An Ethnopharmacological Study of Medicinal Plants of the Kamilaroi and Muruwari Aboriginal Communities in Northern New South Wales

A thesis submitted for the degree of

DOCTOR OF PHILOSOPHY

at

MACQUARIE UNIVERSITY

by

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July, 2006

Declaration

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

Qian Liu

July 2006

Acknowledgements

I would like to thank my supervisors Dr Joanne Jamie, Dr Subramanyam Vemulpad and Dr James Kohen for involving me in this multidisciplinary project and letting me participate in all the different aspects of it, and for their support throughout this study.

I also would like to give my thanks to all the members of the Indigenous Bioresource Research Group at Macquarie University: David Harrington, John Hunter, Mathew Flower, Nynke Brouwer and Thomas Dzeha, and Professor Donna Craig and Chris Jones at the Centre for Environmental Law, for their collaboration and help during this study. I am very grateful to Mr and Mrs Roy and June Barker for sharing their customary medicinal plant knowledge with me. I also wish to thank Ms Alison Downing at the Herbarium of Macquarie University for identifying all the plant specimens.

My sincere acknowledgement goes to Professor Jianmin Yue at Shanghai Institute of Materia Medica, Chinese Academy of Sciences, for extending his research facilities to me for a part of this study, and for his support and guidance during my stay in his laboratory. I also want to thank his group members, especially Associate Professor Sheng-Ping Yang, for the helpful discussions on my research work during my overseas study.

I am very thankful for all those people who trained me on all of the instrumentation in the university. I thank Dr Andrew Piggott for the instructions on operating 2D NMR, Dr Isla Hains for the LCMS, Rama Nimmagadda and Mark Tran for the GCMS and Keith Tonkin and Thulasy Jeyendra for the IR and polarimeter.

I made a few very good friends during my study at Macquarie University in Sydney. Since I was away from my family, these friends have been an important part of my life. I would like to express my sincere gratitude to Mr Priambudi Kosim-Satyaputra for his always wise suggestions on the problems I encountered in my work and daily life. My special thanks go to Mr Simone Ciampi who proof read the first chapter of this thesis and helped to improve my English expressions. My thanks also go to Ms Ning Xu, Ms Qiang Xu, Ms Hong Yu, Dr Yabai He, Mr Hua Liu and Mr Dayong Jin, for their friendship and for always being there to cheer me up during my difficult times.

I give my heartfelt thanks to my parents, my parents-in-law, my husband and my daughter, for their love, care and patience. They sacrificed a lot for me during these three years while I was away from home for my PhD study. I couldn't have finished this study without their support and encouragement.

Finally, I thank the Australian government and Macquarie University for providing me with scholarships for this study.

Abstract

The overall objective of this study was to isolate and identify biologically active compounds from Australian medicinal plants with the assistance of customary (traditional and contemporary) medicinal knowledge of Aboriginal communities in northern New South Wales. This study consisted of three interrelated aspects, namely ethnobotanical research, biological studies, and bioassay-guided isolation and characterisation of bioactive constituents from Australian Aboriginal medicinal plants.

An ethnobotanical study of Australian medicinal plants used by the Kamilaroi and Muruwari Aboriginal communities was conducted with the cooperation of members of these communities. The customary medicinal plant knowledge of these two communities, along with scientific research data from published sources, of a total of 35 plants and 2 customary remedies were obtained through interviews and literature studies, and were documented as a database. The ethnobotanical database contributed to the preservation of customary medicinal knowledge of these communities. A series of educational activities were also conducted for Indigenous students as part of the relationship development and benefit sharing with Aboriginal communities in northern New South Wales. The ethnobotanical data were also used as a guide for targeted biological and chemical studies of two Australian medicinal plants, *Eremophila sturtii* and *Exocarpos aphyllus*.

Anti-inflammatory and antimicrobial assays were employed in this study for the evaluation of the biological activities of the selected medicinal plants according to their customary medicinal uses, and were applied throughout the bioactivity-oriented isolation of bioactive agents from these medicinal plants. The biological study also included optimisation and

validation of a fluorescence-based antibacterial assay, the fluorescein diacetate (FDA) assay, to make it suitable for the screening of medicinal plants for antibacterial activity. Antimicrobial and anti-inflammatory activities of *Eremophila sturtii* and *Exocarpos aphyllus* were revealed in this biological study.

Bioassay-guided fractionations of these Aboriginal medicinal plants led to the isolation of two novel compounds, 3,8-dihydroxyserrulatic acid and serrulatic acid, and six known compounds, *β*-sitosterol, sesamin, 3,6-dimethoxy-5,7-dihydroxyflavone, betulin, betulinic acid and oleanolic acid. The structures of the isolated compounds were elucidated using nuclear magnetic resonance (NMR) and mass spectrometric (MS) techniques. Both novel compounds demonstrated antibacterial activity against *Staphylococcus aureus* and anti-inflammatory activity against cyclooxygenase-1 and cyclooxygenase-2 (COX-1 and COX-2). All known compounds demonstrated anti-inflammatory activity against COX-1, COX-2 and 5-lipoxygenase (5-LO). The biological activities of these compounds were consistent with the customary medicinal applications of these Aboriginal medicinal plants. This is the first time that any of these compounds have been isolated from *Eremophila sturtii* and *Exocarpos aphyllus*.

List of Publications

Liu, Q., Harrington, D., Kohen, J. L., Vemulpad, S., Jamie, J. F., 2006. Bactericidal and cyclooxygenase inhibitory diterpenes from *Eremophila sturtii*. Phytochemistry 67(12), 1256-1261.

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(10), 1252-1262.

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(1), 21-30.

Conference Abstracts

Liu, Q., Wanandy, S., Harrington, D., Kohen, J. L., Vemulpad, S., Jamie, J. F., Ethnopharmacological studies of Australian Aboriginal medicinal plants. The Fifth International Chemical Congress of Pacific Basin Societies (Pacifichem), Honolulu, the United States of America, December 2005.

Liu, Q., Harrington, D., Kohen, J. L., Vemulpad, S., Jamie, J. F., *Eremophila sturtii* – an ethnopharmacological investigation of an Australian Aboriginal medicinal plant. Royal Australian Chemical Institute 2005 Natural Products Annual Symposium, Sydney, Australia, September 2005.

Liu, Q., Wanandy, S., Harrington, D., Kohen, J. L., Vemulpad, S., Jamie, J. F., Chemical and biological investigations of Australian Aboriginal medicinal plants. Royal Australian Chemical Institute Connect 2005 International Conference, Sydney, Australia, July 2005.

Liu, Q., Dzeha, T., Brouwer, N., Harrington, D., Flower, M., Hunter, J., Kohen, J. L., Vemulpad, S., Jamie, J. F., Maclean (Yaegl) Land Council, Research Partnerships in Aboriginal Bush Medicine. Royal Australian Chemical Institute 2004 Natural Products Group Annual Symposium, Lismore, Australia, September 2004.

Brouwer, N., **Liu, Q.**, Harrington, D., Kohen, J. L., Vemulpad, S., Jamie, J. F., Ethnopharmacological study of Medicinal Plants in New South Wales. International Conference of Indigenous Knowledge and Bioprospecting, Sydney, Australia, April 2004.

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List of Abbreviations

$[\alpha]_D$	Specific Optical Rotation
1/10 BPYN	Bacterial growth media containing 10 mM BES buffer, peptone 0.2%, yeast extract 0.1% and NaCl 0.1% (w/v)
^{13}C NMR	Carbon Nuclear Magnetic Resonance Spectroscopy
^{1}H NMR	Proton Nuclear Magnetic Resonance Spectroscopy
2D NMR	Two-Dimensional Nuclear Magnetic Resonance Spectroscopy
BES	*N,N*-Bis(2-hydroxyethyl)-2-aminoethanesulfonic acid
BuOH	*n*-Butanol
CFU	Colony Forming Unit
COSY	(Proton – Proton) Correlation Spectroscopy
COX	Cyclooxygenase
DEPT	Distortionless Enhancement by Polarisation Transfer
DMSO	Dimethyl Sulphoxide
EtOAc	Ethyl acetate
FDA	Fluorescein diacetate
HMBC	Heteronuclear Multiple Bond Correlation
HPLC	High Performance Liquid Chromatography
HREIMS	High Resolution Electron Impact Ionisation
HSQC	Heteronuclear Single Quantum Correlation
IR	Infrared
LO	Lipoxygenase
LREIMS	Low Resolution Electron Impact Ionisation
LT	Leukotriene
m.p.	Melting Point
MBC	Minimum Bactericidal Concentration
MIC	Minimum Inhibitory Concentration

MS	Mass Spectrometry
NCCLS	National Committee for Clinical Laboratory Standards
nOe	Nuclear Overhauser effect
PG	Prostaglandin
r.p.m.	Revolution per Minute
ROESY	Rotating Frame Overhauser Effect Spectroscopy
TLC	Thin Layer Chromatography
UV	Ultraviolet

Chapter 1. Introduction

This chapter describes the aims of this project and the significant contributions of ethnobotanical and ethnopharmacological research on medicinal plants to human healthcare and modern drug discovery.

1.1. Aims and scope of this study

Australian Aboriginal people have used the medicinal properties of their native flora for thousands of years and have accumulated a vast reservoir of knowledge on their medicinal plants. This PhD project dealt with both Australian Aboriginal medicinal knowledge and Australian medicinal plants. The major objective of this project was to isolate biologically important compounds from Australian medicinal plants with the assistance of the customary (traditional and contemporary) medicinal plant knowledge of the Kamilaroi and Muruwari Aboriginal communities in northern New South Wales.

This PhD project was composed of three interrelated aspects. Firstly, an ethnobotanical study was conducted on plants used by members of the Kamilaroi and Muruwari Aboriginal communities. This included documentation of first-hand traditional knowledge from these communities as well as information from the literature regarding the medicinal uses and pharmacological activities of the medicinal plants used by these communities. Details of this ethnobotanical study are provided in Chapter 2 of this thesis. Additionally, in recognition of the importance of working with Indigenous people when studying Indigenous knowledge, and in being responsive to their needs, workshops on best practices and benefit sharing were conducted along with a range of educational activities. These are

also detailed in Chapter 2. Secondly, bioassays were developed and validated for the evaluation of the biological properties of Aboriginal plants with significant medicinal potential. This part of the research is presented in Chapter 3. The third aspect of this project was the exploration and characterisation of bioactive constituents from selected Australian Aboriginal medicinal plants using bioassay-guided fractionation. The isolation and structural elucidation of bioactive compounds from two Aboriginal medicinal plants are described in Chapters 4 and 5. In this research project, particular attention was placed on medicinal plants that have been used traditionally to treat ailments of microbial origin or certain inflammatory conditions. These are the main conditions that Australian Aboriginal people use their medicinal plant preparations for, and are also conditions affecting populations worldwide and are in need of new drug treatments [Balick and Cox, 1996; Mahady, 2005; Yung *et al*., 2005].

In this chapter, a literature review is presented as a general background of this ethnopharmacological research project. The contributions of medicinal plants to human healthcare, as well as the importance of medicinal plant knowledge to modern drug discovery, are discussed.

1.2. Plants for human healthcare

Over centuries, humans have depended on plants as a source of food, medicine and to meet a variety of other needs [Flaster, 1996]. Some of the earliest uses of medicinal plants were documented in the Vedas and could be dated back to about 4500 B.C. [Pei, 2001]. The Egyptian pharmaceutical record, the “Ebers Papyrus”, written in about 1500 B.C., listed nearly 700 drugs, most of which were of plant origin [Cragg and Newman, 2001]. The

ancient Chinese started to record traditional herbal medicine information as early as about 2100 B.C., but the first book wholly devoted to the description of medicines, "Shen Nong Ben Cao Jing" (or "Shen-Nong's Herbal Classics"), is believed to have been compiled during 220-25 A.D.. This book contained 365 traditional medicines, with 252 entries being botanical, 67 zoological and 46 mineral [Zhu, 1998]. In the first century A.D., the Greek physician, botanist and pharmacologist Pedanius Dioscorides in his *De Materia Medica* described nearly 600 medicinal plants. *De Materia Medica* is considered to be the precursor of modern pharmacopoeias [Cowan, 1999].

Plants have not only been found useful in the ancient medical systems, they are still playing a crucial role in contemporary healthcare. In countries in Asia and Latin America, people use traditional medicine to help meet some of their primary healthcare needs [WHO, 2003a]. In China, traditional Chinese medicines (which are mostly derived from medicinal plants) are used within the framework of healthcare services, making up 45% of the medicamental market, while Western-style drugs make up around 55% [Xiao, 1994]. In Africa, up to 80% of the population relies on traditional medicines for primary healthcare [WHO, 2003a].

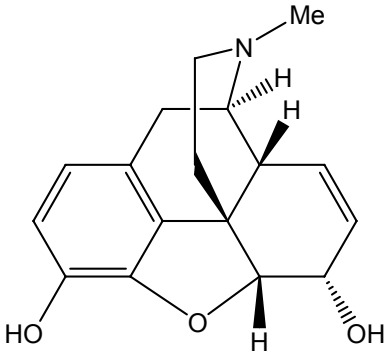
In the industrialised countries, the consumption of plant medicines (as the "complementary medicines") has increased dramatically in recent years. Between 1990 and 1997, the consumption of herbal medicines saw a three times increase in the United States [Morris and Avorn, 2003]. In 2000, about 50 per cent of the Australian population took "natural supplements" [Braun and Cohen, 2005]. In 2005, about 70% of the population in Canada had used "natural health products", and even more people considered natural products as an efficient means to maintain or support health [Nestmann *et al*., 2006].

1.3. Medicinal plants and drug discovery

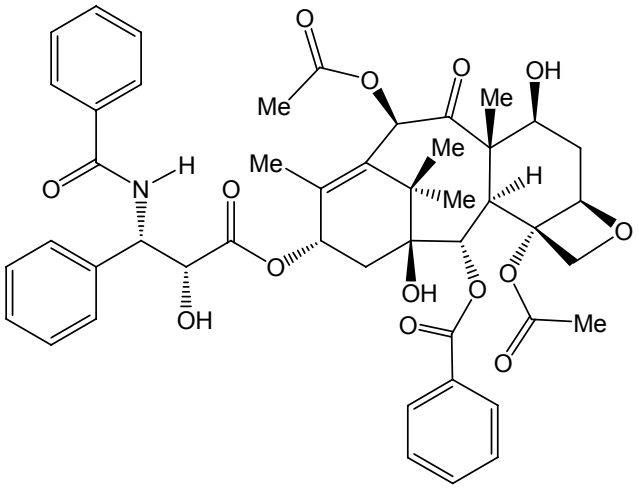
Plants contain a variety of secondary metabolites serving as hormones, attractants, repellents, poisons, and other functional agents of the plants, and a large number of them display pharmacological properties that can be and have been used by humans [Hostettmann and Terreaux, 2000]. The first pure secondary plant compound given in regulated doses to humans was morphine, isolated from the opium poppy in the early nineteenth century [Fellows and Scofield, 1995]. Ever since then, numerous compounds with significant molecular diversity have been discovered from plants as drugs or drug leads. Some more recent examples include the isolation of Vincristine and Vinblastine, two anti-cancer agents for the treatment of Hodgkin's lymphoma and leukaemia, from a Madagascar herbaceous plant, *Catharanthus roseus* [Lee, 2004]. Similarly, Taxol, a chemotherapeutic agent for the treatment of mestastatic ovarian cancer, was isolated from the Pacific yew tree, *Taxus brevifolia* [Suffness and Douros, 1982; Lesney, 2004]. These structurally complex compounds have also been used as leads for the semi-synthesis of further anti-cancer drugs [Newman *et al*., 2000].

Therapeutic effectiveness of medicinal plants in traditional medicinal systems have been verified over centuries, making medicinal plants a valuable source for the modern pharmaceutical industry to explore pharmacologically active principles or lead compounds. Two antimalarial drugs, Quinine and Artemisinin, for example, are pharmaceutically important compounds isolated respectively from the *Cinchona* trees and the plant *Artemisia annua* [Bez *et al*., 2003; Hoffmann *et al*., 2004]. *Cinchona* trees and *Artemisia annua* have been used for centuries in China and other countries to treat fevers and malaria [Klayman, 1985; Balick and Cox, 1996]. The discovery of these two antimalarial drugs was largely due to the traditional medicinal applications of these plants. In the section

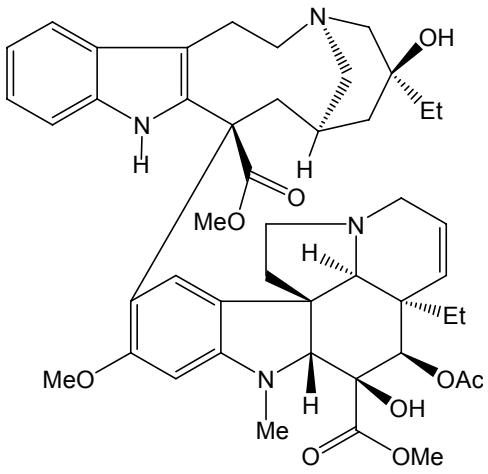
below, the contributions of medicinal plants and traditional medicinal knowledge to modern drug discovery are discussed.



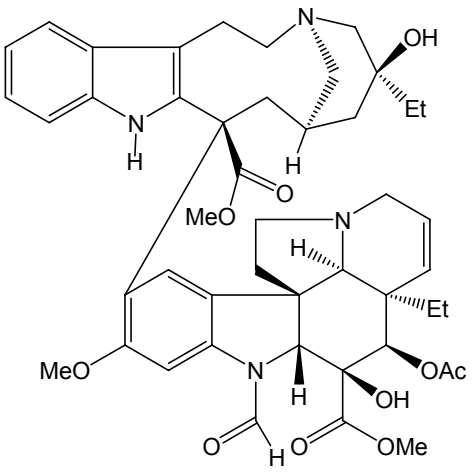
Morphine



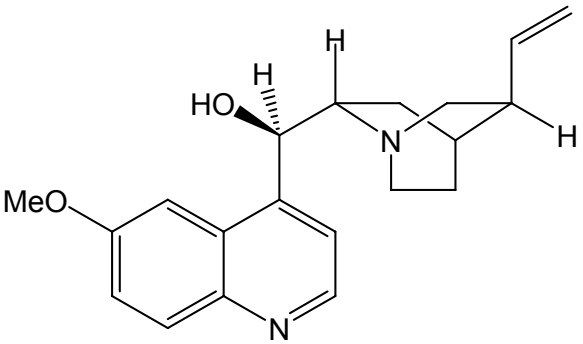
Taxol



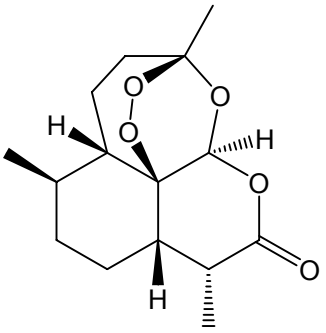
Vinblastine



Vincristine



Quinine



Artemisinin

1.3.1. Ethnobotany and ethnopharmacology

The term "ethnobotany" is one of the most frequently used words to describe the study of traditional knowledge of (medicinal) plants. It was first used by the American botanist John W. Harshberger in 1895 to describe studies of "plants used by primitive and Aboriginal people" [Balick and Cox, 1996]. In over one hundred years, ethnobotany has developed significantly to become a multidisciplinary field of natural science involving aspects of botany, anthropology, phytochemistry, pharmacology, medicine, history, geography and other related sciences and arts [Schultes and von Reis, 1995]. Several specialised terms, such as ethnomedicine and ethnopharmacology, have been created to describe branches of ethnobotany [Schultes and von Reis, 1995].

The term "ethnopharmacology" was first used by Efron *et al.* in 1967 in the book titled "Ethnopharmacologic Search for Psychoactive Drugs" [Heinrich, 2003]. The primary aims of ethnopharmacology are clearly expressed on the homepage of the Journal of Ethnopharmacology, an interdisciplinary journal devoted to the studies of Indigenous drugs, being the "documentation of Indigenous medical knowledge, scientific study of Indigenous medicines in order to contribute in the long-run to improved healthcare in the regions of study, as well as search for pharmacologically unique principles from existing Indigenous remedies". While ethnobotany studies the complex and broad relationships between people and plants, ethnopharmacology is mainly focused on the research of medicinal plants [Heinrich and Gibbons, 2001]. The two research fields, ethnopharmacology and ethnobotany, have contributed to and continue to play an important role in modern drug discovery from plant resources.

It is estimated that only about 6% of the world's approximately 250,000 higher plants have undergone a screening in terms of their pharmacological activities, and about 15% have been phytochemically analysed for their chemical constituents [Fabricant and Farnsworth, 2001]. In the search for pharmacologically active compounds from plants, a few methods of plant selection have been employed. These include the random, phytochemical, chemotaxonomic, ecological and ethnobotanical approaches. The random approach involves the collection of all plants found in a study area irrespective of any prior knowledge; the phytochemical approach requires the collection of all species of a plant family known to be rich in certain classes of compounds; the chemotaxonomic approach focuses on the secondary metabolites in a given family or genus; the ecological approach observes the interaction between the plant and its ecosystem; and finally, in the ethnobotanical approach, plant collection is guided by traditional medicinal information available [Iwu, 2002].

The ethnobotanical approach has demonstrated its advantage over other methods used [Calson, 2002]. Typical examples comparing the ethnobotanical approach with other plant selection methods, in the context of drug discovery, are given below.

1.3.2. Ethnobotanical approach in drug discovery

As a result of an extensive collaboration between the National Cancer Institute (NCI) of the United States and the Central Drug Research Institute (CDRI) in India, more than 39,000 plant species were collected in the 21-year period from 1960 to 1981 in the search for novel anti-cancer drugs. Most of the plants were collected randomly. The plants were screened *in vitro* and *in vivo* for their cytotoxic and anti-tumour activities. From over 110,000 screened plant extracts only 4,897 displayed activities, leading to a poor overall

success rate (4.3%) for the screening program [Suffness and Douros, 1982; Fabricant and Farnsworth, 2001]. Higher hit rates (about 30-50%) were achieved with the plants collected based on ethnobotanical knowledge, compared with that of the taxonomic approach (*e.g.*, about 18% from the Leguminosae family) and that of the random collection approach (about 4%) [Spjut and Perdue, 1976].

A drug discovery program coordinated by the Washington University in the United States used traditional medicinal knowledge to assist the plant selection. Out of nearly 4,000 plants collected between 1996 and 1999, 53.7% of the extracts tested using ethnobotanically-based functional assays showed activity, while only 3.1% were active in the screening where traditional use was not taken into account. The screening for antimalarial drugs resulted in the highest hit rate (91.8%) where 78 out of 85 plants that had been traditionally used as antimalarial remedies showed the expected activity [Lewis *et al*., 2004].

The examples of the NCI and Washington University programs illustrate the relative efficiency of the ethnobotanical *versus* other approaches in the context of drug discovery. In addition, useful and effective drugs derived from plants with a documented history of human use are likely to be safer than active compounds isolated from those with no records of human use [Fabricant and Farnsworth, 2001].

Xiao found that about 200 drugs approved in China have been developed from Chinese medicinal plants, many of which were discovered based on the traditional medicinal knowledge related to these plants [Xiao, 1994]. Similarly, Fabricant and Farnsworth found that more than 120 commercially available therapeutic agents were derived from

approximately 90 plant species, and more than 80% of these drugs are used for the same or related ailments as the original medicinal plants [Fabricant and Farnsworth, 2001].

In summary, the ethnobotanical approach has been shown to be successful in exploring bioactive constituents from plants. Therefore, this approach has been used in the present study for the investigation of bioactive compounds (especially those with antimicrobial and anti-inflammatory properties) from Australian Aboriginal medicinal plants.

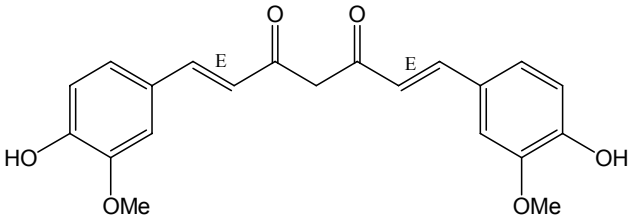
1.3.3. Antimicrobial and anti-inflammatory agents from medicinal plants

By studying medicinal plants used traditionally by humans, compounds with varied applications as therapeutic agents have been discovered and used in a way consistent with the ethnobotanical knowledge [Cox, 1994; Newman *et al*., 2000]. Some examples of clinically used anti-inflammatory and antimicrobial agents isolated based on ethnobotanical studies of traditional medicinal plants are shown below.

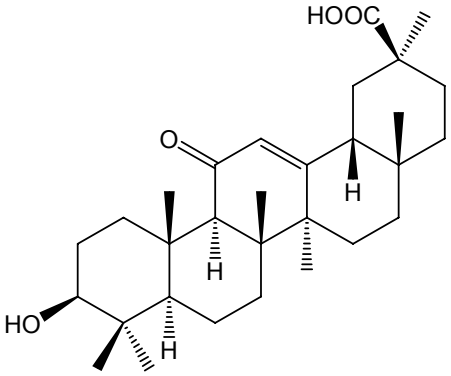
1.3.3.1. Anti-inflammatory agents

Curcumin (diferuloylmethane) is the main component of the rhizome of turmeric, *Curcuma longa* L. (Zingiberaceae), an extensively used spice, food preservative, colouring material and traditional medicine in India, China and South East Asia [Calixto *et al*., 2003]. *Curcuma longa* has been used in these Asian countries as a folk remedy for the treatment of biliary and hepatic disorders, anorexia, cough, diabetic wounds, rheumatism and sinusitis [Chattopadhyay *et al*., 2004]. Curcumin has been shown to possess a broad spectrum of biological and pharmacological actions, displaying anti-inflammatory, antioxidant, anticarcinogenic, antimutagenic, anticoagulant, antifertility, antidiabetic,

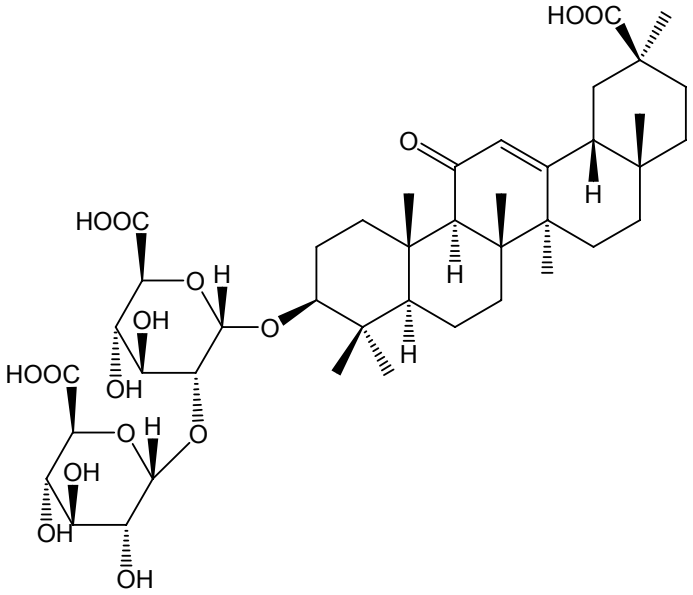
antibacterial, antifungal, antiviral, antifibrotic, antiulcer, hypotensive and hypocholesteremic activities, with little or no toxicity [Chattopadhyay *et al*., 2004]. Its anti-inflammatory property is believed to be mainly a result of its inhibitory effects on inflammation mediators such as cyclooxygenase-2 (COX-2), lipooxygenase (LO), and inducible nitric oxide synthase (iNOS) [Bengmark, 2006]. Its non-steroidal anti-inflammatory drug (NSAID)-like activity has been shown to be associated with its phenolic function [Atsumi *et al*., 2005]. Curcumin has been clinically used for the treatment of post-operative inflammation and rheumatism [Chattopadhyay *et al*., 2004].



Curcumin



Glycyrrhetic acid



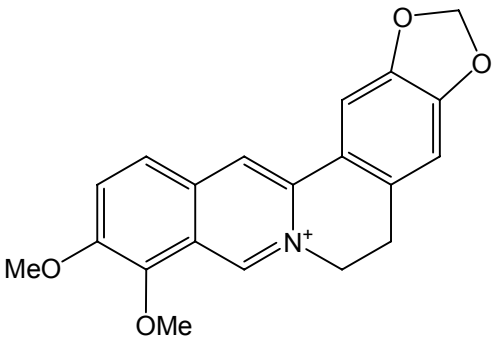
Glycyrrhizinic acid

Glycyrrhetic acid (glycyrrhetin, glycyrrhetinic acid), a pentacyclic triterpene, and its glycoside glycyrrhizin (glycyrrhizinic acid), are the major components isolated from the roots of the common licorice *Glycyrrhiza glabra* and the Chinese licorice *G. urralensis* (Fabaceae) [Wang and Nixon, 2001]. Licorice roots are among the most frequently used materials in Chinese medicine, and the earliest written records describing their uses as health improving agents and treatment for injury or swelling date from 2100 B.C. [Wang and Nixon, 2001]. Pharmaceutical properties of licorice roots include detoxification, antiulcer, anti-inflammation, antiviral, antiatherogenic and anticarcinogenic functions [Wang and Nixon, 2001]. Glycyrrhetic acid and glycyrrhizin have been shown to be inhibitors of lipoxygenase and cyclooxygenase *in vitro*, and possess potent anti-inflammatory effects *in vivo* [Koda *et al*., 1988; Inoue *et al*., 1988]. Glycyrrhetic acid and glycyrrhizin preparations as antiallergic, antihepatitis and peptic ulcer treatments have long been used clinically in Japan [Shibata, 2000]. Glycyrrhetic acid has also been shown to have antibacterial effects, with the minimum inhibitory concentrations (MICs) against *Staphylococcus aureus* and *Actinobacillus actinomycetemcomitans* being 64 and 8 μg/mL, respectively, and the minimum bactericidal concentrations (MBCs) being 64 and 16 μg/mL, respectively [Salari *et al*., 2001].

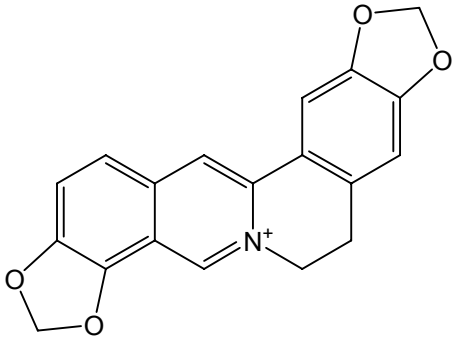
1.3.3.2. Antimicrobial agents

The rhizome of the Chinese Goldthread *Coptis chinensis* Franch (Ranunculaceae) is a traditional Chinese medicine extensively used in the treatment of disorders of bacterial aetiology, including bacillary dysentery, acute enteritis, typhoid, pertussis, tuberculosis, diphtheria, bronchitis and pneumonia [Zhu, 1998]. Berberine, the major alkaloid isolated from the rhizome of *Coptis chinensis*, has been manufactured as chloride or sulphate

tablets and tinctures, and is available over-the-counter in China and other Asian countries [Zhu, 1998]. Berberine has antimicrobial activity against a wide spectrum of microbes, including the Gram positive bacteria *Staphylococcus aureus*, *Bacillus subtilis, Streptococcus mutans* and *Zoogloea ramigera*, the Gram negative bacteria *Pseudomonas aeruginosa*, *Escherichia coli* and *Fusobacterium nucleatum*, and the yeasts *Candida albicans* and *Saccharomyces cerevisiae* [Cernakova and Kostalova, 2002; Hwang *et al*., 2003; Pepeljnjak and Petricic, 1992]. Berberine has also been found to be active against drug-resistant bacteria, with an MIC of 125 μg/mL against methicillin-resistant *S. aureus* (MRSA) and *S. epidermidis* (MRSE) [Ohtani *et al*., 1996]. The antibacterial activity of berberine is believed to be due to its inhibition of the carbohydrate and protein metabolism of bacteria, and its ability to form complexes with bacterial DNA. The quaternary ammonium group present in berberine's structure has been proven to be essential for its antibacterial activity [Zhu, 1998]. Another alkaloid isolated from *C. chinensis* with the quaternary ammonium group, coptisine, is also a potent antibacterial agent, but only exists in small amounts in this plant [Zhu, 1998].



Berberine



Coptisine

1.4. Ethnobotanical and ethnopharmacological research in Australia

With a great variety in its topography and climate, Australia has evolved a biodiversity with unique flora very different from that of the other parts of the world. Australian Aboriginal people, over thousands of years, have maintained their health by using plants as medicines [Isaacs, 1987] and as a result of extensive trials, errors and observations on human patients, Australian Aboriginal people have accumulated a vast empirical knowledge regarding the medicinal value of individual plant species [Isaacs, 1987]. This knowledge has been passed on through generations, mainly with oral transmission [Isaacs, 1987]. Like all other bodies of information built on oral transmission, this accumulated knowledge is being lost and is more vulnerable to cultural changes than the printed word [Sheldon and Balick, 1995]. In recent years, the need for the conservation of Indigenous traditional knowledge has become more urgent and serious, since the loss of Indigenous knowledge systems and plant biodiversity are accelerating throughout the world [Cordell, 2002; Balick and Cox, 1996]. It is essential that this knowledge be conserved because of its historical and cultural value as well as its medicinal significance [Cordell, 2002; Heinrich, 2003].

In New South Wales, due to a greater westernisation of Aboriginal communities, the interest of younger generations in their traditional medicinal knowledge is declining. Furthermore, many elders have died without passing on detailed information, and this traditional knowledge has been lost irretrievably. This has resulted in Aboriginal community members asking researchers to document ethnobiological information on their behalf (Yaegl, Kamilaroi and Murawari communities, personal communications to Dr James Kohen).

A number of ethnobotanical studies have been undertaken to allow documentation and conservation of Australian Aboriginal medicinal plant knowledge. Most of the work has been based on Aboriginal communities in the Northern Territory and Central Australia [*e.g.*, Barr *et al*., 1988; Cribb and Cribb, 1981; Isaacs, 2000; Lassak and McCarthy, 1983]. A database of plants used as bush foods and medicines by New South Wales Aboriginal communities is also available, with much of the information obtained from published sources [Gott, 1996]. In a limited number of the above studies, data on chemical components and pharmacological assay work are also included [*e.g.*, Barr *et al*., 1988]. Some of these projects have been undertaken as collaborative partnerships between researchers and Aboriginal communities. For example, elders of the Mimili community in Central Australia have documented the preparation of their traditional food and medicines, and this information had been published at their request by the Institute for Aboriginal Development [Goddard and Kalotas 2002].

The medicinal value of Australian Aboriginal traditional knowledge is highlighted by the recent finding of novel analgesic compounds from a Kimberley traditional medicinal plant, *Barringtonia acutangula*, following an ethnopharmacological study conducted jointly by researchers and Aboriginal members [Mills *et al*., 2005; Quinn and Mills, 2005]. Several other studies have been conducted on the screening of pharmacological activities of Australian medicinal plants [*e.g.*, Pennacchio *et al*., 2005; Li *et al*., 2003; Sweeney *et al*., 2001; Palombo and Semple, 2001; Semple *et al*., 1998] and some follow-up studies have resulted in the isolation of bioactive constituents [*e.g.*, Li *et al*., 2004; Palombo and Semple, 2002; Semple *et al*., 1999]. These research projects, however, used published ethnobotanical information to guide the selection of medicinal plants.

The specific aims of this PhD study were to 1) undertake an ethnobotanical study on plants used by members of the Kamilaroi and Muruwari Aboriginal communities of Northern New South Wales, with their involvement, 2) explore best ethical practice protocols and benefit sharing strategies, 3) develop and validate biological assays for the evaluation of the biological properties of Aboriginal plants, as guided by the ethnobotanical study, and 4) isolate and structurally elucidate bioactive compounds from selected Australian Aboriginal medicinal plants using bioassay-guided fractionation.

This PhD study was one of only a few projects in Australia that covered the documentation of first-hand medicinal plant knowledge of Aboriginal communities in northern New South Wales through to the isolation of bioactive compounds from Aboriginal medicinal plants with the full cooperation of Indigenous people.

Chapter 2. An Ethnobotanical Study with the Kamilaroi and Muruwari Aboriginal Communities and Relationship Building

This chapter describes 1) an ethnobotanical study on the traditional and contemporary medicinal plant knowledge of the Kamilaroi and Muruwari Aboriginal communities and 2) approaches towards ensuring best ethical practices and benefit sharing with Aboriginal communities of Yaegl (Maclean) and Bundjalung country.

2.1. Introduction

In this study, the first-hand customary medicinal plant knowledge of the Kamilaroi and Muruwari Aboriginal communities in northern New South Wales, along with the contemporary medicinal plant information in the literature, was documented with the cooperation of Aboriginal people in these communities. The participation of Aboriginal communities was an essential component of this research project. This is ethically important and it promotes two-way exchange of knowledge and skills on native medicinal plant resources and the scientific investigations. Documentation of the Indigenous traditional medicinal plant knowledge assists the preservation of this knowledge and provides information that can be used for Indigenous peoples' own cultural purposes, particularly in education. First-hand traditional medicinal plant knowledge from Aboriginal communities can also be used as a guide for ethnopharmacological studies and facilitate the discovery of new medicinal agents. It is therefore also valuable for the wider scientific community.

As part of a complementary study with Aboriginal communities of Yaegl (Maclean) and Bundjalung country, also in northern New South Wales, workshops were conducted to discuss best ethical practices when undertaking research with Indigenous participants and benefit sharing opportunities were investigated.

As a collaborative research action, both of the above strands of this project required the teamwork of university researchers from different disciplines and the cooperation between researchers and Aboriginal communities in northern New South Wales. Macquarie University's Indigenous Bioresources Research Group (IBRG) was established at the initial stages of this project, bringing together researchers in the Department of Biological Sciences, Chemistry and Biomolecular Sciences, and Health and Chiropractic. The IBRG also worked closely with researchers in Environmental Law and Indigenous Studies. The integration of researchers with multidisciplinary expertise enabled the IBRG to conduct ethnobotanical studies with Aboriginal communities to document and preserve traditional medicinal knowledge and cultural resources, to carry out chemical and biological investigations of Aboriginal medicinal plants, and to develop best practices for collaborative research with Aboriginal communities.

The ethnobotanical study on medicinal plant knowledge of the Kamilaroi and Muruwari Aboriginal communities is described in Section 2.2 and the best practice and benefit sharing work is detailed in Section 2.3.

2.2. Ethnobotanical study of medicinal plants of Kamilaroi and Muruwari Aboriginal communities

In dealing with Aboriginal customary knowledge and Aboriginal people's intellectual properties, all research was conducted in an ethically sound manner. Before any research efforts took place, appropriate human ethics approval (approval number HE27FEB2004-R02750) was obtained from the Macquarie University Ethics Review Committee, to allow the research activities with the Aboriginal communities to be undertaken.

The ethics approval for research with the Kamilaroi and Muruwari Aboriginal communities closely followed the published guidelines of the Participatory Action Research methods and the United Nations declaration on the rights of Indigenous peoples [United Nations, 1994; Tuxill and Nabhan, 2001] and included the following steps:

1. meet with community members with appropriate authority to discuss the project;
2. obtain community authorisation to proceed with the project;
3. meet with community members interested in participating in the project to design research activities;
4. undertake ethnobotanical research jointly (interviews, databases, field work);
5. seek community advice on the best way of storing and transmitting information to the greater community; and
6. discuss with communities expansion of the project (*e.g.* chemical and biological investigations) and in-kind and financial support.

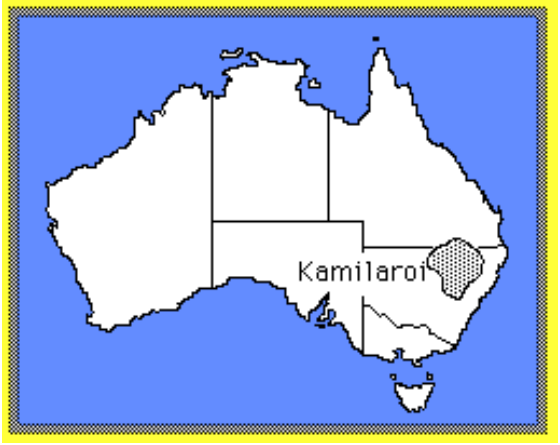
The ethics approval also states that ownership of the Indigenous knowledge will be retained by the Aboriginal people involved and publication of any data requires the consent of the local Aboriginal representative body and shall have community co-authorship/acknowledgment.

The ethnobotanical study was carried out in close association with Kamilaroi and Muruwari Aboriginal people. Initial contact with the Kamilaroi Aboriginal community was made through Mr John Hunter, a Kamilaroi man and also a Masters student in the Department of Indigenous Studies at Macquarie University. In his work devoted to the preservation of cultural heritage of his own community, Mr Hunter had recorded many plants that are currently used by his community members. However, information on food or medicinal uses of these plants has not been included. Further studies of the medicinal uses and preparation methods of these plants were regarded as useful for the documentation of the customary knowledge of the Kamilaroi community. Excellent personal relationships with two elders in the Muruwari Aboriginal community, Mr and Mrs Roy and June Barker, had long been built and maintained by one of the supervisors of this PhD project, Dr James Kohen. Well-informed in bush food and medicines, Mr and Mrs Barker were keen to have their knowledge documented and extended to the whole community. This section of the thesis describes the ethnobotanical studies that have been carried out with the Kamilaroi and Muruwari Aboriginal communities.

The Kamilaroi country (alternative spellings are Gamilaraay, Kamilarai, Gamilaroi, Kamu-laroi) covers a large area of land about 75,400 Km^2 at 140°35'E x 30°15'S in northern New South Wales. The land of the Kamilaroi country extends from Walgett, New South Wales to Nindigully, Queensland, covering Moree, Mungindi, Mogil Mogil, Narrabri, Pilliga, Gunnedah, Bingara, Tamworth, Quirindi, Bundella, Barraba, Gwabegar, and near Talwood and Garah [South Australian Museum, 2000]. The Muruwari (Morowari, Murawari, Murra-Warri) community's land borders in the southwest with the Kamilaroi country. It is an area about 16,400 Km^2 at 146°25'E x 29°0'S in New South Wales, covering Barringun,

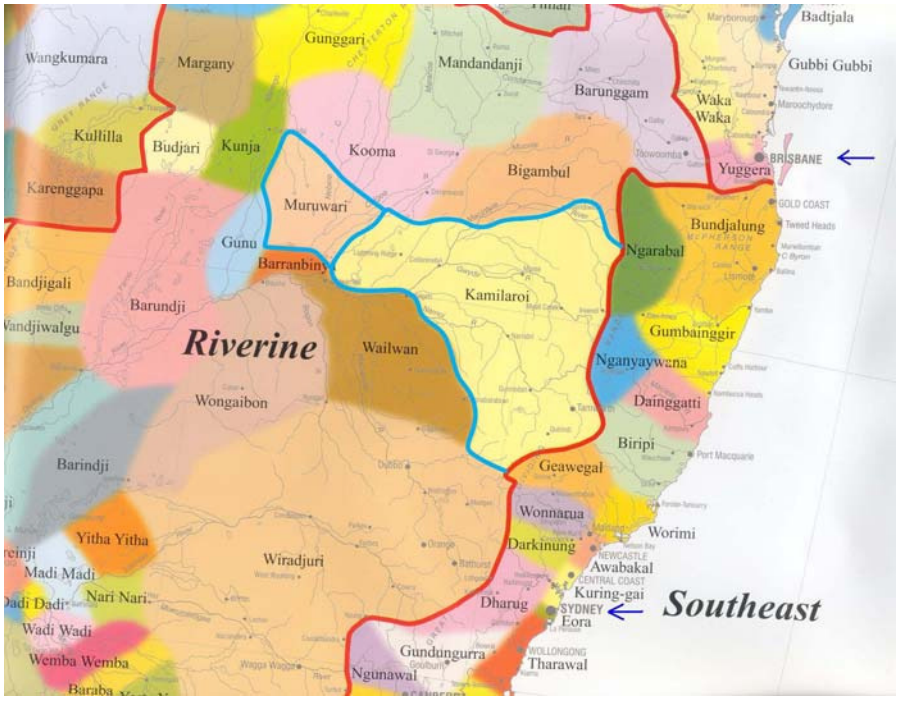
Enngonia, Brenda, Weilmoringle, Milroy, and south to near Collerina, and extending north to Mulga Downs and Weela (Wee-lamurra) in Queensland [South Australian Museum, 2000]. The areas of these two communities are shown in the maps in Figure 2.1 and 2.2.

Figure 2.1. Kamilaroi country [Austin and Nathan, 1998].



* Photo source: Austin, P. and Nathan, D., Kamilaroi/Gamilaraay Web Dictionary. 1998.

Figure 2.2. Kamilaroi and Muruwari Aboriginal communities in northern New South Wales [AIATSIS, 2000].



* Map reprinted from the *Aboriginal Australia map* (3rd Edition). Australian Institute of Aboriginal and Torres Strait Islander Studies (AIATSIS), 2000.

2.2.1. Literature study of plants of the Kamilaroi Aboriginal community

The Kamilaroi man John Hunter had studied the vegetation types in the Kamilaroi country and had compiled a list of 66 plants (with scientific, common and Aboriginal names) that are in use today by his community people [Hunter, 2005]. Although no specific ethnobotanical information of these plants has been included, this list was useful as an original record of plants of Kamilaroi country and was used as the basis for this ethnobotanical study with the Kamilaroi community. A literature study on all plants in this list was carried out, among which 31 plants were reported to have been used as food or medicines. The botanical information, preparation methods, medicinal uses, chemical and biological investigation results of these plants were summarised and put into a Microsoft Access Database. An ethnobotanist, Mr David Harrington (also an IBRG member) supplemented this database by providing valuable first-hand medicinal information obtained through interviews with Aboriginal community members in New South Wales on the *Eremophila* species [Personal communications]. This database became a basic component of the ethnobotanical study with the Kamilaroi community.

2.2.2. Interviews with elders of the Muruwari Aboriginal community

A field trip to Lightning Ridge in December 2005 (together with ethnobotanist Mr Matthew Flower) allowed a face-to-face interview with two elders of Muruwari Aboriginal community, Mr and Mrs Roy and June Barker, from whom the first-hand ethnobotanical information of plants used in the Muruwari community has been obtained, and the medicinal uses of some plants in the Kamilaroi country have been confirmed.

During the interview, Mr and Mrs Barker introduced the native plants around their house, many of which were bush foods. They noted that about 40-50 types of bush foods were commonly taken by them and by members of their community. They also stated that there was no need for them to use medicines, because these bush foods helped them maintain good health. For example, they use the Ruby Saltbush (*Enchylaena tomentosa* R. Br.) as a source of salt to cook emu meat. This saltbush "adds vitamins" to their diet and enhances their health.

Mr Barker pointed out that the method of preparation of bush foods was also very important. For example, the Nardoo (*Marsilea hirsuta* R. Br.), which grows in water, has sporocarps that are generally collected and ground by Aboriginal people to get vitamins. However, the skin of the sporocarps must be taken off as it contains poisons. The edible pink fruit of "Gubigala" (*Apophyllum anomalum* F. Muell.) must be roasted with ash to get rid of the toxin.

Some of the plants were specially mentioned for being important medicines to their community. This included the Quinine Bush (*Alstonia constricta* F. Muell.), named after the anti-malarial quinine due to its bitter taste, which was used for various types of ailments. This plant was appreciated by them and their community members for its healing effects for serious health problems such as diabetes, lung cancer and kidney diseases.

Mr and Mrs Barker also addressed the issue that the knowledge on many of the bush medicines has been lost during the past hundred or so years. This was partly because large areas of bush had been burnt off and Aboriginal people have had to move from place to place, the passing away of community elders who possess most of the medicinal plant knowledge, as well as the adoption of Western life styles and medications. Therefore,

documentation of the knowledge of medicinal plants that were available to their community was essential.

Figure 2.3. Ruby Saltbush: *Enchylaena tomentose* R. Br.

Figure 2.4. Nardoo: *Marsilea hirsuta* R. Br.

Some plant specimens were collected during this interview and were identified by Ms Alison Downing at the Department of Biological Sciences. Voucher specimens of the collected plant samples were deposited in the Herbarium of Macquarie University. These plants included the Wild orange *Capparis mitchelli* Lindl. voucher number 73007961), Nardoo (*Marsilea hirsuta* R. Br. voucher number 73007962), Turpentine bush (*Eremophila sturtii* R. Br. voucher number 73007710), Leopardwood (*Flindersia maculosa* (Lindl.) Benth. (voucher number 73007959) and the Ruby Saltbush (*Enchylaena tomentose* R. Br. voucher number 73007960). The collection was carried out at 140°58'53"E x 29°25'49"S, and all plant specimens were collected within 50 metres of this site.

2.2.3. Ethnobotanical database for the Kamilaroi and Muruwari Aboriginal communities

The information recorded during this interview was put into the database together with the selected plants of the Kamilaroi community, with additional information obtained through literature searches. Some Aboriginal medicinal preparations with materials other than plants, such as river clays and wax from native sugar bees for treatment of skin infections, were mentioned during the interview and were also put into the ethnobotanical database as part of the records. Some of the plants recorded during the interview were not able to be identified according to the common names, and these plants were not entered into the database.

The ethnobotanical database has 37 entries, with 35 being plants that are used as food and/or medicines by Aboriginal people in Kamilaroi and Muruwari communities. Each entry contains information of a plant, including scientific (botanical) name, common name(s), Aboriginal name(s), plant family, description, distribution, applications and

preparation methods in the literature and in these communities, along with phytochemical and biological information available in the literature.

The overall ethnobotanical database is attached as Appendix 1 of this thesis. As a summary, Table 2.1 lists all plants documented in the database with first-hand medicinal information from Kamilaroi and Muruwari communities (in bold font) and from published sources.

A hard copy and an electronic version of the complete ethnobotanical database were given back to Mr Hunter and Mr and Mrs Barker as a record of the ethnobotanical knowledge and scientific research of the plants used by their communities, for them to keep and for the benefit of the whole communities. This database represents one of the contributions of this PhD project to the conservation of valuable ethnobotanical knowledge of the Kamilaroi and Muruwari Aboriginal communities, serving as part of the benefit sharing with these two Aboriginal communities involved in this research.

Table 2.1. Summary of plants documented in the ethnobotanical database with medicinal information from Kamilaroi and Muruwari communities and literature.

Scientific name	Common name	Use	Medicinal uses*	References
Acacia aneura	Mulga, Malga	Food, Medicine	Pain, colds	Barr *et al*., 1993; Isaacs, 1987; Latz, 1995
Acacia cambagei	Gidgee	Medicine	**Wounds, cuts**	NSW Flora Online
Acacia ligulata	Sandhill wattle	Food, Medicine	Cough, dizziness	Isaacs, 1987; Latz, 1995
Acacia salicina	Cooba	Medicine	Cough, sleep	Isaacs, 1987
Acacia stenophylla	River cooba	Food		Isaacs, 1987
Acacia victoriae	Ginderbluey wattle, Bramble wattle	Food		Isaacs, 1987; Latz, 1995
Alstonia constricta	Quinine Bush	Medicine	**"Diabetes", "lung cancer", "kidney diseases".** Malaria, sores, typhoid and dysentery	Isaacs, 1987; Low, 1990
Apophyllum anomalum	Currant Bush	Food		NSW Flora Online
Boerhavia diffusa	Tar vine	Food, Medicine	Expectorant in asthma, diuretic and emetic	Isaacs, 1987
Brachychiton populneum	Kurrajong	Food		Isaacs, 1987
Capparis mitchelli	Wild orange	Food, medicine	"**Women's illnesses"**	Hunter, 2005; Isaacs, 1987; Latz, 1995
Cucumis melo	Ulcardo Melon	Food		Isaacs, 1987; Latz, 1995
Dodonaea attenuata	Narrowleaf hopbush	Medicine	Fever	Latz, 1995
Enchylaena tomentosa	Ruby Saltbush	Food		Australian National Botanic Gardens (online)
Eremophila bignoniiflora	Dogwood	Medicine	Scabies, laxatives, colds, flu, headache, venereal disease	Barr *et al*., 1993; Low, 1990

Scientific name	Common name	Use	Medicinal uses*	References
Eremophila gilesii	Ellangowan poison bush, desert fuschia	Medicine	Scabies	Isaacs, 1987; Latz, 1995
Eremophila longifolia	Berrigan, Emu bush	Medicine	Sores, colds, sore eyes, headaches, insomnia, scabies, boils, general sickness, counter-irritant, antiseptic	Barr *et al*., 1993; Isaacs, 1987; Barr *et al*., 1988; Low, 1990; Latz, 1995
Eremophila mitchellii	Budda	Medicine	Rheumatism	Low, 1990
Eremophila sturtii	Turpentine bush	Medicine	**Skin infections. "Disinfectant".** Cuts, sores, colds, flu, backaches. Fly repellent.	Barr *et al*., 1993; Latz, 1995; Smith, 1991; Low, 1990; Latz, 1995
Eucalyptus camaldulensis	River gum	Medicine	Diarrhoea, colds, fevers, sores, swollen and aching joints, headache. Antimicrobial, antifungal, cough suppression. Antiseptic, decongestant, expectorant, counter-irritant	Barr *et al*., 1993; Isaacs, 1987; Barr *et al*., 1988; Low, 1990
Eucalyptus populnea	Gum tree, Bimble Box	Food		Issacs, 1987
Eucalyptus tessellaris	Carbeen	Medicine	Dysentery	
Euphorbia drummondii	Caustic weed	Medicine	Chronic diarrhoea, dysentery, low fever, rheumatism, skin itches, sores and scabies, chest pains, snakebite, sore eyes	Barr *et al*., 1993; Isaacs, 1987; Barr *et al*., 1988; Low, 1990; Latz, 1995
Exocarpos aphyllus	Stiff cherry	Medicine	Colds, sores	Isaacs, 1987
Flindersia maculosa	Leopardwood	Medicine	**"Scabies".** Diarrhoea, antimicrobial, antiprotozoal	NSW Flora Online
Grevillea striata	Beefwood	Medicine	Wounds, burns	Barr *et al*., 1993
Leichhardtia australis	Wild banana	Food, Medicine	Contraceptive	Isaacs, 1987; Low, 1990

Scientific name	Common name	Use	Medicinal uses*	References
Marsilea hirsuta	Nardoo	Food		Isaacs, 1987; NSW Flora Online
Melaleuca uncinata	Broom bush	Medicine	Catarrh, astringent	Low, 1990
Native sugar bees wax		Medicine	**Skin infections**	
Owenia acidula	Colane	Medicine	Sore eyes, malaria	Low, 1990; Latz, 1995
Pittosporum phillyraeoides	Butter bush Mallee Willow	Medicine	Pain, cramps, eczema, pruritus, colds, bruises, muscle ache and lactagogue	Barr *et al*., 1993; Isaacs, 1987; Latz, 1995
Portulaca oleracea	Common pigweed	Food, Medicine	Diuretic, antiscorbutic, blood cleanser. Prevent scurvy.	Isaacs, 1987; Low, 1990; Latz, 1995
River clays		Medicine	**Skin infections**	
Santalum acuminatum	Quandong	Food, Medicine	Boils, sores, gonorrhoea. Pain relief.	Barr *et al*., 1993; Isaacs, 1987; Low, 1990; Latz, 1995
Santalum lanceolatum	Sandalwood plum	Food, Medicine	Rheumatism, purgative, boils, sores, gonorrhoea, itching, respiratory infections, diabetes, genitourinary infections. Decongestant	Barr *et al*., 1993; Isaacs, 1987; Barr *et al*., 1988; Low, 1990
Ventilago viminalis	Supplejack	Medicine	Toothache, rheumatism and swellings, cuts and sores, hair restorative	Isaacs, 1987; Latz, 1995

* Medicinal uses in bold font are first-hand information from Kamilaroi and Muruwari Aboriginal communities.

2.2.4. Plants selected for biological and chemical investigations

As seen from the ethnobotanical database, some medicinal plants recorded (*e.g.*, *Eremophila sturtii*, *Exocarpos aphyllus*, *Acacia cambagei*, *Acacia ligulata* and *Flindersia maculosa*) have not been extensively studied for their biological activities and chemical

constituents. Owing to their ethnomedicinal uses, these plants were considered worthy of future investigations.

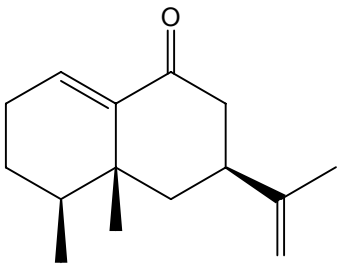
Among all plants listed in the ethnobotanical database, several species of *Eremophila* (Myoporaceae Family) have been recorded in Hunter's list and were also mentioned by the Muruwari elders during the interview. These plants included the Emu bush *Eremophila longifolia*, the Dogwood *E. bignonifolia* and the Turpentine bush *E. sturtii*, all of which are used as medicines by these two Aboriginal communities. Targeted literature research on the *Eremophila* genus revealed that many of its species have been used for medicinal purposes by Australian Aboriginal people to treat skin conditions and other ailments such as wounds, headaches, colds, fever, and general pain and illness [Low, 1990; Barr *et al*., 1988; Latz, 1995; Cribb and Cribb, 1981; Isaacs, 1987]. Details of the ethnobotanical information, biological activities and bioactive constituents of some important *Eremophila* species are reviewed in Section 4.2 of this thesis. Among the above *Eremophila* species, limited information on *E. sturtii* was found in the literature in terms of its chemical constituents and pharmacological activities. However, its ethnomedical applications found in the literature and noted from the interview with Muruwari elders revealed its value for further studies. The ethnomedicinal information of this plant has been used as a guide for the biological and chemical investigations (described in Chapter 4 of this thesis).

2.2.4.1. Ethnobotanical research on **Eremophila sturtii**

Eremophila sturtii R. Br. belongs to the family Myoporaceae. It is known by common names as turpentine bush, kerosene bush, or turpentine emu bush. *Eremophila sturtii* is a sticky shrub typically about 2 m high with narrow and cylindrical leaves and small, hairy,

white, pink or mauve flowers [Latz, 1995; Cribb and Cribb, 1981]. It is endemic to Australia and is distributed in New South Wales, Queensland, Victoria, South Australia and Northern Territory [Cribb and Cribb, 1981].

Eremophila sturtii is reputed to have fly repellent properties and the branches were often used by early European settlers to thatch meat-houses [Latz, 1995; Low, 1990]. Through interviews with the Muruwari elders, it was recorded that this plant is still used to keep off flies and mosquitoes by rubbing the crushed leaves on exposed skin. The fly repellent properties are probably due to the presence of eremophilone, a terpernoid found in *Eremophila* oil, and its derivatives, which have been shown to be insecticidal [Leach *et al*., 2004].



Eremophilone

Water decoctions of the leaves of *E. sturtii* have been taken internally or used as a hot water bath for the treatment of bad colds, diarrhoea, cough, sore eyes, respiratory infection and general sicknesses [Barr *et al*., 1988 and 1993; Palombo and Semple, 2001; Goddard and Kalotas, 2002; Smith, 1991]. The fumes produced from smouldering branches have been used to relieve backaches [Latz, 1995]. An ethanol extract of the stems and leaves of *E. sturtii* has been examined against the Gram positive bacterium *Bacillus cereus* (which is related to food poisoning) at 1 mg/ml in a plate-hole diffusion assay, and a reduced but not complete abolition of growth of the bacterium has been reported [Palombo and Semple, 2001]. The bioactive compounds responsible for this activity, however, have not been

described. The leaves of *E. sturtii* have been reported to contain 3% of tannic acid [Barr *et al*., 1993].

It has been documented that decoctions of *E. sturtii* leaves have been used by the Australian Aboriginal people of the Northern Territory to wash sores and cuts [Smith, 1991; Barr *et al*., 1993]. Our interview with Murawari elders recorded a similar use of water decoctions of crushed leaves of *E. sturtii* "as a disinfectant" to treat skin infections. Since skin infections are generally caused by microbial invasions and often involve inflammatory responses (refer to Chapter 3 for more details), these medicinal uses by the Northern Territory and New South Wales Aboriginal communities suggested that *E. sturtii* possesses antimicrobial and/or anti-inflammatory properties. Characterisation of constituents with antimicrobial and/or anti-inflammatory activities from *E. sturtii* through bioassay-guided fractionation is described in Chapter 4 of this thesis.

Figure 2.5. *Eremophila sturtii* at Lightning Ridge, New South Wales from where the plant specimens were collected.

A plant specimen was collected by David Harrington in Lightning Ridge, New South Wales (140°58'53"E x 29°25'49"S) in March 2004. The specimen was identified by Ms Alison Downing at the Department of Biological Sciences, Macquarie University (Voucher Number 73007710), and was used for the chemical and biological investigations. A second collection from the same site was carried out in December 2005 during the interview with the Muruwari elders (Figure 2.5), and the plant sample was compared by Ms Alison Downing and found to be identical with the voucher specimen. This sample was used for the preparation of the customary water decoction and bioactivity examinations.

2.2.4.2. Ethnobotanical research on **Exocarpos aphyllus**

Exocarpos aphyllus was chosen as another plant for targeted chemical and biological studies because of its traditional medicinal uses. This plant, known by its common names leafless cherry, stiff cherry or leafless ballart, has been used by the Australian Aboriginal people to treat sores and colds by taking the decoctions of mashed stems internally. It has also been used externally. A poultice made by mashed stems of *E. aphyllus* has been used for "wasting disease" (tuberculosis) by placing it on the chest [Lassak and McCarthy, 1983; Cribb and Cribb, 1981; Isaacs, 1987]. Preliminary investigations on the antibacterial activity of *E. aphyllus* had been carried out by two former research students of the group, but the bioactive constituents had not been fully explored [Smith, 1999; Wanandy, 2005].

Exocarpos aphyllus R. Br. belongs to the plant family Santalaceae. *E. aphyllus* is an erect shrub up to 5 m tall, with finely furrowed/striate branches. The leaves are reduced to minute ovate caducous scales, less than 1 mm long, flattened against the branches. The plant flowers throughout the year and the flowers are sessile, with yellowish-green perianth

segments. The fruit is a very small nut resting on the succulent enlarged flower stalk. Other names for this plant include *Exocarpos leptomerioides* F. Muell. Ex Miq. and *Xylophyllos aphyllus* (R. Br.) Kuntze. This plant is distributed mainly in the inland areas of Queensland and New South Wales [Lassak and McCarthy, 1983; Cribb and Cribb, 1981; Isaacs, 1987; Stanley and Ross, 1983; Jessop and Toelken, 1986].

A plant specimen of *E. aphyllus* was collected by David Harrington from Lightning Ridge, New South Wales (140°58'53"E x 29°25'49"S) in March 2004 (Figure 2.6). The specimen was identified by Ms Alison Downing at the Department of Biological Sciences, Macquarie University (Voucher Number 73007709). This plant sample was used for the chemical and biological investigations of *Exocarpos aphyllus*, which are described in detail in Chapter 5 of this thesis.

Figure 2.6. Collected specimen of *Exocarpos aphyllus*.

2.3. Approaches towards ensuring best ethical practices and benefit sharing

In the relationship development with Aboriginal communities in northern New South Wales, a series of social activities, including visits to Aboriginal communities, presentations in Indigenous workshops, contribution to educational activities for community youth, and interviews with community elders, were accomplished as part of research activities in this PhD project. Personal participation in these activities was essential in getting experience in best ethical practice protocols, including being responsible for communities' needs. Some activities undertaken during this project are summarised below.

2.3.1. Relationship Building

A visit to the Yaegl Aboriginal community in northern New South Wales took place in June 2004, aimed at discussing details of the cooperative activities procedures and project research methods for subsequent ethnobotanical studies. A poster describing the proposed biological assays and chemical investigation methods was presented to the representatives of the community and the Local Aboriginal Land Council.

On 16-17th October 2004, a workshop involving all members of the IBRG and representatives from 13 Aboriginal communities in far north New South Wales was held at the Yarrawarra Culture Centre at Corindi Beach, to determine mutually beneficial ways of working together to combine traditional knowledge of medicinal plants with biological and chemical investigations, as well as developing best practices for future research partnerships.

Intellectual property rights of Aboriginal communities, the establishment of cooperative working relationships, scientific approaches to bush medicine research, development of mutual trust, as well as the establishment of in-kind support strategies aimed at providing recompense for the transmission of customary information, were discussed in the workshop.

A poster describing the research methods and participatory action research protocol was compiled for this workshop and presented to all participating Aboriginal communities. Some experiments using household chemicals were organised for these community members, which allowed them to gain hands-on experience in chemistry. A bush walk with community members gave an opportunity to become familiar with the Indigenous life style and a few medicinal plants.

This workshop led to both the establishment of a cooperative relationship and a commitment by the IBRG to collaborate with the local communities in providing educational activities to local Indigenous youth as a contribution to community needs and as part of benefit sharing.

The full story of this workshop was published by the Macquarie University Public Relations and Marketing Unit in Macquarie University News in November 2004 http://www.pr.mq.edu.au/macnews/showitem.asp?ItemID=343 and is reproduced in Figure 2.7.

Figure 2.7. Media coverage of the Yarrawarra workshop [PRMMU, 2004].

Macquarie University and Bundjalung people form bush medicine research partnership

From left: Macquarie University ethnobotanist David Harrington, Deidre Randall and Dale Mercy – both from the Yaegl (Maclean) community.

Representatives of the Maclean, Yamba, Casino, Grafton and Coffs Harbour communities attended the workshop to hear IBRG and Centre for Environmental Law members discuss issues including intellectual property rights and scientific approaches to bush medicine, as well as participate in the Macquarie University hands-on Chemistry Show.

Over the next few months the IBRG and these communities will continue to discuss mutually beneficial ways of working together. These will include developing best practice for future research partnerships, and importantly, addressing the educational needs of Indigenous youth in the region, which the representatives identified as being of real value to their communities.

"The workshop was a stepping stone in developing a pathway for joint partnerships in research between the Aboriginal communities and academic institutions," says Indigenous IBRG member John Hunter. "The proposed joint partnerships model has the potential to become a precedent which may address key Aboriginal issues from the ground up. Academic institutions such as Macquarie University can play a direct and influential role in practical reconciliation and community development to create positive outcomes for the next generation."

The IBRG is a multidisciplinary group which comprises Macquarie staff from the Departments of Chemistry, Biological Sciences and Health and Chiropractic.

The workshop was jointly organised with the Yaegl (Maclean) community and was supported by the Australian Institute for Aboriginal and Torres Strait Islander Studies (AIATSIS) and Macquarie University through a Vice-Chancellor's grant and funding from the Division of Environmental and Life Sciences Outreach Committee.

* Media story reprinted from Macquarie University News, November 2004.

2.3.2. Contributions to Aboriginal communities' education

A major issue raised by Aboriginal communities participating in the Yarrawarra workshop was their concern regarding the education of their community youth. Many Aboriginal teenagers were leaving school before finishing their high school education. Without adequate literacy and numerical skills, these Aboriginal children were facing a substantial challenge to gain employment, posing a serious problem for their families and communities.

In response to the needs of the Aboriginal communities, several educational events involving Indigenous students were subsequently conducted aimed at encouraging Indigenous youth to continue high school and even higher education, and to develop their interest in science, as well as promoting positive images of Aboriginal children by publicising their activities in the local press. A brief description of some educational activities carried out for young Aboriginal students during this PhD research project follows.

- **The Siemens Science Experience (SSE), January 2005 and 2006**

The SSE is a program that involves Year 10 students in a three-day hands-on science education event held annually at Macquarie University. In 2005 a group of 14 Indigenous students from Maclean, Grafton, Casino and Coraki were invited to attend the SSE, with full sponsorship by the IBRG and Macquarie University. This was the first time that a group of Indigenous students was formally involved in any SSE activity run in Australia. These students were given the opportunity to engage in "high-tech and real-world" science in the program. As this event proved to be successful, another group of 7 students and 3 adult mentors from Casino, Grafton and Maclean were sponsored by the IBRG and

Macquarie University to attend the SSE in 2006. Positive feedback was obtained from all the students involved in the SSE activities, commenting that these activities gave them a new perspective on university life and strengthened their resolve to finish high school. Accounts of these activities have been published in Macquarie University News [http://www.pr.mq.edu.au/lighthouse/feb05/story2.htm and http://www.pr.mq.edu.au/macnews/showitem.asp?ItemID=453].

- **The Macquarie University Open Day, October 2005**

Two Indigenous students were invited and sponsored to attend the Macquarie University Open Day in October 2005. They were trained to assist with two chemistry experiments in the "Science 4 Kids" activity, providing demonstration to over 1000 members of the public who attended the activity during the day. In addition, they were given a guided tour of the Macquarie University campus and had a chance to participate in other activities held on the Open Day. After this activity, both students expressed their eagerness to undertake tertiary education after their high school education. The coverage of this activity in the local press, Richmond River Express Examiner, is reproduced in Figure 2.8.

Figure 2.8. Media coverage of the Macquarie University Open Day 2005 [RREE, 2005a].

MEDIA MONITORS
INCISIVE INSIGHT

Richmond River Express Examiner
Wednesday 12/10/2005
Page: 5
Section: General News
Region: Casino NSW Circulation: 12,514
Type: Regional
Size: 185.66 sq.cms.
Published: -T-----

Brief: STUDY_HES

Helping out for science

LEILA WALKER from Casino had never visited a university before this year.

Now she's been to Macquarie University twice.

Last month she, and Teela Barker from Coraki, were invited to the university's Open Day to help out in the Science For Kids tent.

This followed a three-day stint at the uni in January as part of the Siemens Science Experience, where they were among 16 teenagers from the Bundjalung and Yaegl country enjoying science-based, on-campus activities.

Leila, 15, said working on the Open Day was 'really interesting' and she enjoyed chatting to the kids and showing them a science experiment.

David Harrington from the university's Indigenous Bioresources Research Group, who organised the visit, said the two young women were a credit to their communities.

"Leila and Teela were responsible for running two experiments in the tent, showing kids how chemistry can be fun and interesting," he said.

"They were also hosted on a guided tour of the University facilities.

"Both women were fantastic, showing initiative, confidence and patience during their visit."

Leila said the experience hadn't really made her consider science as a career, but now she might possibly think about going to university in the future.

CASINO'S Leila Walker helping out recently in the Science for Kids tent at Macquarie University in Sydney.

Ref: 18733938

* Media story reprinted from *Richmond River Express Examiner*, 12 October 2005. Casino.

- **The Chemistry Road Show, October 2005**

A "Chemistry Road Show" (or "Chemistry Magic Show") consisting of a set of five experiments was developed jointly with another IBRG member for students in two high schools in northern New South Wales. These experiments included slime making, starch detection, pH determination (using home-made turmeric and red cabbage indicators), paper chromatography (separating different colours in ink), and a classroom volcano. All experiments used common household chemicals. An instruction booklet showing experimental procedures and chemical reaction schemes and a result sheet were designed to accompany the experiments. This "Chemistry Road Show" was taken to Casino and Maclean High Schools in October 2005, together with two other members of the IBRG. A total of twelve Aboriginal students were trained in the experiments and were given the opportunity to present their expertise and leadership in their classrooms. These students demonstrated the chemical experiments in front of a total of 360 year 9 students in the Casino and Maclean High Schools with assistance from the IBRG and Local Aboriginal Land Council members. All of these Aboriginal student demonstrators achieved the goals of the standard 40-minute lessons and some of them exhibited strong confidence in managing the classes. Feedback from these two high schools showed that both schools highly appreciated this activity and were eager to have the event repeated in 2006. This activity was published in the Richmond River Express Examiner and the Costal Views, and the media stories are reproduced in Figure 2.9 and 2.10.

All of the above activities have been conducted in this research project as a result of being responsive to the needs of the Aboriginal communities. They demonstrate the commitment of the research group to benefit sharing and establishment of true collaborations with the Aboriginal communities in northern New South Wales.

Figure 2.9. Media coverage of the Chemistry Road Show at the Casino High School [RREE, 2005b].

Tabitha Williams, second from left, and Leila Walker, second from right, together with Casino High School's head science teacher Jim Crowley show Jason Watson, left, and Jacob Lyons how to make slime as part of the Macquarie University's Chemistry Roadshow.

Scientists in Training

Question: What do bush medicines, a chemistry roadshow, improved educational outcomes for Aboriginal youth and Casino all have in common?

Answer: the Macquarie University Indigenous Bioresources Research Unit.

The unit which is working with Aboriginal Land Councils Australia wide, brokered a deal with the local Land council for information on bush foods and medicines in exchange for help to increase the number of Aboriginal students completing year 12.

As a result, the unit invited 14 local Aboriginal students to attend the Siemens Science Experience at the university in January and organised the Chemistry Roadshow to visit Casino High School last week.

The unit also arranged for the Aboriginal students who attended the Siemens Science Experience to help the Chemistry Roadshow presenters in class.

According to Bernie Walker of the Casino based Boolangle Land Council, its vitally important that young Aboriginal people are encouraged to stay on in school.

"The job opportunities for kids leaving school at 14 and 16 are virtually nil and that's across the board in both black and white communities," he said. "By staying on at school many career opportunities open up for them to follow."

* Media story regenerated from the *Richmond River Express Examiner*. 11 Nov 2005. Casino.

Figure 2.10. Media coverage of the Chemistry Road Show at the Maclean High School [Graney, 2005].

Science minds: Samantha Essex, Nikki Wisely and Amber Bennett check their results.

Science at work

Maclean High school students got down and dirty last week learning science.

Macquarie University's Indigenous Bioresources Research group members Dave Harrington, Nynke Brouwer and Qian Liu took funky science experiments back to school to encourage more kids to think about the field.

Called the Chemistry Magic Show, the group taught several MHS students inclusing Dan and Dwayne Randall, to become teachers for a period and teach their fellow students.

"When we do it on the university campus we get to blow stuff up," Dave said."That is cool and gets the attention of the kids.

"The idea is to get kids interested in science again."

And from the looks on the eager faces of the Year 9 students on Tuesday, there may be a few more kids taking science seriously.

"We are trying to get the Goorie kids interested not just in science but university," he said.

* Media story regenerated from the *Coastal Views* (APN Newspaper), 3 November 2005. News was written by Juris Graney.

2.4. Conclusions and future directions

An ethnobotanical research of medicinal plants was carried out together with the Kamilaroi and Muruwari Aboriginal communities. This built on previous established relationships with members of these communities. Medicinal plant knowledge acquired through literature surveys and personal interviews was recorded in a Microsoft Access databases. A total of 35 plants and 2 customary remedies were documented. This database was given to and kept within these communities. The ethnobotanical research contributed to the conservation of traditional medicinal knowledge of these Aboriginal communities.

This is an ongoing study with an intention to update the database as further information becomes available. Such further information could include the parts of the plants used, time of collection, stage of development of the plant (*e.g.* flowering or not; young or old) and possible signs of disease as any of these could influence the levels of bioactive constituents.

Detailed ethnobotanical information of two Aboriginal medicinal plants, *Eremophila sturtii* and *Exocarpos aphyllus*, was obtained in this ethnobotanical study and was used as the basis for further biological and chemical investigations towards the exploration of biological activities and isolation of bioactive constituents.

Some other medicinal plants recorded in this ethnobotanical study (*e.g.*, *Acacia cambagei*, *Acacia ligulata* and *Flindersia maculosa*) have also been found to be important to Aboriginal people as medicines. Due to the time limitations of this project, these plants have not been included in the intensive biological and chemical studies. Owing to their

ethnomedicinal uses, these plants are worthy of future investigations. Chapter 3 describes the development and optimisation of biological assay methods for the evaluation of bioactivities of these Aboriginal medicinal plants.

Discussions and presentations on ensuring best ethical practices and benefit sharing were undertaken with Aboriginal communities of Yaegl (Maclean) and Bundjalung country. This was particularly successful in addressing community needs and led to the development of educational activities for Aboriginal high school students in order to encourage Indigenous youth to continue with their education. This is an in-kind benefit-sharing that has been highly valued by the communities and has strengthened relationships with these communities and laid the foundations for future true research partnerships.

Chapter 3. Biological Assay Methods and Optimisation

This chapter describes the need for new anti-inflammatory and antimicrobial agents, details the assays that were used for the evaluation of biological activities of Australian Aboriginal medicinal plants, and describes the optimisation of a fluorescence based antibacterial assay.

3.1. Introduction

This PhD project had a particular focus on medicinal plants that have been used customarily by Australian Aboriginal people for the treatment of ailments resulting from microbial infections or inflammation. This focus was chosen because skin infections, respiratory tract infections, gastrointestinal disorders, colds, sores and fever are the most common complaints faced by Australian Aboriginal people, and are the main conditions for which their plant remedies have been used [Yung *et al*., 2005; Balick and Cox, 1996]. Development of new antimicrobial agents is a global focus, and new treatments for inflammatory diseases are also largely desired in developed countries [WHO, 2003b; Mahady, 2005; Balick and Cox, 1996].

This chapter describes the need for new antimicrobial and anti-inflammatory agents and provides background to and procedures for the antimicrobial and anti-inflammatory bioassay methods that were used to evaluate the biological activities of selected Australian medicinal plants.

3.2. Inflammation mechanisms and anti-inflammatory assays

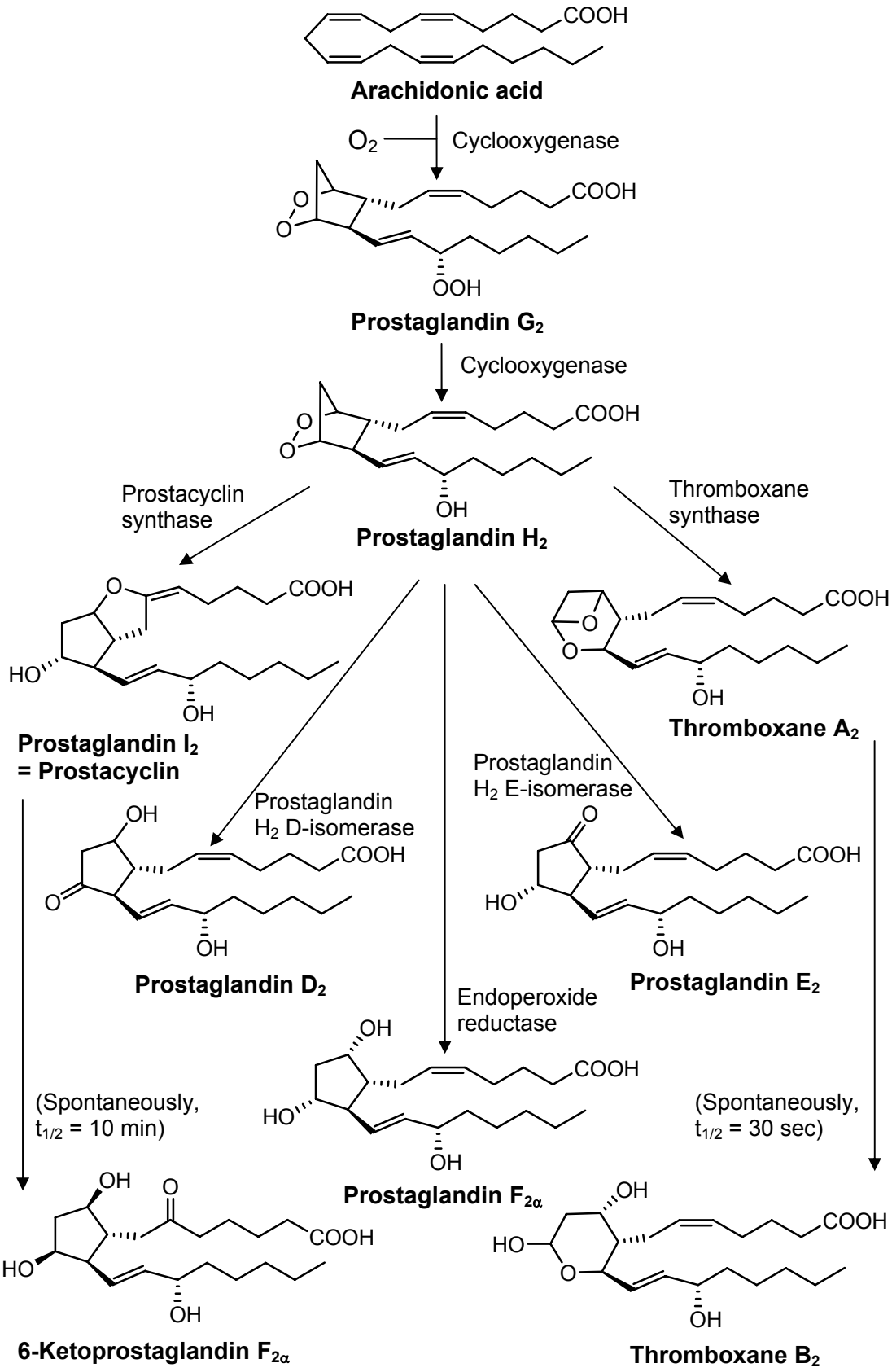
Inflammation is a response of an organism to invasion by a foreign body, such as bacteria, parasites and viruses. It is a complex process involving numerous cellular and plasma mediators with interrelated biological effects. These mediators include kinins, platelet-activating factor, prostaglandins, leukotrienes, amines, purines, cytokines, chemokines and adhesion molecules. Typical characteristics of the inflammatory response include redness, heat, swelling, loss of function and pain [Calixto *et al*., 2003; Vane *et al*., 1994]. Under normal conditions, inflammation serves to isolate the effects of the insult and to remove it rapidly, and causes little damage to host tissues because the production of inflammatory mediators is down-regulated. However, in cases where the inflammatory responses are not effective in removing the invading agent, or are not effectively attenuated, host tissues are likely to be damaged due to the chronic activation of leukocytes [Ali *et al*., 1997]. Diseases such as rheumatoid arthritis, osteoarthritis, asthma and glomerulonephritis, and the more serious Alzheimer's disease and cancer, are related to chronic inflammations [Bochsler and Slauson, 2002]. Inflammatory conditions affect both adults and children. In Australia, asthma is the most common chronic medical condition in childhood and affects one in five children [NH&MRC, 2005].

Prostaglandins and leukotrienes are critical inflammatory mediators with various activities. In general, prostaglandins and leukotrienes lead to increased vascular permeability, increased vasodilation and induction of neutrophil chemotaxis in the inflammatory site, and cause swelling and hyperalgesia [Zhang and Li, 1999]. They are produced at the site of inflammation from arachidonic acid through a series of enzymatic reactions. The first step in the synthesis of prostaglandins is catalysed by prostaglandin H synthase, now most commonly called cyclooxygenase (COX), by converting arachidonic acid (all-*cis*-

5,8,11,14-eicosatetraenoic acid) into prostaglandin H_2, which is the common substrate for specific prostaglandin synthases (Figure 3.1) [Hinz and Brune, 2002]. The production of leukotrienes from arachidonic acid is catalysed by lipoxygenases (LOs) [Zhang and Li, 1999].

Arachidonic acid is a 20:4 omega-6 fatty acid derived from dietary linoleic acid (18:3, n-6) and is one of the essential fatty acids required by the human body. All mammals obtain arachidonic acid directly from vegetable or animal oils, or from the elongation and desaturation of linoleic acid (18:2, n-6) by the liver [Fonteh, 2004]. Arachidonic acid is present in the phospholipids of membranes of the body's cells. Under resting conditions, levels of free arachidonic acid found within cells are very low. When membranes are activated by inflammatory stimuli, arachidonic acid is released by the enzyme phospholipase A_2 and converted to various inflammatory mediators [Zhang and Li, 1999]. Arachidonic acid is recognised as the major fatty acid precursor of a class of lipid mediators including leukotrienes, prostaglandins and thromboxanes, which have been shown to be important as intra- and extra-cellular mediators of cell function [Fonteh, 2004]. The COX and LO pathways underlying the arachidonic acid cascade are described below.

Figure 3.1. The arachidonic acid cascade through the cyclooxygenase pathway [Zhang and Li, 1999].



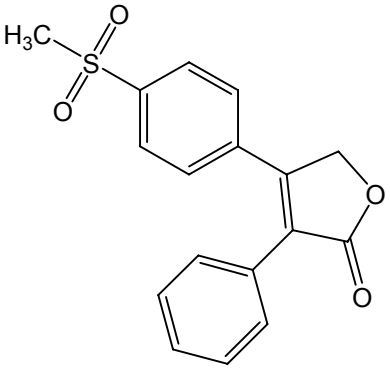
3.2.1. Cyclooxygenase pathway

COX is a bifunctional enzyme that catalyses the first step in the synthesis of the prostaglandins, converting arachidonic acid to prostaglandin G_2 by its fatty acid cyclooxygenase activity, and then to prostaglandin H_2 by its hydroperoxidase activity [Hinz and Brune, 2002]. Prostaglandin H_2 has a short life-time ($t_{1/2}$ < 5 mins), and is converted further to a range of prostanoids (prostaglandins and thromboxanes) by different enzymes in cells [Zhang and Li, 1999]. The arachidonic acid cascade through the COX pathway is shown in Figure 3.1.

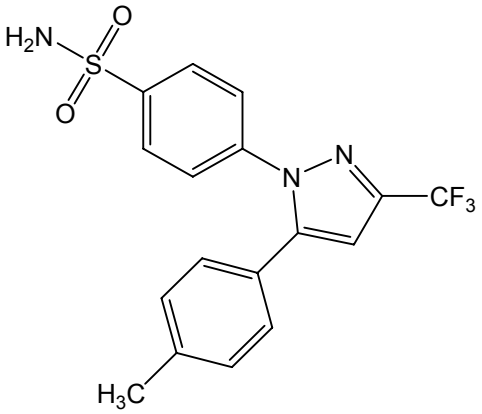
Three isomers of COX have been identified. COX-1, the widely distributed and constitutively expressed isomer, mainly accounts for production of prostanoids in gastric mucosa, kidney and platelets. COX-1 was generally believed to maintain housekeeping functions of these organs [Vane *et al*., 1998], however, COX-1 has also been found to be involved in inflammatory reactions [Siegle *et al*., 1998]. COX-2 is an inducible enzyme and is prominent at sites of inflammation. COX-2 is induced by a variety of mediators including cytokines, growth factors, tumour promoters and UVB irradiation. It is mainly involved in pathophysiological responses including inflammation, arthritis and pain [Fritsche *et al*., 2001; Zhang *et al*., 1997; Galli and Panzetta, 2002]. It has been reported that COX-2-mediated prostaglandin E_2 (PGE_2) production plays a critical neurological role in inflammation and hyperalgesia [Zhang *et al*., 1997]. COX-2 has also been found to be constitutively expressed in the kidney and brain [Galli and Panzetta, 2002]. COX-3 has been proposed to be a COX-1 variant and has been reported to exist in the cerebral cortex and heart [Chandrasekharan *et al*., 2002]. However, its expression has been questioned and its functions in inflammation are still uncertain [Warner and Mitchell, 2002; Schwab *et al*., 2003].

The human COX-1 gene is about 22 kilobase pairs in size and contains 11 exons, while COX-2 is about 8.3 kilobase pairs and contains 10 exons [Fritsche *et al*., 2001]. These two enzymes contain both cyclooxygenase and peroxidase active sites and are similar in catalytic mechanisms, but the cyclooxygenase active site of COX-2 is larger than that of COX-1 [Furse *et al*., 2006; Smith *et al*., 2000].

Many of the non-steroidal anti-inflammatory drugs (NSAIDs), such as aspirin and ibuprofen, used in the treatment of acute and chronic inflammatory disorders (*e.g.*, inflammation, fever, headache, pain, rheumatic arthritis) reduce prostanoid synthesis by inhibiting the activity of both COX-1 and COX-2 enzymes [Masferrer *et al*, 1994]. It is generally believed that the common side effects of NSAIDs, such as gastrointestinal tract toxicity and renal dysfunctions, are mainly due to the inhibition of the COX-1-derived prostanoid production, while the pharmacological effects of NSAIDs are ascribed to the inactivation of COX-2 [Fritsche *et al*., 2001; Zhang *et al*., 1997; Hinz and Brune, 2002]. Some selective COX-2 inhibitors have been manufactured, such as rofecoxib and celecoxib, and have been used to treat inflammatory symptoms including osteoarthritis, acute pain and painful menstruation. However, side effects of these drugs, such as acute renal failure and increased cardiovascular risk in patients, have been recently reported, and some of the COX-2 inhibitors (such as rofecoxib) have been withdrawn from the market or their labels have been requested to be revised by the US Food and Drug Administration after the risk/benefit analysis. Safety issues of these drugs are still under investigation [US Food and Drug Administration, 2005; Galli and Panzetta, 2002; James and Cleland, 2006].



Rofecoxib



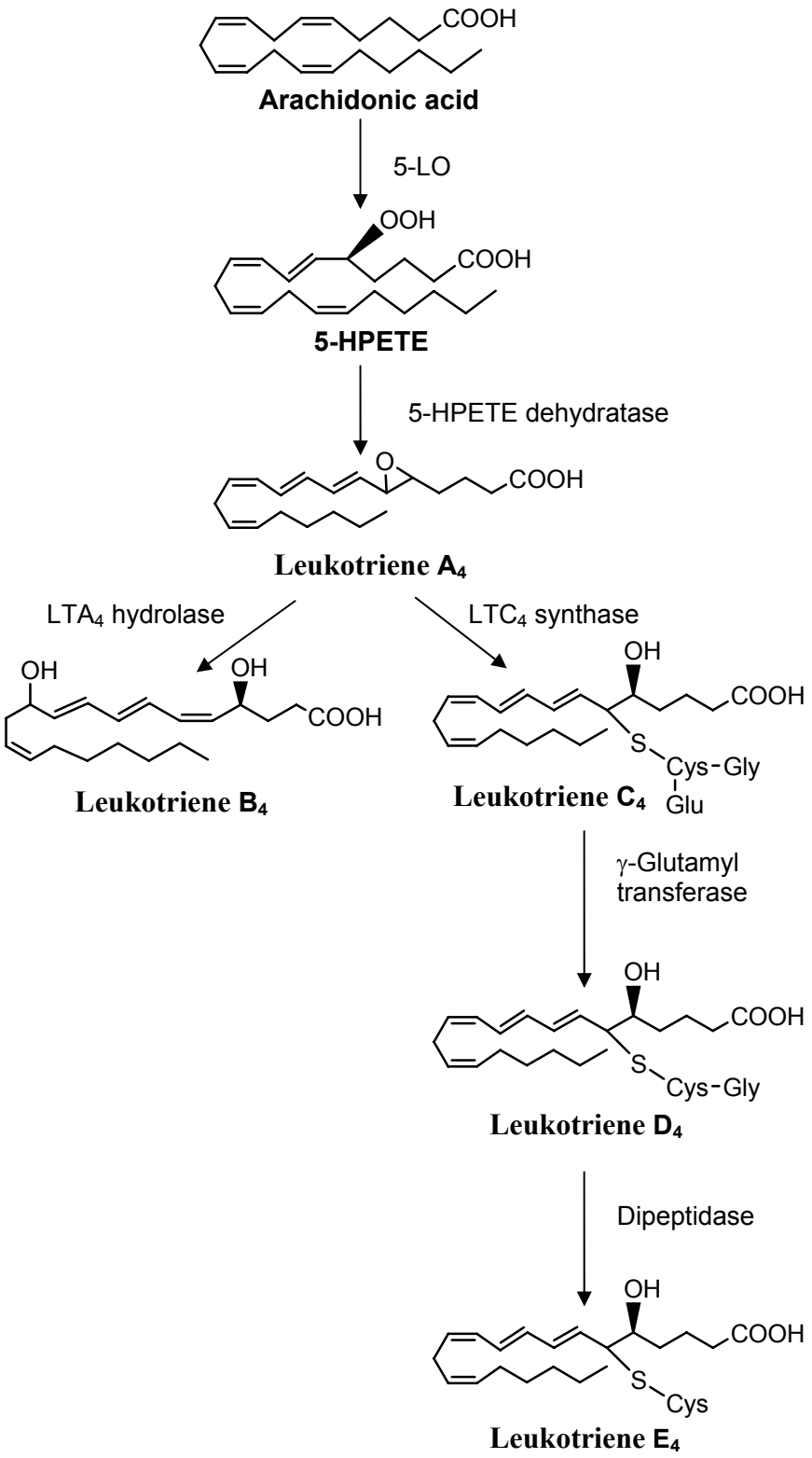
Celecoxib

3.2.2. Lipoxygenase pathway

Lipoxygenases are another important group of enzymes involved in the arachidonic acid cascade. They are responsible for the catalysis of leukotriene production. Leukotrienes are also mediators of inflammation, and have been found to be involved in the pathogenesis of several inflammatory diseases, such as asthma, psoriasis, rheumatoid arthritis, and inflammatory bowel disease [Zhang and Li, 1999; Henderson, 1994].

Lipoxygenases exist in both plants and mammals. The most important lipoxygenase in the human body is 5-LO. This is mainly distributed in lungs, platelets and leukocytes [Zhang and Li, 1999]. 5-LO catalyses the conversion of arachidonic acid to 5-hydroperoxyeicosatetraenoic acid (5-HPETE), which is further converted to a series of leukotrienes. The arachidonic acid cascade through the 5-LO pathway is shown in Figure 3.2.

Figure 3.2. The arachidonic acid cascade through the 5-lipoxygenase pathway [Zhang and Li, 1999; Henderson, 1994].



3.2.3. Targeted enzymes and anti-inflammatory assays

Prostanoids and leukotrienes produced by COX and LO are major mediators involved in inflammation, and inhibition of the activity of these enzymes reduces inflammatory symptoms such as swelling, fever and pain [Masferrer *et al*, 1994; Zhang and Li, 1999]. Since COX and LO use the same substrate, arachidonic acid, inhibition of one enzyme may cause the arachidonic acid cascade to shift to the other pathway. Drugs that act on both the COX and LO pathways have recently attracted the interest of many researchers [*e.g.*, Claria and Romano, 2005; Celotti and Laufer, 2001; Araico *et al*., 2006]. Some dual COX/5-LO inhibitors have been found to be promising in the treatment of rheumatic diseases, with little gastrointestinal damage [Bertilini *et al*., 2002]. Given that only a limited number of these dual inhibitors have entered clinical trials [Julemont *et al*., 2004], the exploration of new dual COX/5-LO inhibitors are greatly desired.

In this study, COX-1, COX-2 and 5-LO have been used as the targeted enzymes for evaluation of anti-inflammatory properties of traditional medicinal remedies used by Australian Aboriginal people, as well as for exploration of new anti-inflammatory agents with inhibitory activity against both COX and 5-LO enzymes. Commercially available COX and 5-LO inhibitor screening assay kits (Cayman Chemicals) were employed. The assay concepts and experimental procedures are described below.

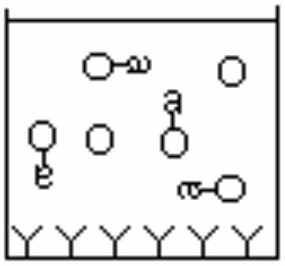
3.2.3.1. COX inhibitor screening assay

The COX inhibitor screening assay measures the percentage inhibition of enzyme activity of COX-1 and COX-2 by test samples (medicinal plant extracts, fractions and pure compounds).

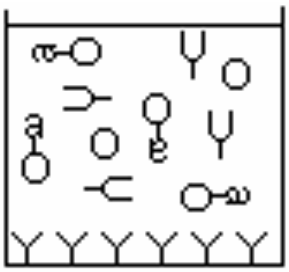
COX-1 and COX-2 are individually treated with test samples before they are reacted with arachidonic acid. The prostaglandin H_2 (PGH_2) produced in the COX reactions is reduced by $SnCl_2$ to the more stable compound prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$). The amount of $PGF_{2\alpha}$ produced by treated COX-1/COX-2 is quantified by an enzyme immunoassay and is compared to that of the untreated COX enzymes. In the enzyme immunoassay (as illustrated in Scheme 3.1), $PGF_{2\alpha}$ is bound to a specific prostaglandin antibody competitively with a PGE_2-AChE tracer (prostaglandin E_2 combined with acetylcholine esterase), and the antibody is then bound to the mouse anti-rabbit IgG, which is coated on the surface of wells of the microtitre plate. After removing all unbound $PGF_{2\alpha}$ and PGE_2-AChE tracer, Ellman's regent (composed of acetylthiocholine and 5,5'-dithio-bis(2-nitrobenzoic acid) [DTNB]) is added to the wells. The bound acetylcholine esterase reacts with acetylthiocholine to form thiocholine, which further reacts with DTNB to form thionitrobenzoic acid, a yellow compound that can be measured quantitatively by absorbance at 405 nm using a spectrophotometer. The above chemical reactions are shown in Figure 3.3.

Scheme 3.1. Enzyme immunoassay in the COX inhibitor screening assay.

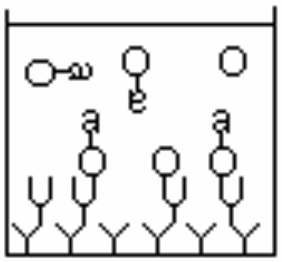
1. Plates are pre-coated with mouse anti-rabbit IgG



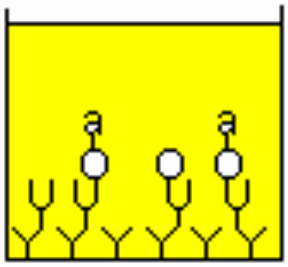
2. $PGF_{2\alpha}$ and PGE_2-AChE tracer are added



3. Prostaglandin antibody is added



4. After 18 hours incubation, unbound reagents are washed off



5. Plates are developed with Ellman's reagent

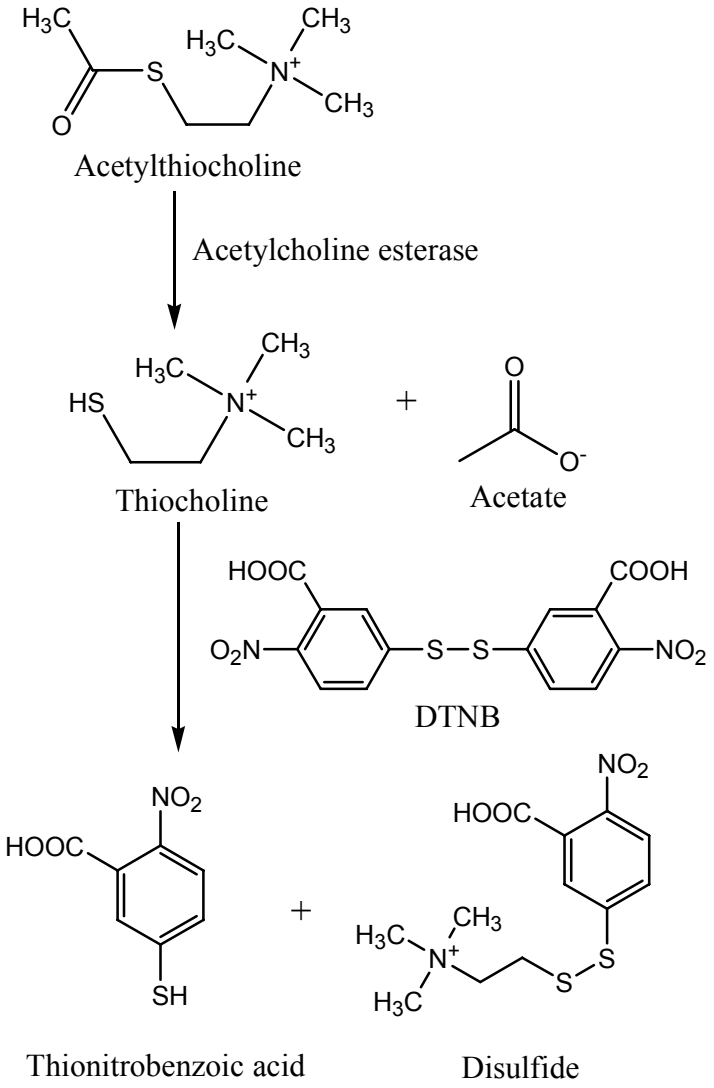
Mouse anti-rabbit IgG

Free $PGF_{2\alpha}$

Acetylcholinesterase coupled with PGE_2 (tracer)

Specific antiserum to $PGF_{2\alpha}$ and PGE_2

Figure 3.3. Reaction scheme for the COX inhibitor screening assay.



3.2.3.2. 5-LO inhibitor screening assay

The 5-LO inhibitor screening assay measures the enzyme activity of 5-LO treated with test samples (medicinal plant extracts, fractions and pure compounds) by detecting 5-HPETE production. Arachidonic acid or linoleic acid is used in this assay, and the amounts of 5-HPETE produced by treated and untreated 5-LO are measured quantitatively by absorbance at 492 nm using a spectrophotometer. Potato 5-LO is employed in this assay

since it is a purified enzyme recommended by the manufacturer of the 5-LO inhibitor screening assay kit, and has also been recognised as a good model because of its similarity to mammalian 5-LO [Cucurou *et al*., 1991; Shimizu *et al*., 1984].

3.3. Microbial infections and the need for new antimicrobial agents

3.3.1. Infectious diseases

Infections are caused by microorganisms, including viruses, bacteria, fungi, helminths and protozoa [Greenwood *et al*., 2002]. Damage to host tissues or organs can result from direct action of the microorganisms or their toxins, or from the host inflammatory and immune responses [Yung *et al*., 2005]. Infectious diseases were once fatal and untreatable, but since the introduction of the first antibiotic, penicillin, into general clinical use in the 1940s, hundreds of antimicrobial agents have been discovered and marketed. Infectious diseases have been largely controlled, and numerous lives have been saved [WHO, 2002; Powers, 2004].

It was once believed that "by the year 2000 most bacterial and viral infections will have been eradicated" (*Times* magazine 1966) [Yung *et al*., 2005]. However, according to the WHO 2003 report, infectious and parasitic diseases caused 11.1 million deaths in 2002, accounting for 19.7% of the total global mortality. HIV/AIDS, diarrhoeal diseases, tuberculosis and childhood diseases (such as measles, pertussis and tetanus) were among the top threats to human life. In addition to these, respiratory infections accounted for 6.7% of the mortality in 2002 [WHO, 2003b].

The serious situation with infectious diseases is largely caused by the increasing development of resistance to antimicrobial agents by many microorganisms, as well as the unpredictable appearance of many new pathogens [Mahady, 2005]. Some of the diseases that were once controlled by antimicrobial agents are appearing again with drug resistance, causing prolonged illness and greater risk of death in humans [WHO, 2002; Levy and Marshall, 2004; Yung *et al*., 2005].

3.3.2. Drug resistance

Resistance developed by microbes to antimicrobial agents has been observed since antibiotics were first used. In the 1930s, sulfonamide-resistant *Streptoccoccus pyogenes* was first found in military hospitals, followed by penicillin-resistant *Staphylococcus aureus* in civilian hospitals in London in the 1940s, shortly after the introduction of penicillin. Multiple drug resistant bacteria, such as *Escherichia coli*, Shigella and Salmonella, appeared since the late 1950s [Levy and Marshall, 2004]. More drug-resistant strains emerged after that. In 1999, 30% of enterococci isolates obtained from clinics in England and Wales were found to be resistant to vancomycin, and about 18% of *Pseudomonas aeruginosa* isolates were resistant to at least one commonly used antibiotic [Livermore, 2004]. In the year 2000, more than 5% *P. aeruginosa* isolates were resistant to 5 out of 6 antibiotics used for the treatment of this bacterium, and some of the strains were resistant to all available antibiotics [Livermore, 2004]. More recently, 40–60% of *Staphylococcus aureus* strains in hospitals in the United States and the United Kingdom were found to be methicillin-resistant, and usually resistant to multiple drugs [Levy and Marshall, 2004].

A survey on the registered antibiotics in Australia conducted by the National Health and Medical Research Council (NH&MRC) in 2003 showed that among about 80 antibacterial drugs available for human use in Australia, 35 were in the category that if resistance develops, severe impact would occur, as there would be very limited or no alternatives available to treat serious bacterial infections [EAGAR, 2003].

The need for developing new therapeutic drugs, especially those with different mechanisms of action, is urgent. Medicinal plants, as a vast reservoir of structurally unique natural products, are a valuable resource for the exploration of new antimicrobial agents. One of the goals of this PhD project was to explore antimicrobial compounds from medicinal plants that have been used by Australian Aboriginal people to treat infections.

In this study, the targeted microbes used in bioassays to screen for potential antimicrobial agents were the human pathogens *Staphylococcus aureus*, *Escherichia coli*, *Pseudomonas aeruginosa* and *Candida albicans*. These microorganisms were selected not only because they have been used commonly in routine antimicrobial screenings, but also because they are associated with many infectious diseases that are common across the world. The section below provides more details about these pathogens.

3.3.3. Targeted microorganisms and causes of infections

A study of the causes of infections in hospitals in Europe showed that *S. aureus* (both methicillin susceptible and resistant), *E. coli* and *P. aeruginosa* were the top three bacteria responsible for in-patient infections, accounting for 33.2%, 18.3% and 10.0% of all isolates from patients in the United Kingdom and Ireland, and 23.0%, 21.1% and 3.2% of bacteraemias (bloodstream infections) in England and Wales in 2001 [Livermore, 2004].

Candida species (including *Candida albicans*) were the fourth most common cause of septicaemias in the United States, accounting for 7.6% of all bloodstream infections in hospitals [Edmond *et al*., 1999].

Staphylococcus aureus is a Gram positive coccus. It is present in the nose of 30% of healthy people, and is one of the common microorganisms residing on human skin. Infections caused by *S. aureus* are mainly found on the skin and soft tissue of humans, as well as other sites such as the bone, joint, brain, lung and blood, resulting in clinical syndromes such as boils, abscesses, impetigo, wound infection, pneumonia and septicaemia [Irving *et al*., 2005].

Escherichia coli is a Gram negative bacterium predominating among the aerobic commensal flora in human gut. *E. coli* is the most common cause of acute urinary tract infections and traveller's diarrhoea [Greenwood *et al*., 2002]. A recent study showed that more than 33% of *E. coli* isolates from human sources in Jamaica were resistant to gentamicin, and about 44% were resistant to tetracycline and kanamycin [Miles *et al*., 2006].

Pseudomonas aeruginosa is a Gram-negative bacillus and an opportunistic pathogen that can infect almost any external site or organ of the human body, causing infections of the lower respiratory tract, ears, eyes, post-operative wounds and burns [Irving *et al*, 2005]. About 1% of *Pseudomonas aeruginosa* strains are resistant to all of the currently available antibiotics [Livermore, 2004].

Candida albicans is a fungus that is found as part of the normal human flora. Superficial Candida infections may occur in the mouth, vagina, skin or nails, and 80-90% of cases are

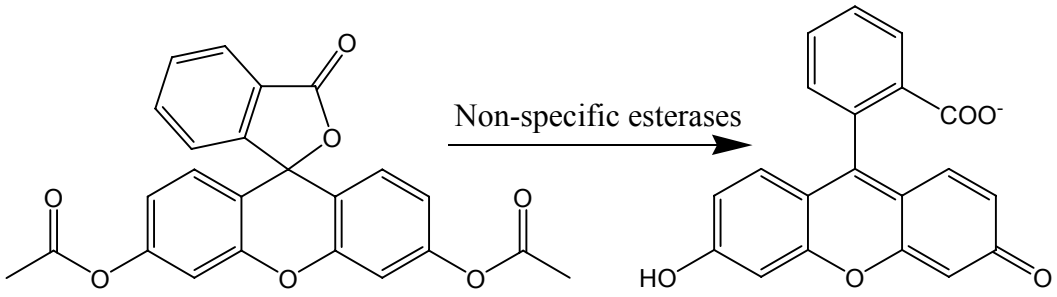
related to *C. albicans*. Invasive infections caused by *C. albicans* (deep candidiasis) may require systemic antifungal or surgical treatment [Irving *et al*, 2005].

Given the various characteristics of and diseases caused by *S. aureus*, *E. coli*, *P. aeruginosa* and *C. albicans*, these pathogens were selected as the microorganisms for targeted antimicrobial screening. In order to evaluate the antimicrobial properties of medicinal plant extracts and their constituents, a valid bioassay method must be employed. As part of this PhD research, the fluorescein diacetate (FDA) antibacterial assay method [Chand *et al*., 1994] was optimised to become suitable for the determination of the antibacterial activity of plant extracts and compounds, and has been used for bioassay-guided fractionation throughout this PhD study. The optimisation of the FDA antibacterial assay method is described below.

3.4. Optimisation of the fluorescein diacetate (FDA) antibacterial assay

A fluorescence-based assay using fluorescence diacetate (FDA) was developed by Chand *et al*. in 1994 for the rapid determination of the antimicrobial activity of natural products [Chand *et al*., 1994]. This assay relies on the measurement of the fluorescence/absorbance following the conversion of the colourless compound FDA to the yellow-green fluorescent compound fluorescein by the non-specific intracellular esterases existing in metabolically active microbial cells (Figure 3.4). The fluorescein production is directly proportional to the microbial population density and esterase activity [Chand *et al*., 1994].

Figure 3.4. Fluorescein diacetate hydrolysis.



Fluorescein diacetate Fluorescein

FDA has long been employed for the determination of the viability of cells of bacteria, fungi, plants, animals and humans, and has been used in a variety of bioassays, including antibacterial, antifungal, immunoassay and cytotoxicity assays [Dorsey and Tchounwou, 2004; Graham-Evans *et al*., 2004; Lu and Higgins, 1999; Sin *et al*., 2006].

Several research groups have used the FDA assay method for the determination of antimicrobial properties of natural products, and achieved satisfactory results [Lindsay *et al*., 2000; Hadacek and Greger, 2000; Qhotsokoane-Lusunzi and Karuso, 2001; Benkendorff *et al*., 2000]. However, problems with this assay have been reported in recent years, such as the production of fluorescein in assay media even without the presence of bacterial cells (*i.e.*, abiotic cleavage) [Clarke *et al*., 2001]. This problem was also experienced by a research student Stephanus Wanandy in our group while working on the antibacterial activity of a medicinal plant extract [Wanandy, 2005]. Wanandy carried out detailed investigations on the FDA antibacterial assay in order to understand the causes of the abiotic cleavage of FDA and the roles of the media components in this cleavage. By choosing appropriate media components, using a 10 times diluted medium and employing a pH buffer, the abiotic cleavage of FDA was successfully minimised, and the viabilities of the tested bacteria (*S. aureus, E. coli* and *P. aeruginosa*) were not severely affected.

However, Wanandy found that the fluorescence intensity produced by *E. coli* was low (*i.e.*, no marked difference to that produced by the medium), even with the presence of further substrates and inducers of esterases in the medium [Wanandy, 2005]. Therefore the FDA assay was thought not to be suitable for *E. coli* [Wanandy, 2005].

In the FDA assay method of Chand *et al*., inoculum (which has an absorbance at 600 nm of 0.11-0.12) is incubated with test samples for 40 minutes (noted as the first incubation time) before FDA is added. Fluorescein production is measured after 3 hours additional incubation [Chand *et al*., 1994]. Wanandy followed this procedure and tested the MIC values of several antibiotics, but found that the results were not consistent with published data [Wanandy, 2005].

One of the aspects of this project was to optimise the FDA assay to make it applicable for use with *E. coli*, and to increase the reliability of the FDA assay to give accurate MIC values of test samples. The results achieved towards the optimisation of the FDA assay are shown in the following sections.

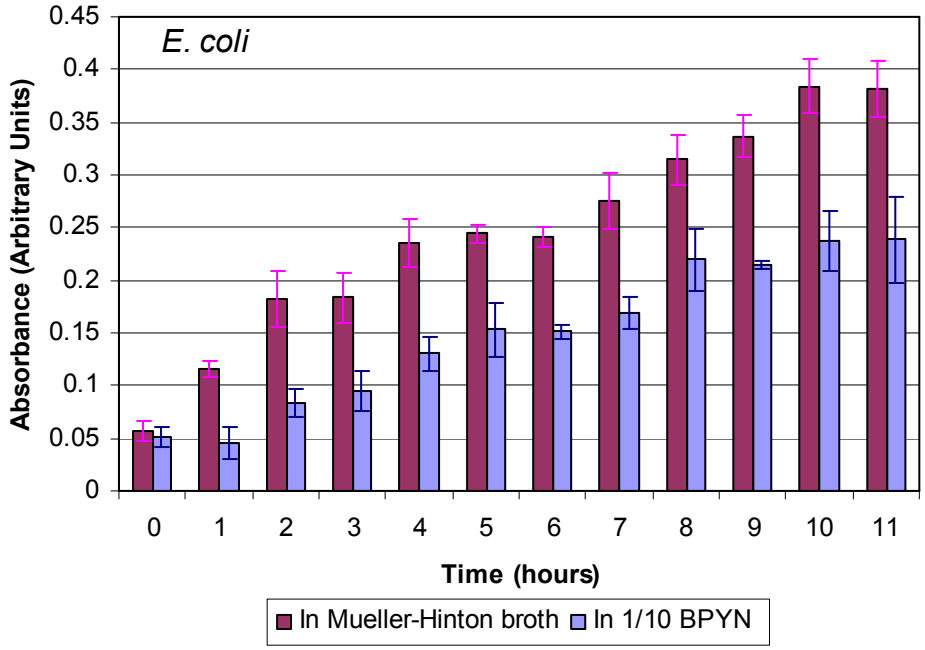
3.4.1. Growth curve of *E. coli*

In testing the effect of media dilution on hydrolysis of FDA with or without bacteria, Wanandy found that a 10 fold diluted BPYN medium (containing 10 mM BES buffer, peptone 0.2% (w/v), yeast extract 0.1% (w/v) and NaCl 0.1% (w/v), referred to as "1/10 BPYN") significantly reduced the background fluorescence (*i.e.*, the abiotic cleavage of FDA), and did not severely affect the viability of *S. aureus* and *P. aeruginosa* (lower fluorescence levels of *S. aureus* and *E. coli* were observed compared to that in the

undiluted medium). However, the fluorescence intensity in the wells of *E. coli* was consistently low and was not distinguishable from the background [Wanandy, 2005].

Since the medium that Wanandy used was a 10 fold dilution of a full strength medium suitable for bacteria growth, the limited nutrient content in the medium was most likely slowing down the growth of *E. coli*. Therefore, a comparison of growth of *E. coli* in the 1/10 BPYN and a full-strength medium, Mueller-Hinton broth, was conducted in this study. To be consistent to Wanandy's studies, the initial cell density of *E. coli* was adjusted to have an absorption (optical density) of 0.08 at 600 nm (OD_{600} 0.08). The inocula were incubated at 37°C and the optical density was measured at different times. The results are shown in Figure 3.5.

Figure 3.5. Growth of *E. coli* in 1/10 BPYN medium and Mueller-Hinton broth.



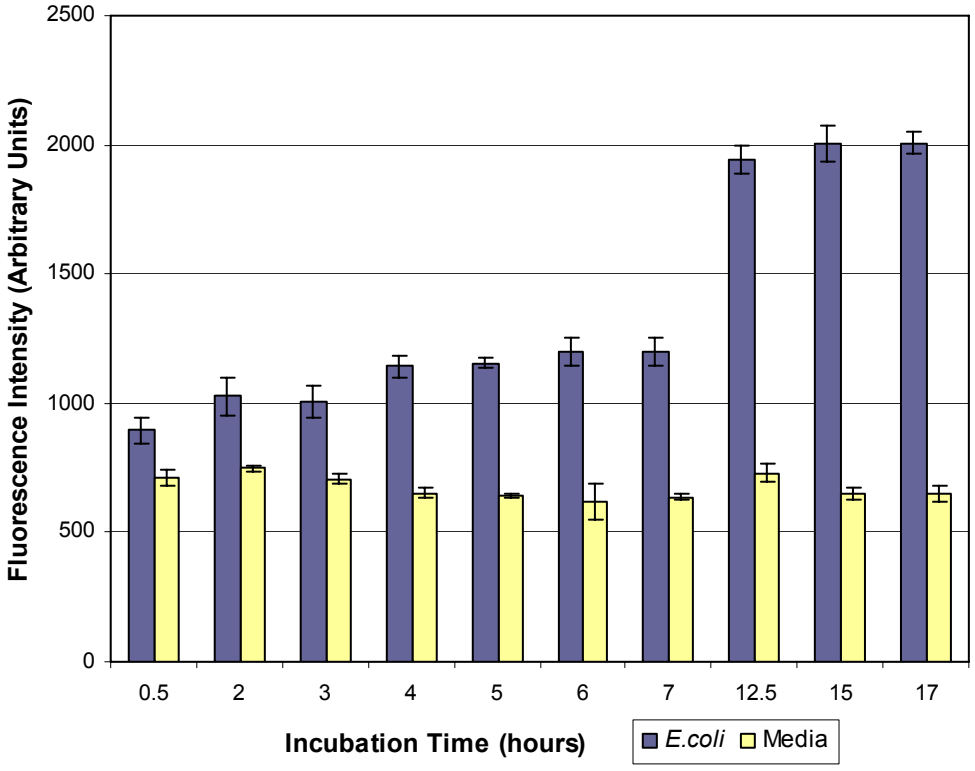
Absorbance was measured at 590 nm with background subtracted. *n*=4. Error bars denote standard deviation.

Figure 3.5 shows clearly that the growth of *E. coli* in the 1/10 BPYN medium was slower than that in the full-strength Mueller-Hinton broth, as indicated by the cell density. In a

short incubation time (1 hour), no distinguishable growth of *E. coli* in 1/10 BPYN was observed. Reasonable growth was achieved with prolonged incubation (*e.g.*, after 10 hours).

The time needed for *E. coli* to hydrolyse FDA under limited nutrient conditions was also tested. An inoculum of *E. coli* in 1/10 BPYN medium (OD_{600} 0.08) was incubated at 37°C and FDA was added at different incubation times. The fluorescence levels of both the inoculum and the medium were measured. The results are shown in Figure 3.6.

Figure 3.6. Effect of incubation time on the fluorescence produced by *E. coli*.



Fluorescence intensity was measured at λ_{ex} 405 nm and λ_{em} 520 nm at 3 hours after adding FDA. *n*=3. Error bars denote standard deviation.

Figure 3.6 shows that the background fluorescence of the medium was consistent during the incubation, while the fluorescence level of *E. coli* increased with incubation time, and

reached maximum values after 15 hours incubation. Within 40 minutes incubation time (as suggested by Chand *et al*.), the fluorescence level with *E. coli* was low and was not much different from the background (only 25% above the background {895 arbitrary units *versus* 714}); however, after 15 hours incubation, the difference was very appreciable (208% increase in fluorescence level {2006 arbitrary units *versus* 652}). Therefore, a prolonged incubation time (such as 15 hours) was found necessary for *E. coli* to attain sufficient growth, and to discern maximum difference between the fluorescence produced from the hydrolysis of FDA by *E. coli* and by the medium. With this notable increase in the fluorescence difference between *E. coli* and the background, it was possible for *E. coli* to be tested with the FDA assay.

The fluorescence measurement was consistent with the growth curve of *E. coli* (Figure 3.5), confirming that the hydrolysis of FDA was corresponding to the esterase activity, and the fluorescence level is a good indication of cell population [Dorsey and Tchounwou, 2004; Graham-Evans *et al*., 2004; Lu and Higgins, 1999; Sin *et al*., 2006].

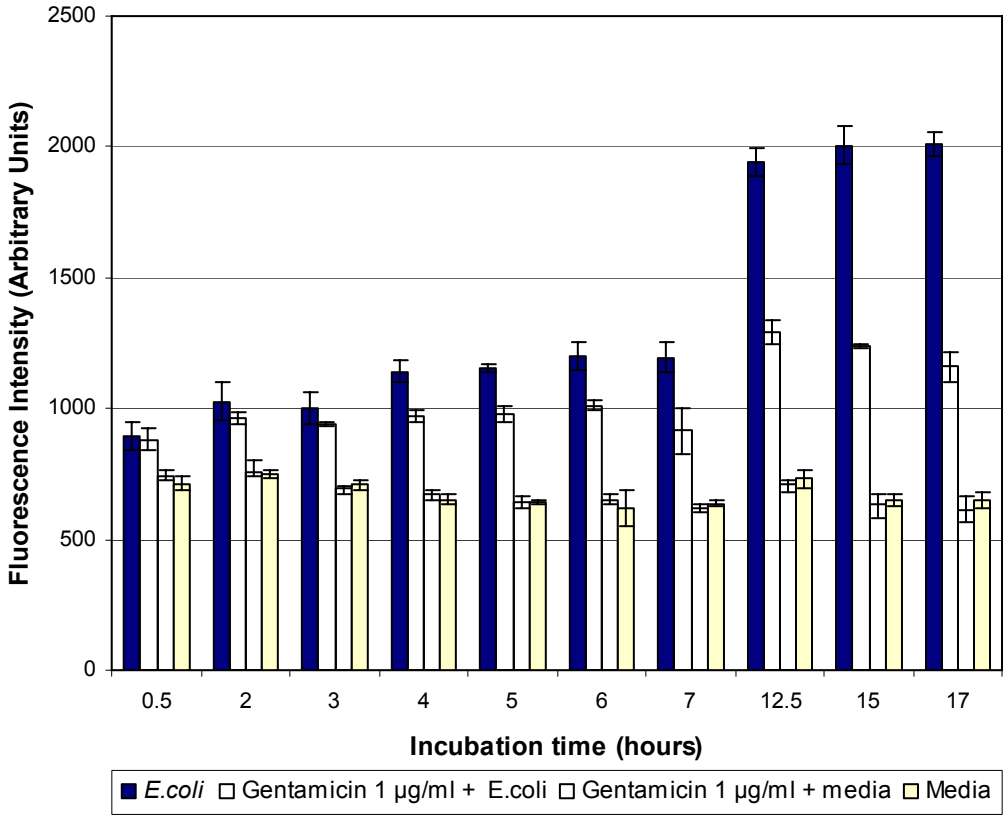
3.4.2. Optimising the incubation time

In the FDA method developed by Chand *et al*., a 40 minutes first incubation time was believed to be sufficient for antimicrobial agents to act with tested microbes [Chand *et al*., 1994]. Wanandy tested the antibacterial activity of gentamicin at a lethal dose (10 μg/ml) on *S. aureus* and *P. aeruginosa* using the diluted medium with a first incubation time of 40 minutes, 3 and 12 hours, and measured the fluorescence intensity 3 hours after adding FDA. It was found that 40 minutes was insufficient for gentamicin to present an obvious antibacterial effect since there was no significant difference between the fluorescence levels of bacteria with or without the antibiotic. Distinctive bactericidal activity of

gentamicin was only observed after 12 hours incubation [Wanandy, 2005]. The reason for this is most likely due to insufficient reaction between the antimicrobial agent and the bacteria in the limited time.

In order to determine the required first incubation time for antibacterial agents to demonstrate antibacterial effects, an inhibitory dose of gentamicin (1 μg/ml) [Sahm and Washington, 1991] was added to *E. coli*, and the fluorescence levels of *E. coli* with or without gentamicin were compared at different incubation times. Gentamicin without inoculum was also included as a background control. The results are shown in Figure 3.7.

Figure 3.7. Difference in fluorescence levels produced from FDA by *E. coli* with or without the presence of gentamicin.



Fluorescence intensity was measured at λ_{ex} 405 nm and λ_{em} 520 nm at 3 hours after adding FDA. n=3. Error bars denote standard deviation.

Similar to Wanandy's finding, with shorter incubation times, the fluorescence level of *E. coli* after addition of FDA with antibacterial treatment was not distinguishable from that without treatment. This indicated that without adequate time to allow antimicrobial agents to react with microorganisms, a false negative result would occur. The difference in fluorescence between gentamicin treated or untreated *E. coli* increased with incubation time, as shown in Figure 3.7, with the maximum difference observed after 17 hours incubation.

In the standardised procedure for the antimicrobial susceptibility test recommended by the (American) National Committee for Clinical Laboratory Standards (NCCLS) using the broth dilution method, 16 to 20 hours incubation time is recommended to allow sufficient reaction between test compounds and microorganisms [Sahm and Washington, 1991]. This study of the growth curve of *E. coli* demonstrated that a short incubation time (40 minutes) was not adequate for *E. coli* to reach sufficient growth and produce fluorescence discernible from the background after addition of FDA, nor was it sufficient for antibacterial agents to exert an antibacterial effect. Therefore, the first incubation time of 15 to 17 hours was more appropriate for this FDA assay. This time scale is consistent with the NCCLS recommendation. Considering the practical aspects in conducting this antibacterial assay, an overnight incubation (15-18 hours) was found to be most convenient, and was therefore used for all subsequent experiments.

3.4.3. Optimising the inoculum density

Chand *et al*. used an inoculum density of OD_{600} 0.11-0.12 (accounting for 10^6 – 10^7 CFU/ml) in the FDA assay [Chand *et al*., 1994]. Wanandy used an inoculum density of

OD_{600} 0.08, and this was also used in the previous experiments on the bacterial growth in this study.

From Figure 3.7 it can be seen that with the initial inoculum density of OD_{600} 0.08, the fluorescence levels of *E. coli* with gentamicin are higher than the gentamicin background at all times. This indicated that the growth of *E. coli* was not totally inhibited by gentamicin at an inhibitory dose of 1 μg/ml (MIC ranges published by NCCLS was 0.25-1.0 μg/ml for *E. coli*) [Sahm and Washington, 1991]. The incomplete inhibition of *E. coli* growth by gentamicin was most likely attributable to the high inoculum density used in the experiment.

As different inoculum densities can significantly affect the determination of MIC values of antimicrobial agents, the NCCLS recommended the inoculum density for the broth dilution assay to be 5×10^5 colony forming units (CFU)/ml [Sahm and Washington, 1991].

The cell densities of *E. coli* and *S. aureus* corresponding to OD_{600} 0.08 were tested by conducting colony counts. The results showed that the cell density of *E. coli* with an OD_{600} 0.08 was 5.8×10^7 CFU/ml, and that of *S. aureus* was 1.0×10^8 CFU/ml. These inoculum densities were 100 to 200 times higher than the recommended value. Similar results were obtained for the cell density of *P. aeruginosa* by another PhD student Nynke Brouwer in this research group. In order to achieve reliable MIC values of tested antimicrobial agents using the FDA assay, all inocula were diluted 100 fold after adjusting the optical density at 600 nm to 0.08 to achieve an inoculum size of 10^5 to 10^6 CFU/ml. This dilution was used in all subsequent experiments.

With the diluted inoculum density and optimised incubation time (15-18 hours or overnight), MIC values of three antibiotics gentamicin, tetracycline and chloramphenicol were measured against *E. coli*. The results agreed perfectly with those published by the NCCLS (see Table 3.1 and section 3.4.4 for details).

In summary, with the optimised incubation time and appropriate inoculum density, the FDA assay was applicable for the measurement of the activity of antibacterial agents on *E. coli*.

3.4.4. Validation of the optimised FDA antibacterial screening assay

The FDA assay originally developed by Chand *et al*. was optimised by using a 1/10 BPYN medium (done by Wanandy), an overnight (15-18 hours) incubation time and a 1/100 diluted inoculum density. In order to validate the quantitative nature of the optimised FDA assay, the antibiotics gentamicin, tetracycline and chloramphenicol were employed as standard controls. MIC values of these antibiotics against *S. aureus*, *E. coli* and *P. aeruginosa* were measured, and the results are presented in Table 3.1. All MIC values were consistent with those published by the NCCLS using the broth microdilution method [NCCLS, 1999]. Repetition of the experiments by colleagues (Wanandy and Brouwer) showed good reproducibility.

Table 3.1. Comparison of MIC results from FDA assay and NCCLS broth microdilution method.

Antibiotics	Minimum Inhibitory Concentration (μg/ml)					
	E. coli		*S. aureus*		*P. aeruginosa*	
	FDA	NCCLS	FDA	NCCLS	FDA	NCCLS
Gentamicin	0.5	0.25-1.0	1.0	0.12-1.0	1.0	0.5-2.0
Tetracycline	0.5	0.5-2.0	0.2	0.12-1.0	5.0	8.0-32
Chloramphenicol	5.0	2.0-8.0	5.0	2.0-8.0	>50	N/A

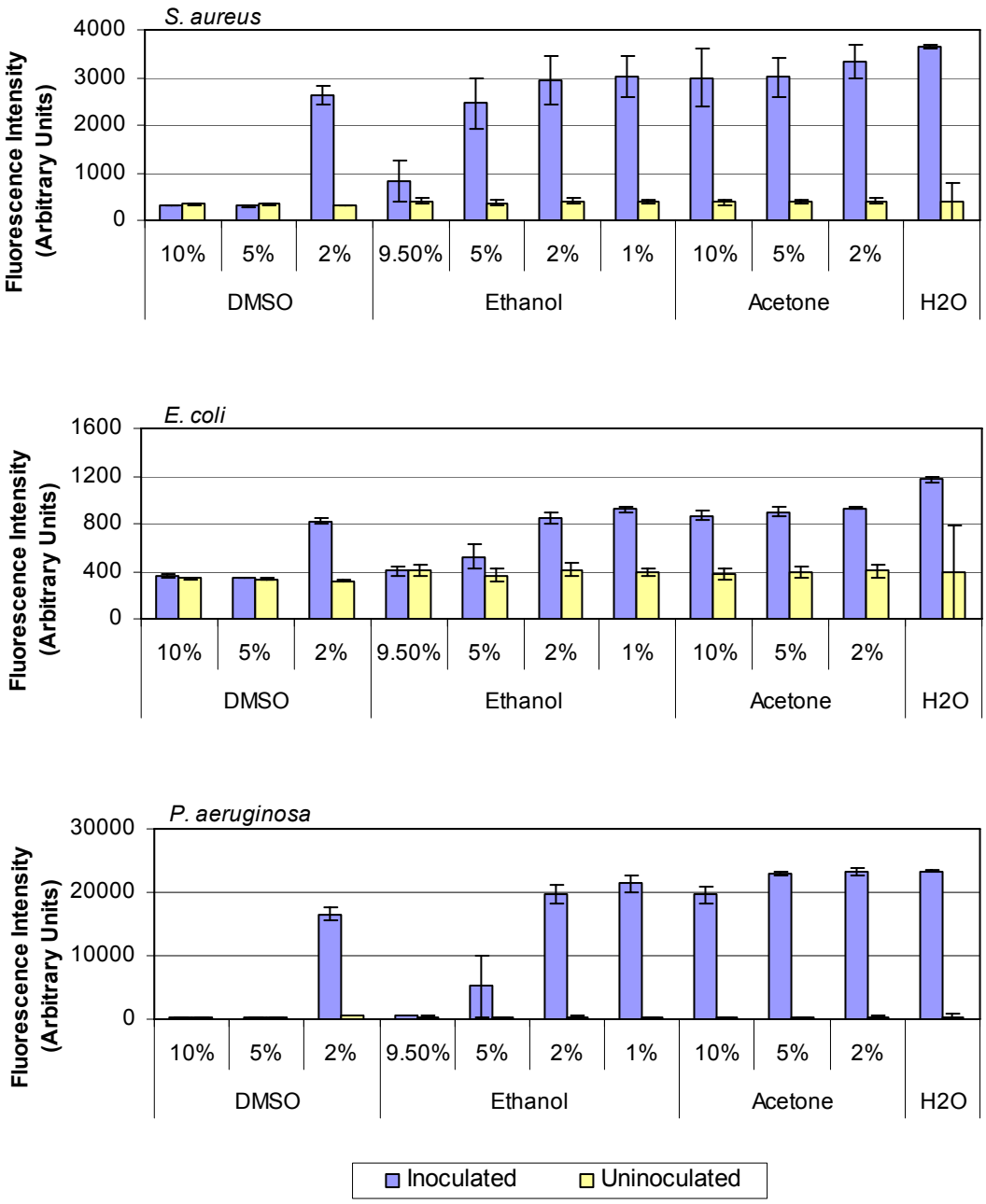
N/A: not available.

3.4.5. Studies on solvent effects

In the determination of MIC values of the antibiotics, acetone was used to dissolve chloramphenicol, as this compound was insoluble in pure water. Given that many natural products are organic compounds that have poor solubility in water (for example, fatty esters and terpenoids), organic solvents would also have to be used to dissolve the natural products from the selected medicinal plants of this study before they could be tested in bioassays.

Some organic solvents are toxic to bacteria, for example, 75% aqueous ethanol is a well-known disinfectant widely used in households, laboratories and hospitals for hygienic purposes. In order to evaluate the effect of organic solvents on bacterial growth, some commonly used water-miscible organic solvents, DMSO, ethanol and acetone, were tested for their effect on the growth of the bacteria at different concentrations. The results are shown in Figure 3.8.

Figure 3.8. Solvent effects on bacterial growth.



Bacterial growth was measured using the optimised FDA assay conditions. Solvent concentrations noted on the graphs were final concentrations (v/v) in wells of microtitre plates. Fluorescence intensity was measured at λ_{ex} 405 nm and λ_{em} 520 nm. *n*=3. Error bars denote standard deviation.

These test results showed that DMSO and ethanol at concentrations of 5% or greater in wells of microtitre plates (v/v) caused a significant decrease in viability of the tested bacteria. Acetone at concentrations up to 10% did not severely affect the bacterial growth.

Collating the solvent effects on the growth of all tested bacteria, it was found that 2% (v/v) final concentration of DMSO and ethanol would be the maximum value tolerated by the bacteria tested. Therefore, 2% (v/v) was the upper limit for these solvents used in the subsequent experiments for the determination of antimicrobial activities of medicinal plant extracts.

3.5. Broth microdilution method for *Candida albicans*

From Section 3.4.4 it was seen that after the optimisation of the assay conditions, the FDA assay was quantitative in measuring the MIC values of antibiotics against bacteria. However, a PhD student in this research group, Nynke Brouwer, found that these FDA assay conditions were not suitable for the screening of antifungal agents against *C. albicans*. Brouwer tested the growth of *C. albicans* in different media and found that only after 30 hours incubation was the growth of this fungus detectable in the 1/10 BPYN medium [Brouwer *et al*., 2006]. Therefore, the 1/10 BPYN medium was not suitable for the growth of *C. albicans*. Without sufficient growth of the test microorganism, the antimicrobial activity as determined by the FDA assay using the diluted medium would be severely affected.

Sabouraud dextrose agar and broth are the recommended media for the growth of fungi and have been commonly used in antifungal assays [Espinel-Ingroff and Pfaller, 2003]. However, Sabouraud dextrose broth (SAB) has been found to cause severe quenching of the fluorescence of fluorescein, even at a 10 fold diluted concentration [Clarke *et al*., 2001]. The significant reduction of fluorescence by SAB medium itself would lead to the report of false positive results, since the fluorescence produced by live cells would become

undetectable and live cells would be regarded as dead. Therefore, SAB was unable to be used as the growth medium for *C. albicans* in the FDA assay. While further investigations on the media suitable to be used in the FDA assay for *C. albicans* were conducted by Brouwer [Brouwer *et al*., 2006], the NCCLS standard broth microdilution assay was employed in this PhD study for the testing of the antifungal activity of the extracts of Australian Aboriginal medicinal plants on *C. albicans* [Espinel-Ingroff and Pfaller, 2003].

In the broth microdilution assay for *C. albicans*, an overnight culture of *C. albicans* in SAB was adjusted to have an OD 0.08 at 600 nm (which accounted for ~ 4 x 10^5 CFU/ml as measured by cell counts), and a 1:100 dilution was used in the broth microdilution assay. This inoculum density was in agreement with the NCCLS recommendation (1 x 10^3 to 5 x 10^3 CFU/ml) for the susceptibility test for yeasts and fungi [Espinel-Ingroff and Pfaller, 2003]. Serial dilutions of test samples (20 μl) were added to the medium with and without the inoculum (180 μl). The medium without the inoculum served as a background control. These were incubated for 24 hours at 37°C. The growth of the yeast was measured by the increased absorbance at 492 nm, compared to that of the background. The spectrophotometric measurement of the growth of fungi at 492 nm was included by the NCCLS as a modification to the visual examination of MIC in the microdilution method, and has been used by other researchers in antifungal assays for *C. albicans* [*e.g.*, Moore *et al*., 2003]. Prior to reading, the microtitre plates were shaken for at least 5 minutes in order to obtain evenly dispersed culture suspensions and avoid any interference from the aggregates formed during the incubation. The lowest concentration of test sample at which 50% growth was inhibited (compared to the drug free control) was recorded as the MIC [Espinel-Ingroff and Pfaller, 2003]. This microdilution method was used in all the subsequent experiments for the determination of antifungal activity of medicinal plant extracts against *C. albicans*.

3.6. Possible interferences in the determination of MIC values of medicinal plant substances by the FDA assay

The optimised FDA antibacterial assay has been demonstrated to be a valid method for the determination of MIC values of some antimicrobial agents (pure compounds), as seen in Section 3.4.4 of this thesis. In this PhD project, this FDA assay was used to evaluate the antibacterial properties of natural products, including not only pure compounds, but also crude extracts and fractions of medicinal plants (refer to chapter 4 and 5). Since the antibacterial activity results were used to guide further separation of active extracts and fractions, it was important that accurate MIC values of crude extracts and fractions were determined, as any invalid results might mislead the separations and chemical investigations, and result in loss of time in tracking inactive components, or miss possible active compounds. From this point of view, several issues that might affect the accuracy of the FDA assay needed to be considered.

Firstly, the abiotic cleavage of FDA by natural products samples could interfere with the fluorescence generated by live cells of bacteria. It has already been demonstrated that nucleophiles such as amino acids or amino acid residues in proteins and peptides are able to cause FDA cleavage [Wanandy, 2005]. Since proteins, peptides and free amino acids are essential components for plant growth and metabolism and exist in large amounts in plants, during the extraction of plant samples, these nucleophiles can be present in the crude extracts, especially when water is used as the solvent. The presence of nucleophilic substances in test samples will cause abiotic cleavage of FDA and result in the production of fluorescence even when bacterial growth is inhibited or bacteria are killed. This will cause a false negative result to be reported.

Secondly, the fluorescence generated by live bacteria could be quenched by natural product samples. Clarke and colleagues [Clarke *et al*., 2001] have reported that some common media could quench the fluorescence of fluorescein, and the quenching effect increased in more coloured and particulate media. Since many of the extracts of plants are coloured (*e.g.*, due to the presence of chlorophylls and phenolic compounds), and sometimes particulate (*e.g.*, due to the forming of precipitates), the fluorescence generated by live bacterial cells could be quenched by the test samples, which would lead to a false positive result.

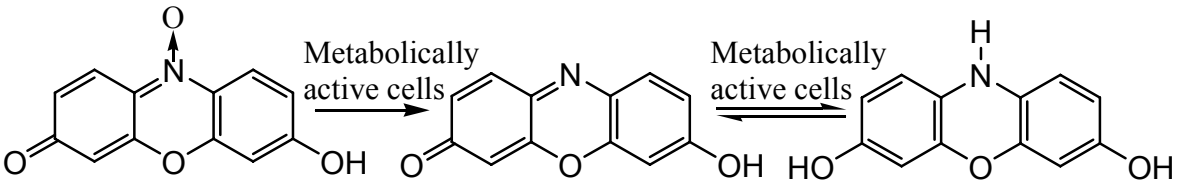
Thirdly, the 10 fold diluted medium used in the FDA assay reduced the growth of bacteria. Wanandy [Wanandy, 2005] has shown that the 10 fold diluted BPYN medium stimulated the esterase activity of *P. aeruginosa*, however, decreased fluorescence production by *S. aureus* and especially *E. coli* did occur, which indicated compromised bacterial growth under the reduced nutrient condition. With the reduced bacterial growth (in other words, lower bacterial density), antibacterial agents tested by the FDA assay may appear to have a "better" inhibitory activity.

With the concerns of the above issues that might affect the accuracy of the MIC determination of medicinal plant extracts by the FDA assay, it was considered useful to employ another antibacterial assay method to compare the results obtained on the same samples. Therefore, another MIC assay, the resazurin antibacterial assay, was incorporated in this study to determine and compare the MIC values of some antibiotics and medicinal plant samples.

3.7. Resazurin antibacterial assay

Resazurin (also known as Alamar Blue or CellTiter-Blue^{TM}) is a redox indicator that is blue (non-fluorescent) under oxidised conditions, and can be irreversibly reduced by metabolically active cells to the pink-coloured fluorescent compound resorufin and further reduced reversibly to the colourless non-fluorescent compound hydroresorufin (Figure 3.9) [O'Brien *et al*., 2000; Mann and Markham, 1998]. Resazurin has been commonly used in the dairy industry since the mid 20th century to monitor the microbiological quality of milk products. It has also been used in many cytotoxicity and proliferation tests and antimicrobial assays in recent years [McBride *et al*., 2005; O'Brien *et al*., 2000; Riss and Moravec, 2003; Mann and Markham, 1998; Shiloh *et al*., 1997]. The reduction of resazurin to resorufin by live cells is believed to be due to oxygen depletion during metabolism or enzyme activity (such as the diaphorase enzyme), but the mechanisms have not been confirmed [O'Brien *et al*., 2000; Zalata *et al*., 1998; Chu *et al*., 2000].

Figure 3.9. Reduction of resazurin to resorufin and hydroresorufin.



Resazurin Resorufin Hydroresorufin

Typically, antimicrobial assays using resazurin as the indicator rely on identifying the endpoint where a distinguishable colour change from blue (indicating no microbial growth) to pink (growth) occurs. The MIC has been reported as the lowest concentration at which a blue colour is maintained [Mann and Markham, 1998]. The resazurin antimicrobial method has been used successfully in determination of MIC values of natural products [Mann and

Markham, 1998]. The production of resorufin can be measured by absorbance (570 nm) or by fluorescence (530 nm excitation and 590 nm emission) [O'Brien *et al*., 2000]. However, since the further reduction of resorufin to colourless hydroresorufin by live cells can cause a decrease in the absorbance or fluorescence reading and lead to an underestimation of microbial growth, the reliability of the MIC value determined by the instrumental measurement has been questioned [Mann and Markham, 1998; O'Brien *et al*., 2000]. Visual determination of the colour change has been used by many researchers, and the results obtained have been in excellent agreement with turbidity measurements and cell counts [Montoro *et al*., 2005; Espinel-Ingroff *et al*., 1995; Mann and Markham, 1998; Davey *et al*., 1998].

To allow comparison to the FDA assay, the resazurin antibacterial assay was conducted to determine MIC values of gentamicin, tetracycline and chloramphenicol against *S. aureus* and *E. coli* (same antibiotics and bacteria as in the FDA assays). The resazurin assay used was based on the general broth microdilution method for the testing of aerobic bacteria [Jorgensen and Turnidge, 2003] and included the standard assay medium Mueller-Hinton broth (which is a full-strength medium for optimal bacterial growth), an inoculum density of ~ 5 x 10^5 CFU/ml, two fold dilutions of test samples, and 16 to 18 hours (overnight) incubation of drug-inoculum mixture at 37°C. Resazurin was added after the overnight incubation, and the colour change was assessed visually following an additional two hours incubation.

MIC values of gentamicin, tetracycline and chloramphenicol against *S. aureus* and *E. coli* measured using the resazurin and optimised FDA assay methods are reported in Table 3.2, along with published values obtained with the NCCLS method [NCCLS, 1999].

Table 3.2. Comparison of MIC results from the FDA and resazurin assays.

Antibiotics	Minimum Inhibitory Concentration (μg/ml)					
	E. coli			*S. aureus*		
	FDA	Resazurin	NCCLS	FDA	Resazurin	NCCLS
Gentamicin	0.5	0.5	0.25-1.0	1.0	1.0	0.12-1.0
Tetracycline	0.5	2.0	0.5-2.0	0.2	0.5	0.12-1.0
Chloramphenicol	5.0	10.0	2.0-8.0	5.0	10.0	2.0-8.0

The MIC values of the antibiotics determined using the resazurin assay agreed well with published data. The MICs of chloramphenicol (10 μg/ml) seemed to be higher than the reference values. This was due to the concentrations of the sample used in the experiment (50, 20, 10, 5.0, 2.0, 1.0, 0.5, 0.2 and 0.1 μg/ml). As is the case in all antimicrobial methods using doubling dilutions, the "real" MIC is somewhere between the concentration inhibiting the microbial growth (10 μg/ml in this experiment) and the next lowest concentration tested (5.0 μg/ml) [Jorgensen and Turnidge, 2003].

The MIC values of gentamicin for *S. aureus* and *E. coli* measured by the resazurin method matched exactly with those by the FDA assay. As to tetracycline and chloramphenicol, the FDA assay gave MIC results that were 1-2 dilutions lower than those tested with the resazurin assay. Although one dilution difference in results in antimicrobial assays is considered insignificant [Moore *et al.*, 2003], comparison of the results obtained by these two methods still suggested a trend that the FDA assay might present lower MIC results, in other words, "better" antibacterial activity, possibly because of the limited nutrient content in the medium used.

Both the resazurin and FDA assay demonstrated in this experiment to be accurate in the measurement of MIC values of antibiotics. In order to assess the accuracy and applicability of the FDA assay in the testing of medicinal plants (considering the potential interference by the medicinal plant extracts), both the resazurin and FDA assays were employed in this study for the determination of MIC values of medicinal plant extracts, fractions and pure compounds, and their results were compared. This is further described in Chapters 4 and 5.

3.8. Conclusions and future directions

There is an urgent need for both new antimicrobial and anti-inflammatory agents due respectively to drug-resistance and the side effects of current anti-inflammatory drugs. Australian Aboriginal medicinal plants are a valuable source for the exploration of novel bioactive compounds.

This chapter described the background and procedures for the cyclooxygenase and lipoxygenase inhibitor screening assays and the FDA and resazurin antimicrobial assays that were subsequently used to evaluate the biological activities of medicinal plants that have been traditionally used by Australian Aboriginal people. It also described the optimisation of a fluorescence based antibacterial assay method (FDA assay), in terms of the optimal incubation time, appropriate inoculum density and solvent effects, to make it suitable for the screening of antibacterial agents. The resazurin antibacterial assay and broth microdilution assay have also been used in this study to avoid any possible problems related to the FDA assay. These biological assays have been used in the bioactivity-oriented isolation of anti-inflammatory and antimicrobial agents from two Australian Aboriginal medicinal plants in this PhD study. The exploration of novel antibacterial and

anti-inflammatory compounds from *Eremophila sturtii* is described in chapter 4 and *Exocarpos aphyllus* in chapter 5.

3.9. Experimental

3.9.1. Reagents and equipment

All reagents were from commercial sources and used without purification unless otherwise noted. All organic solvents (AR grade) were distilled before use. Milli-Q water (Millipore) was used in all antimicrobial experiments and for the media preparation. Sterile filters (Millex®-GP, 0.22 μm, Millipore) were used for filter sterilisation. Optical density (absorbance) of bacterial cultures at 600 nm was measured by a Varian Cary 1 BIO UV-visible spectrophotometer. Fluorescence intensity (λ_{ex} 405 nm and λ_{em} 520 nm) and absorbance (405 nm, 492 nm and 590 nm) were recorded by a BMG Fluostar Galaxy microtitre plate reader.

3.9.2. Microorganisms and inoculum preparation

The Gram positive bacterium *Staphylococcus aureus* ATCC 9144 (obtained from CDS Reference Laboratory, Department of Microbiology, The Prince of Wales Hospital, NSW), the Gram negative *Escherichia coli* JM109 and *Pseudomonas aeruginosa* ATCC 27853, and the yeast *Candida albicans* AMMRL 36.42 (supplied by Associate Professor Michael Gillings, Department of Biological Sciences, Macquarie University, NSW) were used in the antimicrobial assays.

Cultures were stored at -78°C in Muller Hinton broth (or Sabouraud dextrose broth for *C. albicans*) containing 10% v/v glycerol. For the FDA and the resazurin assays, single colonies of *S. aureus*, *E. coli* and *P. aeruginosa* from Mueller-Hinton agar (Becton Dickinson) were inoculated into 1/10 BPYN or Mueller-Hinton broth (Becton Dickinson) and incubated overnight at 37°C. For the broth microdilution assay for *C. albicans*, single colonies from Sabouraud dextrose agar (Oxoid) were inoculated into Sabouraud dextrose broth (Oxoid) and incubated overnight at 37°C. The optical density of each microorganism was adjusted to 0.08 at 600 nm, and a 1/100 dilution of the culture was used in the antimicrobial assays.

3.9.3. Medium preparation for antimicrobial assays

1/10 BPYN medium: Bacteriological peptone (Oxoid) 2 g, yeast extract (ICN and MP Biomedicals) 1 g, and sodium chloride (Univar) 1 g were added to 990 ml Milli-Q water and autoclaved for 20 minutes at 121°C. After cooling, 10 ml of filter sterilised 1 M *N*,*N*-bis(2-hydroxyethyl)-2-aminomethanesulfonic acid (BES) buffer (Sigma) was added to make the final medium with 10 mM BES buffer. The pH value was adjusted to ~ 7.

Mueller-Hinton broth and Sabouraud dextrose broth were prepared according to manufacturers' instructions.

3.9.4. Growth of *E. coli* in 1/10 BPYN and Mueller-Hinton broth

Overnight culture suspensions of *E. coli* in 1/10 BPYN medium and Mueller-Hinton broth were diluted with corresponding fresh media to have an absorbance of 0.08 at 600 nm. 200

μl of inocula were added to a clear flat-bottom 96-well microtitre plate and incubated at 37°C. Uninoculated 1/10 BPYN medium and Mueller-Hinton broth were used as the controls. The absorbance at 590 nm was measured at hourly intervals.

3.9.5. Growth curve of *E. coli* in 1/10 BPNY with or without gentamicin

An overnight culture of *E. coli* in 1/10 BPYN medium was diluted with fresh medium to have an absorbance of 0.08 at 600 nm. 175 μl of the inoculum was added to wells of a black flat-bottom 96-well microtitre plate. 20 μl of sterile Milli-Q water or 1 μg/ml gentamicin solution was added to the inoculum. Uninoculated medium with and without gentamicin were used as the controls. The plate was incubated at 37°C. FDA solution (5 μl, 0.2% w/v in acetone) was added to wells at hourly intervals. The fluorescence intensity (405 nm excitation and 520 nm emission) was measured after an additional 3 hours incubation.

3.9.6. Determination of inoculum density

Overnight culture suspensions of *E. coli* and *S. aureus* in 1/10 BPYN were diluted with fresh medium to have an absorbance of 0.08 at 600 nm. 10 fold serial dilutions of these culture suspensions were used for the colony count. 100 μl of each dilution was spread on the surface of a Mueller-Hinton agar plate and incubated at 37°C. Colonies were counted after the overnight incubation.

3.9.7. Optimised FDA assay procedure

The FDA assay was performed in black flat-bottom 96-well microtitre plates (Greiner). The 1/10 BPYN medium was used in all FDA assay experiments for the growth of *S. aureus*, *E. coli* and *P. aeruginosa*. The inoculum (175 μl) and test sample (20 μl) were added into a microtitre plate and incubated overnight (15-18 hours) at 37°C. Liquid medium (175 μl) and sample (20 μl) were used as the background control. Samples and controls were assayed in triplicates. FDA solution (2 mg/ml in acetone, 5 μl) was added into each well followed by an additional 3 hours incubation. Fluorescence intensity values were recorded at λ_{ex} 405 nm and λ_{em} 520 nm. The lowest concentration of sample at which no increase of fluorescence intensity was observed (compared to the background control) was recorded as the minimum inhibitory concentration (MIC).

3.9.8. Resazurin assay procedure

The resazurin assay was performed in clear flat-bottom 96-well microtitre plates (Greiner). Muller-Hinton broth was used in all resazurin assay experiments for the growth of *S. aureus*, *E. coli* and *P. aeruginosa*. The inoculum (175 μl) and test sample (20 μl) were added into a microtitre plate and incubated overnight (15-18 hours) at 37°C. Liquid medium (175 μl) and sample (20 μl) were used as the background control. Samples and controls were assayed in triplicates. An aqueous solution of resazurin (1 mg/ml, 5 μl) was added into each well followed by an additional 2 hours incubation. The lowest concentration of sample at which a blue colour was maintained was recorded as the minimum inhibitory concentration (MIC).

3.9.9. Validation of the optimised FDA assay

Antibiotics used in the validation of the FDA assay were dissolved in sterile Milli-Q water (for gentamicin and tetracycline) or acetone (for chloramphenicol) to make 1 mg/ml (w/v) stock solutions. The stock solutions were diluted with sterile Milli-Q water to give a series of dilutions. Final concentrations of the antibiotics tested in the FDA and resazurin assays were 50, 20, 10, 5.0, 2.0, 1.0, 0.5, 0.2 and 0.1 μg/ml for tetracycline and chloramphenicol, and 5.0, 2.0, 1.0, 0.5, 0.2, 0.1 and 0.05 μg/ml for gentamicin. Acetone and Milli-Q water were both included as controls.

3.9.10. Broth microdilution assay procedure

The broth microdilution assay was performed in clear flat-bottom 96-well microtitre plates (Greiner) and was used for the test on *C. albicans*. Sabouraud dextrose broth was used for the growth of *C. albicans*. The inoculum (180 μl) and test sample (20 μl) were added into a microtitre plate and incubated for 24 hours at 37°C. Liquid medium (180 μl) and sample (20 μl) were used as the background control. Samples and controls were assayed in triplicates. The absorbance of each well was recorded at 492 nm. The lowest concentration of sample at which no increase of absorbance was observed (compared to the background control) was recorded as the minimum inhibitory concentration (MIC).

Chapter 4. Ethnopharmacological study of *Eremophila sturtii*

This chapter describes the biological and chemical investigations of an Australian Aboriginal medicinal plant, Eremophila sturtii*, under the guidance of its ethnomedicinal uses. The isolation of novel bioactive compounds through bioassay-guided fractionation is presented.*

4.1. Introduction

As introduced in Chapter 2, *Eremophila sturtii* R. Br. has been used by Australian Aboriginal people to treat skin infections, diarrhoea, respiratory infections, sore eyes and general illness [Barr *et al*., 1988 and 1993; Palombo and Semple, 2001; Goddard and Kalotas, 2002; Smith, 1991]. There is no information on the bioactive compounds of this plant species in published literature. This chapter describes the antimicrobial and anti-inflammatory activities of this plant and the characterisation of its bioactive constituents.

Eremophila is a large genus with over 200 species, and many plants of this genus have been used medicinally by Australian Aboriginal people [Ghisalberti, 1994b]. From the chemotaxonomy point of view, it is useful to study the literature for the bioactive components discovered from related species. A literature survey of the *Eremophila* genus, with a special focus on the ethnomedicinal information and the bioactive compounds, is presented below.

4.2. General review of *Eremophila* species

Eremophila is one of the three genera (*Eremophila*, *Myoporum* and *Bontia*) of the Myoporaceae family [Ghisalberti, 1994a]. The name *Eremophila* comes from the Greek: *eremos*, desert; *phileo*, to love, and this genus is very characteristic of the dry interior of the continent, with two-thirds of the *Eremophila* species growing in Western Australia [Cribb and Cribb, 1981; Ghisalberti, 1995]. In general the plants are shrubs or trees with alternate leaves and two-lipped flowers of various colours. *Eremophila* is restricted to Australia [Cribb and Cribb, 1981].

Eremophila species are known to the Australian Aboriginal people as fuchsia bushes or dogwood [Low, 1990], or by Aboriginal names such as *budda* and *Eurah* (Kamilaroi community). The plants are important to the Aboriginal people as medicines and have been prescribed as the "number one medicine" for colds, fever, sores, wounds, headaches, scabies, and almost any kind of ailment [Barr *et al*., 1988; Latz, 1995; Cribb and Cribb, 1981; Low, 1990]. An elder woman in the Kamilaroi Aboriginal community described *Eremophila* as "a shrub … about 3-7m high. It is used for asthma, sores and cuts. The leaves are boiled in water and the patient drank the liquid to cure asthma. More commonly, the liquid was used to wash the infected parts of the body. It could be rubbed into sore eyes, bald patches on the head, to encourage growth of new hair" [Kneale, 1984].

Although various biological activities such as antibacterial, antiviral, neurological, cardioactive and anti-inflammatory activities have been reported with extracts of *Eremophila* species [Palombo and Semple, 2001, 2002; Semple *et al*., 1998; Pennacchio *et al*., 2005; Rogers *et al*., 2000, 2001, 2002; Sweeney *et al*., 2001], only a few bioactive components have been characterised [Shah *et al*., 2004; Pennacchio *et al*., 1996]. A

synopsis of ethnobotanical information, biological activities and bioactive constituents of important species of the Genus *Eremophila* is given below.

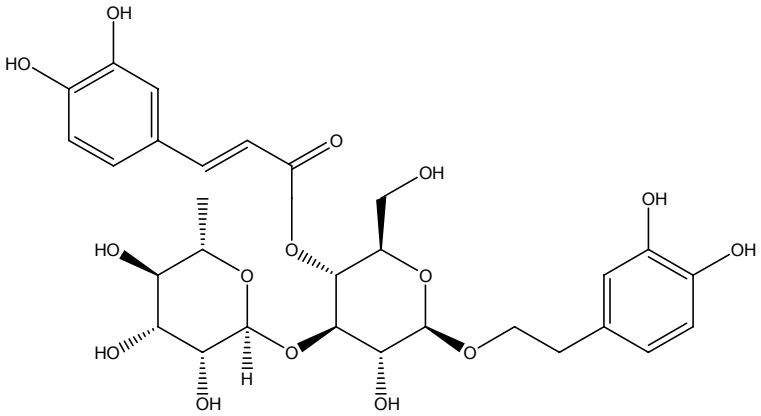
Eremophila alternifolia R. Br.

Eremophila alternifolia is commonly known as narrow-leaf fuchsia bush. Aboriginal people have taken the decoctions of the dried leaves of *E. alternifolia* for coughs, colds, fever, internal pain, and general malaise [Barr *et al*., 1988; Isaacs, 1987]. An infusion of the fresh leaves has been used as a rubbing medicine to treat skin conditions or taken internally for various ailments. It is believed that this plant is able to encourage deep sleep [Latz, 1995].

The leaves of *E. alternifolia* have been found to contain up to 4% of essential oil, with the main components being fenchone and limonene [Barr *et al*., 1988].

An ethanol extract of the stems of *E. alternifolia* has been reported to cause weak inhibition of human cytomegalovirus (HCMV), which is a causative virus of disease in immunosuppressed individuals and is associated with respiratory infections [Semple *et al*., 1998]. An ethanol extract from leaves of *E. alternifolia* was found to have antibacterial activity against methicillin-resistant *Staphylococcus aureus* (MRSA) [Palombo and Semple, 2002]. Given that *S. aureus* is one of the inhabitants of the human upper respiratory tract and is associated with pathological conditions including pneumonia [Brock *et al*., 1994], the inhibitory activity of extracts of *E. alternifolia* against both *S. aureus* and HCMV provides support for the traditional uses of this plant as a remedy for respiratory conditions. The bioactive constituents responsible for the antiviral and antibacterial activities are still unknown.

The methanol extract of leaves of *E. alternifolia* has been found to have cardioactivity, from which the cardioactive compound verbascoside was isolated [Pennacchio *et al*., 1996 and 2005].



Verbascoside

<u>*Eremophila duttonii* R. Br.</u>

Eremophila duttonii is known by its common name as red poverty bush, kangaroo bush or harlequin fuchsia bush [Barr *et al*., 1988; Isaacs, 1987]. A decoction of fresh leaves of *E. duttonii* has been used by Aboriginal people in Northern Territory as a wash for sores, cuts and larger wounds, as well as a lotion for inflamed and sore eyes. It has also been taken internally to treat sore throats [Barr *et al*., 1988].

Eremophila duttonii has been reported to be the most active antibacterial plant in the *Eremophila* genus, with bactericidal activity against many Gram positive bacteria such as *Bacillus cereus, Enterococcus faecalis, Staphylococcus aureus* and *Streptococcus pyogenes,* as well as drug-resistant bacteria such as methicillin-resistant *S. aureus* (MRSA) and vancomycin-resistant enterococci (VRE) [Palombo and Semple, 2001 and 2002]. Its active constituents have not been fully determined, but one antibacterial compound has been tentatively identified as a carotenoid [Shah *et al*., 2004].

Eremophila freelingii R. Br.

Eremophila freelingii is commonly known as emu-bush or rock fuchsia bush [Low, 1990]. Leaves of *E. freelingii* have been used by Aboriginal people to make an antiseptic wash for the treatment of cuts, open sores and scabies, and have been used in steam baths to relieve symptoms of colds or flu, such as fever and congestion in the head and chest [Barr *et al*., 1988]. A decoction of the leaves has been drunk for sores, headaches, chest pains and diarrhoea [Cribb and Cribb, 1981; Latz, 1995; Low, 1990].

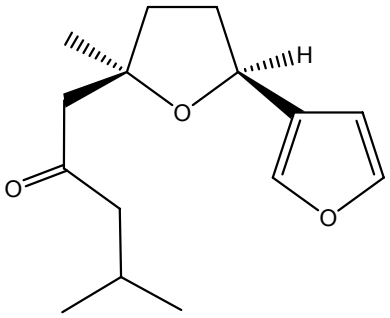
Essential oil mainly composed of α-pinene has been found in the leaves of *E. freelingii* [Barr *et al*., 1988].

Methanol and dichloromethane extracts of *E. freelingii* have been reported to inhibit adenosine diphosphate (ADP) induced human platelet [^{14}C]5-hydroxytryptamine release *in vitro* [Rogers *et al*., 2000]. This biological function of *E. freelingii* is consistent with its traditional medicinal use as a therapeutic agent for migraine and headache [Rogers *et al*., 2000].

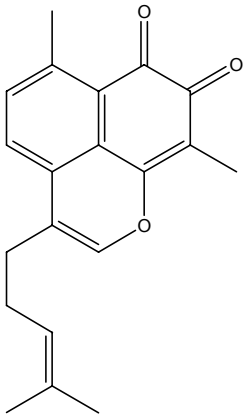
Eremophila latrobei R. Br.

Eremophila latrobei is generally known as native fuchsia or crimson fuchsia bush. A decoction of the leaves of *E. latrobei* has been used by Aboriginal people as a wash for scabies, or drunk or rubbed on the body for the treatment of colds [Low, 1990; Latz, 1995; Isaacs, 1987].

The ethanol extract of the stems of *E. latrobei* has been reported to be effective in inhibiting the Australian endemic Ross River virus, the causative agent of the human disease Ross River fever [Semple *et al*., 1998]. The essential oil of *E. latrobei* has been found to contain a high level of the toxic furanoid sesquiterpene ketone, (-)-ngaione [Hegarty *et al*., 1970]. The antimicrobial compound biflorin has also been isolated from the leaf resin of *E. latrobei* [Forster *et al*., 1986; d'Albuquerque *et al*., 1962].



(-)-Ngaione



Biflorin

<u>*Eremophila longifolia* R. Br.</u>

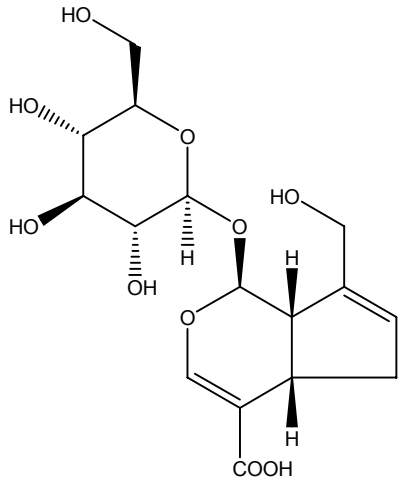
Eremophila longifolia is commonly known as berrigan, emu bush, native plum-tree, or weeping emu bush. It can be found in all mainland states of Australia [Cribb and Cribb, 1981; Latz, 1995; Barr *et al*., 1988].

The decoction or liniment of crushed leaves of *E. longifolia* has been applied on the skin to treat scabies, sores, cuts and boils [Low, 1990; Barr *et al*., 1988; Latz, 1995; Cribb and Cribb, 1981; Isaacs, 1987]. The decoction has been used for general pain and illness including colds and fever, and has also been used as an eye wash [Low, 1990]. Infusions of the fresh leaves have been used for headaches and insomnia [Low, 1990].

Eremophila longifolia is an important medicine to women. Steam from the burnt leaves has been used after childbirth to "smoke" mothers and their newborn babies to stop the mother's bleeding and to strengthen the babies [Isaacs, 1987, Latz, 1995].

The major components of the essential oil of the leaves of *E. longifolia* have been found to be *α*-pinene, *β*-pinene and limonene [Barr *et al*., 1988].

Geniposidic acid, isolated from the methanol extract of *E. longifolia* leaves, has been reported to demonstrate significant cardioactive effects [Pennacchio *et al*., 1996; Rogers *et al*., 2000].



Geniposidic acid

Other *Eremophila* species

In addition to the *Eremophila* species described above, other species of this genus have been used medicinally by the Aboriginal people. *Eremophila gilesii*, *E. goodwinii*, *E. dalyana* and *E. paisleyi* have been used for washing body sores and scabies [Latz, 1995; Cribb and Cribb, 1981; Isaacs, 1987]; *E. bignoniiflora* has been used as a laxative, and a medicine for venereal disease [Barr *et al*., 1993; Low, 1990]; *E. neglecta* (*E. calycina*) and

E. mitchellii have been used to smoke or steam patients to treat rheumatic pain [Low, 1990; Latz, 1995]; and *E. maculata* has been used as a cold remedy [Low, 1990; Cribb and Cribb, 1981]. The methanol extract of the leaves of *E. maculata* has been reported to have antibacterial activity against the Gram-positive bacteria *Staphylococcus aureus*, *Streptococcus pyogenes* and *Bacillus cereus* at a concentration of 10 mg/ml [Pennacchio *et al*., 2005], but the bioactive compounds have not been described.

4.3. Bioassay-guided chemical and biological investigations of *E. sturtii*

The ethnobotanical research had revealed the customary medicinal use of *E. sturtii* as a treatment for skin conditions, diarrhoea, respiratory infections, sore eyes and general sicknesses (refer to Section 2.2.4.1 for details). The customary medicinal uses of *E. sturtii* suggested that this plant might possess antimicrobial and/or anti-inflammatory properties, since skin infections are generally caused by microbial invasions and often involve inflammatory responses [Brock *et al*., 1994; Greenwood *et al*., 2002]. Characterisation of compounds with antimicrobial and/or anti-inflammatory activities from *E. sturtii* through bioassay-guided fractionation is described below.

4.3.1. Antimicrobial and anti-inflammatory activities of the crude extract and fractions of *E. sturtii*

Fresh foliage (648 g) of *E. sturtii* collected from Lightning Ridge, New South Wales was extracted with 70% (v/v) aqueous ethanol to give a crude extract. The dried crude extract (94 g, 14.5% yield) was examined against the human pathogens *Staphylococcus aureus*, *Escherichia coli, Pseudomonas aeruginosa* and *Candida albicans* for its antimicrobial activity, and against the inflammation pathway enzymes COX-1, COX-2 and 5-LO for its

anti-inflammatory activity. The crude extract exhibited antibacterial activity against *S. aureus* with a minimum inhibitory concentration (MIC) of 1.25 mg/ml in the resazurin assay, and of 1.0 mg/ml in the FDA assay, but was ineffective against *E. coli*, *P. aeruginosa* and *C. albicans* at concentrations up to 10 mg/ml, as determined by both assays. The crude extract inhibited COX-1 and COX-2 by 95% and 89%, respectively, at 2 mg/ml. No inhibition of 5-LO was observed at this concentration.

Crude extracts of medicinal plants are generally screened for antimicrobial and anti-inflammatory activities at concentrations between 0.1 and 10 mg/ml [*e.g.*, Kone *et al*., 2004; Camporese *et al*., 2003; Li *et al*., 2003; Shale *et al*., 1999]. Extracts showing activities at concentrations lower than 2 mg/ml in the preliminary screenings are generally considered of interest [*e.g.*, Palombo and Semple, 2001; Lindsey *et al*., 1999], although some researchers have suggested lower cut-off concentrations such as 1 mg/ml as being significant [Rios and Recio, 2005]. Given the positive antibacterial and anti-inflammatory activities demonstrated by the crude extract of *E. sturtii*, further investigations were carried out to isolate the bioactive compounds from this plant.

The crude extract was suspended in water and extracted sequentially with hexane, ethyl acetate and *n*-butanol to give four partition fractions (5.6, 35.2, 36.8 and 7.0 g, respectively). These partition fractions were also examined for their antimicrobial and anti-inflammatory activities. The ethyl acetate partition (Es-EtOAc) was found to be the most active, having an MIC of 0.25 mg/ml against *S. aureus* in both the resazurin and FDA assays (Table 4.1), and 88% and 66% inhibition of COX-1 and COX-2, respectively, at 2 mg/ml. The hexane, *n*-butanol and water partitions also exhibited COX-1 and COX-2 inhibitory activities at 2 mg/ml (Table 4.2). No inhibition of *P. aeruginosa* or *C. albicans* in the antimicrobial assays, nor of 5-LO in the anti-inflammatory assay, was observed with

the partition fractions. The ethyl acetate partition showed inhibition against *E. coli* at 4 mg/ml in the FDA assay, but this activity was not observed in the resazurin assay. Antimicrobial and anti-COX activities of the crude extract and partition fractions are shown in Table 4.1 and 4.2.

The thin layer chromatography (TLC) showed that almost all components in the hexane partition were found in the ethyl acetate partition. To avoid repetitive separation of the same constituents, the hexane partition was combined with the ethyl acetate partition, and the combined fraction was used in subsequent experiments, referred to as the ethyl acetate fraction. The FDA assay on the partition fractions was conducted after the combining and therefore the activity of the hexane partition was not tested separately.

Table 4.1. Antibacterial activities of the ethanol crude extract and partition fractions of *E. sturtii*.

Extract	MIC (mg/ml)			
	Resazurin assay		FDA assay	
	S. aureus	*E. coli*	*S. aureus*	*E. coli*
Es-crude	1.25	> 4	1	> 4
Es-Hex	0.31	> 2.5	N/T	N/T
Es-EtOAc	0.25	> 4	0.25	4
Es-BuOH	2	> 4	2	> 4
Es-water	4	> 4	4	> 4

N/T: not tested.

Table 4.2. COX inhibitory activities of *E. sturtii* crude extract and partition fractions.

Extract	COX-1 inhibition (%)	COX-2 inhibition (%)
Es-crude	95	89
Es-Hex	48	88
Es-EtOAc	88	66
Es-BuOH	77	43
Es-water	69	14

All samples were tested at 2 mg/ml.

The antibacterial activities of the crude extract and fractions of *E. sturtii* were mainly found against the Gram positive bacterium *S. aureus*, and only weak inhibition against Gram negative *E. coli* was observed with the ethyl acetate fraction in the FDA assay. The greater resistance of Gram negative bacteria to plant extracts is not unusual. Palombo and Semple investigated 56 extracts of 39 Australian medicinal plants for antimicrobial activities, amongst which only one extract showed partial inhibition of Gram negative bacteria, whereas all extracts were active against at least one Gram positive bacterium [Palombo and Semple, 2001]. This is because of the differences in cell wall structures between Gram positive and negative bacteria. The Gram positive bacteria generally have a thick and relatively homogeneous layer of peptidoglycan as the cell wall (peptidoglycan constitutes 40-80% of the total weight of the wall), while Gram negative bacteria have a more complex multilayered structure composed of an outer membrane on top of a thin peptidoglycan layer [Volk *et al*., 1996]. This membrane contains many associated proteins whose function includes selective permeability and attachment. The outer membrane protects the peptidoglycan layer, and prevents the entrance of many antibiotics [Greenwood *et al*., 2002].

The ethyl acetate fraction was separated by flash silica gel column chromatography to give 11 fractions (Es-EtOAc-1 to -11). Fractions Es-EtOAc-4 to -11 were examined for their antimicrobial activities against *S. aureus* and *E. coli* (fractions Es-EtOAc-1 to -3 were not tested due to the unavailability of facilities in the overseas laboratory where separations of these fractions were carried out), and all of these fractions demonstrated activity against *S. aureus*. In the FDA assay, fractions Es-EtOAc-5 to -9 were inhibitory to *E. coli* (Table 4.3).

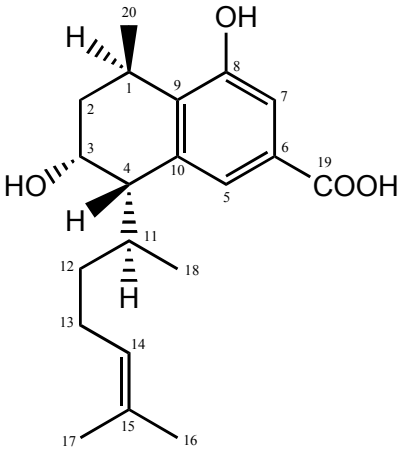
Table 4.3. Antibacterial activities of column chromatography fractions of ethyl acetate fraction of *E. sturtii*.

Extract	MIC (mg/ml)			
	Resazurin assay		FDA assay	
	S. aureus	*E. coli*	*S. aureus*	*E. coli*
Es-EtOAc-4	0.5	> 4	0.25	> 4
Es-EtOAc-5	0.5	> 4	0.5	4
Es-EtOAc-6	0.5	> 4	0.5	4
Es-EtOAc-7	0.125	> 4	0.125	2
Es-EtOAc-8	0.25	> 4	0.25	4
Es-EtOAc-9	0.25	> 4	0.25	4
Es-EtOAc-10	1	> 4	1	> 4
Es-EtOAc-11	4	> 4	4	> 4

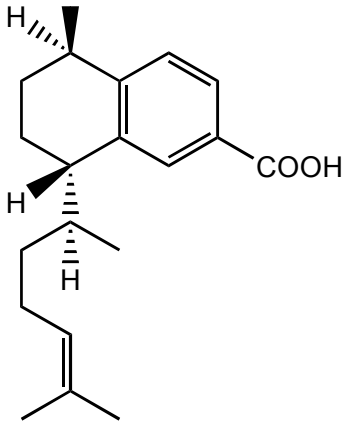
Further separation of fractions Es-EtOAc-1 to Es-EtOAc-5 resulted in the isolation of two novel bioactive compounds, **4.1** and **4.2**, and three bioactive known compounds *β*-sitosterol (**4.3**), sesamin (**4.4**), and 3,6-dimethoxy-5,7-dihydroxyflavone (**4.5**). The bioassay-guided fractionation towards the isolation of these compounds is summarised in Scheme 4.1. The structures of these compounds were elucidated by 1D and 2D NMR and

mass spectra. The NMR and mass spectra of *β*-sitosterol (**4.3**), sesamin (**4.4**), and 3,6-dimethoxy-5,7-dihydroxyflavone (**4.5**) were in agreement with reported data [Kovganko *et al*., 2000; Jayasinghe *et al*., 2003; Buschi *et al*., 1980].

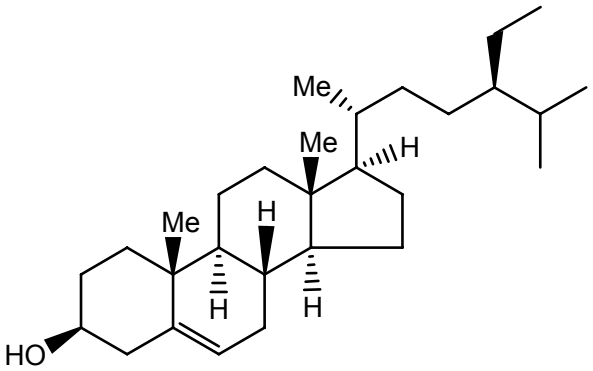
The structural elucidations of the novel compounds **4.1** and **4.2** and their biological activities are described below.



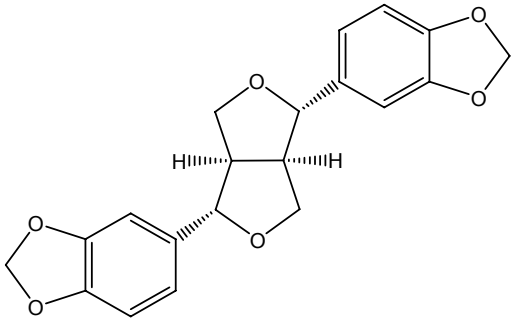
4.1



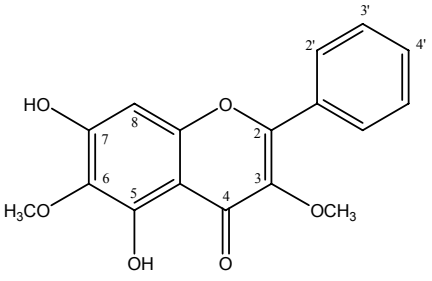
4.2



4.3

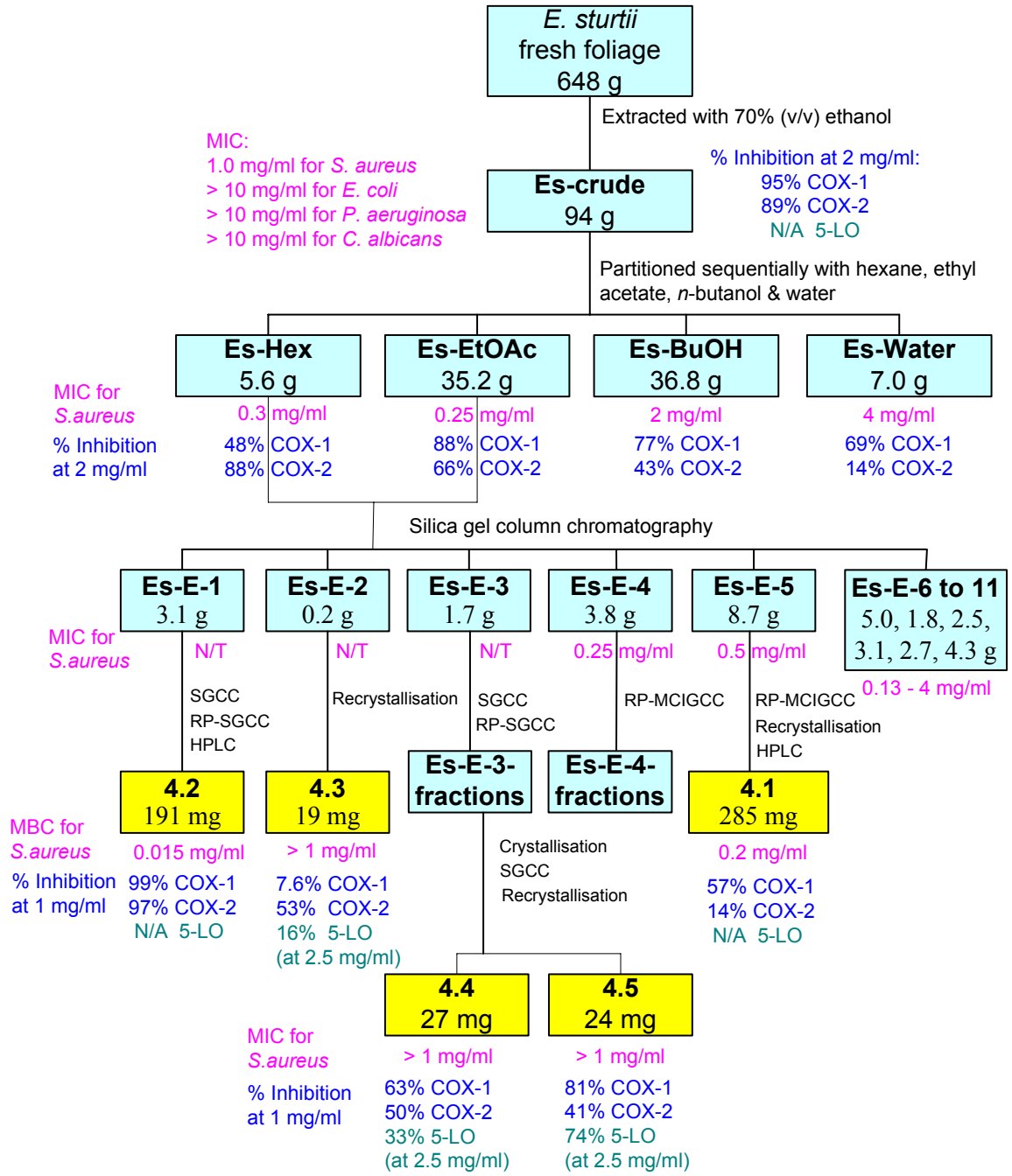


4.4



4.5

Scheme 4.1. Bioassay-guided fractionation of *Eremophila sturtii*.



Antimicrobial activity results (in pink colour) shown in this scheme are from the FDA assay and the broth microdilution assay (for *C. albicans* only). COX inhibitory activity is shown in blue. 5-LO inhibitory activity is shown in green. SGCC: silica gel column chromatography. RP-SGCC: reversed phase silica gel column chromatography. RP-MCIGCC: reversed phase MCI gel column chromatography. N/T: not tested. N/A: not active.

4.3.2. Characterisation of the novel bioactive compounds

Compound **4.1** was isolated as an amorphous white solid, having a molecular formula of $C_{20}H_{28}O_4$ (*m/z* 332.1976, calcd. 332.1988) in the HREIMS. The ^{13}C and DEPT NMR spectra indicated a total of 20 carbons (Table 4.4), with 4 being methyls (δ_C 18.4, 19.7, 22.9 and 26.4), 3 methylenes (δ_C 26.7, 35.5 and 39.7), 3 sp^2 methines (δ_C 113.8, 122.5 and 125.7), 4 sp^3 methines (δ_C 29.1, 30.7, 49.7 and 65.6), and 6 quaternary carbons (δ_C 128.8, 131.2, 134.4, 139.8, 155.6 and 168.5). The ^1H NMR spectrum showed three very broad singlets at δ_H 12.54, 9.58 and 4.70 in DMSO-d_6. These peaks disappeared when D_2O was added.

HMBC correlations showed that two aromatic protons in a *meta* relationship (δ_H 7.19 and 7.07, each *d*, *J* = 1.5 Hz) were located on either side of a quaternary carbon (δ_C 128.8) with a carboxylic acid substituent (δ_H 12.54, *br. s*, δ_C 168.5) and that the aromatic proton at 7.19 ppm was next to a quaternary carbon (δ_C 155.6) with a phenolic group (δ_H 9.58, *br. s*). Two benzylic methine groups (δ_H 3.14, *m*, δ_C 29.1 and δ_H 2.77, *br. d, J* = 4.5 Hz, δ_C 49.7) were found in the ^1H and ^{13}C NMR spectra, with the latter showing a HMBC correlation with the aromatic proton at 7.07 ppm. The HMBC correlations of the aromatic moiety of compound **4.1** are shown in Figure 4.1. The benzylic methine proton at 3.14 ppm, which flanked with a secondary methyl group (δ_H 1.16, *d*, *J* = 6.9 Hz, δ_C 22.9), showed couplings with two protons on a methylene carbon (δ_H 1.88, *ddd*, *J* = 12, ~ 6.5, ~6.5 Hz and δ_H 1.46, *m*; δ_C 35.5) in the COSY spectrum. A hydroxyl bearing methine (δ_H 4.07, *ddd*, *J* = 12, 4.5, ~ 4 Hz, δ_{OH} 4.70, δ_C 65.6) connected the methylene and the benzylic methine group at 2.77 ppm (C-4) to form a bicyclic moiety. The COSY correlations explaining this connection are shown in Figure 4.2.

Figure 4.1. HMBC correlations of the aromatic moiety of compound **4.1**.

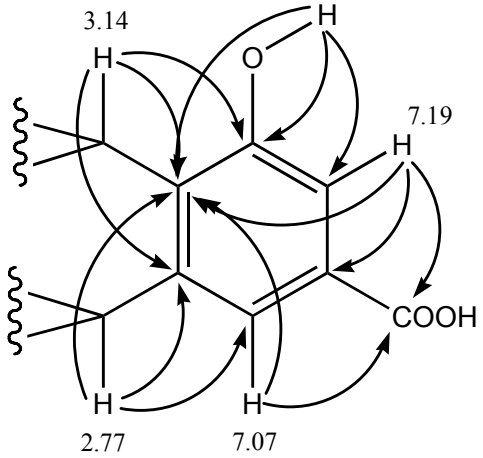
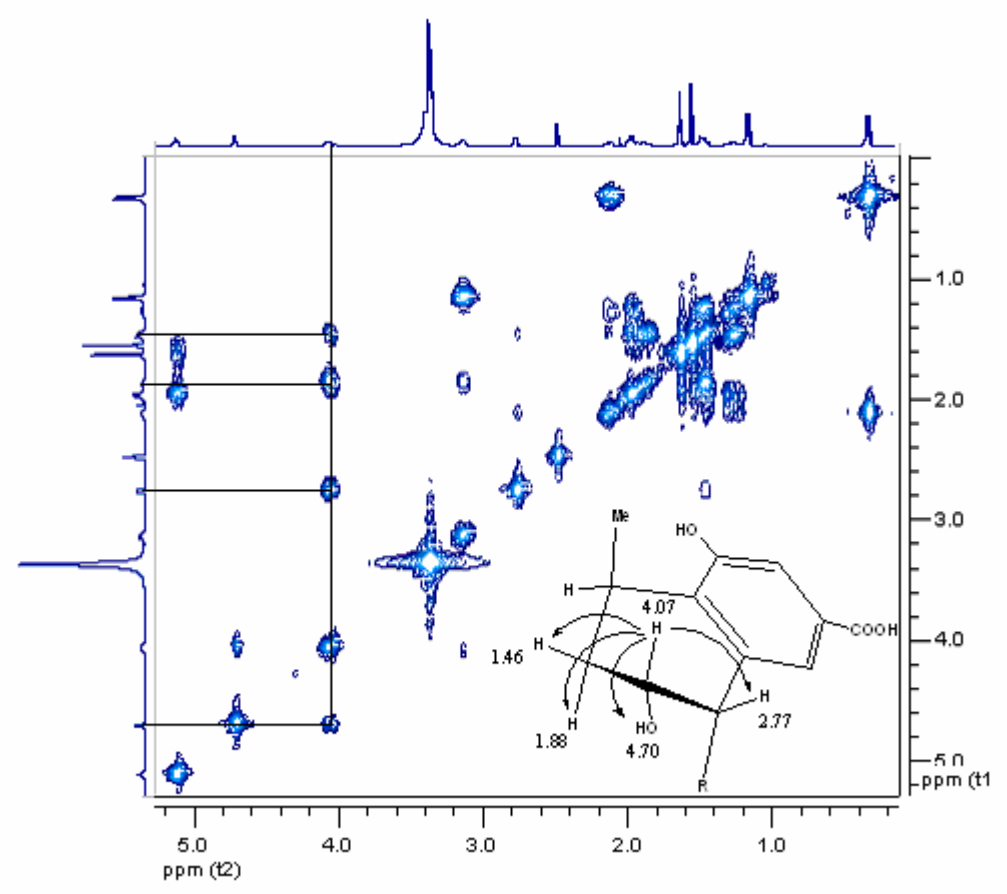


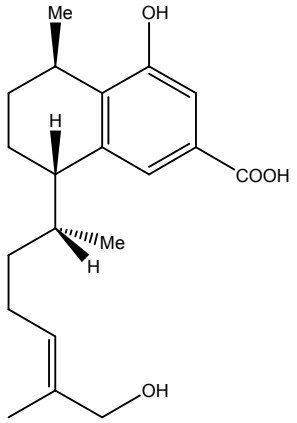
Figure 4.2. COSY correlations of the proton (δ_H 4.07) of compound **4.1**.



The remaining signals in the ^{1}H and ^{13}C NMR spectra were assigned as a side chain connected to the bicyclic moiety at C-4. These signals consisted of a trisubstituted vinyl group (δ_H 5.12, *tqq*, J = 7.1, 1.3, 0.8 Hz, δ_C 125.7 and 131.2), two methylenes (δ_H 1.97, 2H, *m*, δ_C 26.7 and δ_H 1.48, *m*, 1.26, *m*, δ_C 39.7), an alkyl methine (δ_H 2.13, *m*, δ_C 30.7), and

three methyl groups (δ_H 1.55, *d*, *J* = 1.3 Hz, δ_C 18.4; δ_H 1.63, *d*, *J* = 0.8 Hz, δ_C 26.4 and δ_H 0.33, *d*, *J* = 7.0 Hz, δ_C 19.7). The COSY spectrum confirmed the couplings of the olefinic proton with the methylene protons at 1.97 ppm and vinylic methyl protons at 1.55 and 1.63 ppm, and the couplings of the methine proton to the methyl protons at 0.33 ppm and the methylene protons at 1.48 ppm. Attachment of the methine group to the C-4 of the bicyclic system was determined by its HMBC correlations with the hydroxymethine carbon (δ_C 65.6) and a quaternary aromatic carbon (δ_C 139.8).

The spectral data were in agreement with compound **4.1** being a diterpene with a serrulatane skeleton. This compound is a positional isomer of the previously reported dihydroxyserrulatic acid isolated from *Eremophila serrulate* [Croft *et al*., 1979; Forster *et al*., 1986]. To follow the general name used in the previous paper, compound **4.1** was given the trivial name 3,8-dihydroxyserrulatic acid. The assignment of the NMR data of compound **4.1** is shown in Table 4.4.



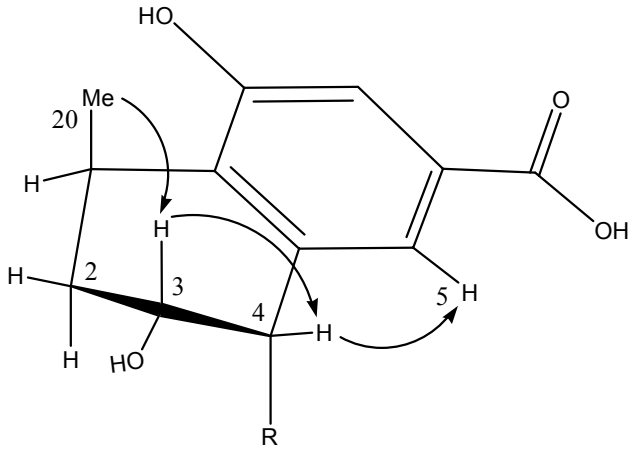
Dihydroxyserrulatic acid

In an attempt to determine the relative stereochemistry of compound **4.1**, the white solid was recrystallised using diethyl ether to give colourless prismatic crystals. X-ray crystal diffraction experiments were organised through another research institute. Unfortunately,

the crystals decomposed and were not suitable for diffraction experiments. Therefore, the determination of the relative stereochemistry of compound **4.1** was mainly based on the 2D NMR data.

The relative stereochemistry of compound **4.1** at C-1, C-3 and C-4 was determined by 2D ROESY NMR experiments and by comparing the coupling constants with published data. In the ROESY spectrum, strong correlations were found between H-3 and H-20, H-3 and H-4, and H-4 and H-5. The coupling constants of $J_{3,4}$ 4.5, $J_{2b,3}$ ~ 4 and $J_{2a,3}$ 12 Hz were consistent with the cyclohexene ring adopting a distorted chair conformation with the methyl group (H-20) and H-3 both pseudoaxial and the 3-OH group and H-4 both pseudoequatorial [Syah and Ghisalberti, 1997; Tippett and Massy-Westropp, 1993]. Furthermore, the significant upfield shift of the H-18 methyl resonance (δ_H 0.33) was in agreement with other serrulatane diterpenes with a secondary hydroxyl group pseudoequatorial at C-3 [Syah and Ghisalberti, 1997; Tippett and Massy-Westropp, 1993]. The nOe correlations of compound **4.1** are shown in Figure 4.3.

Figure 4.3. nOe correlations of compound **4.1**.



R = C_8H_{15} side chain

Table 4.4. NMR data assignments of compounds **4.1** and **4.2**.

	Compound 4.1					**Compound 4.2**	
No.	**δ^{13}C**	**δ^{1}H (*J*)**	**COSY**	**HMBC**	**ROESY**	**δ^{13}C**	**δ^{1}H (*J*)**
1	29.1	3.14, *m*	2a, 20	2, 3, 8, 9, 10, 20	2a, 20	33.7	2.77, *m*
2	35.5	1.88 (2a), *ddd* (12, ~ 6.5, ~ 6.5)	1, 2b, 3	3, 4, 20	1, 2b	31.2	1.90, *m*
		1.46 (2b), *m*	2a, 3	4	2a		1.28, *m*
3	65.6	4.07, *ddd* (12, 4.5, ~ 4)	2a/b, 4		4, 20	22.5	1.82, *m*
							1.48, *m*
4	49.7	2.77, *br. d* (4.5)	3	2, 3, 5, 9, 10, 11, 12, 18	3, 5, 11	43.9	2.77, *m*
5	122.5	7.07, *d* (1.5)		4, 7, 9, 19	4	129.7	7.78, *d* (1.7)
6	128.8					128.9	
7	113.8	7.19, *d* (1.5)		5, 8, 9, 19	OH (δ 9.58)	127.1	7.65, *dd* (8.1, 1.7)
8	155.6			7, 8, 9		128.0	7.31, *d* (8.1)
9	134.4					148.7	
10	139.8					140.4	
11	30.7	2.13, *m*	18	4, 12, 13	4, 18	36.7	2.04, *m*
12	39.7	1.48, *m*	13	13, 18		32.1	0.99, *m*
		1.26, *m*	13	11, 13, 14, 18			
13	26.7	1.97, *m*	12, 14	11, 12, 14, 15		26.5	1.81, *m*
14	125.7	5.12, *tqq* (7.1, 1.3, 0.8)	13	16, 17	13, 16	125.2	4.89, *tqq* (7.0, 1.3, 0.9)
15	131.2					131.7	
16	26.4	1.63, *d* (0.8)		14, 15, 17	14	26.3	1.56, *d* (0.9)
17	18.4	1.55, *d* (1.3)		14, 15, 16		18.3	1.44, *d* (1.3)
18	19.7	0.33, *d* (7.0)	11	4, 11, 12	11	18.7	0.95, *d* (6.7)
19	168.5					168.5	
20	22.9	1.16, *d* (6.9)	1	1, 2, 9	1, 3	22.8	1.21, *d* (6.8)
	-OH	4.70, *br. s*					
	-OH	9.58, *br. s*					
	-COOH	12.54, *br. s*					

Spectra obtained at 400 MHz in DMSO-d_6. HMBC connectivity from H to C. Correlations observed for one bond $J_{C\text{-}H}$ of 145 Hz and long range $J_{C\text{-}H}$ of 10 Hz.

Compound **4.2** was isolated as an amorphous white solid, having a molecular formula of $C_{20}H_{28}O_2$ (*m/z* 300.2090, calcd. 300.2089) in the HREIMS, *i.e.*, two oxygen atoms less than compound **4.1**. The ^{13}C and DEPT NMR identified 20 carbons, with 4 being methyls (δ_C 18.3, 18.7, 22.8 and 26.3), 4 methylenes (δ_C 22.5, 26.5, 31.2 and 32.1), 4 sp^2 methines (δ_C 125.2, 127.1, 128.0 and 129.7), 3 sp^3 methines (δ_C 33.7, 36.7 and 43.9), and 5 quaternary carbons (δ_C 128.9, 131.7, 140.4, 148.7 and 168.5). Compound **4.1** and **4.2** showed similar ^1H NMR spectra, with the main difference being the absence of the phenolic proton (δ_H 9.58) and the hydroxymethyl group (δ_H 4.07 and 4.70) signals for compound **4.2**. Three aromatic protons were found for compound **4.2** (δ_H 7.78, *d*, *J* = 1.7 Hz; 7.65, *dd*, *J* = 8.1, 1.7 Hz and 7.31, *d*, *J* = 8.1 Hz), consistent with a 1, 2, 4 relationship. HMBC correlations confirmed the presence of a carboxylic group (δ_C 168.5) located at C-6, between the two aromatic protons at 7.78 and 7.65 ppm. Two benzylic protons overlapped with each other and gave a multiplet at 2.77 ppm. The bicyclic partial structure of compound **4.2** was confirmed by the coupling of the benzylic protons with two joined methylene groups (δ_H 1.90, *m*, and 1.28, *m*, δ_C 31.2; and δ_H 1.82, *m*, and 1.48, *m*, δ_C 22.5) in the COSY spectrum.

The assignment of the side chain for compound **4.2** was very similar to that of compound **4.1**, with the only significant difference being the methyl group at position 18 resonated at 0.95 ppm. This chemical shift is consistent with other serrulatane diterpenes that lack a secondary hydroxyl group at C-3 [Forster *et al*. 1986; Croft *et al*. 1979 and 1981; Ghisalberti *et al*. 1990]. The assignments of all ^1H and ^{13}C NMR data for compound **4.2** are shown in Table 4.4.

The relative stereochemistry of compound **4.2** was unable to be determined by nOe NMR experiments as both of the benzylic protons (H-1 and H-4) had the same chemical shift and their correlations with vicinal protons were indistinguishable. Due to its similarity to compound **4.1** and other serrulatane diterpenes, the stereochemistry of compound **4.2** was assumed to be the same as that of compound **4.1**. Compound **4.2** is referred to as serrulatic acid to be consistent with the trivial name used by Croft *et al*. [Croft *et al*. 1979].

4.3.3. Biological activities of the novel compounds

The two novel compounds, 3,8-dihydroxyserrulatic acid (**4.1**) and serrulatic acid (**4.2**), were examined for their antimicrobial and anti-inflammatory activities (Table 4.5 and 4.6). Compound **4.1** exhibited bactericidal activity against *S. aureus* with the minimum bactericidal concentration (MBC) of 200 μg/ml in the FDA assay. In the resazurin assay the compound demonstrated inhibition of the growth of *S. aureus* with an MIC of 800 μg/ml. However, the bactericidal activity was not observed with concentrations up to 1 mg/ml. Compound **4.2** demonstrated potent bactericidal activity against *S. aureus* with an MBC of 15 μg/ml (MIC was also 15 μg/ml) in the FDA assay, while in the resazurin assay, it gave an MBC of 30 μg/ml and an MIC of 25 μg/ml (Table 4.5). No activity was observed against *E. coli*, *P. aeruginosa* or *C. albicans* at the highest concentrations tested (1 mg/ml for compound **4.1** and 400 μg/ml for compound **4.2**).

Compound **4.1** had weak COX-1 and COX-2 inhibitory activity with 57% and 14% inhibition, respectively, at 1 mg/ml. Compound **4.2** strongly inhibited both COX-1 and COX-2 at 1 mg/ml with 99% and 97% inhibition, respectively (Table 4.6). IC_{50} values of 27 μg/ml (90 μM) for COX-1 and 73 μg/ml (243 μM) for COX-2 were also obtained for compound **4.2**. The positive control ibuprofen exhibited IC_{50} values of 0.1 μg/ml (0.48 μM)

and 0.6 μg/ml (2.9 μM) for COX-1 and COX-2, respectively. No inhibition of 5-LO was observed with either compound **4.1** or compound **4.2**.

The greater biological activity of compound **4.2** over that of compound **4.1** suggests that the hydroxyl groups present in compound **4.1** are detrimental for activity.

Table 4.5. Minimum bactericidal concentrations (MBCs) and minimum inhibitory concentrations (MICs) of compound **4.1** and **4.2** against *S. aureus*.

Compound	Assay method	MIC (μg/ml)	MBC (μg/ml)
4.1	Resazurin	800	> 1000
	FDA	200	200
4.2	Resazurin	25	30
	FDA	15	15

Table 4.6. Inhibitory activities of compound **4.1** and **4.2** against inflammation pathway enzymes.

Compound	% Inhibition at 1 mg/ml		
	COX-1	COX-2	5-LO
4.1	57	14	N/A
4.2	99	97	N/A

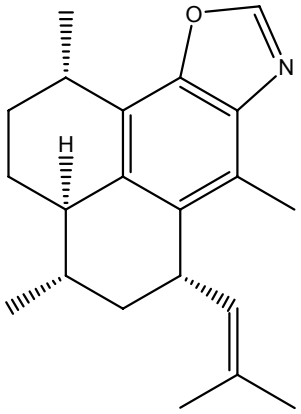
N/A: not active

Since the first isolation of dihydroxyserrulatic acid from *Eremophila serrulata* in 1979, more than twenty serrulatane diterpenes have been discovered [Croft *et al*., 1979], but only a few have been investigated for biological activities, and these compounds were generally isolated from natural resources other than *Eremophila* species. The previously mentioned

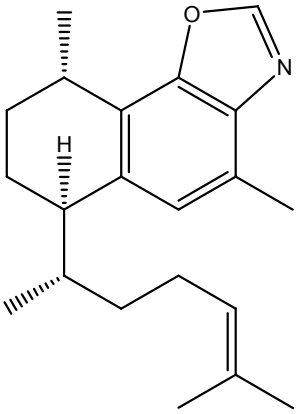
bioflorin, a serrulatane diterpene found in *E. latrobei*, was first isolated from the roots of *Capraria biflora* (Scrophulariaceae), and has been reported to have antibacterial activity of about 0.8 μg/ml against *Bacillus subtilis* [de Lima and d'Albuquerque, 1958]. Its cytotoxic and antioxidant properties were reported recently [Vasconcellos *et al*., 2005].

A few compounds with a serrulatane skeleton, such as pseudopteroxazole, seco-pseudopteroxazole, erogorgiaene and 7-hydroxyerogorgiaene, were isolated from the West Indian gorgonian octocoral *Pseudopterogorgia elisabethae*, and have been demonstrated to have antibacterial activity against *Mycobacterium tuberculosis* H_{37}Rv at concentrations of 12.5 and 6.25 μg/ml [Rodriguez and Ramirez, 2001].

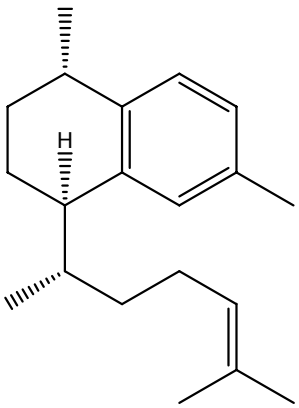
A series of pentoseglycosides of serrulatane diterpenes, namely pseudopterosins, isolated from the above coral, have shown potent anti-inflammatory and analgesic properties [Look *et al*., 1986, Look and Fenical, 1987]. Typical examples of this class of diterpene-pentosides are pseudopterosin A and seco-pseudopterosin A, both possessing anti-inflammatory and analgesic potencies near or exceeding those of several commercial drugs, such as indomethacin, in the *in vivo* as well as *in vitro* bioassays [Look *et al*., 1986, Look and Fenical, 1987]. Although the mechanism of the anti-inflammatory property of these compounds is still undefined, it has been proven not to be due to the inhibition of cyclooxygenases [Look *et al*., 1986]. Antimicrobial activities of the seco-pseudopterosins against a wide variety of bacterial and fungal pathogens have been mentioned, but the only value reported was that of the seco-pseudopterosin A, which demonstrated an MIC against *Staphylococcus aureus* of 8 μg/ml [Look and Fenical, 1987].



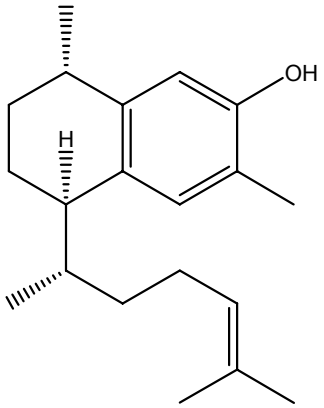
Pseudopteroxazole



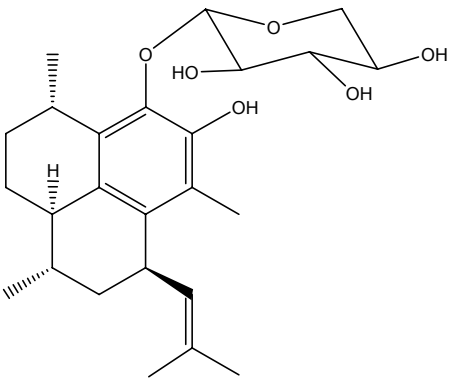
Seco-pseudopteroxazole



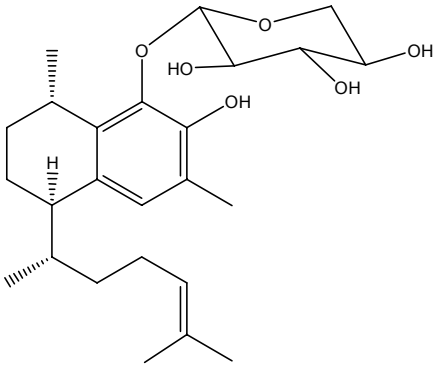
Erogorgiaene



7-Hydroxyerogorgiaene



Pseudopterosin A



Seco-pseudopterosin A

From the above literature survey on the biological activities of serrulatane diterpenes and their derivatives, it can be concluded that this class of natural products possesses important

pharmacological properties. For those serrulatane diterpenes that have already been isolated from *Eremophila* species, their biological activities are worthy of investigation, as compounds with a serrulatane skeleton could be promising as lead structures in the design of new drugs, especially antimicrobial and anti-inflammatory drugs.

4.3.4. Biological activities of known compounds

The known compounds *β*-sitosterol (**4.3**), sesamin (**4.4**) and 3,6-dimethoxy-5,7-dihydroxyflavone (**4.5**) were tested for their antimicrobial activity in the resazurin and FDA assays, but none of them showed antimicrobial activity against the tested microbes at concentrations up to 1 mg/ml.

Anti-inflammatory assays demonstrated that these three compounds possessed cyclooxygenase and lipoxygenase inhibitory activities. *β*-Sitosterol (**4.3**) showed an inhibitory activity against COX-2 (53%) at 1 mg/ml and a weak inhibition of COX-1 (7.6%). Sesamin (**4.4**) and 3,6-dimethoxy-5,7-dihydroxyflavone (**4.5**) demonstrated inhibitory activity against both COX-1 and COX-2 (Table 4.7). At 2.5 mg/ml, all of these three compounds demonstrated 5-LO inhibitory activity (16 - 74% inhibition) (Table 4.7).

Anti-inflammatory activity of *β*-sitosterol (**4.3**) and sesamin (**4.4**) has been previously reported [Rasadah *et al*., 2004; Singh *et al*., 2002; Utsunomiya *et al*., 2000]. To the best of our knowledge, this is the first time that the anti-inflammatory activity of 3,6-dimethoxy-5,7-dihydroxyflavone (**4.5**) has been reported.

Table 4.7. Anti-inflammatory activity of *β*-sitosterol (**4.3**), sesamin (**4.4**) and 3,6-dimethoxy-5,7-dihydroxyflavone (**4.5**).

Compound	% Inhibition		
	COX-1	COX-2	5-LO
4.3	7.6	53	16
4.4	63	50	33
4.5	81	41	74

Compounds were tested at 1 mg/ml in the COX assay and at 2.5 mg/ml in the 5-LO assay.

4.3.5. Antimicrobial activity of customary preparation of *E. sturtii*

From the records of the interview with the Muruwari elders it was clear that the water decoction of *E. sturtii* has been used for medicinal purposes (see Section 2.2.4.1). A water extract of fresh leaves and branches was prepared according to the customary method of this community and its antimicrobial activity was evaluated. The customary preparation exhibited antibacterial activity against *S. aureus* with an MIC of 1 mg/ml in both the resazurin and FDA assays. It was also found to be bactericidal at 2 mg/ml. No inhibition of the growth of *E. coli*, *P. aeruginosa* and *C. albicans* was seen at concentrations up to 4 mg/ml.

The customary preparation also demonstrated anti-inflammatory activity against all three inflammation pathway enzymes used in this study. At 1 mg/ml, it demonstrated a complete inhibition of COX-1, and 88% inhibition of COX-2. At 0.1 mg/ml, it still showed inhibitory activity against both enzymes (69% and 35%, respectively). At 2.5 mg/ml, it inhibited 67% of the 5-LO activity. Given that *S. aureus* antibacterial activity and COX-1, COX-2 and 5-LO inhibitory activity were observed for partitions and pure compounds

derived from the ethanol extract, it is likely that some of the activity seen in the water extract is derived from the same bioactive components. Due to the time constraints of this study, no further chemical investigation of the customary preparation was conducted.

The bactericidal and anti-inflammatory activities of the customary preparation found in this study provide support for the use of *E. sturtii* as a remedy by the Australian Aboriginal people to treat skin conditions.

4.4. Comparison of the FDA and resazurin antibacterial assays

In this study of the antimicrobial activity of *Eremophila sturtii*, a total of 2 crude extracts, 11 fractions and 5 pure compounds were tested using both the FDA and resazurin assays against *S. aureus*, *E. coli* and *P. aeruginosa*. Since none of these samples showed antibacterial activity against *P. aeruginosa* in the resazurin assay, only MIC values obtained by both assays on *S. aureus* and *E. coli* were compared. MIC values with 1 dilution difference were considered as equivalent [Moore *et al*., 2003].

For the crude extracts and fractions, agreement was achieved on all MIC data tested by both the FDA and resazurin assays on *S. aureus*. In the testing of *E. coli*, no inhibitory activity was demonstrated by any of the samples tested by the resazurin assay at the highest concentration. However, 6 fractions tested by the FDA assay showed inhibitory activity against *E. coli*, with at least 1 fraction presenting an MIC more than 2 dilutions lower than that of the resazurin assay. The abiotic cleavage of FDA by tested samples (*i.e.*, higher background fluorescence level) was not observed in this study.

As to the pure compounds, the MIC and MBC values of 3,8-dihydroxyserrulatic acid (**4.1**) against *S. aureus* tested by the FDA assay were 2 or more dilutions lower than that by the resazurin assay. For serrulatic acid (**4.2**), lower MIC and MBC values were also observed with the FDA assay, but the difference was not as significant as that of the 3,8-dihydroxyserrulatic acid (**4.1**).

The observations of lower MIC and MBC values determined by the FDA assay on the pure compounds against *S. aureus* and on some of the fractions (mixtures) against *E. coli*, supported the suggestion made in Chapter 3 that the reduced nutrient content in the media used in FDA assay caused a reduced growth of tested bacteria and increased susceptibility when exposed to antimicrobial agents.

As a summary, the FDA assay is in general a suitable method for the screening of antibacterial agents from medicinal plants. It gave comparable MIC results on the crude extracts, fractions and some pure compounds (including antibiotics). Lower MIC values of some fractions and pure compounds in the FDA assay were also observed compared to the resazurin assay. The difference in MIC values obtained by the FDA and resazurin assays was generally within 1-2 dilutions and was not considered significant.

4.5. Conclusions and future directions

Chemical and biological studies of *Eremophila sturtii* collected in New South Wales have been carried out. The ethnobotanical information on *E. sturtii* has been used as the basis for the exploration of its bioactive constituents.

Biological investigations of the extracts and fractions of *E. sturtii* revealed they had antimicrobial and anti-inflammatory activities. Bioassay-guided fractionations have led to the isolation of two bioactive novel serrulatane diterpenes, 3,8-dihydroxyserrulatic acid (**4.1**) and serrulatic acid (**4.2**), and three known compounds, *β*-sitosterol (**4.3**), sesamin (**4.4**) and 3,6-dimethoxy-5,7-dihydroxyflavone (**4.5**). Both of the novel serrulatane diterpenes demonstrated antimicrobial and anti-inflammatory activities.

This is the first report of the chemical and bioactive constituents of *E. sturtii*, and the first description of their antimicrobial and anti-inflammatory activities. All the results of this study were provided to Mr and Mrs Barker.

The FDA antibacterial assay was shown in this study to be a general method suitable for the screening of antibacterial agents from medicinal plants. In some cases, lower MIC values of test samples were determined by the FDA assay compared to the resazurin assay. This is most likely due to the nutrient limitation of the medium used in the FDA assay. However, the difference between MIC values determined using the FDA and the resazurin assays was generally within 1-2 fold dilutions and was not considered significant.

There are several more fractions from the ethyl acetate partition (Es-EtOAc-6 to -11) showing biological activities. Column chromatography was carried out on fractions Es-EtOAc-4, -6 and -7, resulting in the collection of more bioactive fractions, but no more pure compounds have been isolated due to time constraints. There is potential to isolate more compounds from these bioactive fractions in the future. The water extract of *E. sturtii*, obtained following the partitions, demonstrated antibacterial and anti-inflammatory activity and could also be further investigated in future. A customary preparation (water decoction) also exhibited antibacterial and anti-inflammatory activity. Future studies

relating the levels of the bioactive compounds identified to the activity of the customary preparation could provide further evidence for the efficacy of *E. sturtii* as a traditional medicine.

4.6. Experimental

4.6.1. General

Merck silica gel 60, Fluka reversed phase C_{18} silica gel 100 and Supelco MCI gel CHP20P were used for column chromatography. High performance liquid chromatography (HPLC) experiments were performed on Shimadzu LC-10A and LC-10A *vp* systems with Waters Sunfire analytical (4.6 mm x 150 mm) and semi-preparative (10 mm x 150 mm) HPLC columns. 1D and 2D NMR spectra were acquired on a Bruker DPX400 spectrometer. IR spectra were measured on a Perkin Elmer Paragon 1000 PC FT-IR spectrometer. UV spectra were recorded on a Varian Cary 1 BIO UV-visible spectrophotometer. Melting points (uncorrected) were determined by a Stuart Scientific SMP10 apparatus. Optical rotations were measured on a Jasco P-1010 polarimeter. High resolution electron ionisation mass spectra (HREIMS) were obtained on a VG AutoSpec M series sector (EBE) Mass Spectrometer (resolving power: 8000 Volts / 70 eV; source temperature: 250 °C) by Mr John Allen at Australian National University, and low resolution electron ionisation mass spectra (LREIMS) were acquired on a Shimadzu GCMS-QP5000 Gas Chromatograph Mass Spectrometer using probe injection (ion energy: 70 eV; source temperature: 275 °C).

All organic solvents (AR grade) were distilled prior to use. Distilled water was used for the extraction and partition.

4.6.2. Plant material

Plant sample (fresh foliage) of *Eremophila sturtii* was collected from Lightning Ridge, New South Wales (147°58'53"E x 29°25'49"S) in March 2004. A voucher specimen was deposited in the herbarium of Macquarie University (voucher number 73007710).

4.6.3. Extraction and isolation

Fresh foliage of *Eremophila sturtii* (648 g) was blended with 70% (v/v) ethanol and water (1.6 L) and stirred occasionally at room temperature for 1 day before being filtered. The extraction was repeated twice with increasing stirring time and the filtrates were combined and evaporated under reduced pressure to give 94 g of dark green gum as the crude extract. The crude extract was suspended in 250 ml water and partitioned with hexane (3 x 150 ml), ethyl acetate (3 x 150 ml) and *n*-butanol (4 x 100 ml) sequentially to give four fractions, Es-Hex (5.6 g, green gum), Es-EtOAc (35.2 g, dark green solid), Es-BuOH (36.8 g, dark brown sticky gum) and Es-water (7.0 g, brown solid after lyophilisation).

4.6.3.1. Isolation of 3,8-dihydroxyserrulatic acid (4.1)

The hexane and ethyl acetate partitions were combined and subjected to flash silica gel column chromatography with petroleum ether and acetone with increasing polarities (100:1 to 1:100, v/v). 11 fractions were collected based on their TLC pattern and labelled as Es-EtOAc-1 to Es-EtOAc-11 in order of increasing polarity (3.1, 0.2, 1.7, 3.8, 8.7, 5.0, 1.8, 2.5, 3.1, 2.7 and 4.3 g, respectively).

Fraction Es-EtOAc-5 (8.7 g, eluted with petroleum ether and acetone 5:1, v/v) was subjected to flash silica gel column chromatography with petroleum ether and ethyl acetate (7:1 to 1:3, v/v) and 8 fractions Es-EtOAc-5-1 to Es-EtOAc-5-8 were collected in order of increasing polarity. Fraction Es-EtOAc-5-5 (2.1 g) was separated by reversed phase MCI gel column chromatography using methanol and water 55:45 to 90:10 (v/v) and 9 fractions Es-EtOAc-5-5-1 to Es-EtOAc-5-5-9 were collected in order of elution. Es-EtOAc-5-5-3 (488 mg, eluted with methanol and water 80:20, v/v) was further separated by reversed phase RP-18 column chromatography using methanol and water 55:45 to 75:25 (v/v) and 4 fractions Es-EtOAc-5-5-3-1 to Es-EtOAc-5-5-3-4 were collected. The major component Es-EtOAc-5-5-3-2 (collected with methanol and water 65:35, v/v) was then dissolved in methanol and crystallised by adding water until a white solid just appeared. After an overnight storage at 4°C, 285 mg of 3,8-dihydroxyserrulatic acid (**4.1**) was obtained as a white solid. The white solid was further crystallised using diethyl ether and colourless prism crystals were obtained. M.p. 173-174°C; $[\alpha]^{23}_{D}$ +14.1° (methanol, *c* 0.155); UV λ_{max} nm (methanol, log ε): 293 (3.2), 242 (3.8), 212 (4.5); IR (KBr) ν_{max} cm^{-1}: 2968, 2928, 1685, 1588, 1424, 1236, 1056, 702; HREIMS *m/z*: 332.1976 $[M]^+$; LREIMS *m/z* (rel. int.): 332 $[M]^+$ (2), 314 $[M\text{-}H_2O]^+$ (1), 247 (1), 232 (15), 217 (4), 204 (3), 189 (2), 91 (2), 82 (7), 69 (32), 41 (100). ^1H and ^{13}C NMR data are shown in Table 4.4.

4.6.3.2. Isolation of serrulatic acid (4.2)

Fraction Es-EtOAc-1 (3.1 g, eluted with petroleum ether and acetone 100:1 to 20:1, v/v) was subjected to flash silica gel column chromatography with petroleum ether and ethyl acetate (15:1 to 1:5, v/v). 15 fractions Es-EtOAc-1-1 to Es-EtOAc-1-15 were collected in order of polarity. Fraction Es-EtOAc-1-8 (0.5 g, collected with petroleum ether and

acetone 10:1, v/v) was then separated by a reversed phase RP-18 silica gel column chromatography with methanol and water (60:40 to 97:3, v/v) and 10 fractions Es-EtOAc-1-8-1 to Es-EtOAc-1-8-10 were collected in order of elution. Es-EtOAc-1-8-4 (collected with methanol and water 95:5, v/v) was further cleaned by semi-preparative HPLC repeatedly (acetonitrile:water 88:12, v/v, 4 ml/min) to give 191 mg of serrulatic acid (**4.2**) as a white solid. M.p. 82-83°C; $[\alpha]^{22}_{D}$ -32.2° (methanol, *c* 0.102); HREIMS *m/z*: 300.2090 $[M]^+$; LREIMS *m/z*: 300 $[M]^+$ (2), 255 (1), 244 (1), 216 (21), 189 (17), 145 (18), 129 (15), 117 (13), 115 (11), 91 (11), 69 (44), 41 (100). ^{1}H and ^{13}C NMR data are shown in Table 4.4.

4.6.3.3. Isolation of β-sitosterol (4.3)

Fraction Es-EtOAc-2 (0.19 g) was washed with methanol until colourless and recrystallised in acetone to afford *β*-sitosterol (19 mg) as needles. M.p. 132-134°C (lit. 135°C) [Pacheco *et al*., 1973]. ^{13}C NMR ($CDCl_3$): 11.8 (C-18), 12.0 (C-21), 18.8 (C-19), 19.0 (C-11), 19.4 (C-26), 19.8 (C-27), 21.1 (C-23), 13.1 (C-15), 24.3 (C-16), 26.1 (C-25), 28.2 (C-2), 29.2 (C-7), 31.7 (C-8), 31.8 (C-20), 31.9 (C-22), 33.9 (C-10), 36.1 (C-1), 36.5 (C-24), 37.2 (C-12), 39.8 (C-4), 42.3 (C-13, C-9), 45.8 (C-14), 50.1 (C-17), 56.1 (C-3), 56.8 (C-6), 71.8 (C-5), 121.7 (C-28), 140.7 (C-29). These data were in agreement with those reported in the literature [Kovganko *et al*., 2000].

4.6.3.4. Isolation of sesamin (4.4)

Fraction Es-EtOAc-3 (1.7 g) was purified by normal and reversed phase silica gel column chromatography to give a yellowish solid. This solid was a mixture of two major compounds. It was recrystallised with methanol repeatedly, and colourless needle crystals of sesamin (27 mg) were obtained. M.p. 122-123°C (lit. 124°C) [Jayasinghe *et al*., 2003]. ^{1}H-NMR ($CDCl_3$): 3.05 (2H, *m*, H-1, H-5), 3.86 (2H, *dd*, *J* = 9.2, 3.6 Hz, H-4, H-8), 4.23 (2H, *m*, H-4, H-8), 4.71 (2H, *d*, *J* = 4.1 Hz, H-2, H-6), 5.96 (4H, *s*, -OCH_2O-), 6.78 (2H, *d*, *J* = 8.0 Hz, H-5', H-5"), 6.79 (2H, *dd*, *J* = 8.0, 1.3 Hz, H-6', H-6"), and 6.85 (2H, *d*, *J* = 1.3 Hz, H-2', H-2"). ^{13}C NMR ($CDCl_3$): 54.7 (C-1, C-5), 72.1 (C-4, C-8), 86.2 (C-2, C-6), 101.5 (-OCH_2O-), 106.9 (C-2', C-2"), 108.6 (C-5', C-5"), 119.8 (C-6', C-6"), 135.5 (C-1', C-1"), 147.5 (C-4', C-4"), 148.4 (C-3', C-3"). LREIMS m/z (% rel. int.): 354 $[M]^+$ (13), 203 (11), 178 (8), 161 (27), 150 (32), 149 (100), 135 (45), 131 (35), 122 (24), 121 (19), 117 (10), 115 (12), 103 (13), 91 (9), 77 (17), 65 (20), 63 (14). NMR and mass spectral data were identical to those published in the literature [Jayasinghe *et al*., 2003; Pelter and Ward, 1976].

4.6.3.5. Isolation of 3,6-dimethoxy-5,7-dihydroxyflavone (4.5)

The methanol solutions obtained from the recrystallisation of sesamin (**4.4**) were combined and evaporated under reduced pressure. The solid was recrystallised with chloroform repeatedly to give 3,6-dimethoxy-5,7-dihydroxyflavone (**4.5**) as a yellow solid. M.p. 172-174°C (lit. 175-176°C) [Buschi *et al*., 1980]. LREIMS *m/z* (% rel. int.): 315 $[M+1]^+$ (9), 314 $[M]^+$ (63), 299 $[M-CH_3]^+$ (29), 296 $[M-H_2O]^+$ (19), 281 (4), 271 (45), 253 (14), 241 (4), 228 (13), 214 (3), 200 (10), 183 (4), 167 (8), 139 (11), 118 (18), 115 (20), 105 (44), 89

(21), 77 (60), 69 (100), 51 (39). ^{1}H NMR (DMSO-d_6): 12.62 (OH, *s*, 5-OH), 10.82 (OH, *s*, 7-OH), 7.97 (2H, *m*, H-2', H-6'), 7.52 (3H, *m*, H-3', H-4', H-5'), 6.56 (1H, *s*, H-8), 4.04 (3H, *s*, 6-OCH_3) and 3.86 ppm (3H, *s*, 3-OCH_3). The ^{1}H NMR data were consistent with published data [Buschi *et al*., 1980]. ^{13}C NMR: 156.1 (C-2), 139.3 (C-3), 179.3 (C-4), 153.3 (C-5), 132.1 (C-6), 158.5 (C-7), 95.0 (C-8), 152.7 (C-9), 105.7 (C-10), 103.9 (C-1'), 129.1 (C-2', C-6'), 129.6 (C-3', C-5'), 131.9 (C-4'). UV λ_{max} (methanol): 270, 321 nm.

4.6.4. Preparation of customary decoction

Fresh leaves and small branches of *E. sturtii* (44 g) were crushed by hand and boiled in water (200 ml) for 30 minutes to give a brownish green water extract. The water extract was filtered and evaporated under reduced pressure. The extract was lyophilised and a brownish grey solid (2.6 g) was obtained.

4.6.5. Antimicrobial assays

The antimicrobial assays (FDA, resazurin and broth microdilution assays) were performed following the procedures described in Chapter 3. Gentamicin and tetracycline (Sigma) were used as positive controls. The solvent used to dissolve samples (ethanol) was included in the growth controls. Samples and controls were assayed in triplicates.

For the crude extract and fractions, two fold dilutions of samples were used in the assays. For pure compounds, progressive dilutions were used. 3,8-Dihydroxyserrulatic acid (**4.1**) was tested at concentrations between 100 and 1000 μg/ml with increments of 100 μg/ml.

Serrulatic acid (**4.2**) was measured at concentrations between 5 and 50 μg/ml with increments of 5 μg/ml.

To determine whether the activity was bacteriostatic or bactericidal, samples from microwells showing inhibition were plated out and viable counts were compared with those from control microwells containing no inhibitors.

4.6.6. Anti-inflammatory assays

Cyclooxygenase-1 (ovine), cyclooxygenase-2 (human recombinant) and 5-lipoxygenase (potato) screening enzyme, were used as the targeted enzymes in the anti-inflammatory assays (Cayman Chemical, Catalogue No. 60100, 60122, 60401, respectively). Cyclooxygenase inhibitor screening assay kit (Cayman Chemical, Catalogue No. 560131) and Lipoxygenase inhibitor screening assay kit (Cayman Chemical, Catalogue No. 760700) were used in the anti-inflammatory assays.

4.6.6.1. Cyclooxygenase inhibitor screening assay

The cyclooxygenase screening assay was performed according to the manufacturer's instructions [Cayman Chemical, 2005a]. In brief, COX-1 and COX-2 were incubated separately with test samples in a reaction buffer (100 mM Tris-HCl buffer, pH 8.0) containing 1 μM heme for 10 minutes at 37°C. Arachidonic acid was added (final concentration of 100 μM in the reaction) to initiate the reaction. HCl solution (1 M) was added after 2 minutes to terminate the reaction, and saturated $SnCl_2$ solution was added to reduce the reaction products to $PGF_{2\alpha}$. The amounts of $PGF_{2\alpha}$ produced in the COX

reactions were quantified using an enzyme immunoassay. $PGF_{2\alpha}$ and PGE_2-acetylcholine esterase tracer were added to a 96-well plate coated with mouse anti-rabbit IgG and the specific antibody (rabbit anti- $PGF_{2\alpha}$ and PGE_2) was added. The plate was incubated for 18 hours at room temperature. 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 covered with a plastic film and developed in the dark for about 60 minutes, until the maximum binding control yielded an absorbance of 0.3-0.8 A.U. at 405 nm [Cayman Chemical, 2005a]. Ibuprofen was used as a control in the COX assays.

4.6.6.2. Lipoxygenase inhibitor screening assay

The lipoxygenase screening assay was performed according to the manufacturer's instructions [Cayman Chemical, 2005b]. In brief, test samples (10 μl) and purified potato 5-LO (90 μl) were added to a 96-well plate with the assay buffer (0.1M Tris-HCl, pH 7.4, 100 μl). Linoleic acid (1 mM, 10 μl) was added to initiate the reaction. The enzyme reaction was terminated and the plate was developed by the addition of Chromogen after 5 minutes. The plate was shaken for 5 minutes and the absorbance was measured at 492 nm. The rate of inhibition was calculated by comparing the absorbance with that of the 100% Initial Activity control containing no inhibitors (solvent was included as a control) [Cayman Chemical, 2005b]. Samples were assayed in duplicates.

Chapter 5. Ethnopharmacological study of *Exocarpos aphyllus*

This chapter describes the isolation of biologically active constituents from Exocarpos aphyllus *under the guidance of its ethnomedicinal uses.*

5.1. Introduction

Exocarpos aphyllus R. Br. was chosen as one of the plants for a targeted ethnopharmacological study because of its ethnomedicinal uses and because preliminary investigations had already been conducted on its antibacterial properties by former research students (Cathy Smith, Hons, 1999; Wanandy, MSc, 2005). Extracts of the bark and stem of this plant were found to possess antibacterial activity against *S. aureus* and *E. coli*, but the active compounds had not been fully studied [Smith, 1999; Wanandy, 2005]. For this PhD study, along with the isolation of bioactive compounds, the ethnobotanical information on *E. aphyllus* has been obtained, and its ethnomedicinal uses by Aboriginal people of the Kamilaroi community have been confirmed [John Hunter, personal communication].

This chapter describes the isolation of bioactive constituents from *E. aphyllus* through bioassay-guided isolation. The ethnobotanical and phytochemical information relating to *E. aphyllus* within the public domain, and the previous findings of the research group on this plant, are summarised below.

5.2. General review of *Exocarpos* species

Exocarpos aphyllus R. Br. is one of the species of the *Exocarpos* genera in the plant family Santalaceae [Lassak and McCarthy, 1983]. *Exocarpos* was first scientifically described and recorded by the French naturalist Jacques-Julien Houtou de Labillardière in 1792 following his observation of *E. cupressiformis* as "an evergreen tree, which has its nut situated, like that of the acajou (cashew), upon a fleshy receptacle much larger than itself" [Low, 1989]. The name *Exocarpos* comes from the Greek words *exo* meaning outside and *karpos* meaning fruit, as the seeds of *Exocarpos* species are external to the fruit [Baines, 1981; Daw *et al*., 1997]. The generic name was also previously spelt as *Exocarpus*, but *Exocarpos* is now accepted [Low, 1989]. Twenty six *Exocarpos* species are found in Australia, south-east Asia and the Pacific Islands, with nine endemic to Australia [Baines, 1981].

Figure 5.1. Fruit of *Exocarpos aphyllus*.

Photo courtesy of Horst Weber, Australian Plants Online [Weber, 1998].

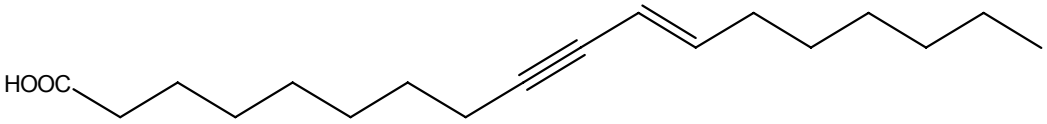
Exocarpos aphyllus is known to the Australian Aboriginal people by the common names leafless ballart or stiff cherry, or by Aboriginal names *ballee* and *ballat* (in Gippsland), *pallert* (at Lake Condah) and *balad* (in Victoria) [Isaacs, 1987]. Apart from its edible fruit,

this plant has been used traditionally as medicine for colds, sores, and tuberculosis [Lassak and McCarthy, 1983; Cribb and Cribb, 1981; Isaacs, 1987].

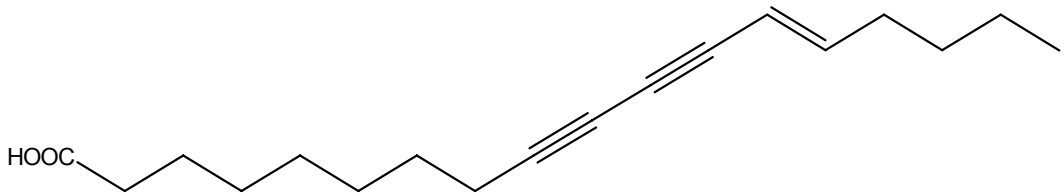
A related species in the *Exocarpos* genus, *Exocarpos latifolius* (broad-leaved native cherry, black sandalwood, mistletoe tree) has also been used for similar medicinal purposes by Australian Aboriginal people [Kyriazis, 1995; Barr *et al*, 1993]. Decoctions of the leafy branches have been used for treating infants' flu and colds. The rough bark and roots of *E. latifolius* have been scraped and crushed and mixed with water to make a preparation for the treatment of blind boils and open sores [Kyriazis, 1995; Barr *et al*, 1993]. *Exocarpos latifolius* has also been used for other medicinal purposes. A water infusion of scraped inner bark of *E. latifolius* was drunk by Aboriginal women to prevent pregnancy [Brock, 1988; Levitt, 1981]. Another *Exocarpos* species used for treatment of sores is *E. phyllanthoides*. Its decoctions have been used for abscesses [Ewart, 1930]. The most widely known cherry ballat or native cherry, *E. cupressiformis*, is a plant used in both Aboriginal and colonial medicines. A decoction of the twigs of *E. cupressiformis* has been used as a bitter tonic and astringent [Lassak and McCarthy, 1983; Low, 1990; Cribb and Cribb, 1981]. As the stem bark has been found to contain 15-22% tannic acid, this is probably responsible for the astringent properties of *E. cupressiformis* [Maiden, 1975; Lassak and McCarthy, 1983].

Not much information on the chemical constituents of *E. aphyllus* has been reported. Seeds of *E. aphyllus* have been found to contain 10% protein and 21% oil, with santalbic acid (*trans*-11-octadecen-9-ynoic acid) as the major fatty acid [Sundarrao *et al*., 1992]. The whole plant has also been reported to contain 0.05% of alkaloids [Collins *et al*., 1990], but the structure or biological activity of these alkaloids has not been described.

It has been found that the *Exocarpos* species contain a number of acetylenic fatty acids. Exocarpic acid (*trans*-octadec-13-en-9,11-diynoic acid) has been detected in the root oils of *E. cupressiformis* and *E. strictus* [Hatt *et al*., 1959], and stearolic acid (9-octadecynoic acid) has been found in the seed oil of *E. cupressiformis* [Morris and Marshall, 1966]. The structural similarity of these conjugated acetylenic acids suggested that santalbic and exocarpic acids are both biologically derived from stearolic acid [Morris and Marshall, 1966]. A furan ring-containing fatty acid, 8-(5-hexyl-2-furyl)octanoic acid, has been isolated from the seed oil of *E. cupressiformis* [Morris *et al*., 1966; Elix and Sargent, 1968].

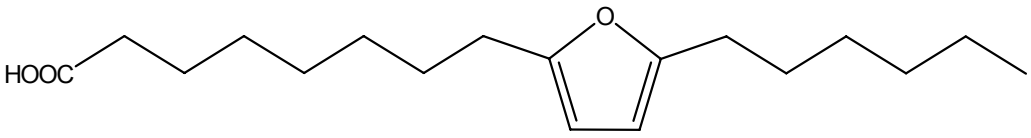


Santalbic acid (Ximenynic acid)



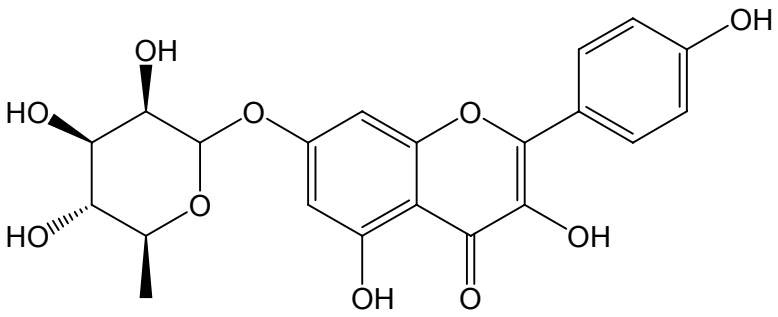
Exocarpic acid

Stearolic acid

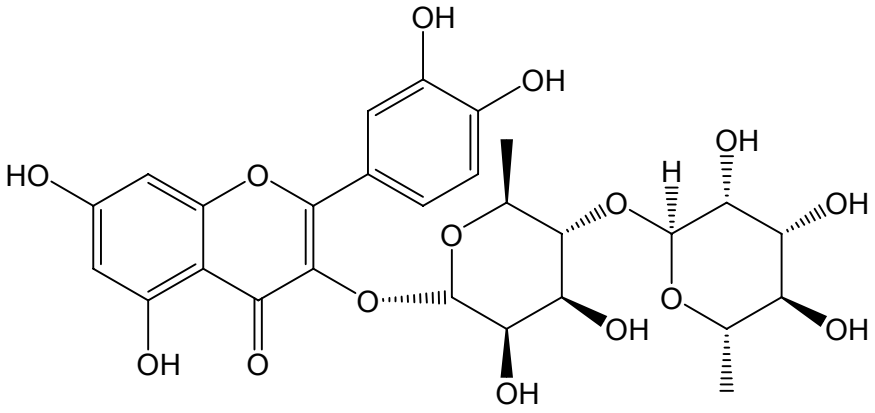


8-(5-Hexyl-2-furyl)octanoic acid

Apart from the fatty acids, several flavonoid glycosides, such as kaempferol-7-rhamnoside and quercetin-3-rhamnobioside, as well as oleanolic acid and its saponin, have been isolated from the leaves and twigs of *E. cupressiformis* [Cooke and Haynes, 1960].

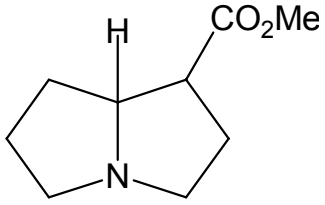


Kaempferol-7-rhamnoside



Quercetin-3-rhamnobioside

Alkaloids have been detected in the leaves of *E. sparteus* and *E. cupressiformis*, and the alkaloid 1-methoxycarbonylpyrrolizidine has been isolated from *E. cupressiformis*. [Collins *et al*, 1990].



1-Methoxycarbonylpyrrolizidine

5.3. Prior studies of chemical constituents and antibacterial activity of *E. aphyllus*

Extracts of *E. aphyllus* have been previously found to have antibacterial activity by two students in this research group [Smith, 1999; Wanandy, 2005]. In their studies, similar extraction and separation procedures were followed. The bark and stems of *E. aphyllus* were extracted sequentially with hexane, dichloromethane (DCM), methanol and water to produce a number of crude extracts. Smith's studies showed that most of the extracts were active against *S. aureus* and *E. coli* at a concentration of 10 mg/ml, with the greatest bactericidal activity against *S. aureus* observed on the bark hexane and DCM extracts [Smith, 1999]. The antibacterial assay method used in Smith's study was the original FDA method developed by Chand *et al*. [Chand *et al*., 1994]. Wanandy used both the optimised FDA assay [Wanandy *et al*., 2005] and disc diffusion methods [NCCLS, 2001] to test the extracts at a single concentration (2.5 mg/ml for the DCM extracts and 5 mg/ml for all the other extracts) against *S. aureus*, *E. coli* and *P. aeruginosa*. All of the methanol extracts of the bark and stems showed antibacterial activity against *S. aureus*. All hexane extracts were found to be inactive against the three bacteria. No inhibition of *P. aeruginosa* was observed for any of the extracts. The bark DCM extracts were found to be active against *S. aureus* and *E. coli* in the FDA assay, but the activity was not replicated in the disc diffusion assay. Wanandy explained the different results obtained with the two assay methods for *E. coli* as being most likely due to the difficulty for non-polar components to migrate across the polar surface of the nutrient agar [Wanandy, 2005].

Combining the findings from previous studies on the antibacterial activities of *E. aphyllus*, it can be summarised that the methanol extracts of the bark and stems of *E. aphyllus*

possess antibacterial activity against *S. aureus*, and the DCM extract of the bark could be active against both *S. aureus* and *E. coli*.

Both Smith and Wanandy found that free fatty acids from the bark DCM extract were the most active fraction against *S. aureus*. The content of the total free fatty acids in the bark DCM extract was found to be only 0.7% (which accounted for 0.006% of the dried bark mass) [Wanandy, 2005]. Wanandy further explored the fatty acids content by saponifying the hexane and DCM extracts. The mixture of fatty acids obtained in this way was found to be active against *S. aureus*. The mixture was analysed by GC-MS and was found to contain docosanoic (C22), octadecenoic (C18:1), exocarpic, tetracosanoic (C24), eicosanoic (C20), hexadecanoic (C16), octadecanoic (C18) and eicosenoic (C20:1) acids [Wanandy, 2005].

Components from the polar extracts (methanol and water extracts) of *E. aphyllus*, which demonstrated substantial antibacterial activity, were not investigated in their studies.

Along with antibacterial properties, the ethnomedicinal applications of *E. aphyllus* suggest potential antiviral (use as a cold remedy) and anti-inflammatory (use as a wash for sores) activities. Semple and colleagues have examined an ethanol extract of the stems of *E. aphyllus* against three types of viruses related to the common cold, but did not observe any antiviral activity [Semple *et al*., 1998]. No anti-inflammatory activity has been reported with *E. aphyllus*.

The aim of this research was to further explore the bioactive constituents of *E. aphyllus*, with a particular focus on its antimicrobial and anti-inflammatory activities. The isolation and characterisation of bioactive constituents of *E. aphyllus* are described in the following sections.

5.4. Bioassay-guided investigation of bioactive constituents of *Exocarpos aphyllus*

A fresh branch of *Exocarpos aphyllus* (660 g) was cut into small portions and blended with 70% (v/v) aqueous ethanol. This solvent was chosen to extract most of the components with higher polarities. The ethanol layer was filtered and evaporated under reduced pressure to give the crude extract (84 g, 13% yield). The dried crude extract was examined for its antimicrobial activity against the human pathogens *S. aureus*, *E. coli*, *P. aeruginosa* and *C. albicans*, and for its inhibitory activity against inflammation pathway enzymes COX-1, COX-2 and 5-LO. All bioassay methods used in this study have been described previously in Chapter 3 and 4 of this thesis.

5.4.1. Characterisation of antimicrobial components of *E. aphyllus*

The crude ethanol extract of *E. aphyllus* (Ea-crude) demonstrated antimicrobial activity against the Gram positive bacterium *S. aureus*, with an MIC of 1 mg/ml in the resazurin assay and 0.25 mg/ml in the FDA assay. The crude extract also inhibited the growth of the Gram negative bacterium *E. coli* at higher concentrations, with an MIC of 4 mg/ml in both assays (Table 5.1). No inhibition of *P. aeruginosa* or *C. albicans* was observed at concentrations up to 4 mg/ml. Higher concentrations of the crude extract resulted in a large amount of precipitation in serial aqueous dilutions and were not tested.

The crude extract was suspended in water and extracted with ethyl acetate and *n*-butanol sequentially to give three partition fractions, namely Ea-EtOAc (42.6 g), Ea-BuOH (12.5 g) and Ea-water (24.5 g). The *n*-butanol and water partitions demonstrated antibacterial activities. In both the resazurin and FDA assays, Ea-BuOH showed the most potent activity

against *S. aureus*, having an MIC of 0.25 mg/ml. Ea-water was also active against *S. aureus*, having an MIC of 0.5 mg/ml in both assays. It also demonstrated inhibition of *E. coli* in the FDA assay, showing an MIC of 4 mg/ml (Table 5.1). No inhibition of *P. aeruginosa* and *C. albicans* was observed by these partition fractions at concentrations up to 4 mg/ml. The results showed that the components responsible for the antibacterial activity were of higher polarity. These are consistent with the findings obtained earlier by colleagues for the methanol extracts of bark and stems of *E. aphyllus* [Smith, 1999; Wanandy, 2005].

Table 5.1. Antimicrobial activity of the crude extract and partition fractions of *E. aphyllus*.

Sample	MIC (mg/ml)			
	Resazurin assay		FDA assay	
	S. aureus	*E. coli*	*S. aureus*	*E. coli*
Ea-crude	1	4	0.25	4
Ea-EtOAc	> 4	> 4	> 4	> 4
Ea-BuOH	0.25	> 4	0.25	> 4
Ea-water	0.5	> 4	0.5	4

Further separations of the active fractions Ea-BuOH and Ea-water were carried out to identify the antibacterial components. The *n*-butanol partition was extracted with 4:1 (v/v) chloroform/methanol to give a medium polarity extract (Ea-BuOH-CM) and a high polarity residue (Ea-BuOH-Res). The antimicrobial assay results for these two extracts indicated that the activity of the *n*-butanol partition was mainly due to the high polarity components, which showed an MIC against *S. aureus* at 0.5 mg/ml (Table 5.2). No

inhibitory activity was observed with these fractions against *E. coli*, *P. aeruginosa* or *C. albicans* at concentrations up to 1 mg/ml.

Table 5.2. Antibacterial activity of fractions from the *n*-butanol partition of *E. aphyllus*.

Sample	MIC (mg/ml)	
	Resazurin assay	FDA assay
	S. aureus	*S. aureus*
Ea-BuOH-CM	> 1	> 1
Ea-BuOH-Res	0.5	0.5

As phenolic compounds (including polyphenols and tannins) are polar natural products possessing antimicrobial activities, the polar fractions of *E. aphyllus* were examined for the presence of phenolic compounds by a $FeCl_3$ colour-change reaction [Xu and Chen, 1983]. The active high-polarity residue from the *n*-butanol partition (Ea-BuOH-Res) and the water partition (Ea-water) were dissolved in 25% aqueous methanol and reacted with an aqueous 1% $FeCl_3$ solution. A dark green colour appeared in both solutions. The results indicated that both of these fractions contained phenolic compounds. In another test, these two fractions were reacted with a saturated aqueous lead acetate solution. Both fractions yielded a large amount of pale-yellow precipitate, indicating polyphenols [Xu and Chen, 1983].

Polyphenols and simple phenolic compounds (consisting of a single substituted phenolic ring) are well documented to have microbicidal activities against a large number of pathogenic microorganisms including fungi and bacteria [Karou *et al*., 2005; Scalbert, 1991]. Their mechanisms of toxicity against microbes have also been well studied. The

mechanisms proposed to explain the antimicrobial activities of polyphenols and simple phenolic compounds include deprivation of the substrates required for microbial growth (such as metal ions), inhibition of extracellular microbial enzymes (such as proteases and carbohydrolases), inactivation of microbial adhesions and cell envelope transport proteins, direct action on microbial metabolism through inhibition of oxidative phosphorylation, complexation with cell wall (proteins and polysaccharides) and membrane disruption [Scalbert, 1991; Cowan, 1999].

As phenolic compounds are a part of the plant defence mechanisms against invasions from the surroundings (*e.g.*, microorganisms) and are found in almost every plant part, *i.e.*, the bark, wood, leaves, fruits and roots [Cowan, 1999], it is not surprising that the phenolic-rich fractions of *E. aphyllus* exhibited antibacterial activity against *S. aureus*. However, many microorganisms may detoxify polyphenols. For example, tannins can be detoxified through synthesis of tannin-complexing polymers, oxidation, tannin biodegradation or synthesis of siderophores by microorganisms [Scalbert, 1991; Basaraba, 1966]. The microorganisms capable of growing on tannins include *E. coli* and some strains of *Pseudomonas* and *Candida* [Scalbert, 1991; Basaraba, 1966]. The ability of certain microorganisms to detoxify polyphenols might explain the lack of activity of the polyphenol-rich fractions of *E. aphyllus* (*e.g.*, Ea-BuOH-Res) against these microorganisms (see Table 5.2).

In summary, the antibacterial activity of the ethanol crude extract of *E. aphyllus* was most likely due to the presence of phenolic components, especially polyphenols. Thousands of polyphenols and simple phenolic compounds from traditional medicinal plants have been isolated and structures determined, and their chemical, biological and pharmacological properties systematically analysed [Okuda, 2005; Haslam, 1996]. Due to the time

constraints of this study, no further isolation of phenolic compounds from *E. aphyllus* was performed.

5.4.2. Isolation and characterisation of anti-inflammatory compounds of *E. aphyllus*

The cyclooxygenase and lipoxygenase inhibitory properties of *E. aphyllus* were evaluated in this study. The crude ethanol extract of *E. aphyllus* was observed to be inhibitory to all of the tested enzymes. At 2 mg/ml, the crude extract inhibited the enzyme activities of COX-1 and COX-2 almost completely (98% and 97% inhibition, respectively). At 0.2 mg/ml, the inhibition rates were lowered to 29% and 80% (Table 5.3). The crude extract of *E. aphyllus* also exhibited 51% inhibition of 5-LO activity at a concentration of 2.5 mg/ml (Table 5.4). The inhibitory activity of the crude extract of *E. aphyllus* against these three major inflammation pathway enzymes demonstrated the anti-inflammatory property of this medicinal plant. This activity is consistent with its ethnomedicinal uses as a treatment for sores.

The ethyl acetate, *n*-butanol and water partitions (Ea-EtOAc, Ea-BuOH and Ea-water) were assayed for their anti-inflammatory activities. Each of these fractions was examined against COX-1 and COX-2 at a single concentration of 1 mg/ml, and against 5-LO at 2.5 mg/ml. All of the partitions appeared to be active against the three inflammation pathway enzymes, especially COX-1, whose enzyme activity was totally inhibited. These partitions also exhibited high inhibition of COX-2 (98%, 93% and 74%, respectively), as shown in Table 5.3. A greater inhibitory effect on 5-LO was observed with increasing sample polarity, with the water partition inhibiting the enzyme activity of 5-LO completely (Table 5.4). Due to the cost of the assay kits, the inhibitory activities of fractions at lower concentrations were not tested. Since the ethyl acetate partition contained the largest mass

of material, it was chosen for further separations to isolate the anti-inflammatory compounds.

Table 5.3. Cyclooxygenase inhibitory activity of the crude extract and partition fractions of *E. aphyllus*.

Sample	% Inhibition		
	Concentration	COX-1	COX-2
Ea-Crude	2 mg/ml	98	97
Ea-Crude	0.2 mg/ml	29	80
Ea-EtOAc	1 mg/ml	~ 100	98
Ea-BuOH	1 mg/ml	~ 100	93
Ea-Water	1 mg/ml	~ 100	74

Table 5.4. 5-Lipoxygenase inhibitory activity of the crude extract and partition fractions of *E. aphyllus*.

Sample	% Inhibition of 5-LO
Ea-Crude	51
Ea-EtOAc	4
Ea-BuOH	76
Ea-Water	~ 100

Samples were tested at a single concentration of 2.5 mg/ml.

The ethyl acetate partition of *E. aphyllus* was further separated by normal phase silica gel column chromatography, eluting with an increasing gradient of petroleum ether and acetone (10:1 to 1:2, v/v), to give 6 fractions with yields being 1.8, 4.8, 22.8, 5.0, 6.3 and 5.3%, respectively. At 1 mg/ml, 5 out of the 6 fractions demonstrated high inhibitory activity against COX-1 and COX-2 (Table 5.5). The fraction Ea-EtOAc-3, collected with

petroleum ether/acetone 2:1 (v/v), also demonstrated 64% inhibition of the 5-LO activity at 2.5 mg/ml (Table 5.5). Due to lack of access to the 5-LO assay at the time, further separations were conducted on fractions Ea-EtOAc-4 and Ea-EtOAc-5 without assaying for 5-LO inhibitory activity.

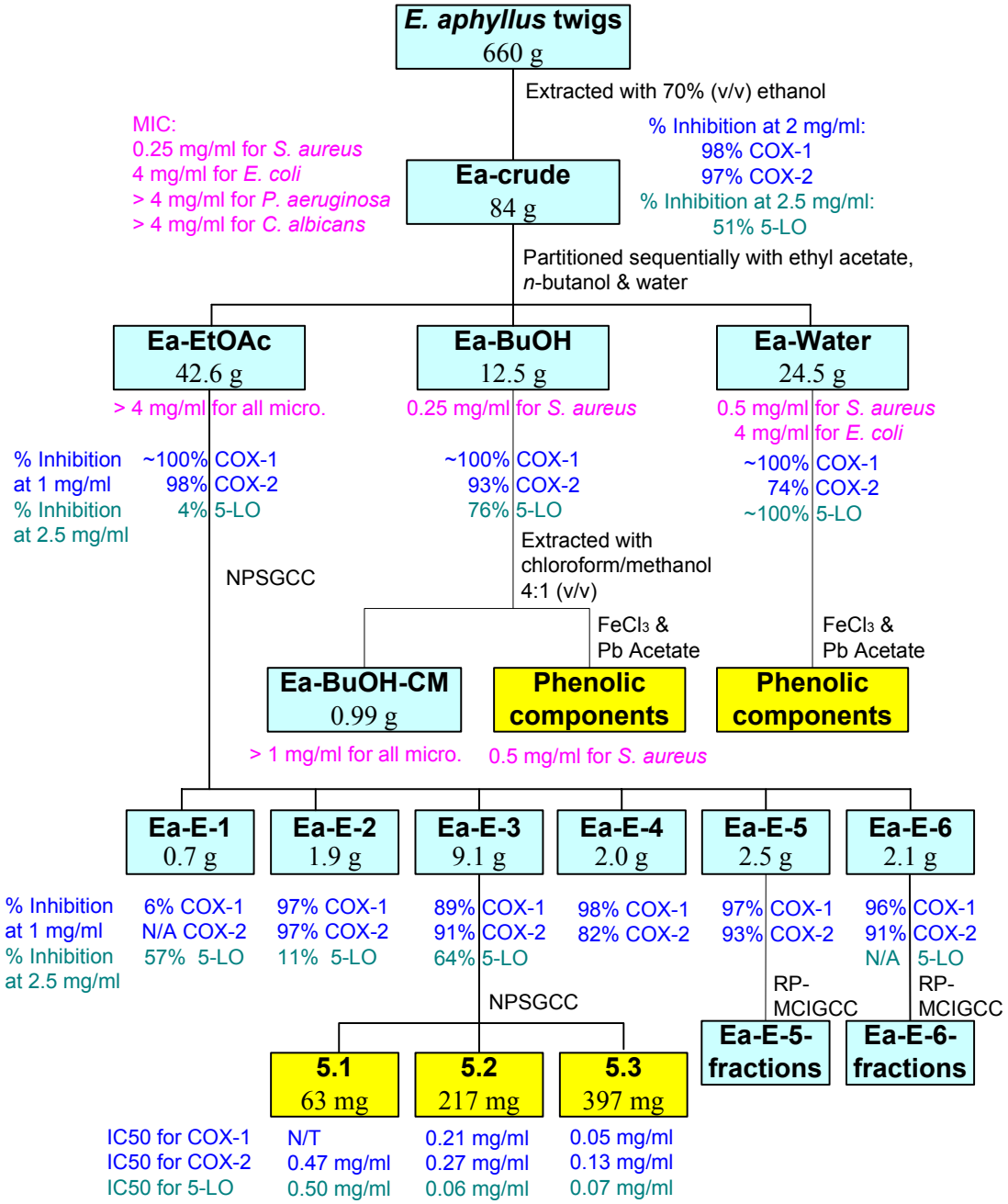
Table 5.5. Anti-inflammatory activity of column chromatography fractions of the ethyl acetate partition of *E. aphyllus*.

Sample	% Inhibition		
	COX-1	COX-2	5-LO
Ea-EtOAc-1	6	-	57
Ea-EtOAc-2	97	97	11
Ea-EtOAc-3	89	91	64
Ea-EtOAc-4	98	82	NT
Ea-EtOAc-5	97	93	NT
Ea-EtOAc-6	96	91	N/A

Samples were tested at 1 mg/ml against COX-1 and COX-2, and at 2.5 mg/ml against 5-LO. NT: not tested; N/A: not active.

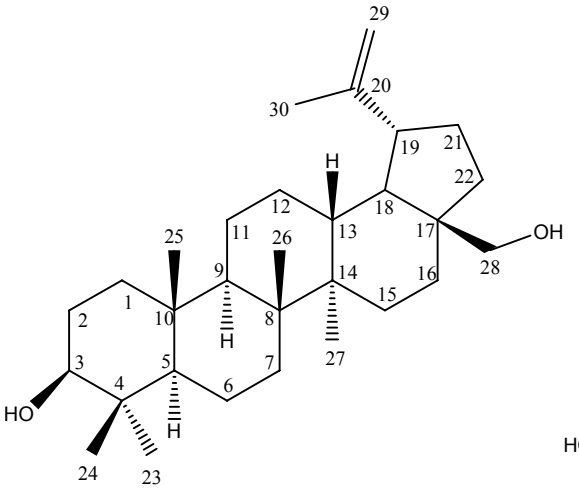
Ea-EtOAc-3 was found to contain a large amount of white solid, which, when tested by silica gel thin layer chromatography, was composed of three major constituents with Rf values being 0.47, 0.28 and 0.23, respectively, on normal phase silica gel TLC developed with 100:1 chloroform and methanol (v/v). These three compounds were isolated using normal phase silica gel column chromatography, eluting with chloroform and methanol, to give betulin (**5.1**), betulinic acid (**5.2**) and oleanolic acid (**5.3**) in order of elution (increasing polarity). Betulin (**5.1**) was collected with 100% chloroform, betulinic acid (**5.2**) with 1% methanol in chloroform and oleanolic acid (**5.3**) with 3% methanol in chloroform (v/v). The procedure for the isolation of these compounds is shown in Scheme 5.1.

Scheme 5.1. Bioassay-guided fractionation of *E. aphyllus*.

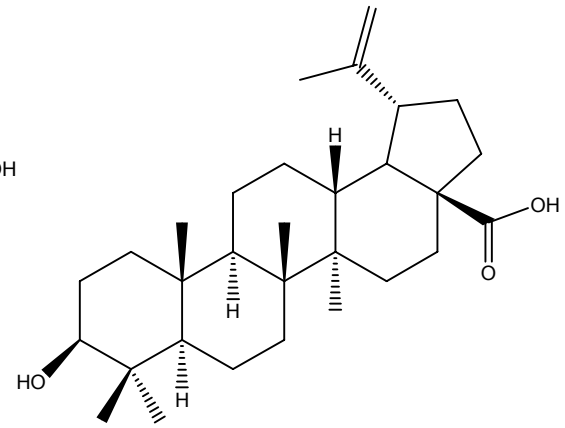


Antibacterial activity results shown in this scheme (in pink) are from the FDA assay and the broth microdilution assay (for *C. albicans* only). COX inhibitory activity is shown in blue. 5-LO inhibitory activity is shown in green. NPSGCC: normal phase silica gel column chromatography. RP-MCIGCC: reverse phase MCI gel column chromatography. N/T: not tested. N/A: not active.

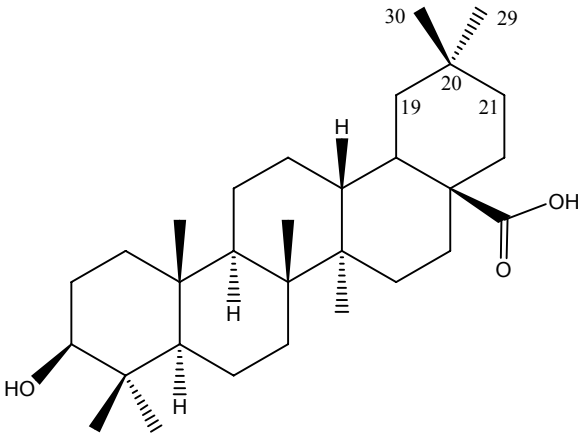
The structures of the isolated compounds were elucidated by NMR and MS analyses, and were found to be consistent with the reported data in the literature [Tinto *et al*., 1992; Siddiqui *et al*., 1988; Ikuta and Itokaw, 1988; Seebacher *et al*., 2003]. The assignments of the ^{13}C NMR data of these compounds are shown in Table 5.6.



5.1



5.2



5.3

Table 5.6. ^{13}C NMR data assignments of compounds **5.1**, **5.2** and **5.3**.

Carbon No.	5.1	5.2	5.3
C-1	39.1	38.8	38.8
C-2	27.8	26.8	27.1
C-3	79.4	78.5	79.2
C-4	39.3	38.7	39.0
C-5	55.7	55.4	55.6
C-6	18.7	18.2	18.7
C-7	34.6	34.3	33.0
C-8	41.3	40.6	39.6
C-9	50.8	50.5	48.0
C-10	37.6	37.1	37.3
C-11	21.2	20.8	23.3
C-12	25.6	25.5	122.6
C-13	37.7	38.3	144.2
C-14	43.1	42.4	42.0
C-15	27.4	30.5	28.0
C-16	29.6	32.2	23.7
C-17	48.2	56.2	46.7
C-18	49.1	49.1	41.5
C-19	48.2	47.0	46.3
C-20	150.9	150.6	31.0
C-21	30.1	29.6	34.2
C-22	34.4	37.0	32.9
C-23	28.4	27.7	28.3
C-24	15.8	15.2	15.9
C-25	16.5	15.9	15.6
C-26	16.4	15.7	17.1
C-27	15.2	14.1	26.2
C-28	60.9	179.0	181.4
C-29	110.1	109.3	33.4
C-30	19.5	19.0	23.8

Data acquired at 100 MHz in *d*-chloroform. A few drops of *d*-methanol were added to dissolve **5.2** and **5.3**.

Betulin (**5.1**), betulinic acid (**5.2**) and oleanolic acid (**5.3**) are pentacyclic triterpenes occurring across a multitude of taxonomically diverse genera. Betulin and betulinic acids are lupane type of triterpenes, and oleanolic acid is of oleanane type.

Thin layer chromatography revealed that the major components of fraction Ea-EtOAc-2 were betulin (**5.1**), betulinic acid (**5.2**) and a less polar compound. Ea-EtOAc-4 was found to contain mainly betulinic acid (**5.2**) and oleanolic acid (**5.3**). Ea-EtOAc-5 and Ea-EtOAc-6 contained compounds other than these three described. These fractions also showed good anti-inflammatory activities. Column chromatography of fractions Ea-EtOAc-5 and Ea-EtOAc-6 were conducted, but no pure compounds have been isolated so far because of the complexity of their components and time constraints. These fractions are worthy of further investigation in the future.

5.4.2.1. Anti-inflammatory activity of pure compounds

The pure compounds isolated from *E. aphyllus*, betulin (**5.1**), betulinic acid (**5.2**) and oleanolic acid (**5.3**), were examined for their anti-inflammatory activities again COX-1, COX-2 and 5-LO. IC_{50} values of these compounds were determined.

All three compounds presented anti-inflammatory activity against the tested enzymes. Oleanolic acid was found to be the most active compound in the assays and exhibited IC_{50} values of 53, 129 and 70 μg/ml against COX-1, COX-2 and 5-LO, respectively. The anti-inflammatory activities of these pure compounds are shown in Table 5.7. The IC_{50} value of betulin against COX-1 was not obtained. However, its inhibitory activity against COX-1 was determined at 1 mg/ml as 38%.

Table 5.7. Anti-inflammatory activities of pure compounds isolated from *E. aphyllus*.

	IC_{50} (mM)		
Compound	COX-1	COX-2	5-LO
Betulin (**5.1**)	N.A.	1.06	1.12
Betulinic acid (**5.2**)	0.45	0.59	0.14
Oleanolic acid (**5.3**)	0.12	0.28	0.15

N.A.: not available.

Betulin (**5.1**), betulinic acid (**5.2**) and oleanolic acid (**5.3**) have been reported to have anti-inflammatory activity in the literature. Their anti-inflammatory properties have been verified in a number of *in vitro* and *in vivo* model systems including the serotonin-, bradykinin- or carrageenin-induced mouse paw edema tests, 12-*O*-tetradecanoyl-13-acetate (TPA)- or 12-deoxyphorbol-13-phenylacetate (DPP)-induced eye edema tests, and neutrophil and macrophage based assays [Recio *et al*., 1995; Mukherjee *et al*., 1997; Cichewicz and Kouzi, 2004; Patocka, 2003]. It has been suggested that these compounds possess moderate anti-inflammatory properties [Cichewicz and Kouzi, 2004]. Betulin has been reported to inhibit the production of nitric oxide and prostaglandin E_2 by inducible nitric oxide synthase and COX-2 in mouse macrophages, with IC_{50} values being 5.0 and 12.9 μM, respectively [Reyes *et al*., 2006]. A study showed that betulinic acid caused weak inhibition of bovine prostaglandin synthase (cyclooxygenase) *in vitro* with an IC_{50} of 101 μM [Huang *et al*., 1995], but a higher value (200 μg/ml, equal to 438 μM) has also been reported by other researchers [Dunstan *et al*., 1998]. Some researchers have suggested that the anti-inflammatory effects of betulin and betulinic acid are due to their glucocorticoidal and non-neurogenic mechanisms [Recio *et al*., 1995; Cichewicz and Kouzi, 2004]. Oleanolic acid in an *in vitro* test (by measuring the initial rate of O_2 uptake) showed a 10% inhibition of the COX-2 enzyme activity at 200 μg/ml (438 μM), but did not show any

inhibition of COX-1 [Zhang *et al*., 2004]. Oleanolic acid has also been reported to reduce the production of leukotriene B_4 from the leukocytes from the rat peritoneal cavity with an IC_{50} of 17 μM, and to inhibit the phospholipase A_2-induced edema [Giner-Larza *et al*., 2001]. The difference between the reported data and the findings achieved in this study on the inhibitory activities of betulin, betulinic acid and oleanolic acid against COX and LO enzymes is most likely due to the different test methods used in the assays. The values are therefore not directly comparable with each other.

Betulin (**5.1**), betulinic acid (**5.2**) and oleanolic acid (**5.3**) are also renowned for possessing various biological properties other than anti-inflammation, such as cytotoxic, hepatoprotective, analgesic, anticancer and antiviral activities [Patocka, 2003; Szuster-Ciesielska and Kandefer-Szerszen, 2005]. Betulinic acid (**5.2**) has been reported to induce apoptosis selectively in human melanoma, neuroblastoma and brain tumour cells [Hata *et al*., 2003; Selzer *et al*., 2000; Liu *et al*., 2004]. Its antiproliferative activity toward MCF-7 human breast cancer cells has been found to be more potent than the anticancer drug 5-fluorouracil [Amico *et al*., 2006]. Betulin, betulinic acid, oleanolic acid and their derivatives have also been shown to have potent anti-HIV-1 activity [Kashiwada *et al*., 1996; Soler *et al*., 1996; Kashiwada *et al*., 1998]. Antiviral activity of betulin and betulinic acid against herpes simplex type 1 (HSV-1) and ECHO 6 viruses has also been reported [Baltina *et al*., 2003; Pavlova *et al*., 2003].

From the above literature survey of betulin, betulinic and oleanolic acids and the anti-inflammatory bioassay results obtained in the present study, it can be seen that these compounds reduce inflammation *via* a variety of mechanisms. Although each of these three triterpenes did not exhibit potent inhibitory activity against all of the inflammation pathway enzymes tested, due to the coexistence of these compounds in *E. aphyllus* and

their abundance in the plant extract, the combination of these triterpenes might result in a better overall anti-inflammatory effect. Given that the Australian Aboriginal people use mixtures (*e.g.*, decoctions) instead of pure compounds for medicinal purposes, the use of *E. aphyllus* as an anti-inflammatory treatment for washing sores is supported by this study.

The anti-inflammatory activity of extracts of *E. aphyllus* has been demonstrated for the first time in this study. This is also the first time that betulin and betulinic acid have been isolated from any *Exocarpos* species, and the first time that oleanolic acid has been isolated from *E. aphyllus*.

5.5. Conclusions and future directions

The ethnomedicinal knowledge on *Exocarpos aphyllus* was used as a guide for targeted chemical and biological investigations of this plant. The anti-inflammatory and antimicrobial activities of extracts of *E. aphyllus* have been demonstrated in this study. The antimicrobial activity has been shown to be caused in part by the phenolic components in this plant. Future investigations of these fractions using LCMS profiling could lead to the identification of these phenolic compounds. The triterpenes betulin, betulinic acid and oleanolic acid were isolated from *E. aphyllus* as anti-inflammatory components. This is the first time that betulin and betulinic acid have been isolated from any *Exocarpos* species, and the first time that oleanolic acids have been isolated from *E. aphyllus*. Further separation of active fractions, such as the *n*-butanol and water fractions of *E. aphyllus*, could lead to the isolation of more compounds with antimicrobial and anti-inflammatory activities. Examination of a customary preparation of *E. aphyllus* to relate levels of any bioactive compounds isolated to bioactivity of the preparation could also provide further

evidence for the efficacy of *E. aphyllus* as a traditional medicine. All the results of this study were provided to Mr and Mrs Barker.

In conclusion, these research findings provide strong support for the traditional medicinal uses of *Exocarpos aphyllus* by Australian Aboriginal people.

5.6. Experimental

5.6.1. General

All reagents, material and instruments used in this study are described in Section 4.6.1.

5.6.2. Plant material

Fresh twigs and branches (stems) of *Exocarpos aphyllus* were collected from Lightning Ridge, New South Wales (140°58'53"E x 29°25'49"S) in December 2003. The plant sample was identified and a voucher specimen has been deposited in the herbarium of Macquarie University (voucher number 73007709).

5.6.3. Extraction and isolation

Fresh branches (stems) of *Exocarpos aphyllus* (660 g) were cut into small portions and blended with 70% (v/v) ethanol and water (2.2 L). The mixture was stirred occasionally at room temperature for 5 days before being vacuum filtered. The ethanol soluble extract was evaporated *in vacuo* and 32.4 g of dark green solid was obtained. The extraction was

repeated twice and the filtrates were combined with the first extract to give a total of 84 g of dark green crude extract (13% yield). 4.5 g of the crude extract was saved for further biological tests. The rest of the extract (79.5 g) was suspended in 500 ml water and partitioned with ethyl acetate (4 x 500 ml), followed by *n*-butanol (4 x 250 ml). Three partition fractions, Ea-EtOAc (42.6 g, 53.6% yield, light green solid), Ea-BuOH (12.5 g, 15.7% yield, crimson sticky gum) and Ea-water (24.5 g, 30.8% yield, crimson sticky gum) were obtained.

The ethyl acetate fraction (40 g) of *E. aphyllus* was subjected to normal phase flash silica gel column chromatography, eluting with petroleum ether and acetone with increasing polarity (10:1 to 1:2, v/v). The eluants were checked by TLC and combined to give 6 fractions, namely Ea-EtOAc-1 to Ea-EtOAc-6. Ea-EtOAc-1 (0.70 g, 1.8% yield) was an orange-brown oil, and Ea-EtOAc-2 to Ea-EtOAc-6 were green or dark green solids (1.9, 9.1, 2.0, 2.5 and 2.1 g, respectively). Yields of Ea-EtOAc-2 to Ea-EtOAc-6 were 4.8%, 22.8%, 5.0%, 6.3% and 5.3%, respectively.

The green solid of Ea-EtOAc-3 (eluted from the column with 2:1 petroleum ether and acetone, v/v) was washed with petroleum ether to remove most of the chlorophylls. The resultant white solid was found to contain three major components with Rf values being 0.47, 0.28 and 0.23, respectively, on normal phase TLC developed with 100:1 chloroform and methanol (v/v), and a few minor components. The solid was subjected to normal phase flash silica gel column chromatography, eluting with mixtures of chloroform and methanol with increasing polarities (100% chloroform to 3% methanol in chloroform, v/v). This afforded three compounds, betulin (**5.1**), betulinic acid (**5.2**) and oleanolic acid (**5.3**).

5.6.3.1. Betulin (5.1)

Betulin (**5.1**) was isolated as a white solid (63 mg, eluted with 100% chloroform). M.p. 251-253°C. LREIMS *m/z* (% rel. int.): 443 $[M+1]^+$(3.5), 442 $[M]^+$(11), 427 $[M-15]^+$ (6), 424 $[M-H_2O]^+$ (5), 411 (31), 393 (7), 385 (7), 381 (3), 288 (8), 234 (25), 220 (19), 203 (68), 189 (93), 175 (33), 43 (100). ^{1}H NMR (*d*-chloroform): 1.65 (*m*, H-1a), 0.89 (*m*, H-1b), 1.59 (*m*, H-2a), 1.52 (*m*, H-2b), 3.18 (*dd*, *J* = 11.2, 5.0 Hz, H-3), 0.67 (*m*, H-5), 1.52 (*m*, H-6a), 1.38 (*m*, H-6b), 1.38 (*m*, H-7), 1.26 (*m*, H-9), 1.39 (*m*, H-11a), 1.19 (*m*, H-11b), 1.64 (*m*, H-12a), 1.04 (*m*, H-12b), 1.60 (*m*, H-13), 1.70 (*m*, H-15a), 1.07 (*m*, H-15b), 1.92 (*m*, H-16a), 1.21 (*m*, H-16b), 1.57 (*m*, H-18), 2.38 (*ddd*, *J* = 5.9, 10.8, 10.8 Hz, H-19), 1.97 (*m*, H-21a), 1.39 (*m*, H-21b), 1.84 (*m*, H-22a), 1.04 (*m*, H-22b), 0.96 (*s*, H-23), 0.75 (*s*, H-24), 0.81 (*s*, H-25), 1.01 (*s*, H-26), 0.97 (*s*, H-27), 3.79 (*dd*, *J* = 10.8, 1.5 Hz, H-28a), 3.32 (*d*, *J* = 10.8 Hz, H-28b), 4.67 (*d*, *J* = 2.2 Hz, H-29a), 4.57 (*dd*, *J* = 2.2, 1.4 Hz, H-29b), 1.67 (*s*, H-30). ^{13}C NMR data of Betulin (**5.1**) are listed in Table 5.6.

5.6.3.2. Betulinic acid (5.2)

Betulinic acid (**5.2**) was isolated as a white solid (217 mg, eluted with 1% methanol in chloroform, v/v). M.p. 272-273°C (decomp.), LREIMS *m/z* 456 $[M]^+$ (9), 438 $[M-H_2O]^+$(6), 423 $[M-H_2O-15]^+$ (5), 410 (3), 395 (5), 377 (2), 316 (4), 302 (5), 259 (6), 248 (43), 234 (19), 220 (25), 207 (51), 189 (100), 175 (29). ^{1}H NMR (*d*-chloroform with a few drops of *d*-methanol): 1.63 (*m*, H-1a), 0.86 (*m*, H-1b), 1.52 (*m*, H-2), 3.10 (*dd*, *J* = 9.8, 6.3 Hz, H-3), 0.64 (*m*, H-5), 1.48 (*m*, H-6a), 1.34 (*m*, H-6b), 1.33 (*m*, H-7), 1.24 (*m*, H-9), 1.38 (*m*, H-11a), 1.18 (*m*, H-11b), 1.64 (*m*, H-12a), 0.97 (*m*, H-12b), 2.20 (*m*, H-13), 1.88 (*m*, H-15a), 1.32 (*m*, H-15b), 2.19 (*m*, H-16a), 1.33 (*m*, H-16b), 1.54 (*m*, H-18), 2.96 (*ddd*, *J* = 4.6, 10.8,

10.7 Hz, H-19), 1.46 (*m*, H-21a), 1.11 (*m*, H-21b), 1.87 (*m*, H-22a), 1.37 (*m*, H-22b), 0.90 (*s*, H-23), 0.70 (*s*, H-24), 0.78 (*s*, H-25), 0.90 (*s*, H-26), 0.94 (*s*, H-27), 4.67 (*br s* H-29a), 4.54 (*br s*, H-29b), 1.64 (*s*, H-30). ^{13}C NMR data of betulinic acid (**5.2**) are listed in Table 5.6.

5.6.3.3. Oleanolic acid (5.3)

Oleanolic acid (**5.3**) was isolated as a white solid (397 mg, eluted with 3% methanol in chloroform, v/v). M.p. 268°C (decomp.), LREIMS *m/z* (% rel. int.): 456 $[M]^+$ (1), 438 $[M\text{-}H_2O]^+$ (1), 423 $[M\text{-}H_2O\text{-}15]^+$ (1), 395 (1), 300 (1), 257 (1), 248 (99), 233 (10), 203 (100), 190 (12), 189 (16), 175 (9). ^{1}H NMR (*d*-chloroform with a few drops of *d*-methanol): 1.51 (*m*, H-1a), 0.87 (*m*, H-1b), 1.48 (*m*, H-2), 3.10 (*dd*, *J* = 10.2, 5.8 Hz, H-3), 0.64 (*m*, H-5), 1.44 (*m*, H-6a), 1.28 (*m*, H-6b), 1.35 (*m*, H-7a), 1.21 (*m*, H-7b), 1.46 (*m*, H-9), 1.87 (*ddd*, *J* = 13.5, 3.9, 3.4 Hz, H-11a), 1.52 (*m*, H-11b), 5.17 (*t*, *J* = 3.4 Hz, H-12), 1.61 (*m*, H-15a), 0.99 (*m*, H-15b), 1.76 (*m*, H-16), 2.72 (*dd*, *J* = 13.8, 3.9 Hz, H-18), 1.52 (*m*, H-19a), 1.03 (*m*, H-19b), 1.24 (*m*, H-21a), 1.11 (*m*, H-21b), 1.64 (*m*, H-22a), 1.46 (*m*, H-22b), 0.87 (*s*, H-23), 0.67 (*s*, H-24), 0.80 (*s*, H-25), 0.67 (*s*, H-26), 0.82 (*s*, H-27), 0.79 (*s,* H-29), 0.82 (*s*, H-30). ^{13}C NMR data of oleanolic acid (**5.3**) are listed in Table 5.6.

5.6.4. Separation of the *n*-butanol fraction

The *n*-butanol fraction (6.4 g) was ground to a powder and mixed with 25 ml of 4:1 chloroform and methanol (v/v). The mixture was sonicated for 20 minutes and centrifuged at 3000 r.p.m. for 30 minutes. The supernatant was collected and the residue was extracted again using the same procedure. The supernatants were combined and filtered. The solvent

was removed under reduced pressure to give a pale yellow solid (0.99 g, Ea-BuOH-CM). The residue was dried under reduced pressure and a brownish solid (4.2 g) was obtained (Ea-BuOH-Res).

5.6.5. Detection of phenolic compounds

Samples of the water partitions and Ea-BuOH-Res (about 20 mg each) were dissolved in 4 ml of 25% aqueous methanol. 1 ml of each solution was transferred in to a test tube and a drop of 1% aqueous $FeCl_3$ solution was added. Both solutions turned green. To test if the phenolic compounds were polyphenols, 1 ml of each original solution was added to a test tube and saturated aqueous lead acetate solution was added. A large amount of precipitate was produced, which indicated the presence of polyphenols.

5.6.6. Antimicrobial and anti-inflammatory assays

Antimicrobial and anti-inflammatory assays were performed using the assay procedures described in Chapters 3 and 4.

Chapter 6. General Conclusions

This PhD study covered the documentation of first-hand medicinal plant knowledge of Aboriginal communities in northern New South Wales through to the isolation and characterisation of bioactive compounds from Aboriginal medicinal plants.

The ethnobotanical research was conducted with the Kamilaroi and Muruwari Aboriginal communities in northern New South Wales. The traditional applications and preparation methods of 35 medicinal plants and 2 traditional remedies were recorded following interviews with Aboriginal community members and literature survey, and were documented in a database along with a literature summary of their biological activities and bioactive constituents. This database was provided to the Kamilaroi and Muruwari Aboriginal communities as a medium for the preservation of Indigenous traditional medicinal knowledge. A series of educational activities were also conducted for the Indigenous youth, serving as part of relationship development and benefit sharing with these communities.

Eremophila sturtii and *Exocarpos aphyllus* were chosen from the documented Aboriginal medicinal plants for comprehensive chemical and biological investigations due to their customary medicinal applications.

A few biological assays have been used in this study for the evaluation of the antimicrobial and anti-inflammatory activities of these medicinal plants, and have been used to guide the fractionation and isolation of the bioactive constituents. The fluorescein diacetate (FDA) assay was optimised in this study to suit the determination of the antibacterial activity of

plant extracts, fractions and pure compounds against the human pathogens *Staphylococcus aureus*, *Escherichia coli* and *Pseudomonas aeruginosa*. The results obtained by the FDA assay were found to be comparable with those determined by the resazurin antibacterial assay. A broth microdilution assay has been used for the examination of the antifungal activity of the medicinal plants against the yeast *Candida albicans*. The anti-inflammatory properties of these medicinal plants have been investigated against the inflammation pathway enzymes cyclooxygenase-1 and -2 and 5-lipoxygenase using commercially available enzyme immunoassays.

The bioassay-guided fractionation of *Eremophila sturtii* resulted in the isolation of two novel bioactive compounds, 3,8-dihydroxyserrulatic acid and serrulatic acid, and three known bioactive compounds, *β*-sitosterol, sesamin and 3,6-dimethoxy-5,7-dihydroxyflavone. Their structures were elucidated by 1D and 2D NMR and mass spectrometric data. Both of the novel compounds exhibited bactericidal activity against *Staphylococcus aureus*, with the minimum bactericidal concentration being 200 μg/ml and 15 μg/ml, respectively (as tested by the FDA assay). The novel compounds also demonstrated anti-inflammatory activity against cyclooxygenase-1 and -2. At 1 mg/ml, 3,8-dihydroxyserrulatic acid inhibited the activity of cyclooxygenase-1 and -2 by 57% and 14%, respectively. Serrulatic acid inhibited cyclooxygenase-1 and -2 with the IC_{50} value being 27 μg/ml (90 μM) and 73 μg/ml (243 μM), respectively. All the known compounds, *β*-sitosterol, sesamin and 3,6-dimethoxy-5,7-dihydroxyflavone, showed anti-inflammatory activity against cyclooxygenase-1 and -2 and 5-lipoxygenase.

Three triterpenes, namely betulin, betulinic acid and oleanolic acid, have been isolated from *Exocarpos aphyllus* through bioassay-guided fractionation. All of these compounds demonstrated anti-inflammatory activity against cyclooxygenase-1 and -2 and

5-lipoxygenase. The antimicrobial activity of *Exocarpos aphyllus* was found to be partly due to the presence of phenolic components.

The biological activities demonstrated by the medicinal plants *Eremophila sturtii* and *Exocarpos aphyllus* were consistent with their traditional medicinal applications. The ethnobotanical information therefore provided a successful lead towards the isolation of bioactive compounds from these Australian Aboriginal medicinal plants.

For all of the compounds identified, this is the first report of their isolation from *Eremophila sturtii* and *Exocarpos aphyllus*. This is also the first time any antibacterial and anti-inflammatory compounds have been reported for *Eremophila sturtii* and any anti-inflammatory compounds have been identified for *Exocarpos aphyllus*.

In recognition of the value of Indigenous knowledge and in line with working as true research partners, if any commercial potential arose as a result of the biological and chemical investigations, then a process of further negotiation would ensue with the communities in order to guarantee joint ownership and protection of Intellectual Property.

In conclusion, this PhD research has successfully achieved its primary aims of conservation of traditional and contemporary Aboriginal medicinal knowledge through to the discovery of novel and bioactive compounds from Australian Aboriginal medicinal plants.

Appendices

Appendix 1. The ethnobotanical database for the Kamilaroi and Muruwari Aboriginal communities.

Genus and Species Name	Common Name
Acacia aneura F. Muell. ex Benth.	Mulga, Malga

Aboriginal Name	Family
Malga	Mimosaceae

Description of Plant	Collection
A shrub or tree 5-12m high with rough, fissured, grey bark. Carries pods from 2.5 to 3.75 cm long [5].	

Distribution
Arid areas of QLD, NSW, SA, WA and NT

Use (Food, Medicine)	Food Value
Food, Medicine	(Seeds) high in energy, protein and carbohydrate

Preparation in the Kamilaroi and Muruwari Communities

Preparation in the Literature
Ash mixed with dried and powdered Isotoma petraea was used for colds and as a pain killer [32]. Smokes from leaves and twigs were used for new-born child to promote good health [6]. Seeds were soaked or roasted, ground and eaten as food, or eaten raw [26].

Medicinal Use in the Kamilaroi and Muruwari Communities

Medicinal Use in the Literature
Pain killer, colds [32].

Studies Performed in the Literature
Three flavonoid aglycons apigenin, rhamnetin and fisetin were identified [52].

References
Barr *et al*., 1993; Isaacs, 1987; Latz, 1995; Australian National Botanic Gardens (online); Saleem, 1992.

Genus and Species Name	Common Name
Acacia cambagei R. T. Baker	Gidgee

Aboriginal Name	Family
Gidjirr	Mimosaceae

Description of Plant	Collection
A long-lived small tree to 15m with a wide dense crown and deeply furrowed bark. Leaves silver grey, densely hairy. Pods to 14cm long, 1-2cm wide [37].	

Distribution
NSW, QLD, SA, NT

Use (Food, Medicine)	Food Value
Medicine	

Preparation in the Kamilaroi and Muruwari Communities
White ashes were used for wounds, cuts, also used to settle mud down from muddy water so that water could be drunk afterwards.

Preparation in the Literature

Medicinal Use in the Kamilaroi and Muruwari Communities
Wounds, cuts. For settling mud down from muddy water [25]

Medicinal Use in the Literature

Studies Performed in the Literature

References
New South Wales Flora Online; Hunter, 2005.

Genus and Species Name	Common Name
Acacia ligulata A.Cunn. ex Benth.	Sandhill wattle

Aboriginal Name	Family
Girran.biiyan	Mimosaceae

Description of Plant	Collection
A bushy, dome shaped shrub up to 3m high. Widespread on dry alkaline soils or costal dunes [16].	

Distribution
NSW, VIC, Central desert

Use (Food, Medicine)	Food Value
Food, Medicine	Seeds roasted and eaten. Gum eaten.

Preparation in the Kamilaroi and Muruwari Communities

Preparation in the Literature
Bark was soaked or boiled in water and decoction was used as a cough medicine and as a medical wash. Also good for dizziness, nerves and fits [32]. Seeds can be ground and roasted and eaten as food [16, 26]. Ashes mixed with Pituri (*Duboisia hopwoodii*) before use as a narcotic [16].

Medicinal Use in the Kamilaroi and Muruwari Communities

Medicinal Use in the Literature
For cough, dizziness [32].

Studies Performed in the Literature

References
Isaacs, 1987; Latz, 1995; Cunningham *et al*., 1981.

Genus and Species Name	Common Name
Acacia salicina Lindl.	Cooba

Aboriginal Name	Family
Dhulan	Mimosaceae

Description of Plant	Collection
Spreading tree to 14 m high with pendulous habit and narrow-elliptical phyllodes to 20 cm. Bears yellow ball-shaped flowers in winter and spring [16].	

Distribution
Drier areas, but usually near rivers, in QLD, NSW, VIC, SA, and NT.

Use (Food, Medicine)	Food Value
Medicine, fish poison	

Preparation in the Kamilaroi and Muruwari Communities

Preparation in the Literature
Bark was soaked or boiled in water and decoction was used as a cough medicine. Leaves were burnt with *Duboisia hopwoodii* and ash was smoked to produce drunkeness, drowsiness or dopiness and finally deep and lengthy sleep [26]. Leaves were added to waterholes and creeks as a fish poison [16].

Medicinal Use in the Kamilaroi and Muruwari Communities

Medicinal Use in the Literature
For cough, sleep [26].

Studies Performed in the Literature

References
Isaacs, 1987; Cunningham *et al*., 1981.

Genus and Species Name	Common Name
Acacia stenophylla A.Cunn. ex Benth.	River cooba

Aboriginal Name	Family
Gurrulay	Mimosaceae

Description of Plant	Collection
An erect or spreading shrub or small tree to 20 m tall with a rounded crown. It varies in form over its distribution but is usually single stemmed with pendulous branchlets [5].	

Distribution
VIC, NSW, WA

Use (Food, Medicine)	Food Value
Food	

Preparation in the Kamilaroi and Muruwari Communities

Preparation in the Literature
Seed pods roasted and seeds eaten [26].

Medicinal Use in the Kamilaroi and Muruwari Communities

Medicinal Use in the Literature

Studies Performed in the Literature

References
Isaacs, 1987; Australian National Botanic Gardens (online)

Genus and Species Name	Common Name
Acacia victoriae Benth.	Ginderbluey wattle, Bramble wattle

Aboriginal Name	Family
ngadul, ngaduwi	Mimosaceae

Description of Plant	Collection
A branched shrub or small tree up to 5m high. Flowers pale yellow, pods flat until the seeds are formed.	

Distribution
Central desert, most common on river flats

Use (Food, Medicine)	Food Value
Food	High in energy, protein and carbohydrates

Preparation in the Kamilaroi and Muruwari Communities

Preparation in the Literature
Seeds ground and roasted for damper. The white gum exuding from the trunk was also eaten.

Medicinal Use in the Kamilaroi and Muruwari Communities

Medicinal Use in the Literature

Studies Performed in the Literature
Saponins avicins D and G isolated from the seed pods of *Acacia victoriae* exhibited potent cytotoxicity (apoptosis) against human T-cell leukemia (Jurkat cells) in vitro [28]. Avicins obtained from *Acacia victoriae* suppressed the development of human skin cancer and other epithelial malignancies [24].

References
Isaacs, 1987; Latz, 1995; Jayatilake *et al*., 2003; Hanausek *et al*., 2001.

Genus and Species Name	Common Name
Alstonia constricta F.Muell.	Quinine Bush

Aboriginal Name	Family
Gardibunduu	Apocynaceae

Description of Plant	Collection
A shrub or tree up to 12m high. Leaves opposite, lanceolate, dark green and glossy. Prefers sandy and loamy soils, also in rainforest clearings [16]. Contains reserpine - useful in treating high blood pressure.	

Distribution
QLD, NSW

Use (Food, Medicine)	Food Value
Medicine	

Preparation in the Kamilaroi and Muruwari Communities
Quinine Bush boiled and liquid drunk for kidney, lung and skin disease. Sap used for diabetes [25].

Preparation in the Literature
A tincture prepared by extraction of 100g of bark with 750ml of proof spirit was used as a febrifuge and tonic (dosage: five to ten drops). Pills made from powdered bark and licorice. Latex or sap used for infectious sores [26,34]. Poison.

Medicinal Use in the Kamilaroi and Muruwari Communities
Various types of ailments. Diabetes, lung cancer, and kidney diseases [25]

Medicinal Use in the Literature
Antiperiodic, cerebro-spinal stimulant, antimalaria, sores. For typhoid and dysentery. A deadly poison.

Studies Performed in the Literature
Alkaloids were found in the stem bark [3]. Reserpine was found in a tissue culture of *Alstonia constricta* [12].

References
Isaacs, 1987; Low, 1990; Allam *et al*., 1987; Carew, 1965, Cunningham *et al.* 1981. Hunter, 2005.

Genus and Species Name	Common Name
Apophyllum anomalum F.Muell.	Currant Bush

Aboriginal Name	Family
Gubigala	Capparaceae

Description of Plant	Collection
Shrub mostly 3-5 m high, sometimes taller and tree-like, older branches leafless, glabrous, sometimes drooping. Fruit a berry, 5mm, black when ripe [37, 16].	

Distribution
NSW, QLD

Use (Food, Medicine)	Food Value
Food	

Preparation in the Kamilaroi and Muruwari Communities
Fruit eaten, roasted with ash to get rid of the toxin [25].

Preparation in the Literature

Medicinal Use in the Kamilaroi and Muruwari Communities

Medicinal Use in the Literature

Studies Performed in the Literature

References
New South Wales Flora Online; Cunningham *et al*., 1981. Hunter, 2005.

Genus and Species Name	Common Name
Boerhavia diffusa L.	Tar vine

Aboriginal Name	Family
Wudhugaa	Nyctaginacceae

Description of Plant	Collection
A prostrate perrenial creeper or herb up to several metres long. Leaves opposite [16].	

Distribution
On bare areas of warm regions. All states except Tasmania.

Use (Food, Medicine)	Food Value
Food, Medicine	Moderate energy, high water and carbohydrates

Preparation in the Kamilaroi and Muruwari Communities

Preparation in the Literature
Ground creeper with edible root roasted in fire was eaten as food [26]. Used as an expectorant in asthma. In India, the root and dried herb are used for diuretic and emetic action.

Medicinal Use in the Kamilaroi and Muruwari Communities

Medicinal Use in the Literature
Expectorant in asthma, diuretic and emetic

Studies Performed in the Literature
Aqueous leaf extact was found non toxic in albino rats [40]. Punernavine isolated from the root showed powerful diuretic activity [58]. Root extract showed antifungal activity [1]. Leaves extract showed antidiabetic and antioxidant effects in alloxan diabetic rats [43, 53].

References
Isaacs, 1987; Orisakwe *et al*., 2003; Wahi *et al*., 1997; Agrawal *et al*., 2004; Pari *et al*., 2004; Satheesh *et al*., 2004

Genus and Species Name	Common Name
Brachychiton populneum (Schott. & Endl.) R. Br.	Kurrajong

Aboriginal Name	Family
Nhungga	Sterculiaceae

Description of Plant	Collection
Tall tree with distinctive, fiborous bark. Leaves 3 or 5 lobed. Flowers, red carried in summer. Fruit a woody capsule10-15cm long, containing numerous black seeds within a mass of irritating hairs [16].	

Distribution
VIC, NSW, Central desert

Use (Food, Medicine)	Food Value
Food	High energy, protein, fat, carbohydrates

Preparation in the Kamilaroi and Muruwari Communities

Preparation in the Literature
Young roots, gum and seeds eaten: seeds are hard, but can be crushed [26].

Medicinal Use in the Kamilaroi and Muruwari Communities

Medicinal Use in the Literature

Studies Performed in the Literature

References
Isaacs, 1987, Cunningham *et al*., 1981.

Genus and Species Name	Common Name
Capparis mitchelli Lindl.	Wild orange

Aboriginal Name	Family
Bambul	Capparaceae

Description of Plant	Collection
A small compact tree about 3.5m high with dark green, somewhat leathery leaves. Flowers open at night, wither before the end of the next day. Small desert fruit, grows on a large shrub, only found infrequently in summer [16, 25].	Date: December 2005 Venue: Lightning Ridge, New South Wales (140°58'53"E x 29°25'49"S) Voucher number: 73007961

Distribution
NSW, NT, QLD, WA, SA

Use (Food, Medicine)	Food Value
Food, medicine	A good source of Vitamin C and thiamine

Preparation in the Kamilaroi and Muruwari Communities
Leaves were boiled in water and used as a wash to cure venereal disease. The flowers were also used in woman's ceremony [25].

Preparation in the Literature
Eaten fresh as a food [26, 32].

Medicinal Use in the Kamilaroi and Muruwari Communities
Women's illnesses [25].

Medicinal Use in the Literature

Studies Performed in the Literature

References
Hunter, 2005; Isaacs, 1987; Latz, 1995; Cunningham *et al*., 1981.

Genus and Species Name	Common Name
Cucumis melo L.	Ulcardo Melon

Aboriginal Name	Family
Baaya	Cucurbitaceae

Description of Plant	Collection
An annual twining creeper, tough to the touch, with small yellow flowers and green fruits which mature to a yellowish colour. Small round fruit.	

Distribution
NSW, QLD, WA, NT, SA

Use (Food, Medicine)	Food Value
Food	

Preparation in the Kamilaroi and Muruwari Communities

Preparation in the Literature
Cooked by boiling, or wrapped in paperbark in hot sand – Kimberley [26, 32].

Medicinal Use in the Kamilaroi and Muruwari Communities

Medicinal Use in the Literature

Studies Performed in the Literature
An aqueous solution was shown to inhibit human platelet aggregation [4]. Antifungal activity was reported [56].

References
Isaacs, 1987; Latz, 1995; Altman *et al*., 1985; Sproston *et al*., 1948

Genus and Species Name	Common Name
Dodonaea attenuata A.Cunn.	Narrowleaf hopbush

Aboriginal Name	Family
Yiilay	Sapindaceae

Description of Plant	Collection
A slender shrub up to 3m tall. Leaves are narrow with parallel sides. Fruit a 3 winged papery capsule [16].	

Distribution
Widespread throughout Australia.

Use (Food, Medicine)	Food Value
Medicine	

Preparation in the Kamilaroi and Muruwari Communities

Preparation in the Literature
Infusion of the foliage was used for sponging of the forehead and body to relieve fever [16, 32].

Medicinal Use in the Kamilaroi and Muruwari Communities

Medicinal Use in the Literature
Fever

Studies Performed in the Literature
Prosapogenins, Lup-20(29)-ene-3b, 11b-diol and lupeol have been isolated from *D. attenuata* [21].

References
Latz, 1995; Han *et al*., 1995; Ghisalberti *et al*., 1973; Cunningham *et al*., 1981.

Genus and Species Name	Common Name
Enchylaena tomentosa R. Br.	Ruby Saltbush

Aboriginal Name	Family
Binamayaa	Chenopodiaceae

Description of Plant	Collection
A compact, greenish grey, scaly, hairy shrub up to 1m tall. Perianth enlarges after flowering forming a suculent covering to the fruit, green to red in colour [5, 16].	Date: December 2005 Venue: Lightning Ridge, New South Wales (140°58'53"E x 29°25'49"S) Voucher number: 73007960

Distribution
In salt marsh habitats in tropical, sub-tropical, and temperate regions.

Use (Food, Medicine)	Food Value
Food	

Preparation in the Kamilaroi and Muruwari Communities
Used as salt to cook emu meat and add Vitamines to diet [25].

Preparation in the Literature

Medicinal Use in the Kamilaroi and Muruwari Communities

Medicinal Use in the Literature

Studies Performed in the Literature

References
Australian National Botanic Gardens (online); Cunningham *et al*., 1981. Hunter, 2005.

Genus and Species Name	Common Name
Eremophila bignoniiflora (Benth.) F. Muell.	Dogwood

Aboriginal Name	Family
Yuurraa	Myoporaceae

Description of Plant	Collection
A small erect tree with drooping branches and a rough grey bark. Along creeks or flooded flats [16].	

Distribution
NSW, QLD, VIC, WA, SA, NT.

Use (Food, Medicine)	Food Value
Medicine	

Preparation in the Kamilaroi and Muruwari Communities
Boil together with the Leopardwood and apply on skin to treat scabies [25]

Preparation in the Literature
A decoction of fruit was used as a laxative. A decoction of leaves was applied as a body wash to relieve symptoms of colds and flu. Boiled leaves were wrapped around head to alleviate headache associated with nasal congestion and sinusitis [6, 34]

Medicinal Use in the Kamilaroi and Muruwari Communities
Scabies [25]

Medicinal Use in the Literature
Laxatives, colds, flu, venereal disease [6, 34]

Studies Performed in the Literature
E. bignoniiflora showed promise as a drug for neurological disorders [48]

References
Barr *et al*., 1993; Low, 1990; Rogers *et al*., 2002. Hunter, 2005. Cunningham *et al*., 1981.

Genus and Species Name	Common Name
Eremophila gilesii F. Muell.	Ellangowan poison bush, desert fuschia

Aboriginal Name	Family
Burrgul.biyan	Myoporaceae

Description of Plant	Collection
A small shrub usually 80 cm high, with narrow leaves and blue flowers, growing under or near mulga [16].	

Distribution
NT, NSW

Use (Food, Medicine)	Food Value
Medicine	

Preparation in the Kamilaroi and Muruwari Communities

Preparation in the Literature
Plant heated or boiled and liquid used as a wash for scabies [26, 32].

Medicinal Use in the Kamilaroi and Muruwari Communities

Medicinal Use in the Literature
Scabies [26, 32]

Studies Performed in the Literature
Phenylethanoid glycosides verbascoside and poliumoside [22], and two new cembrene diterpenes have been isolated from *Eremophila gilesii* [20]

References
Isaacs, 1987; Latz, 1995; Grice *et al*., 2003; Ghisalberti *et al*., 1994. Cunningham *et al*., 1981.

Genus and Species Name	Common Name
Eremophila longifolia (R. Br.) F. Muell.	Berrigan, Emu bush

Aboriginal Name	Family
Ngawil	Myoporaceae

Description of Plant	Collection
A tall erect shrub, up to 6m high, with drooping branches. On limestone soils; continental Australia [16]	

Distribution
NT, NSW

Use (Food, Medicine)	Food Value
Medicine	

Preparation in the Kamilaroi and Muruwari Communities

Preparation in the Literature
Decoction of leaves as a body wash was applied to sores and cuts [6, 7, 32, 34] and drank for colds . A medicinal bath for general pain and sickness [6, 7, 32, 34]. Mashed leaves rubbed onto scabies or used as a dressing for boils and carbuncles [6, 7, 32, 34]. Also used in smoke or steam treatment [26].

Medicinal Use in the Kamilaroi and Muruwari Communities

Medicinal Use in the Literature
Sores, colds, sore eyes, headaches, insomnia, scabies, boils, general sickness, antiseptic, counter-irritant [6, 7, 26, 32, 34]

Studies Performed in the Literature
Extract of *Eremophila longifolia* potently inhibited ADP induced human platelet [14C]5-HT release in vitro (for headache treatment) [49]. The cardioactive compound Geniposidic acid has been isolated from *E. longifolia* leaves [44].

References
Barr *et al*., 1993; Isaacs, 1987; Barr *et al*., 1988; Low, 1990; Latz, 1995; Rogers *et al*., 2000; Pennacchio *et al*., 1996; Cunningham *et al*., 1981.

Genus and Species Name	Common Name
Eremophila mitchellii Benth.	Budda

Aboriginal Name	Family
Budha	Myoporaceae

Description of Plant	Collection
Shrub, small tree of alluvial deposits and low lying areas. Timber yields a unique-smelling aromatic oil [16].	

Distribution
NSW, QLD

Use (Food, Medicine)	Food Value
Medicine	

Preparation in the Kamilaroi and Muruwari Communities

Preparation in the Literature
Steaming [34]

Medicinal Use in the Kamilaroi and Muruwari Communities

Medicinal Use in the Literature
Rheumatism [34]

Studies Performed in the Literature
Two sesquiterpenes, 8a-hydroxy-7a(H)-eremophila-1,11-dien-9-one and 8a-hydroxy-7a(H)-eremophila-10,11-dien-9-one were isolated from *E. Mitchelli* wood oil [35].

References
Low, 1990; Massy-Westropp *et al*., 1966; Cunningham *et al*., 1981.

Genus and Species Name	Common Name
Eremophila sturtii R. Br.	Turpentine bush

Aboriginal Name	Family
Murawaal	Myoporaceae

Description of Plant	Collection
A sticky shrub about 2m high. Leaves are narrow with a small hook at the tip, usually crowded at the end of the branches. Flowers can be white, pink or mauve. Leaves have a faint but pleasant odour when crushed [16].	Date: December 2005; March 2004 Venue: Lightning Ridge, New South Wales (140°58'53"E x 29°25'49"S) Voucher number: 73007710

Distribution
NSW, VIC, QLD, WA, NT

Use (Food, Medicine)	Food Value
Medicine	

Preparation in the Kamilaroi and Muruwari Communities
Crushed leaves applied topically to treat skin infections. Disinfectant. Fly repellent.

Preparation in the Literature
A decoction of leaves was used to wash cuts and open sores. A hot water bath with leaves was used to treat colds and flu [6, 32, 34]. Fumes from smouldering branches were used to relieve backaches [32].

Medicinal Use in the Kamilaroi and Muruwari Communities
Skin infections. Disinfectant. Fly repellent.

Medicinal Use in the Literature
Fly repellent. Wash for cuts and sores, colds and flu. Relieve backaches [6, 32, 34].

Studies Performed in the Literature
E. sturtii showed antibacterial activity against *Bacillus cereus* [42]. Two novel compounds, 3,8-dihydroxyserrulatic acid and serrulatic acid, and three known compounds, *β*-sitosterol, sesamin and 3,6-dimethoxy-5,7-dihydroxyflavone have been isolated from *E. sturtii* in this study.

References
Barr *et al*., 1993; Latz, 1995; Smith, 1991; Low, 1990; Palombo and Semple, 2001; Cunningham *et al*., 1981.

Genus and Species Name	Common Name
Eucalyptus camaldulensis Dehnh.	River gum

Aboriginal Name	Family
Yarran	Myrtaceae

Description of Plant	Collection
A medium-sized tree, up to 20m with wide, spreading branches with grey-white bark and red timber. Widely distributed throughout mainland Australia [16].	

Distribution
Widespread, inland of all states except Tasmania.

Use (Food, Medicine)	Food Value
Medicine	

Preparation in the Kamilaroi and Muruwari Communities

Preparation in the Literature
Kino (0.3-1.3g) was mixed with water and drunk for diarrhoea. Infusion of leaves and twigs was used to bathe the head for colds and fevers. Sap was collected and boiled till dissolved. The solution was rubbed on sores and cuts as a disinfectant [6, 7, 26, 34].

Medicinal Use in the Kamilaroi and Muruwari Communities

Medicinal Use in the Literature
Diarrhoea, colds, fevers, sores, swollen and aching joints, headache. Antimicrobial, antifungal, cough suppression. A powerful antiseptic. Decongestant, expectorant, counter-irritant [6, 7, 32, 34].

Studies Performed in the Literature
Eucalyptus camaldulensis showed antibacterial activities against Gram-positive (including *S. aureus*) and Gram-negative microorganisms and antifungal activity against *Candida albicans* [2, 41]. The cough suppression activity of *Eucalyptus camaldulensis* has been reported [39].

References
Barr *et al*., 1993; Isaacs, 1987; Barr *et al*., 1988; Low, 1990; Alkofahi *et al*., 1996; Oyedeji *et al*., 1999; Nguyen *et al*., 1994; Cunningham *et al*., 1981.

Genus and Species Name	Common Name
Eucalyptus populnea F. Muell.	Gum tree, Bimble Box

Aboriginal Name	Family
Bibil	Myrtaceae

Description of Plant	Collection
Tree to 20 m high; bark persistent on trunk and larger branches, grey with whitish patches, fibrous-flaky ('box'), smooth above, glossy, grey, shedding in short ribbons [16, 37].	

Distribution
NSW, QLD

Use (Food, Medicine)	Food Value
Food	

Preparation in the Kamilaroi and Muruwari Communities

Preparation in the Literature
Water trees whose root are sometimes tapped for water [26].

Medicinal Use in the Kamilaroi and Muruwari Communities

Medicinal Use in the Literature

Studies Performed in the Literature
The compound L-quercitol was found in *E. populnea* [45].

References
Issacs, 1987; New South Wales Flora Online; Plouvier, 1961; Cunningham *et al*., 1981.

Genus and Species Name	Common Name
Eucalyptus tessellaris F. Muell.	Carbeen

Aboriginal Name	Family
Gaabin	Myrtaceae

Description of Plant	Collection
A medium to large tree, to 30m with a rough bark with square cracks [16].	

Distribution
QLD and northern NSW.

Use (Food, Medicine)	Food Value
Medicine	

Preparation in the Kamilaroi and Muruwari Communities

Preparation in the Literature
Bark soaked in water and infusion drank for dysentery. Kino used too.

Medicinal Use in the Kamilaroi and Muruwari Communities

Medicinal Use in the Literature
Dysentery

Studies Performed in the Literature
E. tessilaris has been found to contain both ursolic acid and eucalyptin and volatile leaf oils [8]

References
Dayal, 1982; Bignell *et al*., 1997,

Genus and Species Name	Common Name
Euphorbia drummondii Boiss.	Caustic weed

Aboriginal Name	Family
Minan	Euphorbiaceae

Description of Plant	Collection
A small much-branched prostrate herb with a thick tap-root. Prostrate annual herb with milky sap and bright green leaves. Inland areas of all mainland states [16].	

Distribution
VIC, NSW, WA, SA, QLD

Use (Food, Medicine)	Food Value
Medicine	

Preparation in the Kamilaroi and Muruwari Communities

Preparation in the Literature
Infusion of herb was used for chronic diarrhoea, dysentery, low fever and rheumatism. Decoction was applied to skin itches, sores and scabies, and drunk for gonorrhoea. Sap was applied for veneral diseases and skin complains, also to remove warts [6, 7, 26, 32, 34].

Medicinal Use in the Kamilaroi and Muruwari Communities

Medicinal Use in the Literature
For chronic diarrhoea, dysentery, low fever, rheumatism, skin itches, sores and scabies, chest pains, snakebite, and sore eyes [6, 7, 26, 32, 34].

Studies Performed in the Literature
Euphorbia drummondii has been found to have weak inhibition against human cytomegalovirus [54]. It has been reported to be ineffective against cyclooxygenase-1 [33].

References
Barr *et al*., 1993; Isaacs, 1987; Barr *et al*., 1988; Low, 1990; Latz, 1995; Semple *et al*., 1998; Li *et al*., 2003; Cunningham *et al*., 1981.

Genus and Species Name	Common Name
Exocarpos aphyllus R. Br.	Stiff cherry

Aboriginal Name	Family
	Santalaceae

Description of Plant	Collection
An erect shrub up to 3m high with finely furrowed bark. Branches rigid, appearing leafless. Leaves reduced to tiny scales. Parasitic [16].	Date: December 2003 Venue: Lightning Ridge, New South Wales (140°58'53"E x 29°25'49"S) Voucher number: 73007709

Distribution
Inland of QLD and NSW.

Use (Food, Medicine)	Food Value
Medicine	

Preparation in the Kamilaroi and Muruwari Communities

Preparation in the Literature
Decoction was used internally for colds and sores, also as a poultice on the chest for 'wasting diseases' [26].

Medicinal Use in the Kamilaroi and Muruwari Communities

Medicinal Use in the Literature
Colds, sores [26]

Studies Performed in the Literature
Seeds have been found to contain 21.3% oil with santalbic acid being the major fatty acid [57]. Three triterpenes betulin, betulinic acid and oleanolic acid have been isolated from *E. aphyllus* in this study.

References
Isaacs, 1987; Sundarrao *et al*., 1992; Cunningham *et al*., 1981.

Genus and Species Name	Common Name
Flindersia maculosa (Lindl.) Benth.	Leopardwood

Aboriginal Name	Family
Bagala	Rutaceae

Description of Plant	Collection
A tangled scrubby shrub to small tree, can grow up to 15m high, with spotted trunk. Leaves grey grey, lanceolate, 7cm long [16, 37].	Date: December 2005 Venue: Lightning Ridge, New South Wales (140°58'53"E x 29°25'49"S) Voucher number: 73007959

Distribution
Central & western NSW and QLD.

Use (Food, Medicine)	Food Value
Medicine	

Preparation in the Kamilaroi and Muruwari Communities
Boiled together with the dogwood bush and applied on skin to treat scabies [25]

Preparation in the Literature
The resinous exudate has been used by bushman against diarrhoea [26].

Medicinal Use in the Kamilaroi and Muruwari Communities
Scabies

Medicinal Use in the Literature
Diarrhoea [26], antibacterial, antiprotozoal

Studies Performed in the Literature
Alkaloids were found in the bark of *F. maculosa* [46, 11]. Mild antimicrobial activity was found [38].

References
NSW Flora Online; Prager *et al*., 1960; Newbold *et al*., 1997; Brown *et al*., 1954, Cunningham *et al*., 1981; Hunter, 2005; Issacs, 1987.

Genus and Species Name	Common Name
Grevillea striata R. Br.	Beefwood

Aboriginal Name	Family
Mubu	Proteaceae

Description of Plant	Collection
A tree up to 30m high. Young branches covered with fine silky hair with dark, rough bark on trunk [16].	

Distribution
Inland northern Australia & coastal QLD & WA

Use (Food, Medicine)	Food Value
Medicine	

Preparation in the Kamilaroi and Muruwari Communities

Preparation in the Literature
Resin exuded by the tree was used as a substitute for pitch in the preparation of ointments and plasters. Dried gum was ground and dusted over burns to sweep sores. Charcoal from this tree has been used to stop bleeding from certain spear wounds [6].

Medicinal Use in the Kamilaroi and Muruwari Communities

Medicinal Use in the Literature
Wounds, burns [6]

Studies Performed in the Literature
Potential cardiovascular activity was reported [51]. A phenol, striatol, was isolated from the wood of *G. striata* [47].

References
Barr *et al*., 1993; Roufogalis *et al*., 1999; Rasmussen *et al*., 1968; Cunningham *et al*., 1981.

Genus and Species Name	Common Name
Leichhardtia australis	Wild banana

Aboriginal Name	Family
Gaaguluu	Asclepiadaceae

Description of Plant	Collection
A twining creeper, long and slender leaves, up to 5cm long. Green, egg-shaped fruit 8 cm long, tastes like young peas [6].	

Distribution
Arid regions of Australia but not in QLD.

Use (Food, Medicine)	Food Value
Food, Medicine	

Preparation in the Kamilaroi and Muruwari Communities

Preparation in the Literature
The dried and ground seeds were used as an oral contraceptive [34]. Fruits are cooked before eating when mature. Young pods are eaten raw along with flowers and leaves. Honey from flowers is also eaten and leaves are steamed and eaten [6].

Medicinal Use in the Kamilaroi and Muruwari Communities

Medicinal Use in the Literature
Contraceptive

Studies Performed in the Literature
Leichhardtia australis had a high level of thiamin at 4236 and 2935 mg/100 g [27]

References
Isaacs, 1987; Low, 1990; James, 1983

Genus and Species Name	Common Name
Marsilea hirsuta R. Br.	Nardoo

Aboriginal Name	Family
Bal	Marsileaceae

Description of Plant	Collection
An aquatic fern of inland Australia. Rhizome creeping, covered with pale brown hairs at the apex. Stems slender and floating [37].	Date: December 2005 Venue: Lightning Ridge, New South Wales (140°58'53"E x 29°25'49"S) Voucher number: 73007962

Distribution
Inland Australia

Use (Food, Medicine)	Food Value
Food	Vitamins

Preparation in the Kamilaroi and Muruwari Communities
Sporocarps collected and ground. Sporocarps' skin must be taken off [5].

Preparation in the Literature
Sporocarps were collected and roasted, cases were discarded, then the spores was ground to make cakes [26].

Medicinal Use in the Kamilaroi and Muruwari Communities

Medicinal Use in the Literature

Studies Performed in the Literature

References
Isaacs, 1987; NSW Flora Online; Hunter, 2005.

Genus and Species Name	Common Name
Melaleuca uncinata R. Br.	Broom bush

Aboriginal Name	Family
Biibaaaya	Myrtaceae

Description of Plant	Collection
A spindly shrub 1-2m high with alternate narrow cylindrical leaves. Flowers, numerous, brush like, pale cream or yellow [16].	

Distribution
Arid inland VIC, SA& western NSW

Use (Food, Medicine)	Food Value
Medicine	

Preparation in the Kamilaroi and Muruwari Communities

Preparation in the Literature
Leaves have been chewed to alleviate catarrh [34].

Medicinal Use in the Kamilaroi and Muruwari Communities

Medicinal Use in the Literature
Catarrh, astringent [34]

Studies Performed in the Literature
The oil of *Melaleuca uncinata* has been found to contain alpha-pinene (89%) and terpinen-4-ol [10].

References
Low, 1990; Lassak *et al*., 2004; Brophy *et al*., 1992; Cunningham *et al*., 1981.

Genus and Species Name	Common Name
Native sugar bees wax	

Aboriginal Name	Family

Description of Plant

Distribution

Use (Food, Medicine)	Food Value
Medicine	

Preparation in the Kamilaroi and Muruwari Communities

Preparation in the Literature

Medicinal Use in the Kamilaroi and Muruwari Communities
Skin infections [25]

Medicinal Use in the Literature

Studies Performed in the Literature

References
Hunter, 2005.

Genus and Species Name	Common Name
Owenia acidula F. Muell.	Colane

Aboriginal Name	Family
Guwi	Meliaceae

Description of Plant	Collection
A small tree with dark rough bark, dense dark foliage, sticky young shoots and a rounded crown [37].	

Distribution
Southern QLD, Northern SA, northern NSW

Use (Food, Medicine)	Food Value
Medicine	

Preparation in the Kamilaroi and Muruwari Communities

Preparation in the Literature
Decoction of wood was used to bathe or wash sore eyes [32, 34].

Medicinal Use in the Kamilaroi and Muruwari Communities

Medicinal Use in the Literature
Sore eyes [32, 34], malaria

Studies Performed in the Literature
The seeds of *Owenia acidula* have been found to contain a simple limonoid and a derivative of the cyclopropane protolimonoid glabretal [36].

References
Low, 1990; Latz, 1995; Mulholland *et al*., 1992; NSW Flora Online

Genus and Species Name	Common Name
Pittosporum phillyraeoides DC.	Butter bush Mallee Willow

Aboriginal Name	Family
Miyaymiyaay	Pittosporaceae

Description of Plant	Collection
A slender shrub with pendulous branchlets and rough bark up to 10m high. Fruit a hard, bright orange capsule10-15mm, containing black seeds in a sticky pulp [16].	

Distribution
Arid regions all states mainland Australia

Use (Food, Medicine)	Food Value
Medicine	Gums high in carbohydrates

Preparation in the Kamilaroi and Muruwari Communities

Preparation in the Literature
Infusion of seeds, fruit pulp, leaves or wood was used to relief pain and cramps [26]. Decoction of fruits was drunk and applied for eczema and pruritus [7]. Warmed leaves were placed on breast of new mothers to induce milk flow [26]. Gum from branches was eaten [32].

Medicinal Use in the Kamilaroi and Muruwari Communities

Medicinal Use in the Literature
Pain, cramps, eczema, pruritus, colds, bruises, muscle ache and lactagogue [7, 26, 32].

Studies Performed in the Literature
Triterpenoid sapogenins have been found in *P. phillyraeoides* leaves [18].

References
Barr *et al*., 1993; Isaacs, 1987; Latz, 1995; Errington *et al*., 1988; Cunningham *et al*., 1981.

Genus and Species Name	Common Name
Portulaca oleracea L.	Common pigweed

Aboriginal Name	Family
Ganhan, dhamu	Portulacaceae

Description of Plant	Collection
A prostrate, succulent annual, reddish or brown stems and alternate leaves. Stems sprawl along the ground. Small yellow flowers appear in summer. Each plant bears a large quantity of seeds [16, 26]	

Distribution
Widespread from the coast to the inland desert, gorws along sandy riverbanks. Inland and northern areas.

Use (Food, Medicine)	Food Value
Food, Medicine	Protein, water, dietary fibre and trace element

Preparation in the Kamilaroi and Muruwari Communities

Preparation in the Literature
Seeds were stored as a supply of food in times of drought. Leaves and stems were used as vegetables. Whole plant was ground to form a thick green mush and eaten immediately [26, 32]. In India, it has been used internally for spitting blood.

Medicinal Use in the Kamilaroi and Muruwari Communities

Medicinal Use in the Literature
A cooling diuretic, antiscorbutic, a blood cleanser. Prevent scurvy [34].

Studies Performed in the Literature
Flavonoids, fatty acids, alicyclic hydrocarbons, and isoprenoids were identified in *Portulaca oleracea* [59, 29]. Antioxidant and hypoglycemic properties were reported [13, 19].

References
Isaacs, 1987; Low, 1990; Latz, 1995; Xu *et al*., 2006; Choi *et al*., 2005; Jirovetz *et al*., 1993; Eskander *et al*., 1995; Cunningham *et al*., 1981.

Genus and Species Name	Common Name
River clays	

Aboriginal Name	Family

Description of Plant

Distribution

Use (Food, Medicine)	Food Value
Medicine	

Preparation in the Kamilaroi and Muruwari Communities
Wet river clay was put on skin to treat infections [25]

Preparation in the Literature

Medicinal Use in the Kamilaroi and Muruwari Communities
Skin infections [25]

Medicinal Use in the Literature

Studies Performed in the Literature

References
Hunter, 2005.

Genus and Species Name	Common Name
Santalum acuminatum (R. Br.) A. DC.	Quandong

Aboriginal Name	Family
Guwadhaa	Santalaceae

Description of Plant	Collection
A tall shrub up to 10m high with pale green leathery leaves. Globular red fruit 2-3 cm long. Parasitic [16].	

Distribution
Central desert, VIC, NSW. In sandy soils of the dry interior of Australia.

Use (Food, Medicine)	Food Value
Medicine, Food	High in energy, protein, fat, Vitamin C

Preparation in the Kamilaroi and Muruwari Communities

Preparation in the Literature
Ground seed kernels has been used as a liniment rubbed into affected part to relieve the pain of swelling and bruises, sprains and backache. Pounded leaves have been used for boils, sores and gonorrhoea [7]. Fruit eaten immediately and dried for later use, seeds pounded for oils [26, 32, 34]

Medicinal Use in the Kamilaroi and Muruwari Communities

Medicinal Use in the Literature
For boils, sores, gonorrhoea. Pain relief.

Studies Performed in the Literature
Santalum acuminatum was found to cause significant inhibition of platelet 5-HT release [50]. Kernels of *Santalum acuminatum* contain Santalbic acid which was reported to be an inhibitor of Gram-positive bacteria and a nunber of pathogenic fungi [30].

References
Barr *et al*., 1993; Isaacs, 1987; Low, 1990; Latz, 1995; Rogers *et al*., 2001; Jones *et al*., 1995; Cunningham *et al*., 1981.

Genus and Species Name	Common Name
Santalum lanceolatum R. Br.	Sandalwood plum

Aboriginal Name	Family
ngamanbirra	Santalaceae

Description of Plant	Collection
A small tree or erect shrub to 6m with leathery, opposite leaves. Fruit a dark blue succulent berry, 10mm. Parasitic [16].	

Distribution
Over much of continental Australia. Central desert.

Use (Food, Medicine)	Food Value
Medicine, Food	High water content, some protein, fat and energy

Preparation in the Kamilaroi and Muruwari Communities

Preparation in the Literature
Berries were eaten [26, 34], decoction of leaves & bark was drunk as purgative [26]. Decoction of scraped outer wood was drunk for sickness of the chest [6]. Leaves were used for boils, sores & gonorrhoea [7]. Infusion from mashed roots was applied for rheumatism & itching [26].

Medicinal Use in the Kamilaroi and Muruwari Communities

Medicinal Use in the Literature
Rheumatism, purgative, boils, sores, gonorrhoea, itching. Respiratory infections, diabetes, genitourinary infections. Decongestant, a tonic [6, 7, 26].

Studies Performed in the Literature
Lanceol was found in the oil of *Santalum lanceolatum* [9].

References
Barr *et al*., 1993; Isaacs, 1987; Barr *et al*., 1988; Low, 1990; Bradfield *et al*., 1936; Cunningham *et al*., 1981.

Genus and Species Name	Common Name
Ventilago viminalis Hook.	Supplejack

Aboriginal Name	Family
Ganayanay	Rhamnaceae

Description of Plant	Collection
A small tree up to 6m high with a dense leafy crown. Drooping leaves are bright green and flowers are pale green [16].	

Distribution
Arid regions of northern Australia, western NSW. Central desert.

Use (Food, Medicine)	Food Value
Medicine	

Preparation in the Kamilaroi and Muruwari Communities

Preparation in the Literature
Roots and bark were mashed in water for toothache, rheumatism, swellings, cuts and sores, and as a hair restorative. Its ashes were mixed with native tobacco to make the taste stronger [26, 32].

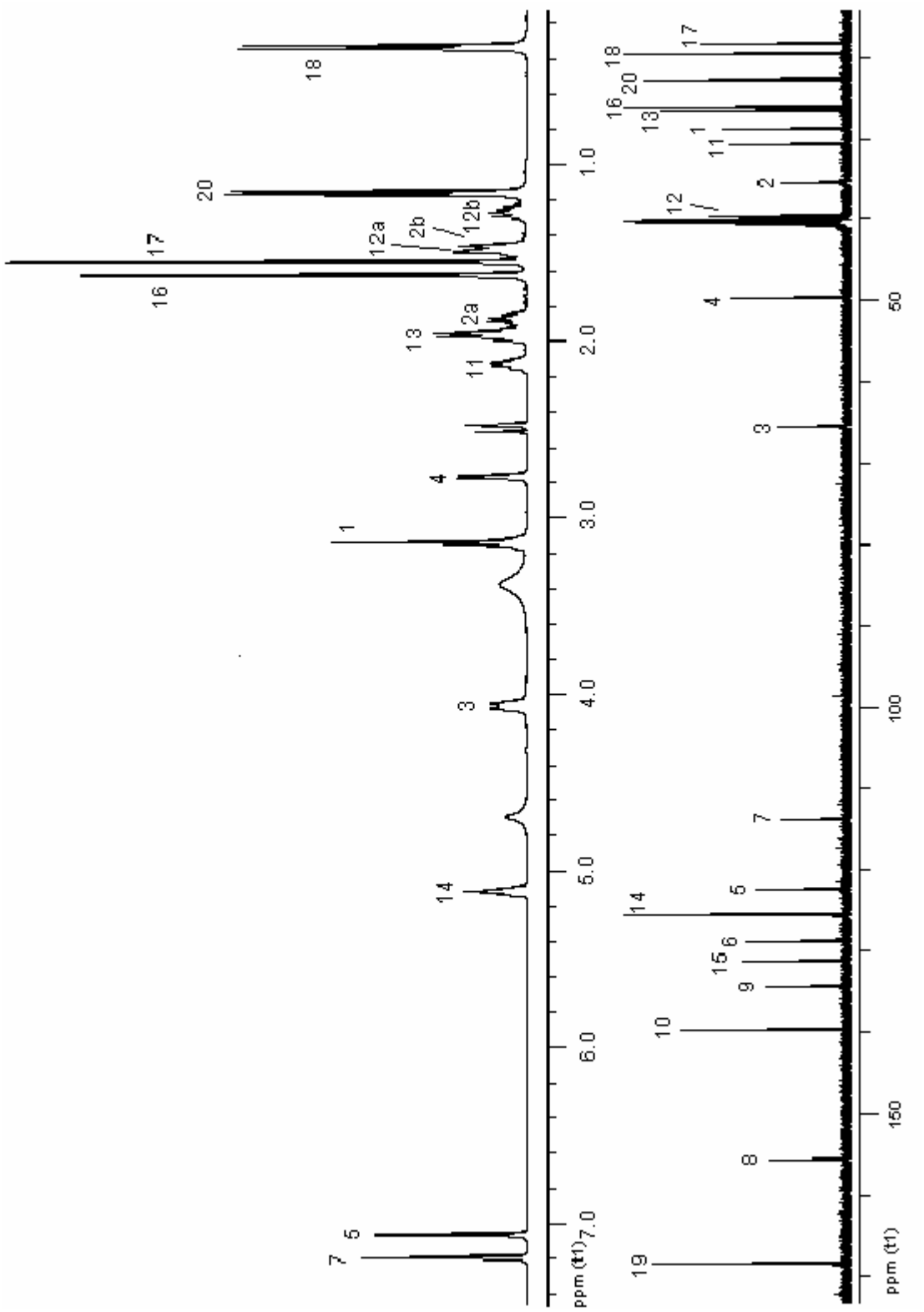
Medicinal Use in the Kamilaroi and Muruwari Communities

Medicinal Use in the Literature
Toothache, rheumatism and swellings, cuts and sores, hair restorative.

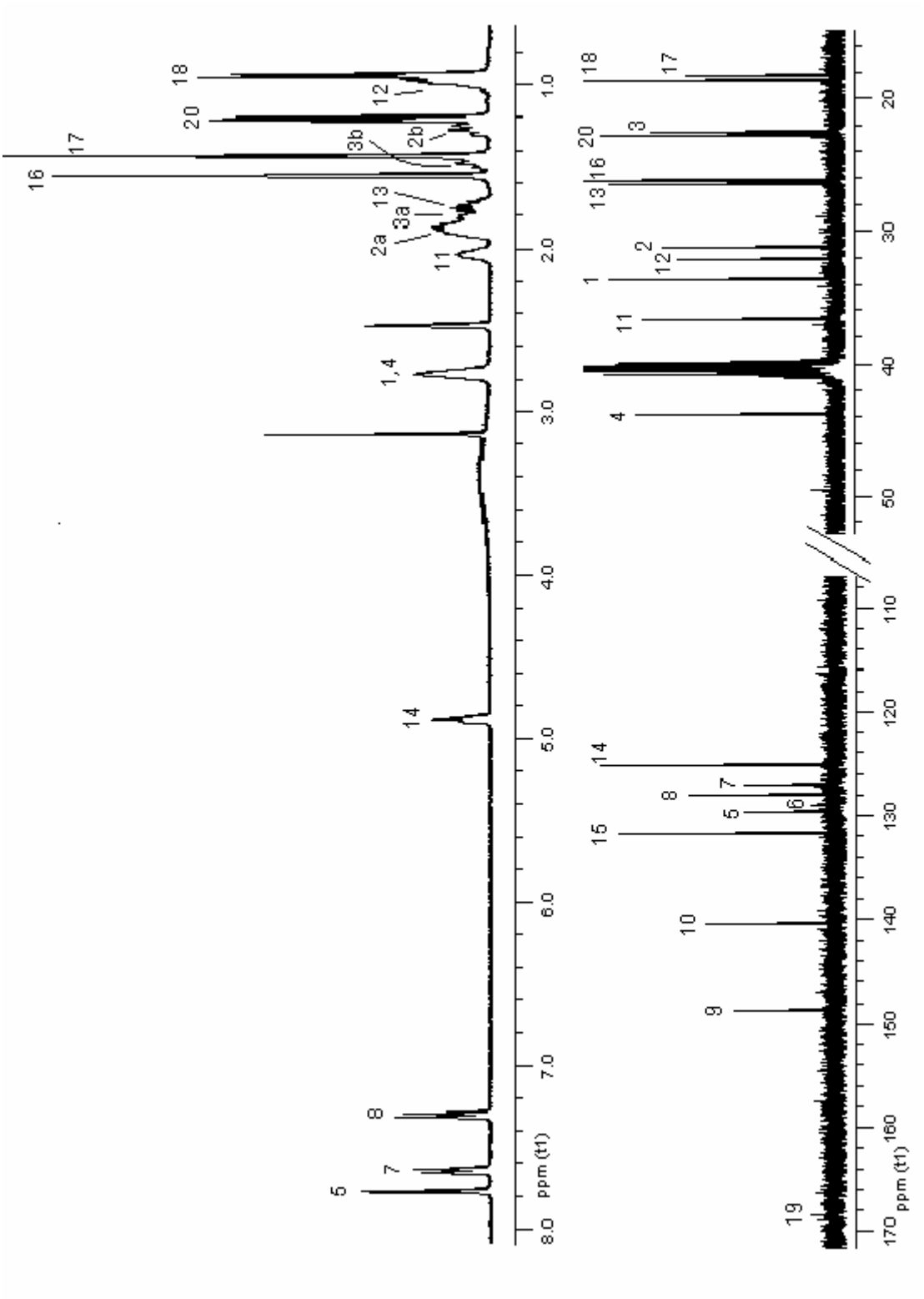
Studies Performed in the Literature
A naphthoquinone Ventilagone and several anthraquinones were found in the root-bark of *V. viminalis* [14].

References
Isaacs, 1987; Latz, 1995; Cooke *et al*., 1965 & 1963; Cunningham *et al*., 1981.

Appendix 2. ^{1}H and ^{13}C NMR of 3,8-dihydroxyserrulatic acid (**4.1**).



Appendix 3. ^{1}H and ^{13}C NMR of serrulatic acid (**4.2**).



Appendix 4. Journal article in Phytochemistry (2006).

Appendix 5. Journal article in Journal of Microbiological Methods (2005).

Appendix 6. Journal article in Molecules (2005).

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