Biological and Phytochemical Analysis of Three Medicinally Important Plants: *Prunus persica*, *Diospyros lanceifolia* and *Holboellia latifolia*

Teresa Malewska

Bachelor of Plants Biochemistry

Master of Science in Microbiology, University of Lodz, Poland,

MPhil in Chemistry and Biomolecular Sciences, Macquarie University, Sydney

A thesis submitted in partial fulfilment of the requirements for the degree

DOCTOR OF PHILOSOPHY

Department of Molecular Sciences

MAQUARIE UNIVERSITY

SYDNEY

2019

DECLARATION

The work presented in this thesis has not been submitted, either in the whole or in part, for a higher degree to any other university or institution, and to the best of my knowledge is my own and original work, except as acknowledged in the text.

Teresa Malewska

December 2019

ACKNOWLEDGEMENTS

First and utmost, I would like to thank A/Profs Joanne Jamie and Subra Vemulpad for providing me with this PhD opportunity. I am very grateful to Joanne and Subra for all the help and support I have received. It has been my privilege to work closely with them. I have been honoured to watch and learn from their knowledge and experience. Their insights and patience will always be remembered and appreciated. I am delighted we could work together and am proud of what we achieved. Thank you!

I am also indebted to Prof. Zbigniew Sidorczyk and Dr Krystyna Zych whose counsel led me through my postgraduate studies at University of Lodz, Poland.

I will always remember my Departmental friends past and present who have made my study enjoyable: Tarannum Naz (Sony), Mikolaj Gad (Nick), Kaisarun Akter (Kalpa), Jason Smith, Meyanungsang (Meza) Kichu and Rayan Al Omar.

My special thanks go to A/Prof. Paul Prenzler from Charles Sturt University of Wagga Wagga for all his hospitality and help with the metabolomics research. I am grateful to Donald Cameron, Ben Hanssen and Sally Noushini for their assistance with GC-MS research.

GC-MS work in this thesis was also performed at the University of New South Wales by Dr Joseph Brophy. I am very grateful to Dr Brophy for his assistance. I also wish to express my thanks to Dr Russell Barrow for organising the HR-MS of the isolated compounds.

I am incredibly indebted to my whole family for their unrelenting help, support and belief in me. Completion of this degree would not have been possible without them!

LIST OF ABREVIATIONS

The following abbreviations are used throughout the text:

ATCC	American Type Culture Collection
br	broad
$CDCl_3$	Deuterated chloroform
CD_3COCD_3	Deuterated acetone
CD_3OD	Deuterated methanol
Cfu	Colony Forming Units
$CHCl_3$	Chloroform
COSY	(Proton-Proton) Correlation Spectroscopy
d	doublet (MNR)
DAD	Diode array detector
DCM	Dichloromethane
DMSO	Dimethyl sulfoxide
EtOAc	Ethyl Acetate
EI	Electronic impact
EIC	Extracted ion chromatogram
ESI	Electrospray Ionisation
EtOH	Ethanol
GC-MS	Analytical gas chromatography mass spectroscopy
GMF	Generated molecular formula
HMBC	Heteronuclear Multiple Bond Correlation
HR-MS	High Resolution Mass Spectrometry
HSQC	Heteronuclear Single Quantum Correlation
J	Coupling constant
LC-MS	Liquid chromatography-mass spectrometry
m	Multiptet (NMR)
mg	Milligram
m/z	Mass to charge ratio
MDRSA	Multidrug resistant *Staphylococcus aureus*
MeOH	Methanol
MH II	Muller Hinton II
MIC	Minimum inhibitory concentration

MIQ	Minimum inhibitory quantity
MRSA	Methicillin resistant *Staphylococcus aureus*
MTT	3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazoliun bromide
NIST	National Institute of Standards and Technology
NMR	Nuclear Magnetic Resonance
NOESY	Nuclear Overhauser effect spectroscopy
QTOF-MS	Quadrupole time-of-flight mass spectrometry
R_f	Retention factor
RT	Retention time
s	Singlet (NMR)
SEC	Size exclusion chromatography
t	Triplet (NMR)
TLC	Thin layer chromatography
TIC	Total ion chromatogram
UV-Vis	Ultraviolet-Visible
2D NMR	Two-Dimensional Nuclear Magnetic Resonance Spectroscopy
µg	Microgram

TABLE OF CONTENTS

ABSTRACT

The plants *Prunus persica*, *Holboellia latifolia* and *Diospyros lanceifolia* have been used as traditional medicines by several Indigenous communities. This includes the use of the roots of *P. persica* and leaves of *H. latifolia* and *D. lanceifolia* for treatment of skin infections and other ailments of a likely microbial origin. These plant parts have limited reported investigations on their phytochemistry and/or antimicrobial properties.

Screening of the 70% aqueous ethanolic extracts of the roots of *P. persica* and leaves of *H. latifolia* and *D. lanceifolia* using the 3-(4,5-dimethyldthiazol-2-yl)-2,5-diphenyltetrazolium bromide MTT microdilution assay showed promising activity against the human pathogens *Staphylococcus aureus*, *Pseudomonas aeruginosa*, *Streptococcus pyogenes* and *Salmonella typhimurium*. The highest inhibitory activities were exhibited by the *P. persica* root extract, with MIC (minimum inhibitory concentration) values of 156 µg/mL for antibiotic susceptible *S. aureus*, methicillin resistant *S. aureus* (MRSA) and multidrug resistant *S. aureus* (MDRSA). The leaf extract of *D. lanceifolia* had MIC values of 156 µg/mL for *S. aureus* and *P. aeruginosa*, 625 µg/mL for MRSA and MDRSA, and 312 µg/mL for antibiotic susceptible *E. coli.* The leaf extract of *H. latifolia* had MIC values of 156 µg/mL for the susceptible *S. aureus*, 1250 µg/mL for MRSA and MDRSA and 625 µg/mL for *S. typhimurium*.

The dried 70% aqueous ethanolic extracts of *P. persica*, *H. latifolia* and *D. lanceifolia* were partitioned against water and sequentially *n*-hexane, dichloromethane and ethyl acetate. The strongest antibacterial activity of the partitioned extracts was observed for the ethyl acetate partition of *P. persica* with MIC 312 µg/mL (susceptible as well as resistant strains of *S. aureus*) and *n*-hexane partition of *P. persica* (MIC 625 µg/mL for susceptible *S. aureus*, and 312 µg/mL for MRSA, MDRSA, susceptible *E. coli* and *P. aeruginosa* strains). The *n*-hexane partition of *D. lanceifolia* showed antibacterial activity against susceptible *S. aureus* and *P. aeruginosa* with MICs of 156 µg/mL, antibiotic sensitive *E. coli*, MRSA and MDRSA with MICs of 312 µg/mL, and *S. typhimurium* with MIC 625 µg/mL. The dichloromethane partition of the *H. latifolia* extract showed activity against antibiotic sensitive as well as resistant strains of *S. aureus* (156 µg/mL for *S. aureus* and 1250 µg/mL for both, MRSA and MDRSA) as well as against *P. aeruginosa* (MIC 1250 µg/mL).

The *n*-hexane and ethyl acetate partitions of *P. persica* were subjected to TLC bioautography guided isolation by normal phase chromatography, size exclusion chromatography and preparative TLC. This led to the isolation of α-cyanobenzyl benzoate, β-sitosterol and stigmast-4-en-3-one from the *n*-hexane partition and gallic acid and caffeic acid phenylethyl ester from the ethyl acetate partition. α-Cyanobenzyl benzoate, assayed by TLC bioautography (MIQ - minimum inhibitory quantity) and

MTT microdilution tests (MIC), showed excellent antimicrobial properties (MIQ/MIC 78 µg/mL) against all tested *S. aureus* strains, MIQ/MIC of 312 µg/mL against β lac- *E. coli*, and MIQ/MIC of 612 µg/mL against *P. aeruginosa*. Gallic acid showed MIQ/MIC values of 156 µg/mL against antibiotic susceptible *S. aureus*, MIQ/MIC of 312 µg/mL against MRSA and MIQ/MIC of 2500 µg/mL against β lac- *E. coli* as well as *P. aeruginosa*. Caffeic acid phenylethyl ester and β-sitosterol showed activity against MRSA and MDRSA (MIQ/MIC 625 µg/mL), and activity against antibiotic sensitive *S. aureus* (MIQ/MIC 312 µg/mL). Additionally, both compounds showed antibacterial activity against susceptible strains of *E. coli*, with a MIQ/MIC values for β-sitosterol of 2500 µg/mL and for caffeic acid phenylethyl ester 312 µg/mL. Stigmast-4-en-3-one was active against *S. aureus*, MRSA and β lac- *E. coli* with MIQ/MIC values of 2500 µg/mL, as well as *P. aeruginosa* with MIQ/MIC values of 1250 µg/mL. This is the first report of isolation of caffeic acid phenylethyl ester and stigmast-4-en-3-one from *P. persica* plant material, the second report of α-cyanobenzyl benzoate being present in the *Prunus* genus and the first report of antibacterial activity of stigmast-4-en-3-one (this compound was previously reported not active).

TLC bioautography guided isolation of the *n*-hexane partition of *D. lanceifolia* led to the isolation of lupeol and plumbagin, whose structures were confirmed by spectral analyses. Lupeol showed antimicrobial activity by TLC bioautography against *P. aeruginosa* (MIQ 156 µg/mL) and *E. coli* (MIQ 312 µg/mL), against *S. aureus* and MRSA (MIQ 2500 µg/mL) and *S. typhimurium* (MIQ 625 µg/mL). Plumbagin, due to its very small amount, was tested only by the bioautography method against one microorganism, susceptible *S. aureus*, and showed excellent activity (MIQ 62 µg/mL). GC-MS analysis of the *n*-hexane partition of *D. lanceifolia* also showed the presence of the quinones plumbagin and 7-methyljuglone, which have been found in this and other *Diospyros* species and have both been previously reported for possessing antimicrobial activity.

GS-MS analysis of *H. latifolia* showed the presence of hexanal and nonanal, which have been previously reported as volatile compounds from many plant species including those of medicinal value.

In order to further investigate the phytochemistry of the antibacterially active extracts of *P. persica*, *H. latifolia* and *D. lanceifolia*, an LC-UV-MS metabolomics approach was used. The following compounds were tentatively identified from the *P. persica* ethanolic extract and/or dichloromethane and ethyl acetate partitions: afzelechin, caffeic acid phenylethyl ester, catechin, *ent*-epiafzelechin-(2α→O→7',4α→8')-(-)-*ent*-afzelechin, *ent*-epiafzelechin-(2α→7,4α→8)-epicatechin and/or *ent*-epiafzelechin-(2α→7,4α→8)-catechin and/or 3'''-hydroxyafzelechin-(2β→7,4β→8)-catechin, epicatechin, eriodictyol, gallic acid, gallocatechin, kaempferol, leucocyanidin, mandelic acid β-D-glucopyranoside, persicogenin, proanthocyanidin A1 or A2 type, quinic acid and taxifolin-7-*O*-

rhamnoside. All compounds except mandelic acid β-D-glucopyranoside and taxifolin-7-*O*-rhamnoside were previously isolated from the *Prunus* genus.

In the crude aqueous ethanolic extract of *D. lanceifolia* only one peak was identified with an acceptable degree of confidence, and it was identified as either ursolic, oleanolic or betulinic acid, which have all been previously reported from the *Diospyros* genus. In the *n*-hexane partition, scopoletin or isoscopoletin, epiisoshinalone or shinalone, and ricinoleic acid were tentatively identified. Those compounds were previously reported to be present in the *Diospyros* genus. No compounds could be confidently identified in the crude ethanolic extract or dichloromethane partition of *H. latifolia*, however some possible candidates were proposed.

All of the compounds tentatively identified by the metabolomics approach, as well as the isolated compounds from *P. persica* roots and *D. lanceifolia* and *H. latifolia* leaves, align with the traditional applications of those plant parts by Indigenous communities.

Chapter 1 - Preface

This chapter introduces the importance of medicinal plants used by Indigenous peoples, *i.e.* traditional medicinal plants, and of the need for new antimicrobial agents, and provides examples of antimicrobial compounds that have been isolated from traditional medicinal plants. This chapter concludes with an outline of the aims of the research conducted.

1.1 Human use of natural products

Fossil records date human use of plants as medicines to at least the Middle Palaeolithic era some 60,000 years ago. The earliest written information on the medicinal usage of plants comes from China in 5000 B.C. (Greathead 2003), Mesopotamia in 2600 B.C. (Newman et al. 2012), India in 2000 B.C. (Ramawat et al. 2009), and Egypt in 1550 B.C. (Newman et al. 2012). Hippocrates (in the late fifth century B.C.) described 300 to 400 medicinal plants (Thomson et al. 1978). *De Materia Medica*, written between 50 and 70 B.C. by Dioscorides, became the prototype for modern pharmacopoeias, describing medicinal preparations of over 600 plants (Cowan 1999). The Bible also refers to approximately 30 plants applicable for healing purposes (Cowan 1999). Humans have continued over the centuries to use plants to treat various disorders and many of these traditional medicines are still included as part of medicinal practices by Indigenous people globally (Fabricant et al. 2001, Ríos et al. 2005). The recent rapid emergence of pathogenic bacteria resistant to available antimicrobial drugs has resulted in the urgent need for new antimicrobial agents. The vast diversity of unstudied natural products, particularly from medicinal plants, provides an ideal field for the discovery of novel active chemical entities (Fabricant et al. 2001, Ríos et al. 2005).

1.2 Antimicrobial resistance

The World Health Organization (WHO) has stated that antimicrobial resistance is currently the most urgent issue for human health (Tagliabue and Rappuoli 2018). Many pathogenic bacteria have become resistant to multiple antibiotics and some species are on the verge of becoming untreatable. Globally, antimicrobial resistance causes around 700,000 deaths/year, and the forecast is that in 2050 it will cause 10 million deaths/year, higher than the 8.2 million deaths caused by cancer today (Tagliabue and Rappuoli 2018). International organisations such as the WHO, the United Nations General Assembly, the World Bank and the G20 have called for action to fight antimicrobial resistance, including through the development of new drugs (Brown et al. 2016, Tagliabue and Rappuoli 2018).

It is now well recognised that natural products can make an enormous contribution to drug discovery especially through the unmatched structural diversity nature provides (Cragg and Newman 2018), and there is increasing interest in the study of medicinal plants for the discovery of new medicines, including those that can provide new antimicrobial agents (Tagliabue and Rappuoli 2018).

1.3 Ethnopharmacological approach

Ethnopharmacology research focuses on the use of traditional medicines, especially medicinal plants, used by Indigenous peoples. An ethnopharmacological approach towards drug discovery has proven to be very effective, with many of the current pharmaceutical drugs derived from higher plants having been discovered in an ethnobotanical context (Cox 2008). Fabricant and Farnsworth reported in 1991 that out of 156 bioactive compounds used as current medicines, 122 were discovered from traditional medicinal plants. Furthermore, 80% of those plants were used by Indigenous communities for the same (or related) medicinal purposes (Fabricant and Farnsworth 2001).

Indigenous peoples have been using plants as medicines for centuries and have developed significant cultural knowledge as to which plants in their region are most effective for medicinal purposes. Plants with documented ethnomedicinal uses are thus more likely to contain valuable bioactive compounds that are also likely to be safer than bioactive compounds isolated from plants with no records of human use (Fabricant and Farnsworth 2001, Cox 2008, Schwikkard and Mulholland 2014). The ethnopharmacological approach has been used to guide the study presented in this thesis to discover antimicrobial fractions and compounds from medicinal plants used by Indigenous peoples.

1.4 Compounds from plants as potential antimicrobials.

Plants synthesise a vast range of phytochemicals that can be divided into primary and secondary metabolites. Primary metabolites are required for the survival of the plant, while secondary metabolites are often helpful for the survival of the plant in specific environments (Kliebenstein 2004). The function of secondary metabolites in plants is increasingly attracting attention due to their key roles in protecting plants from herbivores and microbial infection, as attractants for pollinators and seed-dispersing animals, and as signal molecules in the formation of nitrogen-fixing root nodules in legumes, amongst other factors (Crozier et al. 2008). Secondary metabolites are also viewed as potential sources of novel drugs, insecticides and herbicides as well as safe disinfectant agents (Crozier et al. 2006, Upadhyay et al. 2013, Alamgir 2017)

There is considerable interest in plants employed in traditional medicine as a source of antibacterial natural products for clinical and commercial usage, and this is an area of intense investigation (Shahid et al. 2009, Angiolella 2018). Cowan et al. stated in 1999 (Cowan et al. 1999) “While 25 to 50% of

current pharmaceuticals are derived from plants, none are used as antimicrobials, however, traditional healers have long used plants to prevent or cure infectious conditions; Western medicine has recognised that potential and is trying to understand and utilise that success." While the clinical use of antibacterial plant natural products is still limited, plant derived antimicrobials including *trans*-cinnamaldehyde present in cinnamon bark (*Cinnamomum zeylandicum*), carvacrol and thymol present in oregano oil obtained from *Origanum glandulosum*, and eugenol from the oil of cloves (*Eugenia caryophyllus*), have been shown to be effective in killing mature *Listeria monocytogenes* biofilms and are classified as safe by the U.S. Food and Drug Administration (Adams et al. 1990). *L. monocytogenes* is responsible for the foodborne disease listeriosis, with a mortality rate of about 24% (Farber et al. 1991).

Examples of classes of secondary plant metabolites that have potent antimicrobial properties are provided below.

1.4.1 Phenolic compounds

Phenols and polyphenols are widely distributed in plants, with an excess of 8000 phenolic structures reported from the plant kingdom (Ferreira et al. 2017). Phenolic compounds are characterised by having at least one aromatic ring with one or more hydroxyl groups attached (Crozier et al. 2006). The site(s) and number of hydroxyl groups on the aromatic ring have been shown to correlate with the level of toxicity to microorganisms, with increased hydroxylation commonly leading to increased toxicity (Cowan 1999, Sher 2004). The mechanisms responsible for phenolic antimicrobial activity include enzyme inhibition and other interactions with essential proteins (Cowan 1999) that lead to loss of protein function. Probable targets in the microbial cell are surface exposed adhesions, cell wall polypeptides, and membrane-bound enzymes (Naz et al. 2013).

Flavonoids are the largest, most numerous and widespread group of the phenolic compounds (Harborne and Mabry 2013). Many studies have reported on the remarkable antimicrobial activity of this class of compounds against a wide range of Gram-positive as well as Gram-negative bacteria (Cushnie et al. 2007, Cushnie and Lamb 2011). The flavonols quercetin, myricetin and kaempferol, and the flavones luteolin and apigenin (Figure 1.1), are amongst the most commonly occurring flavonoids in plants (Puupponen-Pimiä et al. 2001), and they have been extensively reported to be active against many pathogenic bacterial strains including *S. aureus*, *Escherichia coli*, *Pseudomonas aeruginosa*, *Bacillus cereus, Bacillus subtilis, Salmonella typhy* and *Shigella dynsenteriae* (Senio et al. 2018, Sganzerla et al. 2018) and fungal strains (Hameed et al. 2018). Highly lipophilic flavonoids are thought to disrupt microbial membranes (Hameed et al. 2018). Flavonoids have been shown to affect the cell-wall permeability of the porins in the outer membrane of microorganisms by blocking

the charges of the amino acids in porins (Cowan 1999). The activity of flavonoids may also be due to their ability to interact with extracellular and soluble proteins, and in turn, with bacterial cell walls (Cushnie et al. 2011).

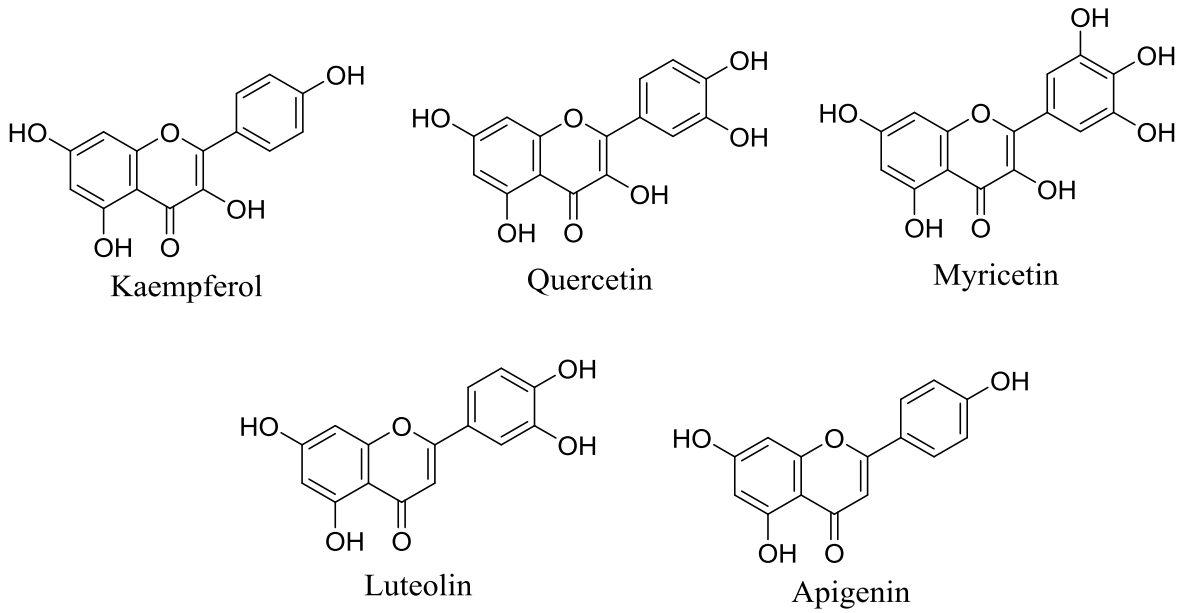


Figure 1.1 Examples of antimicrobially active flavonoids.

Coumarins, also known as benzopyrones, are phenolic substances with a fused benzene and α-pyrone ring, and are often hydroxylated, alkylated and/or alkoxylated (O'Kennedy and Thornes 1997). Coumarins are present in many plants. Simple coumarins and their analogues are a large class of compounds that have attracted attention due to their various biological activities such as antitumor, anti-HIV, anticoagulant and anti-inflammatory, as well as antimicrobial activity (Matos et al. 2013). Scopoletin, psoralen and herniarin are amongst the most potent antimicrobial coumarins, with activity against pathogenic fungal and bacterial strains reported (Ojala et al. 2000, Gnonlonfin et al. 2012). Generally, coumarins have been found to be macrophage stimulants, which is thought to have an indirect negative effect on infections. More specifically, coumarin has been used to prevent recurrences of cold sores caused by HSV-1in humans (Sher 2009).

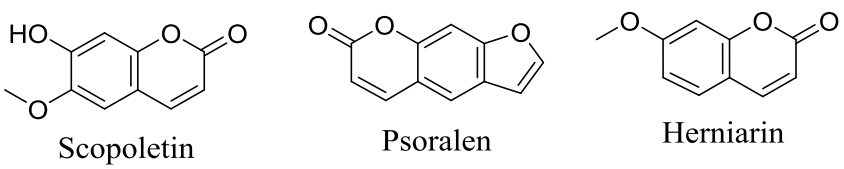


Figure 1.2 Examples of antimicrobially active coumarins.

Hydroxycinnamates are products of the phenylpropanoid pathway and are referred to collectively as phenylpropanoids. The most common antimicrobially active hydroxycinnamates are *p*-coumaric,

caffeic and ferulic acids and their derivatives (Figure 1.3). These compounds have all been reported to be active against a variety of pathogenic bacteria including *Listeria monocytogenes*, *Pseudomonas aeruginosa*, *Enterococcus faecalis*, *Bacillus subtilis* and *Staphylococcus aureus* (Aljadi and Yusoff 2003, Popova et al. 2005, Ayaz et al. 2008, Borges et al. 2013). Their antimicrobial activity is believed to be due to their ability to destabilise and permeabilise the cytoplasmatic membrane, inhibition of enzymes involved in the radical generation and also the inhibition of the synthesis of nucleic acids (Rajasekaran et al. 2018).

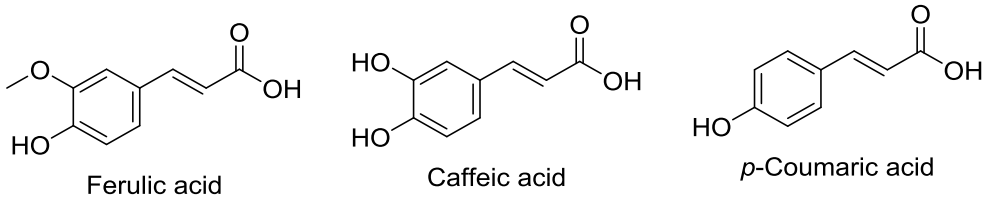


Figure 1.3 Examples of antimicrobially active hydroxycinnamates.

Stilbenes, alike the hydroxycinnamates, are also derived from the phenylpropanoid pathway. Many stilbenes are phytoalexins, which are compounds that are produced by plants in response to attack by fungal, bacterial or viral pathogens. Stilbenes have been subjected to intense investigation because of their potential in human health (Aggarwal and Shishodia 2005). The most common stilbene is resveratrol (Crozier et al. 2006). It can occur in both the *cis*- and the *trans*-isomeric forms and is present in plant tissues primarily as *trans*-resveratrol-3-*O*-glucoside, which is also known as piceid or polydatin (Crozier et al. 2006). Resveratrol has been shown to possess antimicrobial activity against a range of bacterial strains including multidrug resistant *S. aureus* and *P. aeruginosa* (Paulo et al. 2010) and fungal species including *Trichophyton mentagrophytes*, *Epidermophyton floccosum* and *Microsporum gypseum* (Chan 2002).

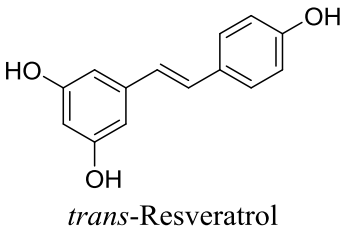


Figure 1.4 Example of antimicrobially active stilbene.

1.4.2 Terpenes

Terpenes are based on the isoprene unit and include diterpenes, triterpenes and tetraterpenes (C20, C30 and C40, respectively), as well as hemiterpenes (C5), monoterpenes (C10), and sesquiterpenes (C15) (Cowan 1999). When the compounds contain additional elements, usually oxygen, they are termed terpenoids. Terpenoids are synthesised from acetate units, thus they originate from fatty acids.

They differ from fatty acids in that they contain extensive branching and are cyclised. Triterpenoids are the most abundant group of phytochemicals, as they comprise more than 20,000 recognised molecules (Jesus et al. 2015). Various triterpenic skeletons have been described, including pentacyclic triterpenoids that have displayed antibacterial, antiviral and antiprotozoal effects. Food scientists have found terpenoids present in essential oils of plants to be useful in the control of *Listeria monocytogenes* (Aureli et al 1992). Oil of basil, a commercially available herbal, was found to be as effective as 125 ppm chlorine in disinfecting lettuce leaves (Aureli et al. 1992).

The common plant pentacyclic triterpenoids oleanolic acid and ursolic acid have been shown to be effective against a wide range of bacteria (Jesus et al. 2015). Studies conducted on the antimicrobial activity of three monoterpenes, namely linalyl acetate, (+)-menthol and thymol against *S. aureus* and *E. coli*, suggested that the antimicrobial effects could be attributed to a perturbation of the plasma membranes by the lipophilic compounds, resulting in alterations of membrane permeability and in leakage of intracellular materials (Jesus et al. 2015).

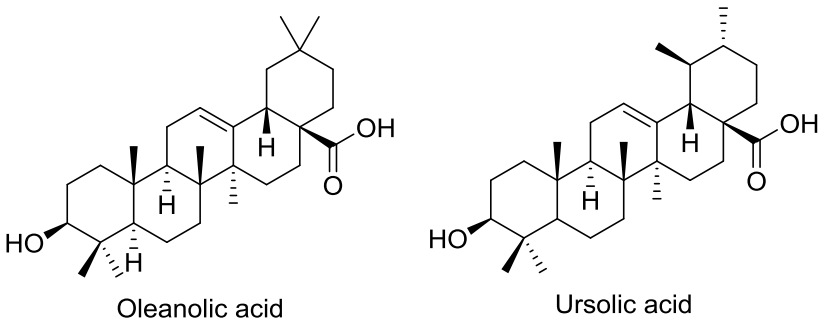


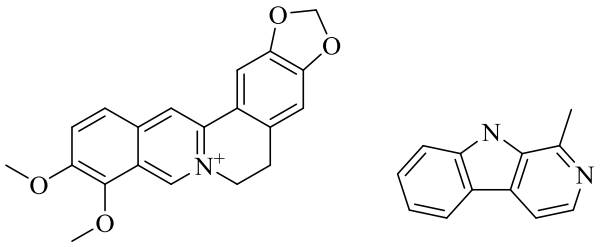
Figure 1.5 Examples of antimicrobially active terpenoids.

1.4.3 Alkaloids

Amongst secondary metabolites produced by plants, alkaloids figure as a very prominent class of compounds. Structurally, alkaloids are heterocyclic nitrogen containing compounds (Cowan 1999). Over 21,000 alkaloids have been identified, constituting the largest group among nitrogen containing secondary metabolites. Alkaloids are widely distributed in the plant kingdom, especially among angiosperms (Fattorusso and Taglialatela-Scafati 2008). One of the main functions of alkaloids in plants is that of chemical defence against herbivores or predators. Some alkaloids possess antimicrobial, antifungal and antiviral properties (Fattorusso and Taglialatela-Scafati 2008).

Berberine and harmane are commonly occurring and important representatives of the alkaloid group (Al-Bayati and Al-Mola 2008). Berberine has been shown to be active against methicillin-resistant *S. aureus* (Freile et al. 2003, Yu et al. 2005), *E. coli, P. aeruginosa* and *B. subtilis* (Čerňáková and Košťálová 2002) and active against *C. albicans* (Freile et al. 2003). Harmane has been shown to be

active against *B. subtilis*, *S. aureus*, *E. coli* and *Proteus vulgaris* and the fungi *Aspergillus niger* and *C. albicans* (Nenaah 2010). The mechanism of action of highly aromatic planar quaternary alkaloids such as berberine and harmane could be explained by their ability to interact with bacterial DNA (Cowan 1999).



Berberine Hermane

Figure 1.6 Examples of antimicrobially active alkaloids.

1.5 Methods used for the identification of antimicrobial compounds

1.5.1 TLC bioautography guided isolation

TLC bioautography is one of the most widely used methods for targeting active compounds (Islam et al. 2003). This method is a combination of thin layer chromatographic (TLC) separation and activity determination that enables the localisation of active constituents present in mixtures (Shahverdi et al. 2007). TLC bioautography was therefore chosen as a method for bioassay guided isolation studies described in this thesis.

1.5.2 Metabolomic screening

Metabolomics is the study of the "global metabolite profile" in a system under a given set of conditions (Rochfort 2005). Application of metabolomics to any type of organism, but most notably of higher plants, is developing into an essential tool in natural sciences specifically with the goal to qualitatively and/or quantitatively analyse metabolites in an organism (Rochfort 2005). An aim of metabolomics is to assess a complex mixture and in doing so allow for the identification of the constituents present in a mixture before or instead of the isolation process (Inui et al. 2012). This approach has been applied in the presented PhD study.

Continued advances in hardware, software and biostatistics are enabling us to generate ever-advancing, detailed insights into the chemical composition of plants and how this is influenced by genetical and environmental perturbation. Over the last decade, plant metabolomics has been transformed from a purely theoretical concept into a highly valued and widely exploited technology, and there are now many examples of metabolomics focused studies on traditionally used medicinal

plants (Hall 2018). This is an excitingly advancing field, but it is also recognised that there are still challenges to overcome (De Vos et al. 2007, Gu et al. 2013, Hall 2018).

1.6 Background and objectives of this study

Previous Master of Philosophy (MPhil) studies conducted by the author (Malewska 2015), and PhD studies by a former member of the research team (Kichu 2010), have reported on the use of the leaves of *Begonia picta* Smith (*Begoniaceae*), *Diospyros lanceifolia* Roxb. (*Ebenaceae*) and *Holboellia latifolia* Wall. (*Lardizabalaceae*) and roots of *Albizia lucidior* (Skud) Hara (*Mimosaceae*) and *Prunus persica* (L.) Stokes (*Rosaceae*) by the Chungtia villagers of Nagaland, North East India for skin related treatments. The aim of the MPhil study was to investigate these five plants for their antimicrobial properties against dermatologically relevant pathogens and undertake phytochemical analysis of the most active species, *Prunus persica*.

In the MPhil study, MTT microdilution assays were used to determine the antimicrobial activities of 70% aqueous ethanolic extracts of leaves of *B. picta*, *D. lanceifolia* and *H. latifolia* and roots of *A. lucidior* and *P. persica* against *Staphylococcus aureus*, *Escherichia coli*, *Pseudomonas aeruginosa*, *Streptococcus pyogenes*, *Salmonella typhimurium* and *Candida albicans*.

It was found that all the five plants aqueous ethanolic extracts exhibited antibacterial activity against *S. aureus*, with the greatest activity exhibited by *P. persica* root extract followed by *D. lanceifolia* and *H. latifolia* leaves. Those plants therefore were chosen for further investigation. The *P. persica* crude extract was further successively partitioned between water and *n*-hexane, dichloromethane and ethyl acetate. All partitions of *P. persica* showed antibacterial activity, with the ethyl acetate partition being the most significant with MIC 312 µg/mL (susceptible as well as resistant strains of *S. aureus*), followed by *n*-hexane with MIC 625 µg/mL (susceptible *S. aureus*) and MIC 312 µg/mL (MRSA and MDRSA and antibiotic susceptible strains of *E. coli* and *P. aeruginosa*). Bioassay guided fractionation of the *n*-hexane partition resulted in the isolation of β-sitosterol (MIQ 312 µg for *S. aureus*, 625 µg for MRSA and MDRSA, 2500 µg for *E. coli*). Bioassay guided fractionation of the ethyl acetate partition resulted in the isolation of *ent*-epiafzelechin-(2α→O→7, 4α→8)-(-)-*ent*-afzelechin (MIQs 156 µg for susceptible *S. aureus*, 312 µg for MRSA and MDRSA, 2500 µg for antibiotic sensitive *E. coli*, *S. typhimurium* and *P. aeruginosa*) and afzelechin (no antimicrobial activity). The antimicrobial properties of the isolated compounds were determined by the minimum inhibitory quantity (MIQ) TLC bioautography method and the structures were elucidated by spectroscopic techniques (Malewska 2015). The partitioning of *D. lanceifolia* and *H. latifolia* was not conducted within the MPhil studies.

The overall objective of this PhD project was to undertake chemical and biological investigations on *D. lanceifolia* and *H. latifolia* as well as to continue the work on the *P. persica* antimicrobially active fractions. For the three plants and parts chosen, beyond the studies within the research group (Malewska 2015 and Kichu 2010), literature reviews identified limited phytochemistry and bioactivity studies, as described below.

Roots of Prunus persica (L.) Batsch (Peach) subfamily *Prunoideae* of the family *Rosaceae* have been used orally to treat typhoid and tonsillitis, and topically to treat skin related infections (Barua 2011, Kichu et al. 2015). While the leaves, seeds and fruits of this plant are widely reported for their phytochemistry and diverse pharmacological properties, there is one phytochemical report on the root reporting the presence of afzalechin and five proanthocyanidin stereoisomers (Ohigashi et al. 1982) There are no other reports on pure compounds isolated from the roots.

Leaves of *Diospyros lanceifolia* (Roxb) of the *Ebenaceae* family have been used to treat various teeth complaints and skin disorders of likely microbial ethnology (Kalita et al. 2016). Leaf extracts have been reported to possess strong antioxidant property, with the isolation of gallic acid attributed in part to this activity (Kalita et al. 2016). The leaves have also been shown to have antibacterial activity against *Staphylococcus aureus*, *Escherichia coli* and *Pseudomonas aeruginosa*, and the antibacterial compounds plumbagin and 7-methyljuglone have been isolated from the leaves (Kichu 2010). No further phytochemical or biological studies have been reported on the leaves.

Leaves of *Holboellia latifolia* Wall. of *Lardizabalaceae* family have been used for the treatment of burns (Begum and Nath 2000, Kichu et al. 2015) and wounds (Juyal and Ghildiyal 2013). Only one report on the phytochemistry of *H. latifolia* has been found (Jian 2013), and no biological studies have been reported.

In order to understand more on the above underexplored traditional medicinal plants, the specific aims of this PhD project for *Prunus persica* roots, *Diospyros lanceifolia* leaves and *Holboellia latifolia* leaves were to conduct bioassay guided isolation studies on extracts and structural elucidation studies to identify antimicrobially active compounds, and use metabolomics studies to further explore the phytochemistry of the plant extracts (and fractions therein).

1.7 References

Adams, T., S. Vartivarian and R. Cowart (1990). "Iron acquisition systems of *Listeria monocytogenes." Infection and Immunity* 58(8): 2715-2718.

Aggarwal, B. B. and S. Shishodia (2005). Resveratrol in health and disease, CRC Press. Taylor & Francis Group, 2006.

Alamgir, A. N. M. (2017). Cultivation of herbal drugs, biotechnology, and *in vitro* production of secondary metabolites, high-value medicinal plants, herbal wealth, and herbal trade. Therapeutic Use of Medicinal Plants and Their Extracts: Volume 1: Pharmacognosy. A. N. M. Alamgir. Cham, Springer International Publishing: 379-452.

Al-Bayati, F. A. and H. F. Al-Mola (2008). "Antibacterial and antifungal activities of different parts of *Tribulus terrestris* L. Growing in Iraq." *Journal of Zhejiang University Science B* **9**(2): 154-159.

Aljadi, A. M. and K. M. Yusoff (2003). "Isolation and identification of phenolic acids in Malaysian honey with antibacterial properties." *Turkish Journal of Medical Sciences* **33**(4): 229-236.

Angiolella, L., G. Sacchetti and T. Efferth (2018). "Antimicrobial and antioxidant activities of natural compounds." *Evidence-Based Complementary and Alternative Medicine* **2018**: 3.

Aureli, P., A. Costantini and S. Zolea (1992). "Antimicrobial activity of some plant essential oils against *Listeria monocytogenes*." *Journal of Food Protection* **55**(5): 344-348.

Ayaz, F. A., S. Hayırlıoglu-Ayaz, S. Alpay-Karaoglu, J. Grúz, K. Valentová, J. Ulrichová and M. Strnad (2008). "Phenolic acid contents of kale (*Brassica oleraceae* L. var. acephala DC.) extracts and their antioxidant and antibacterial activities." *Food Chemistry* **107**(1): 19-25.

Barua, I. (2011). "Indigenous herbal medicine among the Sonowal Kachari tribe: A Study in a forest village in Dibrugarh, Assam, India." *NeBIO - An International Journal of Environment and Biodiversity* **2**(4): 31-35.

Begum, D. and S. C. Nath (2000). "Ethnobotanical review of medicinal plants used for skin diseases and related problems in northeastern India." *Journal of Herbs, Spices & Medicinal Plants* **7**(3): 55-93.

Borges, A., C. Ferreira, M. J. Saavedra and M. Simoes (2013). "Antibacterial activity and mode of action of ferulic and gallic acids against pathogenic bacteria." *Microbial Drug Resistance* **19**(4): 256-265.

Brown, L., C. Langelier, M. J. Reid, R. L. Rutishauser and L. Strnad (2016). "Antimicrobial resistance: a call to action!" *Clinical Infectious Diseases* **64**(1): 106-107.

Čerňáková, M. and D. Košťálová (2002). "Antimicrobial activity of berberine—A constituent of *Mahonia aquifolium*." *Folia Microbiologica* **47**(4): 375-378.

Chan, M. M.-Y. (2002). "Antimicrobial effect of resveratrol on dermatophytes and bacterial pathogens of the skin." *Biochemical Pharmacology* **63**(2): 99-104.

Cowan, M. M. (1999). "Plant Products as Antimicrobial Agents." *Clinical Microbiology Reviews* **12**(4): 564-582.

Cox, P. A. (2008). "Etnnopharmacology and the Search for New Drugs." Bioactive Compounds From Plants, John Wiley & Sons.

Cragg, G. M. and D. J. Newman (2018). Natural products as sources of anticancer agents: current approaches and perspectives. Natural Products as Source of Molecules with Therapeutic Potential, Springer: 309-331.

Crozier, A., M. N. Clifford and H. Ashihara (2008). Plant secondary metabolites: occurrence, structure and role in the human diet, John Wiley & Sons.

Crozier, A., I. B. Jaganath and M. N. Clifford (2006). "Phenols, polyphenols and tannins: an overview." Plant secondary metabolites: Occurrence, structure and role in the human diet **1**(1)342-354.

Cushnie, T., V. Hamilton, D. Chapman, P. Taylor and A. Lamb (2007). "Aggregation of *Staphylococcus aureus* following treatment with the antibacterial flavonol galangin." *Journal of Applied Microbiology* **103**(5): 1562-1567.

Cushnie, T. T. and A. J. Lamb (2011). "Recent advances in understanding the antibacterial properties of flavonoids." *International Journal of Antimicrobial Agents* **38**(2): 99-107.

De Vos, R. C., S. Moco, A. Lommen, J. J. Keurentjes, R. J. Bino and R. D. Hall (2007). "Untargeted large-scale plant metabolomics using liquid chromatography coupled to mass spectrometry." *Nature Protocols* **2**(4): 778.

Duke, J. and M. Bogenschutz-Godwin (1987). "Handbook of Medicinal Herbs, CRC, Press." Inc., Boca, Raton, Florida.

Fabricant, D. S. and N. R. Farnsworth (2001). "The value of plants used in traditional medicine for drug discovery." *Environmental Health Perspectives* **109**(Suppl 1): 69-75.

Farber, J. and P. Peterkin (1991). "*Listeria monocytogenes*, a food-borne pathogen." *Microbiology and Molecular Biology Reviews* **55**(3): 476-511.

Fattorusso, E. and O. Taglialatela-Scafati (2008). Modern alkaloids: structure, isolation, synthesis, and biology, John Wiley & Sons.

Ferreira, I. C. F. R., N. Martins and L. Barros (2017). "Chapter one - phenolic compounds and it's bioavailability: *In vitro* bioactive compounds or health promoters?" *Advances in Food and Nutrition Research* **82**: 1-44.

Freile, M., F. Giannini, G. Pucci, A. Sturniolo, L. Rodero, O. Pucci, V. Balzareti and R. Enriz (2003). "Antimicrobial activity of aqueous extracts and of berberine isolated from *Berberis heterophylla."* *Fitoterapia* **74**(7-8): 702-705.

Gnonlonfin, G. B., A. Sanni and L. Brimer (2012). "Review scopoletin–a coumarin phytoalexin with medicinal properties." *Critical Reviews in Plant Sciences* **31**(1): 47-56.

Greathead, H. (2003). "Plants and plant extracts for improving animal productivity*." Proceedings of the Nutrition Society* **62**(2): 279-290.

Gu, D., Y. Yang, M. Bakri, Q. Chen, X. Xin and A. Aisa Haji (2013). "A LC/QTOF–MS/MS Application to investigate chemical compositions in a fraction with protein tyrosine phosphatase 1B inhibitory activity from *Rosa Rugosa* flowers." *Phytochemical Analysis* **24**(6): 661-670.

Hall, R. D. (2018). "Plant metabolomics in a nutshell: potential and future challenges." *Annual Plant Reviews* online: 1-24.

Hameed, R. H., G. J. Mohammed and I. H. Hameed (2018). "*Matricaria chamonbmilla*: bioactive compounds of methanolic fruit extract using GC-MS and FTIR techniques and determination of its antimicrobial properties."*Indian Journal of Public Health Research & Development* **9**(3).

Harborne, J. B. and T. J. Mabry (2013). The flavonoids: advances in research, Springer.

Inui, T., Y. Wang, S. M. Pro, S. G. Franzblau and G. F. Pauli (2012). "Unbiased evaluation of bioactive secondary metabolites in complex matrices." *Fitoterapia* **83**(7): 1218-1225.

Islam, N., S. Parveen, N. Nakazawa, A. Marston and K. Hostettmann (2003). "Bioautography with the Fungus Valsa ceratosperma in the Search for Antimycotic Agents." Pharmaceutical Biology 41(8): 637-640.

Jesus, J. A., J. H. G. Lago, M. D. Laurenti, E. S. Yamamoto and L. F. D. Passero (2015). "Antimicrobial activity of oleanolic and ursolic acids: an update." *Evidence-Based Complementary and Alternative Medicine* **2015**.

Jian, G. (2013). "Phytochemical study on *Holboellia latifolia*." *Natural Product Research and Development* **10**: 1-8.

Juyal, P. and J. C. Ghildiyal (2013). "Indigenous animal health care practices from Garhwal Himalaya." *Journal of Medicinal Plants Studies* **1**(4): 148-151.

Kalita, D., N. Devi and D. Baishya "Foliar nutraceutical and antioxidant property of *Diospyros lanceifolia* Roxb. (*Ebenaceae*)–An important medicinal plant of Assam, India." *International Advanced Research Journal in Science, Engineering and Technology* **3**(3): 20-22

Kichu, M. (2010). "Documentation and biological and phytochemical analysis of Chungtia medicinal plants of Nagaland, India." Dissertation, Chemistry and Biomolecular Sciences, Macquarie University, Sydney (PhD): 1-179.

Kichu, M., T. Malewska, K. Akter, I. Imchen, D. Harrington, J. Kohen, S. R. Vemulpad and J. F. Jamie (2015). "An ethnobotanical study of medicinal plants of Chungtia village, Nagaland, India." *Journal of Ethnopharmacology* **166**: 5-17.

Kliebenstein, D. (2004). "Secondary metabolites and plant/environment interactions: A view through *Arabidopsis thaliana* tinged glasses." *Plant, Cell & Environment* **27**(6): 675-684.

Matos, M. J., S. Vazquez-Rodriguez, L. Santana, E. Uriarte, C. Fuentes-Edfuf, Y. Santos and A. Muñoz-Crego (2013). "Synthesis and structure-activity relationships of novel amino/nitro substituted 3-arylcoumarins as antibacterial agents." *Molecules* **18**(2): 1394-1404.

Naz T. (2013). "Chemical and biological studies of medicinal plants used by the Yaegl Aboriginal community of Australia" Dissertation, Chemistry and Biomolecular Sciences, Macquarie University, Sydney (PhD): 1-236.

Nenaah, G. (2010). "Antibacterial and antifungal activities of (beta)-carboline alkaloids of *Peganum harmala* (L) seeds and their combination effects." *Fitoterapia* **81**(7): 779-782.

Newman, D. J. and G. M. Cragg (2012). "Natural products as sources of new drugs over the 30 years from 1981 to 2010." *Journal of Natural Products* **75**(3): 311-335.

O'Kennedy, R. and R. D. Thornes (1997). Coumarins: biology, applications, and mode of action, John Wiley & Sons.

Ohigashi, H., S. Minami, H. Fukui, K. Koshimizu, F. Mizutani, A. Sugiura and T. Tomana (1982). "Flavanols, as plant growth inhibitors from roots of peach, *Prunus persica* Batsh. cv. 'Hakuto'." *Agricultural and Biological Chemistry* **46**(10): 2555-2561.

Ojala, T., S. Remes, P. Haansuu, H. Vuorela, R. Hiltunen, K. Haahtela and P. Vuorela (2000). "Antimicrobial activity of some coumarin containing herbal plants growing in Finland." *Journal of Ethnopharmacology* **73**(1–2): 299-305.

Paulo, L., S. Ferreira, E. Gallardo, J. A. Queiroz and F. Domingues (2010). "Antimicrobial activity and effects of resveratrol on human pathogenic bacteria." *World Journal of Microbiology and Biotechnology* **26**(8): 1533-1538.

Popova, M., S. Silici, O. Kaftanoglu and V. Bankova (2005). "Antibacterial activity of Turkish propolis and its qualitative and quantitative chemical composition." *Phytomedicine* **12**(3): 221-228.

Puupponen-Pimiä, R., L. Nohynek, C. Meier, M. Kähkönen, M. Heinonen, A. Hopia and K. M. Oksman-Caldentey (2001). "Antimicrobial properties of phenolic compounds from berries." *Journal of Applied Microbiology* **90**(4): 494-507.

Rajasekaran, R. and Y. Gebrekidan (2018). "A review on antibacterial phytochemical constitutions present in *Aerva lanata* and their mode of action against bacterial biofilm." *International Journal of Pharmaceutical & Biological Archives* **9**(1): 16-30.

Ramawat, K., S. Dass and M. Mathur (2009). The chemical diversity of bioactive molecules and therapeutic potential of medicinal plants. Herbal drugs: ethnomedicine to modern medicine, Springer: 7-32.

Ríos, J. L. and M. C. Recio (2005). "Medicinal plants and antimicrobial activity." *Journal of Ethnopharmacology* **100**(1): 80-84.

Rochfort, S. (2005). "Metabolomics reviewed: a new "omics" platform technology for systems biology and implications for natural products research." *Journal of Natural Products* **68**(12): 1813-1820.

Scalbert, A. (1991). "Antimicrobial properties of tannins." *Phytochemistry* **30**(12): 3875-3883.

Shahid, M., A. Shahzad, F. Sobia, A. Sahai, T. Tripathi, A. Singh and H. Khan (2009). "Plant natural products as a potential source for antibacterial agents: recent trends." *Anti-Infective Agents in Medicinal Chemistry (Formerly Current Medicinal Chemistry-Anti-Infective Agents)* **8**(3): 211-225.

Shahverdi, A. R., F. Abdolpour, H. R. Monsef-Esfahani and H. Farsam (2007). "A TLC Bioautographic Assay for the Detection of Nitrofurantoin Resistance Reversal Compound." *Journal of Chromatography* 850(1): 528-530.

Sher, A. (2009). "Antimicrobial activity of natural products from medicinal plants." *Gomal Journal of Medical Sciences* **7**(1): 72-78.

Schwikkard, S. L. and D. A. Mulholland (2014). "Useful methods for targeted plant selection in the discovery of potential new drug candidates." *Planta Medica* **80**(14): 1154-1160.

Senio, S., C. Pereira, J. Vaz, M. Sokovic, L. Barros and I. C. Ferreira (2018). "Dehydration process influences the phenolic profile, antioxidant and antimicrobial properties of *Galium aparine* L." *Industrial Crops and Products* **120**: 97-103.

Sganzerla, W. G., P. C. Beling, J. P. Ferrareze, R. A. Komatsu, M. R. Nunes and A. P. de Lima Veeck (2018). "Nutritional, physicochemical and antimicrobial properties of uvaia pulp (*Eugenia pyriformis* Cambess)." *Communications in Plant Sciences* **8**: 1-7.

Tagliabue, A. and R. Rappuoli (2018). "Changing priorities in vaccinology: antibiotic resistance moving to the top." *Frontiers in Immunology* **9**: 1068-1068.

Thomson, W. A. and R. E. Schultes (1978). Medicines from the Earth, McGraw-Hill.

Toda, M., S. Okubo, H. Ikigai, T. Suzuki, Y. Suzuki, Y. Hara and T. Shimamura (1992). "The protective activity of tea catechins against experimental infection by *Vibrio cholerae* O1." *Microbiology and Immunology* **36**(9): 999-1001.

Upadhyay, A., I. Upadhyaya, A. Kollanoor-Johny and K. Venkitanarayanan (2013). "Antibiofilm effect of plant derived antimicrobials on *Listeria monocytogenes*." *Food Microbiology* **36**(1): 79-89.

Yu, H.-H., K.-J. Kim, J.-D. Cha, H.-K. Kim, Y.-E. Lee, N.-Y. Choi and Y.-O. You (2005). "Antimicrobial activity of berberine alone and in combination with ampicillin or oxacillin against methicillin-resistant *Staphylococcus aureus*." *Journal of Medicinal Food* **8**(4): 454-461.

Chapter 2 - *Prunus persica*

Prunus persica (L.) Batsch roots have been used traditionally to treat skin infections (topical application), orally for typhoid and tonsillitis, and *via* nasal administration for nose bleeds (Kefalew et al. 2015). Unlike other parts of this plant that have been widely reported for their phytochemistry and diverse pharmacological properties, there are limited biological or phytochemistry reports concerning the roots of *P. persica* (Layne et al. 2008, Malewska 2015).

The author has previously reported on the root extracts showing good antibacterial activity and the bioassay guided isolation of three antibacterial compounds (Malewska 2015). This chapter provides a literature review of traditional medicinal uses of *P. persica*, its bioactivity and phytochemistry, and extends the previous bioassay guided isolation studies of *P. persica* for identification of further antibacterial constituents.

Figure 2.1. *Prunus persica* (photo by Nagaland man Meyanungsang Kichu).

2. *P. persica* literature review

2.1 Taxonomy and botany

Prunus persica (L.) Batsch (Peach) is a deciduous tree of the subfamily *Prunoideae* of the family *Rosaceae*. It is commonly cultivated in West Asia, Europe, the Himalayas and India, and is a commercially harvested stone fruit (Gilani et al. 2010). *P. persica* can reach a height of 5 to 10 metres. The leaves are linear with acute tips and finely serrated margins. The flowers are produced in early spring before the leaves; they are solitary or paired, pink, with five petals. The fruit has yellow or whitish flesh, and a delicate aroma. The single large seed is red-brown, oval and is surrounded by a wood-like husk (Gilani et al. 2010).

2.2 Traditional uses and aligned biological activities

Virtually all parts of *P. persica* have been used as traditional medicines. The leaves and fruits are the most commonly applied, followed by the seeds (Kant et al. 2018).

Leaves of the species have been used in many countries to treat a range of conditions including skin and eye infections and wounds (Tamang and Sedai 2016), inflammatory disorders such as painful joints (Lone and Bhardwaj 2013), to relieve symptoms of cold (Kumar et al. 2015), as well as digestive disorders such as abdominal pain, diarrhoea and stomach ache (Uniyal et al. 2011).

Fruits of *P. persica* have been applied for healing of wounds (Sharma et al. 2017), fungal infections (Rashid et al. 2015), relief of inflammation and irritation of skin and mucous membranes, as well as for rheumatism and arthritis (Pieroni et al. 2015), digestive disorders and as an anthelmintic (Lone and Bhardwaj 2013), for diabetes (Yaseen et al. 2015), urinary and reproductive system disorders, for colds, flu, fever and headaches (Shah and Hussain 2012, Getaneh and Girma 2014, Khan and Ahmad 2015, Yaseen et al. 2015).

Seeds of *P. persica* have been used as a treatment for skin infections and wound healing and to treat urticaria (hives) (Ahmad and Ali 2006), for digestive disorders (Wangchuk et al. 2011, Kichu et al. 2015), rheumatism (Sharma and Painuli 2011), malaria (Giday et al. 2007), constipation, as an anthelmintic (Trak 2017) and for relief of headache (Chauhan 2016).

There are limited reports on use of the bark and roots of *P. persica*. Bark of the plant is used in Africa to treat infectious diseases such as HIV (Amusan et al. 2005) and in Nepal to treat skin conditions such as infected wounds (Ghimire and Bastakoti 2009, Kant et al. 2018).

The Ao tribe of Nagaland, India, consume the liquid from fresh roots soaked in water to treat typhoid and the seed endosperm to treat dysentery and diarrhoea (Kichu et al. 2015). The liquid from the roots and aqueous decoctions of the leaves are also used to treat skin related infections (Kichu 2010). Roots of *P. persica* are used by the Sonowal Kachari Tribe, Assam, India to treat tonsillitis, and by the Ada'a District of Ethiopia to treat nose bleeds (Kefalew et al. 2015). Roots are also used by the Rungwe tribe of Tanzania to relieve the symptoms of chronic cough (Kibonde. 2018, Kant et al. 2018).

The biological properties and phytochemistry of leaves, fruit, bark, seeds and the pericarp of the plant have been very well documented. Due to the overwhelming amount of data available, the author focuses here only on those activities aligned with the traditional medicinal applications of the species.

Methanolic extracts of the bark of *P. persica* have been shown to possess antimicrobial properties against Gram-negative as well as Gram-positive bacterial species; namely *Klebsiella pneumoniae*, *Enterococcus faecalis*, *Escherichia coli*, *Staphylococcus aureus* and *Bacillus subtilis*, as well as against fungi such as *Aspergillus flavus*, *Microsporum canis* and *Fusarium solani*. These results support the usage of the plant by Indigenous communities of Nepal, who use the bark to treat boils, cuts and wounds (Ghimire and Bastakoti 2009). The exact compounds responsible for the antimicrobial activities have not been determined.

Methanolic extracts of the seeds have been found to have antitumour activity, and well documented anticancer compounds have been isolated from the extract, including amygdalin, prunasin, amygdalinic acid, mandelic acid β-D-glucopyranoside, benzyl β-gentiobioside and benzyl β-D-glucopyranoside (Fukuda et al. 2003). These findings support the traditional application of *P. persica* seeds as an ingredient in "Kampo" preparations used by native tribes of Japan and China to treat breast and other cancers (Lim 2012).

The protective effect of *P. persica* pericarp (fruit flesh and the outer layer of the seed) ethanolic extract against hepato- and nephro-toxicity has been demonstrated. This supports Indigenous usage of the fruit by Pakistani people to treat jaundice and kidney problems (Shah and Hussain 2012, Heidari-Soreshjani et al. 2017). The compounds responsible for these properties have not been identified (Lee Chang et al. 2007, Heidari-Soreshjani et al. 2017).

The ethanolic extract of *P. persica* leaves has been shown to stimulate isolated rabbit jejunum and guinea pig ileum preparations (Kim et al. 2003) and further studies have indicated that the leaves contain spasmogenic (cholinomimetic) and spasmolytic (calcium antagonist) constituents. Although the chemical entities responsible for the activities have not been isolated (Suh et al. 2006), these studies are supportive of the leaves being used as a treatment for constipation by Indigenous people of Nagaland, India (Hazarika 2018).

P. persica flowers are traditionally applied in Asian countries to whiten skin and to treat skin disorders (Kim et al. 2002). Ethanolic extracts of *P. persica* fruit and flowers have been found to contain 2-methoxy-5-(2-methylpropyl)pyrazine, which attenuates UV-B-induced changes in procollagen (Han et al. 2010), as well as kaempferol 3-*O*-[β-D-glucopyranosyl(1→4)-α-L-rhamnopyranoside], kaempferol 3-*O*-β-D-galactopyranoside, kaempferol 3-*O*-α-L-rhamnopyranoside and kaempferol 3-*O*-β-D-glucopyranoside, which have all been shown to scavenge free radicals (Kim 2002). The properties of these compounds support the traditional uses of the flowers.

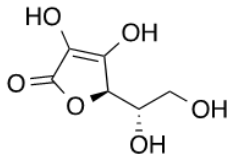
Even though much research has been devoted to the biological properties of nearly all parts of *P. persica*, no data have been found on the biological properties of *P. persica* roots.

2.3 Phytochemistry

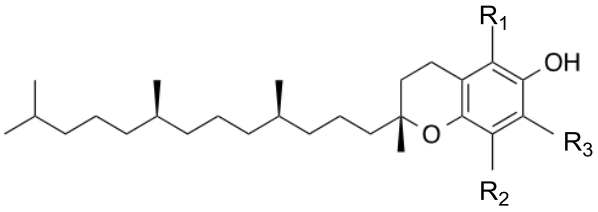
P. persica phytochemistry has been intensely investigated with many compounds having been isolated from the fruits, leaves, seeds and bark.

The fruits are a rich source of antioxidant vitamins and phenolic compounds, containing ascorbic acid (2.1), α-tocopherol (2.2), β-tocopherol (2.3) and citric acid (2.4), and the carotenoids zeaxanthin (2.5) and β-carotene (2.6) (Carbonaro et al. 2002). The fruits have also been found to be a good source of flavonoids, including apigenin (2.7), luteolin (2.8), kaempferol (2.9), quercetin (2.10), eriodictyol (2.11), naringenin (2.12), aromadendrin (2.13) and prunetin (2.14) (Rahman and Bhatnagar 1968, Jang et al. 2016), and the flavanol glycosides trifolin (2.15) and afzelin (2.16). All the above compounds have also been isolated from the leaves of *P. persica*, which have additionally been found to contain phenolic acids including caffeic acid (2.17), ferulic acid (2.18), chlorogenic acid (2.19) and gallic acid (2.20) (Kim 2002, Ravi Kant. et al. 2108). The cyanogenic glucosides amygdalin (2.21) and prunasin (2.22) have been isolated from the seeds and bark of *P. persica* (Jang et al. 2018, Ravi Kant. et al. 2108). Other than the phenolic compounds, the seeds also contain triterpenes including ursolic acid (2.23), and β-sitosterol (2.24) (Ravi Kant. et al. 2018). All the above compounds are predominantly responsible for the broad spectrum of biological activities of this species.

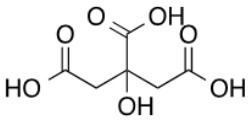
Only two reports concerning the isolation of bioactive compounds from roots have been found. Ohigagashi et al. reported on the presence of proanthocyanidin A (2.25) and afzelechin (2.26) (Ohigashi et al. 1982) and Kichu reported the isolation of afzelechin and *ent*-epiafzelechin-(2α→*O*→7,4α→8)-(-)-*ent*-afzelechin (2.27) (Kichu, 2010). Compounds 2.26 and 2.27 were also isolated in the previous Master of Philosophy (MPhil) studies conducted by the author of this thesis (Malewska 2015). The structures of all these isolated compounds are presented below (Figure 2.2).



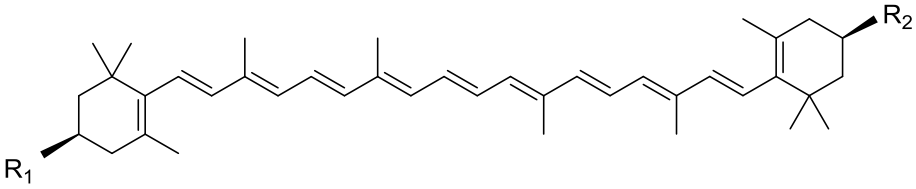
2.1



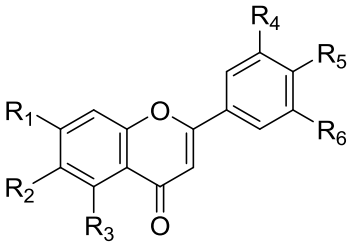
2.2 $R_1=R_2=CH_3$, $R_3=H$
2.3 $R_1=R_2=R_3=CH_3$

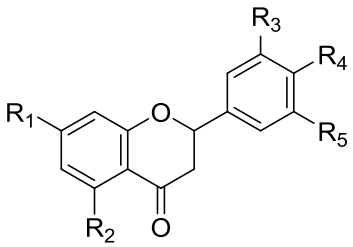


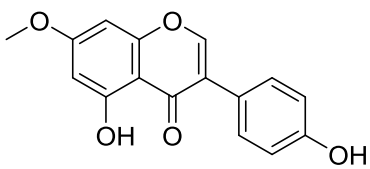
2.4



2.5 $R_1=R_2=OH$
2.6 $R_1=R_2=H$







2.14

2.7 $R_1=R_3=R_5=OH$, $R_2=R_4=R_6=H$
2.8 $R_1=R_3=R_4=R_5=OH$, $R_2=R_6=H$
2.9 $R_1=R_3=R_5=OH$, $R_2=R_4=R_6=H$
2.10 $R_1=R_3=R_5=R_6=OH$, $R_2=R_4=H$

2.11 $R_1=R_2=R_3=R_5=OH$, $R_4=H$
2.12 $R_1=R_2=R_4=OH$, $R_5=H$
2.13 $R_1=R_2=R_3=R_4=OH$, $R_5=H$

Figure 2.2. Compounds isolated from *P. persica* species.

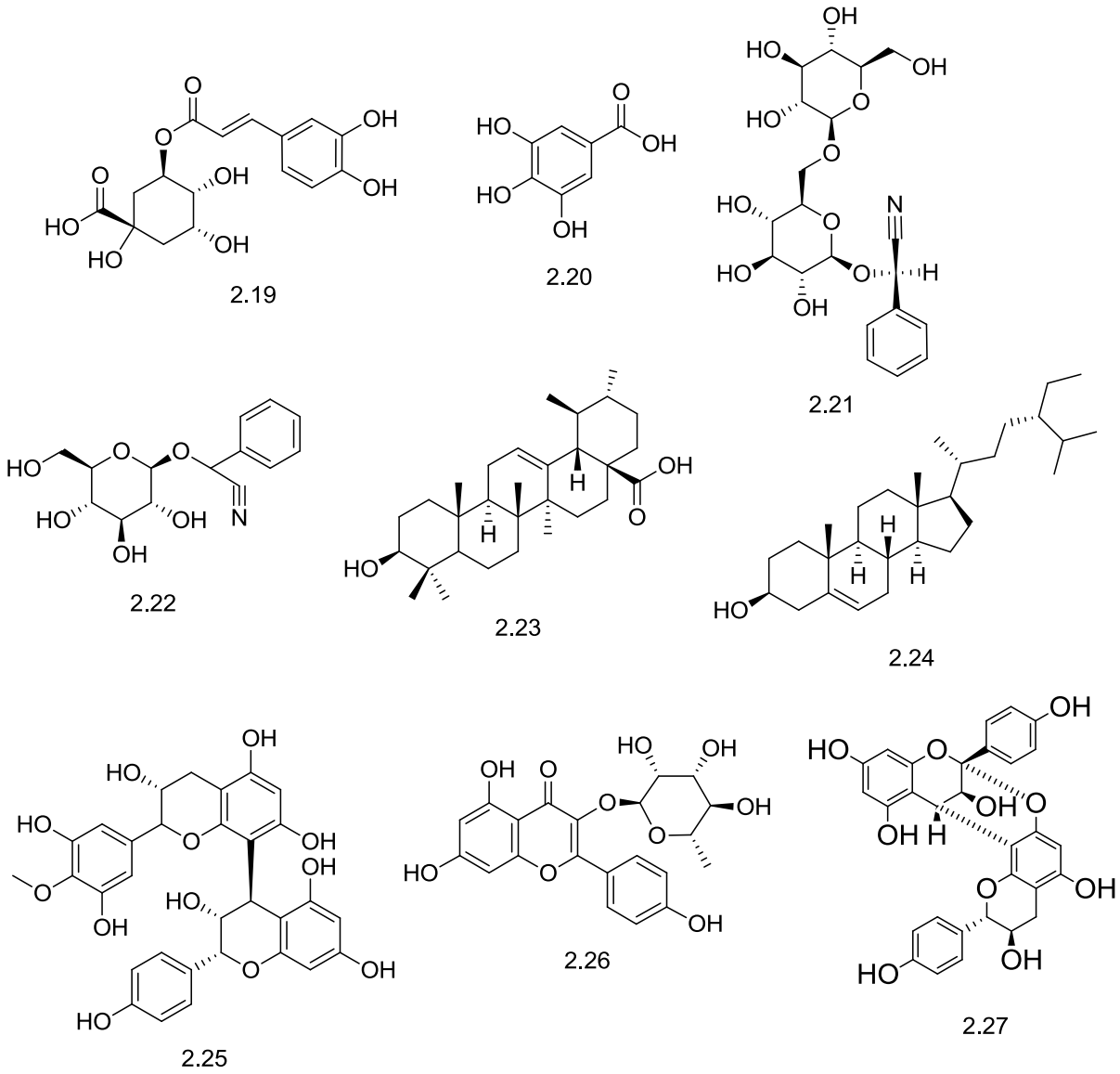


Figure 2.2. Compounds isolated from *P. persica* species (continued).

2.4 Background of the past MPhil project

During the MPhil project of the author (Malewska 2015), the following studies were conducted. Dried powdered roots of *P. persica* were extracted with 70% aqueous ethanol. This extraction method was used based on the traditional mode of preparation (fresh root is soaked in water overnight and taken orally to treat typhoid), suggesting a polar nature of active compounds. The dried extract was resuspended in water and partitioned between *n*-hexane, dichloromethane (DCM) and ethyl acetate (EtOAc); with the remaining fraction being called "water". The partitioned extracts were screened for antimicrobial activity by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) microdilution assay (Akter et al. 2016) against the skin pathogens *Staphylococcus aureus* (susceptible *S. aureus*), methicillin resistant *S. aureus* (MRSA) and multidrug resistant *S. aureus*

(MDRSA), β-lactamase negative *Escherichia coli* (β- *E. coli*), β-lactamase positive (antibiotic resistant) *E. coli* (β+ *E. coli*), *Pseudomonas aeruginosa*, *Streptococcus pyogenes*, *Salmonella typhimurium* and *Candida albicans* (Malewska 2015). The *n*-hexane extract showed antimicrobial activity against *S. aureus* with minimum inhibitory concentrations (MIC) of 625 µg/mL and MIC of 312 µg/mL for MRSA and MDRSA, β- *E. coli* and *P. aeruginosa*. The partition also showed antimicrobial activity against antibiotic sensitive *E. coli* (MIC 312 µg/mL). The dichloromethane partition showed antimicrobial activity against the antibiotic sensitive strain of *S. aureus,* MRSA and MDRSA (MIC 625 µg/mL), and the ethyl acetate partition showed antimicrobial properties against antibiotic susceptible as well as antibiotic resistant strains of *S. aureus* (MIC 312 µg/mL) for all strains. None of the extracts showed antimicrobial activity against the remaining bacterial strains. The obtained results (Table 2.1) agree with those previously reported (Kichu 2010).

Table 2.1 Antimicrobial activity of *P. persica* partitions by MTT microdilution assay (MPhil work).

Fractions and compounds	Minimum inhibitory concentration (MIC)						
	SA	MRSA	MDRSA	β- EC	ST	PA	CA, SP, β+ EC
n-Hexane	625	312	312	312	na	312	na
DCM	625	625	625	na	na	na	na
EtOAc	312	312	312	na	na	na	na
Water	1250	1250	1250	na	na	na	na
Vancomycin	15.6	31.2	31.2	nt	nt	nt	nt
Gentamycin	nt	nt	nt	15.6*	15.6	15.6	15.6*, 31.2**
Fluconazole	nt	nt	nt	nt	nt	nt	31.2***

MIC values were determined as the wells with the lowest concentration of the samples that displayed no yellow to blue change of the MTT colour. *MIC values for β-EC, β+EC *E. coli* (β-, β+), PA (*P. aeruginosa*) and (ST) *S. typhimurium*. **MIC value for SP (*S. pyogenes*). ***MIC value for CA (*C. albicans*). nt: not tested; na: not active. DCM: dichloromethane; EtOAc: ethyl acetate; MeOH: methanol. SA (*S. aureus*), MRSA (methicillin resistant *S. aureus*), MDRSA (multidrug resistant *S. aureus*); MIC: minimum inhibitory concentration.

Based on the antimicrobial activity results, the *n*-hexane and ethyl acetate partitions were chosen for bioassay guided chromatographic separation. Initial fractionation of the *n*-hexane partition was conducted with normal phase silica gel column chromatography, eluting with increasing polarity mixtures of petroleum ether:diethyl ether (100:0 to 10:90). Further chromatographic fractionation of antibacterially active fractions (as confirmed by TLC bioautography) (Akter et al. 2016) using a combination of normal phase and size exclusion chromatography (Sephadex LH-20), led to the isolation of β-sitosterol (2.24, Figure 2.2). For the ethyl acetate partition, initial fractionation was

conducted with normal phase silica gel column chromatography, eluting with increasing polarity mixtures of chloroform:methanol (100:0 to 30:70). Further TLC bioautography guided chromatographic separation led to the isolation of afzelechin and *ent*-epiafzelechin-(2α→*O*→7',4α→8')-(-)*ent*-afzelechin (2.26 and 2.27, Figure 2.2). There were two remaining sub-fractions (EtOAc 2a1 and EtOAc 2a3, Figure 2.3) also considered of interest, but the chromatographic separation was not conducted during the MPhil project due to time restrictions. The flow chart of the key steps that led to isolation of antibacterially active compounds from the ethyl acetate partition as well as the sub-fractions (EtOAc 2a1 and EtOAc 2a3), during the MPhil studies, is presented below.

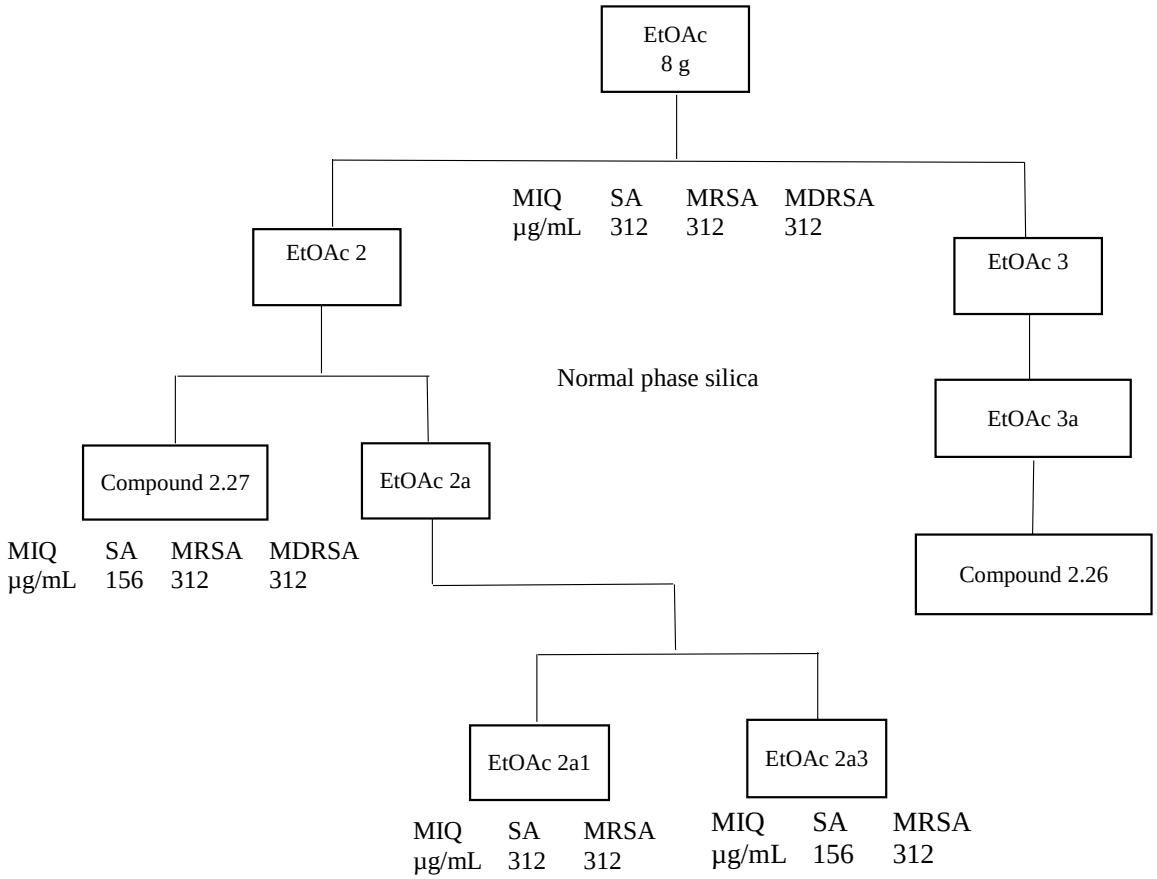


Figure 2.3 Key steps of large scale bioautography guided fractionation of EtOAc partition of *Prunus persica* roots (Malewska 2015). Minimum inhibitory quantity (MIQ) by TLC bioautography method.

2.5 Results and Discussion

The antibacterially active *n*-hexane partition and EtOAc 2a1 and EtOAc 2a3 fractions obtained from the MPhil studies (Malewska 2015) of the *P. persica* roots had been stored dried and under nitrogen at -20 °C and were subsequently used for this PhD study. They were found to have consistent TLC and NMR profiles to that seen previously for the freshly obtained samples, as well as similar

antibacterial activity (MIC values) supporting no significant change of the samples had occurred during storage. Additionally, minimum bactericidal concentration (MBC) was measured for all samples of interest and a slightly lower value of MBC for *n*-hexane fraction (625 µg/mL for MRSA and MDRSA strains) was noted. The same MIC and MBC values for the EtOAc, 2a1 and 2a3 fractions were seen (Table 2.2). EtOAc 2a1 and EtOAc 2a3 were relatively clean by normal phase silica TLC (254 nm visualisation) and of sufficient quantity for further analysis. Therefore, further bioassay guided isolation studies of the *n*-hexane partition and EtOAc 2a1 and EtOAc 2a3 were conducted.

Table 2.2 Antimicrobial activity of *P. persica* partitions and fractions by MTT microdilution assay.

Fractions and compounds	Minimum inhibitory concentration/minimum bactericidal concentration (MIC/MBC µg/mL)						
	SA	MRSA	MDRSA	β-EC	ST	PA	CA, SP, β+ EC
n-Hexane	625	312/625	312/625	312	na	312	na
EtOAc	312	312	312	na	na	na	na
2a1	312	312	na	na	na	na	na
2a3	156	312	na	na	na	na	na
Vancomycin	15.6	31.2	31.2	nt	nt	nt	nt
Gentamycin	nt	nt	nt	15.6*	15.6	15.6	15.6*, 31.2**
Fluconazole	nt	nt	nt	nt	nt	nt	31.2***

MIC values were determined as the wells with the lowest concentration of the samples that displayed no yellow to blue change of the MTT colour. *MIC values for β-EC, β+EC *E. coli* (β-, β+), PA (*P. aeruginosa*) and (ST) *S. typhimurium*. **MIC value for SP (*S. pyogenes*). ***MIC value for CA (*C. albicans*). nt: not tested; na: not active. DCM: dichloromethane; EtOAc: ethyl acetate; MeOH: methanol. SA (*S. aureus*), MRSA (methicillin resistant *S. aureus*), MDRSA (multidrug resistant *S. aureus*); MIC: minimum inhibitory concentration; MBC: minimum bactericidal concentration. Single values indicate that MIC = MBC.

2.5.1 Isolation and antimicrobial activity of compounds from *n*-hexane partition

The antibacterially active *n*-hexane partition (MIC value 625 µg/mL against susceptible strain of *S. aureus*, and 312 µg/mL against MRSA, MDRSA, susceptible *E. coli* and *P. aeruginosa* strains) was subjected to TLC bioautography guided normal phase silica column chromatographic separation (Figure 2.4). Forty fractions were collected and combined into five major sub-fractions (Hex-1 – Hex-5) according to similar R_f values of active spots. The fractions Hex-1, Hex-4 and Hex-5 showed antibacterially active spots by TLC bioautography with *S. aureus*, thus were considered of interest. The test results are summarised in Table 2.3.

Table 2.3 TLC bioautography assay results of sub-fractions 1 to 5 of the *n*-hexane partition.

Fraction No.	Yield mg	Antibacterial activity (bioautography)					
		Susceptible *S. aureus*		MRSA		MDRSA	
		activity	R_f	activity	R_f	activity	R_f
Hex-1	150	a	0.45	a	0.45	a	0.45
Hex-2	130	na	na	na		na	na
							na
Hex-3	65	na	na	na		na	na
Hex-4	126	a	0.77	a	0.77	a	0.77
Hex-5	82	a	0.79	a	0.79	a	0.79

*R_f range where inhibition zones were observed. Samples were run on normal phase TLC plates using (petroleum ether b.p. 40 ºC:diethyl ether 3:7). a – active in the TLC bioautography test; na - not active. MRSA (methicillin resistant *S. aureus*, MDRSA (multidrug resistant *S. aureus*).

Further purification of fraction Hex-1 by Sephadex LH-20 chromatography yielded compound 1 (1.5 mg). Recrystallisation with methanol of fraction Hex-4 yielded compound 2 (65 mg). Further purification of fraction Hex-5 by Sephadex LH-20 chromatography yielded compound 3 (1.7 mg). Spectral analysis of compound 2 (Section 2.6.2) was consistent with it being β-sitosterol (2.24, Figure 2.2), which has been previously found in *P. persica* (Ravi Kant. et al. 2018).

The antimicrobial activities of compounds 1-3 were tested against susceptible as well as resistant strains of *S. aureus* and *E. coli*, susceptible strains of *P. aeruginosa*, *S. typhimurium*, *S. pyogenes* and the fungus *C. albicans* by the MTT microdilution assay (Table 2.4, Section 2.5.2). Compound 1 showed excellent antibacterial properties (MIC 78 µg/mL) against all tested *S. aureus* strains, ans some activity against β- *E. coli* (MIC 312 µg/mL) and *P. aeruginosa* (MIC 625 µg/mL). Compound 2 showed some activity against MRSA and MDRSA (MIC 625 µg/mL for each) and antibiotic sensitive *S. aureus* (MIC 312 µg/mL) as well as the susceptible strain of *E. coli* (MIC 2500 µg/mL). Compound 3 showed weak antibacterial activity (MIC 2500 µg/mL) against susceptible *S. aureus* and MRSA and susceptible *E. coli* as well as *P. aeruginosa* (MIC 1250 µg/mL).

The flow diagram of the bioassay guided isolation process key steps is presented in Figure 2.4.

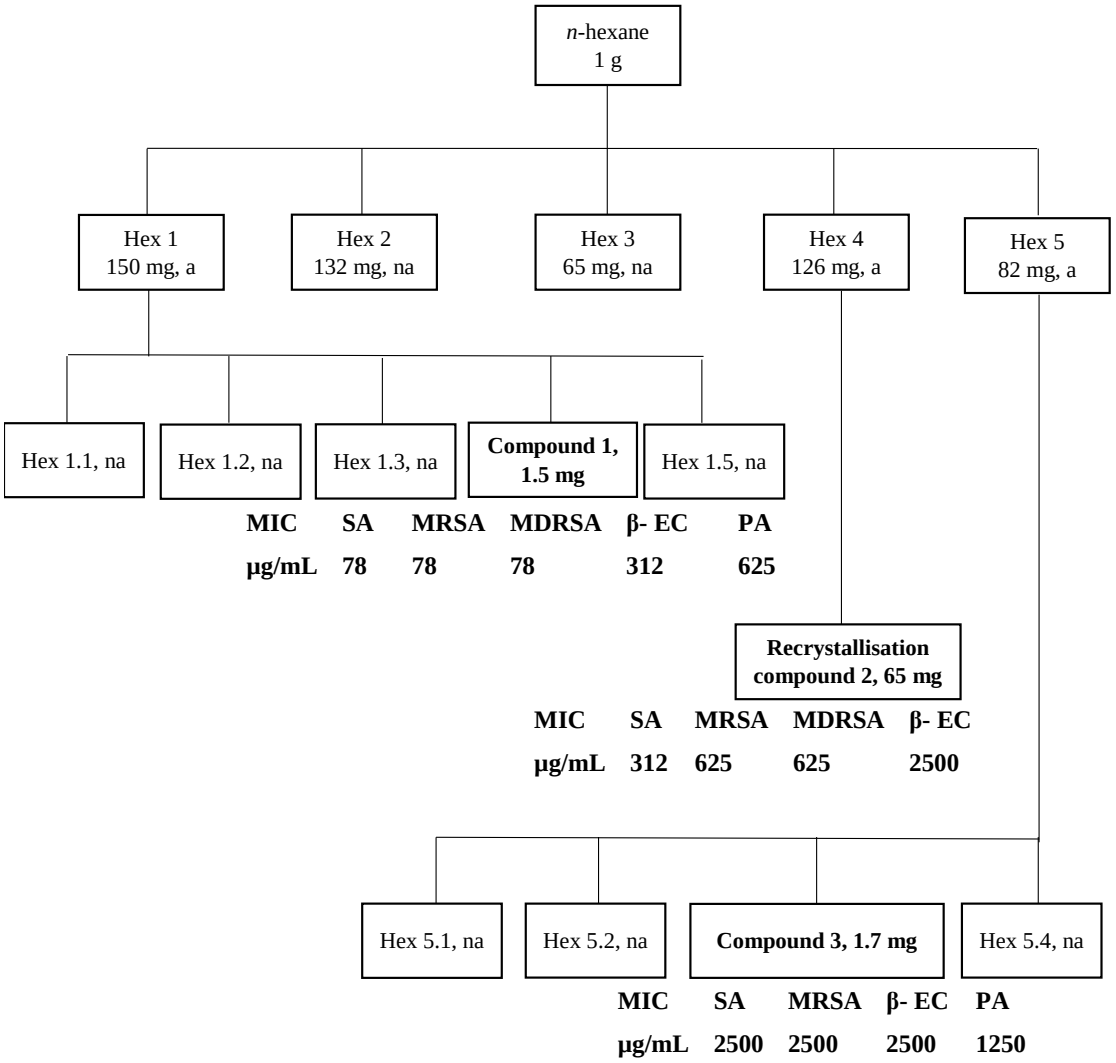


Figure 2.4. TLC bioautography guided fractionation of *n*-hexane partition of *Prunus persica*. a: active, na: not active. MIC: minimum inhibitory concentration. Fractions 1 to 5 tested against *S. aureus* (SA), methicillin resistant *S. aureus* (MRSA) and multidrug resistant *S. aureus* (MDRSA). Fractions 1.1 to 1.5 and 5.1 to 5.4 were tested against *S. aureus* only. Compounds isolated and their activities are bolded. Compounds 1, 2 and 3 were tested against all pathogenic strains - only positive activity is noted.

2.5.2 Isolation and antimicrobial activity of compounds from ethyl acetate partition

The antibacterially active fractions EtOAc 2a1 and 2a3 were subjected to normal phase silica column chromatography, followed by repeated Sephadex LH-20 chromatography, all guided by TLC bioautography with antibiotic susceptible *S. aureus*. This afforded compound 4 (11 mg, purified from EtOAc 2a1) and compound 5 (16 mg, purified from EtOAc 2a3). Spectral analysis of compound 4

(Section 4.1) was consistent with it being gallic acid (2.20, Figure 2.2), which has been previously found in *P. persica* (Kim 2002, Ravi Kant. et al. 2018).

MICs and MBCs of isolated compounds were found to be identical. Compound 4 was found to possess antibacterial activity against susceptible *S. aureus* with an MIC/MBC of 156 µg/mL, activity against MRSA with an MIC/MBC of 312 µg/mL and weak activity against β- *E. coli* and *P. aeruginosa* with MIC/MBCs of 2500 µg/mL for both of the strains. Compound 5 was active against susceptible *S. aureus* and β- *E. coli* with an MIC/MBC of 312 µg/mL. Compound 5 was also found to be active against MRSA and MDRSA, with an MIC/MBC of 625 µg/mL.

The antimicrobial activities of the isolated compounds from the *n*-hexane partition (1, 2 and 3) and ethyl acetate sub-fractions EtOAc 2a1 (4) and EtOAc 2a3 (5) are summarised in Table 2.4.

Table 2.4 Antimicrobial activity of *P. persica* isolated compounds by MTT microdilution assay.

Compound	Minimum inhibitory concentration and minimum bactericidal concentration (MIC/MBC µg/mL)						
	SA	MRSA	MDRSA	β-EC	ST	PA	CA, SP, β+EC
1	78	78	78	312	nt	625	na
2	312	625	625	2500	nt	na	na
3	2500	2500	na	2500	nt	1250	na
4	156	312	na	2500	nt	2500	na
5	312	625	625	312	nt	na	na
Vancomycin	15.6	31.2	31.2	nt	nt	nt	nt
Gentamycin	nt	nt	nt	15.6*	15.6	15.6	15.6*, 31.2**
Fluconazole	nt	nt	nt	nt	nt	nt	31.2***

MIC values were determined as the wells with the lowest concentration of the samples that displayed no yellow to blue change of the MTT colour. *MIC values for β- EC, β+ EC *E. coli* (β-, β+), PA (*P. aeruginosa*) and (ST) *S. typhimurium*. **MIC value for SP (*S. pyogenes*). ***MIC value for CA (*C. albicans*). nt: not tested; na: not active. DCM: dichloromethane; EtOAc: ethyl acetate; MeOH: methanol. SA (*S. aureus*), MRSA (methicillin resistant *S. aureus*), MDRSA (multidrug resistant *S. aureus*). MIC: minimum inhibitory concentration; MBC: minimum bactericidal concentration. Single values indicate that MIC = MBC.

2.6 Structural elucidation of isolated compounds

2.6.1 α-Cyanobenzyl benzoate

Compound 1 was isolated as a yellow oil. The electron-impact mass spectrum (EI-MS) showed an odd molecular ion at *m/z* 237, which suggested the presence of a nitrogen atom, and was consistent with the molecular formula of $C_{15}H_{11}NO_2$. The MS showed a fragmentation pattern consistent with

loss of a $C_6H_5COO\cdot$ group (*m/z* 116) and loss of $C_8H_6N\cdot$ (*m/z* 121), in agreement with the literature for α-cyanobenzyl benzoate (Nuylert et al. 2018). The ^{13}C NMR together with DEPT 135 showed a total of 11 different carbons; four quaternary and seven methine signals. The downfield quaternary carbon signal at δ 165.9 is characteristic of a carbonyl of an ester and the signal at δ 117.6 is consistent with the presence of a CN group (Nair et al. 2008, Mohareb et al. 2012). The ^1H NMR spectrum showed ten aromatic proton signals, consistent with two monosubstituted aromatic rings, along with a deshielded methine signal at δ 6.93 (H-7). HMBC showed connectivities consistent with the deshielded methine being positioned next to an oxygen and nitrile moiety. The ^{13}C and ^1H NMR data were in agreement with the literature for α-cyanobenzyl benzoate (also known as mandelonitrile benzoate) (Hoffmann et al. 1990).

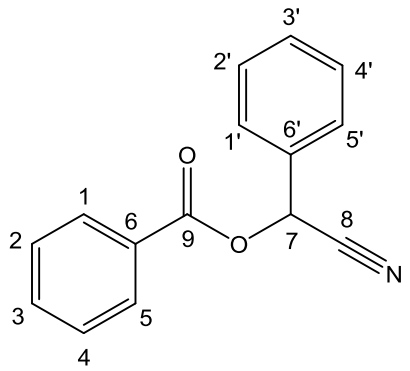


Figure 2.5 The structure of α-cyanobenzyl benzoate (1).

Table 2.5 NMR data of compound 1; key HMBC and COSY correlations (400 MHz, CD_3OD), δ C reference (Hoffmann et al. 1990).

C#	δ C	δ C reference	δ H *J* in Hz	HMBC	Multiplicity
1, 5	129.8	129.3	8.01, *dd*, 7.4, 2.0	C-2/4, C-3, C-6, C-9	CH
2, 4	127.8	127.8	7.57, *dd*, 7.4, 7.4	C-3, C-1/5, C-6, C-9	CH
3	134.7	134.0	7.73, *td*, 7.4, 2.0	C-1/5	CH
6	131.3	131.3			C
7	63.5	63.4	6.93, *s*	C-8, C-9, C-1'/5', C-6'	CH
8	117.6	116.3			C
9	165.9	164.5			C
1', 5'	129.1	128.6	7.70, *dd*, 7.7, 2.0	C-7	CH
2', 4'	130.2	130.8	7.53, *m*	C-3'	CH
3'	129.6	129.6	7.40, *m*	C-2', C-1', C-5'	CH
6'	135.2	135.2			C

2.6.2 β-Sitosterol

Compound 2 (or 2.24) was isolated as a white crystalline solid [melting point 138-139 ºC; $[\alpha]^{25}_D$: -41° (chloroform)]. The structure was tentatively identified from the EI-MS, which showed a molecular

ion at *m/z* 414 and a fragmentation pattern consistent with β-sitosterol ($C_{29}H_{50}O$) based on the NIST library database (95% similarity). High resolution EI-MS (HR-EI-MS supported the molecular formula of $C_{29}H_{50}O$ (found: 414.3855, calculated: 414.3862). The melting point and optical rotation were also consistent with the published melting point and optical rotation for β-sitosterol [136-139 ºC, lit. (Kircher and Rosenstein 1973); $[\alpha]^{25}_{D}$: -41° (in chloroform), lit. (Choi et al. 2002)].

The EI-MS showed fragment ions consistent with loss of water (*m/z* 396), plus loss of a methyl group (*m/z* 381, M-CH_3-HOH), the loss of the side chain of β-sitosterol (*m/z* 273), and loss of the side chain and water (*m/z* 255), which was consistent with the reported MS for β-sitosterol (Jamaluddin et al. 1994). The infrared (IR) spectrum showed a broad band at 3400 cm^{-1}, indicative of an OH group. No other characteristic functional group bands were seen in the IR spectrum.

The ^{13}C NMR spectrum showed the presence of twenty nine aliphatic signals. The DEPT spectrum showed the presence of eleven methylene, nine methine, six methyl and three quaternary carbon atoms. The ^{1}H NMR spectrum showed two singlets of three hydrogens each, at δ 0.61 (H-18) and 0.93 (H-19), consistent with shielded methyl groups attached to quaternary carbons, along with three doublets of three hydrogens each at δ 0.85 (*J* 6.9 Hz, H-21) 0.76 (*J* 7.4 Hz, H-26) and 0.74 (*J* 7.4 Hz, H-27), consistent with methyl groups next to methines and a triplet of three hydrogens at δ 0.77 (*J* 7.2 Hz, H-29) consistent with a methyl group. These were in agreement with ^{1}H NMR signals reported for the methyl groups of β-sitosterol (Joshi and Poudel 2011). A multiplet at δ 5.27 (1H, Hz, H-6) was consistent with an olefinic proton. A multiplet at δ 3.50 (H-3) for a single proton was assigned to a proton adjacent to an OH group. The remaining protons appeared as multiplets at δ 0.91-2.27. The ^{1}H and ^{13}C NMR assignments were supported by COSY and HSQC experiments, while connectivities were verified by HMBC experiments. The spectral data were all in agreement with published reports for β-sitosterol (Joshi and Poudel 2011, Rani et al. 2014, Habib et al. 2007).

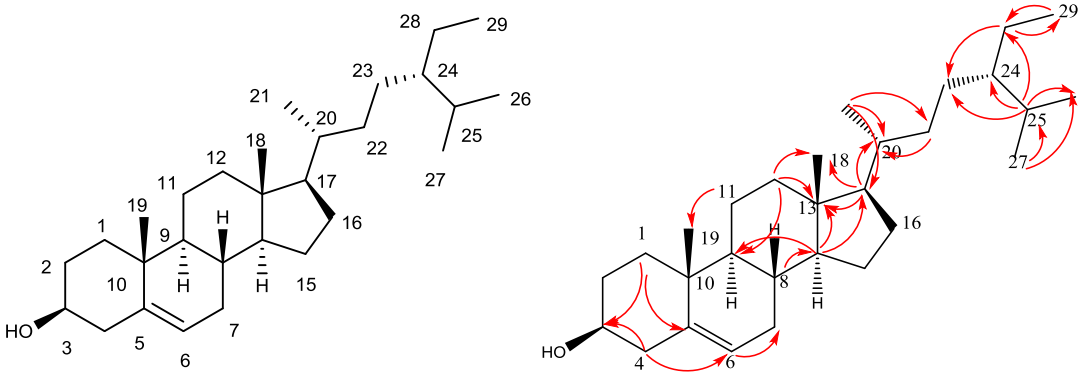


Figure 2.6 The structure of β-sitosterol and its key HMBC correlations.

Table 2.6 NMR data of compound 2 (2.24); key HMBC and COSY correlations (400 MHz, $CDCl_3$), δ C reference (Joshi and Poudel 2011).

C#	δ C	δ C reference	δ H *J* in Hz	HMBC	Multiplicity	COSY
1	37.2	36.1	1.77, *m*	C-3, C-5	CH_2	H-2
2	21.0	21.0	1.42, *m*		CH_2	H-1
3	71.6	71.8	3.50, *m*		CH	H-2, H-4
4	42.3	42.3	2.20, 2.16, *m*	C-3, C-5, C-6	CH_2	H-6, H-3
5	140.8	140.7			C	
6	121.7	121.7	5.27, *m*	C-4, C-7	CH	H-4, H-7
7	31.6	31.6	1.76, 1.43, *m*		CH_2	H-6
8	31.9	28.2	1.90, *m*	C-9	CH	
9	50.1	50.1	0.86, *m*		CH	
10	36.5	36.4			C	
11	19.0	20.9	0.74, *m*		CH_2	H-12
12	39.7	39.6	1.94, *m*	C-18, C-13, C-9	CH_2	H-11
13	42.3	41.9			C	
14	56.7	56.6	0.92, *m*	C-9, C-13, C-17	CH	
15	24.3	24.3	1.50, *m*		CH_2	H-16
16	28.2	29.6	1.19, *m*		CH_2	H-15
17	56.0	56.3	1.03, *m*		CH	
18	11.8	11.9	0.61, *s*	C-12, C-13, C-17	CH_3	
19	19.4	19.4	0.93, s	C-1, C-5, C-9	CH_3	H-20
20	36.1	36.2	1.28, *m*		CH	H-21
21	18.7	18.7	0.85, *d*, 6.9	C-17, C-20, C-22	CH_3	
22	33.9	33.7	1.25, *m*		CH_2	
23	26.0	26.5	1.08, *m*	C-28	CH_2	
24	45.8	45.5	0.85, *m*	C-22	CH	
25	29.2	29.1	1.59, *m*	C-26, C-27	CH_2	H-26
26	19.8	19.8	0.76, *d*, 7.4	C-25, C-27	CH_3	H-25
27	19.8	19.8	0.74, *d*, 7.4	C-25, C-26	CH_3	
28	23.0	24.2	1.18, *m*	C-29, C-23, C-25, C-24	CH_2	H-29
29	11.9	11.8	0.77, *t*, 7.2	C-28, C-24	CH_3	H-28

2.6.3 Stigmast-4-en-3-one

Compound 3 was isolated as an amorphous cream solid [melting point 89-91 ºC; $[\alpha]^{27}{}_D$: +71° (chloroform)]. The ^{1}H NMR spectrum showed it was structurally very similar to compound 2 (β-sitosterol), with the major difference being the presence of a vinylic proton signal at δ 5.62 (and the absence of a peak at δ 3.50). The ^{13}C NMR spectrum showed a carbonyl carbon at δ 198.6 and peaks at δ 124.21 and 71.53. The IR spectrum showed a carbonyl peak at 1740 cm^{-1}. This combined data suggested the presence of an α,β-unsaturated carbonyl. The EI-MS showed a molecular ion at *m/z* 412 and a fragmentation pattern consistent with stigmast-4-en-3-one based on the NIST library database (90% similarity). The ^{1}H and ^{13}C NMR spectral data were also in agreement with the

literature for this compound (Jamaluddin et al. 1995). Melting point as well as optical rotation were consistent with the literature (Lin et al. 2003)].

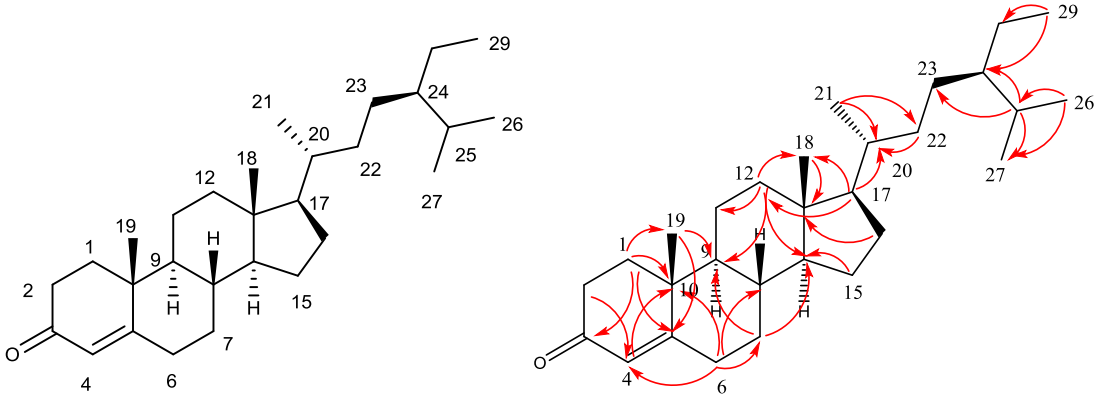


Figure 2.7 The structure of stigmast-4-en-3-one and its key HMBC correlations.

Table 2.7 NMR data of compound 3; key HMBC and COSY correlations (400 MHz, CD_3COCD_3), δ C reference (Jamaluddin et al. 1995).

C#	δ C	δ C reference	δ H *J* in Hz	HMBC	Multiplicity	COSY
1	36.1	36.5	2.02, 1.65, *m*	C-19, C-2, C-10, C-3, C-5	CH_2	H-2
2	34.6	34.1	2.18, 2.40, *m*	C-4	CH_2	H-1
3	198.6	198.6			C	
4	124.2	124.3	5.62, *m*	C-2, C-10, C-3, C-6, C-5	CH	H-6
5	71.5	71.5			C	
6	33.4	33.4	2.26, 2.45, *m*	C-4, C-10, C-7, C-8	CH_2	H-4, H-7
7	33.0	33.1	1.01, 1.87, *m*	C-9, C-14, C-6, C-5	CH_2	H-6, H-7
8	34.7	34.7	1.39, *m*		CH	
9	54.9	54.8	0.93, *m*		CH	
10	39.3	38.9	-		C	
11	21.8	21.7	1.56, 1.48, *m*		CH_2	H-12
12	40.6	40.6	1.17, 2.03, *m*	C-18, C-11, C-13, C-9, C-14	CH_2	H-11
13	43.2	43.2			C	
14	56.6	56.6	1.05, 1.15, *m*		CH	
15	24.0	24.1	1.15, 1.63, *m*	C-14	CH_2	H-16
16	29.0	29.1		C-13	CH_2	H-15
17	56.1	56.1	1.15, *m*	C-18, C-20, C-12, C-13	CH	
18	12.3	12.3	0.75, *s*	C-12, C-13, C-17	CH_3	
19	17.6	17.7	1.22, *s*		CH_3	
20	37.0	36.9	1.34, *m*		CH	H-21
21	19.2	19.2	0.89, *d*, 7.0	C-22, C-20, C-17	CH_3	H-20
22	36.4	36.4	0.98, *m*	C-20	CH_2	
23	26.8	26.8	1.13, 0.98, *m*		CH_2	
24	46.7	46.7	0.96, *m*		CH	
25	29.0	29.1	1.68, *m*	C-27, C-28, C-23, C-24	CH_2	H-26
26	20.1	20.1	0.84, *d*, 7.2	C-27, C-25	CH_3	H-25
27	19.4	19.7	0.93, *d*, 7.2		CH_3	
28	24.8	24.4	1.23, *m*	C-29, C-23, C-25, C-24	CH_2	H-29
29	12.3	12.1	0.82, *t*, 7.0	C-28, C-24	CH_3	H-28

2.6.4 Gallic acid

Compound 4 (2.20) was obtained as white crystals (melting point 251-253 ˚C). The electrospray ionisation-mass spectrum (ESI-MS) in negative mode showed a pseudo molecular ion at *m/z* 169 $[M-H]^-$, which was also the base peak and in agreement with the molecular formula $C_7H_6O_5$. The UV-Vis spectrum in methanol showed λ_{max} at 271, 350 and 372 nm (Sarria-Villa 2017). The compound also showed a green colour upon treatment with ferric chloride, confirming its phenolic nature (Sharma et al. 1998).

The IR spectrum showed a strong and very broad OH peak at 3465 cm^{-1} and a band at 1706 cm^{-1} corresponding to a C=O (Sarria-Villa 2017). The data were consistent with a carboxylic acid and phenolic groups being present. The ^{13}C NMR together with DEPT 135 spectra showed the presence of three different aromatic quaternary carbons (δ 120.96, 138.4 and 145.8), a quaternary carbon at δ 167.9, and one aromatic methine carbon, indicating the presence of symmetry and a tetrasubstituted aromatic ring. The quaternary carbons at δ 138.4 and 145.8 were consistent with aromatic carbons being connected to oxygen, while the quaternary carbon at δ 167.9 was characteristic of a carboxylic acid carbonyl. The ^1H-NMR spectrum ($CDCl_3$) showed only one peak at δ 6.46, which showed HMBC correlations with all of the quaternary carbons. NMR, IR, MS and melting point data were consistent with literature data for gallic acid (Eldahshan 2011, Sarria-Villa 2017).

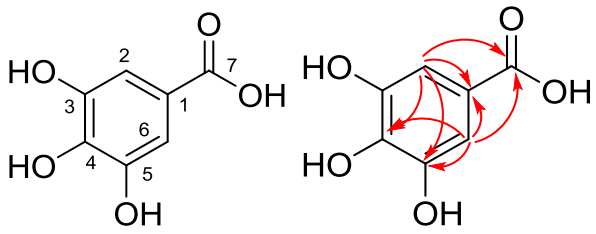


Figure 2.8 The structure of gallic acid and its key HMBC correlations.

Table 2.8 NMR data of compound 4 (2.20) (400 MHz, $CDCl_3$), δ C reference (Sarria-Villa 2017).

C#	δ C	δ C reference	δ H	HMBC	Multiplicity
1	120.9	120.9			C
2, 6	109.1	109.1	6.46, *s*	C-4, C-3, C-7	CH
3, 5	145.8	145.8			C
4	138.4	138.4			C
7	167.9	167.9			C

2.6.5 Caffeic acid phenylethyl ester

Compound 5 was isolated as a cream coloured amorphous solid (melting point 127-130 ˚C). ESI-MS in negative mode showed a pseudo molecular ion at *m/z* 283 $[M-H]^-$, consistent with the molecular formula of $C_{17}H_{16}O_4$. The UV-Vis spectrum in methanol showed λ_{max} at 325 nm, consistent with hydroxycinnamates (Grunberger et al. 1988). The IR spectrum showed strong bands at 3495 cm^{-1} and 1685 cm^{-1}, indicating the presence of OH and C=O groups, respectively (Grunberger et al. 1988). Similar to gallic acid, the compound showed a green colour upon treatment with ferric chloride. The ^{13}C NMR together with DEPT 135 spectra showed the presence of fourteen different carbon signals, indicating some symmetry. This included a quaternary carbon at δ 167.77 characteristic of an ester carbonyl (Nair et al. 2008); four aromatic quaternary carbons (δ 126.2, 138.0, 145.5 and 148.2 – the latter two consistent with attachment to oxygen); and six aromatic methine carbons (δ 113.6, 115.0, 121.5, 126.1, 128.1 and 128.5) consistent with two aromatic rings; two vinylic methine carbons (δ 113.7 and 145.4) consistent with an α,β-unsaturated carbonyl; and two deshielded aliphatic methylene groups at δ 34.79 and 64.73 consistent with being next to an oxygen and aromatic ring, respectively. The ^1H NMR spectrum in $CDCl_3$ showed a total of 14 proton signals, all with HSQC correlations. Coupling patterns and integration indicated the presence of a monosubstituted aromatic ring (δ 7.20-7.28, *m*, 5H) and a 1,3,5-trisubstituted aromatic ring (δ 7.02, 1H, *d*, *J* 2.0 Hz, H-2; 6.92, 1H, *dd*, *J* 8.2, 2.0 Hz, H-6; and 6.77, 1H, *d*, *J* 8.2 Hz, H-5). Two doublets of 16 Hz of one proton each at δ 6.22 (H-8, *d*, *J* 16 Hz) and 7.50 (H-7, *d*, *J* 16 Hz) confirmed the presence of a *trans*-substituted double bond located next to the carbonyl group (Martin et al. 2003). The deshielded triplet of two protons at δ 4.36 (H-8', *t*, *J* 7.0 Hz) was consistent with the methylene group being next to an oxygen and coupled to the methylene at δ 2.99 (H-7', *t*, *J* 7.0 Hz). HMBC correlations confirmed that the monosubstituted aromatic ring was connected to the methylene at position 7' and the trisubstituted aromatic ring to the vinylic carbon at position 7, identifying the compound as caffeic acid phenylethyl ester. All spectral data and the melting point were in agreement with the literature (Kart et al. 2010).

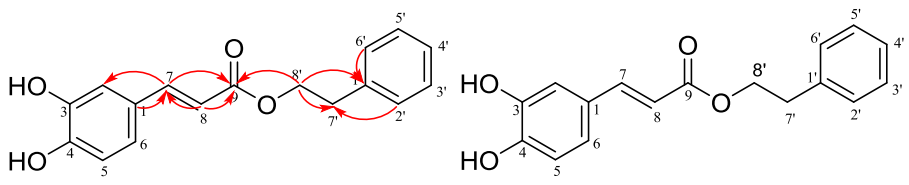


Figure 2.9 The structure of caffeic acid phenylethyl ester and its key HMBC correlations.

Table 2.9 NMR data of compound 5; key HMBC and COSY correlations (400 MHz, $CDCl_3$), δ C reference (Kart et al. 2010).

C#	δ C	δ C reference	δ H *J* in Hz	HMBC	Multiplicity	COSY
1	126.2	126.2			C	
2	113.6	113.5	7.02, *d*, 2.0	C-6, C-3, C-4	CH	H-3
3	145.5	145.5			COH	
4	148.2	148.3			COH	
5	115.0	115.1	6.77, *d*, 8.2	C-1, C-3, C-4	CH	H-6
6	121.5	121.5	6.92, *dd*, 8.2, 2.0	C-2, C-7, C-4	CH	H-5, H-2, H-5
7	145.4	145.4	7.50, *d*, 16	C-6, C-1, C-2, C-9	CH	H-8
8	113.7	113.6	6.22, *d*, 16	C-1, C-9, C-7	CH	H-7
9	167.7	167.7			C	
8’	64.7	64.7	4.36, *t*, 7	C-7’ C-9, C-1’	CH_2	H-7’
7’	34.7	34.5	2.99, *t*, 7	C-8’, C-2’, C-1’	CH_2	H-8’, H-6’
1’	138.0	138.1			C	
2’	128.1	128.1	7.28, *m*	C-1’	CH	H-3’
3’	128.5	128.5	7.27, *m*	C-4’	CH	H-2’, H-4’
4’	126.1	126.1	7.20, *m*	C-2’, C-5’	CH	H-3’, H-5’
5’	128.5	128.5	7.27, *m*	C-4’	CH	H-6’, H-4’
6’	128.1	128.1	7.28, *m*	C-7’, C-1’	CH	H-7’, H-5’

2.7 Natural occurrence and reported antimicrobial properties of isolated compounds

Compound 1, α-cyanobenzyl benzoate, also known as mandelonitrile benzoate, is a natural product commonly present in the antimicrobial defensive secretion of millipedes and has practical interest as an insecticide (Roncadori et al. 1985, Makarov et al. 2010, Shear 2015, Kuwahara et al. 2017).

α-Cyanobenzyl benzoate has been rarely reported in plants, being only isolated from *Piper reticulatum* (the whole plant) (Whitehead et al. 2013) and *Melania oleifera* branches and leaves (Liu et al. 2007). It has also been identified by GC-MS in volatile oils liberated from *Prunus* biomass, along with mandelonitrile, benzoic acid, benzaldehyde, benzyl alcohol and hydrocyanic acid (Ohigashi et al. 1982). This is the first report of α-cyanobenzyl benzoate being isolated from *P. persica*.

As shown in Table 2.4, α-cyanobenzyl benzoate showed very good antibacterial properties against all tested *S. aureus* strains with MIC/MBC values of 78 µg/mL, activity against β- *E. coli* with an MIC/MBC of 312 µg/mL and against *P. aeruginosa* with an MIC of 612 µg/mL. α-Cyanobenzyl benzoate has been reported as a broad-spectrum bactericide, being active against a variety of bacteria such as *Ralstonia*, *Agrobacterium*, *Xanthomonas*, *Erwinia*, *Enterococcus*, *Staphylococcus*, *Streptococcus*, *Bacillus*, *Corynebacterium*, *Listeria*, *Escherichia*, *Salmonella*, *Pseudomonas* and

Helicobacter however, the mechanism of antimicrobial activity has not been reported (Roncadori et al. 1985, Si et al. 2005).

Compound 2, β-sitosterol, has been isolated from many plant sources, including *P. persica*. It has been reported to possess many biological activities such as anti-inflammatory, anticancer, hypocholesterolemic, angiogenic, genotoxic, analgesic, anthelmintic, immunomodulatory, antioxidant and antidiabetic (Saeidnia et al. 2014).

In the present study, β-sitosterol showed some activity against MRSA and MDRSA (MIC/MBC 625 µg/mL for each) and against antibiotic sensitive *S. aureus* (MIC/MBC 312 µg/mL). This is consistent with the findings of Mokbel et al., also using the MTT assay (Mokbel and Hashinaga 2005). In contrast, there are reports using disc diffusion assays of β-sitosterol exhibiting no antibacterial activity against either *S. aureus, E. coli* or *P. aeruginosa* (Hess et al. 1995; Beltrame et al. 2002) and direct TLC bioautography assays showing no activity against *B. subtilis* and *E. coli* (Hamburger and Cordell 1987). Mokbel and Hashinaga (2005) did a comparative study of β-sitosterol's activity using the disc diffusion and the MTT microdilution methods and found much better antimicrobial properties against *S. aureus, B. subtilis, B. cereus, S. enteritidis* and *E. coli* using the MTT method. The insensitivity of the disc diffusion assay to β-sitosterol is most likely due to the non-polar nature of the steroid making it difficult to diffuse into the aqueous agar medium (Valgas et al. 2007). The possible mode of antimicrobial activity has not been proposed.

Compound 3, stigmast-4-en-3-one, showed weak antimicrobial properties against susceptible *S. aureus* and MRSA, the susceptible strain of *E. coli* (MIC/MBC 2500 µg/mL), as well as *P. aeruginosa* (MIC/MBC 1250 µg/mL). Stigma-4-en-3-one has been isolated from many plants (Misra et al. 2012). This is the first report of the compound being present in the *Prunus* genus. A report concerning its activity against *Bacillus subtilis, S. aureus, Staphylococcus epidermidis, Enterococcus faecalis, E. coli, Proteus mirabilis, Klebsiella pneumoniae, P. aeruginosa* and *C. albicans* by the disc diffusion method showed the compound to be inactive against these strains (Ulubelen 2003). In the present study, the compound was found weakly active against *S. aureus, E. coli* and *P. aeruginosa* by the MTT microdilution method. The reported lack of antimicrobial activity in the disc diffusion assay may be due to the compound's poor aqueous solubility and microbial strain differences.

Compound 4, gallic acid, is commonly present in many plants (Nayeem 2016) and has been previously isolated from *P. persica* leaves and fruits (Cantín et al. 2009, Yang et al. 2014, Mokrani and Madani 2016). The compound was reported to possess strong antiinflammatory as well as antioxidant properties (Yen et al. 2002). Gallic acid was found to possess antibacterial activity against

S. aureus with an MIC/MBC of 156 µg/mL, against MRSA with an MIC/MBC of 312 µg/mL, as well as weak activity against β- *E. coli* and *P. aeruginosa* with MIC/MBCs of 2500 µg/mL for both of the strains. Antimicrobial properties of gallic acid have been well documented. Gallic acid has been shown to prevent the proteolytic activities of selected periodontopathic bacteria, such as *Streptococcus mutans, Bacteroides gingivalis, Bacteroides intermedius* and *Treponema denticola* (Kang et al. 2008) as well as common bacteria of medical significance such as *E. coli, P. aeruginosa, S. aureus* and *Listeria monocytogenes* (Borges 2013). Eighteen susceptible as well as antibiotic resistant strains (7 susceptible and 11 MRSA) of *S. aureus* wound isolates (9 from furuncle lesions and 9 from impetigo) have been reported to be susceptible to gallic acid (Akiyama et al. 2001). It was suggested that gallic acid causes irreversible changes in bacterial cellular membrane properties (charge, intra and extracellular permeability and physicochemical properties) through hydrophobicity changes, decrease of negative surface charge, and occurrence of local rupture or pore formation in the cell membranes with consequent leakage of essential intracellular constituents (Borges et al. 2013).

Compound 5, caffeic acid phenethyl ester, is a widely distributed natural product isolated from a number of plants (Tolba et al. 2013). It was found to be active against susceptible *S. aureus* and β- *E. coli* with an MIC/MBC of 312 µg/mL, as well as active against MRSA and MDRSA, with an MIC/MBC of 625 µg/mL. Caffeic acid phenethyl ester is well known for its antimicrobial properties (Kishimoto et al. 2005). It has been reported to be active against the bacteria *B. subtilis, E. coli,* S. *aureus, P. aeruginosa* and *L. monocytogenes* (Mirzoeva et al. 1997) and the fungi *Fusarium culmorum* and *Saccharomyces cerevisiae* (Merkl et al. 2010). Its antimicrobial properties are attributed to its polyphenolic structure and specifically catechol ring (Kartal et al. 2003). Its mechanism of action has been suggested to be due to, at least in part, to the synthesis of reactive oxygen species (ROS) that destroy the outer membrane of bacteria (Lee et al. 2013). This is the first report of the presence of caffeic acid phenethyl ester in *P. persica*.

2.8 Conclusions

Prunus persica has been used in traditional medicine since ancient times for the treatment of various ailments. The plant's leaves, flowers, fruits and bark are used to treat a wide range of conditions, including skin infections, and *in vitro* and *in vivo* biological studies have supported many of their traditional applications. Phytochemical studies have reported the presence of many important classes of active phytoconstituents including alkaloids, flavonoids, polyphenolics, cyanogenic glycosides, sterols and triterpenoids, which influence the pharmacological actions of the plant (Aziz 2013). While

the roots of *P. persica* have been used traditionally, there are limited studies on their phytochemistry and biological activities.

The *n*-hexane partition derived from the 70% aqueous ethanolic extract of *P. persica* roots, and fractions derived from the ethyl acetate partition, were subjected to TLC bioautography guided chromatographic separation. The *n*-hexane partition led to isolation of α-cyanobenzyl benzoate (1), β-sitosterol (2 or 2.24) and stigmast-4-en-3-one (3), and two compounds, gallic acid (4 or 2.20) and caffeic acid phenylethyl ester (5), were isolated from the ethyl acetate fractions. All compounds were characterised using spectroscopic methods and were confirmed through comparision with literature data.

The antimicrobial activities of the five compounds were tested against susceptible as well as resistant strains of *S. aureus* and *E. coli*, susceptible strains of *P. aeruginosa*, *S. typhimurium*, *S. pyogenes* and the fungus *C. albicans* by the MTT microdilution assay. α-Cyanobenzyl benzoate showed very good antibacterial properties against all tested *S. aureus* strains, good activity against β- *E. coli* and some activity against *P. aeruginosa*. β-Sitosterol showed some activity against MRSA, MDRSA, susceptible *E. coli* and antibiotic sensitive *S. aureus*. Stigmast-4-en-3-one showed weak antimicrobial properties against susceptible and resistant *S. aureus*, susceptible *E. coli* and *P. aeruginosa*. Gallic acid showed some antibacterial properties against *S. aureus*, MRSA, antibiotic sensitive *E. coli* and *P. aeruginosa*. Caffeic acid phenylethyl ester showed some activity against MRSA, MDRSA and antibiotic sensitive *S. aureus* and *E. coli* strains. This is the first report of isolation of gallic acid and stigmast-4-en-3-one from *P. persica* roots, the second report of isolation of α-cyanobenzyl benzoate from *P. persica* plant material, and the first report of antibacterial activity of stigmast-4-en-3-one (this compound was previously reported not active).

The finding of antibacterial compounds from the 70% aqueous ethanolic extract of *P. persica* roots supports the traditional use of the aqueous plant root extract topically to treat skin diseases, wounds and infections of a likely microbial origin. In order to gain a better understanding of the chemical composition of antibacterial partitions and fractions, a metabolomics approach was applied (Chapter 5).

2.9 References

Ahmad, S., A. and H. Ali (2006). "Ethnobotanical studies on some medicinal plants of Booni Valley, District Chitral, Pakistan." *Pakistan Journal of Weed Science Research* **12**(3): 183-190.

Akiyama, H., K. Fujii, O. Yamasaki, T. Oono and K. Iwatsuki (2001). "Antibacterial action of several tannins against *Staphylococcus aureus*." *Journal of Antimicrobial Chemotherapy* **48**(4): 487-491.

Akter, K., E. C. Barnes, W. L. Loa-Kum-Cheung, P. Yin, M. Kichu, J. J. Brophy, R. A. Barrow, I. Imchen, S. R. Vemulpad and J. F. Jamie (2016). "Antimicrobial and antioxidant activity and chemical characterisation of *Erythrina stricta* Roxb. (*Fabaceae*)." *Journal of Ethnopharmacology* **185**: 171-181.

Amusan, N. A. Sukati and M. S. Shongwe (2005). "Some phytomedicines from Shiselweni region of Swaziland." *Journal of Natural Remedies* **5**(1): 19-25.

Aziz, S. and Habib-ur-Rahman (2013). "Biological activities of *Prunus persica* L. batch " *Journal of Medicinal Plants Research* **7**(15): 947-951.

Beltrame, F. L., G. L. Pessini, D. L. Doro, B. P. Dias Filho, R. B. Bazotte and D. A. G. Cortez (2002). "Evaluation of the antidiabetic and antibacterial activity of *Cissus sicyoides*." *Brazilian Archives of Biology and Technology* **45**(1): 21-25.

Borges, A. (2013). "Antibacterial activity and mode of action of ferulic and gallic acids against pathogenic bacteria." *Microbial Drug Resistance* **19**(4): 256-265.

Bumrela, S. B. and S. R. Naik (2011). "Identification of β-carotene and β-sitosterol in methanolic extract of *Dipteracanthus patulus* (Jacq) nees and their role in antimicrobial and antioxidant activity." *International Journal of Phytomedicine* **3**(2): 204-215.

Cantín, C. M., M. A. Moreno and Y. Gogorcena (2009). "Evaluation of the antioxidant capacity, phenolic compounds, and vitamin C content of different peach and nectarine (*Prunus persica* (L.) Batsch) breeding progenies." *Journal of Agricultural and Food Chemistry* **57**(11): 4586-4592.

Carbonaro, M., M. Mattera, S. Nicoli, P. Bergamo and M. Cappelloni (2002). "Modulation of antioxidant compounds in organic vs conventional fruit (peach, *Prunus persica* L., and pear, *Pyrus communis* L.)." *Journal of Agricultural and Food Chemistry* **50**(19): 5458-5462.

Chauhan, P. (2016). "Ethnobotanical study of wild fruits in Pabbar Valley, District Shimla, Himachal Pradesh." *Journal of Medicinal Plants Studies* **4**(2): 216-220.

Choi, S., K.-W. Kim, J.-S. Choi, S.-T. Han, Y.-I. Park, S.-K. Lee, J.-S. Kim and M.-H. Chung (2002). "Angiogenic activity of β-sitosterol in the ischaemia/reperfusion-damaged brain of Mongolian gerbil." *Planta Medica* **68**(04): 330-335.

Eldahshan, O. (2011). "Isolation and structure elucidation of phenolic compounds of carob leaves grown in Egypt." *Current Research Journal of Biological Sciences* **3**(1): 52-55.

Fukuda, T., H. Ito, T. Mukainaka, H. Tokuda, H. Nishino and T. Yoshida (2003). "Anti-tumor promoting effect of glycosides from *Prunus persica* seeds." *Biological and Pharmaceutical Bulletin* **26**(2): 271-273.

Getaneh, S. and Z. Girma (2014). "An ethnobotanical study of medicinal plants in Debre Libanos Wereda, Central Ethiopia " *African Journal of Plant Science* **8**(7): 366-379.

Ghimire, K. and R. R. Bastakoti (2009). "Ethnomedicinal knowledge and healthcare practices among the Tharus of Nawalparasi District in Central Nepal." *Forest Ecology and Management* **257**(10): 2066-2072.

Giday, M., T. Teklehaymanot, A. Animut and Y. Mekonnen (2007). "Medicinal plants of the Shinasha, Agew-awi and Amhara peoples in northwest Ethiopia." *Journal of Ethnopharmacology* **110**(3): 516-525.

Gilani, S. A., R. A. Qureshi, A. M. Khan and D. Potter (2010). "Morphological characterization of the pollens of the selected species of Genus *Prunus* Linn. from Northern Pakistan." *African Journal of Biotechnology* **9**(20): 2872-2879.

Grunberger, D., R. Banerjee, K. Eisinger, E. Oltz, L. Efros, M. Caldwell, V. Estevez and K. Nakanishi (1988). "Preferential cytotoxicity on tumor cells by caffeic acid phenethyl ester isolated from propolis." *Experientia* **44**(3): 230-232.

Habib, M., F. Nikkon, M. Rahman, Z. Haque and M. Karim (2007). "Isolation of stigmasterol and ß-sitosterol from methanolic extract of root." *Pakistan Journal of Biological Scinces* **10**(22): 4174-4176.

Hamburger, M. O. and G. A. Cordell (1987). "A direct bioautographic TLC assay for compounds possessing antibacterial activity." *Journal of Natural Products* **50**(1): 19-22.

Han, S., K.-K. Park, W.-Y. Chung, S. K. Lee, J. Kim and J.-K. Hwang (2010). "Anti-photoaging effects of 2-methoxy-5-(2-methyl propyl) pyrazine isolated from peach (*Prunus persica* (L.) Batsch)." *Food Science and Biotechnology* **19**(6): 1667-1671.

Hazarika, T. (2018). "Potential wild edible fruits of Nagaland, North-east India and its significance in the livelihood and nutritional security of rural, indigenous people." *Genetic Resources and Crop Evolution* **65**: 199-215.

Heidari-Soreshjani, S., M. Asadi-Samani, Q. Yang and A. Saeedi-Boroujeni (2017). "Phytotherapy of nephrotoxicity-induced by cancer drugs: an updated review." *Journal of Nephropathology* **6**(3): 254-263.

Hess, S. C., R. L. Brum, N. K. Honda, A. B. Cruz, E. Moretto, R. B. Cruz, I. Messana, F. Ferrari and R. A. Yunes (1995). "Antibacterial activity and phytochemical analysis of *Vochysia divergens* (*Vochysiaceae*)." *Journal of Ethnopharmacology* **47**(2): 97-100.

Hoffmann, H., Z. Ismail, R. Hollweg and A. R. Zein (1990). "DABCO-mediated synthesis and biological activity of cyanohydrin esters." *Bulletin of the Chemical Society of Japan* **63**(6): 1807-1810.

Jamaluddin, F., S. Mohamed and M. N. Lajis (1994). "Hypoglycaemic effect of *Parkia speciosa* seeds due to the synergistic action of β-sitosterol and stigmasterol." *Food Chemistry* **49**(4): 339-345.

Jamaluddin, F., S. Mohameda and M. N. Lajis (1995). "Hypoglycaemic effect of stigmast-4-en-3-one, from Parkia speciosa empty pods." *Food Chemistry* **54**(1): 9-13.

Jang, G. H., H. W. Kim, M. K. Lee, S. Y. Jeong, A. R. Bak, D. J. Lee and J. B. Kim (2018). "Characterization and quantification of flavonoid glycosides in the *Prunus* genus by UPLC-DAD-QTOF/MS." *Saudi Journal of Biological Sciences* **25**(8): 1622-1631.

Joshi, S. and T. N. Poudel (2011). "Isolation and characterization of the chemical constituents of *Sonchus wightianus* of Nepalese origin." *Journal of Nepal Chemical Society* **28**(7): 115-120.

Kang, M.-S., J.-S. Oh, I.-C. Kang, S.-J. Hong and C.-H. Choi (2008). "Inhibitory effect of methyl gallate and gallic acid on oral bacteria." *The Journal of Microbiology* **46**(6): 744-750.

Kant, R., R. K. Shukla and A. Shukla (2018). "A review on Peach (*Prunus persica*): an asset of medicinal phytochemicals." *International Journal for Research in Applied Science & Engineering Technology* **6**(1): 2186-2200.

Kart, A., Y. Cigremis, M. Karaman and H. Ozen (2010). "Caffeic acid phenethyl ester (CAPE) ameliorates cisplatin-induced hepatotoxicity in rabbit." *Experimental and Toxicologic Pathology* **62**(1): 45-52.

Kartal, M., S. Yıldız, S. Kaya, S. Kurucu and G. Topçu (2003). "Antimicrobial activity of propolis samples from two different regions of Anatolia." *Journal of Ethnopharmacology* **86**(1): 69-73.

Kefalew, A., Z. Asfaw and E. Kelbessa (2015). "Ethnobotany of medicinal plants in Ada'a District, East Shewa Zone of Oromia Regional State, Ethiopia." *Journal of Ethnobiology and Ethnomedicine* **11**(1): 25.

Khan, M. P. Z. and M. Ahmad (2015). "Traditional preference of wild edible fruits (WEFs) for digestive disorders (DDs) among the indigenous communities of Swat Valley-Pakistan." *Journal of Ethnopharmacology* ***174***: 339-354.

Kibonde, S. (2018). "Ethnobotanical study of medicinal plants used to manage HIV/AIDS opportunistic infections in Rungwe, Mbeya Region, Tanzania." *Journal of Medicinal Plants Research* **12**(2): 32-41.

Kichu, M. (2010). "Documentation and biological and phytochemical analysis of Chungtia medicinal plants of Nagaland, India." Dissertation, Chemistry and Biomolecular Sciences, Macquarie University, Sydney (PhD): 1-179.

Kichu, M., T. Malewska, K. Akter, I. Imchen, D. Harrington, J. Kohen, S. R. Vemulpad and J. F. Jamie (2015). "An ethnobotanical study of medicinal plants of Chungtia village, Nagaland, India." *Journal of Ethnopharmacology* **166**: 5-17.

Kim, Y.-K., B.-S. Koo, D.-J. Gong, Y.-C. Lee, J.-H. Ko and C.-H. Kim (2003). "Comparative effect of *Prunus persica* L. Batch-water extract and tacrine (9-amino-1,2,3,4-tetrahydroacridine hydrochloride) on concentration of extracellular acetylcholine in the rat hippocampus." *Journal of Ethnopharmacology* **87**(2): 149-154.

Kim, Y. H., H. E. Yang, B. K. Park, M. Y. Heo, B. K. Jo and H. P. Kim (2002). "The extract of the flowers of *Prunus persica*, a new cosmetic ingredient, protects against solar ultraviolet-induced skin damage in vivo." *Journal of Cosmetic Science* **53**(1): 27-34.

Kircher, H. W. and F. U. Rosenstein (1973). "Purification of sitosterol." *Lipids* **8**(3): 97-100.

Kishimoto, N., Y. Kakino, K. Iwai, K. Mochida and T. Fujita (2005). "*In vitro* antibacterial, antimutagenic and anti influenza virus activity of caffeic acid phenethyl esters." *Biocontrol Science* **10**(4): 155-161.

Kumar, K., Y. P. Sharma, R. K. Manhas and H. Bhatia (2015). "Ethnomedicinal plants of Shankaracharya Hill, Srinagar, J&K, India." *Journal of Ethnopharmacology* **170**: 255-274.

Kuwahara, Y., M. Morita, Y. Ichiki, T. Tanabe and Y. Asano (2017). "1-Phenyl-2-pentanone and methyl salicylate: new defense allomone components and their content shift during ontogenetic development of the millipede *Nedyopus tambanus mangaesinus* (Polydesmida: *Paradoxosomatidae*)." *Applied Entomology and Zoology* **52**(3): 447-455.

Layne, D. R. and D. Bassi (2008). *The each: botany, production and uses*, CAB International.

Lee Chang, K., K. Park Kwang, K. Hwang Jae, K. Lee Sang and Y. Chung Won (2007). "The extract of *Prunus persica* flesh (PPFE) attenuates chemotherapy-induced hepatotoxicity in mice." *Phytotherapy Research* **22**(2): 223-227.

Lee, H. S., S. Y. Lee, S. H. Park, J. H. Lee, S. K. Ahn, Y. M. Choi, D. J. Choi and J. H. Chang (2013). "Antimicrobial medical sutures with caffeic acid phenethyl ester and their *in vitro*/*in vivo* biological assessment." *MedChemComm* **4**(5): 777-782.

Lim, T. K. (2012). Edible Medicinal and Non-Medicinal Plants: Volume 4, Fruits, Springer.

Lin, W.-Y., Y.-H. Kuo, Y.-L. Chang, C.-M. Teng, E.-C. Wang, T. Ishikawa and I.-S. Chen (2003). "Anti-platelet aggregation and chemical constituents from the rhizome of *Gynura japonica*." *Planta Medica* **69**(08): 757-764.

Liu, X., W. Li, P. Li and Y. Zhou (2007). "Extraction and chemical components of essential oils of *Malania Olceifera* chum." *Chinese Journal of Applied Chemistry* **24**(8): 970.

Lone, P. A. and A.K. Bhardwaj (2013). "Traditional herbal based disease treatment in some rural areas of Bandipora District of Jammu and Kashmir, India." *Asian Journal of Pharmaceutical and Clinical Research* **6**(4): 162-171.

Makarov, S. E., B. P. Ćurčić, V. V. Tešević, M. B. Jadranin, L. V. Vujisić, S. B. Ćurčić, B. M. Mandić, T. L. Sekulić and B. M. Mitić (2010). "Defensive secretions in three species of polydesmids (*Diplopoda, Polydesmida, Polydesmidae*)." *Journal of Chemical Ecology* **36**(9): 978-982.

Malewska, T. (2015). "Biological and phytochemical analysis of Chungtia medicinal plants of Nagaland, India." Dissertation, Chemistry and Biomolecular Sciences, Macquarie University, Sydney (MPhil): 1-226.

Martin, N. H., N. W. Allen III, J. D. Brown, D. M. Kmiec Jr and L. Vo (2003). "An NMR shielding model for protons above the plane of a carbonyl group." *Journal of Molecular Graphics and Modelling* **22**(2): 127-131.

Murtaza, G., S. Karim, M. R. Akram, S. A. Khan, S. Azhar, A. Mumtaz, B. Asad and M. H. Hassan (2014). "Caffeic acid phenethyl ester and therapeutic potentials." *BioMed Research International* **2014**.

Merkl, R., I. Hrádková, V. Filip and J. Šmidrkal (2010). "Antimicrobial and antioxidant properties of phenolic acids alkyl esters " *Czech Journal of Food Science* **28**(4): 275–279.

Mirzoeva, O. K., R. N. Grishanin and P. C. Calder (1997). "Antimicrobial action of propolis and some of its components: the effects on growth, membrane potential and motility of bacteria." *Microbiological Research* **152**(3): 239-246.

Misra, L., P. Mishra, A. Pandey, R. S. Sangwan and N. S. Sangwan (2012). "1, 4-Dioxane and ergosterol derivatives from *Withania somnifera* roots." *Journal of Asian Natural Products Research* **14**(1): 39-45.

Mohareb, R. M., N. N. El-Sayed and M. A. Abdelaziz (2012). "Uses of cyanoacetylhydrazine in heterocyclic synthesis: novel synthesis of pyrazole derivatives with anti-tumor activities." *Molecules* **17**(7): 8449-8463.

Mokbel, M. S. and F. Hashinaga (2005). "Antibacterial and antioxidant activities of banana (Musa, AAA cv. Cavendish) fruits peel." *American Journal of Biochemistry and Biotechnology* **1**(3): 125-131.

Mokrani, A. and K. Madani (2016). "Effect of solvent, time and temperature on the extraction of phenolic compounds and antioxidant capacity of peach (*Prunus persica* L.) fruit." *Separation and Purification Technology* **162**: 68-76.

Nair, V., S. Devipriya and E. Suresh (2008). "Construction of heterocycles via 1,4-dipolar cycloaddition of quinoline–DMAD zwitterion with various dipolarophiles." *Tetrahedron* **64**(16): 3567-3577.

Nayeem, N. (2016). "Gallic acid: A promising lead molecule for drug development." *Journal of Applied Pharmacy* **8**(2): 2-4.

Nuylert, A., Y. Kuwahara, T. Hongpattarakere and Y. Asano (2018). "Identification of saturated and unsaturated 1-methoxyalkanes from the Thai millipede *Orthomorpha communis* as potential "Raincoat Compounds"." *Scientific Reports* **8**(1): 11730.

Ohigashi, H., S. Minami, H. Fukui, K. Koshimizu, F. Mizutani, A. Sugiura and T. Tomana (1982). "Flavanols, as plant growth inhibitors from roots of peach, *Prunus persica* Batsh. cv. 'Hakuto'." *Agricultural and Biological Chemistry* **46**(10): 2555-2561.

Pieroni, A., A. Nedelcheva and Y. Dogan (2015). "Local knowledge of medicinal plants and wild food plants among Tatars and Romanians in Dobruja (South-East Romania)." *Genetic Resources and Crop Evolution* **62**(4): 605-620.

Rahman, W. and S. Bhatnagar (1968). "Flavonoids from *Prunus persica* Batsch (peach bark)." *Australian Journal of Chemistry* **21**(2): 539-541.

Rani, M. P., K. G. Raghu, M. S. Nair and K. P. Padmakumari (2014). "Isolation and identification of α-glucosidase and protein glycation inhibitors from *Stereospermum colais*." *Applied Biochemistry and Biotechnology*: 4(173): 946–956.

Rashid, S., M. Ahmad, M. Zafar, S. Sultana, M. Ayub, M. A. Khan and G. Yaseen (2015). "Ethnobotanical survey of medicinally important shrubs and trees of Himalayan region of Azad Jammu and Kashmir, Pakistan." *Journal of Ethnopharmacology* **166**: 340-351.

Ravi Kant., R. K. Shukla and A. Shukla (2108). "A review on peach (*Prunus persica*): an asset of medicinal phytochemicals." *International Journal for Research in Applied Science & Engineering Technology* **6**(1): 2186-2200.

Roncadori, R., S. Duffey and M. Blum (1985). "Antifungal activity of defensive secretions of certain millipedes." *Mycologia* **77**(2): 185-191.

Sarria-Villa, R. A. (2017). "Isolation of catechin and gallic acid from colombian bark of *Pinus patula*." *Chemical Sciences Journal* **8**(4): 1-11.

Shah, S. M. and F. Hussain (2012). "Ethnomedicinal plant wealth of mastuj valley, Hindukush Range, District Chitral, Pakistan." " *Journal of Medicinal Plants Research* **6**(26): 4328-4337.

Sharma, I., C. Kanta, S. Semwal and N. Goswami (2017). "Wild fruits of Uttarakhand (India): ethnobotanical and medicinal uses." *International Journal of Complementary and Alternative Medicine* **8**(3): 00260.

Sharma, J. and R. Painuli (2011). "Plants used for the treatment of rheumatism by the Bhoxa tribe of District Dehradun, Uttarakhand, India." *International Journal of Medicinal and Aromatic* **1**(1): 28-32.

Sharma, O. P., T. K. Bhat and B. Singh (1998). "Thin-layer chromatography of gallic acid, methyl gallate, pyrogallol, phloroglucinol, catechol, resorcinol, hydroquinone, catechin, epicatechin, cinnamic acid, *p*-coumaric acid, ferulic acid and tannic acid." *Journal of Chromatography* **822**(1): 167-171.

Saeidnia, S., A. Manayi, A. R. Gohari and M. Abdollahi (2014). "The story of β-sitosterol-a review." *European Journal of Medicinal Plants* **4**(5): 590.

Shear, W. A. (2015). "The chemical defenses of millipedes (*Diplopoda*): biochemistry, physiology and ecology." *Biochemical Systematics and Ecology* **61**: 78-117.

Si, W., J. Gong, R. Tsao, T. Zhou, H. Yu, C. Poppe, R. Johnson and Z. Du (2005). "Antimicrobial activity of essential oils and structurally related synthetic food additives towards selected pathogenic and beneficial gut bacteria." *Journal of Applied Microbiology* **100**(2): 296-305.

Suh, S.-J., B.-S. Koo, U.-H. Jin, M.-J. Hwang, I.-S. Lee and C.-H. Kim (2006). "Pharmacological characterization of orally active cholinesterase inhibitory activity of *Prunus persica* L. Batsch in rats." *Journal of Molecular Neuroscience* **29**(2): 101-107.

Tamang, R. and D. R. Sedai (2016). "Documentation of ethnomedicinal knowledge on plant resources used by Baram community in Arupokhari Vdc, Gorkha District, Central Nepal." *Plant Resousces* **38**: 60-64.

Tolba, M. F., S. S. Azab, A. E. Khalifa, S. Z. Abdel-Rahman and A. B. Abdel-Naim (2013). "Caffeic acid phenethyl ester, a promising component of propolis with a plethora of biological activities: a review on its anti-inflammatory, neuroprotective, hepatoprotective, and cardioprotective effects." *Life* **65**(8): 699-709.

Trak, H. (2017). "Inventory of the plants used by the tribals (Gujjar and Bakarwal) of district Kishtwar, Jammu and Kashmir (India)." *Indian Journal of Scientific Research* **13**(1): 104-115.

Ulubelen, A. (2003). "Cardioactive and antibacterial terpenoids from some *Salvia* species." *Phytochemistry* **64**(2): 395-399.

Uniyal, S. K., V. Sharma and P. Jamwal (2011). "Folk medicinal practices in Kangra District of Himachal Pradesh,Western Himalaya." *Human Ecology* **39**(4): 479-488.

Valgas, C., S. M. d. Souza, E. F. Smânia and A. Smânia Jr (2007). "Screening methods to determine antibacterial activity of natural products." *Brazilian Journal of Microbiology* **38**(2): 369-380.

Wangchuk, P., S. G. Pyne and P. A. Keller (2011). "Ethnobotanical authentication and identification of Khrog-sman (Lower Elevation Medicinal Plants) of Bhutan." *Journal of Ethnopharmacology* **134**(3): 813-823.

Whitehead, S., C. Jeffrey, M. Leonard, C. Dodson, L. Dyer and M. Bowers (2013). "Patterns of secondary metabolite allocation to fruits and seeds in *Piper reticulatum*." *Journal of Chemical Ecology* **39**(11-12): 1373-1384.

L. Zhang, L. Xu, M. Hu, X. Liu, F. Shi and Z. Gu (2014). "Antihypertensive effects of extract from flower buds of *Coreopsis tinctoria* on spontaneously hypertensive rats." *Chinese Herbal Medicines* **6**(2): 103-109.

Yaseen, G., M. Ahmad, M. Zafar, S. Sultana, S. Kayani, A. A. Cetto and S. Shaheen (2015). "Traditional management of diabetes in Pakistan: Ethnobotanical investigation from Traditional Health Practitioners." *Journal of Ethnopharmacology* **174**: 91-117.

Yen, G.-C., P.-D. Duh and H.-L. Tsai (2002). "Antioxidant and pro-oxidant properties of ascorbic acid and gallic acid." *Food Chemistry* **79**(3): 307-313.

Chapter 3 - *Diospyros lanceifolia*

The aims of this study were to evaluate the crude 70% aqueous ethanolic extract as well as partitioned fractions of *Diospyros lanceifolia* Roxb. (Ebenaceae) leaves for their antimicrobial properties and to investigate the active extracts for their major active compounds.

3.1 Introduction

Diospyros Roxb. is a large genus of the Ebenaceae family. Over 700 trees and shrubs have been confirmed as belonging to this genus (Kalita et al. 2016). Most *Diospyros* species are used as traditional medicines. Their uses include treatment of muscle and joint pain (Chang et al. 2011), gastrointestinal issues (Martínez-Las Heras et al. 2017), and fungal and bacterial infections (Baravalia et al. 2009).

Diospyros lanceifolia Roxb. (Ebenaceae) is a large tree that grows up to 27 metres tall. Its twigs are reddish brown when young, and blackish or dark brown when mature. Its stem bears clusters of up to 10 flowers. The fruits are round and up to 2.5 cm in diameter. The *lanceifolia* epithet comes from the Latin meaning "lance-shaped leaves" (Kalita et al. 2016). *D. lanceifolia* can be found in the lowland and hill rainforests of India, Indonesia and the Philippines. The Naga people of Chungtia village, Nagaland, use the fruits and roots of *D. lanceifolia* as a fish poison and the seeds are crushed and applied to the skin for treatment of skin diseases (Dominic and Ramanujam 2012, Kichu et al. 2015). The bark, leaves and fruits have also been used by the Sumi people of Nagaland as a fish poison and to treat various teeth problems and skin disorders (Dominic and Ramanujam 2012, Kalita et al. 2016).

Figure 3.1 *Diospyros lanceifolia* (photo by Nagaland man Meyanungsang Kichu).

Some of the reported biological activities of the Diospyros genus include the antifungal activity of *D. mafiensis* roots (Mmongoyo et al. 2017), antidiabetic activity of *D. melanoxylon* leaves (Harun Al Rashid et al. 2018), cytotoxic activity of *D. undulata* stem bark (Suchaichit et al. 2018) and

neuroprotective activity of *D. kaki* leaves (Zhang et al. 2018). Antimicrobial activity has been reported for the bark of *D. maritima* (Gu et al. 2004) and leaves of *D. monbuttensis* against *Staphylococcus aureus, Escherichia coli, Micrococcus luteus* and *Aspergillus niger* (Ayepola and Olasehinde 2018). The leaves of *D. lanceifolia* have been reported to possess strong antibacterial activity (Kichu 2010, Kalita et al. 2016).

More than 130 *Diospyros* species have so far been screened phytochemically (Kalita et al. 2016). The genus *Diospyros* is a rich source of 1,4-naphthoquinones and about 75% of the phytochemical reports on *Diospyros* are on the detection and isolation of these (Bringmann et al. 2001, Rischer et al. 2002, Suchaichit et al. 2018, Nematollahi et al. 2012). Plumbagin (3.1) and 7-methyljuglone (3.2) are the most widely distributed monomeric naphthoquinones in the *Diospyros* genus and they are mainly responsible for the cytotoxic (stem bark) (Dzoyem et al. 2011), antimicrobial (bark, roots) (Lajubutu et al. 1995, Evans et al. 1999), antifungal (stem bark) (Dzoyem et al. 2007), anthelmintic (bark) (Flota-Burgos et al. 2017, Nematollahi et al. 2012), antiviral (leaves, roots, flowers) (Babula et al. 2005) and anti-inflammatory (stem bark) (Cesari et al. 2013) activities reported for this genus (Rischer et al. 2002, Suchaichit et al. 2018). Other compounds include 8-hydroxy-1,2-naphthoquinone (3.3), ismailin (3.4), diosindigo (3.5), isodiospyrin (3.6), maritinone (3.7) (Gu et al. 2004, Salae et al. 2010, Cesari et al. 2013, Ruphin et al. 2014, Suchaichit et al. 2018), *cis*-isoshinanolone (3.8) and 2-hydroxymethyl-5-methoxy-1,4-naphthoquinone (3.9) (Bringmann et al. 2001, Suchaichit et al. 2018). Other compounds isolated from the *Diospyros* genus include the coumarins umckalin (3.10) and scopoletin (3.11) (Cesari et al. 2013, Suchaichit et al. 2018), the steroids campestrol (3.12) and stigmasterol (3.13), and ellagic acid (3.14) (Gao et al. 2013 (Nematollahi et al. 2012). The structures of the major compounds isolated from the *Diospyros* genus are presented in Figure 3.2.

Despite the extensive phytochemistry research conducted on other *Diospyros* species, there are only two records on the phytochemistry of *D. lanceifolia* (Kichu 2010, Kalita et al. 2016). Gallic acid was isolated from the aqueous methanolic extract of leaves (Kalita et al. 2016) and plumbagin as well as 7-methyljuglone have been isolated from the 70% aqueous ethanolic extract of the leaves by a former member of our research group (Kichu 2010). The work presented in Section 3.2 is a continuation of the research done by Kichu (Kichu 2010) and the MPhil studies of the author (Malewska 2015).

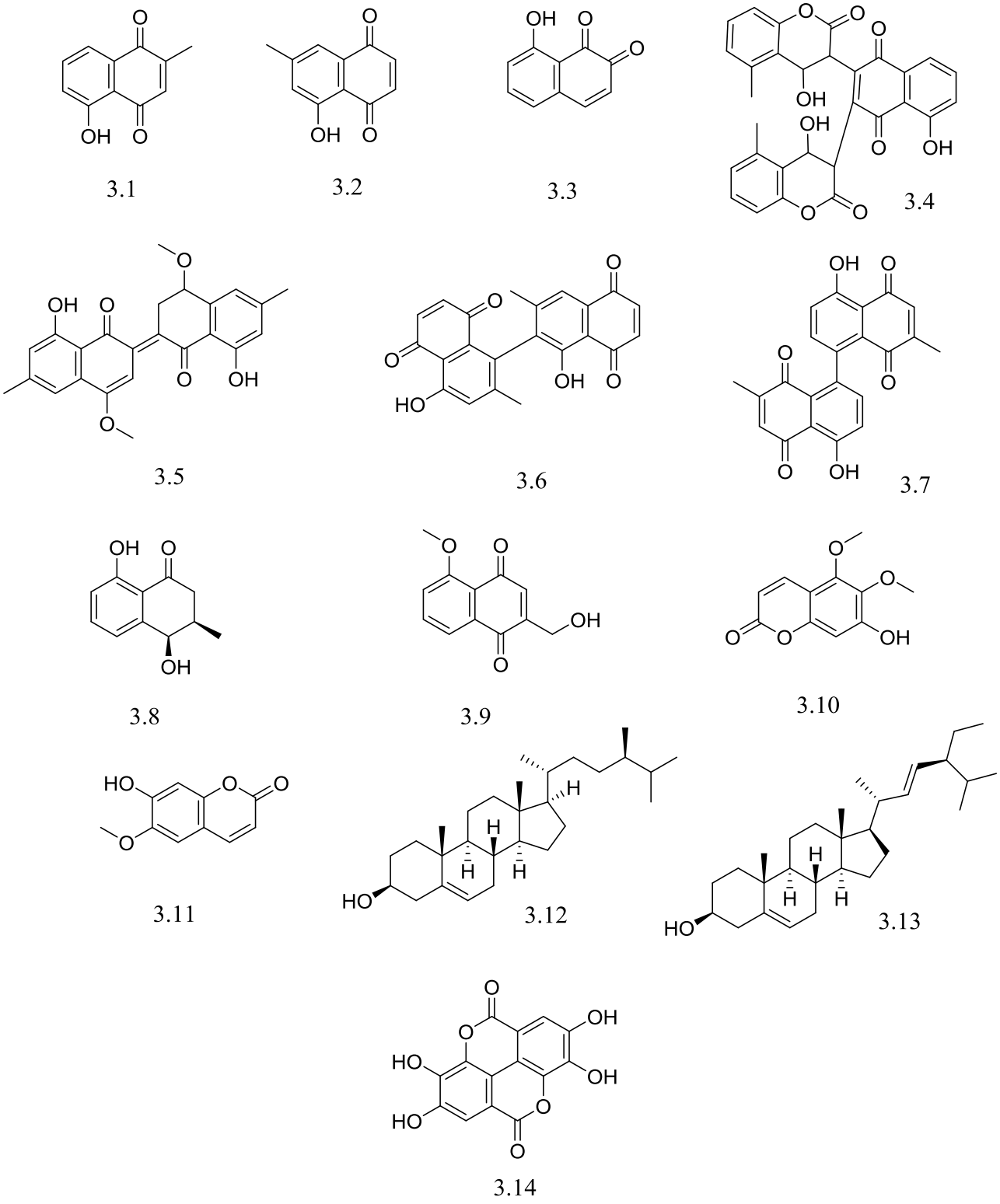


Figure 3.2 Compounds reported from the *Diospyros* genus.

3.2 Results and Discussion

The dried 70% aqueous ethanolic leaf extract of *D. lanceifolia* obtained from the MPhil studies (Malewska 2015), which had been stored at -20 ^{0}C under nitrogen, was used for this PhD study. The extract was resuspended in water and partitioned between *n*-hexane, dichloromethane and ethyl

acetate. The crude extract as well as the partitions and the remaining water fraction were screened using the MTT microdilution assay against the skin pathogens *Staphylococcus aureus* (susceptible *S. aureus*), methicillin resistant *S. aureus* (MRSA) and multidrug resistant *S. aureus* (MDRSA), β lactamase negative (antibiotic susceptible) *Escherichia coli* (*E. coli* β-), β lactamase positive (antibiotic resistant) *E. coli* (*E. coli* β+), *Pseudomonas aeruginosa*, *Streptococcus pyogenes*, *Salmonella typhimurium* and *Candida albicans* (Akter et al. 2016). The results found were all in agreement with that previously determined for freshly extracted leaf material and partitioned samples (Kichu 2010).

The *n*-hexane extract showed the best activity against all strains of *S. aureus* and *P. aeruginosa*, with minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) ranging from 156 µg/mL to 312 µg/mL (Table 3.1) and activity against antibiotic sensitive *E. coli* (312 µg/mL) and against *S. typhimurium* (625 µg/mL). The dichloromethane and ethyl acetate partitions showed weak activity against the antibiotic sensitive strain of *S. aureus* only (2500 µg/mL). The water fraction was inactive and none of the extracts showed activity against the remaining bacterial or fungal strains.

Table 3.1 Antimicrobial activity of *D. lanceifolia* partitions by MTT microdilution assay.

Fractions	MIC/MBC µg/mL					
	SA	MRSA	MDRSA	EC β-	PA	CA, SP, EC β+, ST
n-Hexane	156	312	312	312	156/312	625†
DCM	2500	na	na	na		na
EtOAc	2500/na	na	na	na		na
Water	na	na	na	na		na
Vancomycin	15.6	31.2	31.2	nt		nt
Gentamycin	nt	nt	nt	15.6*		15.6*, 31.2**
Fluconazole	nt	nt	nt	nt		31.2***

MIC values were determined as the wells with the lowest concentration of the samples that displayed no yellow to blue change of the MTT colour. *MIC values for *E. coli* (β-, β+), (PA) *P. aeruginosa* and (ST) *S. typhimurium*. **MIC value for (SP) *S. pyogenes*. (SA) *S. aureus*, (MRSA) methicillin resistant *S. aureus*, (MDRSA) multidrug resistant *S. aureus*. ***MIC value for (CA) *C. albicans;* †MIC value for *S. typhimurium* for *n*-hexane partition. nt: not tested; na: not active. DCM: dichloromethane; EtOAc: ethyl acetate; MeOH: methanol. MIC: minimum inhibitory concentration; MBC: minimum bactericidal concentration; single value in a cell indicates MIC=MBC.

Based on the assay results, the *n*-hexane fraction (10 g) was chosen for bioassay guided chromatographic separation. Initial fractionation was conducted with normal phase silica gel using 1 g of the material and automated Biotage column chromatography. Eluting with increasing polarity mixtures of petroleum ether (b.p. 40 °C):chloroform (100:0 to 30:70), seven fractions were collected

based on similar R_f values. Even though 2D TLC using normal phase silica plates did not show any potential issues of degradation of compounds, sample recovery was poor with ~60% mass recovery and significant loss of activity against the susceptible strain of *S. aureus* as seen in the TLC bioautography. TLC bioautography with susceptible *S. aureus* showed that, in order of elution, fraction two (260 mg) was weakly active at 2500 µg/mL, while fraction four (53 mg) had good activity (MIC 156 µg/mL). Both fractions were further purified by size exclusion chromatography (Sephadex LH-20). The chromatographic separation of fraction two led to the isolation of what appeared to be three pure compounds (1-3; 80.2 mg, 5.5 mg and 3.1 mg, respectively) based on different retention times by gas chromatography-mass spectrometry (GC-MS) using an Rxi-5ms (fused silica) column. The normal phase silica column chromatographic separation of fraction four led to the isolation of one impure compound (4, 0.5 mg). The flow diagram of key steps of the isolation process is presented in Figure 3.3.

The minimum inhibitory quantity (MIQ) values of the isolated compounds were determined by the method described by Akter et al. (Akter et al. 2016). Compounds 1-3 from fraction two showed activity against *P. aeruginosa* (MIQ 156 µg/mL) and against the antibiotic sensitive strains of *E. coli* (MIQ 312 µg/mL) and weak activity against the antibiotic susceptible *S. aureus* as well as MRSA (MIQ 2500 µg/mL). The compounds also possessed activity against *S. typhimurium* with an MIQ of 625 µg/mL. The very significant abundance of compound 1 (80 mg) was noted. Compound 4, due to its very small quantity, was tested only against the susceptible strain of *S. aureus.* It exhibited an excellent MIQ value of 62 µg/mL.

Analysis of compounds 1-3, using GC-MS, in electron impact (EI) mode, showed single peaks for each with molecular ions at *m/z* 426. Comparison of the MS fragmentation patterns with the NIST database showed that all three compounds had high similarity with the triterpenoid lupeol. Compounds 1 and 2 had virtually identical retention times (12.004 and 12.005, respectively); however, compound 3 under the same experimental conditions showed a different retention time (15.595 minutes) (Appendix 1). This initially made the author believe that at least compound 3 was different to compounds 1 and 2. Analysis of the literature revealed that the isomers 19-β-lupeol (lupeol), 19-α-lupeol and 3-α-lupeol (epilupeol) have been reported to have identical fragmentation patterns and only slightly different NMR profiles. Careful analysis of compounds 1-3 against an authentic sample of lupeol (Sigma-Aldrich), including comparison of NMR, melting point and optical rotation, TLC co-spotting and GC-MS co-injecting, was conducted to establish the identity of the compounds. Despite being isolated separately, all data indicated that all three samples were the same compound.

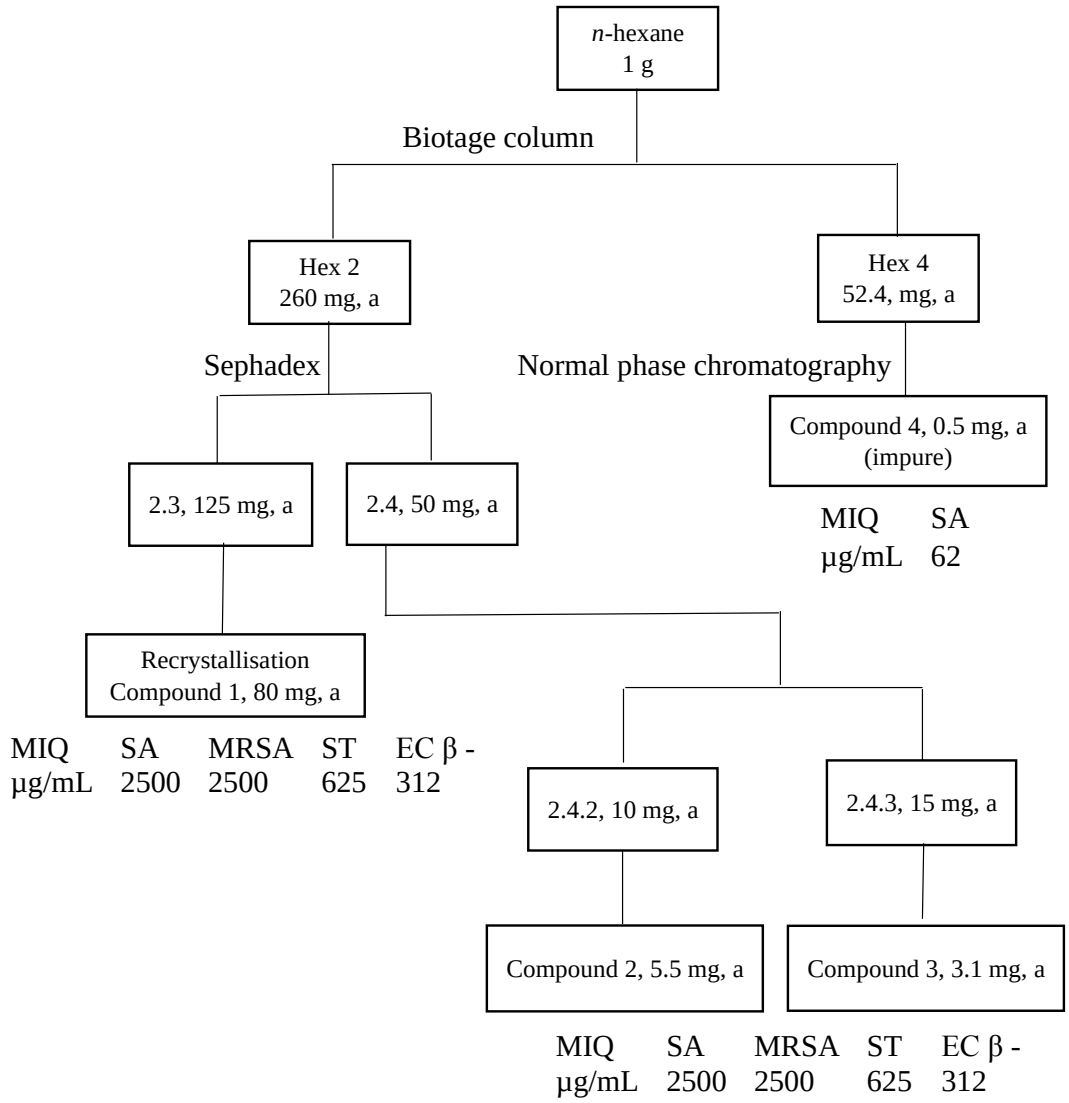


Figure 3.3 Key steps in the isolation process of *Diospyros lanceifolia* compounds. Samples 1, 2 and 3 are the same compound. Samples 1, 2 and 3 were tested against all bacteria but only positive results are shown. Compound 4 was tested against sensitive strain of *S. aureus* only due to the limited amount of the sample.

3.3 Structural elucidation of compounds 1 to 3

Compounds 1, 2 and 3 were isolated as white crystalline solids. As described above, their MS fragmentation patterns, TLC profiles and ^{1}H and ^{13}C NMR data were virtually identical, as well as melting points (213-215 °C), supporting them being the same compound. GC-MS (EI) analysis showed a high level of similarity with the triterpenoid lupeol ($C_{30}H_{50}O$) when compared with the NIST library database as well as with the literature (Gallo et al. 2009). ^{1}H and ^{13}C NMR data were also consistent with the literature spectra of lupeol (Gallo et al. 2009) (Table 3.2, Figure 3.4).

Previous studies have reported the isolation of lupeol-like compounds, namely 19-α-H lupeol 19-β-H lupeol (commonly referred to as lupeol) and epilupeol (Figure 3.4) having the same MS

fragmentation patterns and very similar NMR spectroscopic data (Ahmad et al. 1985, Manzano et al. 2013). As a result of this, data for compounds 1-3 were carefully analysed against that for lupeol, its 19-α-H isomer and epilupeol, to be able to confidently identify their structure.

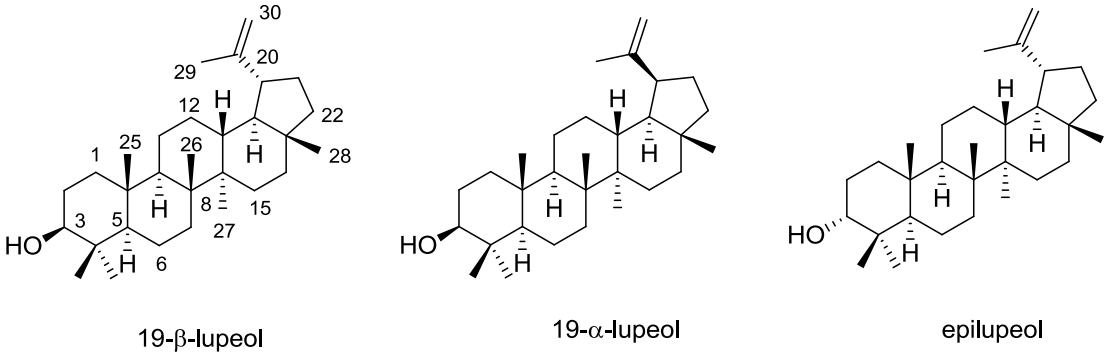


Figure 3.4 The structures of 19-β-lupeol (lupeol), 19-α-lupeol and 3-α-lupeol (epilupeol).

Initial comparison supported the identity of the isolated compounds being lupeol (19-β-H lupeol). Lupeol has a reported melting point of 213-215 °C (Agarwal and Rangari 2003), which was consistent with the isolated compounds, while 19-α-H lupeol has a melting point of 204-206 °C (Puapairoj et al. 2005) and epilupeol 198-201 °C (Hui and Fung 1969). The optical rotation was also consistent with lupeol, with all three compounds having an $[\alpha]_D^{20}$ +27° ($CHCl_3$, *c* 0.006 g/mL), consistent with the literature value of +27° ($CHCl_3$, *c* 0.006 g/mL, Zhong et al. 1984). The optical rotation for epilupeol has been reported to be $[\alpha]_D^{20}$ +14° and +15° ($CHCl_3$, *c* 0.006 g/mL) (Puapairoj et al. 2005); reports concerning optical rotation for 19-α-H lupeol were not found. While showing high similarity, the ^1H and ^{13}C NMR profiles have distinct differences for 19-α-H lupeol, 19-β-H lupeol and epilupeol (Manzano et al. 2013). Manzano et al. reported that the main distinguishing feature between epilupeol (3-α-H) and 3-β-H-lupeol (lupeol) was that the doublet of doublets assignable to the geminal proton of a secondary alcohol at H-3 was at δ 3.40 for epilupeol (in $CDCl_3$) and at δ 3.16 for lupeol (Manzano et al. 2013). The spectrum for epilupeol also showed a characteristic signal for C-3 at δ 76.4 ppm, while for lupeol it was reported to be at 79.1 ppm. Careful spectral analysis was performed on the three isolated compounds as described below.

The IR spectra showed the presence of an hydroxyl group at 3346 cm^{-1} and a double bond at 1639 cm^{-1}. The ^{13}C and DEPT NMR spectra indicated the presence of 30 carbons: 7 methyls, 11 methylenes, 6 methines and 6 quaternary carbons. A double bond was confirmed by the presence in the ^{13}C NMR of the chemical shifts at δ 109.33 (C-30) and δ 151.00 (C-29), and a carbon connected to oxygen was confirmed by the methine at δ C 79.01. The ^1H NMR spectrum (in $CDCl_3$) indicated the presence of 49 hydrogens. The hydroxyl proton, accounting for the remaining hydrogen of the triterpenoid formula $C_{30}H_{50}$, was not seen in the NMR experiments, but was consistent with the IR and ^{13}C chemical shifts. Diagnostic regions in the ^1H NMR included two olefinic resonances at δ 4.54

(H-30) and 4. 66 ppm (H-30), a doublet of doublets at δ 3.16 (1H, *dd, J* 0.9, 5.3 Hz, H-3) assignable to a geminal proton of a secondary alcohol (Manzano et al. 2013), and seven singlets between δ 1.7 and 0.6 ppm consistent with seven methyl groups. HSQC confirmed the two olefinic protons were geminal (C-30). With the oxygen representing a hydroxyl function (IR = 3346 cm^{-1}), the index of hydrogen deficiency was consistent with the structure containing additionally 5 rings.

In the HMBC spectra, the olefinic hydrogens at δ 4.66 and 4.54 (HMBC δ C 109.33, C-30) showed correlations with the methyl carbon at δ 19.31 (C-29), methine carbon at 47.99 (C-19) as well as the quaternary carbon at 151.00 (C-20). The proton attached to the carbon at δ 19.31 (C-29) also showed a correlation with the methine carbon at 47.99 (C-19), the methylene carbon at 109.33 (C-30) as well as the quaternary carbon at 151.00 (C-20). This indicated the connectivity of the disubstituted double bond and the C-19 methine to the carbon at δ 151.00 (C-20). The methine hydrogen at δ 2.35 (HSQC δ C 47.99, C-19) also showed correlation with the same quaternary carbon at δ 151.00 (C-20), the methine carbon at 109.33 (C-30) as well as the methylene at 29.85 (C-21) and methine carbon at 48.30 (C-18). The HSQC experiment showed the hydrogen at δ 2.35 is attached to the methine carbon at 47.99. There was also a COSY correlation between the proton at 2.35 and at 1.33 ppm (HSQC δ C 48.30).

In the HMBC spectra, the carbon at δ 29.85 (C-21) was correlated to the hydrogen at δ 2.35 (H-19) as well as the methylene hydrogens at δ 1.35 and 1.16 (in the HSQC those hydrogens were connected to the methylene carbon at δ 40.01, C-22). The hydrogen at δ 1.16 (H-22) was correlated to the carbon at δ 48.30 (C-18) and 18.01 (H-28). Additionally, the hydrogen at δ 1.88 showed a correlation to the proton at 1.35 (HSQC δ C 40.1, C-22). These HMBC correlations supported the closure of the ring, thus accounting for one of the five rings and the second degree of unsaturation.

Further HMBC correlation of the carbon at δ 40.01 (C-22) was observed to the methyl hydrogens at δ 0.76 (H-28). These methyl hydrogens also showed an HSQC correlation to the methyl carbon at δ 18.01 (C-28) and HMBC correlations to the quaternary carbon at δ 43.01 (C-17) and 48.30 (C-18), suggesting this methyl group is attached to the already closed cyclopentane ring. Further HMBC correlation of the carbon at δ 48.30 were to the methylene hydrogens at δ 1.44 (HSQC δ C 35.59, C-16). In the HSQC spectra, the carbon at δ 48.30 (C-18) correlated with the methine hydrogen at δ 1.33 (H-18). That hydrogen showed correlations to the methylene carbon at 35.59 (HSQC δ H 1.44, 1.34, H-16) and the quaternary carbon at δ 42.84 (C-14). The δ 1.34 hydrogen also showed an HMBC correlation to the methylene carbon at 27.45 (C-12, HSQC δ H 0.97, 1.58). These HMBC correlations supported the closure of a ring that fused to the first ring, thus accounting for the third degree of unsaturation and second of the five rings.

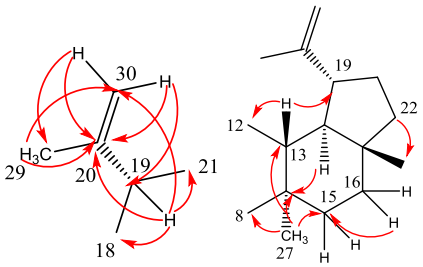


Figure 3.5 The HMBC closure of rings 1 and 2.

The methyl hydrogen at δ 1.00 (δ C 15.98, C-26) showed HMBC correlations with the quaternary carbons at δ 42.84 (C-14) and 40.83 (C-8), methylene carbon at δ 34.28 (C-7) and methine carbon at 55.30 (C-5). The hydrogen signals at δ 1.24 and 1.38 (*m*, H-11) showed correlation with the methine carbon δ 50.44 (C-9). These HMBC correlations supported the third ring being fused to the second ring, thus accounting for one additional degree of unsaturation, and the third of the five rings.

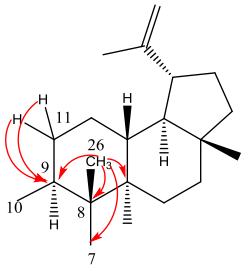


Figure 3.6 The HMBC closure of the third ring.

The methyl hydrogen at δ 0.65 (HSQC 55.30, C-5) showed HMBC correlations with the quaternary carbons at δ 37.17 (C-10) and 38.71 (C-4), methyl carbons at δ 27.99 (C-23) and 15.38 (C-24), methylene carbons at δ 18.32 (C-6) and 34.28 (C-7) and methine carbon at δ 50.44 (C-9). The methyl carbon at δ 16.13 (C-25) showed correlations with the quaternary carbon at δ 38.71 (C-4) and the two methine carbons at δ 55.30 (C-5) and 50.44 (C-9). The HMBC correlations supported the closure of the fourth ring, being fused to the third ring, thus accounting for one additional degree of unsaturation, and the fourth of the five rings.

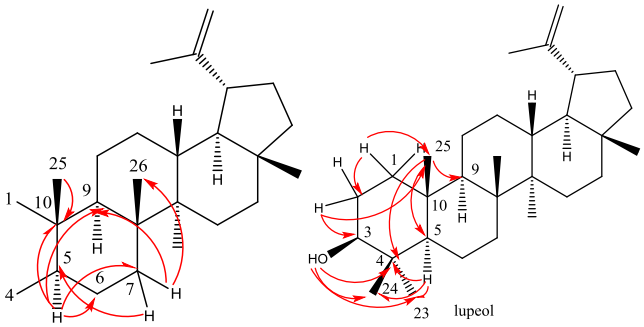


Figure 3.7 The HMBC closure of rings 4 and 5.

The chemical shift of the methine carbon at δ 79.02 (C-3) indicated that an oxygen group is likely to be attached to that carbon. This accounted for one hydroxyl group. The methylene hydrogen at δ 1.63

(HSQC 38.05, C-1) showed correlations to the methyl carbon at δ 25.14 (C-2) and methyl carbon at δ 16.13 (C-25). The methyl hydrogen at δ 0.80 (HSQC 16.13, C-25) showed correlations to the quaternary carbon at δ 38.71 (C-4) and two methine carbons at δ 55.30 (C-5) and 50.44 (C-9). The methine hydrogen at δ 3.16 (HSQC 79.02, C-3) showed correlations to the quaternary carbon at δ 38.71 (C-4) and two methyl carbons at δ 27.99 (C-23) and 15.38 (C-24). The HMBC correlations supported the closure of a ring, which accounted for the sixth degree of unsaturation and final ring. The NMR data is presented in Table 3.2. All spectral data were in agreement with the literature for 19-β-lupeol (Sholichin et al. 1980).

Table 3.2 NMR data of 19-β-lupeol and authentic sample (noted as ref) (400 MHz, $CDCl_3$).

C#	δ C	δ H (*J* in Hz)	HMBC	Multiplicity	COSY	C (ref) †	H (ref)†
1	38.05	1.63	C-25, C-2	CH_2		38.05	1.62
2	25.14	1.64, 1.03	C-25, C-3	CH_2		25.14	1.64, 1.03
3	79.02	3.16, *dd*, 10.9, 5.3	C-24, C-23, C-4	CHOH	C-2	79.01	3.17, *dd*, 10.9, 5.3
4	38.71			C		38.71	
5	55.30	0.65	C-6, C-23, C-7, C-10, C-4, C-9	CH	C-6	55.30	0.65
6	18.32	1.49, 1.36		CH_2		18.32	1.49, 1.37
7	34.28	1.36	C-26, C-9, C-5	CH_2		34.28	1.36
8	40.83	-		C		40.84	
9	50.44	1.24	C-25	CH		50.44	1.24
10	37.17	-		C		37.17	
11	20.93	1.24, 1.38	C-25	CH_2		20.93	1.25, 1.38
12	27.42	0.98, 1.60		CH_2		27.41	0.98, 1.60
13	38.87	-		CH_2		38.86	
14	42.84	-		C		42.84	
15	27.45	0.97, 1.58		CH_2		27.45	0.97, 1.58
16	35.59	1.44, 1.34	C-15	CH_2		35.59	1.45, 1.36
17	43.01	-		C		43.01	
18	48.30	1.33	C-16, C-14, C-20	CH		48.31	1.33
19	47.99	2.35	C-21, C-18, C-30, C-20	CH	C-18	47.99	2.35
20	151.00	-		C		151.00	
21	29.85	1.88, 1.30	C-19, C-22, C-20, C-28	CH_2	C-18	29.85	1.90, 1.31
22	40.01	1.35, 1.16	C-18, C-28	CH_2	C-21	40.01	1.35, 1.16
23	27.99	0.94	C-24, C-4, C-5, C-3	CH_3		27.99	0.94
24	15.38	0.73	C-25, C-23, C-5, C-4, C-3	CH_3		15.37	0.73
25	16.13	0.80	C-10, C-4, C-9, C-5	CH_3		16.12	0.80
26	15.98	1.00	C-7, C-8, C-14, C-9	CH_3		15.98	1.00
27	14.55	0.92	C-15, C-13, C-8, C-14	CH_3	C-15	14.55	0.92
28	18.01	0.76	C-16, C-22, C-17, C-18	CH_3		18.00	0.77
29	19.31	1.65	C-19, C-30, C-20	CH_3		19.31	1.65
30	109.33	4.66, 4.54 *d*, 2.25	C-29, C-21, C-19, C-20	CH	C-29	109.32	4.66, 4.54 *d*, 2.25

†Reference sample of 19-β-lupeol from Sigma-Aldrich.

3.4 Structural elucidation of compound 4 and analysis of fraction 4.1

Compound 4 was obtained as an orange solid. EI-MS showed a molecular ion at *m/z* 188, consistent with the molecular formula of $C_{11}H_8O_3$. The fragmentation pattern was consistent with the NIST database for plumbagin, as well as with published literature (Wenche and Einar, 1995). Plumbagin was also previously isolated from the plant material (Kichu 2010). The compound however was of insufficient quantity for adequate NMR analysis. The fraction 4.1 (Figure 3.3) that compound 4 was derived from was subjected to 1D and 2D NMR and the data compared with the NMR of an authentic sample of plumbagin (Sigma-Aldrich). Even though the fraction was impure, the peaks for plumbagin were easily distinguishable.

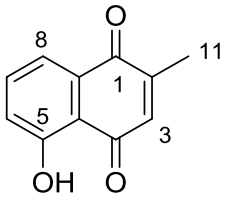


Figure 3.8 The structure of plumbagin.

The ^{1}H NMR spectrum of the fraction showed three aromatic protons (δ 7.73, 7.59 and 7.28) with coupling indicative of them being adjacent to each other and the aromatic ring being 1,2,3-trisubstituted; a deshielded methyl group (δ 2.19) with allylic coupling to an olefinic proton (δ 6.92); and a chelated hydroxyl proton (δ 11.97). The ^{13}C NMR spectrum showed the presence of one methyl, four methine, six quaternary carbons and two carbonyl groups (δ 184.33 and 190.63). The spectral data were indicative of a 1,4-naphthoquinone skeleton. HMBC correlations placed the methyl group at C-2 (δ 149.82). The ^{1}H and ^{13}C NMR data (see Table 3.3) exactly matched the literature for plumbagin (Gu et al. 2004, Lim et al. 2007) as well as the spectra of an authentic sample.

Table 3.3 NMR data of compound 4 (in fraction 4.1) and authentic sample of plumbagin (noted as ref) (400 MHz, $CDCl_3$).

C#	δ C	δ H (*J* in Hz)	HMBC	Multiplicity	δ C (ref) †	δ H (ref)†
1	184.33			C	184.32	
2	149.82			C	149.82	
3	135.14	6.92, *q*, 1.5	C-1, C-10, C-11	CH	135.13	6.92, *q*, 1.5
4	190.63			C	190.62	
5	161.03			C	161.03	
6	123.60	7.28, *dd*, 8.0, 1.5	C-5, C-8, C-10	CH	123.60	7.28, *dd*, 8.0, 1.5
7	135.14	7.59, *dd*, 8.0, 7.5	C-5, C-9	CH	135.14	7.59, *dd*, 8.0, 7.5
8	118.66	7.73, *dd*, 7.5, 1.5	C-1, C-6, C-10	CH	118.65	7.73, *dd*, 7.5, 1.5
9	132.33			C	132.33	
10	115.09			C	115.09	
11	15.41	2.19, *d*, 1.5	C-1, C-2, C-3	CH_3	15.40	2.16, *d*, 1.5
OH-5		11.98	C-5, C-6, C-10			11.98

†Reference sample of plumbagin from Sigma-Aldrich.

3.5 Biological activity of lupeol

As described in Section 3.2, compounds 1-3, which were all confirmed as lupeol, exhibited activity against *P. aeruginosa* (156 µg/mL), and against antibiotic sensitive strains of *E. coli* (312 µg/mL) and weak activity against MRSA and antibiotic susceptible *S. aureus* (2500 µg/mL) for both strains as well as activity against *S. typhimurium* (625 µg/mL). Lupeol has been reported to be active against *S. aureus, E. coli, P. aeruginosa* and *S. typhi* (Singh and Singh 2003, Ajaiyeoba et al. 2003) as well as *Enterococcus faecalis, Klebsiella pneumoniae* (Gallo, Miranda et al. 2009), *Bacillus subtilis* and *Micrococcus luteus* (Sushila et al. 2010). The mechanism of action has been associated with increase of membrane fluidity/permeability, disruption of membrane-embedded proteins, and change of ion transport processes in both Gram-positive and Gram-negative bacteria (Rajasekaran et al. 2018).

Lupeol has been rarely reported in the fungal or animal kingdom (Lutta 2008) but has been shown to occur in many plant families (Connolly and Hill 1989). Lupeol has been studied for its various bioactivities since the early 19th century (Gallo, Miranda et al. 2009). During the early days, the majority of the published articles were related to lupeol's synthesis, phytochemical investigations and biological activities such as anticancer, chemo preventative, antiprotozoal, anti-inflammatory, cardio protective, hepatoprotective, antiviral and antimicrobial activities (Fotie, Bohle et al. 2006, Gallo, Miranda et al. 2009). However, in the beginning of the 21st century, the number of articles on it increased considerably with the uprise occurring throughout the past 5 years with an average of over 50 articles per year (Scifinder and Web of Science databases), mostly attributable to Lupeol's very strong anticancer activities (Gallo, Miranda et al. 2009). Recently, research regarding biotransformation, chemoprevention, mechanism of action, derivative synthesis and methods of detection and quantitation, in addition to conventional studies, have been carried out on the compound (Gallo, Miranda et al. 2009).

3.6 Biological activity of plumbagin

As described in Section 3.2, compound 4, plumbagin, exhibited excellent activity against a sensitive strain of *S. aureus*. Due to the small quantity of the compound, the testing against a full suite of bacteria was not possible.

According to the literature, one of the unique and most noticeable properties of plumbagin is its antibacterial activity. It manifests very strong effects against both Gram-positive and Gram-negative bacteria (Nematollahi et al. 2012). Plumbagin has been shown to have strong antibacterial activity against *Clostridium perfringens* (Lim et al. 2007), *Helicobacter pylori* (Park and Lee 2006), *S. aureus*

(de Paiva et al. 2003), *P. aeruginosa*, *E. coli*, *Salmonella typhi*, *Klebsiella pneumonia*, *Serratia marcescens*, *Bacillus subtilis* and *Proteus vulgaris* (Jeyachandran et al., 2009).

There are several mechanisms described for plumbagin's of antimicrobial activity. Plumbagin has been shown to intercalate with microbial DNA and cause breaking of single and double stranded DNA (Asche, 2005). It has also been reported that plumbagin has the capacity to chelate several trace metals which may also contribute to its antimicrobial properties (Padhyeetal, 2012). Bhattacharyaetal demonstrated plumbagin inhibits cytokinesis in *Bacillus subtilis* by inhibiting FtsZ assembly (Bhattacharyaetal, 2013). It was also demonstrated that it can induce reactive oxygen species production. Because plumbagin acts on multiple targets to exert antimicrobial activity it may be more effective than conventional antibiotics or antifungal agents in controlling microbial growth.

3.7 GC-MS analysis of *n*-hexane extracts

Due to the limited success of the isolation studies, the *n*-hexane extract of *D. lanceifolia* was analysed by analytical GC-MS on a Shimadzu GC17A gas chromatograph with a BP-5 column, with the assistance of Dr Joseph Brophy (University of New South Wales, Sydney, Australia). Compounds were identified by matching their GC retention times and retention indices relative to *n*-alkanes, and by comparison of their mass spectra with the NIST library database and other published data. This identified 7-methyljuglone (*m*/z 188.10, retention time 31.483 minutes) and plumbagin (compound 4, *m/z* 188.05, retention time 31.983 minutes), which were previously isolated from the leaves of the plant by Kichu (Kichu 2010).

Plumbagin and 7-methyljuglone have been isolated from many plants, including those that have been used traditionally for the treatment of bacterial infections (Baravalia et al. 2009). 5 Hydroxy-2-methyl-1,4-naphthoquinone, *i.e.* plumbagin and 7-methyljuglone are primary skeletons of many monomeric and oligomeric naphthoquinones (Nematollahi et al. 2012).

7-Methyljuglone was isolated in 1982 from *Diospyros* spp (*Diospyros hebecarpa* A. Cunn. ex Benth.) for the first time (Ohigashi et al. 1982). Later, it was isolated from other species of this genus (Nematollahi et al. 2012). One of the interesting effects of this compound is its antituberculotic activity (Mahapatra et al. 2007). 7-Methyljuglone has also been previously found active against *S. aureus*, *E. coli* and *P. aeruginosa* (Lajubutu et al. 1995).

The presence of plumbagin and 7-methyljuglone support the antimicrobial properties found in the *n*-hexane partition of *D. lanceifolia* leaves.

3.8 Conclusions

The *n*-hexane partition of leaves of *D. lanceifolia* was found to be active against antibiotic susceptible *S. aureus*, MRSA, MDRSA and *E. coli* in the MTT microdilution assay. Bioassay guided studies with normal phase silica column chromatography and size exclusion chromatography led to the isolation of two antibacterially active compounds, lupeol and plumbagin. The structure of lupeol was confirmed by EI-MS, ^{1}H, ^{13}C and 2D NMR analysis and by comparison with an authentic sample and published literature. Plumbagin was tentatively identified by subsequent GC-MS analyses of the *n*-hexane partition. Its presence was confirmed by ^{1}H, ^{13}C and 2D NMR analysis and by comparison with the NMR data of an authentic sample and reported data. GC-MS analysis of the *n*-hexane fractions also supported the presence of 7-methyljuglone.

Lupeol showed activity against antibiotic sensitive strains of *P. aeruginosa* (MIQ 156 µg/mL) and *E. coli* (312 µg/mL), and against *S. typhimurium* (625 µg/mL), with weak activity against antibiotic susceptible *S. aureus* as well as MRSA (2500 µg). Plumbagin, due to very low quantity, was tested only against the susceptible strain of *S. aureus* and was found to possess good activity of 62 µg/mL against this bacterium.

Many biological and phytochemical studies have been done on the genus *Diospyros* (Kalita et al. 2016), however, very limited studies have been conducted on *D. lanceifolia*. The bioassay guided isolation studies presented in this chapter did not expand the knowledge about this plant much; therefore, antibacterially active extracts of the plant were further analysed using a metabolomics approach, as described in Chapter 5.

3.9 References

Agarwal, R. and V. Rangari (2003). "Antiinflammatory and antiarthritic activities of lupeol and 19 alpha-H lupeol isolated from *Strobilanthus callosus* and *Strobilanthus ixiocephala* roots." *Indian Journal of Pharmacology* **35**(6): 384-387.

Ahmad, V. U., S. Bano and F. V. Mohammad (1985). "Nepehinol - a new triterpene from *Nepeta hindostana*." *Planta Medica* **51**(06): 521-523.

Ajaiyeoba, E., P. Onocha, S. Nwozo and W. Sama (2003). "Antimicrobial and cytotoxicity evaluation of *Buchholzia coriacea* stem bark." *Fitoterapia* **74**(7-8): 706-709.

Akter, K., E. C. Barnes, W. L. Loa-Kum-Cheung, P. Yin, M. Kichu, J. J. Brophy, R. A. Barrow, I. Imchen, S. R. Vemulpad and J. F. Jamie (2016). "Antimicrobial and antioxidant activity and chemical characterisation of *Erythrina stricta* Roxb. (*Fabaceae*)." *Journal of Ethnopharmacology* **185**: 171-181.

Ayepola and G. I. Olasehinde (2018). "*In-vitro* antimicrobial activity of crude extracts of *Diospyros monbuttensis*." *African Journal of Clinical and Experimental Microbiology* **19**(2): 84-91.

Babula, P., R. Mikelova, D. Potesil, V. Adam, R. Kizek, L. Havel and Z. Sladky (2005). "Simultaneous determination of 1,4-naphthoquinone, lawsone, juglone and plumbagin by liquid chromatography with UV detection." *Biomedical Papers* **149**: 25-28.

Baravalia, Y., M. Kaneria, Y. Vaghasiya, J. Parekh and S. Chanda (2009). "Antioxidant and antibacterial activity of *Diospyros ebenum* Roxb. leaf extracts." *Turkish Journal of Biology* **33**(2): 159-164.

Bringmann, G., K. Messer, W. Saeb, E.-M. Peters and K. Peters (2001). "The absolute configuration of (+)-isoshinanolone and *in situ* LC–CD analysis of its stereoisomers from crude extracts." *Phytochemistry* **56**(4): 387-391.

Cesari, I., M. Hoerle, C. Simoes-Pires, P. Grisoli, E. Queiroz, C. Dacarro, L. Marcourt, P. Moundipa, P.-A. Carrupt and M. Cuendet (2013). "Anti-inflammatory, antimicrobial and antioxidant activities of *Diospyros bipindensis* (Gürke) extracts and its main constituents." *Journal of Ethnopharmacology* **146**(1): 264-270.

Chang, T.-N., S.-S. Huang, Y.-S. Chang, C.-I. Chang, H.-L. Yang, J.-S. Deng, Y.-H. Kuo and G.-J. Huang (2011). "Analgesic effects and mechanisms of anti-inflammation of taraxeren-3-

one from *Diospyros maritima* in mice." *Journal of Agricultural and Food Chemistry* **59**(17): 9112-9119.

Connolly, J. D. and R. A. Hill (1989). "Triterpenoids." *Natural Product Reports* **6**(5): 475-501.

De Paiva, S. R., M. R.Figueiredo, T. V. Aragao and M. A. Kaplan (2003). "Antimicrobial activity *in vitro* of plumbagin isolated from *Plumbago* species." *Mem Inst Oswaldo Cruz, Rio de Janeiro* **98**: 959-961.

Dominic, R. and S. N. Ramanujam (2012). "Traditional knowledge and ethnobotanical uses of piscicidal plants of Nagaland, North east India." *Indian Journal of Natural Products and Resources* **3**(4): 582-588.

Dzoyem, J., F. Kechia, V. Kuete, A. Pieme, C. Akak, J. Tangmouo and P. Lohoue (2011). "Phytotoxic, antifungal activities and acute toxicity studies of the crude extract and compounds from *Diospyros canaliculata*." *Natural Product Research* **25**(7): 741-749.

Dzoyem, J., J. Tangmouo, D. Lontsi, F. Etoa and P. Lohoue (2007). "*In vitro* antifungal activity of extract and plumbagin from the stem bark of *Diospyros crassiflora* Hiern (*Ebenaceae*)." *Phytotherapy Research* **21**(7): 671-674.

Evans, P. H., W. S. Bowers, M. Litaudon and T. Sevenet (1999). "Plumbagin from *Diospyros olen*." *Molecules* **4**(4): M93.

Flota-Burgos, G., J. Rosado-Aguilar, R. Rodríguez-Vivas and K. Arjona-Cambranes (2017). "Anthelminthic activity of methanol extracts of *Diospyros anisandra* and *Petiveria alliacea* on cyathostomin (*Nematoda: Cyathostominae*) larval development and egg hatching." *Veterinary Parasitology* **248**: 74-79.

Fotie, J., D. S. Bohle, M. L. Leimanis, E. Georges, G. Rukunga and A. E. Nkengfack (2006). "Lupeol long-chain fatty acid esters with antimalarial activity from *Holarrhena floribunda*." *Journal of Natural Products* **69**(1): 62-67.

Gallo, M., B. Miranda and J. Sarachine (2009). "Biological activities of lupeol." *International Journal of Biomedical and Pharmaceutical Sciences* **3**(1): 46-66.

Gao, W., Q. Li, J. Chen, Z. Wang and C. Hua (2013). "Total synthesis of six 3,4-unsubstituted coumarins." *Molecules* **18**(12): 15613-15623.

Gu, J.-Q., T. N. Graf, D. Lee, H.-B. Chai, Q. Mi, L. B. S. Kardono, F. M. Setyowati, R. Ismail, S. Riswan, N. R. Farnsworth, G. A. Cordell, J. M. Pezzuto, S. M. Swanson, D. J. Kroll, J. O. Falkinham, M. E. Wall, M. C. Wani, A. D. Kinghorn and N. H. Oberlies (2004). "Cytotoxic and antimicrobial constituents of the bark of *Diospyros maritima* collected in two geographical locations in Indonesia." *Journal of Natural Products* **67**(7): 1156-1161.

Harun Al Rashid, M., S. Majumder, V. Mandal, S. C. Mandal and R. A. Thandavarayan (2018). "In search of suitable extraction technique for large scale commercial production of bioactive fraction for the treatment of diabetes: The case of *Diospyros melanoxylon* Roxb." *Journal of Traditional and Complementary Medicine* **9**(6): 106-118.

Herath, W. W., N. D. Rajasekera, M. U. S. Sultanbawa, G. P. Wannigama and S. Balasubramaniam (1978). "Triterpenoid, coumarin and quinone constituents of eleven *Diospyros* species (*Ebenaceae*)." *Phytochemistry* **17**(5): 1007-1009.

Hui, W. and M. Fung (1969). "An examination of the euphorbiaceae of Hong Kong. Part VI. Isolation and structure of glochidonol, a new triterpene ketol from *Glochidion wrightii* Benth." *Journal of the Chemical Society C: Organic* **13**: 1710-1712.

Jeyachandran, R., A. Mahesh, L. Cindrella, S. Sudhakar and K. Pazhanichamy (2009). "Antibacterial activity of plumbagin and root extracts of *Plumbago zeylanica* L. *Acta Biologica Cracoviensia Series Botanica* **51**: 17-22.

Kalita, D., N. Devi and D. Baishya (2016). "Foliar nutraceutical and antioxidant property of *Diospyros lanceifolia* Roxb.(*Ebenaceae*) – an important medicinal plant of Assam, India." *International Advanced Research Journal in Science, Engineering and Technology* **3**(3): 20-22.

Khan, M. and E. Rwekika (1992). "Triterpenoids from the leaves of four species of family *Ebenaceae*." *Fitoterapia* **63**: 375-376.

Kichu, M. (2010). "Documentation and Biological and Phytochemical Analysis of Chungtia Medicinal Plants of Nagaland, India." Dissertation, Chemistry and Biomolecular Sciences, Macquarie University, Sydney (PhD): 1-179.

Kichu, M., T. Malewska, K. Akter, I. Imchen, D. Harrington, J. Kohen, S. R. Vemulpad and J. F. Jamie (2015). "An ethnobotanical study of medicinal plants of Chungtia village, Nagaland, India." *Journal of Ethnopharmacology* **166**: 5-17.

Lajubutu, B., R. Pinney, M. Roberts, H. Odelola and B. Oso (1995). "Antibacterial activity of diosquinone and plumbagin from the root of *Diospyros mespiliformis* (Hostch) (*Ebenaceae*)." *Phytotherapy Research* **9**(5): 346-350.

Lim, M. Y., J. H. Jeon, E. Y. Jeong, C. H. Lee, and H. S. Lee (2007). "Antimicrobial activity of 5-hydroxy-1,4-naphthoquinone isolated from *Caesalpinia sappan* toward intestinal bacteria." *Food Chemistry* **100**: 1254-1258.

Mahapatra, A., S. P. Mativandlela, B. Binneman, P. B. Fourie, C. J. Hamilton, J. J. M. Meyer, F. Van der Kooy, P. Houghton and N. Lall (2007). "Activity of 7-methyljuglone derivatives against *Mycobacterium tuberculosis* and as subversive substrates for mycothiol disulfide reductase." *Bioorganic & Medicinal Chemistry* **15**(24): 7638-7646.

Malewska, T. (2015). "Biological and phytochemical analysis of Chungtia medicinal plants of Nagaland, India." Dissertation, Chemistry and Biomolecular Sciences, Macquarie University, Sydney (MPhil): 1-226.

Manzano, P. I., M. Miranda, J. Abreu-Payrol, M. Silva, O. Sterner and E. L. Peralta (2013). "Pentacyclic triterpenoids with antimicrobial activity from the leaves of *Vernonanthura patens* (*Asteraceae*)." *Emirates Journal of Food and Agriculture*: **25**(7): 539-543.

Martínez-Las Heras, R., A. Pinazo, A. Heredia and A. Andrés (2017). "Evaluation studies of persimmon plant (*Diospyros kaki*) for physiological benefits and bioaccessibility of antioxidants by *in vitro* simulated gastrointestinal digestion." *Food Chemistry* **214**: 478-485.

Mmongoyo, J. A., M. G. Nair, J. E. Linz, F. Wu, J. K. Mugula, A. A. Dissanayake, C. Zhang, D. M. Day, J. M. Wee and G. M. Strasburg (2017). "Bioactive compounds in *Diospyros mafiensis* roots inhibit growth, sporulation and aflatoxin production by *Aspergillus flavus* and *Aspergillus parasiticus*." *World Mycotoxin Journal* **10**(3): 237-248.

Nematollahi, A., N. Aminimoghadamfarouj and C. Wiart (2012). "Reviews on 1,4-naphthoquinones from *Diospyros* L." *Journal of Asian Natural Products Research* **14**(1): 80-88.

Ohigashi, H., S. Minami, H. Fukui, K. Koshimizu, F. Mizutani, A. Sugiura and T. Tomana (1982). "Flavanols, as plant growth inhibitors from roots of peach, (*Prunus persica*) Batsh. cv. 'Hakuto'." *Agricultural and Biological Chemistry* **46**(10): 2555-2561.

Park, J.-H. and H.-S. Lee (2018). "*In vivo* fungicidal properties of *Diospyros kaki*-isolated compound and its analogues." *Applied Biological Chemistry* **61**(4): 383-388.

Puapairoj, P., W. Naengchomnong, A. Kijjoa, M. M. Pinto, M. Pedro, M. S. J. Nascimento, A. M. Silva and W. Herz (2005). "Cytotoxic activity of lupane-type triterpenes from *Glochidion sphaerogynum* and *Glochidion eriocarpum* two of which induce apoptosis." *Planta Medica* **71**(03): 208-213.

Rajasekaran, R. and Y. Gebrekidan (2018). "A review on antibacterial phytochemical constitutions present in *Aerva lanata* and their mode of action against bacterial biofilm." *International Journal of Pharmaceutical & Biological Archives* **9**(1): 16-30.

Rischer, H., A. Hamm and G. Bringmann (2002). "*Nepenthes insignis* uses a C2-portion of the carbon skeleton of l-alanine acquired via its carnivorous organs, to build up the allelochemical plumbagin." *Phytochemistry* **59**(6): 603-609.

Roy, A. and N. Bharadvaja (2018). "Biotechnological approaches for the production of pharmaceutically important compound: plumbagin." *Current Pharmaceutical Biotechnology* **19**(5): 372-381.

Ruphin, F. P., R. Baholy, R. Emmanuel, R. Amelie, M.-T. Martin and N. Koto–te-Nyiwa (2014). "Isolation and structural elucidation of cytotoxic compounds from the root bark of *Diospyros quercina* (Baill.) endemic to Madagascar." *Asian Pacific Journal of Tropical Biomedicine* **4**(3): 169-175.

Salae, A.-W., C. Karalai, C. Ponglimanont, A. Kanjana-Opas and S. Yuenyongsawad (2010). "Naphthalene derivatives from *Diospyros wallichii*." *Canadian Journal of Chemistry* **88**(9): 922-927.

Sholichin, M., K. Yamasaki, R. Kasai and O. Tanaka (1980). "Nuclear magnetic resonance of lupane-type triterpenes, lupeol, betulin and betulinic Acid." *Chemical and Pharmaceutical Bulletin* **28**(3): 1006-1008.

Singh, B. and S. Singh (2003). "Antimicrobial activity of terpenoids from *Trichodesma amplexicaule* Roth." *Phytotherapy Research* **17**(7): 814-816.

Suchaichit, N. P., N. Suchaichit, K. Kanokmedhakul, K. Poopasit, P. Moosophon and S. Kanokmedhakul (2018). "Two new naphthalenones from *Diospyros undulata* stem bark and their cytotoxic activity." *Phytochemistry Letters* **24**: 132-135.

Sumsakul, W., T. Plengsuriyakarn, W. Chaijaroenkul, V. Viyanant, J. Karbwang and K. Na-Bangchang (2014). "Antimalarial activity of plumbagin in vitro and in animal models." BMC *Complementary and Alternative Medicine* **14**(1): 15.

Sushila, R., A. Deepti, R. Permender, T. Madhavi, R. Dharmender and D. Rathee (2010). "Cytotoxic and antibacterial activity of *Basella alba* whole plant: A relatively unexplored plant." *Pharmacologyonline* **3**: 651-658.

Van der Vijver, L. M. (1972). "Distribution of plumbagin in the *Plumbaginaceae*." *Phytochemistry* **11**(11): 3247-3248.

Wenche, G. S. and J. Einar (1995). "Structural determination of 1,4-naphthoquinones by mass spectrometry." *Journal of Mass Spectrometry* **30**: 1126-1132.

Zhang, Y., L. Zhao, S.-W. Huang, W. Wang and S.-J. Song (2018). "Triterpene saponins with neuroprotective effects from the leaves of *Diospyros kaki* Thunb." *Fitoterapia* **129**: 138-144.

Zhong, S.-M., P. G. Waterman and J. A. D. Jeffreys (1984). "Naphthoquinones and triterpenes from african *Diospyros* species." *Phytochemistry* **23**(5): 1067-1072.

Chapter 4 - *Holboellia latifolia*

The aim of this chapter was to evaluate the crude 70% aqueous ethanolic extract as well as partitioned fractions of *Holboellia latifolia* Wall. (Lardizabalaceae) leaves for their antimicrobial properties and to investigate the active extracts for their major active compounds.

4.1 Introduction

Holboellia latifolia also known as *Stauntonia latifolia* (Wall.) is a species belonging to the Lardizabalaceae family. The trunk of the plant can be up to 6 metres high, with the bark vertically fissured when old. Its branches are glabrous and striated, and the leaves are 3-5-foliate. The flowers are monoecious (the male and female flowers are produced on the same plant (Christenhusz 2012). Flowering occurs in Spring and the flowers are greenish-white or purple in colour and grow in clusters. The fruit is an edible berry that is oblong with a rounded base and apex. The fruit contains many seeds that are orbicular and straight, and embedded in the pulp (Wang et al. 2009). The natural occurrence of this species is restricted to the Himalayas and China.

Figure 4.1 *Holboellia latifolia* (photo by Nagaland man Meyanungsang Kichu).

Indigenous communities of the North-Eastern part of India, comprising the states of Arunachal Pradesh, Assam, Manipur, Nagaland, Meghalaya and Mizoram, use the foam and the juice from crushed or ground leaves of *H. latifolia* to treat burns (Begum and Nath 2000, Kichu et al. 2015). The Garhwal community of India apply a paste of the leaves to treat cattle wounds (Juyal and Ghildiyal 2013).

Only one phytochemical study on *H. latifolia* has been previously reported. This study investigated the 95% ethanol extract of the aerial parts of *H. latifolia* and led to the isolation and structural elucidation of lup-20(29)-en-3-one (4.1), lupeol (4.2), β-sitosterol (4.3), oleanolic acid (4.4), ursolic acid (4.5), β-daucosterol (4.6), eleutheroside K (4.7), hederagenin3-*O*-α-L-rhamnopyranosyl-(1→2)-α-L-arabinopyranoside (4.8) and 2-(naphthalen-1-yl)acetic acid (4.9) (Gu et al. 2013) (Figure 4.1).

β-Sitosterol's antimicrobial activity is described in Chapter 2 and lupeol's in Chapter 3. β-Daucosterol was previously reported to be active against *Staphylococcus aureus* and *Escherichia coli* (Chen et al. 2015). Oleanolic acid and ursolic acid have also been widely reported for their antimicrobial properties. Both have been shown to have good activity against *Mycobacterium tuberculosis*, a bacterium that causes the infection responsible for many deaths worldwide (Jiménez-Arellanes et al. 2007). Oleanolic acid and ursolic acid have also been shown to have antibacterial activity against other human pathogens, such as *Streptococcus pneumoniae*, methicillin-sensitive and methicillin-resistant *S. aureus* (Woldemichael et al. 2003), *Bacillus subtilis*, *Bacillus cereus*, *Enterococcus faecalis*, *Enterococcus faecium* and *Pseudomonas aeruginosa* (Horiuchi et al. 2007, Cunha et al. 2010) and *Listeria monocytogenes* (Kurek et al. 2010).

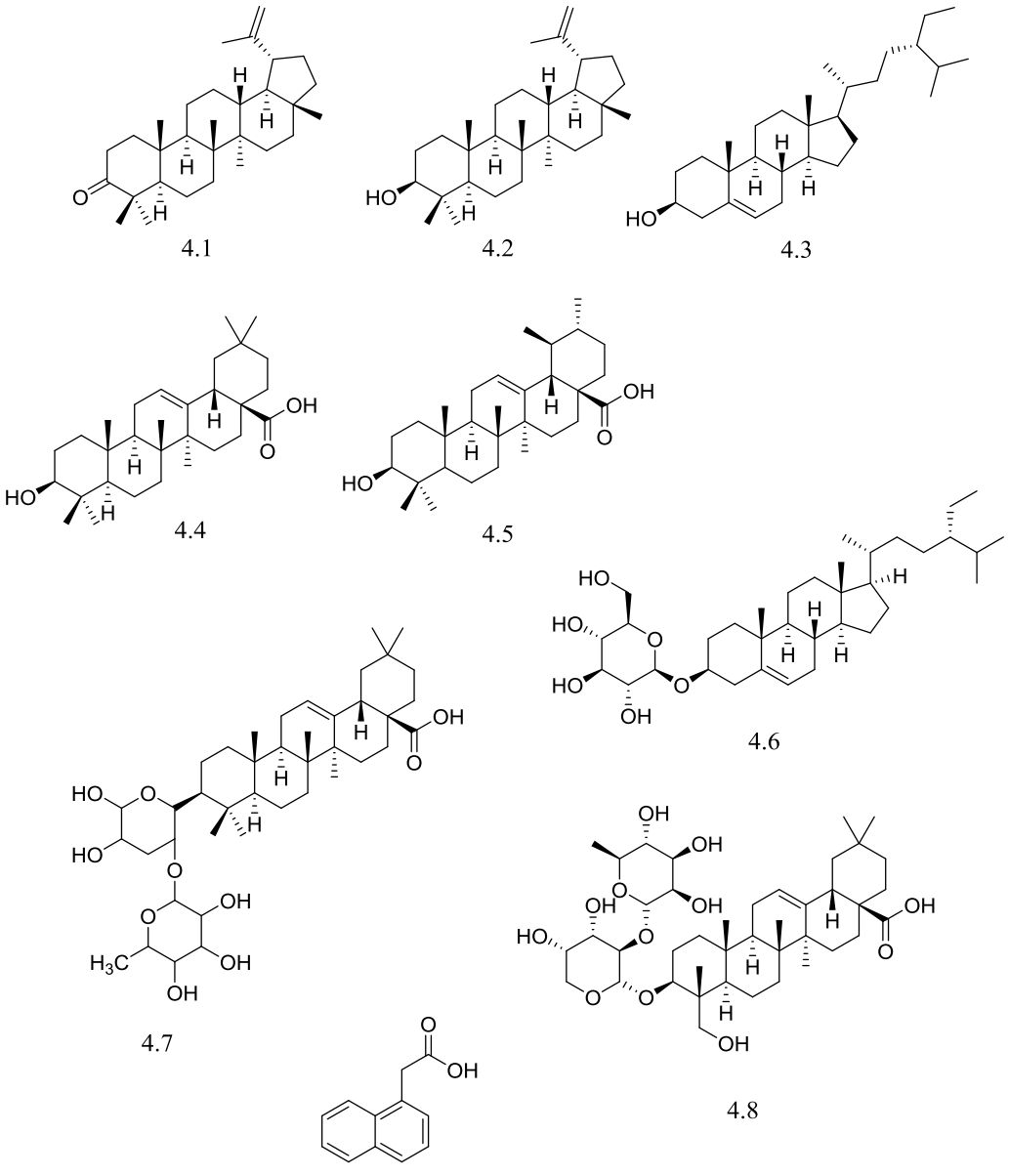


Figure 4.1. Compounds previously isolated from *Holboellia latifolia*.

Reports regarding *H. latifolia* are extremally scarce, however, phytochemistry reports show that triterpenoid saponins are commonly found in the Holboellia genus. Fu et al. reported the isolation of five triterpenoid saponins, fargosides A, B, C, D and E from an ethanolic extract of the roots of *Holboellia fargesii* (Fu et al. 2001). Three noroleanane triterpenoid saponins, akebonoic acid, holboelliside A and holboelliside B, were isolated from the methanolic extract of the stems of *H. coriacea* (Ding et al. 2016), and coriacea saponin A, kalopanax saponin D, patrinia glycosides B-II, leonticin E, guaiacin C and mateglycoside A were isolated from an ethanolic extract of *H. coriacea* roots (Zhu et al. 2015).

4.2 Results and discussion

The leaves of *H. latifolia* were collected and ground and powdered in Nagaland, as described in Chapter six (experimental) (Kichu 2010). The total amount of the material available was only 120 g. The 70% crude ethanolic extract of the powder (yield 8.2 g) was prepared and subsequently partitioned between *n*-hexane (yield 3.5 g), dichloromethane (1.1 g), ethyl acetate (0.5 g) and water (1.5 g).

The partitioned extracts were screened for antimicrobial activity by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) microdilution assay (Akter et al. 2016) against the skin pathogens *S. aureus* (susceptible *S. aureus*), methicillin resistant *S. aureus* (MRSA) and multidrug resistant *S. aureus* (MDRSA), β-lactamase negative (antibiotic susceptible) *E. coli* (β- *E. coli*), β-lactamase positive (antibiotic resistant) *E. coli* (β+ *E. coli*), *P. aeruginosa, S. pyogenes, S. typhimurium* and *C. albicans*, as described in Chapter 2 (Ncube et al. 2008). The *n*-hexane extract showed weak antimicrobial activity against antibiotic sensitive *S. aureus* as well as weak activity against *P. aeruginosa* with an MIC value of 2500 µg/mL. Better activity was exhibited by the dichloromethane extract, which was found to be active against antibiotic sensitive as well as resistant strains of *S. aureus* (MIC 156 µg/mL for antibiotic sensitive *S. aureus* and 1250 µg/mL for the resistant strains) as well as *P. aeruginosa* (MIC 1250 µg/mL). The ethyl acetate and water partitions showed no antibacterial activity.

Due to exhibiting the most promising antibacterial activity, the dichloromethane partition was chosen for bioassay guided isolation. 2D TLC indicated that normal phase silica gel column chromatography would be an appropriate method of separation. Fractionation was therefore attempted with the partition using 1 g of the sample. The column was eluted with *n*-hexane:chloroform (10:90 to 100%, column loaded with a 100 g of normal phase silica) to afford 20 fractions. Upon drying, the yield of the fractions was extremally poor (total weight 0.5 g) and showed little separation of components. Fractions 8 to 14, in order of elution, showed bioactivity by TLC bioautography with antibiotic

sensitive *S. aureus*. The fractions 8 to 14 were pooled together (90 mg) and size exclusion chromatography (Sephadex LH-20, 100% methanol) was attempted. While almost full recovery of material was obtained, size exclusion chromatography did not result in any further separation of compounds.

To obtain further information of the phytochemistry of the dichloromethane partition, GC-MS analysis was conducted by Dr Joseph Brophy (University of New South Wales), using the same conditions as described in Chapter 3). This resulted in the detection of hexanal and nonanal (Figure 4.2).

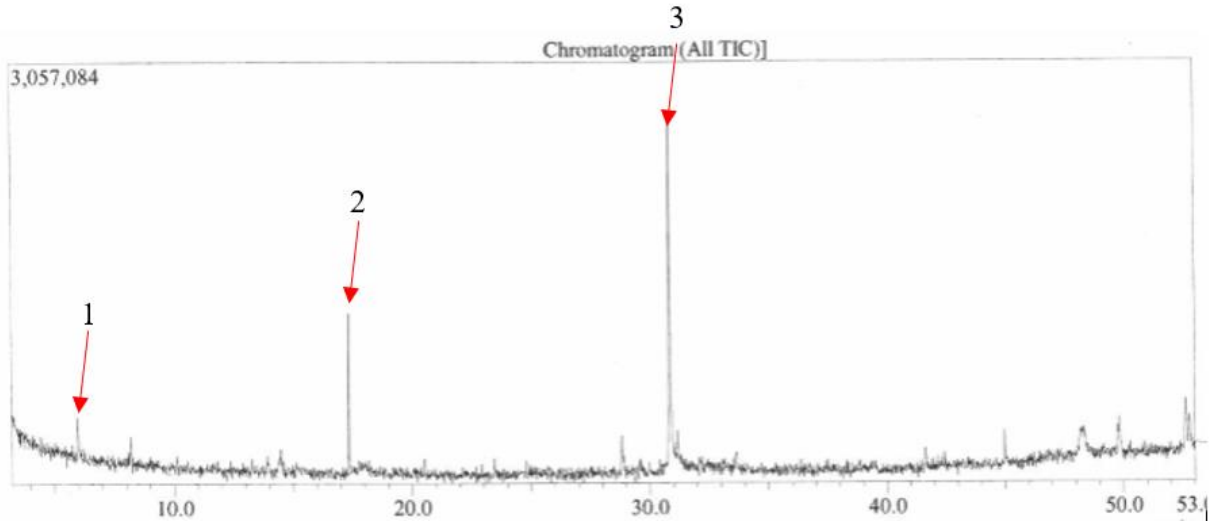


Figure 4.2 GC-MS trace of *Holboellia latifolia* dichloromethane partition. Peak 1: hexanal; Peak 2: nonanal; Peak 3: diethyl phthalate (column contamination).

Both hexanal and nonanal (Figure 4.3) are found in many plant species. Hexanal is a component of volatile oils of citrus species such as *Citrus limon*, *Citrus paradise*, *Coriandrum sativum* and *Caryophyllus aromaticus* (Misharina and Samusenko 2008). Nonanal has been found in *Artemisia ludoviciana* (Zavala-Sanchez et al. 2002), *Solanum aculeastrum* (Koduru et al. 2006), *Medicago sativa* (Buttery and Kamm 1980) and *Prunus domestica* (Ismail et al. 1977). Nonanal has been described as responsible for the antidiarrhoeal properties of *Artemisia ludoviciana* (Zavala-Sanchez et al. 2002).

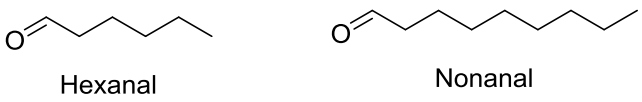


Figure 4.3 The structures of hexanal and nonanal.

Given the limited quantity available, and the poor separation results, the remaining dichloromethane partition was subjected to metabolomics studies, as described in Chapter 5.

4.3 Conclusions

Holboellia latifolia plant parts, including the leaves, have been traditionally used by Indigenous communities of India as a treatment for conditions of a likely microbial origin, but reported biological and phytochemical studies on this species and genus are extremally scarce. The dichloromethane partition obtained from the 70% ethanolic extract of the leaves showed antibacterial activity against antibiotic sensitive (MIC value of 156 µg/mL) *S. aureus*, against resistant strains of *S. aureus* (MIC values for MRSA and MDRSA 1250 µg/mL) and against *P. aeruginosa* (MIC 1250 µg/mL). Normal phase chromatography and size exclusion isolation studies were attempted but were not successful. GC-MS studies of the dichloromethane partition identified hexanal and nonanal to be present. Both volatile compounds are present in many plant species.

4.4 References

Akter, K., E. C. Barnes, W. L. Loa-Kum-Cheung, P. Yin, M. Kichu, J. J. Brophy, R. A. Barrow, I. Imchen, S. R. Vemulpad and J. F. Jamie (2016). "Antimicrobial and antioxidant activity and chemical characterisation of *Erythrina stricta* Roxb. (*Fabaceae*)." *Journal of Ethnopharmacology* **185**: 171-181.

Begum, D. and S. C. Nath (2000). "Ethnobotanical review of medicinal plants used for skin diseases and related problems in Northeastern India." *Journal of Herbs, Spices & Medicinal Plants* **7**(3): 55-93.

Buttery, R. G. and J. A. Kamm (1980). "Volatile components of alfalfa: possible insect host plant attractants." *Journal of Agricultural and Food Chemistry* **28**(5): 978-981.

Chen, W.-H., W.-J. Liu, Y. Wang, X.-P. Song and G.-Y. Chen (2015). "A new naphthoquinone and other antibacterial constituents from the roots of *Xanthium sibiricum*." *Natural Product Research* **29**(8): 739-744.

Christenhusz, M. J. (2012). "*Stauntonia latifolia*." *Curtis's Botanical Magazine* **29**(3): 297-302.

Cunha, W. R., G. X. de Matos, M. G. M. Souza, M. G. Tozatti, M. L. Andrade e Silva, C. H. Martins, R. d. Silva and A. A. Da Silva Filho (2010). "Evaluation of the antibacterial activity of the methylene chloride extract of *Miconia ligustroides*, isolated triterpene acids, and ursolic acid derivatives." *Pharmaceutical Biology* **48**(2): 166-169.

Ding, W., Y. Li, G. Li, H. He, Z. Li and Y. Li (2016). "New 30-noroleanane triterpenoid saponins from *Holboellia coriacea* Diels." *Molecules* **21**(6): 734.

Fu, H., K. Koike, Q. Zheng, K. Mitsunaga, Z. Jia, T. Nikaido, W. Lin, D. Guo and L. Zhang (2001). "Fargosides A-E, triterpenoid saponins from *Holboellia fargesii*." *Chemical and Pharmaceutical Bulletin* ***49***(8): 999-1002.

"Gu, J., G.-y. Li, T. Yang, D.-m. Fang, T.-f. Huang, G.-l. Zhang and X.-z. Chen (2013). "Phytochemical Study on *Holboellia latifolia*." *Natural Product Research and Development*(10): 10."

Horiuchi, K., S. Shiota, T. Hatano, T. Yoshida, T. Kuroda and T. Tsuchiya (2007). "Antimicrobial activity of oleanolic acid from *Salvia officinalis* and related compounds on vancomycin-resistant *Enterococci* (VRE)." *Biological and Pharmaceutical Bulletin* **30**(6): 1147-1149.

Ismail, H. M., G. A. Brown, O. G. Tucknott, P. J. Holloway and A. A. Williams (1977). "Nonanal in epicuticular wax of golden egg plums (*Prunus domestica*)." *Phytochemistry* **16**(6): 769-770.

Jian, G. (2013). "Phytochemical Study on *Holboellia latifolia*." *Natural Product Research and Development* **10**: 1-8.

Jiménez-Arellanes, A., M. Meckes, J. Torres and J. Luna-Herrera (2007). "Antimycobacterial triterpenoids from *Lantana hispida* (*Verbenaceae*)." *Journal of Ethnopharmacology* **111**(2): 202-205.

Juyal, P. and J. C. Ghildiyal (2013). "Indigenous animal health care practices from Garhwal Himalaya." *Journal of Medicinal Plants Studies* **1**(4): 148-151.

Kichu, M. (2010). "Documentation and Biological and Phytochemical Analysis of Chungtia Medicinal Plants of Nagaland, India." Dissertation, Chemistry and Biomolecular Sciences, Macquarie University, Sydney (PhD): 1-179.

Kichu, M., T. Malewska, K. Akter, I. Imchen, D. Harrington, J. Kohen, S. R. Vemulpad and J. F. Jamie (2015). "An ethnobotanical study of medicinal plants of Chungtia village, Nagaland, India." *Journal of Ethnopharmacology* **166**: 5-17.

Koduru, S., O. Asekun, D. Grierson and A. Afolayan (2006). "Isolation of volatile compounds from *Solanum aculeastrum* (*Solanaceae*)." *Journal of Essential Oil Bearing Plants* **9**(1): 65-69.

Kurek, A., A. M. Grudniak, M. Szwed, A. Klicka, L. Samluk, K. I. Wolska, W. Janiszowska and M. Popowska (2010). "Oleanolic acid and ursolic acid affect peptidoglycan metabolism in *Listeria monocytogenes*." *Antonie van Leeuwenhoek* **97**(1): 61.

Misharina, T. and A. Samusenko (2008). "Antioxidant properties of essential oils from lemon, grapefruit, coriander, clove, and their mixtures." *Applied Biochemistry and Microbiology* **44**(4): 438-442.

Ncube, N., A. Afolayan and A. Okoh (2008). "Assessment techniques of antimicrobial properties of natural compounds of plant origin: current methods and future trends." *African Journal of Biotechnology* **7**(12): 1797-1806.

Wang, H.-F., C. R. Friedman, Z.-X. Zhu and H.-N. Qin (2009). "Early reproductive developmental anatomy in *Decaisnea* (*Lardizabalaceae*) and its systematic implications." *Annals of Botany* **104**(6): 1243-1253.

Woldemichael, G. M., S. G. Franzblau, F. Zhang, Y. Wang and B. N. Timmermann (2003). "Inhibitory effect of sterols from *Ruprechtia triflora* and diterpenes from *Calceolaria pinnifolia* on the growth of *Mycobacterium tuberculosis*." *Planta Medica* **69**(07): 628-631.

Zavala-Sanchez, M. A., S. Pérez-Gutiérrez, C. Perez-González, D. Sanchez-Saldivar and L. Arias-García (2002). "Antidiarrhoeal activity of nonanal, an aldehyde isolated from *Artemisia ludoviciana."* *Pharmaceutical Biology* **40**(4): 263-268.

Zhu, Z., T. Chen, H.-F. Pi, H.-L. Ruan, J.-Z. Wu and P. Zhang (2015). "A new triterpenoid saponin and other saponins from the roots of *Holboellia coriacea*." *Chemistry of Natural Compounds* **51**(5): 890-893.

Chapter 5 - Metabolomics studies on *Prunus persica*, *Diospyros lanceifolia* and *Holboellia latifolia*

Chapters two, three and four focused on the bioassay guided isolation of bioactive compounds from *Prunus persica*, *Diospyros lanceifolia* and *Holboellia latifolia*. This proved moderately successful for *P. persica*, had limited success for *D. lanceifolia* and was unsuccessful for *H. latifolia*. As a result, this chapter explores the use of modern techniques through a metabolomics approach to examine the complex mixtures of extracts and partitions of these three plants.

5.1 Introduction

Metabolomics has emerged as a valuable tool for the comprehensive profiling and comparison of metabolites in biological systems (De Vos et al. 2007). Recent advances in mass spectrometric instrumentation combined with powerful chemometric tools has greatly extended the capacity for rapid compound screening of natural products (Hall 2018). Several methods based on liquid chromatography (LC) coupled with a UV-Vis diode-array detector (DAD) and mass spectrometry (MS) have been applied to identify phytochemicals within complex plant mixtures (Garcia et al. 2016). More comprehensive methods of metabolome characterisation have been recently developed applying methodologies such as nuclear magnetic resonance (NMR) (Markley et al. 2017) and quadrupole time-of-flight mass spectrometry (QTOF-MS) (Garcia et al. 2016).

As described in the earlier chapters, the crude 70% aqueous ethanolic extracts as well as *n*-hexane, dichloromethane and ethyl acetate partitions of *P. persica* roots, *n*-hexane partition of *D. lanceifolia* leaves, and dichloromethane partition of *H. latifolia* leaves, collected from Nagaland, exhibited promising antibacterial activity. However, attempts to isolate compounds from the partitions had limited success. To understand more about the phytochemistry of these medicinally important plants, a metabolomics approach was adapted for the extracts and partitions. To the best of the author's knowledge, metabolomic studies on *P. persica* root extract as well as *D. lanceifolia* and *H. latifolia* leaf extracts have not been reported so far.

Even though NMR is in principle the most reliable spectroscopic technique for the unequivocal identification of unknown compounds, NMR is less sensitive than MS, and MS-based platforms are the most widely applied in plant metabolomics (Hall 2006, Garcia et al. 2016). Gas chromatography coupled to electron impact time-of-flight (TOF) MS was the first approach used in large-scale plant metabolomics and is a powerful approach for the detection of volatile compounds and their identification when coupled to databases such as the NIST database (De Vos et al. 2007). LC-MS-based techniques, however, are of particular importance due to the greater ease of LC for separating

and detecting semi-polar and polar secondary metabolites and the capability of coupling LC with soft ionisation MS techniques such as electrospray ionisation or atmospheric pressure chemical ionisation for molecular mass determination (De Vos et al. 2007, Dunn et al. 2013). It has been observed that LC with UV–Vis detection, in combination with high resolution MS (*e.g*. TOF-MS) or MS/MS, enables the tentative characterisation of several hundreds of compounds in a single crude plant extract (De Vos et al. 2007). With continually improving tools for data acquisition, processing and mining, LC-MS will certainly grow in value for biochemical profiling and metabolite identification. Making use of improved separation technologies such as ultra-high-performance LC (UHPLC) coupled to MS will further improve the potential to identify metabolites and to provide an even more detailed metabolite profile of plant extracts (Garcia et al. 2016).

5.2 Results and discussion

5.2.1 Metabolomic profiling and extraction of molecular features

The crude 70% aqueous ethanolic extracts of *P. persica* roots, leaves of *H. latifolia* and *D. lanceifolia*, as well as the dichloromethane and ethyl acetate partitions of *P. persica*, dichloromethane partition of *H. latifolia* and *n*-hexane partition of *D. lanceifolia***,** were chosen to be analysed by ultra-high-performance liquid chromatography-quadrupole time-of-flight mass spectroscopy (UHPLC-DAD-Q-ToF-MS) with a UV-Vis diode array detector. As there was no specific group of target analytes, instrument parameters were set in order to separate and tentatively identify as many compounds as possible (Garcia et al. 2016). All compounds were identified by comparison of molecular ions, retention times and UV-Vis spectra with those published in the literature. Accurate ion mass of each peak was obtained from the total ion chromatograms (TICs). TIC data analysis was carried out using extracted ion chromatograms (EIC) of the deprotonated and protonated molecular ions when available. When the retention times of the peaks in the positive and negative mode EICs align, this is good evidence that the ions observed are indeed the molecular ions. In some cases, peaks were only seen in one mode, making identification of the molecular ion less reliable. Once the molecular ions are identified, accurate mass values can be used to generate likely molecular formulae.

UV-Vis chromatograms were examined for each LC peak for diagnostic wavelengths between 250-290 and 310-350 nm for flavones, 250-290 and 350-385 nm for flavonols, 277-295 and 300-330 nm for flavanones and dihydroflavonols (two absorbance bands each), 275-325 nm for cinnamic acid derivatives, 540 nm for anthocyanins and 210 nm for compounds lacking the phenolic moiety (Tsimogiannis et al. 2007). Where diagnostic UV-Vis absorbances were observed, the retention time of the molecular ion peak (from EICs) was checked so that it matched the retention time corresponding to the UV-Vis peak.

The exact theoretical masses were based on the formula calculated by the molecular weight calculator tool of Mass Hunter software. Molecular formulae were generated from the accurate masses and isotopic patterns using an algorithm that combined C (0-60), H (0-120), O (0-30) and N (0-20). Other elements such as Cl or Br or S were not considered since they are rarely present in plants, thereby reducing the number of possibilities. The generated molecular formulae (GMF) were compared to those registered in commonly used online databases including ChemSpider, KEGG, CheEBI, LIPID MAPS, Google Scholar and ScienceDirect (Garcia et al. 2016). Tables 5.1-5.7 list masses of molecular ions (*m/z*), the retention times (RT), generated molecular formulae (GMF), measured and calculated monoisotopic masses and their mass differences (diff) in parts per million (ppm). A variation in ppm across the product ion masses should ideally differ no more than 2 ppm, although 7 ppm is still acceptable due to the instrument peak spacing tolerance recommended setting of 7 ppm for the detection of "common organic molecules" (Hopfgartner et al. 1999, Brenton and Godfrey 2010, Garcia et al. 2016). The parameters obtained were used to create lists of possible compounds for *P. persica*, *D. lanceifolia* and *H. latifolia*. These lists were then compared with the published literature on the phytochemistry of the species and genus to shortlist likely candidate compounds (Schwikkard and Mulholland 2014). Figure 5.1 provides a summary of this "identification" process.

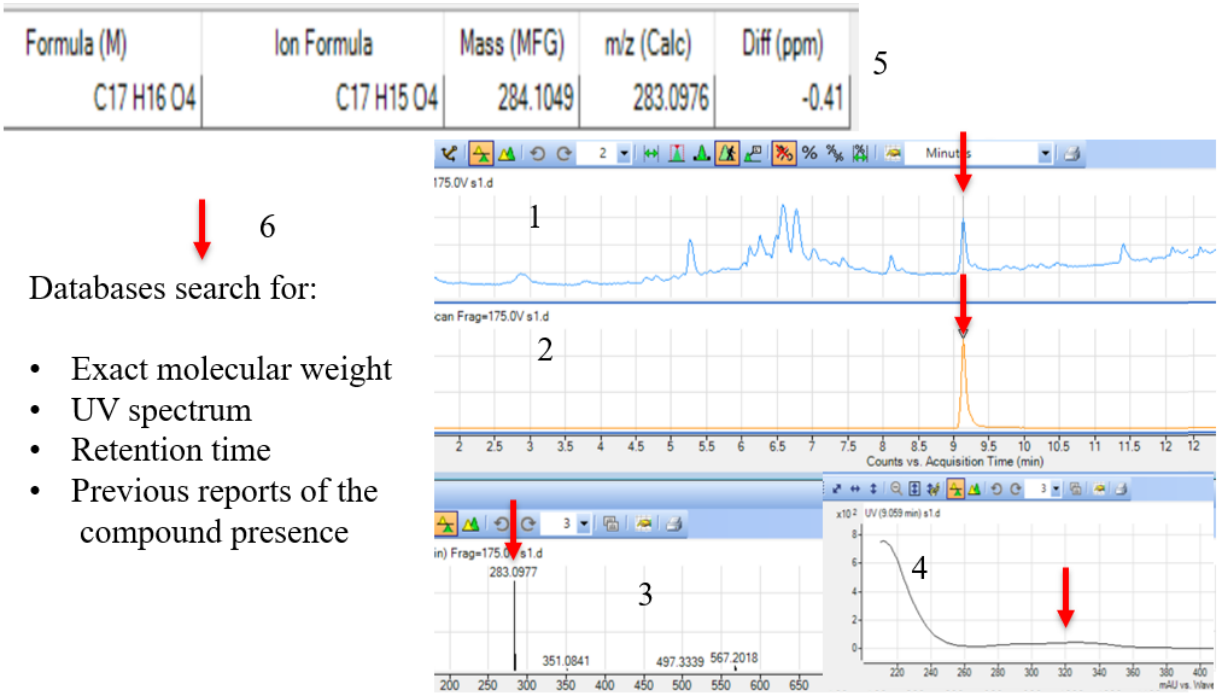


Figure 5.1. Summary of the tentative identification process. Accurate mass of each peak obtained from the total ion chromatograms (TICs, caption 1) was analysed using extracted ion chromatograms (EIC, caption 2). UV-Vis spectra (caption 4) at diagnostic wavelengths were generated for the selected mass peak (caption 3). The mass of a peak at its most intense was used to generate a molecular formula [Formula (M), caption 5] and calculate the exact mass of that formula [Mass (MFG)]. The UV-Vis spectrum, formula (M) and exact mass (MFG) were compared with the literature (caption 6).

In the absence of standards for most compounds (with the exception of gallic acid and catechin), the term "identification" for all investigated plants refers to "tentative identification" (Hanafy et al. 2017). Compounds where the molecular mass, GMF and UV-Vis absorbance did not correspond to those found in the literature, or the compounds for which UV-Vis spectra were not detected, are referred to as "unknown".

5.2.2 Tentative "identification" - challenges

P. persica, *D. lanceifolia* and *H. latifolia* crude ethanolic extracts as well as antibacterially active partitions were subjected to analysis by UHPLC-DAD-Q-ToF-MS, using electrospray ionisation (ESI) for the MS. This combination allows the detection of thermally labile, non-volatile and polar compounds (Friedrich et al. 2000), covering many important classes of secondary metabolites such as alkaloids, saponins, phenolic acids, phenylpropanoids, flavonoids and polyamines (De Vos et al. 2007). While the soft ionisation of ESI allows for calculation of the molecular masses, and in turn, possible molecular formulae, it does not allow definitive identification of compounds, and is hampered especially by the possibility of isomers (Dunn et al. 2013, Gu et al. 2013). While UV-Vis analysis can assist in identifying particular classes of compounds, this method is often not distinguishing enough for the identification of certain subgroups of compounds (Tsimogiannis et al. 2007).

5.2.3 Tentative characterisation of *P. persica* roots compounds

Phytochemistry of various parts of *P. persica* including leaves, fruits and flowers has been intensely studied and well reported. However, only one publication on the chemical composition of the roots (the focus of this study) has been found. As mentioned in Chapter 2, the publication reported on the isolation of afzelechin and five proanthocyanidin stereoisomers (Ohigashi et al. 1982).

Sixteen compounds were tentatively identified from the LC-MS analysis of the *P. persica* crude aqueous ethanolic root extract (Table 5.1, Figure 5.2), eight from the dichloromethane partition (Table 5.2, Figure 5.3) and eleven from the ethyl acetate partition (Table 5.3, Figure 5.4). Consistent with the reports concerning *P. persica* leaves, fruits, flowers and bark phytochemical composition, many compounds were found to be of phenolic or polyphenolic character.

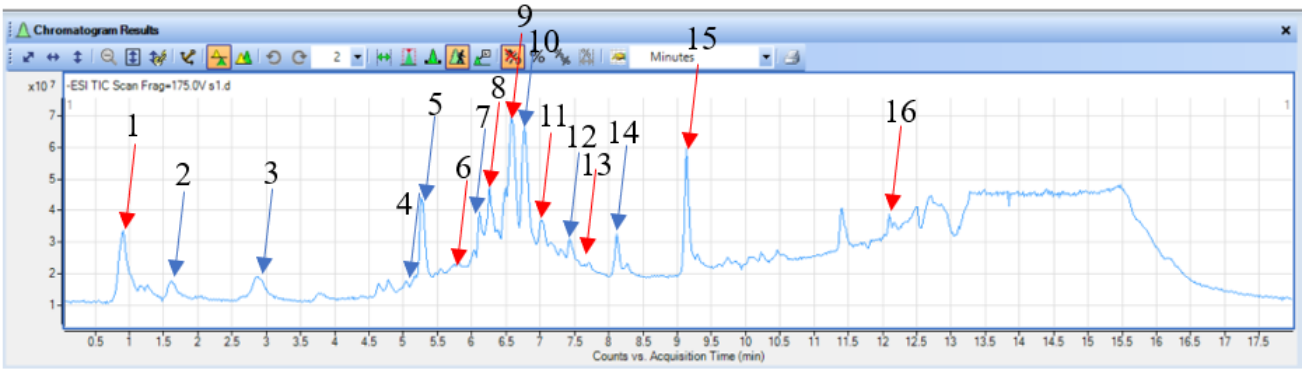


Figure 5.2. ESI (negative mode) TIC chromatogram of *P. persica* crude aqueous ethanolic root extract. Red arrows indicate compounds that had no corresponding peaks in ESI positive mode.

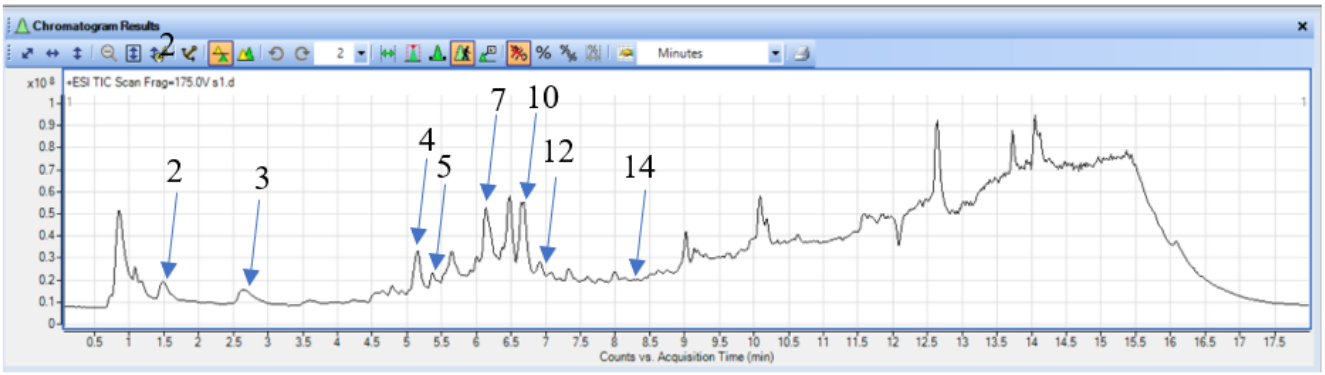
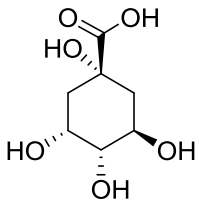


Figure 5.3. ESI (positive mode) TIC chromatogram of *P. persica* crude ethanolic extract.

In the crude aqueous ethanolic extract, in order of retention time (RT), based on their mass, GMF and UV-Vis profile, compounds were tentatively identified as follows (Table 5.1):

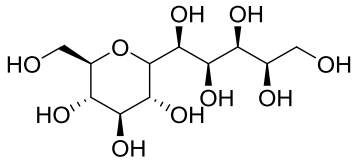
Peak number 1 most closely matched quinic acid (mass 192.0634, $C_7H_{12}O_6$, λ_{max} 230, 262 nm, 5.1) (Liu et al. 2006, Anouar et al. 2012). The UV-Vis spectrum was consistent with some reports for quinic acid (Lai et al. 2007), but UV-Vis spectral data differed significantly across the literature (López-Cobo et al. 2016). This compound was previously isolated from *P. persica* fruit (Dirlewanger et al. 1999, Etienne et al. 2002, Quilot et al. 2004, Montevecchi et al. 2012).



Quinic acid

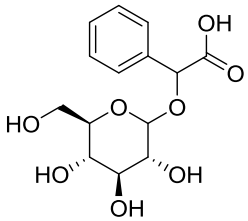
Only one report was found matching the mass of peak number 2 (mass 314.1135, $C_{11}H_{22}O_{10}$, 5.2). This was identified from published literature as the C-glycoside diospyrodin, which was previously isolated from *Diospyros nigra* (Dinda et al. 2006). However, as the compound was not previously

found in the *Prunus* genus, and the mass difference to that obtained experimentally was -8.96 ppm, which set it beyond the "acceptable" level, the peak was referred to as unknown.



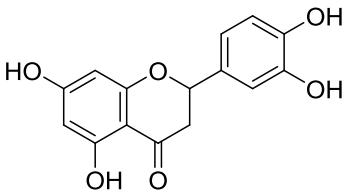
Diospyrodin

Peak number 3 was identified as mandelic acid β-D-glucopyranoside (mass 314.1002, $C_{14}H_{18}O_8$, λ_{max} 280 nm, 5.3), The exact mass, molecular formula and UV-Vis spectrum were in agreement with the literature (Geibel and Feucht 1991, Songsong et al. 2015). This compound has been previously isolated from *P. persica* seeds (Fukuda et al. 2003).



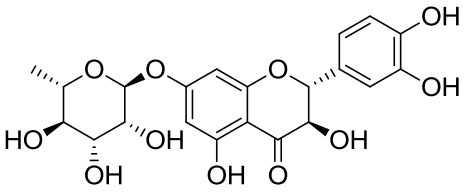
Mandelic acid β-D-glucopyranoside

Peaks number 4 and 11 (RT 5.146 and 7.034 minutes, respectively) had the same GMF, mass and UV-Vis absorption profiles (mass 288.0634, $C_{15}H_{12}O_6$, λ_{max} 289 and 324 nm), indicating they were isomers of each other. The data were in agreement with the literature for eriodictyol (5.4) (Anouar et al. 2012, Gaggeri et al. 2012). This compound has been previously found in *P. persica* bark (Rahman and Bhatnagar 1968), sapwood (Pacheco 1960), stem bark (Jang et al. 2018) and whole plant (Jiménez-Atiénzar et al. 2006, Jang et al. 2018). Attempts to elucidate which of peak 4 and 11 were likely eriodictyol from reported elution times were unsuccessful due to significant variation in reported data (Garcia et al. 2016).



Eriodictyol

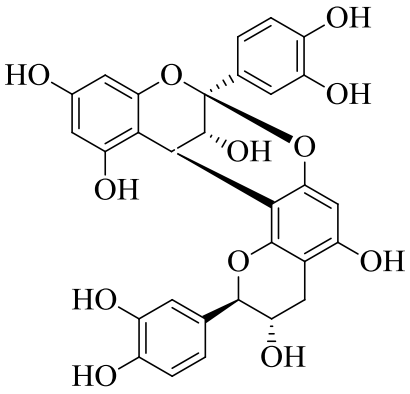
Peak number 5 was characterised as taxifolin-7-*O*-rhamnoside (mass 450.1162, $C_{21}H_{22}O_{11}$, λ_{max} 280 and 365 nm, 5.5), with all data in agreement with the literature (Tsimogiannis et al. 2007). This compound has not been previously found in *P. persica.* However, taxifolin has been isolated from other *Prunus* species such as *P. aequinoctialis, P. nipponica, P. maximowiczii* and *P. avium* (Hasegawa 1957). Of course, other regeoisomers are possible.



Taxifolin-7-*O*-rhamnoside

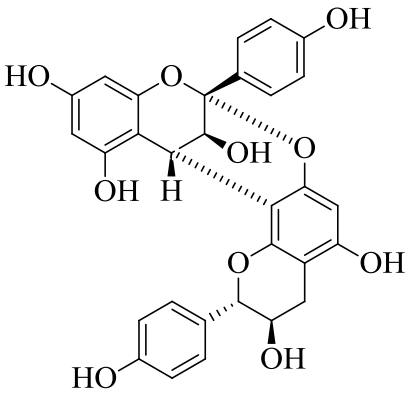
Peak number 6 (mass 480.1268, $C_{22}H_{24}O_{12}$, λ_{max} 280 nm, 5.6) was consistent with 3′-*O*-methylcatechin-5-*O*-glucuronide (Liang et al. 2014) using the database Pubchem. Moreover, the reported UV-Vis absorptions for this compound (Wu et al. 2009, Liang et al. 2014) were consistent with the one obtained experimentally. However, no reports on the isolation of 3′-*O*-methylcatechin-5-*O*-glucuronide from plant sources have been found. Due to insufficient data, peak number 6 was regarded as an unknown.

Peak number 8 ($C_{30}H_{24}O_{12}$, 576.1268, λ_{max} 280 nm, 5.8) (Friedrich et al. 2000), was identified as either proanthocyanidin A1 or A2. The molecular weight, formula and UV-Vis absorptions were consistent with those published for A1 type and A2 type proanthocyanidins (Friedrich et al. 2000), neither of which can be distinguished by this method. Both of these compounds were previously isolated from *P. persica* roots (Ohigashi et al. 1982) and fruits (Moing et al. 2003).



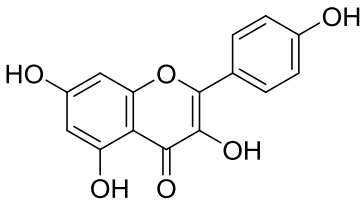
Proanthocyanidin A1

Peak number 10 was consistent with another proanthocyanidin *ent*-epiafzelechin-(2α→O→7',4α→8')-(-)-*ent*-afzelechin (mass 544.1290, $C_{30}H_{24}O_{10}$, λ_{max} 273 nm, 5.10) (Ferreira and Li 2000), another proanthocyanidin. It was isolated from *P. persica* roots by the author through the course of previous studies (Malewska 2015) and by Kichu (Kichu 2010) from the same plant material and has been previously reported from roots of *P. armeniaca* (Ferreira and Li 2000).

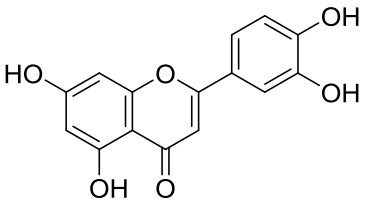


ent-Epiafzelechin-(2α→O→7',4α→8')-(-)-*ent*-afzelechin

The mass and molecular formula for peak number 14 (mass 286.0477, $C_{15}H_{10}O_6$, λ_{max} 265 and 365 nm, 5.14) were consistent with kaempferol (flavonol) or luteolin (flavone), however, luteolin (flavone) absorbs at around 260 and 350 nm (Gardana et al. 2007) while kaempferol (flavonol) absorbs around 265 and 365 nm (Gardana et al. 2007). The peak was therefore tentatively identified as kaempferol. The compound has been previously isolated from *P. persica* fruit (Lee Chang et al. 2007), leaves (Kazan et al. 2014) and pericarp (Lee et al. 2008). Luteolin has been isolated from *P. cerasus* bark (Geibel and Feucht 1991) and fruit (Terry 2011).

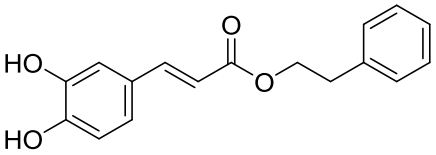


Kaempferol



Luteolin

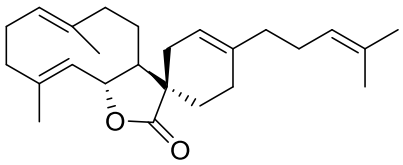
Peak number 15 was identified as caffeic acid phenylethyl ester (mass 284.1049, $C_{17}H_{16}O_4$, λ_{max} 325 nm, 5.15). The mass, GMF and UV-Vis spectra were in agreement with the literature (Gardana et al. 2007). This compound has been previously isolated from *P. jamasakura* bark (Matsuoka et al. 2011) and was isolated by the author (as described in Chapter 2) from *P. persica* for the first time.



Caffeic acid phenylethyl ester

Peak number 16 was identified as the sesterterpene γ-lactone genepolide (mass 368.2715, $C_{25}H_{36}O_2$, 5.16). The exact molecular weight was consistent with genepolide. However, this compound has not been previously reported in the *Prunus* genus. It has been isolated from *Artemisia umbelliformis*

(whole plant) (Appendino et al. 2009). This is the first report of this compound potentially being present in the *Prunus* genus.



Genepolide

For the peaks 7, 9, 12 and 13, no corresponding compounds were found in the literature. These peaks have the potential to represent new chemical entities.

In the dichloromethane partition, in order of retention time, compounds were tentatively identified as follows (Table 5.2, Figures 5.4 and 5.5):

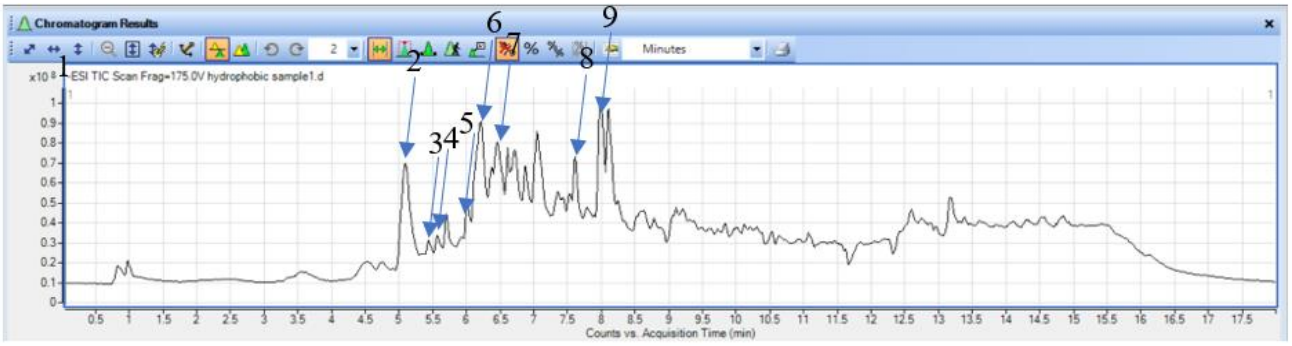


Figure 5.4. EIC (negative mode) TIC of *P. persica* dichloromethane extract.

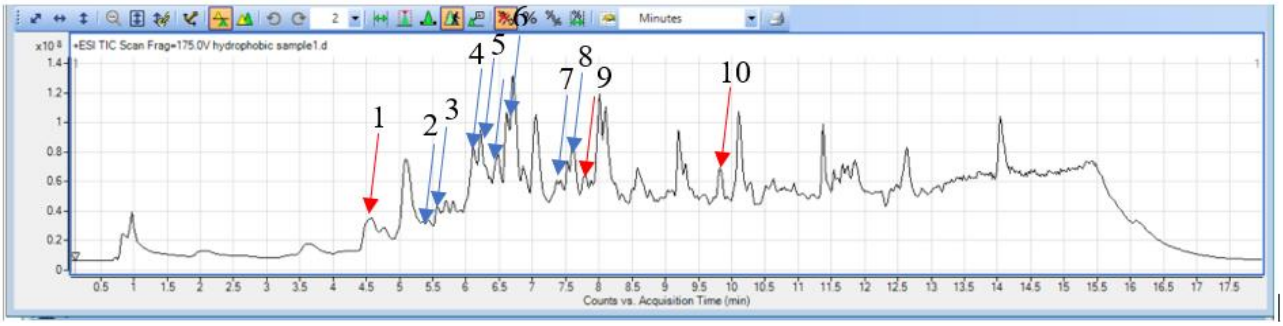
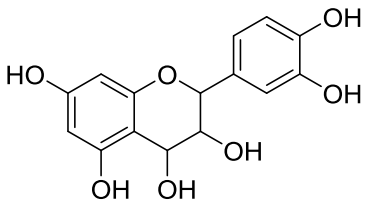


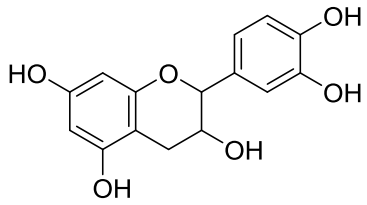
Figure 5.5. EIC (positive mode) TIC of *P. persica* dichloromethane extract. Red arrows indicate compounds present in positive mode only.

Peak number 1 was identified as leucocyanidin (mass 306.074, $C_{15}H_{14}O_7$, λ_{max} 280 nm, 5.17), with all data in agreement with the literature (Yang et al. 2012). This compound was previously isolated from *P. persica* fruit, stems and roots (Ye 2017). This peak was only detected in positive mode.



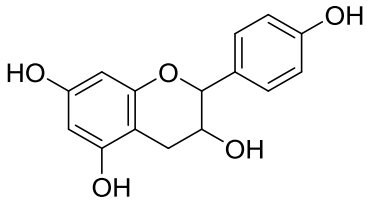
Leucocyanidin

Obtained data for peak number 2 (mass 290.079, $C_{15}H_{14}O_6$, λ_{max} 285 nm, 5.18) were consistent with the literature (Zu et al. 2006) in being either catechin (Sudjaroen et al. 2012, Jang et al. 2018) or its diastereoisomer epicatechin (Sudjaroen et al. 2012). Both of those compounds have also been previously found in *P. persica* pulp (Jang et al. 2018). The presence of catechin was confirmed using a co-injected standard. Catechin and epicatechin often both occur together in plants, with catechin dominating. The ratio varies depending on various influencing factors including plant variety and climate. The EICs showed only one peak with an *m/z* 291 in positive mode and *m/z* 289 negative mode, suggesting epicatechin levels were below the detection limits.



Catechin

Peak number 3 was identified as afzelechin (mass 274.0841, λ_{max} 260 nm, $C_{15}H_{14}O_5$, 5.19), with data being consistent with the literature (Ohigashi et al. 1982). This compound was previously isolated from *P. persica* roots (Kichu 2010) and in the MPhil studies conducted by the author (Malewska 2015).



Afzelechin

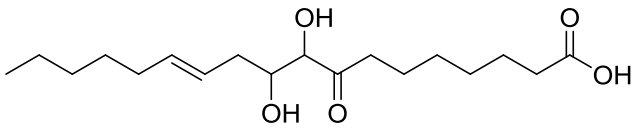
Peak number 4 was tentatively identified as either proanthocyanidin A1 or A2, both likely candidates indistinguishable by the method used [mass 576.1268, $C_{30}H_{24}O_{12}$, λ_{max} 280 nm, 5.20 (5.8, Section 5.2.8)]. The potential presence of these compounds was also seen in the crude aqueous ethanolic extract.

Peak number 5 was consistent with 4′-methoxy-7-*O*-β-D-glucopyranosyl-8,3′-dihydroxyflavanone (mass 464.1319, $C_{22}H_{24}O_{11}$, λ_{max} 230 and 295 nm, 5.21) through data being in agreement with the

literature (Silva et al. 2013). The (-) enantiomeric form has been previously isolated from *Bidens gardneri* (Silva et al. 2013). Since the compound has not been previously identified from *Prunus* genus, the peak is referred to as unknown.

Peak number 6 and peak number 7 (mass 560.1319, $C_{30}H_{24}O_{11}$, λ_{max} 230 and 275 nm, 5.22 and 5.23) were consistent with *ent*-epiafzelechin-(2α→7,4α→8)-epicatechin, *ent*-epiafzelechin-(2α→7,4α→8)-catechin and 3'''-hydroxyafzelechin-(2β→7,4β→8)-catechin, which have been reported in various *Prunus* species, and could not be distinguished (Buckingham 2015).

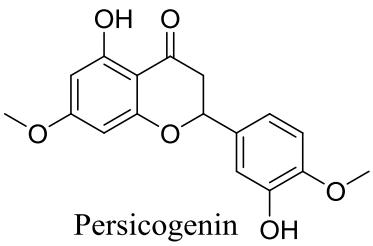
Peak number 8 could only be characterised from its exact molecular weight and GMF using the database Pubchem, as the peak did not show any UV-Vis absorption spectra. The peak was possibly identified as 9,10-dihydroxy-8-oxo-12-octadecenoic acid (mass 328.225, $C_{18}H_{34}O_5$, 5.24), which has been found in *Musa acuminata* leaves (Sonibare et al. 2018). However, there are many possible isomers, and due to the lack of further confirmation, the peak is referred to as unknown.



9,10-Dihydroxy-8-oxo-12-octadecenoic acid

Peak number 9 (mass 330.2406, $C_{18}H_{34}O_5$, 5.25) was consistent with trihydroxy-12*Z*-octadecenoic acid, as identified from the exact mass and GMF and the database Pubchem. The stereoisomer in the 9*S*,10*S*,11*R*-form has been isolated from the leaves of *Lactuca sativa* (Garcia et al. 2016) and *Tasmannia lanceolata* (Paran et al. 2016). Lack of further characterisation led to the compound being named unknown.

Peak number 10 was identified as persicogenin (mass 316.0947, $C_{17}H_{16}O_6$, λ_{max} 286 nm, 5.26), with data being consistent with the literature (Li et al. 2005). This was previously isolated from *P. persica* bark (Rahman and Bhatnagar 1968) and stem (Jang et al. 2018).



Persicogenin

Peak number 11 was characterised as glutinolic acid (mass 519.3322, $C_{30}H_{48}O_7$, 5.27) using the database Pubchem. This compound has been previously isolated from *Rehmannia glutinosa* roots (Lee et al. 2011). However, the compound did not show a UV-Vis absorption at 210 nm, which is typical for ursane type terpenoids (Luo et al. 2017) and was therefore regarded as an unknown.

In the ethyl acetate partition, in order of retention time, compounds were identified as follows (Table 5.3, Figures 5.6 and 5.7):

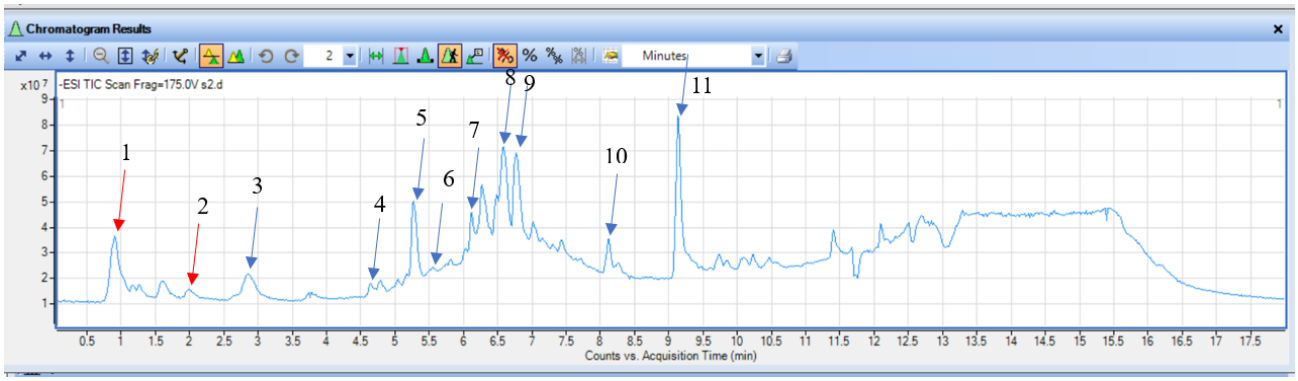


Figure. 5.6. EIC (negative mode) TIC of *P. persica* ethyl acetate extract. Red arrows indicate compounds present in negative mode only.

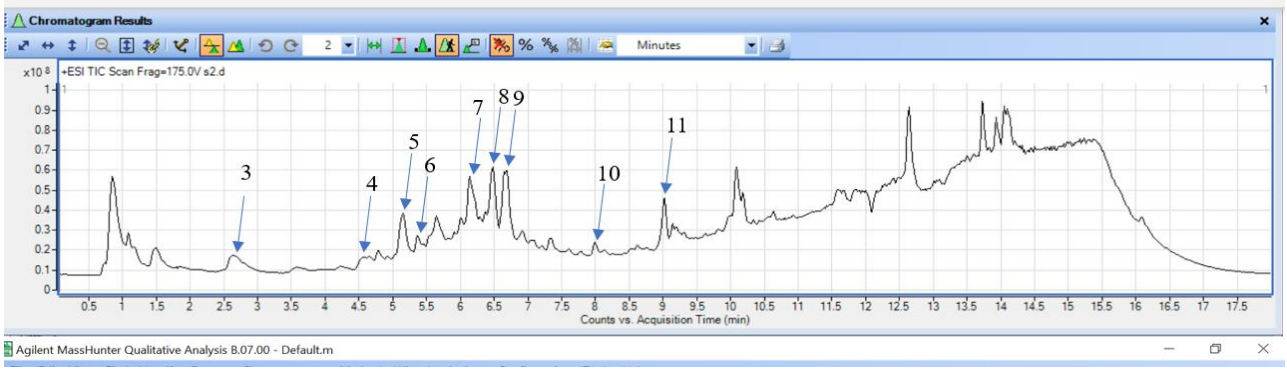


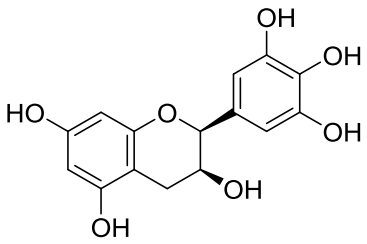
Figure 5.7. EIC (positive mode) TIC of *P. persica* ethyl acetate extract.

Peak number 1 was identified as quinic acid [mass 192.0634, $C_7H_{12}O_6$, λ_{max} 325 nm, 5.28 (5.1)], which was already tentatively identified in the crude aqueous ethanolic extract.

Peak number 2 was identified as gallic acid [mass 170.0215, λ_{max} 270 nm, $C_7H_6O_5$, 5.29 (2.4)], which was found in the isolation study (Chapter 2, Section 5), as well as having been previously reported from *P. persica* fruit (Cantín et al. 2009). The presence of gallic acid was confirmed using an authentic standard.

Peak number 3 was identified as mandelic acid β-D-glucopyranoside [mass 314.1002, $C_{14}H_{18}O_8$, λ_{max} 275 nm, 5.30 (5.3)] (Songsong et al. 2015), which was already reported from the crude aqueous ethanolic extract as well as previously isolated from *P. persica* seeds (Fukuda et al. 2003) and *P. jamasakura* bark (Matsuoka et al. 2011).

Peak number 4 was characterised as gallocatechin (mass 306.074, $C_{15}H_{14}O_7$, λ_{max} 280 nm, 5.31), with data being in agreement with the literature (Chandra Sekhar et al. 2017). This was previously found in *P. persica* seeds (Fukuda et al. 2003).



Gallocatechin

Peak number 5 was tentatively identified as catechin [mass 290.079, $C_{15}H_{14}O_7$, λ_{max} 285 nm, 5.32 (5.18)]. The presence of catechin was also tentatively identified in the dichloromethane partition, as well as consistent with the retention time with the co-running of a standard of catechin. Catechin has been previously isolated from *P. persica* stem bark (Cantín et al. 2009, Jang et al. 2018).

Peak number 7 was tentatively identified as either proanthocyanidin A1 or A2 and peak number 8 as one of *ent*-epiafzelechin-(2α→7,4α→8)-epicatechin, *ent*-epiafzelechin-(2α→7,4α→8)-catechin or 3’’’-hydroxyafzelechin-(2β→7,4β→8)-catechin, similar to that seen for the crude aqueous ethanolic extract as well as the dichloromethane partition.

Peak numbers 9, 10 and 11 were identified as *ent*-epiafzelechin-(2α→*O*→7,4α→8)-(-)-*ent*-afzelechin, afzelechin and caffeic acid phenylethyl ester, respectively. The first two compounds have been previously shown to be present in the crude aqueous ethanolic extract as well as dichloromethane partition, while caffeic acid phenylethyl ester was isolated in this study (Chapter 2, Section 5) and seen in the crude ethanolic extract. Only peak number six (5.33) was not found in any database or literature reports.

Table 5.1. Compounds tentatively identified from the crude ethanolic extract of *P. persica*.

Peak	RT	λ_{max} (nm)	ESI^- $[M-H]^-$	ESI^+ $[M+Na]^{+\dagger}$ $[M+H]^+$	Mass (MFG)	Molecular formula	*m/z* (calc) for $[M-H]^-$	Diff (ppm)	Tentative identity
1	0.907	230 262	191.0567		192.0634	$C_7H_{12}O_6$	191.0561	-3.06	5.1: Quinic acid; found in *P. persica* fruits (Dirlewanger et al. 1999, Etienne et al. 2002, Quilot et al. 2004, Montevecchi et al. 2012)
2	1.606	260 365	313.1090	337.1090†	314.1135	$C_{11}H_{22}O_{10}$	312.1062	-8.96	5.2: Unknown
3	2.888	275	313.0925	337.0903†	314.1002	$C_{14}H_{18}O_8$	313.0929	1.25	5.3: Mandelic acid β-D-glucopyranoside; found in *P. persica* seeds (Fukuda et al. 2003)
4	5.146	285 330	287.0564	289.0677	288.0634	$C_{15}H_{12}O_6$	287.0561	-1	5.4: Eriodictyol±; found in *P. persica* whole plant (Jiménez-Atiénzar et al. 2006), *P. persica* stem bark (Jang et al. 2018)
5	5.269	275 360	449.1089	451.1206	450.1162	$C_{21}H_{22}O_{11}$	449.1089	0.08	5.5: Taxifolin-7-*O*-rhamnoside (Wong 2015); taxifolin found in *P. aequinoctialis*, *P. nipponica*, *P. maximowiczii* and *P. avium* (Hasegawa 1957)
6	5.818	280	479.1197	-	480.1268	$C_{22}H_{24}O_{12}$	479.1195	-0.42	5.6: Unknown
7	6.118	280	575.1158	577.1340	576.1268	$C_{30}H_{24}O_{12}$	575.1195	6.42	5.7: Unknown
8	6.268	280	575.1196		576.1268	$C_{30}H_{24}O_{12}$	575.1195	-0.17	5.8: Proanthocyanidin A1 or A2 (Zhao et al. 2015); both isolated from *P. persica* fruit
9	6.584	360	433.1078		434.1154	$C_{28}H_{18}O_5$	433.1081	0.8	5.9: Unknown
10	6.767	273	543.1287	545.1481	544.1290	$C_{30}H_{24}O_{10}$	543.1290	0.3	5.10: *ent*-Epiafzelechin-(2α→*O*→7',4α→8')-(-)-*ent*-afzelechin; isolated from *P. persica* root (Kichu 2010, Malewska 2015), *P. armeniaca* roots (Ferreira and Li 2000)
11	7.034	285 330	287.0558		288.0634	$C_{15}H_{12}O_6$	287.0561	1.08	5.11: Unknown (possible isomer of 5.4)±
12	7.433	290	813.1820	815.1982	814.1898	$C_{45}H_{34}O_{15}$	813.1825	0.61	5.12: Unknown
13	7.450	280	461.1084		462.1162	$C_{22}H_{22}O_{11}$	461.1089	1.16	5.13: Unknown

14	8.289	260 365	285.0385	287.0548	286.0477	$C_{15}H_{10}O_6$	285.0405	6.86	5.14: Kaempferol (Campillo et al. 2015); isolated from *P. persica* fruit (Lee Chang et al. 2007), leaves (Kazan et al. 2014), pericarp (Lee et al. 2008)
15	9.132	325	283.0977		284.1049	$C_{17}H_{16}O_4$	283.0976	0.41	5.15 (2.5): Caffeic acid phenylethyl ester (isolated in this study) as well as from *P. jamasakura* bark (Matsuoka et al. 2011)
16	12.112	215	367.2643	-	368.2715	$C_{25}H_{36}O_2$	367.2643	-0.13	5.16: Unknown

[±]Likely isomers. RT - retention time. ESI^- - electrospray ionisation (negative mode), ESI^+ - electrospray ionisation (positive mode). $[M-H]^-$ - deprotonated molecular mass, $[M+H]^+$ protonated molecular mass, †$[M+Na]^+$ - Na adduct (positive mode). λ_{max} (nm) – UV-Vis. MGF - generated molecular formula, *m/z* (calc) - calculated molecular ions for $[M-H]^-$. Diff (ppm) - the difference between measured and calculated masses in parts per million (ppm) for $[M-H]^-$.

Table 5.2 Compounds identified from the DCM partition of *P. persica.*

Peak	RT	λ_{max} (nm)	ESI^- $[M-H]^-$	ESI^+ $[M+Na]^{+\dagger}$ $[M+H]^+$	Mass (MFG)	Molecular formula	*m/z* (calc) for $[M-H]^-$	Diff (ppm)	Tentative identity
1	4.567	280	-	307.0795	306.074	$C_{15}H_{14}O_7$	307.0812	5.65	5.17: Leucocyanidin (Yang et al. 2012); found in *P. persica* fruit (Lim 2012), fruit, stems, roots (Ye 2017)
2	5.448	285	289.0735	291.0866	290.079	$C_{15}H_{14}O_6$	289.0718	-5.99	5.18: Catechin (Sudjaroen et al. 2012); found in *P. persica* fruit (Jang et al. 2018)
2		285			290.079	$C_{15}H_{14}O_6$			5.18: Epicatechin (Sudjaroen et al. 2012); found in *P. persica* fruit (Jang et al. 2018)
3	5.564	260	273.0791	275.0892	274.0841	$C_{15}H_{14}O_5$	273.0768	-8.22	5.19: Afzelechin; isolated from roots of *P. persica* (Ohigashi et al. 1982, Kichu 2010, Malewska 2015)
4	6.014	280	575.1156	577.1347	576.1268	$C_{30}H_{24}O_{12}$	575.1195	6.77	5.20 (5.8): Proanthocyanidin A1 or A2; both found in *P. persica* roots (Ohigashi et al. 1982), *P. prostrata* aerial parts (Bilia et al. 1996)
5	6.213	230 295	463.1246	487.1209†	464.1319	$C_{22}H_{24}O_{11}$	463.1246	-0.03	5.21: Unknown
6 & 7	6.463	230 275	559.1217	561.1365	560.1319	$C_{30}H_{24}O_{11}$	559.1246	5.15	5.22 and 5.23: *ent*-Epiafzelechin-(2α→7,4α→8)-epicatechin OR *ent*-

									epiafzelechin-(2α→7,4α→8)-catechin OR 3'''-hydroxyafzelechin-(2β→7,4β→8)-catechin; found in *Prunus* species (Buckingham 2015)
8	7.396	280	327.2181	351.2139†	328.225	$C_{18}H_{32}O_5$	327.2177	-1.23	5.24: Unknown
9	7.612	385	329.2338	353.2297†	330.2406	$C_{18}H_{34}O_5$	329.2333	1.05	5.25: Unknown
10	9.173	286	-	317.1014	316.0947	$C_{17}H_{16}O_6$	317.102	1.97	5.26: Persicogenin; found in *P. persica* bark (Rahman and Bhatnagar 1968), stem bark (Jang et al. 2018)
11	9.829	-	-	520.3411	519.3322	$C_{30}H_{48}O_7$	520.3395	-3.17	5.27: Unknown

RT - retention time. ESI^- - electrospray ionisation (negative mode), ESI^+ - electrospray ionisation (positive mode). $[M-H]^-$ - deprotonated molecular mass, $[M+H]^+$ protonated molecular mass, †$[M+Na]^+$ - Na adduct (positive mode). λ_{max} (nm) – UV-Vis. MGF - generated molecular formula, *m/z* (calc) - calculated molecular ions for $[M-H]^-$. Diff (ppm) - the difference between measured and calculated masses in parts per million (ppm) for $[M-H]^-$.

Table 5.3. Compounds identified from the EtOAc extract of *P. persica*.

Peak	RT	λ_{max} (nm)	ESI^- $[M-H]^-$	ESI^+ $[M+Na]^{+\dagger}$ $[M+H]^+$	Mass (MFG)	Molecular formula	*m/z* (calc) for $[M-H]^-$	Diff (ppm)	Tentative identity
1	0.907	210	191.0567	-	192.0634	$C_7H_{12}O_6$	191.482	-3.06	5.28 (5.1): Quinic acid (Dirlewanger et al. 1999, Etienne et al. 2002, Quilot et al. 2004, Montevecchi et al. 2012); found in *P. persica* fruits
2	2.006	270	169.0145	-	170.0215	$C_7H_6O_5$	169.0142	-1.49	5.29 (2.4): Gallic acid; found in *P. persica* fruit (Cantín et al. 2009) (isolated in this study)
3	2.864	275	313.0923	337.0907†	314.1002	$C_{14}H_{18}O_8$	313.0929	1.88	5.30 (5.3): Mandelic acid β-D-glucopyranoside (Songsong et al. 2015); found in *P. persica* seeds (Fukuda et al. 2003), *P. jamasakura* bark (Matsuoka et al. 2011)
4	4.563	280	305.0660	307.0797	306.074	$C_{15}H_{14}O_7$	305.0667	2.21	5.31: Gallocatechin (Chandra Sekhar et al. 2017); found in *Terminalia arjuna* bark

									(Chandra Sekhar et al. 2017), *P. persica* seeds (Fukuda et al. 2003)
5	5.029	280	289.0735	291.00	290.079	$C_{15}H_{14}O_6$	289.0718	-5.99	5.32 (5.18): Catechin; found in *P. persica* stem bark (Cantín et al. 2009, Jang et al. 2018)
6	5.628	545	400.1499	402.1605	401.1542	$C_{29}H_{21}O_2$	400.1469	-7.53	5.33: Unknown
7	6.178	545	575.1156	577.1347	576.1268	$C_{30}H_{24}O_{12}$			5.34: Proanthocyanidin A1 or A2; found in *P. persica* roots (Ohigashi et al. 1982), *P. prostrata* aerial parts (Bilia et al. 1996)
8	6.444	230 275	559.1217	561.1365	560.1319	$C_{30}H_{24}O_{11}$	559.1246	5.15	5.35: *ent*-Epiafzelechin-(2α→7,4α→8)-epicatechin OR *ent*-epiafzelechin-(2α→7,4α→8)-catechin OR 3'''-hydroxyafzelechin-(2β→7,4β→8)-catechin; found in *Prunus* species (Buckingham 2015)
9	6.767	235 285	543.1278	561.1413†	544.1369	$C_{30}H_{24}O_{10}$	543.1297	3.44	5.36: *ent*-Epiafzelechin-(2α→*O*→7, 4α→8)-(-)-*ent*-afzelechin (isolated in this study) as well as reported present in the crude aqueous ethanolic extract
10	8.25	260	273.0791	275.0761	274.0841	$C_{15}H_{14}O_5$	273.0768	-8.22	5.37: Afzelechin isolated in this study as well as already reported present in the crude ethanolic extract
11	9.13	325	283.0977	323.0871†	284.1049	$C_{17}H_{16}O_4$	283.0976	-0.41	5.38: Caffeic acid phenylethyl ester isolated in this study as well as reported present in the crude aqueous ethanolic extract

RT - retention time. ESI^- - electrospray ionisation (negative mode), ESI^+ - electrospray ionisation (positive mode). $[M-H]^-$ - deprotonated molecular mass, $[M+H]^+$ protonated molecular mass, †$[M+Na]^+$ - Na adduct (positive mode). λ_{max} (nm) – UV-Vis. MGF - generated molecular formula, *m/z* (calc) - calculated molecular ions for $[M-H]^-$. Diff (ppm) - the difference between measured and calculated masses in parts per million (ppm) for $[M-H]^-$.

5.2.4 Tentative identification of compounds found in *H. latifolia* leaves

All peaks in the TICs were analysed for the crude aqueous ethanolic extract and dichloromethane partition (Tables 5.4 and 5.5, Figures 5.8, 5.9, 5.10 and 5.11) of *H. latifolia*, however, the lack of overlap between the EICs of the positive and negative mode made confident mass analysis impossible for most of the peaks. Five compounds in the crude aqueous ethanolic extract and five in the dichloromethane partition did have sufficient data for tentative identification. Due to the fact that none of these compounds were previously found in the *Holboellia* genus, all the compounds will be referred to as unknown. However, some possible candidates that matched published literature are discussed below. It is also acknowledged that without further MS/MS fragmentation or comparison with authentic standards, in all cases, the actual compounds could also be isomers of those described.

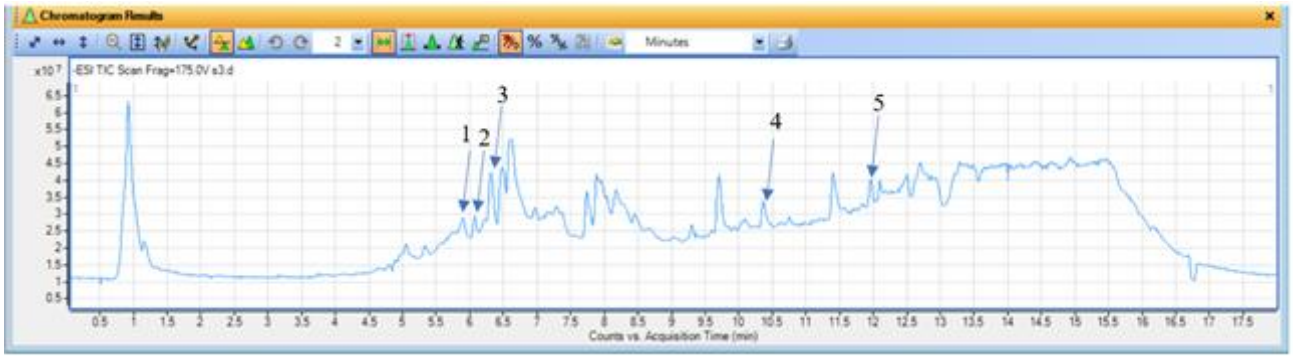
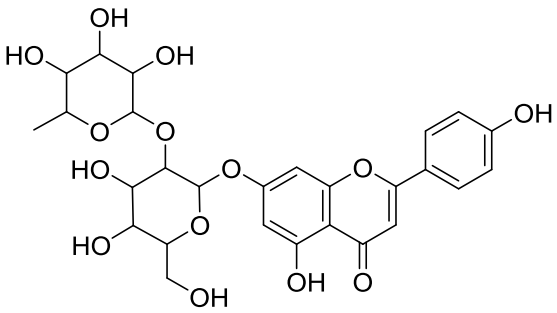


Figure 5.8. EIC (negative mode) TIC of *H. latifolia* 70% aqueous ethanolic extract.

Figure. 5.9. EIC (positive mode) TIC of *H. latifolia* 70% aqueous ethanolic extract.

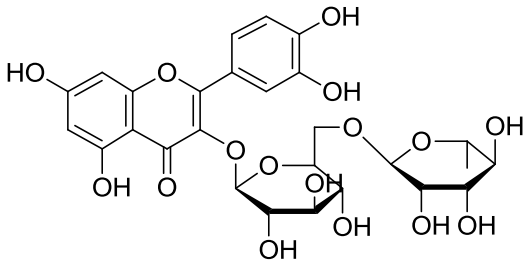
The compounds were tentatively identified from the aqueous ethanolic extract, in order of retention time, as follows (Table 5.4):

Peak number 1 (mass 578.1636, $C_{27}H_{30}O_{14}$, λ_{max} 270, 338 nm, 5.39) was tentatively identified as apigenin 7-*O*-neohesperidoside as all data aligned with the literature (Abad-García et al. 2007). This compound is commonly found in citrus and other plants (Abad-García et al. 2012, Spínola et al. 2015).



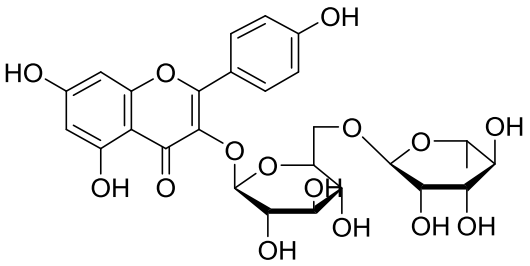
Apigenin 7-*O*-neohesperidoside

Peak number 2 was consistent with rutin (mass 610.1534, $C_{27}H_{30}O_{16}$, λ_{max} 257 and 355 nm, 5.40) due to its characteristic UV-Vis spectrum (Zu et al. 2006). This compound is commonly present in many plants (Vallverdú-Queralt et al. 2014).



Rutin

Peak number 3 could be kaempferol-3-*O*-rutinoside (mass 594.1585, $C_{27}H_{30}O_{15}$, λ_{max} 265 and 348 nm, 5.41) (Avula et al. 2015), which has been previously isolated from *Acalypha wilkesiana* and *A. hispida* leaves (Adesina et al. 2000).

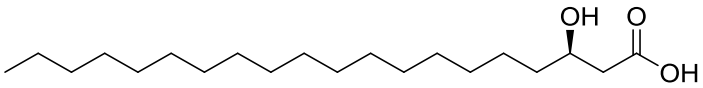


Kaempferol-3-*O*-rutinoside

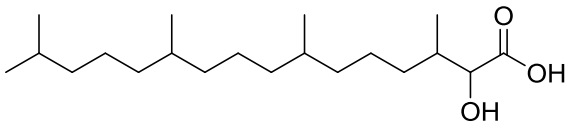
Peak number 4 (mass 298.2508, $C_{18}H_{34}O_3$, 5.42) did not show any UV-Vis absorption. Kumar et al., reported the presence of 79 natural products that matched that molecular mass as well as GMF (Kumar et al. 2018). None of those compounds have been previously reported from *Holboellia* species.

Peak number 5 (mass 328.2977, $C_{20}H_{40}O_3$, 5.29) presented the same difficulties as described above. Database searching with Pubmed suggested that this peak could possibly be 3*R*-hydroxyeicosanoic acid or 2-hydroxyphytanic acid or 3*R*-hydroxyphytanic acid (Kumar et al. 2018). 3*R*-Hydroxyeicosanoic acid was previously identified in *Diascia purpurea*, *D. vigilis*, *D. cordata*, *D.*

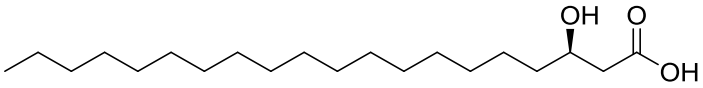
megathura and *D. integerrima* flowers (Dumri 2018). Hydroxyphytanic acid was previously found in *Holarrhena antidysenterica* bark (Kumar et al. 2018).



3*R*-Hydroxyeicosanoic acid



2-Hydroxyphytanic acid



3*R*-Hydroxyeicosanoic acid

In the dichloromethane partitions, peaks were tentatively identified, in order of retention time, as follows:

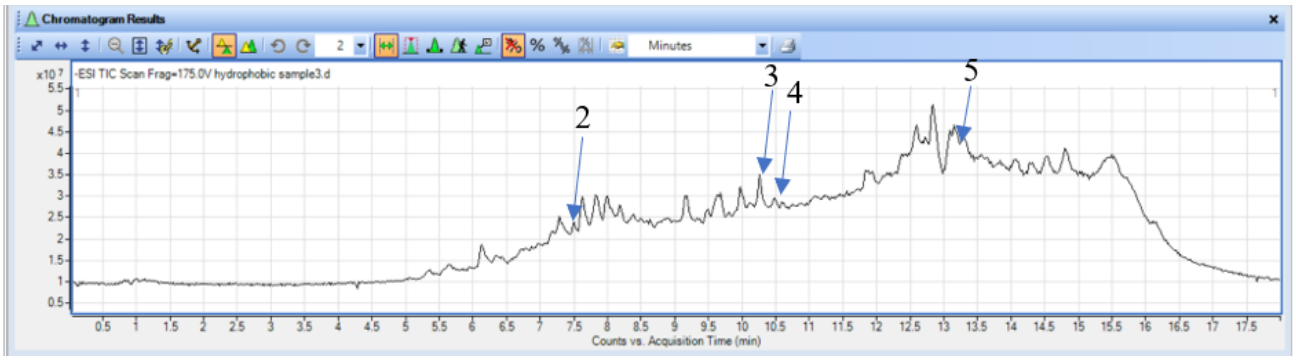


Figure 5.11. EIC (negative mode) TIC of *H. latifolia* dichloromethane extract.

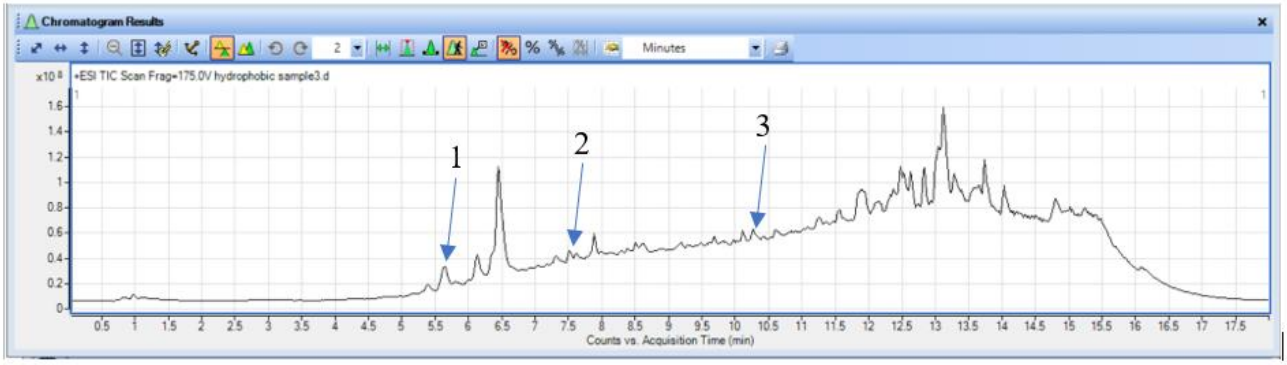
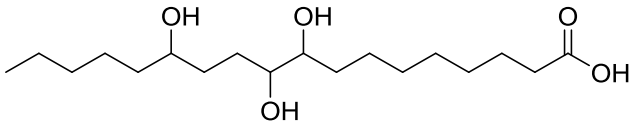


Figure 5.10. EIC (positive mode) TIC of *H. latifolia* dichloromethane extract.

Peak number 1 (mass 292.1099, $C_{19}H_{16}O_3$, λ_{max} 280 nm, 5.44) was consistent with 1,7-bis(4-hydroxyphenyl)-2,4,6-heptatrien-3-one, which has been previously isolated from roots of *Etlingera*

elatior (Habsah et al. 2005). However, the UV-Vis profile did not match the reported values for either of these compounds (Hussain et al. 2012).

Peak number 2 (mass 332.2563, $C_{18}H_{36}O_5$, 5.45), based on the exact mass and database searches, was tentatively identified as 9,10,13-trihydroxyoctadecenoic acid, which is very commonly present in plants (Funk and Powell 1983, Masui et al. 1989, Kobayashi et al. 1993)

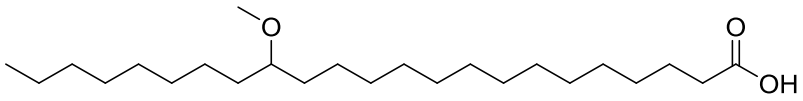


9,10,13-Trihydroxyoctadecenoic acid

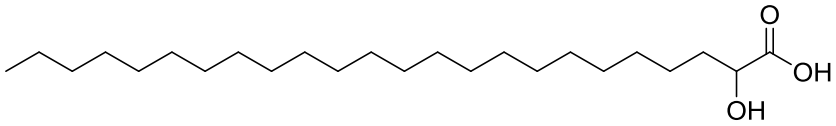
Peak number 3 (mass 346.1053, $C_{18}H_{18}O_7$, λ_{max} 260 and 340 nm, 5.45) from the exact mass and GMF was tentatively identified as 7,3′-dihydroxy-6,2′,4′-trimethoxyisoflavanone or 5,7,4′-trihydroxy-8-ethoxycarbonylflavanol. The absorbance for peak 3 showed a maximum at 260 nm with a shoulder at 340 nm, which is characteristic for flavones (Tsimogiannis et al. 2007). Flavanols have a maximum absorbance at non-specific wavelengths between 270 and 290 nm, at which many phenolics absorb. Thus, further distinction was not possible (Tsimogiannis et al. 2007). (3*R*)-7,3′-Dihydroxy-6,2′,4′-trimethoxyisoflavanone has been previously isolated from *Dalbergia odorifera* (Wang et al. 2014) and 5,7,4′-trihydroxy-8-ethoxycarbonylflavanol has been previously isolated from *Daphne genkwa* (Zhang et al. 2007).

Peak number 4 (mass 286.0477, $C_{15}H_{10}O_6$, λ_{max} 260, 325 nm, 5.46) had an exact mass, molecular formula and UV-Vis spectrum consistent with the common plant compound luteolin (María et al. 2007, García-Villalba et al. 2012, Yuan et al. 2012, Petar et al. 2015, López-Gutiérrez et al. 2016).

Peak number 5 (mass 384.3603, $C_{24}H_{48}O_3$, 5.47) could be either 15-methoxytricosanoic acid or 2-hydroxytetracosanoic acid. 2-Hydroxytetracosanoic acid as well as 15-methoxytricosanoic acid are commonly present in plants (Nagarajan et al. 2001, Carballeira 2002, Davoli et al. 2004, Mishra and Sree 2007, Guzman et al. 2008).



15-Methoxytricosanoic acid



2-Hydroxytetracosanoic acid

Table 5.4 Compounds reported in the crude ethanolic extract of *H. latifolia*.

Peak	RT	λ_{max} (nm)	ESI^- $[M-H]^-$	ESI^+ $[M+Na]^{+\dagger}$ $[M+H]^+$	Mass (MFG)	Molecular formula	*m/z* (calc) $[M-H]^-$	Diff (ppm)	Tentative identity
1	5.891	270 338	577.1561	579.1726	578.1636	$C_{27}H_{30}O_{14}$	577.1563	0.31	5.39: Unknown
2	6.074	257 355	609.1460	611.1612	610.1534	$C_{27}H_{30}O_{16}$	609.1461	0.18	5.40: Unknown
3	6.307	265 350	593.1494	595.1675	594.1585	$C_{27}H_{30}O_{15}$	593.1512	3.02	5.41: Unknown
4	10.370	-	297.2437	319.2274†	298.2508	$C_{18}H_{34}O_3$	297.2435	-0.61	5.42: Unknown
5	11.968	-	327.2907		328.2977	$C_{20}H_{40}O_3$	327.2905	-0.7	5.43: Unknown

RT - retention time. ESI^- - electrospray ionisation (negative mode), ESI^+ - electrospray ionisation (positive mode). $[M-H]^-$ - deprotonated molecular mass, $[M+H]^+$ protonated molecular mass, †$[M+Na]^+$ - Na adduct (positive mode). λ_{max} (nm) – UV-Vis. MGF - generated molecular formula, *m/z* (calc) - calculated molecular ions for $[M-H]^-$. Diff (ppm) - the difference between measured and calculated masses in parts per million (ppm) for $[M-H]^-$.

Table 5.5 Compounds identified in the DCM fraction of *H. latifolia*.

Peak	RT	λ_{max} (nm)	ESI^- $[M-H]^-$	ESI^+ $[M+Na]^{+\dagger}$ $[M+H]^+$	Mass (MFG)	Molecular formula	*m/z* (calc) for $[M-H]^-$	Diff (ppm)	Tentative identity
1	5.631	280		293.114	292.1099	$C_{19}H_{16}O_3$	293.1172	11.03	5.44: Unknown
2	7.545	-	331.2487	355.2452†	332.2563	$C_{18}H_{36}O_5$	331.249	0.9	5.45: Unknown
3	10.259	340	345.0999	347.1148	346.1053	$C_{18}H_{18}O_7$	545.098	-5.56	5.46: Unknown
4	10.492	260 325	285.0412	-	286.0477	$C_{15}H_{10}O_6$	285.0405	-2.58	5.47: Unknown
6	13.172	-	383.3533	-	384.3603	$C_{24}H_{48}O_3$	383.3513	-0.6	5.48: Unknown

RT - retention time. ESI^- - electrospray ionisation (negative mode), ESI^+ - electrospray ionisation (positive mode). $[M-H]^-$ - deprotonated molecular mass, $[M+H]^+$ protonated molecular mass, †$[M+Na]^+$ - Na adduct (positive mode). λ_{max} (nm) – UV-Vis. MGF - generated molecular formula, *m/z* (calc) - calculated molecular ions for $[M-H]^-$. Diff (ppm) - the difference between measured and calculated masses in parts per million (ppm) for $[M-H]^-$.

5.2.5 Tentative identification of compounds found in *D. lanceifolia* leaves

For *D. lanceifolia*, seven compounds were tentatively identified in the crude aqueous ethanolic extract and seven in the *n*-hexane partition (Tables 5.6 and 5.7, Figures 5.12 and 5.13).

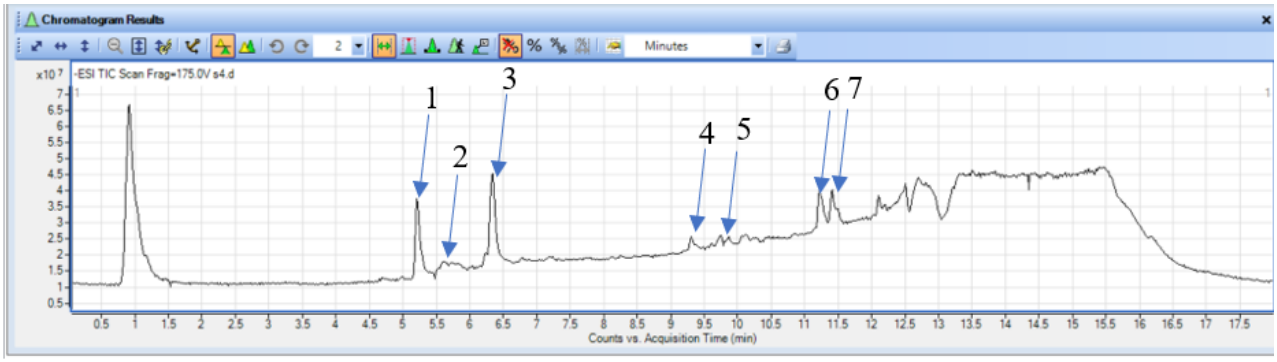


Figure 5. 12. EIC (negative mode) TIC of *D. lanceifolia* 70% ethanolic water extract.

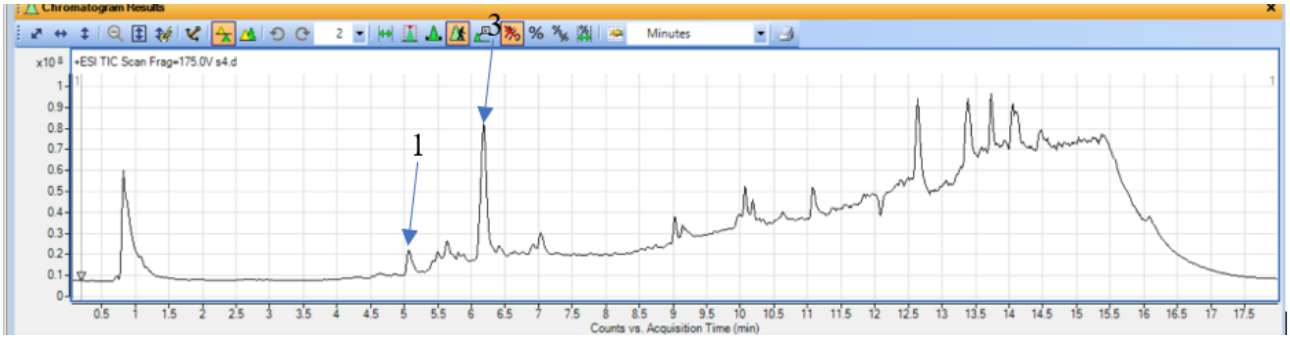
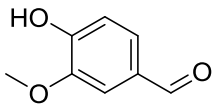


Figure 5. 13. EIC (positive mode) TIC of *D. lanceifolia* 70 % ethanolic water extract.

The *Diospyros* genus is very well known to be a rich source of triterpenes, naphthoquinones and coumarins (Herath et al. 1978, Zhong et al. 1984). Zhong et al. reported on the investigation of the bark and/or wood of seventeen African species of *Diospyros* for the presence of triterpenes and naphthoquinones and found the triterpenes lupeol, betulin and betulinic acid in all samples and the common dimeric naphthoquinones diospyrin, isodiospyrin and diosindigo-A were isolated from fourteen of the seventeen species.

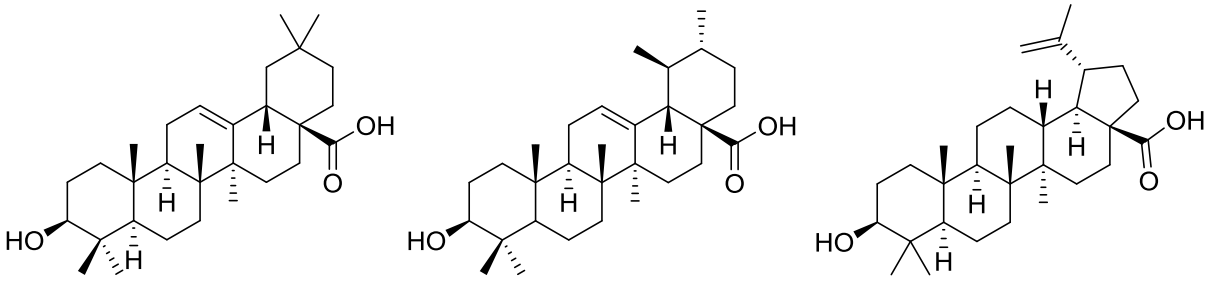
In the crude aqueous ethanolic extract, the peaks were identified, in order of retention time, as follows (Table 6):

Peak number 1 (mass 153.0473, $C_8H_8O_4$, λ_{max} 210 and 290 nm, 5.49) was tentatively identified as vanillin. All data were consistent with the compound (Pereira et al. 2010), which is commonly found in many plants (Sinha et al. 2008).



Vanillin

Peak number 6 (mass 456.3603, $C_{30}H_{48}O_3$, 5.55) was tentatively identified as either ursolic acid, oleanolic acid or betulinic acid (Lee et al. 2006, Ren et al. 2016). All three compounds have been previously isolated from *D. kaki* fruit (Zhou et al. 2010), and ursolic acid has also been previously isolated from *D. leucomelas* leaves, and betulinic acid is also commonly present in other *Diospyros* species (Zhong et al. 1984). The absence of UV-Vis spectra for those compounds was consistent with the literature (Zhong et al. 1984).



Oleanolic acid Ursolic acid Betulinic acid

Five peaks from the crude extract (2, 3, 4, 5 and 7) had no corresponding mass and molecular formulae to that reported in the literature.

In the *n*-hexane partition, the following compounds were tentatively identified, in order of retention time (Table 7):

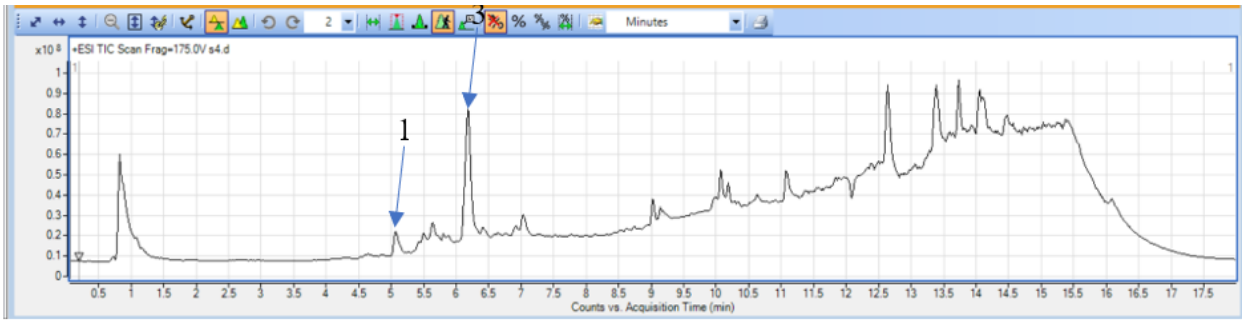


Figure 5.15. EIC (negative mode) TIC of *D. lanceifolia* *n*-hexane extract.

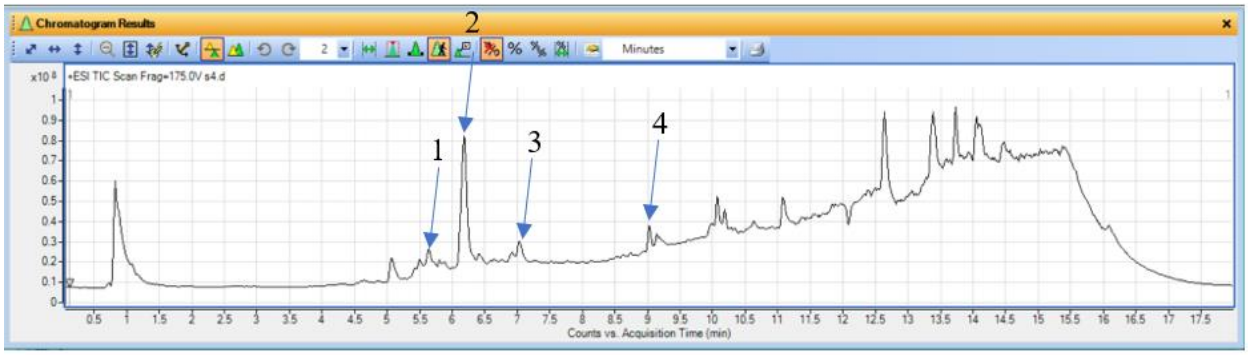
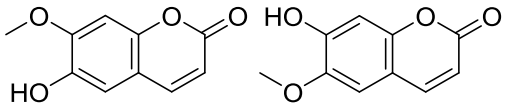


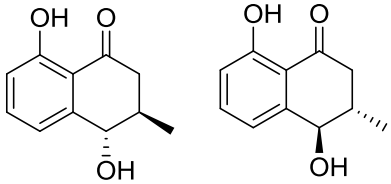
Figure 5.14. EIC (positive mode) TIC of *D. lanceifolia* *n*-hexane extract.

Peak number 2 (mass 192.0423 $C_{10}H_8O_4$, λ_{max} 258 and 344 nm, 5.57) was tentatively identified as 6-hydroxy-7-methoxycoumarin (isoscopoletin) (Liu et al. 2016) or its isomer scopoletin (Abad-García et al. 2007). 6-Hydroxy-7-methoxycoumarin has been previously reported from *D. kaki* leaves (Sheikh 2016) and scopoletin has been previously isolated from *D. kaki* fruit (Liu et al. 2012) as well as *D. lotus* whole plant (Yadav 2016) and D. *celebica* wood (Brown et al. 1965). Herath et al. reported on the investigation of eleven *Diospyros* species for the presence of coumarins. The coumarin scopoletin has been isolated from the bark and timber extracts of six of them, namely *D. hirsuta*, *D. moonii*, *D. quaesita*, *D. spinescens*, *D. thwaitesii* and *D. walkeri* (Herath et al. 1978).



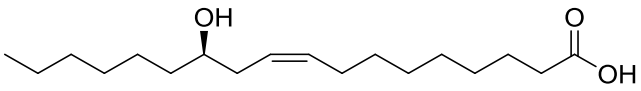
Isoscopoletin Scopoletin

Peak number 4 (mass 192.0786, $C_{11}H_{12}O_3$, λ_{max} 260 and 330 nm, 5.59) was identified as isoshinanolone, or its isomer epiisoshinanolone. Isoshinanolone, as the (1*R*,2*R*) stereoisomer, was previously isolated from *D. canaliculata* (Zhong et al. 1984). Epiisoshinanolone has also been reported from *Plumbago scandens* (Jetty et al. 2010) and isoshinanolone from *P. capensis* (Ariyanathan et al. 2011).



Isoshinanolone Epiisoshinanolone

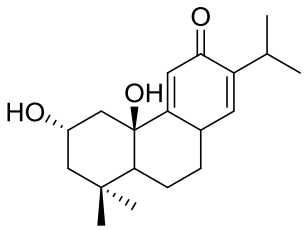
Peak number 6 was identified as ricinoleic acid (mass 298.2508, $C_{18}H_{34}O_3$, 5.61) (Kumar et al. 2018). This has been found in *D. blancoi* leaves (Howlader 2012). $C_{18}H_{34}O_3$ corresponds to many compounds, but out of the 79 listed natural products of this mass (Kumar et al. 2018), only ricinoleic acid has been reported from *Diopyros* spp.



Ricinoleic acid

Peak number 7 (mass 456.3603, $C_{30}H_{48}O_3$, 5.62) was already found in the crude ethanolic extract, being identified as either betulinic, ursolic or oleanolic acid, which have all been reported to be present in many *Diospyros* species (Zhong et al. 1984).

Peak number 8 (mass 304.2038, $C_{24}H_{48}O_3$, λ_{max} 240 nm, 5.63), based on obtained data and its comparison with Pubchem, was tentatively identified as salvipiliferol, which has been previously isolated from aerial parts of *Salvia pilifera*. However, due to the lack of reports concerning the presence of that compounds in *Diospyros* species, it was deemed an unknown.



Salvipiliferol

For peak numbers 1, 4 and 6, the mass and GMF did not correspond to any compounds reported in the databases searched.

Table 5.6. Compounds identified in the crude aqueous 70 % ethanolic extract of *D. lanceifolia*

Peak	RT	λ_{max}	ESI^- $[M-H]^-$	ESI^+ $[M+Na]^+$ $[M+H]^+$	Mass (MFG)	Molecular formula	*m/z* (calc) for $[M-H]^-$	Diff (ppm)	Tentative identity
1	5.074	210 290		153.0546	152.0473	$C_8H_8O_4$	153.0546	0.14	5.49: Vanillin (Pereira et al. 2010); commonly isolated from plants (Sinha et al. 2008)
2	5.225	280	196.0369	-	197.045	$C_9H_9O_5$	196.0377	4.17	5.50: Unknown
3	6.324	-	177.0129	179.0475	178.0188	$C_9H_5O_4$	177.0115	-7.87	5.51: Unknown
4	9.304	-	625.1480	-	266.1518	$C_{15}H_{22}O_4$	625.1445	-13.03	5.52: Unknown
5	9.737		311.1673		312.1725	$C_{20}H_{24}O_3$	311.1653	-6.51	5.53: Unknown
6	11.219	-	455.3528		456.3603	$C_{30}H_{48}O_3$	455.3531	0.59	5.54: Ursolic acid (Lee et al. 2006, Ren et al. 2016); isolated from *D. leucomelas* leaves and *D. kaki* fruit (Zhou et al. 2010) OR Oleanolic Acid (Lee et al. 2006, Ren et al. 2016); isolated from *D. kaki* fruit (Zhou et al. 2010) OR Betulinic Acid (Lee et al. 2006, Ren et al. 2016); isolated from *D. kaki* fruit (Zhou et al. 2010)
7	11.502		475.2489	-	476.2563	$C_{30}H_{36}O_5$	475.249	0.21	5.55: Unknown

RT - retention time. ESI^- - electrospray ionisation (negative mode), ESI^+ - electrospray ionisation (positive mode). $[M-H]^-$ - deprotonated molecular mass, $[M+H]^+$ protonated molecular mass, †$[M+Na]^+$ - Na adduct (positive mode). λ_{max} (nm) – UV-Vis. MGF - generated molecular formula, *m/z* (calc) - calculated molecular ions for $[M-H]^-$. Diff (ppm) - the difference between measured and calculated masses in parts per million (ppm) for [M-H].

Table 5.7. Compounds identified in the *n*-hexane fraction of *D. lanceifolia*

Peak	RT	λ_{max}	ESI- [M-H]-	ESI+ $[M+Na]^+$ $[M+H]^+$	Mass (MFG)	Molecular formula	*m/z* (calc) for $[M-H]^-$	Diff (ppm)	Tentative identity
1	5.633	280 340	-	475.3269	474.3193	$C_{25}H_{46}O_8$	475.3265	-0.75	5.56: Unknown
2	6.133	260 340	-	193.498	192.0423	$C_{10}H_8O_4$	193.0495	-1.38	5.57: 6-Hydroxy-7-methoxycoumarin (isoscopoletin) (Liu et al. 2016); found in *D. kaki* leaves (Sheikh 2016)
		258 344			192.0423	$C_{10}H_8O_4$			5.57: Scopoletin (Abad-García et al. 2007); found in *D. kaki* fruit (Liu et al. 2012), *D. lotus* whole plant (Yadav 2016), *D. celebica* (Brown et al. 1965)
3	7.032	260 330	-	193.0864	192.0786	$C_{11}H_{12}O_3$	193.0859	-2.5	5.58: Epiisoshinanolone; found in *D. canaliculata* bark (Zhong et al. 1984)
		258 333			192.0786	$C_{11}H_{12}O_3$			5.58: Isoshinanolone; found in *D. canaliculata* bark (Zhong et al. 1984)
4	8.647	260 420	-	475.3272	474.3193	$C_{25}H_{46}O_8$	475.3265	-1.38	5.59: Unknown
5	10.145	-	297.2447	-	298.2508	$C_{18}H_{34}O_3$	297.2435	-3.96	5.60: Ricinoleic acid (Kumar et al. 2018) ; found in *D. blancoi* leaves (Howlader 2012)
6	11.077	-	455.3539	-	456.3603	$C_{30}H_{48}O_3$	455.3531	-1.82	6.61: Betulinic, ursolic or oleanolic acids; found in many *Diospyros* species (Zhong et al. 1984)
7	11.676	240	303.1968	-	304.2038	$C_{19}H_{28}O_3$	303.1966	-0.76	5.61: Unknown

RT - retention time. ESI^- - electrospray ionisation (negative mode), ESI^+ - electrospray ionisation (positive mode). $[M-H]^-$ - deprotonated molecular mass, $[M+H]^+$ protonated molecular mass, †$[M+Na]^+$ - Na adduct (positive mode). λ_{max} (nm) – UV-Vis. MGF - generated molecular formula, *m/z* (calc) - calculated molecular ions for $[M-H]^-$. Diff (ppm) - the difference between measured and calculated masses in parts per million (ppm) for [M-H].

The crude 70% aqueous ethanolic extracts of *P. persica* roots, leaves of *H. latifolia* and *D. lanceifolia*, as well as the dichloromethane and ethyl acetate partitions of *P. persica*, dichloromethane partition of *H. latifolia* and *n*-hexane partition of *D. lanceifolia,* were analysed by UHPLC-DAD-Q-ToF-MS. All extracts were analysed under the same conditions. Only proanthocyanidins were found common for all three *P. persica* extracts. Eriodictyol, taxifolin-7-*O*-rhamnoside and kaempferol were tentatively identified only in the crude extract. Leucocyanidin, and persicogenin were exclusive to dichloromethane partition and gallic acid and gallocatechin were tentatively identified only in the ethyl acetate fraction. For *H. latifolia* crude ethanolic extract and dichloromethane extract, all compounds were found unknown. None of the peaks found in crude extract were present in the dichloromethane partition. In the crude ethanolic and hexane fractions of *D. lanceifolia*, the only common peak was tentatively identified as either ursolic, oleanolic or betulinic acid. Vanillin was tentatively identified in the crude ethanolic extract only and scopoletin or isoscopoletin, isoshinanolone, or its isomer epiisoshinanolone and ricinoleic acid were tentatively identified in the hexane partition.

Potentially, all compounds found in the partitions should be present in the crude extract. That wasn't the author's founding. Possibly, this effect could be due to the low, below the noise level, concentration of the "absent" compounds in the crude ethanolic extract.

5.3 Reported antimicrobial properties of the tentatively identified compounds

From the metabolomics approach presented in this chapter, a range of compounds with known biological activities relevant to traditional uses were tentatively identified from the extracts/partitions of the three plants, as summarised below.

Of the compounds tentatively identified from *P. persica* roots, many have activities consistent with the traditional uses of the roots for treating skin infections (topical application), and orally for typhoid and tonsillitis. Eriodictyol and catechin have been found active against *Bacillus cereus, Escherichia coli, Listeria monocytogenes, Pseudomonas aeruginosa, Proteus vulgaris, Staphylococcus aureus, Salmonella enteritidis, Yersinia enterocolitica* and *Shigella sonnei* (Yaltirak et al. 2009). Catechin, taxifolin and eriodictyol also possess anti-inflammatory and antioxidant properties (Tripoli et al. 2007). Taxifolin-7-*O*-rhamnoside was found active against *E. coli, Bacillus subtilis, Enterobacter aerogenes, Klebsiella pneumonia, Shigella flexnerii, Salmonella enterica, S. aureus, Staphylococcus epidermidis, P. aeruginosa* and *Streptococcus pyogenes* (Samaga and Rai 2013). Proanthocyanidin A1 and A2 have been reported to be active against *Enterococcus faecalis, E. coli* and *S. Typhimurium* (Puupponen-Pimiä et al. 2001), and MRSA, *P. aeruginosa* and *Candida albicans*, as well as exhibit

strong antioxidant properties (Karioti et al. 2011, Zang et al. 2013). Kaempferol has also been reported to be active against *S. aureus, E. coli, P. aeruginosa, B. subtilis* and *C. albicans* (Süzgeç et al. 2005), as well as *Helicobacter pylori* (Kataoka et al. 2001). Leucocyanidin is active against *S. aureus, B. subtilis, P. aeruginosa, Proteus vulgaris, Salmonella paratyphi,* and *Klepsiella pneumoniae* (El-Kamali and Mahjoub. 2009), and persicogenin against *S. aureus, E. faecalis, Mycobacterium intracellulare, Mycobacterium chelonei* and *C. albicans*. Diospyridin was reported active against *S. aureus, E. coli, P. aeruginosa* and *B. subtilis* (Dinda et al. 2006).

For *D. lanceifolia,* antimicrobial properties of all the tentatively identified compounds are well known and widely reported. Ursolic acid, oleanolic acid and betulinic acid have been reported to be active against common skin pathogens such as *P. aeruginosa, E. coli, S. aureus, Enterococcus faecalis, E. faecium, Serratia marcescens* and *Streptococcus pneumoniae* as well as *Mycobacterium tuberculosis* (Horiuchi et al. 2007, Tanachatchairatana et al. 2008). Vanillin has been reported to be active against *E. coli* and *Listeria innocua* (Fitzgerald et al. 2004), *L. monocytogenes* (Cava-Roda et al. 2012) and *C. albicans* (Boonchird and Flegel 1982). Ricinoleic acid has been found to be active against *E. coli, P. aeruginosa, S. typhi, S. aureus, M. tuberculosis, C. albicans* and *Aspergillus niger* (Neogi et al. 2008, D'Oca et al. 2010).

5.4 Conclusions

A metabolomics approach was used to gain a better understanding of the phytochemistry of the medicinal plants *Prunus persica* (roots), *Holboellia latifolia* (leaves) and *Diospyros lanceifolia* (leaves). QTOF-MS profiling enabled tentative identification of metabolites that were not previously reported from these three plants or plant parts.

From this study, thirteen metabolites have been tentatively identified in *P. persica* roots. Fourteen were deemed "unknown". The identified compounds include taxifolin-7-*O*-rhamnoside and 3′-*O*-methylcatechin-5-*O*-glucuronide, which have been identified, albeit tentatively, from *P. persica* species for the first time. The compounds were previously reported to be present in the *Prunus* genus. Ten compounds have been detected in *H. latifolia* leaf extracts. Due to the lack of literature confirming the presence of these compounds within the genus, all were regarded as "unknown". Fourteen compounds have been tentatively identified from the leaf extracts of *D. lanceifolia*. Gallic acid, caffeic acid phenyl ethyl ester, afzelechin and *ent*-epiafzelechin-(2α→*O*→7, 4α→8)-(-)-*ent*-afzelechin were isolated and characterised prior to the metabolomic studies. Many of the compounds tentatively identified from *P. persica, D. lanceifolia* and *H. latifolia* have been reported to be antimicrobially active. The biological activities of those compounds support traditional applications of these plants.

For all plants, identification of compounds was tentative due to the reliance on generated molecular formulae, retention time and UV-Vis profiles (in most cases). The use of LC-MS using ESI mode allowed information to be gathered on a range of molecules within the complex mixtures and provided confident molecular mass identification. However, the gentle ionisation mode of ESI did not allow for direct fragmentation patterns that could give further structural information to enable more definitive identification of compounds. Future work using modified MS conditions for mass spectral fragmentation to be obtained, as well as use of authentic standards for LC-MS comparison, would enable greater confidence in establishing the structures of the metabolites (Friedrich et al. 2000). This was not conducted due to time constraints and equipment availability but would be a worthwhile future direction to undertake.

5.5 References

Abad-García, B., L. Berrueta, D. López-Márquez, I. Crespo-Ferrer, B. Gallo and F. Vicente (2007). "Optimization and validation of a methodology based on solvent extraction and liquid chromatography for the simultaneous determination of several polyphenolic families in fruit juices." *Journal of Chromatography* **1154**(1-2): 87-96.

Abad-García, B., S. Garmón-Lobato, L. A. Berrueta, B. Gallo and F. Vicente (2012). "On line characterization of 58 phenolic compounds in Citrus fruit juices from Spanish cultivars by high-performance liquid chromatography with photodiode-array detection coupled to electrospray ionization triple quadrupole mass spectrometry." *Talanta* **99**: 213-224.

Adesina, S., O. Idowu, A. Ogundaini, H. Oladimeji, T. Olugbade, G. Onawunmi and M. Pais (2000). "Antimicrobial constituents of the leaves of *Acalypha wilkesiana* and *Acalypha hispida*." *Phytotherapy Research* **14**(5): 371-374.

Anouar, E. H., J. Gierschner, J.-L. Duroux and P. Trouillas (2012). "UV/Visible spectra of natural polyphenols: A time-dependent density functional theory study." *Food Chemistry* **131**(1): 79-89.

Appendino, G., O. Taglialatela-Scafati, A. Romano, F. Pollastro, C. Avonto and P. Rubiolo (2009). "Genepolide, a sesterpene γ-Lactone with a novel carbon skeleton from mountain wormwood (*Artemisia umbelliformis*)." *Journal of Natural Products* **72**(3): 340-344.

Ariyanathan, S., A. Saraswathy and G. V. Rajamanickkam (2011). "Phytochemical investigation of *Plumbago capensis* Thunb." *International Journal of Pharmacy and Life Sciences* **2**(4): 670-673.

Avula, B., S. Sagi, S. Gafner, R. Upton, Y.-H. Wang, M. Wang and I. A. Khan (2015). "Identification of *Ginkgo biloba* supplements adulteration using high performance thin layer chromatography and

ultra high performance liquid chromatography-diode array detector-quadrupole time of flight-mass spectrometry." *Analytical and Bioanalytical Chemistry* **407**(25): 7733-7746.

Bilia, A. R., I. Morelli, M. Hamburger and K. Hostetmann (1996). "Flavans and A-type proanthocyanidins from *Prunus prostrata*." *Phytochemistry* **43**(4): 887-892.

Boonchird, C. and T. Flegel (1982). "In vitro antifungal activity of eugenol and vanillin against *Candida albicans* and *Cryptococcus neoformans*." *Canadian Journal of Microbiology* **28**(11): 1235-1241.

Brenton, A. G. and A. R. Godfrey (2010). "Accurate mass measurement: terminology and treatment of data." *Journal of the American Society for Mass Spectrometry* **21**(11): 1821-1835.

Brown, A. G., J. C. Lovie and R. H. Thomson (1965). "432. *Ebenaceae* extractives. Part I. Naphthalene derivatives from macassar ebony (*Diospyros celebica* Bakh)." *Journal of the Chemical Society* 2355-2361.

Buckingham, J. (2015). "Dictionary of Flavonoids with CD-ROM." CRC Press.

Campillo, N., P. Viñas, G. Férez-Melgarejo and M. Hernández-Córdoba (2015). "Dispersive liquid–liquid microextraction for the determination of flavonoid aglycone compounds in honey using liquid chromatography with diode array detection and time-of-flight mass spectrometry." *Talanta* **131**: 185-191.

Cantín, C. M., M. A. Moreno and Y. Gogorcena (2009). "Evaluation of the antioxidant capacity, phenolic compounds, and vitamin C content of different peach and nectarine *Prunus persica* (L.) Batsch breeding progenies." *Journal of Agricultural and Food Chemistry* **57**(11): 4586-4592.

Carballeira, N. (2002). "New advances in the chemistry of methoxylated lipids." *Progress in Lipid Research* **41**(6): 437-456.

Cava-Roda, R. M., A. Taboada-Rodríguez, M. T. Valverde-Franco and F. Marín-Iniesta (2012). "Antimicrobial activity of vanillin and mixtures with cinnamon and clove essential oils in controlling *Listeria monocytogenes* and *Escherichia coli* O157: H7 in milk." *Food and Bioprocess Technology* **5**(6): 2120-2131.

Chandra Sekhar, Y., G. Phani Kumar and K. R. Anilakumar (2017). "*Terminalia arjuna* bark extract attenuates picrotoxin-induced behavioral changes by activation of serotonergic, dopaminergic, GABAergic and antioxidant systems." *Chinese Journal of Natural Medicines* **15**(8): 584-596.

D'Oca, C. D. R. M., T. Coelho, T. G. Marinho, C. R. L. Hack, R. da Costa Duarte, P. A. da Silva and M. G. M. D'Oca (2010). "Synthesis and antituberculosis activity of new fatty acid amides." *Bioorganic & Medicinal Chemistry Letters* **20**(17): 5255-5257.

Davoli, P., V. Mierau and R. Weber (2004). "Carotenoids and fatty acids in red yeasts *Sporobolomyces roseus* and *Rhodotorula glutinis*." *Applied Biochemistry and Microbiology* **40**(4): 392-397.

De Vos, R. C., S. Moco, A. Lommen, J. J. Keurentjes, R. J. Bino and R. D. Hall (2007). "Untargeted large-scale plant metabolomics using liquid chromatography coupled to mass spectrometry." *Nature Protocols* **2**(4): 778.

Dinda, B., A. Bhattacharya, U. C. De, S. Arima, H. Takayanagi and Y. Harigaya (2006). "Antimicrobial *C*-glucoside from aerial parts of *Diospyros nigra*." *Chemical and Pharmaceutical Bulletin* **54**(5): 679-681.

Dirlewanger, E., A. Moing, C. Rothan, L. Svanella, V. Pronier, A. Guye, C. Plomion and R. Monet (1999). "Mapping QTLs controlling fruit quality in peach (*Prunus persica* (L.) Batsch)." *Theoretical and Applied Genetics* **98**(1): 18-31.

Dumri, K. (2018). "Chemical analyses of non-volatile flower oils and related bee nest cell linings." *Natural Product Reports* **18**(7): 20-26.

Dunn, W. B., A. Erban, R. J. Weber, D. J. Creek, M. Brown, R. Breitling, T. Hankemeier, R. Goodacre, S. Neumann and J. Kopka (2013). "Mass appeal: metabolite identification in mass spectrometry-focused untargeted metabolomics." *Metabolomics* **9**(1): 44-66.

El-Kamali, H. H. and S. A.-T. Mahjoub (2009). "Antibacterial activity of *Francoeuria crispa*, *Pulicaria undulata*, *Ziziphus spina-christi* and *Cucurbita pepo* against seven standard pathogenic bacteria." *Ethnobotanical Leaflets* **2009**(6): 6.

Etienne, C., C. Rothan, A. Moing, C. Plomion, C. Bodenes, L. Svanella-Dumas, P. Cosson, V. Pronier, R. Monet and E. Dirlewanger (2002). "Candidate genes and QTLs for sugar and organic acid content in peach *Prunus persica* (L.) Batsch." *Theoretical and Applied Genetics* **105**(1): 145-159.

Ferreira, D. and X.-C. Li (2000). "Oligomeric proanthocyanidins: naturally occurring *O*-heterocycles." *Natural Product Reports* **17**(2): 193-212.

Fitzgerald, D., M. Stratford, M. Gasson, J. Ueckert, A. Bos and A. Narbad (2004). "Mode of antimicrobial action of vanillin against *Escherichia coli*, *Lactobacillus plantarum* and *Listeria innocua*." *Journal of Applied Microbiology* **97**(1): 104-113.

Friedrich, W., A. Eberhardt and R. Galensa (2000). "Investigation of proanthocyanidins by HPLC with electrospray ionization mass spectrometry." *European Food Research and Technology* **211**(1): 56-64.

Fukuda, T., H. Ito, T. Mukainaka, H. Tokuda, H. Nishino and T. Yoshida (2003). "Anti-tumor promoting effect of glycosides from *Prunus persica* seeds." *Biological and Pharmaceutical Bulletin* **26**(2): 271-273.

Funk, C. D. and W. S. Powell (1983). "Metabolism of linoleic acid by prostaglandin endoperoxide synthase from adult and fetal blood vessels." *Biochimica et Biophysica Acta (BBA)-Lipids and Lipid Metabolism* **754**(1): 57-71.

Gaggeri, R., D. Rossi, M. S. Christodoulou, D. Passarella, F. Leoni, O. Azzolina and S. Collina (2012). "Chiral flavanones from *Amygdalus lycioides* Spach: Structural elucidation and identification of TNFalpha inhibitors by bioactivity-guided fractionation." *Molecules* **17**(2): 1665-1674.

García-Villalba, R., A. Carrasco-Pancorbo, C. Oliveras-Ferraros, J. A. Menéndez, A. Segura-Carretero and A. Fernández-Gutiérrez (2012). "Uptake and metabolism of olive oil polyphenols in human breast cancer cells using nano-liquid chromatography coupled to electrospray ionization–time of flight-mass spectrometry." *Journal of Chromatography* **898**: 69-77.

Garcia, C. J., R. García-Villalba, Y. Garrido, M. I. Gil and F. A. Tomás-Barberán (2016). "Untargeted metabolomics approach using UPLC-ESI-QTOF-MS to explore the metabolome of fresh-cut iceberg lettuce." *Metabolomics* **12**(8): 138.

Gardana, C., M. Scaglianti, P. Pietta and P. Simonetti (2007). "Analysis of the polyphenolic fraction of propolis from different sources by liquid chromatography–tandem mass spectrometry." *Journal of Pharmaceutical and Biomedical Analysis* **45**(3): 390-399.

Geibel, M. and W. Feucht (1991). "Flavonoid 5-glucosides from *Prunus cerasus* bark and their characteristic weak glycosidic bonding." *Phytochemistry* **30**(5): 1519-1521.

Gu, D., Y. Yang, M. Bakri, Q. Chen, X. Xin and H. A. Aisa (2013). "A LC/QTOF–MS/MS application to investigate chemical compositions in a fraction with protein tyrosine phosphatase 1B inhibitory activity from *Rosa rugosa* flowers." *Phytochemical Analysis* **24**(6): 661-670

Guzman, E., C. Pérez, M. Zavala, K. Acosta-Viana and S. Pérez (2008). "Antiprotozoal activity of (8-hydroxymethylen)-trieicosanyl acetate isolated from *Senna villosa*." *Phytomedicine* **15**(10): 892-895.

Habsah, M., A. M. Ali, N. H. Lajis, M. A. Sukari, Y. H. Yap, H. Kikuzaki and N. Nakatani (2005). "Antitumour-Promoting and Cytotoxic Constituents of *Etlingera Elatior*." *The Malaysian Journal of Medical Sciences* **12**(1): 6-12.

Hall, R. D. (2006). "Plant metabolomics: from holistic hope, to hype, to hot topic." *New Phytologist* **169**(3): 453-468.

Hall, R. D. (2018). "Plant metabolomics in a nutshell: potential and future challenges." *Annual Plant Reviews* online: 1-24.

Hanafy, D. M., P. D. Prenzler, G. E. Burrows, D. Ryan, S. Nielsen, S. A. El Sawi, T. S. El Alfy, E. H. Abdelrahman and H. K. Obied (2017). "Biophenols of mints: antioxidant, acetylcholinesterase, butyrylcholinesterase and histone deacetylase inhibition activities targeting Alzheimer's disease treatment." *Journal of Functional Foods* **33**: 345-362.

Hasegawa, M. (1957). "Flavonoids of various *Prunus* species. VI. The flavonoids in the wood of *Prunus aequinoctialis*, *P. nipponica*, *P. Maximowiczii* and *P. avium*." *Journal of the American Chemical Society* **79**(7): 1738-1740.

Herath, W. W., N. D. Rajasekera, M. U. S. Sultanbawa, G. P. Wannigama and S. Balasubramaniam (1978). "Triterpenoid, coumarin and quinone constituents of eleven *Diospyros* species (*Ebenaceae*)." *Phytochemistry* **17**(5): 1007-1009.

Hopfgartner, G., I. V. Chernushevich, T. Covey, J. B. Plomley and R. Bonner (1999). "Exact mass measurement of product ions for the structural elucidation of drug metabolites with a tandem quadrupole orthogonal-acceleration time-of-flight mass spectrometer." *Journal of the American Society for Mass Spectrometry* **10**(12): 1305-1314.

Horiuchi, K., S. Shiota, T. Hatano, T. Yoshida, T. Kuroda and T. Tsuchiya (2007). "Antimicrobial activity of oleanolic acid from *Salvia officinalis* and related compounds on vancomycin-resistant *enterococci* (VRE)." *Biological and Pharmaceutical Bulletin* **30**(6): 1147-1149.

Howlader, M. S. I. (2012). "Characterization of chemical groups and study of antioxidant, antidiarrhoeal, antimicrobial and cytotoxic activities of ethanolic extract of *Diospyros blancoi* (Family: *Ebenaceae*) leaves." *Journal of Pharmacy Research* **5**(6): 3050-3052.

Hussain, T., H. H. Siddiqui, S. Fareed, M. Vijayakumar and C. V. Rao (2012). "Chemopreventive evaluation of *Tephrosia purpurea* against *N*-nitrosodiethylamine-induced hepatocarcinogenesis in Wistar rats." *Journal of Pharmacy and Pharmacology* **64**(8): 1195-1205.

Jang, G. H (2018). "Characterization and quantification of flavonoid glycosides in the *Prunus* genus by UPLC-DAD-QTOF/MS." *Saudi Journal of Biological Sciences* **25**(8): 1622-1631.

Jetty, A., C. Subhakar, D. Rajagopal, M. Jetty, M. Subramanyam and M. Marthanda Murthy (2010). "Antimicrobial activities of neo-and 1-epineo-isoshinanolones from *Plumbago zeylanica* roots." *Pharmaceutical Biology* **48**(9): 1007-1011.

Jiménez-Atiénzar, M., J. Escribano, J. Cabanes, F. Gandía-Herrero and F. García-Carmona (2006). "The flavonoid eriodictyol as substrate of peach polyphenol oxidase." *Journal of Food Science* **70**(9): C540-C544.

Karioti, A., M. Sokovic, A. Ciric, C. Koukoulitsa, A. R. Bilia and H. Skaltsa (2011). "Antimicrobial properties of *Quercus ilex* L. proanthocyanidin dimers and simple phenolics: evaluation of their synergistic activity with conventional antimicrobials and prediction of their pharmacokinetic profile." *Journal of Agricultural and Food Chemistry* **59**(12): 6412-6422.

Kataoka, M., K. Hirata, T. Kunikata, S. Ushio, K. Iwaki, K. Ohashi, M. Ikeda and M. Kurimoto (2001). "Antibacterial action of tryptanthrin and kaempferol, isolated from the indigo plant (*Polygonum tinctorium* Lour.), against *Helicobacter pylori*-infected Mongolian gerbils." *Journal of Gastroenterology* **36**(1): 5-9.

Kazan, A., H. Koyu, I. C. Turu and O. Yesil-Celiktas (2014). "Supercritical fluid extraction of *Prunus persica* leaves and utilization possibilities as a source of phenolic compounds." *The Journal of Supercritical Fluids* **92**: 55-59.

Kichu, M. (2010). "Documentation and Biological and Phytochemical Analysis of Chungtia Medicinal Plants of Nagaland, India." Dissertation, Chemistry and Biomolecular Sciences, Macquarie University, Sydney (PhD): 1-179.

Kobayashi, N., H. Kaneda, Y. Kano and S. Koshino (1993). "The production of linoleic and linolenic acid hydroperoxides during mashing." *Journal of Fermentation and Bioengineering* **76**(5): 371-375.

Kumar, D., G. Kumar and V. Agrawal (2018). "Green synthesis of silver nanoparticles using *Holarrhena antidysenterica* (L.) Wall.bark extract and their larvicidal activity against dengue and filariasis vectors." *Parasitology Research* **117**(2): 377-389.

Lai, J.-P., Y. H. Lim, J. Su, H.-M. Shen and C. N. Ong (2007). "Identification and characterization of major flavonoids and caffeoylquinic acids in three compositae plants by LC/DAD-APCI/MS." *Journal of Chromatography* **848**(2): 215-225.

Lee Chang, K., K. Park Kwang, K. Hwang Jae, K. Lee Sang and Y. Chung Won (2007). "The extract of *Prunus persica* flesh (PPFE) attenuates chemotherapy-induced hepatotoxicity in mice." *Phytotherapy Research* **22**(2): 223-227.

Lee, C. K., K.-K. Park, J.-K. Hwang, S. K. Lee and W.-Y. Chung (2008). "The pericarp extract of *Prunus persica* attenuates chemotherapy-induced acute nephrotoxicity and hepatotoxicity in mice." *Journal of Medicinal Food* **11**(2): 302-306.

Lee, S. Y., J. S. Kim, R. J. Choi, Y. S. Kim, J.-H. Lee and S. S. Kang (2011). "A new polyoxygenated triterpene and two new aeginetic acid quinovosides from the roots of *Rehmannia glutinosa*." *Chemical and Pharmaceutical Bulletin* **59**(6): 742-746.

Lee, W. S., K.-R. Im, Y.-D. Park, N.-D. Sung and T.-S. Jeong (2006). "Human ACAT-1 and ACAT-2 inhibitory activities of pentacyclic triterpenes from the leaves of *Lycopus lucidus*." *Biological and Pharmaceutical Bulletin* **29**(2): 382-384.

Li, W.-X., C.-B. Cui, B. Cai, H.-Y. Wang and X.-S. Yao (2005). "Flavonoids from *Vitex trifolia* L. inhibit cell cycle progression at G2/M phase and induce apoptosis in mammalian cancer cells." *Journal of Asian Natural Products Research* **7**(4): 615-626.

Liang, J., F. Xu, Y. Z. Zhang, X. Y. Zang, D. Wang, M. Y. Shang, X. Wang, D. H. Chui and S. Q. Cai (2014). "The profiling and identification of the metabolites of (+)-catechin and study on their distribution in rats by HPLC-DAD-ESI-IT-TOF-MSn technique." *Biomedical Chromatography* **28**(3): 401-411.

Lim, T. K. (2012). *Prunus persica* var. persica. edible medicinal and non-medicinal plants: Volume 4, Fruits. T. K. Lim. Dordrecht, Springer Netherlands**:** 498-508.

Liu, C., S. Kurakane, J. Takita, R. Itano, T. Soga, A. Oikawa and K. Igarashi (2012). "Antihypertensive effects of unripe persimmon (*Diospyros kaki* L. cv. Hiratanenashi) fruit and its component in spontaneously hypertensive rats." *Food Science and Technology Research* **18**(3): 391-398.

Liu, Q., Z. Jiao, Y. Liu, Z. Li, X. Shi, W. Wang, B. Wang and M. Zhong (2016). "Chemical profiling of San-Huang decoction by UPLC–ESI-Q-TOF-MS." *Journal of Pharmaceutical and Biomedical Analysis* **131**: 20-32.

Liu, R., W. Zhang, Z. Gu, C. Zhang, J. Su and X. Xu (2006). "Two new iridoids from roots of *Patrinia scabra*." *Natural Product Research* **20**(9): 866-870.

López-Cobo, A., A. M. Gómez-Caravaca, F. Pasini, M. F. Caboni, A. Segura-Carretero and A. Fernández-Gutiérrez (2016). "HPLC-DAD-ESI-QTOF-MS and HPLC-FLD-MS as valuable tools for the determination of phenolic and other polar compounds in the edible part and by-products of avocado." *LWT -Food Science and Technology* **73**: 505-513.

López-Gutiérrez, N., R. Romero-González, J. L. Martínez Vidal and A. G. Frenich (2016). "Determination of polyphenols in grape-based nutraceutical products using high resolution mass spectrometry." *LWT - Food Science and Technology* **71**: 249-259.

Luo, Y., L.-Z. Cheng, Q. Luo, Y.-M. Yan, S.-M. Wang, Q. Sun and Y.-X. Cheng (2017). "New ursane-type triterpenoids from *Clerodendranthus* spicatus." *Fitoterapia* **119**: 69-74.

Malewska, T. (2015). "Biological and phytochemical analysis of Chungtia medicinal plants of Nagaland, India." Dissertation, Chemistry and Biomolecular Sciences, Macquarie University, Sydney (MPhil): 1-226.

María, G. R., A. R. David, M. T. Rosario, G. S. Patricia, S. C. Antonio and F. G. Alberto (2007). "Antioxidant compounds of propolis determined by capillary electrophoresis–mass spectrometry." *Journal of Separation Science* **30**(4): 595-603.

Markley, J. L., R. Brüschweiler, A. S. Edison, H. R. Eghbalnia, R. Powers, D. Raftery and D. S. Wishart (2017). "The future of NMR-based metabolomics." *Current opinion in biotechnology* **43**: 34-40.

Masui, H., T. Kondo and M. Kojima (1989). "An antifungal compound, 9,12,13-trihydroxy-(*E*)-10-octadecenoic acid, from *Colocasia antiquorum* inoculated with *Ceratocystis fimbriata*." *Phytochemistry* **28**(10): 2613-2615.

Matsuda, H., T. Morikawa, K. Ninomiya and M. Yoshikawa (2001). "Hepatoprotective constituents from *Zedoariae* rhizoma: absolute stereostructures of three new carabrane-type sesquiterpenes, curcumenolactones A, B, and C." *Bioorganic & Medicinal Chemistry* **9**(4): 909-916.

Matsuoka, N., T. Ikeda, M. El-Aasr, H. Manabe, Y. Murakami, H. Deguchi and T. Nohara (2011). "Study of the chemical constituents of Pruni cortex and its related parts." *Journal of Natural Medicines* **65**(1): 166-171.

Mishra, P. M. and A. Sree (2007). "Antibacterial activity and GCMS analysis of the extract of leaves of *Finlaysonia obovata* (a mangrove plant)." *Asian Journal of Plant Sciences* **6**: 168-172.

Moing, A., J.-L. Poëssel, L. Svanella-Dumas, M. Loonis and J. Kervella (2003). "Biochemical basis of low fruit quality of *Prunus davidiana*, a pest and disease resistance donor for peach breeding." *Journal of the American Society for Horticultural Science* **128**(1): 55-62.

Montevecchi, G., G. V. Simone, F. Masino, C. Bignami and A. Antonelli (2012). "Physical and chemical characterization of Pescabivona, a Sicilian white flesh peach cultivar (*Prunus persica* (L.) Batsch)." *Food Research International* **45**(1): 123-131.

Nagarajan, S., L. Jagan Mohan Rao and K. Gurudutt (2001). "Chemical composition of the volatiles of *Decalepis hamiltonii* (Wight & Arn)." *Flavour and Fragrance Journal* **16**(1): 27-29.

Neogi, U., R. Saumya, R. K. Mishra and K. Raju (2008). "Lipid content and *in vitro* antimicrobial activity of oil seeds of some Indian medicinal plants." *Current Research in Bacteriology* **1**: 1-6.

Ohigashi, H., S. Minami, H. Fukui, K. Koshimizu, F. Mizutani, A. Sugiura and T. Tomana (1982). "Flavanols, as plant growth inhibitors from roots of peach, *Prunus persica* Batsh. cv. 'Hakuto'." *Agricultural and Biological Chemistry* **46**(10): 2555-2561.

Pacheco, H. (1960). "The structure of a glucoside of aromadendrin extracted from sapwood of *P. mahaleb* and *P. persica vulgaris*." *Compte Rendu Hebdomadaire des Seances de l'Academie des Sciences* **251**(16): 1653-1654.

Paran, R., M. Ben, D. Pauline Mc and C. Ian Edwin (2016). "Phytochemical analysis of *Tasmannia lanceolata* extracts and inhibition of *Giardia duodenalis* proliferation." *Pharmacognosy Journal* **8**(3) 291-299.

Pereira, V., J. S. Câmara, J. Cacho and J. C. Marques (2010). "HPLC-DAD methodology for the quantification of organic acids, furans and polyphenols by direct injection of wine samples." *Journal of Separation Science* **33**(9): 1204-1215.

Petar, R., T. Jelena, G. Uroš, A. Filip, N. Nebojša, T. Živoslav and M. O. Dušanka (2015). "Ultrahigh-performance liquid chromatography and mass spectrometry (UHPLC–LTQ/Orbitrap/MS/MS) study of phenolic profile of serbian poplar type propolis." *Phytochemical Analysis* **26**(2): 127-136.

Puupponen-Pimiä, R., L. Nohynek, C. Meier, M. Kähkönen, M. Heinonen, A. Hopia and K. M. Oksman-Caldentey (2001). "Antimicrobial properties of phenolic compounds from berries." *Journal of Applied Microbiology* **90**(4): 494-507.

Quilot, B., B. H. Wu, J. Kervella, M. Génard, M. Foulongne and K. Moreau (2004). "QTL analysis of quality traits in an advanced backcross between *Prunus persica* cultivars and the wild relative species *P. davidiana*." *Theoretical and Applied Genetics* **109**(4): 884-897.

Rahman, W. and S. Bhatnagar (1968). "Flavonoids from *Prunus persica* Batsch (peach bark)." *Australian Journal of Chemistry* **21**(2): 539-541.

Ren, Q., Y.-l. Wang, M.-l. Wang and H.-y. Wang (2016). "Screening and identification of the metabolites in rat urine and feces after oral administration of *Lycopus lucidus* Turcz extract by UHPLC− Q-TOF-MS mass spectrometry." *Journal of Chromatography* **1027**: 64-73.

Samaga, P. V. and V. R. Rai (2013). "Evaluation of pharmacological properties and phenolic profile of *Hypericum japonicum* Thunb. from Western Ghats of India." *Journal of Pharmacy Research* **7**(7): 626-632.

Schwikkard, S. L. and D. A. Mulholland (2014). "Useful methods for targeted plant selection in the discovery of potential new drug candidates." *Planta Medica* **80**(14): 1154-1160.

Sheikh, I. A. (2016). "Phytochemical and biotechnological studies on *Diospyros kaki* (family *Ebenaceae*) cultivated in Egypt." Master in Pharmaceutical Sciences thesis.

Silva, D. B., L. T. Okano, N. P. Lopes and D. C. R. de Oliveira (2013). "Flavanone glycosides from *Bidens gardneri* Bak. (*Asteraceae*)." *Phytochemistry* **96**: 418-422.

Sinha, A. K., U. K. Sharma and N. Sharma (2008). "A comprehensive review on vanilla flavor: extraction, isolation and quantification of vanillin and others constituents." *International Journal of Food Sciences and Nutrition* **59**(4): 299-326.

Songsong, W., X. Haiyu, M. Yan, W. Xuguang, S. Yang, H. Bin, T. Shihuan, Z. Yi, L. Defeng and L. Rixin (2015). "Characterization and rapid identification of chemical constituents of NaoXinTong capsules by UHPLC-linear ion trap/Orbitrap mass spectrometry." *Journal of Pharmaceutical and Biomedical Analysis* **111**: 104-118.

Sonibare, M. A., I. O. Ayoola, B. Gueye, M. T. Abberton, R. D'Souza and N. Kuhnert (2018). "Leaves metabolomic profiling of *Musa acuminata* accessions using UPLC–QTOF–MS/MS and their antioxidant activity. "*Journal of Food Measurement and Characterization* **12**(2): 1093–1106.

Spínola, V., J. Pinto and P. C. Castilho (2015). "Identification and quantification of phenolic compounds of selected fruits from Madeira Island by HPLC-DAD–ESI-MSn and screening for their antioxidant activity." *Food Chemistry* **173**: 14-30.

Sudjaroen, Y., W. E. Hull, G. Erben, G. Würtele, S. Changbumrung, C. M. Ulrich and R. W. Owen (2012). "Isolation and characterization of ellagitannins as the major polyphenolic components of Longan (*Dimocarpus longan* Lour) seeds." *Phytochemistry* **77**: 226-237.

Süzgeç, S., A. H. Meriçli, P. J. Houghton and B. Çubukçu (2005). "Flavonoids of *Helichrysum compactum* and their antioxidant and antibacterial activity." *Fitoterapia* **76**(2): 269-272.

Tanachatchairatana, T., J. B. Bremner, R. Chokchaisiri and A. Suksamrarn (2008). "Antimycobacterial activity of cinnamate-based esters of the triterpenes betulinic, oleanolic and ursolic acids." *Chemical and Pharmaceutical Bulletin* **56**(2): 194-198.

Terry, L. A. (2011). "Health-promoting Properties of Fruits and Vegetables." CABI google books: 238-259.

Tripoli, E., M. La Guardia, S. Giammanco, D. Di Majo and M. Giammanco (2007). "Citrus flavonoids: molecular structure, biological activity and nutritional properties: A review." *Food Chemistry* **104**(2): 466-479.

Tsimogiannis, D., M. Samiotaki, G. Panayotou and V. Oreopoulou (2007). "Characterization of flavonoid subgroups and hydroxy substitution by HPLC-MS/MS." *Molecules* **12**(3): 593-606.

Vallverdú-Queralt, A., J. Regueiro, M. Martínez-Huélamo, J. F. Rinaldi Alvarenga, L. N. Leal and R. M. Lamuela-Raventos (2014). "A comprehensive study on the phenolic profile of widely used culinary herbs and spices: rosemary, thyme, oregano, cinnamon, cumin and bay." *Food Chemistry* **154**: 299-307.

Wang, H., W.-l. Mei, Y.-b. Zeng, W.-j. Zuo, Z.-k. Guo, L.-l. Chen, H.-m. Zhong and H.-f. Dai (2014). "Phenolic compounds from *Dalbergia odorifera*." *Phytochemistry Letters* **9**: 168-173.

Wong, L. L. (2015). "Ingredient authentication of commercial Xihuangcao herbal tea by a microscopic technique combined with UPLC-ESI-QTOF-MS/MS." *Analitycal Methods* **7**: 4257-4268

Wu, Z. J., X. Z. Chen, D. M. Fang, H. Y. Qi, W. J. Ren and G. L. Zhang (2009). "Analysis of phenolic glycosides from *Ilex litseaefolia* using electrospray ionization quadrupole time-of-flight mass spectrometry." *Rapid Communications in Mass Spectrometry* **23**(23): 3881-3885.

Yadav, D. K. (2016). "Importance of Neem Leaf: An insight into its role in combating diseases." *Indian Journal of Experimental Biology* **54**(11): 708-718.

Yaltirak, T., B. Aslim, S. Ozturk and H. Alli (2009). "Antimicrobial and antioxidant activities of Russula delica Fr." *Food and Chemical Toxicology* **47**(8): 2052-2056.

Yang, J., D. Qian, S. Jiang, E.-x. Shang, J. Guo and J.-a. Duan (2012). "Identification of rutin deglycosylated metabolites produced by human intestinal bacteria using UPLC–Q-TOF/MS." *Journal of Chromatography* **898**: 95-100.

Ye, J. F. (2017). "Original article molecular cloning and characterization of an anthocyanidin synthase gene in *Prunus persica* (L.) Batsch." *Notulae Botanicae Horti Agrobotanici Cluj-Napoca* **45**(1): 28-35.

Yuan, L., Z. Xiaocui, W. Tongbo, Z. Meiping and L. Huwei (2012). "Rapid and sensitive detection of auxins and flavonoids in plant samples by high-performance liquid chromatography coupled with tandem mass spectrometry." *Journal of Separation Science* **35**(19): 2559-2566.

Zang, X., M. Shang, F. Xu, J. Liang, X. Wang, M. Mikage and S. Cai (2013). "A-Type proanthocyanidins from the stems of *Ephedra sinica* (*Ephedraceae*) and their antimicrobial activities." *Molecules* **18**(5): 5172-5189.

Zhang, W., Q. Shi, Y. Shen and H. Chen (2007). "A new flavanol from *Daphne genkwa*." *Fitoterapia* **78**(7): 596-597.

Zhao, X., W. Zhang, X. Yin, M. Su, C. Sun, X. Li and K. Chen (2015). "Phenolic composition and antioxidant properties of different peach *Prunus persica* (L.) Batsch cultivars in China." *International Journal of Molecular Sciences* **16**(3): 5762-5778.

Zhong, S.-M., P. G. Waterman and J. A. D. Jeffreys (1984). "Naphthoquinones and triterpenes from african *Diospyros* species." *Phytochemistry* **23**(5): 1067-1072.

Zhou, C., Y. Sheng, D. Zhao, Z. Wang and J. Tao (2010). "Variation of oleanolic and ursolic acid in the flesh of persimmon fruit among different cultivars." *Molecules* **15**(9): 6580-6587.

Zu, Y., C. Li, Y. Fu and C. Zhao (2006). "Simultaneous determination of catechin, rutin, quercetin kaempferol and isorhamnetin in the extract of sea buckthorn (*Hippophae rhamnoides* L.) leaves by RP-HPLC with DAD." *Journal of Pharmaceutical and Biomedical Analysis* **41**(3): 714-719.

Chapter 6 - Experimental

6.1 Reagents and equipment

All solvents used for extraction, chromatographic separations and HPLC, GC and MS analysis were of HPLC grade and were used without further purification. This included ethanol, *n*-hexane, dichloromethane (DCM), ethyl acetate (EtOAc), chloroform, diethyl ether and petroleum ether (b.p. 40 ^{0}C) (Merck, Germany), methanol (Sigma-Aldrich) and acetonitrile (VWR BDH, Australia). Formic acid, sulfuric acid, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), vanillin (99%), gallic acid, catechin, β-sitosterol and plumbagin (95%) were from Sigma-Aldrich (Australia), and were used without further purification. Dimethyl sulfoxide (DMSO) was purchased from Cambridge Isotope Laboratories Inc., USA. Water used was purified by a Barnstead, GenPure xCAD Plus Ultrapure Water Purification System (Thermo Fisher Scientific, USA). Filter paper discs (6 mm) used for disc diffusion assays were from Whatman (UK). Kanamycin, gentamycin, vancomycin and fluconazole were obtained from Amresco (USA). Mueller Hinton II agar, horse blood agar, Sabouraud dextrose (SAB) broth, potato dextrose agar, Todd Hewitt (TH) broth and Mueller Hinton (MH) II broth were purchased from Bacto laboratories Pty Ltd (Australia).

Analytical normal phase thin layer chromatography (TLC) was performed on fluorescent Merck silica gel F_{254} plates (Germany). Preparative TLC (PTLC) was carried out using Uniplate preparative TLC plates (Sigma-Aldrich, Australia). The TLC plates were visualised using UV light (254 nm and 365 nm) and vanillin-sulfuric acid spray reagent. Vanillin-sulfuric acid reagent was prepared by mixing 6 g vanillin with 2.5 mL concentrated H_2SO_4 in 250 mL ethanol. Ferric chloride was used for detection of phenols. This was prepared by dissolving ferric chloride (0.5 g in 10 mL of methanol). The mixture was filtered before use. UV-Visible spectra were recorded on a CARY 1 Bio spectrophotometer (Varian, USA). IR spectra were recorded on a NICOLET iS10 (Thermo Fisher Scientific, USA) instrument. Optical rotations were measured using a P-1010 polarimeter (JASCO, Japan). A Stuart Scientific melting point detector (Stuart Scientific, UK) was used for determining melting points. A Shimadzu 2010 LC-MS system was used for electrospray ionisation mass spectrometry (ESI-MS). A Shimadzu GC-17 system was used for electron impact mass spectrometry (EI-MS). Analytical gas chromatography (GC) was carried out on a Shimadzu GC-17A gas chromatograph with an FID detector as well as Shimadzu GCMS-QP2010 (Shimadzu, Japan) with a split-splitless injector and a Restek Rxi-5Ms fused silica capillary column (30 m x 0.25 mm, 0.25 µm film). Normal phase column chromatography was performed using silica gel 60 (0.040-0.063 mm, Merck, Germany). Size exclusion chromatography (SEC) was carried out using Sephadex LH-20 (18-111 µm, GE Healthcare Bio-sciences AB, Sweden) or using a Biotage column (25 g normal phase

silica SNAP cartridge, flow rate 30 mL/min, Biotage Isolera). Organic solvents were evaporated using a Büchi rotary evaporator (Switzerland). Freeze drying was conducted with a CHRIST alpha 1-4 LD*plus* (Labconco, USA) freeze drier.

^{1}H, ^{13}C, COSY, HSQC, HMBC and NOESY NMR spectra were recorded on Bruker Avance AMX 400 and Bruker DRX600K 600 MHz NMR Spectrometers (Germany) using standard pulse sequences. The ^{1}H and ^{13}C chemical shifts were referenced relative to the residual chloroform (^{1}H δ 7.24 and ^{13}C δ 77.2), acetone (^{1}H δ 2.09 and δ C 205.8 and 30.6), methanol (^{1}H δ 3.31 and ^{13}C δ 49.0) and DMSO (^{1}H δ 2.50 and ^{13}C δ 39.51) solvent (Cambridge Isotope Laboratories, USA) signals (deuterated solvents). *J* values were measured in Hz. High resolution mass spectrometry (HR-MS) was determined using a Bruker Apex 3 instrument.

6.1.1 Preparation of plant material for antimicrobial testing

All plant materials (*Prunus persica* (L.) Stokes (Rosaceae) roots, voucher number 69503, *Diospyros lanceifolia* Roxb. (Ebenaceae) leaves, voucher number 69544 and *Holboellia latifolia* Wall. (Lardizabalaceae) leaves, voucher number 69500) were collected from Chungtia village, Nagaland, India, by the villagers with the assistance of Mr Anungba Jamir, a Chungtia Senso Mokokchung Town (CSMT) representative. The collections were done between the months of June and August 2007 and 2009.

All plant materials were thoroughly inspected by Mr Anungba Jamir for precise identification of the species and dried in the shade for 10 to 20 days. Voucher specimens for each plant were deposited at Botanical Survey of India (BSI), Shillong Branch, India. For further processing, *D. lanceifolia* and *H. latifolia* were transported to Chennai, India to Dr Velmurugan, while *P. persica* (which was collected and dried at a different time), was transported to Dr Udaya Sankar of Central Food Technological Research Institute (CFTRI), located in Mysore, India. Upon receipt by Dr Velmurugan or Dr Sankar, the plant materials were separated from foreign particles, washed with clean water, dried in the shade for 24 hours, then dried in a vacuum drier at 75 to 85 ^{o}C. After 48 hours of drying under vacuum, the plant materials were kept in the shade for three days and rechecked for foreign particles. After confirming that there were no foreign particles, the plant materials were chopped and passed through a micro pulveriser. The process was repeated until 130-200 mesh size was obtained. The powders were then sieved and dried again under vacuum to ensure that they did not contain any moisture. The plant materials were allowed to cool in the shade and packed into plastic bags, which in turn were packed in plastic containers and sealed. The sealed containers were couriered to Australia, under the import permit IP12012991from Department of Agriculture, Fisheries and Forestry (DAFF).

6.1.2 Preparation of dried plant materials for large scale extraction

Powdered dried root of *P. persica* and leaves of *D. lanceifolia* and *H. latifolia* (100 g each) were separately suspended in 70% aqueous ethanol (1 L) and shaken overnight (orbital shaker) at room temperature. The extracts were then filtered, and the process repeated three times. After removal of ethanol by rotary evaporation (Büchi) at 37 °C, the remaining aqueous residues (approximately 900 mL) were successively partitioned with *n*-hexane (3 x 1 L), dichloromethane (3 x 1 L) and ethyl acetate (3 x 1 L). This afforded four partitions for each plant material, *i.e. n*-hexane, dichloromethane and ethyl acetate partitions and the remaining water residue which was deemed the water partition. The partitions were evaporated to dryness by rotary evaporation, then freeze dried. Weights, appearances and yields of the extracts are presented in Table 6.1.

Table 6.1 Weight of dried extracts from large scale extraction.

Plant sample	Weight	Crude extracts yield and appearance				
		Crude	*n*-Hexane	DCM	EtOAc	water
P. persica roots	100 g	25 g (dark green)	2.2 g (dark green)	6.3 g (reddish green)	8.2 g (red)	5.5 g (red)
D. lanceifolia leaves	100 g	32.6 g (green)	12.8 g (green)	4.2 g (yellowish green)	6.8 g (dark green)	4.2 g (green)
H. latifolia leaves	100 g	8.2 g (dark green)	3.5 g (light green)	1.1 g (green)	0.5 g (dark green)	1.5 g (dark green)

6.2 Bioassay: methods and materials

6.2.1 Microbes and culture preparation

The use of all microbial strains was approved by the Macquarie University Biosafety Committee (approval references TEM170512BHA and 5201600535). All cultures were provided by Dr John Merlino (Department of Microbiology, Concord Hospital, Sydney). These included a susceptible strain of *Staphylococcus aureus* (ATCC 29213), community acquired methicillin resistant (MRSA) *Staphylococcus aureus* (ATCC BAA 1026), wild multidrug resistant (MDRSA) clinical isolate of *Staphylococcus aureus*, β lactamase negative (β-; sensitive to β-lactam antibiotics) *Escherichia coli* (ATCC 25922), β lactamase positive (β+; resistant to β-lactam antibiotics) *Escherichia coli* (ATCC 35218), *Pseudomonas aeruginosa* (ATCC 27853; sensitive to common antibiotics), clinical isolates of *Streptococcus pyogenes* and *Salmonella typhimurium*, as well as a clinical isolate of *Candida albicans*. Stock cultures of the bacterial strains were maintained in MH II broth containing 10% *v/v* glycerol. The stock culture of the fungus was maintained in SAB broth containing 10% *v/v* glycerol. Fresh subcultures were made by inoculating the bacterial cultures into MH II broth with the exception

of *S. pyogenes* which was inoculated into TH broth and the fungus in to SAB broth, followed by an overnight incubation at 37 ºC (bacteria) and 30 ºC (fungus). After overnight incubation the optical density at 600 nm (OD600) was measured and the density was adjusted to 0.08 with fresh MH II, TH or SAB broths, as appropriate. This resulted in microbial densities, as noted below in Table 6.2. Vancomycin and kanamycin were used as positive controls for *S. aureus* strains (susceptible *S. aureus*, MRSA and MDRSA) and gentamycin was used for β-, β+ *E. coli*, *P. aeruginosa*, *S. typhimurium* and *S. pyogenes*. Fluconazole was used as a positive control for *C. albicans*. The MTT microdilution assay was used for the screening of the crude and partitioned extracts. TLC bioautography was used for the identification of active column fractions and to determine minimum inhibitory quantity (MIQ) of the purified compounds.

Table 6.2 Microbial strains used in antimicrobial assays of crude plant materials, partitioned extracts and partly purified and purified compounds.

Organism	Strain[a]	Characteristics	cfu/mL (A_{600} = 0.08)[b]
Escherichia coli (β-)	ATCC 25922	β lactamase negative sensitive to common antibiotics	2.54×10^7
Escherichia coli (β+)	ATCC 35218	β lactamase positive resistant to β-lactam antibiotics	5.32×10^7
Pseudomonas aeruginosa	ATCC 27853	sensitive to common antibiotics	6.97×10^7
Salmonella typhimurium		clinical isolate	8.59×10^7
Streptococcus pyogenes		clinical isolate	3.70×10^7
Staphylococcus aureus (susceptible)	ATCC 29213	sensitive to common antibiotics	9.62×10^7
Staphylococcus aureus (MRSA)	ATCC BAA 1026	community acquired methicillin resistant *S. aureus* (MRSA)	6.38×10^7
Staphylococcus aureus (MDRSA)		wild multidrug resistant (MDRSA) clinical isolate	3.76×10^8
Candida albicans		fungus, clinical isolate	4.56×10^5

[a]American Type Culture Collection (ATCC) strain designation where applicable. [b]The inoculum sizes (cfu/mL) at optical density 0.08 (λ 600 nm) were estimated using the spread plate colony count method.

6.2.2 Determination of minimum inhibitory concentration (MIC)

The MTT microdilution assay was used to quantify the minimum inhibitory concentration (MIC) values of the plant extracts (Awouafack et al. 2013). The plant samples (10 mg/mL) or the antibiotic (1 mg/mL) were dissolved in 200 µL DMSO and diluted with MH II broth to a final volume of 1 mL for all bacterial strains, except for *S. pyogenes* for which TH broth was used. SAB broth was used for *C. albicans*. Using a 96 well microtitre plate (Greiner), 100 µL of suitable broth was dispensed into wells 1-11 (from left to right) for each row, 100 µL of the samples or antibiotic was added to well 1 (in different rows for each sample) and mixed thoroughly, after which 100 µL was taken out and

dispensed into the next well (*i.e.* well 2). This process of two-fold serial dilution was carried out until well 10, and skipping well 11, with the final volume dispensed into well 12. 100 µL each of the microbial inoculum was dispensed into wells 1 to 11, leaving well 12. Since well 11 was free of the test sample or the antibiotic, this acted as a positive control for the growth of the inoculum and well 12 being free of inoculum served as the sterile control of the assay. 2% DMSO/H_2O was also included as a solvent control. The plate was incubated at 37 °C for 18 to 20 hours. 20 µL of MTT solution in methanol (5 mg/mL) was added to each well followed by incubation for 30 minutes. The MIC value was determined as the lowest concentration of the test sample or antibiotic that showed no visible colour change from yellow to blue. All wells with the samples that showed no microbial growth (*i.e.* yellow wells) were assessed for their minimum bactericidal concentration (MBC) against the bacterial strains and minimum fungicidal concentration (MFC) against *C. albicans* by subculturing the tested material onto fresh agar plates. Plates were incubated overnight at 37 °C. The lowest concentrations showing no growth were identified as the MBCs or MFCs. All assays were done in duplicate.

6.2.3 TLC bioautography analysis

For the TLC bioautography, the method described by Rahalison *et al.* (Rahalison et al. 1991) with modifications was used. An inoculum of each test microbe was prepared in the broth by overnight incubation. After incubation, optical density of the culture was measured and adjusted to 0.16 by diluting with the broth (1:50), then an equal volume of molten agar (warm MH II agar) was added, resulting in a final dilution of 0.08 optical density. Approximately 10 mL of the inoculum was rapidly distributed over the TLC plates (10 × 10 cm). After solidification of the medium, the overlayed TLC plate was incubated overnight at 37 ºC. The bioautogram was then gently covered with a methanolic solution (2.5 mg/mL) of MTT with a sterile micropipette, and then incubated for 5 minutes at 37 ºC for visualisation of the results. The minimum inhibitory quantity (MIQ) values of the pure compounds were defined as the minimum quantity of the compounds that showed clear zones of inhibition. MIQ values were calculated as follow:

MIQ (µg) = C (µg/µL) x V (µL)
C - the concentration of the compound used
V- the volume of compound solution applied onto the TLC plate

All assays were done in duplicate.

6.3 Phytochemical analysis

The *n*-hexane and ethyl acetate partition of *P. persica*, *n*-hexane partition of *D. lanceifolia* and dichloromethane partition of *H. latifolia* (10 mg/mL, 20 µL in methanol) were applied on TLC plates

in duplicate and developed with the following solvent systems: chloroform:methanol 7:3 for the *P. persica* ethyl acetate partition; petroleum ether (b.p. 40 ^{0}C):diethyl ether 6:4 for the *P. persica* *n*-hexane partition; petroleum ether:chloroform (6:4) for the *D. lanceifolia* *n*-hexane partition; and EtOAC:chloroform:methanol 3:1:1 for the *H. latifolia* dichloromethane partition. Chemical constituents were detected by visualisation at 254 and 365 nm, and by spraying with vanillin-sulfuric acid reagent and heating of the plates at 100 ºC for 1-2 minutes.

6.4 GC-MS analysis

Analytical gas chromatography (GC) of the *n*-hexane fraction of *D. lanceifolia* and dichloromethane fraction of *H. latifolia* was carried out on a Shimadzu GC17A gas chromatograph with a BP-5 column (30 m×0.25 mm×25 µm) that was programmed from 35-250 ºC at 3 ºC/min with helium as the carrier gas. The injector and detector were both programmed at 220 ºC. GC integrations were performed on an SMAD electronic integrator. GC-MS was carried out on a Shimadzu GCMS-QP5000 mass spectrometer operating at 70 eV ionisation energy. Mass spectra were recorded in electron impact (EI) mode at 70 eV, scanning the 41-450 *m/z* range. Compounds were identified by their matching GC retention indices relative to *n*-alkanes and by comparison of their mass spectra with either known compounds (NIST database) or published spectra.

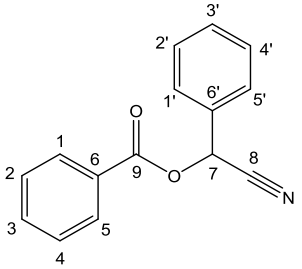
GC-MS analyses of the isolated compounds lupeol, plumbagin, β-sitosterol, stigmast-4-en-3-one and α-cyanobenzyl benzoate were done using the Shimadzu GC-MS-QP2010 (Shimadzu Corporation, Japan) with a split-splitless injector and a Restek Rxi-5Ms fused silica capillary column (30 m x 0.25 mm, 0.25 µm film) and in EI mode. Helium gas (BOC, NSW, Australia) (99.999%) was used as a carrier gas for the TD-20 and GC-MS, while dry nitrogen gas (liquid N_2 boil-off, BOC) was used for the TD-20 purge. The desorption method consisted of first sampling the tubes for 15 minutes at a flow rate of 60 mL min^{-1} with the secondary trap set to 0 °C, before heating the trap to 300 °C for 12 minutes. During both sampling and desorption from the secondary trap, the valve, transfer lines and the interface to the GC-MS were heated to 275 °C. The initial column temperature was 70 °C and held for 1 minute. Thereafter it was increased to 280 °C at a rate of 10 °C min^{-1} before being held at 280 °C for 2 minutes. The split ratios ranged from 1:20 to 1:200. The ion source and interface temperatures in the MS were 200 °C and 290 °C, respectively. The data was acquired either in scan mode at a scan speed of 5000 with an *m/z* range of 45 to 300 or by single ion monitoring (SIM). The data were processed using GC-MS solution Software (V2.72) from Shimadzu to obtain the peak areas.

6.5 Chemical study: methods and materials

6.5.1 Bioautography guided isolation of active compounds from *P. persica n*-hexane partition

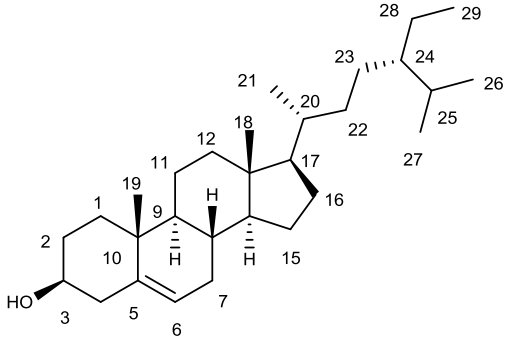
The *n*-hexane partition of *P. persica* (1 g) was dissolved in methanol (2 mL), mixed with silica (0.5 g) and evaporated to dryness by rotary evaporation at 37 ºC. The sample was then applied to the top of a normal phase silica gel column (1000 mL, 270 g silica) prepared with 100% petroleum ether (b.p. 40 ^{0}C). The column was eluted with a gradient of petroleum ether/diethyl ether 0 to 100%. Forty fractions (20 mL each) were collected, spotted in duplicate on TLC plates and developed with petroleum ether (b.p. 40 ^{0}C):diethyl ether 3:7. The initial 40 fractions were combined into five major sub-fractions (Hex-1 – Hex-5 in order of increasing polarity) according to similar TLC R_f values. One of the TLC plates was then stained with vanillin-sulfuric acid reagent, and the other was used for overlay TLC bioautography testing against susceptible *S. aureus,* MRSA and MDRSA. Hex-1 (150 mg, yellow oil, three merged spots, R_f 0.30-0.58; 0.45 active), Hex-2 (130 mg, white solid, four spots, R_f 0.25, 0.46, 0. 65, 0.70, no activity), Hex-3 (65 mg, white solid, four spots, R_f 0.65, 0.70, 0.75, 0.85, no activity), Hex-4 (126 mg, two spots, R_f 0.58, 0.77, no activity) and Hex-5 (82 mg, three spots, R_f 0.72, 0.73, 0.79; 0.79 active). Further purification of Hex-1 was done using Sephadex LH-20 (50 g in MeOH) column chromatography that was prepared and eluted with methanol. This yielded α-cyanobenzyl benzoate (**1**, 1.5 mg, R_f 0.45, petroleum ether:diethyl ether 3:7). Recrystallisation with methanol of fraction Hex-4 yielded β-sitosterol (**2**, 65 mg R_f 0.77, petroleum ether:diethyl ether 3:7). Further purification of Hex-5 by Sephadex LH-20 column chromatography, eluting with methanol, yielded stigmast-4-en-3-one (**3**, 1. 7 mg, R_f 0.79, petroleum ether:diethyl ether 3:7).

α-Cyanobenzyl benzoate **(1)**: Yellow oil; IR (neat) υ_{max} (cm^{-1}): 2248, 1732, 1248; EI-MS *m/z* 237, 115, 105, 77, 51; ^{1}H NMR (400 MHz, CD_3OD) δ 8.01 (2H, *dd*, *J* 7.4, 2.0, H-1 and H-5), 7.73 (1H, *td*, *J* 7.4, 2.0 H-3), 7.70 (2H, *dd*, *J* 7.7, 2.0, H-1' and H-5'), 7.57 (2H, *dd*, 7.4, 7.4 H-2 and H-4), 7.53 (2H, *m*, H-2' and H-4'), 7.40 (1H, *m*, H-3'), 6.93 (1H, *s*, H-7); ^{13}C NMR (150 MHz, CD_3OD) δ 63.5 (C-7), 117.6 (C-8), 127.8 (C-2 and C-4), 129.1 (C-1' and C-5'), 129.6 (C-3'), 129.8 (C-1 and C-5), 130.2 (C-2' and C-4'), 131.3 (C-6), 134.7 (C-3), 135.2 (C-6'), 165.9 (C-9).

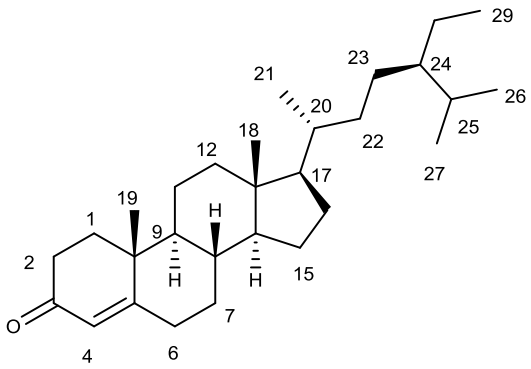


β-Sitosterol **(2)**: White needle-like crystals, m.p. 138-139 ºC, lit. 136-139 ºC (Kircher et al. 1973). $[\alpha]^{25}_D$: -41°, chloroform lit. $[\alpha]^{25}_D$: -41°, in chloroform (Kircher et al. 1973). IR (neat) υ_{max} (cm^{-1}):

3400 (broad, strong), 2955, 2918, 2850, 1735, 1463, 1377, 1057; EI-MS *m/z* 414, 396, 381, 367, 354, 341, 329, 315, 303, 288, 273, 255, 241, 231, 213, 199, 185, 173, 159, 145, 133, 119, 105, 95, 81, 55; fragmentation pattern identical with the authentic sample (Sigma-Aldrich). HREI-MS *m/z* 414.3855 (calc. 414.3862 for $C_{29}H_{50}O$); ^{1}H NMR (400 MHz, $CDCl_3$) δ 5.27 (1H, *m*, H-6), 3.50 (1H, *m*, H-3), 2.20 (1H, *m*, H-4), 2.16 (1H, *m*, H-4), 1.94 (1H, *m*, H-12), 1.90 (2H, *m*, H-8), 1.77 (2H, *m*, H-1), 1.76 (1H, *m*, H-7), 1.43 (1H, *m*, H-7), 1.42 (2H, *m*, H-2), 1.59 (2H, *m*, H-25), 1.50 (2H, *m*, H-15) 1.28 (1H, *m*, H-20), 1.25 (2H, *m*, H-22), 1.29 (2H, *m*, H-22), 1.18 (2H, *m*, H-28), 1.19 (2H, *m*, H-16), 1.08 (2H, *m*, H-23), 1.03 (1H, *m*, H-17), 0.93 (3H, *s*, H-19), 0.92 (1H, *m*, H-14), 0.86 (1H, *m*, H-9), 0.85 (3H, *d*, *J* 6.9, H-21), 0.85 (1H, *m*, H-24), 0.61 (3H, *s*, H-18); ^{13}C NMR (150 MHz, $CDCl_3$) δ 37.22 (C-1), 31.64 (C-2), 71.80 (C-3), 42.27 (C-4), 140.73 (C-5), 121.72 (C-6), 31.87 (C-7), 31.89 (C-8), 50.09 (C-9), 36.48 (C-10), 21.06 (C-11), 39.74 (C-12), 42.30 (C-13), 56.74 (C-14), 24.28 (C-15), 28.23 (C-16), 56.01 (C-17), 11.84 (C-18), 19.81 (C-19), 36.13 (C-20), 18.76 (C-21), 33.91 (C-22), 26.01 (C-23), 45.79 (C-24), 29.10 (C-25), 19.00 (C-26), 19.38 (C-27), 23.03 (C-28), 11.96 (C-29).



Stigmast-4-en-3-one **(3):** White solid, m.p. 89-91 ºC, lit. 89-91 ºC (Kolak et al. 2001). $[\alpha]^{27}_{D}$: +71° (chloroform); lit. +72° in chloroform (Choi et al. 2002). EI-MS *m/z* 412, 397, 370, 327, 288, 271, 229, 207, 147, 135, 124, 107, 95, 55; ^{1}H NMR (600 MHz, CD_3COCD_3) δ 5.62 (1H, *s*, H-4), 2.45 (1H, *m*, H-6), 2.40 (1H, *m*, H-2), 2.26 (1H, *m*, H-6), 2.18 (1H, *m*, H-2), 2.03 (1H, *m*, H-12), 2.02 (1H, *m*, H-1), 1.87 (1H, *m*, H-7), 1.68 (1H, *m*, H-25), 1.65 (1H, *m*, H-1), 1.56 (1H, *m*, H-11), 1.48 (1H, *m*, H-11), 1.27 (1H, *m*, H-12), 1.22 (3H, *s*, H-19), 1.15 (1H, *m*, H-14), 1.05 (1H, *m*, H-17), 1.01 (1H, *m*, H-7), 0.96 (1H, *m*, H-24), 0.93 (1H, *m*, H-9), 0.84 (3H, *d*, *J* 7.2, H-26), 0.83 (3H, *d*, *J* 7.2, H-27), 0.75 (3H, *s*, H-18); ^{13}C NMR (150 MHz, CD_3COCD_3) δ 198.62 (C-3), 171.53 (C-5), 124.21 (C-4), 56.65 (C-14), 56.11 (C-17), 54.91 (C-9), 46.71 (C-24), 43.21 (C-13), 40.61 (C-12), 39.33 (C-10), 37.01 (C-20), 36.41 (C-22), 36.10 (C-1), 34.72 (C-8), 34.64 (C-2), 33.49 (C-6), 33.00 (C-7), 29.09 (C-16), 29.05 (C-25), 26.80 (C-23), 24.80 (C-28), 21.85 (C-11), 20.12 (C-26), 19.40 (C-27), 19.22 (C-21), 17.63 (C-19), 12.30 (C-18), 12.33 (C-29).

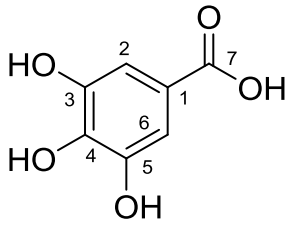


6.5.2 TLC bioautography guided isolation of active compounds from *P. persica* EtOAc fractions

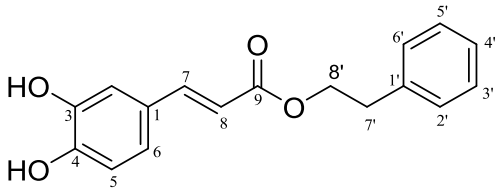
The sub-fraction EtOAc 2a (800 mg) obtained from the MPhil project (Malewska 2015) was dissolved in chloroform (0.25 mL), mixed with silica (200 mg) and subjected to normal phase silica gel column chromatography, eluting with methanol:chloroform 0 to 80% (every 100 mL, increasing 10%). This afforded 40 sub-fractions (20 mL each) that were then combined into three major sub-fractions based on their R_f values and TLC bioautography with antibiotic susceptible *S. aureus*: EtOAc 2a1 (220 mg, yellowish solid, one elongated merged spot from baseline to R_f 0.66, chloroform:methanol:water 7:3:0.2), EtOAc 2a2 (1 mg, white crystals, one smeared spot, R_f 0.63, chloroform:methanol:water 7:3:0.2), EtOAc 2a3 (220 mg, white solid, three main merged spots and many smeared faint ones, R_f from baseline to 0.45, 0.63 and 0.66, chloroform:methanol:water 7:3:0.2).

The fractions EtOAc 2a1 and EtOAc 2a3 were further purified separately by normal phase silica column chromatography with 10% to 90% (every 100 mL, increasing 10%) methanol:chloroform to afford 35 sub-fractions for 2a1 and 40 fractions for 2a3, respectively. Sub-fraction 2a1 was then combined into five fractions 2a1.1-2a1.5 and sub-fraction 2a3 was combined into fractions 2a3.1-2a3.4. Sub-fractions EtOAc 2a1.4 and EtOAc 2a3.2, which were active by TLC bioautography (antibiotic susceptible *S. aureus*), were further purified by Sephadex LH-20 column chromatography using chloroform:methanol (1:1, 300 mL) and then 100% methanol (300 mL) to afford gallic acid (**4**, 11 mg) and sub-fraction EtOac2a1. EtOac2a1 was further purified by Sephadex LH-20 column chromatography using chloroform:methanol (1:1, 500 mL) and then 100% methanol (100 mL) to afford 30 fractions. Fractions 19-25 were combined to afford caffeic acid phenylethyl ester (**5**, 16 mg).

Gallic acid (**4**): White crystals, m.p. 251-253 ºC, lit. 251-253 ˚C (Faried et al. 2007). IR υ_{max} (cm^{-1}): 3465 (strong, broad), 1706; UV-vis (MeOH) λ_{max} 271, 350, 372 nm; ESI-MS (negative mode) *m/z* 169 $[M-H]^-$; ^1H NMR (400 MHz, $CDCl_3$) δ 6.46 (2H, *s*, H-2 and 6); ^{13}C NMR (150 MHz, $CDCl_3$) δ 167.9 (C-7), 145.8 (C-3 and C-5), 138.4 (C-4), 109.16 (C- 2 and C-6).



Caffeic acid phenylethyl ester (**5**): Creamish amorphous solid, m.p. 127-130 ºC, lit. 128-130 ºC (Kart et al. 2010). IR υ_{max} (cm^{-1}): 3495 (strong), 1685; UV-vis (MeOH) λ_{max} 325 nm; ESI-MS (negative mode) *m/z* 283 $[M-H]^-$; ^1H NMR (400 MHz, $CDCl_3$) δ 7.50 (1H, *d, J* 16, H-7), 7.27 (2H, *m*, H-3' and H-5'), 7.28 (2H, *m*, H-2' and 6'), 7.20 (1H, *m*, H-4'), 7.02 (1H, *d, J* 2.0, H-2), 6.92 (1H, *dd*, *J* 8.2, 2.0, H-6), 6.77 (1H, *d*, *J* 8.2, H-5), 6.22 (1H, *d, J* 16, H-8), 4.36 (2H, *t, J* 7.0, H-8'), 2.99 (2H, *t, J* 7.0, H-7'). ^{13}C NMR (150 MHz, $CDCl_3$) δ 167.7 (C-9), 148.2 (C-4), 145.5 (C-3), 145.4 (C-7), 138.0 (C-1'), 128.5 (C-3'and 5'), 128.1 (C-2' and 6'), 126.1 (C-4'), 126.2 (C-1), 121.5 (C-6), 115.0 (C-5), 113.7 (C-8), 113.6 (C-2), 64.7 (C-8'), 34.7 (C-7').



6.5.3 TLC bioautography guided isolation of *D. lanceifolia n*-hexane fraction

The *n*-hexane partition of *D. lanceifolia* (1.0 g) was dissolved in methanol (1 mL), mixed with silica gel (0.5 g) and dried by rotary evaporation at 37 ºC. The sample was then subjected to Biotage column chromatography (25 g silica SNAP cartridge, flow rate 10 mL/min) eluting with a mobile phase of petroleum ether (b.p. 40 ºC):chloroform (100:0 to 30:70) yielding 20 sub-fractions. The sub-fractions (40 mL each) were collected, spotted in duplicate on TLC plates and developed with petroleum ether (b.p. 40 ºC):chloroform (4:6). One of the TLC plates was then stained with vanillin-sulfuric acid reagent, the other was used for overlay TLC bioautography testing against susceptible *S. aureus*. The sub-fractions were then combined into seven major sub-fractions according to similar TLC R_f values and TLC bioautography results to give, in order of increasing polarity, Hex-1 (sub-fractions 1-3, 23.2 mg, many spots, R_f 0.35-0.72, no activity), Hex-2 (sub-fractions 4 and 5, 260 mg, three major spots, R_f 0.60, 0.74, 0.82; 0.74 and 0.60 active), Hex-3 (sub-fractions 6-9, 20.5 mg, many spots, R_f 0.45-0.65, no activity), Hex-4 (sub-fractions 10-14, 52.7 mg, many spots, R_f 0.42-0.60; 0.60 active), Hex-5 (sub-fractions 14 and 15, 45.2 mg, two major spots, R_f 0.65, 0.77, no activity), Hex-6 (sub-fraction 16, 255 mg, many spots, R_f 0.55-0.82, no activity) and Hex-7 (sub-fractions 17-20, 156 mg, many spots, R_f 0.47-0.65, no activity).

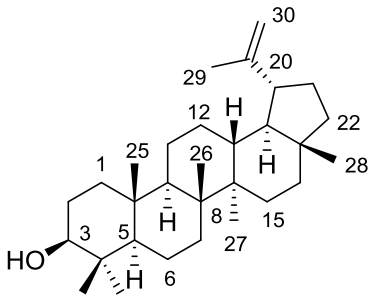
Hex-2 (260 mg) was dissolved in methanol, mixed with silica (1 g) and rotary evaporated at 37 ºC to dryness. The solid was applied on top of a normal phase silica column (200 g silica) prepared with 100% petroleum ether (b.p. 40 ºC). Elution with an increasing polarity gradient of 100% petroleum ether:chloroform yielded 16 sub-fractions, which were spotted in duplicate on TLC plates and developed with petroleum ether (b.p. 40 ºC):chloroform (4:6). One plate was visualised with vanillin-sulfuric acid reagent and the other tested by TLC bioautography. The fractions were combined based on their TLC R_f values and bioautography results into five sub-fractions: Hex 2.1 (sub-fractions 1-5, 5 mg, three major spots, R_f 0.60, 0.72, 0.80, no activity), Hex 2.2 (2.5 mg, sub-fractions 6-8, three major spots, R_f 0.60, 0.74, 0.80; 0.74 active), Hex 2.3 (125 mg sub-fractions 7-10, one major spot, R_f 0.74 active), Hex 2.4 (50 mg, sub-fractions 10-11, two major spots, R_f 0.60 and 0.74; both active), Hex 2.5 (4.5 mg, sub-fractions 12-16, many spots, R_f 0.45-0.65, no activity). Crystallisation of Hex 2.3 with methanol gave compound **1** as a yellow crystalline solid (80 mg).

Sub-fraction Hex 2.4 (50 mg) was dissolved in chloroform:methanol 1:1 (0.10 mL) and subjected to size exclusion column chromatography (1000 mL column, Sephadex LH-20, 50 g, chloroform:methanol 1:1), eluting with chloroform:methanol 1:1. This afforded 20 sub-fractions that were combined to give 4 sub-fractions based on similar R_f values: Hex 2.4.1 (10 mg, many spots, spots R_f 0.72-0.82, no activity), Hex 2.4.2 (10 mg, one major spot; 0.74 active), Hex 2.4.3 (15 mg, smeared spot, R_f 0.70-0.74; 0.74 active) and Hex 2.4.4 (10 mg, one smeared spot, R_f 0.70-0.72, no activity). Sub-fraction 2.4.2 was recrystallised with methanol to afford **1** as a pure compound (2 mg). Sub-fraction 2.4.3 was further purified by Sephadex LH-20 using chloroform:methanol 1:1 to afford compound **1** (3 mg).

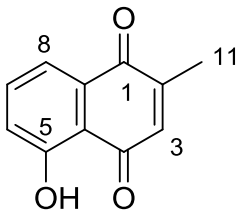
Hex-4 (52.7 mg) was dissolved in methanol and subjected to Sephadex LH-20 size exclusion column chromatography, eluting with methanol. This yielded 16 sub-fractions, which were spotted in duplicate on TLC plates and developed, one being visualised with vanillin-sulfuric acid reagent, the other tested by TLC bioautography (antibiotic sensitive *S. aureus*). The fractions were combined based on their TLC R_f values and bioautography results into two major sub-fractions, Hex 4.1 and Hex 4.2, which both had one major spot by TLC along with slight impurities. Further chromatographic separation of fraction Hex 4.1 yielded compound **2** (0.5 mg, R_f 0.60, petroleum ether:chloroform (4:6), active).

Lupeol (1): Yellowish crystals, m.p. 212-214 ºC, lit. 213-215 ºC (Agarwal and Rangari 2003). IR υ_{max} (cm^{-1}): 3346 (broad, strong), 2934; EI-MS *m/z* 426; ^{1}H NMR ($CDCl_3$, 400 MHz) δ 4.66, 4.54 (2H, *d*, *J* 2.25, H-30a and H-30b), 3.16 (1H, *dd*, *J* 10.9, 5.3, H-3), 2.35 (1H, *m*, H-19), 1.88 (1H, *m*, H-21), 1.64 (3H, *s*, H-29), 1.64 (2H, *m*, H-2), 1.63 (2H, *m*, H-1), 1.60 (1H, *m*, H-12a), 1.48 (1H, *m*, H-6), 1.36 (1H, *m*, H-6), 1.38 (1H, *m*, H-11), 1.23 (1H, *m*, H-11), 0.97 (1H, *m*, H-12b), 1.56 (2H, *m*,

H-15), 1.44 (1H, *m*, H-16), 1.36 (2H, *m*, H-7), 1.34 (1H, *m*, H-22b), 1.34 (1H, *m*, H-16), 1.16 (1H, *m*, H-22a), 1.30 (2H, *m*, H-21), 1.33 (1H, *m*, H-18), 1.23 (1H, *m*, H-9), 1.00 (3H, *s*, H-26), 0.94 (3H, *s*, H-23) 0.92 (3H, *s*, H-27), 0.80 (3H, *s*, H-25), 0.76 (3H, *s*, H-28), 0.73 (3H, *s*, H-24), 0.65 (1H, *m*, H-5); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 151.00 (C-20), 109.33 (C-30), 79.02 (C-3), 55.30 (C-5), 50.44 (C-9), 48.30 (C-18), 47.99 (C-19), 43.01 (C-17), 42.84 (C-14), 40.83 (C-8), 40.01 (C-22), 38.87 (C-13), 38.71 (C-4), 38.05 (C-1), 37.17 (C-10), 35.59 (C-16), 34.28 (C-7), 29.85 (C-21), 27.99 (C-23), 27.45 (C-15), 27.42 (C-12), 25.14 (C-2), 20.93 (C-11), 19.31 (C-29), 18.32 (C-6), 18.01 (C-28), 16.13 (C-25), 15.98 (C-26), 15.38 (C-24), 14.55 (C-27).



Plumbagin (fraction 4.1): Orange solid. EI-MS *m/z* 188; ^{1}H NMR ($CDCl_3$, 400 MHz) δ 11.98 (1H, *s*, OH), 7.73 (1H, *dd*, *J* 7.5, 1.5, H-8), 7.59 (1H, *dd*, *J* 8.0, 7.5, H-7), 7.28 (1H, *dd*, *J* 8.0, 1.5 Hz, H-6), 6.92 (1H, *q*, *J* 1.5, H-3), 2.19 (H-3, *d*, *J* 1.5, CH_3); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 190.6 (C-4), 184.3 (C-1), 161 (C-5), 149.8 (C-2), 135.14 (C-3 and C-7), 132.3 (C-9), 123.60 (C-6), 118.6 (C-8), 115.0 (C-10), 15.4 (C-11).



6.5.4 TLC bioautography guided isolation of *H. latifolia* dichloromethane fraction

The dichloromethane partition of *H. latifolia* (1.0 g) was dissolved in methanol (1 mL), mixed with silica gel (0.5 g) and dried by rotary evaporation at 37 ºC. The sample was then subjected to normal phase silica gel chromatography. A mobile phase of chloroform:*n*-hexane (10:90 to 100%, column loaded with a 100 g of normal phase silica) yielded 20 sub-fractions. The sub-fractions (20 mL each) were collected, spotted in duplicate on TLC plates and developed with chloroform:*n*-hexane 3:7. One of the TLC plates was then stained with vanillin-sulfuric acid reagent, and the other was used for overlay TLC bioautography testing against susceptible *S. aureus*. The sub-fractions did not show any separation. Size exclusion chromatography was used (50 g of the gel, eluted with 100% methanol, appx 200 mL). The size exclusion chromatography did not show separation.

6.6 UHPLC/ Q-TOF analysis

The crude 70% aqueous ethanolic extracts of *P. persica* roots and leaves of *H. latifolia* and *D. lanceifolia*, as well as the *n*-hexane and ethyl acetate partitions of *P. persica*, dichloromethane partition of *H. latifolia* leaves and *n*-hexane partition of *D. lanceifolia*, were analysed by ultra high-performance liquid chromatography-quadrupole time-of-flight mass spectroscopy with a diode array detector (UHPLC-DAD-Q-ToF-MS) using a Kinetex F5 100Å column. The flow rate was 0.3 mL/min and the injection volume was 2 µL/min. The mobile phase consisted of solvent A (0.1% formic acid in water) and solvent B (0.1% formic acid in acetonitrile). The solvent system started at 5% solvent B and 95% Solvent A, increasing to 100% solvent B at 10 minutes, remaining isocratic for 25 minutes, to 5% solvent B and 95% Solvent A over the last 1 minute. The system was allowed to equilibrate for 4.5 minutes between runs. Diode array detection was set to record chromatograms at 210, 280, 360 and 540 nm. The eluent from the DAD was directed to a 6530 Accurate-Mass Q-TOF equipped with an ESI interface. MS analysis was performed in negative and positive ion mode (*m/z* 50-1700) under the following conditions: nitrogen gas, 300 °C; gas flow rate 10L/min; nebuliser pressure 45 psig; capillary voltage 3.5 kV; column temperature 25 °C, in the extended dynamic range mode. As there was no specific group of target analytes (untargeted analysis) generic settings were applied to obtain as many compounds as possible. To ensure mass accuracy during the MS analysis, calibration was performed at the beginning of the sample runs introducing a mixture of reference compounds (Tuning Mix). Data were analysed using Agilent MassHunter workstation version B.07.00 (Agilent Technologies, USA).

6.7 References

Agarwal, R. and V. Rangari (2003). "Antiinflammatory and antiarthritic activities of lupeol and 19 alpha-H lupeol isolated from *Strobilanthus callosus* and *Strobilanthus ixiocephala* roots." *Indian Journal of Pharmacology* **35**(6): 384-387.

Awouafack, M. D., L. J. McGaw, S. Gottfried, R. Mbouangouere, P. Tane, M. Spiteller and J. N. Eloff (2013). "Antimicrobial activity and cytotoxicity of the ethanol extract, fractions and eight compounds isolated from *Eriosema robustum* (*Fabaceae*)." *BMC Complementary and Alternative Medicine* **13**(1): 289.

Choi, S. (2002). "Angiogenic activity of β-sitosterol in the ischaemia/reperfusion-damaged brain of Mongolian gerbil." *Planta Medica* **68**(04): 330-335.

Faried, A., D. Kurnia, L. Faried, N. Usman, T. Miyazaki, H. Kato and H. Kuwano (2007). "Anticancer effects of gallic acid isolated from Indonesian herbal medicine, *Phaleria macrocarpa* (Scheff.) Boerl, on human cancer cell lines." *International Journal of Oncology* **30**(3): 605-613.

Kart, A., Y. Cigremis, M. Karaman and H. Ozen (2010). "Caffeic acid phenethyl ester (CAPE) ameliorates cisplatin-induced hepatotoxicity in rabbit." *Experimental and Toxicologic Pathology* **62**(1): 45-52.

Kircher, H. W. and F. U. Rosenstein (1973). "Purification of Sitosterol." *Lipids* **8**(3): 97-100.

Kolak, U., S. Ari, H. Birman, S. Hasancebi and A. Ulubelen (2001). "Cardioactive diterpenoids from the roots of *Salvia amplexicaulis*." *Planta Medica* **67**(8): 761-762.

Malewska, T. (2015). "Biological and phytochemical analysis of Chungtia medicinal plants of Nagaland, India." Dissertation, Chemistry and Biomolecular Sciences, Macquarie University, Sydney (MPhil): 1-226.

Rahalison, L., M. Hamburger, K. Hostettmann, M. Monod and E. Frenk (1991). "A bioautographic agar overlay method for the detection of antifungal compounds from higher plants." *Phytochemical Analysis* **2**(5): 199-203.

Chapter 7 - Conclusions

Plants used as traditional medicines are a proven source of drugs and drug leads. This project was focused on understanding the antimicrobial properties of and phytochemistry (especially with regards to antimicrobially active compounds) of the roots of *Prunus persica* and leaves of *Diospyros lanceifolia* and *Holboellia latifolia*. These three plant parts have all been used in traditional medicinal applications for treatment of conditions of a likely microbial origin.

Screening of the 70% aqueous ethanolic extracts of the plant parts and partitions was conducted against nine human pathogenic microorganisms; antibiotic susceptible *Staphylococcus aureus* (susceptible *S. aureus*), methicillin resistant *S. aureus* (MRSA) and multidrug resistant *S. aureus* (MDRSA), susceptible beta-lactamase negative *Escherichia coli* (β- *E. coli*), β- lactamase positive (antibiotic resistant) *E. coli* (β+ *E. coli*), *Pseudomonas aeruginosa*, *Streptococcus pyogenes*, *Salmonella typhimurium* and *Candida albicans*. The highest inhibitory activities were exhibited by the *P. persica* root extract, with MIC values of 156 µg/mL for all strains of *S. aureus*, followed by the leaf extract of *D. lanceifolia* with MIC values of 156 µg/mL for the susceptible strain of *S. aureus* and 625 µg/mL for MRSA and MDRSA, 312 µg/mL for antibiotic susceptible *E. coli* and 156 µg/mL for *P. aeruginosa*. The leaf extract of *H. latifolia* had MIC values of 156 µg/mL for the susceptible *S. aureus*, 1250 µg/mL for MRSA and MDRSA and 625 µg/mL for *S. typhimurium*.

The 70% aqueous ethanolic extracts of *P. persica* root, and *D. lanceifolia* and *H. latifolia* leaves, were subjected to partitioning with *n*-hexane, dichloromethane and ethyl acetate and tested for their antimicrobial properties. The results indicated that the *n*-hexane (MIC 625 µg/mL *S. aureus*; 312 µg/mL MRSA, MDRSA, susceptible *E. coli* and *P. aeruginosa* strains) and ethyl acetate partitions (MIC 312 µg/mL for all strains of *S. aureus*) of *P. persica*, *n*-hexane extract of *D. lanceifolia* (MIC 156 µg/mL for *S. aureus* and *P. aeruginosa*; 312 µg/mL MRSA, MDRSA as well β- *E. coli*; 625 µg/mL for *S. typhimurium*) and DCM extract of *H. latifolia* (MIC 156 µg/mL *S. aureus* and 1250 µg/mL for MRSA as well as MDRSA) possess the strongest antibacterial properties.

Normal phase silica and size exclusion chromatography of the *n*-hexane partition of *P. persica* led to the isolation of α-cyanobenzyl benzoate, β-sitosterol and stigmast-4-en-3-one, while gallic acid and caffeic acid phenylethyl ester were isolated from the ethyl acetate partition. Similar chromatographic separation of the *n*-hexane partition of *D. lanceifolia* led to the isolation of lupeol and plumbagin. The structures of these compounds were elucidated based on 1D and 2D NMR, MS, IR and UV-Vis spectral analyses and comparison with the literature. Attempts to isolate compounds from *H. latifolia* were unsuccessful due to insufficient separation and loss of material during normal phase silica chromatography.

α-Cyanobenzyl benzoate showed strong antibacterial activity with MIC/MIQ 78 µg/mL for susceptible and resistant *S. aureus* strains, MIC/MIQ 312 µg/mL for the susceptible strain of *E. coli* and MIC/MIQ 625 µg/mL for *P. aeruginosa*. β-Sitosterol was active against the susceptible *S. aureus* (MIC/MIQ 312 µg/mL), MRSA and MDRSA (MIC/MIQ 625 µg/mL) and the susceptible strain of *E. coli* (MIC/MIQ 2500 µg/mL). Stigmast-4-en-3-one was weakly active against *S. aureus* and MRSA and *E. coli* with MIC/MIQ of 2500 µg/mL for all three strains as well as an MIC/MIQ of 1250 µg/mL for *P. aeruginosa* with. Gallic acid was active against the susceptible strain of *S. aureus* (MIC/MIQ 156 µg/mL), MRSA (MIC/MIQ 312 µg/mL) and the susceptible strain of *E. coli* and *P. aeruginosa* (MIC/MIQ 2500 µg/mL for each). Caffeic acid phenylethyl ester showed activity against the susceptible *S. aureus* and *E. coli* (MIC/MIQ 312 µg/mL) as well as MRSA and MDRSA (MIC/MIQ 625 µg/mL). Lupeol showed good antibacterial activity against *P. aeruginosa* (MIQ 156 µg/mL) and *E. coli* (MIQ 312 µg/mL), weak activity against *S. aureus* and MRSA (MIQ 2500 µg/mL) and *S. typhimurium* (MIQ 625 µg/mL). Plumbagin was tested against susceptible *S. aureus* only and exhibited an MIQ of 62 µg/mL.

GS-MS analysis of the *n*-hexane partition of *D. lanceifolia* showed the presence of the quinones plumbagin and 7-methyljuglone, which have been previously reported from this and other *Diospyros* species and have both been previously found to have antimicrobial activity.

GC-MS analysis of *H. latifolia* showed the presence of the common plant volatiles, hexanal and nonanal. Nonanal was previously isolated from *Artemisia ludoviciana* and specifically reported for antidiarrheal properties.

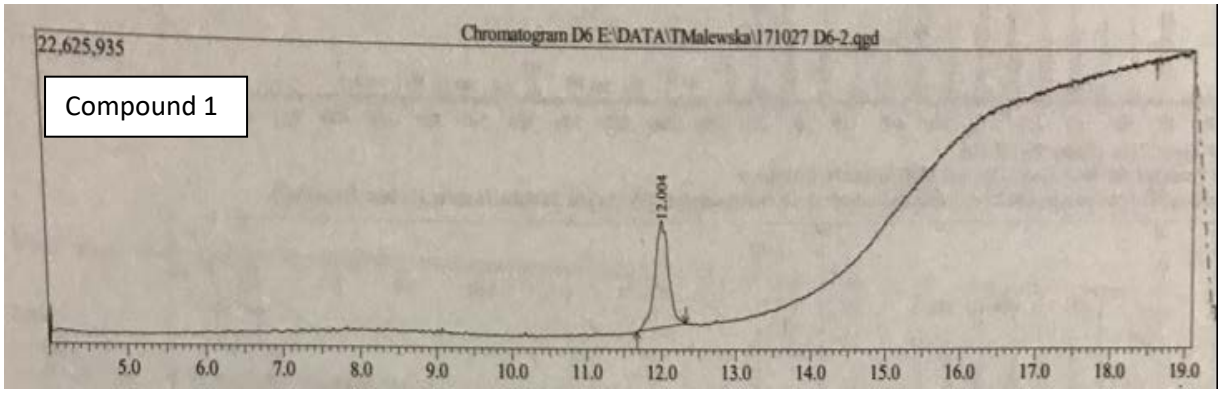
In order to better understand the phytochemistry of the antibacterially active extracts of *P. persica*, *H. latifolia* and *D. lanceifolia*, a metabolomics approach was used. An aim of metabolomics is to rapidly assess a complex mixture and to allow for the identification of the constituents, independently from the isolation process. Twenty seven metabolites in total (including 13 unassignable peaks) were tentatively identified in *P. persica* roots. Taxifolin-7-*O*-rhamnoside and 3′-*O*-methylcatechin-5-*O*-glucuronide were tentatively identified from *P. persica* species for the first time. Genepolide, (−)-4′-methoxy-7-*O*-β-D-glucopyranosyl-8,3′-dihydroxyflavanone, 9,10-dihydroxy-8-oxo-12-octadecenoic acid, 9*S*,10*S*,11*R*-trihydroxy-12*Z*-octadecenoic acid and glutinolic acid were also tentatively identified for the first time from the *Prunus* genus. Ten compounds were tentatively identified from *H. latifolia* leaf extracts and partitions, but due to insufficient data all peaks were deemed unknown. Fourteen compounds in total were tentatively identified from the leaf extract of *D. lanceifolia*. Eight peaks were found unassignable.

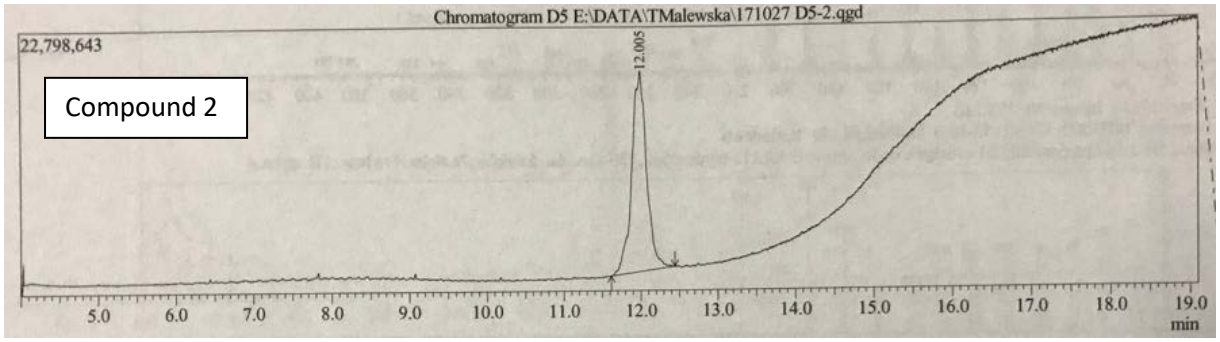
In conclusion, this project has resulted in the isolation of five antibacterially active compounds from *P. persica* roots and two from *D. lanceifolia* leaves using bioassay guided isolation approach, as well as tentative characterisation of 27 compounds from *P. persica*, ten from *H. latifolia* and fourteen from *D. lanceifolia* using a metabolomics approach. Many of these compounds are well reported for their antibacterial and other biological properties. Two antibacterially active compounds (lupeol and plumbagin) previously isolated from *D. lancelifolia* and two compounds (hexanal and nonanal) not previously reported from *H. latifolia* were characterised in these plants by GC-MS analysis. The metabolomic screening indicated the potential for the isolation of novel compounds from all three plants. Due to the fact that the characterisation of the compounds in the metabolomics study was based only on the comparison of the exact molecular mass, generated molecular formulae and UV-visible absorption spectra with published literature concerning the species and genus phytochemical composition alone, the detected compounds could only be partially characterised. Further work looking at MS/MS fragmentation of the peaks and authentic standards is necessary for more conclusive identification of compounds.

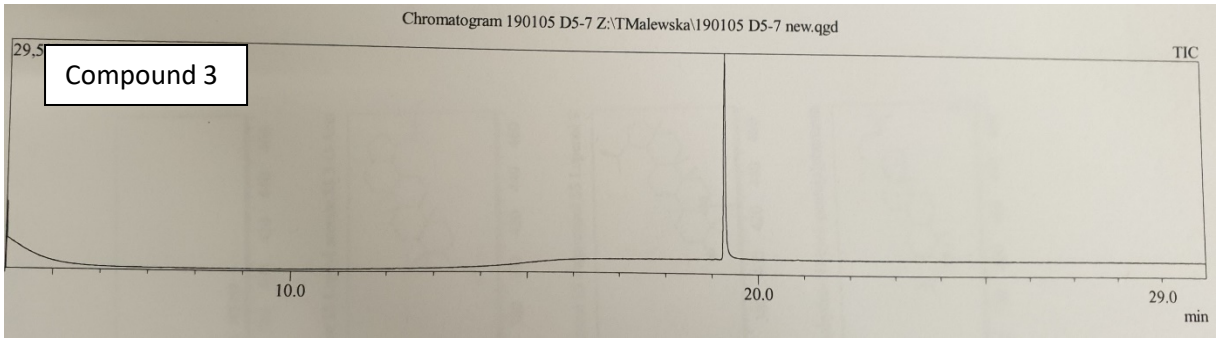
This research also highlighted the pros and cons of bioassay guided isolation and metabolomics for identification of bioactive compounds from complex plant mixtures. Bioassay guided isolation and structural elucidation is very time consuming, requires reasonable amounts of material, and may lead to the isolation and identification of only a relatively limited number of compounds, however NMR and other spectroscopic methods of the isolated compounds allow identification of the compounds with high precision. A metabolomics approach enables rapid screening of the natural products from small amounts of plant material and potentially characterisation of hundreds of compounds in a single crude plant extract, however, characterisation without standards is only "tentative" and has several pitfalls, as described in detail in the thesis. The combination of these two approaches is most promising, especially when applied to metabolomics and bioassay guided isolation of novel bioactive compounds.

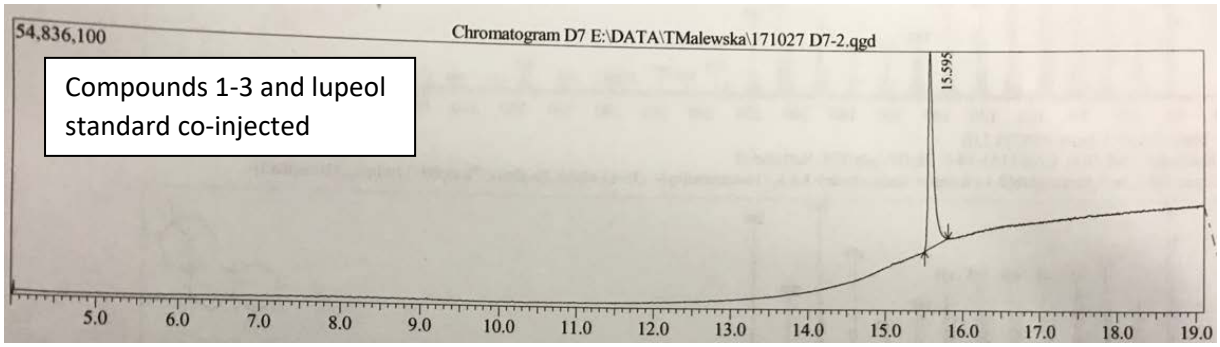
APPENDIX 1

GC-MS traces of lupeol (compounds 1-3) isolated from *Diospyros lanceifolia*

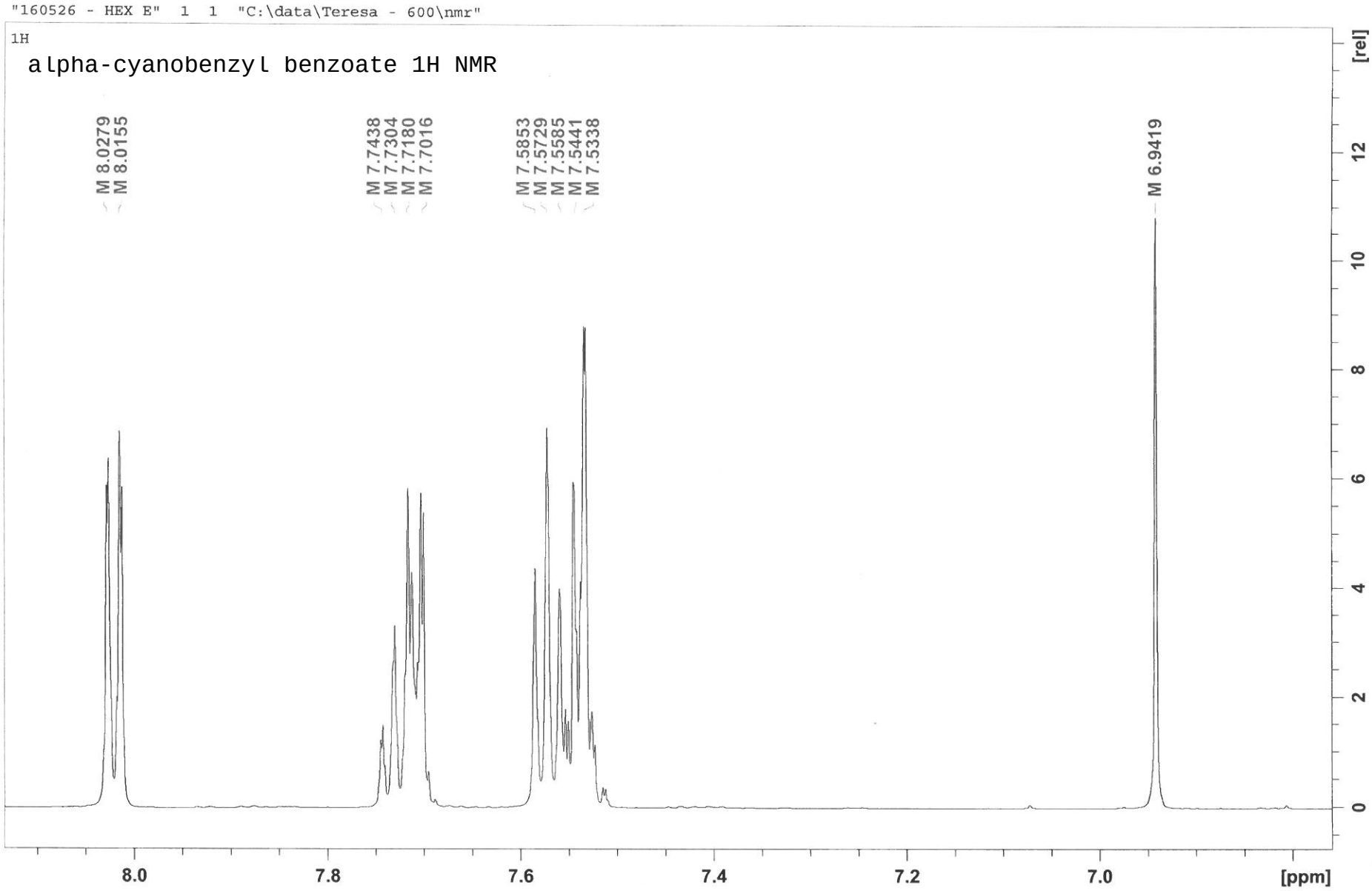


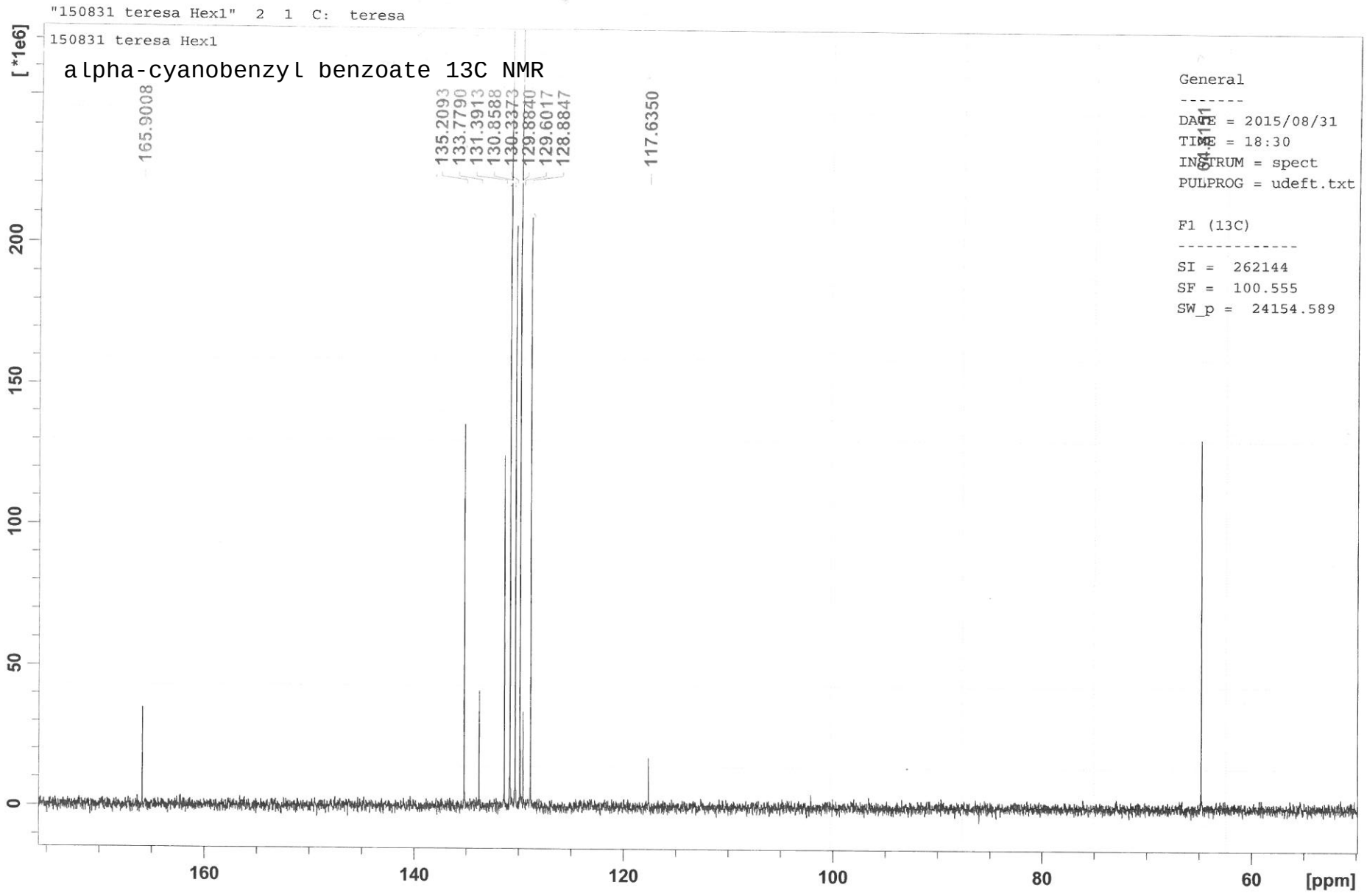


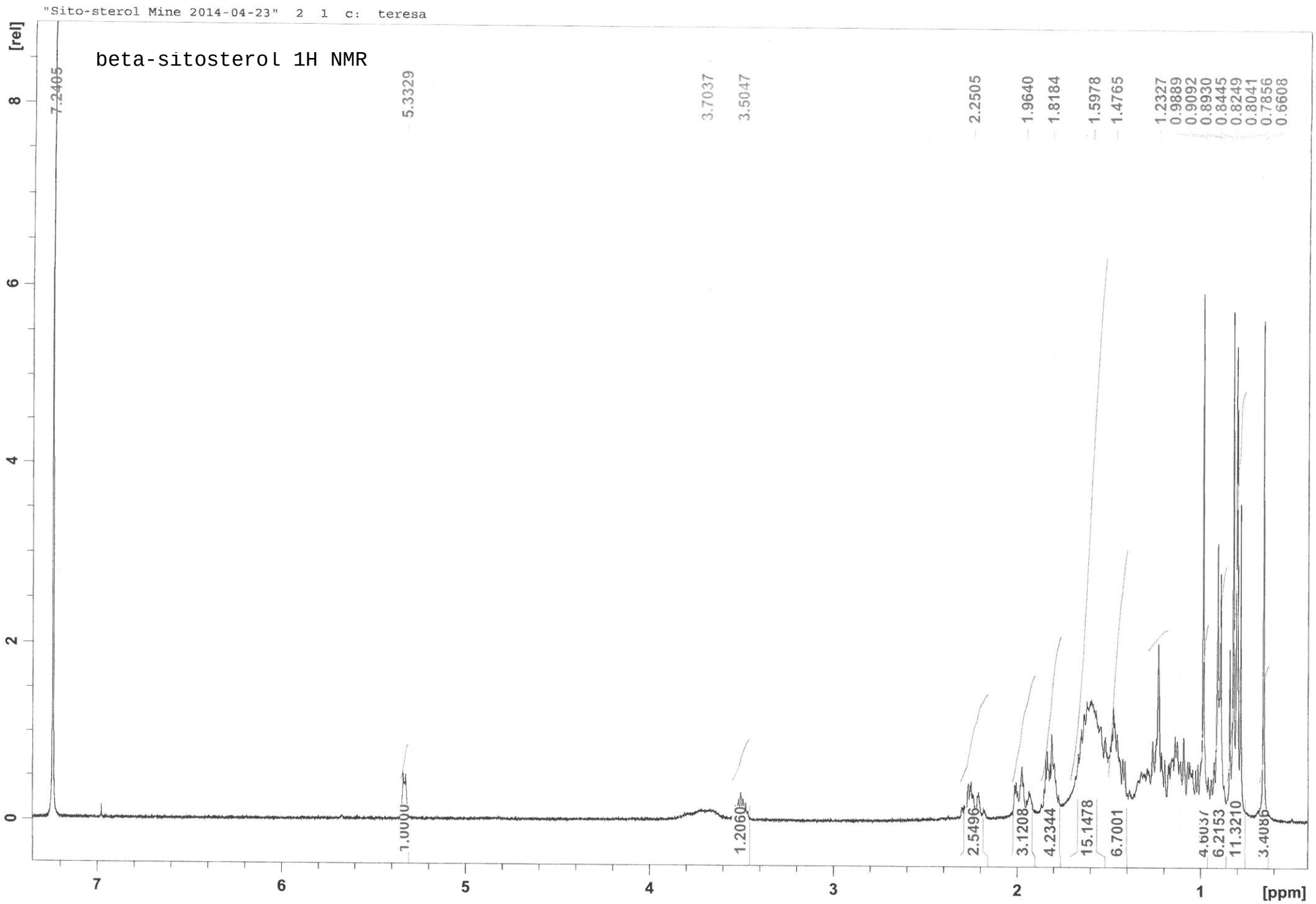


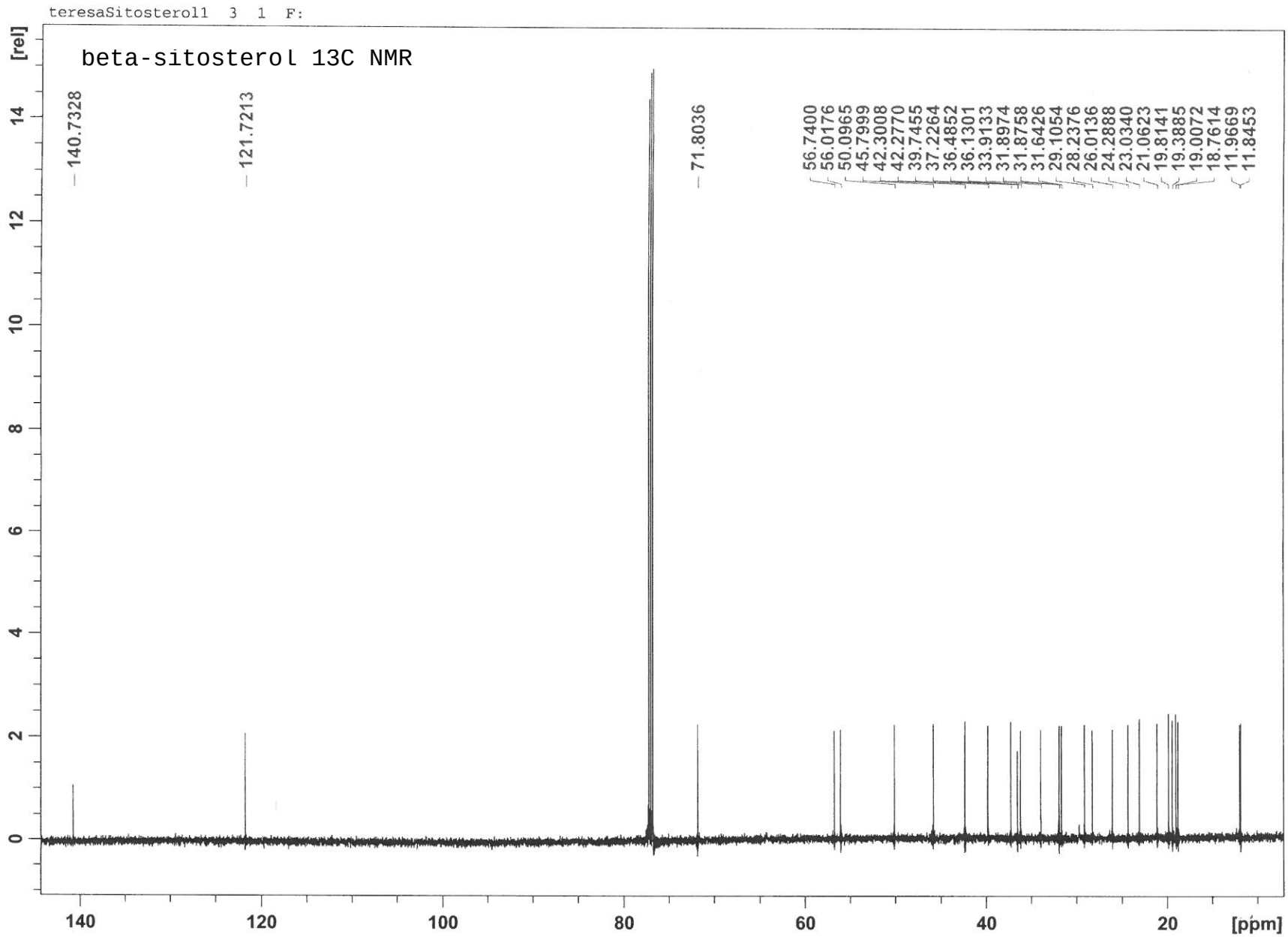


Appendix 2

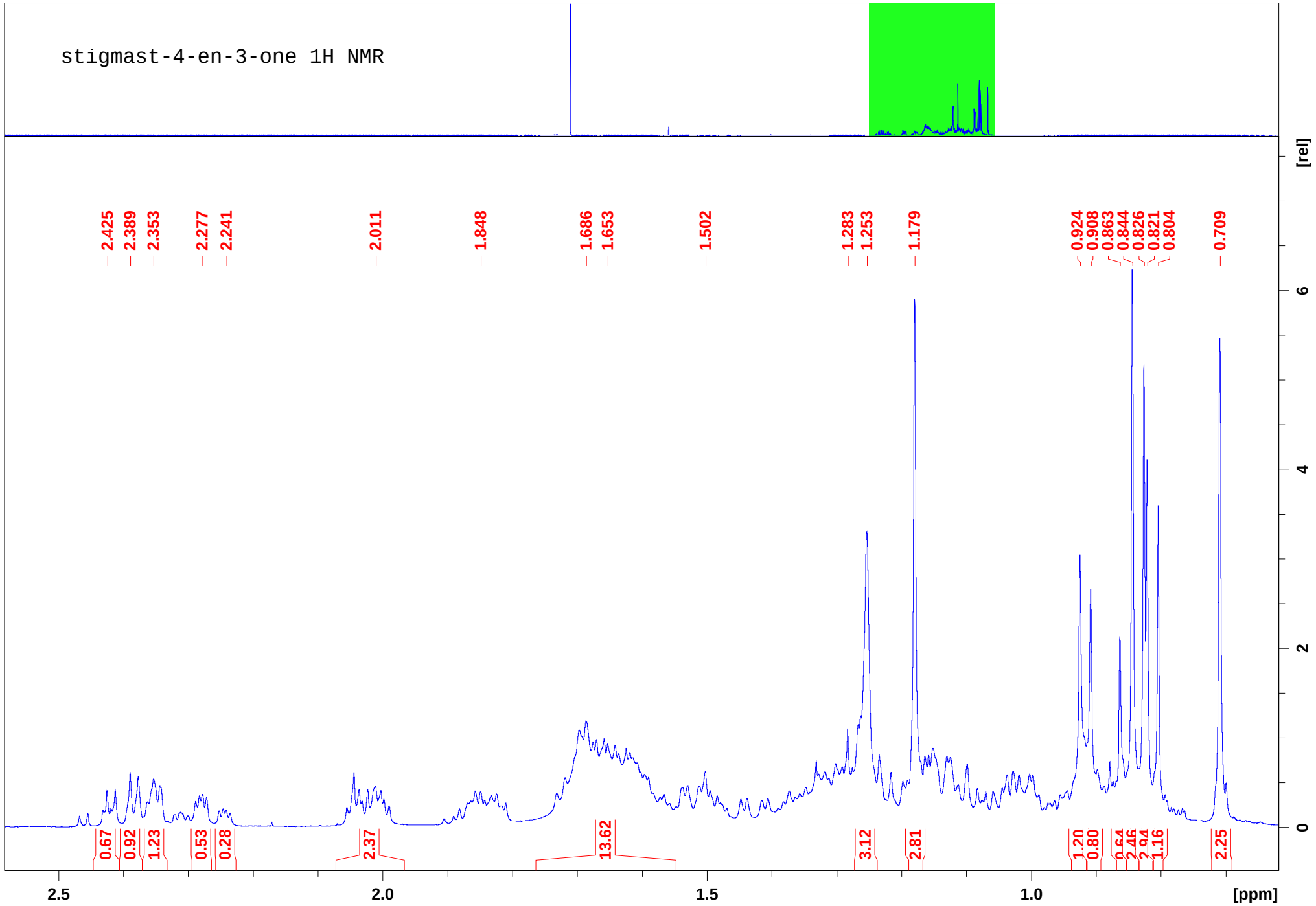

"160526 - HEX E" 1 1 "C:\data\Teresa - 600\nmr"
1H
alpha-cyanobenzyl benzoate 1H NMR
M 8.0279
M 8.0155
M 7.7438
M 7.7304
M 7.7180
M 7.7016
M 7.5853
M 7.5729
M 7.5585
M 7.5441
M 7.5338
M 6.9419
[rel]
0
2
4
6
8
10
12
8.0
7.8
7.6
7.4
7.2
7.0
[ppm]


"150831 teresa Hex1" 2 1 C: teresa
150831 teresa Hex1
alpha-cyanobenzyl benzoate 13C NMR
165.9008
135.2093
133.7790
131.3913
130.8588
130.3373
129.8840
129.6017
128.8847
117.6350
77.1511
General
DATE = 2015/08/31
TIME = 18:30
INSTRUM = spect
PULPROG = udeft.txt
F1 (13C)
SI = 262144
SF = 100.555
SW_p = 24154.589
[*1e6]
200
150
100
50
0
160
140
120
100
80
60
[ppm]

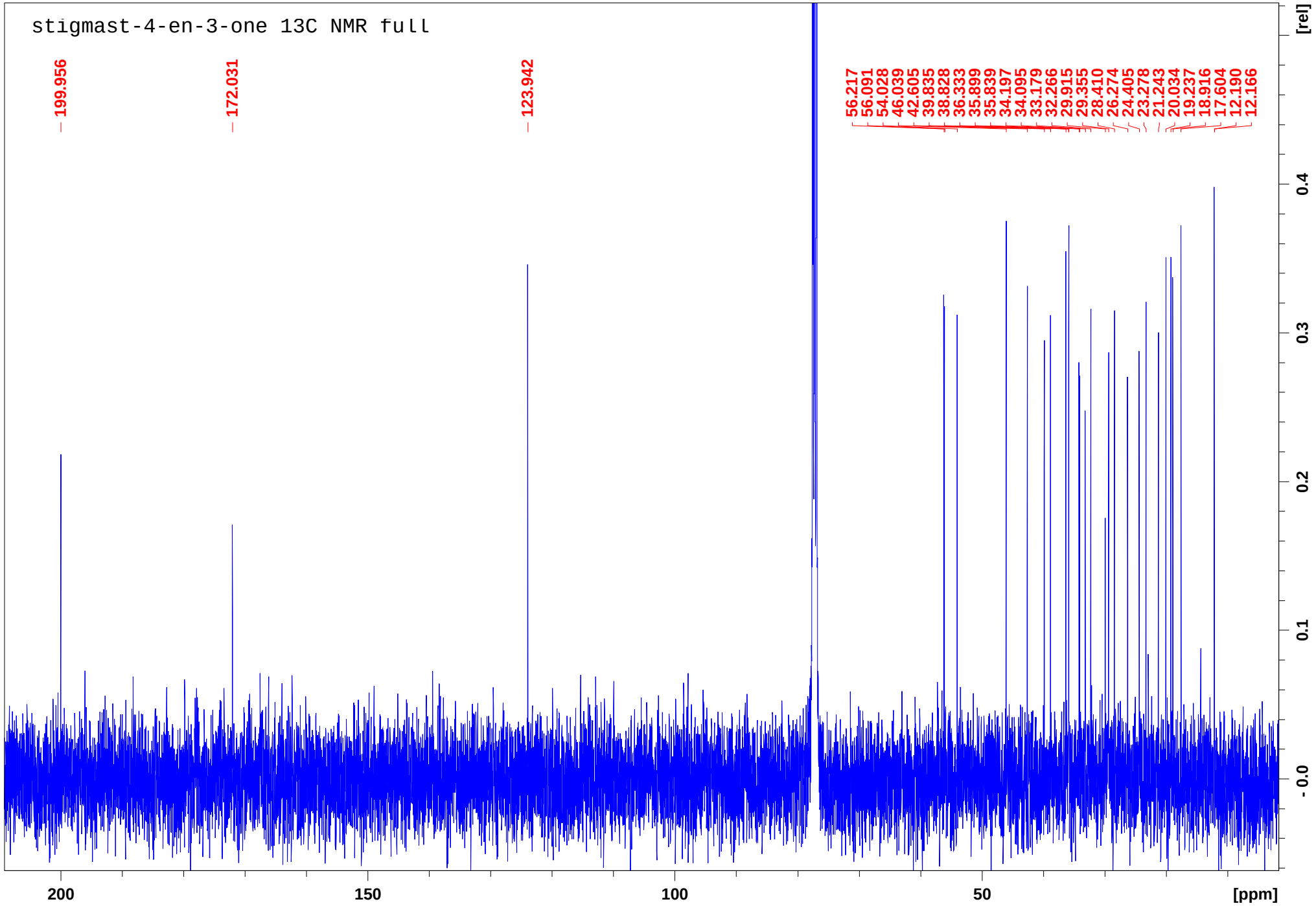

"Sito-sterol Mine 2014-04-23" 2 1 c: teresa
beta-sitosterol 1H NMR
[rel]
[ppm]
7.2405
5.3329
3.7037
3.5047
2.2505
1.9640
1.8184
1.5978
1.4765
1.2327
0.9889
0.9092
0.8930
0.8445
0.8249
0.8041
0.7856
0.6608
1.0000
1.2060
2.5496
3.1208
4.2344
15.1478
6.7001
6.2153
11.3210
3.4086


teresaSitosterol1 3 1 F:
beta-sitosterol 13C NMR
[rel]
140.7328
121.7213
71.8036
56.7400
56.0176
50.0965
45.7999
42.3008
42.2770
39.7455
37.2264
36.4852
36.1301
33.9133
31.8974
31.8758
31.6426
29.1054
28.2376
26.0136
24.2888
23.0340
21.0623
19.8141
19.3885
19.0072
18.7614
11.9669
11.8453
0
2
4
6
8
10
12
14
140
120
100
80
60
40
20
[ppm]

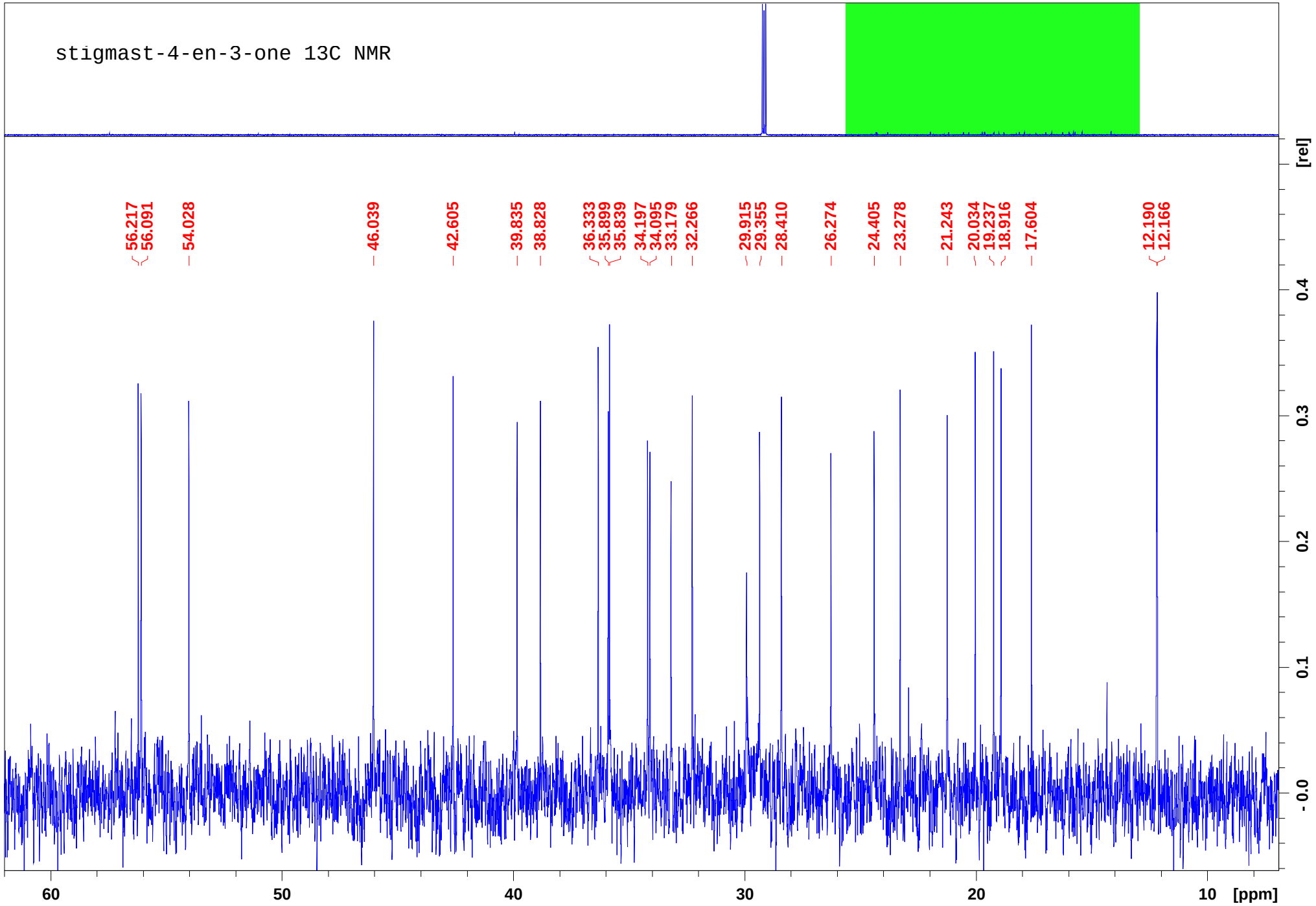
"171017-prunus sigmasterone in chloroform" 1 1 C:\Bruker\TopSpin3.5pl7\examdata



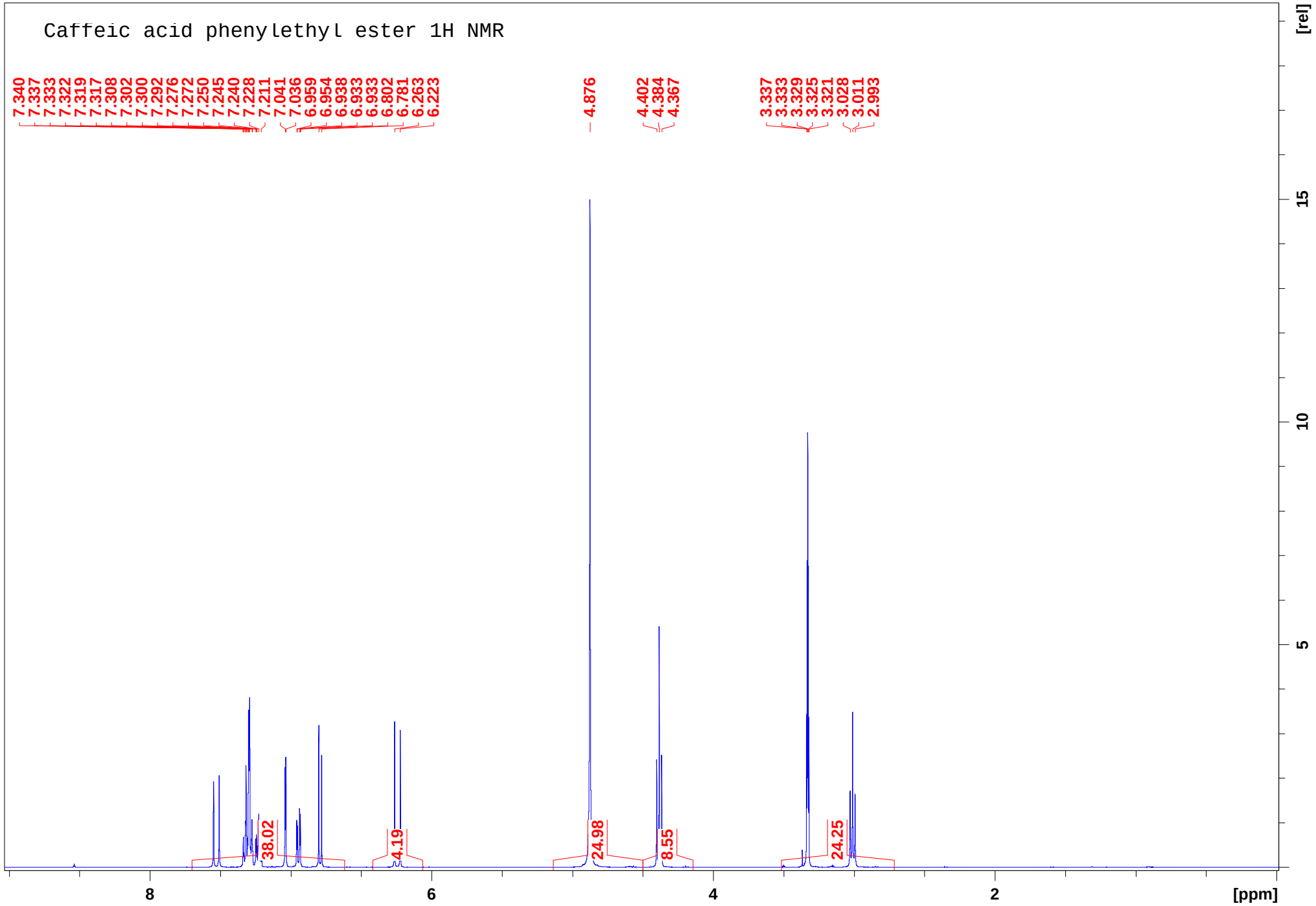
"171017-prunus sigmasterone in chloroform" 2 1 C:\Bruker\TopSpin3.5pl7\examdata

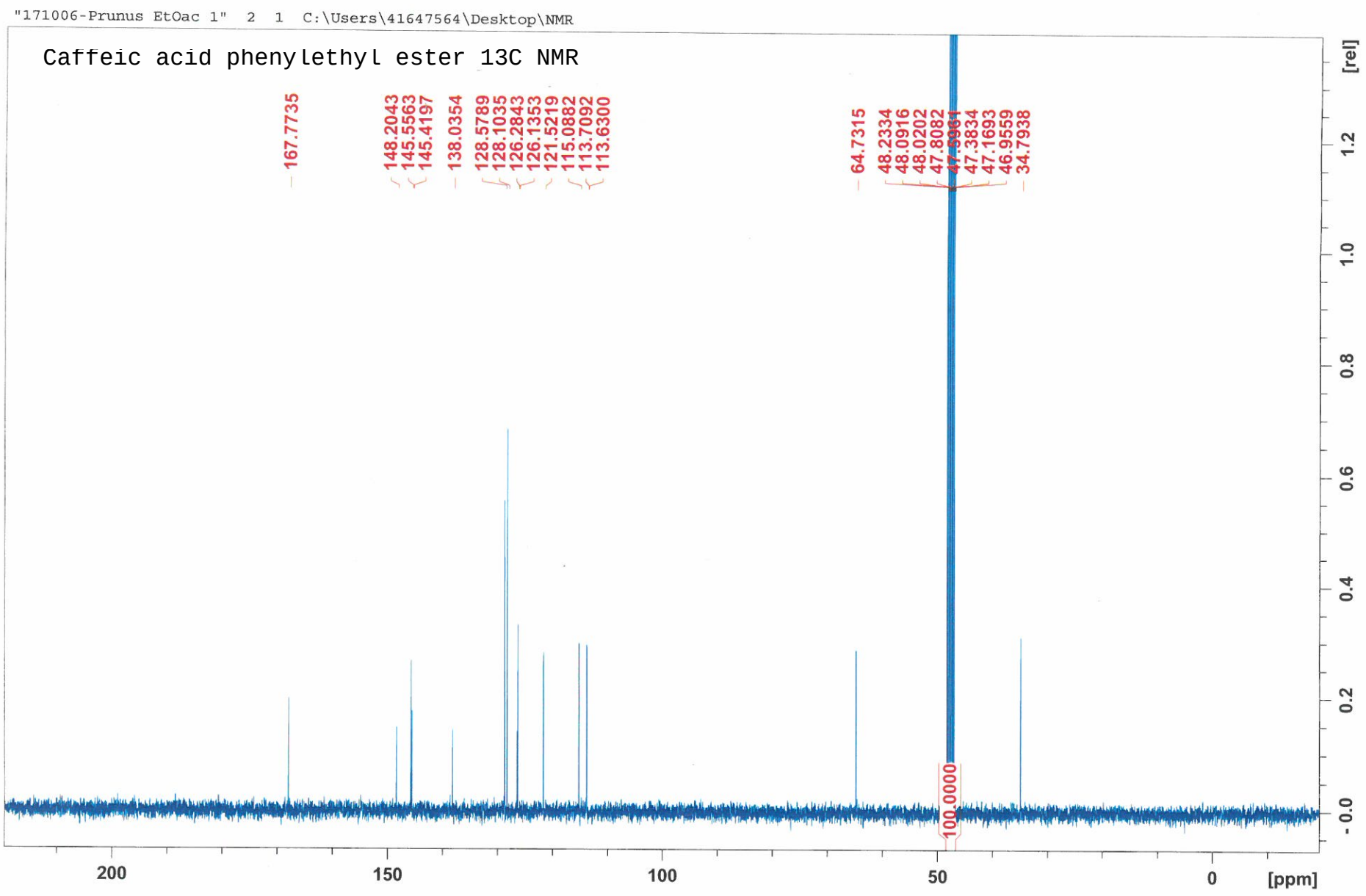


"171017-prunus sigmasterone in chloroform" 2 1 C:\Bruker\TopSpin3.5pl7\examdata

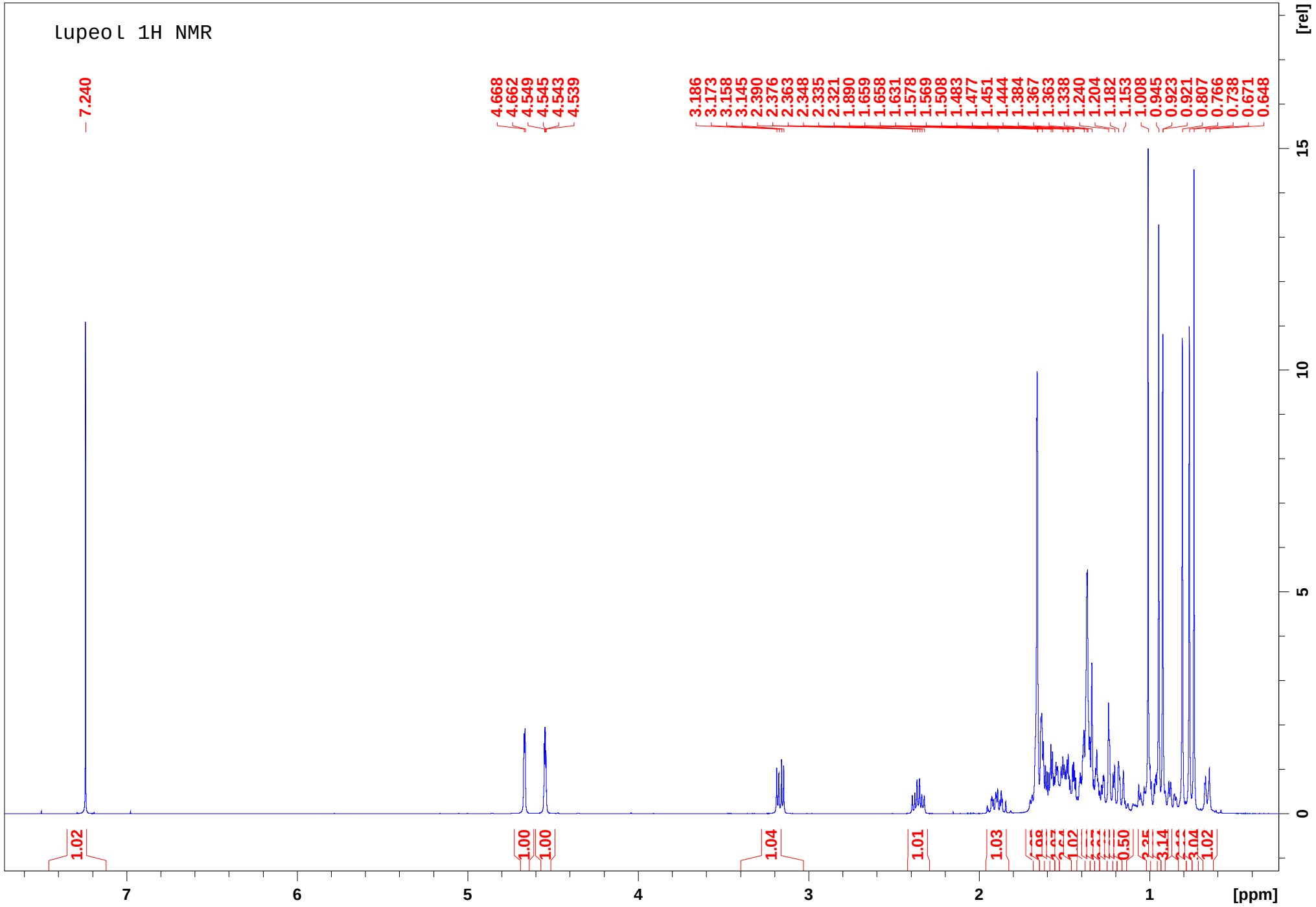


"171006-Prunus EtOac 1" 1 1 C:\Bruker\TopSpin3.5pl7\examdata




"171006-Prunus EtOac 1" 2 1 C:\Users\41647564\Desktop\NMR
Caffeic acid phenylethyl ester 13C NMR
167.7735
148.2043
145.5563
145.4197
138.0354
128.5789
128.1035
126.2843
126.1353
121.5219
115.0882
113.7092
113.6300
64.7315
48.2334
48.0916
48.0202
47.8082
47.5961
47.3834
47.1693
46.9559
34.7938
100.0000
200
150
100
50
0
[ppm]
- 0.0
0.2
0.4
0.6
0.8
1.0
1.2
[rel]

"180528-diospyros yellow cristals" 4 1 C:\Bruker\TopSpin3.5pl7\examdata



"180528-diospyros yellow cristals" 5 1 C:\Bruker\TopSpin3.5pl7\examdata