

Chemical and Biological Studies of Medicinal Plants Used by the Yaegl Aboriginal Community of Australia

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ABSTRACT

This PhD study was based on the ethnomedicinal knowledge of the Yaegl Aboriginal community of northern NSW, Australia. It follows previous investigations of the Indigenous Bioresources Research Group (IBRG) on first-hand documentation of and preliminary screening of some medicinal plants used by the Yaegl community for treatment of wounds, sores and skin infections. The overall aim of this project was to isolate and indentify bioactive components from two medicinal plants of the Yaegl community.

Two Indigenous Australian medicinal plants, *Lophostemon suaveolens* and *Alphitonia excelsa* were selected for detailed chemical and biological studies. This selection was on the basis of a literature review on the Yaegl medicinal plants documented by the IBRG for the treatment of wounds, sores and skin infections, preliminary screening results of previous IBRG researchers, and specific Yaegl community requests. Antimicrobial activity (disc diffusion, MTT microdilution and TLC bioautography assays), anti-inflammatory activity (NO, TNF- α , PGE₂ and COX inhibitory assays) and antioxidant activity (ORAC assay) of these two plants were evaluated.

Leaves of *L. suaveolens* were extracted sequentially with *n*-hexane, DCM, EtOAc and MeOH. Significant antimicrobial activity was observed with the *n*-hexane and DCM extracts with IC₉₀ <50 μ g/mL against antibiotic sensitive and resistant strains of *Staphylococcus aureus* (MRSA and MDRSA) and *Streptococcus pyogenes* in the MTT microdilution assay. Both extracts also showed significant NO inhibitory activity in RAW264 cells with IC₅₀ 43.9 μ g/mL and 4.6 μ g/mL, respectively, for the *n*-hexane and DCM extracts. These extracts (*n*-hexane and DCM) did not show TNF- α or PGE₂ inhibitory activity. GC-MS analysis of the oily *n*-hexane extract identified 16 components including the well known bioactive compounds aromadendrene (15.47%), spathulenol (12.46%), globulol (4.47%), epiglobulol (2.69%), phytol (2.84%), β -caryophyllene (2.53%) and α -humulene (1.52%). These compounds were reported for having antimicrobial (aromadendrene, spathulenol, globulol, β -caryophyllene, α -humulene and phytol), anti-inflammatory (β -caryophyllene) and cytotoxic (β -caryophyllene, spathulenol and α -humulene) properties. Further fractionation of the DCM extract by normal phase silica gel column chromatography yielded five major bioactive fractions that showed good antimicrobial (IC₉₀ <50-1000 μ g/mL range against *Streptococcus pyogenes* and methicillin sensitive and

resistant strains of *Staphylococcus. aureus*), NO inhibitory ($IC_{50} < 12 \mu\text{g/mL}$), PGE_2 inhibitory ($< 20 \mu\text{g/mL}$) and modest antioxidant ($1000\text{--}2700 \mu\text{M TE/g}$) activities. Further bioassay guided studies on these active fractions using different chromatographic procedures led to the isolation of betulinic acid and eucalyptin from the *n*-hexane and DCM extracts. Eucalyptin has been previously reported to have antimicrobial properties and betulinic acid is a well known anti-inflammatory compound. This is the first report of bioassay studies and isolation of bioactive compounds from *L. suaveolens*.

Leaves of *A. excelsa* were extracted sequentially with *n*-hexane, DCM, EtOAc and MeOH. They were also extracted with water to mimic some preparation practices of the Yaegl community. The EtOAc extract showed promising antimicrobial activity ($IC_{90} < 50 \mu\text{g/mL}$ against *S. pyogenes* and $IC_{90} 500\text{--}1000 \mu\text{g/mL}$ against antibiotic sensitive and resistant strains of *S. aureus*) in the MTT microdilution assay. The EtOAc extract also showed potent nitric oxide (NO) inhibition ($IC_{50} 10.7 \mu\text{g/mL}$) and promising antioxidant ($3.70 \times 10^3 \mu\text{M TE/g}$) activities. The MeOH extract of *A. excelsa* displayed moderate levels of antimicrobial ($IC_{90} 1000$ and $62.5 \mu\text{g/mL}$ against *S. aureus* and *S. pyogenes*, respectively, in MTT microdilution assay) and anti-inflammatory ($IC_{50} 30.5 \mu\text{g/mL}$ in NO inhibition assay) activities. The water extract showed good anti-inflammatory activity in the COX inhibitory assay and modest antioxidant activity in the ORAC assay.

Further bioassay guided isolation of the EtOAc extract led to the isolation of two bioactive flavonoids, kaempferol and quercetin. Both isolated compounds showed antimicrobial activity ($IC_{90} \leq 62.5 \mu\text{g/mL}$ against *S. pyogenes*, sensitive and resistant strains of *S. aureus*) in the MTT microdilution assay. This is the first report of isolation of these two compounds from *A. excelsa*.

Nurturing and sustaining a strong relationship with the Yaegl Indigenous people and providing capacity strengthening opportunities for the community was an essential part of this research. This is aligned with the best ethical practices essential for working with Indigenous people on their knowledge systems. For this PhD study, this included participation in meetings and workshops with the Yaegl community members; presenting and providing feedback on the research work and inviting feedback by the community to guide the research; and organising and participating in an education program with the local youth to enhance educational outcomes, as requested by the Yaegl community.

DECLARATION

I declare that the study in this thesis was carried out by myself. 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 preparation of the thesis itself have been appropriately acknowledged.

The research presented in this thesis was approved by the appropriate committees of Macquarie University as follows:

Biosafety Committee: Ref: 08/06/LAB

Biohazards Committee: Ref: TAN180512BHA

Human Research Ethics Committee:

- An Ethnopharmacological Study of Medicinal Plants in New South Wales, Ref: HE27JUL2007-R05356.
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LIST OF ABBREVIATIONS

The following abbreviations are used throughout the text:

ATCC	American Type Culture Collection
AU	Arbitrary Unit
CDCl₃	Deuterated chloroform
CFU	Colony Forming Units
CHCl₃	Chloroform
COSY	(Proton-Proton) Correlation Spectroscopy
COX	Cyclooxygenase
DCM	Dichloromethane
DMSO	Dimethyl sulfoxide
EtOAc	Ethyl acetate
EI	Electron Impact
EtOH	Ethanol
HMBC	Heteronuclear Multiple Bond Correlation
HPLC	High Performance Liquid Chromatography
HRESIMS	High Resolution Electrospray Ionisation Mass Spectrometry
HSQC	Heteronuclear Single Quantum Correlation
IBRG	Indigenous Bioresources Research group
IC₅₀	Inhibitory concentration capable of inhibiting 50% growth
IC₉₀	Inhibitory concentration capable of inhibiting 90% growth
ISEP	Indigenous Science Education Program
<i>J</i>	Coupling constant
LC-MS	Liquid Chromatography-Mass Spectrometry
5-LO	5-Lipoxygenase

m	Multiplet (NMR)
<i>m/z</i>	Mass to charge ratio
MDR	Multi-Drug Resistant
MeOH	Methanol
MHII	Muller Hinton II
MIC	Minimum Inhibitory Concentration
MRSA	Methicillin Resistant <i>Staphylococcus aureus</i>
MS	Methicillin Sensitive
MS	Mass Spectrometry
MTT	3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
NCCLS	National Committee for Clinical Laboratory Standards
NISEP	National Indigenous Science Education Program
NIST	National Institute of Standards and Technology
NMR	Nuclear Magnetic Resonance
PTLC	Preparative Thin-Layer Chromatography
RP	Reversed Phase
SEC	Size Exclusion Chromatography
SPE	Solid Phase Extraction
TLC	Thin Layer Chromatography
UN	United Nations
UV	Ultra Violet
2D NMR	Two Dimensional Nuclear Magnetic Resonance

CHAPTER ONE

Introduction

This chapter describes the importance of medicinal plant research in the process of drug discovery guided by ethnomedicinal use. The specific aims of this project are also described.

Overview

Through the ages humans have relied on nature for many of their needs, including as medicines. Plants in particular have formed the basis of sophisticated traditional medicine systems that have been in existence for thousands of years (Butler and Newman, 2008). Every race in the world practised the ancient art of herbal medicine and over the centuries traditional medicinal plant knowledge has played an important role in the discovery of new medicines. Although drug development in its modern understanding focuses on pure chemical entities, local and traditional medicinal knowledge, especially that of plants, remains an essential starting point (Heinrich *et al.*, 2010).

In Australia, the Indigenous people have over 40,000 years of knowledge of unique flora as sources of food, resources and healing agents (Pennacchio *et al.*, 2005). This knowledge is a very important cultural resource as well as of immense value in drug discovery (van Holst Pellekaan and Clague, 2005). The unique Australian medicinal flora is relatively unexplored. Some recent studies of Australian medicinal plants have identified biological activities aligned with their medicinal uses and new bioactive molecules (Pennacchio *et al.*, 2005, Shou *et al.*, 2012, Palombo and Semple, 2001, Liu *et al.*, 2006). Therefore, the study of Australian flora using Indigenous medicinal knowledge can be a good avenue for discovering new drugs.

This thesis presents the biological and chemical investigations of two Indigenous medicinal plants used by the Yaegl Aboriginal community of northern New South Wales (NSW), Australia. The choice of these plants was guided by first-hand accounts of the Yaegl elders, as part of a larger ongoing partnership with them and other community groups.

1.1. Brief history of use of plants as medicines

The use of natural products, especially plants, for healing is an ancient and universal practice. Ancient traditional medicine systems, such as Chinese, Ayurveda and Egyptian, especially relied on natural products (Sarker *et al.*, 2006) for their healthcare. Globally, traditional medicines are still routinely used. According to the World Health Organization (WHO), approximately 80% of the world's inhabitants rely mainly on traditional medicines for their primary healthcare. Natural products also play an important role in the healthcare systems of

the remaining 20% of the population, mainly residing in developed countries (Cragg and Newman, 2002). According to a survey by Ramawat and Goyal, American consumers spent US \$5.1 billion on herbal medicines in 1997 (Ramawat and Goyal, 2008). Another study by Nahin has shown that in 2007, Americans spent about US \$14.8 billion to purchase non-vitamin non-mineral natural products (Nahin, 2010). A brief summary of the history of plants as medicines is presented below.

The first records, written on clay tablets in cuneiform, are from Mesopotamia and date from 2600 BCE, while Egyptian medicine dates from 2900 BCE, with the best known Egyptian pharmaceutical record being the *Ebers Papyrus* of 1500 BCE (Mann, 2000, Butler and Newman, 2008). The Chinese *Materia Medica* has been extensively documented over the centuries, with the first record (Wu Shi Er Bing Fang) containing 52 prescriptions, dating from 1100 BCE, though the records from the *Pent'sao* are reputed to be even earlier (~2700 BCE) (Butler and Newman, 2008, Cowan, 1999). Documentation of Indian Ayurvedic systems dates from 1000 BCE (Susruta and Charaka) and Ayurveda *Materia Medica* documented over 1500 herbs and 10,000 formulations (Patwardhan and Mashelkar, 2009). In the ancient Western world, the Greeks contributed substantially to the rational development of the use of herbal drugs. The philosopher and natural scientist, Theophrastus (~300 BCE), in his *History of Plants*, described several plants and animals that were sources of medicine. Dioscorides, a Greek physician (100 CE), recorded the collection, storage and use of medicinal herbs in *De Materia Medica*, which described more than 600 medicinal plants. Galen (130-200 CE), who practiced and taught pharmacy and medicine in Rome, published almost 30 books on these subjects and is well known for his complex prescriptions and formulas for compounding drugs, sometimes containing dozens of ingredients (galenicals) (Butler and Newman, 2008, Ramawat *et al.*, 2009, Heinrich *et al.*, 2004).

During the Dark and middle Ages (5th to 12th Centuries), Arabs were responsible for the preservation of much of the Greco-Roman expertise and for expanding it to include the use of their own resources, together with Chinese and Indian herbs unknown to the Greco-Roman world. The Arabs were the first to establish privately owned drug stores in the 8th Century and the Persian pharmacist, physician, philosopher and poet, Avicenna, described the Greco-Roman medicines of the time in his book *Canon Medicinae*, which is regarded as the final

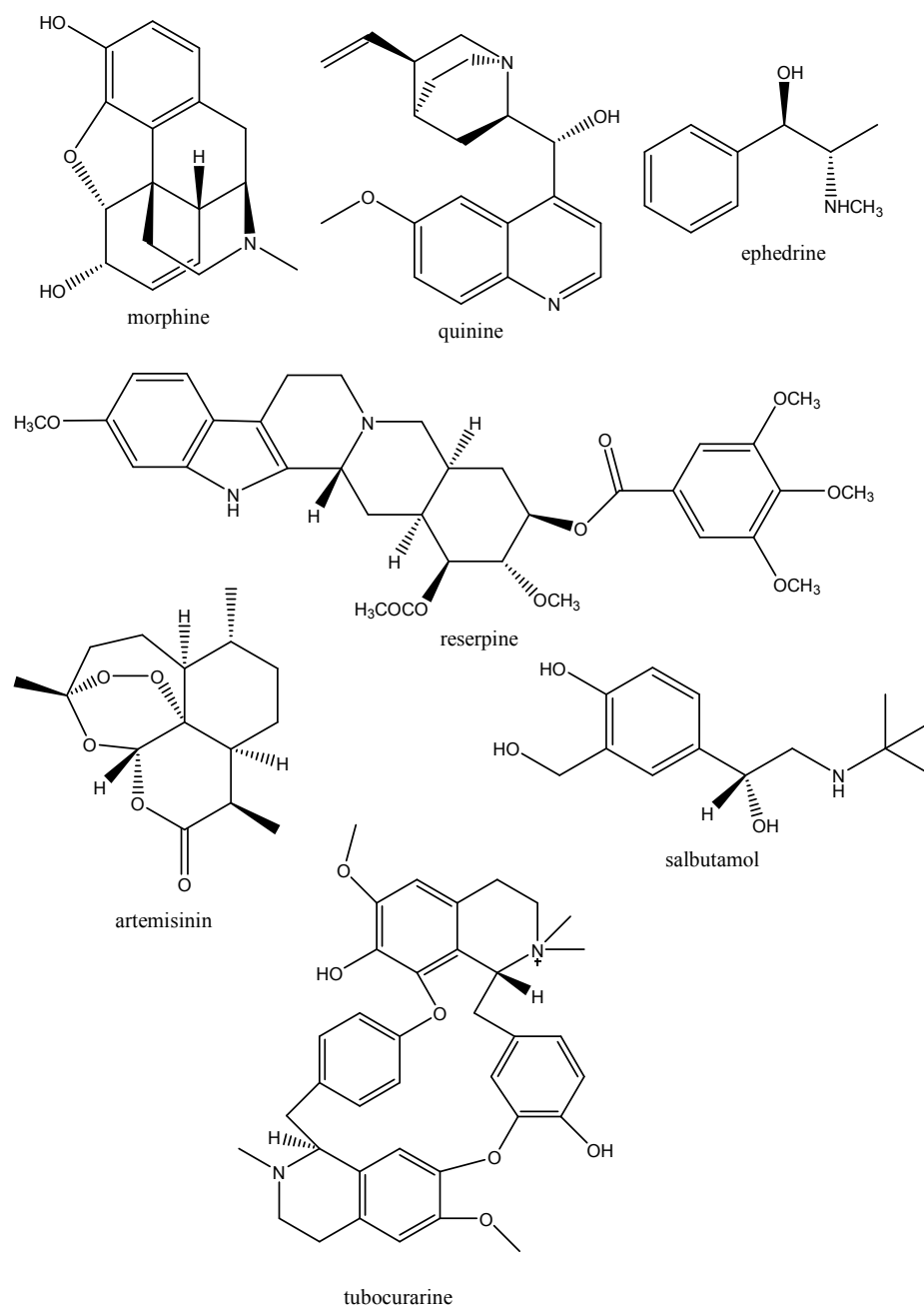
collection of all Greco-Roman medicines (Butler and Newman, 2008, Sarker *et al.*, 2006, Ramawat *et al.*, 2009).

1.2. Natural products as a source of valuable modern medicines

Natural products have always been a source of valuable modern medicines (Butler and Newman, 2008). According to Newman and Cragg (Newman and Cragg, 2007), 39% of the 520 new approved drugs between 1983 and 1994 were natural products or their derivatives and 60-80% of antibacterial and anticancer drugs were from natural origins. In 2000, approximately 60% of all drugs in clinical trials for the multiplicity of cancers had natural origins. In 2001, eight (simvastatin, pravastatin, amoxicillin, clavulanic acid, azithromycin, ceftriaxone, cyclosporine and paclitaxel) of the 30 top selling medicines were natural products or their derivatives. Of the modern drugs that we still use today, a large percentage of these were identified based on their use in traditional medicine. Some examples are presented below.

The opium poppy, *Papaver somniferum* (Papaveraceae), was reported to be used as an euphoriant by ancient Sumerians and also by other ancient societies in the Arab world, India and China. The *Ebers Papyrus* (1500 BCE) described the use of opium (dried latex of the poppy) as “the remedy to prevent excessive crying of children” (Brownstein, 1993). In 1804, Serturmer (Germany) isolated the active ingredient in opium and named it morphine. Morphine was first chemically characterised in 1817 as an alkaloid and its structure was finally established in 1923 by Gulland and Robinson (Heinrich *et al.*, 2010). The isolation of the antimalarial drug quinine, from the bark of *Cinchona officinalis*, was reported in 1820 by the French pharmacists Caventou and Pelletier. The bark had long been used by Indigenous groups in the Amazon region for the treatment of fevers, including malaria, and was first introduced into Europe in the early 1600s for the treatment of malaria. Another plant used in the treatment of fevers in traditional Chinese medicine, *Artemisia annua* (Qinhaosu), has yielded artemisinin and its derivatives, artemether and artether, which are effective against resistant strains of the malarial parasite (Buss and Waigh, 1995). Other significant drugs developed from the study of traditional medicinal plants include the antihypertensive agent, reserpine, isolated from *Rauwolfia serpentina*, which was used in Ayurvedic medicine for the treatment of snakebite and other ailments (Kapoor, 1990); ephedrine, first isolated in 1887

from *Ephedra sinica* (Ma Huang), a plant long used in traditional Chinese medicine and the lead compound for the anti-asthma agents salbutamol and salmetrol (Buss and Waigh, 1995); and the muscle relaxant, tubocurarine, isolated from the plant *Chondrodendron tomentosum* that was used by Indigenous groups of the Amazon as the basis for the arrow poison, curare (Buss and Waigh, 1995). Structures of some of these drugs derived from the study of traditional medicines are shown below (Sarker *et al.*, 2006, Heinrich *et al.*, 2010).



1.3. **Ethnopharmacological approach - an effective avenue for drug discovery**

The process of drug discovery and development, no matter how rationally approached, is generally recognised as a high risk/high pay-off endeavour. Drug discovery from natural sources costs about US \$500 million and takes 15-20 years to go from the natural product to a new medicine (Ramawat *et al.*, 2009). Two thirds of these costs go to the leads that fail during the clinical trial (Ramawat *et al.*, 2009). According to the experience of the pharmaceutical company Merck, for every 10,000 substances that are evaluated in a battery of biological assays, 20 are selected for animal testing. Of these 20, 10 will be evaluated in humans and only one will be approved by the food and drug administration (FDA) in the United States for sale as a drug. Merck also claims that this process requires about 12 years at a cost of US \$231 million (Vagelos, 1991).

Different approaches have been developed to discover new drugs from plants. These includes random screening, the chemotaxonomic approach and the ethnopharmacological approach, which are among the more common and well documented approaches (Fabricant and Farnsworth, 2001, Cordell, 2000, Verpoorte, 2000, Snader and McCloud, 1994).

- *Random screening*

The principle of the random screening approach is to have a large pool of plant material from a given locality that provides chemical diversity, therefore increasing the possibility of obtaining a novel, biologically active molecule (Walton and Brown, 1999). This approach is best suited to specific target screens of a particular disease, for example cancer. The well known anticancer drugs, taxol (from *Taxus brevifolia*) and camptothecin (from *Camptotheca acuminata*) were discovered by random screening performed by the National Cancer Institute, USA (Wall and Wani, 1996).

- *Chemotaxonomic approach*

The chemotaxonomic approach aims to target plant material for specific compounds based on their chemotaxonomic distribution (Fabricant and Farnsworth, 2001, Cordell, 2000). Knowledge about the genus to which a plant species belongs and the family to which the genus belongs helps to predict the spectrum of compounds that can be found in the species

(Walton and Brown, 1999). For example, indole alkaloids in Apocynaceae, coumarins in the Rutaceae and sesquiterpene lactone in the Compositae (Farnsworth, 1994). The chemotaxonomic approach is a useful method for accessing drug leads and analogues of compounds that can not be readily synthesised in the laboratory (Gurib-Fakim, 2006).

- *Ethnopharmacological approach*

The ethnopharmacological approach is one of the oldest methods of drug discovery. According to Bruhn and Holmstedt, ethnopharmacology can be defined as “*the interdisciplinary scientific exploration of biologically active agents traditionally employed or observed by man*” (Heinrich and Gibbons, 2001). This approach utilises Indigenous knowledge for the selection of target plant species for screening.

Successful hit rates of about 30-50% have been achieved with plants collected based on ethnomedicinal knowledge, compared with significantly lower rates for that of the chemotaxonomic approach (e.g. about 18% from Leguminosae family) and that of the random collection approach (about 4%) (Spjut and Perdue, 1976). A study by Washington University, where traditional medicinal knowledge was used for the selection of medicinal plants, found that out of 4,000 plants collected between 1996 and 1999, 53.7% of extracts showed activity aligned with ethnomedicinal use, while only 3.1% were active in the screening where traditional use was not taken into account (Lewis *et al.*, 2004).

The ethnopharmacological approach has not only lead to higher hit rates in screens than the other methods, many drugs valuable in modern society (e.g. atropine, ephedrine, tubocurarine, digoxin, reserpine), have been discovered from ethnopharmacology based research (discussed in Section 1.2). Some of these drug (morphine, physostigmine, quinidine, theophylline, emetine) have acted as leads to the development of more effective and less toxic medicines (Heinrich and Gibbons, 2001). In addition, drugs derived from plants with a documented history of human ethnomedicinal use are likely to be safer than active compounds isolated from those with no records of human use (Fabricant and Farnsworth, 2001, Patwardhan, 2005).

As highlighted above, the ethnopharmacological approach has proven successful in the discovery of new drugs. This approach has been used in the study presented in this thesis to discover bioactive components from medicinal plants used by the Yaegl Aboriginal community of northern NSW, Australia.

1.4. The Aboriginal people in Australia

Aboriginal Australians have lived on the Australian continent for at least 40 to 50 thousand years (Kohen, 1995). Australia began its slow separation from 'Gondwana' some 65 million years ago and evolved with a unique fauna and flora compared to much of the rest of the world (Crotty and Crotty, 1995). Aboriginal Australians have a vast knowledge of Australia's unique flora and have used plants as medicine for thousands of years (Pennacchio *et al.*, 2005). Aboriginal people have occupied every possible habitat, from the arid central desert to the alpine areas of New South Wales, Victoria and Tasmania. Therefore, a wide range of different food and medicinal resources, particularly plants, has been exploited by different Aboriginal groups.

1.4.1. The Yaegl Aboriginal community

The Yaegl people are the traditional custodians of the land around the towns of Maclean and Yamba, situated along the banks of the Clarence River, in the northern region of New South Wales (NSW), Australia. The first residents of the area were the Yaygir tribe after whom the neighbouring Yuragiyir national park was named and the Yaegl people derived their name (McSwan, 1992).

Ulgundahi Island is one of the numerous river islands of the Clarence River located off the township of Maclean. The island has been occupied by Aboriginal people since 1880. It was gazetted as an Aboriginal Reserve in 1904, and accommodated a school, store and a church. It remained populated by many Aboriginal families until the late 1950s. The Aboriginal families were encouraged to be self sufficient through farming and 'living off the land' with strict rationing of foods and medicines that were not provided during the flood times. The recurrent flood episodes eventually drove the families from the island to the mainland and the coastal town of Yamba, where there was plenty of bush foods and medicines to draw on. The Yaegl community and descendants of those who lived on the island maintain a strong connection with the place. The island continues to be used by the Yaegl Aboriginal community for educational tours and organic farming (van Holst Pellekaan and Clague, 2005).

Like other Aboriginal communities of Australia, the Yaegl Aboriginal community also have a long history of using plants as medicines, but this is gradually diminishing due to integration

into an urban lifestyle and the passing away of elder custodians of the customary knowledge. Customary medicinal knowledge can be described as the knowledge that is based on the traditional knowledge of a community that has evolved over time to incorporate modern innovations and access (Packer *et al.*, 2011a).

An ethnobotanical survey on medicinal plants used by the Yaegl people (Packer *et al.*, 2012), documented thirty two plants used by the Yaegl community, with many of these used topically for the treatment of wounds, sores, skin infections, burns, bites and stings. Topical treatments of wounds and infections are the most common application of Australian traditional medicines (Webb, 1969).

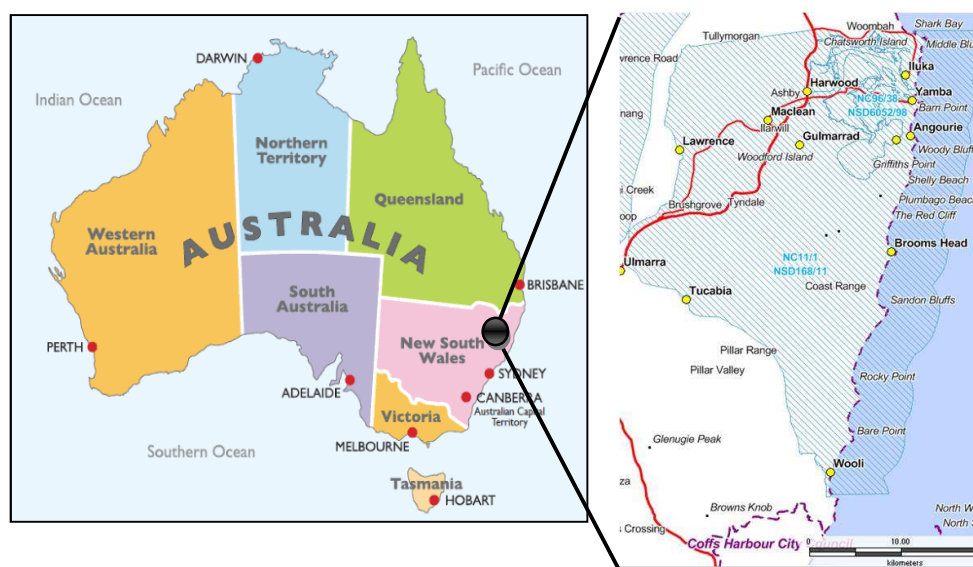


Figure 1.4.1: Map of Australia (left) and enclosed shaded area (right) denotes Yaegl traditional land as defined by Native title tribunal decision (National Native Title Tribunal, 2011).

1.4.2. Collaboration with the Yaegl Aboriginal community

The Indigenous Bioresources Research Group (IBRG) at Macquarie University established a collaboration with the Yaegl elders in 2004 in response to concerns of community members regarding the loss of valuable medicinal plant knowledge and a desire to have the medicinal value of these plants investigated to support their use of them. The author has been an important member of this group, especially with regards to exploring the medicinal properties of two of the Yaegl medicinal plants.

The primary objective of the partnership between the academically founded IBRG and the Yaegl Aboriginal Community represented by the Yaegl Local Aboriginal Land council is to preserve the valuable and disappearing medicinal plant knowledge possessed by the Yaegl community and to use this specialised local knowledge to identify the medicinal properties and bioactive compounds of some of the medicinal plants used by the community. A focus is on those plants used for the treatment of wounds, sores and microbial infections, aligned with their ethnomedicinal uses. Additionally, the IBRG and the community hope that the Indigenous medicinal plant knowledge of Aboriginal people will assist in the discovery of novel therapeutic agents.

1.4.3. Best ethical practices with the Yaegl community

The study presented in this thesis has been performed with a focus on best ethical practices. The IBRG and the Yaegl elders have definite aims and goals for the partnership, identified by a written collaborative research agreement between the Yaegl Local Aboriginal Land Council (representative of the Yaegl community) and Macquarie University (employer of the IBRG researchers). The agreement acknowledges and ensures the attribution of customary knowledge and intellectual property to the Yaegl elders, as the custodians of this knowledge. The agreement also ensures joint ownership of any scientific outcomes and that capacity strengthening for the Yaegl community is implicit. Outcomes of this partnership have included production of a bush medicine handbook for the community (Packer *et al.*, 2011b), joint authorship of scientific publications (Packer *et al.*, 2012, Brouwer *et al.*, 2005), and development of an educational program with the local school, youth and Aboriginal community to enhance educational outcomes and transmit and celebrate the Yaegl scientific and cultural knowledge. As part of this PhD project, the author actively worked in collaboration with the Yaegl elders and the local community, using best ethical practice protocols.

1.5. Necessity of searching for new drugs/leads for the treatment of skin infections and wounds

In rural areas of developing countries, wounds and dermatological conditions constitute one of the five most common reasons for people seeking medical care (Ryan, 1992). Rural people

sustain injuries working in the fields, burns from cooking and sleeping near fires and injuries incurred during conflicts (Bodeker *et al.*, 1999, Ryan, 1992). Chronic wounds are also a major cause of morbidity in developed countries. Chronic wounds affect more than 1% of the UK population, with treatment costs of at least 1 billion pounds per year (Edwards and Harding, 2004). In the USA, chronic wounds are also a major health problem, costing approximately US \$5 billion a year (Beitz, 2001) and global wound care expenditure is US \$13 to \$15 billion annually (Fonder *et al.*, 2008). In Australia, about 200,000 Australians suffer from chronic wounds at any one time (Minnis, 2008) and the wound care expenditure of Australia is \$2.6 billion a year (CRC, 2013).

When wounds and infections occur, it usually takes 3 to 14 days for complete recovery. The normal wound healing process has three phases: inflammation, proliferation and remodelling. During the inflammation phase, neutrophil and macrophages accumulate in the wounded area to phagocytise bacteria and debris. In the proliferation phase, fibroblasts produce a collagen matrix, new blood vessels invade the granular tissue and epidermal cells migrate across the wound surface to close the breach. In the remodelling phase, fibroblasts remodel the collagen matrix to enhance tissue strength and decrease wound thickness (Singer and Clark, 1999). The wound healing process is illustrated in Figure 1.5.1.

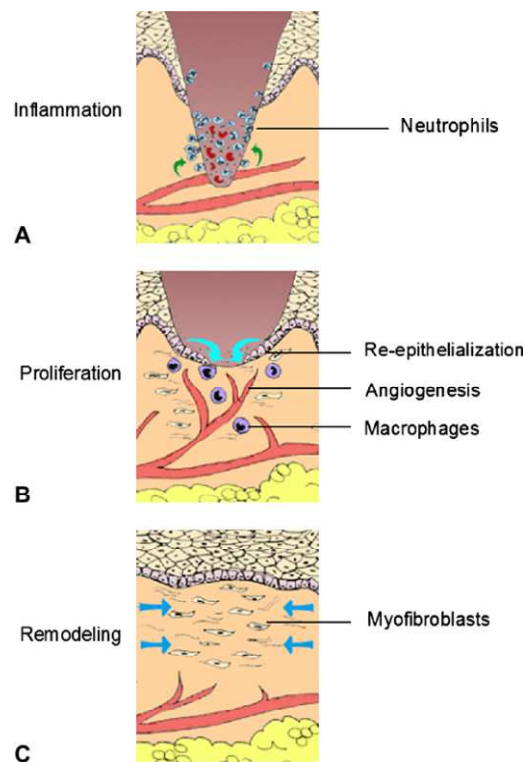


Figure 1.5.1: Normal wound healing process. Source: (Fonder *et al.*, 2008).

When any of the components of wound healing progress is compromised, the healing is delayed. There are a number of factors responsible for delayed wound healing that can result in chronic wounds. Chronic wounds are a serious health problem globally (James *et al.*, 2008). One of the most important factors responsible for skin infection and delayed wound healing is bacteria. All chronic wounds are colonised by bacteria (Edwards and Harding, 2004). The progression from wound colonisation to infection depends not only on the bacteria present but also on the host immune response. There is increasing evidence that bacteria in chronic wounds live within bio-films and get protected from host defences and thus develop resistance to antibiotic treatment (Edwards and Harding, 2004). Non-healing wounds are also associated with prolonged inflammation (Cutting and White, 2005, Gardner *et al.*, 2001). Besides bacteria and inflammatory responses, low antioxidant levels is another important factor that is often associated with delayed wound healing. With increased oxidative stress, the wound healing process becomes stagnant in the inflammatory phase, impairing the ability of dermal fibroblasts and keratinocytes to migrate, proliferate and synthesise extracellular matrix components. This delays the wound healing process (Soneja *et al.*, 2005). Therefore, bacterial

infection, inflammatory responses and low antioxidant levels are often interrelated and are the most common reasons for delayed healing of skin infections, sores and wounds. Finding new antimicrobial, anti-inflammatory or antioxidant agents is thus beneficial for the treatment of skin infections and chronic wounds. Plant based treatments are particularly attractive, given the desire of many people in both developing and developed countries to use natural remedies for their healthcare.

1.5.1. Antimicrobials

The discovery of broad spectrum antibiotics began with the penicillins, the first β -lactams, and heralded the “golden age” (1940-1962) of antibiotic discovery. During this period, many classes of novel natural product antibiotics were identified. These included phenylpropanoids (e.g. chloramphenicol), polyketides (e.g. tetracyclines), aminoglycosides (e.g. gentamycin), macrolides (e.g. erythromycin), glycopeptides (e.g. vancomycin) and streptogramins (e.g. quinpristin), and also second generation β -lactams (e.g. cephalosporins). A third class of β -lactams (carbapenems) was discovered in the early 1970s (Singh and Barrett, 2006). The timeline of discovery of these novel classes of antibiotics is shown in Figure 1.5.2.

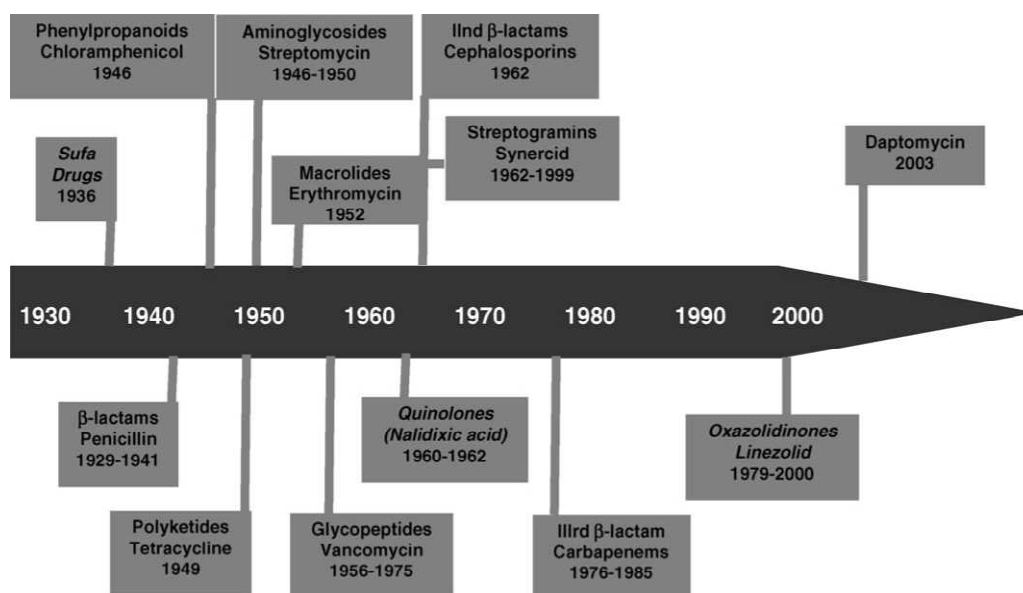


Figure 1.5.2: Timeline of the discovery of novel classes of antibiotics. Source: (Singh and Barrett, 2006).

With the passing of the golden age of antibiotic drug discovery, many natural product-based antibiotics and their scaffolds from the 1950s and 1960s are becoming less useful due to the

evolution of clinically significant resistance to those antibiotics (Alanis, 2005). It is clear that we are in a race to develop new antimicrobials to supplement our dwindling antibiotic arsenal for combating the growing emergence of antibiotic resistant strains. Currently we are losing the race. The Infectious Disease Society of America (ISDA) estimates that 70% of hospital acquired infections in the USA are resistant to one or more antibiotics (Clatworthy *et al.*, 2007). In the USA, antibiotic resistant infections are responsible for US \$20 billion per year in excess healthcare costs, US \$35 billion per year in societal costs and US \$8 billion additional hospital days per year (Bush *et al.*, 2011). Yet, with the exception of the development of the narrow spectrum drugs daptomycin and linezolid, there have been no new classes of clinically relevant antimicrobials discovered in over 40 years (Clatworthy *et al.*, 2007). Realising this problem, the ISDA has proposed a goal of “10 new antibiotics for 2020” (Anonymous, 2010).

The current status of antifungal drugs is also concerning. Fungal infection is considered as one of the major health problems (Loeffler and Stevens, 2003). The mortality rates for invasive infections by the three most common species of fungal pathogens are *Candida albicans*, 20-40% (Lai *et al.*, 2008), *Aspergillus fumigates*, 50-90% (Lai *et al.*, 2008) and *Cryptococcus neoformans*, 20-40% (Park *et al.*, 2009). The incidence of fungal infection is most pronounced in solid organ transplantation (liver 40% and heart 35%), leukemia (30%), and AIDS patients (84%) (Loeffler and Stevens, 2003). Like bacteria, fungi can develop resistance to antifungal drugs rapidly. For example, flucytosine resistance usually develops rapidly when this drug is used alone for the fungal infection (Herbrecht *et al.*, 2005). Bone marrow transplant recipients who are on long term treatment with fluconazole are also likely to encounter antifungal drug resistance by *C. albicans* (Eliopoulos *et al.*, 2002). The timeline of antifungal drug discovery is presented in Figure 1.5.3. As this figure highlights, it took 30 years for the newest class of antifungal drugs, the echinocandins (Butts and Krysan, 2012), to be developed. With the increasing evidence of antifungal drug resistance, the low number of new antifungal drugs developed, and the significant timeframe for this development, is particularly concerning.

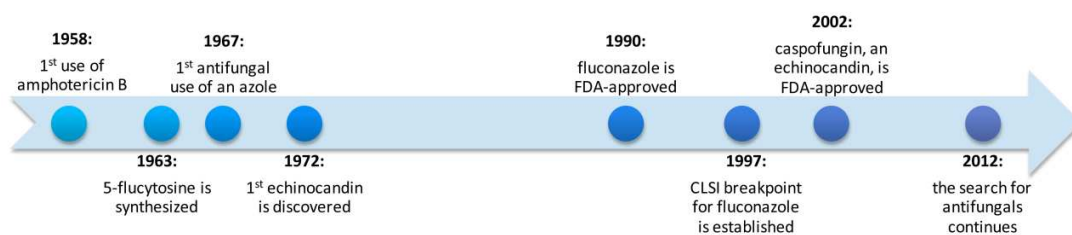


Figure 1.5.3: The timeline of antifungal drug discovery. Source: (Butts and Krysan, 2012).

The discovery of new antimicrobial and antifungal drugs is necessary. Ethnopharmacology based research can be a useful avenue for this.

1.5.2. Anti-inflammatory agents

Inflammation is defined as one of the first innate immune responses to tissue injury and various pathological stimuli. It is also considered as the first phase of the wound healing process (Lai *et al.*, 2013). The inflammatory process consists of many coordinated yet complex biochemical reactions. Inflammation protects the body against infection and injury but can itself become dysregulated with deleterious consequences (e.g. tissue destruction and many inflammatory diseases) to the host (Rai *et al.*, 2011). It may exist on an acute or chronic level depending on the duration of the event. Causes of acute inflammation are mechanical trauma, thermal or electrical injury, chemical burns and biological factors (such as bacterial, viral and fungal infection). This may last from several days up to a few weeks until homeostasis is achieved. Under these conditions the inflammatory response serves to heal the damaged tissue as quickly as possible. However, when this event does not occur, chronic inflammation may result. Chronic inflammation can be described as the inability of the body to reach homeostasis within the affected area (Praveen and Knaus, 2008, Ali *et al.*, 1997). The symptoms resulting from inflammation include pain, redness, swelling (oedema) and temperature (heat) and often cause considerable suffering (Praveen and Knaus, 2008).

A range of therapies are available for the treatment of inflammation-driven diseases. These include steroids (e.g. prednisone, betamethasone and hydrocortisone), non-steroidal anti-inflammatory drugs (NSAIDs) (e.g. aspirin, ibuprofen, indomethacin, diclofenac and celecoxib), antihistamines and the newer biopharmaceuticals such as tumour necrosis factor (TNF)- α neutralising therapies, anti-IgE and anti-CD20 antibodies. However, neither the

established therapies nor the newer biopharmaceutical approaches are without their shortcomings (Ward, 2008). For example, steroids can cause osteoporosis and impair wound healing. Cyclooxygenase (COX) inhibitors (NSAIDs) have ulcerogenic effects and have been reported to increase the risk of coronary thrombosis and stroke by selective COX 2 inhibition (Wong *et al.*, 2005). Moreover, the use of new biopharmaceuticals such as TNF- α - and integrin- α 4 β 7 neutralising therapies has also led to several complications (Edwards *et al.*, 2004, Kremer *et al.*, 2003). Given the limitations of existing pharmaceuticals, there remains a clear need for identification and validation of new safe anti-inflammatory drugs, which may be derived from natural products (O'Neill, 2006, Heinrich *et al.*, 2010, Gossau *et al.*, 2011).

Plants have always been a source of valuable drugs, including anti-inflammatory drugs. For example, the well known drug aspirin has its origin in the bark of the willow tree (Rai *et al.*, 2011). The common classes of anti-inflammatory agents from plants are phenolics, alkaloids, terpenoids and steroids. Some prominent examples are the flavonoid wogonin from *Scutellaria baicalensis* (Kim *et al.*, 2004), curcumin from *Curcuma longa* (Khanna *et al.*, 2007, Bremner and Heinrich, 2005), the alkaloid colchicine from *Colchicum autumnale* (Kiraz *et al.*, 1998), the monoterpene eucalyptol from *Eucalyptus* spp. (Juergens *et al.*, 1998, Juergens *et al.*, 2004) and the steroid guggulsterone from *Commiphora mukul* (Khanna *et al.*, 2007) (Figure 1.5.4).

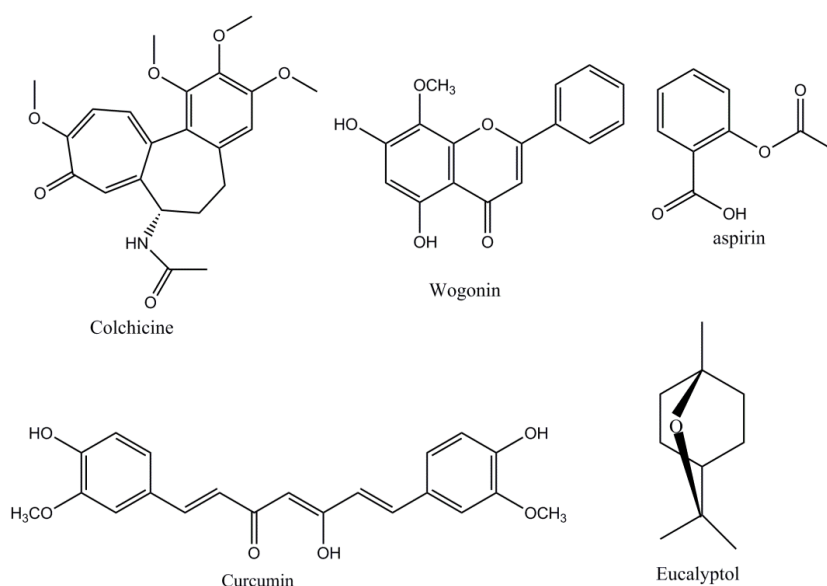


Figure 1.5.4: Anti-inflammatory drugs.

1.5.3. Antioxidants

Free radicals (including hydroxyl, peroxy radicals and singlet oxygen) are reactive species that are capable of damaging molecules such as DNA, proteins and carbohydrates (Li *et al.*, 2008) and contribute towards chronic disease and aging. Antioxidants are compounds that can react with these free radicals, minimising their damage.

During inflammation, reactive oxygen species (ROS) such as superoxide anion radical ($O_2^{\bullet-}$) and hydrogen peroxide (H_2O_2) are continuously being generated to provide adequate host defence against invading pathogens. Normally ROS and potentially pathogenic organisms are detoxified in the presence of the body's endogenous antioxidants, for example, superoxide dismutase removes superoxide (Kapoor *et al.*, 2005) and catalase removes hydrogen peroxide. However, for chronic wounds, there is a decrease in normal endogenous antioxidant levels and increase in the consequences of free radical damage (Lee *et al.*, 2010). In oxidative stress conditions (overproduction of free radicals), 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, therefore the healing process is delayed (Soneja *et al.*, 2005). The use of antioxidants for treatment of chronic wounds may be beneficial in reducing the increased levels of oxidation and can help the wound healing process during injury and infections.

Different types of antioxidants are available. Natural products such as vitamin C and vitamin E are well known for their antioxidant activity (Hernández *et al.*, 2009). Phenolics including kaempferol, quercetin, catechins, tannins and phenolic acids constitute the most important class of natural antioxidants and are also reported to be used widely (Lopez-Velez *et al.*, 2003, Kapoor *et al.*, 2005) (Figure 1.5.5). Synthetic compounds such as butylhydroxyanisole (BHA) and butylhydroxytoluene (BHT) are also commonly used, but have potential health risks and toxicity (Li *et al.*, 2008). Therefore, it is of value to find new sources of inexpensive antioxidants. Searching for antioxidant compounds from medicinal plants that have been used effectively for treatment of chronic wounds is a useful avenue for this.

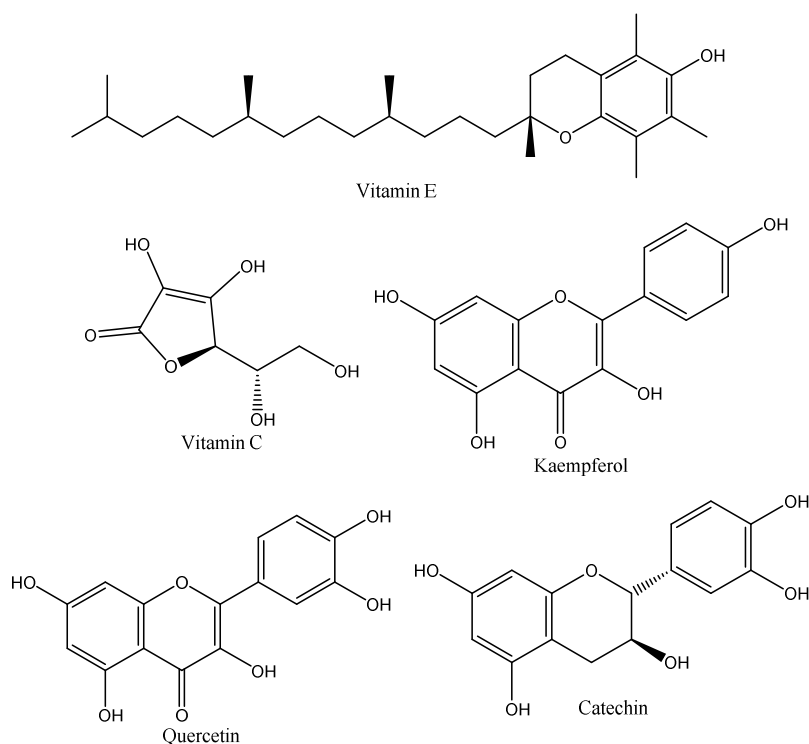


Figure 1.5.5: Antioxidants from plants.

1.6. Objectives of this study

The overall objectives of this PhD project were to undertake chemical and biological investigations, in partnership with the Yaegl elders, guided by their knowledge on medicinal plants they have used for the treatment of skin infections, wounds and sores.

The specific objectives of this PhD project were to:

- Conduct a literature review of plants used by the Yaegl elders for the treatment of sores, wounds and skin infections to allow a selection of medicinal plants for chemical and biological investigations.
- Investigate antibacterial, anti-inflammatory and antioxidant properties of two northern New South Wales (NNSW) medicinal plants based on their customary (traditional and contemporary) use by the Yaegl elders for the treatment of sores, wounds and skin infections.

- Identify and characterise the compound(s) responsible for any antibacterial, anti-inflammatory and antioxidant properties of these medicinal plants.
- Establish a strong relationship between the Yaegl community and the IBRG through working in collaborative partnership and providing capacity strengthening opportunities.

1.7. Thesis overview

It was hypothesised that investigation of medicinal plants used by the Yaegl Aboriginal community of Australia for treatment of sores, wounds and skin infections could assist in the identification of plant extracts and bioactive compounds with antimicrobial, anti-inflammatory and/or antioxidant properties. The finding of such biological activities within the Yaegl medicinal plants was regarded as of value not only to the Yaegl community but potentially also to the wider public in providing natural treatments or drug leads that could assist the urgent treatment of ailments with a microbial, inflammatory and/or oxidant association.

Chapter two introduces medicinal plants customarily used by the Yaegl community for the treatment of skin infections, sores and wounds, along with biological and chemical studies already reported in the literature for these plants and preliminary biological studies conducted by previous IBRG members. This is followed by a justification for the selection of the two Yaegl medicinal plants, *Alphitonia excelsa* and *Lophostemon suaveolens* for further chemical and biological investigations, and an introduction to the proposed biological screening methods.

Chapter three describes the detailed chemical and biological studies conducted on *L. suaveolens*. This includes antibacterial, anti-inflammatory and antioxidant screening of crude extracts and fractions and bioassay guided isolation of bioactive compounds.

Chapter four describes the detailed chemical and biological studies conducted on *A. excelsa*. This includes antibacterial, anti-inflammatory and antioxidant screening of crude extracts and fractions and bioassay guided isolation of bioactive compounds.

Chapter five describes aspects of the IBRG approach and methods used for sustaining a strong relationship with the Yaegl Indigenous people and providing capacity strengthening

opportunities for the community. This includes participation in meetings and workshops with the community members, presenting and providing feedback on the research work and also organising and participating in an education program with the local youth to enhance educational outcomes.

Chapter six provides a summary and future directions.

CHAPTER TWO

Selection of plants and methods for screening and isolation of bioactive compounds

This chapter describes the rationale behind the selection of two medicinal plants, Alphonimia excelsa and Lophostemon suaveolens, for detailed chemical and biological studies, along with the methods to be used for the antimicrobial, anti-inflammatory and antioxidant testing of extracts, fractions and bioassay guided isolation of bioactive compounds from these plants.

2.1. Introduction

This PhD project was part of a larger project between the IBRG (Indigenous Bioresources Research Group) of Macquarie University and several Indigenous custodians of traditional knowledge, including the Yaegl community of northern New South Wales (NNSW). It followed on from the PhD studies of Drs Nynke Brouwer (Brouwer, 2006) and Joanne Packer (Packer, 2012), who worked with Yaegl community elders to help preserve their medicinal plant knowledge and identify plants of medicinal potential for further chemical and biological investigations. Brouwer and Packer undertook first-hand interviews of Yaegl elders to document their valuable ethnobotanical information of Yaegl bush medicines. They subsequently undertook preliminary biological screening of plants used by the Yaegl community for the treatment of wounds and skin infections. Both water (modelling customary – traditional and contemporary preparations) and ethanol extracts of plant material were tested against the common pathogenic microorganisms *Staphylococcus aureus*, *Escherichia coli* and *Pseudomonas aeruginosa*, as summarised in Table 2.1.1 and Table 2.1.2. Some extracts were also tested for anti-inflammatory activity.

Brouwer identified 30 medicinal plants used by the Yaegl community. These were used for a variety of conditions, including wounds, sores and skin infections. Leaves of *Duboisia myoporoides*, *Alphitonia excelsa* and *Ipomoea pes-caprae* were used for the treatment of wounds, sores, pain relief and scabies and were tested for antibacterial and anti-inflammatory activity guided by ethnomedicinal uses. According to Brouwer's preliminary screening results (Table 2.1.1), crude ethanol (EtOH) and water (H₂O, at 80 °C) extracts of *D. myoporoides* and *A. excelsa* leaves showed promising antibacterial activity using the fluorescein diacetate (FDA) assay (Wanandy *et al.*, 2005), with MIC values being less than 5.0 mg/mL against *S. aureus* and *E. coli*. The crude extracts of *A. excelsa* also showed good anti-inflammatory activity in COX (cyclooxygenase) inhibition assays (H₂O and EtOH extracts showed 92% and 90% inhibition of COX 1, and 93% and 91% inhibition of COX 2, respectively, at 240 µg/mL) (Brouwer, 2006).

Packer documented 32 medicinal plants that were used by the Yaegl community. This included *Lophostemon suaveolens*, *Syncarpia glomulifera*, *Ipomoea pes-caprae*, *Canavalia rosea*, *Alocasia brisbanensis*, *Smilax glyciphylla*, *Alphitonia excelsa* and *Hibbertia scandens*,

which were all used for wounds, sores and/or skin infections by the Yaegl community. H₂O (room temperature) and 80% aqueous EtOH extracts of the stems and leaves of *I. pes-caprae* and of the leaves of *L. suaveolens*, *S. glomulifera*, *C. rosea*, *A. brisbanensis*, *S. glyciphylla*, and *H. scandens* were tested for antimicrobial activity against *S. aureus*, *P. aeruginosa* and *E. coli* (Table 2.1.2) (Packer, 2012). The 80% aqueous EtOH extract of *L. suaveolens* and *S. glomulifera* had the highest levels of antibacterial activity with MIC values <125 µg/mL against *S. aureus* in MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide] microdilution assay as well as good activity by the disc diffusion and turbidity assays (Packer, 2012) (Table 2.1.2). *I. pes-caprae* was tested by both researchers (Packer and Brouwer). The H₂O extract was more active in the study of Brouwer, most likely due to the use of a higher temperature for the extraction. Additionally, *I. pes-caprae* leaves and stems were used in Packer's study and only leaves for the Brouwer study, providing a further differentiating feature.

Table 2.1.1: Antimicrobial activities of Yaegl medicinal plants using FDA assay (Brouwer, 2006).

Plant ^a	Ext ^b	MIC (mg/mL) ^c		
		EC	PA	SA
<i>Alphitonia excelsa</i> (Fenzl) Benth (MQ 73007870)	H ₂ O	5	>10.0	5
	EtOH	>3.1	>3.1	1.6
<i>Duboisia myoporoides</i> (R. Br) (MQ 73007957)	H ₂ O	9.0	9.0	≤9.0
	EtOH	1	0.5-1.0	0.5-1.0
<i>Ipomoea pes-caprae</i> ssp. <i>Brasiliensis</i> (MQ 73007958)	H ₂ O	1.6-8.4	8.4	1.6-8.4
	EtOH	>8.2	>8.2	>8.2

^aLeaves of plants used. ^bExtraction method used: H₂O - hot water extract at 80 °C, EtOH - 100% ethanol extract. FDA method was used (Wanandy *et al.*, 2005) to test the MIC, high activity ≤1.0 mg/mL, moderate activity >1.0-10 mg/mL. Tetracycline was used as a control. Organisms tested: EC: *Escherichia coli* (JM109), PA: *Pseudomonas aeruginosa* (ATCC 27853), SA: *Staphylococcus aureus* (ATCC 9144). SA 0.12 µg/mL, EC 0.5 µg/mL and PA 5.0 µg/mL. Experiments were performed in triplicate. *A. excelsa* and *D. myoporoides* were collected from Maclean, NSW in March 2005 and May 2003, respectively, and *I. Pes-caprae* was collected from Yamba, NSW in May 2003.

Table 2.1.2: Antimicrobial activities of Yaegl medicinal plants using disc diffusion, MTT microdilution and turbidity assay methods (Packer, 2012).

Plant ^a	Ext ^b	DD ^c /MTT ^d /Turbidity ^e		
		EC	PA	SA
<i>Alocasia brisbanensis</i> Domin (MQ 73008737)	H ₂ O	- / - / -	- / - / -	- / - / -
	EtOH	- / - / -	- / - / +	- / - / -
<i>Canavalia rosea</i> (Sw.) DC. (MQ 73008909)	H ₂ O	- / - / -	- / - / -	- / - / -
	EtOH	- / - / -	- / - / +	- / - / -
<i>Hibbertia scandens</i> (Willd.) Gilg (MQ 73008905)	H ₂ O	- / - / -	- / - / +	- / + / +
	EtOH	- / - / -	- / - / +	- / - / +
<i>Ipomoea pes-caprae</i> (L.) Sweet (MQ 73007958) [#]	H ₂ O	- / - / -	- / - / + [^]	- / - / -
	EtOH	- / - / -	- / - / + [^]	- / - / -
<i>Lophostemon suaveolens</i> (Sol. ex Gaertn.) Peter G. Wilson & J.T. Waterh (MQ 73008908)	H ₂ O	- / - / +	+ / - / ++ [^]	++ / + / ++
	EtOH	- / - / +	- / - / ++	++ / +++ / +++
<i>Smilax glycyphylla</i> Sm. (NSW 792380) (narrow leaf)	H ₂ O	- / - / -	- / - / +	- / - / +
<i>Syncarpia glomulifera</i> subsp. <i>glomulifera</i> (Sm.) Nied. (MQ 73009066)	H ₂ O	- / - / -	+ / - / ++	++ / + / +
	EtOH	- / - / -	- / - / ++ [^]	++ / +++ / +++
Antibiotic control*		+++ / +++ / +++	+++ / +++ / +++	+++ / +++ / +++

^aLeaves of plants used in all cases except where indicated, [#]leaves and stems. ^bExtraction method used: H₂O - room temperature, EtOH - 80% aqueous ethanol. ^cDegree of activity as determined by diameter of complete inhibition, including 6 mm disc diameter - good: >16 mm (+++), moderate: 10–16 mm (++), slight: <10 mm (+). ^dMIC was determined as the well with the lowest concentration of sample that displayed no yellow-blue colour change: ≤125 µg/mL (+++), >125 µg/mL (+). ^eActivity based on change in turbidity (A₆₀₀) as a percentage of growth control: <10% = good (+++), 10–50% = moderate (++) inhibition at 125 µg/mL extract, slight (+) = activity at concentrations >125–1000 µg/mL. (–) No activity detected at 1000 µg/mL. Organisms tested: EC: *Escherichia coli* (β lactamase negative, ATCC 25922), SA: *Staphylococcus aureus* methicillin sensitive (ATCC 29213), PA: *Pseudomonas aeruginosa* (ATCC 27853). Activity based on median values (n ≥ 4). [^]Three repeats did not fall within one dilution of median.

2.2. Review of literature of promising medicinal plants

As described in the Introduction chapter (Chapter 1), this PhD study focused on screening for and identifying antimicrobial, anti-inflammatory and antioxidant components from Yaegl medicinal plants, guided by their ethnomedicinal use. The preliminary antimicrobial screening by Brouwer and Packer showed that the crude extracts of *L. suaveolens*, *S. glomulifera*, *C. rosea*, *A. brisbanensis*, *A. excelsa*, *H. scandens*, *D. myoporoides* and *I. pes-caprae* all exhibited some level of bioactivity (Packer, 2012, Brouwer, 2006). *A. excelsa*, *D. myoporoides*, *L. suaveolens* and *S. glomulifera* were most promising (Section 2.1). Additionally, *A. excelsa* extracts (EtOH and H₂O) showed significant anti-inflammatory activity in the COX assays (Section 2.1).

For all the plants that Brouwer and Packer showed had activity, *i.e.* *L. suaveolens*, *S. glomulifera*, *C. rosea*, *A. brisbanensis*, *A. excelsa*, *H. scandens*, *D. myoporoides* and *I. pes-caprae*, a literature review on customary uses of them and chemical and biological studies that have been conducted, was undertaken to assist in the selection of two of the Yaegl medicinal plants for further biological and chemical investigations.

I. Alocasia brisbanensis (or Alocasia macrorrhiza)

Local medicinal uses: Leaves and stems of the plant have been used by the Yaegl community for treatment of burns and boils, cuts, sores, green ant bites and open wounds (Packer *et al.*, 2012). The plant has also been reported to be used in the treatment of boils, burns, ulcers and sores in other parts of Australia (Lassak and McCarthy, 2008, Webb, 1969). In Chinese folk medicine, the roots have been used for treatment of cancers (Fang *et al.*, 2012).

Reported Biological studies: The tuber of the plant has been reported to have *in vitro* proliferation inhibition and apoptosis effects on human hepatocellular carcinoma and *in vivo* hepatoma growth (Fang *et al.*, 2012) and the leaves have antioxidant, antinociceptive, anti-inflammatory (Mulla *et al.*, 2010) and hepatoprotective (Mulla *et al.*, 2009) activities.

Reported Chemical constituents: Indole alkaloids, alocasins (A-E), hyrtosin B and hyrtiosulawesin have been isolated from the rhizome. Among the isolated compounds, hyrtiosulawesin and alocasin A, B, D and E showed antiproliferative activity (Zhu *et al.*, 2012a). Glycosmistic acid, *N-trans*-feruloyltyramine, grossamide, protocatechuic acid, borneol

acetate, vanillic acid and methyl 4-hydroxybenzoate have been isolated (Zhu *et al.*, 2012b). Vanillic acid has been reported to have antibacterial activity (Aziz *et al.*, 1997, Naz *et al.*, 2006).

II. *Alphitonia excelsa*

Local medicinal uses: Leaves of the plant are commonly used as an antiseptic hand wash by the Yaegl community (Packer *et al.*, 2012). Leaves have been used for fishing where throwing the crushed leaves into the water would make fish come floating to the surface (Brouwer, 2006). Leaves are also used for sore eyes and chewed in the case of an upset stomach. The bark, root and wood have been used to make an infusion to rub on the body as a liniment for body pains. The bark and wood have also been employed to make a decoction for use as a gargle for toothache (Lassak and McCarthy, 2008). The crushed leaves have been used for the treatment of headache by bathing with the leaves in water and, similar to the Yaegl community, leaves have been used by other communities in the making of instant bush soap by crushing the leaves (Lassak and McCarthy, 2008, Cribb and Cribb, 1984, Barr *et al.*, 1990, Low, 1990).

Reported Biological studies: Leaves have been reported to inhibit platelet aggregation properties (Rogers *et al.*, 2000). No antibacterial, anti-inflammatory or antioxidant activities have been reported.

Reported Chemical constituents: Triterpenoid saponins (Lassak and McCarthy, 2008), alphitonin, ceanothic acid and betulinic acid, have been isolated from the bark (Branch *et al.*, 1972, Guise *et al.*, 1962). Structures are provided in Section 2.5.3. Betulinic acid is reported in the literature as having good anti-inflammatory activity (Nguemfo *et al.*, 2009, Tsai *et al.*, 2011, Viji *et al.*, 2011) and ceanothic acid is reported to have antibacterial activity (Li *et al.*, 1997).

III. *Canavalia rosea* (or *Canavalia obtusifolia* or *Canavalia maritima* or *Dolichos roseus*)

Local medicinal uses: Leaves have been reported to be used for boils and sores by the Yaegl community (Packer, 2012). The roots are reported to be used in the treatment of rheumatism, aches and pains (Lassak and McCarthy, 2008, Cribb and Cribb, 1984, Low, 1990). Leaves

have been also reported to be used to relieve pain and to promote healing of burns (Prabhu *et al.*, 2010).

Reported Biological studies: The leaves have been reported to have antibacterial activity (Prabhu *et al.*, 2010) and the isolated lectins were reported to have anticancer properties (Pinto *et al.*, 2010).

Reported Chemical constituents: Canarosine, β -sitosterol, stigmasterol, rutin and epi-inositol 6-O-methyl ether have been isolated from aerial parts (Pattamadilok *et al.*, 2008), 2-hydroxy-4,9-dimethoxypterocarpin, medicarpin, 4-hydroxy-3-methoxy-8,9-methylene-dihydroxypterocarpan, 7-hydroxy-2'4'-dimethoxy isoflavan, 7-hydroxy-4'-methoxy-isoflavone, 3,7-dihydroxy-6-methoxyflavone and quercetin from leaves and stems (Huang *et al.*, 2012), and lectins (Pinto *et al.*, 2010) from seeds. The guanidine alkaloid, canarosine is reported to have dopamine receptor inhibitory activity (Pattamadilok *et al.*, 2008). Quercetin (Martínez-Flores *et al.*, 2005, García-Mediavilla *et al.*, 2007, Calderon-Montano *et al.*, 2011) and rutin (Guardia *et al.*, 2001, La Casa *et al.*, 2000) are well known for having anti-inflammatory and antioxidant activities.

IV. *Duboisia myoporoides*

Local medicinal uses: Fruits are used for treatment of ringworm (fungal infection of skin) and leaves are used as a topical anaesthetic, antiseptic and also used for bronchitis and coughs by the Yaegl community (Brouwer, 2006, Packer *et al.*, 2012). Leaves are also reported to be used as a fish poison by other communities (Isaacs, 1987, Boydrón-Le Garrec *et al.*, 2005).

Reported Biological studies: The plant is reported to have mydriatic, analgesic and antispasmodic activities (Khanam *et al.*, 2001).

Reported Chemical constituents: A wide range of alkaloids including hyoscyne and (-)-hyoscyamine (Hills *et al.*, 1954), nicotine, anabasine (Gritsanapan and Griffin, 1991), hygrine, valeroidine, valtropine, tigloidine, tropine, butropine, norscopolamine, norhyoscyamine, homatropine, isoporoidine, (phenyl)-acetyltropine, poroidine, aposcopolamine, noratropine, atropine and tetramethylputrescine (Gritsanapan and Griffin, 1991) have been isolated. Nicotine has well documented uses and can be classified as a toxin. Hyoscyne is used for travel sickness and amnesia, while hyoscyamine and atropine are

reported to have anticholinergic activity (Woolley, 2001, Shamsa *et al.*, 1999). The presence of these compounds in *D. myoporoides* correlates with traditional uses of this plant as an anaesthetic, for treatment of bronchitis and as a fish poison.

V. *Hibbertia scandens*

Local medicinal uses: The plant has been used for treatment of sores and rashes by the Yaegl community (Packer, 2012). However, there was little that could be recalled about the use of the plant by the Yaegl elders and the preparation method was not known.

Reported Biological studies: No biological studies have so far been reported.

Reported Chemical constituents: No chemical studies have so far been reported.

VI. *Ipomoea pes-caprae* ssp. *brasiliensis*

Local medicinal uses: The plant has been used by the Yaegl community as a poultice for boils to relieve pain and also to relieve headache (Brouwer, 2006). The leaves have been reported to be used externally to relieve pain in colic (Lassak and McCarthy, 2008). Rogers *et al.* mentioned the use of the whole plant for the treatment of headaches, aches and pains and marine stings (Rogers *et al.*, 2000). The leaves have been also reported to be used in the treatment of boils (Lassak and McCarthy, 2008, Isaacs, 1987, Cribb and Cribb, 1984).

Reported Biological studies: A wide range of bioactivities has been reported. This includes antioxidant (Umamaheshwari *et al.*, 2012) antibacterial (Escobedo-Martinez *et al.*, 2010) and anti-inflammatory (Pongprayoon *et al.*, 1991) activities. It is also reported to have collagenase (Teramachi *et al.*, 2005) and platelet aggregation inhibitory activities (Rogers *et al.*, 2000), and antinociceptive (de Souza *et al.*, 2000) and antihistamine activities (Wasuwat, 1970).

Reported Chemical constituents: This plant has been widely studied. Pescapreins XVIII-XX, murucoidin VI, pecapreins II, III and stoloniferins III (Escobedo-Martinez *et al.*, 2010), along with several resin glycosides (pescaproside B, pescapreins V-IX, stoloniferin III) (Yu *et al.*, 2011, Tao *et al.*, 2008, Escobedo-Martinez and Pereda-Miranda, 2007), quinic acid ester, 3,5-di-*O*-caffeoyl-4-*O*-coumaroylquinic acid, 4,5-di-*O*-caffeoyl-1,3-di-*O*-coumaroylquinic acid (Teramachi *et al.*, 2005) and pescaproside A, pescapreins I-IV (Pereda-Miranda *et al.*, 2005) have been isolated. Pescaprein XVIII is a good inhibitor of multidrug resistant *S. aureus*

(Escobedo-Martinez *et al.*, 2010). Pescapreins X-XVII are reported to have antibacterial, antifungal and cytotoxic activities (Tao *et al.*, 2008).

VII. *Lophostemon suaveolens*

Local medicinal uses: Milky sap and ash from the bark have been used for antiseptic purposes by the Yaegl community (Packer *et al.*, 2012).

Reported Biological studies: No biological study has so far been reported for this plant.

Reported Chemical constituents: The volatile oil composition has been reported by Brophy *et al.* and the main identified compounds were α -pinene (4-33%), β -caryophyllene (3-19%), aromadendrene (7-13%), *allo*-aromadendrene (2-4%), globulol (5-14%), viridiflorol (1-3%) and spathulenol (5-11%) (Brophy *et al.*, 2000).

VIII. *Smilax glyciphylla*

Local medicinal uses: Leaves have been used for treatment of pain, arthritis and for skin problems (as a face wash in pimples), as a revitalising tonic and for treatment of diabetes by the Yaegl community (Packer *et al.*, 2012). The use of the plant for the treatment of coughs is widely reported (Lassak and McCarthy, 2008, Webb, 1969).

Reported Biological studies: The leaves have been reported to have antioxidant activities (Cox *et al.*, 2005).

Reported Chemical constituents: Linoleic, oleic and palmitic acid have been isolated from the seeds of the plant (Morice, 1970).

IX. *Syncarpia glomulifera*

Local medicinal uses: The milky latex has been used for treatment of cuts and sores and ash of the bark has been used as an antiseptic powder by the Yaegl community (Packer, 2012).

Reported Biological studies: The chloroform extract of the bark has been reported to have antibacterial and cytotoxic properties (Setzer *et al.*, 2000).

Reported Chemical constituents: The bark has been reported to contain betulinic acid, oleanolic acid-3 acetate and ursolic acid-3-acetate (Setzer *et al.*, 2000). A study on the volatile

oil composition has shown that the leaf oil contains α -pinene (30-50%), along with aromadendrene and globulol (Brophy *et al.*, 1996). The leaf wax coating of *S. glomulifera* has been reported to contain eucalyptin and 8-desmethyleucalyptin (Courtney *et al.*, 1983). As already discussed in Section 2.2 (*A. excelsa*), betulinic acid is also reported in the literature for having anti-inflammatory activity and eucalyptin is reported for having antibacterial activity (Takahashi *et al.*, 2004).

2.3. Selection of two medicinal plants for detailed chemical and biological studies

The choice of plants for further biological and chemical studies was based on investigation of relatively unexplored Australian flora, the desires of the Yaegl community themselves to which plants and plant parts should be further investigated and the results of the preliminary screenings by Brouwer and Packer (Table 2.1.1 and Table 2.1.2). Using these criteria, *A. excelsa* and *L. suaveolens* were chosen as the most significant candidates for investigation within this study. Other Yaegl medicinal plants and plant parts could be worthy of future investigations.

According to the literature review (Section 2.2), *A. excelsa* and *L. suaveolens* had no (or very limited) reports of biological activities relevant to their medicinal uses by the Yaegl community. Additionally, only one chemical study, examining the volatile oil composition of the leaves of *L. suaveolens*, has so far been reported. In contrast, apart from *H. scandens*, the other Yaegl plants reviewed had either been found to have biological activities relevant to their ethnomedicinal uses and/or had significant chemical studies already conducted. Furthermore, in the preliminary screening, leaf extracts of *A. excelsa* had promising antibacterial and anti-inflammatory activities when tested by Brouwer (Brouwer, 2006) (Section 2.1).

The promising antibacterial and anti-inflammatory activity of *A. excelsa* leaf extracts, along with it being the most commonly used medicinal plant of the Yaegl elders, prompted its further investigation. *A. excelsa* is very well known to the Yaegl community and the leaves have been commonly used for cuts, sores and scabies by them. The elders had a particular desire for this plant to be further examined. It had only been tested for one specific class of

inflammatory enzymes (COX) and not tested for antioxidant activity. Additionally, this plant was in plentiful supply in northern NSW and locally in Sydney. Thus, *A. excelsa* leaves were chosen for further biological and chemical investigations in this study.

As described earlier, the ash from the bark and the sap or latex of *L. suaveolens* was used traditionally in the treatment of wounds by the Yaegl community. Bark ash was tested for antibacterial activity by Packer, with no activity being detected against *S. aureus*, *E. coli* and *P. aeruginosa* (Packer, 2012). Latex or sap was not able to be collected for the preliminary screenings or subsequent studies and the Yaegl elders requested leaves to be collected and tested. The leaf extract was found to have excellent antibacterial activity when tested by Packer (Packer, 2012). These factors, along with the lack of reports of chemical and biological activity studies in the published literature, led to the leaves of *L. suaveolens* being chosen for biological and chemical investigations in this study.

Both *L. suaveolens* and *A. excelsa* are endemic to Australia and the investigation of these plants within this PhD study was also regarded as important in increasing the knowledge on these unexplored Australian flora. Both plants are reviewed in more detail below.

2.4. *Lophostemon suaveolens*

Lophostemon suaveolens belongs to the genus *Lophostemon*. Common names of *L. suaveolens* include swamp mahogany, swamp, swamp box, swamp turpentine, mahogany, paperbark, paperbark mahogany (Australian Tropical Rainforest Plants). This plant is known as the apple gum tree by the Yaegl community (Packer, 2012). The genus *Lophostemon* belongs to the sub-family Leptospermoideae of the family Myrtaceae. There are four species of *Lophostemon* (The Plant List, 2010b) and all of them are native to Australia. All species are trees or tree like shrubs (Brophy *et al.*, 2000). The four *Lophostemon* species are:

- *Lophostemon confertus* (synonym: *Tristania conferta*)

This species grows in open or closed forests on the east coast of Australia from north of Cooktown (Queensland) to the northern side of the Hunter river (New South Wales). This species forms a large tree up to 25 m in height (Brophy *et al.*, 2000).

- *Lophostemon grandiflorus*

This species has a discontinuous distribution across northern Australia from Queensland to Western Australia. It is closely related to *L. confertus*, but is usually a smaller tree (Brophy *et al.*, 2000).

- *Lophostemon lactifluus*

This species grows in woodland close to water or in ground subject to flooding. It occurs in the Northern Territory north of the Victoria river (Brophy *et al.*, 2000).

- *Lophostemon suaveolens* (synonym: *Tristania suaveolens*)

It grows in woodlands and open forests in southern New Guinea and eastern Australia from Cape York (Queensland) to Scott's Head (New South Wales) (Brophy *et al.*, 2000).

Scientific classification of *Lophostemon suaveolens*

Kingdom:	<i>Plantae</i>
Division:	<i>Magnoliophyta</i>
Class:	<i>Magnoliopsida</i>
Order:	<i>Myrtales</i>
Family:	<i>Myrtaceae</i>
Genus:	<i>Lophostemon</i>
Species:	<i>suaveolens</i>

2.4.1. Description of *Lophostemon suaveolens*

L. suaveolens is a large tree with a red-brown, fibrous-papery, persistent bark. The leaves are broad and rounded at the base, tapering towards the end. They are 9–15 cm long and 4–5 cm wide, leathery, light green and either smooth or covered with soft, shaggy unmatted hairs. The leaf stalk is 10–20 mm long. Old leaves turn a bright orange red prior to falling. Clusters of white flowers 3–5 mm long are produced in early summer. The petals are circular, 4–5 mm long and the flowers have numerous stamens (Wilson and Waterhouse, 1982).



Figure 2.4.1: *L. suaveolens* tree (left) and *L. suaveolens* leaves (right).

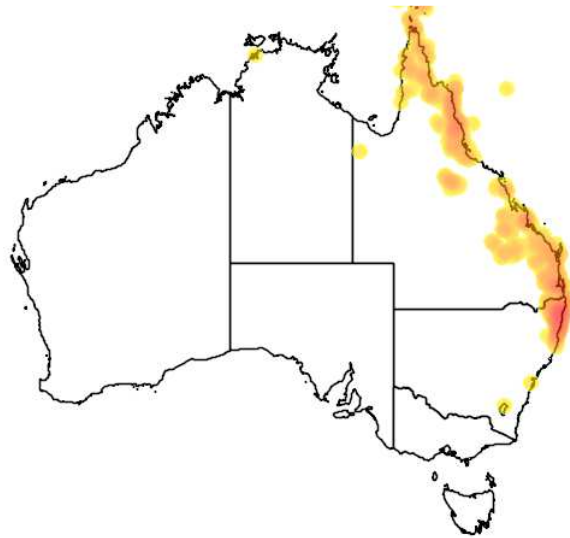


Figure 2.4.2: Distribution of *L. suaveolens* in Australia (Atlas of living Australia).

2.4.2. Medicinal uses of *Lophostemon suaveolens*

According to the interviews conducted by Brouwer and Packer (Brouwer, 2006, Packer, 2012), the local medicinal uses of *L. suaveolens* by the Yaegl community are:

- Ash from the bark is used as an antiseptic powder.
- The milky sap is used as an antiseptic face wash for acne and bad skin blemishes.
- The sap is used for skin diseases and for ringworm.

No reports have been found in the published literature regarding the medicinal uses of *L. suaveolens* by any other community.

2.4.3. Previous chemical and biological studies on *Lophostemon suaveolens*

GC-MS analysis of essential oils of leaves of *L. suaveolens* has been reported (Brophy *et al.*, 2000). According to this study, the major compounds were α -pinene (4-33%), β -caryophyllene (3-19%), aromadendrene (7-13%), *allo*-aromadendrene (2-4%), globulol (5-14%), viridiflorol (1-3%) and spathulenol (5-11%).

Compounds responsible for the bioactivity of this plant have so far not been reported.

2.4.4. Studies on other species of *Lophostemon*

According to the literature, the genus *Lophostemon* has not been well explored. Similar to the studies on *L. suaveolens*, the essential oils of the leaves of *L. grandiflorus*, *L. confertus* and *L. lactifluus* have been examined by GC-MS by Brophy *et al.* (Brophy *et al.*, 2000) with α -pinene, aromadendrene, *allo*-aromadendrene, globulol and spathulenol as the major volatile components. Courtney *et al.* found that the leaf wax contained the unusual C-methyl flavone, 8-desmethyleucalyptin and friedelin in *L. confertus* (Courtney *et al.*, 1983). Yao *et al.* have reported the presence of the flavonoids tricetin, luteolin and quercetin as major components and kaempferol, quercetin 3-methyl ether and 8-methoxy kaempferol in *L. confertus* (Yao *et al.*, 2004). Ritchie *et al.* have reported arjunolic acid in the wood of *L. confertus* (Ritchie *et al.*, 1961).

2.5. *Alphitonia excelsa*

Alphitonia excelsa belongs to the family Rhamnaceae. It is commonly known as soap bush by the Yaegl community (Packer *et al.*, 2012). Other common names of *A. excelsa* are red ash or mountain ash, leather jacket, Coopers' wood, white leaf, humbug and soap tree (Lassak and McCarthy, 2008). Around Australia it is known under different names, including as *murr-rung* in the Illawarra district, as *nono-groyinandie* in the Clarence River, as *culger-culgera* in northern New South Wales and as *mee-a-mee* and *an-na* in Queensland (Lassak and McCarthy, 2001). In the Northern Territory *A. excelsa* is known as *minjirrajirda* in Burarra, *bani* or *buwalawal* in Djambarrpuyngu and *mitjirribiya* in Emi (Barr *et al.*, 1990).

Closely related to *A. excelsa* are the other *Alphitonia* species: *A. carolinensis*, *A. erubescens*, *A. ferruginea*, *A. franguloides*, *A. incana*, *A. macrocarpa*, *A. marquesensis*, *A. moluccana*, *A. neocaledonica*, *A. obtusifolia*, *A. petriei*, *A. philippinensis*, *A. ponderosa*, *A. rubiginota*, *A. whitei*, *A. xerocarpa* and *A. zizyphoides* (The Plant List, 2010a). Three of these species (*A. incana*, *A. petriei* and *A. zizyphoides*) have been used by Indigenous societies in traditional medicine; others have been used for timber (e.g. *A. philippinensis*). Only *A. excelsa* and *A. petriei* are endemic to Australia (Brouwer, 2006). Geographic distributions of ethnomedicinally important *Alphitonia* species are described below.

- *A. excelsa*

It is a native Australian medicinal plant. It grows in or near rainforests in New South Wales, Queensland and the Northern Territory (ANBG, 2012).

- *A. petriei*

It is also a native Australian medicinal plant, distributed in New South Wales and in Queensland (Royal Botanic Gardens Melbourne, 2013).

- *A. incana*

It occurs in Western Australia, Northern Territory, Cape York peninsula and Queensland (Australian Tropical Rainforest plants, 2013).

Scientific classification of *Alphitonia excelsa*

Kingdom	<i>Plantae</i>
Division	<i>Magnoliophyta</i>
Sub-division	<i>Magnoliophytina</i>
Class	<i>Rosopsida</i>
Sub-class	<i>Rosidae</i>
Super-order	<i>Rhamnae</i>
Order	<i>Rhamnales</i>
Family	<i>Rhamnaceae</i>
Genus	<i>Alphitonia</i>
Species	<i>excelsa</i>

2.5.1. Description of *Alphitonia excelsa*

A. excelsa is a tall tree with a pale grey bark and tomentose young branches. The leaves are very characteristic, being dark green above and white and hairy beneath and are often seen to be ‘moth-eaten’ as a result of insect attack (Figure 2.5.1) (Lassak and McCarthy, 2008, Cribb and Cribb, 1984).



Figure 2.5.1: *A. excelsa* leaves.



Figure 2.5.2: Distribution of *A. excelsa* in Australia (ANBG, 2012).

2.5.2. Medicinal uses of *Alphitonia excelsa*

According to the interviews done by Brouwer and Packer (Brouwer, 2006, Packer, 2012), the local medicinal uses of *A. excelsa* by the Yaegl community are:

- The leaves have been used as a skin disinfectant or antiseptic detergent by Yaegl community people. For this purpose, a few leaves of the plant are rubbed between the hands with a little water (Packer *et al.*, 2012).
- Leaves have been used for fishing where throwing the crushed leaves into the water would make fish come floating up to the surface (Brouwer, 2006).

A. excelsa has also been reported to be used traditionally by other Aboriginal communities in a variety of remedies and treatments (Lassak and McCarthy, 2008, Cribb and Cribb, 1984, Low, 1990). The leaves of *A. excelsa* have been used for sore eyes and young leaves have been chewed in the case of an upset stomach. The bark, roots and wood have been used to make an infusion to rub on the body as a liniment for body pains. The bark and wood have also been employed to make a decoction for use as a gargle for toothache (Lassak and McCarthy, 2008). The crushed leaves have been used for the treatment of headache by bathing with the leaves in water and similarly to the Yaegl community, leaves have been used by other communities in the making of instant bush soap by crushing the leaves (Lassak and McCarthy, 2008, Cribb and Cribb, 1984, Barr *et al.*, 1990, Low, 1990).

The uses of *A. excelsa*, reported both in the literature and by the Yaegl community, suggested that antibacterial, anti-inflammatory and antioxidant compounds might be present in the plant.

2.5.3. Previous chemical studies on *Alphitonia excelsa*

Chemical analyses of different parts (wood, bark and leaves) of *A. excelsa* have shown that triterpenoid saponins have been found in all parts of *A. excelsa* (Lassak and McCarthy, 2008). Din *et al.* found a strongly positive result for the presence of saponins (Din *et al.*, 2002). Phytochemical screening by collaborators of the Northern Territory Aboriginal Pharmacopoeia, resulted in a very strong test for saponins in the leaves, a negative test for alkaloids in the leaves and bark, and a yield of 4% tannins in the bark (Barr *et al.*, 1990). Alphonin has been identified as one of the main compounds in the wood, ceanothic acid (emmollic acid) and betulinic acid have also been identified in the wood in smaller quantities. The bark of *A. excelsa* contains mainly betulinic acid and smaller amounts of alphitolic acid, betulin and alphitoxolide. The leaves contain ceanothic acid, betulinic acid, alphitolic acid and salicylic acid (Guise *et al.*, 1962, Branch *et al.*, 1972). Figure 2.5.3 shows the chemical structures of the compounds isolated from *A. excelsa* (Branch *et al.*, 1972).

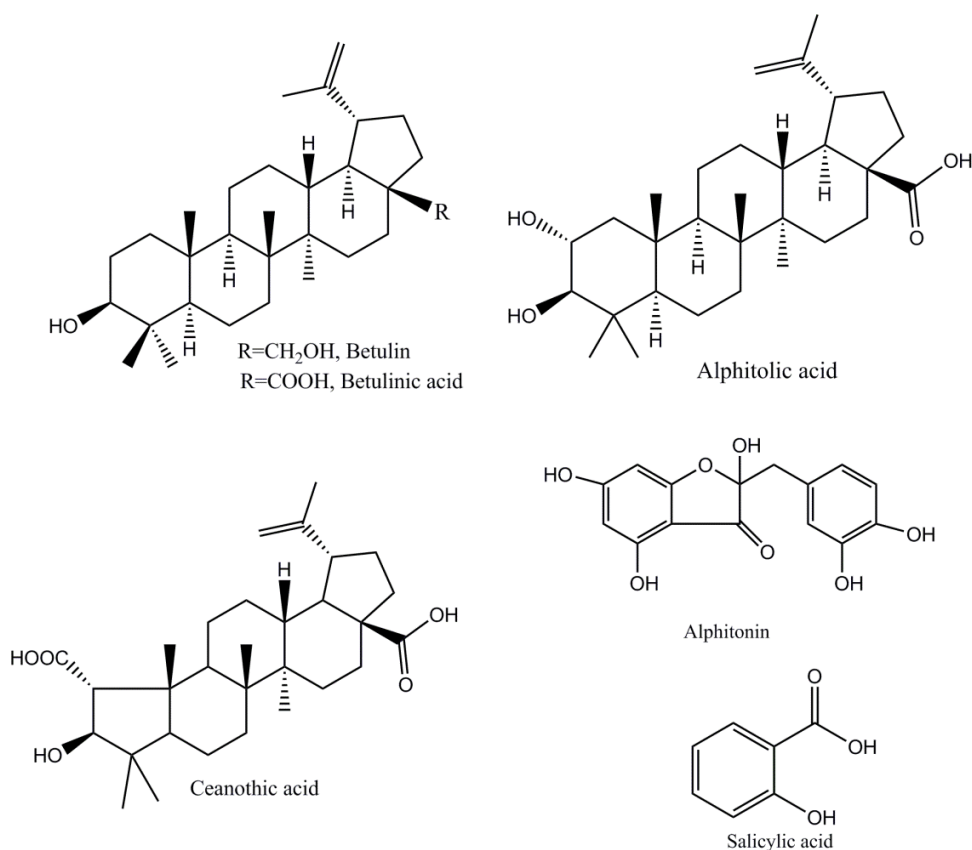


Figure 2.5.3: Compounds present in *A. excelsa*.

2.5.4. Previous biological studies on *Alphitonia excelsa*

Few biological studies have so far been reported on *A. excelsa*. Rogers *et al.* have found that the methanol extract of leaves of *A. excelsa* caused significant inhibition of platelet 5-HT release and inhibition of platelet aggregation (Rogers *et al.*, 2000). In another study an extract from the leaves and branches of *A. excelsa* was found to have mild xanthine oxidase inhibitory activity (5%). 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).

2.5.5. Previous chemical, biological studies and uses of other species of *Alphitonia*

As described in Section 2.5, three species of *Alphitonia* (*A. incana*, *A. petriei* and *A. zizyphoides*) have been reported with ethnomedicinal uses; others are used for timber. Although ethnomedicinal use of *A. philippinensis* could not be found in the literature, there is a report of isolation of important bioactive molecules from this plant. Table 2.5.1 shows a review of literature on chemical and biological studies of four *Alphitonia* species, *A. incana*, *A. petriei*, *A. zizyphoides* and *A. philippinensis*.

Table 2.5.1: Traditional uses and chemical and biological studies of some important species of *Alphitonia*

Different species of <i>Alphitonia</i>	Traditional uses	Chemical and Biological studies
<i>Alphitonia incana</i>	Used to treat bites of the death adder and the small-eyed snake (Mebs, 1999).	No chemical or biological activity studies reported.
<i>Alphitonia petriei</i>	<p>A decoction of the bark has been used externally for relief of body pains (Lassak and McCarthy, 2001).</p> <p>The tree has also been used to treat sores, boils or ringworm by burning the wood and mixing the ash with water to form a paste (Isaacs, 1987).</p>	<p>Alphitonin, ceanothic acid, betulinic acid, alphaltolic acid (Guise <i>et al.</i>, 1962), salicylic acid, lupeol (Branch <i>et al.</i>, 1972), 2-ketobetulonic acid and betulinic acid (Setzer <i>et al.</i>, 2004).</p> <p>CHCl₃ extract of <i>A. petriei</i> showed cytotoxicity against MDA-MB-231 (human mammary adenocarcinoma), MCF-7 (human mammary adenocarcinoma), and 5637 (human primary bladder carcinoma) cells. The extract also showed antibacterial activity against <i>Bacillus cereus</i> (Setzer <i>et al.</i>, 2004).</p>
<i>Alphitonia zizyphoides</i>	<p>Used to treat inflammation, gastro-intestinal and urogenital disorders in Samoan medicine (Dunstan <i>et al.</i>, 1997).</p> <p>Used as a tonic for internal distress (Cox, 1993).</p>	<p>The bark contains alphaltol (Dunstan <i>et al.</i>, 1998) and saponins, zizyphosides C, D and E (I-III) (Li <i>et al.</i>, 1994).</p> <p>Dried bark extract inhibited prostaglandin biosynthesis <i>in vitro</i> as well as the formation of experimentally induced ear oedema in rats (Dunstan <i>et al.</i>, 1997, Dunstan <i>et al.</i>, 1998).</p>
<i>Alphitonia philippinensis</i>		<p>Flavonol glycosides: isorhamnetin 3-<i>O</i>-(6''-<i>O</i>-(<i>Z</i>)-<i>p</i>-coumaroyl)-β-D-glucopyranoside, quercetin 3-<i>O</i>-α-L-rhamnopyranosyl-α-L-arabinopyranosyl-α-L-rhamnopyranoside and quercetin 3-<i>O</i>-α-L-arabinopyranosyl-α-L-rhamnopyranoside have been isolated.</p> <p>The isolated flavonoid glycosides showed cytotoxicity against PC-3 cells and hepatoma HA22T cells and inhibition of replication on herpes simplex virus type-1 (Jou <i>et al.</i>, 2004).</p>

2.6. Selection of methods for biological testing of extracts and for isolation of bioactive compounds

As discussed in Chapter 1, microbial infections, inflammation and lower antioxidant levels at wound sites are the major conditions associated with chronic wounds and infection, which are a global healthcare concern. It is vital to discover new antimicrobial, anti-inflammatory and antioxidant compounds to combat these. Therefore, *A. excelsa* and *L. suaveolens* were chosen to be examined for antimicrobial, anti-inflammatory and antioxidant activities (Chapters 3 and 4). For leaves of *A. excelsa*, this is directly aligned with their medicinal uses by the Yaegl community, while the finding of such activity for *L. suaveolens* leaves could provide a more accessible source of medicine from *L. suaveolens* for the community.

To examine antimicrobial activity, three assay methods were chosen, *i.e.* disc diffusion, MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide] microdilution and TLC bioautography. The COX inhibitory assay, nitric oxide inhibition assay, TNF- α assay and PGE₂ assays were selected to evaluate anti-inflammatory activities and the ORAC (oxygen radical absorbance capacity) assay to evaluate antioxidant activity. A combination of different antimicrobial and anti-inflammatory assays were regarded as necessary in the present study to confirm the bioactivities and to overcome the limitations of each method. A summary of each of these assays is provided below.

2.6.1.1. Antimicrobial activity study

2.6.1.1.1. Disc diffusion assay

The disc diffusion assay is a simple and still widely used method for testing antimicrobial activity. In this method small filter paper discs impregnated with the compound or extract to be tested are placed on the surface of a suitable agar plate that has been inoculated with the microorganism under investigation. After incubation, the presence of a zone of inhibition in the growth of microorganism around the filter disc indicates the effect of the tested sample (Maidment *et al.*, 2006). While being a very simple method, the disc diffusion assay is not effective for non polar or large molecules as this method relies on the diffusion of the active component into the agar (Barry and Thornsberry, 1991). The disc diffusion assay also requires moderate amounts of sample for testing, which is not always possible to obtain when investigating natural products. Determination of the minimum inhibitory concentrations (MIC)

is also not possible in the disc diffusion assay. Therefore a combination of antimicrobial assays has been performed in this PhD study.

2.6.1.1.2. *MTT microdilution assay*

The MTT microdilution assay is a popular method for determining MICs of extracts and pure compounds. It is a rapid, low cost and reliable colourimetric assay that can be conducted in a microtitre plate for ease of handling many samples. It is based on the tetrazolium salt 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT), which changes from yellow to the blue formazan upon reductive cleavage by reductase enzymes of living cells (Martin *et al.*, 2005) (Figure 2.6.1). If a sample or compound to be tested has antimicrobial activity, MTT will not be converted into MTT formazan (blue colour) and the MIC can be determined as the lowest concentration where no growth is observed (yellow colour).

Although the MTT microdilution assay is a useful method for obtaining quantitative data for MIC determination, plant extracts may interfere with the colourimetric analysis. It is also dependent on the solubility of the test samples in a predominantly aqueous medium. Therefore, the disc diffusion assay and TLC bioautography assays (see below) were also used in this study to test the antimicrobial activity of extracts/fractions/and pure compounds.

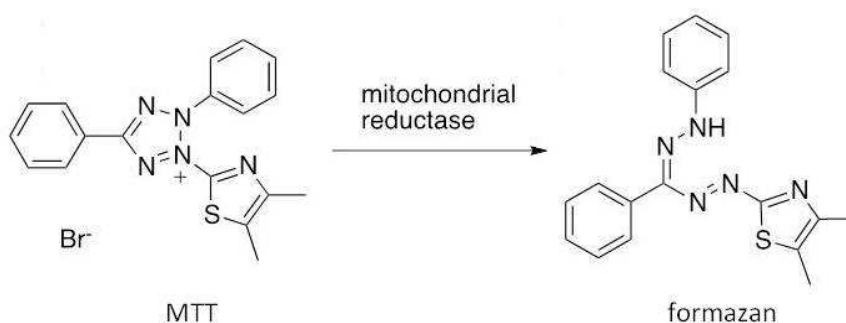


Figure 2.6.1: MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide) (yellow) is converted to MTT formazan (blue) in the presence of live bacteria or yeast.

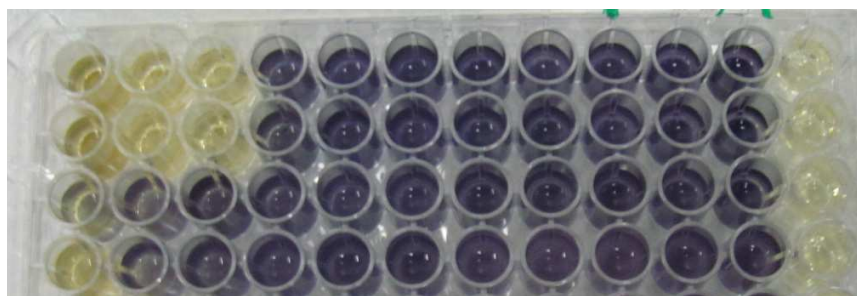


Figure 2.6.2: MTT microdilution assay plate. MIC was determined as the well with the lowest concentration of sample that displayed no yellow-blue colour change of MTT.

2.6.1.1.3. *TLC bioautography*

TLC bioautography uses the simplicity and ability of TLC to separate mixtures quickly with little expense, to allow the detection of bioactive components on a TLC plate. It has proved exceptionally popular owing to its ease of use, cost, rapidity, and ability to assess antimicrobial activity of a large number of samples (Gibbons, 2005). This method is particularly useful for bioassay guided isolation of bioactive compounds and gives an idea of which compound on a TLC plate to target for isolation and identification. This method is also useful for determining antimicrobial activity of samples that are not soluble in the predominantly aqueous environment of the MTT microdilution assay. In this assay, TLC plates are run with the samples in an appropriate solvent system. After drying the solvents, the plate is placed on an agar plate and covered with a growth medium containing the microorganism. After incubation, a detecting agent can be added. For this study, MTT was chosen due to its ability to convert MTT (colourless/yellow) into MTT formazan (blue) in the presence of reductase enzymes of microbes. Therefore, a clear spot on the TLC plate indicates where MTT is not converted into MTT formazan and that the components at that R_f have antibacterial activity.



Figure 2.6.3: A simple TLC bioautography assay.

2.6.1.2. **Anti-inflammatory activity study**

As already discussed in Chapter 1, inflammation is a complex biochemical process of the human body (Section 1.5.2). Several chemical species such as eicosanoids (prostaglandins and leukotrienes), chemokines, cytokines, kinins, amines and proinflammatory mediators nitric oxide (NO), tumor necrosis factor alpha (TNF- α) and necrosis factor kappa B (NF- κ B) are involved in mediating and regulating the response (Praveen and Knaus, 2008, Ward, 2008, Tripathi *et al.*, 2007). Therefore, several methods have been developed to inhibit these mediators. The most recognised and widely used *in vitro* anti-inflammatory assay methods involve measurement of the inhibition of NO, TNF- α , NF- κ B, interleukin 6 (IL-6), prostaglandin E₂ (PGE₂) and cyclooxygenase (COX) enzymes (Shou *et al.*, 2012, Yuan *et al.*, 2013, Park, 2013, Choodej *et al.*, 2013, Ruangnoo *et al.*, 2012, Zhang *et al.*, 2011).

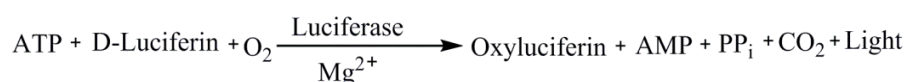
This study, *in vitro* anti-inflammatory activity was examined by measuring the production of nitric oxide (NO) and TNF- α in RAW264 murine macrophages, PGE₂ production in 3T3 Swiss albino fibroblast cells and by measuring inhibition of cyclooxygenase enzymes (COX 1 and 2).

2.6.1.2.1. *Cytotoxicity assay (ATPlite™ assay)*

In vitro cytotoxicity assays provide a means of establishing whether a test drug, extract or compound is toxic to cells in culture. By measuring an indicator of metabolic activity such as adenosine triphosphate (ATP), the number of viable cells remaining after a defined incubation

period can be determined (Riss *et al.*, 2011). ATP concentration is a valid marker of cell viability and the ATPlite™ assay is extremely sensitive, detecting as few as 10 cells in a well of a microtitre plate (Germain *et al.*, 2003). Proliferating cells express high amounts of ATP, but cell death by necrosis or apoptosis causes a reduction of ATP (Germain *et al.*, 2003). By investigating a range of concentrations, the IC₅₀ (the concentration that causes 50% inhibition) can be determined (Vlachy *et al.*, 2009).

The ATP assay system is based on the production of light caused by the reaction of ATP with added luciferase and D-luciferin. Luciferin is converted by MgATP²⁺ to a form that can be catalytically oxidised by luciferase. Then, cellular ATP can be measured by direct lysis of cells. The ATP concentration is directly proportional to the emitted light. This is illustrated in the following reaction scheme:



2.6.1.2.2. Nitric oxide (NO) inhibition assay

Nitric oxide plays an important role in normal wound healing. However, excess production in the wound environment can increase the susceptibility of wounds and infection and prolonged inflammation (Schulz and Stechmiller, 2006). Cells of the innate immune system, macrophages, neutrophils and natural killer cells, release NO to inhibit replication of pathogens (Tripathi *et al.*, 2007). Increased production of NO is an outcome often seen in chronic wounds and has been associated with recurrent ulcers in diabetic patients (Blakytyn and Jude, 2006). Therefore, any extract or compound that inhibits the production of NO may have potential therapeutic value in the treatment of wounds and inflammation.

The effect of plant extracts and isolated pure compounds on NO production in lipopolysachharide (LPS) activated RAW264 cells were evaluated in the present study. The basic principle of this assay can be described as follows:

Addition of LPS induces the production of NO in RAW264 cells. NO is readily metabolised to nitrite (NO₂⁻), which can be measured by the Griess reaction (Figure 2.6.4). In this reaction, nitrite reacts under acidic conditions quantitatively with sulfanilic acid to form a diazonium

hydrochloric acid) is added. The intensity of the colour measured (450 nm) is in proportion to the amount of mouse TNF- α bound in the initial step.

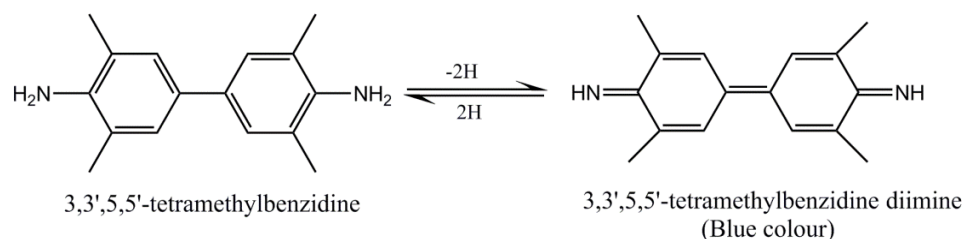


Figure 2.6.5: Oxidation of 3,3',5,5'-tetramethylbenzidine to 3,3',5,5'-tetramethylbenzidine diimine.

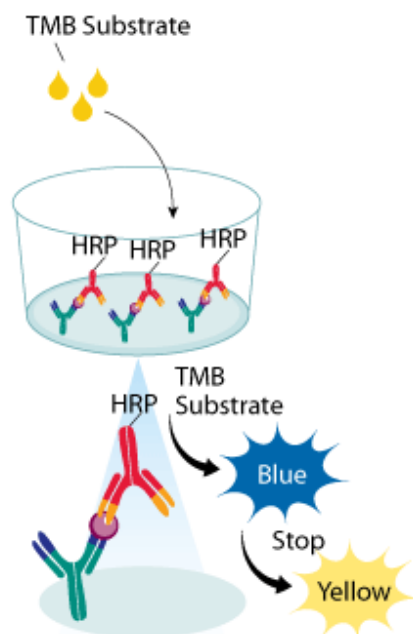


Figure 2.6.6: Principle of TNF- α assay. Source: (R&D Systems, 2013).

2.6.1.2.4. *Inhibition of PGE₂*

Prostaglandins are one of the critical inflammatory mediators that lead to increased vascular permeability and increased vasodilation in the inflammatory site and cause swelling and increased sensitivity to pain (Williams and Peck, 1977). PGE₂ inhibition can be assayed in the presence of test samples and controls using a PGE₂ enzyme immunoassay (EIA) assay kit.

The stimulation of 3T3 Swiss albino mouse embryonic fibroblast cells with a calcium ionophore induces the production of PGE₂. The PGE₂ can be measured quantitatively using a

commercially available prostaglandin E₂ EIA kit. This assay is based on the competition between PGE₂ and a PGE₂-acetylcholinesterase (AChE) conjugate (known as PGE₂ tracer) for a limited amount of monoclonal antibody. The amount of PGE₂ tracer that is able to bind to the PGE₂ monoclonal antibody will be inversely proportional to the concentration of PGE₂ in the well. This antibody-PGE₂ complex binds to goat polyclonal anti-mouse IgG that has been previously attached to the well. The plate is washed to remove any unbound reagents and after that Ellman's reagent (composed of acetylthiocholine and 5,5'-dithio-bis(2-nitrobenzoic acid) [DTNB]) is added to the well. The bound acetylcholine esterase reacts with acetylthiocholine to form thiocholine, which further reacts with DTNB to form thionitrobenzoic acid which is yellow in colour and can be measured quantitatively at 412 nm. The intensity of the colour is proportional to the amount of PGE₂ tracer bound to the well, which is inversely proportional to the amount of free PGE₂ present in the well. The reaction scheme for the conversion of acetylthiocholine into thionitrobenzoic acid is shown in Figure 2.6.7.

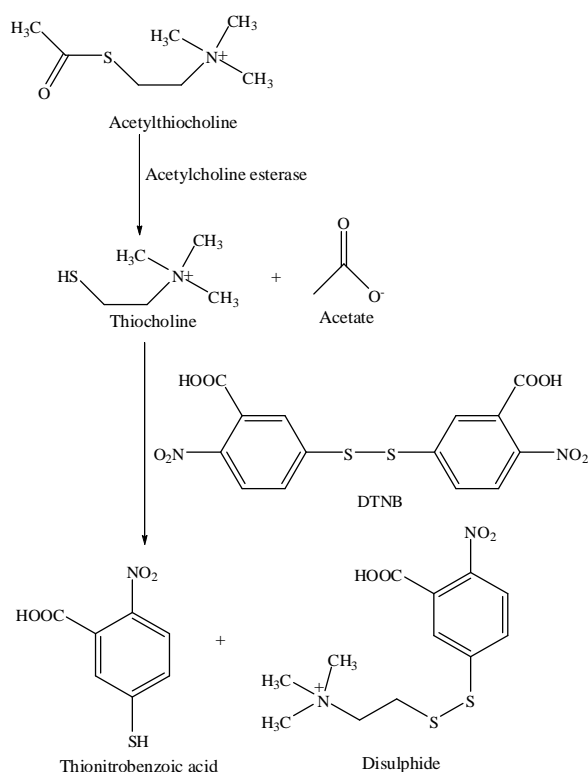


Figure 2.6.7: Reaction for conversion of acetylthiocholine into thionitrobenzoic acid.

2.6.1.2.5. Cyclooxygenase (COX) inhibitor screening assay

As already discussed in Chapter 1, prostaglandins and leukotrienes are the important inflammatory mediators that lead to increased vascular permeability and increased vasodilation in the inflammatory site and cause swelling and increase the sensitivity to pain (Williams and Peck, 1977). Arachidonic acid is the precursor for prostaglandins and leukotrienes (Irvine, 1982). The first step in the synthesis of prostaglandins is catalysed by the COX enzyme, which converts arachidonic acid into prostaglandin H_2 which is a common substrate for specific prostaglandin synthesis (Vane *et al.*, 1998). Any substance that inhibits the COX enzyme is considered to have anti-inflammatory activity.

Commercially available COX inhibitor screening assay kits are used to assess COX inhibition. In this assay, COX 1 and COX 2 are treated with the test samples and then allowed to react with arachidonic acid. The amount of prostaglandin H_2 (PGH_2) formed in this reaction depends on the enzyme inhibitory activity of the test sample. The PGH_2 formed is reduced by $SnCl_2$ to the more stable compound prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$). The amount of $PGF_{2\alpha}$ produced by the treated COX 1 or COX 2 is quantified by the enzyme immunoassay (Figure 2.6.8) (Maclouf *et al.*, 1988) and is compared to that of the untreated COX enzymes. This enzyme immunoassay is based on the competitive affinities of $PGF_{2\alpha}$ and a PG acetyl cholinesterase conjugate (PG tracer) for a limited amount of PG antiserum. The concentration of the PG tracer is maintained constant whereas the concentration of $PGF_{2\alpha}$ will vary depending upon the COX inhibitory activity. Hence, the amount of PG tracer that can bind to PG antiserum is inversely proportional to the amount of $PGF_{2\alpha}$ formed. The antibody-PG complex formed in the wells binds to a mouse anti-rabbit monoclonal antibody that is coated inside the wells. The plate is washed to remove all unbound $PGF_{2\alpha}$ and PG tracer reagents and then Ellman's reagent is added to the well. The conversion of acetylthiocholine (component of Ellman's reagent) to thionitrobenzoic acid (yellow colour) is similar to that of the PGE_2 assay (Section 2.6.1.2.4) and the amount of thionitrobenzoic acid formed (yellow colour) is directly proportional to the amount of PG tracer bound to the well, which is inversely proportional to the amount of PG formed, *i.e.* a higher inhibitory activity, more intense yellow colour and vice versa.

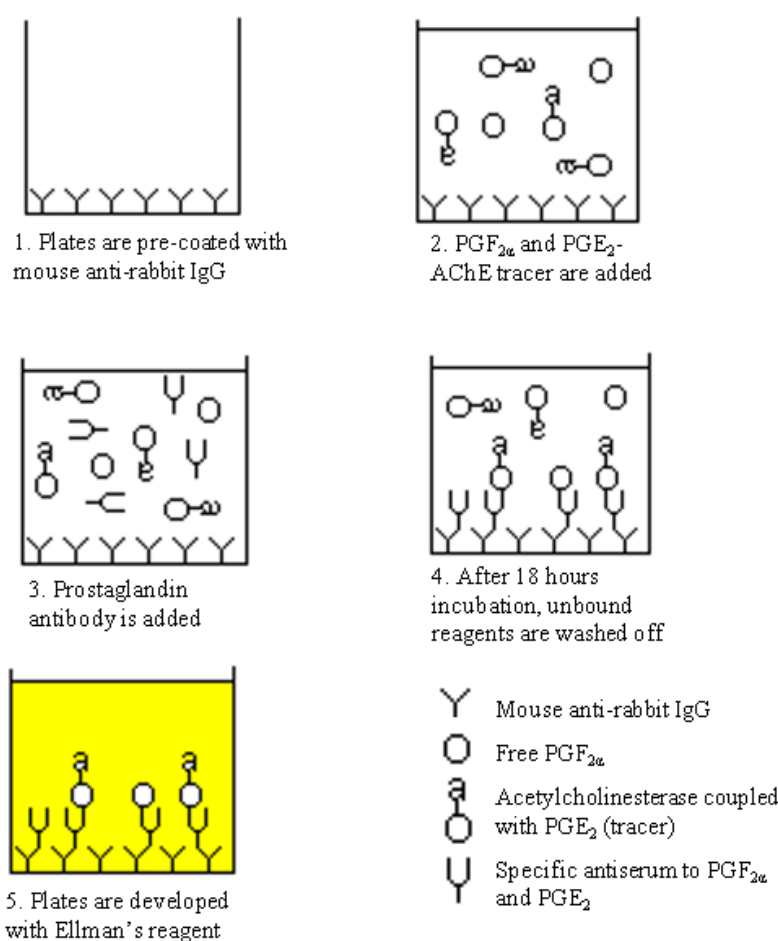


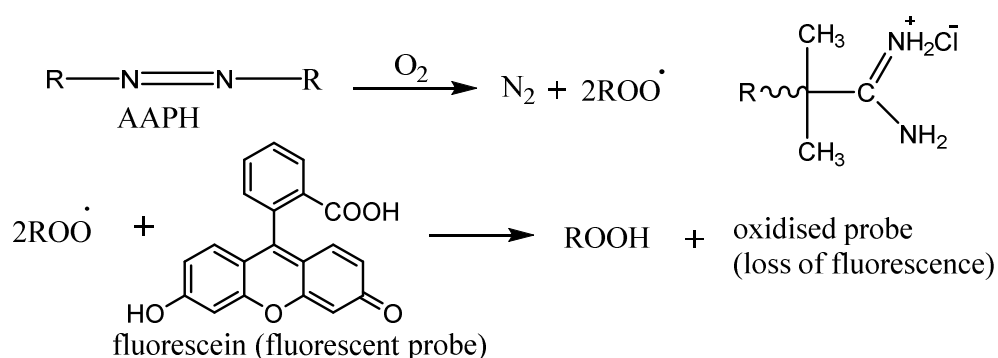
Figure 2.6.8: Enzyme immunoassay in the COX inhibitor screening assay.

2.6.1.3. Antioxidant assay (ORAC assay)

Chronic wound conditions are often accompanied by low antioxidant levels and increased markers of free radical damage (Lee *et al.*, 2010). Different methods have been used to measure the antioxidant capacity of foods, pharmaceuticals or natural products, for example: oxygen radical absorbance capacity (ORAC) assay, Folin-Ciocalteu method, DPPH (2,2-diphenyl-1-picrylhydrazyl) radical scavenging activity assay and the ABTS [2,2'-azinobis (3-ethylbenzothiazoline-6-sulfonic acid)] method (Prior *et al.*, 2005, Djeridane *et al.*, 2006, Moyo *et al.*, 2010). The Folin-Ciocalteu assay is based on the transfer of electrons in alkaline medium from phenolic compounds to phosphomolybdic/phosphotungstic acid complexes, which are determined spectroscopically at 765 nm (Ainsworth and Gillespie, 2007). The DPPH assay method is based on the scavenging of the stable DPPH by an antioxidant. The

absorbance of the radical is in the range of 515-520 nm (Noipa *et al.*, 2011). The ABTS method is based on the scavenging ability of the long-life radical anion ABTS. ABTS is oxidised to its radical cation, which is intensely coloured and the antioxidant capacity is measured as the ability of the test compounds to decrease the colour reacting directly with the ABTS radical (Prior *et al.*, 2005).

The ORAC assay is the most widely used and recognised method of measuring antioxidant capacity. It involves a hydrogen atom transfer reaction mechanism, which is most relevant to human biology (Prior *et al.*, 2005, Shou *et al.*, 2012, Gillespie *et al.*, 2007). This assay measures the antioxidant activity of the test samples against peroxy radicals induced by 2,2'-azobis-2-methyl-propanimidamide dihydrochloride (AAPH) at 37 °C, with fluorescein used as the fluorescent probe. The antioxidant potential is determined from the area under the curve (AUC) of fluorescence versus time. The fluorescence signal is decreased by the addition of the reactive oxygen species (ROS) generator AAPH.



Antioxidants are considered to protect the fluorescent molecule from oxidative degeneration. The degree of protection is measured using a fluorometer. The degeneration of fluorescein is measured as the presence of the antioxidant slows the fluorescence decay. Decay curves (fluorescence intensity vs time) (Figure 2.6.9) are recorded and the difference in area between the two decay curves (with or without antioxidant) is calculated. The degree of protection is quantified using the antioxidant trolox (a water soluble analogue of vitamin E) as a standard. Different concentrations of trolox are used to make a standard curve and samples are compared to this. Results for the test samples have been published as 'trolox equivalents' or TEs (Huang *et al.*, 2005, Garrett *et al.*, 2010).

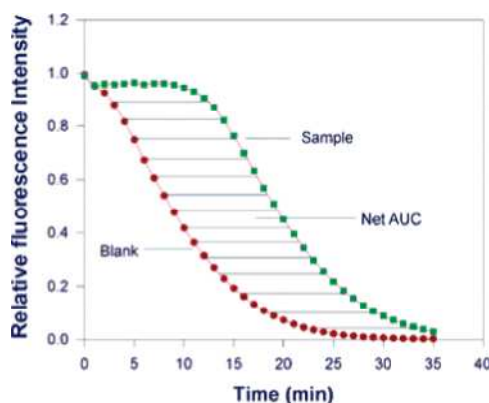


Figure 2.6.9: ORAC antioxidant activity of tested sample expressed as the net area under the curve (AUC). Source: (Prior *et al.*, 2005).

2.6.2. Selection of methods for chemical studies

For bioassay guided chemical studies, chromatographic procedures including normal and reversed phase and size exclusion chromatography were selected to isolate bioactive compounds. Structures of the isolated compounds were elucidated by 1D and 2D NMR, mass spectrometry, UV and IR.

2.7. Concluding remarks

The aim of the PhD study was to isolate and indentify antimicrobial, anti-inflammatory and antioxidant activity and bioactive compounds from medicinal plants used by the Yaegl Aboriginal community of NSW. Two potential medicinal plants were selected, guided by the Yaegl elders knowledge and desires for further investigation, a review of the literature and preliminary biological screening. A combination of well recognised antibacterial, anti-inflammatory and antioxidant assay methods were selected to test the antibacterial, anti-inflammatory and antioxidant potential of these plants. Likewise, a range of modern chemical study methods were selected to isolate the bioactive compounds. Chapters 3 and 4 describe the chemical and biological studies on *Lophostemon suaveolens* and *Alphitonia excelsa*, respectively.

CHAPTER THREE

Chemical and biological studies on *Lophostemon* *suaveolens*

This chapter describes the studies carried out with extracts of Lophostemon suaveolens leaves to determine antimicrobial, anti-inflammatory and antioxidant properties and to isolate and identify bioactive fractions and molecules.

3.1. Introduction

As introduced in Chapter 2, *Lophostemon suaveolens* (apple gum) is a plant endemic to Australia, distributed on the eastern coast, including in northern NSW. It is regarded as an important medicinal plant of the Yaegl community, with first-hand accounts of the bark ash, sap and latex having been customarily used as an antiseptic. There have been no reports of biological activity studies for any part of this plant in the published literature and only one chemical study examining the volatile oil composition of the leaves (Brophy *et al.*, 2000). Following requests of the Yaegl elders for *L. suaveolens* leaves to be collected and tested, preliminary screening assays (Packer, 2012) (Chapter 2, Section 2.1) of the leaf extracts showed potent activity against *S. aureus*, with some activity also observed for *E. coli* and *P. aeruginosa*. Therefore, leaves of *L. suaveolens* were chosen for further antibacterial screening, along with anti-inflammatory and antioxidant studies, and investigations of the bioactive constituents aligned with these activities. Determining whether the leaves have any of these biological properties, which are relevant to application for skin infections, sores and wounds, was regarded as valuable because positive findings could provide a more accessible source of medicine (*i.e.* leaves, rather than the sap) from *L. suaveolens* for the Yaegl community. These investigations would also add to the scientific knowledge of this important Australian plant.

This chapter describes the antibacterial, anti-inflammatory and antioxidant activities of the leaves of *L. suaveolens* and identification, isolation and characterisation of bioactive constituents.

3.2. Experimental

3.2.1. General experimental procedures

All the solvents used for extraction and chromatographic separations were of analytical HPLC grade. Analytical normal phase thin layer chromatography (TLC) was performed on fluorescent Merck silica gel F₂₅₄ plates (Germany) and reversed phase TLC on Merck Silica gel 60 RP-18 F_{254s} plates. Size exclusion chromatography (SEC) was carried out using Sephadex LH-20 (Sigma-Aldrich). The TLC plates were visualised using UV light (254 nm and 365 nm) and different spray reagents. Reversed phase (C₁₈) solid phase extraction was

carried out using Waters Sep Pak Vac 35cc (2g or 10 g) cartridges. Preparative TLC (PTLC) was carried out using Uniplate preparative TLC plates (Sigma-Aldrich). The ^1H , ^{13}C , HSQC, COSY and HMBC NMR spectra were recorded on a Bruker Avance AMX 400 and Bruker DRX600K 600 MHz NMR Spectrometer (Germany) using standard pulse sequences. Chemical shifts were calculated relative to the chloroform (^1H δ 7.24 and ^{13}C δ 77.2), acetone (^1H δ 2.09 and ^{13}C δ 205.8 and 30.6), methanol (^1H δ 3.31 and ^{13}C δ 49.0) and DMSO (^1H δ 2.50 and ^{13}C δ 39.51) solvent signals. A Shimadzu LC 10 AVP HPLC system was used for chromatographic separations and a Shimadzu 2010 LC-MS system was used for electrospray ionisation mass spectrometry (ESI MS) analysis. A Shimadzu GC-17 system was used for electron impact mass spectrometry (EIMS) analysis. A Stuart Scientific melting point detector (UK) was used for determining melting points. High resolution mass spectrometry (HRMS) was determined using a Bruker Apex 3 instrument. Betulinic acid (90%) was purchased from Sigma-Aldrich.

3.2.2. Plant material

Fresh mature leaves of *Lophostemon suaveolens* were collected by the author with the help of IBRG ethnobotanist Mr David Harington from the corner of Angourie road and Deering street, Yamba, NSW 2464 (29°26'32.85", 153°21'06.23") on May 2011. A voucher specimen was lodged at the Macquarie University Herbarium and identified by Mrs Alison Downing (voucher specimen number 73009156).

3.2.3. Preparation of extracts

Freshly collected leaves of *L. suaveolens* were chopped with a Waring heavy duty blender (John Morris scientific) to give coarse plant material suitable for extraction. Fresh plant material was extracted separately with water (method 1) and into four different fractions using solvents of increasing polarity (sequential solvent extraction, method 2).

Method 1 (preparation of water extract): Freshly chopped *L. suaveolens* leaves (100 g) were extracted with water (3x300 mL) at 25 °C for 24±1 h with agitation. After every 24 h, the extracts were decanted and filtered *in vacuo* through Whatman No. 1 filter paper (Whatman, UK) and the residual solid plant material further extracted in water (for a total of three extractions). The filtered extracts were combined, concentrated using a Buchi rotary evaporator (Germany) with a water bath at 40-42 °C and the residue subsequently freeze dried

(Labconco (USA) freeze dryer) to remove the remaining water. This provided the water extract LS-water (yellowish green solid, 2.03 g, 2.03% w/w).

Method 2 (preparation of sequential solvent extracts): Freshly chopped *L. suaveolens* leaves were sequentially extracted with *n*-hexane, dichloromethane (DCM), ethyl acetate (EtOAc) and methanol (MeOH). In brief, *L. suaveolens* leaves (1003 g) were extracted with *n*-hexane (3x2 L) at 25 °C with agitation (80 rpm) for 24±1 h. After every 24 h, the extracts were decanted and filtered *in vacuo* through Whatman No. 1 filter paper (Whatman, UK). The residual solid plant material was then extracted in an identical manner with DCM, followed by EtOAc and MeOH. The filtered extracts of the same solvent system were combined, concentrated using a Buchi Rotary evaporator (Germany) with a water bath at 40-42 °C and the residues subsequently freeze dried to remove the residual water. This afforded the *n*-hexane, DCM, EtOAc and MeOH extracts LS-Hex (yellow oil, 9.42 g, 0.9% w/w), LS-DCM (dark green solid, 24.08 g, 2.4% w/w), LS-EA (dark green solid, 16.51 g, 1.6% w/w) and LS-MeOH (blackish green solid, 32.76 g, 3.2% w/w), respectively.

Long term storage of all the dried extracts was done in a -20 °C freezer.

3.2.4. Bioassays: methods and materials

3.2.4.1. Selection of microorganisms for antibacterial study

Extracts, fractions and pure compounds isolated from *L. suaveolens* were assayed for antibacterial activity against a number of pathogenic Gram-positive and Gram-negative bacteria (Table 3.2.1).

The use of all microbial strains (Table 3.2.1) was approved by the Macquarie University Biosafety Committee (Approval References 08/06/LAB, TAN180512BHA). All cultures were kindly provided by Dr John Merlino (Department of Microbiology, Concord Hospital, Sydney). The inoculum sizes (cfu/mL) at optical density 0.08 (λ 600 nm) were estimated using the spread plate colony count (Willey *et al.*, 2011) (Table 3.2.1). In this technique 0.1 mL of bacterial suspension was placed in the centre of agar plate. Streaking was performed using a sterilised bent glass rod back and forth across the plate. Streaking was also performed by turning the plate 90 and 45 degrees. The plate was covered and was placed in an incubator for incubation. Colonies were counted using a plate counter.

Table 3.2.1: Bacterial strains used in antibacterial screening assays.

Organism	Strain ^a	Characteristics	cfu/mL (A ₆₀₀ = 0.08) ^b
<i>Escherichia coli</i> (β-)	ATCC 25922	β lactamase negative – sensitive to common antibiotics	2.54 x 10 ⁷
<i>Escherichia coli</i> (β+)	ATCC 35218	β lactamase positive	5.32 x 10 ⁷
<i>Pseudomonas aeruginosa</i>	ATCC 27853	sensitive to common antibiotics	6.97 x 10 ⁷
<i>Salmonella</i> ser. Typhimurium		clinical isolate	8.59 x 10 ⁷
<i>Streptococcus pyogenes</i>		clinical isolate	3.70 x 10 ⁷
<i>Staphylococcus aureus</i> (MS)	ATCC 29213	sensitive to common antibiotics	9.62 x 10 ⁷
<i>Staphylococcus aureus</i> (CMR)	ATCC BAA 1026	community acquired methicillin resistant <i>S. aureus</i> (MRSA)	6.38 x 10 ⁷
<i>Staphylococcus aureus</i> (MDR)		wild multidrug resistant (MDRSA) clinical isolate	3.76 x 10 ⁸

^aAmerican Type Culture Collection strain designation where applicable. ^bThe inoculum sizes (cfu/mL) at optical density 0.08 (λ 600 nm) were estimated using the spread plate colony count (Willey *et al.*, 2011). These cell counts were used for the agar based disc diffusion assay and diluted one hundred fold for use in the microdilution assays.

3.2.4.2. Disc diffusion assay for antibacterial activity

Dried plant extracts and antibiotics were dissolved in 20% DMSO/H₂O to get a concentration of 100 mg/mL for extracts and 0.1 mg/mL for a suitable antibiotic [vancomycin (Amresco, Ohio) for Gram-positive bacteria and gentamycin (Amresco, Ohio) for Gram-negative bacteria]. Filter sterilisation of solutions was performed by passing through 0.45 µm sterilisation filters (Sartorius Stedim Biotech, Göttingen). 20 µL of plant extract or antibiotic was applied on sterile discs (6 mm) (Whatman, Maidstone) resulting in a final concentration of 2 µg of antibiotic or 2 mg of plant extract per disc. Negative control discs were also prepared as above with 20 µL of 20% DMSO/H₂O. All discs were dried for half an hour after application of the material. All tests were performed in duplicate.

Overnight cultures of organisms in Mueller Hinton II (MHII) broth (Bacto Laboratories Pty Ltd) were swabbed over MHII agar (for all tested bacteria except *S. pyogenes*) or horse blood agar (only for *S. pyogenes*), dried discs were placed on the agar plates and the plates were incubated overnight at 37 °C. Antibacterial activity was evaluated by measuring the diameter (including 6 mm disc) of the zone of inhibition.

3.2.4.3. TLC bioautography

TLC bioautography was conducted based on the method by Rahalison (Rahalison *et al.*, 1991), with minor modifications. Normal phase silica gel F₂₅₄ aluminium backed TLC plates were UV sterilised for half an hour. The samples (50-100 µg) were spotted on the TLC plates with appropriate solvent systems in aseptic conditions in a biosafety cabinet. 5 µg of antibiotic was also spotted on the same TLC plate as a positive control and a solvent spot acted as a negative control. The chromatograms were dried with a hair dryer for complete removal of solvents. All TLC plates were run in duplicate, one of them being used as the reference chromatogram. Compounds visible under UV light (254 nm) and by spraying were detected and recorded on the reference chromatogram. The duplicate chromatograms were placed on agar plates (12×12 cm) with the silica gel facing upwards. An inoculum of bacteria (methicillin sensitive and resistant strains of *S. aureus*) was prepared with MHII broth by overnight incubation. After incubation, optical density (OD) of the culture was measured and was adjusted to 0.08 by diluting with the molten MHII agar (40-45 °C). Approximately 20 mL (depending on the size of the TLC plate) of the inoculum was rapidly distributed over the TLC plate (10×10 cm). After solidification of the medium, the agar plate with TLC plate was incubated overnight at 37 °C. The bioautogram was covered with 1-2 mL methanolic solution (2.5 mg/mL) of MTT (methyl thiazolyl tetrazolium bromide) (from Sigma-Aldrich) by gently adding the MTT solution with a sterile micropipette, and then incubated for 1 h at 37 °C. Antibacterial activity was observed as a clear zone, against a purple background.

3.2.4.4. MTT microdilution (turbidity and MTT) assay

The microdilution assay was primarily based on the method outlined by Appendino (Appendino *et al.*, 2008) with minor modifications done in our laboratory (Packer, 2012). Dried plant extracts were dissolved in 20% DMSO/H₂O to make a concentration of 10 mg/mL for each extract and 1 mg/mL for a suitable antibiotic (vancomycin for Gram-positive bacteria and gentamycin for Gram-negative bacteria). Using a clear, flat-bottom 96-well microtitre plate (well volume 340 µL), 20 µL of MHII broth was dispensed into wells 2-11, 40 µL of the filter sterilised test sample or appropriate antibiotic was dispensed into well 1 and serially diluted across the plate, omitting well 11 for the growth control. The final volume (20 µL) was dispensed into well 12, which, being free of bacteria, served as the sterile control. 175 µL of inocula ($A_{600} = 0.08$ diluted 1/100 in MHII broth) were added to wells 1-11. 175 µL of sterile

medium was added to well 12. A DMSO control was also included. The plate was read at A_{600} to control for pre-incubation turbidity of the samples and the plates were incubated for 18 h at 37 °C. After this step, the procedure was different for the turbidity and MTT assays.

- a) For turbidity, absorbance of the microtitre plate after 18 h incubation was measured at 600 nm using a microplate reader (Spectramax). The percentage of antimicrobial activity (IC_{90}) was determined using the following equation:

$$\text{Inhibition (\%)} = [1 - (A_{\text{sample}} - A_{\text{blank}}) / \text{average } A_{\text{culture control}}] * 100$$

- b) For the MTT assay, after the 18 h incubation 5 μ L of a methanolic solution (5 mg/mL) of MTT was added to each well and the plate was further incubated at 37 °C for 1 h to detect the bacterial growth. A blue colour in the wells meant bacterial growth and yellow or no colour meant no bacterial growth. The minimum inhibitory concentration (MIC) values were defined as the lowest concentration of the test samples that inhibited the visible growth of microorganisms (first well in decreasing concentration with no blue colour).

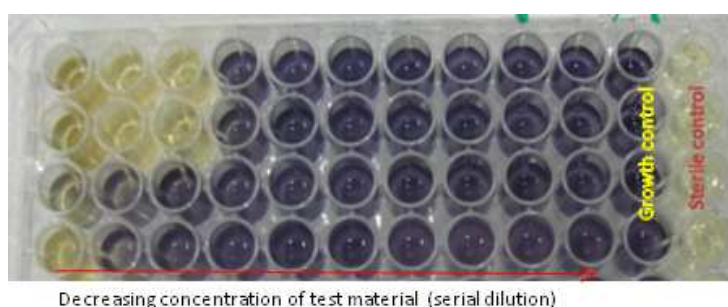


Figure 3.2.1: MTT microdilution assay plate. MIC is shown as the lowest concentration of the test sample that inhibited growth (yellow well) of organism.

The absorbance of the working cultures was read using a UV-Mini 1240 UV-Vis Spectrophotometer (Shimadzu). Filter sterilisation of solutions was performed by passing through 0.45 μ m sterilisation filters (Sartorius Stedim Biotech, Göttingen).

c) Bactericidal and bacteriostatic effect determination

Plant extracts having antibacterial activity as determined by the MTT assay were assessed for their bacteriostatic or bactericidal properties by subculturing onto fresh agar after the assay (Karaman *et al.*, 2003). 5 μ L from wells of interest were taken from the MTT test plates and spotted onto MHII agar plates. Plates were inverted and incubated overnight at 37 °C. Aliquots taken from yellow (no growth) wells of the MTT assay, exhibiting microbial growth after subculture, were considered to result from the plant extracts' bacteriostatic activity. Aliquots that did not produce colonies were deemed to have been affected by the plant extract by a bactericidal mechanism. This assay was generally performed on at least two separate occasions and an average was taken of the minimum bactericidal concentration (MBC), as the last well showing no further growth.

3.2.4.5. Anti-inflammatory and antioxidant assays

3.2.4.5.1. Cytotoxicity assay

Cytotoxicity in RAW264 murine leukemic monocyte macrophages (ATCC, Manassas, VA, USA) was assayed in 96-well plates using the ATP-lite assay kit (PerkinElmer, Glen Waverly, Australia). The well known cytotoxic drug, chlorambucil (Sigma C0253) (Habtemariam, 1995), was used as a positive control. Cells were grown in clear 96-well microtitre plates. The growth medium consisted of colour free Dulbecco's modified Eagle's medium containing 10% (v/v) foetal bovine serum (FBS; Interpath, Heidelberg, Australia), L-glutamine (2 mM), sodium pyruvate (1 mM), penicillin (200 U/mL) and streptomycin (200 μ g/mL) (all from Invitrogen, Mulgrave, Australia). Cells were plated at a concentration of 30,000 cells/well (90 μ L of cell suspension/well). Test and control compounds were dissolved in DMSO at six concentrations and further diluted 20-fold in the medium. These were added to the cell suspension at 10 μ L/well. Initial concentration for extracts and fractions was 20 mg/mL and for pure compound was 2.5 mg/mL, leading to concentrations of 100, 33.3, 11.1, 3.7, 1.2 and 0.4 μ g/mL for extracts and fractions in the assay wells and 12.5, 4.1, 1.3, 0.46, 0.15 and 0.05 μ g/mL for the pure compounds. The plate was incubated at 37 °C with 5% CO₂ for 24 h. Following incubation, cell lysates were assayed for ATP as per the kit manufacturer's instructions. Briefly, all kit components were equilibrated to room temperature. Mammalian cell lysis solution (50 μ L) was added to each well of the cell culture microplate, the plate was shaken on an orbital microplate shaker (500 rpm, 5 min), then substrate solution (50 μ L/well)

was added, and the plate was further shaken (500 rpm, 5 min). The plate was dark adapted for 10 min, and luminescence measured on a Wallac 1450 Microbeta luminescence counter (Wallac, Turku, Finland). Half-maximum inhibitory concentration (IC₅₀) values were calculated using GraphPad prism version 4 (La Jolla, CA, USA). Samples were assayed in triplicate.

3.2.4.5.2. *Nitric oxide inhibitory assay*

RAW264 cells were cultivated as described for the cytotoxicity assay. Cell suspension (120 µL/well, 10⁶ cells/mL) was added to the wells of a 96 well microtitre plate and incubated for 20 h (37 °C, 5% CO₂), after which test compounds (dissolved in DMSO and further diluted 20-fold in the medium) were added to the cell suspension at 10 µL/well. Extracts were tested from 0.294 µg/mL to 71.4 µg/mL and pure compounds were tested from 0.04 µg/mL to 8.93 µg/mL. Following incubation for 1 h, lipopolysaccharide (LPS) solution (10 µL/well, 10 µg/mL) was added and the plate incubated for a further 20 h. Following this incubation, the plate was centrifuged (1500 g, 3 min), and 90 µL of the supernatant was transferred to a clear flat bottom assay plate (ParkinElmer, Glen Waverley, VIC, Australia) and assayed immediately for nitrite. Nitrite standards (0-100 µM) were prepared in the medium. Then 90 µL of each standard and cell supernatant were transferred to a flat-bottom 96-well microtitre plate (well volume 340 µL) (Greiner Bio-One, Frickenhausen, Germany) and 90 µL of Griess reagent (0.1% *N*-1-naphthylethylenediamine dihydrochloride, 1% sulfanilic acid in 5% phosphoric acid) was added to each well, followed by incubation at 23 °C for 20 min on an orbital shaker. Following incubation, the absorbance was read at 550 nm in a Wallac Victor 2 plate reader (Wallac, Turku, Finland). In this assay, dexamethasone was used as a positive control.

Standard curves were plotted for nitrite standards and R² values determined to verify linearity. Mean and standard deviations were calculated for replicates. The nitric oxide (as measured by nitrite) production in the sample wells was calculated as a percentage of that produced in solvent control wells.

3.2.4.5.3. *TNF-α Assay*

RAW264 cells were prepared as described in the cytotoxicity assay (Section 3.2.4.5.1). Cells were incubated overnight to allow adherence before samples were added. All samples were

tested at sub-cytotoxic levels in the presence and absence of LPS. Solvent control wells were also included. Following 1 h pre-incubation with samples, LPS was added to the wells, and cells were further incubated for approximately 20 h (37 °C, 5% CO₂). Following incubation, the plate was centrifuged at 1500 g for 3 min. Supernatants were collected and frozen (-80 °C) until measurement of TNF- α concentration at a later time.

TNF- α was quantified using a Quantikine Mouse TNF- α immunoassay kit (R&D Systems, Minneapolis, MN, USA) according to the manufacturer's instructions. Briefly 50 μ L of assay diluent was added to all wells on the immunoassay plate. A TNF- α standard curve with a range of 0-1500 pg/mL was prepared in calibrator diluents provided in the kit. Samples were diluted prior to the addition to the immunoassay plate. Samples tested in the absence of LPS were diluted 1/10 in calibrator diluents (10 μ L sample + 90 μ L calibrator diluents). Samples tested in the presence of LPS were diluted 1/100 (1/10 in calibrator diluents, 1/10 in media). 50 μ L of diluted samples, standards and kit control were added in triplicate to the assay plate. 100 μ L/well of mouse TNF- α conjugate solution was added to the plate, followed by 2 h incubation. After washing the plate with the washer provided in the kit, 100 μ L of substrate solution/well was added to the plate followed by 2 h incubation. After washing the plate, 100 μ L/well stop solution was added followed by 30 min incubation in the dark. Absorbance was read at 450 nm on a Wallac Victor 2 multilabel counter (Wallac, Turku, Finland). Absorbance at 550 nm was also read and Abs₅₅₀ subtracted from Abs₄₅₀ to correct for optical imperfections in the plate. A TNF- α standard curve was constructed and the TNF- α production in sample wells was calculated as a percentage of the production in solvent control wells.

3.2.4.5.4. *PGE₂ Assay*

3T3 Swiss albino mouse embryonic fibroblast cells (ATCC, Manassas, VA, USA) were used for the PGE₂ inhibitory activity assay. Cells were grown at 37 °C in the presence of 5% CO₂ in Dulbecco's modified Eagle medium containing 10% FBS, 5% newborn calf serum, L-glutamine (2 mM), sodium pyruvate (1 mM), penicillin (50 U/mL) and streptomycin (50 μ g/mL). Cells were seeded into 96-well cell culture plates (Interpath, Heidelberg, VIC, Australia) at a concentration of 27×10^3 cells/well in the medium as for the maintenance medium without phenol red. Cells were allowed to incubate overnight. Test samples dissolved in DMSO were added to the cells and incubated for 3 h at 37 °C in the presence of 5% CO₂. The DMSO concentration was 0.5% in all wells. Calcium ionophore A23187 (0.5 mM, 10

μL/well) (Sigma-Aldrich, St Louis, MO, USA) was added to the wells to stimulate PGE₂ production and the cells were incubated for a further 15 min. Culture plates were centrifuged (1500 g, 3 min), and the supernatant was separated and stored at -80 °C until assayed for PGE₂. Cayman chemical prostaglandin E₂ monoclonal EIA kit (Cayman chemical company, Ann Arbor, MI, USA) was used according to the manufacturer's protocol for the determination of PGE₂. The cell culture supernatant was diluted 81-fold in the kit assay buffer before determination of PGE₂ content. A standard curve was plotted, and the curve was fitted to a four-parameter logistic equation using Graph-Pad Prism software. The percentage of inhibition of PGE₂ production by each sample (assayed in triplicate) was calculated relative to the DMSO control.

3.2.4.5.5. *Selectivity Index*

The index was calculated as the ratio of the concentration that reduces cell viability to 50% divided by the IC₅₀ value for inhibition of NO, TNF-α or PGE₂ synthesis (Cos *et al.*, 2001, Jabit *et al.*, 2009).

3.2.4.5.6. *ORAC assay*

This assay was carried out in black-well fluorescence assay plates (Interpath, Heidelberg, VIC, Australia). All samples were diluted by combining 40 μL of samples in DMSO (20 mg/mL) with 960 μL of phosphate buffer (75 mM, pH 7.4) to give a concentration of 0.8 mg/mL. Further 2-fold serial dilutions were performed in 75 mM phosphate buffer/2% DMSO to give a range of eight sample concentrations. Based on sample concentrations that gave area under the curve (AUC) values within the range of the Trolox (Fluka) standard curve (12.5-100 μM), two or three concentrations were repeated in subsequent assays.

Fluorescein (Sigma-Aldrich) solution (10 μL, 5 μM), 20 μL of sample, Trolox standard, epicatechin, or solvent control solution, and AAPH (2,2'-azobis-2-methylpropanimidamide dihydrochloride) solution (170 μL, 20 mM) were added into each well. Immediately after addition of the AAPH solution the assay plates were placed in a Wallac Victor 2 plate reader (Wallac, Turku, Finland) and the fluorescence was recorded at 37 °C every minute for 35 min. The fluorescence readings were referenced to solvent blank wells. Final ORAC values were determined using a regression equation for Trolox concentration versus net area under the fluorescein decay curve. Antioxidant activity was based on the mean value for two sample

concentrations and expressed in micromoles of Trolox equivalents per gram of sample. All assays were performed in quadruplicate. Epicatechin (Sigma-Aldrich, St Louis, MO, USA) was used as a positive control in this assay.

3.2.5. Preliminary phytochemical screening

Crude extracts (LS-Hex and LS-DCM) of *L. suaveolens* leaves were tested on TLC plates for the preliminary detection of terpenoids, steroids, unsaturation and alkaloids. An iodine chamber, and vanillin-sulphuric acid, Dragendorff's, anisaldehyde and permanganate spray reagents were used to detect steroids, alkaloids, terpenoids and unsaturated compounds. TLC chromatograms were developed on normal phase silica plates in different solvent systems.

3.2.5.1. Preparation of staining agents for TLC plates

Vanillin-sulfuric acid (Johnsson *et al.*, 2007): Vanillin-sulfuric acid is a universal spray reagent that can detect a wide range of compounds. It was prepared by mixing 6 g vanillin (Sigma-Aldrich) with 2.5 mL H₂SO₄ in 250 mL ethanol. After spraying the plate with vanillin-sulfuric acid, plates were heated at 100 °C for 1-2 min. Many terpenes give a red or blue colour and steroids stain pink or purple (Gibbons, 2005).

Permanganate: Permanganate spray reagent was prepared by mixing 40 g K₂CO₃ with 6 g KMnO₄ in 600 mL of water, followed by addition of 5 mL of 10% NaOH. Permanganate solution is useful for detection of unsaturated compounds, which appear as brown spots on a pink background (Munavalli and Pannella, 1988).

Anisaldehyde (Simpson, 2011): Anisaldehyde reagent was composed of 95 mL ethanol, 2.5 mL acetic acid, 2.5 mL sulphuric acid and 0.5 mL anisaldehyde. Terpenoids appear as purple coloured spots with anisaldehyde reagent.

Dragendorff's reagent (Appendino *et al.*, 2008, Gibbons, 2004): Dragendorff's reagent is a traditional method for alkaloid detection. It is prepared by adding 10 mL of a 40% aqueous solution of KI to 10 mL of a solution of 0.85 g bismuth subnitrate in acetic acid (10 mL) and distilled water (50 mL). The resulting solution was diluted with acetic acid and water in the ratio 1:2:10. Alkaloids appear as dark orange or red spots with Dragendorff's reagent.

Iodine chamber: An iodine vapor chamber was made by adding a mixture of iodine crystals powdered with dry silica gel in a sealed glass container. After developing the TLC plate,

solvents were evaporated and the plate was inserted into the chamber. Compounds with double or triple bonds appear as brown spots on the TLC plate.

3.2.6. GC-MS analysis

Analytical gas chromatography (GC) was carried out on a Shimadzu GC17A gas chromatograph with a BP-5 column (a silicone stationary phase in which 5% of the silicon atoms have a phenyl group attached and other 95% are methyl group) (30 m×0.25 mm×25 µm) that was programmed from 35-250 °C at 3 °C/min with helium as the carrier gas. The injector and detector were both programmed at 220 °C. GC integrations were performed on a SMAD electronic integrator. GC-MS was carried out on a Shimadzu GCMS-QP5000 mass spectrometer operating at 70 eV ionisation energy. Some analyses were also carried out by GC-MS on a BP-20 column (carbowax column, stationary phase is polyethylene glycol, molecular weight 20,000) (60 m×0.25 mm×0.25 µm) programmed from 50-220 °C at 3 °C/min with helium as the carrier gas. Mass spectra were recorded in electron impact (EI) mode at 70 eV, scanning the 41-450 *m/z* range. Compounds were identified by their matching GC retention times and retention indices relative to *n*-alkanes (Hnawia *et al.*, 2012) by comparison of their mass spectra with either known compounds or published spectra (Adams, 2007, Joulain and Konig, 1998, Stenhagen *et al.*, 1974, Swigar and Silverstein, 1981).

3.2.7. Chemical study methods and materials

3.2.7.1. Isolation of bioactive compounds from LS-DCM extract

3.2.7.1.1. *Small scale fractionation of LS-DCM extract*

The DCM extract (2.6 g) of *L. suaveolens*, LS-DCM, was subjected to size exclusion chromatography (SEC) with a Sephadex LH-20 column (160 g, 45×3.5 cm), eluting with MeOH. This yielded eight major fractions on the basis of their normal phase TLC profiles; LSS-1 (10 mg, yellow solid), LSS-2 (13 mg, yellow solid), LSS-3 (782 mg, greenish yellow solid), LSS-4 (135 mg, green solid), LSS-5 (286 mg, green solid), LSS-6 (116.8 mg, blackish green solid), LSS-7 (115 mg, yellowish green solid) and LSS-8 (96 mg, green solid), in order of elution from the column. TLC bioautography (normal phase TLC plate, *n*-hexane:EtOAc, 2:1) against antibiotic sensitive and resistant bacteria (MS, MRSA and MDRSA) identified bioactive compounds in the LSS-4 and LSS-5 fractions.

LSS-4 was subjected to normal phase preparative thin layer chromatography (PTLC) (*n*-hexane:EtOAc, 3:1). Compounds were UV inactive and were visualised by spraying on one vertical edge of the plate with anisaldehyde spray reagent. Compound LS-29 (20 mg) was isolated as a white solid with minor impurities. Further purification by recrystallisation with MeOH afforded **LS-29** (10 mg) as white needle-like crystals ($R_f = 0.28$ in *n*-hexane:EtOAc, 3:1, normal phase).

Purification of LSS-5 (200 mg) was performed using normal phase silica gel (141 g) column chromatography. The column was eluted using a gradient of *n*-hexane:EtOAc (100:0 to 0:100) and finally with EtOAc:MeOH (100:0 to 0:100). Fractions were combined to afford 4 major fractions on the basis of their TLC profiles (*n*-hexane:EtOAc, 1:1, normal phase); LSS-5-I (12.5 mg, light yellow, solid), LSS-5-II (11.1 mg, green solid), LSS-5-III (55 mg, gray solid) and LSS-5-IV (8.7 mg, yellow solid), in order of elution from the column. Bioactive fraction LSS-5-III (55 mg) showed a single spot on the TLC plate ($R_f = 0.5$, *n*-hexane:EtOAc, 2:1, normal phase) that was visualised by UV light (254 nm) and stained blue with anisaldehyde spray reagent (indicating terpenoids), with additional impurities. Further normal phase PTLC, eluting with *n*-hexane and EtOAc (1:1), yielded a mixture LSS-5-IIIA with R_f 0.4 (*n*-hexane:EtOAc, 2:1, normal phase) (~3.0 mg, white powder) that stained blue with anisaldehyde reagent. This mixture was not further purified.

3.2.7.1.2. *Large scale fractionation of LS-DCM extract*

LS-DCM (8 g) was dissolved in DCM (7 mL) and then mixed with silica gel (6-8 g) and evaporated to dryness by rotary evaporation at 45 °C to make a free flowing sample. The solid mixture was then applied to the top of a normal phase silica column (270 g) (with initial solvent *n*-hexane). The column was eluted using a gradient of *n*-hexane:EtOAc (100:0 to 0:100) and finally with EtOAc:MeOH (100:0 to 0:100). According to TLC profiles, similar R_f fractions were combined to afford 12 major sub-fractions; LSL-1 (12 mg, pale yellow solid), LSL-2 (353 mg, yellow solid), LSL-3 (212 mg, greenish yellow solid), LSL-4 (160 mg, light green solid), LSL-5 (120 mg, green solid), LSL-6 (10 mg, green solid), LSL-7 (210 mg, green jelly), LSL-8 (369 mg, green solid), LSL-9 (50 mg, blackish green solid), LSL-10 (768 mg, green solid), LSL-11 (674 mg, brown solid), LSL-12 (1234 mg, brown solid), in order of elution. Among these 12 sub-fractions, six fractions (LSL-4, LSL-5, LSL-7, LSL-10, LSL-11

and LSL-12) were selected for further purification according to the bioactivity results of the TLC bioautography and MTT microdilution assays.

LSL-11 (360 mg), which was one of the most active antibacterial fractions, was subjected to column chromatography using Sephadex LH-20 (96 g) and MeOH as the eluting solvent. This yielded five sub-fractions according to their TLC profiles (*n*-hexane:EtOAc, 1:1, normal phase); LSL-11-I (~10 mg, pale yellow solid), LSL-11-II (40 mg, pale yellow solid), LSL-11-III (~30 mg, brown solid), LSL-11-IV (10 mg, greenish yellow) and LSL-11-V (10 mg, brown solid), in order of elution. While concentrating LSL-11-II using rotary evaporation, a white solid was deposited on the bottom of the round bottom flask. This was separated from the liquid by decanting the liquid and washing with a small amount of MeOH (1 mL). This white solid (~10 mg collected) was checked by TLC bioautography, but it was inactive against bacteria. The remaining liquid portion was further evaporated to give a brownish white solid (~25 mg), LSL-11-II, which was active by TLC bioautography. Further separation using normal phase PTLC (*n*-hexane:EtOAc, 2:1) yielded a compound mixture LSL-11-IIA ($R_f = 0.45$ in *n*-hexane:EtOAc, 2:1, normal phase) as a white solid (2.5 mg). LSL-11-IIA was found to be inactive by TLC bioautography. Other active fractions of LSL-11 (LSL-11-IV and LSL-11-V) were not further purified.

LSL-12 (700 mg) was subjected to column chromatography using Sephadex LH-20 (96 g), eluting with MeOH. This yielded four sub-fractions according to their TLC profiles (*n*-hexane:EtOAc, 1:1, normal phase); LSL-12-I (101 mg, greenish yellow solid), LSL-12-II (50 mg, pale white solid), LSL-12-III (150 mg, brown solid) and LSL-12-IV (100 mg, brown solid) in order of elution. LSL-12-II was active by TLC bioautography and showed a single spot on TLC (*n*-hexane:EtOAc, 1:1, normal phase) visualised by UV (256 nm), with minor impurities. The spot also appeared blue with anisaldehyde reagent. Further normal phase PTLC with *n*-hexane:EtOAc (1:1) yielded a mixture, LSL-12-IIA ($R_f = 0.6$ in *n*-hexane:EtOAc, 1:1, normal phase) as a white solid (1.5 mg), which was active by TLC bioautography against methicillin sensitive and resistant *S. aureus*.

Repeated column chromatography of LSL-5 using Sephadex LH-20 (90 g) with MeOH yielded LS-22 as yellow crystals (5 mg) with minor impurities. LS-22 was further purified by recrystallisation using MeOH and DCM to afford yellow crystals of compound **LS-22** (3 mg, $R_f = 0.5$, *n*-hexane:EtOAc, 1:1, normal phase).

Washing the jelly like material of LSL-7 (210 mg) with *n*-hexane (6x5 mL) yielded a white solid (170 mg). Repeated recrystallisation of the white solid (170 mg) with MeOH (4 times) afforded **LS-29** (115 mg) as white needle-like crystals ($R_f = 0.28$, *n*-hexane:EtOAc, 3:1, normal phase).

3.2.7.2. Isolation of bioactive compounds from LS-Hex extract

LS-Hex (3.3 g) was dissolved in petroleum ether (Bp. 40-60 °C) (5 mL) and applied to the top of a normal phase silica column (260 g) prepared with petroleum ether. After application of the sample and elution with petroleum ether (50 mL), a yellowish waxy solid was deposited on the top of the column. The waxy solid, LS-Hex-A, was scraped out from the column and dried under high vacuum to give 400 mg of a pale yellow waxy solid. The waxy material was initially washed with petroleum ether (6x5 mL) then recrystallised with MeOH (3 times) to yield **LS-22** (15 mg) as a yellow crystalline solid.

The fraction remaining adsorbed on the silica column was collected by eluting with *n*-hexane and DCM (1:1). Evaporation of the solvents using rotary evaporation at 40 °C and subsequent removal of the remaining solvent under high vacuum, afforded LS-Hex-B (2.3 g). Further normal phase silica gel column chromatography (260 g) of LS-Hex-B (2.3 g) starting with petroleum ether:DCM (100:0 to 0:100) and finally with EtOAc:MeOH (0:100 to 100:0) yielded 6 major sub-fractions according to the TLC profiles to give Hex-1 (15 mg, orange oil, $R_f = 0.8-0.9$, petroleum ether:DCM, 3:1, normal phase), Hex-2 (30 mg, light yellow oil, $R_f = 0.7-0.9$, petroleum ether:DCM, 3:1, normal phase), Hex-3 (50 mg, yellow solid, $R_f = 0.3-0.6$, petroleum ether:DCM, 3:1, normal phase), Hex-4 (110 mg, greenish yellow solid, $R_f = 0.2-0.4$, petroleum ether:DCM, 3:1, normal phase), Hex-5 (150 mg, dark green solid, $R_f = 0.2-0.7$, petroleum ether:DCM, 2:1, normal phase) and Hex-6 (400 mg, blackish green solid $R_f = 0.2-0.8$, petroleum ether:DCM, 3:1, normal phase). Only Hex-3, Hex-4 and Hex-5 were active by TLC bioautography. Further washing of Hex-5 (140 mg) with *n*-hexane (3x5 mL) afforded a light green-yellow solid and recrystallisation afterward with MeOH (3 times) afforded yellow crystals of **LS-22** (31.0 mg, $R_f = 0.5$, hexane:EtOAc, 1:1, normal phase). Normal phase PTLC (petroleum ether:DCM, 2:1) of Hex-3 yielded an impure bioactive compound mixture, LS-Hex-15 (2 mg, yellow oil, $R_f = 0.6$, petroleum ether:DCM, 3:1, normal phase) that stained blue with anisaldehyde reagent.

4',7-Dimethoxy-6,8-dimethyl-5-hydroxyflavone (LS-22): Yellow crystals; mp 184-186 °C (lit. 184-185 °C) (Courtney *et al.*, 1983). HRESIMS: m/z 326.1154 for C₁₉H₁₈O₅ (calcd. 326.1154). IR (neat) ν_{\max} (cm⁻¹): 3077, 2918, 2838, 1650, 1634, 1607, 1471, 1441, 1425, 1317, 1254, 1214, 1122, 1030, 892, 828. ¹H NMR (600 MHz, CDCl₃): δ 12.85 (1H, s, 5-OH), 7.86 (2H, d, J = 8.8 Hz, H-2', 6'), 7.02 (2H, d, J = 8.8 Hz, 3', 5'), 6.60 (1H, s, H-3), 3.87 (3H, s, 4'-OCH₃), 3.77 (3H, s, 7-OCH₃), 2.37 (3H, s, 8-CH₃), 2.19 (3H, s, 6-CH₃). ¹³C NMR (150 MHz, CDCl₃): δ 183.5 (C-4), 164.1 (C-2), 162.8 (C-9), 162.8 (C-4'), 157.5 (C-5), 153.1 (C-7), 128.2 (C-2', 6'), 124.1 (C-1'), 114.8 (C-3', 5'), 114.2 (C-6), 109.0 (C-8), 107.5 (C-10), 104.3 (C-3), 60.6 (C-7-OCH₃), 55.8 (C-4'-OCH₃), 8.8 (C-8-CH₃), 8.5 (C-6-CH₃).

Betulinic acid (LS-29): White crystals, mp 283-285 °C (lit. 282-285 °C) (Zhu *et al.*, 2003). $[\alpha]_D^{24.5} +5^\circ$ (0.05 g/100 mL, 10% CH₃OH in CHCl₃) (lit. $[\alpha]_D +4^\circ$ (CHCl₃) (Koudou *et al.*, 1994). IR (neat) ν_{\max} (cm⁻¹): 3440, 2868, 1682, 1447. HRESIMS: m/z 455.3526 for C₃₀H₄₇O₃ (calcd. m/z 455.3525). ESI MS (-) m/z 455 [M-H]⁻. ¹H NMR (600 MHz, DMSO-*d*₆): δ 4.68 (1H, d, J = 2.4 Hz, H-29b), 4.55 (1H, d, J = 2.4 Hz, H-29a), 4.25 (1H, d, J = 5.2 Hz, H-3-OH), 2.21 (1H, ddd, J = 3.6, 12.7, 12.7 Hz, H-13), 2.93 (1H, m, H-19), 2.96 (1H, m, H-3), 1.64 (3H, s, H-30), 0.92 (3H, s, H-27), 0.867 (3H, s, H-26), 0.864 (3H, s, H-24), 0.76 (3H, s, H-25), 0.64 (3H, s, H-23). ¹³C NMR (150 MHz, DMSO-*d*₆): δ 177.3 (C-28), 150.2 (C-20), 109.7 (C-29), 76.8 (C-3), 55.3 (C-17), 54.9 (C-5), 49.8 (C-9), 48.4 (C-18), 46.5 (C-19), 42.0 (C-14), 40.1 (C-8), 38.5 (C-4), 38.1 (C-1), 37.6 (C-13), 36.6 (C-10), 36.2 (C-22), 33.9 (C-7), 31.7 (C-16), 30.1 (C-21), 29.2 (C-15), 28.1 (C-26), 27.2 (C-2), 25.1 (C-12), 20.3 (C-11), 18.8 (C-30), 17.9 (C-6), 15.8 (C-25), 15.7 (C-23), 15.6 (C-24), 14.4 (C-27).

3.3. Results and discussion

3.3.1. Preliminary screening and extraction of *Lophostemon suaveolens* leaves

As described earlier (Chapter 2), 80% aqueous ethanol (EtOH) and water (H₂O) extracts (room temperature) of the leaves of *L. suaveolens* collected from northern NSW by Packer (Packer, 2012) exhibited good antibacterial activity against a methicillin sensitive strain of *Staphylococcus aureus* and moderate activity against *P. aeruginosa*. For this study, fresh leaves of *L. suaveolens* were also collected from northern NSW. To ensure they had relevant

antibacterial activity, a water extract (LS-water) of the leaves (100 g) was made (method 1) in an analogous manner to the water extract of the Packer study (LS-water JP) and screened for antibacterial activity using the disc diffusion and MTT microdilution assays against methicillin sensitive and resistant strains of *Staphylococcus aureus*, *Streptococcus pyogenes* and *E.coli*. The disc diffusion assay was performed along with that of the water extracts (which had been stored for one year at -80 °C) from Packer's studies. LS-water JP and LS-water were found to have identical assay results by the disc diffusion assay, with both samples giving 10 mm diameter zones of inhibition (at 2 mg/disc) against *S. aureus* (Figure 3.3.1). However, some differences were noted in the activities of LS-water JP and LS-water in the MTT microdilution assay. In this assay, LS-water JP showed better activity (IC₉₀ 188 µg/mL) in Packer's study (Packer, 2012) than LS-water (IC₉₀ 1000 µg/mL) against *Staphylococcus aureus* and LS-water showed modest activity against *Streptococcus pyogenes* whereas LS-water JP did not show activity against *Streptococcus pyogenes* (Table 3.3.1). However, LS-water showed similar antibacterial activities to that found previously by Packer against MRSA and MDRSA (Packer, 2012) (Table 3.3.1).

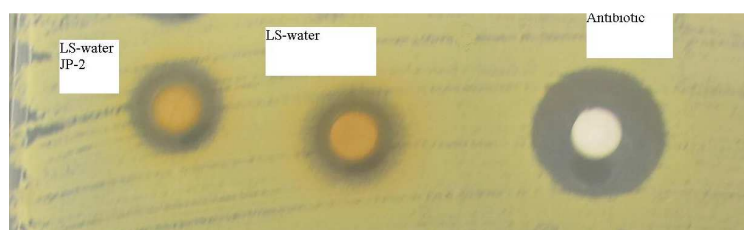


Figure 3.3.1: Comparison of two water extracts (LS-water JP and LS-water, 2 mg/disc) by disc diffusion method against *S. aureus*. Vancomycin (2 µg/disc) was used as a positive control.

Table 3.3.1: Comparison of antimicrobial activity of water extracts among different collection batches.

Water extracts of <i>L. suaveolens</i>	IC ₉₀ (µg/mL)				
	<i>Staphylococcus aureus</i>	MRSA	MDRSA	<i>Streptococcus pyogenes</i>	<i>E. coli</i> (β+) & (β-)
LS-water JP	188	1000	1000	na	na
LS-Water	1000	1000	1000	1000	na

LS-water JP represents water extract of leaves of Packer's collection batch (Packer, 2012) and LS-water represents water extract of leaves of the author's collection batch. MIC was determined as the well with the lowest concentration of sample that displayed no yellow-blue colour change of MTT and also by turbidity (absorbance). Two repeats were performed; values are average of observed results. na: Not active against tested bacteria.

Following the finding of antimicrobial activity in the water extract of fresh leaves of *L. suaveolens*, a large scale extraction (1003 g) was conducted to assist fractionation and bioassay guided isolation of bioactive compounds. Fresh leaves of *L. suaveolens* were ground and sequentially extracted with *n*-hexane, DCM, EtOAc and MeOH (Houghton and Raman, 1998) to afford four different extracts (LS-Hex, LS-DCM, LS-EA and LS-MeOH). Table 3.3.2 summarises the yields and characteristics of these extracts, along with the water extract LS-water.

Table 3.3.2: Yields (w/w %), appearances and TLC R_f values of extracts of *L. suaveolens*.

Extracts	Percent yield (w/w)	Physical appearance	R _f (normal phase TLC)
LS-water	2.03%	Yellowish green powder	0.1-0.3 (<i>n</i> -hexane:EtOAc, 1:1)
LS-Hex	0.9%	Yellow oily gum	0.2-0.9 (<i>n</i> -hexane:EtOAc, 1:1)
LS-DCM	2.4%	Dark green solid	0.2-0.9 (<i>n</i> -hexane:EtOAc, 1:1)
LS-EA	1.6%	Dark green solid	0.1-0.3, 0.5-0.8 (<i>n</i> -hexane:EtOAc, 1:1)
LS-MeOH	3.2%	Blackish green solid	0.1-0.3 (<i>n</i> -hexane:EtOAc, 1:1)

3.3.2. Antibacterial screening of extracts of *Lophostemon suaveolens*

3.3.2.1. Disc diffusion assay

The extracts from the sequential solvent extraction (LS-Hex, LS-DCM, LS-EA and LS-MeOH) and LS-water (2 mg/disc) were tested for antibacterial activity using the disc diffusion assay. A range of Gram-positive and Gram-negative bacteria including antibiotic sensitive strains [*S. aureus*, *S. pyogenes*, *S. typhimurium*, *P. aeruginosa* and *E. coli* (β lactamase negative) and also antibiotic resistant strains (MRSA, MDRSA and *E. coli* (β lactamase positive))] (Table 3.3.3) were used in this assay. These bacteria used in the present study are representative of pathogens important in human infections. The diameter of zone of inhibition was measured in millimetres (mm) including the 6 mm diameter of the disc. Comparing the published literature of disc diffusion assay, extracts (2 mg/disc) were considered as having ‘good’ (>16 mm), ‘moderate’ (10-15 mm) or ‘low’ (<10 mm) levels of antimicrobial activity (Kirmizigul *et al.*, 1996, Rabe and van Staden, 1997, Lin *et al.*, 1999). The highest activity against the Gram-positive organisms was demonstrated by LS-DCM, followed by LS-Hex, LS-EA and the LS-water extracts. The LS-MeOH extract was inactive against all the tested Gram-positive and Gram-negative organisms (Table 3.3.3).

Table 3.3.3: Antibacterial activity of *L. suaveolens* extracts by disc diffusion assay.

Extracts (2.5 mg/disc)	Diameter of zone of inhibition (mm)				
	<i>S. aureus</i>	<i>MRSA</i>	<i>MDRSA</i>	<i>S. pyogenes</i>	<i>S. typhimurium</i> , <i>P. aeruginosa</i> , <i>E. coli</i> (β +) & (β -)
LS-Hex	15	17	16	13	na
LS-DCM	17	17	17	11	na
LS-EA	13	14	13	na	na
LS-MeOH	na	na	na	na	na
LS-water	10	10	10.5	na	na
Vancomycin (2 μ g)	12	12.5	11.5	16	na
Gentamycin (2 μ g)					17, 12, 15, 15 [#]

*Two repeats were performed on at least two separate occasions. Zone of inhibition was determined by diameter of complete inhibition, including 6 mm disc diameter. Values are average of two results. na: not active against the tested bacteria. [#]Values are diameters of zone of inhibition against *S. typhimurium*, *P. aeruginosa*, *E. coli* (β +) & (β -), respectively.

3.3.2.2. MTT microdilution assay

Antibacterial activities of LS-Hex, LS-DCM, LS-EA and LS-MeOH extracts were also evaluated by the MTT microdilution method (Appendino *et al.*, 2008, Cherigo *et al.*, 2009). As described previously (Chapter 2), this assay uses the tetrazolium salt (MTT), which is converted from yellow to the blue formazan by metabolically active bacteria (Martin *et al.*, 2005). According to Rios and Recio (Rios and Recio, 2005) and Gibbons (Gibbons, 2004), crude plant extracts having MIC values below 1000 μ g/mL are considered worthy of further investigation. In the present study, a plant extract with an IC₉₀ of <50 μ g/mL was considered as having good (***) antimicrobial activity, those with IC₉₀ of 50-125 μ g/mL were considered as having moderate (**) antimicrobial activity and those with IC₉₀ of 126-1000 μ g/mL were considered as having low (*) levels of antimicrobial activity.

In the MTT microdilution assay, LS-Hex and LS-DCM extracts showed significant antimicrobial activity against all tested sensitive and resistant strains of *S. aureus* and *S. pyogenes* (IC₉₀ <125 μ g/mL) (Table 3.3.4) (Figure 3.3.2). Although LS-EA showed antibacterial activity (13 mm) in the disc diffusion assay at a concentration of 2 mg/disc, it

showed no activity at a concentration of 1000 µg/mL in the MTT microdilution assay. It was observed on the TLC plate that there was some overlap of components in both the LS-DCM and LS-EA extracts (Table 3.3.2). Therefore, some bioactive components of LS-DCM are also likely to be present in the LS-EA extract and may contribute to its antibacterial activity in the disc diffusion assay. LS-MeOH did not show any activity in the disc diffusion or MTT microdilution assays. The active extracts (LS-Hex, LS-DCM, LS-EA and LS-water) were also tested for their bactericidal (killing of bacteria) or bacteriostatic (prevention of multiplication of bacteria without killing them) effects (Hnawia *et al.*, 2012, Vagelos, 1991). LS-Hex, LS-DCM and LS-water were found to have bactericidal effects on the tested bacteria (*S. aureus*, MRSA, MDRSA and *S. pyogenes*) (Table 3.3.4). It was also revealed from this study that promising antibacterial activity was observed in less polar extracts (LS-Hex and LS-DCM), whereas the more polar extracts (LS-EA and LS-MeOH) showed less or no activity in the disc diffusion or MTT assays. The separately prepared LS-water extract also showed less activity than the less polar extracts (LS-Hex and LS-DCM) (Table 3.3.4) (Figure 3.3.2). Accordingly, LS-Hex and LS-DCM extracts of *L. suaveolens* were selected for further chemical and antibacterial bioassay guided investigations.

Table 3.3.4: Antibacterial activity of extracts of *L. suaveolens* by the MTT microdilution assay and bacteriostatic or bactericidal effects.

Extracts of <i>Lophostemon suaveolens</i>	MIC and IC ₉₀ values (µg/mL)				
	<i>S. aureus</i>	MRSA	MDRSA	<i>S. pyogenes</i>	<i>S. typhimurium</i> , <i>P. aeruginosa</i> , <i>E. coli</i> (β+) & (β-)
LS-Hex	31.25*** (C)	31.25*** (C)	31.25*** (C)	15.62*** (C)	na
LS-DCM	1.9*** (C)	1.9*** (C)	1.9*** (C)	62.5*** (C)	na
LS-EA	na	na	na	na	na
LS-MeOH	na	na	na	na	na
LS-Water	1000* (C)	1000* (C)	1000* (C)	1000* (C)	na
Vancomycin	1.25*** (C)	1.25*** (C)	1.25*** (C)	0.31*** (C)	na

***IC₉₀ <50 µg/mL, **IC₉₀ 50-125 µg/mL, *IC₉₀ 126-1000 µg/mL. C: bactericidal effect on the microorganisms. MIC was determined as the well with the lowest concentration of sample that displayed no yellow-blue colour change of MTT and also by turbidity (absorbance). Two repeats were performed on at least two separate occasions; values are average of observed results. na: not active against the tested bacteria at 1000 µg/mL.

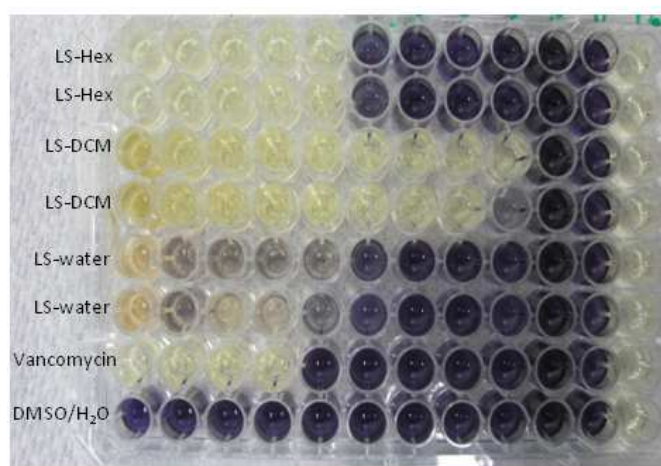


Figure 3.3.2: MTT microdilution assay plate of *L. suaveolens* extracts against MDRSA. First well sample concentration for LS-Hex, LS-DCM, LS-water, vancomycin and DMSO control were 500, 500, 1000, 10 µg/mL and 2% DMSO/H₂O, respectively. MIC was determined as the well with the lowest concentration of sample that displayed no yellow-blue colour change of MTT.

3.3.3. Phytochemical screening

The antibacterial extracts LS-Hex and LS-DCM were subjected to preliminary phytochemical screening using specific tests on TLC plates to get an initial idea about the types of compounds present. Preliminary screening is often helpful in selecting methods for isolation of targeted compounds. The staining agents used were: vanillin-sulfuric acid, anisaldehyde, permanganate, iodine and Dragendorff's reagent. Vanillin-sulfuric acid is an universal spray reagent that can detect steroids (pink or violet spot) and terpenes (blue or red coloured spot) (Gibbons, 2005). Anisaldehyde reagent is useful for detecting terpenoids (blue or green spot) (Wagner, 1996), while permanganate and iodine are useful for detecting monoterpenes and other unsaturated compounds (Davies and Johnson, 2007). Dragendorff's reagent is useful for detecting the presence of alkaloids (dark orange to red spot) (Gibbons, 2005).

Figure 3.3.3 shows the distinctive staining seen with anisaldehyde reagent, with blue-green colouration suggesting the presence of terpenoids (a); pink, blue and green spots observed with vanillin stain suggesting the presence of terpenes and steroids (b); and yellow-brown spots on a pink plate when sprayed with permanganate stain indicating the presence of monoterpenes and unsaturated compounds (c). Both extracts (LS-Hex and LS-DCM) did not show any orange or red spots upon spraying with Dragendorff's reagent (Table 3.3.5). These results indicate the presence of terpenes, terpenoids, steroids and unsaturated compounds and absence of alkaloids in the LS-Hex and LS-DCM extracts of *L. suaveolens*.

Table 3.3.5: Preliminary phytochemical study of LS-Hex and LS-DCM extracts of *L. suaveolens*.

Bioactive extracts of <i>L. suaveolens</i>	Terpenoids	Steroids	Alkaloids	Unsaturated compounds
LS-Hex	✓	✓	×	✓
LS-DCM	✓	✓	×	✓

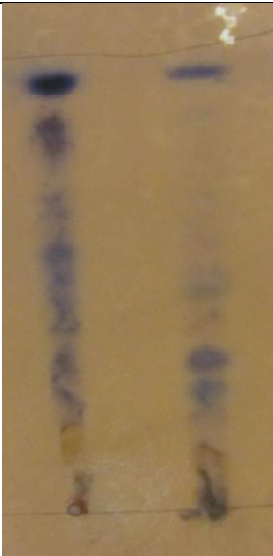


		
<p>(a) Anisaldehyde reagent, <i>n</i>-hexane:EtOAc (2:1), normal phase TLC, LS-Hex (left), LS-DCM (right).</p>	<p>(b) Vanillin-sulfuric acid stain, <i>n</i>-hexane:EtOAc (2:1), normal phase TLC, LS-Hex (left), LS-DCM (right).</p>	<p>(c) Permanganate spray, <i>n</i>-hexane:EtOAc (2:1), normal phase TLC, LS-Hex (left), LS-DCM (right).</p>

Figure 3.3.3: TLC Chromatograms of LS-Hex and LS-DCM extracts after spraying with spray reagents.

3.3.4. GC-MS analysis of LS-Hex extract of *Lophostemon suaveolens*

Following the promising antibacterial activity, the LS-Hex extract was selected for further investigation to identify the components responsible for its bioactivity. TLC of the yellow oil of the LS-Hex extract upon spraying with permanganate stain (which is useful in detecting monoterpenes e.g. limonene, carvone) (Davies and Johnson, 2007) showed most of the components as yellow-brown spots against a purple background, indicating the possible presence of monoterpenes.

A GC-MS study on the volatile oil composition has reported the presence of a number of monoterpenes in the leaves of all four species of the *Lophostemon* genus (*L. suaveolens*, *L. confertus*, *L. grandiflorus* and *L. lactifluus*) (Brophy *et al.*, 2000). Therefore, the LS-Hex extract was examined by GC-MS. The GC-MS analysis was performed at two places (Organic Geochemistry Laboratory, Macquarie University, Sydney and School of Chemistry, University of New South Wales). Percentages of identified components were determined at University of New South Wales (UNSW). Data analysis at Macquarie University was performed by the

author with the help of Prof. Simon George and data analysis at UNSW was performed by Dr Joe Brophy.

Sixteen phytochemicals were identified from the combined GC-MS analyses (Table 3.3.6 and Figure 3.3.4). These compounds were identified by comparing their GC retention times and retention indices relative to *n*-alkanes (C₅-C₂₆) and also by comparison of their mass spectra with either known compounds or published spectra (Adams, 2007, Joulain and Konig, 1998, Stenhagen *et al.*, 1974, Swigar and Silverstein, 1981). Results obtained from the GC-MS analysis are presented in Table 3.3.6. The major constituents identified in the LS-Hex extract of *L. suaveolens* leaves were aromadendrene (15.4%), spathulenol (12.46%), *allo*-aromadendrene (7.04%), globulol (4.47%), *t*-calamenene (3.15%), epiglobulol (2.69%), phytol (2.84%), β -caryophyllene (2.53%), α -humulene (1.52%) and ledol (1.22%) (Figure 3.3.4) (Table 3.3.6). As described in Section 3.3.2, the *n*-hexane extract (LS-Hex) showed remarkable antibacterial activity (MIC values were less than 50 μ g/mL against *S. pyogenes* and sensitive and resistant strains of *S. aureus*) in the MTT microdilution assay (Table 3.3.4). According to the literature, aromadendrene (Dorman and Deans, 2000), spathulenol (Bougatsos *et al.*, 2004), β -caryophyllene (Ozturk *et al.*, 2009), α -humulene (Dorman and Deans, 2000), globulol (Tan *et al.*, 2008), phytol (Rajab *et al.*, 1998) and α -pinene (Inouye *et al.*, 2001, Dorman and Deans, 2000) have good antimicrobial properties. The antimicrobial activity of the LS-Hex extract of *L. suaveolens* is therefore likely to be associated with the high percentage of these bioactive components. α -Humulene and β -caryophyllene were reported for having cytotoxic effects on cancer cell lines (Sylvestre *et al.*, 2007), while spathulenol has immunomodulatory effects and has also been reported to inhibit proliferation of the lymphocytes and induce apoptosis (Ziaei *et al.*, 2011). β -caryophyllene has also been reported to have good anti-inflammatory activity in the NO inhibition assay (Tung *et al.*, 2008).

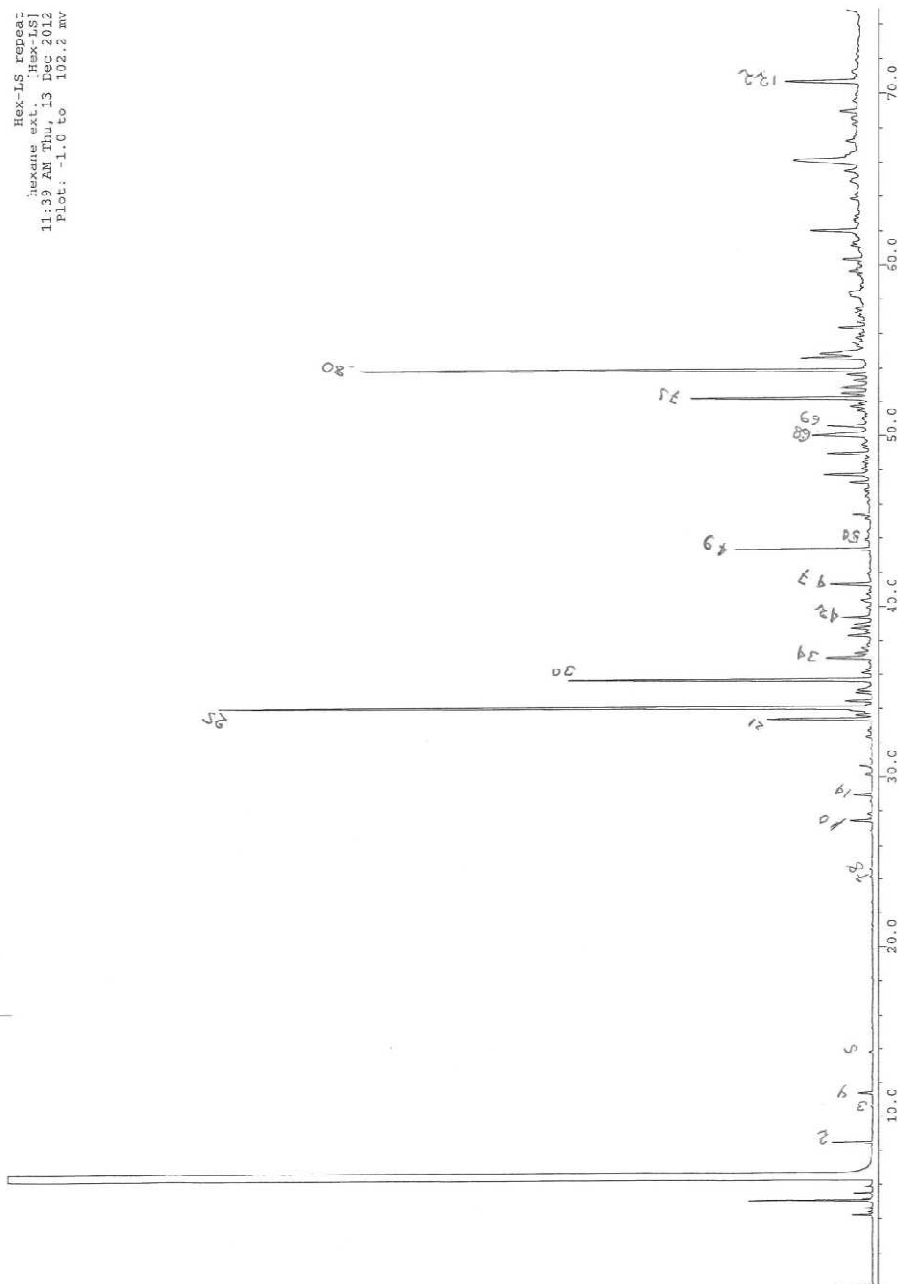


Figure 3.3.4: GC-MS total ion chromatogram (TIC) of the LS-Hex extract of *L. suaveolens* using a BP-20 column. The compounds labelled in the chromatogram correspond to the Arabic numerals given in Table 3.3.6 as peak positions.

Table 3.3.6: GC-MS analysis of LS-Hex extract of *L. suaveolens* using BP-20 and DB-5 columns.

S. No.	Compounds	Peak Position (BP-20 column)	Retention time (min) (BP-20 column)	LRI Values (BP-5 column)	Methods of identification ^a Published literature data* ^b LRI index	% of identified compounds (BP-20 column)
1	α -Pinene	2	8.52	-	a	0.67
2	α -Cubebene	10	27.41	1351	a, b	0.49
3	α -Copaene	14	28.92	1377	a, b	0.46
4	β -Caryophyllene	21	33.41	1411	a, b	2.53
5	Aromadendrene	25	34.06	1442	a, b	15.47
6	<i>Allo</i> -Aromadendrene	30	35.68	1460	a, b	7.04
7	α -Humulene	34	36.98	1453	a, b	1.52
8	Bicyclogermacrene	42	39.36	1499	a, b	0.90
9	Cadino-1,4 diene	47	41.30	1535	a, b	0.91
10	<i>t</i> -Calamenene	49	43.34	1524	a, b	3.15
11	Benzyl alcohol	50	43.91	-	a	0.15
12	Epiglobulol	68	50.022	-	a	2.69
13	Ledol	69	50.59	1594	a, b	1.22
14	Globulol	75	52.20	1585	a, b	4.47
15	Spathulenol	80	53.88	1579	a, b	12.46
16	Phytol	122	70.67	-	a	2.84

*(Adams, 2007, Joulain and Konig, 1998, Stenhagen *et al.*, 1974, Swigar and Silverstein, 1981), NIST and Wiley library data. -: LRI (linear retention indices) value could not be determined. a: confirmed at UNSW and Macquarie University. b: determined at UNSW. S. No.: serial number.

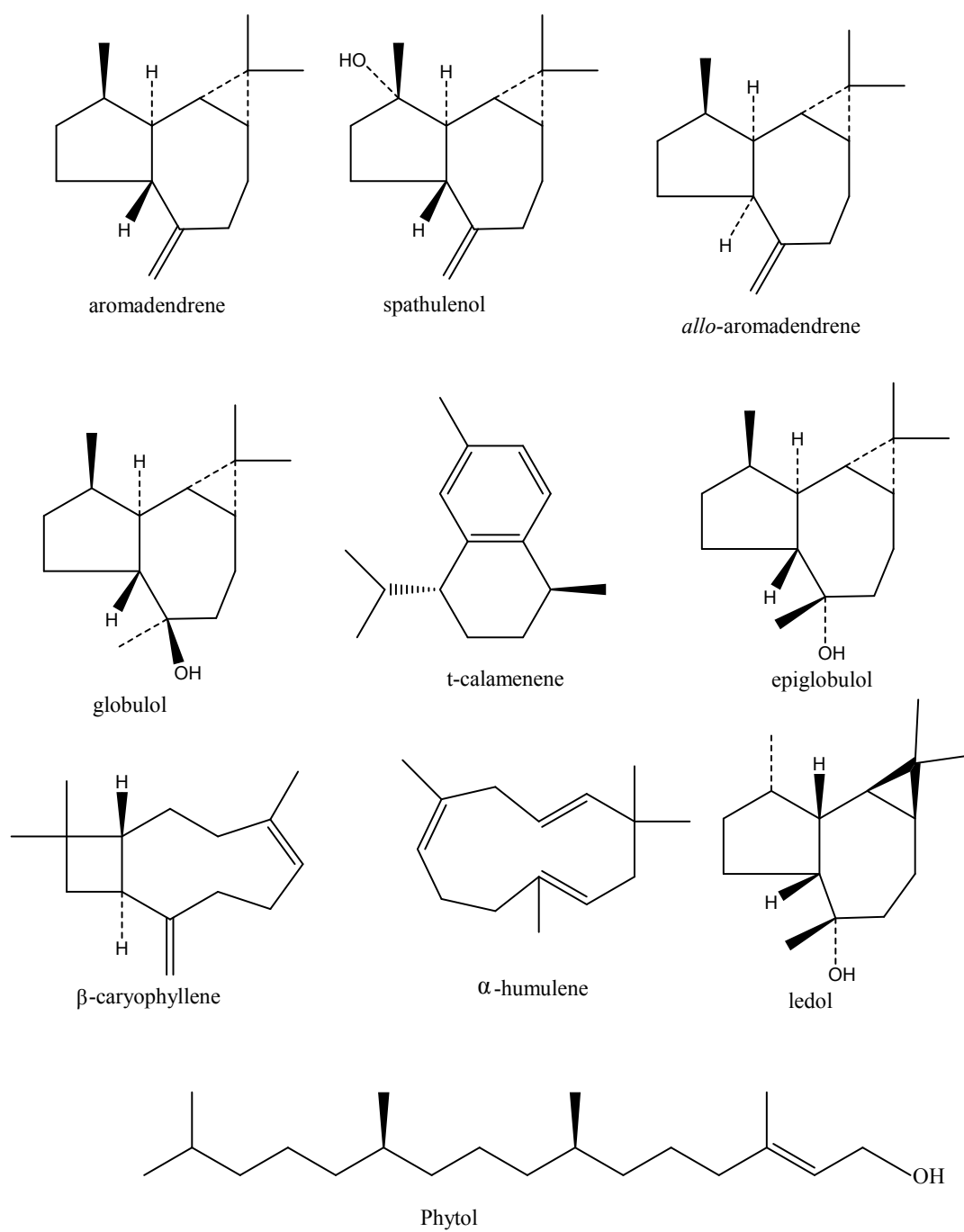


Figure 3.3.5: Structures of major components of LS-Hex extract identified by GC-MS analysis.

3.3.5. **Bioassay guided isolation of bioactive compounds from LS-Hex and LS-DCM**

Following on from finding potent antibacterial activity in the LS-DCM and LS-Hex extracts with the disc diffusion and MTT microdilution assays, different chromatographic techniques were performed to isolate bioactive components from these extracts. The MTT microdilution assay and TLC bioautography were used to detect bioactivity. From the preliminary phytochemical screening, it was inferred that terpenoids, flavonoids and steroids might be the principal components in the LS-Hex and LS-DCM extracts. TLC bioautography is a widely used method for its ability to detect antimicrobial activity rapidly (already discussed in Chapter 2). It helps in targeted compound isolation and also helps to detect antimicrobial activity of extracts, fractions or pure compounds using small amounts of sample (Gibbons, 2005). Several procedures have been published for performing the TLC bioautography. In this study, some of these methods were trialled and finally an optimised method was obtained. This optimised method was used extensively to detect bioactive fractions and compounds from the LS-DCM and LS-Hex extracts (Section 3.3.5.1) and also for studies with *A. excelsa* (see Chapter 4).

3.3.5.1. **Optimisation of TLC bioautography**

As already described in Chapter 2 (Section 2.6.1.1.3), TLC bioautography is a useful technique for bioassay guided isolation of bioactive compounds and gives an idea about which compound/fraction on a TLC plate to target before it gets isolated and identified.

The basic principle of this method is extracts or fractions or pure compounds are run on two TLC plates under identical conditions with an appropriate developing solvent system or without developing with a solvent (in cases where extracts or fractions do not give good separation on the TLC plate). One of these plates is visualised under UV light and/or stained to observe the compounds. This TLC plate is then used as a reference chromatogram. After drying the solvents, the plate is covered with a growth medium containing the microorganism. After incubation, a detecting agent is added. MTT (a colourless to pale yellow compound) is commonly used as the detecting agent and is converted into the blue MTT formazan in the presence of viable microorganisms. Therefore, a clear spot on the TLC plate indicates compounds or fractions in that region having antimicrobial activity.

Several procedures have been published for performing TLC bioautography. In the present study, three methods were trialled and finally method 4, which is a slight modification of the method developed by Rahalison *et al.* (Rahalison *et al.*, 1991) was decided on as the most effective and reproducible method for the detection of the antibacterial activity of extracts and fractions in this study. The methods that were trialled are described below.

Method 1: This method was originally described by Hamburger and Cordell (Hamburger and Cordell, 1987). To perform this method, extracts, fractions or pure compounds were run on TLC plates with an appropriate developing solvent. A suspension of microorganisms in a suitable broth was applied to the developed TLC plates using a sterile pippette. The TLC plates were incubated overnight in a box containing wet cotton wool. Incubation in a humid atmosphere permits the growth of the bacteria. After incubation, zones of inhibition were visualised by adding the MTT reagent (2.5 mg/mL MeOH solution). While performing this method it was observed that although bioactive compounds were visible on the TLC plate, often, an uneven growth of bacteria was observed on the TLC plate, especially in the middle of the TLC plate, leading to difficulties in obtaining reproducible results. Figure 3.3.6 shows the TLC bioautography performed by method 1.



Figure 3.3.6: TLC bioautography (method 1). Yellow arrow indicates less bacterial growth.

Method 2: TLC bioautography was also tried by the method described by Gibbons *et al.* (Gibbons, 2005). Extracts or fractions or pure compounds were run on TLC plates with an appropriate developing solvent (as described before). An inoculum of bacteria in Mueller

Hinton broth II was prepared and then 7.5 g/l nutrient agar was added to the broth to thicken the medium. The final titre of the prepared inoculum was typically 10^5 cfu/mL. The TLC plates were then placed on the agar base, silica side up, and covered with the inoculum. After overnight incubation, inhibition zones were visualised by adding the MTT solution for 15 min. Although this method was successful in detecting the antibacterial compounds on the TLC plate, it was seen that while working with the aluminium backed TLC plates, the silica gel sometimes swelled and cracked, making it difficult to determine the antibacterial activity (Figure 3.3.7).

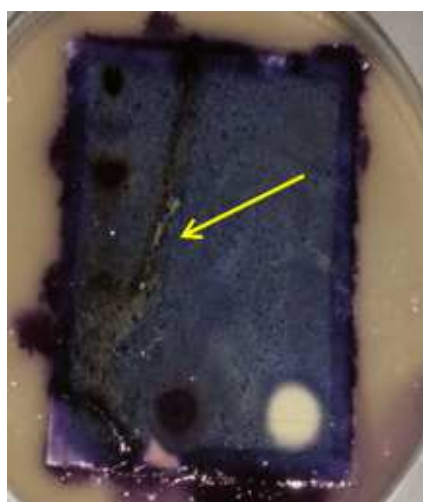


Figure 3.3.7: TLC bioautography (method 2). Yellow arrow indicates the cracked area.

Method 3: To overcome the difficulty of the above methods, another method described by Rahalison *et al.* (Rahalison *et al.*, 1991) was tried. In this method, an overnight culture of the microorganism was prepared in a suitable broth. The molten agar was kept in a water bath at 45 °C to maintain the molten state. The final titre of the microorganism was adjusted to an $OD_{600} = 1$ in the molten solid medium. The developed TLC plates were then placed on a hot plate at 35 °C, and the inoculum rapidly distributed over the plates. After solidification of the medium, the TLC plates were then incubated overnight in a box lined with wet cotton wool. The inhibition zones were visualised using MTT solution (as described before). Generally this method was very good for the detection of the antimicrobial components on the TLC plate. However, while using this method it was observed that sometimes the agar dried and the bacterial growth on the TLC plate was not uniform (Figure 3.3.8). To overcome this difficulty,

slight modifications were made in our laboratory. The modified TLC bioautography (method 4) proved to be a very useful and reproducible method for bioassay guided isolation of bioactive compounds.

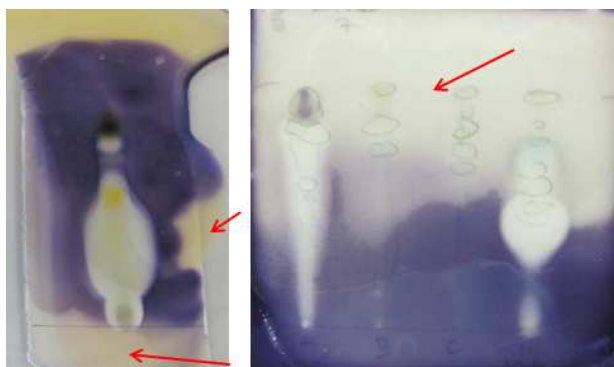


Figure 3.3.8: TLC bioautography (method 3). Red arrows indicate the area where the agar dried up, the bacteria did not grow and the MTT did not convert into the blue formazan.

Method 4: For the present study, the method as described in detail in the materials and methods Section was used. This is very similar to the method described by Rahalison *et al.* (as described above), with the exception that, instead of placing the TLC plates on the hot plate, the developed TLC plates were placed on an agar plate and then the inoculum was added. This provided a perfect environment for the growth of bacteria and an even growth of bacteria was observed all over the plates. It also gave reproducible results (Figure 3.3.9). This method was used for all subsequent TLC bioautography assays reported in this Chapter, along with the majority of the TLC bioautography assays reported in Chapter 4 for *A. excelsa*.



Figure 3.3.9: TLC bioautography (method 4). The bacteria grew evenly all over the plate and the clear area indicates no bacterial growth due to the presence of antibacterial compounds.

3.3.5.2. Isolation of bioactive fractions and compounds from LS-DCM

LS-DCM was the most active extract among the sequential solvent extracts of *L. suaveolens* (Table 3.3.3 and Table 3.3.4). A small scale size exclusion column was performed using 2.6 g of LS-DCM. This yielded 8 major fractions (LSS-1 to LSS-8) according to their normal phase TLC profiles. Antibacterial activities of these 8 major fractions were determined by TLC bioautography using methicillin sensitive (MS) and methicillin resistant strains of *S. aureus* (MRSA and MDRSA) to assist targeted compound isolation. Only LSS-4 and LSS-5 were active by TLC bioautography against MS, MRSA and MDRSA. Further purification of LSS-4 using normal phase PTLC and recrystallisation yielded the pure compound **LS-29**. Further fractionation of LSS-5 (200 mg) using normal phase silica gel afforded four major fractions (LSS-5-I, LSS-5-II, LSS-5-III, LSS-5-IV), in order of elution and according to their TLC profiles. Among them, LSS-5-III and LSS-5-IV were active by TLC bioautography against MS, MRSA and MDRSA. On the normal phase TLC plate (*n*-hexane:EtOAc, 2:1) LSS-5-III showed one main component and some other minor impurities at the baseline and near the solvent front. Further normal phase PTLC of LSS-5-III removed the greenish impurities near the baseline and solvent front. This gave what appeared to be a single spot by TLC, LSS-5-IIIA (3.0 mg), but was found to be a mixture, as detected by ^1H and ^{13}C NMR. The compound mixture was non UV active and gave a positive test for terpenoids (with anisaldehyde spray reagent). ^1H NMR also showed features consistent with the presence of terpenoids (unresolved upfield methyl and methylene protons) along with some other characteristic groups (doublet of doublets at 2.8 ppm and multiplet at 5.2 ppm, the latter suggesting the presence of a double bond). Normal phase preparative HPLC might purify the fraction, but was not available. Moreover, the amount of sample was also very small. Normal and reversed phase TLC of LSS-5-IV showed the presence of several components within a very close R_f region (difference from each other was 0.1-0.2). Reversed phase HPLC (using $\text{H}_2\text{O}:\text{CH}_3\text{CN}$ from 90:10 to 10:90) also showed the presence of at least eight compounds. Purification of LSS-5-IV was not carried out due to time constraints and the limited quantity and complexity of the sample.

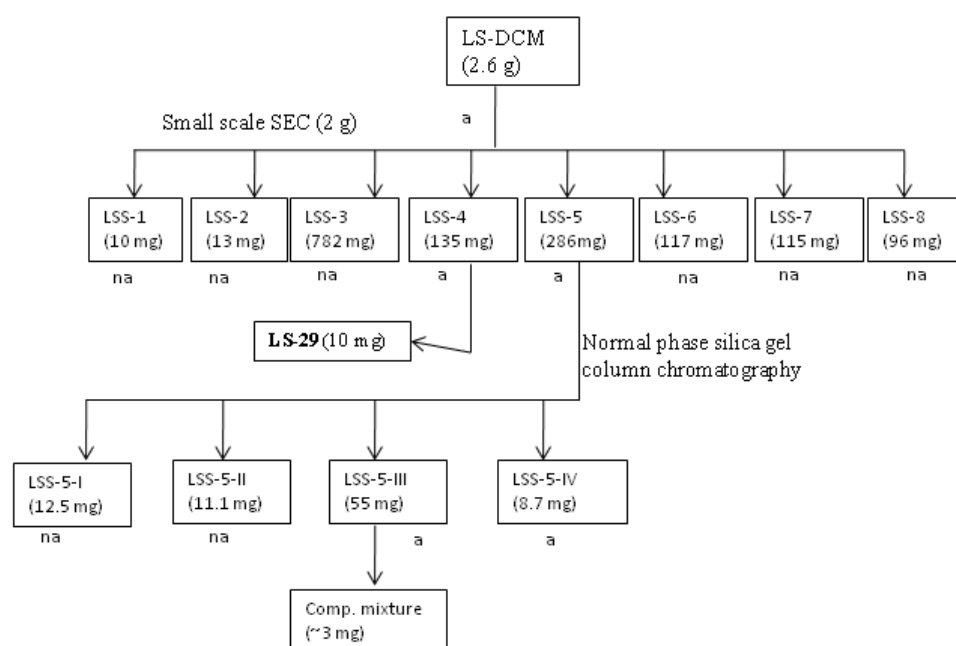


Figure 3.3.10: Small scale fractionation of LS-DCM extract. a: active in TLC bioautography against methicillin sensitive and resistant strains of *S. aureus*. na: not active in TLC bioautography.

Large scale normal phase silica gel column chromatography was conducted on the LS-DCM extract (8.0 g) after finding that components were stable on a normal phase silica gel 2D TLC plate (Leonard *et al.*, 2013). This afforded 12 fractions (LSL-1 to LSL-12) based on their TLC profiles. Antimicrobial activity of the fractions was determined by the MTT microdilution assay and TLC bioautography (Table 3.3.7 and Figure 3.3.12). Fractions LSL-10 to LSL-12 showed the most promising antibacterial activity against all the tested Gram-positive bacteria (methicillin sensitive *S. aureus*, MRSA, MDRSA) and *S. pyogenes* ($IC_{90} < 50 \mu\text{g/mL}$) (Table 3.3.7). Fractions LSL-4 and LSL-5 exhibited moderate to low levels of antibacterial activity (IC_{90} 125-1000 $\mu\text{g/mL}$). All major fractions (LSL-1 to LSL-12) were inactive against the tested Gram-negative bacteria *S. typhimurium*, *P. aeruginosa*, *E. coli* (β lactamase positive) and *E. coli* (β lactamase negative) in the MTT microdilution assay (Table 3.3.7). The fractions were also examined for their bactericidal or bacteriostatic effect.

It was found that fractions LSL-4, 5, 10, 11 and 12 (active fraction in the MTT microdilution assay) were bactericidal against *S. pyogenes* and methicillin sensitive and resistant strains of *S. aureus* (Table 3.3.7). MIC values of fractions LS-7 and LS-8 could not be determined because

of forming a gel in 20% DMSO/H₂O during sample preparation. Even after sonication and vortexing, gelling occurred in the well, making it impractical to pipette the sample in the assay plate for serial dilution. However, antibacterial activities of those fractions (LSL-7 and LSL-8) as well as of the other fractions were also evaluated using TLC bioautography (Figure 3.3.12).

Fractions LSL-1 to LSL-12 were tested for antibacterial activity by TLC bioautography. LSL-4, 5, 7, 10, 11 and 12 showed antibacterial activity against *S. aureus*, MRSA and MDRSA (Figure 3.3.12). Only methicillin sensitive and resistant strains of *S. aureus* were selected for testing by TLC bioautography as none of the extracts or fractions of *L. suaveolens* were active against the tested Gram-negative bacteria in the disc diffusion or MTT microdilution assays. *S. pyogenes* was not used for TLC bioautography as the blood agar required for its growth would interfere with the colour change in the MTT assay.

The most promising fraction, LSL-11, was subjected to size exclusion chromatography. This yielded five sub-fractions, LSL-11-I to LSL-11-V, based on their similarity in normal phase TLC profiles (*n*-hexane:EtOAc, 1:1). LSL-II, LSL-IV and LSL-V were active by TLC bioautography. While concentrating LSL-11-II using rotary evaporation, a white solid (~10 mg) was deposited on the bottom of the round bottom flask. This white solid was separated and checked by TLC bioautography against Gram-positive and Gram-negative bacteria (MS, MRSA, MDRSA, *E. coli* and *P. aeruginosa*). The solid was found to be inactive against all the bacteria. The remaining portion (~25 mg) was a greenish solid and was active by TLC bioautography. It did not show any separation by reversed phase TLC and exhibited a tailing in reversed phase solvents systems (e.g. CH₃CN:H₂O or MeOH:H₂O in different ratios). On normal phase TLC it showed a single spot (upon spraying with anisaldehyde reagent) along with some impurities near R_f region 0.8 (*n*-hexane:EtOAc, 2: 1) and near the baseline. Normal phase PTLC (*n*-hexane: EtOAc, 2:1) removed the impurities near the baseline and other impurities and yielded LSL-11-IIA (2.5 mg) (R_f = 0.45 in *n*-hexane:EtOAc, 2:1, normal phase). ¹H NMR and LC-MS showed that this was a mixture. LC-MS (ESI MS) (negative ion mode) showed the presence of at least three components (*m/z* 456, 368 and 541). The sample with mass *m/z* 456 was the major component, and was consistent with betulinic acid, which was also found in the fractions LSS-4 and LSL-7 (see Section 3.3.7.2). The ¹H NMR spectrum was also in agreement with the presence of betulinic acid, along with a complex aliphatic mixture, as seen by the complex clutter of unresolved signals in the aliphatic region (0.5-3.0

ppm). LSL-11-IIA was found to be inactive by TLC bioautography against methicillin sensitive and resistant *S. aureus*. None of the other fractions of LSL-11 (IV and V) showed any distinctive separation by TLC using normal phase silica with a variety of solvents (e.g. *n*-hexane:EtOAc, CHCl₃:MeOH, without and with 0.1% formic or acetic acid in the solvent system) and reversed phase (CH₃CN:H₂O with and without adding formic acid). These fractions always appeared with major tailing on the TLC plate.

LSL-12, which also showed good antimicrobial activity, was also subjected to size exclusion chromatography. This afforded four sub-fractions LSL-12-I, LSL-12-II, LSL-12-III and LSL-12-IV, in order of elution according to similar TLC profiles. Only LSL-12-II and LSL-12-IV were active by TLC bioautography. Attempted purification of LSL-12-II using normal phase PTLC yielded another mixture LSL-12-IIA (1.5 mg), which was visible by UV light (254 nm) and showed a single spot by TLC under various solvent systems (e.g. *n*-hexane:EtOAc, *n*-hexane:DCM). LSL-12-IIA was active by TLC bioautography against methicillin sensitive and resistant *S. aureus*. ¹H NMR showed one phenolic hydroxyl group or carboxyl group (13.0 ppm), several aromatic hydrogens (6.8-8 ppm), and a complex clutter of signals in the aliphatic region (0.8-2.6 ppm). Therefore, purification of LSL-12-IIA (1.5 mg) was not carried out any further. Although LSL-12-IV was active by TLC bioautography, even after trying different normal and reversed phase solvent systems (*n*-hexane:EtOAc, petroleum ether:Et₂O, CH₃CN:H₂O or MeOH: H₂O) compounds in that fraction were tailing on the TLC plate, instead of separating into discrete spots.

Purification of LSL-5 using Sephadex LH-20 provided a yellow crystalline substance (5 mg). Further recrystallisation using MeOH yielded pure compound **LS-22** (3 mg) (eucalyptin) as yellow needles (Section 3.3.7.1). Eucalyptin (LS-22) has already been reported in the literature for having antibacterial activity against methicillin sensitive and resistant strains of *S. aureus* (Takahashi *et al.*, 2004). Eucalyptin appeared as a bioactive compound on the TLC plate in the TLC bioautography assay. The MIC of eucalyptin (LS-22) could not be determined in the MTT assay at 100 µg/mL. During sample preparation with 20% DMSO/H₂O (v/v) for the assay, the eucalyptin formed a suspension. The assay was still conducted, but eucalyptin was seen as a cloudy suspension in the wells of the assay plate and did not exhibit any inhibition in the MTT microdilution assay.

Washing of the gel like material of LSL-7 (Figure 3.3.11) with *n*-hexane and repeated recrystallisation with MeOH yielded further LS-29 (betulinic acid) (115 mg). The isolated compound **LS-29** (betulinic acid) was found to be inactive in both the TLC bioautography (at 100 µg) and MTT microdilution assay (at 250 µg/mL).

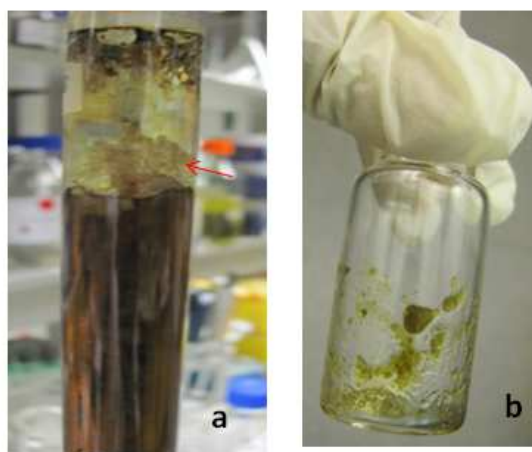


Figure 3.3.11: LSL-7 fraction, which formed a gel when DMSO/H₂O mixtures were added. 'a' represents the gel formation of LSL-7 in a test tube after column chromatography and 'b' represents a small portion of LSL-7 in the DMSO/H₂O mixture.

Fractionation of LSL-4 and LSL-10 was not carried out as both of them showed no separation by TLC in normal or reversed phase systems (*n*-hexane:EtOAc, petroleum ether:Et₂O, CH₃CN:H₂O or MeOH:H₂O) and time was limited. Future work can be carried out on these fractions by trying other methods of separation, such as MCI gels or normal phase HPLC. MCI gel CHP 20P can be especially useful for isolation of terpenoids (Kashiwada *et al.*, 1998, Fujioka *et al.*, 1994, Xiao *et al.*, 2013).

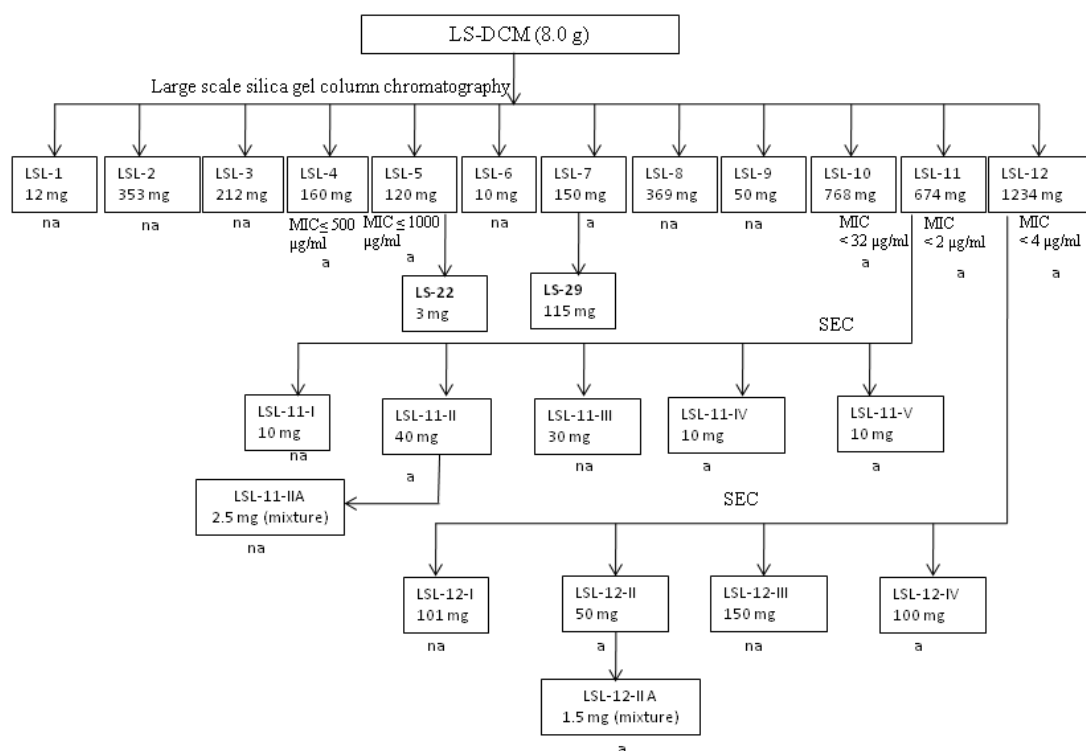


Figure 3.3.12: Large scale fractionation of LS-DCM extract. a: active in TLC bioautography against methicillin sensitive and resistant strains of *S. aureus*. na: not active in TLC bioautography. MIC represents IC₉₀ value in MTT microdilution assay against methicillin sensitive and resistant strains of *S. aureus*.

3.3.5.3. Isolation of bioactive compounds from LS-Hex extract

2D TLC showed LS-Hex was stable to normal phase silica gel. Therefore, LS-Hex (3.3 g) was attempted to be purified by normal phase silica chromatography. After application of the sample with petroleum ether (Bp. 40-60 °C) and elution with petroleum ether, a yellowish waxy solid was deposited on the top of the column. As a result, the solvent was not able to pass through the column and the column got dried and cracked. The waxy solid, LS-Hex-A (~400 mg) was scraped out from the column and dried on a vacuum pump. Washing the separated waxy material with petroleum ether and recrystallisation with MeOH provided pure **LS-22** (15 mg).

After removing the waxy substance, the remaining LS-Hex-B extract (2.3 g) was further subjected to silica gel chromatography. This yielded six major fractions (Hex-1 to Hex-6, in order of elution) (Figure 3.3.13). Hex-3, Hex-4 and Hex-5 were active by TLC bioautography against antibiotic sensitive and resistant strains of *S. aureus*. Washing of Hex-5 with *n*-hexane

and recrystallisation with MeOH also yielded **LS-22** (31 mg). Normal phase PTLC of another bioactive fraction, Hex-5, yielded an impure compound mixture LS-Hex-15 (2 mg). This was visible at 254 nm and showed up as a blue spot upon spraying with anisaldehyde reagent. Normal phase PTLC removed the impurities near the baseline and showed as a single spot on the TLC plate ($R_f = 0.6$, petroleum ether:DCM, 2:1). However, it appeared as a mixture of compounds by ^1H NMR. In the ^1H NMR spectrum, there were well defined signals at ~10 ppm, consistent with aldehydic protons, but there was no distinct separation of signals in the aliphatic region (0.5-3 ppm), which appeared as a complex mixture. Later TLC bioautography revealed LS-Hex-15 to be inactive against methicillin sensitive and resistant *S. aureus*. Purification of Hex-3 (50 mg) was not performed because of time constraints.

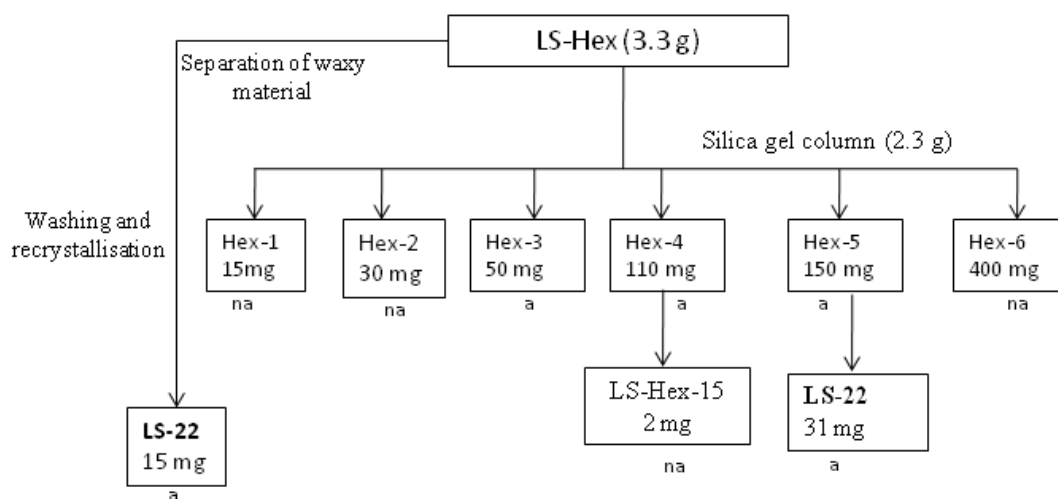


Figure 3.3.13: Isolation of bioactive fractions from LS-Hex extract. a: active in TLC bioautography against methicillin sensitive and resistant strains of *S. aureus*. na: not active in TLC bioautography.

Table 3.3.7: MIC of large scale column chromatography partitioned fractions from the DCM extract of *L. suaveolens* in MTT microdilution assay and bacteriostatic or bactericidal effect.

Fractions from LS-DCM	MIC values (µg/mL)				
	<i>S. aureus</i>	MRSA	MDRSA	<i>S. pyogenes</i>	<i>S. typhimurium</i> , <i>P. aeruginosa</i> , <i>E. coli</i> (β+) & (β-)
LSL-1	na	na	na	na	na
LSL-2	na	na	na	na	na
LSL-3	na	na	na	na	na
LSL-4	500* (C)	500* (C)	500* (C)	125** (C)	na
LSL-5	1000* (C)	1000* (C)	na	15.6**** (C)	na
LSL-6	na	na	na	na	na
LSL-7	#	#	#	#	#
LSL-8	#	#	#	#	#
LSL-9	na	na	na	na	na
LSL-10	31.25**** (C)	31.25**** (C)	7.8**** (C)	0.98**** (C)	na
LSL-11	1.9**** (C)	1.9**** (C)	1.9**** (C)	0.24**** (C)	na
LSL-12	1.9**** (C)	3.9**** (C)	1.9**** (C)	0.49**** (C)	na
LS-22	#	#	#	#	#
LS-29	#	#	#	#	#
Vancomycin	1.25**** (C)	1.25**** (C)	1.25**** (C)	1.25**** (C)	na
DMSO (2%)	na	na	na	na	na

***IC₉₀ <50 µg/mL, **IC₉₀ 50-125 µg/mL, *IC₉₀ 126-1000 µg/mL. C: bactericidal effect on the microorganisms.

MIC was determined as the well with the lowest concentration of sample that displayed no yellow-blue colour change of MTT and also by turbidity (absorbance). Two repeats were performed on at least two separate occasions; values are average of observed results. na: not active against the tested bacteria at 1000 µg/mL. # MIC could not be determined because of forming gels/suspension in initial 20% DMSO/H₂O.

3.3.6. Summary of bioassay guided studies

A summary of bioassay guided studies and isolation of bioactive compounds from *L. suaveolens* have been presented in Figure 3.3.14.

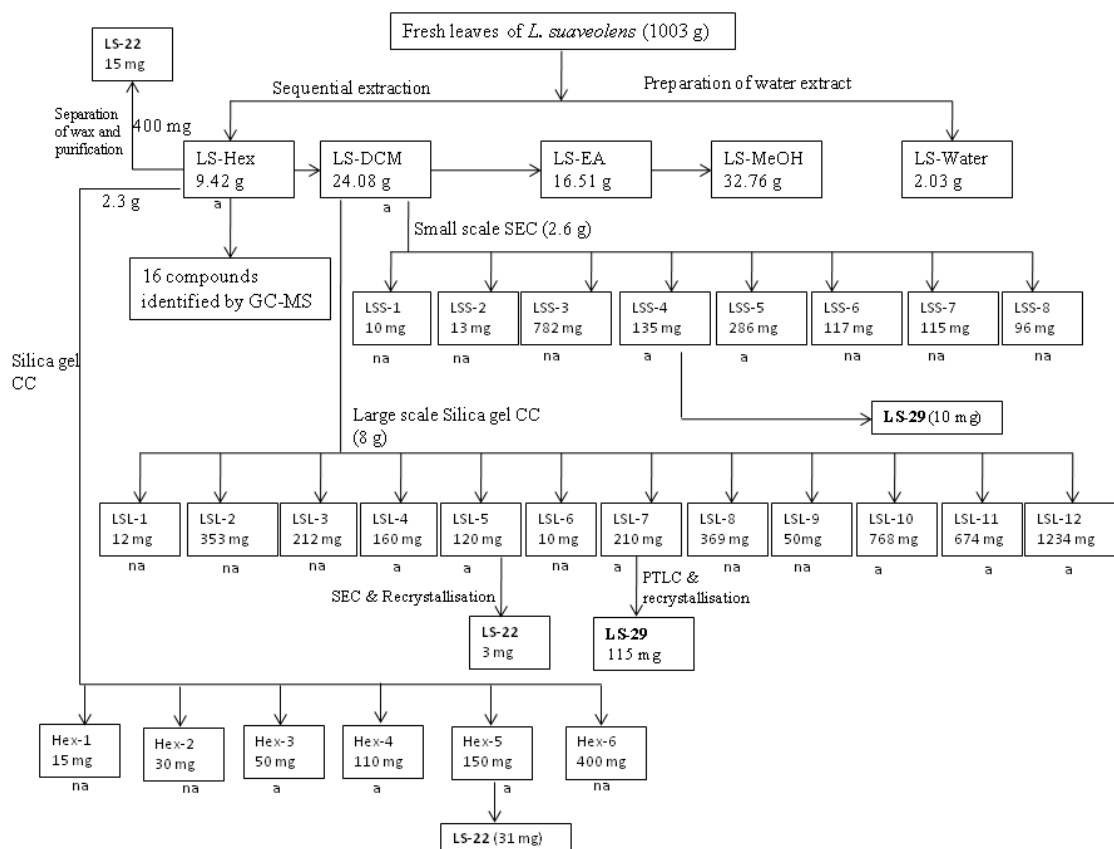


Figure 3.3.14: Bioassay guided isolation of bioactive compounds from *L. suaveolens*. a: active in TLC bioautography against methicillin sensitive and resistant strains of *S. aureus*. na: not active in TLC bioautography.

3.3.7. Characterisation of bioactive compounds

The structures of the bioactive compounds LS-22 and LS-29 (Figure 3.3.15, Figure 3.3.18) were elucidated by 1D and 2D NMR and mass spectrometry and also by comparison with reported data. The structure elucidation is described below.

3.3.7.1. 4',7-Dimethoxy-6,8-dimethyl-5-hydroxyflavone (LS-22)

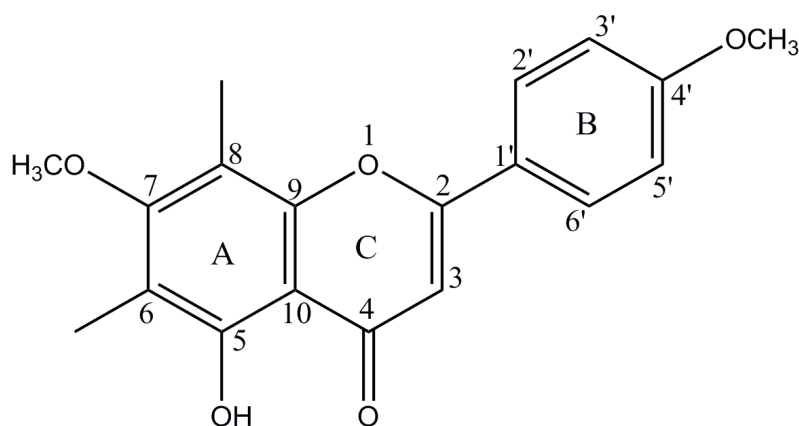


Figure 3.3.15: 4',7-Dimethoxy-6,8-dimethyl-5-hydroxyflavone.

Compound LS-22 was isolated from the LS-DCM and LS-Hex extracts, with the greatest quantity being from the LS-Hex extract (5.7% w/w) as yellow crystals (184-185 °C). The molecular ion peak of compound LS-22 by HREIMS was at m/z 326.1154. This is consistent with a formula of $C_{19}H_{18}O_5$ (calcd. 326.1154). Its EIMS (Figure 3.3.16) showed two significant fragment ions at m/z 311 and m/z 296, which is characteristic of the loss of CH_3 groups. The IR spectrum showed a highly conjugated carbonyl stretching band at 1650 cm^{-1} .

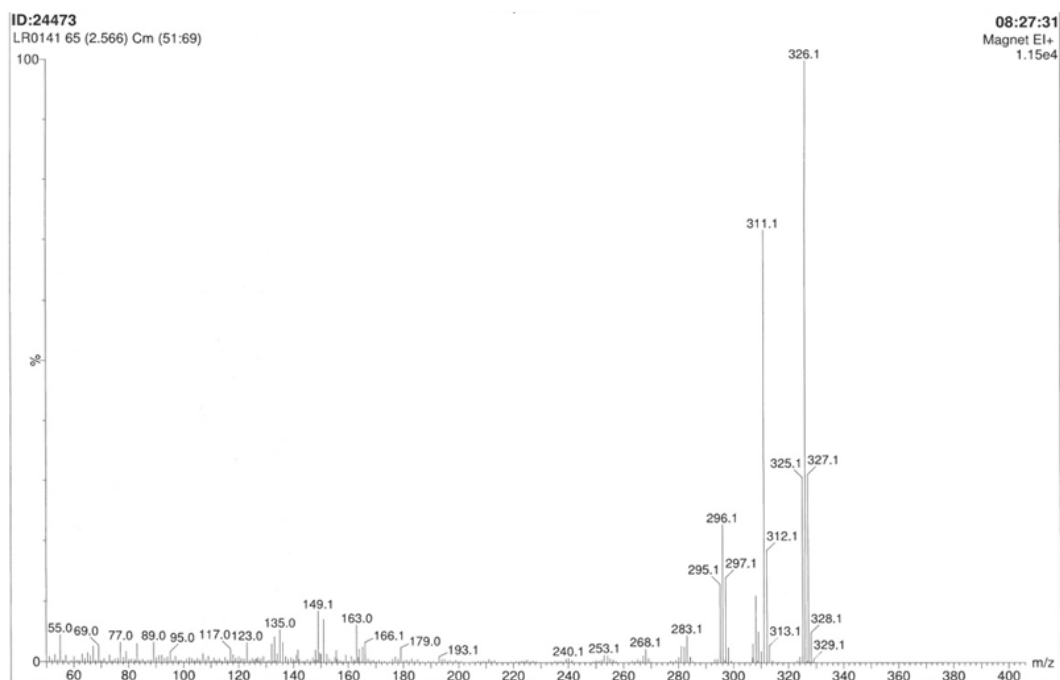


Figure 3.3.16: EIMS fragmentation of LS-22.

The ^1H NMR spectrum gave signals for two CH_3 groups at δ_{H} 2.37 and δ_{H} 2.19, consistent with attachment to an aromatic ring, and two OCH_3 groups at δ_{H} 3.77 and δ_{H} 3.87, a hydrogen bonded hydroxyl proton at δ_{H} 12.85 and an olefinic proton at δ_{H} 6.60. A characteristic *para* disubstitution pattern in the aromatic region (δ_{H} 7.86, d, $J = 8.8$ Hz and δ_{H} 7.02, d, $J = 8.8$ Hz) was also seen in the ^1H NMR.

The ^{13}C NMR spectrum showed a total of 17 signals for 19 carbons. The 1D spectrum and DEPT 135 NMR identified signals for a carbonyl carbon at (δ_{C} 183.5), two methyl carbons (δ_{C} 8.5 and δ_{C} 8.8) and two methoxy carbons (δ_{C} 55.8 and δ_{C} 60.6).

The ^{13}C NMR along with HSQC data identified ten quaternary carbons at δ_{C} 164.1 (C-2), δ_{C} 183.5 (C-4), δ_{C} 157.5 (C-5), δ_{C} 114.2 (C-6), δ_{C} 153.1 (C-7), δ_{C} 109.0 (C-8), δ_{C} 162.8 (C-9), δ_{C} 107.5 (C-10), δ_{C} 124.1 (C-1') and δ_{C} 162.8 (C-4'). The presence of a very deshielded resonance of a hydrogen bonded hydroxyl proton at δ_{H} 12.85 (5-OH), suggested that it was *peri* to the carbonyl group at δ_{C} 183.5 (C-4). Two aromatic methyl protons at δ_{H} 2.19 (s, 6- CH_3) and δ_{H} 2.37 (s, 8- CH_3) showed HSQC correlations to the signals at δ_{C} 8.5 and δ_{C} 8.8, respectively. The methyl group at δ_{H} 2.19 (6- CH_3) was assigned *ortho* to the hydrogen-bonded

hydroxyl group (5-OH) and the methoxy group (7-OCH₃) according to its $^3J_{CH}$ HMBC correlation (Figure 3.3.17) with δ_c 157.5 (C-5) and δ_c 153.1 (C-7). While the methyl group at δ_H 2.37 (8-CH₃) was assigned as at C-8 and *ortho* to 7-OCH₃ due to $^3J_{CH}$ HMBC correlations with δ_c 153.1 (C-7) and δ_c 162.8 (C-9).

Two aromatic protons appeared at δ_H 7.86 (2H, d, J = 8.8 Hz, H-2', H-6') and two protons at δ_H 7.02 (2H, d, J = 8.8 Hz, H-3', H-5') with a characteristic *para* disubstitution pattern. These protons showed HSQC correlations to the signals at δ_c 128.2 (C-2' and C-6') and δ_c 114.8 (C-3' and C-5'), respectively. In the HMBC spectrum, both aromatic protons showed correlations with each other and also with a carbon at δ_c 162.8 (C-4'). The singlet at δ_H 6.60 was assigned to an olefinic proton (H-3) and this was confirmed from the observation of an HMBC correlation with δ_c 164.1 (C-2), δ_c 124.1 (C-1') and δ_c 107.5 (C-10). The NMR data is presented in (Table 3.3.8).

The HMBC spectrum also showed correlations of the protons at δ_H 7.86 (H-2' and H-6') to the carbon signal at δ_c 164.1 (C-2), confirming the phenyl group attachment to C-2 of a C ring flavonoid skeleton. Other positions were confirmed by HSQC and HMBC data, which are presented Table 3.3.8 and Figure 3.3.17.

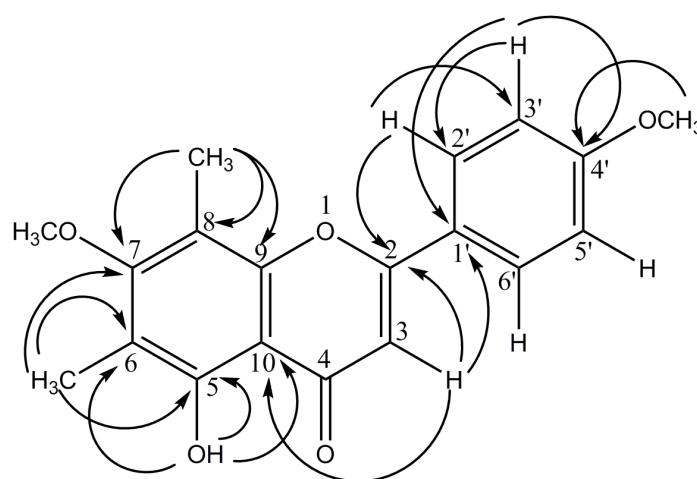


Figure 3.3.17: HMBC correlations of 4',7-dimethoxy-6,8-dimethyl-5-hydroxyflavone.

Comparison of the melting point of LS-22 with that of the published literature (lit. mp. 184-185 °C), also confirmed LS-22 as 4',7-dimethoxy-6,8-dimethyl-5-hydroxyflavone or eucalyptin (Huq and Misra, 1997, Courtney *et al.*, 1983).

C-Methylated flavones (methyl group directly connected to carbon, e.g. in eucalyptin, CH₃ connected with C-6 and C-8) are relatively rare. It appears that C-methyl flavones are distributed throughout both sub-families of the Myrtaceae: the Myrtoideae (*Eugenia*, *Myrcia*) and Leptospermoideae (*angophora*, *eucalyptus*) and they are quite distinctive of the whole family (Courtney *et al.*, 1983, Sarker *et al.*, 2001). This is the first report of isolation and characterisation of 4',7-dimethoxy-6,8-dimethyl-5-hydroxyflavone from *L. suaveolens* and thus adds new phytochemical data of chemotaxonomic significance.

Table 3.3.8: NMR data of 4',7-dimethoxy-6,8-dimethyl-5-hydroxyflavone or eucalyptin (LS-22).

Position	δ_C	δ_H multiplicity	Carbon multiplicity	HMBC
2	164.1		C	
3	104.3	6.60 s	CH	C-10, C-1', C-2
4	183.5		C	
5	157.5		C	
5-OH		12.85 s		C-5, C-6, C-10
6	114.2		C	
6-CH ₃	8.5	2.19 s	CH ₃	C-5, C-6, C-7
7	153.1		C	
7-OCH ₃	60.6	3.77 s	CH ₃	
8	109.0		C	
8-CH ₃	8.8	2.37 s	CH ₃	C-8, C-7, C-9
9	162.8		C	
10	107.5		C	
1'	124.1		C	
2', 6'	128.2	7.86, d, $J = 8.8$ Hz	CH	C-3', 5', C-2, 4'
3', 5'	114.8	7.02, d, $J = 8.8$ Hz	CH	C-2', 6', C-1', C-4'
4'	162.8		C	
4'-OCH ₃	55.8	3.87 s	CH ₃	C-4'

Data acquired in CDCl₃ 600 and 150 MHz for ¹H and ¹³C NMR, respectively.

3.3.7.2. Betulinic acid (LS-29)

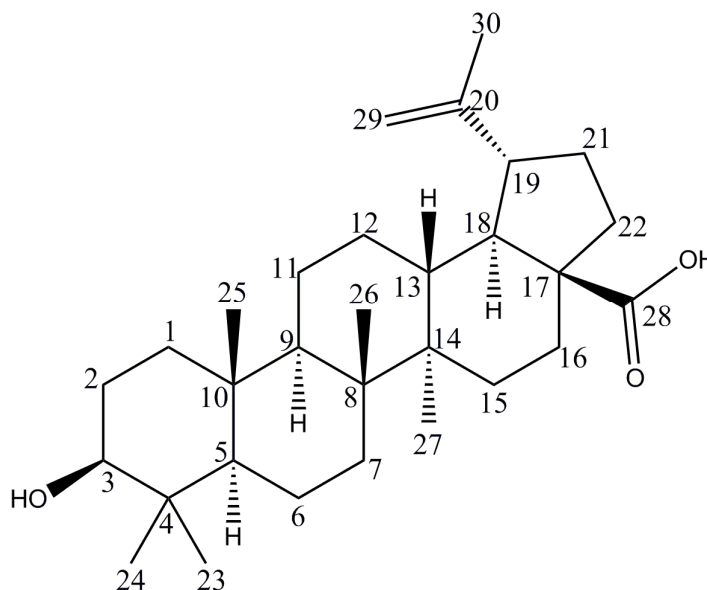


Figure 3.3.18: Betulinic acid.

Compound LS-29 was isolated from the LS-DCM extract as white crystals with mp 283-285 °C. The molecular ion peak of compound LS-29 by HREIMS (negative ion mode) was at m/z 455.3526. This is consistent with a formula of $C_{30}H_{47}O_3$ (calculated m/z 455.3525) and supports the molecular formula $C_{30}H_{48}O_3$. Electrospray ionisation mass spectrometry (ESI MS) (negative ion mode) indicated a deprotonated molecular ion at m/z 455 $[M-H]^-$, which is also consistent with the formula $C_{30}H_{48}O_3$. EIMS also showed a molecular ion peak at m/z 456.

The ^{13}C NMR data confirmed the presence of 30 carbon atoms, including a carboxyl carbon at δ_C 177.26 and an olefinic methylene carbon at δ_C 109.66. DEPT 135 showed eleven (11) CH_2 and a total of twelve (12) CH and CH_3 groups. Therefore, the remaining 7 carbons observed in the ^{13}C NMR spectrum should be quaternary carbons.

The 1H NMR spectrum showed the molecule contains six (6) methyl groups at δ_H 0.64 (s, 3H, CH_3), δ_H 0.76 (s, 3H, CH_3), δ_H 0.864 (s, 3H, CH_3), δ_H 0.867 (s, 3H, CH_3), δ_H 0.92 (s, 3H, CH_3) and δ_H 1.64 (s, 3H, CH_3). These exhibited HSQC correlations to δ_C 15.7, δ_C 15.8, δ_C 15.6, δ_C 28.1, δ_C 14.4 and δ_C 18.8, respectively. In combination with the DEPT 135 analysis, this confirmed the presence of six (6) CH_3 and six (6) CH groups. The characteristic chemical shift

in the ^{13}C NMR spectrum at δ_{C} 177.3 (C=O, C-28) was assigned to a carboxyl carbon. The IR spectrum gave absorption bands at 2939 cm^{-1} (strong, -COO-H) and 1682 cm^{-1} (strong, C=O), confirming a carboxylic acid group present in the molecule.

The chemical shifts at δ_{C} 109.7 ($=\text{CH}_2$, C-29) and δ_{C} 150.2 ($=\text{C}$, C-20) in the ^{13}C NMR spectrum were assigned to olefinic carbons. The methyl group at δ_{H} 1.64 (s, 3H, CH_3 , δ_{C} 18.93) in the ^1H NMR spectrum is characteristic of a methyl directly attached to a double bond. Two doublets at δ_{H} 4.55 (d, 1H, $J = 2.4\text{ Hz}$), δ_{H} 4.68 (d, 1H, $J = 2.4\text{ Hz}$) were consistent with two asymmetric protons on the same carbon of a double bond. HSQC confirmed both δ_{H} 4.55 and δ_{H} 4.68 are connected to a carbon at δ_{C} 109.7 (CH_2 in DEPT 135, C-29). The connectivity of $\text{CH}_2=\text{C}(\text{CH}_3)-$ was further confirmed by HMBC experiments in which the δ_{H} 1.64 (δ_{C} 18.8, H-30) exhibited strong correlations to δ_{C} 46.5 (CH, C-19), δ_{C} 109.7 ($=\text{CH}_2$, C-29) and δ_{C} 150.2 ($=\text{C}$, C-20), as well as a weak correlation to δ_{C} 30.1 (CH_2 , C-21).

The ^1H NMR and ^{13}C NMR DEPT 135 showed no aromatic rings or further double bonds present in the molecule. A calculation of index of hydrogen deficiency (IHD) for the molecular formula of ($\text{C}_{30}\text{H}_{48}\text{O}_3$), however, gave a total IHD of 7 [$\text{N}=(30\times 2+2-48)/2$]. After subtracting two unsaturated bonds (C=C and C=O), the remaining number of IHD in the molecule is 5. The presence of six (6) CH carbons and an additional ten (10) CH_2 strongly suggested the presence of five rings to account for the remaining for 5 IHD.

The assignments of the five rings were largely based on HMBC correlations and the direct bonding of C-H as seen by HSQC. The starting points for assigning the ring E (C-17, C-18, C-19, C-21, C-22) were the already assigned functional groups, namely -COOH (C-28) and - $\text{CH}(\text{CH}_3)\text{C}=\text{CH}_2$ (C-19, C-30, C-20, C-29) (Figure 3.3.20).

In the HMBC spectrum, δ_{H} 2.93 (δ_{C} 46.5, m, 1H, CH, H-19) correlated strongly to δ_{C} 150.2 (C, C-20), δ_{C} 48.4 (δ_{H} 1.51, CH, C-18), δ_{C} 109.7 (δ_{H} 4.55, 4.68, CH_2 , C-29), δ_{C} 30.1 (δ_{H} 1.31, 1.78, CH_2 , C-21), δ_{C} 18.8 (δ_{H} 1.64, CH_3 , C-30) and δ_{C} 37.6 (δ_{H} 2.2, CH, C-13). The signal with the chemical shift at δ_{H} 1.78 (m, 1H, CH_2 , H-21) correlated to δ_{C} 36.2 (δ_{H} 1.42, 1.78, CH_2 , C-22), δ_{C} 46.5 (δ_{H} 2.93, CH, C-19), δ_{C} 48.4 (δ_{H} 1.51, m, CH, C-18) and δ_{C} 55.3 (C, C-17). From COSY, H_A -21 (δ_{H} 1.78, $J = 11.5\text{ Hz}$) was assigned *cis* to H-19 (δ_{H} 1.78) and H_B -21 (δ_{H} 1.31, $J = 11.5\text{ Hz}$) was assigned *trans* to H-19 (δ_{H} 1.78). The signal with the chemical shift at δ_{C} 55.3 (C, C-17) is consistent with a carbon adjacent to a carboxylic acid group.

Collectively, these data allowed the assignment of ring E containing C-17, C-18, C-19, C-21 and C-22 as shown in Figure 3.3.20 (a) (Table 3.3.9).

The assignments of the chemical shifts for the six-membered ring D (C-13, C-14, C-15, C-16, C-17, C-18) adjacent to the ring E were made based on the correlations of H-18, H-13 and H-27. The signal with the chemical shift at δ_{H} 1.51 (δ_{C} 48.4, CH, H-18) showed HMBC correlations to δ_{C} 18.8 (δ_{H} 1.64, CH₃, C-30), δ_{C} 31.7 (δ_{H} 2.1, 1.36, CH₂, C-16), δ_{C} 37.6 (δ_{H} 2.21, CH, C-13), δ_{C} 42.0 (C, C-14), δ_{C} 46.5 (δ_{H} 2.93, CH, C-19), δ_{C} 55.3 (C, C-17), δ_{C} 150.2 (=C, C-20) and δ_{C} 177.3 (C=O, C-28). The chemical shift at δ_{H} 2.21 (δ_{C} 37.6, CH, H-13) showed HMBC correlations to δ_{C} 14.4 (δ_{H} 0.92, CH₃, C-27), δ_{C} 25.1 (δ_{H} 1.60, 0.97, CH₂, C-12), δ_{C} 42.0 (C, C-14), 55.3 (C, C-17) and δ_{C} 48.4 (δ_{H} 1.51, CH, C-18). The signal with the chemical shift at δ_{H} 0.92 (δ_{C} 14.4, CH₃, H-27) correlated to δ_{C} 29.2 (δ_{H} 1.35, 1.4, CH₂, C-15), δ_{C} 37.6 (δ_{H} 2.21, CH, C-13), δ_{C} 40.1 (C, C-8) and δ_{C} 42.0 (C, C-14). These collectively gave the connectivity of the ring D containing C-13, C-14, C-15, C-16 and C-17 and its connectivity to C-18, C-12 and C-27, as shown in Figure 3.3.20 (b) (Table 3.3.9).

The IR spectrum gave an absorption band at 3440 cm⁻¹ (strong, broad), which is characteristic of an alcohol -OH group. Using the -OH group as the starting point, the A ring containing C-1, C-2, C-3, C-4, C-5 and C-10 and the associated methyl groups C-23, C-24 and C-25 were assigned. In the ¹³C NMR spectrum, the carbon at δ_{C} 76.77 was assigned to the carbon directly attached to the -OH group. In the HMBC experiment, the proton of the -OH at δ_{H} 4.25 (d, *J* = 5.2 Hz) correlated to δ_{C} 76.8 (δ_{H} 2.96, CH, C-3), δ_{C} 38.5 (C, C-4) and δ_{C} 27.2 (δ_{H} 1.42, CH₂, C-2). The signal with the chemical shift at δ_{H} 2.96 (δ_{C} 76.8, CH, H-3) correlated to δ_{C} 15.7 (δ_{H} 0.64, CH₃, C-23) and δ_{C} 15.6 (δ_{H} 0.864, CH₃, C-24, being *cis* to OH). The methyl groups at δ_{H} 0.64 and δ_{H} 0.864 were found to be directly attached to δ_{C} 15.7 and δ_{C} 15.6 by HSQC. They showed strong HMBC correlations to δ_{C} 76.8 (δ_{H} 2.96, CH, C-3) and to δ_{C} 54.9 (δ_{H} 0.62 m, CH, C-5) and were assigned as δ_{C} 15.7 (δ_{H} 0.64, CH₃, C-23) and δ_{C} 15.6 (δ_{H} 0.864, CH₃, C-24, being *cis* to OH). The signal with the chemical shift at δ_{H} 0.62 m (δ_{C} 54.9, CH, H-5) showed HMBC correlations to δ_{C} 15.7 (δ_{H} 0.64, CH₃, C-23), δ_{C} 15.6 (δ_{H} 0.864, CH₃, C-24), δ_{C} 15.8 (δ_{H} 0.76, C-25) and δ_{C} 17.9 (δ_{H} 1.44, 1.3 m, CH₂, C-6). These gave the connectivity of the A ring and the methyl groups at C-23, C-24 and C-25, as shown in Figure 3.3.21 (c) (Table 3.3.9).

The six-membered B ring containing C-5, C-6, C-7, C-8, C-9 and C-10, the methyl groups on the ring at C-25 and C-26, and their connectivity with C-8 and C-11, were assigned using the connectivity of H-5, H-6, H-9, H-25 and H-26 with the carbons.

The signal with the chemical shift at δ_{H} 0.76 (δ_{C} 15.8, CH₃, H-25) showed HMBC correlations to δ_{C} 15.6 (δ_{H} 0.864, CH₃, C-24), δ_{C} 28.1 (δ_{H} 0.867, CH₃, C-26), δ_{C} 36.6 (C, C-10), δ_{C} 38.1 (δ_{H} 1.55, 0.82, CH₂, C-1), δ_{C} 49.8 (δ_{H} 1.23, CH, C-9) and δ_{C} 54.9 (δ_{H} 0.62 m, CH, C-5). The signal with the chemical shift at δ_{H} 1.23 (δ_{C} 49.8, CH, H-9) correlated to δ_{C} 15.8 (δ_{H} 0.76, CH₃, C-25), δ_{C} 20.3 (δ_{H} 1.3, 1.23, CH₂, C-11), δ_{C} 36.6 (C, C-10) and δ_{C} 40.1 (C, C-8). The signal with the chemical shift at δ_{H} 0.867 (δ_{C} 28.1, CH₃, H-26) correlated to δ_{C} 33.9 (δ_{H} 1.34, CH₂, C-7), δ_{C} 40.1 (C, C-8), δ_{C} 49.8 (δ_{H} 1.23, CH, C-9) and δ_{C} 42.0 (C, C-14). These gave the connectivity of the B ring, the methyl groups in the ring at C-25 and C-26 and their connectivity with C-8 and C-11, therefore assigning the final six-membered C ring containing C-8, C-9, C-11, C-12, C-13 and C-14, as shown in Figure 3.3.21 (d) (Table 3.3.9).

The EIMS showed significant fragment ions at m/z 189 (84.7% relative abundance) and 207 (37.14% relative abundance) (Figure 3.3.19). These fragment ions have been reported as characteristic fragmentations of betulinic acid and other lupane-type triterpenes (Ryu *et al.*, 1992, Macías *et al.*, 1998) also supported the assignment of compound LS-29 as betulinic acid.

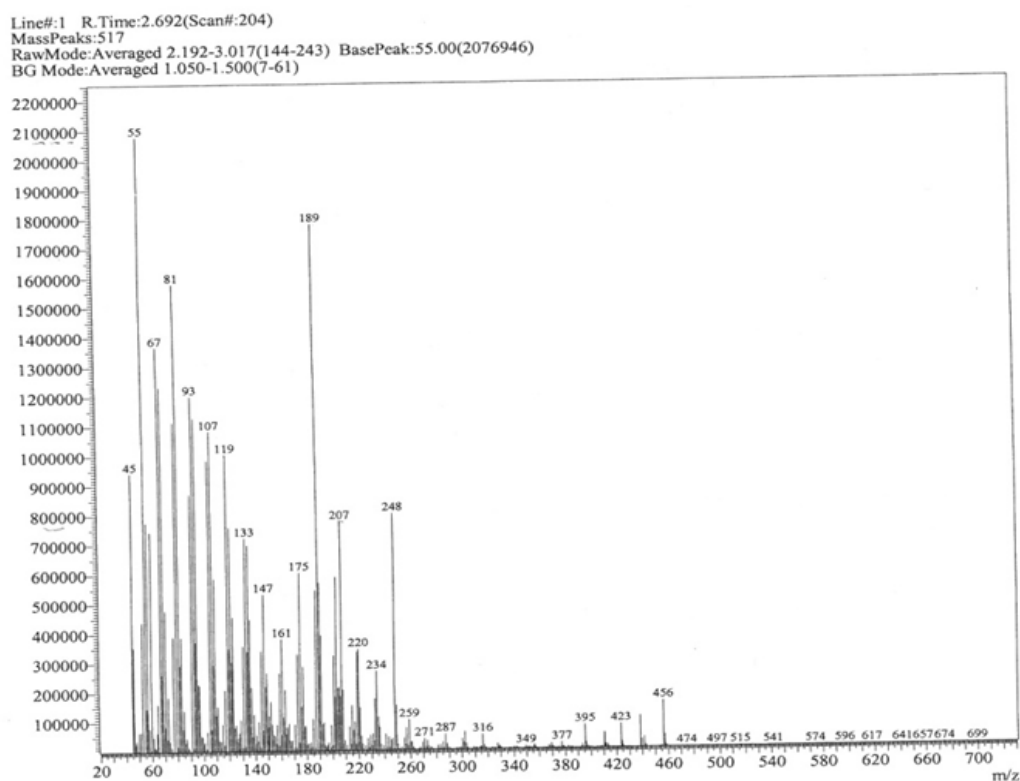


Figure 3.3.19: EIMS fragmentation of LS-29.

Betulinic acid is a well known anti-inflammatory, anticancer and anti-HIV compound (Nguemfo *et al.*, 2009, Tsai *et al.*, 2011, Fulda, 2009, Viji *et al.*, 2011, Potterat and Hamburger, 2008). Betulinic acid was previously isolated from the bark of the white birch (*Betula pubescens*) from which it gets its name and also from the ber tree (*Ziziphus mauritana*), selfheal (*Prunella vulgaris*), jambul (*Syzygium formosanum*), flowering quince (*Chaenomeles sinensis*) and rosemary (Gao *et al.*, 2003, Tan *et al.*, 2003, Zuco *et al.*, 2002). This is the first report of isolation of betulinic acid from *L. suaveolens*.

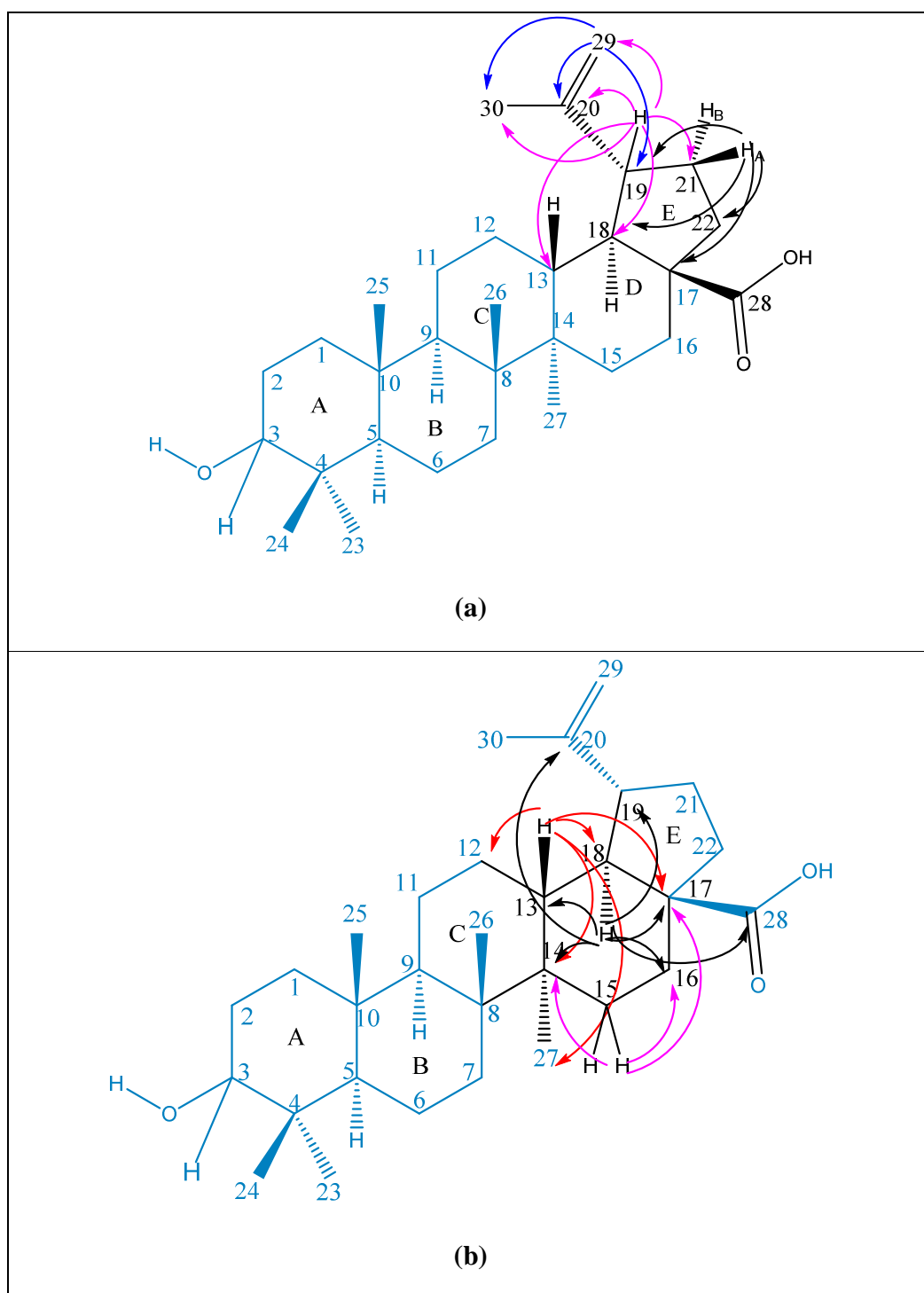


Figure 3.3.20: HMBC of compound LS-29 (a and b).

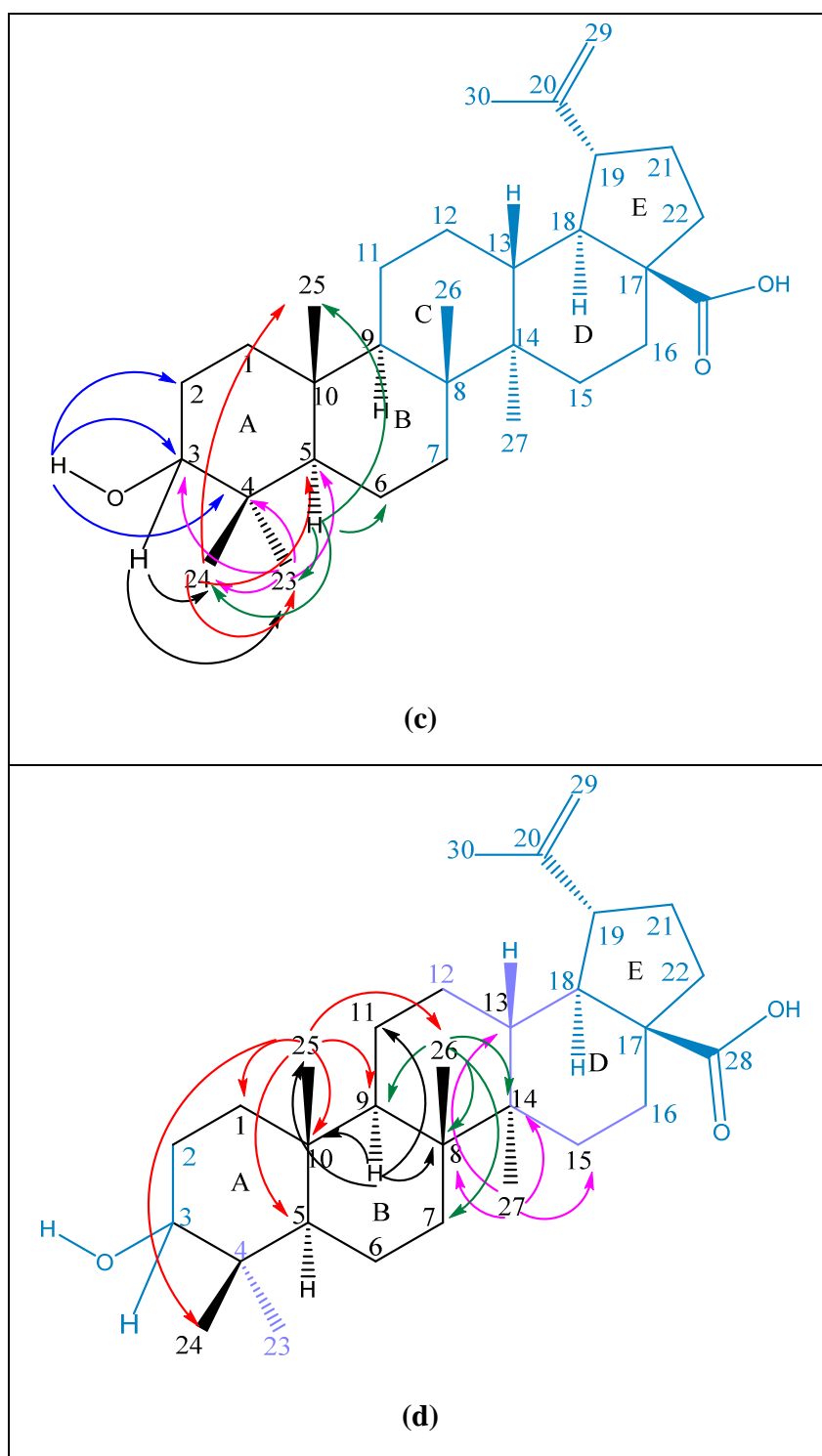


Figure 3.3.21: HMBC of compound LS-29 (c and d).

Table 3.3.9: NMR data assignments of compound LS-29 and commercially available betulinic acid.

Position	¹³ C (Bet. acid)*	¹³ C (LS-29)	δ _H multiplicity	Carbon multiplicity	HSQC data	HMBC
1	38.1	38.1		CH ₂	1.55 m, 0.82 m	
2	27.1	27.2		CH ₂	1.42	
3	76.8	76.8	2.96 m	CH	2.96 m	C-23, C-24
3-OH			4.25 d, <i>J</i> = 5.2 Hz	-	-	C-2, C-3, C-4
4	38.5	38.5		qC	-	
5	54.8	54.9	0.62 m	CH	0.62 m	C-23, C-24, C-25, C-6
6	17.9	17.9		CH ₂	1.44 m, 1.3 m	
7	33.9	33.9		CH ₂	1.34 m	
8	40.2	40.1		qC	-	
9	49.8	49.8		CH	1.23 m	C-25, C-11, C-10, C-8
10	36.6	36.6		qC		
11	20.4	20.3		CH ₂	1.3 m, 1.23 m	
12	25.1	25.1		CH ₂	1.60 m, 0.97 m	
13	37.6	37.6	2.21 ddd (<i>J</i> = 3.6 Hz, 12.7 Hz, 12.7 Hz)	CH	2.21 ddd	C-27, C-14, C-18, C-17, C-12
14	41.9	42.0		qC	-	
15	29.2	29.2		CH ₂	1.4 m, 1.35 m	C-16, C-17, C-14
16	31.7	31.7		CH ₂	2.10 m, 1.36 m	
17	55.4	55.3		qC		
18	48.4	48.4		CH	1.51 m	C-13, C-14, C-16, C-17, C-19, C-30, C-20, C-28
19	46.5	46.5	2.93 m	CH	2.93 m	C-20, C-29, C-18, C-13, C-30, C-21
20	150.3	150.2		qC	-	
21	30.1	30.1		CH ₂	1.78 m, 1.31 m	C-22, C-19, C-18, C-17
22	36.2	36.2		CH ₂	1.42 m, 1.78 m	
23	15.8	15.7	0.64 (s)	CH ₃	0.64 (s)	C-24, C-4, C-5, C-3
24	15.7	15.6	0.864 (s)	CH ₃	0.86 (s)	C-23, C-5, C-25
25	15.8	15.8	0.76 (s)	CH ₃	0.76 (s)	C-24, C-26, C-10, C-1, C-9, C-5
26	28.1	28.1	0.867 (s)	CH ₃	0.86 (s)	C-7, C-8, C-9, C-14
27	14.3	14.4	0.92 (s)	CH ₃	0.92 (s)	C-15, C-13, C-8, C-14
28	177.3	177.3		qC	-	
29	109.7	109.7	4.55 d (<i>J</i> = 2.4 Hz) and 4.68 d (<i>J</i> = 2.4 Hz)	CH ₂	4.55 d, 4.68 d	C-30, C-19, C-20
30	18.8	18.8	1.64 (s)	CH ₃	1.64 s	C-19

*Commercially available betulinic acid (Sigma-Aldrich). Data acquired in DMSO-*d*₆ 600 MHz.

3.3.8. Evaluation of anti-inflammatory and antioxidant activities of *L. suaveolens*

As described earlier, the finding of antibacterial, anti-inflammatory and antioxidant activities in the leaves of *L. suaveolens* could provide a more accessible source of medicine (*i.e.* leaves, rather than the sap) for the Yaegl community for the treatment of skin infections, sores and wounds. Following the determination of antibacterial activity in the LS-Hex and LS-EA extracts and fractions of fresh leaves of *L. suaveolens*, these were examined for anti-inflammatory and antioxidant activities, along with the active fractions LSL-5, 7, 10, 11 and 12 and the isolated compound eucalyptin. While analysis of all the extracts and fractions would have been ideal, these samples were chosen as these assays are very laborious to conduct and there was only a limited timeframe available for these to be run. Anti-inflammatory activity was performed using the NO, TNF- α and PGE₂ inhibition assays and antioxidant activity was performed using the ORAC assay. The basic principles of each assay have been described in Chapter 2. The assays were performed by the author at Southern Cross University under the guidance of Prof. Hans Wohlmuth and with the help of Mr Dane Renshaw and Dr Joshua Smith of the same university.

3.3.8.1. NO inhibition and cytotoxicity assay using RAW264 macrophages

LS-Hex, LS-DCM and LS-water extracts, along with the semi-purified fractions LSL-5, 7, 10, 11 and 12 and pure compound eucalyptin were tested for NO inhibition using the NO inhibition assay (Chapter 2) in RAW264 cells. The extracts and fractions were tested at a concentration range of 0.294 to 71.4 $\mu\text{g/mL}$. Eucalyptin was tested at a concentration range of 0.04 to 8.93 $\mu\text{g/mL}$. Well recognised anti-inflammatory drugs (indomethacine, curcumin, ibuprofen) possess $\text{IC}_{50} < 10 \mu\text{g/mL}$ (Brouet and Ohshima, 1995). Eucalyptin was unable to be tested at a higher concentration due to limited sample availability. In the cytotoxicity study, LS-Hex, LS-DCM and LS-water, and the semi-purified fractions LSL-5, 7, 10, 11 and 12 were tested at a concentration range of 0.05 to 100 $\mu\text{g/mL}$. Cytotoxicity was tested at a higher concentration range (0.05-100 $\mu\text{g/mL}$) than the NO inhibition testing range to check whether extracts/fractions or eucalyptin had any toxic effects that could contribute to NO inhibition in the RAW264 cells.

LS-Hex and LS-DCM extracts inhibited the synthesis of nitric oxide in the LPS stimulated RAW264 macrophages in a dose dependent manner (Table 3.3.10 and Figure 3.3.23) with IC_{50}

values of 43.9 $\mu\text{g/mL}$ and 4.6 $\mu\text{g/mL}$, respectively (Cos *et al.*, 2001). Plant extracts, fractions or compounds that are claimed to have anti-inflammatory activity possess $\text{IC}_{50} < 100 \mu\text{g/mL}$ (Ren and Chung, 2007, Shou *et al.*, 2012). Although both extracts (LS-Hex and LS-DCM) showed good anti-inflammatory activity in the NO inhibition assay, the LS-Hex extract showed a lower cytotoxic concentration (IC_{50} cytotoxicity 5.1 $\mu\text{g/mL}$) than the NO inhibition concentration (IC_{50} 43.9 $\mu\text{g/mL}$) in this assay. Therefore the selectivity index of LS-Hex was less than 1 (meaning cytotoxic) (Table 3.3.10). The selectivity index (SI) can be defined as the ratio of the concentration that reduces cell viability to 50% divided by the IC_{50} value for the inhibition of nitric oxide (Cos *et al.*, 2001, Shou *et al.*, 2012, Jabit *et al.*, 2009). α -Humulene (1.53%) and β -caryophyllene (2.52%) were present as major components in the LS-Hex fraction, as identified by GC-MS analysis. These compounds have been reported to have cytotoxic activities on cancer cell lines (Sylvestre *et al.*, 2007) and β -caryophyllene is reported to have NO inhibitory activity in RAW cells (Tung *et al.*, 2008). It can be assumed that the presence of these components might contribute to the anti-inflammatory and cytotoxic activity of the LS-Hex extract.

The IC_{50} value for the cytotoxic concentration (IC_{50} 9.7 $\mu\text{g/mL}$) of LS-DCM was twice that for the NO inhibition (IC_{50} 4.6 $\mu\text{g/mL}$), therefore, the SI for LS-DCM was more than 2 in the present assay. LS-water did not show any NO inhibition or cytotoxicity in the tested concentration range (Table 3.3.10).

Bioactive fractions from the silica gel column of LS-DCM (*i.e.* LSL-5, 7, 10, 11 and 12), were also tested for NO inhibition and for cytotoxicity. All these fractions were found to have IC_{50} values $< 12 \mu\text{g/mL}$ in the NO inhibition assay. Fraction LSL-5 was the most promising among the tested fractions (IC_{50} 3.7 $\mu\text{g/mL}$). Eucalyptin (LS-22) was isolated from the LSL-5 fraction. There appears to be no reports on whether or not eucalyptin has anti-inflammatory activity; therefore testing of its NO inhibitory activity was of particular interest. However, eucalyptin was unable to be dissolved initially at 2.5 mg/mL with 100% DMSO for the stock solution. Vortexing and sonication also did not help in dissolving. Later the stock solution was diluted (200 fold) with media and cell culture to give the highest tested concentration of 12.5 $\mu\text{g/mL}$ for the cytotoxicity study. This gave a cloudy suspension of eucalyptin, which was used in the cytotoxicity assay and NO inhibitory assay. Nitric oxide (NO) inhibition by eucalyptin was not found in this study at the tested concentration range 0.04-8.93 $\mu\text{g/mL}$. It is

possible that the apparent lack of activity of eucalyptin in the assays is attributable to its poor solubility, but it could also be due to not having any NO inhibitory activity.

Fraction LSL-7 also showed good NO inhibitory activity (IC_{50} 7.1 $\mu\text{g/mL}$) with cytotoxicity (IC_{50} 6.0 $\mu\text{g/mL}$) in RAW264 macrophages. Betulinic acid was the major compound of the LSL-7 fraction. Betulinic acid and its analogues have been reported to have good NO inhibitory activity (Honda *et al.*, 2006, Reyes *et al.*, 2006, Ryu *et al.*, 2000), and other anti-inflammatory activity (Nguemfo *et al.*, 2009, Tsai *et al.*, 2011, Viji *et al.*, 2011). Betulinic acid is also known to have anticancer properties (Pisha *et al.*, 1995, Fulda, 2009). Because the NO inhibitory activity of betulinic acid is known, it was not tested further for anti-inflammatory activity.

The NO inhibitory activity and cytotoxic activity study results and selectivity index for extracts, fractions and eucalyptin (LS-22) are shown in Table 3.3.10, Figure 3.3.22, Figure 3.3.23 and Figure 3.3.24.

The order of anti-inflammatory potential among the tested extracts and fractions in the NO inhibition assay was: LSL-11 > LSL-5 > LSL-12 > LS-DCM > LSL-10 > LSL-7 > LS-Hex. LS-Hex was the most toxic among the tested samples with a selectivity index less than 1, which indicates the effective concentration is higher than the cytotoxic concentration. LS-water did not show any cytotoxicity (up to a concentration of 100 $\mu\text{g/mL}$) or NO inhibition (up to a concentration of 71.4 $\mu\text{g/mL}$).

Table 3.3.10: Cytotoxicity and nitric oxide synthesis inhibitory activity of extracts, fractions and eucalyptin from of *L. suaveolens*.

Samples	Cytotoxicity ($\mu\text{g/mL}$)	Inhibition of NO synthesis ($\mu\text{g/mL}$)	Selectivity index
LS-Hex	5.1 (3.9-6.7)	43.9 (23.1-83.6)	0.1
LS-DCM	9.7 (7.2-13.0)	4.6 (2.9-7.5)	2.1
LS-Water	>100	>71.4	-
LSL-5	23.4 (13.0-42.0)	3.7 (2.4-5.7)	6.3
LSL-7	6.0 (5.3-6.8)	7.1 (4.5-11.2)	0.85
LSL-10	22.4 (9.06-56.0)	11.6 (8.1-16.6)	1.93
LSL-11	81.6 (33.8- 100)	7.0 (4.6-11.7)	11.6
LSL-12	24.95 (21.0-29.5)	8.9 (5.2-15.2)	2.8
Eucalyptin	#	#	-
Dexamethasone	-	0.0062 (0.0025-0.0157)	-
Chlorambucil	8.0 (5.8-11.1)	-	-

Inhibition was measured as IC_{50} (95% confidence intervals); - not relevant, # IC_{50} values could not be found in 0.04 $\mu\text{g/mL}$ to 12.5 $\mu\text{g/mL}$ concentration range for cytotoxicity study, 0.04 $\mu\text{g/mL}$ to 8.93 $\mu\text{g/mL}$ concentration range for NO inhibitory activity and also samples could not be dissolved properly during sample preparation. Dexamethasone and chlorambucil were used as positive controls for NO inhibition assay and cytotoxicity study, respectively. n=3 for both cytotoxicity and NO inhibition activity study.

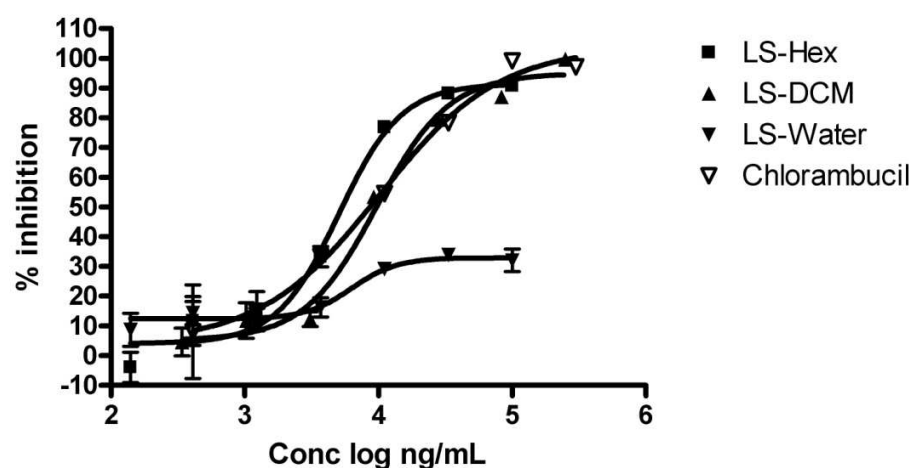


Figure 3.3.22: Cytotoxic effects of LS-Hex, LS-DCM, LS-water and the positive control chlorambucil on RAW264 cells.

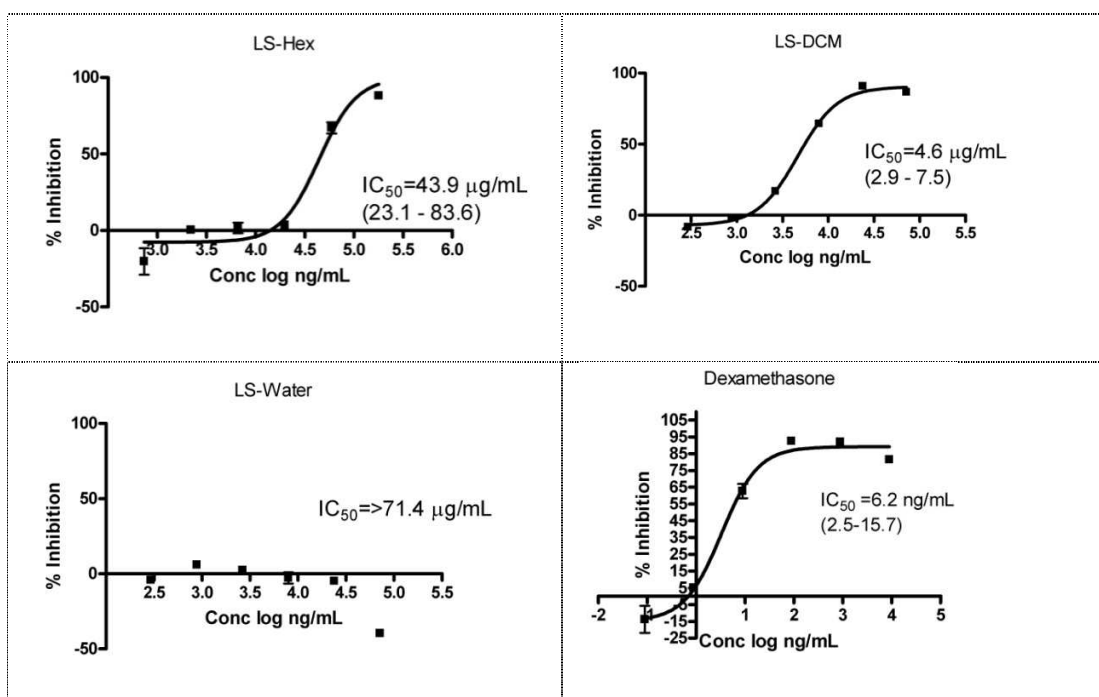


Figure 3.3.23: Inhibition of nitric oxide synthesis in RAW264 cells; dose response curves for LS-Hex, LS-DCM, LS-water and dexamethasone.

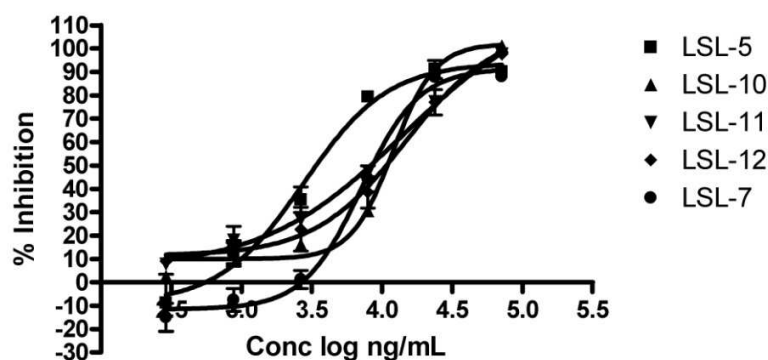


Figure 3.3.24: Inhibition of nitric oxide synthesis in RAW264 cells; dose response curves for fractions (LSL-5, 7, 10, 11 and 12).

3.3.8.2. Inhibition of TNF- α production

LS-Hex, LS-DCM and LS-water extracts and the fractions LSL-5, LSL-7, LSL-10, LSL-11 and LSL-12 were tested for TNF- α inhibitory activity in LPS-stimulated RAW264 macrophages. According to the published literature, extracts, fractions and pure compounds that are considered as having TNF- α inhibitory activity possess $IC_{50} < 100 \mu\text{g/mL}$ (Wang and Mazza, 2002, Ban *et al.*, 2004). Bioactive extracts and fractions were tested at a concentration range of 0.29 to $71.4 \mu\text{g/mL}$. Eucalyptin was tested at a concentration range of 0.04 to $8.93 \mu\text{g/mL}$. Negligible inhibition of TNF- α was seen for all the tested samples (Figure 3.3.25).

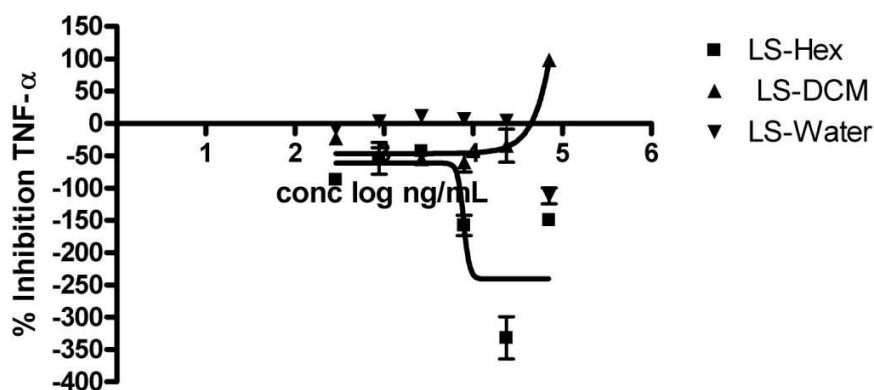


Figure 3.3.25: Effect of extracts of *L. suaveolens* on TNF- α production in LPS-stimulated RAW264 macrophages.

3.3.8.3. Inhibition of PGE₂ synthesis

LS-Hex, LS-DCM, LS-water and the fractions LSL-5, 7, 10, 11 and 12 were also tested for PGE₂ inhibitory activity in calcium ionophore stimulated 3T3 cells using a prostaglandin E₂

monoclonal EIA kit. The concentration range used was 0.41 to 100 µg/mL. Indomethacin (IC₅₀ 78.1 nM) was used as a positive control in this assay. Indomethacin is a well known PGE₂ and cyclooxygenase inhibitor and is widely used as a positive control for PGE₂ inhibition assays (Delamere *et al.*, 1994, Kalgutkar *et al.*, 2000). LS-Hex and LS-DCM showed limited inhibition of PGE₂ synthesis and a standard curve could not be produced to calculate the IC₅₀ values.

Although the crude extracts LS-Hex, LS-DCM and LS-water did not show any activity in the PGE₂ inhibitory assay in stimulated 3T3 cells, fractions LSL-5, 10, 11 and 12 showed good inhibition (IC₅₀ 2.8-19.7 µg/mL) of PGE₂ production in the assay (Table 3.3.11) (Figure 3.3.27). LSL-5 was the most promising fraction among the tested samples (IC₅₀ 2.8 µg/mL). According to the published literature, extracts or fractions that inhibit PGE₂ by 50% at a concentration ≤10 µg/mL are claimed as potent PGE₂ inhibitors (Wohlmuth *et al.*, 2010). None of the extracts (LS-Hex, LS-DCM and LS-water) or fractions (LSL-5, 10, 11 and 12) exhibited cytotoxic effects in the 3T3 cells at a concentration of 100 µg/mL.

Table 3.3.11: PGE₂ inhibitory activity of extracts, fractions and eucalyptin from *L. suaveolens*.

Extracts/ fractions/ compounds	Cytotoxicity 3T3 (µg/mL)*	Inhibition of PGE ₂ synthesis 3T3 (µg/mL)*
LS-Hex	na	na
LS-DCM	na	na
LS-water	na	na
LSL-5	na	2.8 (2.1-3.6)
LSL-7	na	na
LSL-10	na	19.7 (13.9-28.0)
LSL-11	na	7.3 (5.6-9.5)
LSL-12	na	11.5 (6.5-20.4)
Eucalyptin	#	#
Indomethacine	na	78.1 nM (59.9-101.8)

*Cytotoxicity and PGE₂ inhibitory activity were expressed as IC₅₀ (95% confidence intervals) values in µg/mL except indomethacine which was expressed in nM. na: not active. # samples could not be dissolved in 0.5% DMSO/media (v/v), n=3.

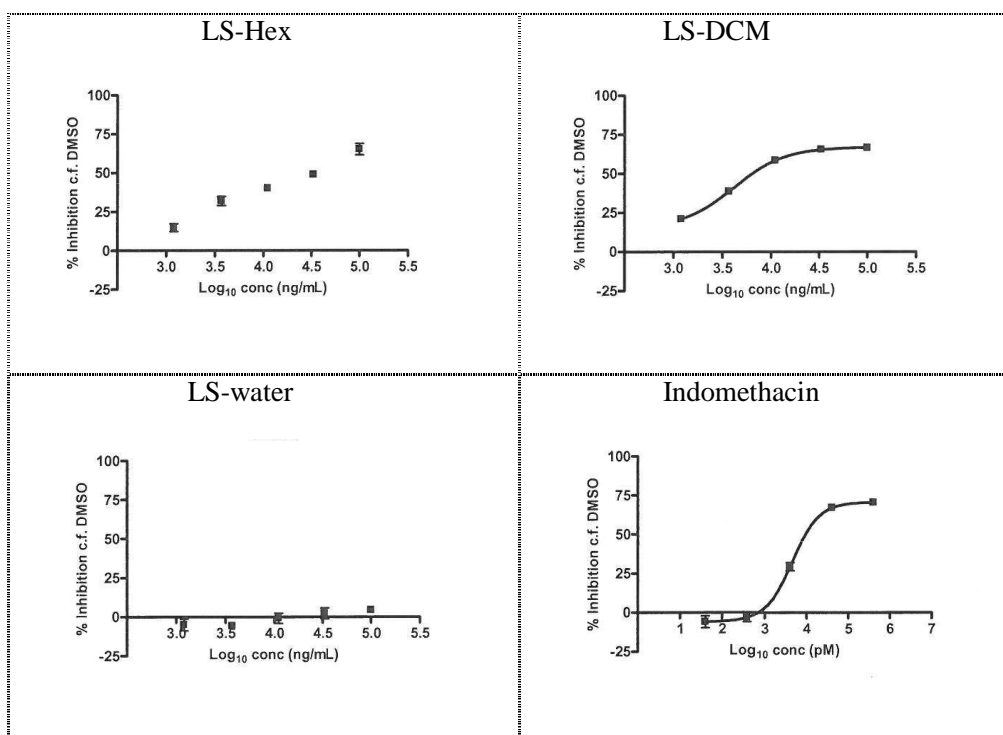


Figure 3.3.26: PGE₂ inhibitory activity of extracts of *L. suaveolens*.

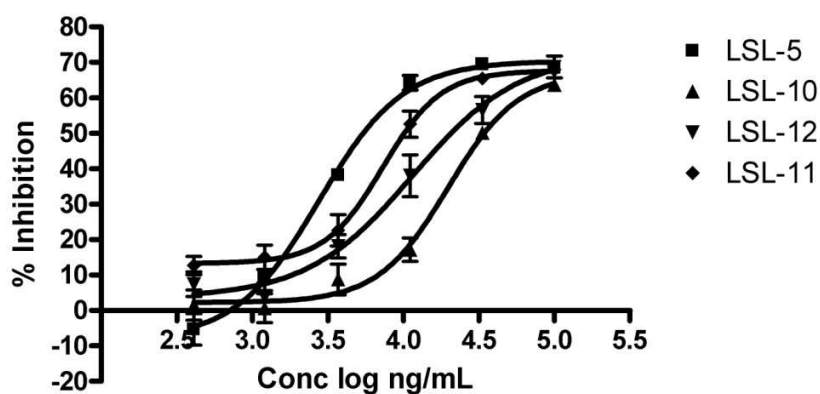


Figure 3.3.27: Inhibition of PGE₂ activity in 3T3 cells; dose response curves for active fractions of *L. suaveolens*.

3.3.8.4. ORAC assay

The ORAC assay, which is described in Chapter 2, was used to test the antioxidant activity of the antibacterial extracts and fractions. Except LSL-7, all the other tested extracts and fractions showed antioxidant activity in the ORAC assay. However, all the tested extracts and fractions showed antioxidant activity that was much lower than for the positive control epicatechin (2.98×10^4 $\mu\text{M TE/g}$), and were therefore considered to have modest antioxidant

activity (Table 3.3.12). Antioxidant activity is expressed as Trolox equivalents per gram (TE/g). The LS-water extract showed the highest antioxidant activity (2.66×10^3 TE/g) among the tested extracts and fractions and the antioxidant activity in decreasing order was was: LS-water > LSL-5 > LSL-11 > LSL-12 > LS-DCM > LS-Hex > LSL-10 (Table 3.3.12).

Table 3.3.12: Antioxidant activity of extracts and fractions of *L. suaveolens* in ORAC assay.

Samples	ORAC Assay mean \pm SD μ M TE/g
LS-Hex	$9.83 \pm 1.61 \times 10^2$
LS-DCM	$1.32 \pm 0.06 \times 10^3$
LS-Water	$2.66 \pm 0.34 \times 10^3$
LSL-5	$2.08 \pm 0.46 \times 10^3$
LSL-7	na
LSL-10	$1.81 \pm 0.30 \times 10^2$
LSL-11	$1.58 \pm 0.03 \times 10^3$
LSL-12	$1.55 \pm 0.12 \times 10^3$
Epicatechin	$2.98 \pm 0.40 \times 10^4$

na = not active. Antioxidant activity is expressed as micromoles of Trolox equivalent per gram.
n = 4.

3.3.9. Concluding remarks

L. suaveolens is a plant that is important to the Yaegl elders, having been used customarily for treatment of skin diseases, infections and for the treatment of ringworm. While the leaves have not been customarily used for sores, wounds and infections, the elders requested them to be examined. EtOH and H₂O extracts of leaves of this plant showed potent antibacterial activity against methicillin sensitive and resistant strains of *S. aureus* and against *S. pyogenes* in preliminary antimicrobial screening (Chaper 2) (Packer, 2012). A review of the literature showed that there is only one report on volatile oil composition of the leaves (Brophy *et al.*, 2000) and no biological studies has so far been reported on this native Australian medicinal plant. The water extract of Packer's collection and the author's collection showed promising

antimicrobial activity. Leaves of this plant were selected for detailed chemical and biological studies. Sequential solvent extraction yielded four extracts (LS-Hex, LS-DCM, LS-EA and LS-MeOH). Among these, LS-Hex and LS-DCM showed potent antibacterial activity ($IC_{90} < 50 \mu\text{g/mL}$) in the MTT assay. The TLC bioautography and disc diffusion assay results also confirmed the antibacterial activity of LS-Hex and LS-DCM. Therefore, LS-Hex and LS-DCM were selected for phytochemical analysis and bioassay guided isolation of antibacterial compounds. GC-MS analysis of the LS-Hex fraction identified 16 phytochemicals, including aromadendrene (15.4%), spathulenol (12.46%), β -caryophyllene (2.53%), α -humulene (1.52%), globulol (4.47%), phytol (2.84%) and α -pinene (0.67%). These compounds are well reported in the literature for having antibacterial activity. Using bioassay guided isolation with various chromatographic and antibacterial assay techniques, two bioactive compounds, betulinic acid and eucalyptin, were isolated from the LS-DCM and LS-Hex extracts. They were characterised using NMR and mass spectrometry. This is the first report of isolation betulinic acid and eucalyptin from *L. suaveolens*. A number of promising antibacterial fractions (LSL-10, 11 and 12) (with $IC_{90} < 50 \mu\text{g/mL}$ against *S. pyogenes* and methicillin sensitive and resistant strain of *S. aureus*) were also isolated.

Bioactive extracts, fractions and pure compounds were also tested for anti-inflammatory and antioxidant activities. LS-Hex and LS-DCM showed anti-inflammatory activity in the NO inhibition assay, but LS-Hex had greater cytotoxic activity than NO inhibitory activity on RAW264 cells. α -Humulene and β -caryophyllene, which were identified in the LS-Hex extract (by GC-MS analysis), have reported cytotoxic activity (Sylvestre *et al.*, 2007). The antibacterial fractions (LSL-5, 7, 10, 11 and 12) also showed good NO inhibitory activity. LS-Hex and LS-DCM extracts were inactive in the PGE_2 inhibition assay, while LSL-5, 10, 11 and 12 displayed good PGE_2 inhibitory activity (IC_{50} 2.8-19.7 $\mu\text{g/mL}$). No bioactive compounds could be isolated from LSL-11 and 12 fractions using different normal phase, reversed phase and size exclusion chromatographic procedure. Because of time constraints further exploration of these fractions was not performed. However, further chemical studies could be carried out in future to explore the bioactive component(s) from the antibacterial and anti-inflammatory fractions (LSL-10, 11 and 12). Except for LSL-7, all the tested extracts (LS-Hex, LS-DCM and LS-water) and fractions (LSL-5, 7, 10, 11 and 12) showed modest levels of antioxidant activity in the ORAC assay. In future it would be worthwhile to examine

other extracts (LS-EA and LS-MeOH) or fractions (LSL-4, Hex-3, 4 and 5) for anti-inflammatory and antioxidant activities.

This is the first report of bioactive compound isolation and bioactivity studies of *L. suaveolens* and thus extends the current knowledge of this relatively unexplored Australian plant. Although promising findings in this study cannot be linked directly with community use of leaves, the selection of this plant was made based on the traditional use of another part of the plant in the treatment of skin infection and as an antiseptic. The selection was also dictated by community interest. The finding of the various biological activities in the leaf extracts that are relevant to application for skin infections, sores and wounds, suggests that *L. suaveolens* leaves could provide a more accessible source of medicine (*i.e.* leaves, rather than the sap) for the Yaegl community.

CHAPTER FOUR

Chemical and biological studies on *Alphitonia excelsa*

This Chapter describes different methods of extraction and bioassays as well as isolation of bioactive components from the bioactive extract of Alphitonia excelsa.

4.1. Introduction

Alphitonia excelsa is a native Australian plant that is well documented as an Aboriginal medicinal plant (Brock, 2001). It is commonly used as a medicinal plant by the Yaegl community of northern NSW as an antiseptic hand wash, whereby leaves of the plant are rubbed between the hands with a little water (Packer, 2012, Brouwer, 2006) (see Section 2.5). Yaegl elders expressed a particular interest in this plant being investigated scientifically. Previous preliminary antibacterial (fluorescein diacetate assay) and anti-inflammatory screening (COX inhibitory assay method) performed by Brouwer showed that EtOH and H₂O extracts of *A. excelsa* leaves collected from northern NSW had good antibacterial (against *S. aureus* and *E. coli*) and anti-inflammatory activity (Section 2.1). Along with its use by the Yaegl community, as already described in Chapter 2, other Aboriginal communities of Australia have used the leaves of *A. excelsa* for the treatment of headaches and sore eyes and the bark and wood for the treatment of body pains and toothache. According to the literature, very few chemical and biological studies have so far been performed on *A. excelsa* (Branch *et al.*, 1972, Guise *et al.*, 1962, Rogers *et al.*, 2000). There have also been no reports of antimicrobial, anti-inflammatory and antioxidant activity studies and the chemical investigations done so far have not been guided by the traditional uses of this native Australian medicinal plant. Thus, given the lack of previous scientific investigations, the finding of biological activity in preliminary studies by the IBRG, and the requests of the Yaegl elders for further investigations, *A. excelsa* was examined to evaluate antimicrobial, anti-inflammatory and antioxidant activity of leaf extracts and to isolate bioactive compounds.

This Chapter describes the antibacterial, anti-inflammatory and antioxidant activities of the leaves of *A. excelsa* and identification, isolation and characterisation of bioactive constituents.

4.2. Experimental

4.2.1. Reagents and equipment

All the solvents used for extraction, chromatographic separations and for HPLC were HPLC grade. Organic solvents were evaporated using a Buchi rotary evaporator. Analytical normal phase thin layer chromatography (TLC) was performed on fluorescent Merck silica gel F₂₅₄

plates (Germany) and reversed phase TLC on Merck Silica gel 60 RP-18 F₂₅₄s plates. Size exclusion chromatography was carried out using LH20100 Sephadex LH-20 (Sigma-Aldrich). The TLC plates were visualised using UV light (254 nm and 365 nm). Reversed phase (C₁₈) solid phase extraction was carried out using Waters Sep Pak Vac 35cc (2 g) cartridges. A Shimadzu LC 10 AVP HPLC system was used for chromatographic separations. The ¹H (400 MHz), ¹³C (100 MHz), HSQC, COSY and HMBC NMR spectra were recorded on a Bruker Avance AMX 400 using standard pulse sequences. Chemical shifts were calculated relative to the chloroform (¹H δ 7.26 and ¹³C δ 77.2), methanol (¹H δ 3.31 and ¹³C δ 49.0) and DMSO (¹H δ 2.50 and ¹³C δ 39.51) solvent signals. A Shimadzu 2010 LC MS system was used for ESI MS analysis. A Shimadzu GC-17 system was used for GC analysis. A Stuart Scientific melting point detector (UK) was used for determining melting points. A Labconco (USA) freeze dryer was used for removal of water.

4.2.2. **Plant material**

Fresh leaves of *Alphitonia excelsa* were collected by the author with the help of IBRG ethnobotanist Mr David Harrington from Cumberland State Forest, Sydney, Australia (33°44'39'', 151°2'26'') in August 2008. The voucher specimen was deposited at Macquarie University Herbarium (voucher specimen number MQ73008737).

A second collection of *A. excelsa* leaf material was made in March 2009 from Lake Boulevard, Yamba, NSW 2464. The voucher specimen (number NSW792677) and details of plant location (29°28'19'', 153°21'17'') were recorded and lodged at the Macquarie University Herbarium, Sydney.

4.2.3. **Preparation of extracts**

Freshly collected leaves of *A. excelsa* were chopped with a Waring heavy duty blender (John Morris scientific) to obtain coarse plant material suitable for extraction. Fresh plant material from the the 2008 collection (first batch) was extracted with solvents of increasing polarity using accelerated solvent extraction (ASE; method 1), 24 h repeated (3 times) extractions at room temperature and atmospheric pressure (method 2) and also extracted separately with water (method 3).

Freshly chopped *A. excelsa* leaves from the 2009 collection (second batch) were only extracted with solvents of increasing polarity using sequential solvent extraction at room temperature and atmospheric pressure. A water extract was also prepared separately with the second batch of *A. excelsa*.

Method 1 (ASE sequential solvent extraction method): Accelerated solvent extraction (ASE) was performed with a Dionex ASE 300 instrument (Dionex, USA) equipped with a solvent controller. The parameters were: preheat time 1 min; static extraction per cycle 5 min; flush 100% solvent of cell volume; purge 120 sec with nitrogen; temperature 70 °C; pressure 120 bar. In the present experiment 100 mL sample cells (the highest available size for Dionex ASE 300) were used to perform ASE. Freshly chopped *A. excelsa* leaves (175 g, ~15 g plant material for each of 12 sample cells) was mixed with sand (~4 g per sample cell) and sequential solvent extraction was performed starting with *n*-hexane and followed by DCM, EtOAc and MeOH. 2 l of each solvent was required for each individual solvent extraction cycle. The combined extracts for each solvent were concentrated using a Buchi rotary evaporator (Germany) with a water bath at 40-42 °C and the residues subsequently freeze dried (Labconco freeze dryer, USA) to remove the remaining water. This afforded the *n*-hexane, DCM, EtOAc and MeOH extracts, ASE-Hex (yellow dense solid, 0.88 g, 0.5% w/w), ASE-DCM (green solid, 4.8 g, 2.7% w/w), ASE-EA (dark green solid, 2.0 g, 1.14% w/w) and ASE-MeOH (dark green solid, 8.9 g, 5.08% w/w), respectively.

Method 2 (room temperature sequential solvent extraction method): Freshly chopped *A. excelsa* leaves (1447 g) were extracted with *n*-hexane (3x2 l) at 25 °C with agitation (80 rpm) using an orbital mixture incubator for 24±1 h. After every 24 h, the extracts were decanted and filtered *in vacuo* through Whatman No. 1 filter paper (Whatman, UK) twice. The residual solid plant material was then extracted in an identical manner with DCM, EtOAc and MeOH. The filtered extracts of the same solvent system were combined, concentrated using a rotary evaporator with a water bath at 40-42 °C, and the solvent was evaporated under vacuum and subsequently freeze dried to remove the remaining water. This afforded the *n*-hexane, DCM, EtOAc and MeOH extracts, (yellow dense solid, 5.4 g, 0.3% w/w), Aex-DCM (green solid, 10.1 g, 0.7% w/w), Aex-EA (dark green solid, 10.7 g, 0.73% w/w) and Aex-MeOH (dark green solid, 22.4 g, 1.5% w/w), respectively.

Freshly chopped *A. excelsa* leaves (303 g) collected from northern NSW (second collection) were also extracted sequentially (method 3, as above) using 3x1 l of each solvent at 25 °C with agitation (80 rpm) for 24±1 h. After every 24 h, the extracts were decanted and filtered *in vacuo* through Whatman No. 1 filter paper. The filtered extracts of the same solvent system were combined, concentrated using a Buchi rotary evaporator with a water bath at 40-42 °C and the residues subsequently freeze dried to remove the remaining water. This afforded the *n*-hexane, DCM, EtOAc and MeOH extracts, Aex-NSW-Hex (yellow dense solid, 0.87 g, 0.28% w/w), Aex-NSW-DCM (green solid, 1.19 g, 0.39% w/w), Aex-NSW-EA (dark green solid, 3.1 g, 1.02% w/w) and Aex-NSW-MeOH (dark green solid, 7.7 g, 2.5% w/w) extracts, respectively.

Method 3 (preparation of water extract): Freshly chopped *A. excelsa* leaves (262 g) from the first collection was extracted with water (3×400 mL) at 25 °C for 24±1 h with agitation (80 rpm). After every 24 h, extracts were decanted and filtered *in vacuo* through Whatman no. 1 filter paper twice and residual plant material was further extracted in water (3 times in total). The filtered extracts were combined, concentrated using a Buchi rotary evaporator with a water bath at 40-42 °C and the residues subsequently freeze dried to remove the remaining water. This afforded Aex-water (greenish yellow powder, 1.3 g, 0.5% w/w).

Chopped leaves (110 g) from the second collection batch were also extracted with water (3×400 mL) following the same procedure as for the first collection batch. This provided Aex-NSW-water extract (greenish yellow powder, 2.6 g, 2.3% w/w).

Long term storage of all the dried extracts was in a -20 °C freezer.

4.2.4. Bioassays: methods and materials

4.2.4.1. Selection of microorganisms for antimicrobial activity

Extracts, fractions and pure compounds isolated from *A. excelsa* were assayed for antimicrobial activity against a number of pathogenic organisms. The bacteria used have been described (in Section 3.2.4.1). In addition, *Candida albicans* (clinical isolate) was provided by Dr John Merlino, Department of Microbiology, Concord Hospital, Sydney. The inoculum size of *C. albicans* was 4.56×10^5 cfu/mL at a spectrophotometric absorbance of 0.08 (wavelength of 600 nm). The use of all microbial strains was approved by the Macquarie University Biosafety Committee (Approval Reference 08/06/LAB, TAN180512BHA).

4.2.4.2. **Disc diffusion assay for antibacterial activity**

Dried plant extracts of *A. excelsa* were tested for antimicrobial activity by the disc diffusion assay using the same protocol and assay procedures as described in Section 3.2.4.2. Potato dextrose agar (PDA, Oxoid, Basingstoke, UK) and DifcoTM Sabouraud dextrose broth (SAB) was used to grow *C. albicans*. Fluconazole was used as a positive control for *C. albicans*. Dried plant extracts and antibiotics were dissolved in 20% DMSO/H₂O (v/v) to get a concentration of 100 mg/mL for extracts and 0.1 mg/mL for a suitable antibiotic [vancomycin (Amresco, Ohio) for Gram-positive bacteria, gentamycin (Amresco, Ohio) for Gram-negative bacteria and fluconazole (MP Biomedicals, France) for *Candida albicans*].

4.2.4.3. **MTT microdilution assay for antibacterial activity**

MIC of extracts and pure compounds of *A. excelsa* using MTT microdilution was also performed using the same protocol and assay procedures as described in Section 3.2.4.4.

4.2.4.4. **Anti-inflammatory and antioxidant assays**

Anti-inflammatory activity was evaluated using a combination of COX, NO, TNF- α , PGE₂ inhibition and cytotoxicity assays. Antioxidant activity was performed using the ORAC assay. The anti-inflammatory (NO, TNF- α , PGE₂ inhibition and cytotoxicity) and antioxidant (ORAC) assays were performed by the author at Southern Cross University under the guidance of Prof. Hans Wohlmuth and with the help of Mr Dane Renshaw and Dr Joshua Smith. The COX assay was performed by the author at Macquarie University with the assistance of Unnikrishnan Kuzhiumparambil.

4.2.4.4.1. **COX inhibition assay**

The COX inhibition assay was performed according to the manufacturer's (Cayman Chemicals, USA) protocol. All extracts (Aex-Hex, Aex-DCM, Aex-EA, Aex-MeOH and Aex-water extract) were tested at two different concentrations (100 μ g/mL and 50 μ g/mL). In this assay, COX 1 and COX 2 were incubated separately with the plant extracts and controls in a reaction buffer (100 mM Tris-HCl buffer, pH 8.0) containing 1 μ M heme, for 10 min at 37 °C. Arachidonic acid was added (final concentration of 100 μ M) to initiate the reaction. HCl solution (1 M) was added after 2 min to terminate the reaction, followed by addition of saturated SnCl₂ solution to reduce the reaction products to PGF_{2 α} . The amounts of PGF_{2 α} produced in the COX reactions were quantified using an enzyme immunoassay. PGF_{2 α} and

PGE₂-acetylcholine esterase tracer were added to a 96-well plate coated with mouse anti-rabbit IgG and the specific antibody (rabbit anti-PGF_{2α} and PGE₂) was added. The plate was incubated for 18 h at room temperature. After the incubation period, the plate was washed 5 times with 10 mM potassium phosphate buffer containing 0.05% Tween 20 to remove any unbound reagents. Ellman's reagent was added to each well and the plate was developed in the dark for about 60 min and absorbance was read at 405 nm. The samples were tested in duplicate. Ibuprofen and celecoxib were used as controls (Selvam *et al.*, 2004). Ibuprofen was purchased from Sigma-Aldrich and celecoxib was obtained as a gift from Ranbaxy Lab, Mumbai, India. A Spectramax spectrophotometer was used for absorbance measurements.

4.2.4.4.2. *NO inhibitory and cytotoxicity assay using RAW264 macrophage*

NO inhibitory and cytotoxicity assays of extracts of *A. excelsa* was performed using RAW264 macrophages. The assay method and protocol used for these studies were similar to the methods described in Sections 3.2.4.5.1 and 3.2.4.5.2. For the cytotoxicity study, extracts of *A. excelsa* (Aex-EA, MeOH and Aex-water) were tested at 0.4 to 100 µg/mL concentration range. For the NO inhibitory assays, the extracts (Aex-EA, MeOH and Aex-water) were tested from 0.294 µg/mL to 71.4 µg/mL.

4.2.4.4.3. *Inhibition of TNF-α*

Inhibitory activity of *A. excelsa* extracts (Aex-EA, MeOH and Aex-water) on TNF-α production in RAW264 cells was determined using the same protocol and assay method described in Section 3.2.4.5.3.

4.2.4.4.4. *Inhibition of PGE₂ synthesis*

PGE₂ inhibitory activity of *A. excelsa* extracts (Aex-EA, MeOH and Aex-water) using 3T3 Swiss albino mouse embryonic fibroblast cells was performed using the assay method described in Section 3.2.4.5.4.

4.2.4.4.5. *Selectivity Index*

The selectivity index was calculated as the concentration that reduces cell viability to 50% divided by the IC₅₀ value for inhibition of NO, TNF-α or PGE₂ synthesis (Cos *et al.*, 2001, Jabit *et al.*, 2009).

4.2.4.4.6. *ORAC assay*

Antioxidant activity of Aex-EA, MeOH and Aex-water extracts were determined using the ORAC assay. The assay method has been described in Section 3.2.4.5.6.

4.2.5. **Preliminary phytochemical screening**

Crude extracts of *A. excelsa* leaves were tested on TLC plates for the preliminary detection of terpenoids, steroids, unsaturation and alkaloids. Vanillin-sulphuric acid (Johnsson *et al.*, 2007), Dragendorff's reagent (Gibbons, 2004), anisaldehyde (Wagner, 1996) and permanganate spray reagents (Davies and Johnson, 2007) were used to detect steroids, alkaloids, terpenoids and unsaturated compounds. TLC plates were developed on normal phase silica plates with *n*-hexane and EtOAc. Methods of preparation of the spray reagents are described in Section 3.2.5.1.

4.2.6. **Chemical study methods and materials**

The Aex-EA fraction of *A. excelsa* (2.6 g) was subjected to size exclusion chromatography (SEC) with Sephadex LH-20 using MeOH as an eluting solvent. Fractions with similar TLC profiles in the solvent system (DCM:EtOAc, 1:1, normal phase) were combined to get 14 major fractions, in order of elution, Aex-1 (213 mg, green solid, 8.2% w/w), Aex-2 (3 mg, green solid, 0.1% w/w), Aex-3 (101.4 mg, dark green solid, 3.9% w/w), Aex-4 (330.2 mg, greenish brown solid, 12.7% w/w), Aex-5 (228.6 mg, yellowish green solid, 8.7% w/w), Aex-6 (269.4 mg, greenish brown solid, 10.2% w/w), Aex-7 (235.3 mg, greenish brown solid, 9.7% w/w), Aex-8 (50 mg, greenish brown solid, 1.9% w/w), Aex-9 (309.8 mg, brown solid, 11.8% w/w), Aex-10 (201.5 mg, yellowish green solid, 7.75% w/w), Aex-11 (251.0 mg, greenish yellow solid, 9.6 % w/w), Aex-12 (13.04 mg, yellow solid, 5.01% w/w), Aex-13 (14 mg, yellow solid, 0.5% w/w) and Aex-14 (3.4 mg, brownish yellow solid, 0.13% w/w). The major fractions (Aex-1 to Aex-14) were tested for antibacterial activity by TLC bioautography (as described in Section 3.2.4.3) against methicillin sensitive and resistant strains of *S. aureus*.

Purification of Aex-11 (200 mg) by normal phase PTLC with DCM:EtOAc (1:1) yielded pure compound **AE72** (9 mg, yellow powder, $R_f = 0.5$ in DCM:EtOAc, 2:1, normal phase TLC). Washing Aex-13 (14 mg) with MeOH (1.5 mL×3) yielded **AE81** (10 mg, yellow powder, $R_f = 0.2$ in DCM:EtOAc, 2:1, normal phase TLC) as a pure compound.

Bioactive fraction Aex-5 (150 mg), which was a mixture of at least three components, as seen on reversed phase TLC plate ($R_f = 0.2-0.7$, $\text{CH}_3\text{CN}:\text{MeOH}$, 4:1), was subjected to solid phase extraction (SPE) (Water Sep pack C_{18} -2 g) using gradient elution of $\text{MeOH}:\text{CH}_3\text{CN}$ (100:0 to 0:100) to give 14 fractions. Fractions with similar R_f profiles (reversed phase) were further combined to give 3 major fractions Fr1 (50 mg, yellow solid, $R_f = 0.3$ in $\text{CH}_3\text{CN}:\text{MeOH}$, 4:1, reversed phase TLC), Fr2 (23 mg, greenish yellow solid, $R_f = 0.4$ in $\text{CH}_3\text{CN}:\text{MeOH}$, 4:1, reversed phase TLC) and Fr3 (20 mg, blackish green solid). Fr1 and Fr2 were active against methicillin sensitive and resistant *S. aureus* by TLC bioautography. Further purification of Fr1 was attempted using reversed phase HPLC using a Shimadzu HPLC system consisting of a LC-10-AVP photodiode array detector and an autosampler. Chromatographic separation was performed at 23-25 °C on a Synergi 10 u Hydro RP 80 A column (250×3 mm i.d., 4 μ , Phenomenex). The mobile phase consisted of H_2O (A) and CH_3CN (B) and was eluted in a step gradient mode (0.01 min - 90% A, 10 min - 70% A, 35 min - 50% A, 45 min - 20% A continued until 50 min). The injection volume was 10 μL (10 mg/mL) and run time was 50 min at a flow rate of 1.0 mL/min. Overlapping peaks on HPLC and a mixture of compounds in LC-MS were noted.

Kaempferol (AE72): Yellow amorphous powder, mp. 278 °C (lit. 278-279 °C) (Ali *et al.*, 2010). UV (MeOH): λ_{max} 266 and 366 nm. ESI MS m/z 287 $[\text{M}+\text{H}]^+$. ^1H NMR (400 MHz, $\text{DMSO}-d_6$): δ 12.45 (1H, br s, 5-OH), 8.03 (2H, d, $J = 8.8$ Hz, H-2', 6'), 6.91 (2H, d, $J = 8.8$ Hz, H-3',5'), 6.41 (1H, d, $J = 1.9$ Hz, H-8), 6.17 (1H, d, $J = 1.9$ Hz, H-6). ^{13}C NMR (100 MHz, $\text{DMSO}-d_6$): δ 177.3 (C-4), 165.7 (C-7), 162.7 (C-5), 160.6 (C-4'), 158.5 (C-9), 148.0 (C-2), 137.3 (C-3), 123.8 (C-1'), 130.7 (C-2',6'), 116.5 (C-3',5'), 104.4 (C-10), 99.4 (C-6), 94.5 (C-8).

Quercetin (AE81): Yellow amorphous powder, mp. 318 °C (lit. 316-318 °C) (Li and Xu, 2008). UV (MeOH): λ_{max} 258 and 362 nm. ESI MS m/z 303 $[\text{M}+\text{H}]^+$. ^1H NMR (400 MHz, $\text{DMSO}-d_6$): δ 12.48 (1H, br s, 5-OH), 9.4 (3H, br s, 3',4', 3-OH), 7.66 (1H, d, $J = 2.2$ Hz, H-2'), 7.53 (1H, dd, $J = 8.4, 2.2$ Hz), 6.87 (1H, d, $J = 8.4$ Hz, H-5'), 6.39 (1H, d, $J = 2.04$ Hz, H-8), 6.17 (1H, d, $J = 2.04$ Hz, H-6). ^{13}C NMR (100 MHz, $\text{DMSO}-d_6$): δ 175.8 (C-4), 164.1 (C-7), 160.7 (C-5), 156.0 (C-9), 147.7 (C-4'), 146.7 (C-2), 145.0 (C-3'), 135.7 (C-3), 121.8 (C-1'), 119.8 (C-6'), 115.4 (C-5'), 115.0 (C-2'), 102.9 (C-10), 98.2 (C-6), 93.2 (C-8).

4.3. Results and Discussion

As described in Chapter 2, *A. excelsa* is very well known to the Yaegl community and its leaves have been commonly used for washing wounds and also for treatment of cuts and sores (Packer *et al.*, 2012). The leaves have also been reported to be used for pain and inflammatory conditions (Lassak and McCarthy, 2008). These ethnomedicinal uses suggested the possibility of antimicrobial, anti-inflammatory and antioxidant activities of the leaves. Previous studies in the IBRG laboratory by Brouwer showed that H₂O extracts (extracted at 80 °C) and 100% EtOH extracts of the leaves (extracted at room temperature) had good antibacterial activity against the common pathogenic bacteria *S. aureus* and *E. coli* (using the disc diffusion and FDA assays) and also had good cyclooxygenase inhibitory activity (Chapter 2, Section 2.1) (Brouwer, 2006).

4.3.1. Extraction of *A. excelsa* leaves using sequential solvent extraction method

Following on from the findings of antibacterial and COX inhibitory activity in leaf EtOH extracts by Brouwer and also in accordance with the Yaegl elders' requests to examine this plant further, fresh leaves of *A. excelsa* were extracted for detailed biological and chemical investigations. To assist in fractionation of bioactive compounds for further bioassay guided isolation of compounds of interest, two different extraction methods, both using increasing solvent polarity were employed: accelerated solvent extraction (ASE) at higher temperature (method 1) and pressure and sequential solvent extraction at room temperature and pressure (method 2).

In this PhD study, a 'traditional preparation' water extract (Aex-water) was also prepared at room temperature to mimic the use of the leaves by the Yaegl community as an antiseptic hand wash where they simply rub fresh leaves between their hands with a little water (Packer, 2012).

4.3.2. Comparison of efficiency between ASE (method 1) and room temperature sequential extraction (method 2)

Plants are complex matrices and produce a range of secondary metabolites with different functional groups and polarities, e.g. fatty acids, terpenoids, steroids, essential oils, phenolics, alkaloids and glycosides (Seidel, 2006). The extraction process often affects the ease of isolation and bioactivity of plant extracts. Therefore choosing the right extraction process is an important part of the bioassay guided isolation process. Sequential solvent extraction with solvents of increasing polarity is often regarded as a useful method for the initial separation of bioactive components according to their polarity (Eloff, 1998, Simpson *et al.*, 2011). In the present study, sequential solvent extraction was performed by two methods: accelerated solvent extraction (ASE), which employs high temperature and pressures (method 1) and sequential solvent extraction at room temperature and atmospheric pressure (method 2).

Accelerated solvent extraction (ASE) is a relatively recent extraction technique (Basalo *et al.*, 2006). This extraction technique in principle overcomes some of the drawbacks such as long extraction time, labour intensive manual procedures, relatively high solvent consumption and the unsatisfactory reproducibility of traditional extraction methods currently in use (Benthin *et al.*, 1999). ASE uses solvents at elevated pressure and temperature in order to increase the efficiency of the extraction process. Increased temperature accelerates the extraction kinetics and elevated pressure keeps the solvent in the liquid state, thus enabling safe and rapid extractions (Kaufmann and Christen, 2002).

ASE sequential extraction of 175 g of *A. excelsa* leaves (starting from low polar to high polar solvents) using *n*-hexane, DCM, EtOAc and MeOH (method 1) afforded four different extracts ASE-Hex (yellow dense solid, 0.88 g, 0.5% w/w), ASE-DCM (green solid, 4.8 g, 2.7% w/w), ASE-EA (dark green solid, 2.0 g, 1.14% w/w) and ASE-MeOH (dark green solid, 8.9 g, 5.08% w/w), respectively.

This ASE method was found to be relatively quick for doing the extraction on a small scale (e.g. 100-200 g plant material), taking only 24-48 h for the sequential extraction (using four different solvents) with three repeats for each solvent. However, some difficulties were also observed for the ASE method. For example, for the facility available, it was not possible to put any more plant material (more than 175 g) in one cycle. One of the important requirements of

ASE is it needs material to be free flowing as otherwise the solvent cannot pass through the material resulting in poor extraction. To make fresh plant material free flowing, it needs to be mixed with sand and therefore a large scale extraction becomes difficult. Moreover, the sample cells also require cleaning and ultrasonication after each extraction cycle. The preparation of sample, loading samples into the sample cell, operation of the extraction process and cleaning afterwards took about 48 h. For the ASE facility available, several extraction cycles (multiple repetition using fresh plant material) are required for doing large scale (more than 1 kg) extraction. After performing sequential solvent extraction by ASE with fresh crushed leaves of *A. excelsa*, it was observed that the method was not very convenient for large scale extraction.

Sequential solvent extraction at room temperature and atmospheric pressure was also performed on a large scale (1447 g of fresh leaves of *A. excelsa*) starting from low polar to high polar solvents (*n*-hexane, DCM, EtOAc and MeOH, 36±3 h and 6 l for each solvent) (method 2). This yielded four extracts, Aex-Hex (yellow dense solid, 5.4 g, 0.3% w/w), Aex-DCM (green solid, 10.1 g, 0.7% w/w), Aex-EA (dark green solid, 10.7 g, 0.73% w/w) and Aex-MeOH (dark green solid, 22.4 g, 1.5% w/w). Despite using more solvent and taking longer than the ASE method, the author found this extraction procedure to be more convenient than the ASE method. Table 4.3.1 provides the yields and appearances of the extracts following both extraction methods. The advantages and disadvantages of the two sequential extraction methods observed during working with fresh leaves of *A. excelsa* are summarised in Table 4.3.2.

Table 4.3.1: Percentages of yields and appearances of first collection batch extracts of *A. excelsa* obtained by two sequential solvent extraction methods.

Extracts	Extraction method*	Percent yield (w/w)	Physical appearance	R _f (normal phase TLC)
ASE-Hex	Method 1	0.50%	Yellow dense solid	0.7-0.8 (<i>n</i> -hexane:EtOAc, 1:1)
Aex-Hex	Method 2	0.30%	Yellow dense solid	0.6-0.8 (<i>n</i> -hexane:EtOAc, 1:1)
ASE-DCM	Method 1	2.70%	Dark green solid	0.5, 0.8-0.9 (<i>n</i> -hexane:EtOAc, 1:1)
Aex-DCM	Method 2	0.70%	Dark green solid	0.2-0.5, 0.7-0.9 (<i>n</i> -hexane:EtOAc, 1:1)
ASE-EA	Method 1	1.14%	Dark green solid	0.2, 0.7-0.8 (DCM:EtOAc, 1:1)
Aex-EA	Method 2	0.73%	Dark green solid	0.1-0.4, 0.6-0.9 (DCM:EtOAc, 1:1)
ASE-MeOH	Method 1	5.08%	Blackish green solid	0.2-0.8 (DCM:EtOAc, 1:1)
Aex-MeOH	Method 2	1.50%	Blackish green solid	0.3-0.8 (DCM:EtOAc, 1:1)

*Method 1 refers to ASE sequential solvent extraction; method 2 refers to room temperature sequential solvent extraction.

Table 4.3.2: Advantages and disadvantages of the two sequential extraction methods (ASE and room temperature sequential solvent extraction) for *A. excelsa* leaf extracts.

ASE sequential solvent extraction method	Room temperature sequential solvent extraction method
<p>Advantages</p> <p>Suitable for small scale extraction with one solvent.</p> <p>More suitable for dry powdered plant material as this system requires free flowing material for better separation.</p> <p>It can perform small scale extraction with one solvent quickly.</p> <p>No need to do filtration after extraction.</p> <p>Yield of extract is higher than room temperature sequential solvent extraction method.</p>	<p>Advantages</p> <p>Suitable for both small and large scale extraction.</p> <p>Suitable for fresh as well as dry plant material.</p> <p>It is performed at room temperature, therefore safe for thermolabile compounds.</p> <p>For sequential extraction with large scale of plant material, this method is easy to set up.</p>
<p>Disadvantages</p> <p>It involves extraction at higher temperature (60-70 °C), therefore some compounds might be degraded during the extraction process.</p> <p>For sequential extraction with large scale of fresh plant material, this method is more laborious and time consuming as compared to the room temperature sequential solvent extraction method.</p> <p>Result of initial separation of fresh plant material using the ASE method was not good as compared to the room temperature sequential solvent extraction method.</p>	<p>Disadvantages</p> <p>It requires filtration at every step and is more labour intensive.</p> <p>For small scale plant material, this method requires more time than the ASE method.</p>

4.3.3. **Bioactivity studies of ASE and room temperature sequential solvent extracts**

Following the extraction of the fresh plant material collected from Cumberland State Forest, Sydney (first collection batch), by the ASE method and the room temperature sequential extraction method, antimicrobial activity was performed using the disc diffusion assay (2 mg/disc). Extracts from the ASE sequential solvent extraction method (ASE-Hex, ASE-DCM, ASE-EA and ASE-MeOH) and extracts from the room temperature sequential solvent extraction methods (Aex-Hex, Aex-DCM, Aex-EA and Aex-MeOH) were tested against the common pathogenic strains *S. aureus*, *E. coli* and *P. aeruginosa*. For the ASE method, the methanol extract (ASE-MeOH) contained all the antibacterial activity, with a 12 mm diameter of zone of inhibition against *S. aureus*. For the room temperature/normal atmospheric pressure extraction method, both the EtOAc extract (Aex-EA) and the MeOH extract (Aex-MeOH) showed antibacterial activity. Aex-EA was the most active extract, with a 10 mm diameter of zone of inhibition against *S. aureus* (Table 4.3.3), while Aex-MeOH showed a 9 mm zone of inhibition against *S. aureus*. Despite the higher yield in the ASE method, these results suggested greater separation of different bioactive compounds into different fractions using the room temperature extraction method (Table 4.3.1 and Table 4.3.3). Moreover, on normal phase TLC plates, better separation was seen between the different extracts in the room temperature sequential extraction method verses the ASE method. Therefore, considering the poorer separation and difficulty of the large scale extraction in the ASE method, the room temperature sequential solvent extracts were chosen for further assays and bioassay guided isolation of bioactive compounds.

Table 4.3.3: Antibacterial activity of sequential ASE and sequential room temperature extracts in disc diffusion assay.

Extracts (2 mg/disc)	Diameter of zone of inhibition (mm)		
	<i>S. aureus</i>	<i>E. coli</i>	<i>P. aeruginosa</i>
Extracts of ASE sequential solvent extraction method			
ASE-Hex	na	na	na
ASE-DCM	na	na	na
ASE-EA	na	na	na
ASE-MeOH	12	na	na
Extracts of room temperature sequential solvent extraction method			
Aex-Hex	na	na	na
Aex-DCM	na	na	na
Aex-EA	10	na	na
Aex-MeOH	9	na	na
Vancomycin (2 µg)	12	na	na
Gentamycin (2 µg)	na	12	12

*Two repeats were performed on at least two separate occasions. Zone of inhibition was determined by diameter of complete inhibition, including 6 mm disc diameter. na: not active against the tested bacteria.

4.3.4. MTT microdilution assay

Initially antibacterial activity of the room temperature sequential solvent extracts, Aex-Hex, Aex-DCM, Aex-EA and Aex-MeOH were evaluated by the disc diffusion method. Later, the extracts were tested for the determination of minimum inhibitory concentration (MIC) using the MTT microdilution method (this method has been described in Chapter 2 and 3). The disc diffusion assay is often useful for initial qualitative screening, but it is not capable of determining MIC and antimicrobial activity in the disc diffusion assay depends on the test samples capacity to diffuse into the agar. Therefore, to determine the MIC of the room temperature sequential extracts, MTT microdilution was used. According to Rios and Recio (Rios and Recio, 2005) and Gibbons (Gibbons, 2004), crude plant extracts having MIC values

below 1000 µg/mL are considered worthy of further investigation. In the present study, a plant extract with an IC₉₀ of <50 µg/mL was considered as having good (***) antimicrobial activity, those with IC₉₀ of 50-125 µg/mL were considered as having moderate (**) antimicrobial activity and those with IC₉₀ value of 126-1000 µg/mL were considered as having low (*) levels of antimicrobial activity.

Initially, the disc diffusion assay was performed against three bacterial strains (*S. aureus*, *P. aeruginosa* and *E. coli*) that are known to be susceptible to common antibiotics. The MTT microdilution assay was performed using an extended range of medically important Gram-positive and Gram-negative bacteria including antibiotic sensitive strains (*S. aureus*, *S. pyogenes*, *S. typhimurium*, *P. aeruginosa* and *E. coli* β lactamase negative) and also antibiotic resistant strains (MRSA, MDRSA and *E. coli* β lactamase positive). The bacteria used in the present study are representative of pathogens important in human infections.

The EtOAc extract, Aex-EA, showed the highest activity (IC₉₀ 31.25 µg/mL against *S. pyogenes* and 500-1000 µg/mL against methicillin sensitive and resistant *S. aureus*), followed by the MeOH extract, Aex-MeOH, (IC₉₀ 62.5 µg/mL against *S. pyogenes* and 1000 µg/mL against methicillin sensitive *S. aureus*) (Table 4.3.4). The Aex-Hex and Aex-DCM extracts did not show any antimicrobial activity at 1000 µg/mL and none of the extracts showed any activity against the tested Gram-negative bacteria and fungi. The antimicrobial extracts Aex-EA and Aex-MeOH were also tested for their bactericidal or bacteriostatic effects and both extracts were found to have bactericidal effects on the tested bacteria (Table 4.3.4).

Table 4.3.4: Antibacterial activity of extracts of *A. excelsa* by the MTT microdilution assay and bacteriostatic or bactericidal effects.

Extracts	MIC values (µg/mL)				
	<i>S. aureus</i>	MRSA	MDRSA	<i>S. pyogenes</i>	<i>S. typhimurium</i> , <i>P. aeruginosa</i> , <i>E. coli</i> (β+) & (β-), <i>C. albicans</i>
Aex-Hex	na	na	na	na	na
Aex-DCM	na	na	na	na	na
Aex-EA	500* (C)	1000* (C)	1000* (C)	31.25*** (C)	na
Aex-MeOH	1000* (C)	na	na	62.5** (C)	na
Vancomycin	1.25*** (C)	1.25*** (C)	1.25*** (C)	0.31*** (C)	na

***IC₉₀ <50 µg/mL, **IC₉₀ 50-125 µg/mL, *IC₉₀ 126-1000 µg/mL. C: bactericidal effect on the microorganisms. MIC was determined as the well with the lowest concentration of sample that displayed no yellow-blue colour change of MTT and also by turbidity (absorbance). Two repeats were performed on at least two separate occasions; values are average of observed results. na: not active against the tested bacteria at 1000 µg/mL.

4.3.5. Evaluation of anti-inflammatory and antioxidant activities

The ethnomedicinal use of *A. excelsa* leaves for treatment of cuts and sores (Packer *et al.*, 2012) and for pain and inflammatory conditions (Lassak and McCarthy, 2008) suggests the possibility of anti-inflammatory and antioxidant activity. Moreover, a previous study in this laboratory using the COX assay (performed by Brouwer, described in Chapter 2) showed that EtOH and H₂O extracts of *A. excelsa* leaves had good cyclooxygenase (COX) inhibitory activity. Therefore, extracts of *A. excelsa* were tested for anti-inflammatory and antioxidant activities. Initially, the extracts Aex-Hex, Aex-DCM, Aex-EA and Aex-MeOH and the ‘traditional preparation’ (*i.e.* water extract; Aex-water) were tested for anti-inflammatory activity by the author using the Cayman COX inhibitory assay kit. However, it was reasoned that a combination of different anti-inflammatory and ORAC antioxidant assays could be very useful for investigating *A. excelsa* for compounds of relevance for the treatment of skin infections, sores and wounds (Chapter 2). Therefore, anti-inflammatory activity of Aex-EA,

Aex-MeOH and Aex-water was investigated using other well recognised anti-inflammatory assays, including NO, TNF- α and PGE₂ inhibition assays and the antioxidant activity was investigated using the ORAC assay. Although, analysis of all the extracts was ideal, only Aex-EA, Aex-MeOH and Aex-water were chosen, as these assays are very laborious to conduct and there was only a limited timeframe available for these to be run. The assays were performed by the author at Southern Cross University under the guidance of Prof. Hans Wohlmuth and with the help of Mr Dane Renshaw and Dr Joshua Smith.

4.3.5.1. **Anti-inflammatory activity using COX inhibitory assay**

Extracts of *A. excelsa* were tested initially for COX inhibitory activity using the commercially available Cayman COX inhibitory assay kits (Selvam and Jachak, 2004), which test for COX 1 and COX 2 inhibition. According to the literature, crude extracts of plants are generally screened for COX anti-inflammatory activity at concentrations between 0.1 and 10 mg/mL and those extracts that exhibit activities at concentrations <2 mg/mL are generally considered to be worthy of further investigation (Lindsey *et al.*, 1998, Palombo and Semple, 2001). The results of this screening of *A. excelsa* leaf extracts (Aex-Hex, Aex-DCM, Aex-EA and Aex-MeOH) are presented in Table 4.3.5. It was found that Aex-DCM showed the most potent activity, inhibiting COX 1 and COX 2 by 81% and 70%, respectively, at 50 μ g/mL, whereas at 100 μ g/mL the inhibition observed was 99% and 82% for COX 1 and COX 2, respectively. Aex-water, Aex-MeOH and Aex-EA also showed promising COX 1 inhibitory activity (inhibited 94%, 85% and 81%, respectively, at 50 μ g/mL, and 100% for all extracts at 100 μ g/mL) (Table 4.3.5). All tested extracts (except Aex-DCM) showed negligible COX 2 inhibitory activity (Table 4.3.5) and Aex-Hex was the least active extract in the COX inhibitor screening assay. For positive controls, ibuprofen was used as a selective inhibitor for COX 1 and celecoxib was used as an inhibitor for COX 2.

Table 4.3.5: COX inhibitory activities of extracts of *A. excelsa* leaves.

Extracts	Conc. µg/mL	% Inhibition of	
		COX 1	COX 2
Aex-Hex	50	63	41
	100	72	21
Aex-DCM	50	81	70
	100	99	82
Aex-EA	50	81	27
	100	100	na
Aex-MeOH	50	85	2.7
	100	100	49
Aex-water	50	94	21
	100	100	na
Ibuprofen	6	80	
Celecoxib	1.8		100

Assay was performed in duplicate and values are average of two results.

4.3.5.2. Nitric oxide (NO) inhibition and cytotoxicity assay

Antibacterial and anti-inflammatory extracts (Aex-EA, Aex-MeOH) and the ‘traditional preparation’ extract (Aex-water) were tested for NO inhibitory activity in RAW264 cells. The extracts were tested at a concentration range from 0.294 µg/mL to 71.4 µg/mL for NO inhibitory activity. All extracts (Aex-EA, Aex-MeOH and Aex-water) were tested at a concentration range from 0.05 µg/mL to 100 µg/mL for cytotoxicity study. Cytotoxicity was tested at a higher concentration range (0.05-100 µg/mL) than the NO inhibition testing range to check whether the extracts/fractions or compounds had any toxic effects that could contribute to NO inhibition in the RAW264 cells.

In the NO inhibition assay, Aex-EA showed promising anti-inflammatory activity (IC₅₀ 10.7 µg/mL) in comparison to its cytotoxic concentration (IC₅₀ 60 µg/mL) and the selectivity index was 5.6 (Table 4.3.6, Figure 4.3.1 and Figure 4.3.2). Aex-MeOH also showed moderate NO inhibitory activity (IC₅₀ 30 µg/mL) and did not produce any cytotoxic effects on the RAW264 cells even at 100 µg/mL. Aex-water was considered inactive in the NO assay as it did not produce any inhibition at the highest concentration tested (71.4 µg/mL) (Table 4.3.6 and Figure 4.3.2).

Table 4.3.6: Cytotoxicity and nitric oxide synthesis inhibitory activity of extracts of *A. excelsa*.

Samples	Cytotoxicity ($\mu\text{g/mL}$)	Inhibition of nitric oxide (NO) synthesis ($\mu\text{g/mL}$)	Selectivity index
Aex-EA	60 (44.2-80.8)	10.7 (6.2-18.5)	5.6
Aex-MeOH	>100	30.5 (19.6-47.4)	-
Aex-water	>100	>71.4	-
Chlorambucil	8.0	-	-
Dexamethasone	-	0.0062 (0.0025-0.0157)	-

Inhibition was measured as IC_{50} (95% confidence intervals); -: not relevant; Dexamethasone and chlorambucil were used as positive controls for NO inhibition assay and cytotoxicity study, respectively. $n=3$ for both cytotoxicity and NO inhibition activity study.

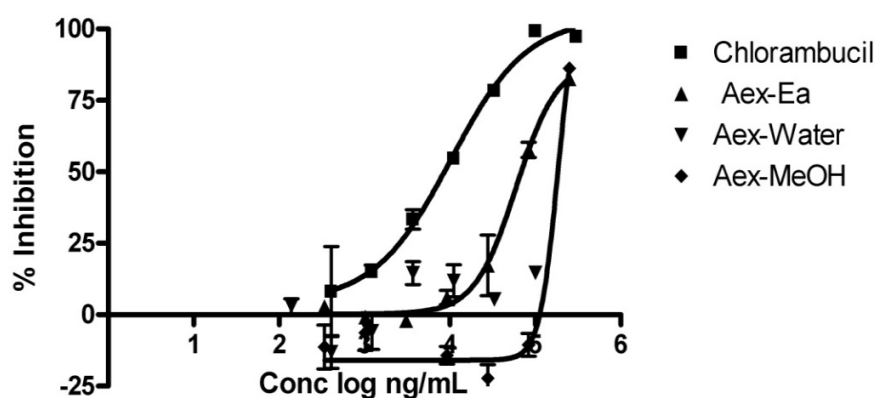


Figure 4.3.1: Cytotoxic effects of extracts (Aex-EA, Aex-MeOH and Aex-water) and cytotoxic drug chlorambucil on RAW264 cells.

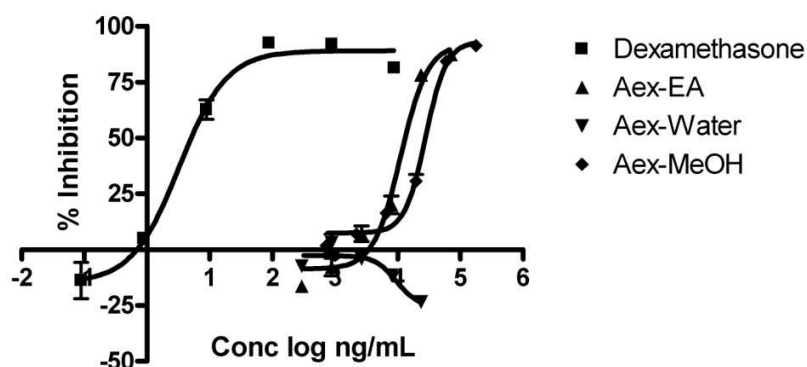


Figure 4.3.2: Inhibition of nitric oxide synthesis in RAW264 cells; dose response curves for *A. excelsa* extracts.

4.3.5.3. Inhibition of TNF- α production

Aex-EA, Aex-MeOH and Aex-water were tested for TNF- α inhibitory activity in LPS-stimulated RAW264 macrophages. A range of concentrations (0.29 μ g/mL to 71.4 μ g/mL) was tested to in an attempt to establish IC₅₀ values (Figure 4.3.3). However negligible inhibition of TNF- α production was observed from the tested extracts and dose response curves could not be produced to calculate IC₅₀ values (Figure 4.3.3).

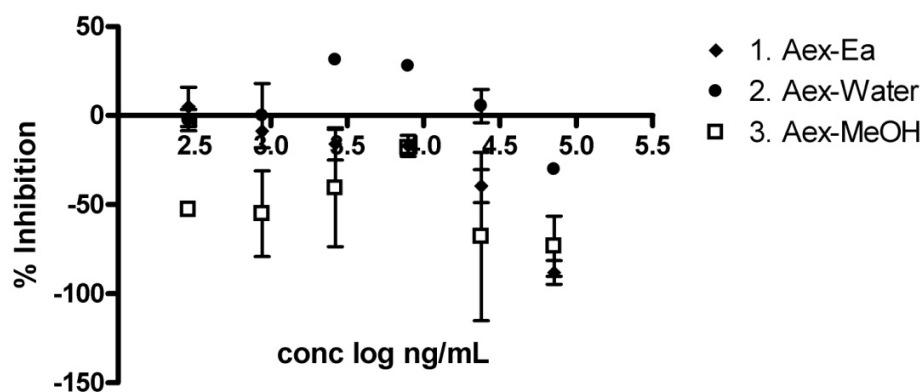


Figure 4.3.3: Effects of extracts of *A. excelsa* on TNF- α production in LPS-stimulated RAW264 macrophages.

4.3.5.4. Inhibition of PGE₂ synthesis

Aex-EA, Aex-MeOH and Aex-water were tested for PGE₂ inhibition using the PGE₂ inhibition assay in calcium ionophore stimulated 3T3 cells using a PGE₂ monoclonal EIA kit.

The concentration range used was 0.41 µg/mL to 100 µg/mL. All the tested extracts showed limited inhibition of PGE₂ synthesis.

4.3.5.5. ORAC assay

Aex-EA, Aex-MeOH and Aex-water were tested for antioxidant activity by the ORAC (oxygen radical absorbance capacity) assay. All the tested samples showed reasonable antioxidant activity in this assay (Table 4.3.7). Aex-EA showed the highest activity of 3.70×10^3 µM TE/g, which is considered modest in comparison to the positive control epicatechin (2.98×10^4 µM TE/g). Although Aex-water did not show any antibacterial or anti-inflammatory activity in the present study, Aex-water showed reasonable antioxidant activity in the ORAC assay (1.6×10^3 µM TE/g).

Finding of antioxidant activity in the Aex-water extract also proves that lack of activity in certain assays (antimicrobial and anti-inflammatory) does not necessarily mean that it is not effective in the customary way. Antioxidant activity of the Aex-water extract provides some support for the traditional use of the plant in treatment of inflammatory conditions and wounds by the Indigenous community.

Table 4.3.7: Antioxidant activity of extracts of *A. excelsa* in ORAC assay.

Samples	ORAC assay (mean ± SD µM TE/g)
Aex-EA	$3.70 \pm 0.36 \times 10^3$
Aex-MeOH	$7.50 \pm 0.28 \times 10^2$
Aex-water	$1.6 \pm 0.23 \times 10^3$
Epicatechin	$2.98 \pm 0.40 \times 10^4$

Antioxidant activity is expressed as micromoles of Trolox equivalent per gram, n=4.

4.3.6. Phytochemical screening

The preliminary antibacterial study showed that the EtOAc extract Aex-EA had the highest activity amongst the tested extracts against the Gram-positive organisms methicillin sensitive *S. aureus*, MRSA, MDRSA and *S. pyogenes*. Therefore, Aex-EA was selected for further studies to isolate bioactive compounds. To assist in identifying the types of compounds

present, Aex-EA was subjected to preliminary phytochemical screening using specific tests on TLC plates (as described in Section 3.2.5). Preliminary screening is often helpful in detecting types of compounds present in extracts or fractions initially. The staining agents used for the detection of the types of compounds present in *A. excelsa* were: vanillin-sulfuric acid, anisaldehyde, permanganate, iodine and Dragendorff's reagent. Figure 4.3.4 shows the distinctive staining seen with anisaldehyde reagent, with blue-green colouration suggesting the presence of terpenoids (a) (Wagner, 1996); and brown, blue and green spots observed with vanillin-sulfuric acid suggesting the presence of terpenes (b) (Gibbons, 2005) and yellow spots on a pink plate when sprayed with permanganate stain indicating the presence of monoterpenes and unsaturated compounds (c) (Davies and Johnson, 2007). These results suggested the presence of terpenes, terpenoids, steroids and unsaturated compounds in the EtOAc extracts of *A. excelsa*. Aex-EA did not show any orange or red spot upon spraying with Dragendorff's reagent, indicating the absence of alkaloids.




 <p style="text-align: right;">(a)</p>	 <p style="text-align: right;">(b)</p>	 <p style="text-align: right;">(c)</p>
Anisaldehyde spray. DCM:EtOAc (1:1), normal phase TLC.	Vanillin-sulfuric acid stain, DCM:EtOAc (1:1), normal phase TLC.	Permanganate spray, DCM:EtOAc (1:1), normal phase TLC.

Figure 4.3.4: TLC chromatograms of Aex-EA after spraying with spray reagents.

4.3.7. Bioassay guided isolation of bioactive compounds from Aex-EA

Size exclusion chromatography (SEC) using the lipophilic organic resin Sephadex LH-20 is a commonly used method for the purification of flavonoids, steroids and some other classes of compounds. It separates on the basis of molecular size and the separated molecules do not interact or form bonds with the Sephadex resin. Typically high recovery of compounds is obtained using Sephadex (Gutzeit *et al.*, 2007, Flamini *et al.*, 2001, Lo and Cheung, 2005, Carr *et al.*, 1971). According to the literature, chemical constituents so far isolated from *A. excelsa* leaves are alphitolic acid, betulinic acid, salicylic acid and ceanothic acid (described in Section 2.5.3). All these compounds have either a hydroxyl group (OH) or a carboxyl group (COOH) or both. The silica gel surface consists of exposed silanol groups and polar compounds containing carboxylic acids, amines and amides can be strongly adsorbed on to silica gel in normal phase silica gel chromatography (Reid and Sarkar, 2006). Therefore, it was decided to do size exclusion chromatography (SEC) for the initial separation of Aex-EA. SEC of Aex-EA yielded 14 major fractions based on TLC R_f profiles. These were subjected to TLC bioautography to assist isolation of targeted compounds (Sections 3.2.4.3 and 3.3.5.1).

The major fractions obtained from SEC (Aex-1 to Aex-14, in order of elution) were tested for antibacterial activity by TLC bioautography against methicillin sensitive and resistant strains of *S. aureus*. Only these strains were used in this assay as none of the extracts were active against the Gram-negative bacteria used (β lactamase positive and negative strains of *E. coli* and *P. aeruginosa*) or the fungus (*C. albicans*). *S. pyogenes* was also not used in this assay as the blood agar required for its growth would interfere with the colour change in the MTT assay. For TLC bioautography, 100 μ g of fractions were spotted on the TLC plate and then run with an appropriate solvent system for a good separation. The fractions that did not give good separation on the TLC plate were also tested directly for antibacterial activity after spotting 100 μ g of sample on the TLC plate and without running with a solvent system.

Aex-4 to Aex-7, Aex-9, Aex-11, Aex-12 and Aex-13 showed antimicrobial activity against antibiotic sensitive and resistant strains of *S. aureus* (*S. aureus*, MRSA and MDRSA) in the TLC bioautography assay. Among these active fractions, Aex-11, Aex-12, Aex-13 and Aex-5 were the most promising regarding separation on the TLC plate and antimicrobial activity. These active fractions were therefore selected for further purification.

Further fractionation of Aex-11 (200 mg) by normal phase preparative TLC yielded a pure bioactive compound, **AE72**, as a yellow amorphous powder (9 mg). Washing of the solid Aex-13 (14 mg, light greenish yellow) with MeOH yielded a pure bioactive compound, **AE81** (10 mg, yellow powder).

The bioactive fraction Aex-5 (150 mg) on a normal phase silica gel TLC plate showed overlapping spots with tailing in different solvent systems (different ratios of *n*-hexane:EtOAc, CHCl₃:MeOH or DCM:EtOAc with and without 0.1% formic or acetic acid). However, on a reversed phase TLC plate it showed three separated spots when eluted with CH₃CN:MeOH (4:1). Reversed phase (C₁₈) solid phase extraction (SPE) of Aex-5 yielded three major fractions Fr1 (50 mg), Fr2 (23 mg) and Fr3 (5 mg). Only Fr1 and Fr2 were active by TLC bioautography against methicillin sensitive and resistant *S. aureus*. Further attempts at purification of Fr1 using reversed phase HPLC and CH₃CN and H₂O mixtures with 0.1% TFA (90:10 to 20:80) did not show any distinct peaks and also showed a mixture of poorly resolved compounds in LC-MS (reversed phase). LC-MS showed two major peaks and each corresponded to a mixture of several compounds, with ESI MS (negative mode) showing masses at *m/z* 502, 458, 365, 301 and 257. ¹H NMR showed many unresolved peaks in the 0.2-2 ppm region, indicating the presence of aliphatic compounds. Further purification could not be performed due to time constraints. Fr2 did not show any separation on normal or reversed phase TLC plates and LC-MS (ESI MS) (negative ion mode) showed a mixture of several components (*m/z* 485, 469, 455, 243, 227 and 213).

Fraction Aex-4 did not show any distinct separation on normal or reversed phase TLC plates using normal (*n*-hexane:EtOAc, CHCl₃:MeOH or DCM:EtOAc) and reversed phase solvent systems (CH₃CN:H₂O or MeOH:H₂O with and without 0.1% formic or acetic acid). It consistently showed tailing on TLC plates.

Fractions Aex-6, Aex-7 and Aex-9 showed little antimicrobial activity and were not explored any further. Figure 4.3.5 shows the antibacterial activity of Aex-5, 6, 7 and 9 against methicillin sensitive *S. aureus* by TLC bioautography (method 1, unoptimised method, described in Section 3.3.5.1) after spotting 100 µg of fractions on the TLC plate (without running with solvent system).

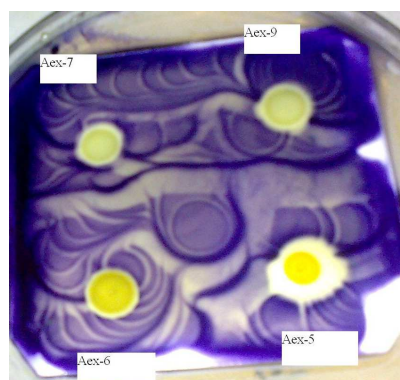


Figure 4.3.5: TLC bioautography of fractions of *A. excelsa* against methicillin sensitive *S. aureus*.

A summary of the bioassay guided studies from extraction to isolation of antibacterial compounds is presented in Figure 4.3.6.

4.3.8. Summary of bioassay guided studies

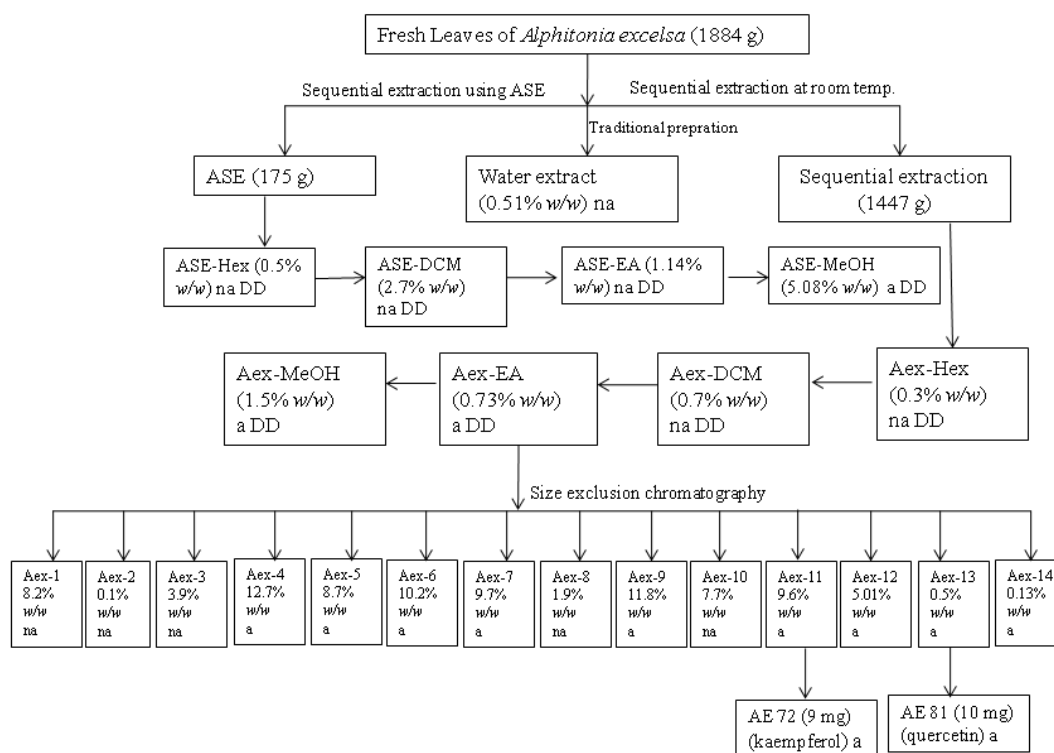


Figure 4.3.6: Extraction flowchart of *A. excelsa*. a: active in TLC bioautography against methicillin sensitive and resistant *S. aureus*. na: not active against the tested bacteria in TLC bioautography. a DD: active in disc diffusion assay against *S. aureus*. na DD: not active in disc diffusion assay against *S. aureus*.

4.3.9. Characterisation of bioactive compounds

Two antibacterially active compounds, AE72 and AE81, were isolated from Aex-EA. Their structures (Figure 4.3.7 and Figure 4.3.10) were elucidated by 1D and 2D NMR and mass spectrometry and also by comparison with reported data. The structure elucidations are described below.

4.3.9.1. Kaempferol (AE72)

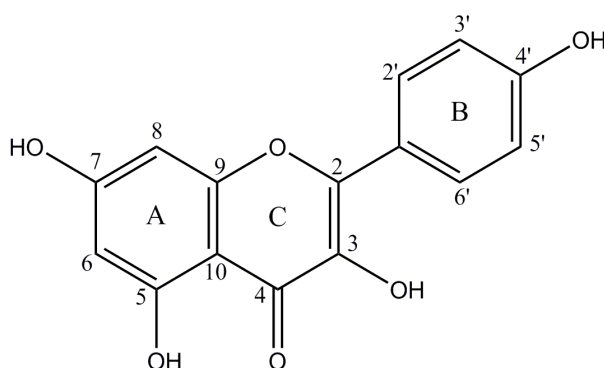


Figure 4.3.7: Kaempferol (AE72).

AE72 was obtained from the Aex-EA extract as a yellow powder (9.0 mg, 1.44% *w/w*, mp 278 °C). ESI MS (positive ion mode) indicated a protonated molecular ion at m/z 287 $[M+H]^+$ consistent with the molecular formula $C_{15}H_{10}O_6$. Electron impact MS (EIMS) also showed the molecular ion peak at m/z 286. The UV spectrum showed λ_{max} at 266 and 366 nm, indicative of a flavonoid type molecule (Xiao *et al.*, 2006).

The ^{13}C NMR along with HSQC and 1H NMR data identified 15 carbon atoms in agreement with one carbonyl and two aromatic rings (6 methines, 9 quaternary carbons). The 1H NMR spectrum (recorded in $DMSO-d_6$) also confirmed the presence of 6 aromatic protons. A broad downfield proton was seen at δ_H 12.45 (1H, s). This is typical of the hydrogen bonded 5-OH group of flavonoids (Markham, 1982). A broad downfield-shifted peak of two protons was also seen at δ_H 10.06, showing the presence of two other hydroxyl groups.

One of the aromatic rings contained only two aromatic proton signals, at δ_H 6.41 (1H, *d*, J = 1.9 Hz, H-8) and δ_H 6.17 (1H, *d*, J = 1.9 Hz, H-6), with characteristic *meta* coupling (J = ~2Hz). Since there were no other proton signals on this ring, this suggested the aromatic ring was tetrasubstituted. Both protons showed HMBC correlations to the quaternary carbons at δ_C

104.4 (C-10) and δ_c 165.7 (C-7) (Table 4.3.8 and Figure 4.3.8). Additionally, the proton at δ_H 6.17 (H-6) showed a correlation to a quaternary carbon at δ_c 162.7 (C-5), while the proton at δ_H 6.41 (H-8) showed a correlation to the carbon signal at δ_c 158.5 (C-9).

Similarly, coupled resonances at δ_H 8.03 (2H, d, $J = 8.8$ Hz, H-2', 6') and δ_H 6.91 (2H, d, $J = 8.8$ Hz, H-3', H-5') were typical of H-2'/6' and H-3'/5' of the flavonoid B-ring, respectively (Harborne, 1993). These protons showed HSQC correlations to the signals at δ_c 130.7 (C-2' and C-6') and δ_c 116.5 (C-3' and C-5'), respectively, and along with their HMBC correlations to the signal at δ_c 160.6 (C-4'), were consistent with a 4'-oxygenated phenyl group of the B ring of a flavonoid. The HMBC spectrum showed correlations of the protons at δ_H 8.03 (H-2' and H-6') to the carbon signal at δ_c 148.0 (C-2), confirming the phenyl group attachment to C-2 of ring C of the flavonoid skeleton. There was no HMBC connectivity between the A and C rings. The presence of a very deshielded resonance of a hydrogen bonded hydroxyl proton at δ_H 12.45 (5-OH), suggested that it was *peri* to the carbonyl group at δ_c 177.3 (C-4). Other positions were confirmed by HSQC and HMBC data, which are presented in Table 4.3.8 and Figure 4.3.8.

Therefore, by comparing the melting point, 1H NMR and ^{13}C NMR data of compound AE72 with the published literature (Veitch *et al.*, 2005, Ali *et al.*, 2010), and also by comparing the EIMS spectrum with NIST (National Institute of Standards and Technology) library data (>99% match) (Figure 4.3.9), the isolated pure compound AE72 was assigned as kaempferol. This is the first report of isolation of kaempferol from *A. excelsa*.

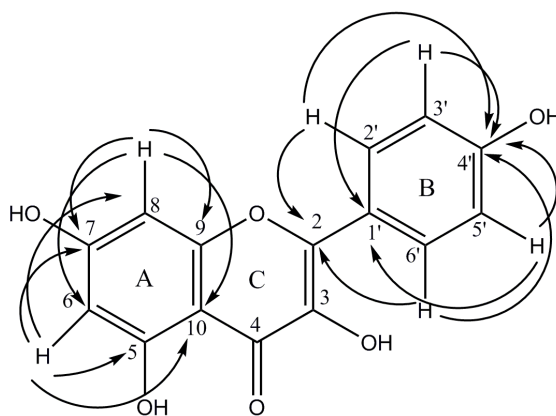


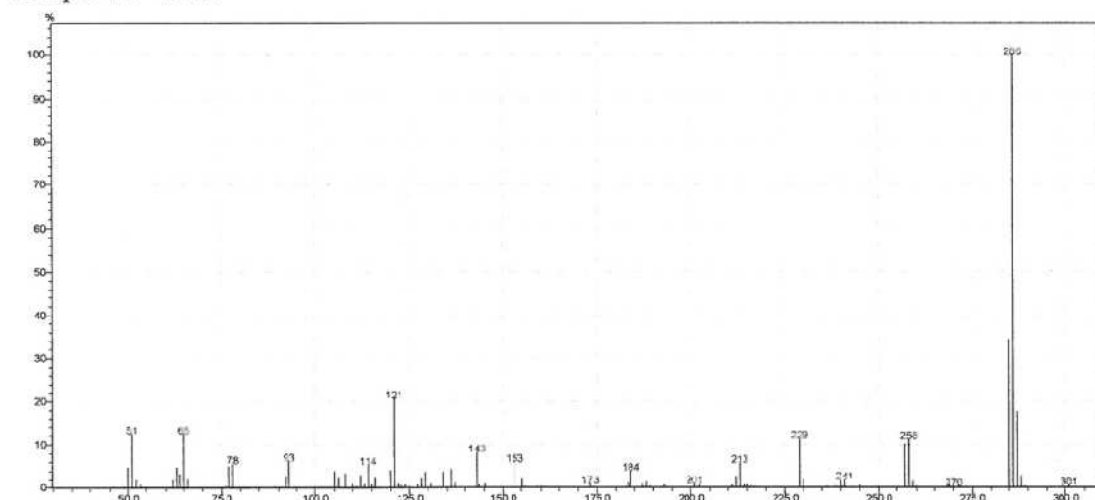
Figure 4.3.8: HMBC correlations of kaempferol (AE72).

Table 4.3.8: ^1H and ^{13}C NMR data of Compound AE72 (kaempferol).

Position	δ_{C}	δ_{H} multiplicity	HMBC
2	148.0		
3	137.3		
4	177.3		
5	162.7		
5-OH		12.45 s	
6	99.4	6.17 d ($J = 1.9$ Hz)	C-5, C-7, C-8, C-10
7	165.7		
8	94.5	6.41 d ($J = 1.9$ Hz)	C-6, C-7, C-9, C-10
9	158.5		
10	104.4		
1'	123.8		
2', 6'	130.7	8.03 d ($J = 8.8$ Hz)	C-4', C-2', 6', C-2
3', 5'	116.5	6.91 d ($J = 8.8$ Hz)	C-1', C-3', 5', C-4'
4'	160.6		
3,4'-OH		10.06 br s	

* recorded in $\text{DMSO}-d_6$

Sample 72 - Scan



NIST Database

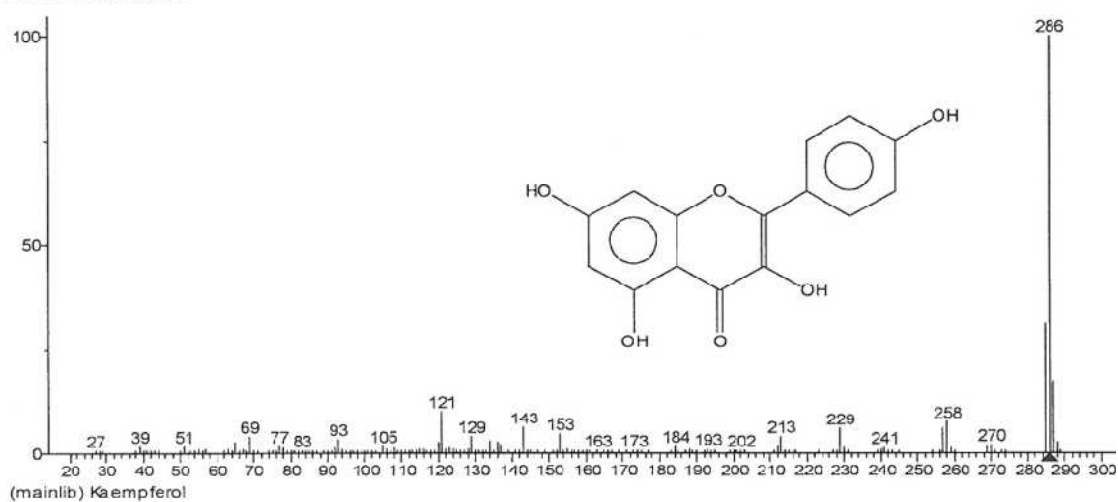


Figure 4.3.9: Comparison of EIMS spectrum of AE72 with the NIST library data.

4.3.9.2. Quercetin (AE81)

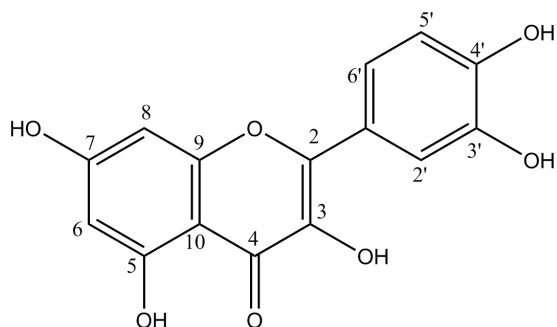


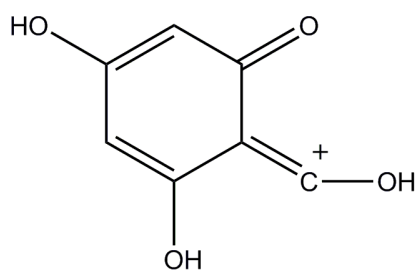
Figure 4.3.10: Quercetin (AE81).

Compound AE81 was isolated from the Aex-EA extract as a yellow powder (10.0 mg, 1.52% w/w, mp 318 °C). ESI MS (positive ion mode) showed a protonated molecular ion peak at m/z 303 $[M+H]^+$, consistent with the molecular formula of AE81 as $C_{15}H_{10}O_7$. EIMS also showed a molecular ion peak at m/z 302. The UV spectrum showed λ_{max} at 258 and 362 nm, indicative of a flavonoid type molecule (Li and Xu, 2008).

The ^{13}C NMR along with HSQC and 1H NMR data identified 15 carbon atoms in agreement with one carbonyl and two aromatic rings (5 methines, 10 quaternary carbons). The 1H NMR spectrum (recorded in $DMSO-d_6$) also confirmed the presence of 5 aromatic protons. A broad downfield-shifted peak of three protons that did not show any correlations in HSQC data was also seen at δ_H 9.4 (br, s), indicating the presence of phenolic hydroxyl groups.

Like compound AE72 (kaempferol), one of the aromatic rings contained only two aromatic proton signals, at δ_H 6.39 (1H, d, $J = 2.0$ Hz, H-8) and δ_H 6.17 (1H, d, $J = 2.0$ Hz, H-6), with characteristic *meta* coupling ($J = 2$ Hz), implying the presence of a 5,7-dihydric flavone in an A ring (Markham, 1982). Both protons showed HMBC correlations to the quaternary carbons at δ_C 102.9 (C-10) and δ_C 164.1 (C-7). Additionally, the proton at δ_C 6.17 (H-6) showed correlations to a quaternary carbon at δ_C 160.7 (C-5), while the proton at δ_H 6.39 (H-8) showed a correlation to the carbon signal at δ_C 156.0 (C-9). Additionally, the proton at δ_H 6.17 (H-6) showed a HMBC correlation to a quaternary carbon at δ_C 160.7 (C-5), while the proton at δ_H 6.39 (H-8) showed a correlation to the carbon signal at δ_C 156.0 (C-9). The 1H NMR spectrum also showed resonances of a hydrogen bonded hydroxyl proton (5-OH, δ_H 12.48), suggesting that it was *peri* to the carbonyl carbon group (Table 4.3.9).

The second aromatic ring showed a doublet at δ_{H} 6.87 (1H, d, $J = 8.4$ Hz, H-5'), a doublet of doublets at δ_{H} 7.53 (1H, dd, $J = 8.4, 2.2$ Hz, H-6') and an *meta* coupled doublet at δ_{H} 7.66 (1H, $J = 2.2$ Hz, H-2'), consistent with a 1,3,4-trisubstitution pattern. The carbon resonances at δ_{C} 145.0 and δ_{C} 147.7 (C-3' and C-4') and a broad peak of three protons at δ_{H} 9.4 ppm suggested the presence of three hydroxyl group at C-3', C-4' and C-3 positions. The HMBC spectrum showed correlations of the protons at δ_{H} 7.53 (H-6') and δ_{H} 7.66 (H-2') to the carbon signal δ_{C} 146.7 (C-2), confirming that the aromatic ring was attached to C-2 of ring C of the flavonoid skeleton. Although, there was no HMBC connectivity between the A and C rings, the characteristic signal at δ_{C} 102.9 (C-10) supported its connectivity to the carbonyl carbon of a flavonoid. A characteristic fragment at m/z 153 (A_1+1)⁺ in the EIMS spectrum also supported a dihydroxylated A ring of a flavonoid (Hedin and Phillips, 1992) (Figure 4.3.12).



m/z 153 ($A_1 + 1$)

Other positions were also confirmed by ^1H and ^{13}C NMR, along with HSQC and HMBC data, which are presented in Table 4.3.9 and Figure 4.3.11.

Therefore, by comparing ^1H , ^{13}C NMR data and melting point of the isolated pure compound AE81 with that of the published literature and also by comparing EIMS data of AE81 with NIST library data (>99% match) (Figure 4.3.12), the isolated compound AE81 was assigned as quercetin (Li and Xu, 2008, Fathiazad *et al.*, 2006, Kang, 1981). This is the first report of isolation of quercetin from *A. excelsa*.

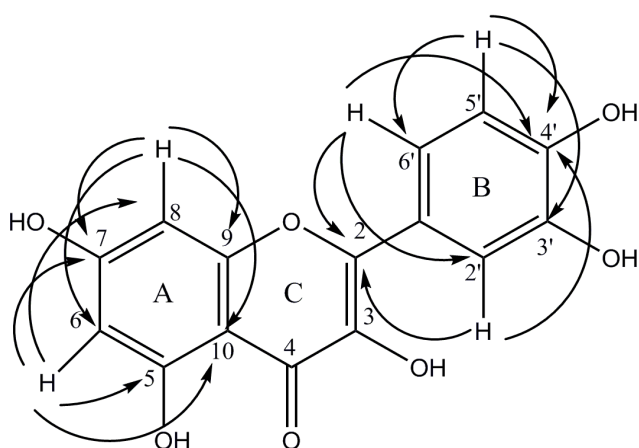


Figure 4.3.11: HMBC correlations of quercetin (AE81).

Table 4.3.9: ^1H and ^{13}C NMR data of compound AE81 (quercetin).

Position	δ_{C}	δ_{H} multiplicity	HMBC
2	146.7		
3	135.7		
4	175.8		
5	160.7		
5-OH		12.48 br s	
6	98.2	6.17, d, $J = 2.0$ Hz	C-5, C-7, C-8, C-10
7	164.1		
8	93.2	6.39, d, $J = 2.0$ Hz	C-6, C-7, C-9, C-10
9	156.0		
10	102.9		
1'	121.8		
2'	115.0	7.66, d, $J = 2.2$ Hz	C-3', C-4', C-6', C-2
3'	145.0		
4'	147.7		
5'	115.4	6.87, d, $J = 8.4$ Hz	C-1', C-3', C-4'
6'	119.8	7.53, dd, $J = 8.4, 2.2$ Hz	C-4', C-5', C-2
3',4', 3-OH		9.4, br s	

* recorded in $\text{DMSO}-d_6$

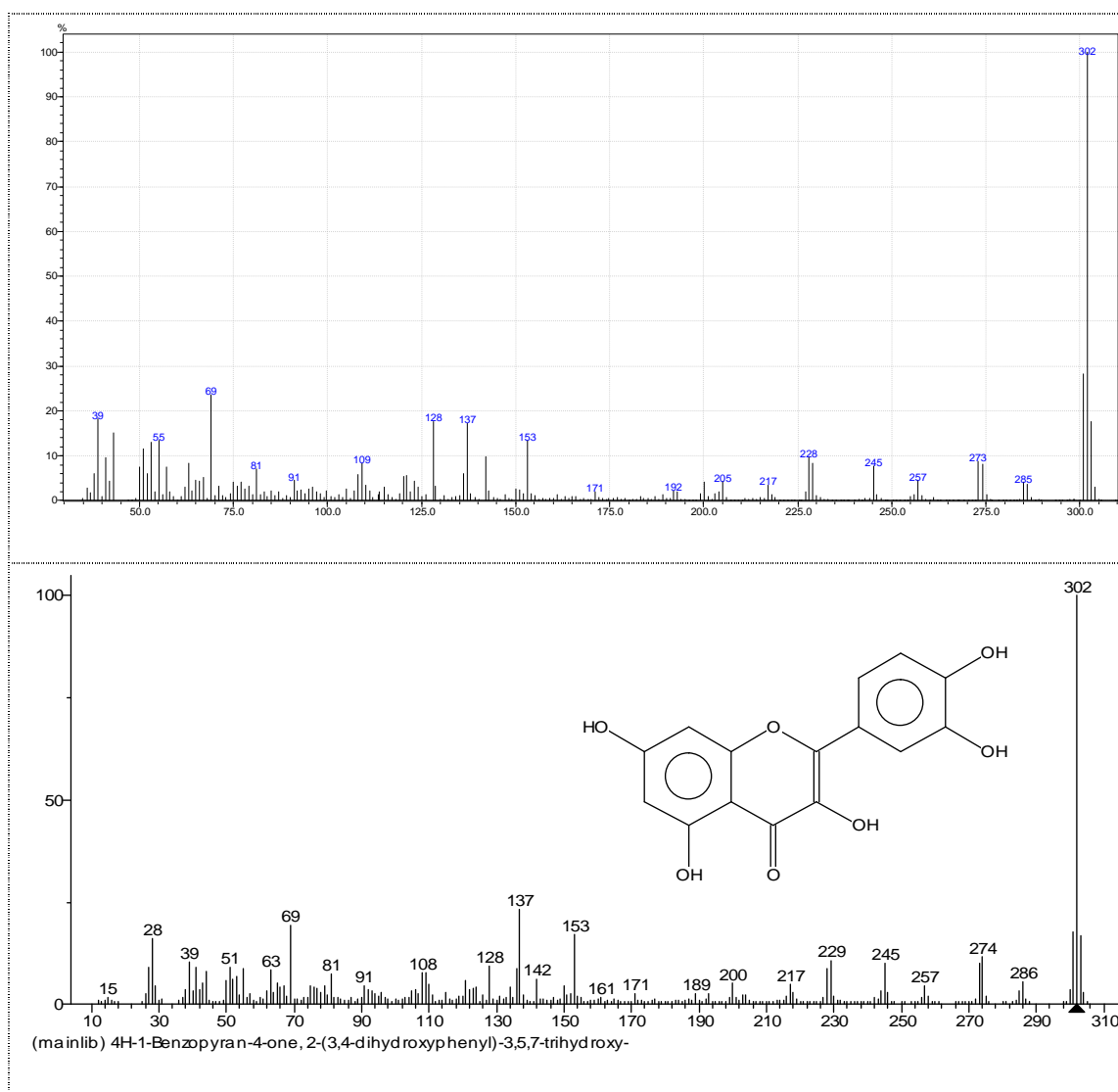


Figure 4.3.12: Comparison of EIMS spectrum of AE81 with the NIST library data.

4.3.10. Determination of MIC of isolated compounds using MTT microdilution assay

Both isolated pure compounds AE72 (kaempferol) and AE81 (quercetin) are widely reported for their antibacterial properties against a range of Gram-positive and Gram-negative bacteria including *S. aureus*, *Bacillus subtilis*, *B. cereus*, *Proteus vulgaris* and *E. coli* using different antibacterial activity studies (e.g. broth microdilution, disc diffusion, direct TLC bioautography assays) (Calderon-Montano *et al.*, 2011, Li and Xu, 2008, Hamburger and Cordell, 1987, Kang *et al.*, 2006). In the present study, antibacterial activity of kaempferol and quercetin were evaluated using the MTT microdilution assay along with bacteriostatic or bactericidal determination assays.

Both compounds showed good antibacterial activity with $IC_{90} < 100 \mu\text{g/mL}$ against *S. pyogenes* and methicillin sensitive and resistant strains of *S. aureus* (Table 4.3.10). They also showed bactericidal activity on the tested bacteria MS, MRSA, MDRSA and *S. pyogenes* and did not show any antimicrobial activity against the tested Gram-negative bacteria (*P. aeruginosa*, β lactamase positive and negative strains of *E. coli*). Antibacterial activity of both isolated compounds supports the reported antibacterial activity of these compounds and also supports the antibacterial activity of the fractions (Aex-11 and Aex-13) observed by TLC bioautography.

Table 4.3.10: MIC of isolated pure compounds (AE72 and AE81) in MTT microdilution assay.

Compounds	MIC values ($\mu\text{g/mL}$)				
	<i>S. aureus</i>	MRSA	MDRSA	<i>S. pyogenes</i>	<i>P. aeruginosa</i> , <i>E. coli</i> ($\beta+$) & ($\beta-$)
AE77	62.5** (C)	62.5** (C)	62.5** (C)	31.5*** (C)	na
AE82	62.5** (C)	62.5** (C)	62.5** (C)	31.5*** (C)	na
Vancomycin	1.25*** (C)	1.25*** (C)	1.25*** (C)	0.31*** (C)	na

*** $IC_{90} < 50 \mu\text{g/mL}$, ** $IC_{90} 50-125 \mu\text{g/mL}$, * $IC_{90} 126-1000 \mu\text{g/mL}$. C: bactericidal effect on the microorganisms. MIC was determined as the well with the lowest concentration of sample that displayed no yellow-blue colour change of MTT and also by turbidity (absorbance). Two repeats were performed on at least two separate occasions; values are average of observed results. na: not active against the tested bacteria at $250 \mu\text{g/mL}$.

Kaempferol and quercetin were not tested for anti-inflammatory activity (COX, NO, TNF- α and PGE₂ inhibition) due to limited quantities of the samples. However, there have been several reports in the literature in support of these two compounds possessing NO inhibitory and other anti-inflammatory activity (Boots *et al.*, 2008, Comalada *et al.*, 2006, García-Mediavilla *et al.*, 2007, Martínez-Flores *et al.*, 2005, Lin *et al.*, 2003).

Both compounds (kaempferol and quercetin) were not able to be tested for antioxidant activity due to sample limitations. They have been reported in the literature for having antioxidant properties (Calderon-Montano *et al.*, 2011, Rice-Evans *et al.*, 1995, Silva *et al.*, 2002, Sivasothy *et al.*, 2013) and their presence in Aex-EA (kaempferol 1.44% w/w and quercetin 1.52% w/w) might explain some of the antioxidant activity of the Aex-EA extract. The finding of antioxidant activity provides some support for the traditional use of *A. excelsa* leaves in wounds and infection related conditions.

4.3.11. Phytochemical and antimicrobial activity comparison of two extracts of *A. excelsa* leaves from different locations and seasons

The first collection of *A. excelsa* leaves was performed in August 2008 from Cumberland State Forest, NSW. The leaves were found in damaged condition (extensive herbivory, evident by numerous holes and other damage in the leaf blade). Literature also reports that leaves are often seen to be ‘moth-eaten’ as a result of insect attack (Lassak and McCarthy, 2008, Cribb and Cribb, 1984). A second batch of *A. excelsa* leaves was collected in March 2009, when the leaves were in a fresh condition without any obvious insect attack. This collection was from Lake Boulevard, Yamba, northern NSW. Freshly ground leaves (303 g) of *A. excelsa*, from the northern NSW collection, were extracted by the room temperature sequential solvent extraction method, similarly to the first collection, batch. This provided Aex-NSW-Hex, Aex-NSW-DCM, Aex-NSW-EA and Aex-NSW-MeOH extracts. Table 4.3.11 summarises the yields and characteristics of the extracts from both collections. The appearances were similar for extracts from both of the collection batches, but the percentage yields were greater for the second collection.

Table 4.3.11: Percentages of yields and appearances of sequential solvent extracts of *A. excelsa* from different collection batches.

Extracts*	Collection batch ^a	Percent yield (w/w)	Physical appearance
Aex-Hex	First	0.30%	Yellow dense solid
Aex-DCM	First	0.70%	Dark green solid
Aex-EA	First	0.73%	Dark green solid
Aex-MeOH	First	1.50%	Blackish green solid
Aex-water	First	0.51%	Yellowish green fluffy powder
Aex-NSW-Hex	Second	0.28%	Yellow dense solid
Aex-NSW-DCM	Second	0.39%	Dark green solid
Aex-NSW-EA	Second	1.02%	Dark green solid
Aex-NSW-MeOH	Second	2.50%	Blackish green solid
Aex-NSW-water	Second	2.3%	Yellowish green fluffy powder

*Extracts were prepared using the room temperature solvent extraction method (method 2), except for Aex-water and Aex-NSW-water, which were prepared by room temperature extraction with water. ^aRefers to collection batches described in Section 4.2.2.

The main reason for the second batch collection of *A. excelsa* leaves was to check whether the bioactivity of leaves without insect damage was any different to leaves in damaged condition. It was also of interest to have a collection of plant material from the local region of the Yaegl elders. Extracts of both collection batches were stored for about two years at -20 °C freezer until tested for bioactivity. Disc diffusion and MTT microdilution assays were performed using a range of medically important Gram-positive and Gram-negative bacteria including antibiotic sensitive strains (*S. aureus*, *S. pyogenes*, *S. typhimurium*, *P. aeruginosa* and *E. coli* β lactamase negative), antibiotic resistant strains (MRSA, MDRSA and *E. coli* β lactamase positive) and a pathogenic fungus *C. albicans*. The bacteria used in this study are representative of pathogens important in human infections.

The extracts were checked by disc diffusion assays (2 mg/disc) against *S. aureus*, MRSA, MDRSA, *S. pyogenes*, *S. typhimurium*, *P. aeruginosa*, *E. coli* β lactamase positive and negative and *C. albicans*. The EtOAc extracts, Aex-EA and Aex-NSW-EA, were the most active extracts (10-12.5 mm zone of inhibition against tested Gram-positive bacteria) in both batches of collection (Table 4.3.12). Aex-NSW-EA of the second batch showed slightly larger zones of inhibition than the Aex-EA of the first batch (from Cumberland) against the antibiotic

resistant strains of *S. aureus* and no activity against Gram-negative bacteria (Table 4.3.12). Aex-MeOH extract also showed antimicrobial activity (8-9 mm zone of inhibition) against the Gram-positive bacteria.

Table 4.3.12: Comparison of antibacterial activity of extracts of *A. excelsa* by disc diffusion method.

Name of extracts (2 mg/disc)	Diameter of zone of inhibition (mm)				
	<i>S. aureus</i>	<i>MRSA</i>	<i>MDRSA</i>	<i>S. pyogenes</i>	<i>S. typhimurium</i> , <i>P. aeruginosa</i> , <i>E. coli</i> (β^+) & (β^-), <i>C. albicans</i>
Extracts from Cumberland					
Aex-Hex	na	na	na	na	na
Aex-DCM	na	na	na	na	na
Aex-EA	10	11	12.0	11	na
Aex-MeOH	8	9	10	10	na
Aex-water	na	na	na	na	na
Extracts from northern NSW					
Aex-NSW-Hex	na	na	na	na	na
Aex-NSW-DCM	na	na	na	na	na
Aex-NSW-EA	10	11.5	12.5	11	na
Aex-NSW-MeOH	8	9	11	10	na
Aex-NSW-water	na	na	na	na	na
Vancomycin (2 μ g/disc)	11	11.5	11	16	-
Gentamycin (2 μ g/disc)	-	-	-	-	17, 12, 15, 15 [#]
Fluconazole (2 μ g/disc)					18 [^]

*Two repeats were performed on at least two separate occasions. Zone of inhibition was determined by diameter of complete inhibition, including 6 mm disc diameter. Values are average of two results. na: not active against the tested bacteria. [#]Diameters of zone of inhibition against *S. typhimurium*, *P. aeruginosa*, *E. coli* (β^+) & (β^-), respectively. [^]Diameter of zone of inhibition against *C. albicans*.

The MICs of the extracts (Aex-Hex, Aex-DCM, Aex-EA, Aex-MeOH, Aex-NSW-Hex, Aex-NSW-DCM, Aex-NSW-EA, Aex-NSW-MeOH) from the two batches of *A. excelsa* collected were determined using the MTT microdilution assay against the methicillin sensitive and resistant strains of *S. aureus* (*S. aureus*, MRSA, MDRSA), *S. typhimurium*, β lactamase positive and negative strains of *E. coli*, *P. aeruginosa* and *C. albicans*.

Table 4.3.13 summarises the results from these assays. Aex-Hex, Aex-DCM and Aex-water from the first and second collection batches showed no activity in the MTT microdilution assay. The EtOAc extract was the most active in both collections. However, the EtOAc extract of the second batch (Aex-NSW-EA) showed better activity than the first batch (IC₉₀ of Aex-NSW-EA against MRSA was 500 μ g/mL, whereas IC₉₀ of Aex-EA was 1000 μ g/mL. The EtOAc and MeOH extracts (Aex-EA, Aex-NSW-EA, Aex-MeOH and Aex-NSW-MeOH) showed the highest activity against *S. pyogenes* (IC₉₀ 31.25 μ g/mL and 62.5 μ g/mL for Aex-EA and Aex-MeOH, respectively). Antibacterial extracts (EtOAc and MeOH extracts) were also found to have bactericidal effects on the tested bacteria (Table 4.3.13). None of the extracts showed any activity against the tested Gram-negative bacteria and *Candida albicans*.

Table 4.3.13: Comparison of antimicrobial activities of extracts of *A. excelsa* collected from Cumberland State Forest and northern NSW in the MTT microdilution assay.

Extracts	MIC values (µg/mL)				
	<i>S. aureus</i>	MRSA	MDRSA	<i>S. pyogenes</i>	<i>S. typhimurium</i> , <i>P. aeruginosa</i> , <i>E. coli</i> (β+) & (β-), <i>C. albicans</i>
From Cumberland					
Aex-Hex	na	na	na	na	na
Aex-DCM	na	na	na	na	na
Aex-EA	500* (C)	1000* (C)	1000* (C)	31.25*** (C)	na
Aex-MeOH	1000* (C)	na	na	62.5** (C)	na
Aex-water	na	na	na	na	na
From northern NSW					
Aex-NSW-Hex	na	na	na	na	na
Aex-NSW-DCM	na	na	na	na	na
Aex-NSW-EA	500* (C)	1000* (C)	500* (C)	31.25*** (C)	na
Aex-NSW-MeOH	1000* (C)	na	na	62.5** (C)	na
Aex-NSW-water	na	na	na	na	na
Vancomycin	1.25*** (C)	1.25*** (C)	1.25*** (C)	0.31*** (C)	na

***IC₉₀ <50 µg/mL, **IC₉₀ 50-125 µg/mL, *IC₉₀ 126-1000 µg/mL. C: bactericidal effect on the microorganisms. MIC was determined as the well with the lowest concentration of sample that displayed no yellow-blue colour change of MTT and also by turbidity (absorbance). Two repeats were performed on at least two separate occasions; values are average of observed results. na: not active against the tested bacteria at 1000 µg/mL.

Direct TLC comparison of Aex-NSW-EA to Aex-EA (using DCM:EA (1:1), normal phase) was also conducted with vanillin-H₂SO₄, anisaldehyde, permanganate, iodine and Dragendorff's reagent. Figure 4.3.13 shows the staining seen with anisaldehyde reagent (a), vanillin-sulfuric acid (b) and permanganate stain (c). These results suggested the presence of similar components in both of the EtOAc extracts.

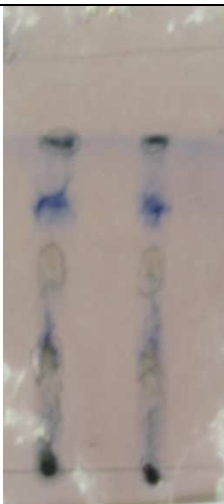
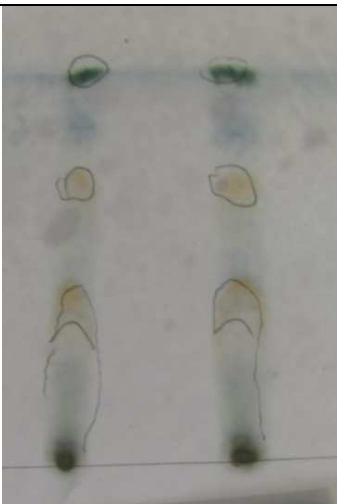

 <p style="text-align: center;">(a)</p>	 <p style="text-align: center;">(b)</p>	 <p style="text-align: center;">(c)</p>
<p>Anisaldehyde spray, DCM:EtOAc (1:1), normal phase TLC. Left: Aex-EA extract, right: Aex-NSW-EA extract.</p>	<p>Vanillin-H₂SO₄ stain, DCM:EtOAc (1:1), normal phase TLC. Left: Aex-EA extract, right: Aex-NSW-EA extract.</p>	<p>Permanganate spray, DCM:EtOAc (1:1), normal phase TLC. Left: Aex-EA extract, right: Aex-NSW-EA extract.</p>

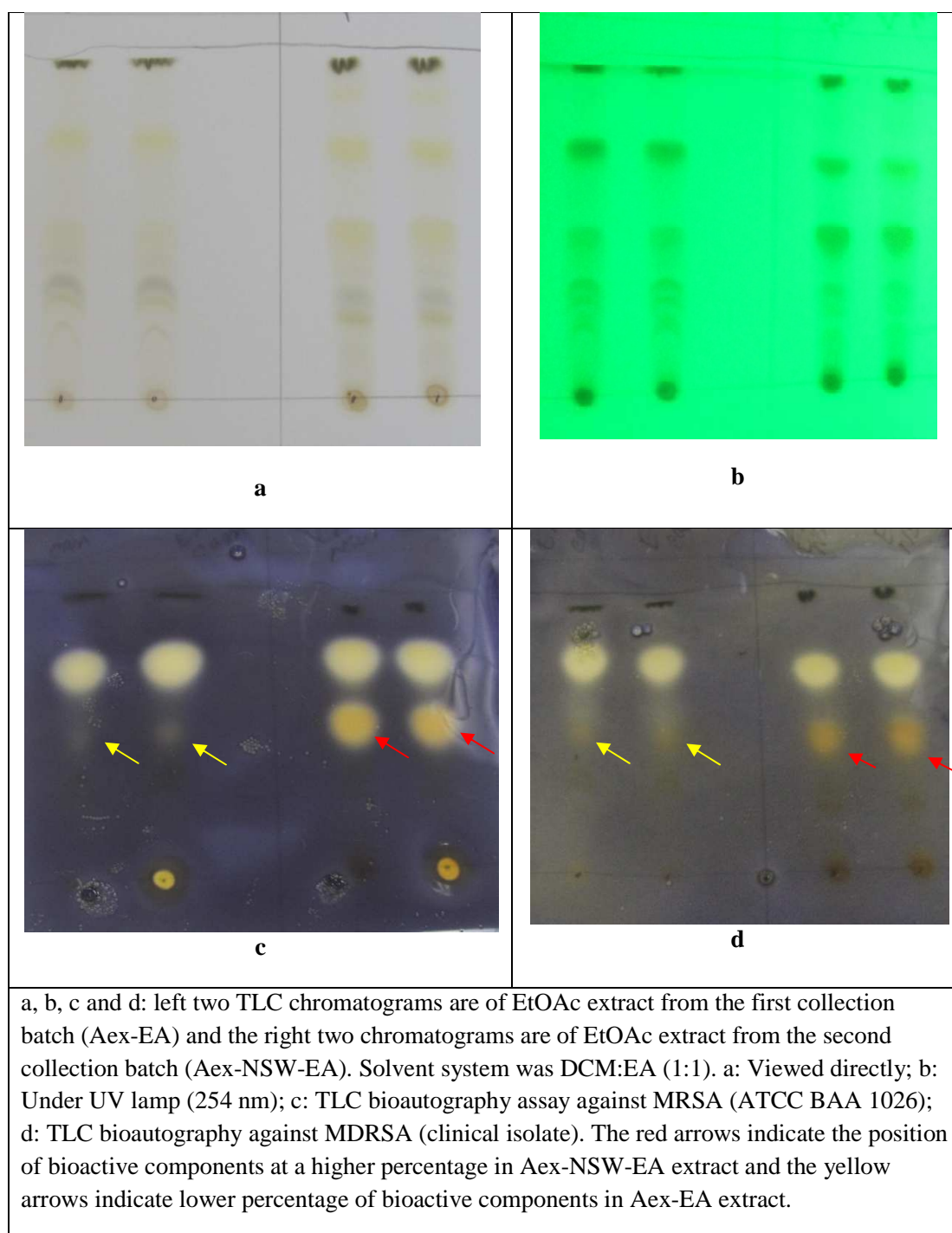
Figure 4.3.13: TLC chromatograms of Aex-EA and Aex-NSW-EA extracts after spraying with spray reagents.

Aex-EA and Aex-NSW-EA were also compared by TLC bioautography using methicillin sensitive and resistant *S. aureus*. TLC bioautography has been used to detect the effect of collection time on antimicrobial activity of plant extracts (Buwa and Van Staden, 2007). Figure 4.3.14 shows the TLC chromatogram for Aex-EA and Aex-NSW-EA and the TLC bioautography assay plate. Both extracts were prepared identically, with 100 µg of each sample spotted (dissolved in EtOAc and dried afterward) being placed on the same plate and eluted with DCM:EA (1:1). The solvent system DCM:EtOAc (1:1) was used in the TLC bioautography, as it was observed while doing the bioassay guided isolation of bioactive compounds from Aex-EA extract (Section 4.3.7) that most bioactive compounds were visible

on the normal phase TLC plate in this solvent system. Similar R_f components were seen on the TLC plate, as visualised by UV light and spray reagents for both EtOAc extracts. The northern NSW extract (Aex-NSW-EA) gave larger and more distinct zones of inhibition in the TLC bioautography against methicillin resistant *S. aureus* (MRSA and MDRSA), indicating that Aex-NSW-EA had a higher percentage of some active components (Figure 4.3.14).

Both EtOAc extracts showed similar components (with similar R_f values) and similar staining properties in the preliminary phytochemical screening (Figure 4.3.13). The different activity results could be due to the different locations of the plant collections, season or quality of the plants – the latter collection having visibly better quality leaves. Many studies in the literature support the fact that location and seasonal variation can affect biological activity and composition of plant extracts (Celiktaş *et al.*, 2007, Hussain *et al.*, 2008, Buwa and Van Staden, 2007). In future it would be interesting to test more rigorously, the effect of visible leaf quality, seasonal variations, different geographical locations or long term storage on the chemical composition or bioactivity of leaves of this plant.

Figure 4.3.14: TLC bioautography of EtOAc extracts of *A. excelsa* leaves.



4.4. Concluding remarks

Alphitonia excelsa is the most widely used medicinal plant of the Yaegl community of northern NSW. Leaves have been used by the community for the treatment of wounds, sores and inflammation. Preliminary antimicrobial and anti-inflammatory screening (performed by Brouwer) showed that extracts of the leaves had antimicrobial activities against *S. aureus* and *E. coli* and anti-inflammatory activity in COX assays (Brouwer, 2006). A literature review on this plant also showed that only limited chemical and biological studies have so far been reported on this native medicinal plant of Australia. Therefore, leaves of *A. excelsa* were selected for more detailed chemical and biological studies to investigate the antimicrobial, anti-inflammatory and antioxidant potential of this plant and to begin to identify compounds responsible for those activities.

In the present study, sequential extraction of the leaves with increasing polarities of solvent, *i.e.* *n*-hexane, DCM, EtOAc and MeOH, were examined using room temperature and atmospheric pressure extraction (stirring 24 h per extract) and accelerated solvent extraction (ASE) at higher temperature and pressure. Due to better separation of bioactive fractions and convenience, the extracts from the room temperature extraction method was chosen for further studies to isolate bioactive compounds.

Antimicrobial screening of extracts (Aex-Hex, Aex-DCM, Aex-EA and Aex-MeOH) obtained from the sequential solvent extraction using disc diffusion and MTT microdilution assays revealed that Aex-EA was the most promising among the tested extracts. *A. excelsa* leaves have been commonly used by the Yaegl community for treatment of wounds and sores. Leaves are also reported to be used in inflammatory conditions by the Yaegl and other communities (Lassak and McCarthy, 2008, Cribb and Cribb, 1984, Brouwer, 2006, Packer *et al.*, 2012). Therefore, the extracts Aex-Hex, Aex-DCM, Aex-EA and Aex-MeOH and the ‘traditional preparation’ (water extract; Aex-water) were tested for COX, NO, TNF- α and PGE₂ inhibition all measures of anti-inflammatory activity. Aex-DCM showed good activity in the COX assay (81% and 70% inhibition of COX 1 and COX 2, respectively, at 50 μ g/mL). Aex-water, Aex-MeOH and Aex-EA also showed promising COX 1 inhibitory activity (inhibited 94%, 85% and 81%, respectively, at 50 μ g/mL). Aex-Hex showed negligible COX inhibitory activity.

Aex-EA, Aex-MeOH and Aex-water were also tested for NO, TNF- α and PGE₂ inhibition and antioxidant activity (ORAC assay). Aex-EA showed good NO inhibitory activity (IC₅₀ 10.7 μ g/mL) in comparison to its cytotoxic concentration (IC₅₀ 60 μ g/mL) and a modest antioxidant (3.70×10^3 μ M TE/g) activity. Aex-MeOH showed mild NO inhibition (IC₅₀ 30 μ g/mL) without showing any cytotoxicity on RAW264 cells (at 100 μ g/mL). None of the tested extracts showed any inhibition of TNF- α or PGE₂ production. The 'traditional preparation' extract, Aex-water, showed a modest activity in the ORAC assay (1.6×10^3 μ M TE/g).

The most promising antibacterial and anti-inflammatory extract, Aex-EA, was subjected to size exclusion chromatography and gave a number of bioactive fractions. Two bioactive compounds, kaempferol and quercetin, were isolated from two bioactive fractions (Aex-11 and Aex-13), following detection by TLC bioautography against methicillin sensitive and resistant strains of *S. aureus*. IC₉₀ values for both of the compounds were ≤ 62.5 μ g/mL against methicillin sensitive and resistant strains of *S. aureus* and against *S. pyogenes* in the MTT microdilution assay. Both compounds are very well known for having antimicrobial, anti-inflammatory and antioxidant properties. This is the first report of the isolation of kaempferol and quercetin from *A. excelsa*. No more bioactive compounds could be identified from this plant due to the complexity in separation of compounds in fractions and due to time constraints.

As described above, Aex-water was inactive in the antimicrobial studies, but it showed modest antioxidant activity and COX 1 inhibitory activity. The finding of antioxidant and anti-inflammatory activity (COX assay) supports the traditional use of *A. excelsa* in inflammatory conditions by the Yaegl and other Indigenous communities. Aex-DCM showed good anti-inflammatory activity in the COX assay and no activity in the antimicrobial assays. This extract was not tested for NO, TNF- α and PGE₂ inhibition or antioxidant activity. Anti-inflammatory and antioxidant activities of the Aex-DCM extract are worthy of future investigations. Future work on the bioactive fractions Aex-4 to Aex-7 of the Aex-EA extract and fractionation of the MeOH extract would be of value.

Leaves of *A. excelsa* appear different during different times of the year (numerous holes and other damage to the leaf blade possibly due to extensive herbivory during August-September; mostly undamaged leaves during March-April). Two collections (first in August from Cumberland, State Forest, Sydney and second in March from northern NSW) were compared

following sequential solvent room temperature extraction. It was found from this study that similar compositions were seen in the TLC profile of extracts from both batches of collection, but higher antimicrobial activity was observed with the EtOAC extract of the leaves from northern NSW (Aex-NSW-EA) when compared to the EtOAC extracts from the Cumberland collection batch (Aex-EA). TLC bioautography suggested that some active components were present in a higher quantity in visibly undamaged leaves collected from northern NSW. In future it will be interesting to know whether seasonal variations, different geographical locations or long term storage have any impact on the composition or bioactivity of this plant. This could be done by collecting the plant material from different locations and in different seasons and doing the extraction and bioassays using identical methods, both directly following extraction of the plant material and after storage. Although ASE was not found to be very convenient for large scale extraction of *A. excelsa* leaves, it may be useful in such a comparative study as it is possible to maintain identical conditions and parameters for extraction by this method.

The antimicrobial, anti-inflammatory and antioxidant activities of extracts of *A. excelsa* leaves and the isolation of two known antimicrobial, anti-inflammatory and antioxidant flavonoids (kaempferol and quercetin) support the traditional use of this plant in wounds and infection related conditions by the Yaegl community.

CHAPTER FIVE

Ethical engagement with community and capacity strengthening

This Chapter describes the community engagement aspect of this PhD project, which is a vital part of an ethnopharmacological based research program.

5.1. Introduction

In general, community engagement refers to the process of collaborative work with relevant partners who share common goals and interests (Nakibinge *et al.*, 2009). It involves building authentic partnerships, including with mutual respect and active, inclusive participation, power sharing and equity (Zakus and Lysack, 1998, Tindana *et al.*, 2007). According to Dickert *et al.*, the four ethical goals of community engagement in research are: enhancing protection, enhancing benefits, creating legitimacy and sharing responsibility, all facilitated through the incorporation of community views and the community's participation in research (Dickert and Sugarman, 2005).

This PhD study was guided by the use of medicinal plants customarily (traditionally and contemporarily) used by the Yaegl Aboriginal elders. Best ethical practice for such research that encompasses Indigenous knowledge needs to be inclusive of the Indigenous community and be responsive to their needs. This Chapter presents some of the issues relevant to best ethical practice and benefit sharing approaches with the Indigenous communities. It also describes how this research project has been conducted using best ethical practice with the Yaegl people and their local (Maclean) community and details the capacity strengthening educational opportunities the author provided to the Yaegl/Maclean community and to Indigenous youth from other communities.

5.2. Recognition of the importance of traditional medicinal plant knowledge

Traditional knowledge developed from experience gained over time and adapted to a local culture and environment has always played, and is still playing, an important role in the daily lives of the majority of people globally (Cragg and Newman, 2002). It is also an essential part of cultural identity. Traditional knowledge is vital to the health of millions of people in developing countries (O'Connor, 2003). In many developing countries, traditional medicines provide the only affordable treatment available to the poor people (Maroyi, 2013). Traditional medicinal knowledge based treatments are also popular in developed countries (Zollman and Vickers, 1999, Molassiotis *et al.*, 2005). A study has shown that in 2007, Americans spent

about US \$14.8 billion to purchase non vitamin and non mineral natural products (Nahin, 2010). Another study has shown that about 36% of adults in the USA used complementary and alternative medicine in 2002, and among these adults, 18.9% used natural products (Barnes *et al.*, 2004). Traditional medicinal plant knowledge has also been a source of many modern medicines (O'Connor, 2003, Butler and Newman, 2008). The valuable drug leads provided by the study of traditional knowledge save time, money and investment for the modern pharmaceutical industry in any research and product development. Therefore, it is essential to acknowledge and assign the share of benefits to the “creators” and “holders” of traditional knowledge.

Protection and recognition of traditional knowledge has also gained the attention of several International communities, for example, the World Intellectual Property Organization (WIPO) and the United Nations Educational, Scientific and Cultural Organization (UNESCO) (O'Connor, 2003). In Australia, several medicinal plant research collaborative ventures have been established between organisations and Indigenous Australian communities to protect and recognise traditional medicinal plant knowledge of the Aboriginal people. For example, the IBRG (Indigenous Bioresources Research Group) of Macquarie University and the Yaegl Aboriginal community of northern NSW (Packer *et al.*, 2012, Brouwer *et al.*, 2005, Gaikwad *et al.*, 2008); the University of South Australia and Chuulangun Aboriginal corporation Kaanju medicinal plant project (Simpson *et al.*, 2010) and Griffith University and Jarlmadangah Buru Aboriginal community (Quinn and Mills, 2009). The collaborative partnership between the IBRG of Macquarie University and the Yaegl Aboriginal community of northern NSW was established in 2004 with the goals of preservation and recognition of their customary medicinal plant knowledge, conducting chemical and biological investigations of Yaegl medicinal plants and ensuring capacity strengthening opportunities for the Yaegl elders and their local (Maclean/Yaegl) community.

5.3. Best ethical practice with the Yaegl community

It is imperative for the research community to engage with Indigenous people in a spirit of cooperation, consultation and support. Therefore, the researcher must follow best ethical practices by conforming to rigorous ethical principles and developing trust in relationships with the individual communities. Guidelines on best practices have been developed by the

international community (UN, 1994). The participatory action research methods published by UNESCO and the NH&MRC (Tuxill and Nabhan, 2001, NH&MRC and AVCC, 2007) have been adopted by the IBRG for developing relationships and trust with the Aboriginal communities. For the Yaegl community (and similarly for other communities the IBRG has engaged with), the following steps were followed by the IBRG.

- Meetings were held on a regular basis with community members who constituted the appropriate authorising persons to discuss the project.
- Authorisation to proceed with the project was obtained from the community.
- Meetings were held with community members to design research activities and to identify interested participants.
- The study plan was implemented with the participants.
- Data was summarised with input from participants and preserved.
- Discussions were held for possible expansion of the project and community advice was sought as to how best to provide in kind support strategies to the community as recompense for their efforts.
- Meetings were held to find the most appropriate way of storing and transmitting the information to the greater community as deemed appropriate by the elders.
- Presentations were made by the IBRG to the community members to update the latter with the ongoing research work and to discuss the future directions with them.

In Australia, strategies of best practice of collaborative work with Indigenous community groups have also gained attention in recent years. “The Aboriginal plan’ developed by traditional owners of the Wet Tropics Natural Resource Management (WTNRM) region, with support from various government and non-government organisations, was established to address issues of cultural and natural resource management in that region. As a part of the collaborative and benefit sharing approach, strategies and aspirations of the community were documented. One of the strategies aimed to “develop and implement programs and projects that document knowledge and facilitate the transmission of cultural knowledge and practices”. The following action points were recommended from this report (WTAPPT, 2005).

- Record and document knowledge of country *via* books, databases and CDs (with appropriate intellectual and cultural property rights protection).
- Develop educational materials (books, CDs, internet, etc) for both Aboriginal people and the broader community.
- Develop and implement cultural programs, including camps, to bring elders and young people together on country to facilitate transmission of knowledge and cultural practices.

The IBRG have been highly committed to working in true collaborative partnership with the Yaegl community, and other communities, in accordance with the national and international protocols of working with Indigenous people on traditional knowledge systems. For this PhD project, all stages of the project were performed with collaborative participation of the Yaegl community and all stages were approved by the Human Ethics Committee at Macquarie University for human research and for collaborative research work with Indigenous people (HE27JUL2007-R05361 and 5201200763).

5.4. Participation in community engagement and capacity strengthening with the Yaegl community

As a member of the IBRG, the author actively participated in different activities as a part of community engagement and capacity strengthening with the Yaegl community of northern NSW. Some of these activities are detailed below.

- The author was introduced to the Yaegl elders in 2008 and discussed with them the plans and goals for exploring their medicinal plants as part of her project.
- The author presented her research findings to date at a meeting with the Yaegl elders during a visit of the elders at Macquarie University in mid 2009. This meeting involved presentations by all members of the IBRG team about their research activities and discussions with the elders on ongoing research activities, future plans and expected outcomes of the project. The author also facilitated a laboratory visit by the Yaegl elders, along with two Aboriginal youth from Maclean, in 2009 (Figure 5.4.1). This laboratory visit was filmed and contributed to a story on the IBRG-

Yaegl/Maclean research partnership as part of a Caring for Country special broadcast by ABC television channel's 'Message Stick' program. The story was broadcast on ABC National Television on 28 August 2010. During that laboratory visit, the author described in layman's terms about the laboratory work with the Yaegl medicinal plants to give the elders and youth a better idea about the hands-on research.



Figure 5.4.1: Extraction and purification procedures were shown to the elders and youth of the Yaegl/Maclean community by the candidate in 2009.

- The author also participated in a workshop that was arranged by the IBRG in Maclean with the community members in 2011. A photo-collage of the extraction and purification process of the medicinal plants was also shown to give the participants of the workshop an update of the biological and chemical analyses being conducted by the author at Macquarie University.
- The author made an oral presentation to Yaegl community members at the Yaegl Local Aboriginal Land council meeting (April 2013) to provide information on the medicinal plant research that had been done to date by the candidate and to introduce possible partnership opportunities to enhance the research (Figure 5.4.2). This included discussion on the value of collaboration with Prof. Hans Wohlmuth's research group at the Southern Cross University for anti-inflammatory and antioxidant activity. The Yaegl elders present were happy for this collaboration with Prof. Wohlmuth to develop and provided in principle verbal agreement. Written agreement for this partnership

with Prof. Wohlmuth was provided subsequently on June 5, 2013, following a formal meeting in Maclean of the Yaegl elders with Prof. Wohlmuth and IBRG leaders A/Profs Joanne Jamie and Subra Vemulpad.



Figure 5.4.2: The author presenting updated research work at the Yaegl Local Aboriginal Land council meeting (April 2013). Photo courtesy: A/Prof Subra Vemulpad.

- Finally in August 2013, the author presented her research findings as a whole in front of the Yaegl elders who came to Sydney to take part in the National Indigenous Science Experience program (NISEP) at the Redfern Community Centre during National Science Week 2013 (Figure 5.4.3).



Figure 5.4.3: The author along with the Yaegl elders and the NISEP Co-Director A/Prof Joanne Jamie during National Science Week event, August 2013.

As indicated by the above examples, the author has actively participated in several events and programs during the whole course of this PhD study. All the activities with the author were performed as a part of the collaborative research program of the IBRG with the Yaegl community, in which both parties were regularly informed of the research taking place and their outcomes and discussed together on the future research directions.

5.5. Benefit sharing and capacity strengthening

The Convention of Biological Diversity (CBD), adopted at the 1992 Earth Summit in Rio de Janeiro, Brazil, aims to guarantee the conservation and sustainable use of biodiversity by ensuring that its traditional custodians are rewarded, and hence encouraged to promote conservation (Schroeder, 2009). The term “benefit sharing”, in the context of traditional knowledge, was popularised by CBD. It is a relatively new idea in international law. The Hoodia case is a classic example of benefit sharing and recognition of traditional knowledge of the San people. The San tribe is one of the oldest communities in South Africa and they are holders of traditional knowledge on the use of *Hoodia gordonii*, which is found in the Kalahari Desert. Historically, the San people consumed the plant to stave off hunger during times of famine, low food supply or long nomadic hunting trips. The San people were initially unaware that the South African Council for Scientific and Industrial Research (CSIR) had been granted a patent for the research work on “an appetite suppressant derived from the extract of *Hoodia gordonii*”. Later, with the involvement of NGOs, the San people and CSIR negotiated one of the first benefit sharing agreements, which gives the San people as holders of the traditional knowledge, a share of royalties derived from the sale of patented products from *Hoodia gordonii* (Moyer-Henry, 2008).

Capacity building (or capacity strengthening, which is becoming the more accepted terminology) goes hand in hand with benefit sharing. Capacity building has been defined by Chaskin (2001) as “the interaction of human capital, organizational resources, and social capital existing within a given community that can be leveraged to solve collective problems and improve or maintain the well being of the community” (Chaskin, 2001).

The IBRG of Macquarie University is fully aware of benefit sharing and capacity strengthening and the importance of the recognition of the custodians of the traditional

medicinal knowledge aspects of this project. This PhD research was based right from its onset on collaboration and two-way exchange of knowledge, skills and benefits. Collaborative research partnership agreements were co-developed between the IBRG and the Yaegl community (with the Yaegl Local Aboriginal Land Council as the authorising body). Both parties (Yaegl community members and IBRG) strictly followed the terms and conditions of the agreements. The IBRG and the Yaegl elders and members of the Maclean community also developed several initiatives towards capacity strengthening opportunities for the Yaegl/Maclean community. A few examples of these, in which the candidate also participated, are presented below.

5.5.1. Co-authorship in publications

The IBRG acknowledge the ownership of the customary knowledge of the Yaegl elders and from the commencement of their collaborative program have been committed to documenting this through co-authorship on relevant publications that arise from work on their medicinal plants (Packer *et al.*, 2012, Brouwer *et al.*, 2005, Packer *et al.*, 2011b).

5.5.2. Integration of medicinal knowledge and research findings into an online database (CMKb)

Considering the importance of documenting and conserving the medicinal plant knowledge possessed by Indigenous communities, the IBRG has developed an online database that is known as the Customary Medicinal Knowledgebase (CMKb). CMKb was initially established for collating, disseminating, visualising and analysing public domain data on customary medicinal plants. The database can be accessed online at <http://biolinfo.org/cmkb/>. The database stores information related to taxonomy, phytochemistry, biogeography, biological activities of customary medicinal plant species as well as images of individual species (Gaikwad *et al.*, 2008). The database also stores literature information on the use and preparation of customary medicines. The CMKb also has a private password protected component for community members that allows documentation of their medicinal plant preparations and uses, along with media files and audiovisual recording clips from the interviews of elders (with written consent). This community specific component of the database is currently being updated in consultation with the Yaegl elders to ensure the information is presented in an appropriate manner for their community use. They are

particularly keen to use this information towards the education of their youth on their customary practices. Information obtained first-hand from communities will remain password accessible to only community members, unless community elders request for public availability. The author has provided her research findings on the two medicinal plants, *Alphitonia excelsa* and *Lophostemon suaveolens*, for the CMKb database. This will not only help to conserve the valuable medicinal plant knowledge of the Yaegl elders, but it will also help in contributing to science and to the younger generation.

5.5.3. Cultural immersion program

The cultural immersion program was designed to ‘close the gap’ of information exchange between the Yaegl elders, Maclean High School students and teachers, and the IBRG in a sustainable and integrated format. The main aim is to encourage Maclean High School students to gain a greater understanding of their local culture. The cultural immersion program between the IBRG, Maclean school staff and Yaegl elders commenced in 2010 in a true spirit of collaboration. This program constitutes a series of excursions for the Maclean school students to go with the elders out on to country to experience storytelling about their culture, including bush food and medicines. Resources for this program were developed jointly by the IBRG and the Yaegl elders. The elders take the students to sites that have an important cultural value, including quarries for stone tools, fish traps and hunting sites. The students are also taught traditional Indigenous sports and games. This has run annually since 2010 and has become very popular amongst the students, school staff and elders.

5.5.4. Indigenous Science Education Program

According to the Australian Bureau of Statistics, a remarkable discrepancy exists between the high school completion rates of Indigenous versus non-Indigenous students. The high school completion rates for year 10 have increased for Indigenous students by more than ten percentage points in 10 years from 2001 to 2011, to 99%, nearing the perfect completion rates of non-Indigenous students (Australian Bureau of Statistics, 2013). However, there remains a large discrepancy in the final year (year 12) completion rates between these two groups, with 81% of non-Indigenous students completing their final year of high school, while the completion rate of Indigenous students was only 49% in 2011 (Australian Bureau of Statistics, 2013). In 2007, only 32% of the NSW Indigenous population stayed in school until year 12,

compared to 72% of the non-Indigenous population. Progression to tertiary education was also disproportionately low, with nationally only 6% of Indigenous 20-24 year olds engaged in full time university study, compared with 24% of the non-Indigenous population (Long *et al.*, 2009).

Concerned by the alarmingly low numbers of local Indigenous students completing their secondary education and higher education, the Yaegl Aboriginal elders requested the IBRG to assist with strategies to enhance the educational outcomes of their Indigenous youth. In particular they hoped that the IBRG and others within Macquarie University could help to motivate the younger generation of Aboriginal people in their community to engage in their formal schooling and increase their exposure to further education and career options. To achieve this goal, the IBRG with the collaboration of Aboriginal communities and high school staff of northern NSW established a program in 2005 called the Indigenous Science Education Program (ISEP) (available online on facebook and youtube at <http://www.facebook.com/NISEP.MQ> and <http://www.youtube.com/user/mqNISEP>). The ISEP, now known as National Indigenous Science Education Program (NISEP), has been running science shows and other science and technology focused activities since 2005. Initiatives of NISEP include interactive science shows encouraging secondary student including Indigenous youth involvement in demonstration of 'household' science to their peers, junior students and local community. The major aim of this was to provide these secondary student demonstrators with leadership skills and the confidence and motivation to complete their high school studies and a pathway to tertiary education. Additionally, having direct interaction with the NISEP University staff and undergraduate and postgraduate students, coordinating these shows, the school students are exposed to the opportunities available to them through tertiary education.

As an active member of the IBRG team, the author was involved with arranging and running the science shows from 2008 to 2013 at Maclean and the nearby Casino High school, Western Sydney Schools and Macquarie University (Figure 5.5.1). The author also participated in other events with Indigenous students from Maclean, Casino and Western Sydney High Schools, as part of NISEP. These includes during National Science Week events in Sydney in 2012 and 2013, Macquarie University Open Days in 2011 and 2012, along with several other events performed by NISEP from 2008 to 2013.

The main responsibilities performed by the author for NISEP included:

- Preparation of reagents for chemistry science shows activities.
- Organising necessary items required for running the science shows.
- Setting up the science show activities at the science show event venue.
- Participating as a demonstrator and also training new demonstrators – the secondary school students - for running the science shows to up to 200 junior students per event.

This education work was also approved by the Macquarie University ethics committee (Human ethics approval number HE27JUL2007-R05361, 5201200891).



Figure 5.5.1: The author's participation in a science show with an Indigenous demonstrator. Photo courtesy: Erin Rozgonyi.

5.5.5. Outcomes of NISEP

The activities of ISEP/NISEP, especially the science shows, have been successful and popular activities among the student demonstrators and student participants, elders and teachers as well as the participating Macquarie University volunteers. These shows helped to establish a good relationship of trust among the Indigenous and scientific community. It also helped the Indigenous students demonstratorstor grow in confidence (Figure 5.5.4 and Figure 5.5.5) and want to enhance their educational outcomes. These students became role models for junior

Indigenous students who saw them as leaders, and this was enhanced by the excellent media coverage on a national and international level of these youth conducting these activities.

5.5.5.1. Impact on Indigenous students

An analysis and consolidation of pre-event student demonstrator surveys and surveys completed post-event by the participating students and teachers on the impact of the activities on the student demonstrators indicated that the Indigenous students overcame their initial nervousness and left with positive impressions. There were significant impacts of the NISEP activities among the overall Indigenous community members.

According to the data from students of five schools (who participated in the NISEP program from 2007 to 2013), before participating in the activities, 65% of the participating students intended to finish school in years 10 or 12 and/go to TAFE; 36% intended to go to university. Post-event surveys (after participation in the NISEP events) revealed that 42% students intended to go to university. Approximately 6% students reported a new intention to attend university. These students had previously intended to finish school in years 10/12 and/or go TAFE. The survey results are presented in the following chart (Figure 5.5.2).

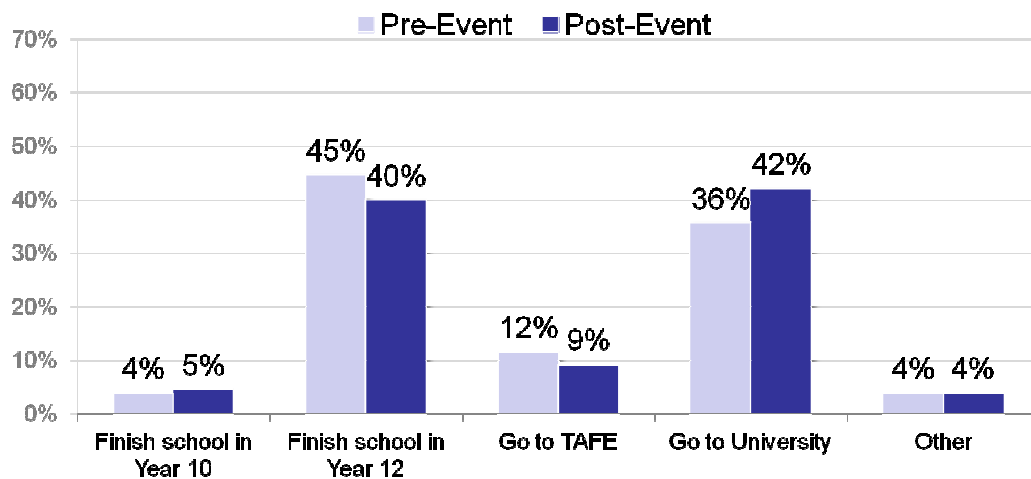


Figure 5.5.2: Accumulative pre- and post-event surveys on intention to undertake further studies among school students who participated in NISEP program from 2007-2013. n=300.

NISEP also encouraged the participating students towards science. About 81% of all students surveyed reported an increased interest in science as a result of the program (28% reported a great increase, 53% reported a slight increase) from 2007 to 2013 (Figure 5.5.3).

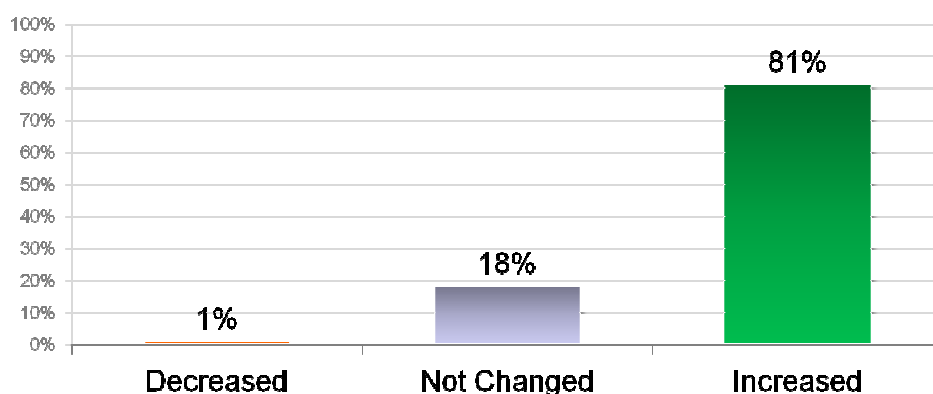


Figure 5.5.3: Accumulative pre- and post-event surveys on interest in science among school students that participated in the NISEP program as demonstrators from 2007-2013. n=314.

Most telling has been the remarkable changes the teachers and Aboriginal Education Assistants have seen in the students involved as demonstrators in the NISEP activities, especially the student demonstrators. Teachers and Aboriginal Education Assistant surveys in the months following NISEP activities noted clear improvements in student learning and engagement, with positive changes in concentration and behaviour up 60% and 61%, respectively; completion of work up 68%; and motivation, willingness to work with others and enthusiasm for learning up 73%, 73% and 80%, respectively. It was also noted that several of the students had significantly improved on their assessment scores.

“... is definitely more motivated and more positive about his schooling ... devoting more time, at home in particular, to work”.

“There has been a definite improvement in behaviour and attitude ... showing more interest and making a serious attempt to complete work”.

Maclean High school is one of the participating schools that have been involved with the NISEP program from the beginning (2005). Maclean High school is located in the area where

the Yaegl community members reside. NISEP has significantly influenced the Indigenous students of Maclean High School towards higher education and learning science. The Former Head Science teacher and Deputy Principal of Maclean High School commented in 2010 on the impact of the program: *“I have noticed a dramatic change in perception of the whole school community who now see Indigenous students as academic role models within the school”*.



Figure 5.5.4: Student demonstrators from Maclean High School with the NISEP Co-Director A/Prof. Joanne Jamie, 2013. Photo courtesy: Joanne Packer.



Figure 5.5.5: Jordan Walker (an Indigenous student) demonstrating the properties of carbon dioxide to the Australian Chief Scientist, Professor Penny Sackett (2010). Photo courtesy: Teresa Malewska.

5.5.6. Media exposure

The IBRG and the Indigenous community established a collaborative model of research between an academic institution (Macquarie University) and the Yaegl Aboriginal community. It has also gained media attention locally, nationally and internationally. Some examples are:

- **TV coverage:** Australian Broadcasting Corporation (2009) Indigenous Medicine – NSW. Message Stick Documentary – (05 min 28 sec) screened on ABC National Television, 28 August 2010.
- **TV coverage:** King, S. (2009) Indigenous Students Make Science Fun. (01 min 13 sec) screened on NBN News local television (Northern Rivers), 02 June 2010.
- **Print media coverage:** Students learning science can be fun, Express Examiner, Casino, NSW; 26 August 2009.
- **Print media coverage:** Brady, E. (2010) Yaegl project success, Daily Examiner, Grafton, NSW; 03 February 2010.
- **Online media coverage:** Yaegl research collaboration hits new heights, Macquarie University Newsroom, www.mq.edu.au, 01 February 2010.
- **Online media coverage:** Modernising the magic of Indigenous medicine, Medical Observer, www.medicalobserver.com.au, 08 February 2010.
- **Online media coverage:** Researchers develop Australian traditional herbs, Chinese Radio International, www.english.cri.cn, 19 July 2010.
- **Online media coverage:** Traditional Healing, euro news, www.euronews.net. 19 July 2010.
- **Print media coverage:** Indigenous science program helps enhance learning, Northern Rivers Echo, Lismore, NSW; 04 August 2011.
- **TV coverage:** Indigenous science education. Screened on ABC National Television program - Catalyst, <http://www.abc.net.au/catalyst/stories/3576726.htm>, 30 August 2012.

- **Print media coverage:** Collins, S, Medicine goes native, The Age National, 25 March 2013.

These types of media coverage helped to publicise the science activities and the bush medicine project, as well as helped to acknowledge the achievements of the Yaegl community members and boost their self-confidence.

5.6. Conclusions

The present PhD study is based on the ethnomedicinal knowledge of the Yaegl Aboriginal community of northern New South Wales. This study was performed in a collaborative manner by bringing Indigenous communities and research scientists together on one platform. This Chapter presented the ethical approach followed to strengthen the relationship and highlighted the capacity strengthening activities provided to the community and the candidate's role in these activities. In future, the IBRG team (including the author) plans to explore making some simple preparations (e.g. antiseptic soap or a decoction from leaves) that the community can market, backed by the collaborative research findings. This type of collaborative and co-operative partnership between the IBRG and the Yaegl community will also help to motivate other research groups to work with Indigenous communities for the preservation and utilisation of valuable medicinal plant knowledge, which can aid discovery of new medicines, and can provide capacity strengthening opportunities for the community.

CHAPTER SIX

Conclusions and future directions

This PhD project was based on a collaborative research partnership between the Indigenous Bioresources Research Group (IBRG) of Macquarie University and the Yaegl Aboriginal community of northern NSW. It followed on from previous IBRG research by Drs Brouwer and Packer who documented first-hand knowledge of medicinal plants used for treatment of wounds, sores and infection by the Yaegl Aboriginal community of northern NSW and undertook preliminary screening of some of these plants. This PhD study focused on identifying and isolating bioactive compounds from two Yaegl medicinal plants used for the treatment of wounds, sores and skin infections, following a bioassay guided isolation approach.

Chapter 1 described the importance of medicinal plant research in the process of drug discovery guided by ethnomedicinal use. The specific aims of this PhD project were also described.

Chapter 2 described the rationale for selecting the two native Australian medicinal plants, *Lophostemon suaveolens* and *Alphitonia excelsa*, for chemical and biological investigations. The selection was made on the basis of community interest, a detailed literature review on chemical and biological studies undertaken on the Yaegl medicinal plants, and preliminary biological screening results. This Chapter also highlighted the selection of biological and chemical methods chosen to evaluate the antimicrobial, anti-inflammatory and antioxidant activities.

Chapter 2 summarised preliminary antimicrobial and anti-inflammatory screening results conducted by Drs Brouwer and Packer on the Yaegl medicinal plants used for treatment of wounds and skin infections. This led to the identification of eight medicinal plants, *Lophostemon suaveolens*, *Syncarpia glomulifera*, *Canavalia rosea*, *Alocasia brisbanensis*, *Alphitonia excelsa*, *Hibbertia scandens*, *Duboisia myoporoides* and *Ipomoea pes-caprae* with promising biological activities. A detailed review of the chemical and biological investigations previously reported assisted in the selection of *L. suaveolens* and *A. excelsa* for further studies.

Leaves of *A. excelsa* have been widely used by the Yaegl community as an antiseptic hand wash. They are also used for sores and an upset stomach by other communities. Preliminary screening results of Brouwer showed promising antibacterial and anti-inflammatory activities of EtOH and H₂O extracts of leaves of *A. excelsa*. According to the literature, there have been

limited chemical studies on *A. excelsa* and no antimicrobial or anti-inflammatory activity studies.

L. suaveolens is an important medicinal plant for the Yaegl elders. Ash from the bark and milky sap of *L. suaveolens* are used by the Yaegl community for skin diseases, infections and for the treatment of ringworm. There have been no reports of biological activity studies for any part of this plant in the published literature and only one chemical study of examining the volatile components of the leaves has been reported. Sap could not be collected in sufficient quantity for testing, but the elders requested the leaves be investigated. Preliminary screening by Packer found that EtOH and H₂O extracts of leaves of *L. suaveolens* displayed potent antimicrobial activity against *S. pyogenes* and antibiotic sensitive and resistant strains of *S. aureus* and moderate activity against *P. aeruginosa*.

Thus, the leaves of both *A. excelsa* and *L. suaveolens* were selected for antimicrobial, anti-inflammatory and antioxidant activity studies, followed by bioassay guided isolation of bioactive compounds, as described in Chapters 3 and 4. Antimicrobial screening was performed against a range of pathogenic bacteria (*Streptococcus pyogenes*, methicillin sensitive, resistant and multidrug resistant strains of *Staphylococcus aureus*, β lactamase positive and negative strains of *Escherichia coli*, *Salmonella typhimurium* and *Pseudomonas aeruginosa*) and a pathogenic yeast *Candida albicans* using disc diffusion, TLC bioautography and MTT microdilution assay methods. Anti-inflammatory activity was tested using COX, nitric oxide, TNF- α and PGE₂ inhibition assays. Antioxidant activity was tested using the ORAC assay.

Preliminary screening studies and the literature review of plants used by the Yaegl community also revealed that *Syncarpia glomulifera* and *Hibbertia scandens* would be worthy of further exploration biologically and chemically.

Chapter 3 described the chemical and biological studies on *Lophostemon suaveolens*. Sequential solvent extraction of *L. suaveolens* yielded four extracts, *n*-hexane, DCM, EtOAc and MeOH extracts. A water extract (LS-water) was also separately prepared. *n*-hexane (LS-Hex) and DCM (LS-DCM) extracts showed significant antibacterial activity with MIC (IC₉₀) <50 μ g/mL against *Streptococcus pyogenes* and antibiotic sensitive and resistant strains of *S. aureus*. Five major bioactive fractions (LSL-5, 7, 10, 11 and 12) obtained from fractionation

of LS-DCM, showed potent antimicrobial activity (IC_{90} <50-1000 $\mu\text{g/mL}$ range against *Streptococcus pyogenes* and methicillin sensitive and resistant strains of *S. aureus*). Fractionation and purification of the LS-Hex and LS-DCM extracts led to the isolation of two bioactive compounds, betulinic acid and eucalyptin. The structures were elucidated by 1D and 2D NMR and mass spectrometric data. This is the first report of isolation of betulinic acid and eucalyptin from *L. suaveolens*. Both of these isolated compounds have been reported as having antibacterial and anti-inflammatory activities.

GC-MS analysis of the *n*-hexane extract (LS-Hex) identified 16 compounds. These included the well known bioactive compounds aromadendrene (15.4%), spathulenol (12.46%), globulol (4.47%), epiglobulol (2.69%), phytol (2.84%), β -caryophyllene (2.53%) and α -humulene (1.52%). These compounds have earlier been reported for having antibacterial properties. The significant antimicrobial activity of the oily *n*-hexane extract of *L. suaveolens* might be associated with the presence of these well known bioactive compounds.

The antibacterially active extracts LS-Hex, LS-DCM and LS-water and fractions (LSL-5, 7, 10, 11 and 12) were also tested for anti-inflammatory and antioxidant activity using NO, TNF- α inhibition (in RAW264 cells), PGE₂ inhibition (in 3T3 mouse fibroblast cells) and ORAC (for antioxidant activity) assays. Both the LS-Hex and LS-DCM extracts of *L. suaveolens* showed NO inhibitory activity (LS-Hex IC_{50} 43.9 $\mu\text{g/mL}$ and LS-DCM IC_{50} 4.6 $\mu\text{g/mL}$). Although the selectivity index for LS-Hex was less than 1 (toxic), LS-DCM was more promising with a selectivity index >2. The fractions with antibacterial activity (LSL-5, 7, 10, 11 and 12) showed promising NO inhibitory (IC_{50} <12 $\mu\text{g/mL}$) and PGE₂ inhibitory (<20 $\mu\text{g/mL}$) activity. The ORAC assay also revealed that some extracts and fractions (LS-DCM, LS-water, LSL-5, 11 and 12) had modest antioxidant activity (1000-2700 $\mu\text{M TE/g}$). None of the tested extracts, fractions or pure compounds showed any activity in the TNF- α inhibition assay. Betulinic acid, which was isolated from LSL-7, showed good anti-inflammatory activity. Betulinic acid is well known for its anti-inflammatory and cytotoxic activities.

β -Caryophyllene (identified by GC-MS in LS-Hex) has been reported to have anti-inflammatory activity and may contribute to the anti-inflammatory activity of the LS-Hex extract. β -Caryophyllene, along with α -humulene and spathulenol (also identified by GC-MS in LS-Hex) are also known to be cytotoxic and may therefore contribute to the cytotoxic activity of the LS-Hex extract. This is the first report of chemical and biological studies of *L.*

suaveolens. Although the promising findings of this study cannot be linked directly with community use of leaves, the selection of this plant was made based on the traditional use of another part of the plant in the treatment of skin infections and as an antiseptic. The selection was also dictated by community interest. The present findings also suggest that *L. suaveolens* leaves could provide a more accessible source of medicine (*i.e.* leaves, rather than the sap) for the treatment of skin infections and wounds for the Yaegl community.

Due to time constraints and complexity of the samples, several of the fractions that had antibacterial, anti-inflammatory or antioxidant activities (LSL-4, 10, 11 and 12) were not further investigated for their bioactive compounds. There is therefore a potential to isolate more bioactive compounds from *L. suaveolens* leaves in future studies.

Chapter 4 described the chemical and biological studies performed on *Alphitonia excelsa*. Fresh leaves of *A. excelsa* were initially extracted by two different methods, accelerated solvent extraction (ASE) and sequential solvent extraction at room temperature and atmospheric pressure to compare the efficiency of each method. TLC, along with disc diffusion assays, indicated that better initial fractionation of bioactive components occurred using room temperature sequential extraction rather than the ASE sequential extraction method. Due to this better separation and convenience, the room temperature sequential solvent extraction method was chosen for further studies to isolate bioactive compounds.

Large scale sequential solvent extracts were tested for antibacterial activity by the disc diffusion and MTT microdilution assays. The EtOAc extract (Aex-EA) was the most active extract in the disc diffusion assay (10-12 mm diameter zone of inhibition at 2 mg/disc against *S. pyogenes* and antibiotic sensitive and resistant strains of *S. aureus*) and in the MTT microdilution assay (with MICs (IC₉₀) of 500-1000 µg/mL against sensitive and resistant strains of *S. aureus* and MIC <100 µg/mL against *S. pyogenes*). The MeOH extract (Aex-MeOH) also displayed antimicrobial activity in the MTT microdilution and disc diffusion assays. Further bioassay guided isolation of Aex-EA led to the isolation of two bioactive flavonoids, kaempferol and quercetin (IC₉₀ ≤62.5 µg/mL against *S. aureus*, MRSA, MDRSA and *S. pyogenes*). Both isolated compounds are well known for their antibacterial, anti-inflammatory and antioxidant properties. This is the first report of isolation of these two bioactive flavonoids from *Alphitonia excelsa*.

To evaluate anti-inflammatory activity, all extracts (Aex-Hex, Aex-DCM, Aex-EA, Aex-MeOH and Aex-water) were tested using a COX inhibition assay. It was observed that Aex-DCM and Aex-MeOH had good COX 1 inhibition (>80% inhibition at 50 µg/mL concentration and 100% inhibition at 100 µg/mL). Aex-EA showed potent nitric oxide (NO) inhibition (IC₅₀ 10.7 µg/mL) with a high selectivity index (>5). Aex-EA also showed modest antioxidant activity in the ORAC assay (3.70×10³ µM TE/g). This study is the first report of antibacterial, anti-inflammatory and antioxidant activity for *A. excelsa*.

While only two compounds (kaempferol and quercetin) were isolated from Aex-EA (EtOAc extract of *A. excelsa*), its promising activity, as well as that of the other extracts and several fractions warrants further studies on this plant in future.

Leaves of *A. excelsa* appeared different during different times of the year (numerous holes and other damage to the leaf blade possibly due to extensive herbivory during August-September; mostly undamaged fresh leaves during March-April). Two collections (first in August from Cumberland State Forest, Sydney and second in March from northern NSW) were performed. Similar compositions were seen in the TLC profiles of extracts from both collections. However, higher antimicrobial activity was observed with the EtOAc extract from northern NSW (Aex-NSW-EA) than the extracts from Cumberland, Sydney (Aex-EA) in both the disc diffusion and MTT microdilution assays against *S. pyogenes*, methicillin sensitive, resistant and multidrug resistant strains of *S. aureus*. TLC bioautography suggested that some bioactive components were present in a higher quantity in the visibly undamaged leaves collected from northern NSW. In future it will be interesting to know whether seasonal variations, different geographical locations or long term storage have any impact on the composition or bioactivity of this plant. This could be done by collecting the plant material from different locations and in different seasons and doing the extraction and bioassays using identical methods, both directly following extraction of the plant material and after storage. Although ASE was not found to be very convenient for large scale extraction of *A. excelsa* leaves, it may be useful in such a comparative study as it is possible to maintain identical conditions and parameters for extraction by this method.

A water extract (Aex-water) for *A. excelsa* was also prepared to reflect the Yaegl customary preparation of the plant – leaves are rubbed between the hands with a small amount of water. The extract was tested for antibacterial, anti-inflammatory and antioxidant properties. It

showed modest antioxidant activity ($>1000 \mu\text{M TE/g}$) in the ORAC assay and good inhibition against COX 1 (94% inhibition at $50 \mu\text{g/mL}$).

The antimicrobial, anti-inflammatory and antioxidant activities of extracts of *A. excelsa* leaves and the isolation of two known antimicrobial, anti-inflammatory and antioxidant flavonoids (kaempferol and quercetin) support the traditional use of this plant in wounds and infection related conditions by the Yaegl community.

Chapter 5 focused on ethical engagement and capacity strengthening opportunities with the Yaegl community and more broadly. Best ethical procedures were followed towards relationship building with the Yaegl community. Capacity strengthening and benefit sharing approaches included co-authorship in publications, consolidating the research findings into an online database and encouraging Indigenous students to complete their secondary studies and consider higher education. The author's participation and contribution to these relationship building and benefit sharing activities were detailed in this Chapter. In future, the IBRG is planning to develop some preparations (e.g. antiseptic soaps made from leaves or decoction from leaves) with the Yaegl community to provide further tangible benefits for the community following this medicinal plant study.

In conclusion, this PhD study has achieved the primary goal of conducting chemical and biological investigations on two endemic Australian medicinal plants used by the Yaegl community for the treatment of wounds, sores and skin infections. This study identified the presence of bioactive fractions and compounds (antibacterial, anti-inflammatory and antioxidant) in the two selected medicinal plants (*L. suaveolens* and *A. excelsa*) and contributed to the understanding of the chemistry and biological properties of these underexplored Australian flora. This study was also conducted with best ethical practices and provided capacity strengthening opportunities to the Yaegl elders and their local community.

REFERENCES

- Adams, R. P. 2007. *Identification of Essential oils components by gas Chromatography/Quadrupole Mass spectroscopy*, Allured Pub. Corporation.
- Ainsworth, E. A. & Gillespie, K. M. 2007. Estimation of total phenolic content and other oxidation substrates in plant tissues using Folin-Ciocalteu reagent. *Nature Protocols*, 2, 875-877.
- Alanis, A. J. 2005. Resistance to Antibiotics: Are We in the Post-Antibiotic Era? *Archives of Medical Research*, 36, 697-705.
- Ali, B., Imran, M., Hussain, R., Ahmed, Z. & Malik, A. 2010. Structural determination of abutilins A and B, new flavonoids from *Abutilon pakistanicum*, by 1D and 2D NMR spectroscopy. *Magnetic Resonance in Chemistry*, 48, 159-163.
- Ali, H., Haribabu, B., Richardson, R. M. & Snyderman, R. 1997. Mechanisms of inflammation and leukocyte activation. *The Medical Clinics of North America*, 81, 1.
- ANBG. 2012. *Alphitonia excelsa* [Online]. Canberra. Available: <http://www.anbg.gov.au/gnp/gnp6/alph-exc.html> [Accessed 29 June 2013].
- Anonymous 2010. The 10 x '20 Initiative: pursuing a global commitment to develop 10 new antibacterial drugs by 2020. *Clinical Infectious Diseases*, 50, 1081-3.
- Appendino, G., Gibbons, S., Giana, A., Pagani, A., Grassi, G., Stavri, M., Smith, E. & Rahman, M. M. 2008. Antibacterial Cannabinoids from *Cannabis sativa*: A Structure-Activity Study. *Journal of Natural Products*, 71, 1427-1430.
- Atlas of Living Australia. *Lophostemon suaveolens* [Online]. Available: <http://bie.ala.org.au/species/Swamp+Turpentine> [Accessed 5 January 2012].
- Australian Bureau of Statistics. 2013. *Education: School Retention* [Online]. Canberra. Available: <http://www.abs.gov.au/AUSSTATS/abs@.nsf/lookup/4704.0Chapter350Oct+2010> [Accessed 18 July 2013].
- Australian Tropical Rainforest Plants. *Lophostemon suaveolens* [Online]. Available: http://keys.trin.org.au/key-server/data/0e0f0504-0103-430d-8004-060d07080d04/media/Html/taxon/Lophostemon_suaveolens.htm [Accessed 5 January 2012].
- Australian Tropical Rainforest Plants. 2013. *Alphitonia oblata* [Online]. Available: http://keys.trin.org.au/key-server/data/0e0f0504-0103-430d-8004-060d07080d04/media/Html/taxon/Alphitonia_oblata.htm [Accessed 29 June 2013].
- Aziz, N., Farag, S., Mousa, L. & Abo-Zaid, M. 1997. Comparative antibacterial and antifungal effects of some phenolic compounds. *Microbios*, 93, 43-54.
- Ban, H. S., Suzuki, K., Lim, S. S., Jung, S. H., Lee, S., Ji, J., Lee, H. S., Lee, Y. S., Shin, K. H. & Ohuchi, K. 2004. Inhibition of lipopolysaccharide-induced expression of inducible nitric oxide synthase and tumor necrosis factor- α by 2'-hydroxychalcone derivatives in RAW 264.7 cells. *Biochemical Pharmacology*, 67, 1549-1557.
- Barnes, P. M., Powell-Griner, E., Mcfann, K. & Nahin, R. L. 2004. Complementary and alternative medicine use among adults: United States, 2002. *Seminars in Integrative Medicine*, 2, 54-71.
- Barr, A., Chapman, J., Smith, N. & Beveridge, M. 1990. *Traditional Bush Medicine*, Northern Territory, Australia, Greenhouse Publications Pty Ltd.
- Barry, A. & Thornsberry, C. 1991. Susceptibility tests: diffusion test procedures.

- Basalo, C., Mohn, T. & Hamburger, M. 2006. Are extraction methods in quantitative assays of pharmacopoeia monographs exhaustive? A comparison with pressurized liquid extraction. *Planta Medica*, 72, 1157-1162.
- Beitz, J. 2001. Overcoming barriers to quality wound care: a systems perspective. *Ostomy/Wound Management*, 47, 56.
- Benthin, B. R., Danz, H. & Hamburger, M. 1999. Pressurized liquid extraction of medicinal plants. *Journal of Chromatography A*, 837, 211-219.
- Blakytyn, R. & Jude, E. 2006. The molecular biology of chronic wounds and delayed healing in diabetes. *Diabetic Medicine*, 23, 594-608.
- Bodeker, G. C., Ryan, T. J. & Ong, C. K. 1999. Traditional approaches to wound healing. *Clinics in Dermatology*, 17, 93-8.
- Boots, A. W., Wilms, L. C., Swennen, E. L. R., Kleinjans, J. C. S., Bast, A. & Haenen, G. R. M. M. 2008. In vitro and ex vivo anti-inflammatory activity of quercetin in healthy volunteers. *Nutrition*, 24, 703-710.
- Bougatsos, C., Ngassapa, O., Runyoro, D. K. B. & Chinou, I. B. 2004. Chemical composition and in vitro antimicrobial activity of the essential oils of two *Helichrysum* species from Tanzania. *Zeitschrift für Naturforschung C-Journal of Biosciences*, 59, 368-372.
- Boydron-Le Garrec, R., Benoit, E., Sauviat, M.-P., Lewis, R. J., Molgó, J. & Laurent, D. 2005. Ability of some plant extracts, traditionally used to treat ciguatera fish poisoning, to prevent the in vitro neurotoxicity produced by sodium channel activators. *Toxicon*, 46, 625-634.
- Branch, G. B., Burgess, D. V., Dunstan, P. J., Foo, L. Y., Green, G. H., Mack, J. P. G., Ritchie, E. & Taylor, W. C. 1972. Constituents of *Alphitonia* species. III. Alphitexolide, a new triterpene, and other extractives. *Australian Journal of Chemistry*, 25, 2209-16.
- Bremner, P. & Heinrich, M. 2005. Natural products and their role as inhibitors of the pro-inflammatory transcription factor NF- κ B. *Phytochemistry Reviews*, 4, 27-37.
- Brock, J. 2001. *Native plants of northern Australia*, Sydney, Australia, Reed New Holland.
- Brophy, J. J., Goldsack, R. J., Bean, A. R., Forster, P. I. & Fookes, C. J. R. 1996. The leaf essential oils of the genus *Syncarpia* Ten. (Myrtaceae). *Flavour Fragrance Journal*, 11, 361-366.
- Brophy, J. J., Goldsack, R. J. & Forster, P. I. 2000. Essential oils of the genus *Lophostemon* (Myrtaceae). *Flavour Fragrance Journal*, 15, 17-20.
- Brouet, I. & Ohshima, H. 1995. Curcumin, an Anti-tumor Promoter and Anti-inflammatory Agent, Inhibits Induction of Nitric Oxide Synthase in Activated Macrophages. *Biochemical and Biophysical Research Communications*, 206, 533-540.
- Brouwer, N. 2006. *Traditional medicinal knowledge of the Yaegl Aboriginal community of New South Wales: An ethnobotanical, biological and chemical study*. PhD, Macquarie University.
- Brouwer, N., Liu, Q., Harrington, D., Kohen, J., Vemulpad, S., Jamie, J., Randall, M. & Randall, D. 2005. An ethnopharmacological study of medicinal plants in New South Wales. *Molecules*, 10, 1252-1262.
- Brownstein, M. J. 1993. A brief history of opiates, opioid peptides, and opioid receptors. *Proceedings of the National Academy of Sciences of the United States of America*, 90, 5391.
- Bush, K., Courvalin, P., Dantas, G., Davies, J., Eisenstein, B., Huovinen, P., Jacoby, G. A., Kishony, R., Kreiswirth, B. N. & Kutter, E. 2011. Tackling antibiotic resistance. *Nature Reviews Microbiology*, 9, 894-896.

- Buss, A. & Waigh, R. 1995. In: WOLFF, M. (ed.) *Burgers Medicinal Chemistry and Drug Discovery*. 5 ed. New York: Wiley.
- Butler, M. S. & Newman, D. J. 2008. Mother nature's gifts to diseases of man: the impact of natural products on anti-infective, anticholestemics and anticancer drug discovery. *Progress in Drug Research*, 65, 1, 3-44.
- Butts, A. & Krysan, D. J. 2012. Antifungal Drug Discovery: Something Old and Something New. *PLoS Pathogens*, 8, e1002870.
- Buwa, L. V. & Van Staden, J. 2007. Effects of collection time on the antimicrobial activities of *Harpephyllum caffrum* bark. *South African Journal of Botany*, 73, 242-247.
- Calderon-Montano, J. M., Burgos-Moron, E., Perez-Guerrero, C. & Lopez-Lazaro, M. 2011. A review on the dietary flavonoid kaempferol. *Mini Reviews in Medicinal Chemistry*, 11, 298-344.
- Carr, B., Mikhail, G. & Flickinger, G. 1971. Column chromatography of steroids on Sephadex LH-20. *Journal of Clinical Endocrinology and Metabolism*, 33, 358-360.
- Celiktas, O. Y., Kocabas, E. E. H., Bedir, E., Sukan, F. V., Ozek, T. & Baser, K. H. C. 2007. Antimicrobial activities of methanol extracts and essential oils of *Rosmarinus officinalis*, depending on location and seasonal variations. *Food Chemistry*, 100, 553-559.
- Chaskin, R. J. 2001. Building community capacity a definitional framework and case studies from a comprehensive community initiative. *Urban Affairs Review*, 36, 291-323.
- Cherigo, L., Pereda-Miranda, R. & Gibbons, S. 2009. Bacterial resistance modifying tetrasaccharide agents from *Ipomoea murucoides*. *Phytochemistry*, 70, 222-227.
- Choodej, S., Sommit, D. & Pudhom, K. 2013. Rearranged limonoids and chromones from *Harrisonia perforata* and their anti-inflammatory activity. *Bioorganic and Medicinal Chemistry Letters*, 23, 3896-3900.
- Clatworthy, A. E., Pierson, E. & Hung, D. T. 2007. Targeting virulence: a new paradigm for antimicrobial therapy. *Nature Chemical Biology*, 3, 541-548.
- Comalada, M., Ballester, I., Bailón, E., Sierra, S., Xaus, J., Gálvez, J., Medina, F. S. D. & Zarzuelo, A. 2006. Inhibition of pro-inflammatory markers in primary bone marrow-derived mouse macrophages by naturally occurring flavonoids: Analysis of the structure-activity relationship. *Biochemical Pharmacology*, 72, 1010-1021.
- Cordell, G. A. 2000. Biodiversity and drug discovery-a symbiotic relationship. *Phytochemistry*, 55, 463-480.
- Cos, P., Calomme, M., Sindambiwe, J.-B., De Bruyne, T., Cimanga, K., Pieters, L., Vlietinck, A. J. & Berghe, D. V. 2001. Cytotoxicity and lipid peroxidation-inhibiting activity of flavonoids. *Planta Medica*, 67, 515-519.
- Courtney, J. L., Lassak, E. V. & Speirs, G. B. 1983. Leaf wax constituents of some myrtaceous species. *Phytochemistry*, 22, 947-9.
- Cowan, M. M. 1999. Plant products as antimicrobial agents. *Clinical Microbiology Reviews*, 12, 564-582.
- Cox, P. A. 1993. Saving the ethnopharmacological heritage of Samoa. *Journal of Ethnopharmacology*, 38, 181-8.
- Cox, S. D., Jayasinghe, K. C. & Markham, J. L. 2005. Antioxidant activity in Australian native sarsaparilla (*Smilax glycyphylla*). *Journal of Ethnopharmacology*, 101, 162-8.
- Cragg, G. M. & Newman, D. J. 2002. Drugs from nature: past achievements, future prospects. In: MAURICE, I. M. & WOOTTON, J. C. (eds.) *Ethnomedicine and drug discovery*. Elsevier.

- CRC. 2013. *Wound Management Innovation Cooperative Research Centre* [Online]. Brisbane. Available: <http://www.awma.com.au/wuwhs2016-australiabid/wound-management.html> [Accessed 26 June 2013].
- Cribb, A. B. & Cribb, J. W. 1984. *Wild Medicine in Australia*, Sydney, Collins.
- Crotty, M. & Crotty, R. H. 1995. *Finding a way-The religious world of today*, Melbourne, Australia, Collins Dove.
- Cutting, K. F. & White, R. J. 2005. Criteria for identifying wound infection--revisited. *Ostomy Wound Manage*, 51, 28-34.
- Davies, D. R. & Johnson, T. M. 2007. Isolation of three components from spearmint oil: An exercise in column and thin-layer chromatography. *Journal of Chemical Education*, 84, 318.
- De Souza, M. M., Madeira, A., Berti, C., Krogh, R., Yunes, R. A. & Cechinel-Filho, V. 2000. Antinociceptive properties of the methanolic extract obtained from *Ipomoea pes-caprae* (L.) R. Br. *Journal of Ethnopharmacology*, 69, 85-90.
- Delamere, F., Holland, E., Patel, S., Bennett, J., Pavord, I. & Knox, A. 1994. Production of PGE₂ by bovine cultured airway smooth muscle cells and its inhibition by cyclo-oxygenase inhibitors. *British Journal of Pharmacology*, 111, 983-988.
- Dickert, N. & Sugarman, J. 2005. Ethical goals of community consultation in research. *American Journal of Public Health*, 95, 1123-1127.
- Din, L. B., Yusoff, N. I., Samsuddin, M. W., Mat, S. K., Ibrahim, A. Z., Latiff, A. & Said, I. M. 2002. A preliminary phytochemical survey of plants in Crocker range, Sabah, Malaysia. *ASEAN Review of Biodiversity and Environmental Conservation (ARBEC)*.
- Djeridane, A., Yousfi, M., Nadjemi, B., Boutassouna, D., Stocker, P. & Vidal, N. 2006. Antioxidant activity of some algerian medicinal plants extracts containing phenolic compounds. *Food Chemistry*, 97, 654-660.
- Dorman, H. J. D. & Deans, S. G. 2000. Antimicrobial agents from plants: antibacterial activity of plant volatile oils. *Journal of Applied Microbiology*, 88, 308-316.
- Dunstan, C. A., Liu, B., Welch, C. J., Perera, P. & Bohlin, L. 1998. Alphitol, a phenolic substance from *Alphitonia zizyphoides* which inhibits prostaglandin biosynthesis in vitro. *Phytochemistry*, 48, 495-497.
- Dunstan, C. A., Noreen, Y., Serrano, G., Cox, P. A., Perera, P. & Bohlin, L. 1997. Evaluation of some Samoan and Peruvian medicinal plants by prostaglandin biosynthesis and rat ear oedema assays. *Journal of Ethnopharmacology*, 57, 35-56.
- Edwards, J. C. W., Szczepanski, L., Szechinski, J., Filipowicz-Sosnowska, A., Emery, P., Close, D. R., Stevens, R. M. & Shaw, T. 2004. Efficacy of B-cell-targeted therapy with rituximab in patients with rheumatoid arthritis. *The New England Journal of Medicine*, 350, 2572-2581.
- Edwards, R. & Harding, K. G. 2004. Bacteria and wound healing. *Current Opinion in Infectious Diseases*, 17, 91-6.
- Eliopoulos, G. M., Perea, S. & Patterson, T. F. 2002. Antifungal resistance in pathogenic fungi. *Clinical infectious diseases*, 35, 1073-1080.
- Eloff, J. 1998. Which extractant should be used for the screening and isolation of antimicrobial components from plants? *Journal of Ethnopharmacology*, 60, 1-8.
- Escobedo-Martinez, C., Cruz-Morales, S., Fragoso-Serrano, M., Rahman, M. M., Gibbons, S. & Pereda-Miranda, R. 2010. Characterization of a xylose containing oligosaccharide, an inhibitor of multidrug resistance in *Staphylococcus aureus*, from *Ipomoea pes-caprae*. *Phytochemistry*, 71, 1796-801.

- Escobedo-Martinez, C. & Pereda-Miranda, R. 2007. Resin glycosides from *Ipomoea pes-caprae*. *Journal of Natural Products*, 70, 974-8.
- Fabricant, D. S. & Farnsworth, N. R. 2001. The value of plants used in traditional medicine for drug discovery. *Environ. Health Perspect. Suppl.*, 109, 69-75.
- Fang, S., Lin, C., Zhang, Q., Wang, L., Lin, P., Zhang, J. & Wang, X. 2012. Anticancer potential of aqueous extract of *alocasia macrorrhiza* against hepatic cancer in vitro and in vivo. *Journal of Ethnopharmacology*, 141, 947-956.
- Farnsworth, N. R. 1994. Ethnopharmacology and drug development. *Ethnobotany and the Search for New Drugs*, 185, 2-11.
- Fathiazad, F., Delazar, A., Amiri, R. & Sarker, S. D. 2006. Extraction of flavonoids and quantification of rutin from waste tobacco leaves. *Iranian Journal of Pharmaceutical Research*, 5, 223-227.
- Flamini, G., Antognoli, E. & Morelli, I. 2001. Two flavonoids and other compounds from the aerial parts of *Centaurea bracteata* from Italy. *Phytochemistry*, 57, 559-564.
- Fonder, M. A., Lazarus, G. S., Cowan, D. A., Aronson-Cook, B., Kohli, A. R. & Mamelak, A. J. 2008. Treating the chronic wound: A practical approach to the care of nonhealing wounds and wound care dressings. *Journal of American Academy of Dermatology*, 58, 185-206.
- Fujioka, T., Kashiwada, Y., Kilkuskie, R. E., Cosentino, L. M., Ballas, L. M., Jiang, J. B., Janzen, W. P., Chen, I. S. & Lee, K. H. 1994. Anti-AIDS agents, 11. Betulinic acid and platanic acid as anti-HIV principles from *Syzygium claviflorum*, and the anti-HIV activity of structurally related triterpenoids. *Journal of Natural Products*, 57, 243-7.
- Fulda, S. 2009. Betulinic acid: a natural product with anticancer activity. *Molecular Nutrition and Food Research*, 53, 140-6.
- Gaikwad, J., Khanna, V., Vemulpad, S., Jamie, J., Kohen, J. & Ranganathan, S. 2008. CMKb: a web-based prototype for integrating Australian Aboriginal customary medicinal plant knowledge. *BMC Bioinformatics*, 9, S25.
- Gao, H., Wu, L., Kuroyanagi, M., Harada, K., Kawahara, N., Nakane, T., Umehara, K., Hirasawa, A. & Nakamura, Y. 2003. Antitumor-promoting constituents from *Chaenomeles sinensis* Koehne and their activities in JB6 mouse epidermal cells. *Chemical and Pharmaceutical Bulletin*, 51, 1318-1321.
- García-Mediavilla, V., Crespo, I., Collado, P. S., Esteller, A., Sánchez-Campos, S., Tuñón, M. J. & González-Gallego, J. 2007. The anti-inflammatory flavones quercetin and kaempferol cause inhibition of inducible nitric oxide synthase, cyclooxygenase-2 and reactive C-protein, and down-regulation of the nuclear factor kappaB pathway in Chang Liver cells. *European Journal of Pharmacology*, 557, 221-229.
- Gardner, S. E., Frantz, R. A. & Doebbeling, B. N. 2001. The validity of the clinical signs and symptoms used to identify localized chronic wound infection. *Wound Repair Regen*, 9, 178-86.
- Garrett, A. R., Murray, B. K., Robison, R. A. & O'Neill, K. L. 2010. Measuring antioxidant capacity using the ORAC and TOSC assays. *Methods in Molecular Biology (Totowa, NJ, U. S.)*, 594, 251-262.
- Germain, M., Hatton, A., Williams, S., Matthews, J., Stone, M., Fisher, J. & Ingham, E. 2003. Comparison of the cytotoxicity of clinically relevant cobalt–chromium and alumina ceramic wear particles in vitro. *Biomaterials*, 24, 469-479.
- Gibbons, S. 2004. Anti-staphylococcal plant natural products. *Nat. Prod. Rep.*, 21, 263-277.
- Gibbons, S. 2005. An Introduction to planar chromatography. In: SARKAR, S. D., LATIF, Z. & GRAY, A. I. (eds.) *Natural Products Isolation*. 2 ed. Totowa: Humana Press Inc.

- Gillespie, K. M., Chae, J. M. & Ainsworth, E. A. 2007. Rapid measurement of total antioxidant capacity in plants. *Nature Protocols*, 2, 867-870.
- Gossiau, A., Li, S., Ho, C.-T., Chen, K. Y. & Rawson, N. E. 2011. The importance of natural product characterization in studies of their anti-inflammatory activity. *Molecular Nutrition and Food Research*, 55, 74-82.
- Gritsanapan, W. & Griffin, W. J. 1991. Alkaloid variation within *Duboisia myoporoides*. *Phytochemistry*, 30, 2667-9.
- Guardia, T., Rotelli, A. E., Juarez, A. O. & Pelzer, L. E. 2001. Anti-inflammatory properties of plant flavonoids. Effects of rutin, quercetin and hesperidin on adjuvant arthritis in rat. *Il farmaco*, 56, 683-687.
- Guise, G. B., Ritchie, E. & Taylor, W. C. 1962. Further constituents of *Alphitonia* species. *Australian Journal of Chemistry*, 15, 314-21.
- Gurib-Fakim, A. 2006. Medicinal plants: traditions of yesterday and drugs of tomorrow. *Molecular Aspects of Medicine*, 27, 1-93.
- Gutzeit, D., Wray, V., Winterhalter, P. & Jerz, G. 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, 1-7.
- Habtemariam, S. 1995. Cytotoxicity of diterpenes from *Premna schimperi* and *Premna oligotricha*. *Planta Medica*, 61, 368-9.
- Hamburger, M. O. & Cordell, G. A. 1987. A direct bioautographic TLC assay for compounds possessing antibacterial activity. *Journal of Natural Products*, 50, 19-22.
- Han, Y.-P., Tuan, T.-L., Wu, H., Hughes, M. & Garner, W. L. 2001. TNF-alpha stimulates activation of pro-MMP2 in human skin through NF-(kappa) B mediated induction of MT1-MMP. *Journal of Cell Science*, 114, 131-139.
- Harborne, J. 1993. The Flavonoids: Advances in research from 1986. *Champion & Hall: London, UK*.
- Hedin, P. A. & Phillips, V. A. 1992. Electron impact mass spectral analysis of flavonoids. *Journal of Agricultural and Food Chemistry*, 40, 607-611.
- Heinrich, M., Editors-in-Chief: Lewis, M. & Hung-Wen, L. 2010. 3.12 - Ethnopharmacology and Drug Discovery. *Comprehensive Natural Products II*. Oxford: Elsevier.
- Heinrich, M. & Gibbons, S. 2001. Ethnopharmacology in drug discovery: an analysis of its role and potential contribution. *Journal of Pharmacy and Pharmacology*, 53, 425-432.
- Heinrich, M., Kinghorn, A. D., Phillipson, J. D., Maizels, D. & Gibbons, S. 2004. *Fundamentals of Pharmacognosy and Phytotherapy*, Churchill Livingstone Elsevier, UK.
- Herbrecht, R., Nivoix, Y., Fohrer, C., Natarajan-Ame, S. & Letscher-Bru, V. 2005. Management of systemic fungal infections: alternatives to itraconazole. *Journal of Antimicrobial Chemotherapy*, 56, i39-i48.
- Hernández, I., Alegre, L., Van Breusegem, F. & Munné-Bosch, S. 2009. How relevant are flavonoids as antioxidants in plants? *Trends in Plant Science*, 14, 125-132.
- Hills, K. L., Bottomley, W. & Mortimer, P. I. 1954. Variation in the main alkaloids of *Duboisia myoporoides* and *Duboisia leichhardtii*. V. Seasonal changes and some environmental factors. *Australian Journal of Applied Science*, 5, 292-7.
- Hnawia, E., Brophy, J. J., Craven, L. A., Lebouvier, N., Cabalion, P. & Nour, M. 2012. An examination of the leaf essential oils of the endemic *Melaleuca* (Myrtaceae) species of New Caledonia. *Journal of Essential Oil Research*, 24, 273-278.

- Honda, T., Liby, K. T., Su, X., Sundararajan, C., Honda, Y., Suh, N., Risingsong, R., Williams, C. R., Royce, D. B., Sporn, M. B. & Gribble, G. W. 2006. Design, synthesis, and anti-inflammatory activity both in vitro and in vivo of new betulinic acid analogues having an enone functionality in ring A. *Bioorganic and Medicinal Chemistry Letters*, 16, 6306-6309.
- Houghton, P. & Raman, A. 1998. Laboratory handbook for fractionation of natural extracts. . Chapman and Hall, London.
- Huang, D., Ou, B. & Prior, R. L. 2005. The Chemistry behind Antioxidant Capacity Assays. *Journal of Agricultural and Food Chemistry*, 53, 1841-1856.
- Huang, X., Mu, B., Lin, W. & Qiu, Y. 2012. Pterocarpin and isoflavan derivatives from *Canavalia maritima* (Aubl.) Thou. *Records of Natural Products*, 6, 166-170.
- Huq, F. & Misra, L. N. 1997. An alkenol and C-methylated flavones from *Callistemon lanceolatus* leaves. *Planta Medica*, 63, 369-370.
- Hussain, A. I., Anwar, F., Hussain Sherazi, S. T. & Przybylski, R. 2008. Chemical composition, antioxidant and antimicrobial activities of basil (*Ocimum basilicum*) essential oils depends on seasonal variations. *Food Chemistry*, 108, 986-995.
- Inouye, S., Takizawa, T. & Yamaguchi, H. 2001. Antibacterial activity of essential oils and their major constituents against respiratory tract pathogens by gaseous contact. *Journal Antimicrobial Chemotherapy*, 47, 565-573.
- Irvine, R. F. 1982. How is the level of free arachidonic acid controlled in mammalian cells? *Biochemical Journal*, 204, 3.
- Isaacs, J. 1987. *Bush Food: Aboriginal Food and Herbal Medicine*, Sydney, Lansdowne Publishing.
- Jabit, M. L., Wahyuni, F. S., Khalid, R., Israf, D. A., Shaari, K., Lajis, N. H. & Stanslas, J. 2009. Cytotoxic and nitric oxide inhibitory activities of methanol extracts of *Garcinia* species. *Pharmaceutical Biology*, 47, 1019-1026.
- James, G. A., Swogger, E., Wolcott, R., Secor, P., Sestrich, J., Costerton, J. W. & Stewart, P. S. 2008. Biofilms in chronic wounds. *Wound Repair and Regeneration*, 16, 37-44.
- Johnsson, R., Träff, G., Sundén, M. & Ellervik, U. 2007. Evaluation of quantitative thin layer chromatography using staining reagents. *Journal of Chromatography A*, 1164, 298-305.
- Jou, S.-J., Chen, C.-H., Guh, J.-H., Lee, C.-N. & Lee, S.-S. 2004. Flavonol glycosides and cytotoxic triterpenoids from *Alphitonia philippinensis*. *Journal of Chinese Chemical Society (Taipei, Taiwan)*, 51, 827-834.
- Joulain, D. & König, W. A. 1998. *The Atlas of Spectral Data of Sesquiterpene Hydrocarbons*, E.B.-Verlag.
- Juergens, U., Stöber, M. & Vetter, H. 1998. Inhibition of cytokine production and arachidonic acid metabolism by eucalyptol (1,8-cineole) in human blood monocytes in vitro. *European Journal of Medical Research*, 3, 508.
- Juergens, U. R., Engelen, T., Racké, K., Stöber, M., Gillissen, A. & Vetter, H. 2004. Inhibitory activity of 1, 8-cineol (eucalyptol) on cytokine production in cultured human lymphocytes and monocytes. *Pulmonary Pharmacology & Therapeutics*, 17, 281-287.
- Kalgutkar, A. S., Marnett, A. B., Crews, B. C., Rimmel, R. P. & Marnett, L. J. 2000. Ester and amide derivatives of the nonsteroidal antiinflammatory drug, indomethacin, as selective cyclooxygenase-2 inhibitors. *Journal of Medicinal Chemistry*, 43, 2860-2870.
- Kang, S. S. 1981. Flavonoids from the leaves of *Polygonum sachalinense* Fr. Schm. I. *Soul Taehakkyo Saengyak Yonguso Opjukjip*, 20, 55-7.

- Kang, S. S., Kim, J.-G., Lee, T.-H. & Oh, K.-B. 2006. Flavonols inhibit sortases and sortase-mediated *Staphylococcus aureus* clumping to fibrinogen. *Biological and Pharmaceutical Bulletin*, 29, 1751-1755.
- Kapoor, L. 1990. *CRC handbook of Ayurvedic medicinal plants*, CRC Press LLC.
- Kapoor, M., Clarkson, A., Sutherland, B. & Appleton, I. 2005. The role of antioxidants in models of inflammation: emphasis on L-arginine and arachidonic acid metabolism. *Inflammopharmacology*, 12, 505-519.
- Karaman, I., Sahin, F., Gulluce, M., Ogutcu, H., Sengul, M. & Adiguzel, A. 2003. Antimicrobial activity of aqueous and methanol extracts of *Juniperus oxycedrus* L. *Journal of Ethnopharmacology*, 85, 231-5.
- Kashiwada, Y., Wang, H.-K., Nagao, T., Kitanaka, S., Yasuda, I., Fujioka, T., Yamagishi, T., Cosentino, L. M., Kozuka, M., Okabe, H., Ikeshiro, Y., Hu, C.-Q., Yeh, E. & Lee, K.-H. 1998. Anti-AIDS agents. 30. Anti-HIV activity of oleanolic acid, pomolic acid, and structurally related triterpenoids. *Journal of Natural Products*, 61, 1090-1095.
- Kaufmann, B. & Christen, P. 2002. Recent extraction techniques for natural products: microwave-assisted extraction and pressurised solvent extraction. *Phytochemical Analysis*, 13, 105-113.
- Khanam, N., Khoo, C., Close, R. & Khan, A. G. 2001. Tropane alkaloid production by shoot culture of *Duboisia myoporoides* R. Br. *Phytochemistry*, 56, 59-65.
- Khanna, D., Sethi, G., Ahn, K. S., Pandey, M. K., Kunnumakkara, A. B., Sung, B., Aggarwal, A. & Aggarwal, B. B. 2007. Natural products as a gold mine for arthritis treatment. *Current Opinion in Pharmacology*, 7, 344-351.
- Kim, H. P., Son, K. H., Chang, H. W. & Kang, S. S. 2004. Anti-inflammatory plant flavonoids and cellular action mechanisms. *Journal of Pharmacological Sciences (Tokyo, Jpn.)*, 96, 229-245.
- Kiraz, S., Ertenli, I., Arici, M., Calgüneri, M., Haznedaroglu, I., Celik, I., Pay, S. & Kirazli, S. 1998. Effects of colchicine on inflammatory cytokines and selectins in familial Mediterranean fever. *Clinical and Experimental Rheumatology*, 16, 721-724.
- Kirmizigul, S., Anil, H., Ucar, F. & Akdemir, K. 1996. Antimicrobial and antifungal activities of three new triterpenoid glycosides. *Phytother. Res.*, 10, 274-276.
- Kohen, J. L. 1995. *Aboriginal Environmental Impacts*, Sydney, Australia, University of New South Wales Press.
- Koudou, J., Roblot, G., Wylde, R. & Heitz, A. 1994. Constituents of the hexane extract from *Dichrostachys glomerata*. *Planta medica*, 60.
- Kremer, J. M., Westhovens, R., Leon, M., Di, G. E., Alten, R., Steinfeld, S., Russell, A., Dougados, M., Emery, P., Nuamah, I. F., Williams, G. R., Becker, J.-C., Hagerty, D. T. & Moreland, L. W. 2003. Treatment of rheumatoid arthritis by selective inhibition of T-cell activation with fusion protein CTLA4Ig. *The New England Journal of Medicine*, 349, 1907-1915.
- La Casa, C., Villegas, I., Alarcon De La Lastra, C., Motilva, V. & Martin Calero, M. 2000. Evidence for protective and antioxidant properties of rutin, a natural flavone, against ethanol induced gastric lesions. *Journal of Ethnopharmacology*, 71, 45-53.
- Lai, C.-C., Tan, C.-K., Huang, Y.-T., Shao, P.-L. & Hsueh, P.-R. 2008. Current challenges in the management of invasive fungal infections. *Journal of Infection and Chemotherapy*, 14, 77-85.
- Lai, P. K.-K., Chan, J. Y.-W., Cheng, L., Lau, C.-P., Han, S. Q.-B., Leung, P.-C., Fung, K.-P. & Lau, C. B.-S. 2013. Isolation of Anti-Inflammatory Fractions and Compounds from the Root of *Astragalus membranaceus*. *Phytotherapy Research*, 27, 581-587.

- Lassak, E. V. & McCarthy, T. 2001. *Australian Medicinal Plants*, Sydney, Reed New Holland.
- Lassak, E. V. & McCarthy, T. 2008. *Australian Medicinal Plants*, Sydney, New Holland Publishers.
- Lee, B. Y., Noori, A., Stubbs, D., Wendell, K., Butler, G., Thia, A. & Ali, A. 2010. Ultra-low microcurrent in the management of diabetes mellitus, hypertension and chronic wounds: report of twelve cases and discussion of mechanism of action. *International Journal of Medical Sciences*, 7, 29.
- Leonard, J., Lygo, B. & Procter, G. 2013. *Advanced practical organic chemistry*, CRC Press.
- Lewis, W., Vaisberg, A., Lamas, G., Sarasara, C. & Elvin-Lewis, M. 2004. Advantage of ethnobotanically-based research for searching new pharmaceuticals. *Ethnobotany*, 16, 10-15.
- Li, D., Owen, N. L., Perera, P., Andersson, C., Bohlin, L., Cox, P. A., Pugmire, R. J., Mayne, C. L. & Grant, D. M. 1994. Structure elucidation of three triterpenoid saponins from *Alphitonia zizyphoides* using 2D NMR techniques. *Journal of Natural Products*, 57, 218-24.
- Li, H.-B., Wong, C.-C., Cheng, K.-W. & Chen, F. 2008. Antioxidant properties in vitro and total phenolic contents in methanol extracts from medicinal plants. *LWT - Food Science and Technology*, 41, 385-390.
- Li, M. & Xu, Z. 2008. Quercetin in a lotus leaves extract may be responsible for antibacterial activity. *Archives of Pharmacal Research*, 31, 640-644.
- Li, X.-C., Cai, L. & D.Wu, C. 1997. Antimicrobial compounds from *Ceanothus americanus* against oral pathogens. *Phytochemistry*, 46, 97-102.
- Lin, H.-Y., Juan, S.-H., Shen, S.-C., Hsu, F.-L. & Chen, Y.-C. 2003. Inhibition of lipopolysaccharide-induced nitric oxide production by flavonoids in RAW264. 7 macrophages involves heme oxygenase-1. *Biochemical Pharmacology*, 66, 1821-1832.
- Lin, J., Opoku, A. R., Geheeb-Keller, M., Hutchings, A. D., Terblanche, S. E., K. Jäger, A. & Van Staden, J. 1999. Preliminary screening of some traditional zulu medicinal plants for anti-inflammatory and anti-microbial activities. *Journal of Ethnopharmacology*, 68, 267-274.
- Lindsey, K., Jäger, A. K., Raidoo, D. M. & Van Staden, J. 1998. Screening of plants used by Southern African traditional healers in the treatment of dysmenorrhoea for prostaglandin-synthesis inhibitors and uterine relaxing activity. *Journal of Ethnopharmacology*, 64, 9-14.
- Liu, Q., Harrington, D., Kohen, J. L., Vemulapad, S. & Jamie, J. F. 2006. Bactericidal and cyclooxygenase inhibitory diterpenes from *Eremophila sturtii*. *Phytochemistry*, 67, 1256-1261.
- Lo, K. M. & Cheung, P. C. K. 2005. Antioxidant activity of extracts from the fruiting bodies of *Agrocybe aegerita* var. *alba*. *Food Chemistry*, 89, 533-539.
- Loeffler, J. & Stevens, D. A. 2003. Antifungal drug resistance. *Clinical Infectious Diseases*, 36, S31-S41.
- Long, M., North, S. & Australia, R. How young Indigenous people are faring: key indicators 1996-2006. 2009. Dusseldorp Skills Forum.
- Lopez-Velez, M., Martinez-Martinez, F. & Valle-Ribes, C. D. 2003. The study of phenolic compounds as natural antioxidants in wine.
- Low, T. 1990. *Bush medicine: a pharmacopoeia of natural remedies*, North Ryde, NSW, Angus and Robertson.

- Macías, F. A., Simonet, A. M., Galindo, J. C. G., Pacheco, P. C. & Sánchez, J. A. 1998. Bioactive polar triterpenoids from *Melilotus messanensis*. *Phytochemistry*, 49, 709-717.
- Macclouf, J., Grassi, J. & Pradelles, P. 1988. Development of enzyme-immunoassay techniques for measurement of eicosanoids. *Prostaglandin and Lipid Metabolism in Radiation Injury*. Springer.
- Maidment, C., Dyson, A. & Haysom, I. 2006. A study into the antimicrobial effects of cloves (*Syzygium aromaticum*) and cinnamon (*Cinnamomum zeylanicum*) using disc-diffusion assay. *Nutrition and Food Science*, 36, 225-230.
- Mann, J. 2000. *Murder, magic, and medicine*, Oxford University Press, USA.
- Markham, K. R. 1982. *Techniques of Flavonoid Identification*, Academic Press.
- Maroyi, A. 2013. Traditional use of medicinal plants in south-central Zimbabwe: review and perspectives. *Journal of Ethnobiology and Ethnomedicine*, 9, 31.
- Martin, A., Morcillo, N., Lemus, D., Montoro, E., Da Silva Telles, M. A., Simboli, N., Pontino, M., Porras, T., Leon, C. & Velasco, M. 2005. Multicenter study of MTT and resazurin assays for testing susceptibility to first-line anti-tuberculosis drugs. *The International Journal of Tuberculosis and Lung Disease*, 9, 901-906.
- Martin, T., Mufson, E. & Mesulam, M. 1984. The light side of horseradish peroxidase histochemistry. *Journal of Histochemistry & Cytochemistry*, 32, 793-793.
- Martínez-Flores, S., Gutiérrez-Fernández, B., Sánchez-Campos, S., González-Gallego, J. & Tuñón, M. 2005. Quercetin prevents nitric oxide production and nuclear factor kappa B activation in interleukin-1 β -activated rat hepatocytes. *Journal of Nutrition*, 135, 1359-1365.
- Mcswan, E. H. 1992. *Maclean - The first fifty years*, Maclean, NSW, Clarence Press.
- Mebs, D. 1999. Notes on the traditional use of plants to treat snake bite in northern Papua New Guinea. *Toxicon*, 38, 299-302.
- Minnis, A. 2008. Substantive theory to explain the impact of living with a chronic wound whilst receiving conflicting or inappropriate advice or care.
- Molassiotis, A., Fernandez-Ortega, P., Pud, D., Ozden, G., Scott, J. A., Panteli, V., Margulies, A., Browall, M., Magri, M. & Selvekerova, S. 2005. Use of complementary and alternative medicine in cancer patients: a European survey. *Annals of Oncology*, 16, 655-663.
- Morice, I. M. 1970. Seed fats of some New Zealand and Australian monocotyledons. *Phytochemistry*, 9, 1829-33.
- Moyer-Henry, K. 2008. Patenting Neem and Hoodia: Conflicting decisions issued by the opposition board of the European Patent Office. *Biotechnology Law Report*, 27, 1-10.
- Moyo, M., Ndhala, A. R., Finnie, J. F. & Van Staden, J. 2010. Phenolic composition, antioxidant and acetylcholinesterase inhibitory activities of *Sclerocarya birrea* and *Harpephyllum caffrum* (Anacardiaceae) extracts. *Food Chemistry*, 123, 69-76.
- Mulla, W., Kuchekar, S., Thorat, V., Chopade, A. & Kuchekar, B. 2010. Antioxidant, Antinociceptive and Anti-inflammatory Activities of Ethanolic Extract of Leaves of *Alocasia indica* (Schott.). *Journal of Young Pharmacists*, 2, 137-43.
- Mulla, W. A., Salunkhe, V. R. & Bhise, S. B. 2009. Hepatoprotective activity of hydroalcoholic extract of leaves of *Alocasia Indica* (Linn.). *Indian Journal of Experimental Biology*, 47, 816-821.
- Munavalli, S. & Pannella, M. 1988. Thin-layer chromatography of mustard and its metabolites. *Journal of Chromatography A*, 437, 423-428.

- Nahin, R. L. 2010. *Costs of Complementary and Alternative Medicine (CAM) and Frequency of Visits to CAM Practitioners: US 2007*, DIANE Publishing.
- Nakibinge, S., Maher, D., Katende, J., Kamali, A., Grosskurth, H. & Seeley, J. 2009. Community engagement in health research: two decades of experience from a research project on HIV in rural Uganda. *Tropical Medicine & International Health*, 14, 190-195.
- National Native Title Tribunal 2011. Native Title Vision. Geospatial Services ed. Perth: Commonwealth of Australia.
- Naz, S., Ahmad, S., Ajaz Rasool, S., Asad Sayeed, S. & Siddiqi, R. 2006. Antibacterial activity directed isolation of compounds from *Onosma hispidum*. *Microbiological Research*, 161, 43-48.
- Newman, D. J. & Cragg, G. M. 2007. Natural Products as Sources of New Drugs over the Last 25 Years. *Journal of Natural Products*, 70, 461-477.
- Nguemfo, E. L., Dimo, T., Dongmo, A. B., Azebaze, A. G. B., Alaoui, K., Asongalem, A. E., Cherrah, Y. & Kamtchouing, P. 2009. Anti-oxidative and anti-inflammatory activities of some isolated constituents from the stem bark of *Allanblackia monticola* Staner L.C (Guttiferae). *Inflammopharmacology*, 17, 37-41.
- NH&MRC & AVCC. 2007. *National statement on ethical conduct in human research* [Online]. Available: <http://www.nhmrc.gov.au/files/nhmrc/publications/attachments/e72.pdf> [Accessed 21 July 2013].
- Noipa, T., Srijaranai, S., Tuntulani, T. & Ngeontae, W. 2011. New approach for evaluation of the antioxidant capacity based on scavenging DPPH free radical in micelle systems. *Food Research International*, 44, 798-806.
- O'Connor, B. 2003. Protecting Traditional Knowledge. *The Journal of World Intellectual Property*, 6, 677-698.
- O'Neill, L. a. J. 2006. Targeting signal transduction as a strategy to treat inflammatory diseases. *Nature Reviews Drug Discovery*, 5, 549-563.
- Ozturk, M., Duru, M. E., Aydogmus-Ozturk, F., Harmandar, M., Mahlicli, M., Kolak, U. & Ulubelen, A. 2009. GC-MS analysis and antimicrobial activity of essential oil of *Stachys cretica* subsp. *smyrnaea*. *Natural Product Communications*, 4, 109-114.
- Packer, J. 2012. *Customary medicines used in the treatment of wounds: an integrated ethnopharmacological approach*. PhD, Macquarie University.
- Packer, J., Brouwer, N., Harrington, D., Gaikwad, J., Heron, R., Yaegl, C. E., Ranganathan, S., Vemulpad, S. & Jamie, J. 2012. An ethnobotanical study of medicinal plants used by the Yaegl Aboriginal community in northern New South Wales, Australia. *J Ethnopharmacology*, 139, 244-55.
- Packer, J., Gaikwad, J., Harrington, D., Ranganathan, S., Jamie, J. & Vemulpad, S. 2011a. *Medicinal plants of New South Wales (NSW), Australia* Boca Raton, CRC Press LLC.
- Packer, J., Harrington, D., Jamie, J., Brouwer, N., Gaikwad, J. & Vemulpad, S. 2011b. Yaegl: medicinal and plant resources handbook| Macquarie University ResearchOnline.
- Palombo, E. A. & Semple, S. J. 2001. Antibacterial activity of traditional Australian medicinal plants. *Journal of Ethnopharmacology*, 77, 151-157.
- Park, B. J., Wannemuehler, K. A., Marston, B. J., Govender, N., Pappas, P. G. & Chiller, T. M. 2009. Estimation of the current global burden of cryptococcal meningitis among persons living with HIV/AIDS. *Aids*, 23, 525-530.

- Park, K. S. 2013. Aucubin, a naturally occurring iridoid glycoside inhibits TNF- α -induced inflammatory responses through suppression of NF- κ B activation in 3T3-L1 adipocytes. *Cytokine*, 62, 407-412.
- Pattamadilok, D., Pengsuparp, T., Phummiratch, D., Ongpipattanakul, B., Meksuriyen, D., Kawanishi, K., Kaneda, N. & Suttisri, R. 2008. Canarosine: A new guanidine alkaloid from *Canavalia rosea* with inhibitory activity on dopamine D1 receptors. *Journal of Asian Natural Products Research*, 10, 915-918.
- Patwardhan, B. 2005. Ethnopharmacology and drug discovery. *Journal of Ethnopharmacology*, 100, 50-52.
- Patwardhan, B. & Mashelkar, R. A. 2009. Traditional medicine-inspired approaches to drug discovery: can Ayurveda show the way forward? *Drug Discovery Today*, 14, 804-811.
- Pennacchio, M., Kemp, A. S., Taylor, R. P., Wickens, K. M. & Kienow, L. 2005. Interesting biological activities from plants traditionally used by Native Australians. *Journal of Ethnopharmacology*, 96, 597-601.
- Pereda-Miranda, R., Escalante-Sanchez, E. & Escobedo-Martinez, C. 2005. Characterization of lipophilic pentasaccharides from beach morning glory (*Ipomoea pes-caprae*). *Journal of Natural Products*, 68, 226-30.
- Pinto, V. P. T., Teixeira, E. H., Teixeira, A. H., Carneiro, V. A., Cristino-Filho, G., Dus, D., Debray, H., Sampaio, A. H. & Cavada, B. S. 2010. Lectins isolated from Brazilian beans as markers of membrane glycoconjugates of human colon cancer cells. *Journal of Cancer Research and Experimental Oncology*, 2, 54-59.
- Pisha, E., Chai, H., Lee, I.-S., Chagwedera, T. E., Farnsworth, N. R., Cordell, G. A., Beecher, C. W. W., Fong, H. H. S., Kinghorn, A. D. & Et, A. 1995. Discovery of betulinic acid as a selective inhibitor of human melanoma that functions by induction of apoptosis. *Nature Medicine (N. Y.)*, 1, 1046-51.
- Pongprayoon, U., Baekstrom, P., Jacobsson, U., Lindstrom, M. & Bohlin, L. 1991. Compounds inhibiting prostaglandin synthesis isolated from *Ipomoea pes-caprae*. *Planta Medica*, 57, 515-8.
- Potterat, O. & Hamburger, M. 2008. *Progress in Drug Research*, Basel (Switzerland), Birkhauser Verlag.
- Prabhu, S., Raj, L. J. M., Britto, S. J. & Senthilkumar, S. R. 2010. Antibacterial activity and preliminary phytochemical analysis of leaf extract of *Canavalia rosea* (Sw.) DC. (Beach Bean). *International Journal of Research in Pharmaceutical Sciences*, 1, 428-434.
- Praveen, R. P. N. & Knaus, E. E. 2008. Evolution of nonsteroidal anti-inflammatory drugs (NSAIDs): cyclooxygenase (COX) inhibition and beyond. *Journal of Pharmacy and Pharmaceutical Sciences*, 11, 81s-110s.
- Prior, R. L., Wu, X. & Schaich, K. 2005. Standardized methods for the determination of antioxidant capacity and phenolics in foods and dietary supplements. *Journal of Agricultural and Food Chemistry*, 53, 4290-4302.
- Quinn, R. & Mills, C. 2009. Analgesic compounds, extracts containing same and methods of preparation. Google Patents.
- R&D Systems. 2013. *Quantikine Colorimetric Sandwich ELISAs* [Online]. Available: http://www.rndsystems.com/product_detail_objectname_quantikine.aspx [Accessed 29 June 2013].
- Rabe, T. & Van Staden, J. 1997. Antibacterial activity of South African plants used for medicinal purposes. *Journal of Ethnopharmacology*, 56, 81-87.

- Rahalison, L., Hamburger, M., Hostettmann, K., Monod, M. & Frenk, E. 1991. A bioautographic agar overlay method for the detection of antifungal compounds from higher plants. *Phytochemical Analysis*, 2, 199-203.
- Rai, M., Acharya, D. & Rios, J. L. 2011. *Ethnomedicinal plants: Revitalizing of traditional knowledge of herbs*, Science Publishers, US.
- Rajab, M. S., Cantrell, C. L., Franzblau, S. G. & Fisher, N. H. 1998. Antimycobacterial activity of (E)-phytol and derivatives. A preliminary structure-activity study. *Planta Medica*, 64, 2-4.
- Ramawat, K., Dass, S. & Mathur, M. 2009. The chemical diversity of bioactive molecules and therapeutic potential of medicinal plants. *Herbal Drugs: Ethnomedicine to Modern Medicine*. Springer.
- Ramawat, K. & Goyal, S. 2008. The Indian herbal drugs scenario in global perspectives. *Bioactive Molecules and Medicinal Plants*. Springer.
- Reid, R. G. & Sarkar, S. D. 2006. Isolation of Natural Products by Low-Pressure Column Chromatography. In: SARKAR, S. D., LATIF, Z. & GRAY, A. I. (eds.) *Natural Products Isolation*. 2 ed.: Humana Press Inc, Totowa, New Jersey.
- Ren, J. & Chung, S. H. 2007. Anti-inflammatory effect of α -linolenic acid and its mode of action through the inhibition of nitric oxide production and inducible nitric oxide synthase gene expression via NF- κ B and mitogen-activated protein kinase pathways. *Journal of Agricultural and Food Chemistry*, 55, 5073-5080.
- Reyes, C. P., Núñez, M. J., Jiménez, I. A., Busserolles, J., Alcaraz, M. J. & Bazzocchi, I. L. 2006. Activity of lupane triterpenoids from Maytenus species as inhibitors of nitric oxide and prostaglandin E2. *Bioorganic and Medicinal Chemistry*, 14, 1573-1579.
- Rice-Evans, C. A., Miller, N. J., Bolwell, P. G., Bramley, P. M. & Pridham, J. B. 1995. The relative antioxidant activities of plant-derived polyphenolic flavonoids. *Free radical research*, 22, 375-383.
- Rios, J. L. & Recio, M. C. 2005. Medicinal plants and antimicrobial activity. *Journal of Ethnopharmacology*, 100, 80-84.
- Riss, T. L., Moravec, R. A. & Niles, A. L. 2011. Cytotoxicity Testing: Measuring Viable Cells, Dead Cells, and Detecting Mechanism of Cell Death. *Mammalian Cell Viability*. Springer.
- Ritchie, E., Snape, D. & Taylor, W. C. 1961. Chemical studies of the Myrtaceae. III. Triterpenoids from the wood of *Tristania conferta*. *Australian Journal of Chemistry*, 14, 471-2.
- Rogers, K. L., Grice, I. D. & Griffiths, L. R. 2000. Inhibition of platelet aggregation and 5-HT release by extracts of Australian plants used traditionally as headache treatments. *Eur. Journal of Pharmaceutical Sciences*, 9, 355-363.
- Royal Botanic Gardens Melbourne. 2013. *Alphitonia petriei*, Royal Botanic gardens Melbourne [Online]. Available: http://www.rbg.vic.gov.au/dbpages/rbgcensus/index.php/census/species_detail/18773 [Accessed 29 June 2013].
- Ruangnoo, S., Jaiaree, N., Makchuchit, S., Panthong, S., Thongdeeying, P. & Itharat, A. 2012. An in vitro inhibitory effect on RAW 264.7 cells by anti-inflammatory compounds from *Smilax corbularia* Kunth. *Asian Pacific Journal of Allergy and Immunology*, 30, 268-274.
- Ryan, T. J. 1992. Worldwide strategy for skin health care in a financial strait-jacket. *International Journal of Dermatology*, 31, 416-21.

- Ryu, S. Y., Lee, C.-K., Lee, C. O., Kim, H. S. & Zee, O. P. 1992. Antiviral triterpenes from *Prunella vulgaris*. *Archives of Pharmacal Research*, 15, 242-245.
- Ryu, S. Y., Oak, M.-H., Yoon, S.-K., Cho, D.-I., Yoo, G.-S., Kim, T.-S. & Kim, K.-M. 2000. Anti-allergic and anti-inflammatory triterpenes from the herb of *Prunella vulgaris*. *Planta medica*, 66, 358-360.
- Sarker, S. D., Bartholomew, B., Nash, R. J. & Simmonds, M. S. J. 2001. Sideroxylin and 8-demethylsideroxylin from *Eucalyptus saligna* (Myrtaceae). *Biochemical Systematics and Ecology*, 29, 759-762.
- Sarker, S. D., Latif, Z. & Gray, A. I. 2006. *Natural Products Isolation*, Humana Press, Totowa, New Jersey.
- Schroeder, D. 2009. Justice and benefit sharing. *Indigenous Peoples, Consent and Benefit Sharing*. Springer.
- Schultz, G. S. & Mast, B. A. 1999. Molecular analysis of the environments of healing and chronic wounds: cytokines, proteases and growth factors. *Primary Intention*, 7, 7-15.
- Schulz, G. & Stechmiller, J. 2006. Wound healing and nitric oxide production: too little or too much may impair healing and cause chronic wounds. *International Journal of Lower Extremity Wounds*, 5, 6-8.
- Seidel, V. 2006. Initial and Bulk Extraction. In: SARKER, S. D., LATIF, Z. & GRAY, A. I. (eds.) *Natural Product Isolation*. 2 ed. Totowa: Humana Press Inc.
- Selvam, C. & Jachak, S. M. 2004. A cyclooxygenase (COX) inhibitory biflavonoid from the seeds of *Semecarpus anacardium*. *Journal of Ethnopharmacology*, 95, 209-212.
- Selvam, C., Jachak, S. M. & Bhutani, K. 2004. Cyclooxygenase inhibitory flavonoids from the stem bark of *Semecarpus anacardium* Linn. *Phytotherapy Research*, 18, 582-584.
- Setzer, W. N., Petty, J. L., Schmidt, J. M., Setzer, M. C., Bates, R. B. & Jackes, B. R. 2004. Bioactive lupane triterpenoids in *Alphitonia petriei* from Paluma, north Queensland, Australia. *Current Topics in Phytochemistry*, 6, 145-148.
- Setzer, W. N., Setzer, M. C., Bates, R. B. & Jackes, B. R. 2000. Biologically active triterpenoids of *Syncarpia glomulifera* bark extract from Paluma, north Queensland, Australia. *Planta Medica*, 66, 176-177.
- Shamsa, F., Ahmadiani, A. & Khosrokhavar, R. 1999. Antihistaminic and anticholinergic activity of barberry fruit (*Berberis vulgaris*) in the guinea-pig ileum. *Journal of Ethnopharmacology*, 64, 161-166.
- Shou, Q., Banbury, L. K., Renshaw, D. E., Lambley, E. H., Mon, H., Macfarlane, G. A., Griesser, H. J., Heinrich, M. M. & Wohlmuth, H. 2012. Biologically active dibenzofurans from *Pilidiostigma glabrum*, an endemic Australian Myrtaceae. *Journal of Natural Products*, 75, 1612-1617.
- Silva, M. M., Santos, M. R., Caroco, G., Rocha, R., Justino, G. & Mira, L. 2002. Structure-antioxidant activity relationships of flavonoids: a re-examination. *Free Radical Research*, 36, 1219-1227.
- Simpson, B. 2011. *Chemical and Pharmacological investigation of Dodonaea polyandra*. PhD PhD, University of South Australia.
- Simpson, B., Claudie, D., Smith, N., Wang, J., Mckinnon, R. & Semple, S. 2010. Evaluation of the anti-inflammatory properties of *Dodonaea polyandra*, a Kaanju traditional medicine. *Journal of Ethnopharmacology*, 132, 340-3.
- Simpson, B. S., Claudie, D. J., Gerber, J. P., Pyke, S. M., Wang, J., Mckinnon, R. A. & Semple, S. J. 2011. In vivo activity of benzoyl ester clerodane diterpenoid derivatives from *Dodonaea polyandra*. *Journal of Natural Products*, 74, 650-657.

- Singer, A. J. & Clark, R. 1999. Cutaneous wound healing. *The New England Journal of Medicine*, 341, 738-746.
- Singh, S. B. & Barrett, J. F. 2006. Empirical antibacterial drug discovery-Foundation in natural products. *Biochemical Pharmacology*, 71, 1006-1015.
- Sivasothy, Y., Sulaiman, S. F., Ooi, K. L., Ibrahim, H. & Awang, K. 2013. Antioxidant and antibacterial activities of flavonoids and curcuminoids from *Zingiber spectabile* Griff. *Food Control*, 30, 714-720.
- Snader, M. & Mccloud, T. G. 1994. Ethnobotany and drug discovery: the experience of the US National Cancer Institute. *Ethnobotany and the Search for New Drugs*, 185, 178.
- Soneja, A., Drews, M. & Malinski, T. 2005. Role of nitric oxide, nitroxidative and oxidative stress in wound healing. *Pharmacological Reports*, 57 Suppl, 108-19.
- Spjut, R. W. & Perdue, R. E., Jr. 1976. Plant folklore: a tool for predicting sources of antitumor activity? *Cancer Treatment Reports*, 60, 979-85.
- Stenhagen, E., Abrahamsson, S. & McLafferty, F. W. 1974. *Registry of mass spectral data*, Wiley.
- Sweeney, A. P., Wyllie, S. G., Shalliker, R. A. & Markham, J. L. 2001. Xanthine oxidase inhibitory activity of selected Australian native plants. *Journal of Ethnopharmacology*, 75, 273-7.
- Swigar, A. A. & Silverstein, R. M. 1981. *Monoterpenes: infrared, mass, ¹H NMR, and ¹³C NMR spectra, and Kovats indices*, Aldrich Chemical Co.
- Sylvestre, M., Pichette, A., Lavoie, S., Longtin, A. & Legault, J. 2007. Composition and cytotoxic activity of the leaf essential oil of *Comptonia peregrina* (L.) Coulter. *Phytotherapy Research*, 21, 536-540.
- Takahashi, T., Kokubo, R. & Sakaino, M. 2004. Antimicrobial activities of eucalyptus leaf extracts and flavonoids from *Eucalyptus maculata*. *Letters in Applied Microbiology*, 39, 60-64.
- Tan, M., Zhou, L., Huang, Y., Wang, Y., Hao, X. & Wang, J. 2008. Antimicrobial activity of globulol isolated from the fruits of *Eucalyptus globulus* Labill. *Natural Product Research*, 22, 569-575.
- Tan, Y., Yu, R. & Pezzuto, J. M. 2003. Betulinic acid-induced programmed cell death in human melanoma cells involves mitogen-activated protein kinase activation. *Clinical Cancer Research*, 9, 2866-2875.
- Tao, H., Hao, X., Liu, J., Ding, J., Fang, Y., Gu, Q. & Zhu, W. 2008. Resin Glycoside Constituents of *Ipomoea pes-caprae* (Beach Morning Glory). *Journal of Natural Products*, 71, 1998-2003.
- Teramachi, F., Koyano, T., Kowithayakorn, T., Hayashi, M., Komiyama, K. & Ishibashi, M. 2005. Collagenase inhibitory quinic acid esters from *Ipomoea pes-caprae*. *Journal of Natural Products*, 68, 794-796.
- The Plant List. 2010a. *Alphitonia* [Online]. Available: <http://www.theplantlist.org/browse/A/Rhamnaceae/Alphitonia/> [Accessed 18 June 2013].
- The Plant List. 2010b. *Lophostemon* [Online]. Available: <http://www.theplantlist.org/browse/A/Myrtaceae/Lophostemon/> [Accessed 28 June 2013].
- Tindana, P. O., Singh, J. A., Tracy, C. S., Upshur, R. E., Daar, A. S., Singer, P. A., Frohlich, J. & Lavery, J. V. 2007. Grand challenges in global health: community engagement in research in developing countries. *PLoS Medicine*, 4, e273.

- Tripathi, P., Tripathi, P., Kashyap, L. & Singh, V. 2007. The role of nitric oxide in inflammatory reactions. *FEMS Immunology and Medical Microbiology*, 51, 443-452.
- Tsai, J.-C., Peng, W.-H., Chiu, T.-H., Lai, S.-C. & Lee, C.-Y. 2011. Anti-inflammatory Effects of *Scoparia dulcis* L. and Betulinic Acid. *The American Journal of Chinese Medicine*, 39, 943-956.
- Tsikas, D. 2007. Analysis of nitrite and nitrate in biological fluids by assays based on the Griess reaction: Appraisal of the Griess reaction in the L-arginine/nitric oxide area of research. *Journal of Chromatography. B: Anal. Technol. Biomed. Life Sci.*, 851, 51-70.
- Tung, Y.-T., Chua, M.-T., Wang, S.-Y. & Chang, S.-T. 2008. Anti-inflammation activities of essential oil and its constituents from indigenous cinnamon (*Cinnamomum osmophloeum*) twigs. *Bioresource Technology*, 99, 3908-3913.
- Tuxill, J. D. & Nabhan, G. P. 2001. *People, Plants and Protected Areas: A Guide to "In Situ" Management*, Earthscan.
- Umamaheshwari, G., Ramanathan, T. & Shanmugapriya, R. 2012. Antioxidant and radical scavenging effect of *Ipomoea pes-caprae* Linn. R.Br. *International Journal of PharmTech Research*, 4, 1177-1180.
- UN. 1994. *Draft United Nations declaration on the rights of indigenous peoples* [Online]. Available: <http://www.unhchr.ch/huridocda/huridoca.nsf/%28Symbol%29/E.CN.4.SUB.2.RES.19.94.45.En>.
- Vagelos, P. R. 1991. Are prescription drug prices high? *Science*, 252, 1080-4.
- Van Holst Pellekaan, S. M. & Clague, L. 2005. Toward health and wellbeing for indigenous Australians. *Postgraduate Medical Journal* 81, 618-624.
- Vane, J., Bakhle, Y. & Botting, R. 1998. CYCLOOXYGENASES 1 AND 2. *Annual Review of Pharmacology and Toxicology*, 38, 97-120.
- Veitch, N. C., Bristow, J. M., Kite, G. C. & Lewis, G. P. 2005. Mildbraedin, a novel kaempferol tetraglycoside from the tropical forest legume *Mildbraediodendron excelsum*. *Tetrahedron Letters*, 46, 8595-8598.
- Verpoorte, R. 2000. Pharmacognosy in the new millennium: leadfinding and biotechnology. *Journal of Pharmacy and Pharmacology*, 52, 253-262.
- Viji, V., Helen, A. & Luxmi, V. R. 2011. Betulinic acid inhibits endotoxin stimulated phosphorylation cascade and pro-inflammatory prostaglandin E(2) production in human peripheral blood mononuclear cells. *British Journal of Pharmacology*, 162, 1291-1303.
- Vlachy, N., Touraud, D., Heilmann, J. & Kunz, W. 2009. Determining the cytotoxicity of catanionic surfactant mixtures on HeLa cells. *Colloids and Surfaces B: Biointerfaces*, 70, 278-280.
- Wagner, H. 1996. *Plant drug analysis: a thin layer chromatography atlas*, Springer.
- Wall, M. E. & Wani, M. C. 1996. Camptothecin and taxol: from discovery to clinic. *Journal of Ethnopharmacology*, 51, 239-254.
- Walton, N. N. J. & Brown, D. D. E. 1999. *Chemical from plants: perspectives on plant secondary products*, World Scientific.
- Wanandy, S., Brouwer, N., Liu, Q., Mahon, A., Cork, S., Karuso, P., Vemulpad, S. & Jamie, J. 2005. Optimisation of the fluorescein diacetate antibacterial assay. *Journal of Microbiological Methods*, 60, 21-30.
- Wang, J. & Mazza, G. 2002. Effects of anthocyanins and other phenolic compounds on the production of tumor necrosis factor α in LPS/IFN- γ -activated RAW 264.7 macrophages. *Journal of Agricultural and Food Chemistry*, 50, 4183-4189.

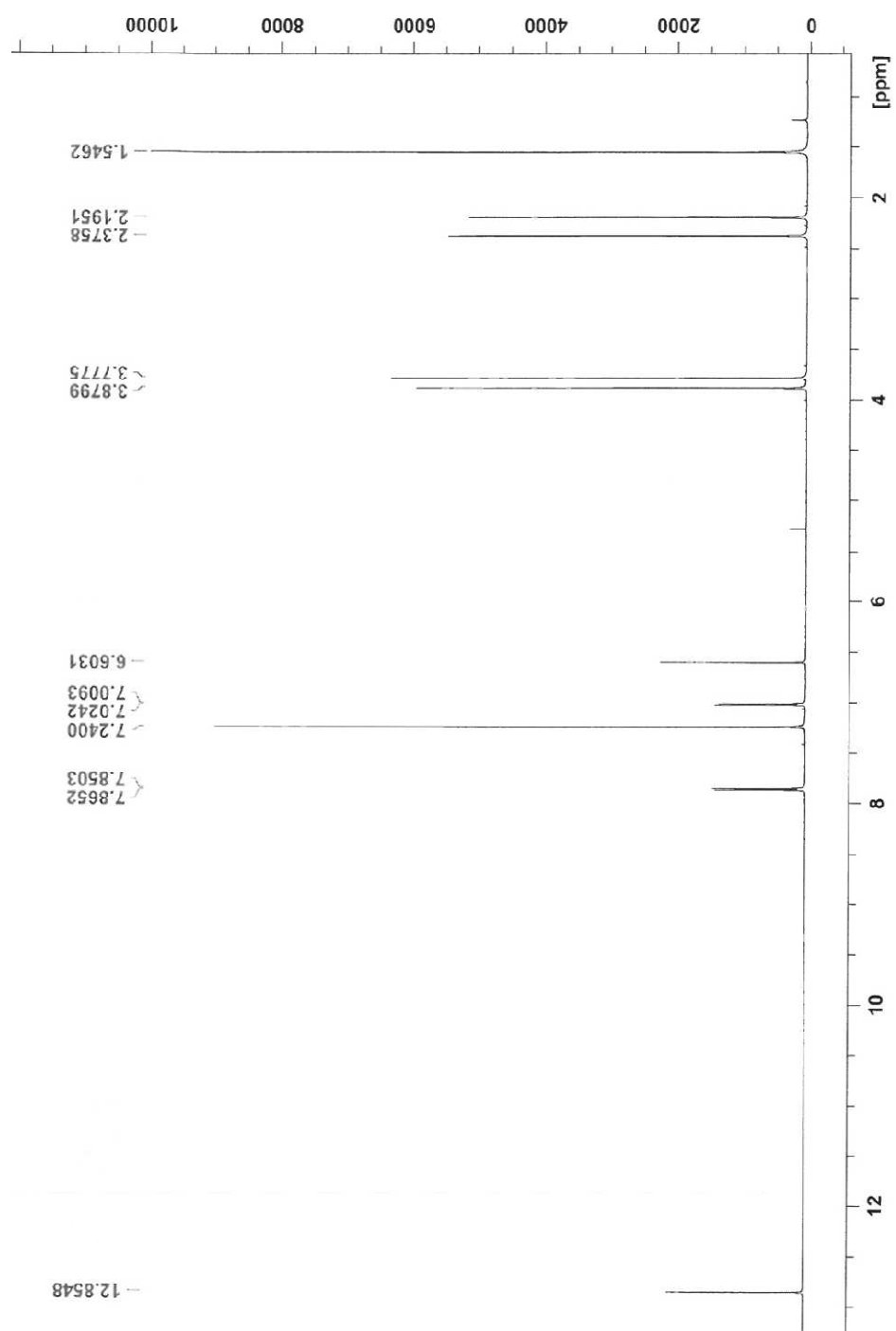
- Ward, S. G. 2008. New drug targets in inflammation: efforts to expand the anti-inflammatory armoury. *British Journal Pharmacology*, 153, S5-S6.
- Wasuwat, S. 1970. Extract of *Ipomoea pes-caprae* (Convolvulaceae) antagonistic to histamine and jelly-fish poison. *Nature (London)*, 225, 758.
- Webb, L. J. 1969. The use of plant medicines and poisons by Australian Aborigines. *Mankind*, 7, 137-146.
- Willey, J. M., Sherwood, L. & Woolverton, C. J. 2011. *Prescott's microbiology*, McGraw-Hill.
- Williams, T. J. & Peck, M. J. 1977. Role of prostaglandin-mediated vasodilatation in inflammation. *Nature (London)*, 270, 530-2.
- Wilson, P. G. & Waterhouse, J. T. 1982. A review of the genus *Tristania* R. Br. (Myrtaceae): a heterogeneous assemblage of five genera *Australian Journal of Botany* 30, 413-446.
- Wohlmuth, H., Deseo, M. A., Brushett, D. J., Thompson, D. R., Macfarlane, G., Stevenson, L. M. & Leach, D. N. 2010. Diarylheptanoid from *Pleuranthodium racemigerum* with in vitro prostaglandin E2 inhibitory and cytotoxic activity. *Journal of Natural Products*, 73, 743-746.
- Wong, D., Wang, M., Cheng, Y. & Fitzgerald, G. A. 2005. Cardiovascular hazard and non-steroidal anti-inflammatory drugs. *Current Opinion in Pharmacology*, 5, 204-210.
- Woolley, J. G. 2001. Plant Alkaloids. *eLS*. John Wiley & Sons, Ltd.
- WTAPPT. 2005. *The Wet Tropics Aboriginal Plan Project Team, caring for country and culture - wet tropics Aboriginal and cultural resource management plan*, Cairns, Rainforest CRC and FNQ NRM Ltd.
- Xiao, S.-J., He, D.-H., Ding, L.-S., Zhou, Y. & Chen, F. 2013. Triterpenes from aerial parts of *Clematoclethra scandens* subsp. *actinidioides*. *Zhongguo Zhong Yao Za Zhi*, 38, 358-61.
- Xiao, Z., Wu, H., Wu, T., Shi, H., Hang, B. & Aisa, H. 2006. Kaempferol and quercetin flavonoids from *Rosa rugosa*. *Chemistry of Natural Compounds*, 42, 736-737.
- Yao, L., Jiang, Y., Singanusong, R., D'arcy, B., Datta, N., Caffin, N. & Rayment, K. 2004. Flavonoids in Australian *Melaleuca*, *Guioa*, *Lophostemon*, *Banksia* and *Helianthus* honeys and their potential for floral authentication. *Food Research International*, 37, 166-174.
- Yu, B.-W., Luo, J.-G., Wang, J.-S., Zhang, D.-M., Yu, S.-S. & Kong, L.-Y. 2011. Pentasaccharide Resin Glycosides from *Ipomoea pes-caprae*. *Journal of Natural Products*, 74, 620-628.
- Yuan, Q., Zhang, X., Liu, Z., Song, S., Xue, P., Wang, J. & Ruan, J. 2013. Ethanol extract of *Adiantum capillus-veneris* L. suppresses the production of inflammatory mediators by inhibiting NF- κ B activation. *Journal of Ethnopharmacology*, 147, 603-611.
- Zakus, J. D. L. & Lysack, C. L. 1998. Revisiting community participation. *Health Policy and Planning*, 13, 1-12.
- Zhang, L., Feng, L., Jia, Q., Xu, J., Wang, R., Wang, Z., Wu, Y. & Li, Y. 2011. Effects of β -glucosidase hydrolyzed products of harpagide and harpagoside on cyclooxygenase-2 (COX-2) in vitro. *Bioorganic and Medicinal Chemistry*, 19, 4882-4886.
- Zhu, H., Tu, P., Chen, Q. & Xu, A. 2003. Studies on chemical constituents of cytotoxicity portion in bark of *Reevesia longipetiolata*. *Zhongcaoyao*, 34, 976-978.
- Zhu, L.-H., Chen, C., Wang, H., Ye, W.-C. & Zhou, G.-X. 2012a. Indole alkaloids from *Alocasia macrorrhiza*. *Chemical and Pharmaceutical Bulletin*, 60, 670-673.
- Zhu, L.-H., Huang, X.-S., Ye, W.-C. & Zhou, G.-X. 2012b. Study on chemical constituents of *Alocasia macrorrhiza* (L.) Schott. *Zhongguo Yaoxue Zazhi (Beijing, China)*, 47, 1029-1031.

- Ziaei, A., Ramezani, M., Wright, L., Paetz, C., Schneider, B. & Amirghofran, Z. 2011. Identification of spathulenol in *Salvia mirzayanii* and the immunomodulatory effects. *Phytotherapy Research*, 25, 557-562.
- Zollman, C. & Vickers, A. 1999. ABC of complementary medicine: What is complementary medicine? *BMJ: British Medical Journal*, 319, 693.
- Zuco, V., Supino, R., Righetti, S. C., Cleris, L., Marchesi, E., Gambacorti-Passerini, C. & Formelli, F. 2002. Selective cytotoxicity of betulinic acid on tumor cell lines, but not on normal cells. *Cancer Letters (Shannon, Irel.)*, 175, 17-25.

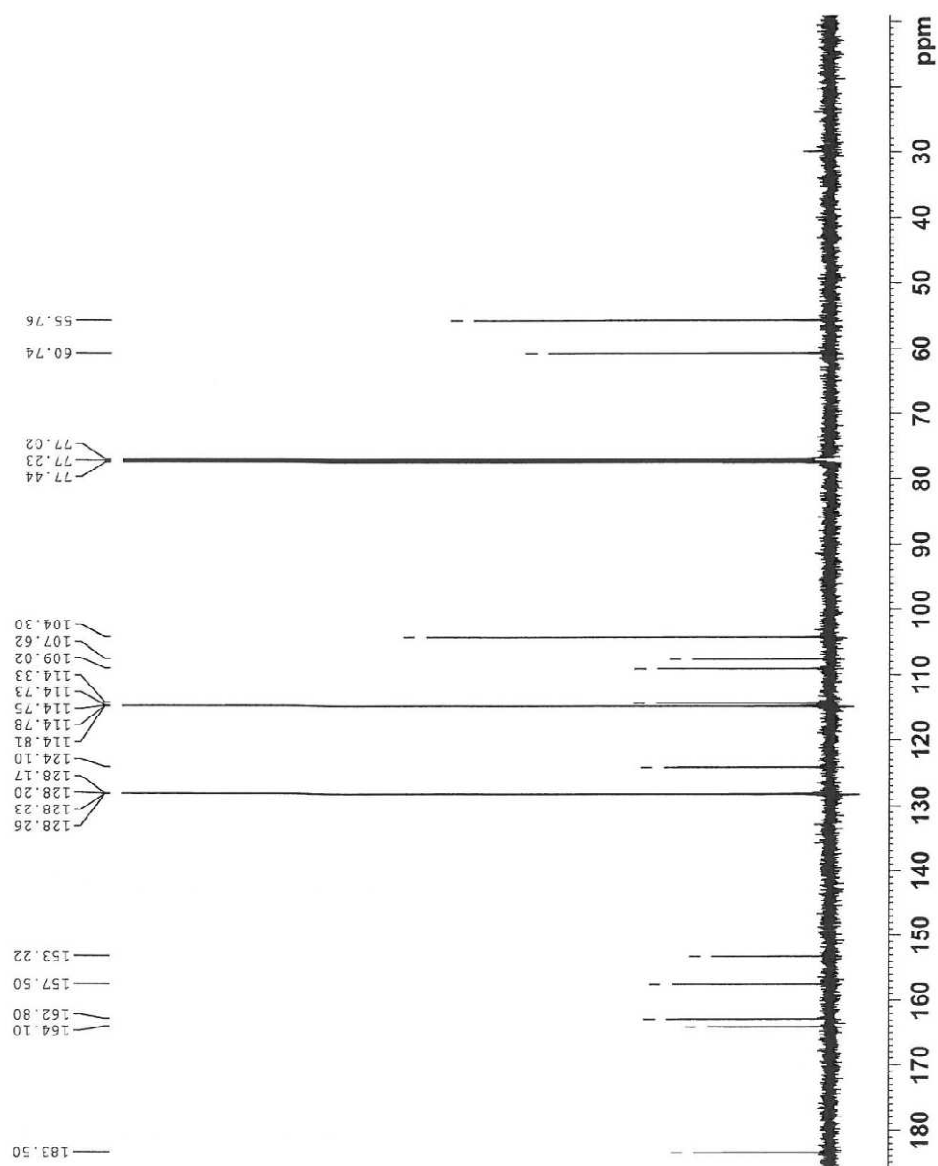
Appendix I (NMR/MS supporting data for compounds LS-22 and LS-29).

1. Compound LS-22 ^1H NMR spectrum.....	213
2. Compound LS-22 ^{13}C NMR spectrum.....	215
3. Compound LS-22 HMBC spectrum.....	217
4. Compound LS-22 high resolution MS.....	219
5. Compound LS-29 ^1H NMR spectrum.....	221
6. Compound LS-29 ^{13}C NMR spectrum.....	223
7. Compound LS-29 HMBC spectrum.....	225
8. Compound LS-29 high resolution MS.....	227

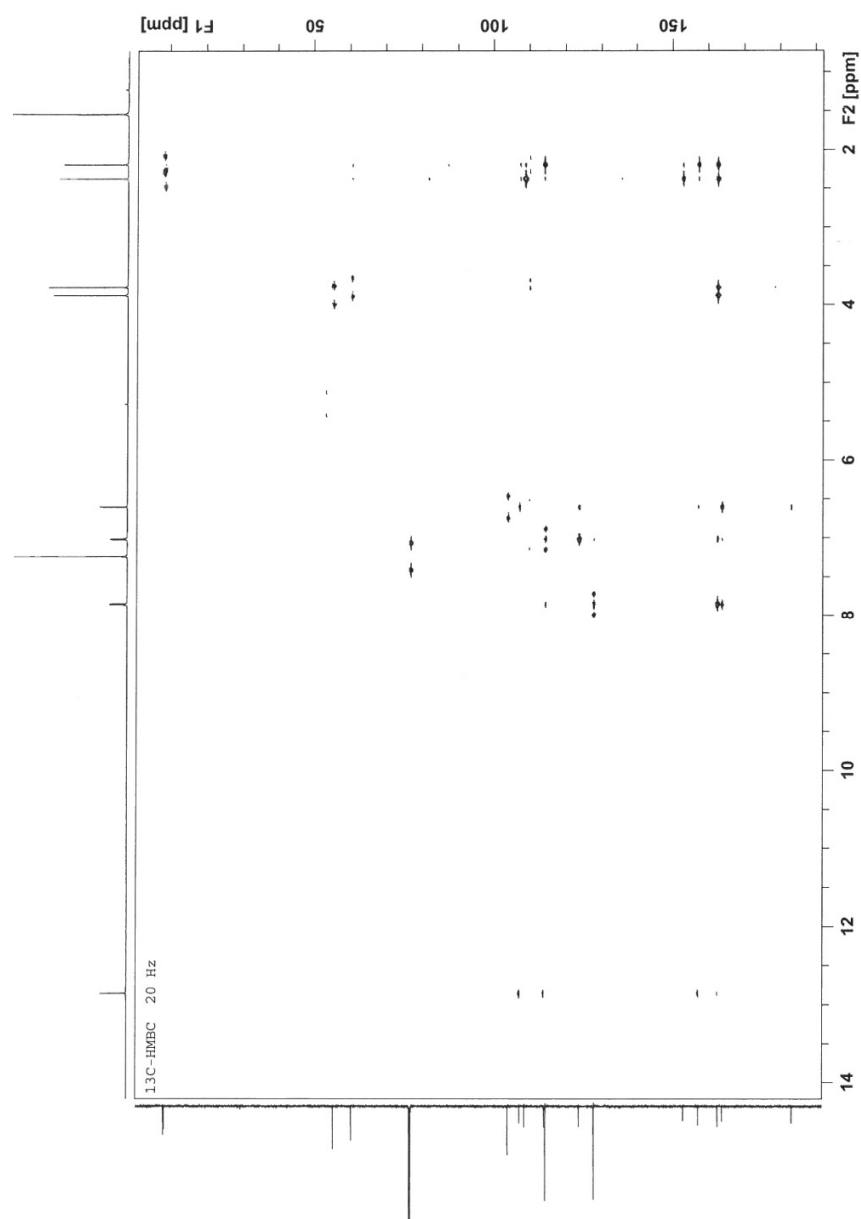
1. Compound LS-22 ^1H NMR (CDCl_3 , 600 MHz) spectrum



2. Compound LS-22 ^{13}C NMR (CDCl_3 , 150 MHz) spectrum



3. Compound LS-22 HMBC (CDCl₃, 600 MHz) spectrum



4. Compound LS-22 high resolution MS

Elemental Composition Report

Page 1

Single Mass Analysis

Tolerance = 5.0 PPM / DBE: min = -1.5, max = 20.0

Selected filters: None

Monoisotopic Mass, Odd and Even Electron Ions

42 formula(e) evaluated with 1 results within limits (up to 50 best isotopic matches for each mass)

Elements Used:

C: 0-50 H: 0-50 O: 0-10

RAB-LS22/AJ

ID:24473

HR0094AFAMM 81 (3.228)

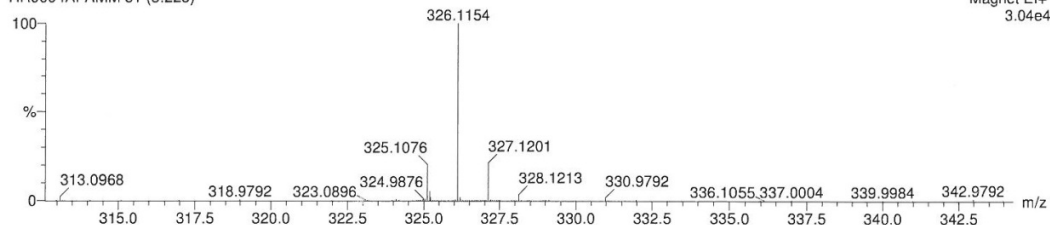
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Magnet EI+

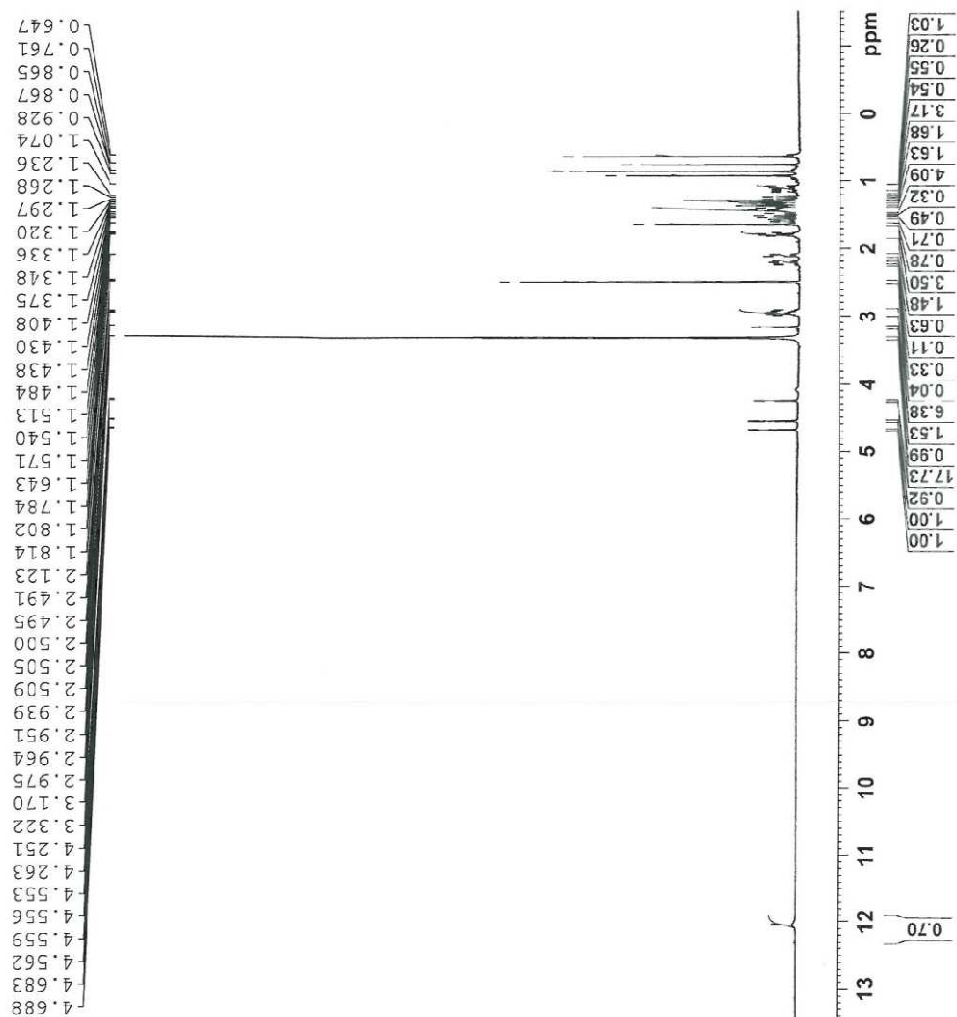
3.04e4



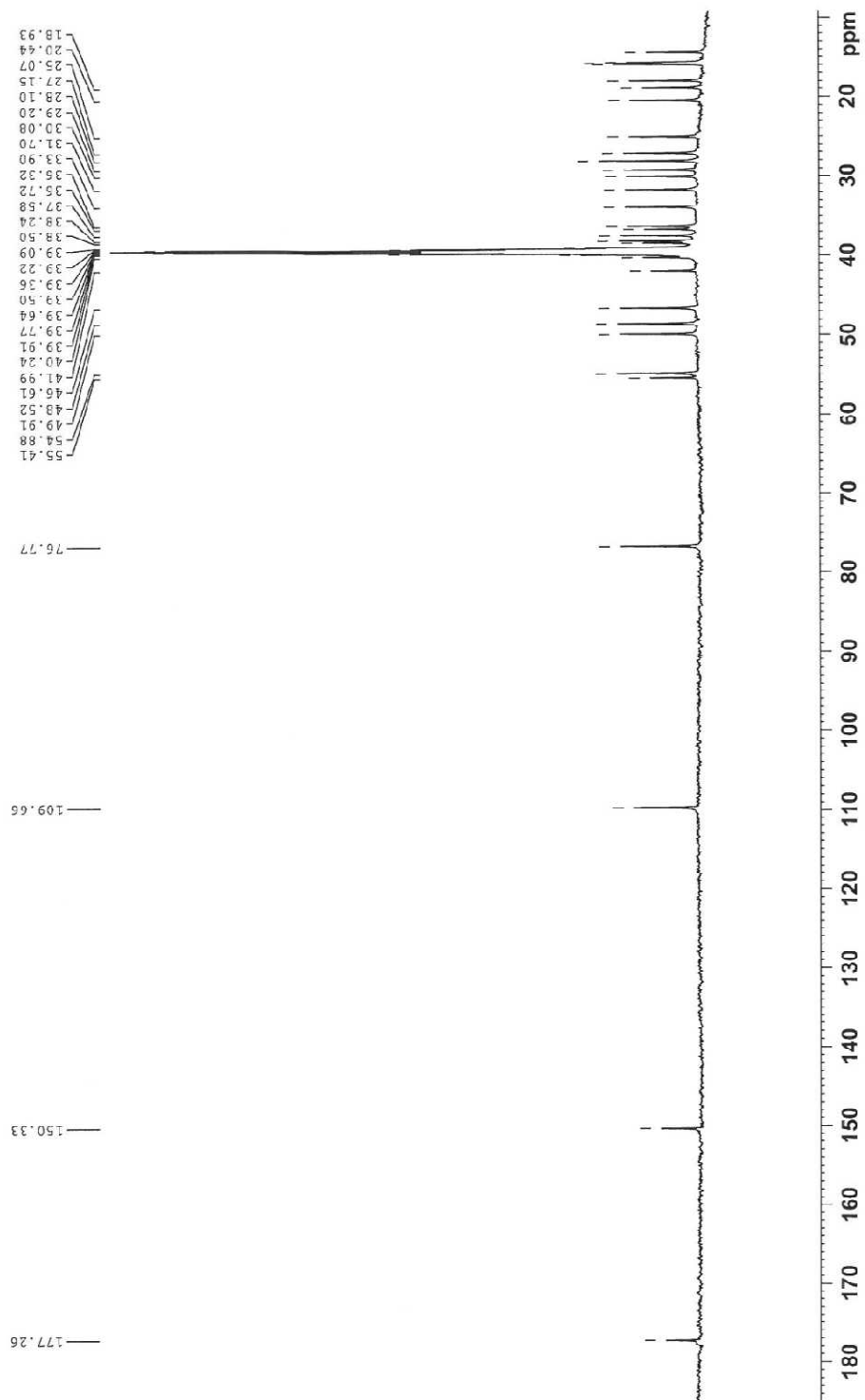
Minimum: -1.5
Maximum: 5.0 5.0 20.0

Mass	Calc. Mass	mDa	PPM	DBE	i-FIT	Formula
326.1154	326.1154	0.0	0.0	11.0	3.5	C19 H18 O5

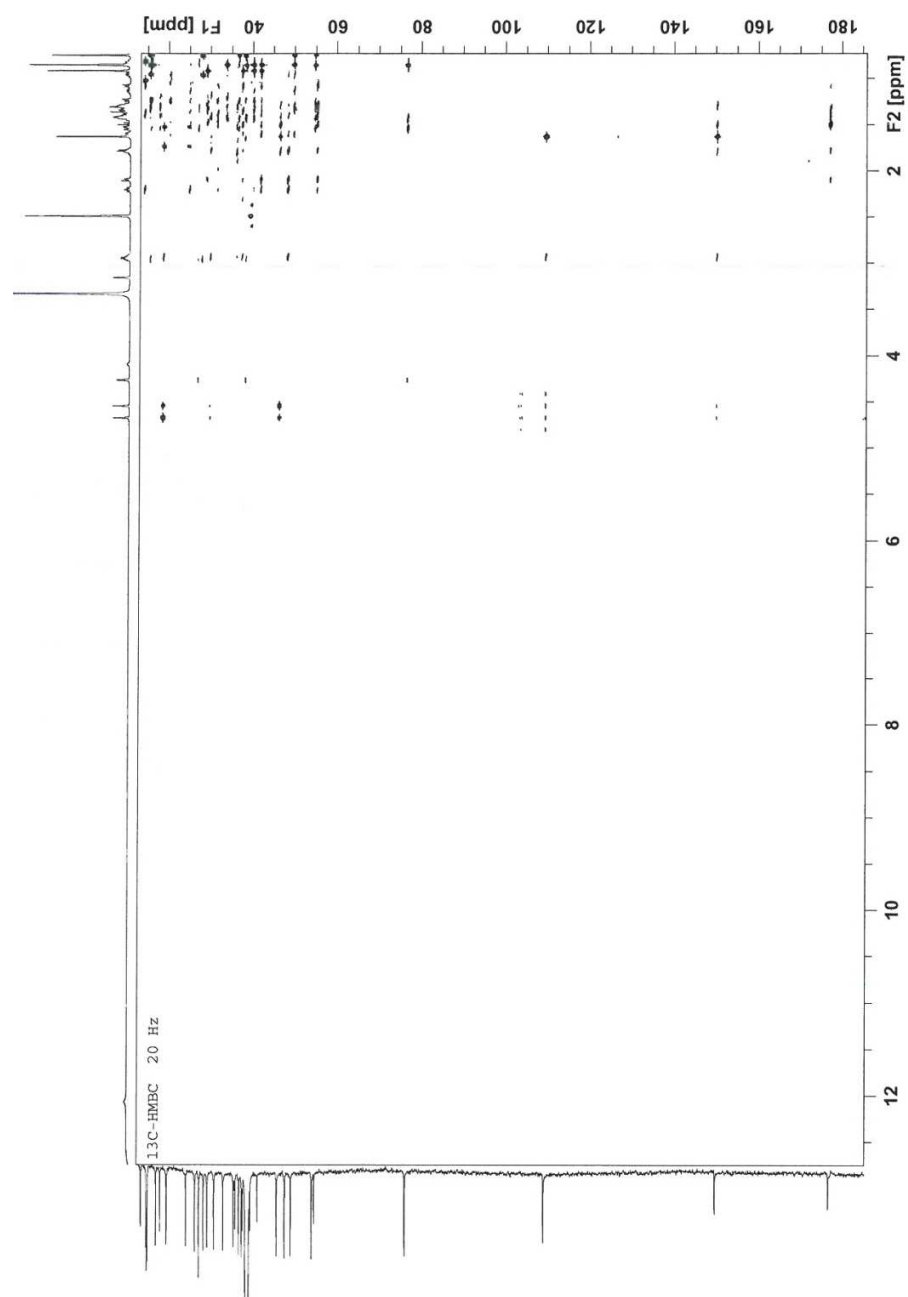
5. Compound LS-29 ^1H NMR (DMSO, 600 MHz) spectrum



6. Compound LS-29 ^{13}C NMR (DMSO, 150 MHz) spectrum



7. Compound LS-29 HMBC (DMSO, 600 MHz) spectrum



8. Compound LS-29 high resolution MS

Elemental Composition Report

Page 1

Single Mass Analysis

Tolerance = 3.0 PPM / DBE: min = -1.5, max = 20.0

Element prediction: Off

Number of isotope peaks used for i-FIT = 3

Monoisotopic Mass, Even Electron Ions

18 formula(e) evaluated with 1 results within limits (all results (up to 1000) for each mass)

Elements Used:

C: 0-50 H: 0-50 O: 0-3

RAB-LS29/AJ

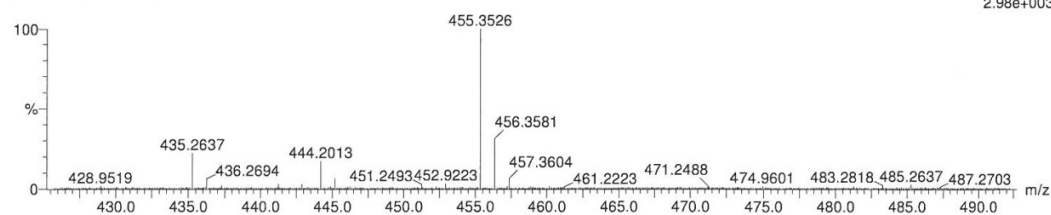
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1: TOF MS ES-
2.98e+003

1570 25 (1.121) Cm (25:29)



Minimum: -1.5
Maximum: 20.0

Mass	Calc. Mass	mDa	PPM	DBE	i-FIT	Formula
455.3526	455.3525	0.1	0.2	7.5	3.5	C30 H47 O3

Appendix II (Ethics approval letters)

1. Human Ethics Approval: An Ethnopharmacological Study of Medicinal Plants in New South Wales.....	231
2. Human Ethics Approval: An Ethnopharmacological Study of Australian Medicinal Plants.....	233
3. Human Ethics Approval: Engaging Indigenous Secondary Students in Science and Technology.....	235

1. Human Ethics Approval: An Ethnopharmacological Study of Medicinal Plants in New South Wales.



13 January 2009

Associate Professor Joanne Jamie
Department of Chemistry and Biomolecular Sciences
Faculty of Science

Reference: HE27JUL2007-R05356

Dear Associate Professor Jamie,

Renewal Approved

Title of project: *"An Ethnopharmacological Study of Medicinal Plants in New South Wales"*

Thank you for your recent correspondence. Approval for renewal of the above application has been granted, effective 1 October 2008.

The following are standard requirements attached to approval of all projects:

1. Approval will be for a period of twelve months. At the end of this period, if the project has been completed, abandoned, discontinued or not completed for any reason you are required to submit a Final Report on the project. If you complete the work earlier than you had planned you must submit a Final Report as soon as the work is completed. This form is available at:
http://www.research.mq.edu.au/researchers/ethics/human_ethics/forms
2. However, at the end of the 12 month period if the project is still current you should instead submit an application for renewal of the approval if the project has run for less than five (5) years. This form is available at:
http://www.research.mq.edu.au/researchers/ethics/human_ethics/forms If the project has run for more than five (5) years you cannot renew approval for the project. You will need to complete and submit a Final Report (see Point 1 above) and submit a new application for the project. (The five year limit on renewal of approvals allows the Committee to fully re-review research in an environment where legislation, guidelines and requirements are continually changing, for example, new child protection and privacy laws).
3. Please remember you must notify the Committee in writing regarding any alteration to the project.
4. You must notify the committee immediately in the event of any adverse effects on participants or of any unforeseen events that might affect continued ethical acceptability of the project.
5. At all times you are responsible for the ethical conduct of your research in accordance with the guidelines established by the University
http://www.research.mq.edu.au/researchers/ethics/human_ethics

Yours sincerely

A handwritten signature in dark ink, appearing to read "Dr Shirley Wyver".

Dr Shirley Wyver
Acting Chair, Ethics Review Committee (Human Research)

ETHICS REVIEW COMMITTEE (HUMAN RESEARCH)
MACQUARIE UNIVERSITY

Level 3, Research HUB, Building C5C

Secretary: Ph: (02) 9850 6848 Fax: (02) 9850 4465 E-mail: ethics.secretariat@vc.mq.edu.au

http://www.research.mq.edu.au/researchers/ethics/human_ethics

2. Human Ethics Approval: An Ethnopharmacological Study of Australian Medicinal Plants.

Macquarie University Student Email and Calendar Mail - Approved- Et... <https://mail.google.com/mail/u/0/?ui=2&ik=3e15136375&view=pt&q=...>



TARANNUM NAZ <tarannum.naz@students.mq.edu.au>

Approved- Ethics application- Jamie (Ref No: 5201200763)

4 messages

Ethics Secretariat <ethics.secretariat@mq.edu.au>

Thu, Jan 24, 2013 at 3:07 PM

To: A/Prof Joanne Jamie <joanne.jamie@mq.edu.au>

Cc: A/Prof Subramanyam Vemulpad <subramanyam.vemulpad@mq.edu.au>, Mr David Harrington <david.harrington@mq.edu.au>, Prof Shoba Ranganathan <shoba.ranganathan@mq.edu.au>, Dr Joanne Michelle Packer <joanne.packer@students.mq.edu.au>, joanne.packer@mq.edu.au, Mrs Tarannum Naz <tarannum.naz@mq.edu.au>, Mrs Kaisarun Akter <kaisarun.akter@students.mq.edu.au>

Dear A/Prof Jamie

Re: "An Ethnopharmacological study of Australian Medicinal plants" (Ethics
Ref: 5201200763)

Thank you for your recent correspondence. Your response has addressed the
issues raised by the Human Research Ethics Committee and you may now
commence your research.

This research meets the requirements of the National Statement on Ethical
Conduct in Human Research (2007). The National Statement is available at
the following web site:

http://www.nhmrc.gov.au/_files_nhmrc/publications/attachments/e72.pdf.

The following personnel are authorised to conduct this research:

A/Prof Joanne Jamie
A/Prof Subramanyam Vemulpad
Mr David Harrington
Mrs Kaisarun Akter
Mrs Tarannum Naz
Ms Joanne Michelle Packer
Prof Shoba Ranganathan

NB. STUDENTS: IT IS YOUR RESPONSIBILITY TO KEEP A COPY OF THIS APPROVAL
EMAIL TO SUBMIT WITH YOUR THESIS.


Please note the following standard requirements of approval:

1. The approval of this project is conditional upon your continuing
compliance with the National Statement on Ethical Conduct in Human Research
(2007).
2. Approval will be for a period of five (5) years subject to the provision
of annual reports.

Progress Report 1 Due: 24 January 2014
Progress Report 2 Due: 24 January 2015
Progress Report 3 Due: 24 January 2016
Progress Report 4 Due: 24 January 2017
Final Report Due: 24 January 2018

NB. If you complete the work earlier than you had planned you must submit a
Final Report as soon as the work is completed. If the project has been
discontinued or not commenced for any reason, you are also required to
submit a Final Report for the project.

3. Human Ethics Approval: Engaging Indigenous Secondary Students in Science and Technology.



3 October 2007

Dr Neil Harrison
School of Education
Australian Centre for Educational Studies

Reference: HE27JUL2007-R05361

Dear Dr Harrison

FINAL APPROVAL

Title of project: *Engaging Indigenous secondary students in science and technology - a pathway to higher education*


Thank you for your recent correspondence. Your responses have satisfactorily addressed the outstanding issue raised by the Committee. You may now proceed with your research.

Please note the following standard requirements of approval:

1. Approval will be for a period of twelve months. At the end of this period, if the project has been completed, abandoned, discontinued or not commenced for any reason, you are required to submit a Final Report on the project. If you complete the work earlier than you had planned you must submit a Final Report as soon as the work is completed. The Final Report is available at <http://www.ro.mq.edu.au/ethics/human/forms>
2. However, at the end of the 12 month period if the project is still current you should instead submit an application for renewal of the approval if the project has run for less than five (5) years. This form is available at <http://www.ro.mq.edu.au/ethics/human/forms>. If the project has run for more than five (5) years you cannot renew approval for the project. You will need to complete and submit a Final Report (see Point 1 above) and submit a new application for the project. (The five year limit on renewal of approvals allows the Committee to fully re-review research in an environment where legislation, guidelines and requirements are continually changing, for example, new child protection and privacy laws).
3. Please remember the Committee must be notified of any alteration to the project.
4. You must notify the Committee immediately in the event of any adverse effects on participants or of any unforeseen events that might affect continued ethical acceptability of the project.
5. At all times you are responsible for the ethical conduct of your research in accordance with the guidelines established by the University (<http://www.ro.mq.edu.au/ethics/human>).

If you will be applying for or have applied for internal or external funding for the above project **it is your responsibility** to provide Macquarie University's Research Grants Officer with a copy of this letter as soon as possible. The Research Grants Officer will not inform external funding agencies that you have final approval for your project and funds will not be released until the Research Grants Officer has received a copy of this final approval letter.

Yours sincerely



P. P. Dr Margaret Stuart
Director of Research Ethics
Chair, Ethics Review Committee [Human Research]

HLTR38.1 : CRO File: 07/892

ETHICS REVIEW COMMITTEE (HUMAN RESEARCH)
MACQUARIE UNIVERSITY (EIA)
SYDNEY, NSW, 2109 AUSTRALIA
Secretary: Ph: (02) 9850 7850 Fax: (02) 9850 4465 E-mail: ethics.secretariat@vc.mq.edu.au
<http://www.ro.mq.edu.au/ethics/human>

Portrait (85%)