2009

Phytochemical studies and bioactivity of Centipeda and Eremophila species

Karren D. Beattie
Southern Cross University

Publication details

Beattie, KD 2009, 'Phytochemical studies and bioactivity of Centipeda and Eremophila species', PhD thesis, Southern Cross University, Lismore, NSW.

Phytochemical Studies and Bioactivity of *Centipeda* and *Eremophila* Species

Thesis submitted by

Karren Deanne Beattie B.Sc. (Hons.).

A thesis submitted in fulfillment
of the requirements for the award
of the degree of
Doctor of Philosophy

School of Natural and Complementary Medicine

Southern Cross University

2009

Thesis Declaration

I certify that the work presented in this thesis is, to the best of my knowledge and belief, original, except as acknowledged in the text, and that the material has not been submitted, either in whole or in part, for a degree at this or any other university.

I acknowledge that I have read and understood the University's rules, requirements, procedures and policy relating to my higher degree research award and to my thesis. I certify that I have complied with the rules, requirements, procedures and policy of the University (as they may be from time to time).

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Preface

Some of the bioassays described in Chapter 6 of this thesis were performed by others;

- The termiticidal, repellency and fumigant properties as well as the barrier studies of the *E. mitchellii* wood oil were performed by Associate Professor Robert Spooner-Hart and Dr Albert Basta of the University of Western Sydney, (Centre for Plant and Food Sciences) Hawkesbury.
- Efficacy of the *E. mitchellii* wood oil fractions against termites as well as investigation of the termiticidal properties of the *E. mitchellii* leaf oil and its constituents were also performed by Associate Professor Robert Spooner-Hart and Dr Albert Basta.
- Determination of the acute dermal toxicity IC_{50} and IC_{95} for the eremophilanes was performed collaboratively with Associate Professor Robert Spooner-Hart and Dr Albert Basta.

Some of the bioassays described in Chapter 4 of this thesis were performed by others;

- Antioxidant and anti-inflammatory assays on *C. cunninghamii* extracts and fractions were performed by Dr Denise Hunter and Ms Kelly Shepherd at the Centre for Phytochemistry and Pharmacology at Southern Cross University.

Some of the spectroscopic analyses described in this thesis were performed by others;

- Optical rotations were performed by Dr Kim Dastlick at the Institute for Molecular Biology at the University of Queensland.
- High Resolution Mass Spectroscopy was performed by Chemical Analysis Laboratories, Bulleen, Victoria.
- X-ray crystallography studies were performed by Dr Donald Craig at the Department of Chemistry, University of New South Wales.
- NMR spectroscopy of compounds from *C. cunninghamii* was performed by Dr Myrna Deseo at the Centre for Phytochemistry, Southern Cross University.

Some photographs in this thesis were provided by others, as credited in the text.

Abstract

The aim of this study was to isolate and characterise biologically active compounds from endemic Australian plants. A total of 6 novel, and 26 known compounds have been isolated throughout the course of this work.

A comprehensive investigation of the GC-MS chemical profile of *C. cunninghamii* leaf essential oil found that thymol (**1**) *cis*-chrysanthenyl acetate (**4**), myrtenyl acetate (**2**), myrtenol (**3**) and *cis*-chrsanthenol (**5**) were the major constituents. The essential oil and crude solvent extracts of *C. cunninghamii* possessed significant antioxidant and anti-inflammatory activity. A 50% aqueous ethanol extract was demonstrated to possess multiple modes of anti-inflammatory action. The crude extract was found to significantly inhibit both COX-1 and COX-2 cyclooxygenases and was comparable to the positive controls; Ibuprofen and Celebrex respectively. The crude extract also exhibited anti-inflammatory activity in the nitric oxide (NO) and tumor necrosis factor-alpha (TNF-α) assays, but did not show inhibition in the lipoxygenase (LO) assay.

A total of seventeen compounds, of which **10**, **6**, **7**, **8** and **9** are novel, have been identified from the aqueous-ethanolic extract of *C. cunninghamii*. Five flavonoids; axillarin (**16**), isokaempferide (**17**), 4',5,7-trihydroxy-3,6-dimethoxyflavone (**18**), jaceidin (**19**), and 2',4',5,7-tetrahydroxy-6-methoxyflavone-3-*O*-β-glucopyranoside (**10**) were isolated from the flowers of *C. cunninghamii*. A series of caffeic acids were isolated as the major component of the stems, these included; chlorogenic acid (**12**) and its methyl ester (**13**), caffeic acid ethyl ester (**11**), isochlorogenic acid A (**14**)

macroantoin G (**15**) and the novel 4ξ,5ξ-di-*O*-caffeoyl-2,6ξ-dihydroxyhept-2-ene-1,7-dioic acid (**6**) and its 1-methyl ester (**7**), 7-methyl ester (**8**) and 1,7-dimethylester (**9**) derivatives. Lastly, arnicolide C (**20**) a sesquiterpene lactone, 3-hydroxykaura-9(11),16-diene-18-oic acid (**21**) and 8-hydroxy-9,10-diisobutyryloxythymol (**23**) were characterised by spectroscopic methods.

All of the compounds were evaluated for anti-inflammatory activity, as determined by the inhibition of prostaglandin E_2 in 3T3 fibroblast cells. All compounds, inhibited PGE_2 production to some extent, at a concentration of 31.25 μg/mL. The flavonoids **10** and **16-19** were the most active compounds. The caffeic acids **6-9**, **12-14** and the thymol derivative **23** also significantly inhibited PGE_2 production. The IC_{50} values were determined for the novel compounds; **10**, **6**, **7**, **8** and **9**, as 1.47, 2.48, 4.73, 5.54 and 1.26 μM, respectively. These novel compounds were more potent than the positive control, aspirin, which was found to inhibit PGE_2 production by 42% at a concentration of 18 μM.

Antioxidant activity, as determined by oxygen radical absorbance capacity (ORAC) has also been attributed to both the flavonoids; **10**, **16-19** and caffeic acid compounds; **6-9** and **12-14**. The antioxidant capacity of these compounds was found to be comparable to epicatechin, a major antioxidant constituent of green tea.

A detailed analysis of the wood, leaf, branch and root oil of *E. mitchellii* was carried out by a combination of GC-FID, GC-MS, LC/MS and NMR spectroscopy. The wood, root, leaf and branch oils were found to be predominantly composed of sesquiterpenes. The three major compounds identified in the leaf oil, which accounted for 44% of the

oil, were α-pinene (**40**), (+) spathulenol (**15**) and an unidentified sesquiterpene alcohol. The composition of the leaf oil was complex and chemically distinct from the wood and root oils, whereas the branch oil was found to exhibit a chemical composition that was intermediate between the leaf and the wood oil.

After fractionation by preparative HPLC six components of the wood oil were characterized and accounted for 80% of the oil. The major constituents of the wood oil were; eremophilone (**30**), 9-hydroxy-7(11),9-eremophiladien-8-one (**36**), santalcamphor (**35**) and the novel 9-hydroxy-1,7(11),9-eremophilatrien-8-one (**42**). Two minor constituents, 8-hydroxy-10,11-eremophiladien-9-one (**32**) and 8-hydroxy-1,11-eremophiladien-9-one (**33**) were also isolated in this study.

The two major constituents of the root oil of *E. mitchellii* were found to be eremophilone (**30**) and the zizaene, sesquithuriferone (**43**). These, together with the minor constituents **32**, **33**, **42**, **35** and **36** accounted for 92% of the root oil.

The insecticidal properties of *E. mitchellii* were evaluated against several species of termites *Nasutitermes walkeri* (Hill), *Nasutitermes exitiosus* (Hill) and *Coptotermes acinaciformis* (Froggatt). Bioassay-guided fractionation of *E. mitchellii* wood oil was undertaken to investigate the termiticidal metabolites. Of the major components, it has been determined that eremophilone (**30**) was the most active constituent of the wood oil followed by 8-hydroxy-1,11-eremophiladien-9-one (**33**), 9-hydroxy-7(11),9-eremophiladien-8-one (**36**) and santalcamphor (**35**).

The methanolic extracts from a total of 36 species have been evaluated for cytotoxicity against $P388D_1$ mouse lymphoblast cells. A collection of fifteen species of *Eremophila* from Western Australia and a further twenty from the Northern Territory were surveyed. Cytotoxicity was found to be largely non-selective across a range of human cancer cell lines, including MCF7 (mammary adenocarcinoma), Hep G2 (hepatocellular carcinoma), A2780 (ovarian carcinoma), A-375 (malignant melanoma) and PC-3 (prostate cancer).

Fractionation of the leaf material of *E. racemosa* afforded the six major metabolites. Isolation and structural elucidation of these polar compounds revealed the cyanogenetic glycoside prunasin (**65**), the flavonoid luteolin (**74**), the furofuran lignans, phillygenin (**75**), its 4-*O*-*β*-D-glucoside phillyrin (**76**), pinoresinol-4-*O*-*β*-D-glucoside (**77**) and epipinoresinol-4-*O*-*β*-D-glucoside (**78**). Fractionation of the leaf material of *E. maculata* var. *brevifolia* afforded piceine (**81**) and epipinoresinol-4-*O*-*β*-D-glucoside (**78**). Quercetin (**79**) and nepetin (**80**) were isolated from the methanolic extracts of *E. bignoniflora.*

Publications

Some of this work has been published previously;

Beattie, K.; Leach, D.; Waterman, P. G.; Flowers, A.; Banbury, L.; Spooner-Hart, R. Biologically Active Eremophilanes from *Eremophila mitchellii*; Poster Presentation. RACI Natural Products Annual Symposium 3/10/2003 (Appendix VI).

Gabriel, B.; Beattie, K.; Leach, D.; Stevenson, L.; Biologically Active Compounds. Australian Patent AU2006319748. November 29, 2006.

Reichelt-Brushett, A.; Leach, D.; Spooner-Hart, R.; Beattie, K.; Wang, H. Declaration of the composition for *Eremophila* oil as the active ingredient in termite control products. APVMA submission. (submitted October 2008).

Acknowledgements

I wish to express my sincere gratitude to Associate Professor David Leach, Professor Peter G. Waterman, and Doctor Andrew Flowers for their invaluable expertise, guidance and encouragement throughout the course of my research. I would like to thank the staff at the Centre for Phytochemistry, Southern Cross University; Don Bruschett, Linda Banbury, Gloria Karagianis, Aaron Pollack and Bill Eickhoff for their technical expertise and assistance.

I would like to thank Matt Kealley of BioProspect, Jon Belling and Ruth Brown of The Alice Springs Desert Park, Greg Leach of the Northern Territory Department Parks and Wildlife, and Paul and Liz Gordon from Wilbertree Homestead Brewarrina NSW, for the collection and provision of plant materials.

I would like to thank Dr Albert Basta and Associate Professor Robert Spooner-Hart of the University of Western Sydney, Centre for Plant and Food Sciences, Hawkesbury for their expertise and assistance with the insecticidal assays.

I am grateful to Bioprospect Ltd. and Bioactives Pty. Ltd. for their financial assistance and the Australian Government for their award of an APA bursary.

Lastly thank you to my family, Johnno and fellow colleagues; Leah, Hilwan, Clynton, Nan, Myrna, Hao, Ben and Heidi for their support and encouragement.

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List of Abbreviations and Acronyms

The following abbreviations are used throughout this thesis.

A	shielded component of an AB system
AAPH	2,2′-azobis(2-methyl)propionamidine dihydrochloride
ACC	acid copper chromate
ACN	acetonitrile
ACQ	ammoniacal copper quaternary
APCIMS	atmospheric pressure chemical ionization mass spectrometry
APL	Australian Phytochemicals Ltd.
APVMA	Australian Pesticide and Veterinary Medicines Authority
ASDP	Alice Springs Desert Park
A:W:A	acetone: water: acetic acid
ATCC	American type culture collection
ATP	adenosine triphosphate
B	deshielded component of an AB system
BHT	butylated hydroxytoluene
BV	bed volume
CCA	copper chromated arsenate
CCB	chromated copper borate
COSY	correlation spectroscopy 2D NMR ($^{2\text{-}4}J_{HH}$)
COX	cyclooxygenase
CPAFS	Centre for Plant and Food Sciences
CPP	Centre for Phytochemistry and Pharmacology
DBE	double bond equivalent

DDT	dichlorodiphenyltrichloroethane
DMEM	Dulbecco's modified eagle medium
DMSO	dimethyl sulfoxide
ECACC	European collection of cell cultures
EI	electron impact (MS)
EIA	enzyme immunoassay
ESI	electrospray ionisation (MS)
EtOAc	ethyl acetate
EtOH	ethanol
F	fraction
FBS	foetal bovine serum
FID	flame ionization detector
GABA	*gamma*-aminobutyric acid
GC-MS	gas chromatography - mass spectrometry
HAT	hours after treatment
HMBC	heteronuclear multiple bond correlation 2D NMR ($^{2\text{-}3}J_{CH}$)
HPLC	high performance liquid chromatography
HSQC	heteronuclear single quantum coherence 2D NMR ($^{1}J_{CH}$)
HR ESIMS	high resolution electrospray mass spectrometry
IC_{50}/IC_{95}	concentration effecting 50/95% inhibition
IPM	integrated pest managment
LC_{50}/LC_{95}	concentration lethal to 50/95% of the test organisms
LD_{50}/LD_{95}	dose lethal to 50/95% of the test organisms
KT_{50}/KT_{95}	period of time to kill 50/95% of the test organisms
L	layer

LC - MS	liquid chromatography - mass spectrometry
LO	lipoxygenase
LPS	lipopolysaccharide
LRESIMS	low resolution electrospray mass spectrometry
mAb	monoclonal antibody
MeOH	methanol
mp	melting point
MQ	milliQ water
MS	mass spectrum
MW	molecular weight
NDGA	nordihydroguaiaretic acid
NMR	nuclear magnetic resonance
NO	nitric oxide
nOe	nuclear Overhauser effect (through space signal enhancement)
NP	normal phase
NSAIDS	non-steroidal anti-inflammatory drugs
OCP	organochlorine pesticide
ORAC	oxygen radical absorbance capacity
prep-HPLC	preparative scale HPLC chromatography
PGE_2	prostaglandin E_2
PGH_2	prostaglandin H_2
RCF	relative centrifugal force
RH	relative humidity
RI A/P	retention indicies apolar/polar column
ROS	reactive oxygen species

RP	reverse phase
RVC	rotation vacuum centrifuge
SCU	Southern Cross University
SEM	standard error of the mean
SPE	solid phase extraction
TE	Trolox equivalent
TEAC	Trolox equivalent antioxidant capacity
TFA	trifluoroacetic acid
TNF-α	tumor necrosis factor-α
TOF-MS	time of flight - mass spectrometry
UV	ultra-violet

Chapter 1

Introduction

1.1 Overview

This introduction covers a brief overview of the field of phytochemistry. An in-depth background to the three phytochemical investigations covered in this thesis is presented at the beginning of the relevant section.

1.1.1 Phytochemistry

Phytochemistry or plant chemistry;

> *"is concerned with the enormous variety of organic substances that are elaborated and accumulated by plants and deals with the chemical structures of these substances, their biosynthesis, turnover and metabolism, their natural distribution and their biological function* (Harborne, 1998)."

It is estimated that worldwide there are over 328 640 species of plants (Raven et al., 2005). Their sessile nature and lack of a sophisticated immune system has necessitated the development of complex chemical systems. Historically, the compounds produced by plants have been categorized into primary and secondary metabolites (Raven et al., 2005). Compounds contributing to fundamental metabolism are termed primary metabolites. In contrast secondary metabolites are limited in their distribution; both throughout the plant and between different species (Raven et al., 2005).

Secondary metabolites were once thought to be waste compounds (Raven et al., 2005). Our understanding of the important in-planta function of many secondary metabolites is gradually expanding. It is being revealed that many of these secondary metabolites are potent bactericidal, repellent, or even toxic agents to pests and herbivores (Dewick, 1997). Semiochemicals are relied on as a means of defense against pathogens and predators, as attractants to lure mobile creatures for fertilization and dissemination and also for aerial allelopathy (interplant communication). Volatile organic compounds and pigments are revealed to be attractive to insects that help with fertilization, or warning colors to defend against predators (Dewick, 1997), whilst other plant pigments can provide protection against environmental damage such as free radicals and UV radiation (Raven et al., 2005). Some secondary products perform signaling functions as plant hormones and pheromones.

Plants produce an incredible array of secondary metabolites and many of these have been developed into economically important products including; oils, gums, resins, tannins, rubber, waxes, pigments, flavors, fragrances, surfactants, preservatives,

pesticides and pharmaceuticals (Raven et al., 2005). Plant secondary metabolites represent a tremendous resource for commerce.

Phytochemists play a fundamental role in the chemical investigation of these plants. Phytochemical studies may be directed towards characterizing the chemical composition of complex essential oils or plant extracts. Phytochemical screening can assist taxonomic classification whilst bioassay guided studies can target and identify biologically active compounds in complex plant extracts (Harborne, 1998).

1.1.2 Aims of the Project

The overall objective of this study was to isolate and identify biologically active compounds from endemic Australian plants. The work presented in this thesis has been directed by commercial interest and is divided in to three sections. The first deals with the bioassay guided isolation and identification of antioxidant and anti-inflammatory compounds from *Centipeda cunninghamii*. The second section deals with the exploration and characterization of the termiticidal properties of *Eremophila mitchellii*. The final section of this thesis details the phytochemical and cytotoxicity investigation of extracts from the genus *Eremophila*.

Chapter 2

Experimental

2.1 General Experimental Procedures

2.1.1 Extraction Techniques

Unless specified the dried plant material was ground in a Waring blender and then extracted in the specified solvents by steeping overnight at room temperature. The resulting extracts were then filtered through a glass frit (Pore 3) and evaporated to dryness on a rotary evaporator. The preliminary screening of each plant was performed by LC-MS. MilliQ (MQ) water and HPLC grade solvents were employed throughout the course of this research.

2.1.2 Chromatographic Techniques

Detailed information for the HPLC, GC-MS and LC-MS parameters are included in Appendix I.

2.1.2.1 Liquid Chromatography – Mass Spectrometry (LC-MS)

Samples were suspended in a suitable solvent and analysed at a concentration of 5-10 mg/mL for crude extracts and 1 mg/mL for pure compounds with an injection volume of 10 μL. The LC-MS system used was an Agilent 1100 Series with binary pump and auto sampler/auto injector. The operation of the LC-MS system was controlled by HP Chemstation software. The column used was a Phenomenex Aqua C18 (125 A, 5 μ, 150 x 4.6 mm I.D) operating at 40°C. Gradient elution was carried out using MQ Water and acetonitrile with 0.005% trifluoroacetic acid (TFA) and a flow rate of 1.0 mL/min. An Agilent photodiode array detector (#G1315B) was used for monitoring absorbance (210 nm, 238 nm, 254 nm, 280 nm and 360 nm). Unless specified an Agilent SL1100 series mass spectrometer detector (#G1946D) was used in atmospheric pressure chemical ionisation (APCI) mode with an ionisation voltage of 150 eV and a scanning range of 100-1200 a.m.u.

2.1.2.2 Gas Chromatography - Mass Spectrometry (GC-MS)

The GC-MS system used was an Agilent GC-MSD system (Agilent Technologies 6890/5973) with helium as the carrier gas at a constant linear velocity of 33 cm/s. The transfer, source and quadrupole temperatures were 280°C, 230°C and 150°C respectively, operating at 70 eV ionisation energy. Unless specified, the column used was an SGE Ltd. BPX5 capillary column (50.0 m x 0.22 mm ID x 1μm film thickness) programmed from 50˚C to 300˚C at 8˚/min. Composition values were recorded as percentage area based on the total ion current chromatogram. Additional information for the GC-MS methods and their operating parameters are included in the appendix.

2.1.2.3 Preparative High Performance Liquid Chromatography

Fractionation of extracts was performed on a Gilson Preparative HPLC system

employing a Gilson 322 binary pump system, a Gilson 156 UV-Vis dual wavelength detector set at 210 nm and 280 nm, and Gilson fraction collector (FC204). A C18 column (Phenomenex Luna C18, 5 μm, 50 mm x 21.2 mm) was used for reverse phase (RP) separations and as specified, acetonitrile/water or methanol/water with or without TFA (0.05%) were used as the mobile phase. Normal phase (NP) separations were achieved using a silica column (Phenomenex Luna 5 μm Silica (2), 50 mm x 21.20 mm) utilizing a hexane/ethyl acetate gradient. Unless specified, the sample loading was between 50-100 mg/injection (RP) or 300-500 mg/injection (NP), the flow rate employed was 15 mL/min operating at ambient temperatures. The prep HPLC was interfaced with Gilson Unipoint v.3.0 software. Unless specified the fractions were dried using a rotation vacuum centrifuge (RVC) (Martin Christ, Germany) and combined as appropriate after verification by LC-MS. Detailed information for the HPLC methods and their mobile phase parameters are included in the relevant sections.

2.1.3 Spectroscopic Techniques

2.1.3.1 Nuclear Magnetic Resonance Spectroscopy

NMR spectra were obtained on a Bruker Avance DRX-500. XWin NMR software was used to analyze the spectral data. The ^{1}H NMR spectra were recorded at 500.13 MHz and the ^{13}C NMR spectra at 125.77 MHz. The chemical shifts (δ) are expressed in parts per million (ppm) as δ values and the coupling constants (J) in Hertz (Hz). COSY, NOESY, HSQC HMBC and nOe diff experiments were acquired using the standard Bruker pulse programs. The experiments were performed in deuterated solvents, chemical shifts were calibrated relative to; the methanol solvent peak (^{1}H δ 3.31 and ^{13}C δ 49.15 ppm), the DMSO solvent peak (^{1}H δ 2.50 and ^{13}C δ 39.51 ppm), the pyridine solvent peak (^{1}H δ 8.74 and ^{13}C δ 150.35 ppm), the acetone solvent peak (^{1}H δ

2.05 and ^{13}C δ 29.92 ppm), the chloroform solvent peak (^{1}H δ 7.27 and ^{13}C δ 77.23 ppm).

2.1.3.2 UV-Visible Spectroscopy

The UV-Visible spectrum of selected compounds were obtained from a Unicam UV 4-100 UV/Visible spectrophotometer. The light sources were deuterium and tungsten lamps. All samples were dissolved in HPLC grade solvents as specified in the text.

2.1.3.3 IR Spectroscopy

The IR spectrum of each compound was obtained from a Vector 33 IR-spectrometer (Bruker). The samples were prepared in KBr disc forms or liquid film on sodium chloride. The IR-spectrometer was operated at spectral range 4000-500 cm^{-1}.

2.1.3.4 Melting Points

Melting points were determined by a Gallenkamp melting point apparatus and are uncorrected.

2.1.3.5 Optical Rotations

$[\alpha]_D$ were performed by Kim Dastlick (IMB, University of Queensland) and determined using a Jasco P-1010 polarimeter fitted with a sodium lamp (589nm). Solvents, concentrations and temperatures are as specified in the text. Concentration c is in g/l00 mL; the units of the specific rotation are $° \cdot mL \cdot g^{-1} \cdot dm^{-1}$.

2.1.3.6 High Resolution Mass Spectrometry

HR-MS analyses were performed by Chemical Analysis Laboratories (Bulleen, Victoria) using an LC-MS (Agilent G1313A) interfaced to a TOF MS (Agilent

G1969A) fitted with an APCI source (Agilent G1978A) scanning at a mass range of 50-1100 a.m.u. The mobile phase consisted of 50% water (0.1% formic) and 50% methanol (0.1% formic) at a flow rate of 0.3 mL/min operating at ambient temperature. Nitrogen was used as the nebulising gas at a pressure of 10 psi and vaporiser temperature, drying gas flow and capillary voltage were set to 350°C, 4 mL/min, 3000 V respectively.

2.2 Experimental on Chemistry of *C. cunninghamii*

2.2.1 Plant Material.

Two collections of *Centipeda cunninghamii* were provided by Bioactives Ltd., on 28/11/05 (5 Kg, CPR#050285) and on the 10/04/06 (5.0 Kg, CPR#060023). The dried milled aerial parts of *Centipeda cunninghamii* (CPR#050285) was used for all of the studies described in this thesis.

2.2.2 Steam Distillation of *C. cunninghammii* Oil

Whole, dried *C. cunninghamii* plant material (1.0 kg) was steam distilled for 24 hours in a 20 litre distillation flask fitted with an oil estimator. An amber coloured oil (2.25 mL, 0.21% yield, w/dry weight) was obtained.

2.2.3 Characterisation of the Oil by Gas Chromatography

The analytical GC-MS system used was an Agilent GC-MSD system (Agilent Technologies 6890/5973) with helium as the carrier gas at a constant linear velocity of 28 cm/s. The transfer, source and quadrupole temperatures were 280°C, 230°C and 150°C respectively, operating at 70 eV ionisation energy. The column used was an SGE Ltd. BPX5 capillary column (50.0 m x 0.22 mm ID x 1μm film thickness)

programmed from 50˚C to 300˚C at 4˚/min and a BP-20 column (50.0 m x 0.22 mm ID x 1μm film thickness) programmed from 50˚C to 260˚C at 4˚/min. Oil samples (20 μL) were diluted with acetone (1000 μL). The injection volume was 0.2 μL, the split ratio was 1:40 and the injector temperature was 280˚C.

The analytical GC system used was a Hewlett Packard GC system (HP6890) fitted with an Agilent 7683 injector using the same instrument parameters as above and an FID detector temperature of 300˚C. The column used was an SGE Ltd. BPX5 capillary column (50.0 m x 0.22 mm ID x 1μm film thickness). Composition values were calculated as percentage area based on the FID chromatogram.

Identification of the individual components was based on; (i) comparison with the mass spectra of authentic reference compounds where possible and by reference to WILEY275, NBS75K, and Adams terpene library (Adams, 2007): (ii) comparison of their retention indices (RI) on a BPX-5 (polar, 5% phenyl polysilphenylene-siloxane) and a BP-20 (polar, polyethylene glycol) column, calculated relative to the retention times of a series of C-8 to C-22 *n*-alkanes, with linear interpolation, with those of authentic compounds or literature data: (iii) by comparison of the MS, NMR and retention time data of the pure compounds isolated in this study.

α-Pinene, β-pinene, *p*-cymene, 1,8-cineole and α-terpineol were obtained from Aldrich chemical Co. Inc. (Castle Hill, NSW). Terpinen-4-ol was identified by comparison of this compound in authentic tea tree oil (*Melaleuca alternifolia*, FPI Oils, Melbourne). *Trans*-β-caryophyllene was identified by comparison with this compound from betel leaf oil (*Piper betle*, FPI Oils, Melbourne). Myrtenyl acetate, myrtenal and myrtenol

were identified by comparison of these compounds from myrtle oil (*Myrtus communis*, FPI Oils, Melbourne). Thymol was identified by comparison with this compound from thyme oil (*Thymus vulgaris*, FPI Oils, Melbourne). Camphene and *p*-cymenene were identified by comparison of these compounds in angelica root oil (*Angelica archangelica*, FPI oils Melbourne).

2.2.4 Preparative HPLC Fractionation

The dried, ground *C. cunninghamii* (32.8 g) was extracted overnight at room temperature in 50% aqueous ethanol. The extract was filtered and evaporated under reduced pressure to yield a resinous, amber coloured extract (5.5 g, 16.8% yield). The crude extract was resuspended in MQ water (36 mL), methanol (4 mL) was added to improve solubility. The extract was then fractionated using reverse phase preparative HPLC (Figure 2.1). A mobile phase system of solvent A (MQ water with 0.05% TFA) and solvent B (acetonitrile with 0.05% TFA) was utilized for all of the isolation work. The eluent was a gradient of 10-95% B over 25 minutes at a flow rate of 15 mL/min. Fractions were collected in 1 minute intervals and fractions and/or compounds were recovered from the eluent by rotary evaporation. Preparative HPLC steps were repeated until adequate quantities (ca. $\geq$ 20 mg) of pure compounds were obtained for bioassay and structure elucidation. Fractions; 2, 11, 14, 16 and 17 were sub-fractionated to obtain pure compounds (Figure 2.1).

Fraction 2 was sub-fractionated by RP prep-HPLC utilizing a gradient of 10-25% B over 15 min at a flow rate of 15 mL/min to afford pure compounds **12** and **13**. Compounds **10** and **11** were further purified from fraction 11 by RP prep-HPLC utilizing a gradient of 20-40% B over 15 min at a flow rate of 15 mL/min. Compounds

14 and **15** were purified from fraction 14 utilizing the same HPLC parameters as fraction 11. Fractions 16 and 17 were combined and sub-fractionated by RP prep-HPLC to obtain pure compounds **6**, **7**, **8** and **9**. The eluent composition was 40-75% B over 16.4 min, followed by an isocratic gradient of 75% B for 3.6 min, at a flow rate of 15 mL/min.

An ethanolic extract of the floral parts was partitioned with hexane to afford an enriched fraction of the non-polar, PGE_2 active compounds (Figure 2.2). The hexane and the ethanol partitions were then evaporated to dryness and subjected to RP-prep HPLC. Compounds **16**, **17**, **18** and **19** were obtained from RP prep-HPLC fractionation of the polar components, a gradient of 20-60% B over 20 min, followed by and isocratic gradient of 60% B for 3 min, at a flow rate of 15 mL/min was employed. Compounds **20**, **21**, and **23** were obtained from RP prep-HPLC fractionation of the non-polar components, a gradient of 40-75% B over 16.4 min, followed by an isocratic gradient of 75% B for 3.6 min, then a gradient 75-90% B over 1 min, and finally an isocratic gradient of 90% B for 7 minutes, at a flow rate of 15 mL/min was utilized.

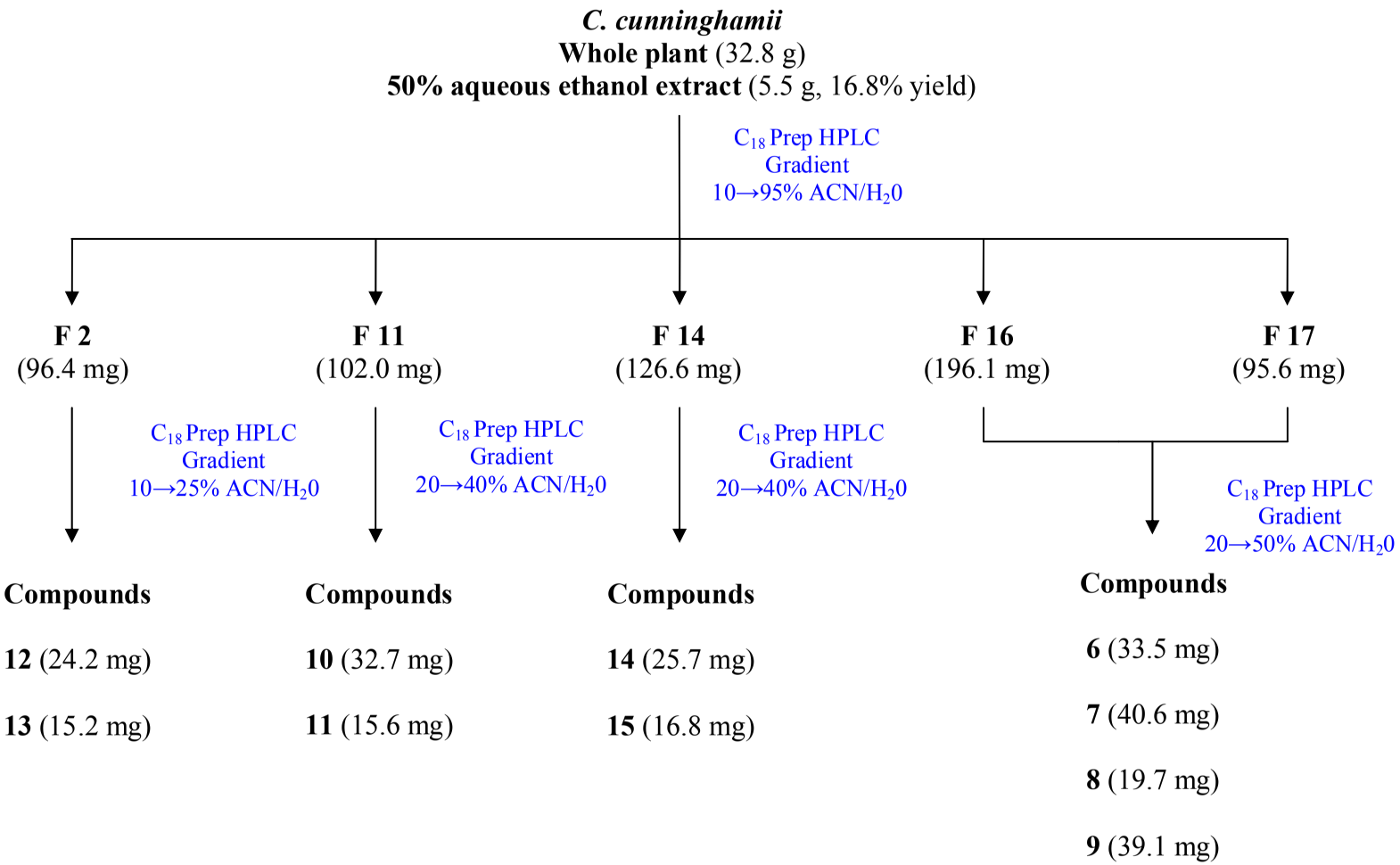


Figure 2.1. Isolation scheme for compounds from *C. cunninghamii* – aerial parts.

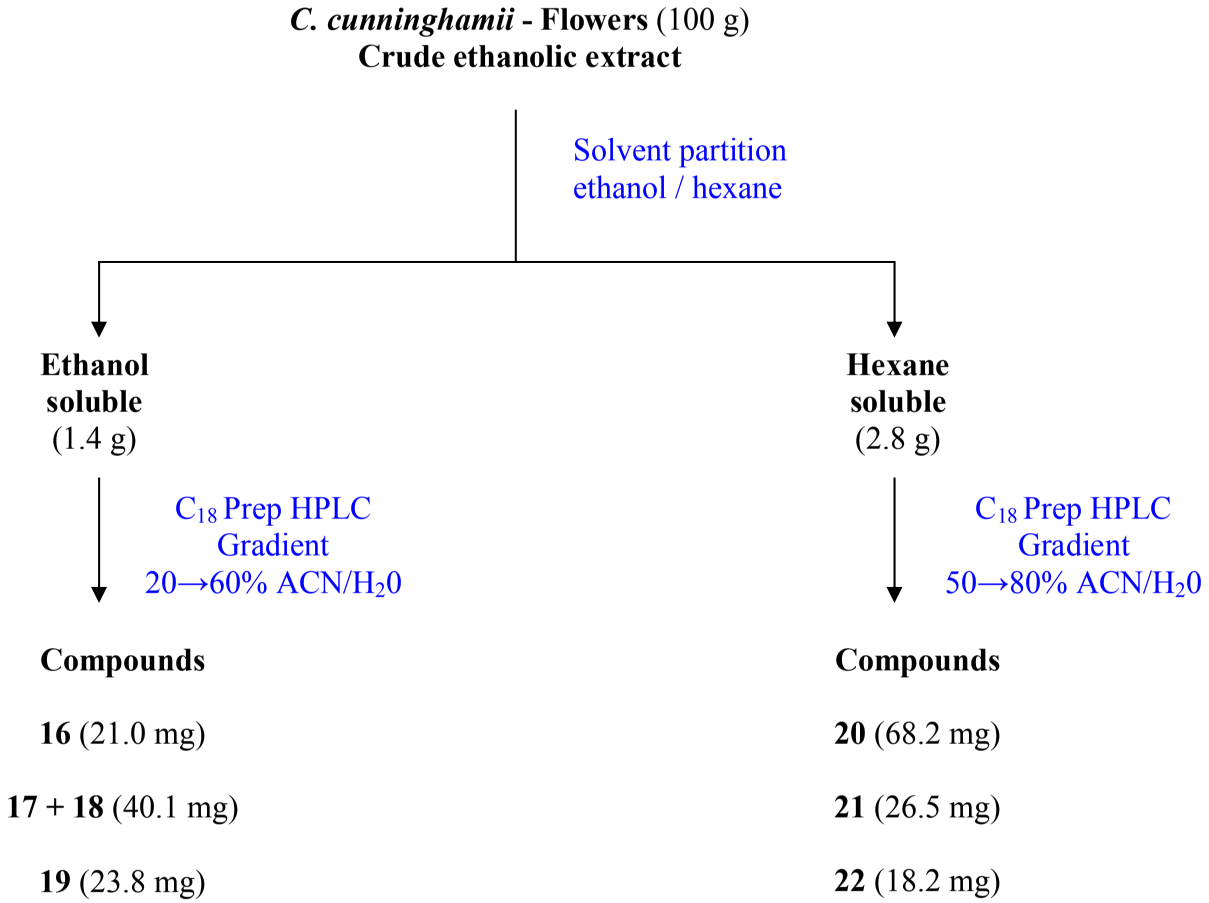


Figure 2.2. Isolation scheme for compounds from *C. cunninghamii* - flowers.

Centipetin-3-glucoside [(10), 2′,4′,5,7-Tetrahydroxy-6-methoxyflavone-3-*O*-*β*-glucopyranoside]: Yellow powder (32.7 mg, 0.59%, not optimised); $[\alpha]_D^{21}$ -12.5° (c 0.06, MeOH); ^{1}H NMR (500 MHz, CD_3OD) see Table 3.4; ^{13}C NMR (126 MHz, CD_3OD) see Table 3.4; (+)-LRAPCIMS *m/z* (rel. int.): 495 [$M+H^+$, 100%], 333 (98), 318 (24); (+)-HRAPCIMS *m/z* (rel. int.): 495.1142 calcd for $C_{22}H_{23}O_{13}$, 495.1138 $[M+H]^+$ (100%), 333 (30).

Myriogenic acid [(6), 4*ξ*,5*ξ*-Di-*O*-caffeoyl-2,6*ξ*-dihydroxyhept-2-ene-1,7-dioic acid): Colourless needles (33.5 mg, 0.61%, not optimised); $[\alpha]_D^{22}$ +46.2° (c 0.375, MeOH); ^{1}H NMR (500 MHz, CD_3OD) see Table 3.2; ^{13}C NMR (126 MHz, CD_3OD) see Table 3.2; (+)-LRAPCIMS *m/z* (rel. int.): 547 [$M+H^+$, 13%], 546 (47), 350 (40), 349 (92), 170 (8), 169 (91), 164 (14), 163 (100), 141 (20), 135 (34), 123 (35), 117 (12); (+)-HRAPCIMS *m/z* (rel. int.): 546.1248 calcd for $C_{25}H_{23}O_{14}$, 547.1087 (Δ0.9839 a.m.u.) [$M+H^+$], (48%) 384 (25), 349 (100);

Caffeic acid ethyl ester [(11), 3-(3,4-Dihydroxyphenyl)-2-propenoic ethyl ester]: Colourless powder (15.6 mg, 3.7%); ^{1}H NMR (500 MHz, CD_3OD): 7.53 (1H, d, *J* = 15.9 Hz, H-3′), 7.03 (1H, d, *J* = 2.1 Hz, H-2), 6.95 (1H, dd, *J* = 2.1, 8.2 Hz, H-6), 6.78 (1H, d, *J* = 8.2 Hz, H-5), 6.20 (1H, d, *J* = 15.9 Hz, H-2′), 4.22 (2H, q, *J* = 7.1 Hz, H-1″), 1.30 (3H, t, *J* = 7.1 Hz, H-2″), Lit. (Gabriel, 2005); ^{13}C NMR (126 MHz, CD_3OD): 169.5 (C, C-1′), 149.7 (C, C-4), 147 (C, C-3), 146.9 (CH, C-3′), 127.9 (C, C-1), 123 (CH, C-6), 116.7 (CH, C-5), 115.5 (CH, C-2′), 115.3 (CH, C-2), 61.5 (CH_2, C-1″), 14.8 (CH_3, C-2″), Lit. (Gabriel, 2005); (+)-LRAPCIMS *m/z* (rel. int.): 209 [$M+H^+$, 66%, calcd for $C_{11}H_{13}O_4$, 209.0814], 181 (29), 163 (100), 145 (5), 135 (32), 117 (11).

Chlorogenic acid [(12), 3-*O*-Caffeoylquinic acid]: Transparent brown oil (24.2 mg, 0.44%, not optimised); ^{1}H NMR (500 MHz, CD_3OD): 7.56 (1H, d, *J* = 16.0 Hz, H-3′), 7.05 (1H, d, *J* = 2.0 Hz, H-5′), 6.95 (1H, dd, *J* = 2.0, 8.2 Hz, H-9′), 6.78 (1H, d, *J* = 8.2 Hz, H-8′), 6.26 (1H, d, *J* = 16.0 Hz, H-2′), 5.33 (1H, ddd, *J* = 4.4, 8.6, 9.2 Hz, H-3), 4.17 (1H, ddd, *J* = 3.3, 8.6, 5.3 Hz, H-5), 3.72 (1H, dd, *J* = 3.2, 8.6 Hz, H-4), 2.23 (1H, dd, *J* = 4.4, 13.3 Hz, H-2a), 2.18 (1H, dd, *J* = 3.3, 14.1 Hz, H-6a), 2.08 (1H, dd, *J* = 5.3, 14.1 Hz, H-6b), 2.05 (1H, dd, *J*=13.3, 9.2 Hz, H-2b), Lit. (Cheminat et al., 1988); ^{13}C NMR (126 MHz, CD_3OD): 177.1 (C, C-7), 168.8 (C, C-1′), 149.7 (C, C-7′), 147.2 (CH, C-3′), 147.0 (C, C-6′), 128.0 (C, C-4′), 123.1 (CH, C-9′), 116.6 (CH, C-8′), 115.5 (CH, C-2′), 115.4 (CH, C-5′), 76.3 (C, C-1), 73.7 (CH, C-4), 72.1 (CH, C-3), 71.5 (CH, C-5), 38.9 (CH_2, C-2), 38.4 (CH_2, C-6), Lit. (Lin et al., 1999); (+)-LRAPCIMS *m/z* (rel. int.): 355 [$M+H^+$, 24%, calcd for $C_{16}H_{19}O_9$, 355.1029], 164 (9), 163 (100), 135 (18), 117 (8).

Chlorogenic acid methyl ester [(13), 3-*O*-Caffeoylquinic acid methyl ester]: Transparent brown oil (15.2 mg, 0.28%, not optimised); ^{1}H NMR (500 MHz, CD_3OD): 7.52 (1H, d, *J* = 15.9 Hz, H-3′), 7.04 (1H, d, *J* = 2.1 Hz, H-5′), 6.95 (1H, dd, *J* = 2.1, 8.2 Hz, H-9′) 6.78 (1H, d, *J* = 8.2 Hz, H-8′), 6.21 (1H, d, *J* = 15.9 Hz, H-2′), 5.28 (1H, ddd, *J* = 4.4, 8.6, 9.2 Hz, H-3), 4.13 (1H, ddd, *J* = 3.2, 6.3, 8.1 Hz, H-5), 3.72 (1H, dd, *J* = 3.2, 8.6 Hz, H-4), 3.70 (3H, s, 7-COOMe), 2.21 (1H, dd, *J* = 14.1, 4.4 Hz, H-2a), 2.14 (1H, dd, *J* = 8.1, 13.5 Hz, H-6a), 2.05 (1H, dd, *J* = 14.1, 9.2 Hz, H-2b), 2.01 (1H, dd, *J* = 6.3, 13.5 Hz, H-6b), Lit. (Zhu et al., 2005a); ^{13}C NMR (126 MHz, CD_3OD): 175.6 (C, C-7), 168.4 (C, C-1′), 149.8 (C, C-7′), 147.3 (CH, C-3′), 147.0 (C, C-6′), 127.8 (C, C-4′), 123.1 (CH, C-9′), 116.7 (CH, C-8′), 115.3 d (CH, C-5′), 115.2 d (CH, C-2′), 75.6 (C, C-1), 72.7 (CH, C-4), 72.3 (CH, C-3), 70.5 (CH, C-5), 53.1 (C, 7-COOMe), 38.2 (CH_2, C-6), 38.0 (CH_2, C-2), Lit. (Zhu et al., 2005a); (+)-LRAPCIMS *m/z* (rel. int.):

369 [M+H$^+$, 42%, calcd for $C_{17}H_{21}O_9$, 369.1185], 164 (12), 163 (100), 135 (17), 117 (9).

Isochlorogenic acid A [(14), 3,5-Di-*O*-caffeoylquinic acid]: Colourless powder (25.7 mg, 0.47%, not optimised); ^{1}H NMR (500 MHz, CD_3OD) 7.62 (1H, d, *J* = 15.9 Hz, H-3′), 7.57 (1H, d, *J* = 15.9 Hz, H-3″), 7.07 (1H, d, *J* = 1.70 Hz, H-5″), 7.07 (1H, d, *J* = 1.7 Hz, H-5′), 6.98 (1H, dd, *J* = 1.7, 8.2 Hz, H-9′), 6.96 (1H, dd, *J* = 1.7, 8.2 Hz, H-9″), 6.78 (1H, d, *J* = 8.2 Hz, H-8″), 6.78 (1H, d, *J* = 8.2 Hz, H-8′), 6.35 (1H, d, 15.9 Hz, H-2′), 6.26 (1H, d, *J* = 15.9 Hz, H-2″), 5.43 (1H, ddd *J* = 3.2, 3.9, 7.1 Hz, H-5), 5.38 (1H, m, H-3), 3.97 (1H, dd, *J* = 3.2, 7.4 Hz, H-4), 2.32 (1H, dd, *J* = 3.9, 13.8 Hz, H-6a), 2.25 (2H, m, H-2), 2.17 (1H, dd, *J* = 7.1, 13.8 Hz, H-6b), Lit. (Kodoma et al., 1998); ^{13}C NMR (126 MHz, CD_3OD): 177.5 (C, C-7), 169.0 (C, C-1″), 168.5 (C, C-1′), 149.8 (C, C-7″), 149.7 (C, C-7′), 147.4 (CH, C-3″), 147.2 (CH, C-3′), 147.0 (C, C-6″), 147.0 (C, C-6′), 128.1 (C, C-4″), 128.0 (C, C-4′), 123.2 (CH, C-9″), 123.1 (CH, C-9′), 116.6 (CH, C-8″), 116.6 (CH, C-8′), 115.8 (CH, C-2″), 115.4 (CH, C-5″), 115.3 (CH, C-5′), 115.3 (CH, C-2′), 74.8 (C, C-1), 72.7 (CH, C-5), 72.3 (CH, C-3), 70.8 (CH, C-4), 38.0 (CH_2, C-2), 36.1 (CH_2, C-6), Lit. (Kodoma et al., 1998); (+)-LRAPCIMS *m/z* (rel. int.): 517 [M+H$^+$, 8%, calcd for $C_{25}H_{25}O_{12}$, 517.1346], 500 (29), 499 (100), 163 (36), 135 (7).

Macroantoin G [(15), 1,4-Dihydroxy-3*R*,5*R*-di-*O*-caffeoylquinic acid methyl ester]: Colourless powder (16.8 mg, 0.31%, not optimised); $[\alpha]_D^{22}$ -100.9° (c 0.25, MeOH); Lit. $[\alpha]_D^{25}$ -81° (c 1, MeOH), Zhang et al., 2000; ^{1}H NMR (500 MHz, CD_3OD) see Table 3.5. Lit. (Zhang et al., 2000); ^{13}C NMR (126 MHz, CD_3OD) see Table 3.5. Lit. (Zhang et al., 2000); (+)-LRAPCIMS *m/z* (rel. int.): 531 [M+H$^+$, 8%], 513(100), 163

(24), 135 (5); (+)-HRAPCIMS *m/z* 531.1497 [M+H$^+$, 100%] (calcd for $C_{26}H_{27}O_{12}$, 531.1502, Δ 0.0005 a.m.u.), 513 (26), 391 (55). Lit. (Zhang et al., 2000).

Myriogenic acid-7-methyl ester [(7), 4*ξ*,5*ξ*-Di-*O*-caffeoyl-2,6*ξ*-dihydroxyhept-2-ene-1,7-dioic acid-7-methyl ester]: Transparent brown oil (40.6 mg, 0.74%, not optimised); $[\alpha]_D^{22}$ +20.6° (c 0.24, MeOH); ^{1}H NMR (500 MHz, CD_3OD) see Table 3.3; ^{13}C NMR (126 MHz, CD_3OD) see Table 3.3; (+)-LRAPCIMS *m/z* (rel. int.): 561 [M+H$^+$, 22%], 560 (73), 364 (23), 363 (100), 183 (42), 163 (51), 155 (25), 135 (12), 117 (5); (+)-HRAPCIMS *m/z* (rel. int.): 560.1399 calcd for $C_{26}H_{25}O_{14}$, 561.1244, (Δ0.9845 a.m.u.), [M+H$^+$] (100%), 391 (23), 363 (75), 349 (34).

Myriogenic acid-1-methyl ester [(8), 4*ξ*,5*ξ*-Di-*O*-caffeoyl-2,6*ξ*-dihydroxyhept-2-ene-1,7-dioic acid-1-methyl ester]: Tan spheres (19.7 mg, 0.36%, not optimised); $[\alpha]_D^{22}$ +22.6° (c 0.11, MeOH); ^{1}H NMR (500 MHz, CD_3OD) see Table 3.3; ^{13}C NMR (126 MHz, CD_3OD) see Table 3.3; (+)-LRAPCIMS *m/z* (rel. int.): 561 [M+H$^+$, 30%], 560 (100), 364 (31), 363 (60), 184, (12), 183 (100), 164 (10), 163 (90), 155 (18), 135 (20), 123 (43), 117 (7); (+)-HRAPCIMS *m/z* (rel. int.): 560.1400 calcd for $C_{26}H_{25}O_{14}$, 561.1244, Δ0.9844 a.m.u.), [M+H$^+$] (70%), 391 (100), 363 (64).

Myriogenic acid dimethyl ester [(9), 4*ξ*,5*ξ*-Di-*O*-caffeoyl-2,6*ξ*-dihydroxyhept-2-ene-1,7-dioic acid-dimethyl ester]: tan oil (39.1 mg, 0.71%, not optimised); $[\alpha]_D^{22}$ +29.1° (c 0.395, MeOH); ^{1}H NMR (500 MHz, CD_3OD) see Table 3.3; ^{13}C NMR (126 MHz, CD_3OD) see Table 3.3; (+)-LRAPCIMS *m/z* (rel. int.): 575 [M+H$^+$, 31%], 574 (96), 378 (27), 377 (100), 198 (12), 197 (99), 169 (42), 164 (6), 163 (49), 137 (7), 135

(12), 123 (10), 117 (6); (+)-HRAPCIMS *m/z* (rel. int.): 574.1559 calcd for $C_{22}H_{23}O_{13}$, 575.1400 (Δ0.9841 a.m.u.), [M+H$^+$] (100%), 560 (12), 391 (19), 377 (66).

Axillarin [(16), 3′,4′,5,7-Tetrahydroxy-3,6-dimethoxyflavone]: Yellow needles (ACN/H_2O) (21.0 mg, 1.5%, not optimised); ^{1}H NMR (500 MHz, CD_3OD): δ 7.62 (1H, d, J = 2.2 Hz, H-2′), 7.53 (1H, dd, J = 2.2, 8.5 Hz, H-6′), 6.89 (1H, d, J = 8.5 Hz, H-5′), 6.50 (1H, s, H-8), 3.88 (3H, s, 6-OCH_3), 3.79 (3H, s, 3-OCH_3), Lit. (Jefferies et al., 1974); ^{13}C NMR (126 MHz, CD_3OD): δ 180.5 (C, C-4), 158.9 (C, C-7),158.3 (C, C-2), 154.0 (C, C-5), 153.9 (C, C-9), 150.1 (C, C-4′), 146.6 (C, C-3′), 139.4 (C, C-3), 132.7 (C, C-6), 123.1 (C, C-1′), 122.5 (CH, C-6′), 116.7 (C, C-5′), 116.6 (CH, C-2′), 106.5 (C, C-10), 95.1 (CH, C-8), 61.1 (CH_3, C6-OMe), 60.5 (CH_3, C3-OMe); (+)-LRAPCIMS *m/z* (rel. int.): 347 calcd for $C_{17}H_{15}O_8$, 347.0767 [M+H$^+$] (100%), 332 (9), 317 (5), 289 (10).

Isokaempferide [(17), 4′,5,7-Trihydroxy-3-methoxyflavone]: Yellow needles (ACN/H_2O) (40.1 mg, as a 1:1 mixture of compound 12 and 13, 2.9%, not optimised); ^{1}H NMR (500 MHz, CD_3OD): δ 7.98 (1H, d, J = 8.9 Hz, H-2′), 7.98 (1H, d, J = 8.9 Hz, H-6′), 6.92 (1H, d, J = 8.9 Hz, H-3′), 6.92 (1H, d, J = 8.9 Hz, H-5′), 6.40 (1H, d, J = 2.1 Hz, H-6), 6.20 (1H, d, J = 2.1 Hz, H-8), 3.78 (3H, s, 3-OCH_3), Lit. (Grouiller et al., 1967); ^{13}C NMR (126 MHz, CD_3OD): δ 180.2 (C, C-4), 166.2 (C, C-7), 163.3 (C, C-5), 161.9 (C, C-4′), 158.6 (C, C-9), 158.2 (C, C-2), 139.5 (C, C-3), 131.6 (CH, C-6′), 131.6 (CH, C-2′), 122.8 (C, C-1′), 116.6 (CH, C-5′), 116.6 (CH, C-3′), 106.1 (C, C-10), 100.0 (CH, C-6), 94.9 (CH, C-8), 60.7 (CH_3, C3-OMe); (+)-LRAPCIMS *m/z* (rel. int.): 301, calcd for $C_{16}H_{13}O_6$, 301.0712 [MH$^+$] (100%).

4′,5,7-Trihydroxy-3,6-dimethoxyflavone (18): Yellow needles (ACN/H_2O); ^{1}H NMR (500 MHz, CD_3OD): δ 7.98 (1H, d, J = 8.9 Hz, H-6′), 7.98 (1H, d, J = 8.9 Hz, H-2′), 6.92 (1H, d, J = 8.9 Hz, H-5′), 6.92 (1H, d, J = 8.9 Hz, H-3′), 6.51 (1H, s, H-8), 3.88 (3H, s, 6-OCH_3), 3.78 (3H, s, 3-OCH_3), Lit. (Herz et al., 1975); ^{13}C NMR (126 MHz, CD_3OD): δ 180.5 (C, C-4), 161.9 (C, C-4′), 159.1 (C, C-9), 158.3 (C, C-5), 158.2 (C, C-2), 154.0 (C, C-7), 139.5 (C, C-3), 132.6 (C, C-6), 131.6 (CH, C-6′), 131.6 (CH, C-2′), 122.8 (C, C-1′), 116.6 (CH, C-5′), 116.6 (CH, C-3′), 106.5 (C, C-10), 95.2 (CH, C-8), 61.1 (CH_3, C6-OMe), 60.7 (CH_3, C3-OMe); (+)-LRAPCIMS *m/z* (rel. int): 331, calcd for $C_{17}H_{15}O_7$, 331.0818 [MH^+] (100%).

Jaceidin [(19), 4′,5,7-Trihydroxy-3,3′,6-trimethoxyflavone]: Yellow needles (ACN/H_2O) (23.8 mg, 1.7%, not optimised); ^{1}H NMR (500 MHz, CD_3OD): δ 7.71 (1H, d, J = 2.0 Hz, H-2′), 7.63 (1H, dd, J = 2.0, 8.4 Hz, H-6′), 6.94 (1H, d, J = 8.4 Hz, H-5′), 6.53 (1H, s, H-8), 3.94 (3H, s, 3′-OCH_3), 3.88 (3H, s, 6-OCH_3), 3.80 (3H, s, 3-OCH_3), Lit. (Roitman and James, 1985); ^{13}C NMR (126 MHz, CD_3OD): δ 180.5 (C, C-4), 158.9 (C, C-7), 158.1 (C, C-2), 153.9 (C, C-5), 153.7 (C, C-9), 151.3 (C, C-4′), 149.1 (C, C-3′),139.5 (C, C-3), 132.8 (C, C-6), 123.9 (CH, C-6′), 123.1 (C, C-1′), 116.0 (CH, C-5′), 113.1 (CH, C-2′), 106.5 (C, C-10), 95.2 (CH, C-8), 61.1 (CH3, C6-OMe), 60.8 (CH_3, C3-OMe), 56.7 (CH_3, C3′-OMe); (+)-LRAPCIMS *m/z* (rel. int.): 361 [$M+H^+$, 100%, calcd for $C_{18}H_{17}O_8$, 361.0923], 346 (7), 303 (7).

Arnicolide C [(20), 6-*O*-(2-Methylpropanoyl)-4-oxo-2-pseudoguaien-12,8-olide]: Colourless crystals (ACN/H_2O) (68.2 mg, 2.4%, not optimised); $[\alpha]_D^{22}$ -81.7° (c 0.42, MeOH); $[\alpha]_D^{20}$ -91.1 (c 0.25, MeOH), Lit.(Poplawski et al., 1971); ^{1}H NMR (500 MHz, CD_3OD): δ 7.89 (1H, dd, J = 1.8, 6.1 Hz, H-2), 6.05 (1H, dd, J = 3.1, 6.1 Hz, H-3), 5.40

(1H, s, H-6), 4.86 (1H, dt, *J* = 6.1, 1.9 Hz, H-8), 3.26 (1H, dq, *J* = 7.5, 7.5 Hz, H-11), 3.12 (1H, ddd, *J* = 2.5, 10.8 Hz, H-1), 2.88 (1H, dd, *J* = 7.5, 10.1 Hz, H-7), 2.46 (1H, ddd, *J* = 2.3, 5.9, 15.5 Hz, H-9a), 2.42 (1H, septet, *J* = 7.1 Hz, H-2′), 2.17 (1H, m, H-10), 1.72 (1H, ddd, *J* = 1.9, 11.5, 15.5 Hz, H-9b), 1.44 (3H, d, *J* = 7.5 Hz, H-13), 1.26 (3H, d, *J* = 6.7 Hz, H-14), 1.06 (3H, d, *J* = 7.1 Hz, 2′-CH_3), 1.02 (3H, d, *J* = 7.1 Hz, H-3′), 1.00 (3H, s, H-15), Lit. (Poplawski et al., 1971); ^{13}C NMR (126 MHz, CD_3OD): *δ* 212.5 (C, C-4), 181.7 (C, C-12), 177.1 (C, C-1′), 165.4 (CH, C-2), 130.1 (CH, C-3), 81.6 (CH, C-8), 73.1 (CH, C-6), 55.9 (C, C-5), 55.7 (CH, C-1), 50.4 (CH, C-7), 42.0 (CH_2, C-9), 41.6 (CH, C-11), 35.3 (CH, C-2′), 27.3 (CH, C-10), 20.1 (CH_3, C-14), 19.4 (CH_3, C-3′), 19.0 (CH_3, 2′-CH_3), 18.3 (CH_3, C-15), 11.3 (CH_3, C-13); (+)-LRAPCIMS *m/z* (rel. int.): 335 calcd for $C_{19}H_{27}O_5$, 335.1859, [$M+H^+$] (18%), 247 (100), 203 (37), 201 (37), 199 (18), 173 (18), 150 (7), 145 (9), 128 (5), 115 (6), 105 (7).

(3*R*,4*S*,5*S*,8*S*,10*R*,13*R*)-3-Hydroxykaura-9(11),16-dien-18-oic acid (21): Colourless needles (ACN/H_2O) (26.5 mg, 1.0%, not optimised); $[\alpha]_D^{22}$ +30.8° (c 0.12, MeOH); ^{1}H NMR (500 MHz, CD_3OD): *δ* 5.26 (1H, t, *J* = 3.3 Hz, H-11), 4.89 (1H, d, *J* = 1.1 Hz, H-17a), 4.77 (1H, br s, H-17b), 3.17 (1H, dd, *J* = 4.4, 12.1 Hz, H-3), 2.74 (1H, br s, H-13), 2.61 (1H, br d, *J* = 14.8 Hz, H-15a), 2.44 - 2.40 (1H, m, H-12a), 2.41 - 2.35 (1H, m, H-6a), 2.29 - 2.16 (1H, m, H-2a), 2.20 - 2.16 (1H, m, H-15b), 2.04 (1H, m, H-1a), 1.99 - 1.95 (1H, m. H-7a), 1.99 - 1.95 (1H, m, H-12b), 1.93 – 1.85 (1H, m, H-6b), 1.68 - 1.74 (1H, m, H-2b), 1.65 – 1.62 (1H, m, H-5), 1.63 - 1.60 (1H, m, H-14a), 1.51 - 1.47 (1H, m, H-7b), 1.51 - 1.47 (1H, m, H-14b), 1.38 - 1.32 (1H, m, H-1b), 1.36 (3H, s, H-18), 1.10 (3H, s, H-20), Lit. (methyl ester derivative, Bohlmann et al., 1982); ^{13}C NMR (126 MHz, CD_3OD): *δ* 180.1 (C, C-19), 159.6 (C, C-16), 157.3 (C, C-9), 116.3 (CH, C-11), 106.2 (CH_2, C-17), 79.4 (CH, C-3), 51.5 (CH_2, C-15), 51.3 (C, C-4), 47.0 (CH, C-

5), 46.2 (CH_2, C-14), 43.6 (C, C-8), 42.8 (CH, C-13), 40.4 (CH2, C-1), 39.7 (C, C-10), 39.1 (CH_2, C-12), 31.0 (CH_2, C-7), 29.9 (CH_2, C-2), 24.6 (CH_3, C-18), 24.4 (CH_3, C-20), 19.8 (CH_2, C-6); (+)-LRAPCIMS *m/z* (rel. int.): 316 [$M+H^+$, 0%], 299 (100), 271 (59), 253 (93), 225 (6); (+)-HRAPCIMS *m/z* (rel. int): 317.2109 calcd for $C_{20}H_{29}O_3$, 317.2118 (Δ0.0009 a.m.u.).

8-Hydroxy-9,10-diisobutyryloxythymol (23): Colourless needles (ACN/H_2O) (18.2 mg, 0.7%, not optimised); ^{1}H NMR (500 MHz, CD_3OD): δ 7.21 (1H, d, J = 8.0 Hz, H-5), 6.65 (1H, br d, J = 8.0 Hz, H-6), 6.60 (1H,br s, H-2), 4.54 (1H, d, J = 11.3 Hz, H-9b), 4.54 (1H, d, J = 11.3 Hz, H-10b), 4.44 (1H, d, J = 11.3 Hz, H-9a), 4.44 (1H, d, J = 11.3 Hz, H-10a), 2.51 (2H, septet, J = 7.0 Hz, H-2′, H-2″), 2.24 (3H, s, H-7), 1.09 (3H, s, H-3″), 1.07 (3H, s, H-3′), 1.06 (3H, s, 3″-CH_3), 1.05 (3H, s, 3′-CH_3), Lit. (Mossa et al., 1997); ^{13}C NMR (126 MHz, CD_3OD): δ 178.4 (C, C-1″), 178.4 (C, C-1′), 156.3 (C, C-3), 140.5 (C, C-1), 128.8 (CH, C-5), 123.5 (C, C-4), 121.3 (CH, C-6), 118.0 (CH, C-2), 77.3 (C, C-8), 68.3 (CH_2, C-9), 68.3 (CH_2, C-10), 35.3 (CH, C-2″), 35.3 (CH, C-2′), 21.1 (CH_3, C-7), 19.4 (CH3, 3′-CH_3), 19.4 (CH3, 3″-CH_3), 19.3 (CH_3, C-3″), 19.3 (CH_3, C-3′); (+)-LRAPCIMS *m/z* (rel. int.): 321 calcd for $C_{18}H_{25}O_5$, 321.17025, [M-H_2O^+] (44%), 145 (100).

2.3 Experimental on Bioactivity of *C. cunninghamii*

2.3.1 Preparation of *C. cunninghamii* Extracts

Four different solvent extracts of *C. cunninghamii* (CPR# 050285) were prepared. The dried plant material was ground in a Waring blender and then extracted by steeping overnight at room temperature. A boiled extract was prepared by boiling the sample in water for 1 hour. The resulting extracts were then filtered through a glass frit and

evaporated to dryness on a rotary evaporator. A description of the extracts and yields is listed in Table 2.1.

Table 2.1. *C. cunninghamii* solvent extracts and yields.

Extract	Solvent	Mass sample	Volume solvent	Yield of extract
1	100% aqueous	25.2 g	250 mL	4.1 g
2	50% aqueous-ethanol	25.2 g	250 mL	3.8 g
3	100% ethanol	27.1 g	250 mL	1.2 g
4	100% aqueous (100°C)	25.8 g	250 mL	4.6 g

2.3.2 Solid Phase Extraction (SPE)

The crude 50% aqueous ethanol extract was partitioned into four fractions using reverse phase SPE columns (Supelco, 60 mL, 10g Supelclean LC-18). SPE columns were first preconditioned with 6-10 bed volumes (BV) of methanol and then equilibrated using 6-10 BV of water. The sample (500 mg) was first dissolved in 1 BV of the starting mobile phase and loaded onto the SPE column. The sample was then eluted with water/acetonitrile (ACN) using a stepwise gradient (Table 2.2).

Table 2.2. Solid phase extraction fractionation parameters.

Fraction	Solvent	Volume
1	100% Water	60 mL
2	20% ACN/Water	60 mL
3	40% ACN/Water	60 mL
4	100% ACN	60 mL

2.3.3 Preparative HPLC Fractionation

The crude 50% aqueous ethanol extract of *C. cunninghamii* was fractionated by RP prep-HPLC. The eluent was a gradient of 10-90% B over 25 minutes, followed by an isocratic gradient of 90% B for 3 min, at a flow rate of 15 mL/min. Fractions were collected in 1 minute intervals and fractions and/or compounds were recovered from the eluent by rotary evaporation. All of the SPE fractions were further fractionated by

RP prep HPLC. A mobile phase gradient of 10-36% B over 12 min at a flow rate of 20 mL/min was utilized to fractionate SPE fraction 2. A mobile phase gradient of 10-60% B over 20 min at a flow rate of 20 mL/min was utilized to fractionate SPE fraction 3. A mobile phase gradient of 40-80% B over 16 min at a flow rate of 20 mL/min was utilized to fractionate SPE fraction 4. Consecutive fractions which produced low yields were combined for the purpose of bioassay evaluation.

2.3.4 Oxygen Radical Absorbance Capacity (ORAC)

The antioxidant assays on *C. cunninghamii* extracts and compounds (Chapter 4) were performed by the staff at the Centre for Phytochemistry and Pharmacology (Southern Cross University). The ORAC assay employed in this study measured the antioxidant scavenging activity of the test samples, against peroxyl radicals induced by 2,2′-azobis(2-methylpropionamidine) dihydrochloride (AAPH, Wako: 01711062) at 37 ºC, fluorescein (Aldrich: 166308) was used as the fluorescent probe. The method used is based on that of Huang et al. (2002a); Huang et al. (2002b) and Prior et al. (2003).

All samples were assayed using the hydrophilic ORAC procedure in four-fold serial dilution with AWA (acetone: water: acetic acid; 70: 29.5: 0.5), and in quadruplicate, starting with a concentration relevant to the sample. Initial sample concentration was selected based on the approximated antioxidant capacity observed from a preliminary screen.

Trolox, a water soluble analogue of vitamin E, was used as a reference standard. A trolox standard curve was established from trolox (Aldrich: 23881-3) standards prepared at 100, 50, 25, and 12.5 μM in AWA. Butylated hydroxytoluene (BHT,

Sigma-Aldrich: B-1378) was used as a positive control, and was assayed at 500, 250, 125, and 62.5 μM.

Ten μL fluorescein (6.0×10^{-7} M), 20 μL samples/standards/control/blank (A:W:A) and 170 μL AAPH (20 mM) were introduced into each well. Immediately after loading, the plate was transferred to the plate reader preset to 37 ºC, and the fluorescence was measured 35 times at one minute intervals. The fluorescence readings were referenced to solvent blank wells. The final ORAC values were calculated using a regression equation between the trolox concentration and the net area under the fluorescein decay curve, and where possible were expressed as trolox equivalents (TE) in μmol/g of sample.

2.3.5 Inhibition of Prostaglandin E_2 Production (PGE_2)

The anti-inflammatory activity of the test sample is measured by its ability to inhibit the ionophore-induced production of PGE_2 by cells. In this assay a suspension of murine swiss albino fibroblast cell (NIH 3T3, Sigma-Aldrich, Castle Hill, NSW: 93061524)consisting of phenol-red free DMEM with 10% FBS and 2mM L-glutamine was plated out into 96-well tissue culture plates (1×10^5 cells/mL, 100 μL/well). The cells were cultured overnight at 37 ºC in 5% CO_2. The extracts and fractions were solubilised in DMSO, and diluted appropriately with media so that the extracts were tested at a final concentration of 1000, 100, and 10 μg/mL. Five μL of sample was added to each well and tests were performed in triplicate. The cells and samples were incubated (37 ºC, 3 hours) before the addition of calcium ionophore A23187 (Sigma-Aldrich: C-7522). Following 20 min incubation, the plate was centrifuged (1000 RCF, 3 min) and the supernatants were removed. A positive control aspirin (100 μM, Sigma:

A-5376), and DMSO control, both with and without calcium ionophore A23187 were included on the plate.

The supernatants were diluted by serial dilution (1:500) in enzyme immunoassay (EIA) buffer, and assayed for PGE_2 using the Prostaglandin E_2 EIA Monoclonal Kit (Cayman Chemical: 514010), according to manufacturer's instructions.

2.3.6 Inhibition of Cyclooxygenase (COX) Gene Expression

The method employed in this experiment detects the intracellular cyclooxygenases (COX-1 & COX-2) in human whole blood monocytes and is adapted from published methods (Ruitenberg and Waters, 2003). A dried 50% aqueous ethanol extract was reconstituted in DMSO, to provide concentrations of 100, 10 and 1 mg/mL. Aliquots of fresh whole human blood were pre-incubated with each extract dilution (final concentrations of 1000, 100 and 10 μg/mL) for two hours at 37 °C. Lipopolysaccharide (LPS, Sigma-Aldrich: L-3880) was then added to each aliquot (except unstimulated controls) for a final LPS concentration of 0.01 μg/mL and then incubated for a further two hours at 37°C. Unstimulated, untreated, dexamethasone (Sigma-Aldrich: D-4902) (positive inhibitor), and solvent (DMSO) controls were also included.

Samples from each aliquot were then stained with CD14 (monocyte marker) monoclonal antibody (mAb) then subsequently stained intracellularly with COX-2 mAb. The percentage of monocytes expressing COX-1 and COX-2 was then determined using a FACSCalibur flow cytometry instrument (BD Biosciences). Testing was performed in duplicate.

2.3.7 Inhibition of Cyclooxygenase (COX) Enzyme Activity

The influence of a 50% aqueous ethanol extract on cyclooxygenase (COX) activity was measured using a commercial COX inhibitor screening assay (Cayman Chemical: 560131). The COX inhibitor screening assay directly measures $PGF_{2\alpha}$ produced by $SnCl_2$ reduction of COX-derived PGH_2. The prostanoid product is quantified using an enzyme immunoassay with a broadly specific antibody that binds to all the major prostaglandin compounds.

Separate reactions were performed to measure the influence of the extract on COX-1 and COX-2. Both reactions were carried out on the sample, in duplicate. The extract was solubilised in DMSO to obtain test concentrations of 60 and 6.0 mg/mL. A 20 μL aliquot of each dilution was included with the COX reactions, providing a final concentration of 1000 and 100 μg/mL. These COX reactions and the EIA were carried out according to the manufacturer's instructions.

Ibuprofen (Cayman Chemical: 70280) (85 μM; 18 μg/mL) was included as a positive control for COX-1 inhibition, and Celebrex (Cayman Chemical: 10008005) (40 μM; 15.3 μg/mL) was included as a positive control for COX-2 inhibition.

2.3.8 Inhibition of Lipoxygenase (LO) Activity

The influence of the 50% aqueous ethanol extract on lipoxygenase (LO) activity was measured using a commercial LO inhibitor screening assay (Cayman Chemical: 760700). Lipoxygenases catalyze the addition of molecular oxygen to fatty acids. Lipoxygenase inhibition was determined by measuring the hydroperoxides produced in the lipoxygenation reaction, using purified 5-lipoxygenase (potato) enzyme. Linoleic

acid (Sigma: L-1268) was used as the substrate for 5-LO, as per the manufacturer's recommendation.

Serial dilutions were performed using the extract solubilised at 21 μg/mL in DMSO, which provided the final concentrations of; 1000, 100, and 10 μg/mL in the well. Each concentration was tested in triplicate. Nordihydroguaiaretic acid (Cayman Chemical: 70300) (NDGA) was included as a positive control for lipoxygenase inhibition at a final concentration of 50 μM (14 μg/mL). The lipoxygenase inhibitor screening assay was carried out according to the manufacturer's instructions.

2.3.9 Inhibition of Nitric Oxide (NO) Production

NO was measured using a nitrate/nitrite fluorometric assay kit (Cayman Chemical: No. 780051). The final products of NO are nitrite (NO_2^-) and nitrate (NO_3^-), and the proportion of each is variable and relatively unpredictable. Therefore, this assay measures the total NO production as the sum of nitrates and nitrites. The assay was carried out according to manufacturer's instructions.

RAW 264 cells (ECACC: 85062803) were routinely cultured in Dulbecco's modified eagle medium (DMEM) with 10% foetal bovine serum (FBS) and 2 mM L-glutamine, at 37ºC and 5% CO_2. Cell culturing media was purchased from Invitrogen, Mt Waverley, VIC. RAW 264 cells were plated out at a concentration of 1×10^6 cells/mL (100 μL/well), in the above media but without phenol red indicator. Cells were allowed to attach overnight before sample addition. The 50% aqueous ethanol extract was tested in triplicate in the presence and absence of lipopolysaccharide (LPS; from *E. coli* serotype 0127:B8). The presence of LPS stimulates NO production. After 1 h pre-incubation with the samples (15 μL; final concentration of 130, 13, and 1.3 μg/mL and

solvent controls), LPS (0.05 μg/mL) was added to the appropriate wells, and the cells were incubated for a further 20 h (37ºC, 5% CO_2). Supernatants were collected following centrifugation (1500 RCF, 3 min), and the concentration of NO was determined by fluorometry using a Victor^2 plate reader.

2.3.10 Inhibition of Tumor Necrosis Factor-α (TNF-α) Production

Raw 264 cells were prepared as described above. Once plated, cells were allowed to attach overnight before sample addition. The 50% aqueous ethanol extract was tested in triplicate in the presence and absence of lipopolysaccharide (LPS; from *E. coli* serotype 0127:B8). The presence of LPS stimulates TNF-α production. Solvent control wells were also included. After 1 h pre-incubation with the samples (15 μL; final concentrations of 130, 13, and 1.3 μg/mL), LPS (0.05 μg/mL) was added to the appropriate wells, and cells were further incubated for 20 h (37ºC, 5% CO_2). Supernatants were collected following centrifugation (1500 RCF, 3 min), and used to measure the concentration of TNF-α. The supernatants were frozen (-80ºC) to measure TNF-α concentration at a later time.

The levels of TNF-α were determined using an Quantikine Mouse TNF-α immunoassay (R&D Systems: MTA00). The assay was carried out according to the manufacturer's instructions.

2.3.11 Inhibitory Concentration at 50% (IC_{50}) Determination

Linear regression between % INH and log concentration of each compound was calculated using SPSS software. Percentage inhibition (% INH) was calculated by formula;

$$\% \text{ INH} = 1 - \left[\frac{\text{LS-LI}}{\text{LC-LI}} \right] \text{x } 100$$

LS = Luminescence count from well contains tested sample

LI = Luminescence count from well with maximum inhibition

LC = Luminescence count from DMSO control (average)

2.4 Experimental on Chemistry of *E. mitchellii*

2.4.1 Plant Materials

Wood and root materials (Refer to Section 2.5.2 for collection details) were ground using a Retsch cutting mill (Retsch GmbH, Haan, Germany: SM 100).

2.4.2 Steam Distillation of *E. mitchellii* Oils

Steam distillation of *E. mitchellii* plant parts was carried out using approximately 500 g of the dried plant material. Whole leaf material and ground wood, root and bark material was steam distilled for 48 h in a 5 L distillation flask that was fitted with a modified Cocking and Middleton trap. A description of the oils and yields are presented in Table 2.3.

Table 2.3. Details of the steam distillation of *E. mitchellii* - different plant parts.

Plant part†	Mass plant material	Yield	Description
leaf	500 g	7.1 mL	black-green oil
twigs	500 g	0.2 mL	dark amber oil, $d \geq 1.0$
roots	766 g	2.0 mL	dark amber oil, $d \geq 1.0$
bark	392 g	yielded no oil	-
red heart wood	647 g	14.3 mL	dark amber oil, $d \geq 1.0$
white outer wood	472 g	yielded no oil	-

d = density; † *E. mitchellii* Specimen # PIF 30486.

2.4.3 Characterisation of the Oil by Gas Chromatography

GC-MS analysis of the oil was undertaken using the same methodology described in section 2.2.3.

α-Pinene, *β*-pinene, *p*-cymene, limonene, linalool, *α*-terpineol and eugenol were obtained from Aldrich chemical Co. Inc. (Milwaukee, WI); *α*-phellandrene, *α*-terpinolene, viridiflorene (ledene), globulol and *α*-bisabolol were obtained from Fluka Chemie (Buchs, Switzerland); aromadendrene was obtained from Sigma-Aldrich (Castle Hill, NSW). Epiglobulol, viridiflorol and *δ*-cadinene were identified by comparison with these componds in authentic tea tree oil (*Melaleuca alternifolia*. FPI Oils, Melbourne). Elemol, *α*- and *β*-eudesmol and *α*- and *β*-selinene were identified by comparison with these compounds from celery seed oil (*Apium graveolens*, Auroma, Melbourne). Cadina-1,4-diene, *β*-phellandrene and *trans*-*β*-caryophyllene were identified by comparison with these compounds from betel leaf oil (*Piper betle*, FPI Oils, Melbourne). *β*-Elemene was identified by comparison with this compound from myrrh oil (*Commiphora myrrha*, FPI Oils, Melbourne). Spathulenol was identified by comparison with this compound from angelica root oil (*Angelica archangelica*, FPI Oils, Melbourne).

2.4.4 Normal Phase Fractionation of *E. mitchellii* Wood Oil

Fractionation of the oil was achieved using normal phase preparative HPLC employing a hexane/ethyl acetate gradient (EREMO4 method) (Figure 2.3). The wood oil (CP020173) was fractionated in this way on three occasions to generate fractions for testing against two spotted mites, termites and to generate pure compounds for LD_{50} and LD_{95} determination on termites. The eluent gradient was 5-40% EtOAc over 20

min, at a flow rate of 20 mL/min. A more efficient, silica column chromatographic method was developed for large scale fractionation of the oil (Figure 2.4).

Normal phase prep HPLC fraction 3 was sub-fractionated by PR prep-HPLC utilizing a gradient of 40-70% B over 15 min, followed by an isocratic gradient of 70% B for 5 min, then a gradient of 70-90% B over 5 min, at a flow rate of 15 mL/min to afford pure compound **36**. Compound **35** was further purified from NP prep-HPLC fraction 5 by recrystallising from MeOH. Compounds **30** and **42** were purified from NP prep-HPLC fraction 4 by RP prep-HPLC utilizing two preparative columns in tandem and an isocratic gradient of 70% MeOH/H_2O over 55 min at a flow rate of 15 mL/min. Compounds **42** and **32** were purified from NP prep-HPLC fraction 6, utilizing a gradient of 10-90% B over 25 min, and a flow rate of 15 mL/min. NP prep fractions 8 and 9 were combined and sub-fractionated by RP prep-HPLC to obtain pure compound **33**. The eluent composition used was 40-70% B over 15 min, followed by an isocratic gradient of 70% B for 5 min, then a gradient of 70-90% B, at a flow rate of 15 mL/min.

2.4.5 Reverse Phase Fractionation of *E. mitchellii* Wood Oil

Fractionation of the oil was achieved using reverse phase preparative HPLC employing a water/acetonitrile gradient (Figure 2.5). Compounds were recovered from the eluent by rotary evaporation. A mobile phase system of solvent A (MQ water with 0.05% TFA) and solvent B (acetonitrile with 0.05% TFA) was utilized for the isolation work. The eluent was a gradient of 40-70% B over 15 min, followed by an isocratic gradient of 70% B for 5 min, then a gradient of 70-90% B over 5 min, at a flow rate of 15 mL/min. Fractions were collected in 1 min intervals and fractions and/or compounds were recovered from the eluent by rotary evaporation.

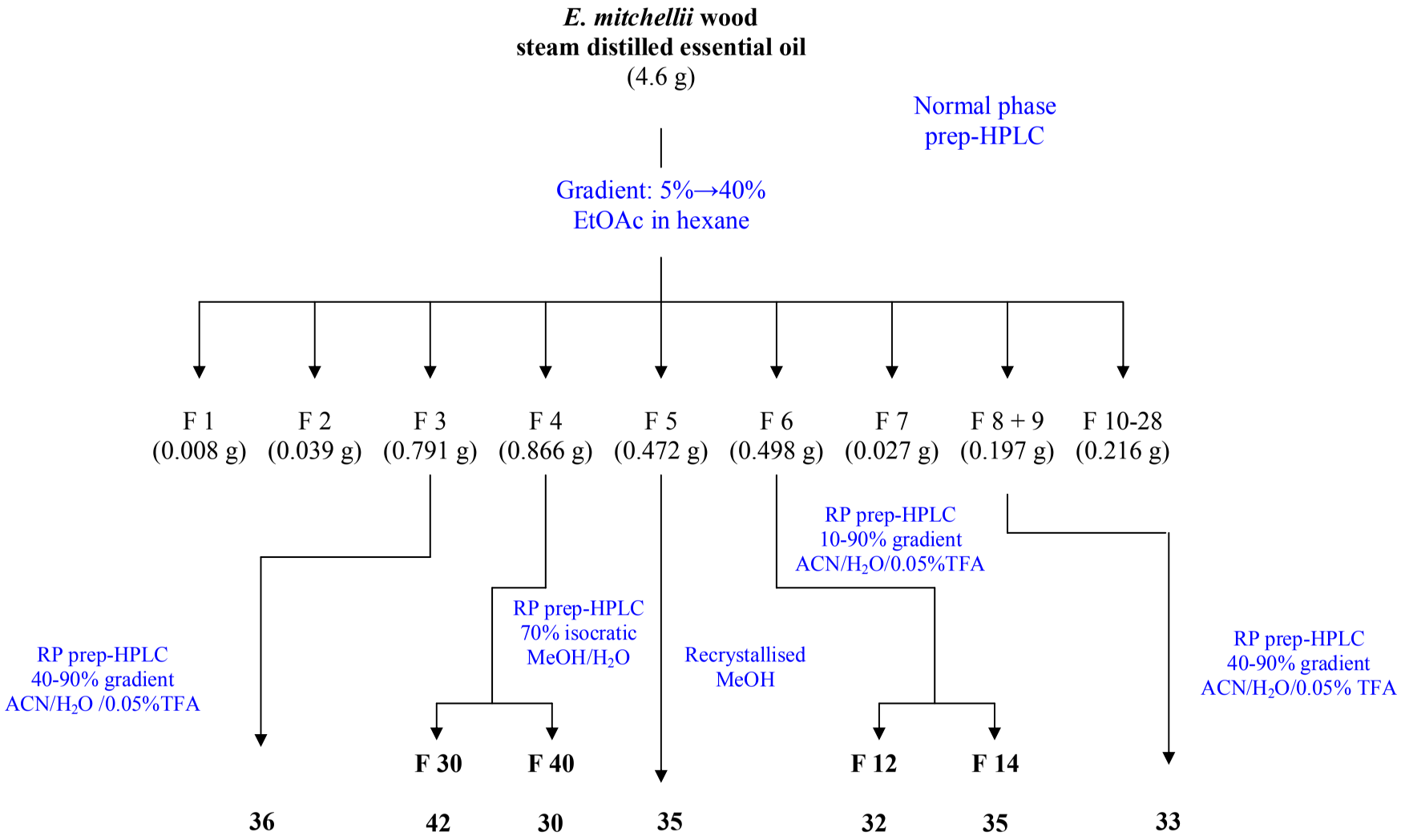


Figure 2.3. Isolation scheme for NP-prep HPLC fractionation of major compounds from *E. mitchellii* wood oil.

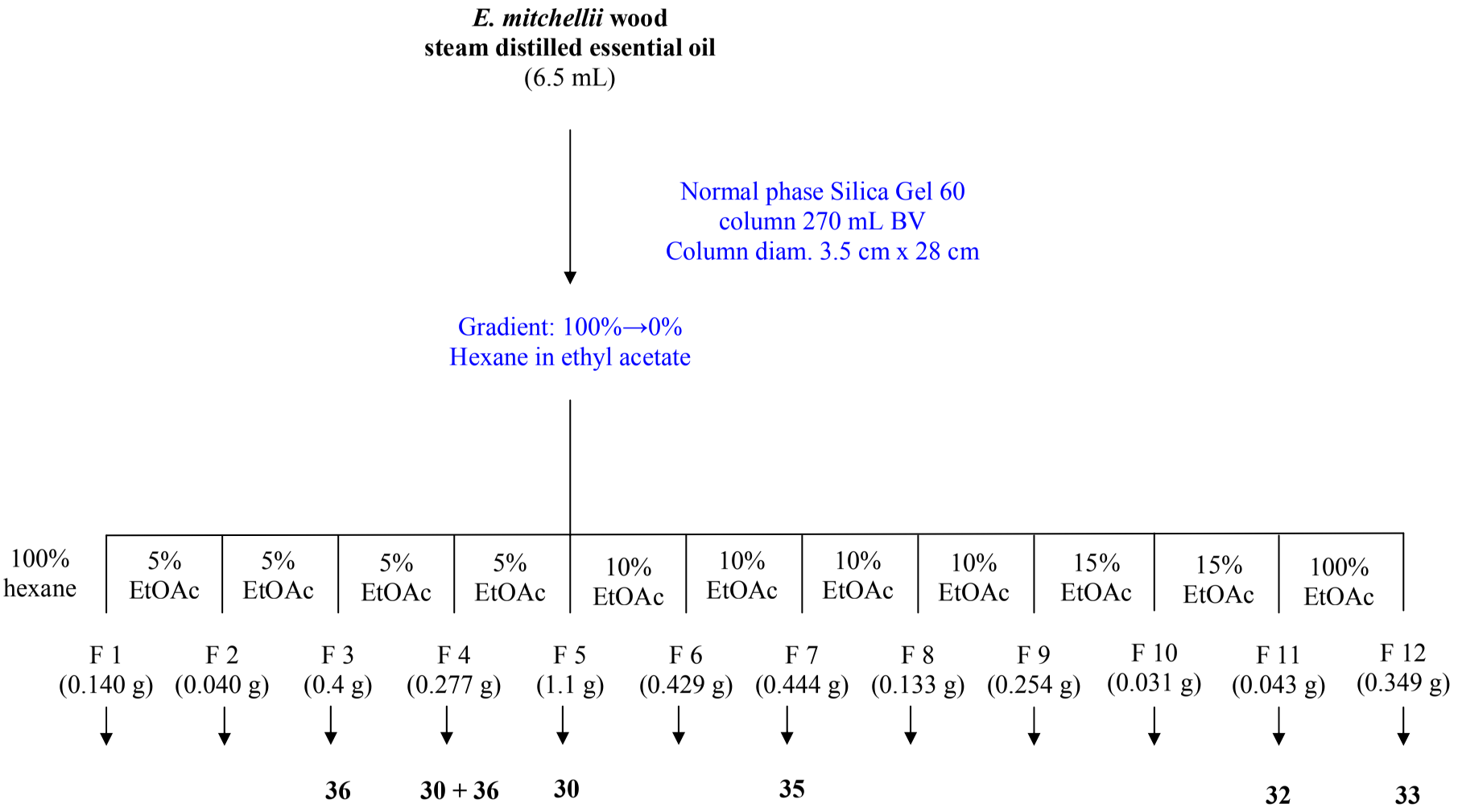


Figure 2.4. Isolation scheme for silica column chromatography of major compounds from *E. mitchellii* wood oil.

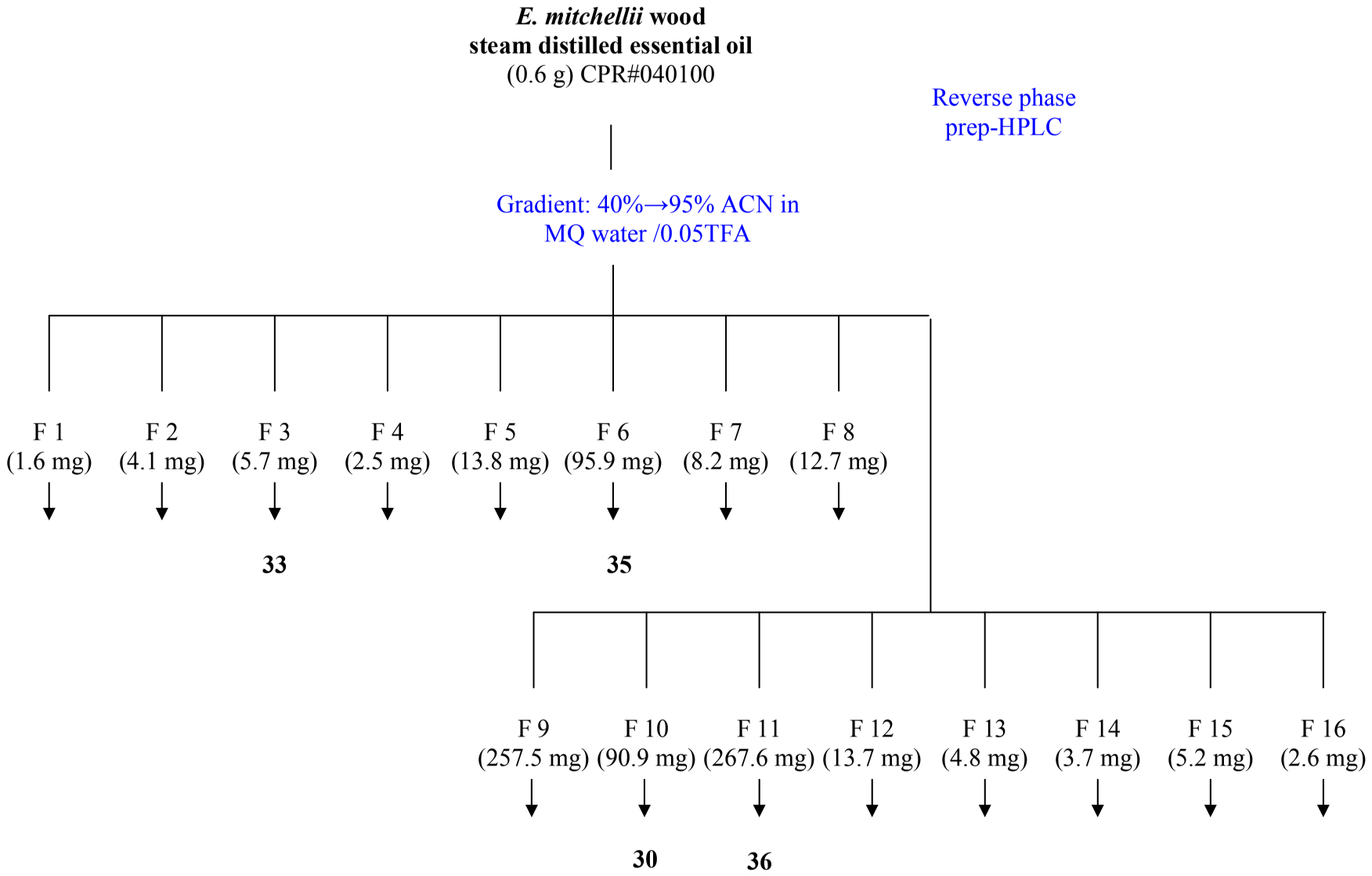


Figure 2.5. Isolation scheme for direct RP-prep HPLC fractionation of major compounds from *E. mitchellii* wood oil.

2.4.6 Fractionation of *E. mitchellii* Root Oil

The isolation scheme for fractionation of the root oil is summarized in Figure 2.6. The root oil (6.1 grams) was first fractionated by column chromatography on silica gel (0.063-0.200 mm, 3.5 cm diam., 300 mL bed volume (BV)). The column was first equilibrated with 3 BV of 100% pentane prior to sample loading. The column was first eluted with 600 mL of pentane to remove unwanted hydrocarbons. The column was then eluted with 2 x 400 mL of 9:1 pentane: diethyl ether (root oil fractions 1 and 2) followed by 2 x 400 mL of 100% diethyl ether (root oil fractions 3 and 4).

The root oil fraction 1 (300 mg) was dissolved in ACN (ca. 2.0 mL) and subjected to C18 preparative HPLC using a gradient of 40-95% B over 15 min, followed by an isocratic gradient of 70% B for 5 min, then a gradient of 70-90% B over 5 min, at a flow rate of 15 mL/min (Figure 2.6). The three major peaks in the HPLC profile were selectively cut from the root fraction 1.

To recover the volatile compounds from the aqueous solutions, each of the RP prep fractions (ca. 30 mL) was diluted up to 150 mL with MQ water, (a cloudy ppt. formed). The solutions were then passed through C18 SPE cartridges and then eluted with ACN (ca. 3 mL). The compounds were then dried under nitrogen and evaluated by GC-MS and LC-MS.

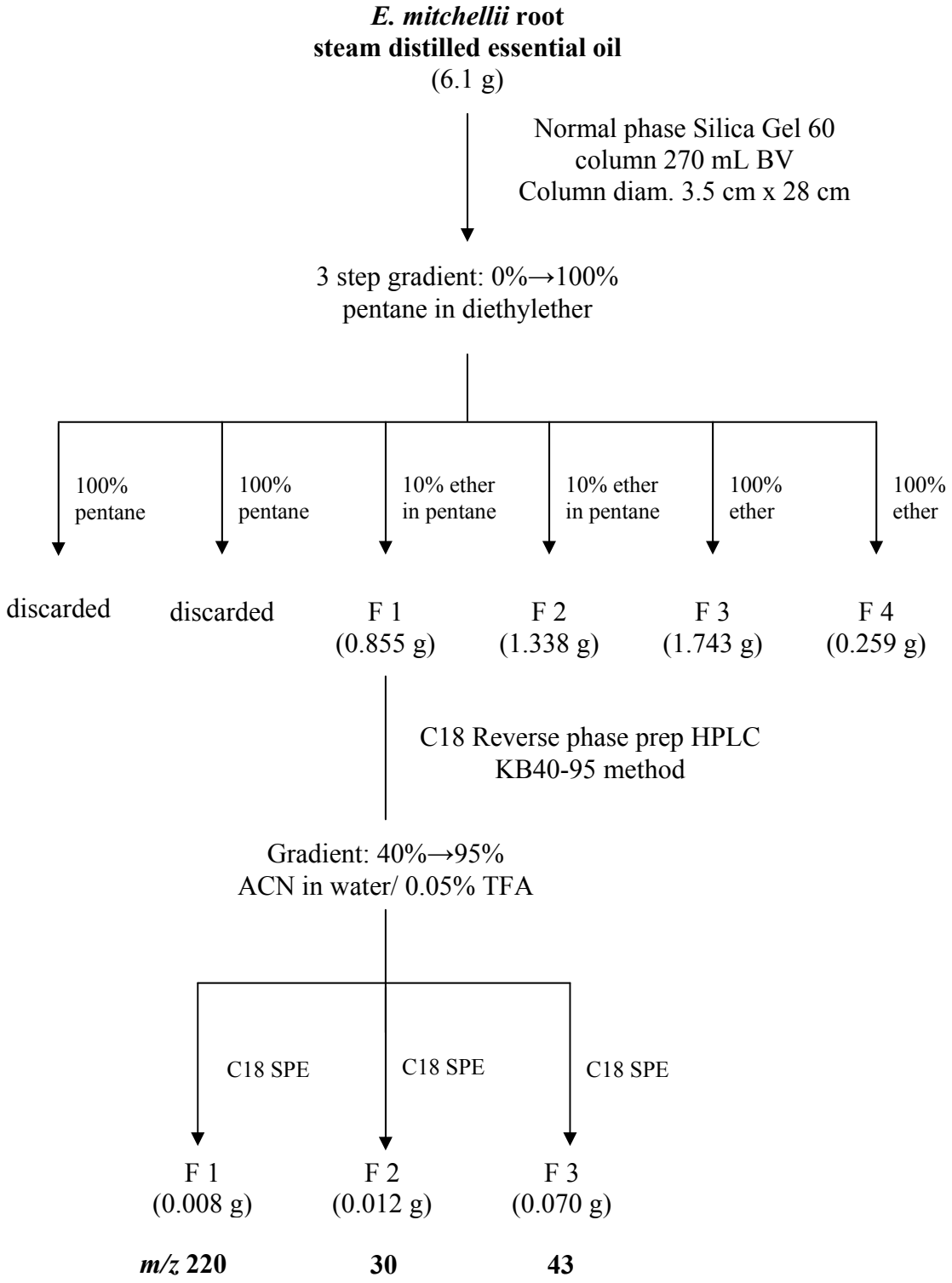


Figure 2.6. Isolation scheme for sesquithuriferone (**43**) from the root oil of *E. mitchellii*.

Eremophilone [(30), 1(10),11-eremophiladien-9-one]: Colourless oil (90.9 mg, 15.2%); $[\alpha]_D^{20}$ -78° (c 0.49, MeOH); $[\alpha]_{Hg}$ -207 (MeOH) Lit.(Bradfield et al., 1932a); ^{1}H NMR (500 MHz, $CDCl_3$) see Table 5.2; ^{13}C NMR (126 MHz, $CDCl_3$) see Table 5.2; EIMS (70eV) *m/z* (rel. int.): 218 (M·, 67), 203 (30), 185 (7), 176 (100), 161 (57), 147 (39), 133 (73), 119 (58), 107 (82), 91 (89), 79 (88), 67 (38), 53 (33), 41 (69).

Santalcamphor [(35), 8-hydroxy-11-eremophilen-9-one]: Colourless needless (95.9 mg, 16.0%); mp 99-100°C; Lit. 102-103°C (Bradfield et al., 1932a); $[\alpha]_D^{20}$ +112° (c 0.26, $CHCl_3$); $[\alpha]_D$ +90.6° ($CHCl_3$) Lit. (Bradfield et al., 1932a); ^{1}H NMR (500 MHz, $CDCl_3$) see Table 5.2; ^{13}C NMR (126 MHz, $CDCl_3$) see Table 5.2; EIMS (70eV) *m/z* (rel. int.): 236 (M·, 29%), 221 (3), 207 (100), 189 (15), 179 (5), 167 (7), 149 (8), 135 (10), 123 (40), 109 (56), 95 (39), 81 (35), 69 (74), 55 (44), 41 (50).

9-Hydroxy-7(11),9-eremophiladien-8-one [(36), 2-Hydroxyeremophilone]: Yellow oil (267.6 mg, 44.6%); $[\alpha]_D^{20}$ +63° (c 2.13, MeOH); $[\alpha]_D^{25}$ +138° (c 2.59, MeOH); Lit. (Pinder and Torrence, 1971); ^{1}H NMR (500 MHz, $CDCl_3$) see Table 5.2; ^{13}C NMR (126 MHz, $CDCl_3$) see Table 5.2; EIMS (70eV) *m/z* (rel. int.): 234 (M·,73%), 219 (48), 201 (25), 191 (57), 177 (43), 163 (100), 153 (91), 137 (42), 124 (12), 115 (19), 105 (24), 91 (55), 77 (38), 67 (37), 55 (39), 41 (81).

9-Hydroxy-1,7(11),9-eremophilatrien-8-one (42): Unstable yellow gum (2.2 mg, 0.4% not optimised); $[\alpha]_D^{20}$ +31° (c 0.32, $CHCl_3$); ^{1}H NMR (500 MHz, $CDCl_3$) see Table 5.3; ^{13}C NMR (126 MHz, $CDCl_3$) see Table 5.3; EIMS (70eV) *m/z* (rel. int.): 232 (M·, 100%), 219 (45), 208 (3), 199 (53), 189 (36), 171 (18), 161 (19), 152 (4), 143 (12),

128 (14), 115 (19), 105 (16), 91 (39), 77 (27), 65 (15), 53 (15), 41 (36); HRAPCIMS *m/z* 233.1545 (calcd for $[C_{15}H_{21}O_2]^+$, 233.1542).

8-Hydroxy-1,11-eremophiladien-9-one (33): Yellow oil (5.7 mg, 0.95%); $[\alpha]_D^{20}$ +167° (c 0.14, $CHCl_3$); $[\alpha]_D^{19}$ +59.3 (c 1.1) Lit. (Massy-Westropp and Reynolds, 1966); ^{1}H NMR (500 MHz, $CDCl_3$) see Table 5.3; ^{13}C NMR (126 MHz, $CDCl_3$) see Table 5.3; EIMS (70eV) *m/z* (rel. int.): 234 (M·, 9%), 216 (5), 207 (15), 191 (10), 173 (14), 159 (6), 150 (16), 137 (14), 121 (24), 107 (93), 93 (100), 77 (52), 67 (40), 55 (38).

Sesquithuriferone [(43), 2,6,6,8-Tetramethyltricyclo[6.2.1.0^{1,5}]undecan-7-one]: White powder (70 mg, 8.2%, not optimised); $[\alpha]_D^{20}$ +5.9° (c 1, $CHCl_3$); $[\alpha]_D$ +5.7° (c 1, $CHCl_3$) Lit. Barrero et al., 2000.; ^{1}H NMR (500 MHz, $CDCl_3$) see Table 5.4; ^{13}C NMR (126 MHz, $CDCl_3$) see Table 5.4; EIMS (70eV) *m/z* (rel. int.): 220 (M·, 35%), 205 (7), 192 (30), 177 (12), 159 (2), 147 (25), 136 (16), 121 (100), 108 (46), 93 (26), 81 (67), 67 (16), 55 (17), 41 (29).

8-Hydroxy-10,11-eremophiladien-9-one (32): Yellow oil (47 mg, 0.7%, not optimised); $[\alpha]_D^{20}$-36° (c 0.22, MeOH); $[\alpha]_D^{20}$ -33.6° (c 0.84, MeOH) Lit. (Massy-Westropp and Reynolds, 1966); ^{1}H NMR (500 MHz, $CDCl_3$) see Table 5.3; ^{13}C NMR (126 MHz, $CDCl_3$) see Table 5.3; EIMS (70eV) *m/z* (rel. int.): 234 (M·, 16%), 219 (10), 205 (91), 191 (28), 177 (16), 163 (31), 149 (20), 137 (67), 121 (34), 109 (86), 91 (69), 79 (62), 67 (55), 55(56), 41 (100).

2.5 Experimental on Bioactivity of *E. mitchellii*

2.5.1 *E. mitchellii* Wood Oil Samples

The termiticidal, repellency and fumigant properties and barrier studies of the *E. mitchellii* wood oil were performed by Associate Professor Robert Spooner-Hart and Dr Albert Basta of the University of Western Sydney (Centre for Plant and Food Sciences) Hawkesbury. Assessment of the acute dermal toxicity IC_{50} and IC_{95} values was determined by the author in collaboration with staff at CPAFS, UWS.

E. mitchellii wood oil and its fractions were tested in mode of action studies against the termite species. Two separate batches of steam distilled *E. mitchellii* wood oil, with similar chemical profiles, CP030102 and AP-778 were utilized**.** Fractions for testing were prepared from the wood oil by normal-phase preparative HPLC. The fractions and their compositions are presented in Table 2.4.

Table 2.4. Fractions and isolated constituents of *E. mitchellii* used for the termiticidal assays.

Fraction	**Major Component**	**Purity %**[†]
BIF F1	Hydrocarbon fraction	46.2
BIF F3	9-hydroxy-7(11),9-eremophiladien-8-one	80.8
BIF F4	eremophilone	86.5
BIF F5/6	santalcamphor	91.8
BIF F8-10	8-hydroxy-1,11-eremophiladienone	86.2
BIF F11-25	Non-volatile residue	

[†] Purity has been assessed on the basis of GC-MS quantitative analysis.

2.5.2 Plant Materials

Plant material was dried in a drying room (30°C) upone receipt and stored at room temperature prior to extraction or in-house steam distillation. Wood and root materials (Table 2.5) were ground using a Retsch cutting mill (Retsch GmbH, Haan, Germany: SM 100). The essential oils were stored at 4 °C. Commercially available vetiver oil was kindly provided by Australian Botanical Products.

Table 2.5. Details of the plant parts and essential oils investigated in this study.

Species	Parts	Area Collected	Mass (kg)	Date	Collector	Herbarium deposited	Voucher #	In House ID
E. mitchellii	Branchlets & Leaves	Lightning Ridge, NSW	0.105	24/4/2003	David Leach	Queensland	N/A	
E. mitchellii	Flowers & Leaves	near Eidsvold SE QLD	0.769	5/12/2005	Paul Forster	Queensland	PIF30486	
E. mitchellii	Wood	near Eidsvold SE QLD	1.760	5/12/2005	Paul Forster	Queensland	PIF30486	
E. mitchellii	Root	near Eidsvold SE QLD	0.766	5/12/2005	Paul Forster	Queensland	PIF30486	
E. mitchellii	Branchlets	near Eidsvold SE QLD	1.012	5/12/2005	Paul Forster	Queensland	PIF30486	
E. mitchellii	Wood oil	West QLD	300 mL	unknown	Cavanagh & Sons	N/A	N/A	CPR040100
E. mitchellii	Wood oil	West QLD	150 mL	unknown	Cavanagh & Sons	N/A	N/A	CP020173
E. mitchellii	Wood Oil	West QLD	150 mL	unknown	Greg Eaton	N/A	N/A	AP-778
E. mitchellii	Wood oil	West QLD	1000 mL	unknown	Cavanagh & Sons	N/A	N/A	CP030102
E. mitchellii	Root oil	Lightning ridge, NSW	100 mL	unknown	SCU	N/A	N/A	
Vetiveria zizanioides (Vetiver) root oil Java			100 mL	9/11/06	Supplied by Australian Botanical Products			Batch# EOVETJ

2.5.3 Termites

Termites for bioassay were field collected from several locations within the Sydney Basin. The two major species used in this study were *Coptotermes acinaciformis* (Froggatt), *Nasutitermes walkeri* (Hill) and *Nasutitermes exitiosus* (Hill). When required, termites were field collected and maintained in large plastic tubs (50 cm x 35 cm x 30 cm high). The tubs were filled with their host timber food source, soil debris as well as stakes of *Pinus radiata* and kept at room temperature 25 ± 2°C and 60 ± 5% RH. The colony was covered in moistened paper towel and moistened soil from the collection site and the culture was maintained in the dark under laboratory conditions. The required number of fully developed and healthy, worker and soldier termites, were removed from the colony immediately prior to the assay. Termites were only retained for a maximum of three weeks before being used in bioassays.

2.5.4 Acute Toxicity of *E. mitchellii* Wood Oil and Fractions Against Termites Via Topical Application

Preliminary range-finding investigations were conducted, followed by more detailed bioassays, to determine LC values. To assess the acute dermal toxicity; test samples, essential oils, crude fractions and pure compounds were prepared as follows: replicate stock solutions of 10000 ppm were made by initially dissolving the test sample in 10 mL absolute alcohol, sonicating for 10 min, then diluting with distilled water containing 200 ppm Triton X-100™ (octylphenolethylene oxide condensate; Union Carbide, Sigma Chemicals, St. Louis, Missouri, USA). The stock solutions were sonicated for 20 min, then serial dilutions were prepared using the Triton-X /distilled water stock solution as diluent. A homogeneous and uniform emulsion was achieved by thorough agitation. All products dissolved well in absolute ethanol and gave uniform emulsions when shaken vigorously prior to application.

In the assay twenty uniform termite workers and one soldier were transferred to a Petri dish (90 mm diam.) lined with filter paper (90 mm diam., Advantec No 2, Toyo Roshi Kaisha, Japan) that had been moistened with distilled water (1.0 mL) to maintain humidity. Test solutions (2.5 mL or 5.0 mL aliquots) were applied to the Petri dish containing termites using a Potter precision spray tower (Burkard, Rickmansworth, Herts, UK) as described by Herron et al. (1995). The spray tower simulates field applications by applying the test solution as a very fine mist with even application. Each spray application was delivered at a pressure of 18.5 psi (ca. 5 s) and the average weight of the test solution sprayed on each dish was determined. Three to five true replicates were normally used, and ethanol (10000 ppm) in 200 ppm Triton X-100/distilled water was applied as a control.

Substances were routinely tested at concentrations between 0.5 - 0.05%. The test was routinely performed in triplicate for each test sample at each concentration. After treatment all of the Petri dishes were covered with their lids and left undisturbed in darkness under a black plastic sheet under laboratory conditions of 25±2°C and 60±5% relative humidity (RH). Behaviour was observed post treatment and mortality was recorded at 24 and 48 hrs after treatment. End point (death) was recognised by the absence of movement of all appendages (legs and antennae) when an individual was turned on its back or side. In some cases additional repeat doses were employed to more accurately determine the key statistics such as LD_{50}. In these cases, both data sets were incorporated into the analyses.

2.5.5 Acute Toxicity of Residues of *E. mitchellii* Wood Oil and Fractions - Fresh Residues

The wood oil of *E. mitchellii* and its fractions F4 and F5/6 were dissolved in pure

acetone (Univar UN No 1090) in 50 mL volumetric flasks to prepare a series of test solutions, with separate test solutions being prepared for each replicate. A filter paper (90 mm diam., Whatman No 1) was dipped for 10 s in a 400 mL beaker containing the required concentration, and acetone-only was used as the untreated control. Treated filter papers were air dried in a fume cabinet for 2 h. The treated filter papers were then transferred to line the lids of the Petri dishes. One mL of distilled water was uniformly distributed to moisten each filter paper and to maintain high ambient humidity. Subsequently, 1 mL of water was added to each Petri dish daily.

Twenty termite workers were then released onto the treated filter paper. The Petri dishes were covered with their lids, and maintained in darkness under black plastic sheeting under laboratory conditions of 25±2°C and 60±5% RH. Termite behaviour was observed post treatment, and mortality was recorded after 24 h.

2.5.6 Fumigant Studies of *E. mitchellii* Wood Oil

Filter papers (Whatman No 1) were immersed into 20000 ppm *E. mitchellii* wood oil in acetone for 10 sec, transferred to aluminium foil and left to air dry in a fume cabinet for 1 h. The assessment container was a 600 mL round glass Kilner jar with dimensions 70 mm diam. (top), 90 mm diam. (bottom) and 95 mm high. Fifteen workers of *C. acinaciformis* were placed onto filter paper (90 mm diam., Whatman No 1) that had been previously laid on the bottom of the glass jar and moistened with 0.5 mL distilled water. The treated filter paper was placed in the lid of a 90 mm diam. plastic Petri dish. The dish lid with filter paper was then used to cover the jar containing termites and sealed with Parafilm (Pechiney Plastic Packaging, Chicago, IL) and afterwards, with plastic sheet wrapping (Glad® wrap The Glad Products Company, Oakland, CA), to

secure a seal. Nine replicates were made for the 20000 ppm *E. mitchellii* wood oil treatment and for the acetone-only control. The containers were maintained under laboratory conditions of 25±2°C and 60±5% RH, under black plastic sheeting. Termite mortality was recorded 1 h, 6 h, 12 h, 24 h and 96 h after sealing the container.

2.5.7 Choice Test Using *E. mitchellii* Wood Oil Using Filter Paper Choice and No Choice Bioassays

Round plastic containers (4 L: 27cm diam. x 7cm height) were used as arenas for these investigations. Concentrations of *E. mitchellii* wood oil and its fractions were made up as previously described for the filter paper fresh residues test (2.1). Filter papers (90 mm diam., Whatman No 1) were immersed in the required concentration of the test liquid or in pure acetone for 10 sec. then left to air dry on a sheet of aluminium foil in a fume cabinet for 2 h. In no-choice tests, two filter papers treated with the same concentration were placed inside each plastic container. In the choice test, a pair of filter papers was placed inside each container, one of which was an acetone-only control and the other was treated in *E. mitchellii* wood oil or its fractions, either with 2000 ppm in the case of *N. exitiosus* or 5000 ppm in the case of *C. acinaciformis*. The two papers were arranged so that they faced each other and were tangential to the container wall. One mL of distilled water was applied uniformly to each filter paper to moisten it before transferring it to the arena. Additional water was applied twice a day throughout the duration (3 days) of the experiment. The arenas were kept covered with their lids, then covered with black plastic sheet to prevent light penetration, and under laboratory conditions of 25±2°C and 60±5% RH. Observations were made 3 days after termites were released. Each container was uncovered and immediately photographed with a digital camera. All images were downloaded onto a computer, enlarged, and the number of termites on each filter paper was counted.

In the no-choice assessment, the numbers of termites on the two treated filter papers in a container were summed, and compared with the summed numbers on two untreated filter papers in a comparative control container. In the choice assessment, the number of termites on the treated filter paper was compared with the number on the untreated filter paper in the same container. The repellency index (RI) was calculated, based on the formula RI = [(Nc–Nt)/Nc]* 100, where Nc is the percentage on the control paper(s), and Nt is the percentage on the treated paper(s). The mean RI values were analyzed by ANOVA. Where significant differences were detected, the means were separated using Duncan's Multiple Range test.

2.5.8 Barrier Studies of *E. mitchellii* Wood Oil

Tests were conducted to determine the efficacy of *E. mitchellii* oil as a barrier treatment to prevent termite incursions. The method used for assessing the barrier efficacy was based on that described by Su et al. (1995) with minor modifications (Figure 6.2). Pyrex medium walled tubes (24 x 200 mm Bibby Sterilin Ltd, Staffordshire ST15OSA, England) were used as bioassay chambers. To make the required barrier material, 90 g samples of autoclaved washed medium river sand were placed into 200 mL beakers and 10 mL aliquots of each serial dilution in acetone were titrated onto the sand while continuously mixing with a spatula. Concentrations of *E. mitchellii* wood oil tested were 0 ppm (acetone/water only), 5000 ppm, 10000 ppm and 20000 ppm. The mixture was then thinly spread on aluminium foil sheet overnight in a fume cabinet to dry.

Wooden sticks (3 x 5cm wooden applicator sticks) and termites (*C. acinaciformis*, 80 workers and 4 soldiers) were paced at the bottom of the test tube. A 3 cm core of 7.0% agar gel (Avocado Research Chemicals Ltd, Heysham, Lancashire) was inserted into the tube until it rested on the wooden sticks (layer 1). Moistened sand (10% water) was

spooned in to the tubes to a height of 3 cm (layer 2). The tube was gently shaken and the sand surface was then lightly tamped and levelled. A 2 cm barrier of freshly treated sand was then transferred from the beakers to the test tube with a small spatula (layer 3) and lightly tamped before inserting a 1 cm core of 7.0% agar over this barrier layer (layer 4). A 1 x 5 cm paper towel food source was placed in the top of each tube. The top of each tube was then covered with aluminium foil (Glad Foil, Padstow NSW 2211, Australia). Eight replicate tubes were made of each treatment concentration. Tubes were held vertically in a cardboard packing box and maintained at 24 ± 2°C and 60 ± 5 % RH.

Vertical length of tunnelling was monitored on days; 1, 4 and 7 post treatment in both treated and untreated sand layers. Seven days after treatment, the majority of termites in the *E. mitchellii* wood oil treatments had died, presumably due to its toxic effect, so the investigations were terminated at this point.

To determine the residual efficacy of *E. mitchellii* wood oil, the remaining treated sand was retained in the laboratory in a 1 L glass beaker covered with Glad® wrap under conditions of 25 ± 2°C and 60 ± 5% RH and normal laboratory illumination. Its efficacy was tested, using the above methodology, up to 14 days old. At this time, termite mortality was observed to be lower in the *E. mitchellii* wood oil treatments, so the period for termites being challenged in the bioassays was increased to 16 d for 38-day old treated sand and to 21 d for 240 day old treated sand. In all cases, except the 240 day old sand the termite species used was *N. exitiosus*; the last bioassay was conducted with *C. acinaciformis*.

2.5.9 Data Analysis

Data was analysed using SPSS® for Windows® Version 14 (SPSS Inc. 2007). Probit analysis was carried out for dose mortality data and heterogeneity of regression was determined by the Pearson chi-squared characteristic with Abbott's (1925) formula used to correct for control mortality prior to analysis. Pearson's test for goodness of fit was performed, and if the result was significant at $p \leq 0.150$ level, a heterogeneity factor was used for calculation of confidence limits. Corresponding lethal concentration (LC_{50} and LC_{95}) and values were calculated with their lower and upper confidence limits (CL, 95%).

2.5.10 Solvent Partitioning of *E. mitchellii* Leaf Oil

The steam distilled leaf oil (1.53 g) was partitioned between hexane (20 mL hexane/5 mL pentane) and aqueous methanol (25 mL of methanol/4 mL water). The hexane layer was evaporated to dryness under nitrogen obtaining a yield of 39% (w/w). Water (800 mL) was added to the aqueous methanol layer until a cloudy precipitate formed. The leaf oil methanol fraction was then recovered by back extracting with diethyl ether. The methanol fraction was dried under nitrogen to obtain a yield of 35% (w/w). The fractions were analysed by GC-MS prior to shipment to CPAFS for termiticidal screening.

2.5.11 Fractionation of *V. zizanioides* Oil by Column Chromatography

Vetiver oil (6.0 g) was first fractionated by column chromatography on silica gel (0.063-0.200 mm, 3.5 cm diam., 29 cm height, 300 mL bed volume). The column was equilibrated with 3 BV of 100% pentane prior to sample loading. The column was then eluted with 2 x 400 mL of pentane. The column was then eluted with 2 x 400 mL of 9:1

pentane: diethyl ether followed by 2 x 400 mL of 100% diethyl ether and lastly 400 mL of acetone. The fractions were evaporated using a rotary evaporator and then analysed by GC-MS. Fraction 4 which contained an enriched mixture of α- and β-vetivone was sent to CPAFS for termiticidal screening.

2.6 Experimental on Chemistry and Cytotoxicity of *Eremophila* sp.

2.6.1 Plant Material

The shipment of Western Australian *Eremophila* specimens were collected by botanists Robert Davis and Matt Kealley of BioProspect Ltd. A second shipment of Northern Territory *Eremophila* specimens was provided by The Alice Springs Desert Park (ASDP). A specimen of *E. bignoniflora* was collected from Wilbertree homestead near Brewarrina NSW a specimen has been deposited in the Southern Cross University Herbarium. A specimen of *E. mitchellii* was collected by Paul Forster from Northern Queensland. Lastly, specimens of *E. macdonaldii* and *E. obovata* collected from NT were kindly provided by Greg Leach. A summary of species and collection data of the plants analysed in this study is summarized in Table 2.6 and 2.7.

2.6.2 Sample Preparation

Fresh plant materials (Table 2.6 and 2.7) were dried in a drying room (40˚C) upon receipt and prior to grinding. Wood and root materials were ground using a Retsch

Table 2.6. Details of the Western Australian *Eremophila* collections investigated in this study.

Species	Plant parts	Mass (kg)	Date	Area Collected	Collector	Herbarium deposited	Voucher #	In House ID	Botanist number
E. racemosa F Muell.	aerial	2.87	3/4/2001	Southern Cross Junction, WA	Robert Davis/ Matt Kealley	Western Australian	05819512	BP202	RD9665
E. miniata C. A. Gardner.	aerial	2.20	12/6/2001	Wubin, WA	Robert Davis/ Matt Kealley	Western Australian	05818680	BP368	RD9763
E. miniata C. A. Gardner.	root	0.66	12/6/2001	Wubin, WA	Robert Davis/ Matt Kealley	Western Australian	05818680	BP369	RD9763
E. oppositifolia ssp. *angustifolia* R. Br.	aerial	2.40	13/6/2001	Wubin, WA	Robert Davis/ Matt Kealley	Western Australian	05825253	BP392	RD9786
E. oppositifolia ssp. *angustifolia* R. Br	root	2.15	13/6/2001	Wubin, WA	Robert Davis/ Matt Kealley	Western Australian	05825253	BP393	RD9786
E. latrobei ssp. *latrobei* F. Muell.	aerial	2.20	13/6/2001	Wubin, WA	Robert Davis/ Matt Kealley	Western Australian	05818710	BP395	RD9788
E. clarkei Oldfield & F. Muell.	aerial	1.87	14/6/2001	Wubin, WA	Robert Davis/ Matt Kealley	Western Australian	05873789	BP406	RD9799
E. clarkei Oldfield & F. Muell.	root	1.60	14/6/2001	Wubin, WA	Robert Davis/ Matt Kealley	Western Australian	05873789	BP407	RD9799
E. deserti (Cunn. Ex. Benth.) Chinnock.	aerial		29/08/2001	Kalbarri, WA	Robert Davis/ Matt Kealley	Western Australian	05921260	BP575	RD9977
E. decipens Ostenf. ssp. *decipiens*	aerial	0.55	17/07/02	N of Noresman, WA	Robert Davis/ Matt Kealley	Western Australian	06207758	BP875	RD10318
E. gibbosa Chinnock.	aerial	0.85	17/07/02	N of Noresman, WA	Robert Davis/ Matt Kealley	Western Australian	06207766	BP876	RD10319
E. oblonga Chinnock.	aerial	0.70	18/07/02	N of Balladonia, WA	Robert Davis/ Matt Kealley	Western Australian	06207804	BP887	RD10330
E. scoparia (R. Br.) F. Muell.	aerial	0.65	19/07/02	N of Balladonia	Robert Davis/ Matt Kealley	Western Australian	06207847	BP897	RD10341
E. ionantha Diels.	aerial	0.70	20/07/02	N of Balladonia	Robert Davis/ Matt Kealley	Western Australian	06207898	BP911	RD10355
E. subfloccosa ssp. *lanata* Chinnock.	aerial	0.45	20/07/02	N of Balladonia	Robert Davis/ Matt Kealley	Western Australian	06139892	BP915	RD10359
E. dempsteri F. Muell.	aerial	0.45	21/07/02	E of Balladonia	Robert Davis/ Matt Kealley	Western Australian	06207960	BP927	RD10373
E. subfloccosa Benth. ssp. *subfloccosa*	aerial	0.75	22/07/02	S of Balladonia	Robert Davis/ Matt Kealley	Western Australian	06208045	BP950	RD10397

Table 2.7. Details of the Northern Territory *Eremophila* collections investigated in this study.

Species	Parts	Area Collected	Mass (kg)	Date	Collector	Herbarium deposited	Voucher #
E. A48866	leaf	ASDP, NT	0.049	27/4/2003	Jon Belling	ASDP	Living collection
E. A09760	leaf	ASDP, NT	0.075	27/4/2003	Jon Belling	ASDP	Living collection
E. alternifolia R. Br.	leaf	ASDP, NT	0.015	27/4/2003	Jon Belling	ASDP	Living collection
E. batti F. Muell.	leaf	ASDP, NT	0.037	27/4/2003	Jon Belling	ASDP	Living collection
E. bignoniflora F. Muell.	leaf	ASDP, NT	0.089	27/4/2003	Jon Belling	ASDP	Living collection
E. christophori F. Muell.	leaf	ASDP, NT	0.060	27/4/2003	Jon Belling	ASDP	Living collection
E. dalyana F. Muell.	leaf	ASDP, NT	0.033	27/4/2003	Jon Belling	ASDP	Living collection
E. duttonii F. Muell.	leaf	ASDP, NT	0.050	27/4/2003	Jon Belling	ASDP	Living collection
E. freelingii F. Muell.	leaf	ASDP, NT	0.195	27/4/2003	Jon Belling	ASDP	Living collection
E. gilesii F. Muell.	leaf	ASDP, NT	0.032	27/4/2003	Jon Belling	ASDP	Living collection
E. gilesii repeat	leaf	ASDP, NT	0.090	27/4/2003	Jon Belling	ASDP	Living collection
E. hybrid A94704	leaf	ASDP, NT	0.024	27/4/2003	Jon Belling	ASDP	Living collection
E. latrobei var. *latrobei* F. Muell.	leaf	ASDP, NT	0.196	27/4/2003	Jon Belling	ASDP	Living collection
E. latrobei var. *glabra* L. S. Sm.	leaf	ASDP, NT	0.195	27/4/2003	Jon Belling	ASDP	Living collection
E. longifolia (R. Br.) F. Muell,	leaf	ASDP, NT	0.068	27/4/2003	Jon Belling	ASDP	Living collection
E. macdonellii F. Muell.	leaf	ASDP, NT	0.046	27/4/2003	Jon Belling	ASDP	Living collection
E. maculata var. *maculata* F. Muell.	leaf	ASDP, NT	0.185	27/4/2003	Jon Belling	ASDP	Living collection
E. maculata var. *brevifolia* Benth.	leaf	ASDP, NT	0.063	27/4/2003	Jon Belling	ASDP	Living collection
E. obovata var. *obovata* L. S. Sm.	leaf	ASDP, NT	0.014	27/4/2003	Jon Belling	ASDP	Living collection
E. willsii F. Muell. ssp. *willsii*	leaf	ASDP, NT	0.092	27/4/2003	Jon Belling	ASDP	Living collection
E. macdonellii F. Muell	leaf	NT			Greg Leach		
E. obovata L. S. Sm.	leaf	NT			Greg Leach		

cutting mill (Retsch GmbH, Haan, Germany: SM 100). The extracts were stored at 4 ºC prior to analysis. The extracts were re-solubilised in the specified solvent by sonicating for 20 minutes.

2.6.3 Cytotoxicity Screening

All cell lines, except A2780, were purchased from American Type Culture Collection (ATCC), Manassas, VA, USA. Cell culturing media was purchased from Invitrogen, Mt Waverley, VIC. *In vitro* cytotoxicity was measured against mouse lymphoblast cells ($P388D_1$, ATCC: CCL-46) using the commercially available ATPLite-M assay (Packard BioScience B. V.). ATP is a marker for cell viability because it is present in all metabolically active cells and the concentration declines very rapidly when the cells undergo necrosis or apoptosis. The cytotoxicity of selected samples was further assessed against human liver carcinoma (Hep G2, ATCC: HB-8065), human mammary adenocarcinoma (MCF7, ATCC: HTB-22), human prostate cancer (PC-3, ATCC: CRL-1435), human ovarian carcinoma (A2780, ECACC, Sigma-Aldrich, Castle Hill, NSW: 93112519) and human malignant melanoma (A-375, ATCC: CRL-1619), cell lines.

The crude extracts, fractions or pure compounds were diluted in DMSO and screened at 3-4 concentrations (ideally 0.1, 0.01, 0.001, mg/mL) and in triplicate using DMSO, media (without additives) and chlorambucil as solvent control, control, and positive control, respectively. Samples were prepared in a 96 well microplate by the addition of cell media (99 μL) and the desired extract (1 μL). The plates were incubated in the dark for a period of 24 h (37 °C, 5% CO_2). The cells were then lysed using mammalian cell lysis solution (50 μL, 0.1 M NaOH) and agitated on a plate shaker for 5 min, and luciferin/luciferase substrate solution (50 μL, lyophilized) was then added. The microplate was again agitated for 5 min and then allowed to equilibrate in the dark for

10 min. ATP luminescence, as an indicator of cell proliferation, was then measured using the Wallac Victor 2 Luminescence counter (Perkin Elmer: Model 1420). Results were then evaluated based on percentage inhibition of $P388D_1$ cell growth versus concentration of crude extract or fraction.

2.6.4 Size Exclusion Chromatography

Sephadex LH-20 (Sigma-Aldrich: LH20100) was used for either improving the purity of fractions or for separation of compounds on the basis of their molecular weight. The Sephadex was activated with methanol for 16-24 h prior to use.

2.6.5 Extraction and Isolation of Compounds from *Eremophila racemosa*

The air-dried leaves (10 g) were ground and subsequently extracted with MeOH (3 x 100 mL) overnight at room temperature, these extracts were then combined and evaporated under reduced pressure. A portion (1.25 g) of the resulting dark green residue (3.3 g) was subjected to RP prep-HPLC using a mobile phase system of solvent A (water with 0.05% TFA) and solvent B (acetonitrile with 0.05% TFA). The eluent composition used was 95% A for 5 minutes followed by a gradient of 5-95% B over 20 min and 95% B for a further 20 min at a flow rate of 21.6 mL/min. Fractions were collected at 4 minute intervals to evaluate the cytotoxic activity. Prep-HPLC fractionation was repeated for purification of compounds, fractions were collected at 1 min intervals to yield 44 fractions.

Prunasin (65): $C_{14}H_{17}O_6N$, MW 295.2947; Peak 1 (R_T 11.23 min, Figure 7.3) was further purified using sephadex LH-20 and methanol as eluent to yield a white powder

(62.8 mg, 1.7%), IR, MS, ^{1}H NMR and ^{13}C NMR spectra were consistent with previously published data (Nakajima, et al., 1998).

Pinoresinol-4-*O*-β-D-glucoside (**77**): $C_{23}H_{32}O_{11}$, MW 484.5049; Peak 2 (R_T 13.24 min, Figure 7.3) was resolved from epipinoresinol-4-*O*-β-D-glucoside (**78**) using RP semi-preparative HPLC eluting with a mobile phase system of solvent C (water) and solvent D (MeOH). The eluent composition used was 50% C for 5 minutes followed by a gradient of 50-75% D over 20 min and 75% D for a further 10 min at a flow rate of 1 mL/min. Fractions were collected at ½ minute intervals to yield **77** as a pale yellow gum (7.2 mg, 0.2%). ^{1}H NMR and 2D COSY spectra were consistent with previously published data (Rahman, et al., 1990) and (Ayres and Loike, 1990).

Epipinoresinol-4-*O*-β-D-glucoside (78): $C_{23}H_{32}O_{11}$, MW 484.5049; Peak 2 (R_T 13.24 min, Figure 7.3) was obtained as a pale yellow gum (6.3 mg, 0.2%). The ^{1}H NMR and ^{13}C NMR data are in agreement with previously published data (Rahman, et al., 1990) and is further supported by 2D COSY, NOESY, HMBC and HSQC data.

Luteolin (74): $C_{10}H_{15}O_6$, MW 286.2434; Peak 4 (R_T 15.39 min, Figure 7.3) was further purified using Sephadex LH-20 and methanol as eluent to yield a yellow powder (3.3 mg, 0.1%). Its mass spectral, ^{1}H NMR and ^{13}C NMR data are in agreement with previously published data (Youssef and Frahm, 1995).

Phillygenin (75): $C_{21}H_{24}O_6$, MW 372.4218; Peak 5 (R_T 17.23 min, Figure 7.3) was further purified using Sephadex LH-20 and methanol as eluent to yield a white gum (52.3 mg, 1.4%). The ^{1}H NMR and ^{13}C NMR data are in agreement with previously

published data (Rahman, et al., 1990) and the assignment is further supported by 2D COSY, NOESY, HMBC and HSQC data.

Phillyrin [(76), Phillygenin-4-*O*-*β*-D-glucoside]: $C_{24}H_{34}O_{11}$, MW 498.5320; Peak 3 (R_T 14.61 min, Figure 7.3) was recrystallised from MeOH to yield colourless needles (38.5 mg, 1.0%). Its melting point, mass spectral, ^{1}H NMR and ^{13}C NMR data are in agreement with previously published data (Rahman, et al., 1990) and the assigned structure is further supported by 2D COSY, NOESY, HMBC and HSQC data.

Chapter 3

The Chemistry of *Centipeda cunninghamii.*

3.1 Introduction

3.1.1 Background of This Study.

Centipeda cunninghamii (DC.) A. Braun & Asch. (Asteraceae) is a native Australian herb that is reputed to possess therapeutic properties. Preparations of *C. cunninghamii* or 'Phytoplenolin' are available commercially in the form of topical preparations to 'heal and rejuvenate' the skin (D'Amelio and Mirhom, 1998). Research conducted by D'Amelio and Mirhom (1998) for the Phytoplenolin patent reported that the steam distilled essential oil possesses an anti-inflammatory action. The Phytoplenolin® preparation was found to reduce inflammation comparable to hyaluronic acid in a chemically induced erythemal response (D'Amelio and Mirhom, 1998). Research conducted at SCU/CPP has found significant antioxidant and anti-inflammatory activity

in the solvent extracts of *C. cunninghamii* (Gabriel, 2005). A scientific approach was used to investigate the pharmacological basis for these claims. The aim of this project was to determine the chemical components that are responsible for the bioactivity. This chapter deals with the isolation and structural elucidation of the bioactive compounds from *C. cunninghamii*. Chapter 4 details the anti-inflammatory and antioxidant properties of the extracts, fractions and purified compounds of the herb.

3.1.2 Ethnobotanical Uses of *Centipeda* Species

C. cunninghamii usually occurs near wet places along banks of dams and creeks and rivers and ditches (Harden, 1993) and it is widespread throughout the temperate regions of Australia. *Centipeda cunninghamii* is commonly referred to as sneeze weed, old man weed or gukwonderuk (Lassak and McCarthy, 1983). The common name "sneezeweed" refers to the powdered leaves which are believed to act like snuff (Lassak and Mc Carthy, 1983).

Aboriginals have reportedly used the herb to make teas or other preparations which have been used for various ailments. The tea could be taken orally to treat illnesses including, tuberculosis (Campbell, 1973). Other known uses of the tea solution or decoction include the treatment of purulent ophthalmia (Maiden, 1975) and sandy blight and for alleviating eye inflammation by bathing the eyes with a cooled solution (Webb, 1948). Additional uses included placing the plant around a person's head to provide relief from colds or when placed around campsites it could repel ants (Cribb, 1988). It has also been administered as an antiprotozoal (D'Amelio and Mirhom, 1998).

In Ayurvedic medicine *C. minima* (synonymous with *C. orbicularis*) is reported to be useful for worms, skin diseases, white patches on the skin, dyspnoea, cough and toxicosis (Sudarshan, 2005). *Centipeda minima* is distributed throughout China and Japan (Quattrocchi, 2000), India (Sudarshan, 2005), Australia, New Zealand and Afghanistan to Asia (Harden, 1993). In contrast, *C. cunninghamii* is indigenous to Australia and New Zealand (Harden, 1993). For this reason it is thought that the *C. cunninghamii* would not have been utilized in Indian or Chinese medicines.

3.1.3 Therapeutic Uses of *Centipeda* Species

More than 30 patents exist for the therapeutic use of plants from the *Centipeda* genus. This may be attributed for the most part to the medicinal properties ascribed to *Centipeda minima* by Ayurvedic (Nepalese Indian) and Traditional Chinese medicine. Patented claims include treatments for; rhinitis (Huang, 1995), cancer (Wu, 1997), bone fractures (Wu, 2004), herpes (Tang, 2003) and topical treatments for anti-aging (Inoue and Yamaguchi, 2000) and pruritis (Takano et al., 2007).

Several therapeutic uses of *C. cunninghamii* have been patented. In 1985 Robert Egan (1985) patented preparations of *C. cunninghamii* for promoting hair growth. Next, D′Amelio and Mirhom (1998) reported on the therapeutic properties of extracts of the herb and patented preparations of the herb for treatment of various skin disorders including; “the relief of itching and dry skin from psoriasis” and also “the anti-inflammatory, antiallergenic, sunscreen and cell renewal properties”. Later, Close (2002) patented the preparation of an alcoholic tincture of the herb. Whilst D’Amelio and Mirhom (2005) patented “preparations containing extracts of *C. cunninghamii* for the treatment of periodontal infections and gingivitis.” The patent of Gupta and Hoyt

(2006) relates to a cosmetic or pharmaceutical preparation of a plant from the *Centipeda* genus "to reduce skin damage caused by aging and/or the environment". Hill (1997) reports that extracts of *C. cunninghamii* possessed antifungal activity. Yu et al. (1994) report on the antiprotozoal activity of *C. minima*.

3.1.4 Phytochemistry of *Centipeda* Species

The active components of *C. cunninghamii* responsible for the medicinal properties are unknown. D'Amelio and Mirhom (1998) reported that the extract is known to contain 'a volatile oil having a bitter principle myriogenin and *cis*-chrysanthenyl acetate'. Myriogenin is derived from the former scientific name of *C. cunninghamii*; *Myriogyne cunninghamii*. It was not possible to identify the compound ascribed as myriogenin in the scientific literature. Pinney and Southwell (1971) reported that the steam distilled volatile oil contained *cis*-chrysanthenyl acetate as the major component. D′Amelio and Mirhom (1998) reported the identification (by GC-MS and HPLC) of the chemical constituents of *C. cunninghamii*. Forty two compounds were identified in the GC-MS profile of the oil (Section 3.2.1) and 25 compounds in the HPLC chromatogram of the extract. These were;

> "brevilin A, arnicolide, arnicolide B, arnicolide C, caryophyllane-2,6-*β*-oxide, florilenalin-angelate, florilenalin-isobutyrate, florilenalin-isovalerate, helenalin, microhelenalin B, 6-*0*-angeloyl plenolin, 6-*0*-senecoyl plenolin, isobutyroyl plenolin, aurantiamide acetate, apigenin, *cis*-chrysanthenyl acetate, kaempferol-7-glucosyl-rhamnoside, lupeol acetate, quercetin, scoparol, *β*-sitosterol, taraxasterol, thymol, 10-isobutyryl-oxy-8,9-epoxy- isobutyrate, 9-epihardwickiic acid".

The nomenclature in the patent is in some cases ambiguous and emphasis is placed on the importance of the sesquiterpene lactones. It is not clear whether the extract of *C. cunninghamii* has been characterized using HPLC with association to compounds identified from *C. minima*. Certainly there are no subsequent reports of the chemical constituents of *C. cunninghamii* in the scientific literature. The LC-MS analysis of the herb extract produced at CPP/SCU was not consistent with the reported chemical profile.

Apart from a study on the essential oil (Pinhey and Southwell, 1971), and an evaluation of the antibacterial (Palombo and Semple, 2001) and antifungal (Wiesner, 1986; Hill, 1997) activity, the chemistry and bioactivity of *C. cunninghamii* is largely unreported.

The chemistry of *C. minima* has been studied in more detail. Murakami and Chen (1970) extracted phytosterols and arnidol. Bohlmann and Mahanta (1979) isolated centapedaoic acid and flavones. Bohlmann and Chen (1984) isolated lupeol acetate, 10-isobutyryloxy-8,9-epoxythymol, arnicolide C, brevifolin, helenanin, florilenanin isobutyrate, florilenanin isovalerate and florilenalin angelate.

3.2 Results and Discussion

The compounds of both the essential oil and the solvent extract of *C. cunninghamii* were examined in this study. A detailed analysis of the composition of the steam distilled essential oil of *C. cunninghamii* by GC-MS is discussed in Section 3.3.2 Isolation and structural elucidation of compounds from the aqueous ethanolic extract of *C. cunninghamii* is discussed in Section 3.2.2.

3.2.1 Composition of *C. cunninghamii* Essential Oil.

A large scale steam distillation of the commercially prepared, cut and dried herb material was carried out. 1.0 kilogram of the dried *C. cunninghamii* material was steam distilled for 24 hours. A light yellow oil having a characteristic odour was obtained in a yield of 0.21% oil (w/dry weight) based on the dry weight of the herb.

The GC-MS profile of the oil produced is presented in Figure 3.1. The major chemical constituents were identified by GC-MS. The components of the essential oil are presented in Table 3.1 and are compared against the chemical composition published by D′Amelio and Mirhom (1998).

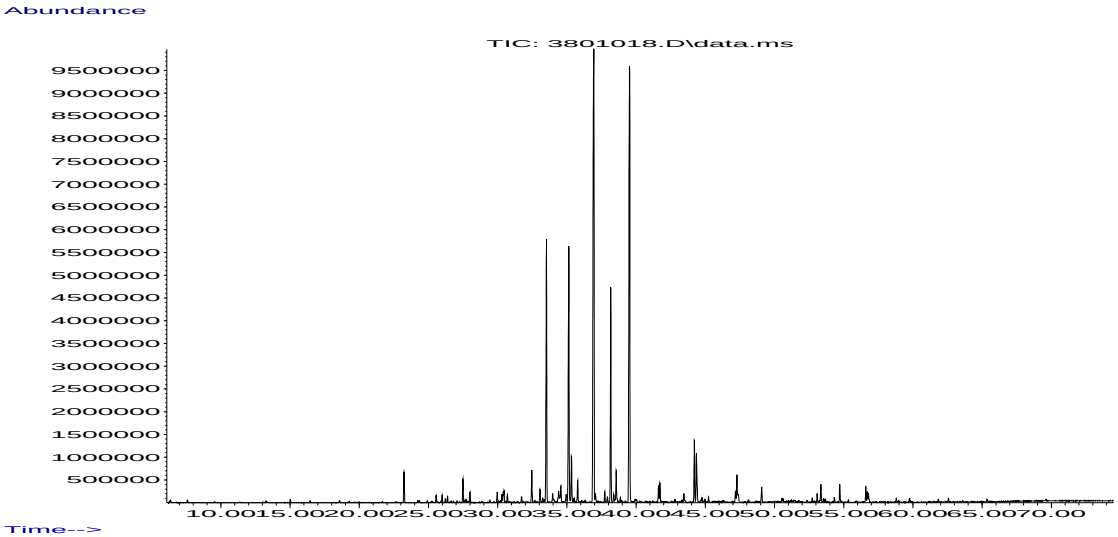


Figure 3.1. The GC-MS profile of the essential oil of *C. cunninghamii*.

The five major constituents identified in the oil were thymol (**1**), myrtenyl acetate (**2**), myrtenol (**3**), *cis*-chrysanthenyl acetate (**4**) and *cis*-chrysanthenol (**5**). The composition of this oil varies somewhat to the Bio-botanica oil. It was found to be higher in *cis*-chrysanthenyl acetate relative to the Bio-botanica oil whilst sabinyl acetate and *cis*-dihydrocarveol were not evident in the SCU oil. Myrtenyl acetate and *cis*-chrysanthenol

Table 3.1. Study of the constituents of the essential oil of *C. cunninghamii* and comparisons with the published results.

Compound	RI P	RI A	SCU Oil	Bio-botanica	Identification
methylbutyl acetate	-	-	nd	0.28	-
n-amyl acetate	-	-	nd	0.75	-
α-pinene	952	823	1.23	0.78	RI, MS, RC, Lit.
camphene	974	835	tr	-	RI, MS, RC
β-pinene	1000	846	0.28	0.22	RI, MS, RC, Lit.
trans-geraniol	-	-	nd	0.12	-
isobutyl isovalerate	1010	866	0.23	0.68	RI, MS, Lit.
isobutyl isovaleric acid ester	1020	867	0.16	1.18	RI, MS, Lit.
p-cymene	1046	881	0.61	-	RI, MS, RC
β-phellandrene	-	-	nd	0.10	-
1,8-cineole	1058	869	0.35	-	RI, MS, RC
γ-terpinene	-	-	nd	0.10	-
benzylethyl carbinol†	-	-	nd	0.18	-
trans-carveol	-	-	nd	0.17	-
amyl valerate	-	-	nd	0.33	-
2-methylbutyl 2-methylbutyrate	-	-	nd	0.64	-
3-methylbutyl ester	-	-	nd	0.67	-
pentyl 3-methylbutanoate	1113	885	0.53	2.09	RI, MS, RC, Lit.
p-cymenene	1115	927	0.28	-	RI, MS, RC
cis-sabinene hydrate	-	-	nd	0.27	-
trans-3(10)-caren-4-ol	-	-	nd	0.92	-
trans-(-)-pinocarveol	-	-	nd	0.36	-
trans-sabinene hydrate	-	-	nd	0.13	-
p-anisaldehyde	-	-	nd	0.13	-
cis-dihydrocarveol	-	-	nd	23.89	-
cis-chrysanthenol	1191	1756	9.70	-	RI, MS
p-mentha-1,5-dien-8-ol	-	-	nd	0.69	-
terpinen-4-ol	1213	1606	0.60	0.94	RI, MS, RC, Lit.

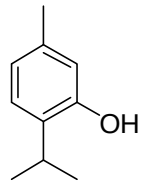
†compound name as transcribed from the Biobotanica patent (D′Amelio and Mirhom, 1998).

Table 3.1 Continued. Study of the constituents of the essential oil of *C. cunninghamii* and comparisons with the published results.

Compound	RI P	RI A	SCU Oil	Bio-botanica	Identification
p-cymene-8-ol	-	-	nd	0.58	-
cis-caran-*trans*-3-ol†	-	-	nd	0.21	-
isoamyl caproate	-	-	nd	1.23	-
α-terpineol	1228	1701	0.27	-	RI, MS, RC
myrtenol	1234	1799	8.20	5.85	RI, MS, RC, Lit.
myrtenal	1239	1636	2.78	0.17	RI, MS, RC, Lit.
cis-cerveol	-	-	nd	0.18	-
3,3-dimethylhexanol	-	-	nd	0.17	-
σ-isoprenylanisole	-	-	nd	0.82	-
cis-chrysanthenyl acetate	1281	1578	30.57	13.54	RI, MS, Lit.
acetanisole†	-	-	nd	0.40	-
ascaridole	-	-	nd	0.14	-
thymol	1314	2191	6.08	4.64	RI, MS, RC, Lit.
2,3-epoxygeranyl acetate	-	-	nd	0.10	-
hexyl caproate	-	-	nd	0.34	-
myrtenyl acetate	1354	1695	23.94	0.23	RI, MS, RC
sabinyl acetate	-	-	nd	22.97	
α-ylangene	-	-	nd	0.29	-
α-copaene	1413	964	0.47	-	RI, MS, RC
β-caryophyllene	1469	1600	0.66	0.17	RI, MS, RC, Lit.
allylanisole	-	-	nd	1.50	-
β-phenylethyl isovalerate	-	-	nd	0.72	-
nerol	-	-	nd	0.53	-
Total (%)			**86.9**	**90.4**	

RI P, RI A: retention indices on polar (BPX-5) and polar (BP 20) column respectively; RC: identification by reference compound. Order of elution and percentages (%) of individual component are derived from the polar column; nd: not detected; †compound name as transcribed from the Biobotanica patent (D′Amelio and Mirhom, 1998).

have not previously been reported as constituents of *C. cunninghamii* oil. Myrtenyl acetate is difficult to distinguish from sabinyl acetate by MS and may have been erroneously identified. In this instance the presence of myrtenyl acetate has been confirmed by comparison against this constituent in commercially available myrtle oil (Lawrence, 1990). The absence of sabinyl acetate has been confirmed by comparison against this constituent in commercially available valerian root oil (Lawrence, 1999). The nomenclature used to describe several constituents of the Bio-botanica oil is not comprehensive, to remove ambiguity, presumably and the authors were referring to 2-methylpropyl 3-methylbutanoate (syn: isobutyl isopentanoic acid ester); 1-phenylpropanol (benzylethyl carbinol); caranol (possibly as *cis*-caran-*trans*-3-ol) and 4-acetanisole (acetanisole).



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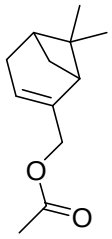

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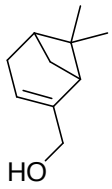

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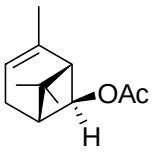

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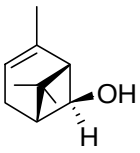


5

3.2.2 Isolation of Compounds from Extracts of *C. cunninghamii*.

The extract was fractionated by reverse phase preparative HPLC. Ten compounds were isolated from the 50% aqueous-ethanol extract as shown in Chapter 2, Figure 2.1. This approach afforded purification of the major metabolites (**6**-**15**) however; minor

constituents were also of interest. Significant anti-inflammatory activity (which is discussed in Chapter 4) was attributed to metabolites occurring in the region 7-15 minutes in the chromatogram (Figures 4.10 and 4.11). Consequently, a more efficient isolation scheme (Chapter 2, Figure 2.2) was proposed to pursue these constituents. An ethanolic extract of the floral parts was partitioned with hexane to afford an enriched fraction of the non-polar, PGE_2 active compounds. The hexane and the ethanol partitions were then evaporated to dryness and subjected to RP prep-HPLC. The pure compounds isolated from the floral parts are summarized in Chapter 2, Figure 2.2.

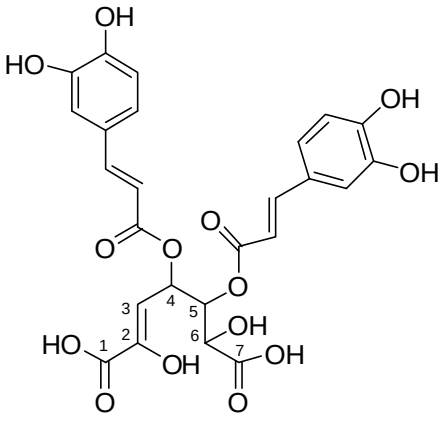
The recoveries of these compounds, does not reflect their abundance in the crude extract. The aim of this work was to obtain > 20 mg of each compound to undertake PGE_2 and ORAC bioassays. Traces of one compound were often discarded during the isolation of another, or derived from a specific fraction of the crude extract.

3.2.2.1 4ξ,5ξ-Di(3,4-dihydroxy-(*E*)-cinnamoyl)-2,6ξ-dihydroxyhept-2-ene-1,7-dioic acid (6) and its Derivatives (7, 8 and 9).

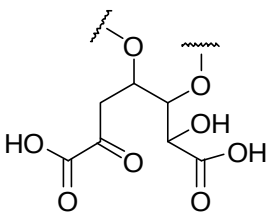
Five novel compounds were isolated from the 50% aqueous ethanol extract of the whole plant (Chapter 2, Figure 2.1). Sub-fractionation of preparative fractions 16 and 17 yielded four compounds. The major compound crystallised as white needles from ACN/H_2O. The (+)-LRAPCIMS molecular ion $[M+H]^+$ *m/z* 547 suggested that compound **6** had the molecular formula $C_{25}H_{22}O_{14}$. Twinning of some of the ^1H NMR signals indicated that the compound was composed of a subunit bearing two identical caffeoyl groups. The remaining ^1H and ^{13}C signals showed a vinylic group (δ_H 6.02 ppm, δ_C 109.3 ppm), three quaternary carbons (δ 65.4, 66.3, 76.3) and three tertiary carbons (δ 164.8, 146.1, 170.1 ppm). Quaternary signals at δ 170.1 and 164.8 ppm could readily be identified as carboxylic acid moieties. From their resonances at δ 65.4,

66.3 and 76.3 ppm, it could be established that three of the tertiary carbons were connected to oxygen. On the basis of the ^{1}H NMR and ^{13}C JMOD spectra the data alluded to a heptenedioic acid subunit. Only partial connectivity between the tertiary carbons in the subunit could be established by ^{1}H-^{1}H COSY. HMBC long range coupling established the order of connectivity between H-3 - H-4 - H-5 and H-6. The magnitude of the coupling between H-3, H-4 and H-5 indicated that the three hydrogens were oriented in a *syn* relationship to each other.

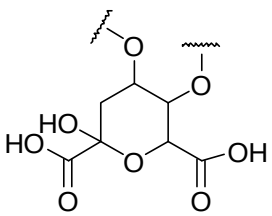
The structure proposed for **6** was 4*ξ*,5*ξ*-di-*O*-caffeoyl-2,6*ξ*-dihydroxyhept-2-ene-1,7-dioic acid, to which we have assigned the trivial name myriogenic acid. Isomerism of compound **6** was observed during fractionation and subsequent LC-MS analysis. It was not possible to determine whether the additional peak was that of the keto-tautomer (**6a**) or of the ring-closed pyranose (hemiacetal) form (**6b**). The ring-opened isomeric form of **6** was further supported on the basis of the theoretical ^{1}H NMR shifts. The observed and predicted ^{1}HNMR resonances for the proposed structure (**6**) and the ketomer (**6a**) and hemiacetal (**6b**) are presented in Table 3.2. Theoretical chemical shifts are derived from theoretical calculations determined by ChemBioDraw Software (version 11, 2007).



6



6a



6b

Table 3.2. ^{1}H NMR and ^{13}C NMR assignments for compound **6**.

	Compound **6** (CD_3OD)		Structure **6** (theoretical)		Structure **6a** (theoretical)		Structure **6b** (theoretical)	
C/H	^{13}C δ ppm	^{1}H δ ppm (i, m, *J* Hz)	^{13}C δ ppm	^{1}H δ ppm	^{13}C δ ppm	^{1}H δ ppm	^{13}C δ ppm	^{1}H δ ppm
1	164.8	-	164.5	-	162.8	-	174.7	-
2	146.1	-	171.8	-	195.0	-	105.9	3.65
3	109.3	6.02 (1H, dd,1.9, 2.2)	121.3	6.93	27.6	3.63, 3.88	37.5	2.35, 2.10
4	65.4	5.97 (1H, m)	60.2	5.23	64.5	4.77	63.0	4.66
5	66.3	5.98 (1H, m)	77.9	4.99	72.8	4.95	74.6	4.98
6	76.3	5.14 (1H, m)	70.9	4.68	70.5	4.68	74.3	5.06
7	170.1	-	176.2	-	176.2	-	173.2	-
1′	168.0	-	166.5	-	166.5	-	166.5	-
2′	114.1	6.30 (1H, d, 15.9)	116.2	6.31	116.2	6.31	116.2	6.31
3′	148.6	7.54 (1H, d, 15.9)	145.1	7.48	145.1	7.48	145.1	7.48
4′	128.0	-	128.0	-	128.0	-	128.0	-
5′	115.4	7.03 (1H, d, 2.0)	115.2	7.17	115.2	7.17	115.2	7.17
6′	147.0	-	145.9	-	145.9	-	145.9	-
7′	150.1	-	146.5	-	146.5	-	146.5	-
8′	116.7	6.76 (1H, d, 8.2)	117.2	6.93	117.2	6.93	117.2	6.93
9′	123.6	6.91 (1H, dd, 2.0, 8.2)	123.2	6.79	123.2	6.79	123.2	6.79
1″	167.9	-	166.5	-	166.5	-	166.5	-
2″	114.3	6.16 (1H, d, 15.9)	116.2	6.31	116.2	6.31	116.2	6.31
3″	148.1	7.50 (1H, d, 15.9)	145.1	7.48	145.1	7.48	145.1	7.48
4″	128.1	-	128.0	-	128.0	-	128.0	-
5″	115.3	6.99 (1H, d, 2.0)	115.2	7.17	115.2	7.17	115.2	7.17
6″	147.0	-	145.9	-	145.9	-	145.9	-
7″	150.0	-	146.5	-	146.5	-	146.5	-
8″	116.6	6.69 (1H, d, 8.2)	117.2	6.93	117.2	6.93	117.2	6.93
9″	123.7	6.81 (1H, dd, 2.0, 8.2)	123.2	6.79	123.2	6.79	123.2	6.79

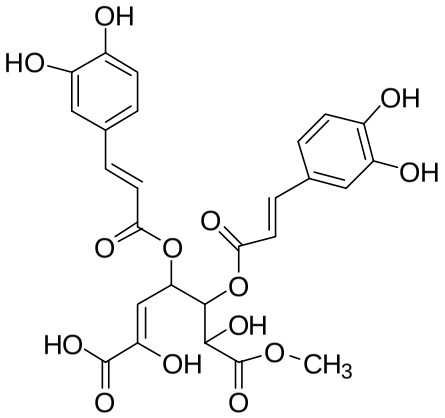
The ^{1}H NMR spectra of the three other compounds; **7**, **8** and **9** suggested that they were derivatives of compound **6**. The 1H NMR spectra were almost identical. The exception being that 3 additional proton singlets were observed in each of the spectra of **7**, **8** and **9** (Table 3.3). This, together with mass spectroscopic data strongly suggested that **7**, **8** and **9** were methyl esters of compound **6**. HMBC data confirmed that **7** was methylated at the 7-COOH position, **8** was methylated at the 1-COOH position and **9** was methylated at both the 1- and 7-COOH positions. The structural assignments are further supported by 2D, COSY, HMBC and HSQC data. These compounds (**6**, **7**, **8** and **9**) are novel, and to each we have assigned trivial names; myriogenic acid-1-methyl ester (**8**), myriogenic acid-7-methyl ester (**7**) and myriogenic acid dimethyl ester (**9**). It is possible that these compounds arise as artefacts from methanol during the isolation process.

Discrepancies were observed for the (+)-HRAPCIMS for myriogenic acid (**6**). The *m/z* value of 546.1248 was observed instead of the expected 547.1087 for the protonated parent ion $[M+H]^{+}$ thus resulting in a discrepancy of Δ0.9839 a.m.u. Equivalent discrepancies of Δ0.984 a.m.u. were also observed for the parent ions for the methyl esters **7** and **8** and dimethyl ester **9**. A molecular ion for these compounds could not be obtained using ES or MALDI TOF techniques. Fourier transform mass spectroscopy will be undertaken prior to the publication of this work.

Elemental analysis confirmed that there was no nitrogen in compound **6**. Hypothetically an alternative structure that could satisfy both the NMR data and mass spectral data and give rise to the apparent odd molecular weight would be a dimer possessing a plane of symmetry across a peroxide bridge. However this was not evident.

Table 3.3. ^{1}H NMR and ^{13}C NMR assignments for compounds **7**, **8** and **9**.

	Compound **7** (CD_3OD)		Compound **8** (CD_3OD)		Compound **9** (CD_3OD)	
C/H	^{13}C δ ppm	^{1}H δ ppm (i, m, *J* Hz)	^{13}C δ ppm	^{1}H δ ppm (i, m, *J* Hz)	^{13}C δ ppm	^{1}H δ ppm (i, m, *J* Hz)
1	164.4	-	163.6	-	163.4	-
2	146.3	-	145.9	-	145.9	-
3	109.1	6.03 (1H, dd, 1.6, 2.1)	109.9	6.01 (1H, dd, 1.8, 2.2)	109.3	6.03 (1H, dd, 1.6, 2.6)
4	66.0	5.93 (1H, m)	66.3	5.95-5.98 (1H, m)	66.0	5.95 (1H, m)
5	65.4	5.89 (1H, m)	65.4	5.95-5.98 (1H, m)	65.4	5.89 (1H, ddd, 1.6, 1.7, 4.6)
6	76.0	5.18 (1H, br s)	76.3	5.11 (1H, br s)	76.1	5.18 (1H, br s)
7	168.9	-	169.9	-	168.8	-
1-COOMe	-		53.2	3.86 (3H, s)	53.2	3.85 (3H, s)
7-COOMe	53.4	3.79 (3H, s)	-		53.4	3.79 (3H, s)
1′	167.9	-	168.0	-	167.9	-
1″	167.9	-	167.9	-	167.9	-
2′	114.0	6.29 (1H, d, 15.9)	114.1	6.29 (1H, d, 15.9)	113.9	6.27 (1H, d, 16.0)
2″	114.2	6.17 (1H, d, 15.9)	114.3	6.16 (1H, d, 15.9)	114.2	6.69 (1H, d, 16.0)
3′	148.7	7.54 (1H, d, 15.9)	148.6	7.53 (1H, d, 15.9)	148.7	7.53 (1H, d, 16.0)
3″	148.2	7.50 (1H, d, 15.9)	148.1	7.49 (1H, d, 15.9)	148.2	7.50 (1H, d, 16.0)
4′	127.6	-	127.7	-	127.7	-
4″	127.7	-	127.7	-	127.7	-
5′	115.4	7.04 (1H, d, 2.0)	115.3	7.03 (1H, d, 2.0)	115.4	7.03 (1H, d, 2.0)
5″	115.1	7.00 (1H, d, 2.0)	115.1	6.99 (1H, d, 2.0)	115.1	6.99 (1H, d, 2.0)
6′	147.0	-	147.0	-	147.0	-
6″	147.0	-	147.0	-	147.0	-
7′	150.1	-	150.1	-	150.1	-
7″	150.0	-	150.0	-	150.0	-
8′	116.7	6.76 (1H, d, 8.2)	116.7	6.76 (1H, d, 8.2)	116.7	6.76 (1H, d, 8.2)
8″	116.6	6.69 (1H, d, 8.2)	116.6	6.69 (1H, d, 8.2)	116.6	6.69 (1H, d, 8.2)
9′	123.6	6.91 (1H, dd, 2.0, 8.2)	123.7	6.91 (1H, dd, 2.0, 8.2)	123.6	6.91 (1H, dd, 2.0, 8.2)
9″	123.7	6.82 (1H, dd, 2.0, 8.2)	123.6	6.81 (1H, dd, 2.0, 8.2)	123.7	6.82 (1H, dd, 2.0, 8.2)



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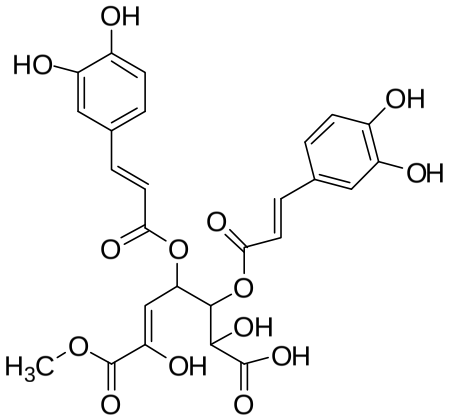

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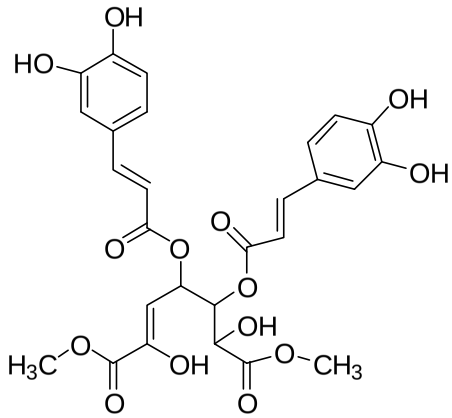


9

Compound **6** reliably crystallised from solution as filamentous crystals that were deemed unsuitable for X-ray crystallography. Powdered X-ray diffraction studies revealed only part of the unit cell. Multiple attempts were made at crystallising the compound from different solvents in order to obtain crystals of dimensions suitable for crystallography. To date, this structural validation route has not been successful.

The discrepancies observed in the (+)-HRAPCIMS may possibly be due to mis-identification of the molecular ion. Distinction between $[M-H]^+$, the molecular ion$[M]^+$, and the isotopes [M+1] and [M+2] is usually determined from the intensities of these ions in the mass spectrum. The theoretical isotopic ratios are derived from natural isotopic abundances and it is expected that; M (100%), M+1 (27%), M+2 (6.58%). However the intensities of the isotopes is not applicable to chemical ionisation techniques due to ion-molecule reactions (Silverstein et al., 1991).

It has been reasoned that the prominent ion observed in the (+)-HRAPCIMS is that of $[M-H]^+$. Support for the proposed structure (**6**) was obtained from the mass spectrum fragmentation pattern. (+)-LRAPCIMS gave rise to ions *m/z* 163 [caffeoyl-OH]$^+$, 164 [caffeoyl -O]+, 349 [M- caffeoyl-OH]$^+$, 350 [M-caffeoyl -O]$^+$, 546 $[M-H]^{\bullet+}$, 547 $[M+H]^+$. (+)-HRAPCIMS gave rise to ions *m/z* 546.1248 (48%), 384.0930 (25), 349.0557 (100). The strongest evidence in support of the heptenedioic acid subunit is derived from an ion occurring at *m/z* 384.0930 corresponding to loss of one of the caffeoyl groups $[M-caffeoyl]^+$.

Further support is garnered from the derivatives of compound **6**. It was observed that myriogenic acid (**6**) readily undergoes esterification in the presence of methanol at

room temperature in the expected ratio of 2:2:1 and progresses to the dimethyl ester over time.

It is anticipated that the dimethyl ester (**9**) will produce more suitable crystals for spectroscopic work. Structural verification of compound **6** may be confirmed by X-ray crystallographic data of the dimethyl ester (**9**). Approximately 1 gram of myriogenic acid has been isolated and esterified. Re-isolation of the dimethyl ester is currently underway, it is intended that the structure of the dimethyl ester will be confirmed prior to publication of this work.

3.2.2.2 2′,4′,5,7-Tetrahydroxy-6-methoxyflavone-3-*O*-*β*-glucopyranoside (10).

The molecular ion $[M+H]^+$ *m/z* 495 suggested that compound **10** had a molecular formula $C_{22}H_{22}O_{13}$ with twelve double bond equivalents. The UV spectrum showed UV maxima at λ_{max} 210, 258, 270 sh, and 354 nm which are characteristic of flavonols (Dey and Harborne, 1989).

The ^{1}H NMR spectrum (Table 3.4) showed three protons in the aromatic region resonating at δ 7.71 (d, J = 2.2 Hz), 6.87 (d, J = 8.5Hz) and 7.59 (dd, J = 2.2, 8.5 Hz) ppm, exhibiting *ortho* and *meta* coupling. A fourth aromatic proton resonated as a singlet (6.52 ppm) indicating it was a substituent of a highly substituted aromatic ring.

The ^{13}C JMOD NMR spectrum (Table 3.4) revealed the presence of a carbonyl signal at δ 179.9 ppm and a methoxy signal present at δ_H 3.88 ppm. The location of the methoxy group could be assigned to C-6 based on the $^3J_{CH}$ correlations in the HMBC spectrum. There were also was also oxygen bearing quaternary carbons present as indicated by the

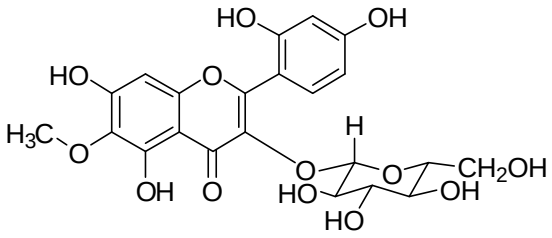
signals at δ 143.1, 153.9, 154.0, 159.4 and 150.0 ppm. From the HMBC and HSQC data the substitution patterns of the aromatic rings, and consequently the partial structure 2′,4′,5,7-tetrahydroxy-6-methoxyflavone, could be deduced.

Table 3.4. ^{1}H and ^{13}C NMR spectral data for compound **10** in CD_3OD.

Compound 10		
C/H	^{13}C δ ppm	^{1}H δ ppm (i, m, *J* Hz)
1	-	
2	146.1	
3	135.5	
4	179.9	
5	153.9	
6	132.9	
6-OCH_3	62.7	3.88 (3H, s)
7	154.0	
8	95.1	6.52 (1H, s)
9	159.0	
10	106.3	
1′	123.3	
2′	159.4	
3′	117.7	7.71 (1H, d, 2.2)
4′	150.0	
5′	123.4	7.59 (1H, dd, 2.2, 8.5)
6′	116.2	6.87 (1H, d, 8.5)
1″	104.4	5.26 (1H, d, 7.6)
2″	75.9	3.49 (1H, dd, 7.6, 9.1)
3″	78.3	3.43 (1H, dd, 8.7, 9.1)
4″	71.4	3.35 (1H, dd, 8.7, 9.6)
5″	78.5	3.22 (1H, ddd, 2.4, 5.4, 9.6)
6a″	62.7	3.77 (1H, dd, 2.4, 11.9)
6b″	-	3.57 (1H, dd, 5.4, 11.9)

On the basis of the resonance at δ_H 5.26 ppm, which was suggestive of an anomeric proton, and the characteristic loss of [M-162] observed in the mass spectrum, the remaining ^{1}H and ^{13}C NMR signals alluded to a sugar moiety. On the basis of ^{1}H-^{1}H COSY and coupling constants all of the hydrogen groups in the sugar moiety were

deduced to be axial. Since both the anomeric and adjacent hydrogen were axial, the sugar moiety could be assigned as *β*-D-glucopyranose. Compound **10** was elucidated as 5,7,2′,4′-tetrahydroxy-6-methoxyflavone-3-*O*-*β*-glucopyranoside to which we have assigned the trivial name centipetin-3-glucoside. The structure (**10**) was partially verified by Gabriel (2005), and the ^{1}H and ^{13}C NMR data were consistent with the reported values.



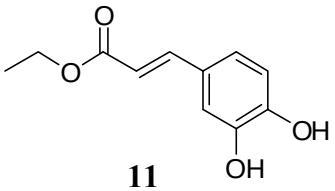
10

3.2.2.3 Caffeic acid ethyl ester (11).

In addition to compounds **6-10**, seven known compounds were isolated from *C. cunninghamii*. Fractionation of the aerial parts of the plant yielded five known caffeic acids, whilst a methanolic extract from the floral parts yielded four flavonoids and three sesquiterpenes.

Compound **11** was isolated from preparative F11 (Figure 2.1). The structure of compound **11** was verified on the basis of ^{1}H, ^{13}C, HMBC and HSQC NMR spectroscopy and was in agreement with the reported data (Gabriel, 2005.). The compound appears to be the esterified product of caffeic acid. Throughout the extraction and isolation process concentrations of compound **11** were observed to

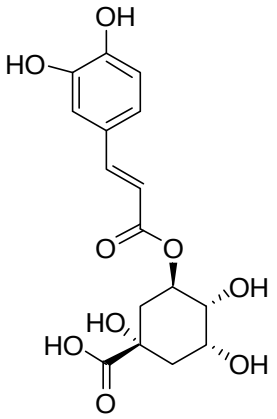
increase over time, indicating that it arises as a breakdown product derived from the caffeic acid constituents.



11

3.2.2.4 Chlorogenic acid (12) and its methyl ester (13).

Two compounds were isolated from prep-HPLC F2 (Figure 2.1). The first was elucidated to be chlorogenic acid (**12**). The ^{1}H and ^{13}C NMR, and stereochemical assignments are in agreement with published data for chlorogenic acid (Cheminat et al., 1988; Lin et al., 1999). The immuno-modulatory properties of **12** have recently been investigated by Lin and co-workers (1999). Chlorogenic acid was shown to potently enhance human mononuclear cell proliferation and interferon-γ production to an extent comparable to the positive control, interleukin-2.



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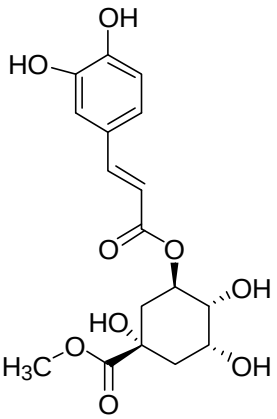
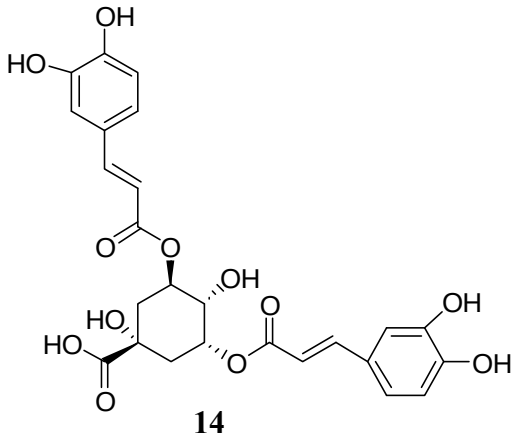


13

The ^{1}H NMR spectrum for the second compound was almost identical with chlorogenic acid (**12**) with the exception being an additional resonance δ_H 3.70 ppm readily identified as a methoxy substituent. The location of the methoxy signal could be determined from the $^{3}J_{CH}$ correlation to C-7 in the HMBC spectrum. Compound **13** was elucidated as caffeic acid methyl ester, ^{1}H and ^{13}C NMR data and stereochemical assignments are in agreement with the reported values (Zhu et al., 2005a), the structure is further supported by 2D COSY, HMBC and HSQC data.

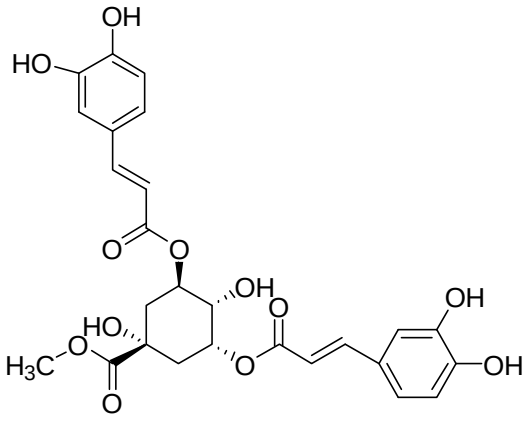
3.2.2.5 Isochlorogenic acid A (14).

Analysis of the ^{1}H and ^{13}C NMR data of two compounds (**14** and **15**) isolated from F14 (Figure 2.1) showed similarities betweeen the two by the presence of two caffeic acid moieties in their structures. The first compound (**14**) was elucidated as isochlorogenic acid A. The ^{1}H and ^{13}C NMR data are in agreement with the reported values (Kodoma et al. 1998) and is further supported by 2D COSY, HMBC and HSQC data. The stereochemical assignments have been derived from the affinity of **14** to compound **15**.



14

3.2.2.6 Revised assignments for macroantoin G (15).



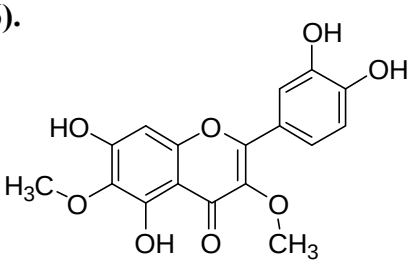
15

The second compound (**15**) isolated from F14 (Figure 2.1) was elucidated as the methyl ester of **14**. Two caffeic acid moieties were observed in the NMR spectra of **15** and in contrast to **14**, an additional methoxy singlet was apparent (δ_H 3.70 ppm). The methoxy protons showed a $^3J_{CH}$ correlation to the carboxylic group at C-7 which indicated the location of the methoxy substituent. The optical rotation and stereochemical assignments are consistent with reported values for macroantoin G (Zhang et al., 2000). ^{1}H and ^{13}C NMR assignments are in agreement with the published values (Table 3.5) with the exception of the assignments for H-2a/H-2b and H-6a/H-6b which have been revised as shown in Table 3.5.

Table 3.5. ^{1}H and ^{13}C NMR spectral data for compound **15** and macroantoin G (Zhang et al., 2000) 300MHz, CD_3OD).

	Compound **15**		Macroantoin G	
C/H	^{13}C δ ppm	^{1}H δ ppm (i, m, *J* Hz)	^{13}C δ ppm	^{1}H δ ppm (i, m, *J* Hz)
1	74.8	-	74.64	-
2a	35.8	2.35 (1H, m)	36.78	} 2.19 (2H, m)
2b	-	2.15 (1H, m)		
3	72.1	5.40 (1H, ddd, 3.6, 4.0, 8.0)	72.08	5.35 (1H, m)
4	69.9	3.98 (1H, ddd, 3.2, 6.5)	69.81	3.98 (1H, dd, 3.2, 6.8)
5	72.4	5.31 (1H, m)	71.98	5.41 (1H, m)
6a	37.0	2.31 (1H, m)	35.63	} 2.21 (2H, m)
6b	-	2.18 (1H, m)		
7	175.8	-	175.59	-
7-COOMe	53.2	3.70 (3H, s)	53.03	-
1′	168.1	-	168.73	-
1″	168.9	-	167.98	-
2′	115.0	6.33 (1H, d, 15.9)	115.38	6.33 (1H, d, 15.9)
2″	115.6	6.22 (1H, d, 15.9)	114.81	6.21 (1H, d, 15.9)
3′	147.3	7.62 (1H, d, 15.9)	147.09	7.62 (1H, d, 15.9)
3″	147.6	7.55 (1H, d, 15.9)	147.37	7.54 (1H, d, 15.9)
4′	127.8	-	127.81	-
4″	128.1	-	127.56	-
5′	115.3	7.06 (1H, d, 2.2)	115.14	7.06 (1H, d, 2.0)
5″	115.3	7.06 (1H, d, 2.2)	115.14	7.06 (1H, d, 2.0)
6′	147.0	-	146.65	-
6″	147.0	-	146.65	-
7′	149.7	-	149.40	-
7″	150.0	-	149.56	-
8′	116.7	6.79 (1H, d, 8.2)	116.45	6.79 (1H, d, 8.1)
8″	116.7	6.79 (1H, d, 8.2)	116.46	6.79 (1H, d, 8.2)
9′	123.1	6.97 (1H, dd, 2.2, 8.2)	122.99	6.96 (1H, dd, 8.1, 2.0)
9″	123.2	6.97 (1H, dd, 2.2, 8.2)	123.05	6.96 (1H, dd, 8.2, 2.0)

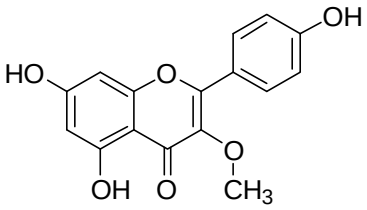
3.2.2.7 Axillarin (16).



16

Three compounds were obtained from the SPE3 fractions (Figure 2.2), all of which were elucidated as known flavonoids. The first pure isolate, SPE3-F1 was elucidated as the flavone axillarin (**16**). ^{1}H NMR data is in agreement with reported the values (Jefferies et al., 1974).

3.2.2.8 Isokaempferide (17) and 3,6-dimethoxy apigenin (18).



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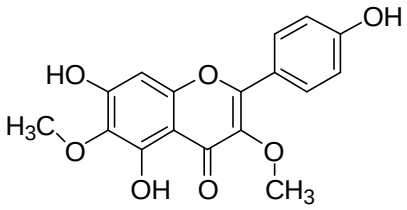
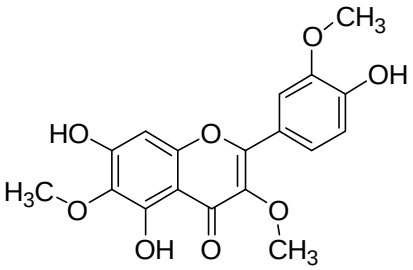


18

The second compound SPE3-F2 (Figure 2.2) was composed of a mixture of two co-eluting compounds (**17** and **18**). Compound **17** was elucidated as isokaempferide and compound **18** as 3,6-dimethoxyapigenin. Spectroscopic data was in agreement with published values for compound **17** (Grouiller et al., 1967) and **18** (Herz et al., 1975). Due to the common occurrence and published biological activity of these flavones it was considered unnecessary to proceed with further purification.

3.2.2.9 Jaceidin (19).

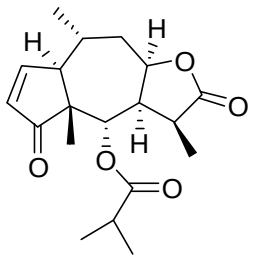
The third isolate from fraction SPE3 was elucidated as jaceidin (**19**). ^{1}H NMR assignments were consistent with published values (Roitman and James, 1985). Structure **19** is further supported by ^{13}C and 2D HMBC, HSQC and COSY data.



19

3.2.2.10 Arnicolide C (20).

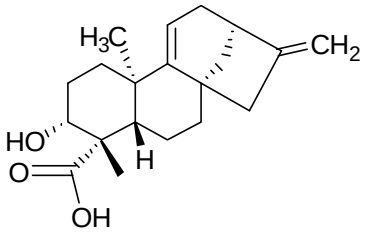
The fractions from the hexane partition of the ethanol extract (SPE4 fractions, Figure 2.2) were mostly terpenoids, plant sterols and fatty acids based on their ^{1}H NMR spectra. Three of the SPE4 fractions were sufficiently pure for structural elucidation work. These were identified as known terpenoid compounds. SPE4-F1 was elucidated to be the known sesquiterpene lactone, arnicolide C (**20**). The structure and stereochemical assignments have been confirmed by X-ray crystallography and optical rotation studies (Poplawski et al., 1971) who reported the isolation of arnicolide C and related sesquiterpene lactones from *Arnica* species.



20

3.2.2.11 3-Hydroxykaura-9(11),16-dien-19-oic acid (21).

The second isolate SPE4-F7 was elucidated as the known kaurane diterpene; 3-hydroxykaura-9(11),16-dien-19-oic acid (**21**). 3-Hydroxy-9(11),16-kauradien-19-oic acid was first reported by Bohlmann and co-workers (1982) as a metabolite from *Ichthyothere terminalis*. The compound was characterised as the methyl ester derivative **22** and stereochemistry was assigned on the basis of ^{1}H NMR studies.



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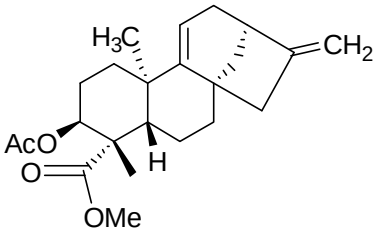

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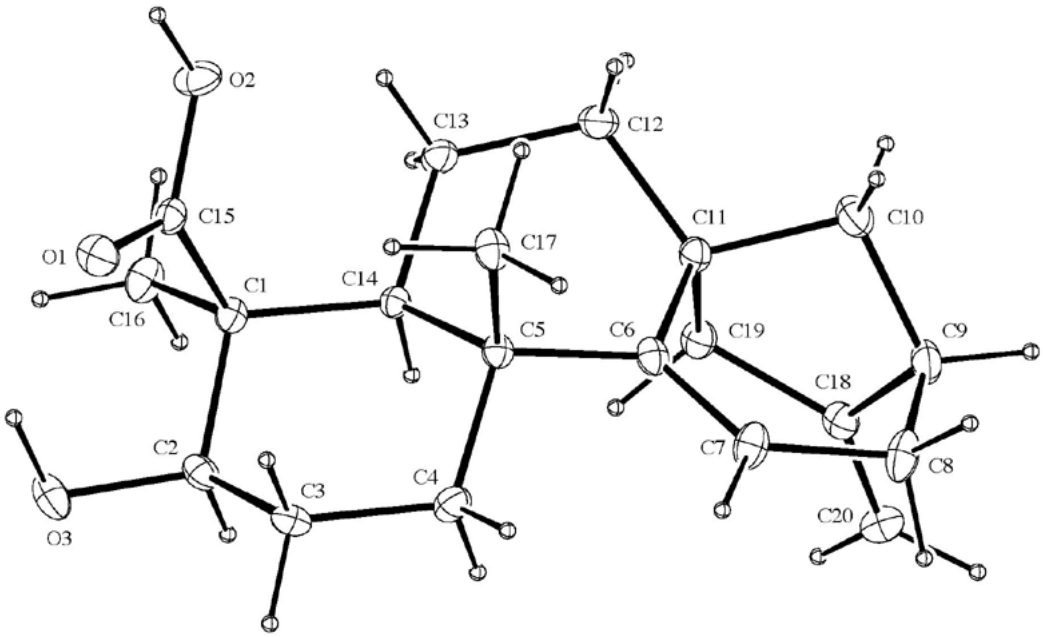
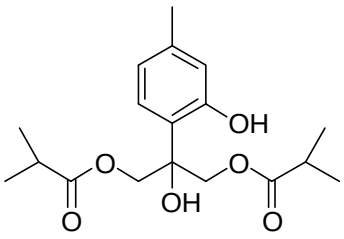


Figure 3.2. X-ray crystal structure of 3-hydroxy-9(11),16-kauradien-19-oic acid (**21**).

X-ray crystallography studies (Figure 3.2) have confirmed the structure (**21**) and relative stereochemical assignments. In contrast to the published structure (Bohlmann et al., 1982) 3-hydroxy-9(11),16-kauradien-19-oic acid was elucidated to possess an *R* configuration at C-3. Compound **21** was elucidated as (*rel*)-(3*R*,4*S*,5*S*,8*S*,10*R*,13*R*)-3-hydroxy-kaura-9(11),16-dien-19-oic acid.

3.2.2.12 8-Hydroxy-9,10-diisobutyryloxythymol (23).

SPE4-F8 (Figure 2.2) was elucidated as 8-hydroxy-9,10-diisobutyryloxythymol (**23**). ^{1}H NMR data is in agreement with published values (Mossa et al., 1997). The occurrence in, and antibacterial activity of this compound has previously been reported from *Centipeda minima* (Liang et al., 2007).



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3.3 Conclusion

Characterisation of the essential oil of *C. cunninghamii* identified *cis*-chrysanthenol (**5**), myrtenol (**3**), chrysanthenyl acetate (**4**), thymol (**1**) and myrtenyl acetate (**2**) as the major constituents. Myrtenyl acetate and *cis*-chrysanthenol have not previously been reported from *C. cunninghamii*. The oil was not found to contain sabinyl acetate as previously reported by D'Amelio and Mirhom (1998).

The chemical composition of solvent extract of *C. cunninghamii* was different from the essential oil and also different from the reported constituents (D'Amelio and Mirhom, 1998). In common with D'Amelio and Mirhom (1998), arnicolide C was isolated in this instance. Of the 25 compounds identified by D'Amelio and Mirhom (1998), from the HPLC chromatogram of the extract, twelve were sesquiterpene lactones. Additional sesquiterpene lactones were isolated but were not sufficiently pure for characterisation. D'Amelio and Mirhom (1998) also reported on the identification of kaempferol-7-glucosyl-rhamnoside, apigenin and quercetin which are different to the flavonoids purified in the course of this work.

Very few parallels were observed between compounds reported from *C. minima* and *C. cunninghamii.* The principal constituents identified from solvent extracts of *C. cunninghamii* were phenolic compounds; caffeic acids and flavonoids. Lipophilic isolates constituted a thymol derivative, a diterpene and a sesquiterpene lactone. Previous chemical investigations of *C. minima* and have been directed more towards the lipophilic components and consequently multiple triterpenoids and sesquiterpene lactones have been characterised from this species.

The bioassay directed fractionation and anti-inflammatory and antioxidant activity of the compounds isolated from *C. cunninghamii* is reported is Chapter 4.

Chapter 4

Bioassay Directed Isolation of Antioxidant and Anti-inflammatory Compounds from *Centipeda cunninghamii*

This investigation encompasses both the anti-inflammatory and antioxidant activity of *C. cunninghamii* extracts, fractions and pure compounds.

4.1 Introduction

4.1.1 Mechanisms of Inflammation and Anti-inflammatory Drugs

Inflammation is an immune response to the invasion of pathogens, chemical irritants, burns, toxins or mechanical injury. The processes of oxidation and inflammation are closely linked, as during the inflammatory response free radicals are produced at the

inflamed site. The mechanism and biological importance of antioxidants is discussed in Section 4.1.2.

Inflammation is initiated at the cellular level by mast cells which release histamine and commence the generation of eicosanoids at the site of injury, inducing localized vasodilation. This serves to increase the number of platelets, leukocytes and crucial plasma proteins at the wound. The increased cellular permeability incidentally gives rise to the physical characteristic of inflammation; redness, swelling, pain, heat and loss of function (Calixto et al., 2003; Vane et al., 1994). It is known that endothelial cells, macrophages, basophils and platelets can respond independently as well as play a concerted role in the inflammatory cascade (Rang et al., 1995). Endothelial cells are capable of generating nitric oxide whilst macrophages can produce nitric oxide and also cytokines.

Inflammation is normally a tightly regulated process. A progression of biochemical events consisting of both cellular and plasma derived mediators which serve to confine the location, remove the insult, and repair the tissue. In cases where the inflammatory response is not effective in removing the insult or where inflammation runs unchecked chronic inflammation can result. This is observed to occur in a cohort of diseases such as hay fever, atherosclerosis, rheumatoid arthritis, asthma, cancer, Alzheimer's and inflammatory bowel disease (Bochsler and Slauson, 2002).

Most anti-inflammatory drugs in clinical use act on the biosynthesis of eicosanoids (Rang et al., 1995). Eicosanoids are implicated in the control of many physiological processes and are among the most important mediators and modulators of the

inflammatory reaction. Eicosanoids arise from arachidonate. Multiple enzymatic pathways exist for the biosynthesis of various eicosanoids and vary by cell type. A summary of the eicosanoid biosynthetic pathways and the drugs that act on them is depicted in Figure 4.1.

Prostaglandin E_2 (PGE_2) is the predominant product released during an inflammatory response and is metabolised via the cyclooxygenase (COX) enzyme via a multi-step process from arachidonic acid. COX has been found to exist in three forms; COX-1, COX-2 and COX-3. COX-1 is found as a constituent of most cells and is thought to synthesize prostanoids that are involved in normal homeostasis such as regulating vascular responses, coordinating the actions of circulating hormones and also inflammation. The COX-2 is an enzyme that is expressed in response to an inflammatory stimulus. COX-3 has been proposed to be a variant of COX-1 and has been reported to exist in the cerebral cortex and heart (Chandrasekharan et al., 2002). Its' expression and function remain uncertain (Warner and Mitchell, 2002; Schwab et al., 2003).

It is thought that the efficacy of traditional NSAIDs such as aspirin and ibuprofen is due to their inhibition of the COX-2 enzyme whilst the unwanted side effects such as gastric and duodenal ulcers, is due to the inhibition of COX-1 (Fritsche et al., 2001; Zhang et al., 1997; Hinz and Brune, 2002). Selective COX-2 inhibitors such as celecoxib (**24**), and rofecoxib (Vioxx) have been developed. However rofecoxib (**25**) has been implicated in cardiovascular health complications such as heart attacks, thrombosis and strokes and has been withdrawn from the market. At this time it is not

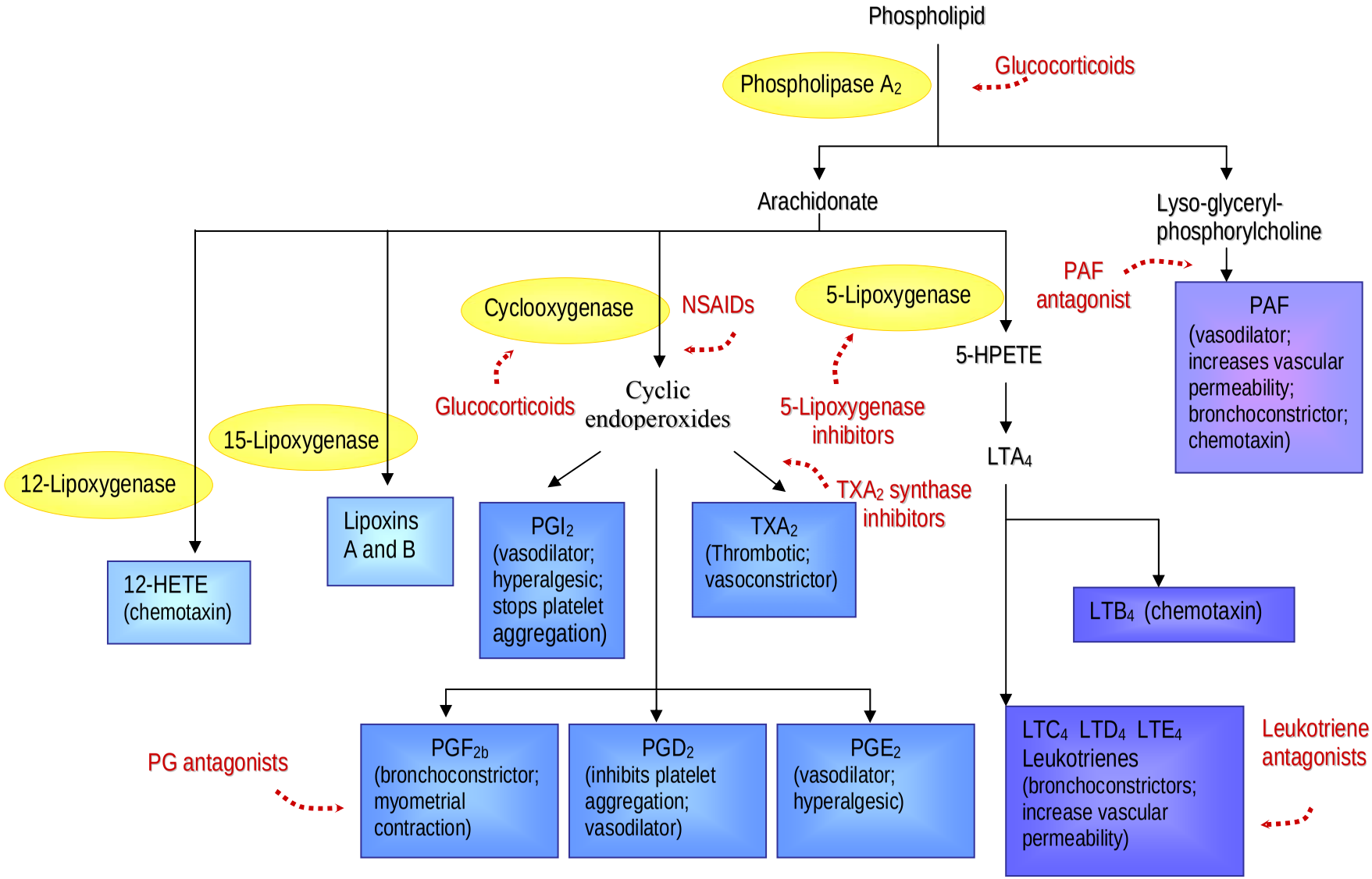
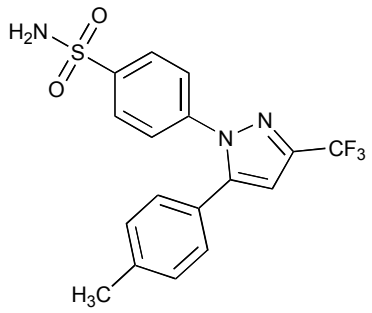


Figure 4.1 Mediators derived from phospholipids and associated anti-inflammatory drugs. Adapted from Rang et al. (1995).

PG=prostglandin; PGI_2=prostacyclin; TX=thromboxane; LT=leukotriene; HETE=hydroxyeicosatetraenoic acid; HPETE=hydroperoxyeicosatetraenoic acid; PAF=platelet activating factor; NSAIDs=non-steroidal anti-inflammatory drugs.

clear whether all COX-2 selective drugs pose an increased cardiovascular risk or just rofecoxib.



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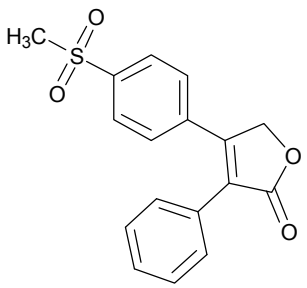

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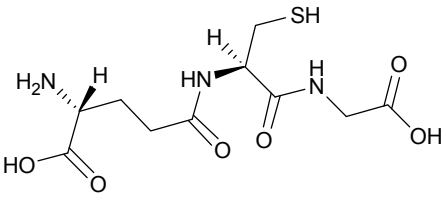
The lipoxygenase (LO) enzymes (Figure 4.1) are found in the lung, platelets, mast cells and white blood cells with 5-LO being the principal enzyme (Rang et al., 1995). The leukotrienes they metabolise have been found to be involved in inflammatory diseases such as asthma, psoriasis, rheumatoid arthritis and inflammatory bowel disease (Zhang and Li, 1999; Henderson, 1994). Since the COX and LO enzymes use the same substrate, arachidonic acid, inhibition of one enzyme may cause the arachidonic acid cascade to shift to the alternative pathway. Drugs that act on both LO and COX pathways have recently attracted interest (Celotti and Laufer, 2001; Claria and Romano, 2005; Araico et al., 2006). Some dual active COX/5-LO inhibitors have been discovered and several have entered clinical trials (Julemont et al., 2004) and are proving to be favourable in the treatment of rheumatic diseases with few gastrointestinal side effects (Bertolini et al., 2002).

Additional inflammatory processes can occur via the actions of macrophages and lymphocytes which can produce cytokines such as the interleukins (IL) and tumor

necrosis factors (TNF). These peptides are produced mainly upon inflammatory activation and possess a wide range of physiological roles. They are responsible for facilitating the immune response, the release of acute phase proteins and can effect fever (Rang, et al., 1995). Drugs that are active against the metabolism of cytokines have also been developed such as the TNF-α inhibitors adalimumab, etanercept, and infliximab. Further to this, nitric oxide (NO) can be stimulated by the production of cytokines, such as TNF-α. Nitric oxide is implicated in many biological processes, but in general increased NO can contribute to disease processes (Rang, et al., 1995).

4.1.2 Free Radicals, Antioxidants and Bioassays

A wide range of reactive oxygen species (ROS) are constantly generated *in vivo* as an integral product of metabolism, by exposure to environmental factors and as a consequence of inflammatory processes. It is recognized that over one hundred diseases, including cancer, cardiovascular disease, diabetes mellitis, male infertility, renal disease, cataracts, liver, lung, neurological and inflammatory diseases give rise to a pathological increase of free radicals. These free radicals circulate freely in the body with access to all organs and tissues. ROS are capable of killing cells and may cause oxidative damage to DNA, lipids, proteins and enzymes. Organisms have developed complex antioxidant systems to counteract ROS. Endogenous antioxidants include glutathione (**26**) and melatonin and enzymes such as catalase, superoxide dismutase and peroxidases. Nutritional antioxidants include vitamin C and α-tocopherol (**27**), carotenoids and polyphenolics. In addition to their biological and medical applications, antioxidants have important industrial uses such as preservatives in food and cosmetics, and in preventing the degradation of gasoline and rubber.



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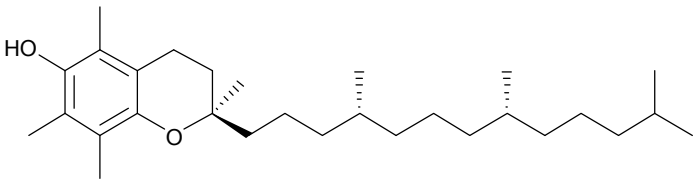

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The antioxidant strength of an extract or compound can be determined by measuring the oxygen radical absorbance capacity (ORAC). The ORAC assay measures the oxygen scavenging ability of a test sample by monitoring the free radical dependant decrease in florescence intensity of the β-pycoerythrin marker protein (Huang et al., 2005). In the ORAC assay 2,2′-azobis(2-methyl)propionamidine dihydrochloride (AAPH) is added to generate free radicals. The decrease in fluorescence is monitored over time (Figure 4.2) in the presence of the test sample, in comparison to trolox, a water soluble vitamin E analogue and for a blank sample. From a plot of the decrease in fluorescence over time the area under the curves can be calculated and the data extrapolated to determine a trolox equivalent value, an indication of the ORAC capacity of the test sample relative to trolox.

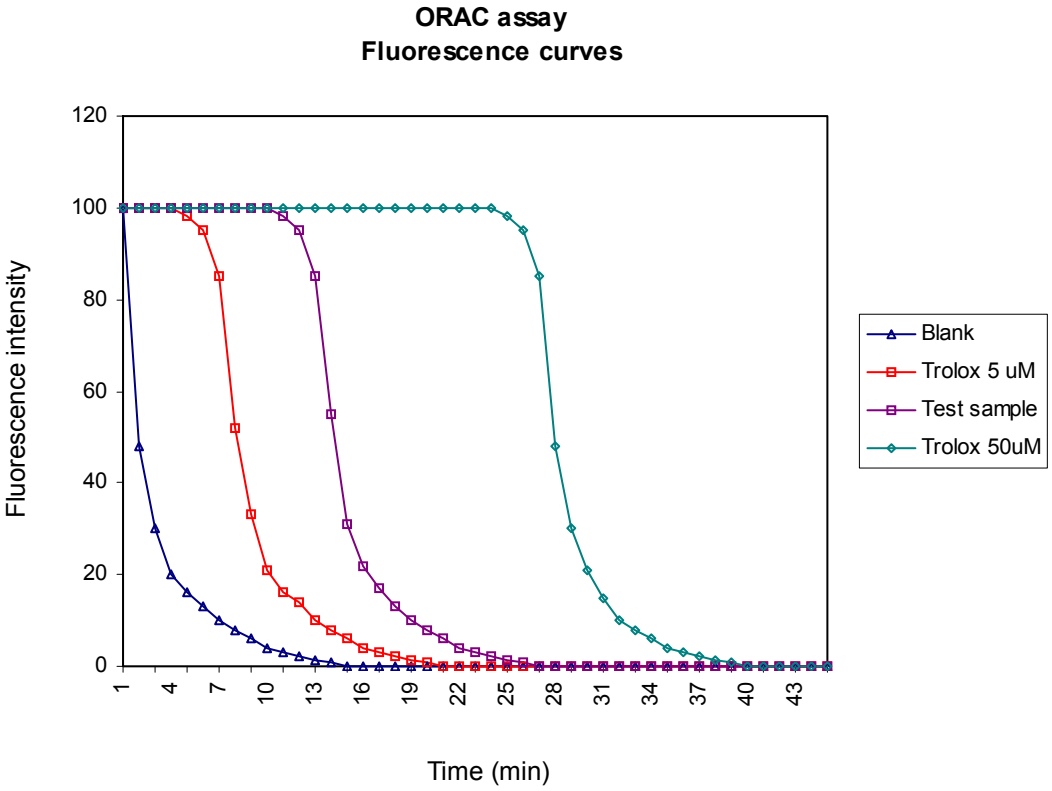

Figure 4.2. ORAC assay fluorescence curves.

4.2 Results and Discussion

4.2.1 The Biological Activity of *C. cunninghamii* Extracts

In this study commercially available COX-1, COX-2, 5-LO, TNF-α, and NO assay kits were selected as the target enzymes to assess the anti-inflammatory properties of *C. cunninghamii* extracts whilst the ORAC hydrophillic assay was selected to assess the antioxidant constituents of the herb.

Three solvent extracts; 100% aqueous (ambient temperature), 50% aqueous ethanol and 100% ethanol were prepared by steeping overnight at room temperature. A 100% aqueous (100°C) extract was prepared by boiling the herb for one hour. The four extracts and oil were analysed by LC-MS to determine their chemical compositions. One kilogram of the dried herb was steam distilled to produce the essential oil.

The chemical profile of the aqueous extract was almost identical to that of the boiled aqueous and 50% ethanolic extract. It was observed that the compounds found in the steam distilled oil may also be extracted at room temperature by the inclusion of ethanol (50-100%). It was considered that the 50% aqueous ethanol extract gave the broadest representation of the chemical profile of *C. cunninghamii* compared to the oil or the aqueous extracts. The 50% aqueous ethanol extract also gave a much greater yield, 15.0% compared to only a 4.4% yield for the 100% ethanol extract. The four solvent extracts and the essential oil were tested against the ORAC and PGE_2 bioassays. The inhibition of PGE_2 and antioxidant activity of the 5 extracts is presented in Table 4.1.

Table 4.1. Anti-inflammatory and antioxidant capacity of *C. cunninghamii* extracts.

	% inhibition of PGE_2		Antioxidant Capacity	
Extract	**Sample Concentration (μg/mL)**	**Average Inhibition ± SEM**	**μmol TE / g extract ± SEM**	**μmol TE / g sample ± SEM**
100 % Ethanol	10 100 1000	22.91 ± 4.30 59.40 ± 5.88 58.41 ± 3.63	1440 ± 96	63.8 ± 4.3
50 % Ethanol	10 100 1000	52.36 ± 13.91 60.65 ± 9.23 68.71 ± 2.44	2030 ± 201	306.1 ± 30.3
100 % Aqueous	10 100 1000	6.37 ± 7.97 -20.82 ± 8.06 84.52 ± 1.49	1277 ± 20	199.6 ± 3.254
Boiled Water	10 100 1000	45.54 ± 1.57 12.74 ± 8.35 63.76 ± 0.69	1455 ± 170	259.4 ± 30.3
Oil	10 100 1000	69.65 ± 3.22 76.55 ± 2.73 69.71 ± 5.31	1086 ± 276	2.3 ± 0.6
Aspirin	18	45.47 ± 5.56	-	-
Green Tea Extract	1 μM	-	7108 ± 434	592 ± 36

All extracts inhibited PGE_2 to some extent, between 6 to 84% (Table 4.1). The 50% aqueous ethanol extract and oil inhibited PGE_2 production to the greatest extent, with the exception of the highest concentration of boiled water extract. Dose responses were poor with many extracts, particularly the oil, and it is likely that this is due to mixed modes of action. Better dose responses were observed for pure compounds. Aspirin was

found to inhibit PGE_2 production by approximately 45% at 18 μg/mL, and it was found that both of the ethanolic extracts and the essential oil exhibited greater inhibition than aspirin 100 μg/mL.

The antioxidant activity was tested using the ORAC (oxygen radical absorbance capacity) method. All extracts displayed antioxidant activity, with the 50% ethanol extract being the most active (Table 4.1). This extract is considered to have the broadest representation of the chemical profile. The antioxidant capacity was also highest for the 50% ethanol extract, when expressed as antioxidant activity per gram of plant tissue (sample), indicating that this solvent extracted the greatest proportion of antioxidants, per gram of plant tissue.

4.2.2 Mode of Anti-inflammatory Action

4.2.2.1 Inhibition of Cyclooxygenase Gene Expression

A 50% aqueous ethanol *Centipeda cunninghamii* extract did not considerably influence COX-2 gene expression (Figure 4.3). The positive control, dexamethasone was found to inhibit COX-2 expression by approximately 60%, compared to stimulated control cells (untreated cells). The scatter plots of the highest concentration of the extract tested (1000 μg/mL) were rendered uninterpretable due to interference of the flow cytometry scatter plots.

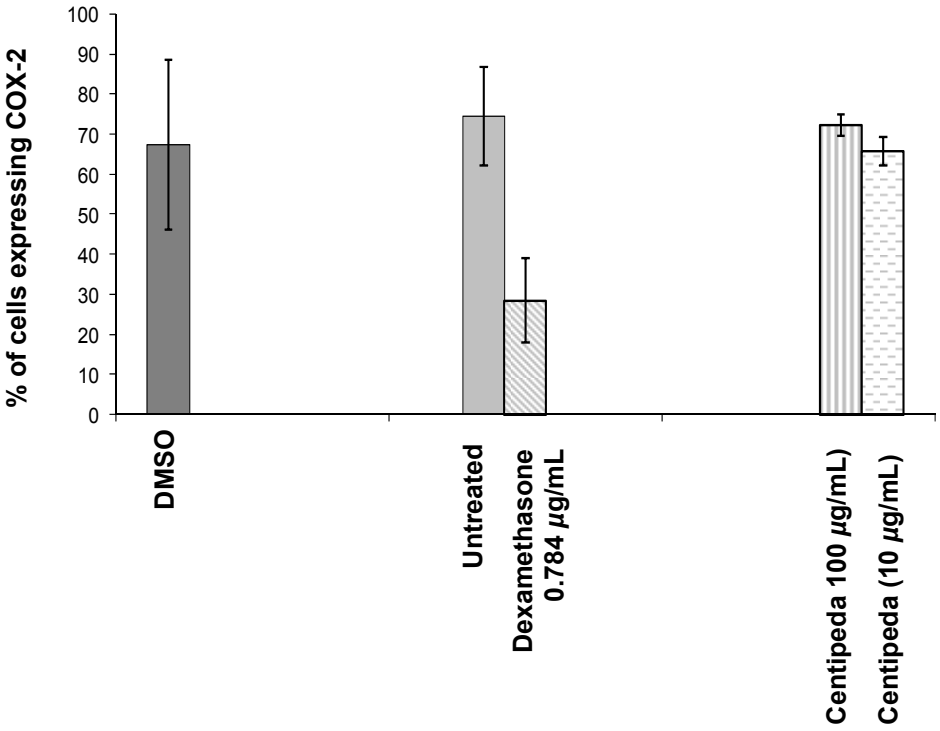

Figure 4.3. Influence of 50% aqueous ethanol *Centipeda cunninghamii* extract on COX-2 expression. Values are mean ± SEM, n=2.

4.2.2.2 Inhibition of Cyclooxygenase Enzyme Activity

At the highest concentration (1000 μg/mL) the 50% aqueous ethanol *Centipeda cunninghamii* extract inhibited both COX-1 and COX-2 activity, to values comparable to ibuprofen (COX-1) and celebrex (COX-2) (Figure 4.4). The biological relevance of such a high concentration requires further investigation. At a low concentration (100 μg/mL) the extract exhibited some selectivity towards COX-2 inhibition.

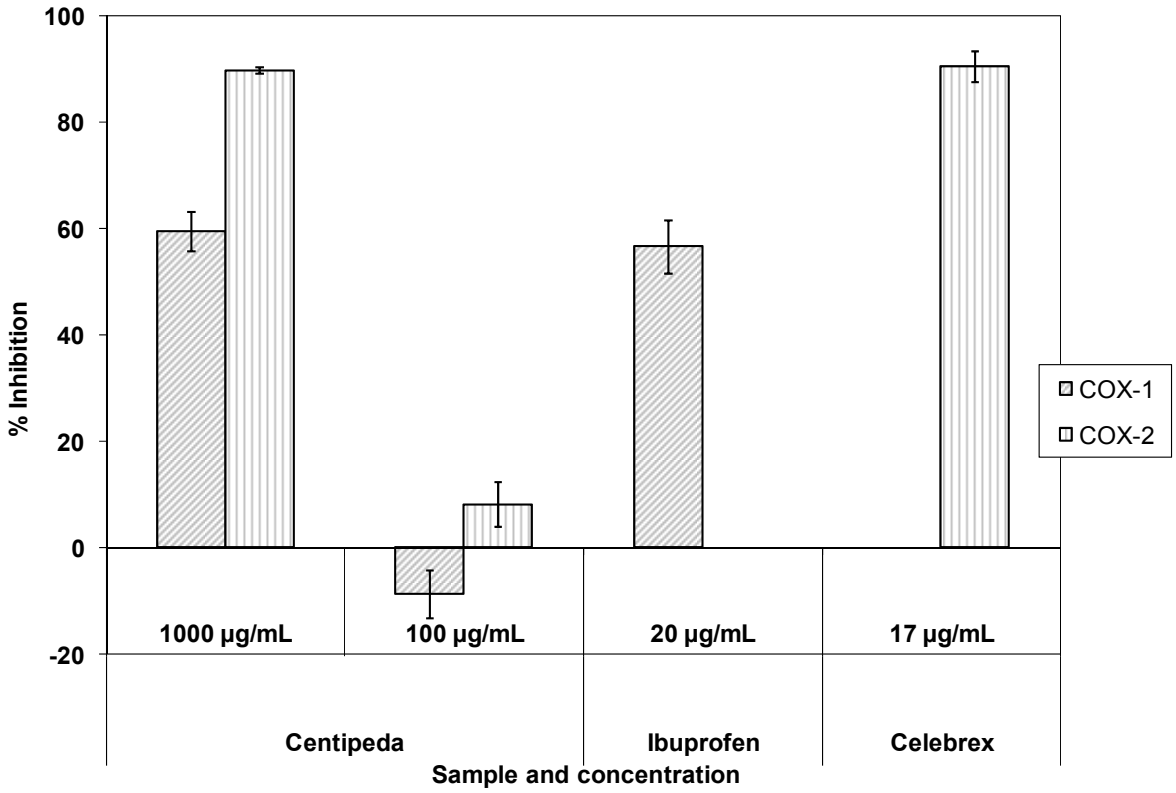

Figure 4.4. Inhibition of COX-1 and COX-2 activity by a 50% aqueous ethanol *Centipeda cunninghamii* extract. Values are mean ± SEM, n=3.

4.2.2.3 Inhibition of Lipoxygenase Enzyme Activity

The 50% aqueous ethanol *Centipeda cunninghamii* extract did not inhibit lipoxygenase (5-LO) activity and at the higher concentrations (100 and 1000 μg/mL) the extract appeared to slightly promote 5-LO activity (Figure 4.5). Nordihydroguaiaretic acid (NGDA) was included as a positive control, at a low concentration NGDA inhibited LO activity by approximately 100%. This result suggests that the anti-inflammatory activity associated with *Centipeda cunninghamii* is due to its influence on the cyclooxygenase inflammatory pathway, not the lipoxygenase inflammatory pathway.

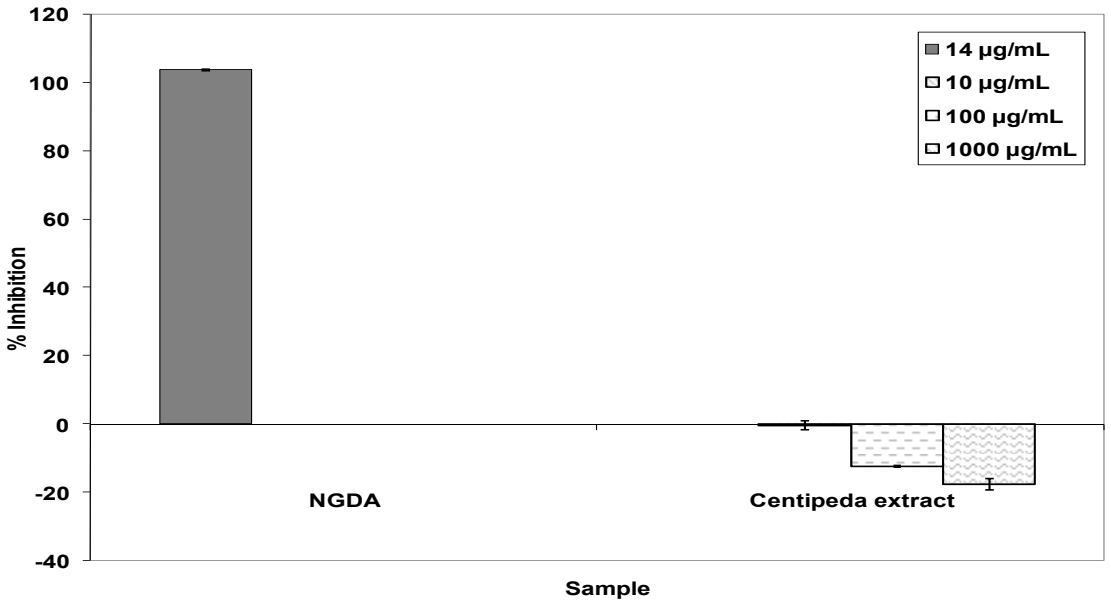

Figure 4.5. Inhibition of 5-lipoxygenase activity by a 50% aqueous ethanol *Centipeda cunninghamii* extract. Values are mean ± SEM, n=3.

4.2.2.4 Inhibition of Tumor Necrosis Factor-*α* Production

RAW 264 cells were stimulated with LPS to induce the production of tumor necrosis factor-*α* (TNF-*α*). The addition of 50% aqueous ethanol *Centipeda cunninghamii* extract (130 *μ*g/mL) to the culture medium considerably inhibited the production of TNF-*α*, reducing production by approximately 33% (Figure 4.6). At lower concentrations (13 and 1.3 *μ*g/mL) the extract was not as efficacious. At 16 *μ*g/mL aspirin inhibited TNF-*α* production by approximately 7%.

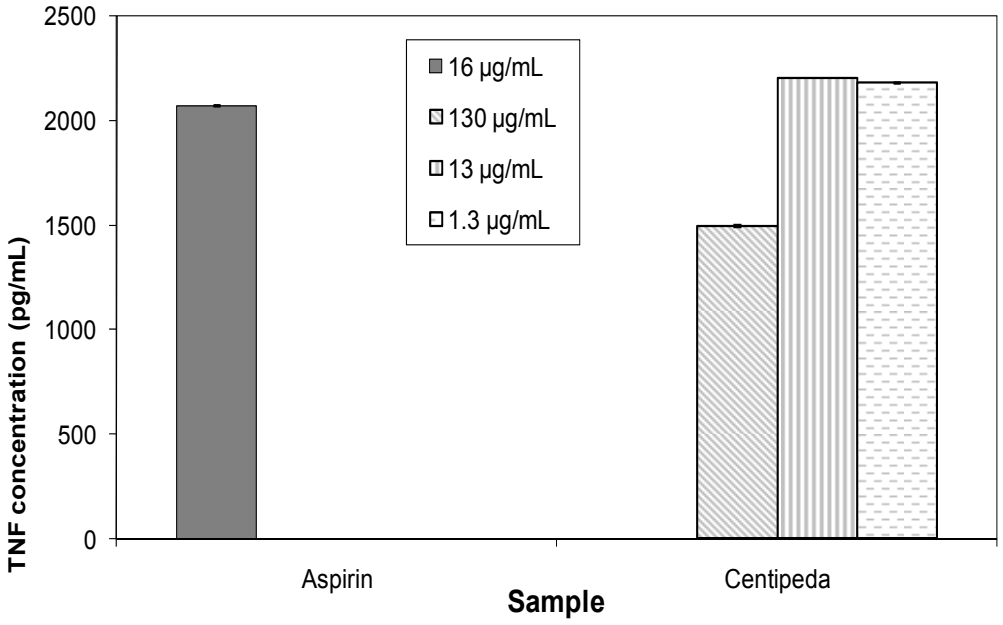

Figure 4.6. The effect of a 50% aqueous ethanol *Centipeda cunninghamii* extract on the production of tumor necrosis factor-*α* by stimulated RAW 264 cells. Values are mean ± SEM, n=3.

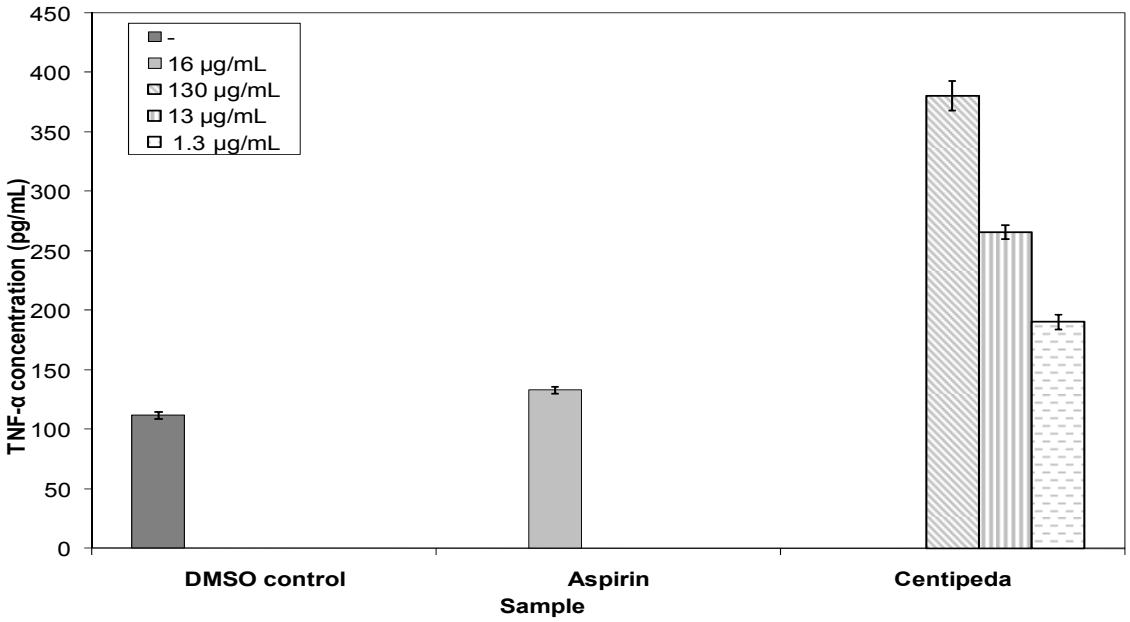

Figure 4.7. The effect of a 50% aqueous ethanol *Centipeda cunninghamii* extract on the production of tumor necrosis factor-*α* by unstimulated RAW 264 cells. Values are mean ± SEM, n=3.

The influence of the 50% aqueous ethanol extract of *C. cunninghamii* on RAW 264 cells not stimulated with LPS was also tested (Figure 4.7). At all concentrations tested the *C. cunninghamii* extract stimulated TNF-*α* production compared to the solvent control (DMSO), and aspirin. This indicated that extracts of *C. cunninghamii* have a stimulatory action in the case of unstimulated cells whereas, in the case of stimulated cells, an inhibitory action was observed. It is noted that the stimulatory action of *C. cunninghamii* (400 *p*g/mL) is considerably less than that of LPS (2000 *p*g/mL). Making inferences from the stimulatory action observed from the crude extract is premature given the inherent complexity of the crude extract. Evaluation of the TNF-*α* activity of the purified compounds would give a clearer understanding of the mode/s of anti-inflammatory action.

4.2.2.5 Inhibition of Nitric Oxide Production

The stimulation of RAW 264 cells with LPS also induces the production of nitric oxide (NO). The addition of a 50% aqueous ethanol extracts of *C. cunninghamii* to stimulated cells inhibited the production of NO, at all concentrations tested (Figure 4.8). At 130 μg/mL, the *C. cunninghamii* extract inhibited NO production by approximately 100%. Whilst increased NO can contribute to disease states, NO has other biological functions; including its' action as a signalling molecule. Therefore, complete inhibition of NO may not be desirable and a more moderate response, such as that achieved with *C. cunninghamii* at 13 and 1.3 μg/mL may be of greater clinical relevance.

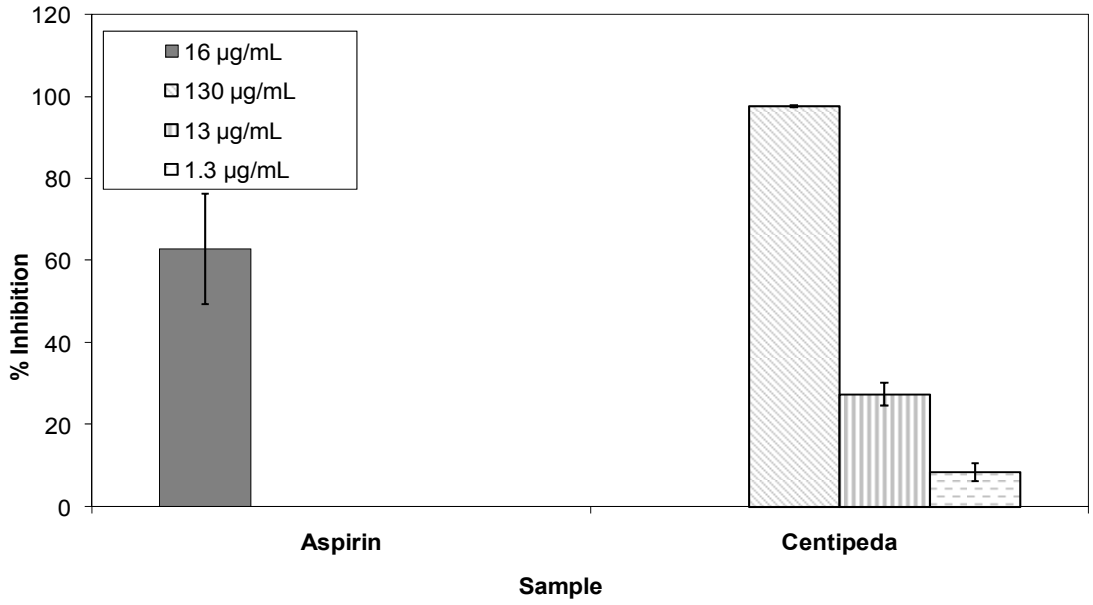

Figure 4.8. The effect of a 50% aqueous ethanol *Centipeda cunninghamii* extract on the production of nitric oxide by stimulated RAW 264 cells. Values are mean ± SEM, n=3.

The influence of the *C. cunninghamii* extract on unstimulated RAW 264 cells was also tested, and it did not stimulate NO production as compared to the DMSO control or aspirin (Figure 4.9).

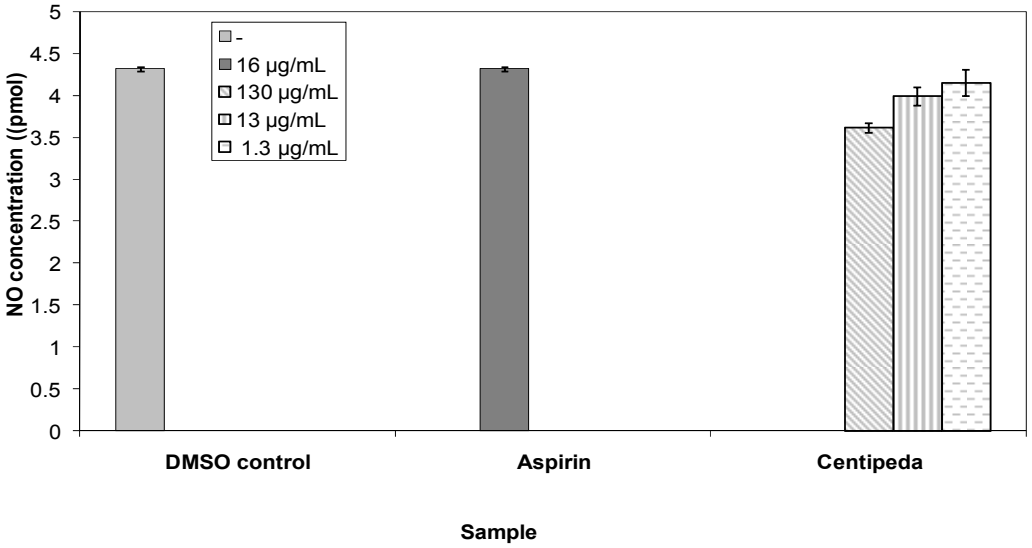

Figure 4.9. The effect of a 50% aqueous ethanol *Centipeda cunninghamii* extract on the production of nitric oxide by unstimulated RAW 264 cells. Values are mean ± SEM, n=3.

4.2.3 Anti-inflammatory & Antioxidant activity of *C. cunninghamii* Fractions

For the purpose of bioassay work the most active extract, derived from 50% aqueous ethanol was fractionated into four fractions using a solid phase extraction technique. Four C18-SPE fractions were generated by eluting with a water/acetonitrile gradient. The mobile phase, volume of eluting solvent and the yield for each fraction is listed in Table 4.2. A sample of the whole extract, together with the four SPE fractions was submitted for bioassay. The fractions were analysed by LC-MS prior to bioassay work.

The four SPE fractions were tested against the ORAC and PGE_2 bioassays. The inhibition of PGE_2, and antioxidant activity of the 4 fractions is presented in Table 4.3.

Table 4.2. Solid phase extraction of *C. cunninghamii* extract; eluent, volumes used and yields for each fraction.

Fraction	Solvent	Volume	Yield (g)
1	100% MQ Water	60 mL	0.306
2	20% ACN/Water	60 mL	0.113
3	40% ACN/Water	60 mL	0.030
4	100% ACN	60 mL	0.071

Table 4.3. Anti-inflammatory and antioxidant capacity of *Centipeda cunninghamii* samples and SPE fractions.

	% inhibition of PGE_2		**Antioxidant Capacity**	
Extract/Fraction	**Sample Concentration (μg/mL)**	**Average Inhibition ± SEM**	**μmol TE / g extract ± SEM**	**μmol TE / g sample ± SEM**
Aspirin	18	26.42 ± 5.32	-	-
Green Tea Extract			7108 ± 434	592 ± 36
SPE F1	10 100 1000	-31.82 ± 5.00 -33.33 ± 11.50 -51.70 ± 3.22	676 ± 39	413.7 ± 23.9
SPE F2	10 100 1000	-38.24 ± 2.31 -38.11 ± 6.85 0.63 ± 3.67	6411 ± 351	1455.3 ± 79.7
SPE F3	10 100 1000	-42.39 ± 5.52 18.74 ± 0.33 34.59 ± 4.70	3382 ± 678	202.9 ± 40.7
SPE F4	10 100 1000	-31.19 ± 0.50 38.11 ± 0.87 29.31 ± 6.17	694 ± 159	99.2 ± 22.7

The results suggest that SPE fractions 3 and 4 contain constituents that act to inhibit PGE_2 production (Table 4.3). Fractions 1 and 2 exhibit little or no influence on PGE_2 production. Fractions 2 and 3 exhibit the greatest antioxidant capacity, and represent ~23% and 6% of the crude extract, respectively. Fraction SPE F1 did not inhibit PGE_2 production or exhibit significant antioxidant capacity, even though its mass accounted for 61% of the extract. SPE fractions 2, 3, and 4 exhibited antioxidant activity, and SPE fractions 3 and 4 inhibited PGE_2 production. It was therefore decided that sub-fractionation of these fractions and re-testing in the bioassays would be of most value.

4.2.4 Anti-inflammatory and Antioxidant Activity of *C. cunninghamii* Sub-fractions

Approximately 3.0 grams of the crude dried extract of *C. cunninghamii* was fractionated by RP SPE in the usual way (Section 2.3.2). The resulting SPE fractions were sub-fractionated by RP prep-HPLC. SPE fraction 2 was divided into ten fractions, SPE fraction 3 was divided into seven fractions and SPE fraction 4 was divided into ten fractions. The prep-HPLC runs were repeated between 10-14 times each to obtain sufficient material for the antioxidant and anti-inflammatory assays. The relevant prep-HPLC fractions were pooled and then dried using a rotary-evaporator and subsequently freeze dried to remove all traces of solvent prior to bioassay.

The 27 sub-fractions were tested against the ORAC and PGE_2 bioassays. The inhibition of PGE_2, and antioxidant activity of these fractions is presented in Tables 4.4–4.6. Sub-fractions F18-F20 were not submitted for bioassay because they were identical in composition, by LC-MS, to sub-fractions F21-F23.

Table 4.4. Anti-inflammatory and antioxidant capacity of *Centipeda cunninghamii* sub-fractions generated from SPE fraction 2

	% inhibition of PGE_2		**Antioxidant Capacity**
Sub-fraction	**Concentration (μg/mL)**	**Average Inhibition ± SEM**	**μmol TE / g fraction ± SEM**
SPE F2 - F1	100 1000	-0.00 ± 4.76 -12.08 ± 5.02	279 ± 47
SPE F2 - F2	100 1000	-2.97 ± 5.32 10.89 ± 0.34	4215 ± 371
SPE F2 - F3	100 1000	1.58 ± 3.37 5.74 ± 6.49	3295 ± 8
SPE F2 - F4	100 1000	-18.42 ± 2.92 51.88 ± 2.93	1922 ± 300
SPE F2 - F5	100 1000	-15.05 ± 1.05 35.05 ± 3.57	2334 ± 111
SPE F2 - F6	100 1000	6.34 ± 2.23 44.16 ± 2.09	3692 ± 241
SPE F2 - F7	100 1000	7.13 ± 3.50 -17.62 ± 3.56	6510 ± 809
SPE F2 - F8	100 1000	9.11 ± 2.47 2.18 ± 1.76	10151 ± 699
SPE F2 - F9	100 1000	-2.18 ± 3.14 21.19 ± 2.77	7699 ± 561
SPE F2 - F10	100 1000	-16.44 ± 6.79 15.45 ± 2.53	4608 ± 767
Aspirin	18 μg/mL	44.16 ± 3.27	-
Green Tea Extract		-	6406 ± 498

Table 4.5. Anti-inflammatory and antioxidant capacity of *Centipeda cunninghamii* sub-fractions generated from SPE fraction 3.

	% inhibition of PGE_2		**Antioxidant Capacity**
Fraction	**Concentration (μg/mL)**	**Average Inhibition ± SEM**	**μmol TE / g fraction ± SEM**
SPE F3 - F11	100 1000	-77.19 ± 11.05 69.39 ± 8.80	4186 ± 287
SPE F3 - F12	100 1000	9.28 ± 14.82 71.47 ± 5.57	8520 ± 587
SPE F3 - F13	100 1000	7.02 ± 4.02 61.32 ± 1.50	5588 ± 694
SPE F3 - F14	100 1000	3.85 ± 10.15 61.55 ± 1.69	3139 ± 502
SPE F3 - F15	100 1000	29.74 ± 12.60 72.19 ± 3.85	2177 ± 328
SPE F3 - F16	100 1000	57.62 ± 2.47 78.97 ± 1.41	4172 ± 499
SPE F3 - F17	100 1000	70.56 ± 1.71 66.64 ± 5.85	1430 ± 282
Aspirin	18 μg/mL	63.99 ± 8.55	-
Green Tea Extract		-	3892 ± 580

Table 4.6. Anti-inflammatory and antioxidant capacity of *Centipeda cunninghamii* sub-fractions generated from SPE fraction 4.

	% inhibition of PGE_2		**Antioxidant Capacity**
Fraction	**Concentration (μg/mL)**	**Average Inhibition ± SEM**	**μmol TE / g fraction ± SEM**
SPE F4 - F21	100 1000	81.00 ± 4.60 81.26 ± 3.24	4936 ± 481
SPE F4 - F22	100 1000	85.23 ± 6.17 83.84 ± 2.49	931 ± 26
SPE F4 - F23	100 1000	61.42 ± 3.00 76.13 ± 2.62	145 ± 17
SPE F4 - F24	100 1000	76.53 ± 2.88 78.61 ± 0.68	226 ± 20
SPE F4 - F25	100 1000	76.28 ± 2.86 76.73 ± 0.70	277 ± 14
SPE F4 - F26	100 1000	57.51 ± 5.96 78.77 ± 0.74	211 ± 12
SPE F4 - F27	100 1000	73.45 ± 3.24 75.12 ± 3.77	2186 ± 507
SPE F4 - F28	100 1000	74.74 ± 1.44 80.16 ± 2.65	1509 ± 545
SPE F4 - F29	100 1000	73.95 ± 2.29 82.80 ± 2.59	600 ± 80
SPE F4 - F30	100 1000	63.24 ± 1.81 71.38 ± 3.88	133 ± 11
Aspirin	18 μg/mL	66.95 ± 7.14	-
Green Tea Extract		-	5282 ± 426

Most sub-fractions demonstrated biological activity. Several fractions exhibited both anti-inflammatory and antioxidant activity.

Sub-fractions F4, F6, F11-F17 and F21-F30 demonstrated reasonable anti-inflammatory activity at the highest concentration tested (1000 μg/mL), inhibiting PGE_2 production comparable to, or better than, aspirin (Tables 4.4-4.6). It should be noted however, that these sub-fractions produced cloudy suspensions at high concentrations when dispensed in the aqueous medium required by the 3T3 cells in the PGE_2 assay. This suggests that the inhibition may have been greater if the samples were able to be completely dissolved at this concentration. The biological relevance of this high concentration is uncertain. More insight was obtained at the lower concentration tested (100 μg/mL). At this concentration, only sub-fractions F21-F30 and sub-fractions F15-F17 demonstrated considerable anti-inflammatory activity.

Sub-fractions F7, F8, F9, F11-F13, F16 and F21 demonstrated considerable antioxidant activity, with ORAC values ranging from 4172 to 10151 μmol TE/g of fraction.

From these results and considering solubility issues, cost and time constraints, it was difficult to prioritize which sub-fractions warranted further investigation since most demonstrated anti-inflammatory and/or antioxidant activity (Figures 4.10 and 4.11).

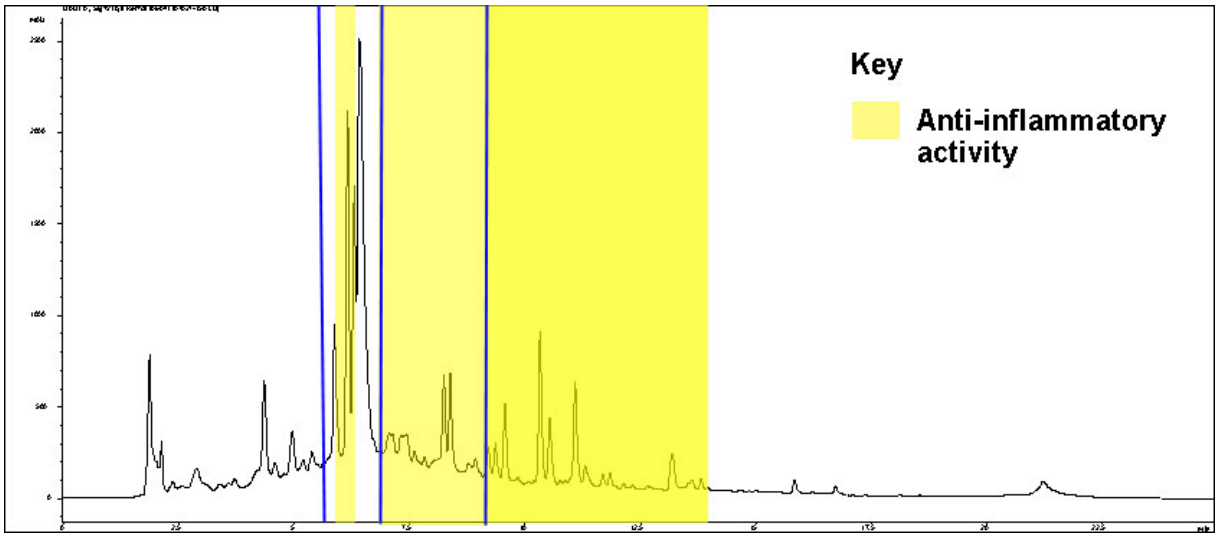

Figure 4.10. The UV-Vis 210 nm chromatogram of the fresh 50 % aqueous ethanol extract of *C. cunninghamii* showing SPE fractionation (blue lines) and location of anti-inflammatory activity (yellow zones derived from sub-fractions F1-F30).

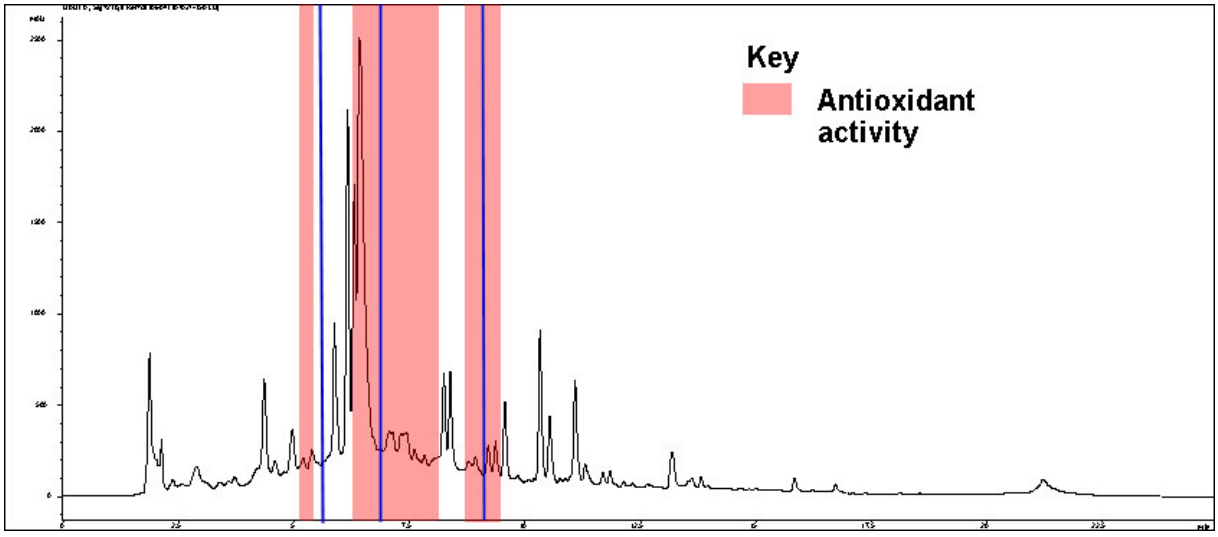

Figure 4.11. The UV-Vis 210 nm chromatogram of the fresh 50 % aqueous ethanol extract of *C. cunninghamii* showing SPE fractionation (blue lines) and location of antioxidant activity (pink zones derived from sub-fractions F1-F30).

The chemical components of the stems and floral parts, and the anti-inflammatory activity contributed by the stems and floral parts were compared against the whole plant extract.

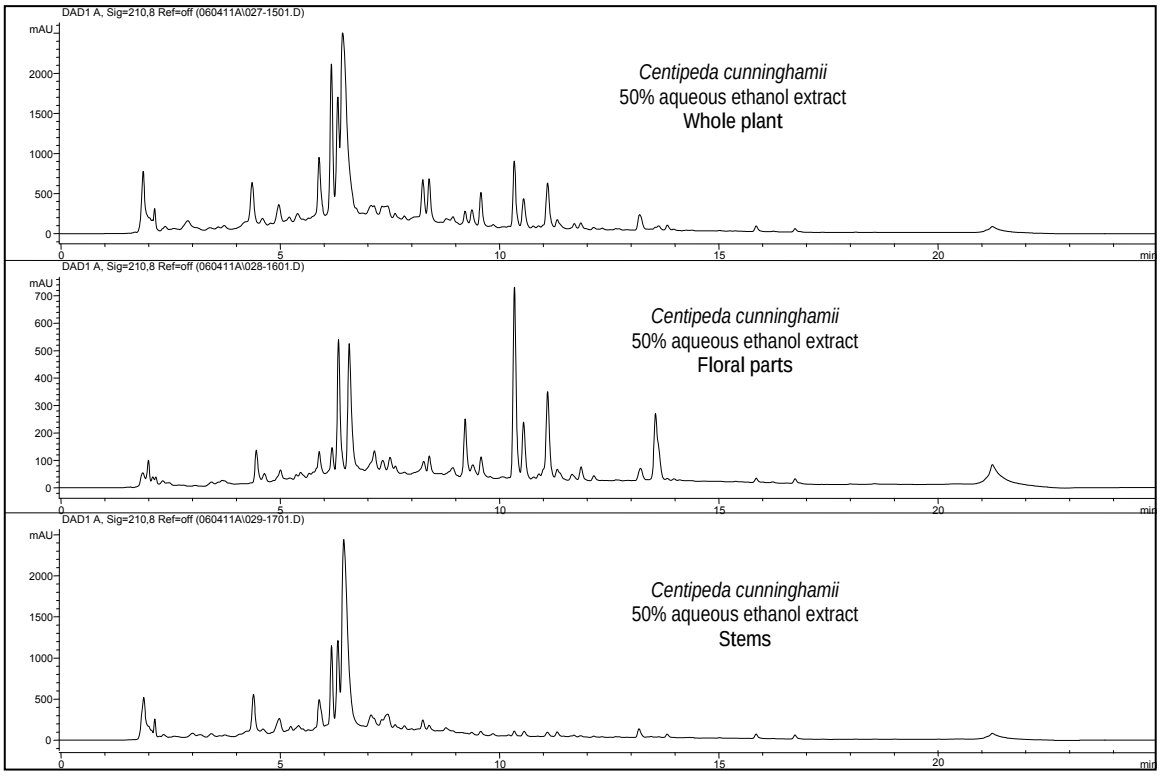

Figure 4.12. The UV-Vis 210 nm chromatogram of the fresh 50 % aqueous ethanol extracts of *C. cunninghamii* whole plant, flowers and stems.

Table 4.7. Inhibition of PGE_2 production by *Centipeda cunninghamii* extracts

Extract	**Concentration** (μg/mL)	**% Inhibition of PGE_2** average inhibition ± SEM
Flowers	10 100 1000	-7.96 ± 8.51 72.71 ± 2.26 73.93 ± 5.64
Stems	10 100 1000	-5.90 ± 10.46 85.72 ± 1.00 63.88 ± 1.38
Aspirin	18	45.47 ± 5.56

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It was anticipated that the biological activity of these extracts would be different, given their different chemical profiles (Figure 4.12), but this outcome was not evident as both samples inhibited PGE_2 production to a reasonable degree (Table 4.7). To pursue the bioactive compounds in an effective manner, it was rationalised that as many compounds as possible would be isolated from SPE fractions 2, 3 and 4 and submitted for bioassay.

4.2.5 Anti-inflammatory Compounds from *C. cunninghamii*

Each of the pure compounds isolated in this study were tested on the PGE_2 bioassay at 1000 μg/mL, however many of the compounds were insoluble, producing cloudy suspensions at this concentration, consequently invalidating the results. All of these compounds and fractions were retested at a much lower, soluble concentration (31.25 μg/mL), (Table 4.8).

A series of flavonoids were found to posses high anti-inflammatory activities. A series of fractions SPE4 F6 through to SPE4 F13 also exhibited strong activity. NMR studies have shown that these fractions contained sesquiterpenes, sterols and fatty acids.

Overall the results for the two concentrations were similar. The flavonoids have potent anti-inflammatory activity and significant activity was observed for the caffeic acids compounds , **6**, **7**, **8**, **14** and **15**.

Table 4.8. PGE_2 anti-inflammatory activity of *Centipeda cunninghamii* compounds and fractions.

Compound/Fraction	**31.25 μg/mL Average (± SD)**
Centipetin-3-glucoside (**10**)	14.0 (± 13.2)
Myriogenic acid (**6**)	19.5 (± 4.6)
Chlorogenic acid (**12**)	9.7 (± 19.2)
Chlorogenic acid methyl ester (**13**)	4.2 (± 22.4)
3,5-Di-*O*-caffeoylquinic acid (**14**)	9.6 (± 10.5)
Macroantoin G (**15**)	16.9 (± 1.4)
Myriogenic acid-7-methyl ester (**7**)	25.5 (± 13.0)
Myriogenic acid-1-methyl ester (**8**)	15.7 (± 13.4)
Myriogenic acid dimethyl ester (**9**)	-3.7 (± 9.0)
Axillarin (**16**)	49.2 (± 6.5)
Isokaempferide (**17**) and 4',5,7-Trihydroxy-3,6-dimethoxyflavone (**18**)	72.8 (± 2.0)
Jaceidin (**19**)	79.9 (± 3.3)
Arnicolide C (**20**)	2.1 (± 4.1)
SPE4 peak 2 (sesquiterpene lactone*)	-13.0 (± 1.0)
SPE4 peak 3 (sesquiterpene lactone*)	-7.7 (± 1.6)
SPE4 peak 4	-12.3 (± 5.2)
SPE4 peak 5	-37.2 (± 21.2)
SPE4 peak 6 (sesquiterpene lactone*)	-24.6 (± 43.6)
3-Hydroxykaura-9(11),16-diene-18-oic acid (**21**)	-43.5 (± 26.2)
8-Hydroxy-9,10-diisobutyryloxythymol (**23**)	40.2 (± 1.9)
SPE4 peak 9	-8.7 (± 1.5)
SPE4 peak 10 (sterol*)	9.2 (± 0.3)
SPE4 peak 11	-6.1 (± 2.7)
SPE4 peak 12 (fatty acid*)	25.4 (± 2.8)
SPE4 peak 13	36.7 (± 0.7)
Aspirin (100 μM)	43.0 (± 11.0)
100% Aq extract	37.8 (± 5.3)

* impure

Five of the compounds were examined in more detail to determine IC_{50} values (Table 4.9 and Figure 4.13). Compounds **6-10** were tested at concentrations of 0.2, 1.0, 5.0, 25.0 and 125 μg/ml. In most cases the compounds were found to inhibit PGE_2 production in a dose dependent manner (Figure 4.13).

Table 4.9. IC_{50} values for PGE_2 anti-inflammatory activity of selected compounds.

Compound	**IC_{50} (μM) (±SD)**	**% inhibition at 5 μg/mL (±SD)**
6	2.48 ± 0.83	67.38 ± 4.73
7	4.73 ± 0.93	73.50 ± 4.08
8	5.54 ± 3.78	45.00 ± 8.94
9	1.26 ± 3.25	73.95 ± 2.52
10	1.47 ± 0.75	52.20 ± 0.91

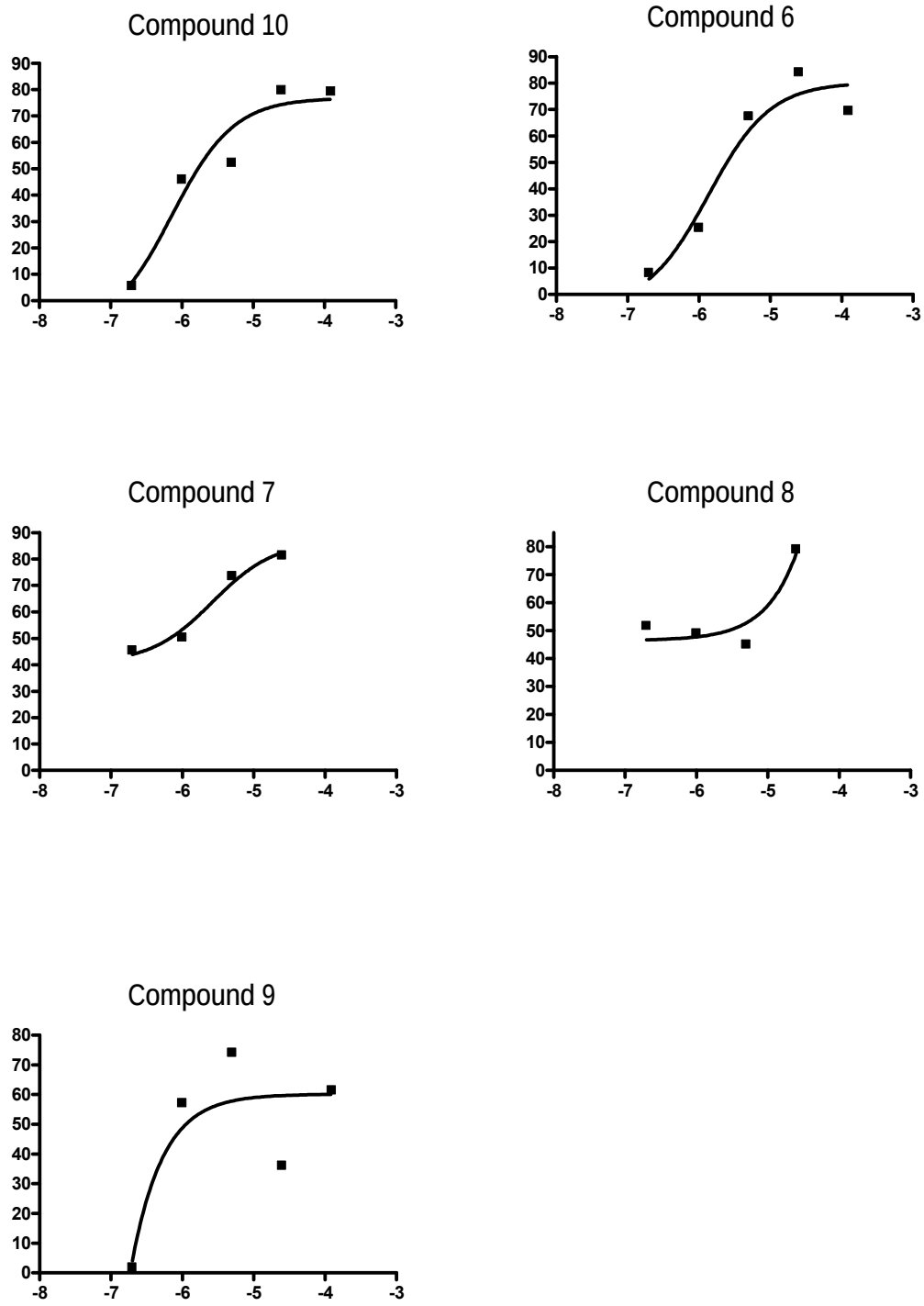


Figure 4.13. IC_{50} dose-response curves for selected compounds.
% Inhibition PGE_2 vs. Log concentration (μg/mL).

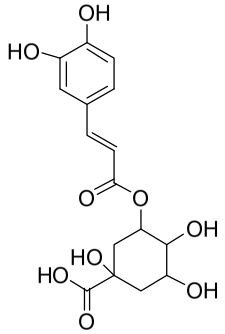
4.2.6 Antioxidant Compounds from *C. cunninghamii*

All of the pure compounds isolated in this study were evaluated for ORAC antioxidant capacity (Table 4.10).

Table 4.10. Antioxidant capacity of *Centipeda cunninghamii* compounds and fractions.

Compound/Fraction	**TE/g compound (± SD)**
Centipetin-3-glucoside (**10**)	12178 (± 942)
Myriogenic acid (**6**)	13781 (± 828)
Chlorogenic acid (**12**)	12077 (± 1698)
Chlorogenic acid methyl ester (**13**)	5558 (± 457)
3,5-Di-*O*-caffeoylquinic acid (**14**)	12290 (± 29)
Macroantoin G (**15**)	6337 (± 718)
Myriogenic acid-7-methyl ester (**7**)	8122 (± 272)
Myriogenic acid-1-methyl ester (**8**)	6822 (± 361)
Myriogenic acid dimethyl ester (**9**)	3957 (± 69)
Axillarin (**16**)	11512 (± 802)
Isokaempferide (**17**) and 4',5,7-Trihydroxy-3,6-dimethoxyflavone (**18**)	15163 (± 925)
Jaceidin (**19**)	7349 (± 306)
Arnicolide C (**20**)	22 (± 2)
SPE4 peak 2 (sesquiterpene lactone*)	211 (± 1)
SPE4 peak 3 (sesquiterpene lactone*)	198 (± 14)
SPE4 peak 4	2772 (± 904)
SPE4 peak 5	1186 (± 347)
SPE4 peak 6 (sesquiterpene lactone*)	566 (± 134)
3-Hydroxykaura-9(11),16-diene-18-oic acid (**21**)	706 (± 227)
8-Hydroxy-9,10-diisobutyryloxythymol (**23**)	4682 (± 277)
SPE4 peak 9	213 (± 48)
SPE4 peak 10 (sterols*)	2492 (± 346)
SPE4 peak 11	898 (± 215)
SPE4 peak 12 (fatty acid*)	107 (± 39)
SPE4 peak 13	53 (± 7)
Green Tea Extract	7108

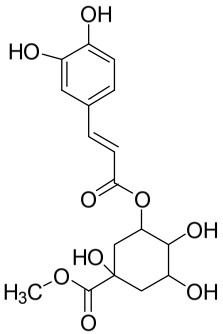
***** impure



Chlorogenic acid (**12**)

12077 T/E/g

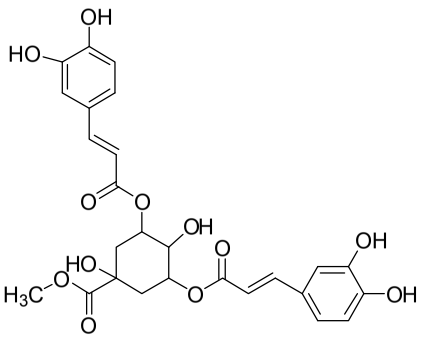
10% PGE_2 inh.



Chlorogenic acid methyl ester (**13**)

5558 T/E/g

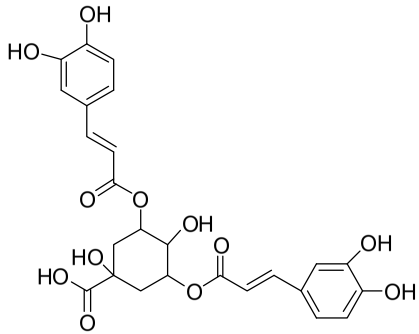
4% PGE_2 inh.



Macroantoin G (**15**)

6337 T/E/g

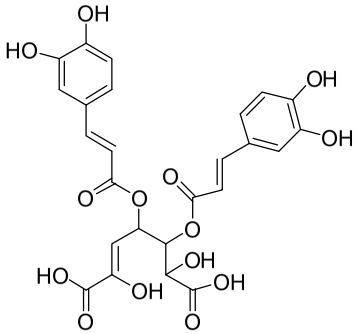
17% PGE_2 inh.



3,5-Di-*O*-caffeoylepiquinic acid (**14**)

12290 T/E/g

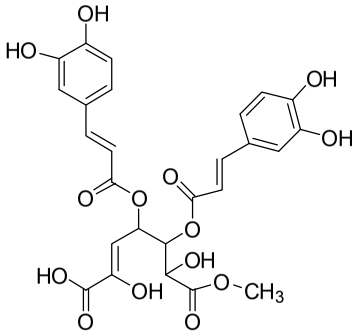
10% PGE_2 inh.



Myriogenic acid (**6**)

14 T/E/g

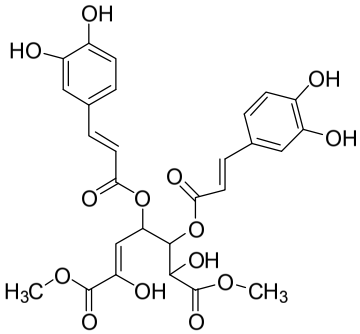
20% PGE_2 inh.



Myriogenic acid-7-methyl ester (**7**)

8122 T/E/g

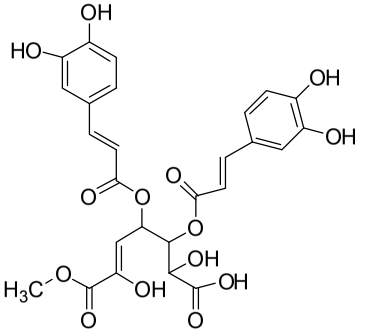
26% PGE_2 inh.



Myriogenic acid dimethyl ester (**9**)

3957 T/E/g

-4% PGE_2 inh.

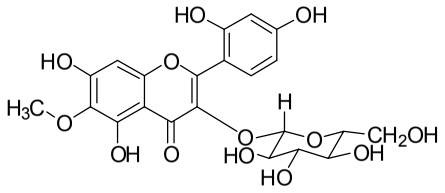


Myriogenic acid-1-methyl ester (**8**)

6822 T/E/g

16% PGE_2 inh.

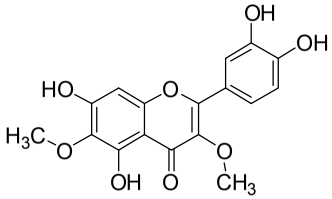
Figure 4.14. Anti-inflammatory and antioxidant capacity of caffeoylquinic acid compounds from *C. cunninghamii*.
% Inhibition PGE_2 at 31.25 μg/mL compared with aspirin control 50% Inh. (18 μg/mL).
ORAC values; Trolox equivalent per gram of compound, in comparison green tea extract 7108 TE/g.



Centipetin-3-glucoside (**10**)

12178 T/E/g

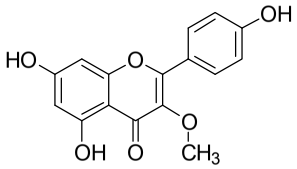
14% PGE_2 inh.



Axillarin (**16**)

11512 T/E/g

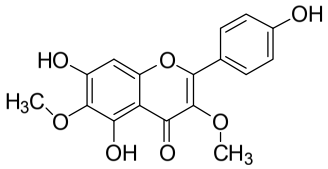
49% PGE_2 inh.



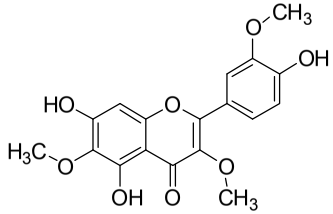
Isokaempferide (**17**)

17 & **18**
(as mixture)
15163 T/E/g

73% PGE_2 inh.



4',5,7-Trihydroxy-3,6-dimethoxyflavone (**18**)



Jaceidin (**19**)

7349 T/E/g

80% PGE_2 inh.

Figure 4.15. Anti-inflammatory and antioxidant capacity of flavonoid compounds from *C. cunninghamii*.
% Inhibition PGE_2 at 31.25 μg/mL compared with aspirin control 50% Inh. (18 μg/mL).
ORAC values; Trolox equivalent per gram of compound, in comparison green tea extract 7108 TE/g.

4.2.7 Discussion

The results from the PGE_2 assay on the pure compounds indicate that the most active compounds appear to be a series of flavonoids and a range of, as yet unidentified, sesquiterpenes, fatty acids and sterols. The flavonoids, compounds **10**, **16-19** and the caffeic acids compounds; **6-9** and **12-15** all exhibited very high antioxidant capacity. For comparison the antioxidant capacity of green tea extract was found to be 7108 TE/gram.

Phenolic compounds are reported to react with proteins and thus can interact with enzymes and the biological processes of cells (Harborne, 1998). Consequently, phenolic compounds are toxic to certain microorganism or animals, can inhibit their growth, or give unpleasant taste sensations to the taste buds of animals (Dey and Harborne, 1989). Phenolic compounds also constitute part of a plants chemical defence mechanism against pathogens and are located in almost every plant part including the roots, leaves, bark, wood and fruit (Cowan, 1999).

Antioxidant activity has been shown to correlate with the phenolic content of traditional Chinese medicinal herb extracts (Cai et al., 2004). The antioxidant capacity of phenolic compounds is essentially due to the ease with which a hydrogen atom from an aromatic hydroxyl group can be donated to a free radical (Duthie and Crozier, 2000). It is not unexpected that the caffeic acids have exhibited antioxidant activity *in vitro*. The anti-inflammatory activity of 3-*O*-caffeoylquinic acid (**12**) has been reported by Lin and co-workers (1999) and has been shown to potently enhance human mononuclear cell proliferation and interferon-γ production.

It was observed that the suite of flavonoids possesses both anti-inflammatory and antioxidant activity. Flavonoids are the major category of compounds reported from plant origin as COX inhibitors (Jachak, 2006). The anti-inflammatory activity of axillarin, (Moscatelli et al., 2006; Williams et al., 1999; Pelzer et al., 1998) 4',5,7-trihydroxy-3,6-dimethoxyflavone (Williams et al., 1999) and jaceidin (Williams et al., 1999) has been published previously.

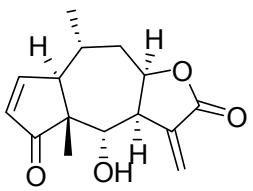
Flavonoids intrinsically exhibit antioxidant capacity. However; owing to their poor bioavailability the direct antioxidant value of dietary flavonoids has been questioned. Lotito and Frei (2007) have determined that the increase in serum antioxidant capacity is most likely is due to an endogenous response to these compounds. The antioxidant activity of axillarin (Kim et al., 2002) isokaempferide (Yoo et al., 2002; Jiang and Sun, 2004; El-Shamy et al., 2001; Cos et al., 2001) and jaceidin (Gil et al., 1999) has previously been reported in the literature.

It is likely both the phytosterols and the flavonoids impart anti-inflammatory properties to the extract (Lagarda et al., 2006). The thymol derivative 8-hydroxy-9,10-diisobutyryloxythymol (**23**), isolated from the ethanolic extract of the herb, also exhibited moderate PGE_2 inhibition.

The essential oil of *C. cunninghamii* which constituted 0.21% of the dried herb also demonstrated notable anti-inflammatory activity and moderate antioxidant activity. The chemical components of the oil were not isolated in this study. It is implied that several chemical constituents of the oil; chrysanthenyl acetate, *cis*-chrysanthenol, myrtenol,

myrtenyl acetate or thymol contribute antioxidant or anti-inflammatory activity, albeit at low levels, in the plant.

The arnicolides belong to the pseudoguiaiene class of sesquiterpene lactones and have been isolated from *Arnica* (Poplawski et al., 1971; Willuhn et al., 1983) and *Centipeda* species (Wu et al., 1991; Wu et al., 1985; Taylor and Towers, 1998; D'Amelio and Mirhom, 1998). D'Amelio and Mirhom (1998) suggest that the anti-inflammatory properties of Phytoplenolin®, an extract of *C. cunninghamii,* may be due in part to the sesquiterpene lactones and flavonoids. However, this assumption was not concluded in our investigation. Arnicolide and several fractions rich in sesquiterpene lactones (indicated by a characteristic fragment ion *m/z* 247^{+}) produced little or no PGE_2 inhibition. The sesquiterpene lactone helenalin (**28**) is closely related to arnicolide C (**20**) and is noted for its antineoplastic activity (Hertz et al., 1962), but has not been considered for clinical evaluation due to the prevailing toxicity of this class of compounds (Fischer, 1991). Plenolin (**29**), a sesquiterpene lactone constituent of *Centipeda minima* has also been noted for its toxicity (Buckingham et al., 2008). The potential toxicity of these compounds, enriched in non-polar extracts of the floral parts, requires consideration.



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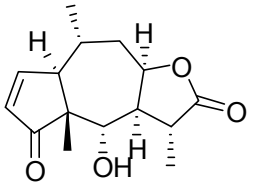

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4.3 Conclusion

The novel caffeic acids are significant antioxidant constituents that also contribute to the anti-inflammatory activity of *Centipeda cunninghamii* extracts.

Significant, additional activity was also observed in both assays for the suite of flavonoids that are concentrated in the floral parts of *C. cunninghamii*. A novel flavonoid glycoside from the herb possessed significant antioxidant activity. Whilst a strong patent position was obtained based on the efficacy of the novel compounds, known compounds in the extract also contribute significantly to the anti-inflammatory activity. The anti-inflammatory and/or antioxidant activity of these flavonoids has been reported in the literature.

Of particular interest in regards to the biological activity of *C. cunninghamii* was the finding that the 50% aqueous ethanol extracts posses an ability to influence several anti-inflammatory processes. This indicates the potential for *C. cunninghamii* to possess diverse modes of anti-inflammatory action.

The phytochemistry of the plant parts; namely the leaf, stem, flower and essential oil varies significantly with all parts conferring some biological activity to the extract. It is the current commercial practice to harvest the plant in flower. It was also noted that steeping the herb for several days at room temperature produced higher recoveries of compound **6** than sonication for 1 hour. The stability and storage of the extract is also a manufacturing concern, whereby degradation via hydrolysis of the caffeic acid compounds was observed to give rise to the caffeic acid ethyl ester (**11**). Fortuitously, the antioxidant capacity is retained. The stability, time of harvest and other agronomic

and post-harvest aspects will require careful appraisal to ensure good quality control in finished products.

4.3.1 Further Studies

Further work is required to understand the extent of biological activity associated with this extract. The anti-inflammatory activity of the non-polar metabolites, sterols, fatty acids and sesquiterpene lactones warrant further investigation. Triterpenoid saponins have also been reported from this genus (Gupta and Singh, 1989; 1990) and it is anticipated that these compounds would exhibit biological activity. The antioxidant and anti-inflammatory activity associated with the compounds present in the oil is also of interest.

The polar fractions from the stems of *C. cunninghamii* were found to be rich in phenolic compounds which are likely to impart microbicidal properties to the plant. The author is aware that the anti-fungal activity of extracts of the plant have been investigated by Hill (1997) and the antibacterial activity reported by Palombo and Semple (2001). The biological activities exhibited by extracts of *C. cunninghamii* are consistent with the ethnobotanical applications of the plant. Commercial applications, utilizing the antimicrobial properties of the herb, are worth pursuing.

Chapter 5

Phytochemical investigation of *Eremophila mitchellii*

5.1 Introduction

Eremophila mitchellii Benth. (Myoporaceae) is a shrub or small tree that occurs in arid inland areas of New South Wales and Queensland. It is known colloquially as bastard sandalwood, buddah, budtha, or native sandalwood and bears a profusion of white flowers in spring (Cunningham, 1992). Its timber is widely described as possessing a very strong scent that is reminiscent of sandalwood and historically its essential oil has been exploited commercially by the perfume industry (Bradfield 1932a; Low, 1990). "*E. mitchellii* is drought resistant, and capable of regenerating from the roots and recovers well after burning, ringbarking, or cutting, and is very difficult to eradicate" (Cunningham, 1992). *Eremophila mitchellii* is an invasive species and in some parts of

Australia it is considered a pest. It is not permitted to be cultivated in Western Australia.

Commercial interest in *E. mitchellii* arose due to reports that the timber, employed mainly for fence posts, was especially durable and resistant to termites (Cribb and Cribb, 1981). Preliminary work by Australian Phytochemicals Ltd. (APL) and the Centre for Plant and Food Sciences (CPAFS) at the University of Western Sydney had determined that the steam distilled oil and solvent extracts of the wood were toxic to termites (Leach et al., 2004). Based on these findings it was anticipated that the essential oil and its active ingredients may be utilized as termite control products.

The aims of this project were to undertake bio-assay guided fractionation to determine the chemical constituents of the timber that are responsible for the termite activity, to determine the toxicity of the oil and its components against a range of organisms and lastly to determine the yield, distribution, and chemical variation of the oil to assist with commercialisation of a wood oil product. This chapter encompasses the chemical investigation of *E. mitchellii* whilst the insecticidal aspects of *E mitchellii* are discussed in Chapter 6.

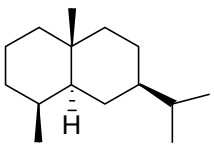
5.1.1 Literature Review

There are several reports on the biological activity and ethnopharmacology of *E. mitchellii* in the literature. The plant has been used by the aboriginal people for the treatment of rheumatism (Low, 1990). Kerr (1951) demonstrated that the wood oil was virtually non-toxic as a fly spray but when incorporated with the pyrethrins it had an adjuvant action. Kerr observed that the wood oil produced a marked increase in fly

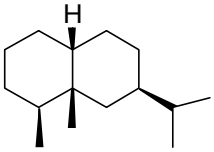
mortality in comparison to the pyrethrins alone. More recently Wilkinson and Cavanagh (2005) have reported on the antimicrobial activity of the wood oil against *C. albicans* and five different bacteria. The undiluted oil showed inhibition against *Escherichia coli*, *Staphylococcus aureus*, *Salmonella typhimurium*, *Alcaligenes faecalis* and *Candida albicans*, no inhibition was observed against *Pseudomonas aeruginosa*.

There are no published reports on the chemical composition of extracts of the leaf, root or branchlets from this plant. The eremophilones were first reported from the wood oil of *E. mitchellii* in 1932 by Bradfield and co-workers (1932a; 1932b). In 1955 Robinson suggested that the eremophilones were in fact the first of a new class of sesquiterpenes, the eremophilanes, and were not based on a eudesmane skeleton as first thought (Robinson 1955).

The elucidation of eremophilone (**30**) predated NMR spectroscopy and took almost 30 years to confirm the structure by chemical methods (Zalkow et al., 1959 and Zalkow et al., 1960). This was controversial at the time because it challenged Ruzicka's famous isoprene rule (Ruzicka, 1959) and demonstrated that methyl migrations were naturally possible in the biosynthesis of terpenoids. The first synthesis of eremophilone (**30**) was achieved by McMurry and co-workers (1975), whilst the stereoselective synthesis was reported by Ficini and Touzin (1977).



eudesmane skeleton



eremophilane skeleton

5.1.2 Eremophilane Biosynthesis

Farnesyl diphosphate gives rise to many mono, bi, and tricyclic sesquiterpene structures. Some 200 skeletal types of sesquiterpenes have been recorded (Harborne, 1998). *Eremophila* species are known to elaborate sesquiterpenes belonging to the bisabolane, eudesmane, eremophilane, spathulane, cadinane, zizaene and elemane classes (Ghisalberti, 1994b). The eremophilanes are a very rare class of bicyclic sesquiterpenes. The eremophilane sesquiterpenes have been demonstrated in *Aspergillus terrus* to arise biosynthetically from cyclization of 2*E*,6*E*-farnesyl pyrophosphate (Figure 5.1 and 5.2) (Dewick, 1997; Cane et al., 1990). Further cyclization via a germacratriene intermediate, followed by ring closure, proton shift and subsequent methyl migration gives rise to the eremophilanes. Unusual sesquiterpenes such as vetispiradiene are biosynthetically closely related to the eremophilanes.

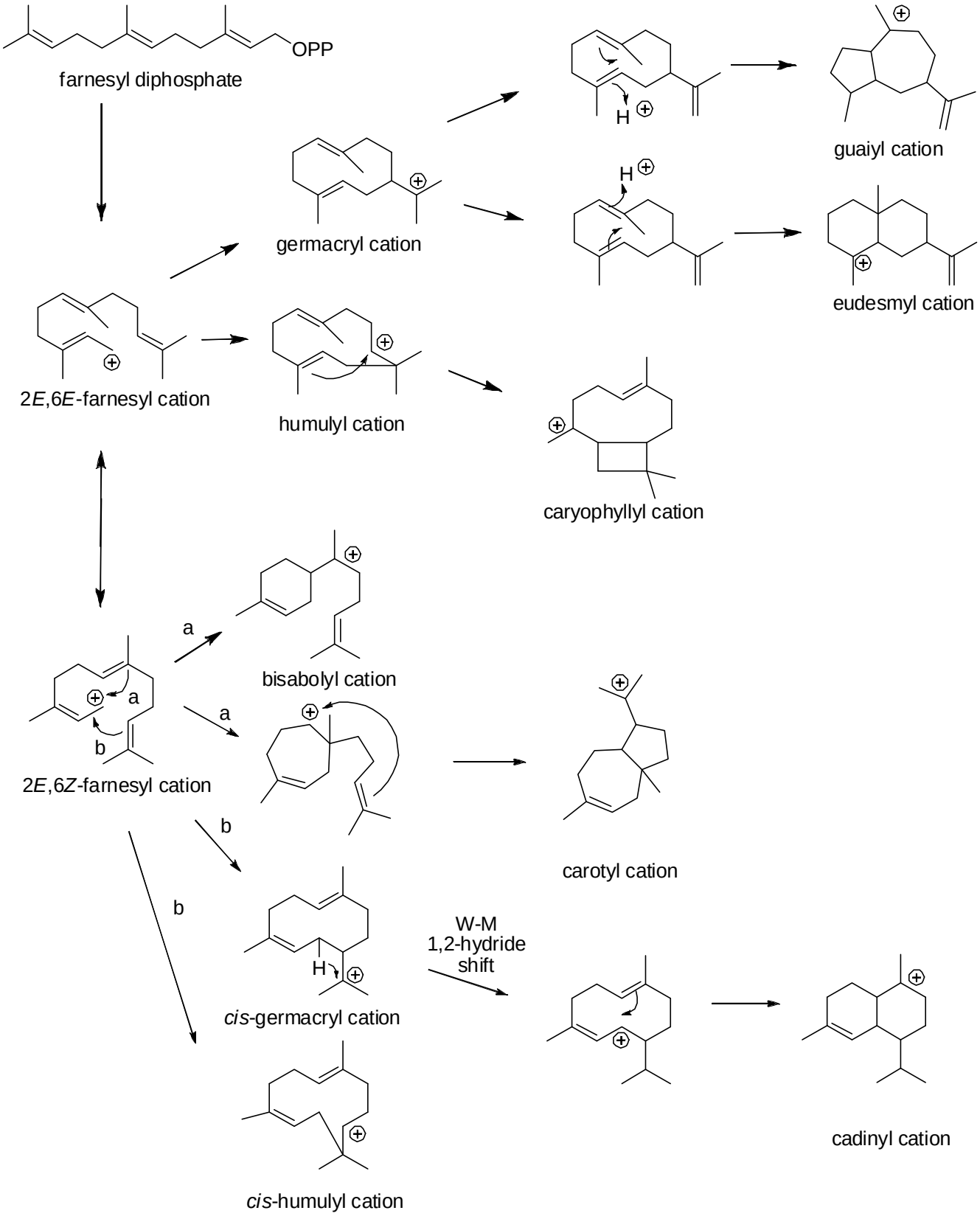


Figure 5.1. Biosynthesis of sesquiterpenes

(reproduced from Dewick, 1997).

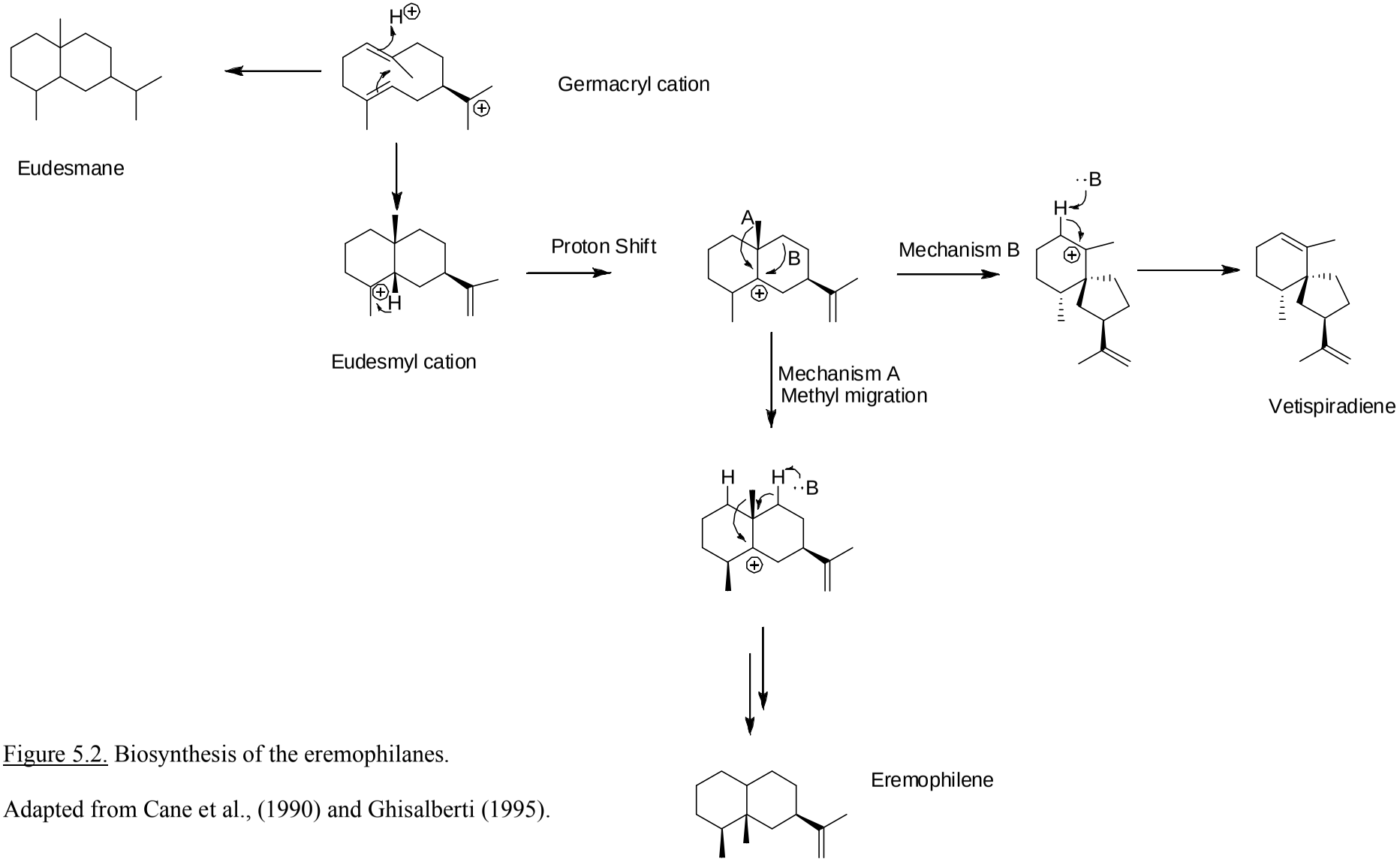
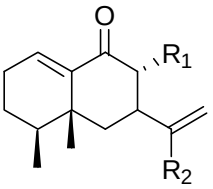


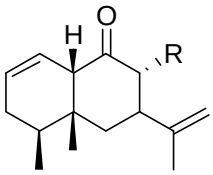
Figure 5.2. Biosynthesis of the eremophilanes.

Adapted from Cane et al., (1990) and Ghisalberti (1995).

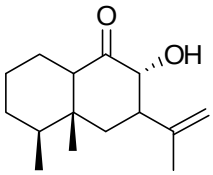
Since the discovery of eremophilone (**30**) and its oxygenated derivatives (**31**, **33**, **35** and **36**) only three additional eremophilanes, the keto aldehyde **32** and the dimers **38** and **39** have been reported from *Eremophila* despite intensive chemical investigation of the genus by Emilio Ghisalberti and co-workers (1994b). The significance of this finding is unclear considering that most of these investigations have focused on leaf extracts and not on the constituents of the wood or roots.



	R_1	R_2
30	H	Me
31	H	CHO
32	OH	Me



33 R = OH
34 R = H



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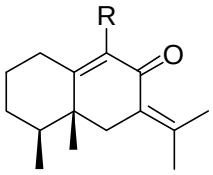
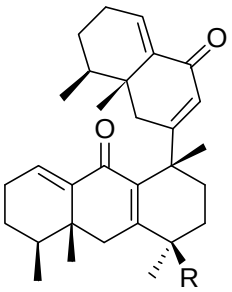


36 R = OH
37 R = H



38 R=H
39 R=OH

This chapter reports the investigation of the distribution, yield, and variation in the chemical composition of the oil in the whole plant and also describes the purification of the major constituents of the steam distilled wood and root oil of *E. mitchellii*. At present, the published NMR data for the eremophilanes is only of low resolution (50 MHz) and incomplete. Consequently, a comprehensive discussion of the NMR data has been reported here.

5.2 Results and Discussion

5.2.1 Distribution of the Oil, Yields and Chemical Composition

To assist with commercialisation of the oil, studies were undertaken to investigate the distribution and chemical variation of the oil in the plant. A whole tree was collected and each of the plant parts were steam distilled separately. The plant parts examined and the relevant yields of these oils are summarised in Figure 5.3. A cross section of the tree trunk revealed a pale yellow timber with a distinctive red-brown heartwood and coarse bark (Figure 5.3). No oil was obtained from distillations of the bark or outer light wood. The essential oils of the leaves, branchlets, wood and roots were analysed by GC-MS (Figure 5.4) More than thirty components have been identified in the essential oils and these are summarised in Table 5.1.

Compound identification was based on comparison with mass spectra and retention indices of authentic reference compounds, and the Adams, Wiley 275 and NIST 98 mass spectral libraries.

It is apparent from this series of chemical profiles that the yield and chemical composition varies greatly depending on the location in the plant. The leaf and branchlet oil are very complex. The leaf oil is chemically distinct from the wood and root oils whereas the branchlet oil exhibits a chemical composition that is intermediate between the leaf and the wood oil.

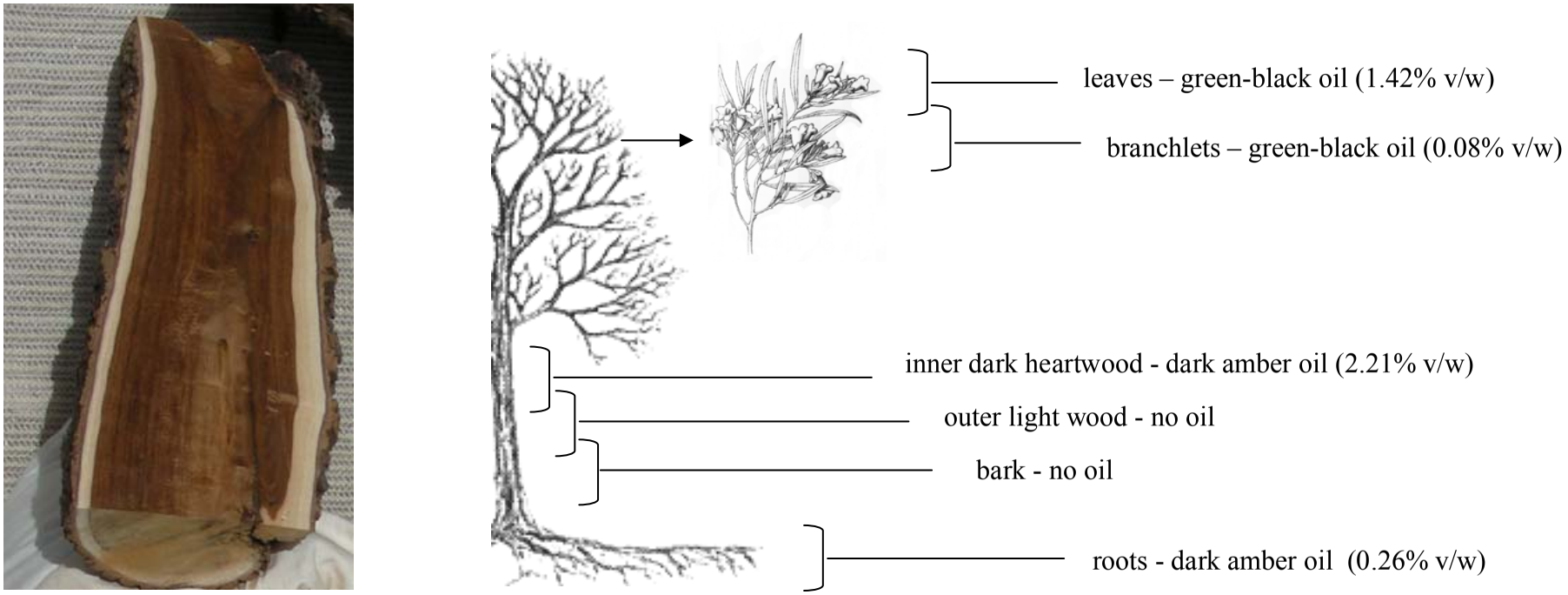


Figure 5.3. Cross section of *E. mitchellii* tree trunk and the distribution of oil in *E. mitchellii* plant parts.

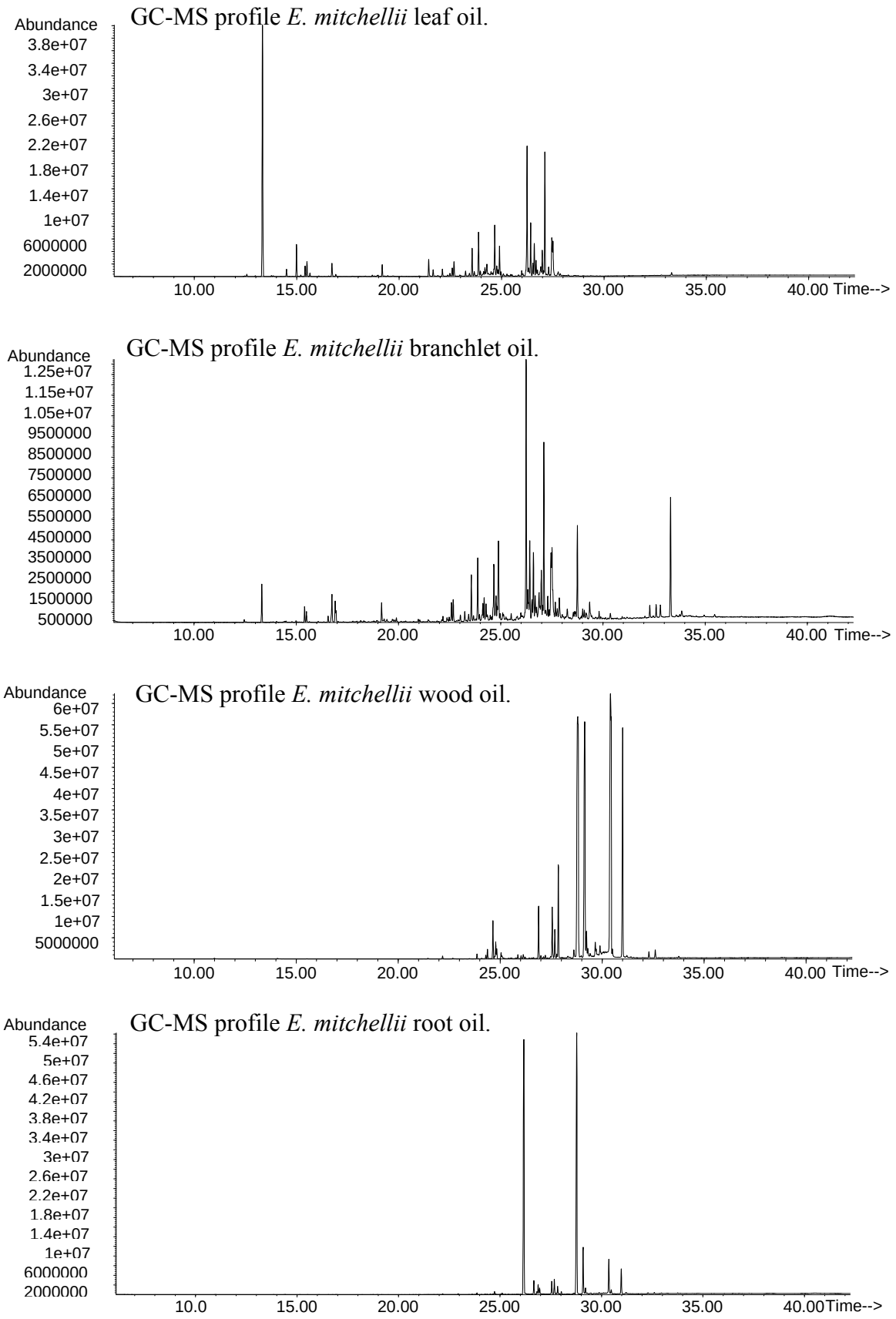


Figure 5.4. GC-MS profiles of steam distilled oil from different *E. mitchellii* plant parts.

Table 5.1. Composition of *Eremophila mitchellii* leaf, branchlet, wood and root essential oils.

Compound	RI P	RI A	Leaf	Branchlet	Wood	Root	Identification
			-				
α-pinene	953	824	22.84	1.84	-	-	RI, MS, RC
β-pinene	1000	846	0.55	tr	-	-	RI, MS, RC
α-phellandrene	1026	860	1.29	-	-	-	RI, MS, RC
p-cymene	1046	881	0.60	0.55	-	-	RI, MS, RC
limonene	1050	867	0.89	0.42	-	-	RI, MS, RC
β-phellandrene	1056	869	0.23	-	-	-	RI, MS, RC
$C_{10}H_{16}O$	1097	889	-	0.24	-	-	
α-terpinolene	1106	883	0.67	-	-	-	RI, MS, RC
linalool	1111	1606	tr	0.76	-	-	RI, MS, RC
$C_{10}H_{12}$	1115	928	0.13	0.70	-	-	
$C_{10}H_{14}O$	1218	1857	0.14	tr	-	-	
$C_{10}H_{16}O$	1225	1519	-	tr	-	-	
α-terpineol	1228	1701	0.65	0.69	-	-	RI, MS, RC
$C_{10}H_{16}O$	1232	nd	-	tr	-	-	
$C_{10}H_{18}O$	1265	932	-	tr	-	-	
$C_{10}H_{14}O$	1326	1855	-	tr	-	-	
$C_{15}H_{24}$	1362	1740	tr	-	-	-	
unknown	1384	1773	-	0.61	0.07	0.10	
eugenol	1387	2177	0.35	-	-	-	RI, MS, RC
$C_{15}H_{24}$	1406	947	0.18	0.17	-	-	
$C_{15}H_{24}$	1413	964	0.47	0.77	-	-	
β-elemene	1421	1592	0.83	0.88	-	-	RI, MS, RC
$C_{15}H_{24}$	1431	1505	-	-	-	0.10	

Table 5.1 continued. Composition of *Eremophila mitchellii* leaf, branchlet, wood and root essential oils.

Compound	RI P	RI A	Leaf	Branchlet	Wood	Root	Identification
$C_{15}H_{24}$	1436	959	-	0.34	-	-	
$C_{15}H_{24}$	1451	1531	0.30	-	-	-	
$C_{15}H_{24}$	1461	1544	0.22	tr	-	-	
trans-β-caryophyllene	1469	1599	1.77	0.21	-	-	RI, MS, RC
$C_{15}H_{24}$	1476	1602	0.28	0.23	-	-	
aromadendrene	1488	1609	2.95	2.77	-	-	RI, MS, RC
unknown	1490	1845	-	-	0.14	0.18	
$C_{15}H_{24}$	1494	1618	0.34	0.25	-	-	
$C_{15}H_{24}$	1503	1648	0.34	0.67	-	-	
$C_{15}H_{24}$	1507	1673	0.60	0.27	-	-	
alloaromadendrene	1513	1649	0.52	0.31	-	-	RI, MS, RC
$C_{15}H_{24}$	1516	1678	0.57	0.55	0.22	-	
$C_{15}H_{24}$	1519	1683	-	-	0.15	-	
$C_{15}H_{24}$	1521	1666	0.29	tr	-	-	
$C_{15}H_{24}$	1529	1671	0.28	-	-	-	
$C_{15}H_{24}$	1531	1712	0.20	-	-	-	
$C_{15}H_{24}$	1536	1718	tr	-	-	-	
$C_{15}H_{24}$	1537	1713	-	0.22	0.40	-	
viridiflorene	1539	1697	3.59	0.72	-	-	RI, MS, RC
unknown	1540	2002	-	-	-	0.36	
β-selinene	1544	1723	1.03	1.74	1.73	-	RI, MS, RC
α-selinene	1548	1723	0.90	2.50	0.77	-	RI, MS, RC
δ-cadinene	1557	1761	1.83	0.63	-	-	RI, MS, RC
$C_{15}H_{24}$	1560	1763	0.26	0.34	-	-	

Table 5.1 continued. Composition of *Eremophila mitchellii* leaf, branchlet, wood and root essential oils.

Compound	RI P	RI A	Leaf	Branchlet	Wood	Root	Identification
$C_{15}H_{22}$	1561	1727	-	-	0.45	-	
$C_{15}H_{22}$	1566	1829	-	-	0.28	0.15	
$C_{15}H_{22}$	1568	1838	0.26	0.50	-	-	
cadina-1,4-diene	1579	2073	0.15	-	-	-	
unknown	1582	1794	0.12	0.06	-	-	
elemol	1591	nd	0.26	-	-	-	
unknown	1593	1922	-	0.29	-	-	
$C_{15}H_{22}O$	1612	nd	-	-	0.18	-	
epiglobulol	1622	2020	0.38	0.25	-	-	RI, MS, RC
unknown	1624	nd	-	0.37	-	-	
unknown	1625	nd	-	0.46	-	-	
unknown	1629	nd	tr	0.96	-	-	
sesquithuriferone	1631	1946	-	-	-	42.61	RI, MS, ^{1}H, ^{13}C, 2DNMR
$C_{15}H_{26}O$	1632	2023	-	-	0.23	-	
$C_{15}H_{26}O$	1635	nd	0.42 sh	tr	-	-	
spathulenol	1639	2134	10.50	15.90	-	-	RI, MS, RC
$C_{15}H_{24}O$	1645	2128	1.21	2.59	-	-	
globulol	1650	2085	4.45	6.00	-	-	RI, MS, RC
unknown	1658	nd	0.88	-	-	-	
unknown	1659	nd	-	0.89	-	-	
viridiflorol	1661	2092	2.93	2.01	-	-	RI, MS, RC
unknown	1663	nd	-	2.27	-	-	
$C_{15}H_{24}O$	1664	2091	-	-		1.50	
$C_{15}H_{24}O$	1669	2114	1.26	2.00	-	-	
$C_{15}H_{24}O$	1672	2114	0.55	tr	-	-	

Table 5.1 continued. Composition of *Eremophila mitchellii* leaf, branchlet, wood and root essential oils.

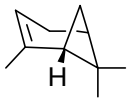
Compound	RI P	RI A	Leaf	Branchlet	Wood	Root	Identification
$C_{15}H_{26}O$	1674	2103	-	-	-	0.27	
$C_{15}H_{26}O$	1676	2066	-	-	0.20	-	
unknown	1678	nd	-	0.54	-	-	
$C_{15}H_{26}O$	1681	2146	-	1.76	1.88	0.98	
unknown	1685	nd	1.11	0.59	-	-	
$C_{15}H_{24}O$	1690	2123	2.33	0.61	-	-	
$C_{15}H_{24}O$	1694	2175	0.48	-	-	-	
unknown	1696	nd	tr	0.54	-	-	
$C_{15}H_{26}O$	1699	2194	10.16	12.35	-	-	
$C_{15}H_{26}O$	1713	nd	0.93	1.62	-	-	
α-eudesmol	1723	2207	~ 1.5	~ 3.7	-	-	RI, MS, RC
β-eudesmol	1725	2210	~ 2.8	~ 6.9			RI, MS, RC
$C_{15}H_{26}O$	1727	2203	-	-	2.25	1.35	
α-bisabolol	1730	2206	2.81	sh	-	-	RI, MS, RC
$C_{15}H_{24}O$	1736	2167	-	1.57	1.24	1.56	
$C_{15}H_{26}O$	1743	2216	-	-	0.18	-	
unknown	1744	2216	-	0.72	-	0.18	
$C_{15}H_{22}O$	1748	2222	-	-	3.90	0.54	
$C_{15}H_{26}O$	1756	2202	-	-	-	0.36	
unknown	1766	nd	-	0.20	-	-	
unknown	1797	nd	-	0.15	-	-	
$C_{15}H_{24}O_2$	1802	nd	-	0.19	0.38		
$C_{15}H_{24}O$	1802	2210	-			0.15	
$C_{15}H_{22}O_2$	1806	nd	-	-	-	0.16	
eremophilone	1816	2238	-	4.90	42.99	41.18	RI, MS, ^{1}H, ^{13}C, 2DNMR
unknown	1818	nd	-	0.22	-	-	

Table 5.1 continued. Composition of *Eremophila mitchellii* leaf, branchlet, wood and root essential oils.

Compound	RI P	RI A	Leaf	Branchlet	Wood	Root	Identification
unknown	1827	nd	-	0.16	-	-	
santalcamphor	1839	2257	-	0.47	17.54	4.40	RI, MS, ^{1}H, ^{13}C, 2DNMR
8-hydroxy-1,11-eremophiladien-9-one (minor)	1839	2257			minor	minor	RI, MS, ^{1}H, ^{13}C, 2DNMR
$C_{15}H_{22}O_2$	1848	2259	-	-	tr	0.11	
$C_{15}H_{22}O_2$	1852	2260	-	-	tr	-	
unknown	1864	2261	-	-	0.14	0.10	
$C_{15}H_{22}O_2$	1879	2262	-	-	0.08	0.09	
$C_{15}H_{22}O_2$	1884	2263	-	-	0.28	-	
$C_{15}H_{22}O_2$	1895	2263	-	-	0.96	0.18	
$C_{15}H_{22}O_2$	1900	2265	-	-	0.43	-	
$C_{15}H_{22}O_2$	1921	nd	-	-	-	0.19	
8-hydroxyeremophilone	1936	2270	-	-	minor	minor	RI, MS, ^{1}H, ^{13}C, 2DNMR
9-hydroxy-7(11),9-eremophiladien-8-one	1937	2270			18.00	0.83	RI, MS, ^{1}H, ^{13}C, 2DNMR
$C_{15}H_{20}O_2$	1943	2273	-	-	tr	0.20	
9-hydroxy-1,7(11),9-eremophilatrien-8-one	1981	2277	-	-	1.04	0.34	RI, MS, ^{1}H, ^{13}C, 2DNMR
unknown	2000	2278	-	-	0.17	tr	
$C_{15}H_{20}O_2$	2086	2282	-	-	0.23	0.10	
$C_{15}H_{20}O_2$	2112	2288	-	-	0.33	0.15	
Total (%)			91.26	91.25	96.84	98.48	

RI P, RI A: retention indices on polar (BPX-5) and polar (BP 20) column respectively; tr <0.10%. RC; identification by reference compound. Order of elution and percentages (%) of individual component are derived from the polar column; - not present; nd: not determined; ~ approximately.

Thirty two compounds have been identified by CGMS from the leaf oil of *E. mitchellii*. The three major compounds identified in the leaf oil were *α*-pinene (**40**), spathulenol (**41**) and an unidentified sesquiterpene alcohol which accounted for 45% of the oil.



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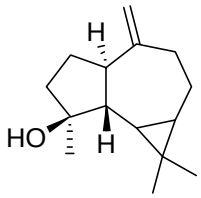

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With the support of spectral data and the corresponding reference compounds it was possible to confirm the presence of the monoterpenes; *α*- and *β*-pinene, *α*- and *β*-phellandrene, *p*-cymene, limonene, *α*-terpinolene, linalool, *α*-terpineol and eugenol on the basis of their prevalence in essential oils. Harborne (1998) reports that *α*- and *β*-pinene, limonene, Δ^3-carene, *α*-phellandrene and myrcene are ubiquitous in leaf oils. This aside, it was observed that the remainder of the chemical profile of the leaf and branchlet oils was predominantly sesquiterpenes.

A rudimentary structural assignment on the basis of the mass spectra and retention indices inevitably indicates the presence of selinenes, humulenes, bisabolols, gurjunenes, maalienes and patchoulenes in the oils. Only a handful of the eremophilane sesquiterpenes are represented in commercial mass spectral libraries. Given the tendency for *Eremophila* species to exhibit unusual stereochemistry (Ghisalberti; 1994b) and the structural similarity between the eremophilanes and other bicyclic sesquiterpenes the identity of many of the minor components can only be assigned with the support of NMR data.

Ghisalberti (1994b) reports that bisabolene, eudesmane, eremophilane, aromadendrane, cadinane, zizaene, spathulenol and elemol classes of sesquiterpenes have all been isolated from the leaf material of *Eremophila* species. With the support of spectral data, reference compounds and inference from metabolites reported from *Eremophila* species it was possible to confirm the presence of aromadendrene, *trans-β*-caryophyllene, *δ*-cadinene, *α*- and *β*-selinene, elemene, epiglubulol, globulol, *α*- and *β*-eudesmol, α-bisabolol, viridiflorene and viridiflorol.

A single ion chromatogram of the sesquiterpene-diene ion, $C_{15}H_{24}$, *m/z* 204 (retention time; 40 - 47 minutes) further supports that there is likely to be multiple classes of sesquiterpenes represented in the leaf oil (Figure 5.5).

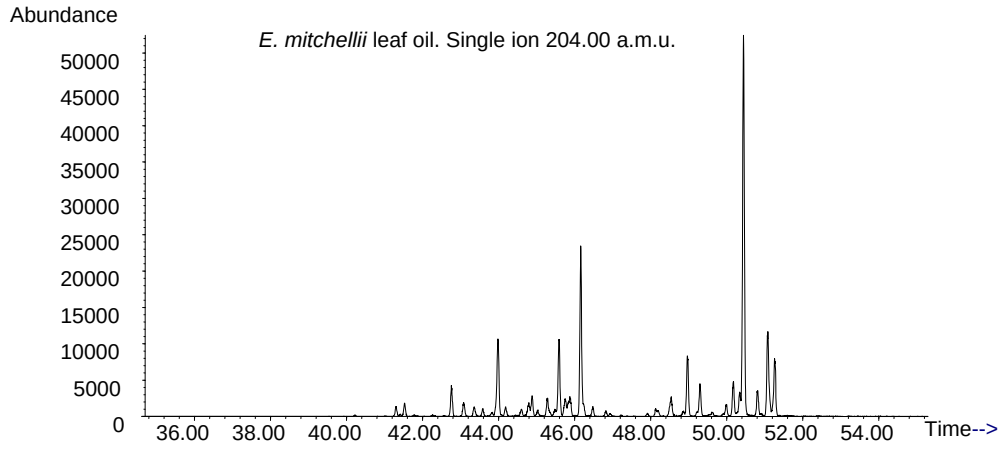


Figure 5.5. Single ion chromatograms of *E. mitchellii* leaf oil.

The wood oil was also predominantly composed of sesquiterpenes. Three of the major constituents identified in the wood oil were eremophilone, santalcamphor, and 9-hydroxy-7(11),9-eremophiladien-8-one and their occurrence in *E. mitchellii* has been well documented. A fourth major constituent, the novel 9-hydroxy-1,7(11),9-

eremophilatrien-9-one proved to be very unstable ($t_{1/2}$ < 12 hours in solution). These four major constituents account for 80% of the wood oil. These components are also observed in the root oil. The minor constituents; the sesquiterpenes and sesquiterpene ketones and alcohols were present at very low concentrations (most <1%). The assignment of α- and β-selinene (synonymous with eudesmenes) in *E. mitchellii* oil was based on comparison with authentic α- and β-selinene from commercial celery seed oil (*Apium graveolens*). This finding is also in accord with the observation that the eudesmenes are the biosynthetic precursors of the eremophilanes.

Several attempts to isolate the minor constituents of the wood oil met with limited success. Their isolation and characterisation is complicated because they share similar molecular weights and many were found to co-elute with the major compounds throughout; HPLC (both normal phase and reverse phase) and GC-MS (both BP20 and BPX-5 columns). Several structural analogues of the eremophilones have been reported in the literature including the aldehyde (**31**) (Abel and Massy-Westropp, 1985), isoeremophilone (**34**) (Chetty and Zalkow, 1969), alloeremophilone (**37**) (Bates and Paknikar, 1966) and the dimers **38** and **39** (Lewis et al., 1979; Lewis et al., 1982). The analogues, if present, appear to be very minor constituents.

It is evident that all of the sesquiterpene ketones have the capacity to tautomerise. The four major constituents can give rise to 11 different tautomers (Figure 5.6). Evidently the major keto-tautomers are thermodynamically favoured. Interestingly, eremophilone (**30**) does not readily convert to its Δ^1 isomer, isoeremophilone (**34**) under mild conditions. The interconversion of the eremophilanes via isomerisation, dehydration

and hydrogenation has been reported by chemical methods (Zalkow and Chetty, 1975; Djerassi et al., 1959).

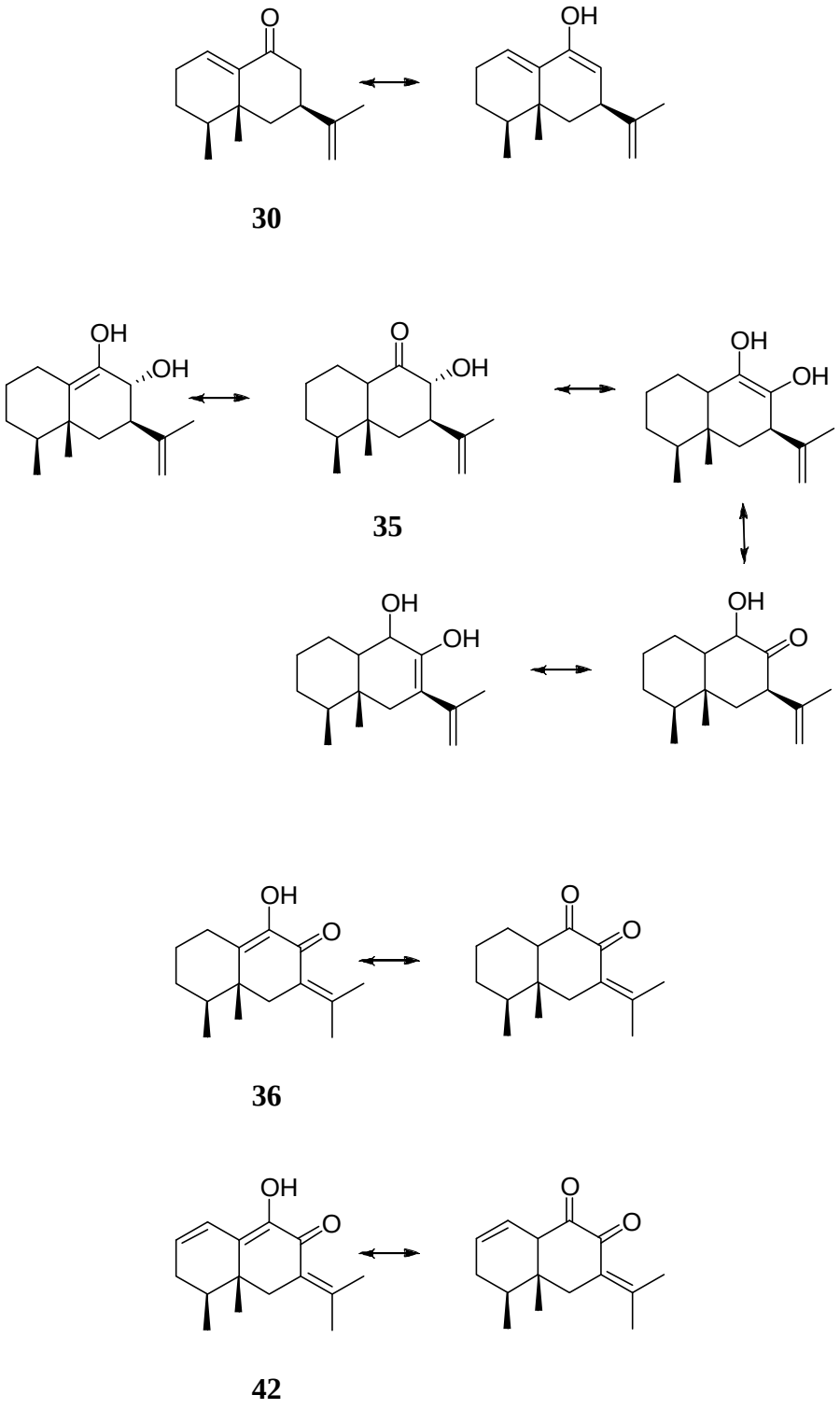


Figure 5.6. Possible tautomers of the major eremophilones.

It is apparent that different biosynthetic pathways are operating in the leaf compared to the wood. Inspection of the single ion chromatogram of the sesquiterpene-diene ion, $C_{15}H_{24}$, *m/z* 204 (retention time; 40 - 47 minutes) for the wood oil indicates that fewer sesquiterpene classes are represented in the wood oil compared to the leaf oil (Figure 5.7). Only six significant peaks are apparent and conceivably, these could all be assigned to eremophilene or selinene analogues. To date, aside from the selinenes, the eleven sesquiterpenes isolated from *E. mitchellii* have been of the eremophilene type, a large number of unknown minor compounds are apparent from the GC-MS chromatogram.

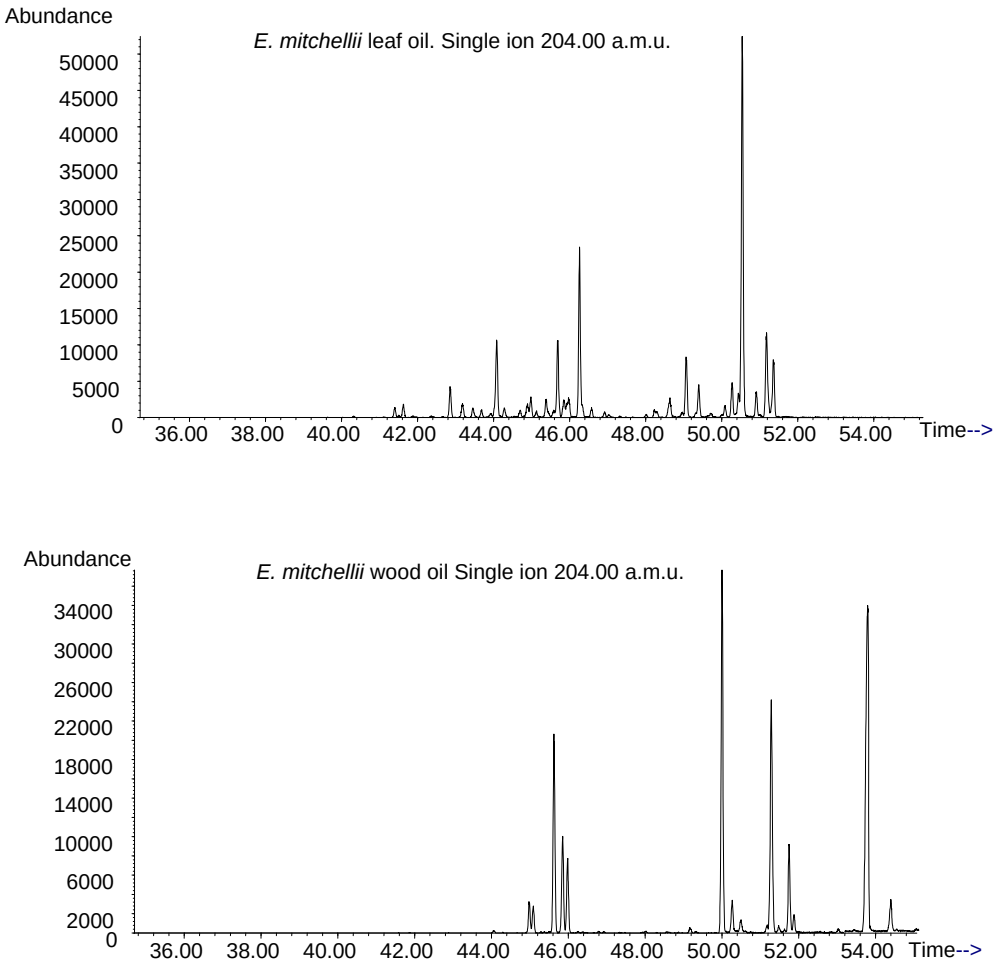


Figure 5.7. Single ion chromatograms of *E. mitchellii* leaf and wood oils.

Structural assignment on the basis of mass spectra is also difficult since many components share the same molecular weight, retention times and similar fragmentation patterns. The mass spectra for all of the compounds isolated in this study are presented in Figure 5.8. Notably santalcamphor (**35**) and 8-hydroxyeremophila-1,11-dien-9-one (**33**) co-elute but may be differentiated by inspection of their molecular ions [M^+] at *m/z* 236 and 234 respectively. Similarly, 9-hydroxy-7(11),9-eremophiladien-8-one (**36**) co-elutes with 8-hydroxy-10,11-eremophiladien-9-one (**32**, synonymous with 8-hydroxyeremophilone). Qualification of the minor constituent **32** is evident by the major fragment ion *m/z* 205 eluting slightly later than the major molecular ion [M^+] at *m/z* 234. Adams (2007) has erroneously ascribed the mass spectrum of **36** as being that of compound **32**. In most cases for compounds bearing an α-hydroxyl group, inspection of the fragmentation pattern reveal a pronounced fragment ion [M-29]$^+$ corresponding to loss of CHO.

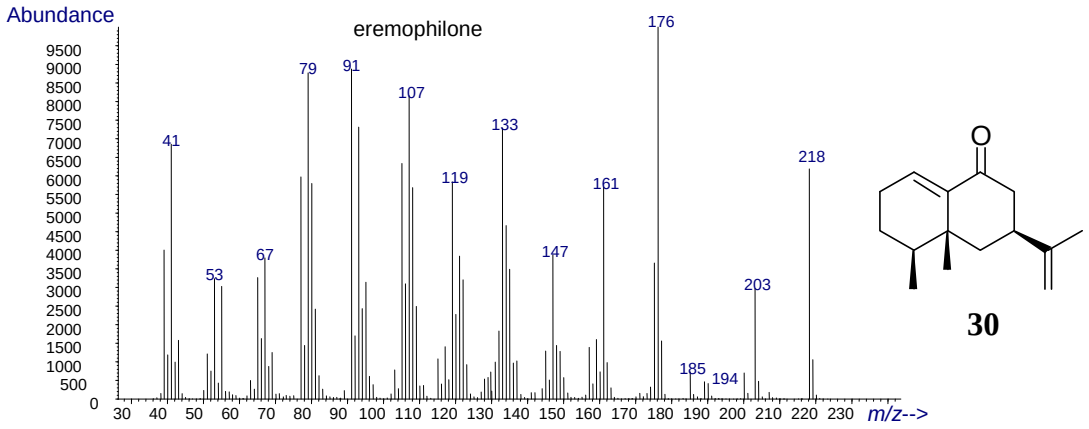
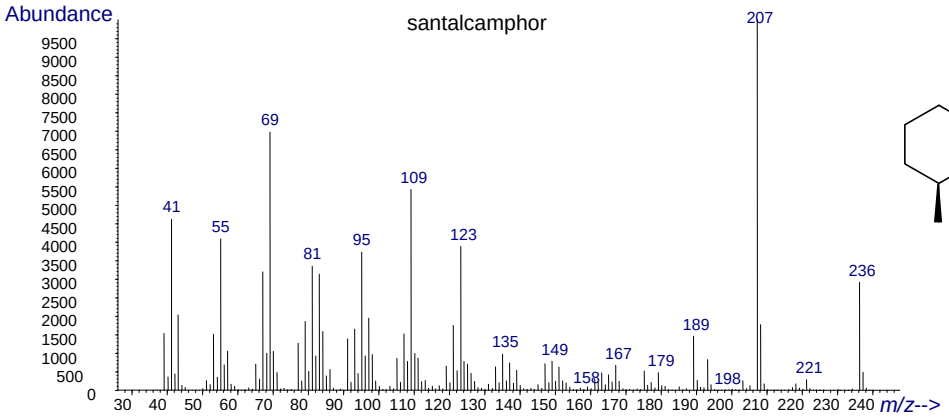
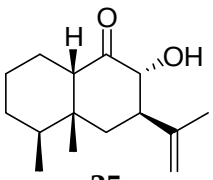


Figure 5.8. Mass spectra of the major eremophilones.





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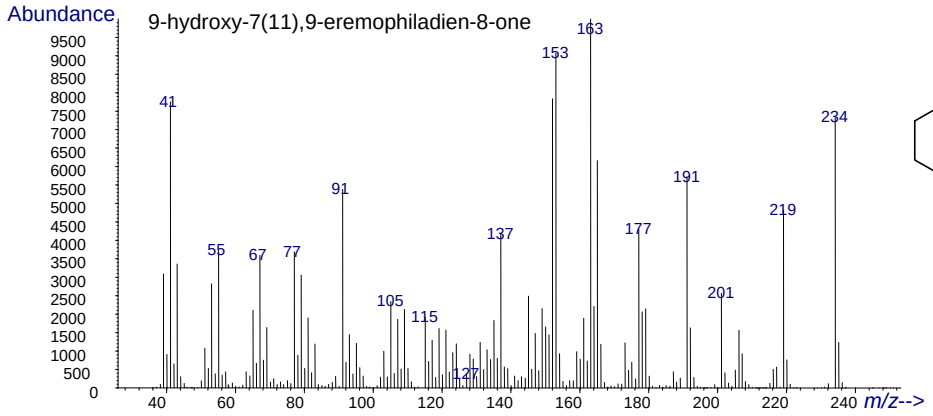

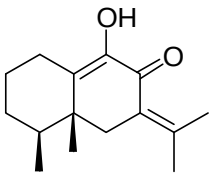

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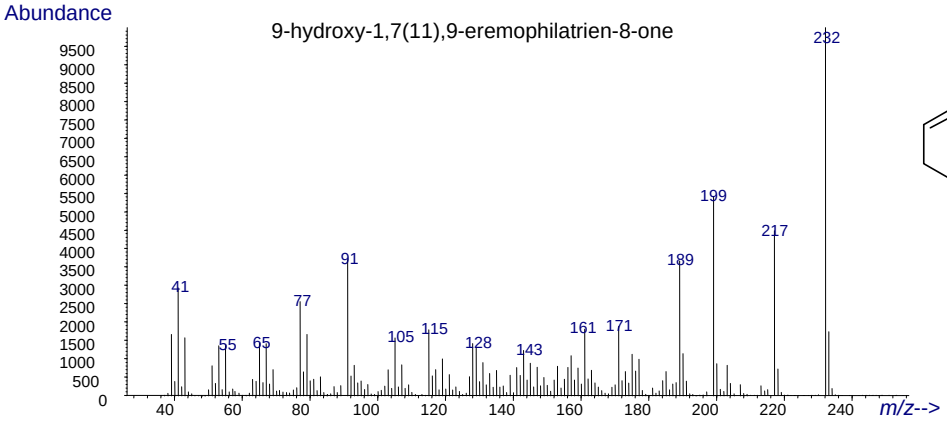
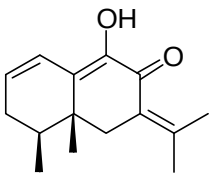




42

Figure 5.8 continued. Mass spectra of the major eremophilones.

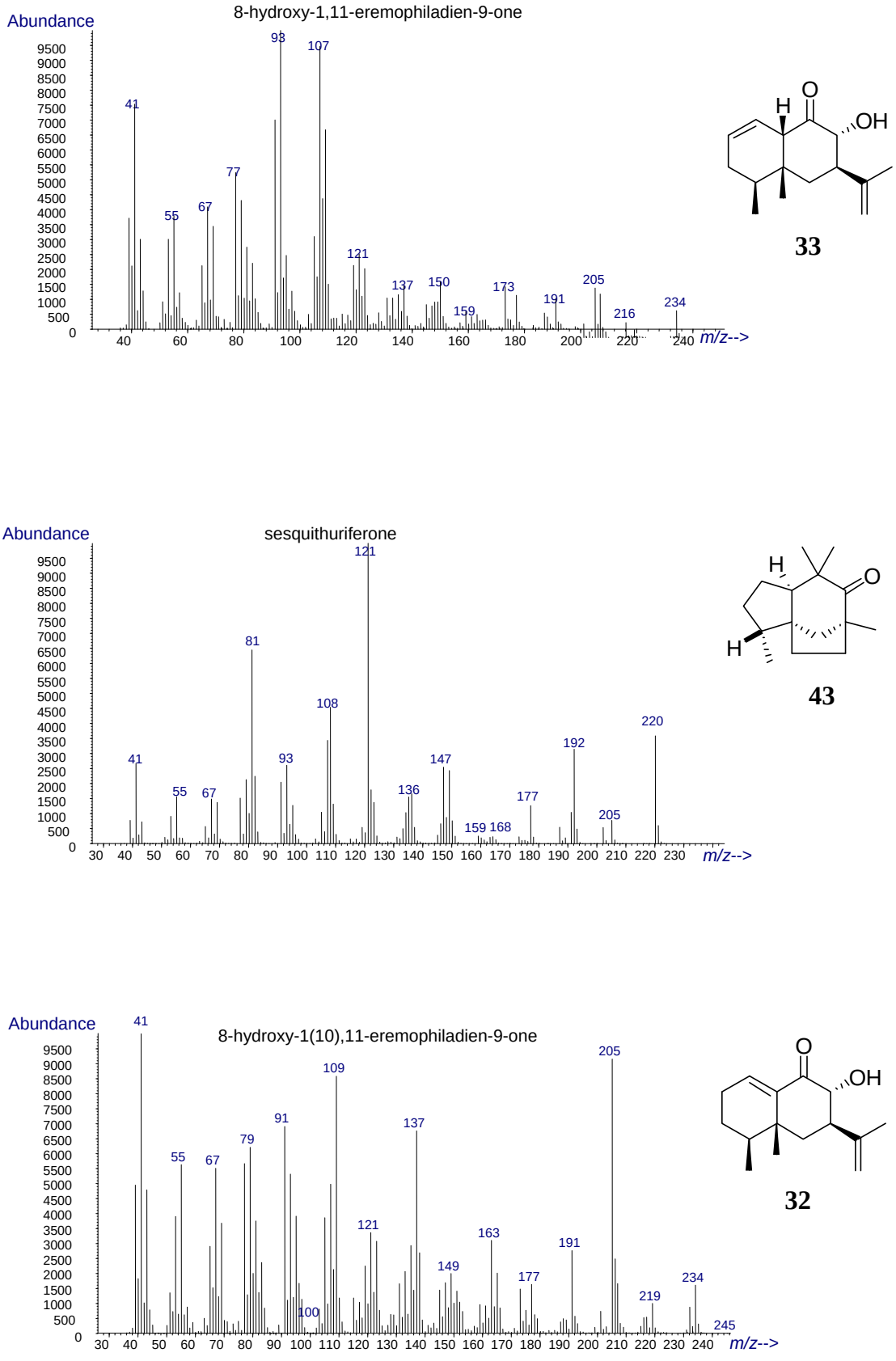


Figure 5.8 continued. Mass spectra of the major eremophilones.

Of interest is the chemical profile of the steam distilled root oil (Figure 5.3), because it only possessed two major constituents. One can readily be identified as eremophilone (**30**) but the second major compound, sesquithuriferone (**43**), is very unusual in that it is a zizaene sesquiterpene. The isolation and structural elucidation of this compound is discussed in section 5.2.4.1.

5.2.2 Fractionation of *E. mitchellii* Wood Oil

Fractionation of the steam distilled wood oil was achieved using normal phase preparative HPLC employing a hexane/ethyl acetate gradient (refer to Chapter 2, Figure 2.3). The wood oil was fractionated in this way on four occasions to generate fractions for testing against two spotted mites and termites and pure compounds for LD_{50} and LD_{95} determination (termites) and structural elucidation work.

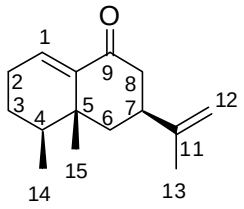
5.2.3 Isolation of Compounds from the Wood Oil of *E. mitchellii*

Large quantities (>1 g) of the purified compounds were required to evaluate the termiticidal activity of each of the pure compounds and to determine LD_{50} and LD_{95} values. Fractionation of more than 5 grams of the oil was achieved using normal phase preparative HPLC, with a hexane/ethyl acetate gradient (95-60% hexane) as eluent, as outlined in the isolation scheme in Figure 2.3. Each fraction was then further fractionated using RP prep-HPLC or purified by means of recrystallisation. A rapid silica column chromatographic method was derived from this method for subsequent large scale fractionations (Figure 2.5). On several occasions a reverse phase preparative HPLC method was utilized to isolate several pure compounds directly from the crude oil (Figure 2.4).

5.2.3.1 Eremophilone

Compound **30** was isolated by normal phase preparative HPLC fractionation as a viscous yellow oil in a yield of approximately 19% (w/w) of the steam distilled wood oil. Further purification of **30** was achieved using RP preparative HPLC employing a 40-95% ACN/H_2O gradient (Figure 2.3). The EI mass spectrum for this compound showed a molecular ion [M^+] at *m/z* 218 which corresponds to the molecular formula $C_{15}H_{22}O$ having 5 double bond equivalents (DBE). The UV spectrum exhibited a λ_{max} at 243 nm consistent with the presence of an *α,β*-unsaturated ketone. The ^{1}H NMR spectrum of compound **30** revealed the presence of a highly deshielded proton (*δ* 6.6 ppm), two vinylic protons (*δ* 4.73 and *δ* 4.77 ppm), two methyl groups situated on quaternary carbons (*δ* 0.97 and *δ* 1.75 ppm) and a third methyl group (*δ* 0.96 ppm) situated on a tertiary carbon. ^{1}H NMR and ^{13}C JMOD data is presented in Table 5.2.

^{13}C JMOD, HSQC, HMBC and COSY data is in accord with the bicyclic eremophilone structure **30** below. The eremophilane skeleton, with methyls at the C-4 and C-5 positions and not at the C-4 and C-10 positions corresponding to eudesmane, can be established from the HMBC data.



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From the multiplicity of the C-15 protons (singlet) it can be established that the methyl group resides on a quaternary carbon. The proximity of the C-14 and C-15 methyls to each other is evidenced by the cluster of HMBC signals that are located to one side of the molecule only. The assignments are presented in Table 5.2 and are consistent with

the low resolution NMR data published by Ziegler et al. (1977) and McMurry et al. (1975) and support the same absolute stereochemical assignments published by Zalkow et al. (1960).

Eremophilone was found to be laevorotatory in agreement with Bradfield et al. (1932b). Discrepancies in the magnitude of rotation may be attributed to the different wavelength and concentrations used for the analysis (sodium at 589 nm and mercury at 365 nm).

$[\alpha]_{Hg}$ -207 (c 2.46 MeOH); (Bradfield et al., 1932b)

Lit; $[\alpha]_D^{20}$ -78° (c 0.49, MeOH);

5.2.3.2 Santalcamphor

Compound **35** readily crystallised from normal phase preparative HPLC fractions 5 and 6 as colourless needles mp 102-103°C in a yield of approximately 20% (w/w) of the oil (Figure 2.3). The compound was purified by recrystallisation in MeOH. The EI mass spectrum for this compound gave a molecular ion [M^+] at *m/z* 236 which suggested a molecular formula $C_{15}H_{24}O_2$ and 4 DBE. The ^{1}H NMR shifts (Table 5.2) bears some resemblance to eremophilone (**30**) with the exceptions being the absence of any highly deshielded protons, a new signal at δ 4.00 ppm and the presence of two methine protons (δ 4.90 and δ 4.93 ppm). In parallel with eremophilone (**30**), compound **35** also possesses two C-quaternary methyls (δ 1.83 and δ 1.06 ppm) and a C-tertiary methyl (δ 0.79 ppm).

The ^{13}C JMOD, HSQC, HMBC and COSY data is in agreement with the structure of santalcamphor (**35**). The doublet at δ 4.00 ppm is indicative of a secondary alcohol. The

Table 5.2. ^{1}H NMR and ^{13}C NMR assignments for compounds **30**, **35** and **36.**

	Compound **30** ($CDCl_3$)		Compound **35** ($CDCl_3$)		Compound **36** ($CDCl_3$)	
C/H	^{13}C δ ppm	^{1}H δ ppm (i, m, J Hz)	^{13}C δ ppm	^{1}H δ ppm (i, m, J Hz)	^{13}C δ ppm	^{1}H δ ppm (i, m, J Hz)
1a	135.5	6.60 (1H, t, 3.8)	21.0	2.08, (1H, m)	23.8	2.98 (1H, m)
1b				1.55 (1H, m)		1.95(1H, m)
2a	25.8	2.23 (2H, m)	22.4	1.55 (1H, m)	25.8	1.42 (1H, m)
2b				1.65 (1H, m)		1.86 (1H, m)
3a	26.7	1.51 (2H, m)	30.3	1.32 (1H, m)	30.8	1.42 (1H, m)
3b				1.43 (1H, m)		1.54 (1H, m)
4	39.0	1.63 (1H, m)	33.9	1.43 (1H, m)	43.1	1.49 (1H, m)
5	36.2	-	41.3	-	39.8	-
6a	41.6	1.97 (1H, m)	40.1	1.55, (1H, m)	40.7	2.11 (1H, m)
6b		1.51 (1H, m)		1.91 (1H, dd, 3.1, 14.5)		2.88 (1H, d, 13.7)
7	39.3	2.36 (1H, m)	48.1	2.42 (1H, dt, 12.2, 3.1)	125.9	-
8	43.4	2.41 (2H, m)	76.7	4.00 (1H d, 11.3)	185.7	-
9	204.0	-	211.9	2.31 (1H, s)	142.7	-
10	144.5	-	54.2	-	137.4	-
11	147.8	-	145.3	-	146.7	-
12a	110.2	4.74 (1H, s)	112.3	4.89 (1H, s)	23.2	2.18 (3H, d, 2.0)
12b		4.77 (1H, t, 1.4)		4.91 (1H, t, 1.4)		
13	20.8	1.75 (3H, s)	19.6	1.82 (3H, s)	23.0	1.90 (3H, s)
14	16.2	0.96 (3H, d, 6.8)	15.4	0.79 (3H, d, 6.5)	16.4	0.94 (3H, d, 6.5)
15	25.0	0.97 (3H, s)	21.5	1.05 (3H, s)	15.7	0.96 (Me, s)

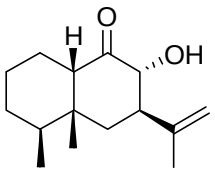
Table 5.3. ^{1}H NMR and ^{13}C NMR assignments for compounds **42**, **33** and **32**.

	Compound **42** ($CDCl_3$)		Compound **33** ($CDCl_3$)		Compound **32** ($CDCl_3$)	
C/H	^{13}C δ ppm	^{1}H δ ppm (i, m, *J* Hz)	^{13}C δ ppm	^{1}H δ ppm (i, m, *J* Hz)	^{13}C δ ppm	^{1}H δ ppm (i, m, *J* Hz)
1	122.0	6.63 (1H, dd, 2.7, 9.9)	122.5	5.67 (1H, m)	140.4	7.11 (1H, t, 3.9)
2	134.3	6.10 (1H, ddd, 2.6, 5.7, 9.8)	129.9	5.88 (1H, m)	26.4	2.31 (2H, m)
3a	32.7	2.20 (1H, m)	32.2	1.76 (1H, m)	26.4	1.48 (2H, m)
3b		2.03 (1H, m)		2.10 (1H, td, 5.0, 18.2)		
4	38.4	1.77 (1H, m)	30.4	1.61 (1H, m)	40.6	1.60 (1H, m)
5	37.0	-	40.0	-	34.9	-
6a	39.6	2.11 (1H, brd, 13.6)	39.1	1.93 (1H, dd, 14.6, 3.0)	40.8	1.80 (2H, m)
6b		2.85 (1H, d, 13.6)		1.66 (1H, d, 13.8)		
7	126.2	-	48.4	2.33 (1H, dt, 3.0, 12.1)	47.7	2.35 (1H, m)
8	185.5	-	77.0	4.12 (1H, dd, 1.3, 11.4)	72.9	4.38 (1H, d, 12.8)
9	131.9	-	c.a 212	-	200.9	-
10	141.9	-	56.3	2.78 (1H, d, 5.1)	139.8	-
11	148.1	-	145.0	-	145.1	-
12a	23.4	2.25 (3H, d, 2.3)	112.6	4.90 (1H, s)	113.2	4.85 (1H, s)
12b				4.93 (1H, t, 1.4)		4.90 (1H, t, 1.5)
13	23.5	1.93 (3H, d, 1.5)	19.4	1.84 (3H, s)	25.7	1.85 (3H, s)
14	15.9	0.98 (3H, d, 6.6)	14.4	0.82 (3H, d, 6.7)	15.7	0.94 (3H, d, 6.7)
15	14.6	0.89 (3H, d, 0.6)	21.0	1.00 (3H, s)	18.4	1.08 (3H, s)

relative stereochemistry of the C-7 isoprenyl and the C-8 hydroxyl is defined as *anti* by the strong coupling constant (*J* 11.3 Hz) between the resident protons (δ 2.43 and δ 4.00 ppm respectively). The aliphatic region of the ^{1}H NMR is quite complex with several overlapping multiplets. NOe difference experiments were utilized to confirm the assignments of the chemical shifts for all of the alkyl protons (Table 5.2). Irradiation of the methyl groups and examination of the resulting nOe difference spectrum indicated the proximity of the respective alkyl groups. The optical rotation was found to be consistent with previously published data (Bradfield et al., 1932a) and assumes the same absolute stereochemical assignments published by Zalkow et al., (1960).

$[\alpha]_D^{20}$ +112° (c 0.26, $CHCl_3$);

Lit; $[\alpha]_D$ +90.6° ($CHCl_3$); (Bradfield et al., 1932a.)



35

5.2.3.3 9-hydroxy-7(11),9-eremophiladien-8-one

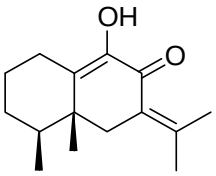
Fraction 3 from the normal phase preparative HPLC yielded a yellow oil composed of a mixture of several compounds. The major compound (**36**) represented approximately 14% of the crude oil (w/w) and was purified further using RP preparative HPLC employing a 40-95% ACN/H_2O gradient (Figure 2.3). The compound (**36**) showed an EI molecular ion [M^+] at *m/z* 234 which suggested a molecular formula of $C_{15}H_{22}O_2$ and 5 DBE. The ^{1}H NMR spectrum (Table 5.2) did not feature any chemical shifts further downfield than a multiplet at δ 2.98 ppm. Four methyl groups were evident. One methyl group (δ 0.94 ppm) was positioned on a tertiary carbon and three were situated

at quaternary positions; one at δ 0.96 ppm and two more deshielded vinylic methyls at δ 1.90 and δ 2.18 ppm.

Inspection of the ^{13}C JMOD spectrum (Table 5.2) revealed the presence of a carbonyl group (δ 185.7 ppm) that was highly shielded. The chemical shift of a carbonyl group can vary depending on the degree and type of conjugation. Unconjugated carbonyls such as that in santalcamphor (**35**) resonate at approximately 212 ppm. In the case of carbonyls that are in conjugation with a double bond, as in eremophilone (**30**)**,** the carbonyl resonance is shielded by approximately 12 ppm, and a resonance at approximately δ 200 ppm can be expected. If the carbonyl is part of a dienone system the second double bond has the effect of either reducing or increasing the magnitude of the deshielding. If the conjugation is in an $\alpha,\beta,\gamma,\delta$ configuration the second double bond reduces the shielding effect caused by the first ($\Delta\delta$ < 12 ppm). In the case of cross conjugated $\alpha,\beta,\alpha',\beta'$-dieneones the shielding effects of both of the double bonds are cumulative and carbonyl chemical shifts of 185-190 ppm can be expected (Ziegler et al., 1977). For compound **3** the carbonyl resonance (δ 185.7 ppm) was indicative of a component in an $\alpha,\beta,\alpha',\beta'$ cross conjugated system. The ^{13}C JMOD (Table 5.2), HSQC, HMBC and COSY data was in agreement with the structure of 9-hydroxy-7(11),9-eremophiladien-8-one (**36**). The ^{1}H chemical shifts are in agreement with the low resolution methyl group assignments published by Pinder and Torrence (1971). A discrepancy was observed for the optical rotation;

$[\alpha]_D^{20}$ +63° (c 2.13, MeOH)

Lit; $[\alpha]_D^{25}$ +138° (c 2.59, MeOH); Lit. Pinder and Torrence (1971).



36

5.2.3.4 9-Hydroxy-1,7(11),9-eremophilatrien-8-one

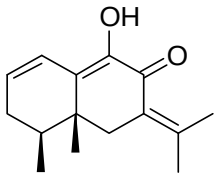
Compound **42** co-eluted as the minor constituent with **30** from normal phase preparative HPLC fraction 4. It was apparent that **42** was not present in some commercial oils, and was highly unstable since only trace levels were present after 24 hours in solution. The chemical stability of **42** was monitored in multiple solvents. It was observed that the stability of **42** could be optimised depending on the solvents selected for isolation work. It was found that the ideal solvents were, in order of increasing stability; ACN, acetone, ACN/0.5% TFA, EtOAc, $CHCl_3$, DMSO, EtOH, MeOH, and hexane. No discernible breakdown products or increases in relative peak intensities were observed by GC-MS.

Purification of **42** was achieved by subjecting a fresh enriched silica fraction to RP preparative HPLC using a 70% MeOH/H_2O isocratic gradient and 2 x tandem preparative columns (Figure 2.3). Compound **42** was purified as a yellow gum in a yield of approximately 0.4% (w/w) of the crude oil. The EI mass spectrum for this compound gave a molecular ion [M^+] at *m/z* 232. HRAPCIMS established the molecular weight [M+H]$^+$ 233.1514 that was consistent with the molecular formula $C_{15}H_{20}O_2$ and 6 DBE.

Compound **36** and **42** showed similar ^{13}C JMOD spectra (Table 5.3) with the main difference being the absence of two methylenes (δ_C 23.8 and 25.8 ppm) and the presence of two additional methines (δ_C 122.0 and 134.3 ppm). Like **36**, **42** possessed a highly deshielded carbonyl group (δ_C 185.5 ppm) situated in an $\alpha,\beta,\alpha',\beta'$ dieneone system. Compared to **36** the ^1H NMR spectrum of **42** showed two additional, highly deshielded, peaks at δ 6.63 and at δ 6.10 ppm as part of an ABX_2 system consisting of two vinylic protons (δ_H 6.63, dd, J = ~2.7, ~9.9 Hz; 6.10 (ddd, J = 2.6, 5.7, 9.8 Hz) adjacent to a methylene group (δ_H 2.20, m; 2.03, m). HSQC and HMBC correlations confirmed the presence of a C-1 - C-2 double bond and was in agreement with the novel compound 9-hydroxy-1,7(11),9-eremophilatrien-8-one (**42**). Owing to the instability of the compound in solution a crystal structure could not be obtained, consequently the stereochemistry of C-4 and C-5 has been assumed on the basis of its affinity to the preceding eremophilanes **30**, **33**, **35** and **36**.

Optical rotation found;

$[\alpha]_D^{20}$ +31° (c 0.32, $CHCl_3$)



42

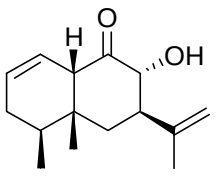
5.2.3.5 8-Hydroxy-1,11-eremophiladien-9-one

Compound **33** was isolated from normal phase preparative HPLC fractions 8 and 9 in a yield of 4.3% (w/w) of the oil. Compound **33** was purified further by RP preparative HPLC using a 40-95% ACN/H_2O gradient (Figure 2.3). Compound **33** showed an EI

molecular ion at [M^+] at *m/z* 234 which suggested a molecular formula of $C_{15}H_{22}O_2$ and 5 DBE. Inspection of the ^{1}H NMR and ^{13}C JMOD spectra suggested that this compound was very similar to santalcamphor (**35**). Compared to **35** the ^{1}H NMR spectrum of compound **33** has gained two additional protons; a 1-H multiplet δ 5.88 ppm, and also a 1-H multiplet (δ 5.67 ppm) that from the COSY spectrum, are coupled to each other. Relative to **35** the 3-(2H) and 10-(1H) protons are more deshielded indicating a C-1 - C-2 double bond and that compound **33** is 8-hydroxy-1,11-eremophiladien-9-one (**33**). This structure was also confirmed by the resonance of the carbonyl (δ 212 ppm) which is unconjugated. The NMR assignments are presented in Table 5.3 and are consistent with the limited data (low resolution NMR) published by Massy-Westropp and Reynolds (1966). The reasons for the discrepancies between the published (Massy-Westropp and Reynolds., 1966) and the observed optical rotation of this compound are unclear.

$[\alpha]_D^{20}$ +167° (c 0.14, $CHCl_3$);

Lit; $[\alpha]_D^{19}$ +59.3 (c 1.1, $CHCl_3$); Massy-Westropp and Reynolds, (1966)



33

5.2.3.6 8-Hydroxy-1(10),11-eremophiladien-9-one

Compound **32**, a minor constituent of the oil, co-eluted with santalcamphor (**35**) from normal phase preparative HPLC fractions 6. Isolation of **32** was effected utilizing RP

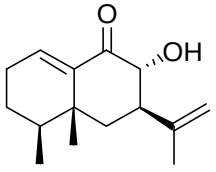
preparative HPLC using a 10-95% ACN/H_2O gradient, yielding **32** as a colourless oil in a yield of approximately 1% (w/w) of the oil. The EI mass spectrum for this compound gave a molecular ion [M^+] at *m/z* 234 which suggested a molecular formula $C_{15}H_{22}O_2$ and 5 DBE. The ^{13}C JMOD spectrum (Table 5.3) bears some resemblance to eremophilone (**30**), featuring a conjugated carbonyl (δ 200.9 ppm) and four sp^2 hybridized carbons (δ 113.2, 139.8, 145.1 and 140.4 ppm). with the exceptions being the loss of the methylene signal at δ 2.41, and a new signal at δ 4.38 ppm indicative of a secondary alcohol. In parallel with eremophilone (**30**), compound **32** also possesses two C-quaternary methyls (δ 1.08 and δ 1.85 ppm) and a C-tertiary methyl (δ 0.94 ppm).

^{13}C JMOD (Table 5.3), HSQC, HMBC and COSY data is in agreement with the structure of 8-hydroxy-1(10),11-eremophiladien-9-one (**32**). The relative stereochemistry of the C-7 isoprenyl and the C-8 hydroxyl is defined as *anti* by the strong coupling constant (*J* 12.8 Hz) between the resident protons (δ 2.35 and δ 4.38 ppm respectively). The ^1H chemical shifts are in agreement with the low resolution assignments published by Massy-Westropp and Reynolds (1966).

Optical rotation found

$[\alpha]_D^{20}$ -36° (c 0.22, MeOH);

Lit; $[\alpha]_D^{20}$ -33.6° (c 0.84, MeOH); Massy-Westropp and Reynolds. (1966)



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5.2.4 Isolation of Compounds from the Root Oil of *E. mitchellii*

The roots of *E. mitchellii* were steam distilled for 48 hours yielding 2.0 mL of dark amber oil (0.26% yield). The GC-MS analysis of the root oil revealed that it contained two major components; one was readily identified as eremophilone (**30**), whilst the second peak showed an EI molecular ion [M^+] at *m/z* 220 corresponding to an unidentified compound with a molecular formula $C_{15}H_{24}O$. This compound was of interest because there have been no reports of a compound of MW 220 being characterized from *E. mitchellii*. To isolate this unknown compound (**43**), the root oil was first fractionated on a silica gel column using a pentane/ether stepwise gradient. Compound **43** was enriched in fraction 1 (Refer to isolation scheme Figure 2.6). Fraction 1 was found to contain predominantly eremophilone and the as yet unidentified compound (MW 220).

The root oil fraction 1 was further fractionated using C18 prep-HPLC. Three peaks were selectively cut from the chemical profile of root fraction 1.

5.2.4.1 Sesquithuriferone

Analysis by LC-MS indicated that compound **43**, the major constituent fraction 1, showed an APCI molecular ion [M+H] at *m/z* 203, Δ18 a.m.u. less than expected resulting from loss of water. Compound **43** was further purified by C18 RP preparative HPLC using an ACN/H_2O/TFA gradient (Figure 2.6). Pure **43** was obtained as a white powder in a yield of 23% (w/w) of root oil fraction 1.

The ^{1}H NMR spectrum of **43** (Table 5.4) revealed the presence of three quaternary methyl groups (δ 1.08, δ 1.08 and δ 1.12 ppm) and a tertiary methyl group (δ 0.88

ppm). The remainder of the proton signals were all in the aliphatic region, with many signals overlapped. The ^{13}C JMOD (Table 5.4) revealed the presence of a carbonyl group and indicated that all of the other carbons were sp^3 hybridised. Compound **43** must be tricyclic on the basis that it has four DBE.

Table 5.4. Experimental and published ^1H and ^{13}C NMR data for sesquithuriferone **43**.

C/H	Compound **43** ($CDCl_3$)		Compound **6** ($CDCl_3$)†
	^{13}C ppm	^1H δ ppm (i, m, *J* Hz)	^1H (δ ppm) (i, m, *J* Hz)
1	54.1	-	
2	40.7	1.86 (1H, *m*)	}
3	31.8	1.16-1.23 (1H, *m*)	}1.12 (6H, *s*)‡
		2.0-2.1 (1H, *m*)	}
4	23.1	1.56-1.64 (2H, *m*)	
5	54.4	1.78-1.82 (1H, *m*)	
6	45.8	-	
7	220.1	-	
8	53.6	-	
9	35.4	1.56-1.64 (1H, *m*)	
		1.68-1.74 (1H, *m*)	
10	33.6	1.44-1.50 (1H, *m*)	
		2.0-2.1 (1H, *m*)	
11	45.5	1.48-1.51 (1H, *m*)	
		1.68 (1H, *d*, 11.6)	
12	20.0	0.88 (3H, *d*, 7.2)	0.92 (3H, *d*, 7.0)
13	29.6	1.08 (3H, *s*)	}
14	24.9	1.08 (3H, *s*)	}1.16 (9H, *s*)
15	21.9	1.12 (3H, *s*)	}

† Source; Carrol et al. (1976) ^1H NMR (90MHz).
‡ Assignments have not been designated to specific protons.

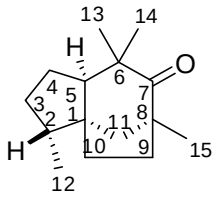
Compound **43** was elucidated as the known zizaene compound, sesquithuriferone on the basis of HSQC, HMBC, TOCSY, COSY and nOe experiments. One dimensional TOCSY experiments were utilized to establish ^1H-^1H connectivities within discrete spin systems. In 1D TOCSY a peak in the spectrum is irradiated and the signal is transferred from it to all of the *J* coupled protons in a stepwise process. Thus over short intervals

(20 ms mixing time) only 1-2 step transfers will be observed whilst longer mixing times (200 ms) allow more transfers (5-7 steps) and can progressively reveal the extent of the spin system. The relative stereochemistry was determined on the basis of nOe difference experiments.

Optical rotation found;

Lit; $[\alpha]_D^{20}$ +5.9° (c 1, $CHCl_3$);

$[\alpha]_D$+5.7° (c 1, $CHCl_3$); Barrero 2000



43

This is the first detailed confirmation of a tricyclic sesquiterpene from *E. mitchellii*. Sesquithuriferone (**43**) has previously been isolated from *E. georgei* (Ghisalberti et al., 1976), *E. metallicorum,* (Ghisalberti et al., 1994b) and *E. subteritifolia* (Carrol et al., 1976). NMR data is limited (Refer to Table 5.4.), and due to lack of material **6** was characterised as its *p*-bromobenzoate derivative (Carrol *et al*; 1976). The structure was confirmed by X-ray diffraction studies (Ghisalberti et al., 1976). At the time of compiling this thesis Adams (2007) reports, on the basis of mass spectral data, that **43** is a component of *E. mitchellii* wood oil. Throughout the course of this research we have observed that sesquithuriferone is prevalent in the root heartwood and that no traces of sesquithuriferone were observed in carefully prepared wood oil.

Chapter 6

Termiticidal Investigations of *Eremophila mitchellii*

6.1 Introduction

Commercial interest in *E. mitchellii* arose due to reports that the timber was especially durable and resistant to termites (Cribb and Cribb, 1981). One of the main uses of the timber has been for fencing posts (Cribb and Cribb, 1981). Preliminary work by Australian Phytochemicals Ltd. (APL) and the Centre for Plant and Food Sciences (CPAFS) at the University of Western Sydney had determined that the steam distilled oil and solvent extracts of the wood were toxic to termites (Leach et al., 2004). This chapter describes the assessment of the wood oil, fractions and compounds for activity against selected termite species.

An ethanolic and a hexane extract as well as the steam distilled essential oil, have been evaluated by CPAFS for termiticidal, antifeedant and repellency against two species of

termites, *Nasutitermes walkeri* (Hill) and *Coptotermes acinaciformis* (Froggatt) (Leach et al., 2004). In direct contact assays it was found that the steam distilled oil was more efficacious than the solvent extracts on a weight per weight basis. Ninety five percent mortality was achieved at concentrations of 0.18% (w/v) against *C. acinaciformis* and 0.11% (w/v) against *N. walkeri* (Leach et al., 2004). This chapter reports on the fumigation, barrier and choice studies performed on the oil to assess the mode of action and the direct dermal toxicity of the compounds from *E. mitchellii*.

6.1.1 Review of Termites and Termiticides

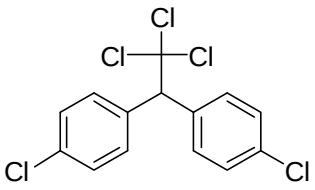
Ecologically termites serve an important role in the ecosystem, they breakdown cellulose and help to recycle soil nutrients. Termites also invade many man-made structures such as buildings, boats, pipelines, underground cables and telegraph poles. Economically, termites are the most destructive timber pests in the world, and have the greatest economic impact in south eastern USA followed by Japan, Australia and South East Asia. In the USA the cost of termite damage is estimated to exceed (US) 2 billion dollars annually (U.S. National Institute of Building Services, 1993). It is reported that the Japanese spend in excess of (US) 800 million dollars annually on termite control (Tsunoda, 2003). In Australia, it is estimated that termites cause damage to one in five buildings and structures, with the cost of this damage and subsequent control measures estimated to exceed 780 million dollars annually (Archicentre, 2003).

Termites may be classified depending on the type of timber they invade, and fall into three categories; dry wood species, damp wood species or subterranean species. It is the subterranean species of termite that are responsible for the destruction of woodwork in Australian buildings. Of these species *Coptotermes* is responsible for the majority of

the damage. Softwoods are their preferred food source but they will ingest many of the hardwood species (Creffield, 1998).

6.1.2. History of Termiticides

In the past, protection of buildings and timber structures from termites has chiefly relied on soil chemical barriers. Traditionally, organochlorine pesticides have been employed for this purpose. Dichlorodiphenyltrichloroethane (DDT) (**44**), developed in 1874, was the first synthetic pesticide. Due to its chemical stability (it has a soil half-life between 2-15 years (U.S. DHHHS., 1994)), it proved to be a highly effective chemical soil barrier for protection against termites. Dieldrin (**45**) and chlordane (**46**) were developed in the 1940s as alternatives to DDT (**44**) and were later followed by heptachlor (**47**)**.**



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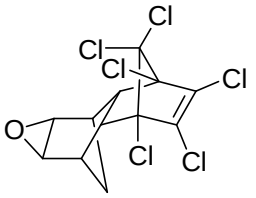

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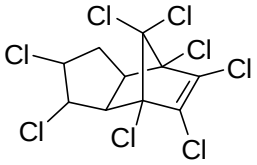

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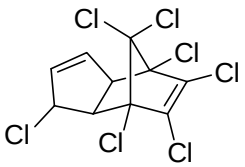

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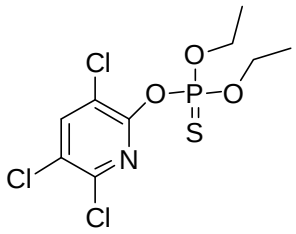
Use of these organochlorine pesticides (OCP) was widespread during the 1950s to early 1970s. They were inexpensive and renowned as broad spectrum insecticides that were used for the control of insect pests in crops and forests as well as in termiticidal

applications. Throughout the 1960's declines in bird populations and other detrimental effects on humans and animals were being correlated to the indiscriminate use of organochlorine pesticides (Carson, 1962). OCP's are persistent pesticides that are toxic to humans and many animals, and are very toxic to aquatic life. They are bio-accumulative, bio-magnified and are transported through the atmosphere and watercourses for long distances. DDT is neuroactive and interferes with cell membrane ion movement, disrupts endocrine function as well as being a suspected carcinogen (Klassen et al., 1996). The organochlorines **45**, **46**, and **47** are also neurotoxic *gamma*-aminobutyric acid (GABA) inhibitors (Coats, 1990).

Although deregistration of the most toxic OCP's such as DDT, dieldrin, chlordane and heptachlor commenced during 1970's, chlordane remained the most successful termite treatment available and was heavily depended upon. Despite its environmental impact and its potential to cause cancer, its use was still permitted for termite applications in the U.S until 1988 (ATSDR., 1997) and in Australia as late as 1995 (CSIRO Entomology, 1995) until effective substitutes could be found. Despite these environmental impacts some countries such as Mexico still permit the regulated use of chlordane for termite control. The effectiveness of the organochlorine chemical soil barriers applied throughout the 1970-80's would have begun to decline throughout the 1990's and their withdrawal has forced an urgent need for safe and effective termite control strategies.

The organochlorines employed as preventative soil chemical barriers, have been superseded by the organophosphate chlorpyrifos (**48**), a cholinesterase inhibitor, and the synthetic pyrethroid bifenthrin (**49**). Chlorpyrifos is the most widely used chemical barrier termiticide in Australia. It is relatively stable, having a half-life of 13

months, a low water solubility and is readily absorbed by organic matter (Murray et al., 2001). Other chemical soil barriers include imidacloprid (**50**), a derivative of nicotine, and insect growth regulators such as hexaflumuron (**51**). These new generation soil termiticides have a shorter service life, usually a minimum of 3-8 years depending on the climate where they are applied. They are also more expensive, less persistent and are widely perceived as being less reliable than the OCP's. Achieving a continuous and uniform chemical soil barrier can be difficult. The frequent rate of re-treatments that are necessary throughout the life of a building have raised public concern and increased scrutiny by regulatory bodies (Ahmed, 2000).



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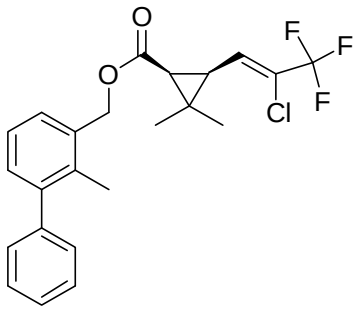

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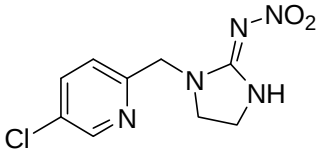

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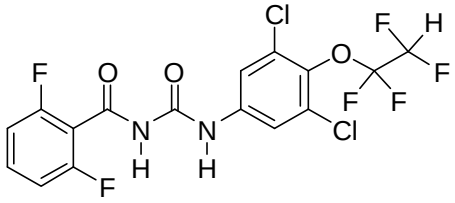

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6.1.3 Current Methods for Termite Control

Current methods for the control of termites encompass an integrated pest management (IPM) strategy that uses an array of ecologically sound complementary methods to significantly reduce or eliminate the use of pesticides. Knowledge of termite biology,

nutritional physiology, foraging behaviour and population dynamics is becoming increasingly fundamental to the design and implementation of highly targeted termiticides or biological controls. IPM draws on; preventative measures, cultivation, pest monitoring, mechanical controls, biological controls and as a last resort chemical controls.

6.1.3.1 Modern Chemical Controls

Chemical controls may be classified into three categories based on their repellency or killing style (Su et al. 1982). Type I termiticides encompass all of the repellent chemicals which may be used as preventative agents and include chemicals that are used for timber preservation. Type II termiticides are those that kill on contact and type III termiticides are those that are slow acting and are non-repellent. Remedial measures for active infestations employ both type II and type III termiticides, whilst baiting and dusting techniques are strictly dependent upon type III termiticides.

6.1.3.2 Preventative Chemical Treatments

Timber may be pressure treated with termiticides to aid in the prevention of termite incursions (USDA, 1960; Tamashiro et al., 1988). The main timber preservatives currently in use are heavy metal formulations such as copper chromated arsenate (CCA) and acid copper chromate (ACC). The use of arsenic not only protects against termites but also inhibit wood rot, moulds and other timber pests. One disadvantage for CCA and ACC is that arsenic and chromium have the capacity to leach from treated timbers and pose a health risk. Consequently, the use of ACC and CCA for timber preservation has been banned since 2004 by the U.S. Environmental Protection Agency. The Australia Pesticides and Veterinary Medicines Authority (APVMA) still

endorses the use of CCA and ACC for the protection of structures. Newer alternatives to CCA pressure treated timber include formulations based on boron such as boric acid and disodium octaborate tetrahydrate, ammoniacal copper chromate (ACC) and ammoniacal copper quaternary (ACQ), copper sulphate, copper azole, chromated copper borate (CCB) and pentachlorophenol (U.S. Environmental Protection Agency, 2006; Lebow, 2004). Borate pressure treatments are inexpensive alternatives that have low mammalian toxicity (refer to Figure 6.1). Borate compounds readily penetrate timber but their use is limited to dry applications. Borates are water-soluble and readily leach into the soil thereby compromising the barrier (Lebow, 2004).

6.1.3.3 Remedial Chemical Treatments

Pest control operators may utilize chemical soil termiticides, baiting systems or dust toxicants to control an active termite infestation. Non repellent liquid termiticides may be injected or trenched into the soil around the perimeter of an infected structure with spot treatment of the infected areas. Chemical soil treatments are time consuming, costly and use large quantities of chemicals to ensure every possible invasion site is treated.

The last decade has seen the intensive development of baiting technology. Baiting systems employ a series of canisters that contain a palatable food source. They serve to aggregate large numbers of termites which, when detected, can then be dusted with or fed a slow acting, non-repellent termiticide. Bait or dust toxicants may be termiticides that are stomach or contact poisons such as the arsenates or perchloropentacyclodecane (Mirex) respectively, insect growth regulators, chitin synthesis inhibitors such as hexaflumuron and potentially, biological controls.

Traditionally organochlorine termiticides have been formulated into dry powders and used as dust toxicants. These have been superseded by arsenates such as arsenic trioxide, which is a slow acting stomach poisons that is topically applied and is effective at reducing termite populations. Grace and Abdallay (1990) have reported on the potential for boron compounds to be used as dust toxicants. Compared to the hundreds of litres of toxic liquids used in soil treatments, baiting or dust systems only require a few grams of the toxin. The environmental hazards associated with termiticidal treatments are greatly reduced by the use of baiting, after treatment the infested structures are pesticide free except for the minute levels of the toxin in the dead termites (Ahmed, 2000).

Of the selection of modern termiticides, most have undesirable consequences due to their toxicity to non-target organisms and their persistence. A comparison is made in Figure 6.1 of the toxicity of termiticides, indicating that there is a trend towards the development of less toxic chemicals. However, data on the negative impact of the modern termiticides is also mounting, resulting in increasing limitations and regulations on their use and progressively many are being banned. Fipronil, a GABA inhibitor (Cole et al. 1993) has recently been banned in France due to its toxicity to bees. It is also highly toxic to aquatic organisms and bird species (Hamon. 1996). The

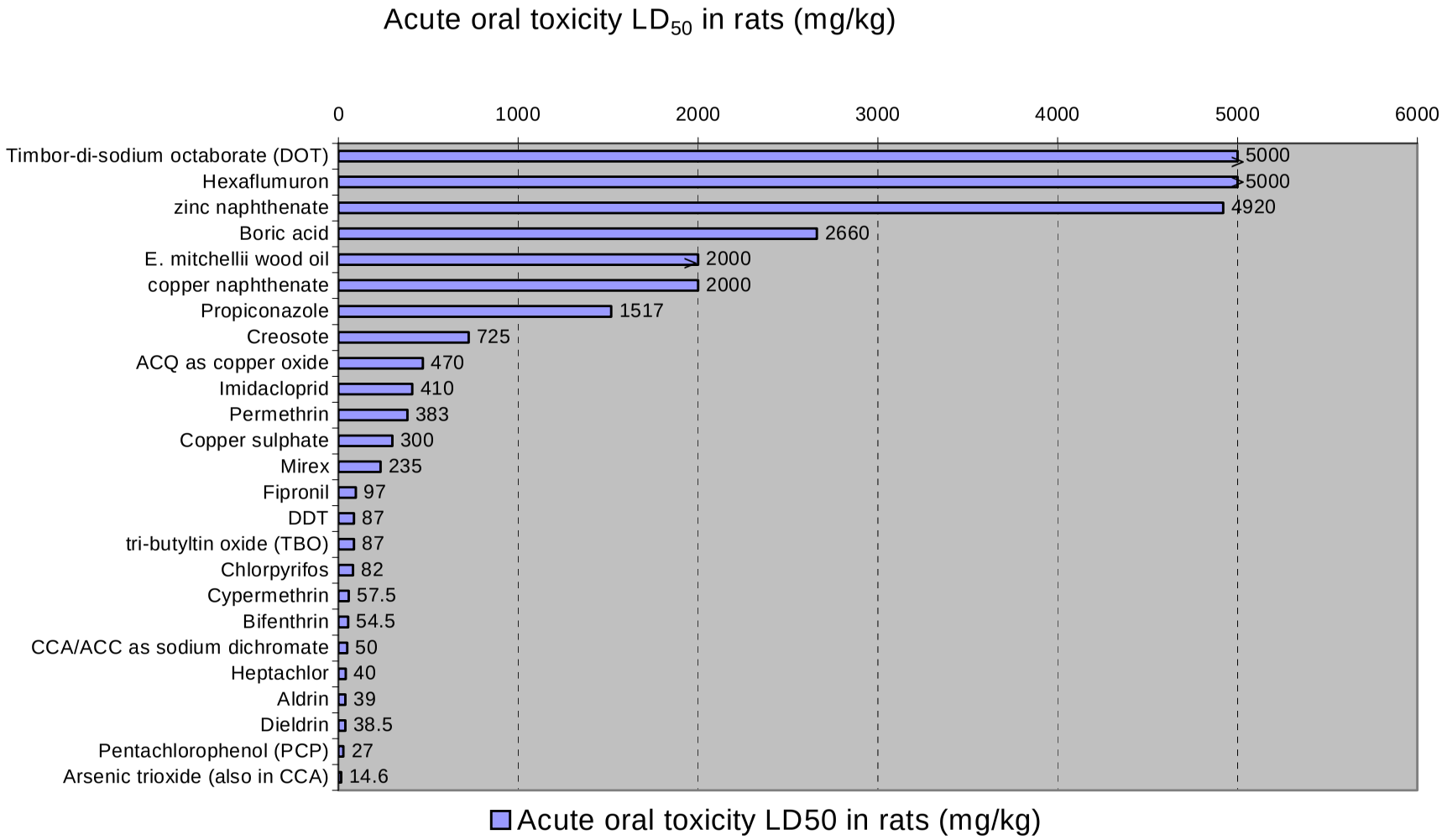


Figure 6.1. Acute oral toxicity LD_{50} in rats of traditional and contemporary termiticides
Adapted from Ahmed (2000) and Bioprospect (2008).

The APVMA is currently reviewing the registration status of fipronil. Chlorpyrifos (**48**) is also extremely toxic to birds and other wildlife and aquatic organisms (U.S. EPA., 1984). The APVMA has reported that rain induced run-off from termiticidal applications employing chlorpyrifos are a common cause of fish kills in Australia.

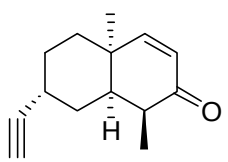
6.1.4 Phytochemicals for Termite Controls

With the withdrawal of OCP's and the progressive banning of the modern termiticides researchers are striving to develop highly targeted, organism specific means of control. The use of baiting systems offer a means for the responsible use of termiticides however there have been mixed reports on the efficacy of these systems (Pawson and Gold, 1996). It is unclear whether the inadequacies of this technique are due to the delivery system or the active ingredients. Historically, chemical soil barriers have outperformed all other modes of treatment. At present, there is a surge of research into new chemical alternatives.

Chemical controls will remain an important tool in termite control programs, whether as repellents or as remedial controls. Natural products are a promising resource for the search for 'softer' chemicals. They have the potential to be less toxic and more specific and to provide novel templates for the development of synthetic termiticides. Plants are being surveyed for termite-active secondary metabolites. Several researchers have investigated the innate termite resistance of timbers. Bultman et al. (1979) evaluated 42 tropical African woods for their termite resistance and suggested that the volatile oils may be a factor in their durability. Studies conducted by the CSIRO in Australia (Thornton et al., 1997) and the University of Hawaii in collaboration with the Forest Research Institute of Malaysia (Grace et al., 1989; Grace et al., 1996; Grace et al.,

1998; Wong et al., 1998; Wong et al., 2001; Grace and Tome, 2005) have identified timber species that are inherently resistant to termites. A comprehensive list of durable timber species is included in Appendix 1. These studies have been directed towards selecting timber for plantation that can be utilized in construction and termite resistant wood products. This list may be a useful starting point for further phytochemical investigations of termite resistant timber species.

Studies addressing the termite-active chemical constituents of the timber are limited. The whole essential oil of cedarwood (Adams, 1991), *Litsea cubeba* (Lin and Yin, 1995a) and *Cinnamomum* spp. (Lin and Yin, 1995b) were demonstrated to be repellent to termites. Vetiver, cassia leaf, clove bud, cedarwood, *Eucalyptus globulus*, *Eucalyptus citriodora*, lemongrass and geranium oils were evaluated by Zhu et al., (2001a), with notable activity observed for vetiver and clove bud oil. Several essential oil components have also been reported to be toxic to termites including chamaecynone (**52**) from *Chamaecyparis pisidera* D. Don (Saeki et al., 1973), the naphthoquinones; 7-methyljuglone (**53**) and its dimer isodiospyrin (**55**) isolated from the wood of *Diospyros virginiana* L. (Carter et al., 1978) and torreyal (**54**) from *Torreya nucifera* Sieb. et Zucc., (Ikeda et al., 1978). Recent work by Ganapatay et al., (2004) on *Diospyros* species has described the termiticidal activity of plumbagin (**56**), microphyllone (**57**) and isodiospyrin (**55**) from the roots of *Diospyros sylvatica*.



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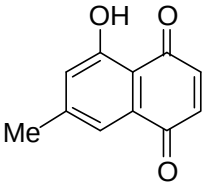

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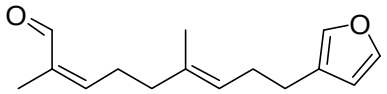

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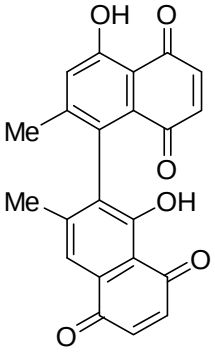

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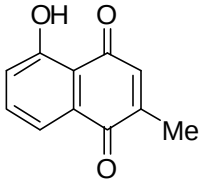

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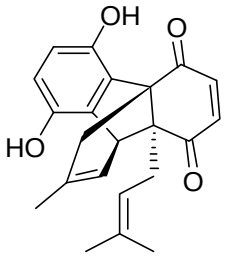

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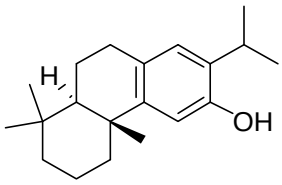

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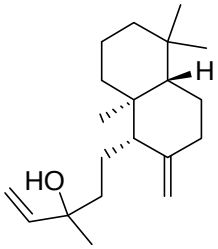

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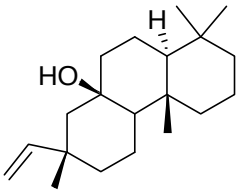

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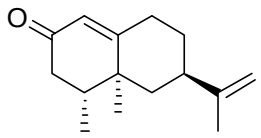
A number of terpenoids from essential oils such as citronellal, citral, geraniol, and eugenol have exhibited repellent properties against the Formosan termite (Cornelius, et al., 1997; Sharma et al., 1994). Scheffrahn and co-workers (1988) isolated ferruginol (**58**), manool (**59**), and nezukol (**60**) from bald cypress, *Taxodium distichum* L. wood that possessed antifeedant properties towards *C. formosanus*. Geranyllinalool caused direct mortality to termites as reported by Nangan and Clement (1990). The azadirachtins, which are the systemic insecticides from Neem (*Azadirachta indica*),

have also shown promise as a natural termiticide (Grace and Yates, 1992; Delate and Grace, 1995b).

Osbrink and co-workers (2005) have evaluated five natural and seventeen conventional termiticides against *C. formosanus* Shiraki. They found that the natural naphthoquinones; menadione, plumbagin, and juglone and the natural benzoquinones; thymoquinone and co-enzyme Q1 were capable of producing 100% mortality at the highest concentrations tested (5% w/w as a dried powdered extract). The natural products possessed minimal toxicity at concentrations less than 0.5% (w/w). With the exception of boric acid, all of the synthetic termiticides evaluated were ten-fold more potent. "The synthetic termiticides; permethrin, *cis*-permethrin, *trans*-permethrin, cypermethrin, *α*-cypermethrin, *β*-cypermethrin, bifenthrin, fenvalerate, cyfluthrin, *β*-cyfluthrin, deltamethrin, tralomethrin, chlorpyrifos, propoxur, imidacloprid, caused 100% mortality at concentrations less than 0.05% (w/w)" (Osbrink et al., 2005.) To date, apart from the synthetic derivatives of the pyrethrins and nicotine no natural products are presently commercially registered for the control of termites.

Towards the completion of our research, we became aware of the discoveries made by a research group at the Louisiana State University Agricultural Centre (LSU Ag. Centre). The group were investigating the termiticidal activity of vetiver oil. Vetiver oil is distinguished by the presence of *α*- and *β*-vetivone, structures **62** and **63** respectively, which are remarkably similar to eremophilone but arise from different biosynthetic pathways. It has been identified that nootkatone (**61**) is the most active constituent of the vetiver root oil (Zhu et al., 2001a; Zhu et al., 2001b; Ibrahim et al., 2004a). Nootkatone (**61**) is used commercially as a flavouring agent (grapefruit) and is a major

component of the termite resistant Alaskan Yellow Cedar (*Chamaecyparis nootkatensis* (Erdtman and Hirose, 1962; Zhu et al., 2001b). It is minor component of vetiver oil (Fergeus, 2007).



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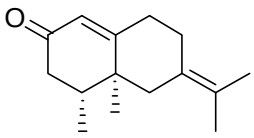

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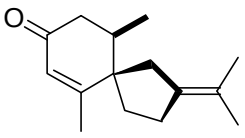

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6.2 Background of These Studies

Extracts of *E. mitchellii* wood were found to be toxic to termites (Leach et al., 2004), a series of investigations were conducted to elucidate the mode(s) of action of the crude wood oil against termites. These included a series of bioassays to determine if the oil could act as a toxicant via fumigation, had a repellent effect or could cause mortality by direct contact with either the fresh oil or dried residues (Spooner-Hart and Basta, 2006). Lastly, studies were conducted to assess whether the oil might be utilized as a chemical soil barrier (Spooner-Hart and Basta, 2006).

6.2.1 Efficacy of *E. mitchellii* Wood Oil against Termites - Topical Application

The whole wood oil was evaluated for acute dermal toxicity via topical application. Test solutions containing between 0 - 5000 ppm of *E. mitchellii* wood oil were used for the initial screening. The relative efficacies of *E. mitchellii* wood oil against *N. exitiosus* and *C. acinaciformis* are given in Table 6.1.

It was apparent that the whole oil possessed significant activity against termites. There was a strong dose-mortality response for both termite species. For *N. exitiosus*, the LC_{50} and LC_{90} values for 2.5 mL aliquots were approximately twice those for 5.0 mL aliquots, confirming the dose-related response. The estimated LC_{50} and LC_{90} values for *C. acinaciformis* for 5.0 mL aliquots were higher than those for *N. exitiosus*, indicating that the latter termite species was more susceptible.

Table 6.1. Relative efficacy of *E. mitchellii* oil against *N. exitiosus* and *C. acinaciformis*

wood oil (vol)	**Termite species**	**N**	**LC_{50} ppm (95%CL)**	**LC_{90} ppm (95%CL)**	**Slope# ±SE**	**$\chi 2$**	**df**
2.5 mL	*N. exitiosus*	800	621 (572-686)	1014 (877-1280)	6.017 ±0.434	92.3	30
5.0 mL	*N. exitiosus*	480	359 (339-378)	465 (440-500)	10.051 ±0.794	41.8	18
5.0 mL	*C. acinaciformis*	400	1357 (1002-1636)	3342 (2787-4456)	3.273 ±0.416	20.7	14

Pearson goodness of fit test

6.2.2 Efficacy of *E. mitchellii* Wood Oil Residues against Termites

The whole wood oil was evaluated for acute dermal toxicity against termites via contact with dried residues of *E. mitchellii* wood oil. Test solutions containing between 0 - 5000 ppm of *E. mitchellii* wood oil were used to test the efficacy of the dried residue. The relative efficacies of the wood oil against *N. exitiosus* and *C. acinaciformis* are given in Table 6.2. A strong dose-mortality response was observed for both termite species. The estimated LC_{50} and LC_{90} values for *C. acinaciformis* were higher than for *N. exitiosus*, indicating that the latter termite species was more susceptible.

Table 6.2. Relative efficacy of dry fresh residues of *E. mitchellii* oil against *N. exitiosus* and *C. acinaciformis.*

Termite species	**N**	**LC_{50} ppm (95% CL)**	**LC_{90} ppm (95% CL)**	**χ^2#**	**Slope ±SE**	**df**
N. exitiosus	700	634 (557-721)	1107 (940-1412)	56.993	5.292 ±0.408	22
C. acinaciformis	500	1357 (1002-1636)	3342 (2787-4457)	20.689	3.273 ±0.416	14

Pearson goodness of fit test

6.2.3 Fumigant Properties of *E. mitchellii* Wood Oil

To assess the fumigant properties of the oil, termites were contained in a Kilner jar and exposed to filter papers treated with *E. mitchellii* wood oil (20000 ppm). The filter papers were fitted to the lid of the jar and consequently the termites had no direct contact with the test solution. Results for fumigant activity of the wood oil are presented in Table 6.3. No termite mortality occurred in either; the oil treatment or in the control 24 h after sealing the jar, but at 96 h 100% mortality had occurred in the *E. mitchellii* wood oil-treated jars, whereas no mortality had occurred in the controls.

Table 6.3. Mortality (%) of workers of *C. acinaciformis* exposed to vapours of *E. mitchellii* wood oil in a Kilner jar.

Conc. ppm	**Time hours**	**R1**	**R2**	**R3**	**R4**	**R5**	**R6**	**R7**	**R8**	**R9**	**Total % Mortality (n=9)**
0.00	24	0	0	0	0	0	0	0	0	0	0.0
	96	0	0	0	0	0	0	0	0	0	0.0
20000	24	0	0	0	0	0	0	0	0	0	0.0
	96	100	100	100	100	100	100	100	100	100	100.0

6.2.4 Termite Repellent Activity of *E. mitchellii* Wood Oil - Choice Tests

In the choice test, one filter paper impregnated with a control solution and another with a solution of *E. mitchellii* wood oil was placed in a termite enclosure. In the no choice assay two filter papers impregnated with the test solution were placed in the termite enclosure and termite behaviour was recorded after 3 days. The results on the repellency studies are given in Table 6.4. In both no-choice and choice investigations, *E. mitchellii* wood oil showed high repellent effect against both *N. exitiosus* (at 2000 ppm) and *C. acinaciformis* (at 5000 ppm), with mean RI values >83%. There were no significant differences between any of the treatments in either choice or no-choice investigations. There were no significant differences between any of the means ($P \leq 0.05$).

Table 6.4. Repellency indices of *E. mitchellii* wood oil against *N. exitiosus* and *C. acinaciformis* workers in choice and no-choice experiments.

Termite species	Test	RI (%) Rep 1	RI (%) Rep 2	RI (%) Rep3	Mean RI (%) ± S.D.
N. exitiosus	No choice	83.40	88.47	86.37	86.08 ± 2.55
	Choice	96.55	100.00	100.00	98.85 ± 2.00
C. acinaciformis	No choice	84.00	88.90	86.36	86.42 ± 2.45
	Choice	96.55	100.00	100.00	98.85 ± 2.00

6.2.5 Barrier Studies of *E. mitchellii* Wood Oil

Tests were conducted to determine the efficacy of *E. mitchellii* oil as a barrier treatment to prevent termite intrusions using modified bioassay tubes containing sand (Figure 6.2), (Su et al., 1995). It was found that termites tunnelled further in the control than in

any of the experiments containing sand barriers treated with *E. mitchellii* wood oil. The results for the effects of a fresh 2 cm sand barrier treated with different concentrations of the wood oil (layer 3: L3, refer to Chapter 2 for a detailed description) on termite tunnelling over a 7 d challenge period are presented in Figure 6.2. L3 was only breached in the control, and there was a strong trend towards reduction in mean vertical length tunnelled with increasing concentration of wood oil in the barrier.

Figure 6.2. Barrier studies for *E. mitchellii* wood oil showing the difference in tunnelling of *N. exitiosus* workers in water treated sand control (bottom 8 replicates) and *E. mitchellii* wood oil (top 8 replicates). (Photograph courtesy of Robert Spooner-Hart and Albert Basta.)

The results for efficacy of aged *E. mitchellii* oil-treated sand barriers are presented in Tables 6.5 and 6.6. L3 was only breached in the control, and even in the 38-day old sand barrier with 20000 ppm wood oil, there was minimal termite incursion into L2, the untreated sand below the barrier, even after challenging for 16 d. With the 240 day old sand barrier, there was tunnelling into L2 after challenging with *C. acinaciformis* for 21 d, but the termites did not reach L3.

Table 6.5. Means (cm ± SD) for length of vertical tunnelling by *N. exitiosus* through layers in bioassay tube in response to different age sand barrier treated with *E. mitchellii* wood oil (L3).

Age of treated sand barrier (d)	**time (d)**	**Control (0 ppm wood oil)** (cm ± SD)				**5000 ppm wood oil** (cm ± SD)				**10000 ppm wood oil** (cm ± SD)				**20000 ppm wood oil** (cm ± SD)			
		L1	**L2**	**L3**	**L4**	**L1**	**L2**	**L3**	**L4**	**L1**	**L2**	**L3**	**L4**	**L1**	**L2**	**L3**	**L4**
0-day old	1	3.00 ±0.0	0.45 ±0.40	0	0	0.20 ±0.18	0	0	0	0.16 ±0.16	0	0	0	0.10 ±0.10	0	0	0
	4	3.00 ±0.0	2.80 ±0.40	1.50 ±0.90	0.75 ±0.46	1.30 ±0.50	0	0	0	0.90 ±0.60	0	0	0	0.56 ±0.40	0	0	0
	7	3.00 ±0.00	3.00 ±0.00	2.00 ±0.00	1.00 ±0.00	1.4 ±0.50	0	0	0	0.94 ±0.60	0	0	0	0.56 ±0.44	0	0	0
14-day old	7	3.00 ±0.00	3.00 ±0.00	2.00 ±0.00	1.00 ±0.00	n.a				n.a				3.00 ±0.00	0.13 ±0.06	0	0
38-day old	16	3.00 ±0.00	3.00 ±0.00	2.00 ±0.00	1.00 ±0.00	n.a				n.a				3.00 ±0.00	0.18 ±0.08	0	0

L1= Bottom agar layer (3cm); L2= Untreated sand layer (3cm); L3= Treated sand layer (2cm); L4= Top agar layer (1cm)

Table 6.6. Means (± SD) for length of vertical tunnelling by *C. acinaciformis* through layers in bioassay tube in response to 240-day old sand barrier originally containing 20000 ppm *E. mitchellii* wood oil.

Treatment	Age of treated sand (d)	Time after termite release (d)	Mean distance tunnelled (cm)				Mean total distance tunnelled (cm) ± SD
			L1	L2	L3	L4	
0 ppm	240	1	3.00	3.00	2.00	1.00	9.00 ± 0.00
20000 ppm Wood oil.	240	1	2.64	0.34	0	0	2.98 ± 0.86
20000 ppm Wood oil	240	21	3.00	1.80	0	0	4.80 ± 1.01

L1= Bottom agar layer (3cm); L2= Untreated sand layer (3cm); L3= Treated sand layer (2cm); L4= Top agar layer (1cm)

6.2.6 Discussion

The wood oil was shown to act as a contact toxicant to all termite species tested, including the economically important subterranean species, *C. acinaciformis* and *N. exitiosus*. However, the susceptibility of termite species differed, as evidenced by their different LC_{50} and LC_{90} values. *C. acinaciformis* was the least susceptible of the species tested. Observations confirmed that when termites and filter papers on which they were located were sprayed with 5 mL aliquots of wood oil at concentrations ≥ 250 ppm for *N. exitiosus* and ≥ 625 ppm for *C. acinaciformis*, the termites began to show signs of intoxication, and at higher concentrations, frequently died within 3-8 h.

The wood oil did not have rapid fumigant activity against termites, with no mortality recorded 24 h after their introduction into the container, although 100% mortality (with no corresponding control mortality) was recorded at 96 h. It was, therefore, not possible to calculate a KT_{50} value.

E. mitchellii oil residues were highly repellent to termites in both choice and no choice investigations, with RI values > 83% being recorded in filter paper tests. The wood oil demonstrated repellent activity against both *N. exitiosus* and *C. acinaciformis*, when added to sand and used as 2 cm barriers in test-tube investigations. In fact, in no case did termites tunnel into any oil-treated barrier, and, commonly, they did not tunnel far into the untreated sand layer directly below the treated layer. Mean vertical termite tunnelling was related to initial wood oil concentration in the barrier. The "barrier repellency" was maintained for sand initially treated with 20000 ppm for ≥ 240 d, under our experimental conditions. Many termites in the test tube bioassays commonly died: in investigations with higher concentrations of fresh *E. mitchellii* oil-treated barriers,

this was probably associated with the toxic activity of the wood oil (such as fumigant effect), but with older age barriers, it was probably a function of starvation/dehydration.

6.3 Results and Discussion

Studies were conducted in collaboration with CPAFS to determine the chemical constituents of the wood oil that were responsible for the activity against termites. Fractionation of the oil was achieved using normal phase preparative HPLC, with a hexane/ethyl acetate gradient as eluent (Figure 2.3). The fractions and subsequent pure compounds were evaluated in a range of termite assays.

6.3.1 Efficacy of *E. mitchellii* Fractions against Termites - Topical Application

The prep HPLC fractions were subjected to a termiticidal assay using *N. exitiosus* as the test organism. Fractions were tested at concentrations ranging from between 0-5000 ppm, which corresponded to application rates of 0-25 mg/5 mL aliquot/64 cm^2 treatment area. The relative efficacies of the wood oil fractions against *N. exitiosus* are given in Table 6.7. Fraction F1 exhibited moderate activity at the highest concentration tested. Analysis of the GC-MS data indicated that fraction F1 was composed of sesquiterpene hydrocarbons, whilst fractions F4-F10 were composed of oxygenated sesquiterpenes. These later fractions possessed mixed efficacy with fractions F4 and F5 exhibiting moderate activity at concentrations less than 3 mg/application. Fraction F11-28 contained higher MW non-volatile compounds that had negligible activity against the termites.

Table 6.7. Relative efficacy of *E. mitchellii* wood oil fractions against *N. exitiosus* at 24 hours HAT.

	Total % Mortality					
Concentration ppm	**F1**	**F3**	**F4**	**F5/6**	**F8-10**	**F11-28**
0	0.0	0.0	0.0	0.0	0.0	0.0
312	0.0	0.0	20.0	0.0	4.0	0.0
625	0.0	5.3	26.6	65.0	0.0	0.0
1250	0.0	1.3	84.0	100.0	0.0	0.0
2500	6.7	20.0	96.0	100.0	0.0	0.0
5000	66.7	49.3	98.7	100.0	0.0	24.0

Mean of 3 replicates, 20 termites per replicate.

6.3.2 Efficacy of *E. mitchellii* Fractions against Termites - Dried Residues

The two most efficacious fractions were F4 and F5/6. The relative efficacies of *E. mitchellii* wood oil and its fractions F4 and F5/6 against *C. acinaciformis* are given in Table 6.8, 24 and 48 h after treatment (HAT). The termite mortality increased with concentration of the tested products, and with exposure time to their residues. Fractions F4 and F5/6 produced similar comparative mortality to the crude wood oil.

Table 6.8. Mortality of workers of *C. acinaciformis* when exposed to filter papers containing fresh dry residues after treatment with 2000 ppm and 5000 ppm *E. mitchellii* whole oil and fractions F4 and F5/6.

Product	Conc. ppm	24 hours (HAT) Total % Mortality ±SD*	48 hours (HAT) Total % Mortality ±SD*
Acetone only control	0	0.00	0.00
Crude oil	2000	20.63±7.29	98.75±2.32
	5000	100.00	100.00
F4	2000	22.50±7.07	86.88±10.33
	5000	97.50±4.63	100.0
F5/6	2000	19.00±8.91	93.13±8.82
	5000	96.25±3.54	100.00

* Means of 8 replicates, 20 termites per replicate

6.3.4 Barrier Studies of *E. mitchellii* Fractions

Barrier studies were also performed on these two fractions. The results for the effects of a fresh 2 cm sand barrier treated with *E. mitchellii* oil and fractions F4 and F5/6 (L3) on

Table 6.9. Means (± SD) for length of vertical tunnelling by *N. exitiosus* efficacy of sand barrier of *E. mitchellii* wood oil fractions F4 and F5/6.

Lapsed time (days)	**Control (n=8)** Means (± SD)				**1000 ppm Fraction 4 (n=5)** Means (± SD)				**1000 ppm Fraction 5/6 (n=5)** Means (± SD)			
	L1	L2	L3	L4	L1	L2	L3	L4	L1	L2	L3	L4
1	3.00 ±0.00	3.00 ±0.00	2.00 ±0.00	1.00 ±0.00	3.00 ±0.00	0.33 ±0.25	0.00	0.00	3.00 ±0.00	0.35 ±0.19	0.00	0.00
7	3.00 ±0.00	3.00 ±0.00	2.00 ±0.00	1.00 ±0.00	3.00 ±0.00	0.33 ±0.25	0.00	0.00	3.00 ±0.00	0.35 ±0.19	0.00	0.00
14	3.00 ±0.00	3.00 ±0.00	2.00 ±0.00	1.00 ±0.00	3.00 ±0.00	0.33 ±0.25	0.00	0.00	3.00 ±0.00	0.35 ±0.19	0.00	0.00
21	3.00 ±0.00	3.00 ±0.00	2.00 ±0.00	1.00 ±0.00	3.00 ±0.00	0.33 ±0.25	0.00	0.00	3.00 ±0.00	0.35 ±0.19	0.00	0.00

L1= Bottom agar layer (3cm); L2= Untreated sand layer (3cm); L3= Treated sand layer (2cm); L4= Top agar layer (1cm)

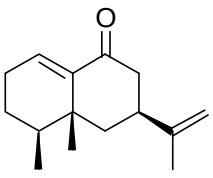
termite, *N. exitiosus*, tunnelling over a 21 d challenge period are presented in Table 6.9. L3 was only breached in the control, indicating that these fractions contain compounds associated with repellent activity of the barriers.

6.3.5 Discussion

The investigations confirmed that *E. mitchellii* oil fractions F4 and F5/6 were highly active against termites, causing similar acute toxicity to the whole oil, via topical application and fresh residues, and repellent activity in both filter paper and barrier trials. The results suggest that these two fractions are important contributors to the modes of termite activity reported here for *E. mitchellii* oil.

6.3.6 LD_{50} and LD_{95} of the Eremophilanes from *E. mitchellii* Wood Oil by Direct Contact Mortality

The purification of the four major components of the oil is described in Chapter 5. Each of the pure compounds (**30**, **33**, **35** and **36**) and the whole oil were evaluated for termiticidal activity against *N. walkeri* (the raw data is included in the appendices). The LD_{50} and the LD_{95} was then determined for each compound (Table 6.10). There was only a sufficient quantity of compound **33** to perform 2 replicates and insufficient quantities of compounds **42** and **32** (minor components of the oil) to include these in the termiticidal assays.



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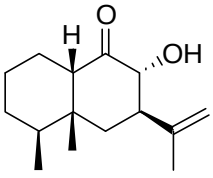

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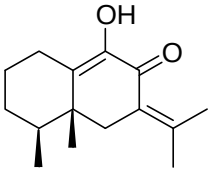

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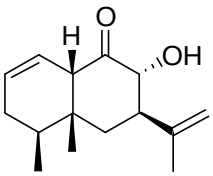

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Table 6.10. Mortality of *N. walkeri* associated with pure compounds 24 hours after treatment (direct contact mortality).

Product	LC_{50} (ppm)[a] (95% CL)[b]	LC_{90} (ppm)[a] (95% CL)[b]	χ^2	df	n[c]	Intercept ± .SE	Slope ± SE	P[d]
Eremophilone (30)	744 (602 – 890)	1619 (1300 – 2317)	19.427	13	266	- 10.900 ± 1.342	3.796 ± 0.455	0.110
8-Hydroxy-11-eremophilen-9-one (35)	3324 (2491 – 5307)	19506 (10058 – 72667)	11.304	12	266	- 5.873 ± 0.949	1.668 ± 0.292	0.503
9-hydroxy-7(11),9-eremophiladien-8-one (36)	2978 (2791 – 3155)	4262 (3926 – 4838)	3.243	10	235	- 28.606 ± 3.822	8.234 ± 1.093	0.975
8-Hydroxyeremophilone (33)	1983 (1761 – 2251)	2838 (2456 – 3714)	2.528	6	173	- 27.131 ±5.157	8.228 ± 1.568	0.865
Whole oil	811 (535 – 1083)	1952 (1452 – 4019)	26.244	10	240	- 9.779 ± 1.343	3.362 ± 0.446	0.003

Probit model: Probit (p) = Intercept + BX (Covariate X are transferred using the base 10.000 logarithm).
[a]LC_{50} and LC_{90} data were determined by probit analysis (SPSS Version 15, 2008); concentration (ppm) in 200 ppm Triton X-100 in distilled water.
[b]CL confidence limits.
[c] number of insects tested.
Statistics based on individual cases differ from statistics on aggregated cases.
[d] Since the significance level is less than 0.150, a heterogeneity factor is used in calculation of confidence limits.
The same three replicates of the untreated control (ethanol in 200 ppm Triton X-100 in distilled water) were included in the calculation of the estimates of the LC_{50} and LC_{90} values for each compound.

It appears from these results (Table 6.10) that eremophilone (**30**) and the 8-hydroxyeremophilone (**33**) possessed the most potent toxicity towards the termites, which is consistent with the activity associated with fraction F4 and F5/6. It was also noted that eremophilone (**30**) possessed similar activity to the crude oil. This may possibly be due to some of the minor constituents contributing to the potency of the whole oil. It was also apparent that activity was not enriched upon isolation suggesting the loss of active components, possibly via irreversible adsorption or degradation during silica chromatography.

6.3.7 Discussion

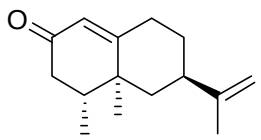
The sesquiterpene ketone, compound **30** possesses the greatest activity followed closely by its 8-hydroxylated compound **33**. Compound **36** is less active whilst activity of compound **35** is greatly diminished. Activity appeared to be related to the presence of an α,β-unsaturated ketone group and having a relatively un-hindered as well as activated oxygen in the carbonyl group at C-8.

It is possible that some compounds, like **35**, may have solubility problem, thus giving inaccurate reults. Compound **35** readily precipitated out of solution during application, this is reflected in the statistically poor correlation between dose and response. Consequently, an accurate determination of LD_{50} and LD_{95} was not possible. The % mortality associated with compound **35** did increase over time and indicates that **35** possesses important activity. It is not unexpected that chemical reactivity correlates with biological activity.

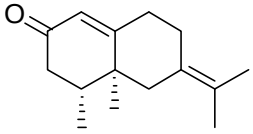
It is emphasized that the termite active oil that we have characterised is only present in the heartwood of the tree and absent in the bark and outer white wood. The tree develops the darker heartwood as it ages implying that younger trees will produce very low oil yields and are not suitable for oil harvesting. The root oil is composed of almost 50% eremophilone, the most active component of the wood oil, suggesting that the root oil will possess activity similar to or greater than the wood oil. Samples of the leaf and root oil were sent to CPAFS together with key components of the leaf oil that were commercially available; α-pinene and limonene. To elaborate on the nature of the activity of the leaf oil, a sample of the oil was separated into polar and non-polar metabolites by partitioning between hexane and methanol.

6.3.8 Other Termite-active Sesquiterpenes

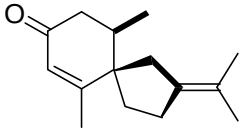
Nootkatone and its derivatives, like eremophilone, have shown insecticidal activity against a broad range of insects and several patents claim the incorporation of nootkatone into an aerosol to repel mosquitoes (Masahiro and Kazumasa, 1999), against termites, midges and mosquitoes (Zhu et al., 2005), and ants, ticks and cockroaches (Henderson et al., 2005). Maistrello et al. (2003) reported that nootkatone possessed a novel mode of action. Lower termites (Thorne, 1998; Pearce, 1997) depend mostly (Doolittle et al., 2007) on their gut microbiota to metabolise cellulose. Nootkatone (**61**) was found to be toxic to the gut protozoa of the termites consequently starving the termites to death.



nootkatone (**61**)



α-vetivone (**62**)



β-vetivone (**63**)

Of greater interest to this project was to compare the activity of *α*-vetivone and nootkatone that possess structural similarities to that of eremophilone. It was decided to include nootkatone, vetiver oil and an enriched fraction of *α*-vetivone in an assay together with eremophilone, the most active component of the wood oil. A commercial sample of vetiver oil was obtained from Australian Botanical Products. GC-MS analysis indicated that the oil contained approximately 10% vetivones (% FID). An enriched fraction of the oil containing approx. 40% vetivones was prepared by column chromatography of the oil. The fraction (F4) containing an enriched mixture of *α*- and *β*-vetivone was sent to CPAFS for termiticidal screening.

The products were evaluated using Potter Spray Tower topical applications to determine their acute dermal toxicity against adult workers of *N. exitiosus*. The products were initially investigated in range-finding assays, and then appropriate concentrations were applied to ensure the range of mortalities required for analysis. Because of the limited amount of product, each was tested with two or three treatment replicates. The raw data and observations are given in the appendices.

Table 6.11. Calculated estimates of LD_{50} and LD_{95} values (ppm (w/v)) with other relevant statistical parameters.

Product	**LD_{50} (ppm) (95% CL)**	**LD_{95} (ppm) (95% CL)**	**χ^2**	**DF**	**Intercept ± SE**	**Slope ± SE**	**P**	***Goodness-of-fit (Chi square)**
(+)-nootkatone	967 (839-1193)	1127 (1397-3562)	47.2	13	-17.8±0.7	6.0 ± 0.7	0.000	Significant
vetiver oil - fraction 4	1184 (1039-1320)	2387 (1992-3345)	42.4	16	-16.6 ± 1.7	5.4 ± 0.5	0.000	Significant
leaf oil methanol	1534 (1229-1780)	3153 (2531-5280)	31.9	11	-16.7 ± 2.1	5.3 ± 0.6	0.001	Significant
leaf oil hexane fraction	1695 (1456-1890)	3478 (3047-4266)	33.3	18	-17 ± 1.7	5.3 ± 0.5	0.015	Significant
***E. mitchelli* leaf oil fraction**	3042 (2615-3451)	8455 (6998-11196)	49.1	24	-12.9±1.1	3.7 ± 0.3	0.002	Significant
***E. mitchellii* root oil**	3615 (3315-3912)	9033 (7841-10976)	24.9	19	-14.7±1.4	4.1 ± 0.4	0.165	Not significant
eremophilone	3789 (3462-4168)	7566 (6412-9814)	24.7	16	-19.6±1.8	5.5 ± 0.5	0.076	Significant
α-pinene	5943 (5415-6127)	9000 (8022-10871)	37.4	19	-31.7 ± 2.9	8.5 ± 0.8	0.007	Significant
vetiver root oil	4577 (3903-5116)	12120 (9960-17168)	9.7	10	-14.2±2.3	3.9 ± 0.6	0.468	Not Significant
limonene	>10000	>10000						

* If Goodness-of-Fit (Chi square) is significant, a heterogeneity factor is used in the calculation of confidence limits

6.3.9 Discussion

The bioassay results and the estimates obtained from the statistical analysis indicate that amongst the products tested the highest efficacy was observed for (+)-nootkatone (LD_{50} and LD_{95} were the lowest; also its regression line slope is high). This is also represented graphically (Figures 6.3 and 6.4), with the most efficacious products appearing on the left hand side of the graphs, with the slope of the dose-response being represented graphically. Considering the LD_{50} values (the best value for comparisons of efficacy) as shown in Table 6.11, it appears that the activity of vetiver oil fraction 4 is very close to that of nootkatone.

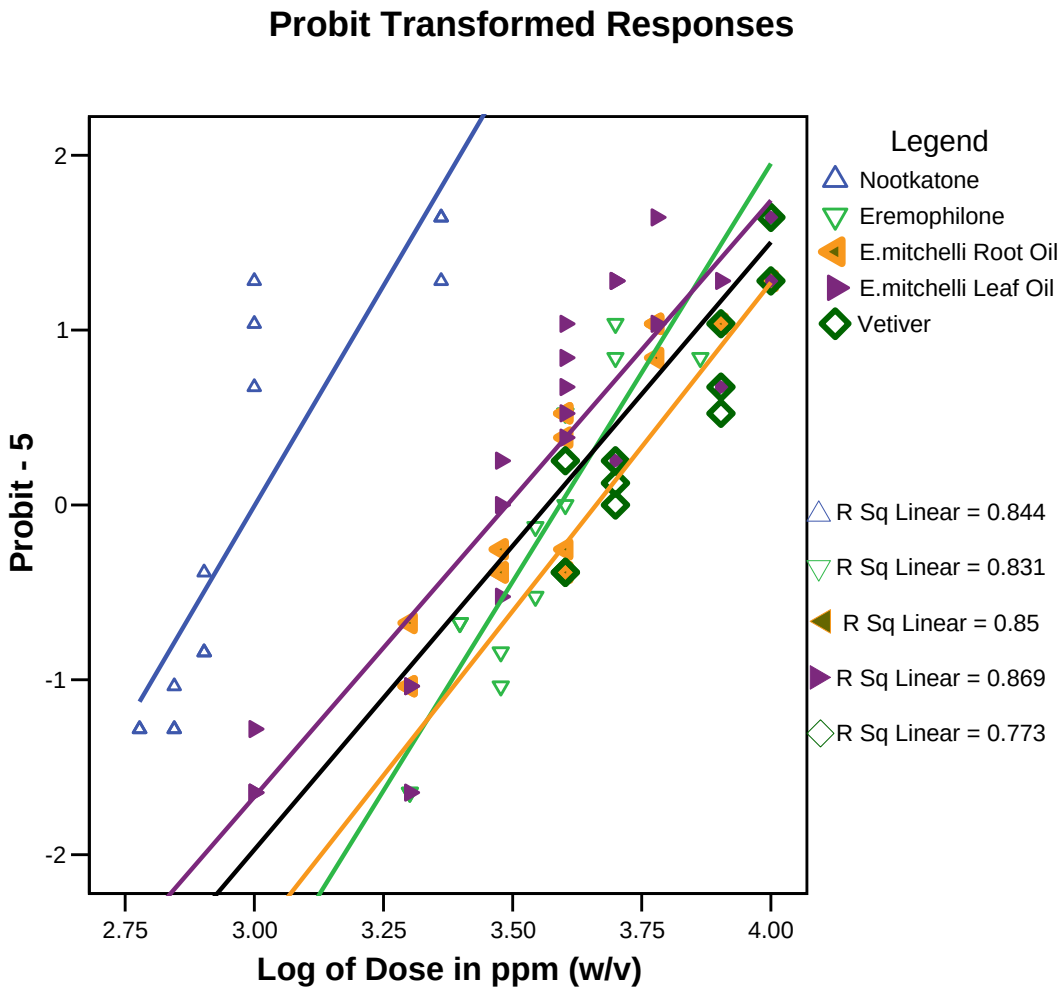


Figure 6.3. Probit analysis plots of termiticidal activity for selected products at 24 hours.

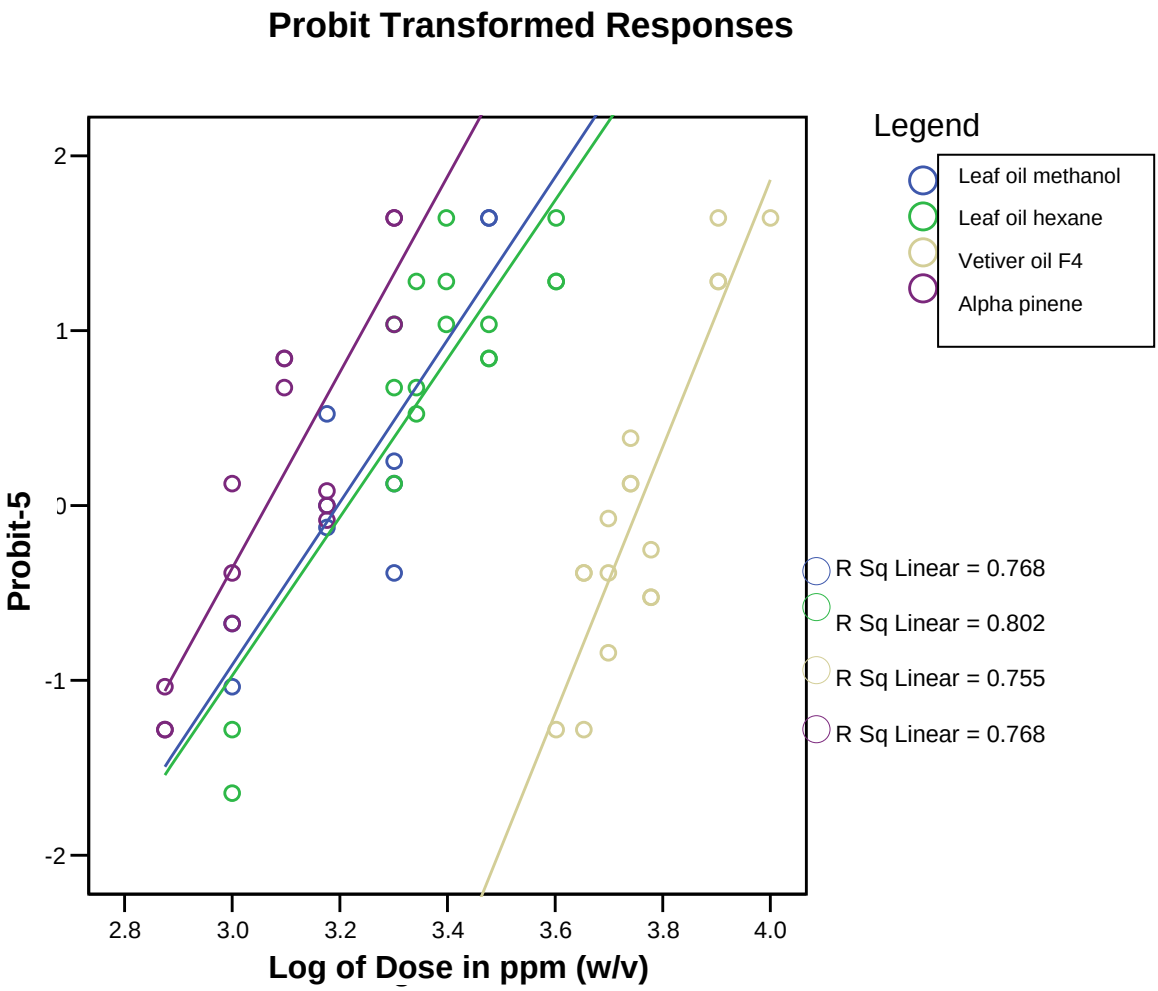


Figure 6.4. Probit analysis plots for termiticidal activity of selected products at 24 hours.

The non-polar (hexane) and polar (methanol) fractions of *E. mitchellii* leaf oil had an efficacy approximately one half of that of (+)-nootkatone. The methanol fraction showed similar efficacy to the hexane fraction, but the speed of action was faster (i.e. symptoms of intoxication were quicker) for the polar metabolites. Referring to Table 6.11, *E mitchellii* root oil, leaf oil and eremophilone appeared to have similar efficacy but all were less than that of (+)-nootkatone and the *E. mitchellii* leaf oil fractions. *α*-Pinene showed good efficacy at concentration levels greater than 5000 ppm (w/v). It appeared that limonene had no efficacy even at 10000 ppm (w/v) against workers of *N.*

exitiosus. Additionally there is a trend towards steeper slopes on the probit regression lines for pure compounds over mixtures. This is also a feature of synthetic (i.e. single active constituent) products and reflects a single and consistent mode of action.

Note that in a number of treatments, the mortality counts conducted 24 h after treatment, by themselves, may not fully reflect the activity of the product. For example, most moribund insects, while not technically dead, did not recover. That is why these data observations are included in the data. Thus, the results partly reflect the speed of action of the products. It was not possible to extend the time period for the mortality measurements to 48 h, as at this time, there was >10% mortality in the controls, which would have lead to rejection of the data sets. The observed continued high levels of moribund insects were one reason for repeating experiments, although as indicated above, both sets of data were included in the analyses, for accuracy of reporting.

It was noted in the last set of termiticidal experiments that the eremophilone (**30**) was not as potent in the assays against *N. exitiosus* as it was against *N. walkeri*. The potency of termiticides is species dependent.

6.4 Conclusion

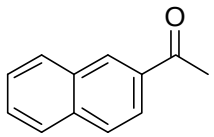
Our results indicate that *E. mitchellii* leaf, wood and root oils possess significant termiticidal activity. Preliminary investigations of the leaf oil have demonstrated that both the polar and non-polar metabolites in the oil are active in direct contact mortality assays. The major constituent of the leaf oil, α-pinene (25%) did not contribute significantly to the activity whilst limonene was not found to be active in this assay. The discovery of termiticidal activity in the leaf oil is interesting and unexpected, and

thus warrants further investigation. It is thought that the leaf oil is unlikely to be utilized in termite products due to the volatility of these compounds, however the fumigant capacity of the leaf oil may be worthy of investigation.

Eremophilone (**30**) and the wood oil are less volatile and possessed significant termiticidal activity in the direct contact mortality assays. The repellency, toxicity and speed of kill observed for *E. mitchellii* wood oil and eremophilone indicate that these products are not suitable for use in baiting systems or as remedial termiticides. It is possible that the heartwood timber may be utilized in termite durable wood products or the oil may be used to impregnate susceptible timbers. Sesquiterpenes are less volatile, a characteristic that is important for chemical soil barriers and timber treatments. The oils possess low water partitions, an important property for their employment in chemical soil barriers and as a timber treatment.

In regards to harvesting the oil for commercial production, the age of the tree is important since the oil is only present in the mature heartwood. The roots could also be harvested to obtain a valuable oil with a high proportion of eremophilone. Methods have been developed to obtain pure compounds from oil. However, this is not justified from a cost benefit perspective, as the purified compounds did not have a marked increase in potency over the whole oil.

Recent investigations of the structure-activity of analogues of the vetivones have arrived at 2-acetonaphthone (**64**) as the critical termiticidal structure (Ibrahim, 2004b; 2004c.). The presence of an α,β-unsaturated ketone is a common feature of all of the natural termiticides discovered to date.



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The simplicity of this structure suggests that the *α,β*-unsaturated carbonyl group is essential for activity and renders the eudesmane/eremophilane/vetivone skeleton argument moot. However, the structural features and functional groups may still impart significant properties such as; the organism-specificity of the compound, the volatility and the chemical stability and other physical properties.

Whilst nootkatone was found to be more potent than eremophilone in our assay, laboratory assays cannot predict how a product will perform *in situ*. Repellency, mode of action, or slower kill rates may be critical to the effectiveness of a termiticide. Many other factors are vitally important to the commercial success of a product such as environmental toxicity, production cost, product half-life, mammalian safety data and organism specificity. *Eremophila mitchellii* oil has the advantage of low production costs and is considered a renewable resource due to its wide distribution, ability to regenerate and weed status. In comparison, vetiver oil is widely available commercially AU$ 192/kg (Australian Botanical Products, 2009) whilst synthetically derived nootkatone costs AU$ 175/g (Sigma-Aldrich, 2009).

Field trials are currently underway to determine the effectiveness of *E. mitchellii* wood oil in field trials. Further studies on the environmental toxicity and safety of the wood oil are planned.

Chapter 7

Cytotoxicity and Phytochemistry of *Eremophila* species

7.1 Introduction

This project arose from commercial interest in the biologically active metabolites from *Eremophila* species. *Eremophila* species are endemic to Australia and have exhibited considerably novel secondary metabolites. The extracts from several *Eremophila* species have demonstrated antibacterial, anti-viral, anti-inflammatory and cardioactive actions and several bio-active compounds have been documented from this genus. The aim of this project was to screen *Eremophila* extracts for cytotoxic activity, pursue interesting bioactivity, and investigate the chemical constituents.

7.1.1 The Genus *Eremophila*

The genus *Eremophila* is one of seven genera, together with *Myoporum*, *Bonita*, *Diocirea*, *Pentacoelium, Calamphoreus* and *Glycocystis* which constitute the small

family Myoporaceae. The family is largely restricted to Australia with only *Myoporum* and *Pentacoelium* occurring in areas including New Zealand, the Pacific Islands, Asia and Japan (Chinnock, 2007). The taxonomy of *Eremophila* has only recently been reviewed by Chinnock (2007), comprising over 350 species, is by far the largest genus in the family. *Eremophila* species are usually small to medium shrubs and are distributed throughout the semi-arid and arid regions with the largest number of species located in Western Australia.

Eremophila species are commonly called fuchsia or emu bushes, dogwood, buddah or eurah. The common name "emu bush" is derived from the erroneous belief that the fruits are commonly eaten by emus and that digestion enhances the rate of seed germination after excretion (Chinnock, 2007). Traditionally the plants have been valued by Aboriginals for ceremonial purposes and feature prominently in their medicinal folklore. More recently, many *Eremophila* have been valued as the main edible shrub species throughout arid regions of Australia (Ghisalberti, 1994b). They have been proven useful in revegetation programs because of their invasive nature and resistance to drought, fire, and grazing. Currently, the genus is being explored for its horticultural potential. Most species of *Eremophila* exhibit ornamental flowers and are valued for their hardiness and drought resistance.

7.1.2 Ethnopharmacology and Ethnobotany

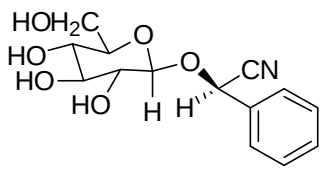
Eremophila species are common and widespread throughout the drier regions of Australia. These plants have been used by Aboriginals to treat colds and influenza, to relieve headaches and pain, as antiseptic washes for cuts and sores, as skin washes to treat scabies, and to promote general well being (Barr, 1988; Low, 1990). To date, the

therapeutic uses of only 17 species of *Eremophila* have been documented, (Richmond and Ghisalberti, 1994) and the medicinal uses of these species has been summarized in Table 7.1.

There have been some attempts to substantiate the traditional uses of *Eremophila* species with scientific evidence. Ghisalberti (1994a) has compiled an extensive review of the ethnopharmacology and phytochemistry of *Eremophila* species and drawn inferences between the phytochemistry and the known biological activity of these compounds.

7.1.3 Chemistry of *Eremophila*

Interest in the chemistry of *Eremophila* arose over 100 years ago, when it was observed that certain species were toxic to livestock. Finnemore and Cox (1930) characterized prunasin (**65**), the cyanogenic glycoside from *E. maculata*. Later Hegarty and co-workers (1970) identified the hepatotoxic furanoid sesquiterpene, (-)-ngainone (**66**) from *E. latrobei*.



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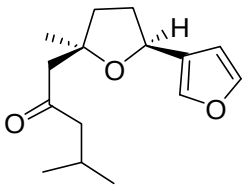

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Table 7.1. Ethnopharmacology of *Eremophila* species. (Adapted from Richmond and Ghisalberti, 1994).

Species	Plant Part	Preparation	Treatment	Reference
E. alternifolia	Leaves	Infusion / ingested	Encourage deep sleep	Tindale, 1937
	Leaves	Decoction	Colds, Influenza, coughs, headaches	Smith. 1991
	Leaves	External	Internal pain	Barr, 1988
	Leaves	External	Septic wounds	Bowen, 1975
	Leaves	Decoction	Body wash for fever	Smith, 1991
E. bignoniflora	Leaves	Decoction	Laxative	Bowen, 1975
	Fruits	Ingested	Purgative	Bowen, 1975
E. cuneifolia	Leaves	Decoction	Colds	Lassak and Mc Carthy, 1983
E. dalyana	Leaves	Body rub	Relieve chest pain	Latz, 1982
	Leaves	Decoction	Body wash for scabies	O'Connell et al., 1983
E. duttonii	Leaves	Antiseptic wash	Sores, cuts, colds, influenza, eye/ear	Smith, 1991
	Leaves	Decoction	Sore throat	Barr, 1988
	Leaves	Decoction	Inflamed and sore eyes, scabies	Knight et al., 1988
	Leaves	External	Insect repellant.	Knight et al., 1988
E. elderi	Leaves	External	Bedding, head rest, colds	O'Connell et al., 1983
E. fraseri	Leaves	Decoction	Colds	Lassak and Mc Carthy, 1983
	Plant	Unknown	Toothache, rheumatism	Lassak and Mc Carthy, 1983
E. freelingii	Leaves	Decoction	Headaches, chest pain	Cleland and Tindale, 1959
	Leaves	Decoction	Antiseptic wash for sores	Smith, 1991
	Leaves	Infusion	Colds	Maconochie, 1970
	Leaves	Decoction	Aches and pains	Low, 1990

Table 7.1.cont. Ethnopharmacology of *Eremophila* species. (Adapted from Richmond and Ghisalberti, 1994).

Species	Plant Part	Preparation	Ailment	Reference
E. freelingii cont.	Leaves	Decoction	Antidiarrhoea	Barr, 1988
	Leaves	Infusion	Headache and rest	Lassak and Mc Carthy, 1983
	Leaves	External	Pillow to promote rest	Meggitt, 1962
	Leaves	Infusion	General well being	Latz, 1982
	Leaves	External	Vapour bath for fever and decongestion	Knight et al., 1988
E. gilesii	Leaves	Decoction	Headaches and chest pain	Cleland and Tindale, 1959
	Leaves	Body wash	Sores	Tynan, 1979
	Leaves	Infusion	Colds, scabies, general well being	Latz, 1982
	Leaves	Pillow	Promote rest	Latz, 1982
E. goodwinii	Leaves	Decoction	Purgative	Latz, 1982
E. latrobei	Leaves	Decoction	Body wash for scabies	Cleland and Tindale, 1959
	Leaves	Infusion	Smoke treatment for newborns	Meggitt, 1962
	Leaves	Decoction	Colds	Smith, 1991
	Leaves	External	Colds and influenza	Smith, 1991
E. longifolia	Leaves	Decoction	Skin/bodywash	Silberbauer, 1971
	Leaves	Infusion	Smoke treatment for mothers, newborns	Cleland and Johnston, 1933, 1937
	Leaves	Decoction	Eyewash	O'Connell et al., 1983
	Leaves	Infusion	Colds	Spencer and Gillen, 1969
	Leaves	Decoction	Counter-irritant	Tynan, 1979
	Leaves/twigs/bark	Infusion	Headache	Spencer and Gillen, 1969
	Leaves	Infusion	Scabies, sores, cuts and boils	Knight et al., 1988
E. maculata	Leaves	Poultice	Colds	Cunningham et al., 1981

Table 7.1.cont. Ethnopharmacology of *Eremophila* species. (Adapted from Richmond and Ghisalberti, 1994).

Species	Plant Part	Preparation	Ailment	Reference
E. mitchellii	Twigs	Smoke	General medicinal purposes	Low, 1990
E. neglecta	Leaves	Infusion	General well-being	Latz, 1982
E. paisleyi	Leaves/twigs	Decoction	Wash for scabies	Latz, 1982
E. sturtii	Branches	Infusion	Backaches	Silberbauer, 1971
	Shrub	Burnt ashes	Backaches	Bowen, 1975
	Leaves	Decoction	Sores and cuts	Smith, 1991
	Leaves	Infusion	Head colds, sore eyes	Barr, 1988
	Leaves	Decoction	Antidiarrhoea	Barr, 1988

Systematic studies on the chemical constituents of *Eremophila* species have occurred since the 1960's (Ghisalberti, 1994b). Consequently many novel sesquiterpenes and diterpenes have been characterized. Much of this work has focused on the leaf constituents. The resins secreted by many *Eremophila* species were found to be composed of a mixture of lipids, flavones and terpenes (Ghisalberti, 1994b). The dominant classes of sesquiterpenes identified in *Eremophila* species are presented in Figure 7.1 and the diterpenes in Figure 7.2.

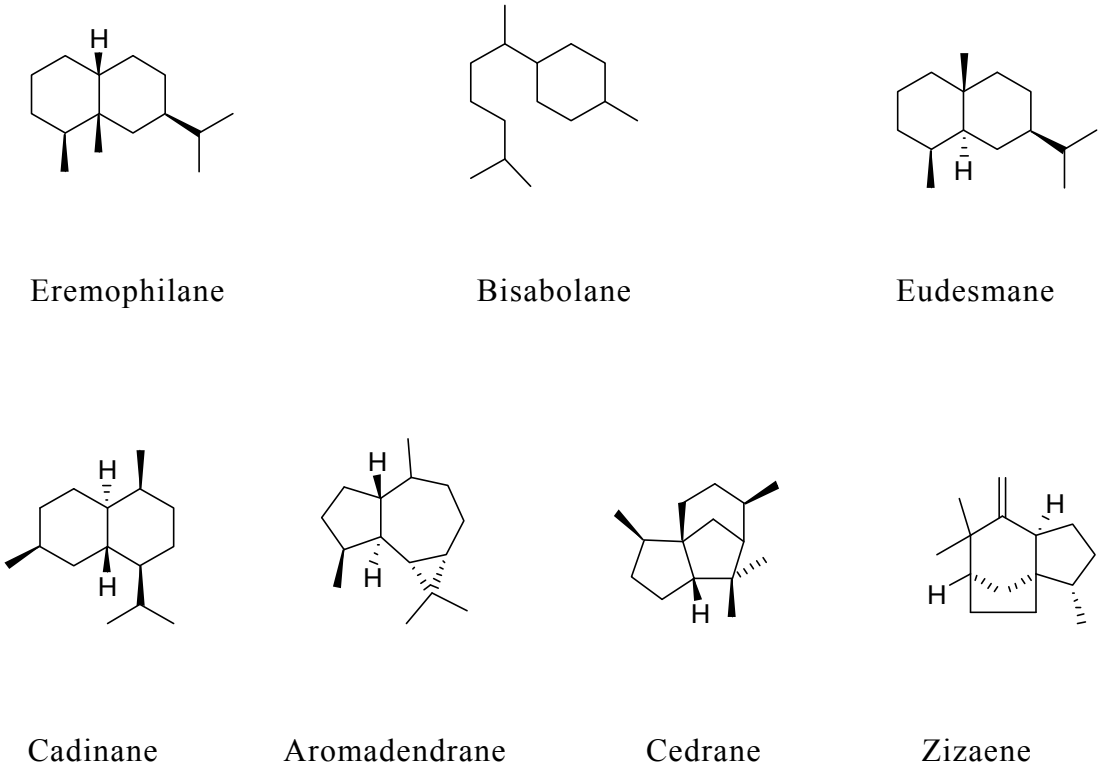


Figure 7.1. Important sesquiterpenes classes from *Eremophila* species.

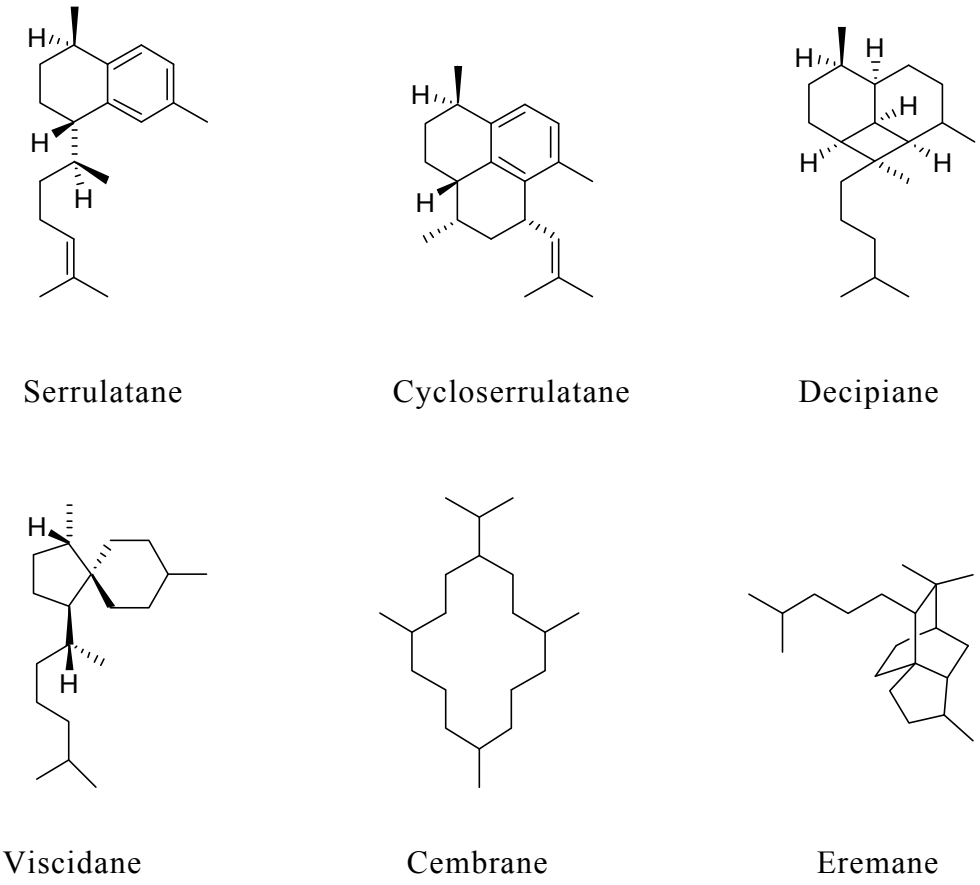
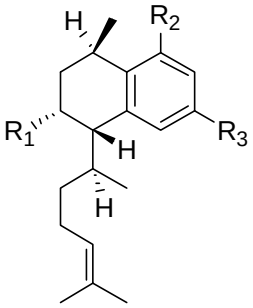


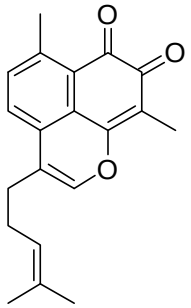
Figure 7.2. Important diterpene classes from *Eremophila* species.

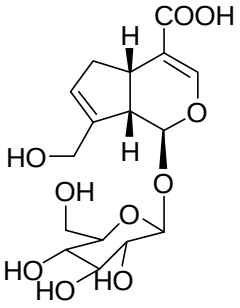
7.1.4 Biologically Active Compounds from *Eremophila* Species

Recently Palombo and Semple (2001;2002), Ndi et al. (2007a), Semple et al., (1998), Penacchio et al., (1996), Rogers et al., (2000) and Sweeney et al. (2001) have described the respective; *in vitro* antibacterial, antimicrobial, anti-viral, anti-inflammatory, neurological and cardioactive properties of extracts of *Eremophila* species. Several bioactive compounds have been described. The furanoid sesquiterpenes prevalent in the essential oils of several species of *Eremophila* have been shown to be hepatotoxic to laboratory animals and stock (Seawright, et al., 1982). Whilst the innocuous prunasin (**65**) is metabolized to cyanide *in vivo* by enzymes that are released when the plant tissues are damaged (ie. livestock grazing). More recently, Shah and co-workers (2004)

have suspected a carotenoid from *E. duttonii* as possessing antibacterial activity. Verbascoside (**73**) and geniposidic acid (**72**) from the leaf extract of *E. alternifolia* is reported to possess significant cardioactive properties (Pennacchio et al. 1996; Rogers et al., 2000). Antibacterial and anti-inflammatory serrulatanes, 3,8-dihydroxyserrulatic acid (**67**) and serrulatic acid (**68**), have been characterised from *E. sturtii* (Liu et al., 2006). Additional serrulatanes **69** and **70**, and biflorin (**71**) from *E. serrulata* (Ndi et al., 2007b) have also demonstrated antimicrobial activity. Smith et al. (2007), reported on the antibacterial activity of serrulat-14-ene-7,8,20-triol and serrulat-14-ene-3,7,8,20-tetraol against *Staphylococcus aureus, Staphylococcus epidermidis* and *Streptococcus pneumoniae.*

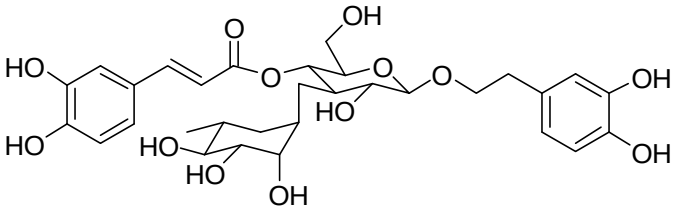






	R_1	R_2	R_3
67	OH	OH	COOH
68	H	H	COOH
69	H	OH	CH_2OH
70	H	OH	COOH

71 **72**



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7.2 Results and Discussion

7.2.1 Cytotoxic Activity of Extracts of Western Australian *Eremophila* Species

Fifteen species of *Eremophila* have been evaluated for cytotoxicity against murine leukemia cells ($P388D_1$). In some cases (where available) comparisons have been made between the distributions of the cytotoxic metabolites in the plant, i.e. roots versus aerial parts. The results for the cytotoxic activity from the methanolic extracts are presented in Table 7.2.

Table 7.2. Cytotoxicity of *Eremophila* crude methanolic extracts against $P388D_1$ cells. Estimates of IC_{50} values (mg/mL).

BP #	Species	IC_{50} (mg/mL)
BP 575	*E. deserti*	0.03
BP 876	*E. gibbosa*	0.04
BP 202	*E. racemosa*	0.05
BP 897	*E. scoparia*	0.05
BP 950	*E. subfloccosa* ssp. *subfloccosa*	0.05
BP 911	*E. ionantha*	0.06
BP 927	*E. dempsteri*	0.06
KB 001	*E. bignoniflora*	0.07
BP 407	*E. clarkei* - root	0.08
BP 875	*E. decipens* ssp. *decipens*	0.08
BP 915	*E. subfloccosa* ssp. *lantana*	0.08
BP 887	*E. oblonga*	0.11
BP 368	*E. miniata* - aerial	> 0.1
BP 369	*E. miniata* - root	> 0.1
BP 392	*E. oppositifolia* ssp. *angustifolia*- aerial	> 0.1
BP 393	*E. oppositifolia* ssp. *angustifolia*- root	> 0.1
BP 395	*E. latrobei* ssp. *latrobei*	> 0.1
BP 406	*E. clarkei* - aerial	> 0.1

On the basis of cytotoxicity (Table 7.2), *E. deserti* (syn. *Myoporum deserti*), *E. gibbosa*, *E. racemosa*, *E. scoparia*, *E. subfloccosa* ssp. *subfloccosa*, *E. ionantha* and *E. dempsteri* warrant further investigation. It was observed that extracts from the aerial parts and root material of *E. miniata* exhibited potent cytotoxicity at low concentrations (not evident in IC_{50} values).

It is noted that toxic furanosesquiterpenes have been identified in (among others) the extracts of *E. deserti*, *E. scoparia*, *E. miniata* and *E. latrobei* (Ghisalberti, 1994b).

Extracts from *E. clarkei*, and *E. latrobei* exhibited relatively low IC_{50} values (Table 7.2) and the chemistry of these two species has been investigated (Coates, et al., 1977; Forster, et al., 1986; Hegarty, et al., 1970; Blackburne, et al., 1972). *E. oppositifolia* ssp. *oppositifolia* also exhibited a relatively low IC_{50} value but may warrant further chemical (but not cytotoxic) investigation on the grounds that only fatty acids have been reported from the closely related *E. oppositifolia* (Ghisalberti, et al., 1979; Jefferies and Knox, 1961).

On the basis of cytotoxicity, published phytochemistry and availability of plant materials *E. maculata* var. *brevifolia*, *E. miniata* and *E. racemosa* from the Bioprospect collection were prioritised for further chemical and/or cytotoxic investigations.

7.2.2 Cytotoxic Activity of Extracts of Central Australian *Eremophila* Species

A total of 20 methanolic extracts representing 20 Northern Australian *Eremophila* species was subjected to cytotoxicity assays against a suite of cell lines. Cytotoxicity

was determined against MCF7 (mammary adenocarcinoma), Hep G2 (hepatoblastoma), A2780 (ovarian carcinoma), A-375 (malignant melanoma) $P388D_1$ (mouse lymphoblasts) and PC-3 (prostate cancer) cell lines. The extracts were tested at two concentrations and the results are presented in Tables 7.3. Previous assays have shown that the two most discernible concentrations for bioassay were 0.1 and 0.01 mg/mL.

Among the extracts tested, five species; *E. duttonii*, *E. gilesii*, *E. willsii* ssp. *willsii*, *E. latrobei* var. *latrobei* and *E. bignoniflora* exhibited cytotoxicity across all of the cell lines tested, indicating that these species possess constituents that have non-selective toxicity. Four species; *E. A48866*, *E. longifolia*, *E. macdonellii* and *E. maculata* var. *maculata* exhibited low to no cytotoxicity across the cell lines tested. Intermediate cytotoxicities were observed for the 11 remaining *Eremophila* species.

For more than half of the extracts, moderate selectivity ($\Delta \geq 50\%$ inhibition at the same concentration) towards specific cell lines was observed. In general higher inhibitions were discernible against ovarian, prostate and melanoma cell lines, whilst lower inhibitions were determined for breast, liver, and mouse lymphoblast cells.

The toxicity of several classes of compounds metabolized by *Eremophila* species has been documented (Ghisalberti, 1994b). The occurrence of furanosesquiterpenes in *E. deserti* (syn. *M. deserti*), *E. latrobei*, *E. maculata*, *E. miniata*, *E. inflata*, *E. rotundifolia*, *E. alternifolia*, *E. latrobei* var. *glabra* and *E. scoparia* has been published (Ghisalberti, 1994b). However correlation between the cytotoxicity and published compounds was not possible on the basis of the limited data available. To date; terpenes are the predominant class of compounds characterised from this species. The HPLC chromatograms of the species evaluated in this study (Appendix V) indicate that the

Table 7.3 Cytotoxicity of ASDP *Eremophila* species. Percentage inhibition of cell growth for methanolic extracts (0.1/0.01 mg/mL).

	Cell Line					
Species	MCF7	Hep G2	A2780	A-375	PC-3	P388D$_1$
E. duttonii	99/0	80/0	100/17	99/11	99/5	99/0
E. gilesii	70/0	50/1	73/30	55/45	71/45	100/39
E. willsii ssp. *willsii*	98/0	36/7	99/12	83/1	94/27	63/0
E. latrobei var. *latrobei*	69/0	46/0	86/15	75/17	69/7	58/8
E. bignoniflora	92/0	14/18	99/16	42/3	74/7	66/10
E. alternifolia	35/0	34/6	77/4	72/0	67/17	53/6
E. A94704 (hybrid)	56/0	25/0	73/16	48/0	63/3	39/1
E. maculata var. *brevifolia*	26/0	19/30	49/23	28/29	28/28	0/0
E. latrobei var. *glabra*	20/0	14/0	65/9	31/11	52/5	53/0
E. A09760	17/0	37/21	39/8	18/0	44/22	7/2
E. freelingii	0/0	0/0	60/0	16/0	41/0	64/0
E. obovata	5/0	0/5	50/16	39/0	31/0	13/0
E. obovata var. *obovata*	18/0	3/0	43/10	22/0	28/5	5/0
E. dalyana	0/0	13/8	47/0	19/0	26/0	13/0
E. christophori	13/0	11/14	38/0	17/1	11/19	0/0
E. batti	0/0	24/6	36/2	16/0	21/5	1/0
E. A48866	0/0	0/0	28/11	4/0	23/0	0/0
E. longifolia	7/0	7/0	20/5	0/0	7/0	2/0
E. macdonellii	0/0	1/0	16/0	0/0	23/0	0/0
E. maculata var. *maculata*	0/0	0/0	13/0	0/0	0/0	0/0

chemistry of *Eremophila* species is very complex and chemically diverse. It was beyond the scope of this thesis to explore the chemical composition of all 35 species in more detail.

A trend could also not be established between the ethnopharmacological uses of *Eremophila* species and the toxicity. Cytotoxic species such as *E. duttonii* and *E. gilesii*, were prepared as decoctions and taken internally (Ghisalberti, 1994a). Likewise, the non-toxic species such as *E. dalyana* and *E. longifolia* determined in this study were utilized to treat infections (colds and boils respectively) (Ghisalberti, 1994a).

Plants were selected for further study on the basis of their availability, chemical profile degree of cytotoxicity and whether any chemical studies had been undertaken previously. Five of these species have been selected for further chemical investigation. *E. racemosa, E. maculata* var. *brevifolia, E. miniata and E. subfloccosa* for cytotoxicity based bioassay guided fractionation. *E. bignoniflora* was selected for chemical investigation because little is known about the chemistry of this species.

7.2.3 Isolation of Compounds from *Eremophila racemosa*

The chromatographic profiles of the methanolic extracts from the leaves, fruit and twigs of *E. racemosa* were initially evaluated using a C18 RP LC-MS system with a water/acetonitrile gradient. The resulting chromatograms (Figures 7.3-7.5) indicated there was little difference between the occurrence and distribution of the plant metabolites. This finding was also paralleled in the cytotoxicity profile of the plant parts (Table 7.4).

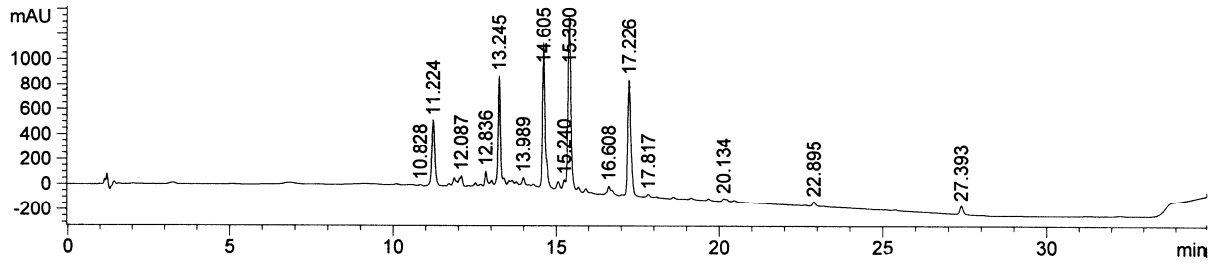


Figure 7.3. Chromatographic profile of *E. racemosa* - leaves
(RP HPLC profile of methanolic extract, λ=280 nm).

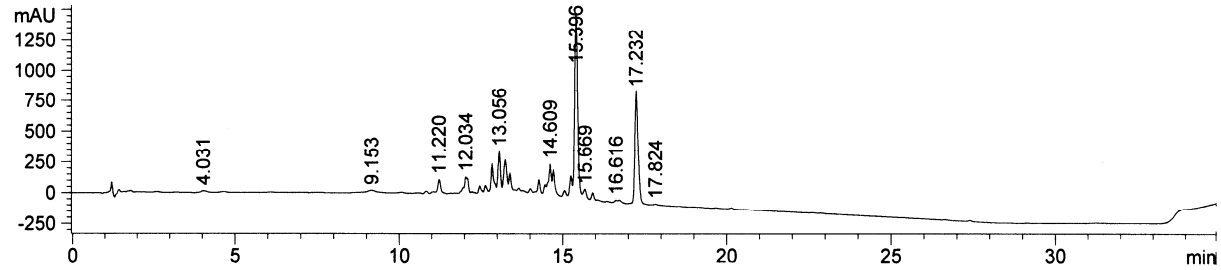


Figure 7.4. Chromatographic profile of *E. racemosa* - fruit
(RP HPLC profile of methanolic extract, λ = 280 nm).

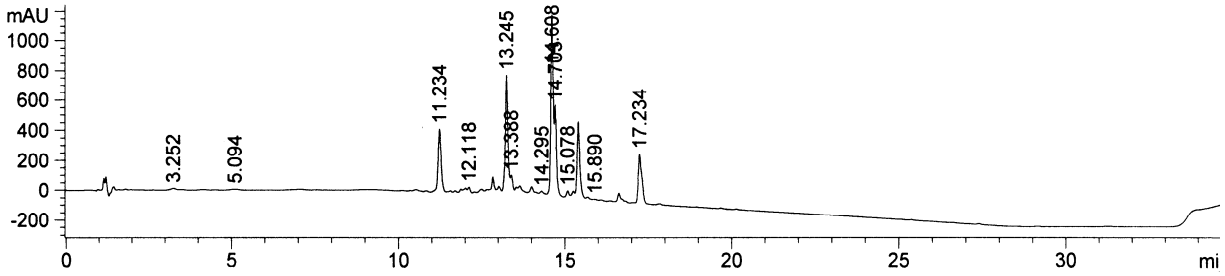


Figure 7.5. Chromatographic profile of *E. racemosa* - stems
(RP HPLC profile of methanolic extract, λ = 280 nm).

Table 7.4. Cytotoxicity of *E. racemosa* methanolic extracts
(% inhibition of P388D$_1$ cell growth, IC_{50} estimates).

Plant Part	IC_{50} (mg/mL)
Stem	0.05
Leaf	0.05
Fruit	0.05

For reasons of the high yield and ease of extraction, the investigation focused on the leaf material. The methanolic extract was divided into 10 fractions using preparative HPLC, with the bulk of the components eluting between fractions 3 and 5. The prep-HPLC fractions were evaluated for cytotoxicity against the $P388D_1$ cell line (Table 7.5).

Table 7.5. Cytotoxicity of *E. racemosa* leaf extract prep-HPLC fractions (% inhibition of $P388D_1$ cell growth, IC_{50} estimates).

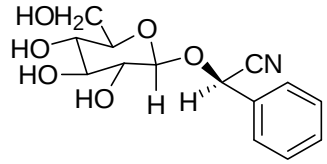
Leaf Extract Fraction #	IC_{50} (mg/mL)
F1	>0.1
F2	0.02
F3	0.06
F4	0.05
F5	0.01
F6	0.03
F7	0.01
F8	0.01
F9	0.02
F10	0.03

For the purpose of isolating compounds the methanolic extract was divided into 44 fractions using preparative HPLC, with the bulk of the components eluting between fractions 9 and 21. The six major polar metabolites were isolated and characterised from the methanolic extract of *E. racemosa*.

7.2.3.1 Prunasin

Further purification by means of semi-preparative HPLC, size exclusion chromatography and recrystallisation yielded prunasin (**65**). IR, MS, ^{1}H NMR and ^{13}C NMR spectra were consistent with previously published data (Nakajima, et al., 1998).

To date, this cyanogenic glycoside has only been reported from one other species of *Eremophila*. The occurrence of prunasin in this species indicates that, similarly to *E. maculata* (Finnemore and Cox, 1930), *E. racemosa* would be toxic to livestock.



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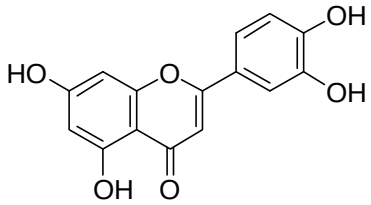
7.2.3.2 Luteolin

Luteolin was isolated from the cytotoxic fraction 5 (Table 7.5). MS, ^{1}H NMR and ^{13}C NMR data are in agreement with previously published data (Youssef and Frahm, 1995). In this study luteolin was found to exhibit 50% inhibition (IC_{50}) against $P388D_1$ cell growth at 160 μM. Luteolin (**74**), occurs commonly in plants (Harborne et al., 1975), and its cytotoxic (Matsuo et al., 2005) antioxidant (Benavente-Garcia et al.; 2000), anti-inflammatory (Ueda et al., 2002) and antimicrobial (Yamamoto and Ogawa, 2002) properties have been reported.



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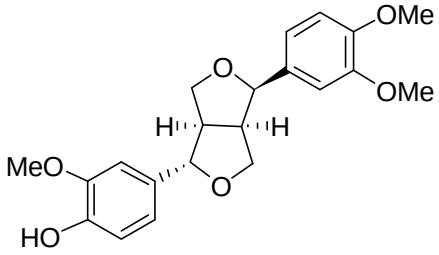
7.2.3.3 Phillygenin

The lignan, phillygenin (**75**) (syn. phillygenol) was also isolated from cytotoxic fraction 5 (Table 7.5). The ^{1}H NMR and ^{13}C NMR data are in agreement with data published

previously (Rahman, et al., 1990) and is further supported by 2D COSY, NOESY, HMBC and HSQC data. In this study, phillygenin was found to exhibit 50% inhibition (IC_{50}) against $P388D_1$ cell growth at 430 μM. The cytotoxic effects of phillygenin and several lignans isolated from *Lancea tibetica* have been studied. Phillygenin showed strong cytotoxicity against human hepatoma cells (SMMC-7721), human uterine cervix carcinoma cells (HeLa), hamster lung fibroblast cells (V79) and mouse melanoma cells (B16), (Zhao et al., 2000). The results of this study also demonstrated that the toxicity of phillygenin was lost when the phenolic hydroxyl groups are glucosylated as is the case for its 4-*O*-*β*-D-glucoside phillyrin (**76**).



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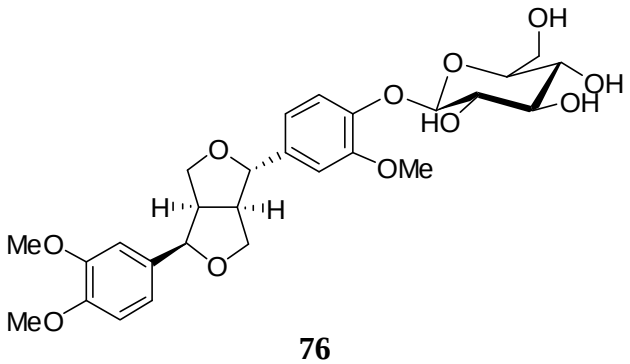
7.2.3.4 Phillyrin

The third major peak (Figure 7.5) was elucidated as phillygenin-4-*O*-*β*-D-glucoside (**6**) (syn, phillyrin). This compound is the only compound that has been published as a constituent of *E. racemosa* (Forster et al., 1986).



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7.2.3.5 Pinoresinol-4-*O*-β-D-glucoside

The second major peak (Figure 7.5) was found to be a mixture of two compounds **77** and **78**. Resolution and further purification of these two compounds was achieved using semi-preparative HPLC.

The ^{1}H NMR spectrum for the early eluting compound **77** showed distinct similarities to that of the lignan glycoside phillyrin (**76**), with two methoxy singlets (δ 3.81 & 3.78 ppm), six aromatic proton signals resonating between δ 6.75 - 7.15 ppm and a characteristic anomeric hemiacetal proton doublet (δ 4.8 ppm). The chemical shift and multiplicities of the six glycosidic protons of this molecule were identical to those of phillyrin (**76**), confirming the sugar residue as a 4-*O*-β-D-glucoside. Several differences are noted in the ^{1}H NMR signals for the eight protons attached to the furofuran ring system, most significantly; only four signals are observed, with each signal integrating for two protons.

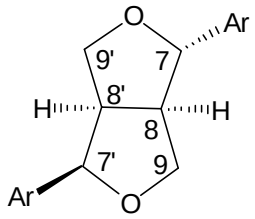


Figure 7.6. Numbering convention for the lignan furofuran ring system.

The furofuran ring system (Figure **7**.6.) possesses four stereogenic centers that give rise to several possible isomers. Useful information regarding the stereochemistry at the C-7 and C-7′ centres came from the observation that, in the ^{1}H NMR the 7-H and 7′-H, 8-H and 8′-H, and 9α-H and 9′α-H were chemically equivalent which indicated a high degree of symmetry for this molecule. It can be established that the aromatic residues at

C-7 and C-7′ are both equatorial from inspection of the chemical shifts of the adjacent C-9 substituents where the chemical shifts of these protons lie within the range δ 3.8 - 4.4 ppm. Whereas in the diaxial model the C-9 β protons are shifted further up field due to shielding by the C-7 aryl substituent, typically δ 3.3 – 4.0 ppm are observed (Ayres and Loike, 1990).

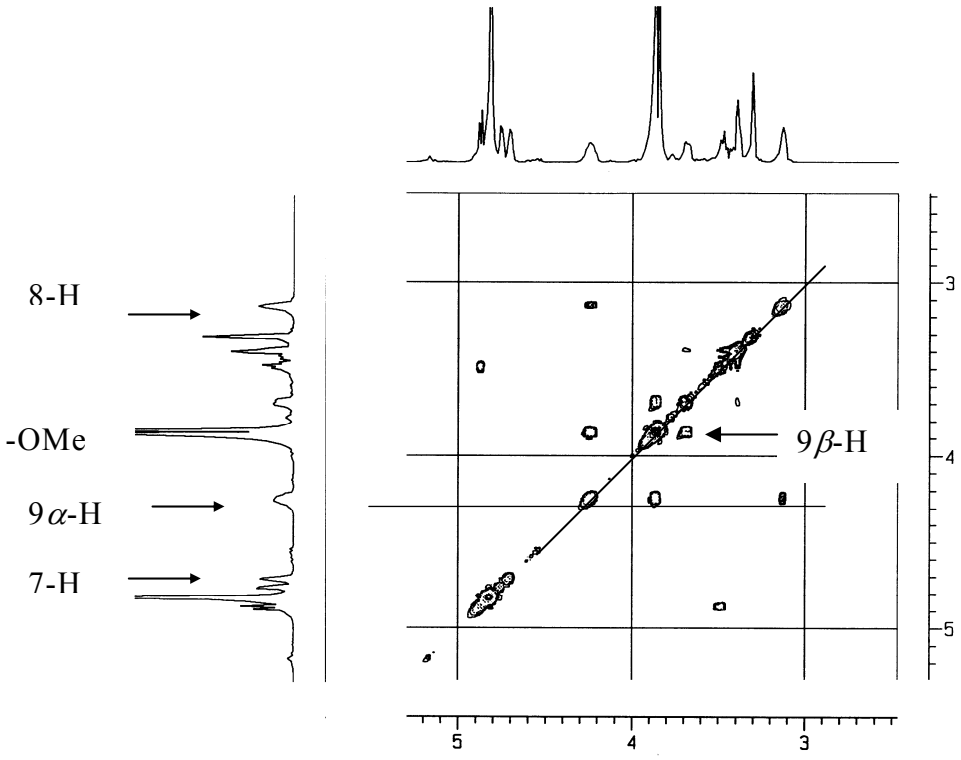
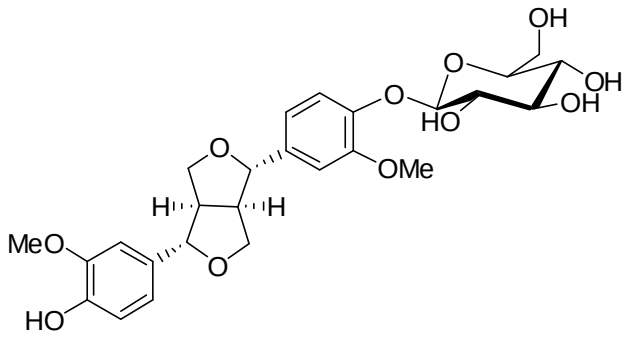


Figure 7.7. Detail of 2D COSY spectrum of compound **77**. (500 MHz, CD_3OD).

The 9β-H and 9′β-H protons of this molecule are overlapped by the strong methoxy peaks at δ 3.9 ppm. Inspection of the 2D COSY spectrum (Figure 7.7.) for this molecule reveals off-diagonal coupling of signals close to δ 3.9 ppm with those of the 9α-H, 9′α-H protons at δ 4.25 ppm, which in turn couple to the 8-H, 8′-H protons at 3.15 ppm. The structure **77**, pinoresinol-4-*O*-β-D-glucoside, is proposed for this compound and is further supported by HMBC, HSQC, NOESY and COSY

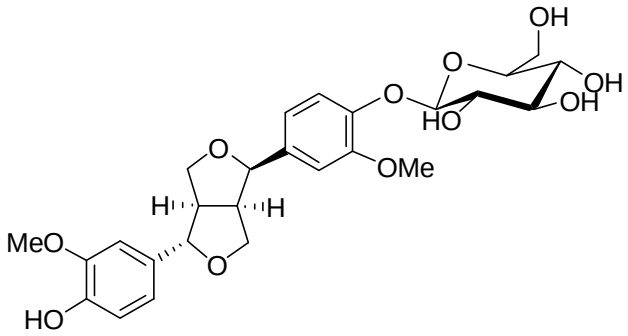
spectroscopic data. The ^{1}H and ^{13}C NMR as well as the 2D COSY spectra were consistent with previously published data (Rahman, et al., 1990) and (Ayres and Loike, 1990).



77

7.2.3.6 Epipinoresinol-4-*O*-*β*-D-glucoside

The ^{1}H NMR of the second lignan glycoside indicated that the sugar residue was also identical to the 4-*O*-*β*-D-glucoside of phillyrin (**76**). The remainder of the spectrum revealed the presence of six aromatic protons resonating between δ 6.78 - 7.15 ppm and two methoxy groups (δ 3.75, 3.87 ppm) consistent with the presence of vanillyl and a glucosylated guacyl group. The asymmetry of the two H-7 protons (δ 4.48, 4.89-4.88 ppm), four nonequivalent H-9 protons (δ 4.13, 3.86, 3.80 and 3.30 ppm) and two non-equivalent H-8 bridgehead protons (δ 3.70 and 2.93 ppm) established the furofuran lignan as the epi isomer. Comparison of the ^{13}C NMR data for this compound with that reported in the literature (Rahman, et al., 1990) suggests that this molecule is the 4-*O*-*β*-D-glucoside of epipinoresinol (**78**). This structure is further supported by 2D COSY, HMBC, HSQC and nOe spectroscopic data.



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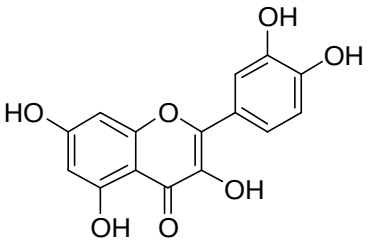
7.2.4 Discussion

Bioassay guided fractionation of the methanolic extracts of *E. racemosa* indicated that both polar and non-polar metabolites were cytotoxic. Purification of fraction 5 (Table 7.5) afforded the cytotoxic flavonoid (**74**) and lignan (**75**). The IC_{50} values were determined for luteolin (**74**) and phillygenin (**75**) against mouse lymphoblast cells ($P388D_1$) were 160 and 430 μM, respectively. It is noted that cytotoxicity was not enriched upon sub-fractionation of fraction 5. This may possibly be due to minor constituents contributing to the cytotoxicity or loss of the active components via irreversible adsorption or degradation during purification steps. Investigation of the cytotoxic lipophillic components was undertaken but is incomplete due to time constraints.

7.2.5 Preliminary Investigations of Several *Eremophila* Species

Bioassay guided fractionation of *E. maculata* var. *brevifolia*, *E. miniata* and *E. subfloccosa* ssp. *subfloccosa* were undertaken. Identification of the cytotoxic metabolites from these species was not completed due to a combination of low yields or purity of the active fractions and time constraints as other aspects of this project were prioritized.

A specimen of *E. bignoniflora* was selected for further chemical investigation because there are only two metabolites reported from this species. Ghisalberti (1994a) indicates that mannitol and verbascoside have been characterised. The ethyl acetate soluble fraction of *E. bignoniflora* was subjected to RP prep-HPLC. Semi-prep of two of these fractions afforded quercetin (**79**) and nepetin (**80**).



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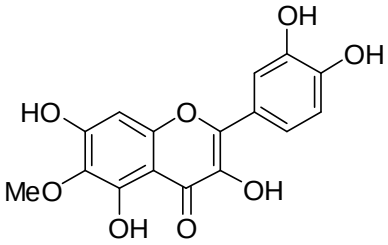

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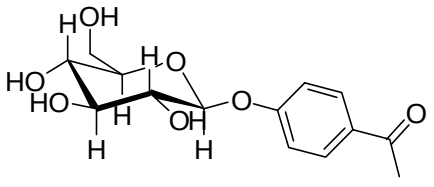
A phenolic glycoside piceine (**81**) and a lignan epipinoresinol-4-*O*-*β*-D-glucoside (**78**) have been isolated from *E. maculata* var. *brevifolia*.



81

7.3 Conclusion

Many of the species evaluated in this study exhibited some degree of cytotoxicity, indicating that *Eremophila* species produce biologically active metabolites. The

chemistry of *Eremophila* species was observed to be very complex and chemically diverse (see Appendix V). To date, phytochemical investigations of the genus have characterised predominantly unusual sesquiterpenes and diterpenes from *Eremophila* species. The pharmacological actions of many of these compounds have not been studied. In addition the chemistry of many *Eremophila* species has not yet been investigated. Correlation between the ethnopharmacological uses and phytochemistry of the *Eremophila* species investigated in this study could not be established owing to limited published data and time constraints. An investigation of this type would need to be undertaken on a case by case basis.

This study characterised six of the major metabolites, predominantly lignans, from *E. racemosa.* Lignans are reported to exhibit a variety of biological activities including; anti-cancer, anti-inflammatory, antioxidant, antimicrobial and immunosuppressive actions (Saleem et al., 2005). The occurrence of lignans at such high concentrations in this species is of interest and worthy of further pharmacological investigation. To date, prunasin (**65**) has only been reported from one other species of *Eremophila*. The occurrence of prunasin in this species indicates that, similarly to *E. maculata* (Finnemore and Cox, 1930), *E. racemosa* would be toxic to livestock. A phytochemical survey for the presence of prunasin in other *Eremophila* species is warranted.

References

Abbott, W. S. A method of computing the effectiveness of an insecticide. *J. Econ. Entomol.* **1925,** 18, 265-287.

Abel, A. D.; Massy-Westropp, R. A. Eremophilane and Serrulatane Terpenoids from *E. rotundifolia. Aust. J. Chem.* **1985,** 38, 1263-1269.

Adams, R. P. Cedar Wood Oil - Analysis and Properties. *Mod. Meth. of Plant Anal.* **1991,** 12, 159-173.

Adams, R. P. *Identification of Essential Oil Components by Gas Chromatography/ Mass Spectrometry*, 4th ed,; Allured Publishing Corp.: Carol Stream, IL, 2007.

Agency for Toxic Substances and Disease Registry Toxicological Profiles on CD-ROM, Chlordane. 1997; Lewis Publishers: Boca Raton, FL.

Ahmed B. M. The Effects of Boron-Treated Timbers Against *Coptotermes Species* in Australia. Ph. D. Dissertation, The University of Melbourne, Melbourne, Aust. 2000.

Araico, A.; Terencio, M. C.; Alcaraz, M. J.; Dominguez, J. N.; Leon, C.; Ferrandiz, M. L. Phenylsulphonyl Urenyl Chalcone Derivatives as Dual Inhibitors of Cyclo-oxygenase-2 and 5-lipoxygenase. *Life Sci.* **2006,** 78(25), 2911-2918.

Archicentre. Australian Termites $780 million Smorgasboard. Royal Australian Institute of Architects. http://www.archicentre.com.au/media/archinews-18012003.htm. (accessed Jan 31, 2007).

Ayres, D. C.; Loike, J. D. *Lignans Chemical, Biological and Clinical Properties*; Cambridge University Press: Cambridge, 1990.

Barr, A. *Traditional Bush Medicines: An Aboriginal Pharmacopoeia. Aboriginal Communities of Northern Territory Australia*; Greenhouse Publications: Richmond, Victoria, 1988.

Barrero, A. F.; del Moral, Q.; J.; Lara, A. Sesquiterpenes from *Juniperus thurifera* L. Stereochemistry in Unudual Cedrane and Duprezianane series. *Tetrahedron* **2000,** 56, 3717-3723.

Bates, R. B.; Paknikar, S. K. Ereremophilone and Alloeremophilone from Hydroxydihydroeremophilone. *Chem. Ind.* **1966,** 2170-2171.

Benavente-Garcia, O.; Castillo, J.; Lorente, J.; Ortuno, A.; Del Rio, J. A. Antioxidant Activity of Phenolics Extracted from *Olea europaea*. *Food Chem.* **2000,** 68(4), 457-462.

Bertolini, A.; Ottani, A.; Sandrini, M. Selective COX-2 Inhibitors and Dual Acting Anti-inflammatory drugs: Critical Remarks. *Curr. Med. Chem.* **2002,** 9, 1033-1043.

Bioprospect Pty Ltd., Termilone - Termite control. http://www.bioprospect.com/-projects_termilone.html (accessed 8-4-09).

Blackburne, I. D.; Park, R. J.; Sutherland, M. D. Myoporone and Dehydromyoporone, Toxic Furanoid Ketones from *Myoporum* and *Eremophila* Species, Terpenoid chemistry XX. *Aust. J. Chem.* **1972,** 25, 1787-96.

Bochsler, P. N.; Slauson, D. O. Inflammation and Repair of Tissue. Reference in: Slauson, D. O., Cooper, B. J. (eds.), *Mechanisms of Disease*; Mosby: St. Louis, Missouri, 2002.

Bohlmann, F.; Chen, Z. New Guaianolides from *Centipeda minima*. *Kexue Tongbao Foreign Lang. Ed.* **1984,** 29(7), 900-903.

Bohlmann, F.; Jakupovic, J.; Schuster, A.; King, R. M.; Robinson, H. Naturally Occurring Terpene Derivatives. Part 440. New Melampolides, Kaurene Derivatives, and Other Constituents from *Ichthyothere* Species. *Phytochemistry* **1982,** 21(9), 2317-2327.

Bohlmann,F.; Mahanta, P. K. Eine Neue Diterpensaure aus *Centipeda orbicularis*. *Phytochemistry* **1979,** 18(6), 1067-1068.

Bowen, S. E. Taxonomic Studies in the Myoporacea. Honours Thesis, The University of New England, 1975.

Bradfield, A. E.; Penfold, A. R.; Simonsen, J. L. The Essential Oil from the Wood of *Eremophila mitchellii* (Bentham). *J. Proc. Roy. Soc. N.S.W.* **1932a,** 66, 420-433.

Bradfield, A. E.; Penfold, A. R.; Simonsen, J. L. The Constitution of Eremophilone and of Two Related Hydroxy-ketones from the Wood Oil of *Eremophila mitchellii. J. Chem. Soc.* **1932b,** 2744-2759.

Buckingham, J.; Macdonald, F. M.; Bradley, H. M.; Cai, Y.; Munasinghe, V. R. N.; Pattenden, C. F. Dictionary of Natural Products on DVD. Chapman and Hall Version 16:2. 2008.CRC Press, 1994 London.

Bultmann, J. D.; Beal, R. H.; Ampong, F. E. K. Natural Resistance of Some Tropical African Woods to *Coptotermes formosanus* Shiraki. *For. Prod. J.* **1979,** 29, 46-51.

Cai, Y.; Luo, Q.; Sun, M.; Corke, H. Antioxidant Activity and Phenolic Compounds of 112 Traditional Chinese Medicinal Plants Associated with Anticancer. *Life Sci.* **2004,** 74(17), 2157-2184.

Calixto, J. B., Otoki, M. F., Santos, A. R. S., Anti-inflammatory Compounds of Plant Origin. Part I. Action on Arachidonic Acid Pathway, Nitric Oxide and Nuclear Factor κB (NF- κB). *Planta Med.* **2003,** 69(11), 973-983.

Campbell, A. Pharmacy of Victorian Aborigines. *Aust. J. Parm.* **1973,** 54, 894-900, Dec.-Jan. 1973-1974.

Cane, D. E.; Prabhakaran, P. C.; Oliver, J. S.; Mc Ilwaine, D. B. J. Aristolochene Biosynthesis. Stereochemistry of the Deprotonation Steps in the Enzymatic Cyclization of Farnesyl Pyrophosphate. *J. Amer. Chem. Soc.* **1990,** 112(8), 3209-3210.

Carrol, P. J.; Ghisalberti, E. L.; Ralph, D. E. Tricyclic Sesquiterpenes from *Eremophila georgei*. *Phytochemistry* **1976,** 15, 777-780.

Carson, R. *Silent Spring*; Houghton Mifflin: Boston, 1962.

Carter, F. L.; Garlo, A. M.; Stanley, J. B. Termiticidal Components of Wood Extracts: 7-methyl-juglone from *Diospyros virginiana*. *J. Agric. Food Chem*. **1978,** 26, 869-873.

Celotti, F.; Laufer, S. Anti-inflammatory Drugs : New Multitarget Compounds to Face an Old Problem. The Dual Inhibition Concept. *Pharmacol. Res.* **2001,** 43(5), 429-436.

Chandrasekharan, N. V.; Dai, H.; Roos, K. L. T.; Evanson, N. K.; Tomsik, J.; Elton, T. S.; Simmons, D. L. COX-3, a Cyclo-oxygenase-1 Variant Inhibited by Acetaminophen and Other Analgesic/Antipyretic Drugs: Cloning, Structure, and Expression. *Proceedings of the National Academy of Sciences of the United States of America.* **2002,** 99(22), 13926-13931.

ChemBioDraw Ultra 11.0, 2007; CambridgeSoft: Cambridge, MA 02140 USA.

Cheminat, A.; Zawatsky, R.; Becker, H.; Brouillard, R. Caffeoyl Conjugates from *Echinacea* Species: Structures and Biological Activity. *Phytochemistry* **1988,** 27(9), 2787-2794.

Chetty, G. L.; Zalkow, L. H. 7a(H)-Eremophila-1,11-dien-9-one. A New Sesquiterpene of the Eremophilane Type. *Tetrahedron Lett.* **1969,** 5, 307-309.

Chinnock, R. J. *Eremophila and Allied Genera: A Monograph of the Myoporaceae*; Rosenberg Publishing: Kenthurst, NSW, 2007.

Claria, J.; Romano, M. Pharmacological Intervention of Cyclo-oxygenase-2 and 5-lipoxygenase Pathways: Impact on Inflammation and Cancer. *Curr. Pharm. Design* **2005,** 11(26), 3431-3447.

Cleland, J. B.; Johnston, T. H. Notes on Native Names and Uses of Plants in the Musgrave Ranges Region. *Oceania* **1937,** 8(2), 208-215.

Cleland, J. B.; Johnston, T. H. The ecology of the Aborigines of Central Australia. *Transactions and Proceedings of the Royal Society of South Australia* **1933,** 57, 113-124.

Cleland, J. B.; Tindale, N. B. Ecological Surrounding of the Ngalia Natives of Central Australia and Native Names and Uses of Plants. *Transactions and Proceedings of the Royal Society of South Australia* **1954,** 77, 81-86.

Close, G. A. *Centipeda* Plant Extract. U.S. Patent, US2002044977, April 18, 2002.

Coates, P.; Ghisalberti, E. L.; Jefferies, P. R. The Chemistry of *Eremophila* spp. VIII. A cembrenetriol from *E. clarkei*. *Aust. J. Chem.* **1977,** 30, 2717-21.

Coats, J. R. Mechanisms of Toxic Action and Structure Activity Relationships for Organochlorine and Synthetic Pyrethroid Insecticides. *Environ. Health Persp.* **1990,** 87, 255-262.

Cole, L.M.; Nicholson, R.A.; Casida, J.E. Action of Phenylpyrazole Insecticides at the GABA-gated Chloride Channel. *Pest. Biochem. Physiol.* **1993,** 46, 47-54.

Cornelius, M.; Grace, J. K.; Yates III, J. R., Toxicity of Monoterpenoids and Other Natural Products to the *Formosan* Subterranean Termite. *J. Econ. Entomol.* **1997,** 90, 320-325.

Cos, P.; Calomme, M.; Sindambiwe, J. B.; Bruyne, T. de.; Cimanga, K.; Pieters, L.; Vlietinck, A. J.; Berghe, D. V. Cytotoxicity and Lipid Peroxidation-inhibiting Activity of Flavonoids. *Planta Med.* **2001,** 67(6), 515-519.

Cowan, M. M. Plant Products as Antimicrobial Agents. *Clin. Microbiol. Rev.* **1999,** 12(4), 564-582.

Creffield, J. The RIC Good Wood Guide. http://www.rainforestinfo.org.au/-good_wood/nat_htrs.htm 1998 (accessed Feb 2, 2007).

Cribb, A. B. *Wild Medicines in Australia*; Collins: Sydney, 1988.

Cribb, A. B.; Cribb, J. W. *Useful Wild Plants in Australia*; William Collins Pty Ltd.: Sydney. 1981.

CSIRO Entomology. Report of Research 1993-95. http://www.ento.csiro.au/histor/rr93-95/sp_intro.htm (accessed Feb 2, 2007).

Cunningham, G. M.; Mueller, W. E.; Milthorpe, P. L.; Leigh, J. H. *Plants of Western New South Wales*; New South Wales Government Printing Office: Sydney, 1981.

Cunningham, G. M.; Mulham, W. E.; Milthorpe, P. L.; Leigh, J. H. *Plants of Western New South Wales*.; Inkata Press: Melbourne, 1992; pp 606 – 616.

D'Amelio, F. S.; Mirhom, Y. W. Therapeautic Composition for Treating Skin Using *Centipeda Cunninghamii* Extract. World Patent WO9838971, Sept 11, 1998.

D'Amelio, F. S.; Mirhom, Y. W. Method and Composition for Treating Oral Bacteria and Inflammation. World Patent WO/2005/084625, Sept 15, 2005.

Delate, K. M.; Grace, J. K. Succeptibility of Neem to Attack by the Formosan Subterranean Termite, *Coptotermes formosanus* Shiraki. (Isoptera: Rhinotermitidae). *J. Appl. Entomol*. **1995b.** 119(2), 93-95.

Dewick, P. M. *Medicinal Natural Products: A Biosynthetic Approach*; John Wiley & Sons Ltd: West Sussex, 1997, Chapter 2.

Dey, P. M.; Harborne, J. B. *Methods in Plant Biochemistry: Vol. 1. Plant Phenolics*; Dey, P. M.; Harborne, J. B., Eds.; Academic Press: San Diego, CA. 1989.

Dey, P. M.; Harbourne, J. B. *Alkaloids and Sulphur Compounds*; Academic Press Inc: London, 1993.

Djerassi, C.; Mauli, R.; Zalkow, L. H. Terpenoids XXXVIII. Interconversion of Eremophilone, Hydroxyeremophilone and Hydroxydihydroeremophilone. The Relative Stereochemistry of Eremophilone and its Reduction Products. *J. Amer. Chem. Soc*. **1959,** 81, 3424-3429.

Doolittle, M.; Ashok, R.; Lax, A.; Boopathy, R. Effect of Natural Products on Gut Endosymbiotic Microbes in Formosan Subterranean Termites. *Int. Biodeter. Biodegr.* **2007,** 59(1), 69-71.

Duthie, G.; Crozier, A. Plant-derived Phenolic Antioxidants. *Curr. Opin. Clin. Nutr.* **2000,** 3(6), 447-451.

Egan, R. A. Hair Growth Promoting Composition Comprising Extract of *Centipeda cunninghamii*. Australian Patent AU3264984, March 7, 1985.

El-Shamy, A. M.; Shehata, A. H.; Sanad, O. A.; El-Halawany, A. M.; El-Latif, H. A. Abd. Biologically Active Flavonoids from *Simmondsia chinensis* (Link) Schneider Growing in Egypt. *Bulletin of the Faculty of Pharmacy (Cairo University)* **2001,** 39(2), 55-63.

Erdtman, H.; Hirose, Y. The Chemistry of the Natural Order Cupressales: 46. The Structure of Nootkatone. *Acta. Chem. Scand*. **1962,** 16, 1311-1314.

Fergeus, John. 2007. Personal comm.

Ficini, J.; Touzin, A. M. Stereoselective Total Synthesis of (±) Eremophilone. *Tetrahedron Lett.* **1977,** 12, 1081-1084.

Finnemore, H.; Cox, C. B. Cyanogenetic Glucosides in Australian plants. II. (a) *Eremophila maculata. J. Proc. Roy. Soc. N.S.W*. **1930,** 63, 172-178.

Fischer, N.H. in Terpenoids. In *Methods in Plant Biochemistry Vol. 7*; Charlwood, B. V.; Banthorpe, D. V., Eds.; Academic Press: London, **1991**, pp 187 - 211.

Forster, P. G.; Ghisalberti, E. L.; Jefferies, P. R.; Poletti, V. M.;Whiteside, N. J. . The Chemistry of *Eremophila* spp. Part 23. Serrulatane Diterpenes from *Eremophila* spp. *Phytochemistry* **1986,** 25, 1377-1383.

Fritsche, E.; Baek, S. J.; King, L. M.; Zeldin, D. C.; Eling, T. E.; Bell, D. A. Functional Characterization of Cyclooxygenase-2 Polymorphisms. *J. Pharmacol. Exp. Ther.* **2001,** 299(2), 468-476.

Gabriel, B. Phytochemistry and Pharmacology of *Centipeda cunninghamii* and *Olea europaea*. Honours Thesis, Leopold-Franzens Universität, Innsbruck, 2005.

Ganapatay, S.; Thomas, P. S.; Fotso, S.; Laatsch, H. Antitermitic Quinones from *Diospyros sylvatica*. *Phytochemistry* **2004,** 65, 1265-1271.

Ghisalberti, E. L., The Ethnopharmacology and Phytochemistry of *Eremophila* Species (Myoporaceae). *J. Ethnopharmacol*. **1994a,** 44, 1-9.

Ghisalberti, E. L. The Phytochemistry of the Myoporaceae. *Phytochemistry* **1994b,** 35(1), 7-33.

Ghisalberti, E. The Chemistry of Unusual Terpenoids from the Genus *Eremophila.* In *Studies in Natural Products Chemistry;* Rahman, A. Ed.; Elseiver B. V.: Amsterdam, 1995; Vol. 15; p 225.

Ghisalberti, E. L.; Jefferies, P. R.; Toia, R. F. Biosynthesis of (2*E*,4*E*,6*E*)-5-acetoxymethyltetradeca-2,4,6-trienoic acid in *Eremophila oppositifolia*. *Phytochemistry* **1979,** 18, 65-9.

Ghisalberti, E. L.; White, A. H.; Willis, A. C. Crystal Structure and Absolute Configuration of 2α,6,6,8-tetramethyltricyclo[6.2.1.$0^{1,5}$]undecan-7β-yl p-bromo-benzoate. *J. Chem. Soc. Perkin Trans II* **1976,** 12, 1300-1303.

Gil, M. I.; Ferreres, F.; Tomás-Barberán, F. A. Effect of Postharvest Storage and Processing on the Antioxidant Constituents (Flavonoids and Vitamin C) of Fresh-Cut Spinach. *J. Agr. Food Chem.* **1999,** 47(6), 2213-2217.

Grace, J. K.; Abdallay, A. Termiticidal Activity of Boron Dusts (Isoptera Rhinotermitidae). *Z. Angew. Entomol./J. Appl. Entomol*. **1990,** 109(3), 283-288.

Grace, J. K.; Ewart, D. M.; Tome, C. H. M. Termite Resistance of Wood Species Grown in Hawaii. *Forest Prod. J.* **1996,** 46(10), 57-60.

Grace, J. K.; Tome, C. H. M. Resistance of Indonesian Heartwoods Bangkirai (*Shorea laevis*) and Merbau (*Intsia palembanica*) to Formosan Subterranean Termite Attack. *Sociobiology* **2005,** 45(2), 503-509.

Grace, J. K.; Wong, A. A. H.; Tome, C. H. M. *Termite Resistance of Malaysian and Exotic Woods with Plantation Potential: Laboratory Evaluation;* IRG Document No. IRG/WP 98-10280; International Research Group on Wood Preservation: Stockholm, Sweden, 1998.

Grace, J. K.; Wood, D. L.; Frankie, G. W. Behaviour and Survival of *Reticulitermes hesperus* Banks (Isoptera: Rhinotermitidae) on Selected Sawdusts and Wood Extracts. *J. Chem. Ecol.* **1989,** 15(1), 129-139.

Grace, J. K.; Yates, J. R. Behavioural Effects of Neem Insecticide on *Coptotermes formosanus* (Isoptera: Rhinotermitidae). *Trop. Pest Manage.* **1992,** 38(2), 176-180.

Grouiller, A; Pacheco, H. Flavonoid Compounds. VI. Nuclear Magnetic Resonance Spectra of some *O*-glucosylflavonals, their Aglycons and Three Synthetic mono- and di-*O*-glucosylflavanones. *Bull. Soc. Chim. Fr.* **1967,** 1938-1943.

Gupta, D.; Singh, J. Triterpenoid Saponins from *Centipeda minima*. *Phytochemistry* **1990,** 29(6), 1945-1950.

Gupta, D.; Singh, J. Triterpenoid Saponins from *Centipeda minima*. *Phytochemistry* **1989,** 28(4), 1197-1201.

Gupta, S. K.; Hoyt, E. G. Cosmetic or Pharmaceutical Compositions for Skin Care. U.S. Patent 2006/023710, March 02, 2006.

Hamon, N.; Shaw, R.; Yang, H. *Worldwide Development of Fipronil Insecticide*. Proceedings of the Beltwide Cotton Conference, Rhone Poulenc Inc. USA. National Cotton Council. 1996;. 2, 759-765.

Harborne, J. B. *Phytochemical Methods: A Guide to Modern Techniques of Plant Analysis*, 3rd ed.; Chapman and Hall: London, 1998.

Harborne, J. B.; Mabry, T. J.; Mabry, H. *The Flavonoids*; Chapman and Hall: London, 1975.

Harden, G. J. *Flora of New South Wales*; NSW University Press: Sydney, 1993.

Hegarty, B. F.; Kelly, J. R.; Park, R. J.; Sutherland, M. D. (-)-Ngainone, a Toxic Constituent of *Myoporum deserti*. The Absolute Configuration (-)-Ngainone. *Aust. J. Chem.* **1970,** 23, 107-117.

Henderson, G.; Heumann, D. O.; Laine, R. A.; Maistrello, L.; Zhu, B. C. R.; Chen, F. Extracts of Vetiver Oil as Repellents and Toxicant to Ants, Ticks, and Cockroaches. U.S. Patent 6906108, Jun 14, 2005.

Henderson, W. R. Jr. The Role of Leukotrienes in Inflammation. *Ann. Intern. Med.* **1994.** 121(9), 684-697.

Herron, G. A.; Beattie, G. A. C.; Parkes, R. A.; Barchia, I. Potter Spray Tower Bioassay of Selected Citrus Pests to Petroleum Spray Oil. *Aust. J. Entomol.* **1995,** 34(3), 255-263.

Herz, W.; Anderson, G. D.; Wagner, H.; Maurer, G.; Maurer, I.; Flores, G. Isolierung, Struktur und Synthese der Flavone von *Ambrosia grayi* Nelson. *Tetrahedron* **1975**, 31, 1577-1581.

Herz, W.; Watanabe, H.; Miyazaki, M.; Kishida, Y. Structures of Parthenin and Ambrosin. *J. Am. Chem. Soc*. **1962,** 84, 2601-2610.

Hill, L. The anti-microbial properties of *C. cunninghamii*. Honours Thesis, University of Ballarat, 1997.

Hinz, B.; Brune, K. Cyclooxygenase-2 – 10 Years Later. *J. Pharmacol. Exp. Ther.* **2002,** 300(2), 367-375.

Huang, D.; Ou, B.; Hampsch-Woodill, M.; Flanagan, J. A.; Deemer, E. K. Development and Validation of Oxygen Radical Absorbance Capacity Assay

for Lipophilic Antioxidants Using Randomly Methylated-β-Cyclodextrin as the Solubility Enhancer. *J. Agric. Food Chem.* **2002a,** 50(16), 1815-1821.

Huang, D.; Ou, B.; Hampsch-Woodill, M.; Flanagan, J. A.; Prior, R. L. High-Throughput Assay of Oxygen Radical Absorbance Capacity (ORAC) Using a Multichannel Liquid Handling System Coupled with a Microplate Fluorescence Reader in 96-Well Format. *J. Agric. Food Chem.* **2002b,** 50(16), 4437-4444.

Huang, H.; Ou, B.; Prior, R. The Chemistry Behind Antioxidant Capacity Assays. *J. Agric. Food Chem.* **2005,** 53, 1841-1856.

Huang, J. Injection for Treatment of Nasosinusitis. Chinese Patent CN1106684 (A), August 16, 1995.

Ibrahim, S. A.; Henderson, G.; Fei, H.; Laine, R. Survivorship, Tunneling and Feeding Behaviours of *Coptotermes formosanus* (Isoptera: Rhinotermitidae) in Response to 2'-acetonaphthone Treated Sand. *Pest Manag. Sci.* **2004c,** 60, 746-754.

Ibrahim, S. A.; Henderson, G.; Fei, H.; Laine, R. Toxic and Repellent Effects of 2'-acetonaphthone on *Coptotermes formosanus* (Isoptera: Rhinotermitidae). *Sociobiology* **2004b,** 43(3), 429-443.

Ibrahim, S. A.; Henderson, G.; Zhu B. C. R.; Fei, H.; Laine, R. Toxicity and Behavioural Effects of Nootkatone, 1,19-dihydronootkatone and Tetrahydronootkatone to the *Formosan* Subterranean Termite (Isoptera: Rhinotermitidae). *J. Econ. Entomol.* **2004a,** 97(1), 102-111.

Ikeda, T.; Takahashi, M.; Nishimoto, K. Antitermitic Components of Kaya Wood, *Torreya nucifera* Sieb. Et Zucc. *Mokuzai Gakkaishi* **1978,** 24, 262-266.

Inoue, S.; Yamaguchi, S. Eraser for Active Oxygen and Skin Cosmetic. Japanese Patent JP2000344653 (A), December 12, 2000.

Jachak, S. M. Cyclooxygenase Inhibitory Natural Products: Current Status. *Curr. Med. Chem.* **2006,** 13, 659-678.

Jefferies, P. R.; Knox, J. R. The Chemistry of *Eremophila* Species Part II. *Aust. J. Chem.* **1961,** 14, 628-636.

Jefferies, P. R.; Knox, J. R.; Price, K. R.; Scaf, B. Constituents of the Tumor-Inhibitory Extract of *Olearia muelleri*. *Aust. J. Chem.* **1974,** 27, 221-225.

Jiang, A. -L.; Sun, L. -Q. Antioxidation Activities of Natural Components from *Dalbergia odorifera* T. Chen. *Jingxi Huagong* **2004,** 21(7), 525-528.

Julemont, F.; Dogne, J. -M., Pirotte, B.; Leval, X. Recent Development in the Field of Dual COX/5-LOX Inhibitors. *Mini-Rev. Med. Chem.* **2004,** 4, 633-638.

Kerr. R. W. Adjuvants for Pyrethrins in Fly Sprays. Section I. The Adjuvant Action of Some Essential Oils and Other Materials from Australian Plants. Bulletin No. 261; CSIRO: Melbourne, 1951.

Kim, S. R.; Park, M. J.; Lee, M. K.; Sung, S. H.; Park, E. J.; Kim, J.; Kim, S. Y.; Oh, T. H.; Markelonis, G. J.; Kim, Y. C. Flavonoids of *Inula britannica* Protect Cultured Cortical Cells from Necrotic Cell Death Induced by Glutamate. *Free Radical Bio. Med.* **2002,** 32(7), 596-604.

Klassen, C. D.; Amdur, M. O.; Doull, J., Ed.; *Cassaret & Doull's toxicology. The Basic Science of Poisons,* 5th Ed.; McGraw-Hill: New York. 1996.

Knight, T.; Barr, A.; Andrews, M.; Alexander, V.; Aboriginal Communities of the Northern Territory of Australia. *Traditional Bush Medicines: an Aboriginal Pharmacopoeia / Aboriginal Communities of the Northern Territory of Australia*; Greenhouse Publications: Richmond, Victoria, 1988.

Kodoma, M.; Wada, H.; Otani, H.; Kohmoto, K.; Kimura, Y. 3,5-Di-*O*-Caffeoylquinic Acid, an Infection-inhibiting Factor from *Pyrus pyrifolia* Induced by Infection with *Alternaria alternata*. *Phytochemistry* **1998,** 47(3), 371-373.

Lagarda, M. J.; García-Llatas, G. Farré, R. Analysis of Phytosterols in Food. *J. Pharmaceut. Biomed.* **2006,** 41, 1486-1496.

Lassak, E. V.; Mc Carthy, T. *Australian Medicinal Plants*; Methuen: Sydney, 1983.

Latz, P. K. Bushfires and Bushtucker. M.A. Thesis, The University of New England, 1982.

Lawrence, B. M. (reference in) *Perfumer and Flavourist*. **1990,** 15, 1-7. CD-Rom compilation. Allured Publishing Corporation. 2000.

Lawrence, B. M. (reference in) *Perfumer and Flavourist*. **1999,** 24, 1-13. CD-Rom compilation. Allured Publishing Corporation. 2000.

Leach, D. N.; Spooner-Hart, R. N; Eaton, G. F.; Eremophilone and Eremophilone Derivatives for Pest Control. World Patent WO2004021784, March 18, 2004.

Lebow, S. Alternatives to Chromated Copper Arsenate (CCA) for Residential Construction. Proceedings of the Environmental Impacts of Preservative-Treated Wood Conference. Orlando, Florida, Feb 8-10, 2004. [online] http://www.fpl.fs.fed.us/documnts/pdf2004/fpl_2004_lebow002.pdf (accessed Feb 3, 2007).

Lewis, D. E.; Massy-Westropp, R. A.; Ingham, C. F.; Wells, R. J. The Structure Determination of Two Related Eremophilone Dimers. *Aust. J. Chem.* **1982,** 35, 809-826.

Lewis, D. E.; Massy-Westropp, R. A.; Snow, M. R. *cis*,*trans*-Tetrahydrometchelladione. *Acta Crystallogr., Sect. B: Struct. Sci.* **1979,** 35, 2253-2255.

Liang, H.; Bao, F.; Dong, X.; Tan, R.; Zhang, C.; Lu, Q.; Cheng, Y. Antibacterial Thymol Derivatives Isolated from *Centipeda minima*. *Molecules* **2007,** 12, 1606-1613.

Lin, L. -C.; Kuo, Y. -C.; Chou, C. -J. Immunomodulatory Principles of *Dichlorocephala bicolor. J. Nat. Prod.* **1999,** 62(3), 405-408.

Lin, T. S.; Yin, H.-W. Effects of *Litsea cubeba* pres Oils on the Control of Termite *Coptotermes formosanus* Shiraki. *Taiwan For. Res. Inst. New Series* **1995a,** 10, 59-63.

Lin, T. S.; Yin, H.-W. The Effects of *Cinnamomum* spp. on the Control of the Termite *Coptotermes formosanus* Shiraki. *Taiwan For. Res. Inst. New Series* **1995b,** 10, 459-464.

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

Lotito, S. B.; Frei, B. Consumption of Flavonoid Rich Foods and Increases in Plasma Antioxidant Capacity in Humans: Cause, Consequence or Epiphenomenon? *Free Radical Bio. Med.* **2006,** 41(12), 727-1746.

Low, T. *Bush Medicine: A Pharmacopoeia of Natural Remedies.*; Angus and Robertson: North Ryde, NSW, Australia. 1990.

Maconochie, J. R. Pitjantjara Names of Some Central Australian Plants. *South Australian Naturalist* **1970,** 44(4), 75-77.

Maiden, J. H. *The Useful Native Plants of Australia (including Tasmania)* Melbourne: Compendium; Turner and Henderson: Sydney, 1975.

Maistrello, L.; Henderson, G.; Laine, R. A. Comparative Effects of Vetiver Oil, Nootkatone and Disodium Octaborate Tetrahydrate on *Coptotermes formosanus* and its Symbiotic Fauna. *Pest Manag. Sci.* **2003,** 59(1), 58-68.

Masahiro, T.; Kazumasa, O. Hematophagous Insect Repellent. World Patent WO9925196(A1), May 27, 1999.

Matsuo, M.; Sasaki, N.; Saga, K.; Kaneko, T. Cytotoxicity of Flavonoids towards Cultured Normal Human Cells. *Biol. Pharm. Bull.* **2005,** 28(3), 253-259.

Massy-Westropp, R. A.; Reynolds, G. D. Eremophilane Sesquiterpenes from *Eremophila mitchellii*. *Aust. J. Chem.* **1966,** 19, 303.

McMurry, J. E.; Musser, J. H.; Ahmad, M. S.; Blaszczak, L. C. The Total Synthesis of Eremophilone. *J. Org. Chem.* **1975,** 40(12), 1829-1832.

Meggitt, M. J. *Desert People: A Study of the Walbiri Aborigines of Central Australia*; Angus and Robertson Publications: Sydney, 1962.

Moscatelli, V.; Hnatyszyn, O.; Acevedo, C.; Megías, J.; Alcaraz, M. J.; Ferraro, G. Flavonoids from *Artemisia copa* with Anti-inflammatory Activity. *Planta Med.* **2006,** 72(1), 72-74.

Mossa, J. S.; El-Feraly, F. S.; Muhammad, I.; Zaw, K.; Mbwambo, Z. H.; Pezzuto, J. M.; Fong, H. S. Sesquiterpene Lactones and Thymol Esters from *Vicoa pentanema*. *J. Nat. Prod.* **1997,** 60(6), 550–555.

Murakami, T.; Chen, C. M. Studies on the Constituents of *Centipeda minima*. *Yakuga. Zasshi.* **1970,** 90(7), 846-9.

Murray, R. T.; von Stein, C.; Kennedy, I.R.; Sanchez-Bayo, F. Stability of Chlorpyrifos for Termiticidal Control in Six Australian Soils *J. Agric. Food Chem.* **2001,** 49(6), 2844-2847.

Nakajima, N.; Ubukata, M. Facile Synthesis of Cyanogen Glycosides (*R*)-Prunasin, Linamarin and (S)-Heterodendrin. *Biosci. Biotechnol. Biochem.* **1988,** 62(3), 453-458.

Nangan, P.; Clement, J. L. Terpenes from the Maritime Pine *Pinus Pinaster*: Toxins for Subterranean Termites of the Genus Reticulitermes (Isoptera: Rhinotermitidae). *Biochem. Syst. Ecol*. **1990,** 18, 13-16.

Ndi, C. P.; Semple, S. J.; Griesser, H. J.; Barton, M. D. Antimicrobial Activity of Some Australian Plant Species from the Genus *Eremophila. J. Ethnopharmacol.* **2007a,** 112(2), 386-393.

Ndi, C. P.; Semple, S. J.; Griesser, H. J.; Pyke, S. M.; Barton, M. D. Antimicrobial Compounds from *Eremophila serrulata. Phytochemistry* **2007b,** 68(21), 2684-2690.

O'Connell, J. F.; Latz, P. K.; Barnett, P. Traditional and Modern Plant Use Among the Alyawara of Central Australia. *Econ. Bot.* **1983,** 31, 80-109.

Osbrink, W. L.; Lax, A. R.; Cantrell, C. L. Comparison of Natural and Conventional Insecticides Against *Formosan* Termites (Isoptera Rhinotermitidae). Proceedings of International Conference on Urban Pests, May 20, 2005, 213-223.

Palombo , E. A.; Semple, S. J. Antibacterial Activity of Australian Plant Extracts Against Methicillin-resistant *Staphylococcus aureus* (MRSA) and Vancomycin-Resistant Enterococci (VRE). *J. Basic Microb.* **2002,** 42(6), 444-448.

Palombo, E. A.; Semple, S. J. Antibacterial Activity of Traditional Australian Medicinal Plants. *J. Ethnopharmacol.* **2001,** 77(2), 151-157.

Pawson, B. M.; Gold, R. E. Evaluation of Baits for Termites (Isoptera Rhinotermitidae) in Texas. *Sociobiology* **1996,** 28, 485-510.

Pearce, M. J. *Termites: Biology and Pest Managment*; CABI Publishing: CAB International, Wallingford, Oxon, U. K., 1997.

Pelzer, L. E.; Guardia, T.; Juarez, A. O.; Guerreiro, E. Acute and Chronic Anti-inflammatory Effects of Plant Flavonoids. *Farmaco,* **1998,** 53(6), 421-424.

Pennacchio, M.; Syah, Y. M.; Ghisalberti, E. L.; Alexander, E. Cardioactive Compounds from *Eremophila* species. *J. Ethnopharmacol.* **1996,** 53, 21-27.

Pinder, A. R.; Torrence, A. K. Total Synthesis of Racemic Fukinone and Natural (+)-Hydroxyeremophilone. *J. Chem. Soc. (C),* **1971,** 3410-3414.

Pinhey, J. T.; Southwell, I. A. Occurrence of (-)-*cis*-Chrysanthenyl Acetate in *Centipeda Cunninghamii*. *Aust. J. Chem.* **1971,** 75(5), 1311-1313.

Poplawski, J.; Holub, M.; Samek, Z.; Herout, V. On Terpenes. CCIX Arnicolides - Sesquiterpenic Lactones from the Leaves of *Arnica montana* L. *Collect. Czech. Chem. C.* **1971,** 36, 2189-2199.

Prior, R. L.; Hoang, H.; Gu, L.; Wu, X.; Bacchiocca, M.; Howard, L.; Hampsch-Woodill, M.; Huang, D.; Ou, B.; Jacob, R. Assays for Hydrophilic and Lipophilic Antioxidant Capacity (oxygen radical absorbance capacity (ORAC)) of Plasma and Other Biological and Food Samples. *J. Agric. Food Chem.* **2003,** 51(11), 3273-3279.

Quattrocchi, U. *CRC World Dictionary of Plant Names: Common Names, Scientific Names, Eponyms, Synonyms, and Etymology*; CRC Press: Boca Raton, 2000.

Rahman, M. M. A.; Dewick, P. M.; Jackson, D. E.; Lucas, J., A. Lignans of *Forsythia intermedia*. *Phytochemistry* **1990,** 29, 1971-1980.

Rang, H. P., Dale, M. M.; Ritter, J. M. *Pharmacology,* 3rd ed.; Churchill Livingstone: United Kingdom, 1995.

Raven, P. H.; Evert, R. F.; Eichhorn, S. E. *Biology of Plants*, 7th ed.; W. H. Freeman and Company: New York, 2005.

Richmond, G. S.; Ghisalberti, E. L., The Australian Desert Shrub *Eremophila* (Myoporacea): Medicinal, Cultural, Horticultural and Phytochemical Uses. *Econ. Bot.* **1994,** 48(1), 35-59.

Robinson, R. *The Structural Relations of Natural Products.* Oxford University Press. 1955, p 12.

Rogers, K. L.; Grice, D.; Griffiths, L. R. Inhibition of Platelet Aggregation and 5-HT Release by Extracts of Australian Plants Used Traditionally as Headache Treatments. *Eur. J. Pharm. Sci.* **2000,** 9(4), 355-363.

Roitman, J. N.; James, L. F. Chemistry of Toxic Range Plants. Highly Oxygenated Flavonol Methyl Ethers from *Gutierrezia microcephala*. *Phytochemistry* **1985,** 24(4), 835-848.

Ruitenberg, J. J.; Waters, C. A. A Rapid Flow Cytometric Method for the Detection of Intracellular Cyclooxygenases in Human Whole Blood Monocytes and a Cox-2 Inducible Human Cell Line. *J. Immun. Methods* **2003**, 274(1-2), 93-104.

Ruzicka, L. History of the isoprene rule: Faraday lecture. *Proc. Chem. Soc.* **1959,** 341-360.

Saeki, L.; Sumimoto, M.; Kondo, T. The Termiticidal Substances from the Wood of *Chamaecyparis pisifera* D. Don. *Holzforschung* **1973,** 27, 93-96.

Saleem, M.; Kim, H. J.; Ali, M. S.; Lee, Y. S. An Update on Bioactive Plant Lignans. *Nat. Prod. Rep.* **2005,** 22, 696-716.

Sankawa, U.; Chun, Y. T. Anti-Allergic Substances from Chinese Medicinal Plants. *Adv. Chin. Med. Mater. Res., Int. Symp.* **1985,** 171-180.

Scheffrahn, R. H.; Hsu, K. C.; Su, N. Y.; Huffman, J. B.; Midlan, S. L.; Sims, J. J. Allelochemical Resistance of Bald Cypress, *Taxodium distichum*, Heartwood to the Subterranean Termite, *Coptotermes formosanus*. *J. Chem Ecol*. **1988,** 14, 765-776.

Schwab, J. M.; Schluesener, H. J.; Laufer, S. COX-3: Just Another COX or the Solitary Elusive Target of Paracetamol? *Lancet* **2003,** 361(9362), 981-982.

Seawright, A. A.; Hrdlicka, J.; Lee, J. A.; Ogunsan, E. A. Toxic Substances in the Food of Animals: Some Recent Findings of Australian Poisonous Plant Investigations. *J. Appl. Toxicol.* 2(2), 75 - 82.

Semple, S. J.; Reynolds, G. D.; O'Leary, M. C.; Flower, R. L. P. Screening of Australian Medicinal Plants for Antiviral Activity. *J. Ethnopharmacol.* 1998, 60(2), 163-172.

Shah, A.; Cross, R. F.; Palombo, E. A. Identification of the Antibacterial Component of an Ethanolic Extract of the Australian Medicinal Plant, *Eremophila duttonii. Phytother. Res.* **2004,** 18(8), 615-618.

Sharma, R. N.; Tungikar, V. B.; Pawar, P. V.; Vartak, P. H. Vapour Toxicity and Repellency of Some Oils and Terpenoids to the Termite, *Odontotermes brunneus*. *Insect Sci. Applic*. **1994,** 15, 495-498.

Silberbauer, G. B. Ecology of the Ernabella Aboriginal Community. *Anthropological Forum* **1971,** 3(1), 21-36.

Silverstein, R. M.; Bassler, G. C.; Morrill, T. C. *Spectrometric Identification of Organic Compounds*. 5th ed.; John Wiley and Sons, Inc.: New York, 1991, Chapter 2.

Smith, J. E.; Tucker, D.; Watson, K.; Jones, G. L. Identification of Antibacterial Constituents from the Indigenous Australian Medicinal Plant *Eremophila duttonii* F. Muell. (Myoporaceae). *J. Ethnopharmacol.* **2007,** 112(2), 386-393.

Smith, N. M. Ethnobotanical Field Notes from the Northern Territory, Australia. *Journal of the Adelaide Botanical Gardens* 1991, 14(1), 1-65.

Spencer, W. B.; Gillen, F. J. *The Native Tribes of Central Australia*; Dover Publications: New York, 1969.

Spooner-Hart, R.; Basta, A. Centre for Plant and Food Sciences. Final Report on the Ausindustry Biotechnology Innovation Fund Grant, 2006.

Su, N. Y.; Wheeler, G. S.; Scheffran, R. Subterranean Termite (Isoptera: Rhinotermidae) Penetration into Sand Treated at Various Thicknesses with Termiticides. *J. Econ. Entomol.* **1995,** 88(6), 1690-1694.

Su, N. –Y.; Tamashiro, M.; Haverty, M. I. Effect of Behaviour on the Evaluation of Insecticides for Prevention of or Remedial Control of the Formosan Subterranean Termite. *J. Econ. Entomol.* **1982,** 75, 188-193.

Sudarshan, S. R. *Encyclopaedia of Indian Medicine, Vol. 4, Materia Medica-Herbal Drugs*; Popular Prakashan: Mumbai, 2005.

Sweeney, A. P.; Wyllie, S. G.; Shalliker, R. A.; Markham, J. L. Xanthine Oxidase Inhibitory Activity of Selected Australian Native Plants. *J. Ethnopharmacol.* **2001,** 75(2-3), 273-277.

Takano, K.; Kurachi, M.; Ohashi, T. Prevention or Therapeutic Agent for Pruritic Dermalogical Disease. Japanese Patent JP2007169177 (A), July 5, 2007.

Tamashiro, M.; Yamamoto, R.; Ebesu. R., *Resistance of ACZA Treated Douglas Fir Heartwood to the Formosan Subterranean Termite*; American Wood Preservers' Association, 1988. [online] http://www.acza.com/ (accessed Feb 2, 2007)

Tang, Y., Herpes Zoster Tincture. Chinese Patent CN1408378 (A), April 9, 2003.

Taylor, A. O.; Wong, E. Quercetagetin 3,6-Dimethyl Ether. *Tetrahedron Lett.* **1965,** 41, 3675-3678.

Taylor, R. S. L.; Towers, G. H. N. Antibacterial Constituents of the Nepalese Medicinal Herb, *Centipeda minima*. *Phytochemistry* **1998,** 47(4), 631-634.

Thorne, B. L. *Biology of Subterranean Termites of the Genus Reticulitermes, NPCA Research Reports on Subterannean Termites Part I*; National Pest Control Association: Dunn Loring, Virginia, 1998.

Thornton, J. D.; Johnson, G. C.; Nguyen, N. *Revised CSIRO Natural Durability Classification in Ground Durability Ratings for Mature Outer Heartwood*; CSIRO: Clayton, Victoria, Australia. June 1997.

Tindale, N. B. *Vocabulary of Pitjandjarra, the Language of the Great Western Desert (Adelaide) 1931-1937*; Typescript, 1937.

Tsunoda, K. Economic Importance of Formosan Termite and Control Practices in Japan (Isoptera: Rhinotermitidae). *Sociobiology* **2003,** 41:1a, 27-36.

Tynan, B. J. Medical Systems in Conflict. A Study of Power. Diploma Thesis, The University of Sydney, 1979.

Ueda, H.; Yamazaki, C.; Yamazaki, M. Luteolin as an Anti-inflammatory and Anti-allergic Constituent of *Perilla frutescens*. *Biol. Pharm. Bull.* **2002,** 25(9), 1197-1202.

U. S. Department of Human Health and Human Services. *Toxicology Profile for 4,4'-DDT, 4,4'-DDE, 4,4'-DDD (update);* Agency for Toxic Substances and Disease Registry, 1994.

U. S. Environmental Protection Agency. *Pesticide Fact Sheet: Chlorpyrifos*. Office of pesticide programs. 1984. U.S government printing office, Washington DC.

U. S. Environmental Protection Agency. Chromated Copper Arsenate (CCA): ACQ – an Alternative to CCA. 2006. http://www.epa.gov/oppad001/-reregistration/cca/-acq.htm (accessed Feb 3, 2007).

United States Department of Agriculture. Subterranean Termites: Their Prevention and Control in Buildings. *Home and Garden Bulletin No. 64.* **1960**.

U. S. National Institute of Building Services. *Wood Protection Guidelines-Protecting Wood from Decay, Fungi, and Termites*; Wood Protection Council: Washington DC, 1993.

Vane, J. R.; Mitchell, J. A.; Appleton, I.; Tomlinson, A.; Bishop-Bailey, D.; Croxtall, J.; Willoughby, D. A., Inducible Isoforms of Cyclooxygenase and Nitric-oxide Synthase in Inflammation. *Proceedings of the National Academy of Sciences of the United States of America.* **1994,** 91(6), 2046-2050.

Warner, T. D.; Mitchell, J. A. Cyclooxygenase-3 (COX-3): Filling in the Gaps Toward a COX Continuum? *Proceedings of the National Academy of Sciences of the United States of America* **2002,** 99(21), 13371-13373.

Webb, L. J. *Guide to Medicinal and Poisonous Plants of Queensland*; Council for Scientific and Industrial Research: Melbourne, 1948.

Wiesner, D. M. Study of an Australian Native Plant: *Centipeda cunninghamii*. *Aust. J. Parm.* **1986,** 67, 785-786.

Wilkinson, J. M.; Cavanagh H. M. A. Antibacterial Activity of Essential Oils from Australian Native Plants. *Phytother. Res.* **2005,** 19(7), 643-646.

Williams, C. A.; Harborne, J. B.; Geiger, H.; Hoult, J. R. S. The Flavonoids of *Tanacetum parthenium* and *T. vulgare* and Their Anti-inflammatory Properties. [Erratum to document cited in CA131:142071]. *Phytochemistry* **1999.** 52(6), 1181-1182.

Willuhn, G.; Kresken, J.; Wendisch, D. Sesquiterpenlactone aus *Arnica chamissonis. Planta Med.* **1983,** 47, 157-160.

Wong, A. A. H., Kee, S.-C. and Grace, J. K. *Laboratory Evaluation of Termite Resistance of Five Lesser-known Malaysian Hardwoods used for Roof and Ceiling Construction;* IRG Document No. IRG/WP 01-10398; International Research Group on Wood Preservation: Stockholm, Sweden, 2001.

Wong, A. A. H.; Grace, J. K.; Kirton, L. G. *Termite Resistance of Malaysian and Exotic Woods with Plantation Potential: Field Evaluation;* IRG Document No. IRG/WP 98-10289; International Research Group on Wood Preservation: Stockholm, Sweden, 1998.

Wu, J B : Chun, Y T : Ebizuka, Y : Sankawa, U. Biologically Active Constituents of *Centipeda minima* Sesquiterpenes of Potential Anti-Allergy Activity. *Chem. Pharm. Bull. (Tokyo),* **1991,** 39(12), 3272-3275.

Wu, J. B.; Chun, Y. T.; Ebizuka, Y.; Sankawa, U. Biologically Active Constituents of *Centipeda minima*: Isolation of a New Plenolin Ester and the Antiallergenic activity of Sesquiterpene Lactones. *Chem. Pharm. Bull.* **1985,** 33(9), 4091-4094.

Wu, X., Medicinal Composition for Treating Bone Fracture. Chinese Patent CN1483436 (A), March 24, 2004.

Wu, Y., Powder Medicine for Tumour. Chinese Patent CN1152463 (A), June 25, 1997.

Yamamoto, H.; Ogawa, T. Antimicrobial Activity of Perilla Seed Polyphenols against Oral Pathogenic Bacteria. *Biosci. Biotechnol. Boichem.* **2002,** 66(4), 921-924.

Yoo, H.; Kim, S. H.; Lee, J.; Kim, H. J.; Seo, S. H.; Chung, B. Y.; Jin, C.; Lee, Y. S. Synthesis and Antioxidant Activity of 3-Methoxyflavones. *B. Kor. Chem. Soc.* **2005,** 26(12), 2057-2060.

Youssef, D.; Frahm, A. W. Constituents of the Egyptian *Centaurea scoparia*; III. Phenolic Constituents of the Aerial Parts. *Planta Med.* **1995,** 61, 570-573.

Yu, H. W.; Wright, C. W.; Cai, Y.; Yang, S. L.; Phillipson, J. D.; Kirby, G. C.; Warhurst, D. C. Antiprotozoal Activities of *Centipeda minima*. *Phytother. Res.* **1994,** 8(7), 436-438.

Zalkow, L. H.; Chetty, G. L. Interconversion of Eremophilone and Isoeremophilone and Related Reactions. *J. Org. Chem*. **1975,** 40(12), 1833-1834.

Zalkow, L. H.; Markley, F. X.; Djerassi, C. Terpenoids XLVIII. The Absolute Configuration of Eremophilone and Related Sesquiterpenes. *J. Amer. Chem. Soc.* **1960,** 82, 6354-6362.

Zalkow, L. H.; Markley, F. X.; Djerassi, C., Terpenoids XL. The Absolute Configuration of Eremophilone. *J. Amer. Chem. Soc*. **1959,** 81, 2914-2915.

Zhang, W. -D.; Tam, H.; Thi Bang, C.; Wan-Sheng, K.; D. -Y.; Li, H. -T.; Wang, Y. -H.; Fouraste, I. Two New Caffeoyl Conjugation from *Erigeron Breviscapus*. *J. Asian Nat. Prod. Res.* **2000,** 2(4), 283-288.

Zhang, W. S.; Li, A. L. *Medicinal Chemistry*; Higher Education Press: Beijing, 1999.

Zhang, Y.; Shaffer, A.; Portanova, J.; Seibert, K.; Isakson, P. C. Inhibition of Cyclooxygenase-2 Rapidly Reverses Inflammatory Hyperalgesia and

Prostaglandin E_2 Production. *J. Pharmacol. Exp. Ther*. **1997,** 283(3), 1069-1075.

Zhao, C.; Qui, R.; Zheng, R. *Lanzhou Daxue Xuebao, Ziran Kexueban* **2000,** 64, 66. Reference 43 in Saleem et al., 2005.

Zhu, B. C. R.; Henderson, G.; Chen, F.; Fei, H.; Laine, R. A. Evaluation of Vetiver oil and Seven Insect Active Essential Oils Against the Formosan Subterranean Termite. *J. Chem. Ecol.* **2001a,** 27(8), 1617-1625.

Zhu, B. C. R.; Henderson, G.; Chen, F.; Maistrello, L.; Laine, R. A. Nootkatone is a Repellent for Formosan Subterranean Termite (*Coptotermes formosanus*). *J. Chem. Ecol.* **2001b,** 27(3), 523-531.

Zhu, B. C. R.; Henderson, G.; Laine, R. A. Dihydronootkatone and Tetrahydronootkatone as Repellents to Arthropods. U.S. Patent US2005/0171213A1, Feb 8, 2005.

Zhu, X.; Dong, X.; Wang, Y.; Ju, P.; Luo, S. Phenolic Compounds from *Viburnum cylindricum*. *Helv. Chim. Acta* **2005a,** 88, 339-342.

Ziegler, F.E.; Reid, G. R.; Studt, W. L.; Wender, P. A. Stereochemistry of Dialkylcuprate Additions to Cyclopropylacrylic Esters. An Application to the Synthesis of (+/-)-Eremophilone. *J. Org. Chem*. **1977,** 42(11), 1991-2001.

Appendix I Experimental Methods

LC/MS

High Performance Liquid Chromatography

Column: Phenomenex Aqua C18 125A, 5µ, 150 x 4.6 mm I.D.
Column Temperature: 40 °C
Mobile Phases: A - MQ Water with 0.005% TFA.
B - Acetonitrile with 0.005%TFA.
Flow Rate: 1.0 mL/min
Injection volume: 10.0µL
Detectors: A. Photodiode array detector (PDA).
B. Mass Spectrometer Detector (MSD).

Agilent SL1100 Series Mass Spectrometer Detector (MSD)

Scan mode: 100 – 1200amu
Ionisation Mode: Atmospheric Pressure Chemical ionisation (APCI)
Ionisation Voltage:150 V
Capillary Voltage:2000 V
Corona Current: 8uA (both modes)
Drying Gas Flow: 5.0L/min
Drying Gas Temperature: 345^{o}C
Vaporiser Temperature: 395^{o}C
Nebuliser Pressure:60psig

Diode Array Detector (DAD)

Detection: 210nm, 238nm, 254nm, 280nm and 360nm
Scan: 190-600nm (store every 2nd spectrum)
Peak width: 0.11min

CI 10-95M Method - Mobile Phase Gradient Timetable:

Time (min)	%A	%B	Flow Rate (mL/min)
0	90	10	1.0
15	5	95	1.0
18	5	95	1.0
20	90	10	1.0
25	90	10	1.0

CI 10-95 Method - Mobile Phase Gradient Timetable:

Time (min)	%A	%B	Flow Rate (mL/min)
0	90	10	1.0
30	5	95	1.0
35	5	95	1.0
40	90	10	1.0
45	90	10	1.0

KB5-95 Method - Mobile Phase Gradient Timetable:

Time (min)	%A	%B	Flow Rate (mL/min)
0	95	5	1.0
5	95	5	1.0
35	5	95	1.0
37	5	95	1.0
40	95	5	1.0
45	95	5	1.0

KB95 Method - Mobile Phase Gradient Timetable:

Time (min)	%A	%B	Flow Rate (mL/min)
0	5	95	1.0
30	5	95	1.0
32.5	95	5	1.0
37.5	95	5	1.0
40	5	95	1.0
45	5	95	1.0

KBE5-95 Method - Mobile Phase Gradient Timetable:

Time (min)	%A	%B	Flow Rate (mL/min)
0	95	5	1.0
5	95	5	1.0
25	5	95	1.0
30	5	95	1.0
32	95	5	1.0
35	95	5	1.0

GC/MS methods

ISO-EXT method

Injector Parameters:
Injection volume: 1.0µL
Gas: He
Inlet Temperature: 250°C
Inlet Pressure: 39.1 psi
Total flow: 24.1 ml/min
Split Ratio: 10:1
Split Flow: 18.9 mL/min

Column Parameters:
Gas: He
Pressure: 39 psi
Flow: 1.9 mL/min
Average velocity: 35 cm/sec

ISO-EXT Oven Temperature Program

Time (min)	Temp	Ramp (°C/min)	Flow (cm/sec)
0.0	50	0.0	35
1.0	50	0.0	35
73.5	300	4.0	35

ISO-EXNT method

Injector Parameters:
Injection volume: 0.1µL
Gas: He
Inlet Temperature: 280°C
Inlet Pressure: 29.1 psi
Total flow: 408 ml/min
Split Ratio: 300:1
Split Flow: 403 mL/min

Column Parameters:
Gas: He
Pressure: 29.1 psi
Flow: 1.3 mL/min
Average velocity: 33 cm/sec

ISO_EXNT Oven Temperature Program

Time (min)	Temp	Ramp (°C/min)	Flow (cm/sec)
0.0	50	0.0	35
1.0	50	0.0	35
73.5	300	4.0	35

MSQCIDE method

Injector Parameters:
Injection volume: 0.1µL
Gas: He
Inlet Temperature: 280°C
Inlet Pressure: 29.1 psi
Total flow: 71.8 ml/min
Split Ratio: 50:1
Split Flow: 67.1 mL/min

Column Parameters:
Gas: He
Pressure: 29.1 psi
Flow: 1.3 mL/min
Average velocity: 33 cm/sec

MSQCIDE Oven Temperature Program

Time (min)	Temp	Ramp (°C/min)	Flow (cm/sec)
0.0	50	0.0	33
2.0	50	0.0	33
33.25	300	8.0	33
43.25	300	0.0	33

EREOM method

Column: SGE BPX5 Capillary column
50.0m x 0.22mm ID x 1µm film thickness

Injector Parameters:
Injection volume: 0.2µL
Gas: He
Inlet Temperature: 280°C
Inlet Pressure: 25.9 psi
Total flow: 33.4 ml/min
Split Ratio: 25:1
Split Flow: 28.9 mL/min

Column Parameters:
Gas: He
Pressure: 25.9 psi
Flow: 1.2 mL/min
Average velocity: 30 cm/sec

EREOM Oven Temperature Program

Time (min)	Temp	Ramp (°C/min)	Flow (cm/sec)
0.0	50	0.0	30
1.0	50	8.0	30
32.5	300	0.0	30
42.5	300	0.0	30

COSOL70 method

Column: SGE BPX5 Capillary column
50.0m x 0.22mm ID x 1μm film thickness

Injector Parameters:
Injection volume: 0.1μL

Gas: He
Inlet Temperature: 300°C
Inlet Pressure: 49.9 psi
Total flow: 68.7 ml/min
Split Ratio: splitless

Column Parameters:
Gas: He

Pressure: 49.89 psi
Flow: 1.3 mL/min
Average velocity: 35 cm/sec

COSOL70 Oven Temperature Program

Time (min)	Temp	Ramp (°C/min)	Flow (cm/sec)
0.0	250	0.0	35
3.0	250	15	35
9.0	340	0	35
41	340	0	35

Preparative HPLC Methods

Initial fractionation of *C. cunninghamii* crude extract.
Mobile Phase Gradient Timetable KB10-95 and BI10-95. Method:

Time (min)	%Water	%ACN	Flow Rate (mL/min)
0	90	10	15.0
25	10	90	15.0
28	10	90	15.0
30	90	10	15.0
35	90	10	15.0

Fractionation of *C. cunninghamii* SPE Fraction 2.
Mobile Phase Gradient Timetable KB10-36. Method:

Time (min)	%Water	%ACN	Flow Rate (mL/min)
0	90	10	20.0
12	64	36	20.0
15	90	10	20.0
18	90	10	20.0

Fractionation of *C. cunninghamii* SPE Fraction 3.
Mobile Phase Gradient Timetable KB10-60. Method:

Time (min)	%Water	%ACN	Flow Rate (mL/min)
0	90	10	20
20	40	60	20
22	90	10	20
25	90	10	20
27	90	10	0.1

Fractionation of *C. cunninghamii* SPE Fraction 4.
Mobile Phase Gradient Timetable KB40-80. Method:

Time (min)	%Water	%ACN	Flow Rate (mL/min)
0	60	40	20
16	20	80	20
18	10	90	20
21	10	90	20

Sub-fractionation of *C. cunninghamii* Fraction 2.
Mobile Phase Gradient Timetable BI10-25. Method:

Time (min)	%Water	%ACN	Flow Rate (mL/min)
0	90	10	15
15	75	25	15
18	90	10	15
20	90	10	15

Sub-fractionation of *C. cunninghamii* Fraction 11 and 14.
Mobile Phase Gradient Timetable BI20-40. Method:

Time (min)	%Water	%ACN	Flow Rate (mL/min)
0	80	20	15
15	60	40	15
18	80	20	15
20	80	20	15

Sub-fractionation of *C. cunninghamii* Fraction 16 and 17.
Mobile Phase Gradient Timetable KB20-50. Method:

Time (min)	%Water	%ACN	Flow Rate (mL/min)
0	80	20	15
15	50	50	15
16	5	95	15
20	5	95	15
21	20	20	15
23	20	20	15

Fractionation of *C. cunninghamii* flowers - ethanol partition.
Mobile Phase Gradient Timetable KB20-60. Method:

Time (min)	%Water	%ACN	Flow Rate (mL/min)
0	80	20	15
20	40	60	15
23	40	60	15
25	80	20	15
28	80	20	15

Fractionation of *C. cunninghamii* flowers - hexane partition.
Mobile Phase Gradient Timetable KB50-80. Method:

Time (min)	%Water	%ACN	Flow Rate (mL/min)
0	60	40	15
16.4	25	75	15
20	25	75	15
21	10	90	15
28	10	90	15
30	60	40	15
33	60	40	15

EREMO4 Method - Mobile Phase Gradient Timetable:

Time (min)	%Hexane	%EtOAc	Flow Rate (mL/min)
0	95	5	20
20	60	40	20
23	80	20	20
25	80	20	20
27	95	5	20

Mobile Phase Gradient Timetable KB05-60. Method:

Time (min)	%Water	%ACN	Flow Rate (mL/min)
0	95	5	20.0
20	40	60	20.0
23	95	5	20.0
28	95	5	20.0
28.5	95	5	1.0

Mobile Phase Gradient Timetable KB10-90. Method:

Time (min)	%Water	%ACN	Flow Rate (mL/min)
0	90	10	15.0
25	10	90	15.0
28	10	90	15.0
30	90	10	15.0
35	90	10	15.0
35.5	90	10	1.0

Mobile Phase Gradient Timetable KB30-60. Method:

Time (min)	%Water	%ACN	Flow Rate (mL/min)
0	70	30	20.0
20	40	60	20.0
25	10	90	20.0
30	10	90	20.0
32	70	30	20.0
37	70	30	1.0

Mobile Phase Gradient Timetable KBISO20. Method:

Time (min)	%Water	%ACN	Flow Rate (mL/min)
0	80	20	20.0
12	80	20	20.0
14	25	75	20.0
16	25	75	20.0
18	80	20	20.0
20	80	20	20.0
20.5	80	20	1.0

Mobile Phase Gradient Timetable KB30-80. Method:

Time (min)	%Water	%ACN	Flow Rate (mL/min)
0	70	30	20.0
20	20	80	20.0
25	10	90	20.0
30	10	90	20.0
32	70	30	20.0
37	70	30	1.0

Mobile Phase Gradient Timetable KB10-60. Method:

Time (min)	%Water	%ACN	Flow Rate (mL/min)
0	90	10	20.0
20	40	60	20.0
22	90	10	20.0
25	90	10	20.0
27	90	10	0.1

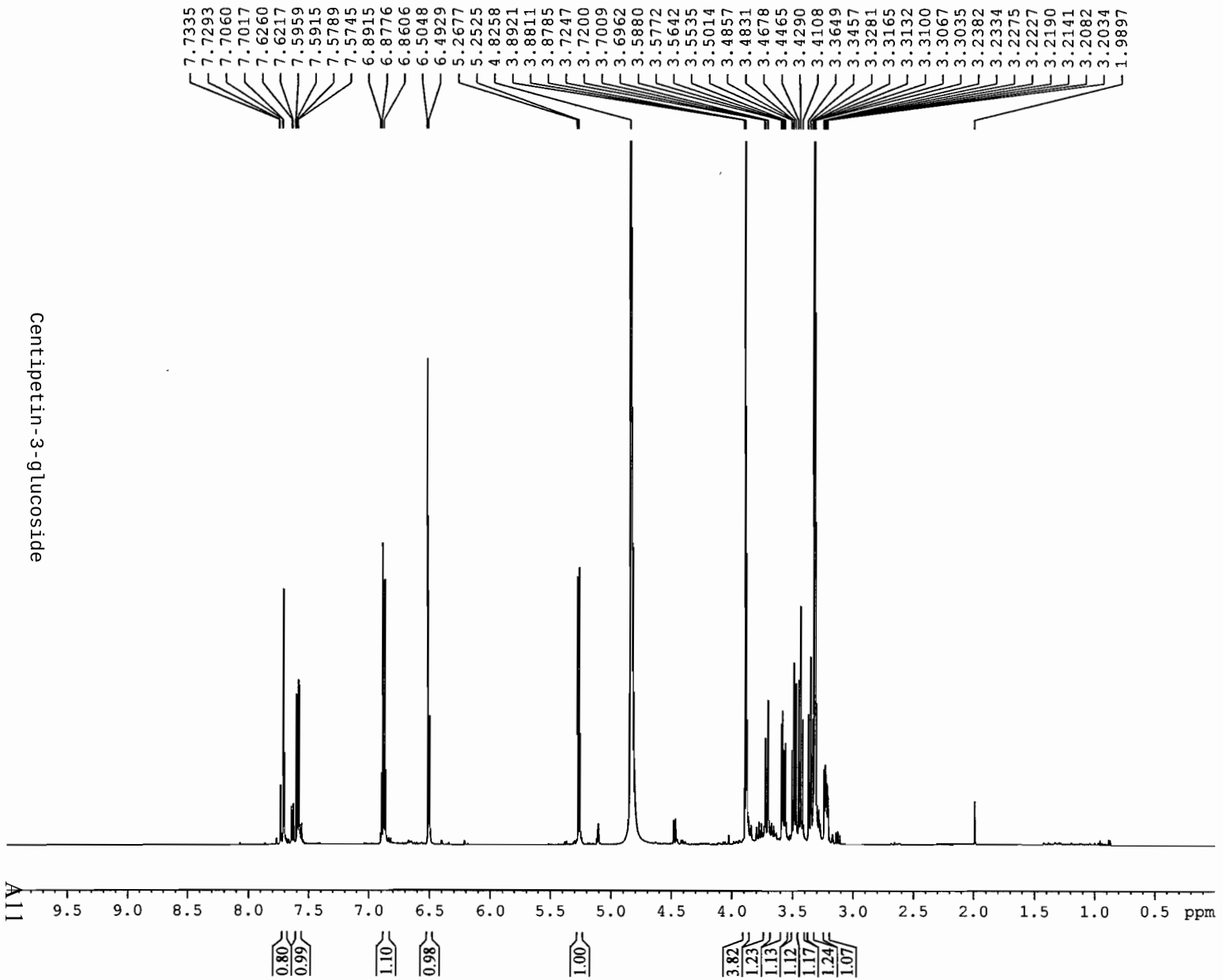
Mobile Phase Gradient Timetable KB40-80. Method:

Time (min)	%Water	%ACN	Flow Rate (mL/min)
0	60	40	20.0
16	20	80	20.0
18	10	90	20.0
21	10	90	20.0

Mobile Phase Gradient Timetable SPISO70. Method:

Time (min)	%Water	%MeOH	Flow Rate (mL/min)
0	30	70	15
55	30	70	15
56	30	70	0.1

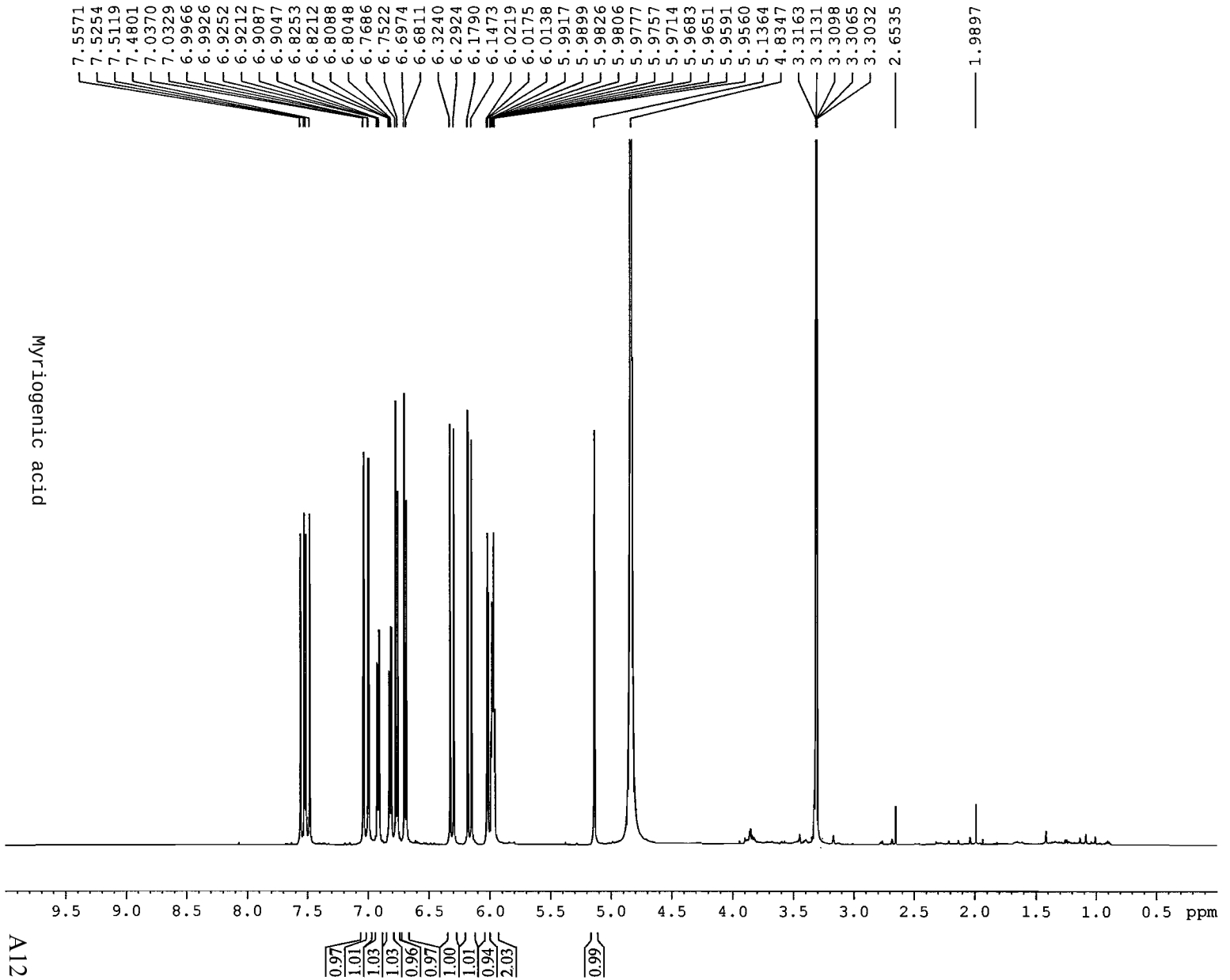
Centre for Phytochemistry, Southern Cross University
1-H NMR spectrum
Sample: Cent-Fr11, 5.4 mg
Solvent: CD3OD



Appendix II

```
Current Data Parameters
NAME                 Sep24-2006
EXPNO                         4
PROCNO                        1

F2 - Acquisition Parameters
Date_                  20060925
Time                      10.24
INSTRUM                   spect
PROBHD   5 mm QNP  1H/1
PULPROG                     zg
TD                       65536
SOLVENT                   DMSO
NS                          32
DS                           2
SWH                  10504.202 Hz
FIDRES                0.160281 Hz
AQ                   3.1195636 sec
RG                         256
DW                    47.600 usec
DE                     5.50 usec
TE                     302.0 K
D1                   2.00000000 sec
MCREST               0.00000000 sec
MCWRK                0.01500000 sec

======== CHANNEL f1 ========
NUC1                        1H
P1                     7.10 usec
PL1                   -4.00 dB
SFO1                500.1345012 MHz

F1 - Acquisition parameters
ND0                          2
TD                         128
SFO1                 500.1325 MHz
FIDRES              46.949928 Hz
SW                     12.016 ppm
FnMODE               undefined

F2 - Processing parameters
SI                       32768
SF                  500.1300110 MHz
WDW                         EM
SSB                          0
LB                    0.30 Hz
GB                           0
PC                        1.00

F1 - Processing parameters
SI                         512
MC2               echo-antiecho
SF                  500.1300083 MHz
WDW                         no
SSB                          2
LB                    0.30 Hz
GB                         0.1
```

Centre for Phytochemistry, Southern Cross University
1-H NMR spectrum
Sample: Cent-Fr17a, 6.9 mg
Solvent: CD3OD



A12

Current Data Parameters

NAME	Sep12-2006
EXPNO	1
PROCNO	1

F2 - Acquisition Parameters

Date_	20060912
Time	9.49
INSTRUM	spect
PROBHD	5 mm QNP 1H/1
PULPROG	zg
TD	65536
SOLVENT	DMSO
NS	32
DS	2
SWH	10504.202 Hz
FIDRES	0.160281 Hz
AQ	3.1195636 sec
RG	181
DW	47.600 usec
DE	5.50 usec
TE	302.0 K
D1	2.00000000 sec
MCREST	0.00000000 sec
MCWRK	0.01500000 sec

======== CHANNEL f1 ========

NUC1	1H
P1	7.10 usec
PL1	-4.00 dB
SFO1	500.1345012 MHz

F1 - Acquisition parameters

ND0	2
TD	128
SFO1	500.1325 MHz
FIDRES	46.949928 Hz
SW	12.016 ppm
FnMODE	undefined

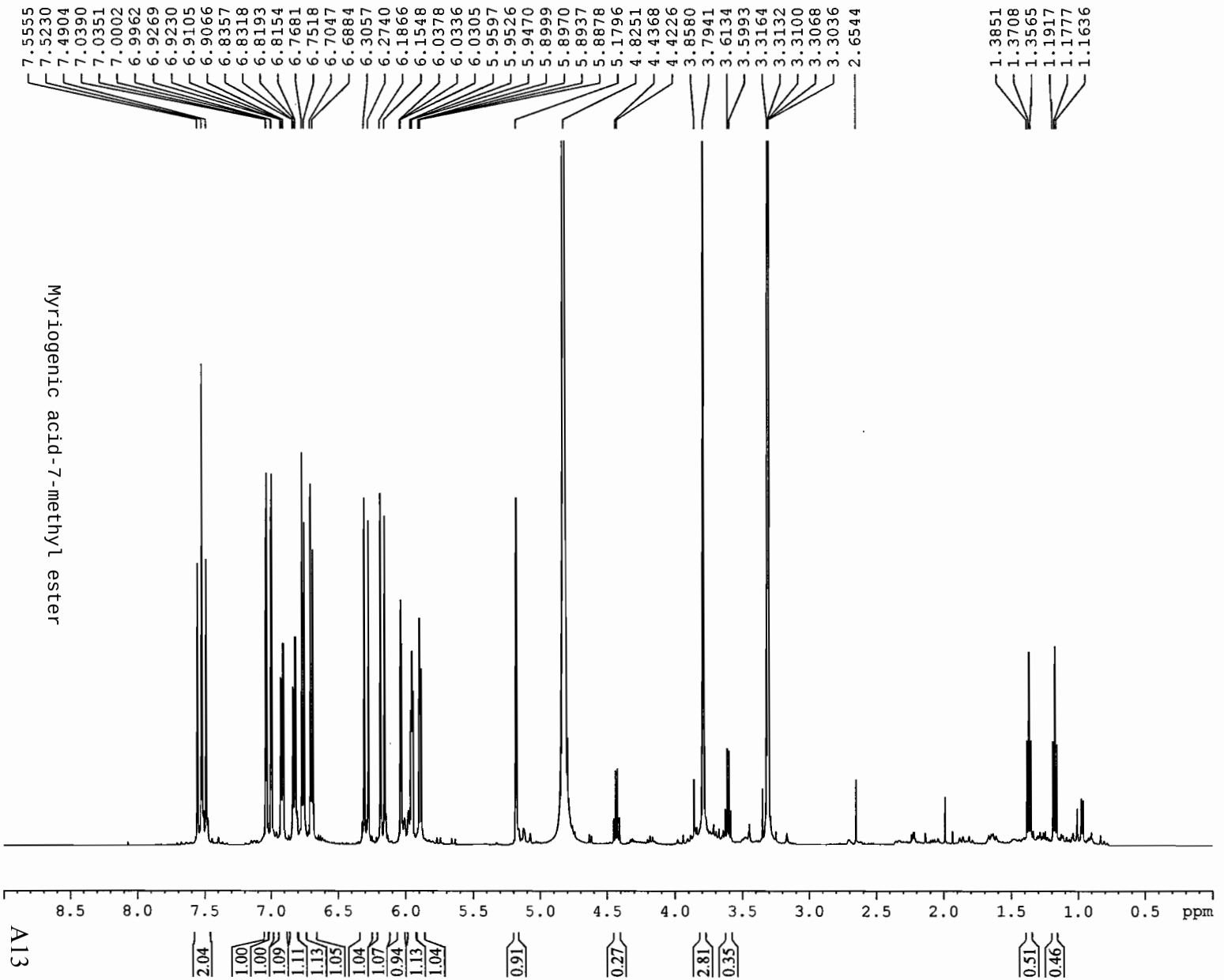
F2 - Processing parameters

SI	32768
SF	500.1300110 MHz
WDW	EM
SSB	0
LB	0.30 Hz
GB	0
PC	1.00

F1 - Processing parameters

SI	512
MC2	echo-antiecho
SF	500.1300083 MHz
WDW	QSINE
SSB	2
LB	0.30 Hz
GB	0.1

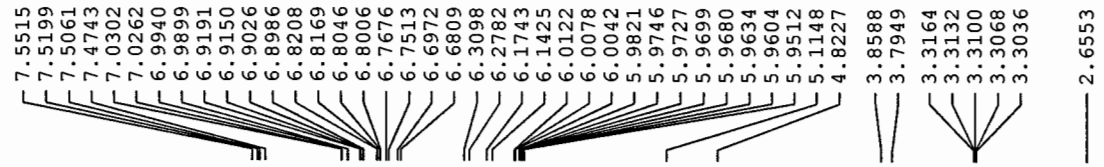
Centre for Phytochemistry, Southern Cross University
1-H NMR spectrum
Sample: Cent-Fr17b
Solvent: CD3OD

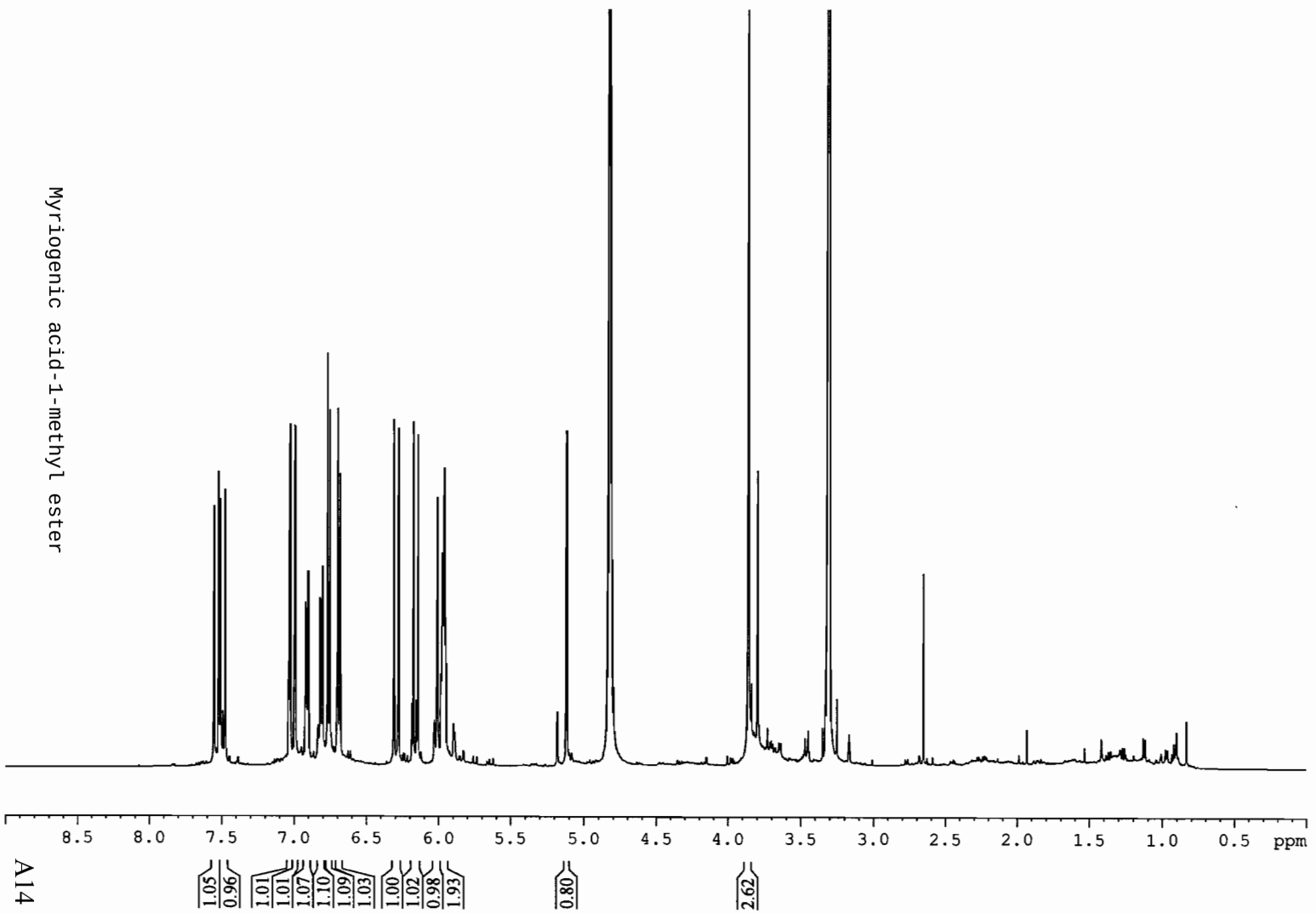


A13

```
Current Data Parameters
NAME          Sep20-2006
EXPNO                  1
PROCNO                 1

F2 - Acquisition Parameters
Date_           20060920
Time              17.12
INSTRUM           spect
PROBHD   5 mm QNP  1H/1
PULPROG             zg
TD                65536
SOLVENT            DMSO
NS                   32
DS                    2
SWH           10504.202 Hz
FIDRES         0.160281 Hz
AQ            3.1195636 sec
RG                  128
DW               47.600 usec
DE                5.50 usec
TE                302.0 K
D1           2.00000000 sec
MCREST       0.00000000 sec
MCWRK        0.01500000 sec

======== CHANNEL f1 ========
NUC1                 1H
P1                7.10 usec
PL1              -4.00 dB
SFO1        500.1345012 MHz

F1 - Acquisition parameters
ND0                  2
TD                 128
SFO1         500.1325 MHz
FIDRES      46.949928 Hz
SW             12.016 ppm
FnMODE       undefined

F2 - Processing parameters
SI                32768
SF          500.1300110 MHz
WDW                  EM
SSB                   0
LB                 0.30 Hz
GB                    0
PC                 1.00

F1 - Processing parameters
SI                  512
MC2        echo-antiecho
SF          500.1300083 MHz
WDW               QSINE
SSB                   2
LB                 0.30 Hz
GB                  0.1
```

Centre for Phytochemistry, Southern Cross University
1-H NMR spectrum
Sample: Cent-Fr17c
Solvent: CD3OD





A14

Current Data Parameters
NAME Sep21-2006
EXPNO 1
PROCNO 1

F2 - Acquisition Parameters
Date_ 20060921
Time 14.42
INSTRUM spect
PROBHD 5 mm QNP 1H/1
PULPROG zg
TD 65536
SOLVENT DMSO
NS 32
DS 2
SWH 10504.202 Hz
FIDRES 0.160281 Hz
AQ 3.1195636 sec
RG 362
DW 47.600 usec
DE 5.50 usec
TE 302.0 K
D1 2.00000000 sec
MCREST 0.00000000 sec
MCWRK 0.01500000 sec

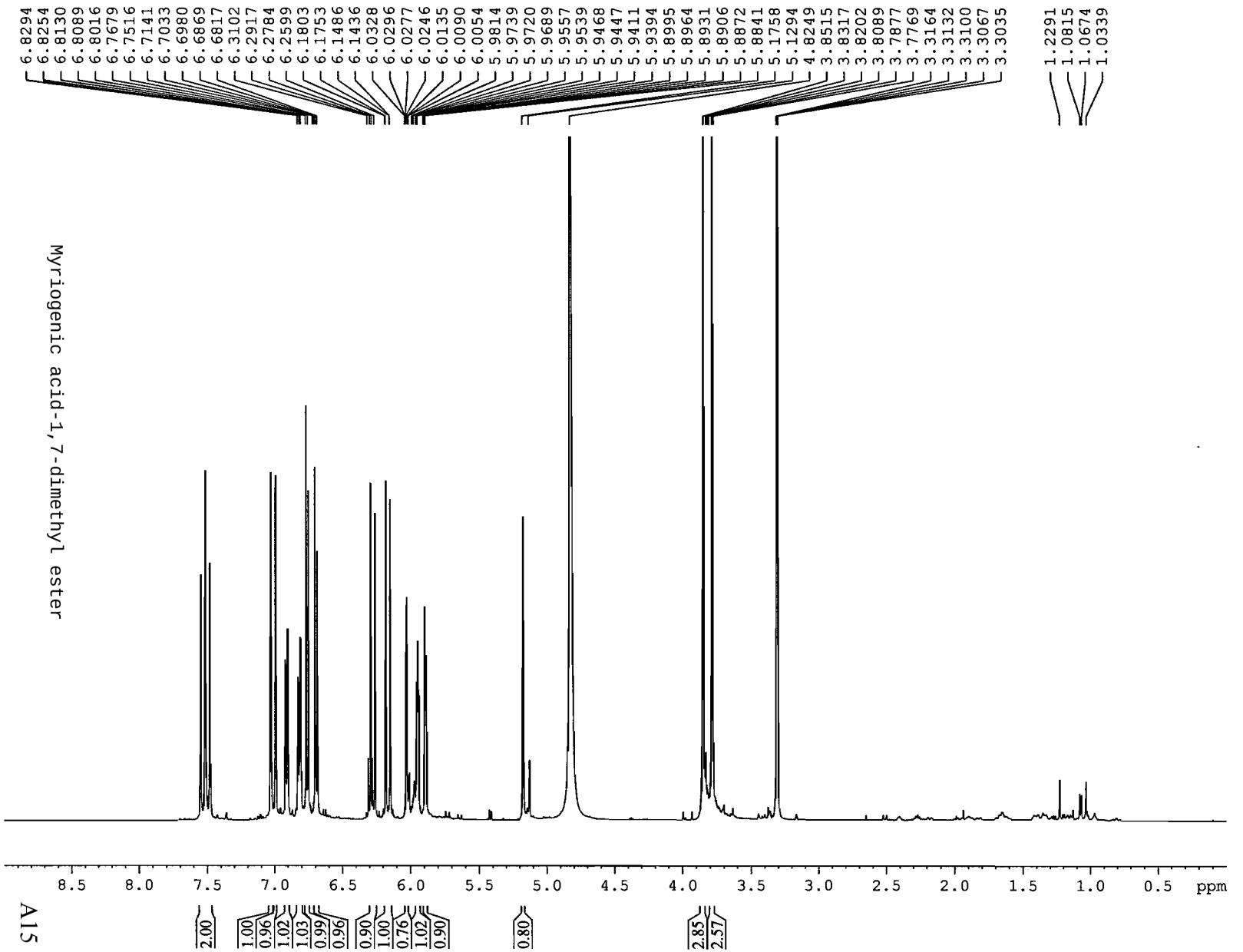
======== CHANNEL f1 ========
NUC1 1H
P1 7.10 usec
PL1 -4.00 dB
SFO1 500.1345012 MHz

F1 - Acquisition parameters
ND0 2
TD 128
SFO1 500.1325 MHz
FIDRES 46.949928 Hz
SW 12.016 ppm
FnMODE undefined

F2 - Processing parameters
SI 32768
SF 500.1300110 MHz
WDW EM
SSB 0
LB 0.30 Hz
GB 0
PC 1.00

F1 - Processing parameters
SI 512
MC2 echo-antiecho
SF 500.1300083 MHz
WDW no
SSB 2
LB 0.30 Hz
GB 0.1

Centre for Phytochemistry, Southern Cross University
1-H NMR spectrum
Sample: Cent-Fr16d
Solvent: CD3OD



A15

Current Data Parameters
NAME Oct10-2006
EXPNO 1
PROCNO 1

F2 - Acquisition Parameters
Date_ 20061010
Time 11.54
INSTRUM spect
PROBHD 5 mm QNP 1H/1
PULPROG zg
TD 65536
SOLVENT MeOH
NS 32
DS 2
SWH 10504.202 Hz
FIDRES 0.160281 Hz
AQ 3.1195636 sec
RG 128
DW 47.600 usec
DE 5.50 usec
TE 302.0 K
D1 2.00000000 sec
MCREST 0.00000000 sec
MCWRK 0.01500000 sec

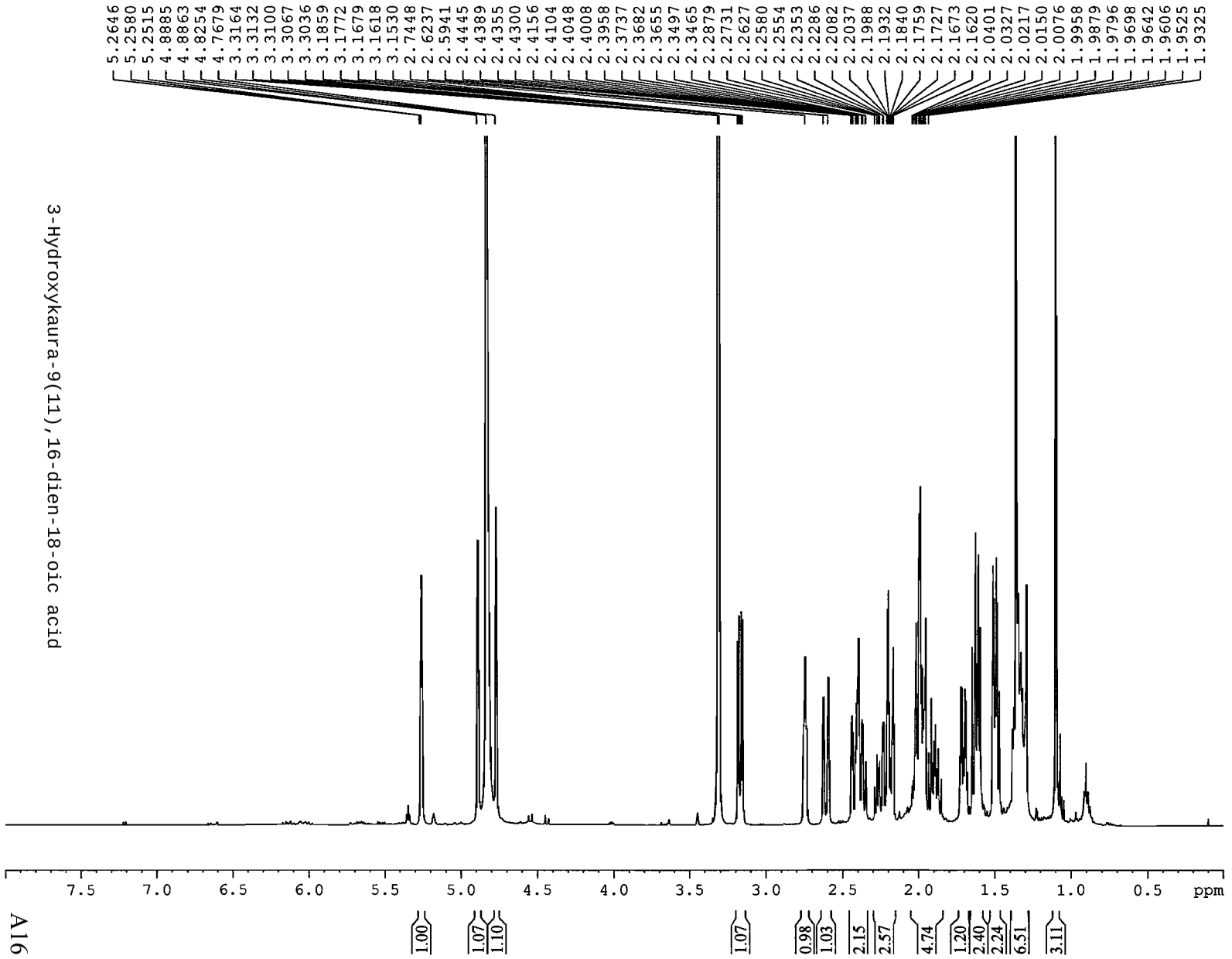
======== CHANNEL f1 ========
NUC1 1H
P1 7.10 usec
PL1 -4.00 dB
SFO1 500.1345012 MHz

F1 - Acquisition parameters
ND0 2
TD 128
SFO1 500.1325 MHz
FIDRES 46.949928 Hz
SW 12.016 ppm
FnMODE undefined

F2 - Processing parameters
SI 32768
SF 500.1300110 MHz
WDW EM
SSB 0
LB 0.30 Hz
GB 0
PC 1.00

F1 - Processing parameters
SI 512
MC2 echo-antiecho
SF 500.1300083 MHz
WDW no
SSB 2
LB 0.30 Hz
GB 0.1

Centre for Phytochemistry, Southern Cross University
1-H NMR spectrum
Sample: Cent SPE-F4 Cpd 7
Solvent: CD3OD



A16

Current Data Parameters
NAME Sep01-2006
EXPNO 1
PROCNO 1

F2 - Acquisition Parameters
Date_ 20060901
Time 17.01
INSTRUM spect
PROBHD 5 mm QNP 1H/1
PULPROG zg
TD 65536
SOLVENT DMSO
NS 32
DS 2
SWH 10504.202 Hz
FIDRES 0.160281 Hz
AQ 3.1195636 sec
RG 161.3
DW 47.600 usec
DE 5.50 usec
TE 302.0 K
D1 2.00000000 sec
MCREST 0.00000000 sec
MCWRK 0.01500000 sec

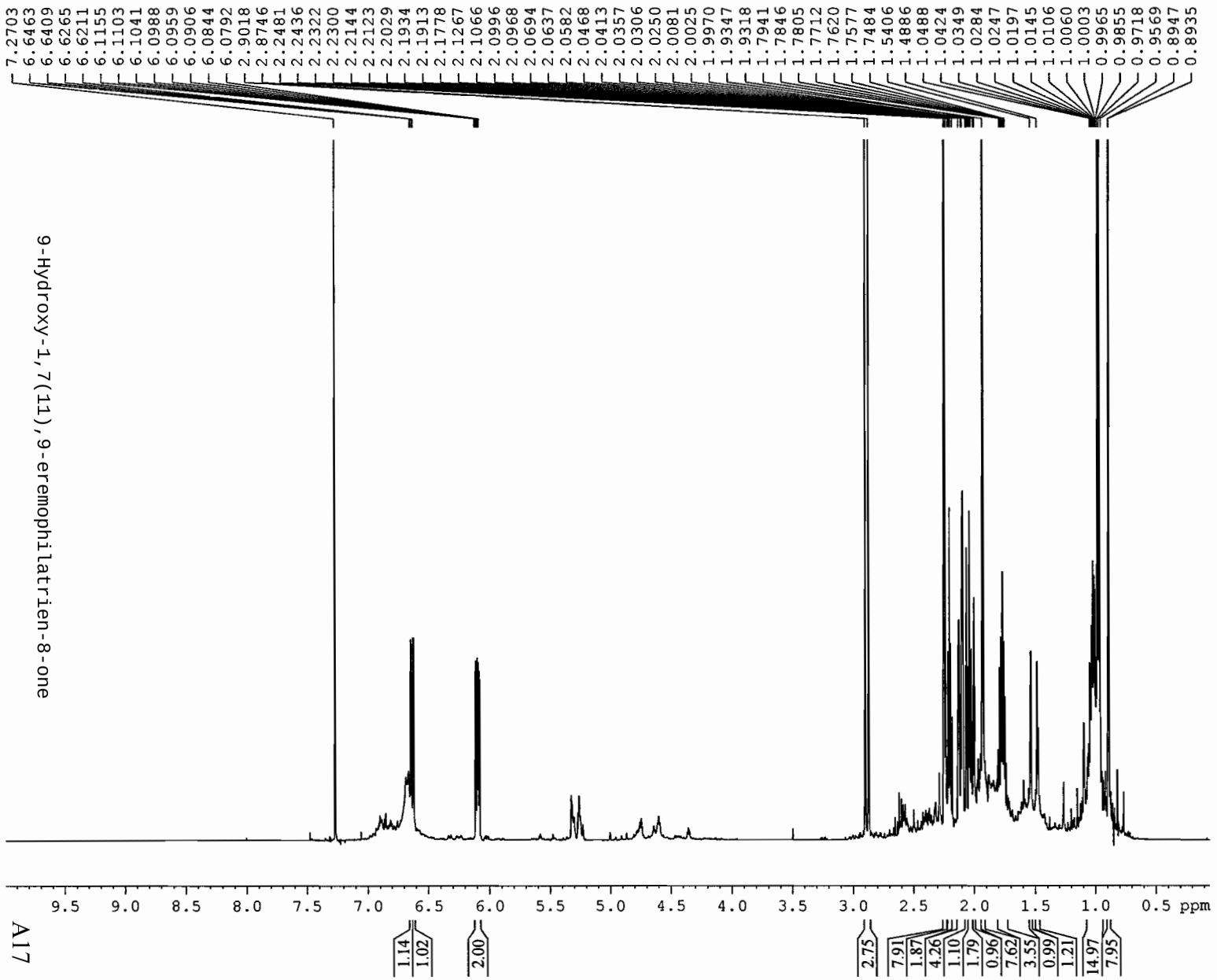
======== CHANNEL f1 ========
NUC1 1H
P1 7.10 usec
PL1 -4.00 dB
SFO1 500.1345012 MHz

F1 - Acquisition parameters
ND0 2
TD 128
SFO1 500.1325 MHz
FIDRES 46.949928 Hz
SW 12.016 ppm
FnMODE undefined

F2 - Processing parameters
SI 32768
SF 500.1300110 MHz
WDW EM
SSB 0
LB 0.30 Hz
GB 0
PC 1.00

F1 - Processing parameters
SI 512
MC2 echo-antiecho
SF 500.1300083 MHz
WDW QSINE
SSB 2
LB 0.30 Hz
GB 0.1

Centre for Phytochemistry, Southern Cross University
1-H NMR spectrum
Sample: EM4 A3 (Karren)
Solvent: CDCl3



A17

Current Data Parameters
NAME Jul05-2007
EXPNO 1
PROCNO 1

F2 - Acquisition Parameters
Date_ 20070705
Time 15.17
INSTRUM spect
PROBHD 5 mm QNP 1H/1
PULPROG zg
TD 65536
SOLVENT CDCl3
NS 8
DS 2
SWH 10504.202 Hz
FIDRES 0.160281 Hz
AQ 3.1195636 sec
RG 90.5
DW 47.600 usec
DE 5.50 usec
TE 302.0 K
D1 2.00000000 sec
TD0 1

======== CHANNEL f1 ========
NUC1 1H
P1 7.10 usec
PL1 -4.00 dB
SFO1 500.1345012 MHz

F1 - Acquisition parameters
ND0 2
TD 128
SFO1 500.1325 MHz
FIDRES 46.949928 Hz
SW 12.016 ppm
FnMODE undefined

F2 - Processing parameters
SI 32768
SF 500.1300082 MHz
WDW no
SSB 0
LB 0.00 Hz
GB 0
PC 1.00

F1 - Processing parameters
SI 512
MC2 echo-antiecho
SF 500.1300083 MHz
WDW QSINE
SSB 2
LB 0.30 Hz
GB 0.1

Appendix III

Termite Resistant Tree Species

Durability figures are based on; the Revised CSIRO natural durability classification in ground durability ratings for mature outer heartwood list compiled by CSIRO Australia (Thornton *et al.*,1997). Other information sources include The University of Hawaii – Department of Plant and Environmental Protection Sciences[a] and review of the literature. This is not intended as an exhaustive list. Information on commercial availability sourced from The Rainforest Information Centre (NSW)/System pest management[b].

(P) Plantation grown in Australia.
(OS) Overseas grown in plantations.
(C) Limited certified sources available.
(X) Not commercially available unless salvaged, recycled, or localised plantation.

Durability is adapted from Thornton *et al.* (1997) who have surveyed the durability of the timber in the presence of decay plus termites,
durability is scored on a scale of 1-4 where;

(1) = non durable, (**2)** = moderately durable, (**3)** = durable, (**4)** = highly durable.

AUSTRALIAN NON-EUCALYPTS

Black Bean [heartwood] *Castanospermum australe* [b]		OS
Brigalow *Acacia harpophylla* [b,c]	**(3)**	X
Brown Penda *Xanthostemen chrysanthus* [b]		X
Bull Oak *Allocasuarina luehmannii* [b, c]	**(3)**	X
Raspberry Jam *Acacia acuminata* [b, c]	**(4)**	X
Red Penda *Xanthostemen whitei* [b]		X
Satinay *Syncarpia hillii* [b, c]	**(2.5)**	OS
Swamp Box *Lophostemon suaveolens* [b, c]	**(3)**	X
Turpentine *Syncarpia glomulifera* [b, c]	**(3)**	OS

AUSTRALIAN EUCALYPT HARDWOODS

Blackbutt	*E. pilularis* [b, c]	**(2)**	P,OS
Blackdown Stringybark	*E. sphaerocarpa* [b]		X
Bloodwood	*E. corymbosa* [b]		X
Broad-leaved Red Ironbark	*E. fibrosa* [b]		X
Broad-leaved Peppermint	*E. dives* [c]	**(2)**	
Brown Mallett	*E. astringens* [b, c]	**(3)**	P
Coast Grey Box	*E. bosistoana* [b, c]	**(3)**	X
Forest Red Gum	*E. blakeleyi/ / E. tereticornis* [b, c]	**(3.5)**	X
Grey Box	*E. moluccana* [b, c]	**(3)**	X
Grey Gum	*E. canaliculata* [b]		X
Grey Ironbark	*E. paniculata* [b, c]	**(4)**	X
Gympie Messmate	*E. cloeziana* [b, c]	**(4)**	P
Jarrah	*E. marginata* [b, c]	**(2.5)**	P
Long-leaved Box	*E. goniocalyx* [b, c]	**(2)**	X
Narrow-leaved Red Ironbark	*E. crebra* [b]		X
New England Blackbutt	*E. andrewsii* [b]		X
Red Bloodwood	*E. gummifera* [b]		X
Red Box	*E. polyanthemos* [b, c]	**(4)**	X
Red Ironbark	*E. sideroxylon* [b, c]	**(4)**	X
Red Mahogany	*E. resinifera* [b, c]	**(3)**	X
Red Stringybark	*E. macrorhyncha* [b, c]	**(3)**	X
River Red Gum	*E. camaldulensis* [b, c]	**(3)**	P,OS
Rose Gum	*E. grandis* [c]	**(2)**	

AUSTRALIAN EUCALYPT HARDWOODS Continued:

Salmon Gum	*E. salmonophloia* [b, c]	**(2)**	X
Scribbly Gum	*E. Haemastoma* [b, c]	**(2)**	X
Southern Mahogany	*E. botryoides* [b, c]	**(2.5)**	X
Spotted Gum	*E. maculata* [b, c]	**(3)**	P
Sugar Gum	*E. cladocalyx* [b, c]	**(4)**	X
Sydney Blue Gum	*E. saligna* [c]	**(2)**	
Tallowwood	*E. microcorys* [a, b, c, f]	**(4)**	P
Tuart	*E. gomphocephala* [b, c]	**(2)**	X
Wandoo	*E. wandoo* [b, c]	**(4)**	X
White Mahogany	*E. acmenoides* [b, c]	**(4)**	X
White Stringybark	*E. eugenoiides* [b, c]	**(2)**	X
Woolybutt	*E. Longifolia* [b, c]	**(3)**	X
Yate	*E. cornuta* [c]	**(2)**	
Yellow Box	*E. melliodora* [b, c]	**(4)**	X
Yellow Gum	*E. leucoxylon* [b, c]	**(2.5)**	X
Yellow Tingle	*E. guilfoylei* [c]	**(2)**	
Yertchuk	*E. consideniana* [b, c]	**(2)**	X

AUSTRALIAN SOFTWOODS

Black Cypress Pine	*Callitris endlicheri* [b]		X
Huon Pine	*Lagarostrobus franklinii* [b, c]	**(1)**	OS
King William Pine	*Athrotaxis selaginoides* [b c]	**(2)**	X
White Cypress Pine	*Callitris columellaris* [b, c]	(3)	OS

IMPORTED SPECIES

Burmese Teak or Laotian Teak *Tectona grandis* [a, b, c, d, e, j]	**(3)**	OS
Coast Redwood *Sequoia sempervirens* [a, b, c, j]	(2)	X
Casuarina pine *Casuarina equisetifolia* [a, d]		
Kamani *Calophyllum inophyllum* [a, f]		
Kempas *Koompassia malaccensis* [a, d]		
Kuo *Cordia subcordata* [a, f]		
Kwila *Intsia bijuga* [b, c]	**(2.5)**	C
Milo *Thespesia populnea* [a, f]		
New Guinea Rosewood *Pterocarpus indicus* [b, c]	**(2)**	C
Pacific Coast Yellow or Alaska Cedar *Chamaecyparis nootkatensis* [a, j]		
Sentang *Azadirachta excelsa* [a, d]		
Sugi *Cryptomeria japonica* [a, f]		
Tualang *Koompassia excelsa* [a, d]		
Western Red Cedar *Thuja plicata* [b, c]	**(2)**	C
Hala *Pandanus tectorius* [f]		
Mempening *Lithocarpus spp.*[g]		
Bangkirai *Shorea laevis* Ridl.[h]		
Merbau *Intsia palembanica* Miq.[h]		
Ponderosa Pine *Pinus ponderosa* [i]		
Lysiloma seemanii [i]		
Gold Trumpet Tree *Tabebuia ochracea* [i]		
Macassar Ebony *Diospyros celebica* [k]		
Persimon, American *Diospyros virginiana* [l]		
Diospyros sylvatica [m]		

IMPORTED SPECIES Continued:

May Chang *Litsea cubeba* [n]

Cinnamon sp. *Cinnamomum* spp.[o]

Sawara, Japanese False Cypress *Chamaecyparis pisidera* [p]

Kaya Wood *Torreya nucifera* [q]

Bald Cypress *Taxodium distichum* [r]

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[a]University of Hawaii: Termite Project. http://www2.hawaii.edu/~entomol/research/r_durable.htm (accessed Oct 26, 2006).

[b] The RIC Good Wood Guide. Pesticides in the Home: Natural Household Treatments. http://www.rainforestinfo.org.au/good_wood/nat_htrs.htm (accessed Oct 26, 2006)

[c] Thornton, J. D.; Johnson, G. C.; Nguyen, N. *Revised CSIRO natural durability classification in ground durability ratings for mature outer heartwood*; CSIRO: Clayton, Victoria, Australia. A3 leaflet. June 1997

[d] Grace, J. K.; Wong, A. A. H.;Tome, C. H. M.Termite resistance of Malaysian and exotic woods with plantation potential: laboratory evaluation. International Research Group on Wood Preservation, Stockholm. Sweden. IRG Document No. IRG/WP 98-10280. 1998.

[e] Wong, A. A. H.; Grace, J. K.; Kirton, L. G. Termite resistance of Malaysian and exotic woods with plantation potential: field evaluation. International Research Group on Wood Preservation, Stockholm. Sweden. IRG Document No. IRG/WP 98-10289. 1998.

[f] Grace, J. K.; Ewart, D. M.; Tome, C. H. M. Termite resistance of wood species grown in Hawaii. *Forest Prod. J.* **1996,** 46(10), 57-60.

[g] Wong, A. A. H.; Kee, S. C.; Grace, J. K. Laboratory evaluation of termite resistance of five lesser-known Malaysian hardwoods used for roof and ceiling construction. International Research Group on Wood Preservation, Stockholm. Sweden. IRG Document No. IRG/WP 01-10398. 2001.

[h] Grace, J. K.; Tome, C. H. M. Resistance of Indonesian heartwoods bangkirai (*Shorea laevis*) and merbau (*Intsia palembanica*) to Formosan subterranean termite attack. *Sociobiology* **2005,** 45(2), 503-509.

[i] Grace, J. K.; Wood, D. L.; Frankie, G. W. Behaviour and survival of *Reticulitermes hesperus* Banks (Isoptera: Rhinotermitidae) on selected sawdusts and wood extracts. *J. Chem. Ecol.* **1989,** 15(1), 129-139.

[j] Grace, J. K.; Yamamoto, R. T. Natural resistance of Alaska-Cedar, redwood, and teak to Formosan subterranean termites. *Forest Prod. J.* **1994,** 44, 41-45.

[k] Sandermann, W.; Dietrichs, H. H. Investigation of termite resistant wood. *Holz Roh Werkst.* **1957,** 15, 281-297.

[l] Carter, F. L.; Garlo, A. M.; Stanley, J. B. Termiticidal components of wood extracts: 7-methyl-juglone from *Diospyros virginiana*. *J. Agric. Food Chem.* **1978,** 26, 869-873.

[m] Ganapatay, S.; Thomas, P. S.; Fotso, S.; Laatsch, H. Antitermitic quinones from *Diospyros sylvatica*. *Phytochemistry* **2004,** 65, 1265-1271.

[n] Lin, T. S.; Yin, H.-W. Effects of *Litsea cubeba* pres oils on the control of termite *Coptotermes formosanus* Shiraki. *Taiwan For. Res. Inst. New Series* **1995,** 10, 59-63.

[o] Lin, T. S.; Yin, H.-W. The effects of *Cinnamomum* spp. on the control of the termite *Coptotermes formosanus* Shiraki. *Taiwan For. Res. Inst. New Series* **1995,** 10, 59-63.

[p] Saeki, L.; Sumimoto, M.; Kondo, T. The termiticidal substances from the wood of *chamaecyparis pisifera* D. Don. *Holzforschung* **1973,** 27, 93-96.

[q] Ikeda, T.; Takahashi, M.; Nishimoto, K. Antitermitic components of Kaya wood, *Torreya nucifera* Sieb. Et Zucc. *Mokuzai Gakkaishi* **1978,** 24, 262-266.

[r] Scheffrahn, R. H.; Hsu, K. C.; Su, N. Y.; Huffman, J. B.; Midlan, S. L.; Sims, J. J. Allelochemical resistance of bald cypress, *Taxodium distichum*, heartwood to the subterranean termite, *Coptotermes formosanus*. *J. Chem Ecol*. **1988,** 14, 765-776.

Table A1. Mortality of *N. walkeri* associated with pure compounds 24 and 48 hours after treatment (direct contact mortality).

Product	Concentration in ppm (w/v)	Observations at 24 Hours							Observations at 48 Hours						
		R 1		R 2		R 3		Remarks	R 1		R 2		R 3		Remarks
		O	D	O	D	O	D		O	D	O	D	O	D	
Compound 3	**2000**	16	**0**	16	**1**	16	**1**	Normal	16	**2**	16	**1**	16	**1**	Normal
	3000	16	**4**	16	**6**	16	**3**	Majority unhealthy	16	**6**	16	**8**	16	**9**	Majority unhealthy
	3500	15	**3**	16	**3**	16	**7**	Majority unhealthy	15	**10**	16	**12**	16	**13**	Moribund
	4000	15	**7**	16	**6**	16	**6**	Majority unhealthy	15	**13**	16	**13**	16	**14**	Moribund
Comopund 2*	**430**	16	**0**	16	**1**	16	**2**	Majority healthy	16	**0**	16	**2**	16	**2**	Majority healthy
	860	16	**1**	16	**0**	16	**4**	Majority healthy	16	**1**	16	**3**	16	**5**	Majority healthy
	1440	x	**x**	16	**11**	13	**3**	Majority unhealthy	x	**x**	16	**11**	13	**3**	Majority unhealthy
	2400	16	**6**	16	**4**	16	**2**	Majority unhealthy	16	**6**	16	**8**	16	**6**	Majority unhealthy
	4000	16	**4**	16	**5**	16	**4**	Moribund	16	**10**	16	**9**	16	**9**	Moribund
Compound 1	**430**	16	**1**	16	**1**	16	**2**	Majority unhealthy	16	**4**	16	**3**	16	**6**	Majority unhealthy
	860	15	**1**	17	**3**	16	**9**	Majority unhealthy	15	**5**	17	**5**	16	**10**	Majority unhealthy
	1440	15	**12**	16	**13**	16	**14**	Moribund	15	**13**	16	**13**	16	**16**	Moribund
	2400	16	**13**	16	**16**	16	**11**	Moribund	16	**16**	16	**16**	16	**16**	Moribund
	4000	16	**14**	16	**14**	15	**15**	Moribund	16	**16**	16	**16**	15	**15**	Moribund
Compound 5	**420**	16	**0**	16	**0**			Normal	16	**0**	16	**0**			Normal
	840	16	**0**	16	**0**			Normal	16	**0**	16	**0**			Normal
	1680	16	**6**	16	**3**			Majority unhealthy	16	**x**	16	**3**			Majority unhealthy
	3360	16	**15**	16	**16**			Moribund	16	**15**	16	**16**			Moribund
***E. mitchellii* whole wood oil**	**500**	16	**4**	16	**2**	16	**0**	Normal	16	**5**	16	**2**	16	**1**	Normal
	1000	16	**6**	16	**5**	16	**4**	Moribund	16	**12**	16	**14**	16	**12**	Moribund
	2000	17	**13**	16	**5**	16	**16**	Moribund	17	**13**	16	**11**	16	**16**	Moribund
	4000	17	**17**	16	**16**	17	**17**	Moribund	17	**17**	16	**16**	17	**17**	Moribund
Control	**0.0**	15	**0**	15	**1**	15	**0**	Normal	15	**0**	15	**1**	15	**0**	Normal

*Note Compound EM2 crystallised out of solution onto paper surface.

R= Replicate, O= Observed, D=Dead, x = result discarded.

Table A2. Mortality of *N. exitiosus* associated with different products 24h after treatment (direct contact mortality).

Product	Concentration in ppm (w/v)	R 1		R 2		R 3		Remarks
		O	D	O	D	O	D	
(+)-nootkatone	**0.0**	20	**1**	20	**0**	20	**1**	Normal
	600	20	**2**	20	**0**	20	**2**	A few unhealthy
	700	20	**3**	20	**2**	20	**2**	Majority unhealthy
	800	20	**4**	20	**7**	20	**4**	Majority unhealthy
	1000	20	**17**	20	**15**	20	**18**	Moribund
	2300	20	**19**	20	**18**	20	**19**	Moribund
Eremophilone	**0.0**	20	**0**	20	**0**	20	**0**	Normal
	2000	20	**1**	20	**1**	20	**0**	Normal
	2500	20	**5**	20	**5**	-	**-**	Normal
	3000	20	**3**	20	**4**	-	**-**	Less than normal feeding and excretion
	3500	20	**9**	20	**6**	-	**-**	Unhealthy
	4000	20	**10**	20	**14**	20	**14**	Unhealthy
	5000	20	**16**	20	**17**	-	**-**	Moribund
	7300	20	**20**	20	**16**	-	**-**	Moribund
	10000	20	**20**	20	**19**	-	**-**	Moribund
***E. mitchellii* root oil**	**0.0**	20	**0**	20	**0**	20	**0**	Normal
	2000	20	**3**	20	**3**	20	**5**	Majority unhealthy
	3000	20	**8**	20	**7**	20	**7**	Majority unhealthy
	4000	20	**7**	20	**8**	20	**7**	Moribund
	4000	20	**13**	20	**14**	20	**14**	Moribund
	6000	20	**17**	20	**16**	20	**17**	Moribund
	8000	20	**20**	20	**20**	20	**17**	Moribund
	10000	20	**20**	20	**20**	20	**18**	Moribund
***E.mitchellii* leaf oil**	**0.0**	20	**0**	20	**0**	20	**0**	Normal
	1000	20	**1**	20	**1**	20	**2**	Normal
	2000	20	**3**	20	**3**	20	**1**	Majority unhealthy
	3000	20	**10**	20	**6**	20	**12**	Moribund
	4000	20	**15**	20	**13**	-	**-**	Moribund
	4000	20	**16**	20	**17**	20	**14**	Moribund
	5000	20	**18**	20	**12**	20	**18**	Moribund
	6000	20	**19**	20	**20**	20	**17**	Moribund
	8000	20	**20**	20	**18**	20	**15**	Moribund
	10000	20	**19**	20	**18**	20	**20**	Moribund
Vetiver oil	**0.0**	20	**0**	20	**0**	20	**0**	Normal
	4000	20	**12**	20	**7**	20	**7**	Majority unhealthy
	5000	20	**12**	20	**10**	20	**11**	Majority unhealthy
	8000	20	**15**	20	**17**	20	**14**	Moribund
	10000	20	**19**	20	**18**	20	**20**	Moribund

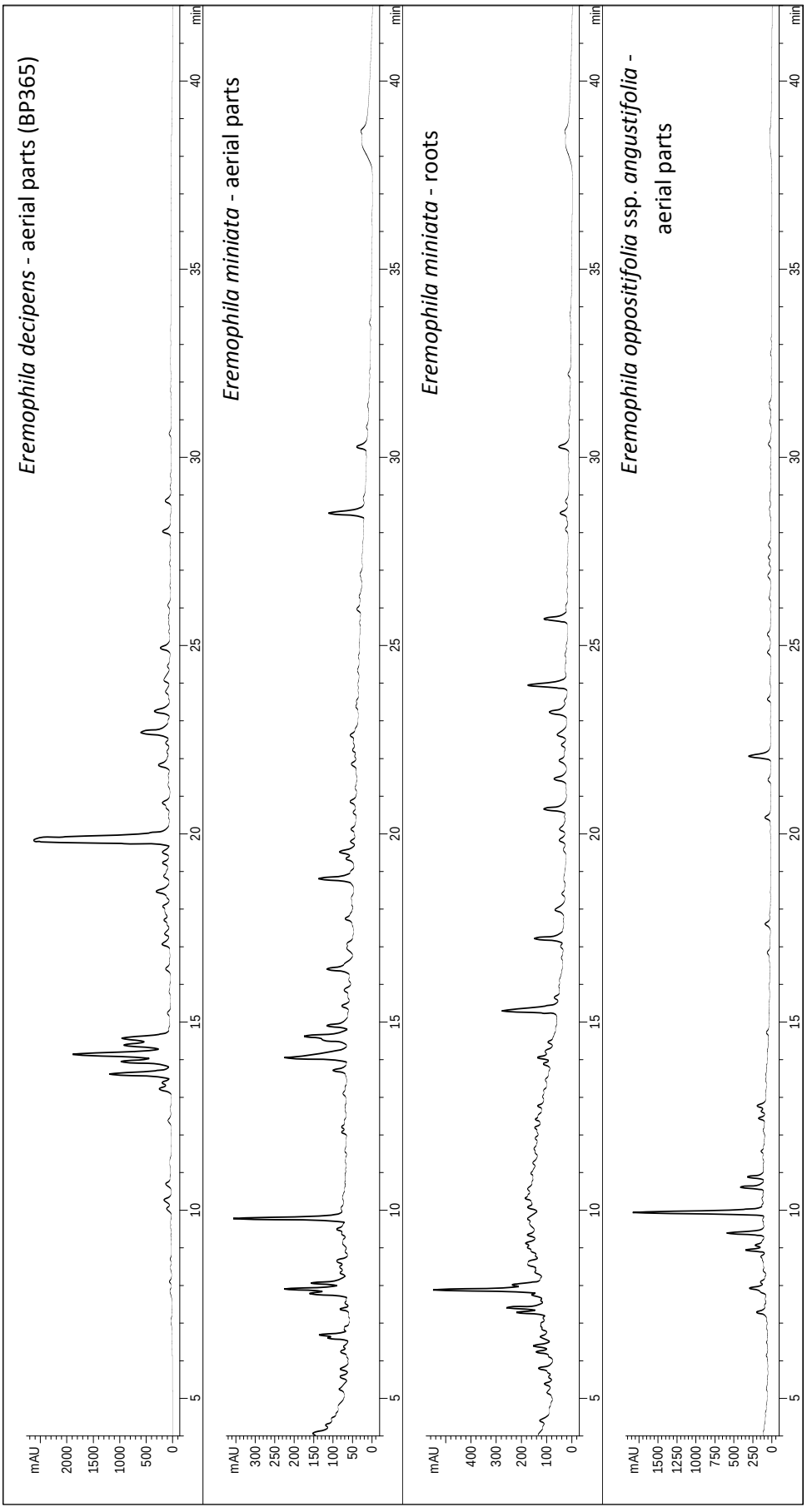
R= Replicate, O= Observed, D=Dead

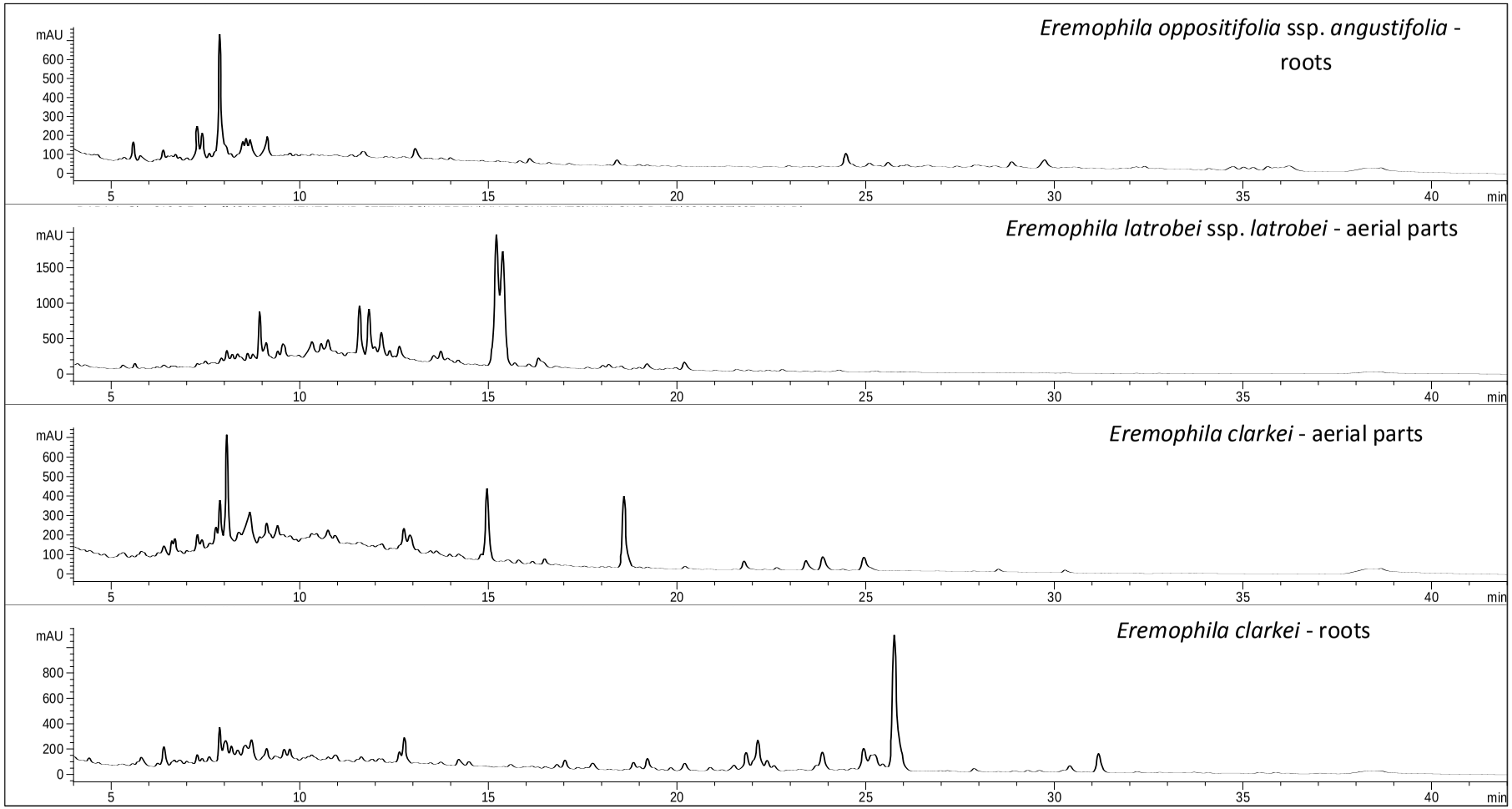
Table A2 Cont. Mortality of *N. exitiosus* associated with different products 24h after treatment (direct contact mortality).

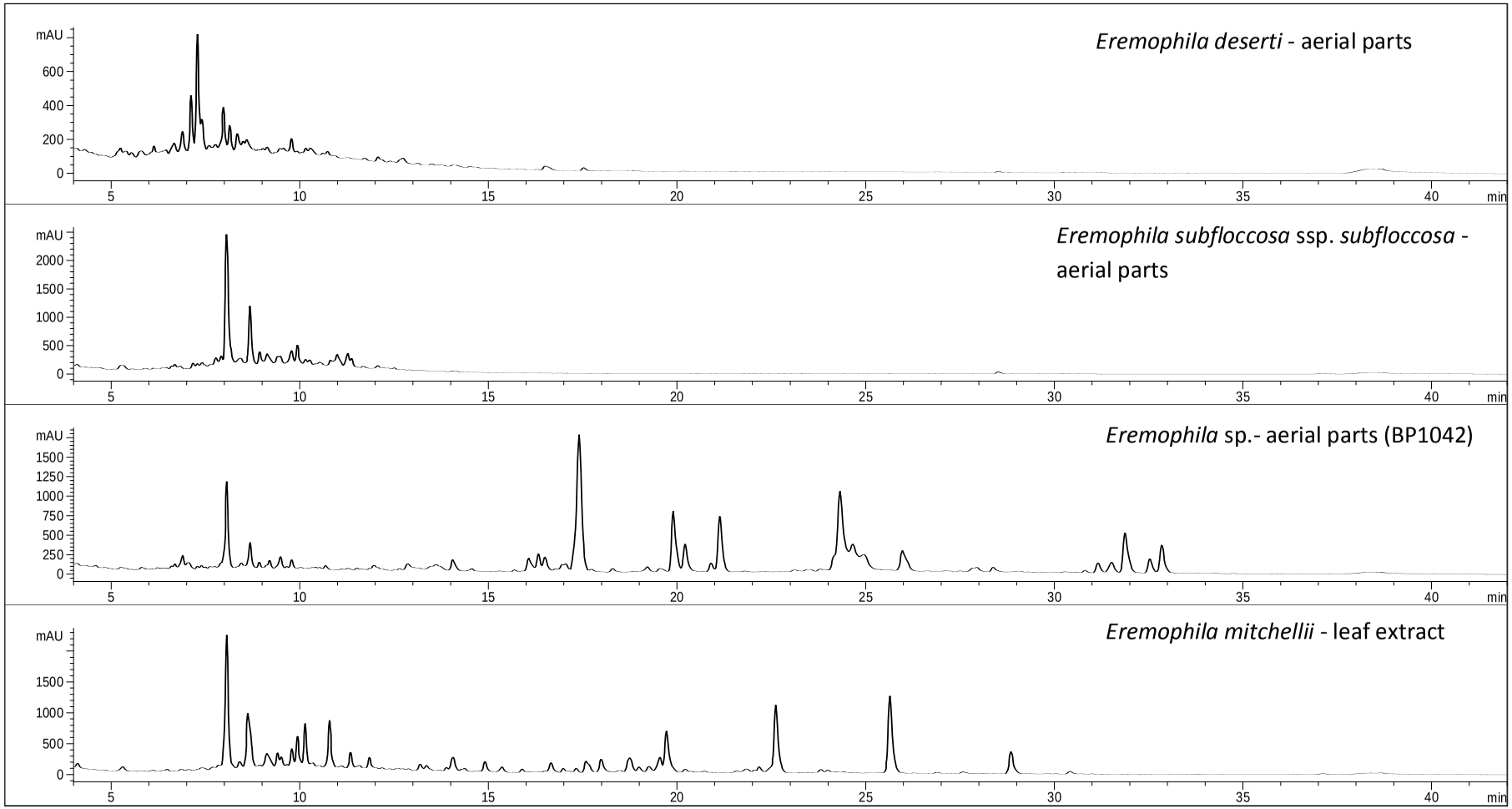
Product	Concentration in ppm (w/v)	R 1		R 2		R 3		Remarks
		O	D	O	D	O	D	
***E. mitchellii* leaf oil - methanol fraction**	**0.00**	20	**0**	20	**0**	20	**0**	Normal
	1000	20	**5**	20	**3**	-	**-**	A few unhealthy
	1500	20	**10**	20	**9**	20	**14**	Majority unhealthy
	2000	20	**7**	20	**11**	20	**12**	Moribund
	2500	20	**20**	20	**20**	20	**20**	All dead
	3000	20	**19**	20	**19**	20	**19**	Moribund
***E. mitchellii* leaf oil - hexane fraction**	**0.00**	20	**0**	20	**0**	20	**0**	Normal
	1000	20	**2**	20	**0**	20	**1**	A few unhealthy
	2000	20	**17**	20	**15**	20	**11**	Majority moribund
	2200	20	**15**	20	**14**	20	**18**	Majority moribund
	2500	20	**17**	20	**18**	20	**19**	Majority moribund
	3000	20	**17**	20	**16**	20	**16**	Majority moribund
	3500	20	**20**	20	**20**	20	**20**	Majority moribund
	4000	20	**19**	20	**18**	20	**18**	Majority moribund
α-pinene	**0.00**	20	**0**	20	**0**	20	**0**	Normal
	4000	20	**0**	20	**0**	20	**2**	A few unhealthy
	4500	20	**7**	20	**7**	20	**2**	A few unhealthy
	5000	20	**4**	20	**7**	17	**8**	A few unhealthy
	5500	20	**11**	20	**13**	20	**11**	Majority moribund
	6000	20	**6**	20	**6**	20	**8**	Majority moribund
	8000	20	**19**	20	**18**	20	**18**	Moribund
	10000	20	**20**	20	**20**	20	**19**	Moribund
vetiver fraction 4	**0.00**	20	**0**	20	**1**	20	**0**	Normal
	750	20	**2**	20	**3**	20	**2**	A few unhealthy
	1000	20	**5**	20	**11**	20	**7**	Moribund
	1250	20	**16**	20	**15**	20	**16**	Moribund
	1500	20	**15**	20	**14**	20	**16**	Moribund
	2000	20	**17**	20	**19**	20	**19**	Moribund
	2500	20	**20**	20	**20**	20	**20**	All dead
limonene	**0.00**	20	**0**	20	**0**	20	**0**	Normal
	2000	20	**0**	20	**0**	20	**0**	Normal
	3000	20	**1**	20	**0**	20	**0**	Normal
	4000	20	**0**	20	**0**	20	**0**	Normal
	6000	20	**0**	20	**0**	20	**0**	Normal
	8000	20	**0**	20	**0**	20	**0**	Normal
	10000	20	**1**	20	**0**	20	**0**	Normal

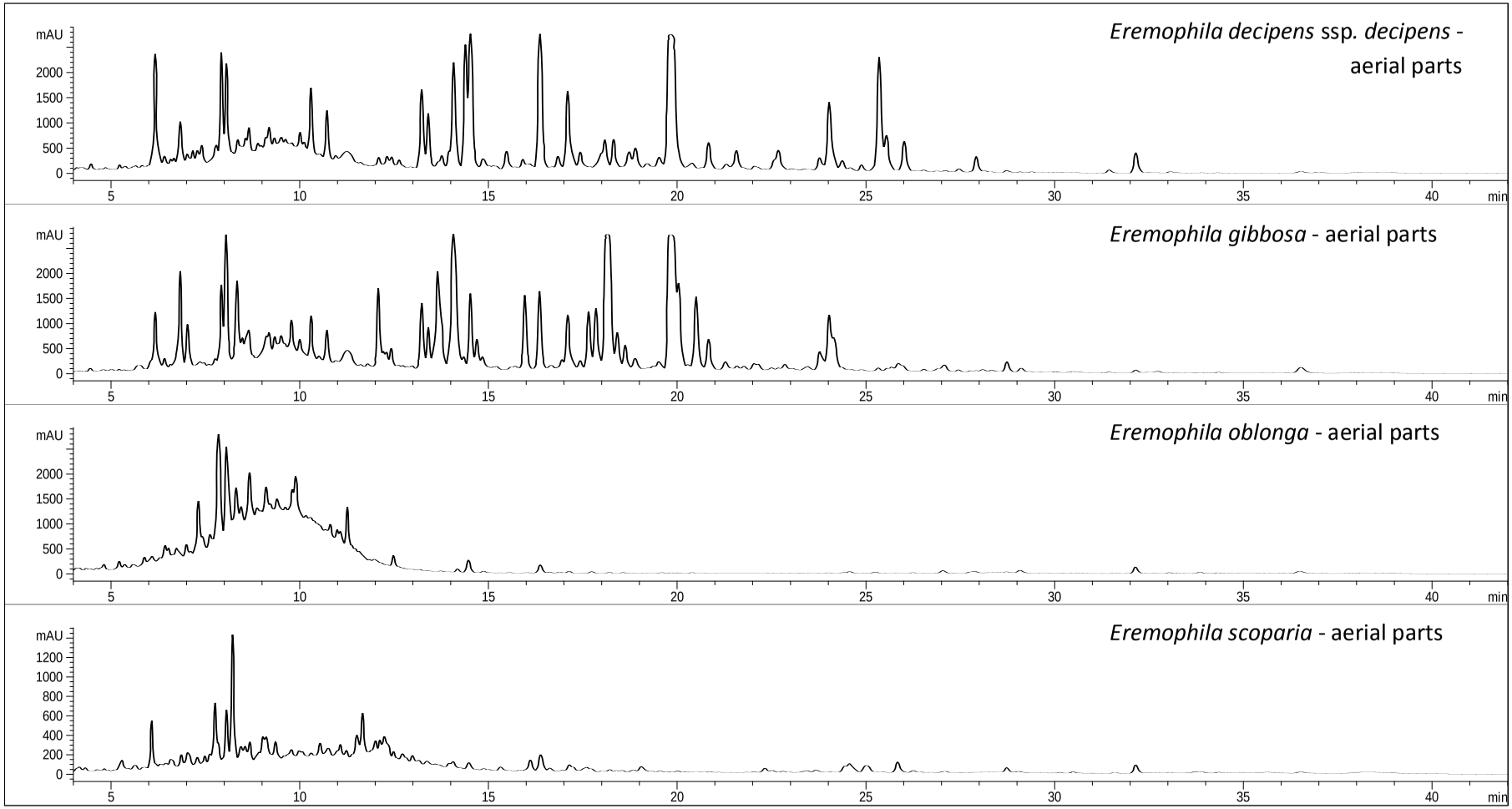
R= Replicate, O= Observed, D=Dead

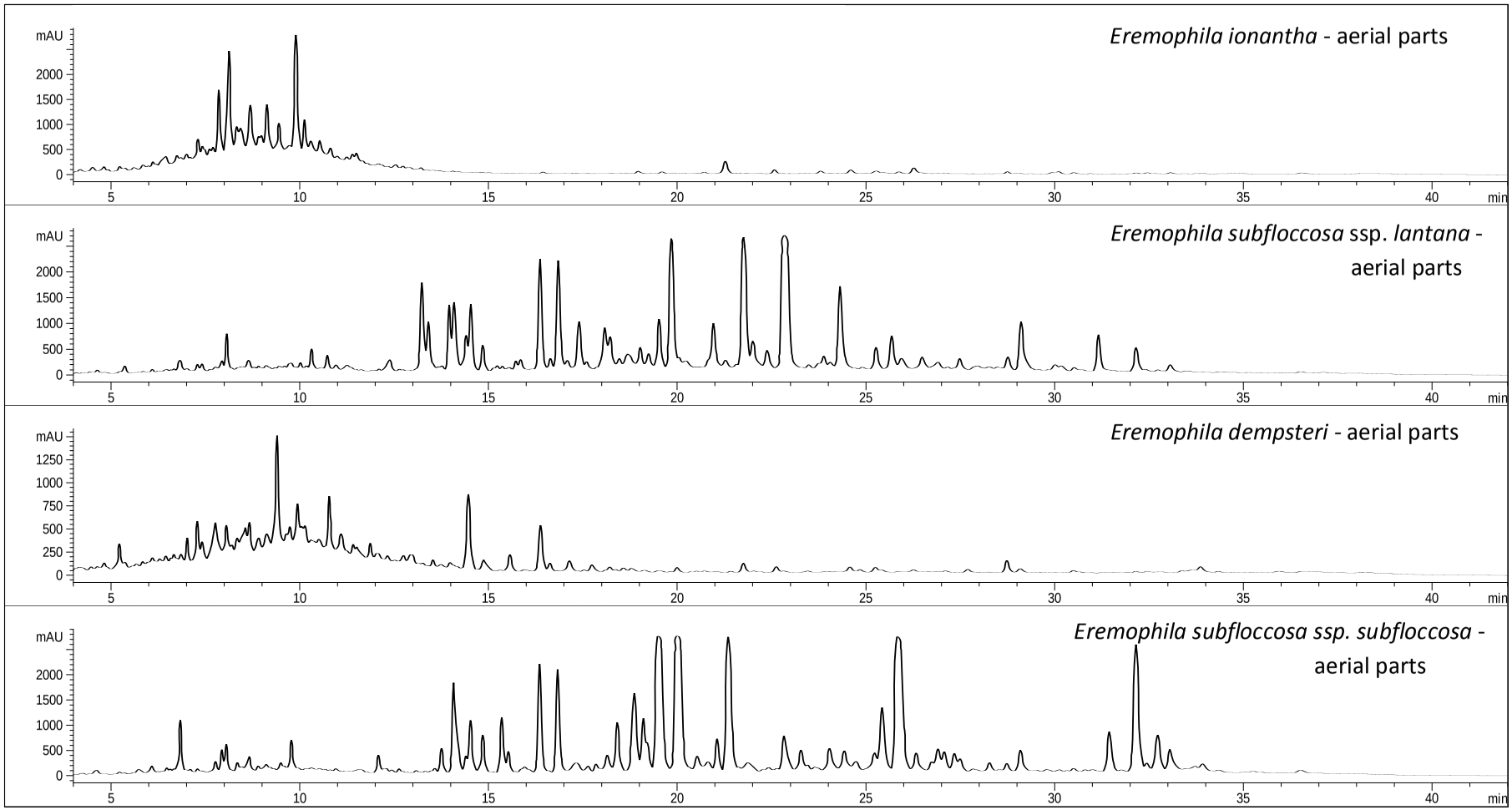
Appendix V

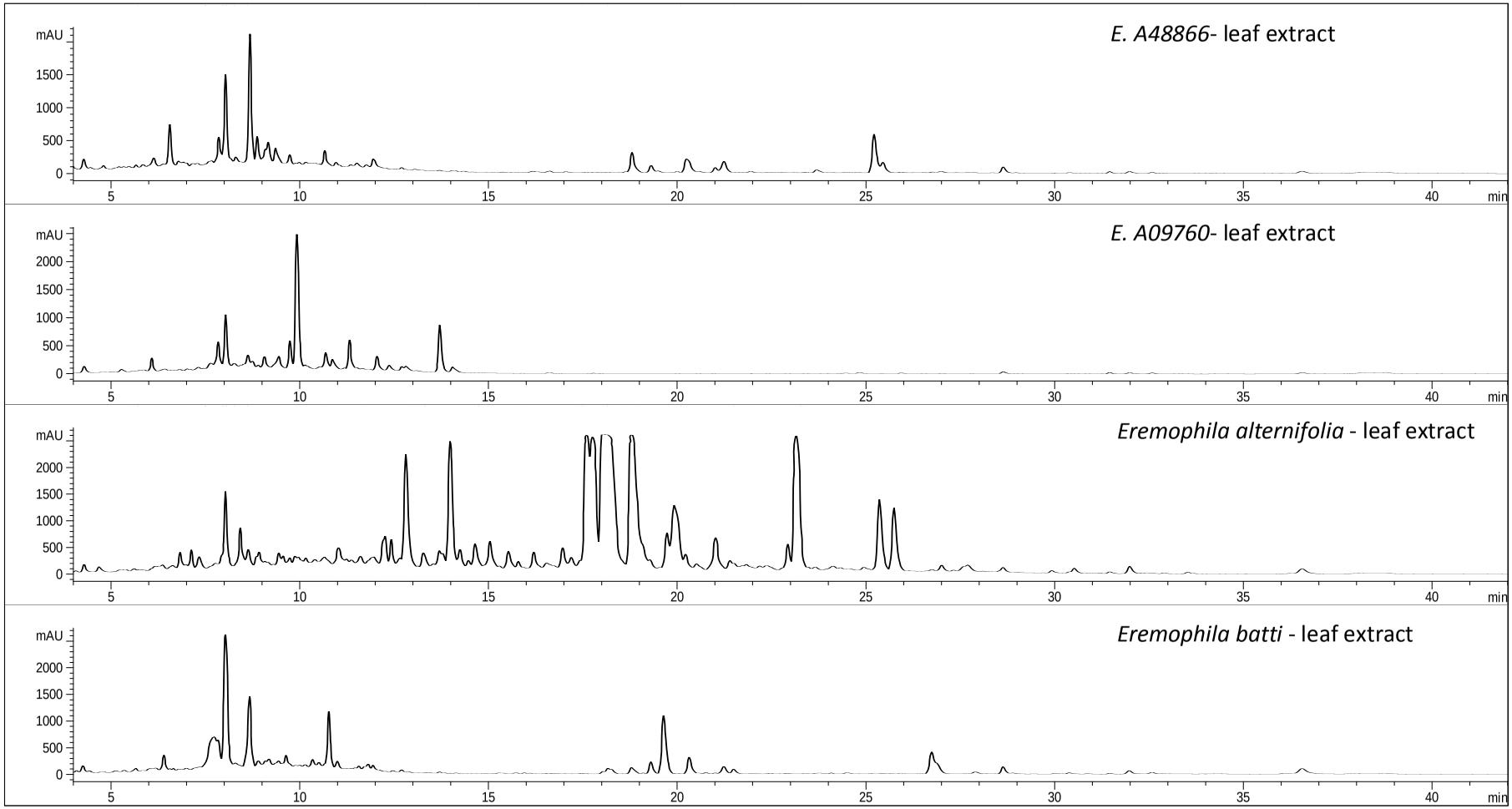

Eremophila decipens - aerial parts (BP365)
Eremophila miniata - aerial parts
Eremophila miniata - roots
Eremophila oppositifolia ssp. angustifolia - aerial parts
mAU
min

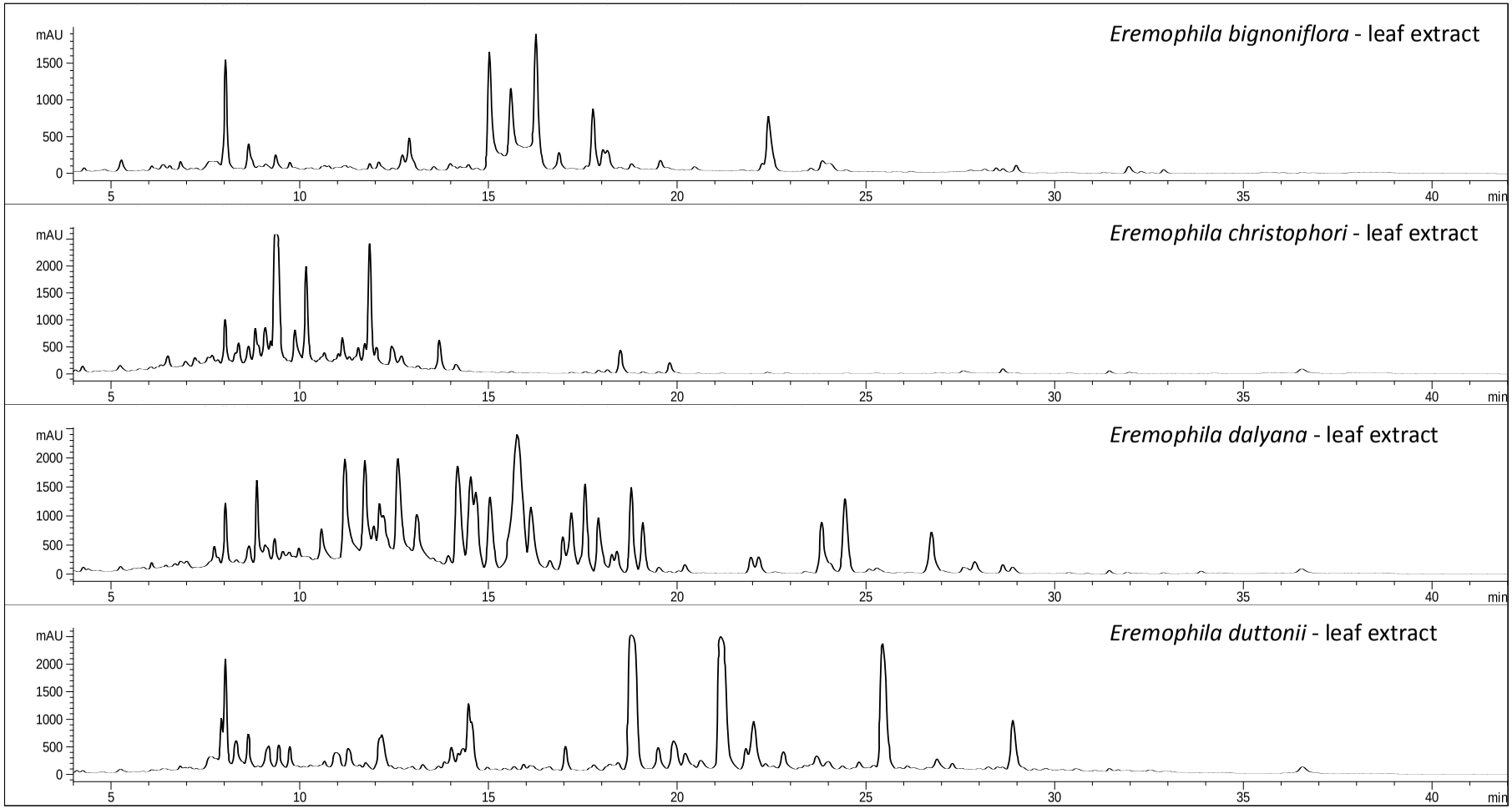

Eremophila oppositifolia ssp. *angustifolia* - roots
Eremophila latrobei ssp. *latrobei* - aerial parts
Eremophila clarkei - aerial parts
Eremophila clarkei - roots
mAU
min


Eremophila deserti - aerial parts
Eremophila subfloccosa ssp. *subfloccosa* - aerial parts
Eremophila sp.- aerial parts (BP1042)
Eremophila mitchellii - leaf extract
mAU
min


Eremophila decipens ssp. *decipens* - aerial parts
Eremophila gibbosa - aerial parts
Eremophila oblonga - aerial parts
Eremophila scoparia - aerial parts
mAU
min


Eremophila ionantha - aerial parts
Eremophila subfloccosa ssp. *lantana* - aerial parts
Eremophila dempsteri - aerial parts
Eremophila subfloccosa ssp. subfloccosa - aerial parts
mAU
min


E. A48866- leaf extract
E. A09760- leaf extract
Eremophila alternifolia - leaf extract
Eremophila batti - leaf extract
mAU
min


Eremophila bignoniflora - leaf extract
Eremophila christophori - leaf extract
Eremophila dalyana - leaf extract
Eremophila duttonii - leaf extract
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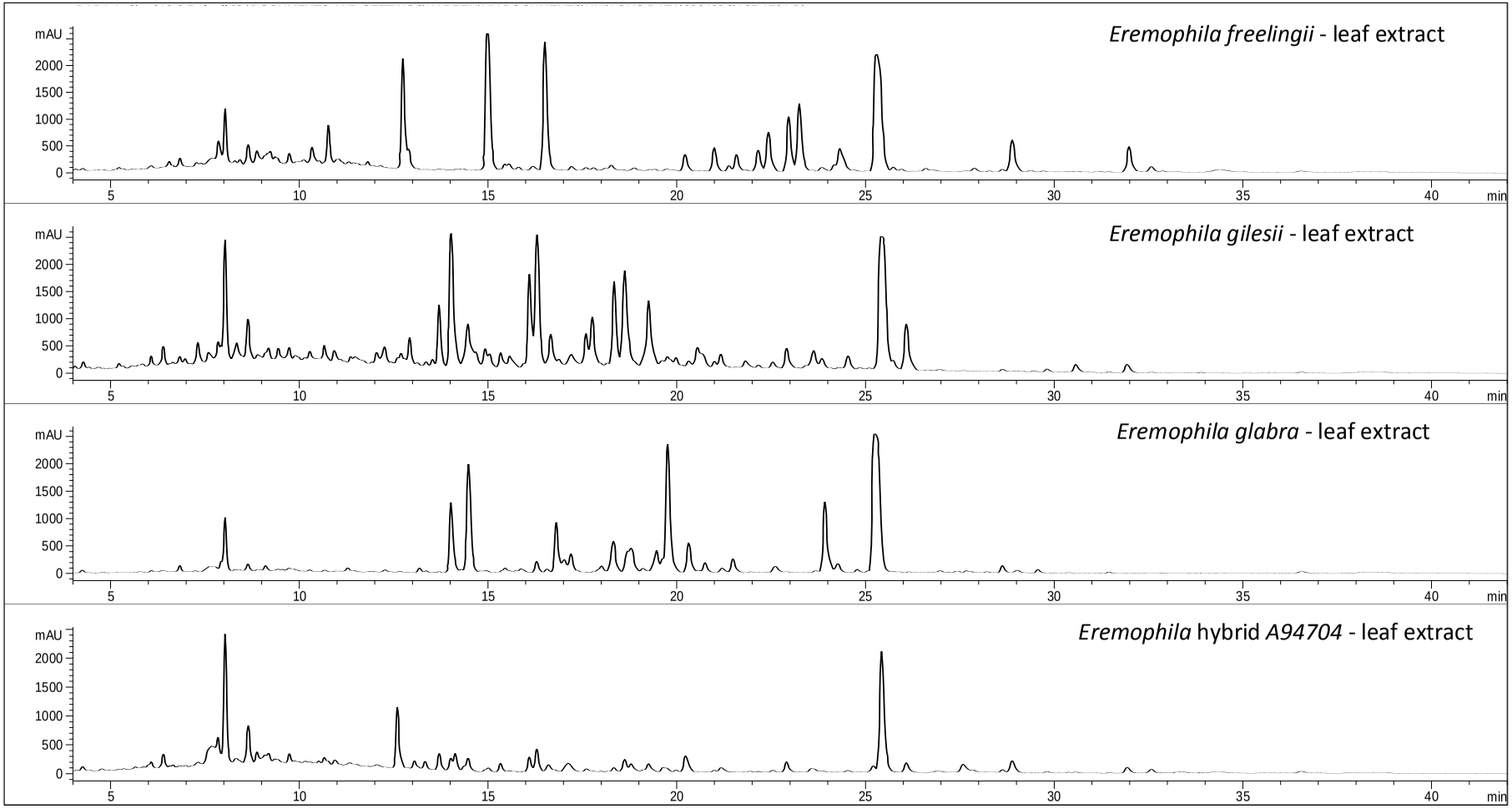
Eremophila freelingii - leaf extract
Eremophila gilesii - leaf extract
Eremophila glabra - leaf extract
Eremophila hybrid *A94704* - leaf extract
mAU
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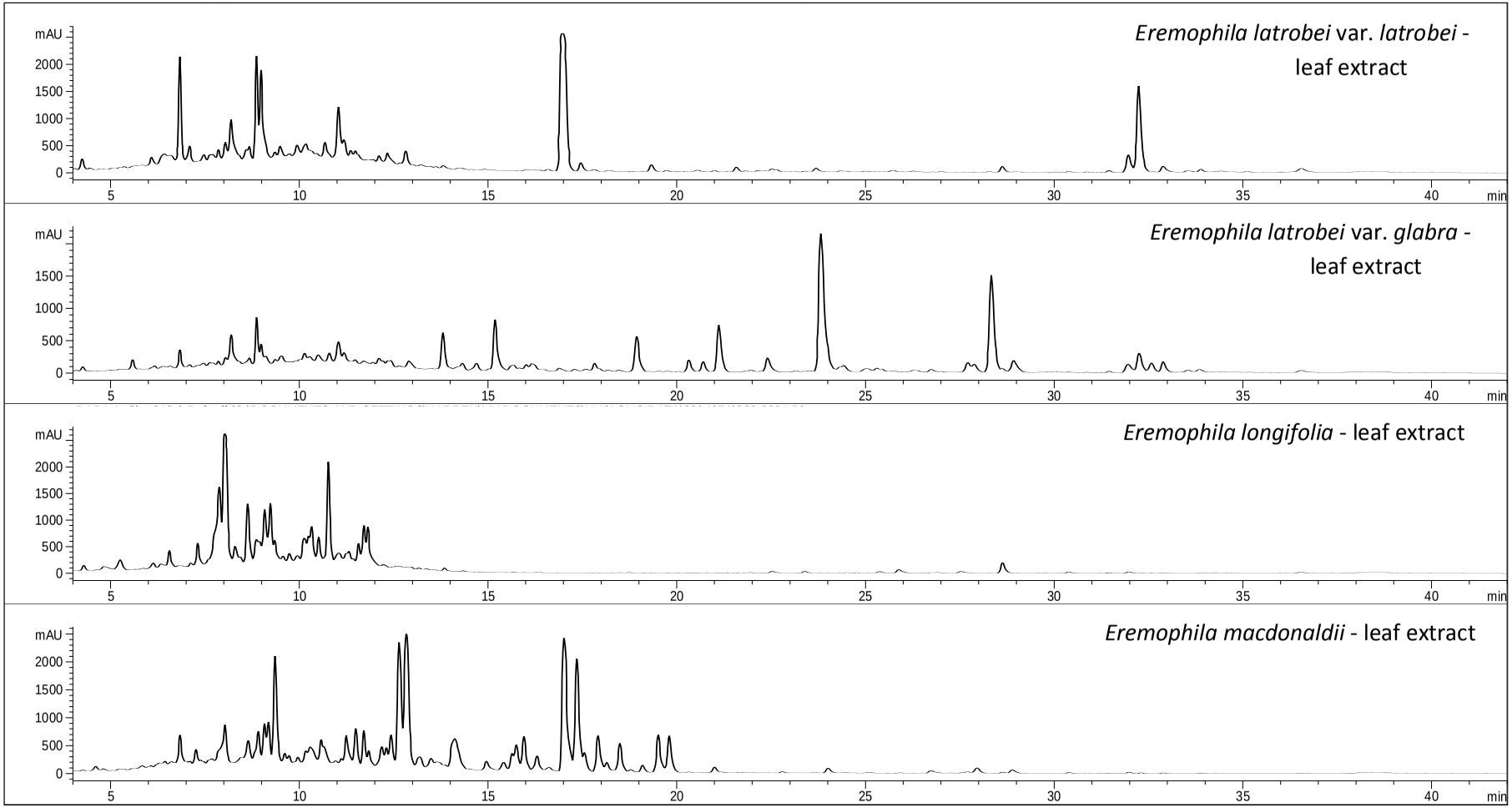
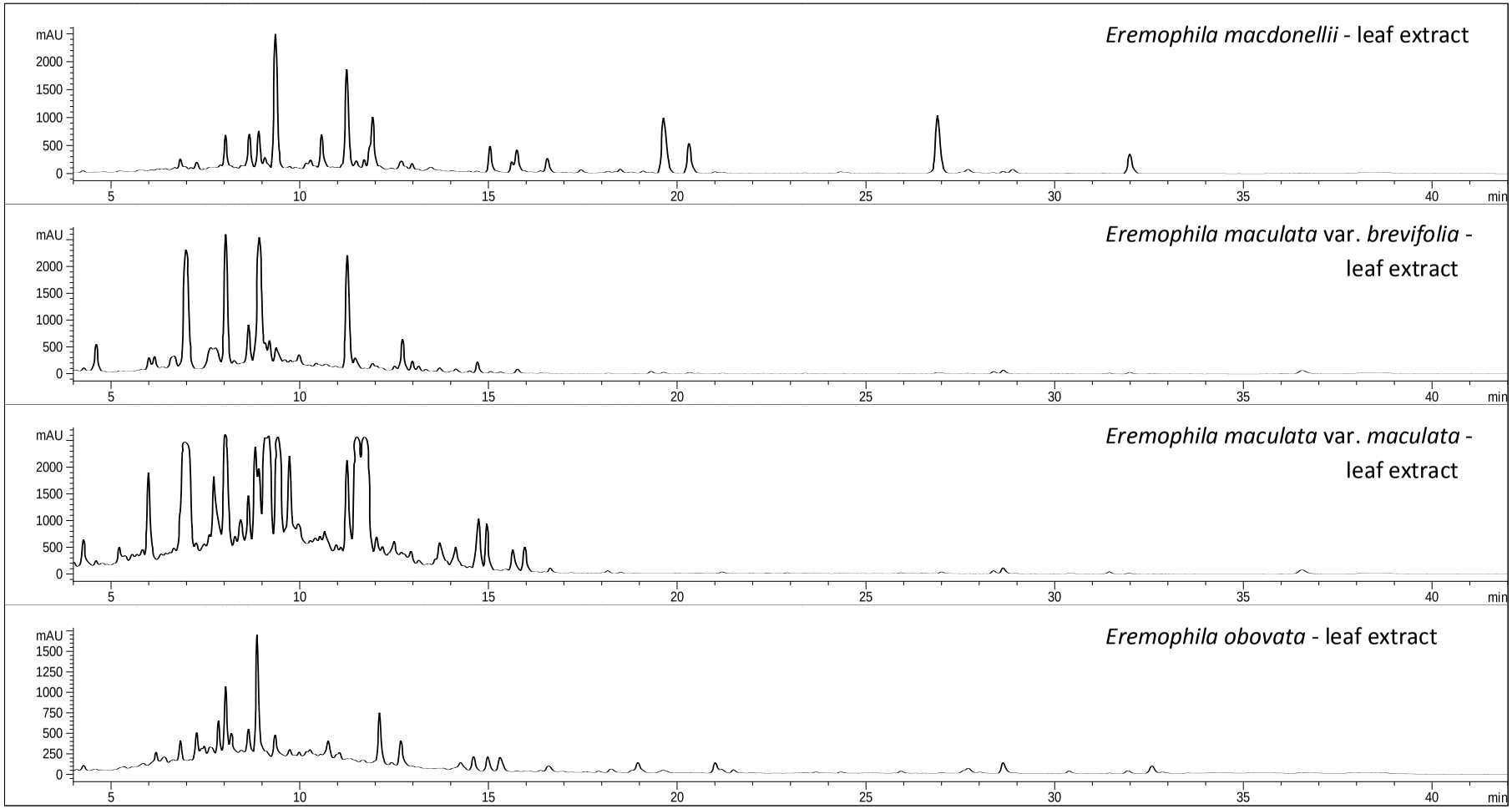
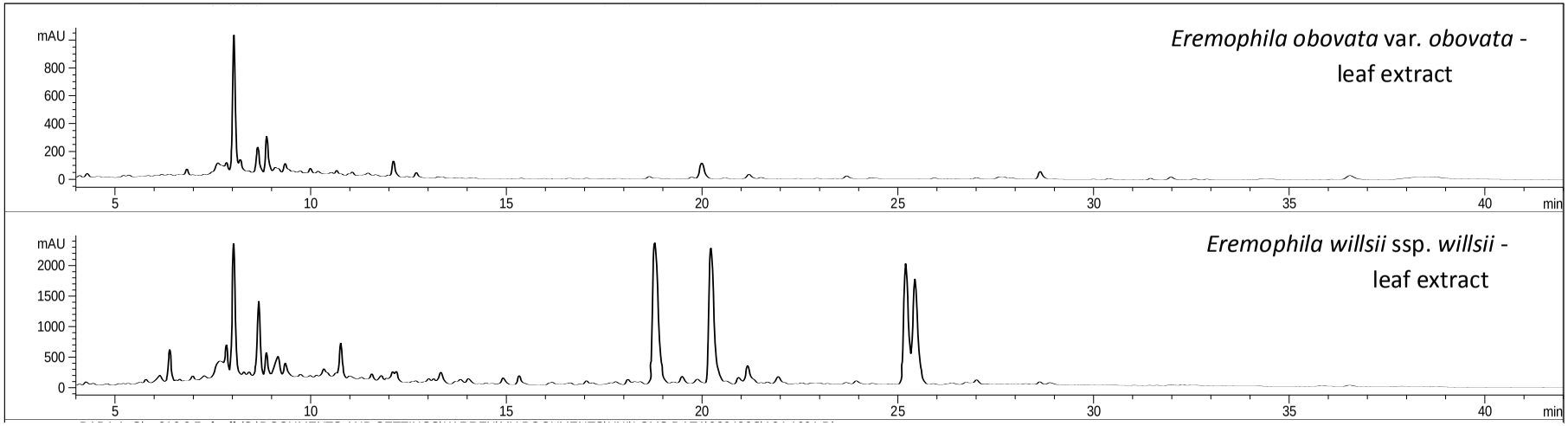
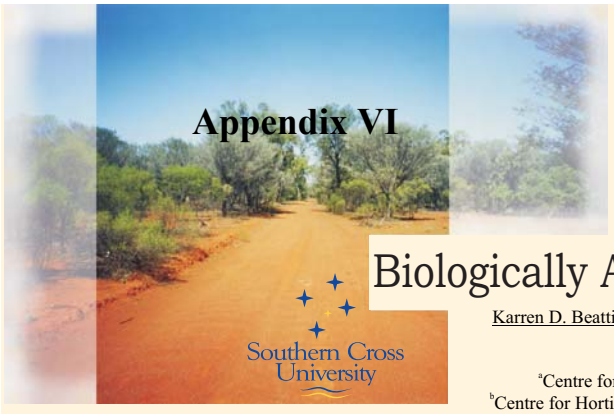
5
10
15
20
25
30
35
40
min


Eremophila latrobei var. *latrobei* - leaf extract
Eremophila latrobei var. *glabra* - leaf extract
Eremophila longifolia - leaf extract
Eremophila macdonaldii - leaf extract
mAU
min


Eremophila macdonellii - leaf extract
Eremophila maculata var. *brevifolia* - leaf extract
Eremophila maculata var. *maculata* - leaf extract
Eremophila obovata - leaf extract
mAU
min


mAU
800
600
400
200
0
5
10
15
20
25
30
35
40
min
Eremophila obovata var. *obovata* - leaf extract
mAU
2000
1500
1000
500
0
5
10
15
20
25
30
35
40
min
Eremophila willsii ssp. *willsii* - leaf extract



Biologically Active Eremophilanes from *Eremophila mitchellii*.

Karren D. Beattie[a], David N. Leach[a], Peter G. Waterman[a], Andrew Flowers[a], Linda Banbury[a], Robert Spooner-Hart[b], and Albert Basta[b].

[a]Centre for Phytochemistry, Southern Cross University, Lismore, NSW 2480, Australia.
[b]Centre for Horticulture and Plant Sciences, University of Western Sydney, Hawkesbury NSW 2753,

Abstract

The timber from *Eremophila mitchellii*, an endemic Australian tree, was noted for its resistance to attack by termites[1]. The steam distilled oil obtained from the wood was subjected to bioassay guided fractionation to identify the active metabolites. The eremophilane sesquiterpenes; eremophilone **1** and 8-hydroxyeremophila-1,11-dienone, **5** were demonstrated to exhibit potent toxicity towards the subterranean termite *Nasutitermes walkeri*. 1,7 (11), 9-Eremophilatrien-8-one **4**, a new natural product has also been isolated from the wood oil.

Introduction

It is estimated that termites cause damage to one in five buildings and structures throughout NSW[2], with the cost of this damage exceeding many millions of dollars anually[3]. Traditionally, organochloride pesticides have been employed for the purpose of termite control. These hazardous pesticides have been deregistered in Australia, creating a need for the development of less toxic methods of termite control.

Materials and Methods

The oil of *E. mitchellii* was obtained by steam distillation of the wood (Yield 1.7%). Fractionation of the oil was achieved using normal-phase preparative HPLC, with a hexane/ethyl acetate gradient (95-60% hexane) as eluent. The fractions obtained in this way were then subjected to a termiticidal assay using *N. walkeri* as the test organism.

For the termiticidal assay, 17 termites were placed on a petri dish containing a disc of filter paper moistened with distilled water. Fractions were then diluted to the desired concentration with water, triton-X and ethanol. 5 mL of the extract was atomised with the aid of a Potter spray tower and applied to the test organisms. Tests were performed in triplicate across a range of concentrations. Mortality and behaviour is observed at 12, 24 and 48 hours. A LD_{50} was then determined for each fraction.

Each fraction was then further purified by means of size-exclusion chromatography or recrystallisation. A LD_{50} was then determined for each compound. (Refer to Table 1.) The structure of these compounds was then determined using NMR spectroscopy.

Results and Discussion

It was evident from these results (Table 1) that eremophilone **1** and the 8-hydroxyeremophiladienone **5** possessed the strongest toxicity towards the termites. It was also noted that the whole oil posseses significant activity.

The eremophilanes **1-5** isolated from *E. mitchellii* are quite rare and unique sesquiterpenes. They were the first terpenes that were discovered to disobey the 'head to tail linkage' isoprene rule. To date Eremophilanes have only been reported in one other species of *Eremophila*.[4]

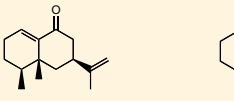
Table 1. LD_{50} and LD_{95} values for compounds from *E. mitchellii* wood oil.

Compound	LD_{50} @ 24 hrs	LD_{95} @ 24 hrs
eremophilone	160 (ug/mL)*	290 (ug/mL)*
santalcamphor	680	1340
2-hydroxyeremophilone	450	700
8-hydroxyeremophiladienone	210	340
E. mitchellii wood oil	170	330

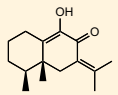
* 0.5 mL delivery per petri dish.

E. mitchelli in flower.
Winged reproductive termites.

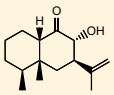
Ethnobotanical field trip Brewarrina NSW 2002.
Termiticidal assay.
Termite infestation.



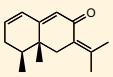
1 Eremophilone



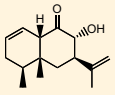
2 2-Hydroxyeremophilone



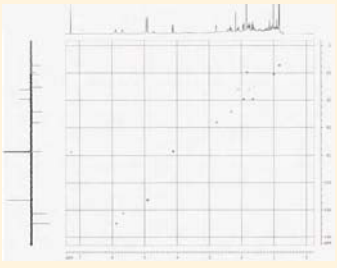
3 Santalcamphor



4 1,7(11),9-Eremophilatrien-8-one



5 8-Hyroxyeremophila-1,11-dienone



HSQC of 8-Hydroxyeremophiladienone **5**.

References

1. "The Australian Desert Shrub Eremophila (Myoporacea) Medicinal, Cultural, Horticultural and Phytochemical Uses." Guy S. Richmond and Emilio L. Ghisalberti, *Economic Botany* 48(1) pp. 35-59. 1994.
2. CSIRO feature article, "The trouble with termites." www.csiro.au/news/features/termites.html 25/9/03.
3. Tanzer pest control webpage.www.tanzerpestcontrol.com.au 25/9/03
4. "The Phytochemistry of the Myoporacea." Emilio L. Ghisalberti, *Phytochemistry*, Vol. 35. No. 1, pp. 7-33, 1994.

Acknowledgements

The author wishes to thank Bio Prospect for their generous financial support, and the staff and students at The Centre for Phytochemistry at Southern Cross University for their assistance.