An Ethnopharmacological Study of Australian Indigenous Medicinal Plants Used in Dermatological and Wound Healing Remedies

A thesis submitted in fulfilment for the degree of

Doctor of Philosophy

University of New England, Armidale, Australia

by

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November, 2011

Declaration

I certify that the substance of this thesis has not been submitted, either in whole or in part, for a higher degree at any other university or institution, and, that to the best of my knowledge, all assistance received in preparing this work and all sources used have been acknowledged.

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November, 2011

Acknowledgements

I wish to thank my academic supervisors Associate Professor Graham Jones and Emeritus Professor Kenneth Watson for their assistance in the preparation of this thesis along with their helpful advice, encouragement and patience throughout my candidature. I also wish to express thanks to Professor David Leach and Associate Professor Carol Morris of the Centre for Phytochemistry and Pharmacology at Southern Cross University (CPP, SCU) Lismore, for their efforts in arranging a short term attachment at the centre to carry out key experimental work. I also wish to especially thank Dr Myrna Deseo of the CPP, SCU for performing NMR spectroscopy and interpretation of spectra, supervision and advice along with Ashley Dowel and Lei Liu for their helpful and valuable advice on chromatographic procedures.

Mr. Ian Telford of the N.C.W. Beadle Herbarium (University of New England, Armidale) provided valuable assistance with confirmation of field identities of collected plant specimens. Assistance and advice was also given by Professor Jeremy Bruhl of the N.C.W. herbarium, along with the loan of essential field equipment and use of herbarium facilities. Assistance and advice on location of plant specimens was also provided by Dr Dale Dixon, Glenn Wightman, Ian Cowie and other staff of the DNA Northern Territory Herbarium along with the loan of field equipment and use of herbarium facilities during fieldwork in the Northern Territory. Advice on the location of certain plant species in the Jodetluk area (Northern Territory) and medicinal use of *Cymbopogon ambiguus* was given by Ms Jane Runyu and Ms Jenny Brown of Jodetluk (Jarwoyn) community, NT. Collection of plant specimens in this area and in the Katherine region were carried out under a permit issued by the Jarwoyn Association Aboriginal Corporation. Assistance with the field collection of some plant specimens in western NSW and preparation of some of the sequential solvent extracts of

plant material used in the study was given by Mr Srdjan Mijajlovic for the purposes of a parallel collaborative research project.

Spreadsheets used to transform or convert raw instrumental data to standard values (i.e. ORAC and cytotoxicity assays, and standard curve calculations in PGE_2 inhibition assays) were modified from those originally conceived and designed by Dr Dion Thompson at the CPP, SCU. Maintenance of mammalian cell cultures used in experimental work was also undertaken by Dr Dion Thompson. NMR spectroscopy and confirmation of identity of compounds isolated from *E. duttonii* (Chapter 5) was performed entirely by Dr Myrna Deseo of the CPP, SCU. NMR spectroscopy of essential oil samples and structural elucidation of karahanaenone from *E. longifolia* essential oil (Chapter 6) and the original structural elucidation of the serrulatane diterpenes isolated from *E. duttonii* (Chapter 5) was performed entirely by Dr David Tucker at the University of New England (School of Science and Technology, Division of Chemistry). Financial support for this PhD candidature was provided with a Commonwealth Australian Postgraduate Award.

Abstract

In this study, relevant *in vitro* biological activities were evaluated in extracts of Australian medicinal plants used in indigenous traditional medicine for the treatment of wounds, sores and other dermatological conditions. A survey of Australian medical ethnobotanical literature was conducted in order to identify suitable species and plant parts for inclusion in the study and to collect data for systematic and quantitative analyses of Australian indigenous medicinal plant use. Thirty-seven aqueous extracts were obtained following traditional methods of preparation from various parts of 23 plant species representing 15 families. Sequential solvent extracts (hexane-dichloromethane-methanol) were also prepared using material from 5 species of *Eremophila* to enable comparison of bioactivity with corresponding traditional aqueous preparations. Extracts were screened using *in vitro* assays for anti-microbial activities, anti-oxidant capacity via DMPD decolourisation and Oxygen Radical Absorbance Capacity (ORAC) assays, P388D1 murine lymphoblast cytotoxicity, and inhibition of prostaglandin E_2 from calcium ionophore-stimulated murine fibroblasts. Total phenolic content of the aqueous preparations was also estimated using a Folin-Ciocalteu type assay.

Anti-bacterial activity was assessed using a broth microdilution technique to measure minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC). A number of the aqueous preparations were found to exhibit a broad spectrum of anti-bacterial activity (i.e. were active against both Gram-negative and Gram-positive organisms) and retained activity at MIC/MBC levels representing concentrations well below that produced using traditional extraction techniques. These included the expressed fruit juice of *Cochlospermum fraseri* (MIC: 0.20 - 25.00 mg · mL^{-1}; MBC: 0.80 - 50.00 mg · mL^{-1} representing 0.002 - 0.53× original concentration of 95.2 mg · mL^{-1}), a decoction of leaves from *Eucalyptus tetrodonta*, (MIC: 0.06 - 4.00 mg · mL^{-1}; MBC: 0.50 - 4.00 mg · mL^{-1},

representing 0.01 - 0.74× the original concentration of 5.4 mg · mL^{-1}), and a decoction of *Buchanania obovata* trunk inner bark (MIC: 0.50 - 16.00 mg · mL^{-1}; MBC: 2.00 - 8.00 mg · mL^{-1}, representing 0.03 - 0.78× the original concentration of 20.4 mg · mL^{-1}). The *C. fraseri* juice and *B. obovata* inner bark preparations also exhibited the highest levels of phenolic content among the aqueous preparations (11947 - 18189 ppm gallic acid equivalents) and antioxidant capacity as measured in Trolox equivalents (TE), in DMPD assays (46.7 - 60.9 mM TE). Highest ORAC values among the preparations were found for the *C. fraseri* juice (68.2 mM TE) and a decoction of *Erythrophleum chlorostachys* trunk inner bark (19.8 mM TE). The majority of preparations tested did not exhibit *in vitro* cytotoxicity over the range of concentrations assayed. Extracts exhibiting notable P388D1 cytotoxicity included a decoction of *Callitris intratropica* inner bark (IC_{50}: 29.4 μg · mL^{-1}) and extracts of *Crinum angustifolium* bulbs (IC_{50}: 28.4 -58 μg · mL^{-1}).

Major use agreement values (MUA), an index used to evaluate the degree of agreement between specific medicinal uses cited by independent ethnobotanic studies, were determined for each of the medicinal preparations examined using data collected in the survey of ethnobotanic literature. MUA values were compared with the results of assays for biological activity, to determine whether any relationship existed between the degree of agreement for specific medicinal uses cited, and any activities observed. Higher MUA values representing citations referring to treatment of wounds and dermal lesions were found to be associated with higher levels of total phenolic content, *in vitro* antimicrobial activity and P-388 murine lymphoblast cytotoxicity among the preparations examined. These findings indicate that phenolic compounds may be of considerable relevance in Australian medicinal plant species and preparations used in the treatment of wounds and dermal lesions, and further studies are required to confirm this finding and to identify whether any specific class or species of phenolic compound predominates in this group of plants.

In previous work, two diterpenes of the serrulatane class, serrulat-14-en-7,8,20-triol (**1**) and serrulat-14-en-3,7,8,20-tetraol (**2**), were identified as the principal anti-bacterial constituents in extracts of the medicinal plant *Eremophila duttonii*. In this study, an attempt was made to isolate and purify the serrulatane diterpenes from a dichloromethane extract of *E. duttonii* leaves, to assay for further bioactivities and use as standards in order to determine whether either compound could be detected in traditional aqueous preparations using this species. As in previous work, partially purified fractions containing both compounds exhibited inhibitory and bactericidal activity against Gram-positive bacteria. MIC against 3 Gram-positive organisms for fractions containing the triol (**1)** ranged from 8 - 15 μg · mL^{-1} and MBC from 8 - 30 μg · mL^{-1}. MIC of fractions containing the tetraol (**2**) ranged from 60 - 120 μg · mL^{-1} and MBC from 120-480 μg · mL^{-1}. Fractions containing **1** and **2** also exhibited high levels of cytotoxicity against P-388 lymphoblasts and HEP-G1 cell lines (**1**: IC_{50}, 7.7 - 13.7 μg· mL^{-1}; **2**: IC_{50}, 11.2 - 17.2 μg· mL^{-1}) and antioxidant capacity in lipophilic ORAC assays (**1**: 10919 ± 854 μmol TE/g; **2**: 13610 ± 989 μmol TE/g). In traditional aqueous decoctions of this species, only the serrulatane tetraol (**2**) was detected. Attempts to estimate quantities of **2** in traditional preparations were complicated by the finding that this compound appeared to be unstable as products of decomposition were found to appear in purified samples following a period of storage. For this reason, an accurate determination of quantity in the traditional preparations could not be obtained with certainty and were reported as tentative estimations (i.e. around 40-68 μg mL^{-1}, corresponding to 0.6 - 0.7% of extract dry weights) based on estimated purity of standards at the time the assays were performed.

Overall, the organic solvent extracts tended to exhibit higher potency and a higher rate of 'positive hits' in biological assays when compared with corresponding traditional aqueous preparations. These results would support the argument that non-polar organic solvent

extracts may be unsuitable for certain investigations (e.g. in seeking to explain or validate medicinal plant use in the 'traditional' context), or that such studies should be followed up with appropriate investigations into the pharmacognosy of traditional preparations where possible.

In this study, evidence was found which showed a high degree of intraspecific phytochemical variation in volatile oil fractions of one of the species examined (*Eremophila longifolia*). A preliminary study was undertaken to examine chemical variation in volatile oils of this species, resulting in identification of three novel chemotypes (isomenthone, karahanaenone and borneol chemotypes) occurring in western regions of the state of NSW. Previous studies had indicated the possibility of volatile oils of this species containing the toxic phenylpropanoid safrole. Interestingly, no safrole or other phenylpropanoids were detected in any of the specimens examined in this study. When traditional decoctions were prepared using material representative of each of the chemotypes identified, significant differences in biological activity and phenolic content were apparent. This species is frequently mentioned in the ethnobotanical literature of Australia and widespread anecdotal evidence exists which suggests that preparations using this species are frequently and routinely employed by contemporaneous indigenous Australian people. Further work may be warranted to characterise the extent of phytochemical variation in this species, and to carry out pharmacognostic studies of preparations and material in areas where the species is currently used for medicinal purposes. The work undertaken in this study is novel in that biological activity or chemistry has not previously been investigated in extracts obtained following traditional methods of preparation, from the majority of species collected. This work is also, to the best of the author's knowledge, the first instance where relevant biological activities have been investigated in Australian medicinal plants used specifically for dermatological conditions and wounds.

Publications arising from this thesis

Smith, J., Tucker, D., Alter, D., Watson, K. and Jones, G. (2010). "Intraspecific variation in essential oil composition of *Eremophila longifolia* F. Muell.(Myoporaceae): Evidence for three chemotypes." *Phytochemistry* **71**(13): 1521-1527.

Smith, J. E., Tucker, D., Watson, K. and Jones, G. L. (2007). "Identification of antibacterial constituents from the indigenous Australian medicinal plant *Eremophila duttonii* F. Muell. (Myoporaceae)." *Journal of Ethnopharmacology* **112**(2): 386-393.

Conference Abstracts

Smith, J. (2009). Accepted oral presentation: *Screening of some Australian indigenous medicinal plants for in vitro biological activities relevant to wound healing*. International Evidence Based Complementary Medicine Conference, University of New England, Armidale, March 2009.

Smith, J., Tucker, D., Jones, G. and Watson, K. (2005). Poster Presentation (P415): *Characterisation and Evaluation of Antibacterial Activity of a New Type of Essential Oil from Eremophila longifolia.* 53rd Annual Congress of the Gesellschaft fur Arzneipflanzenforschung (GA) and joint congress with the Societa Italiana di Fitochimica (SIF), Florence, Italy, 21-25 August 2005.

Smith, J., Tucker, D., Jones, G. and Watson, K. (2005). Poster Presentation (P407): *Isolation and Identification of Antimicrobial Compounds from the Australian Medicinal Plant Eremophila duttonii (Scrophulariaceae).* 53rd Annual Congress of the Gesellschaft fur Arzneipflanzenforschung (GA) and joint congress with the Societa Italiana di Fitochimica (SIF), Florence, Italy, 21-25 August 2005.

List of Abbreviations and Acronyms

AAPH	2,2′-azobis (2-methyl) propionamidine dihydrochloride
Abs	Absorption
ACM	Australian Collection of Microorganisms
ACN	Acetonitrile
APCI	Atmospheric-Pressure Chemical Ionization
APNI	Australian Plant Name Index
ATCC	American Type Culture Collection
ATP	Adenosine triphosphate
aq.	Aqueous
AU	Absorption Units
BA	Blood Agar
BBIz	Broadband Inverse NMR Probe, Z-Gradient
bp	Before Present (Years)
CLSI	Clinical and Laboratory Standards Institute (formerly NCCLS)
COSY	Correlation Spectroscopy (2D NMR)
CPP	Centre for Phytochemistry and Pharmacology
CVPNT	Checklist of the Vascular Plants of the Northern Territory
DCM	Dichloromethane
DMEM	Dulbecco's Modified Eagle Medium
DMPD	N,N-dimethyl-p-phenylenediamine
DMS	Degrees, Minutes, Seconds (Geospatial coordinates)
DMSO	Dimethyl sulfoxide
EIA	Enzyme Immunoassay
EPE	Estimated Position Error (Geospatial coordinates)
ESI	Electrospray Ionisation
EtOAc	Ethyl Acetate
EtOH	Ethanol
FBS	Foetal Bovine Serum
GC	Gas Chromatography
GC-MS	Gas Chromatography-Mass Spectrometry
GMB	Mueller-Hinton Agar with 2% Glucose and 0.5 μg/mL Methylene Blue

HMBC	Heteronuclear Multiple Bond Correlation
HPLC	High Performance Liquid Chromatography
HSQC	Heteronuclear Single Quantum Coherence (2D NMR)
IC_{50}	Concentration Effecting 50% Inhibition
IBRA	Interim Biogeographic Regionalisation of Australia
LC-MS	Liquid Chromatography-Mass Spectrometry
LPS	Lipopolysaccharide
MBC	Minimum Bactericidal Concentration
MeOH	Methanol
MHA	Mueller-Hinton Agar
MHz	Megahertz
MIC	Minimum Inhibitory Concentration
MS	Mass Spectrum
MW	Molecular Weight
NA/NB	Nutrient Agar/Nutrient Broth
NAED	North American Ethnobotanic Database
NCCLS	National Committee for Clinical Laboratory Standards
NSAID	Non-Steroidal Anti-Inflammatory Drug
NSW	New South Wales
NMR	Nuclear Magnetic Resonance
NT	Northern Territory
ORAC	Oxygen Radical Absorbance Capacity
PC	Principal Component
PCA	Principal Component Analysis
PCA	Plate Count Agar
PGE_2	Prostaglandin E_2
PLC	Preparative Thin Layer Chromatography
ppm	Parts Per Million
PYE	Peptone-Yeast Extract
RCF	Relative Centrifugal Force
Rf	Retention Factor
RI	Relative Retention Index/Retention Indices
RMCD	Randomised methylated cyclodextrin
ROS/RNS	Reactive Oxygen/Nitrogen Species

Rt	Retention Time
SCU	Southern Cross University
SPSS	Statistical Package for the Social Sciences
TE	Trolox Equivalent
TFA	Trifluoroacetic Acid
TLC	Thin Layer Chromatography
TSB	Tryptone Soya Broth
TSSA	Tryptone Soya Sloppy Agar
UNE	University of New England
UV	Ultra-Violet
V_e	Elution Volume
YEP	Yeast Extract Peptone

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