DOCUMENTATION AND BIOLOGICAL AND PHYTOCHEMICAL ANALYSIS OF CHUNGTIA MEDICINAL PLANTS OF NAGALAND, INDIA

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by

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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.

Meyanungsang

August 2010

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ABSTRACT

Nagaland is a small State in North-East India. Its Indigenous tribes have a rich plant folklore culture. The Indigenous Bioresources Research Group (IBRG) of Macquarie University have established a collaborative research partnership with Chungtia village, Nagaland, with the objectives of documenting firsthand ethnobotanical plant knowledge of Chungtia village and investigating the chemical and biological properties of selected plants. This study consisted of three interrelated aspects, namely ethnobotanical research, biological studies, and isolation and characterisation of bioactive constituents from Nagaland medicinal plants.

An ethnobotanical research of Nagaland medicinal plants used by Chungtia village was conducted with the cooperation of Chungtia villagers. This resulted in the documentation of 135 plants. These plants were taxonomically identified and voucher specimens were deposited at Botanical Survey of India (BSI), Shillong, India, for future reference. Of these 135 plants, 39 plants have been previously reported for similar ethnomedicinal uses elsewhere in Nagaland, 61 plants have been previously reported in Nagaland for different ethnobotanical purposes, and 35 plants appear to have novel ethnomedicinal uses in Nagaland.

Thirty five plants were documented for their use in skin related treatments and literature searches on these plants indicated 16 plants with either no antibacterial or phytochemical studies previously reported. This led to the selection of eight plants for antibacterial screening with priority given to those plants that were reported as treatments for skin diseases. Three plants were also selected from the literature on Nagaland medicinal plants for antibacterial activity studies.

The antibacterial screenings were performed on 11 plants against three pathogenic microorganisms, *i.e. S. aureus*, *E. coli* and *P. aeruginosa*, by using disc diffusion and MTT microdilution assays. All of the eight plants used by Chungtia villagers showed antibacterial activity against *S. aureus*, at concentrations less than 2.5 mg/ml. The highest inhibitory activities were exhibited by the stem bark of *Erythrina stricta* and the roots of *Prunus persica*, with MIC values of 156 µg/ml and 312 µg/ml, respectively. Based on the antibacterial screening results, the stem bark of *E. stricta*, root bark of *P. persica* and root bark of *Diospyros lanceifolia* were selected for further biological and chemical investigations of their antibacterial constituents.

The bioassay guided isolation of the dichloromethane partition of the ethanolic extract of the stem bark of *E. stricta* led to the isolation of 11 compounds: the flavanones 5-hydroxysophoranone and maackiaflavanone B, the isoflavones chandalone, alpinumisoflavone and lupalbigenin, and the pterocarpans 1-methoxyerythrabyssin II, erythrabyssin II, erystagallin A, phaseollidin, cristacarpin and 2-(γ,γ-dimethylallyl)-6*a*-hydroxyphaseollidin. This is the first reported isolation of any of these 11 compounds from the stem bark of this plant. The antibacterial activities of the isolated compounds were evaluated against four different strains of *S. aureus* including two drug-resistant strains. The most potent inhibition was shown by lupalbigenin. Compounds 1-methoxyerythrabyssin II, erythrabyssin II, erystagallin A, cristacarpin and 2-(γ,γ-dimethylallyl)-6*a*-hydroxyphaseollidin were active against all of the strains.

The ethyl acetate fraction of the root bark of *P. persica* led to the isolation of afzelechin and *ent-epiafzelechin-(2α→O→7,4α→8)-(-)-afzelechin*. This is the first report of *ent-epiafzelechin-(2α→O→7,4α→8)-(-)-afzelechin* isolation from *P. persica*. The two compounds were tested for antibacterial activities against eight bacteria, including both Gram-positive and-Gram negative bacteria and two methicillin resistant *Staphylococcus aureus* (MRSA) strains, but they were found to be not active even at a concentration of 250 μg/ml.

Examination of the *n*-hexane fraction of the root bark of *D. lanceifolia*, which showed activity against *S. aureus*, *E. coli* and *P. aeruginosa* in the disc diffusion assay, led to the isolation of the two antibacterial compounds plumbagin and 7-methyljuglone. Plumbagin and 7-methyljuglone were tested against eight microorganisms including both Gram-negative and Gram-positive bacteria and two MRSA strains to expand the current bioactivity data on these compounds. Plumbagin showed more potent and broad spectrum activity than 7-methyljuglone.

ABBREVIATIONS

$(CD_3)_2CO$	Deuterated acetone
δ	Chemical shift (NMR)
^{13}C NMR	Carbon Nuclear Magnetic Resonance Spectroscopy
^{1}H NMR	Proton Nuclear Magnetic Resonance Spectroscopy
2D NMR	Two-Dimensional Nuclear Magnetic Resonance Spectroscopy
br	Broad (NMR)
BSI	Botanical Survey of India
CBD	Convention on Biological Diversity
$CDCl_3$	Deuterated chloroform
CFU	Colony Forming Unit
$CHCl_3$	Chloroform
COSY	(Proton-Proton) Correlation Spectroscopy
CSMT	Chungtia Senso Mokokchung Town
CVC	Chungtia Village Council
d	Doublet (NMR)
DCM	Dichloromethane
DMSO	Dimethyl Sulphoxide
EI	Electronic impact
EtOAc	Ethyl acetate
EtOH	Ethanol
H_2SO_4	Sulphuric acid
HCl	Hydrochloric acid
HMBC	Heteronuclear Multiple Bond Correlation
HSQC	Heteronuclear Single Quantum Correlation
IBRG	Indigenous Bioresources Research Group
J	Coupling constant
m	Multiplet (NMR)
m/z	Mass to charge ratio
MBC	Minimum Bactericidal Concentration
Me	Methyl

MeOH	Methanol
MHA II	Mueller Hinton Agar II
MIC	Minimum Inhibitory Concentration
min.	Minute
MRSA	Methicillin Resistant *Staphylococcus aureus*
MS	Mass Spectrometry
n-BuOH	*n*-Butanol
NHMRC	National Health and Medical Research Council
NMR	Nuclear Magnetic Resonance
ºC	Degrees Celsius
PE	Petroleum ether
r.p.m.	Revolutions per minute
R_f	Retention factor
s	Singlet (NMR)
SGC	Silica Gel Chromatography
t	Triplet (NMR)
TLC	Thin Layer Chromatography
UNESCO	United Nations Educational, Scientific and Cultural Organisation
UV	Ultraviolet
µg	Microgram

TABLE OF CONTENTS

Chapter 1

Introduction

This chapter provides an introduction into the history of medicinal plant use and their importance in healthcare and drug discovery; the Indian experience post the Convention on Biological Diversity; and the aims of this project.

1.1 Background

1.1.1 History of medicinal plant use

As reflected in all ancient cultures, humans have relied on plants for food, clothing, shelter, and medicines over thousands of years. The earliest fossil records of plants used by humans dates to at least the Middle Paleolithic age, some 60,000 years ago, from the present day Iraq (Solecki, 1975).

Sumerians started to document their medicinal knowledge some 4000 years ago in ancient Mesopotamia in the form of clay tablets. This was followed by the Egyptian pharmaceutical record, called "Ebers Papyrus", which contains approximately 700 drugs (mainly plants), recorded around 1500 B.C. (Cragg and Newman, 2001). At the same time, the Indian subcontinent started to document the value of medicinal plants in a more systematic manner called the *vedas*, "the book of knowledge" and employed a traditional health care system called *ayurveda*, which is currently being practiced in India and around the world. Ayurveda, meaning "knowledge of life", is a holistic approach that collectively encompasses medicinal, psychological, cultural, religious and philosophical concepts to attain a long, healthy and happy life (Patel, 1986).

In ancient China, the oldest form of a herbal medicine book, *Herbal Classics of the Divine Plowman (Shennong Bencao)*, known as "the canon of materia medica" was written by Shennong in the first century B.C. and first century A.D. The book contains 365 kinds of drugs, of which 239 are derived from medicinal plants (Liu, 2005). In the west, Hippocrates (460 B.C. - 377 B.C), the father of ancient Greek medicine and the first physician to separate science from

religion, also observed the therapeutic value of diet and fruits to treat certain ailments and this consequently attributed to the evolution of *Corpus Hippocraticum*, a collection of more than 60 treatise from the fifth century B.C. to the second century A.D. During the first century A.D., a Greek physician by the name of Dioscorides wrote *De Materia Medica*, which contains accurately recorded classified medicinal plants that includes methods of preparation, medicinal uses and dosages. It was during the sixth and ninth century A.D. that the book *De Materia Medica* was translated into Latin and Arabic. This book is considered by many to be the most important representative of the science of herbal drugs in "ancient times" (Reiss et al., 2002; Touwaide, 2005).

1.1.2 Ethnobotany and Ethnopharmacology

Because of the growing interest in traditional medicines, various scientific disciplines have emerged such as ethnomedicine, ethnobotany and ethnopharmacology. The term ethnobotany was coined by Harshberger in the year 1895 and since then several definitions have been assigned to ethnobotany. It is now universally taken as the study of the "direct relationship between humans and plants" (Jain, 1994).

According to Jan G Bruhn and Helmstedt (1980), ethnopharmacology is defined as the "interdisciplinary scientific exploration of biologically active agents traditionally employed or observed by man" and its objectives are "to rescue and document a vast cultural knowledge before it is lost to the world, and to investigate and evaluate the agents employed without any prejudice or bias in order to find the rationale for their use."

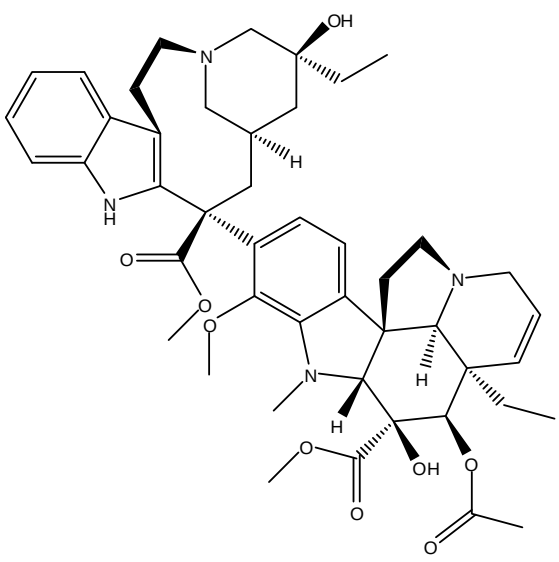
1.1.3 The value of medicinal plants

Over the past few decades, the use of traditional medicines has rapidly gained in popularity around the world and it is estimated that almost 80% of the world's population rely on traditional medicines, particularly in developing countries that have limited access to essential medicines (Farnsworth et al., 1985). In China, the use of herbal preparations accounts for 30-40% of the total medicinal consumption, and in Africa up to 80% of the population uses traditional medicine for primary healthcare (WHO, 2003). Today, more than 1500 herbal medicines are sold worldwide. The global market for herbal industries is approximately US $62 billion annually and is showing strong growth (Patwardhan et al., 2005). The world's economy on the pharmaceutical

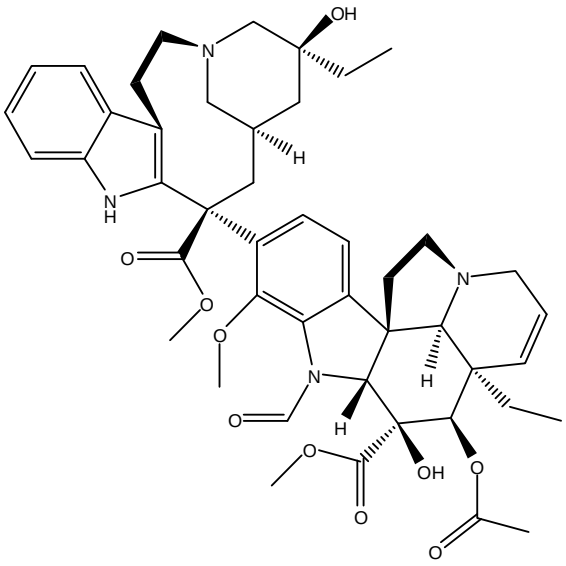
market has also increased dramatically over the years - in 2006, the pharmaceutical market reached a record high of US $643 billion and is projected to approach US $1.3 trillion by the year 2020 (PricewaterhouseCoppers, 2007; Seget, 2007).

1.1.4 Drug discovery from plant sources

There are over 120 different plant derived drugs in the market. Out of these, 80% have had an ethnomedicinal association (Fabricant and Farnsworth, 2001). Classical examples of this include vinblastine and vincristine, discovered from Madagascar's wild rosy periwinkle. In folklore medicine, this plant is reputed for the treatment of diabetes (Noble, 1990). These drug were discovered while working on the leaf extracts for antidiabetic properties (Kadidal, 1993; Noble, 1990). Since then, vinblastine and vincristine have been used for the treatment of cancers like Hodgkin's disease and pediatric lymphocytic leukaemia. It is estimated that Eli Lilly, the corporate producer of these drugs, earns about US $100 million each year from their sale, while Madagascar earns nothing (Kadidal, 1993).



Vinblastine



Vincristine

1.1.5 Convention on Biological Diversity and the Indian experience

The enormous importance and financial value of medicines derived from traditional knowledge systems has led to, in some cases, exploitation of Indigenous people, and this has in turn led to some activists coining the term "biopiracy", which implies inequitable sharing of benefits

between the corporate sector and the countries that supply the raw material along with the traditional knowledge.

It was during the "Earth Summit" in Rio De Janeiro, Brazil, in 1992, that the Convention on Biological Diversity (CBD) was developed. At this time, 167 nations signed the international legal agreement, which accepted the principle that "bio-resources are the sole property of sovereign States and that the States have the freedom to use them as tradable commodities" (Kartal, 2007; Tedlock, 2006).

The CBD is a framework agreement that provides a comprehensive and holistic approach to the conservation of biological diversity, the sustainable use of its components and the fair and equitable sharing of benefits arising from the use of genetic resources (Zedan, 2005). Central to the convention is Article 3 which recognises the "States' sovereign right to exploit their biological and genetic resources for commercial purposes" (Timmermans, 2003). Furthermore, Article 8(j) acknowledges "Indigenous and local communities' contribution to biodiversity conservation and calls for respect and support for the preservation and maintenance of the knowledge, innovations and practices of the local and Indigenous communities, and also confirms Indigenous peoples' rights over the knowledge they hold" (Timmermans, 2003). The convention also focuses in particular on the provisions of protecting genetic resources (traditional knowledge) by establishment of appropriate, national accesses and benefit sharing (ABS), which is highlighted in Article 1 and Article 15 of the CBD. This aims at fair and equitable sharing of benefits arising from the use of genetic resources and that the access to genetic resources shall be subject to prior informed consent from the Contracting Party providing such resources (Van Overwalle, 2005).

India has been a member of the CBD since its inception. To comply with the CBD, India has enacted the Biological Diversity Act 2002. An example of the Indian experience with the CBD is the benefit sharing arrangement between the Kani tribe of Kerala and the Tropical Botanical Garden and Research Institute (TBGRI) for the development of a drug called '*Jeevani'*, which is a restorative, immuno-enhancing, anti-stress and anti-fatigue agent. The people of the Kani tribe provided the knowledge of traditional use of *Trichopus zeylanicus* to the scientists at TBGRI, who isolated 12 active compounds. This drug was then licensed to the Arya Vaidya Pharmacy Ltd., an Indian pharmaceutical manufacturer of Ayurvedic herbal formulations, for

commercialisation of the drug. A Trust Fund was also established to share the benefits arising from the commercialisation of *Jeevani* with the Kani tribe (Anuradha, 1998).

1.2 Aim and scope of this study

The tribal people of Nagaland, North-East India, have relied on medicinal plants for hundreds of years for food, shelter and medicine. Until the advent of the British invasion in the first half of the 19th century, the Nagas, as they are collectively known, have lived in seclusion and their livelihoods were solely dependent on plants and animals. Through this process, they have developed their own traditional knowledge of medicines by experimenting with the source (plants), and this knowledge has been passed down orally for generations. However, as is happening elsewhere in the world (Cordell, 2002; Cox, 2000), Indigenous medicinal plant knowledge is being lost in Nagaland as elderly people with this knowledge are dying and communities are being dislocated and westernised.

This PhD project dealt with establishing a collaborative research partnership between Chungtia village of Nagaland and the Indigenous Bioresources Research Group (IBRG) of Macquarie University, to document the firsthand ethnobotanical knowledge of Chungtia village and to explore the bioactive components of those plants that are recorded for skin related uses. The initiation of this project and the firsthand ethnobotanical survey of Chungtia village are described in Chapter 2. In Chapter 3, the selection of plants for biological investigation from the firsthand ethnobotanical survey of Chungtia village and from literature reports of the medicinal plants of Nagaland are presented. The selected plants are presented with a thorough literature review in this chapter. The antibacterial screening of these selected plants are described in Chapter 4 and the following Chapters, 5, 6 and 7, describe the studies on isolation of bioactive compounds from three plants.

Chapter 2

Ethnobotanical Documentation of Medicinal Plants of Chungtia Village, Nagaland, India

2.1 Introduction

Nagaland is located in North-East India. It lies within the Indo-Burma (Eastern Himalayas) biodiversity hotspot, which is one of the world's richest biodiversity regions (Mao et al., 2009).

Figure 2-1: Indo-Burma hotspot region (demarcated in red colour) highlighting Nagaland (Map source: www. biodiversityhotspots.org)

The population of Nagaland is close to 2 million, with 90% of the population being Indigenous tribal people, collectively known as the 'Nagas'. The origin of the Naga race, and the word 'Naga', is still shrouded in mystery. According to Asoso Yonuo (1974), great hordes of mongoloid stocks allied to the Tibetans and Bhutanese migrated continuously from 2000 B.C. onwards into the plains and mountainous hills of Burma and Assam. By the early centuries A.D, they were known by the different names such as Khasi, Garo, Kachari, Kuki, Naga etc. Some tribes, loosely termed as 'Nagas' speaking the Tibeto-Burman language, migrated from Burma and spread throughout to the present Naga-inhabited areas, where they established permanent small sovereign village-states on the hills and mountain ridges or spurs. Nagaland, at present, is

made up of eleven districts and there are about 700 villages. There are 15 major tribes, distinguishable from each other by their language, culture, and traditions.

Until the advent of the British invasion in the first half of the 19th century, Nagas lived in seclusion and their livelihoods were solely dependent on plants and animals. Through this process, they have developed their own traditional knowledge of medicines by experimenting with the sources (mainly plants) and this knowledge has been passed down orally for generations.

As the world becomes 'smaller' because of globalisation and technological advancements, acculturation is becoming rampant and threatens the very existence of cultures, traditions and languages in the most vulnerable societies, such as in Nagaland. As is happening elsewhere in the world (Cordell, 2002; Cox, 2000), Indigenous medicinal plant knowledge is also being lost in Nagaland as elderly people with this knowledge are dying and communities are being dislocated. There are only a limited number of studies that have been undertaken to document the medicinal plant knowledge in Nagaland (Changkija, 1999; Deorani and Sharma, 2007; Jamir and Rao, 1982; Jamir et al., 1999; Megoneitso and Rao, 1983). There is still much to be documented given the nature of the biodiversity of Nagaland and the cultures and traditions of its people.

This project was established following specific request from elders of Chungtia village, Nagaland, through the author, who is also from the same village, to assist in the preservation of their medicinal plant knowledge and to undertake studies to determine the bioactive constituents of some of their medicinal plants. The motivation of these villagers to document their customary medicinal plant knowledge is particularly strong as in recent years this village has lost several key elders who have been the custodians of significant medicinal knowledge. It is essential that this knowledge be conserved because of its historical and cultural value. Given that the majority of plant-based medicines have arisen from Indigenous knowledge systems (Fabricant and Farnsworth, 2001), the disappearance of this information would also be a significant loss to the wider scientific community and the public.

2.2 Establishment of the collaborative research partnership and authorising body

2.2.1 Chungtia village

Chungtia village is an Ao tribe village in the Mokokchung district, Nagaland. It is situated 18 kms away from Mokokchung town, the largest town in the Mokokchung district. Chungtia village is located at an elevation of 896 m above sea level. It is surrounded by Khensa village on the east, Aliba village and Kinunger village in the south, Mopungchuket in the north and Changki village and Khar village in the west. The State highway passes through village connecting it to Mokokchung town, the headquarters of Mokokchung district, which is 18 kms away and Mariani (a small town located in Jorhat district, Assam), which is 75 kms away. The topography is characterised by a moderate to steep hilly area and the predominant vegetation is of a semi-evergreen forest type. Generally, January and February are the coldest months, where the temperature drops to 2ºC. The summer months are mild, with an average temperature of 27ºC and a maximum of 32ºC. The village is influenced by a strong monsoonic climate and the average annual rainfall is 2,500 mm, which falls over nine months of the year, with July and August receiving the heaviest rainfall. Because of the favourable climatic conditions, the village area is conducive to growth of many varieties of vegetation (Imchen, 1986).

2.2.2 Chungtia Senso Mokokchung Town (CSMT): the authorising body

Over the course of time, Chungtia villagers have migrated and settled in Mokokchung town and at present there are about 230 households of Chungtia villagers, with a population of about 1200 Chungtia villagers in the town. In order to look after the wellbeing of these Chungtian residents, they have established their own governing body, the Chungtia Senso Mokokchung Town (CSMT).

The CSMT was established in the year 1960 and is the sole governing body of these Chungtian residents. The CSMT executive members, headed by the President, are elected by the Chungtian residents and every resident is a *de facto* member of the CSMT. The CSMT functions on its own, within the jurisdiction of Mokokchung town and in turn is supervised by the Chungtia Village Council (CVC). The CVC is the main governing body of Chungtia village in which the executive members are represented by one member each from the 14 clans in Chungtia village. The CVC often seeks help from the CSMT in matters of importance to the communities. For this project,

the CVC authorised the CSMT to enter into a collaborative research partnership with Macquarie University, on behalf of Chungtia village (see Appendix 2-1).

2.2.2 Establishment of the collaborative research partnership

The collaborative research partnership was established between the Indigenous Bioresources Research Group (IBRG) of Macquarie University, Sydney, Australia, and the council of the CSMT, Mokokchung, Nagaland, on behalf of Chungtia village. This partnership was formalised by a collaborative research agreement that followed the principles of the Convention of Biological Diversity (CBD) along with the stepwise Participatory Action Research Methodology (PAR) of UNESCO (Tuxill and Nabhan, 2001) and the ethical guidelines of the National Health and Medical Research Council (NHMRC, 2003) for working with traditional knowledge holders. It was co-developed with the CSMT to ensure that:

- all research took place with the full consent of the CSMT
- all interviewees were informed of the project aims and their participation was entirely voluntary
- ownership of traditional knowledge was respected and confidentiality was maintained concerning any information not in the public domain
- publication of any data was only allowed with the consent of the CSMT
- for any commercial interest, a process of further negotiation would be undertaken for appropriate benefit sharing with the village community.

2.3 Firsthand ethnobotanical documentation of Chungtia village

The CSMT, in consultation with the CVC, had the role of inviting Chungtia village elders (residents of Chungtia village) to be interviewed on their firsthand medicinal plant knowledge. They especially encouraged elders considered to be the key custodians of traditional knowledge to participate, upon their consent. Over a period of two years, the author made two field trips to Chungtia village for the interviews and collection of voucher specimens of documented plants. The first trip was in November 2007 where information was generated from 10 village elders, including two herbal practitioners. While it was clear from the interviews of Chungtia village

elders that their use of medicinal plants is an important part of their culture, it also became evident that this traditional knowledge was on the decline. This can be attributed to several factors including: 1) elders with significant medicinal knowledge having died before passing on their knowledge; 2) the advent of western medicines; 3) fewer diseases prevalent due to improved lifestyle (hence the medicinal knowledge related to the diseases is being lost); and 4) the lack of awareness on the importance and preservation of medicinal plant knowledge.

During the second trip in November 2008, follow up interviews were carried out with three (2 herbal practitioners and 1 village elder) of the 10 village elders, who had extensive knowledge on the medicinal plants. For the other village elders, their knowledge was mainly on the most commonly used medicinal plants of the village. The interviews identified that the villagers had a strong preference for using medicinal plants for treatment of common ailments such as fever, cold, cuts and wounds and gastrointestinal problems, even though conventional treatment is available within their reach. These ailments, however, were generally treated using only a limited number of plants. It was also observed that most of the villagers did not have any specific or major health conditions. According to the data provided by *Sapanya* hospital, which is situated in Chungtia village and looks after three neighbouring villages, the most common symptoms treated were peptic ulcer, gastritis, hypertension, malaria, enteric fever and arthritis (personal communication with Dr Limala, 2010).

During the entire process of this project, all the 10 village elders willingly shared their medicinal knowledge and none of them withdrew their participation. Overall, the interviews resulted in firsthand information on 135 plants. This information is detailed for each plant in Table 2-1 and includes information on their uses and method of preparation. Pictures of each plant were also obtained, along with voucher specimens.

A major concern raised by all the village elders was that not only was the knowledge being lost but the plants were also being lost from their vicinity at an alarming rate. Some informants noted that several medicinal plants that used to grow abundantly near the village were now virtually unseen. This included *Cassia floribunda*, *Scutellaria glandulosa* and *Calotropis gigantean*. They further highlighted that some of the plants are now found only deep in the forest, such as *Diospyros lanceifolia*, *Zanthoxylum acanthopodium* and *Stixis suaveolens*. Concerns were also raised by the informants that if appropriate measures were not taken up for preservation, several

plants will be lost from their village within a period of a few years. These threatened plant species are highlighted in Table 2-2. One example, *Scutellaria glandulosa*, grows on the furrowed land of cultivated paddy fields and since the villagers have either ceased or greatly reduced the practice of jhum (paddy field) cultivation, this plant is now virtually unseen in Chungtia village.

Table 2-1: Ethnobotanical plants documented in Chungtia village, Nagaland
Highlighted in **bold** are plants not previously reported ethnobotanically in Nagaland

#	Scientific name (Botanical family) (C=cultivated/W=wild) (Herbarium accession number, (ASSAM Acc. No)	Local name	Part(s) used (F=fresh, D=dry, B=both)	Recorded uses, preparation and mode of administration
1	*Acacia pennata* (Linn.) Willd. (Mimosaceae) (W) (69649)	Zanghi	Stem (F)	Stem is crushed into river or creek water to poison fish[a].
2	*Acorus calamus* Linn. (Araceae) (W) (69659)	Mukupen	Leaf (F)	Leaf decoction is used in bath for the treatment of influenza.
3	***Adenia trilobata* Engl. (Passifloraceae) (W) (69536)**	**Tenik tepang**	**Leaf (F)**	**Leaf poultice is bandaged onto the knee to relieve pain.**
4	*Adhatoda vasica* Nees. (Acanthaceae) (C) (69665)	Sungjem wa	Leaf (F)	Leaf extract is applied externally for treatment of fever, cold and body ache[a,b,c,d]. Leaves are also used to ward off evil spirits.
5	*Albizia chinensis* (Osb) Merr. (Mimosaceae) (W) (69660)	Mokokwa	Stem and leaf (F)	Leaf or stem bark is crushed into river or creek water to poison fish[d]. Leaves are put in a sack with unripe bananas to assist the ripening process.
6	*Albizia lebbeck* Linn. Benth. (Mimosaceae) (W) (69677)	Moang	Stem (B)	Sack filled with stem bark is crushed with a stone in river for poisoning (killing) fish. For tempering *anok* (machete), the *anok* is wrapped with sun dried stem bark and burned until red hot. It is then immersed in water for 1 second. This results in a hardening of the metal.
7	*Albizia lucidior* (Skud) Hara (Mimosaceae) (W) (69595)	Sunemtong	Root (F)	Root infusion is applied topically to abscesses and boils.
8	*Allium Chinense* G. Don (Liliaceae) (C) (69679)	Alolasung	Bulb (F)	During fever, the bulb, roasted in mustard oil, is rubbed on the body[d]. Fresh bulb is eaten raw for treating high blood pressure. Bulb paste is applied topically to spider and snake bites and skin diseases.

9	*Allium hookeri* Thw. (Liliaceae) (C) (69628)	Repchalasung	Leaf (F)	Leaves are eaten raw for vermifuge.
10	*Allium sativum* Linn. (Liliaceae) (C) (69602)	Lasung	Bulb (B)	During high blood pressure, bulbs are kept in the mouth without chewing for half an hour each day[d]. Bulb paste is applied externally for spider and snake bites.
11	*Alstonia scholaris* (Linn.) R. Br. (Apocynaceae) (C) (69541)	Loomi	Stem, leaf and root (B)	Decoction of each is taken orally to treat gastritis[a], jaundice and also drunk as a liver tonic.
12	***Amaranthus gangeticus* Linn. (Amaranthaceae) (C) (69699)**	**Tsumarlua**	**Leaf (F)**	**Boiled leaves are consumed to treat indigestion and also taken as a laxative.**
13	*Aquillaria agallocha* Roset. (Thymelaeaceae) (C) (W) (69604)	Sungza	Root and stem (F)	Either an infusion or decoction of the root or stem is taken orally thrice a day to treat dysentery and malaria.
14	*Artemisia vulgaris* Linn. (Asteraceae) (W) (69505)	Chinangchibaza	Root (F)	Infusion is taken orally to treat dysentery.
15	***Artocarpus chaplasha* Roxb. (Moraceae) (W) (69688)**	**Unem**	**Ripe fruit (B)**	**Eaten either raw or an infusion taken orally twice a day for liver, kidney and gall bladder problems.**
16	*Artocarpus heterophyllus* Lamk. (Moracea) (C) (69596)	Polong	Sap	Sap is applied topically to treat skin disease.
17	*Asclepias curassavica* Linn. (Asclepiadaceae) (C) (69617)	Noklangchang	Leaf (F)	Leaf paste is applied topically for cuts and wounds[d].
18	*Averrhoa carambola* L. (Averrhoaceae) (C) (69692)	Jarkona	Fruit and leaf (B)	Either the fresh fruits or dried leaves or fruits made into a powder are consumed during high blood pressure, bladder and intestinal problems.
19	*Bambusa tulda* Roxb. (Bambusoideae) (W) (C) (69605)	Longme	Ash, leaf (B) and root (F)	Ash is used as dye, leaf decoction is used in bath during cold and fresh root juice is taken orally as vermifuge.
20	***Basella alba* Linn. (Basellaceae) (W) (69633)**	**Latsung-en**	**Leaf (F)**	**Leaves eaten either raw or boiled to treat gastritis and as a laxative.**

21	*Bauhinia variegata* Linn. (Caesalpiniaceae) (C) (69599)	Owepanghef	Leaf (F)	Boiled immature leaves are consumed during gastrointestinal problems[a]
22	*Begonia picta* Smith (Begoniaceae) (W) (69642)	Tesenlawa	Leaf (F)	Leaves are used to cleanse hands by crushing between palms. Leaves are also used in cooking for their sour taste.
23	***Brassica oleracea* Linn. (Brassicaceae) (C) (69510)**	**Pandacobi**	**Foliage (F)**	**The fresh juice of the foliage is consumed to treat jaundice.**
24	*Cajanus cajan* (Linn.) (Fabaceae) (C) (69672)	Mahajang	Leaf (F)	Leaf decoction is consumed to provide relief from fever.
25	*Calotropis gigantea* Linn. (Asclepiadaceae) (W) (69691)	Kutjak moli	Leaf (F)	Poultice is used topically to treat bone dislocation, body pain, sprain and burns[c,d].
26	*Cannabis sativa* L. (Cannabaceae) (C) (69608)	Ganja	Leaf (F)	Leaf decoction is taken orally for stomach ache[a].
27	*Capsicum annum* Linn. (Solanaceae) (C) (69658)	Metsu	Fruit (F)	Fruit eaten during loss of appetite, indigestion and to purify blood.
28	*Carica papaya* Linn. (Caricaceae) (C) (69626)	Kumita	Sap and fruit (F)	Decoction of unripe fruit is consumed as liver tonic and to treat gastritis. Boiled unripe fruit is consumed as laxative. The sap is used as preservative for citrus juices and extracts of other herbs.
29	*Cassia floribunda* Cav. (Caesalpiniaceae) (W) (69535)	Napongchami	Leaf (F)	Warmed leaves are made into a paste and applied externally for fungal infection[d], eczema, contact dermatitis, allergic reaction, prickly heat and burns. Caution - only for external use.
30	*Catharanthus roseus* (Linn.) G. Don (Apocynaceae) (C) (69517)	Supienaro	Leaf (F)	Leaf decoction is taken orally for gastroenteritis problem and as laxative.
31	*Celosia cristata* L. (Amaranthaceae) (W) (69520)	Alonaro	Flower and leaf (F)	Decoction of flower is taken orally for urinary tract infection. Leaf paste is applied topically for cuts and wounds[d].
32	*Centella asiatica* L. (Apiaceae) (W) (69598)	Longtsukolok	Whole plant (F)	Plant is boiled and consumed for gastrointestinal problems[a,b,c,d].

33	***Chrysanthemum indicum* L. (Asteraceae) (C) (69529)**	**Asurongmang**	**Leaf (F)**	**Leaf paste is applied topically to treat lip scab (angular cheilitis) and scabies.**
34	*Cissampelos pareira* Linn. (Menispermaceae) (W) (69675)	Likhazung	Root (B)	Roots are eaten raw or infusion is taken orally for high blood pressure, malaria, dysentery, piles[d], gastrointestinal problems[d] and diabetes. Powdered dried roots are used for long term storage.
35	*Cissus repens* Lam. (Vitaceae) (W) (69537)	Zerebliwa	Leaves (B)	Leaf decoction is taken orally for high blood pressure, urinary, spleen and kidney problems.
36	***Citrus microcarpa* Bunge (Rutaceae) (C) (69618)**	**Nimbutinga**	**Fruit juice (B)**	**Fresh fruit juice is taken judiciously for stomach ache and gas formation (purgative).**
37	*Clerodendron cordatum* D. Don (Verbenaceae) (W) (C) (69531)	Oremwa	Leaf (F)	Boiled leaves are consumed to treat high blood pressure and also eaten as a delicacy. Caution - ingestion of drupe may induce body swelling and vomiting.
38	*Coix lacryma-jobi* Linn. (Poaceae) (W) (69662)	Jemur	Seed (B)	Necklace made of seeds is worn to treat high blood pressure but long term wearing may induce weight loss.
39	*Costus speciosus* (Koenig ex Retz) JE Smith (Costaceae) (W) (69496)	Aokmejang	Stem (F)	Inner stem is chewed for tooth ache and vermifuge.
40	***Crataeva nurvala* Buch-Ham. (Capparaceae) (W) (69690)**	**Kongkawa**	**Leaf (F)**	**Warmed leaves are applied externally to relieve pain and body swelling. Boiled leaves are consumed as liver tonic.**
41	***Croton Caudatus* Gieseler (Euphorbiaceae) (W) (69664)**	**Khemetsu koila**	**Leaf and root (F)**	**Fresh leaves or roots are chopped into fine pieces and soaked in water overnight and the extract is drunk twice a day for cancer, sinusitis and gastrointestinal problems.**
42	***Cucurbita pepo* L. (Cucurbitaceae) (C) (69682)**	**Moyamatsu**	**Fruit and leaf (F)**	**Cooked and consumed as vitamin source.**
43	***Curculigo capitulata (*Lour.) Kuntze (Hypoxidaceae) (W) (69527)**	**Kurivu**	**Rhizome (F)**	**Outer skin is peeled off and soaked in water until it turns slimy and then consumed for gastritis and squeezed into eyes for treating eye infection (dirt, conjunctivitis)[a,c,d].**

44	*Curanga amara* Juss. (Scrophulariaceae) (W) (C) (69538)	Longri	Leaf (B)	Fresh leaves are chewed or infusion taken orally to treat dysentery, high blood pressure, food poisoning, gastroenteritis and loss of appetite. Caution - extremely bitter.
45	*Cyclea peltata* Diels. (Menispermaceae) (W) (69650)	Tsungrempangmoli	Leaf (B)	Leaf decoction is applied topically to abscesses and boils. Leaves also added to bath to ward off evil spirits.
46	*Datura stramonium* Linn. (Solanaceae) (W) (69528)	Kohima sangjem	Leaf (F)	Warmed leaf is applied externally to relieve back pain.
47	*Debregeasia longifolia* (Burm. f.) Wedd. (Urticaceae) (W) (69504)	Natsulawa	Leaf (F)	Leaf decoction is taken orally to treat diabetes, fever and high blood pressure. Boiled leaves are eaten as a delicacy.
48	***Dendrocnide sinuata* (Bl.) (Urticaceae) (W) (69508)**	**Zaklojawa**	**Stem (F)**	**Outer fresh stem is scraped off and the mucilage secreted is applied on fresh cuts and wounds (haemostatic). Caution - produces extremely painful sting.**
49	*Diospyros lanceifolia* Roxb. (Ebenaceae) (W) (69544)	Urcha	Fruit and root (F)	Fruits are crushed in stream to poison fish[a,c].
50	***Dolichos lablab* L. (Fabaceae) (C) (69523)**	**Napakauv**	**Leaf and pod (B)**	**Cooked pods are consumed to treat diarrhoea, nausea, vomiting and poor appetite. For insect and poisonous spider bites and bee sting, leaf paste is applied topically. Caution - fatal when consumed after a dog bite.**
51	*Drymaria cordata* (Linn.) Willd. (Caryophyllaceae) (W) (69530)	Pipivula	Whole plant (F)	Plants warmed in fire are crushed into a paste and applied topically to treat fungal infection (ringworm), contact dermatitis and lip scab (angular cheilitis). For, sinusitis, leaf paste is inserted into the nostril. To deodorise armpit, plant is wrapped in banana leaves and toasted for 5-10 minutes and then applied to armpits. For ear pain and infection (otitis media), leaves, mustard oil and spider exuvia are pounded, filtered and a few drops instilled into the ear.
52	***Dryopteris filix-mas* (L.) Schott (Dryopteridaceae) (W) (69507)**	**Nachav**	**Whole plant (F)**	**Whole plant is crushed into the stream to poison fish. Infusion is sprayed as pesticide and insecticide. Leaves are laid down in chicken coop for killing chicken ticks/bugs. Leaf paste is used to treat skin irritation and snake and insect bites.**

53	*Duabanga grandiflora* (Roxb. ex DC.) Walp. (Sonneratiaceae) (C) (W) (69686)	Kisati	Stem (F)	Fresh bark is scraped off and applied topically to treat skin diseases[d], cuts and wounds.
54	***Elsholtzia blanda* (Benth.) Benth. (Lamiaceae) (W) (69524)**	**Changjang**	**Leaf (B)**	**Leaf paste is applied to fresh cuts and decoction of leaves is added to bath during cold or fever. Leaf paste is also inserted into the nostril to treat sinusitis.**
55	*Entada pursaetha* DC. (Leguminosae) (W) (69616)	Keling	Seed (B)	Seed extract is used for head wash to treat head lice and dandruff[a,d].
56	*Equisetum ramosissimum* Desf. Subsp. *Debile* Subsp. *Debile* (Equisetaceae) (W) (69499)	Avpenba	Stem (F)	Stem decoction is taken orally for kidney problems.
57	*Eryngium foetidum* Linn. (Apiaceae) (W) (69668)	Aong thonia	Leaf (F)	Leaves are consumed either raw or cooked for indigestion.
58	*Erythrina stricta* Roxb. (Fabaceae) (W) (69629)	Lochet	Stem (F)	Bark paste is applied topically to treat contact dermatitis, eczema and skin infections[d].
59	*Eucalyptus globulus* Labill. (Myrtaceae) (C) (69663)	Eucalyptus	Leaf (B)	Steam from boiling leaves is inhaled for nasal decongestion.
60	*Eupatorium odoratum* Linn. (Asteraceae) (W) (69523)	Zasen	Leaf (F)	Leaf paste is applied topically to fresh cuts and wounds[c,d].
61	*Euphorbia pulcherrima* Willd. ex Klotzsch (Euphorbiaceae) (C) (69615)	Muluchangnaro	Flower and leaf (F)	Death may result when fed accidentally to pigs.
62	*Euphorbia royleana* Boiss. (Euphorbiaceae) (C) (69684)	Takterak	Milky sap (F)	Milky sap is applied topically to treat skin diseases and body pain.
63	*Eurya acuminata* DC. (Theaceae) (W) (69612)	Mesetwa	Fruit and leaf (F)	Leaf infusion is taken orally to treat dysentery/diarrhoea[d]. Leaf paste is applied topically to cuts and wounds. Fresh fruits are crushed and mixed with water and drunk 3 to 4 times to treat gas

				formation.
64	***Ficus elastica* Roxb. ex Hornem. (Moraceae) (C) (69619)**	**Ngisa**	**Root and sap (F)**	**Root juice or sap is applied topically to snake bite and cuts and wounds.**
65	*Garcinia cowa* Roxb. (Guttiferae) (W) (69631)	Songtula	Stem and seed (D)	Decoction of dried bark or seed cover is taken orally to treat dysentery/diarrhoea. Seed is edible.
66	*Garcinia pedunculata* Roxb. Ex Buch.-Ham. (Guttiferae) (W) (69630)	Asong	Stem and seed (D)	Decoction of dried bark or seed cover is taken orally to treat dysentery/diarrhoea. Seed is edible.
67	***Girardinia palmata* (Forsk.) Gaud. (Urticaceae) (W) (69661)**	**Ongpangzaklu**	**Leaf (F)**	**Leaf paste is applied to dog bites. Dogs stung with this plant may die.**
68	***Glycine max* (L.) Merr. (Fabaceae) (C) (69680)**	**Alichami**	**Seed (D)**	**Roasted seeds are made into powder and taken with tea to treat dysentery.**
69	*Gmelina arborea* Roxb. (Verbenaceae) (W) (69518)	Ekong	Drupe (F)	Mesocarp of the drupe is applied topically to treat skin diseases.
70	*Gonatanthus pumilus* (D. Don) Engler and Krause (Araceae) (W) (69498)	Longtong	Leaf and stem (F)	Small quantity of the leaf or stem is mixed with food and given as vermifuge to pigs. Caution - extremely poisonous and death may result if ingested by humans. Rhizome also causes extreme itching when contacted.
71	*Gossypium herbaceum* Linn. (Malvaceae) (C) (69611)	Khumpa	Root (F)	Root decoction is taken orally as diuretic.
72	***Gynocordia odorata* R. Br. (Flacourtiaceae) (W) (69501)**	**Lamen**	**Leaf (F)**	**Leaf paste is applied to bee sting and leaves are used to protect from bee sting during honey harvest. Caution - plant growing near drinking water source or channel can contaminate the drinking water and if consumed result in abnormal enlargement of neck.**
73	*Gynura crepidioides* Benth. (Asteraceae) (W) (69506)	Monglibaza	Leaf (F)	Leaf decoction is taken orally to treat diabetes.

74	*Hedyotis scandens* Roxb. (Rubiaceae) (W) (69539)	Termoli	Leaf and root (B)	Leaf paste is applied topically to cuts and wounds and infusion or decoction is taken orally to treat urinary tract infection, piles, gastrointestinal problems and also taken as laxative. Roots are chewed and applied topically to bee sting.
75	*Hodgsonia macrocarpa* (Blume) Cogn. (Cucurbitaceae) (W) (69667)	Assa	Leaf and seed (F)	Leaf paste is used for massaging body pain and roasted seed is consumed as laxative.
76	***Holboellia latifolia* Wall. (Lardizabalaceae) (W) (69500)**	**Mezetsuk**	**Leaf (F)**	**Foam from crushed leaves is applied topically to burns.**
77	*Houttuynia cordata* Thunb. (Saururaceae) (C) (69532)	Nokna	Whole plant (F)	Eaten raw to treat dysentery/diarrhoea[a,d], gas formation and as vermifuge.
78	***Ipomoea nil* (Linn.) Roth. (Convolvulaceae) (C) (69678)**	**Makenchangnaro**	**Flower and leaf (F)**	**Leaf paste/flowers are applied topically to burns.**
79	*Kalanchoe pinnata* (Lam.) Pers. (Crassulaceae) (C) (69515)	Nokchamoli	Leaf (F)	Warmed leaf paste is applied topically to treat ringworms, skin diseases and burns[d].
80	***Lagenaria siceraria* (Molina) Standl. (Cucurbitaceae) (C) (69521)**	**Aakuf**	**Leaf (F)**	**Juice extract is applied topically to treat skin diseases and inflammation.**
81	*Lantana camara* Linn. (Verbenaceae) (W) (69620)	Aiangketba naro	Whole plant (F)	Plant decoction is taken orally to treat jaundice, cold and fever.
82	*Lasia spinosa* (L.) Thwaites (Araceae) (W) (69655)	Turang	Leaf and stem (F)	Decoction of stem/leaves is taken orally as vermifuge[a,c]. leaf paste is applied topically to treat skin diseases. Young tender leaves are edible[c].
83	*Lycopersicon esculentum* Linn. (Solanaceae) (C) (69654)	Benganatasula	Fruit (F)	Juice from unripe fruits is taken orally twice a day to treat urinary problems and kidney pain.
84	***Luffa acutangula* Linn. (Cucurbitaceae) (C) (69676)**	**Pokka**	**Flower, fruit and leaf (F)**	**Boiled and consumed as laxative and to aid digestion.**

85	***Macropanax undulatus* (Wall. ex G. Don) Seem. (Araliaceae) (W) (69540)**	**Semza**	**Leaf (F)**	**During cold and high fever leaves are laid down and slept on. Leaves can be seen in many bird nests, particularly flamingo and bulbul.**
86	***Maesa indica* (Roxb.) Wall. (Myrsinaceae) (W) (69514)**	**Kensametong**	**Leaf (F)**	**Leaf paste is applied topically to cuts and wounds.**
87	*Manihot esculenta* Crantz. (Euphorbiaceae) (C) (69695)	Alicha	Tuber (B)	Tuber is used with some rice and herbs to produce fermented beer and this is taken for gastrointestinal problems.
88	*Melastoma malabathricum* (Melastomataceae) (W) (69652)	Nemna	Fruit and leaf (F)	Fruit is edible and leaf paste is applied to cuts and wounds[d].
89	*Melia composite* Willd. (Meliaceae) (W) (69669)	Aiet	Fruit and leaf (F)	Fruit/leaves are consumed to expel gas from stomach.
90	*Mentha cordifolia* Opiz ex Fresen. (Lamiaceae) (C) (69683)	Pudina	Leaf (F)	Leaf paste is applied topically to fresh cuts and skin diseases. Crushed leaves are inhaled for nostril decongestion.
91	*Mikania cordata* (Burm. F.) B. L. Rob. (Asteraceae) (W) (69534)	Indialeelang	Leaf and stem (B)	Leaf/stem paste is inserted into the rectum for about 5 minutes to treat dysentery/diarrhoea and piles. Leaf/stem paste is applied topically to treat skin diseases and cuts[d]. Dried powdered leaves are also used with other plants.
92	*Millettia cinerea* Benth. (Fabaceae) (W) (69666)	Suli	Root and vine (F)	Root is crushed into stream or creek to poison fish[a]. Vine extract is used in massages to relieve body pain.
93	*Mimosa pudica* Linn. (Mimosaceae) (W) (69526)	Amidangku-ayaklawa	Leaf (F)	Leaf paste is applied topically to treat inflammation and decoction is taken orally for gastrointestinal problems.
94	*Mirabilis jalapa* Linn. (Nyctaginaceae) (C) (69609)	Chumdangnaro	Leaf and root (F)	Decoction of leaves/roots is taken orally as diuretic and to treat ear ache.
95	*Mussaenda roxburghii* Hk. f. (Rubiaceae) (W) (69502)	Andipeernaro	Leaf (B)	Fresh leaf paste is applied topically to cuts and wounds. Leaves are also used in combination with other herbs to brew rice beer.
96	*Myrica esculenta* Buch.-Ham., ex D. Don (Myricaceae) (C) (69522)	Mediong	Fruit and leaf (F)	Fruit is edible and leaf paste is applied to cuts and wounds.

97	***Nephrolepis cordifolia*** **(Davalliaceae) (W) (69623)**	**Seraenjen**	**Tuber (F)**	**Tubers are crushed and taken orally with water to treat hiccups and sneezing. They are also used as diuretic. A few drops of juice extract are inserted into the nostrils to treat sinusitis. Fresh tubers are crushed and applied topically or a slice of tuber is rubbed into the affected areas for the treatment of skin infections.**
98	*Nerium indicum,* Mill. (Apocynaceae) (C) (69674)	Sharonnaro	Flower (F)	Flower decoction is used to kill lice and insects.
99	*Ocimum basilicum* Linn. (Lamiaceae) (C) (69543)	Nangperra	Whole plant (B)	Infusion is taken orally to treat stomach upset[c] and gas formation. It is also added in bath to treat influenza.
100	*Oroxylum indicum* (Linn.) Vent. (Bignoniaceae) (W) (69637)	Ochamiliau	Bark (F)	Decoction is taken orally to treat dysentery and rheumatism[d].
101	*Paederia foetida* Linn. (Rubiaceae) (W) (69694)	Atsulelang	Stem (F)	Decoction is taken orally to treat intestinal problems[b].
102	*Passiflora edulis* Sim. (Passifloraceae) (C) (69700)	Entsulashi	Leaf (F)	Leaf decoction is taken orally once a day after meal to treat high blood pressure[a,d].
103	*Oxalis acetosella* L. (Oxalidaceae) (W) (69607)	Waroetsu	Leaf (F)	Eaten raw as diuretic, vermifuge and to treat gas formation and dysentery.
104	*Phyllanthus emblica,* Linn. (Euphorbiaceae) (W) (C) (69671)	Lher	Fruit (B)	Fresh or dried fruits are eaten raw or infusion or decoction is taken orally to treat cough[b], high blood pressure, bladder and kidney problems and also taken as a laxative.
105	*Phyllanthus urinaria* Linn. (Euphorbiaceae) (W) (69696)	Asularlir	Fruit and leaf (F)	Fruits or leaves are eaten raw to treat fever, kidney pain, jaundice[d], dysentery[d] and gastrointestinal problems[d]. They are also taken as a laxative.
106	***Physalis alkekengi*** **L. (Solanaceae) (W) (69647)**	**Entsupilvu**	**Fruit (F)**	**Eaten raw or infusion is taken orally for kidney and bladder problems.**
107	*Piper betel* (Piperaceae) (C) (69697)	Patiwa	Leaf (F)	Leaf paste is applied topically to cuts and wounds[a] . Fresh leaves

				are chewed with lime, areca nut and tobacco to treat dental caries.
108	*Plantago erosa* Wall. (Plantaginaceae) (W) (69625)	Sangnem	Leaf (F)	Boiled leaves are consumed as a laxative.
109	*Polygonum hydropiper* Linn. (Polygonaceae) (W) (69641)	Nikmeremlawa	Leaf (F)	Leaf paste is applied topically to treat fungal infections[b] and skin infections.
110	*Prunus persica* (L.) Stokes (Rosaceae) (C) (69503)	Mokori	Leaf, root and seed (F)	Fresh root is soaked in water overnight and taken orally to treat typhoid. It is also used to treat skin related infections. Seed endosperm is consumed to treat dysentery/diarrhoea, and leaf extract is applied topically to treat skin diseases (acne).
111	*Psidium guajava* Linn. (Myrtaceae) (C) (69513)	Monaim	Leaf (F)	Leaf is chewed and swallowed to treat constipation and dysentery/diarrhoea.
112	*Punica granatum* Linn. (Punicaceae) (C) (69621)	Jarem	Fruit and leaf (F)	Leaf decoction is taken twice a day before meals to treat dysentery/diarrhoea. Dried fruit cover is also used for the same purpose.
113	*Rhus javanica* var. *roxburghiana* (Anacardiaceae) (W) (69603)	Tangma	Fruit (B)	Infusion of the fresh or dried fruit (gall) is taken orally once or twice a day to treat gas formation, stomach ache[d], mushroom poisoning[a,c], dysentery, high blood pressure and allergies (rash)[a].
114	***Rhus roxburghii* Hook. f. (Anacardiaceae) (W) (69673)**	**Jarak**	**Whole plant (F)**	**Causes contact dermatitis.**
115	*Ricinus communis* Linn. (Euphorbiaceae) (W) (C) (69516)	Pakawa	Leaf and seeds (F)	A quarter of a roasted seed is chewed and swallowed as a laxative. Caution - high dose causes extreme diarrhoea (not advisable for children). Leaves are used for rearing silk worms.
116	*Saccharum officinarum* Linn. (Poaceae) (C) (69622)	Mostutong	Culm (F)	Juice is taken orally twice a day to treat jaundice[d].
117	***Scutellaria glandulosa* Colebr. (Lamiaceae) (W) (69542)**	**Yimramoli**	**Whole plant (B)**	**Plant infusion is taken orally to treat stomach upset and gas formation. Infusion is also used to bath to treat influenza.**
118	*Solanum indicum* Linn.	Ao longkok	Fruit and leaf (B)	Unripe fruit is roasted and consumed once or twice a day as

	(Solanaceae) (C) (69627)			antihelminthic (pinworm). Fresh leaves, rice and water is ground, dried, powdered and use as brewing cake for rice beer.
119	*Solanum khasianum* Clarke (Solanaceae) (W) (69597)	Atsu longkok	Seed (F)	Fumes from the burning seeds are channelled into the affected area to treat tooth ache.
120	***Sonerila maculata* Roxb. (Melastomaceae) (W) (69509)**	**Alichang**	**Leaf (F)**	**Leaf paste is applied topically to treat insect bites and inflammation.**
121	***Spermacoce scaberrima* Blume (Rubiaceae) (W) (69643)**	**Ongpangentilawa**	**Leaf (F)**	**Leaf paste is applied immediately to snake bites.**
122	***Spermacoce hispida* Linn. (Rubiaceae) (W) (69698)**	**Intilawa**	**Leaf (B)**	**Fresh leaves are chewed and swallowed as a laxative. Dried leaf paste pounded with rice grains is used for brewing rice beer.**
123	*Spilanthes acmella* Linn. (Asteraceae) (W) (69639)	Okensencha	Flower (F)	Flowers are chewed 2 to 4 times a day to treat tooth ache[d].
124	*Spondias pinnata* (Linn. F.) Kurz. (Anacardiaceae) (W) (69519)	Pakho	Drupe and leaf (F)	Fresh immature leaves are eaten raw to treat gastrointestinal problems. Ripe drupe is eaten raw or the juice is taken orally as liver tonic and as appetiser.
125	***Stereospermum chelonoides* (Linn. f.) DC (Bignoniaceae) (W) (69610)**	**Sengpet**	**Stem (F)**	**Stem bark paste is applied to treat cuts and wounds and skin diseases. Stem bark paste is also used as antiseptic and a decoction of stem bark is taken orally to treat allergies.**
126	***Stixis suaveolens* (Roxb.) Pierre (Capparidaceae) (W) (69525)**	**Aiemaluv**	**Fruit, seed and root (F)**	**Root infusion is taken orally to treat spleen enlargement. Fruits and seeds are edible.**
127	*Tagetes erecta,* Linn. (Asteraceae) (C) (69670)	Pesunaro	Whole plant (F)	Plant infusion is taken orally to treat intestinal problem, rheumatic pain[d], boils[d], skin infection and sinusitis.
128	*Terminalia chebula* Retz. (Combretaceae) (W) (C) (69640)	Ningkha	Drupe (B)	Drupe is eaten as a good source of vitamins and to treat stomach ache.
129	*Thunbergia grandiflora* Roxb. (Acanthaceae) (W) (69497)	Koktsuli	Stem (F)	Fluid from stem is added to the eye to treat eye infection.

130	*Tithonia diversifolia* (Hemsl.) A. Gray (Asteraceae) (C) (69687)	Zoninaro	Leaf (F)	Infusion is taken orally to treat high blood pressure, malaria and leaf paste is applied topically to treat abscesses and body pain.
131	*Urtica dioica* L. (Urticaceae) (W) (69601)	Zaklutasula	Leaf (F)	Leaf paste is applied topically to treat dog and snake bites.
132	***Viola betonicifolia* Boj. Ex. baker (Violaceae) (W) (69651)**	**Hingpangmoli**	**Leaf (F)**	**Leaf paste is applied to draw out stings or thorns.**
133	*Wedelia chinensis* (Osb.) Merrill (Asteraceae) (W) (C) (69693)	Enze	Leaf (F)	Fresh leaves are eaten raw in the form of a salad to treat gastrointestinal problems.
134	*Zanthoxylum acanthopodium* DC. (Rutaceae) (W) (C) (69600)	Changpet	Leaf (F)	A mixture of leaves with *Rhus javanica* fruit extract is taken orally to treat stomach ache. Leaves soaked in lukewarm water are used in bath to treat influenza. Leaf infusion is sprayed on pest infected plants as pesticide.
135	*Zanthoxylum rhetsa* (Roxb.) DC. (Rutaceae) (W) (69689)	Ongret	Seed (D)	Seeds are crushed in the stream to poison fish. Leaf infusion is sprayed on pest infected plants as pesticide.

a, b, c, d, uses are consistent with information already reported in Nagaland ethnobotany literature.
a (Changkija, 1999)
b (Jamir, 1999)
c (Jamir and Rao, 1982)
d (Deorani and Sharma, 2007)

Table 2-2: List of threatened species in Chungtia village, Nagaland

#	**Scientific name**	**Local name**
1	*Adenia trilobata*	Tenik tepang
2	*Basella alba*	Latsung-en
3	*Calotropis gigantea*	Kutjak moli
4	*Cassia floribunda*	Napongchami
5	*Coix lacryma-jobi*	Jemur
6	*Curanga amara*	Longri
7	*Cyclea peltata*	Tsungrempangmoli
8	*Diospyros lanceifolia*	Urcha
9	*Equisetum ramosissimum*	Avpenba
10	*Garcinia cowa*	Songtula
11	*Gonatanthus pumilus*	Longtong
12	*Hedyotis scandens*	Termoli
13	*Holboellia latifolia*	Mezetsuk
14	*Lasia spinosa*	Turang
15	*Paederia foetida*	Atsulelang
16	*Physalis alkekengi*	Entsupilvu
17	*Scutellaria glandulosa*	Yimramoli
18	*Solanum khasianum*	Atsu longkok
19	*Viola betonicifolia*	Hingpangmoli
20	*Zanthoxylum acanthopodium*	Changpet

2.5 Ethnobotanical uses of documented plants

The 135 documented plants were represented by 69 families and 123 genera and the most represented family was Asteraceae with 9 species, followed by Euphorbiaceae with 7 species and Solanaceae with 6 species. Table 2-1 details the documented plants with their uses, parts used, mode of preparation and administration. A picture of each plant is presented in Appendix 2-3. The most reported uses were for gastrointestinal problems such as stomach ache, dysentery/diarrhoea,

constipation, gas formation, gastritis and indigestion, which was attributed to 61 plants, followed by dermatological problems such as cuts and wounds, allergies, eczema, contact dermatitis, fungal infection, prickly heat and burns, accounting for 35 plants, followed by 16 plants for musculoskeletal and 13 plants each for hypertension and influenza/fever/colds. Apart from uses for human ailments, seven plants were recorded to poison fish and four plants as pesticides/insecticides. This is consistent with other Indigenous communities where dermatological and gastrointestinal problems were the leading disorders treated by medicinal plants (Frei et al., 1998a; Sanz-Biset et al., 2009; Uprety et al., 2010). Figure 2-1 summarises the findings of this study for the number of plants used in relation to the various ailments.

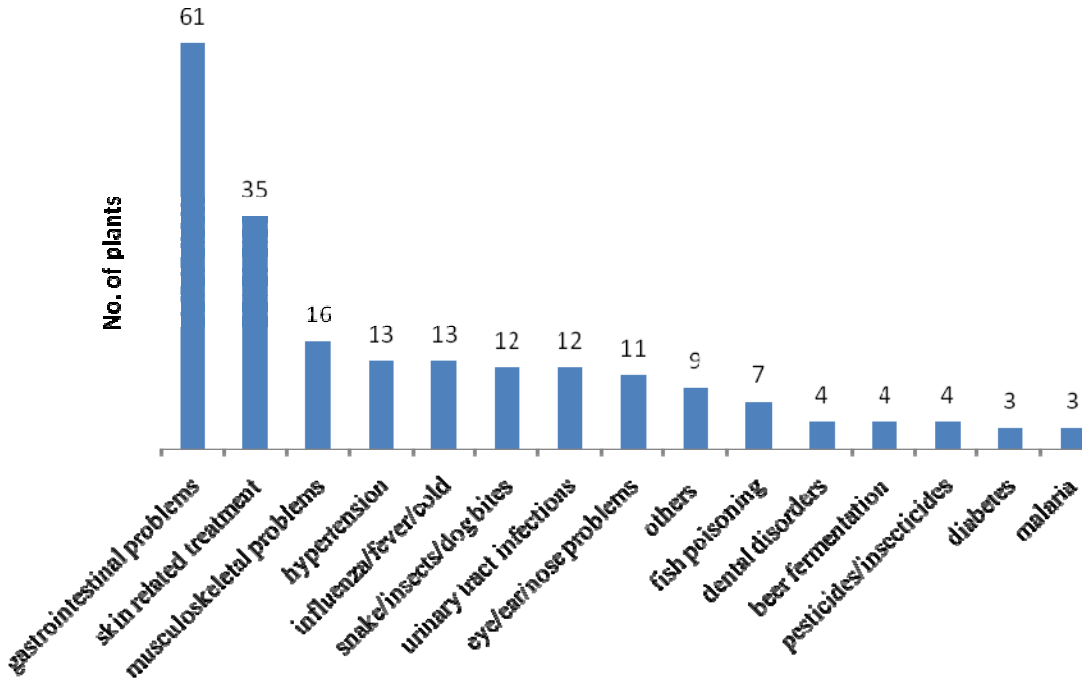


Figure 2-1: Number of plants recorded by their uses

2.6 Habitat, parts used, mode of preparation and administration of plants

Of the 135 plants, 68 plant species were woody in nature (tree, shrub, woody vine or liana) and 67 plants were herbs or herbaceous vines. Seventy eight plants were collected in the wild, while 47 were collected as cultivated species and 10 were collected both as wild and cultivated species (Figure 2-2).

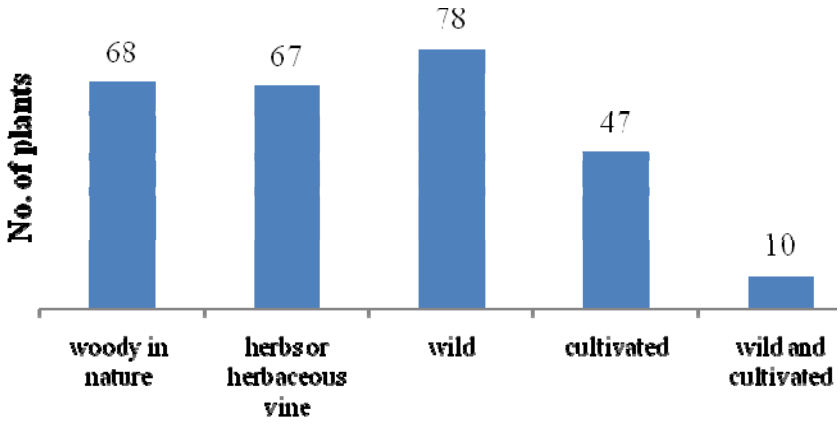


Figure 2-2: Habitat of plants

As presented in Figure 2-3, the most commonly used plant parts were leaves (77 species), followed by fruits (20 species), stems (18 species), roots (15 species), flowers (6 species), sap/latex (3 species), bulbs (2 species), and in some cases, the whole plant was used (11 species). The predominant use of leaves have also been reported elsewhere in Northern and North-East India (Poonam and Singh, 2009; Sajem and Gosai, 2006).

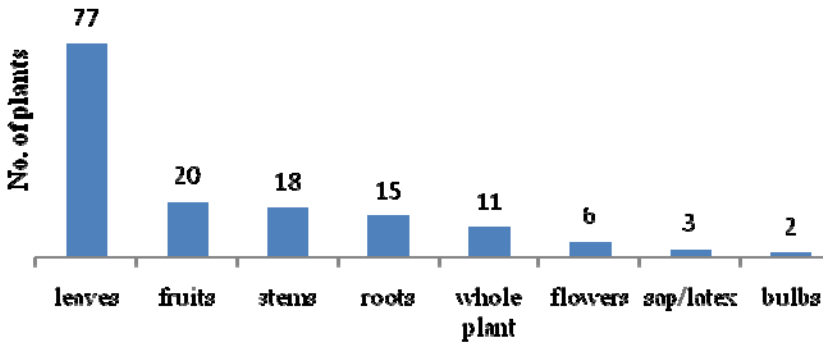


Figure 2-3: Plant parts used

The methods of preparation for medicinal use (Figure 2-4) were in the form of pastes (32 species), oral consumption (29 species), decoctions (27 species), infusions (19 species), juice (11 species), poultices (7 species) and others (21 species). Here decoction refers to boiling the plant material in water and an infusion refers to just soaking it in cold water for a limited period of time (1 to 24 hours). For poultice treatments, banana leaves were generally employed for wrapping the plant material and heating it up before being applied to the affected areas. The methods of administration were mainly oral consumption or internal use, which accounted for 80 medicinal remedies, while topical or external applications accounted for 54 medicinal remedies. In most cases, preparations were drawn from a single plant, but some mixtures of plants were also commonly used. For example, the dried leaf powder of *Ocimum basilicum* is typically mixed with the powder of dried drupes of *Rhus javanica*.

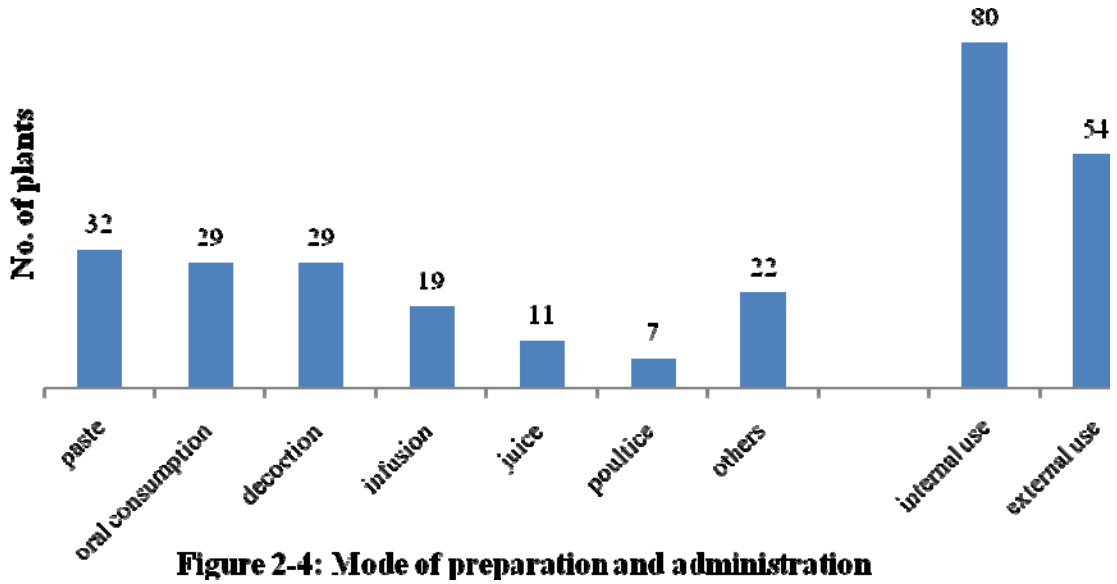


Figure 2-4: Mode of preparation and administration

2.7 Most commonly used plants and their biological activities

A number of remedies were consistently reported by the village elders. This included the use of *Eupatorium odoratum* or *Mikania cordata*, to treat fresh cuts and wounds. These two plants were cited as useful by all the village elders. Since cuts and wounds are common ailments for the villagers and these plants are common weeds in the village, they form the first line of treatment for cuts and wounds. Similar uses of these plants have been reported from the neighbouring state of Mizoram (Bhardwaj and Gakhar, 2005) as well as from southern India (Ayyanar and Ignacimuthu, 2009), Nepal (Rai, 2004) and Nigeria (Akah, 1990). The efficacy of *Eupatorium odoratum* was investigated based on its traditional use and it has been shown to have significant wound-healing properties (Akah, 1990; Phan et al., 1996). Of the other most commonly cited plants, *Mikania cordata* possesses strong analgesic activity and its active ingredients have been isolated (Ahmed et al., 2001). Another important remedy used was a mixture of dried powdered leaves of *Curanga amara* Juss., *Ocimum basilicum*, and the dried drupe powder of *Rhus javanica.* This is used extensively to treat stomach ache, stomach upset, gas formation, high blood pressure and influenza and is highly regarded by the villagers, being found in virtually every household. A preparation of *Rhus javanica* alone is also used for the above mentioned ailments and as an antidote for the poisonous effects of mushrooms or during allergic reactions. These plants are used medicinally in other parts of the world and a variety of biological activities have been reported for these plants.

Ocimum basilicum, is widely employed for a range of ailments (Warrier and Nambiar, 1995) and has been shown to possess antibacterial (Sanni et al., 2008), antifungal (Zhang et al., 2009), antiviral (Chiang et al., 2005), hypotensive and cardiovascular activities (Assaf, 2003). *Curanga amara* is an extremely bitter plant, also used in Chinese folk medicine for the treatment of fever, herpes infection, cancer and inflammation. It has anti-lipid peroxidation activities (Thuan et al., 2007). *Rhus javanica* is also used in Chinese traditional medicine for the treatment of cold, fever, cough and malaria (Wang et al., 2008b), and has strong antiviral activity against the human immunodeficiency virus (Gu et al., 2007; Wang et al., 2008b) and herpes simplex virus (Kurokawa et al., 1999). The traditional use of this plant by Naga tribes for dysentery and diarrhoea has been examined and it has been shown to possess strong anti-diarrhoeal property, thereby validating its efficacy (Tangpu and Yadav Arun, 2004).

2.8 Comparison of the information documented firsthand with published literature on Nagaland Plants

Ethnobotanical surveys of Nagaland have so far resulted in the documenting of over 500 medicinal plants belonging to around 137 families and 327 genera, with the majority of them belonging to the flowering plants of the Asteraceae, Liliaceae, Verbenaceae and Menispermaceae families (Changkija, 1999; Deorani and Sharma, 2007; Jamir and Rao, 1982; Jamir, 1999). No ethnobotanical studies had been previously undertaken in Chungtia village. Comparison of the 135 plants documented in this study with the existing literature on Nagaland plants revealed that 39 plants have already been reported for similar ethnobotanical uses elsewhere in Nagaland. Sixty one plants of the remaining 96 plants documented in our study have been reported to be used by Nagaland people but for different purposes. Thirty five plants were not mentioned in any of the sources consulted and this may therefore constitute novel ethnobotanical information concerning Nagaland plants. This novel information is highlighted in bold in Table 2-1.

2.9 Conclusion

A collaborative research partnership was established between Chungtia village, Nagaland, and Macquarie University, Australia, to document the medicinal plant knowledge of Chungtia village, with a primary aim to aid the preservation of this knowledge. This is the first ethnobotanical study of Chungtia village. Interviews were conducted with 10 village elders chosen to represent the

Chungtia villagers. These village elders highlighted that medicinal plants are still the preferred mode of treatment for the villagers for common ailments such as cuts and wounds, stomach ache, stomach upset, gas formation, high blood pressure, gastritis and influenza, however, the in-depth traditional knowledge of the general villagers was being lost. The interviews resulted in the documentation of firsthand information on 135 plants. Of these, 35 plants were not mentioned in any of the published sources consulted and may therefore constitute novel ethnobotanical information concerning Nagaland plants. This study has helped to document and preserve the important customary medicinal plant knowledge of the Chungtia villagers, assisting them in the continued preservation of their cultural and traditional values.

The following chapter will describe the selection of Nagaland medicinal plants for antibacterial screening from the firsthand ethnobotanical studies of Chungtia village and literature reports from Nagaland.

2.10 Experimental

2.10.1 Collaborative research partnership between CSMT and IBRG

Human research ethics approval (Ref: HE22JUN2007-R05316) was obtained from Macquarie University, Sydney, Australia on 10th of July 2007. The agreement, on behalf of Chungtia village, was signed by the President of Chungtia Senso Mokokchung Town (CSMT) on the 13th of August 2007 (see appendix 2-2).

2.10.2 Data collection and taxonomic identification

Interviews with Chungtia village elders and plant collection for taxonomic identification were carried out from November 2007 - January 2008 and November 2008 - January 2009 with the help of selected representatives from CSMT. Before the first interviews were conducted, a meeting was held with the CSMT representative council members and some Chungtia village council representatives along with the author to select appropriate informants and to select a few representatives from the CSMT to guide and oversee the project. Four CSMT representatives and 10 informants were selected by the CSMT council. The informants were mostly above 50 years of age. Before the interviews, individual consent forms written in English were handed out to the informants and when the informant was not English literate, it was translated and explained in the

local *mongsen* language by those selected CSMT representatives. The first interviews (November 2007 – January 2008) were conducted by means of an open discussion with the 10 informants and four CSMT representatives present, and informants were asked to talk about the knowledge of the medicinal plants in the village, such as where they grow and how they are prepared and used. These were recorded in an audio format in local *mongsen* language and also by filling out a structured questionnaire that included the local plant name, local distribution of plants/habitat, seasonal availability/flowering, medicinal use, preparation, and parts used (see Appendix 2-4). Follow up individual interviews were also carried out in November 2008 – January 2009 with three informants (2 traditional herbal practitioners and 1 village elder) that were part of the 10 informants.

For the collection of plants, the informants were asked to accompany the author and the CSMT representatives to places where the discussed plants were located and these plants were collected for herbarium preparation following standard procedures (Jain and Rao, 1977). In some cases, young experienced hunters from the village were employed for plant collections when the plants were found only deep in the forest. Those plants were later identified by the informants, who shared their knowledge on these plants. Some plant collections were also done throughout the year during flowering or fruiting seasons, by a representative of the CSMT (A. Anungba Jamir), for voucher specimens.

2.10.3 Taxonomic identification of documented plants

Prepared herbarium specimens were identified with the assistance of Prof. N. S Jamir (Nagaland University), Dr Alemmeren (Fazl Ali College, Mokokchung), Bendangchuchang and Wangshikokla (Nagaland University), and later authenticated by the Botanical Survey of India (BSI), Eastern Circle, Shillong, India. The voucher specimens were deposited at the BSI herbarium for future reference.

Chapter 3

Selection of Nagaland Medicinal Plants for Antibacterial Screening

3.1 Background

The firsthand ethnobotanical studies of Chungtia village resulted in documentation of 135 plants. A literature search on Nagaland ethnobotanical studies also provided information on more than 500 plants used ethnomedicinally in Nagaland. The aim of the study presented in this chapter was to select plants for antibacterial studies and to provide a thorough review on the selected plants using the firsthand information and literature reports from Nagaland. The selection of plants was guided by their uses for treatment of conditions of a likely bacterial origin.

3.2 Selection of plants for antibacterial studies

3.2.1 Search for antibacterial agent

The search for newer antibacterial agents is crucial as in recent years the increase in the number of multidrug-resistant bacteria has led to the prediction that we are being thrown back into the pre-antibiotic era (Ash, 1996). It is a stark reality that bacteria are also acquiring virulence genes, thus transforming commonly occurring bacteria into potent pathogens. This predicament has been compounded by the cessation, or downsizing, of antibacterial drug discovery efforts by large pharmaceutical companies (Fernandes, P. 2006). It is estimated that ~80% of the population in developing countries rely mainly on traditional medicines (mostly from plants) for their primary healthcare (WHO, 2005). Natural products from medicinal plants used traditionally are also the most consistently successful source of structurally diverse and novel drugs (Harvey, 2000; Newman and Cragg, 2007). Studies focussed on traditional Indigenous medicinal plant use are also associated with a high hit rate of non-toxic bioactive mixtures or molecules. This is believed to be a result of a 'filtering' over centuries of effective plants from those that are ineffective or toxic (Clark, 1996). Indigenous medicinal plants are therefore a major resource for safe alternative medicines and the discovery of new drugs.

3.2.2 Selection of plants from firsthand information

From the firsthand information of Chungtia village, plants that were used for cuts and wounds, skin diseases, sores, boils, fire burns, scabies, abscesses, fungal infections and as a hand cleansing agent, were categorised under skin related treatments. This resulted in 35 plants being selected. For these plants, a literature search was performed on each plant to identify those that have not been previously investigated for their antibacterial properties.

Table 3-1 presents the 35 plants with their firsthand documented uses for skin related conditions, along with their reported biological activities.

The literature review highlighted 15 plants that have not been reported for antibacterial activities. The selection priority was based on plant availability for large scale collections. This resulted in selection of seven plants, *i.e. Begonia picta*, *Cassia floribunda*, *Erythrina stricta*, *Lasia spinosa*, *Nephrolepis cordifolia, Polygonum hydropiper* and *Prunus persica*. Additionally, *Cissampelos pareira* was included in the list for antibacterial studies. Some of the elders requested for this plant to be studied biologically due to its importance to them, despite it not having been used by them in skin related treatments. A literature survey indicated no antibacterial studies (see below) on this plant and hence it was included in the list for antibacterial studies.

Table 3-1: List of plants categorised under skin related treatments from firsthand information of Chungtia village and their reported biological activities

Scientific name (Botanical family)	Firsthand recorded uses for skin related treatments	Reported biological activities
Albizia lucidior (Mimosaceae)	Root infusion is applied topically to abscesses and boils.	Anticancer (Cai et al., 2002; Wang et al., 2005).
Allium Chinense (Liliaceae)	Bulb paste is applied topically for skin diseases.	Cardioprotective effect (Ren et al., 2010), antifungal (Meng et al., 2005), antibacterial (Sun et al., 2004).
Artocarpus heterophyllus (Moracea)	Sap is applied topically to treat skin diseases.	Antibacterial (Khan et al., 2003b), cytotoxic (Arung et al., 2010), anti-inflammatory (Fang et al., 2008).
Asclepias curassavica (Asclepiadaceae)	Leaf paste is applied topically for cuts and wounds.	Coagulant activity (Shivaprasad et al., 2009), anticancer (Li et al., 2009b), antifungal (Moulin-Traffort et al., 1990).
Begonia picta (Begoniaceae)	Leaves are used to cleanse hands by crushing between palms.	No biological or phytochemical studies.
Cassia floribunda (Caesalpiniaceae)	Warmed leaves are made into a paste and applied externally for fungal infections, eczema, contact dermatitis, allergic reactions, prickly heat and burns.	Antiprotozoal (Mesia et al., 2008).
Celosia cristata (Amaranthaceae)	Leaf paste is applied topically for cuts and wounds.	Antiviral (Balasubrahmanyam et al., 2000; Begam et al., 2006), antibacterial (Gnanamani et al., 2003).
Chrysanthemum indicum (Asteraceae)	Leaf paste is applied topically to treat lip scab and scabies.	Antibacterial (Aridogan et al., 2002; Jung, 2009).

Cyclea peltata (Menispermaceae)	Leaf decoction is applied topically for abscesses and boils.	Antiulcer (Shine et al., 2009), antitumour (Kupchan et al., 1973).
Dendrocnide sinuata (Urticaceae)	Outer fresh stem is scraped off and the mucilage secreted is applied on fresh cuts and wounds.	Antibacterial (Tanti et al., 2010).
Drymaria cordata (Caryophyllaceae)	Toasted plants are crushed into a paste and applied topically to treat fungal infection (ringworms), contact dermatitis and lip scabs.	Anti-inflammatory (Mukherjee et al., 1998), antitussive (Mukherjee et al., 1997), antibacterial (Pulok et al., 1997).
Duabanga grandiflora. (Sonneratiaceae)	Fresh bark is scraped off and applied topically to skin diseases and cuts and wounds.	Skin-whitening, anti-aging, anti-inflammatory (Tsukiyama et al., 2010).
Elsholtzia blanda (Lamiaceae)	Leaf paste is applied to fresh cuts.	Anti-ischemic effect (Ling and Lou, 2005).
Erythrina stricta (Fabaceae)	Bark paste is applied topically to treat contact dermatitis, eczema and skin infections.	Xanthine oxidase inhibitory activity (Umamaheswari et al., 2009), antimycobacterial and cytotoxic activity (Rukachaisirikul et al., 2007b).
Eupatorium odoratum (Asteraceae)	Leaf paste is applied topically to fresh cuts and wounds.	Antimicrobial (Chomnawang et al., 2005; Chomnawang et al., 2009; Inya-agha et al., 1987), antineoplastic agent (Arene et al., 1978).
Eurya acuminata (Theaceae)	Leaf paste is applied topically to cuts and wounds.	Antibacterial (Grosvenor et al., 1995).
Ficus elastica (Moraceae)	Root juice or sap is applied topically for cuts and wounds.	Anti-inflammatory (Sackeyfio and Lugeleka, 1986), antibacterial (Almahy et al., 2003).
Gmelina arborea (Verbenaceae)	Mesocarp of the drupe is applied topically to treat skin diseases.	Antioxidant (Patil et al., 2009), antifungal (Kawamura and Ohara, 2005; Kawamura et al., 2004).

Hedyotis scandens (Rubiaceae)	Leaf paste is applied topically to cuts and wounds.	No biological studies reported.
Holboellia latifolia (Lardizabalaceae)	Foam from crushed leaves is applied topically to burns.	No biological studies reported.
Ipomoea nil (Convolvulaceae)	Leaf paste is applied topically to burns.	Antitumour (Ko et al., 2004), antifungal (Koo et al., 2000).
Kalanchoe pinnata (Crassulaceae)	Warmed leaf paste is applied topically to treat ringworms, skin diseases and burns.	Immunodulatory activity (Gomes et al., 2010), antileishmanial (Muzitano et al., 2006), antimicrobial activity (Akinpelu, 2000) .
Lagenaria siceraria (Cucurbitaceae)	Juice extract is applied topically to treat skin diseases and inflammation.	Cytotoxic (Ghosh et al., 2009), antihyperlipidemic (Agrawal et al., 2008), analgesic and anti-inflammatory (Ghule et al., 2006).
Lasia spinosa (Araceae)	Leaf paste is applied topically to treat skin diseases. Young tender leaves are edible.	Free radical scavenging activity (Maisuthisakul et al., 2006).
Maesa indica (Myrsinaceae)	Leaf paste is applied topically to cuts and wounds.	No biological studies reported .
Mentha cordifolia (Lamiaceae)	Leaf paste is applied topically to fresh cuts and skin diseases.	Antibacterial (Ragasa et al., 2001), analgesic (Villasenor and Sanchez, 2009), antioxidant and antihypertensive (Pakdeechote et al., 2009).
Mikania cordata (Asteraceae)	Leaf paste is applied topically to treat skin diseases and cuts.	Analgesic (Ahmed et al., 2001), antiulcer (Paul et al., 2000), anticarcinogenic (Bishayee and Chatterjee, 1994).
Mussaenda roxburghii (Rubiaceae)	Fresh leaf paste is applied topically to cuts and wounds.	No biological studies reported.

Myrica esculenta (Myricaceae)	Leaf paste is applied to cuts and wounds.	Antioxidant and radical scavenging activities (Chen et al., 2007).
Nephrolepis cordifolia (Davalliaceae)	Tubers are rubbed on skin for skin diseases.	No biological studies reported.
Piper betel (Piperaceae)	Leaf paste is applied topically to cuts and wounds.	Antifungal (Ali et al., 2010), antibacterial (Nalina and Rahim, 2007) antioxidant (Dasgupta and De, 2004).
Polygonum hydropiper Linn. (Polygonaceae)	Leaf paste is applied topically to treat fungal infections and scabies.	Antimicrobial (Duraipandiyan et al., 2010b), antioxidant (Kiem et al., 2008b; Peng et al., 2003).
Prunus persica Stokes (Rosaceae)	Leaf juice extract is applied topically to treat skin diseases.	Anti-inflammatory activity (Rho et al., 2007).
Stereospermum chelonoides (Bignoniaceae)	Bark paste is applied to treat cuts and wounds and skin diseases. It is used as antiseptic.	Cytotoxic and antimicrobial (Haque et al., 2006).
Tagetes erecta (Asteraceae)	Plant infusion is applied topically to treat boils and skin infections.	Antinociceptive and anti-inflammatory (Chakraborthy, 2009), antibacterial activity (Chatterjee and Ali, 2010; Chomnawang et al., 2009).

3.2.3 Selection of plants from Nagaland literature

There are more than 500 medicinal plants used ethnomedicinally in Nagaland that are documented in the literature. Although not exhaustive, 20 plants have been reported for use for skin diseases or skin related treatments of possible bacterial origin. Table 3-2 presents literature searches on these plants for antibacterial or phytochemical studies. Three plants were identified that had either no antibacterial or phytochemical studies reported in the literature. The plants were *Albizia chinensis*, *Sarcandra glabra* and *Diospyros lanceifolia*.

Table 3-2: List of plants selected from the documented Nagaland medicinal plants along with their reported biological activities

Scientific name (Botanical family)	**Recorded Nagaland uses for skin related treatment**	**Reported biological activities**
Albizia chinensis (Osb) Merr. (Mimosaceae)	Leaf extract is applied to skin diseases (Deorani and Sharma, 2007).	Cytotoxic against human tumour cell lines (Liu et al., 2009).
Alstonia scholaris (Apocynaceae)	Latex used for sexually transmitted diseases, fungal infections and ear aches (Changkija, 1999).	Hepatoprotective effect (Lin et al., 1996), antibacterial (Khan et al., 2003a), cytotoxic to human cancer cell lines (Keawpradub et al., 1997), antiplasmodial (Keawpradub et al., 1999).
Aphanamixis polystachya (Meliaceae)	Used for skin diseases (Jamir and Rao, 1982).	Cytotoxic to human colon carcinoma cell lines (Ramachandran et al., 2006), and human breast carcinoma (Rabi et al., 2007).
Artemisia indica Willd. (Asteraceae)	Used for treatment of skin diseases (Deorani and Sharma, 2007).	Antibacterial (Poiata et al., 2009), antioxidant (Temraz and El-Tantawy, 2008), antimalarial (Chanphen et al., 1998), antiplasmodic and bronchodilator (Khan and Gilani Anwarul, 2009) activities.
Bauhinia purpurea (Caesalpiniaceae)	Used for skin diseases (Changkija, 1999).	Antinociceptive, anti-inflammatory and antipyretic (Zakaria et al., 2007).
Bidens Pilosa Linn. Asteraceae	Used to cure leprosy and various skin diseases (Deorani and Sharma, 2007).	Antibacterial, antimalarial (Tobinaga et al., 2009), antidiabetic (Chien et al., 2009), anticancer (Kumari et al., 2009).
Calotropis gigantea (Aselepiadaceae)	Leaf juice used for skin diseases (Jamir and Rao, 1982).	Anti-diarrhoeal (Chitme et al., 2004), anti-inflammatory (Manoranjan Adak and Gupta, 2006).

Carica papaya (Caricaceae)	Latex used for skin diseases and ringworm (Changkija, 1999).	Antibacterial (Emeruwa, 1982), antifungal (Echeverri et al., 1996; Giordani et al., 1996), seeds affect reproductive efficiency on male rats (Ikpeme et al., 2007; Lohiya and Goyal, 1992).
Costus speciosus (Costaceae)	Used for skin diseases (Changkija, 1999; Deorani and Sharma, 2007; Jamir and Rao, 1982).	Antispasmodic, cardiotonic (Bhattacharya et al., 1973), antifertility activity (Tewari et al., 1973), antioxidant and antibacterial (Malabadi, 2005; Vijayalakshmi and Sarada, 2008).
Curculigo capitulata (Amaryllidaceae)	Plant paste used as antiseptic (Changkija, 1999; Jamir and Rao, 1982).	Antibacterial (Choudhury and Shil, 2005).
Diospyros lanceifolia Roxb. (Ebenaceae)	Seeds are used to treat skin diseases (Jamir, 1999).	No biological or phytochemical studies reported.
Holarrhena pubescens (Apocynaceae)	Latex applied for skin diseases and ringworm (Changkija, 1999).	Antibacterial (Chakraborty and Brantner, 1999), hypotensive activity (Siddiqui et al., 1995), antiplasmodial activity (Simonsen et al., 2001).
Hypericum japonicum (Hypericaceae)	Plant paste are used to kill bacteria (Changkija, 1999).	Hepatoprotective and jaundice-relieving effects (Li et al., 2007a; Wang et al., 2008a), antimalarial (Gu et al., 1988), antimicrobial (Ishiguro et al., 1990a; b).
Jatropha curcas (Euphorbiaceae)	Seeds used in skin diseases (Deorani and Sharma, 2007).	Antifungal (Saetae and Suntornsuk, 2010; Wei et al., 2005), antitumour (Lin et al., 2003).
Leonurus japonicus (Lamiaceae)	Used as antibacterial agent (Deorani and Sharma, 2007).	Antibacterial (Ahmed et al., 2006), anti-inflammatory (Islam et al., 2005; Shin et al., 2009), analgesic (Islam et al., 2005).
Ricinus communis (Euphorbiaceae)	Leaf paste is applied on boils and pimples (Changkija, 1999).	Antifungal (Sitton and West, 1975), anti-fertility effect (Makonnen et al., 1999; Sandhyakumary et al., 2003), anti-inflammatory (Ilavarasan et al., 2006).

Rubia cordifolia (Rubiaceae)	Paste made from the plant is applied to ringworm, and other skin diseases (Changkija, 1999).	Antibacterial (Qiao et al., 1990), anti-inflammatory (Kasture et al., 2001), hepatoprotective activity (Babita et al., 2007), anticancer properties (Adwankar and Chitnis, 1982; Adwankar et al., 1980; Son et al., 2008; Takeya et al., 1993).
*Sarcandra glabra** (Chloranthaceae)	Plant paste is used for infected wounds (Changkija, 1999).	Antiviral (Ma et al., 2002), anticancer (Kang et al., 2008).
Wrightia Tinctoria (Apocynaceae)	Bark is used for skin diseases, ringworm and leprosy (Deorani and Sharma, 2007).	Antibacterial (Khyade and Vaikos, 2009; Murugananthan et al., 2008), Anti-inflammatory (Sethuraman et al., 1984), antinociceptive activity (Reddy et al., 2002).
Zanthoxylum armatum DC. (Rutaceae)	Used for skin diseases (Deorani and Sharma, 2007).	Hepatoprotective (Verma and Khosa, 2010), piscicidal (Ramanujam and Ratha, 1983)

*Antibacterial activity was reported after the commencement of this study (Yuan et al., 2008)

3.3 Literature review of selected plants

Overall, 11 plants were selected for antibacterial studies based on firsthand documentation from Chungtia village and Nagaland ethnobotany literature searches. These plants were: *Albizia chinensis*, *Begonia picta*, *Cassia floribunda*, *Cissampelos pareira*, *Diospyros lanceifolia, Erythrina stricta*, *Lasia spinosa*, *Nephrolepis cordifolia, Polygonum hydropiper*, *Prunus persica*, and *Sarcandra glabra.* The literature reviews of each plant are presented below.

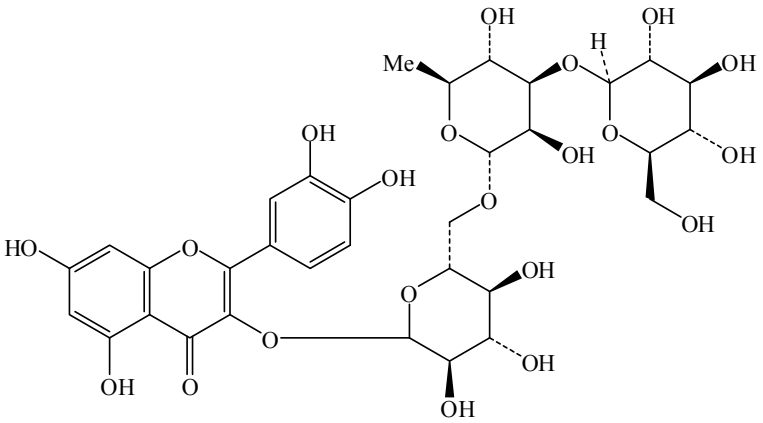
3.3.1 *Albizia chinensis*

Albizia chinensis belongs to the family Mimosaceae. It is a deciduous tree, up to 40 m tall, with dark gray smooth bark. Leaves are bipinnate and flowers are yellowish-white occurring in clusters either in the leaf axils or at the end of the branches. *A. chinensis* is known by the common name silk tree in English and Kala siris in Hindi (India). It is native to India, Myanmar, Thailand, Indonesia, China, Laos Vietnam and Cambodia and occurs commonly in secondary forests of mixed deciduous forest (Faridah Hanum et al., 1997).

Immature plant of *A. chinensis*

Because it is a fast growing tree legume, *A. chinensis* is generally planted for slope stabilisation and soil improvement and is also used as a shade tree in tea and coffee plantations (Faridah Hanum et al., 1997). The leaves are used as fodder for cattle, while the bark, which is rich in saponins, is hardly touched by the cattle (Faridah Hanum et al., 1997). The wood is generally light in weight and thus its uses are limited to house building, light furniture, tea chests and veneers, and in India, additionally for boat building and as firewood (Lalfakzuala et al., 2007). The Naga people use the bark powder to treat helmintic infections and for fish poisoning and also the leaf extract is applied to skin diseases (Deorani and Sharma, 2007). The firsthand information from Chungtia village detailed the use of this plant for fish poisoning, where the leaves or the stem is crushed into the stream. They also use the leaves to assist the ripening process of bananas.

The leaf extracts have been phytochemically studied and the ethanolic extract has led to the isolation of eight known compounds namely: quercetin 3'-*O*-β-D-glucopyranosyl-3-*O*-rutinoside (1), kaempferol 3,7-di-*O*-β-D-glucopyranoside (2), rutin (3), D-pinitol (4), luteolin 7-*O*-β-D-glucopyranoside (5), (+)-lyoniresinol 3α-*O*-β-D-glucopyranoside (6), (-)-lyoniresinol 3α- *O*-β-D-glucopyranoside (7) and syringin (8) (Liu R et al., 2009). The ethanolic stem bark extract has also led to the isolation of three new oleanane-type triterpene saponins, albizosides A-C (9,10, 11), which exhibited cytotoxicity against human tumour cell lines and showed hemolytic activity against rabbit erythrocytes (Liu et al., 2009).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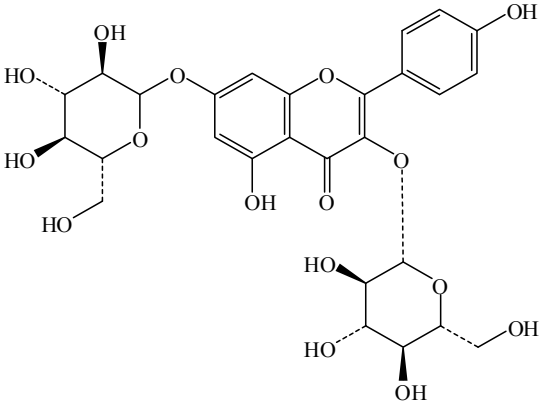
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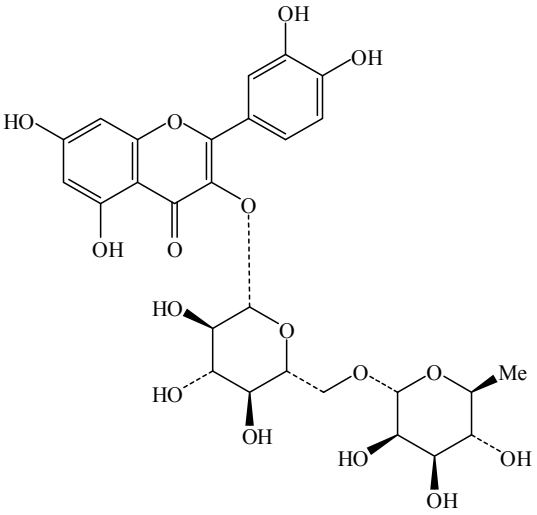
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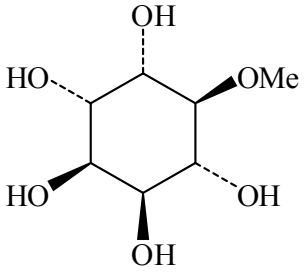
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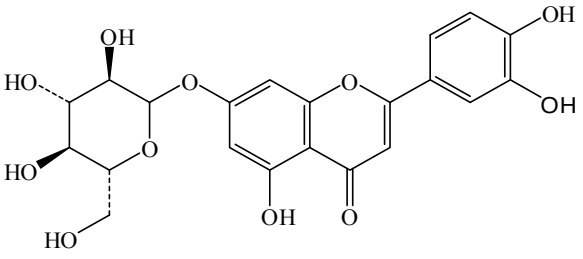
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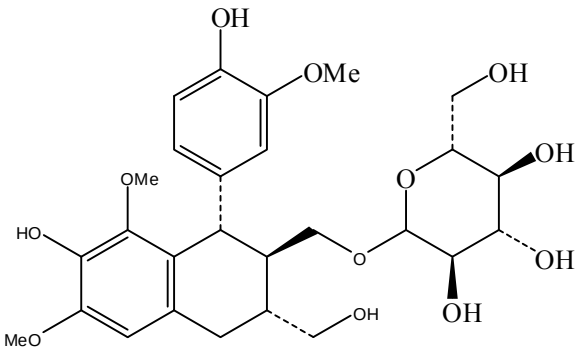
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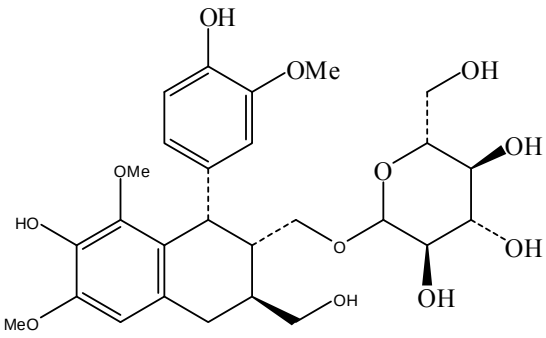
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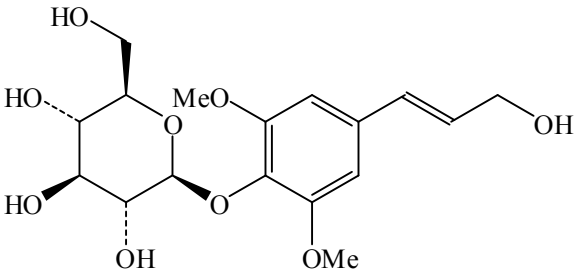
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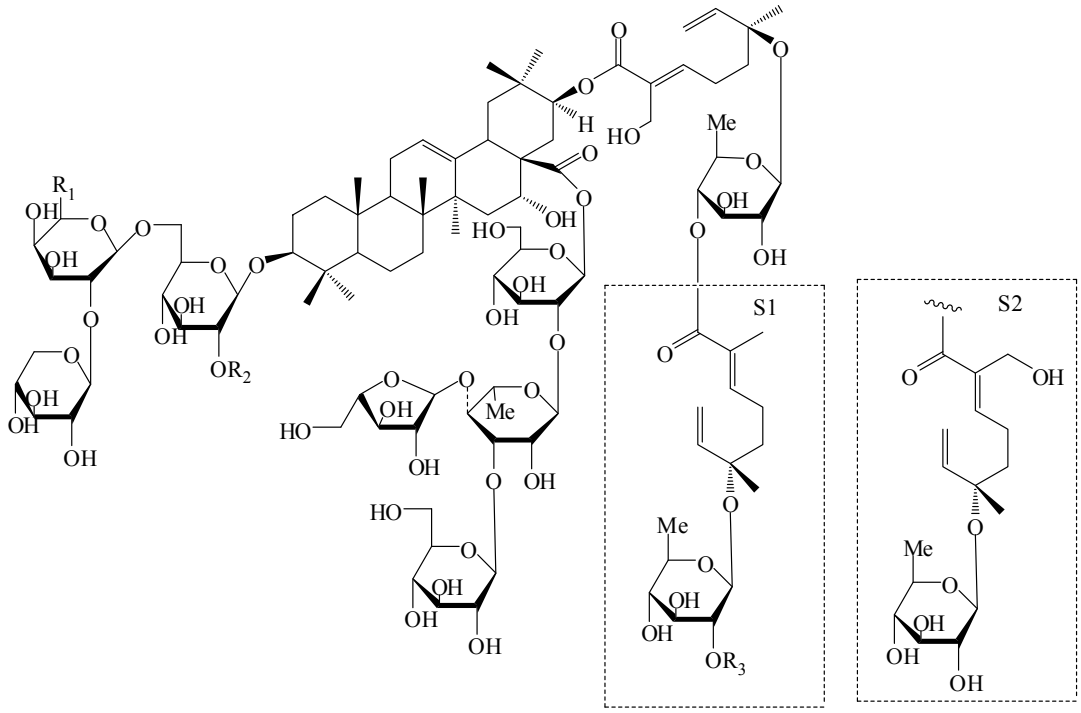


	R_1	R_2	R_3
9	Me	H	S2
10	Me	Glc	S2
11	H	Glc	H

3.3.2 *Cissampelos pareira*

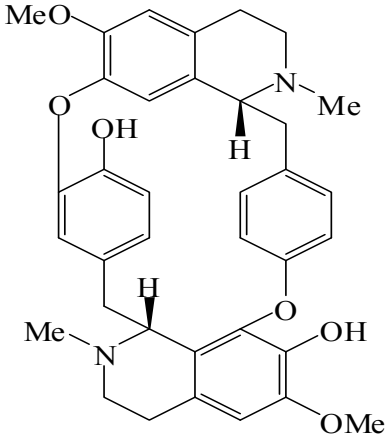
The genus *Cissampelos* (Greek word meaning “ivy-vine”) comprises of about 20 species belonging to the family Menispermaceae (Schmelzer and Gurib-Fakim, 2008). *Cissampelos pareira* is the most important species of the genus *Cissampelos* and has significant mentions of its medicinal uses in most pharmacopeias. *C. pareira* is a woody climbing shrub with round, smooth stems and leaves that when fully grown are smooth above and silky pubescence underneath. It also bears small yellow flowers (Pereira, 1854). It occurs in rainforests, coastal evergreen and deciduous bushland that persist on cleared ground or in secondary vegetation, occurring up to 2300 m in altitude (Schmelzer and Gurib-Fakim, 2008).

C. pareira bearing fruits

The traditional use of *C. pareira* has been reported from many parts of the world. In India, this plant is mentioned in the classical text (referred to as *paatha* in Ayurvedic and Unani, *appatta* in Siddha medical system), where the roots and leaves are used internally for fever, urinary disorders, diarrhoea, piles, indigestion, loss of appetite, persistent nausea, colic pain, intestinal catarrh and vaginal discharges, and a decoction is used externally as a cleansing and antiseptic agent for wounds and ulcers (Amresh et al., 2007d; Khare, 2004). In the Chinese traditional system, it is used externally on wound surfaces to relieve pain for the treatment of traumatic injuries and rheumatitis, and to treat asthma and cardiac diseases (Huang and Williams, 1999). In Africa, an infusion of the bitter rhizome or leaves or stem is used for gastrointestinal complaints, urogenital problems, and a decoction of the above parts are used for skin infections such as sores, boils, scabies and childhood eczema (Schmelzer and Gurib-Fakim, 2008). The Naga people use the juice of the leaves for eye troubles, skin burns and wounds (Deorani and Sharma, 2007), and our firsthand information from Chungtia village recorded the use of this plant to treat high blood pressure, malaria, dysentery, piles, gastrointestinal problems and diabetes.

C. pareira possesses significant biological activities including anti-inflammatory (Amresh et al., 2007c), antioxidant and immunomodulatory (Amresh et al., 2007a; Bafna and Mishra, 2010), antifertility (Ganguly et al., 2007), antinociceptive and antiarthritic (Amresh et al., 2007d), anticancer (Amresh et al., 2007b; Kupchan et al., 1965; Morita et al., 1993b; Morita et al., 1993c),

antidiarrhoea (Amresh et al., 2004), anti-trypanosomiasis (Ramirez et al., 2003), diuretic (Caceres et al., 1987), neuromuscular blocking (Basu, 1970; Kupchan et al., 1960; Mukerji and Bhandari, 1959; Patnaik et al., 1973; Roy et al., 1952), and β-glucosidase inhibition (Sanchez-Medina et al., 2001) properties. This plant is a rich source of alkaloids and the entire plant contains up to 2.5% alkaloids (Kupchan et al., 1965). Several alkaloids have so far been isolated, namely hayatine (12) (Bhattacharji et al., 1955; Mukerji and Bhandari, 1959), hayatinine (13) (Bhattacharji et al., 1962; Mukerji and Bhandari, 1959), *d*-isochondrodendrine (14) (Kupchan et al., 1960; Srivastava and Khare, 1964), cissampareine (15) (Kupchan et al., 1965), protoberberine (16) (Anwer et al., 1968), pareirubrines A (17) and B (18) ((Morita et al., 1993b; Morita et al., 1993c) and pareitropone (19) (Morita et al., 1995), and the majority of these alkaloids possess potent curare-like activity (Basu, 1970; Mukerji and Bhandari, 1959) and antitumour or cytotoxic activities (Kupchan et al., 1965; Morita et al., 1993a; Morita et al., 1993b; Morita et al., 1993c; Morita et al., 1995). A chalcone-flavone dimer, cissampeloflavone (20), has also been isolated from the aerial parts of *C. pareira* and showed good activity against *Trypanosoma cruzi* and *T. brucei rhodesiense* and low toxicity to human KB cell lines (Ramirez et al., 2003). The toxicity of this plant has been investigated and it was reported to be safe when tested for acute and subacute toxicities in experimental animals (Amresh et al., 2008). The antimicrobial nature of the leaf and stem extracts have also been investigated, where the growth of *S. aureus* was inhibited (Sanchez-Medina et al., 2001).



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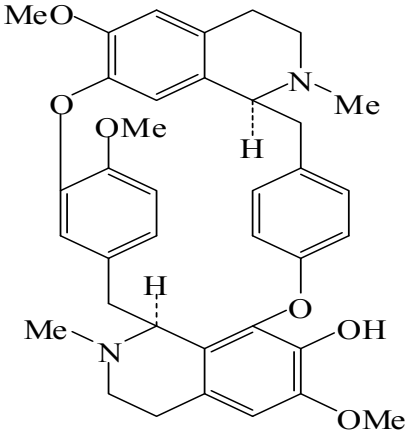

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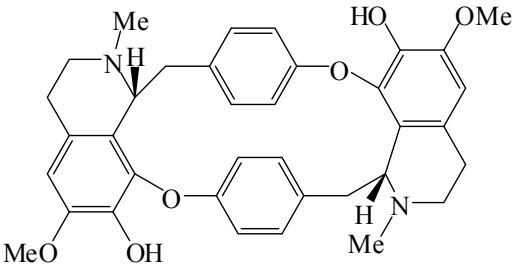

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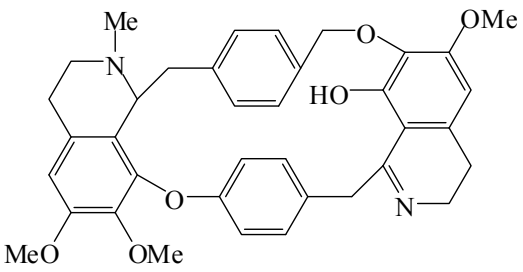

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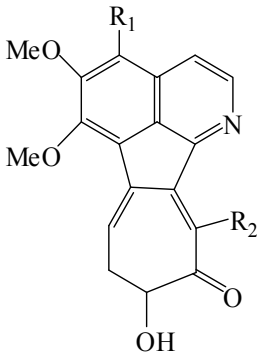
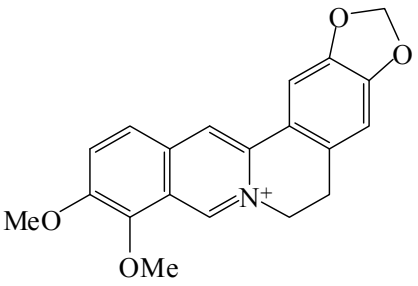


	R_1	R_2
17	OMe	OMe
18	H	H



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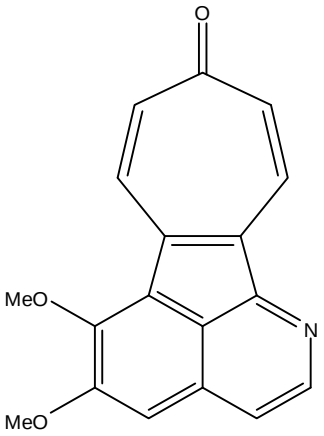

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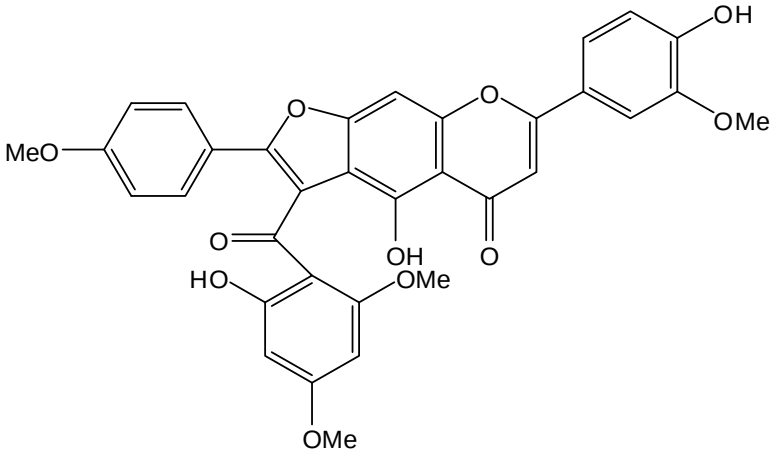

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Cissampelos pareira Linn. was included in the list for antibacterial studies based on the specific requests of the villagers, even though this plant was not recorded for skin related treatments from

Chungtia village. Since the root of this plant is recorded elsewhere to treat sores and boils (Schmelzer and Gurib-Fakim, 2008), which are generally caused by pathogenic microorganisms, the root of this plant was selected for screening of antibacterial activity.

3.3.3 *Begonia picta*

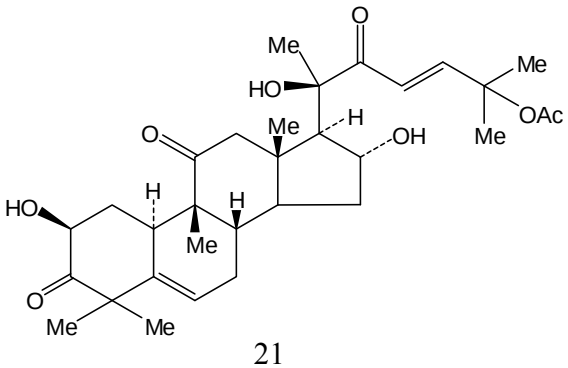
The genus *Begonia* (family Begoniaceae) consists of more than 1400 species distributed widely in the tropical and subtropical regions of the world, with approximately 150 species in Africa, more than 600 species in Central and South America, and more than 600 species in Asia (Gu et al., 2010), including 64 species in India (Hooker, 1879). *Begonia picta* is a deciduous herb with broad ovate-cordate and slightly asymmetric leaves, pink flowers, thick rhizomes and a short reddish stem (sometimes absent) and 3-winged obovoid capsules (one wing more elongated than the others) (Gu et al., 2010). This plant is mainly found in Northern and North-East India, Nepal and Myanmar, where it grows in the forest margins and on rocks in shaded moist environments near streams and slopes (Gu et al., 2010).

B. picta

B. picta has been traditionally used in Nepal and India (Chauhan, 1999; Manandhar and Manandhar, 2002; Shrestha and Dhillion, 2003; Singh et al., 2002). In Nepal, juice is used to treat head aches, peptic ulcers and conjunctivitis, crushed leaves are applied to sore nipples and wounds and sterile animals are fed on this plant to conceive (Manandhar and Manandhar, 2002; Shrestha and Dhillion, 2003). In Northern India, fresh leaves of the plant are used to treat ulcers of the

mouth and tongue (Singh et al., 2002) and leaves are crushed and consumed with cold water to treat pain related to the navel (Chauhan, 1999). In Nagaland, the luke warm juice is used to treat ulcers and bristles of the mouth or stomatitis, juice is drunk to cure diarrhoea or dysentery, leaves are eaten as a vegetable and mature root stalks are used in the making of red dyes with the leaves of *Impatiens sp* (Changkija, 1999; Deorani and Sharma, 2007; Jamir and Rao, 1982). Our firsthand information from Chungtia village recorded the use of this plant as a cleansing agent for hand washing. The Chhattisgarh State Medicinal Plant Board, India, enlisted this species under the List of Endangered Taxa of the State (www.cgvanoushadhi.gov.in).

Biological or phytochemical studies have not been reported for *B. picta*, however some other *Begonia* species have been studied biologically and phytochemically. With regard to biological studies, several species have been reported for their antimicrobial (Frisby et al., 1953; Maridass, 2009; Ramesh et al., 2002), antitumour (Doskotch and Hufford, 1970; Doskotch et al., 1969; Frei et al., 1998c; Wu et al., 2004), anti-HIV (Wu et al., 2004), xanthine oxidase inhibitory (Nguyen et al., 2004) and anti-diabetic (Pandikumar et al., 2009) activities. The most notable compounds to be isolated from this genus are the cucurbitacin based derivatives (cucurbitacin B (21) and D (22)), isolated from *B. tuberhybrida*, *B. heracleifolia*, *B. fimbristipulata* and *B. nantoensis* (Cai and Wang, 1999; Doskotch et al., 1969; Frei et al., 1998c; Wu et al., 2004), which are considered to be one of the bitterest tastes to humans and are potent inhibitors of cancer cells (Blaskovich et al., 2003; Doskotch et al., 1969; Frei et al., 1998b; Wu et al., 2004). Several flavonoids have also been isolated from *B. fagifolia*, *B. erythrophylla* and *B. fimbristipulata* (Cai and Wang, 1999; Ensemeyer and Langhammer, 1984; Vereskovskii et al., 1987a; Vereskovskii et al., 1987b).



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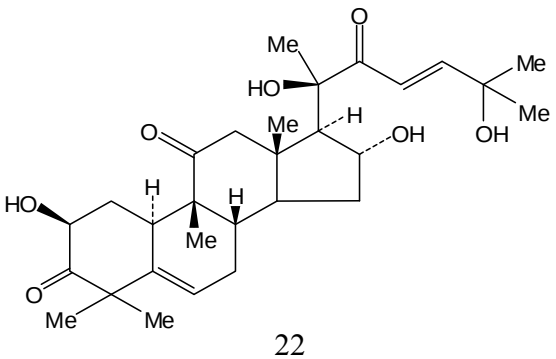

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3.3.4 *Lasia spinosa*

Lasia spinosa is an herbaceous plant belonging to the family Araceae, which has 107 genera. The genus *Lasia* comprises of just two species, namely *L. spinosa* and *L. concinna*. *Lasia spinosa* (synonym: *Dracontium spinosum* L., *Lasia aculeata* Lour., *Lasia crassifolia* Engl., *Lasia descisens* Schott., *Lasia heterophylla* Schott., *Lasia roxburgii* Griff., *Lasia zollingeri* Schott., *Pothos heterophyllus* Roxb.) is a stout marshy plant up to 1.4 m tall with creeping rhizomes and spiny stems. Its juvenile leaves are shaped like an arrow-head and as they mature they become more deeply pinnately lobed. Flowers occur near the end of the plant and the spadix (a type of inflorescence characteristic of the Araceae family) is cylindrical, yellow to pale orange and 3 to 5 cm long (Giesen et al., 2007). This species is mainly found in the tropical forests around the regions of tropical Sikkim Himalayas, Assam, Bengal and Southern parts of India and to Sri Lanka, China, Malaysia, Indonesia, Vietnam and Singapore (Hong Van et al., 2006; Khare, 2008; Lok and Tan, 2008; Seidemann, 2005). *Lasia spinosa* is often confused with *Cryptosperma merkusii* where the only difference lies in the leaves, with the former being deeply divided into 4 to 6 pairs of narrow side-lobes, while the latter is arrow-shaped with prickly nerves (Lok and Tan, 2008).

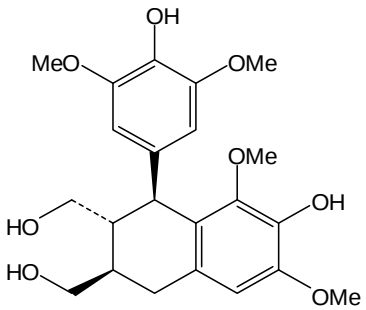
Lasia spinosa

The young and tender leaves and stems are used in curries (Dassanayake and Fosberg, 1988). In Sri Lanka, a salad prepared with fresh rhizomes or rhizomes cooked with coconut milk is given to diabetic patients (Ediriweera and Ratnasooriya, 2009). In India, the root is used as a remedy for throat infections (Nadkarni, 2005), the entire plant is used in colic and intestinal diseases and the

leaves are used for stomach aches (Khare, 2008). The fresh juice of *L. spinosa* is also used by the local population of Jharkhand, India, to induce permanent sterility in women (Faridah Hanum et al., 1997). In Vietnam, the whole plant is used to treat inflammation and rheumatism (Nguyen et al., 2004). In Nagaland, the young tender leaves and stems are eaten as a vegetable. The plant is also used to treat cuts and wounds and is commonly used in the treatment of intestinal worm infections in humans and domesticated animals (Changkija, 1999; Jamir and Rao, 1982). From our firsthand information from Chungtia village, *L. spinosa* was reported for its use as a vermifuge and for treating skin diseases.

Following the ethnobotanical reports of Nagaland, the antihelmintic activity of *L. spinosa* leaf extracts has been investigated against tapeworms and found to be comparable with that of the known antihelmintic praziquantel (Temjenmongla and Yadav, 2006). Based on its ethnomedicinal uses in Vietnam, *L. spinosa* has also been examined for xanthine oxidase inhibition and was found to have weak inhibitory activity (Nguyen et al., 2004). So far biological activity guided isolation of active compounds has not been reported.

From the whole plant several compounds have been isolated, namely lyoniresinol (23), vitexin 2''-*O*-β-D-glucopyranoside (24), *p*-hydroxybenzoic acid, 4-methoxyphenethyl alcohol, *p*-hydroxybenzaldehyde, isovanillic acid, 3'-methyl quercetin-3-*O*-α-L-rhamnopyranosyl-(1→6)β-D-glucopyranoside (25), vitexin and meridinol (26) (Hong Van et al., 2006).



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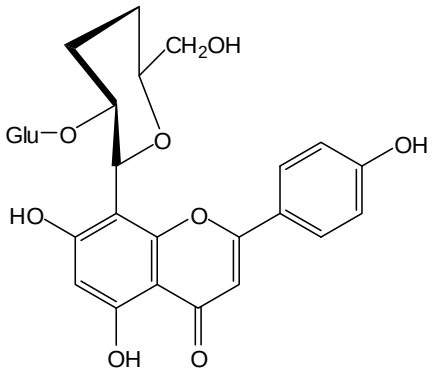

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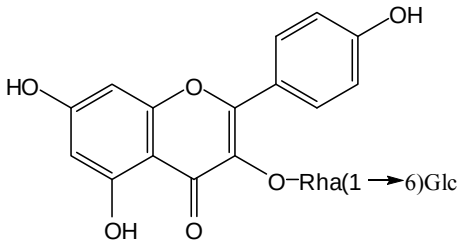

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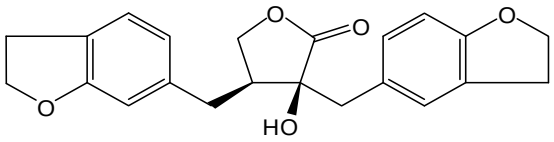

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3.3.5 *Sarcandra glabra*

Sarcandra glabra belongs to the family Chloranthaceae, which is the oldest family in fossil records (Singh, 2004). *S. glabra* is an evergreen sub-shrub that grows to about 50 to 150 cm tall. Its stems are cylindrical, erect, glabrous and it has swollen nodes. The leaves are elliptic or ovate to ovate-lanceolate or broadly elliptic to oblong and they are leathery or papery. The flowers are bisexual and blooms yellowish green. The drupes are green when young and shiny red or yellowish red at maturity and either globose or ovoid (3-4 mm in diameter) (Hu, 1999). *S. glabra* is found in forests, thickets, valleys, ravines, slopes, streamsides, swamps and sandy soil up to 2000 m elevation. This plant is found in China, Cambodia, India, Japan, Korea, Laos, Malaysia, Philippines, Sri Lanka, North Thailand and Vietnam (Hu, 1999).

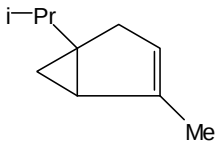
Sarcandra glabra

S. glabra is very popular in Chinese medicine, where it is known by the local name *Jiujiefeng*. It is consumed as an herbal tea or food supplement to enhance mental efficiency and to recover from stress or fatigue (He et al., 2009) and is used as an antibacterial and antitumour agent (Hu et al., 2009; Zhu et al., 2008), and the volatile oil from the plant is used in tooth paste and chewing gum to inhibit oral bacteria (He et al., 2009). In North-East Asia, this plant is used to treat pneumonia,

appendicitis, gastroenteritis, traumatic fractures, rheumatic arthritis, epidemic influenza, encephalitis, dysentery and cancer of the pancreas, stomach, rectum, liver and oesophagus (Kimura et al., 1998). In Nagaland, the plant paste is used to treat dog bites and also for infected wounds inflicted by tigers (Changkija, 1999). Our firsthand information did not document any medicinal use of this plant but generally the villagers used this plant to decorate churches or houses during the Christmas season when the drupes have matured into their beautiful red colour.

Numerous biological activity studies have been carried out on *S. glabra*, mainly in China, and an excellent review on this plant has been published in the Chinese Materia Medica (Chang and But, 2001) with regards to biological activities, clinical studies and toxicity, with information derived from numerous scientific papers and medical reports from Chinese journals, which are not easily available in the West. Here is a summary of the main biological activities from the review. Crude extracts of the herb and the volatile oils have been found to have potent anticancer activities in *in vivo* rat experiments and possess immunosuppressive action against macrophages and T and B lymphocytes. The leaves have been found to have strong antibacterial activity, with the stem and root also having some activity. The herb also exhibited anti-ulcer activity in mice. Clinical studies have shown that the herbal preparation had good antineoplastic activity against carcinomas of the pancreas, stomach, rectum, liver and oesophagus, leukaemia and Hodgkin's disease. Since this review more biological studies has been reported on this plant for antiviral (Ma et al., 2002), hepatoprotective (Li et al., 2006a), anticancer (Kang et al., 2008; Li et al., 2007b; Su et al., 2009), anti-stress (He et al., 2009) and antibacterial activities (Yuan et al., 2008).

S. glabra contains volatile oils (eg origanene (27), germacra-1(10), 4, 7(11)-triene (28)) (Yang et al., 2008), sesquiterpene lactones (eg sarcandralactones A (29) and B (30), (-)-istanbulin A (31), chloranthalactone G (32)) (He et al., 2010; Wang et al., 1988; Tsui and Brown, 1996), sesquiterpene glucosides, (eg sarcaglabosides A (33) and F (34)), (Hu et al., 2009; Li et al., 2006a), dimeric sesquiterpenoids (eg sarcandrolides A (35) and C (36)) (He et al., 2010), triterpenoid saponins (Luo et al., 2005), flavonoid glycosides and chalcones (Huang et al., 2008; Li et al., 2006b; Yuan et al., 2008), caffeic acid derivatives (Li et al., 2009a) and isofraxidin and its derivatives (Huang et al., 2007). The *n*-butanol extract of the plant led to the isolation of seven compounds of which kaempferol-3-*O*-β-D-glucuronide, isofraxidin-7- *O*-β-D-glucopyranoside (37) and kaempferol (38) showed antibacterial activity against *S. aureus* (Yuan et al., 2008).



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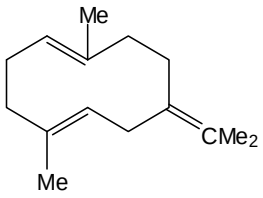

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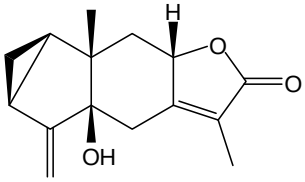

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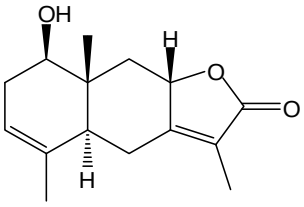

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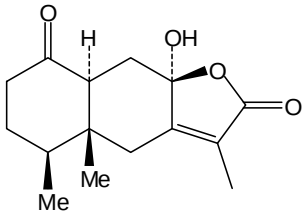

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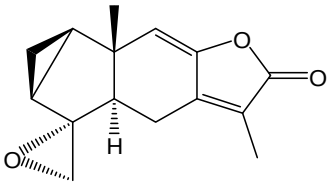

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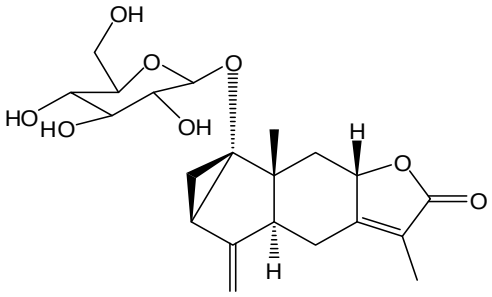

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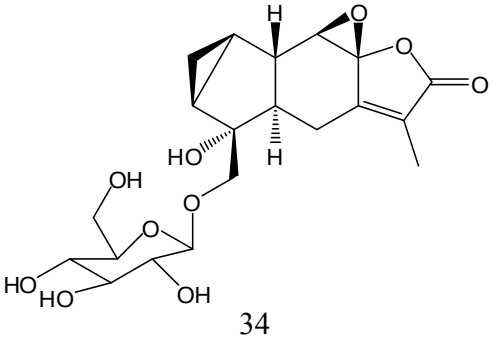

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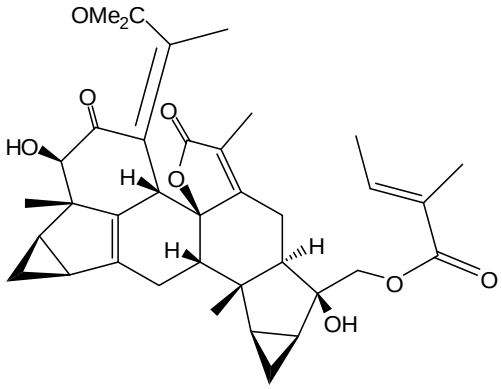

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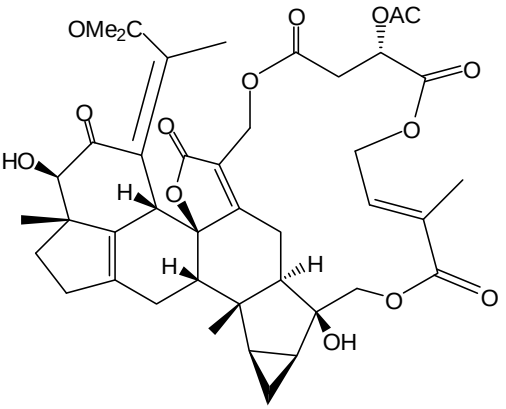

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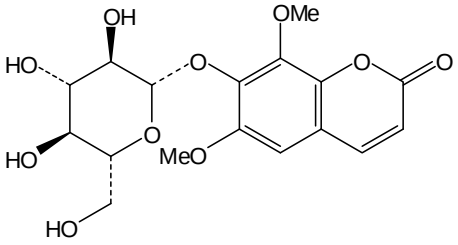

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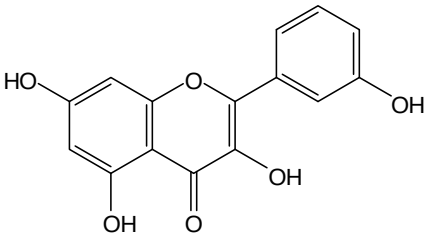

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3.3.6 *Polygonum hydropiper*

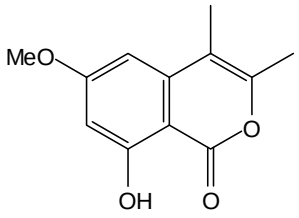
Polygonum hydropiper belongs to the family Polygonaceae. It is commonly known as water-pepper or smartweed because of its habitats and peppery taste experienced on chewing the leaves. The stems are erect or assurgent, often purple-red, branched or unbranched, glabrous and up to 55 cm tall. The leaves are ovate-lanceolate to narrowly lanceolate, 2-9 cm long, acute or acuminate at the apex and narrow at the base and fringed with short bristles. They are very acrid and peppery. The flowers are greenish and often tinged with purple and bordered with white (Abrams and Ferris, 1990; Mohlenbrock and Thomson, 2009). *P. hydropiper* is native to Southeast Asia, Europe and North America and grows as a weed in moist or wet grounds (Mohlenbrock and Thomson, 2009; Peng et al., 2003).

Polygonum hydropiper

P. hydropiper has been used traditionally as a food spice in Japan, China and Malaysia (Fukuyama et al., 1982; Ono et al., 1998; Peng et al., 2003). It is used medicinally to treat various ailments such as head ache, pain, tooth ache, liver enlargement, gastric ulcers, dysentery, loss of appetite, wounds, skin diseases and carbuncles (Rahman et al., 2002). In China, it is also commonly prescribed to combat indigestion, dysentery and enteritis (Huang and Williams, 1999). The historical use of this plant by the Indian tribes of North America has been systematically documented for its uses in curing wounds and sores, head pain and rheumatism (Erichsen-Brown, 1989).

The biological activity and phytochemistry of *P. hydropiper* has been well studied. Extracts of the whole plant have been shown to have antinociceptive activity (Rahman et al., 2002), while the roots have encouraging antifertility activity and leaf and root extracts have nematostatic properties (Sukul, 1970). The leaves have also been found to possess strong antioxidant activity attributed to their flavonoids (Haraguchi et al., 1992; Peng et al., 2003; Yagi et al., 1994) and phenylpropanoid esters (Kiem et al., 2008a). Root extracts have yielded polygonolide (39), which possesses anti-inflammatory activity (Furuta et al., 1986). Leaf extracts have led to the isolation of warburganal (40), which exhibits potent cytotoxic, antifeedent, antibiotic and molluscicidal activities (Fukuyama et al., 1982), and polygodial (41), which is responsible for the pungency of this plant (Barnes and Loder, 1962; Ohsuka, 1962). The leaf essential oils contain confertifolin (42), which has antimicrobial activity with significant activity towards fungi (Duraipandiyan et al., 2010a). Sprouts of this plant have also been found to contain taxifolin, a tyrosinase inhibitor. Other

compounds present in the plant include norsesquiterpene aldehyde, sesquiterpene hemiacetal (Asakawa and Takemoto, 1979), drimane-type sesquiterpenes (Fukuyama et al., 1985), hydropiperoside (43) (Fukuyama et al., 1983) and roseoside (44) (Murai et al., 2001).



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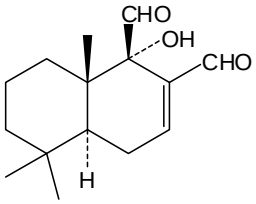

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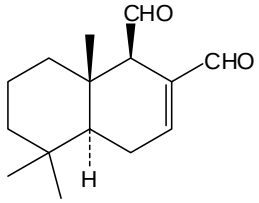

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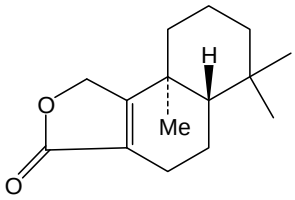

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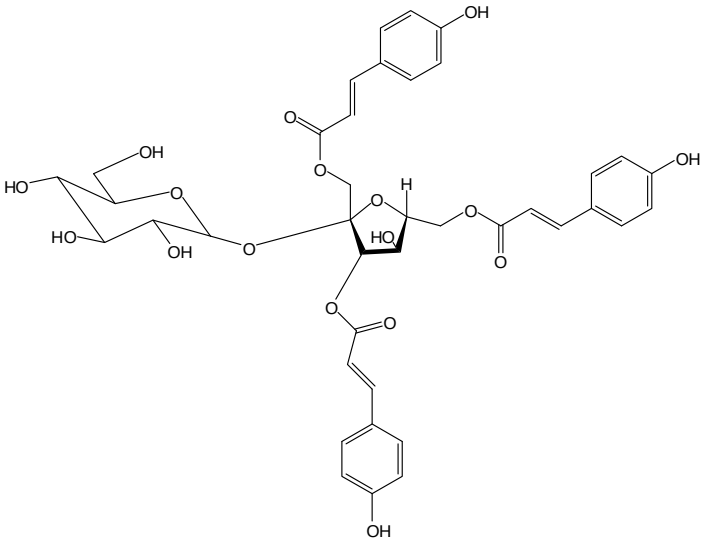

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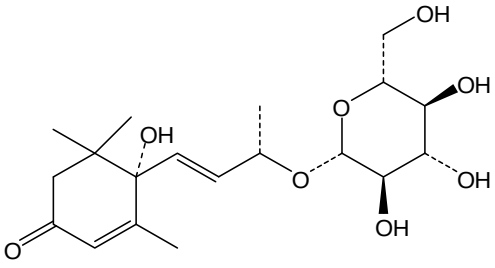

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3.3.7 *Cassia floribunda*

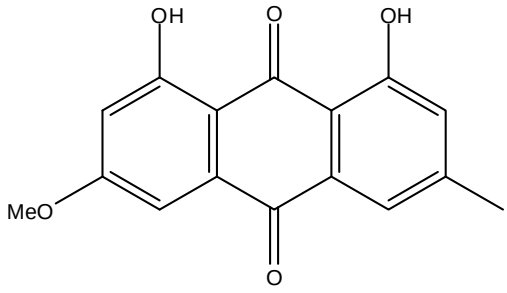
Cassia floribunda belongs to the family Leguminosae. It is a shrub that grows up to 3 m tall. The leaves are stalked and pinnate and contain 3-5 pairs of leaflets. The leaflets are elliptic to lanceolate, acuminate, entire, with glabrous glands on rachis between the leaflets. The flowers are yellow in colour and the pods (fruit) are cylindrical (6-10 cm x 8-15 mm) and the seeds ovoid (6-8 mm in diameter) and shiny olive green (Faridah Hanum et al., 1997; Manandhar and Manandhar, 2002). The flowers bloom in June-July and fruits in August-November (Manandhar and Manandhar, 2002). *C. floribunda* is found on roadsides, cultivated fields, forest edges and plantations from 900-2500 m altitude (Faridah Hanum et al., 1997). It is mainly distributed in South and Central America (possibly originating from Mexico), Nepal, India, Indonesia, and is widespread in the tropics (Faridah Hanum et al., 1997).

Cassia floribunda

In Nepal, the tender fruits are cooked as a vegetable, a paste is made of seeds and applied topically to treat scabies, and seeds are roasted and chewed to treat coughs (Manandhar and Manandhar, 2002). The tree is often used as a shade plant for coffee plantations and in Guatemala the seeds are used as a coffee substitute. The young leaves are eaten as a vegetable in Sumatra (Faridah Hanum et al., 1997), and in India, its tender shoots and pods are cooked as vegetables by the North-West Himalayan tribals, Gujjars and Gaddies (Singh, 1996). The plant is poisonous to livestock, causing haemorrhagic gastroenteritis and lungs, kidney and liver congestion (Bizimana, 1994; Neuwinger,

1996). The Naga tribes use the leaf paste to treat ringworms (Deorani and Sharma, 2007), and from our firsthand information, it was documented that a leaf poultice can be applied externally to the affected areas for treating fungal infections (ringworms), eczema, contact dermatitis, allergic reactions, prickly heat and burns. It was also noted that it should not be internally consumed because of its poisonous nature.

Two new bianthraquinones, physcion (45) and spermidine alkaloid (46) have been isolated (Alemayehu et al., 1988) from the leaves of *C. floribunda*. Only limited biological and phytochemical studies have been conducted on this plant, but the genus *Cassia* has been extensively studied. A review of *Cassia sp* traditionally used in Africa is presented in the book 'African Ethnobotany: Poisons and Drugs' (Neuwinger, 1996). Anthraquinones (eg alatinone (47)), (Hemlata and Kalidhar, 1993; Kazmi et al., 1994; Singh and Singh, 1987; Singh et al., 1980; Sob et al., 2010), alkaloids, such as cassiadinine (48), cassine (49), carnavaline (50) (Biswas and Mallik, 1986; Bolzani et al., 1995; Christofidis et al., 1977; Lythgoe and Vernengo, 1967), and flavanoids (El-Sayed et al., 1992; Gupta and Singh, 1991; Hatano et al., 1999; Tiwari and Bajpai, 1977; Tiwari and Singh, 1977) are the major constituent of this genus and several of its species including *C. alata* (Ibrahim and Osman, 1995; Khan et al., 2001; Sangetha et al., 2009; Somchit et al., 2003), *C fistula* (Duraipandiyan and Ignacimuthu, 2007) and *C. singueana* (Endo and Naoki, 1980) have antimicrobial activity.



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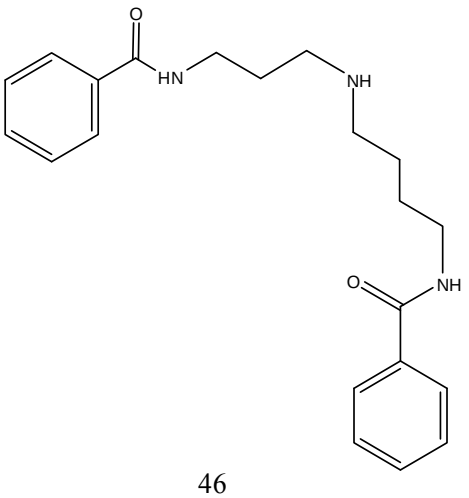

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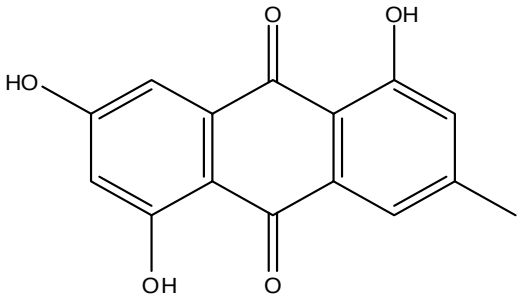

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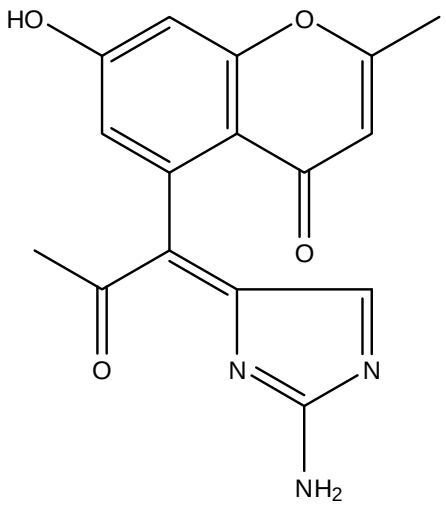

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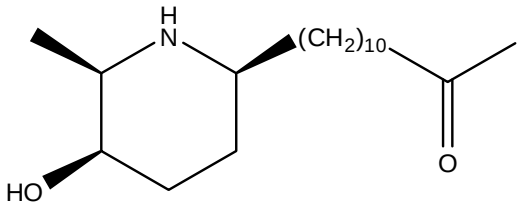

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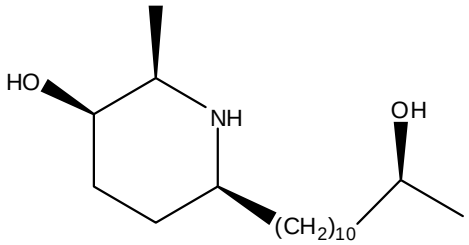

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3.3.8 *Prunus persica*

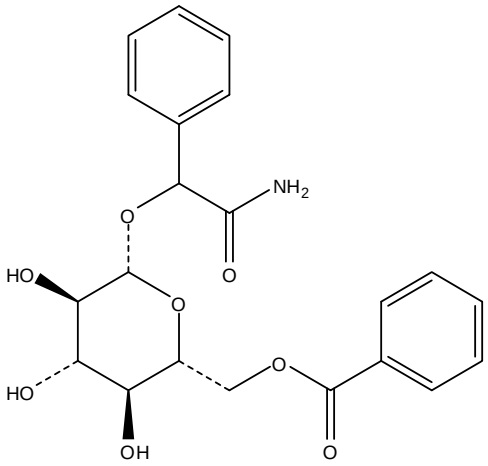
Prunus persica (Peach) belongs to the Rosaceae family. Peaches were among the first crops to be domesticated in China about 4000 years ago. Peaches were moved on to Persia (Iran) along silk trading routes. The epithet *Persica* denotes Persia, which is where Europeans thought peaches originated. *P. persica* is a shrub or a tree and grows to a height of about 1.8 to 3 m in cultivation. The leaves are linear, with acute tips and finely serrated margins. They are sickle shaped and 5 to 15 cm in length. The flowers are light pink to carmine to purplish in colour and 2.5 to 4 cm in diameter, and are borne singly in short peduncles from lateral buds with usually one to two flower buds per node. The peach fruit is a drupe, variable in size and shape and the bony endocarp surrounds a single, large, ovate seed (Rieger, 2006).

Prunus persica

In China, *P. persica* is used traditionally for general disturbances of the digestive tract and is also a mild helpful agent in constipation (Li, 2003). The seeds are used in traditional Chinese herbal drugs for the treatment of gynaecological disorders such as hypermenorrhea, dysmenorrhoea and infertility (Khare, 2008). In North-East Asia, the seeds are used for blood circulation disorders, mass formation in the abdomen, constipation, traumatic injury and contusions, the leaves are used to treat malaria, hemorrhoid, eczema, vaginal trichomoniasis and dermatitis, and the flowers are used to treat edema, ascites and constipation (Kimura et al., 1998). The tea made from the leaves also helps to ease kidney ailments and urinary infections and to clear the body of toxins, especially after a debilitating illness (Roberts, 2000). In Nagaland, the leaves are used as a laxative and antihelmintic and an infusion of the leaves or the bark is given for whooping cough (Deorani and Sharma, 2007). From our firsthand information from Chungtia village, an infusion of the root is taken for typhoid infection, the seeds are consumed to treat dysentery/diarrhoea, and the juice of the leaves or root extracts is applied to treat skin related infections.

Extracts of the flesh of the fruit of *P. persica* have been found to have potent antitumour activity (Chang et al., 2009), while extracts of the flowers protect the skin from UVB-induced DNA damage (Lee et al., 2002) and have potent anti-cholineesterase activity (Suh et al., 2006). The seeds contain the alkaloid persicaside (51), which selectively inhibits cyclooxygenase-2 (COX-2) over COX-1 enzyme activity (Rho et al., 2007). The seeds also contain antitumour compounds including the cyanogenic glycosides amygdalin (52) and prunasin (53), and mandelic acid *β*-D-glucopyranoside, benzyl *β*-gentiobioside and benzyl *β* -D-glucopyranoside (Fukuda et al., 2003).

Several antifungal compounds have been isolated from *P. persica* including 4-naphthylnaphthoquinone from the leaves (Mishra et al., 1993) and seven triterpenoid phytoalexins including 1β,2α,3α,24-tetrahydroxyurs-12-en-28-oic acid (54), 1β,2α,3α,24-tetrahydroxyolean-12-en-28-oic acid (55), from the peel of unripe fruits (El Lahlou et al., 1999). Several compounds including hexanal, *trans*-2-hexenal, *trans*-3-hexen-1-ol, benzaldehyde, nonanal, methyl salicylate, eugenol, 1,2-dihydro-1,1,6-trimethylnaphthalene, 1,2,3,4-tetrahyrdo-1,1,6-trimethylnaphthalene (Kemp et al., 1971) and persicaxanthin (56) (Molnár et al., 1987) have been reported from the fruits, and cyclododecyne (57), henicosane, dotriacontane, tritetracontane, 1-dotriacontanol, vitamin E, β-sitosterol, squalene, kaempferol and quercetin from the leaves (Lin and Lin, 1992).



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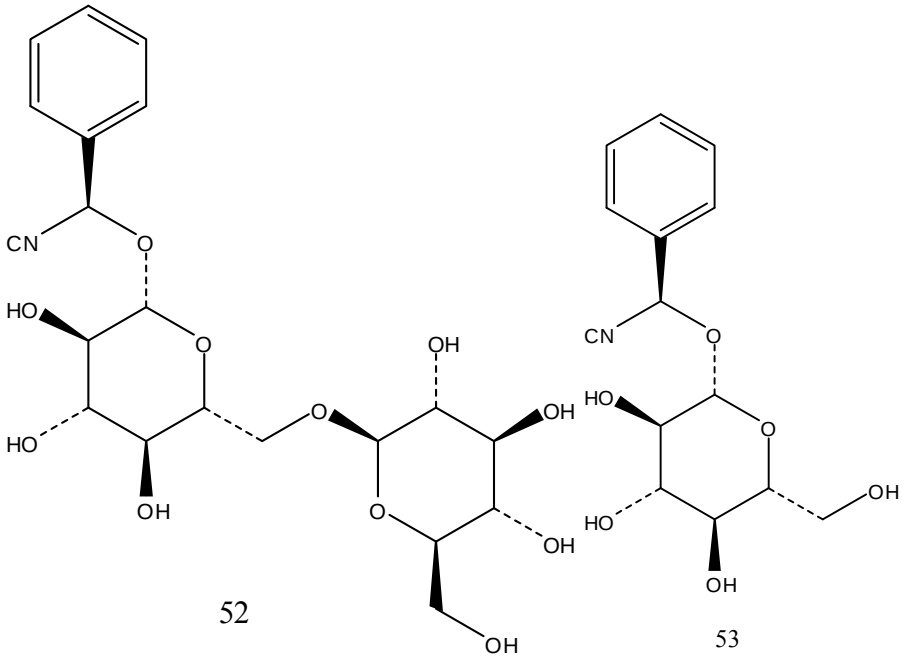

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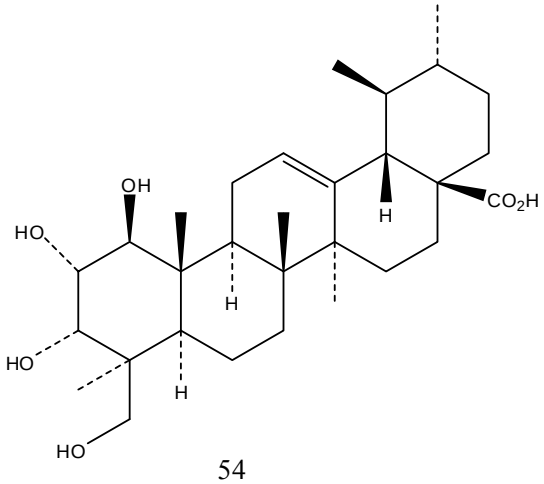

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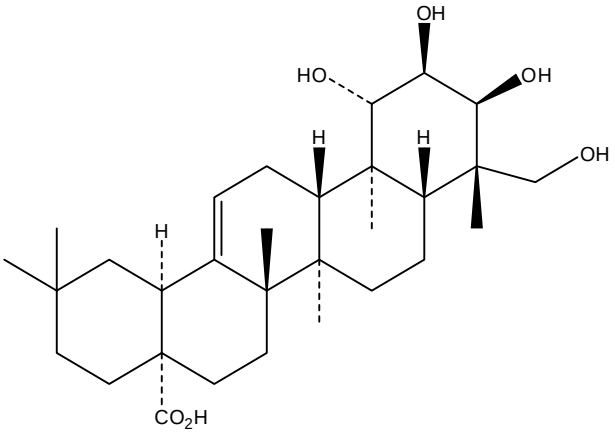

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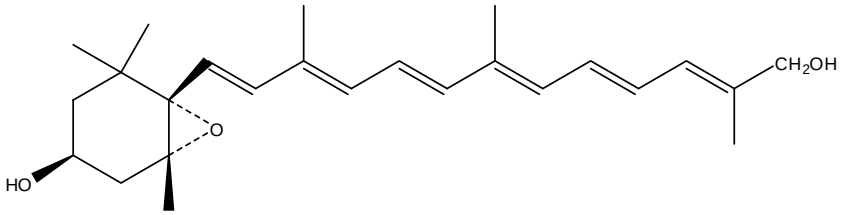

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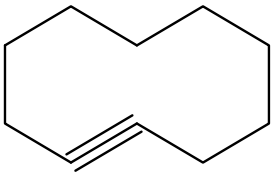

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3.3.9 *Nephrolepis cordifolia*

Nephrolepis cordifolia is a popular garden plant that grows well in many climates, particularly in subtropical and tropical climates, and is found predominantly in scrubs and wetlands. This plant is native to Australia and the Himalayan mountains. It grows on the ground as well as on trees (epiphytic) and rocks (epilithic). It is a common weed that has been introduced to many parts of the world and is referred to by many names such as Boston fern, erect sword fern, fish-bone fern, southern sword fern, tube ladder fern and tuber sword fern. This weed is also invasive and prevents the regeneration of other native plants (Hoshizaki and Moran, 2001).

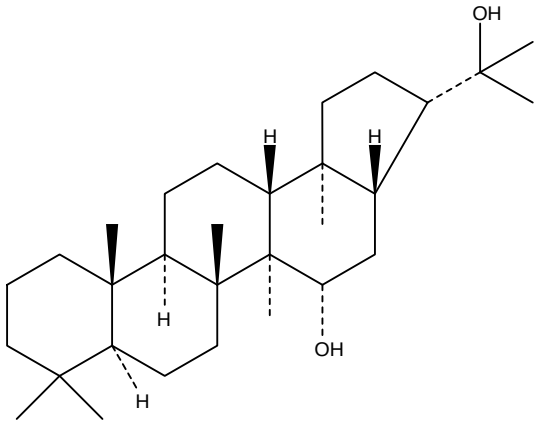
Nephrolepis cordifolia

N. cordifolia is classified under the fern family Davalliaceae and belongs to the genus *Nephrolepis*, which contains about 30 species. *N. cordifolia* (synonym *Nephrolepsis tuberosa*) is characterised by the presence of orange-brown to pale-brown tubers, fronds to 1 m long and 7 cm wide, petioles to 20 cm long with pale brown scales, and indusia ranging in shape from kidney-to cresent-shaped to rounded-triangular (Langeland, 2009). In many instances, it is often confused with the species *N. exaltata*, which never bears tubers and has more sharply pointed leaflet tips than *N. cordifolia*. The fleshy tubers are developed from the thick rhizomes during the mid-summer and are about 0.5 to 1.5 cm in diameter. Their main purpose is for storing water (Harshberger, 1908). This fern is also very common in the jungles of Nagaland where it abundantly grows as an epiphytic plant, particularly on the palm tree *Livistona jenkinsiana* Griff. (Local name: *Sura*). In Chungtia village, it is known by the local name *sura enjen* (enjen = fern).

N. cordifolia is used as a source of food and medicine in many parts of the world (Manandhar and Manandhar, 2002; Misra and Dutta, 2003; Nwosu, 2002; Rao et al., 2007). In Nepal, the tubers are eaten to quench thirst and the juice from tubers are used to treat fever, indigestion, headache and haematuria (presence of blood in the urine) (Manandhar and Manandhar, 2002). The tuber content has been studied and it contains 93.5% w/w water (Ducloux and Awshalom, 1924). In some parts of India the tubers are eaten as food and also taken to treat cough and skin diseases (Misra and Dutta, 2003; Rao et al., 2007). In Southern Nigeria, the tubers are boiled with salt and water and eaten as food, and the infusion of fronds is administrated to the elderly for treatment of amnesia

(Nwosu, 2002). In Chungtia village, the uses of this plant are limited to the tubers, which are used for various ailments including sinusitis, urinary problems and skin diseases.

Essential oils have also been identified from *N. cordifolia*, with the main components being hexadecanoic acids and linoleic acids (Wang et al., 2004). Dustanin (58), fern-9(11)-ene (59), β-sitosterol (60), β-sitosterol-β-D-glucoside, oleanolic acid, myristic acid octadecylesters, hentriacontanoic acid and triacontanol have also been isolated from the leaves and stem (Banerjee et al., 1988; Liang et al., 2008). (22*S*)-Hopan-30-oic acid and tuberosic acid (61), a new pentacyclic triterpene, have been isolated from the Tubers (Dutta et al., 1993). To date, no biological activity studies have been reported for this plant.



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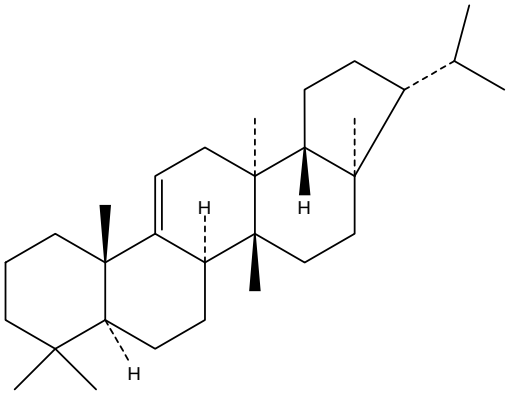

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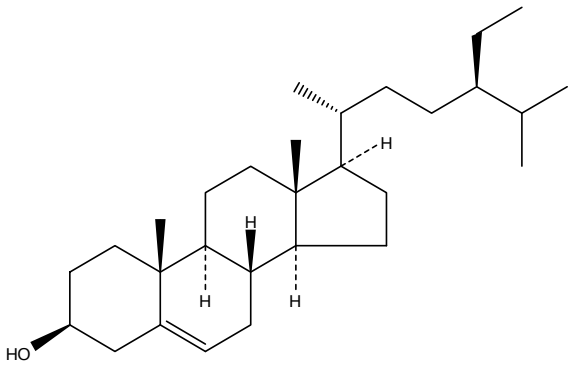

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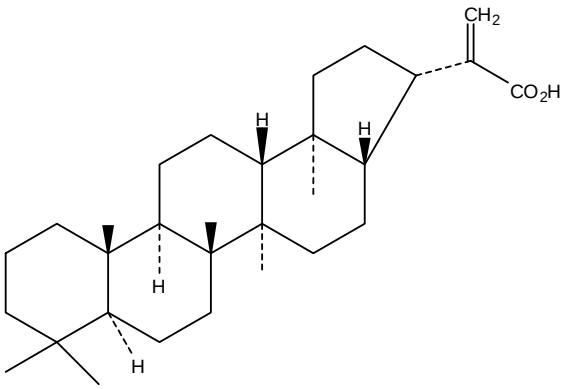

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3.3.10 *Diospyros lanceifolia*

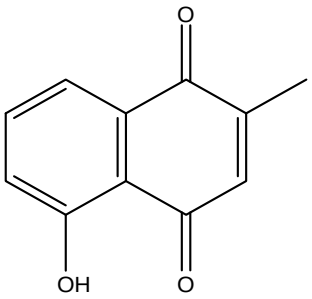
Diospyros lanceifolia belongs to the genus *Diospyros*, under the family Ebenaceae. The genus *Diospyros* has approximately 350 species. Many of these are of economic importance due to their edible fruits (*e.g.* persimmon, *D. kaki*), ebony (*D. ebenum*) and valuable timbers (Mallavadhani et al., 1998). In India, the leaves of *D. melanoxylon* are highly esteemed for wrapping *bidis* (cigarettes) (Mallavadhani et al., 1998). *D. lanceifolia* is a large tree that grows up to 20 m. It is found in the lowland and hill rainforests of India, Indonesia, the Philippines, Singapore and Malaysia (Wiart, 2006). The leaves are simple, oblong-elliptical to lanceolate with a base-pointed apex acuminate. The bark is brown to black, smooth, or with fine cracks and the inner bark is bright yellow. The male flowers are sessile, very small and salver-shaped and the female flowers are solitary, small, sub-sessile and urseolate. The fruits are ovoid or globose with a short apical beak. They are approximately 2 cm in diameter and seated on a shallow 3-5 lobed calyx (Hooker, 1879; Wiart, 2006).

Diospyros lanceifolia

The genus *Diospyros* is particularly used as fish poisons in many parts of Southeast Asia (Wiart, 2006). In Indonesia, the seeds of *D. lanceifolia* are used as a fish poison (Wiart, 2006). In Nagaland both the fruits and the seeds are used to poison fish (Changkija, 1999; Jamir, 1999) and the seeds are used for skin diseases (Jamir, 1999). The wood is also used in crafts and construction

by the Naga people and the young fruits and seeds are eaten (Changkija, 1999). In Chungtia village, the roots and seeds are used as a fish poison.

Several species of the genus *Diospyros* have been studied, leading to interesting biological and pharmacological activities, and of the 350 plants in this genus, more than 130 species have so far been screened phytochemically (Mallavadhani et al., 1998). Some of the recent reported pharmacological activities are the antidiabetic and antioxidant activities of *D. peregrine* fruit (Dewanjee et al., 2009), antioxidant and antiproliferative activities of *D. lotus* (Loizzo et al., 2009) and the neuroprotective effects of *D. kaki* leaves (Bei et al., 2009). The genus *Diospyros* is a rich source of 1,4-naphthquinones and about 75% of the phytochemical reports on *Diospyros* are on the detection and isolation of these 1,4-naphthquinones, which include several monomers, dimers, a few trimers and tetramers (Mallavadhani et al., 1998). Plumbagin (62) and 7-methyljuglone (63) are the most widely distributed monomeric naphthquinones that are found to accumulate in significant quantities and they are mainly responsible for the broad spectrum biological activities of this genus. Other than the naphthoquinones (8-hydroxy-1,2-naphthoquinone (64), ismailin (65), diosindigo B (66), chromenone acid (67), pentacyclic quinine (68), batacanone (69), xylospyrin (70), bis-isodiospyrin (71)), terpenoids (lupine (72), ursane (73), oleanane (74), friedelane (75)), steroids (campestrol (76), stigmasterol (77)), benzopyrones (scopoletin (78), ellagic acid (79)), naphthalene-based aromatics (3-methyl-naphthalene 1,8-diol (80), diospyriol (81)) hydrocarbons and lipids have been reported from this genus (Mallavadhani et al., 1998). Despite the extensive studies on this genus, there are no reports on biological or phytochemical studies of *D. Lanceifolia*.



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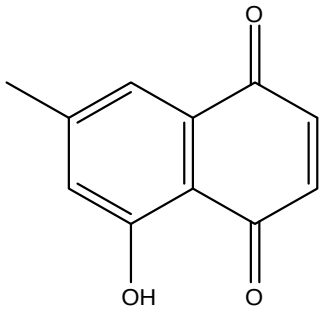

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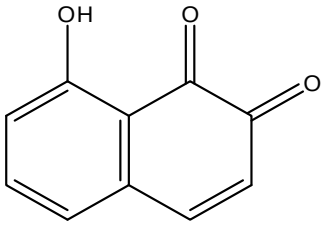

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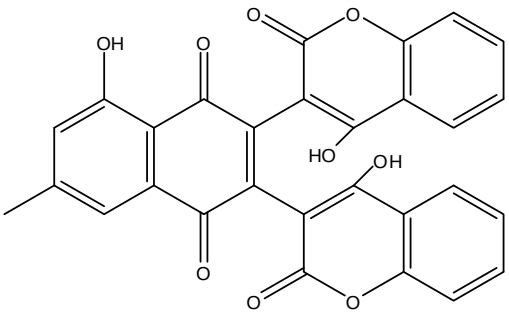

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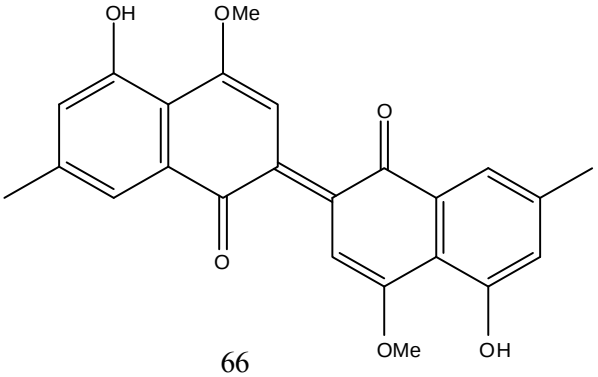

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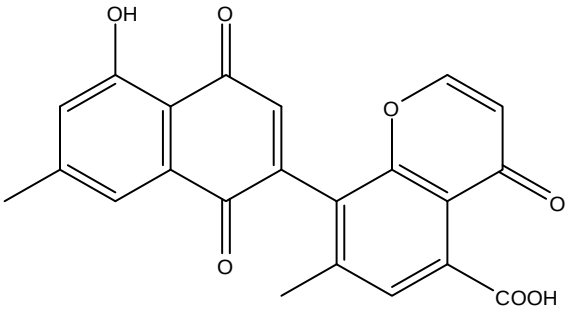

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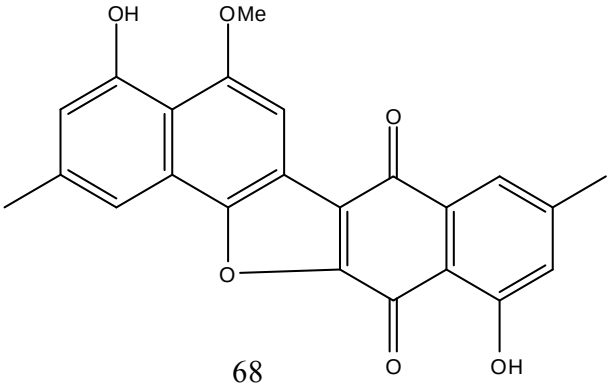

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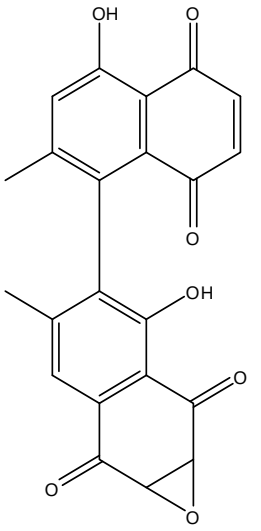

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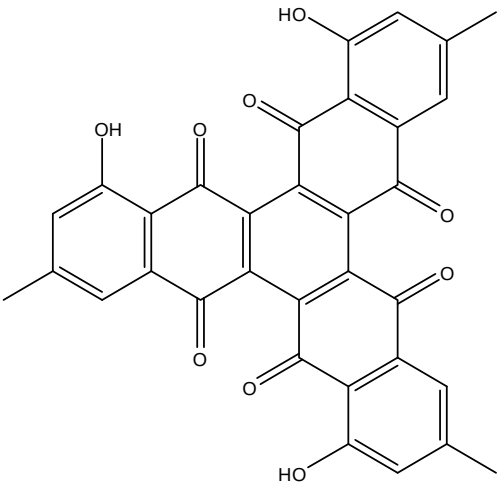

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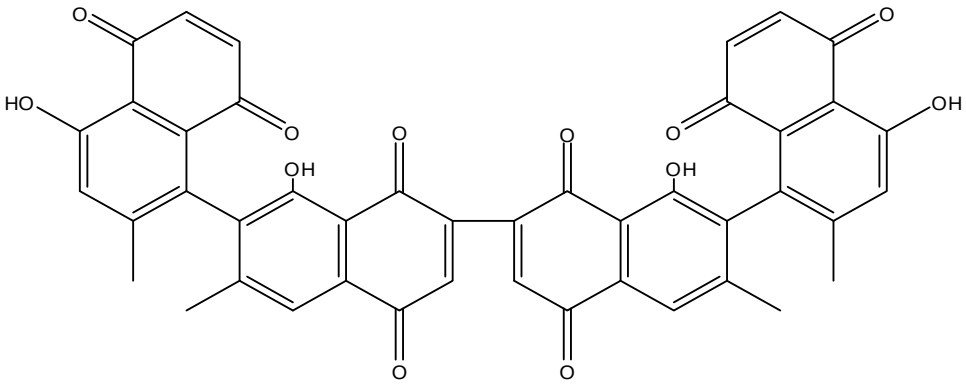

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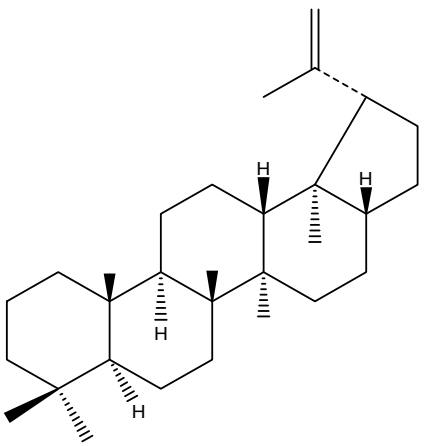

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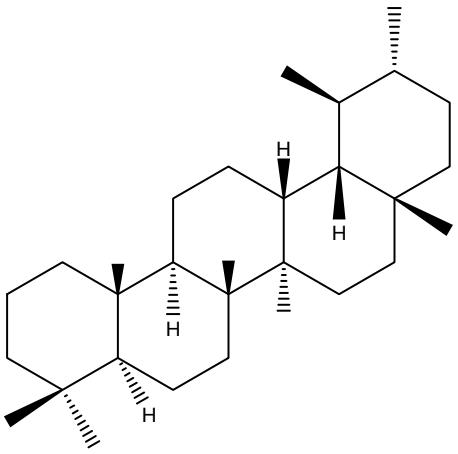

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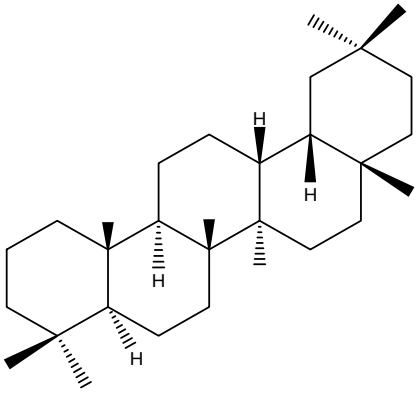

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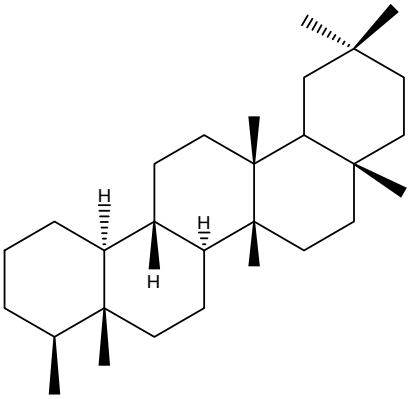

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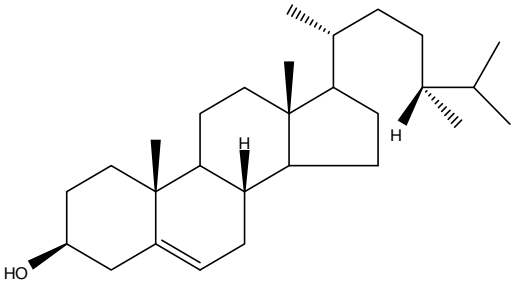

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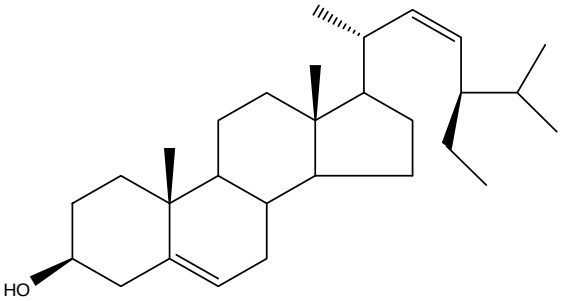

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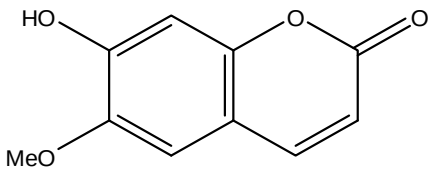

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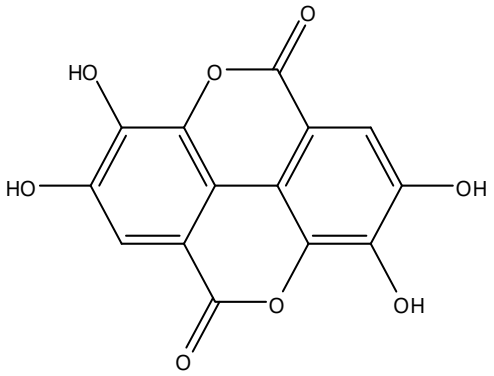

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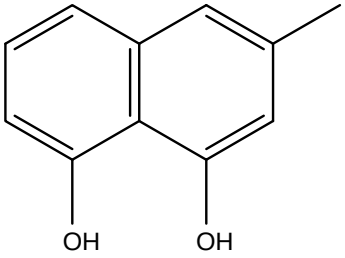

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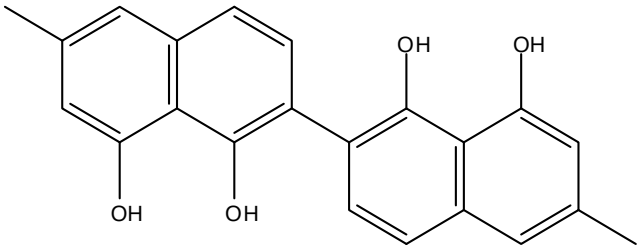

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3.3.11 *Erythrina stricta*

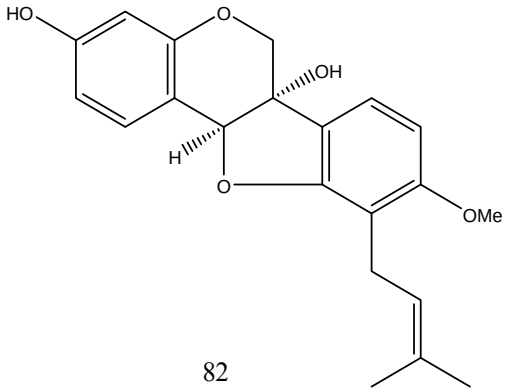
Erythrina stricta belongs to the family Leguminosae. The genus *Erythrina* comprises more than 100 species occurring in tropical and subtropical regions worldwide. In Nagaland, 4 species of Erythrina, *i.e. E. arborescnes*, *E. crista-galli*, *E. stricta* and *E. variegata*, have been documented based on their ethnobotanical uses (Deorani and Sharma, 2007). *E. stricta* is a large tree with light grey bark and whitish prickles. The leaves are stalked, trifoliolate and the leaflets are stalked, broadly ovate, shiny on the upper side, 5 to 13 cm long and 3 to12 cm wide. The flowers appear in dense spike-like clusters in terminal racemes and the pods are 10 to 12 cm long, spindle shaped and contain red kidney shaped seeds. *E. stricta* is distributed throughout Nepal to about 1600 m and is common in cultivated areas in India, Eastern Tibet and China (Manandhar and Manandhar, 2002).

Erythrina stricta

E. stricta has been reported for the treatment of joint pain, ear ache, eye infection and tooth ache where the leaves are used, and the bark powder is also used in rheumatism, epilepsy and asthma (Sivarajan and Balachandran, 1994). In Nepal, the juice of the bark is applied to treat skin diseases (Manandhar and Manandhar, 2002). In Nagaland, the flowers are used as a tonic and a decoction of the bark is used for skin diseases (Deorani and Sharma, 2007). From our ethnobotanical survey of Chungtia village, this plant has been documented for its uses in treating various skin related symptoms such as contact dermatitis (caused by touching the plant of *Drimycarpus racemosus* or *Rhus griffithii*), eczema, and any form of skin infection, where a paste of the stem bark is applied topically or as an infusion.

Ethnomedicinal-directed biological studies on the use of *E. stricta* for rheumatism have shown that the leaf extracts have significant xanthine oxidase inhibitory activity (Umamaheswari et al., 2009). Several biologically active compounds have been isolated from the roots, including erythrabissin I (82), erythrabyssin II (83), erystagallin A (84) and 5-hydroxysophoranone (85), which showed strong antiplasmodial, antimycobacterial and cytotoxic activities (Rukachaisirikul et al., 2007b). A new alkaloid, 11-acetylerysotrine (86), has been isolated from the flowers (Hussain, 2002) and the alkaloids erythraline (87), erythrinine (88), erysodine (89), erysopine (90), 11-hydroxyerysodine and 11-hydroxyerysovine have been isolated from the seeds (Games et al., 1974). 7-Methoxy-8-(15-hydroxypentadecyl)-coumarin (93), the alkaloids erysovine (91), erysodine and hypaphorine

(92), alkyl ferulates, sitosterol and stigmasterol have been isolated from the stem bark (Singh et al., 1981).



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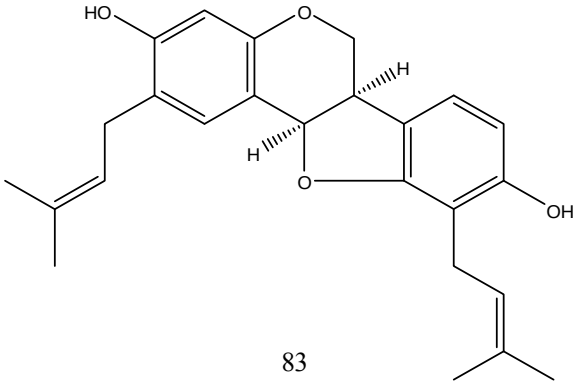

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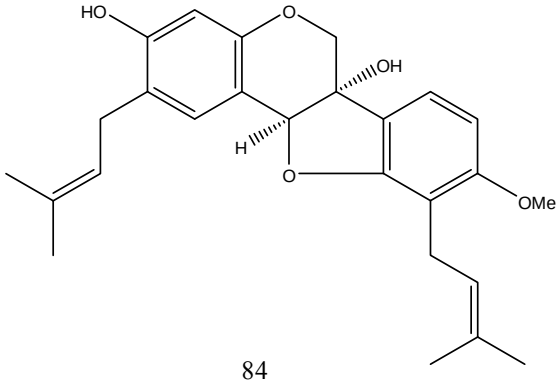

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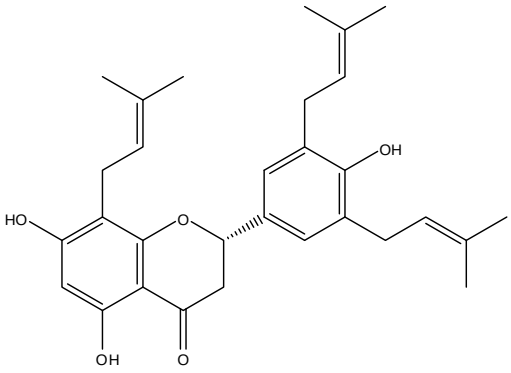

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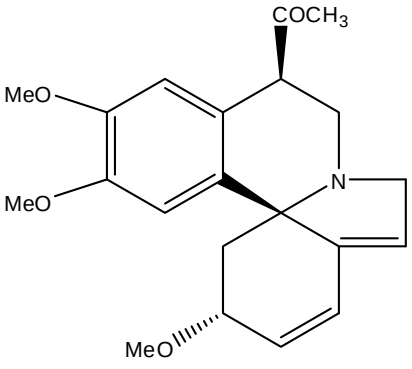

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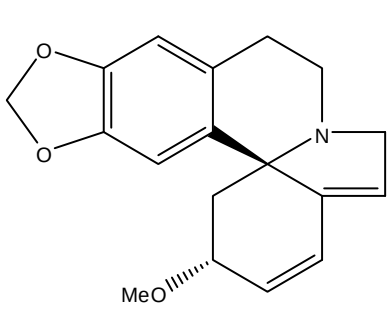

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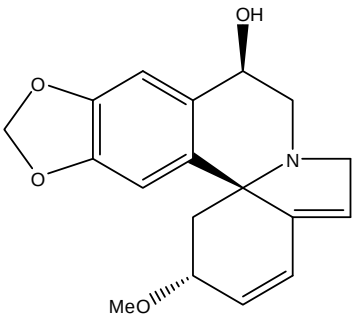

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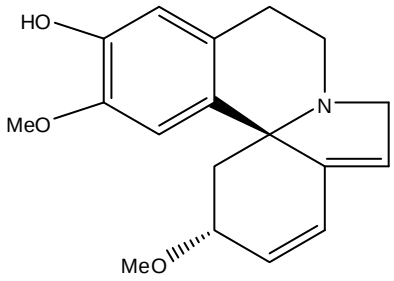

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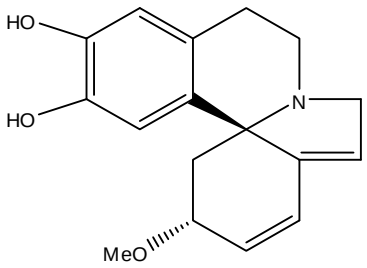

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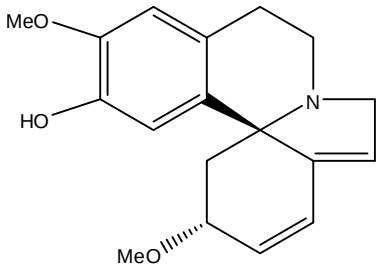

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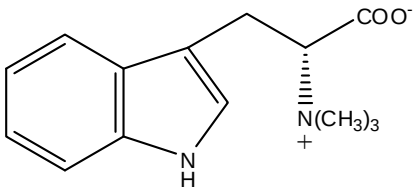

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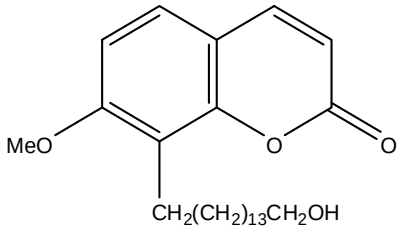

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3.4 Conclusion

The plants for antibacterial studies were selected following examination of the firsthand ethnobotanical information of Chungtia village and the literature reports from Nagaland. The

selection was guided by the plants uses for treatment of conditions of a likely bacterial origin. From the firsthand information, literature reviews of the 35 plants used for skin related conditions identified 15 plants that had no previous reported antibacterial activity studies. Seven plants were selected from these 15 for further biological studies, *i.e. Begonia picta*, *Cassia floribunda*, *Erythrina stricta*, *Lasia spinosa*, *Nephrolepis cordifolia, Polygonum hydropiper* and *Prunus persica*, with the selection priority based on plant availability for large scale collections. Additionally, *Cissampelos pareira* was included for antibacterial studies, based on the specific requests of the villagers. From the literature reports from Nagaland, 20 plants were identified as having been used for skin diseases or skin related treatments of possible bacterial origin. Literature reviews of these plants identified three plants *i.e. Albizia chinensis*, *Sarcandra glabra* and *Diospyros lanceifolia*, that had no previous reported antibacterial activity studies.

Overall, 11 plants were selected for antibacterial studies based on the firsthand documentation from Chungtia village and Nagaland ethnobotany literature searches, and a thorough review on the selected plants was performed. Chapter 4 describes the antibacterial studies on these 11 plants.

Chapter 4

Screening of Nagaland Medicinal Plants for Antibacterial activity

4.1 Background

The firsthand ethnobotanical documentation of Chungtia village and literature studies on Nagaland medicinal plants, as described in Chapter 3, identified 11 plants for further investigation due to their ethnomedicinal uses suggesting possible antibacterial properties. The aim of the section of research presented in this chapter was to screen the selected plants for antibacterial activity to support their traditional uses and highlight candidates for further phytochemical investigations.

4.2 Selection of model microorganisms for antibacterial testing

Three human pathogenic microorganisms were selected for the preliminary screening of Nagaland medicinal plants, *i.e. Staphylococcus aureus*, *Escherichia coli* and *Pseudomonas aeruginosa*. These are the most frequently isolated pathogens from a variety of clinical conditions. *S. aureus* and *E. coli* are the pathogens responsible for significant morbidity (Fluit et al., 2001). According to the SENTRY Antimicrobial Surveillance Program, in Europe *S. aureus* ranked first for skin and soft tissue and nosocomial pneumonia (37% and 22% of the isolates, respectively) and *E. coli* for bloodstream and urinary tract infections (21% and 49% of the isolates, respectively), while *P. aeruginosa* ranked second for skin and soft tissue and nosocomial pneumonia, third for urinary tract infections and fourth for bloodstream infections (Fluit et al., 2001). In Latin America and Brazil, *S. aureus* ranked first for bloodstream infections and skin and soft tissue infections, followed by *E. coli* and *P. aeruginosa* (Sader et al., 2004). In the U.S.A and Canada, *S. aureus, E. coli* and *P. aeruginosa* were ranked first, second and seventh, respectively, for bloodstream infections (Pfaller et al., 1998). For the above reasons, *S. aureus*, *E. coli* and *P. aeruginosa* are ideal candidates to be used for screening antimicrobial agents.

4.3 Antibacterial screening of selected plants

4.3.1 Selected plant list for antibacterial screening and preparation to import Australia

As described in Chapter 3, eleven plants were identified for antibacterial screening because of their use in skin diseases that may have originated from bacterial infections. Eight of these plants *i.e. Erythrina stricta*, *Cissampelos pareira, Begonia picta*, *Polygonum hydropiper, Cassia floribunda, Prunus persica, Lasia spinosa* and *Nephrolepis cordifolia*, were highlighted from the firsthand interviews and 3 of the plants *i.e. Diospyros lanceifolia, Sarcandra glabra* and *Albizia chinensis* were from Nagaland literature.

Plants were collected in two batches. The first collection of plants (November 2007 - January 2008) included *D. lanceifolia* (leaves and root bark), *S. glabra* (leaves), *B. picta* (aerial parts) and *N. cordifolia* (tubers and leaves). These plants were collected by the author, with assistance from the villagers. They were dried, ground and brought to Australia by the author. The second collection of plants (June 2008 - August 2008) included *D. lanceifolia* (root bark), *C. pareira* (roots), *P. persica* (root bark and leaves), *L. spinosa* (aerial parts), *E. stricta* (stem bark), *P. hydropiper* (aerial parts), *C. floribunda* (leaves) and *A. chinensis* (stem bark and leaves). These plants were collected and dried by the villagers, then sent to Dr R. Velmurugan who processed the plant materials to a fine powder and sent them to Australia. The tubers of *N. cordifolia* could not be imported due to difficulties in meeting Australian Quarantine and Inspection Service (AQIS) conditions for importing. The tubers were therefore collected from the grounds of Macquarie University, where they were plentiful. Similarly, the seeds of *D. lanceifolia* could not be imported to Australia due to AQIS restrictions on importing seeds. Since there are no biological or phytochemical studies reported for *D. lanceifolia*, the root bark and leaves were collected instead and imported to Australia.

4.3.2 Plant preparation for antibacterial assays

There are several methods that can be used to extract plant materials (Cannell, 1998). For this study, the plant materials were extracted with 70% aqueous ethanol and the solvents were evaporated under reduced pressure and low temperature (40ºC) to afford the crude extracts. Further partitioning of the crude extracts was carried out on the first batch of plant collected where the

crude extracts was partitioned with increasing polarity of solvent to afford the *n*-hexane (Hex), ethyl acetate (EtOAc) and *n*-butanol (*n*-BuOH) and water (Water) fractions.

4.3.3 Disc diffusion assay

For the plants collected on the first Nagaland trip (first batch of plant collections), the crude extracts (AQOH) and partitioned fractions was screened for antibacterial activity using the disc diffusion assay. The disc diffusion assay is the most widely used method for determining microorganisms' susceptibility to antibiotics. It is commonly used for antibacterial screening of medicinal plants (Perumal Samy and Ignacimuthu, 2000; Rabe and Van Staden, 1997; Van Vuuren, 2008) because of its simplicity and low cost. The disc diffusion assay involves adsorbing the test sample on to a disc on an agar plate with a lawn of bacteria and relies on it diffusing into the agar where it forms a gradient of concentration around the disc. If the extract or test compound is active against the tested microbes, a clear zone of inhibition can be seen around the disc after incubation. The zone of inhibition reflects the antimicrobial activity and this can be measured easily.

The crude extracts and the partitioned fractions of *D. lanceifolia* (leaves and root bark), *S. glabra* (leaves), *B. picta* (aerial parts) and *N. cordifolia* (tubers and leaves), were screened against *S. aureus*, *E. coli* and *P. aeruginosa*. Generally, a concentration between 1 to 5 mg of crude plant extract per disc is ideal to evaluate antimicrobial activity (Jeevan Ram et al., 2004; Kuete et al., 2008). In this study, the quantity of extract per disc was 2 mg, except for *N. cordifolia,* which was at 0.5 mg due to solubility issues. An arbitrary scoring system was used to interpret the activity in which a zone of inhibition larger than 15 mm was scored as high activity, between 10 and 15 mm as moderate activity and less than 10 mm as weak activity. The results indicated that all four plants and their plant parts, except for the leaf extracts of *D. lanceifolia*, showed inhibitory activity to at least one of the three tested microorganisms (*S. aureus*, *E. coli* and *P. aeruginosa*). The zones of inhibition of these active plant parts are presented in Table 4-1.

The crude extract and the EtOAc and *n*-butanol partitions of *S. glabra* showed weak inhibitory activity against *S. aureus*. *S. glabra* is used for wound treatment in Nagaland (Changkija, 1999). When this study was being conducted, the antibacterial activity of this plant was reported against *S. aureus* and three active compounds were isolated from the *n*-butanol extract (Yuan et al., 2008).

The leaves of *B. Picta* are used by Nagaland people to treat ulcers of the mouth (Changkija, 1999) and Chungtia villagers use the aerial parts of the plant as a hand cleansing agent. The crude extract and water partition of *B. picta* showed weak inhibitory activity, and only against *S. aureus*.

The crude extract and *n*-hexane partition of the root of *D. lanceifolia* showed high activity against *S. aureus*, whereas the other partitioned fractions showed weak activity against *E. coli* and *P. aeruginosa*. Although, the seeds are used for skin diseases in Nagaland, as mentioned earlier, the seeds could not be tested and the roots and leaves were tested instead because there were no biological activities or phytochemical studies reported on this plant.

Table 4-1: Disc diffusion assay results of the first collection of plants

Plant sample	Zone of inhibition (diameter in mm)		
	S. aureus	*E. coli*	*P. aeruginosa*
Sarcandra glabra leaves			
AQOH	11	na	na
Hex	na	na	na
EtOAc	8	na	na
n-BuOH	9	na	na
Water	na	na	na
Begonia picta aerial parts			
AQOH	9	na	na
Hex	na	na	na
EtOAc	na	na	na
n-BuOH	na	na	na
Water	9	na	na
Diospyros lanceifolia root bark			
AQOH	15	7	8
Hex	16	7	8
EtOAc	10	7	7
n-BuOH	10	na	na
Water	Na	10	na
Nephrolepis cordifolia leaves			
AQOH	14	na	na
Hex	8	na	na
EtOAc	8	na	na
n-BuOH	9	na	na
Water	11	na	na
Nephrolepis cordifolia tuber*			
AQOH	20	15	na
Hex	Na	na	na
EtOAc	13	11	na
n-BuOH	18	14	na
Water	15	na	na

Kanamycin	30	40	20

Sample concentration: 2 mg/disc or *0.5 mg/disc
Kanamycin 30 µg/disc
Disc size: 6 mm, na = not active

The crude extract and the *n*-butanol and water partitions of the tuber of *N. cordifolia* showed high activity against *S. aureus* and the EtOAc and *n*-butanol partitions showed moderate activity against *E. coli*. This result may support the use of the tubers by Chungtia villagers for skin diseases and partly for urinary problems because *E. coli* is responsible for most urinary tract infections (Fluit et al., 2001; Tambekar et al., 2006). However, since the tubers were collected in Australia, these results cannot be extrapolated unless testing is done with plant material sourced from Nagaland.

4.3.4 MTT Microdilution assay

Although the disc diffusion assay is a commonly used method for antibacterial screening because of its simplicity and low cost, the results of the activity measured as zone of inhibition depends on many variables (*e.g*., disc content, diffusibility, inoculum, *etc*.) and this method is less suitable for hydrophobic compounds because of their diffusibility barrier in agar medium. Another method that is gaining wide popularity is the MTT microdilution assay.

Microdilution assays are often preferred over the disc diffusion assay as they consume less sample and allow the testing of a range of sample concentrations in a single microtitre plate. MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) is a monotetrazolium salt (a yellow tetrazole) and is reduced to purple formazan by living cells. It has been exploited to measure cell proliferation and cytotoxicity (Mosmann, 1983) and for antimicrobial assays of natural products (Appendino et al., 2008; Cherigo et al., 2009; Dickson et al., 2007). Although the mechanism is unknown for bacteria, in eukaryotic cells, the mechanism of cellular MTT reduction to MTT formazan is due to mitochondrial dehydrogenases (Liu et al., 1997) and possibly some other non-

mitochondrial dehydrogenases or xanthine oxidase (Burdon et al., 1993) (Figure 4-1).

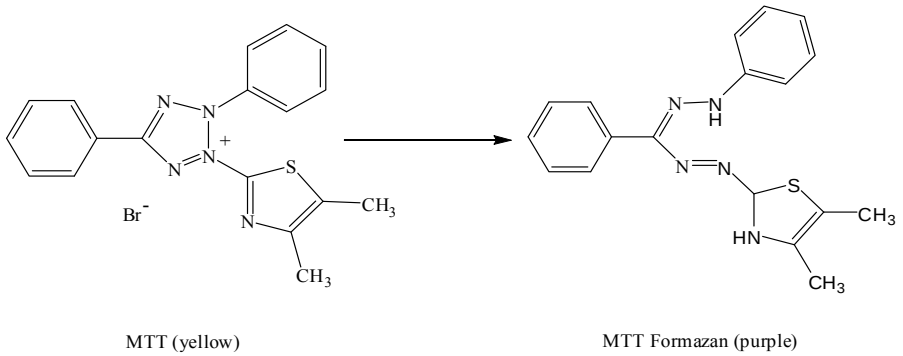


Figure 4-1: enzymatic conversion of MTT to MTT formazan by living cells.

In the MTT microdilution method, a known volume of liquid medium is dispensed into the wells of a microtitre plate (typically 96 well), the test compound is added to the first well and a series of serial dilutions is made. A suspension of known microbial density is dispensed into the wells and incubated at 37ºC for 18 to 20 hrs. Finally, a solution of MTT is dispensed into each well to detect the bacterial growth by a colour change from yellow to dark blue (dark blue wells indicate bacterial growth and yellow indicates no bacterial growth) and the minimum inhibitory concentration (MIC) is determined as the lowest concentration at which no growth (yellow colour) is observed. A section of a 96 well plate showing the MTT microdilution assay is presented in Figure 4-2.

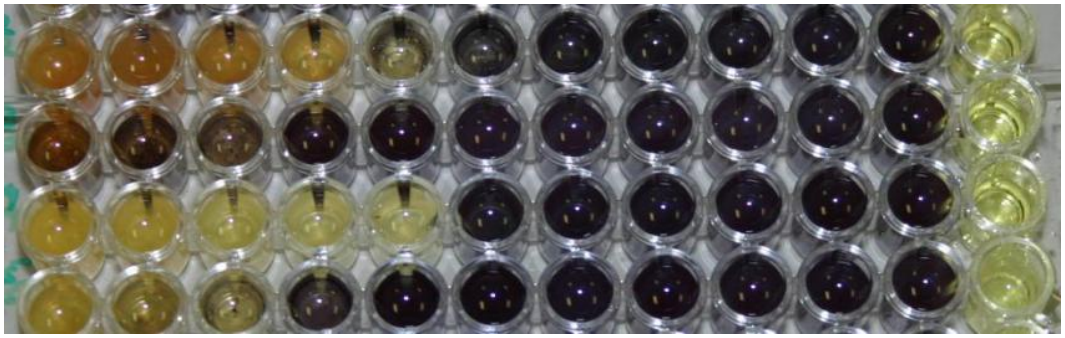


Figure 4-2: A section of a 96 well plate. The left hand side (yellow) wells show antibacterial activity while the right hand side wells show no antibacterial activity (dark blue). The last column to the right is the sterile control. (Picture taken by author, 2009)

The plants from the second collection were screened for antibacterial activity using the MTT microdilution assay. This included 10 plant materials. The crude extracts of the root bark of *D.*

lanceifolia, roots of *C. pareira*, root bark of *P. persica*, leaves of *P. persica*, aerial parts of *L. spinosa*, stem bark of *E. stricta*, aerial parts of *P. hydropiper*, leaves of *C. floribunda*, stem bark of *A. chinensis* and leaves of *A. chinensis* were tested against *S. aureus*, *E. coli* and *P. aeruginosa* using the MTT microdilution assay. Two-fold serial dilutions of the crude extracts (10 mg/ml) were used providing a range of concentrations (4.9 µg/ml to 2.5 mg/ml). The results of the antibacterial activities are presented in Table 4-2.

Table 4-2: MTT microdilution assay results of the second collection of plants

Extract	Minimum inhibitory concentration (MIC, µg/ml) against *S. aureus*
Diospyros lanceifolia: root bark	625
Cissampelos pareira: roots	2500
Prunus persica: root bark	312
Prunus persica: leaves	1250
Lasia spinosa: aerial parts	625
Erythrina stricta: stem bark	156
Polygonium hydropiper: aerial parts	1250
Casia floribunda: leaves	1250
Albizia chinensis: stem bark	Not active@ 2.5
Albizia chinensis: leaves	Not active@2.5
Kanamycin (control)	15.63

Extracts from 7 plants (8 plant parts) showed activity against *S. aureus*, whereas none of the plant extracts showed activity against the Gram-negative bacteria *E. coli* and *P. aeruginosa*. The most potent inhibitory activity was observed with the stem bark extract of *E. stricta*, followed by the root bark extract of *P. persica,* with MIC values of 156 µg/ml and 312 µg/ml, respectively. Generally, crude plant extracts showing inhibitory activity at a concentration lower than 1 mg/ml are considered to have very good activity (Ríos and Recio, 2005). Thus, four plant extracts, *i.e.* the root bark of *D. lanceifolia*, root bark of *P. persica*, aerial parts of *L. spinosa* and stem bark of *E. stricta*, showed good inhibitory activity against *S. aureus*. The strong antibacterial activity possessed by *E. stricta* and *P. persica* is consistent with the strong reliance of the Chungtia villagers on the use of these plants for skin related infections. The high MIC value for the *C. pareira* crude extract was not surprising given that its ethnomedicinal use by the villagers is for hypertension. It was however tested for antibacterial activity as some of the villagers requested for

its biological activity to be examined and it has been documented that the root has been used elsewhere to treat sores, boils, scabies and childhood eczema (Schmelzer and Gurib-Fakim, 2008).

The root of *D. lanceifolia* exhibited activity against all the three tested microbes (although weak activity against *E. coli* and *P. aeruginosa*) in the disc diffusion assay, but the inhibitory activity in the MTT microdilution assay was limited only to *S. aureus* (MIC 625 µg/ml).

The plants *P. hydropiper* and *C. floribunda* were documented from Chungtia village for their uses in skin related treatments and in particular to treat fungal infections. These two plants showed similar inhibitory activities against *S. aureus* (MIC 1250 µg/ml) and were not active against *E. coli* and *P. aeruginosa*. The testing of antifungal activity on these plants was not performed due to time constraints, but would be valuable to examine in future studies.

The use of *L. spinosa* to treat skin diseases by Chungtia villagers is also supported by the strong antibacterial activity against *S. aureus* (MIC 625 µg/ml). The plant *A. chinensis* (from literature search) did not show any inhibitory activity at a concentration of 2500 µg/ml.

4.4 Conclusion

The antibacterial activity of 8 plants used by Chungtia villagers to treat skin related infections and 3 plants selected through a literature search on Nagaland medicinal plants were analysed by the disc diffusion assay and the MTT microdilution assay.

All of the eight plants used by the Chungtia villagers showed antibacterial activity against *S. aureus* at concentrations less than 2.5 mg/ml. However, they were not active against *E. coli* and *P. aeruginosa*. The antibacterial activity possessed by these plants strongly supports the traditional use of these plants by Chungtia villagers to treat skin related infections. The highest inhibitory activities were exhibited by the stem bark of *E. stricta* and the roots of *P. persica*, with MIC values of 156 µg/ml and 312 µg/ml, respectively.

The only plant that did not show antibacterial activity was *A. chinensis* (from literature search). *S. glabra* (from literature search) exhibited antibacterial activity against *S. aureus*. The antibacterial activity of this plant against *S. aureus* was recently reported, with the isolation of three antibacterial compounds (Yuan et al., 2008). The leaves of *D. lanceifolia* did not show any activity whereas the root of *D. lanceifolia*, although not used traditionally, showed significant activity

against *S. aureus* and weak activity against *E. coli* and *P. aeruginosa*. This is the first report of biological activity related to this plant. So far there are no phytochemical studies on this plant.

Based on these results, the stem of *E. stricta*, roots of *P. persica* and roots of *D. lanceifolia* were selected for further biological and chemical investigations of the antibacterial constituents. The results of these studies are presented in Chapters 5, 6 and 7, respectively.

4.5 Experimental

4.5.1 Reagents and equipment

All organic solvents were AR grade and were re-distilled before use, except for *n*-butanol and *n*-hexane. Distilled water was used in the preparation of all aqueous extracts and partitions. Mueller Hinton II broth and Mueller Hinton II agar were from Bacto laboratories (Australia). Filter paper discs (6 mm) used for disc diffusion assays were from Whatman (UK). Kanamycin was obtained from Amresco (U.S.A.) and chloramphenicol from Boehringer Manheim GmbH (Germany). Flat bottomed 96 well plates were from Greiner. MTT (Thiazolyl Blue Tetrazolium Bromide) was from Sigma-Aldrich. The orbital mixer incubator was from Ratek (Australia) and the freeze dryer system was from Labconco (USA).

4.5.2 Collection, preparation and importation of plant materials to Australia

All the plant materials except for the tubers of *N. cordifolia* were collected in Chungtia village, Nagaland, India, by the villagers under the assistance of Mr Anungba Jamir (AJ), a CSMT representative. All plant collections were done between November 2007 and January 2008, and June and August 2008. The collected materials were thoroughly inspected by AJ for precise identification of the species and these plant materials were dried under shade for 10 to 20 days. Voucher specimens were prepared for all the plants during the interviewing process as well as for all the collected plants, as described in Chapter 2 Experimental. One representative of each plant of the prepared voucher specimens were deposited at Botanical Survey of India (BSI), Shillong Branch, India. The plants collected between November 2007 and January 2008 (first plant collection) were *D. lanceifolia* (leaves and root bark), *S. glabra* (leaves), and *B. picta* (aerial parts). These plant materials were dried, ground and brought to Australia by the author during his first field trip. Plants collected between June and August 2008 (second plant collection) were *D.*

lanceifolia (root bark), *C. pareira* (roots), *P. persica* (root bark and leaves), *L. spinosa* (aerial parts), *E. stricta* (stem bark), *P. hydropiper* (aerial parts), *C. floribunda* (leaves) and *A. chinensis* (stem bark and leaves). After the plant materials were dried, these plant materials were transported by two CSMT representatives to Chennai, India, where Dr Velmurugan further processed the plant materials.

The protocol used by Dr Velmurugan for processing of plants was as follows:

> *The plants were procured from Nagaland and brought to the processing station. There, all the plants were separated from foreign particles and unwanted materials. They were washed with clean water. Then these plants were dried in the shade for 24 hours. The next day these plants were dried in a vacuum drier at 75 to 85 ^{o} C.*
>
> *After 48 hours of drying under vacuum, they were kept in the shade for cooling for three days. Again the plant materials were checked for foreign particles.*
>
> *After confirming that there were no foreign particles, the plant materials were chopped and passed through micropulverizer for grinding. The process was repeated until 130-200 mesh size. Then the powders were sieved and dried again in the vacuum to confirm that they did not contain any humidity. Then the material was allowed to cool in shade and packed in plastic bags, which in turn were packed in plastic containers and sealed. The sealed containers were couriered to Australia.*

Prior to the collection and shipment, AQIS permits (08003967) to import these plant materials to Australia were sought and permission was granted for all the plant parts except for the tubers of *N. cordifolia* and seeds of *D. lanceifolia*.

After the shipments were procured from India, the plant materials were stored in the laboratory (cupboard) until used.

4.5.3 Preparation of crude extracts for antibacterial assays

The dried plant material was suspended in 70% aqueous ethanol, shaken overnight at room temperature and the extracts vacuum filtered. The extraction process was carried out three times and the final volume of solvent was removed by rotary evaporation at 40ºC after which the

residues were freeze dried overnight to afford crude extracts (AQOH). For the disc diffusion assay, the crude extract was suspended in water and successively partitioned with *n*-hexane, EtOAc and *n*-butanol to give four fractions, Hex, EtOAc, BuOH and Water extract. Table 4-3 shows the partitioning of the crude extracts with different polarity solvent and table 4-4 shows the afforded partitioned fractions with yield and appearance. The crude extracts yield for MTT microdilution assay are shown in Table 4-5.

Table 4-3

Plant sample	Weight	Vol. of 70% aqueous ethanol	Partitioning of crude extracts (volume of solvent used)			
			Water (ml)	*n*-Hexane (ml)	EtOAc (ml)	*n*-Butanol (ml)
D. lanceifolia leaves	15 g	100 ml x 3	100	100 x3	100 x 3	100 x 3
D. lanceifolia root	15 g	100 ml x 3	100	100 x 3	100 x 3	100 x 3
S. glabra leaves	15 g	100 ml x 3	100	100 x 3	100 x 3	100 x 3
B. picta aerial parts	15 g	100 ml x 3	100	100 x 3	100 x 3	100 x 3
N. cordifolia leaves	2.2 kg	2 l x 3	400	600 x 3	600 x 3	600 x 3
N. cordifolia tubers	1.2 kg	3.5 l x 3	300	500 x 3	500 x 3	500 x 3

Table 4-4

Plant sample	Weight of sample	Partition of crude extracts yield (% w/w) and appearance				
		AQOH	Hex	EtOAc	BuOH	water
D. lanceifolia leaves	15 g	3.7 g (24.7%) dark green	0.14 g (0.9%) dark green	0.42 g (2.8%) light green	0.82 g (5.5%) yellow	1.4 g (9.3%) dark brown
D. lanceifolia	15 g	3.8 g	0.06 g (0.4%)	0.35 g (2.3%)	0.22 g (1.5%)	1.6 g (10.7)

root		(25.4%) dark brown	orange	dark brown	creamish yellow	dark brown
S. glabra leaves	15 g	3.2 g (21.5%) dark green	0.48 (3.2%) dark green	0.5 g (3.4%) dark green	0.81 g (5.4%) light yellow	0.53 g (3.5%) brown
B. picta aerial parts	15 g	1.0 g (7.1%) dark purple	0.19 g (1.2%) dark green	0.07 g (0.5%) light green	0.13 g (0.8%) purple	0.52 g (3.5%) purple
N. cordifolia leaves	2.2 kg	134.6 g (6.1%) dark green	1.7 g (0.1%) dark green	14.5 g (0.7%) dark green	57.3 g (2.6%) creamish yellow	65.4 g (3%) brown
N. cordifolia tubers	1.2 kg	49.6 g (4.1%) Brown	2.1 g (0.8%) light brown	1.0 g (0.08%) light brown	2 g (0.7%) brown	32.2 g (2.7%) light brown

Table 4-5

Plant sample	Weight of sample (g)	Vol. of 70%aqueous ethanol (ml)	Weight of crude extract and appearance (%w/w)
E. stricta stem	10	100 x 3	0.51 g (5%) dark brown
C. pareira root	10	100 x 3	1.34 g (13.4%) dark brown
C. floribunda leaves	10	100 x 3	1.25 g (12.5%) dark green
P. persica leaves	10	100 x 3	1.71 g (17.1%) dark green
P. persica root	10	100 x 3	2.25 g (22.5%) dark brown
A. chinensis leaves	10	100 x 3	1.3 g (13%) dark green

A. chinensis stem	10	100 x 3	1.91 g (19.1%) dark brown
L. spinosa aerial parts	10	100 x 3	0.47 g (4.7%) dark green
P. hydropiper aerial parts	10	100 x 3	0.4 g (4%) dark green

4.5.4 Antibacterial assays

Biosafety approval was obtained from the Macquarie University Biosafety Committee (approval number Biosafety - 05/14/LAB).

The strains of bacteria used were *Staphylococcus aureus* ATCC 9144 (obtained from the CDS Reference Laboratory, Department of Microbiology, The Prince of Wales Hospital, NSW), *Escherichia coli* JM109 and *Pseudomonas aeruginosa* ATCC 27853 (both obtained from Professor Michael Gillings, Department of Biological Sciences, Macquarie University). Stock cultures of these strains were maintained in Mueller Hinton II broth containing 10% v/v glycerol. Fresh subcultures were made by inoculating the cultures in Luria Broth (LB) followed by an overnight incubation (37 ºC). For the disc diffusion assay, the overnight cultures were diluted 1/100 with distilled water before use. For the MTT microdilution assay, all bacterial strains were grown overnight in MH II broth and the optical density at 600 nm (OD_{600}) was adjusted to 0.08-0.1 to produce an inoculum density of 1.0×10^8 cfu/mL (OD).

4.5.4.1 Disc diffusion assay

The Mueller Hinton II Agar (MHIIA) medium was used throughout. The medium was prepared as per the manufacturer protocol (suspended 38 g of the powder in 1 l of purified water, mixed thoroughly, heated with frequent agitation until the powder was completely dissolved.) and autoclaved at 121ºC for 20 min. The molten medium was poured into petri plates (10 to 15 ml) and allowed to dry overnight.

Using a sterile cotton swab, the diluted culture of the bacteria was applied gently on the entire surface of the MHAII plate to provide a lawn of microbes. The plant extracts were prepared to the appropriate concentration by suspending in their respective partitioning solvent. The antibiotic samples were prepared in water. The samples were then impregnated into the filter paper discs (6 mm) by addition of appropriate volumes to allow 2 mg on the discs (or in the case of *N. cordifolia*,

0.5 mg). Kanamycin in water (30 µg/disc) was used as the positive control. The impregnated filter discs were kept at room temperature until the solvents were evaporated; *n*-butanol extracts were kept overnight, and were then placed on the inoculated plate by pressing down. The plates were incubated at 37 ºC for 18 hours, and the diameter of the zones of inhibition were measured with a ruler.

4.5.4.2 MTT Microdilution assay

The plant extracts (10 mg) or the antibiotic (1 µg) was dissolved in 200 µl DMSO and the final volume was made up to 1 ml with distilled water. Using a 96 well microtitre plate, 100 µl each of double strength MHB II was dispensed into wells 1-11 (from left to right) for each row, 100 µl of the test plant extract or antibiotic was added to well 1 (in different rows for each extract) and mixed thoroughly, after which 100 µl was taken out and dispensed to the next well (*i.e.* well 2). This process of two-fold serial dilution was carried out until well 10, and skipping well 11, the final volume was dispensed into well 12. Again, 100 µl each of the bacterial inoculum was dispensed into wells 1 to 11 leaving well 12. Since well 11 was free of the test compound or the antibiotic, this acted as a positive control for the growth of the inoculum and well 12 being free of inoculum served as the sterile control of the assay. 5% DMSO was also included as a negative control. The plate was incubated at 37°C for 18 to 20 hours and finally 20 µl of a methanolic solution of MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazoliium bromide) (5 mg/ml) was added to each well and again incubated for 30 minutes. The MIC was determined as the lowest concentration of the test compound or antibiotic that showed no visible colour change from yellow to blue/purple.

Chapter 5

Bioassay Guided Isolation of Antibacterial Compounds from Erythrina stricta

5.1 Background

As described in Chapter 4, the aqueous ethanolic extract of the stem bark of *Erythrina stricta*, which has been documented for use in skin related infections, showed significant inhibitory activity towards *S. aureus*. This extract exhibited the highest inhibitory activity of all the tested plant materials in this study with an MIC value at 156 µg/ml and was one of the three plants selected for phytochemical analysis. The aim of this chapter was to present the studies on the bioassay guided isolation of antibacterial compounds from this plant.

As mentioned in the preceding chapter, the stem bark of *E. stricta* was collected in Chungtia village by the villagers and further processed by Dr Velmurugan into a fine powder and shipped to Australia. The dried powder was extracted with 70% aqueous ethanol and the aqueous residue, after evaporation of ethanol, was successively partitioned with increasing polarities of solvents to afford five partitioned fractions, *i.e. n*-hexane, DCM, EtOAc, *n*-butanol and water fractions.

5.2 Bioassay guided investigation of DCM partitioned fractions

The partitioned fractions were analysed against *S. aureus, E. coli* and *P. aeruginosa* using the MTT microdilution assay to determine the fractions with the greatest antibacterial activity. Consistent with the crude aqueous extracts of the stem bark, the partitions did not show activity against *E. coli* and *P. aeruginosa*.

The partitioned fractions of *n*-hexane, DCM and EtOAc showed inhibitory activity against *S. aureus* with MIC values of 2500, 156 and 312 µg/ml, respectively, while the *n*-butanol and water fractions were inactive. The DCM partitioned fraction, being the most active amongst the three fractions, was selected for bioassay guided phytochemical analysis. A portion of the DCM fraction was subjected to silica gel column chromatography, resulting in 15 fractions (A1 to A15), based on order of elution. These fractions were then analysed by the MTT microdilution assay to determine the active fractions. Of these fractions, fractions A6 to A11 showed activity with A6 possessing the

most potent activity. In order to simplify the bioassay guided isolation of the active compounds, the use of TLC-bioautography was investigated. This method allows detection of antimicrobially active constituents by R_f on a TLC plate, therefore greatly assisting the choice of subsequent chromatographic conditions for isolation of the active compounds.

5.3 TLC-bioautography method

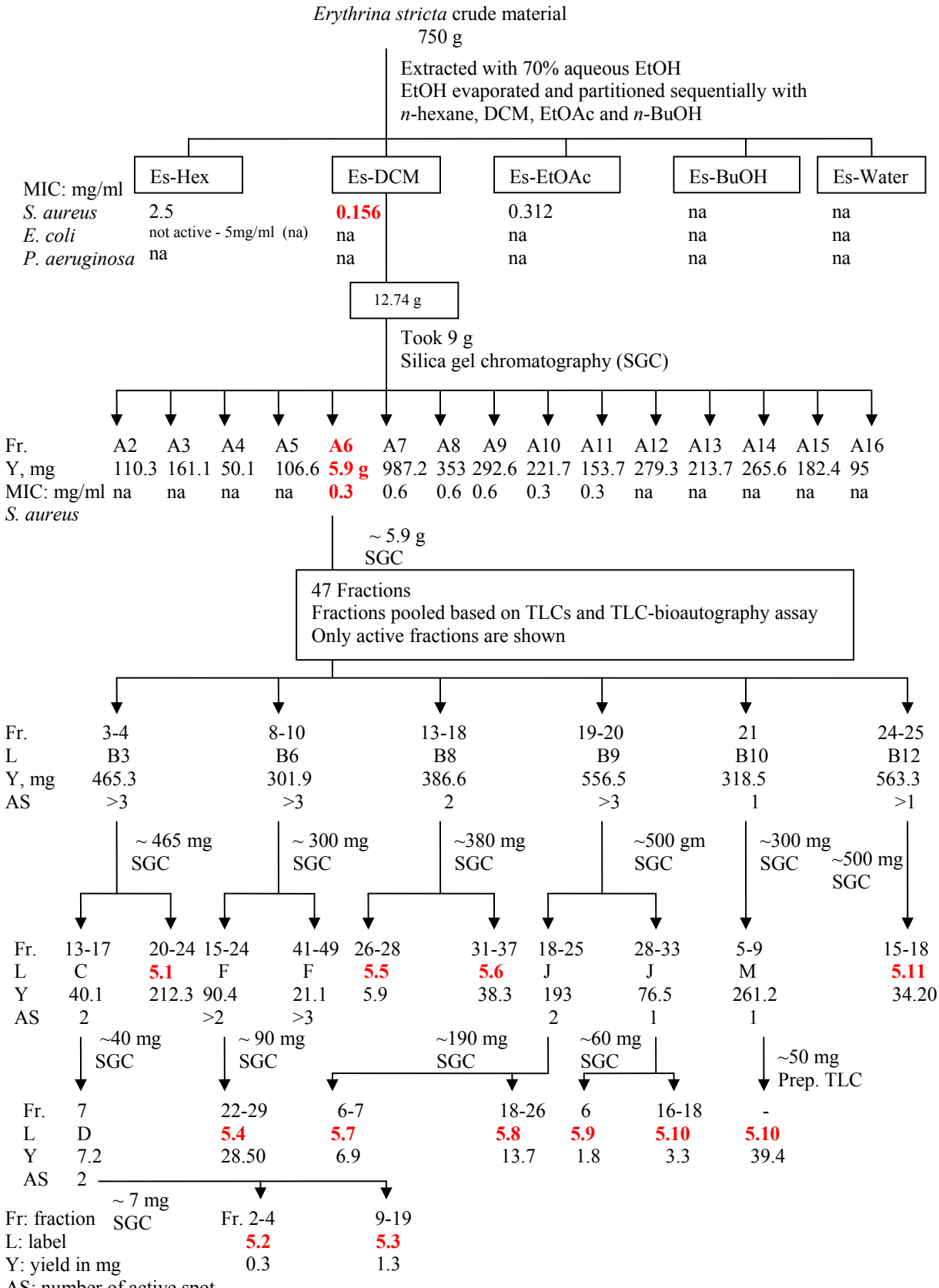
The bioautographic method to localise antibacterial activity on a chromatogram was initiated in the 1960's. For this method, samples (containing antibacterial compounds) that have been run on a TLC plate are transferred to an inoculated agar plate through a diffusion process and the zones of inhibition are then visualised by appropriate stains (Hamburger and Cordell, 1987). This method is called contact bioautography. The disadvantage of this method is the problem of differential diffusion of compounds from the chromatogram into the agar plate. This problem was overcome by the introduction of immersion/overlay bioautography and direct bioautography. In immersion/overlay bioautography, the chromatogram (TLC plate) is covered with an agar medium containing the desired microbial strain and after incubation, the zones of inhibition are then visualised by appropriate stains (Schmourlo et al., 2005; Smith et al., 2007). The main disadvantage of this method is again the dilution of the antibacterials in the agar layer, causing lower sensitivity. In direct bioautography, the chromatogram (TLC plate) is either dipped in the suspension of microorganisms growing in a suitable broth or the suspension is sprayed onto the TLC plate (Hamburger and Cordell, 1987; Moulari et al., 2006; Rahalison et al., 1991). In this method, the TLC plate is incubated in a moist chamber and after incubation, the zones of inhibition are visualised by appropriate stains, mainly MTT. This method is currently the most preferred method and has found widespread usage for antibacterial screening (Suleiman et al., 2010; Tasdemir et al., 2004) and isolation of antibacterial compounds (Jassbi et al., 2002; Rabe et al., 2002; Rodrigo et al., 2005). It has also been developed for antifungal screening (Rahalison et al., 1991) and modified further for the detection of acetylcholinesterase and butyrylcholinesterase inhibitors in plants (Marston et al., 2002), detection of estrogenic compounds from the environment (Müller et al., 2004), and detection of xanthine oxidase inhibitors and superoxide scavengers (Ramallo et al., 2006).

For this study, direct bioautography method was trialled with crude extracts of 10 plants samples (from the second collection, see Chapter 4) and also with standard antibiotics (kanamycin and

chloramphenicol). However, some minor issues were encountered with this method. One of the issues was the uneven distribution of microorganisms on the TLC plate following the dipping method. This problem was overcome by using a floating method, in which a suspension of microorganisms was poured into a sterile petri plate and the TLC plate (with the antibacterials agent facing inward) was gently placed on the surface of the suspension. This allowed even distribution of microorganisms in the TLC plate. The second issue was the growth of microorganisms in the moist chamber. This was overcome by incubating the inoculated TLC plate (with the antibacterial agents and microorganisms and facing outward) onto a petri plate containing agar medium. This provided optimal growth for the microorganisms used (*S. aureus*).

5.4 TLC-bioautography guided isolation of compounds from the A6 fraction

Fractions A2 to A16 were analysed by TLC-bioautography to determine their activities. Similar to the results of the MTT microdilution assay, fraction A6 to A11 showed clear zones of inhibition and some overlap of active spots between fractions. Fraction A6 had the greatest number of active spots and also had the maximum yield, and therefore was subjected to normal phase silica gel chromatographic separation to afford 47 fractions. These fractions were further analysed by TLC-bioautography and based on the results, the fractions were pooled to 13 fractions (B1 to B13). Fraction B3, which contained more than 3 active spots, was again subjected to normal phase chromatographic separation to afford compound 5.1. Similarly, repeated chromatographic separations lead to the isolation of 10 other antibacterially active compounds (5.2 – 5.11). A flow chart of the process of isolation of compounds is shown in Scheme 5-1.



Scheme 5-1: Bioassay-guided fractionation of the stem bark of *Erythrina stricta*

5.5 Characterisation of isolated compounds

5.5.1 Structural elucidation of 5-hydroxysophoranone (5.1)

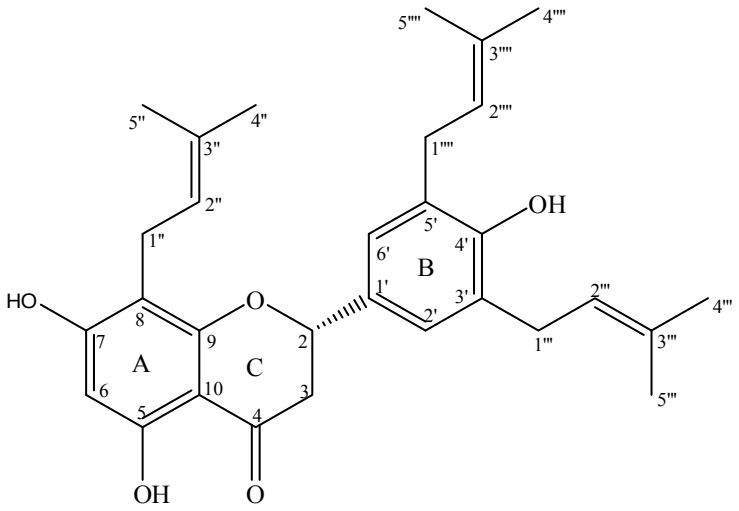
Compound 5.1 was obtained as a white amorphous solid (212 mg). The negative-ion HRESI-MS showed an $[M-H]^-$ peak at *m/z* 475.2479, corresponding to the molecular formula $C_{30}H_{36}O_5$. From ^1H and ^{13}C NMR spectra, a flavanone skeleton was deduced based on the proton signals at δ 5.28 (*m*, H-2), 2.79 (*dd*, *J*=17.2, 3.0 Hz, H-3_{eq}) and 3.05 (*dd*, *J*=17.2, 13.0 Hz, H-3_{ax}) and carbon signals at δ 79.1 (C-2), 43.3 (C-3) and 196.8 (C-4) (Jang et al., 2008; Li et al., 2009c). The proton signals at δ 3.30 (br *d*, *J*=7.3 Hz, H-1"), 3.37 (br *d*, *J*=7.3 Hz, H-1"', H-1""), 5.22 (*m*, H-2"), 5.32 (*m*, H-2"', H-2""), 1.73 (*d*, *J*=1.1 Hz, H-4", H-5") and 1.77 (*s*, H-4"', H-5"', H-4"", H-5"") indicated the presence of three sets of isoprenyl groups. The observation of a proton singlet at δ 6.0 (H-6) and two proton singlets at δ 7.05 (H-2', H-6') in the aromatic region were indicative of a pentasubstituted and trisubstituted flavanone skeleton. Also present in the ^1H NMR spectrum were two aromatic hydroxyl protons (δ 6.16, H-7; 5.49, H-4') and a chelated hydroxyl proton (δ 12.01).

The ^{13}C NMR signals at δ 21.9 (C-1"), 29.8 (C-1"', C-1""), 121.8 (C-2"), 121.7 (C-2"', C-2""), 135 (C-3"), 134.9 (C-3"', C-3""), 17.9 (C-4"), 18.0 (C-4"', C-4"") and 25.9 (C-5", C-5"',C -5"") confirmed the presence of the three isoprenyl groups. The carbonyl signal at δ 196.8 (C-4) and a quaternary carbon attached to an oxygen at δ 162.3 (C-5) confirmed the presence of the chelated hydroxyl group. The presence of two aromatic hydroxyl groups was confirmed by the HSQC spectrum, which had no correlations between the hydroxyl protons and attached aromatic carbons. All the ^1H and ^{13}C NMR signals were uniquely assigned by H-H-COSY, HSQC and HMBC experiments.

The assignment of one isoprenyl group was made at ring A at C-8 following HMBC correlations in which proton H-1" correlated with C-7, C-8, C-9, C-2" and C-3". Similarly, the other two isoprenyl groups were placed at the C ring at C-3' and C-5', where H-1"' correlated with C-6', C-4' and C-5' and H-1"" correlated with C-3', C-4' and C-3"". The aromatic proton at δ 6.0 (H-6) was assigned to C-6 (δ 96.8) by the HSQC spectrum and the other two protons at δ 7.05 (H-2', H-6') were assigned to C-2' (125.8) and C-6' (127.5). This was confirmed by HMBC correlations in which H-6 correlated with C-5, C-8 and C-10; H-2' correlated with C-2, C-2"', C-4' and C-1"'; and H-6' correlated with C-2, C-6', C-4' and C-1"". The two hydroxyl groups (δ 6.16 and 5.49) were

assigned to ring A at C-7 and ring B at C-4', respectively. The hydroxyl group at C-7 showed long-range correlations to C-6, C-7 and C-8, whereas the hydroxyl group at C-4' showed correlations to C-4' and C-6'. The CD curve showed a high-amplitude positive Cotton effect and a negative Cotton effect at 334 nm and 291 nm, respectively, which are the diagnostic wavelength regions for 2*S* flavanones (Gaffield, 1970).

This compound was identified as 5-hydroxysophoranone (5.1) and had identical ^{1}H NMR data to the literature (Kulesh and Denisenko, 2003; Matsuura et al., 1994). The first isolation of 5-hydroxysophoranone was from *Millettia pulchra* (Baruah et al., 1984), and this plant was collected in Meghalaya State, which is the neighbouring state of Nagaland. Even though this compound has been isolated several times from other plants (Kulesh and Denisenko, 2003; Matsuura et al., 1994), including from the roots of *Erythrina stricta* (Rukachaisirikul et al., 2007b), the ^{13}C NMR data has not been previously reported. The 1 and 2-D NMR data is presented in Table 5-1. This is the first reported isolation of this compound from the stem bark of *E. stricta*.



5-Hydroxysophoranone (5.1)

Table 5-1: NMR spectra of compound 5.1

Compound 5.1				
Position	δ_H multiplicity (*J* in Hz)	δ_C	HMBC	COSY
2	5.28 *m,* 1H	79.1	C-1', C-2', C-6'	3ax, 3eq
3_{ax}	3.05 *dd,* (17.2, 13.0), 1H	43.3	C-2, C-1'	
3_{eq}	2.79 *dd,* (17.2, 3.0), 1H	43.3	C-4, C-10	
4		196.8		
5	12.01 *s*, OH, 1H	162.3	C-5, C-6, C-10	
6	6.00 *s*, 1H	96.8	C-5, C-8, C-10	
7	6.16 *s*, OH, 1H	163.7	C-6, C-7, C-8	
8		106.2		
9		160.0		
10		103.3		
1'		130.3		
2'	7.05 *s*, 1H	125.8	C-2, C-2''', C-4', C-1'''	
3'		127.5		
4'	5.49 *s*, OH, 1H	153.1	C-4', C-6'	
5'		127.5		
6'	7.05 *s*, 1H	125.8	C-2, C-6', C-4', C-1''''	
1''	3.30 *br d,* (7.32), 2H	21.9	C-7, C-8, C-9, C-2'', C-3''	5'', 2''
2''	5.22 *m*, 1H	121.7	C-4'', C-1'', C-5'', C-8	5'', 1''
3''		135.0		
4''	1.73 *d,* (1.1), 3H	18.0	C-5'', C-2'', C-3''	2''
5''	1.73 *d,* (1.1), 3H	25.9	C-4'', C-2'', C-3''	2''
1'''	3.37 *br d,* (7.32), 2H	29.8	C-6', C-4', C-5'	
2'''	5.32 *m,* 1H	121.7	C1'''' C-4'''', C-5'''', C-3'	4''',1'''
3'''		134.9		
4'''	1.77 *m*, 3H	18.0	C-5''', C-2''', C-3'''	2'''
5'''	1.77 *m*, 3H	25.9	C-4''', C-2''', C-3'''	
1''''	3.37 *br d,* (7.32), 2H	29.8	C-3', C-4', C-3''''	
2''''	5.32 *m,* 1H	121.7	C1'''' C-4'''', C-5'''', C-3'	4'''', 1''''
3''''		134.9		
4''''	1.77 *m*, 3H	18.0	C-5'''', C-2'''', C-3''''	2''''
5''''	1.77 *m*, 3H	25.9	C-4'''', C-2'''', C-3''''	2''''

Data acquired at 400 MHz for ^{1}H and 100 MHz for ^{13}C in *d*-chloroform

5.5.2 Structural elucidation of maackiaflavanone B (5.3)

Compound 5.3 was initially obtained as a pale yellow solid that was a mixture (~1:1) of compounds 5.2 and 5.3. Initial 1 and 2-D NMR spectral analyses were conducted on the mixture before further purification steps were carried out. Once the compounds were purified, ^{1}H NMR spectra were obtained for the compounds and these were used as a guide to extract out the ^{13}C, HMBC and HSQC data for each compound from the mixture. ESI-MS spectral analysis was conducted on the purified samples.

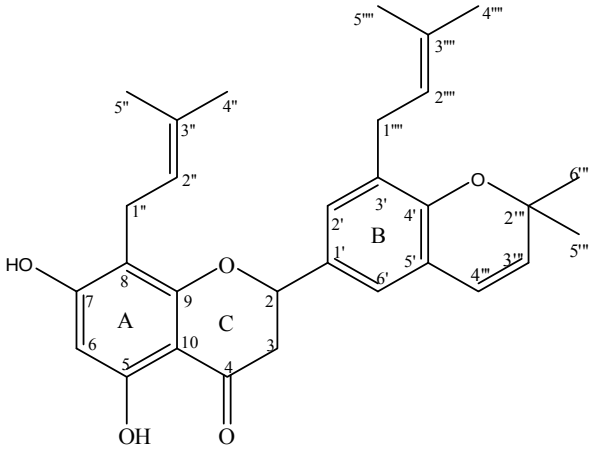
Compound 5.3 was obtained as a pale yellow solid (1.3 mg). ESI-MS indicated a protonated molecular ion at *m/z* 475 $[M+H]^+$, consistent with the molecular formula $C_{30}H_{34}O_5$.

The ^{1}H NMR spectrum was similar to that of compound 5.1, indicating the close structural similarity of these two compounds. The only significant difference observed was the absence of one isoprenyl group and the spectrum indicated the presence of a 2,2-dimethylpyran [δ 1.44 (6H, *s*), 5.64 (1H, *d*, *J*=9.1 Hz), 6.32 (1H, *d*, *J*=9.1 Hz)]. The carbon resonances were derived from the HSQC and HMBC spectra, and the signals and their correlations matched exactly with that of compound 5.1 except for the chemical shifts in ring B. The presence of a 2,2-dimethylpyran was confirmed by the carbon signals at δ 28.3 (C-5''', C-6'''), 131.5 (C-3''') and 122.5 (C-4''') (Ichimaru et al., 1996).

The two *meta*-coupled proton signals at δ 7.02 (*d*, *J*=2.1 Hz, H-2') and 6.90 (*d*, *J*=2.1 Hz, H-6') showed HSQC correlations to δ 127.6 (C-2') and 122.1 (C-6'), respectively. The long-range correlations from H-2' to C-2 (δ 79.4), C-6' (122.1) and C-1'''' (21.9) indicated that one of the isoprenyl groups was adjacent to H-2' and since C-1' (129.9) is linked to the C-2 of ring C, the only possible attachment was at C-3'. This was confirmed by the HMBC correlations from H-1'''' with C-2', C-3', C-3'''' and C-4' and thus, the isoprenyl group was attached to C-3', the other isoprenyl being attached to C-8 of ring A. Likewise, H-6' correlated to C-2, C-4''' (δ122.5), C-2' (δ127.6) and C-4' (δ 151.1) in the HMBC spectrum. This led to the placement of the 2,2-dimethylpyran ring at C-5' and C-4', and was confirmed by HMBC correlations arising from H-4''' and H-3''', which correlated with C-2''', C-5', C-4' and C-5''', C-6''', C-2''', C-5', respectively.

The ^{1}H and ^{13}C NMR and MS data were identical to that reported for maackiaflavanone B (5.3), which has been isolated from the stem bark of *Maackia amurensis* (Li et al., 2009c). However, the

absolute stereochemistry could not be ascertained due to insufficient sample. ^{1}H and ^{13}C NMR data are presented in Table 5-3 and Table 5-4, respectively.



Maackiaflavanone B (5.3)

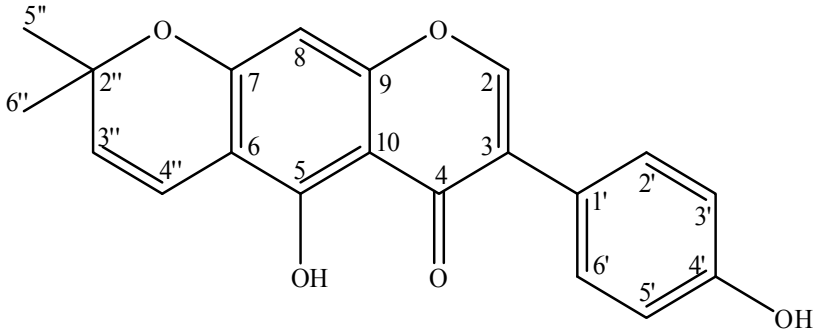
5.5.3 Structural elucidation of alpinumisoflavone (5.4)

Compound 5.4 was obtained as a pale yellow solid (28.5 mg). ESI-MS indicated a protonated molecular ion at *m/z* 337 $[M+H]^{+}$, consistent with the molecular formula $C_{20}H_{16}O_5$. The ^{1}H NMR spectrum showed characteristic signals of a 2,2-dimethylpyran ring [δ 1.47 (s, 6H), 5.62 (*d*, *J*=10 Hz, 1H), 6.72 (*d*, *J*=10 Hz, 1H)], a chelated hydroxyl proton [δ 13.10 (*s*, 1H)], one AA'BB' spin system [δ 7.36 (*d*, *J*=8.5 Hz, 2H), 6.86 (*d*, *J*=8.5 Hz, 2H)] and two proton singlets [δ 6.33 (*s*, 1H), 7.80 (*s*,1H)]. The presence of a one-proton singlet at δ 7.80 was characteristic of an isoflavone skeleton (Ferrari et al., 2005; Mizuno et al., 1990).

The HSQC spectrum indicated the correlations between the singlet proton at δ 7.80 (H-2) and 152.8 (C-2), thereby confirming the isoflavone skeleton. The HSQC spectrum also confirmed the 2,2-dimethylpyran by its correlations of proton signals to the corresponding carbon signals at δ 28.5 (C-5", C-6"), 78.2 (C-2"), 115.7 (C-4") and 128.4 (C-5"), respectively. The HMBC spectrum showed correlations of the aromatic proton at δ 6.33 to C-9 (δ 157.4), C-10 (106.4), C-7 (159.5) and C-6 (105.8), indicating its presence in ring A, and it correlated to C-8 (δ95.1) in the HSQC spectrum. The two olefinic protons [δ 6.72 (H-4") 5.62 (H-3")], arising from the 2,2-dimethylpyran moiety, showed HMBC correlations to C-6 and H-4" to C-7, therefore the 2,2-dimethylpyran moiety was fused to C-6 and C-7. The signals arising at δ 7.36 (2H) and 6.86 (2H) were assigned

to the B ring at C-2' and C-6', and C-3' and C-5', respectively, based on the HSQC and HMBC spectra and their long-range correlations to a biogenetically expected oxygenation at C-4' (δ 156.2) (Li et al., 2009c; Yenesew et al., 2004).

This compound was identified as alpinumisoflavone (5.4) (Jackson et al., 1971) and spectral data were in agreement with the literature (El-Masry et al., 2002; Han et al., 2005). The crystal structure of this compound (Harrison et al., 2009) and synthesis (Rao et al., 1987) have been reported. ^{1}H and ^{13}C NMR data are presented in Table 5-3 and Table 5-4, respectively. This is the first reported isolation of this compound from *E. stricta*.



Alpinumisoflavone (5.4)

5.5.4 Structural elucidation of chandalone (5.2)

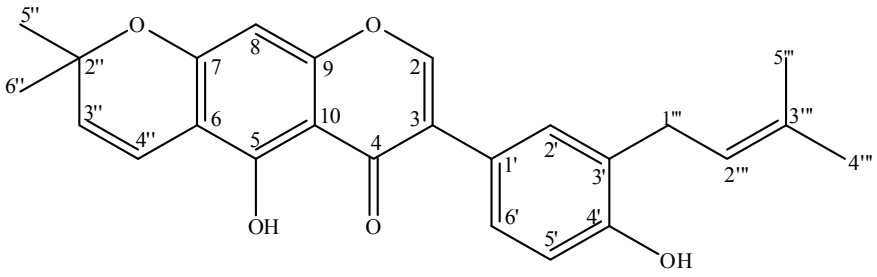
Compound 5.2 was obtained as a pale yellow solid (0.3 mg). ESI-MS indicated a protonated molecular ion at *m/z* 405 $[M+H]^{+}$, consistent with the molecular formula $C_{25}H_{24}O_{5}$.

This compound was obtained as a mixture with compound 5.3 (~1:1 mixture) and further separation provided a clear ^{1}H NMR spectrum (although some traces of compound 5.3 was still evident). The structural determination was achieved following careful analysis of the HSQC and HMBC spectra obtained on the mixture with compound 5.3.

The ^{1}H NMR spectrum showed a characteristic signal at δ 7.80 for an isoflavone skeleton (Ferrari et al., 2005; Mizuno et al., 1990). The spectrum also indicated a 2,2 dimethylpyran moiety [δ 1.47 (*s*, 2H), 5.62 (*d*, *J*=10 Hz), 6.72 (*d*, *J*=10 Hz)], an isoprenyl group [δ 1.78 (*s*, 3H), 1.79 (*s*, 3H),

3.40 (*br d*, *J*=7.2, 2H), 5.32 (*m*, 1H)], four aromatic proton signals [δ 6.33 (*s*, 1H), 7.26 (2H), 6.87 (*d*, *J*=9 Hz, 1H)], an aromatic hydroxyl proton [δ 5.21 (*s*, 1H)] and a chelated hydroxyl proton [δ 13.17 (*s*, 1H)]. The HSQC and HMBC correlations were identical with the 'tricyclic system' of alpinumisoflavone and the only difference observed was the attachment of an isoprenyl group at the B ring. The aromatic proton at δ 7.26 (H-2') showed long-range correlations to C-1''' (δ 30.1) of the isoprenyl unit, the proton (δ 3.40) attached to that carbon (C-1''') showed long-range correlations to C-2''' and C-4'''. This confirmed the isoprenyl group was attached at C-3'.

This compound was identified as chandalone (Falshaw et al., 1969). There have been four reported isolations of chandalone from plants (Deachathai et al., 2005; Mahabusarakam et al., 2004; Tahara et al., 1989). Only the ^{1}H NMR data, which was recorded at low field (100 MHz), has been reported (Tahara et al., 1989). ^{1}H and ^{13}C NMR data are presented in Table 5-2. This is the first reported isolation of this compound from the genus *Erythrina*.



Chandalone (5.2)

Table 5-2: NMR data of Chandalone (5.2)

Compound 5.2			
Position	δ_C	δ_H multiplicity (*J* in Hz)	HMBC
2	152.8	7.80 *s*	C-3, 9, 4
3	124.0		
4	181.3		
5	157.1	13.17 *s*	C-5, 6, 10
6	105.6		
7	159.5		
8	95.2	6.33 *s*	C-7, 9, 6, 10
9	157.4		
10	106.0		
1'	123.2		
2'	130.8	7.26*	C-1'", 1', 6', 4'
3'	127.2		
4'	154.9	5.21 *s*	C-5', 3', 4'
5'	116.1	6.87 *d* (9.0)	C-1', 3', 4'
6'	128.5	7.26*	C-,1'", 1', 2', 4'
2"	78.2		
3"	128.4	5.62 *d* (10.0)	C-2", 6, 5", 6"
4"	115.7	6.72 *d* (10.0)	C-2", 5, 6, 7
5"	28.5	1.47 *s*	C-6", 2", 3"
6"	28.5	1.47 *s*	C-5", 2", 3"
1'"	30.15	3.40 *br d* (7.2)	C-2'", 3', 2', 3'", 4'"
2'"	121.6	5.32 *m*	C-4'", 5'"
3'"	135.3		
4'"	26.08	1.78 *s*	C-5'", 2'", 3'"
5'"	18.19	1.79 *s*	C-4'", 2'", 3'"

Data acquired at 400 MHz for ^{1}H and 100 MHz for ^{13}C in *d*-chloroform
*signal overlapping with residual $CHCl_3$

5.5.5 Structural elucidation of lupalbigenin (5.7)

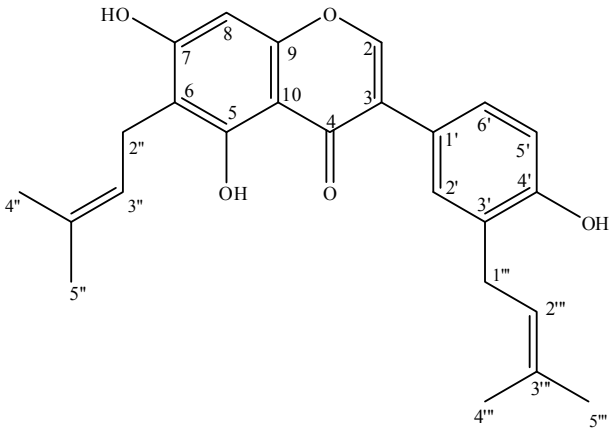
Compound 5.7 was obtained as a white amorphous solid (6.9 mg). The negative-ion HRESI-MS spectrum showed an $[M-H]^-$ peak at *m/z* 405.1701, corresponding to the molecular formula $C_{25}H_{26}O_5$.

The ^{1}H NMR spectrum showed a characteristic signal for an isoflavone skeleton by the presence of a proton singlet at δ 8.07 (Ferrari et al., 2005; Mizuno et al., 1990). From the proton spectrum two isoprenyl groups [δ 5.35 (*m*, 1H), 5.35 (*m*, 1H), 3.31 (*br d*, *J*=7.3 Hz, 4H), 1.75 (*s*, 3H), 1.70 (*s*, 3H), 1.68 (*s*, 3H), 1.62 (*s*, 3H)], an ABX aromatic spin system [δ 7.31 (*d*, *J*=2.1 Hz, 1H), 7.24 (*dd*, *J*= 8.2, 2.1 Hz, 1H), 6.86 (*d*, *J*=8.2 Hz, 1H)], an aromatic singlet (δ 6.46), and a chelated hydroxyl

proton [δ 13.33 (*s*)] were observed. These proton signals closely resemble that of a diprenylated 5,7,4'-trihydroxyisoflavone (Singhal et al., 1980). The only significant difference observed was the presence of an ABX aromatic spin system, which indicated that the B ring was 3',4'-disubstituted. Since hydroxylation at C-4' is expected on biogenetic grounds, the attachment of the one isoprenyl group was deduced to be at C-3' of ring B, rather than both at ring A.

The ^{13}C NMR spectrum showed the presence of five carbons bearing oxygen (δ 153.9, 160.6, 162.6, 155.9, 156.8), of which two are from C-2 and C-9 of the isoflavone skeleton. This indicated the presence of three hydroxylated groups and as expected biogenetically, one hydroxyl group at C-4', and since an ABX aromatic spin system was observed from B ring, the two other hydroxylated groups had to be at ring A. This was confirmed by the appearance of an aromatic proton singlet at δ 6.46 arising from ring A and therefore, the two hydroxylated groups were at C-5 and C-7.

Literature examination revealed that the compound was lupalbigenin (Singhal et al., 1980) and the NMR data were in agreement with the published literature (Maximo et al., 2002; Sekine et al., 1999; Yamamoto et al., 2002). ^{1}H and ^{13}C NMR data are presented in Table 5-3 and Table 5-4, respectively. This is the first reported isolation of this compound from *E. stricta*.



Lupalbigenin (5.7)

Table 5-3: Proton NMR data of flavanone and isoflavone compounds

Position	**δ_H multiplicity (*J* in Hz)**		
	Compound 5.3	Compound 5.4	Compound 5.7
2	5.28 *m*	7.80 *s*	8.07 *s*
3	2.78 *dd* (3.0, 17.2)		
3	3.04 *dd* (17.2, 13.0)		
4			
5		13.10 *s*	13.33 *s*
6	6.01 *s*		
7	6.12 *s*, OH		
8		6.33 *s*	6.46 *s*
9			
10			
1'			
2'	7.02 *d* (2.1)	7.36 *d* (8.5)	7.31 *d* (2.1)
3'		6.86 *d* (8.5)	
4'			
5'		6.86 *d* (8.5)	6.86 *d* (8.2)
6'	6.90 *d* (2.1)	7.36 *d* (8.5)	7.24 *dd* (2.1, 8.2)
1"	3.30 *d* (7.6)		3.31 *br d* (7.3)
2"	5.21 *t* (7.3)		5.25 *t* (7.3)
3"		5.62 *d* (10)	
4"	1.73 *s*	6.72 *d* (10)	1.75 *s*
5"	1.73 *s*	1.47 *s*	1.70 *s*
6"		1.47 *s*	
1"'			3.31 *br d* (7.3)
2"'			5.35 *t* (7.3)
3"'	5.64 *d* (9.1)		
4"'	6.32 *d* (9.1)		1.68 *s*
5"'	1.44 *s*		1.62 *s*
6"'	1.44 *s*		
1""	3.28 *d* (7.5)		
2""	5.28 *m*		
3""			
4""	1.73 *s*		
5""	1.73 *s*		

Data acquired at 400 MHz in *d*-chloroform

Table 5-4: Carbon NMR data of flavanone and isoflavone compounds

position	Compound 5.3	Compound 5.4	Compound 5.7
2	79.4	152.8	153.9
3	43.3	123.8	123.1
4	196.9	181.1	181.7
5	162.4	157.0	160.6
6	96.9	105.8	112.4
7	163.8	159.6	162.6
8	106.2	95.1	93.8
9	160.1	157.5	155.9
10	103.3	106.4	108.0
1'	129.9	123.1	123.3
2'	127.6	130.5	131.2
3'	129.6	115.8	124.2
4'	151.1	156.2	156.8
5'	121.8	115.8	115.5
6'	122.1	130.5	128.5
1"	21.9		29.1
2"	122.7	78.3	123.1
3"	135.1	128.4	132.4
4"		115.7	25.8
5"		28.8	17.9
6"		28.8	
1"'			22.0
2"'	76.6		123.6
3"'	131.5		131.6
4"'	122.5		25.9
5"'	28.3		17.8
6"'	28.3		
1""	21.9		
2""	121.6		
3""	132.6		
4""	26.2		
5""	18.11		

Data acquired at 400 MHz in *d*-chloroform

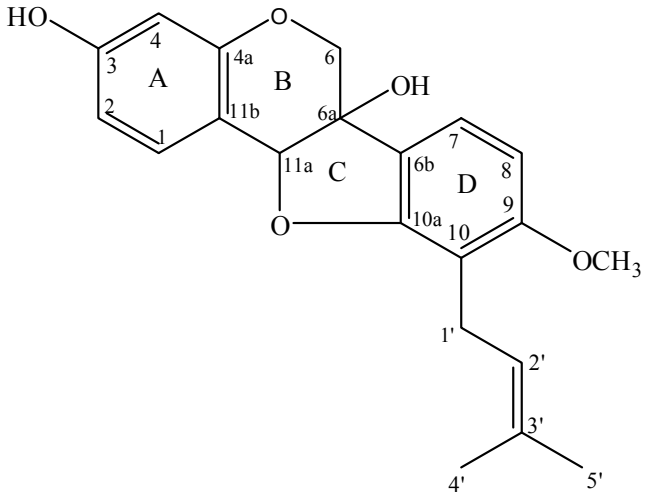
5.5.6 Structural elucidation of cristacarpin (5.10)

Compound 5.10 was obtained as a pale yellow amorphous solid (39.4 mg). The negative-ion HRESI-MS spectrum showed an $[M-H]^-$ peak at *m/z* 353.1387, corresponding to the molecular formula $C_{21}H_{22}O_5$.

The ^{1}H NMR spectrum showed signals at δ 4.18 (*d*, *J*=11.5 Hz, 1H), 3.97 (*d*, *J*=11.5Hz, 1H) and 5.23 (*s*, 1H) that were consistent with the presence of a 6a-hydroxypterocarpan skeleton (Tanaka et al., 1997). An ABX aromatic spin system [δ 7.36 (*d*, *J*=8.4 Hz, 1H), 6.54 (*dd*, *J*=8.4, 2.3 Hz, 1H), 6.35 (*d*, *J*=2.3 Hz, 1H)], two *ortho*-coupled aromatic protons [δ 7.12 (*d*, *J*=8.2 Hz, 1H), 6.48 (*d*, *J*=8.2 Hz, 1H)], a methoxy group [δ 3.80 (*s*, 3H)] and an isoprenyl group [δ 5.19 (*m*, 1H), 3.25 (*d*, *J*=7.1 Hz, 2H), 1.73 (*s*, 3H), 1.63 (*s*, 3H)] were also indicated in the proton spectrum.

The HSQC and HMBC spectra confirmed the 6a-hydroxypterocarpan skeleton. The proton signals at δ 4.18 (H-6), 3.97 (H-6) and 5.23 (H-11a) correlated to the carbon signals at δ 110.5 (C-6) and 84.4 (C-11a), respectively, in the HSQC spectrum, and showed long-range correlations to C-6a (δ 77.0), C-6b (δ 120.5) , C-10a (δ 158.7), C-11b (δ 112.9) and C-4a (δ 155.7) in the HMBC spectrum. The ABX spin system was assigned to ring A based on HSQC and HMBC correlations. These aromatic protons also correlated to a carbon bearing hydroxyl group (δ 157.1), which was assigned to C-3. The protons of the methoxy group correlated only to C-9 (δ 160.1) and hence the methoxy group was placed at C-9. Based on this information, the attachment of the isoprenyl group was either at C-7, C-8 or C-10. Since an *ortho*-coupled aromatic system was evident from the ^{1}H NMR spectrum, the only possible attachment for the isoprenyl group was at C-10. This was confirmed by HMBC correlations in which the methylene protons (δ 3.25) of the isoprenyl group correlated to C-9, C-10 and C-10a.

The spectroscopic data were in agreement with the published data of cristacarpin (Ingham and Markham, 1980; Dagne et al., 1993; Tanaka et al., 1996). However, the absolute stereochemistry could not be ascertained due to insufficient sample. ^{1}H and ^{13}C NMR data are presented in Table 5-5 and Table 5-6, respectively. This is the first reported isolation of this compound from *E. stricta*.



Cristacarpin (5.10)

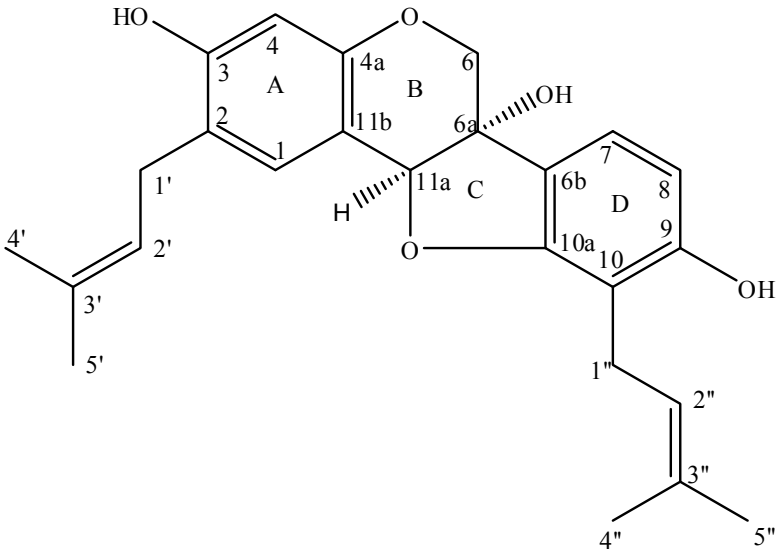
5.5.7 Structural elucidation of 2-(γ,γ-dimethylallyl)-6a-hydroxyphaseollidin (5.11)

Compound 5.11 was obtained as a pale yellow amorphous solid (34.2 mg). The negative-ion HRESI-MS spectrum showed an $[M-H]^-$ peak at *m/z* 407.1857, corresponding to the molecular formula $C_{25}H_{28}O_5$.

The signals from the ^1H NMR spectrum were consistent with a 6a-hydroxypterocarpan skeleton where the characteristic signals matched with that of cristacarpin (5.10). The additional spectral signal were observed for an isoprenyl group [δ 5.35 (*t*, *J*=7.3 Hz, 1H), 3.27 (*d*, *J*=7.3 Hz, 2H), 1.72 (*s*, 6H)], and two aromatic proton singlets [δ 6.31 (*s*, 1H), 7.18 (*s*, 1H)]. This additional isoprenyl group was placed at C-2 based on HMBC correlations observed from the methylene proton (δ 3.27, H-1') to C-1 and C-3 and the aromatic proton (δ 7.18, H-1) to the methylene carbon (C-1'). The latter also correlated to C-11a, thereby confirming the placement of the aromatic proton signal at δ 7.18 to C-1 and not at C-4.

This compound was identified as 2-(γ,γ-dimethylallyl)-6*a*-hydroxyphaseollidin (5.11) (O'Neill et al., 1986). The absolute stereochemistries at C-6a (*S*) and C-11a (*S*) were established from the specific optical rotation of -184.2º, which was in agreement with the literature (Ingham and Markham, 1980; Tanaka et al., 1997). NMR data were also in agreement with the published data

(Tanaka et al., 1997). ^{1}H and ^{13}C NMR data are presented in Table 5-5 and Table 5-6, respectively. This is the first reported isolation of this compound from *E. stricta*.

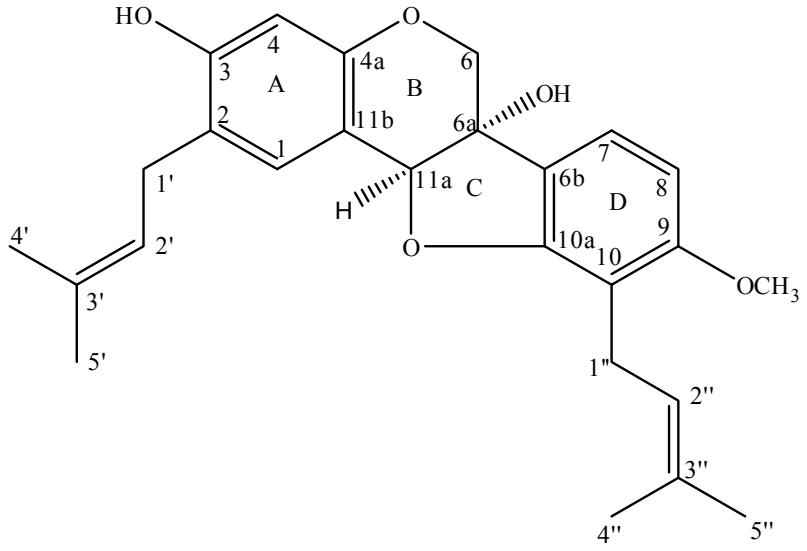


2-(γ,γ-Dimethylallyl)-6*a*-hydroxyphaseollidin (5.11)

5.5.8 Structural elucidation of erystagallin A (5.8)

Compound 5.8 was obtained as a pale yellow amorphous solid (13.7 mg). The negative-ion HRESI-MS spectrum showed an $[M-H]^-$ peak at *m/z* 421.2018, corresponding to the molecular formula $C_{26}H_{30}O_5$. The ^{1}H and ^{13}C NMR spectra were almost identical to that of compound 5.11. The only additional peak observed was the presence of a methoxy group. HSQC and HMBC correlations and comparison with compound 5.11 indicated the methoxy group was attached to C-9.

This compound was identified as erystagallin A (5.8) (Tanaka et al., 1997). The absolute stereochemistries at C-6a (*S*) and C-11a (*S*) were confirmed by the CD spectrum, which displayed positive (293 nm) and negative (249 nm) Cotton effects that were consistent with the literature for erystagallin A (Ingham and Markham, 1980). This was further supported by a negative specific optical rotation (Ingham and Markham, 1980; Tanaka et al., 1997). NMR data were in agreement with the literature. ^{1}H and ^{13}C NMR data are presented in Table 5-5 and Table 5-6, respectively. This is the first reported isolation of this compound from the stem bark of *E. stricta*.



Erystagallin A (5.8)

5.5.9 Structural elucidation of 1-methoxyerythrabyssin II (5.5)

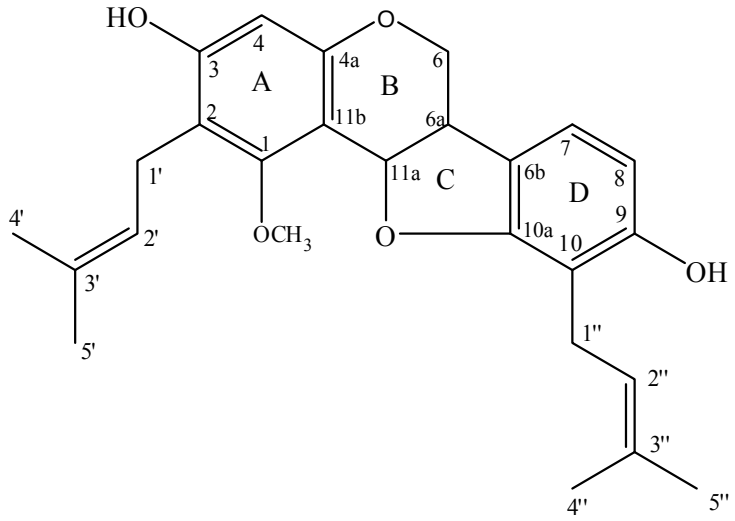
Compound 5.5 was obtained as a pale yellow amorphous solid (5.9 mg). The negative-ion HRESI-MS spectrum showed an $[M-H]^-$ peak at *m/z* 421.2014, corresponding to the molecular formula $C_{26}H_{30}O_5$.

The ^1H NMR spectrum of compound 5.5 showed signals at δ 4.14 (*dd*, *J*= 11.0, 4.9 Hz, 1H), 3.57 (*t*, *J*=11.0 Hz, 1H), 3.38 (*m*, 1H), and δ5.60 (*d*, *J*=6.4 Hz, 1H), which were consistent with a 6a-dehydroxypterocarpan skeleton (Sakurai et al., 2005; Subarnas et al., 1991). Further signals observed in the proton spectrum were two sets of isoprenyl groups [δ 5.23 (*t*, *J*=7.2 Hz, 1H), 3.50 (*m*, 2H), 1.82 (*s*, 3H), 1.75 (*s*, 3H) and 5.29 (*t*, *J*=7.2 Hz, 1H), 3.32 (*m*, 2H), 1.76 (*s*, 3H), 1.72 (*s*, 3H)], two *ortho*-coupled doublets [6.94 (*d*, *J*=8 Hz, 1H), 6.35 (*d*, *J*=8 Hz, 1H)], an aromatic singlet (δ 6.26, 1H), two broad singlet hydroxyl groups (δ 5.51, 1H and 5.31, 1H) and a methoxy group [δ 3.96 (*s*, 3H)].

Other than the proton signals for the 6a-dehydroxypterocarpan skeleton, the rest of the signals were almost identical to that of compound 5.11. The only additional feature was a methoxy group, which from HSQC and HMBC data indicated attachment at C-1.

The spectroscopic data matched the data of 1-methoxyerythrabyssin II (5.5) (Rukachaisirikul et al., 2008), which was recently isolated from the bark of *Erythrina subumbrans*. However, the absolute

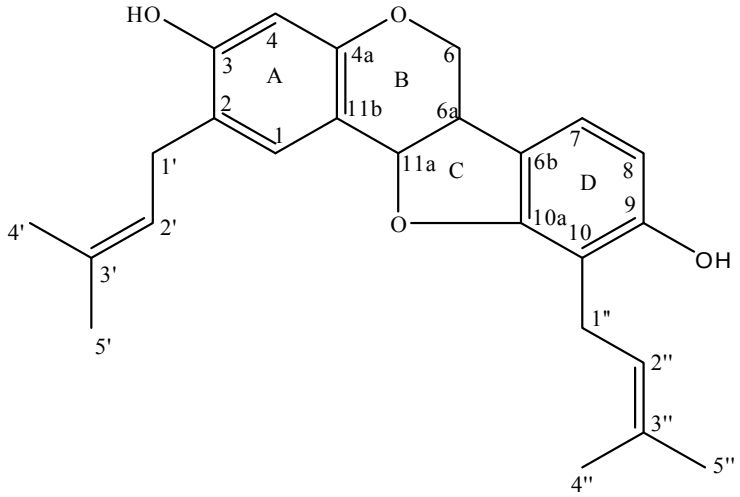
stereochemistry could not be ascertained due to insufficient sample. ^{1}H and ^{13}C NMR data are presented in Table 5-5 and Table 5-6, respectively. This is the first reported isolation of this compound from *E. stricta*.



1-Methoxyerythrabyssin II (5.5)

5.5.10 Structural elucidation of erythrabyssin II (5.6)

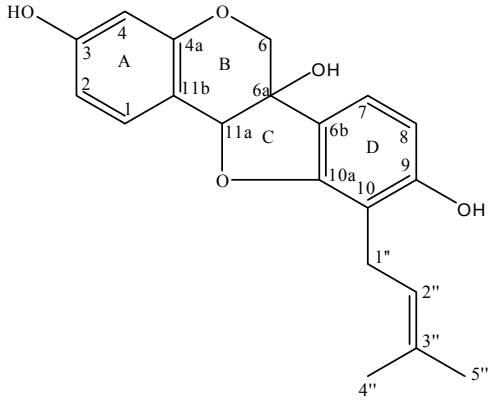
Compound 5.6 was obtained as a pale yellow amorphous solid (38.3 mg). The negative-ion HRESI-MS spectrum showed an $[M-H]^-$ peak at *m/z* 391.1907, corresponding to the molecular formula $C_{25}H_{28}O_4$. The ^{1}H NMR spectrum was almost identical to that of compound 5.5. The only difference was the lack of the methoxy group and an additional aromatic singlet at δ 7.25. The structure was deduced using HSQC and HMBC correlations. The spectroscopic data matched the reported data for erythrabyssin II (5.6) (Kamat et al., 1981; Tanaka et al., 1998). However, the absolute stereochemistry could not be ascertained due to insufficient sample. ^{1}H and ^{13}C NMR data are presented in Table 5-5 and Table 5-6, respectively. This is the first reported isolation of this compound from *E. stricta*.



Erythrabyssin II (5.6)

5.5.11 Structural elucidation of phaseollidin (5.9)

Compound 5.9 was obtained as a pale yellow amorphous solid (1.8 mg). The negative-ion HRESI-MS spectrum showed an $[M-H]^-$ peak at *m/z* 339.1233, corresponding to the molecular formula $C_{20}H_{20}O_5$. The ^1H NMR spectrum indicated a pterocarpan skeleton and a closer examination of the spectrum revealed that some of the peaks were almost identical to that of compound 5.6, with the only difference being the absence of the peaks for one isoprenyl group and the presence of an aromatic ABX spin system. The lack of the isoprenyl group was also evident by its molecular mass at *m/z* 324, which is one less isoprenyl group than for compound 5.6. The correlations observed from the HSQC and HMBC spectra for the aromatic protons of the ABX spin system to the carbons of ring A and B, confirmed the ABX spin system to be part of ring A and suggested that the isoprenyl group was part of ring D. This was confirmed by the methine proton signal (δ 5.26) from the isoprenyl group correlating to C-10. The spectroscopic data precisely matched the data for phaseollidin (5.9) (Tanaka et al., 1997). However, the absolute stereochemistry could not be ascertained due to insufficient sample. ^1H and ^{13}C NMR data are presented in Table 5-5 and Table 5-6, respectively. This is the first reported isolation of this compound from the stem bark of *E. stricta*.



Phaseollidin (5.9)

Table 5-5: Proton NMR data of pterocarpan compounds

(δ_H multiplicity (*J* in Hz)

No.	5.10 $CDCl_3$	5.11 Acetone D6	5.8 $CDCl_3$	5.5 $CDCl_3$	5.6 $CDCl_3$	5.9 $CDCl_3$
1	7.36 *d (*8.4)	7.18 *s*	7.23 *s*		7.25 *s*	7.34 *d* (8.3)
2	6.54 *dd* (8.4, 2.3)					6.56 *dd* (8.3, 2.4)
3						
4	6.35 *d* (2.3)	6.31 *s*	6.38 *s*	6.26 *s*	6.41 *s*	6.35 *d* (2.4)
4a						
6	4.18 *d* (11.5)	4.08 *d* (11.3)	4.10 *d* (11.3)	3.57 *t* (11.0)	3.59 t (11.0)	3.58 *t* (10.3)
6	3.97 *d* (11.5)	3.97 *d* (11.3)	3.99 *d* (11.3)	4.15 *dd* (11.0, 4.9)	4.20 *dd* (5.1, 11)	4.23 *m*
6*a*			2.35 *s*, OH	3.38* *m*	3.49 *m*	3.53 *m*
6*b*						
7	7.12 *d* (8.2)	6.99 *d* (8.0)	7.14 *d* (8.2)	6.94 *d* (8.0)	6.95 *d* (8.1)	6.95 *d* (8.0)
8	6.48 *d* (8.2)	6.42 *d* (8.0)	6.49 *d* (8.2)	6.35 *d* (8.0)	6.37 *d* (8.1)	6.38 *d* (8.0)
9						
10						
10*a*						
11*a*	5.23 *s*	5.22 *s*	5.24 *s*	5.6 *d* (6.4)	5.44 *d* (6.7)	5.46 *d* (6.6)
11*b*						
1'	3.25 *d* (7.1)	3.27 *d* (7.3)	3.33 *d* (7.3)	3.5* *m*	3.34 *d* (7.3)	3.26 *d* (7.4)
2'	5.19 *m*	5.35 *t* (7.3)	5.32 *t* (7.3)	5.23 *t* (7.2)	5.34 *m*	5.26 *t* (7.4)
3'						
4'	1.63 *s*	1.72 *s*	1.78 *s*	1.82 *s*	1.81* *s*	1.61 *s*
5'	1.73 *s*	1.72 *s*	1.78 *s*	1.75 *s*	1.80* *s*	1.73 *s*
1"		3.22 *d* (7.3)	3.27 *d* (7.3)	3.32* *m*	3.40 *m*	
2"		5.22 *m*	5.20 t (7.3)	5.29 *m*	5.26 *m*	
3"						
4"		1.72 *s*	1.74 *s*	1.76 *s*	1.79* *s*	
5"		1.59 *s*	1.64 *s*	1.72 *s*	1.75* *s*	
OMe	3.80 *s*		3.80 *s*	3.96 *s*	5.26 *br s*	
OH		4.79 *s*		5.55 *s*		
OH		8.42 (2H) *br s*		5.31 *s*		

*= interchangeable, Data acquired at 400 MHz

Table 5-6: Carbon NMR data of pterocarpan compounds

No.	5.10 $CDCl_3$	5.11 Acetone (D6)	5.8 $CDCl_3$	5.5 $CDCl_3$	5.6 $CDCl_3$	5.9 $CDCl_3$
1	132.5	132.5	132.2	159.6	132.0	133.1
2	110.5	123.0	121.7	114.2	121.0	110.4
3	157.1	156.8	155.6	157.4	155.1	157.0
4	103.8	103.3	104.0	100.3	103.9	103.9
4*a*	155.7	154.8	154.1	155.4	155.7	157.7
6	69.7	70.4	69.6	66.5	66.6	67.2
6*a*	77.0	77.1	77.0	39.6	40.1	41.0
6*b*	120.5	121.3	120.8	118.8	118.8	119
7	120.9	121.8	120.6	122.3	122.4	122.7
8	104.1	108.6	104.3	108.0	108.2	108.2
9	160.1	157.7	159.8	155.8	155.9	156.7
10	113.9	111.9	113.7	110.2	110.2	110.2
10*a*	158.7	159.9	158.6	158.8	158.4	158.1
11*a*	84.4	85.7	84.4	75.8	78.2	79.0
11*b*	112.9	113.2	112.8	107.4	112.4	113.3
1'	22.8	28.4	29.2	23.2	29.3	23.5
2'	122.1	123.9	121.9	122.3	121.4	123.6
3'	132.0	132.3	135.2	135.3	134.9	131.3
4'	18.0	17.8	18.1	18.1	17.9	18.2
5'	26.0	25.9*	25.7	25.9	25.8	25.9
1"		23.0	22.4	23.4	23.2	
2"		123.3	122.2	121.6	121.9	
3"		131.2	131.9	135.2	135.2	
4"		17.8	17.7	18.0	17.9	
5"		25.8*	25.7	25.9	25.8	
OMe	56.1		56.3	63.6		

*= interchangeable, Data acquired at 100 MHz

5.6 Antimicrobial activities of isolated compounds from published literature

A literature search indicated that all the compounds, with the exception of maackiaflavanone B (just recently isolated), have been reported to exhibit antimicrobial activities. The antimicrobial activities of these compounds, along with their MICs, are tabulated in Table 5-7. With the exception of alpinumisoflavone, a γ-γ-dimethylallyl (prenyl) group is present in all of these compounds. The presence of the prenyl group at specific positions in flavonoids has been shown to influence the biological activities of these molecules (Chi et al., 2001; Rukachaisirikul et al., 2007a; Tanaka et al., 2002; Tsuchiya et al., 1996).

The literature reviews indicated that compounds 5.2, 5.6, 5.7, 5.8, 5.9, 5.10 and 5.11 possessed strong inhibitory activities against MRSA strains, with an MIC range from 0.78 to 100 μg/ml, and the most potent inhibition of the MRSA strains was exhibited by compound 5.6, which had an MIC range from 0.78-3.13 μg/ml.

Since the isolation of these compounds was directed by bioassay guided isolation using *S. aureus*, and also as some of the compounds have not yet been reported for their activities against methicillin resistant *Staphylococcus aureus* (MRSA), it was decided to test the isolated compounds against the *S. aureus* strains, including two MRSA strains (the MRSA strains used for the study were different from the strains reported in the literature).

Table 5-7: The reported antimicrobial activities of the 11 isolated compounds

Compound	Microorganisms tested	MIC range (μg/mL)	References
5-Hydroxysophoranone (5.1)	*Streptococcus agalactiae* 16442 *S. agalactiae* 11159 *S. sobrinus*	25 0.78 6.25	(Rukachaisirikul et al., 2007a)
Chandalone (5.2)	*S. aureus* ATCC 25923 MRSA SK1	128 16	(Mahabusarakam et al., 2004)
Maackiaflavanone B (5.3)	Not reported		
Alpinumisoflavone (5.4)	*Mycobacterium smegmatis* *Enterobacter cloacae* *Morganella morganii* *Proteus mirabilis* *S. aureus* *Bacillus stearothermophilus* *Candida albicans*	19.53 39.06 39.06 78.12 39.06 39.06 78.12	(Kuete et al., 2008)
1-Methoxyerythrabyssin II (5.5)	*Mycobacterium tuberculosis* (H37Ra strain)	50	(Rukachaisirikul et al., 2008)
Erythrabyssin II (5.6)	*Streptococcus sp* *S. aureus* and drug resistant strains MRSA and VRSA)	1.56-0.78 3.13-0.78	(Rukachaisirikul et al., 2007a)
Lupalbigenin (5.7)	*S. aureus* ATCC 25923 MRSA SK1	8-2 8-4	(Deachathai et al., 2005; Mahabusarakam et al., 2004)
Erystagallin A (5.8)	*Streptococcus sp* *Actinomyces viscosus* *Lactobacillus casei* *S. aureus* and drug resistant strains MRSA and VRSA)	12.5-0.78 3.13 12.5 6.25-0.78	(Rukachaisirikul et al., 2007a; Sato et al., 2003)
Phaseollidin (5.9)	*E. coli* *S. aureus* *B. subtilis* *C. mycoderma* Methicillin resistant *S. aureus*	5* 0.5* 0.5* 0.1* 25	(Chacha et al., 2005; Tanaka et al., 2002)
Cristacarpin (5.10)	*E. coli* *S. aureus* *B. subtilis* *C. mycoderma* Methicillin resistant *S. aureus*	0.10* 0.01* 0.01* 0.5* 100	(Chacha et al., 2005; Tanaka et al., 2002)
2-(γ,γ-Dimethylallyl)-6a-hydroxyphaseollidin (5.11)	Methicillin resistant *S. aureus* *Streptococcus sp* *A. viscosus* *L. casei*	12.5-6.25 25-6.25 3.13 12.5	(Sato et al., 2003; Tanaka et al., 2002)

* minimum inhibitory amount of compound in μg

5.7 Antibacterial studies with isolated compounds

Four strains of *S. aureus*, including two MRSA strains, were used to further determine the antibacterial nature of the isolated compounds. The MTT microdilution assay was used for this study and the isolated compounds were tested at various concentrations, with the maximum concentration at 250 µg/ml, to record the MICs. Compounds 5.7 and 5.9 could only be tested against three strains due to their low quantities. The antibiotics kanamycin, chloramphenicol and gentamicin were used as positive controls. The results of the antibacterial activities are tabulated in Table 5-8 with their respective MIC values.

Except for compounds 5.2 and 5.3, antibacterial activity was observed for all the isolated compounds against *S. aureus* ATCC 9144 strain (non-resistant). Compounds 5.2 and 5.3 were initially isolated as a mixture that showed a clear zone of inhibition in the TLC-bioautography assay. Because of the low yield of compound 5.2, after separation from compound 5.3, the maximum concentration that was tested was 25 µg/ml. Compounds 5.4, 5.6, 5.8, 5.10 and 5.11 showed inhibitory activities against the four strains while compound 5.7, which was tested only against three strains due to limited sample, showed the highest level of inhibitory activities against the three strains with MICs in the range of 9.38 and 18.75 µg/ml. The MICs of compounds 5.4, 5.6 and 5.11 against the two strains of MRSA were 31.25 µg/ml and 62.5 µg/ml each, respectively. The antibiotics gentamicin and kanamycin did not show inhibitory activities against the MRSA strains even at a concentration of 250 µg/ml, while the MIC of chloramphenicol was 31.25 µg/ml. Compound 5.5 was not active except against *S. aureus* ATCC 9144, which was the strain used in the bioassay guided assay. The minimum bactericidal concentrations (MBC) were also determined and except for compound 5.1, in all cases, the MBCs were equivalent to MIC values.

Table 5-8: Antibacterial activities (MIC, µg/mL) of isolated compounds from *Erythrina stricta*

Compound	MICs (µg/ml)			
	S. aureus ATCC 9144	*S. aureus* ATCC 29213 Methicillin sensitive	*S. aureus* ATCC BAA 1026 MRSA CMRSA	*S. aureus* wild MDR MRSA clinical isolate
5-Hydroxysophoranone (5.1)	62.5	na @ 250	na @ 250	250
Maackiaflavanone B (5.3)	na @ 250	na @ 250	na @ 250	na @ 250
Alpinumisoflavone (5.4)	31.25	31.25	31.25	31.25
Chandalone (5.2)	na @ 25	na @ 25	na @ 25	na @ 25
Lupalbigenin (5.7)	9.38	9.38	18.75	nt
Cristacarpin (5.10)	62.5	62.5	125	125
2-(γ,γ-Dimethylallyl)-6*a*-hydroxyphaseollidin (5.11)	31.25	31.25	62.5	62.5
Erystagallin A (5.8)	31.25	31.25	250	250
1-Methoxyerythrabyssin II (5.5)	250	na @ 250	na @ 250	na @ 250
Erythrabyssin II (5.6)	31.25	62.5	62.5	62.5
Phaseollidin (5.9)	100	100	na @ 200	Nt
Kanamycin	15.63	31.25	na @ 250	na @ 250
Chloramphenicol	nt	31.25	31.25	31.25
Gentamicin	nt	3.91	na @ 250	na @ 250

na=not active, nt=not tested

To conclude, the inhibitory activities based on the skeletal structures, for the isoflavones, compound 5.7 had more pronounced activity than compound 5.2 and compound 5.4; for the flavanones, compound 5.1 was active against two strains (one MRSA strain) whereas compound 5.3 was not active; and for the pterocarpans compounds 5.6 and 5.11 showed similar activities whereas for compounds 5.5 and 5.8 (methyl ether substitutions of compounds 5.6 and 5.11, respectively) antibacterial activities were significantly reduced, and compound 5.9 was not active against MRSA strains.

5.8 Conclusions and future directions

The bioassay guided isolation of the DCM partition of the ethanolic extract of the stem bark of *Erythrina stricta* led to the isolation of 11 compounds. This is the first reported isolation of any of these 11 compounds from the stem bark of this plant. Compounds 5.4, 5.5, 5.6, 5.7, 5.9, 5.10 and 5.11 have not been previously reported from this species and compounds 5.2 and 5.3 have not been reported from the genus *Erythrina*.

The literature reviews on these isolated compounds showed that all the compounds, except for compound 5.3, have been reported to exhibit antimicrobial activities. In our antibacterial study, the antibacterial activities of the isolated compounds were evaluated against 4 different strains of *S. aureus* including two drug-resistant strains. The most potent inhibition was shown by Lupalbigenin (5.7). Compounds 5.4, 5.6, 5.8, 5.10 and 5.11 were active against all the four strains and compounds 5.2 and 5.3 were not active at 25 μg/ml and 250 μg/ml, respectively. This study supports the use of this plant by the Naga people, including Chungtia villagers, for treating skin related infections.

Even though most of the active compounds have been isolated and identified from the DCM extract, the EtOAc extract, which was also active, should be further examined for its antibacterial components. A TLC-bioautography study of the DCM and EtOAc extracts indicated the presence of different active components for both. Therefore, there is potential to isolate other antimicrobial compounds from the EtOAc extract in the future.

5.9 Experimental

5.9.1 Reagents and equipment

All the solvent used for chromatography were of analytical grade and re-distilled before use. Normal phase column chromatography was performed using silica gel 60 (0.040-0.063 mm) from Merck (Germany). Analytical normal phase thin layer chromatography (TLC) was performed on fluorescent Merck silica gel F_{254} plates (Germany). The TLC plates were visualised using UV light (254 nm and 365 nm) or developed by spraying with vanillin stain (3 g vanillin in 100 ml absolute ethanol and 0.5 ml sulphuric acid) and heating (120ºC). Organic solvents were evaporated using a

Büchi rotary evaporator (Germany) under reduced pressure and 40ºC and traces of solvent were removed using a Pascal 2005 SD high vacuum pump from Alcatel.

The ^{1}H (400 MHz), ^{13}C (100 MHz), HSQC and HMBC NMR spectra were recorded on a Bruker Avance AMX 400 spectrometer (Germany) using standard pulse sequences. Chemical shifts were calculated relative to the chloroform (^{1}H δ 7.26 and ^{13}C δ 77.2) and acetone (^{1}H δ 2.04 and ^{13}C δ 29.8) solvent peaks.

5.9.2 Preparation of plant material of *Erythrina stricta*

The powdered stem bark (750 g) was extracted successively with 70% aqueous EtOH (3 x 2.5 l) and the EtOH was evaporated leaving the aqueous extract, which was then partitioned with *n*-hexane (2 x 0.5 l), DCM (2 x 0.5 l), EtOAc (4 x 0.5 l) and *n*-BuOH (3 x 0.5 l) to afford five partitioned fractions. Each fraction (150 ml) were concentrated to dryness, under reduced pressure and dried under high vacuum, to afford their corresponding fractions as Es-hex (yellow gum, 15.2 mg), Es-DCM (dark yellow solid, 25.6 mg), Es-EtOAc (dark yellow solid, 29.3 mg), Es-BuOH (dark brown solid, 34.6 mg) and Es-Water (dark brown solid, 43.2 mg). The fractions were then evaluated for antibacterial activity against *E. coli*, *S. aureus* and *P. aeruginosa,* by the microdilution MTT and TLC-bioautography assays.

5.9.3 Bioassay guided isolation of antibacterial compounds from DCM fraction

The remaining DCM fraction (850 ml) was dried following a similar protocol above to yield 12.8 g of a dark yellow solid, labelled as Es-DCM extract. A TLC-bioautography assay (see section 5.8.6) was performed on the Es-DCM extract, with the TLC plate developed in chloroform and methanol (19:1), against *S. aureus*. A portion of the Es-DCM extract (9.2 g) was subjected to column chromatography (silica gel; $CHCl_3$-MeOH gradient) to afford 16 fractions (A1 to 16), and TLC-bioautography was performed on all the fractions. Fraction A6 (5.9 g), which contained 6 clear active spots by TLC-bioautography (hexane:EtOAc, 4:1), was subjected to further CC (silica gel; hexane-EtOAc, 3:1, 1:1 and 1:3 EtOAc and DCM-MeOH, 2:1) to afford 46 fractions and following TLC-bioautography results of the fractions, they were pooled to 13 fractions (B1 to 13).

Fraction B3 was further subjected to column chromatography (silica gel; hexane-acetone, 8:2, 2:1) to afford 29 fractions (C1 to 29). Fractions C20 to 24 afforded compound **5.1** (220.5 mg).

Fractions C13 to 17 (40 mg) was further subjected to column chromatography (silica gel; toluene) to afford 10 fractions (D1 to D10). Fraction D7 (7 mg) was again subjected to column chromatography (silica gel; hexane-EtOAc, 8:2 and EtOAc) to afford E1-E35 fractions. Fractions E2-4 afforded compound **5.2** (0.3 mg) and fractions E5-19 afforded a mixture of compound **5.2** and compound **5.3** (1.3 mg, 1:1).

Fraction B6 (300 mg), which showed two major active spots on TLC-bioautography (hexane: EtOAc, 3:1), was subjected to column chromatography (silica gel; toluene-EtOAc) to afford 49 fractions (F1-F49). Fractions F15-F24 (90 mg) was subjected to column chromatography (silica gel; $CHCl_3$) to afford 36 fractions (G1-G36). Fractions G22-G29 afforded compound **5.4** (28.50 mg).

Fraction B8 (380 mg), which showed two major active spots on TLC-bioautography (hexane:EtOAc, 2:1), was subjected to column chromatography (silica gel; DCM and DCM-MeOH, 50:1, 20:1) to afford 57 fractions (H1-H57). Fractions H26-H28 afforded compound **5.5** (5.9 mg) and fraction H31-H33 afforded compound **5.6** (23.5 mg).

Fraction B9 (500 mg), which showed 4 active spots close to each other on TLC-bioautography (hexane:EtOAc, 6:4), was chromatographed further on column chromatography (silica gel; hexane-acetone, 3:1) to afford 49 fractions (J1-J49). Fractions J18-J25 (190 mg) was subjected to column chromatography (silica gel; DCM and DCM-MeOH, 50:1) to afford 48 fractions (K1-K48). Fractions K6-K7 afforded compound 6-7 (6.9 mg) and fractions K18-K26 afforded compound **5.8** (13.7 mg). Fractions J28-J33 (76.5 mg) was subjected to column chromatography (silica gel; DCM-acetone, 50:1) to afford 40 fractions (L1-L40). Fraction L6 afforded compound **5.9** (1.8 mg) and fractions L16-L18 afforded compound **5.10** (3.3 mg).

Fraction B10 (300 mg), which showed a large active spot on TLC-bioautography (hexane:EtOAc, 6:4), was chromatographed on column chromatography (silica gel; $CHCl_3$-MeOH gradient) to afford 43 fractions (M1-43). Fractions M5-9 (50 mg) was subjected to normal phase preparative TLC silica plate (solvent system; $CHCl_3$-MeOH, 20:1, R_f band from 0.2 to 0.6) to afford compound **5.10** (39.4 mg).

Fraction B12 (500 mg), which showed a large active spot on TLC-bioautography (hexane:EtOAc, 6:4), was subjected to column chromatography (silica gel; $CHCl_3$-MeOH, 40:1) to afford 25 fractions (N1-N25). Fractions N15-N18 afforded compound **5.11** (34.2 mg).

5-Hydroxysophoranone (5.1): white amorphous solid; ESI-MS *m/z* 476$[M+H]^+$; HRESI-MS *m/z* 475.2479 $[M-H]^-$ (calc. for $C_{30}H_{35}O_5$, *m/z* 475.2484). ^1H, ^{13}C and 2-D NMR data ($CDCl_3$), see Table 5-1. CD (MeOH) $[\theta]_{334}$ 6964.3, $[\theta]_{291}$ -33343.6. $[\alpha]_D$ -10.62º (MeOH). Mp 139-140º.

Maackiaflavanone B (5.3): pale yellow solid; ESI-MS *m/z* 475 $[M+H]^+$. ^1H and ^{13}C NMR data are presented in Table 5-3 and Table 5-4, respectively.

Alpinumisoflavone (5.4): pale yellow solid; ESI-MS *m/z* 337 $[M+H]^+$. ^1H and ^{13}C NMR data are presented in Table 5-3 and Table 5-4, respectively.

Chandalone (5.2): pale yellow solid; ESI-MS *m/z* 405 $[M+H]^+$. ^1H, ^{13}C and 2-D NMR data ($CDCl_3$), see Table 5-2.

Lupalbigenin (5.7): white amorphous solid; ESI-MS *m/z* 407 $[M+H]^+$; HRESI-MS *m/z* 405.1701 $[M-H]^-$ (calc. for $C_{25}H_{25}O_5$, *m/z* 405.1702). ^1H and ^{13}C NMR data are presented in Table 5-3 and Table 5-4, respectively.

Cristacarpin (5.10): pale yellow amorphous solid; ESI-MS *m/z* 355 $[M+H]^+$; HRESI-MS *m/z* 353.1387 $[M-H]^-$ (calc. for $C_{21}H_{21}O_5$, *m/z* 353.1389). ^1H and ^{13}C NMR data are presented in Table 5-5 and Table 5-6, respectively.

2-(γ,γ-Dimethylallyl)-6*a*-hydroxyphaseollidin (5.11): pale yellow amorphous solid; ESI-MS *m/z* 409 $[M+H]^+$; HRESI-MS *m/z* 407.1857 $[M-H]^-$ (calc. for $C_{25}H_{27}O_5$, *m/z* 407.1858). ^1H and ^{13}C NMR data are presented in Table 5-5 and Table 5-6, respectively. $[\alpha]_D$ -184.2º (MeOH).

Erystagallin A (5.8): pale yellow amorphous solid; ESI-MS *m/z* 423 $[M+H]^+$. HRESI-MS *m/z* 421.2018 $[M-H]^-$ (calc. for $C_{26}H_{29}O_5$, *m/z* 421.2015). ^1H and ^{13}C NMR data are presented in Table 5-5 and Table 5-6, respectively. $[\alpha]_D$ -112.1º (MeOH).

1-Methoxyerythrabyssin II (5.5): pale yellow amorphous solid; HRESI-MS *m/z* 421.2014 $[M-H]^-$ (calc. for $C_{26}H_{29}O_5$, *m/z* 421.2015). ^1H and ^{13}C NMR data are presented in Table 5-5 and Table 5-6, respectively.

Erythrabyssin II (5.6): pale yellow amorphous solid; ESI-MS *m/z* 393 $[M+H]^+$; HRESI-MS *m/z* 391.1907 $[M-H]^-$ (calc. for $C_{25}H_{27}O_4$, *m/z* 391.1909). ^1H and ^{13}C NMR data are presented in Table 5-5 and Table 5-6, respectively.

Phaseollidin (5.9): pale yellow amorphous solid; HRESI-MS negative-ion mode: *m/z* 339.1233 $[M-H]^-$ (calc. for $C_{20}H_{19}O_5$, *m/z* 339.1232). ^1H and ^{13}C NMR data are presented in Table 5-5 and Table 5-6, respectively.

5.9.4 Antibacterial assays of fractions and isolated compounds

5.9.5 MTT-microdilution assay

The initial fractions were tested for antibacterial activities against *S. aureus* by the MTT microdilution assay, and the isolated compounds were tested against four *S. aureus* strains *i.e*. *S. aureus* ATCC 9144, *S. aureus* ATCC 29213 methicillin sensitive, *S. aureus* ATCC BAA 1026 MRSA CMRSA and *S. aureus* MDR MRSA clinical isolate. The same procedure was followed as described in Chapter 4 for the MTT microdilution assay.

5.9.6 TLC-bioautography assay

Normal phase silica gel TLC plates were heated at 100ºC for 2 hours prior to use. The samples were spotted on the TLC plates and developed with an appropriate solvent. The solvents were allowed to dry at room temperature for a few hours or if the solvent was *n*-butanol it was dried overnight. A single colony of the appropriate microorganism was picked form the stock culture and transferred into the liquid medium (Mueller Hinton II broth) and allowed to grow overnight at 37ºC, shaking at 150 rpm. After incubation, the optical density (OD) of the growth culture was adjusted to 0.08 by diluting it with fresh liquid medium. A small volume of this adjusted suspension of microorganisms was poured into a watch glass or a petri plate under sterile condition and the developed TLC plate (front side of TLC plate facing inward) was gently placed on the surface of this liquid medium and allowed to stay for 5 to 10 seconds. Afterwards, this inoculated TLC plate was transferred to a petri plate containing an agar medium (Mueller Hinton II agar), with the front side of the TLC plate facing outward. This was incubated at 37ºC for 18 to 20 hours and after incubation, a methanolic solution of MTT (5 mg/ml) was gently poured on the surface of

the TLC plate using a micropipette. The clear zones of inhibition were observed against a dark purple background, after incubating for 15 to 30 mins.

Chapter 6

Antibacterial studies and isolation of compounds from Prunus persica

6.1 Background

Prunus persica is used by the Chungtia villagers to treat typhoid disease, dysentery, diarrhoea and skin related infections. A literature review on *P. persica* is presented in Chapter 3. The results of the antibacterial screening studies (Chapter 4) indicated that the 70% aqueous ethanolic root extract of *P. persica* possessed strong inhibitory activity against *S. aureus* (MIC 312 µg/ml). Therefore, the root extract of *P. persica* was selected for further studies to isolate antibacterial compounds and to evaluate the antibacterial activity of the isolated compounds

6.2 Isolation of compounds from root extract of *Prunus persica*

The dried powdered root of *P. persica* was extracted with 70% aqueous ethanol in a similar manner to that described in Chapter 4, but on a larger scale, and the aqueous portion, after evaporating the ethanol, was successively partitioned with *n*-hexane, DCM, EtOAc, and *n*-butanol to give five partitioned fractions (Pp-Hex, Pp-DCM, Pp-EtOAc, Pp-BuOH and Pp-Water extract, respectively).

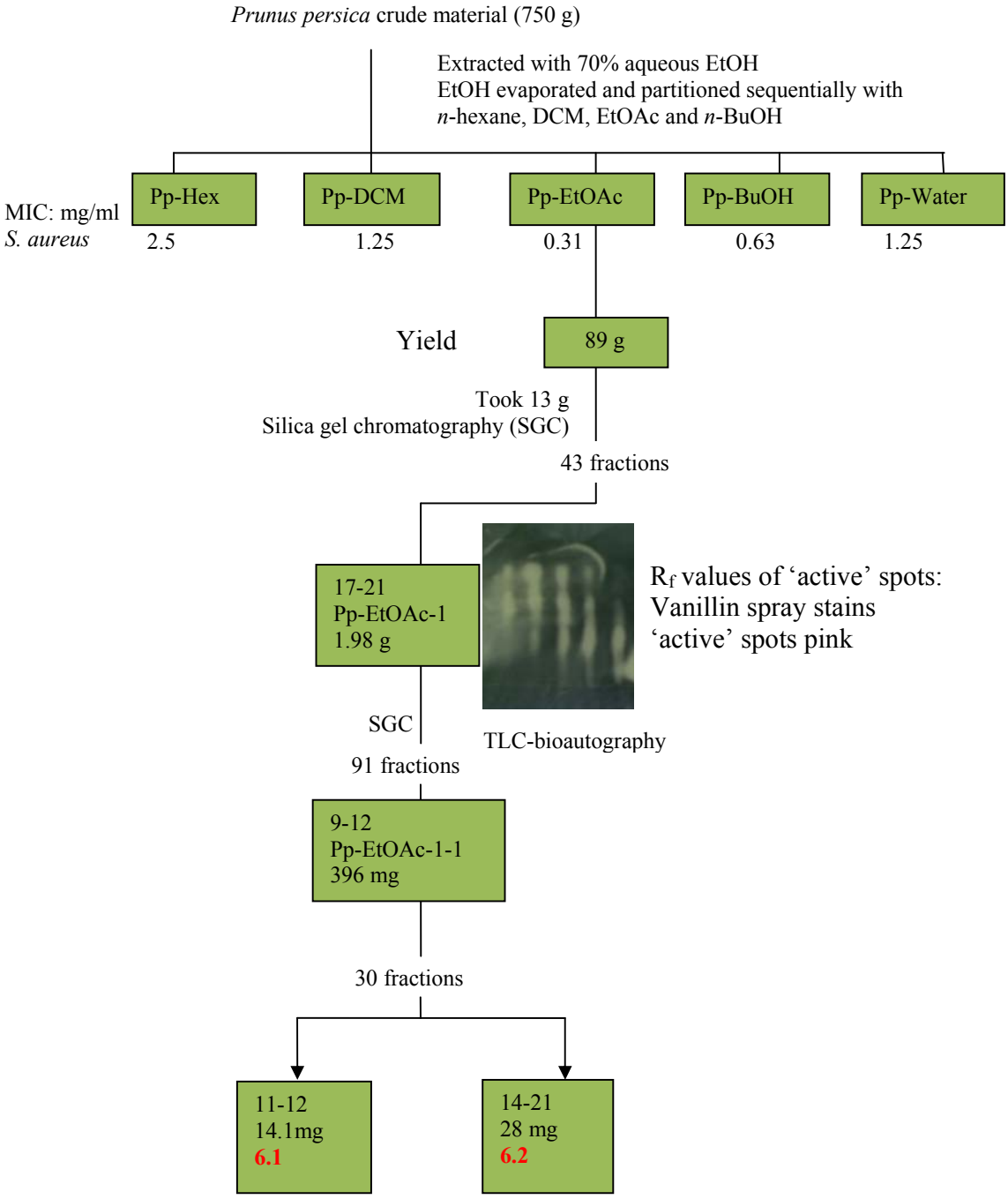
The antibacterial activities of the partitioned fractions were tested against *S. aureus* by the MTT microdilution assay. *E. coli* and *P. aeruginosa* were not tested because the crude extract did not show any activity against these two microorganisms. The results of the antibacterial activities of the partitioned fractions are provided in Table 6-1.

Table 6-1: Antibacterial activities of the partitioned fractions of *P. persica*

Extract	MIC (µg/ml) *S. aureus*
Pp-Hex	2500
Pp-DCM	1250
Pp-EtOAc	312
Pp-BuOH	625
Pp-Water	1250
Kanamycin (control)	2

All partitioned fractions were found to exhibit activity, with the ethyl acetate partition (Pp-EtOAc) being the most active, with an MIC of 312 µg/ml. The ethyl acetate fraction was therefore separated by normal phase silica gel column chromatography, eluting with chloroform and methanol of increasing polarity. This gave 43 fractions and each fraction was tested against *S. aureus* by TLC-bioautography. The fractions 17 to 21 showed four active spots on the TLC chromatogram and they were pooled (Pp-EtOAc-1). The R_f values of these active spots were compared with the R_f values of the spots (pink) produced by vanillin stain on the same fraction.

These R_f values were consistent with the R_f values of the active spots in the TLC-bioautography. Therefore, in order to minimise time, further analyses of the active fractions were carried out using the vanillin stain as a guide, rather than the TLC-bioautography. Fraction Pp-EtOAc-1 was further separated by silica gel column chromatography, eluting with chloroform, then chloroform and methanol mixtures of increasing polarities, to afford 91 fractions. Fractions 9 to 12 (Pp-EtOAc-1-1) showed two pink spots on the TLC plate and so they were pooled together and further separation was done with silica gel column chromatography. This led to the isolation of compounds 6.1 and 6.2. The flow chart of the isolation is presented in Scheme 6-1.



Scheme 6-1: Fractionation of *Prunus persica*

6.3 Identification of compounds

6.3.1 Structural elucidation of afzelechin (6.1)

Compound 6.1 was obtained as a creamish amorphous solid. ESI-MS indicated a protonated molecular ion at *m/z* 275 $[M+H]^+$, consistent with the molecular formula $C_{15}H_{15}O_5$.

In the ^{1}H NMR spectrum, the proton signals at δ 4.59 (*d*, *J*=8.0 Hz, H-2), 4.00 (*m*, H-3), 2.95 (*dd*, *J*=5.5, 16.1 Hz, H-4eq) and 2.53 (*dd*, *J*=8.5, 16.1 Hz, H-4ax) were characteristic of a flavan C ring of a flavan-3-ol skeleton with a phloroglucinol pattern for ring A [δ 6.02 (*d*, *J*=2.1 Hz, H-8) and 5.87 (*d*, *J*=2.1 Hz, H-6)] (Hussein et al., 1999; Zhou et al., 2005). The coupling constant (*J*=8.0 Hz) of the flavan H-2 signal indicated a 2,3-*trans* configuration (Min-Won et al., 1992) and this was confirmed by the chemical shift appearance of a C-2 signal at δ 82.7 (Vdovin et al., 1997). The ^{1}H NMR spectrum also revealed AA'BB' spin system at δ 7.25 (*d*, *J*=8.5 Hz, H-2', H-6') and 6.82 (*d*, *J*=8.5 Hz, H-3', H-5'). In the HMBC spectrum, the proton signal at δ 7.25 correlated to the carbon signal at δ 82.7, which was assigned to C-2 due to correlation to the proton signal at δ 4.59 (H-2) in the HSQC spectrum. Based on this spectroscopic evidence and by comparison with the literature, the compound was identified as afzelechin (6.1) (Saijyo et al., 2008; Wan and Chan, 2004). However, the absolute stereochemistry could not be ascertained due to insufficient sample. This was first isolated from *Corymbia calophylla* (formerly *Eucalyptus calophylla*) from Australia (Hillis and Carle, 1960) and since then, has been isolated from other plants (Baek et al., 1994; Balde et al., 1995). This compound has also been synthesised (Wan and Chan, 2004). NMR data are provided in Table 6-2.

6.3.2 Structural elucidation of [*ent-epiafzelechin-(2α→O→7,4α→8)-(-)-afzelechin*] (6.2)

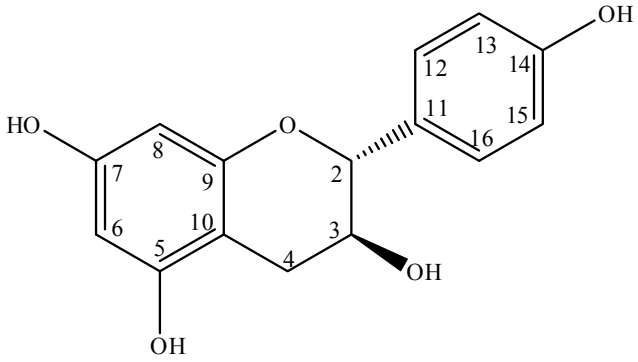
Compound 6.2 was obtained as white crystals. The negative-ion HRESI-MS spectrum showed an $[M-H]^-$ peak at *m/z* 543.1291, corresponding to the molecular formula $C_{30}H_{24}O_{10}$.

The diagnostic feature of the ^{1}H NMR spectrum was the presence of an isolated AB system in the heterocyclic proton region at δ 4.24 (*d*, *J*=3.5 Hz, H-3) and 4.26 (*d*, *J*=3.5 Hz, H-4), which are characteristic of the C ring protons of A-type proanthocyanidins (Kolodziej et al., 1991). The other signals observed were at δ 4.14 (*m*, H-3'), 3.06 (*dd*, *J*=5.5, 16.5 Hz, H-4'), 2.61 (*dd*, *J*=9.0, 16.5

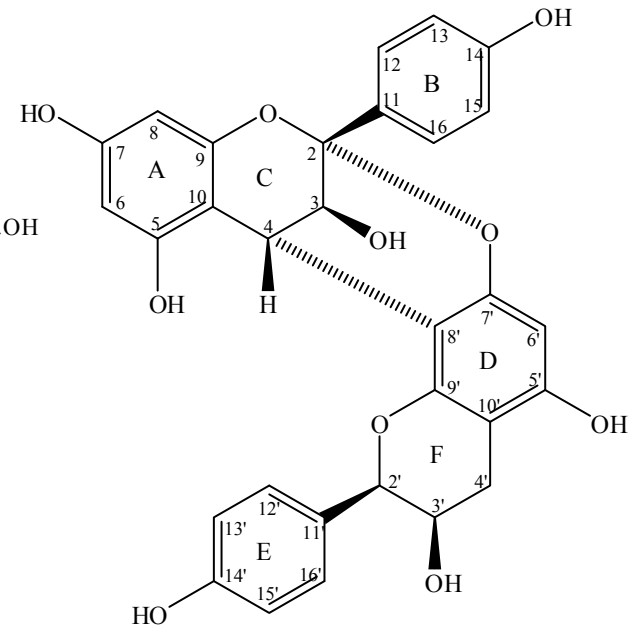
Hz, H-4') and 4.83 (*d*, *J*=8.2 Hz, H-2'), which are the signals arising from the lower flavan-3-ol moiety of A-type proanthocyanidins. The ^{1}H NMR spectrum also showed signals for two *meta*-coupled doublets [δ 5.92 (*d*, *J*=2.2 Hz, H-6) and 6.07 (*d*, *J*=2.2 Hz, H-8)] (characteristic with a phloroglucinol pattern for ring A of a flavan-3-ol skeleton), two AA'BB' spin system [δ 7.55 (*d*, *J*=8.6 Hz, H-12, H-16), 6.86 (*d*, *J*=8.6 Hz, H-13, H-15), 7.40 (*d*, *J*=8.6 Hz, H-12', H-16') and 6.90 (*d*, *J*=8.6 Hz, H-13', H-15')] and an aromatic proton at δ 6.14 (*s*).

The ^{13}C NMR spectrum displayed the presence of six aliphatic and 20 aromatic signals. The carbon resonances at δ 100.1 (C-2) and 28.8 (C-4) indicated that the two flavan-3-ol units are joined *via* a C-4 carbon-carbon and a C-2 ether linkage (Drewes et al., 1992; Liu et al., 2007; Prasad, 2000). In the HMBC spectrum, the proton at δ 4.26 (H-4) showed a $^{2}J_{CH}$ interaction with C-3, C-10 and C-8', and a $^{3}J_{CH}$ interaction with C-2, C-5, C-9, C-7' and C-9', confirming the presence of a dimeric linkage at C-4 and C-2. In the CD spectrum, a strong negative Cotton effect in the diagnostic wavelength region (233 nm) was observed, which reflected a 2α,4α configuration in the C ring (Kolodziej et al., 1991).

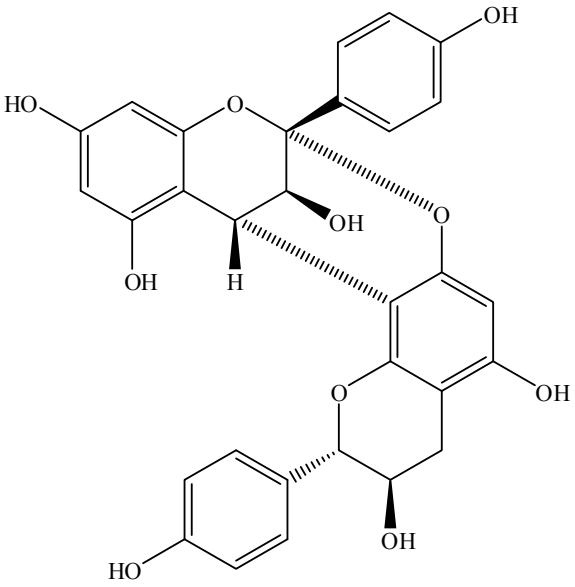
Overall the ^{1}H and ^{13}C NMR data closely resembled that of mahuannin A (Hikino et al., 1982), except for the chemical shifts and ^{1}H NMR coupling constants of the F-ring protons and carbons. In this compound, the appearance of a doublet at δ 4.83 (*d*, *J*=8.2 Hz, H-2') (rather than a broad singlet as in the case of mahuannin A) indicated that the 'lower' constituent flavan-3-ol moiety is a 2,3-*trans* configuration. Therefore, this compound was regarded as an epimer of manhuannin A and the possibilities of two compound structures arose: either compound 6.2 or compound 6.3. These three compounds were isolated from the roots of *Prunus armeniaca* by Rawat, Prasad, *et al*. (Rawat et al., 1999) and they deduced the stereochemistry of the F ring from NOESY and CD spectra. Based on their ^{1}H and ^{13}C NMR data and CD spectrum, the compound was identified as 6.2 [*ent-epiafzelechin-(2α→O→7,4α→8)-(-)-afzelechin*]. NMR data are provided in Table 6-2. This is the first report of the isolation of this compound from *P. persica* and is only the second time that compound 6.2 has been isolated from a natural source.



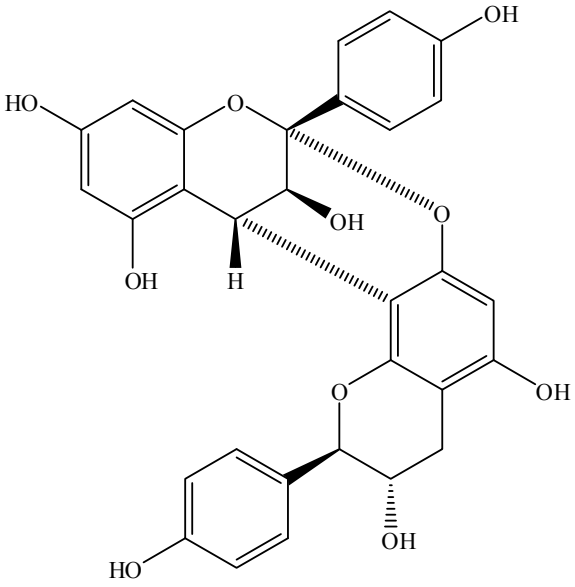
Afzelechin (6.1)



mahuannin A



Compound 6.2



Compound 6.3

Table 6-2: NMR data of compound 6.1 and compound 6.2 (400 MHz, $(CD_3)_2CO$)

Compound 6.1				Compound 6.2		
Position	δ_C	δ_H (*J* in Hz)	HMBC	δ_C	δ_H (*J* in Hz)	HMBC
2	82.7	4.59 *d* (8.0)	C-3, C-4, C-9, C-1', C-2', C-6'	100.1		
3	68.4	4.00 *m*		67.1	4.24 *d* (3.5)	
4	29.3	2.95 *dd* (5.5, 16.1) 2.53 *dd* (8.5, 16.1)	C-2, C-3, C-5, C-9, C-10	28.8	4.26 *d* (3.5)	C-3, C-2, C-10, C-8', C-9', C-7', C-9, C-5
5	157.3			156.5		
6	95.5	5.87 *d* (2.1)	C-5, C-8	97.8	5.92 *d* (2.2)	C-10, C-5, C-7
7	157.8			157.9		
8	96.2	6.02 *d* (2.1)	C-6, C-9. C-10	96.1	6.07 *d* (2.2)	C-6, C-10, C-9
9	157.0			153.8		
10	100.8			103.7		
11				131.4		
12				129.3	7.55 *d* (8.6)	C-2, C-14, C-16
13				115.1	6.86 *d* (8.6)	C-15, C-11, C-14
14				158.4		
15				115.1	6.86 *d* (8.6)	C-13, C-11, C-14
16				129.3	7.55 *d* (8.6)	C-2, C-12, C-14
1'	131.5					
2'	129.7	7.25 *d* (8.5)	C-2,C-6', C-4'	83.6	4.83 *d* (8.2)	C-4', C-3', C-11', C-12', C-9'
3'	115.8	6.82 *d* (8.5)	C-1', C-4', C-5'	67.7	4.14 *m*	
4'	158.1			29.1	3.06 *dd* (5.5, 16.5) 2.61 *dd* (9.0, 16.5)	C-3', C-2', C-10', C-9', C-5'
5'	115.8	6.82 *d* (8.5)	C-1', C-4', C-3'	155.5		
6'	129.7	7.25 *d* (8.5)	C-2, C-2', C-4'	96.4	6.14 *s*	C-10', C-8', C-7', C-5'
7'				151.8		
8'				106.3		
9'				150.5		
10'				102.6		
11'				129.8		
12'				129.7	7.40 *d* (8.6)	C-2', C-11', C-14'
13'				116.0	6.90 *d* (8.6)	C-11', C-14', C-15'
14'				158.4		
15'				116.0	6.90 *d* (8.6)	C-11', C-13', C-14'
16'				129.7	7.40 *d* (8.6)	C-2', C-11', C-14'

6.4 Biological studies of isolated compounds

There are no literature reports on the antibacterial activities of compounds 6.1 and 6.2. Other biological activities have been reported for compound 6.1, but none for compound 6.2. Compound 6.1 has earlier been isolated as one of the antioxidant compounds from *Eysenhardtia subcoriacea* through an antioxidant activity assay-guided chemical analysis (Narváez-Mastache et al., 2008). It has also been shown to have neuroprotective and free radical scavenging activities (Li et al., 2005) and α-glucosidase activity (Saijyo et al., 2008).

The antibacterial activities of these two compounds were tested against 8 bacteria *i.e.*, *E. coli* lactamase negative, *E. coli* lactamase positive, *Salmonella typhimurium*, *Streptococcus pyogenes*, *P. aeruginosa*, *S. aureus* methicillin sensitive, *S. aureus* CMRSA and *S. aureus* wild multidrug resistant MRSA, by the MTT microdilution assay. The results indicated that neither compound 6.1 nor 6.2 showed inhibitory activity against the tested microorganisms, even at a concentration of 250 μg/ml. Although the initial stages of the isolation was carried out guided by TLC-bioautography assays, in the latter stage of fractionation, vanillin stain was used to save time. It was anticipated that the stain would be appropriate as the R_f profile of the active spots from the TLC-bioautography assay were consistent with the R_f profile of the pink spots produced by the vanillin stain. Since the isolated compounds were not antibacterial in nature, it can be inferred that the 'antibacterial components' were not staining pink with the vanillin stain and because the isolation was guided by the vanillin stain rather than the TLC-bioautography assay, the antibacterial compounds were sidelined as 'inactive components'.

6.5 Conclusions and future directions

P. persica is used by the Chungtia villagers for treatment of typhoid, skin diseases, dysentery and diarrhoea. The antibacterial studies revealed strong activity for the root extracts against *S. aureus*. The MIC of the crude extract was 312 μg/ml. Following partitioning of the crude extracts with different polarity solvents, the ethyl acetate fraction was found to be the most antibacterial (MIC 312 μg/ml). Therefore, the ethyl acetate fraction was selected to study and isolate the active components. Following repeated silica gel column chromatographic separations, two compounds were isolated and their structures determined by 1 and 2D NMR and CD spectroscopy. This led to

the isolation of afzelechin (6.1) and *ent-epiafzelechin-(2α→O→7,4α→8)-(-)-afzelechin* (6.2). This is the first report of isolation of compound 6.2 from *P. persica*.

The two compounds were tested for antibacterial activities against 8 bacteria, including both Gram-positive and Gram-negative bacteria and MRSA strains, but the two compounds were found to be not active at a concentration of 250 µg/ml. Nevertheless, the antibacterial activity possessed by both the leaves and root extracts of this plant strongly support their traditional use for skin diseases.

The ethyl acetate fraction possessed strong inhibitory activity against *S. aureus*. In future studies, the isolation of active components should be strictly guided by bioassays, such as TLC-bioautography to ensure isolation of the active compounds from the ethyl acetate fraction.

6.7 Experimental

6.7.1 Reagents and equipment

This was the same as Section 5.8.1

6.7.2 Preparation of plant material of *Prunus persica*

The powdered dried root bark of *P. persica* (750 g) was suspended in 70% aqueous ethanol (2.5 l) and shaken overnight at room temperature. The extract was then filtered and the extraction process was repeated three times. The ethanol was then removed under reduced pressure and the remaining aqueous residue was successively partitioned with *n*-hexane (1 l x 2), DCM (1 l x 2), EtOAc (1 l x 2) and *n*-butanol (1 l x 2). This afforded five fractions *i.e.* Pp-Hex, Pp-DCM, Pp-EtOAc, Pp-BuOH and Pp-Water.

For the MTT microdilution assay of the partitioned fractions, 150 ml each of the fractions were evaporated to dryness under reduced pressure at 40 ºC and the residues were freeze dried overnight, to afford the following partitioned fractions: Pp-Hex (light brown gum, 26.7 mg), Pp-DCM (light brown gum, 30.2 mg), Pp-EtOAc (dark brown solid, 42.5 mg), Pp-BuOH (dark yellow solid, 56. 8 mg) and Pp-Water (dark yellow solid, 89.6 mg).

For compound isolation, the remaining ethyl acetate fraction (Pp-EtOAc) was evaporated following a similar protocol to above to afford 89 g of a dark brown solid.

6.7.3 Isolation of compounds from ethyl acetate fraction

The ethyl acetate fraction (12 g) was subjected to silica gel chromatography, eluting with increasing solvent polarities of chloroform and methanol (10:1, 3:1, 2:1, 1:1 and 1:2; 500 ml each) to afford 43 fractions. Fractions 17 to 21 in order of elution (eluting with 3:1 of chloroform and methanol) were pooled together based on TLC-bioautography results to afford 1.98 g of a yellow hygroscopic solid (Pp-EtOAc-1). Fraction Pp-EtOAc-1 was further separated by silica gel chromatography with increasing solvent polarities of chloroform and methanol (3:1, 1:1 and 1:2; 500 ml each) to afford 91 fractions. Fractions 9 to 12 were pooled together to afford 396 mg of a yellow hygroscopic solid (Pp-EtOAc-1-1). Fraction Pp-EtOAc-1-1 was again separated by silica gel chromatography with an isocratic solvent system of chloroform and methanol (10:1; 500 ml) to afford 30 fractions (Pp-EtOAc-1-1-1 to 30). Fraction Pp-EtOAc-1-1-11 and 12 produced compound 6.1 and fraction Pp-EtOAc-1-1-14 to 21 produced compound 6.2.

Afzelechin (6.1): creamish amorphous solid; ESI-MS *m/z* 275 $[M+H]^+$. ^1H, ^{13}C and 2D NMR data, see Table 6-2.

Ent-epiafzelechin (2α→O→7,4α→8)-(-)-afzelechin (6.2): white crystals; HRESI-MS negative-ion mode: *m/z* 543.1291 $[M-H]^-$ (calc. for $C_{30}H_{23}O_{10}$, *m/z* 543.1291). ^1H, ^{13}C and 2D NMR data, see Table 6-2. CD (MeOH) $[\theta]_{232}$ – 45600.8. $[\alpha]_D$ -113.7º (MeOH).

6.7.4 MTT microdilution and TLC-bioautography assays

The assay conditions were identical to that described in Chapter 4 for the MTT microdilution assay and Chapter 5 for the TLC-bioautography assay experimental.

Chapter 7

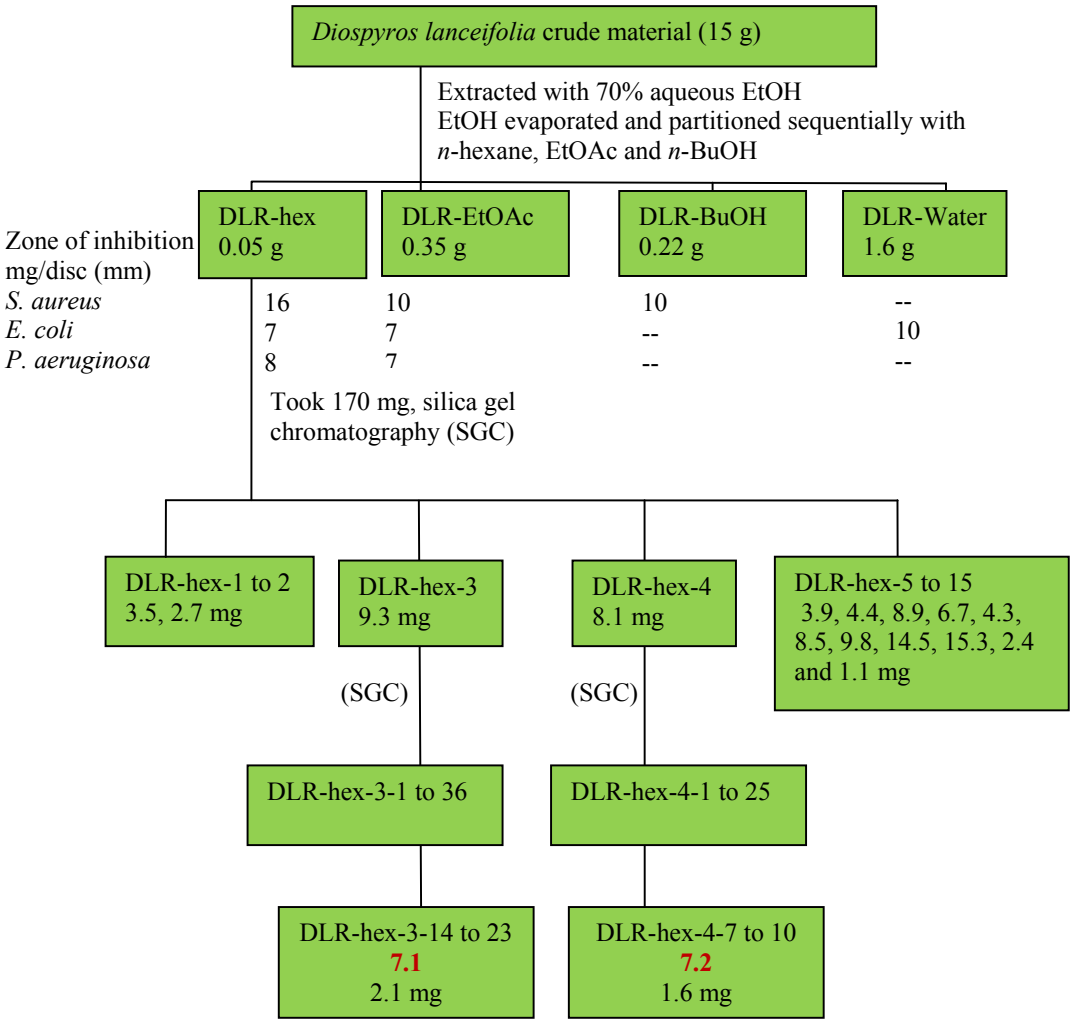
Isolation of antibacterial compounds from Diospyros lanceifolia Roxb.

7.1 Background

The review of the Nagaland ethnobotanical literature (Chapter 3) identified *Diospyros lanceifolia* as an important plant for Nagaland people. The roots have been documented to be used as a fish poison and the seeds for treatment of skin diseases (Changkija, 1999; Jamir and Rao, 1982). The people of Chungtia village also use the roots and fruits of *D. lanceifolia* to poison fish. There have been no reports on the biological or phytochemical studies of this plant. As described in Chapter 3, antimicrobial screening of the seeds of *D. lanceifolia* was of particular interest for this study due to their use for skin diseases, but the seeds were unable to be tested due to import restrictions to Australia. Since there were no reports on the biological or phytochemical studies of this plant, the roots and leaves were therefore collected by the villagers, dried and ground and then transported to our laboratory for antibacterial screening. The *n*-hexane fraction was active against *S. aureus*, *E. coli* and *P. aeruginosa* in the disc diffusion assay (Chapter 4). The aim of the study was to investigate the *n*-hexane fraction of *D. lanceifolia* for its major components and to evaluate their antibacterial activities.

7.2 Isolation of compounds from the *n*-hexane fraction

The *n*-hexane fraction (50 mg) was separated by normal phase silica gel column chromatography, eluting with increasing polarities of *n*-hexane and toluene. This resulted in 15 fractions based on TLC R_f profiles. Fraction 3 (DLR-hex-3, orange needles) was further separated by silica gel column chromatography with increasing polarities of petroleum ether (40-60ºC) and chloroform to afford 36 fractions. Fractions 14 to 23 were pooled to afford compound 7.1 (plumbagin) as orange needles. The DLR-hex-4 fraction showed 4 spots on the TLC plate (petroleum ether:chloroform; 1:2) and this was separated by silica gel column chromatography, eluting with increasing polarities of petroleum ether and chloroform, to afford 25 fractions. Fractions 7 to 10 were pooled to afford compound 7.2 (7-methyljuglone) as red needles. The fractionation and isolation of these compounds are summarised in Scheme 7-1.



Scheme 7-1: Fractionation of the root bark of *Diospyros lanceifolia*

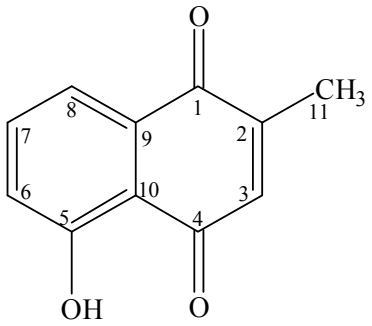
7.3 Identification of compounds

7.3.1 Structural elucidation of plumbagin 7.1

Compound 7.1 was obtained as orange needles. EI-MS showed a molecular ion at *m/z* 188 [M^+], consistent with the molecular formula for plumbagin of $C_{11}H_8O_3$.

The ^{1}H NMR spectrum of compound 7.1 showed an ABC spin system of three aromatic protons [δ 7.63 (*dd*, *J*=7.5, 1.5 Hz, H-8), 7.60 (*dd*, *J*=8.0, 7.5 Hz, H-7) and 7.25 (*dd*, *J*=8, 1.5 Hz, H-6)], an olefinic proton at δ 6.80 ppm (*q*, *J*=1.5 Hz, H-3) and a chelated hydroxyl proton (δ 11.97, *s*). The

^{13}C NMR spectrum showed the presence of two carbonyl groups (δ 184.9 and 190.4). The spectral data were indicative of a 1,4-naphthoquinone skeleton. Additionally, the ^{1}H NMR spectrum indicated the presence of a methyl group [δ 2.19 (d, *J*=1.5 Hz)] and the placement of this was made at C-2 (δ 149.7) by HSQC and HMBC correlation studies, where the methyl protons correlated to C-1 (δ 184.9), C-2 (149.7) and C-3 (135.9). The ^{1}H and ^{13}C NMR data (see Table 7-1) exactly matched the literature for plumbagin (Gu et al., 2004; Lim et al., 2007). The EI-MS fragmentation pattern showed prominent peaks at *m/z* 188 (M), 173 (M – CH_3), 160 (M – CO), 132 (M – 2CO), 131 (M – CO – HCO), 120 (M – CO – CH_3CCH), 92 (M – 2CO – CH_3CCH) and was in agreement with reported MS data for plumbagin (Wenche and Einar, 1995).

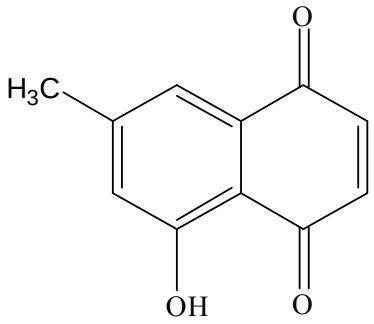


Plumbagin

7.3.2 Structural elucidation of 7-methyljuglone 7.2

Compound 7.2 was obtained as red needles. EI-MS showed a molecular ion at *m/z* 188 [M^+], consistent with the molecular formula for 7-methyljuglone of $C_{11}H_8O_3$. The EI-MS fragmentation pattern was similar to plumbagin except for an additional peak at *m/z* 134 (M – CO – HCCH). The presence of this peak, along with a peak at 132 (M – 2CO), are characteristic features of compounds without substituents on the quinoid moiety (Wenche and Einar, 1995).

The ^{1}H NMR spectrum confirmed the absence of substitution in the quinonoid moiety by the appearance of a single resonance peak of two aromatic protons at δ 6.91 (2H, *s*, C-2 and C-3). The ^{1}H NMR spectrum also showed the presence of a chelated hydroxyl proton at δ 11.86 (*s*), two aromatic protons at 7.44 (*m*) and 7.09 (*m*) and a methyl group at 2.44 (3H, *t*). The ^{1}H NMR and EI-MS data were identical with the literature reports for 7-methyljuglone (Budzianowski, 1995). Furthermore, the compound was confirmed by total synthesis (see Section 7.4).



7-methyljuglone

Table 7-1: NMR data for plumbagin and 7-methyljuglone

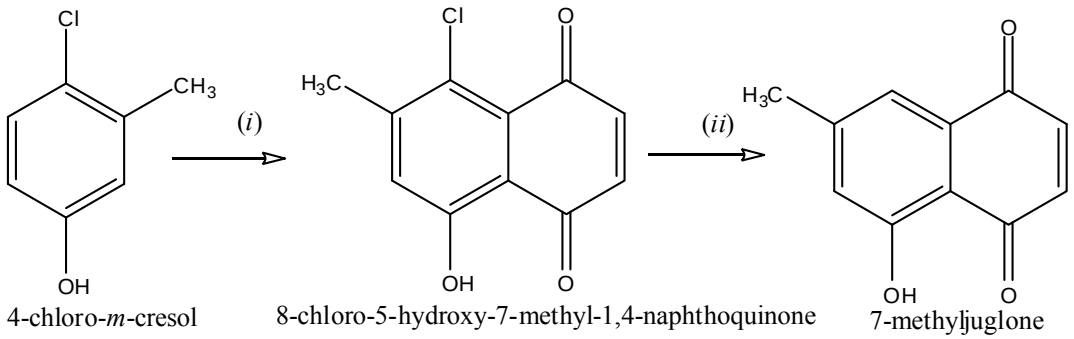
Plumbagin				7-Methyljuglone
Position	δ_C	δ_H multiplicity (*J* in Hz)	HMBC	δ_H multiplicity (*J* in Hz)
1	184.8	--		--
2	149.8	--		6.91 *s*
3	135.9	6.80 *q* (1.5)	C-1, C-10, C-11	6.91 *s*
4	190.4	--		--
5	161.3	--		--
6	124.4	7.25 *dd* (8.0,1.5)	C-5, C-8, C-10	7.09 *m*
7	135.9	7.60 *dd* (8.0, 7.5)	C-5, C-9	--
8	119.1	7.63 *dd* (7.5, 1.5)	C-1, C-6, C-10	7.44 *m*
9	132.1	--		--
10	115.4	--		--
11(CH_3)	16.7	2.19 *d* (1.5)	C-1, C-2, C-3	2.44 *t* (0.6)
OH-5	--	11.97 *s*	C-5, C-6, C-10	11.86 *s*

400 MHz, $CDCl_3$

Since the quantities of the two isolated compounds were very small (2.1 and 1.6 mg), plumbagin was purchased from commercial source and 7-methyljuglone was synthesised to allow structural confirmation and for performing antibacterial studies. The NMR spectral data of plumbagin from the commercial source were consistent with the isolated sample.

7.4 Synthesis of 7-methyljuglone

7-Methyljuglone was first synthesised in 1953 by a five-step procedure (Cooke and Dowd, 1953; Cooke et al., 1953). In this method, a Friedel-Crafts acylation was performed in which maleic anhydride was reacted with 4-chloro-*m*-cresol in the presence of an aluminium chloride-sodium chloride melt to give 8-chloro-5-hydroxy-7-methyl-1,4-naphthoquinone as the major product. Replacement of the chlorine atom with hydrogen was achieved by a four-step procedure, providing 7-methyljuglone in very low yield. This method was reinvestigated in 2001 (Musgrave and Skoyles, 2001) in which the dechlorination of 8-chloro-5-hydroxy-7-methyl-1,4-naphthoquinone was achieved in a one-step procedure by treating it with tin(II) chloride in tetrahydrofuran (THF) and hydrochloric acid followed by iron(III) chloride to give 7-methyljuglone.



4-chloro-*m*-cresol — 8-chloro-5-hydroxy-7-methyl-1,4-naphthoquinone — 7-methyljuglone

Scheme 7-1. Reagents and conditions:
(i) maleic anhydride, $AlCl_3$, NaCl, 180^{o} C, 2 min;
ii) $SnCl_2$, 4 M HCl/THF, 60 ^{o} C, 2–4 h.

The synthesis of 7-methyljuglone was successfully achieved by following the procedure of Musgrave and Skoyles, with no modification. The final product, 7-methyljuglone, was confirmed by ^{1}H NMR, which was identical to the ^{1}H NMR data of the isolated 7-methyljuglone.

7.5 Antibacterial activities of plumbagin and 7-methyljuglone

Plumbagin has been isolated from many plants, including those that have been used traditionally for the treatment of bacterial infections. For example, plumbagin has been isolated from *Drosera peltata* following antibacterial bioassay guided isolation (Didry et al., 1998). Plumbagin possesses strong inhibitory activity against *Clostridium perfringens* (intestinal bacteria) (Lim et al., 2007), *Helicobacter pylori* (Park et al., 2006), *S. aureus* (de Paiva et al., 2003), *E. coli, Salmonella typhi,*

Klebsiella pneumonia, Serratia marcescens, Bacillus subtilis, Proteus vulgaris and *P. aeruginosa* (Jeyachandran et al., 2009). Plumbagin and 7-methyljuglone also possess strong antimycobacterial activity (Mahapatra et al., 2007). Both of these compounds also exhibit strong antifungal activity (Dzoyem et al., 2007; Sinha et al., 2009).

While plumbagin has been extensively studied for its antibacterial activity, 7-methyljuglone has only been studied for its activity against mycobacterium (Bapela et al., 2006; Mahapatra et al., 2007). Furthermore, the activities of both these compounds towards MRSA have not been reported. Therefore, to broaden the understanding of their antibacterial activities, plumbagin and 7-methyljuglone were further tested against 8 microorganisms, including two strains of MRSA, in which the strains selected for this assay were different from the strains reported in the literature. The antibacterial activities were analysed by the MTT microdilution assay and the results are shown in Table 7-2.

Table 7-2: Antimicrobial activity of plumbagin (7.1) and 7-methyljuglone (7.2)

Microorganisms	7-1 MIC (µg/ml)	7-2 MIC (µg/ml)	Gentamicin MIC (µg/ml)	Chloromphenicol MIC (µg/ml)
1. *E. coli* ATCC 25922 B lactamase negative	62.5	250	3.91	-
2. *E. coli* ATCC 35218 B-lactamase positive	125	na	7.81	-
3. *Salmonella typhimurium* (Group B) CI	250	na	15.63	-
4. *Streptococcus pyogenes* (Group A strep) CI	3.91	3.91	0.98	-
5. *P. aeruginosa* ATCC 27853	125	na	7.81	-
6. *S. aureus* ATCC 29213 Methicillin sensitive	1.95	7.81	3.91	-
7. *S. aureus* ATCC BAA 1026 MRSA CMRSA	15.63	15.63	na	31.25
8. *S. aureus* wild Multidrug resistant MRSA CI	7.81	15.63	na	31.25

na=not active

Plumbagin was active against all the tested microorganisms. The MIC values were in the range of 1.95 to 250 µg/ml. The most potent inhibitory activity was observed for *S. aureus* methicillin sensitive strain (MIC 1.95 µg/ml), followed by *S. pyogenes* (MIC 3.91 µg/ml) and *S. aureus*

multidrug resistant (MRSA) (MIC 7.81 µg/ml). The activities against Gram-negative bacteria (*E. coli*, *S. typhimurium* and *P. aeruginosa*) were significantly weaker (MIC range 62.5-250 µg/ml) than the corresponding Gram-positive bacteria (1.95-15.63 µg/ml).

7-Methlyjuglone showed antibacterial activity similar to that of plumbagin against Gram-positive bacteria (MIC range 3.91 - 15.63 µg/ml) but the activity was much weaker than plumbagin against Gram-negative bacteria, with activity only against lactamase negative *E. coli* (MIC 250 µg/ml). The highest inhibitory activity was observed against *S. pyogenes* (MIC 3.91 µg/ml), followed by the *S. aureus* methicillin sensitive strain (MIC 7.81 µg/ml) and *S. aureus* multidrug resistant strains (MRSA) (MIC 15.63 µg/ml).

Both plumbagin and 7-methyljuglone showed much better activities than the antibiotic used against the MRSA strains; gentamicin and ampicillin were not active at 250 µg/ml, kanamycin showed an MIC at 250 µg/ml and chloromphenicol showed an MIC at 31.25 µg/ml.

7.5.1 Other biological activities of plumbagin and 7-methyljuglone

Other than the antimicrobial activities, plumbagin is a strong anticancer agent (Aziz et al., 2008; Shieh et al., 2010; Shih et al., 2009; Xu and Lu, 2010) and also has anti-inflammatory (Checker et al., 2009), antifeedant (Tokunaga et al., 2004), antifertility (Kini et al., 1997), ichthyotoxic (Ogihara et al., 1997), hypolipidemic and antiatherosclerotic (Sharma et al., 1991) (Gujar, 1990), antianaphylactic (Wurm and Geres, 1982) and anticoagulant (Santhakumari et al., 1978) activity. 7-Methyljuglone has been reported to additionally have termiticidal activity (Carter et al., 1978). The use of roots by Nagaland people to poison fish is supported by the fact that plumbagin, being a constituent of the root, has known ichthyotoxic activity.

7.6 Conclusion

Examination of the *n*-hexane fraction of the root of *D. lanceifolia*, which showed activity against *S. aureus*, *E. coli* and *P. aeruginosa* in the disc diffusion assay, led to the isolation of the two antibacterial compounds plumbagin and 7-methyljuglone. The isolation was achieved through repeated silica gel chromatography and the identification of the compounds was done by EI-MS, ^{1}H, ^{13}C and 2D NMR analysis and by comparison with the reported data from the literature and authentic samples.

The quantities of isolated compounds were low, therefore, for antibacterial studies, 7-methyljuglone was synthesised following a published method and plumbagin was purchased from a commercial source. Plumbagin and 7-methyljuglone were tested against 8 microorganisms including both Gram-negative and Gram-positive bacteria and two MRSA strains to expand the current bioactivity data on these compounds. Plumbagin showed more potent and broad spectrum activity, exhibiting activity against all the tested microorganisms with MIC values ranging from 1.95 to 250 µg/ml. 7-Methyljuglone showed significant activity against the Gram-positive bacteria but it was less active against Gram-negative bacteria, showing activity only against β-lactamase negative *E. coli*. Overall, the two compounds were much more active than the antibiotics tested (ampicillin, chloromphenicol, kanamycin and gentamicin) against the two MRSA strains. The MIC values of plumbagin and 7-methyljuglone ranged from 1.95 to15.63 µg/ml.

This is the first report of any biological or phytochemical studies of *Diospyros lanceifolia*.

7.7 Future directions

The genus *Diospyros* has been extensively studied biologically and phytochemically (Mallavadhani et al., 1998), but *D. lanceifolia* has not been studied biologically or phytochemically prior to now. An exhaustive phytochemical analysis could not be achieved due to limited time. Future antibacterial studies on this plant should focus on the *n*-hexane and EtOAc roots extracts, which showed strong antibacterial activities against *S. aureus*, *E. coli* and *P. aeruginosa*. Large scale extraction and bioassay guided fractionation will perhaps lead to isolating more antimicrobial compounds (particularly in the ethyl acetate fraction) and the possibility of isolating novel compounds cannot be excluded. Furthermore, interest should also focus on the seeds due to their specific ethnomedicinal use in skin diseases.

7.8 Experimental

7.8.1 Reagents and equipment

All the solvents used for chromatography were of analytical grade and re-distilled before use. Normal phase column chromatography was performed using silica gel 60 (230-400 mesh) from Merck (Germany). Analytical normal phase thin layer chromatography (TLC) was performed on

fluorescent Merck silica gel F_{254} plates (Germany). The TLC plates were visualised using UV light (254 nm and 365 nm) or developed by spraying with vanillin stain (3 g vanillin in 100 ml absolute ethanol and 0.5 ml sulphuric acid) and heating (120ºC). Organic solvents were removed using a Büchi rotary evaporator (Germany) under reduced pressure and traces of solvent were removed using a Pascal 2005 SD high vacuum pump from Alcatel.

The ^{1}H (400 MHz), ^{13}C (100 MHz), HSQC and HMBC NMR spectra were recorded on a Bruker Avance AMX 400 spectrometer (Germany) using standard pulse sequences. Chemical shifts were calculated relative to the chloroform solvent peak (^{1}H δ 7.26 and ^{13}C δ 77.2). Low resolution electron impact mass spectra (EI-MS) were acquired on a Shimadzu GCMS-QP5000 Gas Chromatograph Mass Spectrometer using probe injection with an ion energy of 70 eV.

7.8.2 Isolation of compounds from *D. lanceifolia n*-hexane fraction

The *n*-hexane fraction (170 mg) was subjected to silica gel column chromatography with increasing polarities of *n*-hexane and toluene. This afforded 15 fractions, based on their R_f profiles, labelled as DLR-hex-1 to DLR-hex-15 in order of increasing polarity (3.5, 2.7, 9.3, 8.1, 3.9, 4.4, 8.9, 6.7, 4.3, 8.5, 9.8, 14.5, 15.3, 2.4 and 1.1 mg, respectively).

DLR-hex-3 (9.3 mg) was further purified by silica gel chromatography using increasing polarities of petroleum ether (40-60ºC) and chloroform (100:0, 50:1, 10:1, 5:1, 1:1, 1:4, 1:50 and 0:100) and resulted in 36 fractions (DLR-hex-3-1 to DLR-hex-3-36). Fractions DLR-hex-3-14 to DLR-hex-3-23 were evaporated to afford plumbagin (2.1 mg).

DLR-hex-4 (8.1 mg) was further purified by silica gel column chromatography using increasing polarities of petroleum ether (40-60ºC) and chloroform (1:1, 1:2, 0:100), and chloroform and methanol (1:1). This resulted in 25 fractions (DLR-hx-4-1 to DLR-hx-4-25) and fractions DLR-hx-4-7 to DLR-hx-4-10 after evaporation afforded 7-methyljuglone (1.6 mg).

Plumbagin (7.1): orange needles; MS: [EI, 70 eV] (*m/z*, relative intensity, %) 188 (M, 100), 173 (M – CH_3, 23), 160 (M – CO, 21), 132 (M – 2CO, 24), 131 (M – CO – HCO, 37), 120 (M – CO – CH_3CCH, 22), 92 (M – 2CO – CH_3CCH, 29). ^{1}H, ^{13}C and 2D NMR data, see Table 7-1.

7-Methyljuglone (7.2): red needles; [EI, 70 eV] (*m/z*, relative intensity, %) 188 (M, 89), 173 (M – CH_3, 7), 160 (M – CO, 18), 134 (M – CO –HCCH, 31), 132 (M – 2CO, 51), 131 (M – CO – HCO, 56). ^1H and ^{13}C NMR data, see Table 7-1.

7.8.3 Synthesis of 7-methyljuglone

The procedure as originally described by Musgrave and Skoyles (2001) was followed. Briefly, a mixture of maleic anhydride (4.0 g, 41 mmol) and 4-chloro-*m*-cresol (1.5 g, 11 mmol) was added to a melt of anhydrous aluminum chloride (40.0 g, 300 mmol) and sodium chloride (8.0 g, 140 mmol) at 180˚C with vigorous stirring for 2 mins. A mixture of 150 ml of 12 N HCl with an equal quantity of ice was added to the dark orange mixture and kept at room temperature for 30 mins. The dark orange precipitate was filtered and allowed to dry overnight at room temperature. The solid obtained was ground into a fine powder with a mortar and pestle and then extracted with *n*-hexane by stirring at 50˚C for 1 hr (repeated 3 times) and filtered. The extract was concentrated under reduced pressure to afford 803 mg of an orange amorphous solid. The solid was allowed to crystallise from hot methanol (~20 ml) (overnight at room temperature) to afford purple-red crystals. The crystals were filtered, washed with cold methanol and dried under vacuum to afford 8-chloro-5-hydroxy-7-methyl-1,4-naphthoquinone as purple-red crystals (480 mg, 32%). ^1H NMR: $CDCl_3$, δ_H 7.19 (1H, s, ArH), 6.89 (2H, s, 2-H and 3-H), 2.75 (1H, s, ArOH), 2.47 (3H, s, ArMe). Spectral data were identical with the reported literature (Musgrave and Skoyles, 2001).

A solution of 8-chloro-5-hydroxy-7-methyl-1,4-naphthoquinone (200 mg, 0.89 mmol) in THF (20 ml) was added dropwise to a solution of $SnCl_2$ (1.0 g, 5.4 mmol) in 4 M HCl (70 ml) and THF (20 ml) at 60ºC and stirred for 3 hrs. It was then cooled and filtered into a solution of $FeCl_3$ (5 g in 40 ml H_2O) and the resultant orange precipitate was collected. The precipitate was triturated with chloroform and the orange liquid evaporated to dryness to give an orange amorphous solid (56.4 mg). This was further purified by silica gel chromatography eluting with *n*-hexane and chloroform (1:1) to afford 11 fractions. Fractions 2 to 5 were pooled together based on the TLC profile to afford 7-methyljuglone (13 mg, 6.5%). The ^1H NMR spectral data were consistent with the literature (Budzianowski, 1995) and the isolated sample from *D. lanceifolia*.

7.8.4 MTT microdilution assay

The assay conditions were identical to that described in Chapter 4 Experimental. Additional microorganisms were included for the assay: *E. coli* ATCC 35218 β-lactamase positive, *Salmonella typhimurium* (Group B) CI, *Streptococcus pyogenes* (Group A strep) CI, *S. aureus* ATCC 29213 methicillin sensitive, *S. aureus* ATCC BAA 1026 MRSA CMRSA and *S. aureus* wild multidrug resistant MRSA CI.

Conclusions

This PhD study covered the initiation of a collaborative research partnership between Macquarie University (IBRG) and Chungtia village (CSMT), Nagaland, and documentation of firsthand ethnobotanical information of Chungtia village through to the biological investigation and isolation of bioactive constituents from Nagaland medicinal plants.

An ethnobotanical research of Nagaland medicinal plants used by Chungtia village was conducted with the cooperation of Chungtia villagers and this resulted in the documentation of 135 plants. These plants were taxonomically identified and voucher specimens were deposited at Botanical Survey of India (BSI), Shillong, India, for future reference. Of these 135 plants, 35 plants appear to have novel ethnomedicinal uses in Nagaland.

Thirty five plants were documented for their use in skin related treatments and literature searches on these plants indicated 15 plants with either no antibacterial or phytochemical studies reported. This led to the selection of 8 plants from the 15 plants, with priority, for antibacterial screening. Three plants out of the 20 plants that were recorded for their uses in skin diseases from the literature on Nagaland medicinal plants were also selected for antibacterial screening.

The antibacterial screening was performed on these 11 plants using disc diffusion and MTT microdilution assays against three human pathogenic microorganisms: *Staphylococcus aureus*, *Escherichia coli* and *Pseudomonas aeruginosa*. All of the 8 plants used by the Chungtia villagers showed antibacterial activity against *S. aureus*, at concentrations less than 2.5 mg/ml. The highest inhibitory activities were exhibited by the stem bark of *Erythrina stricta* and the roots of *Prunus persica*, with MIC values of 156 µg/ml and 312 µg/ml, respectively. The root extract of *D. lanceifolia* also showed activity against *S. aureus*, *E. coli* and *P. aeruginosa*. Only one plant did not show antibacterial activity (*Albizia chinensis* from Nagaland medicinal plant literature). Based on the antibacterial screening results, the stem bark of *E. stricta*, root bark of *P. persica* and root bark of *D. lanceifolia* were selected for further biological and chemical investigations of their antibacterial constituents.

The bioassay guided isolation of the dichloromethane partition of the ethanolic extract of the stem bark of *E. stricta* led to the isolation of 11 compounds: flavanones 5-hydroxysophoranone (5.1) and maackiaflavanone B (5.3), the isoflavones chandalone (5.2), alpinumisoflavone (5.4) and lupalbigenin (5.7), and the pterocarpans 1-methoxyerythrabyssin II (5.5), erythrabyssin II (5.6), erystagallin A (5.8), phaseollidin (5.9), cristacarpin (5.10) and 2-(γ,γ-dimethylallyl)-6*a*-hydroxyphaseollidin (5.11). This is the first reported isolation of any of these 11 compounds from the stem bark of this plant. The antibacterial activities of the isolated compounds were evaluated against 4 different strains of *S. aureus* including two drug-resistant strains. The most potent inhibition was shown by compound 5.7. Compounds 5.4, 5.6, 5.8, 5.10 and 5.11 were active against all the four strains.

The ethyl acetate fraction of the root bark of *P. persica* led to the isolation of afzelechin (6.1) and *ent-epiafzelechin-(2α→O→7,4α→8)-(-)-afzelechin* (6.2). This is the first report of compound 6.2 isolation from *P. persica*. The two compounds were tested for antibacterial activities against 8 bacteria, including both Gram-positive and Gram-negative bacteria and two MRSA strains, but they were found to be not active even at a concentration of 250 μg/ml.

Examination of the *n*-hexane fraction of the root bark of *D. lanceifolia*, which showed activity against *S. aureus*, *E. coli* and *P. aeruginosa* in the disc diffusion assay, led to the isolation of the two antibacterial compounds plumbagin (7.1) and 7-methyljuglone (7.2). Plumbagin and 7-methyljuglone were tested against eight microorganisms including both Gram-negative and Gram-positive bacteria and two MRSA strains to expand the current bioactivity data on these compounds. Plumbagin showed more potent and broad spectrum activity than 7-methyljuglone. This is the first report on biological and phytochemical studies of *D. lanceifolia*.

In conclusion, this PhD study has successfully achieved its primary aims to both document the Chungtia medicinal plants knowledge for its preservation and cultural value and to scientifically understand the medicinal properties of some of these plants.

APPENDIX 2-1

Office of The

CHUNGTIA VILLAGE COUNCIL

MOKOKCHUNG NAGALAND. 798601

Ref. No. — Date 7-7-07

Ref: Research collaboration

Dear Dr. Joanne Jamie,

In pursuance of your letter E- mail: Joanne.jamie@mq.edu.au,
I on behalf of the Chungtia Village Council, Mokokchung District, Nagaland, India herewith confirm the Chungtia Village Council authorizes Chungtia Senso Mokokchung Town to enter into the research agreement with Macquarie University in respect of the medicinal plants project under the signature of President and Secretary.

Yours sincerely,
(Supongsashi)
Chairman
Chungtia Village Council, Nagaland

W. Supong
Chairman
Village Council
Chungtia
Date 7-7-07

APPENDIX 2-2

MACQUARIE UNIVERSITY STANDARD COLLABORATIVE RESEARCH AGREEMENT
BETWEEN CHUNGTIA SENSO MOKOKCHUNG TOWN AND MACQUARIE UNIVERSITY

EXECUTED in two originals on the days and dates written below

SIGNED for and on behalf of	SIGNED by
University	**Chungtia Senso Mokokchung Town**
Signature:	Signature:
Name:	Name: K. LUEN. LONGKUMER.
Capacity:	president CSMT.
Date:	Date: 13/08/07

President
Chungtia Senso
Mokokchung Town

APPENDIX 2-3

Acacia pennata (Linn.) Willd.

(Mimosaceae)

Zanghi

Acorus calamus Linn.

(Araceae)

Mukupen

Adenia trilobata Engl.

(Passifloraceae)

Tenik tepang

Adhatoda vasica Nees.

(Acanthaceae)

Sungjem wa

Albizia chinensis (Osb) Merr.

(Mimosaceae)

Mokokwa

Albizia lebbeck Linn. Benth.

(Mimosaceae)

Moang

Albizia lucidior (Skud)

Hara (Mimosaceae)

Sunemtong

Allium Chinense G. Don

(Liliaceae)

Alolasung

Allium hookeri Thw.

(Liliaceae)

Repchalasung

Allium sativum Linn.

(Liliaceae)

Lasung

Alstonia scholaris (Linn.) R. Br.

(Apocynaceae)

Loomi

Amaranthus gangeticus Linn.

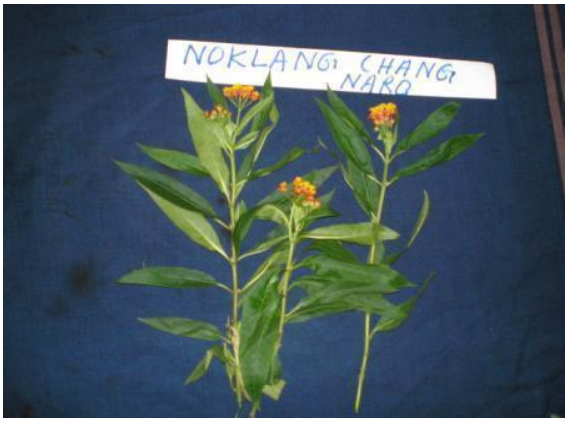
(Amaranthaceae)

Tsumarlua

Aquilaria agallocha Roset.
(Thymelaeaceae)
Sungza

Artemisia vulgaris Linn.
(Asteraceae)
Chinangchibaza

Artocarpus heterophyllus Lamk.
(Moracea)
Polong



Asclepias curassavica Linn.
(Asclepiadaceae)
Noklangchang

Averrhoa carambola L.
(Averrhoaceae)
Jarkona

Bambusa tulda Roxb.
(Bambusoideae)
Longme

Basella alba Linn.
(Basellaceae)
Latsung-en

Bauhinia variegata Linn.
(Caesalpiniaceae)
Owepanghef

Begonia picta Smith
(Begoniaceae)
Tesenlawa

Brassica oleracea Linn.
(Brassicaceae)
Pandacobi

Cajanus cajan (Linn.)
(Fabaceae)
Mahajang

Calotropis gigantea Linn.
(Asclepiadaceae)
Kutjak moli

Cannabis sativa L.
(Cannabaceae)
Ganja/kancha

Capsicum annum Linn.
(Solanaceae)
Metsu/Mersu

Carica papaya Linn.
(Caricaceae)
Kumita/Kometa

Cassia floribunda Cav.
(Caesalpiniaceae)
Napongchami

Catharanthus roseus (Linn.) G. Don
(Apocynaceae)
Supienaro

Celosia cristata L.
(Amaranthaceae)
Alonaro

Centella asiatica L.
(Apiaceae)
Longtsukolok

Chrysanthemum indicum L.
(Asteraceae)
Asurongmang

Cissampelos pareira Linn.
(Menispermaceae)
Likhazung

Cissus repens Lam.
(Vitaceae)
Zerebliwa

Citrus microcarpa Bunge.
(Rutaceae)
Nimbutinga

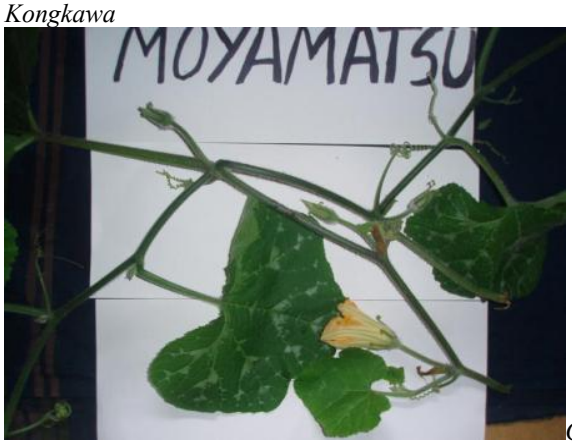
Clerodendron cordatum D. Don
(Verbenaceae)
Oremwa

Coix lacryma-jobi Linn.
(Poaceae)
Jemur

Costus speciosus (Koenig ex Retz) JE Smith
(Costaceae)
Aokmejang

Crataeva nurvala Buch-Ham
(Capparaceae)
Kongkawa

Croton Caudatus Gieseler
(Euphorbiaceae)
Khemetsu koila



Cucurbita pepo L.
(Cucurbitaceae)
Moyamatsu

*Curculigo capitulata (*Lour.) Kuntze
(Hypoxidaceae)
Kurivu

Curanga amara Juss.
(Scrophulariaceae)
Longri

Cyclea peltata Diels.
(Menispermaceae)
Tsungrempangmoli

Datura stramonium Linn.
(Solanaceae)
Kohima sangjem

Debregeasia longifolia (Burm. f.) Wedd.
(Urticaceae)
Natsulawa

Dendrocnide sinuata (Bl.)
(Urticaceae)
Zaklojawa

Diospyros lanceifolia Roxb.
(Ebenaceae)
Urcha

Dolichos lablab L.
(Fabaceae)
Napakauv

Drymaria cordata (Linn.) Willd.
(Caryophyllaceae)
Pipivula

Dryopteris filix-mas (L.) Schott
(Dryopteridaceae)
Nachav

Duabanga grandiflora (Roxb. ex DC.) Walp.
(Sonneratiaceae)
Kisati

Elsholtzia blanda (Benth.) Benth.
(Lamiaceae)
Changjang

Endata pursaetha DC.
(Leguminosae)
Keling

Equisetum ramosissimum Subsp. *Debile*
(Equisetaceae)
Avpenba

Eryngium foetidum Linn.
(Apiaceae)
Aong thonia

Erythrina stricta Roxb.
(Fabaceae)
Lochet

Eucalyptus globulus Labill.
(Myrtaceae)
Eucalyptus

Eupatorium odoratum linn.
(Asteraceae)
Zasen

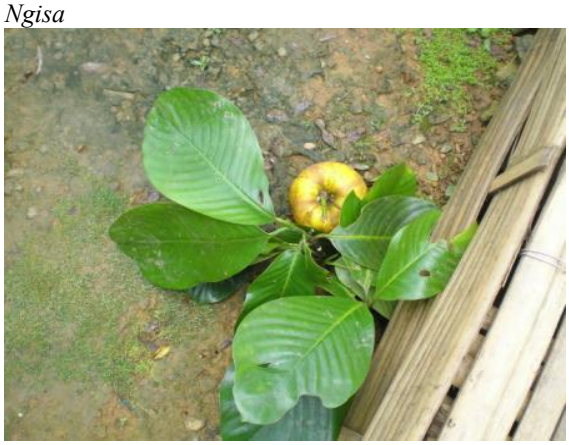
Euphorbia pulcherrima Willd. ex Klotzsch (Euphorbiaceae)
Muluchangnaro/Molojangnaro

Euphorbia royleana Boiss.
(Euphorbiaceae)
Takterak

Eurya acuminata DC.
(Theaceae)
Mesetwa

Ficus elastica Roxb. ex Hornem.
(Moraceae)
Ngisa

Garcinia cowa Roxb.
(Guttiferae)
Songtula



Garcinia pedunculata Roxb. Ex Buch.-Ham. (Guttiferae)
Asong

Girardinia palmata (Forsk.) Gaud.
(Urticaceae)
Ongpangzaklu

Glycine max (L.) Merr.
(Fabaceae)
Alichami

Gmelina arborea Roxb.
(Verbenaceae)
Ekong

Gonatanthus pumilus (D.Don) Engler & Krause
(Araceae)
Longtong

Gossypium herbaceum Linn.
(Malvaceae)
Khumpa/Kumba

Gynura crepidioides Benth.
(Asteraceae)
Monglibaza

Hedyotis scandens Roxb.
(Rubiaceae)
Termoli

Hodgsonia macrocarpa (Blume) Cogn.
(Cucurbitaceae)
Assa

Holboellia latifolia Wall.
(Lardizabalaceae)
Mezetsuk

Houttuynia cordata Thunb.
(Saururaceae)
Nokna

Ipomoea nil (Linn.) Roth.
(Convolvulaceae)
Makenchangnaro

Kalanchoe pinnata (Lam.) Pers.
(Crassulaceae)
Nokchamoli

Lagenaria siceraria (Molina) Standl.
(Cucurbitaceae)
Aakuf

Lantana camara Linn.
(Verbenaceae)
Aiangketba naro

Lasia spinosa (L.) Thwaites
(Araceae)
Turang

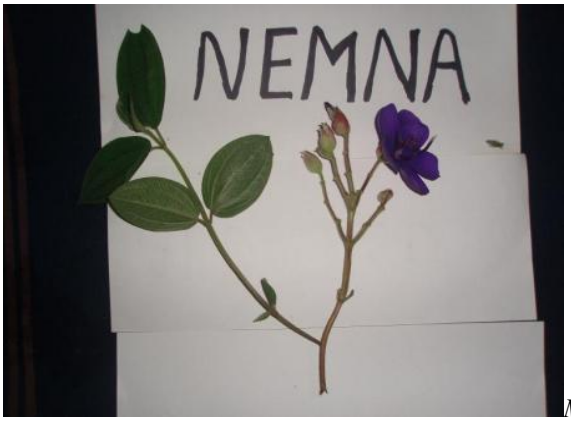
Lycopersicon esculentum Linn.
(Solanaceae)
Benganatasula

Luffa acutangula Linn.
(Cucurbitaceae)
Pokka

Macropanax undulatus (Wall.ex G. Don) seem. (Araliaceae)
Semza

Maesa indica (Roxb.) Wall.
(Myrsinaceae)
Kensametong

Manihot esculenta Crantz.
(Euphorbiaceae)
Alicha



Melastoma malabathricum
(Melastomataceae)
Nemna

Melia composite Willd.
(Meliaceae)
Aiet

Mentha cordifolia Opiz ex Fresen.
(Lamiaceae)
Pudina

Mikania cordata (Burm. F.) B.L. Rob.
(Asteraceae)
Indialeelang

Millettia cinerea Benth.
(Fabaceae)
Suli

Mimosa pudica Linn.
(Mimosaceae)
Amidangku- ayaklawa

Mirabilis jalapa Linn.
(Nyctaginaceae)
Chumdangnaro

Mussaenda roxburghii Hk.f.
(Rubiaceae)
Andipeernaro

Myrica esculenta Buch.-Ham., ex D. Don
(Myricaceae)
Mediong

Nephrolepis cordifolia
(Davalliaceae)
Seraenjen

Nerium indicum, Mill.
(Apocynaceae)
Sharonnaro

Ocimum basilicum Linn.
(Lamiaceae)
Nangperra

Oroxylum indicum (Linn.) Vent.
(Bignoniaceae)
Ochamiliaf/Ojamelavu

Paederia foetida Linn.
(Rubiaceae)
Atsulelang

Passiflora edulis Sim.
(Passifloraceae)
Entsulashi

Oxalis acetosella L.
(Oxalidaceae)
Waroetsu

Phyllanthus emblica, Linn.
(Euphorbiaceae)
Lher

Phyllanthus urinaria Linn.
(Euphorbiaceae)
Asularlir

Physalis alkekengi L.
(Solanaceae)
Entsupilvu

Piper betel
(Piperaceae)
Patiwa/Pateo

Plantago erosa Wall.
(Plantaginaceae)
Sangnem

Polygonum hydropiper Linn.
(Polygonaceae)
Nikmeremlawa

Prunus persica (L.) Stokes
(Rosaceae)
Mokori

Psidium guajava Linn.
(Myrtaceae)
Monaim

Punica granatum Linn.
(Punicaceae)
Jarem

Rhus javanica var. *roxburghiana*
(Anacardiaceae)
Tangma

Rhus roxburghii Hook. f.
(Anacardiaceae)
jarak

Ricinus communis Linn.
(Euphorbiaceae)
Pakawa

Saccharum officinarum Linn.
(Poaceae)
Motsutong/Motsu

Scutellaria glandulosa Colebr.
(Lamiaceae)
Yimramoli

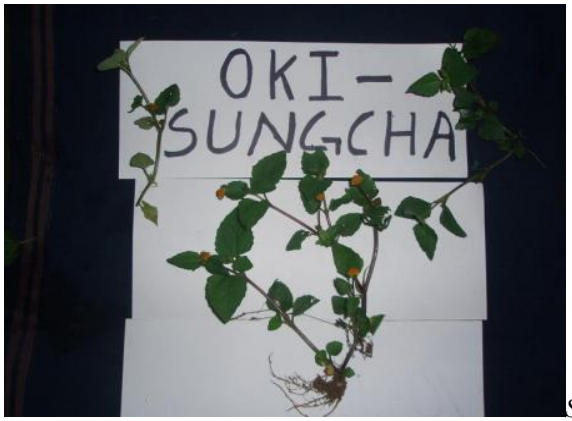
Solanum indicum Linn.
(Solanaceae)
Ao longkok

Solanum khasianum Clarke
(Solanaceae)
Atsu longkok

Sonerila maculata Roxb.
(Melastomaceae)
Alichang

Spermacoce scaberrima Blume
(Rubiaceae)
Ongpangentilawa

Spermacoce hispida Linn.
(Rubiaceae)
Intilawa



Spilanthes acmella Linn.
(Asteraceae)
Okensencha

Spondias pinnata (Linn. F.) Kurz.
(Anacardiaceae)
Pakho

Stereospermum chelonoides (Linn. f.) DC. (Bignoniaceae)
Sengpet

Stixis suaveolens (Roxb.) Pierre
(Capparidaceae)
Aiemaluv

Tagetes erecta, Linn.
(Asteraceae)
Pesunaro

Terminalia chebula Retz.
(Combretaceae)
Ningkha

Thunbergia grandiflora Roxb.
(Acanthaceae)
Koktsuli

Tithonia diversifolia (Hemsl.) A. Gray
(Asteraceae)
Zoninaro

Urtica dioica L.
(Urticaceae)
Zaklutasula

Viola betonicifolia Boj. Ex baker
(Violaceae)
Hingpangmoli/Engpangmoli

Wedelia chinensis (Osb.) Merrill
(Asteraceae)
Enze

Zanthoxylum acanthopodium DC.
(Rutaceae)
Changpet

Zanthoxylum rhetsa (Roxb.) DC.

(Rutaceae)

Ongret

One to one interview with a village elder

Interview in progress

Voucher specimens preparation

Processing of plant materials

Chungtia village 2008

Pictures taken by author and A. Anungba Jamir

APPENDIX 2-4

Name of the person	**Sex/ Age**
Date	**Location**
Tribal Plant Name	**Scientific name**
Local Distribution of Plants/Habitat	**Seasonal Availability/Flowering**
Medicinal/Food use in Chungtia Village	
Medicinal purpose:	**Food:**
Preparation in the Chungtia Village Communities	
Parts used: **Fresh/Dry:** **Mode of preparation:** **Dosage and Healing Time:**	
Comments if any:	

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