraction for the two series of eluting solvents using SPE

The relative area of each standard for a series is displayed in Figure 27 with the composition of the solvent changed by 15% CH₃CN for each fraction. The second series is offset by 7.5% CH₃CN from the first series in order to determine the range of solvent compositions that are able to elute each solute. In both series, the Milli-Q water fraction contained 26% of the phenol

with the remaining phenol not initially adsorbing and passing through in the sample solvent during loading. In most cases, a fraction contained only one or two solutes indicating that the 15% step in solvent composition had sufficient separation resolution for the range of test solutes that were chosen. This is not surprising as the chromatogram in Figure 26 shows that the test solute retention times are relatively evenly distributed.

When the two series are combined (Figure 28), it can be seen that acetophenone ($\log P=1.66 \pm 0.22$) elutes over a range of CH₃CN compositions between 10% and 33%, with 40% of the acetophenone eluting in the 25% CH₃CN fraction. For anisole ($\log P=2.13 \pm 0.20$), 50% was extracted in the 40% CH₃CN fraction and was distributed in the fractions between 33% and 48% CH₃CN. Toluene ($\log P=2.70 \pm 0.17$) extracted in fractions between 40% and 63% CH₃CN with most eluting in the 48% CH₃CN fraction. Both biphenyl ($\log P=3.98 \pm 0.23$) and pyrene ($\log P=5.17 \pm 0.17$) were extracted in the non-polar solvent range between 63% and 85% CH₃CN fractions. The biphenyl is evenly distributed in the 63% and 70% CH₃CN fractions with 50% of pyrene extracted in the 78% CH₃CN fraction.

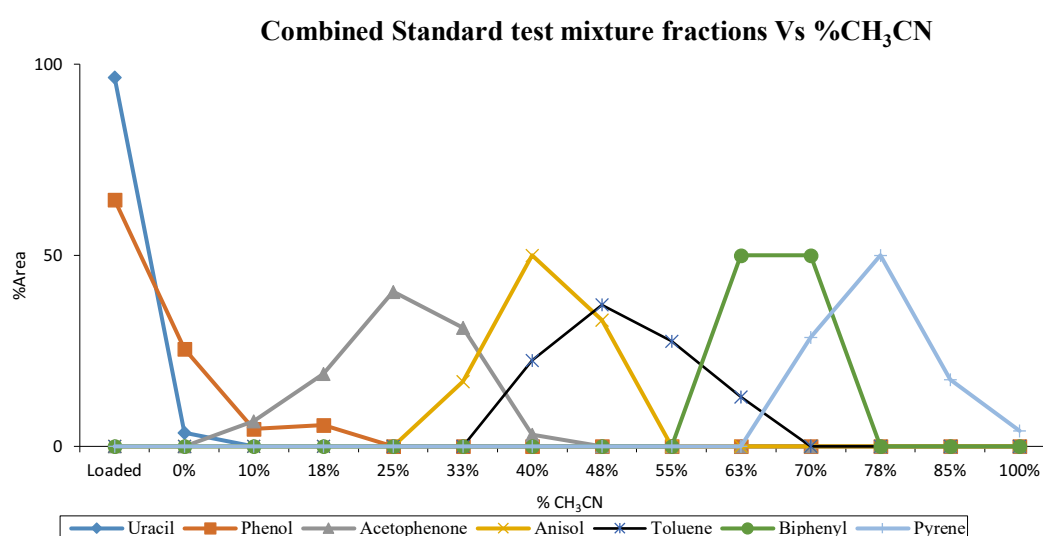


Figure 28: SPE results for the standard test mixture

This study has demonstrated that a crude primary stage fractionation will be able to produce fractions that contain a relatively narrow polarity range for the solutes in each fraction. However, as will become apparent in Section 3.4.2.2, primary fractionation of plant extracts will still contain a large number of solutes.

3.4.3.2 Secondary fractionation SPE of the standard test mixture

To further examine the need to produce fractions that contained fewer compounds, the same standard test mixture of compounds was loaded (20 mL) onto a conditioned SPE cartridge. Similar to the primary fractionation, elution was carried out using 20 mL volumes of Milli-Q water containing 10% CH₃CN and 25% CH₃CN. However, the elution with 40% CH₃CN was performed in 1 mL aliquots for 20 times with each 1 mL aliquot collected in a microcentrifuge tube. To observe how this influenced the extraction process, the primary fractionation was continued as in elution Series 1 with 20 mL volumes of 55%, 70%, 85% and 100% CH₃CN added and collected, respectively. To examine the profile, all the fractions were transferred into HPLC glass vials and analysis was completed using analytical HPLC.

Figure 29 shows that reasonably similar results were obtained as for the first four fractions from Series 1 of the the primary fractionation in Figure 27. When the sample was loaded onto the conditioned SPE column, 92% of uracil remained in the solvent and the majority of the phenol has eluted in the 10% CH₃CN fraction. Only some acetophenone has started to elute in the 25% CH₃CN fraction whereas in Figure 28, most of it eluted in this fraction.

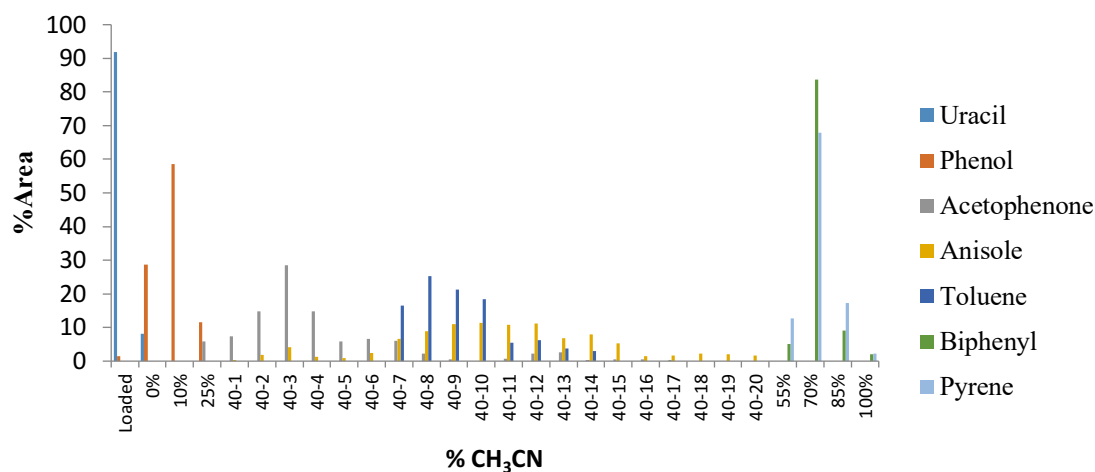


Figure 29: Secondary fractionation of the standard test mixture with 40% acetonitrile/Milli-Q water

The minor variability was due to the manual control of the eluting solvent flow rate. Secondary fractionation was performed for the 40% CH₃CN eluting solvent where it was eluted in twenty 1 mL aliquots (Figure 29). It can be seen that the remaining acetophenone was eluted in fractions up to 40%-5 CH₃CN. It is interesting to observe that the anisole is distributed between fractions 40%-2 CH₃CN and 40%-20 CH₃CN having only been present in the 40% CH₃CN fraction in the primary Series 1. The fractionation of toluene spreads over a narrower region between the 40%-7 CH₃CN and 40%-14 CH₃CN fractions. As in the earlier Series 1, a considerable amount of biphenyl eluted in 70% CH₃CN fraction and pyrene was also quite similar.

3.4.4.3 *Eremophila longifolia* plant extract separation

The primary fractionation strategy was used for the whole plant extract for *E. longifolia* and the HPLC chromatogram is displayed in Figure 30 with the primary fractionation results displayed in the HPLC chromatograms in Figure 31. Due to the similarity in the stationary phases for the SPE cartridges and the HPLC column, the fractions contain groups of solutes of similar polarity. This enables some predictive interpretation of each fraction depending on whether the bioassay

of a fraction results in a positive result. As an example, based on the standard test mixture, if a solute has a retention time between 0 and 2.5 min, then it is most likely they will be a polar compound with a log P value less than 1.5, similar to phenol. Likewise, if the retention time is in the 2.5-5 min time range, corresponding to the 18% to 40% CH₃CN fractions, then the log P values would be similar to acetophenone. The 48%-55% CH₃CN fractions have retention times from 5 to 7.5 min time and log P values of approximately 2.1. The group of solutes with retention times between 7.5-10 min from fractions 48% to 63% CH₃CN will have log P values around 2.7. Solutes with retention times around 12 min from fractions 55%-70% CH₃CN will log P values between 4 and 5.2 based on comparison to biphenyl and pyrene.

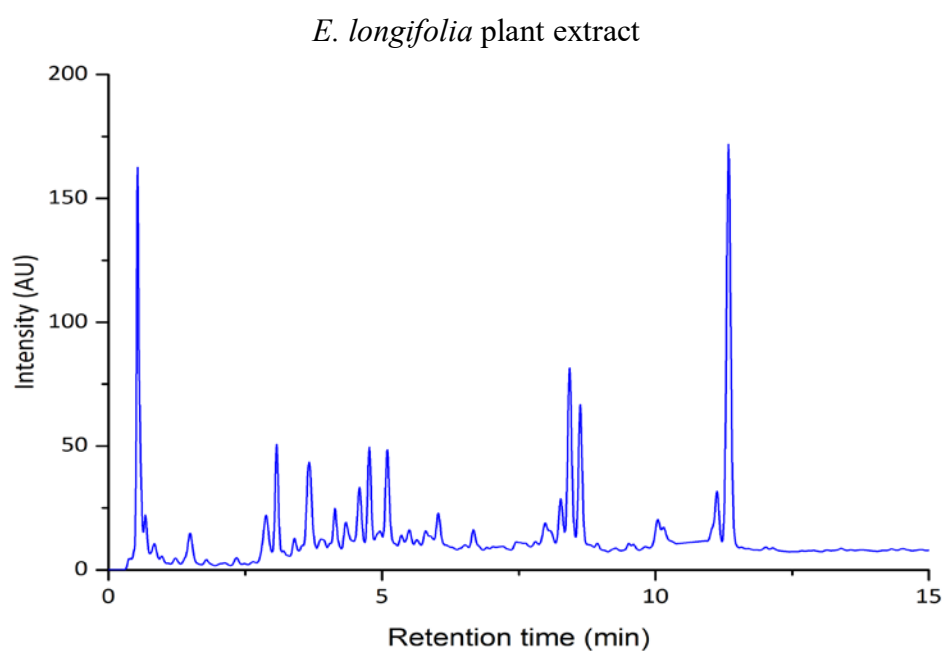


Figure 30: The analytical HPLC chromatogram of crude plant extract from *E. longifolia*

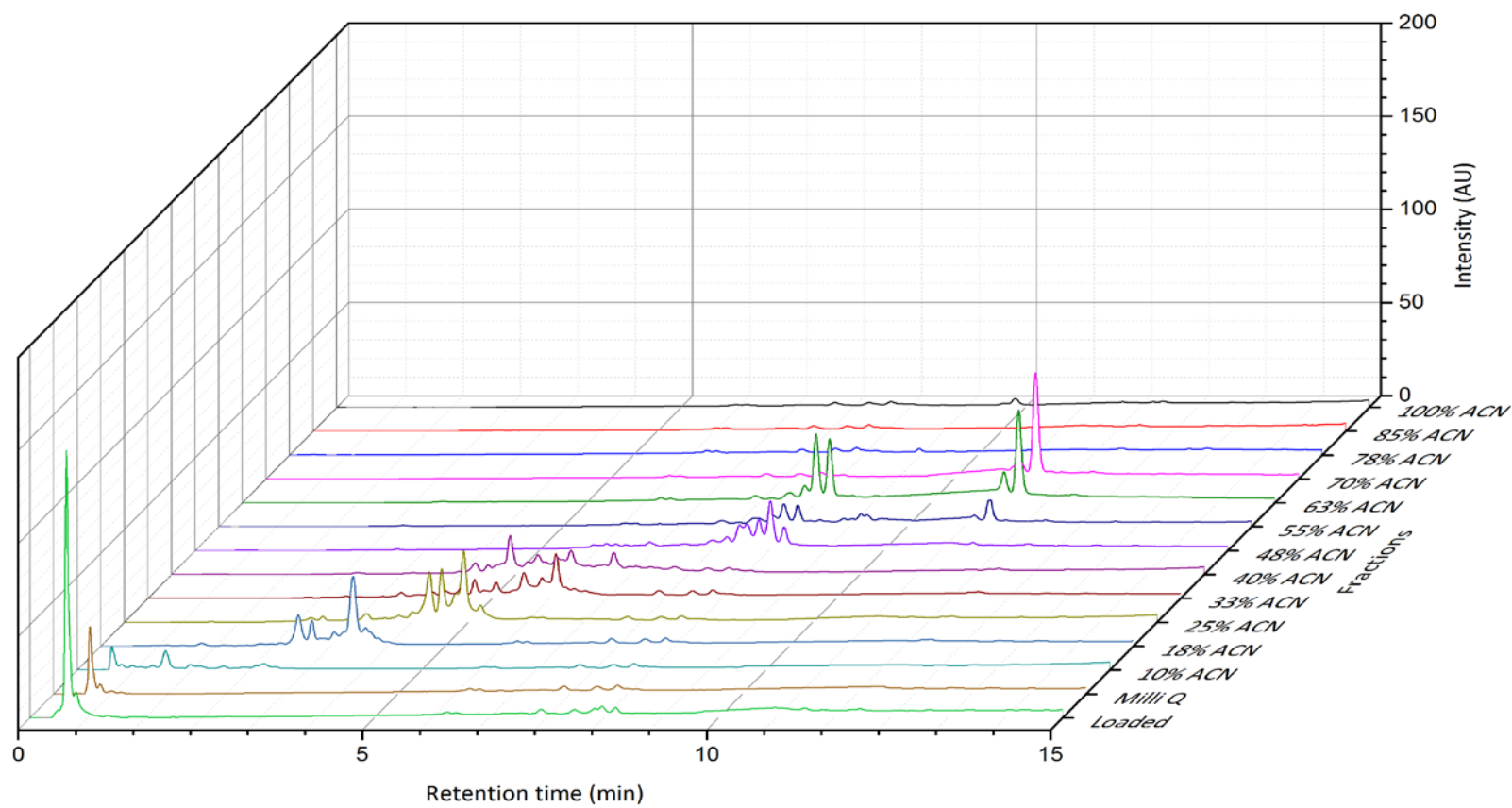


Figure 31: Analytical HPLC chromatogram of SPE eluted *E. longifolia* fractions

3.5 Conclusions

Plant extracts are a complex mixture containing molecules with a wide range of polarities from very polar, and possibly ionisable, to relatively non-polar with some selectivity introduced by the extracting solvent. Generally, bioactivity-guided fractionation to isolate the most active agent has primarily been attempted using large scale column chromatography techniques with the goal of isolating quantities on the tens of milligram scale but modern analytical techniques can easily cope with much less. The SPE based technique has been thoroughly characterised using known organic compounds with a range of polarities that include uracil, phenol, acetophenone, anisole, toluene, biphenyl and pyrene. For the selection of these compounds, the overall polarity of each molecule was estimated on the basis of $\log P$ values with polar compounds having a $\log P < 2$; moderate polarity compounds between 2 and 4 and non-polar compounds having a $\log P > 4$. It was found that this correlated well with the ability to separate the mixture of compounds using a binary combination of solvents that span the polarity range.

The overall observation from the secondary fractionation is that it is possible to have fractions with fewer components but the disadvantage is that the amount of each component is greatly decreased and this is common with most separation strategies. However, the fractionation provides similar amounts that are distributed over a wider range of fractions. If this amount is sufficient to maintain bioactivity, then statistical correlations between the measured amounts and bioactivity could be a useful way of identifying the bioactive compounds, as will be explored with *E. longifolia* and *B. lanceolata* plant extracts.

Chapter 4

Antimicrobial activity of *Eremophila longifolia* F. Muell

4.1 Introduction

The aims of this chapter are to evaluate the antimicrobial properties of the *E. longifolia* plant leaves extract and to determine the appropriate extraction method suitable to maximize the antimicrobial activity. *Eremophila longifolia* (Scrophulariaceae) is an Australian native plant that has been used traditionally by Aboriginal elders in order to treat various infective diseases. An infusion prepared by brewing the leaves of *Eremophila* can be taken as a treatment for headaches, to reduce fever and can act as an analgesic ^[84]. In addition, inhalation of the smoke from leaves placed on a fire was thought to cure microbial and fungal infections ^[95].

4.2 Plant collection

Details regarding the plant material are described in Section 3.2.

4.2.1 Sample preparation

Collected plant samples were stored at -20 °C in a freezer until required. The leaves were inspected for signs of degradation and stems were separated prior to extraction. The selected leaves were stored in an incubator for 7 days at a temperature of 60 °C in order to remove excess water. A commercial grinder was used to grind the dried leaves into a fine powder and the leaf powder was stored at -20 °C in a freezer prior to extraction.

4.3 Extraction of leaves

4.3.1 Different extraction techniques

Numerous extraction methods have been used for obtaining leaf extracts. In the literature, the most popular extraction techniques are Soxhlet extraction, conventional (traditional) immersion extraction and accelerated solvent extraction (ASE). The conventional immersion

and ASE methods were investigated in this study to determine the optimal extraction conditions based on antibacterial assays as summarised in Figure 32.

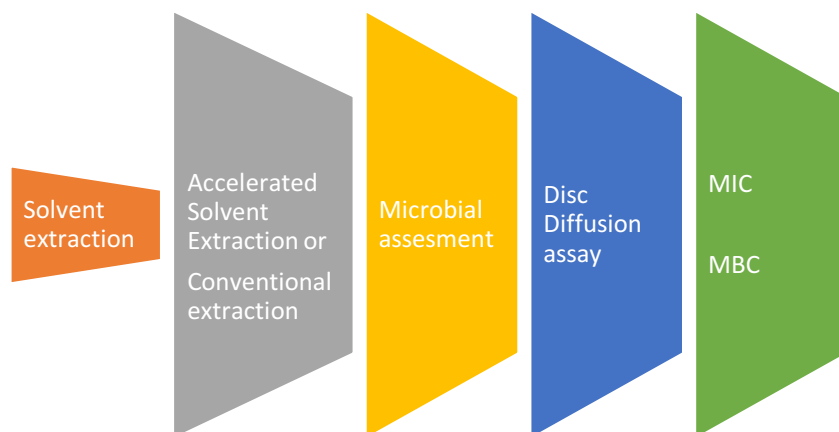


Figure 32: Flow chart of extractions and microbial assessment

4.3.1.1 Conventional extraction method

This extraction method is basically an immersion of the sample in the desired solvent with occasional gentle agitation over a period of days. This technique has been widely used in recent decades, as no specialised extraction equipment is required. Approximately 10 g of finely ground leaves was submerged in separate 250 mL bottles containing either 50 mL CH₃CN (Merck Gradient grade) or 50 mL EtOH (Merck Analytical grade) ^[175], which were then stored at ambient temperature (approximately 21 °C) for 5 days with occasional stirring.

4.3.1.2 Conventional extraction method-at different time scales

A consideration of the conventional extraction approach is the uncertainty on how much time is required to extract the targeted compounds from the plants and this needs to be explored. Separate amounts of approximately 30 g of finely ground *Eremophila* leaves were immersed into separate bottles containing either 150 mL of CH₃CN or 150 mL of EtOH, where each was

stored at room temperature for 7 days for extraction with continuous shaking (170 rpm). Periodically, 10 mL aliquots of the crude plant extract were removed from each sample at consecutive intervals of 24, 48, 72, 96, 120, and 168 hours.

The crude extraction aliquot was filtered through a 0.45 µm nylon syringe filter and centrifuged at 1058 RCF (Relative Centrifugal Force) (Hettich EBA 20) with the supernatant collected. The supernatant was transferred into a weighed 50 mL Falcon tube and placed in a Speed Vac (RVC-2-8) solvent evaporator at 40 °C for approximately 8 hours where the solvent was completely evaporated. The residues were accurately weighed and resuspended in a known volume of DMSO before the antimicrobial activity was assessed. Another sample series was prepared for HPLC and GC-MS analysis using EtOH as the solvent with the initial concentration adjusted to 100 mg/mL for the both analyses. These extracts were stored at -20 °C until required for analysis.

4.3.1.3 Hot water extraction (100 °C boiling water)

This method is particularly relevant to the way that the Australian indigenous people used the leaves to form a decoction that was consumed to cure headaches and colds, and applied to sores to treat infections. About 10 g of the ground plant sample was placed in a beaker with 50 mL of water and boiled at 100 °C for 10 min. The extract was left to cool to room temperature and then filtered through a 0.45 µm nylon filter. The water was evaporated using a rotary evaporator and the residue was stored at -20 °C until required. The residues were accurately weighed and resuspended in a known volume of DMSO before antimicrobial evaluation.

4.3.1.4 Accelerated Solvent Extraction using different temperatures

A pressurised Dionex ASE 100 ACE was used that uses nitrogen gas with elevated pressures (>1000 psi) coupled with temperature regulation (40-200 °C) in order to pass the desired solvent through the sample. The ASE system has programmable options that include settings for preferred temperature, extraction time and number of static cycles (number of times the solvent passes through the sample while heating). These options generally enabled the extraction procedure to be kept to less than one hour as opposed to the conventional method extraction time, which was five days. About 10 g of the ground leaf sample was placed in a 34 mL ASE sample cell. The static time was constant (10 min) for all the extractions and the variable temperatures used in the separate extractions were 40, 80 and 120 °C for both CH₃CN and EtOH solvents.

4.4 Extraction efficiency analysis

4.4.1 Assessment of antimicrobial activity

4.4.1.1 Bacterial strains and media

Eleven bacterial isolates and one fungal strain were used in this study. The bacterial and fungal strains were collected from the Microbiology Laboratory at Swinburne University of Technology. Apart from the bacteria described in Section 3.3.3, two clinical isolates and two more laboratory bacterial strains were utilised. *E. coli* (O157:H7) and *Vancomycin*-Resistant *Enterococci* (VRE) were employed as clinical isolates and the facultative anaerobic Gram-positive *Streptococcus mutans* and aerobic *Streptococcus sobrinus* were tested.

The bacteria were subcultured on Brain Heart Infusion agar slants as described in Section 3.3.4. Fresh cultures of *E. coli* and VRE were prepared by inoculating approximately 2 mL of Brain Heart Infusion Broth with a loopful of microorganism from the slant cultures. Bacterial broth

cultures were incubated at 37 °C overnight under aerobic conditions as previously described for the other bacteria isolates used in inhibitory assays. *S. sobrinus* was incubated at 37 °C for 48 hours in aerobic conditions and *S. sobrinus* was grown at 37 °C for 48 hours in anaerobic conditions using the candle jar technique ^[176].

There are a number of approaches that are commonly employed for the evaluation of antibacterial activity of plant extracts. Disc diffusion assay (DDA) and plate-hole diffusion assay (PHDA) are the most effective techniques employed in microbial assessments ^[177]. In this study, DDA was used to assess the antimicrobial activity with commercially available 6 mm diameter sterile discs used as controls. The test culture for the DDA was prepared by adjusting the concentration with sterile water to a 0.5 McFarland standard and was transferred to the agar in a Petri dish using a cotton swab. A volume of 20 µL of the extracts from the different extraction methods, where the concentration was 100 mg/mL in DMSO, was added to the sterile disc and the discs were then placed on the agar plate. The agar plates were then incubated at a specific temperature depending on the organism, as already described, and the zones of inhibition for bacterial growth were measured in terms of a circular diameter in mm ^[178]. A commercial tetracycline (30 µg) antibiotic disc was used as the positive control and 20 µL of DMSO was applied to a sterile disc as the blank in these experiments. All the assays were performed in triplicate.

The microplate reader technique is a rapid alternative method for testing biological activity and the instrument is designed to measure the optical density of samples at a desired wavelength in a microtitre plate. This method is particularly useful in determining MIC and MBC values for test compounds ^[178-179] and is suitable as a biological assessment technique for assessing crude plant extracts ^[180]. General aspects in the determination of MIC and MBC values for bacteria

using this technique are described in Sections 3.3.4.2 and 3.3.4.3. However, additional specific details for these experiments are that the initial concentration of the plant extract was 100 mg/mL and 100 μ L of the plant extract was added to the first well. The doubling serial dilutions started from the 100 mg/mL well and continued to 0.0488 mL with 10 μ L of fresh bacterial cultures (grown overnight and adjusted to a 0.5 McFarland standard) added into each well. Tetracycline (1 mg/mL initial concentration) was applied as a positive control and DMSO was the blank. The 96 well microtitre plates were incubated according to the desired temperatures of the test bacteria and fungi. The activity of each well was estimated based on the measurement of the absorbance at a wavelength of 600 nm ^[181]. All the assays were performed in triplicate and the median of the MIC and MBC values were taken into the consideration.

4.4.2 HPLC analysis

The HPLC method that was used in these experiments is described in Section 3.3.6.2.

4.4.3 GC-MS analysis

General aspects related to GC-MS are contained in Section 3.3.6.1. However, the temperature program used for these experiments was as follows: Initially 50 °C for 2 min, followed by an increase of temperature to 280 °C (at a heating rate of 20 °C min⁻¹) that was then held for 28 min.

4.5 Results and Discussion

4.5.1 Solvent optimisation

The choice of the solvent in an extraction can influence the compounds that are extracted as dissolution of the compounds will depend on their relative solubility in the extraction solvent. The solvents CH₃CN and EtOH were examined as the primary solvents because they are the

most compatible with reversed phase liquid chromatography, which was the main analytical tool utilised. Previous literature shows that the crude *E. longifolia* extracts were obtained with EtOH and MeOH ^[85]. EtOH is a common polar protic solvent that is used for extraction with a dielectric constant of 24.55 and a dipole moment of 1.69 D ^[182]. The polarity is due to the alcohol group (-OH) present in the molecule, which contains the highly electronegative oxygen atom that is able to bond with other molecules. The molecule also has an ethyl group (-C₂H₅), that has relatively non-polar characteristics as well ^[183]. Thus, EtOH can extract both polar and non-polar compounds from plant leaves ^[184].

Acetonitrile is a widely used solvent in HPLC but it has not been employed as a solvent in the extraction of *Eremophila* species. CH₃CN is an intermediate polar aprotic solvent with a higher dielectric constant than EtOH of 38.8 and also has a larger dipole moment of 3.92 D. In this sense, it can be expected that it should extract a wider range of polar and nonpolar compounds ^[185].

Extraction efficiencies of each solvent using the conventional immersion approach and hot water extraction are presented in Table 4. The data indicate that the bioactivity assays of the crude extract obtained with CH₃CN displayed significantly lower MIC and MBC values compared to EtOH and the hot water extracts.

Table 4: Minimum inhibitory, bactericidal and fungal concentrations for *E. longifolia* leaves extracted using different solvents and hot water decoction extraction against various organisms

Extraction Type		Concentration (µg/mL) ^c									
		<i>E. coli</i>	<i>P. aeruginosa</i>	<i>S. enterica</i>	<i>M. catarrhalis</i>	<i>S. pyogenes</i>	<i>B. cereus</i>	<i>S. aureus</i>	<i>C. albicans</i>	<i>S. mutans</i>	<i>S. sobrinus</i>
EtOH Extract ^a	MIC	1563	391	1563	781	1563	391	391	3125	781	781
	MBC/MFC	1563	391	3125	781	1563	391	391	3125	1563	1563
CH ₃ CN Extract ^b	MIC	781	195	781	391	781	195	98	391	391	781
	MBC/MFC	781	195	3125	391	781	195	98	391	1563	1563
Hot water Extract	MIC	1563	781	3125	781	3125	≥6250	≥6250	6250	1563	≥6250
	MBC/MFC	1563	781	3125	781	3125	≥6250	≥6250	6250	3125	≥6250

MBC, minimum bactericidal concentration; MFC, minimum fungicidal concentration; MIC, minimum inhibitory concentration.

^{a, b} *E. longifolia* leaf extraction using the conventional method.

Tetracycline (1 mg/mL initial concentration) and DMSO were applied as controls.

^c Average of triplicate results.

The MIC and MBC values against *E. coli* for the CH₃CN plant extract were both 781 µg/mL whereas the EtOH and hot water extracts produced slightly higher results with MIC and MBC values of 1563 µg/mL. For *P. aeruginosa*, the CH₃CN plant extract once again had the lowest MIC and MBC concentration of 195 µg/mL followed by EtOH with 391 µg/mL and hot water with 781 µg/mL. The CH₃CN plant extract displayed the lowest MIC against *S. enterica* compared to other solvent extracts with the MBC values being the same for the all extraction solvents at 3125 µg/mL. Ethanol and hot water extracts showed similar MBC and MIC concentrations of 781 µg/mL against *M. catarrhalis* and the CH₃CN extract had MBC and MIC concentrations of 781 µg/mL.

Considering the Gram-negative bacteria, all of the extracts showed the lowest MBC and MIC against *P. aeruginosa* and *M. catarrhalis*. For the Gram-positive bacteria, the CH₃CN extract had MIC and MBC that are remarkably low for *S. aureus*, with a value of 98 µg/mL, whereas the hot water extract has values greater than 6250 µg/mL. The other bacteria, *S. pyogenes* and *B. cereus*, along with the fungi *C. albicans* showed a similar pattern to the *S. aureus* isolate against the extracts.

The cariogenic species *S. mutans* and *S. sobrinus* ^[186] are closely associated with formation of dental caries within the teeth, especially in pre-school children ^[187]. Some research illustrated that both of these bacteria isolates are considered to be the major etiologic agents for tooth decay ^[188], which is related to their acid tolerance capacity ^[189]. Table 4 shows that the hot water extract had a comparatively insignificant effect against either of *S. mutans* and *S. sobrinus*. However, the EtOH and CH₃CN extracts displayed similar MBC and MIC values against both bacteria.

In summary, when comparing three different extraction methods, the CH₃CN extract showed the lowest MBC and MIC values and the hot water extract has displayed the highest values against the broad range of selected pathogenic Gram-positive, Gram-negative and yeast isolates. Thus, further extractions were limited to the solvents CH₃CN and EtOH.

4.5.2 Maximising extraction yield using different extraction techniques

4.5.2.1 Conventional extraction method versus ASE with different extraction temperatures

ASE is the most rapid technique for extracting compounds from plant materials and the whole extraction can be done in 30 minutes when using elevated temperature and pressure ^[190]. The results in Table 5 illustrate that the EtOH extraction solvent produces the highest percentage yields under all conditions compared to extractions with CH₃CN. For the conventional extraction method, both EtOH and CH₃CN extraction percentage yields were comparatively lower than the ASE producing 7.63% and 2.14%, respectively. The ASE extraction yield generally increases with temperature except for conditions using CH₃CN at a temperature of 80 °C but it appears that the yields for 40 °C and 80 °C are quite similar and only significantly increases at 120 °C.

Table 5: Yield extraction efficiency for different extraction methods

Extraction Method	Extraction Efficiency (%)	
	EtOH	CH ₃ CN
Conventional extraction	7.63	2.14
ASE 40 °C	12.50	6.53
ASE 80 °C	13.22	5.18
ASE 120 °C	20.23	9.14

Figure 33 contains HPLC chromatograms of the crude plant extract from *E. longifolia* using CH₃CN as the solvent for different temperatures from ASE and the conventional method. Comparison of the three chromatograms A, B and C with D indicates a higher intensity for the ASE extractions relative to the conventional method with slightly below 1×10^6 AU for the largest peak compared to only 4×10^5 AU for the same peak. The profiles for A to C are quite similar but there are noticeable differences compared to chromatogram D where some peaks are much smaller, such as around 8 minutes, and some peaks at around 16 minutes are much larger. Furthermore, it was noticeable that the ASE extracts were much greener compared to the conventional extract reflecting the greater yields for the ASE method. All of the extracts were prepared at the same concentration for analysis using HPLC.

4.5.2.2 HPLC analysis

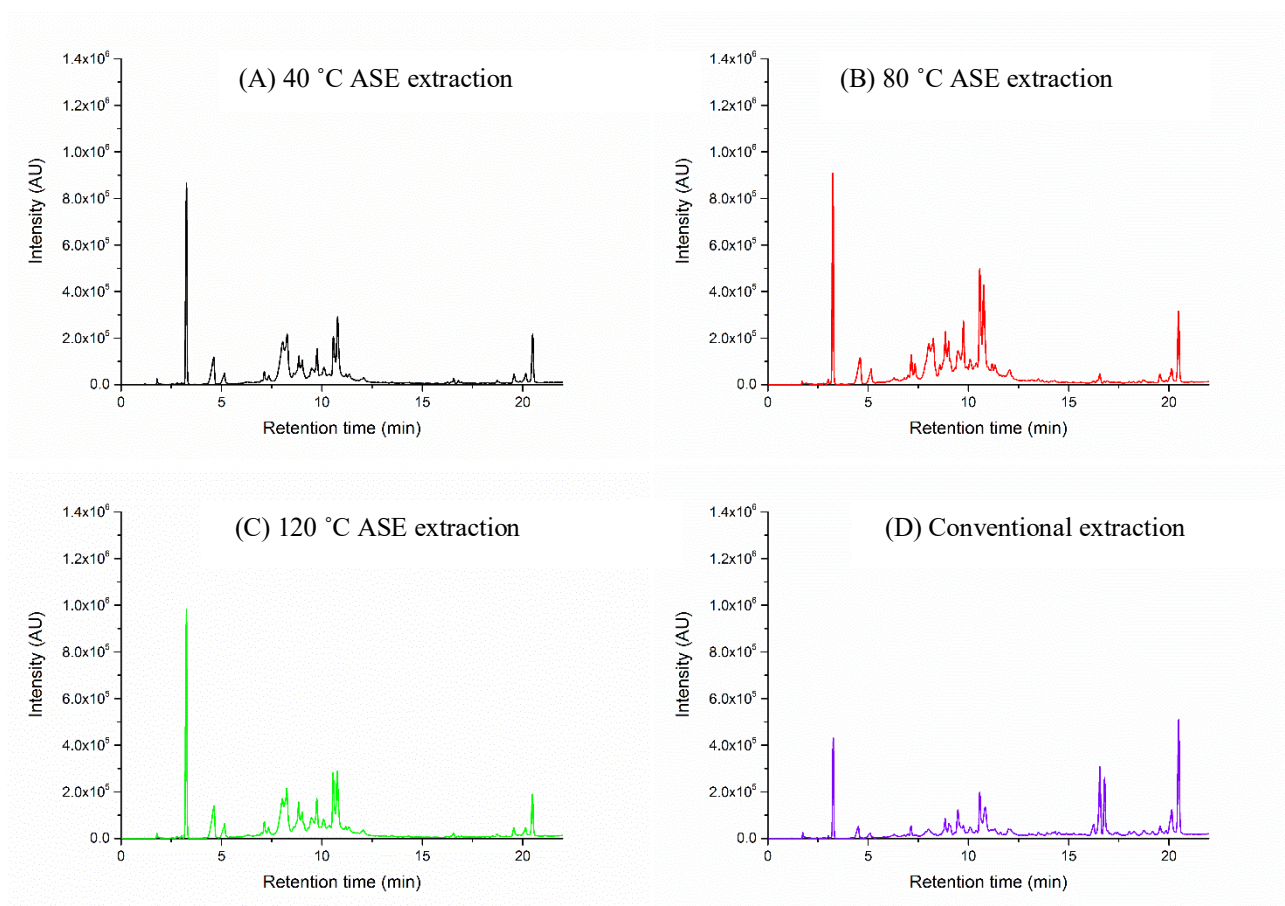


Figure 33: HPLC chromatograms with CH₃CN for *E. longifolia* leaves extraction at (A to C) different temperatures for ASE and (D) the conventional extraction method

The corresponding HPLC chromatograms for leaves extracted with EtOH as the solvent are displayed in Figure 34 with the different temperatures using the ASE in Figures A, B and C with chromatogram D obtained for the conventional method. The intensities for the ASE extracted samples are quite similar to the conventional method extract with the largest peak intensities of around 1.3×10^6 AU. However, once again, there was a clear difference between the profiles for ASE and conventional method extracts, especially around a retention time of 8 minutes.

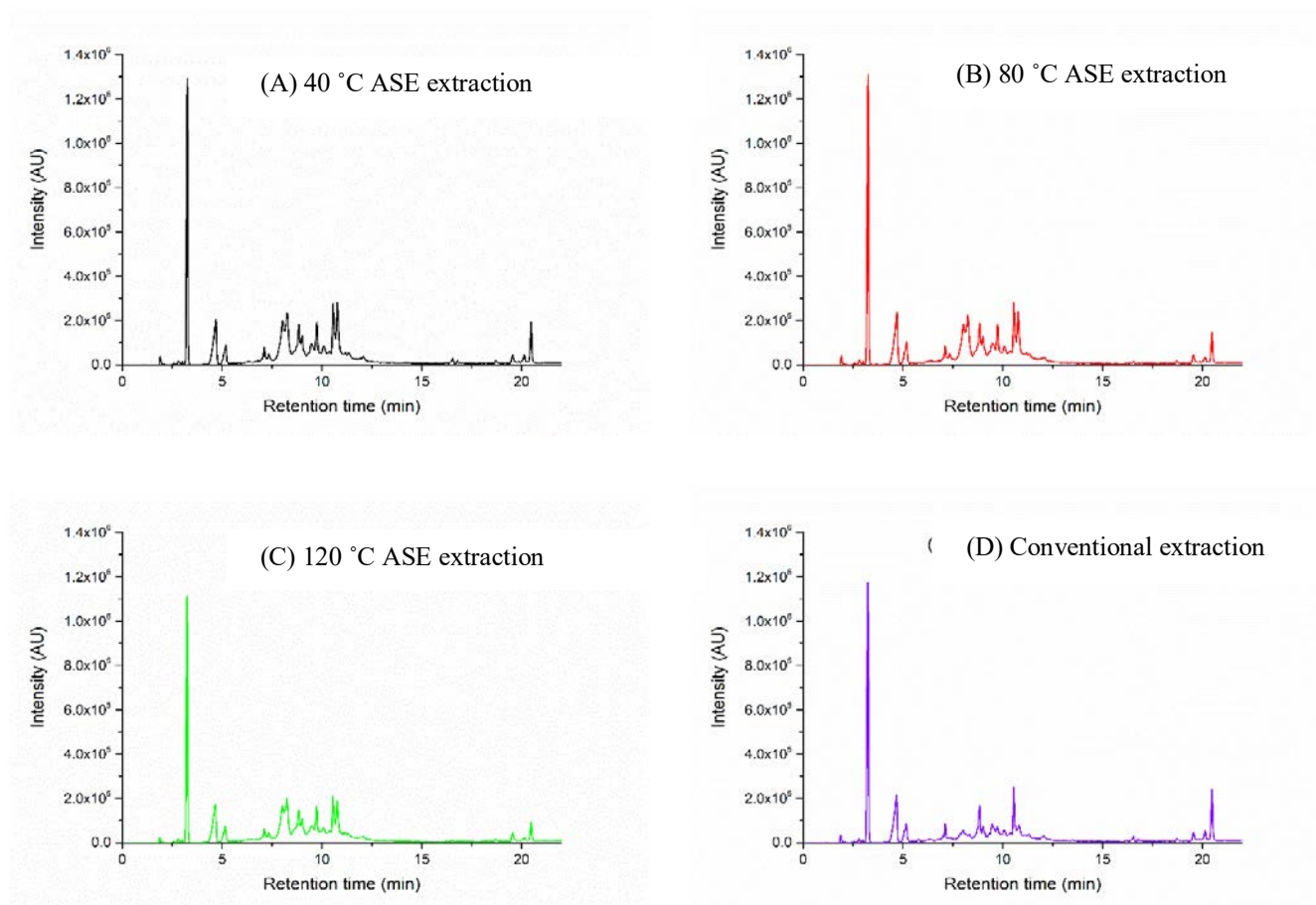


Figure 34: HPLC chromatograms with EtOH for *E. longifolia* leaves extraction at (A to C) different temperatures for ASE and (D) the conventional extraction method

4.5.2.2 GC-MS analysis

The corresponding GC-MS chromatogram from extractions using CH_3CN as the solvent are displayed in Figure 35. Chromatograms A, B, and C show the ASE extractions at different temperatures and the conventional extraction is presented in chromatogram D. Compared to the HPLC chromatograms for the same sample in Figure 33, there are substantially more peaks due to the greater resolution available in gas chromatography^[191].

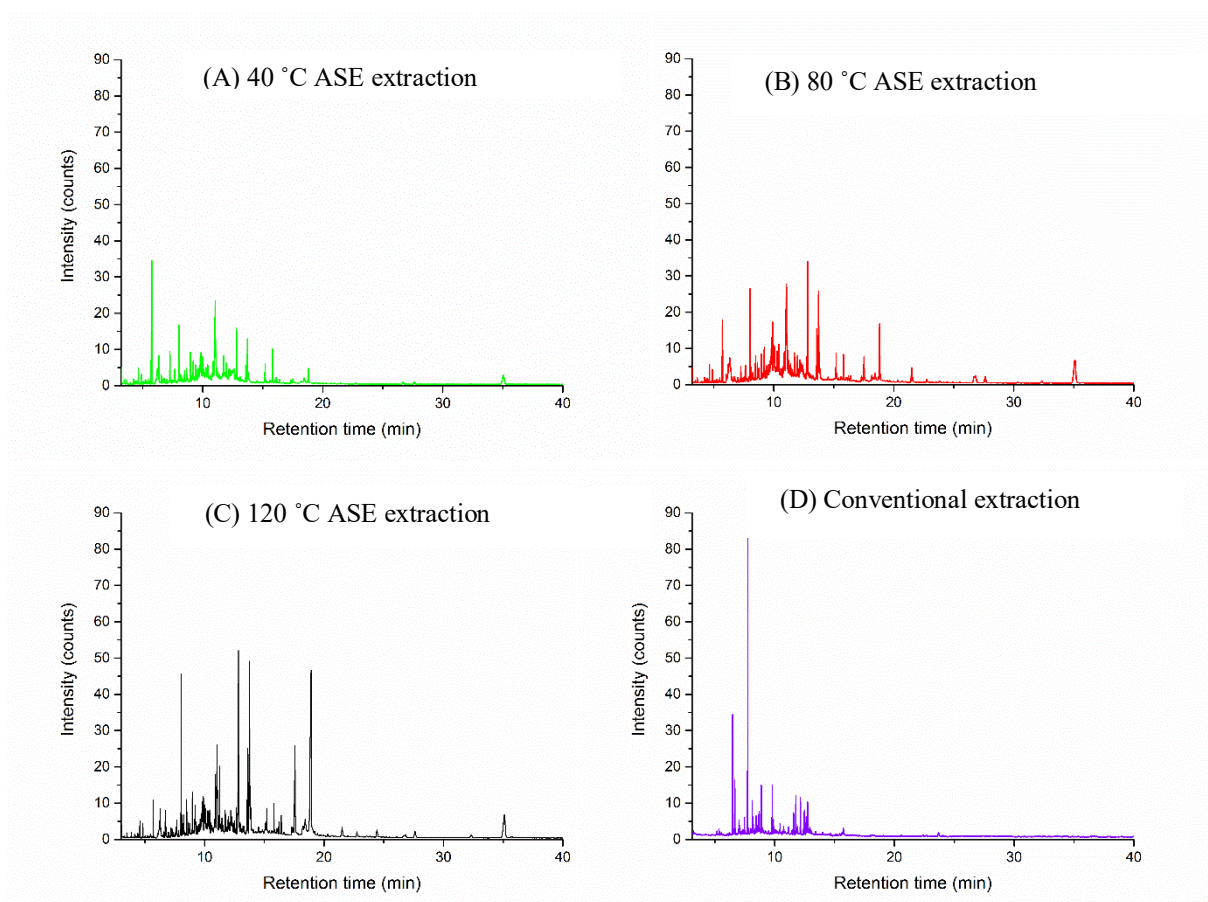


Figure 35: GC-MS chromatograms with CH₃CN for *E. longifolia* leaves extraction at (A to C) different temperatures for ASE and (D) the conventional extraction method

Comparison of the profiles for the ASE extracts shows that there are noticeable differences, particularly for the highest temperature. However, these differences are relative minor when they are compared with the chromatogram obtained for the conventional method.

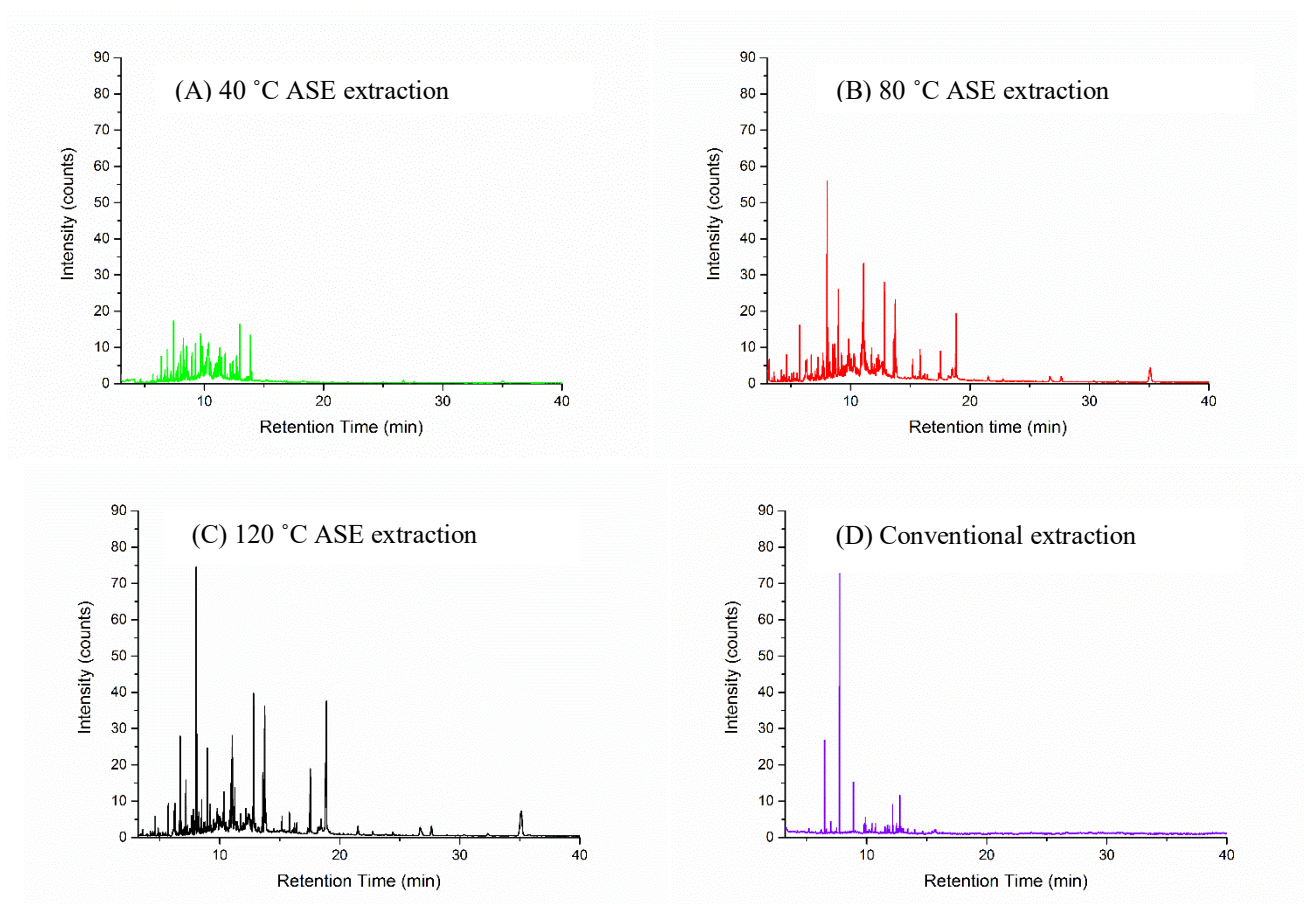


Figure 36: GC-MS chromatograms with EtOH for *E. longifolia* leaves extraction at (A to C) different temperatures for ASE and (D) the conventional extraction method

GC-MS chromatograms for extractions using EtOH as the solvent are contained in Figure 36 with chromatograms A, B and C obtained from ASE extractions and chromatogram D resulting from the conventional method. A similar behavior pattern was observed in EtOH as was seen with CH₃CN.

4.5.2.3 Bioguided identification assays

4.5.2.3.1 Disc Diffusion Assays

The bioactivity of the various extracts was initially tested using two Gram-positive and one Gram-negative bacteria with the DDA method. Average zones of inhibition from triplicate

measurements are presented in Figure 37 and reported in Table 6. Tetracycline was used as the positive control with dried ethanol and acetonitrile discs used as negative controls.

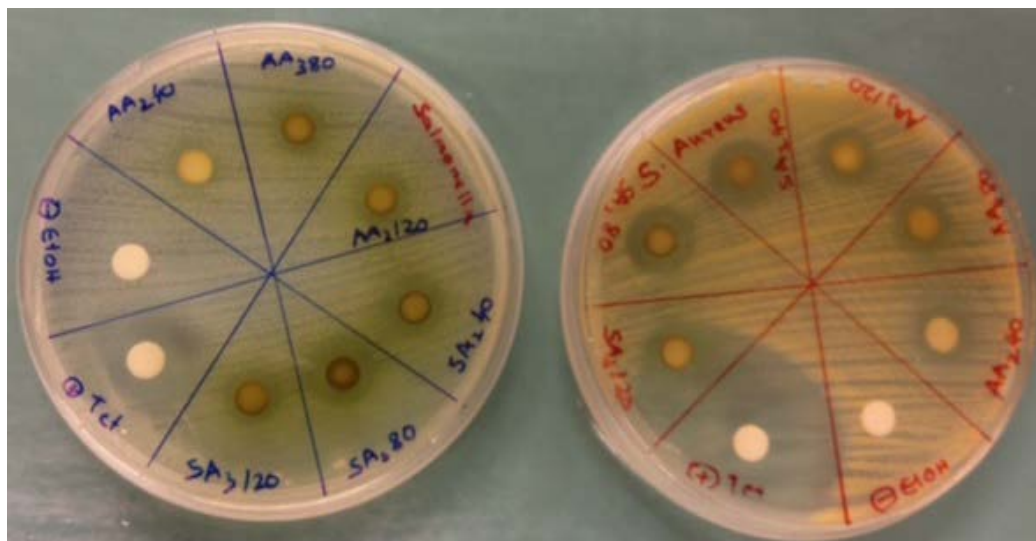


Figure 37: Examples for antibacterial activity using DDA for different organisms (*S. enterica* and *S. aureus*) and a range of extracts. SA refers to the CH₃CN extracts and AA are the EtOH extracts

Table 6: Antibacterial activity for different organism from various extracts obtained using DDA

Extraction Method	<i>B. cereus</i>	<i>S. enterica</i>	<i>S. aureus</i>
<i>Zone of inhibition in mm</i>			
EtOH	-	-	-
Conventional extraction	12	12	15
ASE 40 °C	9	-	15
ASE 80 °C	9	-	13
ASE 120 °C	9	-	14
CH₃CN	-	-	-
Conventional extraction	16	20	19
ASE 40 °C	12	10	25
ASE 80 °C	10	9	18
ASE 120 °C	8	12	15
Tetracycline (30µg)	28	25	35

Extract concentration is 100 mg/mL and 20 µL of extract was added to each disc. Pure solvents were used as negative controls.

The conventional method for both CH₃CN and EtOH extracts produced larger zones of inhibition against the bacteria tested compared to the ASE extracts, with the exception of the CH₃CN ASE 40 °C extract against *S. aureus*. It is also apparent that an increased temperature for the ASE method actually decreased the bioactivity for these extracts. The highest temperature resulted in the highest yield of extract but clearly the extra mass that is extracted is not bioactive and indicates that the external part of the plant contains the most antimicrobially active compounds. This is not surprising as most microbial exposure will occur on the external surfaces and this is where the defensive chemicals need to be located.

For the two solvents used for the conventional method, it is the CH₃CN solvent that generally showed higher zones of inhibition compared to EtOH. However, it is noted that the single largest zone of inhibition is observed against *S. aureus* for the ASE method at 40 °C but because it is less effective against the other two organisms compared to the conventional CH₃CN extraction, it has not been considered further. Therefore, based on these results, CH₃CN is the best solvent using the conventional method for extractions of *E. longifolia* leaves.

4.5.2.3.2 Assessment of MIC and MBC/MFC

To confirm the observations from the DDA experiments described in the previous section, a more elaborate and detailed study was undertaken with an increase in the range of organisms tested with values for MIC and MBC/MFC measured. Following the format of the results from the previous DDA, the measured MIC and MBC/MFC results are listed in Table 7.

The extract obtained from the convention extraction method using CH₃CN displayed the lowest MBC and MIC values against Gram-negative *E. coli*, *P. aeruginosa*, and *M. catarrhalis*. Similar results were obtained for the Gram-positive bacteria, namely *B. cereus*, *S. aureus*, *S. mutans*, *S. sobrinus* and *S. pyogenes* with MIC values ranging from 98 µg/mL to 781 µg/mL and MBC values ranging from 98 µg/mL to 1563 µg/mL. Likewise, the yeast *C. albicans* behaved in a similar manner with both MIC and MFC values being 391 µg/mL. Overall, the lowest MBC and MIC values were 98 µg/mL against *S. aureus*.

Table 7: Minimum inhibitory, bactericidal, and fungicidal concentrations of *E. longifolia* leaf extracts using various extraction conditions

Solvent	Extraction Type		Concentration (µg/mL)									
			<i>E.coli</i>	<i>P.aeruginosa</i>	<i>S. enterica</i>	<i>M. catarrhalis</i>	<i>S. pyogenes</i>	<i>B. cereus</i>	<i>S. aureus</i>	<i>C.albicans</i>	<i>S. mutans</i>	<i>S. sobrinus</i>
EtOH	Conventional	MIC	1563	391	781	781	1563	391	391	3125	1563	781
		MBC/MFC	1563	391	1563	781	1563	391	391	3125	3125	781
	ASE 40 °C	MIC	1563	391	781	781	3125	781	391	3125	781	781
		MBC/MFC	1563	391	1563	781	3125	781	391	3125	1563	781
	ASE 80 °C	MIC	1563	185	781	781	3125	391	781	3125	1563	781
		MBC/MFC	1563	185	1563	781	3125	391	781	3125	3125	781
	ASE 120 °C	MIC	1563	391	781	195	3125	391	391	3125	391	781
		MBC/MFC	1563	391	1563	195	3125	391	391	3125	781	781
CH ₃ CN	Conventional	MIC	781	195	781	391	781	195	98	391	391	391
		MBC/MFC	781	195	1563	391	781	195	98	391	1563	391
	ASE 40 °C	MIC	1563	391	781	195	1563	391	195	1563	391	781
		MBC/MFC	1563	391	781	195	1563	391	195	1563	1563	781
	ASE 80 °C	MIC	1563	391	781	195	3125	781	391	781	391	781
		MBC/MFC	1563	391	1563	195	3125	781	391	781	1563	781
	ASE 120 °C	MIC	781	391	781	391	3125	781	1563	3125	781	1563
		MBC/MFC	781	391	1563	391	3125	781	1563	3125	1563	781

MBC, minimum bactericidal concentration; MFC, minimum fungicidal concentration; MIC, minimum inhibitory concentration.

Tetracycline (1 mg/mL initial concentration) and DMSO were applied as controls.

Averages of triplicate results.

The ASE extracts were less active and the DDA result observed for the ASE extract at 40 °C against *S. aureus* has not been repeated. Once again, the EtOH extracts were less active than CH₃CN extracts.

4.5.2.3.3 Assessment of MIC and MBC/MFC against clinical isolates

To further establish the preferred extraction solvent and method, these extracts were tested against clinical isolates and the results are included in Table 8. The isolate *E. coli* O157:H7 is not a common bacterium used in teaching laboratories as it can be responsible for cases of severe gastroenteritis and has caused Haemolytic uraemic syndrome in America and Europe, with children suffering from acute renal failure ^[192]. Another clinical isolate, Vancomycin-Resistant *Enterococcus* (VRE) is a Gram-positive bacterium that is resistant to vancomycin and can cause infection in the urinary tract, heart conditions like endocarditis, secondary infections in wounds and blood poisoning ^[193].

The results for all extraction conditions against *E. coli* O157H7 displayed similar MIC and MBC values of 781 µg/mL and 1563 µg/mL, respectively. The variation in the values previously observed for *E. coli* using the various extraction methods that are listed in Table 7 has not been repeated in Table 8 for this clinical isolate but, clearly, the *E. longifolia* extracts have bioactivity.

The VRE susceptibility was higher in all the EtOH extracts with MIC and MBC values ranging between 781 µg/mL and 3125 µg/mL, with the majority being 1563 µg/mL. However, the extract from the conventional method using CH₃CN has the lowest MIC and MBC values at 781 µg/mL. It is interesting to note that the ASE extraction at 120 °C showed the highest MIC and MBC values of 1563 µg/mL and 6250 µg/mL, respectively.

Table 8: Minimum inhibitory and bactericidal concentrations of *E. longifolia* leaf extracts using various extraction conditions

Solvent	Extraction Type	Concentration ($\mu\text{g/mL}$) ^c against		
			<i>E. coli</i> O157:H7	VRE M259849
EtOH	Conventional	MIC	781	781
		MBC	1563	1563
	ASE 40 °C	MIC	781	1563
		MBC	1563	3125
	ASE 80 °C	MIC	781	1563
		MBC	1563	1563
	ASE 120 °C.	MIC	781	1563
		MBC	1563	1563
CH ₃ CN	Conventional	MIC	781	781
		MBC	1563	781
	ASE 40°C	MIC	781	781
		MBC	1563	1563
	ASE 80°C	MIC	781	781
		MBC	1563	1563
	ASE 120°C	MIC	781	1563
		MBC	1563	6250

MBC, minimum bactericidal concentration; MIC, minimum inhibitory concentration.

Tetracycline (1 mg/mL initial concentration) and DMSO were applied as controls.

Averages of triplicate results

In summary, all of these studies consistently demonstrated that extractions based on the conventional method produced an extract that showed more inhibitory activity against the tested microorganisms compared to ASE methods. However, the CH₃CN traditional method extract displayed the lowest MIC and MBC values in all of the antimicrobial testing. The conclusion was to use CH₃CN as the solvent for the conventional extraction method in further experiments.

4.5.2.4 Time dependence for the conventional method

Conventional extraction of *E. longifolia* using CH₃CN as the solvent for 120 hours was found to be the most effective conditions to obtain maximum bioactivity. However, a further study to identify the optimum extraction time was undertaken with times of 24, 48, 72, 96, 120 and 168 hours. The extracts were analysed using HPLC, GC-MS and bioactivity assays.

4.5.2.4.1 Chromatographic analysis

The HPLC results are included in Figure 38 with the chromatograms for 24, 48, 72 and 96 hours of extractions being quite similar but with slightly smaller peaks compared to 120 and 168 hours. Figure 39 contains the GC-MS chromatograms with extracts for 120 and 168 hours showing the most peaks.

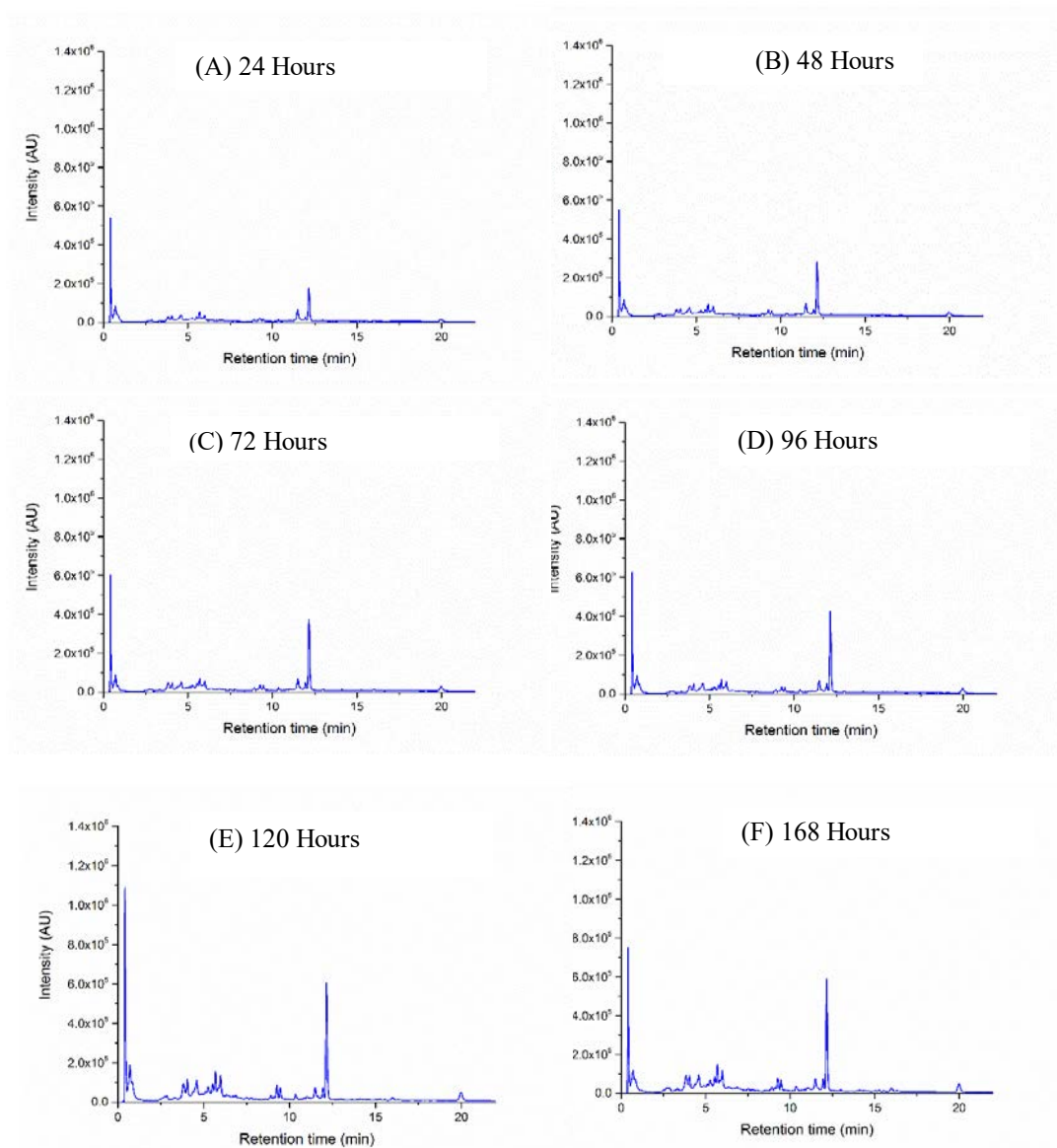


Figure 38: HPLC chromatograms from the conventional extraction of *E. longifolia* leaves using CH_3CN with various times represented in A to F

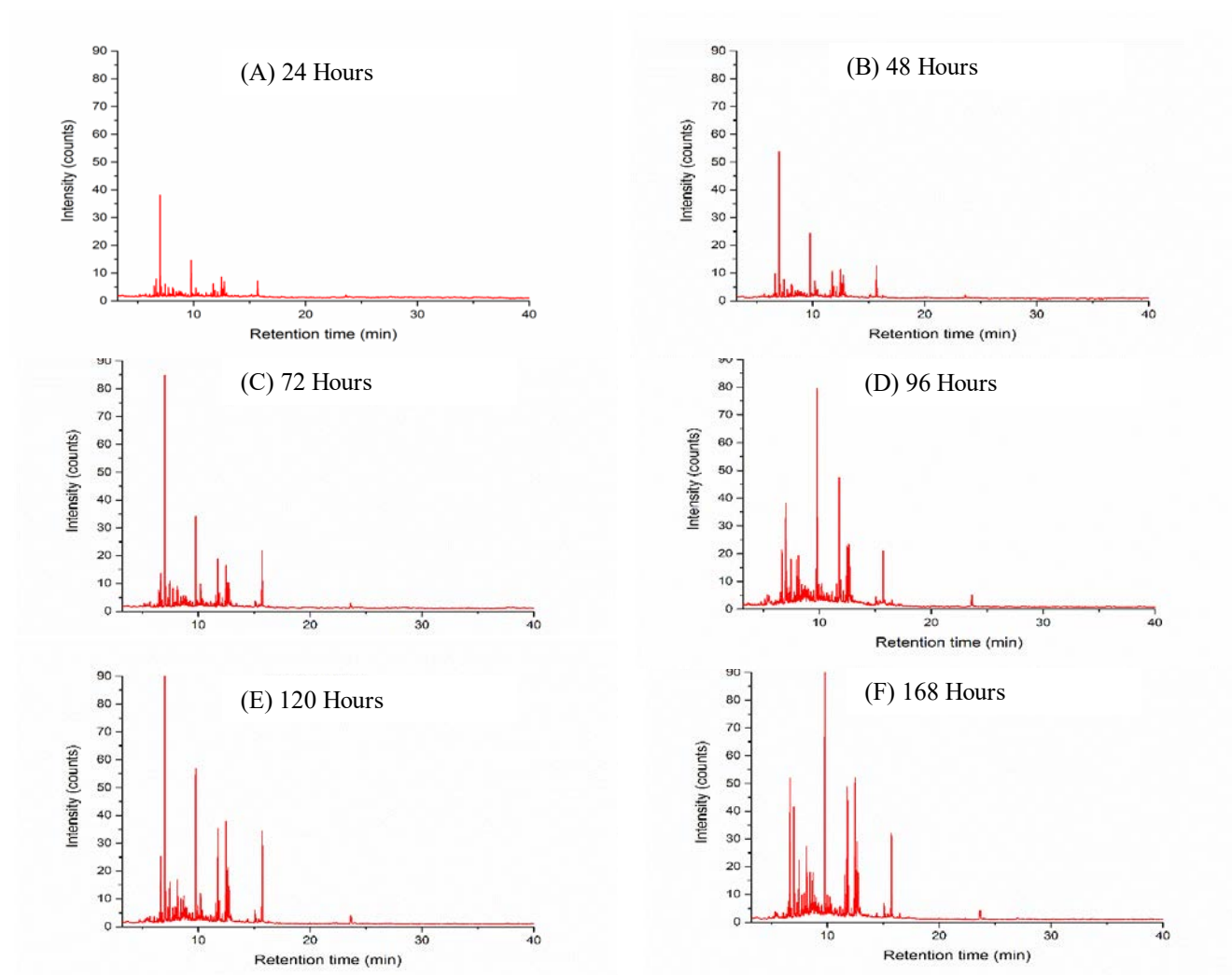


Figure 39: GC-MS chromatograms from the conventional extraction of *E. longifolia* leaves using CH₃CN with various times represented in A to F

4.5.2.4.2 Bioactivity assays

The extracts were tested for their MIC and MBC/MFC against a range of pathogens and the results are presented in Table 9. It appears that the extraction time has had no effect on activity against *M. catarrhalis* and the fungus *C. albicans* with both MIC and MBC values being consistent for all of the extraction times. The extracts showed decreased activity as the extraction time increased against *E. coli*, *S. enterica*, *B. cereus* and *S. aureus*, but mostly for their MIC values. A similar pattern of activity was observed against *P. aeruginosa*, *S. pyogenes* and *S. mutans*, except that activity was restored after extended extraction times, with the optimum time being 95-120 hours. As these tests are based on an equal initial concentration of the total extract, this suggest that extended extraction times result in inactive material being extracted and subsequent dilution of the active compounds. It is interesting to note that the maximum activity against *S. sobrinus* was achieved with the shortest extraction time of 24 hours and then decreases with a plateau as the extraction time increases.

Table 9: Minimum inhibitory, bactericidal and fungicidal concentrations of *E. longifolia* leaf extracts using different times with conventional CH₃CN extraction.

CH ₃ CN Extraction		Concentration (µg/mL)									
		<i>E. coli</i>	<i>P. aeruginosa</i>	<i>S. enterica</i>	<i>M. catarrhalis</i>	<i>S. pyogenes</i>	<i>B. cereus</i>	<i>S. aureus</i>	<i>C. albicans</i>	<i>S. mutans</i>	<i>S. sobrinus</i>
24 hours	MIC	3125	781	781	391	1563	3125	391	781	1563	195
	MBC/MFC	6250	1563	1563	781	3125	≥6250	3125	1563	6250	195
48 hours	MIC	3125	781	781	391	781	3125	391	781	6250	781
	MBC/MFC	≥6250	1563	1563	781	1563	≥6250	3125	1563	6250	781
72 hours	MIC	1563	391	781	391	781	3125	391	781	6250	391
	MBC/MFC	6250	1563	1563	781	1563	≥6250	3125	1563	6250	391
96 hours	MIC	3125	391	781	391	781	3125	391	781	6250	391
	MBC/MFC	3125	1563	1563	781	1563	6250	3125	1563	6250	391
120 hours	MIC	781	391	391	391	3125	781	195	781	6250	391
	MBC/MFC	1563	781	1563	781	3125	6250	3125	3125	6250	391
168 hours	MIC	781	391	391	391	3125	781	195	781	6250	391
	MBC/MFC	1563	1563	1563	781	3125	6250	3125	3125	≥6250	391

MBC, minimum bactericidal concentration; MFC, minimum fungicidal concentration; MIC, minimum inhibitory concentration.

Tetracycline (1 mg/mL initial concentration) and DMSO were applied as controls.

Averages of triplicate results.

4.6 Conclusions

In this chapter, the extraction conditions for *E. longifolia* leaves were optimised based on antimicrobial assays. The effects of solvent, time, temperature and automation were investigated and the conventional CH₃CN immersion method at room temperature for 120 hours (5 days) was found to produce the best bioactivity results. Although the ASE was very efficient based on the extraction yields, subsequent testing showed that much of these extracts were inactive. Furthermore, the inactive compounds increased the complexity of the sample and would likely make the identification of the active components more challenging.

Chapter 5

Phytochemical identification of *Eremophila longifolia*

F. Muell

5.1 Introduction

It was demonstrated in Chapter 4 that extracts of *E. longifolia* have antimicrobial activity against a broad range of microorganisms. The extraction conditions for obtaining the maximum bioactivity were optimised and, in this chapter, the separation, isolation and characterisation of the bioactive compounds will be described. Experiments based on bioguided fractionation followed by instrumental analysis have identified a number of bioactive compounds.

Preliminary fractionation of the crude plant extract was completed using a solid phase extraction method with these extracts analysed using GC-MS. Further bioguided fractionation was accomplished by using an automated HPLC fraction collector. Identification was determined from information obtained from NMR and LC-HRMS.

5.2 Extraction, isolation and characterisation

5.2.1 Fractionation of the *E.longifolia* plant extract

Crude plant extracts are very complex mixtures that contain hundreds of different secondary metabolites and the nature of these different metabolites varies considerably for any given extract. It follows that it is very challenging to isolate the active antimicrobial compounds without any primary bioguided fractionation. Preliminary fractionation was performed in order to separate the complex mixture of compounds in plant extracts as described in Section 3.3.1. For the primary fractionation, 100 mg of crude plant extract was loaded onto a solid phase cartridge. The antimicrobial activity of the fractions was measured to identify the fraction where the compounds responsible for the inhibitory activity had been isolated.

5.2.2 GC-MS analysis

General aspects related to GC-MS are contained in Section 3.3.6.1. However, the temperature program used for these experiments was as follows: linear temperature ramp from 50 °C to 250 °C over 12 min and then hold at 250 °C for a further 18 min. All fractions were evaporated to dryness and reconstituted in 900 µL ethanol with 1 µL injected into the GC-MS. The mass scan range was set to 50–600 amu and the solvent delay time was 5 min to avoid detector saturation

5.2.3 NMR Analysis

NMR analysis was performed using a Bruker Avance-600 MHz spectrometer equipped with a TXI cryoprobe using standard pulse sequences with the resonances referenced to TMS (δ_H 0.00 and δ_C 0.0 ppm). NMR spectra for 1H (600 MHz) and ^{13}C (150 MHz) were obtained in $CDCl_3$.

5.3 Antimicrobial and antifungal assays

5.3.1 Assessment of biological inhibitory activity of separated fractions

5.3.1.1. Disc diffusion assay

The solvent for the separated fractions was removed and the extract was resuspended in 100 µL of DMSO and 20 µL was deposited onto blank discs placed on freshly prepared *B. cereus* spread over Nutrient Agar plates as described in Section 3.3.4.1. Zones of inhibition were recorded for each fraction.

5.3.1.2 MIC antimicrobial assays

Bacteria were obtained from American Type Culture Collection (ATCC; Manassas, VA, US) or Network on Antimicrobial Resistance in *S. aureus* (NARSA). The pathogenic microorganism isolates used for the bioguided fractionation are:

- Gram-positive bacteria, *B. cereus* (ATCC 11778), *S. aureus* (MRSA ATCC 43300, GISA NRS17, GISA MRSA NRS1, VRS10 GRSA), *Streptococcus pneumoniae* (MDR ATCC700677), *Enterococcus faecalis* (*vanA* clinical isolate) and *Enterococcus faecium* (MDR *vanA* 15559)
- Gram-negative bacteria, *E. coli* (FDA control ATCC 25922) and *Acinetobacter baumannii* (ATCC 19606)

The tested bacteria strains were cultured in Mueller-Hinton broth (MHB) (Bacto Laboratories, cat. no. 211443) at 37 °C overnight with shaking (200 RPM). A sample of each culture was then diluted 40-fold in fresh MHB and incubated with shaking (200 RPM) at 37 °C for 2-3 hours. A dose response MIC assay was performed using a standard microdilution assay according to the Clinical and Laboratory Standard Institutes (CLSI) guidelines. The compounds were serially diluted two-fold across the wells of 96-well plates (Corning; cat. no. 3370, polystyrene plates), with concentrations ranging from, 0.06–128 µg/mL, plated in duplicate. The resultant mid-log phase cultures were diluted to the final concentration of 1×10^6 CFU/mL, then 50 µL was added to each well of the compound-containing plates, giving a final compound concentration range of 0.03–64 µg/mL and a final cell density of 5×10^5 CFU/mL. Plates were covered and incubated at 37 °C for 24 hours. MIC values were determined visually, being defined as the lowest concentration showing no visible growth. Vancomycin. HCl (Sigma 861987), daptomycin (Molekula 64342447), colistin sulfate (Sigma C4661) and polymyxin B sulfate (Sigma P4932) were used as control inhibitors. All work was conducted in a biosafety cabinet class II.

5.3.1.3 Antifungal assays

Fungal strains *C. albicans* (ATCC 90028) and an encapsulated yeast *Cryptococcus neoformans* (H99 ATCC 208821) were obtained from American Type Culture Collection (ATCC; Manassas, VA, US). An 8-point dose response MIC assay was performed using a standard microdilution assay. The compounds were serially diluted two-fold across the wells of 384-well plates (Corning; cat. no. 3680, polystyrene plates), with concentrations ranging from, 1–128 µg/mL, plating 25 µL in duplicate. Fungi were cultured for 3 days on Yeast Extract-Peptone Dextrose (YPD) agar at 30 °C. A yeast suspension of 1×10^6 to 5×10^6 cells/mL (as determined by OD₅₃₀) was prepared from five colonies. These stock suspensions were diluted with Yeast Nitrogen Base (YNB) broth to a final concentration of 2.5×10^3 CFU/mL. Then, 25 µL of the fungal suspension was added to each well of the 384-well compound-containing plates, giving final concentrations in the range of 0.5–64 µg/mL for the tested samples. Plates were covered and incubated at 35 °C for 24 hours without shaking. To aid visualization of *C. neoformans* growth, 0.001% resazurin (Sigma R7017) was added to each well and incubated for a further 2 hours at 35 °C. MIC values were then determined visually for both strains, being defined as the lowest concentration showing no visible growth. Fluconazole (Sigma; F8929) was used as a control inhibitor. All work was conducted in a biosafety cabinet class II.

5.3.1.4 Cytotoxicity assays

HEK293 human embryonic kidney cells (ATCC® CRL-1573) and HepG2 hepatocellular carcinoma cells (ATCC® HB-8065) were seeded as 6000 and 5000 cells/well, respectively, in clear bottom 384-well plates (Corning® 3712) in a volume of 20 µL in DMEM medium (GIBCO-Invitrogen, cat. no. 11330032), in which 10% FBS was added. Cells were incubated for 24 hours at the temperature of 37 °C in 5% CO₂ to allow the cells to attach to the plates. Active compounds were dissolved in DMSO at 10 mg/mL and a dilution series of 1:3 fold steps

in cell culture medium was created with a final compound concentration range of 50–0.39 µg/mL in a 40 µL final assay volume. The final DMSO concentration was 1%. The cells were incubated with the compounds for 24 hours at 37 °C and 5% CO₂. After incubation, 10 µM resazurin in PBS (Sigma R7017) was added to each well and the plates were incubated for a further 3 hours at 37 °C, 5% CO₂. The fluorescence intensity was read using a TECAN Infinite M100 Pro Plate reader with excitation/emission at 560/590 nm. The data from four replicates were analyzed using GraphPad Prism software (version 6) and the results were calculated using the following equation:

$$\% \text{Viability} = 100 (FI_{\text{TEST}} - FI_{\text{NEGATIVE}}) / (FI_{1\% \text{DMSO}} - FI_{\text{NEGATIVE}}) \quad (1)$$

Tamoxifen (Sigma T5648) was the positive control and had an IC₅₀ of 23 µg/mL.

5.4 Results and discussion

5.4.1 Primary fractionation

As described in Section 3.3.5.1.1, two different SPE fractionation series using different ratios of CH₃CN and H₂O were carried out and the results of the DDA for antimicrobial activity against *B. cereus* for Series 1 are shown in Figure 40 with Figure 41 showing Series 2. Table 10 contains a summary of the measured zones of inhibition. The highest activity was found in the 25% CH₃CN/H₂O fraction from Series 1 with a 14 mm zone of inhibition. This is similar to Series 2 where the 18% CH₃CN/H₂O fraction has a zone of 11 mm. When considering both series together, the activity covers the range of 18% to 55% CH₃CN/H₂O and when compared to the standard test mixture that was used in Section 3.4.1, these results indicate that most of the active compounds are probably in the moderately non-polar range with log *P* values between 1.9 and 2.5.

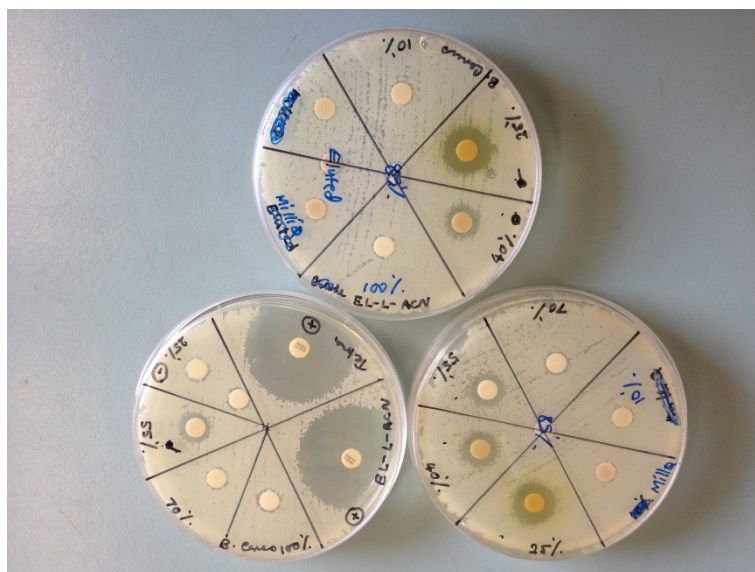


Figure 40: Bioactivity against *B. cereus* from the Series 1 fractions in DDA.

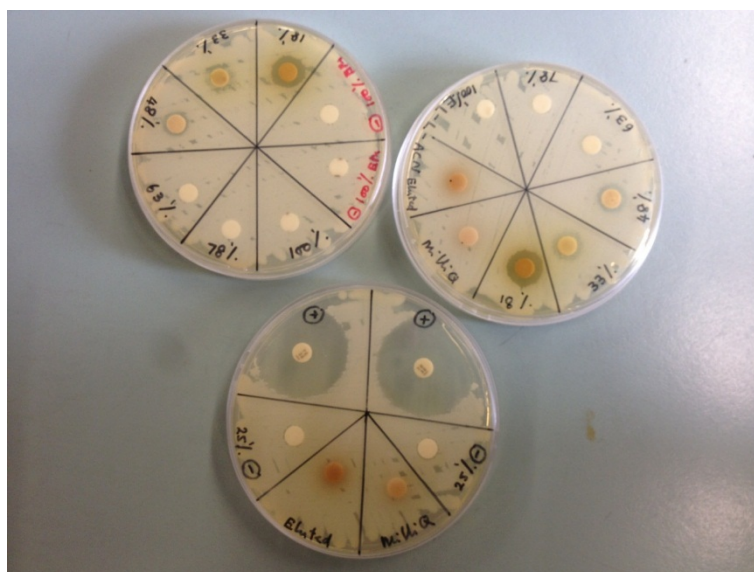


Figure 41: Bioactivity against *B. cereus* from the Series 2 fractions in DDA.

Table 10: DDA bioactivity against *B. cereus* of CH₃CN/H₂O from Series 1 and 2 fractions.

Series 1 CH ₃ CN/H ₂ O ratio	Zone of inhibition (mm)	Series 2 CH ₃ CN/H ₂ O ratio	Zone of inhibition (mm)
Eluted	-		
Milli-Q water	-	Eluted	-
10%	-	Milli-Q water	-
25%	14	18%	11
40%	11	33%	9
55%	10	48%	9
70%	-	63%	-
85%	-	78%	-
100%	-	100%	-

5.4.2 GC-MS analysis

GC-MS has two main advantages compared to conventional HPLC, with GC having higher chromatographic resolution compared to LC and the GC-MS having a searchable mass spectral database whereas the HPLC has a diode array detector that provides only rudimentary UV-Visible spectra. For these reasons, GC-MS analysis was completed on all of the fractions and the chromatograms were compared for the active and non-active fractions in order to identify the bioactive compounds in the plant extract. A comparison of the GC-MS chromatograms for an active fraction in Figure 42 with an inactive fraction in Figure 43 showed that the biggest change in intensity occurred for a peak with a retention time of 8.98 min and a mass spectral database search identified 2-methoxy-4-vinyl phenol as a potential compound of interest. The Wiley GC-MS library database match was identified with a very high probability of 95% and the chemical structure is presented in Figure 44.

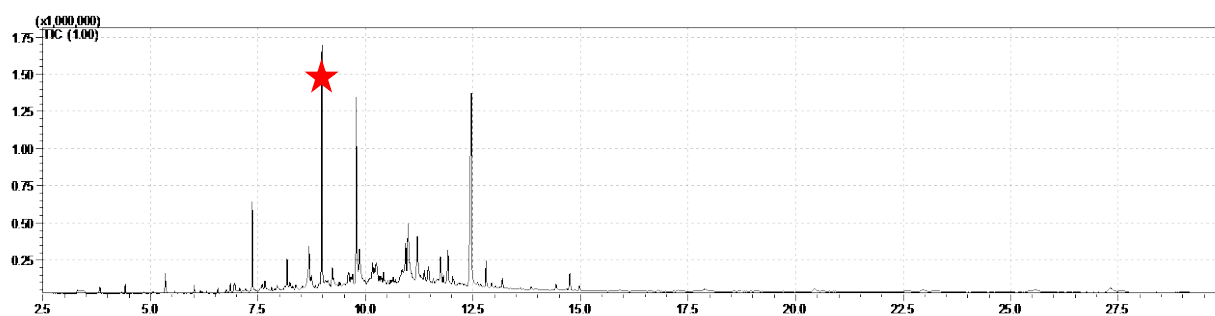


Figure 42: GC-MS chromatogram of an active fraction of *E. longifolia* against *B. cereus*

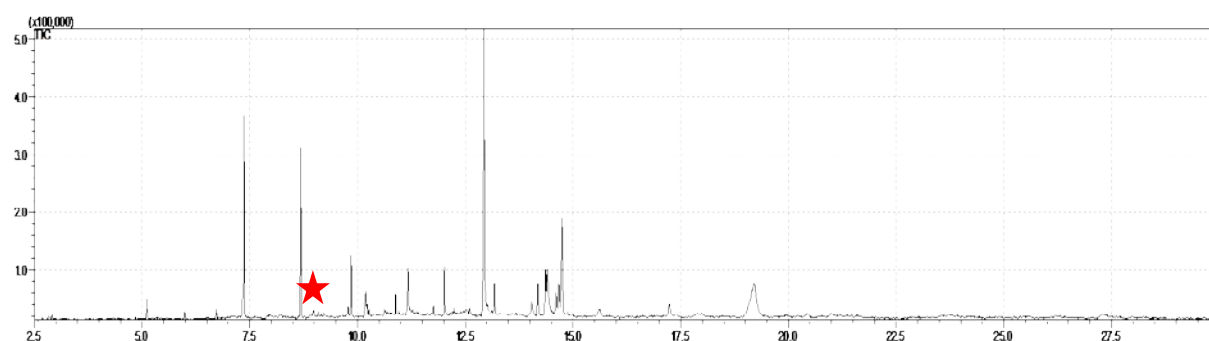


Figure 43: GC-MS chromatogram of non-active fraction of *E. longifolia* against *B. cereus*. In this figure the red star indicates 2-methoxy-4-vinyl phenol

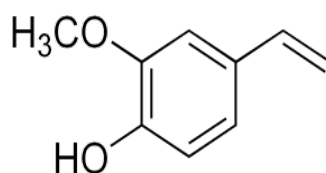


Figure 44: Chemical structure of 2-methoxy-4-vinyl phenol

This is a commercially available chemical and to confirm the database identification, the pure compound was analysed under the same conditions as shown in Figures 45 and the retention time was also 8.98 minutes.

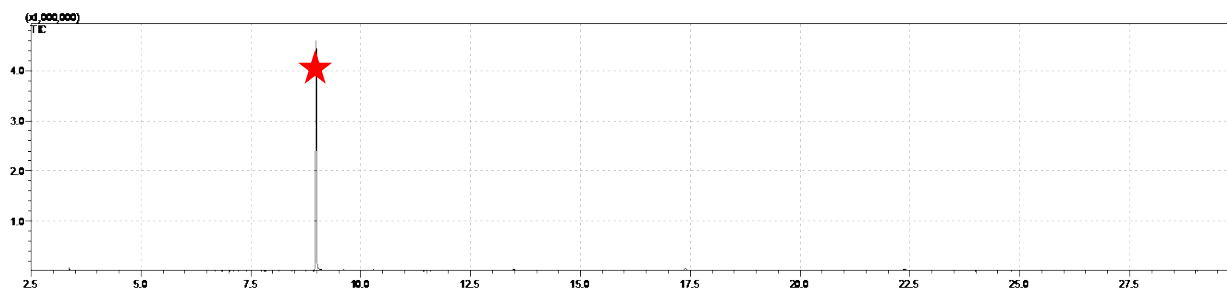


Figure 45: GC-MS chromatogram of 2-methoxy-4-vinyl phenol

The GC-MS analysis confirmed that the 2-methoxy-4-vinyl phenol retention time and spectra were identical for the pure standard and the active fraction. The microbial inhibitory activity of 2-methoxy-4-vinyl phenol was tested against *B. cereus* in a DDA (Figure 46). A 20 μ L aliquot of a 200 mg/mL solution of 2-methoxy-4-vinyl phenol in EtOH was tested along with a range of further diluted solutions.

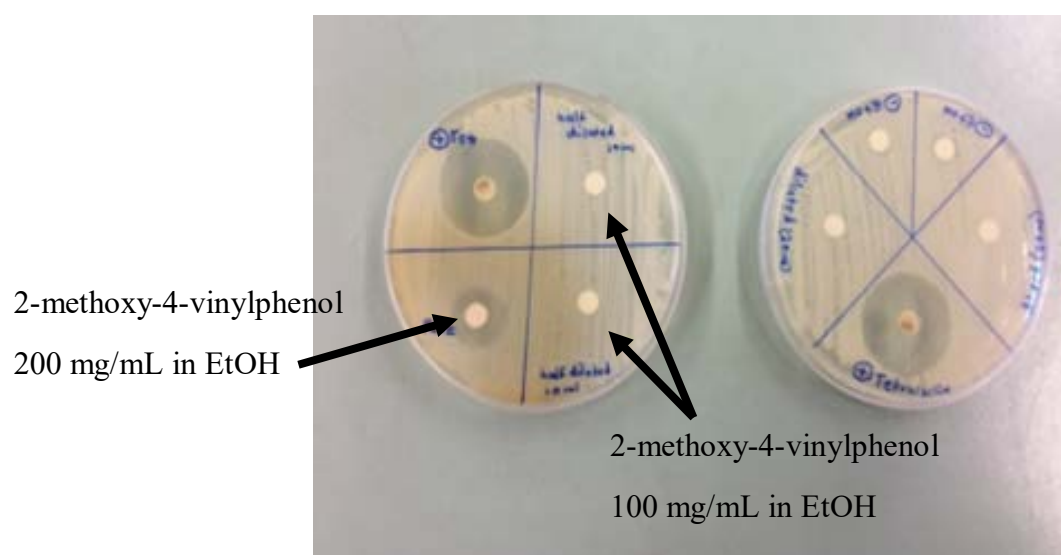


Figure 46: Disc diffusion assay of 2-methoxy-4-vinylphenol against *B. cereus*

The assays show that 2-methoxy-4-vinylphenol at a concentration of 200 mg/mL is able to inhibit the growth of *B. cereus* producing a 15 mm of zone of inhibition This is compared to a commercially available tetracycline disc (inhibiting the growth and killing of viable bacteria cells) ^[194] used as the positive control (30 μ g) with a 33 mm zone of inhibition and EtOH

(negative control) which showed no activity. It is apparent that the amount of bioactivity observed for the pure 2-methoxy-4-vinyl phenol is substantially less than expected compared to DDA for the extract fractions and it was concluded that other unknown compounds that are not included in the GC-MS database might be present. It was also feasible that the active compound has decomposed and fragmented due to the temperature of the GC-MS injector and this will be further explored later in this chapter.

5.4.3 Further separation, identification and characterisations of microbially active compounds

In collaboration with Dr Mark Butler and his associates in the Institute of Molecular Biosciences at the University of Queensland, a more sophisticated approach was undertaken utilising this research group's expertise in natural products isolation and identification.

5.4.3.1 Automated HPLC based fractionation

Separated leaves were dried in an incubator for 7 days at 60 °C and ground into a fine powder (51.1 g). The fine powder was extracted at constant temperature of 21 °C for 5 days with 250 mL CH₃CN with occasional stirring. The solvent was removed by rotary evaporation to give approximately 1.195 g of crude extract. A portion of the extract (500 mg) was dissolved in 20 mL of (1:1) CH₃CN and Milli-Q H₂O with separation obtained using a Shimadzu HPLC consisting of a Prominence Modular High Performance Liquid Chromatograph coupled to an UV-Vis Photo-Diode Array detector (SPD-M20A). An Agilent Phenyl XDB HPLC column (100 × 20 mm, 5 µm) served as the stationary phase and a mobile phase of 40% CH₃CN at a flow rate 0.2 mL/min produced 14 mg of an active fraction. This fraction was further separated on a Phenomenex C8 HPLC column (250 × 9.4 mm, 5 µm) and the collected sample fraction

was freeze dried and stored in a -20 °C refrigerator until required for further experimental analysis.

5.4.3.2 Purification and characterisation

Fraction purification was achieved using a Gilson® benchtop PLC 2020 Personal Purification System with an interchangeable pump head. The flow rate was 1 mL/min and the mobile phase was 0.05% formic acid in water (A) and 0.05% formic acid in CH₃CN (B).

Compound purity was analyzed using a Shimadzu LCMS 2020 LC/MS with a SPD-M20A UV-vis detector (λ = 200-400 nm), using a Zorbax Eclipse XDB phenyl column (3.0 × 100 mm, 3.5 μ m, flow rate 1 mL/min, 40 °C). The mobile phase was prepared using 0.05% formic acid in water (A) and 0.05% formic acid in CH₃CN (B). High-resolution mass spectrometry (HRMS) was performed on a Bruker Micro TOF mass spectrometer using (-)-ESI mode calibrated to sodium formate.

As described in Section 5.3.1.2, further antimicrobial assays were performed against different strains of Gram positive and Gram negative bacterium including different strains of *S. aureus* (ATCC 43300), *S. aureus* (NRS 17 GISA), *S. aureus* (NRS 1 GISA MRSA), *S. aureus* (VRS 10 GRSA), *S. pneumonia* (ATCC 700677), *E. faecalis* (Clinical isolate vanA) and *E. faecium* (ATCC 51559). The results for the crude extract and isolated fractions are displayed in Table 11. Whereas the controls have relatively broad spectrum activity, the fractions show excellent and specific activity against vancomycin-resistant organisms, which is quite promising.

Table 11: MIC of the crude extract and active fractions against a panel of Gram-positive and Gram-negative bacteria.

8 point dose response - 0.015 to 32 µg/mL, n=2		GP_020	GP_030	GP_035	GP_065	GP_023	GP_011	GP_025
		<i>S. aureus</i>	<i>S. aureus</i>	<i>S. aureus</i>	<i>S. aureus</i>	<i>S. pneumoniae</i>	<i>E. faecalis</i>	<i>E. faecium</i>
Polystyrene Plates 96w (Corning 3370)		ATCC 43300	NRS 17	NRS 1	VRS 10	ATCC 700677	clinical isolate	ATCC 51559
		MRSA	GISA	GISA/MRSA	GRSA	MDR	VanA	MDR/VanA
MCC ID	Sample Name	MIC (µg/mL)						
MCC_0000095:02	Vancomycin	1	8	8	2	2	>32	>32
MCC_0000561:03	Daptomycin	1, 2	8	16	4	2	8	16
Crude extract		>32	>32	>32	>32	>32	>32	>32
Active fraction	DE7359_32_108	>32	>32	32	32	32	8, 16	8
Active fraction	DE7359_32_109	>32	>32	>32	>32	>32	8	8

Abbreviations: MDR - Multi-drug resistant *S. aureus*, MRSA - Methicillin Resistant *S. aureus*, GISA - Glycopeptide intermediate *S. aureus*, GRSA – Glycopeptide resistant *S. aureus*, VRE VanA - Vancomycin-resistant *Enterococcus* carrying VanA gene.

Vancomycin and Daptomycin were used as positive controls

Figure 47 shows two compounds that have been isolated and they are identified as **(1)** neryl ferulate and **(2)** neryl *p*-coumarate, which are closely related and new antimicrobial compounds.

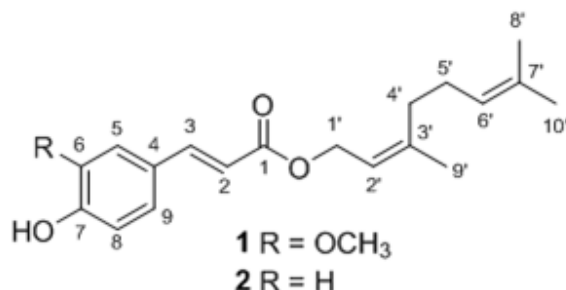


Figure 47 : Structures of (1) neryl ferulate and (2) neryl *p*-cinnamate.

Table 12: Chemical details for the structures in Figure 47

	(1) Neryl ferulate	(2) Neryl <i>p</i> -cinnamate
Chemical formula	C ₂₀ H ₂₆ O ₄	C ₁₉ H ₂₄ O ₃
Exact Mass	330.1831	300.1725
Molecular Mass	330.4240	300.3980

The purification of **(1)** and **(2)** was hampered by their close HPLC retention times and the gradual *in situ* peroxide formation and subsequent reduction to the alcohols as described in Figure 48. Singlet oxygen ene-type peroxidation of isoprene units in natural products was proposed by Kadota and co-workers in 2000 ^[195] and this reaction has been used widely in synthetic chemistry ^[196-197]. Although there is a debate about whether autoxidation occurs in the organism or upon isolation, there is evidence to show that terpenes such as α -santalene ^[198], linalool ^[199] and geraniol ^[200], which is the *E* isomer of the nerol subunit found in **1** and **2** can undergo autoxidation. We found that no autoxidation occurred when the fractions and pure compounds were stored under argon at -20 °C.

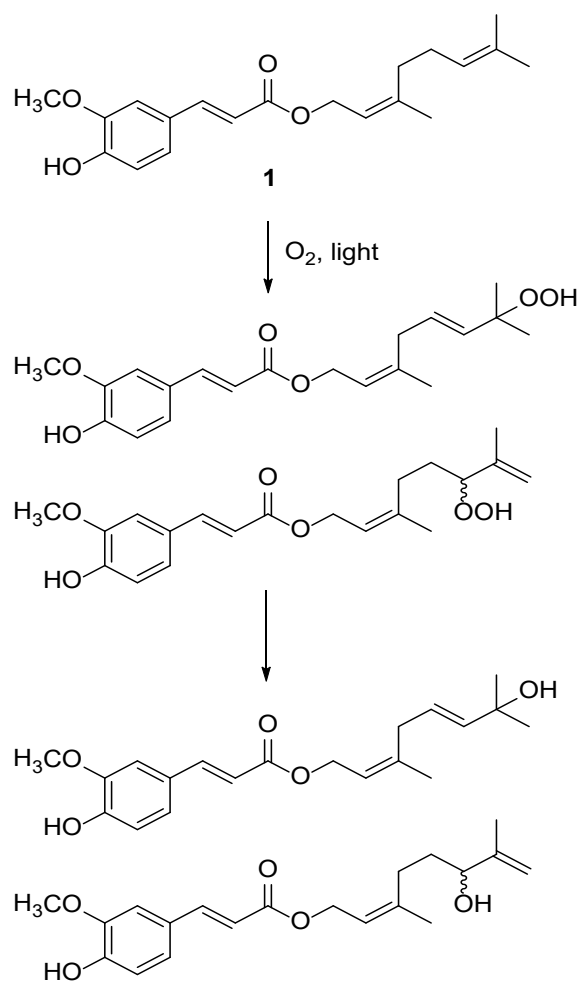


Figure 48: Proposed autoxidation mechanism of neryl ferulate (**1**)

The molecular formula of (**1**) was determined to be $\text{C}_{20}\text{H}_{26}\text{O}_4$ by HRMS and examination of the ^1H , ^{13}C , COSY, HSQC and HMBC NMR spectra indicated the presence of methoxy and hydroxy-substituted cinnamic acid with a linear C10 monoterpene, assignments are listed in Table 13 with spectra contained in Appendix B,C and D. The C10 monoterpene unit was determined to be nerol on the basis of the NMR data (C-9' δ_{C} 23.6; δ_{C} 24.0 in nerol compared to δ_{C} 17.1 in geraniol^[201]), which was attached to the carboxy group of the cinnamic acid with HMBC correlations from H₂-1' (δ_{H} 4.69, dd, J = 0.9, 7.2 Hz) to C-1 (δ_{C} 167.3). HMBC correlations from OCH₃-6 (δ_{H} 3.92, s) and H-8 (δ_{H} 6.91, d, J = 8.2 Hz) to C-6 (δ_{C} 146.7) indicated that the cinnamic acid unit was ferulic acid. Therefore, the structure of (**1**) was

determined to be neryl ferulate, which has only been reported previously as a synthetic product [202].

The molecular formula of **(2)** was determined to be $C_{19}H_{24}O_3$, which was OCH_3 less than **(1)**. Analysis of the NMR data shows that **(2)** still had the nerol monoterpene unit but had a *p*-coumaric acid in place of ferulic acid. Therefore, the structure of **(2)** was determined to be neryl *p*-cinnamate, which has not been previously reported.

Table 13: NMR spectroscopic data (600 MHz, $CDCl_3$) for (1) neryl ferulate and (2) neryl *p*-cinnamate

(1) Neryl ferulate			(2) neryl <i>p</i> -cinnamate	
Position	δ_C , type	δ_H , mult. (<i>J</i> in Hz)	δ_C , type	δ_H , mult. (<i>J</i> in Hz)
1	167.3, C		167.3, C	
2	115.6, CH	6.30, d (15.9)	115.8, CH	6.31, d (15.9)
3	144.7, CH	7.61, d (15.9)	144.2, CH	7.63, d (15.9)
4	127.1, C		127.5, C	
5	109.2, CH	7.03, d (1.9)	129.9, CH	7.42, br d (8.5)
6	146.7, CH		115.8, CH	6.83, br d (8.5)
CH ₃ O-6	55.9, CH ₃	3.92, s	-	-
7	147.7, C		157.4, C	6.83, br d (8.5)
8	114.7, CH	6.91 d (8.2)	115.8, CH	6.91 d (8.2)
9	123.1, CH	7.06, dd (1.9, 8.2)	129.9, CH	7.42, br d (8.5)
OH-7		5.84, s		5.17, br s
1'	61.1, CH	4.69, dd (0.9, 7.2)	61.1, CH	4.69, dd (0.9, 7.2)
2'	119.3, CH	5.44, br t (7.2)	119.3, CH	5.43, br t (7.3)
3'	142.8, C		142.7, C	
4'	32.2, CH ₂	2.16, m	32.2, CH ₂	2.16, m
5'	26.7, CH ₂	2.10, dt (7.0, 7.0)	26.7, CH ₂	2.10, dt (7.0, 7.0)
6'	123.6, CH	5.12, tq (7.0, 1.4, 1.4)	123.6, CH	5.11, tq (7.0, 1.5, 1.5)
7'	132.2, C		132.2, C	
8'	17.7, CH ₃	1.61, br s	17.7, CH ₃	1.61, br s
9'	23.6, CH ₃	1.79, br s	23.6, CH ₃	1.79, br s
10'	25.7, CH ₃	1.69, br s	25.7, CH ₃	1.69, br s

The isolated compounds **(1)** neryl ferulate and **(2)** neryl *p*-cinnamate were profiled against a panel of Gram-positive and Gram-negative bacteria, fungi and the human kidney-derived cell lines HEK293 and HepG2 with the results presented in Table 14. Both **(1)** and **(2)** had no activity against Gram-negative bacteria and only **(1)** displayed marginal antifungal activity against *Cryptococcus neoformans*. **(1)** Neryl ferulate showed activity against a small panel of antibiotic-resistant Gram-positive bacteria in the range of 8 to 32 µg/mL but showed some cytotoxicity against the HEK293 and HepG2 human kidney cell lines at around 90 µg/mL. **(2)** Neryl *p*-cinnamate was only active against *E. faecium* and showed a similar cytotoxicity profile.

The only antibacterial activity of related compounds was the recent report of very weak antibacterial activity of geranyl cinnamate against *S. aureus* and *E. coli* (MIC 5000 µg/mL) [203]. Other biological activities of related compounds include the inhibition of lipid absorption and accumulation by geranyl caffeate [204] and inhibition of LPS-induced production of nitric oxide in murine macrophages by *E* and *Z*-geranyl ferulate [205], as well as geranyl caffeate and farnesyl caffeate [206]. Farnesyl caffeate and farnesyl *p*-coumarate were reported to show cytotoxicity (GI₅₀ 1.1–2.5 and 4.5–6.4 µg/mL respectively), [207] while farnesyl caffeate showed *in vitro* antitrypanosomal activity (IC₅₀ 0.23 µg/mL) and cytotoxicity (IC₅₀ 13.3 µg/mL) against the MRC5 cell line [208].

Table 14: Biological activity of (1) Neryl Ferulate and (2) Neryl *p*-Cinnamate against a panel of bacteria, fungi and cell lines

Bacterial and fungal strains, and cell lines	Compounds		
	(1)	(2)	Controls
Gram negative bacteria	MIC (µg/mL)		
<i>E. coli</i> ATCC 25922	> 64	> 64	0.3 (COL), 0.3 (PMXB)
<i>A. baumannii</i> ATCC 19606	> 64	> 64	0.5/1 (COL), 0.3 (PMXB)
Gram positive bacteria	MIC (µg/mL)		
<i>S. aureus</i> ATCC 43300 (MRSA)	16	> 64	1 (VAN), 4 (DAP)
<i>S. aureus</i> NARSA NRS 17 (GISA)	16	> 64	>32 (VAN), 16/32 (DAP)
<i>S. aureus</i> NARSA VRS 1 (VRSA)	16/32	> 64	>32 (VAN), 8 (DAP)
<i>S. pneumoniae</i> ATCC 700677 (MDR)	8/16	64	2 (VAN), 8 (DAP)
<i>E. faecium</i> ATCC 51559 (VRE VanA)	8	8	>32 (VAN), 32 (DAP)
Fungi	MIC (µg/mL)		
<i>C. albicans</i> ATCC 90028	> 64	> 64	0.5/0.3 (FLU)
<i>C. neoformans</i> H99 ATCC 208821	64/32	>64/32	8 (FLU)
Cell lines	CC₅₀ (µg/mL)		
HEK293 ATCC CRL-1573	90	90	23 (TAM)
HepG2 ATCC HB-8065	90	47	23 (TAM)

Abbreviations: MDR - Multi-drug resistant, MRSA - Methicillin Resistant *S. aureus*, GISA - Glycopeptide intermediate *S. aureus*, VRSA - Vancomycin Resistant Intermediate *S. aureus*. VRE VanA - Vancomycin-resistant *Enterococcus* carrying VanA gene. HEK293 - human embryonic kidney cells, HepG2 - hepatocellular carcinoma cells. Controls: Colistin (COL), polymyxin B (PMXB), vancomycin (VAN), daptomycin (DAP) and tamoxifen (TAM).

5.4.4 Thermal degradation experiments

As discussed in Section 5.4.2, it was suspected that the extract underwent thermal degradation when exposed to the high temperature of the GC-MS injector with 2-methoxy-4 vinyl phenol identified as a bioactive compound. In order to investigate this further, a thermal degradation experiment was performed and the results have proven to be very interesting. HPLC analysis was used to compare the chromatograms for an active fraction before the heat treatment with the same fraction after the heat treatment where the sample was exposed to a temperature of 230 °C for 30 minutes in a vacuum oven. A further chromatogram of the pure 2-methoxy-4 vinyl phenol was obtained with the concentration being 1 mg/mL. The resulting HPLC chromatograms are displayed in Figure 49 with a dashed line that shows a peak with the same retention time as 2-methoxy-4 vinyl phenol forms after the heat treatment of the extract

indicating that it is the product of thermal degradation. It is also quite noticeable the sizes of other peaks in the extract are dramatically decreased due to the heat treatment.

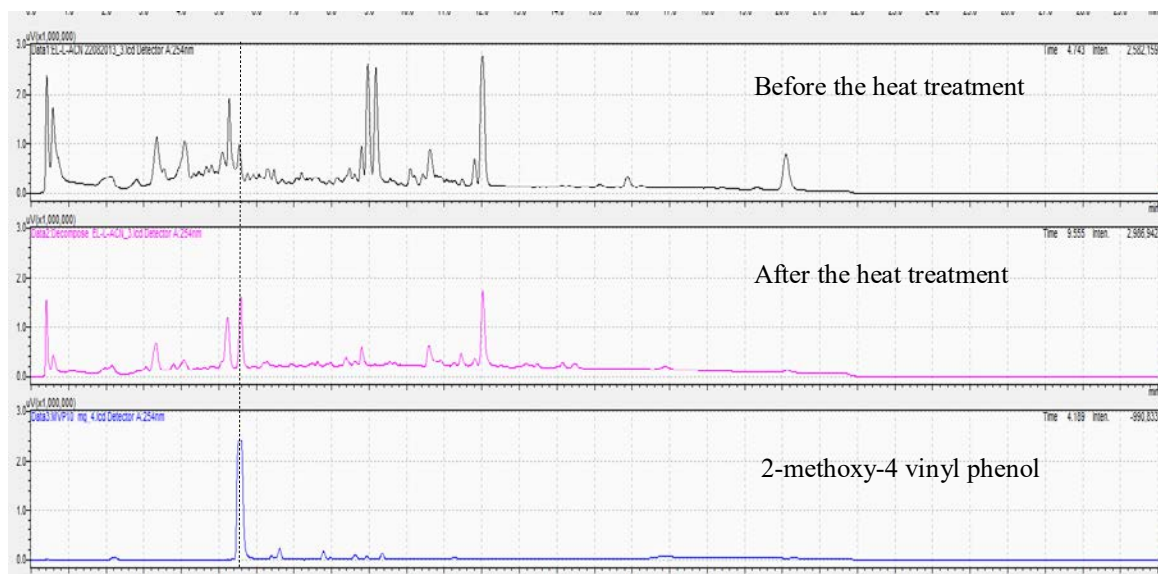


Figure 49: HPLC chromatograms of the thermal degradation experiment for an active fraction from *E. longifolia*

Examination of the molecular structures for the identified compounds **(1)** neryl ferulate and **(2)** neryl *p*-cinnamate shows that both compounds contain the 2-methoxy-4 vinyl phenol fragment as shown in (Figure 50) for **(1)** neryl ferulate.

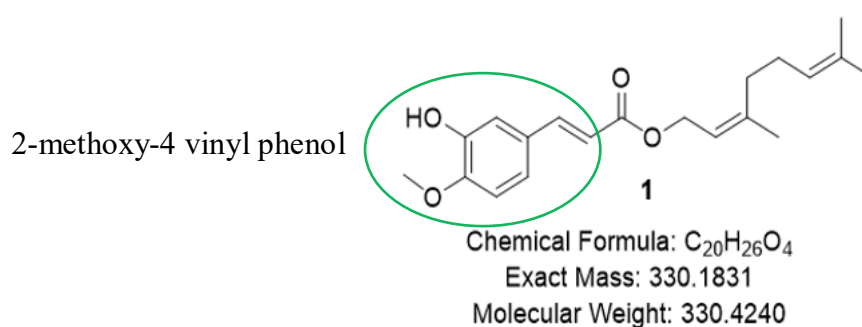


Figure 50: Chemical structure of **(1)** neryl ferulate and with the thermal degradation product 2-methoxy-4 vinyl phenol circled in green

5.5 Conclusion

In conclusion, two novel natural compounds, neryl ferulate and neryl *p*-coumarate, have been identified using bioguided isolation from the leaves of the indigenous Australian medicinal plant *E. longifolia*. Neryl ferulate displayed moderate activity against various Gram-positive bacteria, while neryl *p*-coumarate was only active against *E. faecium*. As both compounds displayed weak cytotoxicity effects upon mammalian cells and had issues with autoxidation, they were not pursued further as antibacterial lead compounds. However, their discovery has further advanced our knowledge of an important indigenous medicine. Furthermore, both of these compounds easily degraded thermally to form 2-methoxy-4 vinyl phenol at higher temperatures and this product might be responsible for the effectiveness of the smoke inhalation treatment when the plant is used as an indigenous medicine.

Chapter 6

Screening of ethnomedicinal Southeast Asian plants for antimicrobial and antifungal activities

6.1 Introduction

Historically, Eastern Malaysian indigenous tribes have used traditional herbal remedies or medicinal plants for centuries to cure infectious diseases ^[106]. These days, they inhabit the Malaysian province of Sarawak, which is situated on the island of Borneo. Borneo is the third biggest island in the world, well known as its rugged, dense rainforest and home to some of the most endangered animals and trees ^[209]. Most of the forested areas are protected by the Malay government ^[210] and are World Heritage listed by the United Nations Educational, Scientific and Cultural Organization (UNESCO) ^[211]. The plant extracts and herbal remedies are quite distinctive with frequent use by several indigenous tribes in Sarawak ^[212]. The Sarawak Biodiversity Centre in Sarawak has undertaken intensive work with tribal leaders to document the ethnomedicinal knowledge about the plants that are still in use.

In collaboration with the Sarawak Biodiversity Centre, this research was performed to evaluate the ethnomedicinal importance of extracts obtained from plants that are native to the province of Sarawak in Malaysia. As most of the plants are used to treat infective illnesses, it is important to establish that they possess antibacterial properties against Gram-positive and Gram-negative pathogenic bacteria, as well as their potential antifungal properties. Initially, fifty crude plant extracts were examined using antimicrobial assays and six plants were identified as the most appropriate plant species for further analysis, namely *Baccaurea lanceolata* (Miq.) Müll. Arg., *Fibraurea tinctoria* Lour, *Goniothalamus tapisoides* Mat Salleh, *Goniothalamus velutinus* Airy Shaw, *Polyalthia hookeriana* King and *Pyrenaria serrata* Bl. var *masocarpa* (Korth) H. Keng.

6.2 Material and methods

All plants tested have been fully documented for their traditional medicinal use to treat symptoms of infectious diseases by the SBC. Preliminary screening of fifty crude plant extracts identified six extracts with potential antimicrobial activities. These were the main focus of this chapter.

6.2.1 Preparation of extracts

The crude extracts were prepared according to Section 3.3.2.

6.2.2 Bacterial and fungal isolates and media used

Bacterial and fungal isolates that were used in this study have been discussed in Section 3.3.4. In addition, two additional strains of *C. albicans*, clinical isolate (SC5314) and laboratory isolate (DAY286), were also employed.

6.2.3 Antibacterial and antifungal assays

All the microbial assays were performed according to the protocols described in Section 3.3.4 and include:

- Disc diffusion assay (DDA)
- Determination of minimum inhibitory concentration (MIC)
- Determination of bacterial and fungicidal concentration (MBC/MFC)

6.3 Results and discussion

6.3.1 Disc diffusion assay

Antimicrobial activity was evaluated using the disc diffusion method with the median diameter of the zone of inhibition measured ^[164]. The disc used in this assay was 5 mm in diameter and the negative control was DMSO. Although all of these plants have been documented due to

their use as natural medicines, according to the preliminary results from the disc diffusion assays presented in Table 15, it was found that only six of the plants were identified as having potentially antibacterial activity. Information obtained from the SBC identified them as *B. lanceolata*, *F. tinctoria*, *G. tapisoides*, *G. velutinus*, *P. hookeriana* and *P. serrata*.

Table 15: Summary of DDA results for the Sarawak extracts

No	Special Barcode	Voucher specimen	Scientific name (Family)	Antimicrobial activity		
				<i>E. coli</i>	<i>S. aureus</i>	<i>C. albicans</i>
1	A000461020301			-	-	-
2	A000461010301	SABC 0242	<i>Fibraurea tinctoria</i> (Menispermaceae)	+	+	+
3	A000492010301			-	-	-
4	A000602010301	SABC 0422	<i>Polyalthia hookeriana</i> (Annonaceae)	+	+	+
5	A000602020301	SABC 0422	<i>Polyalthia hookeriana</i> (Annonaceae)	+	+	+
6	A000617040103			-	-	-
7	A000760020301			-	-	-
8	A000803010103	SABC0782	<i>Mussaenda elmeri</i> (Rubiaceae)	-	-	-
9	A001001010103			-	-	-
10	A001042030103	SABC 1106	<i>Pyrenarea serrata</i> (Theaceae)	+	+	+
11	A001066020103			-	-	-
12	A001347040103	SABC 1979	<i>Baccaurea lanceolata</i> (Euphorbiaceae)	+	+	+
13	A001349040103	SABC1753	<i>Trigonopleaura malayana</i> (Euphorbiaceae)	-	-	-
14	A001373010103	SABC1768	<i>Santiria apiculata</i> (Burseraceae)	-	-	-
15	A001989010103			-	-	-
16	A002427010103			-	-	-
17	A002576020103			-	-	-
18	A002644010103			-	-	-
19	A002657010103			-	-	-
20	A002677020103			-	-	-
21	A002748020103			-	-	-

Summary of DDA results for the Sarawak extracts. (cont.)

No	Special Barcode	Voucher specimen	Scientific name (Family)	Antimicrobial activity		
				<i>E. coli</i>	<i>S. aureus</i>	<i>C. albicans</i>
22	A002749040103			-	-	-
23	A002753030103	SABC 3373	<i>Baccaurea lanceolata</i> (Euphorbiaceae)	+	+	+
24	A002791020103	SABC 1989	<i>Goniothalamus tapisoides</i> (Annonaceae)	+	+	+
25	A002791030103			-	-	-
26	A002793020103	SABC 1992	<i>Goniothalamus velutinus</i> (Annonaceae)	+	+	+
27	A002805020103			-	-	-
28	A002805010103			-	-	-
29	A002805030103	SABC1984	<i>Anisophyllea disticha</i> (Anisophylleaceae)	-	-	-
30	A002814030103	SABC1996	<i>Trigonopleaura malayana</i> (Euphorbiaceae)	-	-	-
31	A002822020103	SABC1988	<i>Trivalvaria macrophylla</i> (Euphorbiaceae)	-	-	-
32	A002822040103			-	-	-
33	A002828010103			-	-	-
34	A002837050103			-	-	-
35	A002890010103			-	-	-
36	A002895030103			-	-	-
37	A002895020103	SABC3970	<i>Baccaurea angulata</i> (Euphorbiaceae)	-	-	-
38	A002970030103	SABC3809	<i>Tetracera macrophylla</i> (Dilleniaceae)	-	-	-
39	A002970010103			-	-	-
40	A002981010103			-	-	-
41	A003161010103	SABC1528	<i>Calophyllum lanigerum</i> (Clusiaceae)	-	-	-
42	A003161040103	SABC1528	<i>Calophyllum lanigerum</i> (Clusiaceae)	-	-	-
43	A003546020103	SABC4492	<i>Albizia corniculata</i> (Fabaceae)	-	-	-

Summary of DDA results for the Sarawak extracts. (cont.)

No	Special Barcode	Voucher specimen	Scientific name (Family)	Antimicrobial activity		
				<i>E. coli</i>	<i>S. aureus</i>	<i>C. albicans</i>
44	A003561020103			-	-	-
45	A003699010103			-	-	-
46	A003703010103			-	-	-
47	A003807010103			-	-	-
48	A003857010103			-	-	-
49	A003857030103			-	-	-
50	A003865010103			-	-	-

+ Indicates plant species active against bacterial or fungal isolate.

- Indicates plant species not active against bacterial or fungal isolate.

Empty spaces in Voucher specimen number column and scientific name column indicate confidential information belonging to the SBC

These selected plant species were subjected to a more extensive panel of microorganisms and the observations of the DDA are listed in Table 16. *G. velutinus* and *G. tapisoides* exhibited some inhibitory effects against both Gram-negative and Gram-positive bacteria that include *E. coli*, *S. enterica* serovar Typhimurium, *M. catarrhalis*, *B. cereus* and *S. aureus*. Most of the plant extracts showed antimicrobial activity against the *B. cereus* strain. The extract from *F. tinctoria* displayed antimicrobial activity against only two Gram-positive bacteria, *B. cereus* and *S. aureus*. The two plant extracts from *B. lanceolata* (a and b) showed wide activity against all the bacterial strains tested with the extract.

Since the extracts obtained from *P. serrata* species displayed antifungal activity against the *C. albicans* isolate, further testing was performed using other laboratory and clinical strains of *C. albicans*. Figure 51 (A and B) show the disc diffusion assays against the *C. albicans* clinical isolate (SC5314) and laboratory isolate (DAY286), with different concentrations of Amphotericin B used as the positive control as reducing number of viable cell count ^[213] and DMSO used as a negative control. The effectiveness of the *P. serrata* extract against the *C. albicans* isolates was indicated by zones of inhibition of 12 mm and 12.5 mm for the clinical and laboratory strains, respectively. The zone of inhibition of the plant extract was actually larger than that of the positive control.

Table 16: DDA antimicrobial screening of selected plant extracts

Plant Name	Zone of inhibition (mm)							
	<i>E. coli</i>	<i>P. aeruginosa</i>	<i>S. enterica</i>	<i>M. catarrhalis</i>	<i>S. pyogenes</i>	<i>B. cereus</i>	<i>S. aureus</i>	<i>C. albicans</i>
<i>F. tinctoria</i>	0.0	0.0	0.0	0.0	0.0	10.0	10.0	0.0
<i>P. hookeriana</i>	7.0	0.0	0.0	9.0	0.0	11.3	8.0	0.0
<i>P. serrata</i>	0.0	0.0	0.0	7.0	0.0	10.0	0.0	12.5
<i>B. lanceolata</i> ^a	7.0	8.3	7.3	12.0	7.6	12.0	8.0	0.0
<i>B. lanceolata</i> ^b	7.0	8.3	7.0	10.6	7.0	9.6	7.0	0.0
<i>G. tapisoides</i>	7.0	0.0	8.0	8.0	0.0	10.6	8.0	0.0
<i>G. velutinus</i>	10.0	0.0	9.3	8.0	0.0	11.3	9.0	0.0
Tetracycline ^c	24.0	15.3	21.6	40.6	19.6	30.6	30	0.0
Amphotericin B ^c	0.0	0.0	0.0	0.0	0.0	0.0	0.0	18.0

The discs used in this study were impregnated with 400 µg of the plant extracts.

^{a,b} Two separate samples of *B. lanceolata* were collected from the same location during different months.

^c Tetracycline (30 µg) and Amphotericin B (1.38 µg) were used as positive controls for antibacterial and antifungal susceptibility tests, respectively.

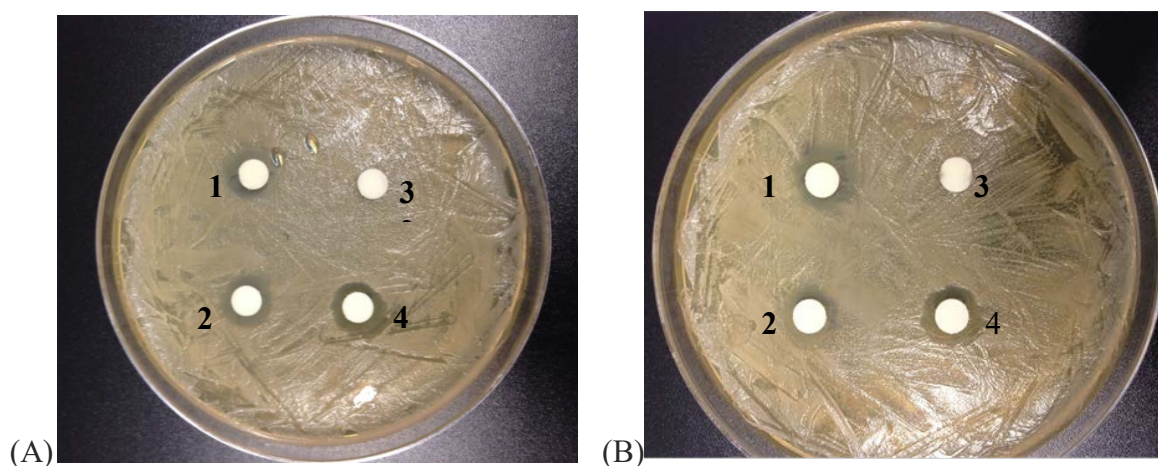


Figure 51: (A) Disc diffusion assay for *C. albicans* clinical isolate (SC5314) (B) Disk diffusion assay for *C. albicans* laboratory isolate (DAY286)

Disc 1: Impregnated with Amphotericin B (10µg)

Disc 2: Impregnated with Amphotericin B (1µg)

Disc 3: Impregnated with DMSO

Disc 4: Impregnated with 100µg/mL *P. serrata* extract

6.3.2 MIC and MBC/MFC determination for the selected extracts.

MIC and MBC/MFC analysis was performed using the Mueller Hinton broth dilution method with doubling serial dilution (50, 25, 12.5, 6.25 and 3.13 µg/mL). The initial concentration of the plant extracts used was 100 µg/mL. Absorbance readings and subculturing methods were conducted as described in Section 3.3.4.2 to determine MICs and Section 3.3.4.3 for the determination of MBCs/MFCs. The results summary in Table 17 displays the MIC and MBC/MFC values for the plant extracts against a range of microorganisms.

Table 17: MIC and MBC/MFC values for selected plant extracts

Plant Name	Concentration (µg/mL)	<i>E. coli</i>	<i>P. aeruginosa</i>	<i>S. enterica</i>	<i>M. catarrhalis</i>	<i>S. pyogenes</i>	<i>B. cereus</i>	<i>S. aureus</i>	<i>C. albicans</i>
<i>F. tinctoria</i>		n/a	n/a	n/a	n/a	n/a	25	50	n/a
	MBC	n/a	n/a	n/a	n/a	n/a	≥100.00	100	n/a
<i>P. hookeriana</i>	MIC	25	n/a	n/a	25	n/a	12.5	25	n/a
	MBC	50	n/a	n/a	50	n/a	50	50	n/a
<i>P. serrata</i>	MIC	n/a	n/a	n/a	25	n/a	12.5	n/a	25
	MBC/MFC	n/a	n/a	n/a	100	n/a	≥100.00	n/a	50
<i>B. lanceolata</i> ^a	MIC	25	12.5	25	25	25	12.5	50	n/a
	MBC	100	50	100	50	100	≥100.00	100	n/a
<i>B. lanceolata</i> ^b	MIC	25	12.5	25	25	25	12.5	50	n/a
	MBC	100	50	100	50	100	≥100.00	100	n/a
<i>G. tapisoides</i>	MIC	25	n/a	25	12.5	n/a	12.5	25	n/a
	MBC	50	n/a	100	50	n/a	100	100	n/a
<i>G. velutinus</i>	MIC	25	n/a	25	12.5	n/a	12.5	25	n/a
	MBC	50	n/a	100	100	n/a	≥100.00	50	n/a

MBC, minimum bactericidal concentration; MFC, minimum fungicidal concentration; MIC, minimum inhibitory concentration; n/a, no activity.

^{a,b} Two separate samples of *B. lanceolata* were collected from the same location during different months.

The plant extracts from *P. hookeriana*, *B. lanceolata*^a, *B. lanceolata*^b, *G. tapisoides* and *G. velutinus* produced MIC values of 25 µg/mL and MBC values between 50 to 100 µg/mL against *E. coli*. Only the two *B. lanceolata* extracts showed activity against *P. aeruginosa* with MIC and MBC values of 12.5 µg/mL and 50 µg/mL, respectively. The *Goniothalamus* and *Baccaurea* extracts were active against *S. enterica* serovar Typhimurium with respective MIC and MBC values of 25 µg/mL and 100 µg/mL. All of the plant extracts, except *F. tinctoria*, showed antibacterial activity against *M. catarrhalis* with MIC values in the range 12.5 µg/mL to 25 µg/mL and MBC values between 50 µg/mL and 100 µg/mL with the *Goniothalamus* extract exhibiting the strongest activity. The *B. lanceolata* extract was the most effective inhibitory agent for *S. pyogenes* with MIC and MBC levels of 25 µg/mL and 100 µg/mL, respectively.

Most of the plant extracts, except *F. tinctoria*, had the same MIC values against *B. cereus* and the MBC values were more than 100 µg/mL except for *G. tapisoides* with a resulting MIC of 100 µg/mL. Except for the *P. serrata* extract, all the other plants showed acceptable inhibitory activity against *S. aureus*. The strongest candidates were *G. velutinus* and *P. hookeriana* with the MIC and MBC values of 25 µg/mL and 50 µg/mL, respectively. Overall, the two extracts from *B. lanceolata* exhibited the lowest MIC and MBC values thereby showing the greatest activity towards all the bacteria used in this study.

Only the *P. serrata* extract exhibited any antifungal activity against *C. albicans* with MIC and MBC values of 25 µg/mL and 50 µg/mL, respectively. A major problem associated with this pathogen is the formation of biofilms on the surface of medical devices inside the human body (cardiac valves, catheters, etc.) [214], which promotes systemic fungal infection (candidiasis) that usually occurs in immune-compromised patients [215]. Subject to further research and

analysis, it may be found that a drug developed from *P. serrata* can be used as an anticandidal agent.

In summary, out of the 50 plant extracts that were screened, only six exhibited broad spectrum activity against several strains of bacteria and fungi. Similar to literature observations, plant extracts generally show more inhibitory activity against Gram-positive than Gram-negative bacteria against selected plant extracts ^[216]. However, in most cases there was a significant effect against both Gram-positive and Gram-negative bacteria.

All of the selected plant extracts showed considerable inhibitory activity against pathogenic organisms and could be active against infections when used as medicines. For example, stomachaches, diarrhoea and enteritis most probably result from bacterial infections caused by *E. coli*, *Salmonella*, *B. cereus* and *S. aureus* due to contaminated food and water ^[217]. This correlates well with the use of medicines derived from extracts of *B. lanceolata*. In general, headaches and fever are often a consequence of bacterial infections and treatment using an extract of *G. velutinus* shows strong broad spectrum activity that supports its use. Also, any snake or insect bite-related infections can be triggered by the above mentioned pathogens ^[218] with most skin infections being caused by *S. aureus*, the results confirm the effectiveness of using extracts from *F. tinctoria*. Vaginitis is often due to infection from *C. albicans*, but there has been no recorded traditional knowledge that describes the use of *P. serrata* for this purpose. However, the results show that any treatment based on *F. tinctoria* will only be effective if the infection is due to *S. aureus*.

6.4 Conclusions

The use of these plant species by the indigenous peoples of Borneo in the treatment of various microbial illnesses can be supported by the reported findings. The results confirm that extracts from these plants have inhibitory effects on pathogenic microorganisms responsible for some common diseases such as diarrhoea, stomachache, fever, headaches, coughs and inflammation. Based on this evidence, it is reasonable to suggest that some traditional medicinal plants could be more widely utilised as novel therapeutic agents for the treatment of microbial diseases, including those caused by antibiotic-resistant microorganisms. The results also reveal the need for urgency in collecting and documenting plant specimens, as the traditional knowledge of plants used for medicinal purposes is slowly vanishing.

Plant extracts derived from *F. tinctoria*, *P. hookeriana*, *P. serrata*, *B. lanceolata*, *G. tapisoides* and *G. velutinus* demonstrated antimicrobial properties. *P. serrata* showed significant antifungal activity against *C. albicans* with minimum inhibitory and minimum fungicidal concentrations of 25 µg/mL and 50 µg/mL respectively. The outcomes of this study have highlighted the importance of using the traditional knowledge to develop highly potent herbal remedies for protection against human and animal pathogens. Of all the extracts, *B. lanceolata* consistently showed the greatest antimicrobial activity and further effort to identify the specific chemicals in the extract that provide the bioactivity is described in Chapter 7.

Chapter 7

Application of analytical and statistical approaches for isolation and identification of bioactive compounds from *Baccaurea lanceolata*

7.1 Introduction

The screening undertaken in Chapter 6 suggests that the *B. lanceolata* crude plant extract has the highest activity against a wide panel of microorganisms and the active compounds will be targeted in this Chapter. Isolation was attempted using the Solid Phase Extraction approach with the primary separation completed with two different series of CH₃CN/H₂O ratios and bioassays identified the most active of these fractions. In this case, the Milli-Q water fraction was identified as the most active fraction against *S. aureus* and then secondary fractionation was performed on this fraction. The identification and characterisation was completed using GC-MS and LC-MS, in conjunction with a statistical correlation analysis.

The interpretation of quantitative dose-response data for a plant extract is complicated because the active compound(s) have both unknown concentration(s) and mode(s) of action. However, assuming that a greater concentration will result in a stronger response, a straightforward ranking test based on Spearman correlation ^[219] between the relative chemical concentrations in the fractions and the measured bioactive response for each fraction should enable the most bioactive compound(s) to be identified. Spearman's correlation coefficient (ρ) is the most appropriate instrument to distinguish the source of bioactivity as the data employed in this analysis have a non-linear and monotonic relationship.

7.2 Experimental

7.2.1 Preparation of crude extract

The *B. lanceolata* plant was collected in 2007 by the Sarawak Biodiversity Centre as a part of its Traditional Knowledge Documentation Program. The initial botanical identification of the collected plant was performed by the Sarawak Biodiversity Centre and the voucher specimen (SABC3373) was deposited in the Centre's herbarium. The fruit from the plant was initially

dried in an oven, then ground into a powder and the resulting material was extracted using 1:1 (v/v) dichloromethane and methanol as described in Section 3.3.2.

7.2.2 Primary fractionation by Solid Phase Extraction

The details are as described in Section 3.3.5 with 110.4 mg of the crude plant extract loaded onto the SPE cartridge. The solvent was removed in a RVC 2-18 Rotor Evaporator at 40 °C (Martin Christ Rotational Vacuum Concentrator) and the residue was reconstituted in 200 µL of Milli-Q water for antimicrobial susceptibility testing.

7.2.3 Secondary fractionation by Solid Phase Extraction

Based on the bioactivity results from the primary fractionation, it was found that the Milli-Q water fraction was the most active. Further plant extract (116.4 mg) was loaded onto a conditioned SPE cartridge and elution was performed using Milli-Q water in 1 mL aliquots repeated 20 times. Each 1 mL aliquot was collected in a micro-centrifuge tube and subjected to freeze drying for 24 hrs. The freeze-dried fractions were then resuspended in 200 µL of Milli-Q water and subjected to antimicrobial testing.

7.2.4 Antimicrobial susceptibility tests

7.2.4.1 DDA antimicrobial assays

Overnight nutrient broth cultures of Gram-positive bacteria *B. cereus* (ATCC 11778), *S. aureus* (ATCC 12600), *S. pyogenes* (ACM 178) and the Gram-negative bacteria *E. coli* (ATCC 25922), *P. aeruginosa* (ATCC 10145), *S. enterica* serovar Typhimurium (ATCC 13311) were used for antimicrobial assays after both the primary and secondary fractionations. The details for this testing are described in Section 3.3.4.1 with 10 µL of sample applied to the discs.

7.2.4.2 Determination of the relative MIC for the active fractions against *S.aureus*

The MIC of the fractions was determined by the broth micro-dilution method as described in Section 3.3.4.3. Two-fold serial dilution of the fractions was performed in a 96-well plate using Mueller Hinton Broth (MHB). A 10 μ L aliquot of *S. aureus* that had been previously adjusted to match a 0.5 McFarland standard was added to each well and the plates were incubated at 37 °C for 24 hrs. Tetracycline was used as a positive control and Milli-Q water as the negative control.

7.2.4.3 Determination of colony-forming units (CFU) using *E. coli*

To assess the antibacterial effect of secondary fractions on Gram-negative bacteria, *E. coli* cultures were adjusted to match a 0.5 McFarland standard ($\sim 1 \times 10^8$ CFU/mL). A volume of 90 μ L of *E. coli* culture was seeded on MHB agar plates with 10 μ L of two-fold serially diluted aqueous fractions of the extract. Seeded MHB agar plates without extracts cultured under the same conditions were used as controls. All the seeded plates were incubated at 37 °C for 24 hrs and the number of colonies was then counted.

7.2.5 Chromatographic analysis

7.2.5.1 GC-MS analysis of the fractions

The GC-MS analyses were completed on a Shimadzu GC Chromatograph GC 2010 with the methodology described in Section 3.3.6.1.

7.2.5.2 Derivatisation the active fractions

Bioactive secondary fractions were derivatised according to the method described in Section 3.3.6.1.1.

7.2.5.3 UPLC-Q-Exactive Orbitrap Mass Spectrometry

LC-MS analysis was conducted in collaboration with Thermofisher Scientific Australia at their Demonstration Laboratory in Scoresby, Melbourne, Australia. The details are described in Section 3.3.6.3.

7.3 Statistical Analysis

All the experiments were performed in triplicate except where otherwise stated. The data are presented as mean \pm standard deviation (mean \pm SD). Statistical analysis was performed using an online statistical package, MetaboAnalyst 3.0 (TMIC, Edmonton, Canada). Spearman correlation analysis was used with data from GC-MS analysis and LC-MS analysis in both positive and negative modes. A *P*-value of < 0.05 was considered statistically significant.

7.4 Results and discussion

7.4.1 Primary fractionation

A stepwise separation of the extract using SPE with increasingly non-polar fractions enabled the optimal solvent mixture to be selected on the basis of bioguided fractionation. Figure 52 shows the results from DDA testing against *B. cereus*.



Figure 52: Primary SPE fractionation and inhibitory activity against *B. cereus*

The Milli-Q water fraction was found to be the only fraction that exhibited antimicrobial activity against the array of bacteria tested and indicates that the most bioactive components of the plant extract are relatively polar. Consideration of all the DDA results showed that the Milli-Q water fraction was active against both Gram-negative and Gram-positive bacteria. Amongst the Gram-negative bacteria, the antimicrobial activity decreased in the order: *E. coli* > *P. aeruginosa* > *S. enterica* and amongst the Gram-positive bacteria, *B.cereus* was more susceptible than *S. pyogenes* and *S. aureus*. The DDA results are presented in Figure 53 and, based on these results, secondary fractionation was carried out on the Mill-Q water fraction.

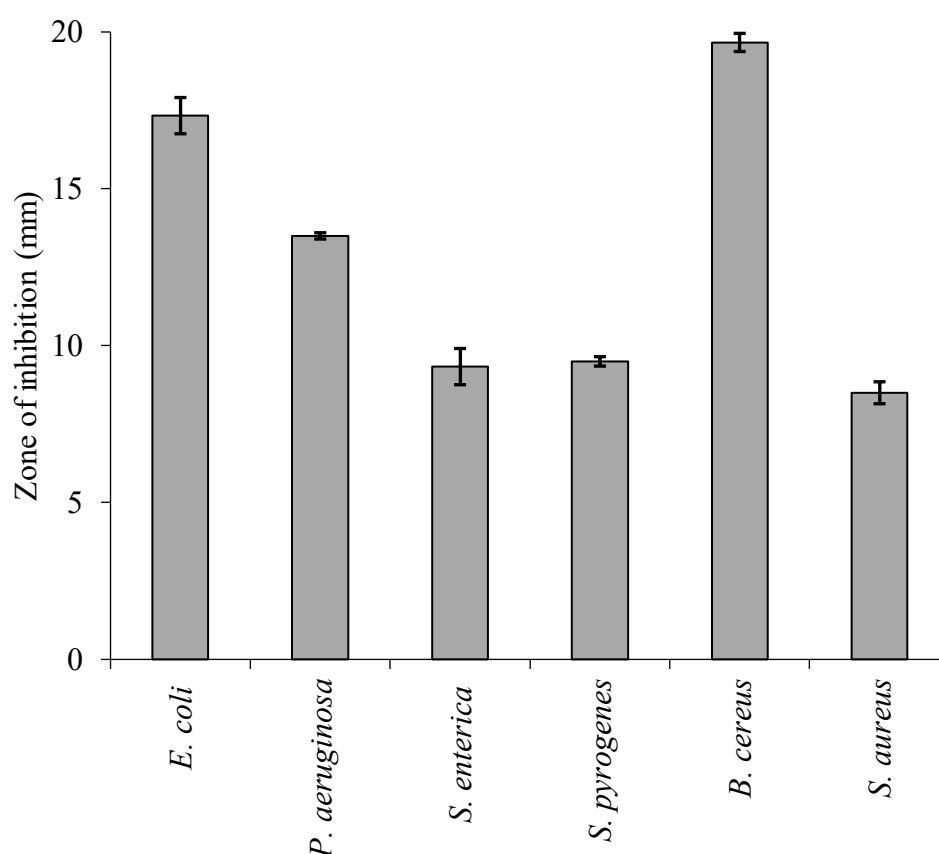


Figure 53: Disc diffusion assay results showing antimicrobial activity of the Milli-Q water fraction with various bacterial cultures

7.4.2 Secondary fractionation

7.4.2.1 Relative MIC and MBC values

Secondary fractionation was performed using only Milli-Q water as the eluting solvent. Twenty fractions were collected and DDA was performed for each fraction against *S. aureus*. Figure 54 illustrates the inhibitory microbial activities for the first seven fractions against *S. aureus* as the remaining thirteen fractions were found to be inactive under the conditions used in the DDA. The results indicated that the second fraction exhibited the highest activity against *S. aureus*. The antimicrobial activity decreased in the order: Fraction 2 > Fraction 3 > Fraction 4 > Fraction 1 \approx Fraction 5 where Fractions 6 and 7 did not show any activity.

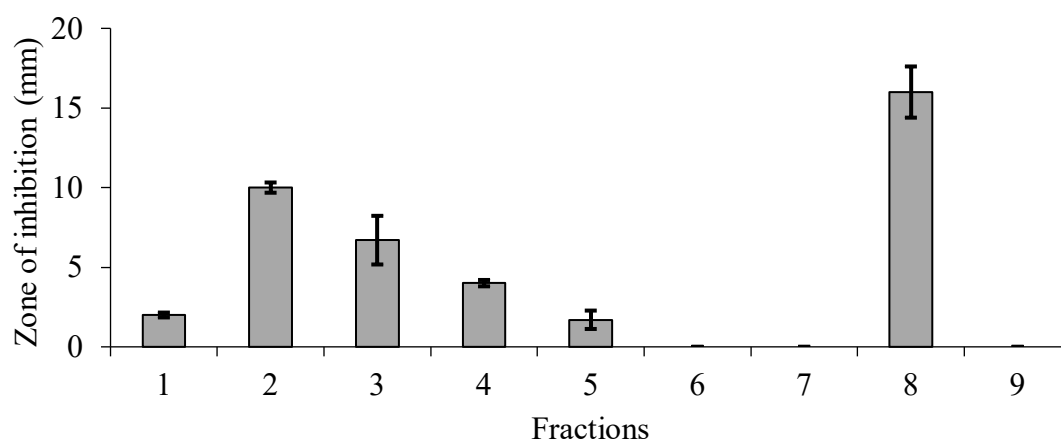


Figure 54: Antimicrobial activity of secondary fractions (Fractions 1 to 7) of *B. lanceolata* against *S. aureus*. Fraction 8 is the positive control (Tetracycline) and Fraction 9 is the negative control (Milli-Q water) in disc diffusion assay

Figure 55 shows the relative MIC and MBC of the active fractions when tested against *S. aureus*. The results showed that, similar to the DDA, the relative MIC and MBC were found to be the highest for Fraction 2. However, the relative MIC decreased in the following order: Fraction 2 > Fraction 1 \approx Fraction 6 > Fraction 3 \approx Fraction 5 > Fraction 4 and Fraction 7 did not show any activity with the trend for the MBC being slightly different: Fraction 2 > Fraction

3 \approx Fraction 5 > Fraction 1 \approx Fraction 4 > Fraction 6 with Fraction 7 not showing any inhibitory activity.

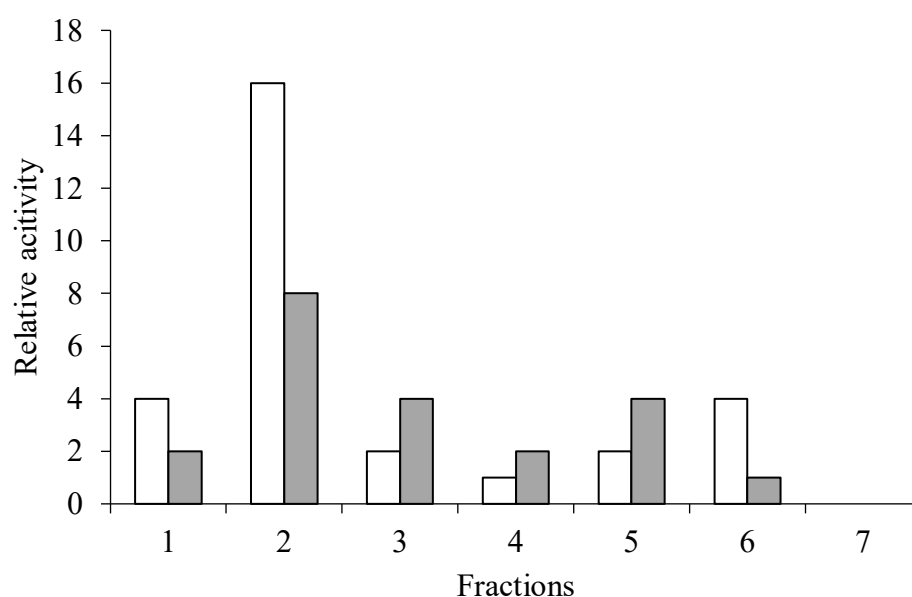


Figure 55: Relative activities of secondary fractions (Fraction 1-7) of *B. lanceolata* against *S. aureus*. The white bars represent relative MIC and those in grey represent MBC

7.4.2.2 Comparing antibacterial activity of the fractions based on the colony-forming units (CFU) method.

In order to obtain more robust quantitative data for antimicrobial testing, fractions were tested against *E. coli* using an agar plate-based assay that allowed CFU values to be obtained. The results are presented in Figure 56 and in this case, Fraction 2 showed the lowest CFU values compared to the other fractions and the activity declined in the following order: Fraction 2 > Fraction 1 > Fraction 3 > Fraction 4 > Fraction 5.

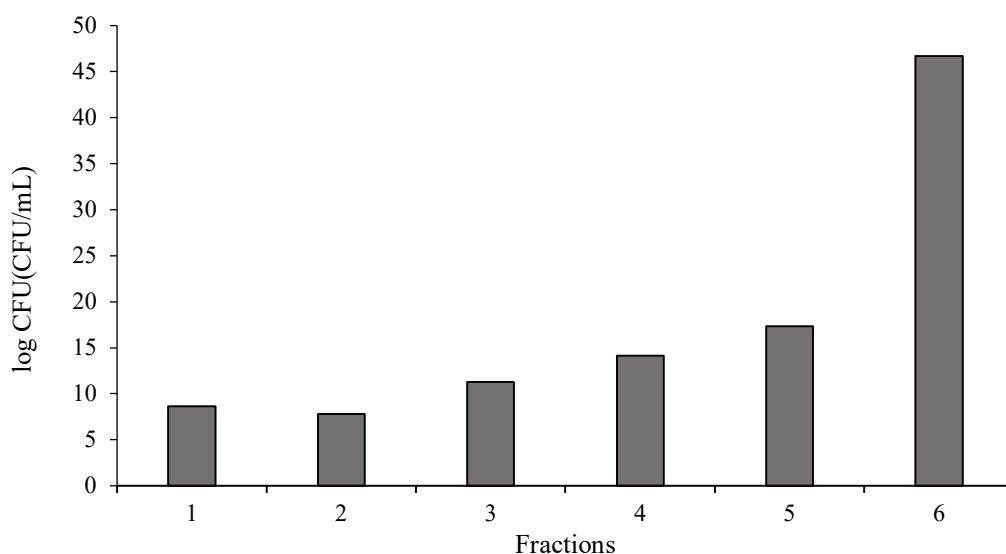


Figure 56. CFU values against *E. coli* for active Fractions 1 to 5 with 6 being the negative control

7.4.3 Compound identification and verification

7.4.3.1 GC-MS analysis

Direct GC-MS analysis of the secondary fractions was generally uninformative due the polar nature of the eluted compounds but dimethyl tartrate was observed eluting as the peak at 15.6 minutes in Figure 57. BSTFA derivatisation of the six fractions followed by analysis with GC-MS increased the number of observed peaks with the chromatograms shown in Figure 58. The variation in MBC for each secondary fraction was mapped against the variation in peak area for each peak in the chromatograms from the GC-MS. The reproducibility of the retention time and the similarity of the mass spectra enabled the retention time to be used as the identifier for each peak.

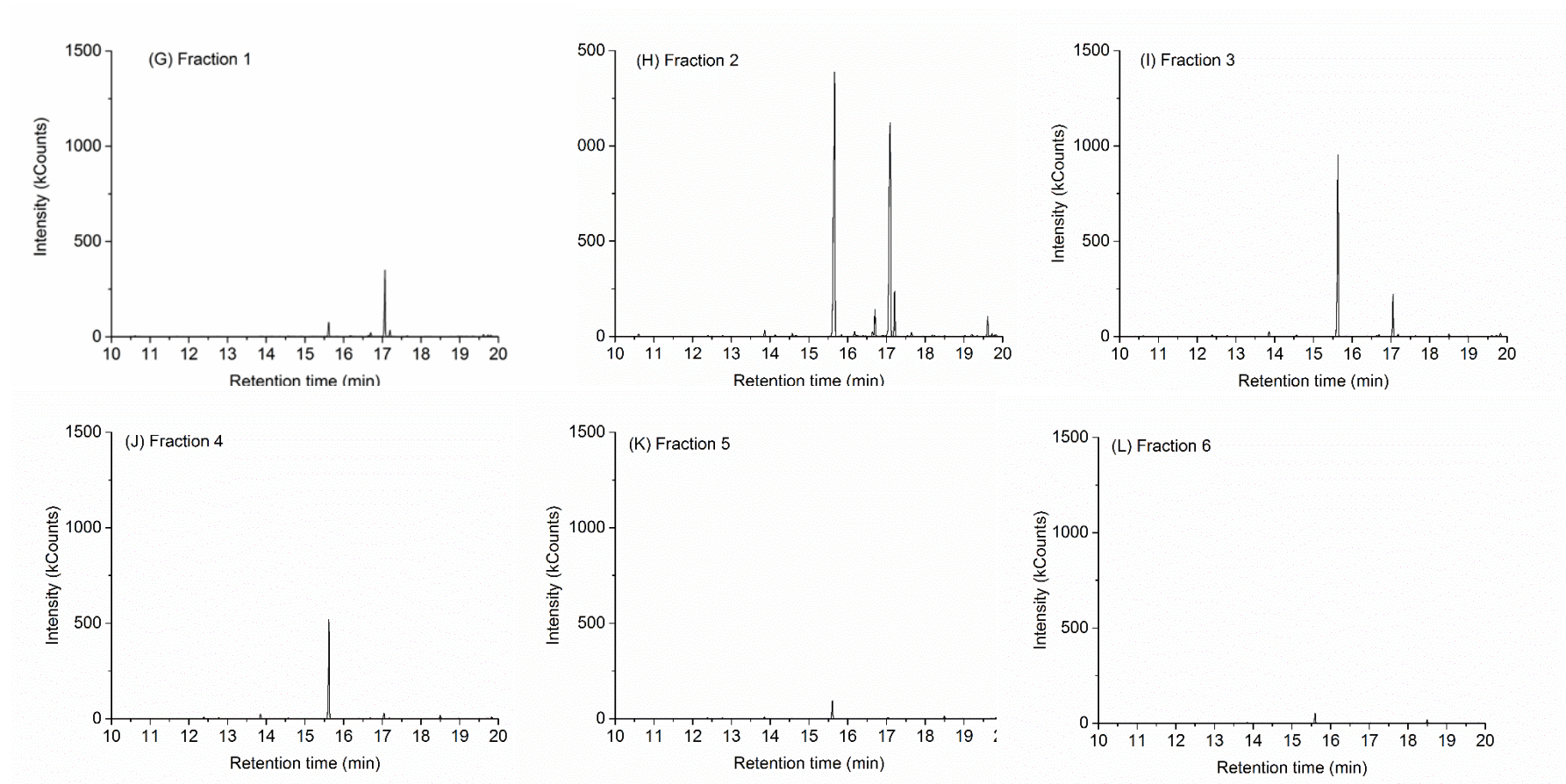


Figure 57: Direct GC-MS analysis of the Milli-Q water fractions (1 to 6) with dimethyl tartrate eluting at 15.6 minutes

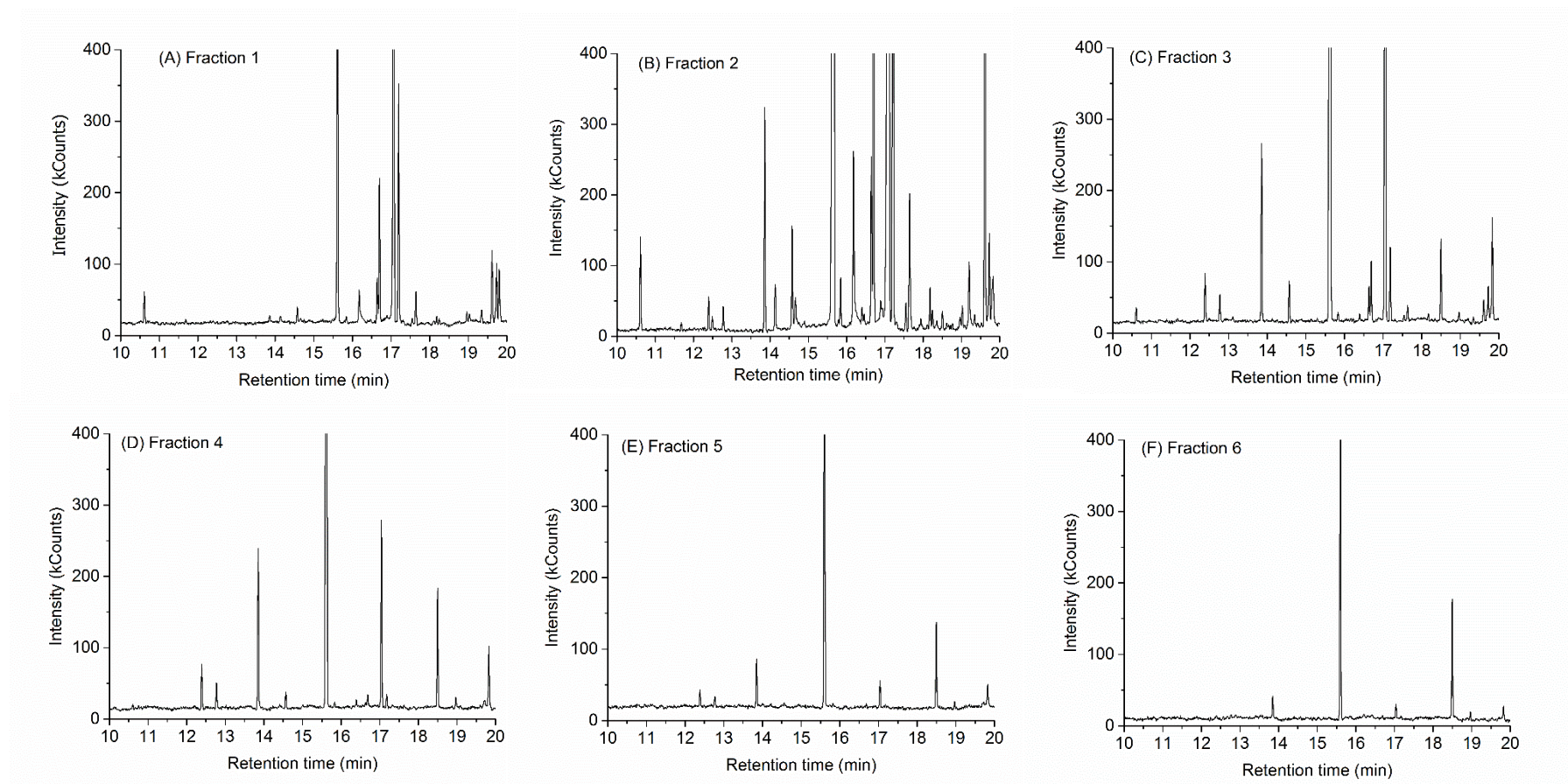


Figure 58: Derivatised GC-MS analysis of the Milli-Q water fractions (1 to 6)

A listing of the Spearman's rank correlation coefficients is given in Table 18 where the compounds are identified by their retention time. The critical value for the Spearman's rank correlation coefficient for seven fractions is 0.714 for a one-sided test of significance ($\alpha = 5\%$) and absolute correlations greater than 0.786 for a two-sided test of significance ($\alpha = 5\%$) [220]. Of the twenty-seven compounds detected, only three compounds showed a significant correlation between the MBC and peak area.

Table 18: Spearman's rank correlation coefficients for chromatographic peak area versus MBC for each fraction

GC-MS Retention time (min)	Spearman's rank- correlation coefficient
10.58	0.619
12.36	0.629
12.75	0.711
13.84*	0.820
14.54*	0.842
14.83	0.619
15.61	0.619
15.80*	0.756
16.15	0.619
16.23	0.644
16.43	0.569
16.67	-0.374
16.88	0.186
16.96	0.710
17.04	0.584
17.16	0.619
17.42	0.615
17.64	0.642
18.47	0.619
18.94	0.486
19.61	0.576
19.70	0.619
19.80	0.619
20.79	0.464
20.86	0.664
22.07	0.619
23.54	0.435

*Spearman's rank correlation coefficient greater than 0.714 ($\alpha = 5\%$).

Based on the critical test value, three compounds were identified and are listed in Table 19 along with their GC-MS library match probability; their chemical structures are given in Figure 59.

Table 19 : Compounds identified in the most active fractions from the secondary fractionation stage using derivatised GC-MS analysis

Retention time (min)	Compound name	Probability %
13.84	Dimethyl 2,3-bis[(trimethylsilyl)oxy]succinate	96
14.54	Arabino-hexos-2-ulose,3,4,5,6-tetrakis-o-(trimethylsilyl)-, bis(dimethyl acetal)	80
15.80	Bis(trimethylsilyl)2,3-bis[(trimethylsilyl)oxy]succinate	92

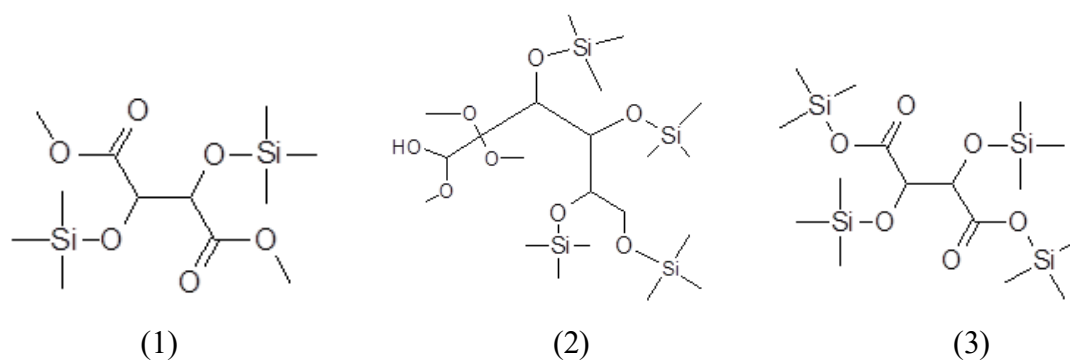


Figure 59: Chemical structures of the compounds with Spearman's rank correlation coefficients greater than 0.716. (1) Dimethyl 2, 3-bis[(trimethylsilyl)oxy]succinate, (2) Arabino-hexos-2-ulose, 3, 4, 5, 6-tetrakis-o-(trimethylsilyl)-, bis(dimethyl acetal) and (3) Bis(trimethylsilyl) 2,3-bis[(trimethylsilyl)oxy]succinate

It is noticeable that first structure is the trimethylsilyl derivative of dimethyl tartrate and the third structure is derivatized tartaric acid. It was subsequently found that the arabino-hexos-2-

ulose, 3, 4, 5, 6-tetrakis-O-(trimethylsilyl)-, bis (dimethyl acetal) is a by-product from the derivatisation process when commercially available authentic standards of dimethyl tartrate and tartaric acid were analysed using the same conditions. The availability of pure substances enabled confirmation of the derivatised identities from GC-MS based on retention times and mass spectral library data.

The MIC and MBC values of pure dimethyl tartrate and tartaric acid were obtained against *S. aureus*. Dimethyl tartrate showed MIC and MBC values of 15 mg/mL and 7.5 mg/mL, respectively. Tartaric acid had MIC and MBC values of 940 µg/mL and 470 µg/mL, respectively. Tartaric acid is an alpha hydroxyl, short chain carboxylic acid and is a chiral compound. It acts as a pH lowering agent during the fermentation step in the wine making process that reduces the levels of spoilage bacteria and also acts as a preservative during wine storage in barrels ^[221]. The bacterial species *Lactobacillus sp.* and *Pediococcus sp.* are the main causes for the wine spoilage, which reduces the quality of the wine and decreases its value ^[222].

According to published research, it has been found that there are some inhibitory effects on *Salmonella typhimurium* by a few carboxylic acids that included tartaric acid ^[223]. *Salmonella typhimurium* is responsible as the main food borne pathogen ^[224] that contaminates meat products including poultry and beef ^[224-225]. Blended mixtures of tartaric, lactic, malic and citric acids are used as a dietary acidifier in poultry and piggery diets. The gastrointestinal tract contains acid tolerant bacteria that includes pathogenic *E. coli*, *Salmonella typhimurium* and *Campylobacter* but the growth of these bacteria can be reduced significantly by adjusting the digesta pH (pKa 3 to 5) in the gut, which can positively influence the growth of chicken and piglets ^[223].

7.4.3.2 LC-MS analysis

As tartaric acid is not detectable in direct GC-MS analysis, high resolution LC-MS analysis was performed to confirm the identities of tartaric acid and the dimethyl tartrate. It was found that both were present in the active fractions but the amount of tartaric acid was considerably higher compared to the dimethyl tartrate in the plant extract. Quantitative analysis of the *B. lanceolata* fruit plant extract shows 0.8% of dimethyl tartrate and 7% of tartaric acid in the crude extract. These results indicate that the tartaric acid exhibits considerably higher activity against *S. aureus* compared to dimethyl tartrate and is the main contributor to the bioactivity.

Unfortunately, the extract was obtained using a general extraction procedure developed at the Sarawak Biodiversity Centre with methanol used as one of the solvents. It is suspected that the dimethyl tartrate is the result of an esterification reaction between methanol and tartaric acid. However, at this stage it was not possible to obtain the fruit for direct analysis to detect dimethyl tartrate and examine whether changing the extraction solvent composition influences the distribution of compounds that are extracted but this should be further investigated.

7.5 The presence of tartaric acid in other plants.

L-Tartaric acid is a plant based derived metabolite that can be found in plants due to a naturally occurring biosynthetic pathway that converts glucose to tartaric acid ^[226]. Tartaric acid is produced in grapes during the ripening process where the acid content increases around 50 days after flowering ^[227]. It is also found in most fruits including oranges ^[228], bananas and tamarinds. A study from Thailand found that rural plant leaves such as from Cowa, Moringa, Neem tree, Noni, Tamarind, Soap pod, Indian gooseberry, Ceylon Spinach and Gac have considerable levels of tartaric acid. However, the Neem leaves showed the highest concentration of tartaric acid compared to others ^[229]. It has been measured in the leaves and berries of *Vitis labrusca* cv,

Delawa grapes and leaves of *Parthenocissus quinquefolia* L. (Virginia creeper) ^[230]. Tartaric acid can be used as a food additive to create a sour taste ^[231] and as an antioxidant ^[141].

7.6 Conclusions

Overall, the *B. lanceolata* fruit extract contains bioactive compounds that can suppress the growth of various bacterial strains and the observed medicinal properties that are apparent for many infectious diseases can be mostly attributed to the presence of tartaric acid in this extract.

Chapter 8

Summary and conclusions

8.1 Summary of conclusions and further directions

This research has validated the importance of plant based natural medicines from indigenous Australian communities and the Malay ethnic groups from Sarawak. The accumulated knowledge from past centuries regarding plants from their surrounding environments that have been used to treat everyday ailments has been confirmed in many cases. To some extent, the research supports the continued collection of knowledge about plant medicines from tribal elders as it is important to secure their awareness of ethnomedicinal significant plants and to protect them for future generations.

Method development

Solid Phase Extraction was used to extract compounds in complex mixtures based on a method that was characterised using a standard mixture of known compounds with known log P values. Elution with various ratios of water and acetonitrile where the polarity of the mixed solvent was systematically altered enabled complex sample mixtures to be fractionated into narrow bands of similar polarity. A multi-stage SPE method was developed and the distribution of the bioactivity over a series of fractions was correlated with the composition of each fraction to identify the source of bioactivity in plant extracts.

E. longifolia extraction and characterisation

As plant material was used for this study, a part of this study was to optimise the yield of potentially bioactive compounds using extraction methods that included Accelerated Solvent Extraction (ASE) and the conventional immersion process. The variables that were studied included the type of solvent, temperature and the time of extraction. Extraction efficiency was evaluated based on the effectiveness of each plant extract against selected microorganisms

including bacteria (both Gram-positive and Gram-negative) and fungi. The key findings from this study are as follows:

- Extracts produced using the conventional immersion method exhibited better antimicrobial activities compared to ASE.
- Acetonitrile was found to be the most suitable extraction solvent.
- Optimal activity was found with extracts produced using the conventional immersion technique at 21 °C and 5 days.

Two novel compounds were identified, namely neryl ferulate and neryl *p*-cinnamate, using nuclear magnetic resonance (NMR) and liquid chromatography with high resolution mass spectrometry (LC-MS). These novel compounds were tested for antimicrobial activity against selected microbes with neryl ferulate showing borderline inhibitory activity against antibiotic-resistant Gram-positive bacteria including *S. aureus* ATCC 3300 (MRSA), *S. aureus* NARSA NRS 17 (GISA), *S. aureus* NARSA VRS 1 (VRSA), *S. pneumoniae* ATCC 700677 (MDR), *E. faecium* ATCC 51559 (VRE VanA) and *C. neoformans* H99 ATCC 208821. Neryl *p*-cinnamate was only active against *E. faecium*. The extracted compounds were also tested against the human kidney-derived cell lines HEK293 and HepG2 to determine any cytotoxicity effects. Cytotoxic effects were observed against the HEK293 and HepG2 human kidney cell lines at concentrations of 90 µg/mL and above.

Southeast Asian plant screening

Fifty plant extracts from the Sarawak Biodiversity Centre were screened based on information obtained from their Traditional Knowledge Documentation program. After initial screening for microbial inhibitory activity, only six plants were selected for the further antipathogenic analysis: *Baccaurea lanceolata* (Miq.) Müll.Arg, *Fibraurea tinctoria* Lour, *Goniothalamus tapisoides* Mat Salleh, *Goniothalamus velutinus* Airy Shaw, *Polyalthia hookeriana* King,

Pyrenaria serrata Bl. var *masocarpa* (Korth) H. Ken. *Baccaurea lanceolata* produced the best results and was targeted for further phytochemical analysis.

Southeast Asian plant characterisation

The two *B. lanceolata* extracts, derived from plants collected at different times of the year from the same location showed comparable activity indicating that this plant species produces similar antimicrobial chemicals, regardless of the time of collection. The *B. lanceolata* fruit extract contains bioactive compounds that can inhibit the growth of numerous bacterial strains and this can be attributed to the presence of dimethyl tartrate and tartaric acid.

8.2 Potential for future work

Automated fraction collection

All the fractionation work was performed using manual flow control and was a highly labor intensive process. An inconsistent flow rate affects the reproducibility during the fractionation process and impedes increasing the amount of substance that can be collected from repeat experiments. Going forward, it is recommended to use an automated fraction collector that allows for more consistent flow rate control, is less labor intensive and will reduce experimental time. Repeated experiments will increase the sensitivity of the bioactivity testing as this was a major disadvantage with the current methodology.

Cell culture and animal studies

Of all fifty plants tested, *B. lanceolata* showed microbial activity against a wide range of bacteria with *P. serrata* exhibiting activity against *C. albicans* and these two plants might have future potential to be used as antimicrobial and antifungal agents. Therefore, it is important to assess the safety of extracts by undertaking bioactivity assays against specific cell lines. Subject

to the *in vitro* findings, *in vivo* animal studies would be recommended to confirm safety and efficacy.

Hyperglycemia related testing

Following the world-wide trend, it has been found that Australians are increasingly diagnosed with Type 2 diabetes. Hyperglycemia related testing of the Australian endemic tree *E. longifolia* and the Southeast Asian plants could provide useful treatments. Previous studies in our laboratory have shown that traditional plants have anti-hyperglycemic activity. Understanding these effects for *E. longifolia* would be beneficial.

Providing feedback to Sarawak Biodiversity Centre

The general extraction procedure used by the Sarawak Biodiversity Centre is a mixed solvent that provides for a wide range of chemical polarities to be collected. However, the use of methanol as one of the extraction solvents for the extraction of *B. lanceolata* fruit could be altering the chemical constituents due to the esterification of carboxylic acids. According to the chromatographic analysis, tartaric acid and dimethyl tartrate were identified as the active constituents of *B. lanceolata*. However, as reported in Chapter 7, it was suspected that dimethyl tartrate is the result of an esterification reaction between methanol and tartaric acid. Therefore, the suggestion is to extract the *B. lanceolata* fruit using water as the solvent to remove the possibility of compositional changes.

Reflective Overview

The analytical approach that was implemented due to the advancement in instrumental techniques and the small sample amounts has been demonstrated to be a feasible strategy. The use of the Accelerated Solvent Extraction system shows that it is too efficient when extracting

antibacterial substances from plant material. In hindsight this should have been foreseen because if the antibacterial substances are part of a protective defense response for the plant then these substances need to be closer to the external part of the plant.

The SPE fractionation approach was useful but requires further automation to be reproducible and the chromatographic separations were effective but the value of these instrumental separations is greatly enhanced if highly specific detection, such as high resolution mass spectrometry, is available. Unfortunately access to LC-NMR was not possible and, in our case, a more traditional approach was required to obtain structural information about the active compounds but it is apparent that the high throughput screening of natural products is mostly limited by the bioassay component of the process.

References

- [1] I. Hamdan, F. Afifi, *Studies on the in vitro and in vivo hypoglycemic activities of some medicinal plants used in treatment of diabetes in Jordanian traditional medicine*, *J. Ethnopharmacol.* **2004**, 93, 117-121.
- [2] S. Chatterjee, A. Banerjee, I. Chandra, *Hemidesmus indicus: A Rich Source of Herbal Medicine*, *Journal of Medicinal & Aromatic Plants* **2014**, 3, 1-3.
- [3] A. A. Abdullahi, *Trends and challenges of traditional medicine in Africa*, *African Journal of Traditional, Complementary and Alternative Medicines* **2011**, 8.
- [4] M. S. Micozzi, *Culture, anthropology, and the return of" complementary medicine"*, *Med. Anthropol. Q.* **2002**, 16, 398-403.
- [5] V. Londhe, *Role of garlic (allium sativum) in various diseases an overview*, *Journal of pharmaceutical research & opinion* **2014**, 1, 129-134.
- [6] M. Alzweiri, A. Al Sarhan, K. Mansi, M. Hudaib, T. Aburjai, *Ethnopharmacological survey of medicinal herbs in Jordan, the Northern Badia region*, *J. Ethnopharmacol.* **2011**, 137, 27-35.
- [7] T. Bartram, *Bartram's Encyclopedia of Herbal Medicine*, Hachette UK, **2013**.
- [8] E. Roeder, H. Wiedenfeld, *Plants containing pyrrolizidine alkaloids used in the Traditional Indian Medicine including Ayurveda*, *International Journal of Pharmaceutical Sciences* **2013**, 68, 83-92.
- [9] G. Bodeker, K. Who, C. Ong, C. Grundy, G. Burford, K. Shein, *Word Health Organisation Kobe Centre, Japan*, **2005**, pp. 1-347.
- [10] A. L. Rice, L. Sacco, A. Hyder, R. E. Black, *Malnutrition as an underlying cause of childhood deaths associated with infectious diseases in developing countries*, *Bulletin of the World Health Organization* **2000**, 78, 1207-1221.
- [11] L. H. Taylor, S. M. Latham, E. Mark, *Risk factors for human disease emergence*, *Journal of Philosophical Transactions of the Royal Society of London Biological Sciences* **2001**, 356, 983-989.
- [12] L. Silver, K. Bostian, *Discovery and development of new antibiotics: the problem of antibiotic resistance*, *Journal of Antimicrobial agents and chemotherapy* **1993**, 37, 377-383.
- [13] J. L. Ríos, M. C. Recio, *Medicinal plants and antimicrobial activity*, *J. Ethnopharmacol.* **2005**, 100, 80-84.
- [14] J. Nguyen Pouplin, H. Tran, T. Phan, C. Dolecek, J. Farrar, T. Tran, P. Caron, B. Bodo, P. Grellier, *Antimalarial and cytotoxic activities of ethnopharmacologically selected medicinal plants from South Vietnam*, *J. Ethnopharmacol.* **2007**, 109, 417-427.
- [15] R. Lee, *The eureka! moment: 100 key scientific discoveries of the 20th century*, Routledge, **2016**.
- [16] A. Tomasz, *Multiple antibiotic resistant pathogenic bacteria.*, *N. Engl. J. Med.* **1994**, 330, 1247-1251.
- [17] M. Shnit Orland, A. Kushmaro, *Coral mucus associated bacteria: a possible first line of defense*, *Journal of FEMS Microbiology Ecology* **2009**, 67, 371-380.
- [18] H. Nikaido, *Multidrug resistance in Bacteria*, *Journal of Annual Review of Biochemistry* **2009**, 78, 119-146.
- [19] X. Z. Li, H. Nikaido, *Efflux-mediated drug resistance in bacteria*, *Drugs* **2009**, 69, 1555-1623.
- [20] J. Martinez, F. Baquero, *Mutation frequencies and antibiotic resistance*, *Antimicrob. Agents Chemother.* **2000**, 44, 1771-1777.

- [21] C. Marshall, T. Kossmann, S. Wesselingh, D. Spelman, *Methicillin - Resistant Staphylococcus aureus and beyond: what's new in the world of the 'golden staph' ?*, *ANZ J. Surg.* **2004**, 74, 465-469.
- [22] J. C. Baumgartner, T. Xia, *Antibiotic susceptibility of bacteria associated with endodontic abscesses*, *J. Endod.* **2003**, 29, 44-47.
- [23] P. Schneider, S. Hawser, K. Islam, *Iclaprim, a novel diaminopyrimidine with potent activity on trimethoprim sensitive and resistant bacteria*, *Journal of Bioorganic & Medicinal Chemistry Letters* **2003**, 13, 4217-4221.
- [24] V. Schaar, T. Nordstrom, M. Morgelin, K. Riesbeck, *Vol. 55*, National Center for Biotechnology Information, **2011**, pp. 3845-3853.
- [25] L. M. Weigel, D. B. Clewell, S. R. Gill, N. C. Clark, L. K. McDougal, S. E. Flannagan, J. F. Kolonay, J. Shetty, G. E. Killgore, F. C. Tenover, *Genetic analysis of a high-level Vancomycin-Resistant isolate of Staphylococcus aureus*, *Journal of Science Magazine* **2003**, 302, 1569-1571.
- [26] D. J. H. Kilgus, D.J.Strang, A, *Results of periprosthetic hip and knee infections caused by resistant bacteria*, *Journal of Clinical Orthopaedics and Related Research* **2002**, 404, 116-124.
- [27] M. C. Roberts, *Tetracycline resistance determinants: mechanisms of action, regulation of expression, genetic mobility, and distribution*, *Journal of FEMS Microbiology Ecology* **1996**, 19, 1-24.
- [28] H. Seppälä, T. Klaukka, J. Vuopio-Varkila, A. Muotiala, H. Helenius, K. Lager, P. Huovinen, *The effect of changes in the consumption of macrolide antibiotics on erythromycin resistance in Group A Streptococci in Finland*, *N. Engl. J. Med.* **1997**, 337, 441-446.
- [29] B. H. Heintz, J. Halilovic, C. L. Christensen, *Vancomycin-Resistant Enterococcal urinary tract infections*, *Journal of Pharmacotherapy* **2010**, 30, 1136-1149.
- [30] A. Mottalib, M. Hossain, I. Khalil, S. Islam, A. Hossain, *Drug susceptibility pattern of Mycobacterium tuberculosis isolates against conventional anti-tuberculosis drugs in Dhaka, Bangladesh*, *Saudi Medical Journal* **2011**, 32, 484-488.
- [31] E. Skyman, Doctoral thesis, University of Gothenburg. Sahlgrenska Academy. **2014**.
- [32] T. Poovelikunnel, G. Gethin, H. Humphreys, *Mupirocin resistance: clinical implications and potential alternatives for the eradication of MRSA*, *J. Antimicrob. Chemother.* **2015**, 70, 2681-2692.
- [33] F. J. Angulo, P. Collignon, J. H. Powers, T. M. Chiller, A. Aidara-Kane, F. M. Aarestrup, *World Health Organization ranking of antimicrobials according to their importance in human medicine: a critical step for developing risk management strategies for the use of antimicrobials in food production animals*, *Journal of Clinical Infectious Diseases* **2009**, 49, 132-141.
- [34] N. Tabassum, M. Hamdani, *Plants used to treat skin diseases*, *Pharmacognosy reviews* **2014**, 8, 52.
- [35] K. Harada, M. Saito, T. Sugita, R. Tsuboi, *Malassezia species and their associated skin diseases*, *J. Dermatol.* **2015**, 42, 250-257.
- [36] J. Sardi, L. Scorzoni, T. Bernardi, A. Fusco-Almeida, M. M. Giannini, *Candida species: current epidemiology, pathogenicity, biofilm formation, natural antifungal products and new therapeutic options*, *J. Med. Microbiol.* **2013**, 62, 10-24.
- [37] N. Tabassum, M. Hamdani, *Plants used to treat skin diseases*, *Journal of Pharmacognosy Reviews* **2014**, 8, 52-60.
- [38] N. Smith, D. Denning, *Underlying conditions in chronic pulmonary aspergillosis including simple aspergilloma*, *Eur. Respir. J.* **2011**, 37, 865-872.

- [39] D. Mahmoud, N. Hassanein, K. Youssef, A. Zeid, *Antifungal activity of different neem leaf extracts and the nimonol against some important human pathogens*, *Braz. J. Microbiol.* **2011**, 42, 1007-1016.
- [40] Y. P. Ran, X. X. Wang, Y. L. Dai, X. M. Peng, G. P. Zhou, *Facial tinea corporis in an infant due to Microsporum gypseum: a case report*, *Chinese Journal of Mycology* **2006**, 1, 1-8.
- [41] N. R. Bhalodia, V. Shukla, *Antibacterial and antifungal activities from leaf extracts of Cassia fistula : An ethnomedicinal plant*, *Journal of Advanced Pharmaceutical Technology & Research* **2011**, 2, 104-109.
- [42] K. Saki, M. Bahmani, M. Rafieian Kopaei, H. Hassanzadazar, K. Dehghan, F. Bahmani, J. Asadzadeh, *The most common native medicinal plants used for psychiatric and neurological disorders in Urmia city, northwest of Iran*, *Asian Pacific Journal of Tropical Disease* **2014**, 4, 895-901.
- [43] K. P. Hill, *Medical marijuana for treatment of chronic pain and other medical and psychiatric problems: a clinical review*, *The Journal of the American Medical Association* **2015**, 313, 2474-2483.
- [44] K. A. Belendiuk, L. L. Baldini, M. O. Bonn Miller, *Narrative review of the safety and efficacy of marijuana for the treatment of commonly state-approved medical and psychiatric disorders*, *Journal of Addiction Science & Clinical Practice* **2015**, 10, 1-10.
- [45] S. A. Karamati, H. Hassanzadazar, M. Bahmani, M. Rafieian Kopaei, *Herbal and chemical drugs effective on malaria*, *Asian Pacific Journal of Tropical Disease* **2014**, 4, 599-601.
- [46] V. Lohsiriwat, *Hemorrhoids: from basic pathophysiology to clinical management*, *World J. Gastroenterol.* **2012**, 18, 2009-2017.
- [47] E. C. Evans, *Hemodialysis-Related Cramps and Nocturnal Leg Cramps-What Is Best Practice ?*, *J. Nephrol. Nurs.* **2013**, 40, 549-553.
- [48] A. S. Botsaris, *Plants used traditionally to treat malaria in Brazil: the archives of Flora Medicinal*, *Journal of Ethnobiology and Ethnomedicine* **2007**, 3, 1-8.
- [49] A.-M. Carroll, D. J. Kavanagh, F. P. McGovern, J. W. Reilly, J. J. Walsh, *Nature's Chiral Catalyst and Anti-Malarial Agent: Isolation and Structure Elucidation of Cinchonine and Quinine from Cinchona calisaya*, *J. Chem. Educ.* **2012**, 89, 1578-1581.
- [50] J. Achan, A. O. Talisuna, A. Erhart, A. Yeka, J. K. Tibenderana, F. N. Baliraine, P. J. Rosenthal, U. D'Alessandro, *Quinine, an old anti-malarial drug in a modern world: role in the treatment of malaria*, *Malar. J.* **2011**, 10, 1-12.
- [51] B. Brinkhaus, J. Hummelsberger, R. Kohnen, J. Seufert, C. H. Hempen, H. Leonhardy, R. Nögel, S. Joos, E. Hahn, D. Schuppan, *Acupuncture and chinese herbal medicine in the treatment of patients with seasonal allergic rhinitis: a randomized controlled clinical trial*, *J. Allergy Clin. Immunol.* **2004**, 59, 953-960.
- [52] J. C. Jeong, M. S. Choi, S. H. Yoon, D. I. Kim, D. I. Kim, S. H. Jang, J. Y. Leem, S. K. Ku, B. Y. Seo, M. H. Jeon, *Analysis of the result of Korean medicine treatment for female subfertility using herbal medicine, Acupuncture and moxibustion treatment*, *Journal of Korean Medicine* **2015**, 36, 1-7.
- [53] Y. Sui, H. Zhao, V. Wong, N. Brown, X. Li, A. Kwan, H. Hui, E. Ziea, J. Chan, *A systematic review on use of Chinese medicine and acupuncture for treatment of obesity*, *Journal of Obesity Reviews* **2012**, 13, 409-430.
- [54] G. S. Liao, M. K. Apaya, L. F. Shyur, *Herbal medicine and acupuncture for breast cancer palliative care and adjuvant therapy*, *Journal of Evidence Based Complementary and Alternative Medicine* **2013**, 2013, 1-17.
- [55] M. Lafferty, S. Phillips, *Acupuncture as a beneficial treatment for insomnia and depression*, *J. Altern. Complement. Med.* **2014**, 146, 1-18.

- [56] S. Su, *Observation of the clinical effect of acupuncture and Chinese medicine treatment of bacterial vaginosis*, *International Journal of Clinical Acupuncture* **2015**, 24, 172-174.
- [57] M. P. Coleman, D. Forman, H. Bryant, J. Butler, B. Rachet, C. Maringe, U. Nur, E. Tracey, M. Coory, J. Hatcher, C. E. McGahan, D. Turner, L. Marrett, M. L. Gjerstorff, T. B. Johannesen, J. Adolfsson, M. Lambe, G. Lawrence, D. Meechan, E. J. Morris, R. Middleton, J. Steward, M. A. Richards, *Cancer survival in Australia, Canada, Denmark, Norway, Sweden, and the UK, 1995–2007 (The International Cancer Benchmarking Partnership): an analysis of population-based cancer registry data*, *The Lancet* **2011**, 377, 127-138.
- [58] F. R. Siakholak, M. Ghoncheh, R. Pakzad, H. S. Gandomani, F. Ghorat, H. Salehiniya, *Epidemiology, incidence and mortality of oral cavity and lips cancer and their relationship with the human development index in the world*, *Journal of Biomedical Research and Therapy* **2016**, 3, 872-888.
- [59] M. P. Heron, in *National Vital Statistics Reports (NVSR)*, Vol. 64, National Vital Statistics Reports, **2015**, pp. 1-93.
- [60] A. Coates, in *Cancer Council Australia for Health Professionals Working in Cancer Control. Journal of the Clinical Oncological Society of Australia*, Vol. 37, Australia, **2013**, pp. 1-136.
- [61] J. F. Perz, G. L. Armstrong, L. A. Farrington, Y. J. Hutin, B. P. Bell, *The contributions of Hepatitis B virus and Hepatitis C virus infections to cirrhosis and primary liver cancer worldwide*, *J. Hepatol.* **2006**, 45, 529-538.
- [62] H. Mirghani, F. Amen, F. Moreau, J. Guigay, M. Ferchiou, A. E. Melkane, D. M. Hartl, J. L. St Guily, *Human papilloma virus testing in oropharyngeal squamous cell carcinoma: what the clinician should know*, *Journal of Oral Oncology* **2014**, 50, 1-9.
- [63] M. A. Nobbenhuis, J. M. Walboomers, T. J. Helmerhorst, L. Rozendaal, A. J. Remmink, E. K. Risse, H. C. van der Linden, F. J. Voorhorst, P. Kenemans, C. J. Meijer, *Relation of Human papilloma virus status to cervical lesions and consequences for cervical-cancer screening: a prospective study*, *The Lancet* **1999**, 354, 20-25.
- [64] J. S. Smith, R. Herrero, C. Bosetti, N. Munoz, F. X. Bosch, J. Eluf-Neto, X. Castellsague, C. J. Meijer, A. J. Van den Brule, S. Franceschi, *Herpes simplex virus 2 as a Human papilloma virus cofactor in the etiology of invasive cervical cancer*, *The National Cancer Institute* **2002**, 94, 1604-1613.
- [65] V. Tsouris, M. K. Joo, S. H. Kim, I. C. Kwon, Y. Y. Won, *Nano carriers that enable co-delivery of chemotherapy and RNAi agents for treatment of drug-resistant cancers*, *Journal of Biotechnology Advances* **2014**, 32, 1037-1050.
- [66] U. Shah, R. Shah, S. Acharya, N. Acharya, *Novel anticancer agents from plant sources*, *Chinese Journal of Natural Medicines* **2013**, 11, 16-23.
- [67] J. W. Lee, W. B. Lee, W. Kim, B. I. Min, H. Lee, S. H. Cho, *Traditional herbal medicine for cancer pain: A systematic review and meta-analysis*, *Journal of Complementary Therapies in Medicine* **2015**, 23, 265-274.
- [68] S. Wang, X. Wu, M. Tan, J. Gong, W. Tan, B. Bian, M. Chen, Y. Wang, *Fighting fire with fire: poisonous Chinese herbal medicine for cancer therapy*, *J. Ethnopharmacol.* **2012**, 140, 33-45.
- [69] A. Faried, D. Kurnia, L. Faried, N. Usman, T. Miyazaki, H. Kato, H. Kuwano, *Anticancer effects of gallic acid isolated from Indonesian herbal medicine, Phaleria macrocarpa (Scheff.) Boerl, on human cancer cell lines*, *Int. J. Oncol.* **2007**, 30, 605-614.
- [70] M. Moudi, R. Go, C. Y. S. Yien, M. Nazre, *Vinca alkaloids*, *International journal of Preventive Medicine* **2013**, 4, 1231-1235.

- [71] L. Chiu, I. Hsin, T. Yang, W. Sung, J. Chi, J. Chang, J. Ko, G. Sheu, *The ERK–ZEB1 pathway mediates epithelial–mesenchymal transition in pemetrexed resistant lung cancer cells with suppression by vinca alkaloids*, *Journal of Oncogene* **2016**, 36, 242-253.
- [72] S. Howat, B. Park, I. S. Oh, Y. W. Jin, E. K. Lee, G. J. Loake, *Paclitaxel: biosynthesis, production and future prospects*, *Journal of New Biotechnology* **2014**, 31, 242-245.
- [73] R. Victor, *On the use of biotechnology for biodiversity conservation in arid countries with special reference to Oman*, *International Journal of Environmental Studies* **2013**, 70, 900-908.
- [74] G. M. Cragg, *Paclitaxel (Taxol®): a success story with valuable lessons for natural product drug discovery and development*, *Journal of Medicinal Research Reviews* **1998**, 18, 315-331.
- [75] S. Kusari, S. Zühlke, M. Spiteller, *An endophytic fungus from *Camptotheca acuminata* that produces camptothecin and analogues*, *J. Nat. Prod.* **2009**, 72, 2-7.
- [76] G. M. Cragg, D. J. Newman, *Plants as a source of anti-cancer agents*, *J. Ethnopharmacol.* **2005**, 100, 72-79.
- [77] P. M. Woster, *Foreword: American Chemical Society Division of Medicinal Chemistry. Celebrating 100 Years of Excellence*, *J. Med. Chem.* **2009**, 52, 7333-7338.
- [78] A. Cirila, J. Mann, *Combretastatins: from natural products to drug discovery*, *Journal of Natural Product Reports* **2003**, 20, 558-564.
- [79] H. X. Li, X. R. Liu, W. P. Zhang, C. Zhang, H. B. Sun, *A review of current status of combretastatin A-4 phosphate (CA4P)*, *Chinese Journal of New Drugs and Clinical Remedies* **2010**, 11, 1-5.
- [80] P. Bradley, *British herbal compendium: A handbook of scientific information of widely used plant drugs*, Vol. 2, British Herbal Medicine Association, **2006**.
- [81] U. Quattrocchi, *CRC world dictionary of medicinal and poisonous plants: common names, scientific names, eponyms, synonyms, and etymology*, Vol. 5, CRC Press, New York, **2012**.
- [82] E. C. Barnes, A. R. Carroll, R. A. Davis, *Mitchellenes A–E, Cyclic sesquiterpenes from the Australian plant *Eremophila mitchellii**, *J. Nat. Prod.* **2011**, 74, 1888-1893.
- [83] A. W. Claridge, J. M. Trappe, D. J. Paull, in *Desert Truffles*, Springer, **2014**, pp. 203-214.
- [84] G. S. Richmond, E. L. Ghisalberti, *The Australian desert shrub *Eremophila* (myoporaceae): Medicinal, cultural, horticultural and phytochemical uses*, *Econ Bot* **1994**, 48, 35-59.
- [85] A. N. Singab, F. S. Youssef, M. L. Ashour, M. Wink, *The genus *Eremophila* (Scrophulariaceae): An ethnobotanical, biological and phytochemical review*, *J. Pharm. Pharmacol.* **2013**, 65, 1239-1279.
- [86] ANPSA. Australian Native Plants Society 2014, from <http://asgap.org.au>
- [87] B. D. Cooke, *Planning Landscape-scale Rabbit Control*, **2013**.
- [88] R. J. Chinnock. (1992). PlantNET New South Wales flora online. 2018, from <http://pngplants.org/web2printer/web2printer4.php?img=0&lnk=3&style=/style.css>
- [89] E. M. Curtis, A. Leigh, S. Rayburg, *Relationships among leaf traits of Australian arid zone plants: alternative modes of thermal protection*, *Australian Journal of Botany* **2012**, 60, 471-483.
- [90] G. Richmond, E. Ghisalberti, *Seed dormancy and germination mechanisms in *Eremophila* (Myoporaceae)*, *Australian Journal of Botany* **1994**, 42, 705-715.
- [91] B. Barlow, *Cytogeography of the genus *Eremophila**, *Australian Journal of Botany* **1971**, 19, 295-310.
- [92] C. Williams, Vol. 4, Rosenberg Publishing, **2013**.

- [93] N. J. Sadgrove, S. Mijajlovic, D. J. Tucker, K. Watson, G. L. Jones, *Characterization and bioactivity of essential oils from novel chemotypes of Eremophila longifolia (F. Muell) (Myoporaceae): a highly valued traditional Australian medicine*, *Flavour and Fragrance Journal* **2011**, 26, 341-350.
- [94] J. Smith, D. Tucker, D. Alter, K. Watson, G. Jones, *Intraspecific variation in essential oil composition of Eremophila longifolia F. Muell. (Myoporaceae): Evidence for three chemotypes*, *Journal of Phytochemistry* **2010**, 71, 1521-1527.
- [95] N. J. Sadgrove, G. L. Jones, *A possible role of partially pyrolysed essential oils in Australian Aboriginal traditional ceremonial and medicinal smoking applications of Eremophila longifolia (R. Br.) F. Muell (Scrophulariaceae)*, *J. Ethnopharmacol.* **2013**, 147, 638-644.
- [96] Q. Liu, D. Harrington, J. L. Kohen, S. Vemulpad, J. F. Jamie, *Bactericidal and cyclooxygenase inhibitory diterpenes from Eremophila sturtii*, *Journal of Phytochemistry* **2006**, 67, 1256-1261.
- [97] C. P. Ndi, S. J. Semple, H. J. Griesser, S. M. Pyke, M. D. Barton, *Antimicrobial compounds from Eremophila serrulata*, *Journal of Phytochemistry* **2007**, 68, 2684-2690.
- [98] I. J. Biva, C. P. Ndi, H. J. Griesser, S. J. Semple, *Antibacterial constituents of Eremophila alternifolia: An Australian aboriginal traditional medicinal plant*, *J. Ethnopharmacol.* **2016**, 182, 1-9.
- [99] E. C. Barnes, A. M. Kavanagh, S. Ramu, M. A. Blaskovich, M. A. Cooper, R. A. Davis, *Antibacterial serrulatane diterpenes from the Australian native plant Eremophila microtheca*, *Journal of Phytochemistry* **2013**, 93, 162-169.
- [100] A. Abell, E. Horn, G. Jones, M. Snow, R. Massywestropp, R. Riccio, *The structure of a stable serrulatane diterpenoid acetal from Eremophila rotundifolia*, *Australian Journal of Chemistry* **1985**, 38, 1837-1845.
- [101] K. Croft, E. Ghisalberti, P. Jefferies, G. Proudfoot, *The chemistry of Eremophila spp. XVI. New serrulatanes from Eremophila sp.*, *Australian Journal of Chemistry* **1981**, 34, 1951-1957.
- [102] Y. M. Syah, E. L. Ghisalberti, *Serrulatane diterpenes from a new Eremophila species*, *Journal of Phytochemistry* **1997**, 45, 1479-1482.
- [103] Y. Tahtah, S. G. Wubshet, K. T. Kongstad, A. M. Heskes, I. Pateraki, B. L. Møller, A. K. Jäger, D. Staerk, *High resolution PTP1B inhibition profiling combined with high performance liquid chromatography high resolution mass spectrometry solid phase extraction nuclear magnetic resonance spectroscopy: Proof of concept and antidiabetic constituents in crude extract of Eremophila lucida*, *Journal of Fitoterapia* **2016**, 110, 52-58.
- [104] E. Della, P. Jefferies, *The chemistry of Eremophila species. The essential oil of Eremophila longifolia F. Muell*, *Australian Journal of Chemistry* **1961**, 14, 663-664.
- [105] M. Pennacchio, Y. M. Syah, E. L. Ghisalberti, E. Alexander, *Cardioactive compounds from Eremophila species*, *J. Ethnopharmacol.* **1996**, 53, 21-27.
- [106] J. Kulip, *An ethnobotanical survey of medicinal and other useful plants of Muruts in Sabah, Malaysia*, *Telopea* **2003**, 10, 81-98.
- [107] T. Lim, *Edible medicinal and non-medicinal Plants*, Springer, **2012**.
- [108] R. Nagarajan, M. Jonathan, P. D. Roy, L. Wai Hwa, M. Prasanna, S. Sarkar, M. Navarrete López, *Metal concentrations in sediments from tourist beaches of Miri City, Sarawak, Malaysia (Borneo Island)*, *The International Journal for Marine Environmental Scientists, Engineers, Administrators, Politicians and Lawyers* **2013**, 73, 369-373.
- [109] P. P. K. Chai, *Medicinal plants of Sarawak*, Lee Miing Press, **2006**.

- [110] U. Quattrocchi, *CRC World Dictionary of Medicinal and Poisonous Plants: Common Names, Scientific Names, Eponyms, Synonyms, and Etymology*, Vol. 5, CRC Press, UK, **2015**.
- [111] K. C. Wong, S. W. Wong, S. S. Siew, D. Y. Tie, *Volatile constituents of the fruits of *Lansium domesticum correa* (Duku and Langsat) and *Baccaurea motleyana* (Muell. Arg.) Muell. Arg. (Rambai), Flavour and Fragrance Journal* **1994**, 9, 319-324.
- [112] X. W. Yang, H. P. He, Y. L. Ma, F. Wang, Y. Q. Zuo, H. Lin, S. L. Li, L. Li, X. J. Hao, *Three new vanilloid derivatives from the stems of *Baccaurea ramiflora*, Planta Med.* **2010**, 76, 88-90.
- [113] D. Ibrahim, N. Hazali, N. Jauhari, M. N. Yahya, I. A. Ahmed, M. A. Mikail, M. Ibrahim, *Sub-chronic toxicological evaluation of the *Baccaurea angulata* (Belimbing Dayak) fruit juice in rats, International Journal of Applied Research in Natural Products* **2013**, 6, 23-32.
- [114] D. Arbain, J. R. Cannon, K. Kartawinata, R. Djamal, A. Bustari, A. Dharma, H. Rivai, D. Basir, M. Sjafar, R. Nawfa, *Survey of some West Sumatran plants for alkaloids, Econ Bot* **1989**, 43, 73-78.
- [115] A. Latiff, I. M. Turner, K. Mat Salleh, *Taxonomic realignment of Malaysian vascular plants in Burkill's monumental dictionary, The Malayan Nature Journal* **2014**, 65, 171-229.
- [116] C. R. Su, Y. F. Chen, M. J. Liou, H. Y. Tsai, W. S. Chang, T. S. Wu, *Anti-inflammatory activities of furanoditerpenoids and other constituents from *Fibraurea tinctoria*, Bioorg. Med. Chem.* **2008**, 16, 9603-9609.
- [117] P. Rojsanga, W. Gritsanapan, W. Leelamanit, S. Sukrong, *Molecular authentication of Thai medicinal plant Khamin khrua by PCR-RFLP analysis, Planta Med.* **2008**, 74, 1327-1333.
- [118] N. Eswani, K. A. Kudus, M. Nazre, A. A. Noor, M. Ali, *Medicinal plant diversity and vegetation analysis of logged over hill forest of Tekai Tembeling Forest Reserve, Jerantut, Pahang, Journal of Agricultural Science* **2010**, 2, 189-210.
- [119] J. Siwon, R. Verpoorte, A. B. Svendsen, *Studies on Indonesian medicinal plants VI further alkaloids from *Fibraurea chloroleuca*, Planta Med.* **1981**, 41, 65-68.
- [120] C. R. Su, Y. F. Ueng, N. X. Dung, M. Vijaya Bhaskar Reddy, T. S. Wu, *Cytochrome P3A4 inhibitors and other constituents of *Fibraurea tinctoria*, J. Nat. Prod.* **2007**, 70, 1930-1933.
- [121] R. M. Ali, *ASEAN Herbal and Medicinal Plants*, ASEAN Secretariat, **2010**.
- [122] R. P. T. Kim, V. Bihud, K. Bin Mohamad, K. H. Leong, J. Bin Mohamad, F. Bin Ahmad, H. Hazni, N. Kasim, S. N. A. Halim, K. Awang, *Cytotoxic and antioxidant compounds from the stem bark of *Goniothalamus tapisoides* Mat Salleh, Molecules* **2012**, 18, 128-139.
- [123] F. Ahmad, B. A. Moharm, I. Jantan, *A Comparative study of the constituents of the essential oils of *Goniothalamus tapis* Miq. and *G. tapisoides* Mat Salleh from Borneo, Journal of Essential Oil Research* **2010**, 22, 499-502.
- [124] R. B. Gardens, M. B. G. Kew. (2012). The Plant List 1.1, from <http://www.theplantlist.org/>
- [125] K. Mat-Salleh, A. Latiff, *Tumbuhan Ubatan Malaysia*, Pusat Pengurusan Penyelidikan, UKM, **2002**.
- [126] F. B. Ahmad, N. K. N. M. Sallehuddin, Z. Assim, *Chemical constituents and antiviral study of *Goniothalamus velutinus*, Journal of Fundamental Sciences* **2010**, 6, 72-75.
- [127] S. Omar, C. L. Chee, F. Ahmad, J. X. Ni, H. Jaber, J. Huang, T. Nakatsu, *Phenanthrene lactams from *Goniothalamus velutinus*, Journal of Phytochemistry* **1992**, 31, 4395-4397.

- [128] B. Fassihuddin, *Phytochemical and biological studies on Goniiothalamus spp. in Borneo*, *Iranian Journal of Pharmaceutical Research* **2004**, 3, 13-14.
- [129] H. Keng, R.-S. L. Keng, *The concise flora of Singapore: gymnosperms and dicotyledons, Vol. 1*, NUS Press, **1990**.
- [130] I. Turner, *New species of Polyalthia (Annonaceae) from Borneo and their relationship to Polyalthia cauliflora*, *Nordic Journal of Botany* **2010**, 28, 267-279.
- [131] G. M. Cragg, D. J. Newman, K. M. Snader, *Natural products in drug discovery and development*, *J. Nat. Prod.* **1997**, 60, 52-60.
- [132] A. L. Harvey, *Natural products in drug discovery*, *Drug Discov. Today* **2008**, 13, 894-901.
- [133] R. L. Bielecki, N. A. Turner, *Separation and estimation of amino acids in crude plant extracts by thin layer electrophoresis and chromatography*, *Journal of Analytical Biochemistry* **1966**, 17, 278-293.
- [134] A. Gupta, M. Naraniwal, V. Kothari, *Modern extraction methods for preparation of bioactive plant extracts*, *International Journal of Applied and Natural Sciences* **2012**, 1, 8-26.
- [135] A. Nostro, M. Germano, V. D'angelo, A. Marino, M. Cannatelli, *Extraction methods and bioautography for evaluation of medicinal plant antimicrobial activity*, *Lett. Appl. Microbiol.* **2000**, 30, 379-384.
- [136] F. E. Ahmed, *Analyses of pesticides and their metabolites in foods and drinks*, *Trend in Analytical Chemistry* **2001**, 20, 649-661.
- [137] D. E. Breithaupt, *Simultaneous HPLC determination of carotenoids used as food coloring additives: applicability of accelerated solvent extraction*, *Journal of Food Chemistry* **2004**, 86, 449-456.
- [138] F. F. Cantwell, M. Losier, in *Comprehensive Analytical Chemistry*, Vol. 37, Elsevier, **2002**, pp. 297-340.
- [139] J. N. Eloff, *A sensitive and quick microplate method to determine the minimal inhibitory concentration of plant extracts for bacteria*, *Journal of Planta Medica* **1998**, 64, 711-713.
- [140] L. Berrueta, B. Gallo, F. Vicente, *A review of solid phase extraction: basic principles and new developments*, *Journal of Chromatographia* **1995**, 40, 474-483.
- [141] J. Dai, R. J. Mumper, *Plant phenolics: Extraction, analysis and their antioxidant and anticancer properties*, *Journal of Molecules* **2010**, 15, 7313-7352.
- [142] S. Babić, M. Petrović, M. Kaštelan-Macan, *Ultrasonic solvent extraction of pesticides from soil*, *J. Chromatogr. A* **1998**, 823, 3-9.
- [143] V. F. Péres, J. Saffi, M. I. S. Melecchi, F. C. Abad, R. de Assis Jacques, M. M. Martinez, E. C. Oliveira, E. B. Caramão, *Comparison of soxhlet, ultrasound assisted and pressurized liquid extraction of terpenes, fatty acids and Vitamin E from Piper gaudichaudianum Kunth*, *J. Chromatogr. A* **2006**, 1105, 115-118.
- [144] L. Wang, C. L. Weller, *Recent advances in extraction of nutraceuticals from plants*, *Trends in Food Science & Technology* **2006**, 17, 300-312.
- [145] G. Sapkale, S. Patil, U. Surwase, P. Bhatbhage, *Supercritical fluid extraction*, *International Journal of Chemical Sciences* **2010**, 8, 729-743.
- [146] C. S. Eskilsson, E. Björklund, *Analytical-scale microwave assisted extraction*, *J. Chromatogr. A* **2000**, 902, 227-250.
- [147] C. D. Stalikas, *Extraction, separation, and detection methods for phenolic acids and flavonoids*, *J. Sep. Sci.* **2007**, 30, 3268-3295.
- [148] M. J. Wells, J. L. Michael, *Reversed-phase solid-phase extraction for aqueous environmental sample preparation in herbicide residue analysis*, *J. Chromatogr. Sci.* **1987**, 25, 345-350.

- [149] C. F. Poole, *New trends in solid-phase extraction*, *Trends in Analytical Chemistry* **2003**, 22, 362-373.
- [150] D. M. Pavlović, S. Babić, A. J. Horvat, M. Kaštelan Macan, *Sample preparation in analysis of pharmaceuticals*, *Trends in Analytical Chemistry* **2007**, 26, 1062-1075.
- [151] K. M. Li, L. P. Rivory, S. J. Clarke, *Solid Phase Extraction (SPE) techniques for sample preparation in clinical and pharmaceutical analysis: a brief overview*, *Current Pharmaceutical Analysis* **2006**, 2, 95-102.
- [152] J. Płotka Wasylka, N. Szczepańska, M. de la Guardia, J. Namieśnik, *Modern trends in solid phase extraction: new sorbent media*, *Trends in Analytical Chemistry* **2016**, 77, 23-43.
- [153] S. I. Mussatto, L. F. Ballesteros, S. Martins, J. A. Teixeira, *Extraction of antioxidant phenolic compounds from spent coffee grounds*, *Journal of Separation and Purification Technology* **2011**, 83, 173-179.
- [154] Y. Wen, L. Chen, J. Li, D. Liu, L. Chen, *Recent advances in solid-phase sorbents for sample preparation prior to chromatographic analysis*, *Trends in Analytical Chemistry* **2014**, 59, 26-41.
- [155] F. Pena-Pereira, I. Lavilla, C. Bendicho, *Miniaturized preconcentration methods based on liquid-liquid extraction and their application in inorganic ultratrace analysis and speciation: A review*, *Spectrochimica Acta Part B: Atomic Spectroscopy* **2009**, 64, 1-15.
- [156] M. Santiago, S. Strobel, in *Methods Enzymol.*, Vol. 533, Elsevier, **2013**, pp. 303-324.
- [157] J. Sherma, in *A Century of Separation Science*, CRC Press, **2001**, pp. 65-86.
- [158] I. Molnar, C. Horváth, *Reverse-phase chromatography of polar biological substances: separation of catechol compounds by high-performance liquid chromatography*, *Journal of Clinical Chemistry* **1976**, 22, 1497-1502.
- [159] S. K. Bharti, R. Roy, *Quantitative ¹H NMR spectroscopy*, *Trends in Analytical Chemistry* **2012**, 35, 5-26.
- [160] L. P. Koh, D. S. Wilcove, *Is oil palm agriculture really destroying tropical biodiversity ?*, *Conservation Letters* **2008**, 1, 60-64.
- [161] H. Girish, S. Satish, *Antibacterial activity of important medicinal plants on human pathogenic bacteria-a comparative analysis*, *Journal of World Applied Sciences* **2008**, 5, 267-271.
- [162] A. G. Hise, J. Tomalka, S. Ganesan, K. Patel, B. A. Hall, G. D. Brown, K. A. Fitzgerald, *An essential role for the NLRP3 inflammasome in host defense against the human fungal pathogen *Candida albicans**, *Journal of Cell Host & Microbe* **2009**, 5, 487-497.
- [163] P. A. Hunter, S. Dawson, G. L. French, H. Goossens, P. M. Hawkey, E. J. Kuijper, D. Nathwani, D. J. Taylor, C. J. Teale, R. E. Warren, *Antimicrobial-resistant pathogens in animals and man: prescribing, practices and policies*, *J. Antimicrob. Chemother.* **2010**, 65, 3-17.
- [164] J. H. Jorgensen, J. D. Turnidge, *Susceptibility Test Methods: Dilution and Disk Diffusion Methods**, 11 ed., American Society of Microbiology, **2015**.
- [165] C. Kamaraj, A. A. Rahuman, C. Siva, M. Iyappan, A. V. Kirthi, *Evaluation of antibacterial activity of selected medicinal plant extracts from south India against human pathogens*, *Asian Pacific Journal of Tropical Disease* **2012**, 2, 296-301.
- [166] B. A. Bannister, N. T. Begg, S. H. Gillespie, in *Infectious Disease*, Blackwell Science Pty Ltd, **2000**, pp. 35-51.
- [167] R. Gherardi, R. Becerril, C. Nerin, O. Bosetti, *Development of a multilayer antimicrobial packaging material for tomato puree using an innovative technology*, *Journal of LWT-Food Science and Technology* **2016**, 72, 361-367.

- [168] G. Caprioli, L. Navarini, M. Cortese, M. Ricciutelli, E. Torregiani, S. Vittori, G. Sagratini, *Quantification of isoflavones in coffee by using solid phase extraction (SPE) and high performance liquid chromatography tandem mass spectrometry (HPLC - MS/MS)*, *J. Mass Spectrom.* **2016**, 51, 698-703.
- [169] N. M. Garrido, I. G. Economou, A. J. Queimada, M. Jorge, E. A. Macedo, *Prediction of the n - hexane/water and 1-octanol/water partition coefficients for environmentally relevant compounds using molecular simulation*, *AIChE Journal* **2012**, 58, 1929-1938.
- [170] K. Ansari, C. Lal, *Synthesis, physicochemical properties and antimicrobial activity of some new benzimidazole derivatives*, *European Journal of Medicinal Chemistry* **2009**, 44, 4028-4033.
- [171] K. Valko, C. Du, C. Bevan, D. P. Reynolds, M. H. Abraham, *Rapid method for the estimation of octanol/water partition coefficient (log P_{oct}) from gradient RP-HPLC retention and a hydrogen bond acidity term (Sigma α_{2H})*, *Curr. Med. Chem.* **2001**, 8, 1137-1146.
- [172] M. Anastassiades, S. J. Lehotay, D. Štajnbaher, F. J. Schenck, *Fast and easy multiresidue method employing acetonitrile extraction/partitioning and "dispersive solid-phase extraction" for the determination of pesticide residues in produce*, *J. AOAC Int.* **2003**, 86, 412-431.
- [173] D. J. Creek, A. Jankevics, R. Breitling, D. G. Watson, M. P. Barrett, K. E. Burgess, *Toward global metabolomics analysis with hydrophilic interaction liquid chromatography mass spectrometry: improved metabolite identification by retention time prediction*, *Journal of Analytical chemistry* **2011**, 83, 8703-8710.
- [174] B. A. Olsen, *Hydrophilic interaction chromatography using amino and silica columns for the determination of polar pharmaceuticals and impurities*, *J. Chromatogr. A* **2001**, 913, 113-122.
- [175] E. J. Hayhoe, E. A. Palombo, *Extracts of Eremophila longifolia inhibit the cariogenic activities of Streptococcus mutans and Streptococcus sobrinus*, *Journal of medicinal plant research* **2011**, 5, 2476-2482.
- [176] M. E. Saravia, P. Nelson-Filho, R. A. B. Silva, A. De Rossi, G. Faria, L. A. B. Silva, C.-G. Emilson, *Recovery of Streptococcus mutans on MSB, SB-20 and SB-20M agar media*, *Arch. Oral Biol.* **2013**, 58, 311-316.
- [177] C. Kenny, A. Furey, B. Lucey, *A post-antibiotic era looms: can plant natural product research fill the void ?*, *Br. J. Biomed. Sci.* **2015**, 72, 191-200.
- [178] K. Hegstad, C. G. Giske, B. Haldorsen, E. Matuschek, K. Schønning, T. M. Leegaard, G. Kahlmeter, A. Sundsfjord, *Performance of the EUCAST disk diffusion method, the CLSI agar screen method, and the Vitek 2 automated antimicrobial susceptibility testing system for detection of clinical isolates of Enterococci with low and medium level VanB type vancomycin resistance: a multicenter study*, *J. Clin. Microbiol.* **2014**, 52, 1582-1589.
- [179] D. E. Yang, X. Y. Lin, Y. L. Wang, Z. G. Yang, Q. Wei, *Structure-functional relationship of chitosans against bacteria*, *Gaodeng Xuexiao Huaxue Xuebao/Chemical Journal of Chinese Universities* **2006**, 27, 1277-1281.
- [180] U. Mabona, A. Viljoen, E. Shikanga, A. Marston, S. Van Vuuren, *Antimicrobial activity of southern African medicinal plants with dermatological relevance: From an ethnopharmacological screening approach, to combination studies and the isolation of a bioactive compound*, *J. Ethnopharmacol.* **2013**, 148, 45-55.
- [181] P. Velmurugan, K. Anbalagan, M. Manosathyadevan, K.-J. Lee, M. Cho, S. M. Lee, J. H. Park, S. G. Oh, K. S. Bang, B. T. Oh, *Green synthesis of silver and gold nanoparticles using Zingiber officinale root extract and antibacterial activity of silver*

- nanoparticles against food pathogens, *Journal of Bioprocess and Biosystems Engineering* **2014**, 37, 1935-1943.
- [182] M. Mohsen-Nia, H. Amiri, B. Jazi, *Dielectric constants of water, methanol, ethanol, butanol and acetone: Measurement and computational study*, *Journal of Solution Chemistry* **2010**, 39, 701-708.
- [183] X. Yuan, J. Wang, G. Zeng, H. Huang, X. Pei, H. Li, Z. Liu, M. Cong, *Comparative studies of thermochemical liquefaction characteristics of microalgae using different organic solvents*, *Energy* **2011**, 36, 6406-6412.
- [184] J. S. Kirkwood, C. Maier, J. F. Stevens, *Simultaneous, untargeted metabolic profiling of polar and nonpolar metabolites by LC - Q - TOF Mass Spectrometry*, *Curr. Protoc. Toxicol.* **2013**, 4, 1-4.
- [185] J. Coetzee, G. Padmanabhan, *Properties of bases in acetonitrile as solvent. IV. Proton acceptor power and homoconjugation of mono-and diamines*, *J. Am. Chem. Soc.* **1965**, 87, 5005-5010.
- [186] G. Conrads, H. J. de Soet, L. Song, K. Henne, H. Sztajer, I. Wagner Döbler, A. P. Zeng, *Comparing the cariogenic species *Streptococcus sobrinus* and *S. mutans* on whole genome level*, *Journal of Oral Microbiology* **2014**.
- [187] M. Okada, Y. Soda, F. Hayashi, T. Doi, J. Suzuki, K. Miura, K. Kozai, *PCR detection of *Streptococcus mutans* and *S. sobrinus* in dental plaque samples from Japanese pre-school children*, *J. Med. Microbiol.* **2002**, 51, 443-447.
- [188] S. D'eroles, M. Tieri, D. Martinelli, D. Tripodi, *The effect of swimming on oral health status: competitive versus non-competitive athletes*, *Journal of Applied Oral Science* **2016**, 24, 107-113.
- [189] M. M. Nascimento, J. A. Lemos, J. Abranches, R. B. Gonçalves, R. A. Burne, *Adaptive acid tolerance response of *Streptococcus sobrinus**, *J. Bacteriol.* **2004**, 186, 6383-6390.
- [190] J. Gan, S. Papiernik, W. Koskinen, S. Yates, *Evaluation of Accelerated Solvent Extraction (ASE) for analysis of pesticide residues in soil*, *Journal of Environmental Science & Technology* **1999**, 33, 3249-3253.
- [191] W. Jennings, *Qualitative analysis of flavor and fragrance volatiles by glass capillary gas chromatography*, Elsevier, **2012**.
- [192] H. Pennington, *Escherichia coli O157*, *The Lancet* **2010**, 376, 1428-1435.
- [193] R. E. Rosenberg Goldstein, S. A. Micallef, S. G. Gibbs, A. George, E. Claye, A. Sapkota, S. W. Joseph, A. R. Sapkota, *Detection of Vancomycin Resistant Enterococci (VRE) at four U.S. wastewater treatment plants that provide effluent for reuse*, *Sci. Total Environ.* **2014**, 466, 404-411.
- [194] M. L. Nelson, S. B. Levy, *The history of the tetracyclines*, *Ann. N. Y. Acad. Sci.* **2011**, 1241, 17-32.
- [195] A. H. Banskota, Y. Tezuka, K. Q. Tran, K. Tanaka, I. Saiki, S. Kadota, *Thirteen novel cycloartane-type tri terpenes from *Combretum quadrangulare**, *J. Nat. Prod.* **2000**, 63, 57-64.
- [196] H. H. Wasserman, J. L. Ives, *Singlet oxygen in organic synthesis*, *Tetrahedron* **1981**, 37, 1825-1852.
- [197] M. Zamadar, A. Greer, in *Handbook of Synthetic Photochemistry*, Wiley-VCH Verlag GmbH & Co. KGaA, **2010**, pp. 353-386.
- [198] K. S. Ngo, G. D. Brown, *Autoxidation of a santalene*, *Journal of Chemical Research* **2000**, 2000, 68-70.
- [199] M. Sköld, A. Börje, E. Harambasic, A. T. Karlberg, *Contact allergens formed on air exposure of linalool. Identification and quantification of primary and secondary oxidation products and the effect on skin sensitization*, *Chem. Res. Toxicol.* **2004**, 17, 1697-1705.

- [200] L. Hagvall, C. Bäcktorp, S. Svensson, G. Nyman, A. Börje, A. T. Karlberg, *Fragrance compound geraniol forms contact allergens on air exposure. Identification and quantification of oxidation products and effect on skin sensitization*, *Chem. Res. Toxicol.* **2007**, 20, 807-814.
- [201] J. W. de Haan, L. J. M. Van de Ven, *Configurations and conformations in acyclic, unsaturated hydrocarbons. A ¹³C NMR study*, *Organic Magnetic Resonance* **1973**, 5, 147-153.
- [202] K. Akhtar, S. B. Khan, L. Khan, *Synthesis and characterization of new derivatives of cinnamic acid*, *Organic Chemistry: An Indian Journal* **2009**, 5, 142-147.
- [203] M. Zanetti, T. K. Carniel, A. Valério, J. V. d. Oliveira, D. d. Oliveira, P. H. H. d. Araújo, H. G. Riella, M. A. Fiori, *Synthesis of geranyl cinnamate by lipase catalyzed reaction and its evaluation as an antimicrobial agent*, *J. Chem. Technol. Biotechnol.* **2017**, 92, 115-121.
- [204] M. Imai, T. Kumaoka, M. Hosaka, Y. Sato, C. Li, M. Sudoh, Y. Tamada, H. Yokoe, S. Saito, M. Tsubuki, N. Takahashi, *Inhibitory effects of hydroxylated cinnamoyl esters on lipid absorption and accumulation*, *Bioorg. Med. Chem.* **2015**, 23, 3788-3795.
- [205] S. S. Hong, J. S. Oh, *Phenylpropanoid ester from Zingiber officinale and their inhibitory effects on the production of nitric oxide*, *Arch. Pharm. Res.* **2012**, 35, 315-320.
- [206] K. Uwai, Y. Osanai, T. Imaizumi, S.-i. Kanno, M. Takeshita, M. Ishikawa, *Inhibitory effect of the alkyl side chain of caffeic acid analogues on lipopolysaccharide-induced nitric oxide production in RAW264.7 macrophages*, *Bioorg. Med. Chem.* **2008**, 16, 7795-7803.
- [207] A. A. Hussein, D. A. Olmedo, Y. Vasquez, P. D. Coley, P. N. Solis, M. P. Gupta, *New cytotoxic cinnamic acid derivatives from leaves of Bonamia trichantha*, *Revista Latinoamericana de Quimica* **2005**, 33, 90-95.
- [208] K. Otoguro, M. Iwatsuki, A. Ishiyama, M. Namatame, A. Nishihara Tsukashima, H. Kiyohara, T. Hashimoto, Y. Asakawa, S. Ōmura, H. Yamada, *In vitro antitrypanosomal activity of some phenolic compounds from propolis and lactones from Fijian Kawa (Piper methysticum)*, *Journal of Natural Medicines* **2012**, 66, 558-561.
- [209] D. L. Gaveau, D. Sheil, M. A. Salim, S. Arjasakusuma, M. Ancrenaz, P. Pacheco, E. Meijaard, *Rapid conversions and avoided deforestation: examining four decades of industrial plantation expansion in Borneo*, *Journal of Scientific Reports* **2016**, 6, 1-14.
- [210] D. L. Gaveau, M. Kshatriya, D. Sheil, S. Sloan, E. Molidena, A. Wijaya, S. Wich, M. Ancrenaz, M. Hansen, M. Broich, *Reconciling forest conservation and logging in Indonesian Borneo*, *Journal of PLOS One* **2013**, 8, 1-11.
- [211] H. Goh, *Influence of park governance on tourism development In Kinabalu Park, Malaysia Borneo*, *Journal of WIT Transactions on Ecology and the Environment* **2015**, 193, 929-939.
- [212] B. Fasihuddin, L. B. Din, *Medicinal plants used by various ethnic groups in Sarawak*, *Malaysian Journal of Science* **2017**, 18, 7-11.
- [213] P. Uppuluri, A. Srinivasan, A. Ramasubramanian, J. L. Lopez-Ribot, *Effect of fluconazole, amphotericin B and caspofungin against Candida albicans biofilms under conditions of flow and on biofilm dispersion*, *Antimicrob. Agents Chemother.* **2011**, AAC. 01701-01710.
- [214] G. Ramage, K. V. Walle, B. L. Wickes, J. L. Lopez-Ribot, *Characteristics of biofilm formation by Candida albicans*, *Revista iberoamericana de micología* **2001**, 18, 163-170.
- [215] D. M. MacCallum, in *Pathogenic Yeasts* (Eds.: R. Ashbee, E. M. Bignell), Springer Berlin Heidelberg, **2010**, pp. 41-67.

- [216] P. Lambert, *Cellular impermeability and uptake of biocides and antibiotics in Gram - positive bacteria and mycobacteria*, *J. Appl. Microbiol.* **2002**, 92.
- [217] K. Molbak, J. E. Oslen, H. C. Wegener, in *Foodborne Infections*, 3rd ed. (Eds.: H. P. Riemann, D. O. Cliver), Academic Press, London, **2006**.
- [218] A. Garg, S. Sujatha, J. Garg, N. S. Acharya, S. C. Parija, *Wound infections secondary to snakebite*, *The Journal of Infection in Developing Countries* **2009**, 3, 221-223.
- [219] J. Hauke, T. Kossowski, *Comparison of values of Pearson's and Spearman's correlation coefficients on the same sets of data*, *Quaestiones geographicae* **2011**, 30, 87-93.
- [220] J. H. Zar, *Biostatistical Analysis*, Prentice-Hall, **1984**.
- [221] W. Pan, D. Jussier, N. Terrade, R. Y. Yada, R. M. De Orduna, *Kinetics of sugars, organic acids and acetaldehyde during simultaneous yeast-bacterial fermentations of white wine at different pH values*, *Food research international* **2011**, 44, 660-666.
- [222] E. Bartowsky, *Bacterial spoilage of wine and approaches to minimize it*, *Lett. Appl. Microbiol.* **2009**, 48, 149-156.
- [223] E. Mani-López, H. S. García, A. López-Malo, *Organic acids as antimicrobials to control Salmonella in meat and poultry products*, *Food Research International* **2012**, 45, 713-721.
- [224] K. Over, N. Hettiarachchy, M. Johnson, B. Davis, *Effect of organic acids and plant extracts on Escherichia coli O157: H7, Listeria monocytogenes, and Salmonella Typhimurium in broth culture model and chicken meat systems*, *J. Food Sci.* **2009**, 74, 515-521.
- [225] J. C. Buzby, P. D. Frenzen, B. Rasco, *Product liability and microbial foodborne illness*, US Department of Agriculture, Economic Research Service, **2001**.
- [226] S. DeBolt, D. R. Cook, C. M. Ford, *L-Tartaric acid synthesis from vitamin C in higher plants*, *Journal of Proceedings of the National Academy of Sciences* **2006**, 103, 5608-5613.
- [227] K. Saito, Z. Kasai, *Accumulation of tartaric acid in the ripening process of grapes*, *Plant Cell Physiol.* **1968**, 9, 529-537.
- [228] H. Cen, Y. Bao, Y. He, D.-W. Sun, *Visible and near infrared spectroscopy for rapid detection of citric and tartaric acids in orange juice*, *Journal of Food Engineering* **2007**, 82, 253-260.
- [229] P. Muangthai, P. Nookaew, *Monitoring on some organic acids in fresh and processed rural plant leaves in Thailand*, *Asian Journal of Natural & Applied Sciences* **2015**, 4, 82-89.
- [230] K. Saito, F. A. Loewus, *Formation of tartaric acid in vitaceous plants: Relative contributions of L-ascorbic acid inclusive and non inclusive Pathways*, *Plant Cell Physiol.* **1989**, 30, 905-910.
- [231] S. Yamaguchi, K. Ninomiya, *Umami and Food Palatability*, *The Journal of Nutrition* **2000**, 130, 921-926.

Appendix

Table A1: Survey of the therapeutic significance of some *Eremophila* species ^[85]

Species	Part used	Folk therapeutic uses
<i>E. alternifolia</i>	Leaves	cold, flu related symptoms and wounds
<i>E. cuneifolia</i>	Leaves	cold and headaches
<i>E. dalyana</i>	Leaves	cold and Respiratory Tract Infections (RTI)
		scabies
<i>E. duttonii</i>	Leaves	scabies
		cold and flu related symptoms
		insect repellents
<i>E. fraseri</i>	Leaves	cold and flu related symptoms
		toothache and rheumatisms
<i>E. freelingii</i>	Leaves	cold and flu related symptoms
		infectious gastroenteritis
<i>E. gilesii</i>	Leaves	cold and skin infections
<i>E. latrobei</i>	Stems and leaves	cold and skin infections
<i>E. longifolia</i>	Leaves	RTI and headaches
		skin infections
<i>E. maculata</i>	Leaves	cold
<i>E. sturtii</i>	Leaves and branches	RTI , headache , sores and cuts
<i>E. paisley</i>	Twigs and leaves	Antiseptic for most of the skin infections

Table A2: Volatile oil composition of *E. longifolia* specimens collected from various locations in western NSW, Australia ^[94]

NSW Bioregion		MD	MD	MD	MD	MD	BHC	MDD	CP
Voucher ID		JES019	SM003	SM004	SM005	SM012	SM017	SM018	SM019
Month of collection		Aug	Dec	Dec	Dec	Dec	Dec	Dec	Dec
Yield (% w/w)		3.1	5.7	4.9	5.6	5.1	1.9	0.5	0.4
Compounds	RRI	Quantity (%)	Identification						
Bornylene	902	–	–	–	–	–	–	–	1.3
α -Pinene	935	–	–	–	–	–	–	–	0.4
α -Fenchene	950	–	–	–	–	–	–	–	0.5
Camphene	952	–	–	–	–	–	–	–	2.5
Sabinene	974	–	–	–	–	–	–	–	5
β -pinene	979	–	–	–	–	–	–	–	0.2
β -Myrcene	990	–	–	–	–	–	–	–	1.3
4-Carene	998	0.1	0.4	0.4	0.4	0.4	–	–	–
α -Phellandrene	1006	–	–	–	–	–	–	–	0.2
α -Terpinene	1017	–	0.3	0.3	0.3	0.4	0.1	–	0.4
p-Cymene	1026	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.7
Limonene	1030	0.2	0.8	0.7	0.7	0.8	2.1	0.9	9.9
β -Phellandrene	1032	–	–	–	–	–	–	–	0.8
β -Ocimene (Z)	1048	–	–	–	–	–	–	–	0.5
γ -Terpinene	1058	–	–	–	–	–	–	–	0.6
Terpinolene	1084	–	–	–	–	–	0.3	–	4.7
Fenchone	1087	–	–	–	–	–	–	–	0.4
Linalool	1088	0.3	0.6	0.1	0.4	0.3	1	–	–
Fenchol	1117	–	–	–	–	–	–	–	19.7

Table A2: Volatile oil composition of *E. longifolia* specimens collected from various locations in western NSW, Australia. (cont.)

NSW Bioregion		MD	MD	MD	MD	MD	BHC	MDD	CP
Voucher ID		JES019	SM003	SM004	SM005	SM012	SM017	SM018	SM019
Month of collection		Aug	Dec	Dec	Dec	Dec	Dec	Dec	Dec
Yield (% w/w)		3.1	5.7	4.9	5.6	5.1	1.9	0.5	0.4
Compounds	RRI	Quantity (%)	Identification						
Menth-2-en-1-ol	1125	0.1	0.1	0.1	0.2	0.1	0.1	0.1	0.3
Limonene oxide	1132	–	–	–	–	–	–	–	0.4
Isomenthone	1168	75.2	67.5	61.1	74.5	86.7	0.3	10.1	1.2
Borneol	1175	–	–	–	–	–	–	–	31.7
4-Terpineol	1187	–	0.1	0.2	0.2	0.2	–	–	4.4
α -Terpineol	1196	8.3	8.8	11	11	8.8	11.7	4.1	1.3
Exo-Fenchyl acetate	1218	–	–	–	–	–	–	–	0.1
(S)-Carvone	1247	–	–	–	–	–	–	0.1	–
Piperitone	1259	1.6	3.8	2.8	1.7	0.5	0.4	0.1	–
Bornyl acetate	1284	–	–	–	–	–	–	–	7.2
Unidentified	–	5.4	0.3	0.4	1	0.7	2.9	2.2	2.8
Total identified	–	94.6	99.7	99.6	99	99.3	97.1	97.8	97.2

Abbreviations: RRI: Kovats retention indices, relative to C9–C24 alkanes on non-polar column (ZB-5 ms); (%): Relative percentage obtained from peak area; MD: Mootwingee Downs bioregion; BHC: Broken Hill Complex bioregion; MDD: Murray–Darling Depression bioregion; CP: Cobar penepain bioregion; Identification: MS: Mass spectra; RI: Retention

Table A3: Diverse names for *Baccaurea lanceolata* (Miq.) Müll.Arg

<i>Sarawak anthology</i>	<i>Synonyms</i>	<i>Vernacular names</i> (According to the Tribe)	<i>Common/English name</i>
Pisau (Bidayuh)	<i>Adenocrepis lancealatus</i> (Miq.) Müll. Arg	Limpasu (Banjarese: Bundu Tuhan) Ampusu (Bidayuh)	Lampaong
Limpa'ong (Iban)	<i>Baccaurea pyrrhodasya</i> (Miq.) Müll. Arg	Asam pauh, Empaong, Lampaong Lampawong, Lampong (Iban)	
Bua'pau (Kelabit)	<i>Baccaurea glabriflora</i> Pax & K. Hoffm	Buah Lepasu Lipasu, Nipassu (Dusun) Kalampesu, Lempahong (Kalimantan)	
Lepesu (Penan)	<i>Hedycarpus lancealatus</i> Miq. (basionym) <i>Pierardia pyrrhodasya</i> Miq.	Buah Lipauh (Kelabit) Kelepesoh (Kenyah) Tampoy (Malay) Buah Lepesuh (Punan)	

Table A4: Diverse names for *F.tinctoria* Lour^[109, 115]

<i>Synonyms</i>	<i>Vernacular names</i>
<i>Fibraurea chloroleua</i>	Akar kunyit, randau merkunyit (Iban) Lang pempuan (Kedayan) Akar penawar (Penan) Akbah binak (Kayn) Laka lete (Penan Totoh)

Figure B: Supporting Information antibacterial neryl ferulate from the Australian Plant *Eremophila longifolia*

Figure B1: ¹H NMR spectrum of neryl ferulate (1) (CDCl₃, 600 MHz)

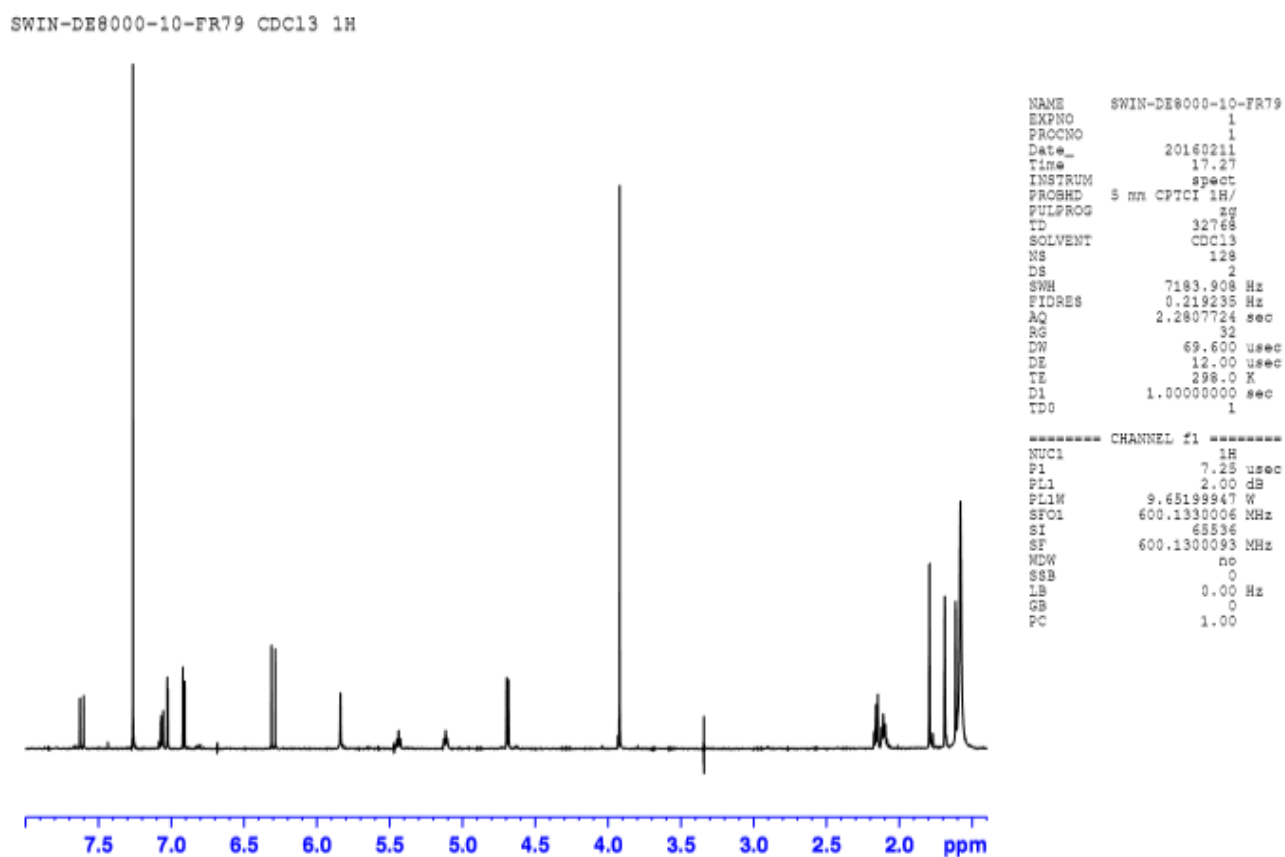


Figure B2: Expansion of ^1H NMR spectrum of neryl ferulate (1) (CDCl_3)

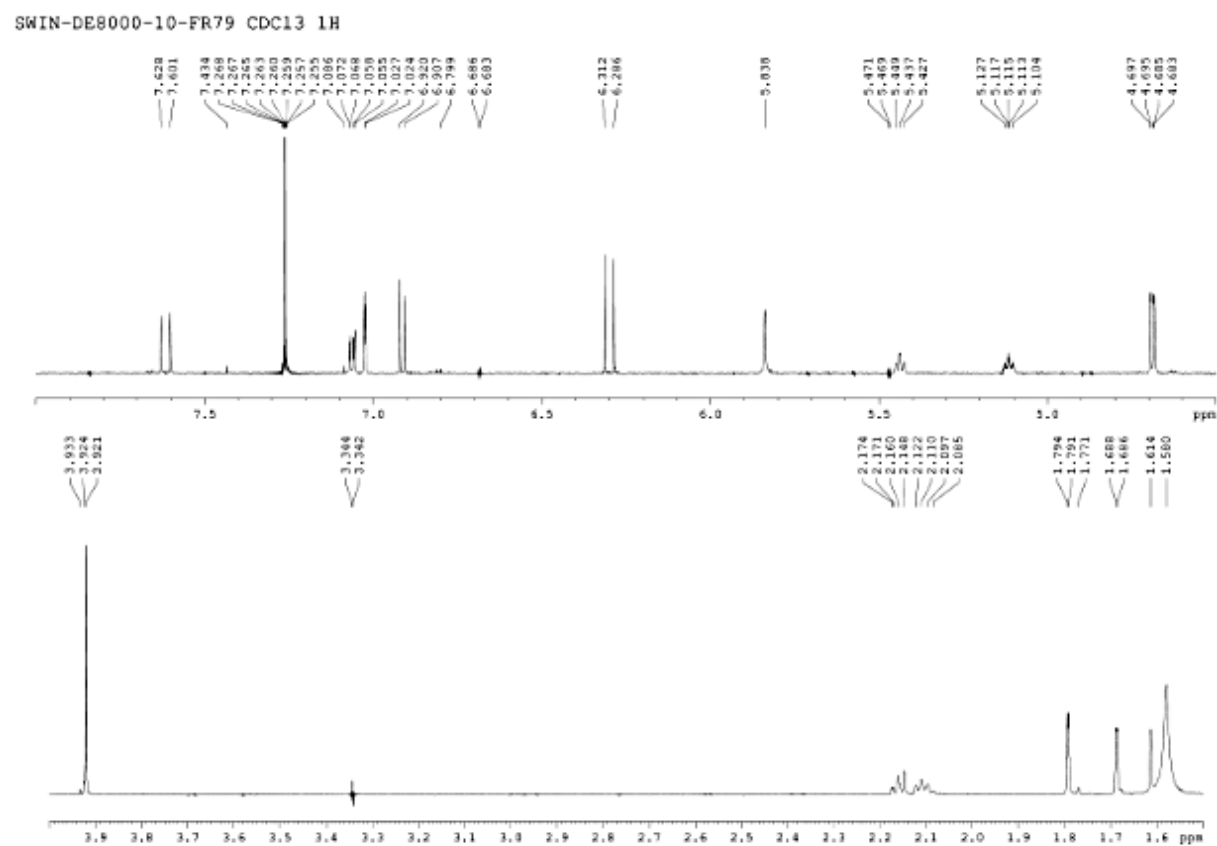


Figure B3: ^{13}C NMR spectrum of neryl ferulate (1) (CDCl_3 , 150 MHz)

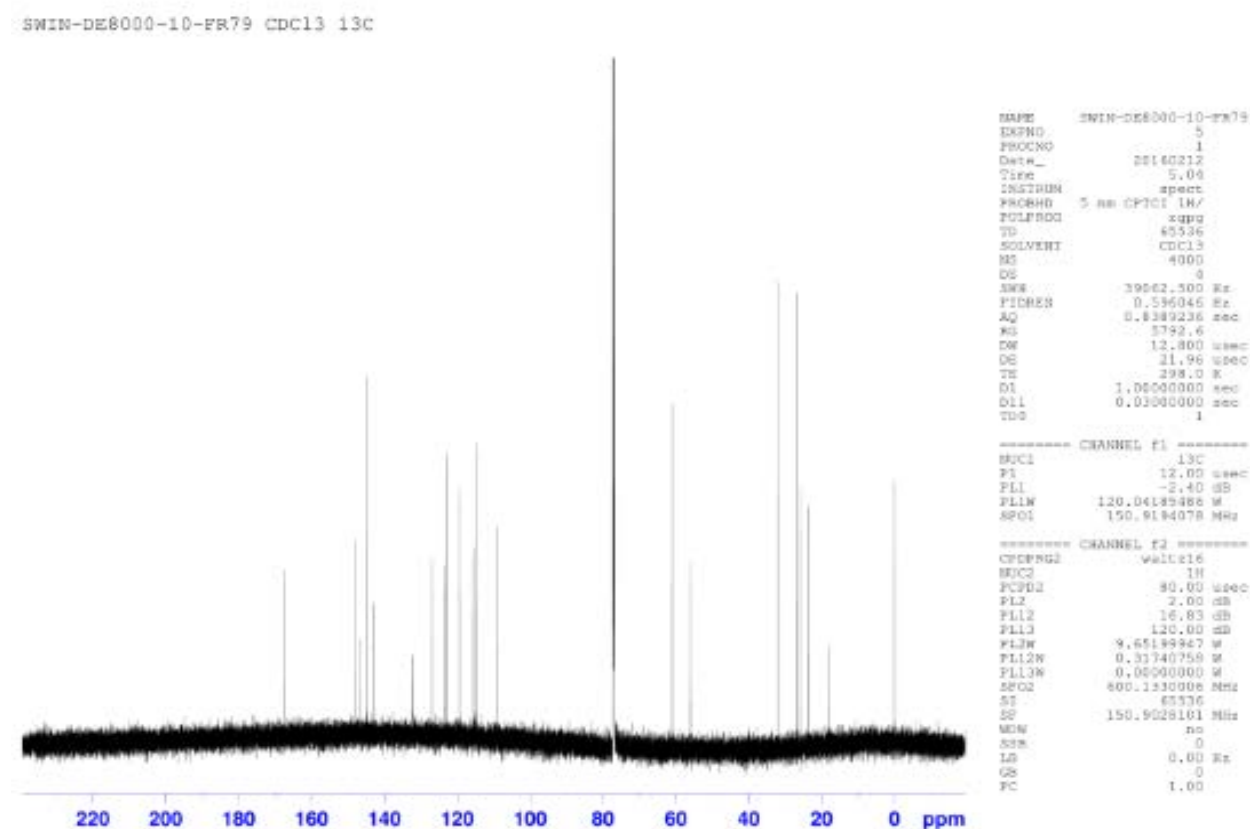


Figure B4: Expansion of ^{13}C NMR spectrum of neryl ferulate (1) (CDCl_3 , 150 MHz)

SNIN-DE8000-10-FR79 CDCl_3 ^{13}C

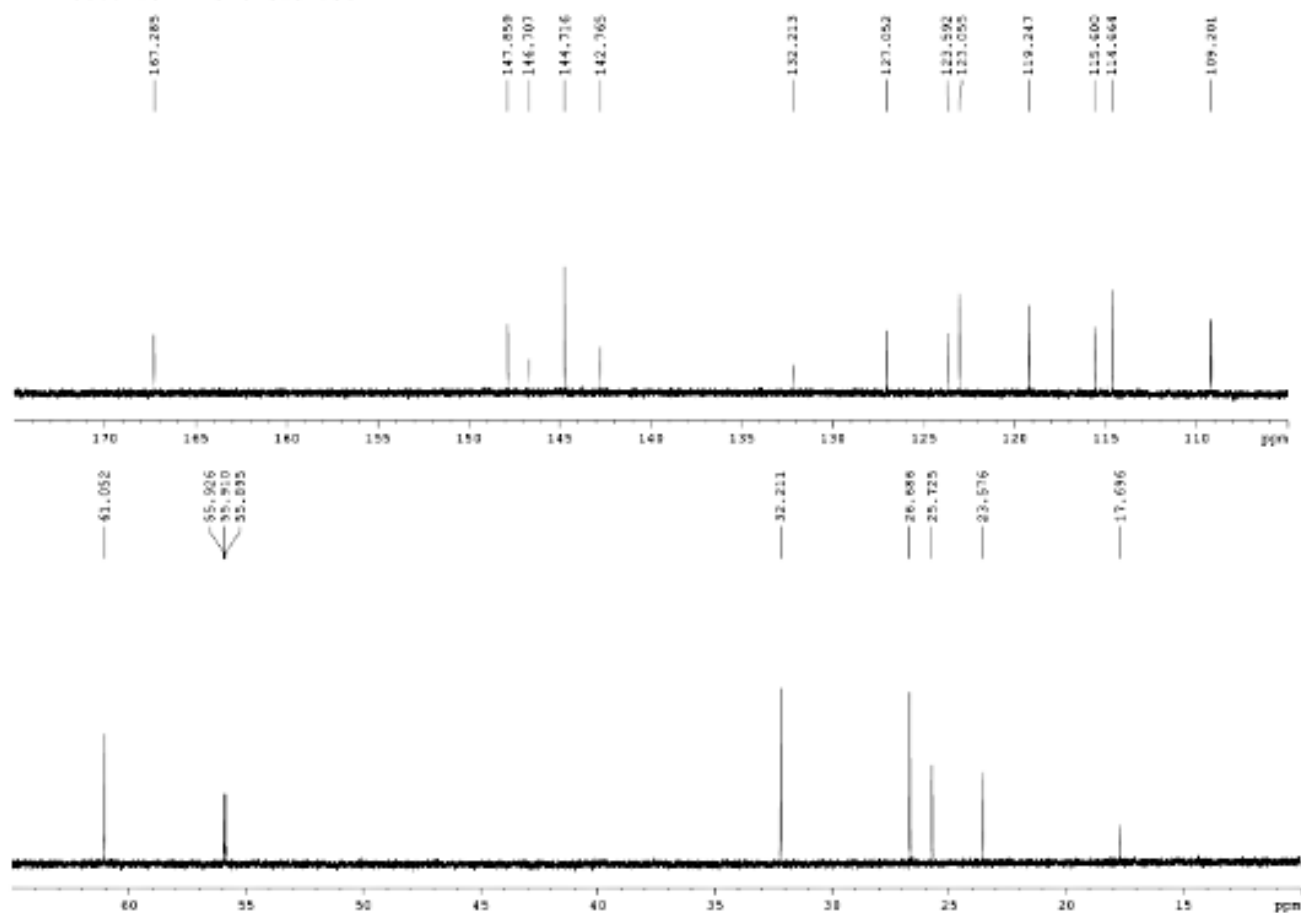
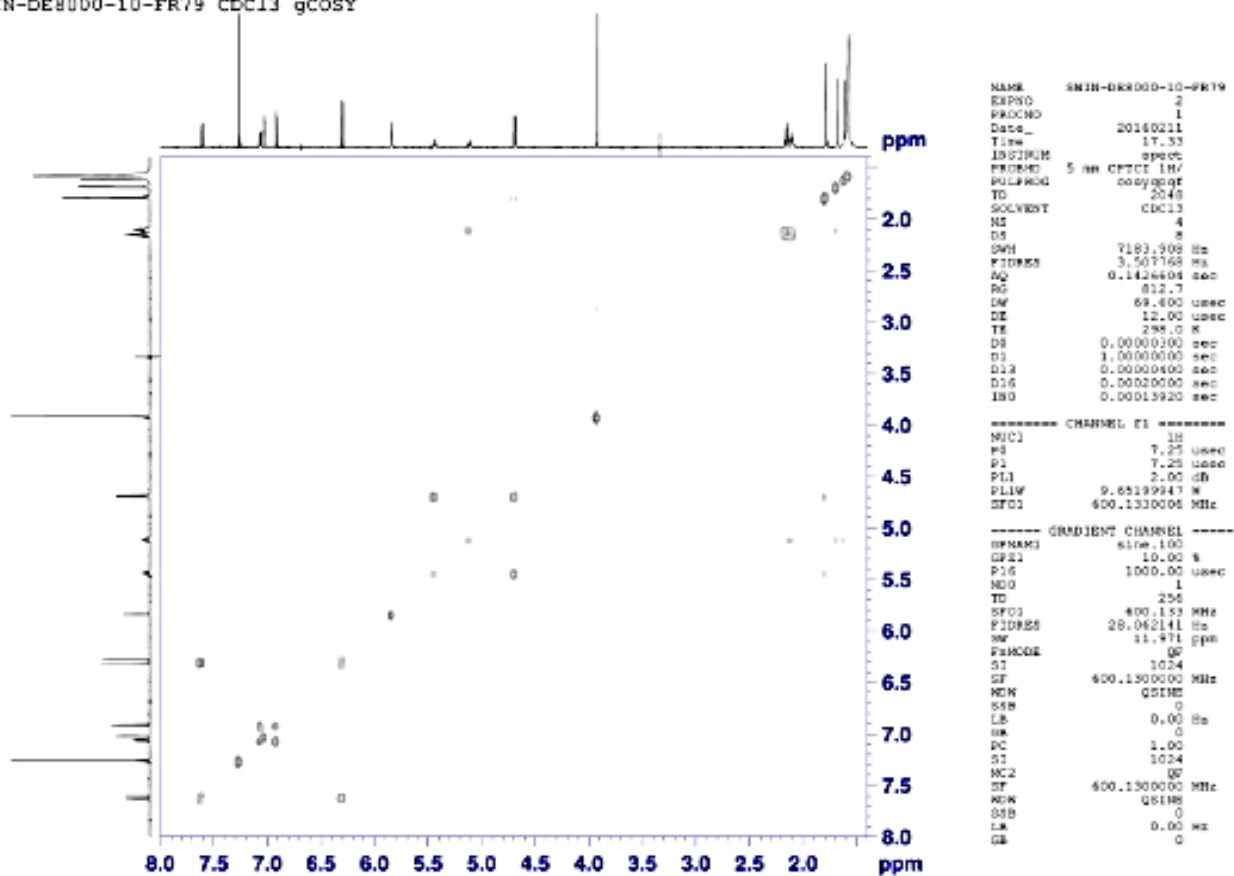


Figure B5: COSY NMR spectrum of neryl ferulate (1) (CDCl₃)

SWIN-DE8000-10-FR79 CDCl₃ gCOSY



SWIN-DE8000-10-FR79 CDCl3 edrSQC

2D NMR spectrum (HSQC) showing correlations between ^1H (horizontal axis, 8.0 to 2.0 ppm) and ^{13}C (vertical axis, 20 to 140 ppm) signals. The spectrum displays several cross-peaks indicating scalar coupling between protons and carbons. Key peaks are labeled with their ^{13}C chemical shifts: 150.0, 140.0, 130.0, 120.0, 110.0, 100.0, 90.0, 80.0, 70.0, 60.0, 50.0, 40.0, 30.0, 20.0.

Figure B7: HMBC NMR spectrum of neryl ferulate (1) (CDCl₃)

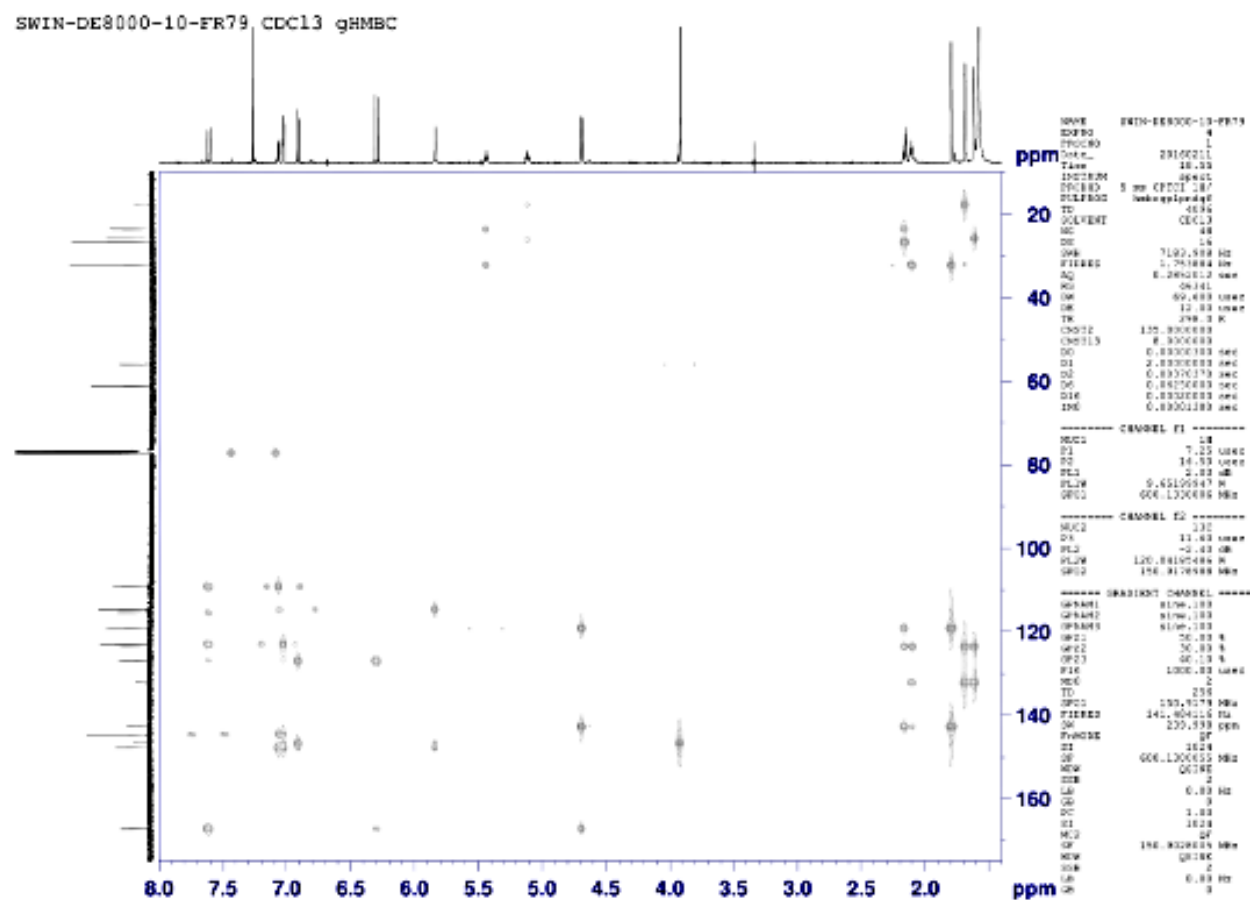
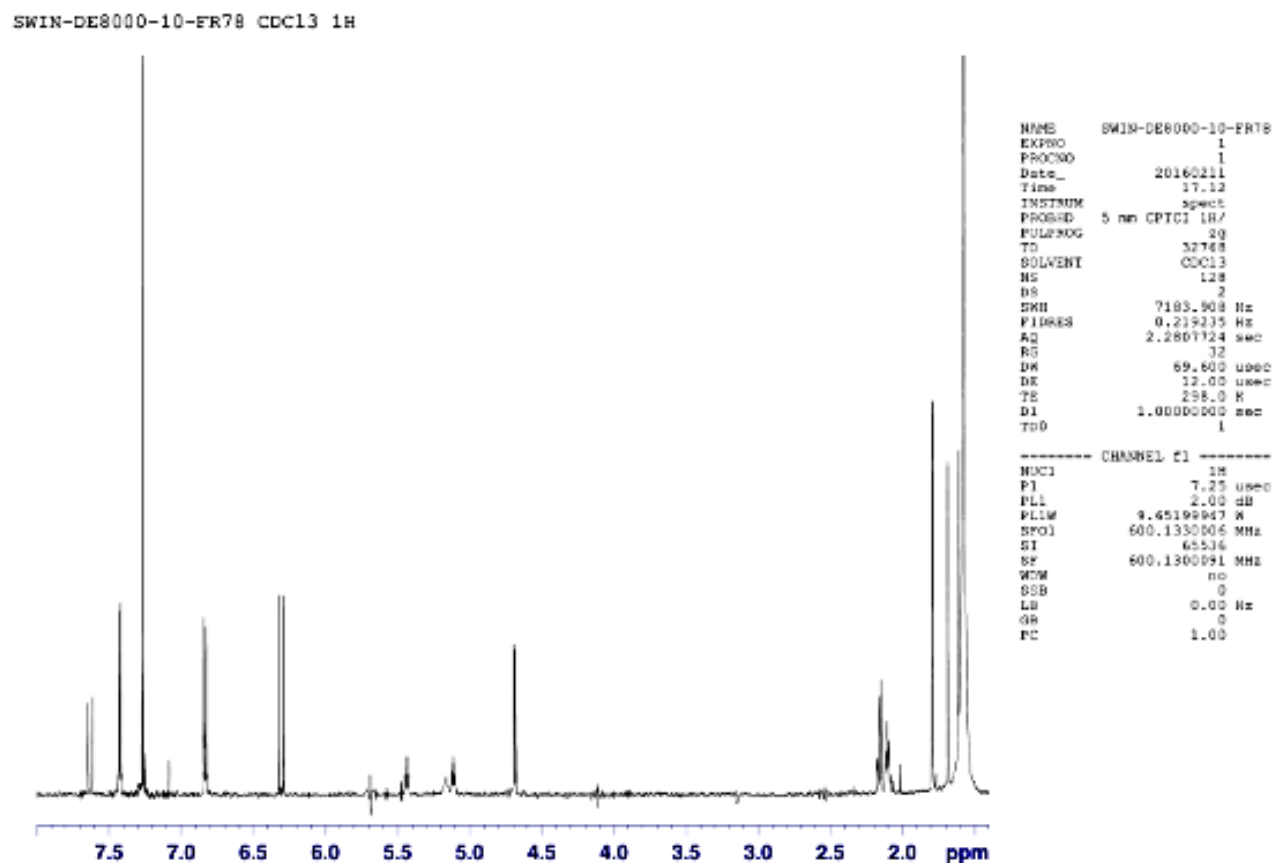


Table C: Supporting Information antibacterial neryl *p*-coumarate from the Australian Plant *Eremophila longifolia*

Figure C1: ^1H NMR spectrum of neryl *p*-coumarate (2) (CDCl_3 , 600 MHz)



SWIN-DE8000-10-FR78 CDCl3 1H

7.435, 7.431, 7.428, 7.420, 7.417, 7.392, 7.382, 7.378, 7.273, 7.271, 7.270, 7.268, 7.267, 7.264, 7.263, 7.260, 7.256, 7.255, 7.253, 7.252, 7.251, 7.250, 7.250, 7.248, 7.247, 7.231, 6.894, 6.892, 6.892, 6.889, 6.887, 6.359, 6.359, 6.293, 5.713, 5.689, 5.685, 5.579, 5.576, 5.474, 5.472, 5.446, 5.436, 5.424, 5.158, 5.129, 5.123, 5.114, 5.114, 5.111, 5.111, 5.102, 5.099, 4.723, 4.484, 4.692, 4.682, 4.680, 4.130, 4.106

7.5, 7.0, 6.5, 6.0, 5.5, 5.0, 4.5 ppm

2.593, 2.530, 2.537, 2.192, 2.190, 2.186, 2.186, 2.119, 2.107, 2.085, 2.082, 2.071, 2.061, 2.053, 1.781, 1.780, 1.780, 1.686, 1.685, 1.652, 1.575

3.9, 3.8, 3.7, 3.6, 3.5, 3.4, 3.3, 3.2, 3.1, 3.0, 2.9, 2.8, 2.7, 2.6, 2.5, 2.4, 2.3, 2.2, 2.1, 2.0, 1.9, 1.8, 1.7, 1.6, 1.5 ppm

Figure C3: ^{13}C NMR spectrum of neryl *p*-coumarate (2) (CDCl_3)

SWIN-DE8000-10-FR78 CDCl_3 ^{13}C

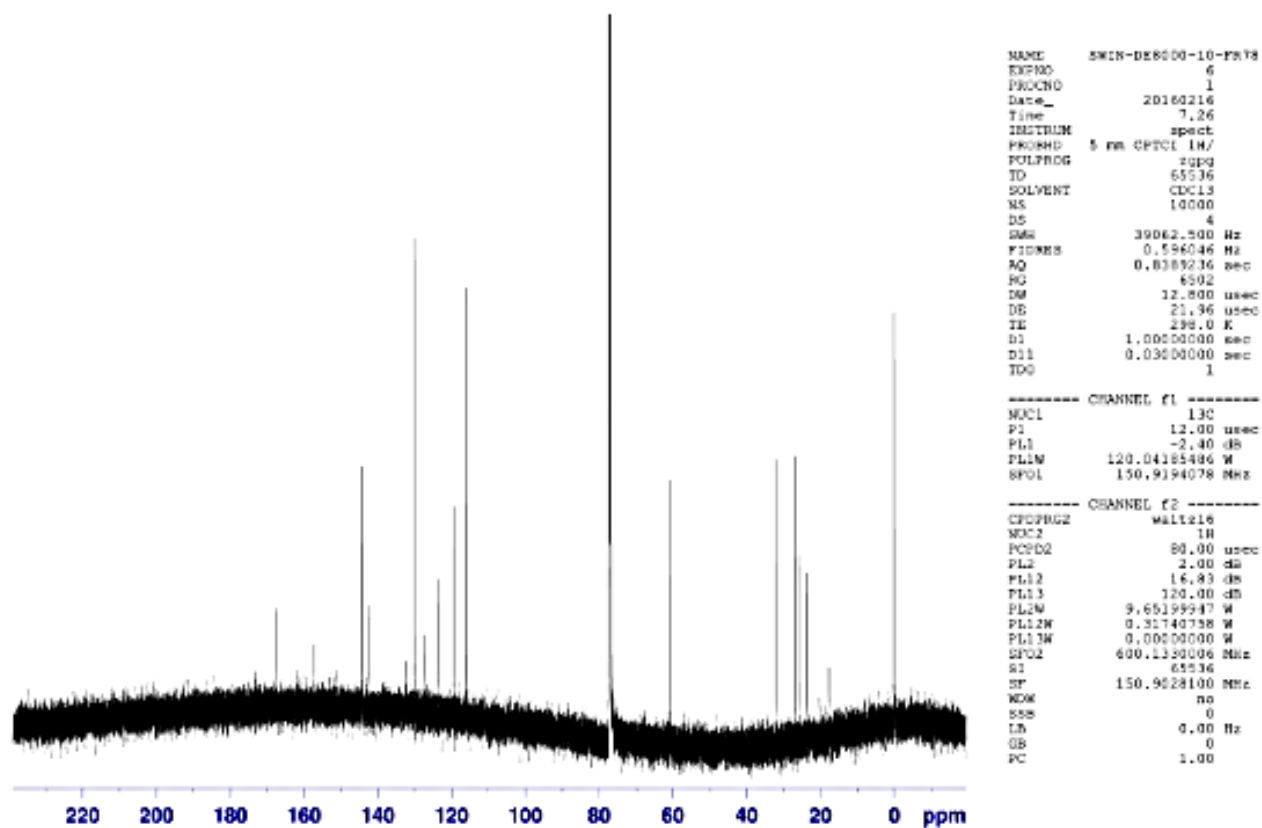


Figure C4: Expansion of ^{13}C NMR spectrum of neryl *p*-coumarate (2) (CDCl_3)

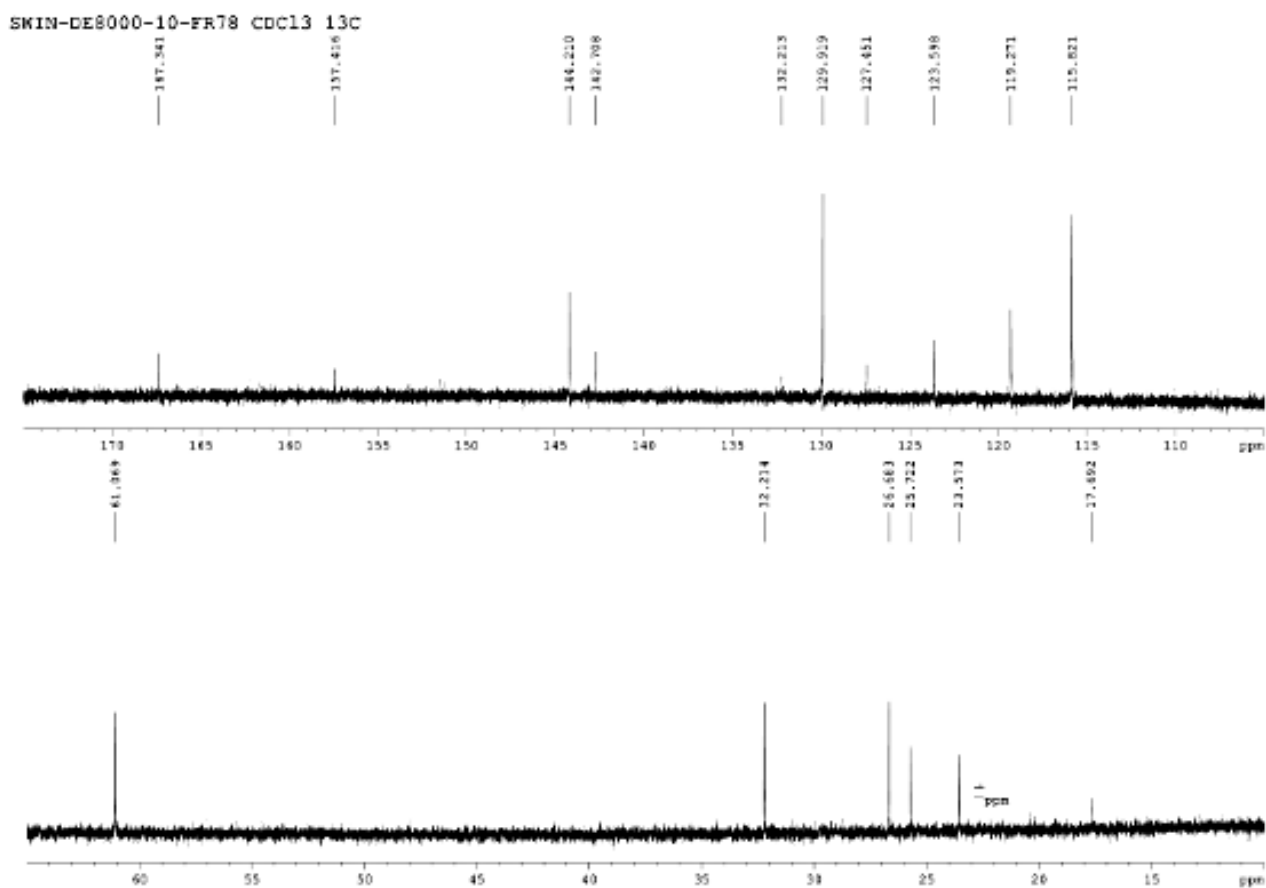
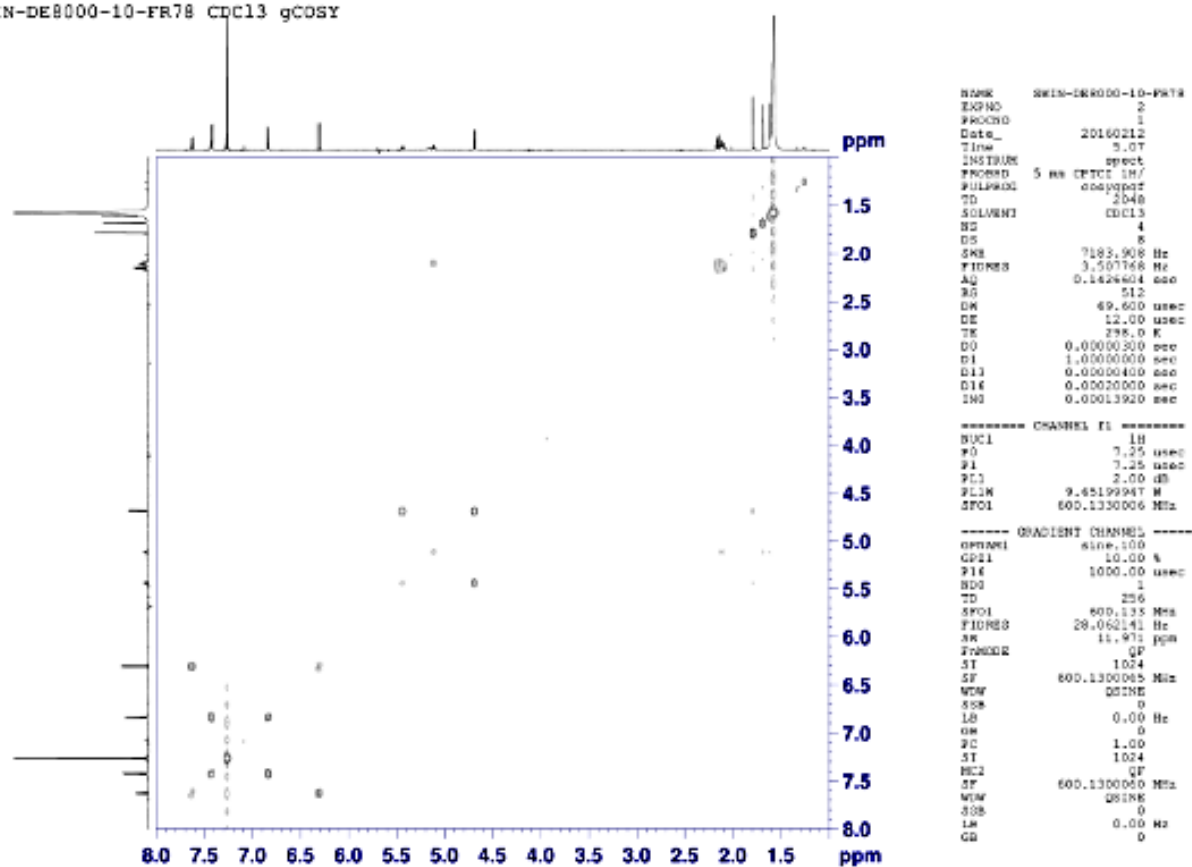
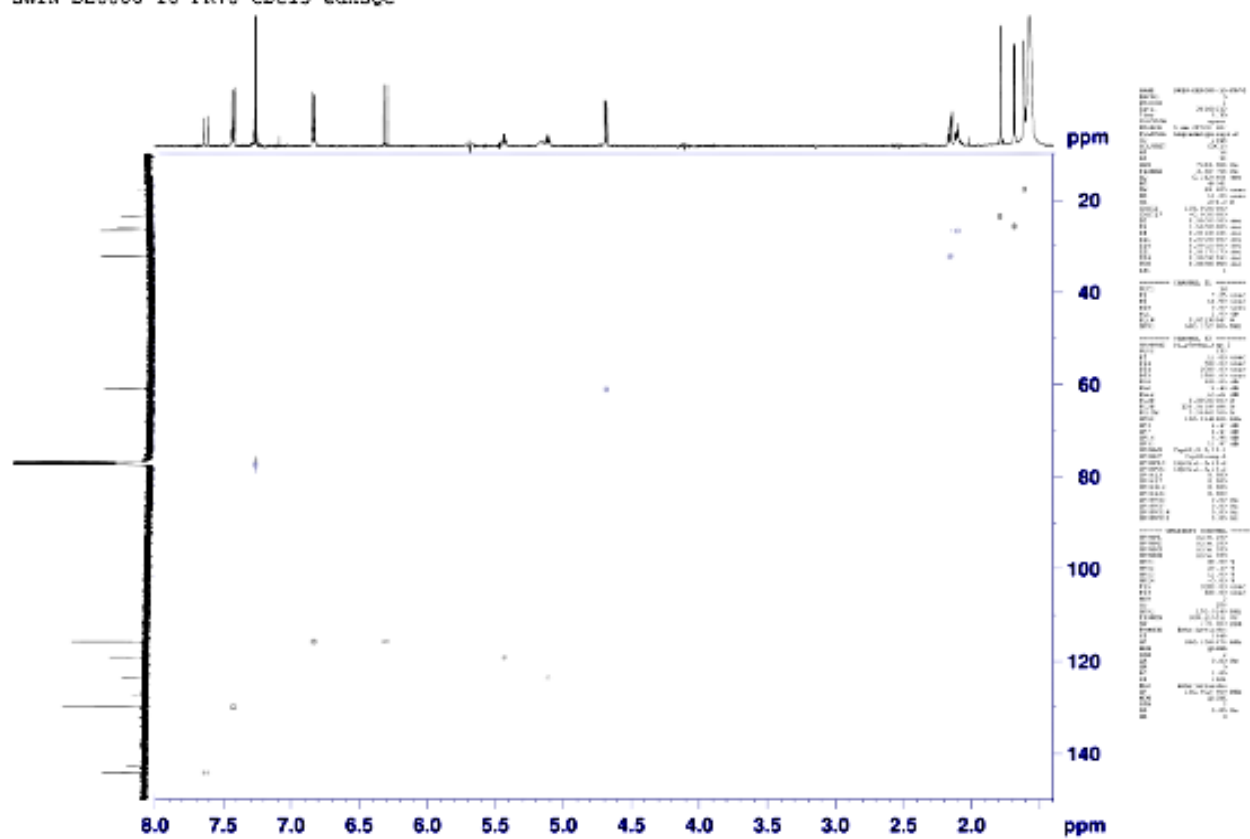


Figure C5: COSY NMR spectrum of neryl *p*-coumarate (2) (CDCl₃)

SWIN-DE8000-10-FR78 CDCl₃ gCOSY



SWIN-DE8000-10-FR78 CDC13 edHSQC



SWIN-DE8000-10-FR78 CDC13 HMBC

NAME SWIN-DE8000-10-FR78
EXPNO 3
PROCNO 3
DATE_ 20160213
TIME 19.25
INSTRUM spect
PROBHD 5 mm CPDQ1 1H/
PULPROG zgpg30
TD 65536
FIDRES 0.094
AQ 0.2800112
RG 655.36
GC 16.943
DM 69.660 sec
DE 12.00 dB
TE 300.2 K
CHCT2 158.8830000
CHCT13 8.8830000
EO 0.0000000 sec
E1 2.0000000 sec
E3 0.0000000 sec
E8 0.0000000 sec
E15 0.0000000 sec
IM0 0.0001388 sec
===== CHANNEL f1 =====
NUC1 1H
P1 7.20 usec
PL1 14.00 dB
PC1 2.00 dB
PS1W 9.0019947 W
SFO1 500.1320000 MHz
===== CHANNEL f2 =====
NUC2 13C
P2 11.40 usec
PL2 -2.40 dB
PC2W 100.6418486 W
SFO2 100.6217000 MHz
===== GRABBER CHANNEL =====
CPDPRG2 gnmr.100
GPNMR2 gnmr.100
GPMNR3 gnmr.100
GP13 50.00 M
OP13 00.00 Hz
GPC3 40.00 Hz
P15 1000.00 kHz
TD 274
SFO3 150.9175 MHz
ZM 141.454116 Hz
JMOD 279.976 cps
FWDPRG2 CF
SI 1024
SF 500.1320000 MHz
GQ1PR2 GQ1PR2
DD0 0
LS 0.00 Hz
GB 0
PC 1.00
SI 1024
MC2 CF
SP 150.9027942 MHz
MEW QNP1H
KCN 0
LN 0.00 Hz
GR 0

Figure D: Supporting NMR spectrums of neryl ferulate autooxidation

Figure D1: Structure of neryl ferulate (1) autooxidation products and peak assignments in the HSQC spectrum

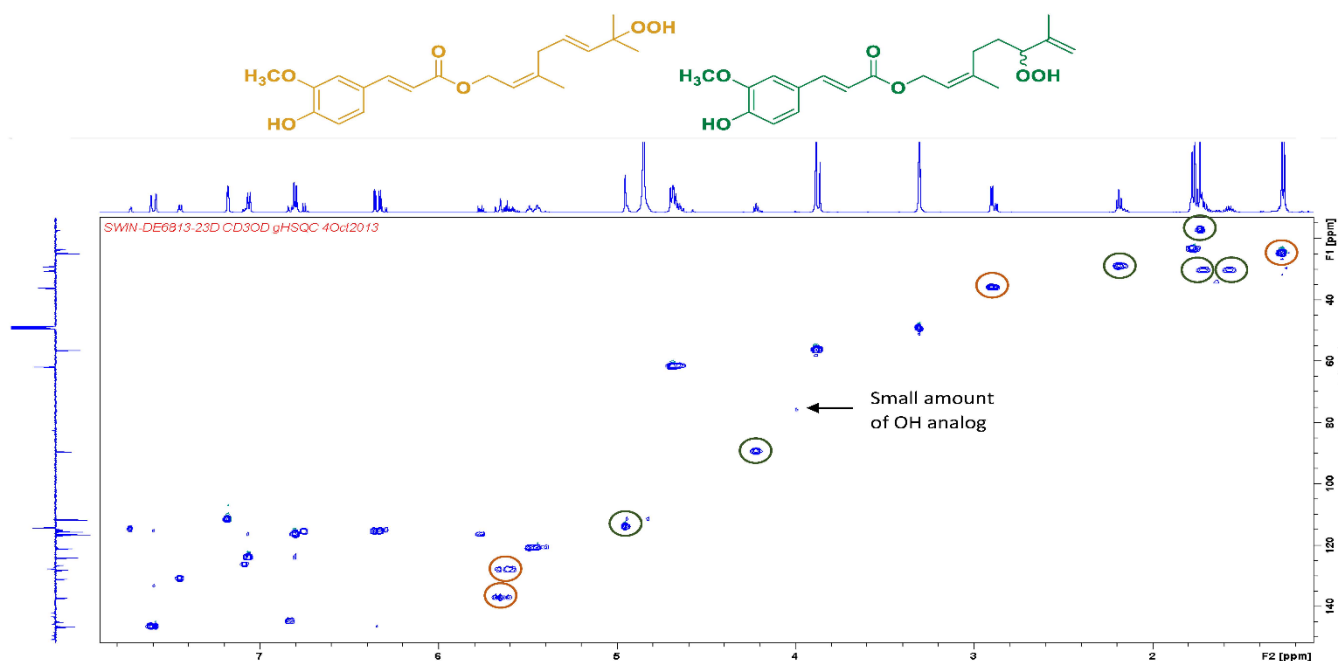


Figure D2: ^1H NMR spectrum of neryl ferulate (1) autooxidation products (CD_3OD , 600 MHz)

SWIN-DE6813-23D CD3OD 1H 4Oct2013

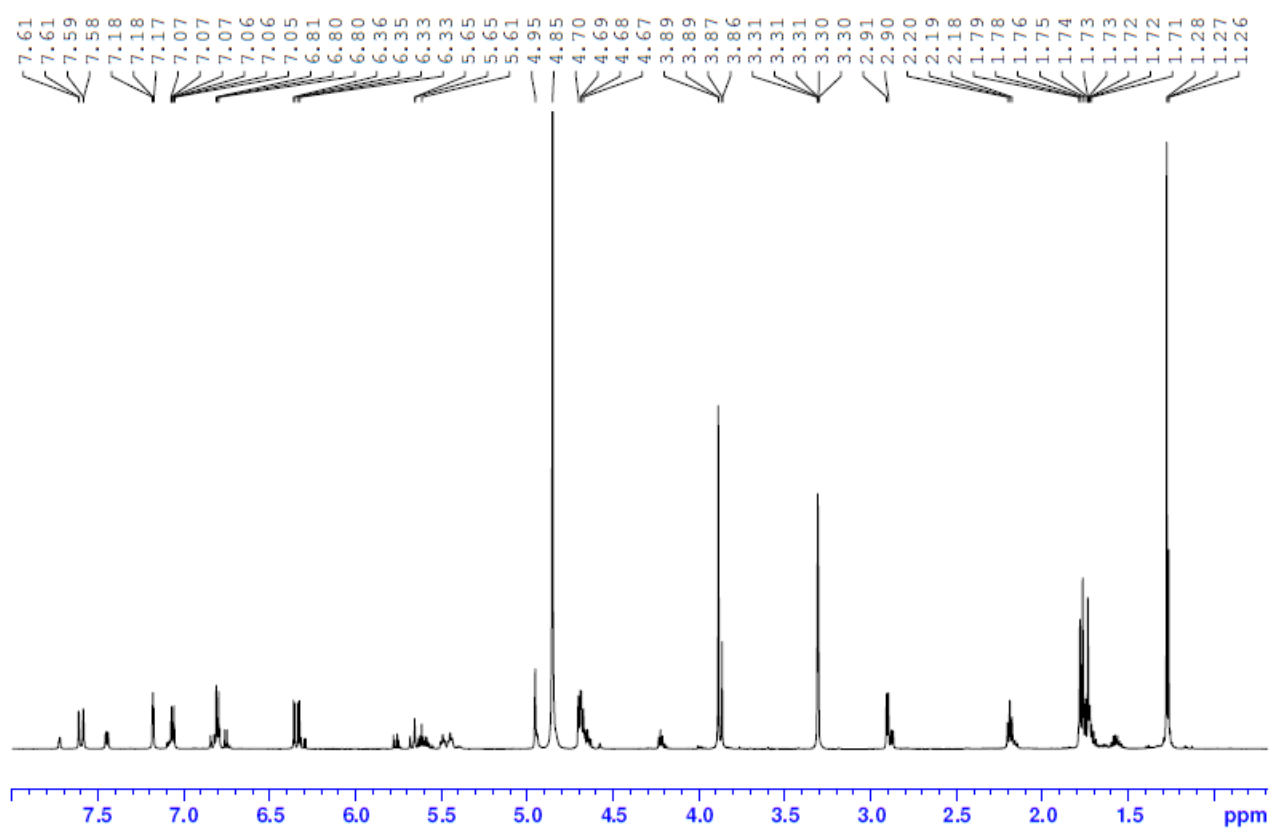


Figure D3: JMOD NMR spectrum of neryl ferulate (1) autooxidation products (CD₃OD, 125 MHZ)

SWIN-DE6813-23D CD3OD JMOD 4Oct2013

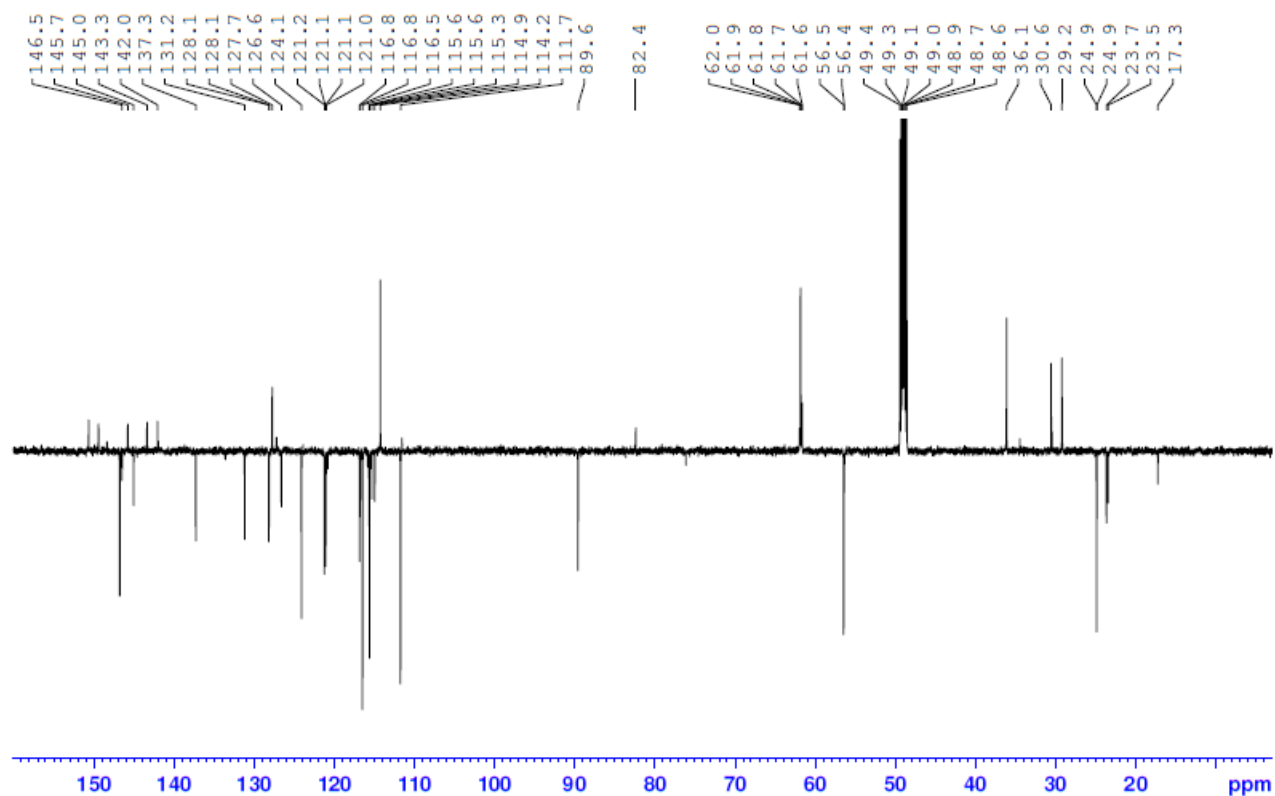


Figure D4: COSY NMR spectrum of neryl ferulate (1) autoxidation products (CD₃OD)

SWIN-DE6813-23D CD3OD gCOSY 40Oct2013

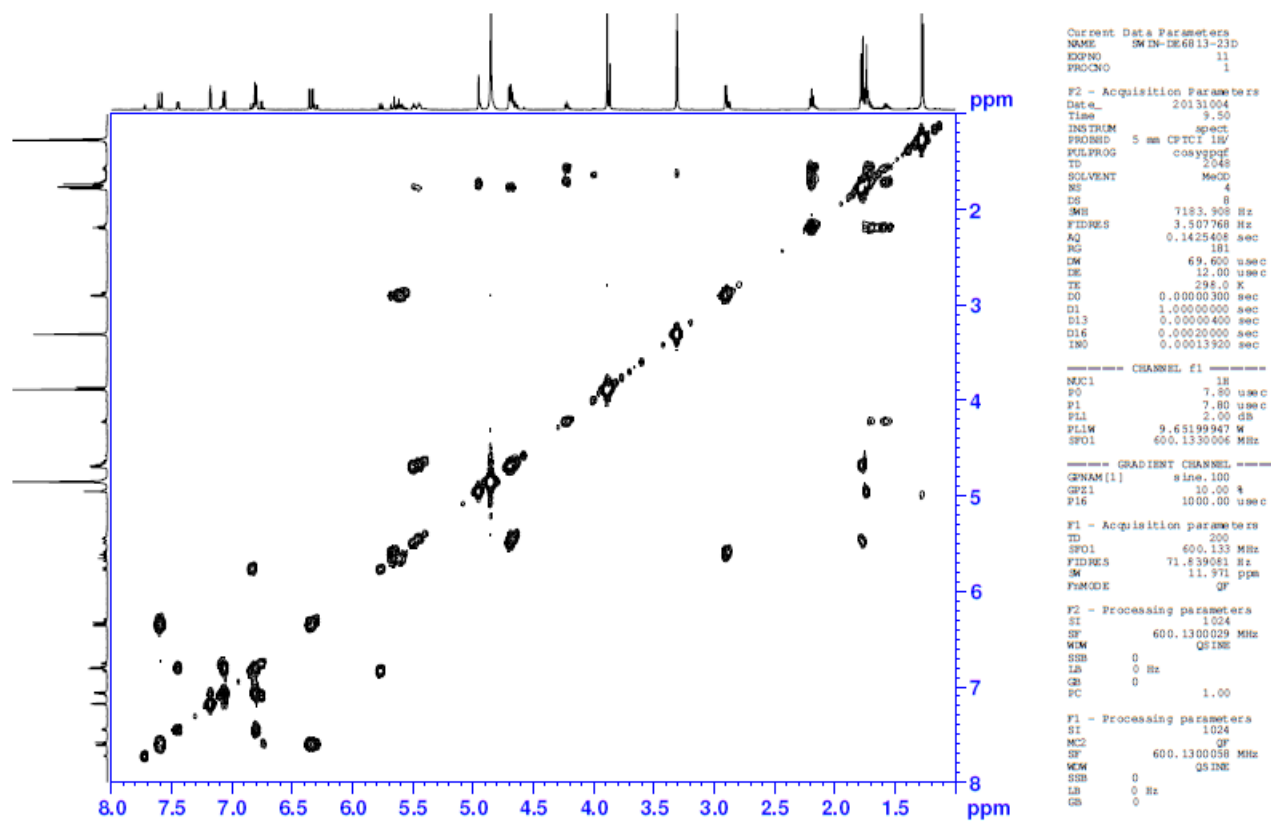


Figure D5: HMBC NMR spectrum of neryl ferulate (1) autoxidation products (CD₃OD)

SWIN-DE6813-23D CD3OD gHMBC 40Oct2013

