Chemical and Biological Investigations of the Yaegl Medicinal Plant *Alphitonia excelsa*

Nazma Akter Tithi

Department of Chemistry and Biomolecular Sciences

Macquarie University

This thesis is submitted in partial fulfilment of the requirements for the degree of

Master of Research (MRes)

Date of Submission: 24 April 2017

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

DECLARATION OF ORIGINALITY

I, Nazma Akter Tithi, declare that the work presented in this thesis has not previously been submitted for a degree, nor has it been submitted as part of requirements for a degree to any other university or institution other than Macquarie University.

I also certify that the thesis is an original piece of research and it has been written by me. Any help and assistance that I have received in my research work and in the preparation of the thesis itself have been appropriately acknowledged.

The research presented in this thesis was approved by the Biosafety Committee of Macquarie University with an approval number 5201600535.

Nazma Akter Tithi (Student No. 44701888)

24 April 2017

ACKNOWLEDGEMENTS

I would like to express my profound gratitude to my supervisors A/Prof Joanne Jamie and A/Prof Subramanyam Vemulpad for giving me the opportunity to be a part of their research group. I would like to thank Joanne and Subra, for their constant support, guidance, motivation, encouragement and invaluable scholarly feedback throughout the study and thesis writing. I am also indebted to Dr Emma Barnes for her cordial effort in structure elucidation and identification of the obtained compounds and teaching me the techniques of NMR data interpretation.

I would also like to thank to all of our research group members, Kaisarun Akter, Teresa Malewska, and other fellow members, Masoud Kazem-Rostami, Donald Cameron, Ben Hanssen and Soo Jean Park for their support in every need during the work. I am grateful to Mr David Harrington for the collection of Yaegl medicinal plant, *Alphitonia excelsa* and associated vouchering work.

I would like to express my special thanks to Macquarie University for awarding me an International Research Training Pathway (iRTP) scholarship for the MRes program without which this study would not have been possible by me. Appreciation also goes to the NMR Facility of Macquarie University and other laboratory facilities to make my work more effective. I also thank other staff members of the Department of Chemistry and Biomolecular Sciences, Macquarie University for their support.

Finally I would like to extend my heartfelt thanks to my wonderful family, including my parents and siblings, for their support and love. I am grateful to my husband, Saugata Bose, for helping me survive throughout all the stressful times during the study.

LIST OF ABBREVIATIONS

1D	One dimensional
2D	Two dimensional
ATCC	American type culture collection
CFU	Colony forming unit
COSY	(Proton-proton) correlation spectroscopy
DCM	Dichloromethane
DMSO	Dimethyl sulfoxide
DPPH	2,2-Diphenyl-1-picrylhydrazyl
ESIMS	Electrospray ionisation mass spectrometry
EtOAc	Ethyl acetate
EI	Electron impact
FDA	Food and Drug Administration
FRAP	Ferric reducing antioxidant power
GC-MS	Gas chromatography-mass spectrometry
HCl	Hydrochloric acid
HMBC	Heteronuclear multiple bond correlation
H_2SO_4	Sulfuric acid
H_2O	Water
HSQC	Heteronuclear single quantum correlation
IBRG	Indigenous Bioresources Research Group
MDRSA	Multidrug resistant *Staphylococcus aureus*
MeOH	Methanol
MIC	Minimum inhibitory concentration
MH	Müller Hinton
MRSA	Methicillin resistant *Staphylococcus aureus*
MSSA	Methicillin sensitive *Staphylococcus aureus*
MTT	3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide
NMR	Nuclear magnetic resonance

NP	Normal phase
NSW	New South Wales
RP	Reversed phase
SEC	Size exclusion chromatography
SGC	Silica gel column
TLC	Thin layer chromatography
TPTZ	2,4,6-Tripyridyl-s-triazine
TROLOX	(±)-6-Hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid
UV	Ultraviolet

ABSTRACT

Alphitonia excelsa leaves are used by the Australian Aboriginal people of Yaegl Country for the treatment of sores, wounds and skin infections. Structural elucidation of compounds previously isolated from the *n*-hexane and dichloromethane sequential extracts of *A. excelsa* leaves identified the well-known antibacterial and antioxidant compounds β-sitosterol, betulin aldehyde, betulinic acid, lupeol, quercetin and kaempferol. This is the first report of lupeol and betulin aldehyde in any *Alphitonia* species and the first report of β-sitosterol in *A. excelsa*. LC-MS studies of over 600 column chromatography fractions obtained from previous studies did not lead to any further compounds of interest. GC-MS analysis of freshly extracted leaves identified the bioactive compounds γ-sitosterol, nonanal, *n*-tetracontane, docosane, 1,54-dibromotetrapentacontane, tetradecane and hexadecane from the *n*-hexane and dichloromethane extracts. The dichloromethane extract also showed moderate activity against antibiotic sensitive and resistant *Staphylococcus aureus* (MIC 312.5 – 625 µg/mL) and promising antioxidant activity, while the *n*-hexane extract showed moderate antibacterial activity (MIC 1250 – 2500 µg/mL). Preliminary fractionation of the *n*-hexane extract provided one active fraction against *S. aureus* and four semi-pure fractions. This study has extended the phytochemical and biological knowledge of *A. excelsa* and shown that it is a worthy plant for further chemical and biological investigations.

Chapter 1: Introduction

Since ancient times, people have been using natural products, especially medicinal plant preparations, to overcome infections, diseases and for general wellbeing (Ramalingum and Mahomoodally 2014). The knowledge of usage of these medicinal preparations, often referred to as traditional medicinal knowledge, remains an essential starting point for drug discovery and development (Heinrich 2013). Australian Aboriginal people have a vast knowledge on their local medicinal plants, much of which has been underexplored scientifically (Packer, Gaikwad et al. 2011). The study of the usage of Australian Aboriginal medicinal plants is therefore an important avenue for drug discovery and healthcare development.

1.1 Historical Review of Usage of Medicinal Plants

An approximately 5000 years old Sumerian clay slab from Nagpur, India, provides one of the earliest written records on the usage of plants as medicines (Petrovska 2012). *Pen T'Sao*, a Chinese book written by Emperor Shen Nung circa 2500 BC, documents 365 preparations made from the dried parts of plants, many of which are still in use in the Chinese traditional medicinal system (Sarker, Latif et al. 2006). Ebers Papyrus, an Egyptian pharmaceutical record written around 1550 BC, describes 800 plant preparations used medicinally from over 700 plants (Butler and Newman 2008, Petrovska 2012). In 1100 BC, *Materia Medica* recorded 52 prescriptions from plants as medicines (Butler and Newman 2008). From 1000 BC the Ayurvedic medicinal system presented more than 1500 plants with over 10,000 medicinal preparations (Patwardhan and Mashelkar 2009). The Greeks contributed substantially to the discovery and development of herbal drugs. The work of Hippocrates in 459 - 370 BC documented 300 traditional medicines that were classified by their physiological actions (Butler and Newman 2008). Dioscorides was one of the most prominent plant drug writers in ancient times and gained the epithet of "father of pharmacognosy". In 77 AD he wrote a classical work, *de Materia Medica*, which has been translated many times, and is a significant source of information on medicinal plants including plant appearance, locality, collection, preparation and therapeutic usage (Butler and Newman 2008, Petrovska 2012). Another major contribution came from Pliny the Elder (23 AD – 79 AD), who described the preparation and uses of more than 1000 medicinal plants (Petrovska 2012).

Throughout the dark and middle ages (5^{th} to 12^{th} centuries), the Arabs contributed to the preservation of Greco-Roman medicinal plant usage expertise by recording over 1000 medicinal plants in "*De Re Medica*" by John Mesue (850 AD), "*Canon Medicinae*" by Avicenna (980 - 1037), and "*Liber Magnae Collectionis Simplicum Alimentorum et Medicamentorum*" by Ibn Baitar (1197 - 1248) (Petrovska 2012). The "*Canon Medicinae*" was considered as a complete record of Greco-Roman traditional knowledge (Sarker, Latif et al. 2006, Butler and Newman 2008).

1.2 Medicinal Plants - A Successful Source of Drug Discovery

Natural products play a significant role in drug discovery and development (Butler 2008, Harvey 2008, Newman and Cragg 2016) In a review of new drugs approved over 1981 to 2014, it was reported that almost half of the new chemical entities approved came from a natural product origin (Newman and Cragg 2016). Natural products from plants have been particularly important in drug discovery. In 2008 Harvey reported that more than 100 natural product derived compounds had undergone clinical trials and almost 100 similar compounds were in the preclinical stage in 2007. Almost 50% of these compounds in both clinical and preclinical stages were of plant origin (Butler 2008). This study also showed that at least 25% of the naturally derived compounds in the clinical trials were anti-infectives, including antibacterial and antifungal agents. Many of the pharmaceutical drugs in current clinical use derived from plants were initially used in traditional medicine systems (Fabricant and Farnsworth 2001, Heinrich 2013) and natural products from traditional medicinal plants are regarded as one of the most consistently successful sources of structurally diverse and novel drugs (Butler 2004, Heinrich 2013, Newman and Cragg 2016). Examples of plant-derived drugs that are in current clinical use include quinine, derived from *Cinchona officinalis*, and digoxin, derived from *Digitalis purpurea* (Lip and Li-Saw-Hee 1998, Regev, Berho et al. 2002).

1.3 Topical Wounds and Skin Infections: A Global Burden

Wounds and skin infections may cause the integrity of skin to be broken and allow pathogenic organisms to access the dermal tissue (O'Dell 1998). Chronic wounds affect 6.5 million people in the USA and cost more than USD 25 billion per year to treat (Sen, Gordillo et al. 2009). In the UK

chronic wounds affect more than 1% of the population and cost at least £1 billion annually (Sen, Gordillo et al. 2009). According to the Australian Wound Management Association (AWMA), at least 270,000 Australians are currently living with chronic wounds. The burden of skin infections is unknown but is serious according to the AWMA. At a global level, skin conditions were the fourth leading cause of non-fatal disease burden in 2010 (Hay, Johns et al. 2014). This burden is rapidly increasing worldwide because of the rise in bacterial resistance and its impact on mortality, morbidity and healthcare costs (Brusselaers, Vogelaers et al. 2011, Kirby 2015). One of the most important factors responsible for skin infections and delayed wound healing is bacteria. Bacteria cause contamination through colonisation, on to local infection, and spread the infection which manifests itself through cellulitis or septicaemia (Edwards and Harding 2004). Oxidative stress (increased free radicals) also plays an important role in delaying wound healing (Clark 1996, Eming, Krieg et al. 2007). Under oxidative stress conditions, the wound healing process becomes inactive in the inflammatory phase, impairing the ability of dermal fibroblasts and keratinocytes to migrate, proliferate and synthesise extracellular matrix components, delaying the healing process (Soneja, Drews et al. 2005). The increasing incidences of antibiotic resistant bacterial infections and limited antioxidant therapies including for wound healing (Fitzmaurice, Sivamani et al. 2011) highlight the need for the development of new antibacterial and antioxidant agents.

1.4 Organisms Associated with Wounds and Skin Infections

Chronic wounds and skin infections support diverse microbial flora (Edwards and Harding 2004). Gram positive bacteria such as *Staphylococcus aureus* (*S. aureus*) initiate wound infection. Over time, different pathogens can colonise the wound bed (Sapico, Canawati et al. 1980). Gram negative bacteria such as *Pseudomonas aeruginosa* (*P. aeruginosa*) affect wounds that are not deep, but can cause serious harm due to the production of tissue-damaging enzymes (Kingsley 2003). Bacteria in chronic wounds live within bio-films that protect them from the host's defenses, making their elimination difficult (Edwards and Harding 2004). Such bacteria are more resistant to conventional antibiotic treatments (Ceri, Olson et al. 1999, Stewart and Costerton 2001).

S. aureus is one of the five most common pathogens that causes skin infections and often causes post-surgical wound infections (Thompson, Cabezudo et al. 1982). *P. aeruginosa* uses a wide range of organic materials to grow and thus it can infect damaged tissue easily. This bacterium is commonly isolated from chronic wound samples (Turner, Everett et al. 2014). *Escherichia coli* (*E.*

coli) is also important in post-surgical wound infections. There are many more organisms that can be associated with wounds and skin infections such as *Streptococcus pyogenes* (*S. pyogenes*) and the fungus *Candida albicans* (*C. albicans*) (Lamagni, Darenberg et al. 2008).

1.5 Discovery of Antibacterial and Antioxidant Agents from Nature

Historically, plants have been a great resource for the discovery of effective anti-infective agents (Mitscher, Drake et al. 1987, Clardy and Walsh 2004). Phytomedicines (derived from plants) remain highly efficient as antibacterial and antioxidant agents to treat wounds and skin infections (Dorman and Deans 2000, Moreno, Scheyer et al. 2006). Antibacterial natural products from plants include saponins, tannins, alkaloids, alkenyl phenols, glycoalkaloids, flavonoids, sesquiterpene lactones, terpenoids and phorbol esters (Abdallah 2011). Natural products such as ascorbic acid and its derivatives and α-tocopherols are well known for their antioxidant activity (Ogunlesi, Okiei et al. 2009). Phenolics including kaempferol, quercetin, catechins, tannins and phenolic acids constitute the most important classes of natural plant-based antioxidants (Singleton, Orthofer et al. 1999). Examples of plant-derived antimicrobial and antioxidant drugs that are in current clinical use include thymol, derived from *Thymus vulgaris*, which is used in oral hygiene (Klarik et al. 2006) and nordihydroguaiaretic acid, derived from *Larrea divaricate* (Lundberg, Halvorson et al. 1944).

1.6 Ethnopharmacology - Successful Avenue for Drug Discovery from Nature

The process of drug discovery and development is complex, time consuming and expensive. In the USA in 2001 it was estimated that for every 10,000 compounds that were biologically active only 20 of them would be tested in an animal model, 10 of those would get to clinical trials and only one would gain FDA approval for commercialisation (Fabricant and Farnsworth 2001, Schwikkard and Mulholland 2014). The time required for this is estimated as 10 years at a cost of 231 million USD (Fabricant and Farnsworth 2001, Schwikkard and Mulholland 2014).

To optimise the drug discovery and development process, the effective selection of plants for further study is crucial. Different strategies have been used by researchers with different levels of success (Schwikkard and Mulholland 2014). Random screening, chemotaxonomy and ethnopharmacology are approaches that have been found to be valuable for plant selection for drug

discovery (Fabricant and Farnsworth 2001, Katiyar, Gupta et al. 2012). The random screening approach depends on selecting and screening plants from a large library of plant materials that provides chemical diversity (Katiyar, Gupta et al. 2012). This has had success, including in the identification of the important anticancer agents taxol and camptothecin, but is often costly and time consuming (Cragg and Newman 2001, Cuong, Hsieh et al. 2009, Katiyar, Gupta et al. 2012). The chemotaxonomic approach focuses on the principle that compounds of a particular structural class are likely to occur in multiple species of plants belonging to the same genus and family (Walton and Brown 1999). It has been useful for isolation of quinine and alkaloids, for example, but limits the discovery of novel drugs (Michael 2008).

Ethnopharmacology is the oldest method for effective plant selection and involves observation, description, and experimental investigation of Indigenous knowledge of medicinal plants (Rates 2001). Indigenous peoples around the world have a rich knowledge on local plants as medicines as they have been using those plants as medicines for thousands of years (Heinrich 2010). This knowledge has accumulated through a long period of observation and experience from generation to generation (Gadgil, Berkes et al. 1993). The ethnopharmacology approach can have a high hit success rate of non-toxic and bioactive molecules, due to their long history of human use (Schwikkard and Mulholland 2014). A study revealed that 83% of plants selected through an ethnopharmacology approach had antimicrobial activities aligned with their traditional uses, whereas only 42% of plants that had no recorded Indigenous knowledge provided such bioactivity (Lewis, Vaisberg et al. 2004). Numerous molecules have originated from an ethnopharmacology study. These include artemisinin and its derivatives for antimalarial treatment, bacosides for mental retention, picrosides for hepatic protection, phyllanthins as antivirals and curcumins for inflammation (Patwardhan and Mashelkar 2009). Other examples include morphine (*Papaver somniferum*) as an analgesic, epipodophyllotoxin (*Podophyllum peltatum* and *Podophyllum emodi*) for antitumor activity and pilocarpine (*Pilocarpus Jaborandi*) for glaucoma treatment (Dias, Urban et al. 2012, Newman and Cragg 2007).

1.7 Australian Indigenous Knowledge on Medicinal Plants

Australian Aboriginal people have one of, if not, the oldest living civilisations in the world, estimated as 40,000 – 60,000 years old (Kijas 2009). Over the generations they have identified effective medicinal plants and plant preparations to treat a range of conditions (Webb 1969). Their

knowledge has developed to incorporate contemporary practices and is best referred to as customary (traditional and contemporary) knowledge (Packer, Brouwer et al. 2012). Unfortunately this customary knowledge is being rapidly eroded due to the death of Elder custodians and communities being dislocated and westernised (Packer, Brouwer et al. 2012). This represents a significant loss to the cultural and health practices of Australian Aboriginal communities as well as to drug discovery research. Thus, ethnopharmacological investigations of medicinal plants of Australian Aboriginal communities are vital to conserve this valuable knowledge and understand its medicinal significance.

1.8 The Yaegl Community

The Yaegl Aboriginal people are the traditional custodians of the coastal region incorporating the towns of Yamba, Iluka and Maclean (collectively Yaegl Country, Figure 1.1), situated near the Clarence river of northern New South Wales (NSW), Australia. The first residents of the area were the Yaygir tribe, from which Yaegl derived its name (McSwan 1992). In the late 1880s, the Aboriginal Protection Board started to segregate the Aboriginal and non-Aboriginal settlement and in 1904 they forced the Yaegl community to move to a separate Aboriginal reserve on Ulgundahi Island, on the Clarence river. They lived there throughout the first half of the twentieth century. In 1945, Yaegl Elders Rocky and Bella Laurie, were the first to permanently move their family back to Yamba (Walker and Coutts 1989). The community has continued to grow there and in Iluka and Maclean since.

Figure 1.1: Map of Australia (left) and enclosed shaded area (right) denotes Yaegl traditional land as defined by the Native title tribunal decision (National Native Title Tribunal, 2011)

1.9 Partnership between Macquarie University and the Yaegl Aboriginal Community

The Indigenous Bioresources Research Group (IBRG) of Macquarie University, Australia, works in close collaboration with Indigenous people, including from Yaegl Country, to aid the preservation of their medicinal plant customary knowledge and to investigate the chemical and biological properties, especially of plants used for the treatment of wounds and skin infections. This is a partnership based on two-way exchange of knowledge, skills and benefits. Focusing on best ethical practice, a written agreement has been developed between both parties that acknowledges the customary knowledge as the intellectual property of the Yaegl Elders, ensuring equal ownership for research outcomes and providing capability strengthening opportunities (Packer, Brouwer et al. 2012).

As part of this partnership, the IBRG has documented Yaegl medicinal plant use from first-hand interviews with Yaegl Aboriginal Elders (Packer, Brouwer et al. 2012), and conducted chemical and biological investigations aligned with customary uses of these plants (Akter, Barnes et al. 2016). The ethnobotanical study involved interviews with a total of nineteen Elder custodians over two periods, 2004 - 2005 and 2009 - 2011. The study revealed the usage of 32 plants from 21 families used against a range of ailments by the Yaegl people (Packer, Brouwer et al. 2012). Among them, nine plants were associated with the topical treatment of sores, wounds and skin infections – the area of focus for the IBRG. One of these plants, *Alphitonia excelsa*, which is a very important plant to the Yaegl people, has limited reports of chemical and biological investigations and therefore was of great interest for further studies.

1.10 *Alphitonia excelsa*

Alphitonia excelsa (Figure 1.1) is a tall tree that reaches a height of 7 – 25 metres and a width of 5 – 10 metres (Burrows and Balciunas 1997). The leaves are dark green on the top and gray and hairy underneath and often appear ‘moth-eaten’ due to insect attack (Packer, Brouwer et al. 2012). The bark is pale gray in colour and the tree bears small greenish white flowers in late autumn and early winter. The fruit is very small and contains two seeds.

Figure 1.2: *Alphitonia excelsa* (www.anbg.gov.au)

A. excelsa belongs to the Rhamnaceae family and is endemic to Australia, being found in NSW, Queensland, Northern Territory and the northeastern tip of Western Australia. It grows in various forests and rainforests of NSW from Mount Gulaga northwards along the coast and inland to the Pilliga scrub, through Queensland and the Northern Territory and into the northeast of Western Australia (Harden 1990). This tree is also known as red ash or mountain ash, leather jacket, Coopers' wood, white leaf, red almond, humbug and soap tree (Packer, Brouwer et al. 2012). It is commonly known as soap bush by the Yaegl community (Packer, Brouwer et al. 2012).

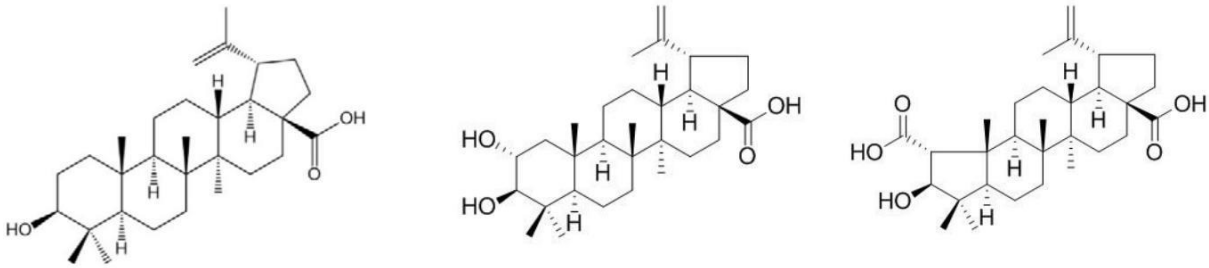
Figure 1.3: Distribution of *A. excelsa* throughout Australia (www. florabank.org.au)

1.10.1 Customary uses of *Alphitonia excelsa*

Crushed leaves of *A. excelsa* are used by the Yeagl community as an antiseptic handwash and for skin infections, sores and wounds, as well as a fishing aid by throwing them into water to make fish come floating to the surface (Packer, Harrington et al. 2011, Packer, Brouwer et al. 2012). Other NSW Aboriginal communities use the leaves of *A. excelsa* for sore eyes and as a bush soap, chew the leaves to fix stomach upsets and mix the crushed leaves with water for bathing to reduce headache (Guise, Ritchie et al. 1962). The root, bark and wood have also been used by different Australian Aboriginal communities to reduce body pain and toothache (Lassak and McCarthy 2008). The customary uses of the leaves of *A. excelsa* by the Aboriginal communities including the Yaegl people, especially topically for treatment of skin infections, sores and wounds, indicate the possibility of the presence of antimicrobial and antioxidant agents in the leaves.

1.10.2 Compounds Reported in *Alphitonia excelsa*

Triterpenoid saponins (Guise, Ritchie et al. 1962) and saponins have been discovered in all parts of *A. excelsa*, including leaves, stem, bark and roots. Other compounds including ceanothic acid, betulinic acid, alphitolic acid and salicylic acid have also been found in the leaves (Low 1990). In the bark, betulinic acid has been shown to be a major component with smaller quantities of alphitolic acid, betulin and alphitexolide, along with a 4% yield of tannins (Low 1990). Alphitonin has been found in the wood along with a smaller quantity of ceanothic acid and betulinic acid (Low 1990). The existence of alphitonin in wood extract could however be an artefact in light of literature on the action of alkali or heat on quercetin (Braune et al, 2001). Betulinic acid has been previously reported for antibacterial activity against *Bacillus subtilis* and *E. coli*, whilst alphitolic acid has been shown to have growth inhibition activity against *S. aureus* and *C. albicans* (Braca et al. 1999).



Betulinic acid Alphitolic acid Ceanothic acid

Figure 1.4: Previously reported compounds from *A. excelsa*

1.10.3 Biological Studies Reported for *Alphitonia excelsa*

Very few biological studies have been conducted on *A. excelsa*. A study in 2000 revealed that the methanol extract of the leaves of this plant exhibited 73% inhibition of ADP-induced 5-hydroxytryptamine release and 53% inhibition of platelet aggregation (Rogers, Grice et al. 2000). This is associated with the anti-inflammatory properties of the plant. Another study in 2001 (Sweeney et al. 2001) suggested that the ethanol extract of the leaves and branches together showed mild xanthine oxidase inhibitory activity (5% inhibition). Xanthine oxidase is an enzyme involved in the formation of uric acid from the purines hypoxanthine and xanthine, and is responsible for gout. The enzyme is also involved in the production of oxygen-derived free radicals, which are often involved in inflammation, atherosclerosis, cancer and aging (Sweeney et

al. 2001). The methanolic extract of *A. excelsa* leaves has also been shown to be bacteriostatic against *Micrococcus luteus*, *Salmonella typhimurium* and methicillin resistant *S. aureus* (Smyth, Ramachandran et al. 2009). Previous preliminary studies by the IBRG (P. Yin and M. Petite, unpublished data) have also shown that crude extracts of *A. excelsa* leaves following sequential extraction with *n*-hexane and dichloromethane (DCM) exhibited antibacterial activity against *S. aureus* (*n*-hexane extract: MTT assay MIC 2500-1250 μg/mL and DCM extract: MTT assay MIC 625-312 μg/mL). Isolation studies afforded a range of fractions along with four compounds from the *n*-hexane extract and three from the DCM extract, albeit in very small quantity. Only one compound, heptyl heptanoate (from the *n*-hexane extract), was structurally elucidated due to limited time.

1.10.4 Potential of *Alphitonia excelsa* for Discovery of Antibacterial and Antioxidant Agents

A. excelsa is a promising natural resource for the discovery of antibacterial and antioxidant agents. Both the customary uses and chemotaxonomical analyses indicate the potential of this plant as a source of compounds for the treatment of wounds and skin infections. As described above, the customary uses of *A. excelsa* indicate the possibility of the presence of bioactive compounds with antibacterial and/or antioxidant activity, and indeed antimicrobial activities have been seen for leaf extracts. From a chemotaxonomical point of view, there are several reports of antimicrobial and antioxidant agents present in the *Alphitonia* genus. Three flavonol glycosides, isorhamnetin 3-*O*-(6″-*O*-(*Z*)-*p*-coumaroyl)-β-D-glucopyranoside, quercetin 3-*O*-α-L-rhamnopyranosyl(1→2)-α-L-arabinopyranosyl(1→2)-α-L-rhamnopyranoside and quercetin 3-*O*-α-L-arabinopyranosyl(1→2)-α-L-rhamnopyranoside have been isolated from the stems of *A. philippinensis* (Jou, Chen et al. 2004), although no bioactivity was found associated with these compounds. Another report identified three triterpenoid saponins from *A. zizyphoides* (Li, Owen et al. 1994). A number of flavonoids and terpenoids, along with a previously undescribed *nor*lupane triterpenoid, have also been isolated from *A. xerocarpus* and *A. neocaledonica* (Muhammad, Lalun et al. 2016). These compounds include betulin, ceanothenic acid, sitosterol, rutin, 60-heptadecanoyl-3-*O*- -D-glucopyranosylsitosterol, 3-*O*- -L-rhamnopyranosyl-(1→2)-[4-*O*-(sodiumsulfonato)- -D-glucopyranosyl-(1→3)]- -L-arabinopyranosyljujubogenin, 3-*O*- -L-rhamnopyranosyl-(1→2)-

[-D-glucopyranosyl-(1→3)]- -L-arabinopyranosyljujubogenin,3-*O*- -D-glucopyranosyl-(1→2)--D-glucopyranosyl-(1→3)-[-L-rhamnopyranosyl-(1→2)]- -L-arabinopyranosyljujubogenin, kaempferol 3-*O*-rutinoside, 3-*O*- -L-arabinopyranosyl-(1→2)- -L-rhamnopyranosylkaempferol and 3-*O*- -D-xylopyranosyl-(1→2)- -L-rhamnopyranosylkaempferol. Among all these compounds, *nor*lupane triterpenoid and ceanothenic acid showed promising antibacterial activity against *S. aureus* and *Enterococcus faecalis* (Muhammad, Lalun et al. 2016).

Thus, the customary use of *A. excelsa*, and studies of it and related species, along with the limited exploration of its chemical and biological properties aligned with its customary use, support it being a plant, worthy of further study.

1.11 Objectives of this Study

The overall objectives of this MRes study were to further understand the phytochemistry of *A. excelsa* leaves and begin to provide an evidence-base for the customary uses by the Yaegl Aboriginal community of the leaves for the treatment of skin related conditions.

The specific objectives of this project were to:

Structurally elucidate the compounds isolated previously by the IBRG from the leaves of *A. excelsa* and to examine the fractions obtained from the *n*-hexane and DCM extracts (obtained sequentially) of the leaves for further compounds of interest.

Extract and fractionate fresh *A. excelsa* leaves to be able to isolate more of the compounds previously obtained for quantification and biological testing to correlate to the bioactivity of the plant extracts and identify other constituents present in the leaves.

Investigate the antibacterial and antioxidant activities of the leaf extracts and of any fractions and compounds isolated.

Chapter 2: Evaluation of Previously Isolated Compounds and Fractions from *Alphitonia excelsa*

As described in Chapter 1, *Alphitonia excelsa* has been used by Yaegl and other Australian Aboriginal people as a medicine and is a promising natural resource for the discovery of antibacterial and antioxidant agents. This MRes study followed on from previous research by members of the Indigenous Bioresources Research Group (IBRG), in which leaves of *A. excelsa* were extracted and subjected to chromatographic purification to afford seven pure compounds along with more than 600 fractions in total. In this Chapter, an overview of the previous study, is first given (2.1), to provide context for this MRes work. This is followed by a description of the structure elucidation and identification of the isolated compounds and the evaluation of the 600 fractions, as undertaken in this MRes project.

2.1 Overview of Previous IBRG Study (Dr P. Yin and M. Petite)

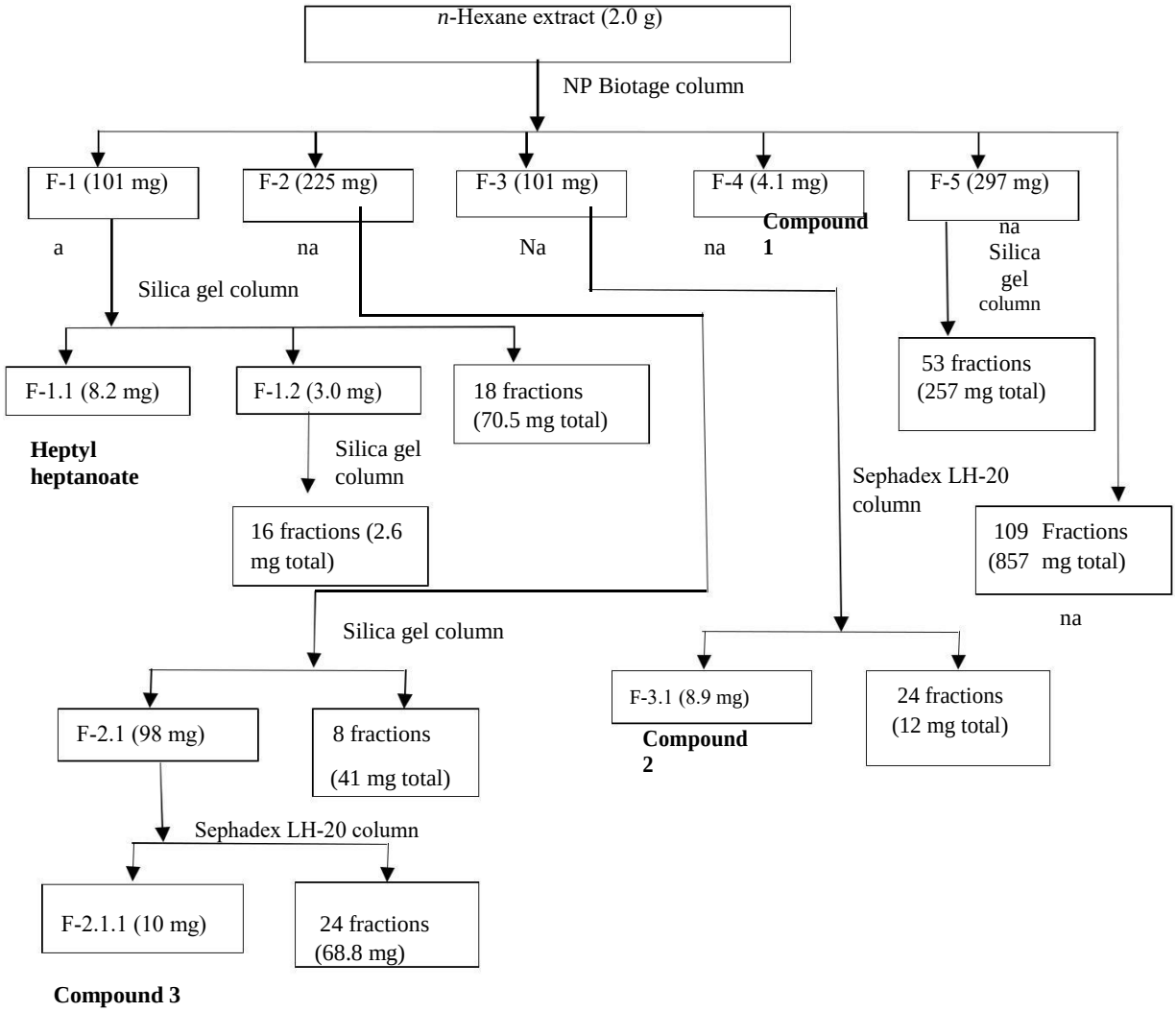
Leaves of *A. excelsa* (1.2 kg) were collected from the Cumberland State Forest, NSW and sequentially extracted with *n*-hexane, dichloromethane (DCM), ethyl acetate (EtOAc) and methanol (MeOH) (Table 2.1). Leaves were also extracted directly with water to partly mimic their customary use as an antiseptic hand wash. Antibacterial activity of the extracts was evaluated against *E. coli*, *P. aeruginosa* and methicillin sensitive *S. aureus* (MSSA) using the disc diffusion and the MTT microdilution assays (Bauer 1966, Appendino, Gibbons et al. 2008). The *n*-hexane and DCM extracts exhibited moderate activity against *S. aureus* in both these assays, whereas the EtOAc extract only showed activity (moderate) in the disc diffusion assay (Table 2.1). The water extract was not active. No antibacterial activity was seen for *E. coli or P. aeruginosa.*

The *n*-hexane and DCM extracts were chosen for further fractionation due to their antibacterial activities. This led to the isolation of four pure compounds from the *n*-hexane extract and three from the DCM extract (Schemes 2.1 and 2.2). Dr Yin identified one compound from the *n*-hexane extract as heptyl heptanoate, but due to limited time the remaining compounds were not identified.

Table 2.1: Summary of previous study of leaves of *A. excelsa*

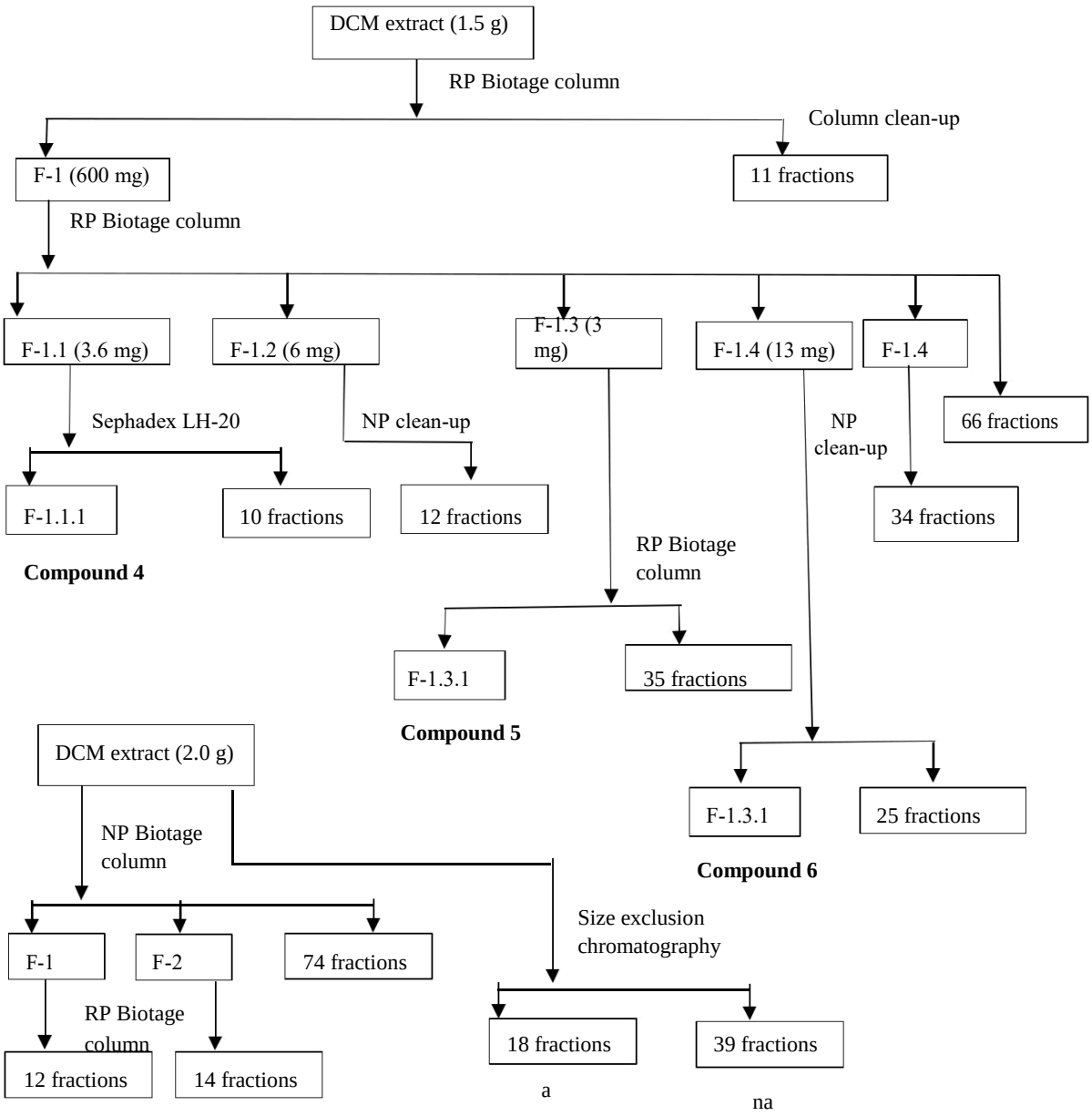
		Biological activities against MSSA		
Extracts	**Yield (g, *w/w* %)**	**Zone of inhibition**	**MIC (µg/mL)**	**Isolated compounds**
n-Hexane	6.5 g, 0.54%	15 mm	2500 - 1250	Heptyl heptanoate, 3 more compounds
DCM	6.8 g, 0.57%	10.5 mm	625 - 312	3 compounds
EtOAc	8.7 g, 0.73%	13 mm	not active	not done
MeOH	25.5 g, 2.15%	not active	not active	not done
H_2O	mass not recorded	not active	not active	not done

MIC = minimum inhibitory concentration



a = active against MSSA; na = not active against MSSA; NP = normal phase

Scheme 2.1. Summary of fractionation of *n*-hexane extract from previous IBRG studies



a = active against MSSA, na = not active against MSSA, NP = normal phase, RP = reversed phase; masses not recorded where not stated

Scheme 2.2. Summary of fractionation of DCM extract from previous IBRG studies

2.2 Characterisation of Isolated Compounds

The unidentified compounds from the study of Dr Yin and Petite (**1**, **2** and **3** from the *n*-hexane extract and **4**, **5** and **6** from the DCM extract) were identified in the current study by analysis of 1D and 2D NMR experiments and mass spectrometry (MS) and by comparison with reported data. Compounds **1-6** were identified as β-sitosterol, betulin aldehyde, lupeol, quercetin, kaempferol and betulinic acid, respectively. The structure elucidation of these compounds is described below. ^{1}H and ^{13}C NMR spectral data for each compound (**1** - **6**) and COSY, HSQC and HMBC spectra of compound (**4** and **5**) are provided in the Appendix.

2.2.1 β-Sitosterol (1)

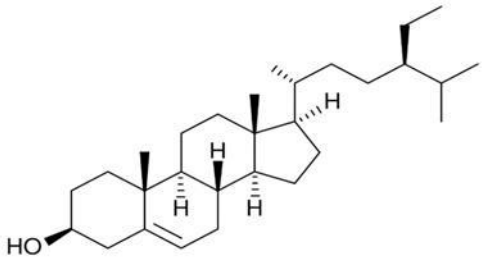


Figure 2.1: β-Sitosterol (**1**)

Compound **1** was obtained from the *n*-hexane extract of *A. excelsa* (Scheme 2.1). A positive result was obtained in a preliminary phytochemical screening of the crude DCM extract for steroids. The ESIMS of **1** showed a molecular ion at 415 $[M+H]^{+}$, consistent with the molecular formula of **1** as $C_{29}H_{50}O$. Twenty-nine carbon resonances were identified in the ^{13}C NMR spectrum. Analyses of the ^{1}H NMR, ^{13}C NMR and HSQC spectra (recorded in chloroform-*d*) indicated the presence of six methyl, eleven methylene, nine methine and three quaternary carbons. Distinctive signals could be seen for one olefin proton (δ_H 5.36, δ_C 121.4), a H-C-O- moiety (δ_H 3.54, δ_C 72.3), and six methyl groups, that appeared as two singlets (δ_H 0.65 and 0.97), three doublets (δ_H 0.78, 0.81 and 0.89) and a triplet (δ_H 0.85) (Figure 2.2).

A dereplication process was used to identify the complete structure of **1**. Using SciFinder Scholar, a structure search of the identified moieties (Figure 2.2) was undertaken and refined using the molecular weight (414) established from the MS data. The results obtained were further refined by searching for known compounds that have been isolated from the genus *Alphitonia*. This search indicated compound **1** to be β-sitosterol. The structure of β-sitosterol was also found to correspond to the number of methyl groups and their multiplicities as established using the ^{1}H NMR spectrum.

Comparison of the ^{1}H, ^{13}C NMR and MS data with that reported in the literature confirmed that compound **1** was β-sitosterol (Kojima, Sato et al. 1990).

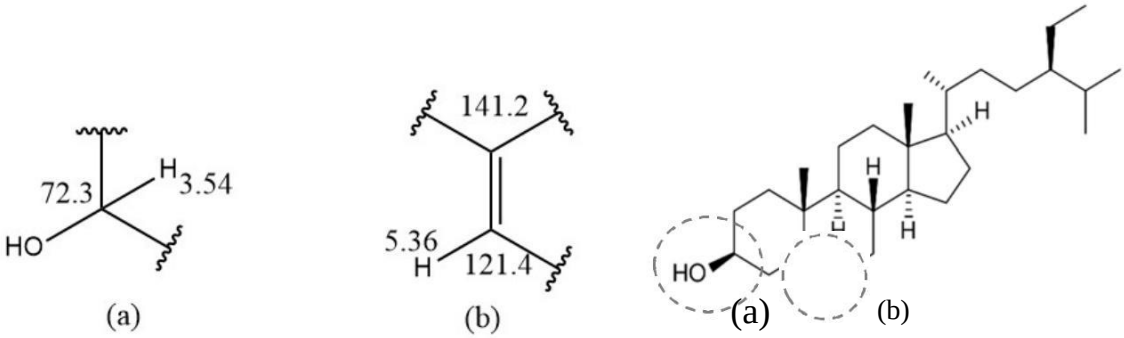


Figure 2.2: Identified moieties (a) and (b) of β-sitosterol (**1**)

2.2.2 Betulin Aldehyde (2)

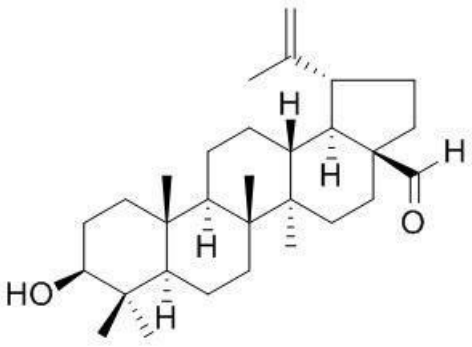


Figure 2.3: Betulin aldehyde (**2**)

Compound **2** was obtained from the *n*-hexane extract of *A. excelsa* (Scheme 2.1). The ESIMS showed a molecular ion at *m/z* 441 $[M+H]^{+}$, consistent with the molecular formula of **2** as $C_{30}H_{48}O_2$. The ^{1}H NMR spectrum showed six methyl groups (δ_H 0.78 (s), 0.72 (s), 0.86 (s), 0.84 (d), 0.92 (d) and 1.66 (s). These exhibited HSQC correlations to δ_C 17.3, 17.4, 17.5, 29.2, 16.0 and 19.5, respectively. The ^{13}C NMR, HSQC and HMBC spectra indicated the presence of 30 carbons. Analyses of these spectra along with the ^{1}H NMR data (recorded in chloroform-*d*) revealed the presence of an olefin (δ_C 110.9 and 152.5), aldehyde (δ_C 207.3) and H-C-O- moieties (δ_H 3.13, δ_C 78.4) within the molecule (Figure 2.4). Analysis of the HSQC spectrum demonstrated that two protons at δ_H 4.59 and δ_H 4.71 were connected to the carbon at δ_C 110.9, indicating they

were at the terminal position of the olefin moiety (Figure 2.4, fragment b). These protons possessed ^{1}H-^{1}H COSY correlations to each other and HMBC signals to δ_C 152.5, giving further evidence for fragment b in Figure 2.4.

Similar to β-sitosterol (**1**), a dereplication process was used to identify the complete structure of **2**. Using SciFinder Scholar, a structure search of the identified moieties (Figure 2.10) was undertaken and refined using the molecular weight (440) established from the MS data. This search indicated that compound **2** was betulin aldehyde. The structure of betulin aldehyde was also found to correspond to the number of methyl groups as established using the ^{1}H NMR spectrum. Comparison of the ^{1}H, ^{13}C NMR and MS data with that reported in the literature confirmed that compound **2** was betulin aldehyde (Barthel, Stark et al. 2008, Pohjala, Alakurtti et al. 2009).

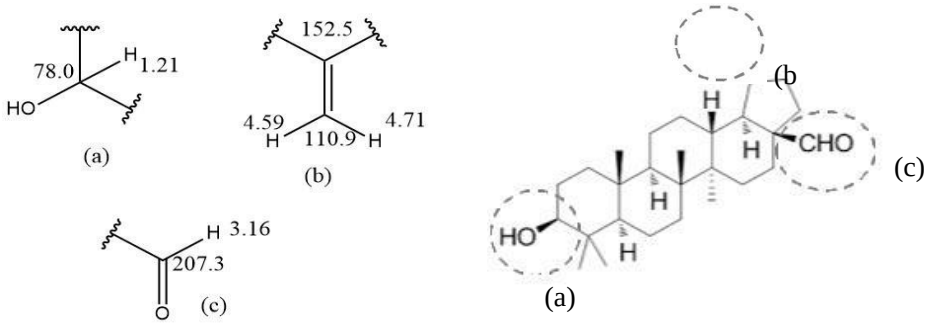


Figure 2.4: Identified fragments (a), (b) and (c) of betulin aldehyde (**2**)

2.2.3 Lupeol (3)

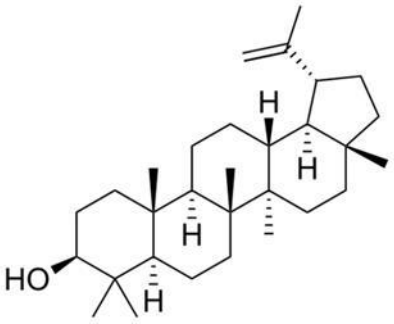


Figure 2.5: Lupeol (**3**)

Compound **3** was isolated from the *n*-hexane extract of *A. excelsa* (Scheme 2.1). The ESIMS spectra showed an ion at *m/z* 427 $[M+H]^+$, consistent with the molecular formula of **3** as $C_{30}H_{50}O$. Compound **3** possessed similar ^{1}H NMR and ^{13}C NMR data to betulin aldehyde (**2**).

The main differences included an additional methyl group and the lack of a carbonyl carbon and the aldehyde hydrogen in the case of compound **3** compared to **2**. The ^{13}C NMR, HSQC and HMBC data of **3** indicated the presence of 30 carbons, including olefinic carbons at δ_C 109.7 and 151.3.

The ^{1}H NMR spectrum (recorded in chloroform-*d*) showed seven methyl groups at δ_H 1.07, 0.76, 0.80, 0.82, 0.89, 1.09 and 1.69. These exhibited HSQC correlations to δ_C 16.9, 15.9, 17.5, 29.9, 14.5, 23.0 and 19.7, respectively. The ^{1}H and ^{13}C NMR and HSQC spectra identified the presence of a H-C-O- moiety (δ_H 3.20, δ_C 77.3) within the molecule (Figure 2.6, fragment a). The HSQC spectrum confirmed both δ_H 4.56 and δ_H 4.70 were connected to the carbon at δ_C 109.7, indicating they were in a terminal position on an olefin moiety (Figure 2.6, fragment b). These protons possessed a ^{1}H-^{1}H COSY correlation to each other and HMBC signals to δ_C 151.3, giving further evidence for fragment b in Figure 2.6.

Like β-sitosterol (**1**) and betulin aldehyde (**2**), a dereplication process was followed to identify the complete structure of compound **3**. Using SciFinder Scholar, a structure search of the identified moieties (Figure 2.6) was undertaken and refined using the molecular weight (427) established from the MS data. This search indicated that compound **3** was lupeol. The structure of lupeol was also found to correspond to the number of methyl groups as established using the ^{1}H NMR spectrum. Comparison of the ^{1}H, ^{13}C NMR and MS data with that reported in the literature confirmed compound **3** as lupeol (Fotie, Bohle et al. 2006).

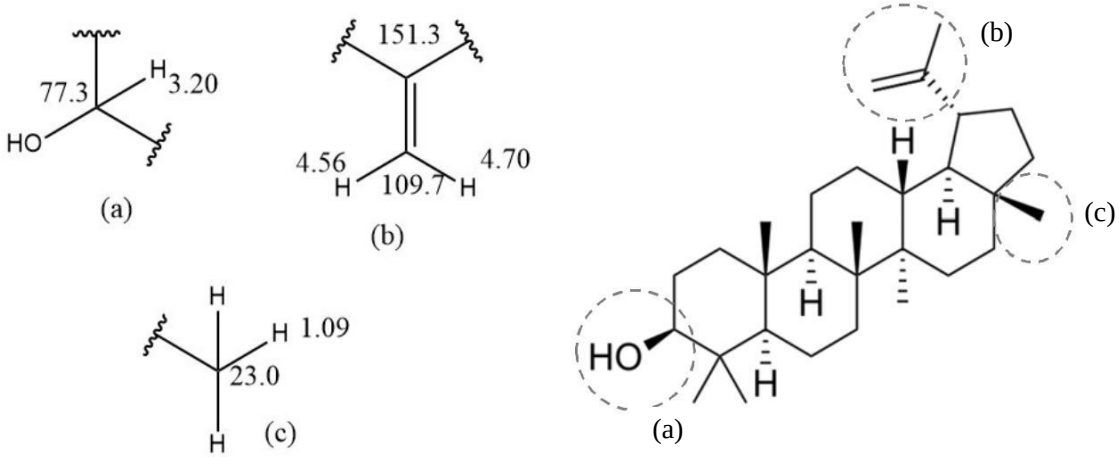


Figure 2.6: Identified fragments (a), (b) and (c) of lupeol (**3**)

2.2.4 Quercetin (4)

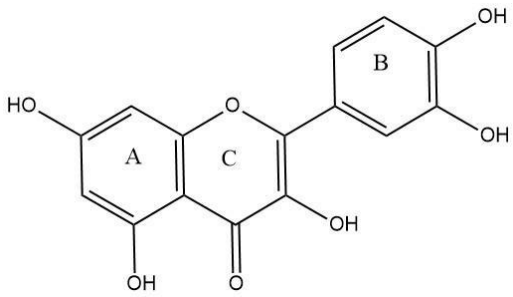


Figure 2.7: Quercetin (**4**)

Compound **4** was isolated from the DCM extract of *A. excelsa* (Scheme 2.2). The ESIMS showed a molecular ion at *m/z* 301 $[M-H]^-$, consistent with the molecular formula of **4** as $C_{15}H_{10}O_7$. In the HPLC analysis, the UV spectrum showed peaks at λ_{max} 210 and 254 nm, indicative of a flavonoid type molecule (Xu and Li 2008). Signals in the ^{1}H NMR spectrum of **4** indicated the presence of five aromatic protons at δ_H 6.25 (H-6), 6.51 (H-8), 6.98 (H-5′), 7.68 (H-6′) and 7.81 (H-2′). Analyses of the ^{13}C NMR and HSQC spectra established that there were five aromatic methine carbons [δ_C 99.2 (C-6), 94.5 (C-8), 116.3 (C-5′), 121.4 (C-6′), 115.7 (C-2′)], nine quaternary carbons [δ_C 147.1 (C-2), 150.0 (C-3), 162.0 (C-5), 165.3 (C-7), 157.8 (C-9), 104.1 (C-10), 123.6 (C-1'), 145.9 (C-3'), 148.6 (C-4')] and one carbonyl carbon [δ_C 176.5 (C-4)] in the molecule (Table 2.2).

HMBC correlations from δ_H 6.25 (H-6) to δ_C 94.5 (C-8) and δ_C 165.3 (C-7) and from δ_H 6.51 (H-8) to δ_C 99.2 (C-6) and δc 165.3 (C-7) suggested that these two protons were separated by an C-O-H moiety, due to the downfield chemical shift of δc 165.3 (C-7). Both these protons additionally showed HMBC resonances to δc 104.1, which was therefore positioned at C-10. An HMBC correlation between δ_H 6.25 (H-6) and δc 162.0 (C-5) along with a signal from δ_H 6.51 (H-8) to δc 157.8 (C-9) allowed the aromatic ring to be closed to give ring A (Figure 2.8). This also allowed for a phenolic group to be positioned at C-5 (δc 162.0) due to the downfield chemical shift of this carbon.

A ^{1}H-^{1}H COSY correlation between δ_H 6.98 (H-5′) and δ_H 7.68 (H-6′) allowed these protons to be positioned as neighbours. This was supported by the observation of an *ortho* ^{1}H-^{1}H coupling constant between H-5′ and H-6′ in the ^{1}H NMR spectrum ($J_{5',6'}$ = 8.4 Hz). HMBC correlations from δ_H 7.81 (H-2′) to δ_C 121.4 (C-6′) and δ_C 148.6 (C-4′), from δ_H 7.68 (H-6′) to δ_C 115.7 (C-2′) and δ_C 148.6 (C-4′), and from δ_H 6.98 (H-5′) to δ_C 123.6 (C-1′) and δ_C 145.9 (C-3′), allowed for

ring B (Figure 2.8) to be constructed. The carbon resonances at δc 145.9 and δc 148.6 suggested the presence of two phenolic groups at C-3′ and C-4′, due to their downfield chemical shifts. A HMBC signal from δ_H 6.98 (H-5′) to δc 123.6 (C-1′) allowed for ring B to be formed (Figure 2.8). The HMBC spectrum also showed correlations between δ_H 7.68 (H-6′) and δ_H 7.81 (H-2′) to δc 147.1, which was therefore positioned at C-2.

The downfield chemical shifts of δ_C 157.8 (C-9) and δ_C 147.1 (C-2), indicated they were neighbouring an oxygen. A four-bond correlation was observed from H-8 (δ_H 6.51) to the carbonyl carbon at δ_C 176.5 (C-4). The remaining carbon resonance at δ_C 150.0 also possessed a downfield chemical shift, suggesting it was bonded to a hydroxyl group, and was tentatively placed at C-3, which is characteristic for flavonols (Pretsch et al. 2009). This gave a tentative structure matching that of quercetin (**1**). This structure corresponded to the data obtained by ESIMS. Comparison of the NMR and MS data with that in the literature confirmed that the structure was that of quercetin (**1**) (Li, Owen et al. 1994, Fathiazad, Delazar et al. 2010).

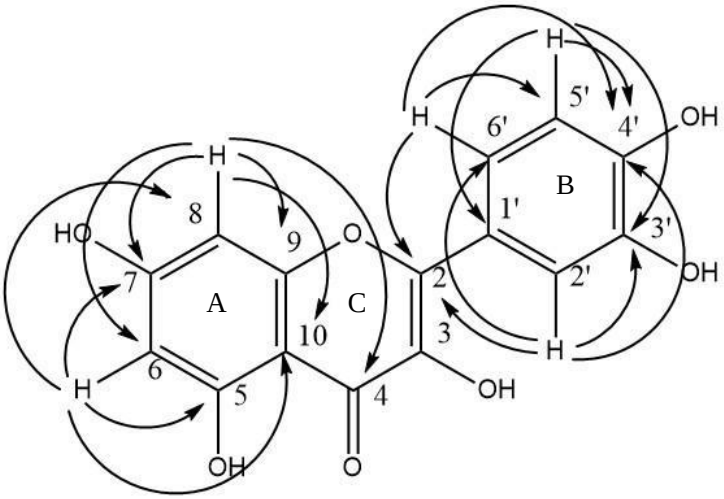


Figure 2.8: HMBC correlations of quercetin (**4**)

Table 2.2: NMR data of quercetin (**4**), recorded in acetone-d_6

Position	δ_H (mult., int., *J* in Hz)	δ_C, mult.	COSY	HMBC
2		147.1, C		
3-OH		150.0, C		
4		176.5, C		
5-OH		162.0, C		
6	6.25 (d, 1H, 1.8)	99.2, CH		5, 7, 8, 10
7-OH		165.3, C		
8	6.51 (d, 1H, 1.8)	94.5, CH		6, 7, 9, 10, 4
9		157.8, C		
10		104.1, C		
1'		123.6, C		
2'	7.81 (d, 1H, 1.8)	115.7, CH	6'[a]	3', 4', 6', 2
3'-OH		145.9, C		
4'-OH		148.6, C		
5'	6.98 (d, 1H, 8.4)	116.3, CH	6'	1', 3', 4'
6'	7.68 (dd, 1H, 8.4, 2.4)	121.4, CH	2'[a], 5'	4', 5', 2

a = four-bond correlation

2.2.5 Kaempferol (5)

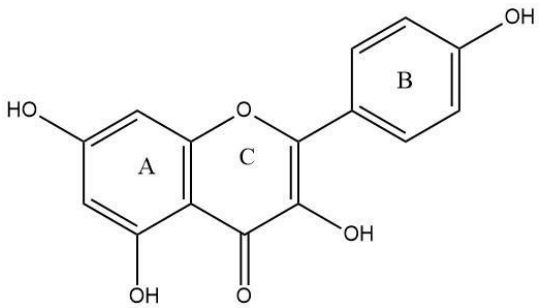


Figure 2.9: Kaempferol (**5**)

Compound **5** was obtained from the DCM extract of *A. excelsa* (Scheme 2.2). The ESIMS indicated a molecular ion at *m/z* 286 $[M-H]^-$, consistent with the molecular formula of **5** as $C_{15}H_{10}O_6$. The UV spectrum in the HPLC analysis showed λ_{max} at 210 nm, which is indicative of a flavonoid type

molecule (Xiao *et al.*, 2006). The ^{1}H NMR spectrum (recorded in methanol-d_4) indicated the presence of 6 aromatic protons at δ_H 6.07 (d, *J* = 1.8, H-6), 6.29 (d, *J* = 1.8, H-8), 6.79 (d, *J* = 9.0, 2H, H-3′, H-5′) and 7.99 (d, *J* = 8.7, 2H, H-2′, H-6′). The ^{13}C NMR and HSQC data allowed for the identification of four aromatic methine carbons [δ_C 99.6 (C-6), 94.8 (C-8), 116.7 (C-3', C-5′), 130.8 (C-2′, C-6')], eight quaternary carbons [δ_C 160.9 (C-2), 177.0 (C-3), 162.7 (C-5), 166.0 (C-7), 158.7 (C-9), 104.8 (C-10), 124.1 (C-1'), 148.4 (C-4')] and one carbonyl carbon [δ_C 180.4 (C-4)] (Table 2.3).

The ^{1}H-^{1}H COSY spectrum showed a resonance between δH 6.79 (H-3') and 7.99 (H-2'), positioning these protons as neighbours. This was supported by the observation of an *ortho* ^{1}H-^{1}H coupling constant between H-2′ and H-3′ in the ^{1}H NMR spectrum (*J*= 8.7 Hz). The HSQC spectrum revealed the attachment of these protons to be to δ_C 116.7 (C-3') and δ_C 130.8 (C-2'), respectively. HMBC correlations from the two protons at δ_H 6.79 (H-3' and H-5') with δ_C 116.7 and the two protons at δ_H 7.99 (H-2' and H-6') with δ_C 130.8 suggested a symmetrical aromatic system. HMBC signals from δ_H 6.79 (H-3') to δ_C 124.1 (C-1') and from 7.99 (H-2') to δ_C 148.4 (C-4') allowed for the completion of ring B (Figure 2.10). The downfield resonance of C-4' (δ_C 148.4) indicated that a phenolic group was positioned at this carbon. Additional HMBC signals were observed from both δ_H 6.79 (H-3') and 7.99 (H-2') to δ_C 160.9, which was thus positioned at C-2. Similar to quercetin (**4**), HMBC correlations from δ_H 6.07 (H-6) to δ_C 94.8 (C-8) and δ_C 166.0 (C-7) and from δ_H 6.29 (H-8) to δ_C 99.6 (C-6) and δc 166.0 (C-7) suggested that the two protons were separated by an C-OH moiety, due to the downfield chemical shift of δc 166.0 (C-7). Both these protons additionally showed HMBC resonances to δc 104.8, which was therefore positioned at C-10. HMBC correlations between δ_H 6.07 (H-6) and δc 162.7 (C-5) and between δ_H 6.29 (H-8) and δc 158.7 (C-9) allowed the aromatic ring to be closed to give ring A (Figure 2.10). The downfield chemical shift of C-5 (δc 162.7) allowed for a phenolic group to be positioned at this carbon.

The downfield chemical shifts of δc 168.7 (C-9) and δc 160.9 (C-2) suggested that they were attached to an oxygen. The presence of a carbonyl signal at δc 180.4 (C-4), which possessed a four-bond HMBC correlation to δ_H 6.29 (H-8), suggested ring C (Figure 2.10) and an overall flavonoid skeleton for compound **5**. The downfield resonance observed for C-3 (δc 177.0) indicated a hydroxyl group was attached to this carbon, which is characteristic of flavonols (Pretsch et al. 2009). This gave a tentative structure matching that of kaempferol (**5**). This structure corresponded to the data obtained by

ESIMS. Comparison of the NMR and MS data with that in the literature (Veitch, Tibbles et al. 2005) confirmed that the structure was that of kaempferol (**5**).

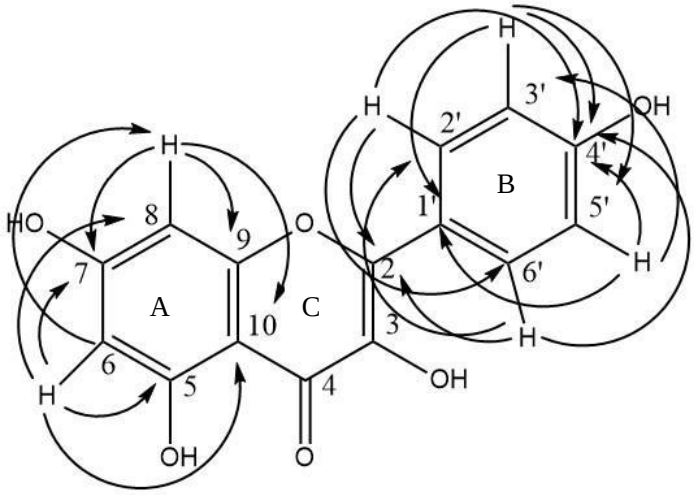


Figure 2.10: HMBC correlations of kaempferol (**5**)

Table 2.3: The NMR data of kaempferol (**5**), recorded in methanol-d_4

Position	δ_H (mult., int., *J* in Hz)	δ_C, mult.	COSY	HMBC
2		160.9, C		
3		177.0, C		
4		180.4, C		
5		162.7, C		
6	6.07 (d, 1H, 1.8)	99.6, CH		5, 7, 8, 10
7		166.0, C		
8	6.29 (d, 1H, 1.8)	94.8, CH		6, 7, 9, 10
9		158.7, C		
10		104.8, C		
1'		124.1, C		
2', 6'	7.99 (d, 2H, 8.7)	130.8, CH	3', 5'	4', 2', 6', 2
3', 5'	6.79 (d, 2H, 8.7)	116.7, CH	2', 6'	1', 3', 5', 4'
4'		148.4, C		

2.2.6 Betulinic Acid (6)

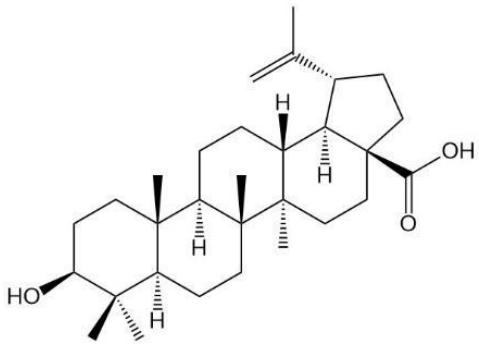


Figure 2.11: Betulinic acid (**6**)

Compound **6** was isolated from the DCM extract of *A. excelsa* (Scheme 2.2). ESIMS of this compound gave molecular ion peaks at *m/z* 457 $[M+H]^+$ and *m/z* 455 $[M-H]^-$, consistent with the molecular formula of **6** as $C_{30}H_{48}O_3$, suggesting a molecular mass of 456. The NMR data of this compound closely resembled that of betulin aldehyde (**2**) and lupeol (**3**). The ^{13}C NMR, HSQC and HMBC data indicated the presence of 30 carbons. The ^{1}H NMR spectrum possessed signals for two olefinic protons (δ_H 5.49 and 5.35) and six methyl groups (δ_H 1.45, 1.56, 1.65, 1.67, 1.73 and 2.44). The methyl ^{1}H NMR resonances exhibited HSQC correlations to δ_C 17.3, 17.4, 17.5, 29.2, 16.0 and 19.5, respectively. The ^{13}C NMR spectrum also indicated the presence of a carboxylic acid (δ_C 178.9) and an olefin moiety (δ_C 111.2 and 151.9) within the molecule.

The HSQC spectrum demonstrated that δ_H 5.49 and δ_H 5.35 were both connected to the carbon at δ_C 111.2, indicating that they were at a terminal position on an olefin moiety (Figure 2.12, fragment b). These protons possessed a COSY correlation to each other and HMBC signals to δ_C 151.9, giving further evidence for fragment b in Figure 2.8. Analyses of the ^{1}H and ^{13}C NMR and HSQC spectra revealed the presence of a H-C-O- moiety (δ_H 3.74, δ_C 78.4) within the molecule. The fragment ions seen in Figure 2.8 have been reported as characteristic fragmentations of betulinic acid and other lupane type triterpenes (Ryu, Lee et al. 1992).

Similar to β-sitosterol (**1**), betulin aldehyde (**2**) and lupeol (**3**), a dereplication process was used to identify the complete structure of **6.** Using SciFinder Scholar, a structure search of the identified moieties (Figure 2.12) was undertaken and refined using the molecular weight (456) established from the MS data. This search indicated that compound **6** was betulinic acid. The structure of betulinic acid was also found to correspond to the number of methyl groups as established using

the ^{1}H NMR spectrum. Comparison of the ^{1}H, ^{13}C NMR and MS data with that reported in the literature confirmed that compound **6** was betulinic acid (Siddiqui, Hafeez et al. 1988).

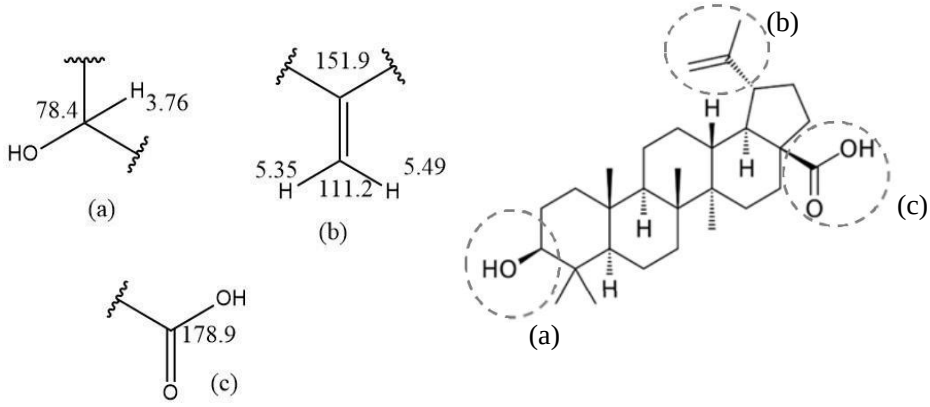


Figure 2.12: Identified fragments (a), (b) and (c) of betulinic acid (**6**)

2.3 Significance of the Isolated Compounds

2.3.1 β-Sitosterol (1)

β-Sitosterol is an important phytosterol found widely in the plant kingdom. It has previously been isolated from *Salvadora persica* (Farag, Fahmy et al. 2017), *Anthocephalus cadamba* (Khandelwal, Bhatia et al. 2016), *Jacaranda oxyphylla* (Pereira, Silva et al. 2016), *Ficus racemose* (Yadav, Nandy et al. 2015) and many more medicinal plants. β-Sitosterol has previously been isolated from different species of *Alphitonia* including *Alphitonia petriei*, *Alphitonia whitei* (Guise, Ritchie et al. 1962) and *Alphitonia xerocarpus* (Muhammad, Lalun et al. 2016). This is the first report of the isolation of β-sitosterol from *A. excelsa*.

β-Sitosterol has been reported as an antibacterial and antifungal agent against *E. coli*, *P. aeruginosa*, *S. aureus* and *Klebsiella pneumoniae* (Saeidnia, Manayi et al. 2014) and *S. typhimurium* (Pereira, Silva et al. 2016). It also stimulates antioxidant enzymes (Saeidnia, Manayi et al. 2014) and enhances pancreatic antioxidant levels (Gupta, Nath et al. 1980). In addition to these antimicrobial and antioxidant activities, β-sitosterol has been reported to have activity against ailments such as heart disease, hypercholesterolemia, rheumatoid arthritis, tuberculosis, cervical cancer, hair loss and benign prostatic hyperplasia and in modulating the immune system (Saeidnia, Manayi et al. 2014). Other bioactivities of this compound include anti-inflammatory, antitumor, anticholesteremic, analgesic, antivenom and larvicidal (Rahuman, Gopalakrishnan et al. 2008).

2.3.2 Betulin Aldehyde (2), Lupeol (3) and Betulinic Acid (6)

Betulinic acid, betulin aldehyde and lupeol are pentacyclic lupine-type triterpenoids. Betulinic acid has been previously isolated from *Dillenia indica*, *Orthosiphon stamineus*, *Engelhardtia serrata*, *Bischofia javanica* and *Nerium oleander*, amongst many other plant species (Moghaddam, Ahmad et al. 2012). It has also been isolated from different species of *Alphitonia* including *A. petriei*, *A. whitei*, *A. zizyphoides* and all parts of *A. excelsa* including the leaves (Guise, Ritchie et al. 1962, Branch, Burgess et al. 1972, Raju, Gunawardena et al. 2016). Betulinic acid is well known for its biological activity, including against Gram positive *Bacillus subtilis* and Gram negative *E. coli* (Chandramu, Manohar et al. 2003, Moghaddam, Ahmad et al. 2012) and *C. albicans* secreted aspartic proteases (Zhang, ElSohly et al. 2002). This compound is also well known for its anti-inflammatory, anticancer, anti-HIV and antimalarial activities (Cicheqicz et al. 2004; Steele, 1999).

Lupeol has been previously isolated from *Alhagi maurorum* (Laghari, Memon et al. 2011), *Himatanthus sucuuba* (Wood, Lee et al. 2001), *Bombax ceiba* (You, Nam et al. 2003) and *Parkia biglobosa* (Tringali, Spatafora et al. 2000). It has been found to act as an effective antibacterial agent when tested against both Gram positive and Gram negative bacteria (Ahmed, Sohrab et al. 2010). These include *Bacillus cereus*, *Bacillus megaterium*, *B. subtilis*, *S. aureus*, *Sarcina lutea*, *Salmonella paratyphi*, *Salmonella typhi*, *Shigella boydii*, *Shigella dysenteriae*, *Vibrio mimicus*, *Vibrio parahaemolyticus*, *E. coli*, *Saccharopolyspora spinose*, *K. pneumoniae*, *P. aeruginosa* and *Micrococcus flavus* (Ahmed, Sohrab et al. 2010). Lupeol has also been shown to inhibit the growth of a variety of fungal species such as *Sporothrix schenckii*, *Microsporum canis*, *Aspergillus fumigatus*, *C. albicans*, *Cryptococcus neoformans* and *Candida guilliermondi* (Ahmed, Sohrab et al. 2010). Skin protective effects have been observed for lupeol and have been associated with its potential to enhance the skin antioxidant system (Saleem 2009). This compound is also known to possess anticancer, anti-HIV and anti-angiogenic activities (You, Nam et al. 2003, Palanimuthu, Baskaran et al. 2012) and also has beneficial effects against cardiovascular diseases, diabetes, inflammation, arthritis, renal diseases and liver toxicity (Siddique 2011). This is the first report of the isolation of lupeol from the genus *Alphitonia*.

Betulin aldehyde has been previously isolated from *Melilotus messanenis* (Macías, Simonet et al. 1994), *Tectona grandis* (Goel, Pathak et al. 1987) and *Doliocarpus dentatus* (Alakurtti, Bergström et al. 2010). Betulin aldehyde has demonstrated low antibacterial activity against *Enterobacter*

aerogenes, *E. coli* and *P. aeruginosa* and moderate antibacterial activity against *Enterococcus faecalis* (Alakurtti, Bergström et al. 2010). It has also shown antitumour and anti-leishmanial activities (Haavikko, Nasereddin et al. 2014). This is the first report of the isolation of betulin aldehyde from the genus *Alphitonia*.

2.3.3 Quercetin (4) and Kaempferol (5)

Kaempferol has previously been isolated from a wide range of plants including the medicinal plants *Euonymus alatus* (Fang, Gao et al. 2008), *Camellia oleifera*, *Polygonum tinctorium* (Iwaki, Koya-Miyata et al. 2006) and *Acacia nilotica* (Iwaki, Koya-Miyata et al. 2006). Similarly, quercetin has been previously isolated from a number of plant species including *Citrullus colocynthis* (Meena and Patni 2008), *Psidium guajava* (Arima 2002) and *Prunus armeniaca* (Williams and Wender 1953). This is the second report of quercetin (**1**) and kaempferol (**2**) being isolated from the leaves of *A. excelsa*.

Quercetin and kaempferol have been reported to possess activity in a wide range of screens including antimicrobial, antioxidant, anti-inflammatory, antiviral and anticancer. Quercetin is known to exhibit antibacterial effects against a large range of bacterial strains, particularly those affecting the dermal systems. The chemical structure of flavonoids such as quercetin and kaempferol allows them to stabilise free radicals (Pietta 2000), thus, they have proven to be effective antioxidants. Both compounds have been reported to prevent oxidation of low density lipoproteins by scavenging free radicals (Moreno, Scheyer et al. 2006).

2.4 Evaluation of Unanalysed Fractions Obtained from *n*-Hexane and DCM Extracts

The pool of unanalysed fractions from the *n*-hexane and DCM extracts obtained from the preliminary studies of Yin and Petit (602 fractions in total) were analysed using reversed phase HPLC (at 210 - 450 nm) and LC-MS to identify promising fractions for further purification and/or structure elucidation. Because of the excessive number of fractions, TLC was performed first to facilitate the combination of similar fractions by their R_f values, evaluated by visualising the spots at 254 nm.

Among 167 fractions of the *n*-hexane extract (252 fractions before combination, 0.5 - 3 mg each), four samples showed distinctive peaks in the HPLC at 210 nm, indicative of organic compounds

(Silverstein, Webster et al. 2014). These samples were analysed by LC-MS (direct injection, electron impact) to identify the mass/molecular ion peaks. Unfortunately, the spectra did not show useful molecular or fragment ions. The ^{1}H NMR spectra (recorded in methanol–d_4 on a 400 MHz NMR spectrometer) of these samples did not show any characteristic proton signals beyond long chain fatty acids (all signals were observed between δ_H 1 - 4 ppm). These fatty acids were not of any interest to this study and were not further investigated as they are essential components of eukaryotes (Bach 2010), and hence would be present in every plant species.

Among the 96 fractions from the DCM extract (350 fractions before combination, 0.5 - 5 mg each), 16 samples showed peaks at 210 nm. After analysis by LC-MS (direct injection, electron impact), five samples were evaluated by ^{1}H NMR (recorded in methanol–d_4 on a 400 MHz NMR spectrometer). Similar to the *n*-hexane samples, they showed long chain fatty acid signals and were not further examined.

2.5 Concluding Remarks

One phytosterol (β-sitosterol), three triterpenoids (lupeol, betulinic acid and betulin aldehyde) and two flavonols (quercetin and kaempferol), previously isolated by the IBRG from *n*-hexane and DCM extracts of *A. excelsa* leaves, were structurally elucidated following spectral analysis and comparison with published literature. This is the first report of lupeol and betulin aldehyde in any *Alphitonia* species and the first report of β-sitosterol in *A. excelsa*. All six compounds have been reported to be beneficial against a wide range of medical conditions, and have a range of biological activities, including antibacterial and antioxidant activities. The presence of these antibacterial and antioxidant compounds thus provides support for the customary use of the leaves of this plant as an antiseptic hand wash and for skin infections, sores and wounds. Analysis of fractions from the *n*-hexane and DCM extracts obtained in the previous IBRG studies did not identify any compounds of interest, although this was hampered by the limited quantity of material.

To allow isolation of compounds in sufficient amount and identify further compounds to quantify their presence in the leaves, as well as to ascertain their relative importance towards the bioactivity and their overall significance in the customary use as an antiseptic, chemical and biological studies of a new collection of *A. excelsa* leaves were conducted and are described in Chapter 3.

Chapter 3: Chemical and Biological Investigations of *A. excelsa* Leaf Extracts

Chapter 2 presented the findings of antibacterial activity in extracts of *A. excelsa* leaves obtained after sequential extraction with *n*-hexane and DCM, along with the isolation of seven pure compounds in total from these extracts. Six of these compounds were identified in this MRes study as β-sitosterol, betulin aldehyde, betulinic acid, lupeol, quercetin and kaempferol. Heptyl heptanoate was previously identified by Dr Ping Yin. Unfortunately, none of these compounds were still available for this study and there was limited quantity of the crude extracts. Chapter 3 describes preliminary chemical and biological studies on a new collection of *A. excelsa* leaves in order to identify other natural products, whether biologically active or not, and to isolate compounds in sufficient quantity to quantify their presence in the extracts and ascertain their relative importance towards any antibacterial and antioxidant activities of these extracts.

3.1 Sequential Extraction of Leaves of *A. excelsa*

The extraction process can impact on the bioactivity of the extracts and also influence the ease of isolation of compounds from the extracts (Delfanian, Esmaeilzadeh Kenari et al. 2015). Therefore, choosing the right extraction process is an important part of chemical and biological investigations of natural products. Sequential extraction with solvents of increasing polarity is often regarded as a useful method for obtaining a wide range of compounds, as it separates components according to their polarity. In the earlier IBRG studies, *A. excelsa* leaves were ground to a fine powder with a food processor and extracted sequentially with *n*-hexane, DCM, EtOAc and MeOH. This gave reasonably good fractionation and the protocol was therefore used for the newer extraction of 1.41 kg of *A. excelsa* leaves, with the modification of grinding the leaves to a fine powder in a Waring blender and addition of a final water extraction step. This yielded five crude extracts; *n*-hexane, DCM, EtOAc, MeOH and water sequential extracts. The *n*-hexane, DCM, EtOAc and MeOH extracts had similar physical appearances and normal phase TLC profiles (based on R_f and visualised at 254 and 365 nm) to the extracts from the previous IBRG study (Table 3.1). As compared to the previous study, higher yields were obtained for the newer extracts. The difference in yields may have occurred due to the collection of the leaves in different seasons (previous

collection: March 2015, new collection: November 2016) or simply due to more efficient extractions in the newer procedure.

Table 3.1: Crude extracts obtained from sequential extraction of leaves of *A. excelsa*

Leaves of ***A. excelsa*** (previous: 1.2 kg, new: 1.41 kg)	Mass		Mass Yield		Physical appearances (new and previous)
	New extraction	Previous extraction	New extraction (*w/w*)	Previous extraction (*w/w*)	
n-Hexane	13.0 g	6.5 g	0.9%	0.54%	Yellowish green gum
DCM	26.7 g	6.8 g	1.9%	0.57%	Dark green solid
EtOAc	18.5 g	8.7 g	1.3%	0.73%	Dark green solid
MeOH	56.4 g	25.5 g	4.02%	2.15%	Blackish green solid
H_2O extract*	29.2 g	na	2.08%	na	Yellow fluffy powder

na = not applicable; *H_2O extract was not sequential in previous study

3.2 Phytochemical Studies of Crude Extracts of *A. excelsa* Leaves

Qualitative phytochemical screening (Akter, Barnes et al. 2016) (as described in Chapter 4: Experimental) was conducted to assist in identifying the presence of alkaloids, flavonoids, steroids, terpenoids, tannins, saponins and anthraquinone glycosides in the crude extracts obtained previously by Dr Yin and Petit and those obtained in this study. The extracts were evaluated for their phytochemical profiling by direct colour-change tests rather than using TLC plates and reagent stains. This was to avoid false positive results due to colour superimposition, which had been observed by the researchers in the previous *A. excelsa* study. Both *n*-hexane extracts showed the presence of steroids. The DCM extracts showed the presence of terpenoids, with the previous DCM extract also showing a positive result in the flavonoid test. Terpenoids were also found in the EtOAc and MeOH extracts. Tannins and saponins were found in the MeOH extracts. No alkaloids or anthraquinone glycosides were detected in any extract. Flavonoids, steroids, terpenoids, tannins and saponins are all known to possess a variety of biological activities including antibacterial and antioxidant (Tringali, Spatafora et al. 2000).

3.3 GC-MS Analyses of *n*-Hexane and DCM Extracts

The *n*-hexane and DCM extracts were selected for preliminary investigation by gas chromatography-mass spectrometry (GC-MS) to identify volatile components present in these extracts. Compounds were tentatively identified by observing the percentage similarity of the mass spectra for each

compound peak with the NIST library database. This led to the preliminary identification of nonanal, 1,54-dibromotetrapentacontane and docosane from the *n*-hexane extract, γ-sitosterol from the DCM extract, and tetracontane, tetradecane and hexadecane from both extracts (Table 3.3). Among all these phytoconstituents, hexadecane, nonanal, docosane and γ-sitosterol have previously been reported for their antimicrobial and antioxidant activities (Table 3.3). This is the first report on the GC-MS analysis of any plant part of *A. excelsa*.

Table 3.3: GC-MS analyses of *n*-hexane and DCM extracts of *A. excelsa* leaves

Phytoconstituents	% similarity of MS with standard stored in NIST library		Reported bioactivities of the identified phytoconstituents
	n-hexane	DCM	
Tetradecane	95%	97%	Antibacterial, antifungal (Akpuaka, Ekwenchi et al. 2013)
Hexadecane	95%	94%	Antifungal, antibacterial, antioxidant (Chin, Godwin et al. 2014)
Nonanal	89%	x	Antidiarrhoeal, antifungal (Zhang, ElSohly et al. 2002); antibacterial (Akpuaka, Ekwenchi et al. 2013)
Tetracontane	96%	96%	Not found
1,54-dibromotetrapentacontane	92%	x	Not found
Docosane	89%	x	Antibacterial (Chin, Godwin et al. 2014)
γ-Sitosterol	x	86%	Antibacterial, antifungal, antiviral, anti-inflammatory (Zhang and Jiang 2015)

x = not present

3.4 Bioassays of Crude Extracts of *A. excelsa* Leaves

As discussed in Chapter 1, bacterial infections and increased oxidative stress are major causes of delayed wound healing and skin infections. It is vital to discover antibacterial and antioxidant compounds to combat these conditions as they are global healthcare concerns. The leaves of *A. excelsa* were chosen for investigation of their antibacterial and antioxidant activities due to the plant's medicinal use by the Yaegl community as an antiseptic wash and for topical treatment of skin infections, sores and wounds. To examine the antibacterial activity of the earlier and newer extracts, disc diffusion (Bauer 1966) and MTT microdilution (Appendino, Gibbons et al. 2008) assays were performed, with slight modification. The DPPH free radical scavenging (Adedapo, Jimoh et al. 2008) and ferric reducing antioxidant power (FRAP) (Wang, Wang et al. 2013) assays were conducted to evaluate the antioxidant activities of all the extracts of *A. excelsa*.

3.4.1 *In Vitro* Antibacterial Activity of Crude Extracts of *A. excelsa* Leaves

3.4.1.1 Selection of bacteria for antibacterial study

Methicillin sensitive *S. aureus* (MSSA), methicillin resistant *S. aureus* (MRSA) and multidrug resistant strains of *S. aureus* ((MDRSA) were selected for antibacterial screening of the extracts as they are commonly associated with wounds and skin infections (Giacometti, Cirioni et al. 2003, Purohit, Solanki et al. 2013). *E. coli* and *P. aeruginosa* which are often used for such screening, were not chosen as the studies with the previous extracts showed they were inactive against these strains.

3.4.1.2 Disc Diffusion Assay

The disc diffusion assay is the most widely used method for determining preliminary antibacterial activity due to its simplicity, rapidity and low cost (Valgas, Souza et al. 2007). In this method, small filter paper discs, impregnated with samples at the desired concentration, are placed on the surface of a suitable agar plate which is then incubated with the selected bacteria under suitable conditions. In this project, a zone of inhibition for 1 mg of extract on the disc of greater than 15 mm, including the 6 mm disc, was considered as good antibacterial activity, between 10 mm and 15 mm as moderate activity and below 10 mm as low activity (Lin, Opoku et al. 1999, Kirmizigul, Koz et al. 2003)

Unlike the previous study, where the *n*-hexane extract showed the highest activity against MSSA, followed by the EtOAc extract, the DCM extract demonstrated the most promising antibacterial activity (10 mm) against both MSSA and MRSA, followed by the EtOAc extract (Table 3.4). In contrast, the EtOAc extract showed greater activity (10 mm) against the MDRSA strain, followed by the DCM and MeOH extracts (Table 3.4). Although the previous study did not show antibacterial activity for the MeOH extract, the current study found it to exhibit low activity (7 - 8 mm) against all three bacterial strains.

Table 3.4: Comparison of disc diffusion assay results of new and earlier *A. excelsa* extracts

Extract of *A. excelsa* leaves (extract: 1 mg/disc; control: 0.1 mg/disc)	**Diameter of zone of inhibition (in mm)[#]**					
	MSSA		**MRSA**		**MDRSA**	
	New extract	**Earlier extract**	**New extract**	**Earlier extract**	**New extract**	**Earlier extract**
n-Hexane	7 mm	15 mm	8 mm	nd	7 mm	nd
DCM	10 mm	10.5 mm	10 mm		9 mm	
EtOAc	8 mm	13 mm	9 mm		10 mm	
MeOH	8 mm	na	7 mm		8 mm	
Vancomycin*	13 mm	18 mm	13 mm		13 mm	

[#]Zone of inhibition was determined by diameter of complete inhibition of bacterial growth on agar plates, including the 6 mm disc diameter; *positive control; tests were performed in duplicate; na = not active; nd = not done

While being a very simple technique, the disc diffusion assay is often not effective for non-polar or large molecules, as it is based on the ability of the sample to diffuse into the agar (Valgas, Souza et al. 2007). Whatman filter paper discs, which are commonly used, may also influence the results (Valgas, Souza et al. 2007). Due to the cellulose content of the paper discs containing free hydroxyl groups, the surface of the discs are hydrophilic, allowing polar compounds to be adsorbed and limiting diffusion into the medium (Burgess, Jordan et al. 1999, Valgas, Souza et al. 2007). Non-polar compounds would not be influenced by the hydroxyls of the discs' surface, but due to their hydrophobic nature they may not diffuse through the aqueous medium. The size of the molecules also affects the assay results as larger molecules often diffuse poorly. Moreover, this method also requires a moderate amount of sample, which is not always possible to obtain in the case of natural product investigations. Furthermore, the MIC of the samples can not be determined using the disc diffusion assay, hence this method can only be used for qualitative evaluations.

3.4.1.3 MTT Microdilution Assay

Microdilution assays allow for the testing of a wide range of samples with different concentrations in a single microtitre plate. The MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] microdilution assay relies on the principle that yellow MTT is reduced to purple MTT formazan in the presence of viable cells (Figure 3.1). This procedure is widely used to measure cell proliferation and cytotoxicity (Mosmann 1983) and to evaluate the antibacterial activity of compounds (Appendino, Gibbons et al. 2008). Although the mechanism of action is unknown within microorganisms, the mechanism for cellular MTT reduction to MTT formazan is mostly due to mitochondrial dehydrogenases (Liu, Peterson et al. 1997) and possibly other non-mitochondrial dehydrogenases and xanthine oxidase (Burdon and Gill 1993).

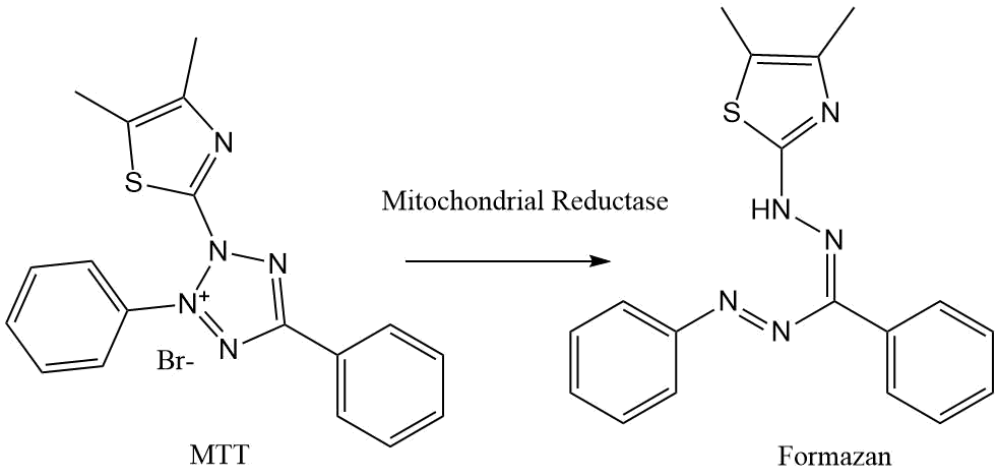


Figure 3.1: Reduction of MTT (yellow) to MTT formazan (purple) by viable cells

The MIC can be determined by the MTT microdilution assay as the lowest concentration at which no bacterial growth (yellow in colour) is observed. According to published reports, crude plant extracts having MIC values below 1000 µg/mL are considered worthy of further investigation (Rios and Recio 2005, Appendino, Gibbons et al. 2008). An MIC below 2000 µg/mL in preliminary screens should also be considered of interest (Palombo and Semple 2001). In this study, MIC values of 312.5 µg/mL or less were considered as being of good activity, 625 - 1250 µg/mL as moderate activity and more than 1250 µg/mL as low antibacterial activity.

The MTT assays were done on the extracts from the earlier and newer extractions and were similar across the different batches for the *n*-hexane, DCM and MeOH extracts. The EtOAc extracts were not as consistent. EtOAc Not showing activity against MRSA is unusual, as had showed activity against both MSSA and MDRSA. Both DCM extracts showed good activity, being the highest activity amongst the extracts (Table 3.5). All extracts showing antibacterial activity were subjected to evaluation for their bactericidal and bacteriostatic effects (Karaman, Sahif et al. 2003). All were found to have bacteriostatic effects on the tested bacteria (Table 3.5).

Table 3.5: Antibacterial activity of *A. excelsa* extracts

Extracts	Minimum inhibitory concentration (MIC) in µg/mL					
	MSSA		MRSA		MDRSA	
	New extract	Old extract	New extract	Old extract	New extract	Old extract
n-hexane	1250 (bc)		1250 (bc)		2500 (bc)	
DCM	625 (bc)		**312.5** (bc)		**312.5** (bc)	
EtOAc	625 (bc)	1250 (bc)	Na	1250 (bc)	625 (bc)	1250 (bc)
MeOH	1250 (bc)		625(bc)		1250 (bc)	
Vancomycin*	0.39 (bc)		0.78 (bc)		0.78 (bc)	

*positive control; na = not active; bc = bacteriostatic; all tests were performed in triplicate

The MTT microdilution assay gives quantitative antibacterial activity results and is generally regarded as a low cost and reliable colourimetric assay (Abate, Mshana et al. 1998). Although the accuracy of the assay can be compromised by samples that are coloured, redox active and/or samples that are not soluble in the predominantly aqueous medium, it is considered the most reliable and widely used method to determine the antibacterial activity of natural products (Han, Niu et al. 2010).

3.4.2 *In Vitro* Antioxidant Activity Study of Crude Extracts of *A. excelsa* Leaves

3.4.2.1 DPPH Free Radical Scavenging Assay

1,1-Diphenyl-2-picrylhydrazyl (DPPH) is a stable free radical (Mensor, Menezes et al. 2001). Determining the ability of antioxidants to scavenge the DPPH free radical is the basis of the popular DPPH antioxidant assay (Sharma and Bhat 2009), which is the simplest and most widely adopted method to evaluate antioxidant activity of compounds (Adefegha, Oboh et al. 2014). Scavenging of the DPPH radical due to the presence of antioxidants or hydrogen-donating compounds results in reduction of the purple DPPH to the corresponding pale yellow hydrazine (Figure 3.2). The DPPH scavenging activity of antioxidants are evaluated by monitoring the decrease in absorbance at 517 nm.

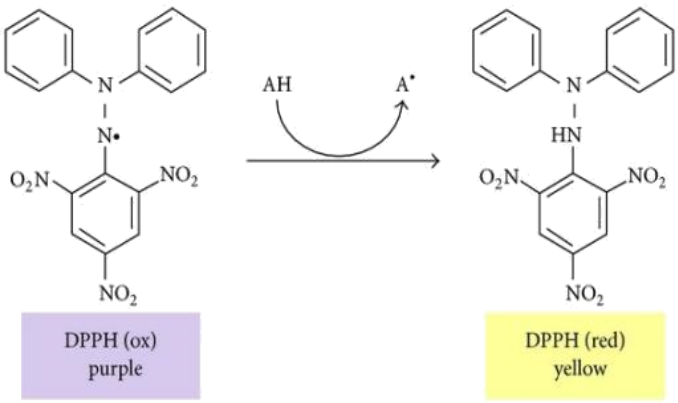


Figure 3.2: Conversion of DPPH free radical (purple) to reduced hydrazine (pale yellow)

The *A. excelsa* extracts from the earlier and newer studies were subjected to the DPPH antioxidant assays at different concentrations (100, 50, 25, 12, 6.25, 3.13, 1.56 and 0.78 µg/mL). L-Ascorbic acid was used as a positive control over the same concentration range. Similar antioxidant activity was seen for the different batches of extracts, hence the activity of only the newer extracts are presented in Figure 3.3 (including the new water extract). The DCM extracts showed very promising DPPH scavenging activity (89%) at 100 µg/mL, followed by MeOH (83%), water (75%), EtOAc (57%) and *n*-hexane (53%) extracts, compared to that of the standard L-ascorbic acid (97%) (Figure 3.3).

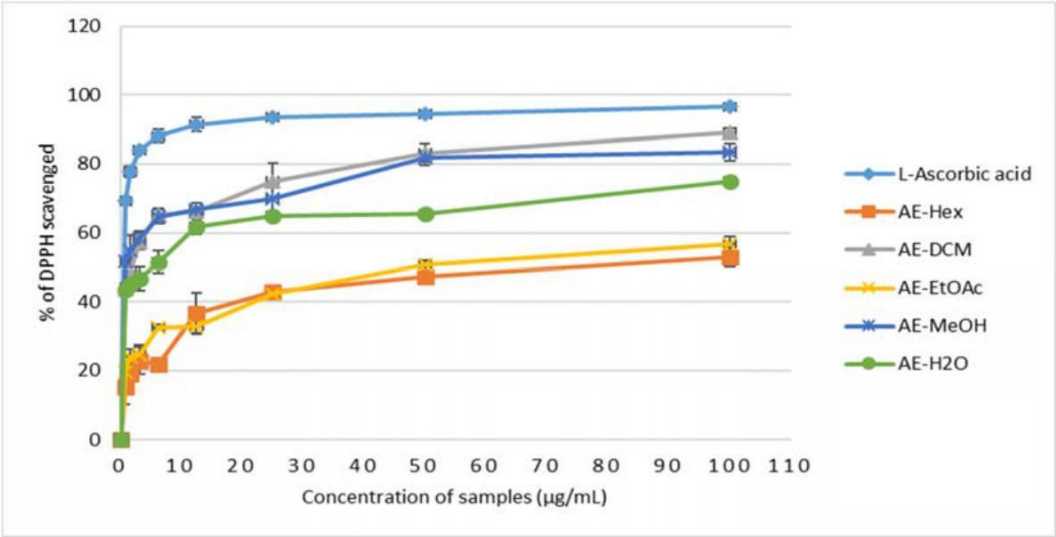


Figure 3.3: DPPH scavenging activity of *A. excelsa* leaves

The steric accessibility of the DPPH radical is a major determinant of the reaction, since small molecules that have better access to the radical site typically have relatively higher antioxidant capacity. Many large antioxidant compounds that react quickly with peroxyl radicals may react slowly or may even be inert in this assay (Prior, Wu et al. 2005). The inexistence of DPPH or similar radicals in biological systems is also a shortcoming to the biological relevance of the assay (Pyrzynska and Pękal 2013). In addition, the spectrophotometric measurements can be affected by compounds, such as carotenoids, that absorb at the wavelength of determination as well as by the turbidity of the sample (Lichtenthaler and Buschmann 2001). Despite these limitations, the DPPH radical is stable, commercially available, and does not have to be generated before the assay. Therefore, it is considered an easy and useful spectrophotometric method with regards to measuring the antioxidant activity of compounds (Adefegha, Oboh et al. 2014).

3.4.2.2 FRAP Assay

The ferric reducing antioxidant power (FRAP) assay is one of the most important methods of quantitating the antioxidant status of natural products. This assay determines the ability of antioxidants to reduce the yellow coloured ferric-tripyridyltriazine complex [Fe(lll)-TPTZ] to the blue coloured ferrous-tripyridyltriazine [Fe(ll)-TPTZ] complex by donation of one electron (Figure 3.4). The ferric reducing ability of antioxidants is evaluated by monitoring the increase in absorbance at 593 nm.

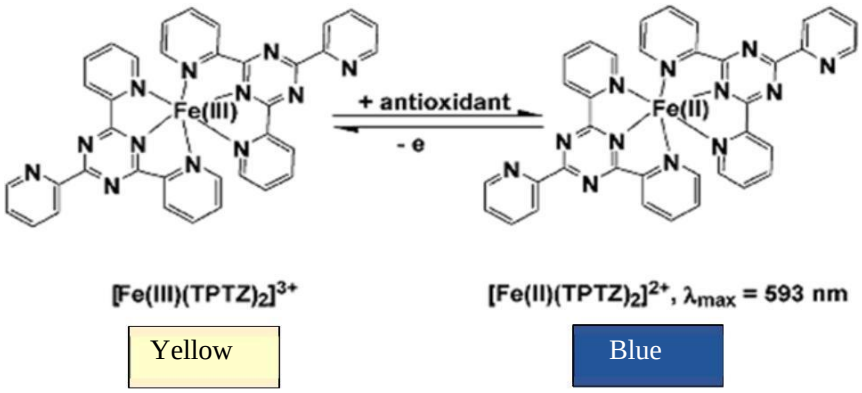


Figure 3.4: Conversion of Fe(lll)-TPTZ complex (yellow) to reduced Fe(ll)-TPTZ complex (blue)

The earlier and newer extracts were prepared at different concentrations (40, 20, 10, 5, 2.5, 1.25, 0.625 and 0.313 µg/mL) and their ferric reduction powers were assessed. Similar to the DPPH scavenging assay, both the earlier and newer extracts showed similar antioxidant activity, hence the activity of only the newer extracts are presented in Figure 3.5 (including the new H_2O extract). The DCM extract, as for the DPPH scavenging assay, showed the highest activity (72%), followed by the water (32%) and MeOH (26%) extracts, compared to that of the standard trolox defined as 100% ferric reducing antioxidant power (Figure 3.5).

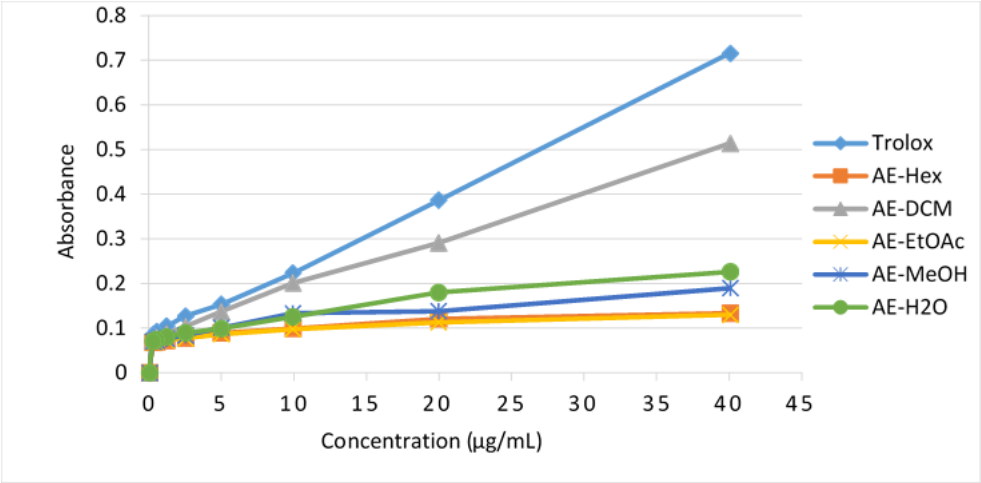


Figure 3.5: Antioxidant activity (%) of extracts of *A. excelsa* leaves by FRAP assay

The FRAP assay relies on the hypothesis that the redox reaction proceeds so rapidly that all reactions are complete within 4 minutes, but this is not always the case (Prior, Wu et al. 2005). The order of reactivity of a series of antioxidants can vary tremendously and even invert, depending on the analysis time. Thus, a single reaction endpoint may not represent the completed activity. The FRAP assay measures only the reducing capability based upon the ferric ion, which is not relevant to antioxidant activity in biological systems mechanistically and physiologically. However, another limitation of this method is that the measured reducing capacity does not necessarily reflect antioxidant activity (Frankel and Meyer 2000). The non-physiological pH (3.6) value is also a limitation (Singh, Singh et al. 2008).

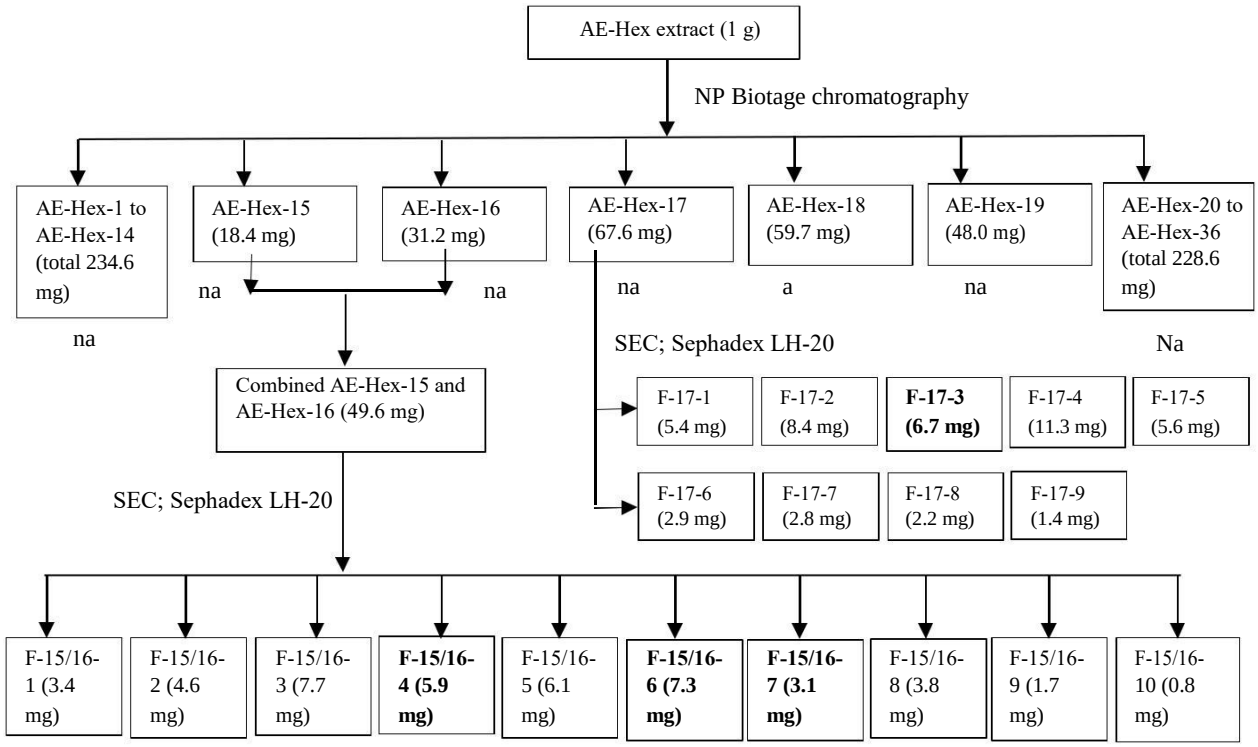
3.5 Commencement of Compound Isolation from *n*-Hexane Extract of *A. excelsa* Leaves

Although the DCM extract looked most promising in terms of bioactivity, the *n*-hexane extract was chosen for chemical studies first; with analysis of the DCM extract being flagged subsequent to this as part of future studies. This choice was made to allow continuation of the previous IBRG study that had already identified a range of interesting molecules through the isolation studies, but had not been comprehensive or quantitative. Investigations on the new *n*-hexane extract was two-fold: to isolate compounds previously obtained for biological testing and quantify their presence in the original extracts to understand their significance in the biological activities of the extracts and customary preparations; and to identify further compounds, whether bioactive or not, to understand the phytochemistry more fully of *A. excelsa*. Targeting the isolation of previously obtained compounds as well as the identification of further compounds of interest, the *n*-hexane extract (1 g) was subjected to normal phase Biotage chromatography. This yielded 36 major fractions based on their normal phase silica TLC R_f profiles (Scheme 3.1), with around 70% recovery of mass. The TLC plates were visualised at 254 nm UV light and seven UV-active fractions (F15 - F21) were identified that showed good separation by TLC with obvious clear spots between R_f 0.3-0.7 with *n*-hexane:EtOAc, 7:3. These fractions were subjected to TLC bioautography (Hamburger and Cordell 1987) against methicillin sensitive *S. aureus* with MTT staining and this identified one bioactive fraction (F18) with one active spot (clear spot against purple background) (Scheme 3.1). Furthermore, the ^{1}H NMR spectra of these fractions (F15 - F21) exhibited interesting signals including in the aromatic region (δ_H 6-8) possibly indicative of flavonoids, along with resonances at δ_H 1 - 3 ppm, which suggested the presence of possible steroids in these fractions. Comparing the ^{1}H NMR and MS data with that of previously identified compounds (**1 - 6**), close similarity was observed with peaks seen for lupeol (**3**) and β-sitosterol (**1**) for fractions (F15 - F19) and these were prioritised for further purification.

As noted above, a 70% mass recovery occurred after the normal phase silica chromatography. While normal phase silica chromatography is commonly used for natural product purifications, the surface of silica gel consists of exposed silanol groups that polar compounds can be strongly adsorbed on to. To aid further purification of the fractions (F15 - F19), and limit loss of sample, size exclusion chromatography (SEC) using the lipophilic organic resin Sephadex LH-20 was conducted. This is a commonly used method for the purification of natural product extracts and can be very useful for separation of molecules grouped together with similar polarities, for example

following normal phase silica chromatography. Sephadex LH-20 separates on the basis of molecular size, with the molecules not directly interacting with the Sephadex resin. Typically, a high recovery rate of compounds is achieved using Sephadex (Gutzeit, Wray et al. 2007).

SEC using Sephadex LH-20 was conducted for fractions F15 and F16 (combined) and for F17. This yielded a total of 19 fractions, with 70% mass recovery (Scheme 3.6). TLC of these fractions showed four semi-pure fractions (F15/16-4, -6 and -7 and F17-3). Further purification of these fractions were not conducted due to the limited time of the present study. Purification of the bioactive fraction (F18) and the other non-active fraction of interest (F19) were also not performed due to the time constraints. These will be the subject of immediate future studies.



NP = normal phase; a = active; na = not active; SEC = size exclusion chromatography; F = fraction

Scheme 3.1: Fractionation of *n*-hexane extract of *A. excelsa* leaves

3.6 Concluding Remarks

Sequential extraction of leaves of *A. excelsa* was conducted with *n*-hexane, DCM, EtOAc, MeOH and water. The extracts showed the presence of steroids, terpenoids, tannins and saponins in

phytochemical screens. The biological activity of these types of compounds are well documented in the literature, including for their antibacterial and antioxidant activities. The GC-MS analyses exhibited the presence of thirteen constituents in the *n*-hexane and DCM extracts, including γ-sitosterol, tetradecane, hexadecane, nonanal, docosane, 1,54-dibromo-tetrapentacontane and tetracontane, which are known to have antibacterial and/or antioxidant activities.

The DCM extract showed good antibacterial activity in both the disc diffusion and MTT microdilution assays (10 mm zone of inhibition and MIC 312.5 µg/mL, respectively) against methicillin sensitive, methicillin resistant and multidrug resistant strains of *S. aureus.* The EtOAc extract also showed considerable antibacterial activity in both assays, followed by the *n*-hexane and MeOH extracts. The DCM extract showed the highest antioxidant activity in both the DPPH free radical scavenging and FRAP assays, with quite impressive activity. The water extract also showed good antioxidant activity in both assays, followed by the MeOH, EtOAc and *n*-hexane extracts.

The *n*-hexane extract was chosen for further fractionation studies as a continuation of the earlier studies on this extract. Normal phase biotage flash chromatography yielded 36 fractions based on their TLC R_f profiles, among which five fractions (F15 - F19) showed ^{1}H NMR and MS spectra of interest. One fraction (F18) showed bioactivity by TLC bioautography against *S. aureus*. Further fractionation by size exclusion chromatography of fractions 15 and 16 (together) and fraction 17 yielded 10 and 9 fractions, respectively, which showed semi-pure compounds by TLC. Due to limited time, no further purification or analysis of these fractions were conducted, however, this will be the subject of future studies.

Chapter 4: Experimental

4.1 Materials and Equipment

All solvents used for extraction, chromatographic separations and analytical techniques were of analytical HPLC grade. EtOAc and MeOH are purchased from Sigma-Aldrich, USA, and acetonitrile, acetone, chloroform, DCM and *n*-hexane were purchased from Merck, Germany. For phytochemical screening and bioactivity testing, acetic anhydride, 10% aqueous ammonia, Dragendorff's reagent, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), 2,2-diphenyl-1-picrylhydrazyl (DPPH), 2,4,6-tri(2-pyridyl)-s-triazine (TPTZ), ferric chloride, 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (trolox), L-ascorbic acid, magnesium metal, sodium acetate trihydrate and vancomycin were all purchased from Sigma-Aldrich, USA; formic acid, glacial acetic acid, hydrochloric acid (HCl, 36%) and sulfuric acid (98%, H_2SO_4) were purchased from Merck, Germany; and Muller Hinton (MH) ll agar and broth for bacterial cultures were purchased from Bacto Laboratories Pty Australia.

Normal phase thin layer chromatography (TLC) was performed on fluorescent silica gel F_{254} plates (Merck, Germany) and the plates were visualised using UV light of 254 nm and 365 nm. The UV–visible data were recorded on a CARY 1 Bio spectrophotometer (Varian, USA). Chromatography was performed using normal phase Biotage columns (50 g silica SNAP cartridges) and a Biotage® Isolera Four system at a flow rate of 40 mL/min or with Sephadex LH-20 (18 – 111 µm, GE Healthcare Biosciences AB, Sweden). Gas chromatography-mass spectrometry (GC-MS) analyses were carried out on a Shimadzu GC-MS-QP2010 and the mass spectra were compared with that stored in the NIST library. Reversed phase HPLC analyses were carried out using an Agilent semi-preparative 1260 Infinity HPLC system with an Agilent ZORBAX SB-C18 column (5 µm, 4.6x150 mm). An Agilent 6130 LC-MS system was used for ESIMS analyses. NMR spectra were recorded on a Bruker AVANCE-400 instrument (^{1}H NMR: 400 MHz, ^{13}C NMR: 100 MHz) in the present study and on a Bruker AVANCE-600 instrument equipped with a cryoprobe (^{1}H NMR: 600 MHz, ^{13}C NMR: 150 MHz) in the previous study. The 1D and 2D ^{1}H chemical shifts were referenced to the residual solvent peaks at δ_H 2.49 for DMSO-d_6, at δ_H 1.93 for acetonitrile-d_3, at δ_H 7.24 for chloroform-*d*, at δ_H 2.09 for acetone-d_6 and at δ_H 3.31 for methanol-d_4. ^{13}C chemical shifts were referenced to the relevant solvent peaks at δ_C 39.5 for DMSO-d_6, at δ_C 1.39 for acetonitrile-d_3, at δ_C 77.0 for chloroform-*d*, at δ_C 205.8

for acetone-d_6 and at δ_C 49.0 for methanol-d_4. *J* values are given in Hz. All bacterial work was performed in a PC2 laboratory in a biosafety cabinet. Chemical risk assessments and biosafety approvals (approved by Macquarie University Biosafety Committee, approval no. 5201600535) were in place before initiation of any experiments. Stocks of methicillin sensitive (MSSA, ATCC 29213), methicillin resistant (MRSA, ATCC BAA 1026) and multidrug resistant (MDRSA, clinical isolate) strains of *S. aureus* were provided by Dr John Merlino from Department of Microbiology, Concord Hospital, Sydney. MH ll agar and broth for bacterial culture were prepared as per the manufacturer's protocols, autoclaving at 121 ^{o}C for 20 minutes. The agar media were poured into petri dishes and allowed to solidify in a biosafety cabinet to prepare the agar plates for bacterial growth. Organic solvents were evaporated under vacuum using a Büchi rotary evaporator (Switzerland) and trace water was dried with the help of liquid nitrogen using a CHRIST alpha 1-4 LD*plus* Freeze Dryer (Germany).

4.2 Previous IBRG Study of *Alphitonia excelsa*

Fresh mature leaves of *A. excelsa* were collected by ethnobotanist Mr David Harrington from Cumberland State Forest, NSW, Australia (33^{o}44'39", 151^{o}2'26") on 27 March, 2014 (voucher specimen no. IBRG00009). The following studies were undertaken by Ms Mathilde Petit and Dr Ping Yin, former researchers of the IBRG. Leaves (1.2 kg) were ground into a fine powder using a food processor (Sunbeam) and divided into 3 portions to facilitate extraction. Each portion of ground leaves was sequentially extracted with *n*-hexane (2 x 4.5 L; 24 hours) at room temperature with continuous shaking using a Rateck orbital mixer incubator at 180 rpm followed by vacuum filtration using Whatman No. 1 filter paper (Whatman, UK). The residual solids were then extracted with DCM (2 x 3 L; 24 hours), followed by vacuum filtration, and further subjected to extraction with EtOAc and MeOH in an identical manner. Water extraction was carried out separately with 200 g of fresh ground leaves with milli-Q water (2 x 1.5 L; 24 hours). The filtered extracts of the same solvent (for *n*-hexane, DCM, EtOAc and MeOH) were combined and concentrated under vacuum, followed by freeze-drying to remove any residual water. For the combined water extract, the water was removed by freeze drying for 48 hours. This afforded crude *n*-hexane (yellow gum, 6.5 g, 0.54% *w/w*), DCM (dark green solid, 6.8 g, 0.57% *w/w*), EtOAc (dark green solid, 8.7 g, 0.73% *w/w*), MeOH (blackish green solid, 25.5 g, 2.15% *w/w*) and water extracts (yellowish green fluffy powder, mass not recorded) (Table 2.1). All crude extracts were

stored in air tight glass containers and kept at -20 ^{O}C for long term storage. Normal phase silica gel, Sephadex LH-20, and normal and reversed phase biotage columns (SNAP) were used for fractionation and isolation of pure compounds from *n*-hexane and DCM extracts (as described in Chapter 2, Scheme 2.1 and 2.2). 1D and 2D NMR experiments, including COSY, HSQC and HMBC, were run by Dr Yin and Mr Ryan Kenny (NMR Operator) and ESIMS was conducted by Ms Kavita Ragini (MS Operator) for the pure compounds.

4.2.1 Characterisation of Pure Compounds Obtained from Previous Study

Pure compounds, previously isolated by Dr Yin, were structurally elucidated using the previously obtained NMR and MS data and confirmed by comparing the spectral data with that of the literature.

4.2.2 Analyses of *n*-Hexane and DCM Fractions Obtained from Previous Study

A total of 602 fractions, previously collected by Dr Yin and MS Petit, were analysed by normal phase TLC using *n*-hexane:EtOAc (7:3) as eluent. Fractions with similar separation on the TLC plates, based on R_f, were combined to give 262 samples that were subjected to analyses by reversed phase HPLC. Samples were prepared for HPLC at 0.5 mg/mL in MeOH followed by filtration. The samples were subjected to semi-preparative HPLC using a C_{18}-bonded silica column at a flow rate of 1 mL/min. Isocratic conditions of 10% acetonitrile/90% water were held for 3 minutes, followed by a linear gradient to acetonitrile over 10 minutes, then isocratic conditions of acetonitrile for 5 minutes. Samples demonstrating strong peaks at 210 nm were analysed at 0.1 mg/mL in MeOH by direct injection with 5% formic acid into an Agilent 6130 LC-MS to determine the mass, with acetonitrile and Milli-Q water as the solvent system, at 7.5:2 ratio for 1 minute. These samples were also subjected to analysis by ^{1}H NMR using methanol-d_4.

4.3 Studies on New Collection of *Alphitonia excelsa*

4.3.1 Collection and Processing of Plant Material

Fresh mature leaves (1.5 kg) of *A. excelsa* were collected from Cumberland State Forest, NSW, Australia (-33.742224, 151.039974) from two adjacent trees on 7th November, 2016 and identified with the expertise of ethnobotanist Mr David Harington (voucher specimen no. IBRG00020 and

IBRG00021). Leaves that appeared moth-eaten (Packer, Brouwer et al. 2012) were removed and the remaining leaves carefully separated and washed with water. After draining the excess water, the wet leaves were spread out in an open space and air-dried at room temperature overnight. The dried leaves were ground into a coarse powder using a Waring heavy duty blender and were subject to extraction without any delay.

4.3.2 Extraction of *A. excelsa* Leaves

Dried coarse powder of the leaves of *A. excelsa* (1.4 kg) was divided into 6 portions (235 g per portion) to facilitate extraction. In brief, the portions of leaves (6 x 235 g) were extracted with *n*-hexane (3 x 1.6 L; 24 hours) at room temperature with agitation at 80 rpm using a Rateck orbital mixer incubator followed by vacuum filtration through Whatman No. 1 filter paper (Whatman, UK). The residual solids were then extracted in an identical manner with DCM, followed by EtOAc, MeOH and water. The filtered extracts of the same solvent (for *n*-hexane, DCM, EtOAc and MeOH) were combined and concentrated under vacuum, followed by freeze drying to remove any residual water. For the combined water extract, the water was removed by freeze drying for 48 hours. This afforded crude *n*-hexane (yellowish green gum, 13.0 g, 0.9% *w/w*), DCM (dark green solid, 26.7 g, 1.9% *w/w*), EtOAc (dark green solid, 18.5 g, 1.3% *w/w*), MeOH (blackish green solid, 56.4 g, 4.0% *w/w*) and water (yellow fluffy powder, 29.2 g, 2.1% *w/w*) extracts. All crude extracts were stored in air tight glass containers and kept at -20 ^{o}C for long term storage.

4.3.3 Phytochemical Screening of Crude Extracts

Qualitative phytochemical screening was conducted for the crude *n*-hexane, DCM, EtOAc, MeOH and water extracts to ascertain the presence of alkaloids, flavonoids, steroids, terpenoids, tannins, saponins and anthraquinone glycosides following published methods (Akter, Barnes et al. 2016). **Identification of alkaloids:** Extracts (0.02 g) were stirred with 2 mL of 1% HCl on a steam bath and filtered through a Whatman No. 1 filter paper. The filtrate (1 mL) was treated with a few drops of Dragendorff's reagent. The presence of alkaloids was indicated by the formation of an orange precipitate.

Identification of flavonoids: Extracts (0.02 g) were dissolved in 1 mL of MeOH and a small chip of magnesium metal was added to the solution, followed by the addition of a few drops of 36% HCl. The presence of flavonoids was identified by a change in the solution's colour to magenta.

Identification of steroids: Extracts (0.02 g) were dissolved in 2 mL of chloroform and filtered through Whatman No. 1 filter paper. H_2SO_4 (98%) was added dropwise to the solution, down the wall of the test tube. Formation of a reddish brown colour at the interface indicated the presence of steroids.

Identification of terpenoids: Extracts (0.02 g) were dissolved in 1 mL of MeOH and filtered through a Whatman No. 1 filter paper. Acetic anhydride (1 mL) was added to the filtrate followed by addition of 2 mL of 98% H_2SO_4 down the wall of the test tube. The presence of terpenoids was confirmed by observing the formation of a reddish brown colour at the interface.

Identification of saponins: Extracts (0.5 g) were stirred with 15 mL of Milli-Q water and shaken vigorously for about 5 minutes. The formation of a stable froth indicated the presence of saponins.

Identification of tannins: Extracts (0.02 g) were dissolved in 2 mL of Milli-Q water and filtered through Whatman filter paper. The filtrate was treated with a few drops of 1% ferric chloride solution. The presence of tannins was confirmed by the formation of a blue colour.

Identification of anthraquinone glycosides: Extracts (0.02 g) were heated with 10% HCl for 2 minutes and 2 mL of chloroform was then added to the mixture. It was shaken continuously for a few minutes then treated with 1 mL of 10% ammonia solution. Formation of a pink colour indicated the presence of anthraquinone glycosides.

4.3.4 GC-MS Analysis of *n*-Hexane and DCM Extracts

GC-MS analyses were performed on the *n*-hexane and DCM crude extracts using a Shimadzu GC QP2010 instrument equipped with a split/splitless injector, a Restek Sil5 fused silica capillary column (30 m x 0.25 mm, 0.25 μm film) and integrated mass spectrometer connected to a NIST library. Helium gas (BOC, North Ryde, NSW, Australia) (99.999%) was used as the carrier gas with a constant flow of 1 mL/min. The temperatures of the injector and detector were set at 270 and 290 °C, respectively. The initial column temperature was set at 50 °C and held for 4 minutes, then increased to 250 °C at a rate of 10 °C/min, then held at 250 °C for 6 minutes. The data acquisition rate was 100 Hz/scan and the data were processed using GC-MS Postrun Analysis

software. Compounds were identified by their identical GC retention times and retention indices and by comparison of their mass spectra with that of pure standards stored in the NIST library.

4.3.5 Bioassays of the Crude Extracts

4.3.5.1 Disc Diffusion Assay

The extracts of *A. excelsa* were prepared at 50 mg/mL in acetone. Similarly, a standard sample of vancomycin at 0.1 mg/mL was prepared as a positive control, whereas acetone served as a negative control. Whatman sterile discs (6 mm) were impregnated with 20 µL of extract, standard vancomycin or acetone per disc. Impregnated discs were then air dried for 30 minutes. All tests were performed in duplicate.

Freshly prepared cultures of the three strains of *S. aureus* in MH ll broth at A_{600} = 0.08 ($10^7 – 10^8$ CFU/mL) were swabbed evenly on the surface of MH ll agar plates and the impregnated discs were placed on the inoculated agar followed by overnight incubation at 37 ^{o}C. The diameter of the clear zone of inhibition of bacterial growth on agar was measured (including the 6 mm disc) to evaluate antibacterial activity of the extracts compared to the standard antibiotic.

4.3.5.2 MTT Microdilution Assay

Each extract (100 µL, 5 mg/mL) and vancomycin (100 µL, 0.1 mg/mL) were mixed with 100 µL of sterile MH ll broth and placed in well 1 of a 96-well microtitre plate. A two-fold serial dilution was carried out for each extract and antibiotic with MH ll broth to give final concentrations of 2500 – 2.44 µg/mL for extracts and 50 – 0.05 µg/mL for vancomycin into wells 1 - 11. Cultures of *S. aureus* (MSSA, MRSA and MDRSA, 100 µL, A_{600} = 0.08; diluted 100-fold in MH II broth) were added to wells 1 - 12, leaving out well 11. Since well 11 was free from bacterial culture, it acted as a sterile control and well 12, being free of extracts or antibiotic, served as a growth control of bacteria. Acetone was treated as above, serving as a negative control. The microtitre plates were incubated at 37 ^{o}C for 18 hours, then 20 µL of a methanolic solution of MTT (5 mg/mL) was added into each well followed by further incubation for 1 hour. The reduction of yellow MTT to purple MTT formazan was used to determine the minimum inhibitory concentrations (MIC) of the samples. The MIC was the lowest concentration of extract or antibiotic that showed no visible colour change from yellow to blue.

Samples showing any antibacterial activity in the MTT microdilution assay were assessed for their bactericidal or bacteriostatic activities against the corresponding bacterial strains by sub-culturing onto fresh agar plates (Karaman, Sahin et al. 2003). Aliquots (5 µL) were taken from yellow (no growth) wells of the MTT test plates and spotted onto fresh MH II agar plates, then incubated overnight at 37°C. Any bacterial growth on the agar plates indicated bacteriostatic activity whereas no growth indicated a bactericidal effect of the samples.

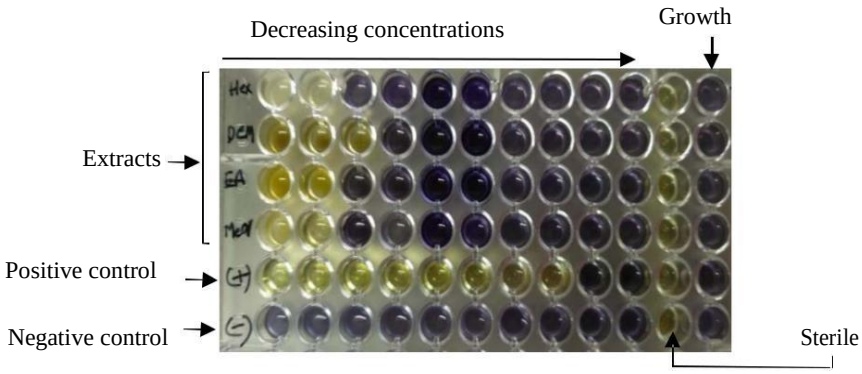


Figure 4.1: Design of MTT assay in 96-well microtitre plates

4.3.5.3 TLC bioautography

TLC bioautography was conducted based on a published method (Hamburger and Cordell 1987). Normal phase silica TLC plates were UV sterilised for 30 minutes. Extracts (50 µg/mL) were dissolved in acetone, then spotted on the TLC plates and air-dried. The plates were developed using *n*-hexane:EtOAc (7:3), under aseptic conditions, in a biosafety cabinet. Vancomycin (5 µg) in 1 mL of solvent was also spotted on the TLC plate as a positive control. The plates were dried through cold air flow using a hair dryer for complete removal of solvents. TLC plates were run in duplicate, with one plate used as a reference chromatogram. UV visible compounds at 254 nm were marked on the reference chromatogram. The duplicate chromatograms were placed on the surface of prepared agar plates with the silica gel of the plates facing upwards and freshly prepared culture of MSSA (A_{600} = 0.08) was spread over the plate followed by overnight incubation at 37 ^{o}C. A methanolic solution of MTT (1 - 2 mL, 5 mg/ml) was gently spread on the bioautogram and allowed to incubate for a further 1 hour at the same temperature. A white zone of inhibition of bacterial growth against a purple background indicated antibacterial activity.

4.3.5.4 DPPH Free Radical Scavenging Assay

DPPH scavenging activity of the crude extracts was determined following the protocol described by Liu, Lin et al. 2009. Extracts were prepared at a concentration range of 100 - 0.78 µg/mL in methanol. Sample solutions (200 µL) were mixed well with 50 µL of 1 mM methanolic DPPH solution (freshly prepared) and the mixture shaken, followed by incubation at room temperature for 30 minutes in the dark. The absorbance of the mixture was measured at 517 nm against methanol (blank). L-Ascorbic acid at the same concentrations was used as the standard and all measurements were done in triplicate. The antioxidant activity of the extracts was evaluated based on the percentage of DPPH scavenged by the extract compared with that of standard ascorbic acid. The percentage of DPPH scavenged was calculated using the following equation:

$$\% \text{ Scavenging of DPPH} = \frac{[(Abs_{control} - Abs_{sample})]}{Abs_{control}} \times 100$$

4.3.5.5 Ferric Reducing Antioxidant Power (FRAP) Assay

The ferric reducing antioxidant power of the crude extracts of the leaves of *A. excelsa* was evaluated according to the method described by Wang, Wang et al. 2013. The FRAP reagent was freshly prepared by mixing 300 mM acetate buffer (3.1 g of sodium acetate trihydrate in 16 mL of glacial acetic acid), 10 mM TPTZ solution in 40 mM HCl (36%), and 20 mM $FeCl_3.6H_2O$ aqueous solution in the ratio of 10:1:1 *v/v*. Extracts were prepared at a concentration range of 40 - 0.313 µg/mL in MeOH. Sample solutions (400 µL) were mixed well with 3 mL of freshly prepared FRAP solution and the mixture was shaken, followed by incubation in a water bath at 37 ^{o}C for 30 minutes. The absorbance of the mixture was measured at 593 nm against methanol (blank) and all measurements were done in triplicate. Commercially available trolox was used as the standard at a concentration range of 40 - 0.313 µg/mL. The ferric reducing antioxidant activity of the extract was evaluated by observing the change in absorbance compared to that of the standard trolox.

4.3.6 Chromatographic Fractionation of *n*-Hexane Extract

4.3.6.1 Normal Phase Biotage Chromatography

The *n*-hexane extract (1.0 g) was applied to a 50 g silica SNAP cartridge that had been equilibrated with 100% *n*-hexane for 3 column volumes (CV). Isocratic conditions of 100% *n*-hexane were held for 3 CV, followed by a linear gradient to EtOAc over 29 CV, then isocratic conditions of 100% EtOAc

for 9 CV was held. 70 mL of each fractions were collected until the automated operation was finished. All collected fractions were dried by rotary evaporation and removal of residual traces of solvent under high vacuum. This afforded fractions AE-Hex F1 – F36 in order of increasing polarity based on TLC. Scheme 3.1 summarises the % *w/w* yield of these fractions and activities detected by TLC bioautography.

4.3.6.1 Size Exclusion Chromatography

Fractions AE-Hex F15, 16 and 17 obtained from the normal phase Biotage chromatography were purified by size exclusion chromatography (SEC) using a Sephadex LH-20 column. Fractions AE-Hex 15 and 16 were combined (49.6 mg), dissolved in 20% *n*-hexane in MeOH, and applied to a column with 25 g of Sephadex LH-20. Eluting with DCM:MeOH, 1:1, afforded 10 UV active fractions that were combined based on similar TLC R_f profiles. Evaporation of the solvent by rotary evaporation and removal of residual traces of solvent under high vacuum afforded fractions AE-Hex-F15/16-1 to F15/16-10. Using a similar protocol, AE-Hex F17 afforded 9 fractions AE-Hex-F17-1 to F17-9. Scheme 3.1 summarises the % *w/w* yield of these fractions.

Chapter 5: Conclusion and Future Directions

5.1 Summary

This MRes project focused on chemical and biological investigations of an underexplored Australian medicinal plant, *Alphitonia excelsa*, which is important to the Yaegl Aboriginal community of northern New South Wales (NSW), Australia and has been traditionally used for the treatment of wounds and skin infections and as an antiseptic hand wash.

Chapter 1 discussed the significant contribution of plants to the discovery of new drug leads and the success of ethnopharmacological research to modern healthcare and drug discovery. This chapter also described the importance of discovery of drug leads for the treatment of wounds and skin infections. A brief review of literature on *A. excelsa* and other plants of the *Alphitonia* genus was also given, highlighting the potential of this plant for discovery of antibacterial and antioxidant agents.

Chapter 2 started with a summary of the preliminary study of leaves of *A. excelsa* conducted by former researchers of the IBRG, followed by the structure elucidation conducted in this study of six compounds isolated from the previous study (three each from the *n*-hexane and DCM extracts). This led to the identification of the two flavonols quercetin and kaempferol, the phytosterol (β-sitosterol) and the three triterpenoids betulinic acid, betulin aldehyde and lupeol. This is the first report of betulin aldehyde and lupeol in the genus *Alphitonia* and of β-sitosterol in *A. excelsa*. Biological activities of all of these compounds are well documented in the literature, including of antibacterial and antioxidant activities. The evaluation of previously separated and unanalysed fractions, focusing on identification of fractions for further purification and/or structure elucidation, did not reveal any further compounds of interest.

Chapter 3 described chemical and biological studies on freshly extracted leaves of *A. excelsa*, including qualitative phytochemical studies, antibacterial and antioxidant screening of crude extracts and commencement of isolation of compounds. Sequential extraction of newly collected leaves of *A. excelsa* with solvents of different polarity yielded *n*-hexane, DCM, EtOAc, MeOH and water extracts. Phytochemical screening revealed the presence of steroids in the *n*-hexane extract, terpenoids in the DCM, MeOH, EtOAc and water extracts and tannins and saponins in the MeOH and water extracts. These classes of compounds are widely recognised for various

biological activities, including antibacterial and antioxidant activities. GC-MS analysis of the *n*-hexane and DCM extracts identified seven major phytoconstituents from the *n*-hexane extract and nine from the DCM extract, including nonanal, tetracontane, docosane, 1,54-dibromo-tetrapentacontane, γ-sitosterol, tetradecane and hexadecane. Some of these compounds have been reported for a wide range of biological activities.

Antibacterial screening was performed against the Gram positive bacterial strains methicillin sensitive, methicillin resistant and multidrug resistant *S. aureus* (MSSA, MRSA and MDRSA), as these are the commonly associated pathogens with wounds and skin infections. The disc diffusion and MTT microdilution assay methods were employed for this purpose. In the disc diffusion assay, the DCM extract showed the greatest antibacterial activity against MSSA and MRSA with the zone of inhibition of 10 mm and the EtOAc extract showed the highest activity against MDRSA also with a 10 mm zone of inhibition. The *n*-hexane and MeOH extracts showed low antibacterial activity (7 - 8 mm zone of inhibition) in this assay. For the MTT assay, the DCM extract showed the greatest bactericidal activity among the extracts against all three strains of *S. aureus* (MSSA, MRSA and MDRSA). It exhibited good activity against MRSA and MDRSA with MIC values of 312.5 µg/mL and moderate activity against MSSA with an MIC of 625 µg/mL. The MeOH and water extracts showed moderate bactericidal activity against the three bacterial strains (MIC 625-1250 µg/mL).

Antioxidant activity of the leaves of *A. excelsa* was evaluated by analysing the DPPH scavenging capability and ferric reducing antioxidant power ability of the extracts. In both analyses, the DCM extract showed very promising antioxidant activity (91% scavenging of DPPH as compared to L-ascorbic acid at 100 µg/mL and 97% ferric reducing ability as compared to Trolox at 40 µg/mL). Although the DCM extract was the most promising based on bioactivity, the *n*-hexane extract was selected for fractionation first to continue on from the earlier studies in being able to gain a more comprehensive understanding of the phytochemistry of this extract and correlate bioactivity of the compounds isolated to the original extract. Normal phase Biotage chromatography of the *n*-hexane extract yielded 36 major fractions based on TLC R_f profiles, including one bioactive fraction against MSSA, as identified by TLC bioautography. Comparing the ^{1}H NMR and MS spectral data of seven fractions that showed good separation by TLC, and comparing characteristic peaks with that seen in the previously isolated compounds, five fractions were identified for further fractionation. Further purification of these fractions using Sephadex LH-20 (size exclusion

chromatography) yielded 19 semi-pure fractions. Further purification and bioactivity evaluation of these sub-fractions as well as the remaining normal phase Biotage chromatography fractions were not conducted due to insufficient time.

Chapter 4 described the methods followed in this study for chemical and biological investigations along with details of general experimental procedures and materials.

In conclusion, the MRes study has achieved the primary goal of identification of previously isolated compounds from the leaves of *A. excelsa* and biological and chemical investigation of extracts and fractions. The preliminary antibacterial and antioxidant activity evaluation of crude extracts supports the traditional uses of the leaves of *A. excelsa* by the Yaegl community for the treatment of skin related ailments. Isolation of sufficient material from *A. excelsa* leaves for further biological and chemical investigations has commenced.

5.2 Future Directions

Immediate future work should focus on identifying compounds from the *n*-hexane extract, followed by the more active DCM extract, and determination of their antibacterial and antioxidant activities. Isolation of compounds to quantify their presence in the original extracts and ascertain their relative significance towards the biological activity of these extracts is important in understanding their overall contribution to the customary use of *A. excelsa* leaves as an antiseptic. Given the limited phytochemical studies that have been conducted on *A. excelsa* as a whole, investigation of all the extracts for their major constituents and novel compounds, independent of their biological activities, is also worthy to explore. Along with isolation studies, metabolomics studies on the extracts and fractions could significantly increase the understanding of the phytochemistry of this plant. More biologically relevant assays and a broader suite of assays could also extend the potential applicability of this plant. Opportunities accessible to the IBRG include functional skin assays and a lipid based antioxidant assay, both highly relevant to biological systems. A greater suite of microbes, as appropriate to skin infections and wounds, would be worthwhile. Examination of seasonal and geographic variation in the phytochemistry and biological activity would also be of value.

REFERENCES

Abate, G., et al. (1998). "Evaluation of a colorimetric assay based on 3-(4, 5-dimethylthiazol-2-yl)- 2, 5-diphenyl tetrazolium bromide (MTT) for rapid detection of rifampicin resistance in *Mycobacterium tuberculosis*." The International Journal of Tuberculosis and Lung Disease **2**(12): 1011-1016.

Abdallah, E.M. (2011). "Plants: An Alternative Source of Antimicrobials". Journal of Applied Pharmaceutical Science **01**(6); 2011: 16-20.

Adedapo, A. A., et al. (2008). "Antibacterial and antioxidant properties of the methanol extracts of the leaves and stems of *Calpurnia aurea*." BMC Complementary and Alternative Medicine **8**(1): 53.

Adefegha, S. A., et al. (2014). "Antihyperglycemic, hypolipidemic, hepatoprotective and antioxidative effects of dietary clove (*Szyzgium aromaticum*) bud powder in a high-fat diet/streptozotocin-induced diabetes rat model." Journal of the Science of Food and Agriculture **94**(13): 2726-2737.

Ahmed, Y., et al. (2010). "Antimicrobial and cytotoxic constituents from leaves of *Sapium baccatum*." Food and Chemical Toxicology **48**(2): 549-552.

Akpuaka, A., et al. (2013). "Biological activities of characterized isolates of *n*-hexane extract of *Azadirachta indica A. Juss* (Neem) leaves." Nature and Science **11**(5): 141-147.

Akter, K., et al. (2016). "Phytochemical profile and antibacterial and antioxidant activities of medicinal plants used by Aboriginal people of New South Wales, Australia." Evidence-Based Complementary and Alternative Medicine **2016**.

Alakurtti, S., et al. (2010). "Anti-leishmanial activity of betulin derivatives." The Journal of Antibiotics **63**(3): 123-126.

Appendino, G., et al. (2008). "Antibacterial cannabinoids from *Cannabis sativa*: a structure−activity study." Journal of Natural Products **71**(8): 1427-1430.

Arima, H., and G. I. Danno (2002). "Isolation of antimicrobial compounds from guava (*Psidium guajava* L.) and their structural elucidation." Bioscience, Biotechnology, and Biochemistry **66**(8): 1727-1730.

Barthel, A., et al. (2008). "Oxidative transformations of betulinol." Tetrahedron **64**(39): 9225-9229.

Bach, A., et al. (2010). "The antioxidant quercetin inhibits cellular proliferation *via* HIF-1-dependent induction of p21WAF." Antioxidants & Redox Signaling **13**(4): 437-448.

Bauer, A. (1966). "Antibiotic susceptibility testing by a standardized single disk method." American Journal of Clinical Pathology **45**: 493-496.

Braca, A., et al. (1999). "Three flavonoids from *Licania heteromorpha*." Phytochemistry **51**(8): 1121-1124.

Branch, G., et al. (1972). "Constituents of *Alphitonia* species. III. Alphitexolide, a new triterpene, and other extractives." Australian Journal of Chemistry **25**(10): 2209-2216.

Brouwer, N., et al. (2005). "An ethnopharmacological study of medicinal plants in New South Wales." Molecules **10**(10): 1252-1262.

Brusselaers, N., et al. (2011). "The rising problem of antimicrobial resistance in the intensive care unit." Annals of intensive care **1**(1): 47.

Burdon, R. H. and V. Gill (1993). "Cellularly generated active oxygen species and HeLa cell proliferation." Free Radical Research Communications **19**(3): 203-213.

Burgess, J. G., et al. (1999). "Microbial antagonism: a neglected avenue of natural products research." Journal of Biotechnology **70**(1): 27-32.

Burrows, D. and J. Balciunas (1997). "Biology, distribution and host-range of the sawfly, *Lophyrotoma zonalis* (Hym. Pergidae), a potential biological control agent for the paperbark tree, *Melaleuca quinquenervia*." Entomophaga **42**(3): 299-313.

Butler, M. S. (2004). "The role of natural product chemistry in drug discovery." Journal of Natural Products **67**(12): 2141-2153.

Butler, M. S. (2008). "Natural products to drugs: natural product-derived compounds in clinical trials." Natural Product Reports **25**(3): 475-516.

Butler, M. S. and D. J. Newman (2008). Mother Nature's gifts to diseases of man: the impact of natural products on anti-infective, anticholestemics and anticancer drug discovery. Natural Compounds as Drugs Volume I, Springer**:** 1-44.

Ceri, H., et al. (1999). "The Calgary Biofilm Device: new technology for rapid determination of antibiotic susceptibilities of bacterial biofilms." Journal of Clinical Microbiology **37**(6): 1771-1776.

Chandramu, C., et al. (2003). "Isolation, characterization and biological activity of betulinic acid and ursolic acid from *Vitex negundo* L." Phytotherapy Research **17**(2): 129-134.

Chin, J. Y., et al. (2014). "Levels and sources of volatile organic compounds in homes of children with asthma." Indoor Air **24**(4): 403-415.

Cichewicz, R. H. and S. A. Kouzi (2004). "Chemistry, biological activity, and chemotherapeutic potential of betulinic acid for the prevention and treatment of cancer and HIV infection." Medicinal Research Reviews **24**(1): 90-114.

Clardy, J. and C. Walsh (2004). "Lessons from natural molecules." Nature **432**(7019): 829-837.

Clark, A. M. (1996). "Natural products as a resource for new drugs." Pharmaceutical Research **13**(8): 1133-1141.

Cragg, G. M. and D. J. Newman (2001). "Natural product drug discovery in the next millennium." Pharmaceutical Biology **39**(sup1): 8-17.

Cuong, N.-V., et al. (2009). "Recent development in nano-sized dosage forms of plant alkaloid camptothecin-derived drugs." Recent Patents on Anti-Cancer Drug Discovery **4**(3): 254-261.

Delfanian, M., et al. (2015). "Influence of extraction techniques on antioxidant properties and bioactive compounds of loquat fruit (*Eriobotrya japonica Lindl.*) skin and pulp extracts." Food Science & Nutrition **3**(3): 179-187.

Dias, D. A., et al. (2012). "A historical overview of natural products in drug discovery." Metabolites **2**(2): 303-336.

Dorman, H. and S. Deans (2000). "Antimicrobial agents from plants: antibacterial activity of plant volatile oils." Journal of Applied Microbiology **88**(2): 308-316.

Edwards, R. and K. G. Harding (2004). "Bacteria and wound healing." Current Opinion in Infectious Diseases **17**(2): 91-96.

Eming, S. A., et al. (2007). "Inflammation in wound repair: molecular and cellular mechanisms." Journal of Investigative Dermatology **127**(3): 514-525.

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.

Fang, X.-K., et al. (2008). "Kaempferol and quercetin isolated from *Euonymus alatus* improve glucose uptake of 3T3-L1 cells without adipogenesis activity." Life sciences **82**(11): 615-622.

Farag, M. A., et al. (2017). "Metabolites profiling reveals for antimicrobial compositional differences and action mechanism in the toothbrushing stick “miswak” *Salvadora persica*." Journal of Pharmaceutical and Biomedical Analysis **133**: 32-40.

Fathiazad, F., et al. (2010). "Extraction of flavonoids and quantification of rutin from waste tobacco leaves." Iranian Journal of Pharmaceutical Research: 222-227.

Fitzmaurice, S., et al. (2011). "Antioxidant therapies for wound healing: a clinical guide to currently commercially available products." Skin Pharmacology and Physiology **24**(3): 113-126.

Fotie, J., et al. (2006). "Lupeol long-chain fatty acid esters with antimalarial activity from *Holarrhena floribunda*." Journal of Natural Products **69**(1): 62-67.

Frankel, E. N. and A. S. Meyer (2000). "The problems of using one-dimensional methods to evaluate multifunctional food and biological antioxidants." Journal of the Science of Food and Agriculture **80**(13): 1925-1941.

Gadgil, M., et al. (1993). "Indigenous knowledge for biodiversity conservation." Ambio: 151-156.

Giacometti, A., et al. (2003). "Prevalence of intestinal parasites among individuals with allergic skin diseases." Journal of Parasitology **89**(3): 490-492.

Goel, R., et al. (1987). "Effect of lapachol, a naphthaquinone isolated from *Tectona grandis*, on experimental peptic ulcer and gastric secretion." Journal of Pharmacy and Pharmacology **39**(2): 138-140.

Guise, G., et al. (1962). "Further constituents of *Alphitonia* species." Australian Journal of Chemistry **15**(2): 314-321.

Gupta, M., et al. (1980). "Anti-inflammatory and antipyretic activities of β-sitosterol." Planta Medica **39**(06): 157-163.

Gutzeit, D. et al. (2007). "Preparative isolation and purification of flavonoids and protocatechuic acid from sea buckthorn juice concentrate (*Hippophae rhamnoides* L. ssp. rhamnoides) by high-speed counter-current chromatography." Chromatographia **65**(1-2): 1-7.

Haavikko, R., et al. (2014). "Heterocycle-fused lupane triterpenoids inhibit *Leishmania donovani* amastigotes." MedChemComm **5**(4): 445-451.

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, P., et al. (2010). "Use of an innovative T-tube maze assay and the proboscis extension response assay to assess sublethal effects of GM products and pesticides on learning capacity of the honey bee *Apis mellifera* L." Ecotoxicology **19**(8): 1612-1619.

Harden, G. J. (1990). Flora of New South Wales, UNSW Press.

Harvey, A. L. (2008). "Natural products in drug discovery." Drug Discovery Today **13**(19): 894-901.

Hay, R. J., et al. (2014). "The global burden of skin disease in 2010: an analysis of the prevalence and impact of skin conditions." Journal of Investigative Dermatology **134**(6): 1527-1534.

Heinrich, M. (2010). "Ethnopharmacology in the 21st century-grand challenges." Frontiers in Pharmacology **1**: 8.

Heinrich, M. (2013). "Ethnopharmacology and drug discovery." Comprehensive Natural Products II: Chemistry and Biology, Development & Modification of Bioactivity **3**: 351-381.

Iwaki, K., et al. (2006). "Antimicrobial activity of *Polygonum tinctorium* Lour: extract against oral pathogenic bacteria." Journal of Natural Medicines **60**(2): 121-125.

Jou, S. J., et al. (2004). "Flavonol glycosides and cytotoxic triterpenoids from *Alphitonia philippinensis*." Journal of the Chinese Chemical Society **51**(4): 827-834.

Karaman, I., et al. (2003). "Antimicrobial activity of aqueous and methanol extracts of *Juniperus oxycedrus* L. Journal of Ethnopharmacology **85**(2): 231-235.

Katiyar, C., et al. (2012). "Drug discovery from plant sources: An integrated approach." AYU (An international quarterly journal of research in Ayurveda) **33**(1): 10.

Khandelwal, V., et al. (2016). "Antimicrobial and antioxidant efficacy of aqueous extract of *Anthocephalus cadamba* leaves." Journal of Pure and Applied Microbiology **10**(1): 209-216.

Kijas, J. (2009). "There were always people here: a history of Yuraygir National Park."

Kingsley, A. (2003). "The wound infection continuum and its application to clinical practice." Ostomy/wound management **49**(7A Suppl): 1-7.

Kirby, T. (2015). "New anribiotic development hailed as game changing." Lancet Infectious Disease **15**(3): 271-272.

Kirimizigul, S., et al. (2003). "Isolation and structure elucidation of novel natural products from Turkish lichens." Turkish Journal of Chemistry **27**(4): 493-500.

Klaric M. S., et al. (2007). "Antifungal activity of thyme (*Thymus vulgaris* L.) essential oil and thymol against moulds from damp dwellings." Letters in Applied Microbiology 44: 36-42.

Kojima, H., et al. (1990). "Sterol glucosides from *Prunella vulgaris*." Phytochemistry **29**(7): 2351-2355.

Laghari, A. H., et al. (2011). "*Alhagi maurorum*: a convenient source of lupeol." Industrial Crops and Products **34**(1): 1141-1145.

Lamagni, T. L., et al. (2008). "Epidemiology of severe *Streptococcus pyogenes* disease in Europe." Journal of Clinical Microbiology **46**(7): 2359-2367.

Lassak, E. V. and T. McCarthy (2000) "Australian Medicinal Plants". New Holland Publishers, Sydney

Lewis, W., et al. (2004). "Advantage of ethnobotanically-based research for searching new pharmaceuticals." Ethnobotany **16**: 10-15.

Li, D., et al. (1994). "Structure elucidation of three triterpenoid saponins from *Alphitonia zizyphoides* using 2D NMR techniques." Journal of Natural Products **57**(2): 218-224.

Lichtenthaler, H. K. and C. Buschmann (2001). "Chlorophylls and carotenoids: Measurement and characterization by UV-VIS spectroscopy." Current Protocols in Food Analytical Chemistry.

Lin, J., et al. (1999). "Preliminary screening of some traditional Zulu medicinal plants for anti-inflammatory and anti-microbial activities." Journal of Ethnopharmacology **68**(1): 267-274.

Lip, G. Y. and F. L. Li-Saw-Hee (1998). "Does hypertension confer a hypercoagulable state?" Journal of Hypertension **16**(7): 913-916.

Liu, S. C. et al. (2009). "Antioxidant properties of various solvent extracts from lychee (*Litchi chinenesis* Sonn.) flowers." Food Chemistry **114**(2): 577-581.

Liu, Y., et al. (1997). "Mechanism of cellular 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) reduction." Journal of Neurochemistry **69**(2): 581-593.

Lo, K. M. and P. C. K. Cheung (2005). "Antioxidant activity of extracts from the fruiting bodies of *Agrocybe aegerita var*. alba." Food Chemistry **89**(4): 533-539.

Low, T. (1990). Bush medicine: a pharmacopoeia of natural remedies, Angus & Robertson.

Lundberg, W., et al. (1944). "The antioxidant properties of nordihydroguaiaretic acid." Journal of the American Oil Chemists' Society **21**(2): 33-35.

Macías, F. A., et al. (1994). "Potential allelopathic lupane triterpenes from bioactive fractions of *Melilotus messanensis*." Phytochemistry **36**(6): 1369-1379.

Mall, T. P. and S. C. Tripathi (2017). "Biosciences and Plant Biology." International Journal of Current Research in Biosciences and Plant Biology **4**(1): 65-76.

McSwan, E. (1992). Maclean-The first fifty years, Clarence Press, Maclean, NSW.

Meena, M. C. and V. Patni (2008). "Isolation and identification of flavonoid "quercetin" from *Citrullus colocynthis* (Linn.) Schrad." Asian Journal of Experimental Science **22**(1): 137-142.

Mensor, L. L., et al. (2001). "Screening of Brazilian plant extracts for antioxidant activity by the use of DPPH free radical method." Phytotherapy Research **15**(2): 127-130.

Michael, J. P. (2008). "Quinoline, quinazoline and acridone alkaloids." Natural Product Reports **25**(1): 166-187.

Mitscher, L. A., et al. (1987). "A modern look at folkloric use of anti-infective agents." Journal of Natural Products **50**(6): 1025-1040.

Moghaddam, M. G., et al. (2012). "Biological activity of betulinic acid: a review." Pharmacology & Pharmacy 3(2): 119-123.

Moreno, S., et al. (2006). "Antioxidant and antimicrobial activities of rosemary extracts linked to their polyphenol composition." Free Radical Research **40**(2): 223-231.

Mosmann, T. (1983). "Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays." Journal of Immunological Methods **65**(1-2): 55-63.

Muhammad, D., et al. (2016). "Triterpenoids from the leaves of *Alphitonia xerocarpus* Baill and their biological activity." Phytochemistry **129**: 45-57.

Murugan, R. et al. (2016). "Aphrodisiac activity of ethanolic activity of *Vitex negundo* fruit of male wistar albino rats." Acta Biomedica Scientia 3(4): 128-130.

Newman, D. J. and G. M. Cragg (2007). "Natural products as sources of new drugs over the last 25 years" Journal of Natural Products **70**(3): 461-477.

Newman, D. J. and G. M. Cragg (2016). "Natural products as sources of new drugs from 1981 to 2014." Journal of Natural Products **79**(3): 629-661.

O'Dell, M. L. (1998). "Skin and wound infections: an overview." American Family Physician **57**(10): 2424-2432.

Ogunlesi, M., et al. (2009). "Determination of the concentrations of zinc and vitamin C in oysters and some medicinal plants used to correct male factor infertility." Journal of Natural Products **2**: 89-97.

Packer, J., et al. (2012). "An ethnobotanical study of medicinal plants used by the Yaegl Aboriginal community in northern New South Wales, Australia." Journal of Ethnopharmacology **139**(1): 244- 255.

Packer, J., et al. (2011). Medicinal plants of New South Wales, Australia. Genetic Resources, Chromosome Engineering, and Crop Improvement: Medicinal Plants, Volume 6, CRC Press**:** 259-296.

Packer, J. M., et al. (2011). "Yaegl: Medicinal and Plant Resources Handbook." Macquarie University.

Palanimuthu, D., et al. (2012). "Lupeol, a bioactive triterpene, prevents tumor formation during 7, 12-dimethylbenz (a) anthracene induced oral carcinogenesis." Pathology & Oncology Research **18**(4): 1029-1037.

Palombo, E. A. and S. J. Semple (2001). "Antibacterial activity of traditional Australian medicinal plants." Journal of Ethnopharmacology **77**(2): 151-157.

Pandey, A. and S. Tripathi (2014). "A Review on pharmacognosy, pre-phytochemistry and pharmacological analysis of *Tridax procumbens*. PharmaTutor, **2**(4): 78-86.

Patwardhan, B. and R. A. Mashelkar (2009). "Traditional medicine-inspired approaches to drug discovery: can Ayurveda show the way forward?" Drug Discovery Today **14**(15): 804-811.

Pereira, V. V., et al. (2016). "Chemical constituents of *Jacaranda oxyphylla* and their acetylcholinesterase inhibitory and antimicrobial activities." Records of Natural Products **10**(3): 392.

Petrovska, B. B. (2012). "Historical review of medicinal plants' usage." Pharmacognosy Reviews **6**(11): 1.

Pietta, P.-G. (2000). "Flavonoids as antioxidants." Journal of Natural Products **63**(7): 1035-1042.

Pohjala, L., et al. (2009). "Betulin-derived compounds as inhibitors of alphavirus replication." Journal of Natural Products **72**(11): 1917-1926.

Pretsch, E., P. Bühlmann and M. Badertscher. "*1*H NMR Spectroscopy." Structure Determination of Organic Compounds. Springer Berlin Heidelberg, 2009. 1-86.

Prior, R. L., et al. (2005). "Standardized methods for the determination of antioxidant capacity and phenolics in foods and dietary supplements." Journal of Agricultural and Food Chemistry **53**(10): 4290-4302.

Purohit, S., et al. (2013). "Evaluation of wound healing activity of ethanolic extract of *Curcuma longa* rhizomes in male albino rats." Asian Journal of Pharmaceutical Research **3**(2): 79-81.

Pyrzynska, K. and A. Pękal (2013). "Application of free radical diphenylpicrylhydrazyl (DPPH) to estimate the antioxidant capacity of food samples." Analytical Methods **5**(17): 4288-4295.

Rahuman, A. A., et al. (2008). "Isolation and identification of mosquito larvicidal compound from *Abutilon indicum* (Linn.) Sweet." Parasitology Research **102**(5): 981-988.

Raju, R., et al. (2016). "Anti-inflammatory chemical profiling of the Australian rainforest tree *Alphitonia petriei* (Rhamnaceae)." Molecules **21**(11): 1521.

Ramalingum, N. and M. F. Mahomoodally (2014). "The therapeutic potential of medicinal foods." Advances in Pharmacological Sciences **2014**.

Rates, S. M. K. (2001). "Plants as source of drugs." Toxicon **39**(5): 603-613.

Regev, A., et al. (2002). "Sampling error and intraobserver variation in liver biopsy in patients with chronic HCV infection." The American Journal of Gastroenterology **97**(10): 2614-2618.

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

Rogers, K. L., et al. (2000). "Inhibition of platelet aggregation and 5-HT release by extracts of Australian plants used traditionally as headache treatments." European Journal of Pharmaceutical Sciences **9**(4): 355-363.

Ryu, S. Y., et al. (1992). "Antiviral Triterpenes from *Prunella vulgaris*." Archives of Pharmacal Research 15(3): 242-245.

Saeidnia, S., et al. (2014). "The story of beta-sitosterol - a review." European Journal of Medicinal Plants 4(5): 590-609.

Saleem, M. (2009). "Lupeol, a novel anti-inflammatory and anti-cancer dietary triterpene." Cancer Letters **285**(2): 109-115.

Sapico, F., et al. (1980). "Quantitative aerobic and anaerobic bacteriology of infected diabetic feet." Journal of Clinical Microbiology **12**(3): 413-420.

Sarker, S., et al. Gray AI, 2006. Natural Product Isolation, Humana Press Inc. Totowa, New Jersey.

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.

Sen, C. K., et al. (2009). "Human skin wounds: a major and snowballing threat to public health and the economy." Wound Repair and Regeneration **17**(6): 763-771.

Sharma, O. P. and T. K. Bhat (2009). "DPPH antioxidant assay revisited." Food Chemistry **113**(4): 1202-1205.

Siddiqui, S., et al. (1988). "Oleanderol, a new pentacyclic triterpene from the leaves of *Nerium oleander*." Journal of Natural Products **51**(2): 229-233.

Siddique, H. R., et al. (2011). "Lupeol, a novel androgen receptor inhibitor: implications in prostate cancer therapy." Clinical Cancer Research **17**(16): 5379-5391.

Silverstein, R. M., et al. (2014). Spectrometric identification of organic compounds, John wiley & sons.

Singh, R., et al. (2008). "Anti-free radical activities of kaempferol isolated from *Acacia nilotica* (L.) Willd. Ex. Del." Toxicology *in vitro* **22**(8): 1965-1970.

Singleton, V. L., et al. (1999). "Analysis of total phenols and other oxidation substrates and antioxidants by means of folin-ciocalteu reagent." Methods in Enzymology **299**: 152-178.

Smyth, T., et al. (2009). "A study of the antimicrobial activity of selected naturally occurring and synthetic coumarins." International Journal ofAantimicrobial Agents **33**(5): 421-426.

Soneja, A., et al. (2005). "Role of nitric oxide, nitroxidative and oxidative stress in wound healing." Pharmacological Reports **57**: 108.

Steele, J. C. P., et al. (1999). "In vitro and in vivo evaluation of betulinic acid as an antimalarial." Phytotherapy Research **13**: 115-119.

Stewart, P. S. and J. W. Costerton (2001). "Antibiotic resistance of bacteria in biofilms." The lancet **358**(9276): 135-138.

Sweeney, A., et al. (2001). "Xanthine oxidase inhibitory activity of selected Australian native plants." Journal of Ethnopharmacology **75**(2): 273-277.

Thompson, R. L., et al. (1982). "Epidemiology of nosocomial infections caused by methicillin-resistant *Staphylococcus aureus*." Annals of Internal Medicine **97**(3): 309-317.

Tringali, C., et al. (2000). "Bioactive constituents of the bark of *Parkia biglobosa*." Fitoterapia **71**(2): 118-125.

Turner, K. H., et al. (2014). "Requirements for *Pseudomonas aeruginosa* acute burn and chronic surgical wound infection." PLoS Genet **10**(7): e1004518.

Uddin, G. H. W., et al. (2011). "Chemical constituents and phytotoxicity of solvent extracted fractions of stem bark of *Grewia optiva* Drummond ex burret." Middle-East Journal of Scientific Research **8**: 85-91.

Valgas, C., et al. (2007). "Screening methods to determine antibacterial activity of natural products." Brazilian Journal of Microbiology 38(2): 369-380.

Veitch, N. C., et al. (2005). "Flavonol tetraglycosides from *Ateleia chicoasensis* (Leguminosae)." Biochemical Systematics and Ecology **33**(12): 1274-1279.

Walker, D. and T. Coutts (1989). "Me and You." The Life Story of Della Walker. Canberra: Aboriginal Studies.

Walton, N. J. and D. E. Brown (1999). Chemicals from Plants: Perspectives on Plant Secondary Products, World Scientific.

Wang, S.S., et al. (2013). "Phytochemical profiles, antioxidant and antimicrobial activities of three *Potentilla* species." BMC Complementary and Alternative Medicine **13**(1): 321.

Webb, L. J. (1969). "The use of plant medicines and poisons by Australian Aborigines." Mankind **7**(2): 137-146.

Williams, B. L. and S. H. Wender (1953). "Isolation and identification of quercetin and isoquercitrin from apricots (*Prunus armeniaca*)." Archives of Biochemistry and Biophysics **43**(2): 319-323.

Wood, C. A., et al. (2001). "A bioactive spirolactone iridoid and triterpenoids from *Himatanthus sucuuba*." Chemical and Pharmaceutical Bulletin **49**(11): 1477-1478.

Xiao Z.P. et al. (2006). "Kaempferol and quercetin flavonoids from *Rosa rugose*." Chemistry of Natural Compounds 42(6): 736-737.

Xu, W. et al. (2008). "Kaempferol-7-*O*-β-d-glucoside (KG) isolated from *Smilax china* L. rhizome induces G 2/M phase arrest and apoptosis on HeLa cells in a p53-independent manner. Cancer Letters **264**(2): 229-240.

Yadav, R. K., et al. (2015). "Phytochemistry, pharmacology, toxicology, and clinical trial of *Ficus racemosa*." Pharmacognosy Reviews **9**(17): 73.

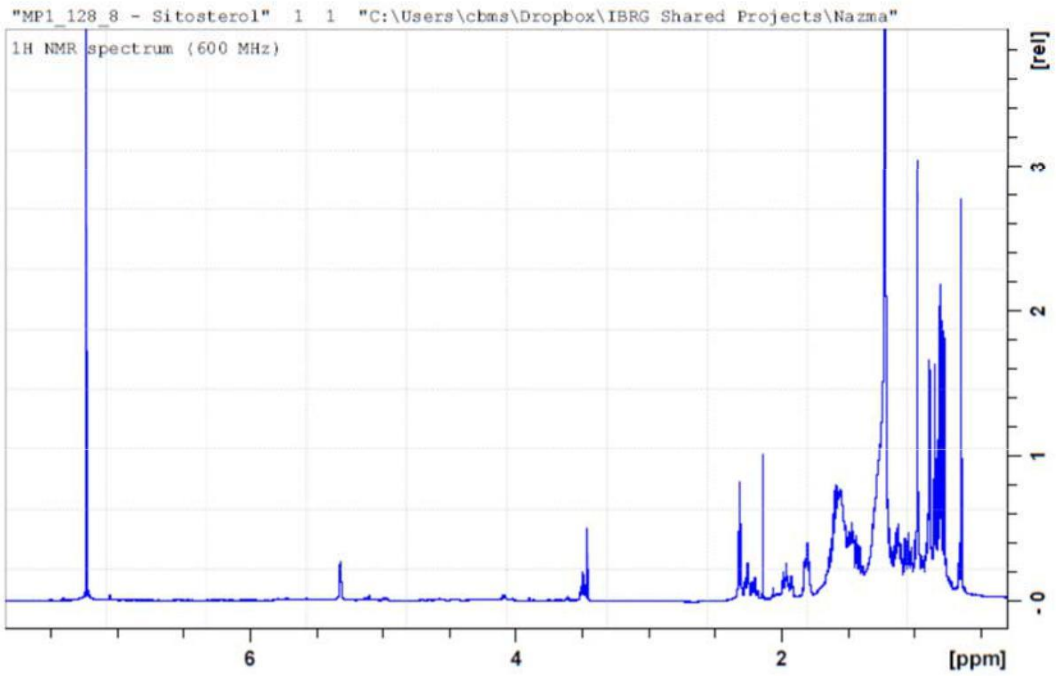
You, Y. J., et al. (2003). "Antiangiogenic activity of lupeol from Bombax ceiba." Phytotherapy Research **17**(4): 341-344.

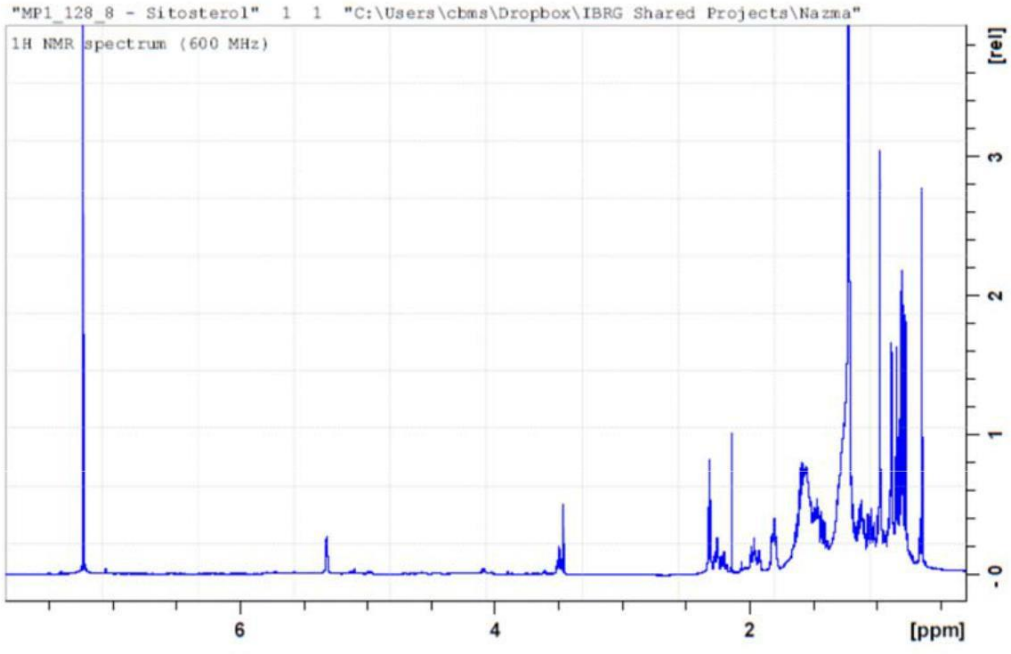
Zhang, T.-T. and J.-G. Jiang (2015). "Analyses on essential oil components from the unripe fruits of *Rubus chingii* Hu by different methods and their comparative cytotoxic and anti-complement activities." Food Analytical Methods **8**(4): 937-944.

Zhang, Z., et al. (2002). "Natural products inhibiting *Candida albicans* secreted aspartic proteases from *Tovomita krukovii*." Planta Medica **68**(01): 49-54.

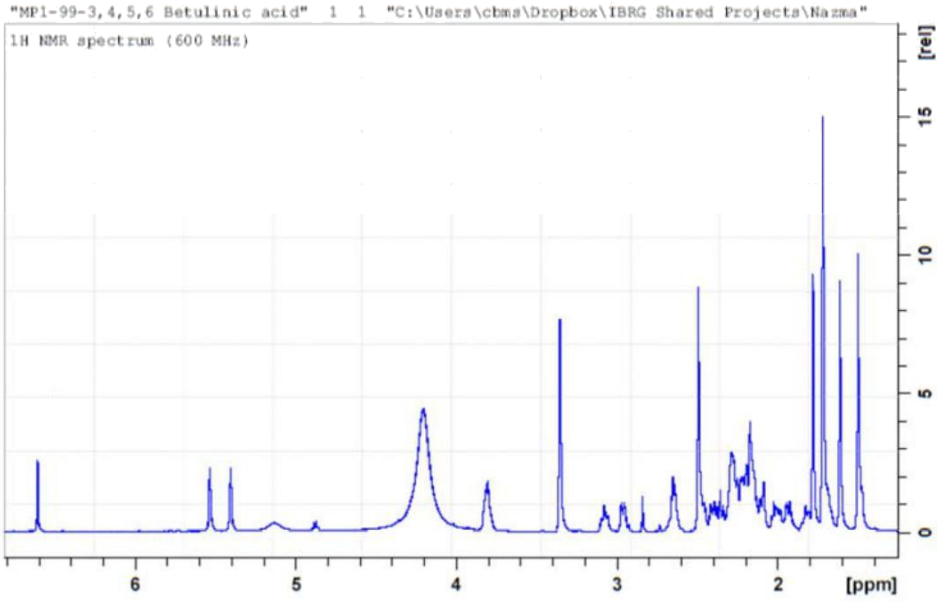
APPENDIX

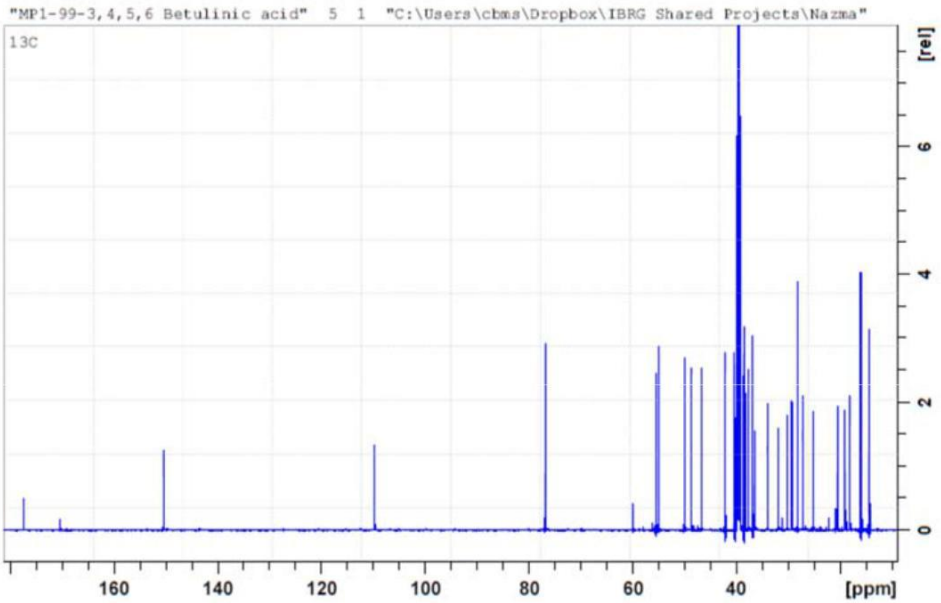
^{1}H and ^{13}C NMR Spectra of β-Sitosterol



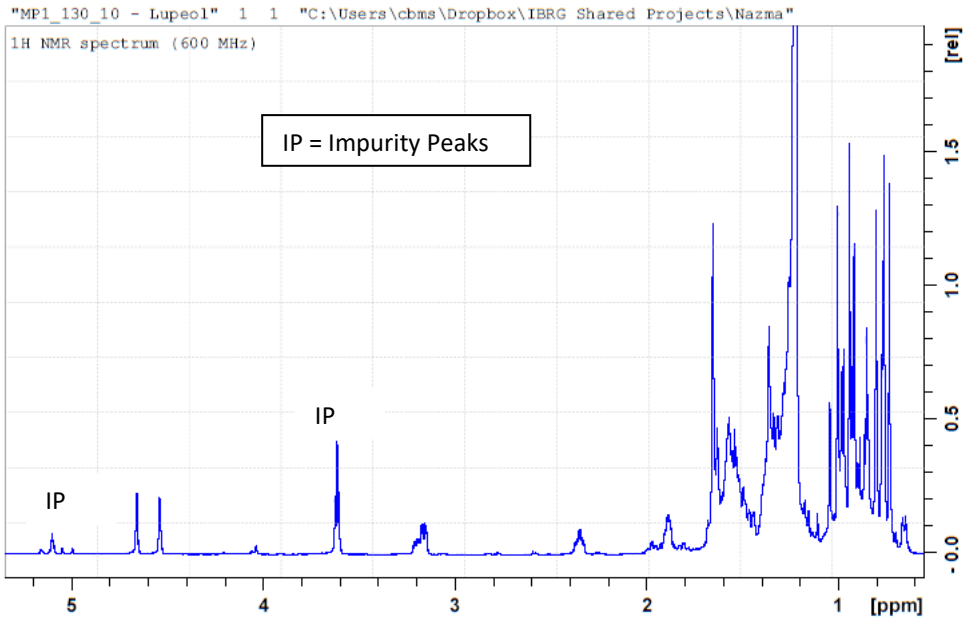


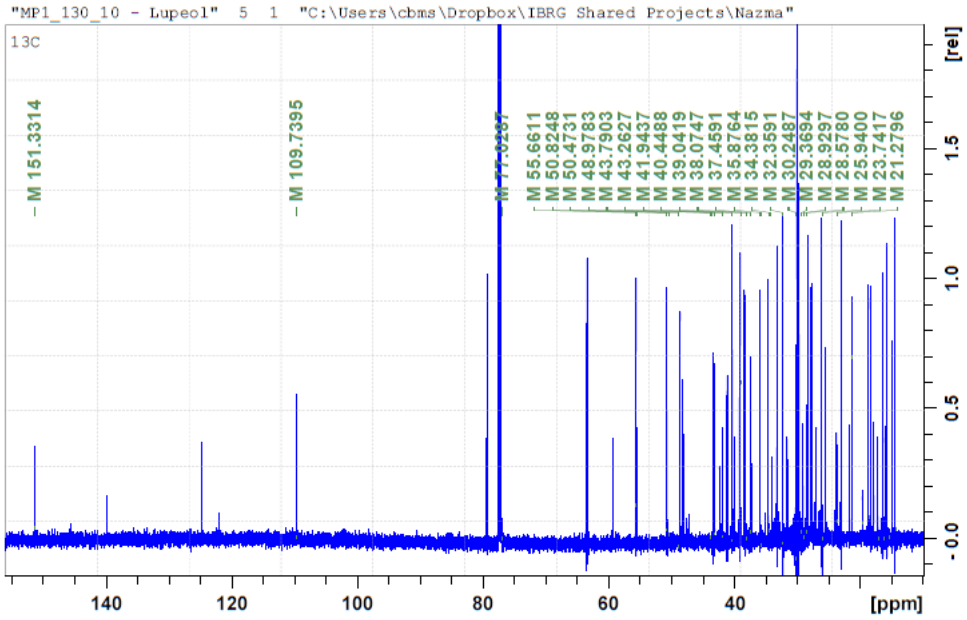
^{1}H and ^{13}C NMR Spectra of Betulinic Acid



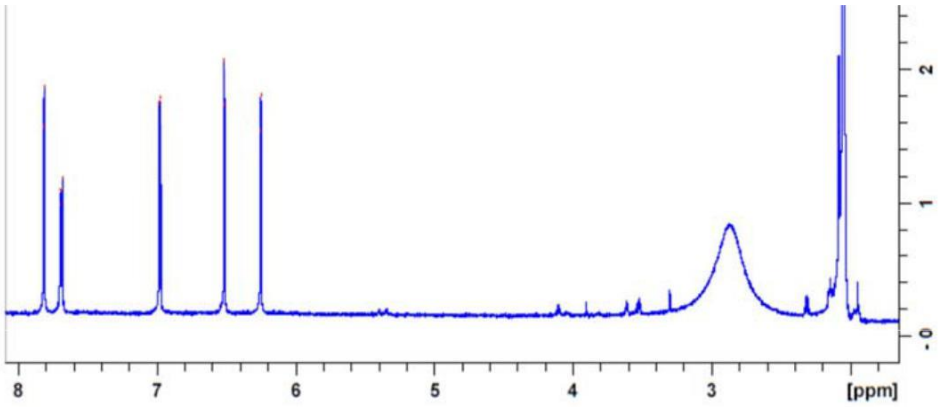


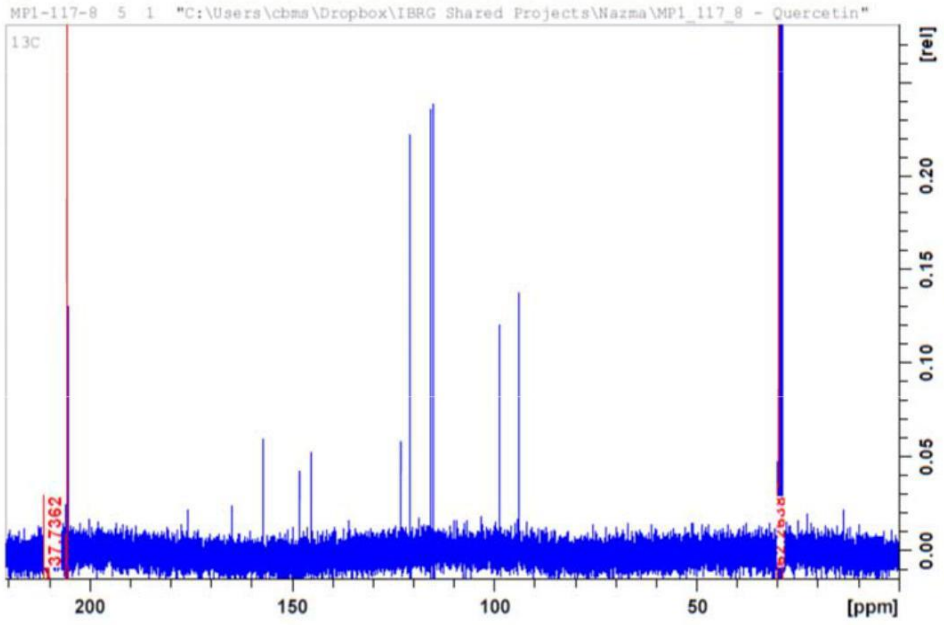
1H and 13C NMR Spectra of Lupeol



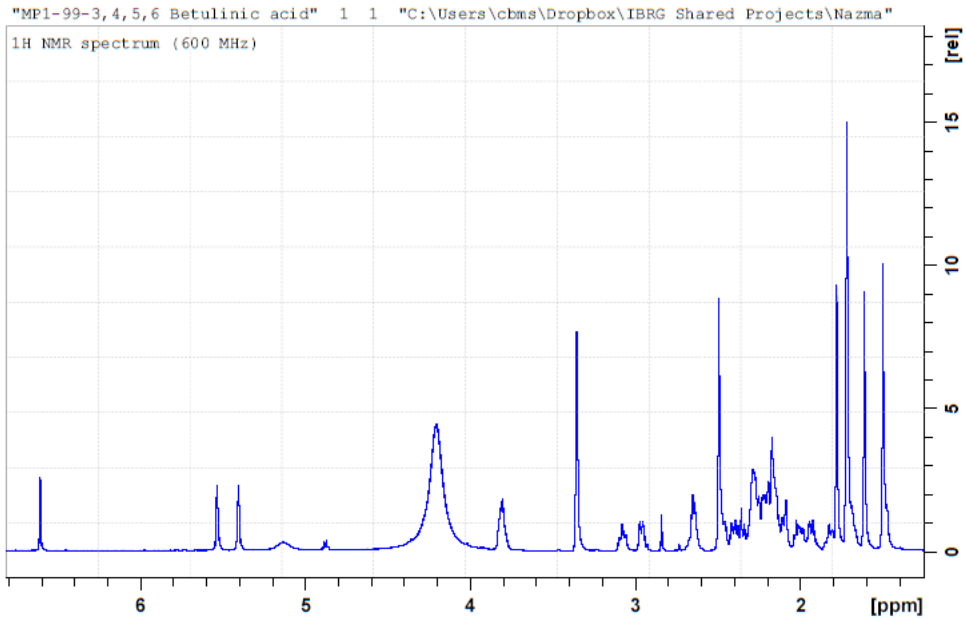


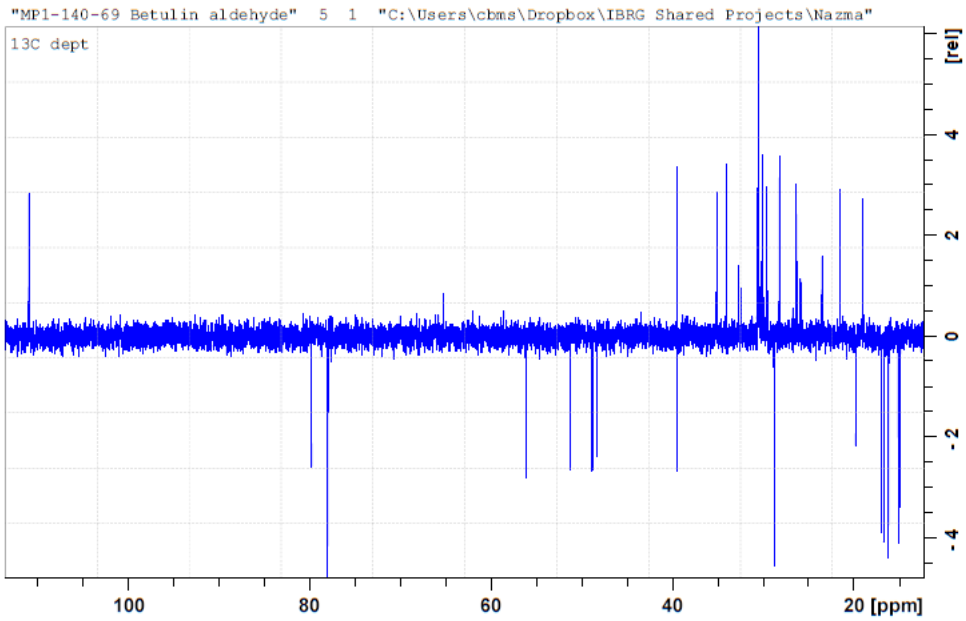
^{1}H and ^{13}C NMR Spectra of Quercetin



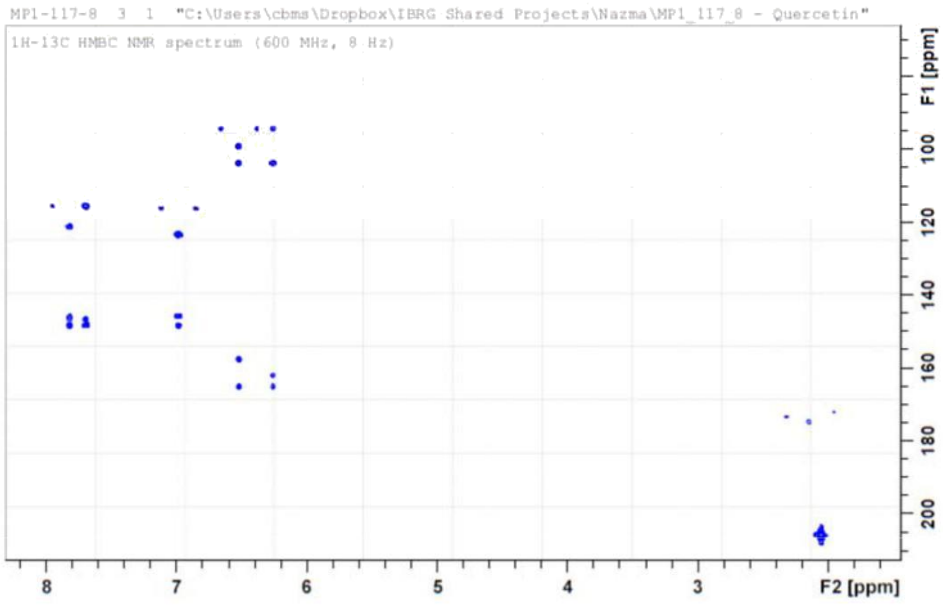


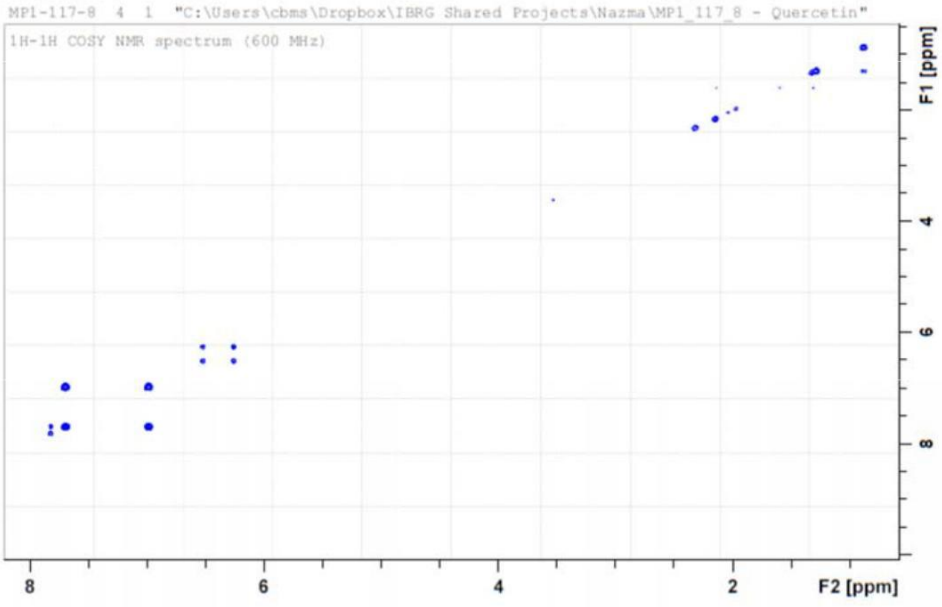
^{1}H and ^{13}C NMR Spectra of Betulin Aldehyde



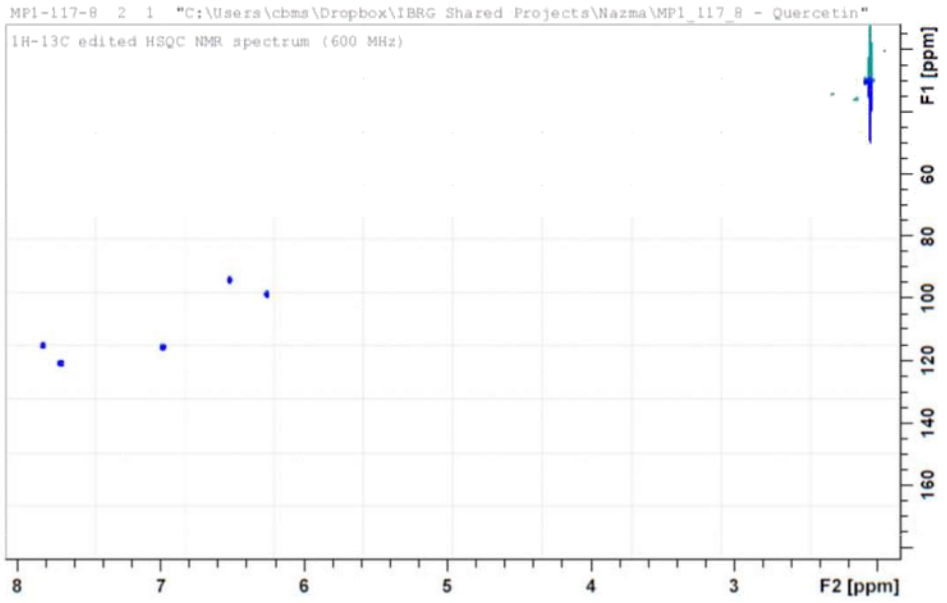


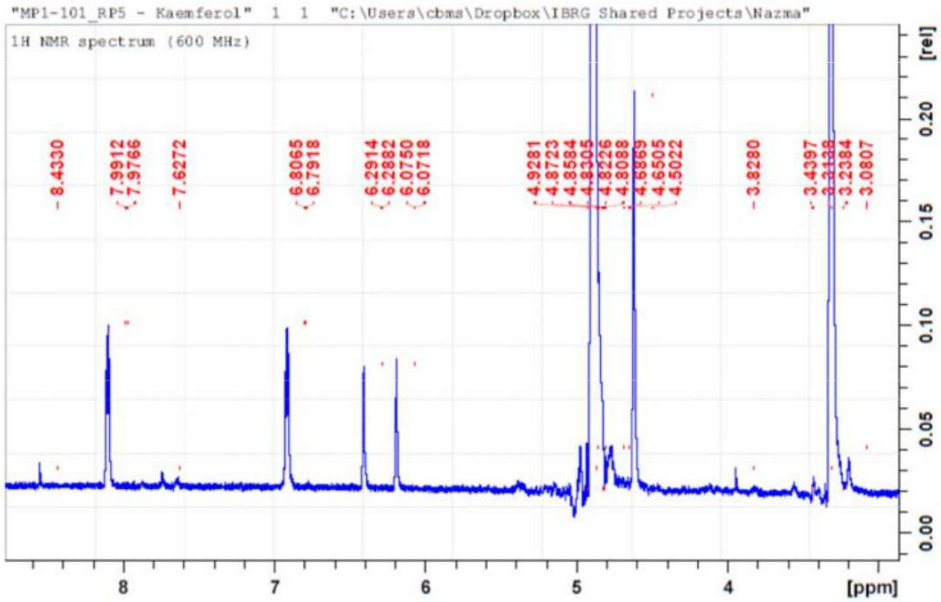
COSY and HMBC Spectra of Quercetin



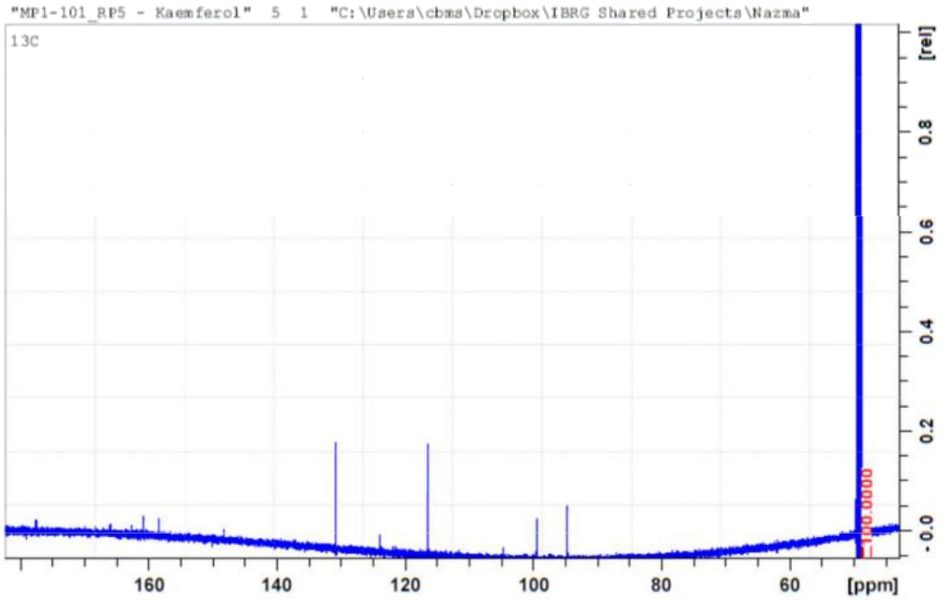


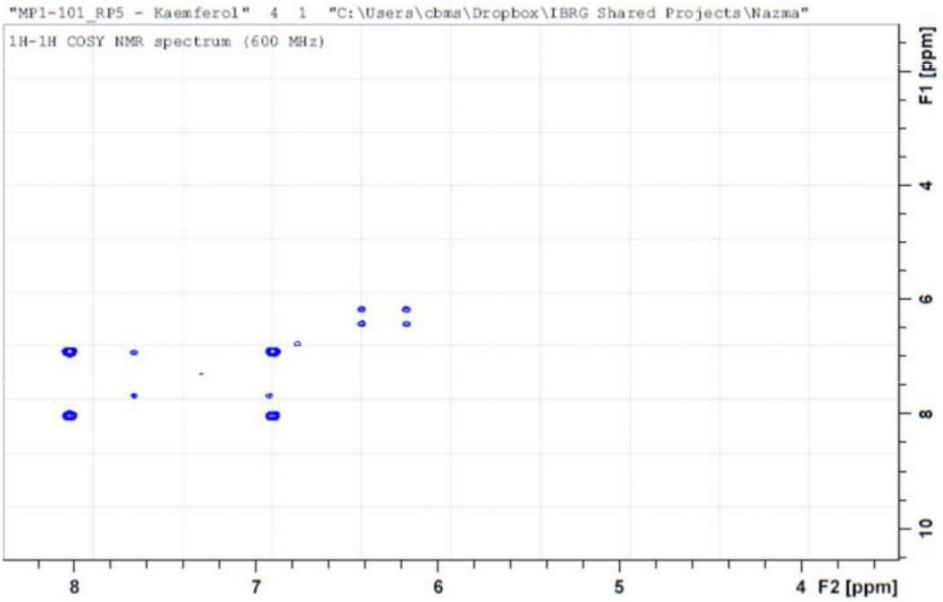
HMBC Spectra of Quercetin and ^{1}H Spectra of Kaempferol



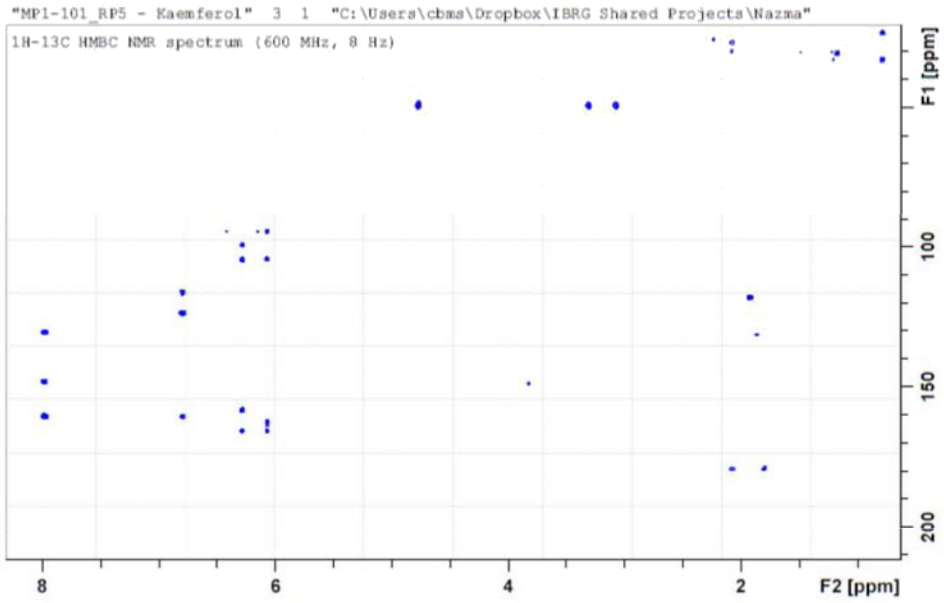


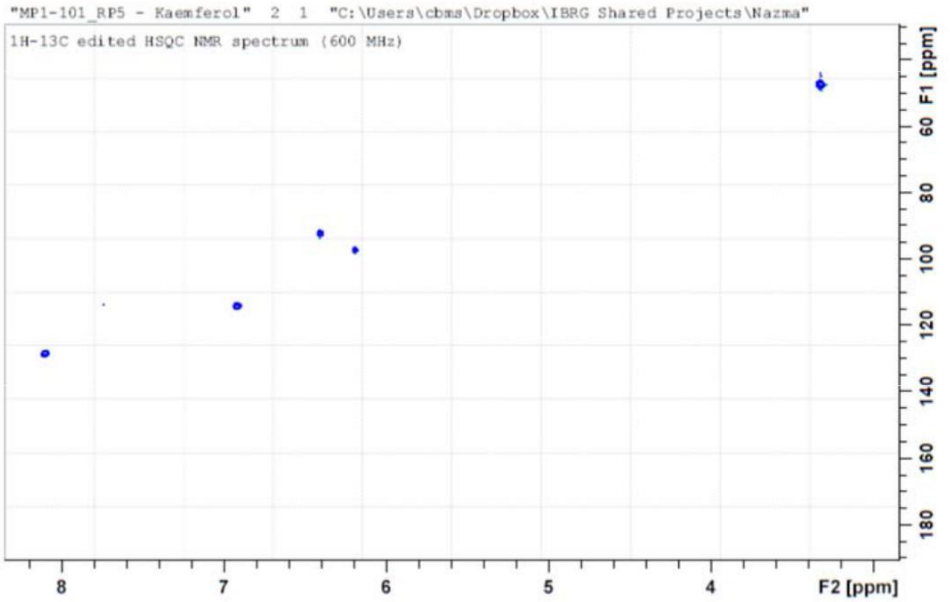
^{13}C and COSY Spectra of Kaempferol





HMBC and HSQC Spectra of Kaempferol





Ethics Approval Letter

MACQUARIE University

Office of the Deputy Vice-Chancellor (Research)
Research Office

C5C Research HUB East, Level 3, Room 324

MACQUARIE UNIVERSITY NSW 2109 AUSTRALIA

Phone +61 (0)2 9850 4063
Fax +61 (0)2 9850 4465

Email biosafety@mq.edu.au

14th September 2016

Dear Associate professor Vemulpad

RE: "Indigenous Bioresources Research Group (IBRG) project: Investigation of antimicrobial and other biological activities of medicinal plants" [5201600535]

Your risk group 2 project has been approved effective 14/09/2016. The risk assessment portion of this project is valid for 5 years. Please ensure a hard copy of this project is made available in your laboratory for reference.

Kind regards,

Biosafety Secretariat
Research Office
Level 3, Research Hub, Building C5C East
Macquarie University
NSW 2109 Australia

T: +61 2 9850 4063
F: +61 2 9850 4465

http://www.mq.edu.au/research

Animal Ethics Application

Application ID :	5201600535
Application Title :	Indigenous Bioresources Research Group (IBRG) project: Investigation of antimicrobial and other biological activities of medicinal plants.
Date of Submission :	03/08/2016
Primary Investigator :	Associate Professor Subramanyam Vemulpad
Other Investigators :	Mrs Kaisarun Akter Mrs Nazma Akter Tithi Tithi Ms Teresa Maria Malewska Associate Professor Joanne Jamie Dr Emma Barnes