Chapter 1: Introduction

In this chapter a brief overview of the project is given along with review of recent Australian ethnopharmacological and medicinal plant research. A survey of published Australian medical ethnobotanical records was conducted and the results of quantitative and botanical systematic analyses carried out using data obtained in the survey are presented and discussed. Rationale is given for the decision specifically to focus on the examination of plant based therapies used for dermal wounds and lesions, and for the selection of the bioassays employed to screen for activity.

1.1 Introduction

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. The objectives of the study were to contribute to the improved understanding of the traditional medicinal use of the species and preparations examined, and, to determine whether references to specific dermatological therapeutic uses in the published Australian ethnobotanical record for these species could be associated with detection of any of the *in vitro* activities examined.

1.1.1 Overview of present study

Plant species used in indigenous Australian ethnomedical practice for the treatment of dermatological complaints were chosen as the focus of the study for a number of reasons. On reviewing published ethnobotanical reports describing the use of medicinal species, it became apparent that plants used in treatment of dermatological complaints formed the largest category of use both in terms of the number of reports referring to these types of use and the overall number of species employed. In addition, a relatively high degree of consensus was

found among separate, independent reports, in the mention of certain taxa for these purposes. The majority of extracts examined in this study were obtained as aqueous extracts, prepared after descriptions given for each species and/or plant part in the ethnobotanical literature. This was carried out in preference to extraction with organic solvents, as is the more common practice in phytochemistry and medicinal plant research, for several reasons. One of the aims of the project was to explore whether any relationship could be established between specific symptoms and indications mentioned in records of traditional use for each species, and any biological activity detected. The use of extracts which closely approximated traditional preparations described in the literature was deemed more appropriate for this purpose, as extracts prepared with organic solvents would be expected to differ significantly in terms of their resulting chemical profiles and spectrum of biological activity, which could potentially obscure any interpretations to this end. In this way the study was designed for the examination of activity of traditional *preparations* rather than of the phytochemical profiles of each plant species or part, as would be represented by a series of standardised or exhaustive extracts. Additionally, one of the goals often cited in ethnopharmacological research is the provision of useful information to indigenous populations who rely on traditional plant based medicinal products in preference to, or in the absence of access to contemporary orthodox 'western' pharmaceuticals (*cf.* Iwu, 1994; Heinrich, 2003). In recent years a number of public health care clinics servicing remote indigenous populations in Australia have initiated programs to make traditional medicinal preparations available as a type of culturally appropriate alternative or complementary therapy, in order to facilitate cultural engagement between indigenous patients and healthcare providers (Albert, 1992; Devanesen, 2000; Belfrage, 2007; INGW, 2010; PMHC, 2010). Virtually no published information exists detailing chemical composition of these types of preparations and to the best of the author's knowledge, only two studies examining biological and/or

pharmacological activities have so far been undertaken: These include the examination of antibacterial and anti-inflammatory activities in traditional decoctions of *Eremophila sturtii* by Liu (2006a) and one unpublished small scale clinical trial undertaken by staff of the Elliot Health Centre in the Northern Territory (NT) to examine the efficacy of topical preparations of *Bauhinia cunninghamii* in the treatment of skin disorders (Devanesen, 2000). The work undertaken in this study is novel in that biological activity or chemistry has not been previously investigated using traditional type extracts for the majority of the species examined. This work is also, to the best of the author's knowledge, the first instance where biological activities relevant to the therapeutic treatment of dermatological conditions and wounds, have been investigated in Australian medicinal plants cited specifically for these purposes.

A survey of Australian medical ethnobotanical literature was conducted 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. Plant parts from 23 species representing 15 botanical families were collected, from which 37 traditional aqueous preparations were obtained. Successive organic solvent extractions were also prepared using leaf material from five species belonging to the genus *Eremophila* for the purposes of comparing activity with corresponding traditional preparations. Extracts were screened for *in vitro* activities relevant to wound healing and other infectious/inflammatory dermal and ophthalmic conditions, for which the plants were reported to be used. Activities examined included antimicrobial activity, antioxidant capacity and anti-inflammatory activity. In addition, preliminary cytotoxicity screening was undertaken for the purposes of establishing appropriate ranges of concentration for subsequent cell-based assays. Aqueous extracts were also assayed for total phenolic content. Each assay utilised is described below (1.4.4) with

results presented in Chapter 3 (antimicrobial activity) and Chapter 4 (cytotoxicity screening, total phenolic content, antioxidant assays and anti-inflammatory activity).

Data obtained from the survey of published ethnobotanic literature was used to generate measurements of relative agreement or consensus among independent studies, for specific medicinal uses described for each preparation, and amongst separate categories of medicinal use. These included major use agreement (MUA) (after a method described by Coelho de Souza *et al.*, 2004) and the 'cultural agreement ratio' (CAR) (after methods described by Moerman, 2007). Use agreement indices were compared with results of bioactivity screening to determine whether any relationship could be found between the relative level of any activity detected, and the relative degree of consensus amongst published records. Botanical systematic analyses were also carried out on data obtained from the survey using Moerman's technique of regression residual analysis (Moerman, 1996). These analyses were performed to determine whether selectivity for particular botanical families could be demonstrated within the medicinal flora of the NT as a whole, and also, among species cited for use in the treatment of dermatological complaints.

In previous work, two diterpenes of the serrulatane class were identified as the principal anti-bacterial constituents in extracts of the medicinal plant *Eremophila duttonii*: serrulat-14-en-7,8,20-triol (**1**) and serrulat-14-en-3,7,8,20-tetraol (**2**) (Smith, 2004; Smith *et al.*, 2007). In this study, an attempt was made to isolate and purify the serrulatane diterpenes from a dichloromethane (DCM) extract of *E. duttonii* leaves to further investigate bioactivity and to use as standards in order to determine whether either compound could be detected in traditional aqueous preparations of this species, and if so, whether in quantities sufficient to account for any biological activity observed in the whole preparations. Results arising from this work are presented and discussed in Chapter 5.

During this study, evidence was obtained which indicated a high degree of intraspecific phytochemical variation in leaf volatile oil composition of one of the species examined (*Eremophila longifolia*). This was considered significant as decoctions of leaf material from this plant are used routinely and frequently by contemporary indigenous people throughout the continent for a variety of therapeutic purposes. In addition, an early report on *E. longifolia* volatile oil composition had indicated that the leaves were rich in the hepatotoxic and carcinogenic phenylpropanoids safrole and methyleugenol (Della and Jefferies, 1961). As a result of this finding, some authors (e.g. Lassack and McCarthy, 1997) have recommended against internal use of traditional decoctions prepared from this plant. A preliminary study was undertaken to examine variation in volatile oil composition and to determine whether toxic phenylpropanoids could be detected in material from populations of the plant occurring in the state of New South Wales (NSW). Examination of volatile oils from 10 specimens collected from several locations in NSW using gas chromatography-mass spectrometry and NMR spectroscopy resulted in characterisation of three novel chemotypes, none of which were found to contain detectable quantities of safrole or other phenylpropanoids. The results of this study are presented and discussed in Chapter 6, along with an assessment of anti-microbial activity of the volatile oil samples obtained.

1.2 **Previous ethnopharmacological investigations of Australian medicinal plants**

Over the past 20 years a number of studies have been undertaken involving screening for biological/pharmacological activity in extracts of native Australian plants selected on the basis of documented medicinal use by indigenous people. Activities which have been examined include anti-viral activity (Semple *et al.*, 1998), anti-bacterial activity, (Barr *et al.*, 1993; Palombo and Semple, 2001; Palombo and Semple, 2002; Palombo and Meilak, 2008; Smyth *et al.*, 2009) and various anti-inflammatory activities (Rogers *et al.*, 2000; Rogers *et*

al., 2001; Li *et al.*, 2003). These studies and instances where active species/extracts were followed up for identification of active constituents are discussed below.

1.2.1 **Previous studies examining anti-inflammatory activities**

Rogers *et al*. (2000) reported screening sequential DCM, methanol (MeOH) and aqueous extracts derived from 18 species for effects on *in vitro* platelet activation/aggregation and inhibition of platelet serotonin (5-hydroxytryptamine - 5-HT) release. In this study, 8 of the species examined were Australian natives which were selected on the basis of documented traditional indigenous use in the treatment of cephalalgia/headache. Significant activity was found predominately in the native species used traditionally to treat headache, with potent inhibition of 5-HT release reported for MeOH extracts of *Alphitonia excelsior*, *Ipomoea pes-caprae*, *Eremophila freelingii*, *Eremophila longifolia*, and *Asteromyrtus symphyocarpa* along with significant inhibition of adenosine diphosphate (ADP) induced platelet aggregation by *I. pes-caprae* extracts. Rogers *et al.* (2001) later investigated the effects of sequential DCM, MeOH and aqueous extracts of an additional 15 species on adenosine diphosphate (ADP) induced platelet aggregation and 5-HT release, reporting significant inhibition of 5-HT release by DCM extracts of *Cymbopogon ambiguus* (whole plant), and *Santalum acuminatum* seeds, and, MeOH extracts of aerial parts of *Eremophila gilesii* and *Erythrina vespertilio* bark. In a subsequent follow up study (Grice *et al.*, 2003), bioactivity guided fractionation of the *E. gilesii* extract led to the isolation of two phenylethanoid glycosides, verbascoside and poliumoside. More recently, Grice *et al.* (2011) reported isolation by bioactivity guided fractionation, of four phenylpropanoids with anti-platelet activity (eugenol, elemicin, eugenol methylether and trans-isoelemicin) from extracts of *C. ambiguus*.

Sweeney *et al*. (2001) have examined *in vitro* xanthine oxidase inhibition by 26 aqueous-ethanol (aq.-EtOH) extracts, which were then further purified by solvent partitioning between

water and ethyl acetate (EtOAc), of various parts of 15 species of Australian plants traditionally used to treat conditions indicative of inflammation. Sweeney *et al*. (2001) reported >25% inhibition by extracts/partitions from 9 species, and potent activity (IC_{50} < 50 μg/mL) by an aq.-EtOH extract of *Stemodia grossa* aerial parts and the EtOAc partitions of the *Clerodendrum floribundum* leaf/branch and *Eremophila maculata* aerial parts extracts.

Li *et al.* (2003) examined *in vitro* inhibition of cyclooxygenase-1 (COX-1) activity by 33 ethanol (EtOH) extracts obtained from 24 Australian and Chinese traditional medicinal plants which had been selected using a combined ethnobotanical and taxonomic approach (i.e. where occurrence of plants of the same genus in separate datasets of Chinese and Australian plants with documented traditional uses indicative of anti-inflammatory activity, was used to select plants for inclusion in the study). In this study the authors found a high rate of 'positive hits' for activity amongst species selected in this manner (21 of 22 extracts derived from Australian plants producing IC_{50} >20% at 3.4 mg/mL) and reported potent inhibition (IC_{50} >80%) by extracts of *Acacia ancistrocarpa* and *Acacia adsurgens* leaves, *Euphorbia drummondii* whole plant, *Ficus racemosa* inner bark and *Tinospora smilacina* stems and root. *T. smilacina* is mentioned in several Australian medical ethnobotanic reports for uses which may be considered suggestive of anti-inflammatory activity (e.g. headache, oedema and joint pain). In a follow up study, Li *et al*. (2004) described further activity of extracts against COX-1 and COX-2, 5-lipoxygenase and phospholipase A2, with bioactivity guided fractionation resulting in identification of an active fraction containing a mixture of C16-20 fatty acids and at least 3 triterpenoids.

1.2.2 **Previous studies examining antimicrobial activities**

Semple *et al*. (1998) reported on the investigation of antiviral activity against human cytomegalovirus AD169, Ross River virus T48, and poliovirus type-1 by 57 EtOH extracts

from 40 plant species used in Australian indigenous medicine for treatment of disease states and symptoms indicative of viral infection. Six extracts exhibiting antiviral activity were identified including those derived from *Pterocaulon sphacelatum* aerial parts and *Dianella longifolia* roots (inhibiting virus induced cytopathic effects of poliovirus), *Eremophila latrobei* subsp. *glabra* stems and leaves and *Pittosporum phylliraeoides* var. *microcarpa* fruit, wood and leaves (inhibiting virus induced cytopathic effects of Ross River virus) and *Euphorbia australis* whole plant, and *Scaevola spinescens* stem and leaf (inhibition of human cytomegalovirus late antigen production). In follow up studies, Semple *et al*. (1999) reported on isolation of the anti-picnoviral compound, 4'-hydroxy-3-methoxyflavone (chrysosplenol-C), from aerial parts of *P. sphacelatum*. 4'-Hydroxy-3-methoxyflavones such as chrysosplenol-C are specific inhibitors of rhinoviruses and preparations of this plant were known to be used traditionally in the treatment of respiratory infections (Semple *et al.*, 1999). Semple *et al.* (2001) have since additionally reported on the isolation of chrysophanic acid from extracts of *D. longifolia*.

Barr *et al*. (1993) conducted basic screening for antimicrobial activity of refluxed EtOH extracts of various parts of 34 of the species identified during the compilation of the Northern Territory Aboriginal Pharmacopoeia using agar dilution assays at two concentrations against the Gram-positive organism *Staphylococcus aureus* and the Gram-negatives *Klebsiella pneumoniae*, *Salmonella typhimurium* and *Escherichia coli*. Barr *et al*. (1993) reported identification of 21 species (62%) exhibiting growth inhibitory activity against two or more bacteria, with broad spectrum activity (inhibition of all four organisms) reported for extracts of *Alphitonia excelsior* bark, *Bauhinia cunninghamii* root, *Callitris glaucophylla* leaves and twigs, *Capparis umbonata* bark, *C. ambiguus* leaves and *E. longifolia* leaves. Palombo and Semple (2001) screened 56 EtOH extracts of various parts of 39 native medicinal plants against a panel of Gram-negative and Gram-positive bacteria using disc diffusion assays and

reported inhibitory activity against at least one organism by extracts of 9 species (23%) and activity against 3 or more organisms by extracts of (in order of decreasing potency) *E. duttonii* leaves, *Acacia kempeana* leaves, *Amyema quandang* leaves, *Lepidosperma viscidum* stem base and *Eremophila alternifolia* leaves. In a separate study, Palombo and Semple (2002) further demonstrated activity for each of these extracts (excepting that of *A. kempeana*) against clinical isolates of methicillin resistant Staphylococci and vancomycin resistant Enterococci. Further work characterising the mode of action and spectrum of activity of *E. duttonii* EtOH extracts has been undertaken by a number of researchers since this time, and a consistent pattern of potent and rapid bactericidal activity against several Gram-positive and food-borne pathogens, including antibiotic resistant clinical isolates, has been demonstrated (Palombo and Semple, 2002; Shah *et al.*, 2004; Tomlinson and Palombo, 2005; Owen and Palombo, 2007). Efforts at identifying active constituents by activity guided fractionation have resulted in the isolation of two diterpenoids of the serrulatane class (Smith *et al.*, 2007) (*cf.* Chapter 5).

Wickens and Pennacchio (2002) have screened tertiary aqueous extracts (after sequential extraction with hexane, DCM and MeOH) of the phyllodes and stems from 8 species of *Acacia* used medicinally by indigenous Australians for antimicrobial activity (using disc diffusion assays), cytotoxicity (using brine shrimp lethality tests) and allelopathy (inhibition of lettuce seed germination). No antimicrobial activity was reported but extracts of *Acacia pruinocarpa* were found to exhibit greatest allelopathic effects and cytotoxicity. Pennacchio *et al*. (2005) have since reported finding bactericidal activities in MeOH extracts of *Acacia auriculiformis*, *Acacia bivenosa* and *Eremophila maculata*.

Palombo and Meilak (2008) examined anti-mycobacterial activity (against *Mycobacterium fortuitum* and *M. smegmatis*) in EtOH extracts of 17 plant species reported to be used for

infections and respiratory conditions. Palombo and Meilak (2008) reported finding inhibitory activity against both organisms by extracts of *E. longifolia* leaves and *E. alternifolia* leaves and stems, and, activity against *M. smegmatis* by extracts of *Acacia ligulata* bark and leaves and *P. sphacelatum* aerial parts.

1.3 **Review of Australian medical ethnobotanical literature**

A survey of commonly available Australian medical ethnobotanical publications was undertaken in order to identify candidate species and plant parts for subsequent collection and to collect data for further botanical systematic and quantitative analyses.

Information was collected from a total of 29 separate sources, together containing 1014 entries describing traditional indigenous medicinal uses of 408 vascular plant species. Entries were recorded in a spreadsheet and included reference to the original source material, location of study and aboriginal language group or community, species name and authority, botanical family and medicinal uses cited. Medicinal uses were recorded as one or more of 332 uses or indications referred to in the original source material. Many of the 332 uses cited referred to what may have been considered to be similar sets of symptoms and indications. The 332 uses/indications were later grouped first into 53 subcategories and then further grouped into 16 broad categories of medicinal use for the purpose of subsequent analyses of use consensus within each use category (see 1.3.3 below). Broad categories of medicinal use were not initially defined in order to preserve information derived from the source material for each entry in the dataset. Details of the original descriptions of use, along with the scheme used for subsequent categorisations are given in Appendix I with the representative number of species and use reports within each sub-category and original description. Each species cited was checked for nomenclatural synonyms in the dataset using the *Australian Plant Name Index* (APNI) online database (CANBR, 2010) to avoid producing separate entries

describing the same botanical species [e.g. the species *Lysiphyllum cunninghamii* (Benth.) De Wit, as cited by Barr *et al.* (1993) is synonymous with *Bauhinia cunninghamii* (Benth.) Benth. as cited by Liddy *et al.* (2006)]. Additionally, a number of entries which cited plants only to the genus level were also removed where the possibility existed of creating separate, duplicate entries. After removal of these entries and reduction of synonyms, the final dataset contained reference to 394 medicinal plants. Organisation of genera into botanical families followed that currently given in APNI entries (CANBR, 2010). Angiosperms represented the majority of species cited in the literature. Only one species representing the Pteridophyta (ferns and fern allies) was mentioned (*Pteridum esculentum* as cited by Zola and Gott, 1992), and two gymnosperms (*Callitris glaucophylla* and *C. intratropica*, cited in several of the sources). Each of the 29 sources included in the survey is listed below in Table 1.1. The majority of sources consisted of primary or firsthand ethnobotanical surveys, each restricted to one region or aboriginal language group. Some information from sources covering broader regions or areas was also included (e.g. descriptions given by Maiden (1889), Webb (1969), Zola and Gott (1992), and Peter Latz's (1995) comprehensive study of central Australian ethnobotany). Information given in compilations of medical ethnobotanical monographs (e.g. Lassack and McCarthy, 1997) or general texts on the subject (e.g. Isaacs, 1987; Low, 1990), was omitted as to avoid the possibility of creating duplicate records. Exceptions included the survey of medical ethnobotanical records from Western Australia compiled by Reid and Betts (1979), from which duplicate records describing the work of Webb (1969) and Crawford (1982), (which were included in the survey) were omitted. The majority of the sources surveyed consisted of the series of reports published by the Northern Territory government and authored by local indigenous peoples in collaboration with the ethnobotanists Glenn Wightman and Nicholas Smith (Northern Territory Botanical Bulletin series). Each work in this series individually described the ethnobotany for specific groups and regions within the

NT of Australia. Another significant source of information was the Northern Territory indigenous pharmacopoeia compiled by Barr *et al*. (1993). This source gives detailed information on species used medicinally by several indigenous groups throughout the Northern Territory of Australia. For the purposes of the quantitative analyses performed on the dataset, information on medicinal plant use from each separate group or community, as individually described by Barr *et al*. (1993), was treated as a separate, independent study. Additionally, some of the information given in this source had been derived from earlier Northern Territory botanical bulletins. In these cases, or where the same indigenous informants were cited, records were omitted or combined for each species listed.

Table 1.1 List of Australian ethnobotanical surveys included in dataset

Reference	Area/Region covered	Language/Group	Total number of species mentioned	Total number of medicinal uses mentioned
(Maiden, 1889)	Various	Various	8	9
(Webb, 1969)†	Various	Various	107	210
(Reid and Betts, 1979)	Various (WA)	Various (WA)	20	55
(Levitt and Lyon, 1981)	Groote Eylandt (NT)	Anindilyakwa	41	104
(Crawford, 1982)	Kalumburu (WA)	Various (WA)	11	16
(O'Connell *et al.*, 1983)	Central Australia	Alyawarra	12	12
(Smith and Wightman, 1989)*	Belyuen (NT)	Emi & Batjamal	15	37
(Wightman and Smith, 1989)*	Milingimbi (NT)	Yolngu	23	62
(Wightman *et al.*, 1991)*	Minyerri (NT)	Alawa	28	96
(Wightman *et al.*, 1992)*	Kulumindini (Elliot) (NT)	Mudburra	34	110
(Zola and Gott, 1992)	Various (Vic.)	Various (Vic.)	10	16
(Wightman *et al.*, 1992)*	Elsey area (Jilkmingan Matalanka) (NT)	Mangarrayi	25	51
(Smith, 1993)*	Bulla (NT)	Ngarinyman	54	136
(Barr *et al.*, 1993)	Various (NT)	Various (NT)	117	608
(Wightman *et al.*, 1994)	Daguragu/Kalkarindji/Wave Hill (NT)	Gurindji	47	92
(Yunupingu *et al.*, 1994)*	Yirrkala (NT)	Rirratjingu	33	91
(Marrfurra *et al.*, 1995)*	Nauiyu Nambiyu (NT)	Ngan'gikurunggur & Ngangiwumirri	21	40
(Latz, 1995)	Central Australia	Various	71	142
(Blake *et al.*, 1998)	Gurig National Park (NT)	Iwaidja	29	41
(Raymond *et al.*, 1999)	Flora River/south-west Katherine region (NT)	Wardaman	36	65
(Renwick, 2000)	Wreck Bay area (ACT)	Booderee	6	19
(Lindsay *et al.*, 2001)	Daly River area (NT)	MalakMalak & Matngala	22	27
(Puruntatameri *et al.*, 2001)	Bathurst/Melville Islands (NT)	Tiwi	33	66
(Goddard and Kalotas, 2002)	Mimili (SA)	Yankunytjatjara	13	18
(Wynjorrotj *et al.*, 2005)	Katherine area (NT)	Jarwoyn	39	87
(Liu, 2006a)	Lightning Ridge area (NSW)	Kamilaroi & Muwarri	6	9
(Liddy *et al.*, 2006)	Mid Daly River area (NT)	Wagiman	26	41
		TOTALS	**394**	**2260**

†: Duplicate records from Angurugu submitted by Dulcie Levitt omitted; *: Duplicate records omitted or combined with equivalent presented in Northern Territory Bush Medicines Pharmacopoeia (Barr, Chapman *et al*. 1993); WA: Western Australia; NT: Northern Territory; SA: South Australia; Vic.: Victoria; ACT: Australian Capital Territory

Areas in the central arid and northern part of the Australian continent were among the last to come under colonial rule and indigenous people in these regions have been less exposed to processes of acculturation than people living in other parts of the continent (Mulvaney and Kamminga, 1999). Indigenous people in these areas have thus maintained a number of cultural practices which may be considered closer to those existing prior to European settlement, which includes traditional medicinal plant use. A 1994 government health survey reported that 22 - 30% of indigenous respondents in the NT had used traditional medicine within the six months prior to the survey, which was a significantly higher figure than in most other parts of the country (typically <6% of respondents) (McLennan, 1997). Mulvaney and Kamminga (1999) note that the ethnographic record tends to be biased toward the northern and central regions, and this bias can be seen to extend to the current Australian ethnobotanical record, and in particular to the more recent work of the past three decades or so, where the majority describes the ethnobotany of groups in these regions. The NT covers an area of around one-fifth of the Australian continent and the current number of vascular plant species listed for this state is similarly proportional (4,702 species, representing around 21% of 22,210 species listed for the entire continent) (Chapman, 2009; Short *et al.*, 2011). Of the 394 documented medicinal species recorded in this study, however, 315 (80%) included species cited by indigenous people of the NT (Table 1.2).

Table 1.2 Proportion of flora with documented records of medicinal use for the Northern Territory and the entire Australian continent

Regions	Documented medicinal species	Available species	Percentage of available flora used medicinally
Northern Territory	315	4702*	6.7%
Australia (entire continent)	394	22210†	1.8%
Percentage of total represented in Northern Territory (%)	80%	21%	

Sources: *: *Checklist of the Vascular Plants of the Northern Territory* (Short *et al.*, 2011);†: *Number of Living Species in Australia and the World* (ABRS Report) (Chapman, 2009). Figures cited include native and naturalised species; Sources for numbers of documented medicinal species given in Table 1.1

Dowell and Morris (2008) have noted the relative paucity of the Australian medical ethnobotanical record when compared to that of other countries, citing that globally, traditional medicinal uses have been documented for an estimated 12.5% of the worlds 422,000 plant species while the documented Australian medicinal flora, in contrast, represents only around 2% of that available. Similar proportions were found for the number of medicinal species listed in the records examined in this study when compared with listed numbers of available species, i.e. 394 medicinal species, representing 1.8% of an estimated 22,210 available species. Lassack and McCarthy's exhaustive monograph compilation (1997), lists 211 species documented as indigenous Australian medicinal plants which includes a further 75 species not mentioned in the literature surveyed in this study. From this, an estimate of a total of 469 species with documented medical uses by indigenous people is reached, however, this figure still represents only 2.1% of the vascular flora available. Moerman (1996) has cited a similar number of available vascular species listed for north America (21,641 species) to that of Australia (22,210 species), yet according to Moerman's north American ethnobotanic database (NAED), records of species used medicinally amounted to around 11.5% of those available (2,564 species) (Moerman, 1996). It is possible that the records of indigenous medicinal plant use of the NT probably represent a more accurate picture of local cultural traditions in this respect than those of the rest of the continent, yet the proportion of documented medicinal species in this state (i.e. around 6.7% as compared to ca. 2% estimated for the whole continent) is still relatively low when compared to that of north America and some other regions of the world (*cf.* Moerman, 1996; Dowell and Morris, 2008).

1.3.1 **Regression residual analyses of medicinal plants of the Northern Territory**

Regression residual analyses were carried out using the data obtained from the survey of literature to analyse the relationship of flora reported to be used medicinally in the NT, to the

available flora of the state. This technique was based on methods described by Moerman (1996) where the number of medicinal species representative of each angiosperm family mentioned in the north American ethnobotanic database (NAED) was compared with that of the available flora of the continent. In this analysis, the number of species representing each botanical family of the available flora forms the independent variable for a least squares regression against the corresponding numbers of representative species used medicinally in a given region or indigenous culture. The analysis can be used to test the assumption that the selection of plants for medicinal purposes is more or less random and thus principally determined by the prevailing flora of any given geographic region (i.e. that the number of species representing each family will be equally proportional to the relative size of that family as predicted by the least squares regression). Significant departures from predicted numbers of representative species (in the form of large residuals) would imply that certain families are instead, either preferentially selected, or, ignored/avoided for medicinal purposes (Moerman *et al.*, 1999). Families can then be ranked according to the residuals representing the difference between the numbers of representative species predicted by the regression equation and the actual or observed number of medicinal species, to determine relative selectivity and identify those which are proportionally under- or over-utilised. The use of regression analysis in this way allows for reduction of the effect of proportional size of each family and enables relative importance of each family to be assessed in terms of selection for medicinal use. The technique has been used for botanical systematic analyses of documented medicinal flora from regions of Korea, Kashmir, Mexico and eastern Ecuador (Moerman *et al.*, 1999), Veracruz (Mexico) (Leonti *et al.*, 2003) and Toledo (Belize) (Amiguet *et al.*, 2006). Wohlmuth and Brooks (2007) have also utilised the technique for the systematic analysis of plant species used in commonly prescribed phyto-therapeutic formulations employed in contemporary complementary medicine systems (including mostly European

and Asian species). Moerman (1996) demonstrated distinct selectivity among families represented in the NAED, specifically finding overrepresentation of the Asteraceae ('sunflower' family) and Apiaceae ('parsley' family) and underrepresentation of Poaceae ('grasses') and Cyperaceae ('sedges'). in further work, (Moerman *et al.*, 1999) found commonalities in the selectivity of certain families between medicinal plants of geographically separate cultures of the northern hemisphere inhabiting regions mostly dominated by holarctic flora (again demonstrating high selectivity of the Asteraceae and avoidance of the Poaceae and Cyperaceae), and, proposed that this could be explained by the relatedness of the flora along with the development of "similarly useful knowledge over broad geographic regions" and/or, the conservative transmission of specific knowledge relating to medicinal plant use, throughout prehistoric migrations of modern humans to the Americas. Other more recent studies have employed the technique for analysis of medicinal flora of Mesoamerican cultures inhabiting neotropical and neotropical/holarctic flora tension zones including Veracruz (Mexico) (Leonti *et al.*, 2003), and Toledo (Belize) (Amiguet *et al.*, 2006).

For the purpose of this study, the regression analysis was restricted to indigenous medicinal plants of the NT as the majority of records used to construct the dataset originated from studies conducted in this state, yet as outlined above, 80% of the available vascular plants of the continent are found outside of the NT. Performing this type of analysis using current records representing medicinal plants of the entire continent may not, at present, produce meaningful results given the lower relative representation of species endemic to regions outside of the NT.

Linear regression analysis was performed using the number of representative species in each of the angiosperm families used medicinally in the NT as the dependent variable, with the

independent variable consisting of the number of representative species listed for each of 163 angiosperm families of the entire state. Number of species representative of each family was taken from the current *Checklist of the Vascular Plants of the Northern Territory* (CVPNT) (Short *et al.*, 2011). Data for the medicinal species was extracted from sources listed above in Table 1.1. A total of 312 species representing 71 angiosperm plant families were mentioned in the literature surveyed. Residuals and standard residuals were calculated from the observed numbers of species representing each family, according to predicted numbers derived from the linear regression equation. For the purposes of this analysis some taxonomic arrangements were altered to conform with that followed in the CVPNT (Mabberley, 2008; cited by Short *et al.,* 2011). Under this arrangement, members of the Tiliaceae, Bombacaceae and Sterculiaceae were treated as Malvaceae, *Vitex* spp. (Verbenaceae) were treated as Lamiaceae, Chenopodiaceae were included in the Amaranthaceae and the species *Trachymene hemicarpa* (F.Muell.) Benth. (Apiaceae) was treated as Araliaceae. Myoporaceae (represented almost entirely by *Eremophila* spp. in the NT) were treated as Scrophulariaceae while *Stemodia* and *Scoparia* spp. (Scrophulariaceae) were treated as Plantaginaceae.

1.3.2 **Regression residual analyses of Australian medicinal plants used to treat dermatological complaints**

Of the 394 species cited for medicinal use in the records examined, more than half (211 species) were reported to be used for treatment of various dermatological complaints and conditions including the treatment of wounds and dermal lesions. A further regression residual analysis was carried out to analyse the relative proportions of plant families represented in this specific group of plants. The same techniques as were used to analyse representation of families in the medicinal flora were used except that representation of families comprising the 'dermatological' use category (see 1.3.3 below) were compared with

those of the medicinal rather than available flora and all vascular plants were included (i.e. gymnosperms and ferns). This was carried out to determine whether any further selectivity for particular families in this use category could be demonstrated relative to all plants used medicinally. For this analysis the independent variable was composed of the number of representative species for each of the 84 vascular plant families of the entire dataset, while the independent variable consisted of the corresponding number of representative species from the dataset containing only plants cited for dermatological uses. One of the plants included in the dataset, the Iwaidja medicinal species "*Garnggurrgarnggurr Garnggurrgarnggurr"* as described by Blake *et al.* (1998), was not included in the analysis as taxonomy has not been completely determined.

1.3.2.1 **Results for regression residual analysis of Northern Territory medicinal flora**

The regression equation using the 4,580 angiosperm species listed in the *Vascular Plants of the Northern Territory* (CVPNT) and the 312 NT medicinal species (NTMS) was as follows:

$$NTMS = 0.3297 + (0.0564 \times CVPNT)$$

Where *NTMS* is the predicted number of species for any given family of angiosperms appearing in the ethnobotanical records examined and *CVPNT* is the number of available species according to the NT flora checklist. Families were ranked by residual to identify those of high or low use in terms of selectivity. The 8 highest and lowest ranking families are listed below in Table 1.3, along with the number of species appearing in the NT flora checklist, predicted and observed numbers of medicinal species, residuals and standard residuals. The regression analysis is also represented graphically below in Figure 1.1. A complete list of the numbers of representative species and residual values for each family used in the analysis is provided in Appendix I.

Table 1.3 High and low use families identified by regression residual analysis of medicinal plants of the Northern Territory

	Number of representative species per family				
Family	NT Vascular Flora Checklist*	Observed NT Medicinal Plants†	Predicted NT Medicinal Plants	Residuals	Standard Residuals
High Use Families					
Myrtaceae	181	37	10.5	26.5	8.0
Fabaceae	619	48	35.2	12.8	3.9
Proteaceae	54	14	3.4	10.6	3.2
Scrophulariaceae	39	11	2.5	8.5	2.6
Solanaceae	62	12	3.8	8.2	2.5
Lamiaceae	74	10	4.5	5.5	1.7
Combretaceae	23	6	1.6	4.4	1.3
Santalaceae	10	5	0.9	4.1	1.2
Loranthaceae	33	6	2.2	3.8	1.2
Moraceae	23	5	1.6	3.4	1.0
Low Use Families					
Poaceae	559	20	31.8	-11.8	-3.6
Amaranthaceae	218	2	12.6	-10.6	-3.2
Cyperaceae	285	6	16.4	-10.4	-3.1
Goodeniaceae	112	2	6.6	-4.6	-1.4
Asteraceae	269	12	15.5	-3.5	-1.1
Dilleniaceae	54	0	3.4	-3.4	-1.0
Convolvulaceae	77	2	4.7	-2.7	-0.8
Stylidiaceae	40	0	2.6	-2.6	-0.8
Portulacaceae	38	0	2.5	-2.5	-0.7
Polygalaceae	37	0	2.4	-2.4	-0.7

Sources: *: *Checklist of the Vascular Plants of the Northern Territory* (Short *et al.*, 2011);†: Sources listed in Table 1.1

Plant families for which disproportionately high use was identified included the Myrtaceae ('Myrtle' family), Fabaceae ('pea' family) and Proteaceae ('Protea' or 'Banksia' family). Disproportionately low use was found for the Poaceae, Amaranthaceae ('Amaranth' family) and Cyperaceae. The highest and lowest ranking families were compared with rankings reported from previous studies (Table 1.4). While some of the taxonomic arrangements followed in this analysis differed from those used in previous studies, most of the major families (e.g. Asteraceae, Fabaceae, Myrtaceae, Poaceae) were equivalent or unchanged in circumscription and were deemed comparable in this sense. The disproportionately low use found for the Poaceae and Cyperaceae was similar to that reported in the studies examining the holarctic and neotropical/holarctic medicinal floras mentioned above and in Table 1.4. Disproportionately low use of these two families among species used in commercial phyto-

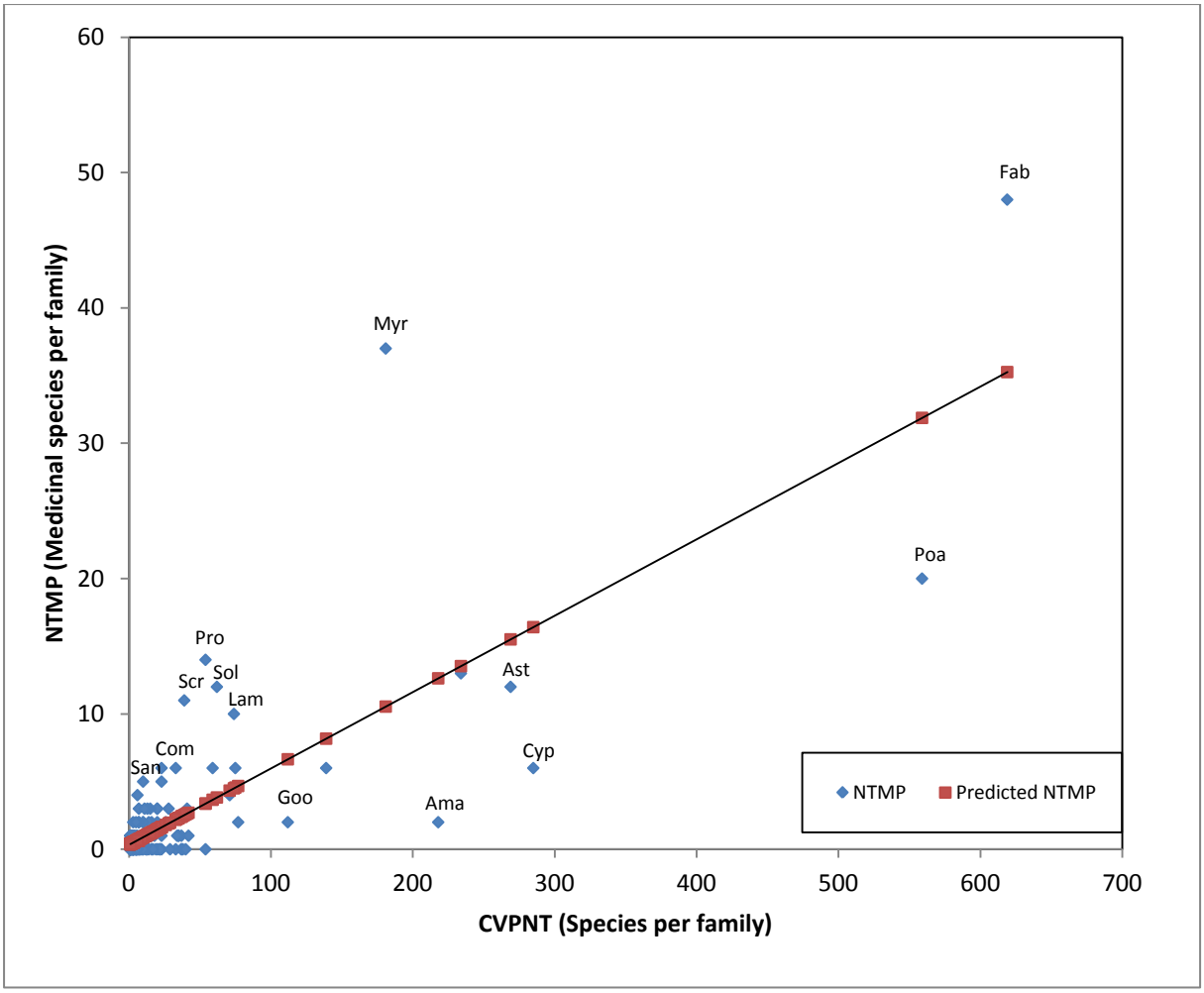

Figure 1.1 Regression residual analysis of medicinal plants and all available plants of the Northern Territory by family. CVPNT: Checklist of the Vascular Plants of the Northern Territory; NTMP: Northern Territory medicinal plants (sources listed in Table 1.1)

therapeutic formulations was also reported by Wohlmuth and Brooks (2007) and has been found in all but one of the medicinal floras from the 7 regions covered in the studies mentioned above (i.e. in Ecuador as reported by Moerman *et al.*, 1996). While low use selectivity for Fabaceae has been reported by some of the studies mentioned above, others have also reported high use selectivity for this family. These include the analysis of plant use in Ecuador and Korea in comparative studies by Moerman *et al.* (1999) and in Veracruz (Mexico) by Leonti *et al.* (2003). Interestingly, the regions reporting overuse of the Fabaceae in addition to Australia include those within the holartic (Korea), holarctic/neotropical tension zone (Mexico) and neotropical floristic regions (Ecuador), yet overuse of this family did not appear to be globally consistent according to the studies reviewed.

Table 1.4 Six highest ranking and five lowest ranking families of the NT medicinal flora with comparison to published data of seven other regions.

Families	Northern Territory, Australia	Chiapas, Mexico[a]	Veracruz, Mexico[b]	Toledo, Belize[c]	Ecuador[a]	Korea[a]	Kashmir[a]	North America[a]
High use families								
Myrtaceae	1	128	79	58	106	-	-	245
Fabaceae	2	131	3	179	2	7	85	253
Proteaceae	3	-	55	95	-	-	-	169
Scrophulariaceae*	4	7	152	202	78	123	15	222
Solanaceae	5	3	87	56	16	24	6	14
Lamiaceae*	6	2	5	22	95	3	4	8
Low use families								
Poaceae	1	1	2	2	67	2	1	1
Amaranthaceae*	2	113	168	151	35	90	94	14
Cyperaceae	3	3	4	3	16	1	6	2
Goodeniaceae	4	-	-	-	-	-	-	120
Asteraceae	5	144	174	207	72	135	100	255
Total families	163	144	174	209	118	136	100	255

a: (Moerman *et al.*, 1999); b: (Leonti *et al.*, 2003); c: (Amiguet *et al.*, 2006); *: Circumscription of family different to that of previous studies; Dashes (-) indicate no representatives of that family among the available flora of the region.

Similarly high rankings as were found in this analysis for the Solanaceae ('nightshade' or 'potato' family) and Lamiaceae ('mint' family), were also seen in a number of previous studies (Table 1.4). The composition of the Lamiaceae in this study, however, differed to that of previous studies (with the inclusion of Verbenaceae) and so this result may not be directly comparable. Also, Leonti *et al.* (2003) has noted that the apparent overuse of the Lamiaceae as was reported for the Popoluca medicinal flora (Veracruz, Mexico), was largely attributable to the fact that many of the medicinal Lamiaceae recorded in this region consisted of cultivated introduced species, and that the ranking of this family would have been lower based on observed numbers of native or wild species.

Selectivity in the Australian/NT medicinal flora differed most strikingly from that previously reported for other parts of the world in the selective overuse of large families, characteristic of the Australian flora and represented by widely radiated and abundant genera. These families included the Myrtaceae (as represented mainly by *Eucalyptus* spp. and *Melaleuca* spp.) and the Proteaceae (as represented mainly by *Hakea* spp. and *Grevillea* spp.). Another

distinctive difference in the selectivity found in this analysis of the NT medicinal flora, was the disproportionally low use of the Asteraceae. The majority of studies cited above have consistently identified the Asteraceae as a disproportionately high use family among plants used in traditional ethnomedicine. Although the Asteraceae includes a number of genera which feature importantly in indigenous Australian ethnomedicine (e.g. *Pterocaulon*, *Streptoglossa* and *Centipeda* spp.) this family was not selectively overused and ranked 159th (i.e. 5th last) among the 163 families according to the residual value (12 medicinal species observed with 15.5 species predicted by regression analysis from the 269 species listed in the CVPNT). The Amaranthaceae were ranked 2nd last according to the residual among the NT medicinal flora, whereas, in most of the studies referred to above, the Amaranthaceae were ranked relatively highly. Under the scheme of classification followed in this study, however, circumscription of the Amaranthaceae has expanded to include a large number of genera previously belonging to the Chenopodiaceae and so this result may not be directly comparable.

Another interesting difference in the selectivity of families within the Australian records analysed, was the high selective utilisation found for the Scrophulariaceae ('figwort' family). In the NT medicinal plant records the Scrophulariaceae are represented entirely by members of the endemic Australian genus *Eremophila*. The importance and prevalence of the *Eremophila* in Australian indigenous medical ethnobotany has long been recognised (Richmond and Ghisalberti, 1994) and Ghisalberti (1993; 1994) has provided comprehensive and extensive review of both the phytochemistry and ethnopharmacology of this genus. The Genus is known to accumulate a variety of secondary metabolites of potential pharmacological interest (e.g. phenylethanoid glycosides) and is particularly known for its elaboration of unusual and often unique diterpenoids. The *Eremophila* are cited for a wide variety of medicinal uses by indigenous Australian people and it has not been uncommon for

members of the genus to be included in studies involving screening of medicinal plant extracts from relatively large numbers of Australian native species. More interestingly, in such studies *Eremophila* spp. are frequently identified as 'positive hits' among plants selected and/or exhibit significant levels of the activity of interest (*cf.* section 1.2 above).

Higher than predicted selectivity for the *Eremophila* is also interesting as some members of the genus used medicinally are not as common or dominant as some of the medicinal Myrtaceae or Fabaceae and it is, therefore, less likely that selectivity may be explained by relative abundance or density. Of particular note in this regard is the relatively rare species *Eremophila alternifolia*, which is restricted in distribution and growth habit (typically only found occupying crests of eroded, stony ridges) yet features prominently in the ethnobotanical literature. Reports indicate that the medicinal virtues of the plant are particularly highly regarded (e.g. the plant is referred to as the "number one medicine" by some indigenous informants) and people may travel long distances to obtain necessary parts which are occasionally dried and stored for later use (Low, 1990; Richmond and Ghisalberti, 1994).

While rankings generated in other studies for the Scrophulariaceae have been given in Table 1.4 these may not be comparable with those generated in this analysis. Studies on the molecular phylogeny of the Scrophulariaceae (Olmstead *et al.*, 2001) have led to substantial revision in the circumscription of the family. Under the scheme followed in this study, the Scrophulariaceae of the NT flora is comprised entirely of plants previously belonging to the Myoporaceae (38 *Eremophila* spp. and 1 species of *Myoporum*), with other genera previously included (i.e. *Scoparia* and *Stemodia*), now belonging to the Plantaginaceae ('plantains'). The Myoporaceae do not occur in most of the regions mentioned in Table 1.4, except for 3 species recorded in the North American flora, none of which are documented for medicinal use

according to the analysis of Moerman *et al.* (1999). There are, however, several interesting phytochemical similarities between the endemic Australian *Eremophila* and some Scrophulariaceae of the Americas. Antimicrobial serrulatane diterpenes similar to those which have been isolated from *Eremophila* spp. (e.g. Liu *et al.*, 2006b; Smith *et al.*, 2007; Ndi *et al.*, 2007b; Ndi *et al.*, 2007c), have also been isolated from the Brazilian medicinal plant *Capraria biflora* (de Lima *et al*., 1958) (now typically excluded from the family and included in the Plantaginaceae) and the Mexican medicinal species *Leucophyllum frutescens* (Molina-Salinas *et al.*, 2011). Pharmacologically active iridoid and phenylethanoid glycosides such as verbascoside, which have been found in medicinal *Eremophila* spp. such as *E. alternifolia*, *E. longifolia* and *E. gillesii* (Ghisalberti, 1993; Pennacchio *et al.*, 1996; Grice *et al.*, 2003) have also been isolated from the Chilean wound healing plant *Buddleja globosa* (Pardo *et al.*, 1993; Liao *et al.*, 1999; Mensah *et al.*, 2001) and *Scrophularia nodosa* (Stevenson *et al*., 2002). Iridoid and phenylethanoid glycosides are also characteristic of the closely related Plantaginaceae (Rønsted *et al.*, 2000).

Other families which appeared to be selectively utilised in the NT included the Santalaceae, represented mainly by *Santalum* spp. ('native sandalwoods'), with 5 representatives used medicinally from 10 available, and the Combretaceae, represented mainly by *Terminalia* spp. ('native plums'). In addition a number of families were found which were represented by very few members, of which most were used medicinally and which are not indicated in the figure and table above. These included the Amaryllidaceae (i.e. *Crinum* spp.) with 4 representatives used medicinally from 6 available, The Picrodendraceae (3 of 7 available species used medicinally), and the Cannabaceae (2 of 3 available species used medicinally). As in previous studies using the technique, gymnosperms were not included in the analysis. Of the 314 NT medicinal species mentioned in the surveyed literature, 2 gymnosperms were recorded, *Callitris glaucophylla* and *Callitris intratropica* (Cupressaceae). While

gymnosperms were not included in the analysis, it is noteworthy that these two species represent all of the Cupressaceae occurring in the NT.

1.3.2.2 Results for regression residual analyses of Australian medicinal plants used to treat dermatological complaints

The regression equation using all 392 medicinal species cited in the sources examined and the 210 of the dermatological complaints category was as follows:

$$DCC = 0.066 + (0.520 \times AMS)$$

Where *DCC* represents the predicted number of species for each family of the dermatological complaints category and AMS (Australian Medicinal Species) represents number of species in each family for all medicinal records. The 7 highest and lowest ranking families are listed below in Table 1.5 and represented graphically below in Figure 1.2. A complete list of the numbers of representative species and residual values for each family used in the analysis is provided in Appendix I.

Table 1.5 High and low use families identified by regression analysis of dermatological use category

	Number of species per family*				
Use category	Number for entire dataset	Observed number for dermatological category	Predicted number for dermatological category	Residuals	Standard residuals
High use families					
Scrophulariaceae	11	9	5.8	3.2	2.5
Euphorbiaceae	13	10	6.8	3.2	2.5
Amaryllidaceae	5	5	2.7	2.3	1.8
Fabaceae	57	32	29.7	2.3	1.8
Combretaceae	6	5	3.2	1.8	1.4
Rubiaceae	6	5	3.2	1.8	1.4
Malvaceae	16	10	8.4	1.6	1.2
Low use families					
Solanaceae	16	3	8.4	-5.4	-4.2
Loranthaceae	6	0	3.2	-3.2	-2.5
Poaceae	21	8	11.0	-3.0	-2.3
Asteraceae	13	4	6.8	-2.8	-2.2
Lamiaceae	15	6	7.9	-1.9	-1.4
Sapinidaceae	7	2	3.7	-1.7	-1.3
Myrtaceae	43	21	22.4	-1.4	-1.1

*: Sources for data listed in Table 1.1

Among the plants used in treatment of dermatological conditions, a number of families were found to exhibit further or increased use selectivity to that found among the NT medicinal species. The families which ranked highest according to residual values included the Scrophulariaceae, Euphorbiaceae ('spurges'), and Amaryllidaceae ('amaryllis family'). Families found to be underutilised or avoided for dermatological purposes included the Solanaceae, Loranthaceae ('mistletoes') and Poaceae.

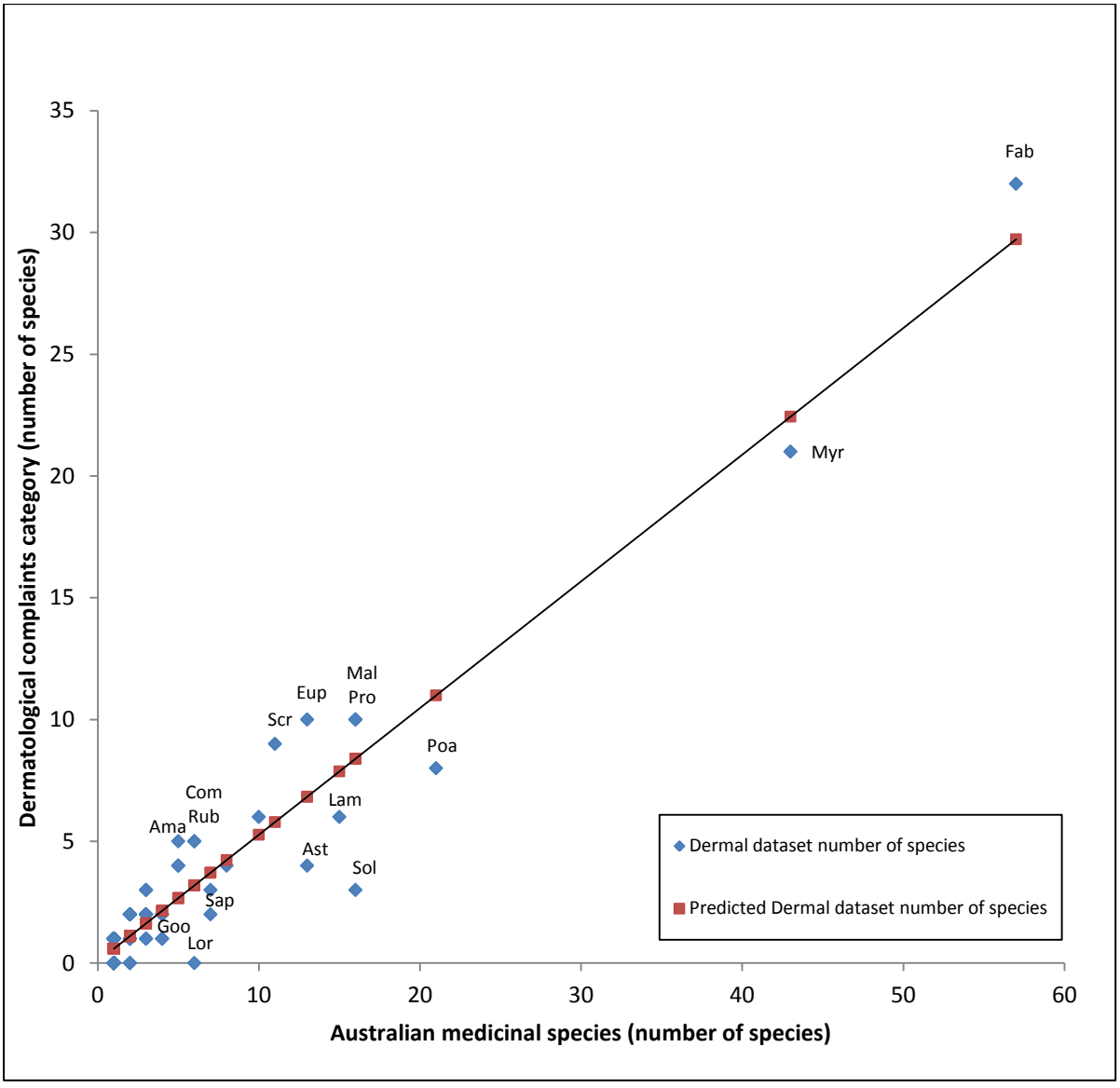

Figure 1.2 Regression analysis of species used for dermatological complaints and medicinal plants of entire dataset by family

Of the 11 medicinal species of Scrophulariaceae appearing in the records examined (represented entirely by *Eremophila* spp.), all but 2 species were cited for various dermatological uses (i.e. *E. alternifolia* and *E. elderi*). Of the 13 species representing the Euphorbiaceae in the dataset, the majority (10 species) were cited for dermatological uses and indications, with around half (6) of the species cited in this context belonging to the genus *Euphorbia*. This result was not altogether surprising, as the common use of the proteolytic latex of some *Euphorbia* spp. for the topical treatment of wounds, dermal lesions and removal of warts is well known (Lassack and McCarthy, 1997). The majority of preparations described for treatment of dermatological conditions and involving use of the Euphorbiaceae, however, more often involved mention of decoction of aerial parts (which would probably denature and render inactive most proteolytic enzymes). It is possible that other factors present in this family, e.g. high tannin content of aerial parts (*cf.* Lassack and McCarthy, 1997; Okuda, 2005), may partly explain the apparent overutilisation of members of this family for dermatological therapies.

All 5 of the *Crinum* spp. (representative of the Amaryllidaceae) mentioned in the records examined were also cited for a variety of dermatological uses. The *Crinum* spp. cited, (in particular *Crinum angustifolium*), were additionally among the species most frequently cited for use in preparations for the treatment of wounds and dermal lesions in the surveyed literature (*cf.* Table 1.7 below). The Rubiaceae ('madder' or 'coffee' family) did not appear to be significantly selected in the medicinal flora of the NT overall, ranking 151st overall according to the residual regression analysis described above. Five of the 6 species mentioned, however, were cited for dermatological medicinal uses (including 3 species of *Gardenia*). The majority of the medicinal Combretaceae (represented entirely by *Terminalia* spp.) were also cited for dermatological medicinal uses (5 of 6 species) and similarly, 4 of the 5 Plantaginaceae used medicinally were also represented in the dermatological use category.

Numbers of species representing the Myrtaceae did not appear to diverge significantly from that predicted in the regression analysis, however, significant differences could be seen at the genus level in the composition of taxa cited for dermatological remedies. The medicinal Myrtaceae were mostly represented by members of the genus *Eucalyptus* ('gums'), or, of *Melaleuca* ('tea-trees'). Of the 15 medicinal species of *Melaleuca* mentioned in the records examined, only one was cited for uses which could be classed as 'dermatological' [i.e. *Melaleuca viridiflora*, along with another unidentified species not included in the dataset, both cited by Levitt and Lyon (1981)]. The remaining medicinal *Melaleuca* were typically cited for uses which involved treatment of the symptoms of respiratory tract infections and topical treatment of joint and skeleto-muscular pain. In contrast, almost all of the medicinal *Eucalyptus* species mentioned were cited for use in dermatological remedies (17 of a total of 21 species mentioned). Similarly the Proteaceae did not appear to be disproportionately represented among the 'dermatological' plants, however, all 6 of the *Hakea* spp. representing this family were cited for the treatment of wounds.

The most strongly avoided family within the dermatological grouping appeared to be the Solanaceae. Only 3 of the 16 medicinal species recorded were cited for dermatological uses including *Nicotiana excelsior* and *N. rosulata* by Yankunytjatjara people of central Australia (Latz, 1995), and *Physalis minima* by MalakMalak/Matngala people of the NT (Lindsay *et al.*, 2001). Aside from including several species which are used as food in many parts of the world (including native Australian species such as *Solanum centrale*), the Solanaceae are generally known to accumulate a number of toxic, alkaloid defensive secondary metabolites, e.g. solanine of *Solanum tuberosum* ('potato') (Harborne, 1993). General toxicity among members of the family may in part explain the low selectivity observed, however, this is not likely to be characteristic of the dermatological category in particular. The majority of Solanaceae mentioned in the records examined (mostly represented by *Nicotiana* and

Solanum spp. and 2 species of *Duboisia which are* also reported to be used as fish/game poisons), are used almost exclusively for purposes which in this study, were classed under the 'neurological complaints and uses' category. Specifically, these uses included uses/indications falling under the sub-categories of 'psychoactive' uses and 'narcotics' and frequently involved the use of *Nicotiana* spp. as substitutes for tobacco and/or *pituri* preparations. These species tended to be cited exclusively for these purposes and secondary uses representing any of the other use categories defined were not often cited.

Interestingly, members of the Asteraceae (which were not found in this study to be highly selected in the general medicinal flora of the NT as has been indicated in the majority of studies reporting results of similar analyses in other regions of the world), appeared to be further avoided in the dermatological category. Only 4 of the 13 species used medicinally were cited for use in the treatment of dermatological conditions, including *Streptoglossa odora* and 3 species of *Pterocaulon*. None of the *Blumea* or *Centipeda* spp. belonging to the Asteraceae appeared to be cited for dermatological uses. Species of *Pterocaulon* cited for dermatological remedies (specifically *P. serrulatum* and *P. sphacelatum*) were, however, among the more frequently cited species for this purpose (i.e. in separate independent ethnobotanic surveys), and in particular, for the treatment of wounds and dermal lesions.

Members of the Goodeniaceae and Poaceae were found to be avoided in the NT medicinal species overall (ranking 160th and 163rd respectively among 163 families according to the residual regression analysis) and also appeared to be further avoided among plants used for dermatological remedies.

1.3.3 Use consensus and major use agreement in the Australian ethnobotanic literature

Quantitative analyses of ethnobotanic data may be used to provide relative measures of the cultural importance within defined categories of use and for specific taxa, e.g. by identifying taxonomic subsets within lists of plants which are used frequently or consistently both within and between cultural groups. For the purposes of this study, two methods of quantitative analysis for consensus and agreement were applied to the data compiled from the published ethnobotanic sources listed in Table 1.1. These included cultural agreement ratio (after Moerman, 2007), and major use agreement (as described by Coelho de Souza *et al.*, 2004).

In the survey of ethnobotanical literature, a total of 339 medicinal uses and indications were recorded. For the purposes of quantitative analyses, these were first grouped into 49 subcategories and then further reduced to a final 16 use categories consisting of dermatological complaints, haemostasis, ophthalmic complaints, bites and stings, dental/buccal complaints, ear/nose/throat complaints, respiratory complaints, fever, bone fractures and disorders, pain and inflammation, urinary tract complaints and venereal disease, gastro-intestinal complaints, reproductive uses and complaints, neurological uses and complaints, 'tonics', and miscellaneous/other uses. The full list of uses and indications given in the literature which were subsequently sorted into sub-categories and categories of therapeutic use is provided in Appendix I.

Cultural agreement ratio (CAR) describes the degree of agreement or consensus for use of specific taxa within a use category, by comparing the number of species used within a given category to the total number of use reports. The method of calculating CAR followed that outlined by Moerman (2007), where similar techniques (i.e. informant agreement ratio and informant consensus factor), developed for the purpose of measuring consensus among use

reports of individual informants (e.g. Trotter and Logan, 1986; Heinrich *et al.*, 1998), were applied to records derived from Moermans' census of native American ethnobotany (Moerman, 2007).

In this study CAR was calculated for each defined use category, using reports derived from the Australian ethnobotanical literature and was calculated as follows:

$$CAR = \frac{n_{ur} - n_t}{n_{ur} - 1}$$

Where n_{ur} is the number of use reports in each category and n_t is the number of taxa used in each category (in this case species). With CAR, values closer to 1.0 represent a higher degree of consensus within any given use category. To give an example, in the literature examined, 26 mentions of uses related to use as a 'soap plant' were found, referring to a total of 10 separate species. CAR was the use category of 'soap plant' was determined as follows:

$$CAR = \frac{26 - 10}{26 - 1} = \frac{16}{25} = 0.64$$

If the 26 use reports had all referred to a single species, used universally for this purpose, then CAR would be 1.0, indicating a very high level of agreement as:

$$CAR = \frac{26 - 1}{26 - 1} = \frac{25}{25} = 1.00$$

On the other hand, if no agreement was found in the literature for the use of specific taxa as soap plants (i.e. all 26 use reports cite a different species for this purpose), then CAR will be 0 as:

$$CAR = \frac{26 - 26}{26 - 1} = \frac{0}{25} = 0.00$$

While the CAR and analogous indices are useful for indicating agreement within defined use categories, information regarding consensus for individual taxa is not provided (Heinrich *et al.*, 2009). For the purpose of measuring consensus for specific uses of individual taxa collected during the study, the method of major use agreement (MUA) as described by Coelho de Souza *et al.* (2004) was employed. MUA describes the degree of consensus between separate informants in citations describing uses for individual species. MUA values for all species included in the study were determined for three separate categories of medicinal use or therapeutic indication. Categories of use for which separate sets of MUA values were calculated were as follows: Citations referring only to treatment of dermal wounds, citations referring only to treatment of dermal lesions or dermatoses (i.e. 'sores'), and all citations referring to treatment of any dermatological and ophthalmic complaints. The method used to calculate MUA was based on that described by Amarozo and Gely (1988), cited by Coelho de Souza, Haas *et al*. (2004) using the following formula:

$$MUA = FL \times CF$$

Where FL (fidelity level) is determined as the percentage ratio between the number of independent surveys citing a specific use category (SU) and the total number of independent surveys citing the species for any use (TU) and CF is a correction factor to account for the relative proportion of total citations for each species (determined as the ratio between TU and the highest number of citations for a given use in a single species (U_{max}) i.e.:

$$FL = \frac{SU}{TU} \times 100$$

$$CF = \frac{TU}{U_{max}}$$

Confusingly, FL is sometimes itself referred to as 'MUA', and MUA as defined here and elsewhere (e.g. Coelho de Souza *et al.*, 2004) as corrected MUA or 'MUA_C' (e.g. Amarozo and Gely, 1988). In this study, the term MUA is always used to refer to the final product of FL and the correction factor.

Major use agreement values are typically calculated based on citations referring to taxa at the species level. For many of the species examined in the study, however, reference to a variety of preparations, each utilising distinct plant parts and modes of preparations was often found. As this study involved the examination of specific 'traditional' plant based preparations, the technique was modified by using only those citations referring to each specific preparation examined. This was possible as in many instances, multiple independent citations were found which described similar modes of preparation of each species/plant part for the three therapeutic use categories considered. In this way each preparation was treated as a distinct entity rather than the species from which particular plant parts used in the preparation were obtained. MUA values thus derived were subsequently referred to as 'MUA for traditional medicinal preparations' (MUA_{TMP}). 'MUA_{TMP}' was used in preference to MUA calculated with all citations referring to each species, in order to assess agreement with a higher degree of specificity for particular uses of each preparation. To give an example of the calculation, one of the preparations examined in the study, a decoction of the trunk inner bark from *Erythrophleum chlorostachys*, was mentioned in 6 separate, independent studies. Five of these studies include reference to use of the preparation as a treatment for dermal lesions ('sores'). Fidelity level (FL) for this purpose then is calculated as 83%:

$$FL = \frac{SU}{TU} \times 100 = \frac{5}{6} \times 100 = 83\%$$

Correction factor is determined using the most frequently cited preparation (U_{max}), in this case, 9 independent citations for use of decoctions of *Protasparagus racemosus* as a remedy for sores, giving CF of 0.67 in respect to the *E. chlorostachys* preparation:

$$CF = \frac{TU}{U_{max}} = \frac{6}{9} = 0.67$$

Finally, MUA_{TMP} is determined as the product of FL and the CF:

$$MUA_{TMP} = FL \times CF = 83 \times 0.67 = 56\%$$

Here it can also be seen that an MUA_{TMP} value relating to the treatment of sores for the *P. racemosus* decoction referred to above (cited in 9 separate reports) would be 100%, as would any other preparation also cited independently for this purpose in 9 separate studies.

MUA has been used to assess relative importance for specific uses amongst different medicinal species in relation to biological activity (e.g. Oliveira *et al.*, 2006) and to guide selection of medicinal plants for specific pharmacological studies (e.g. Coelho de Souza *et al.*, 2004; Oliveira *et al.*, 2011), based on the assumption that the more widely or consistently a species is cited for a particular use, the greater the probability that the plant will be effective for the condition/use cited. The technique was adopted here in order to test this assumption in the confines of the specific *in vitro* pharmacological activities examined and major use agreement measurements derived from the survey of Australian ethnobotanical literature. Oliveira *et al.* (2011) have shown that a higher probability of detecting antimycobacterial and anti-cholinesterase activity can be found among extracts of plants selected on the basis on high MUA for the respective treatment of respiratory illnesses and certain neurological uses, when compared to extracts derived from wild plants collected randomly.

In addition to signifying an increased probability of biomedical efficacy, Heinrich *et al.* (2009) state that frequently cited taxa may also reflect use for a common disease or event, ease of availability of taxa and/or special cultural significance associated with the plant (in addition to any perceived medical effectiveness). Infrequent citations, aside from signifying lowered probability of efficacy may represent artefacts or erroneous recording/translation of cultural uses, the taxa falling into disuse via cultural adaptation, relative rarity or scarceness of the taxa, or, specialised knowledge (Heinrich *et al.*, 2009). As with CAR, MUA was originally developed for analysis of agreement in plant use between individual informants (Amarozo and Gely, 1988) and has been adapted to deal with records of previous ethnobotanic work (Coelho de Souza *et al.*, 2004). Studies where data on medicinal plant use supplied by individual informants are subjected to quantitative analyses are typically limited to a defined geographical area where, presumably, any taxa mentioned or considered are potentially available to all participants/informants. Applying the technique of MUA analysis to surveys of published ethnobotanic literature describing plant use across a wide geographic area (as was done in this study) may be problematic as the distribution of species included may not cover all locations considered. A species which was only available in regions covered by relatively few surveys may then be relatively underrepresented and be assigned a falsely low MUA value. In this study, MUA_{TMP} values were indexed relative to the most frequently cited preparation in the surveyed literature (Decoction of *P. racemosus* root for treatment of sores which was cited in 9 separate reports). The individual distribution of the majority of the species collected was such that in each case an area was covered which encompassed more than 9 locations representing independent studies. Possible exceptions included *Scaevola sericea* (which has limited distribution in the more northerly, coastal areas of the NT) and *Eremophila alternifolia* which is a relatively rare plant with limited distribution to the more central parts of the continent. In any case it is possible that certain

species which are more widely distributed may be more likely to be utilised as medicinal plants and potentially relatively overrepresented in frequency of citation in a similar way in which plants which are common or occur densely may be overrepresented in surveys conducted in geographically limited locations. Some of the more widely distributed species which were collected in this study included *Eremophila longifolia*, *Eremophila latrobei*, and *Acacia holosericea*. Another potential problem with comparing use agreement indices gathered over relatively large geographic areas is the possibility of the occurrence of significant intraspecific phytochemical variation. Occurrence of rare or unusual chemotypic variation in a particular region (i.e. variant accumulating secondary metabolites which have contributed to efficacy or other factors favouring cultural selection, which is not present in other regions from which use data is compared, may be expected to result in a lowered frequency of citation for specific uses or high variability in use reports). MUA_{TMP} values determined for each preparation using data obtained from the survey of literature, are presented and discussed alongside results of assays for biological activity in each relevant chapter (Chapters 3 and 4).

1.3.3.1 **Use categories and consensus in Australian ethnobotanic literature**

Number of species, use reports and the CAR for each use category in the literature reviewed are given below in Table 1.6 and represented graphically in Figure 1.3. CAR ranged from 0.00 to 0.55 with mean value of 0.33. These figures were very similar to those reported in Moerman's (2007) analysis of records forming the comprehensive North American ethnobotanic database, where reported CAR values ranged from 0.00 to 0.65, with mean CAR reported at 0.27. As was noted by Moerman, these values, while generally in the range of those previously reported overall, were slightly lower than those typically reported in other studies using the analogous informant agreement ratio/informant consensus factor (e.g. Heinrich *et al.*, 1998). Moerman has suggested that this is likely to reflect the fact that using

the CAR, species with a narrow distribution, which while used extensively in one society, would not necessarily be available in other regions. A similar effect is likely to be operating in this analysis of the Australian records, where a range of biogeographically and floristically diverse regions were encompassed in the literature from which the dataset was composed. Also, as discussed above, the current Australian ethnobotanical record exhibits a bias toward the documenting of plant use in the NT and Central Australia. Records of traditional plant use outside of these regions include endemic species not found in the NT, and it would be expected that this would result in an increase of numbers of species without a proportional increase in the number of use reports which would in turn, lower overall consensus.

The category containing species used for dermatological complaints was the largest, both in terms of number of species (211) and use reports (450), followed (in order of decreasing number of representative species) by the categories of respiratory complaints, pain and inflammation and gastrointestinal complaints. In terms of consensus, the categories of respiratory complaints and dermal complaints ranked the highest (CAR: 0.55 and 0.53 respectively). Interestingly, several similarities were found in the level of consensus and relative proportion of representative species between use categories defined in this analysis and those reported by Moerman (2007) where similar categorisations of medicinal use from records of the NAED were described. In Moerman's analysis the category including dermatological uses ("dermatological aid") also represented the largest category, representing around 40% of all medicinal species with a CAR value of 0.59. Relatively high CAR (0.56) was also reported in Moerman's category of 'cold remedy' (Moerman, 2007) (similar to the 'respiratory flu-like illness' subcategory of this study with CAR of 0.53). CAR values reported by Moerman (2007) for categories of "febrifuge" (CAR: 0.40) and "Anti-diarrheal" (CAR: 0.35) were also similar to those found here (fever category, CAR: 0.40; diarrhoea/dysentery category, CAR: 0. 37).

Table 1.6 Cultural agreement ratio (CAR) values and frequently cited species for use categories in Australian ethnobotanical records

Use category	Number of use reports	Number of species	Cultural agreement ratio (CAR)	Maximum number of citations	Most frequently cited species in use category (number of independent citations in parentheses)
Dermatological complaints/uses	**450**	**211**	**0.53***	10	*Acacia holosericea* (10), *Erythrophleum chlorostachys* (10), *Ipomoea pes-caprae* (10), *Protasparagus racemosus* (9)
Respiratory illnesses/infections	**297**	**133**	**0.55***	12	*Eucalyptus camaldulensis* (12), *Melaleuca leucadendra* (11), *Morinda citrifolia* (10), *Pterocaulon serrulatum* (10)
Pain and inflammation	175	108	0.39	9	*Tinospora smilacina* (9), *Clerodendrum floribundum* (7), *Eucalyptus camaldulensis* (6), *Eremophila alternifolia* (5), *Ipomoea pes-caprae* (5)
Gastrointestinal Complaints	105	66	0.38	13	*Grewia retusifolia* (13), *Clerodendrum floribundum (4), Nymphaea macrosperma* (4)
Miscellaneous	84	64	0.24	3	*Eucalyptus camaldulensis* (3), *Cymbopogon bombycinus* (3), *Melaleuca leucadendra* (3), *Ventilago viminalis* (3)
Neurological complaints/uses	79	54	0.32	5	*Pterocaulon serrulatum* (5), *Pterocaulon sphacelatum* (5), *Isotoma petraea* (4), *Lobelia quadrangularis* (4), *Duboisia hopwoodii* (3)
Ophthalmic complaints	54	36	0.34	4	*Grewia retusifolia* (4), *Owenia acidula* (3), *Persoonia falcata* (3), *Scaevola taccada* (3)
Reproductive complaints/uses	49	37	0.25	4	*Exocarpos latifolius* (4), *Acacia lysiphloia* (3), *Erythrophleum chlorostachys* (3)
Dental/buccal complaints	49	30	0.40	6	*Buchanania obovata* (6), *Pandanus spiralis* (4), *Grewia retusifolia* (3), *Persoonia falcata* (3)
Ear-nose-throat complaints	49	28	0.44	8	*Morinda citrifolia* (8), *Livistona humilis* (4), *Eremophila latrobei* (3), *Eucalyptus camaldulensis* (3)
Fever	41	25	0.40	5	*Eremophila alternifolia* (5), *Eucalyptus camaldulensis* (5), *Cymbopogon ambiguus* (5)
Tonics	**31**	**28**	**0.10†**	3	*Terminalia carpentariae* (3), *Eucalyptus dichromophloia* (2)
Bites and stings	25	15	0.42	9	*Ipomoea pes-caprae* (9), *Pteridium esculentum* (2), *Tinospora smilacina* (2)
Urinary tract complaints and venereal disease	14	10	0.31	3	*Euphorbia drummondii* (3), *Santalum lanceolatum* (3)
Bone fractures and disorders	**9**	**9**	**0.00†**	1	NA
Haemostasis	8	6	0.29	3	*Ipomoea pes-caprae* (3)

*(bold type): CAR greater than mean plus one unit of standard deviation (0.48); †(bold type): CAR less than mean minus one unit of standard deviation (0.19)

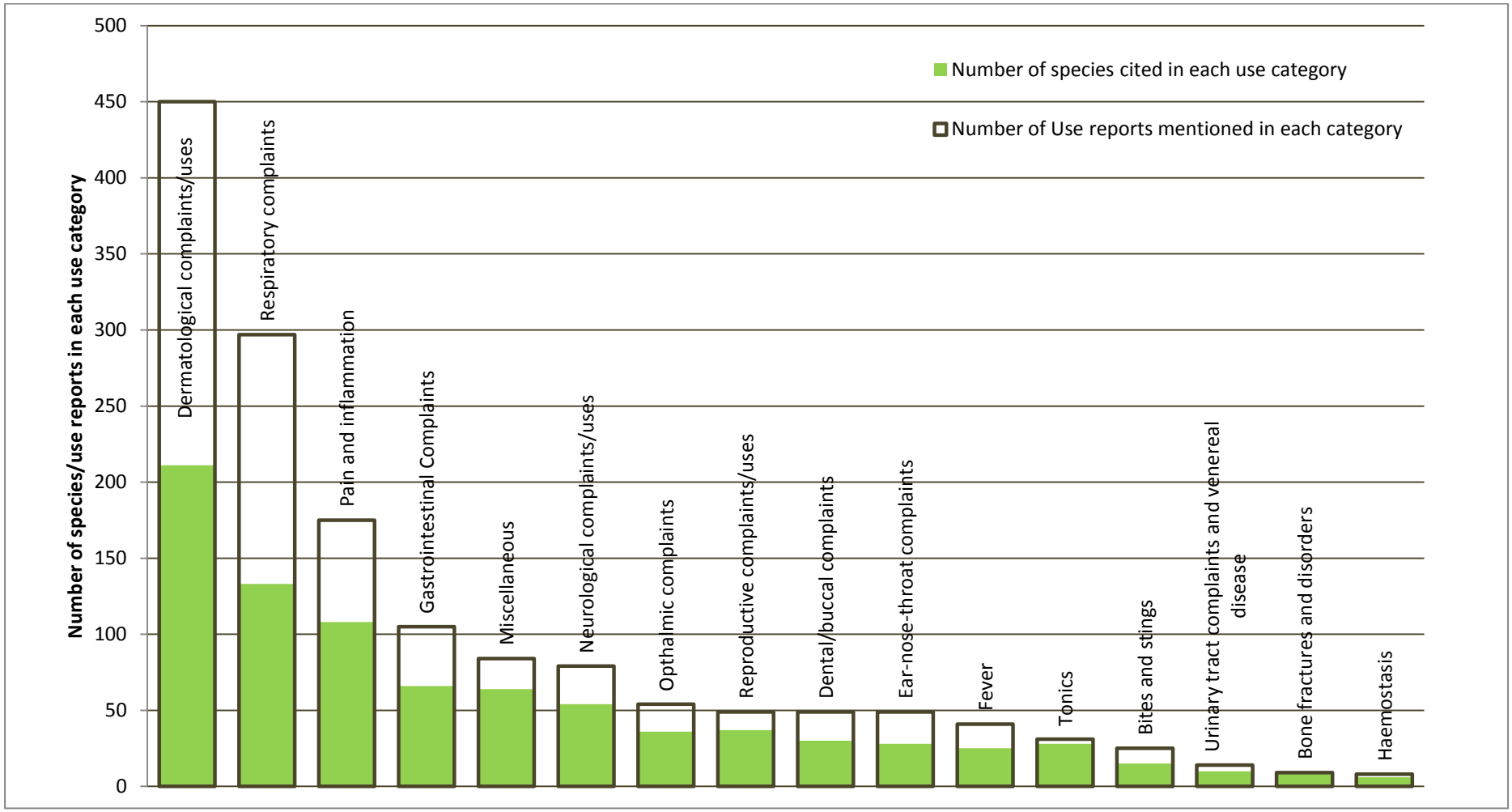

Figure 1.3 Number of species and use reports within 16 use categories from 29 Australian ethnobotanical reports

Low agreement was found amongst use reports of species used for bone fractures and disorders (CAR: 0.00) and the category containing species used as 'tonics' (CAR: 0.10), the category of reproductive complaints/uses (CAR: 0.25), the 'miscellaneous' category (CAR: 0.24) and haemostasis (CAR: 0.29). Moerman reports similarly low consensus for "hemostats" (CAR: 0.21) and "antihemorrhagics" (CAR: 0.26) of the NAED. In this study, only one of the species mentioned for this purpose (*I. pes-caprae*), was cited in more than one of the reports surveyed. In the Australian records examined, several of the references to haemostasis described the use of large or broad leaves applied to the site of bleeding (e.g. Levitt and Lyon, 1981). It is possible that many of the species used for this purpose have been selected simply on the basis of relative leaf size (i.e. large and broad leaves which may be suitable for applying pressure to a superficial haemorrhage, e.g. those of *B. obovata* or *I. pes-caprae*) and may not necessarily act in any other way to expedite haemostasis. Such factors would not limit selection of plants for this purpose to those which provided some distinct or definite action and this may in part explain the low degree of consensus in this category. Plants used for this purpose might also be expected to mostly include species which are very common or easily available in any given region as for haemorrhage, immediate treatment would generally be desired (Burford *et al.*, 2007). Low consensus in this category and for the category of 'bone fractures' may also have resulted as an artefact of the very low numbers of species mentioned within these categories (9 species for bone fractures and 6 for haemostasis). Bone fractures might also be relatively underrepresented as serious fractures might not be expected to occur frequently as a disease event.

The majority of species listed under the 'reproductive uses and complaints' category included those cited for the post-natal 'smoke treatment' of neonates and mothers, and those used in the modification of lactation (both categories exhibiting low consensus). Smoke treatments are

typically described in the ethnobotanic literature as ritualistic events, involving the slow burning or smouldering of green plant material to produce smoke in which the newborn infant is held. Smoking treatment is practiced as a form of preventative therapy which is believed to exert a number of long-term beneficial effects, most often involving the "strengthening" and pacifying of the newborn infant (Pearn, 2005). As a therapeutic practice involving components of ritual and ceremony, it is probable that the selection of particular species for smoke therapy, involves a number of complex, interacting cultural factors, and is not likely to be based primarily on any direct biomedical efficacy or actions. Some of the species cited for this purpose (i.e. *E. chlorostachys* and *E. longifolia*) are also used in a similar manner (smouldered) in other ceremonial or ritual processes. While this practice is in many ways specific to Australian indigenous ethnomedicine, the production of fragrant smoke with the smouldering of plant materials during ritual and ceremonial procedures has been widespread throughout history and across many cultures in the form of incense (Stoddart, 1990). In the literature examined, 20 species were cited for the purpose of smoke treatment of newborns and included a number of essential oil bearing species which may be expected to produce a pleasant odour when burnt, including *Eremophila* spp., *Cymbopogon* spp., and *Santalum lanceolatum* ('northern sandalwood'), along with the resinous 'Spinifex' grass, *Triodia pungens*. Pearn (2005), in contrast, has noted that certain species used in the central parts of the continent for this purpose, (e.g. *Exocarpus latifolius*, *Acacia aneura*), are not known to contain significant quantities of volatile oils and instead are characterised by the presence of triterpenes, saponins and tannins.

Low consensus in the category of plants used to modify lactation may be explained in part by the practice commonly mentioned, which involves topical application the breasts, of white latex (of milk-like appearance) from a range of diverse, and in some cases taxonomically unrelated genera such as *Euphorbia*, *Sarcostemma* and *Alstonia*. It is likely that these

practices represent a doctrine of signatures, or 'like cures like' type approach to the selection of medicinal plants (*cf*. Farnsworth, 1994). Very few species used as abortifacients or contraceptives were mentioned, and are likely to be underrepresented in the recent ethnobotanical literature. These uses may fall into a category of plant-use knowledge which is restricted to individuals (in some cases of specific gender) who have passed certain degrees of initiation and as a result, are not usually recorded or passed into the public domain.

The miscellaneous category contained species with ill-defined uses (e.g. treatments for general illness and malaise). A subcategory within the 'miscellaneous' category, included references used for treatment of what were termed 'contemporary' diseases (i.e. those not typically associated with traditional ethnomedical disease terms, such as cancer, cardiovascular disease and type II diabetes). This use category exhibited nil consensus, with a separate species cited in each instance and low number of species/citations overall (11 species, 11 use citations). Such a low degree of consensus in this category might be expected given that such uses probably represent relatively recent developments in plant use and involve diseases which would not be likely be prevalent amongst, or recognised by pre-literate hunter gather cultures (*cf.* Cox, 1994). Relatively low CAR values were similarly reported by Moerman (2007) for the use category of "cancer treatment" (CAR: 0.07) in the NAED. Table 1.6 lists the maximum number of independent citations for a single species which appeared in each use category along with some of the more frequently cited species. Many of these species can also be seen to be cited across a number of use categories (e.g. *I. pes-caprae*, *G. retusifolia* and *T. smilacina*). One possible explanation for this tendency, if some degree of biomedical efficacy in traditional use of these plants is assumed, would be the occurrence of fairly non-specific activities with the potential to treat, or relieve symptoms in a wide range of diseases and conditions (e.g. antimicrobial, anti-inflammatory and/or analgesic activity). It may also be noted that several of the species indicated in Table 1.6

which have been frequently cited for uses which may be considered indicative of anti-inflammatory activity (e.g. pain and inflammation, bites and stings, fever and dermatological complaints), have also been shown to exhibit significant levels of *in-vitro* anti-inflammatory activities in studies where relatively large numbers of Australian ethnomedical plant species have been screened, e.g. *C. ambiguus*, *E. drummondii* and *I. pes-caprae* (Rogers *et al.*, 2000; Rogers *et al.*, 2001), *M. citrifolia* and *T. smilacina* (Li *et al.*, 2003; Li *et al.*, 2004) (*cf.* section 1.2).

1.3.4 **Plant species used to treat dermatological conditions**

Of the 394 species in the dataset, 211 (over 50%) were recorded with citations for treatment of dermatological conditions in at least one instance. Numbers of species, use reports and CAR values for subcategories within the dermatological use category, are presented below in Table 1.7 and represented graphically in Figure 1.4. The largest of the dermatological subcategories was that including reference to treatment of 'sores' (dermal lesions), encompassing 135 species and 246 use reports cited in the records examined. This use subcategory represented around two thirds (67%) of all 211 species used for dermatological complaints and around a third (34%) of all 394 species in the dataset and was almost equal in number of representative species to the second largest of the 16 use categories defined (respiratory illness category consisting of 133 species or 33% of total). The sores/dermal lesions subcategory may in fact be considered larger if the subcategory of 'infected sores and wounds' is included (this was recorded as a separate sub-category to preserve information in instances where evidence of infection was explicit in the original description of use). The 'infected sores and wounds' subcategory included reference to a further 9 species and 13 use reports not shared across the 'sores' subcategory.

Table 1.6 Table 1.7 Cultural agreement ratio (CAR) values and frequently cited species for subcategories of the dermatological uses and complaints category

Dermatological Use subcategory	Number of use reports	Number of species	Cultural agreement ratio (CAR)	Maximum number of citations	Most frequently cited species in use category (number of independent citations in parentheses)
'Sores' (dermal lesions)	246	135	0.45	9	*Protasparagus racemosus* (9), *Erythrophleum chlorostachys* (7), *Planchonia careya* (6), *Crinum angustifolium* (6), *Eucalyptus tetrodonta* (6), *Buchanania obovata* (5)
Wounds	112	66	0.41	6	*Protasparagus racemosus* (6), *Terminalia carpentariae* (5), *Crinum angustifolium* (5), *Erythrophleum chlorostachys* (4), *Ipomoea pes-caprae* (4)
Scabies lesions/infestation	80	51	0.37	5	*Strychnos lucida* (5), *Buchanania obovata* (4), *Grewia retusifolia* (4) *Ipomoea pes-caprae* (4)
Dermal irritation (Rashes, Eczema, pruritus etc.)	72	49	0.32	5	*Terminalia carpentariae* (5), *Acacia holosericea* (4), *Alphitonia excelsa* (4), *Pterocaulon serrulatum* (4)
"Infected" sores and wounds	69	40	0.43	6	*Protasparagus racemosus* (6), *Terminalia carpentariae* (5), *Erythrophleum chlorostachys* (4), *Grewia retusifolia* (4)
Boils	52	30	0.43	7	*Grewia retusifolia* (7), *Ipomoea pes-caprae* (5), *Tinospora smilacina* (5), *Hibiscus tiliaceus* (3)
Dermal unspecified	**42**	**39**	**0.07†**	2	*Cymbopogon procerus* (2), *Eucalyptus leucophloia* (2), *Sarcostemma australe* (2)
Fungal dermatophytes	34	20	0.42	4	*Alphitonia excelsa* (4), *Camptostemon schultzii* (3), *Crinum angustifolium* (3), *Pterocaulon serrulatum* (3), *Senna alata* (3)
Soap plants	**26**	**10**	**0.64***	6	*Acacia holosericea* (6), *Acacia auriculiformis* (5), *Alphitonia excelsa* (5), *Acacia pellita* (3)
Leprosy sores	25	15	0.42	5	*Crinum angustifolium* (5), *Terminalia carpentariae* (5)
Dermal viral lesions	23	14	0.41	6	*Protasparagus racemosus* (6), *Buchanania obovata* (2), *Excoecaria parvifolia* (2), *Planchonia careya* (2)
Burns	18	13	0.29	3	*Acacia estrophiolata* (3), *Grevillea striata* (2), *Hakea divaricata* (2)
Dermal 'other'	16	12	0.27	3	*Ficus opposita* (3), *Ficus scobina* (2), *Euphorbia australis* (2)
Removal of warts and tumours	10	6	0.44	3	*Acacia tetragonophylla* (3), *Carissa lanceolata* (2), *Euphorbia hirta* (2)

*(bold type): CAR greater than mean plus one unit of standard deviation (0.51); †(bold type): CAR less than mean minus one unit of standard deviation (0.26)

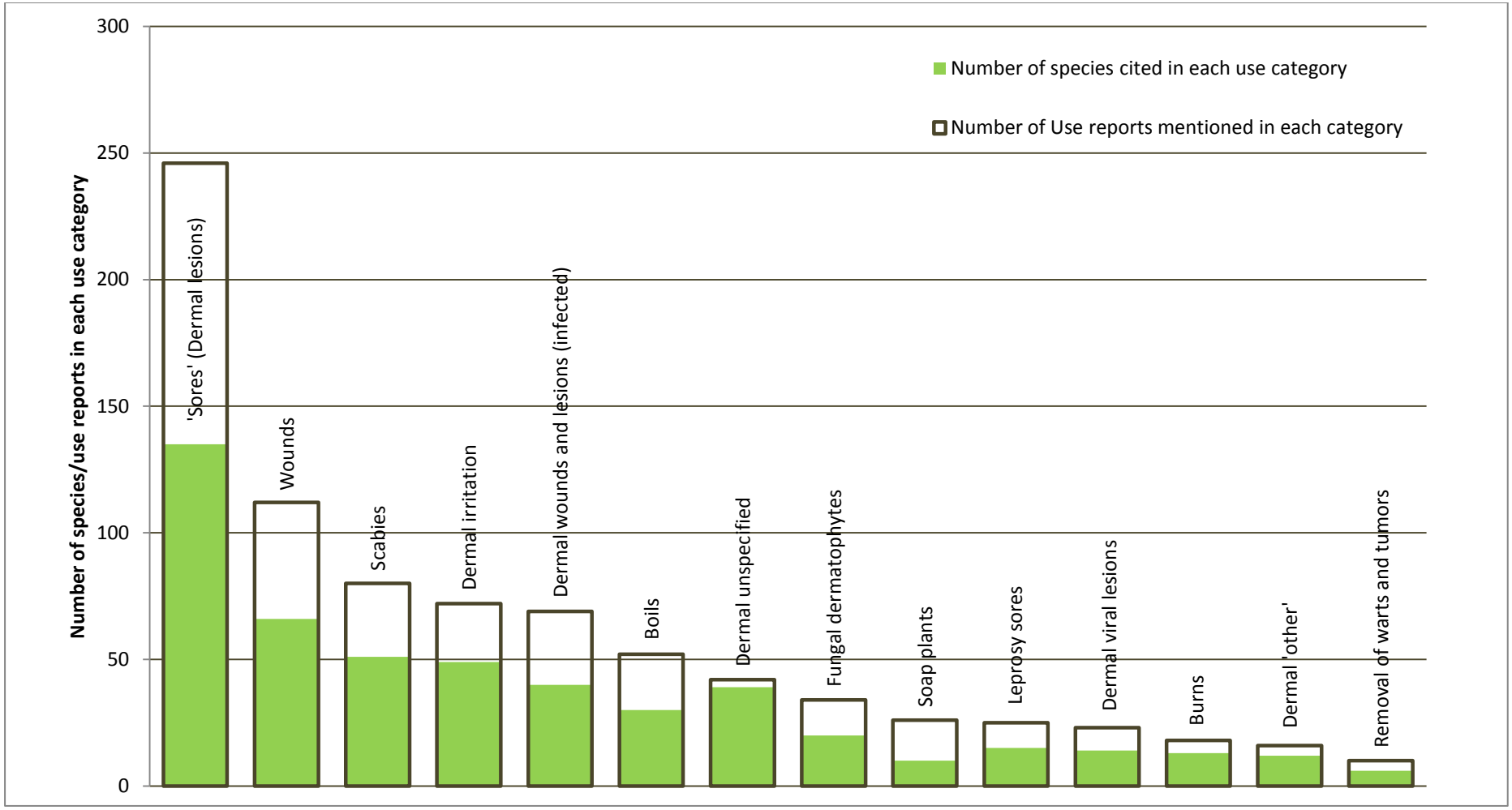

Figure 1.4 Number of species and use reports describing dermatological sub-categories in 29 Australian ethnobotanical reports

The next largest subcategory involved species used for treatment of wounds (66 species with 112 use reports), followed by scabies lesions/infestations (51 species with 80 use reports) and dermal irritation (49 species and 72 use reports including reference to those used to treat conditions such as eczema, pruritus and undefined rashes).

Consensus factors determined for each subcategory in this subset of the data were fairly consistent with the group overall except for the 'unspecified' subcategory (CAR 0.07), representing ambiguous or ill-defined use citations (e.g. species cited as being used for "general dermal complaints") and the category of soap plants (CAR 0.64). The high level of consensus amongst the plants used as detergents or soap may be explained by the high accumulation of saponins, characteristic of most of the species represented (i.e. *Alphitonia excelsior* and several species of *Acacia*), which may be expected to favour selection specifically for this purpose across cultures.

The relatively high representation of species cited for treatment of sores, may in part be explained by the high prevalence of pyoderma (i.e. skin sores or impetigo) amongst remote Australian indigenous communities. Pyoderma is currently endemic in remote indigenous communities, with prevalence in children of up to 70%, and there is evidence to suggest that scabies infestation and dermatophytic infections underlie the majority of cases (Currie and Carapetis, 2000). Scabies is caused by infestation with the parasitic scabies mite (*Sarcoptes scabiei*) which burrows into the stratum corneum of the skin, causing intense nocturnal itching and eczema, and is typically accompanied by the appearance of inflammatory papules and/or non-specific secondary lesions at the site of infestation (Chosidow, 2000). Scabies is highly prevalent amongst remote/rural Australian indigenous populations, largely due to a combination of prevailing socioeconomic inequities and lack of appropriate public health measures (Currie and Carapetis, 2000). Scabies, along with a number of other cutaneous

infectious diseases for which treatment is represented in the ethnobotanic literature (such as fungal dermatophytic infection and leprosy), were unlikely to have been present or sustainable in the context of the hunter gatherer economy and attendant lifestyle existing in Australia prior to European colonisation (Basedow, 1932; Currie and Carapetis, 2000).

If the high prevalence of pyoderma/impetigo amongst indigenous communities and the relatively high representation of medicinal species used to treat dermal sores in the current ethnobotanic record is not coincidental, then the question of whether these practices reflect 'traditional use' may be raised, given that prior to European colonisation and modernisation of indigenous society, rates of pyoderma/impetigo may have been dramatically lower. As pointed out above, the Australian ethnobotanical record represents a smaller proportion of available taxa than that which has been recorded in many other parts of the world, and may be significantly underrepresentative of the actual extent of indigenous medicinal plant use of the recent past and prior to European colonisation, notwithstanding changes which have occurred in the floristic landscape with the advent of pastoralism. Another potential explanation for the high representation of species used to treat dermal sores in the record is that given the currently high prevalence of pyoderma/impetigo, use of these species in particular has been maintained despite the relative paucity of the ethnobotanic record and any ecological or cultural processes which may have led to the loss of knowledge of traditional plant use. If this is the case then it is possible that the ethnobotanic records examined may present a more accurate or complete record of traditional use for plants used in the treatment of sores and other dermatological conditions as compared to other categories and modes of use. It is also possible that the use of some species for the treatment of dermatological conditions has been adopted relatively recently. In particular, it is very likely that this is the case with plants used for the treatment of scabies, fungal dermatophytic infections and other dermatological complaints which can be considered to be of recent emergence in indigenous

populations and this is reflected in the overall pattern of use for species used for this purpose. Very few of the species and preparations cited for dermatological complaints are used exclusively for any of the conditions or indications cited. This is particularly the case among the 51 species cited for the treatment of scabies infestation, where only 4 are described as being used exclusively for this purpose [i.e. *Ceriops tagal* (Puruntatameri *et al.*, 2001) *Flindersia maculosa* (Liu, 2006a), *Eremophila paisleyi*, and *Sida fibulifera* (Latz, 1995)]. Of the remaining 48 species (cited for a variety of other purposes), 43 are cited for use in the treatment of other dermatological conditions. It is likely that the use of these species and their respective medicinal preparations in the treatment of scabies has been adapted from pre-existing uses involving the treatment of conditions with a similar presentation of symptoms (e.g. pruritic conditions and dermal lesions).

1.3.4.1 Residual regression analysis of additional use citations in plants used to treat dermatological complaints

Many of the species mentioned in the surveyed literature were cited for a variety of distinct medicinal uses. Among the 211 species cited for use in treatment of dermatological complaints, 136 were found with additional citations for indications and therapeutic uses outside of the 'dermatological' category. These additional citations included description of uses representing the entire range of categories defined in this analysis.

A further residual regression analysis was carried out to examine the relationship between the dermatological category and the 15 other shared use categories, and to determine whether any of the shared use categories were more or less likely to be represented among the species used for treatment of dermatological conditions. The number of representative species belonging to each of the 16 use categories defined in the entire dataset (besides the category of dermatological complaints) formed the independent variable. The dependent variable

consisted of the corresponding number of species in each category, which had also been cited for 'dermatological' uses. If the selection of plants for preparations used to treat dermatological conditions was more or less random and indiscriminate among the medicinal plants as a whole, then it may be predicted that the numbers of species representing each of the shared use categories would be proportional to those of the entire dataset. As with the regression analysis by family, the predicted number of species representing each shared use category within the dermatological group (UCDC) would be expected to be around half that of the same use category in the entire dataset of Australian medicinal species (UCAMS) and this was reflected in the resulting regression equation:

$$UCDC = 1.613 + (0.524 \times UCAMS)$$

Predicted and observed numbers of species representing each use category for the dermal group along with residuals and standard residuals are presented below in Table 1.8 and represented graphically in Figure 1.5.

Table 1.8 Regression analysis of secondary use categories within dermatological category.
Categories greater or less than one standard residual deviation given in bold type

	Number of species per use category				
Use category	**Entire dataset**	**Dermatological group**	**Predicted number**	**Residuals**	**Standard Residuals**
Pain and inflammation*	**108**	**67**	**58.2**	**8.8**	**1.7**
Ophthalmic complaints*	**36**	**28**	**20.5**	**7.5**	**1.4**
Miscellaneous	64	39	35.1	3.9	0.7
Bites and stings	15	12	9.5	2.5	0.5
Dental/buccal complaints	30	19	17.3	1.7	0.3
Tonics	28	17	16.3	0.7	0.1
Bone fractures and disorders	9	7	6.3	0.7	0.1
Reproductive complaints/uses	37	21	21.0	0.0	0.0
Fever	25	14	14.7	-0.7	-0.1
Haemostasis	6	4	4.8	-0.8	-0.1
Urinary tract complaints and venereal	10	6	6.8	-0.8	-0.2
Ear-nose-throat complaints	28	15	16.3	-1.3	-0.2
Respiratory complaints	133	68	71.3	-3.3	-0.6
Gastrointestinal Complaints†	**66**	**31**	**36.2**	**-5.2**	**-1.0**
Neurological complaints/uses†	**54**	**16**	**29.9**	**-13.9**	**-2.6**

*(Bold type): Greater than or equal to one standard residual; †(Bold type): Less than or equal to one standard residual

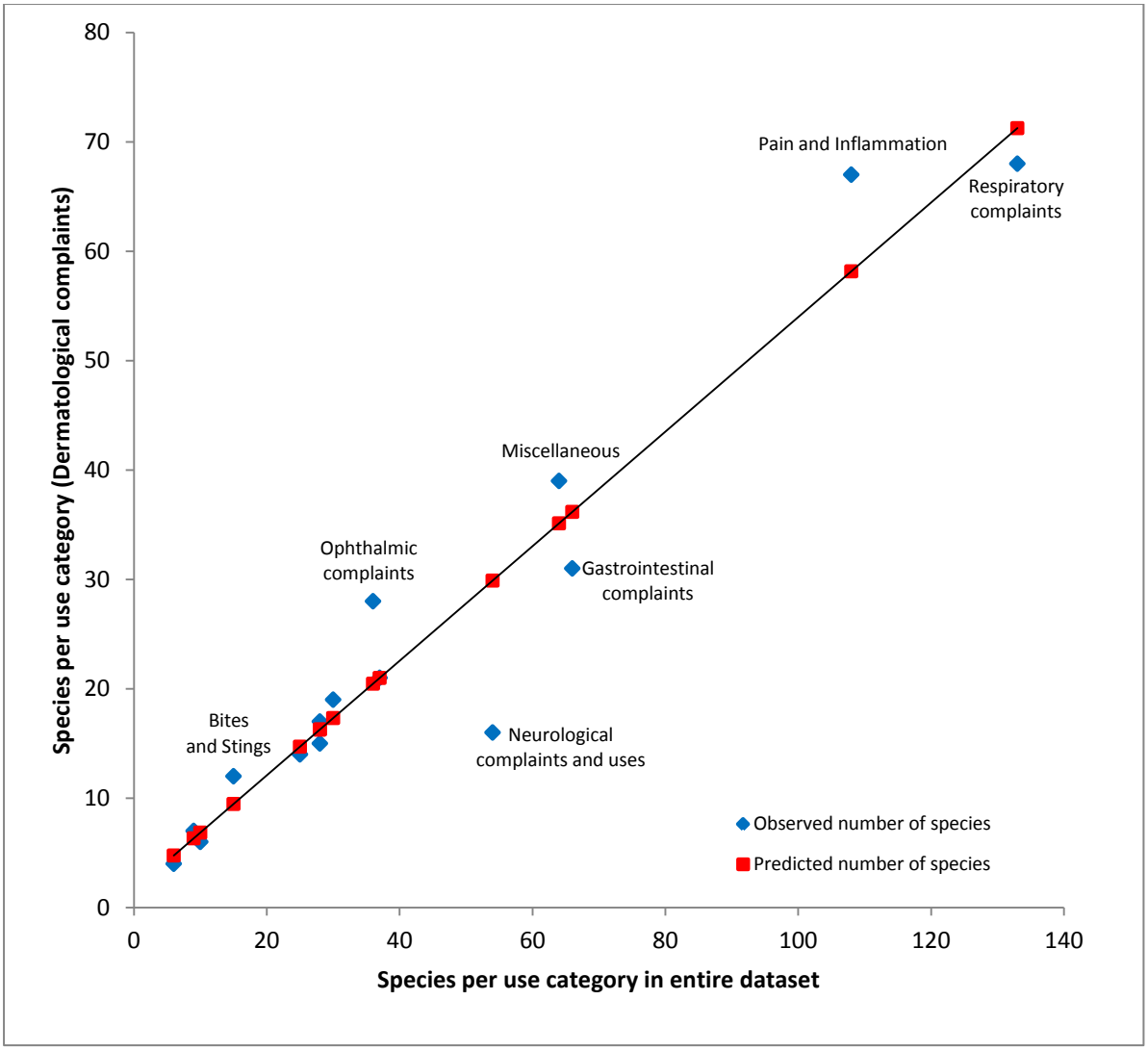

Figure 1.5 Regression analysis by use category of species used for dermatological complaints and medicinal plants of entire dataset

As can be seen in Figure 1.5**,** a slightly greater number of species than was predicted by the regression analysis were found representing the 'pain and inflammation' category (predicted: 58.2, observed: 67, residual = 8.8) and 'ophthalmic complaints' category (predicted: 20.5, observed: 28, residual = 7.5). The observed number of species from the neurological uses and complaints category was about half that predicted by the regression line (predicted: 29.9, observed: 16, residual = 13.9). Species cited for uses classed as 'neurological' included a large number of Solanaceae reported to be used for psychoactive effects (including tobacco and *pituri* substitutes) and this result is reflective of the apparent underuse of this family which was found in the regression residual analysis of the dermatological category by family (*cf.*

1.3.2.2 above). As was discussed earlier, many of the plants used for chewing quids or tobacco substitutes were used almost exclusively for this purpose. As with the apparent underuse of the Solanaceae among plants used for dermatological therapies, the disproportionately low representation of this use category is not therefore likely to represent a specific characteristic of the 'dermatological' medicinal plants.

The higher than predicted number of species with citations for use in pain and inflammation, although proportionally small, is not altogether surprising as perceived activities which may potentially be associated with this type of use or indication (e.g. analgesic or anti-inflammatory activity) would also be expected to be of benefit in the treatment of many of the indications represented in the dermatological category. The majority of species in the dermatological category which were also cited for the treatment of pain and inflammation included those used in preparations for the topical relief of skeleto-muscular pain and headache.

It is interesting to find a greater than predicted number of species concurrently cited for ophthalmic complaints amongst those used for dermatological complaints. Processes of epidermal wound healing which occur in the ocular surface in response to injury are similar to those which take place in the dermis. Also, some of the pharmacological activities which may be expected to treat or ameliorate symptoms of many of the dermatological conditions mentioned may also be similarly beneficial in the treatment of some eye disorders (e.g. antimicrobial and anti-inflammatory activity for infections and ocular irritation). This finding confirmed initial observations made on examining the medical ethnobotanical literature and informed the decision to include citations for ophthalmic uses (along with dermatological uses and indications), as additional criteria in the subsequent selection of plants for the study.

1.3.5 **Conclusions**

The analyses described above were based on data extracted from Australian ethnobotanical reports. As pointed out by the authors of several of these reports (e.g. Barr *et al.*, 1993) these studies are limited in their representation of traditional indigenous plant use and may not represent a complete account of regional indigenous ethnobotanical knowledge at any given time. Additionally, the literature selected did not represent a complete census of all published works of Australian medicinal ethnobotany. Lassack and McCarthy (1997) have produced a collection of monographs listing Australian medicinal species documented up to 1983 (date of first publication), which included 211 species used by indigenous Australians, 75 of which were not included in the records examined in this study. These species were mentioned in a number of older works which were not included in the literature surveyed. In the survey presented here, records mentioning a further 258 species were found (from ethnobotanical reports conducted since 1983) for a total of 394 species. Based on Lassack and McCarthy's (1997) work, an estimate of 469 Australian plant species for which ethnomedical use has been documented may be arrived at. In terms of the number of species covered, the survey presented here represents around 83% of those currently documented. For this reason it must be acknowledged that the analyses presented in this chapter, are limited by the scope of information used to construct the dataset.

Many of the findings arising from the analyses described above confirmed previous observations by ethnobotanists and other researchers, of the prevalence of myrtaceous species along with *Acacia* spp. and *Eremophila* spp. in the Australian medical ethnobotanic record (e.g. Low, 1990; Richmond and Ghisalberti, 1994; Latz, 1995; Lassack and McCarthy, 1997). Applying Moerman's technique of regression residual analysis revealed selectivity for these families above that which may be predicted by the available number of species representing each family. The differences seen between selectivity for certain families in the NT

ethnopharmacopoeias and those reported in other parts of the world were in many ways reflective of the character of the Australian flora. In particular for the high use selectivity seen in families such as the Myrtaceae, Fabaceae and Proteaceae, which in Australia, are relatively large and highly diversified, compared to other regions of the world. The commonalities in the selectivity of medicinal plants found between the Australian, and the neotropical and holarctic floristic kingdoms as reported by Moerman *et al* (1999), Leonti *et al.*, (2003) and Amiguet *et al.* (2006) (e.g. underuse of the Poaceae and Cyperaceae) is also interesting. Australia (as the Pleistocene continent Sahul), is generally thought to have been first colonised by modern humans at a point between 40,000 - 60,000 years before the present (bp), and prior to European settlement, indigenous culture has developed in relative geographical isolation (Mulvaney and Kamminga, 1999). Very recent evidence from genomic analyses has emerged which suggests that Australia may have been first colonised by modern humans via an early dispersal out of Africa ca. 62000 - 75000 years bp, prior to that occurring 25000 - 38000 bp and giving rise to present day Asian and European populations, suggesting that indigenous Australians may represent the oldest continuous population outside of sub-Saharan Africa (Rasmussen *et al.*, 2011). Similarities between the relative selectivity of specific taxa in the ethnopharmacopoeias of such distantly separate cultures (i.e. indigenous Australians and North/Mesoamericans), lends further support the observation of Leonti *et al.*, (2003) that selection of plants for medicinal uses may be governed by a number of global "common selection criteria" along with the tantalising possibility that some elements of knowledge relating to the selection of medicinal species may have arrived with early colonising populations (as was proposed by Moerman *et al.* (1999) in explaining similarities in the taxonomic selectivity of medicinal plants between North America and other cultures inhabiting regions within the holarctic floristic zone across the Bering Strait).

If the majority of plant species used for dermatological conditions had been selected very recently and more or less randomly or 'experimentally' from medicinal plants already in use for other purposes, it would be unlikely that any significant difference would have been found in the proportion of species representative of each family compared to that of the medicinal flora as a whole. The disproportional use which was found among the dermatological group for certain families, and in some instances, certain genera (i.e. *Eucalyptus*), would imply that plants used for this purpose are subject to selectivity, distinguishing this group from the medicinal flora as a whole. Selectivity may also be inferred from the relatively high CAR values found for this group, indicating agreement among separate indigenous groups in use of certain taxa for treatment of dermatological complaints and conditions. Regression residual analysis of shared use categories for taxa within the 'dermatological category' revealed that some use categories were shared disproportionately. More medicinal species cited for treatment of dermatological conditions, were also cited for treatment of pain and inflammation and ophthalmic complaints, than were predicted by the regression equation. On the one hand this may be interpreted as an indication that the symptoms and diseases exhibiting positive residuals may share commonalities in pathophysiology and potential therapeutic targets. Equally, this may also indicate differences in indigenous concepts of disease and treatment of symptoms to that represented by the system of classification used in this study for use categories. As pointed out by Heinrich *et al.* (2009) the classification of indigenous categories of disease and illness for the purpose of quantitative analyses is always prone to some degree of subjectivity. The larger than predicted number of species in the dermatological category that were also cited for treatment of pain and inflammation, could indicate that many of these species have been selected by indigenous people to treat dermatological conditions principally on the basis of perceived analgesia or anti-inflammatory action. This may also be the case for species which shared

citations for the treatment of 'bites and stings' (for which a positive residual was found). Two species (*Avicennia marina* and *I. pes-caprae*) cited in the literature for both the treatment of dermatological lesions and infections, and marine stings, are restricted to coastal and estuarine habitats. The close proximity of these two species to the ocean may partly explain selection for the treatment of stonefish and jellyfish stings.

In addition to providing a list of candidate species for collection and extraction, the detailed survey and analysis of data extracted from the ethnobotanic literature also allowed identification of specific taxa with a high level of agreement and selectivity for dermatological uses. This information was used to guide subsequent collection of species for the study. As discussed above, derivation of agreement indices for specific use of certain taxa such as MUA using published ethnobotanical records is not ideal and the accuracy of such analyses in representing actual agreement in use of plants among the cultures involved would be limited by the scope and range of the existing literature which was consulted. Any analysis using indices derived in such a way may not be expected to be as robust as those where agreement indices have been derived from well designed ethnobotanic surveys, conducted within a single well defined cultural and/or biogeographical region and involving large numbers of informants. On the other hand, few such studies have been conducted or published in Australia and the majority of available literature describing traditional indigenous medicinal plant use does not provide data which would permit analyses of agreement between information given by individual informants. The sources from which citations used to derive the MUA_{TMP} values used in this study, represent the bulk of the published Australian medical ethnobotanical record and have commonly been used for the selection of candidate species in most of the published studies investigating biological activity in Australian medicinal plant extracts referred to in section 1.2 above. Despite the potential problems with applying the technique of MUA to data derived from ethnobotanical

records, the analyses were carried out in an attempt to determine whether such values could be related to any subsequently observed bioactivity relevant to the medicinal uses cited.

1.4 **Dermal lesions and wound healing**

The majority of plant species which were subsequently collected for inclusion in the study have been cited in the ethnobotanical literature for traditional use in the therapeutic treatment of wounds and/or sores (*cf*. Table 3.2 p.114). The biological assays employed in the study were selected to cover pharmacological activities which may lead to resolution, improvement and/or symptomatic relief in these conditions. *In vitro* activities examined included anti-microbial activities, antioxidant capacity and anti-inflammatory activity. For the remainder of this chapter, the physiology/pathophysiology of wound healing will be briefly discussed along with that of some dermatoses to provide rationale for the selection of the biological assays employed, along with review of some previous studies examining relevant *in vitro* biological activities of traditional medicinal plants used for treatment of wounds.

1.4.1 **Dermal lesions**

As discussed above, the indication for treatment of dermal lesions or 'sores' with medicinal plant preparations was one of the most frequent given in the Australian ethnobotanical literature and a relatively large number of species are reportedly used for this purpose (*cf*. 1.3.4). In the majority of cases, these citations refer to dermal lesions in a general sense as 'sores'. As with many of the terms used in the ethnobotanic literature to describe medical indications and symptoms, it is difficult to make a clear distinction as to what this particular term represents in the sense of a defined biomedical disease state or event and many separate dermatoses can be accompanied by lesions exhibiting similar presentation. Topical corticosteroids are commonly indicated in the treatment of a number of dermatoses owing to their anti-inflammatory and immunosuppressive effects (Marley, 2004). Anti-inflammatory

action, local immunosuppression and analgesia may be expected to provide relief from the symptoms and discomfort associated with many dermatoses and these activities may potentially be included among those which may be present in medicinal plant preparations used in the treatment of sores. Bacterial infection can also underlie, or occur with secondary involvement in many dermatoses, and under these conditions, antimicrobial activity may be of potential benefit to resolution of the condition and amelioration of symptoms. Other actions which may be considered of potential benefit in the treatment of sores and dermatoses include antipruitic activity and astringency (for treatment of 'weeping sores' and exuding dermatoses) and for certain specific lesions: antiviral, antifungal and antiparasitic activities.

1.4.2 **Physiological and biochemical events in wound healing**

Dermal wound healing in response to injury involves the sequential actions of a coordinated and interacting set of biochemical and cellular events which culminate in tissue repair and regeneration of the superficial dermal structures. Three to four overlapping stages in the wound healing process are recognised and these include: 1. Haemostasis, 2. The inflammatory phase (when considered as a distinct second phase, separate from initial wound haemostasis), 3. The proliferative phase and 4. Repair or remodelling phase. A short description of some of the events defining each phase (partly adapted from Mauro, 2005) is presented below. The inflammatory phase occurs immediately after initial injury and haemostasis and typically lasts for 3-4 days in a healthy wound. Injury to the blood vessels of the skin initiates the haemostatic cascade resulting in formation of the fibrin clot. The haemostatic response is accompanied by release of inflammatory factors such as histamine and a number o prostaglandins, from platelets participating in clot formation, damaged tissue and activated mast cells, resulting in local vasodilatation and an increase in capillary porosity seen as local redness and oedema. These vascular changes facilitate subsequent entry of leukocytes to the wound site, such as neutrophils, which are attracted by release of

fibronectin and chemotactic growth factors secreted by platelets within the clot. Neutrophils phagocytise bacteria and debris in the wound and release reactive free radicals and proteinases. After 1-2 days (providing infection of the wound has not occurred), the neutrophil population is largely replaced by activated macrophages. Macrophages continue to secrete proteinases and debride damaged, necrotic tissue and extracellular matrix. Macrophages are involved in initiating the subsequent proliferative phase and low partial oxygen pressure in the wound site stimulates macrophages to release cytokines and growth factors (e.g. epidermal growth factor and fibroblast growth factor) promoting angiogenesis and migration and proliferation of fibroblasts. The proliferative phase occurs toward the end of the inflammatory phase, some 4-5 days following initial injury, and may continue for up to four weeks depending on the nature of the wound. The proliferative phase is characterised by proliferation of endothelial cells to form blood vessels and fibroblasts which form a temporary extracellular matrix (ECM) of type III collagen and other fibres, increasing tensile strength and forming the basis for granulation tissue, through which keratinocytes and basal epithelial cells subsequently migrate and proliferate to 'close' the wound. Damaged components of the temporary ECM are partly degraded with metalloproteinases released from migrating keratinocytes during this process. Migration of keratinocytes and epithelial cells progresses from the edges of the wound until each 'sheet' of cell meets. The third and final stage of remodelling is variable according to the nature, severity and location of the wound and may continue from a period of around 3 weeks to over 1 year. Remodelling involves replacement of the type III collagen fibres with type I collagen which aligns along tension lines, further increasing tensile strength across the wound.

Wound healing should occur successfully without intervention when the processes involved are uninterrupted or unimpeded. Barriers to successful resolution of wounds often involve a prolongation of the inflammatory phase, where the shift to proliferative phase (largely

mediated by activated macrophages) is delayed or prevented and inflammation becomes excessive. Some factors which interfere with normal wound healing and prolong inflammation include: Topical infection by pathogenic microorganisms, presence of debris in the wound and drying of the wound bed. Where the inflammatory phase of wound healing is prolonged or chronic, excess production of reactive oxygen species (ROS) and oxidants by neutrophils may occur and impair normal healing and protection of adjacent healthy tissue through a combination of an increase in the activity of oxidant activated metalloproteases and oxidative damage to antiproteases (Mensah *et al.*, 2001). Infection of wounds by bacteria is known to significantly impair wound healing and infection can progress beyond the immediate area of the wound, causing cellulitis and eventually systemic sepsis (AWMA, 2011). Bacteria in a wound environment may produce toxins and destructive enzymes in addition to disturbing the balance of endogenous proteases and growth factors, leading to excess of exudate in the wound and promotion of a chronic inflammatory state (AWMA, 2011). Topical antimicrobial agents for the treatment of wounds can be of limited use in treating established infections but may be useful as prophylaxis, by cleansing the wound and reducing initial contamination and colonisation of the wound (Lehne *et al.*, 1998). Drying of superficial regions of the wound bed prevents cell migration, retarding processes such as angiogenesis, and infiltration of phagocytic leukocytes, resulting in a delay in healing and an increased chance for microbial infection. Excessive pain or pain stimuli at the wound site can also impair healing (AWMA, 2011) and prolong the inflammatory phase.

1.4.3 *In vitro* tests for wound healing potential of medicinal plant extracts

The physiological and biochemical complexity of the wound healing process presents a diverse range of potential pharmacological targets which could conceivably enable amelioration of the discomfort and/or prevent barriers to timely and successful resolution. Given this diverse range of potential targets, it is not unreasonable to expect that any

traditional plant based remedy used for the purpose of treating wounds might exhibit activity against any, if not several, such targets simultaneously. Over the last decade or so, studies focusing on biological activity in traditional plants used for wound healing have utilised a variety of *in vitro* assays for activities relevant to the wound healing process in preference to *in vivo* animal models. One reason for this development is the relative unsuitability of *in vivo* models for any subsequent process of bioactivity guided fractionation for isolation of active compounds from plant extracts (Houghton *et al.*, 2005). *In vitro* activities which have been most commonly examined in studies examing medicinal plants used specifically for treatment of wounds have included antimicrobial activity, antioxidant activity, effects on fibroblast proliferation and migration, and various anti-inflammatory activities. Houghton *et al*. (2005), citing the multiplicity of potential pharmacological targets which any traditional wound healing remedy may be expected to affect, have recommended the application of a "battery" of distinct *in vitro* bioassays, suggesting that at least two relevant activities be present before any scientific acceptance of traditional use for wound healing. Techniques which have been employed to examine antimicrobial activity in plants used for wound healing include agar diffusion type assays (e.g. Chah *et al.*, 2006; Schmidt *et al.*, 2009), agar dilution assays (Marwah *et al.*, 2007), and more commonly, broth microdilution assays (Mensah *et al.*, 2001; Steenkamp *et al.*, 2004; Mensah *et al.*, 2006; Annan and Houghton, 2008; Adetutu *et al.*, 2010). Some *in vitro* tests to determine antioxidant activity of plants used as wound healing remedies have included direct chemical assays of free radical scavenging potential, such as the 1,1-diphenyl-2-picrylhydrazyl (DPPH) assay for hydrogen donating free radical scavengers (e.g. Annan and Houghton, 2008; Jorge *et al.*, 2008; Adetutu *et al.*, 2010), or protective effects on neutrophil or fibroblast cultures challenged with oxidant generators or H_2O_2 (e.g. Mensah *et al.*, 2001; Steenkamp *et al.*, 2004; Mensah *et al.*, 2006; Annan and

Houghton, 2008; Adetutu *et al.*, 2010). Mensah, Houghton *et al*. (2006) have additionally used techniques to examine attenuation of peroxidation of liposomes by plant extracts.

Several studies have examined the effects of plant extracts on stimulation of fibroblast growth *in vitro* utilising cultures of IBR-3 human dermal fibroblasts (Sampson *et al.*, 2000; Annan and Houghton, 2008), auricular dermal fibroblasts (Mensah *et al.*, 2001; Stevenson *et al.*, 2002), and MRC-5 lung fibroblasts (Steenkamp *et al.*, 2004). Effects of wound healing plant extracts on fibroblast migration have been examined using *in vitro* 'scratch assays' where a wound in a fibroblast monolayer culture is produced using a sterile syringe needle or pipette tip and examined microscopically after a period of incubation for evidence of migration of cells into the wound site (e.g. Fronza *et al.*, 2009; Schmidt *et al.*, 2009). Liao *et al*. (1999) have examined the inhibitory activity of extracts and isolated compounds from the Chilean wound healing plant, *Buddleja globosa*, on production of the inflammatory eicosanoids thromboxane B2 and leukotriene B4 in leukocytes (representing inhibition of the cyclooxegenases and 5-lipoxygenase respectively). More recent studies have utilised cell based *in vitro* techniques to detect effects of plants used traditionally in wound healing therapies, on production and/or activity of various inflammatory and apoptotic cytokines and markers. Mensah, Houghton *et al*. (2006) have examined the effects of extracts of plants used therapeutically by Ghana Ashanti for wound healing on activation of the pro-inflammatory mediator, NF-κB, in HeLa cells. Schmidt, Fronza *et al*. (2009) have examined a number of anti-inflammatory activities in several extracts of medicinal plants used in Brazil for treatment of wounds, including association of NF-κB with DNA, release of the pro-inflammatory mediator TNF-α in whole human blood stimulated with bacterial lipopolysaccharide (LPS), and inactivation of the protein kinase p38 MAPK, associated with inflammation, apoptosis and the cellular stress response. Schmidt, Fronza *et al*. (2009) also

examined effects of 'wound healing' plant extracts on release and activity of the serineprotease elastase and on activity of the apoptotic mediator, caspase-3.

1.4.4 **Bioassays employed in the study**

In this study antimicrobial activity, antioxidant capacity and anti-inflammatory activity of organic solvent extracts and aqueous traditional preparations of plants used to treat dermatological and ophthalmic complaints in Australian indigenous traditional medicine were examined. Antimicrobial activity was assessed using agar diffusion techniques for initial screening followed by broth microdilution assays for the determination of minimum inhibitory and minimum bactericidal concentrations (MIC/MBC).

Excess endogenous radicals generated during inflammation have the ability to retard normal process of wound healing and the ability of compounds within medicinal plant extracts to neutralise free radicals or protect cells from free radical mediated damage may contribute to wound healing potential. Antioxidant capacity of the extracts was examined using an oxygen radical absorbance capacity assay (ORAC) and an N,N-dimethyl-p-phenylenediamine (DMPD) radical cation decolourisation assay.

Extracts were screened for ability to inhibit formation of the inflammatory mediator, prostaglandin E_2 (PGE_2) in 3T3 murine fibroblasts stimulated with bacterial calcium ionophore. PGE_2 acts on a family of G-protein coupled eicosanoid receptors, mediating a number of paracrine and autocrine effects. PGE_2 positively mediates vasodilation, pyrogenesis and hyperalgesia. This last effect is particularly relevant to the context of wound healing as excessive pain is known to delay wound healing and tissue repair (AWMA, 2011). To establish appropriate assay concentrations for PGE_2 inhibition experiments, the extracts were assayed for toxicity against murine P-388 lymphocytes. In the next chapter, experimental methods and materials employed throughout the study are described.

Chapter 2: Materials and Methods

2.1 General methods and experimental procedures

2.1.1 Selection of plant species

Plant species collected in the study were selected based on citations in the Australian ethnobotanic literature describing traditional use by indigenous people for the treatment of dermatological and ophthalmic complaints. Criteria for collection/inclusion in the study included at least 1 citation describing use in treatment of dermatological or ophthalmic complaints in the literature initially reviewed (Webb, 1969; Levitt and Lyon, 1981; Barr *et al.*, 1993; Latz, 1995; Wynjorrotj *et al.*, 2005). From these sources a list of 158 candidate species was generated to guide collection. Selection of plants within the list was not entirely random however, as efforts were made to include some species for which a high degree of agreement among separate studies citing dermatological uses had been found (e.g. *Erythrophleum chlorostachys*, *Crinum angustifolium* and *Ipomoea pes-caprae*). Additionally, some preference was also given to the collection of medicinal species of the genus *Eremophila* (Myoporaceae/Scrophulariaceae), given the high degree of representation of this family in the ethnobotanic literature, both generally and specifically for various medical dermatological uses (*cf.* 1.3.2). While some preference was given to frequently cited species, a sufficient number of species with a low frequency of citation and/or agreement among separate ethnobotanical reports were also collected, to facilitate comparison of any subsequently observed bioactivity with major use agreement values among the collection as a whole (e.g. *Eremophila latrobei*, *Morinda citrifolia* and *Cymbopogon ambiguus*).

2.1.2 Collection of plant material

Plant material was collected from a number of localities across western New South Wales (NSW) and the Northern Territory (NT). Many of the species collected were reported to be used for a variety of therapeutic purposes, often employing several distinct plant parts and

tissues for each separate preparation. The specific plant parts collected were restricted to those described as required for medicinal preparations for the treatment of wounds and dermatological conditions. During field collections, plant material was labelled with metal tags inscribed with the unique collector's number and date of collection. In the field, plant material was stored in an insulated cooler and subsequently frozen at -20° C on the same day, remaining frozen until required for extraction. For each individual plant sampled, 2-3 voucher type specimens were collected and pressed where appropriate. Where possible, additional atypical voucher specimens of plant parts required for the extractions were also collected for lodgement (e.g. samples of inner bark material etc.). Collection data recorded followed the template for the NCW Beadle herbarium collections (University of New England, NSW) and included as a minimum: Date of collection, description of individual plant specimen, collection locality and geospatial coordinates as DMS latitude and longitude with elevation and estimated position error (EPE) in meters. For some specimens, additional data was recorded including community type and dominant species, along with photographic records of the plant *in situ*. Voucher specimens were lodged with the NCW Beadle herbarium (UNE, NSW) and field identities were confirmed with the assistance of herbarium staff. Collection localities and geospatial coordinates for each plant specimen collected are provided in Appendix II.

2.2 Extraction procedures

2.2.1 Sources and general methods followed in preparation of traditional type aqueous extracts

Reproduction of traditional type preparations for the experiments followed as closely as possible the descriptions given in the ethnobotanical literature. The main source of information referred to was the Northern Territory indigenous pharmacopoeia compiled by Barr *et al.* (1988; 1993). This work is the result of a number of separate ethnobotanical

studies, conducted by a relatively large multidisciplinary team over several years in close association with several indigenous communities. The long-term scope of this study, and the multidisciplinary approach involved, has enabled the documentation of detailed information on the traditional methods for preparation of plant-based therapeutic preparations (e.g. specific methods of processing of plant, quantities typically used, volumes of solvent, duration of extraction etc.). The text describing indigenous plant uses of Groote Eylandt in the Northern Territory (NT) (Levitt and Lyon, 1981) also contains relatively detailed descriptions of traditional methods for the production of plant based medicinal preparations and was used as a further source of information for the preparation of the extracts examined in this study. Other than these two examples, the majority of Australian ethnobotanical sources unfortunately give very limited information in this respect, which cannot be considered sufficient for suitably accurate reproduction of extracts and preparations (in many instances only providing description of the species, plant parts used and whether the extract is prepared by infusing or boiling). In instances where thorough descriptions of methods of traditional preparation were absent or ambiguous, estimations based on similar preparations were made and noted in the detailed descriptions of each preparation which are provided in Appendix II. For many of the species selected, information describing a number of preparations, each utilising various plant parts and methods of preparation was available. In these cases, only the preparations described specifically for treatment of wounds and other dermal conditions were produced. In instances where descriptions of traditional preparations derived from a particular species were not in agreement, either from independent studies, or by authors describing methods of separate indigenous groups, both or all preparations described were produced. As an example Barr *et al.* (1988; 1993) describe the use of two separate preparations derived from the 'ironwood' tree (*Erythrophleum chlorostachys*) for the treatment of dermal sores and wounds, one consisting of a decoction of leaves and a small

quantity of trunk inner bark and the other consisting of a decoction of trunk inner bark only. In this case both the leaves and inner bark of this species were collected, and both decoctions prepared. In some instances it was not practically feasible to include all relevant preparations as described in the literature among the set of extracts. In particular, preparations composed of solid materials were not included (e.g. pastes of macerated plant tissue or whole plant parts used for topical applications such as poultices or dressings). For the purposes of subsequent biological and chemical assays, preference was instead given to preparations which could be handled as liquids (i.e. typically extractions in water or liquid expressed from plant material). The quantities of plant material required for each preparation were often given in the literature with 'anthropometric' terms of reference (e.g. in number of handfuls of material or units of material sufficient to cover palm of an adult etc.). In these cases, an approximate mass of the quantities specified were estimated to the nearest 5 grams of material by weighing several samples (i.e. handfuls etc.) with a balance for the purpose of determining subsequent extract yields. In cases where quantity was given in units of length (e.g. for stems and branches) or area (e.g. for inner bark), the corresponding mass of material was also recorded. In each case the required quantities of plant material were adjusted as necessary according to the actual volume used for extraction in relation to the volumes cited in the literature. The method most commonly described in the literature for traditional extraction of plant material was by boiling with water (decoctions). The typical container used for this purpose is a small thin walled metal cooking pot with a bail handle for use over campfires, (colloquially referred to in Australia as the 'billycan'). Barr *et al.* (1988) report that the majority of contemporaneous indigenous groups in the NT, typically use a 1.25 kg (1800 mL) capacity can for this purpose (in which powdered milk is sold commercially) and describe traditional extractions as typically being prepared with one, or one half a billycan of water. For most extractions of this type, a 2000 mL capacity Pyrex beaker with similar dimensions

to the powdered milk cans was used. Laboratory magnetic-stirrer hotplates were used to heat the beakers where required. All aqueous extractions were carried out using deionised water. A few of the preparations required processing of the plant material which typically involved the crushing or 'pounding' of material. In some traditional settings this step may be accomplished using 'grinding stones' (stone mortar and pestle or muller/grindstone employed for processing of edible seeds and grains and other plant foods). Where required, plant material was pounded or crushed as directed in a vitreous mortar and pestle. Figure 2.1 below illustrates some stages of a typical simulated traditional preparation (Decoction of new growth terminal stem inner bark and sapwood of *Buchanania obovata* following a description given by Barr *et al.* (1988)). Preparations described in the literature could be classified into three main types which included decoctions, infusions and expressed juices. These terms were adopted for the purpose of discussion and labelling and are defined below:

2.2.1.1 **Decoctions**

In this study, preparations termed decoctions included those where plant material was extracted with boiling water over a specified period of time. Barr *et al.* (1988) report that the typical period of extraction in this manner is around 15 min and this was the case for all decoction type extracts prepared in this study. In one instance an exact duration of extraction was not specified (e.g. decoction of *E. latrobei* leaves as described by Latz (1995) and so an extraction time of 15 min was adopted in this instance.

2.2.1.2 **Infusions**

Preparations termed as infusions included those where plant material was extracted with immersion in water for a specified period of time. Infusions were further classified as either 'cold' (i.e. proceeding at room temperature without application of heat) or 'warm' (where water was warmed to ~60°C prior to addition of material).



1. Recent growth, terminal stems and leaves from juvenile specimen (≤1.5 m height) of *Buchanania obovata*



2. Removal of outer bark from stems



3. Appearance of green inner stem bark after removal of outer bark. (ca. 15 cm length as specified for traditional preparation)



4. Stems crushed by pounding with mortar and pestle.



5. Appearance of crushed terminal stems after pounding .



6. Decoction type extraction with deionised water using 2 L beaker and hotplate.

Figure 2.1 Photographs illustrating steps followed during the preparation of an aqueous 'traditional type' extraction of Buchanania obovata new growth terminal stem inner bark and sapwood (extract JES066TD).

2.2.1.3 **Expressed juices**

Expressed juices were prepared where this was the normal mode of application described in the literature. This type of preparation was typically indicated for topical application to the eye in the treatment of various ophthalmic complaints, usually involving use of watery or succulent tissues such as fruit. In two cases expressed juice was obtained from material normally used with direct topical application (expressed juice of *Cochlospermum fraseri* fruit pulp, and expressed juice of *Morinda citrifolia* fruit flesh, both of which are applied topically by squeezing and rubbing plant material and juice over the affected area (Barr *et al.*, 1988; 1993). In traditional medicinal use, expressed juices were typically described as being squeezed by hand from the plant material as required (Levitt and Lyon, 1981; Barr *et al.*, 1988). For the preparation of the expressed juices examined in this study, juice was extracted on a larger scale using a syringe as a press with extract yield determined as the percentage ratio of the mass of the resulting lyophilised extract, to the mass of fresh material extracted.

2.2.1.4 **Lyophilisation of extracts and determination of yield and concentration**

Following preparation, each extract was immediately filter sterilised into sterile containers under aseptic conditions for storage and lyophilisation. For lyophilisation, a portion of the extract (typically 200-400 mL) was transferred to pre-weighed, individually labelled flasks which were then frozen at -70° C before freeze drying. Once the extract was dried, the flasks were transferred to a sealed container containing a desiccant and mass in grams determined using a four decimal place balance. Dry extract mass was determined by subtracting the mass of the flasks from the combined mass of the flask and extract. Concentration and yields were determined using the volume lyophilised and the total volume of extract. Total final extract volume was determined to the nearest millilitre after extraction using a measuring cylinder (typically volume was less than original quantity of water used for extraction owing to

evaporation occurring over prolonged durations of extraction or when extracts were heated). Prior to estimations of final volume, extracts which had been heated were cooled in a water-bath set at 20°C.

Extracts were vacuum filtered, first using 15 cm diameter Whatman #1 filter paper followed by a 15 cm diameter 14 μm cellulose acetate membrane and finally, into sterile containers using 40 mm, 0.22 μm sterile cellulose acetate membrane. For some of the expressed juices, relatively small volumes were obtained and these were filtered directly into sterile containers under aseptic conditions through 0.22 μm cellulose acetate syringe mounted filters. The pH of a sample of each filtered extract was measured using a calibrated pH electrode. For all subsequent records and labelling of extracts, a shorthand alpha-numeric code was devised which included the unique six-character herbarium collectors number (identifying the particular plant specimen used), and letter codes representing the plant parts used and the method of preparation/extraction.

2.2.1.5 **Sequential solvent extractions of material from *Eremophila* species**

Material from each of the 5 species of *Eremophila* collected was sequentially extracted with hexane, followed by dichloromethane (DCM) and methanol (MeOH). These extracts were prepared for the purposes of comparing bioactivity with the corresponding traditional type aqueous preparations and in the case of *Eremophila duttonii,* for subsequent isolation of the antibacterial serrulatane diterpenes. Where possible, plant material was taken from the same specimens used to prepare the corresponding traditional type extracts. Species extracted included *E. alternifolia*, *E. duttonii*, *E. latrobei*, *E. longifolia* and *E. sturtii*. Plant materials (leaves) were air dried in a solvent extraction cabinet in darkness for three to four days, and extracted without further processing. All solvents used for extraction were of HPLC grade. Material was extracted twice in each solvent over a period of 24 hr using a 10:1 ratio of

solvent volume to g material. At each step the resulting extracts were decanted and solvent removed using a rotary evaporator. Solvent recovered from the first 24 hr extraction was reused for the second extraction and each extract was pooled. Hexane, DCM and MeOH extracts were screened for antimicrobial activity. The MeOH extracts were additionally assayed for prostaglandin E_2 (PGE_2) inhibitory activity and antioxidant capacity (ORAC assay) alongside each corresponding traditional aqueous preparations.

2.3 **Chromatographic vouchering of traditional aqueous preparations**

Traditional aqueous preparations and the 3'MeOH extracts were chromatographed using liquid chromatography - mass spectrometry (LC-MS) for the purpose of obtaining voucher chromatograms. The method employed in all cases was based on a standard protocol developed by staff of the Centre for Phytochemistry and Pharmacology, Southern Cross University, Lismore (CPP SCU) for preliminary chromatographic examination of medicinal plant extracts and natural product samples of unknown composition. The LC-MS system was an Agilent 1100 series with binary pump (G1312A), autosampler/injector (G1313A) and column thermostat/heating block (G1316A) controlled using HP Chemstation software (Rev. A, v09.03). Lyophilised extracts were dissolved in deionised water or MeOH at a concentration of 20 mg · mL^{-1}. Extracts were separated on a 5 μm C-18 column (150 × 4.6 mm I.D.) (Phenomenex luna), operating at 40°C. Injection volume was 10 μL. Flow rate was 1.0 mL· min^{-1}. Mobile phase consisted of: Solvent A: Water (H_2O) with 0.005% trifluoracetic acid (TFA); Solvent B: Acetonitrile (ACN) with 0.005% TFA. Elution gradient program was as follows, 0 to 30 min: 10%- 95% B, 30 - 35 min: Isocratic hold at 95% B; 35 - 40 min: 95% to 10% B; 40 - 45 min: Isocratic hold at 10% B (Table 2.1). Detection included UV/visible absorbance (Agilent photodiode array detector G1315B) and mass spectrometry (Agilent mass spectrometer detector SL1100). UV/Visible absorbance spectra were collected at λ190-

600 nm. Mass spectra were collected using atmospheric pressure chemical ionisation mode (APCI). Ionisation voltage (positive) was 150 and 70 eV. Mass scanning range was 100 - 1000 a.m.u.

Table 2.1 10-95% 30 min Acetonitrile-water gradient timetable used for voucher chromatograms of traditional aqueous preparations. (Solvent system: A: H_2O with 0.005% TFA; B: ACN with 0.005% TFA)

Time (min)	% A	% B	Flow rate (mL·min^{-1})
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

2.4 **Assessment of antimicrobial activity of extracts**

Antimicrobial activity in the traditional type extracts was assessed using a Kirby-Bauer type disc diffusion assay and broth microdilution methods for estimation of minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC). Methods employed were developed from similar techniques developed by the US Clinical Laboratory and Standards Institute (CLSI, formerly NCCLS) for clinical antimicrobial susceptibility testing (NCCLS, 1997; NCCLS, 1997b; NCCLS, 2004) with modifications specific to the testing of plant extracts (Eloff, 1998; Mann and Markham, 1998; Langfield *et al.*, 2004). The use of US standard clinical techniques developed for susceptibility tests for the screening of antimicrobial activity in natural products and plant extracts has become common practice (e.g. Steenkamp *et al.*, 2004; Ndi *et al.*, 2007a) and has been adopted largely for the purposes of standardisation and to enable comparison of results between separate studies.

2.4.1 General methods for assessment of antimicrobial activity

2.4.1.1 Microbial species employed

Standard reference strains for antibiotic susceptibility testing were employed in all assays. Organisms were obtained from the Australian Collection of Microorganisms (ACM, St Lucia, Queensland) and included the Gram-positive bacteria *Staphylococcus aureus* (ATCC 29213), *Staphylococcus epidermidis* (ATCC 12228) and *Bacillus subtilis* (ATCC 6633), and the Gram-negative bacteria *Escherichia coli* (ATCC 25922), *Salmonella typhimurium* (ACM 3598), *Klebsiella pneumoniae* (ATCC10031), *Pseudomonas aeruginosa* (ATCC 27703) along with the unicellular fungi *Candida albicans* (ATCC10231).

2.4.1.2 Media used for assessment of antimicrobial activity

Pre-formulated dehydrated bacteriological media and media components were obtained from Oxoid (Melbourne). Media were prepared as directed with deionised water and sterilised by autoclave (121°C for 20 min). Solid media for culture maintenance and disc diffusion assays were prepared as described and cooled to ca. 45°C prior to dispensing aseptically into 90 mm Petri dishes which were then allowed to solidify in a laminar flow cabinet. Unused media were stored at 4°C and used within 3-4 weeks of production. Each batch of media underwent periodic testing for contamination by incubating test samples at 37°C and 28°C for 48 hr and examining for any visible growth. Microbial growth media employed throughout the study are listed below with a brief description of formulation and specific uses:

Blood agar (BA)

Used as non-selective bacterial media for revival of lyophilised cultures and monitoring culture purity. Prepared with the addition of 950 mL deionised water to 40 gm dehydrated blood agar base (Oxoid CM55), with addition of 50 mL sterile defibrinated horse blood post autoclave sterilisation after cooling to ca. 40°C.

Nutrient broth/agar (NB/NA)

Used for maintenance of bacterial cultures. Nutrient broth (NB) was prepared with the addition of 1000 mL deionised water to 13 gm dehydrated nutrient broth media (Oxoid CM1). For the preparation of nutrient agar (NA), the same procedure was followed with the addition of 1.5% bacteriological agar (15 gm per 1000 mL media).

Yeast extract peptone (YEP)

Used for the maintenance of *C. albicans* cultures. Prepared with addition of 960 mL deionised water to 5 gm bacteriological peptone (Oxoid L37), 5 gm yeast extract (Oxoid L21), 3 gm Potassium dihydrogen orthophosphate (KH_2PO_4) and 3 gm ammonium sulphate ($(NH_4)_2SO_4$) with aseptic addition of 40 mL of a 50% $^{w}/_{v}$ sterile glucose solution post autoclave sterilisation and the addition of 1.5% bacteriological agar for preparation of YEP agar: (15 gm per 1000 mL media).

Peptone yeast extract pH 7.2 (PYE)

Used for maintenance of *P. aeruginosa* cultures. Prepared with addition of 1000 mL deionised water to 10 gm bacteriological peptone (Oxoid L37), 5 gm yeast extract (Oxoid L21), and 5 gm sodium chloride (NaCl). pH was adjusted to 7.2 by titration with addition of a concentrated sodium hydroxide solution before the addition of 15 gm bacteriological agar.

Plate count agar (PCA)

Used for validation of optical density measurements in inoculum solutions and to determine appropriate dilution factors to obtain desired inoculum densities. Also used for subculture of some broth tests for antibacterial activity, to detect bactericidal activity. Prepared with the addition of 1000 mL deionised water to 5 gm bacteriological peptone (Oxoid L37), 2.5 gm

yeast extract (Oxoid L21), 1 gm glucose and 15 gm bacteriological agar, with the addition of 1 gm skim milk powder post autoclave sterilisation.

Mueller-Hinton / Glucose methylene-blue agar (MHA/GMB)

Used for disc diffusion assays. Mueller-Hinton agar (MHA) was used for disc diffusion assays of the bacterial species and was prepared with addition of 1000 mL deionised water to 38 gm dehydrated media. Mueller-Hinton agar + 2% $^{w}/_{v}$ glucose and 0.5 μg/mL methylene blue (GMB) was used for disc diffusion assays with *C. albicans* and was prepared with the addition of 1 mL of a filter sterilised 0.5 mg/mL methylene blue solution and 50 mL of a 40% $^{w}/_{v}$ glucose solution per litre of autoclave sterilised MHA. Methylene blue was incorporated in the media as it has been found to permit easy visualisation of contrast between confluent areas of growth and zones of inhibition post incubation in tests involving *C. albicans* (NCCLS, 2004)].

Tryptone-soya broth/sloppy agar (TSB/TSSA)

Used as assay media for broth microdilution assays (estimation of MIC and MBC). Tryptone soya broth (TSB) was prepared with the addition of 1000 mL deionised water to 30 gm of dehydrated media. TSB was prepared at 10× normal strength for initial broth dilution screening experiments. Tryptone-soya sloppy agar (TSSA) was prepared in the same manner with the addition of 1.5 gm bacteriological agar per 1000 mL of media (0.15%). TSSA was additionally prepared at 2× normal strength for the preparation of aqueous extract stock solutions.

2.4.1.3 Maintenance of microbial cultures

Lyophilised cultures obtained from the supplier were revived in the appropriate growth media, and resulting cultures checked for purity and growth of expected organism using streak agar

cultures and microscopic examination of Gram stained smears. Stock cultures were prepared in TSB containing 20% $^{v}/_{v}$ glycerol and stored at -70°C until required. Working stock cultures were maintained on appropriate media and replaced at intervals not exceeding four weeks during the experiments. Working stock cultures were stored at 4°C prior to use and microscopic and colony growth morphology monitored periodically to detect any culture contamination. For each experiment/assay, the working stock cultures were passaged twice on the appropriate maintenance media for an incubation period of not more than 18 hr each pass.

Table 2.2 Maintenance media, assay media and growth conditions for microorganisms

Species **(Strain)**	**Maintenance media***	**Incubation temperature**	**Assay Media (Solid)***	**Assay Media (liquid)***
Staphylococcus aureus (ATCC 29213)	BA/NA/NB	35°C	MHA	TSB/TSSA
Staphylococcus epidermidis (ATCC 12228)	BA/NA/NB	35°C	MHA	TSB/TSSA
Bacillus subtilis (ATCC 6633)	BA/NA/NB	35°C	MHA	TSB/TSSA
Escherichia coli (ATCC 25922)	BA/NA/NB	35°C	MHA	TSB/TSSA
Salmonella typhimurium (ACM 3598)	BA/NA/NB	35°C	MHA	TSB/TSSA
Klebsiella pneumoniae (ATCC10031)	BA/NA/NB	35°C	MHA	TSB/TSSA
Pseudomonas aeruginosa (ATCC 27703)	BA/PYE	28°C	MHA	TSB/TSSA
Candida albicans (ATCC10231)	YEP	35°C	GMB	-

*: BA: Blood agar; NA: Nutrient agar; NB: Nutrient broth; PYE: Peptone yeast extract media (pH 7.2); YEP: Yeast extract peptone media with 2% glucose; MHA: Mueller-Hinton agar; GMB: Mueller-Hinton agar with 2% glucose and methylene blue; TSB: Tryptone soya broth; TSSA: Tryptone soya sloppy agar. All microbial species grown/maintained under aerobic conditions.

2.4.1.4 **Preparation of inoculum for antimicrobial assays**

Inoculum suspensions were prepared as follows: Five clearly separated colonies from a streak plate culture inoculated ≤18 hr prior to the experiments, were suspended in 5 mL of sterile 0.9% $^{w}/_{v}$ saline to produce a suspension with turbidity slightly greater than that of a $BaSO_4$

0.5 McFarland turbidity standard. The McFarland standard was prepared by adding 50 μL of a 1.175% $^{w}/_{v}$ $BaCl_2 \cdot 2H_2O$ solution to 9.95 mL of 1% $^{v}/_{v}$ H_2SO_4, to yield a $BaSO_4$ precipitate suspension with optical density of ca. 0.132 at λ600 nm. Spectrophotometric optical density of the suspension was read as absorbance (abs) in a 1 cm lightpath cuvette at λ600 nm for bacterial species and λ530 nm for suspensions of *C. albicans*. The suspension was adjusted to match optical density of the $BaSO_4$ 0.5 McFarland standard with addition of sterile 0.9% saline, resulting in suspension of approximately 1×10^8 for bacterial species and $1 - 5 \times 10^6$ cells per mL for *C. albicans*. For disc diffusion assays, the adjusted suspension was used without further dilution for swab inoculation of plates. For broth microdilution assays, the adjusted suspension was further diluted in 0.9% $^{w}/_{v}$ saline or media to achieve a final density in assay wells of ca. 5×10^5 CFU/mL.

2.4.2 **Disc diffusion assays for assessment of antimicrobial activity of extracts**

Disc diffusion assays for traditional type preparations were carried out against all bacterial species and *C. albicans* (ATCC 10231). Extracts was assayed against each organism and experiments were repeated at least three times on separate occasions. Methods followed were as described in NCCLS protocols ((NCCLS, 1997; NCCLS, 1997b; NCCLS, 2004). For the assays, 90 mm disposable Petri dish agar plates were used. MHA media was used for all assays against the bacterial species and GMB media was used for assays against *C. albicans*. Agar plates were placed in a laminar flow cabinet with lids slightly ajar for 30 min to remove excess moisture prior to application of the inoculum and discs. Inoculum was prepared as described above (section 2.4.1.4) and swab inoculated onto the agar surface. Antibiotic susceptibility testing discs were applied to the surface of the agar using a 6-place dispensing apparatus (Oxoid ST6090). Each agar plate contained 5 blank discs and 1 antibiotic reference control disc containing either 10 μg tetracycline (Oxoid CT0053) (for assays against bacterial

species) or 100 μg nystatin (Oxoid CT0073) (for antifungal assays against *C. albicans*). To the blank discs, 6 μL of each filter sterilised crude extract was applied aseptically using an automatic pipette. Agar plates were then left with lids ajar for 15 min to allow the loaded discs to dry before incubating plates in an inverted position for 18 – 24 hr at 35°C before recording results. Antimicrobial activity was seen as zones of inhibition (concentric areas of growth inhibition around the discs against a background of confluent growth). Results were recorded as the diameter of each zone of inhibition to the nearest mm, as measured with callipers.

2.4.3 Microdilution assays for assessment of antimicrobial activity of extracts

2.4.3.1 Initial screening of traditional type plant extracts with broth microdilution assay

Each extract was screened for antibacterial activity against the bacterial species (except for *S. typhimurium*) using a 96-well microplate broth dilution method prior to determination of MIC and MBC. Methods generally followed those outlined in NCCLS guidelines for antibiotic susceptibility testing (NCCLS, 1997b) with some modifications (outlined below). Each assay consisted of a 96-well microplate with wells containing filter sterilised extract added to 10× concentrated media, along with controls for positive growth, media sterility and wells containing antibiotics as reference drugs. Each prepared microplate was inoculated with a suspension of one of the test organisms and subsequently examined for inhibition of growth and integrity of controls after a period of incubation. All experiments were repeated on at least three separate occasions. In addition, an uninoculated microplate prepared with the same configuration of samples as the experimental plates was included in each experiment to monitor sterility of extracts and reagents, and to serve as a control to counter the possibility of false negative growth readings which may have arisen from reduction of the tetrazolium dye indicator (used to detect growth) by compounds present in the extracts.

2.4.3.1.1 Broth microdilution assay procedure

Tryptone soya broth (TSB) was chosen as media for broth dilution assays following methods outlined by Langfield and Scarano *et al.* (2004). TSB is a relatively nutrient rich media and was found in initial testing to support the growth of all organisms used. For each 96-well microplate, 10 μL of 10× TSB was added to wells for assay of extracts, followed by addition, with mixing, of 85 μL of each filter sterilised extract. Internal reference antibiotic controls included cefoxitin, cefazolin, and tetracycline, and were prepared with addition of 95 μL of a 512 $\mu g \cdot mL^{-1}$ aqueous solution to wells containing 95 μL of 2× TSB which were then used as the initial concentration for 12-step, two-fold, 95 μL serial dilutions to give concentrations in the range of 0.13 - 256 $\mu g \cdot mL^{-1}$. Control wells for the purpose of monitoring media sterility and negative or positive growth in the 10× strength media, were prepared with addition of 10 μL of 10× TSB to which 85 μL sterile deionised water was added with mixing. Control wells for 2× strength media were prepared by adding 45 μL of sterile deionised water was to wells containing 50 μL 2× TSB. Inoculum was prepared as described above and was further diluted 1:10 with 0.9% $^{w}/_{v}$ saline to give a suspension of 1×10^7 CFU/mL. To each well containing test extracts, reference antibiotic dilutions and positive growth controls (95 μL), 5 μL of inoculum was added to produce a final inoculum density of ca. 5×10^5 CFU/mL and final volume of 100 μL. To sterility/negative growth control wells (95 μL), 5 μL of sterile 0.9% $^{w}/_{v}$ saline was added (final volume 100 μL). After inoculation, plates were agitated using a plate shaker for 3 min to mix prior to incubation (35°C for 18 - 20 hr). To confirm inoculum density, 10 μL samples were taken from inoculated growth control wells and diluted in 10 mL of sterile 0.9% $^{w}/_{v}$ saline with vortex mixing (1:10,000 dilution). One hundred μL of the diluted sample was spread onto plate count agar (or PYE agar for *P. aeruginosa*) and incubated overnight. Resulting colony counts were used to determine

whether inoculum densities were within an acceptable range (i.e. 30-70 colonies on agar surface indicating density of between 3×10^5 and 7×10^5 CFU/mL). For the uninoculated control microplates used to monitor reagent/extract sterility, 5 μL of the sterile 0.9% $^{w}/_{v}$ saline solution was added to all wells in place of adjusted inoculum.

2.4.3.1.2 Incubation of broth microdilution plates and interpretation of results

Inoculated plates were incubated at 35˚ C for 12 - 18 hr. Following incubation, 50 μL from one the duplicate positive growth control wells was removed aseptically and spread inoculated onto appropriate solid media to confirm growth of each intended microorganism. Growth in test and antibiotic dilution wells (indicating lack of inhibitory activity) was assessed visually by the appearance of turbidity, and with a colorimetric indicator, utilising the metabolic dye p-iodonitrotetrazolium (p-INT) to indicate growth of viable bacteria. After visual examination for turbidity, 40 μL of a 0.2 mg · mL^{-1} p-INT solution was added to all wells, and the plate mixed by agitating on a plate shaker for 3 min. Plates were then incubated for a further 2 - 4 hr to enable reduction of the p-INT. Once the p-INT reaction was complete, plates were removed and results recorded. Inhibitory activity was recorded where no visible reduction of p-INT had taken place. MIC for antibiotic reference serial dilutions was determined as the lowest concentration of antibiotic or test substance producing no visible growth and/or reduction of p-INT ($MIC_{p\text{-}INT}$).

2.4.3.2 Screening of *Eremophila sp.* solvent extracts for antimicrobial activity

The eighteen sequential solvent extracts were screened for antibacterial activity against the panel of six bacteria, using a broth microdilution assay and against *C. albicans* (ATCC 10231), using a disc diffusion assay. General methods followed those used for traditional preparations as outlined above. Extracts were dissolved in DMSO at a concentration of 10 μg · mL^{-1}. For disc diffusion assays, 10 μL was applied to each disc (100 μg extract per disc).

Six discs were applied to each disc diffusion plate including 4 containing the extracts, 1 antibiotic reference control (nystatin -100 μg) and a solvent control (10 μL DMSO). For microplate broth dilution screening assays, 5 μL of each extract was added to duplicate wells containing 90 μL 1.1× TSSA media, prior to the addition of 5 μL inoculum (total well volume: 100 μL, Extract concentration: 500 $\mu g \cdot mL^{-1}$). Growth and sterility controls included wells to which 5 μL DMSO was added (DMSO concentration: 5% $^{v}/_{v}$). All tests were performed in triplicate. Interpretation of results followed methods outlined above.

2.4.3.3 Estimation of MIC and MBC in traditional preparations by broth microdilution assays

Extracts showing inhibitory activity in microdilution screening assays were selected for estimation of MIC and MBC by broth microdilution assays. Stock solutions of lyophilised extract were made to volume with deionised water in sterile volumetric glassware and sterilised by filtration (0.22 μ cellulose acetate). Each extract was assayed using a range of serial dilutions which encompassed the original concentration of the extract as prepared using traditional methods. Stock solutions were prepared at 2.5× the concentration required for the initial well used for serial dilutions (i.e. greater than original concentration as prepared), and in a few instances solubilisation in water at room temperature would not proceed. In these cases, solubilisation was achieved with gentle heating in a sonicating waterbath (<60 °C) and/or, in some instances, addition of DMSO up to a final concentration of 2.5% $^{v}/_{v}$ (to result in a final assay concentration of no more than 1.0% $^{v}/_{v}$ which in earlier testing, did appear to affect growth of the test organisms).

2.4.3.3.1 Estimation of MIC of traditional preparations by broth microdilution assays

MIC was determined using a 96-well format microtitre plate broth dilution assay. Methods followed were generally the same as those employed in the screening phase (2.4.3) with some

exceptions. Stock solution (80 μL) of each extract to be tested was added to wells containing 80 μL of 2× TSSA media and mixed. These wells were then used as the initial step for duplicate 6-step two-fold dilutions into wells containing 80 μL 1× TSSA. Positive growth and media sterility control wells for 2× TSSA consisted of 40 μL TSSA with addition of 40 μL sterile deionised water. Sterility and growth controls for 1× TSSA consisted of 80 μL of 1× TSSA. Solvent vehicle control wells were included to check for inhibitory action of DMSO. These consisted of duplicate growth and sterility control wells to which 80 μL of TSSA containing 1.0% $^{v}/_{v}$ DMSO was added (equivalent to highest concentration of DMSO in extract dilutions). Antibiotic internal reference standards consisted of a 12 step two-fold dilution in range of 0.13 μg/mL - 256 μg · mL^{-1} of cefazolin (except in instances where the test organism used was *P. aeruginosa*, in which case tetracycline was used instead). Once all reagents and test substances had been added, 20 μL of a suspension of the desired microorganism in TSSA was used to inoculate all wells except for sterility controls where 20 μL of sterile saline was added. Suspensions were prepared as described above (Section 2.4.1.4) except that the dilution of the adjusted suspension in saline with media was prepared for a 20 μL addition to result in a final volume of 100 μL (i.e. suspensions adjusted to match turbidity of the 0.5 McFarland standard were diluted in TSSA to give an approximate density of 2.5×10^6 CFU/mL to result in a final density in wells after addition of 20 μL inoculum to 80 μL well contents, of 5×10^5 CFU/mL). Inoculated plates were agitated on a plate shaker to mix and incubated for 12 – 16 hr at 35°C. For each experiment a duplicate plate was prepared without inoculation and incubated along with the test plates to monitor sterility of test substances. After incubation plates were removed and sampled for determination of MBC (see below) before adding 40 μL of 0.2% $^{w}/_{v}$ p-INT to visualise growth inhibition. Each experiment was performed at least three times on separate occasions. $MIC_{p\text{-}INT}$ is defined here

as the lowest concentration of antibiotic or test substance producing no visible growth (MIC) and/or reduction of p-INT ($MIC_{p\text{-}INT}$).

2.4.3.3.2 Estimation of minimum bactericidal concentration of traditional preparations

For estimation of minimum bactericidal concentration (MBC), a 96-well format microtitre plate assay based on methods described by Ndi and Semple *et al.* (2007a) was employed. MBC was determined for all concentrations of extracts exhibiting inhibitory activity. For each MIC assay plate, 190 μL of TSB was added to each well of a 96-well format microtitre plate. A sample of 10 μL was transferred using a multichannel pipette from corresponding wells of MIC microplates, after the incubation period and before addition of the p-INT indicator. Plates were then agitated on a plate shaker to mix and incubated for at least 18 hr before examining for growth. Growth or lack of growth in wells, (indicating bactericidal activity at the concentration of extract or test material in corresponding MIC assay well), was assessed as with previous microplate antimicrobial assays (i.e. with turbid appearance of well contents and reduction of p-INT to violet coloured dye).

2.5 Estimation of antioxidant capacity of extracts

2.5.1 Estimation of antioxidant capacity by DMPD decolourisation assay

The DMPD (N,N-dimethyl-p-phenylenediamine) decolourisation assay developed by Fogliano *et al.* (1999) for the rapid measurement of antioxidant capacity of wine samples, measures the extent of free radical scavenging activity by hydrogen donating antioxidants. The test is similar in principle to other electron transfer decolourising methods such as the ABTS (2,2'-azino-bis(3-ethylbenzthiazoline-6-sulphonic acid) and DPPH (2,2-diphenyl-1-picrylhydrazyl) assays, where the respective coloured radical ions are quenched by radical scavenging antioxidants. In an acidic environment, a stable DMPD radical cation ($DMPD^{\bullet+}$) can be obtained in the presence of an oxidant (e.g. Fe^{3+} ion). The $DMPD^{\bullet+}$ ion is coloured

purple in solution and exhibits a spectral absorption peak at λ505 nm. In the presence of a hydrogen donating antioxidant, the single electron is scavenged from the radical ion to form the colourless DMPD molecule, resulting in a proportional reduction of spectrophotometric absorbance at λ505 nm (Fogliano *et al.*, 1999) (Figure 2.2).

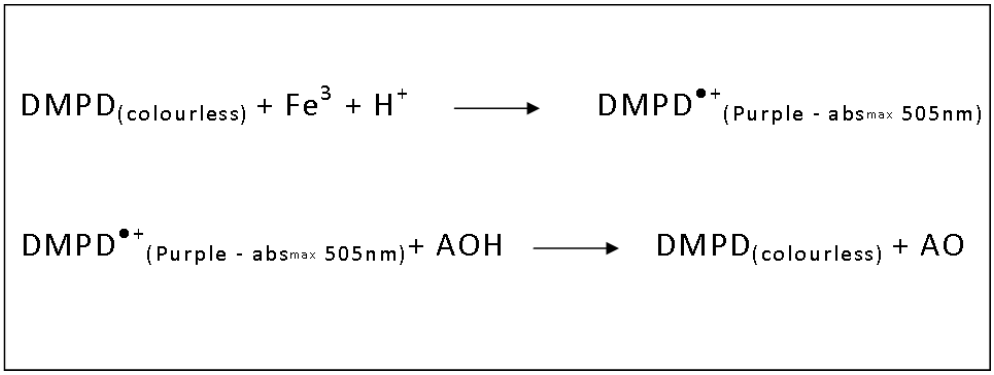

Figure 2.2 Scheme for formation of DMPD radical cation (after Fogliano *et al.*, 1999)

The degree of DMPD decolourisation produced by a sample is compared with that of a standard of the water soluble tocopherol analogue, Trolox (6-hydroxy-2,5,7,8-tetramethychroman-2-carboxylic acid), to derive the Trolox equivalent (TE) antioxidant capacity value. TE values of samples represent the concentration of a solution of Trolox which exerts the same antioxidant capacity as the sample and are typically expressed as equivalents in molar concentration (e.g. mM for liquid samples such as beverages or plasma) or equivalent molar quantities per unit weight of sample (e.g. mmol/g dry or fresh weight for solid samples and foodstuffs). The DMPD decolourisation assay yields TE values which are comparable to the similar ABTS decolourisation assay and offers the advantage that the radical cation is formed relatively quickly and remains stable for an extended period of time. Also, unlike the ABTS and DPPH assays, the end point of antioxidant action is rapid and stable, removing the need to read absorbance of samples and standards at a precise, standardised point in time. For hydrophobic antioxidants however, decreases in assay

sensitivity and reproducibility have been reported and so the use of the method is effectively restricted to the assay of hydrophillic antioxidants (Fogliano *et al.*, 1999).

For the determination of TE antioxidant capacity in the traditional aqueous preparations, methods as described by Fogliano *et al.* (1999) were followed. The extracts (as the portion retained at original concentration without lyophilisation) were initially assayed at dilutions of 1:10, 1:20 and 1:40 in deionised water. Results from these initial assays were used to prepare further dilutions or less diluted samples in order for spectrophotometric absorbance values to fall within the range of decolourisation represented by a Trolox standard curve. For the Trolox standard curve, a 1 mg · mL^{-1} (4 mM) stock solution of Trolox (Aldrich, 238813) in MeOH was prepared and used to construct a standard curve in the range of 0.015 mM - 1.000 mM (resulting in 0.4 - 12.5 μg total Trolox in each spectrophotometric cuvette with 50 μL additions). A 100 mM stock solution of DMPD was prepared by dissolving 209 mg DMPD (Sigma, D4139) in 5 mL deionised water, which was subsequently made to 10 mL. This solution was used to prepare a 1 mM solution in 0.1 M acetate buffer, pH 5.25 (addition of 1 mL 100 mM DMPD/100 mL of 0.1 M acetate buffer). The acetate buffer and DMPD-buffer solutions were kept submerged in a ciculating waterbath set at 25°C. All components of buffers and other reagents were of at least analytical grade and were prepared using stringently clean glassware to avoid unwanted formation of the radical via trace contamination with metal 3+ ions. In each experiment, a sample of the buffered DMPD solution was used as a spectrophometric blank before conversion to the radical form. The DMPD radical cation ($DMPD^{\bullet+}$) was obtained with addition of a 0.05 M ferric chloride (BDH 10110) solution (0.2 mL/100 mL 1 mM buffered DMPD solution), to give a final concentration of 0.1 mM $FeCl_3$. Fifty microlitres of each sample or standard was added to a 1 cm light path cuvette containing 1 mL of buffered $DMPD^{\bullet+}$ solution and mixed with inversion. Absorbance was read spectrophotometrically at λ505 nm 10 min after addition of

test substances. A standard curve for Trolox was plotted as μg addition of Trolox against percentage inhibition of $DMPD^{\bullet+}$ radical at λ505 using the following equation:

$$Percentage\ Inhibition\ (\%) = \left(\frac{1 - Abs_t}{Abs_0}\right) \times 100$$

Equation 1

Where Abs_0 represents absorbance of the uninhibited $DMPD^{\bullet+}$ solution and Abs_t represents the absorbance read 10 min after addition of samples or standards. TE values for the extracts were calculated using a linear regression equation derived from standard curve data and expressed as TE mM (representing relative millimolar equivalence of preparations at original concentrations) and μmol TE/g of fresh weight (FW) of plant material (representing relative Trolox equivalence per gram of plant material extracted).

2.5.2 Estimation of reducing capacity/total phenolic content by Folin-Ciocalteu reagent

The Folin-Ciocalteu reagent contains phosphotungstate-molybdate complexes which undergo a colour change on reduction, and can thus be used to assess the electron transfer reducing capacity of biological samples. The chemistry of the reagent and reaction mechanisms of detection are not completely understood, however, it is believed that heteropolyphoshotunstate-molybdate complexes (e.g. $PMoW_{11}O_{40})^{4-}$ are reduced with the transfer of electrons to the complexed molybdenum, resulting in a shift in valency [Mo(VI) - Mo(V)], accompanied by a colour change to an intense blue with abs_{max} λ760-765 nm (Huang *et al.*, 2005). The reagent is commonly used for the quantitative determination of phenolic compounds and protein content (via reaction with amino acids with phenolic R groups such as tyrosine). Under alkaline conditions, dissociation of the phenolic hydrogen to form the phenolate anion is favoured. The phenolate anion is able to reduce complexes in the

reagent, giving the characteristic colour change (Figure 2.3). Both phenolic and polyphenolic compounds are ubiquitous in plants and together account for around 40% of the organic carbon in the biosphere (Ainsworth and Gillespie, 2007). Phenolic and polyphenolic compounds are commonly reported as constituents of medicinal plants and would be expected to be present in the traditional aqueous extracts prepared from leaves and trunk inner bark. The traditional type extracts were subjected to the Folin-Ciocalteau total phenolic assay, to assess total reducing capacity and to provide an indication of phenolic content.

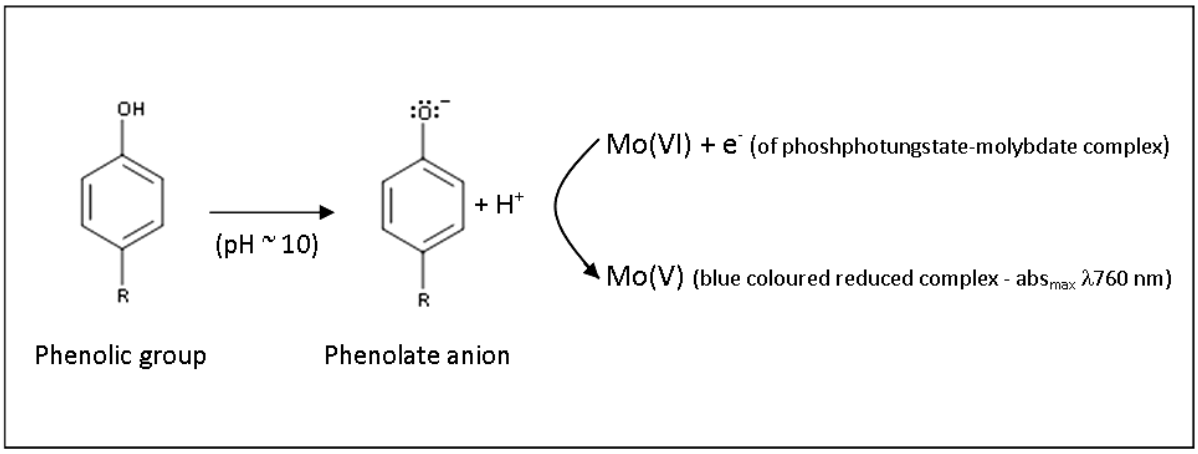

Figure 2.3 Scheme for use of Folin-Ciocalteu reagent to detect phenolic compounds by reducing ability

To perform the test, procedures as described by Fogliano *et al.* (1999) were followed. A sample of each extract was diluted 1:10 with addition of 1 mL of extract to 9 mL deionised water and serially diluted two-fold to produce 1:20 and 1:40 dilutions. Diluted extract samples (0.1 mL) were added to 10 mL capacity stoppered test-tubes containing 4.2 mL deionised water and 0.5 mL Folin-Ciocalteu reagent (Sigma, F9252). The solutions were mixed by vortexing and made alkaline with the addition of 1 mL of 8.0% $^{w}/_{v}$ sodium carbonate, followed by a further 4.2 mL of deionised water, before placing tubes in the dark for 2 hr. A 2 mg · mL^{-1} (2000 ppm) stock solution of gallic acid was prepared and used to prepare a standard curve in the range of 63 - 2000 ppm with 6-step, two-fold serial dilutions. Following incubation, absorbance of samples and standards was measured

spectrophotometrically at λ760 nm in 1 cm light path cuvettes. Initial experiments were used to determine appropriate dilutions of the extracts to produce absorbance values within the range of the standard curves. Total phenolic concentration of samples was determined using a regression equation derived from absorbance values of the gallic acid standard curve and expressed as ppm gallic acid equivalents (GAE) and as mg GAE/g FW of plant material extracted.

2.5.3 **Estimation of antioxidant capacity by oxygen radical absorbance capacity (ORAC) assays**

The oxygen radical absorbance capacity (ORAC) assay directly measures chain breaking antioxidant capacity against peroxyl radicals by following the competitive reaction between a fluorescent probe and hydrogen donating antioxidants for peroxyl radicals generated at steady state rates via thermal decomposition of an azo compound (Huang *et al.*, 2005). Oxidation by peroxyl radicals, consumes the fluorescent probe and the reaction is monitored at regular intervals to yield a fluorescent decay curve representing the kinetics of the competitive reaction. The test involves the monitoring of the fluorescence decay curves for: 1) a mixture of probe, test antioxidant and radical generator, 2) the uninhibited background controls containing probe and azo radical generator and 3) a control to produce 100% relative fluorescent intensity containing the probe without addition of the radical generator. Typical decay curves produced by a fluoroscein/azo blank, and test solution containing an antioxidant are illustrated below in Figure 2.4.

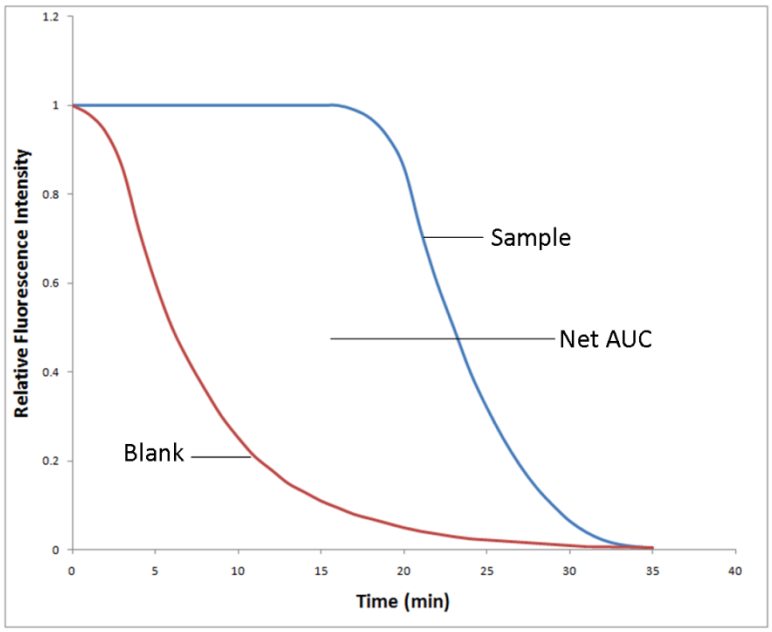

Figure 2.4 Representation of typical ORAC fluorescence decay curve illustrating net area under the curve (AUC). Diagram shows kinetic curves produced by a) blank (fluorescent probe and radical generator to provide background of uninhibited oxidation of the probe), and b) sample, containing the same mixture with added antioxidant (After Huang *et al*., 2002b).

Antioxidant capacity is derived by integrating the area under the curve (AUC), calculated using the following formula (for readings taking place over 35 min at 1 min intervals):

$$AUC = 0.5 + \int_1/\int_0 \ldots + \int_i/\int_0 + \ldots + \int_{34}/\int_0 + 0.5 \times (\int_{35}/\int_0)$$

Equation 2

where $\int_0$ = the initial fluorescence reading at 0 min and $\int_i$ is the fluorescence reading at time *i* (Huang *et al.*, 2002b). The antioxidant activity of a sample is given by net AUC, calculated as AUC of test sample minus AUC of the background (Blank) control:

$$\text{net AUC} = (\text{AUC}_{\text{sample}} - \text{AUC}_{\text{Blank}})$$

Equation 3

The relative Trolox equivalent (TE) ORAC value was calculated using the following formula:

$$ORAC(TE) = \left[\frac{\left(\mathrm{AUC}_{\mathrm{sample}} - \mathrm{AUC}_{\mathrm{Blank}}\right)}{\left(\mathrm{AUC}_{\mathrm{sample}} - \mathrm{AUC}_{\mathrm{Blank}}\right)}\right] \times \left(\frac{[\mathrm{M}]_{\mathrm{Trolox}}}{[\mathrm{M}]_{\mathrm{sample}}}\right)$$

Equation 4

(Huang *et al.*, 2002b)

Where [M] is the molar concentration of either the reference drug Trolox or samples. In this way the ORAC assay combines assessment of both the time kinetics of radical inhibition with the overall degree of inhibition and is thus suitable for the assay of plant and food extracts, which may contain a mixture of compounds with varying degrees of antioxidant ability, and exhibit complicated radical scavenging kinetics (Huang *et al.*, 2005).

For ORAC assays of the extracts, methods based on those described by Prior *et al.* (2003) and Huang *et al.* (2002a; 2002b) were followed, with the exception that sample preparation and dilutions were handled manually. Fluorescein (3',6'-dihydroxyspiro[isobenzofuran-1[3*H*], 9'[9*H*]-xanthen]-3-one) (Aldrich, 166308) was used as the fluorescent probe and 2,2′-azobis(2-methylpropionamidine) dihydrochloride (AAPH), (Wako, 01711062) was used as the peroxyl radical generator. Assays were performed in 96-well format polystyrene fluorescence microplates and kinetic data read using an automated multilabel plate reader (Perkin Elmer, Wallac Victor II 1420).

Extracts were assayed in two-fold serial dilutions, in quadruplicate. Initial concentrations for dilution were chosen based on results of a preliminary screen conducted using four-fold dilutions from an initial concentration of 0.2 mg · mL^{-1}. For each experiment a four step Trolox reference standard curve was prepared using concentrations of 100, 50, 25, and 12.5 μM of Trolox (Aldrich: 23881-3) in deionised water. Epicatechin was used as a secondary internal reference drug and positive control, and was assayed at concentrations of 1.2, 0.6, 0.3,

and 0.15 μM. All samples and standards were diluted/disolved in 75 mM phosphate buffer (pH 7.4). For each experiment, 10 μL of fluorescein (6.0×10^{-7} M) was added to each well of the assay plate followed by 20 μL of samples or standards. For background blank and Fluorescein reference wells, 20 μL of 75 mM phosphate buffer (pH 7.4) was added. Prior to introduction of AAPH, the plates were placed inside the temperature controlled fluorescence reader for 10 min to allow equilibration of temperature to 37ºC. During temperature equilibration of the plate a 20 mM solution of AAPH in 75 mM phosphate buffer was prepared and warmed to 37ºC. One hundred and seventy μL of this solution was added to all wells except for fluorescein reference control wells where 170 μL of phosphate buffer was added, and the plate immediately returned to the plate reader. Fluorescence was measured 35 times at intervals of one minute. Fluorescence readings were referenced to the fluorescein control and background/blank wells and net AUC calculated automatically. ORAC values of samples were derived using a merged linear-hyperbolic standard curve (using methods described by Thompson, 2008), representing AUC response of the Trolox standards. Results were expressed as μmol TE/g FW of plant material extracted, and, mM TE to enable comparison with corresponding TE values generated by the DMPD assay, and to enable ranking of the ORAC antioxidant capacity of the extracts by volume, representing TE of the original concentrations as produced by traditional preparation methods. Fractions containing serrulatane diterprenes isolated from *E. duttonii* were additionally tested using a protocol developed for ORAC assay of lipophilic samples (Huang *et al.*, 2002a). Procedures for the lipophilic ORAC assays were the same as those described above for the assay of hydrophilic samples, except that samples and standards were dissolved in a 7% $^{w}/_{v}$ solution of randomised methylated cyclodextrin (RMCD) in acetone-water (1:1) (prepared by dissolving 7 g of RMCD in 50 mL water, followed by the addition of 50 mL of acetone).

2.6 **Estimation of cytotoxicity against P388D1 lymphoblasts with ATPlite assay**

In vitro cytotoxicity of the extracts, was determined against the murine lymphoblast P388D1 cell line (ATCC: CCL-46). Fractions containing serrulatane diterpenes isolated from *E. duttonii* were additionally assayed for cytotoxic activity against the HepG2 human hepatocarcinoma cell line (ATCC: HB-8065). Cell viability was measured using a commercially available luminescence based adenosine triphosphate (ATP) assay kit (ATPLite-M assay, Packard BioScience B. V.). The assay used detects ATP in samples via emission of light resulting from the ATP dependent activity of firefly luciferase/D-luciferin (Figure 2.5), where the light produced during the reaction is proportional to the quantity of ATP present. Measurement of ATP is a convenient parameter for the assessment of viability in cell cultures as a rapid and immediate decline in ATP concentration occurs with cell death. The assay kit employed utilises a proprietary formulation that extends the normally rapid half life of luciferase light emission to over 5 hr, removing the need for a luminometer equipped with an in assay reagent injection system, thus enabling the addition of reagents prior to the assay. The assay procedure involved addition of a suspension of cells to a luminescence microplate, followed by introduction of an alkaline cell lysis solution. A buffered solution of luciferase/D-luciferin was then added and the plate dark adapted before reading with a luminescence counter.

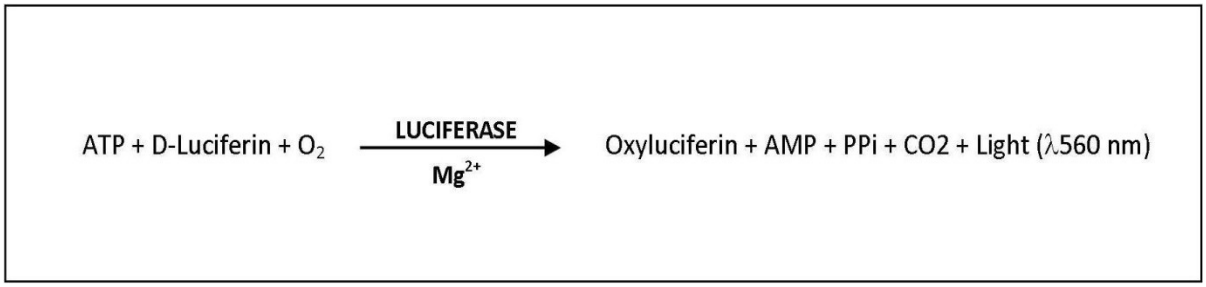

Figure 2.5 Luciferase/D-luciferin reaction scheme

2.6.1.1 **Preparation of samples for cytotoxicity assays**

Samples of the lyophilised traditional aqueous preparations were redissolved at a concentration of 20 mg · mL^{-1} in deionised water with gentle heating and immersion in a sonicating waterbath and filter sterilised (0.2 μm cellulose acetate). Tertiary MeOH extracts were redissolved in sterile DMSO at a concentration of 40 mg · mL^{-1}. Tertiary MeOH extracts of *E. longifolia* (extracts JES021MeOH and JES023MeOH) would not redissolve in DMSO and so were dissolved in DMSO-EtOH (1:1). Immediately prior to the assays, traditional aqueous preparations were further diluted 1:5 in growth medium, and MeOH extracts 1:10, to produce a solution of 2.0 mg · mL^{-1} in media.

2.6.1.2 **P388D1/HepG2 cytotoxicity assay procedure**

Growth medium for the experiments consisted of colour free Dulbecco's modified Eagle's medium (DMEM), containing 2 mM L-glutamine, 100 mM pyruvate, 4.5 g/L glucose, 10% ($^{v}/_{v}$) fetal bovine serum and 100 U/mL penicillin/streptomycin. For each experiment, the culture density of a 24 hr passage of P388D1 cells was determined using a cell counter (Beckman-Coulter ActDiff haematology analyser) and the culture adjusted with growth medium to give an approximate density of 4.2×10^4 cells · mL^{-1}. Ninety-five microlitres of the resulting suspensions were transferred to each well of a white, opaque luminescence 96-well microplate and the plate incubated for 24 hr (37°C, 5% CO_2).

Following incubation of the plates, 5 μL of each diluted standard and extract was added in duplicate to wells containing 95 μL of culture (to give an initial concentration of 200 μg · mL^{-1}), and serially diluted to give final concentrations in a range of 3.13 - 200.00 μg · mL^{-1} (two-fold, 7 step dilution). In addition to growth media controls, vehicle controls consisting of two-fold dilutions of DMSO and DMSO-EtOH (1:1) in growth medium were

prepared in the same manner as the MeOH extracts to produce equivalent concentrations (i.e. 1.00 - 0.02% ($^{v}/_{v}$)). Positive controls/reference drugs consisted of chlorambucil (Sigma, C0253) which was assayed as two-fold serial dilutions on the plate in a range of 7.81 - 500.00 $\mu g \cdot mL^{-1}$, and curcumin (Sigma, C1386), assayed as two-fold serial dilutions in a range of 1.56 - 100.00 $\mu g \cdot mL^{-1}$. Following addition of samples and standards, Plates were incubated for a further 24 hr (37°C, 5% CO_2) before the ATP assay for cell viability.

Assays for ATP were carried out according to the manufacturer's instructions following incubation. Lyophilised kit reagents were prepared with the appropriate buffers/solutions and allowed to equilibrate to room temperature before proceeding. cells were lysed with addition of the mammalian cell lysis solution (50 µL per well) and agitation on a plate shaker for 5 min (700 rpm). Luciferase/D-luciferin buffered substrate solution was then added (50 µL per well) and the plate placed in a sealed opaque bag (to allow equilibration with dark adaptation) and agitated for a further 5 min. Dark adaptation continued for a further 10 min before reading luminescence using a Wallac Microbeta scintillation/luminescence counter (Perkin Elmer). Viability for each well was assessed as percentage inhibition of cell growth relative to controls, calculated from raw data representing luminescence counts. Percentage inhibition and IC_{50} values (as $\mu g \cdot mL^{-1}$) were calculated for each sample and positive control substance using spreadsheets designed by Dr Dion Thompson of CPP, SCU (Lismore NSW).

2.7 **Screening for prostaglandin E_2 inhibitory activity**

Traditional aqueous extracts, tertiary MeOH extracts of the *Eremophila* species and fractions containing serrulatane diterpenes isolated from *E. duttonii* were screened for inhibition of prostaglandin E_2 (PGE_2) production in 3T3 murine fibroblasts stimulated with bacterial calcium ionophore. Assays were carried out in 96-well microplates. PGE_2 levels in the cultures were measured using a commercially available EIA kit (Prostaglandin E2 Enzyme

Immunoassay Kit, Sapphire Bioscience, 133-16359). The PGE_2 EIA kit employed was based on a competitive type EIA reaction where PGE_2 of the test sample competes with an PGE_2 alkaline phosphatase tracer (enzyme linked *antigen*) for a limited amount of a PGE_2 specific monoclonal antibody, bound to polyclonal IgG coating the well surface. In this type of assay the signal produced (chromophore product of alkaline phosphatase activity) is inversely proportional to the amount of PGE_2 in the sample and samples/PGE_2 standards are added to wells before the enzyme linked PGE_2 tracer followed by the monoclonal antibody. All but one of the extracts were screened in triplicate at a single sub-cytotoxic concentration (20 $\mu g \cdot mL^{-1}$), which was determined by examination of cytotoxicity data generated in P388D1 cytotoxicity screening, in order to allow assessment of relative activity. The tertiary MeOH extract of *E. duttonii* was found to exhibit relatively high levels of cytotoxicity (P388D1 IC_{50}: 5.5 $\mu g \cdot mL^{-1}$) and so was assayed at 1.0 $\mu g \cdot mL^{-1}$. Due to budgetary restraints, replicate extracts were not assayed (e.g. duplicate decoction type extracts of the leaves of *Eremophila* species prepared from separate specimens).

2.7.1.1 **Prostaglandin E_2 inhibitory activity assay procedure**

Confluent 3T3 fibroblasts were washed with phosphate buffered saline, pH 7.4 (PBS) and removed from the flask wall with addition of 1 mL of 0.25% trypsin in PBS warmed to 37°C before being suspended in growth media. Growth medium employed was the same colour free medium as described above for P388D1 cytotoxicity screening. Cell suspension was adjusted to 1×10^5 cells $\cdot mL^{-1}$ using the same procedure as described for P388D1 lymphocytes (2.6.1.2 above). Ninety microlitres (90 μL) of the adjusted suspension was added to all wells and the plate was incubated for 20 hr (37°C, 5% CO_2). Samples and controls were added to wells in triplicate following incubation (10 μL). For each test, an unstimulated control (no calcium ionophore) was included to monitor any positive

modulation or up regulation of PGE_2 production by extracts or control substances. Negative controls consisted of growth media and growth media with solvent vehicle where appropriate (DMSO or DMSO-EtOH 1:1 for experiments including *E. alternifolia* MeOH extracts) Positive control/reference drug consisted of Aspirin (Sigma, A5376) at a final concentration of 0.1 mM (10 μL addition of 1.0 mM solution in DMSO). Plates were incubated with samples and controls for a further 3 hr before addition of 5 mM calcium ionophore (calcimycin; Sigma, C7522) in DMSO to the appropriate wells (1 μL) followed by mixing with agitation on a plate shaker for 30 s. Plates were incubated for 20 min following ionophore stimulation and centrifuged for 3 min at 1000 RCF (Hettich Universal 16A Centrifuge). Supernatants were transferred to a fresh microplate where each triplicate test was pooled then diluted 1:500 with the kit TRIS sample buffer and assayed immediately using the PGE EIA kit. One hundred microlitres (100 μL) of each diluted, pooled test was added to wells of the EIA plate in triplicate. For each experiment a standard curve (8 dilutions) of PGE_2 was prepared in TRIS sample buffer with manual two-fold dilution (1 mL) in the range of 4000 - 31 pg · mL-^{1}) and added to the plate in duplicate (100 μL). Fifty microlitres (50 μL) of PGE_2 alkaline phosphatase tracer was added to all wells except blank and total activity wells followed by addition of 50 μL of PGE_2 monoclonal antibody to all except blank, total activity and non specific binding wells. The plate was then sealed and incubated at room temperature with gentle agitation for 4 hr. Following incubation the plate was emptied and washed five times with the reconstituted wash buffer provided with the kit. The plate was then developed with addition of 200 μL of para-nitrophenyl phosphate (*p*-NPP), followed by resealing and enclosing in an opaque bag, and returning to the plate shaker for agitation for a further 90 min. After development, absorbance was read at 405 nm using a Wallac Victor II plate reader (Perkin Elmer, 1420) using a pre-programmed data reduction sequence to return a standard curve and raw data as pg· mL^{-1} PGE_2 of sample and test control wells. Mean,

standard deviation and relative standard deviation were calculated for replicate assays and percentage inhibition derived using the following equation:

$$PGE_2\ Inhibition(\%) = \left(\frac{[PGE_2\ test\ well]}{[PGE_2\ mean\ control\ well]}\right) \times 100$$

Equation 5

PGE_2 inhibition was reported as mean percentage inhibition relative to media controls of triplicate assays for stimulated and unstimulated cells along with standard error.

2.8 Methods and procedures followed for the isolation of serrulatane diterpenes from *Eremophila duttonii*

2.8.1 Column chromatography

The hexane and DCM sequential extracts of *E. duttonii* whole leaves (as described above in section 2.2.1.5) were separated using silica gel (normal phase) low pressure column chromatography with a stepped elution gradient to afford 48 fractions. Stationary phase consisted of silica gel, 60 Å, 230-400 mesh (Merck 227196). To prepare the column the silica gel was prepared as a slurry with a small quantity of hexane and poured into glass chromatography columns (Column bed dimensions: 250 × 25 mm I.D. ca. 500 mL bed volume). Six gm of each extract was dissolved in a small quantity of hexane and applied to the column bed. The extract was eluted with stepwise addition of hexane (100%), followed by hexane-DCM (95% - 10%), DCM (100%), DCM-ethyl actetate (95% - 10%) and finally ethyl acetate (100%) (Table 2.3). Flow rate was maintained at maximum (ca. 15 mL · min^{-1}). Fifty millilitre (50 mL) fractions were collected in pre weighed vials and dried under a stream of nitrogen. After drying the mass of each fraction was calculated by subtracting the vial tare weight from the total mass of the dry fraction and vial in gm as determined using a four place analytical balance.

Table 2.3 Stepped gradient elution for separation of *E. duttonii* extracts with normal phase column chromatography

Fractions	Hexane (%)	DCM (%)	Et-OAc (%)	V_e (mL)
1 - 3	100	-	-	150
4 - 6	100	-	-	300
7 - 9	95	5	-	450
10 - 12	90	10	-	600
13 - 15	70	30	-	750
16 - 18	50	50	-	900
19 - 21	30	70	-	1050
22 - 24	10	90	-	1200
25 - 27	-	100	-	1350
28 - 30	-	95	5	1500
31 - 33	-	90	10	1650
34 - 36	-	70	30	1800
37 - 39	-	50	50	1950
40 - 42	-	30	70	2100
43 - 45	-	10	90	2250
46 - 48	-	-	100	2400

Ve: Elution volume

2.8.2 **Thin layer chromatography of column chromatography fractions**

Column chromatography fractions were further analysed using thin layer chromatography (TLC) to enable identification of those containing the serrulatane diterpenes and to enable recombining of fractions of similar composition. TLC separations were carried out using 20 × 20 cm glass backed, pre coated 0.5 mm silica gel preparative TLC plates (Si-60 non fluorescent: Merck 1.13894) which were activated by heating for 1 hr at 110°C immediately prior to use. The solvent system employed for all separations was hexane-ethyl acetate (3:1) which was freshly prepared for each experiment. In previous work (Smith *et al.*, 2007), hexane-ethyl acetate (3:1) with normal phase TLC had been found to result in optimum separation and resolution of *E. duttonii* serrulatane diterpenes. Each fraction was dissolved in a quantity of DCM to produce a concentration of around 40 mg · mL^{-1}. One and a half microlitres (1.5 μL) of each fraction was loaded 25 mm from the base of the plate using a 5 μL capacity Hamilton syringe (60 μg sample loading). For each plate a line was scored 5

mm from the edge of the opposite side of the plate to mark the final position of the solvent front (total distance travelled by solvent: 170 mm). Following sample loading, plates were allowed to dry to remove residual sample loading solvent and placed in a sealed glass TLC chamber which had been lined with paper for saturation and equilibrated with the solvent system for >1 hr. Development of the plates generally concluded at around 55 - 60 min at which point the plate was removed and dried in a fume extraction hood before applying an acid-anisaldehyde visualising spray reagent. Acid-anisaldehyde visualising spray reagent was chosen for its ability to detect a relatively wide range of n classes of natural product, including terpenoids. The reagent also produces a greater range of colour reactions specific to certain classes of compound when compared to other, similar reagents (e.g. vanillin-sulphuric acid reagent). The reagent was prepared after descriptions by Wagner and Bladt (1996) with the addition of 2 mL p-anisaldehyde (Sigma A0519) to 100 mL of acid alcohol (5% $^{v}/_{v}$ H_2SO_4, 5% $^{v/}{}_{v}$ acetic acid in MeOH). Plates were sprayed with approximately 5 mL of the acid-anisaldehyde reagent using an aspirator connected to a nitrogen source, and placed in a 100°C oven for ca. 5 min for colour development. Resulting chromatograms were immediately recorded using a flatbed scanner at 1200 dpi resolution. Retention factors (R_f) were calculated for each resolved compound visible on the plate as the ratio of distance(s) travelled by each compound, to the distance travelled by the solvent front in mm.

2.8.2.1 Nuclear magnetic resonance spectroscopy of compounds from *Eremophila duttonii*

NMR spectra of fractions containing compounds 1 and 2 were obtained with a Bruker Avance DRX-500. All experiments were performed in deuterated chloroform. 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. COSY, NOESY, HSQC HMBC and nOe diff experiments were acquired using the standard Bruker pulse programs. Chemical shifts (δ)

were calibrated relative to the deuterated chloroform solvent peak (^{1}H δ 7.27 and ^{13}C δ 77.23 ppm) and recorded as parts per million (ppm) with the coupling constants (*J*) in Hertz (Hz). All NMR experiments and subsequent analyses were carried out by Dr. Myrna Deseo (CPP, SCU).

2.8.3 Detection of serrulatane diterpenes in traditional aqueous preparations of *E. duttonii* by LC-MS

Column chromatography fractions containing the serrulatane diterpenes were subjected to separation/analysis by LC-MS using operating parameters as described above (2.3) alongside aqueous extracts of *E. duttonii* leaf material which had been prepared according to traditional methods described in the ethnobotanical literature. Retention times and UV-Vis/mass spectral data obtained in separations of the known compounds were used for the initial qualitative assays for the serrulatane diterpenes in *E. duttonii* traditional aqueous preparations.

2.8.4 Purification of serrulat-14-en-3,7,8,20-tetraol by semi-preparative HPLC

Semi-preparative scale sub-fractionation and purification of column chromatography fractions containing serrulat-14-en-3,7,8,20-tetraol (**2**) was carried out for the purpose of obtaining a sample suitable for use as a standard in subsequent quantitative analyses in traditional aqueous preparations. Method development procedures were carried out using a HPLC system with quaternary pump (G1311A), autosampler/injector (G1313A) and column thermostat/heating block (G1316A) fitted. Absorbance spectra were collected at λ210 nm with an Agilent photodiode array detector (G1315B). The column used for method development was a Phenomenex luna C-18 5 μm 100 Å 150 mm × 4.6 mm I.D. (Phenomenex 00F4252 E0) operating at 50°C. Column loading was up to 50 μg with 10 μL injection volumes. Flow rate was 1.5 mL·min^{-1}. Several mobile phases were trialled including various isocratic and gradient mixtures of isopropanol-MeOH and ACN-water. The mobile phase

found to result in maximal separation between peak of the tetraol **2** and adjacent peaks was an ACN-water gradient programmed as follows: Mobile phase: A = ACN with 0.005% TFA; B= Water with 0.005% TFA. Elution gradient: 0-20 min: 30-60% B, 20-25 min: 60-95% B, 25-30 min: isocratic hold 95% B, 30-35 min: 95-30% B, 35-45 min: Isocratic hold 30% B. Semi-preparative separation was carried out on 89 mg of column chromatography fraction #18 of the DCM extract of *E. duttonii*, using the same HPLC system linked to an automated Gilson 203B fraction collector. Column for semi-preparative purification was a Phenomenex luna C-18 5 μm 100 Å 250 mm × 10.0 mm I.D. (Phenomenex 00G 4041 N0) operating at 50°C. Column loading was 4 mg per injection with 90 μL injection volume. Flow rate was 3.5 $mL \cdot min^{-1}$. The automated fraction collector was programmed to collect fractions at 0.2 min intervals between 18.0 and 26.8 min Rt to collect the major peak eluting between 20.8 and 22.0 min. Fractions were subsequently analysed using an Agilent 1100 Series LC-MSD system (operating parameters as described below for quantitative assay in aqueous preparation). Subfractions 17, 18 and 19 (11 mg, eluting 21.2 - 21.8 min) were initially found to be relatively free of impurities by chromatographic analysis, and were used as standards for subsequent quantitative determinations.

2.8.4.1 Quantitative analysis of serrulat-14-en-3,7,8,20-tetraol in traditional aqueous preparations of E. duttonii by HPLC/LC-MS

HPLC Quantitative assays of **2** in aqueous traditional decoctions of *E. duttonii*, were performed using automated callibration functions of the HP-Chem software (Rev. A, v09.03). Standards and samples were chromatographed using an Agilent 1100 HPLC system with quaternary pump (G1311A), autosampler/injector (G1313A) and column thermostat/heating block (G1316A) fitted. Detection was by UV absorbance at λ210 nm using an Agilent photodiode array detector (G1315B). Column used was Phenomenex luna C-18 5 μm 100 Å 150 mm × 4.6 mm I.D. (Phenomenex 00F4252 E0) operating at 40°C. Injection volume was

5 μL. Flow rate was 0.75 $mL \cdot min^{-1}$. Under these conditions, a 25 min gradient mobile phase of 10-95% ACN-H_2O with 0.005% TFA was found to be ideal for detection and quantitative analysis, as the serrulatane was seen to elute with good separation from neighbouring peaks in the separations of the traditional aqueous preparation (Rt: 17.937 min). Mobile phase/gradient was as follows: A= ACN with 0.005% TFA; B= Water with 0.005% TFA; Elution gradient: 0-25 min: 10-95% B, 25-30 min: 95-10% B, 30-35 min: Isocratic hold 10% B. A sample of Compound **2** was dissolved in MeOH and a standard curve for calibration of detector response as peak area was prepared with manual ten-fold serial dilutions to result in solutions containing 1.184×10^{-3} , 1.184×10^{-2}, 1.184×10^{-1} and 1.184 mg · mL^{-1}. Solutions of aqueous traditional extracts of *E. duttonii* were prepared in deionised water at a concentration of 20 mg · mL^{-1}. All solutions were prepared manually using volumetric glassware which had been previously checked for volumetric accuracy and consistency in delivery with gravimetric analysis. The linear calibration curve was constructed from mean response (peak area at λ210 nm) of three separate injections per standard. Subsequent to the assays, however, it was found that compound **2** may have been unstable and products of decomposition seemed to have formed in the purified sample which was evidenced by a change in appearance on drying. When separations of **2** used to construct the calibration curve were reviewed, a number of peaks not previously present in the initial separations performed to assess purity were found in the most concentrated standard solution. For this reason an accurate estimation of **2** was not obtained and only a tentative determination, based on assay of purity by peak area in the standards could be reported.

2.8.4.2 **Assessment of antimicrobial activity of serrulatane diterpenes**

Antimicrobial activity of fractions containing the serrulatane diterpenes isolated from *E. duttonii*, was assessed as MIC and MBC against *S. aureus* (ATCC 29213)*, S. epidermidis*

(ATCC 12228) and *B. subtilis* (ATCC 6633) using the broth microdilution techniques described above in sections 2.4.3.3.1 and 2.4.3.3.2. Fractions containing compounds **1** and **2** were assayed alongside traditional aqueous extracts prepared using the leaves of *E. duttonii* specimens. *E. duttonii* fraction 9 and fraction 18-17 were first dissolved in a quantity of DMSO before making to volume with sterile deionised water to produce solutions of 2.4 mg · mL^{-1} (highest final concentration of DMSO: 1.0% $^{v}/_{v}$). Solutions were used for the initial additions in 12-step, two-fold serial dilutions to produce final well concentrations ranging from 0.5 to 960 μg · mL^{-1} (This dilution scheme was followed as it would result in a number of well concentrations containing quantities of compound **2** approximately equal to that produced with two-fold dilution of the aqueous extracts, i.e. 60, 30, 15 μg · mL^{-1} etc.).

2.9 Methods and procedures followed for the characterisation of novel *Eremophila longifolia* essential oil chemotypes

2.9.1 Collection of plant material

Aerial parts of mature *E. longifolia* specimens were collected from several localities in the arid and semi-arid regions of western NSW. The majority of specimens were collected during the months of November-December (early summer season) with additional specimens collected during the months of February (late summer season) and August (late winter-early spring). Collection localities covered four biogeographical regions and included the Bynguano Range of Mutawintji National Park in the Mootwingee Downs Bioregion (MD), the plains of the Broken Hill Complex Bioregion (BHC) and the Murray Darling Depression Bioregion (MDD) near to the Darling River and the Cobar Peneplain Bioregion (CP). Geographic bioregion boundaries and nomenclature given here follows that outlined in the *Interim Biogeographic Regionalisation for Australia* (IBRA) (DOTEHWATA, 2006). Field identities of collected plant materials were confirmed by Mr Ian Telford of the NCW Beadle

Herbarium (University of New England, Australia) where voucher specimens for each plant sampled were lodged.

2.9.2 **Isolation of volatile oils**

Whole, unprocessed leaves from each individual specimen (250 – 350 gm) were separated from branches and woody parts and hydrodistilled with deionised water (1000 mL) over 2 hr. Distillation apparatus consisted of a heating mantle, a 2 litre extraction flask, jacketed water cooled condenser and oil receiving flask. Volatile oils were recovered, dried and stored at 4°C prior to and between analyses. Yield was determined as percentage mass of oil recovered for mass of leaf material distilled. As the majority of specimens were found to be very high yielding, distillation of 250 gm of leaf material was deemed adequate in most cases. Material from three specimens however, was not found to yield recoverable amounts of oil with this quantity (Specimens JES001, SM016 and SM019) and so these samples were redistilled using 350 gm of leaves. Of these three specimens, a small quantity of oil was recovered from distillation of SM019 material only.

2.9.3 **Gas Chromatography-Mass Spectrometry**

Analyses were performed using a HP 6890 gas chromatograph coupled with a HP 5973 mass selective detector. Separation was accomplished with a ZB-5ms column (30 m × 0.25 mm i.d., 0.25 μm phase thickness). Operating conditions were as follows: Injector: split ratio 25:1; Temperature: 250°C; carrier gas: helium, 1.0-ml/min, constant flow; column temperature program was as follows: 50°C (6 min), 50-100 ^{o}C at 7 ^{o}C / min (no hold), 100-210°C at 20°C /min (11.36 min). Mass spectra were acquired at -70 eV with mass scan range of 50 - 300 m/z. Volatile oil samples were diluted 1:200 in dichloromethane and injections of 1 μl were performed using an autosampler unit (8 position HP 7673). Quantitative analysis (%) was performed by peak area normalisation measurements of total ion current. Authentic reference

compounds were diluted 1:1000 in dichloromethane (1 mg · ml^{-1} in the case of the solid borneol). A mixture of aliphatic hydrocarbons (C_8 – C_{24}) was injected under same program and operating conditions above to calculate retention indices of separated compounds.

2.9.3.1 **Identification of constituents**

Identification of compounds was performed by comparison of experimental mass spectra and retention indices with those of a mass spectral library database (NIST98) and published mass spectra and RI values (Adams, 2001). Wherever possible, these initial identifications were further verified with co-injection of authentic reference standards.

2.9.3.1.1 Identification of karahanaenone

Oil specimens representing 2 specimens collected in the Broken Hill Complex and Murray-Darling Depression Bioregions were found to contain a compound at relative quantities greater than 80% which could not be identified by comparison with any of the NIST98 library spectra. For these samples, ^{1}H and ^{13}C-NMR spectra of neat oils were obtained. Analyses of NMR spectra were used for structural assignments for this compound. NMR data were consistent with that reported by Naya and Kotake (1968) for the monoterpene ketone karahanaenone (2,2,5,-trimethylcyclohept-4-en-1-one,). Identification of karahanaenone was further verified by comparison of experimental GC retention data and mass spectra with published values (Adams, 2001). NMR spectra were measured using a Bruker Avance 300 spectrometer with a 5 mm BBIz probe, operating at 300.13 MHz (^{1}H) or 75.46 MHz (^{13}C). A 10 mg portion of each sample was dissolved in 0.6 mL $CDCl_3$ and spectra recorded at 30°C using standard Bruker pulse sequences as follows: COSY: (pulse program: cosygpmftp) 1K × 512 data points, 2 scans; HSQC: (pulse program: invieagssi) 1500 × 512 data points, 24 scans; HMBC: (pulse program: inv4gplprnd) 1500 × 512 data points, 12 scans). All NMR

experiments and subsequent structural assignments were carried out by Dr. David Tucker (UNE, Division of Chemistry).

2.9.4 **Multivariate statistical analyses**

Principal component analysis (PCA) was performed using the data set composed of the 8 oil samples transformed into centred and reduced variables (correlation method, varimax rotation). Selected variables consisted of relative percentages of 24 compounds identified in the oil samples occurring in quantities greater than 0.5% of total. Data were analysed using the principal components function of the SPSS v17.0 software (SPSS Inc., Chicago Il.).

2.9.5 **Assessment of antimicrobial activity in *Eremophila longifolia* essential oils**

2.9.5.1 **Estimation of MIC**

MIC of *E. longifolia* essential oils was determined using a broth microdilution method based on techniques described by Mann and Markham (1998) with some modifications. To obtain a homogenous dispersion of the hydrophobic essential oils in the aqueous media, Mann and Markham (1998) recommend use of media containing 0.15% agar (sloppy agar). Agar solutions at this strength enable homogenous dispersion of hydrophobic test substances (in a similar manner to a full-strength solid agar dilution test), while maintaining a level of viscosity low enough to enable manipulations such as pipetting and mixing. General experimental procedures followed for broth microdilution tests (e.g. culture and inoculum preparation, incubation conditions etc.) were as outlined in section 2.4 above, with some modifications noted below. Essential oils were assayed over a range of 0.06% $^{v}/_{v}$ to 2.00% $^{v}/_{v}$ using 6-step, two-fold serial dilutions. To prepare 2.00% $^{v}/_{v}$ test solutions, 200 µL of each oil was added to 9.8 mL of tryptone soya sloppy agar media (TSSA) and vortex mixed at high speed for 30 s to form a well dispersed suspension. 160 µL of each solution was added to wells forming the initial step in serial dilutions into 80 µL TSSA. Cefazolin and tetracycline

were used as reference antibiotics (12 step two-fold serial dilutions in range of 0.13 to 256 $\mu g \cdot mL^{-1}$). Positive growth control wells contained 80 μL TSSA with no addition of oil. Sterility control wells contained 80 μL TSSA to which 20 μL media was subsequently added in place of adjusted bacterial suspensions (prepared with addition of 1 mL 0.9% sterile saline to 9 mL TSSA). For each experiment, replicate plates, to which this solution was added to all wells, were incubated alongside test plates to monitor sterility of the test substances and as a means to detect any chemical reduction of p-INT indicator by the test substances which may potentially have resulted in a false negative result for inhibitory activity. The only major difference between broth microdilution assays of the essential oil samples and those for other extracts in the study was in the practice of sealing the plates. To minimise evaporation of the volatile essential oils, the microtitre plates were sealed at the junction of the base and cover with a strip of parafilm prior to incubation. Following incubation, 40 μL of p-INT solution was added to all wells to visualise growth, or lack of growth (indicating inhibitory activity at a given concentration). MIC for each oil sample was recorded as the lowest concentration at which inhibition was produced or maintained. All experiments were performed in duplicate and repeated on three separate occasions.

2.9.5.2 **Estimation of minimum bactericidal concentration in *Eremophila longifolia* essential oils**

MBC of *E. longifolia* essential oils was determined by examining subcultures of MIC tests for viability which were prepared after the incubation of the tests and prior to addition of the p-INT solution. For the subcultures, 3 μL samples were taken from wells containing oils which had shown inhibitory activity and spot inoculated onto the surface of PCA media in 90 mm Petri dishes, which had been marked to indicate the position of each spot. For the experiments using *P. aeruginosa*, PYE agar was used in place of PCA. Following spot inoculation, plates were left in a laminar flow cabinet with lids ajar for 10-15 min to facilitate

drying of the spots and then incubated under appropriate conditions for at least 18 hr. Following incubation, the agar plates were examined for growth. Positive growth (indicating presence of viable cells remaining in wells and thus lack of bactericidal action) was seen as a region of bacterial growth covering the area where culture had been spot inoculated. Occasionally the region of growth was slightly broken and individual colonies were visible, which suggested that the level of viable cells had been reduced, yet some viable cells still remained. MBC for agar spot inoculated subcultures (MBC_{SIAS}) was defined as the lowest concentration where no growth could be obtained by spot inoculated subculture.

2.10 **Statistical analyses**

Analytical results were in most instances presented as mean values with standard error. Analyses for the comparison of means were achieved with analysis of variance (ANOVA) and student t-tests. Except where indicated, calculation of descriptive statistics, single factor ANOVA, 2 tailed t-tests and tests for correlation, were performed using the Excel 2007 data analysis add in module and/or appropriate worksheet functions (Microsoft Corporation, Redmond, WA). Multivariate statistical analyses, ANOVA with post-hoc tests and other tests for association (Pearson's chi-squared and Fisher's exact tests) were performed using the Statistical Package for the Social Sciences (SPSS) v19 software (IBM Corporation, NY).

Chapter 3: Antimicrobial activity in Australian indigenous medicinal plant extracts used for dermatological and ophthalmic conditions

In this chapter, the plant species collected and extracts prepared for the study are described and results of tests for anti-microbial activity are presented and discussed. Incidence of antimicrobial activity in the group of traditional type preparations was compared with major use agreement values derived from the dataset of Australian medical ethnobotany and discussed in relation to specific indications and uses which were cited in these sources.

3.1 Introduction

Thirty-seven separate aqueous extracts were obtained from various parts of twenty-three plant species following traditional methods of preparation described in the ethnobotanical literature. Each traditional aqueous extract prepared and examined in the study is listed below in Table 3.1 alongside measurements of pH, concentration and yield from fresh weight of plant material. Further detailed information on the preparation of each extract is provided in Appendix II. A summary of therapeutic uses and indications cited in the medical ethnobotanical literature examined for each species collected is given below in Table 3.2. As described in Chapter 1, major use agreement values (MUA_{TMP}) were calculated separately for indications/use in the treatment of wounds, sores (dermal lesions) and all dermatological/ophthalmic complaints. MUA_{TMP} values determined for each specific traditional preparation are given below in Table 3.3. Eighteen extracts of material from the four species of *Eremophila* included in the study were obtained by successive extraction with hexane, followed by dichloromethane (DCM) and methanol (MeOH). Details for the preparation of these extracts are summarised below in Table 3.4.

Table 3.1 Extracts prepared after traditional extraction methods examined in the study

Label code	Species	Family	Description	Source for method of traditional preparation	pH	Concentration ($mg \cdot mL^{-1}$)	Yield ($\%^{w}/_{w}$)
JES30LD	*Acacia auriculiformis*	Fabaceae	Leaf decoction	Barr *et al.,* 1988	5.21	8.0	7.8
JES49LD	*Acacia holosericea*	Fabaceae	Leaf decoction	Barr *et al.,* 1988	5.39	8.7	8.5
JES34LTD	*Avicennia marina*	Acanthaceae	Leaf decoction	Barr *et al.,* 1988	5.73	14.4	7.9
JES42TD	*Bauhinia cunninghamii*	Fabaceae	Root decoction	Barr *et al.,* 1988	5.35	3.8	7.2
JES66TD	*Buchanania obovata*	Anacardiaceae	Recent growth terminal stem decoction	Barr *et al.,* 1988	4.30	4.2	9.5
JES66SE	*Buchanania obovata*	Anacardiaceae	Recent growth terminal stem cold infusion	Levitt and Lyon 1981	4.15	9.7	6.9
JES48BD	*Buchanania obovata*	Anacardiaceae	Trunk inner bark decoction	Levitt and Lyon 1981	4.99	20.4	5.6
JES41TD	*Callitris intratropica*	Cupressaceae	Trunk inner bark decoction	Barr *et al.,* 1988	4.43	13.8	3.9
JES35SE	*Camptostemon schultzii*	Bombacaceae	Charred wood warm infusion	Barr *et al.,* 1988	9.71	6.2	4.3
JES59EJ	*Cochlospermum fraseri*	Bixaceae	Fruit pulp/seed expressed juice	Barr *et al.,* 1988	4.97	95.2	2.4
JES43TS	*Crinum angustifolium*	Amaryllidaceae	Bulb cold infusion	Barr *et al.,* 1988	3.90	18.8	5.6
JES43BD	*Crinum angustifolium*	Amaryllidaceae	Bulb decoction	Barr *et al.,* 1988	5.47	18.0	4.5
JES58AD	*Cymbopogon ambiguus*	Poaceae	Whole plant decoction	Barr *et al.,* 1988	5.12	3.6	4.3
JES23TD	*Eremophila alternifolia*	Myoporaceae	Leaf decoction	Barr *et al.,* 1988	4.91	5.1	8.3
JES21TD	*Eremophila alternifolia*	Myoporaceae	Leaf decoction	Barr *et al.,* 1988	4.66	5.0	8.4
SM10TD	*Eremophila duttonii*	Myoporaceae	Leaf decoction	Barr *et al.,* 1988	4.53	9.7	10.9
SM11TD	*Eremophila duttonii*	Myoporaceae	Leaf decoction	Barr *et al.,* 1988	4.53	6.2	6.8
SM7TD	*Eremophila latrobei*	Myoporaceae	Leaf decoction	Barr *et al.,* 1988; Latz, 1995	3.90	5.4	10.5
SM8TD	*Eremophila latrobei*	Myoporaceae	Leaf decoction	Barr *et al.,* 1988; Latz 1995	3.90	6.1	12.3

Table 3.1 (continued from previous page)

Label code	Species	Family	Description	Source for method of traditional preparation	pH	Concentration ($mg \cdot mL^{-1}$)	Yield ($\%^{w}/_{w}$)
SM3TD	*Eremophila longifolia* (IC)*	Myoporaceae	Leaf decoction	Barr *et al.,* 1988	4.82	7.7	12.1
SM18TD	*Eremophila longifolia* (KC)*	Myoporaceae	Leaf decoction	Barr *et al.,* 1988	4.95	8.1	12.0
SM19TD	*Eremophila longifolia* (BC)*	Myoporaceae	Leaf decoction	Barr *et al.,* 1988	4.49	8.3	12.9
SM1TD	*Eremophila sturtii*	Myoporaceae	Leaf decoction	Barr *et al.,* 1988	4.49	9.3	19.1
JES40LTD	*Erythrophleum chlorostachys*	Fabaceae	Leaf/trunk inner bark decoction	Barr *et al.,* 1988	4.75	4.7	5.8
JES40BTD	*Erythrophleum chlorostachys*	Fabaceae	Trunk inner bark decoction	Barr *et al.,* 1988	4.50	10.1	10.3
JES28LTD	*Eucalyptus tetrodonta*	Myrtaceae	Terminal leaf decoction	Barr *et al.,* 1988	4.18	5.4	10.8
JES28BTD	*Eucalyptus tetrodonta*	Myrtaceae	Trunk inner bark decoction	Barr *et al.,* 1988	3.54	1.2	4.7
JES37SE	*Flagellaria indica*	Flagellariaceae	Stem warm infusion	Levitt and Lyon, 1981	5.43	7.1	3.5
JES37EJ	*Flagellaria indica*	Flagellariaceae	Terminal node expressed juice	Levitt and Lyon, 1981	5.17	118.0	4.9
JES32BHSE	*Hibiscus tiliaceus*	Malvaceae	Stem inner bark hot infusion	Barr *et al.,* 1988	5.77	9.5	2.7
JES32SE	*Hibiscus tiliaceus*	Malvaceae	terminal stem cold infusion	Levitt and Lyon, 1981	5.97	3.3	3.8
JES31SE	*Ipomoea pes-caprae*	Convolvulaceae	Leaf cold infusion	Barr *et al.,* 1988	5.80	0.8	2.1
JES31HSE	*Ipomoea pes-caprae*	Convolvulaceae	Leaf warm infusion	Barr *et al.,* 1988	6.20	1.5	3.2
JES60EJ	*Morinda citrifolia*	Rubiaceae	Fruit expressed juice	Levitt and Lyon, 1981	4.50	51.1	1.6
JES27LBD	*Pandanus spiralis*	Pandanaceae	Leaf base decoction	Barr *et al.,* 1988	5.09	13.6	5.1
JES27EJP	*Pandanus spiralis*	Pandanaceae	Diluted leaf tip expressed juice	Barr *et al.,* 1988	5.45	8.3	1.5
JES63EJ	*Scaevola sericea*	Goodeniaceae	Fruit expressed juice	Barr *et al.* 1988	5.11	75.8	1.8

*: *E. longifolia* chemotypes: IC: Isomenthone; KC: Karahanaenone; BC: Borneol

Table 3.2 Plant species collected and summary of medical ethnobotany

Species (FAMILY)	Plant parts used	Methods of preparation	Uses and Indications cited for therapeutic use	Region/Community (Language/group)	Source
Acacia auriculiformis A. Cunn. ex Benth. (FABACEAE)	Leaves*	Decoction*	Dermal lesions and minor wounds (1), cleaning skin (1)	Maningrida, NT; Kulamindini/Elliott, NT	Barr *et al*., 1993
	Seed pods	Rubbed between hands to produce soapy lather	Soap (2), "skin disorders" (1), pruritus (1)	Nauiyu Nambiyu, NT (Ngan'gikurunggurr / Ngan'giwumirri); Daly River area, NT (MalakMalak/ Matngala); Katherine/Nitmiluk region, NT (Jarwoyn)	Barr *et al.* 1993; Marrfurra *et al*., 1995; Lindsay *et al*., 2001; Wynjorrotj *et al.*, 2005
Acacia holosericea A. Cunn. ex G.Don. (FABACEAE)	Leaves*	Decoction*	Infected skin lesions (2)	Lajamanu, NT, Maningrida, NT	Barr *et al*., 1993
	Leaves	Rubbed between hands to produce soapy lather	Soap (2)	Minyerri, NT (Alawa); Elsey, NT (Mangarrayi)	Wightman *et al*., 1991; Wightman *et al*., 1992
	Leaves and seed pods	Rubbed between hands to produce soapy lather	Soap (3)	Flora River area, NT (Wardaman); Daly River area, NT (MalakMalak/ Matngala); Mid Daly River area, NT (Wagiman)	Raymond *et al*., 1999; Lindsay *et al.*, 2001; Liddy *et al*., 2006
	Ripe seed pods	Rubbed between hands to produce soapy lather	Pruritus (2), soap (1)	Gulin Gulin, NT; Nauiyu Nambiyu, NT	Barr *et al*., 1993; Marrfurra *et al*., 1995
	Root	Macerated and infused in water	Laryngitis (1)	Broome area, WA	Webb, 1969
	Bark	Pliable bark applied directly to head (Inner surface contacting skin)	Headache (1)	Lajumanu, NT	Barr *et al*., 1993
Avicennia marina (Forssk.) Vierh (ACANTHACEAE)	Green bark and leaves*	Decoction*	Dermal and leprotic lesions (1)	Bathurst/Melville Islands, NT (Tiwi)	Puruntatameri *et al*., 2001

*: Shaded entries refer to extracts prepared and examined in this study; NT: Northern Territory; WA: Western Australia; SA: South Australia; NSW: New South Wales; Vic: Victoria; Qld.: Queensland

Table 3.2 (Continued from previous page)

Species (FAMILY)	Plant parts used	Methods of preparation	Uses and Indications cited for therapeutic use	Region/Community (Language/group)	Source
Avicennia marina (Forssk.) Vierh (ACANTHACEAE)	Leaves/young shoots	Chewed and applied directly	Stone fish/ray stings (1)	Milingimbi, NT (Yolngu)	Wightman and Smith, 1989
	Green bark	direct application and taken orally	Stone fish/ray stings (1), wounds (1)	Milingimbi, NT (Yolngu)	Wightman and Smith, 1989; Barr *et al*., 1993
	Timber	Burnt and infused in sea/salt water	Dermal lesions, boils, fungal dermatophytes (1)	Yirrkala, NT (Rirratjingu)	Yunupingu *et al*., 1994; Barr *et al*., 1993
Bauhinia cunninghamii (Benth.) Benth.* (FABACEAE)	Root Inner bark or whole root with outer bark removed*	Decoction*	Dermal lesions (2), wounds (1), dermal ulcers (1), infected dermal conditions (1), herpes dermal eruptions (1), scabies (1) "skin disease" (1), coughs (1) colds (1)	Kulamindini/Elliott, NT; Mid Daly River area, NT (Wagiman)	Wightman *et al*., 1992b; Barr *et al*., 1993; Liddy *et al*., 2006
	Inner Bark (leaves added by Jarwoyn)	Decoction	(Oral): Fever (3), headache (2), malaise and other symptoms of respiratory/flu like illnesses (3) (also used externally in 1 location)	Amanbidji, NT; Bulla, NT (Ngarinyman); Katherine/Nitmiluk region, NT (Jarwoyn)	Barr *et al*., 1993; Smith, 1993; Wynjorrotj *et al*., 2005
	Leaves	Decoction	Dermal lesions (1), "skin disease" (1), coughs (1) colds (1)	Mid Daly River area, NT (Wagiman)	Liddy *et al*., 2006
Buchanania obovata Engl. (ANACARDIACEAE)	Trunk bark*	Decoction*	Scabies (4), dermal lesions (2), dermal ulcers (2), weeping sores (2), eczema (2), infected rashes (2), herpes dermal eruptions (2), (topical) headache (1), (topical) fever (1),	Elsey, NT (Mangarrayi); Minyerri, NT; Borroloola, NT; (Alawa); Flora River area (NT) (Wardaman); Daly River area, NT (MalakMalak/ Matngala); Borroloola, NT	Wightman *et al*., 1991; Wightman *et al*., 1992; Barr *et al*., 1993; Raymond *et al*., 1999; Lindsay *et al*., 2001
	Trunk inner bark/timber	Shavings mixed with small quantity of water and applied directly	Toothache (1)	Yirrkala, NT (Rirratjingu)	Yunupingu *et al*., 1994
	New growth terminal stem (outer bark removed)*	Infusion or decoction*	Toothache (3), wounds and dermal lesions (1), sore eyes (1)	Groote Eyelandt (Warnindilyakwa); Yirrkala, NT (Rirratjingu); Galiwin'ku, NT;	Levitt and Lyon, 1981; Barr *et al*., 1993
	New growth terminal stem (outer bark removed)	Shavings mixed with small quantity of water and applied directly	Toothache (1)	Groote Eyelandt (Warnindilyakwa)	Levitt and Lyon, 1981

*: Shaded entries refer to extracts prepared and examined in this study; NT: Northern Territory; WA: Western Australia; SA: South Australia; NSW: New South Wales; Vic: Victoria; Qld.: Queensland

Table 3.2 (Continued from previous page)

Species (FAMILY)	Plant parts used	Methods of preparation	Uses and Indications cited for therapeutic use	Region/Community (Language/group)	Source
Buchanania obovata Engl. (ANACARDIACEAE)	Terminal twigs, petioles or leaf mid-vein	Outer tissue layers removed and heated part applied directly to cavity	Toothache (5)	Milingimbi, NT (Yolngu); Anguruguru, NT; Galiwin'ku, NT; Maningrida, NT; Minyerri, NT (Alawa)	Wightman and Smith, 1989; Barr *et al*., 1993
	Fruit	Crushed and mixed with water	Constipation (2), gastrointestinal distress (1)	Katherine/Nitmiluk region, NT (Jarwoyn); Mid Daly River area, NT (Wagiman)	Wynjorrotj *et al*., 2005; Liddy *et al*., 2006
	young roots	heated and applied directly	Toothache (1)	Flora River area, NT (Wardaman)	Raymond *et al*., 1999
	Leaves	heated and applied topically	Haemostasis: Acute wounds and post partum (1), lactation inducer (1),'tonic' - (to hasten time in which an infant begins to walk) (1)	Groote Eyelandt, NT (Warnindilyakwa); Flora River area, NT (Wardaman)	Levitt and Lyon, 1981; Raymond *et al*., 1999
Callitris intratropica R.T.Baker & H.G.Sm. (CUPRESSACEAE)	Inner Bark (Pounded)*	Decoction (used topically with direct application of strip of bark when used to treat pain associated with diarrhoea)*	Wounds and dermal lesions (3), pain associated with diarrhoea (3)	Barunga, NT; Galiwin'ku, NT; Maningrida, NT	Barr *et al*., 1993
	Female cones	soaked in water until soft and inserted into birth canal	Haemostasis post partum (1)	Groote Eyelandt, NT (Warnindilyakwa)	Levitt and Lyon, 1981
	Timber	Burnt to produce smoke ('Smoking treatment')	Respiratory/flu like illnesses (1)	Nauiyu Nambiyu, NT	Marrfurra *et al*. 1995; Barr *et al*., 1993
Camptostemon schultzii Mast. (BOMBACACEAE)	Timber*	Infusion of charred wood pieces*	Fungal dermatophytic infection (2), dermal lesions (2), inflamed/irritated skin conditions (2), scabies (2)	Galiwin'ku, NT; Ramingining, NT	Barr *et al*., 1993
	Timber	Charred and ground charcoal applied directly or pieces soaked in sea water to soften and applied directly	Fungal dermatophytic infection	Milingimbi, NT (Yolngu); Galiwin'ku, NT; Ramingining, NT	Wightman and Smith, 1989; Barr *et al*., 1993

*: Shaded entries refer to extracts prepared and examined in this study; NT: Northern Territory; WA: Western Australia; SA: South Australia; NSW: New South Wales; Vic: Victoria; Qld.: Queensland

Table 3.2 (Continued from previous page)

Species (FAMILY)	Plant parts used	Methods of preparation	Uses and Indications cited for therapeutic use	Region/Community (Language/group)	Source
Cochlospermum fraseri Planch. (BIXACEAE)	Immature fruit flesh*	Juice expressed for direct application*	Localised dermal infections e.g. boils (1)	Bulla, NT (Ngarinyman)	Smith, 1993; Barr *et al*., 1993
	Inner bark and flowers	Decoction	Fever (1)	Bulla, NT (Ngarinyman)	Smith, 1993; Barr *et al*., 1993
Crinum angustifolium R.Br. (AMARYLLIDACEAE)	Bulb*	Decoction or infusion (macerated/crushed bulb)*	Dermal lesions (7), wounds (4), blisters (4), boils (4), leprotic lesions (4), fungal dermatophytic infections (3), Joint and muscle pain (2), sunburn (1), herpes lesions (1), rectal lesions and abscess (1), general pain (topical application) (1), respiratory/flu like illnesses (topical application) (1)	Groote Eyelandt, NT (Warnindilyakwa); Minyerri, NT (Alawa); Kulamindini/Elliott, NT (Mudburra); Umbakumba, NT; Kulamindini/Elliott, NT (Mudburra); Anguruguru, NT; Katherine/Nitmiluk region, NT (Jarwoyn)	Levitt and Lyon, 1981; Wightman *et al*., 1991; Wightman *et al*., 1992b; Barr *et al*., 1993; Wynjorrotj *et al*., 2005
	Bulb	Segments applied as dressing	Dermal wounds and lesions (2), rectal dermal lesions and abscess (1), haemostasis of acute wounds (1)	Groote Eyelandt, NT (Warnindilyakwa); Umbakumba, NT	Levitt and Lyon, 1981; Barr *et al*., 1993
Cymbopogon ambiguus (Hack.) A.Camus (POACEAE)	Leaves or entire plant*	Cold or hot infusion*	Fever and symptoms of upper respiratory tract infections (topical application) (5) - (Oral) (3), dermal lesions (1) scabies (1), headache/muscle cramps (topical application) (1), eye wash (1), unspecified oral use ("medicinal drink") (1)	Sandover/Bundey River area, NT (Alyawara); Aputula, NT; Punritjanta, NT; Yuelamu, NT; Yuendumu, NT; Central Australia (Various)	O'Connell *et al*., 1983; Barr *et al*., 1993; Latz, 1995
	Leaves	Crushed and vapour inhaled	Chest (Respiratory) complaints (1)	Central Australia (Various)	Latz, 1995

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Table 3.2 (Continued from previous page)

Species (FAMILY)	Plant parts used	Methods of preparation	Uses and Indications cited for therapeutic use	Region/Community (Language/group)	Source
Eremophila alternifolia R.Br. (MYOPORACEAE)	Leaves (dried or fresh)*	Infusion or decoction*	Respiratory-flu like illnesses (5), fever (5), headache (5), internal pain (5), malaise and pain associated with any illness (2), sedative (1), unspecified topical and oral use ("various ailments") (1)	Kaltukatjara, NT; Tjunti, NT; Tjuntinanta, NT; Wankari, NT; Ulbulla, NT; Central Australia (Various)	Barr *et al*., 1993; Latz, 1995
	Leaves	Poultice of crushed leaf paste applied to head and/or body	Malaise or pain associated with severe or chronic illness (2), 'Internal pain' (1), headache (1)	Wankari, NT; Mimili, SA (Yankunytjatjara)	Barr *et al*., 1993; Goddard and Kalotas, 2002
Eremophila duttonii F.Muell. (MYOPORACEAE)	Leaves*	Decoction*	Infected wounds and dermal lesions (1), painful ears (1), inflamed eyes (1), sore throat (Oral) (1), respiratory-flu like illnesses (Topical application) (1)	Ltyente Purte, NT (Arrernte)	Barr *et al*., 1993
Eremophila latrobei F.Muell. (MYOPORACEAE)	Leaves*	Infusion or decocton*	Symptoms of respiratory-flu like illnesses (4), cough (3), sore throat (3) (Topical and/or Oral), scabies (1), chronic malaise (1)	Aputula, NT; Punritjanta, NT; Wankari, NT; Yuendumu, NT; Central Australia (Arrernte)	Barr *et al*., 1993; Latz, 1995
Eremophila longifolia (R.Br.) F.Muell. (MYOPORACEAE)	Leaves	Leaves burnt to produce smoke	'Smoking' of infants/neonates (1)	Central Australia (Warlpiri)	Latz, 1995
		Crushed and applied directly	Chronic malaise and symptoms of Respiratory-flu like illnesses (1)	Aputula, NT	Barr *et al*., 1993
		Leaves burnt to produce smoke	'Smoking' of neonates and mothers (lactation inducer) (1)	Central Australia (Arandic)	Latz, 1995
	Leaves (Fresh or dried)*	Infusion or decoction (leaves usually 'crushed')*	Headache and chest pain associated with respiratory-flu like illnesses (2) (Topical application), dermal lesions (2), eye wash (2), respiratory-flu like illnesses (Oral application) (1) scabies (1), boils (1), 'dermal ailments' (1), bites and stings (1), relief of pain of aching joints and muscles (Topical) (1), headache (Topical) (1), Topical application for various ailments (1), aid to produce "restful and restorative sleep" (1)	Bogan River area, NSW; Alyawara; Ulbulla, NT; Yuelamu, NT; Ltyente Purte, NT (Arrernte); Central Australia (Various)	Webb, 1969; O'Connell *et al*., 1983; Barr *et al*., 1993; Latz, 1995

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Table 3.2 (Continued from previous page)

Species (FAMILY)	Plant parts used	Methods of preparation	Uses and Indications cited for therapeutic use	Region/Community (Language/group)	Source
Eremophila longifolia (R.Br.) F.Muell. (MYOPORACEAE)	Leaves (fresh or dried)	Pounded to paste with small quantity of water and applied directly	Scabies (1), boils (1)	Ltyente Purte, NT (Arrernte)	Barr *et al*., 1993
	Leaves (dried)	Mixed with animal fat	Scabies (1), "liniment" for swollen tender joints (1)	Ltyente Purte, NT (Arrernte)	Barr *et al*., 1993, 1988
Eremophila sturtii R.Br. (MYOPORACEAE)	Leaves*	Decoction*	Wounds and "open sores" (1)	Ltyente Purte, NT (Arrernte)	Barr *et al*., 1993
	Leaves	Leaves macerated and applied directly	Dermal infections (1)	Lightning Ridge, NSW (Kamilaroi/Muwarri)	Liu, 2006
	Leaves/branches	Leaves/branches burnt to produce smoke	Backache, pain (Smoke vapour) (2), respiratory-flu like illnesses (Smoke vapour inhalation) (1)	Central Australia (Various); Mimili, SA (Yankunytjatjara)	Latz 1995; Goddard and Kalotas, 2002
	Leaves	Added to bathtub	Respiratory-flu like illnesses (bath preparation) (1)	Ltyente Purte, NT (Arrernte)	Barr *et al*., 1993
	Leaves	Boiled in water to produce steam vapour	To relieve soreness of eyes (steam vapour) (1)	Ltyente Purte, NT (Arrernte)	Barr *et al*., 1993
Erythrophleum chlorostachys (F.Muell.) Baill. (FABACEAE)	Inner bark*	Decoction*	Dermal lesions (6), wounds (1), scabies (2), fungal dermatophytic infection (1) dermal rash (1) "skin disorders" (1) venereal disease (1)	Elsey, NT (Mangarrayi); Kulamindini/Elliott, NT (Mudburra); Bathurst/Melville Islands, NT (Tiwi); Gurig NP area, NT (Iwaidja) Katherine/Nitmiluk region, NT (Jarwoyn)	Wightman *et al*., 1992; Wightman *et al*., 1992b; Blake *et al*., 1998; Puruntatameri *et al*., 2001; Wynjorrotj *et al*., 2005
	Leaves and inner bark*	Decoction*	Infected wounds and dermal lesions (3)	Gulin Gulin, NT; Minjilang, NT; Warruwi, NT	Barr *et al*., 1993

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Table 3.2 (Continued from previous page)

Species (FAMILY)	Plant parts used	Methods of preparation	Uses and Indications cited for therapeutic use	Region/Community (Language/group)	Source
Erythrophleum chlorostachys (F.Muell.) Baill. (FABACEAE)	Inner bark and roots	Infusion	Wounds and dermal lesions	Broome area, WA	Webb, 1969
	Leaves	Heated and leaf and/or exuded fluid applied directly	Pain relief (Topical) (2), infected sores (1), pruritic rash (1)	Gunbalanya, NT; Mid Daly River area, NT (Wagiman)	Barr *et al*., 1993; Liddy *et al*., 2006
	Leaves	Cold infusion	Oedema	Milingimbi, NT (Yolngu)	Barr *et al*., 1993
	Leaves	Leaves burnt to produce smoke	'Smoking' of infants/neonates (2)	Nauiyu Nambiyu, NT; Bathurst/Melville Islands, NT (Tiwi)	Marrfurra *et al*., 1995; Puruntatameri *et al*., 2001
	Root bark	Shaved and burnt to produce smoke	Lactation modifier (Smoke vapour)	Galiwin'ku, NT	Barr *et al*., 1993
Eucalyptus tetrodonta F.Muell. (MYRTACEAE)	Juvenile leaves*	Decoction or infusion*	Dermal lesions (6), expectorant (Oral) (1), influenza (Oral) (1)	Numbulwar, NT; Galiwin'ku, NT; Minjilang, NT; Umbakumba, NT; Warruwi, NT; Yirrkala, NT; Gurig NP area, NT (Iwaidja)	Webb, 1969; Barr *et al*., 1993; Blake *et al*., 1998
	Juvenile leaves	Expressed juice applied directly	Dermal lesions (1)	Yirrkala, NT	Barr *et al*., 1993
	Leaves	Heated and applied directly	Relief from pain of aching bones and muscles (1)	Gunbalanya, NT	Barr *et al*., 1993
	Inner bark*	Hot or cold infusion*	Dermal lesions (2), scabies (2), oral infections, red sore toungue (Mouthwash) (1), diarrhoea (Oral) (1)	Groote Eyelandt, NT (Warnindilyakwa); Galiwin'ku, NT; Minjilang, NT; Milingimbi, NT (Yolngu);	Webb, 1969; Wightman and Smith, 1989; Barr *et al*., 1993

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Table 3.2 (Continued from previous page)

Species (FAMILY)	Plant parts used	Methods of preparation	Uses and Indications cited for therapeutic use	Region/Community (Language/group)	Source
Eucalyptus tetrodonta F.Muell. (MYRTACEAE)	Inner bark and sapwood of juvenile plant	Infusion or decoction	Lesions in mouth and throat (1), bronchitis (Oral) (1)	Groote Eyelandt, NT (Warnindilyakwa); Maningrida, NT	Levitt and Lyon, 1981; Barr *et al*., 1993
Flagellaria indica L. (FLAGELLARIACEAE)	Terminal nodes*	Expressed juice applied directly*	"Eye drops" (1)	Groote Eyelandt, NT (Warnindilyakwa)	Levitt and Lyon, 1981
	Stems (outer part occasionally removed)*	Warm infusion or decoction*	Topical treatment for fever and symptoms of respiratory-flu like illnesses (2), pain associated with gastrointestinal disorders (2), irrigation of eye (1)	Groote Eyelandt, NT (Warnindilyakwa); Paru, NT; Bathurst/Melville Islands, NT (Tiwi);	Levitt and Lyon, 1981; Barr *et al*., 1993; Puruntatameri *et al*., 2001
	Leaves	Chewed and transferred to mouth	Oral lesions in infants (1)	Bathurst/Melville Islands, NT (Tiwi)	Puruntatameri *et al*., 2001
Hibiscus tiliaceus L. (MALVACEAE)	Stems (outer bark removed)*	Infusion or heated infusion*	Wounds and compound fracture wounds (1), boils (1)	Groote Eyelandt, NT (Warnindilyakwa)	Levitt and Lyon, 1981
	Stem bark	Expressed juice applied directly or mixed with small quantity of water then boiled	Skin eruptions, e.g. Boils (2)	Yirrkala, NT; Galiwin'ku, NT	Barr *et al*., 1993
	Leaves	Heated and applied directly	Haemostasis for acute wound	Groote Eyelandt, NT (Warnindilyakwa)	Levitt and Lyon, 1981
Ipomoea pes-caprae (L.) R.Br. (CONVOLVULACEAE)	Leaves*	Decoction or infusion (Often of 10 leaves)*	Scabies (4), insect stings and bites (1), respiratory flu like illness (Oral) (1)	Milikapiti, NT; Maningrida, NT; Pularumpi, NT; Paru, NT; Bathurst/Melville Islands, NT (Tiwi)	Barr *et al*., 1988, 1993; Puruntatameri *et al*., 2001
	Leaves	Applied directly (often with heating or boiling prior to application)	Analgesia for insect and marine stings (10), dermal wounds and abrasions, dermal lesions, ulcers (4) haemostasis for acute wounds (3), boils (3), aching muscles (1), snake and spider bites (1), oedema (1), toothache (1)	Aurukun, Qld.; Groote Eyelandt, NT (Warnindilyakwa); Gurig NP area, NT (Iwaidja); Milingimbi, NT (Yolngu); Galiwin'ku, NT; Minjilang, NT; Warruwi, NT; Yirrkala, NT (Rirratjingu); Belyuen (Emi/Batjamal); Umbakumba, NT; Wadeye	Webb, 1969; Levitt and Lyon, 1981; Wightman and Smith, 1989; Barr *et al*., 1993; Yunupingu *et al*., 1994; Blake *et al*., 1998

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Table 3.2 (Continued from previous page)

Species (FAMILY)	Plant parts used	Methods of preparation	Uses and Indications cited for therapeutic use	Region/Community (Language/group)	Source
Ipomoea pes-caprae (L.) R.Br. (CONVOLVULACEAE)	Leaves and stem	Tied around head or affected part	Headache, pain and inflammation (Topical) (4)	Belyuen (Emi/Batjamal); Minjilang, NT; Wadeye, NT; Warruwi, NT	Smith and Wightman, 1989; Barr *et al*., 1993
	Entire plant	Decoction	Venereal disease (Oral) (1)	Aurukun, Qld.	Webb, 1969
	Flowers	Applied directly	Skin disorders (1)	Bathurst/Melville Islands, NT (Tiwi)	Puruntatameri *et al*., 2001
Morinda citrifolia L. (RUBIACEAE)	Unripened green fruit*	Expressed juice applied directly*	Dermal lesions (1)	Groote Eyelandt, NT (Warnindilyakwa)	Levitt and Lyon, 1981
	Ripened fruit	(Flesh eaten)	Respiratory-flu like illnesses (10), cough (9), sore throat (8), asthma (2)	Belyuen, NT (Emi/Batjamal); Milingimbi, NT (Yolngu); Galiwin'ku, NT; Maningrida, NT; Ramingining, NT; Umbakumba, NT; Warruwi, NT; Yirrkala, NT (Rirratjingu); Gurig NP area, NT (Iwaidja); Daly River area, NT (MalakMalak/ Matngala)	Smith and Wightman, 1989; Wightman and Smith, 1989; Barr *et al*., 1993; Yunupingu *et al*., 1994; Blake *et al*., 1998; Lindsay *et al*., 2001
Pandanus spiralis R.Br. †† (PANDANACEAE)	Flesh at leaf base*	Decoction (in small quantity of water)*	Eye drops for "tired and sore eyes", inflammation and infection of eye (1)	Bulla, NT (Ngarinyman)	Smith, 1993; Barr *et al*., 1993
	Flesh at leaf base	Expressed juice diluted with small quantity of water and applied directly	Infected dermal lesions (1)	Galiwin'ku, NT	Barr *et al*., 1993
	Flesh at leaf base	Applied directly or eaten	Infections of mouth and throat (4), abdominal pain and diarrhoea (Oral) (1), wounds (1), backache (Topical) (1)	Groote Eyelandt, NT (Warnindilyakwa); Galiwin'ku, NT; Milingimbi, NT (Yolngu); Ramingining, NT; Yirrkala, NT	Levitt and Lyon, 1981; Barr *et al*., 1993; Yunupingu *et al*., 1994
	Inner part of stems*	Expressed juice applied directly or diluted with small quantity of water*	Toothache/infections of mouth and throat (Mouthwash) (3), dermal lesions of the lips and mouth (1)	Milingimbi, NT (Yolngu); Galiwin'ku, NT; Ramingining, NT; Yirrkala, NT	Barr *et al*., 1993

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Table 3.2 (Continued from previous page)

Species (FAMILY)	Plant parts used	Methods of preparation	Uses and Indications cited for therapeutic use	Region/Community (Language/group)	Source
Pandanus spiralis R.Br. †† (PANDANACEAE)	Inner part of stems	Pounded flat and heated on fire or by boiling and applied directly as poultice	Swollen joints and skeleto-muscular pain (2)	Maningrida, NT; Warruwi, NT	Barr *et al*., 1993
	Inner stem and growing tip	Roasted and chewed	Abdominal pain and diarrhoea (Buccal) (1)	Bathurst/Melville Islands, NT (Tiwi)	Puruntatameri *et al*., 2001
	Stems or leaves	Sections or strips wrapped around stick, heated over fire and applied directly	Abdominal pain (Topical) (1)	Bathurst/Melville Islands, NT (Tiwi)	Puruntatameri *et al*., 2001
	Inner timber	Infusion	"Used as an antiseptic" (1)	Milingimbi, NT (Yolngu)	Wightman and Smith, 1989
	Prop roots	Decoction	Scabies (1)	Bulla, NT (Ngarinyman)	Smith, 1993; Barr *et al*., 1993
	Fruit	Burnt in pit before covering with stems of *Elocharis dulcis* to produce steam and heat	Backache (steam/smoke vapour) (1)	Yirrkala, NT (Rirratjingu)	Yunupingu *et al*., 1994
Scaevola sericea Vahl (GOODENIACEAE)	Ripe fruit*	Juice expressed directly into eye*	Remedy for infection of eye, to relieve redness and soreness of eye (2)	Angurugu, NT; Minjilang, NT	Barr *et al*., 1993
	Ripe fruit and young stems	Juice expressed and applied directly	Treatment of "bites and stings" (1)	Angurugu, NT	Barr *et al*., 1993

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Table 3.3 Major use agreement values (MUA_{TMP}) for three defined categories of use in 32 plant derived medicinal preparations

Species	Extract Description	Total number of studies citing any use (TU)	All dermatological/ophthalmic complaints				'Sores'(Dermal lesions)				Wounds			
			SU	FL	CF	MUA	SU	FL	CF	MUA	SU	FL	CF	MUA
Crinum angustifolium	Cold infusion of bulb	7	7	100	0.8	**78**	7	100	0.8	**78**	4	57	1.2	**67**
Crinum angustifolium	Decoction of bulb	7	7	100	0.8	**78**	7	100	0.8	**78**	4	57	1.2	**67**
Eucalyptus tetrodonta	Terminal leaf decoction	7	6	86	0.8	**67**	6	86	0.8	**67**	0	0	1.2	**0**
Erythrophleum chlorostachys	Trunk inner bark decoction	6	5	83	0.7	**56**	5	83	0.7	**56**	1	17	1.0	**17**
Buchanania obovata	Decoction of inner trunk bark	6	4	67	0.7	**44**	4	67	0.7	**44**	2	33	1.0	**33**
Ipomoea pes-caprae	Hot infusion of leaves	5	4	80	0.6	**44**	0	0	0.6	**0**	0	0	0.8	**0**
Ipomoea pes-caprae	Cold infusion of leaves	5	4	80	0.6	**44**	0	0	0.6	**0**	0	0	0.8	**0**
Callitris intratropica	Decoction of inner trunk bark	3	3	100	0.3	**33**	3	100	0.3	**33**	3	100	0.5	**50**
Erythrophleum chlorostachys	Leaf/inner trunk bark decoction	3	3	100	0.3	**33**	3	100	0.3	**33**	1	33	0.5	**17**
Acacia holosericea	Decoction of leaves	2	2	100	0.2	**22**	2	100	0.2	**22**	0	0	0.3	**0**
Camptostemon schultzii	Cold infusion charred wood	2	2	100	0.2	**22**	2	100	0.2	**22**	0	0	0.3	**0**
Scaevola sericea	Expressed juice of fruit	**2**	2	100	0.2	**22**	0	0	0.2	**0**	0	0	0.3	**0**
Bauhinia cunninghamii	Decoction of root	2	2	100	0.2	**22**	2	100	0.2	**22**	1	50	0.3	**17**
Eucalyptus tetrodonta	Decoction of trunk inner bark	4	2	50	0.4	**22**	2	50	0.4	**22**	0	0	0.7	**0**
Eremophila longifolia	Decoction of leaves	6	2	33	0.7	**22**	2	33	0.7	**22**	0	0	1.0	**0**
Acacia auriculiformis	Decoction of leaves	2	1	50	0.2	**11**	1	50	0.2	**11**	1	50	0.3	**17**
Eremophila duttonii	Decoction of leaves	1	1	100	0.1	**11**	1	100	0.1	**11**	1	100	0.2	**17**
Eremophila sturtii	Decoction of leaves	1	1	100	0.1	**11**	1	100	0.1	**11**	1	100	0.2	**17**
Hibiscus tiliaceus	Warm infusion of stems	1	1	100	0.1	**11**	0	0	0.1	**0**	1	100	0.2	**17**
Hibiscus tiliaceus	Cold infusion of terminal stems	1	1	100	0.1	**11**	0	0	0.1	**0**	1	100	0.2	**17**
Avicennia marina	Decoction of leaves	1	1	100	0.1	**11**	1	100	0.1	**11**	0	0	0.2	**0**
Morinda citrifolia	Expressed juice of unripe fruit	1	1	100	0.1	**11**	1	100	0.1	**11**	0	0	0.2	**0**
Cochlospermum fraseri	Expressed juice of fruit pulp	1	1	100	0.1	**11**	0	0	0.1	**0**	0	0	0.2	**0**
Eremophila latrobei	Decoction of leaves	5	1	20	0.6	**11**	0	0	0.6	**0**	0	0	0.8	**0**
Flagellaria indica	Expressed juice of growing tips	1	1	100	0.1	**11**	0	0	0.1	**0**	0	0	0.2	**0**
Pandanus spiralis	Decoction of leaf bases	2	1	50	0.2	**11**	0	0	0.2	**0**	0	0	0.3	**0**
Buchanania obovata	Cold infusion of terminal stems	3	1	33	0.3	**11**	1	33	0.3	**11**	1	33	0.5	**17**
Buchanania obovata	Decoction of terminal stems	3	1	33	0.3	**11**	1	33	0.3	**11**	1	33	0.5	**17**
Cymbopogon ambiguus	Decoction of whole plant	6	1	17	0.7	**11**	1	17	0.7	**11**	0	0	1.0	**0**
Pandanus spiralis	Expressed juice of stem flesh	3	1	33	0.3	**11**	1	33	0.3	**11**	0	0	0.5	**0**
Flagellaria indica	Warm infusion of stems	3	1	33	0.3	**11**	0	0	0.3	**0**	0	0	0.5	**0**
Eremophila alternifolia	Decoction of leaves	6	0	0	0.7	**0**	0	0	0.7	**0**	0	0	1.0	**0**

SU: Number of independent studies citing specific use; FL: Fidelity level; CF: Correction factor; MUA: Major use agreement. (For sources refer to Table 1.1)

Table 3.4 Sequential extractions of *Eremophila* spp. leaves with hexane, DCM, and MeOH

Species	Collectors number	Plant parts	Extraction /Solvent	Quantity extracted (g)	Extract ID
Eremophila alternifolia	JES021	Leaves	1' Hexane		JES021H
Eremophila alternifolia	JES021	Leaves	2' DCM	125	JES021H
Eremophila alternifolia	JES021	Leaves	3' MeOH		JES021H
Eremophila alternifolia	JES023	Leaves	1' Hexane		JES021H
Eremophila alternifolia	JES023	Leaves	2' DCM	125	JES021H
Eremophila alternifolia	JES023	Leaves	3' MeOH		JES021H
Eremophila duttonii	SM011	Leaves	1' Hexane		SM011H
Eremophila duttonii	SM011	Leaves	2' DCM	268	SM011D
Eremophila duttonii	SM011	Leaves	3' MeOH		SM011M
Eremophila latrobei	SM006	Leaves	1' Hexane		SM011H
Eremophila latrobei	SM006	Leaves	2' DCM	444	SM006D
Eremophila latrobei	SM006	Leaves	3' MeOH		SM006M
Eremophila longifolia	SM003	Leaves	1' Hexane		SM003H
Eremophila longifolia	SM003	Leaves	2' DCM	266	SM003D
Eremophila longifolia	SM003	Leaves	3' MeOH		SM003M
Eremophila sturtii	SM001	Leaves	1' Hexane		SM001H
Eremophila sturtii	SM001	Leaves	2' DCM	250	SM001D
Eremophila sturtii	SM001	Leaves	3' MeOH		SM001M

3.2 **Traditional preparations**

Thirty-four unique traditional extracts were prepared along with replicates for the *Eremophila* species collected, which were prepared using material derived from separate plant specimens (thirty-seven preparations in total). Three extracts were prepared from the leaves of three separate *Eremophila longifolia* specimens, each representative of one of the three essential oil chemotypes identified during the course of the study. These included isomenthone, karahanaenone and borneol oil chemotypes (*cf*. Chapter 6). Of the thirty-seven traditional preparations, twenty-one had been cited for use in the treatment of sores and fourteen for the treatment of wounds. The remainder of the preparations (with one exception) were cited for a range of other therapeutic dermatological/ophthalmic uses (e.g. scabies, boils, pruitis etc.) with 2 preparations cited solely for the treatment of ophthalmic complaints (expressed juice of *Flagellaria indica* terminal nodes and fruit of *Scaevola sericea*). The only extract which was not mentioned for use in treatment of dermatological or ophthalmic complaints in the

literature cited, was the decoction of *Eremophila alternifolia* leaves. This species was originally collected on the basis of a citation appearing in a publication reviewing ethnobotanical uses of *Eremophila* spp., for the use of decoctions in the treatment of 'septic wounds'. On subsequent review of the primary source referenced (Bowen, 1975), it was found that this citation may have been a typographical error, possibly intended to refer instead to the species *Eremophila bignoniflora* as Bowen (1975) describes the indigenous use of only *E. bignoniflora* decoctions for the treatment of "septic wounds" and does not mention *E. alternifolia* in relation to any medicinal or economic purposes. Despite the lack of specific reference to dermatological use, it is very likely that *E. alternifolia* has been used for a much wider variety of purposes than those documented, given that it is held in particularly high regard as a medicinal plant by many indigenous groups and is reported to have been dried, stored and carried on long journeys for use as a general purpose medicine (Ghisalberti, personal communication). Extracts of *E. alternifolia* were retained in the study but, for the sake of consistency in the methods used to determine MUA_{TMP} values, were not considered as preparations used therapeutically for dermatological complaints. Extract concentrations and yields were seen to be dependent on the type and duration of extraction and the quantity of material extracted. Extract concentration ranged from 0.8 mg · mL^{-1} (cold infusion of 10 *Ipomoea pes-caprae* leaves), to 118 mg · mL^{-1} (expressed juice of *F. indica* terminal nodes), while yield ranged from 1.5% (diluted juice of *Pandanus spiralis* stem flesh) to 19.1% (decoction of *Eremophila sturtii* leaves which were highly resinous and viscous). Extraction by expression of juice produced the highest concentrations among the preparations. The mean concentration for the 5 expressed juice preparations was 71.7 ± 17.3 mg · mL^{-1}, which was around 10× greater than that seen for the 24 decoction type extractions (8.4 ± 0.96 mg · mL^{-1}) and 8 infusion type extractions (7.1 ± 2.05 mg · mL^{-1}). ANOVA followed by a Tukey test showed this difference to be significant (p <0.001) (Figure 3.1). Mean concentration of the

decoction and infusion type extractions did not appear to be significantly different and this could be attributed largely to the longer periods of extraction which were typical of the infusions. Many of the infusions were extracted overnight (e.g. 20 hr) as compared to 15 min for the decoctions (e.g. 15 min decoction of 135 gm of *Crinum angustifolium* macerated bulb produced an extract with concentration of 18.8 mg · mL^{-1} while infusion of the same quantity over 20 hr produced a similar concentration of 18.0 mg · mL^{-1}). Also, many of the infusions also involved larger quantities of plant material than were indicated for most of the decoctions and while concentration in these extracts was often higher, yields were typically lower [e.g. 15 min decoction of 17 gm of the inner bark and sapwood of *Buchanania obovata* terminal stems as described by Barr *et al*. (1988), produced an extract of 4.2 mg · mL^{-1} with yield of 9.5% while a 3 hr infusion, as described by Levitt and Lyon (1981) using 70 gm of the same material, produced an extract of 9.7 mg · mL^{-1} with yield of 6.9%]. Mean yield for the 24 decoctions was 8.7 ± 0.73%, which was around twice that seen for the 8 infusions (4.0 ± 0.55%) and 5 expressed juice extractions (2.4 ± 0.63%). ANOVA followed by a Tukey test showed this difference to be significant (p <0.05) (Figure 3.1). As might be expected, when equal quantities of the same plant material were extracted with equal volumes of either hot or cold water (as was the case with the infusions of *I. pes-caprae* leaves), the higher temperature extraction resulted in both greater yield and concentration. The majority of the aqueous extracts were slightly acidic, with pH ranging from 3.54 to 6.20 with the exception of the alkaline extract (pH 9.71) produced by infusion of the charred wood of *Camptostemon schultzii*. This extract was prepared with pieces of timber which had been partially burnt over a flame, after descriptions by Barr *et al*. (1993), of a preparation used for the treatment of fungal dermatophytic infections, sores, scabies and other inflamed/irritated skin conditions in the NT coastal/estuarine communities of Galiwin'ku (Elcho Island) and Ramingining (East Arnhem).

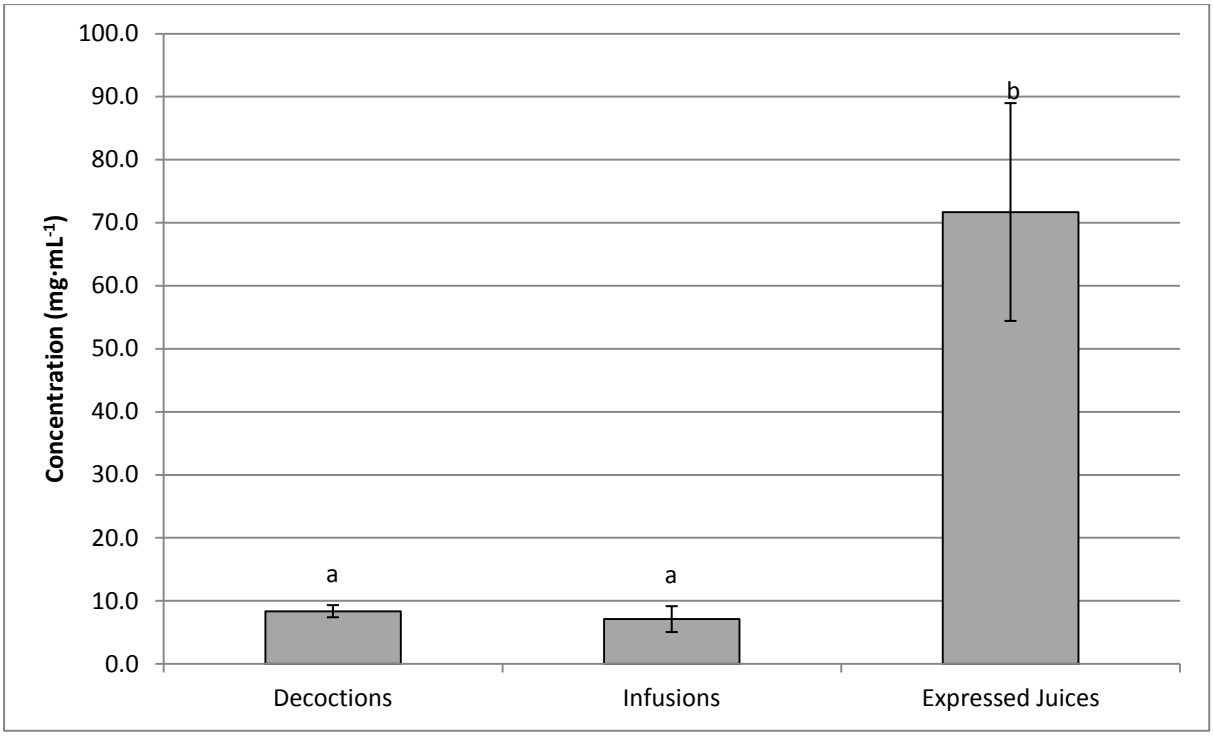


Figure 3.1 Bar chart showing the means with standard error bars of the concentration of three traditional extraction methods. Decoctions n = 24, infusions n = 8, expressed juices n = 5). Letters denote significant differences between groups ($p < 0.001$). Groups denoted by same letter not significantly different

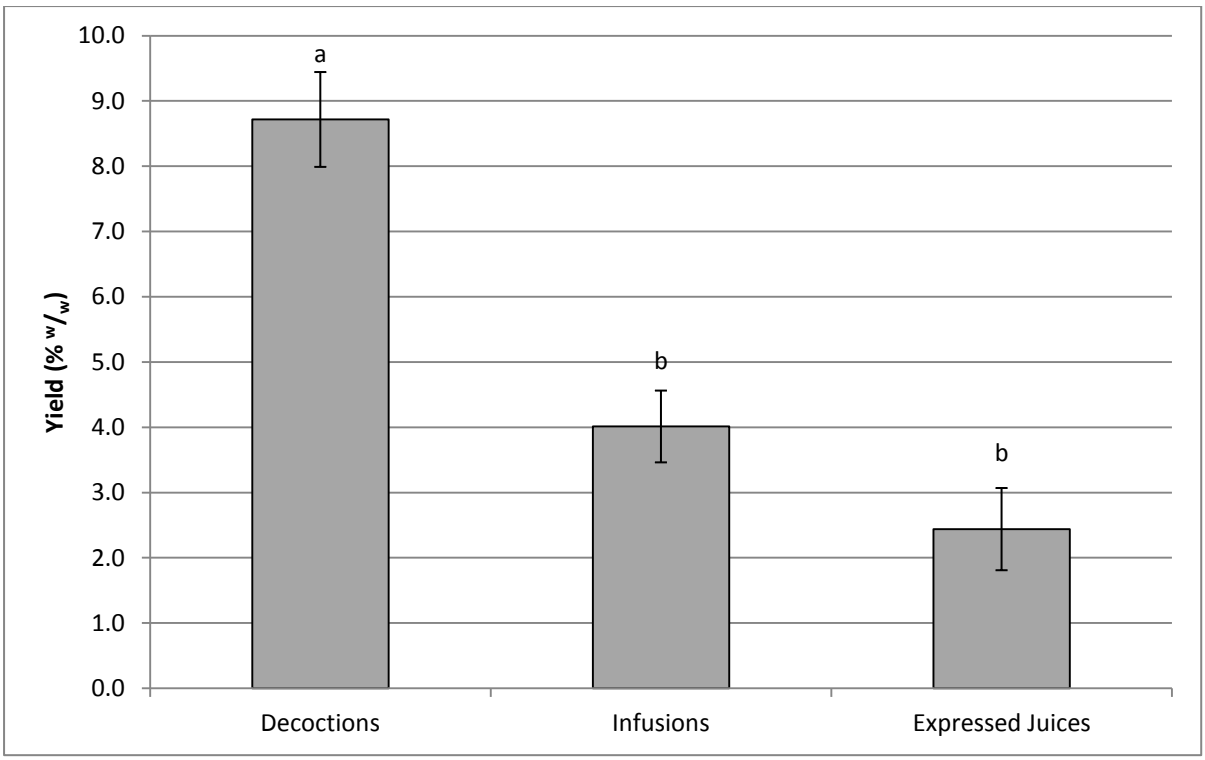


Figure 3.2 Bar chart showing the means with standard error bars of the yield of three traditional extraction methods. Decoctions n = 24, infusions n = 8, expressed juices n = 5). Letters denote significant differences between groups ($p < 0.05$). Groups denoted by same letter not significantly different

Deviations from traditional methods as described in the literature included the freezing of plant material prior to extraction, sterilisation by filtration prior to bioassay, lyophilisation of extracts and storage of extracts at low temperature between bioassays. Freshly collected plant material was frozen as soon as possible following collection in an attempt to preserve the material and prevent drying or decomposition in the period between collection and extraction. While this was considered necessary, the freezing of plant material may have affected composition of the subsequent extracts through degradation of cellular structures not tolerant to freezing (e.g. phospholipid membranes). Freeze-thaw cycles of both the plant material and extracts in solution may also have lead to degradation of constituents susceptible to this effect. To minimise any effects of freeze-thaw cycles, all extracts were divided into small portions (sufficient for at least one assay) prior to freezing which were subsequently thawed once for each assay, with any unused portion discarded. Sterilisation by filtration was necessary for the cell based and microbiological bioassays. Extracts were filtered under reduced pressure using Whatman No. 1 paper and then sterile cellulose acetate filters (0.45 and 0.22 μm). It is acknowledged that some compounds (including those potentially active in subsequent bioassays) may have been retained on the filter material. This was a distinct possibility in at least 2 cases i.e. the decoction of *Erythrophleum chlorostachys* inner bark and extracts of *Hibiscus tiliaceus* stem bark. The extraction of *H. tiliaceus* stem bark produced a viscous, mucilaginous solution which was slippery to the touch. This was considered normal and this quality in infusion type preparations of the stem and stem bark has been noted by Levitt and Lyon (1981). While the filtered extracts retained this quality, it was noted that filtration was relatively slow (i.e. filters were probably blocked) and a fine layer of mucilaginous material had been retained. It is likely that this material consisted partly of mucilaginous polysaccharides from the stem inner bark of this plant. High quantities of mucilaginous polysaccharides are known to accumulate in the edible fruit of other *Hibiscus* species, *H.*

esculentus ('okra') (Woolfe *et al.*, 1977). When the *E. chlorostachys* inner bark was extracted it was noted that a precipitate began to form during cooling (just prior to filtration) which similarly slowed filtration and was seen to be visibly retained on the filter.

Extracts were lyophilised for the purpose of determining concentration and allowing subsequent preparation of solutions of defined concentration. It is possible that lyophilisation of the extracts under vacuum may have resulted in the loss of, or reduction in, concentration of some volatile constituents.

In most cases the specific material required for each preparation (e.g. ripened vs unripened fruit, material from plants of appropriate developmental stage etc.) was able to be collected and certain properties of the extracts generally matched the descriptions given in the sources (e.g. colour, consistency etc.). Material from *Cymbopogon ambiguus*, however, could not be collected in the appropriate stage of life cycle required. *C. ambiguus* was considered for collection based on descriptions by Barr *et al*. (1988), of traditional use of decoctions for the treatment of dermal sores and scabies infestation. Nearly all descriptions of traditional medicinal use of this plant and other *Cymbopogon* spp. in Australia stipulate that only green plants should be used for medicinal preparations. Local indigenous people accompanying the author during field collections at Jodetluk (NT) also expressed this opinion and advised that the season had passed in which the plant should be used for medicinal purposes and that the senescent material was inappropriate for these purposes. At the time of collection (late August - early dry season), all *Cympopogon* sp. had matured and aerial parts were in a state of senescence. The plant was nonetheless collected with the intention that further, more appropriate specimens would be collected the following year, so that any extracts produced could be compared. Unfortunately, budgetary and time constraints prevented any further fieldwork and only the original specimen was included among the plants extracted.

Experimental results obtained with this extract were retained and are presented below, however, it is acknowledged that this preparation may not be adequately representative of typical traditional preparations.

3.3 Antimicrobial activity of traditional type preparations and extracts - Results and discussion

Traditional extracts were screened for antimicrobial activity using disc diffusion and broth dilution assays. Extracts found active in initial screening assays were further tested to determine minimum inhibitory and minimum bactericidal concentrations. Organic solvent extracts were tested in a broth dilution screening assay and disc diffusion assays against the same panel of bacterial species, so that results could be compared with those obtained with the corresponding traditional preparations.

The disc diffusion experiments were successful in that no gross contamination of test cultures was apparent, confluent growth of cultures was achieved and antibiotic positive controls were consistent and as expected. Very little antimicrobial activity among the extracts was detected by disc diffusion screening assays, however, which was in definite contrast to results obtained using broth dilution assays, where activity was detected consistently in over half of the extracts. Discrepancies between results obtained by the two methods will be discussed below. For broth dilution assays, growth, sterility and positive antibiotic controls were typically acceptable and individual experiments were repeated in instances where this was not the case. No evidence of microbial contamination of extracts was seen in replicate un-inoculated plates incubated alongside inoculated test plates and no apparent contamination of inoculated plates was seen when streak subcultures were examined for purity at any stage during the experiments. Some difficulties were encountered with interpreting results for a few extracts which seemed to inhibit reduction of the p-INT to violet tetrazolium dye where growth was otherwise apparent (as indicated by turbidity indicating growth and lack of

inhibitory activity). This was most noticeable in experiments with *Escherichia coli* and *Staphylococcus epidermidis* where the overall intensity of the reduced tetrazolium dye was typically less than that seen with the other organisms employed. Extracts producing this effect included the boiled decoctions of leaf material from *Avicennia marina* and *E. longifolia* isomenthone and borneol chemotypes. Growth in these instances, however, could be easily assessed without addition of p-INT by appearance of turbid growth given that these extracts were completely soluble in media and were seen to remain so throughout the incubation period (as evidenced by comparison with un-inoculated control plates). The same technique could not be applied in those cases where the extract itself was turbid which obscured visual assessment of growth without the addition of p-INT. Subsequent assays for determination of MIC/MBC, however, showed that the possibility of false positive results in these cases due to inhibition of p-INT reduction was extremely unlikely as the serial dilutions reduced appearance of turbidity so that any bacterial growth could have been observed in the absence of p-INT addition and a clear point at which inhibition ceased was seen in all instances. For extracts which were both turbid in media and highly coloured or where colour was close to that of tetrazolium dye (e.g. red coloured extract of *Callitris intratropica* inner bark), a close comparison the with uninoculated control plate was made as suggested by Langfield *et al*. (2004). The additional step of subjecting the control plates to the same conditions of incubation was performed to account for any change in appearance which might have been induced by this process (e.g. gradual precipitation from solution, darkening of coloured extracts etc.). This was also considered a more robust and relevant control for extract sterility within the assays (rather than directly culturing a sample of the extracts), as all steps in manipulation and handling during preparation of assays was replicated alongside test plates. As discussed previously (3.2 above), the majority of the aqueous preparations were found to be slightly acidic (with the exception of the infusion of *C. schultzii* charred timber) with

measured pH values ranging from 3.54 to 6.20 (mean pH: 4.90, SD 0.64). The pH of extracts was not found to correlate with any particular activity examined, including any antibacterial activities subsequently found. In a study examining antimicrobial activity of Hawaiian medicinal plant preparations (extracted with expression of plant parts), Bushnell *et al*. (1950), reported a similar range of pH values amongst extracts and similarly found no correlation between antibacterial activity and pH, and no evidence of activity among controls consisting of a series of sterile buffers prepared to reproduce pH within the range observed in the extracts.

3.3.1 **Disc diffusion assays**

In disc diffusion assays, a sterile filter paper disc containing the substance to be tested is placed on the surface of solid media which has been spread inoculated with the microorganism of interest. During incubation, test substances diffuse outward from the disc into the solid media becoming increasingly diluted as diffusion proceeds. Organisms in the initial spread of inoculum multiply to the point where growth becomes visible as a confluent opaque layer on the agar surface. Circular clear zones (zones of inhibition) concentric to the discs remain where the diffused substance is at a concentration which prevents or inhibits growth. The point where the zone of inhibition begins can be seen to represent the minimum inhibitory concentration and the diameter of the zone of inhibition can thus indicate relative potency and/or initial concentration of the antimicrobial agent(s) when assuming a more or less equal rate of diffusion between separate test substances. Activity of test substances is typically recorded as diameter of the resulting zone of inhibition which in some cases can be correlated experimentally with corresponding MIC values.

Antimicrobial activity was detected in only two of the thirty-seven traditional type extracts tested with disc diffusion assays. These included the boiled decoction of trunk inner bark of *C.*

intratropica and the expressed juice from fruit pulp of *C. fraseri*. The *C. intratropica* extract produced small to moderate zones of growth inhibition against the bacteria *Staphylococcus aureus* (9 mm diameter) and *S. epidermidis* (6 mm diameter). The *C. fraseri* extract produced slightly larger zones of growth inhibition against the bacteria *S. aureus* (12 mm diameter) and *S. epidermidis* (11 mm diameter) and the yeast *Candida albicans* (7 mm) (Table 3.5). No activity by either extract was observed against any of the other microbial species examined.

Table 3.5 Traditional type aqueous preparations active in disc diffusion assays

				Zone of inhibition diameter (mm) for each organism*						
Species	**Plant part**	**Extraction type**	**Extract quantity per disc (µg)**	**SE**	**SA**	**BS**	**KP**	**EC**	**PA**	**CA**
Callitris intratropica	Trunk inner bark	Decoction	83	6	9	-	-	-	-	-
Cochlospermum fraseri	Fruit pulp	Expressed juice	571	11	12	-	-	-	-	7

*: SE: Staphylococcus epidermidis (ATCC 12228); SA: Staphylococcus aureus (ATCC 29213); BS: Bacillus subtilis (ACM 2268); KP: Klebsiella pneumoniae (ATCC 10031); EC: Escherichia coli (ATCC 25922); PA: Pseudomonas aeruginosa (ATCC 27703); CA: Candida albicans (ATCC 10231)

3.3.2 **Microtitre plate broth dilution screening assays**

In broth dilution assays, test substances are dissolved or suspended in liquid media which is then inoculated with the microbial species of interest. Lack of growth following incubation indicates inhibitory activity. Where test components are in solution, growth may be estimated visually by the resulting turbid appearance or with spectrophotometric measurements of optical density. Where tests are initially turbid (e.g. in suspensions of hydrophobic test materials) growth can be detected with use of metabolically active dyes such as reazurin or tetrazolium salts to detect the presence of viable cells. For these experiments tetrazolium violet (p-iodonitrotetrazolium or p-INT) was employed. Oxidised p-INT, which is colourless in solution, is reduced from the tetrazole form with addition of hydrogen by dehydrogenase enzymes of oxidative metabolism, to form a red-violet coloured insoluble formozan. The initial density of inoculum required (ca. 5×10^5 cell/mL) is insufficient to convert visible

quantities of formozan dye at the concentrations used, so that only significant increases in growth (i.e. where lack of inhibition occurs) is detected. The same is true of visual detection of growth as the initial density of 5×10^5 cell/mL is below the detectable threshold. For this reason, such tests cannot distinguish between inhibitory or bacteriostatic activity (where positive growth is prevented) and bactericidal activities (where organism numbers are reduced or completely rendered non-viable). To assess presence of bactericidal activity, a sample of the incubated broth dilution test must be transferred to fresh media and sufficiently diluted to remove any inhibitory effects of the test substance. Lack of subsequent growth in subcultures indicates lack of viable cells in initial inhibitory tests and thus positive bactericidal activity. In this study, broth dilution tests were carried out in 300 μL capacity 96-well microplates to determine minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) of the extracts studied. MIC and MBC may be used to predict effective tissue concentrations for antimicrobials for *in vivo*/clinical situations and are useful for comparing relative activity amongst antimicrobial substances. MIC/MBC values are generally defined as the lowest concentration tested where each respective activity is present. The finite MIC/MBC values which may be generated experimentally are thus always overestimated (with the actual MIC/MBC value occurring at a point less than the lowest concentration where activity was observed and greater than the next concentration tested where activity was not observed).

3.3.2.1 **Microtitre plate broth dilution screening assays - Results and discussion**

Of the 37 extracts screened for antibacterial activity by broth microdilution assays, 20 were found to inhibit growth in two or more species of bacteria (54%). Results are presented in Table 3.6. The majority of positive results were recorded against Gram-positive bacteria (eighteen extracts or 49% active against two or more species) with fewer positive results for

inhibition of Gram-negatives (thirteen extracts or 35% active against two or more species). Virtually all extracts active against Gram-negative species were seen to exhibit a broad spectrum of activity, inhibiting growth across most of the bacterial species tested. Of these, a total of nine extracts (24% of total) were consistently found active against the typically resistant Gram-negative species *Pseudomonas aeruginosa*. These included decoctions of leaf material from *Acacia auriculiformis*, *Acacia holosericea*, and *Eucalyptus tetrodonta*, infusions and decoctions of terminal stems from immature *B. obovata* trees (JES66TD and JES66SE), expressed juice of pulp of unripe fruit of *C. fraseri* and decoctions of the trunk inner trunk of *C. intratropica* and *E. chlorostachys*. The organism most susceptible to the group of extracts in terms of overall numbers of positive results was *S. aureus* (20 positive results) followed by (in order of decreasing susceptibility), *S. epidermidis* (19 positive results), *Bacillus subtilis* (18 positive results), *Klebsiella pneumoniae* (17 positive results), *E. coli* (16 positive results) and *P. aeuginosa* (9 positive results). The purpose of these tests was to establish which extracts to select for subsequent assays to determine MIC and MBC by broth microdilution for each organism. Some extracts did not produce consistent results in that activity was not observed in all repetitions of the experiments. These extracts were, however, included along with all those which had shown any indication of inhibitory activity in subsequent MIC/MBC microdilution assays in order to confirm whether repeatable activity could be observed. One extract (expressed juice of *F. indica* terminal nodes) was omitted from further tests as insufficient material was available for dilutions required for the MIC/MBC assays.

Table 3.6 Broth microdilution screening assays - antibacterial activity against six bacterial species

				Inhibitory activity: +++: Inhibition in all three tests; ++: Inhibition in two of three tests; +: Inhibition in one of three tests.					
Label code	**Species**	**Plant part**	**Extraction type**	***Staphylococcus epidermidis* (ATCC 12228)**	***Staphylococcus aureus* (ATCC 29213)**	***Bacillus subtilis (ACM 2268)***	***Klebsiella pneumoniae* (ATCC 10031)**	***Escherichia coli* (ATCC 25922)**	***Pseudomonas aeruginosa* (ATCC 27703)**
JES30LD	*Acacia auriculiformis*	Leaves	Decoction	+++	+++	+++	+++	++	+++
JES49LD	*Acacia holosericea*	Leaves	Decoction	+++	+++	+++	+++	++	+++
JES34LTD	*Avicennia marina*	Leaves	Decoction		+				
JES42TD	*Bauhinia cunninghamii*	Root	Decoction	+++	+	+++			
JES66TD	*Buchanania obovata*	Terminal stems	Decoctions	+++	+++	+++		+	+++
JES66SE	*Buchanania obovata*	Terminal stems	Cold infusion	+++	+++	+++	+	+	+++
JES48BD	*Buchanania obovata*	Trunk inner bark	Decoction	+++	+++	+++	++	++	+
JES41TD	*Callitris intratropica*	Trunk inner bark	Decoction	++	++	+++	++	++	++
JES35SE	*Camptostemon schultzii*	Wood (Charred)	Warm infusion						
JES59EJ	*Cochlospermum fraseri*	Fruit pulp	Expressed juice	+++	+++	+++	+++	+++	+++
JES43TS	*Crinum angustifolium*	Bulb	Cold infusion						
JES43BD	*Crinum angustifolium*	Bulb	Decoction						
JES58AD	*Cymbopogon ambiguus*	Whole plant	Decoction						
JES23TD	*Eremophila alternifolia*	Leaves	Decoction		+	+			
JES21TD	*Eremophila alternifolia*	Leaves	Decoction			+			
SM10TD	*Eremophila duttonii*	Leaves	Decoction	+++	+++	+++	++		
SM11TD	*Eremophila duttonii*	Leaves	Decoction	+++	+++	+++	++		
SM7TD	*Eremophila latrobei*	Leaves	Decoction						
SM8TD	*Eremophila latrobei*	Leaves	Decoction						

†: *E. longifolia* chemotypes: (IC): Isomenthone chemotype; (KC): Karahanaenone chemotype; (BC): Borneol chemotype

Table 3.6 (continued from previous page)

				Inhibitory activity: +++: Inhibition in all three tests; ++: Inhibition in two of three tests; +: Inhibition in one of three tests.					
Label code	**Species**	**Plant part**	**Extraction type**	***Staphylococcus epidermidis* (ATCC 12228)**	***Staphylococcus aureus* (ATCC 29213)**	***Bacillus subtilis (ACM 2268)***	***Klebsiella pneumoniae* (ATCC 10031)**	***Escherichia coli* (ATCC 25922)**	***Pseudomonas aeruginosa* (ATCC 27703)**
SM3TD	*Eremophila longifolia* (IC)†	Leaves	Decoction						
SM18TD	*Eremophila longifolia* (KC)†	Leaves	Decoction						
SM19TD	*Eremophila longifolia* (BC)†	Leaves	Decoction						
SM1TD	*Eremophila sturtii*	Leaves	Decoction	+	+++	+++	++		
JES40LTD	*Erythrophleum chlorostachys*	Leaves + trunk inner bark	Decoction			++			
JES40BTD	*Erythrophleum chlorostachys*	Trunk inner bark	Decoction	+++	+++	+++	+++	++	+++
JES28LTD	*Eucalyptus tetrodonta*	Terminal leaves	Decoction	+++	+++	+++	+++	++	+++
JES28BTD	*Eucalyptus tetrodonta*	Trunk inner bark	Decoction	+					
JES37SE	*Flagellaria indica*	Stems	Warm infusion						
JES37EJ	*Flagellaria indica*	Terminal nodes	Expressed juice	+++	+++	+++	+++	+++	
JES32BHSE	*Hibiscus tiliaceus*	Stems	Warm infusion						
JES32SE	*Hibiscus tiliaceus*	Terminal stems	Cold infusion						
JES31SE	*Ipomoea pes-caprae*	Leaves	Cold infusion						
JES31HSE	*Ipomoea pes-caprae*	Leaves	Hot infusion						
JES60EJ	*Morinda citrifolia*	Fruit (Unripe)	Expressed juice	+	++	++	++	++	
JES27LBD	*Pandanus spiralis*	Leaf bases	Decoction	+	+		++	++	
JES27EJP	*Pandanus spiralis*	Leaf tips	Expressed juice diluted		+		+		
JES63EJ	*Scaevola sericea*	Fruit	Expressed juice	++	++	+++	++	++	

†: *E. longifolia* chemotypes: (IC): Isomenthone chemotype; (KC): Karahanaenone chemotype; (BC): Borneol chemotype

3.3.3 **Minimum inhibitory concentrations (MIC) of traditional type extracts**

Results for minimum inhibitory concentrations (MIC) of extracts against bacteria were generally reproducible between experiments. All MIC values were in a range of no more than one dilution step from a median value and were typically not more than one step apart. MIC values were recorded as the lowest concentration (mg · mL^{-1}), or range of concentrations at which growth inhibition was found (Table 3.7). The lowest MIC values were found for the decoction of *E. tetrodonta* leaves against *S. epidermidis* (0.06 mg · ml^{-1}) and *S. aureus* (0.25 mg · ml^{-1}) followed by expressed juice preparation of *C. fraseri* fruit (0.20 mg · ml^{-1} against *S. epidermidis* and 0.80 mg · ml^{-1} against *S. aureus*). In order to assess activity in relation to concentration of the original preparations antibacterial potency of each individual preparation was calculated as MIC and MBC values (as mg · mL^{-1}) divided by the original concentration of each preparation as to obtain a figure representing potency as the dilution factor relative to the original concentration at which MIC/MBC was observed. Relative potencies thus obtained are given in Table 3.11 along with major use agreement values. Nine of the extracts produced MIC values against certain bacteria at concentrations less than half that recorded for the original traditional type preparation. The expressed juice preparation from fruit of *C. fraseri* exhibited highest potency in this sense, with activity retained to dilution factor of up to 476 times that of the original preparation (MIC 0.2 mg · ml^{-1} against *S. epidermidis* from original extract concentration of 95.2 mg · ml^{-1}). Other extracts with notable activity in this regard included the *E. tetrodonta* leaf decoction (active at dilution factors of 1 - 90), the inner trunk bark decoctions of *B. obovata* (active at dilution factors of 1 - 41), *C. intratropica* (active at dilution factors of 1 - 40) and *E. chlorostachys* (active at dilution factors of 1 - 13). These extracts also tended to be amongst the few seen to inhibit growth of Gram-negative organisms and to exhibit bactericidal activity. In some cases, an MIC slightly higher than that of the traditional preparation was recorded. In all these instances, however, the original

concentration was found to fall between the two-fold dilution steps where inhibitory activity ceased (e.g. Concentration of *C. intratropica* inner bark decoction was 13.8 mg · ml^{-1} and MIC against *E. coli* was 16.0 mg · ml^{-1} with the next sequential dilution step producing no growth inhibition at 8.0 mg · ml^{-1}). As all of these extracts exhibited activity in initial screening by microplate broth dilution tests it is likely that the actual MIC of these extracts was nearer to the original or typical concentration of the traditional preparation as tested.

3.3.4 **Minimum bactericidal concentrations (MBC) of traditional type extracts**

MBC values were recorded as the lowest concentration where no growth was found to result in subcultures of MIC test wells. Bactericidal activity was detected in around half the extracts initially showing inhibitory activity (twelve extracts active against two or more bacterial species) and tended to be confined to those which showed relatively low MIC values in these assays. In most cases, MBC values were slightly higher (typically by one dilution step) than equivalent MIC values and, in the majority of cases, was nearer to the concentration of the original traditional type extract or preparation. The MBC found for the *C. fraseri* expressed juice extract against *B. subtilis,* however, was relatively high (50.0 mg · mL^{-1}) when compared with MIC (6.13 mg · mL^{-1}). As *B. subtilis* was the only sporulating bacteria included in the experiments it is possible that this result indicated a lack of sporicidal activity. Seven extracts were found to exhibit relatively high bactericidal potency among the Gram-positive organisms and these included the expressed juice preparation of *C. fraseri* (except against *B. subtilis*), decoctions of the trunk inner bark of *B. obovata* and *C. intratropica*, and the decoction of *E. tetrodonta* leaves. As with inhibitory activity, bactericidal activity was generally confined to Gram-positive organisms with only two extracts active against Gram-negative organisms (Expressed juice extracts of *C. fraseri* and *S. sericea* fruit).

Table 3.7 Minimum inhibitory (MIC) and bactericidal concentrations (MBC) of traditional type preparations against six bacterial species (MBC values given in parentheses)

Species	Plant part/preparation	Concentration of original preparation (mg · mL^{-1})	$MIC_{p\text{-}INT}$ / $MBC_{p\text{-}INT}$ (mg · mL^{-1}) (MBC values given in parentheses)					
			S. epidermidis (ATCC 12228)	*S. aureus* (ATCC 29213)	*B. subtilis* (ACM 2268)	*K. pneumoniae* (ATCC 10031)	*E. coli* (ATCC 25922)	*P. aeruginosa* (ATCC 27703)
Acacia auriculiformis	Leaf decoction	**8.0**	2.00 – 4.00 (16.00 - >16.00)*	4.00 (4.00 – 8.00)	8.00 (8.00)	4.00 -	4.00 – 8.00 -	2.00 -
Acacia holosericea	Leaf decoction	**8.7**	2.00 – 4.00 (4.00 – 8.00)	2.00 – 4.00 (2.00 – 4.00)	4.00 – 8.00 (8.00 – 16.00*)	2.00 – 4.00 -	8.00 -	2.00 -
Avicennia marina	Leaf decoction	**14.4**	- -	16.00 - >16.00 -	- -	- -	- -	- -
Bauhinia cunninghamii	Root decoction	**3.8**	2.00 -	2.00 – 4.00* (4.00 – >4.00)*	4.00* (4.00 – >4.00)*	- -	- -	- -
Buchanania obovata	Terminal stem decoction	**4.2**	2.00 (4.00 – 8.00*)	4.00 (8.00)*	4.00 (4.00 – 8.00*)	8.00* (8.00 - >8.00)*	4.00 – 8.00* -	8.00* -
Buchanania obovata	Terminal stem infusion	**9.7**	2.00 – 8.00 (8.00 – 16.00*)	4.00 – 8.00 (8.00 – 16.00*)	8.00 (8.00 – 16.00*)	16.00* -	8.00 – 16.00* -	8.00 – 16.00* -
Buchanania obovata	Trunk inner bark decoction	**20.4**	0.50 (2.00 – 4.00)	1.00 (2.00 – 8.00)	4.00 – 8.00 (4.00 – 8.00)	16.00 – 32.00* -	16.00 -	8.00 -
Callitris intratropica	Trunk inner bark decoction	**13.8**	0.50 – 1.00 (2.00)	1.00 – 2.00 (1.00 - 4.00)	4.00 – 8.00 (2.00 - 8.00)	4.00 -	16.00* -	8.00 – 16.00* -
Camptostemon schultzii	Charred wood infusion	**6.2**	- -	- -	- -	- -	- -	- -
Cochlospermum fraseri	Expressed fruit juice	**95.2**	0.20 (0.78 – 1.56)	0.80 (3.13 – 6.25)	6.13 (50.00)	12.50 – 25.00 (25.00 – 50.00)	3.13-6.25 -	3.13 (25.00)
Crinum angustifolium	Bulb infusion	**18.8**	- -	- -	- -	- -	- -	- -
Crinum angustifolium	Bulb decoction	**18.0**	- -	- -	- -	- -	- -	- -
Cymbopogon ambiguus	Whole plant decoction	**3.6**	- -	- -	- -	- -	- -	- -
Eremophila alternifolia	Leaf decoction	**5.1**	- -	- -	- -	- -	- -	- -
Eremophila alternifolia	Leaf decoction	**5.0**	- -	- -	- -	- -	- -	- -
Eremophila duttonii	Leaf decoction	**9.7**	8.00 (8.00 – 16.00*)	8.00 (8.00)	2.00 - 4.00 (2.00 – 4.00)	16.00* -	- -	- -
Eremophila duttonii	Leaf decoction	**6.2**	4.00 - 8.00* (8.00 - >8.00)*	4.00 - 8.00* (8.00)*	2.00 (2.00)	8.00* -	- -	- -
Eremophila latrobei	Leaf decoction	**5.4**	- -	- -	- -	- -	- -	- -
Eremophila latrobei	Leaf decoction	**6.1**	- -	- -	- -	- -	- -	- -

n.d.: Not determined due to insufficient material; †: *E. longifolia* chemotypes: (IC): Isomenthone chemotype; (KC): Karahanaenone chemotype; (BC): Borneol chemotype. MIC values in parentheses represent measured values exceeding typical extract concentration; -: Not active

Table 3.7 (Continued from overleaf)

Species	Plant part/preparation	Concentration of original preparation (mg · mL^{-1})	$MIC_{p\text{-}INT}$ / $MBC_{p\text{-}INT}$ (mg · mL^{-1}) (MBC values given in parentheses)					
			S. epidermidis (ATCC 12228)	*S. aureus* (ATCC 29213)	*B.subtilis* (ACM 2268)	*K.pneumoniae* (ATCC 10031)	*E. coli* (ATCC 25922)	*P.aeruginosa* (ATCC 27703)
Eremophila longifolia (IC)†	Leaf decoction	**7.7**	- -	- -	- -	- -	- -	- -
Eremophila longifolia (KC)†	Leaf decoction	**8.1**	- -	- -	- -	- -	- -	- -
Eremophila longifolia (BC)†	Leaf decoction	**8.3**	- -	- -	- -	- -	- -	- -
Eremophila sturtii	Leaf decoction	**9.3**	8.00 -	1.00 – 2.00 -	8.00 -	8.00 -	- -	- -
Erythrophleum chlorostachys	Leaf/Trunk inner bark decoction	**4.7**	- -	- -	8.00 - >8.00* -	- -	- -	- -
Erythrophleum chlorostachys	Trunk inner bark decoction	**10.1**	0.50 – 1.00 (2.00)	1.00 – 2.00 (2.00 – 4.00)	4.00 (4.00 – 8.00)	16.00* -	8.00 – 16.00* -	4.00 (16.00)*
Eucalyptus tetrodonta	Terminal leaf decoction	**5.4**	0.06 (1.00)	0.25 (0.50 - 2.00)	2.00 (2.00 – 4.00)	2.00 -	8.00* -	1.00 -
Eucalyptus tetrodonta	Trunk inner bark decoction	**1.2**	- -	- -	- -	- -	- -	- -
Flagellaria indica	Stem infusion	**7.1**	- -	8.00* – >8.00* -	- -	- -	- -	- -
Flagellaria indica	Expressed juice of terminal nodes	**118.0**	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Hibiscus tiliaceus	Stem warm infusion	**9.5**	- -	- -	- -	- -	- -	- -
Hibiscus tiliaceus	Stem cold infusion	**3.3**	- -	- -	- -	- -	- -	- -
Ipomoea pes-caprae	Leaf cold infusion	**0.8**	- -	- -	- -	- -	- -	- -
Ipomoea pes-caprae	Leaf hot infusion	**1.5**	- -	- -	- -	- -	- -	- -
Morinda citrifolia	Expressed unripe fruit juice	**51.1**	64.00* -	64.00* -	64.00* -	16.00 – 32.00 -	8.00 -	- -
Pandanus spiralis	Leaf base decoction	**13.6**	16.00* -	4.00 – 8.00 -	- -	16.00* –>16.00* -	16.00* -	-
Pandanus spiralis	Leaf tip expressed juice diluted	**8.3**	- -	4.00 – 8.00	- -	8.00 - >8.00*	- -	- -
Scaevola sericea	Expressed juice of fruit	**75.8**	32.00 -	128.00* (128.00 - >128.00)*	128.00* 64.00 – (>128.00)*	32.00 -	32.00 -	- -

n.d.: Not determined due to insufficient material; †: *E. longifolia* chemotypes: (IC): Isomenthone chemotype; (KC): Karahanaenone chemotype; (BC): Borneol chemotype. MIC values in parentheses represent measured values exceeding typical extract concentration; -: Not active

3.3.1 Comparison of antibacterial activity of traditional aqueous preparations and sequential organic solvent extractions of *Eremophila* species

With the exception of the *E. longifolia* extracts, all other hexane and DCM extracts exhibited activity against *C. albicans* in disc diffusion assays. Activity was also noted for the MeOH extracts of *Eremophila duttonii* and *Eremophila latrobei*. In contrast, none of the coresponding traditional aqueous preparation were found to exhibit detectable activity. Some consistency was seen between antibacterial activities observed for traditional type preparations and extracts of corresponding material in organic solvents (e.g. for extracts of *Eremophila sturtii* and *E. duttonii*). Activity was, however, present only in relatively higher concentrations in the traditional aqueous extracts (MIC between 1 and 8 mg· mL^{-1} as compared with concentration of 0.5 mg· mL^{-1} at which solvent extractions were tested). In some instances a greater incidence of activity was present in organic solvent extracts when compared with corresponding traditional aqueous preparations. While virtually no activity could be observed for traditional decoctions of *E. alternifolia* leaves, inhibitory activity was recorded for DCM and MeOH extracts of corresponding material. Similarly, no activity could be observed in traditional preparations of *E. latrobei* whereas inhibition of *B. subtilis* was recorded for the corresponding DCM and MeOH extracts (Table 3.9).

Table 3.8 Disc diffusion assay of *Eremophila* sp. solvent extracts with traditional decoctions against *Candida albicans* (ATCC 10231)

			Mean diameter zone of inhibition (mm)			
Species	Plant part	Collectors number	1' Hexane extract	2' DCM extract	3' MeOH extract	Traditional aqueous decoction
Eremophila sturtii	Leaves	SM001	13	7.3	-	-
Eremophila longifolia	Leaves	SM003	-	-	-	-
Eremophila latrobei	Leaves	SM007	7	8	7.7	-
Eremophila duttonii	Leaves	SM011	7	7	8.7	-
Eremophila alternifolia	Leaves	JES021	7	9	-	-
Eremophila alternifolia	Leaves	JES023	7	8	-	-

Table 3.9 Screening of Solvent extracts at 0.5 mg· ml^{-1} using broth microdilution assay. (Results from screening of traditional preparations at near typical concentrations included for comparison)

					Inhibitory activity: +++: Inhibition in all three tests; ++: Inhibition in two of three tests; +: Inhibition in one of three tests.					
Specimen	**Species**	**Plant part**	**Extraction /solvent**	**Conc. mg·mL^{-1}**	**Staphylococcus epidermidis (ATCC 12228)**	**Staphylococcus aureus (ATCC 29213)**	**Bacillus subtilis (ACM 2268)**	**Klebsiella pneumoniae (ATCC 10031)**	**Escherichia coli (ATCC 25922)**	**Pseudomonas aeruginosa (ATCC 27703)**
SM001	*Eremophila sturtii*	Leaves	Hexane	0.5	+++	+++	+++	++	-	+
SM001	*Eremophila sturtii*	Leaves	DCM	0.5	+++	+++	+++	-	-	-
SM001	*Eremophila sturtii*	Leaves	MeOH	0.5	-	-	-	-	-	-
SM001	*Eremophila sturtii*	Leaves	Aq. (TLD)	7.4	+	+++	+++	++	-	-
SM011	*Eremophila duttonii*	Leaves	Hexane	0.5	+++	+++	+++	-	-	-
SM011	*Eremophila duttonii*	Leaves	DCM	0.5	+++	+++	+++	-	-	-
SM011	*Eremophila duttonii*	Leaves	MeOH	0.5	+++	+++	+++	++	-	++
SM011	*Eremophila duttonii*	Leaves	Aq. (TLD)	5.0	+++	+++	+++	++	-	-
SM003	*Eremophila longifolia*	Leaves	Hexane	0.5	-	-	-	-	-	-
SM003	*Eremophila longifolia*	Leaves	DCM	0.5	-	+	-	-	-	-
SM003	*Eremophila longifolia*	Leaves	MeOH	0.5	-	-	-	-	-	-
SM003	*Eremophila longifolia*	Leaves	Aq. (TLD)	6.2	-	-	-	-	-	-
SM006	*Eremophila latrobei*	Leaves	Hexane	0.5	-	-	-	-	-	-
SM006	*Eremophila latrobei*	Leaves	DCM	0.5	-	-	+++	-	-	-
SM006	*Eremophila latrobei*	Leaves	MeOH	0.5	-	-	+	-	-	-
SM007	*Eremophila latrobei*	Leaves	Aq. (TLD)	4.3	-	-	-	-	-	-
JES021	*Eremophila alternifolia*	Leaves	Hexane	0.5	-	-	+++	-	-	-
JES021	*Eremophila alternifolia*	Leaves	DCM	0.5	++	++	+++	-	-	-
JES021	*Eremophila alternifolia*	Leaves	MeOH	0.5	+	-	-	-	-	-
JES021	*Eremophila alternifolia*	Leaves	Aq. (TLD)	4.0	-	-	+	-	-	-
JES023	*Eremophila alternifolia*	Leaves	Hexane	0.5	-	+	+++	-	-	-
JES023	*Eremophila alternifolia*	Leaves	DCM	0.5	+	+++	++	-	-	+
JES023	*Eremophila alternifolia*	Leaves	MeOH	0.5	+	-	-	-	-	-
JES023	*Eremophila alternifolia*	Leaves	Aq. (TLD)	4.1	-	+	+	-	-	-

DCM: Secondary dichloromethane extract; MeOH: tertiary methanol extract; Aq. (TLD): Aqueous traditional leaf decoction preparation

3.3.2 **Antimicrobial activity of traditional type preparations and previous studies**

Antimicrobial activities have previously been reported for extracts of a number of the plant species examined, in particular some of the species of *Acacia* and *Eremophila*, along with some of the more widely distributed pan-tropical species such as *H. tiliaceus* and *Morinda citrifolia*. Palombo and Semple (2001) have screened exhaustive ethanol (EtOH) extracts of *E. alternifolia*, *E. duttonii*, *E. sturtii*, *E. latrobei* (subspecies *glabra*), *E. longifolia* and *C. ambiguus* against a panel of eight Gram-positive and Gram-negative bacteria using disc diffusion assays. Palombo and Semple (2001) reported growth inhibition of most Gram-positive bacteria by extracts of *E. duttonii* and *E. alternifolia,* with some inhibitory activity against *Bacillus cereus* by extracts of *E. sturtii* and *E. latrobei,* reporting no detectable activity for extracts of *E. longifolia*. A similar pattern of activity in the solvent based extracts was seen here (3.3.1) except that all *E. sturtii* extracts, including the traditional preparation, were found to strongly inhibit growth of most of the Gram-positive species. Palombo and Semple (2001) also reported finding no inhibitory activity in extracts of *C. ambiguus*.

During compilation of the Northern Territory indigenous pharmacopoeia, Barr *et al*. (1988; 1993), screened refluxed EtOH extracts of several species collected in the project for antibacterial activity against four organisms using agar dilution assays. Activities reported by Barr *et al*. (1993) in ten EtOH extracts obtained from the same species and plant parts as some of the extracts examined in the present study have been reproduced below alongside corresponding results obtained in antibacterial assays of the traditional preparations in this study (Table 3.10). Results reproduced from Barr *et al*. (1993) include those for activity against the bacterial species *S. aureus*, *E. coli* and *Klebsiella pneumoniae* (strains not specified).

Table 3.10 Comparison between antibacterial activity of traditional aqueous preparations and that reported by Barr *et al*. (1993) for refluxed EtOH extracts of equivalent parts and species

Species	Traditional aqueous preparations (activity indicated by "+") Extraction type, (collectors number), Locality	Organisms* KP	*SA*	*EC*	Activity of EtOH extracts as reported by Barr *et al*. (1993) (activity indicated by "+") Extraction type, (collectors number), Locality	Organisms* KP	*SA*	*EC*
Acacia auriculiformis	Decoction of leaves (JES030) Darwin, NT	+	+	+	EtOH extract of leaves (NMS958) Nauiyu Nambiyu, NT	-	-	-
Avicennia marina	Decoction of leaves (JES034) Darwin, NT	-	+	-	EtOH extract of leaves (NMS1302) Darwin, NT	-	+	-
Bauhinia cunninghamii	Decoction of root (JES042) Katherine, NT	-	+	-	EtOH extract of root (NMS686) Elliot, NT	+	+	+
Crinum angustifolium	Cold infusion of bulb (JES043) Jodetluk, NT	-	-	-	EtOH extract of bulb (NMS978) Elliot, NT	-	+	-
Crinum angustifolium	Decoction of bulb (JES043) Jodetluk, NT	-	-	-	EtOH extract of bulb (NMS978) Elliot, NT	-	+	-
Cymbopogon ambiguus†	Decoction of whole plant (JES058) Jodetluk, NT	-	-	-	EtOH extract of leaves (NMS1394) Alice Springs, NT	+	+	+
Eremophila longifolia	Decoction of leaves (SM003;SM018;SM019) Western NSW	-	-	-	EtOH extract of leaves (NMS1354) Ulbulla, NT	-	+	+
Eremophila longifolia	Decoction of leaves (SM003;SM018;SM019) Western NSW	-	-	-	EtOH extract of leaves (BT3303) Orange Creek, NT	+	+	+
Eucalyptus tetrodonta	Decoction of terminal leaves (JES028) Darwin, NT	+	+	+	EtOH extract of leaves (NMS130) Warruwi, NT	+	+	-
Eucalyptus tetrodonta	Decoction of trunk inner bark (JES028) Darwin, NT	-	-	-	EtOH extract of bark (NMS1158) Darwin, NT	-	-	-

*: KP: *Klebsiella pneumoniae*; SA: *Staphylococcus aureus*; EC: *Escherichia coli*; †: Prepared using senescent material

From the information given above in Table 3.10, it is apparent that overall, a higher incidence of activity in can be seen among the EtOH extracts as reported by Barr *et al*. (1993). Of the ten EtOH extracts described, only two were reported inactive against all three bacterial species as compared with six of the traditional preparations using corresponding plant material which were examined in the present study. The traditional decoction of *A. auriculiformis*, however, was found to inhibit growth of all three bacterial species and overall exhibited a broad spectrum of activity, whereas Barr *et al*. (1993) reported a complete lack of activity by EtOH leaf extracts. Similarly, the decoction of *E. tetrodonta* leaves examined in this study was active against *E. coli* whereas an EtOH extract described by Barr *et al.* (1993) was not. Barr *et al*. (1993) reported broad spectrum inhibitory activity by an EtOH extract of

C. ambiguus, whereas Palombo and Semple (2001) reported a lack of activity in EtOH extracts examined using disc diffusion assays. No activity was exhibited by the decoction examined in this study of *C. ambiguus* over the range of concentrations tested, however, it should be noted that the material used to prepare the decoction was not in accordance with the descriptions followed (*cf.* section 3.2 above). It should also be pointed out that any comparisons between these two sets of results on the basis of extracting solvents and methods, are further complicated by the fact that the susceptibility of the strains used in each study may not be equivalent and in many cases plant specimens have been collected from separate locations in the NT. It is possible that the differences in activity between extracts of the same species, may also be representative of intraspecific chemotypic variations. Variability in *E. longifolia* essential oil composition has been noted by Ghisalberti (1994) and was explored as part of this study. Interestingly, slight variations in the spectrum of activity between EtOH extracts of *E. longifolia* specimens collected from separate areas was recorded by Barr *et al.* (1993). These results also contrast with those reported by Palombo and Semple (2001), (where EtOH extracts of *E. longifolia* leaves were not found to inhibit growth of similar organisms). Hexane, DCM and MeOH extracts of *E. longifolia* leaves (Isomenthone oil chemotype, collected from Mutawintji, western NSW) examined in this study, similarly exhibited a general lack of activity against the microorganisms tested. It is also interesting to note that patterns of antibacterial activity between the aqueous preparations examined in this study and the EtOH extracts reported by Barr *et al.* (1993), were similar in the two cases where specimens had been collected from the same geographic area (leaves of *A. marina* and bark of *E. tetrodonta*).

M. citrifolia is perhaps one of the best studied of the species examined, owing to the popularity and commercial promotion of personal healthcare products derived from the fruit (Commonly referred to as '*noni'* and less commonly, at least in promotional material

associated with products containing extracts of the plant, as 'rotten-cheese fruit'). Interestingly, some of the traditional Polynesian uses for this species (as described by Dixon *et al.*, 1999), are very similar to those reported for indigenous Australians of Groote Eyelandt (i.e. topical application of macerated unripe fruit for treatment of skin disorders, as described by Levitt and Lyon, 1981). In a study conducted on Hawaiian medicinal plants Bushnell *et al*. (1950) have reported activity against *S. aureus, E. coli and P. aeruginosa* by expressed juice extracts of *ripened M. citrifolia* fruit in disc diffusion assays. Some level of inhibitory activity of *unripe* fruit juice was also found in this study against all organisms tested except *P. aeruginosa* in broth dilution assays.

Cochrane (1998) has reported broad spectrum antibacterial activity against a number of microorganisms by EtOH extracts of *H. tiliaceus* leaves. Another study reported significantly greater activity in sequential chloroform and MeOH extracts of *H. tiliaceus* leaf material than that which was found in tertiary aqueous extracts (Hemaiswarya *et al.*, 2010). Smyth *et al.* (2009) have reported broad spectrum inhibitory and bactericidal activity in a MeOH extract of bark of *H. tiliaceus*. In contrast, the traditional aqueous preparations of bark material examined in this study were not found to produce any observable antimicrobial activity over the range of concentrations tested.

Han *et al*. (2007) have reported MIC of 6.25-25.0 $\mu g \cdot mL^{-1}$ against *C. albicans*, *S. aureus* and several species of Mycobacterium, for naphthoquinone derivatives isolated from branchlets of *A. marina*. Broad spectrum antimicrobial activities in EtOH extracts of *A. marina* aerial parts using disc diffusion assays (using 2 mg loadings per disc) have also been reported (Mahasneh, 2002). In contrast, traditional aqueous extracts of *A. marina* leaves examined in this study exhibited very little detectable antimicrobial activity against the organisms examined.

Traditional decoctions of *A. auriculiformis* and *A. holosericea* leaves were found to exhibit relatively modest but broad spectrum inhibitory activity at concentrations representing up to one quarter that of the original preparation and bactericidal activity against Gram-positive species at concentrations close to that of the original preparation. Antibacterial activity has previously been reported in MeOH extracts of *A. auriculiformis* (Pennacchio *et al.*, 2005). Wickens and Pennacchio (2002) reported a lack of antimicrobial activity by extracts of *A. auriculiformis* and *A. holosericea* in an earlier study where tertiary aqueous extracts (after extraction of material with hexane, DCM and MeOH) of several Australian *Acacia* were screened using disc diffusion assays. These species are known to accumulate large quantities of triterpenoid glycosides (saponins) and are used traditionally as 'soap' plants (i.e. detergents) (Barr *et al.*, 1993). Saponins are known to exhibit antimicrobial properties and Burford and Bodeker (2007), in a review of studies describing bioactivy in saponin containing plants used traditionally as 'natural soaps', found that most could be shown to exhibit broad spectrum anti-microbial activities. Antimicrobial activity of the saponins acaciaside A and B isolated from the funicles of *A. auriculiformis* have been reported by Mandal *et al*. (2005).

3.3.3 **Antibacterial activity and records of traditional use**

The anti-bacterial activity found in the extracts was compared with major use agreement (MUA_{TMP}) values as determined for wounds, sores and all dermatological complaints to determine whether activity was associated with increased frequency of citations for these specific uses and indications in the literature examined. MUA_{TMP} values are presented below (Table 3.11) alongside relative antimicrobial potency for each preparation (dilution factors representing MIC and MBC in relation to original concentration). To assess whether a higher incidence of positive activity could be associated with higher MUA_{TMP} values, extracts were divided into two groups, one including those with MUA_{TMP} values corresponding to two or more independent citations for each use/indication and the other including those with

MUA_{TMP} values corresponding to either single independent citations for each use/indication, or the absence of any citation. Relative proportions of extracts exhibiting positive activity in each group were then compared to determine whether any association between activity and MUA_{TMP} could be observed. Results were assessed using Pearson's chi-square and Fisher's exact tests for association. It was not possible to analyse the extracts indicated for treatment of wounds in this way as there was only a relatively small number of species/extracts cited multiple times for this purpose (four extracts in total), which was not suitable for the statistical methods employed. Instead, all plants and extracts with any number of citations for this purpose were assessed as a single group (14 preparations in total) which were compared with remaining species/extracts not cited for this purpose. This technique was additionally applied to extracts cited for treatment of sores as there was in most instances, a sufficient number of species included in the sample which were not cited for this purpose to enable analysis in this way.

Table 3.11 Major use agreement values for traditional preparations with antimicrobial potency (MIC/MBC as dilution factor of original concentration)

Species	Extract Description	Preparation major use agreement values (MUA_{TMP})			Antimicrobial potency (MIC/MBC as multiples of original concentration with MBC values given in parentheses)					
		Wounds	Sores	Dermatological	*SE**	*SA**	*BS**	*KP**	*EC**	*PA**
Crinum angustifolium	Cold infusion of bulb	67	78	78						
Crinum angustifolium	Decoction of bulb	67	78	78						
Callitris intratropica	Decoction of inner trunk bark	50	33	33	18 (7)	9 (9)	2 (2)	3	1	1
Buchanania obovata	Decoction of inner trunk bark	33	44	44	41 (7)	20 (4)	3 (3)	1	1	3
Erythrophleum chlorostachys	Trunk inner bark decoction	17	56	56	13 (5)	7 (3)	3 (2)	1	1	3
Erythrophleum chlorostachys	Leaf/inner trunk bark decoction	17	33	33						
Bauhinia cunninghamii	Decoction of root	17	22	22	2	1 (1)	1 (1)			
Acacia auriculiformis	Decoction of leaves	17	11	11	3	2 (1)	1 (1)	2	1	4
Eremophila duttonii	Decoction of leaves	17	11	11	1 (1)	1 (1)	3 (3)	1		
Eremophila duttonii	Decoction of leaves	17	11	11	1 (1)	1 (1)	2 (2)			
Eremophila sturtii	Decoction of leaves	17	11	11	1	6	1	1		
Buchanania obovata	Cold infusion of terminal stems	17	11	11	2 (1)	2 (1)	1 (1)			
Buchanania obovata	Decoction of terminal stems	17	11	11	2 (1)	1	1 (1)			
Hibiscus tiliaceus	Warm infusion of stems	17	0	11						
Hibiscus tiliaceus	Cold infusion of terminal stems	17	0	11						
Eucalyptus tetrodonta	Terminal leaf decoction	0	67	67	90 (5)	22 (4)	3 (2)	3	1	5
Acacia holosericea	Decoction of leaves	0	22	22	3 (1)	3 (3)	1 (1)	3	1	4
Camptostemon schultzii	Cold infusion charred wood	0	22	22						
Eucalyptus tetrodonta	Decoction of trunk inner bark	0	22	22						
Eremophila longifolia (BC)	Decoction of leaves	0	22	22						
Eremophila longifolia (IC)	Decoction of leaves	0	22	22						
Eremophila longifolia (KC)	Decoction of leaves	0	22	22						
Avicennia marina	Decoction of leaves	0	11	11						
Morinda citrifolia	Expressed juice of unripe fruit	0	11	11	1	1	1	2	6	
Cymbopogon ambiguus	Decoction of whole plant	0	11	11						
Pandanus spiralis	Expressed juice of leaf tips diluted	0	11	11		1				
Ipomoea pes-caprae	Hot infusion of leaves	0	0	44						
Ipomoea pes-caprae	Cold infusion of leaves	0	0	44						
Scaevola sericea	Expressed juice of fruit	0	0	22	2	1	1	2 (1)	2 (1)	
Cochlospermum fraseri	Expressed juice of fruit pulp	0	0	11	476 (81)	119 (20)	16 (2)	5 (3)	20	30 (4)
Eremophila latrobei	Decoction of leaves	0	0	11						
Eremophila latrobei	Decoction of leaves	0	0	11						
Pandanus spiralis	Decoction of leaf bases	0	0	11	1	2		1	1	
Flagellaria indica	Warm infusion of stems	0	0	11						
Eremophila alternifolia	Decoction of leaves	0	0	0						
Eremophila alternifolia	Decoction of leaves	0	0	0						

*: SE: *Staphylococcus epidermidis* (ATCC 12228); SA: *Staphylococcus aureus* (ATCC 29213); BS: *Bacillus subtilis* (ACM 2268); KP: *Klebsiella pneumoniae* (ATCC 10031); EC: *Escherichia coli* (ATCC 25922); PA: *Pseudomonas aeruginosa* (ATCC 27703); n.d.: Not determined

3.3.4 **Major use agreements and antibacterial activity of traditional preparations**

Of the thirty-seven extracts examined, fifteen were documented for use in the treatment of wounds while the remaining twenty-one were not cited for this purpose in the literature examined. When the incidence of activity was compared between each group, a significant association was found between the incidence of antibacterial inhibitory activity, and use citations referring to the treatment of wounds, with the rate of incidence doubling amongst extracts cited for this purpose (i.e. activity was detected in 67% of extracts used for wounds while in the extracts not cited for this purpose, activity was detected in only 29% of cases with significance at the 0.05 level for both Pearson's Chi-square: χ_1^2 = 5.143, and Fisher's exact tests). The association was stronger (χ_1^2 = 8.229) when bactericidal activity was examined, with 60% of 'wound healing' extracts exhibiting activity versus 14% of the 'non wound-healing' extracts (significance at 0.01 level for Chi-square and Fisher's exact tests). No significant association was apparent between inhibitory activity and higher range MUA_{TMP} values derived either from citations describing treatment of sores, or all dermal/ophthalmic complaints. Significant association was, however, found between bactericidal activity and use for sores when activity in *all* extracts cited for this purpose were compared with those not cited (activity detected in 46% of extracts used for sores and 8% in those not cited for this purpose with significance at 0.05 level for Chi-square and Fisher's exact tests). The strength of this association was slightly less than that which was found with the 'wound healing' preparations as assessed by chi-square values (χ_1^2 = 8.229 for wounds and 5.063 for sores).

Table 3.12 Proportion of extracts inhibiting growth of Gram-positive bacteria by groupings greater and less than median MUA_{TMP} values with Pearson's Chi-square and Fisher's exact tests for association

Category	Active preparations	Inactive preparations	Active preparations (%)	χ^2	Fisher's Exact Test (*p*)
≥1 citations for wounds (MUA_{TMP} ≥17)	10	5	67%	*5.143	0.041
=0 citations for wounds (MUA_{TMP} <17)	6	15	29%		
≥2 citations for 'Sores' (MUA_{TMP} ≥22)	6	8	43%	0.023	1.000
<2 citations for 'Sores' (MUA_{TMP} <22)	10	12	45%		
≥2 citations for all D/OC (MUA_{TMP} ≥22)	7	10	41%	0.472	0.525
<2 citations for all D/OC (MUA_{TMP} <22)	10	9	53%		
≥1 citations for 'Sores' (MUA_{TMP} ≥11)	13	11	54%	2.756	0.157
=0 citations for 'Sores' (MUA_{TMP} <11)	3	9	25%		

D D/OC: Dermatological/ophthalmic complaints; *: Pearson's Chi-square test significant at the 0.05 level; ** Pearson's Chi-square test significant at the 0.01 level; †: Expected frequency <5 in Pearson's Chi-square test

Table 3.13 Proportion of extracts with bactericidal activity against Gram-positive bacteria by groupings greater and less than median MUA_{TMP} values with Pearson's Chi-square and Fisher's exact tests for association

Category	Active preparations	Inactive preparations	Active preparations (%)	χ^2	Fisher's Exact Test (*p*)
≥1 citations for wounds (MUA_{TMP} ≥17)	9	6	60%	**8.229	0.010
=0 citations for wounds (MUA_{TMP} <17)	3	18	14%		
≥2 citations for 'Sores' (MUA_{TMP} ≥22)	6	7	46%	0.935	0.471
<2 citations for 'Sores' (MUA_{TMP} <22)	6	17	26%		
≥2 citations for all D/OC (MUA_{TMP} ≥22) (>22%)	6	11	35%	0.056	1.000
<2 citations for all D/OC (MUA_{TMP} <22)	6	13	32%		
≥1 citations for 'Sores' (MUA_{TMP} ≥11)	11	13	46%	*5.063	0.031
=0 citations for 'Sores' (MUA_{TMP} <11)	1	11	8%		

D/OC: Dermatological/ophthalmic complaints; *: Pearson's Chi-square test significant at the 0.05 level; ** Pearson's Chi-square test significant at the 0.01 level; †: Expected frequency <5 in Pearson's Chi-square test

Significant associations between the incidence of activity and upper range MUA_{TMP} values could not be demonstrated, however, extracts within the highest MUA_{TMP} values (33-67) based on citations for wounds which were active, all exhibited relatively high potency (i.e. MIC >10× original concentration against the staphylococci) (e.g. inner bark extracts of *C. intratropica*, *B. obovata* and *E. chlorostachys*). This was even more apparent in the case of MUA_{TMP} values based on number of citations for sores where all but one of the extracts exhibiting high relative potency (expressed juice of *C. fraseri*) were found in the group of

higher range MUA_{TMP} values (56-78%), representing 5-6 citations in independent studies. The extract with the lowest recorded MIC overall (*E. tetrodonta* terminal leaf extract with MIC against Gram-positive organisms of 0.06 - 2.00 mg · mL^{-1}) had one of the highest MUA_{TMP} values (67%) as calculated for use against sores (six independent studies citing use for treatment of sores). In contrast, the extract prepared from inner bark of this species was cited only two times for this purpose in independent studies (MUA_{TMP} 22%) and correspondingly exhibited no detectable antibacterial activity. A similar result was found with the two extracts prepared with material from *E. chlorostachys*. In this case activity was detected in the inner bark decoction which was cited five times for treatment of sores (MUA_{TMP} 56%) while the decoction of leaves and inner bark (in which only a relatively small quantity of inner bark is extracted along with the leaves) produced no detectable activity and was correspondingly cited in less instances for the treatment of sores (three citations, MUA_{TMP} 33%).

While definite associations were found between the overall incidence of activity amongst the extracts and citations for specific uses, a number of species/extracts with high MUA_{TMP} values were not seen to exhibit any antimicrobial activity. Most notably, no activity was detected in the decoction/infusion of *C. angustifolium*, which was the most frequently cited extract with the highest MUA_{TMP} value across all three categories. Similarly, activity was not detected in extracts of *I. pes-caprae*, which were also found to have a relatively high frequency of citation for sores and dermatological uses (four independent citations in each case, MUA_{TMP} of 44%). In addition, antibacterial activity was not exclusively limited to preparations with higher MUA_{TMP} or use citations for wounds and sores. The preparation which was found to exhibit the highest relative potency was not cited for use against either wounds or sores (*C. fraseri* expressed fruit juice with MIC against Gram-positive organisms at 16 - 476 × original concentration). Only a single citation of use for this preparation was

found in the literature surveyed, [i.e. in treatment of boils by people at Bulla, NT (Barr *et al.*, 1993)]. Boils or furuncles arise from a deep, localised infection of hair follicles by bacteria, commonly *S. aureus* (Durupt *et al.*, 2007). Interestingly, topical antimicrobial chemotherapy is not currently considered to be clinically effective for the treatment of furuncles where infection persists below the upper layers of the dermis (McPhee *et al.*, 2011). Currently recommended treatments for furuncles instead include application of systemic antibiotics and, for simple furuncles, moistening the area at the surface with a wet cloth to initiate drainage of accumulated pus to the surface via the follicle to encourage healing (McPhee *et al.*, 2011). Application of *C. fraseri* fruit in the treatment of boils involves crushing the pulp to release the juice prior to rubbing onto the site of infection (Barr *et al.*, 1988) and/or opening the fruit to expose the pulp which is applied as a poultice directly to the boil with the outer layers of the fruit acting as a dressing to keep the pulp in place. Both these methods could be seen as a potentially effective method to initiate drainage by moistening the site of infection, particularly where the outer layer of the fruit is used as an occlusive dressing. It is tempting to speculate that the *in vitro* antibacterial activity of the preparation observed here plays some significant role in the traditional use to treat boils, however, it is possible that this is entirely coincidental or secondary to other modes of activity (such as the moistening/softening of the follicle), including those not investigated in this study (e.g. topical analgesia). It is also worth noting that no antibacterial activity was detected in the eight other preparations included in the study which were cited for treatment of boils (Leaf decoctions of *E. longifolia* and *I. pes-caprae*, decoction of *C. angustifolium* and stem infusions/decoctions of *H. tiliaceus*).

A number of the preparations examined were cited for use in various ophthalmic conditions (most often cited as being used for "sore eyes" or "infected and inflamed eyes"). These included the decoctions of material of *C. angustifolium*, *E. duttonii*, *B. obovata* and the expressed juices of *S. sericea* fruit and *F. indica* terminal stem nodes (with the last two cited

exclusively for this purpose. Antibacterial activity was detected in all but one of these preparations (*C. ambiguus* decoction) with broad spectrum of activity noted for both the *S. sericea* and *F. indica* preparations.

Nine of the preparations examined were documented for treatment of scabies infestation. The majority (6 extracts) of these were found to be inactive. Interestingly the remaining three preparations which were found to exhibit antimicrobial activity (decoctions of *Bauhinia cunninghamii* root and inner trunk bark of *B. obovata* and *E. chlorostachys*) were the only extracts of those cited for scabies which were also cited for treatment of wounds.

MUA_{TMP} values were not determined for specific uses such as the treatment of boils, scabies and ophthalmic complaints, as these indications were only represented in a small number of the extracts/species studied which was insufficient for statistical analyses.

3.4 **General conclusions**

Significant associations were found between antibacterial activity and citations describing use in the treatment of wounds, and dermal lesions (sores). From these results, it could be concluded that a higher probability of the incidence of antibacterial activity may be found in species or plant parts which are cited for use in traditional indigenous Australian ethno-medicine for the treatment of wounds and to a lesser extent: sores/dermal lesions. On the other hand, it is noteworthy that the extract exhibiting the most potent activity (in terms of activity in relation to the original or 'typical traditional' concentration) - *C. fraseri* expressed fruit juice, was not cited for either of these purposes. Measurements of MUA_{TMP} are often used to guide selection of medicinal plants for studies investigating biological activity on the assumption that frequency of citation for particular indications or ailments potentially indicates a greater relative probability of finding meaningful activity. If the species examined here had been restricted to only those used for sores or wounds or only those with a higher

MUA_{TMP} value for dermatological complaints, it is likely that species such as *C. fraseri* or *E. duttonii* may have been excluded from consideration.

Definite association with higher range MUA_{TMP} values, or direct correlation could not be discerned except that overall, relative potency of inhibitory activity did seem to be higher in extracts for which higher MUA_{TMP} values for sores and wounds were obtained. The overall association between activity and frequency of citation for sores, in fact, increased when extracts with only a single citation for this purpose were included.

Antibacterial activity was also seen to occur in almost every species used for ophthalmic complaints and the only extract found inactive in this respect was one where the validity as an accurate reproduction of the traditional preparation was in doubt (i.e. *C. ambiguus* decoction). Conversely very little activity could be found amongst preparations cited for the topical treatment of boils which is not altogether surprising given that this mode of treatment is not known to be an effective treatment for this condition. Of the extracts cited for use in scabies, only those which were also cited for use in treatment of wounds were found to exhibit any antibacterial activity. As pointed out in Chapter 1, it is unlikely that scabies was present, or at least prevalent, in indigenous Australian populations prior to European contact/settlement. It is possible that many of the preparations/species currently used and cited in the ethnobotanical literature for treatment of scabies have been adapted to this use in relatively recent times. As can be seen in Table 3.1, many of the species cited for use in treatment of scabies infestation are in fact also cited for a wide range of other dermatological conditions. In particular many of these preparations/species are also indicated for treatment of sores or dermal lesions (e.g. seven of nine preparations examined in this study). Scabies infestation is typically accompanied by nocturnal pruritus and secondary bacterial infections commonly occur at the site of infestation. Given this range of symptoms, it may be possible to consider

treatments used for scabies as equivalent to those cited for the treatment of other dermal lesions. In the ethnobotanical literature examined, however, it is not always made explicit whether these preparations are intended specifically to treat or provide symptomatic relief from the infected, secondary lesions, or are instead intended to prevent or symptomatically treat infestation at an earlier stage.

While these observations are interesting, it is difficult to draw any firm conclusions regarding the relationship between activity and these uses (i.e. boils, scabies and ophthalmic complaints) given that the number of cases involved in each instance was too small to permit valid statistical analyses of association.

One interpretation of these results is that the presence of antimicrobial activity may be important in determining whether a particular species or preparation is selected for the purpose of treating wound and sores (and possibly ophthalmic complaints) and, subsequently, retained in the ethnopharmacopoeia. On the other hand, antibacterial activity was not seen in all cases including those with a relatively high frequency of citation for these purposes. As discussed in Chapter 1, the conditions for which these preparations are indicated present a number of distinct potential therapeutic targets which might be expected to provide relief or resolution if treated. Such activities potentially include: local analgesic and anti-inflammatory activity, and (for wounds) proliferative and anti-oxidant activities and it is likely that such activities may be found in these preparations. It is also possible that these preparations may exert other actions affecting microbial infection which would be undetected by the *in vitro* assays used including interference with mechanisms of pathogenicity, such as with the ability to form biofilms or via the inhibition of extracellular bacterial proteases and toxins.

In any case, while significant associations could be demonstrated between antibacterial activity and these uses, it cannot be stated with certainty that this activity plays a significant

role in actual (i.e. *in vivo* or ethno-medical) practice. Many of the preparations cited for use in the treatment of sores included extracts of inner bark material, which were also found to contain relatively high levels of phenolic compounds by Folin- Ciocalteu assays (*cf*. Chapter 4). When antibacterial activity was compared with total phenolic content, a weak but significant correlation was found. It is possible that the presence of relatively high levels of phenolic compounds in the species cited for wounds and sores may be medically significant for other reasons (e.g. astringency in extracts containing tannins, antioxidant capacity and redox potential) and that the antimicrobial activity observed here *in vitro*, is coincidental. It is important to note that many of the preparations for which inhibitory activity was recorded (and which formed the 'active' grouping in tests for association between activity and MUA), only exhibited activity at concentrations close to that of the original preparation. It is probably unlikely that these preparations are able to produce a clinically significant level of antibacterial activity even with topical use. The relatively few preparations which were active at concentrations substantially lower than that of the original concentration (such as the decoctions of *E. tetrodonta* leaves and *B. obovata* inner bark), or those active in disc diffusion assays (*C. intratropica* inner bark decoction and *C. fraserii* fruit juice), are probably more likely to potentially involve significant antibacterial action with *in vivo* topical application, if antibacterial activity has been a relevant factor in the traditional medicinal use and selection of these preparations for the treatment of dermatological disorders.

Overall, a higher rate of incidence of antibacterial activity was seen for extractions in organic solvent when compared with corresponding traditional preparations and activity tended to be seen more frequently in the extractions with the relatively non-polar solvents (i.e. hexane and DCM). Positive anti-microbial activity has also been reported in a number of other studies in extracts of species for which the corresponding traditional aqueous preparations examined here were found to be inactive. Based on these observations, it would appear that anti-

microbial activity may generally tend to be over-estimated when medicinal plant parts are extracted with organic solvents in relation to corresponding aqueous traditional preparations. It should be acknowledged, however, that the results cited from other studies using different assay techniques and microbial strains may not be directly comparable with those obtained in the present studies. Also, there is a possibility that the variation in activity noted in these cases may be the result of phytochemical variation within each species. Extraction of medicinal plant parts for phytochemical and biological study with organic solvents has an advantage over aqueous extraction in that it is possible to obtain a more complete or exhaustive extraction of low molecular weight compounds from the plant material and, in this respect, the probability of detecting any biological activity may be increased. This situation may be less desirable, however, where the purpose of any investigation includes providing rationale or validation for, or generally relating activity to documented patterns of traditional use.

Traditional preparations examined in this study were restricted to those which were liquid in form (i.e. aqueous extractions and expressed juices of plant material) in order to facilitate ease of handling and manipulation for *in vitro* bioassay techniques. As a consequence the preparations examined here effectively excluded those such as poultices and pastes of whole or macerated plant material, and ointments prepared with animal fat. Extractions of plant material in organic solvent may in fact be more closely representative of such preparations/applications in terms of chemical profile, particularly in cases where traditional mode of preparation involves extraction into animal fat to produce an unguent or ointment. This is an important point as it is possible that this type of preparation may have been more widely and frequently employed in the past, than may be inferred from information appearing in contemporary and recent Australian ethnobotanical reports. O'Connell *et al* (1983) have indicated that of 28 plant species recorded to be used medicinally by Alyawarr speakers of

central Australia (including three species examined in this study: *E. duttonii*, *E. longifolia* and *C. ambiguus*), all but one were described as being typically prepared *either* as infusions, decoctions *and/or* as "salves" by extraction with animal fat. In the Northern Territory indigenous pharmacopoeias Barr *et al.* (1988; 1993) list 11 of the central Australian medicinal species cited by O'Connell *et al.* (1983), yet indicate that only 2 (*E. longifolia* and *Eucalyptus camaldulensis*) were typically used for the preparation of 'ointments' with animal fat. It is possible that in some indigenous communities where traditional methods of food procurement have been largely displaced by the availability of commercially produced foodstuffs, that the practice of extracting medicinal plant material with animal fat has become less convenient and less common, and so has been reported less frequently in some of the more recent ethnobotanical surveys.

Chapter 4: Phenolic content, antioxidant capacity and anti-inflammatory activity in Australian indigenous medicinal plant preparations

In this Chapter, results from the assays of traditional preparations and extracts for antioxidant activities, cytotoxicity and inhibition of PGE_2 formation activity are presented and discussed. Results of statistical analyses which were performed in order to examine associations between activity and the level of agreement for citations of specific traditional uses mentioned in the ethnobotanical literature are also reported.

4.1 Introduction

4.1.1 Antioxidant activity and wound healing

Oxidative stress in biology involves the progressive damage of cellular macromolecules by reactive free radical species (FRS). Free radicals and reactive oxygen/nitrogen species (ROS/RNS) can be formed in the body as part of normal oxidative metabolism or may arise from photochemical conversion or metabolism of certain drugs and environmental toxins (Kalyanaraman and Sohnle, 1985). FRS can react with cellular lipids to initiate a chain of lipid radical formation and peroxidation, where the initial unstable lipid radical forms a lipid peroxyl which can react with a second lipid molecule to generate a second lipid radical and lipid peroxide and so on. Uncontrolled propagation of radical chain reactions in the body is largely prevented by the endogenous enzymatic systems (e.g. superoxide dismutase, catalase and peroxidase enzymes) and non-enzymatic antioxidant systems (e.g. glutathione), which act to neutralise FRS and ROS/RNS and terminate oxidative chain reactions. Dietary antioxidants, which act by the ability to donate electrons or neutralise FRS without forming unstable intermediates (e.g. ascorbic acid and α-tocopherol), may also play a protective role against oxidative damage (Whitney *et al.*, 2010). Oxidative stress occurs when the rate of

endogenous generation of free radicals and ROS/RNS exceeds the protective capacity of endogenous antioxidant systems (Bors and Michel, 2002). Excess generation of ROS/RNS and resulting oxidative stress has been associated with the development and progression of a wide range of diseases and age-related degenerative processes including atherosclerosis, rheumatoid arthritis, types I and II diabetes mellitus, along with several forms of neurodegenerative disease and cancer (Bors and Michel, 2002). During dermal wound healing, oxidative stress may occur with excessive or inappropriately prolonged inflammation and/or infection at the wound site. Mensah *et al*. (2001) have suggested that an uncontrolled or prolonged generation of ROS by neutrophils during wound healing may impair normal healing and damage adjacent healthy tissues through a combination of increases in activity of oxdidant activated metalloproteases and oxidative damage to antiproteases, and under these conditions, the action of compounds with free radical scavenging and other antioxidant activities may be of potential benefit. For these reasons, scavenging of FRS/ROS/RNS by compounds with antioxidant activity, has been considered as a potential target in the investigation of medicinal plants and natural products used in traditional ethnomedicine for the treatment of wounds (Houghton *et al.*, 2005). Impaired wound healing is a common clinical feature of diabetes mellitus and this is thought to involve damage to cells and extracellular matrix components involved in the wound healing process by the excess production of endogenous ROS by leukocytes which are activated under conditions of peripheral ischemia characteristic of the disease (Altavilla *et al.*, 2001). Administration of raxofelas, an inhibitor of lipid peroxidation in experimental diabetic mice exhibiting impaired wound healing, has been shown to result in improved healing and normalisation of the expression of vascular endothelial growth factor (VEGF) which is deranged in these animals (Altavilla *et al.*, 2001). In the context of normal, uncomplicated wound healing, however, the theoretical potential benefit for antioxidant type therapies to aid treatment is unclear. As with

many of the processes associated with inflammation, the generation of endogenous oxidants throughout certain phases of the wound healing process is normal and in fact may be considered vital to the successful progression of healing and resolution. For example, the respiratory burst of phagocytes, involving rapid generation of superoxide anion, ($O_2^{\bullet-}$) by the NAPDH oxidase enzyme complex partly functions to kill invading microorganisms and prevent wound infection (Sen, 2003). In addition, the majority of non-phagocytic cells directly involved in the wound healing process (e.g. fibroblasts), are known to generate a sustained release of oxidants via the actions of a separate group of oxidases (non-phagocytic NAPDH oxidases) and are thought to increase enzymatic generation of $O_2^{\bullet-}/H_2O_2$ throughout the inflammatory and post-inflammatory phases of wound healing (Sen, 2003; Sen *et al.*, 2006). The significance of this increased rate of generation of oxidants can be explained by the dependence of various processes and signalling events associated with wound healing and tissue repair, on endogenous oxidants as positive modulators or permissive factors. Processes such as angiogenesis, the action of various growth factors, expression of genes involved in synthesis of extracellular matrix components and differentiation of fibroblast to myofibroblasts, are induced or positively modulated with slight elevations in concentration of intracellular H_2O_2 and $O2^{\bullet-}$ (i.e. at concentrations below that which would result in oxidative damage sufficient to impair wound healing) (Sen, 2003; Sen *et al.*, 2006). Roy *et al*. (2006) have monitored H_2O_2 directly in experimental wounds using electrochemical electrodes and reported elevated levels of H_2O_2 during the inflammatory phase and post-inflammatory phases. Roy *et al*. (2006) additionally examined the effect of topical application of H_2O_2 at various concentrations on wound healing using *in* vivo models, and reported improvements in healing outcomes, including a decrease in time of wound closure at concentrations up to 0.15% $^{w}/_{v}$, with higher concentrations (representing those typically used to cleanse wounds in surgical practice), resulting in delayed healing and cellular damage, which was ascribed to

resulting oxidative stress. Roy *et al.* (2006) additionally reported an impairment of wound healing with experimental *in vivo* overexpression of the antioxidant enzyme, catalase in the same study. The potential benefit of substances with antioxidant or free radical scavenging capacities on the wound healing process then, may be limited to situations where normal healing is impaired or delayed by events which produce oxidative overload.

4.1.2 **Measurement of antioxidant capacity**

The i*n vitro* measurement of total antioxidant status or capacity in biological samples can be highly dependent on the specific parameters examined and methodologies employed and while numerous techniques have been developed and applied, no single assay may be considered comprehensive or definitive (Cao and Prior, 1998; Huang *et al.*, 2005). For the initial *in vitro* measurement or screening of antioxidant activity as free radical scavenging potential in biological samples, Huang *et al*. (2005) have thus suggested the use of at least two separate assays, with one based on the mechanism of free radical neutralisation by hydrogen atom transfer and another based on the mechanism of electron transfer. Hydrogen atom transfer methods involve the use of free radicals which can be neutralised with antioxidants capable of donating a proton. Electron transfer methods involve the use of free radicals which can be neutralised by antioxidants capable of donating an electron on deprotonation, and so may be dependent on factors affecting ionisation potential of the antioxidant, such as pH (Miguel, 2010).

In the present studies, free radical scavenging capacity of the extracts was measured using three separate techniques which were the oxygen radical absorbance assay (ORAC), the DMPD (N,N-dimethyl-p-phenylenediamine) decolourisation assay (hydrogen atom transfer methods), and the Folin-Ciocalteu reagent total phenolic content assay (FCR-TPC) (electron transfer type method). The FCR-TPC assay was also used here for the ability to assay total

phenolic content in the extracts. While not exclusively specific for phenolic compounds or functionality, the test is commonly employed for this purpose and is widely accepted as an indicator of total phenolic content. As discussed in Chapter 2, the reagent measures phenolic species via reducing potential and, although molar response is proportional to the overall number of phenolic hydroxyls, reducing capacity is known to be enhanced by ortho or para orientation of hydroxyls (Katalinic *et al.*, 2006).

4.1.3 **Phenolic and polyphenolic compounds in plants**

Phenolic compounds constitute the largest class of plant derived secondary metabolites which are found in virtually all higher plants and include a diverse variety of structures. Phenolic secondary metabolites can be broadly classified as simple phenolics and polyphenolics. Simple phenolics contain a single aromatic ring with one or more hydroxyl and/or hydroxylated hydrocarbon substituents, (e.g. tyrosol, found in olive oil), Some common examples of simple phenolic classes include the phenolic acids (C_6-C_1 structures with a carboxylic acid group such as gallic acid) and C_6-C_3 phenylpropanoids and hydroxycinnamic acids (e.g. caffeic acid). (Andres-Lacueva *et al.*, 2010). Polyphenolics, are characterised by the presence of two or more phenolic rings and may be broadly classified as either flavanoids or non-flavanoids, with further classification based on the number of phenolic rings and other structural elements (Andres-Lacueva *et al.*, 2010). Flavanoids include those with C_6-C_3-C_6 structures and comprise the most abundant and widespread class in nature, comprising 8,405 entries in the Dictionary of Natural Products as of 1999 (Buckingham, 1999, cited by Verpoorte, 2000). Some examples of flavanoid natural products include the catechins and epicatechins found in tea, and the polymeric condensed tannins (e.g. procyanidins of oak leaves). Examples of some non-flavanoid polyphenolics include the hydroxycinnamic acid oligimers [e.g. rosmarinic acid, which is found in several medicinal and culinary Lamiaceae (Zheng and Wang, 2001)], and hydroxystilbenes (e.g. resveratrol, found in peanuts, grapes

and wine). Polyphenolic compounds are known to exhibit a wide range of potentially beneficial biological effects and it is generally assumed that these compounds play a major role in the health benefits associated with high dietary intake of fruits, vegetables and other plant based foods (Robles-Sardin *et al.*, 2010). Almost all polyphenolic compounds exhibit strong antioxidant potential and antioxidant capacity in these compounds has been shown to correlate with the number and arrangement of adjacent hydroxyl groups (Bors and Michel, 2002). Antioxidant capacity may also be influenced by the degree of polymerisation in oligimeric flavanoids and tannins, and these compounds are generally seen as having greater potential activity than their monomeric equivalents (Hagerman *et al.*, 1998).

The term tannin refers to polymeric polyphenols which occur commonly in all plant tissues, and particularly in the leaves and lignified tissue of woody plants (Harborne, 1993). Tannins are able to chelate metal ions and complex strongly with carbohydrates and proteins. The ability of tannins to precipitate protein in solution (i.e. astringency) and crosslink collagen fibres in animal hides underlies the historical use of tannin rich plant extracts to tan and preserve leather, and forms the etymological basis for their name (Scalbert, 1991; Serrano *et al.*, 2009). Tannins can be divided into two classes, termed hydrolysable tannins and condensed tannins. Hydrolysable tannins are derivatives of simple or dimeric phenolic acids such as gallic acid or oxidised derivatives which are esterified with a polyol (typically glucose) (e.g. glucogallin and pentagalloyl glucose). 'Condensed tannins', or proanthocyanidins include oligimers and polymers of hydroxyflavanols (e.g. procyanidin of oak leaves) (Harborne, 1993; Bors and Michel, 2002). Hydrolysable tannins are able to be hydrolysed with acid to the polyol and constituent phenolic acids, whereas proanthocyanidins when treated with acid yield red coloured anthocyanidins (Serrano *et al.*, 2009). Structures for some common simple and polyphenolic compounds, representative of the classes mentioned above, are illustrated below in Figure 4.1.

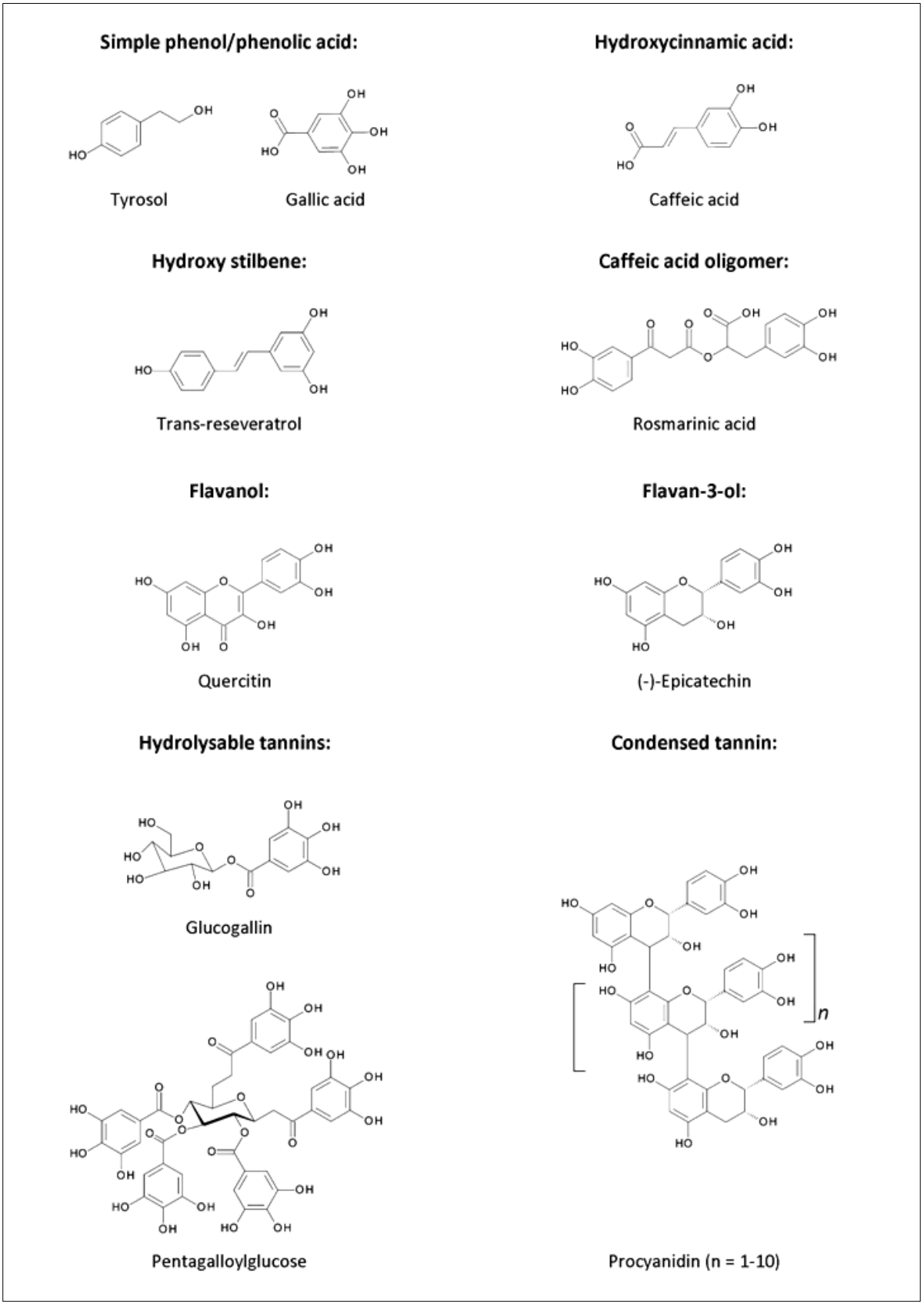


Figure 4.1 Polyphenolics typical of various structural classes for which biological activity has been found. [after Harbourne (1993), and Bors and Michel (2002)]

In addition to their potential antioxidant actions, tannins have been found to exhibit a number of other biological and pharmacological activities including antimicrobial activity (Scalbert, 1991; Serrano *et al.*, 2009), host mediated anti-tumour effects, antiviral activities, anti-inflammatory activities (specifically inhibition of leukotriene production), growth inhibition of *Helicobacter pylori* and potentiation/restoration of the action of β-lactam antibiotic activity against methicillin-resistant *Staphylococcus aureus* strains (Okuda, 2005). Up until the middle of the last century, plant derived tannins (i.e. 'tannic acid' extracts) were used in medicine with reported clinical success for the treatment of severe burn wounds. Use of tannic acid in burns therapy was discontinued due to the emergence of evidence implicating the use of tannic acid in observed increases in mortality associated with liver damage (Hupkens *et al.*, 1995). More recently, critical evaluation of past evidence linking use of tannic acid with liver damage has revealed a number of inconsistencies which suggest that the hepatic damage previously associated with tannic acid treatment may have been a consequence of native burn pathophysiology rather than tannic acid toxicity (Hupkens *et al.*, 1995; Halkes *et al.*, 2001). Additionally, pharmaceutical tannic acid preparations used for burn therapy were typically poorly defined, heterogeneous mixtures of various types of tannin which were derived from various plant species and experimental evidence has shown that toxicity may be restricted to hydrolysable rather than condensed tannins (Hupkens *et al.*, 1995).

A number of studies have demonstrated potentially beneficial effects of tannins and tannin enriched plant extracts toward dermal wound healing using various *in vitro* biological assays and *in vivo* experimental wound models in animals. Agyare *et al*. (2010) have demonstrated stimulation of proliferation in cultured fibroblasts and keratinocytes, and increased formation of extracellular matrix proteins by ellagitannins isolated from the West African medicinal plant *Phyllanthus muellerianus*. Chaudhari and Mengi (2006), have shown improved tensile

strength and epithelialisation of experimental wounds topically treated with tannin enriched extracts of the bark of the Ayurvedic medicinal plant, *Terminalia arjuna*. Kokane *et al*. (2009) have similarly demonstrated improved outcomes using various wound models in rats with topical application of tannin enriched extracts of *Mimosa pudica*.

Khanna *et al*. (2001) have demonstrated positive modulation of the expression of oxidant dependent vascular endothelial growth factor (VEGF) in keratinocytes by a tannin and polyphenol rich grape seed extract. Khanna *et al*. (2002) have described further experiments to examine effects of the extract using *in vivo* wound models and demonstrated improved healing, reporting that treatment was associated with a more well-defined hyperproliferative epithelial region, higher cell density, enhanced deposition of connective tissue, and improved histological architecture, with confirmation that application of the grape seed extract *enhanced* the oxidising environment of the wound site (Khanna *et al.*, 2002). While polyphenolics can exhibit antioxidant and free radical scavenging activities *in vitro,* pro-oxidant activities are also known to occur under some conditions. Oxidation of polyphenols may occur in the presence of metal ions or in solution at high temperature (e.g. in brewed tea) and can result in generation of $O_2^{\cdot -}$ and H_2O_2 (Halliwell, 2008). A tendency toward oxidation of polyphenols and generation of cytotoxic levels of H_2O_2 has been observed in common cell culture media, and this effect has been shown to account for the apparent cytotoxicity of polyphenolic compounds which has been reported in a number of previous studies (Kwee *et al.*, 2003; Halliwell, 2008). Little however, is presently known about either the potential pro-oxidant or anti-oxidant behaviour of phenolic compounds *in vivo* (Aquilano *et al.*, 2008; Halliwell, 2008), and much of the research in this area has been focussed on investigation of dietary polyphenolics in relation to bioavailibility and absorbtion from the ailimentary canal. Khanna *et al*. (2002). have suggested that the oxidant rich environment of the wound site may

favour oxidation of phenolic compounds, leading to induction of oxidant dependent signalling processes involved in tissue repair and regeneration (e.g. expression of VEGF).

4.1.4 **PGE_2 inhibition and cytotoxicity assays**

For screening of anti-inflammatory activity, extracts were examined for the ability to inhibit formation of prostaglandin E_2 (PGE_2) in murine 3T3 fibroblasts stimulated with bacterial calcium ionophore. For the assay of PGE_2, commercial ELISA detection kits were employed. PGE_2 is a prostaglandin eicosanoid which acts as an inflammatory modulator. PGE_2 interacts with the G-protein coupled E_2 prostanoid receptor and is responsible for modulating or inducing a number of physiological responses which include induction of pyresis in response to infection and initiation of uterine contraction during labour. An increase in inducible expression of cycloxygenase-2 (COX-2) activity during inflammation results in increased flux in PGE_2 production by prostaglandin E_2 synthase (Figure 4.2). Local inflammatory actions of PGE_2 relevant to the process of the body's response to injury include vasodilatation and increased capillary permeability (contributing to local oedema, redness and heat) and potentiation of local nocioceptors to produce hyperalgesia. Hyperalgesia or excessive pain at the wound site is known to delay normal wound healing and anti-inflammatory/analgesic activities in general may be expected to provide relief from many of the dermatological and ophthalmic complaints for which the preparations examined are reported to be used to treat (e.g. dermal lesions, pruritis, ophthalmic conditions involving pain and inflammation etc.). Some *in vitro* studies have reported inhibitory actions on fibroblast proliferation migration and differentiation by PGE_2 (Kohyama *et al.*, 2001; Kolodsick *et al.*, 2003), and inhibition of synthesis of components of the extracellular matrix which occur during the proliferative phase of wound repair (Varga *et al.*, 1987). Other studies, however, have provided evidence that elevated PGE_2 levels may stimulate angiogenesis during tissue repair, via induction of expression of basic fibroblast growth factor and VEGF (Cheng *et al.*, 1998; Majima *et al.*,

2000; Sakai *et al.*, 2001). This particular action of PGE_2 has led researchers to investigate the clinical applicability of using selective COX-2 inhibitors as anti-angiogenic therapies in the treatment of cancer (Dempke *et al.*, 2001). The production of PGE_2 occurs toward the endpoint of the inflammatory arachidonic acid/eicosanoid pathways, and attenuation of PGE_2 production (as measured using a cellular assay) may represent inhibition at several of these upstream steps, including inducible COX-2 (Figure 4.2).

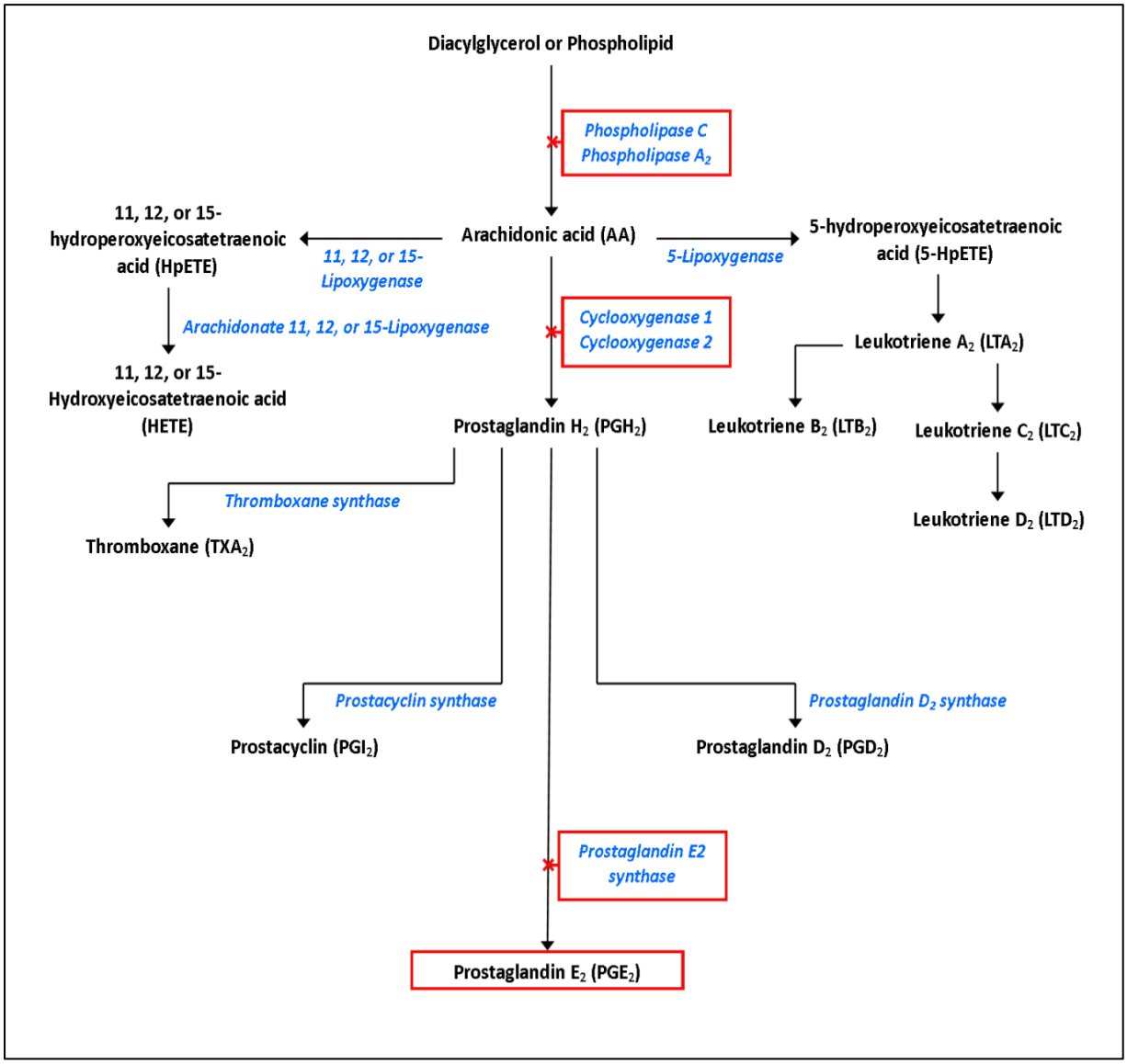


Figure 4.2 Scheme illustrating major elements of the arachadonic acid/eicosanoid pathway. Emphasis on synthesis of prostaglandin E_2 (PGE_2) from membrane phospholipids (substrates and products in black text, enzymes in blue text). Enzymatic steps in the pathway where inhibition may be indicated by the attenuation of PGE_2 production are highlighted in red

Prior to testing the extracts in the PGE_2 inhibitory assay, appropriate sub-cytotoxic assay concentrations were determined. Cytotoxicity was evaluated against cultures of murine P388D1 lymphoblast cell lines incubated in the presence of various concentrations of each extract. The degree of cytotoxicity or subsequent cell viability was determined with the measurement of adenosine triphosphate (ATP) in cell cultures, using a commercial luminescence based assay kit (ATPlite, Packard BioScience B. V.).

4.2 **Results and Discussion**

4.2.1 **Antioxidant capacity and total phenolic content of extracts**

Measurement of total phenolic content (TPC) and antioxidant capacity are reported both as units of reference drug equivalence per unit volume (at the original concentration as prepared using traditional methods) and units of equivalence per unit fresh weight of plant material extracted. Results for the total phenolic assays were reported as gallic acid equivalents (GAE) in parts per million and as mg GAE/g of fresh weight (FW) of plant material extracted. Results for ORAC, and DMPD decolourisation assays are reported as Trolox equivalents (TE) in millmoles and as μmol TE/g FW of plant material extracted, or as μmol/g dry weight of extract for the purpose of comparing traditional aqueous and solvent based extracts. Results for each assay are summarised below in Table 4.1 and Table 4.2. Results were assessed for correlation between the three assays, specifically for that between the FCR assay (as representing total phenolic content) and the other assays of antioxidant capacity. In addition, TPC was compared with measurements of antibacterial MIC (as presented in Chapter 3), in order to determine whether any correlation could be found between antimicrobial activity and total phenolic content. Results obtained in each assay were also compared with MUA_{TMP} values determined for wounds, sores and all dermatological/ophthalmic complaints, in order to determine whether any association could be found between activity and MUA_{TMP} values representing specific uses.

Table 4.1 Total phenolic content and antioxidant capacity of 36 traditional aqueous preparations. Mean values from at least three separate experiments with standard error. Values for Folin-Ciocalteu reagent total phenolic content assay (FCR-TPC) expressed as parts per million of gallic acid equivalents (GAE). ORAC and DMPD-TE values expressed as millimolar (mM) Trolox equivalents. Results ranked in order of highest to lowest TPC (ppm GAE).

Label code	Species	Extract Description	Concentration (mg/mL)	FCR TPC (ppm GAE)		DMPD mM TE		ORAC mM TE	
JES59EJ	*Cochlospermum fraseri*	Expressed juice of fruit pulp	95.2	18189	± 275	60.9	± 4.36	68.2	± 2.19
JES48BD	*Buchanania obovata*	Decoction of inner trunk bark	20.4	11947	± 332	46.7	± 2.91	12.9	± 1.04
JES41TD	*Callitris intratropica*	Decoction of inner trunk bark	13.8	8790	± 210	16.0	± 0.96	16.2	± 1.17
JES40BTD	*Erythrophleum chlorostachys*	Trunk inner bark decoction	10.1	7327	± 206	11.7	± 0.86	19.8	± 0.79
JES66SE	*Buchanania obovata*	Cold infusion of terminal stems	9.7	3017	± 77	16.6	± 1.80	7.6	± 0.65
JES49LD	*Acacia holosericea*	Leaf decoction	8.7	2583	± 26	16.3	± 0.95	6.1	± 0.24
JES30LD	*Acacia auriculiformis*	Leaf decoction	8.0	2528	± 72	14.0	± 0.75	5.9	± 0.18
JES28LTD	*Eucalyptus tetrodonta*	Terminal leaf decoction	5.4	2032	± 57	8.5	± 1.08	8.2	± 0.39
SM18TD	*Eremophila longifolia* (KC)	Decoction of leaves	8.1	1645	± 66	6.2	± 0.20	17.4	± 0.87
JES40LTD	*Erythrophleum chlorostachys*	Leaf/inner trunk bark decoction	4.7	1622	± 48	3.7	± 1.09	5.9	± 0.17
JES66TD	*Buchanania obovata*	Decoction of terminal stems	4.2	1517	± 32	8.8	± 0.36	1.4	± 0.08
SM10TD	*Eremophila duttonii*	Decoction of leaves	9.7	1219	± 40	4.8	± 0.12	16.4	± 0.83
JES42TD	*Bauhinia cunninghamii*	Decoction of root	3.8	1128	± 44	2.4	± 0.29	2.9	± 0.14
SM11TD	*Eremophila duttonii*	Decoction of leaves	6.2	1099	± 65	4.5	± 0.24	12.2	± 0.46
SM19TD	*Eremophila longifolia* (BC)	Decoction of leaves	8.3	1013	± 54	4.0	± 0.17	9.5	± 0.51
SM1TD	*Eremophila sturtii*	Decoction of leaves	9.3	819	± 52	3.1	± 0.27	10.4	± 0.65
SM3TD	*Eremophila longifolia* (IC)	Decoction of leaves	7.7	806	± 39	3.8	± 0.13	9.4	± 0.38
JES63EJ	*Scaevola sericea*	Expressed juice of fruit	75.8	687	± 51	7.8	± 0.89	11.6	± 0.83
SM7TD	*Eremophila latrobei*	Decoction of leaves	5.4	641	± 29	3.5	± 0.10	9.1	± 0.52

(KC): *E. longifolia* karahanaenone chemotype; (IC): *E. longifolia* isomenthone chemotype; (BC): *E. longifolia* borneol chemotype; n.d.: Not determined

Table 4.1 (Continued) Total phenolic content and antioxidant capacity of 36 traditional aqueous preparations. Mean values from at least three separate experiments with standard error are shown. Values for Folin-Ciocalteu reagent total phenolic content assay (FCR-TPC) expressed as parts per million of gallic acid equivalents (GAE). ORAC and DMPD-TE values expressed as millimolar (mM) Trolox equivalents. Results ranked in order of highest to lowest TPC (ppm GAE).

Label code	Species	Extract Description	Concentration (mg/mL)	FCR TPC (ppm GAE)		DMPD mM TE		ORAC mM TE	
JES32BHSE	*Hibiscus tiliaceus*	Warm infusion of stems	9.5	611	± 13	3.9	± 0.08	6.0	± 0.34
JES27LBD	*Pandanus spiralis*	Decoction of leaf bases	13.6	590	± 9	3.0	± 0.31	4.0	± 0.41
JES34LTD	*Avicennia marina*	Decoction of leaves	14.4	590	± 28	1.5	± 0.11	7.7	± 0.68
JES60EJ	*Morinda citrifolia*	Expressed juice of unripe fruit	51.1	544	± 11	6.2	± 0.28	6.1	± 0.72
SM8TD	*Eremophila latrobei*	Decoction of leaves	6.1	498	± 16	3.3	± 0.06	8.2	± 0.45
JES21TD	*Eremophila alternifolia*	Decoction of leaves	5.0	448	± 36	1.7	± 0.13	6.7	± 0.28
JES23TD	*Eremophila alternifolia*	Decoction of leaves	5.1	405	± 11	1.9	± 0.14	10.4	± 0.80
JES43TS	*Crinum angustifolium*	Cold infusion of bulb	18.8	236	± 12	1.7	± 0.03	2.4	± 0.09
JES35SE	*Camptostemon schultzii*	Cold infusion charred wood	6.2	224	± 3	0.3	± 0.07	1.7	± 0.07
JES58AD	*Cymbopogon ambiguus*	Decoction of whole plant	3.6	223	± 11	0.6	± 0.04	2.2	± 0.10
JES28BTD	*Eucalyptus tetrodonta*	Decoction of trunk inner bark	1.2	221	± 10	1.3	± 0.12	1.8	± 0.08
JES37SE	*Flagellaria indica*	Warm infusion of stems	7.1	175	± 6	1.7	± 0.17	2.9	± 0.10
JES27EJP	*Pandanus spiralis*	Expressed juice of leaf tips diluted	8.3	142	± 5	4.2	± 0.14	2.6	± 0.12
JES43BD	*Crinum angustifolium*	Decoction of bulb	18.0	140	± 12	1.7	± 0.05	2.0	± 0.09
JES32SE	*Hibiscus tiliaceus*	Cold infusion of terminal stems	3.3	125	± 10	4.5	± 0.29	2.0	± 0.26
JES31HSE	*Ipomoea pes-caprae*	Hot infusion of leaves	1.5	62	± 2	0.6	± 0.07	1.0	± 0.06
JES31SE	*Ipomoea pes-caprae*	Cold infusion of leaves	0.8	10	± 0.4	0.2	± 0.03	0.2	± 0.01
JES37EJ	*Flagellaria indica*	Expressed juice of growing tips	118.0	n.d	n.d	n.d.	n.d.	n.d	n.d

(KC): *E. longifolia* karahanaenone chemotype; (IC): *E. longifolia* isomenthone chemotype; (BC): *E. longifolia* borneol chemotype; n.d.: Not determined

Table 4.2 Total phenolic content and antioxidant capacity of 36 traditional aqueous preparations (values expressed as equivalents per unit weight of plant material extracted). Mean values from at least three separate experiments with standard error are shown. Values shown as gallic acid equivalents (GAE), or μmol Trolox equivalents (TE) per gm fresh weight of plant material. Results ranked in order of highest to lowest TPC (mg GAE/g FW).

Label code	Species	Extract Description	Extract Yield (% FW)	FCR TPC (mg GAE/g FW)		DMPD (μmol TE/g FW)		ORAC (μmol TE/g FW)	
JES40BTD	*Erythrophleum chlorostachys*	Trunk inner bark decoction	10.3	75.1	± 2.11	120.1	± 8.77	203.1	± 8.06
JES28LTD	*Eucalyptus tetrodonta*	Terminal leaf decoction	10.8	40.6	± 1.14	170.8	± 21.67	163.4	± 7.77
JES66TD	*Buchanania obovata*	Decoction of terminal stems	9.5	33.9	± 0.72	197.7	± 7.99	31.1	± 1.80
JES48BD	*Buchanania obovata*	Decoction of inner trunk bark	5.6	32.9	± 0.91	128.5	± 8.02	35.5	± 2.85
JES49LD	*Acacia holosericea*	Leaf decoction	8.5	25.2	± 0.26	158.9	± 9.29	59.9	± 2.30
JES41TD	*Callitris intratropica*	Decoction of inner trunk bark	3.9	24.9	± 0.59	45.4	± 2.71	46.0	± 3.32
JES30LD	*Acacia auriculiformis*	Leaf decoction	7.8	24.7	± 0.70	136.7	± 7.27	57.7	± 1.79
SM18TD	*Eremophila longifolia* (KC)	Decoction of leaves	12.0	24.3	± 0.98	91.7	± 2.99	257.3	± 12.81
JES66SE	*Buchanania obovata*	Cold infusion of terminal stems	6.9	21.5	± 0.55	118.5	± 12.87	54.0	± 4.64
JES42TD	*Bauhinia cunninghamii*	Decoction of root	7.2	21.4	± 0.84	45.8	± 5.53	55.8	± 2.65
JES40LTD	*Erythrophleum chlorostachys*	Leaf/inner trunk bark decoction	5.8	20.2	± 0.60	46.0	± 13.51	73.6	± 2.16
SM1TD	*Eremophila sturtii*	Decoction of leaves	19.1	16.8	± 1.07	62.8	± 5.61	213.5	± 13.36
SM19TD	*Eremophila longifolia* (BC)	Decoction of leaves	12.9	15.8	± 0.84	62.4	± 2.62	148.5	± 8.03
SM10TD	*Eremophila duttonii*	Decoction of leaves	10.9	13.6	± 0.45	54.2	± 1.35	183.7	± 9.27
SM3TD	*Eremophila longifolia* (IC)	Decoction of leaves	12.1	12.6	± 0.60	58.7	± 1.99	146.5	± 5.92
SM7TD	*Eremophila latrobei*	Decoction of leaves	10.5	12.5	± 0.57	68.5	± 1.90	177.3	± 10.05
SM11TD	*Eremophila duttonii*	Decoction of leaves	6.8	12.0	± 0.71	49.0	± 2.59	133.1	± 5.03
SM8TD	*Eremophila latrobei*	Decoction of leaves	12.3	9.9	± 0.32	66.9	± 1.30	163.7	± 9.07
JES28BTD	*Eucalyptus tetrodonta*	Decoction of trunk inner bark	4.7	8.8	± 0.40	51.6	± 4.70	71.7	± 3.25

*: Extracts of *E. alternifolia* prepared from air dried material. (KC): *E. longifolia* karahanaenone chemotype; (IC): *E. longifolia* isomenthone chemotype; (BC): *E. longifolia* borneol chemotype; n.d.: Not determined

Table 4.2 (Continued) Total phenolic content and antioxidant capacity of 36 traditional aqueous preparations (values expressed as equivalents per unit weight of plant material extracted). Mean values from at least three separate experiments with standard error are shown. Values shown as gallic acid equivalent (GAE) or μmol Trolox equivalents (TE) per gm fresh weight of plant material. Results ranked in order of highest to lowest TPC (mg GAE/g FW).

Label code	Species	Extract Description	Extract Yield (% FW)	FCR TPC (mg GAE/g FW)		DMPD (μmol TE/g FW)		ORAC (μmol TE/g FW)	
JES21TD	*Eremophila alternifolia**	Decoction of leaves*	8.4	*7.6	± 0.60	*28.2	± 2.19	*113.3	± 4.79
JES23TD	*Eremophila alternifolia**	Decoction of leaves*	8.3	*6.7	± 0.18	*30.9	± 2.29	*170.8	± 13.18
JES59EJ	*Cochlospermum fraseri*	Expressed juice of fruit pulp	2.4	4.6	± 0.07	15.3	± 1.10	17.2	± 0.55
JES34LTD	*Avicennia marina*	Decoction of leaves	7.9	3.2	± 0.15	8.3	± 0.60	42.2	± 3.69
JES58AD	*Cymbopogon ambiguus*	Decoction of whole plant	4.3	2.7	± 0.13	7.8	± 0.47	27.0	± 1.15
JES27LBD	*Pandanus spiralis*	Decoction of leaf bases	5.1	2.2	± 0.04	11.4	± 1.17	14.8	± 1.53
JES32BHSE	*Hibiscus tiliaceus*	Warm infusion of stems	2.7	1.7	± 0.04	11.2	± 0.24	17.0	± 0.98
JES35SE	*Camptostemon schultzii*	Cold infusion charred wood	4.3	1.6	± 0.02	2.0	± 0.53	12.0	± 0.52
JES32SE	*Hibiscus tiliaceus*	Cold infusion of terminal stems	3.8	1.4	± 0.11	51.6	± 3.29	23.2	± 3.01
JES31HSE	*Ipomoea pes-caprae*	Hot infusion of leaves	3.2	1.3	± 0.04	11.6	± 1.53	20.9	± 1.20
JES37SE	*Flagellaria indica*	Warm infusion of stems	3.5	0.9	± 0.03	8.1	± 0.83	14.2	± 0.49
JES43TS	*Crinum angustifolium*	Cold infusion of bulb	5.6	0.7	± 0.03	5.1	± 0.09	7.0	± 0.28
JES43BD	*Crinum angustifolium*	Decoction of bulb	4.5	0.4	± 0.03	4.3	± 0.13	5.0	± 0.22
JES27EJP	*Pandanus spiralis*	Leaf tip expressed juice diluted	1.5	0.3	± 0.01	7.6	± 0.26	4.7	± 0.21
JES31SE	*Ipomoea pes-caprae*	Cold infusion of leaves	2.1	0.2	± 0.01	5.8	± 0.77	5.5	± 0.19
JES60EJ	*Morinda citrifolia*	Expressed juice of unripe fruit	1.6	0.2	± 0.00	1.9	± 0.08	1.9	± 0.22
JES63EJ	*Scaevola sericea*	Expressed juice of fruit	1.8	0.2	± 0.01	1.9	± 0.21	2.8	± 0.20
JES37EJ	*Flagellaria indica*	Expressed juice of growing tips	4.9	n.d.	n.d.	n.d.	n.d.	n.d	n.d.

*: Extracts of *E. alternifolia* prepared from air dried material. (KC): *E. longifolia* karahanaenone chemotype; (IC): *E. longifolia* isomenthone chemotype; (BC): *E. longifolia* borneol chemotype; n.d.: Not determined

4.2.2 **Total phenolic content of traditional aqueous preparations by Folin-Ciocalteu reagent assay**

A wide range of values for TPC was found amongst the traditional aqueous extracts. Values expressed per unit volume in relation to the original extract concentration (ppm GAE) ranged from 10 ± 0.2 for the cold infusion of *Ipomoea pes-caprae*, to 18189 ± 275 for the expressed fruit juice of *Cochlospermum fraseri*. The *C. fraseri* extract correspondingly produced the highest ORAC and DMPD-TE values recorded (68.2 ± 2.19 and 60.9 ± 4.36 mM TE respectively). Other extracts exhibiting relatively high TPC (ppm GAE) included the decoctions of inner trunk bark from *Buchanania obovata*, *Callitris intratropica*, and *Erythrophleum chlorostachys*. The high phenolic content measured in *C. fraseri* extracts could be attributed to the relatively high concentration of this preparation, and this was found to be the case with most of the expressed juice preparations (*cf.* 3.2). When TPC was expressed per unit weight of material extracted, C. *fraseri* was found to be relatively low in phenolic content (ranking 22nd overall with TPC of 4.6 ± 0.07 mg GAE/g FW) and higher levels of phenolic content were found to occur amongst extracts of inner bark and leaves (with the highest TPC of 75.1 mg GAE/g FW found in the *E, chlorostachys* inner bark decoction). Results were examined for effects of method of preparation and plant part extracted on TPC. Modes of preparation of the extracts were broadly categorised into three groups which included infusions (9 extracts), decoctions (24 extracts) and expressed juices (3 extracts). Plant parts were categorised into four groups which included leaves (19 extracts), inner bark (including root inner bark) (7 extracts), fruits, and 'others' (including stems, corms and inner fleshy part of leaves and leaf bases of *Pandanus spiralis*) (7 extracts). The *Flagellaria indica* expressed juice extract was not included and so all of the extracts classed as 'expressed juice' preparations were the same as those also classed as fruit extracts. The mean TPC (ppm GAE) of the four groups of plant parts are shown in Figure 4.3.

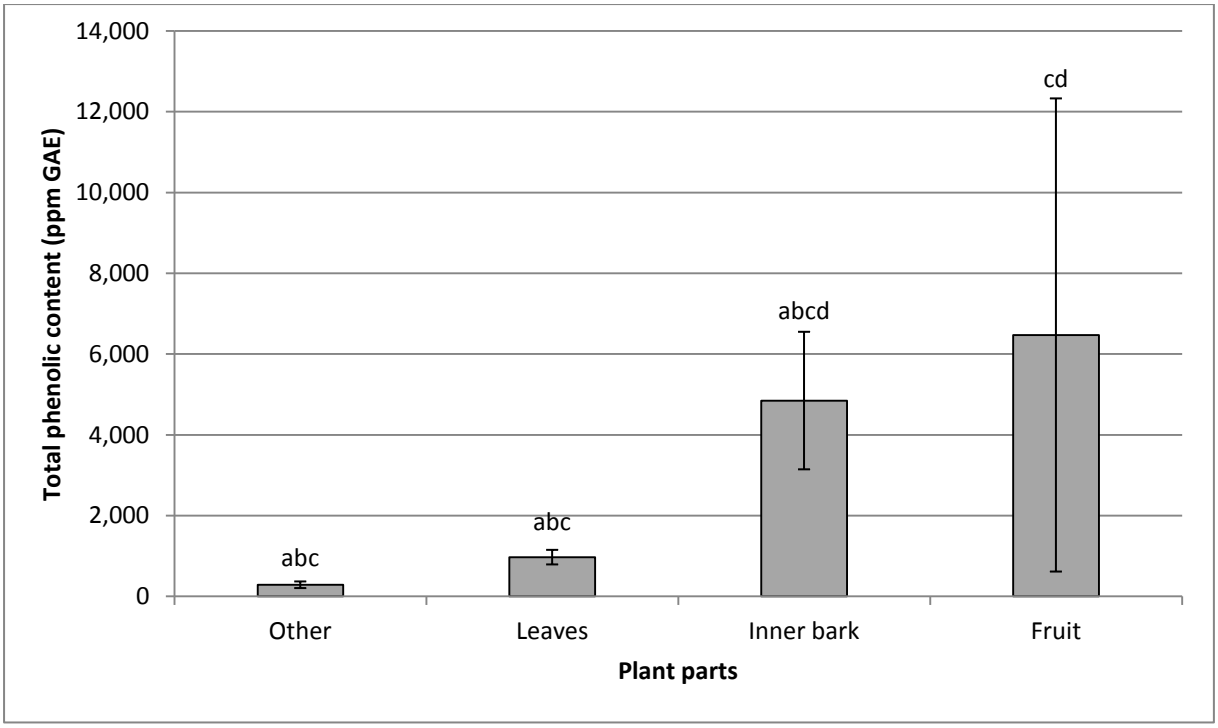


Figure 4.3 Bar chart showing means with standard error bars for total phenolic content (ppm GAE extract) of traditional preparations grouped by plant part extracted. For 'other' (stems, corms etc.) n = 7. For fruit, n = 3. For leaves, n = 19, for inner bark, n = 7. Letters denote significant differences between groups ($p < 0.05$) as determined with one-way ANOVA and Tukey tests. Groups denoted by same letter not significantly different

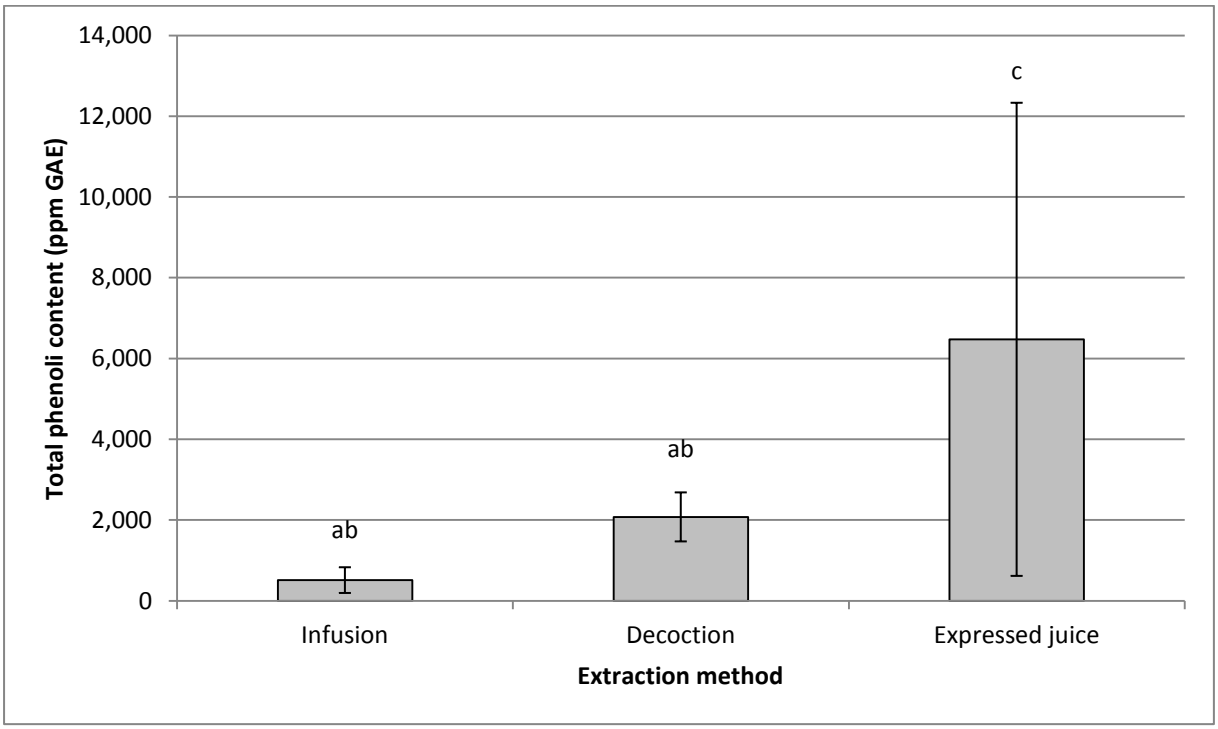


Figure 4.4 Bar chart showing means with standard error bars for total phenolic content (ppm GAE) of traditional preparations grouped by method of extraction. For infusions n = 9. For expressed juices, n = 3. For decoctions, n = 24. Letters denote significant differences between groups ($p < 0.05$) as determined with one-way ANOVA and Tukey tests. Groups denoted by same letter not significantly different.

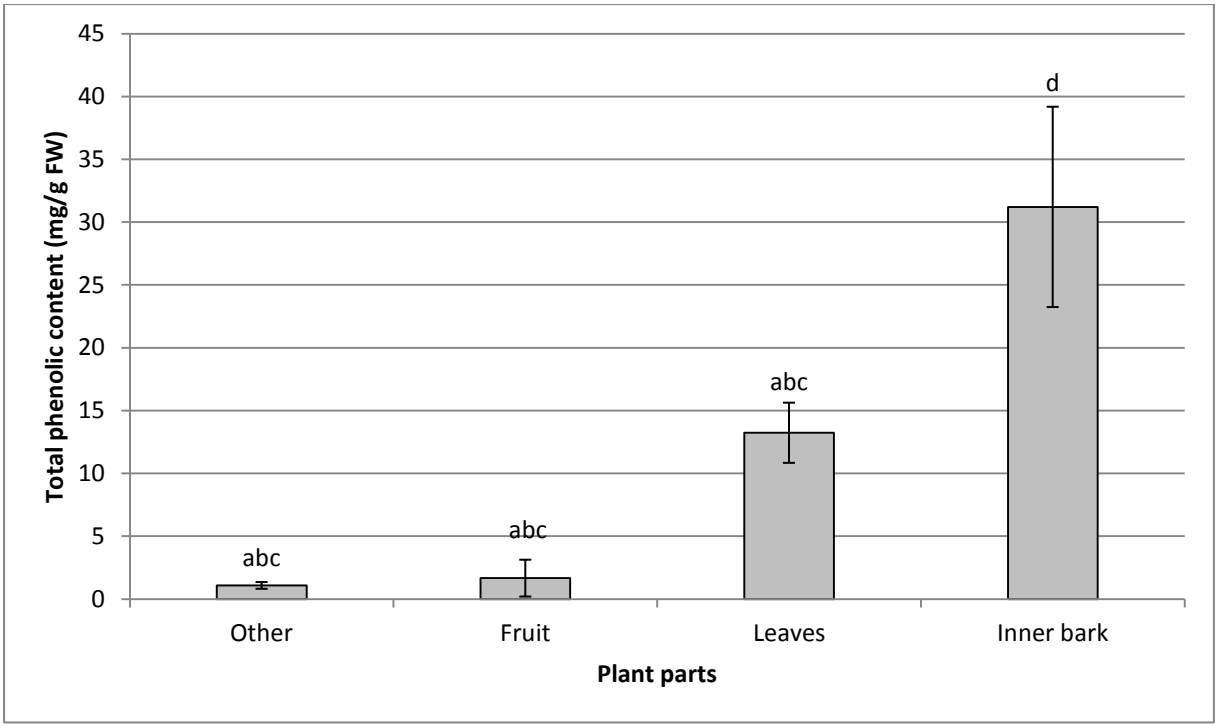


Figure 4.5 Bar chart showing means with standard error bars for total phenolic content (mg GAE/g FW extract) of traditional preparations grouped by plant part extracted. For 'other' (stems, corms etc.) n = 7. For fruit, n = 3. For leaves, n = 19, for inner bark, n = 7. Letters denote significant differences between groups ($p < 0.05$). Groups denoted by same letter not significantly different

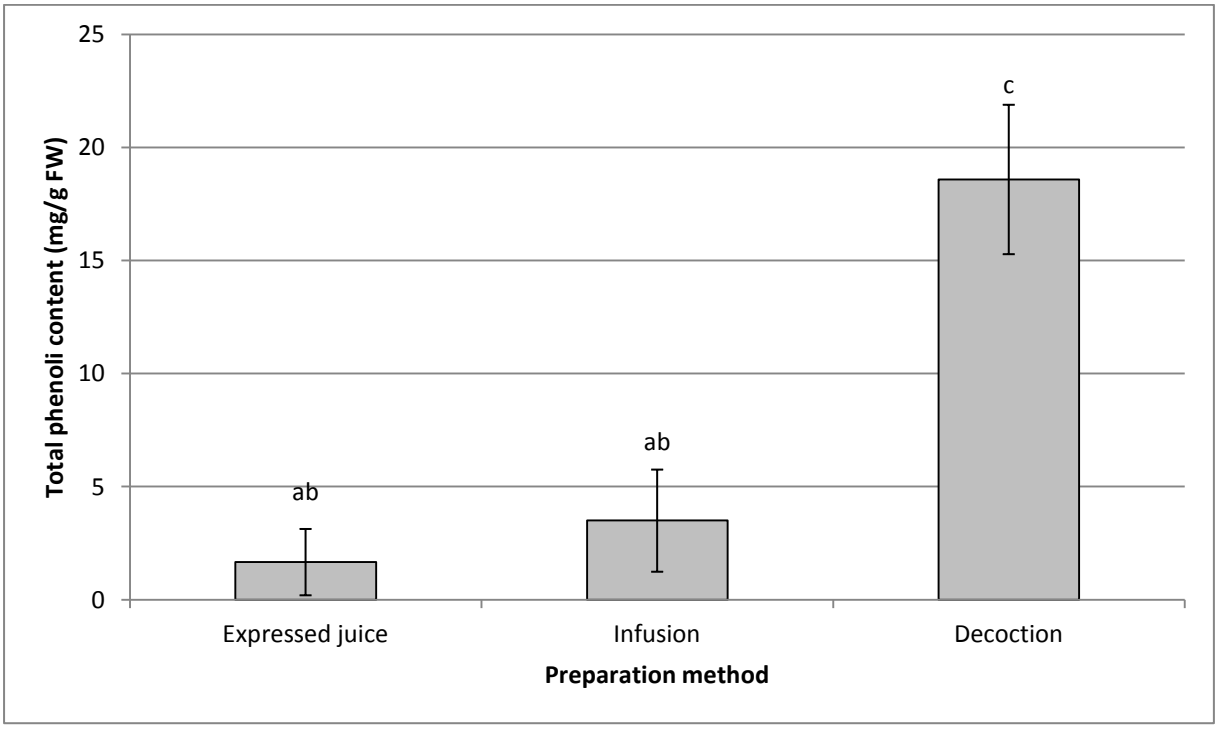


Figure 4.6 Bar chart showing means with standard error bars for total phenolic content (mg GAE/g FW) of traditional preparations grouped general method of extraction. For infusions n = 9. For expressed juices, n = 3. For decoctions, n = 24. Letters denote significant differences between groups ($p < 0.05$). Groups denoted by same letter not significantly different

Among extracts grouped by plant parts, the greatest mean TPC was found in those prepared from fruit (6473 ± 5857), followed by inner bark (4849 ± 1702), then leaves (972 ± 180) then the 'other' category (288 ± 82). A one-way ANOVA followed by a Tukey test showed that the fruit derived extracts exhibited significantly greater TPC levels than extracts derived from leaves and parts of the 'other' category, but not significantly greater than inner bark extracts. A comparison of mean TPC values (ppm GAE) for the three extraction methods is shown above in Figure 4.4. Among extracts grouped by mode of preparation, highest mean TPC was found for the expressed juices (6473 ± 5857), followed by decoctions (2076 ± 607), and then infusions (511 ± 318). Expressed juice extracts were also found to be significantly higher in TPC than either decoctions or infusions. This result, however, may not be accurately representative of fruit juices in general as there were only three extracts examined and variance in TPC was relatively large due to the exceptionally high phenolic content found for the *C. fraserii* extract (as was reflected in the large standard error). TPC of the remaining two expressed juice preparations ranged from 544 ± 11 (*Morinda citrifolia* fruit) to 687± 51 ppm GAE (*Scaevola sericea* fruit). When one-way ANOVA and Tukey tests were repeated on the data excluding all juice/fruit extracts, no significant differences in TPC were found between groups representative of each preparation method, and only the extracts derived from inner bark exhibited significantly higher phenolic content ($p < 0.05$) compared to those derived from other plant parts. As was noted in Chapter 3, extraction by expression of juice produced extracts which were significantly higher in concentration than those obtained by other methods, yet lower in yield relative to fresh weight of plant material. When TPC of extracts was expressed in terms relative to material extracted (i.e. mg GAE/g FW), TPC values of the fruit juice extracts were among some of the lowest recorded. Mean TPC as mg GAE/g FW, of the four plant part categories is shown above in Figure 4.5**.** Greatest mean TPC as mg GAE/g FW was found for extracts prepared from inner bark (31.2 ± 8.0), followed by leaves

(13.2 ± 2.4), then fruit (1.7 ± 1.5) and then the 'other' category (1.1 ± 0.7). One-way ANOVA followed by a Tukey test showed that the inner bark derived extracts exhibited significantly greater TPC levels per unit material extracted than all other types ($p < 0.05$). No significant difference was found among the other groups. Amongst the extraction methods, the greatest mean TPC as mg GAE/g FW was found for decoctions (18.6 ± 3.3), followed by infusions (3.5± 2.3), and expressed juice (1.7 ± 1.5). One-way ANOVA and Tukey tests showed that the decoctions exhibited significantly greater TPC levels relative to yield from material extracted than was found in the infusions or expressed juices ($p < 0.05$), whereas no significant difference was seen between infusions and expressed juices.

A number of extracts were prepared as both decoctions and infusions, using an equal quantity of material from the same plant where descriptions for preparation differed in the sources followed. These included the extracts of *B. obovata* terminal stems, *I. pes-caprae* leaves and *Hibiscus tiliaceus* stems. Lower TPC values were found for infusions of *I. pes-caprae* and *H. tiliaceus* (10 ± 0.2 and 125 ± 10 ppm GAE) than corresponding decoctions (62 ± 2 and 611 ± 10 ppm GAE). TPC measured in infusions of *B. obovata* terminal stem (3017 ± 77 ppm GAE) was higher than that of the corresponding decoction (1517 ± 32 ppm GAE), however, this reflected the higher concentration of the infusion which was seen in this particular case, as the reverse was seen when TPC was expressed as mg GAE/g FW (infusion: 21.5 mg GAE/g FW, decoction: 33.9 mg GAE/g FW).

These results are consistent with observations reported by Katalinic *et al*. (2006), where effects of temperature and time of extraction on phenolic content in aqueous extracts of *Melissa officinalis* leaves ('Melissa', or 'lemon balm') were investigated. Katalinic *et al*. (2006) reported increases in phenolic content of around two-fold, in extractions carried out with water at ca. 98°C as compared with room temperature (20°C) infusions and showed that

almost all extractable phenolics could be found in solution after 10 min of extraction in 98°C water. It was also not surprising that higher phenolic content was found in inner bark and leaf extracts as relatively high levels of flavanoids and tannins are known to accumulate in these tissues (Harborne, 1993). In a study examining phenolic content and antioxidant capacity in extracts of various fruits, vegetables, grains, medicinal herbs and other plant parts, Kahkonen *et al*. (1999) found the highest levels of phenolic content, (as mg GAE/g dry weight, assessed with Folin-Ciocalteu reagent) amongst tree barks and leaves.

TPC values recorded for leaf decoctions prepared from the three chemotypes of *Eremophila longifolia* collected were compared in order to determine whether any differences could be detected. Mean TPC values for each of the extracts are shown below in Figure 4.7. TPC of the decoction prepared using leaves from the karahanaenone chemotype was 1645 ± 66 ppm GAE which was around 1.5 - 2× that found in equivalent decoctions prepared from leaves of the isomenthone and borneol chemotypes (806 ± 39 ppm GAE and 1013 ± 54 ppm GAE respectively). Analysis with one-way ANOVA followed by a post-hoc Tukey test showed this difference to be significant (p <0.05).

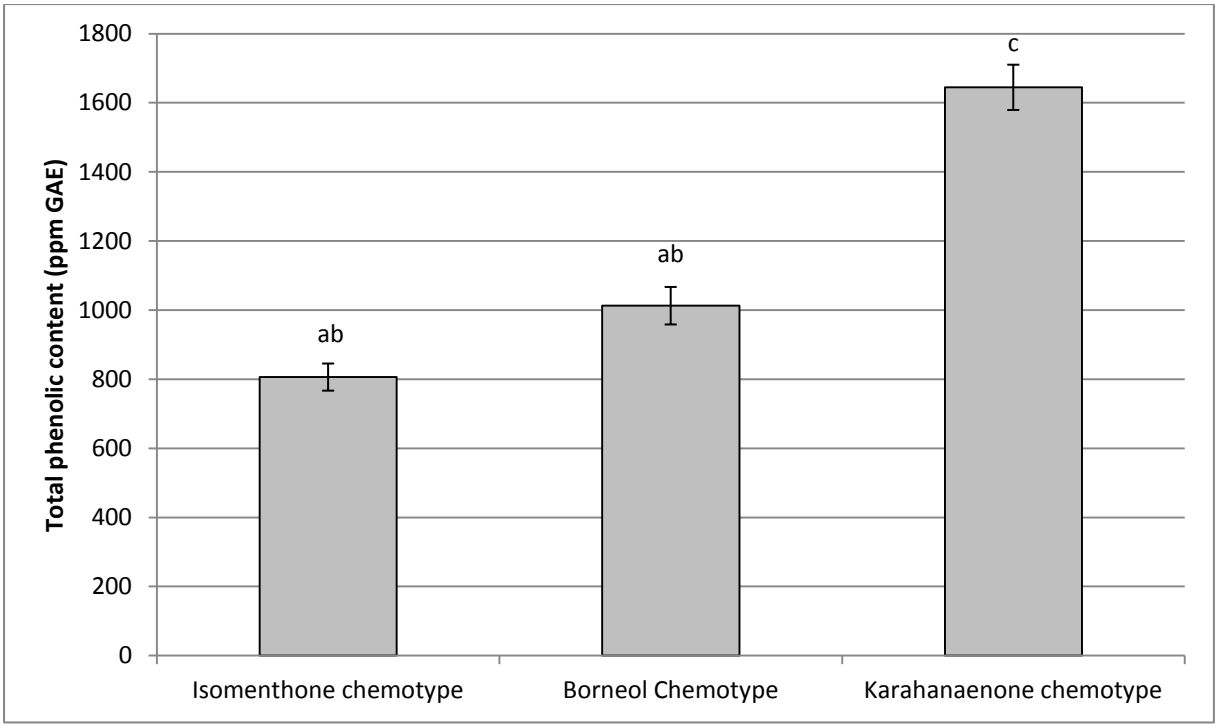


Figure 4.7 Bar chart showing total phenolic content of extracts representative of three separate chemotypes of *E. longifolia*. For all types n = 4. Letters denote significant differences between groups ($p < 0.05$). Groups denoted by same letter not significantly different.

4.2.3 Antioxidant capacity of traditional preparations by DMPD decolourisation assay

Antioxidant capacity of the aqueous preparations as assessed by the DMPD decolourisation assay ranged from 0.2 ± 0.01 mM TE (for *I. pes-caprae* cold infusion) to 60.9 ± 4.36 mM TE (*C. fraserii* expressed juice). Relatively high values were also found in inner bark decoctions of *B. obovata*, *C. intratropica* and *E. chlorostachys*, the cold infusion of *B. obovata* terminal stems and the decoctions of leaf material from *Acacia holosericea* and *Acacia auriculiformis*. The highest TE values by unit mass of plant material extracted were found in the decoctions of *B. obovata* inner bark (197 ± 8 μmol TE/g FW) and *Eucalyptus tetrodonta* leaves (171 ± 22 μmol TE/g FW).

4.2.4 Antioxidant capacity of traditional preparations by ORAC assay

Antioxidant capacity of the aqueous preparations as determined using the ORAC assay ranged from 0.2 ± 0.03 mM TE (for *I. pes-caprae* cold infusion) to 68.2 ± 2.19 mM TE (*C.*

fraserii expressed juice). Other extracts producing relatively high TE values by ORAC assay included, again, the decoction type extracts of trunk inner bark of *B. obovata*, *C. intratropica* and *E. chlorostachys*, (12.9 - 19.8 mM TE), along with the decoctions of leaves from the *E. longifolia* karahanaenone chemotype (17.4 ± 0.9 mM TE) and *Eremophila duttonii* (16.4 ± 0.8 mM TE).

4.2.5 **Correlation between total phenolic content and antioxidant capacity assays**

Significant positive correlation was found between TPC obtained with the FCR assay and DMPD-TE antioxidant capacity assays (37 = 0.944, p <0.001). The relationship between TPC and antioxidant capacity of the extracts as determined using the DMPD decolourisation assay, is shown in Figure 4.8. Correlation between FCR-TPC and ORAC assay values (Figure 4.9) was also significant, but not as strong (37 = 0.830, p <0.001).

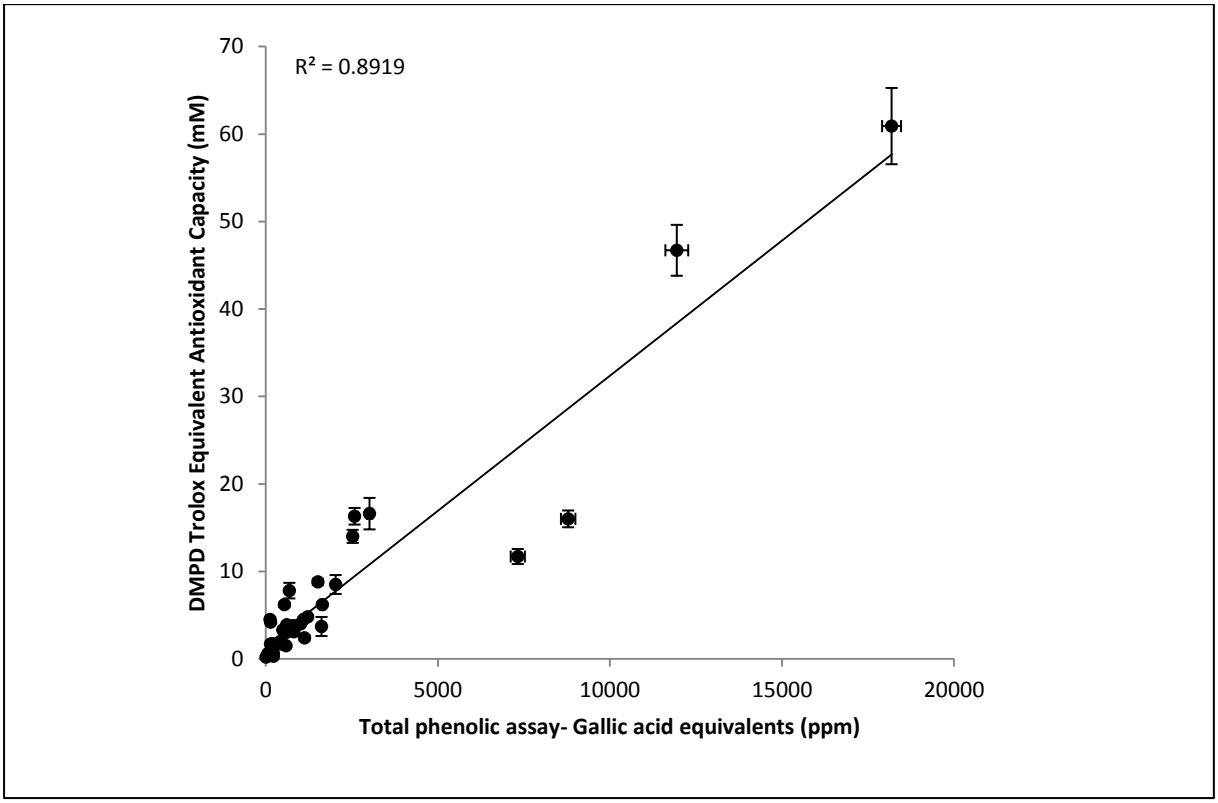


Figure 4.8 Scatter plot showing relationship between Folin-Ciocalteu total phenolic content assay and DMPD TE values (mM) in traditional preparations. (Error bars indicate standard error)

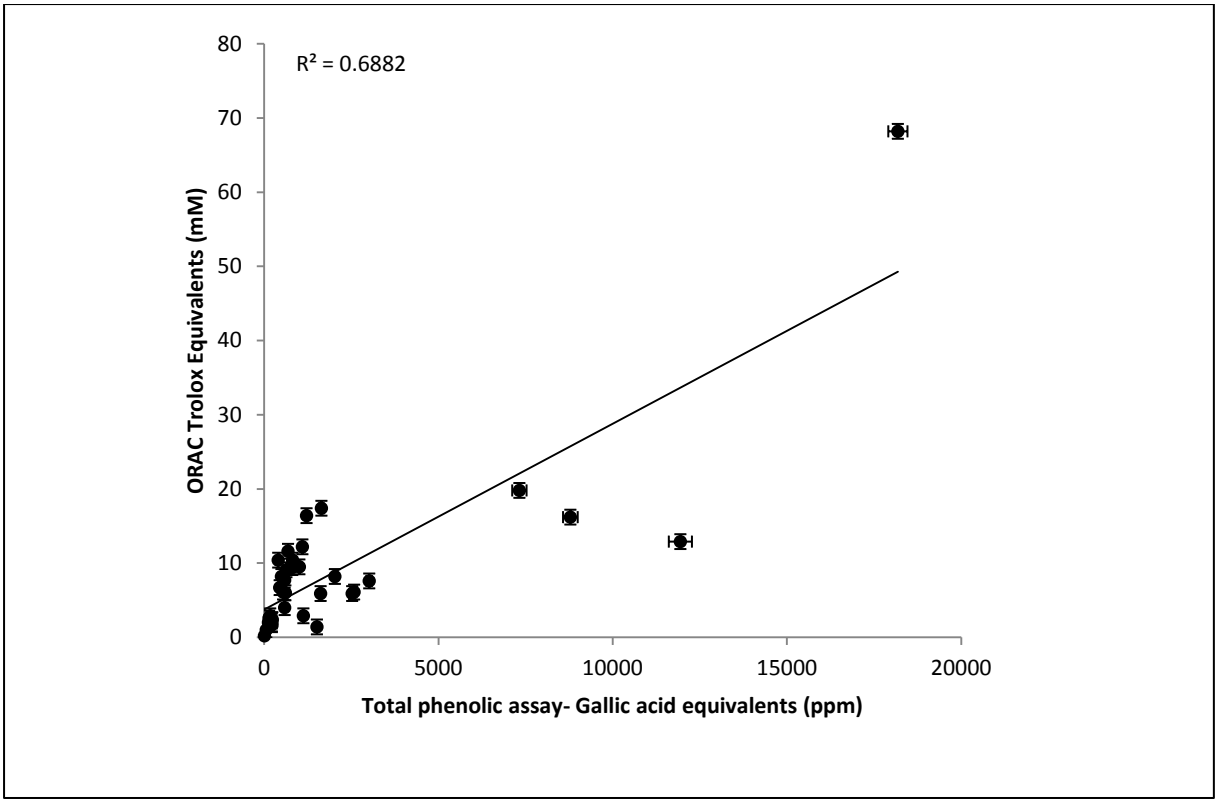


Figure 4.9 Scatter plot showing relationship between Folin-Ciocalteu total phenolic content assay and ORAC values (mM TE). (Error bars indicate standard error)

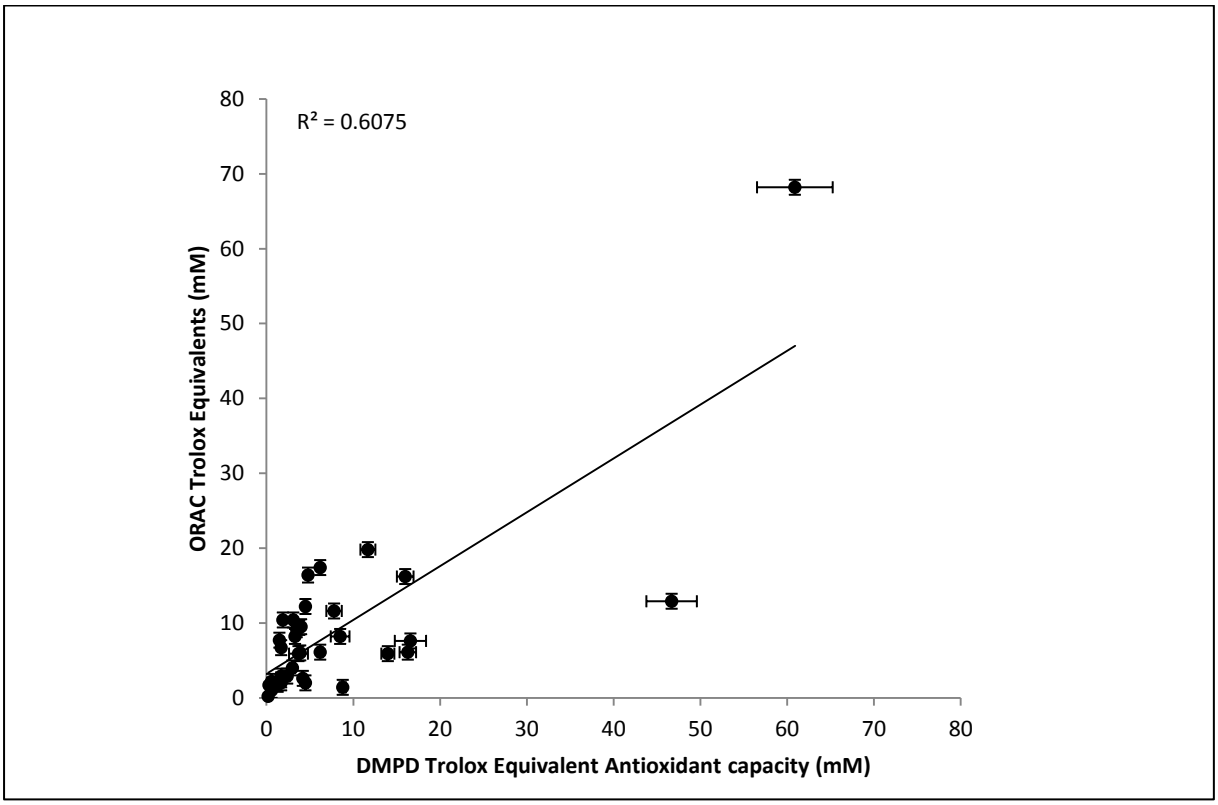


Figure 4.10 Scatter plot showing relationship between DMPD TEAC and ORAC assay values (mM TE) (Error bars indicate standard error).

Table 4.3 Correlation between total phenolic content assay and measurements of antioxidant capacity performed on traditional medicinal preparations (Pearson correlation coefficient (r) with r^2 values in parentheses)

	FCR-TPC (GAE ppm)	DMPD TE mM
FCR-TPC (GAE ppm)	-	
DMPD TE (mM)	**0.944 (0.892)	-
ORAC TE (mM)	**0.830 (0.688)	**0.779 (0.608)

Asterisks denote significance level of correlation; **: $p < 0.01$

A number of previous studies have been conducted to investigate the relationship between total phenolic content (as estimated using FCR) and antioxidant capacity as measured using various techniques, in extracts of medicinal and other plants (e.g. Kahkonen *et al.*, 1999; Zheng and Wang, 2001; Katalinic *et al.*, 2006; Gan *et al.*, 2010). The majority of these studies have also reported finding significant linear correlation between phenolic content and antioxidant capacity, with the exception of the study undertaken by Kahkonen *et al.* (1999), where TPC and antioxidant capacity was assessed in 92 extracts of various plant species and no correlation was found between phenolic content and ability of extracts to inhibit oxidation of methyl lineolate.

While an overall positive correlation was found between the TPA and DMPD assays, a number of extracts with apparently low relative phenolic content were found to be relatively efficient scavengers of DMPD and *vice versa.* For example, the total phenolic content of the decoction of *E. chlorostachys* inner bark was 7327 ± 206 ppm GAE with DMPD-TE of 11.7 mM, in comparison the total phenolic content of the cold infusion of *B. obovata* was less than half that of the *E. chlorostachys* extract, (3017 ± 77 ppm GAE) yet a higher DMPD-TE was recorded (16.6 mM). Similar occurrences were seen amongst ORAC values in relation to TPC. For example, similar ORAC values were found for the decoction of *E. duttonii* leaves and the decoction of inner bark of *B. obovata* (16.4 ± 0.83 and 16.2 ± 1.17 mM TE respectively) despite the fact that measured TPC of the *E. duttonii* extract (1219 ± 40 ppm GAE) was around 1/10th that found for the *B. obovata* extract (11947 ± 332 ppm GAE).

While significant positive correlation was also found between TE values as measured by the ORAC and the DMPD decolourisation assay, in some instances marked divergences were found between TE values obtained using each technique. Most notably, TE values for the *Eremophila* spp. leaf extracts were all found to be greater when measured using the ORAC assay as compared to those obtained using the DMPD assay. As a result, all of the extracts prepared with leaf material from species of *Eremophila* consistently ranked higher within the group of extracts as a whole, when ranked according to ORAC values. For example, TE antioxidant capacity for the decoction of leaves of the karahanaenone chemotype of *E. longifolia* was measured at 6.2 ± 0.20 mM TE using the DMPD assay and was ranked 11th overall in antioxidant capacity by this technique. The same extract, however, ranked 3rd according to results of the ORAC assays (17.4 ± 0.77 mM TE). The leaf decoction of *Avicennia marina* similarly showed noticeably higher ORAC values (7.7 ± 0.68 mM TE) as compared to TE values obtained by the DMPD method (1.5 ± 0.11 mM TE). Conversely, several of the extracts exhibited lower levels of apparent antioxidant capacity in ORAC assays when compared to corresponding results obtained with the DMPD assays. These included the decoction of inner bark from *B. obovata*, (DMPD-TE: 46.7 ± 2.91 mM; ORAC-TE: 12.9 ± 1.04 mM), decoction and infusion type extracts of terminal stems from *B. obovata* (DMPD-TE: 8.8 ± 0.36 mM; ORAC-TE: 1.4 ± 0.08 mM, and DMPD-TE: 16.6 ± 1.80 mM; ORAC-TE: 7.6 ± 0.65 mM) and decoctions of leaves from *A. auriculiformis* and *A. holosericea*. (DMPD-TE: 16.3 ± 0.95 mM; ORAC-TE: 6.1 ± 0.24 mM, and DMPD-TE: 14.0 ± 0.75 mM; ORAC-TE: 5.9 ± 0.18 mM). Similar discrepancies in individual data points despite an overall significant correlation between antioxidant capacity and total phenolic content was reported by Kratchanova *et al*. (2010), where ORAC values and FCR-TPC for aqueous and aqueous-acetone extracts of 25 medicinal plants were compared.

As with TPC assay results, ORAC and DMPD-TE values determined for extracts representing each of the three chemotypes of *E. longifolia* were compared. A similar result to that found for phenolic content was seen, where significantly higher values were found for the extract prepared from material representative of the karahanaenone chemotype. ORAC-TE and DMPD-TE of the karahanenone chemotype decoction was 257.3 ± 12.8 and 91.7 ± 12.8 μmol TE/g FW respectively which was around 40% greater than that measured for equivalent extracts of the borneol and isomenthone chemotypes (ORAC-TE: 146.5-148.5 μmol TE/g FW; DMPD-TE: 58.7-62.4 μmol TE/g FW). One-way ANOVA followed by a Tukey test showed this difference to be significant (p <0.05). Mean ORAC and DMPD-TE values for each of the chemotypes are shown below in Figure 4.11.

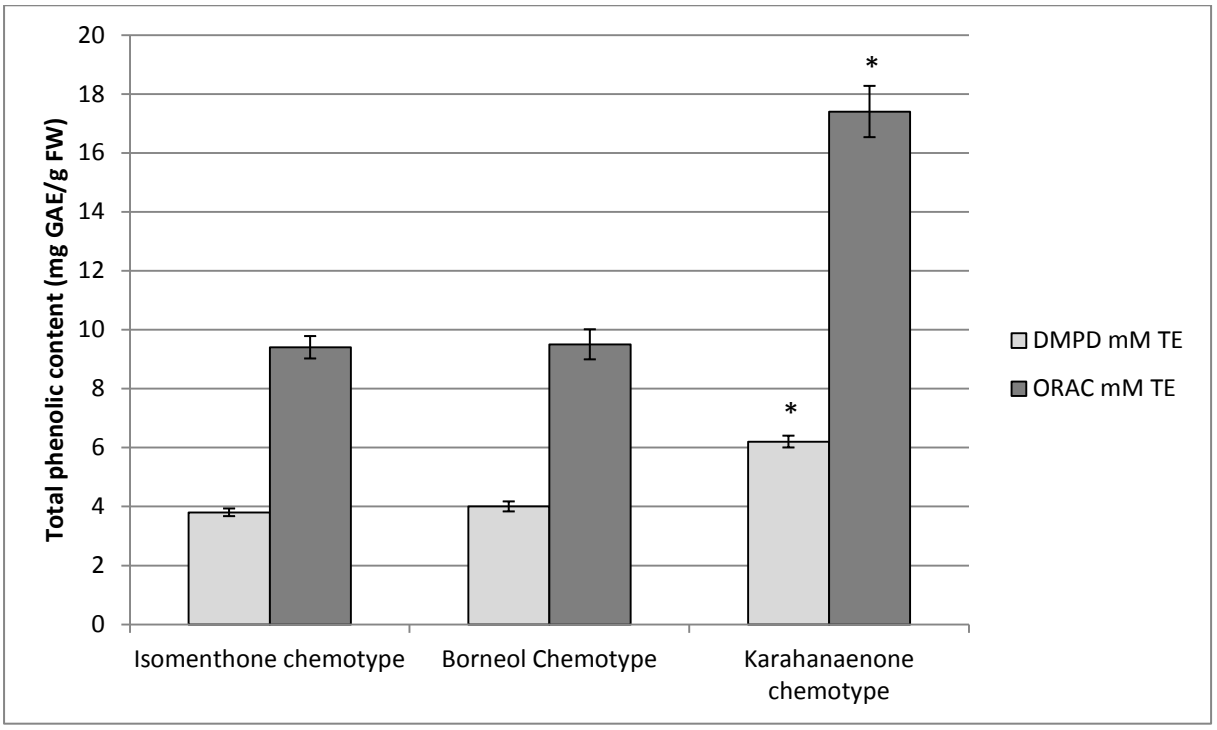


Figure 4.11 Bar chart showing comparison of antioxidant capacity in traditional medicinal preparations representative of three separate chemotypes of *E. longifolia* (ORAC and DMPD decolourisation assays). For data representing ORAC assays n = 4, for data representing DMPD assays n = 3. Asterisk denotes significance of difference between results representing each chemotype, *: $p < 0.05$.

4.2.5.1 **Total phenolic content and antimicrobial activity**

A number of the preparations in which high levels of relative antibacterial activity had been found were also noted to contain correspondingly high levels of phenolic content. Phenolic and polyphenolic natural products are generally known to exhibit antimicrobial activity (Cowan, 1999), and so correlation analyses were carried out to determine whether any relationship existed between TPC in the aqueous preparations and antibacterial inhibitory activity. Correlation coefficients were determined for data representing TPC as ppm GAE and antimicrobial activity as dilution factor of MIC relative to original extract concentration including extracts which exhibited no detectable activity. Correlation coefficients calculated for the dataset representing each organism are presented below in Table 4.4. In each case, an overall positive association between TPC (as ppm GAE) and antimicrobial activity was apparent, however, as with the measurements of antioxidant capacity the relationship was not consistent for all preparations. The *B. obovata* extract of inner trunk bark for example, was found to contain 11947 ppm GAE and inhibited growth of *Staphylococcus epidermidis* to around $1/20^{th}$ of the original concentration. In contrast, the extract of *E. tetrodonta* leaves was found to contain 2032 ppm GAE TPC, yet inhibited growth of *S. epidermidis* down to ca. $1/90^{th}$ of the original concentration. These results would indicate that the antimicrobial activity found in the aqueous preparations (as reported in Chapter 3) can be only partly attributed to the presence of phenolic compounds. It is likely that overall phenolic content, while deemed significant according to linear correlation analysis, relates to potential antimicrobial activity in a very general sense in these extracts. It is possible that these results were representative of variability in the antimicrobial activity of the phenolic species which may have been present in individual extracts and/or that other non-phenolic compounds may be contributing significantly to the activity observed (e.g. particularly in the *E. tetrodonta* extract).

Table 4.4 Pearson correlation coefficient (*r*) of total phenolic content against antimicrobial potency

	Pearson correlation (*r*)
Organism	**FCR GAE ppm with dilution factor of MIC)**
Staphylococcus epidermidis (ATCC 12228)	0.779***
Staphylococcus aureus (ATCC 29213)	0.821***
Bacillus subtilis (ACM 2268)	0.823***
Klebsiella aerogenes (ATCC 10031)	0.598*
Escherichia coli (ATCC 25922)	0.706***
Pseudomonas aeruginosa (ATCC 27703)	0.779***

Asterisks denote significance level in Pearson's correlation;***: Significance at 0.001 level; *: Significance at 0.05 level

4.2.6 Antioxidant capacity in MeOH extracts of *Eremophila* species and comparisons with corresponding traditional preparations

ORAC values obtained for the tertiary MeOH extracts of leaves from the *Eremophila* species are presented below (

Table 4.1) alongside equivalent values obtained for the corresponding traditional aqueous preparations. ORAC values of MeOH extracts were, in the majority of cases, higher than those seen in the traditional aqueous preparations (in most instances around twice that of the value found for aqueous preparations). Greater levels of antioxidant capacity, however, were exhibited by the aqueous preparations of *Eremophila alternifolia*, (1350 - 2048 μmol TE/g) as compared with the corresponding MeOH extracts (922 - 953 μmol TE/g).

Table 4.5 ORAC values (μmol TE/g) for tertiary MeOH extracts of *Eremophila* species and corresponding traditional aqueous preparations

	ORAC (μmol TE/g extract)			
Species	**Tertiary-MeOH extracts**		**Traditional aqueous extracts**	
Eremophila alternifolia (JES021)	953	± 42	2048	± 158
Eremophila alternifolia (JES023)	922	± 36	1350	± 57
Eremophila duttonii	3654	± 134	1957	± 74
Eremophila latrobei	2093	± 36	1336	± 74
Eremophila longifolia	2954	± 45	1212	± 49
Eremophila sturtii	2271	± 82	1119	± 70

4.2.7 **Comparison of traditional use records and MUA_{TMP} values with antioxidant capacity and phenolic content**

Extracts with higher range MUA_{TMP} values or those cited for specific dermatological uses, were examined for association with higher or lower levels of antioxidant activity and phenolic content. The median value of each parameter of antioxidant capacity/phenolic content was determined and used to sort the extracts into two identifiable categories, with one comprised of extracts with antioxidant capacity greater than or equal to the median value (high range category), and the other comprised of all the remaining extracts with antioxidant capacity/phenolic content less than the median value (low range category). Extracts were then divided into two groups, with one containing those with MUA_{TMP} values representing two or more independent citations for each use, and the other containing those with a single, or no citations. The observed frequencies of extracts with either high or low range levels of activity/phenolic content were determined for each MUA_{TMP} grouping and used to construct 2×2 contingency tables, from which expected frequencies were derived. Relative strength and statistical significance of any association of activity/phenolic content with either grouping was assessed using Pearson's chi-square and Fisher's exact tests for association. Only four of the extracts had been cited for treatment of wounds by two or more independent studies. To enable valid statistical tests for association, MUA_{TMP} groups in these analyses instead consisted of one containing extracts with any number of citations for the treatment of wounds and the other of extracts not cited for this purpose. Additional analyses with groups consisting of extracts cited for treatment of sores were also performed in this way as there was a sufficient number of extracts not cited for this purpose to permit valid analyses. MUA_{TMP} values derived from citations for treatment of wounds, sores and all dermatological/ophthalmic complaints in the literature surveyed are listed alongside measurements of each parameter examined in Table 4.6 below.

Table 4.6 Major use agreement values for preparations (MUA_{TMP}) with antioxidant capacity and total phenolic assay data. Ordered by MUA values for dermal lesions ('sores'). Shading indicates extracts exhibiting total phenolic content ≥665 ppm GAE.

Species	Extract Description	Preparation MUA values (MUA_{TMP})			FCR TPA (ppm GAE)	FCR GAE (mg/g FW)	DMPD (mM TE)	DMPD TE (µmol/g FW)	ORAC (mM TE)	ORAC TE (µmol/g FW)
		Wounds	Sores†	Dermatological						
Crinum angustifolium	Cold infusion of bulb	67	78	78	236	0.7	1.7	5.1	2.4	7.0
Crinum angustifolium	Decoction of bulb	67	78	78	140	0.4	1.7	4.3	2.0	5.0
Eucalyptus tetrodonta	Terminal leaf decoction	0	67	67	2032	40.6	8.5	170.8	8.2	163.4
Erythrophleum chlorostachys	Trunk inner bark decoction	17	56	56	7327	75.1	11.7	120.1	19.8	203.1
Buchanania obovata	Decoction of inner trunk bark	33	44	44	11947	32.9	46.7	128.5	12.9	35.5
Callitris intratropica	Decoction of inner trunk bark	50	33	33	8790	24.9	16.0	45.4	16.2	46.0
Erythrophleum chlorostachys	Leaf/inner trunk bark decoction	17	33	33	1622	20.2	3.7	46.0	5.9	73.6
Bauhinia cunninghamii	Decoction of root	17	22	22	1128	21.4	2.4	45.8	2.9	55.8
Acacia holosericea	Leaf decoction	0	22	22	2583	25.2	16.3	158.9	6.1	59.9
Camptostemon schultzii	Cold infusion charred wood	0	22	22	224	1.6	0.3	2.0	1.7	12.0
Eucalyptus tetrodonta	Decoction of trunk inner bark	0	22	22	221	8.8	1.3	51.6	1.8	71.7
Eremophila longifolia (BC)	Decoction of leaves	0	22	22	1013	15.8	4.0	62.4	9.5	148.5
Eremophila longifolia (IC)	Decoction of leaves	0	22	22	806	12.6	3.8	58.7	9.4	146.5
Eremophila longifolia (KC)	Decoction of leaves	0	22	22	1645	24.3	6.2	91.7	17.4	257.3
Acacia auriculiformis	Leaf decoction	17	11	11	2528	24.7	14.0	136.7	5.9	57.7
Eremophila duttonii	Decoction of leaves	17	11	11	1099	12.0	4.5	49.0	12.2	133.1
Eremophila duttonii	Decoction of leaves	17	11	11	1219	13.6	4.8	54.2	16.4	183.7
Eremophila sturtii	Decoction of leaves	17	11	11	819	16.8	3.1	62.8	10.4	213.5
Avicennia marina	Decoction of leaves	0	11	11	590	3.2	1.5	8.3	7.7	42.2
Morinda citrifolia	Expressed juice of unripe fruit	0	11	11	544	0.2	6.2	1.9	6.1	1.9
Buchanania obovata	Cold infusion of terminal stems	17	11	11	3017	21.5	16.6	118.5	7.6	54.0
Buchanania obovata	Decoction of terminal stems	17	11	11	1517	33.9	8.8	197.7	1.4	31.1
Cymbopogon ambiguus	Decoction of whole plant	0	11	11	223	2.7	0.6	7.8	2.2	27.0
Pandanus spiralis	Expressed juice of leaf tips diluted	0	11	11	142	0.3	4.2	7.6	2.6	4.7
Hibiscus tiliaceus	Warm infusion of stems	17	0	11	611	1.7	3.9	11.2	6.0	17.0
Hibiscus tiliaceus	Cold infusion of terminal stems	17	0	11	125	1.4	4.5	51.6	2.0	23.2
Ipomoea pes-caprae	Hot infusion of leaves	0	0	44	62	1.3	0.6	11.6	1.0	20.9
Ipomoea pes-caprae	Cold infusion of leaves	0	0	44	10	0.2	0.2	5.8	0.2	5.5
Scaevola sericea	Expressed juice of fruit	0	0	22	687	0.2	7.8	1.9	11.6	2.8
Cochlospermum fraseri	Expressed juice of fruit pulp	0	0	11	18189	4.6	60.9	15.3	68.2	17.2
Eremophila latrobei	Decoction of leaves	0	0	11	641	12.5	3.5	68.5	9.1	177.3
Eremophila latrobei	Decoction of leaves	0	0	11	498	9.9	3.3	66.9	8.2	163.7
Flagellaria indica	Expressed juice of growing tips	0	0	11	n.d	n.d	n.d.	n.d	n.d	n.d
Pandanus spiralis	Decoction of leaf bases	0	0	11	590	2.2	3.0	11.4	4.0	14.8
Flagellaria indica	Warm infusion of stems	0	0	11	175	0.9	1.7	8.1	2.9	14.2
Eremophila alternifolia	Decoction of leaves	0	0	0	448	7.6	1.7	28.2	6.7	113.3
Eremophila alternifolia	Decoction of leaves	0	0	0	405	6.7	1.9	30.9	10.4	170.8

†: List sorted in descending order based on MUA_{TMP} of use for 'sores (dermal lesions); n.d.: Not determined; (KC): *E. longifolia* karahanaenone chemotype; (IC): *E. longifolia* isomenthone chemotype; (BC): *E. longifolia* borneol chemotype

In addition, mean values for each parameter of antioxidant capacity/phenolic content were compared between groupings of extracts with higher range MUA_{TMP} values (MUA_{TMP} ≥33) and lower range values (MUA_{TMP} <33). Significance of any differences were assessed using one-way ANOVA.

4.2.7.1 **Total phenolic content and MUA_{TMP} values**

The median TPC for all extracts tested was 665 ppm GAE. An increased probability of detecting higher range TPC (i.e. ≥665 ppm GAE), was found with extracts with higher level MUA_{TMP} values for sores and wounds. Higher levels of TPC were found to be less likely to be associated with preparations cited in one instance, or not at all for these purposes in the surveyed literature. Exceptions included the expressed juice of *S. sericea* fruit (687 ppm GAE) and, most notably, the expressed juice of *C. fraseri*, which was not cited for either purpose and in fact represented the extract for which the highest TPC was recorded (18189 ppm GAE). This value was unusually high when compared to that found for both the preparations within the low range MUA_{TMP} values and the group as a whole. A Grubb's test carried out using TPC values recorded for all of the aqueous extracts identified the *C. fraseri* extract as a potential outlier (n = 36; Z = 4.28; $p < 0.01$). The value was not removed from analyses for association or comparison of means between groups, however, additional separate analyses where means were compared between high and low ranging MUA_{TMP} groups in which the TPC values obtained from *C. fraseri* were omitted, were performed for comparison.

A significantly greater proportion of high range TPC values was found amongst the extracts cited for use in treatment of sores than amongst those not cited for this purpose (χ_1^2=8.000, $p < 0.01$; Fisher's exact test: p <0.05); of the 24 extracts used for sores, 16 (67%) were found with high range TPC and 8 (33%) with low range TPC (< 665 ppm GAE) whereas, of the 12 extracts not cited for this purpose only 2 (17%) were found with high range TPC and 10

(83%) with low range TPC. A greater proportion of high range TPC values was also found amongst the 14 extracts cited in two or more studies for treatment of sores (10 extracts, 73%) than amongst the 22 cited once or not at all (8 extracts, 36%), however, the association was not as strong (χ_1^2=4.375, p <0.05) owing to the greater number of higher range values in the group with lower MUA_{TMP}, and did not reach significance when analysed using Fisher's exact test (p >0.05). Despite this it may be noted that some of the highest values for TPC were associated with highest MUA_{TMP} values (with the exception of extracts of *C. angustifolium* for which relatively low TPC was measured) and this was reflected in higher mean TPC value for this group (see below). For extracts with MUA_{TMP} values between 33 and 67, TPC ranged from 1128 to 11947. This was also the case with extracts frequently cited for wounds (i.e. values of 8790 and 11947 for the two extracts independently cited in 3-5 instances, excluding the extracts of *C. angustifolium*). Two other preparations cited for the treatment of sores in more than one source exhibited lower range TPC values and included the infusion of *Camptostemon schultzii* charred timber (224 ppm GAE) and the decoction of *E. tetrodonta* inner bark (221 ppm GAE) (interestingly, these were also the extracts which failed to exhibit any detectable antimicrobial activity).

A significantly greater proportion of high range TPC values was also found amongst the extracts cited for use in treatment of wounds than amongst those not cited for this purpose (χ_1^2=5.800, p <0.05; Fisher's exact test: p <0.05); of 15 extracts cited for wounds, 11 (73%) were found with high range TPC values and 4 (27%) with low range TPC values, whereas of the 21 extracts not cited for this purpose only 7 (33%) were found with high range TPC values, and 14 (66%) with low range TPC values. The 4 extracts cited for wounds and for which lower range MUA_{TMP} values were found included, again, the 2 extracts of C. *angustifolium* bulbs (140 - 236 ppm GAE) and *H. tiliaceus* stems/stem bark (125 - 611 ppm GAE).

No significant association could be demonstrated between higher range MUA_{TMP} values determined on the basis of citations for all dermatological/ophthalmic complaints, and high or low range TPC values. As with the other use categories, however, the highest TPC values seemed to associate with the highest MUA_{TMP} values (again with the exception of the *C. angustifolium* and *I. pes-caprae* extracts). Observed number of categories in each group and results of tests for association are given below in Table 4.7.

Mean TPC values were determined for groupings of preparations which had been sorted into those with MUA_{TMP} greater than or equal to 33, and less than 33, for each of the use categories defined. Mean TPC values were found to be around 2 - 3 times higher in the preparations with MUA_{TMP} values ≥ 33% across all use categories. The greatest difference was seen between the groups representing high and low levels of agreement for the treatment of sores (4585 ± 1782 ppm GAE for sores $MUA_{TMP} \geq 33\%$, 1440 ± 615 ppm GAE for lower sores MUA_{TMP} values). One-way ANOVA showed that this difference was significant, ($p < 0.05$). Analyses were repeated using datasets where the exceptionally high TPC values obtained for the expressed juice extract of *C. fraseri* were treated as outliers and omitted. When this was carried out, TPC values in higher range MUA_{TMP} groups were around 4 - 5 times higher and significance probability was lower in each case. Results of analyses for each MUA_{TMP} category are shown below in Table 4.8.

Table 4.7 Proportion of extracts with phenolic content ≥665 ppm GAE by groupings greater than, and less than median MUA_{TMP} values with Pearson's Chi-square and Fisher's exact tests for association

Category	Preparations ≥665 ppm GAE	Preparations <665 ppm GAE	Preparations ≥665 ppm GAE (%)	χ^2	Fisher's Exact Test (*p*)
≥1 citations for wounds (MUA_{TMP} ≥17)	11	4	73%	*5.800	0.041
=0 citations for wounds (MUA_{TMP} <17)	7	14	33%		
≥2 citations for 'Sores' (MUA_{TMP} ≥22)	10	4	71%	*4.375	0.080
<2 citations for 'Sores' (MUA_{TMP} <22)	8	14	36%		
≥2 citations for all D/OC (MUA_{TMP} ≥22)	11	6	65%	2.786	0.181
<2 citations for all D/OC (MUA_{TMP} <22)	7	12	37%		
≥1 citations for 'Sores' (MUA_{TMP} ≥11)	16	8	67%	**8.000	0.012
=0 citations for 'Sores' (MUA_{TMP} <11)	2	10	17%		

D/OC: Dermatological/ophthalmic complaints; *: Pearson's Chi-square test significant at the 0.05 level; ** Pearson's Chi-square test significant at the 0.01 level; †: Expected frequency <5 in Pearson's Chi-square test

Table 4.8 Mean total phenolic content for groupings of preparations greater than, and less than 33% MUA_{TMP}. Analyses using all data, or with results from *C. fraseri* extract omitted shown

MUA_{TMP} group	n†	Mean TPC (ppm GAE) All data	Mean TPC (ppm GAE) Data from *C. fraseri* extract omitted
MUA_{TMP} < 33 ('Sores')	29 (28)	1440 ± 615	842 ± 149
MUA_{TMP} ≥ 33 ('Sores')	7	*4585 ± 1782	***4585 ± 1782
MUA_{TMP} < 33 (All dermatological/ophthalmic complaints)	27 (26)	1544 ± 657	904 ± 154
MUA_{TMP} ≥ 33 (All dermatological/ophthalmic complaints)	9	3574 ± 1516	**3574 ± 1516
MUA_{TMP} < 33 (wounds)	32 (31)	1648 ± 586	1115 ± 251
MUA_{TMP} ≥ 33 (Wounds)	4	5278 ± 3009	*5278 ± 3009

†: Values in parentheses represent datasets where TPC for the *C. fraseri* extract was omitted. Asterisks denote significance of difference between groups of preparations with MUA_{TMP} greater than or less than 33, *: $p < 0.05$; **: $p < 0.01$; ***: $p < 0.001$.

4.2.7.2 Antioxidant capacity and MUA_{TMP}

The median ORAC value determined for all extracts tested was 6.0 mM TE. ORAC values ≥ 6.0 mM TE were categorised as high range values while those < 6.0 mM TE were categorised as low range values. No significant associations were found between any of the MUA_{TMP} values and ORAC values of the preparations, however, a slightly greater frequency than expected for extracts with higher range ORAC values (> 6.0 mM TE) was observed amongst extracts which were not cited in the literature examined for treatment of wounds than for extracts cited for this purpose (Table 4.9).

Table 4.9 Proportion of extracts with ORAC values ≥ 6.0 mM TE, by groupings greater than, and less than median MUA_{TMP} values with Pearson's Chi-square and Fisher's exact tests for association

Category	Preparations >6.0 mM TE ORAC	Preparations <6.0 mM TE ORAC	Preparations >6.0 mM TE ORAC (%)	χ^2	Fisher's Exact Test (*p*)
≥1 citations for wounds (MUA_{TMP} ≥17)	7	8	47%	0.823	0.500
=0 citations for wounds (MUA_{TMP} <17)	13	8	62%		
≥2 citations for 'Sores' (MUA_{TMP} ≥22)	8	6	57%	0.023	1.000
<2 citations for 'Sores' (MUA_{TMP} <22)	12	10	55%		
≥2 citations for all D/OC (MUA_{TMP} ≥22)	9	8	56%	0.006	1.000
<2 citations for all D/OC (MUA_{TMP} <22)	11	8	55%		
≥1 citations for 'Sores' (MUA_{TMP} ≥11)	14	10	58%	0.225	0.729
=0 citations for 'Sores' (MUA_{TMP} <11)	6	6	50%		

D/OC: Dermatological/ophthalmic complaints; *: Pearson's Chi-square test significant at the 0.05 level; ** Pearson's Chi-square test significant at the 0.01 level; †: Expected frequency <5 in Pearson's Chi-square test

Table 4.10 Mean TE (ORAC) of preparations greater than, and less than 33% MUA_{TMP} for all data and with results from *C. fraseri* extract omitted

MUA_{TMP} group	n†	Mean ORAC TE (mM) All data	Mean ORAC TE (mM) Data from *C. fraseri* extract omitted
MUA_{TMP} < 33 ('Sores')	29 (28)	8.7 ± 2.3	6.6 ± 0.9
MUA_{TMP} ≥ 33 ('Sores')	7	9.6 ± 2.6	9.6 ± 2.6
MUA_{TMP} < 33 (All dermatological/ophthalmic complaints)	27 (26)	9.3 ± 2.4	7.0 ± 0.9
MUA_{TMP} ≥ 33 (All dermatological/ophthalmic complaints)	9	7.6 ± 2.4	7.6 ± 2.4
MUA_{TMP} < 33 (wounds)	32 (31)	8.9 ± 2.1	7.0 ± 0.9
MUA_{TMP} ≥ 33 (Wounds)	4	8.4 ± 3.6	8.4 ± 3.6

†: Values in parentheses represent datasets where ORAC values for *C. fraseri* were omitted. Asterisks denote significance between groups of preparations with MUA_{TMP} > or < 33%, *: $p < 0.05$; **: $p < 0.01$; ***: $p < 0.001$.

The median value of results obtained by the DMPD assays was determined as 4.0 mM TE and DMPD-TE values ≥ 4.0 mM were categorised as high range values while those < 4.0 mM were categorised as low range values. Marginally greater numbers than expected of extracts with high range DMPD-TE values (> 4.0 mM TE), were noted in groups cited for wounds and sores than as seen in those not cited for these purposes, however, these proportions were not significantly greater than expected as assessed by Pearson's chi squared and Fisher's exact tests for association (Table 4.9).

Table 4.11 Proportion of extracts with DMPD assay values > 4.0 mM TE, by greater than, and less than median MUA_{TMP} values with Pearson's Chi-square and Fisher's exact tests for association

Category	Preparations >4.0 mM TE DMPD	Preparations <4.0 mM TE DMPD	Preparations >4.0 mM TE DMPD (%)	χ^2	Fisher's Exact Test (*p*)
≥1 citations for wounds (MUA_{TMP} ≥17)	9	6	60%	2.520	0.175
=0 citations for wounds (MUA_{TMP} <17)	7	14	33%		
≥2 citations for 'Sores' (MUA_{TMP} ≥22)	6	8	43%	0.023	0.499
<2 citations for 'Sores' (MUA_{TMP} <22)	10	12	45%		
≥2 citations for all D/OC (MUA_{TMP} ≥22)	7	10	53%	0.139	0.749
<2 citations for all D/OC (MUA_{TMP} <22)	9	10	58%		
≥1 citations for 'Sores' (MUA_{TMP} ≥11)	13	11	54%	2.756	0.157
=0 citations for 'Sores' (MUA_{TMP} <11)	3	9	25%		

D/OC: Dermatological/ophthalmic complaints; *: Pearson's Chi-square test significant at the 0.05 level; ** Pearson's Chi-square test significant at the 0.01 level; †: Expected frequency <5 in Pearson's Chi-square test

Table 4.12 Mean TE (DMPD) of preparations greater than, and less than 33% MUA_{TMP} for all data and with results from *C. fraseri* extract omitted

MUA_{TMP} group	n†	Mean DMPD TE (mM) All data	Mean DMPD TE (mM) Data from *C. fraseri* extract omitted
MUA_{TMP} < 33 ('Sores')	29 (28)	6.6 ± 2.1	4.7 ± 0.8
MUA_{TMP} ≥ 33 ('Sores')	7	12.9 ± 6.0	*12.9 ± 6.0
MUA_{TMP} < 33 (All dermatological/ophthalmic complaints)	27 (26)	7.1 ± 2.2	5 ± 0.9
MUA_{TMP} ≥ 33 (All dermatological/ophthalmic complaints)	9	10.1 ± 4.9	10.1 ± 4.9
MUA_{TMP} < 33 (wounds)	32 (31)	6.7 ± 1.9	5.0 ± 0.8
MUA_{TMP} ≥ 33 (Wounds)	4	16.5 ± 10.6	**16.5 ± 10.6

†: Values in parentheses represent datasets where DMPD TE values for *C. fraseri* were omitted. Asterisks denote significance between groups of preparations with MUA_{TMP} > or < 33%, *: $p < 0.05$; **: $p < 0.01$; ***: $p < 0.001$.

Mean DMPD and ORAC TE values were determined for groupings of preparations which had been sorted into those with MUA_{TMP} greater than or equal to 33, and less than 33, for each of the use categories defined. No significant difference in mean ORAC values was found between groupings, either when results obtained from *C. fraseri* extracts were omitted or included in the dataset (Table 4.11). While mean DMPD values for preparations with higher levels of agreement were marginally greater than those determined for the low agreement groups, the difference was not significant owing to large within group variance. When results representing antioxidant capacity of *C. fraseri* extracts were omitted, however, mean DMPD TE values for preparations with higher levels of agreement (MUA_{TMP} values ≥

33%) in citations for treatment of wounds and sores were around 2 times higher than in the low agreement group and one-way ANOVA showed that these differences were significant (Table 4.12).

4.2.8 **PGE_2 inhibitory activity of traditional preparations**

Screening of the extracts for PGE_2 inhibitory activity were considered as preliminary results as only one experiment was carried out for each sample. Results were presented as the mean of assays performed in triplicate with standard error and expressed as percentage inhibition of PGE_2 formation relative to controls consisting of stimulated or unstimulated cells incubated in the absence of any test material. In some instances the ELISA assays produced results of unacceptable variability, and for a few samples a clear indication of activity could not be obtained. Results where percentage relative standard deviation (RSD) of replicate tests was greater than 20% were deemed inconclusive and discarded and in some cases, single outlying results were excluded. Unfortunately, tests for these particular samples could not be repeated due to constraints of assay cost and time. Extracts for which results were thus deemed inconclusive in tests against ionophore stimulated cells included the cold infusion of *I. pes-caprae* leaves, infusions of *C. schultzii* charred wood and *B. obovata* terminal stems, and the decoctions of *A. marina* and *E. longifolia* karahanaenone chemotype leaves. Tests which were deemed inconclusive for unstimulated cells included those for the *E. duttonii* and *E. alternifolia* leaf decoctions.

Of the remaining 29 extracts, 9 were found to produce some degree of significant inhibition of PGE_2 formation in calcium ionophore stimulated fibroblasts at a concentration of 20 $\mu g \cdot mL^{-1}$ relative to controls. Percentage inhibition relative to controls ranged from 47% ± 5.1 (decoction of *E. chlorostachys* inner trunk bark) to 15% ± 5.1 (warm infusion of *F. indica* stem). Other extracts exhibiting significant inhibition of PGE_2 formation included the

decoction of *E. duttonii* leaves (42% ± 5.0), decoctions of both the inner bark and leaves of *E. tetrodonta* (41% ± 2.8 for inner bark decoction and 34% ± 1.3 for leaf decoction), the decoction of *Cymbopogon ambiguus* whole plant (34% ± 1.4), decoction of leaves of the borneol chemotype of *E. longifolia* (30% ± 2.3) and the decoction of *E. alternifolia* leaves (18% ± 2.1). Several of the extracts were seen to elicit a positive *increase* in the level of PGE_2 formation in the unstimulated fibroblast control assays. Extracts producing significant increases in PGE_2 included the heated infusion of *H. tiliaceus* stems (83% ± 1.5), decoction of leaves of the karahanaenone chemotype of *E. longifolia* (63% ± 1.8) and the cold infusion of *I. pes-caprae* leaves (55% ± 9.3).

The cold infusion of *C. angustifolium* bulb seemed to exhibit a potentiating effect on PGE_2 formation in the ionophore stimulated cells (increase of 44% ± 5.6 relative to media only controls stimulated with ionophore). This result may have been due to some error or experimental artefact as a similar effect was not seen in either the unstimulated control, or in experiments using the corresponding decoction type extract which had been derived from an equal quantity of the same material (extract JES043BD).

Table 4.13 Inhibition of PGE_2 formation in calcium ionophore stimulated and unstimulated 3T3 fibroblasts by traditional aqueous preparations at 20 μg · mL^{-1}

			PGE_2 percentage inhibition (%)			
			Ionophore stimulated cells		Unstimulated control	
Species	**ID**	**Description of preparation**				
Erythrophleum chlorostachys	JES040BTD	Trunk inner bark decoction	*47	± 5.1	-	-
Eremophila duttonii	SM011TD	Leaf decoction	*42	± 5.0	n.d.	n.d.
Eucalyptus tetrodonta	JES028BTD	Trunk inner bark decoction	*41	± 2.8	*62	± 1.4
Eucalyptus tetrodonta	JES028LTD	Terminal leaf decoction	*34	± 1.3	*62	± 0.9
Cymbopogon ambiguus	JES058AD	Whole plant decoction	*34	± 1.4	*44	± 4.4
Buchanania obovata	JES066SE	Terminal stem decoction	34	± 1.3	-	-
Buchanania obovata	JES048BD	Trunk inner bark decoction	33	± 0.6	-	-
Eremophila longifolia (BC)	SM019TD	Leaf decoction	*30	± 2.3	-	-
Erythrophleum chlorostachys	JES040LTD	Leaf/trunk inner bark decoction	*30	± 0.9	*49	± 2.7
Bauhinia cunninghamii	JES042TD	Root decoction	22	± 4.0	-	-
Eremophila alternifolia	JES023TD	Leaf decoction	*18	± 1.2	*59	± 10.1
Flagellaria indica	JES037SE	Stem warm infusion	*15	± 3.2	*48	± 5.6
Flagellaria indica	JES037EJ	Expressed juice of growing tips	-	-	*37	± 3.1
Eremophila sturtii	SM001TD	Leaf decoction	-	-	*24	± 4.6
Crinum angustifolium	JES043TS	Bulb cold infusion	-	-	*22	± 0.4
Eremophila longifolia (IC)	SM003TD	Leaf decoction*	-	-	22	± 1.6
Avicennia marina	JES034LTD	Leaf decoction	n.d.	n.d.	*21	± 2.1
Pandanus spiralis	JES027EJP	Expressed juice of leaf tips diluted	-	-	-37	± 8.3
Pandanus spiralis	JES027LBD	Leaf base decoction	-	-	-41	± 9.7
Morinda citrifolia	JES063EJ	Expressed juice of unripe fruit	-	-	-44	± 14.5
Ipomoea pes-caprae	JES031SE	Leaf cold infusion	n.d.	n.d.	*-55	± 9.3
Eremophila longifolia (KC)	SM018TD	Leaf decoction	n.d.	n.d.	*-63	± 1.8
Hibiscus tiliaceus	JES032HSE	Stem warm infusion	-	-	*-83	± 1.5
Crinum angustifolium	JES043TD	Bulb decoction	*-44	± 5.6	-	-

-: Indicates lack of detectable activity; KC: *E. longifolia* karahanaenone chemotype; n.d.: Not determined due to test failure; Asterisk denotes significance of difference relative to controls, *: p <0.05.

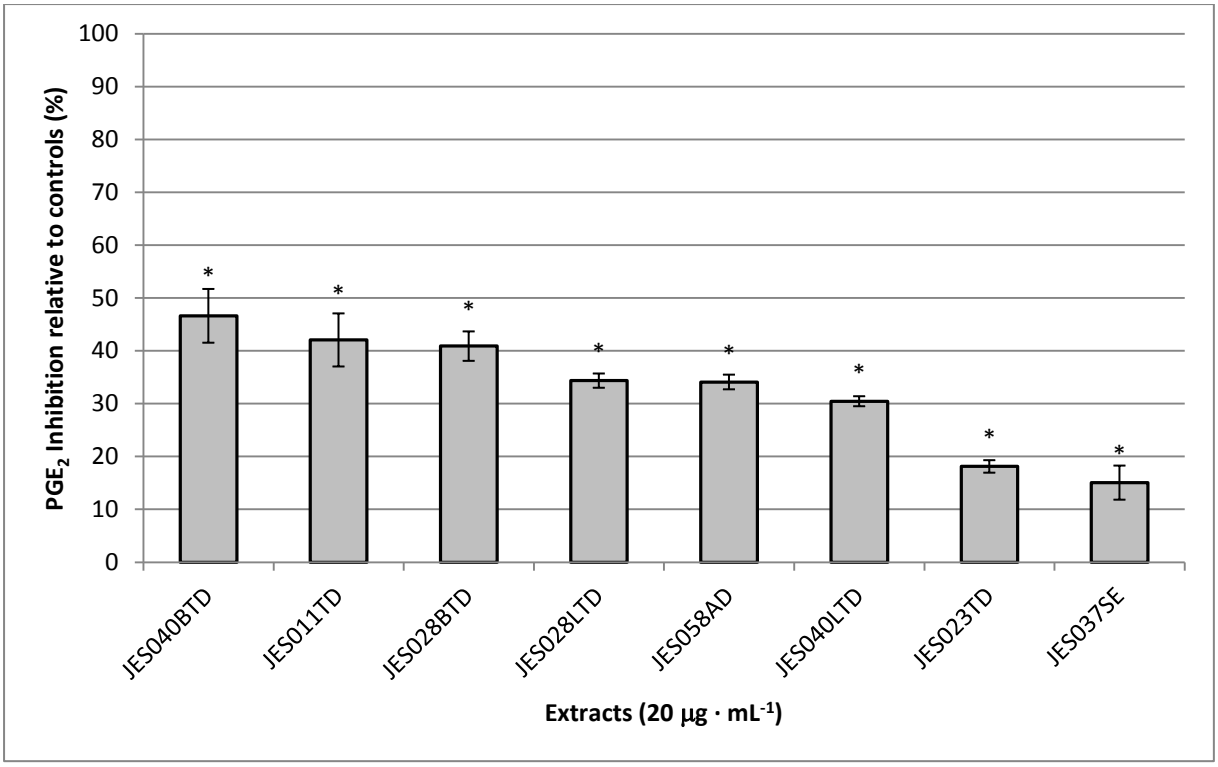


Figure 4.12 Extracts exhibiting significant inhibition of PGE2 formation in calcium ionophore stimulated 3T3 fibroblasts at 20 µg · mL-1 (Asterisks denote significance of difference relative to control;*: $p < 0.05$).

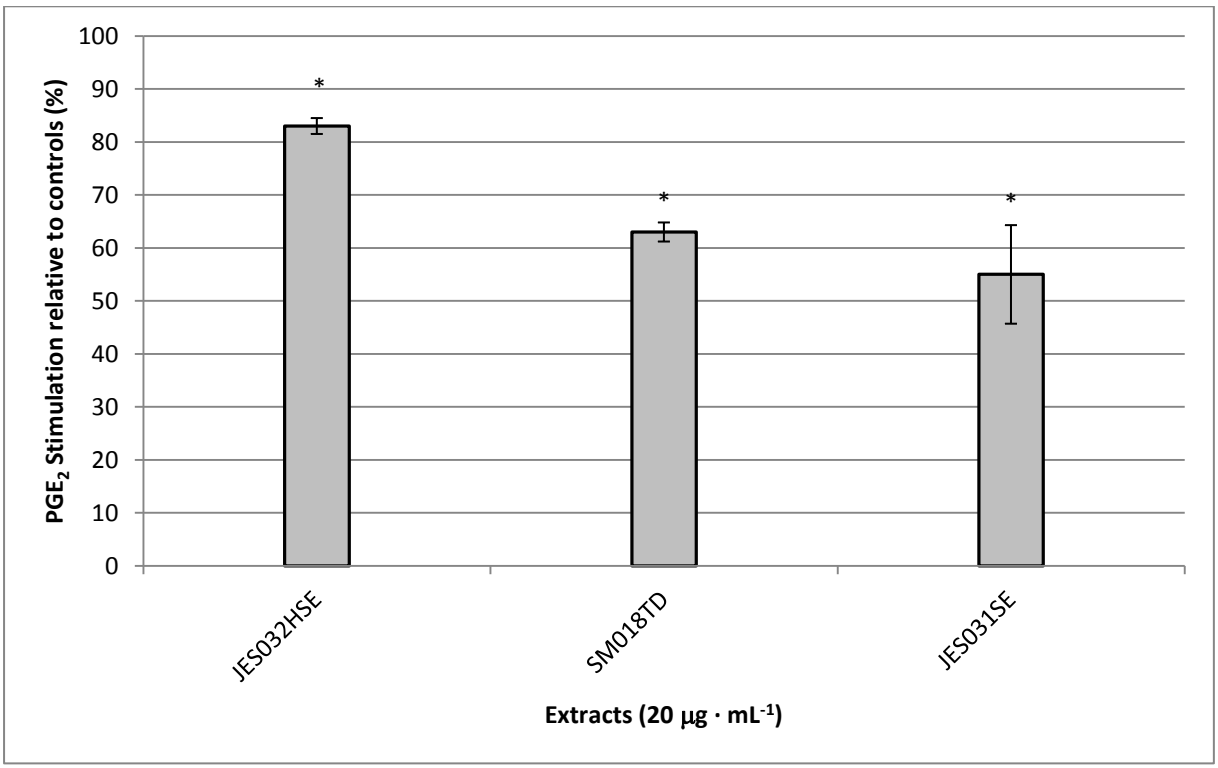


Figure 4.13 Extracts exhibiting significant positive increase in PGE_2 formation in unstimulated 3T3 fibroblasts at 20 µg · mL^{-1}. (Asterisks denote significance of difference relative to control;*: $p < 0.05$).

4.2.8.1 PGE_2 inhibitory activity of solvent extractions of *Eremophila* spp. and comparison with traditional preparations

PGE_2 inhibitory activity by tertiary MeOH extracts of five *Eremophila* species was compared with that of corresponding traditional aqueous preparations. Results are summarised below in Table 4.15. Generally, the incidence of activity was higher amongst MeOH extracts than for aqueous preparations, however, a similar degree of inhibition was found between the MeOH and traditional aqueous of *E. alternifolia*, (MeOH extract: 25% ± 4.2, aqueous decoction of leaves: 18% ± 1.2). Activity was detected in both the MeOH and aqueous extracts of *E. duttonii*, however, these results were not directly comparable as the MeOH extract was assayed at a concentration $1/20^{th}$ that of the corresponding aqueous extract (1.0 $\mu g \cdot mL^{-1}$) owing to the relatively high levels of cytotoxicity found to be exhibited by this extract (*cf.* Table 4.16 below). No significant inhibitory activity was detected in either of the *Eremophila latrobei* extracts. Traditional aqueous preparations of *Eremophila sturtii* and *E. longifolia* were not found to exhibit any significant activity relative to controls, whereas the MeOH extract of *E. longifolia* (isomenthone chemotype) was found to inhibit PGE_2 formation (26% ± 3.3) and the *E. sturtii* MeOH extract to apparently increase ionophore stimulated PGE_2 formation (increase of 74% ± 13.8).

Table 4.14 Percentage inhibition of PGE_2 in calcium ionophore stimulated 3T3 fibroblasts by tertiary MeOH extracts of *Eremophila* species and corresponding traditional aqueous preparations

	PGE_2 percentage inhibition (%)			
Species	**3'-MeOH extracts**		**Traditional aqueous extracts**	
Eremophila alternifolia	25	±4.2	18	±1.2
*Eremophila duttonii**	32	±6.3	42	±5.0
Eremophila latrobei	-	-	-	-
Eremophila longifolia	26	±3.3	-	-
Eremophila sturtii	-74	±13.8	-	-

*: *E. duttonii* MeOH extract tested at 1.0 $\mu g \cdot mL^{-1}$ owing to high P-388 D-1 cytotoxicity exhibited by this extract.

4.2.9 **Results of P388D1 cytotoxicity screening assays**

Results of assays for P388D1 cytotoxicity of the traditional aqueous preparations were expressed as IC_{50} values ($\mu g \cdot mL^{-1}$) (concentration resulting in 50% reduction in culture viability), and are presented below alongside MUA_{TMP} values in Table 4.16. Dose response curves for some of the extracts tested are illustrated below in Figure 4.14. Of the traditional aqueous preparations assayed, around half (16 of 34 extracts tested) did not exhibit any measurable cytotoxicity up to the highest concentration tested (200.0 $\mu g \cdot mL^{-1}$). IC_{50} values for the remainder ranged from 28.4 to 183.38 $\mu g \cdot mL^{-1}$. IC_{50} values less than 50.0 $\mu g \cdot mL^{-1}$ were found in three of the extracts including the cold infusion of *C. angustifolium* bulb (IC_{50} 28.4 $\mu g \cdot mL^{-1}$), the decoction of inner trunk bark of *C. intratropica* (IC_{50} 29.4 $\mu g \cdot mL^{-1}$) and the decoction of leaves of *A. holosericea* (IC_{50} 47.2 $\mu g \cdot mL^{-1}$). The decoction of *C. angustifolium* bulb similarly exhibited relatively high levels of cytotoxicity (IC_{50} 58.0 $\mu g \cdot mL^{-1}$). Cytotoxicity was also exhibited by both the combined leaf/inner bark extract and inner bark only extracts of *E. chlorostachys*. Certain populations of *E. chlorostachys* are known to be toxic to stock and cytotoxic alkaloids have been isolated from both the bark and leaves (Griffin *et al.*, 1971; Loder *et al.*, 1974; Loder and Nearn, 1975) including diterpene alkaloid esters of the type found in *Erythrophleum guineense*, a central African species used as an ordeal poison (Griffin *et al.*, 1971).

IC_{50} values generated were used to select a concentration at which to screen extracts prior to the assays for PGE_2 inhibitory activity using 3T3 fibroblasts. A concentration of 20 $\mu g \cdot mL^{-1}$ was adopted as no P388D1 cytotoxicity was observed at this concentration for the majority of extracts. Samples taken from the fibroblast assay cultures during PGE_2 inhibition tests revealed no significant decreases in cell viability (as determined using the ATP assay), using the assay screening concentration of 20 $\mu g \cdot mL^{-1}$.

Table 4.15 P388 D1 cytotoxicity (IC_{50}, mg · mL^{-1}) and major use agreement values (MUA_{TMP}) for aqueous preparations. List sorted in descending order based on MUA_{TMP} of use citations for 'sores (dermal lesions). Shading indicates extracts in which activity was detected

Species	Extract Description	Preparation major use agreement values (MUA_{TMP})			P388D1 IC_{50} (μg · mL^{-1})
		Wounds	Sores	Dermatological	
Crinum angustifolium	Cold infusion of bulb	67	78	78	28.4
Crinum angustifolium	Decoction of bulb	67	78	78	58
Eucalyptus tetrodonta	Terminal leaf decoction	0	67	67	75.1
Erythrophleum chlorostachys	Trunk inner bark decoction	17	56	56	64.5
Buchanania obovata	Decoction of inner trunk bark	33	44	44	136.3
Callitris intratropica	Decoction of inner trunk bark	50	33	33	29.4
Erythrophleum chlorostachys	Leaf/inner trunk bark decoction	17	33	33	72.8
Bauhinia cunninghamii	Decoction of root	17	22	22	**>200.0**
Acacia holosericea	Leaf decoction	0	22	22	47.2
Camptostemon schultzii	Cold infusion charred wood	0	22	22	**>200.0**
Eucalyptus tetrodonta	Decoction of trunk inner bark	0	22	22	95.7
Eremophila longifolia (BC)	Decoction of leaves	0	22	22	183.4
Eremophila longifolia (IC)	Decoction of leaves	0	22	22	125.9
Eremophila longifolia (KC)	Decoction of leaves	0	22	22	71.9
Acacia auriculiformis	Leaf decoction	17	11	11	108
Eremophila duttonii	Decoction of leaves	17	11	11	79.4
Eremophila duttonii	Decoction of leaves	17	11	11	72.1
Eremophila sturtii	Decoction of leaves	17	11	11	182.4
Morinda citrifolia	Expressed juice of unripe fruit	0	11	11	**>200.0**
Buchanania obovata	Cold infusion of terminal stems	17	11	11	76.7
Buchanania obovata	Decoction of terminal stems	17	11	11	90.1
Cymbopogon ambiguus	Decoction of whole plant	0	11	11	**>200.0**
Pandanus spiralis	Diluted juice of leaf tips	0	11	11	**>200.0**
Hibiscus tiliaceus	Warm infusion of stems	17	0	11	**>200.0**
Hibiscus tiliaceus	Cold infusion of terminal stems	17	0	11	**>200.0**
Ipomoea pes-caprae	Hot infusion of leaves	0	0	44	**>200.0**
Ipomoea pes-caprae	Cold infusion of leaves	0	0	44	**>200.0**
Scaevola sericea	Expressed juice of fruit	0	0	22	**>200.0**
Cochlospermum fraseri	Expressed juice of fruit pulp	0	0	11	**>200.0**
Eremophila latrobei	Decoction of leaves	0	0	11	**>200.0**
Eremophila latrobei	Decoction of leaves	0	0	11	**>200.0**
Pandanus spiralis	Decoction of leaf bases	0	0	11	**>200.0**
Flagellaria indica	Warm infusion of stems	0	0	11	**>200.0**
Eremophila alternifolia	Decoction of leaves	0	0	0	**>200.0**

BC: *E. longifolia* borneol chemotype; IC: *E. longifolia* isomenthone chemotype; KC: *E. longifolia* karahanaenone chemotype

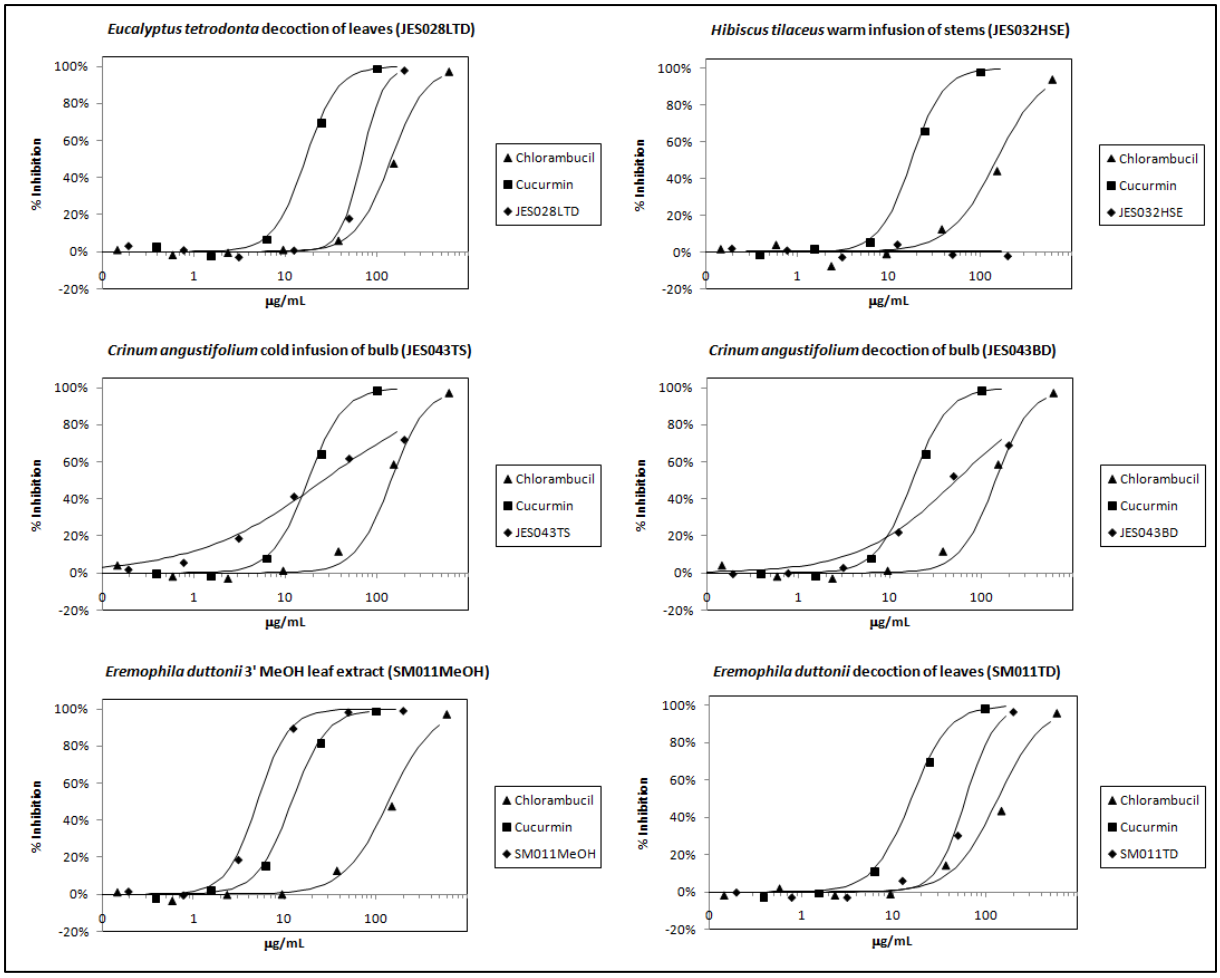


Figure 4.14 Cytotoxicity dose-response curves for selected extracts against P388D1 murine lymphoblasts

4.2.9.1 P388D1 lymphoblast cytotoxicity of *Eremophila* spp. MeOH extracts and comparison with traditional preparations

Results for cytotoxicity screening of *Eremophila* spp. MeOH extracts are presented alongside results obtained for corresponding traditional preparations below in Table 4.17. In all instances the tertiary MeOH extracts were seen to exhibit higher levels of cytotoxicity than the traditional extracts prepared with corresponding plant material. The MeOH extract of *E. duttonii* in particular was found to exhibit exceptionally high cytotoxicity (IC_{50} 5.5 µg · mL^{-1}), which could probably be attributed to presence of the serrulatane diterpenes in this extract (*cf.* Chapter 5).

Table 4.16 Cytotoxicity for P388D1 murine lymphoblasts by tertiary MeOH extracts of *Eremophila* species and corresponding traditional aqueous preparations (IC_{50} μg · mL^{-1})

	P388D1 cytotoxicity IC_{50} (μg · mL^{-1})	
Species	Tertiary MeOH extracts	Traditional aqueous extracts
Eremophila alternifolia	91.8	>200.0
Eremophila duttonii	5.5	79.4
Eremophila latrobei	100.7	>200.0
Eremophila longifolia	63.9	125.9
Eremophila sturtii	70.9	182.4

4.2.10 PGE_2 inhibition, P388D1 cytotoxicity and MUA_{TMP}

Among the aqueous preparations, higher levels of cytotoxic activity against P388D1 lymphoblasts were found to be strongly associated with higher levels of agreement in citations for medicinal use in the treatment of wounds, sores and other dermatological conditions (Table 4.19). Unlike as with other activities examined in relation to MUA_{TMP}, few 'outlying exceptions' to any otherwise significant patterns of association were found. Specifically the extracts of *C. angustifolium* (which were the most frequently cited preparations across all three of the use categories considered) exhibited relatively high levels of cytotoxicity, whereas relatively low levels of activity were observed for extracts of this species in experiments examining phenolic content, antimicrobial activity and antioxidant activity. Similarly, relatively low cytotoxicity was exhibited by the expressed juice extract of *C. fraserii* (for which only a single citation was recorded, for treatment of boils), whereas this extract tended to exhibit the highest levels of activity observed in the other activities/parameters examined when activity was considered in terms of the concentration of the extracts as originally prepared.

A significantly greater proportion of extracts with IC_{50} < 200 μg · mL^{-1} was found amongst those cited for use in treatment of sores than amongst those not cited for this purpose (χ_1^2=18.293); of the 23 extracts cited for treatment of sores, 18 (78%) were found with IC_{50}

<200 μg · mL^{-1} and 5 (22%) with IC_{50} >200 μg · mL^{-1}, whereas none of the 11 extracts not cited for this purpose were found with IC_{50} <200 μg · mL^{-1}. A significantly greater proportion of extracts with IC_{50} <200 μg · mL^{-1} was also found amongst the extracts cited two or more times for use in treatment of sores than amongst those cited less than two times for this purpose, however, the overall association was not as strong as with extracts all cited for this purpose (χ_1^2=10.261, p <0.001; Fisher's exact test: p <0.01); of the 14 extracts cited 2 or more times for sores, 12 (86%) were found with IC_{50} <200 μg · mL^{-1} and 2 (24%) with IC_{50} >200 μg · mL^{-1}, whereas of the 21 extracts not cited for this purpose, only 6 (29%) were found with IC_{50} <200 μg · mL^{-1} and 14 (71%) with IC_{50} >200 μg · mL^{-1}. Significant association was also found between higher levels of cytotoxicity and citation of use for wounds (χ_1^2=7.889, p <0.01; Fisher's exact test: p <0.01); of the 15 extracts cited for the treatment of wounds, 12 (80%) were found with IC_{50} <200 μg · mL^{-1} and 3 (20%) with IC_{50} >200 μg · mL^{-1}, whereas of the 19 extracts not cited for this purpose, only 6 (32%) were found with IC_{50} <200 μg · mL^{-1} (i.e. 32%) and 13 (68%) with IC_{50} >200 μg · mL^{-1}. Significant association was also seen between higher levels of cytotoxicity and MUA_{TMP} values representing 2 or more citations describing any dermatological or ophthalmic use, however association was weaker than that seen across other use categories (χ_1^2=4.250, p < 0.05; Fisher's exact test: p > 0.05).

Extracts for which a high level of agreement was recorded for citations referring to use in the treatment of wounds exhibited high levels of cytotoxicity. Mean IC_{50} values of extracts with MUA_{TMP} values >33, were around twice that of those with lower levels of agreement (MUA_{TMP} <33) across all three use categories. Mean IC_{50} values of extracts independently cited 5 or more times for wounds (MUA_{TMP} ≥ 55%) were around 40% lower than those cited less than 5 times. A one-way ANOVA test showed this difference to be significant (Table 4.18).

Table 4.17 Proportion of extracts exhibiting P388D1 cytotoxicity (IC_{50} <200 mg/mL) by groupings greater and less than median MUA of preparations (MUA_{TMP})

Category	Preparations IC_{50} <200 μg/mL	Preparations IC_{50} ≥200 μg/mL	Proportion IC_{50} <200 μg/mL (%)	χ^2	Fisher's Exact Test (*p*)
≥1 citations for wounds (MUA_{TMP} ≥17)	12	3	80%	**7.889	0.007
=0 citations for wounds (MUA_{TMP} <17)	6	13	32%		
≥2 citations for 'Sores' (MUA_{TMP} ≥22)	12	2	86%	**10.261	0.002
<2 citations for 'Sores' (MUA_{TMP} <22)	6	14	30%		
≥2 citations for all D/OC (MUA_{TMP} ≥22)	12	5	71%	*4.250	0.084
<2 citations for all D/OC (MUA_{TMP} <22)	6	11	35%		
≥1 citations for 'Sores' (MUA_{TMP} ≥11)	18	5	78%	***18.293	0.000
=0 citations for 'Sores' (MUA_{TMP} <11)	0	11	0%		

*: Pearson chi-squared significance at 0.05 level; **Pearson chi-squared significance at 0.01 level; †: Expected frequency <5 in Pearson chi-square test

Table 4.18 Mean IC_{50} (μg · mL^{-1}), P388D1 cytotoxicity of preparations greater and less than 50% MUA_{TMP}

MUA_{TMP} group	n	Mean IC_{50} P388D1 cytotoxicity (μg · mL^{-1})
MUA_{TMP} < 50 (Sores and All D/OC)†	14	98.0 ± 12.2
MUA_{TMP} ≥ 50 (Sores and All D/OC)†	4	56.5 ± 10.0
MUA_{TMP} < 33 (Sores and All D/OC)†	11	103 ± 13.4
MUA_{TMP} ≥ 33 (Sores and All D/OC)†	7	66.4 ± 13.7
MUA_{TMP} < 50 (Wounds)	15	98.8 ± 10.7*
MUA_{TMP} ≥ 50 (Wounds)	3	38.6 ± 9.7*
MUA_{TMP} < 33 (Wounds)	14	96.1 ± 11.1
MUA_{TMP} ≥ 33 (Wounds)	4	63 ± 25.4

†: Mean values and n equal for mean of MUA groupings for citations referring to sores and all dermatological/ophthalmic complaints Asterisks denote significance of difference between groups of preparations with MUA_{TMP} > or < 33%, *: $p < 0.05$; **: $p < 0.01$; ***: $p < 0.001$.

4.2.11 Major use agreement and PGE_2 inhibitory activity

No statistically significant association was found between the incidence of PGE_2 inhibitory activity and level of agreement for the three use citation categories. A marginally greater proportion of extracts exhibiting some level of inhibitory activity, however, was found amongst the extracts cited for use in treatment of sores than amongst those not cited for this purpose (Table 4.20); Of the 19 extracts assayed which were cited for sores, activity was detected in 10 (53%), whereas of the 10 extracts assayed which were not cited for this purpose, activity was only found in 2 extracts (20%). Similarly, a marginally greater

proportion of extracts exhibiting inhibitory activity was found amongst the extracts cited two or more times for treatment of sores than amongst those cited with less frequency, with activity detected in 7 of the 12 extracts cited in more than 2 instances (50%), and in 5 of the 17 cited in less than two instances (29%). Similarly, mean PGE_2 percent inhibition was slightly higher in the grouping of preparations with a higher level of agreement for citations referring to the treatment of sores ($MUA_{TMP} \geq 33$) (21% ± 8) compared to the group with lower agreement (11% ± 8), however, significance was not reached (Table 4.21).

Table 4.19 Major use agreement values of preparations with percentage inhibition of PGE_2 formation in ionophore stimulated 3T3 fibroblasts. List sorted in descending order based on MUA_{TMP} of use citations for 'sores (dermal lesions). Shading indicates extracts in which activity was detected

		Preparation major use agreement values (MUA_{TMP})			PGE_2 percent inhibition at 20 μg · mL^{-1}
Species	**Extract Description**	**Wounds**	**Sores**	**Dermatological**	**(%)**
Crinum angustifolium	Cold infusion of bulb	67	78	78	-
Crinum angustifolium	Decoction of bulb	67	78	78	-
Eucalyptus tetrodonta	Terminal leaf decoction	0	67	67	34
Erythrophleum chlorostachys	Trunk inner bark decoction	17	56	56	47
Buchanania obovata	Decoction of inner trunk bark	33	44	44	33
Callitris intratropica	Decoction of inner trunk bark	50	33	33	-
Erythrophleum chlorostachys	Leaf/inner trunk bark decoction	17	33	33	30
Bauhinia cunninghamii	Decoction of root	17	22	22	22
Acacia holosericea	Leaf decoction	0	22	22	-
Eucalyptus tetrodonta	Decoction of trunk inner bark	0	22	22	41
Eremophila longifolia (BC)	Decoction of leaves	0	22	22	30
Eremophila longifolia (IC)	Decoction of leaves	0	22	22	-
Acacia auriculiformis	Leaf decoction	17	11	11	-
Eremophila duttonii	Decoction of leaves	17	11	11	42
Eremophila sturtii	Decoction of leaves	17	11	11	-
Morinda citrifolia	Expressed juice of unripe fruit	0	11	11	-
Buchanania obovata	Decoction of terminal stems	17	11	11	34
Cymbopogon ambiguus	Decoction of whole plant	0	11	11	34
Pandanus spiralis	Diluted juice of leaf tips	0	11	11	-
Hibiscus tiliaceus	Warm infusion of stems	17	0	11	-
Hibiscus tiliaceus	Cold infusion of terminal stems	17	0	11	-
Ipomoea pes-caprae	Hot infusion of leaves	0	0	44	-
Scaevola sericea	Expressed juice of fruit	0	0	22	-
Cochlospermum fraseri	Expressed juice of fruit pulp	0	0	11	-
Eremophila latrobei	Decoction of leaves	0	0	11	-
Flagellaria indica	Expressed juice of growing tips	0	0	11	-
Pandanus spiralis	Decoction of leaf bases	0	0	11	-
Flagellaria indica	Warm infusion of stems	0	0	11	15
Eremophila alternifolia	Decoction of leaves	0	0	0	18

BC: *E. longifolia* borneol chemotype; IC: *E. longifolia* isomenthone chemotype

Table 4.20 Proportion of extracts exhibiting inhibition of PGE_2 formation in ionophore stimulated fibroblasts by groupings greater and less than median MUA of preparations (MUA_{TMP})

Category	Preparations active	Preparations inactive	% Active	χ^2	Fisher's Exact Test (p)
≥1 citations for wounds (MUA_{TP} ≥17)	6	7	46%	0.221	0.716
=0 citations for wounds (MUA_{TP} <17)	6	10	38%		
≥2 citations for 'Sores' (MUA_{TP} ≥22)	7	5	58%	2.426†	0.120
<2 citations for 'Sores' (MUA_{TP} <22)	5	12	29%		
≥2 citations for all D/OC (MUA_{TP} ≥22)	7	7	50%	0.829	0.462
<2 citations for all D/OC (MUA_{TP} <22)	5	10	33%		
≥1 citations for 'Sores' (MUA_{TP} ≥11)	10	9	53%	2.876†	0.126
=0 citations for 'Sores' (MUA_{TP} <11)	2	8	20%		

*: Pearson chi-squared significance at 0.05 level; Pearson chi-squared significance at 0.01 level; †: Expected number <5 in Pearson chi-square test

Table 4.21 Mean inhibition of PGE_2 formation in ionophore stimulated fibroblasts of preparations greater than, and less than 33% MUA_{TMP}

MUA_{TMP} group	n	Mean Inhibition (%)
MUA_{TMP} < 33 ('Sores')	22	11 ± 3
MUA_{TMP} ≥ 33 ('Sores')	7	21 ± 8
MUA_{TMP} < 33 (All dermatological/ophthalmic complaints)	21	11 ± 4
MUA_{TMP} ≥ 33 (All dermatological/ophthalmic complaints)	8	18 ± 7
MUA_{TMP} < 33 (wounds)	25	14 ± 3
MUA_{TMP} ≥ 33 (Wounds)	4	8 ± 8

Asterisks denote significance of difference between groups of preparations with MUA_{TMP} > or < 33%, *: $p < 0.05$; **: $p < 0.01$; ***: $p < 0.001$.

4.3 **Discussion and conclusions**

A number of previous studies have reported values for total phenolic content and antioxidant capacity for large samples of medicinal and other plant extracts (e.g. Kahkonen *et al.*, 1999; Zheng and Wang, 2001; Cai *et al.*, 2004; Katalinic *et al.*, 2006; Surveswaran *et al.*, 2007; Gan *et al.*, 2010; Kratchanova *et al.*, 2010). It is difficult, however, to make direct comparisons with the results of this study with those of previous studies given the variety of methods of extraction which were used (i.e. following traditional methods and thus prepared with individual variability in the ratio of solvent to plant material, extraction temperatures

and pre-extraction processing methods). The majority of studies reporting on phenolic content and antioxidant capacity of plant extracts have typically done so using standardised methods of processing and extraction. One aspect commonly reported in all of the studies cited above, however, and also seen in this study, was the wide range of variability in phenolic content found among extracts. On the one hand, such a high degree of variability could be interpreted to imply that phenolic content is not directly relevant to medicinal use or activity, particularly in cases where species have been selected for investigation on the basis for use in a particular condition or indication [e.g. as in this study and in the work of Gan *et al*. (2010), where plants were selected on the basis of use in traditional Chinese medicine for the treatment of rheumatism]. On the other hand, phenolic content in medicinal plant specimens has been shown, on average, to be higher than that found in common fruit and vegetables and other plants and generally, the highest values reported for medicinal species in the large scale surveys mentioned above, have been reported to exceed those of many edible and other 'non-medicinal' plants [with the exception of highly coloured, anthocyanidin rich berries and tissues of some tree species (Kahkonen *et al.*, 1999)]. While the phenolic content of the traditional preparations was variable, significant associations were found between higher levels of phenolic content in the sample of extracts and higher levels of agreement in the ethnobotanical literature surveyed, for citations referring to use in the treatment of wounds and sores.

Higher levels of agreement and frequency of citation in the traditional therapuetic treatment of sores and wounds by Australian indigenous groups for the medicinal preparations examined, was found to be most strongly associated with higher levels of phenolic compounds and *in vitro* cytotoxicity against P388D1 murine lymphoblasts. These associations tended to be stronger or more apparent when MUA_{TMP} was determined using citations only refering to the treatment of dermal sores than when derived from citations

describing treatment of wounds. No significant association was found between groupings based on phenolic content and groupings ordered by MUA_{TMP} values calculated using citations refering to all dermatological/ophthalmic complaints. The major difference between this list of MUA_{TMP} values and those describing MUA_{TMP} relating to sores and wounds only, was the relatively high ranking of extracts from *I. pes-caprae* (cited in four independent studies for use in treatment of scabies) and the juice of *S. sericea* fruit, (cited in two independent studies for the treatment of infected and red eyes), which were both found to exhibit relatively low levels of TPC. This pattern was seen across most of the activities examined, where stronger or more significant associations of activity were found in groupings based on MUA_{TMP} values calculated using citations describing the relatively specific indications of dermal sores and wounds.

Overall these findings would suggest that probability of finding Australian plant species which yield extracts of higher phenolic content in phytochemical or ethnopharmacological surveys, may be increased with the selection of species frequently or specifically cited in the ethnobotanical literature for these purposes (particularly in the treatment of sores). This finding would also suggest that relatively high phenolic content in plant based medicinal preparations contributes in some way to biological effectiveness in the treatment of sores and wounds, and that species which may yield preparations with high phenolic content may be more likely than those lacking this characteristic to have been selected for these specific purposes over time, and incorporated into established traditional cultural practice. Phenolic content in the traditional preparations was partly dependent on mode of preparation and partly dependent on specific plant part extracted, with higher quantities generally found in woody tissues and on extraction at higher temperatures. In the sample of preparations examined which were cited frequently for treatment of wounds and sores, there was a predominace of extracts requiring inner bark of the trunks of trees. de Sousa *et al*. (2008)

have presented very similar findings in a report detailing results of an ethnobotanical and phytochemical survey conducted within several communities of the Altinho municipality, within the semi-arid Caatinga region of Pernambuco state, north-eastern Brazil. de Sousa *et al*. (2008) examined total phenolic, tannin and flavonoid content in a sample of medicinal plants in relation to measurements of cultural consensus of use mentions referring to symptoms and indications relating to wound-healing and inflammation, and reported strong association between tannin content and the degree of cultural consensus for this purpose. de Sousa *et al*. (2008) also reported that the species with greater wound-healing/anti-inflammatory consensus were not found to be associated with variation in flavonoid content among the species studied. In this study identity of phenolic compounds in the extracts was not established, however, it is possible that the high total phenolic content found in the preparations may be representative of high tannin content. The location in which the study described by de Sousa *et al*. (2008) was undertaken, shares some climatic and ecological similarities with the regions of northern Australia from which the majority of the plant specimens examined in this study were collected (i.e. semi-arid dry-monsoonal with marked seasonal rainfall and dry-forest communities). It is tempting to speculate that relatively high phenolic content in medicinal plants used for wound healing, as was found in this study, and as reported by de Sousa *et al*. (2008), may represent a universal or common phenomenon in similar and perhaps even other, ecologically distinct regions. de Albuquerque *et al*. (2005; 2010) have presented evidence indicating that phenolic compounds (principally tannins) are unusually predominant in much of the dry-land Caatinga medicinal flora, and have shown that in this region, a general preference amongst local people is seen for medicinal plants or plant parts from long lived perennial or woody species which are constantly available (i.e. not subject to annual seasonal fluctuations in growth or appearance). It is possible that the same may be generally true in northern Australia, where similar climatic conditions occur. On the

other hand, several examples can be found in the Australian ethnobotanical record, which describe selection of specific plants and plant parts which are seasonally available for relatively short periods. In this study for example, at least two preparations could be classified as such, including the extracts of *B. obovata* green terminal stems and *E. tetrodonta* juvenile leaves, both representing ephemeral, new growth tissues which only appear during and toward the end of the wet (high rainfall summer) season. Interestingly, in this study, relatively high levels of phenolic content were found in both of these preparations. Accumulation of high levels of defensive secondary metabolites in juvenile or growing plant tissues has been noted in a number of species, and may function to protect otherwise vulnerable structures from predation (Harborne, 1993; Verpoorte, 2000). It is possible that these plant parts may differ significantly in phytochemical makeup from analogous mature tissues (i.e. the mature sclerophyllous leaves of *E. tetrodonta* and sections of woody branches more proximal to the trunk of *B. obovata*), given the requirements for these specific parts in their respective medicinal preparations.

Presence of tannins or other polyphenolics may explain any preference for the use of the species/plant parts examined in medicinal preparations intended for the treatment of sores and wounds. As noted earlier in this chapter, a number of studies have reported significant improvements in parameters indicative of healing with *in vivo* experimental wound models following treatment with plant derived tannin containing extracts (e.g. Chaudhari and Mengi, 2006; Kokane *et al.*, 2009) and activity of tannins and tannin rich extracts affecting biochemical and molecular processes related to wound healing has been demonstrated (e.g. Khanna *et al.*, 2001; Agyare *et al.*, 2010). The property of astringency common to most tannins could potentially be of benefit in the treatment of exudating sores or weeping dermatoses, as could other activities which have been reported in this group of compounds, e.g. antimicrobial activity (Scalbert, 1991; Serrano *et al.*, 2009), and anti-inflammatory

activity (Okuda, 2005). As pointed out by de Albuquerque (2010), the demonstrated prevalence of tannins in certain medicinal systems (i.e. in the Caatinga semi-arid region of NE Brazil) or in association with certain traditional uses (such as wound healing or dermatoses), may also be indicative of other, as yet unknown biological activities which may be a feature in this group of compounds.

The association of high phenolic content with higher levels of agreement and frequency of citation for sores and wounds while significant was, like most of the parameters and activities examined, not entirely consistent. Several preparations with relatively high MUA_{TMP} values did not exhibit high phenolic content. Most notably, extracts of *C. angustifolium*, for which the highest MUA_{TMP}, all three use categories was found, exhibited very low phenolic content as compared to others in the sample. Similarly, extracts of *C. schultzii* charred timber and *E. tetrodonta* inner bark (both cited for treatment of sores) exhibited relatively low phenolic levels. Interestingly, most of these particular preparations were not seen to exhibit activity across most of the assays utilised (with the exception of the *E. tetrodonta* inner bark extract, for which some inhibition of PGE_2 formation was found). The range of activities examined in the study, however, was relatively narrow when the potential range of therapeutic targets which may provide relief or resolution in conditions for which they are used traditionally are considered. The results of the few assays presented in this study therefore cannot be interpreted to represent a complete picture of potential pharmacological activity in these preparations. It is likely that these preparations may act via other pharmacological activities along with, or instead of, those examined (e.g. local analgesic or anti-nocioceptive activity, proliferative activities and modes of anti-inflammatory activity other than inhibition of prostaglandin synthesis).

It is difficult to explain the very strong and consistent association which was observed between citation of the preparations for treatment of wounds and sores, and P388D1 cytotoxicity. Cytotoxicity may be considered an undesirable trait in pharmaceutical agents intended for the treatment and management of wounds and would be expected to delay healing due to negative effects on proliferation of fibroblasts and keratinocytes, and depletion of leukocytes and macrophages at the wound site. It is possible, however, that the *in vitro* cytotoxicity observed for the preparations may not be predicitive or indicative of potential cytotoxic effects with traditional *in vivo* use. A number of topical antimicrobial preparations which have been shown to exert potent *in vitro* cytotoxic activity toward fibroblast and keratinocyte cultures (e.g. povidone iodine, bacitracin, neomycin and polymyxin B sulphate), have been shown to improve wound healing outcomes in human and porcine *in vivo* studies, with no evidence of cytotoxic effects, and these discrepancies have been ascribed to the relative susceptibility of *in vitro* cell cultures to environmental disturbances as compared with cells *in vivo* (Sullivan *et al.*, 2001). Additionally, P-388 lymphocytes are known to be particularly sensitive to cytotoxic action and are for this reason, often employed as a pre-screen for cytotoxicity studies involving large numbers of samples. Culture media (in particular DMEM, which was used in the experiments), are known to favour the oxidation of polyphenolic compounds, resulting in generation of cytotoxic levels of H_2O_2 and formation of cytotoxic quinone and semi-quinone derivatives (Kwee *et al.*, 2003). This tendency may have been a factor in the cytotoxicity observed for the extracts which were high in phenolic content, but does not explain the high levels of cytotoxicity seen in extracts for which TPC was relatively low (e.g. *C. angustifolium* extracts). It is possible that the significantly higher levels of P-388 cytotoxicity observed in the preparations which were frequently cited for wounds and sores, may be indicative of some other factor common to these particular preparations, which is not involving of significant *in vivo* cytotoxicity.

Very little association was found between MUA_{TMP} of the uses examined and *in vitro* antioxidant capacity, particularly with results obtained using the ORAC assay. Some marginal association was found between the incidence of higher range TE values as obtained by DMPD assay and citation for sores and wounds. Mean DMPD TE was also found to be significantly higher in extracts with MUA_{TMP} values > 33 for sores ($p < 0.05$) and wounds ($p < 0.01$) when results obtained from the *C. fraseri* extract were treated as outlyers and excluded from analyses. These results are reflective of the relationship found between total phenolic content and antioxidant activity which, while statistically significant, were not consistent and stronger for results obtained using the DMPD assay as compared with those of ORAC assays. It is possible that the phenolic compounds detected in the extracts, while capable of free radical scavenging, contribute to overall activity in some other way which may benefit the wound healing process. There is some evidence that antioxidant based therapies may improve wound healing where derangement of normal endogenous processes which counter oxidative overload are impaired or overwhelmed (e.g. in advanced diabetes mellitus, where destruction of the microvasculature produce local wound hypoxia, leading to subsequent oxidative stress and impairment of wound healing). Oxidative signalling, however, has been shown to represent a vital part of wound healing and tissue repair/regeneration and it is conceivable that agents able to quench or scavenge free radicals, may be capable of impairing healing through interference with these processes. Roy *et al*. (2006) have shown that manipulation of the redox environment of wound sites may affect healing, demonstrating improved outcomes with topical application of H_2O_2 and impaired healing with experimental overexpression of catalase *in vivo*. Moreover, Khanna *et al*. (2001) have shown oxidant dependent expression of VEGF in keratinocytes can be stimulated with tannin and polyphenol rich grape seed extracts and have further demonstrated evidence of this activity *in vivo* along with improved healing and confirmation that the grape seed extract *enhanced* the

oxidising environment of the wound site (Khanna *et al.*, 2002). Tannins and flavanoids are generally known for their potential antioxidant and free radical scavenging abilities and this apparently paradoxical effect was explained by the ability of some phenolic compounds to participate in redox cycling and act as pro-oxidants under certain conditions (e.g. in the oxidant rich environment of the wound)(Khanna *et al.*, 2002). It is possible that the ability of polyphenolics to potentially act as pro-oxidants, may represent a principal factor in any tendency for higher levels of these compounds to be found in preparations used traditionally for wound healing. The relatively weak associations that were found between use agreement for wound healing and *in vitro* antioxidant capacity may simply be reflective of phenolic content in the extracts and may not be predictive of the behaviour or pharmacological action of these preparations when applied clinically in the treatment of wounds and dermatoses.

As was noted earlier in the chapter, the results for PGE_2 inhibitory activity were derived from single experiments and must be interpreted with caution. Tests for inhibition of PGE_2 were carried out using a single concentration, and as was the case with the tests for cytotoxicity, results were not transformed or considered in terms of the original concentration of each extract as produced using methods of traditional preparation (as was done in the case of the other tests for biological activity and TPC). While no statistically significant associations were found between inhibition of PGE_2 formation and any of the MUA_{TMP} rankings, marginally higher incidences of activity were noted in all groupings of higher range MUA_{TMP} representing 2 or more citations, than in lower MUA_{TMP} rang groupings (particularly with preparations cited for treatment of sores). Anti-inflammatory drugs commonly form a component of treatment in the clinical management of many dermatoses (e.g. in the form of topical glucocorticoid preparations) (Marley, 2004). It would not be unreasonable to expect that preparations/plant species containing anti-inflammatory compounds would be more likely to be employed for this purpose in traditional medicine. Several of the preparations

which were found to exhibit activity included those which had exhibited relatively high activity in other tests and for which frequent independent citations for wounds, sores etc had been noted (e.g. *E. chlorostachys* inner bark extract, *E. tetradonta* terminal leaf extract). In a few instances, preparations for which activity in other tests had not been observed or was unremarkable, exhibited PGE_2 inhibtory activity (e.g. extracts of *E. alternifolia* leaves, *F. indica* stems and *C. ambiguus* whole plant). Unlike as with most of the other activities examined (i.e. antibacterial and antioxidant capacity), no particular relationship beteen PGE_2 inhibion and phenolic content was seen. Citations for traditional use of *E. alternifolia* include indications which may be considered suggestive of anti-inflammatory activity (e.g. use of topical use of poultice of macerated leaves for the tretament of headache as described by Goddard and Kalotas (2002) and treatments for pain, fever and symptoms of flu-like respiratory illnesses in various regions of central Australia as described by Barr *et al*. (1993) and Latz (1995). *E. alternifolia* was in fact, among the most frequently cited species within the febrifuge category of the literature surveyed (5 independent citations for this purpose). The *C. ambiguus* extract was also cited frequently for this purpose (5 independent citations recorded) along with the *F. indica* stem infusion (2 independent citations for fever). Induction of fever in response to infection is one of the primary functions of PGE_2 in the body and many drugs used as febrifuges in contemporary medicine (i.e. non-steroidal anti-inflammatory drugs, such as paracetamol) act by inhibiting PGE_2 formation (typically by inhibition of cyclooxygenase activity) (Rang *et al.*, 2007). The sample of plant species and their respective preparations were primarily selected on the basis of use mentions pertaining to dermatological conditions. Other uses, such as in treatment of pyresis and symptoms of respiratory illness were not as frequently represented in the sample as a whole and so it is difficult to draw any firm conclusions regards to citation for these uses and observed biological activity. It is interesting however, that all four of the preparations for which pyresis

or fever was explicitly cited as an indication of use in the literature surveyed, also exhibited PGE_2 inhibitory activity (i.e. those mentioned above along with the *B. obovata* inner trunk bark decoction).

Overall, it may not be considered altogether surprising that little association was found between PGE_2 inhibition and agreement/frequency of citation for wounds and sores. While anti-inflammatory activity may be considered as a relevant activity in the treatment of wounds and dermatoses (and therefore might be expected to occur frequently in traditional therapies used for these conditions), inflammation is also a component in the pathophysiology of nearly all diseases and illnesses (Rang *et al.*, 2007) and might therefore be equally expected to be found in medicinal plant preparations used traditionally for almost any purpose. Most of the preparations examined have been cited for a number of seemingly distinct therapuetic uses, and several for which low level MUA_{TMP} values were found (relating to deramtological uses), were otherwise cited frequently for other purposes. This was particularly so for the preparations of *E. alternifolia*, *C. ambiguus* and *F. indica* extracts (where single citation for treatment of sores and scabies was recorded for the *C. ambiguus* decoction, a single citation for treatment of ophthalmic complaints for *F. indica* preparation and no dermatological uses recorded for the *E. alternifolia* preparation).

Detection of PGE_2 inhibitory activity in the *C. ambiguus* extract was not entirely unexpected. Rogers, Grice *et al*. (2000; 2001), have previously demonstrated dose dependent inhibition of arachidonic acid induced platelet activation by extracts of *C. ambiguus* and have recently reported isolation of four phenylpropanoids with anti-platelet activity from *C. ambiguus* (eugenol, elemicin, eugenol methylether and trans-isoelemicin) (Grice *et al.*, 2011). Eugenol has been shown to inhibit arachodonic acid mediated increases in intracellular calcium (Chen *et al.*, 1996) and COX-1 and COX-2 inhibitory activity (Everts *et al.*, 2000). What was

perhaps unexpected in this study, was evidence of relevant activity given that this particular extract was prepared from senescent material (descriptions of traditional use specifically state that only relatively young, green plants are suitable for medicinal preparations) (e.g. Barr *et al.* 1993). All parts of *C. ambiguus* are typically strongly aromatic and recovery of steam distilled essential oils has been reported by Barr *et al*. (1988; 1993). Active compounds described by Grice *et al*. (2011) included volatile phenylpropanoids, which would likely be absent or significantly reduced in the odourless, senescent material. It is possible that the activity reported in this study may either be underestimated, or, that other compounds which remain in senescent tissue were responsible for the activity observed.

One unexpected result was the lack of activity exhibited by extracts of I. *pes-caprae*. *I. pes-caprae* ('beach morning glory') is one of the most frequently cited species in the Australian indigenous ethnobotanical literature, and many of the uses mentioned include those which could be interpreted to suggest anti-inflammatory or analgesic activity (e.g. topical analgesia for marine stings, headache, oedema and inflammation). A high level of agreement was found in the literature surveyed, for citations referring to use of leaves in the treatment of marine stings (e.g. jellyfish). Additionally, very similar traditional uses of the species in Thai traditional medicine have been recorded (e.g. treatment of marine stings) and extracts have been shown to be clinically effective for treatment of jellyfish stings (Pongprayoon *et al.*, 1991b). *in vitro* anti-inflammatory activities have been demonstrated for extracts of *I. pes-caprae* (Pongprayoon *et al.*, 1991a) and a number of compounds inhibiting prostaglandin synthesis have been identified including 2-hydroxy-4,4,7-trimethyl-1(4*H*)-naphthalenone, (-)-mellein, eugenol, and 4-vinylguaiacol (Pongprayoon *et al.*, 1991). The lack of activity found in the aqueous extracts examined in this study may in part be explained by the specific method of preparation followed. The preparation involved infusion or decoction of only ten leaves in water and the resulting extract was of very low yield and low concentration. It is

possible that this method of preparation was not suitable for sufficient extraction of any compounds which may have potentially exhibited activity in the PGE_2 inhibition assay, particularly those which might exhibit a low degree of water solubility. This preparation was chosen as it was a liquid solution, and was frequently cited for treatment of scabies infestation. The majority of other preparations of the plant appearing in the Australian ethnobotanic literature and referring to treatment of marine stings, pain and inflammation and ulcers describe direct application of leaves which may represent a more effective method of delivering active (or lipophillic) principles to the site of inflammation or injury. As pointed out previously, the range of plant-based medicinal preparations examined in this study was somewhat limited by the need to restrict selection to those which could be handled as liquids. In all cases this involved extraction in water or expression of the (aqueous) juice of various plant materials. For this reason the sample of preparations examined is possibly biased toward the representation of the more polar, or hydrophillic compounds which may be present in the species examined.

Chapter 5: Isolation of serrulatane diterpenes from *Eremophila duttonii*

In this chapter, an attempt to isolate and purify serrulatane diterpenes from extracts of Eremophila duttonii is described. Results of assays for biological activity in partially purified fractions containing these compounds, along with attempts at performing quantitative assays in traditional aqueous medicinal preparations, are also presented and discussed

5.1 **Introduction**

Eremophila duttonii is a medium shrub (1-3 m), endemic to the arid and semi-arid regions of Australia. Leaves and aerial parts of the plant are reported to be used medicinally by a number of indigenous groups in the central and northern regions of Australia (O'Connell *et al.*, 1983; Barr *et al.*, 1993; Lassack and McCarthy, 1997). Previously, ethanol (EtOH) extracts of *E. duttonii* material have been shown to exert potent antibacterial activity against Gram-positive organisms (Palombo and Semple, 2001; Palombo and Semple, 2002; Shah *et al.*, 2004). In earlier work, the principal antibacterial constituents were identified with activity guided fractionation from a hexane extract of *E. duttonii* leaves as the serrulatane diterpenoids: serrulat-14-en-7,8,20-triol (**1**), and serrulat-14-en-3,7,8,20-tetraol (**2**) (Smith, 2004; Smith *et al.*, 2007). In the present study, an attempt was made to isolate the serrulatanes **1** and **2** from a DCM extract of *E. duttonii* leaves, using normal phase column chromatography and semi-preparative scale HPLC. Partially purified fractions containing compounds **1** and **2** were assessed for antimicrobial activity and antioxidant capacity**,** and preliminary testing was carried out to establish whether cytotoxicity and PGE_2 inhibitory activity could be detected. Antimicrobial activity was compared with that reported for other serrulatanes isolated from *Eremophila* species and an attempt was made to determine whether either of these compounds could be detected in traditional aqueous decoctions of *E. duttonii*

leaves, which had been prepared according to descriptions in the ethnobotanical literature. Partially purified fractions containing **1** and **2** were used as analytical standards for the initial detection in the aqueous preparations and an attempt was made to purify a sample of the tetraol (**2**) for use as a standard for subsequent quantitative assays by HPLC.

5.1.1 **Ethnopharmacology and phytochemistry of *E. duttonii***

Barr *et al.* (1988; 1993) have described the use of decoctions of *E. duttonii* leaves by indigenous people of the Ltyentye Purte community (NT) for the topical treatment of infected cuts and open sores, scabies, pain and inflammation of the eyes or ears, and internally as a treatment for sore throat, and for general malaise associated with viral respiratory illnesses. Unspecified medicinal use of the species by Pitjantjatjara, Anmatyerre and Alyawarr people of Central and Northern Australia has also been cited by O'Connell *et al.*(1983) and Latz (1995). Barr *et al.* (1993) have indicated that *E. duttonii* is highly valued by indigenous people as a medicinal plant, and, in certain locations, preparations derived from the plant are used in preference to other available proprietary pharmaceutical products.

Palombo and Semple (2001) reported on screening for antimicrobial activity in 56 ethanol extracts of 39 Australian medicinal plants selected on the basis of traditional use in treatment of symptoms of viral and cutaneous infection, and reported that extracts of *E. duttonii* exhibited the highest levels of potency among those tested against Gram-positive organisms. Palombo and Semple (2002) and additionally Shah *et al.*, (2004) further demonstrated that activity was characterised by very rapid bacteriolytic actions (< 1 hr for total reduction of culture viability), and reported activity against strains of methicillin resistant *Staphylococcus aureus* and vancomycin resistant *Enterococci*. In a more recent study, these extracts were shown to exert rapid bactericidal activity against *Listeria monocytogenes* in food and milk homogenates (Owen and Palombo, 2007). Tomlinson and Palombo (2005) have provided

evidence indicating that the mechanism of antibacterial action in EtOH extracts of this species may involve disruption of bacterial cell membrane integrity, resulting in increased membrane permeability, leading to bacterial lysis and culminating in cell death. This mode of activity may be associated with the action of antimicrobial terpenoids (Cristani *et al.*, 2007) and is consistent with the earlier observations by Palombo and Semple (2002) and Shah *et al.* (2004), of rapid bacteriolytic action. In earlier work the principal antibacterial constituents present in EtOH and other solvent extracts of *E. duttonii* leaves were identified with activity guided fractionation as two known diterpenes of the serrulatane class: serrulat-14-en-7,8,20-triol (**1**) and serrulat-14-en-3,7,8,20-tetraol (**2**) (Smith, 2004; Smith *et al.*, 2007). Both compounds had previously been identified as components of the resinous exudates forming the characteristically viscid leaf cuticle of this species (Tippett and Massy-Westropp, 1993) and the serrulatane triol (**1**) from extracts of the allied species *Eremophila linearis* (Croft *et al.*, 1981). Structures representing the basic serrulatane skeleton and compounds **1** and **2** are illustrated below in Figure 5.1.

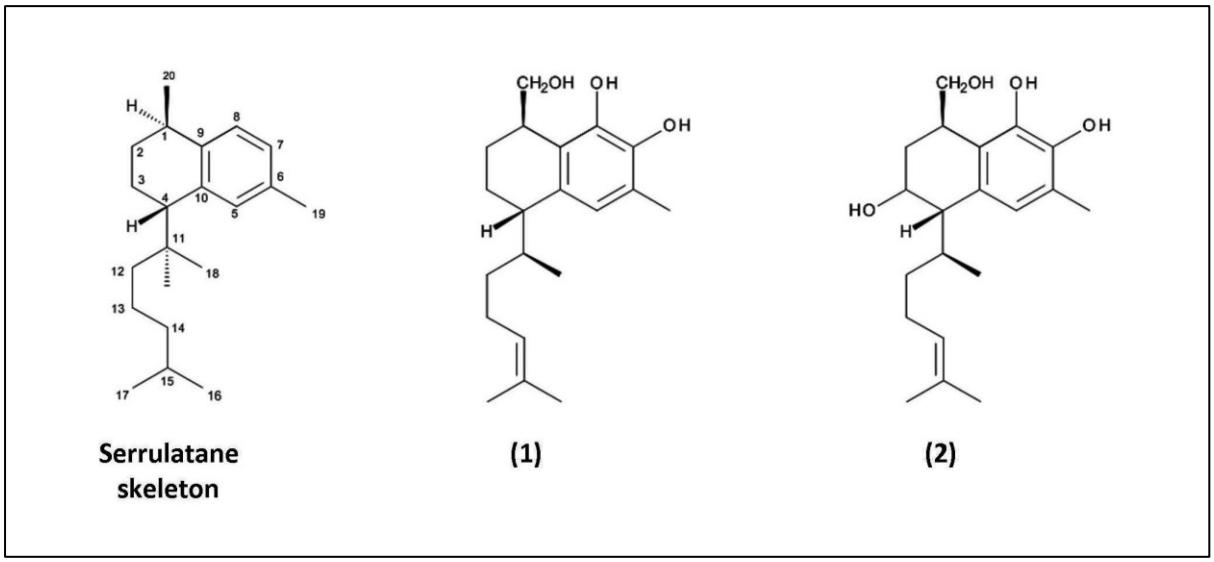


Figure 5.1 Basic serrulatane skeleton with serrulat-14-en-7,8,20-tetraol (1) and serrulat-14-en-3,7,8,20-triol (2) identified in extracts of *Eremophila duttonii*

5.1.2 **Serrulatane diterpenes exhibiting antimicrobial activity**

Of the 8 classes of diterpenoids known to accumulate in *Eremophila* spp., the bicarboyclic serrulatane class is the most common (Ghisalberti, 1993), and a number of recent studies have reported on isolation of a number of serrulatane diterpenes, and structurally related o-naphthoquinones exhibiting antibacterial activity from extracts of *Eremophila* species. Liu *et al.*, (2006a; 2006b) reported isolation of two antibacterial serrulatic acids (serrulat-14-en-19-oic acid and 3,8-dihydroxyserrulat-14-en-19-oic acid), with activity guided fractionation of a 70% aq. ethanol extract of *Eremophila sturtii*. Ndi *et al.* (2007b; 2007c) have described isolation of similar 19-oic serrulatic acids, along with other antibacterial serrulatanes and o-napthoquinones by activity guided fractionation of extracts of *Eremophila neglecta* and *Eremophila serrulata*, after identifying significant inhibitory and bactericidal actions against methicillin resistant strains of *S. aureus* in extracts of these species during a previous study in which 72 *Eremophila* species were screened for antibacterial activity (Ndi *et al.*, 2007a). Antibacterial serrulatanes described by Ndi *et al.* (2007a; 2007b) include 8-hydroxyserrulat-14-en-19-oic acid, 8,19-dihydroxyserrulat-14-ene and biflorin from *E. neglecta* and 8,20-dihydroxyserrulat-14-en-19-oic acid, 20-acetoxy-8-hydroxyserrulat-14-en-19-oic acid, 8,20-diecetoxyserrulat-14-en-19-oic and the o-napthoquinone: 9-methyl-3-(4-methyl-3-pentenyl)-2,3-dihydronaphtho[1,8-*bc*]pyran-7,8-dione from *E. serrulata* (Figure 5.2). 8-Hydroxyserrulat-14-en-19-oic acid has been shown to retain antibacterial activity when covalently attached to polymers and ceramics, and applications for use in antimicrobial surface coatings for medical devices such as catheters and implantable devices, are currently being investigated (Griesser *et al.*, 2008; Vasilev *et al.*, 2009). The o-naphthoquinone biflorin was first described as a constituent of the Brazilian medicinal plant *Capraria biflora* (Scrophulariaceae) and inhibitory activity against Gram-positive bacteria and Mycobacteria has been reported (de Lima *et al.*, 1958). Biflorin also has been isolated from extracts of the

Australian medicinal species *E. latrobei* (cited by Forster *et al.*, 1986). Interestingly, *E. duttonii* and *E. latrobei*, though morphologically distinct, are reported to be referred to by the same name by Alyawarr speaking people: (Aly. *Akwenthey*) (Latz, 1982).

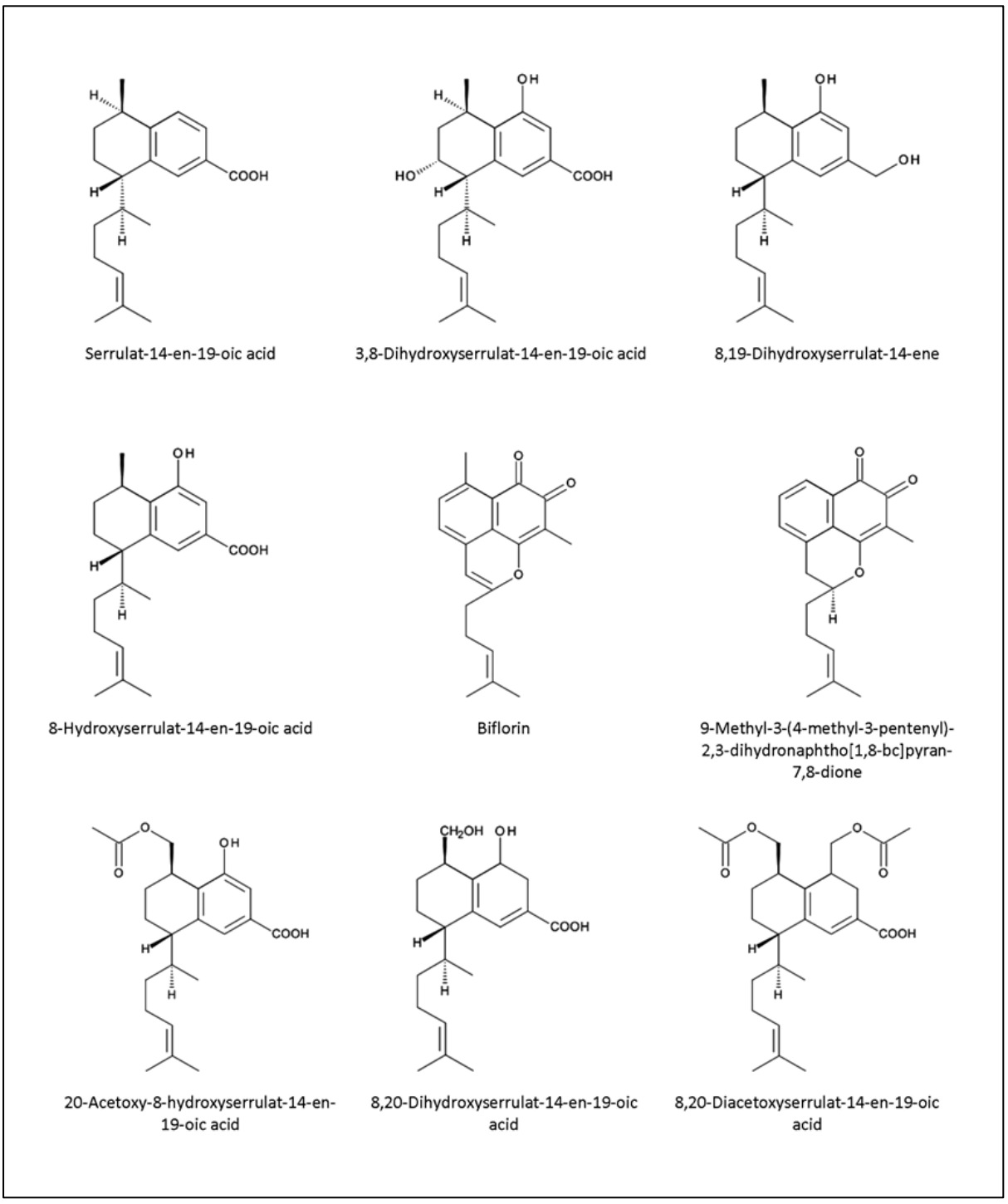


Figure 5.2 Serrulatane type diterpenes and o-naphthoquinones exhibiting antimicrobial activity isolated from *Eremophila* species

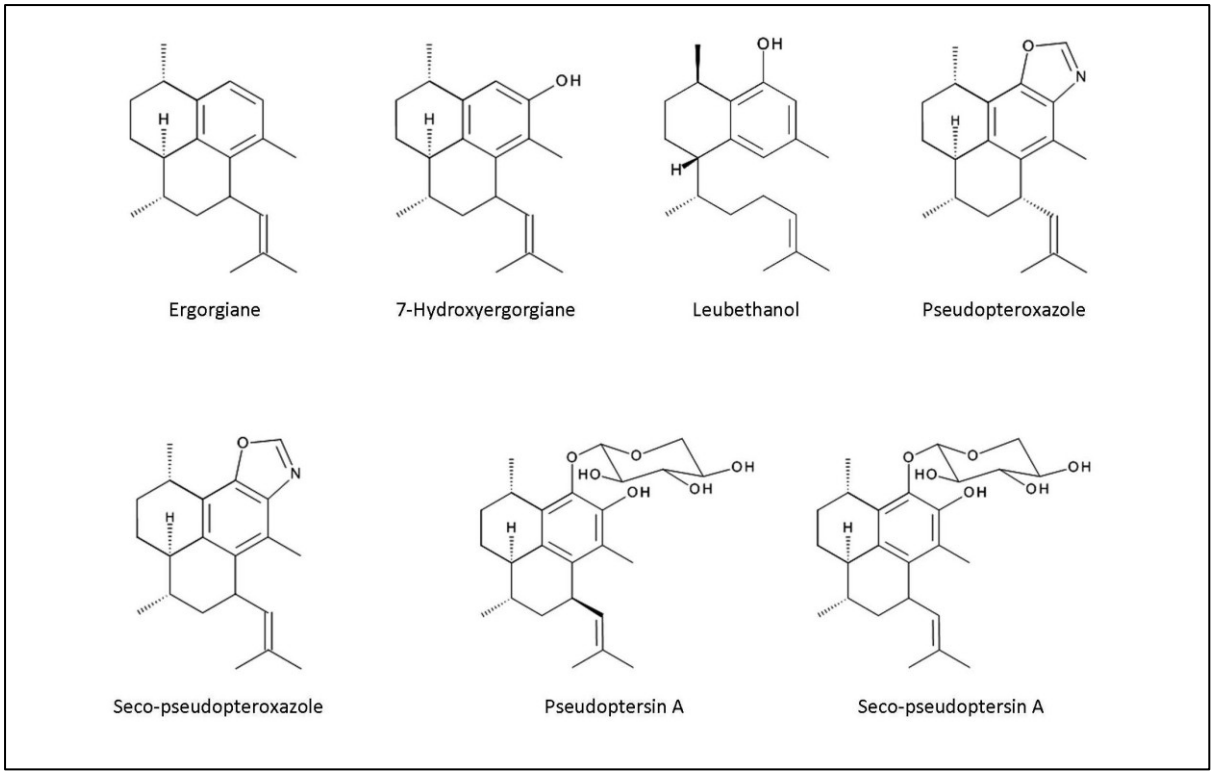


Figure 5.3 Biologically active pseudopterosins isolated from the soft coral *Pseudopterosin elisabethae* and the anti-mycobacterial diterpene leubethanol from *Leucophyllum frutescens* (Scrophulariaceae)

Structurally similar terpenoids possessing the serrulatane skeleton and exhibiting a number of biological activities have been isolated from the soft coral *Pseudopterogorgia elisabethae*. Potent anti-mycobacterial activity has been reported for several of these compounds, including the benzoxazole alkaloids pseudopteroxazole and seco-pseudopteroxazole (Rodríguez *et al.*, 1999) and the seco-pseudopterosins ergorgiane and 7-hydroxyergorgiane (Rodriguez and Ramirez, 2001). Recently, Molina-Salinas *et al.* (2011) reported isolation of an antimycobacterial serrulatane (leubethanol) exhibiting structural similarities to ergorgiane, from *Leucophyllum frutescens*, a Mexican medicinal plant of a genus closely allied to the *Eremophila* (Scrophulariaceae). A series of marine derived seco-pseudopterosin pentose glycosides with potent anti-inflammatory activities, exceeding that of reference drugs such as indomethacin and thought to involve inhibitory action against phospholipase-A2 and the lipoxygenases, have also been described (Look *et al.*, 1986; Look and Fenical, 1987; Roussis

et al., 1990; Potts *et al.*, 1992). Structures of the marine derived serrulatane analogues and leubethanol are illustrated above in Figure 5.3.

5.1.3 **Outline of present study**

The aim of the present study was to determine whether compounds **1** and **2** could be detected in traditional type aqueous extracts of *E. duttonii* and if so, whether in quantities sufficient to explain the antibacterial activities which were observed in these preparations during testing described in Chapter 3. Partially purified samples of each of the compounds were additionally tested for cytotoxic activity against P388D1 murine lymphoblast and HepG2 cell lines and for inhibition of PGE_2 formation in calcium ionophore stimulated 3T3 fibroblasts. Antioxidant capacity was assessed using lipophilic and hydrophillic ORAC assays, and minimum inhibitory and bactericidal concentrations (MIC/MBC) were determined using broth microdilution assays. To establish whether either compound could be detected in the traditional preparations, analytical chromatographic separations of the aqueous traditional preparations by HPLC/LC-MS were performed alongside fractions containing serrulatanes **1** and **2** which had been isolated from a DCM extract using normal-phase column chromatography. HPLC/LC-MS analytical separations revealed the presence of detectable quantities of the serrulatane tetraol (**2**) in the aqueous preparations. The serrulatane triol (**1**), however, was not found to be present in detectable quantities in either of the aqueous preparations examined. An attempt was made to purify a sample of the tetraol **2** using semi-preparative HPLC separation of the crude column chromatography fractions, to employ as a standard for quantitative determinations in the traditional aqueous extracts by HPLC. Subsequent to the experimental work to determine quantity of **2** in the aqueous preparations, however, it was discovered that **2** appeared to be unstable and purity of the isolated sample had apparently deteriorated. For this reason, quantity was estimated based on values corrected for relative peak area of **2** in the standard at the time the determinations were performed.

5.2 **Results**

5.2.1 **Fractionation of *E. duttonii* extracts with column chromatography**

Hexane and DCM extracts of *E duttonii* described in Chapter 2 (2.2.1.5), were separated using normal phase silica gel column chromatography. Separation of the hexane and DCM extracts each afforded 47 fractions. Fractions were analysed using thin layer chromatography (TLC) protocols developed previously for optimum separation and resolution of serrulatane diterpenes from *E. duttonii* (Smith *et al.*, 2007). Monitoring of fractions with TLC enabled preliminary identification of those containing the serrulatanes **1** and **2** by retention factor. Compound (**1)** was found to elute with fractions 26-29 of the hexane extract and fractions 8-11 of the DCM extract (R_f: 0.61-0.70). Compound (**2**) eluted with fractions 31-32 of the hexane extract and fractions 16-19 of the DCM extract (R_f: 0.37-0.39). As in earlier work, compounds **1** and **2** appeared to comprise the two most abundant constituents of the DCM extract (*cf.* Figure 5.4, 5.6 below). Fractions obtained from separation of the DCM extract were selected for further work as this extract was found to contain significantly higher quantities of the serrulatane tetraol (**2**) and separation of each compound appeared to be slightly improved when compared with results obtained with separation of the hexane extract. Yield of fractions from separation of the DCM extract is shown below as mass recovered in Figure 5.4 and TLC chromatograms are illustrated in Figure 5.5.

Fractions which had been shown to contain **1** and **2**, with separation by TLC, were analysed using analytical HPLC. Fractions 9 (F-9) and 18 (F-18) were found to be relatively free of co-eluting compounds (Figure 5.6) and were selected for NMR spectroscopy to confirm identity. Comparison of resulting ^{1}H and ^{13}C chemical shifts with those previously published for **1** and **2** (Smith *et al.*, 2007), confirmed the identity of compound **1** as serrulat-14-en-7,8,20-triol, and compound **2** as serrulat-14-en-3,7,8,20-tetraol.

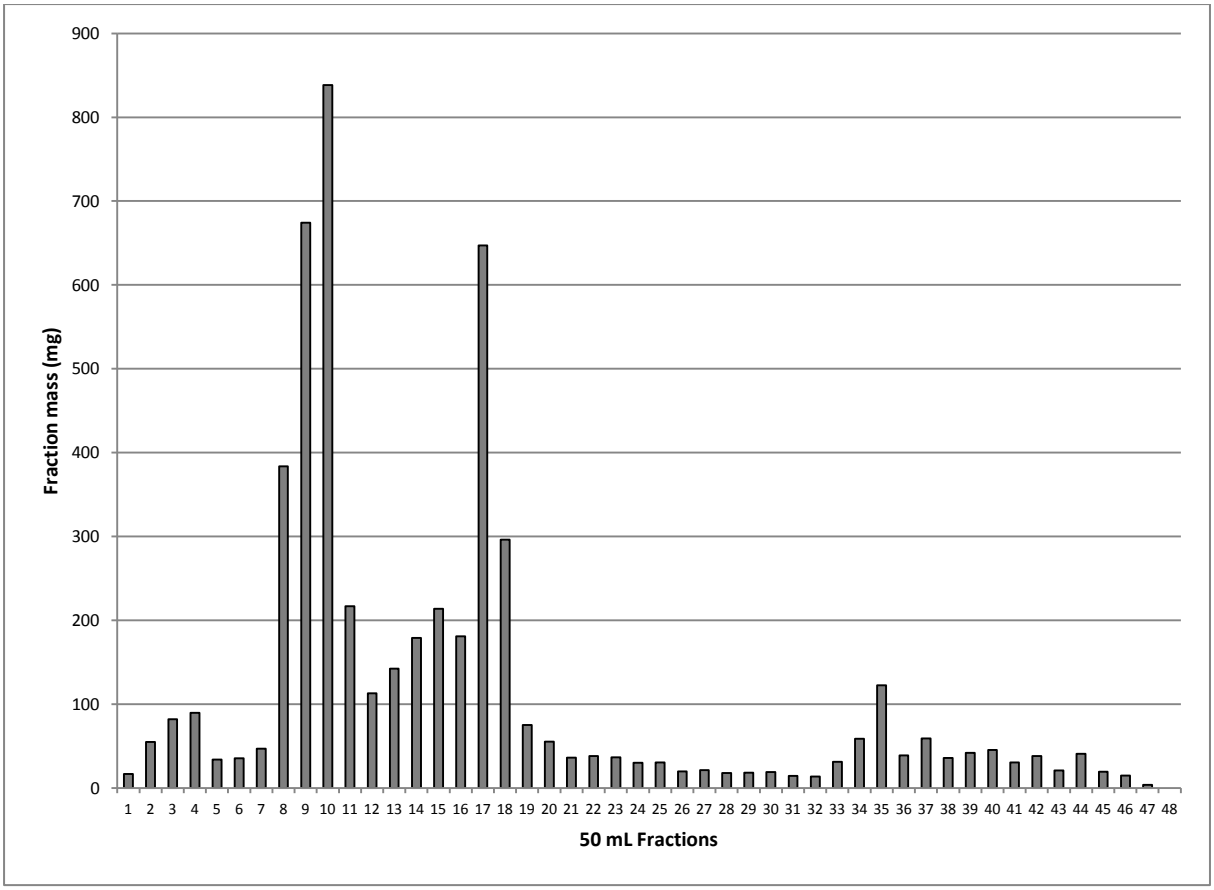


Figure 5.4 Yield (gm) of fractions eluted from separation of *E. duttonii* secondary DCM extract with normal phase silica gel column chromatography (compound 1 eluting with fractions 8-11; compound 2 eluting with fractions 16-19).

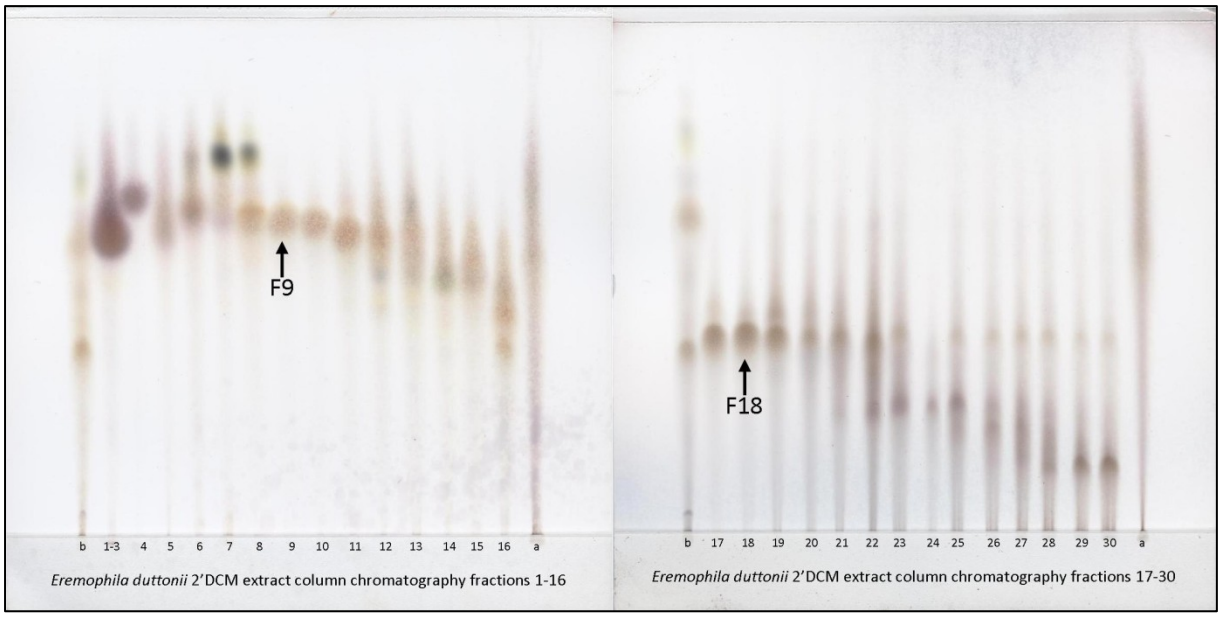


Figure 5.5 Thin layer chromatograms of *E. duttonii* secondary DCM extract column chromatography fractions (Stationary phase: Si-60; Mobile phase: hexane-ethyl acetate (3:1); Loading: 30 µg; F9: Fraction 9; F18: Fraction 18; a: Primary hexane extract; b: Secondary DCM extract; Detection: Acid anisaldehyde spray reagent)

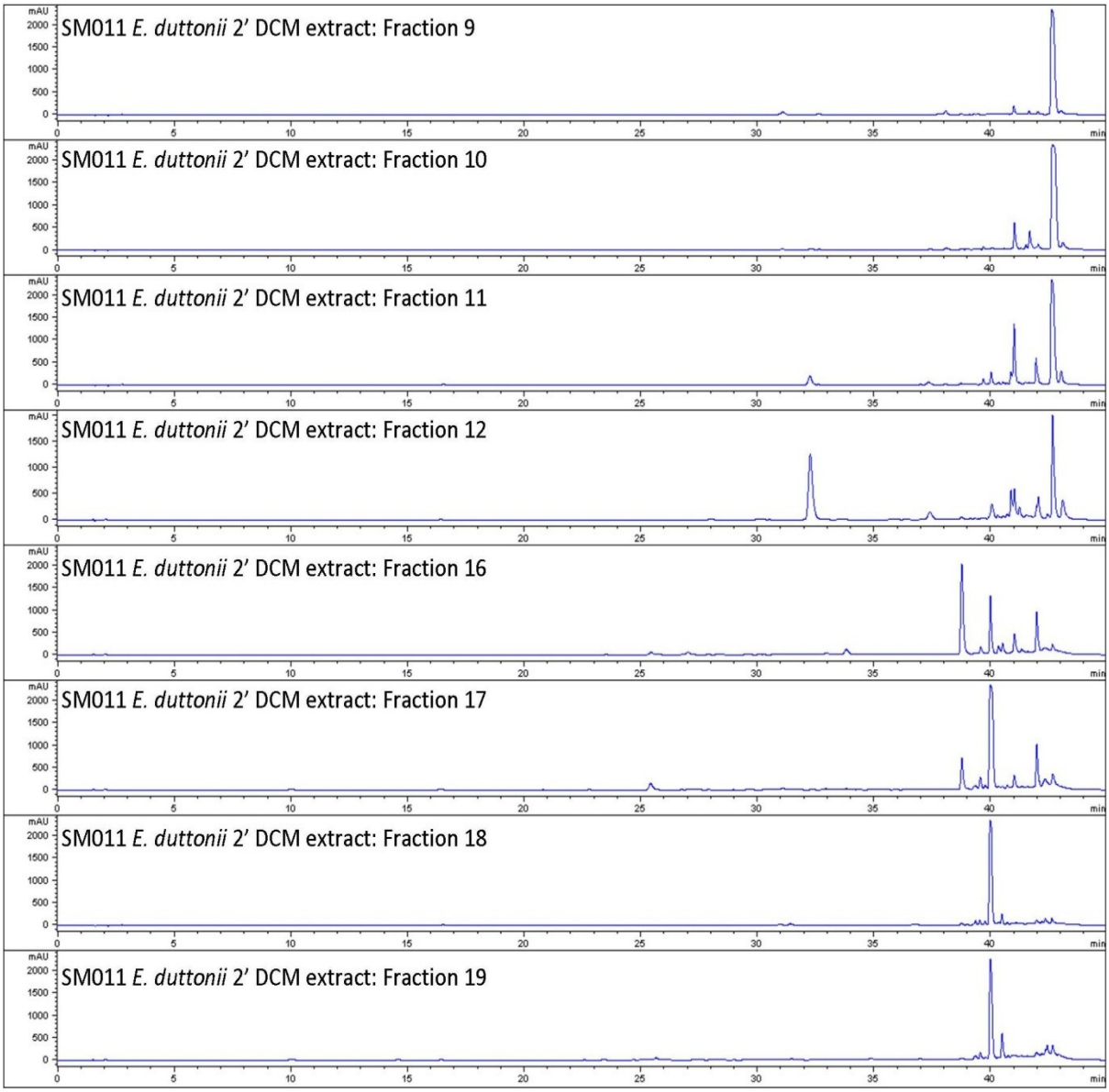


Figure 5.6 Analytical HPLC separations of *E. duttonii* secondary DCM extract column chromatography fractions containing serrulatanes 1 and 2 (Gradient: 10-95% ACN, 30 min; Detection: mAU at λ210 nm with equal scaling of chromatograms)

5.2.2 Detection of serrulatane diterpenes in traditional aqueous preparations of *E. duttonii* by HPLC/LC-MS

To determine whether compounds **1** and **2** were present in the aqueous decoctions, samples of the two traditional type extracts of *E. duttonii* were separated using analytical HPLC/LC-MS alongside F-9 and F-18. The HPLC method employed for these separations was the 30 min 10-95% ACN-H_2O gradient which had been used to generate voucher chromatograms of the traditional aqueous extracts as described in Chapter 2 (2.3).

Separation of F-9 revealed a major peak eluting with retention time (Rt) of 42.64 min (compound **1**). UV/Visible absorbance spectra exhibited maximal absorbance at λ210 nm and λ280 nm (consistent with phenolic groups of **1**) with a shoulder peak at λ228 nm. The mass spectral fragmentation pattern (positive APCI at 150 eV) showed an ion at 318.3 *m/z*, possibly representing the molecular ion and corresponding to the formula weight of the serrulatane triol ($C_{20}H_{30}O_3$: 318.45; Molecular mass: 318.2195). Base peak of the spectrum consisted of a fragment ion at 301.4 corresponding to a loss of ~17 Da. Separation of F-18 revealed a major peak at 39.97 min (compound **2**). The UV/Visible absorbance spectrum was virtually indistinguishable from that of **1,** with maximal absorbance at λ210 and λ280 nm with a shoulder at λ228 nm. The mass spectral fragmentation pattern was similar to that of **1** with the presence of what appeared to be the molecular ion at 334.4 *m/z,* corresponding to the formula weight of the serrulatane tetraol ($C_{20}H_{30}O_4$: 334.45; Molecular mass: 334.2145) with the base peak (317.4 *m/z*) again representing a fragmentation ion with loss of ~17 Da. UV/Visible absorption spectra and 150 eV mass spectral fragmentation patterns of **1** and **2** obtained by analysis of F-9 and F-18 by LC-MS are provided in Appendix III.

The aqueous preparations were separated under the same operating parameters and a peak with Rt corresponding to that of compound **2** was found (Rt 39.98 min) (Figure 5.7). Comparison of mass spectral fragmentation patterns between the peak at Rt 39.9 min produced by separation of the aqueous extract and that of F-18 were used to confirm identity of the peak as the serrulatane tetraol (**2**). The serrulatane triol (**1**) could not be detected in either of the traditional preparations examined at the quantities analysed (100 μg column loading). Fraction 18 was subjected to further purification using semi-preparative scale HPLC, in an attempt to obtain a sample of suitable purity for use as a quantitative standard.

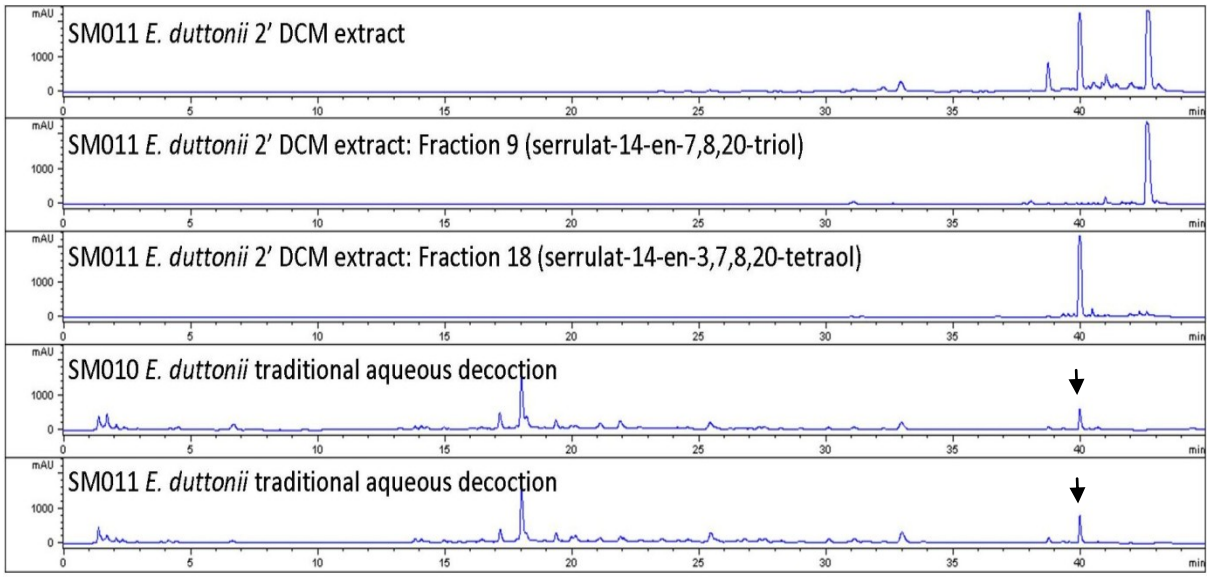


Figure 5.7 HPLC separations of *E. duttonii* traditional aqueous decoctions alongside column chromatography fractions containing compounds 1 and 2, and secondary DCM extract. (Gradient: 30 min 10-95% ACN-H_2O; Detection: MAu at λ210 nm). Arrows indicate elution of compound 2 at Rt of approximately 40 min.

Semi preparative scale sub-fractionation was carried out using 89 mg of F-18 to afford 44 fractions. A chromatogram illustrating a cycle of the semi-preparative fractionation of F-18 is shown below in Figure 5.8. The central portions of the major peak representing the serrulatane tetraol **2** were collected in subfractions 16 - 19 at Rt 21.0 - 21.8 min with the greatest portion found in subfraction 17 (Fractions 18-17, Rt 21.2-21.4 min). Subfractions were lyophilised using a rotary vacuum centrifuge. Fractions 18-17 and 18-18 appeared as colourless fine needles on drying. These fractions were assessed by HPLC/LCMS to be of sufficient purity for use as standards for quantitative assay of **2** in the aqueous preparations (>99% purity by peak area with photodiode array detection at λ210nm) (Figure 5.9).

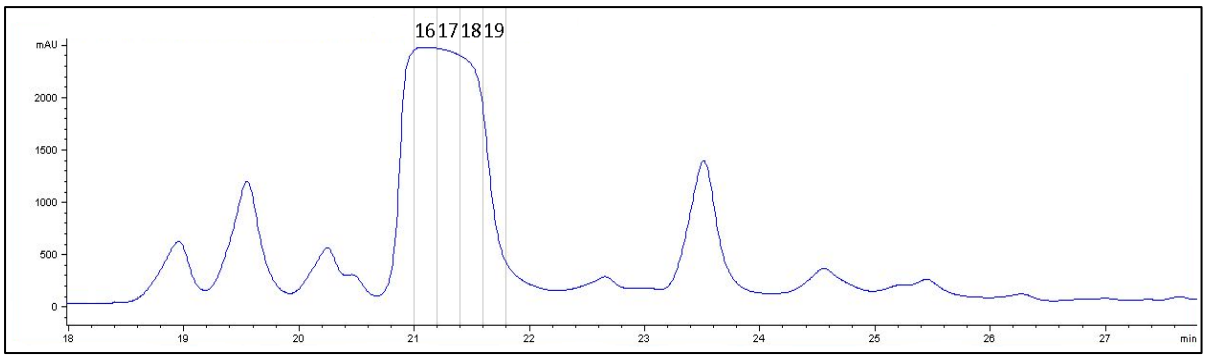


Figure 5.8 Chromatogram representing 1 cycle of semi preparative purification of serrulat-14-en-3,7,8,20-tetraol. Elution of subfractions 16,17,18 and 19 indicated by vertical lines. (Gradient: 20 min, 30-60% ACN-H_2O; 20-25 min, 60-95% ACN-H_2O; Detection: mAU at λ210 nm).

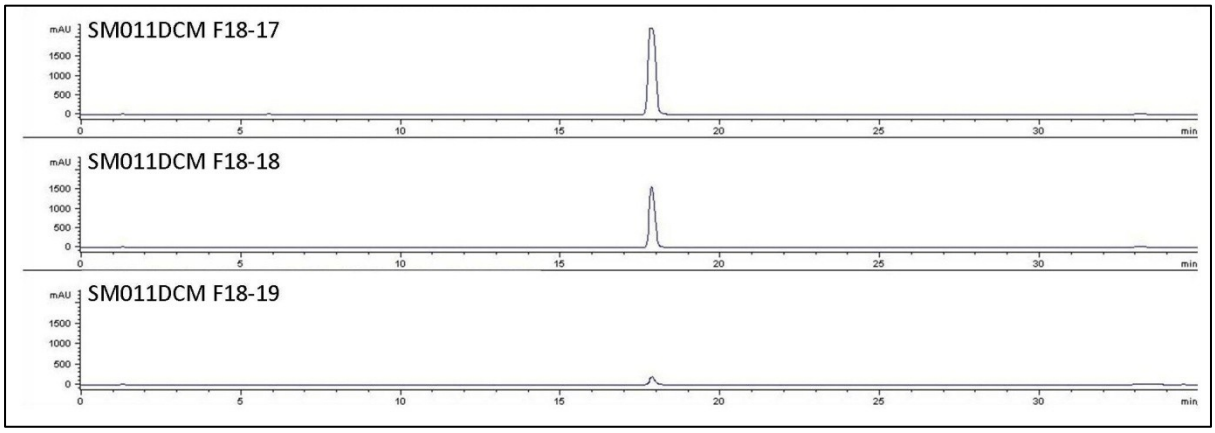


Figure 5.9 HPLC chromatograms of semi-preparative sub-fractions 18-16 to 18-19. (Gradient: 25 min 10-95% ACN-H_2O; Detection: mAU at λ210 nm).

5.2.3 Quantitative assay of serrulat-14-en-3,7,8,20-tetraol in traditional aqueous preparations of *E. duttonii* by HPLC/LC-MS

For quantitative assays of serrulat-14-en-3,7,8,20-tetraol (**2**) by HPLC, a 25 min gradient of 10-95% ACN-H_2O with 0.005% TFA was used. This gradient was found to result in good separation of **2** (Rt: 17.94 min) from other compounds present in the aqueous preparations (Figure 5.10). Sample concentration of 20 mg· mL^{-1} with 5 μL injection volume, was found to produce peaks at Rt 17.9 min, with areas within the range of those of the calibration curve. Calibration of detector response for standards and assay in samples by peak area was performed automatically using HP Chem software integration functions (Rev. A, v09.03). The calibration curve and regression equation used to derive concentration of **2** in the aqueous preparations is given in Appendix III.

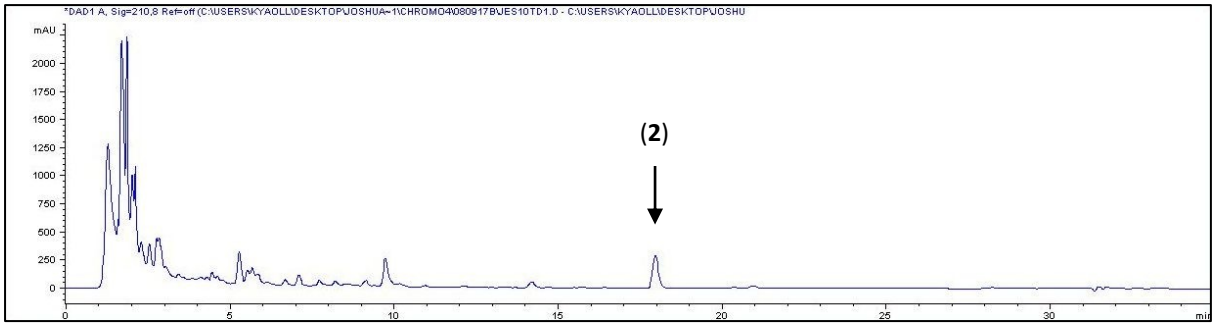
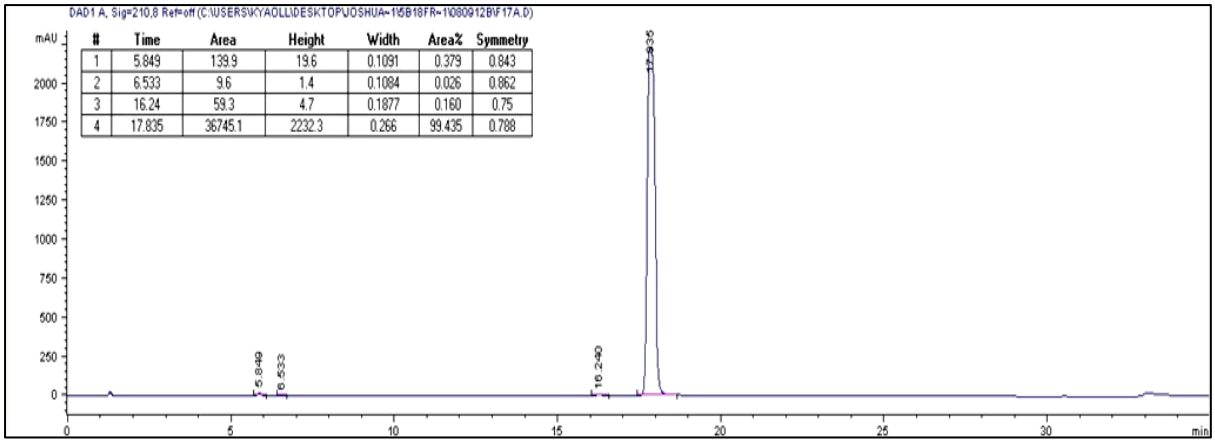


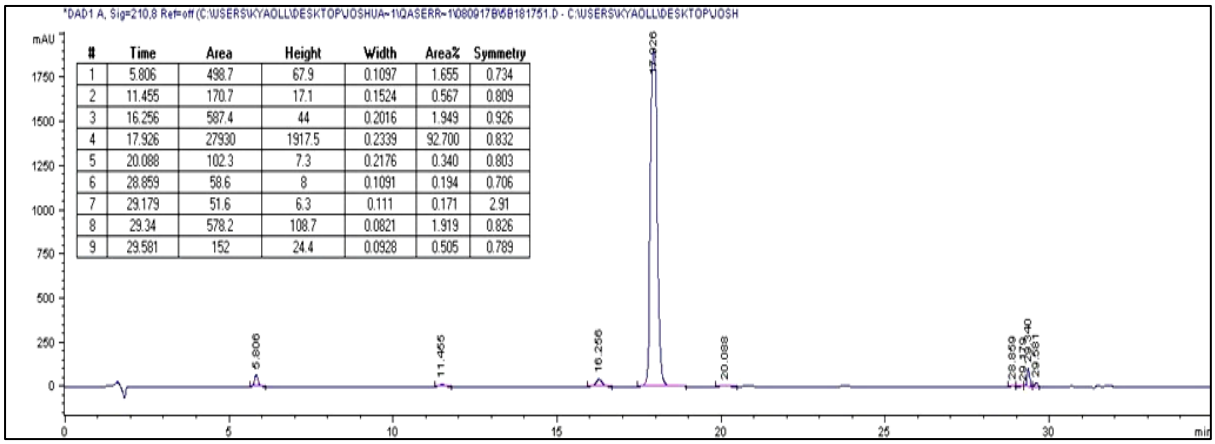
Figure 5.10 Separation of *E. duttonii* aqueous preparation (extract SM010TD) with 25 min 10-95% ACN-H_2O gradient employed for quantitative assays. Arrow indicates elution of serrulatane tetraol (**2**)

Before performing assays for antimicrobial activity, the samples of the purified serrulatane tetraol (**2**) (F-18-17) which had been dissolved in MeOH for quantitative assays were pooled and dried under a stream of nitrogen in order to determine mass of the compound remaining. When the samples were dried, however, the fraction seemed to lose the appearance of a colourless solid and reverted to immobile orange oil, of similar appearance to the partially purified fractions collected originally from the silica gel column. Although separations of the purified standard had appeared superficially to be composed only of the tetraol (**2**), when separations representing the highest concentration of **2** (1.184 mg · mL^{-1}) were examined closely, a number of small peaks which were not present in separations carried out immediately after the compound was isolated had appeared (Figure 5.11, 5.12). It was unlikely that these peaks had resulted from contamination of samples subsequent to isolation, as peaks with similar Rt appeared to be present in the DCM extract and the partially purified fractions. One of the peaks (present at Rt 16.25 min in quantitative separations and ca. Rt 19.5 min in semi-preparative separations) exhibited absorbance maxima at λ445 nm, and was likely to have been responsible for the orange colour of the partially purified fractions and the sample post storage, as this was the only peak found in separations of F-18 exhibiting significant absorbance over visible wavelengths. Additionally, the semi-preparative fractions where this compound eluted between Rt 19.2 and 19.6 also appeared to be orange coloured on elution and exhibited the same UV/visible absorbance spectra).



#	Time	Area	Height	Width	Area%	Symmetry
1	5.849	139.9	19.6	0.1091	0.379	0.843
2	6.533	9.6	1.4	0.1084	0.026	0.862
3	16.24	59.3	4.7	0.1877	0.160	0.75
4	17.835	36745.1	2232.3	0.266	99.435	0.788

Figure 5.11 Assay for purity of serrulatane tetraol in fraction 17-18 performed immediately following purification by semi-preparative HPLC (table inset shows peak area at λ210 nm)



#	Time	Area	Height	Width	Area%	Symmetry
1	5.806	498.7	67.9	0.1097	1.655	0.734
2	11.455	170.7	17.1	0.1524	0.567	0.809
3	16.256	587.4	44	0.2016	1.949	0.926
4	17.926	27930	1917.5	0.2339	92.700	0.832
5	20.088	102.3	7.3	0.2176	0.340	0.803
6	28.859	58.6	8	0.1091	0.194	0.706
7	29.179	51.6	6.3	0.111	0.171	2.91
8	29.34	578.2	108.7	0.0821	1.919	0.826
9	29.581	152	24.4	0.0928	0.505	0.789

Figure 5.12 Assay for purity of serrulatane tetraol in fraction 17-18 (sample used as standard during quantitative assays) performed five days following purification by semi-preparative HPLC (note impurities appear to be present in greater quantity despite lower quantity injected compared to separation shown in Figure 5.11)

Given that compound **2** seemed unstable and had apparently partly decomposed by the time the quantitative assays were performed, an accurate determination of quantity in the traditional preparations was not obtained. Estimations were made instead based on assay of purity by peak area at λ210 nm of the standards at the time of the determination. This estimation is unlikely to be accurate, however, as while all compounds detected in the standard absorbed strongly in the far UV part of the spectrum, the magnitude of detector response for each was unknown. The revised estimations of quantity were obtained by multiplying the original values by the ratio of percent purity determined from the 3 injections of the most concentrated standard solution (i.e. 92.45% ±0.05).

Table 5.1 Quantitative assay of serulat-14-en-3,7,8,20-tetraol (2) in traditional aqueous decoctions of *E. duttonii* leaves (mean and SE derived from two separate determinations).

Extract	Extract concentration ($mg \cdot mL^{-1}$)	Serrulat-14-en-3,7,8,20-tetraol concentration ($\mu g \cdot mL^{-1}$)	(μM)	(% extract dry weight)
E. duttonii aqueous leaf decoction a (extract SM010TD)	9.74	68.4 ± 0.18	214.9 ± 0.55	0.70%
E. duttonii aqueous leaf decoction b (extract SM011TD)	6.22	39.7 ± 0.04	124.5 ± 0.12	0.64%

Quantity of the serrulatane tetraol (**2**) in the traditional preparations determined using this method ranged from 39.7 ± 0.04 $\mu g \cdot ml^{-1}$ (extract SM011TD) to 68.4 ± 0.19 $\mu g \cdot ml^{-1}$ (extract SM010TD) (Table 5.1). The difference in the quantity of **2** seen between the two extracts could probably be attributed mainly to difference in overall concentration of each extract as when expressed as percentage of extract dry weight, quantity was similar for both extracts (extract SM010TD: 0.70 %; Extract SM011TD: 0.64 %). The mean quantity of the serrulatane tetraol (**2**) derived from these determinations was 54 $\mu g \cdot ml^{-1}$ (168 μM or 0.67 % of extract dry weights).

5.2.4 **Antimicrobial activity**

MIC and MBC were determined for samples of F-9 (**1**) and F-18-17 (2). Values determined for F-18-17 containing the serrulatane tetraol (**2**) against the three Gram-positive organisms ranged from 60-120 $\mu g \cdot ml^{-1}$ while MBC ranged from 120-480 $\mu g \cdot ml^{-1}$. MIC and MBC values found for the fraction containing the triol (**1**) were substantially lower (MIC: 8-15 $\mu g \cdot ml^{-1}$; MBC: 8-30 $\mu g \cdot ml^{-1}$). *Bacillus subtilis* (ACM 2268) was found to be the most susceptible organism and the MIC values obtained against this organism were equal to MBC. The aqueous traditional preparations were assayed alongside the fractions containing the serrulatanes, and the estimated concentration of the tetraol (**2**) was calculated for each of the dilutions tested.

Table 5.2 Minimum inhibitory and minimum bactericidal concentrations against Gram-positive bacteria for fractions containing serrulatane diterpenes (1) and (2) and aqueous preparations with concentration of (2) at inhibitory/bactericidal dilutions of traditional preparations (MBC shown in parentheses)

Extract/Compound	MIC (MBC) (μg·mL^{-1})					
	Staphylococcus aureus **(ATCC 29213)**		***Staphylococcus epidermidis*** **(ATCC 12228)**		***Bacillus subtilis*** **(ACM 2268)**	
E. duttonii leaf decoction (SM010TD)	4000	(8000)	2000-4000	(8000)	1000-2000	(2000-4000)
E. duttonii leaf decoction (SM011TD)	4000	(8000)	2000-4000	(8000)	2000-4000	(2000-4000)
E. duttonii DCM-F9 (Serrulat-14-en-7,8,20-triol)	8-15	(15-30)	8-15	(15-30)	8	(8)
E. duttonii DCM-F18-17 (Serrulat-14-en-3,7,8,20-tetraol)	60	(120-480)	60	(240)	120	(120)
Concentration of serrulat-14-en-3,7,8,20-tetraol *in E. duttonii* leaf decoction (SM010TD)	28	(56)	14-28	(56)	7-14	(14-28)
Concentration of serrulat-14-en-3,7,8,20-tetraol *in E. duttonii* leaf decoction (SM011TD)	26	(51)	13-26	(51)	13-26	(13-26)

(): Denotes minimum bactericidal concentration (MBC)

MIC recorded for the two aqueous preparations assayed ranged from 1-4 mg · ml^{-1}, which corresponded to a concentration of the serrulatane tetraol (**2**) of around 8-30 μg · ml^{-1}. MBC for aqueous preparations ranged from 2-8 mg · ml^{-1} (corresponding to a serrulatane tetraol (**2**) concentration of around 15-30 μg · ml^{-1}). MIC and MBC values found for each fraction and the aqueous preparations, along with the estimated quantity of **2** at the point MIC/MBC was found in each aqueous preparation, are summarised above in Table 5.2.

5.2.4.1 Cytotoxicity, PGE_2 inhibitory activity and antioxidant capacity

Antioxidant capacity of the partially purified fractions containing the serrulatanes (**1**) and (**2**) was determined using hydrophilic and lipophilic ORAC assays. ORAC values are reported as μmol TE/g sample ± SE. ORAC values generated by the hydrophilic ORAC assay for F-9 (**1**) and F-18 (**2**) were 1842 ± 270 and 5325 ± 145 μmol TE/g respectively. ORAC values generated by the lipophilic ORAC assay were notably higher for both samples (F-9 (**1**): 10919 ± 854 μmol TE/g; F-18 (**2**): 13611 ± 989 μmol TE/g). When assayed with the

hydrophilic ORAC assay, F-18 containing the serrulatane tetraol (**2**) was found to exhibit significantly greater antioxidant activity (ca. 3-fold increase) when compared to F-9 containing the triol (p <0.001). In contrast, no significant difference was found between ORAC values generated for each compound when assayed using the lipophilic ORAC assay (p >0.05).

Cytotoxicity of fractions containing the serrulatanes **1** and **2** against murine P388D1 lymphoblasts and human hepatocarcinoma HepG2 cell lines was assessed using the ATPlite assay. Results were reported as $\mu g \cdot ml^{-1}$ IC_{50} (concentration resulting in 50% reduction of culture viability) ± SE. IC_{50} for F-9 (**1)** against P388D1 cells was 7.7 ± 1.55 $\mu g \cdot ml^{-1}$ and 13.7 ± 0.47 $\mu g \cdot ml^{-1}$ against the HepG2 cell line. F-18 (**2)** was found to exhibit slightly lower levels of cytotoxicity against both cell lines tested (P388D1 IC_{50}: 11.2 ± 1.69 $\mu g \cdot ml^{-1;}$ HepG2 IC_{50}: 17.2 ± 1.46 $\mu g \cdot ml^{-1}$). Dose response curves for F-9, F-18 and the reference drug curcumin against each cell line tested, are illustrated below in Figure 5.13.

The partially purified fractions were tested for the ability to inhibit formation of the inflammatory mediator PGE_2 in calcium ionophore stimulated 3T3 murine fibroblasts. F-9 and F-18 were tested at assay concentrations of 0.5 and 5.0 $\mu g \cdot ml^{-1}$ following methods outlined in Chapter 2 (2.7.1.1). PGE_2 in cultures incubated in presence of F-9 and F-18, was measured using a competitive enzyme immunoassay and percent inhibition determined relative to controls containing only media and solvent. Results were presented as mean percentage inhibition ± SE. While both fractions were seen to produce apparent inhibition of PGE_2 relative to controls, significance was not reached (p >0.05). Furthermore no significant increase or decrease in measured activity could be discerned between the two concentrations assayed. Results expressed as mean ± SE for the partially purified fractions containing

compounds **1** and **2** in assays of cytotoxicity, lipophilic and hydrophilic ORAC and PGE_2 inhibition are summarised below in Table 5.3.

Table 5.3 ORAC values (μmol/TE/g), PGE_2 Inhibition (% inhibition at two concentrations) and P388D1/HepG2 cytotoxicity IC_{50} values ($\mu g \cdot mL^{-1}$) determined for fractions containing serrulatane diterpenes 1 and 2

Activity/Assay (units)	*E. duttonii* DCM-F-9 (Serrulat-14-en-,7,8,20-triol) (1)	*E. duttonii* DCM-F-18 (Serrulat-14-en-3,7,8,20-tetraol) (2)
$ORAC_H$ (μmol TE/g ± SE)	1842 ±270	5325 ±145
$ORAC_L$ (μmol TE/g ± SE)	10919 ±854	13611 ±989
PGE_2 Inhibition 0.5 $\mu g \cdot mL^{-1}$ (% ± SE)	29 ±6.9	28 ±7.5
PGE_2 Inhibition 5.0 $\mu g \cdot mL^{-1}$ (% ± SE)	29 ±6.6	36 ±4.6
P388D1 Cytotoxicity (IC_{50} $\mu g \cdot mL^{-1}$ ± SE)	7.7 ±1.55	11.2 ±1.69
HepG2 Cytotoxicity (IC_{50} $\mu g \cdot mL^{-1}$ ± SE)	13.7 ±0.47	17.2 ±1.46

$ORAC_H$: Hydrophilic ORAC assay; $ORAC_L$: Lipophilic ORAC assay

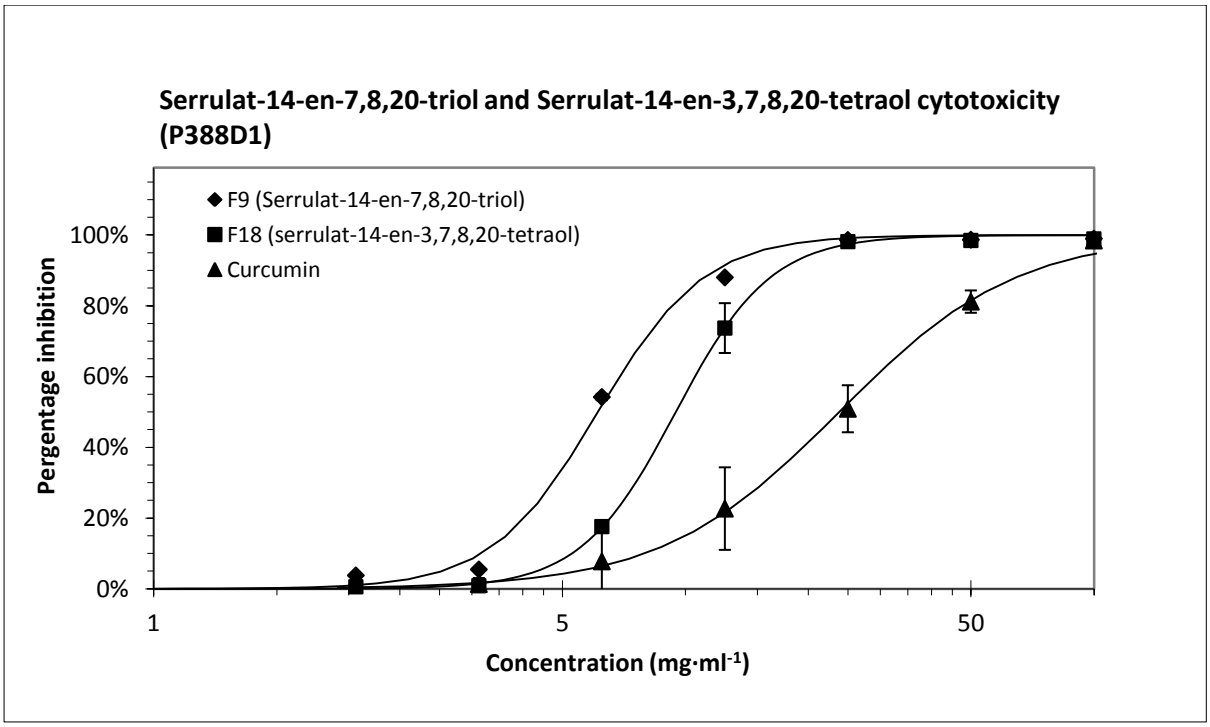


Figure 5.13 Cytotoxicity dose response curves for fractions containing serrulatane triol (1) and tetraol (2) with curcumin (as reference drug) against murine P388D1 cell line

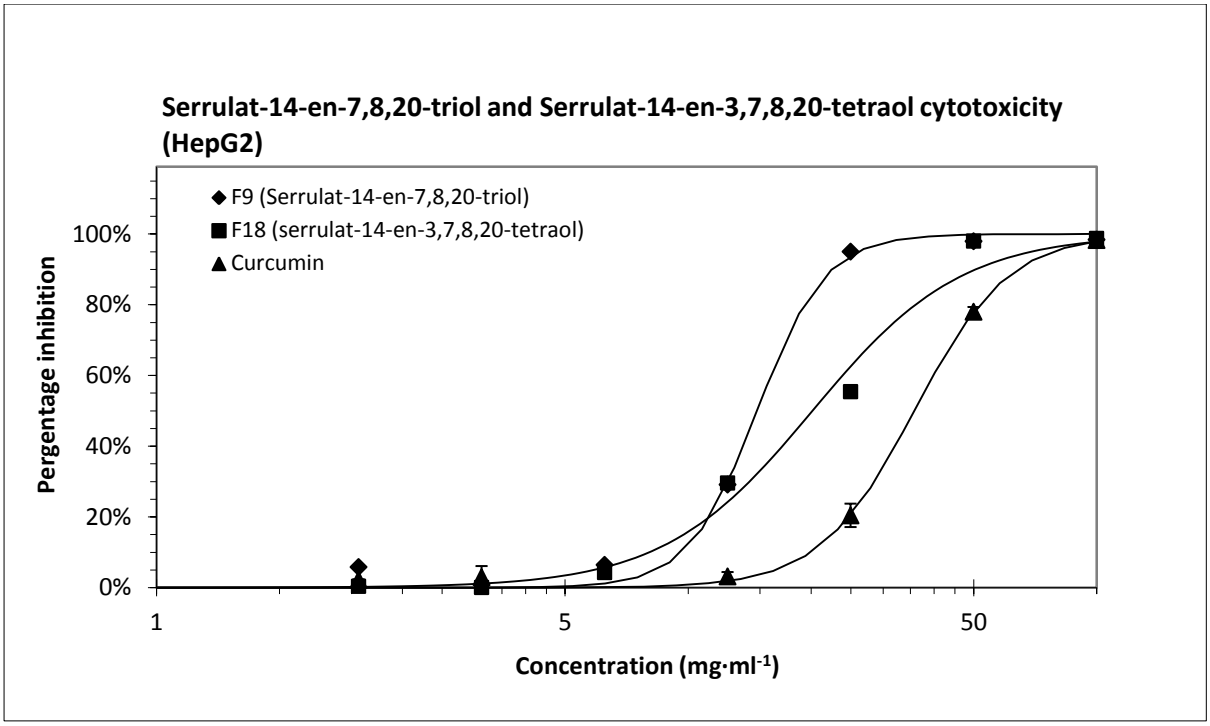


Figure 5.14 Cytotoxicity dose response curves for fractions containingserrulatane triol (1) and tetraol (2) with curcumin (as reference drug) against human HepG2 cell line

5.3 Discussion and conclusions

5.3.1 Antimicrobial activity of serrulatane diterpenes

Antibacterial activity exhibited by the partially purified fractions containing the serrulatane diterpenes (**1**) and (**2**), was comparable to that previously reported for serrulatanes and serrulatic acids isolated from other *Eremophila* species. Ndi, Semple *et al.* (2007b; 2007c) reported values for broth microdilution MIC and MBC against *S. aureus* (ATCC 29213), ranging from 15.7 - 250 $\mu g \cdot ml^{-1}$ for serrulatic acids, and MIC/MBC of 25.8 μM (8 $\mu g \cdot ml^{-1}$) for 3,8-dihydroxyserrulat-14-ene isolated from *E. neglecta* and *E. serrulata*. Liu (2006a) reported MIC of serrulatic acids isolated from *E. sturtii* against *S. aureus* (ATCC 9144) ranging from 15 - 200 $\mu g \cdot ml^{-1}$ and MBC ranging from 25->1000 $\mu g \cdot ml^{-1}$ (fluorescein diacetate microdilution technique). The MIC and MBC values reported for other serrulatanes and obtained using similar techniques and Staphylococcal strains are listed below in Table 5.4.

Table 5.4 Summary of reported MIC/MBC values against *Staphylococcus aureus* for serrulatanes and napthoquinones isolated from *Eremophila* species

Compound	MIC ($\mu g \cdot mL^{-1}$)	MBC ($\mu g \cdot mL^{-1}$)	Organism	Reference
8,19-Dihydroxyserrulat-14-ene*	8	8	*S. aureus* (ATCC 29213)	Ndi *et al.* (2007b)*
Biflorin*	8	64	*S. aureus* (ATCC 29213)	Ndi *et al.* (2007b)*
7,8,20-Trihydroxyserrulat-14-ene‡	12	23	*S. aureus* (ATCC 29213)	This Report†
Serrulat-14-en-19-oic acid	15	15	*S. aureus* (ATCC 9144)	Liu (2006a)
9-methyl-3-(4-methyl-3-pentenyl)-2,3-dihydronaptho[1,8-*bc*]pyran-7,8-dione	15.6	125	*S. aureus* (ATCC 29213)	Ndi *et al.* (2007c)
8-Hydroxyserrulat-14-en-19-oic acid*	16	16	*S. aureus* (ATCC 29213)	Ndi *et al.* (2007b)*
3,7,8,20-Tetrahydroxyserrulat-14-ene‡	60	300	*S. aureus* (ATCC 29213)	This Report†
20-Acetoxy-8-hydroxyserrulat-14-en-19-oic acid	125	n.r	*S. aureus* (ATCC 29213)	Ndi *et al.* (2007c)
3,8-Hydroxyserrulat-14-en-19-oic acid	200	200	*S. aureus* (ATCC 9144)	Liu (2006a)
8,20-Dihydroxyserrulat-14-en-19-oic acid	250	n.r	*S. aureus* (ATCC 29213)	Ndi *et al.* (2007c)
8,20-Diecetoxyserrulat-14-en-19-oic acid	250	n.r	*S. aureus* (ATCC 29213)	Ndi *et al.* (2007c)

*: Values converted from concentrations reported as μM MIC/MBC; †: Mean of MIC/MBC ranged values reported in this study and determined using partially purified samples; n.r.: Values not reported

The serrulatic acids described by both Ndi *et al.* (2007b) and Liu (2006a), were in most cases reported to exhibit antibacterial MIC equalling MBC. This was not seen to be the case in this study where MBC was consistently measured at higher concentrations than corresponding MIC (i.e. typically by 1-3 two-fold dilution steps in assays, representing a 2-8 fold increase in concentrations required for bactericidal activity). MIC values estimated for fractions containing compounds **1** and **2** were also significantly lower than those reported previously (i.e. by a factor representing ca. 3-4 two-fold dilution steps). Previously, MIC of the serrulatane triol (**1**) was measured at 94 $\mu g \cdot ml^{-1}$, while the serrulatane tetraol (**2**) was found to be active to 375-750 $\mu g \cdot ml^{-1}$ (Smith *et al.*, 2007). These values were closer to the MBC values found in the present study for each compound. While it was acknowledged that the compounds were assayed as partially purified fractions, in each instance compounds **1** and **2** formed the majority of F-9 and F-18 and the same was the case with the material described previously, where examination of NMR spectra had indicated that **1** and 2 were present at quantities of around 90% in the material examined. It is possible that this discrepancy in MIC

values reflects the difference in methodologies used in each instance. The method used in earlier studies was similar except that a substantially larger inoculation density was used (ca. 5×10^7 CFU/mL) than that of the modified NCCLS microdilution technique employed for the experiments described in this study (ca. 5×10^5 CFU/mL) and wells contained a larger proportion of DMSO (up to 5% $^{v}/_{v}$). Inoculation density is known to affect experimental MIC values (NCCLS, 1997b) and it is likely that the greater number and density of bacteria in the test wells of the earlier experiments resulted in the higher MIC values being recorded in these tests.

The antimicrobial activity exhibited by terpenoids is generally thought to involve actions at microbial phospholipid membranes that result in an increase in disorder and destabilisation, leading to increased permeability, ion leakage and disruption of membrane associated energy transfer systems (Cox *et al.*, 2000; Cristani *et al.*, 2007). Lipophilic terpenoids have been shown to readily partition from aqueous solution into the lipid fraction of membrane bilayers and may act to disrupt membrane integrity by acting as impurities, intercalating between the flexible acyl chains of membrane lipids (Trombetta *et al.*, 2005; Cristani *et al.*, 2007). Antimicrobial activity among monoterpenoids has been found to be associated with increased oxygenation and the presence of some degree of water solubility (Griffin *et al.*, 1999) and the lack of activity generally seen among the relatively hydrophobic monoterpene hydrocarbons, has been attributed to their inability to traverse the layer of water associated with the hydrophillic outer surface of membrane phospholipid bilayers (Griffin *et al.*, 1999; Trombetta *et al.*, 2005; Cristani *et al.*, 2007). Griffin *et al.* (1999), have additionally shown that activity among monoterpenoids against Gram-negative organisms can be associated with low molecular size and surface area (e.g. as with acyclic monoterpenoids such as citral), and have suggested that this may result from the exclusion of larger terpenoid molecules by porin channels of the outer membrane characteristic of this group of microorganisms. Selective

activity among these compounds may also be affected by factors such as the lipid composition, and net surface charge of microbial membranes (Trombetta *et al.*, 2005; Cristani *et al.*, 2007).

It is possible that disruption of bacterial cell membrane integrity is involved in the antimicrobial activity exhibited by the serrulatane diterpenes as this mode of activity has been demonstrated in EtOH extracts of *E. duttonii* (Tomlinson and Palombo, 2005). If this is the case, then the lack of antibacterial activity against Gram-negative organisms among the serrulatanes may be partly explained by their relatively large size and bicarboyclic structure, given that variability in inhibition of Gram-negative bacteria has been reported among monoterpenoids of lower molecular weight and surface area (Griffin *et al.*, 1999). As was the case in earlier work where antibacterial activity in these compounds was examined, the fraction containing the serrulatane triol (**1**) was found to exhibit significantly greater antibacterial potency than that containing the 3'-hydroxylated tetraol (**2**). Liu (2006a) also noted significant reductions in relative antibacterial potency with increased hydroxylation in the two antibacterial serrulatic acids isolated from *E. sturtii*. On the basis of these results, it would appear that an increase in lipophilicity in these compounds may potentially be associated with greater antibacterial potency.

Cristani *et al.* (2007) have suggested that the amphipathic character of some terpenoids may explain their ability to intercalate with membrane lipids (i.e. with polar regions interacting with the hydrophilic heads of membrane phospholipids and hydrophobic regions with the acyl chains). Some amphipathic character may be inferred from the structure of the serrulatane diterpenes, where polar substituents are typically found in the ring system, with a non-polar hydrocarbon moiety comprising carbons 11-18 (carbons 10-15 in o-napthoquinones such as biflorin, containing an oxygen heterocyclic ring).

5.3.2 **Antibacterial activity of *E. duttonii* traditional aqueous preparations and serrulatane content**

Attempts to assess biological activity in the serrulatane tetraol (**2**), and to determine quantity in the aqueous preparations, were complicated by the apparent instability of this compound, which meant that these estimations could only be reported tentatively, given that the exact composition of the purified sample was uncertain at the time of the assays. Additionally, despite efforts to ensure preservation of the aqueous medicinal preparations (e.g. with lyophilisation, storage at low temperature etc.) it is possible that the concentration of **2** in these extracts may not have been representative of 'typical' freshly prepared extracts given the apparent instability of this compound. For these reasons it is difficult to draw firm conclusions on the possible role of this compound in the traditional aqueous preparations. If the MIC and MBC values obtained for the aqueous decoctions and sample of serrulatane tetraol (**2**) examined in this study are accepted, then it would appear that this compound may contribute only partly to the activity exhibited by the aqueous preparations. The estimated concentration of serrulatane tetraol (**2**) in the two aqueous preparations assayed was 40 $\mu g \cdot ml^{-1}$ (extract SM011TD) and 68 $\mu g \cdot ml^{-1}$ (extract SM010TD). MIC for the sample of serrulatane tetraol (**2**) against the Staphylococci was determined at 60 $\mu g \cdot ml^{-1}$, with MBC of 120-480 $\mu g \cdot ml^{-1}$, and MIC 120 $\mu g \cdot ml^{-1}$, with MBC of 120 $\mu g \cdot ml^{-1}$ against *B. subtilis*. Based on these results, it could be concluded that the serrulatane tetraol (**2**) was present at a concentration sufficient for inhibition of the Staphylococcal strains tested in only one of the two extracts assayed (SM011TD), but not for bactericidal activity or for any type of activity against *B. subtilis*. It is possible, however, that the concentration of serrulatane in the other extract assayed (SM011TD, containing 39 $\mu g \cdot ml^{-1}$ of compound **2**) may have been sufficient for inhibitory activity given that the actual MIC value in the 2-fold serial dilutions used in the assays, could be assumed to fall at some point between 60 and 30 $\mu g \cdot ml^{-1}$.

Additionally, the MIC and MBC recorded for the aqueous preparations seemed to fall below that which could be attributed solely to the activity observed in the sample of serrulatane tetraol (**2**) by 1-2 dilution steps, particularly in relation to the concentrations of **2** required for bactericidal activity. The most likely explanation for this would be the presence of other compounds with antimicrobial activity which may have exerted an additive contribution to the activity observed in the aqueous decoctions. The preparations were seen to contain around 30-35 detectable constituents on separation with analytical HPLC, 6-7 of which were clearly at concentrations in excess of compound **2** (Appendix III). In earlier work (Smith *et al.*, 2007), bioautography TLC separations of EtOH and other organic solvent extracts of this species had indicated that at least another 4 (unidentified) compounds with detectable antibacterial activity were present apart from the three identified, and this observation is supported with similar results reported by Owen and Palombo (2007) where bioautography of an EtOH extract of leaves was described. All of these compounds, however, were seen to migrate further than the tetraol (**2**) in normal phase TLC (Smith *et al.*, 2007), and so are presumably more lipophilic than this compound, which was extracted in relatively low quantities in water. It is therefore unlikely that any of these particular compounds were present in the aqueous preparations examined in this study. Interactions between separate components of essential oils involving effects on aqueous solubility, have been shown to affect antimicrobial activity exhibited by individual terpenoids (Cox *et al.*, 2001) and it is also possible that the antibacterial activity of the serrulatane tetraol (**2**) may have been enhanced or potentiated by the presence of other constituents in the aqueous preparations and/or their effect on the chemical environment of the assays.

Compounds **1** and **2** have been found in relatively high quantities in the viscid leaf cuticle of *E. duttonii* (Tippett and Massy-Westropp, 1993) and were seen as the most abundant compounds in the hexane and DCM extracts of leaves examined in this study (*cf*. Figure 5.7).

Given the relative abundance of these compounds in the leaves of *E. duttonii*, it is possible that the concentrations of **2** found in the aqueous decoctions of this material may be grossly representative of the limit of aqueous solubility of this compound. While aqueous solubility of the tetraol (**2**) was not determined directly, predicted LogP values (octanol-water partition coefficient) obtained for this structure using the ALOGPS v2.1 software (48.2 - 67.4 $\mu g \cdot ml^{-1}$) (Tetko *et al.*, 2001) were broadly in agreement with experimental measurements in the leaf decoctions (40 – 68 $\mu g \cdot ml^{-1}$). The difference in the concentration of **2** between the two extracts examined, however, also seemed to be dependent on extract yield, with the greater quantity found with an increase in total extract yield. This result would suggest that other extractable compounds, may have facilitated the extraction and solubility of **2** [e.g. possibly saponins or other compounds with amphipathic character, *cf.* Farnsworth, cited in Balick, (1994)]**.** It is therefore possible, that aqueous decoctions prepared with higher yielding material, or using a greater ratio of plant material to solvent (water) may contain higher concentrations of the tetraol **2** than were indicated by the results obtained for preparations examined in this study.

5.3.3 **Antioxidant, cytotoxic and PGE_2 inhibitory activities**

The partially purified fractions containing **1** and **2** were found to exhibit high levels of antioxidant capacity using the lipophilic ORAC ($ORAC_L$) assays. $ORAC_L$ values for F-9 (**1**) and F-18 (**2**) were around 3 times the potency of the reference compound Trolox on a gram for gram basis (assuming an ORAC value for Trolox, mw: 250.1, of around 4000 μmol TE/g). $ORAC_L$ values obtained for F-18, containing the serrulatane tetraol (**2**) were slightly higher than those found for F-9, containing the triol (**1**), however this difference was not significant (p >0.05 with ANOVA). Corresponding values obtained using the hydrophilic ORAC assays ($ORAC_H$) were substantially lower in comparison and a significant difference in antioxidant capacity was found between the two fractions (p <0.05 with ANOVA). The apparent

discrepancies seen in values obtained with the hydrophilic ORAC assay ($ORAC_H$) may have been representative of a lack of solubility of the serrulatanes in this assay system, particularly given that a significant difference in antioxidant capacity measured using this assay was found between fractions containing the triol and tetraol forms. If this was the case then is possible that the values obtained with the lipophilic ORAC assay may represent a more accurate indication of relative activity between the two compounds.

Fractions containing the serrulatanes were found to exhibit relatively high levels of cytotoxicity against P388D1 and HepG2 cell lines, in both cases exceeding the IC_{50} of the reference drugs chlorambucil (P388D1: 361.0 ± 13.13 $\mu g \cdot ml^{-1}$) and curcumin (P388D1: 26.8 ± 3.03 $\mu g \cdot ml^{-1}$; HepG2: 39.2 ± 1.83 $\mu g \cdot ml^{-1}$). The presence of a cytotoxic compound in a preparation used traditionally for the topical treatment of wounds seems counterintuitive, particularly given that the quantities of **2** found in the simulated traditional preparation (ca. 60 $\mu g \cdot ml^{-1}$) exceeded, or were close to that seen to result in total loss of culture viability in these assays (ca. 15 - 60 $\mu g \cdot ml^{-1}$). On the other hand, as discussed previously in Chapter 4 (4.3), *in vitro* cell cultures tend to be more susceptible to cytotoxic actions than cells *in vivo* (Sullivan *et al.*, 2001) and these values are probably unlikely to be representative of significant *in vivo* toxicity.

Fractions 9 (**1**) and 18 (**2**) were also tested for PGE_2 inhibitory activity as significant inhibitory activity was detected in a sample of the traditional decoction (Chapter 4, Section 4.2.8). Additionally, serrulatic acids isolated from *E. sturtii* have been shown to inhibit the cyclooxygenases, COX-1 and COX-2 (Liu *et al.*, 2006b). Inhibition of COX-1 and COX-2 activity may be expected to result in a lowered rate of PGE_2 production, and could possibly be detected in the assays used in this study as a reduction in PGE_2 levels produced by ionophore stimulated and unstimulated fibroblasts. While some measurable inhibition was

found for both F-9 (**1**) and F-18 (**2**), results did not reach significance and dose dependent effects could not be established. As with the other PGE_2 screening assays described, only one experiment was performed per sample due to constraints of time and assay cost, so it is difficult to draw a definite conclusion on the activity of the compounds in this respect. It is possible that the serrulatanes **1** and **2**, assayed here in partially purified form and at sub-cytotoxic concentrations (0.5 and 5.0 $\mu g \cdot mL^{-1}$), may possess COX-1/COX-2 inhibitory activity on par with that reported by Liu *et al.* (2006b), where the serrulatic acids were assayed at a significantly higher concentration ($1 mg \cdot mL^{-1}$) using an COX inhibition assay.

Activity against Gram-positive bacteria, including those examined here, is consistent with many of the traditional uses of preparations derived from *E. duttonii*. Disease states and symptoms which preparations of the plant are employed to treat (e.g. minor wounds, sore throat, pain and inflammation of eyes and ears) may be associated with infection by a number of Gram-positive species (e.g. *S. aureus* and *S. epidermidis* in infection of dermal wounds and *S. aureus* and *Streptococcus pneumoniae* in pharyngitis, conjunctivitis and otitis). Antibacterial activity exhibited by the aqueous decoctions examined in this study, however, was relatively moderate in comparison to that seen in some of the other preparations and inhibitory activity was not found to persist at dilutions significantly below those representing the concentration of decoctions prepared following methods described in the ethnobotanical literature. If it is assumed that the *in vitro* antibacterial activity exhibited by the decoctions can be extrapolated to *in vivo* medicinal use, and that the extracts examined in this study are typical of those produced by indigenous people more directly knowledgeable in the usual methods of preparation, then it is possible that antibacterial activity may not play a significant role in the traditional use of these preparations to treat the conditions cited above. In any case, the absence of antibacterial activity does not preclude the possibility of other activities which may be beneficial in the treatment of the conditions for which these preparations are used. As

was discussed in Chapters 3 and 4, it is possible that the practice of preparing unguents or ointments by mixing macerated plant material with animal fat was more widespread and common in the past than may be inferred from more recent Australian ethnobotanical reports. Such preparations made using the leaves of *E. duttonii* may be expected to contain greater quantities of the antibacterial serrulatane diterpenes, including the triol (**1**), and may possibly form a more effective vehicle for the delivery of any active constituents with topical application.

During the final stages of preparation of this thesis, work was published by Ndi *et al.* (2011) describing instability of the *E. duttonii* serrulatanes and attempts to isolate the coloured compounds. Ndi *et al.* (2011) described oxidation of the serrulatane diterpenes with formation of orange colour in collection tubes on elution of these compounds from sephadex after liquid-liquid partitioning and initial fractionation with normal phase vacuum chromatography from a diethyl ether extract of fresh leaves. In this study, however, the orange compounds were seen to be present as bands in the initial column chromatography from the beginning of the separation and until **2,** which was obtained as a colourless solid, was seen to apparently decompose, it was assumed that the orange compound represented an intensely coloured 'impurity' which had co-eluted with the tetraol (**2**) in the initial separations of the extract by column chromatography. It is possible that these coloured products had formed in the extracts used in this study before separation was attempted, or did so relatively rapidly on the silica gel surface during separation. In this study, when semi-preparative purification of **2** was attempted on the reverse phase column with ACN-H_2O, no apparent immediate formation of the orange product was noted as reported by Ndi *et al.* (2011), despite the collecting tubes remaining open and at room temperature for several hours as the fractionation proceeded. It is possible that the serrulatane may have been partially protected from oxidation by the solvents used in the separation (fraction collecting tubes were

relatively long and narrow) and that any formation of the oxidised orange product was not visible due to the small quantities isolated in this instance. Ndi *et al.* (2011) have suggested that the coloured products are likely to represent products of oxidation of the serrulatane diterpenes to quinonoid forms and have further suggested that the antibacterial mode of antimicrobial activity in these compounds may involve redox activity rather than via effects on membrane fluidity which have been associated with other antimicrobial terpenoids. In this study, the orange compounds exhibited absorption spectra with maxima at λ445 nm which would be consistent with their identity as quinonoids as suggested by Ndi *et al.* (2011). While these compounds were found to be present in relatively small quantities in the partially purified fractions as compared to the hydroxylated forms, it is not known whether further or continuous oxidation occurred when the samples were introduced into media or other assay solutions. The possibility of the serrulatanes forming quinonoid oxidation products may explain the high levels of cytotoxicity observed in fractions containing these compounds as some phenolic species are known to oxidise in the presence of metal ions in media containing foetal calf serum such as DMEM to quinones and/or semi-quinones, which may be capable of generating cytotoxic levels of H_2O_2 and $O_2^{\bullet-}$ (Halliwell, 2008). The same may be true of the behaviour of these compounds in microbial media and in other solvent environments of test systems which may contain trace quantities of metal ions or which may favour oxidation of the hydroxylated serrulatanes to a quinonoid form. This possibility is interesting from the standpoint of traditional medicinal use of this species to treat wounds, as the potential ability of phenolic compounds to act in this way was the mechanism suggested by Khanna *et al.* (2002) to explain apparently beneficial increases seen in H_2O_2 concentration in experimental wounds treated with a tannin rich grape seed extract (*cf.* Chapter 4).

Varying levels of antibacterial potency have been reported among the serrulatane diterpenes examined in this and other studies. Given the terpenoid structure of these compounds and

reports of bacteriolytic action reported for extracts of *E. duttonii*, it is possible that the serrulatanes act at least partly through destabilisation and disruption of bacterial phospholipid membranes. Several serrulatanes have been shown to exhibit antibacterial potency comparable to that of other available pharmaceutical antimicrobial agents (i.e. MIC <10 μg mL^{-1}). Given the potential application for these compounds as components of antibacterial surface coatings in medical devices as demonstrated by Griesser *et al.* (2008) and Vasilev *et al.* (2009), and the structural diversity that exists among those identified so far (*cf.* Ghisalberti, 1993), further studies to elucidate structure-activity relationships in the serrulatanes are warranted.

Chapter 6: Novel essential oil chemotypes of *Eremophila longifolia*

In this chapter, variability in composition of essential oils from the Australian indigenous medicinal plant Eremophila longifolia is described and three novel essential oil chemotypes are proposed. Results for screening of E. longifolia essential oil samples for anti-microbial activities are also presented and discussed

6.1 Introduction and background

Investigation into the variability of chemical composition in *Eremophila longifolia* essential oils was initiated after noting distinct variations in the odour and tuberculate gland morphology of wild specimens during field collection of plant material in western New South Wales (NSW). In particular, specimens found in the Bynguano Range (BR) biogeographic region in the vicinity of Mutawintji National Park, were noted to exhibit numerous dense aggregations of prominent tuberculate oil glands on the leaf surfaces and stems. It was also observed that the leaves of these plants produced a strong ethereal peppermint-like odour which was not noted in other specimens occurring at localities further south and east of this region. The possibility of intraspecific chemotypic variation in this species was considered significant and warranted further investigation for a number of reasons. Firstly, *E. longifolia* is one of the more frequently cited species in the Australian ethnobotanic literature, where a wide variety of therapeutic applications has been described (e.g. Webb, 1969; O'Connell *et al.*, 1983; Low, 1990; Barr *et al.*, 1993; Latz, 1995). Secondly, there is anecdotal evidence to suggest *E. longifolia* is one of the more commonly and routinely used of the Australian native medicinal plants by contemporaneous indigenous Australian people and interest in the commercial production of 'cosmeceutical' products incorporating extracts from the plant has developed among some indigenous groups in recent years.

A previous report describing essential oil composition in this species had indicated that leaf essential oils contained large quantities of the phenylpropanoids safrole and methyleugenol (Della and Jefferies, 1961), both of which are hepatotoxic and suspected carcinogens (IARC, 1976; Miller *et al.*, 1983; Burkey *et al.*, 2000). As a result concerns have arisen over the potential toxicity of preparations derived from this species which are intended for internal use (Lassack and McCarthy, 1997). In this study, it was further observed that none of the specimens encountered during collection of plant material in western NSW were found to produce the characteristic aniseed or nutmeg like odours which may be associated with the presence of safrole and other phenylpropanoids. The aim of the work undertaken here was to examine and characterise any intraspecific variation in volatile oil composition which might be present among specimens collected in semi-arid, western regions of NSW, and to determine whether or not safrole or methyleugenol could be detected in *E. longifolia* populations in these regions.

6.1.1 **Outline of present study**

Aerial parts of ten *E. longifolia* specimens were collected from localities covering several biogeographic regional divisions of western NSW (Table 6.1). Biogeographic regions from which specimens were collected included Mootwingee Downs (MD), Broken Hill Complex (BHC), Murray Darling Depression, (MDD) and Cobar Peneplain (CP). The extent of the bioregions covered and specimen collection localities are illustrated below in Figure 6.1. Volatile oils were obtained by hydrodistillation and analysed using gas chromatography-mass spectrometry (GC-MS). Nuclear magnetic resonance spectroscopy (NMR) of neat oil samples was used to identify a major constituent (karahanaenone) found in two of the samples, which could not initially be identified using retention data or the GC-MS spectral library employed in the analyses. Multivariate statistical analysis of chemical composition determined for the

volatile oil samples enabled a tentative identification and classification of three novel chemotypes among the specimens examined.

Table 6.1 Collection localities of *E. longifolia* specimens in western NSW

Collection ID	Date	IBRA Biogeographic region	Geographic coordinates for collection localities	
			Latitude S, Longitude E (DMS)	Elevation (m)
JES019	28-Aug-03	Mootwingee Downs	31°16'33.0"S, 142°18'07.1"E	216
SM003	29-Nov-04	Mootwingee Downs	31°16'32.7"S, 142°18'07.7"E	275
SM004	29-Nov-04	Mootwingee Downs	31°16'45.1"S, 142°18'07.3"E	219
SM005	29-Nov-04	Mootwingee Downs	31°16'40.2"S, 142°18'07.4"E	209
SM012	30-Nov-04	Mootwingee Downs	31°17'30.0"S, 142°17'59.7"E	216
SM016	01-Dec-04	Broken Hill Complex	31°43'51.2"S, 142°30'24.9"E	187
SM017	01-Dec-04	Broken Hill Complex	31°43'31.1"S, 142°40'53.1"E	227
SM018	01-Dec-04	Murray-Darling Depression	31°41'47.7"S, 143°44'19.7"E	194
JES001	23-Feb-03	Murray-Darling Depression	32°06'06.2"S, 144°11'42.0"E	92
SM019	01-Dec-04	Cobar Peneplain	31°32'37.5"S, 145°17'42.3"E	199

6.1.2 **Description and medical ethnobotany**

The endemic Australian plant *E. longifolia* occurs as a medium woody shrub (1-3 m) throughout the arid and semi-arid regions of the continent. The genus *Eremophila* has been the object of phytochemical interest due to its unusual terpenoid chemistry and prevalence in the medical ethnobotanic literature of Australia (Ghisalberti, 1993; Ghisalberti, 1994; Richmond and Ghisalberti, 1994), where it is one of the most frequently mentioned of the medicinal genera, next to the prolific and widely radiated *Acacia*, *Eucalyptus*, and *Melaleuca*. As was demonstrated in Chapter 1 (1.3.2.1) using regression residual analyses, the *Eremophila* also appear to be selectively utilised in the Northern Territory for medicinal purposes, more so than would be predicted by the number of species available in this state. Among the *Eremophila*, *E. longifolia* is one of the most frequently cited owing to widespread use amongst several indigenous groups for a variety of therapeutic purposes.

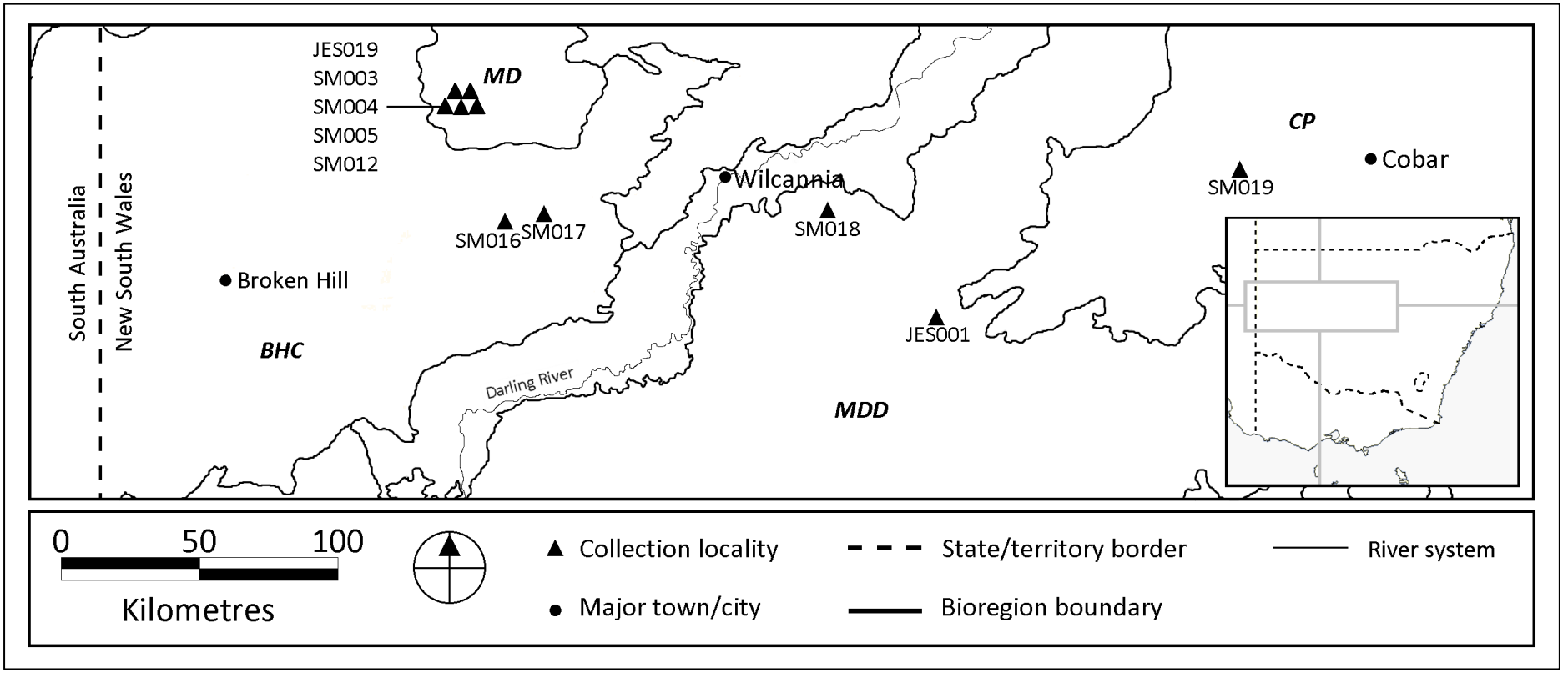


Figure 6.1 Map (expanded from inset) showing collection localities of *E. Longifolia* specimens in western NSW and extent of IBRA bioregions covered. MD: Mootwingee Downs bioregion; BHC: Broken Hill Complex bioregion; MDD: Murray-Darling Depression bioregion; CP: Cobar Peneplain bioregion.

Reported traditional therapeutic uses of preparations derived from the plant include oral use of aqueous preparations for the treatment of symptoms associated with respiratory tract infections (joint pain, fever), gastro-intestinal distress and diarrhoea and headache while topical preparations are reported to be employed for treatment of various dermatological lesions and conditions, ophthalmic complaints (painful or infected eyes), infestation by the scabies mite (*Sarcopetes scabiei*), headache, symptoms of viral respiratory illness, chronic malaise and to 'produce restful and restorative sleep' (Bonney, 1884; Webb, 1969; O'Connell *et al.*, 1983; Barr *et al.*, 1988; Low, 1990; Barr *et al.*, 1993; Latz, 1995). The use of liniments for joint and skeleto-muscular pain, which are prepared by mixing crushed leaves with animal fat, has also been described by Barr *et al*. (1988) along with the practice of inhalation of steam from heated leaves in the treatment of respiratory tract infections and general illness (Low, 1990). The plant is often described in medical ethnobotanic literature as exhibiting a distinctly pleasant odour and authors frequently ascribe the assumed therapeutic benefits to the actions of volatile constituents (Barr *et al.*, 1988; Low, 1990; Ghisalberti, 1994). In addition to its medicinal use, *E. longifolia* is also highly regarded by several groups of indigenous Australians as a plant of significant ritual and mystical importance. The ethnobotanist Peter Latz (1995) has described *E. longifolia* as "*the most sacred, mystical or magical of all central Australian plants*". Recorded ceremonial uses of the plant include the ritual smoking of newborn infants and mothers post-partum (where the infant is held in smoke generated from leaves and branches placed over the hot coals of a fire), smoking of initiates and ritual objects during circumcision rites, lining of graves and funerary shrouding and decorative body use during other ceremonial procedures (Low, 1990; Latz, 1995).

6.1.3 **Previous studies on ethnopharmacology and bioactivity**

Ghisalberti (1993) has provided extensive review of *Eremophila* phytochemistry and has attempted to correlate traditional therapeutic uses with potentially active chemical constituents known to accumulate in species with documented medicinal uses (Ghisalberti, 1994). Since this time a number of reports have been published, describing studies which have examined bioactivity and pharmacological activities of *E. longifolia* extracts. Palombo and Meilak (2008), as part of a study where plants were selected on the basis of traditional indigenous use in the treatment of respiratory infections, have demonstrated *in vitro* inhibition of *Mycobacterium fortuitum* and *Mycobaterium smegmatis* by an ethanol extract of *E. longifolia* leaves. Rogers *et al.* (2000) have reported inhibition of ADP induced *in vitro* platelet aggregation and serotonin release by a MeOH extract of leaves and flowers, in a study where plants were selected on the basis of their use in indigenous traditional medicine as headache remedies. Pennachio *et al*. (1996) have demonstrated *in vitro* and *in vivo* cardioactive effects of MeOH leaf extracts and have identified the corresponding active constituent as the iridoid glycoside geniposidic acid. Hayhoe and Palombo (2011) have more recently reported on anticariogenic properties of an ethanol extract of *E. longifolia* stem, demonstrating inhibitory and bactericidal activity against cariogenic bacteria (*Streptococcus mutans* and *Streptococcus sobrinus*), inhibition of glycolytic pH drop associated with the activity of oral Streptococci, and interference with bacterial adhesion.

6.1.4 **Previous work on volatile oil composition**

Della and Jefferies (1961) described the chemical composition of a sample of steam distilled volatile leaf oil obtained from an *E. longifolia* specimen occurring in the Murchison biogeographic division of Western Australia (WA) (Sandstone airstrip), which was shown to consist almost entirely of the phenylpropanoids, methyleugenol (76%) and safrole (32%). Oil

yield for this specimen was reported at 5.18% $^{g}/_{g}$. Safrole and methyleugenol are hepatotoxic and suspected carcinogens (IARC, 1976; Miller *et al.*, 1983; Burkey *et al.*, 2000), and, as a consequence, authors have urged caution in the internal use of traditional preparations derived from this species (Lassack and McCarthy, 1997). In contrast to the report of Della and Jefferies (1961), a separate study reporting on essential oil composition of an *E. longifolia* specimen collected from the Northern Territory as part of the Northern Territory indigenous pharmacopoeia project (Barr *et al.*, 1988; Barr *et al.*, 1993) described an oil characterised by a dominance of monoterpene hydrocarbons : α-pinene (55%), limonene (17%), β-pinene (11%), sabinene (3.4%), α-phellandrene (2.4%) and α-terpinolene (2.4%) from 0.025% $^{g}/_{g}$ yield, with no evidence of the presence of safrole or methyleugenol.

6.1.5 **Examination of antimicrobial activity of *E. longifolia* essential oils**

Antimicrobial activity of plant essential oils has been well studied and numerous reports on activity of commercial essential oils and their common constituents against a range of microorganisms have been published (e.g. Lis-Balchin *et al.*, 1998; Griffin *et al.*, 1999; Dorman and Deans, 2000). As the *E. longifolia* hydrodistilled oils were found to contain a number of constituents with known antimicrobial activity, assays for inhibitory and bactericidal activity were carried out against a panel of 7 bacterial species and the unicellular fungus, *Candida albicans*. Minimum inhibitory concentrations (MIC) of oil samples were determined using the broth microdilution method described in Chapter 2 (2.4.3.3.1). Minimum bactericidal concentrations (MIC) were determined by examining agar spot subcultures of broth MIC tests for growth to detect presence of viable cells after incubation with the oil samples.

Disc diffusion methods for assessment of antimicrobial activity of essential oils are generally not recommended, mainly due to differences in the ability of individual constituents to

diffuse via the aqueous environment of solid agar media (Mann and Markham, 1998). Additionally, Inouye *et al*. (2006) have shown that a large proportion of the activity which may be measured using diffusion methods can in fact be attributed to the actions of vaporised components (evaporating from the disc reservoir and redepositing on the agar surface), and have demonstrated that high levels of vapour activity tend to be associated with the less polar constituents which are less likely to diffuse into aqueous media. For this reason, disc diffusion assays of essential oils and volatile constituents cannot be considered comparable in terms of assessing relative activity as the relationship between diameters of inhibitory zones and concentration of test substances relies on a more or less equal rate of diffusion for test substances. Despite this, an attempt was made to use a disc diffusion assay for initial antimicrobial screening so that a gross estimation of activity could be made in order to select oils for further experiments (i.e. estimation of MIC/MBC). This strategy was eventually abandoned, however, as results were found to be highly erratic and non-reproducible. Differences of up to 13 mm in diameter of inhibition zones were recorded for repeated experiments and in some cases, zones of inhibition were too large to be measured accurately (i.e. greater than 40 mm diameter for karahanaenone and borneol chemotypes in assays against *Salmonella typhimurium* ACM 3598) which obscured any observation of activity from adjacent discs which may have been present. Additionally, the borneol rich oil of specimen SM019, when included in the assays, seemed to cause a slight inhibition of the confluent culture across the entire surface of the plates, resulting in very scant growth overall. The latter effect was most likely caused by redepositing of the oil components which had vaporised from the disc over the course of incubation. Even when reduction of background growth was minimal, the presence of the oils seemed to exert a potentiating effect on the nystsatin reference control discs, where activity was seen to be significantly increased. For example, the mean diameter of the zone of inhibition of 100 μg nystatin discs used as a

reference antimicrobial for *Candida albicans* was 21.8 mm ± 0.24 SE in tests including the traditional aqueous preparation and solvent based extracts whereas the mean value in assays including the essential oils increased to 24.2 mm ± 0.40 SE (two tailed t-test significance: $p < 0.01$). While some of these problems may have been ameliorated with dilution of the oil, or use of vapour inhibition assays (i.e. as described by Inouye *et al.*, 2006), the decision was made to abandon screening by disc diffusion given that only a relatively low number of samples were to be assayed (also given the possibility of vapour activity affecting adjacent tests, only one sample could be included per agar plate for diffusion assays to be valid, thus negating any potential advantages in relative cost and rapidness of testing). Instead, all of the oil samples were subsequently included for assay of MIC by broth microdilution tests without any screening steps. Only the results for screening of *C. albicans* are shown as activity against this organism was not tested using broth dilution methods and while relatively little activity against this organism was found in terms of relative potency (as assessed by diameter of inhibition zones), results were seen to be more reproducible than those obtained in tests involving the bacterial organisms.

6.2 **Results**

6.2.1 **Essential oil composition**

From the volatile oils, a total of 33 compounds were identified with analysis of the GC-MS and NMR data. Compounds identified and method of identification are listed alongside percentage quantity, oil yield, sample ID and geographic collection locality for each specimen examined, in Table 6.2. Structures for some of the compounds identified in the oils are illustrated below in Figure 6.4. Two of the ten specimens collected were not found to produce a recoverable quantity of volatile oil with hydrodistillation of up to 350 gm fresh leaves. These included a single specimen collected within the Murray-Darling Depression bioregion (JES001), and a specimen collected within the Broken Hill Complex bioregion

(SM016). Of the volatile oils recovered and analysed, considerable variation in both chemical composition and oil yield was found between samples representative of specimens collected from each study locality or geographic bioregion. All oils examined were terpenoid in nature and no safrole or other phenylpropanoids were detected in any of the samples.

Hydrodistillation of specimens collected in the Mootwingee Downs bioregion (Mutawintji National Park) afforded volatile oils at relatively high yields (3.1 - 5.7 % $^{w}/_{w}$) which were dominated by the *p*-menth monoterpene ketones, isomenthone (**3**) (61.1% - 86.7%) and menthone (**4**) (0.9% - 22.6%). Other constituents found in high quantities included α-terpineol (**5**) (8.4% - 11.0%) and piperitone (**6**) (0.5% - 3.8%). These oils also contained a number of monoterpene hydrocarbons including limonene (**7**) (0.2% - 0.8%), 4-carene (**8**) (0.1% - 0.4%) and α-terpinene (**9**) (0.3% - 0.4%), along with the monoterpene alcohols linalool (**10**) (0.1 % - 0.6 %), 4-terpineol (**11**) (0.1 % - 0.2 %) and menth-2-en-1-ol (**12**) (0.1 - 0.2%). These oils all exhibited a pleasant ethereal peppermint-like odour, which was most likely a result of the relatively high quantities of isomenthone and menthone, along with other *p*-menthane terpenoids which were found in abundance in these oils. These specimens were additionally noted to exhibit unique morphological features compared to the other specimens collected, including a narrower leaf, with rugose surfaces and obvious, dense aggregations of large, distended tuberculate secretory glands on the leaf surface and stems. These features were notably absent in the other lower yielding specimens collected in regions further south and east of the MD bioregion, where leaves tended to appear finely pubescent and the rugose surface and visible prominent tubercules were absent (Figure 6.2).

Hydrodistillation of two specimens collected from Broken Hill Complex and Murray-Darling depression bioregions near to the Darling river (50-100 km southeast of the Bynguano ranges) afforded oils at relatively lower yields (0.5 - 1.9 % $^{w}/_{w}$), which were found to be dominated

by the monoterpene trimethyl-heptene ketone, karahanaenone (**13**) (81.0 - 82.2 %). The composition of the remaining minor and trace constituents was generally similar to that found in the isomenthone rich oils of specimens collected from the Mootwingee Downs bioregion (e.g. α-terpineol at 4.1-11.7 % and limonene at 0.9 - 2.1 %). Karahanaenone was not initially able to be identified using the NIST spectral library for preliminary identification. NMR spectra obtained from a sample of the oil was used instead to initially identify this compound with comparison to published spectral data (Naya and Kotake, 1968). Identification of karahanaenone as the major peak in GC-MS separations was subsequently reconfirmed by comparison of other published MS and retention data (Adams, 2001).

Hydrodistillation of one specimen collected from the Cobar Peneplain bioregion (collected ca. 200 km East of the Darling River), produced an oil at a relatively lower yield (0.4 % $^{w}/_{w}$), which differed markedly in composition to both the isomenthone and karahanaenone rich oils. The oil of this specimen was found to be dominated by bicyclic heptane monterpenoids including borneol (**14**) (31.7 %), fenchol (**15**) (19.7 %) and bornyl acetate (**16**) (7.2 %), along with significant quantities of limonene (9.9 %), sabinene (**17**) (5.0 %), terpinolene (**18**) (4.7%) and 4-terpineol (4.4 %). Some of the monoterpene hydrocarbons (limonene, sabinene and terpinolene) found in the oil of the Cobar Peneplain bioregion specimen (SM019) were also reported by Barr *et al.* (1988) as constituents of oil derived from leaves of a similarly low-yielding (0.025 % $^{w}/_{w}$) specimen collected in the Northern Territory. The oil described in this study, however, differed with the presence of bicyclic heptane monterpenoids such as borneol and fenchol as major constituents, whereas, α-pinene was identified as the major constituent (reported at 55%), along with β-pinene (11%) in the oil described by Barr *et al*. (1988). While α-pinene and β-pinene were detected in the oil of SM019, both compounds were only detected at trace levels in this specimen (0.2 and 0.4% respectively).



Figure 6.2 Variation in leaf and stem morphology of *Eremophila longifolia* specimens A: Morphology typical of the isomenthone type specimens collected in the Mootwingee Downs bioregion, with prominent tubercules (distended secretory cavities) on stems and leaves with rugose leaf surfaces (Specimen JES019); B: Morphology typical of low yielding specimens occurring in other areas of the study with pubescent leaves and stems, and absence of prominent visible tubercules and rugose leaf surface (Specimen JES001).

Table 6.2 Volatile oil composition of *Eremophila longifolia* specimens

NSW Bioregion		MD	MD	MD	MD	MD	BHC	MDD	CP	
Voucher ID		JS019	SM003	SM004	SM005	SM012	SM017	SM018	SM019	
Yield (% $^{w}/_{w}$)		3.1	5.7	4.9	5.6	5.1	1.9	0.5	0.4	
Compounds	**RRI**				**Quantity (%)**					**Identification**
Bornylene	902	-	-	-	-	-	-	-	1.3	MS, RI
α-Pinene	935	-	-	-	-	-	-	-	0.4	MS, RI, CI
α-Fenchene	950	-	-	-	-	-	-	-	0.5	MS, RI
Camphene	952	-	-	-	-	-	-	-	2.5	MS, RI
Sabinene	974	-	-	-	-	-	-	-	5.0	MS, RI
β-Pinene	979	-	-	-	-	-	-	-	0.2	MS, RI
β-Myrcene	990	-	-	-	-	-	-	-	1.3	MS, RI, CI
4-Carene	998	0.1	0.4	0.4	0.4	0.4	-	-	-	MS, RI
α-Phellandrene	1006	-	-	-	-	-	-	-	0.2	MS, RI, CI
α-Terpinene	1017	-	0.3	0.3	0.3	0.4	0.1	-	0.4	MS, RI, CI
p-Cymene	1026	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.7	MS, RI, CI
Limonene	1030	0.2	0.8	0.7	0.7	0.8	2.1	0.9	9.9	MS, RI, CI
β-Phellandrene	1032	-	-	-	-	-	-	-	0.8	MS, RI
β-Ocimene (Z)	1048	-	-	-	-	-	-	-	0.5	MS, RI
γ-Terpinene	1058	-	-	-	-	-	-	-	0.6	MS, RI, CI
Terpinolene	1084	-	-	-	-	-	0.3	-	4.7	MS, RI
Fenchone	1087	-	-	-	-	-	-	-	0.4	MS, RI
Linalool	1088	0.3	0.6	0.1	0.4	0.3	1.0	-	-	MS, RI, CI
Fenchol	1117	-	-	-	-	-	-	-	19.7	MS, RI
Menth-2-en-1-ol	1125	0.1	0.1	0.1	0.2	0.1	0.1	0.1	0.3	MS, RI
Limonene oxide	1132	-	-	-	-	-	-	-	0.4	MS, RI
trans-p-Menth-2-en-1-ol	1143	-	-	-	-	-	-	-	0.3	MS, RI
Camphor	1150	-	-	-	-	-	-	-	0.9	MS, RI
Menthone	1159	8.8	17.1	22.6	9.5	0.9	-	-	0.6	MS, RI, CI
Karahanaenone	1157	-	-	-	-	-	81.0	82.2	-	MS, RI*
Isomenthone	1168	75.2	67.5	61.1	74.5	86.7	0.3	10.1	1.2	MS, RI, CI
Borneol	1175	-	-	-	-	-	-	-	31.7	MS, RI, CI
4-Terpineol	1187	-	0.1	0.2	0.2	0.2	-	-	4.4	MS, RI, CI
α-Terpineol	1196	8.3	8.8	11.0	11.0	8.8	11.7	4.1	1.3	MS, RI, CI
exo-Fenchyl acetate	1218	-	-	-	-	-	-	-	0.1	MS, RI
(S)-Carvone	1247	-	-	-	-	-	-	0.1	-	MS, RI
Piperitone	1259	1.6	3.8	2.8	1.7	0.5	0.4	0.1	-	MS, RI, CI
Bornyl acetate	1284	-	-	-	-	-	-	-	7.2	MS, RI
Unidentified	-	5.4	0.3	0.4	1	0.7	2.9	2.2	2.8	
Total identified	-	94.6	99.7	99.6	99.0	99.3	97.1	97.8	97.2	

RRI: Kovats retention indices, relative to C_9 – C_{24} alkanes on non-polar column (ZB-5ms); (%): Relative percentage obtained from peak area; MD: Mootwingee Downs bioregion; BHC: Broken Hill Complex bioregion; MDD: Murray-Darling Depression bioregion CP: Cobar Peneplain bioregion; Identification: MS: Comparison with library and published mass spectra; RI: Retention index; CI: Co-injection of authentic reference compound; *: Initial identification by NMR spectroscopy

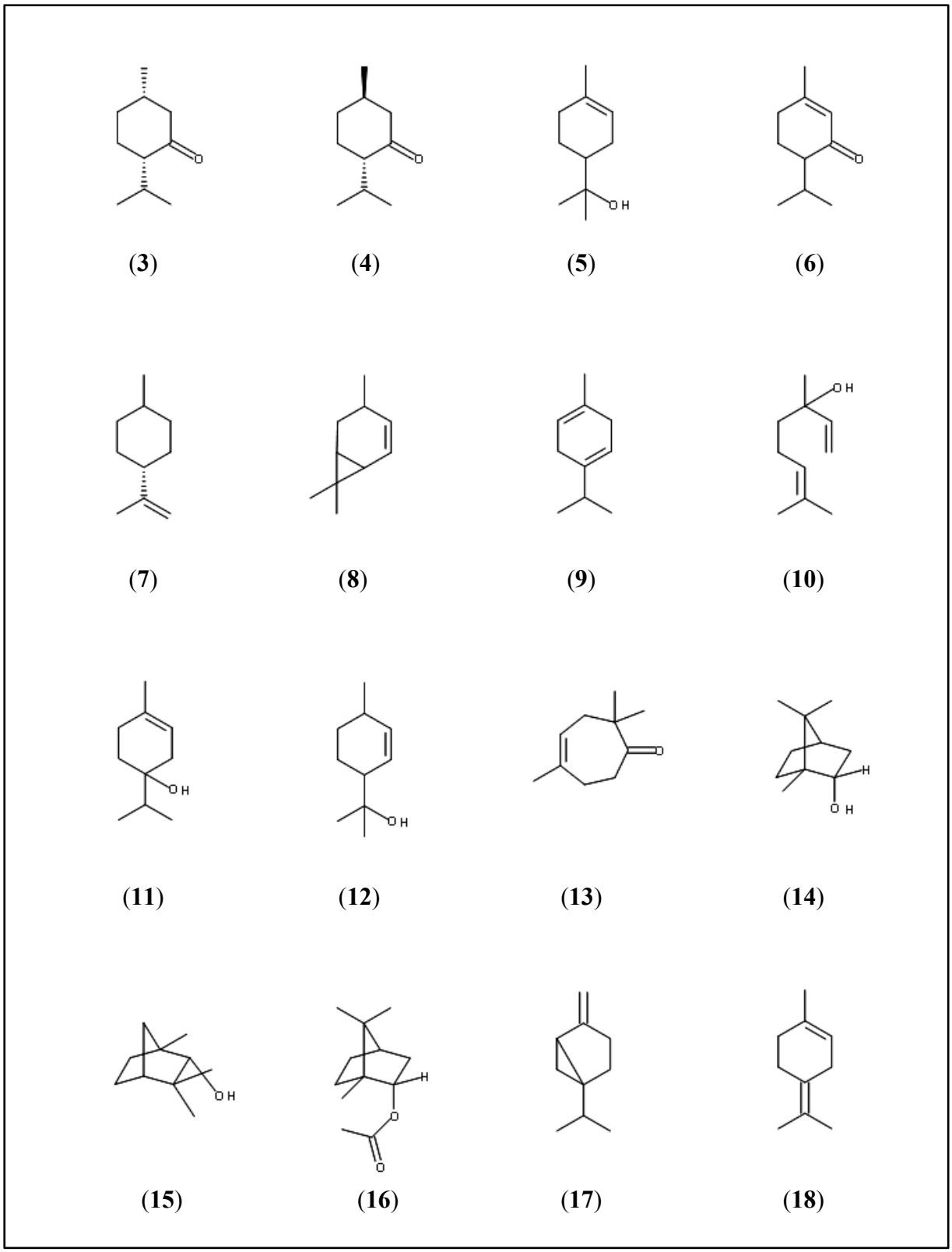


Figure 6.3 Constituents identified in volatile oils of *E. longifolia*: Isomenthone (**3**), menthone (**4**), α-terpineol (**5**), piperitone (**6**), limonene (**7**), 4-carene (**8**), α -terpinene (**9**), linalool, (**10**), 4-terpineol (**11**), menth-2-en-1-ol (**12**), karahanaenone (**13**), borneol (**14**), fenchol (**15**), bornyl acetate (**16**), sabinene (**17**), terpinolene (**18**).

6.2.2 **Principal component analysis**

The data set composed of 8 oil samples and 24 compounds (present in quantities ≥0.5%) was subjected to multivariate statistical analysis using principal component analysis (PCA). Although it was acknowledged that the data set was small for multivariate analysis (in terms of proportion of number of cases to number of variables), PCA was used in this instance to visualise associations between cases (oil samples of specimens from various geographic bioregions) and chemical composition, and to establish a preliminary basis for a scheme of volatile oil chemotypic classification of *E. longifolia* in NSW. The two-dimensional PCA plot, along with factor loadings of transformed variables on principal component axes 1 and 2, are given in Figure 6.3A,B, along with the score plot. Principal components 1 (PC1) (81%) and 2 (PC2) (11%), cumulatively explained 92 % of total variance. PC1 distinguished the oil of the single specimen (collected in the CP bioregion), which was characterised by high levels of bicyclic monoterpenes (borneol, fenchol, bornylene and bornyl acetate) and a comparatively higher proportion of limonene, from the population of both karahanaenone and isomenthone/menthone rich oils, while loadings for PC2 correlated positively with karahanaenone content and negatively with the *p*-menth monoterpene ketones (isomenthone, menthone and piperitone), separating the two specimens collected in the Broken Hill Complex and Murray Darling Depression bioregion (karahanaenone type) from a clustering of specimens collected in the Mootwingee Downs bioregion (Isomenthone type).

Table 6.3 Variables used for principal component analysis and loading for principal components 1 and 2 (All compounds present at ≥0.5% in oil samples)

Variables	PC1	PC2	Variables	PC1	PC2	Variables	PC1	PC2
p-Cymene	0.983	0.113	Camphene	0.977	0.122	4-Terpineol	0.976	0.088
Terpinolene	0.979	0.160	Sabinene	0.977	0.122	Limonene	0.971	0.224
Fenchol	0.977	0.122	β-pinene	0.977	0.122	Menthone	-0.186	-0.868
Bornyl acetate	0.977	0.122	β-Myrcene	0.977	0.122	Piperitone	-0.244	-0.855
Borneol	0.977	0.122	β-Phellandrene	0.977	0.122	Linalool	-0.268	0.073
Bornylene	0.977	0.122	β-Ocimene (Z)	0.977	0.122	Karahanaenone	-0.286	0.867
α-Pinene	0.977	0.122	γ-Terpinene	0.977	0.122	Isomenthone	-0.465	-0.752
α-Fenchene	0.977	0.122	Camphor	0.977	0.122	α-Terpineol	-0.627	-0.349

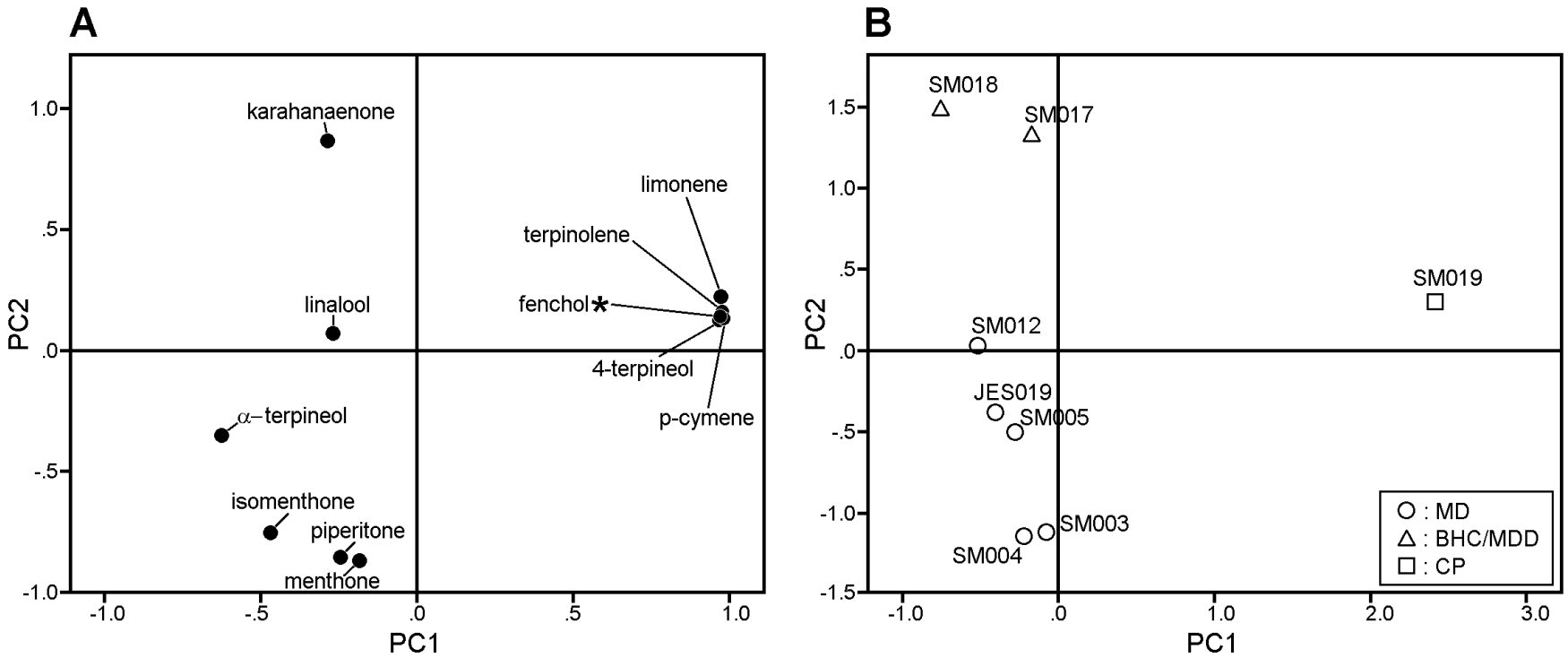


Figure 6.4 Factor loadings of variables (A), and Score plot (B) of oil samples by bioregion on axes 1 and 2 derived from principal component analysis (PCA) on chemical composition of *E. longifolia* volatile oils. MD: Mootwingee Downs bioregion; BHC/MDD: Broken Hill Complex/Murray-Darling Depression bioregions; CP: Cobar Peneplain bioregions. *: For other variables with equal loading at this point see Table 6.3

6.2.3 **Antimicrobial activity of *E. longifolia* essential oils**

E. longifolia essential oils exhibited weak to moderate antibacterial activity against most of the organisms tested. $MIC_{p\text{-}INT}$ for susceptible organisms was typically between 1.0 % $^{v}/_{v}$ and 2.0% $^{v}/_{v}$ for most oils. MBC_{SIAS} values were typically the same as $MIC_{p\text{-}INT}$ values or greater by a factor of 1^{2} (i.e. one dilution step higher). The only oil producing inhibition and bactericidal activity against all organisms tested was that of specimen SM019 (borneol chemotype). This oil also produced the lowest MIC/MBC values overall. No significant differences were noted between activity of the isomenthone and karahanaenone type oils against susceptible organisms except for in tests against *Salmonella typhimurium* (ACM 3598), which appeared to be slightly more susceptible to actions of the isomenthone type oils ($MIC_{p\text{-}INT}$ of 0.5 to 2.0% $^{v}/_{v}$ for isomenthone type oils and 1.0 to 2.0% $^{v}/_{v}$ for karahanaenone oils).

The results were unusual in that Gram-negative organisms were generally seen to be more susceptible to the inhibitory/bactericidal actions of the oils than the Gram-positive species. Of the Gram-positive species tested, only *Bacillus subtilis* was susceptible and no inhibition by the isomenthone or karahanaenone type oils against *Staphylococcus aureus* or *Staphylococcus epidermidis* was seen at the highest concentration tested (2.0 % $^{v}/_{v}$) (for these species, activity was only seen from the single specimen of borneol rich oil -SM019). In contrast, all four Gram-negative species (including the typically resistant species, *Pseudomonas aeruginosa*) were susceptible to all of the oils tested. The most susceptible organism was *Salmonella typhimurium* (ACM 3598) where $MIC_{p\text{-}INT}$ for isomenthone and borneol type oils ranged from 0.5 to 2.0 % $^{v}/_{v}$ (MBC_{SIAS} 1.0 - 2.0% $^{v}/_{v}$). $MIC_{p\text{-}INT}$ and MBC_{SIAS} values for each microorganism are presented in Table 6.4. Figure 6.5 shows the appearance of some of the broth microdilution assays along with corresponding agar MBC_{SIAS} subculture plates.

Table 6.4 Minimum inhibitory concentration ($MIC_{p\text{-}INT}$) and minimum bactericidal concentration (MBC_{SIAS}) for essential oils of three *Eremophila longifolia* chemotypes against 7 microorganisms

		$MIC_{p\text{-}INT}$ with values representing MBC_{SIAS} in parentheses (% $^{v}/_{v}$ oil)						
Specimen (Collectors number)	**Essential oil chemotype**	*Staphylococcus epidermidis* (ATCC 12228)	*Staphylococcus aureus* (ATCC 29213)	*Bacillus subtilis* (ACM 2268)	*Klebsiella aerogenes* (ATCC 10031)	*Escherichia coli* (ATCC 25922)	*Pseudomonas aeruginosa* (ATCC 27703)	*Salmonella typhimurium* (ACM 3598)
E. longifolia (SM003)	Isomenthone	-	-	2.0 (2.0 - **>2.0**)	2.0 (2.0)	1.0 (1.0 - **>2.0**)	2.0 (2.0)	0.5 - 2.0 (1.0-2.0)
E. longifolia (SM004)	Isomenthone	-	-	2.0 (2.0 - **>2.0**)	2.0 (2.0)	1.0 - 2.0 (1.0 - **>2.0**)	2.0 (2.0)	0.5 - 2.0 (1.0-2.0)
E. longifolia (SM005)	Isomenthone	-	-	2.0 (2.0 - **>2.0**)	2.0 (2.0 - **>2.0**)	1.0 - 2.0 (1.0 - 2.0)	2.0 (2.0)	0.5 - 2.0 (0.5 - 2.0)
E. longifolia (SM012)	Isomenthone	-	-	2.0 - **>2.0** (2.0 **- >2.0)**	2.0 (2.0 - **>2.0**)	1.0 - 2.0 (1.0 - **>2.0**)	2.0 (2.0)	0.5 - 2.0 (1.0 - 2.0)
E. longifolia (SM017)	Karahanaenone	-	-	2.0 -	2.0 - **>2.0** (2.0 **- >2.0)**	1.0 (1.0)	2.0 (2.0 - **>2.0**)	1.0 - 2.0 (1.0 - **>2.0**)
E. longifolia (SM018)	Karahanaenone	-	-	2.0 (2.0 - **>2.0**)	2.0 (2.0 - **>2.0**)	1.0 - 2.0 (2.0 - **>2.0**)	2.0 (2.0)	1.0 - 2.0 (1.0 - **>2.0**)
E. longifolia (SM019)	Borneol	1.0 - 2.0 (2.0)	1.0 - 2.0 (2.0)	0.5 (1.0)	2.0 (2.0)	1.0 (1.0)	1.0 (1.0 - 2.0)	0.5 - 2.0 (0.5 - 2.0)

-: Not active up to highest concentration tested (2.0% $^{v}/_{v}$)

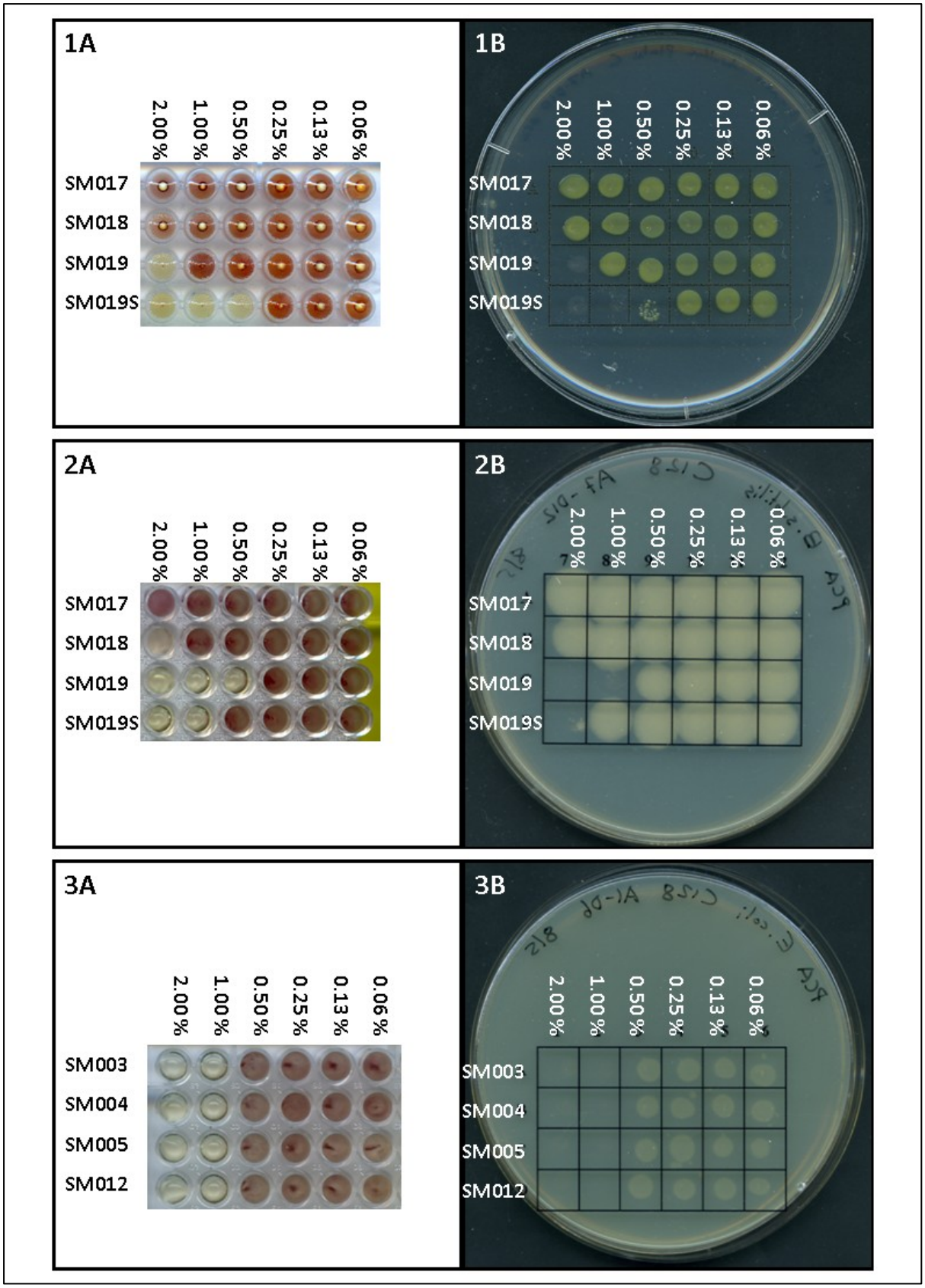


Figure 6.5 Examples of MIC microdilution tests and corresponding subcultures for MBC. A: Microdilution wells after incubation and addition of p-INT; B: Spot agar subcultures; 1: *Staphylococcus aureus* (ATCC 29213); 2: *Bacillus subtilis* (ACM 2268); 3: *Escherichia coli* (ATCC 25922). Essential oil sample and well concentration (% $^{v}/_{v}$) indicated on figure.

Results of disc diffusion assays for inhibitory action against *C. albicans* are shown below in Table 6.5. Some inhibition of *C. albicans* was exhibited by all of the isomenthone and karahanaenone type oils, while the single example of the borneol type oil appeared to be inactive against this organism. These results were in contrast to those obtained with the assays for antibacterial activity, where the borneol type oil was seen to exhibit activity against all organisms tested. Inhibition was relatively weak overall (zones of inhibition <10 mm diameter). The largest inhibitory zones were produced by oil of specimen SM012 (isomenthone chemotype). The oil of this specimen differed to that of the other isomenthone chemotypes in that a higher ratio of isomenthone:menthone was found (Specimen JES012 oil: isomenthone: 86.9%, menthone: 0.9%; Other isomenthone type specimens: isomenthone: 61.1 - 74.5%, menthone: 8.8 - 22.6%).

Table 6.5 Disc diffusion assays of *Eremophila longifolia* essential oil chemotypes against *Candida albicans* (ATCC 10231)

Specimen	Essential oil chemotype	Zone of inhibition diameter (mm)
E. longifolia (SM003)	Isomenthone	7.7 ±1.45
E. longifolia (SM004)	Isomenthone	7.7 ±1.76
E. longifolia (SM005)	Isomenthone	6.0 ±1.00
E. longifolia (SM012)	Isomenthone	9.7 ±0.88
E. longifolia (SM017)	Karahanaenone	7.7 ±1.33
E. longifolia (SM018)	Karahanaenone	7.7 ±1.34
E. longifolia (SM019)	Borneol	-
Nystatin (100 mg)	(NA)	24.2 ±0.40

-: Indicates lack of activity/zone of inhibition

6.3 **Discussion and conclusions**

The findings presented in this study provide further evidence for a high degree of intraspecific variability in leaf volatile oil composition for *E. longifolia*, and provide preliminary evidence for the occurrence of three novel chemovarieties (isomenthone, karahanaenone and borneol chemotypes). The variability in chemical composition of the oils

appeared to coincide with geographic location of specimens with the high yielding isomenthone type restricted to the Bynguano ranges of the Mootwingee Downs bioregion, the karahanaenone type to areas of the Broken Hill Complex and Murray-Darling depression bioregion bordering the Darling river and the low-yielding borneol chemotype to the Cobar Peneplain bioregion, further west of the Darling river. Intraspecific variation in volatile oil yield and composition of wild and cultivated plants is a well recognised phenomenon and can arise as the result of genetic and/or environmental factors. These can include interspecies hybridisation, plant nutrition or soil chemistry, water and temperature stresses, season and climatic conditions, altitude, age and ontogeny of plant. Variations in chemical composition generally may also be representative of adaptive defensive responses to stressors such as insect and animal herbivory, microbial infection and other environmental cues. While a detailed investigation into the cause or causes of variability in this instance was beyond the scope of the study, it can be inferred the differences in volatile oil composition described here are unlikely to represent any ontogenic or age related compositional differences as all sampling took place from mature, well established plants. Similarly, compositional variations due to seasonal effects are unlikely to account for the differences described here as many of the specimens were sampled within a period of 5 days during the months of November-December (late spring/early summer season) and this collection included representatives of each proposed chemotype. An additional specimen (JES019) collected earlier in the year during the month of August from the Mootwingee Downs bioregion (late winter/early spring season) was also seen to conform to the isomenthone chemotype exhibited by the other specimens sampled in this region. All of the collection localities receive similar levels of average annual rainfall (222 – 260 mm). The differences in altitude between the collection localities was also negligible (Mootwingee Downs specimens collected at 209 – 275 meters

above sea level, Broken Hill Complex/Murray-Darling Depression specimens collected at 187 – 227 metres and Cobar Peneplain specimen collected at 199 metres).

While the possibility that the variability seen here is the result of other environmental factors (e.g. as a response to herbivory and predation) cannot be discounted, the high degree of chemovariability reported here and elsewhere may also be suggestive of genetic divergences among populations of this species. *E. longifolia* is recognised as a polymorphic species and the variations in morphology of the indumentum seen among the specimens collected for this study (i.e. superficial surface coverings such as hairs and tubercules) may be considered normal for the species (Chinnock, 2007). In this study dense coverings of large tubercules and rugose leaf surface were associated with high yielding isomenthone chemotypes and Della and Jefferies (1961) also reported large tubercules in the high yielding safrole/methyleugenol type from the Murchison district of WA. While these features might be expected to be associated with specimens producing high volatile oil yield (i.e. enlarged secretory structures and leaf surface rugosity) and therefore may not represent a fixed morphology, it is less clear as to the possible reasons for the relative narrowness seen in leaves of the isomenthone types. Polyploidy is known to occur in the Myoporaceae and is thought to have facilitated colonisation and adaptation of the *Eremophila* to the arid parts of the continent (Barlow, 1971). In *E. longifolia*, tetraploid (n 36) and diploid (n 18) forms are known, however, the less common relic diploid form is only known to occur at the far western part of the species distribution (i.e. WA) (Barlow, 1971) and it is likely that the eastern populations sampled in this study all represent the tetraploid form. Although it is very unlikely that any of the specimens collected represent individual hybrids, interspecific and intergeneric hybridisation is known among the Myoporaceae, albeit rarely (Chinnock, 2007). While no evidence of recorded hybridisations involving *E. longifolia* were found, Chinnock (2007:557) has indicated that the single existing type specimen of *Eremophila graciliflora* (F.

Muell) (recorded at 'Yattoo' in the Murchison region of WA, 1859) may represent a hybrid of the species *E. longifolia* and *E. oldfieldii*.

Monoterpenoids are known to exhibit a diverse array of pharmaceutical and therapeutic properties, and any extracts or traditional preparations derived from plant material variable in monoterpenoid content may be expected to result in corresponding differences in bioactivity or efficacy. Given the diversity in volatile oil yield and composition in *E. longifolia*, some differences in the bioactivity of traditional therapeutic preparations may be expected. Such effects, however, would be dependent on the quantity of the largely lipophillic monoterpenoids extracted into traditional preparations derived from this plant. In the case of aqueous preparations, this may depend on specific methods of preparation, the water solubility of each compound in addition to the presence of other compounds in the plant material which may facilitate or enhance aqueous solubility. Variations in the content and quantity of monoterpenoids in this species may be more relevant in cases where plant parts and preparations are applied topically or mixed with animal fat to form ointments (Ghisalberti, 1994), particularly as monoterpenoids, in addition to any direct pharmacological actions, are known to permit or enhance trans-dermal penetration of other non-lipophilic compounds and drugs which do not normally penetrate the upper layers of the skin (Williams and Barry, 2004). Also, as pointed out by Ghisalberti (1994), content of the volatile monoterpenoid fraction of this species may be particularly relevant in cases where the plant material is heated and vaporised for inhalation as a treatment for symptoms of upper respiratory tract infections. It can be assumed that some quantity of the major oil constituents were present in the aqueous extracts prepared from specimens collected in this study as each decoction was found to exhibit odours characteristic of each type, even in portions which had been lyophilised. An attempt was made to detect the major constituents and any other volatile compounds which may have been extracted in each aqueous extract, by extracting a small

portion (0.5 gm) with hexane for 3 hr and separating the resulting partition using GC-MS with a splitless injection method. This attempt was unsuccessful as none of the expected monoterpenoids were apparently present in the hexane partitions in quantities which could be detected despite being apparently present in quantities above odour threshold. While the chemistry of most of the traditional aqueous preparations examined in this study were not investigated in detail, 'voucher' HPLC separations were obtained as a record for each extract. Separations of aqueous decoctions prepared from material representing each of the three novel chemotypes proposed in this study, carried out under equivalent chromatographic conditions and column loading are shown in Figure 6.6. Even from this relatively limited information, gross quantitative differences, and possibly some qualitative differences in chemical composition can be seen between the extracts representing each of the three chemotypes.

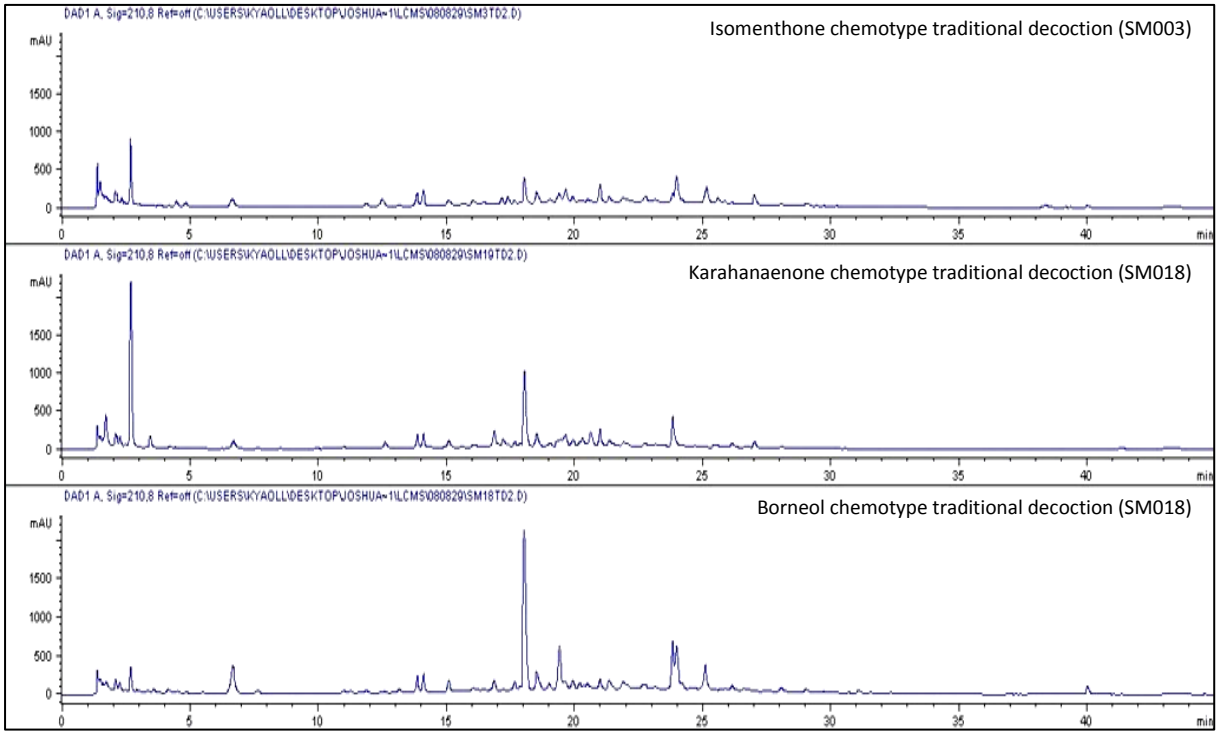


Figure 6.6 HPLC voucher separations of *E. longifolia* traditional aqueous decoctions representative of three chemotypes. (Gradient: 30 min 10-95% ACN-H_2O; Detection: MAu at λ210 nm).

Several of the major constituents found in the volatile oils of each *E. longifolia* chemotype identified here (e.g. menthone, a-terpineol, borneol, limonene) have been shown to exhibit antibacterial and antifungal activities (Griffin *et al*., 1999). Isomenthone, menthone and similar p-menth ketones found in relatively high quantities in the *E. longifolia* specimens collected in the Mootwingee Downs bioregion, have also been shown to exhibit significantly high levels of free radical scavenging capacity (Mimica-Dukic *et al.*, 2003) Antimicrobial activities, if present in traditional preparations, could partly provide rationale for therapeutic use in the treatment of minor wounds and skin lesions. The ketones isomenthone and menthone have also been shown to exhibit acaricidal activity (Tak *et al.*, 2006) and have been identified as major constituents of the volatile oils of species traditionally used as vermifuges in other parts of the world (Senatore *et al.*, 1998). Insecticidal or acaricidal activity due to the presence of terpenoid constituents may help to explain the traditional use of *E. longifolia* preparations in the therapeutic treatment of dermal infestations by the scabies mite (*Sarcoptes scabiei*). Borneol, which is found in a number of medicinal herbs and has been used in Chinese and Japanese traditional medical systems for anti-inflammatory and analgesic purposes, has been shown to possess potential sedative actions via the positive modulation of $GABA_A$ receptors. (Granger *et al.*, 2005).

6.3.1 **Karahanaenone in *E. longifolia* essential oils**

Karahanaenone, found as the major constituent in the oils of specimens collected from the Broken hill Complex and Murray-Darling Depression bioregions, was first reported as a constituent of the volatile oil of hops (*Humulus lupulus*) (Naya and Kotake, 1968), and later as a constituent of essential oils of *Tanacetum lingulatum*, *Nigella sativa* seed and the leaves of several *Cupressus* spp. (Cool *et al.*, 1994; Afsharypuor and Jahromy, 2003; Benkaci-Ali *et al.*, 2006). Karahanaenone is a member of a relatively uncommon class of natural products (oxygenated 1,4,4-trimethylcycloheptanes) and its secondary alcohols and other derivatives

are utilised by the fragrance and cosmetic industries, where interest has arisen in developing methods of synthesis from a variety of commonly available terpenoid precursors (e.g. Bazhina and Manukov, 1995). To the best of the authors knowledge, the *E. longifolia* specimens found occurring near the Darling River in the Broken Hill Complex and Murray Darling Depression bioregions in which large quantities of this constituent were found, potentially represent the richest natural source of this compound so far reported (at 81.0 % - 82.2 % of volatile oils corresponding to yields of 0.4 - 1.5% of fresh leaves by weight).

6.3.2 **Conclusions**

The results presented in this study, together with the earlier reports of Della and Jefferies (1961) (high yielding phenylpropanoid type of WA) and Barr *et al*. (1988) (low yielding α-/β-pinene type of Northern Territory), would suggest that five separate essential oil chemotypes of *E. longifolia* may be tentatively recognised at present. It is interesting to note that while the plant is cited frequently for medicinal uses, a wide variety of distinct medicinal uses are represented for which little agreement seems to exist among the examples of literature examined in this study. As a consequence, the MUA values for dermatological uses calculated in this study tended to be relatively low, as would probably be the case for any estimated measurement of agreement where very specific classes of medicinal use were recognised (*cf*. Chapter 3, Table 3.2). Given the widespread traditional use of this species along with the diverse range of therapeutic applications which have been reported, it is tempting to suggest the possibility that regionally distinct therapeutic applications may be correlated with local chemotypic variation. At present, however, this would be very difficult for a variety of reasons. As discussed earlier, the most detailed and comprehensive reports on traditional use for this species have emerged from studies conducted in the Northern Territory and central Australia. The low yielding α-/β-pinene type reported by Barr *et al*. (1988) which

described analysis of a single sample, remains the only published account detailing essential oil chemistry of the plant in this region. This particular specimen was collected from the Mt Allan station in central Australia (Northern Territory) Specific traditional use reported for this area (Anmatyerre language group members of Yuelamu community) involves topical application of leaf decoctions for the treatment of inflammatory pain and general malaise associated with respiratory viral illness (Barr *et al.*, 1988). While a variety of medicinal applications have been reported for other nearby regions (e.g. treatment of dermatological lesions and infections etc.), no corresponding examination of essential oil chemistry has taken place. In the eastern state of NSW very few formal published reports of regional ethnobotany exist. From anecdotal information, decoctions of the plant appear to be used in some parts of the study area primarily as a 'washing medicine' (rubbed into the body and used for general well being and health) [i.e. in Mutawintji and the Darling River/Menindee lakes area occupied primarily by *Baakantji* groups where where *E. longifolia* is sometimes referred to as *kuyimurra* (Martin, 2001), and where the karahanaenone and isomenthone types were found]. Historical records refer to the use of *E. longifolia* in this region as a topical remedy for headache (Bonney, 1884, cited by Martin, 2001). The borneol type (for which PGE_2 inhibition by samples of the traditional aqueous decoctions was found) occurs nearest to a region (Bogan River area of southwestern NSW) where records describe of decoctions for the treatment of sores (Webb, 1969). A more meaningful analysis of correlation between site specific chemistries and specific traditional medicinal uses may only be possible if more detailed and comprehensive ethnobotanical information becomes available for the relevant areas in NSW, and, studies on the range and occurrence of specific chemotypes (including in central and northern parts of Australia in addition to NSW) are performed.

In recent years, there have been movements in health care centres and clinics in Australia servicing indigenous populations, to develop programs making traditional indigenous

medicines available as complementary therapies in addition to orthodox treatments. The possibility of traditional preparations derived from *E. longifolia* containing safrole or other toxic principles is concerning given the apparent widespread use and popularity of these preparations. The high oil yielding safrole containing specimen described by Della and Jefferies (1961) which was the first published description of essential oil composition in this species, may well be representative of an uncommon or site-specific chemotype restricted to the Murchison area of WA. While the evidence presented here suggests that safrole is not present in the leaves of *E. longifolia* occurring in NSW (and possibly those occurring in the Northern Territory) it would nonetheless be recommended to advise general caution to medical clinics engaging in programs using complimentary traditional indigenous therapies, along with groups engaging in commercial production of cosmeceuticals etc., and to seek laboratory testing for phenylpropanoid content in *E. longfolia* material and preparations which may be employed for these purposes.

Given the potential for economic interest in this plant and its continuing widespread traditional medicinal use, further studies may be warranted to characterise more fully the precise geographic range and chemotaxonomic significance of chemical variations which may be found in this species and to elucidate the causes or conditions under which these occur.

Chapter 7: General Conclusions

7.1 General conclusions

In this study, a number of *in vitro* biological activities in a sample of plant based medicinal preparations used for the treatment of dermatological complaints in Australian indigenous medicine were investigated. Apart from the possibility of identifying species exhibiting significant levels of activity as candidates for further investigation, the aim of the project was to contribute toward a more complete understanding of any biomedical reasons behind the selection and traditional medicinal use of these species and preparations. Data on documented indigenous Australian medicinal plant use was compiled from existing ethnobotanical sources and used to generate indices of agreement for specific dermatological uses of the preparations and species examined in the study. One of the purposes of these analyses was to investigate whether the degree of agreement among citations from separate ethnobotanical sources, could be associated with the incidence of, or degree of relative potency in any of the *in vitro* biological activities and other parameters exhibited.

Additional work was carried out in order to determine whether the serrulatane diterpenes identified in previous work as the compounds principally responsible for antibacterial activity in extracts of *Eremophila duttonii* could be detected in traditional aqueous preparations, and if so, whether in quantities sufficient to account for any biological activities observed. Also, during the course of previous work, evidence had emerged which suggested a high degree of variation in volatile oil composition in one of the species under investigation (*Eremophila longifolia*). Two previous reports on essential oil composition in this species had together also been indicative of variability, with one report indicating that the volatile oil of this species contained the toxic and carcinogenic phenylpropanoids safrole and methyleugenol. *E. longifolia* has been cited relatively frequently in the Australian ethnobotanical literature for a

wide variety of medicinal uses and anecdotal evidence suggests that contemporary use is common, routine and widespread. Additionally, indigenous groups in NSW have at times expressed interest in developing commercial products containing extracts of the species. A preliminary study was undertaken to characterise the apparent variation in populations occurring in NSW and to determine whether the toxic phenylpropanoids could be detected in essential oils obtained from specimens in this state. Additionally, aqueous extracts were prepared after traditional methods as described in the ethnobotanical literature, using material representative of each of the three NSW chemotypes subsequently identified, to determine whether any significant differences in biological activity could be observed.

7.1.1 **Analysis of records of Australian indigenous medicinal plants use**

The regression residual analysis performed on the list of documented medicinal flora of the Northern Territory (NT) indicated that certain families appeared to be disproportionately under- or over-utilised. Some aspects in the pattern of selectivity found were similar to those reported in other studies, where the medicinal flora of cultures inhabiting regions within the neotropical and holarctic floristic kingdoms (e.g. Moerman, 1996; Moerman *et al.*, 1999; Leonti *et al.*, 2003; Amiguet *et al.*, 2006) and among species used in contemporary commercial phyto-therapeutic preparations (Wohlmuth and Brooks, 2007) was examined. In particular, the analysis revealed that members of the Poaceae and Cyperaceae seemed to be disproportionally underutilised in the NT flora, as was similarly reported in the majority of the studies cited above. Selective use of the NT medicinal flora differed from that reported in previous studies with the apparent underutilisation of the available members of the Asteraceae, and the selective overutilisation of certain families, which among the Australian flora specifically, are characteristically large and represented by widely radiated genera (i.e. Myrtaceae, as represented mostly by *Eucalyptus*/*Corymbia* spp. and *Melaleuca* spp., the Fabaceae, as represented mostly by *Acacia* spp. and the Proteaceae as represented mostly by

Hakea spp. and *Grevillea* spp.). Selective overuse was also found for the Scrophulariaceae (Represented in the NT entirely by members of the genus *Eremophila*). Selective overuse of this genus is of particular interest as the *Eremophila* are not typically as common or dominant as some members of the Myrtaceae or Fabaceae and it may be less likely that such selectivity may be explained by the relative abundance or availability of these plants. The *Eremophila* are characterised by unusual diterpenoid chemistry (Ghisalberti, 1993) and a number of interesting biological activities have been found in extracts of, and compounds isolated from, several species, including those for which medicinal uses have been documented.

Further selective use of certain families was also demonstrated among plants which were cited in the literature examined, specifically for the treatment of various dermatological conditions. Among the plants cited for these purposes, greater than predicted numbers of species representing the Euphorbiaceae, Schrophulariaceae (*Eremophila*) and Proteaceae (mostly as *Hakea* spp.) were found, with lower than predicted use of members of the Solanaceae, and further underuse of the Poaceae and Asteraceae. The apparent underuse of the Solanaceae was not likely to be related to dermatological use in any particular way as records of traditional medicinal/ethnochemical uses of members of this family tended to describe exclusive use as 'psychoactive' agents and fish/game poisons.

Cultural agreement ratios (CAR) derived from the Australian ethnobotanical literature (representing the degree of consensus in the use of particular species within certain categories of symptoms or indications of medicinal plant use), were comparable in terms of the range of values obtained to those reported by Moerman (2007) where the results of similar analyses of historical north American ethnobotanic records were described. Values were generally lower than those reported in studies where the analogous 'informant agreement ratio' (IAR) or 'informant consensus factor' (F_{IC}) were derived using data obtained from informants across

relatively smaller geographic ranges (expressing consensus between reports of medicinal plant use of individual informants rather than separate cultures or studies). As explained by Moerman (2007), given the relatively broad range of landscapes and ecological conditions covered by the literature used to build the dataset, it is likely that this effect has arisen from the inclusion of several species with relatively narrow distribution, which would increase the number of species listed within the use categories without a proportional increase in the number of use reports, thus lowering consensus among the categories. Also, in the case of this study specifically, the disproportionately lower representation of documented ethnobotanical data for regions outside of the NT may have similarly contributed to the comparatively low CAR values, as these records are likely to present a less complete representation of either historical or present day plant use by indigenous communities. While the CAR values derived in the analysis of data from the published literature were not directly comparable with those citing IAR/F_{IC}, values exhibited sufficient variability to enable comparison between separate use categories and to identify categories exhibiting relatively high levels of consensus (such as the dermatological uses category, for which the second highest CAR value was found). Also, these values were broadly in agreement with other assessments of ethnomedical plant use in that high levels of agreement, and relatively high numbers of species and use mentions, were found in categories representing use for treatment of respiratory illnesses, gastrointestinal problems and dermatological complaints (e.g. Amarozo and Gely, 1988; Cox, 1994; Heinrich *et al.*, 1998; Moerman, 2007).

7.1.2 Use of traditional aqueous preparations in the study and comparison with non-polar organic solvent extracts

Overall, a higher incidence of positive anti-microbial activity was found among the organic solvent extracts when compared with the corresponding traditional type aqueous preparations of the five *Eremophila* species. This was also found to be the case when results were

compared with those reported in other studies describing antibacterial activity in ethanolic extracts of material derived from the same species (i.e. Barr *et al.*, 1993; Palombo and Semple, 2002). Similarly, values obtained in tests for antioxidant capacity, inhibition of PGE_2 formation and P388D1 cytotoxicity, were in most instances, greater among the sequential tertiary methanol extracts in comparison to corresponding aqueous preparations.

In this study traditional methods of preparation were used in an attempt to produce extracts with a chemical profile that may have been more closely representative of those used in actual ethnomedical practice in comparison to exhaustive or standardised extracts in organic solvents. This was carried out on the assumption that if active compounds which were responsible for the ethnomedical use and selection of these species were present, then these compounds should be expected to occur in quantities sufficient to produce meaningful levels of biological activity at the extract concentrations produced by 'traditional' methods of preparation (notwithstanding the possibility of pro-drug molecules as 'active constituents' or possible failures of *in vitro* test systems to adequately mimic *in vivo* conditions). Exhaustive extraction of medicinal plant parts in organic solvents for biological study offers the advantage of obtaining a more complete phytochemical profile of any given plant material and is thus an appropriate method for studies where discovery of novel compounds as candidates for industry or drug development forms the central aim of investigation. Such extracts, however, may be less suitable for the purpose of providing rationale for, or corroboration of traditional indigenous medicinal uses. While a detailed investigation into the chemical composition of extracts was not pursued in this study, HPLC voucher chromatograms of each aqueous preparation were obtained. The chromatogram obtained from separation of the decoction of *E. alternifolia* leaves is shown below alongside separation of the corresponding tertiary MeOH extract under the same conditions in Figure 7.1, to illustrate some of the differences in chemical composition which were observed between the

two types of extract. Between these two extracts, it can be seen that a number of the more non-polar constituents (peaks eluting between Rt 38 and 45 min), are present in significantly greater quantities in the MeOH extract and a large peak at ca. Rt 42.7 min appears to be absent from the aqueous preparation. Conversely, many of the more polar constituents eluting earlier from the reverse phase column are, not surprisingly, present at greater quantities in the aqueous preparation.

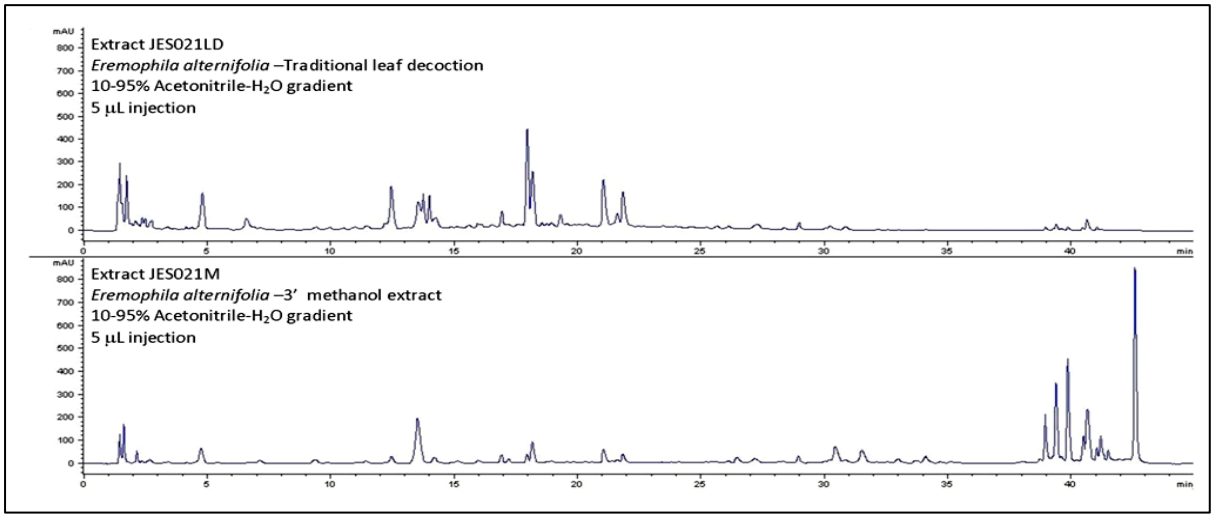


Figure 7.1 HPLC chromatogram of *Eremophila alternifolia* traditional decoction of leaves with tertiary methanol extract of same material for comparison (Detector: UV abs λ254nm, chromatograms scaled equally)

While the aqueous preparations examined in this study were almost certainly more closely representative of those used in indigenous traditional medicine than the solvent extracts, it was acknowledged that the preparations were produced following indirect descriptions from the ethnobotanical literature and may not have been adequately representative of those produced by indigenous people more knowledgeable in these cultural practices. Additionally, during preparation of the aqueous extracts, a number of steps were carried out which while unavoidable in the context of the planned experimental work, may have significantly affected chemical composition (e.g. sterilisation by filtration, lyophilisation). In many instances, aqueous preparations of the type examined in this study are typically used soon after preparation in traditional medicinal practice. While steps were taken to generally minimise

exposure of the extracts between preparation and various tests (e.g. by lyophilisation and storage at low temperature), as aqueous extracts, it is possible that some constituents may have been subject to enzymatic degradation or conversion (particularly among the infusions where extraction took place at low temperatures and where it is possible that enzymes were extracted and present in an active or non-denatured form). It was also acknowledged that preparations examined in the study were restricted to those which were liquid in form (i.e. aqueous extractions and expressed juices of plant material) in order to facilitate ease of handling and manipulation for the purposes of *in vitro* bioassay techniques. As a consequence, preparations such as poultices and pastes of whole or macerated plant material, and unguents were effectively excluded. Extractions of plant material in relatively non-polar organic solvents may in fact be more closely representative of such preparations in terms of chemical profile, particularly in cases where traditional mode of preparation involves extraction into animal fat to produce an ointment.

7.1.3 **Biological activity and major use agreement**

Among the medicinal plant preparations, those cited for use in the treatment of sores and wounds were found to be associated with a greater incidence of and in some cases, higher level of biological activity. Significant associations were found between use agreement for these purposes and total phenolic content, antimicrobial activity and P388D1 cytotoxicity. Assuming the *in vitro* activity observed here is predictive of the *in* vivo situation, it is possible that the presence of antimicrobial activity and phenolic compounds may partly explain the use of these preparations in traditional indigenous medicine, particularly in those preparations where antibacterial activity was retained at high dilution. On the other hand, antimicrobial activity was found partly to be associated with total phenolic content and phenolic content was itself strongly associated with agreement in the use of individual preparation for the treatment of sores and wounds. It is possible, therefore, that the

antimicrobial activity observed may represent an *in vitro* artefact resulting from the concentration of phenolic species in each preparation. This may also be potentially true of the antioxidant capacity as measured using the DMPD assay which was also found to associate with use agreement/citation for wounds and sores. The potential benefit of free radical scavenging or antioxidant activity in the treatment of wounds is unclear. While some studies using *in vivo* wound models have demonstrated improvements in healing with the application of antioxidant based therapies, others have provided evidence that increasing oxidative stress in the wound environment can lead to improved healing outcomes via modulation of oxidant dependent signalling processes. Interestingly, no association at any level could be demonstrated between agreement and frequency of citation for any documented dermatological use and antioxidant capacity as measured by the ORAC assay (which provides a measure of total antioxidant capacity in samples). The significance of the strong association found between agreement in the ethnobotanic record for treatment of sores and wounds among the preparations and cytotoxicity could not be explained and it is possible that this represents an artefact of the *in vitro* test procedures. It would be very unlikely that these preparations would have been selectively chosen for treatment of wounds and dermatoses if significant cytotoxic action was operating *in vivo* with use in traditional ethnomedical practice. While some association seemed to occur between agreement for treatment of sores and inhibition of PGE_2 formation, significance was not reached and a number of preparations were not included in the analyses due to failure of the assays which could not be repeated. Interestingly, some level of PGE_2 inhibition was seen for all four preparations for which records of traditional use in the treatment of fever was found. While this association could not be shown to be statistically significant due to the small number of cases involved, it is reasonable to suggest that inhibition of PGE_2 formation may be a likely possibility in drugs of unknown composition/mode of action which are used as febrifuges in traditional

ethnomedicine. In the ethnobotanical records surveyed a total of 25 species were explicitly cited as treatments for fever and many more indirectly as remedies for symptoms of flu-like illnesses and other febrile conditions. Screening of extracts of these species for PGE_2 inhibition and associated activities (e.g. cyclooxygenase inhibition) may be valuable, particularly given that there is continuing interest in the discovery and development of novel COX-2 inhibitors (Everts *et al.*, 2000).

Overall, these findings would suggest that probability of finding Australian plant species which yield extracts of higher phenolic content may be increased by selection of species frequently or specifically cited in the ethnobotanical literature for the treatment of sores and wounds. This finding would also suggest that relatively high phenolic content in plant based medicinal preparations, may contribute in some way to biological effectiveness in the treatment of sores and wounds and that plants or plant parts capable of yielding preparations with high phenolic content, may be more likely to have been selected specifically for these purposes over time and incorporated into established traditional practice than those lacking this characteristic. These results are supportive of similar findings described by de Sousa Araújo *et al.* (2008), where a strong association between tannin content and effects attributed to wound healing and anti-inflammatory activity were found among plants used medicinally by communities of the Altinho municipality of north-western Brazil. As a form of illness or disease, wounds caused by mechanical injury may be expected to occur in all cultures, irrespective of differences in climate and geography, and it is likely that all traditional medicinal systems and ethnopharmacopoeias contain at least some plant-based remedies for the treatment of wounds. It is possible that the association between phenolic content and traditional use for the treatment of wounds and sores described in this study [and that between tannin content and use for wound healing as described by de Sousa Araújo *et al.* (2008)], may occur more or less globally, and may represent a general feature of medicinal

plants used for this purpose. If this is the case then these results may have important implications for both future studies investigating medicinal plants used traditionally for these purposes, and/or for bioprospecting efforts aimed at characterisation of phenolic compounds and tannins outside of these regions. These results may also provide indirect support for the paradigm of the involvement of oxidants in wound healing as advanced by Sen *et al.* (2006) and potential for oxidant based therapies, as was demonstrated with tannin containing grape seed extracts (Khanna *et al.*, 2001; Khanna *et al.*, 2002), if the possibility of polyphenolics oxidising to quinonoids in the wound environment, and acting to generate oxidants such as H_2O_2 is accepted.

It should be noted, however, that the associations described above, while statistically significant, were not entirely consistent and some exceptions were seen where species/prepations with a high level of agreement were not found to contain high TPC or to exhibit significant biological activity. Similarly, a number of preparations for which low levels of agreement for citations describing the treatment of wounds and sores did exhibit high TPC and biological activity. The parameters examined in this study, however, represent only a relatively narrow range of biological activities which could potentially explain traditional medicinal use and the presence or absence of any activity in any case would not necessarily preclude any other. As discussed in Chapter 1, high or low levels of agreement in the specific medicinal use of taxa either between individual informants or separate ethnobotanical reports, while generally thought of as indicative of perceived efficacy in traditional medicine, may equally be representative of a number of ecological and/or culture specific factors exclusive of any actual biomedical efficacy.

The application of MUA to previously recorded ethnobotanical data encompassing large geographical areas as was attempted in this study, may be subject to similar distortions as

described above for the CAR. For example, species which are uncommon, or narrowly distributed may be relatively underepresented in the literature and be given a falsely low ranking which does not reflect the actual cultural importance in areas in which they occur and are used. Additionally, MUA applied in this manner would not account for the significance of any intraspecific phytochemical variability which may have led to medicinal use in specific regions and may be expected to occur more frequently as larger geographic areas are covered. Despite these inherent problems, application of the technique seemed to produce meaningful results in the context of this study and it is interesting that associations between use agreement and biological activity etc. were demonstrable for extracts prepared following documented traditional methods.

For the activities and parameters that were examined in the study, relatively high levels of biological activity and total phenolic content were exhibited by several of the traditional preparations screened. These included: The expressed juice of *C. fraseri* fruit pulp (relatively high TPC and antioxidant capacity and high levels of antimicrobial activity), the decoction of juvenile terminal leaves of *E. tetradonta*, decoction of inner trunk bark of *B. obovata* and the decoction of trunk inner bark from *E. chlorostachys* (relatively high antimicrobial activity, TPC and antioxidant capacity, and PGE_2 inhibitory activity). Further phytochemical work combined with bioactivity guided fractionation to identify compounds responsible for activity in extracts of these species may be warranted.

The plants chosen for investigation in this study did not represent an exhaustive sample and potential exists for further work to both verify and/or further test and refine these findings (e.g. with other species frequently cited for treatment of dermatological conditions in Australian indigenous ethnomedicine which were not examined in this study such as *Protasparagus racemosus*, *Planchonia careya* and *Terminalia carpenteriae* etc.).

Additionally, while higher levels of phenolic content were found to be associated with agreement in the ethnobotanical literature for treatment of wounds and sores, further chemical work aimed toward identification of specfic classes or compounds was not pursued. Identification of phenolic compounds in the species/extracts examined here, and others frequently cited for treatment of sores and/or wounds may be valuable in order to determine whether any particular class or type predominates in this group of plants. It may be useful for example to determine whether tannins, or monomeric flavonoids predominate in this group of plants, and whether potential differences in the redox chemistry of any predominating class or compound are significant (given that this property may be relevant to wound healing and that differences in redox behaviour have been demonstrated amongst polyphenol classes). Such information could be used to further understanding of the traditional use as therapies for wound healing and treatment of dermatoses in general, (i.e. with previously characterised biological activities of any identified compounds/classes) or, even to suggest other, as yet unexplored, activities in these compounds.

7.1.4 **Serrulatane diterpenes in traditional aqueous preparations of *E. duttonii***

The relatively modest antibacterial activity exhibited by the traditional aqueous decoctions of *Eremophila duttonii* stands in contrast to findings of previous studies, in which ethanolic and other relatively non-polar organic solvent extracts have been shown to exhibit exceptional activity. This result followed the general tendency which was seen in this study where organic solvent extracts exhibited a higher incidence of antibacterial activity and potency when compared to corresponding traditional type aqueous preparations.

Only one of the diterpenes previously identified was detected in the aqueous extracts (serrulatat-14-en-3,7,8,20-tetraol) which in this study was found to exhibit slightly less antibacterial potency than the fraction containing the serrulatane triol. Estimations of

biological activity of fractions containing these compounds and quantitative assays in traditional decoctions were complicated by the fact that these compounds were found to be unstable in solution. For this reason an accurate estimation of quantity in traditional aqueous decoctions was not obtained as products appeared in the sample used as a quantitative standard between purification and quantitative assays. Despite the problems which were encountered, it can be stated with relative certainty that the serrulatane tetraol was detected in the decoctions in quantities which were associated with inhibitory activity (particularly as it is likely that the activity exhibited by the purified fraction itself was potentially underestimated, given that oxidation of this compound was found to occur after separation). Given that the serrulatanes appear to be unstable, it is possible that actual traditional preparations may typically contain greater quantities of this compound for an equivalent quantity of plant material extracted than were seen in the preparations examined in this study (which while lyophilised and stored at low temperature immediately following extraction, were nonetheless stored for periods up to 18 months before these assays were conducted). Interestingly, Barr *et al.* (1993) have indicated that traditional preparations of this plant are used relatively quickly following preparation as it is considered by indigenous users to "deteriorate" with storage.

Fractions containing the serrulatane diterpenes were also found to exhibit relatively high levels of antioxidant capacity and *in vitro* cytotoxicity. During the final stages of preparation of this thesis, work was published describing instability of the serrulatanes from *E. duttonii* and attempts to isolate the coloured compounds (Ndi *et al.*, 2011) (which in this study were present in the column chromatography fractions containing the serrulatanes prior to purification and apparently reformed after storage of the purified material). Ndi *et al.* (2011) have suggested that the degradation products may represent products of oxidation of the serrulatane diterpenes to quinonoid forms and have further suggested that the antibacterial

mode of antimicrobial activity in these compounds may involve redox activity rather than via effects on membrane fluidity which have been associated with other antimicrobial terpenoids. The possibility of the serrulatanes forming quinonoid oxidation products may explain the high levels of cytotoxicity observed in fractions containing these compounds as some phenolic species are known to oxidise to quinones and semi-quinones in the presence of metal ions in media containing foetal calf serum, which then may be capable of generating cytotoxic levels of H_2O_2 and $O_2^{\bullet-}$. The same may be true of the behaviour of these compounds in microbial media and in other solvent environments of test systems which may favour oxidation of the hydroxylated serrulatanes to a quinonoid form.

The variability in antimicrobial activity among the serrulatane diterpenes described here and in other studies warrants further studies to examine structure-activity relationships within this group of compounds, particularly given the structural variety of the currently known serrulatanes and the potential usefulness of these compounds as outlined by Griesser *et al.* (2008) and Vasilev *et al.* (2009), along with the investigation of the potential for anti-mycobacterial activity among members of this class, as has been demonstrated in compounds of similar structure (Molina-Salinas *et al.*, 2011).

7.1.5 **Novel essential oil chemotypes of *Eremophila longifolia***

Investigation of variability in essential oil composition of *E. longifolia* resulted in the tentative identification of three new proposed chemotypes. While chemotype was determined, no further investigations into possible causes of the variation observed were conducted and it is not known whether the variations observed represent the results of local environmental factors on biosynthesis (e.g. as an alleopathic response to predation etc.), or stable genetic differences in these populations. Aqueous traditional decoctions prepared after descriptions given by Barr *et al.* (1988) from material representative of each of the three chemotypes

identified were found to exhibit significant differences in levels of total phenolic content and antioxidant capacity. In particular, the decoction prepared using leaves of the karahanaenone type was found to exhibit significantly higher TPC and antioxidant capacity. From anecdotal information, decoctions of the plant appear to be used in some parts of the study area primarily as a 'washing medicine' (rubbed into the body and used for general well being and health). In regions where the karahanaenone and isomenthone types were found, historical records refer to the use of *E. longifolia* as a topical treatment for headache (Bonney, 1884; Cited in Martin, 2001). The borneol type (for which detectable levels of PGE_2 inhibitory activity was found in decoctions) occurs nearest to a region where records describe of decoctions for the treatment of sores (i.e. Bogan River region, NSW) (Webb, 1969). Apart from these examples, however, little ethnobotanical information was found describing medicinal use among people of NSW where the plants were collected, and so it is difficult to speculate as to whether the differences observed in chemical composition are reflected in any specific documented traditional medicinal uses.

The range of chemotypes and chemical variability described in this study was derived using a relatively small sample of plant specimens. It is likely that the chemo-variability in this species is far more complex than is indicated by these results. Given the apparently popular and widespread contemporary use of this species, and the possibility that some populations may contain toxic phenylpropanoids, further investigation to fully characterise the extent and geographic range of chemical variability may be warranted. It may also be valuable to investigate pharmacognosy and biological activity of typical preparations in other areas where this species is used and where more robust and detailed documentation of traditional medicinal use exists. While safrole and methyleugenol have been detected in plants in WA there is at present no evidence to suggest that these compounds accumulate in the leaves of populations in the NT and NSW. Earlier cautions against use of these preparations due to the

possibility that they may contain safrole may therefore be unjustified in certain regions where the plant remains in use. Additionally, safrole is known to be heat labile and it is possible that extraction by decoction may reduce or remove this compound from such preparations. Reynertson *et al.* (2005) have shown that traditional preparation of material from the Polynesian medicinal plant *Cinnamomum carolinense* with boiling in water, completely eliminates the safrole which can be detected in low temperature and organic solvent extracts of the wood of this species. It is possible that decoctions prepared from *E. longifolia* material containing safrole may in fact be similarly free of the compound.

Several Australian plant species and indigenous medicinal plant preparations are reported to be used in preference to contemporary orthodox pharmaceuticals in the treatment of some minor conditions by both indigenous people and in some instances by people of other cultural groups (e.g. use of *Bauhinia cunninghamii* root decoctions by non-indigenous people at Elliot, NT) (Wightman *et al.*, 1992; Barr *et al.*, 1993). Given that in some locations, indigenous plant based medicinal preparations are now being incorporated into orthodox medical practice as a form of cultural engagement, further studies on the pharmacognosy and biological activity of these materials are warranted. Apart from contributing to a better understanding of these preparation within the sphere of health and medical practice in regions servicing indigenous populations, a more complete knowledge of the preparations used in traditional ethnomedicine and the rationale behind selection of specific taxa, can also be used to refine and inform investigations seeking to explore the medicinal flora for the purpose of finding novel leads for drug discovery and industry.

With the exception of the traditional aqueous decoctions of *Eremophila sturtii* and *Bauhinia cunninghamii*, the work presented in this thesis represents the first report on biological activity and chemical composition in traditional type aqueous extracts of the species

examined. In conclusion, the work carried out in this study has provided evidence for the association of a number of biological activities and phenolic content in Australian medicinal species with traditional use in the treatment of sores and wounds and also for the potential usefulness of using agreement present in the contemporary Australian ethnobotanic literature to aid in the selection of plant species for biological and chemical studies.

References

Adams, R. P. (2001). *Identification of Essential Oil Components by Gas Chromatography/Quadrupole Mass Spectrometry*. Carol Stream, IL, Allured.

Adetutu, A., Morgan, W. and Corcoran, O. (2010). "Antibacterial; antioxidant and fibroblast growth stimulation activity for crude extracts of *Bridelia ferruginea* leaf; a wound-healing plant of Nigeria." *Journal of Ethnopharmacology* **133**(1): 116-119.

Afsharypuor, S. and Jahromy, M. M. (2003). "Constituents of the essential oil of Tanacetum lingulatum (Boiss.) Bornm." *Journal of Essential Oil Research* **15**: 74-76.

Agyare, C., Lechtenberg, M., Deters, A., Petereit, F. and Hensel, A. (2010). "Ellagitannins from *Phyllanthus muellerianus* (Kuntze) Exell.: Geraniin and furosin stimulate cellular activity, differentiation and collagen synthesis of human skin keratinocytes and dermal fibroblasts." *Phytomedicine* **18**(7): 617-624.

Ainsworth, E. A. and Gillespie, K. M. (2007). "Estimation of total phenolic content and other oxidation substrates in plant tissues using Folin–Ciocalteu reagent." *Nature Protocols* **2**(4): 875-877.

Albert, C. (1992). "How Bush Medicine is Still Used Today in Kulumindi." *Aboriginal and Islander Health Worker Journal* **16**(6): 4-5.

Altavilla, D., Saitta, A., Cucinotta, D., Galeano, M., Deodato, B., Colonna, M., Torre, V., Russo, G., Sardella, A. and Urna, G. (2001). "Inhibition of lipid peroxidation restores impaired vascular endothelial growth factor expression and stimulates wound healing and angiogenesis in the genetically diabetic mouse." *Diabetes* **50**(3): 667.

Amarozo, M. C. M. and Gely, A. (1988). "Uso de plantas medicinais por caboclos do baixo Amazonas Barcarena, PA, Brasil." *Boletim do Museu Paraense Emilio Goeldi, Serie Botanica* **4**: 47-131.

Amiguet, V. T., Arnason, J. T., Maquin, P., Cal, V., Pablo, S. Á. V. and Luis, P. A. (2006). "A regression analysis of q'eqchi'Maya medicinal plants from southern Belize." *Economic Botany* **60**(1): 24-38.

Andres-Lacueva, C. A., Medina-Remon, A., Llorach, R., Urpi-Sarda, M., Khan, N., Chiva-Blanch, G., Zamora-Ros, R., Raotches-Ribalta, M. and Lamuela-Raventos, R. M. (2010). Phenolic compounds: Chemistry and occurence in Fruits and vegetables. *Fruit and Vegetable Phytochemicals: Chemistry, Nutritional Value, and Stability*. L. A. de la Rosa, E. A. Parrilla and G. A. Gonzalez-Aguilar. Ames, Wiley-Blackwell**:** 53-88.

Annan, K. and Houghton, P. J. (2008). "Antibacterial, antioxidant and fibroblast growth stimulation of aqueous extracts of *Ficus asperifolia* Miq. and *Gossypium arboreum L*., wound-healing plants of Ghana." *Journal of Ethnopharmacology* **119**(1): 141-144.

Aquilano, K., Baldelli, S., Rotilio, G. and Ciriolo, M. R. (2008). "Role of nitric oxide synthases in Parkinson's disease: a review on the antioxidant and anti-inflammatory activity of polyphenols." *Neurochemical research* **33**(12): 2416-2426.

AWMA (2011). *Position Document of the Australian Wound Management Association: Bacterial impact on wound healing: From contamination to infection*. West Leederville, Cambridge Publishing.

Barlow, B. A. (1971). "Cytogeography of the genus *Eremophila*." *Australian Journal of Botany* **19**(3): 295-310.

Barr, A., Chapman, J., Smith, N. and Beveridge, M. (1988). *Traditional Bush Medicines: An Aboriginal Pharmacopoeia*. Melbourne, Greenhouse Publications.

Barr, A., Chapman, J., Smith, N. and Wightman, G. M. (1993). *Traditional Aboriginal Medicines in the Northern Territory of Australia*. Darwin, Conservation Commission of the Northern Territory of Australia.

Basedow, H. (1932). "Diseases of the Australian Aborigines." *Journal of Tropical Medicine and Hygiene* **35**(18): 117-193.

Bazhina, G. N. and Manukov, É. (1995). "Oxygen-containing terpenoids of the 1, 1, 4-trinethylcycloheptane series." *Chemistry of Natural Compounds* **31**(4): 423-434.

Belfrage, M. (2007). "Why "culturally safe" health care." *Medical Journal Of Australia* **186**(10): 2.

Benkaci-Ali, F., Baaliouamer, B. Y. and Meklati, B. Y. (2006). "Kinetic study of microwave extraction of essential oil of Nigella sativa L. seeds." *Chromatographia* **64**: 227-331.

Blake, N. M., Wightman, G. and Williams, L. (1998). *Iwaidja Ethnobotany. Aboriginal Plant Knowledge from Gurig National Park, Northern Australia*. Northern Territory Botanical Bulletin no. 23. Darwin, Parks and Wildlife Commision of the Northern Territory.

Bonney, F. (1884). "On Some Customs of the Aborigines of the River Darling, New South Wales." *Journal of the Anthropological Institute of Great Britain and Ireland* **13**: 122-137.

Bors, W. and Michel, C. (2002). "Chemistry of the antioxidant effect of polyphenols." *Annals of the New York Academy of Sciences* **957**(1): 57-69.

Bowen, S. E. (1975). Taxonomic studies in the Myoporaceae. *Department of Botany*. Armidale, The University of New England. **BSc (Hons)**.

Buckingham, J. (1999). Dictionary of Natural Products, Chapman & Hall/CRC. **10**.

Burford, G., Bodeker, G. and Ryan, T. J. (2007). Skin and wound care: Traditional, complementary and alternative medicine in public health dermatology. *Traditional, complementary and alternative medicine: policy and public health perspectives*. G. Bodeker and G. Burford. London, Imperial College Press**:** 311 - 347.

Burkey, J. L., Sauer, J. M., McQueen, C. A. and Glenn Sipes, I. (2000). "Cytotoxicity and genotoxicity of methyleugenol and related congeners - a mechanism of activation for methyleugenol." *Mutation Research/Fundamental and Molecular Mechanisms of Mutagenesis* **453**(1): 25-33.

Bushnell, O. A., Fukuda, M. and Makinodan, T. (1950). "The antibacterial properties of some plants found in Hawaii." *Pacific Science* **4**: 168-181.

Cai, Y., Luo, Q., Sun, M. and Corke, H. (2004). "Antioxidant activity and phenolic compounds of 112 traditional Chinese medicinal plants associated with anticancer." *Life Sciences* **74**(17): 2157-2184.

CANBR. (2010). "Australian Plant name Index." *IBIS Database*, 2010, from http://www.cpbr.gov.au/cgi-bin/apni.

Cao, G. and Prior, R. L. (1998). "Comparison of different analytical methods for assessing total antioxidant capacity of human serum." *Clinical Chemistry* **44**(6): 1309-1315.

Chah, K., Eze, C., Emuelosi, C. and Esimone, C. (2006). "Antibacterial and wound healing properties of methanolic extracts of some Nigerian medicinal plants." *Journal of Ethnopharmacology* **104**(1-2): 164-167.

Chapman, A. D. (2009) *Number of Living Species in Australia and the World* D. o. S. E. W. P. a. Communities Canberra, Australian Biological Resources Study/ Genetic Resources Management

Chaudhari, M. and Mengi, S. (2006). "Evaluation of phytoconstituents of *Terminalia arjuna* for wound healing activity in rats." *Phytotherapy Research* **20**(9): 799-805.

Chen, S. J., Wang, M. H. and Chen, I. J. (1996). "Antiplatelet and calcium inhibitory properties of eugenol and sodium eugenol acetate." *General Pharmacology: The Vascular System* **27**(4): 629-633.

Cheng, T., Cao, W., Wen, R., Steinberg, R. H. and LaVail, M. M. (1998). "Prostaglandin E_2 induces vascular endothelial growth factor and basic fibroblast growth factor mRNA expression in cultured rat Müller cells." *Investigative Ophthalmology & Visual Science* **39**(3): 581-591.

Chinnock, R. J. (2007). *Eremophila and Allied Genera: A Monograph of the plant family Myoporaceae*. Sydney, Rosenberg.

Chosidow, O. (2000). "Scabies and pediculosis." *The Lancet* **355**(9206): 818-818.

Cochrane, C. B. (1998). *Antibacterial and Antifungal Screening of Florida's Exotic Invasive Plant Species*. 1998 joint symposium of the Florida Exotic Pest Plant Council and the Florida Native Plant Society, Palm Beach Gardens, Florida, South Florida Water Management District.

Coelho de Souza, G., Haas, A., Von Poser, G., Schapoval, E. and Elisabetsky, E. (2004). "Ethnopharmacological studies of antimicrobial remedies in the south of Brazil." *Journal of Ethnopharmacology* **90**(1): 135-143.

Cool, L. G., Jiang, K. and Zavarin, E. (1994). "Karahanaenone in coastal Californian Cupressus species." *Biochemical Systematics and Ecology* **22**: 857.

Cowan, M. M. (1999). "Plant products as antimicrobial agents." *Clinical microbiology reviews* **12**(4): 564-582.

Cox, P. A. (1994). The ethnobotanical approach to drug discovery: strengths and limitations. *Ethnobotany and the Search for New Drugs - Ciba Foundation Symposium 185*. D. J. Chadwick, J. Marsh and G. T. Prance. Chichester, Wiley**:** 25-42.

Cox, S., Mann, C., Markham, J., Bell, H., Gustafson, J., Warmington, J. and Wyllie, S. (2000). "The mode of antimicrobial action of the essential oil of *Melaleuca alternifolia* (tea tree oil)." *Journal of Applied Microbiology* **88**(1): 170-175.

Cox, S. D., Mann, C. M. and Markham, J. L. (2001). "Interactions between components of the essential oil of *Melaleuca alternifolia*." *Journal of Applied Microbiology* **91**(3): 492-497.

Crawford, I. M. (1982). *Traditional Aboriginal Plant Resources in the Kalumburu Area: Aspects in Ethno-economics*. Perth, Western Australian Museum.

Cristani, M., D'Arrigo, M., Mandalari, G., Castelli, F., Sarpietro, M. G., Micieli, D., Venuti, V., Bisignano, G., Saija, A. and Trombetta, D. (2007). "Interaction of four monoterpenes contained in essential oils with model membranes: implications for their antibacterial activity." *Journal of Agricultural and Food Chemistry* **55**(15): 6300-6308.

Croft, K., Ghisalberti, E., Jefferies, P. and Proudfoot, G. (1981). "The chemistry of *Eremophila* spp. XVI. New serrulatanes from *Eremophila* spp." *Australian Journal of Chemistry* **34**(9): 1951-1957.

Currie, B. J. and Carapetis, J. R. (2000). "Skin infections and infestations in Aboriginal communities in northern Australia." *Australasian journal of dermatology* **41**(3): 139-143.

de Albuquerque, U. P. (2010). "Implications of ethnobotanical studies on bioprospecting strategies of new drugs in semi-arid regions." *The Open Complementary Medicine Journal* **2**: 21-23.

de Albuquerque, U. P., Andrade, L. H. C. and Silva, A. C. O. (2005). "Use of plant resources in a seasonal dry forest (northeastern Brazil)." *Acta Botanica Brasilica* **19**(1): 27-38.

de Lima, G. O., D'Albuquerque, I. L., Magalhaes Neto, B. and Albuquerque, M. M. (1958). "Breve nota sobre a atividade antimicrobiana da biflorina purificada por partição Craig." *Revista do Instituto de Antibióticos* **1**(2): 95-98.

de Sousa Araújo, T. A., Alencar, N. L., de Amorim, E. L. C. and de Albuquerque, U. P. (2008). "A new approach to study medicinal plants with tannins and flavonoids contents from the local knowledge." *Journal of Ethnopharmacology* **120**(1): 72-80.

Della, E. W. and Jefferies, P. R. (1961). "The Chemistry of *Eremophila* Species III: The Essential oil of *Eremophila longifolia* F. Muell." *Australian Journal of Chemistry* **14**: 663-664.

Dempke, W., Rie, C., Grothey, A. and Schmoll, H. J. (2001). "Cyclooxygenase-2: a novel target for cancer chemotherapy?" *Journal of Cancer Research and clinical Oncology* **127**(7): 411-417.

Devanesen, D. (2000). *Traditional Aboriginal Health Practice in the Northern Territory.* International Symposium on Traditional Medicine, Awaji Island, Japan,

Dixon, A. R., McMillen, H. and Etkin, N. L. (1999). "Ferment this: the transformation of Noni, a traditional Polynesian medicine (*Morinda citrifolia*, Rubiaceae)." *Economic Botany* **53**(1): 51-68.

Dorman, H. and Deans, S. (2000). "Antimicrobial agents from plants: Antibacterial activity of plant volatile oils." *Journal of Applied Microbiology* **88**(2): 308-316.

DOTEHWATA. (2006, 01/06/2010). "Australia's Bioregions." Retrieved 01/06/2010, 2010, from http://www.environment.gov.au/parks/nrs/science/ibra.html.

Dowell, A. and Morris, C. A. (2008). *Opportunities for Endemic Medicinal Plants Listed with the Therapeutic Goods Administration.* (Invited Oral Presentation). CRCDK Business Showcase and Symposium, Alice Springs, Australia,

Durupt, F., Mayor, L., Bes, M., Reverdy, M. E., Vandenesch, F., Thomas, L. and Etienne, J. (2007). "Prevalence of *Staphylococcus aureus* toxins and nasal carriage in furuncles and impetigo." *British Journal of Dermatology* **157**(6): 1161-1167.

Eloff, J. N. (1998). "A sensitive and quick microplate method to determine the minimal inhibitory concentration of plant extracts for bacteria." *Planta medica* **64**(8): 711.

Everts, B., Währborg, P. and Hedner, T. (2000). "COX-2-specific inhibitors–the emergence of a new class of analgesic and anti-inflammatory drugs." *Clinical rheumatology* **19**(5): 331-343.

Farnsworth, N. R. (1994). Ethnopharmacology and drug development. *Ethnobotany and the Search for New Drugs - Ciba Foundation Symposium 185*. D. J. Chadwick, J. Marsh and G. T. Prance. Chichester, Wiley**:** 42-59.

Fogliano, V., Verde, V., Randazzo, G. and Ritieni, A. (1999). "Method for measuring antioxidant activity and its application to monitoring the antioxidant capacity of wines." *Journal of Agricultural and Food Chemistry* **47**(3): 1035-1040.

Forster, P. G., Ghisalberti, E. L., Jefferies, P. R., Poletti, V. M. and Whiteside, N. J. (1986). "Serrulatane diterpenes from *Eremophila* spp." *Phytochemistry* **25**(6): 1377-1383.

Fronza, M., Heinzmann, B., Hamburger, M., Laufer, S. and Merfort, I. (2009). "Determination of the wound healing effect of *Calendula* extracts using the scratch assay with 3T3 fibroblasts." *Journal of Ethnopharmacology* **126**(3): 463-467.

Gan, R. Y., Kuang, L., Xu, X. R., Zhang, Y., Xia, E. Q., Song, F. L. and Li, H. B. (2010). "Screening of natural antioxidants from traditional Chinese medicinal plants associated with treatment of rheumatic disease." *Molecules* **15**(9): 5988-5997.

Ghisalberti, E. (1994). "The ethnopharmacology and phytochemistry of *Eremophila* species (Myoporaceae)." *Journal of Ethnopharmacology* **44**(1): 1-9.

Ghisalberti, E. L. (1993). "The phytochemistry of the Myoporaceae." *Phytochemistry* **35**(1): 7-33.

Goddard, C. and Kalotas, A. (2002). *Punu, Yankunytjatjara Plant Use: Traditional Methods of Preparing Foods, Medicines, Utensils and Weapons from Native Plants*. Alice Springs, Jukurrpa Books/IAD.

Granger, R. E., Campbell, E. L. and Johnston, G. A. R. (2005). "(+)-And (-)-borneol: efficacious positive modulators of GABA action at human recombinant a1ß2?2L GABAA receptors." *Biochemical Pharmacology* **69**(7): 1101-1111.

Grice, I., Garhnam, B., Pierens, G., Rogers, K., Tindal, D. and Griffiths, L. (2003). "Isolation of two phenylethanoid glycosides from *Eremophila gilesii*." *Journal of Ethnopharmacology* **86**(1): 123-125.

Grice, I. D., Rogers, K. and Griffiths, L. R. (2011). "Isolation of Bioactive Compounds That Relate to the Anti-Platelet Activity of *Cymbopogon ambiguus*." *Evidence-Based Complementary and Alternative Medicine* **2011**: 1-8.

Griesser, H. J., Ys, H., Ndi, C. P., Britcher, L., Vasilev, K., Jasieniak, M., Griesser, S. S. and Semple, S. J. (2008). "Combating infections at biomedical implants and devices by antibacterial coatings." *Chemistry in Australia* **75**(10): 5-8.

Griffin, S. G., Wyllie, S. G., Markham, J. L. and Leach, D. N. (1999). "The role of structure and molecular properties of terpenoids in determining their antimicrobial properties." *Flavour and Fragrance Journal* **14**(5): 322-332.

Griffin, W., Phippard, J., Culvenor, C., Loder, J. and Nearn, R. (1971). "Alkaloids of the leaves of *Erythrophleum chlorostachys*." *Phytochemistry* **10**(11): 2793-2797.

Hagerman, A. E., Riedl, K. M., Jones, G. A., Sovik, K. N., Ritchard, N. T., Hartzfeld, P. W. and Riechel, T. L. (1998). "High molecular weight plant polyphenolics (tannins) as biological antioxidants." *Journal of Agricultural and Food Chemistry* **46**(5): 1887-1892.

Halkes, S. B. A., Van den Berg, A. J. J., Hoekstra, M. J., Du Point, J. S. and Kreis, R. W. (2001). "The use of tannic acid in the local treatment of burn wounds: Intriguing old and new perspectives." *Wounds* **13**(4): 144-158.

Halliwell, B. (2008). "Are polyphenols antioxidants or pro-oxidants? What do we learn from cell culture and in vivo studies?" *Archives of biochemistry and biophysics* **476**(2): 107-112.

Han, L., Huang, X., Dahse, H. M., Moellmann, U., Fu, H., Grabley, S., Sattler, I. and Lin, W. (2007). "Unusual naphthoquinone derivatives from the twigs of *Avicennia marina*." *Journal of Natural Products* **70**(6): 923-927.

Harborne, J. B. (1993). *Introduction to Ecological Biochemistry*. San Diego, Academic Press.

Hayhoe, E. J. and Palombo, E. A. (2011). "Extracts of *Eremophila longifolia* inhibit the cariogenic activities of *Streptococcus mutans* and *Streptococcus sobrinus*." *Journal of Medicinal Plants Research* **5**(12): 2476-2482.

Heinrich, M. (2003). "Ethnobotany and natural products: the search for new molecules, new treatments of old diseases or a better understanding of indigenous cultures?" *Current Topics in Medicinal Chemistry* **3**(2): 141-154.

Heinrich, M., Ankli, A., Frei, B., Weimann, C. and Sticher, O. (1998). "Medicinal plants in Mexico: Healers' consensus and cultural importance." *Social Science & Medicine* **47**(11): 1859-1871.

Heinrich, M., Edwards, S., Moerman, D. E. and Leonti, M. (2009). "Ethnopharmacological field studies: A critical assessment of their conceptual basis and methods." *Journal of Ethnopharmacology* **124**(1): 1-17.

Hemaiswarya, S., Poonkothai, M., Raja, R. and Anbazhagan, C. (2010). "Comparative study on the antimicrobial activities of three Indian medicinal plants." *Egyptian Journal of Biology* **11**: 52-57.

Houghton, P., Hylands, P., Mensah, A., Hensel, A. and Deters, A. (2005). "*In vitro* tests and ethnopharmacological investigations: wound healing as an example." *Journal of Ethnopharmacology* **100**(1-2): 100-107.

Huang, D., Ou, B., Hampsch-Woodill, M., Flanagan, J. A. and Deemer, E. K. (2002a). "Development and validation of oxygen radical absorbance capacity assay for lipophilic

antioxidants using randomly methylated-cyclodextrin as the solubility enhancer." *Journal of Agricultural and Food Chemistry* **50**(7): 1815-1821.

Huang, D., Ou, B., Hampsch-Woodill, M., Flanagan, J. A. and Prior, R. L. (2002b). "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." *Journal of Agricultural and Food Chemistry* **50**(16): 4437-4444.

Huang, D., Ou, B. and Prior, R. L. (2005). "The chemistry behind antioxidant capacity assays." *Journal of Agricultural and Food Chemistry* **53**(6): 1841-1856.

Hupkens, P., Boxma, H. and Dokter, J. (1995). "Tannic acid as a topical agent in burns: historical considerations and implications for new developments." *Burns* **21**(1): 57-61.

IARC (1976). *IARC Monographs on the Evaluation of Carcinogenic Risk of Chemicals to Man, Safrole, Isosafrole, and Dihydrosafrole*. Lyon, France, International Agency for Research on Cancer.

INGW. (2010, 12/11/2011). "Bush Medicine Knowledge." *Indigenous news government webpages* Retrieved 18/01/2011, 2011, from: http://www.indigenous.gov.au/index.php/stories/75_bush_medicine_knowledge/.

Inouye, S., Uchida, K., Maruyama, N., Yamaguchi, H. and Abe, S. (2006). "A novel method to estimate the contribution of the vapor activity of essential oils in agar diffusion assay." *Nihon Ishikin Gakkai Zashi* **47**(2): 91-98.

Isaacs, J. (1987). *Bush food: Aboriginal food and herbal medicine*. Sydney, Landsdowne.

Iwu, M. M. (1994). African medicinal plants in the search for new drugs based on ethnobotanical leads. *Ethnobotany and the search for new drugs: Ciba Foundation Symposium 185* D. J. Chadwick, J. Marsh and G. T. Prance. Chichester, John Wiley and Sons. **185:** 116-129.

Jorge, M. P., Madjarof, C., Ruiz, A. L. T. G., Fernandes, A. T., Rodrigues, R. A. F., de Oliveira Sousa, I. M., Foglio, M. A. and de Carvalho, J. E. (2008). "Evaluation of wound

healing properties of *Arrabidaea chica* Verlot extract." *Journal of Ethnopharmacology* **118**(3): 361-366.

Kahkonen, M. P., Hopia, A. I., Vuorela, H. J., Rauha, J. P., Pihlaja, K., Kujala, T. S. and Heinonen, M. (1999). "Antioxidant activity of plant extracts containing phenolic compounds." *Journal of Agricultural and Food Chemistry* **47**(10): 3954-3962.

Kalyanaraman, B. and Sohnle, P. G. (1985). "Generation of free radical intermediates from foreign compounds by neutrophil-derived oxidants." *Journal of Clinical Investigation* **75**(5): 1618-1622.

Katalinic, V., Milos, M., Kulisic, T. and Jukic, M. (2006). "Screening of 70 medicinal plant extracts for antioxidant capacity and total phenols." *Food Chemistry* **94**(4): 550-557.

Khanna, S., Roy, S., Bagchi, D., Bagchi, M. and Sen, C. K. (2001). "Upregulation of oxidant-induced VEGF expression in cultured keratinocytes by a grape seed proanthocyanidin extract." *Free Radical Biology and Medicine* **31**(1): 38-42.

Khanna, S., Venojarvi, M., Roy, S., Sharma, N., Trikha, P., Bagchi, D., Bagchi, M. and Sen, C. K. (2002). "Dermal wound healing properties of redox-active grape seed proanthocyanidins." *Free Radical Biology and Medicine* **33**(8): 1089-1096.

Kohyama, T., Ertl, R. F., Valenti, V., Spurzem, J., Kawamoto, M., Nakamura, Y., Veys, T., Allegra, L., Romberger, D. and Rennard, S. I. (2001). "Prostaglandin E_2 inhibits fibroblast chemotaxis." *American Journal of Physiology- Lung Cellular and Molecular Physiology* **281**(5): L1257.

Kokane, D. D., More, R. Y., Kale, M. B., Nehete, M. N., Mehendale, P. C. and Gadgoli, C. H. (2009). "Evaluation of wound healing activity of root of *Mimosa pudica*." *Journal of Ethnopharmacology* **124**(2): 311-315.

Kolodsick, J. E., Peters-Golden, M., Larios, J., Toews, G. B., Thannickal, V. J. and Moore, B. B. (2003). "Prostaglandin E_2 inhibits fibroblast to myofibroblast transition via E. prostanoid receptor 2 signaling and cyclic adenosine monophosphate elevation." *American Journal of Respiratory Cell and Molecular Biology* **29**(5): 537-544.

Kratchanova, M., Denev, P., Ciz, M., Lojek, A. and Mihailov, A. (2010). "Evaluation of antioxidant activity of medicinal plants containing polyphenol compounds. Comparison of two extraction systems." *Acta Biochimica Polonica* **57**(2): 229-234.

Kwee, L. M., Long, L. H., Whiteman, M. and Halliwell, B. (2003). "Factors affecting the ascorbate and phenolic dependent generation of hydrogen peroxide in Dulbeccos modified eagles medium." *Free radical research* **37**(10): 1123-1130.

Langfield, R. D., Scarano, F. J., Heitzman, M. E., Kondo, M., Hammond, G. B. and Neto, C. C. (2004). "Use of a modified microplate bioassay method to investigate antibacterial activity in the Peruvian medicinal plant *Peperomia galioides*." *Journal of Ethnopharmacology* **94**(2-3): 279-281.

Lassack, E. V. and McCarthy, T. M. (1997). *Australian Medicinal Plants*. Sydney, Reed New Holland.

Latz, P. K. (1995). *Bushfires & Bushtucker. Aboriginal Plant Use in Central Australia*. Alice Springs, IAD Press.

Lehne, R. A., Moore, L. A., Crosby, L. J. and Hamilton, D. B. (1998). *Pharmacology for nursing care*. Philadephia, Saunders.

Leonti, M., Fernando, R. R., Sticher, O. and Heinrich, M. (2003). "Medicinal flora of the Popoluca, Mexico: A botanical systematical perspective." *Economic Botany* **57**(2): 218-230.

Levitt, D. and Lyon, K. (1981). *Plants and People: Aboriginal Uses of Plants on Groote Eylandt*. Canberra, Australian Institute of Aboriginal Studies.

Li, R. W., Leach, D. N., Myers, S. P., Leach, G. J., Lin, G. D., Brushett, D. J. and Waterman, P. G. (2004). "Anti-inflammatory activity, cytotoxicity and active compounds of *Tinospora smilacina* Benth." *Phytotherapy Research* **18**(1): 78-83.

Li, R. W., Myers, S. P., Leach, D. N., Lin, G. D. and Leach, G. (2003). "A cross-cultural study: anti-inflammatory activity of Australian and Chinese plants." *Journal of Ethnopharmacology* **85**(1): 25-32.

Liao, Y. H., Houghton, P. J. and Hoults, J. (1999). "Novel and known constituents from *Buddleja* species and their activity against leukocyte eicosanoid generation." *Journal of Natural Products* **62**(9): 1241-1245.

Liddy, L. G., Martin, L. D., Huddlestone, J. G., Liddy, L. J., Liddy, H. I., McMah, C. G., Huddlestone, G. J., Harvey, M. and Wightman, G. (2006). *Wagiman Plants and Animals: Aboriginal Knowledge of Flora and Fauna from the Mid Daly River Area, Northern Australia*. Northern Territory Botanical Bulletin no. 30. Darwin, Department of Natural Resources, Environment and the Arts.

Lindsay, B. Y., Waliwararra, K., Milijat, F., Kuwarda, H., Pirak, R., Muyung, A., Pambany, E., Marryridj, J., Marrfurra, P. and Wightman, G. (2001). *MalakMalak and Matngala Plants and Animals: Aboriginal Flora and Fauna Knowledge from the Daly River Area, Northern Australia*. Northern Territory Botanical Bulletin no. 26. Darwin, Parks and Wildlife Commission of the Northern Territory.

Lis-Balchin, M., Deans, S. and Eaglesham, E. (1998). "Relationship between bioactivity and chemical composition of commercial essential oils." *Flavour and Fragrance Journal* **13**(2): 98-104.

Liu, Q. (2006a). An ethnopharmacological study of medicinal plants of the Kamilaroi and Muruwari aboriginal communitites in northern New South Wales. *Division of Environmental and Life Sciences, Dept. of Chemistry and Biomolecular Science*. Sydney, Macquarie University. **PhD:** 249.

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

Loder, J., Culvenor, C., Nearn, R., Russell, G. and Stanton, D. (1974). "Tumour inhibitory plants. New alkaloids from the bark of *Erythrophleum chlorostachys* (Leguminosae)." *Australian Journal of Chemistry* **27**(1): 179-185.

Loder, J. and Nearn, R. (1975). "Structure of norerythrostachaldine, a cytotoxic alkaloid from *Erythrophleum chlorostachys* (Leguminosae)." *Australian Journal of Chemistry* **28**(3): 651-656.

Look, S. A. and Fenical, W. (1987). "The seco-pseodopteosins, new anti-inflammatory diterpene-glycosides from a Caribbean gorgonian octocoral of the genus *Pseudopterogorgia*." *Tetrahedron* **43**(15): 3363-3370.

Look, S. A., Fenical, W., Jacobs, R. S. and Clardy, J. (1986). "The pseudopterosins: anti-inflammatory and analgesic natural products from the sea whip *Pseudopterogorgia elisabethae*." *Proceedings of the National Academy of Sciences of the United States of America* **83**(17): 6238.

Low, T. (1990). *Bush Medicine: A Pharmacopoeia of Natural Remedies*. Sydney, Angus and Robertson.

Mabberley, D. J. (2008). *Mabberley's plant-book: a portable dictionary of plants, their classification and uses*. Cambridge, Cambridge University Press.

Mahasneh, A. M. (2002). "Screening of some indigenous Qatari medicinal plants for antimicrobial activity." *Phytotherapy Research* **16**(8): 751-753.

Maiden, J. H. (1889). *The Useful Native Plants of Australia* Sydney, Turner & Henderson.

Majima, M., Hayashi, I., Muramatsu, M., Katada, J., Yamashina, S. and Katori, M. (2000). "Cyclooxygenase 2 enhances basic fibroblast growth factor induced angiogenesis through induction of vascular endothelial growth factor in rat sponge implants." *British Journal of Pharmacology* **130**(3): 641-649.

Mandal, P., Sinha Babu, S. and Mandal, N. (2005). "Antimicrobial activity of saponins from *Acacia auriculiformis*." *Fitoterapia* **76**(5): 462-465.

Mann, C. M. and Markham, J. L. (1998). "A new method for determining the minimum inhibitory concentration of essential oils." *Journal of Applied Microbiology* **84**(4): 538-544.

Marley, J. (2004). *Therapeutic Guidelines: Dermatology (Version 2)*. Melbourne, Therapeutic Guidelines Limited.

Marrfurra, P., Akanburru, M., Wawul, M., Kumunerrin, T., Adya, H., Kamarrama, K., Kanintyanyu, M., Waya, T., Kannyi, M. and Wightman, G. (1995). *Ngan'gikurunggur and Ngangiwumirri Ethnobotany: Aboriginal Plant Use from the Daly River Area, Northern*

Australia. Northern Territory Botanical Bulletin no. 22. Darwin, Conservation Commission of the Northern Territory.

Martin, S. (2001) *Aboriginal Cultural Heritage of the Menindee Lakes Area Part 1: Aboriginal Ties to the Land. (A Report to the Menindee Lakes Ecologically Sustainable Development Project Steering Committee* Buronga, NSW Department of Land and Water Conservation

Marwah, R. G., Fatope, M. O., Mahrooqi, R. A., Varma, G. B., Abadi, H. A. and Al-Burtamani, S. K. S. (2007). "Antioxidant capacity of some edible and wound healing plants in Oman." *Food Chemistry* **101**(2): 465-470.

Mauro, T. (2005). Natural Course of Wound Repair Versus Impaired Healing in Chronic Skin Ulcers. *Wound Healing and Ulcers of the Skin: Diagnosis and Therapy: The Practical Approach*. A. Shai and H. I. Maibach. Berlin, Springer.

McLennan, W. (1997). 1994 National Aboriginal and Torres Strait Islander Survey: Social Atlas. Canberra, Australian Bureau of Statistics, Australian Government Publishing Service.

McPhee, S. J., Papadakis, M. A., Gonzales, R., Zeiger, R. and Rabow, M. W. (2011). *Current Medical Diagnosis and Treatment 2011*. New York, Lange Medical Books/McGraw-Hill.

Mensah, A., Houghton, P., Dickson, R., Fleischer, T., Heinrich, M. and Bremner, P. (2006). "*In vitro* evaluation of effects of two Ghanaian plants relevant to wound healing." *Phytotherapy Research* **20**(11): 941-944.

Mensah, A., Sampson, J., Houghton, P., Hylands, P., Westbrook, J., Dunn, M., Hughes, M. and Cherry, G. (2001). "Effects of *Buddleja globosa* leaf and its constituents relevant to wound healing." *Journal of Ethnopharmacology* **77**(2-3): 219-226.

Miguel, M. (2010). "Antioxidant activity of medicinal and aromatic plants." *Flavour and Fragrance Journal* **25**: 291-312.

Miller, E. C., Swanson, A. B., Phillips, D. H., Fletcher, L., Liem, A. and Miller, J. A. (1983). "Structure-activity studies of the carcinogenicities in the mouse and rat of some naturally

occurring and synthetic alkenylbenzene derivatives related to safrole and estragole." *Cancer Research* **43**(3): 1124-1134.

Mimica-Dukic, N., Bozin, B., Sokovic, M., Mihajlovic, B. and Matavulj, M. (2003). "Antimicrobial and antioxidant activities of three *Mentha* species essential oils." *Planta Medica* **69**(5): 413-419.

Moerman, D. E. (1996). "An analysis of the food plants and drug plants of native North America." *Journal of Ethnopharmacology* **52**(1): 1-22.

Moerman, D. E. (2007). "Agreement and meaning: Rethinking consensus analysis." *Journal of Ethnopharmacology* **112**(3): 451-460.

Moerman, D. E., Pemberton, R. W., Kiefer, D. and Berlin, B. (1999). "A comparative analysis of five medicinal floras." *Journal of Ethnobiology* **19**(1): 49-70.

Molina-Salinas, G. M., Rivas-Galindo, V. M., Said-Fernandez, S., Lankin, D. C., Munoz, M. A., Joseph-Nathan, P., Pauli, G. F. and Waksman, N. (2011). "Stereochemical Analysis of Leubethanol, an Anti-TB-Active Serrulatane, from *Leucophyllum frutescens*." *Journal of Natural Products* **74**: 1842-1850.

Mulvaney, D. J. and Kamminga, J. (1999). *Prehistory of Australia*. Sydney, Allen and Unwin.

Naya, T. and Kotake, M. (1968). "New monoterpenoids from hop oil." *Tetrahedron Letters* **13**: 1645-1649.

NCCLS (1997). Performance standards for antimicrobial disk susceptibility tests. 6th ed. Approved standard. NCCLS document M2-A6. Wayne, National Committee for Clinical Laboratory Standards

NCCLS (1997b). Methods for dilution antimicrobial susceptibility testing for bacteria that grow aerobically. 4th ed. Approved standard. NCCLS document M7-A4. Wayne, National Committee for Clinical Laboratory Standards

NCCLS (2004). Method for antifungal disk diffusion susceptibility testing of yeasts. Approved guideline. NCCLS document M44-A. Wayne, National Committee for Clinical Laboratory Standards.

Ndi, C. P., Semple, S. J. and Griesser, H. J. (2011). "Instability of Antibacterial Serrulatane Compounds from the Australian Plant Species Eremophila duttonii." *Australian Journal of Chemistry*((Ahead of Print e-Publication)).

Ndi, C. P., Semple, S. J., Griesser, H. J. and Barton, M. D. (2007a). "Antimicrobial activity of some Australian plant species from the genus *Eremophila*." *Journal of Basic Microbiology* **47**(2): 158-164.

Ndi, C. P., Semple, S. J., Griesser, H. J., Pyke, S. M. and Barton, M. D. (2007b). "Antimicrobial compounds from the Australian desert plant *Eremophila neglecta*." *Journal of Natural Products* **70**(9): 1439-1443.

Ndi, C. P., Semple, S. J., Griesser, H. J., Pyke, S. M. and Barton, M. D. (2007c). "Antimicrobial compounds from *Eremophila serrulata*." *Phytochemistry* **68**(21): 2684-2690.

O'Connell, J. F., Latz, P. K. and Barnett, P. (1983). "Traditional and modern plant use among the Alyawara of central Australia." *Economic Botany* **37**(1): 80-109.

Okuda, T. (2005). "Systematics and health effects of chemically distinct tannins in medicinal plants." *Phytochemistry* **66**(17): 2012-2031.

Oliveira, D. R., Leitão, G. G., Coelho, T. S., Silva, P. E., Lourenço, M. C. S. and Leitão, S. G. (2011). "Ethnopharmacological versus random plant selection methods for the evaluation of the antimycobacterial activity." *Revista Brasileira de Farmacognosia* (Ahead of print e-publication): 0-0.

Oliveira, D. R., Leitao, G. G., Santos, S. S., Bizzo, H. R., Lopes, D., Alviano, C. S., Alviano, D. S. and Leitao, S. G. (2006). "Ethnopharmacological study of two *Lippia* species from Oriximiná, Brazil." *Journal of Ethnopharmacology* **108**(1): 103-108.

Olmstead, R. G., dePamphilis, C. W., Wolfe, A. D., Young, N. D., Elisons, W. J. and Reeves, P. J. (2001). "Disintegration of the Scrophulariaceae." *American Journal of Botany* **88**(2): 348-361.

Owen, R. J. and Palombo, E. A. (2007). "Anti-listerial activity of ethanolic extracts of medicinal plants, *Eremophila alternifolia* and *Eremophila duttonii*, in food homogenates and milk." *Food Control* **18**(5): 387-390.

Palombo, E. A. and Meilak, M. (2008). "Anti-mycobacterial activity of extracts derived from Australian medicinal plants." *Research Journal of Microbiology* **3**(7): 535-538.

Palombo, E. A. and Semple, S. J. (2001). "Antibacterial activity of traditional Australian medicinal plants." *Journal of Ethnopharmacology* **77**(2-3): 151-157.

Palombo, E. A. and Semple, S. J. (2002). "Antibacterial activity of Australian plant extracts against methicillin resistant *Staphylococcus aureus* (MRSA) and vancomycin resistant Enterococci (VRE)." *Journal of Basic Microbiology* **42**(6): 444-448.

Pardo, F., Perich, F., Villarroel, L. and Torres, R. (1993). "Isolation of verbascoside, an antimicrobial constituent of *Buddleja globosa* leaves." *Journal of Ethnopharmacology* **39**(3): 221-222.

Pearn, J. (2005). "Medical Ethnobotany of Australia: Past and Present." *The Linnean* **21**(4): 16-24.

Pennacchio, M., Kemp, A. S., Taylor, R. P., Wickens, K. M. and Kienow, L. (2005). "Interesting biological activities from plants traditionally used by Native Australians." *Journal of Ethnopharmacology* **96**(3): 597-601.

Pennacchio, M., Syah, Y. M., Ghisalberti, E. L. and Alexander, E. (1996). "Cardioactive compounds from *Eremophila* species." *Journal of Ethnopharmacology* **53**(1): 21-27.

PMHC. (2010). "Bush Medicine." Retrieved 18/01/2011, 2011, from http://www.pmhc.org.au/Programs/Bush_Medicine/index00320.html.

Pongprayoon, U., Baeckstrom, P., Jacobsson, U., Lindstrom, M. and Bohlin, L. (1991). "Compounds inhibiting prostaglandin synthesis isolated from *Ipomoea pes-caprae*." *Planta Medica* **57**(6): 515-518.

Pongprayoon, U., Bohlin, L., Soonthornsaratune, P. and Wasuwat, S. (1991a). "Antiinflammatory activity of *Ipomoea pes caprae* (L.) R. Br." *Phytotherapy Research* **5**(2): 63-66.

Pongprayoon, U., Bohlin, L. and Wasuwat, S. (1991b). "Neutralization of toxic effects of different crude jellyfish venoms by an extract of *Ipomoea pes-caprae* (L.) R. Br." *Journal of Ethnopharmacology* **35**(1): 65-69.

Potts, B. C. M., Faulkner, D. J. and Jacobs, R. S. (1992). "Phospholipase A2 inhibitors from marine organisms." *Journal of Natural Products* **55**(12): 1701-1717.

Prior, R. L., Hoang, H., Gu, L., Wu, X., Bacchiocca, M., Howard, L., Hampsch-Woodill, M., Huang, D., Ou, B. and Jacob, R. (2003). "Assays for hydrophilic and lipophilic antioxidant capacity (oxygen radical absorbance capacity ($ORAC_{FL}$)) of plasma and other biological and food samples." *Journal of Agricultural and Food Chemistry* **51**(11): 3273-3279.

Puruntatameri, J., Puruntatameri, R., Pangiraminni, A., Burak, L., Tipuamantymirri, C., Tipakalippa, M., Puruntatameri, J., Puruntatameri, P., Pupangamirri, J. B., Kerinaiua, R., Tipiloura, D., Orsto, M., Kantilla, B., Kurrupuwu, M., Puruntatameri, P. F., Puruntatameri, T. D., Puruntatameri, L., Kantilla, K., Wilson, J., Cusack, J., Jackson, D. and Wightman, G. (2001). *Tiwi Plants and Animals: Aboriginal Flora and Fauna Knowledge from Melville and Bathurst Islands, North Australia*. Northern Territory Botanical Bulletin no. 24. Darwin, Parks and Wildlife Commission of the Northern Territory.

Rang, H. P., Dale, M. M., Ritter, J. M., Flower, R. J. and Henderson, G. (2007). *Pharmacology*. Edinburgh, Churchill Livingstone.

Rasmussen, M., Guo, X., Wang, Y., Lohmueller, K. E., Rasmussen, S., Albrechtsen, A., Skotte, L., Lindgreen, S., Metspalu, M. and Jombart, T. (2011). "An Aboriginal Australian Genome Reveals Separate Human Dispersals into Asia." *Science* **334**(6052): 94-98.

Raymond, E., Blutja, J., Gingina, L., Raymond, M., Raymond, O., Raymond, L., Brown, J., Morgan, Q., Jackson, D., Smith, N. and Wightman, G. (1999). *Wardaman Ethnobiology: Aboriginal plant and animal knowledge from the Flora River and south-west Katherine region, north Australia*. Northern Territory Botanical Bulletin no. 23. Palmerston, Parks and Wildlife Commission of the Northern Territory.

Reid, E. J. and Betts, T. J. (1979). "Records of Western Australian plants used by Aboriginals as medicinal agents." *Planta Medica* **36**(2): 164-173.

Renwick, C. (2000). *Geebungs and Snake Whistles: Koori People and Plants of Wreck Bay*. Canberra, Aboriginal Studies Press for the Australian Institute of Aboriginal and Torres Strait Islander Studies.

Reynertson, K. A., Balick, M. J., Lee, R., Raynor, W., Pelep, Y. and Kennelly, E. J. (2005). "A traditional method of *Cinnamomum carolinense* preparation eliminates safrole from a therapeutic Pohnpean tea." *Journal of Ethnopharmacology* **102**(2): 269-274.

Richmond, G. S. and Ghisalberti, E. L. (1994). "The Australian desert shrub *Eremophila* (Myoporaceae): Medicinal, cultural, horticultural and phytochemical uses." *Economic Botany* **48**(1): 35-59.

Robles-Sardin, A. E., Bolanos-Villar, A. V., Aguilar, G. A. G. and De la Rosa, L. (2010). Flavanoids and Their Relation to Human Health. *Fruit and Vegetable Phytochemicals: Chemistry, Nutritional Value, and Stability*. L. A. de la Rosa, E. A. Parrilla and G. A. Gonzalez-Aguilar. Ames, Wiley-Blackwell**:** 155-176.

Rodriguez, A. D. and Ramirez, C. (2001). "Serrulatane Diterpenes with Antimycobacterial Activity Isolated from the West Indian Sea Whip *Pseudopterogorgia elisabethae*." *Journal of Natural Products* **64**(1): 100-102.

Rodríguez, A. D., Ramírez, C., Rodríguez, I. I. and González, E. (1999). "Novel Antimycobacterial Benzoxazole Alkaloids, from the West Indian Sea Whip *Pseudopterogorgia elisabethae*." *Organic Letters* **1**(3): 527-530.

Rogers, K. L., Grice, I. D. and Griffiths, L. R. (2000). "Inhibition of platelet aggregation and 5-HT release by extracts of Australian plants used traditionally as headache treatments." *European Journal of Pharmaceutical Sciences* **9**(4): 355-363.

Rogers, K. L., Grice, I. D. and Griffiths, L. R. (2001). "Modulation of in vitro platelet 5-HT release by species of *Erythrina* and *Cymbopogon*." *Life Sciences* **69**(15): 1817-1829.

Rønsted, N., Göbel, E., Franzyk, H., Jensen, S. R. and Olsen, C. E. (2000). "Chemotaxonomy of *Plantago*. Iridoid glucosides and caffeoyl phenylethanoid glycosides." *Phytochemistry* **55**(4): 337-348.

Roussis, V., Wu, Z., Fenical, W., Strobel, S. A., Van Duyne, G. D. and Clardy, J. (1990). "New anti-inflammatory pseudopterosins from the marine octocoral *Pseudopterogorgia elisabethae*." *The Journal of Organic Chemistry* **55**(16): 4916-4922.

Roy, S., Khanna, S., Nallu, K., Hunt, T. K. and Sen, C. K. (2006). "Dermal wound healing is subject to redox control." *Molecular Therapy* **13**(1): 211-220.

Sakai, Y., Fujita, K., Sakai, H. and Mizuno, K. (2001). "Prostaglandin E_2 regulates the expression of basic fibroblast growth factor messenger RNA in normal human fibroblasts." *Kobe Journal of Medical Sciences* **47**(1): 35-45.

Sampson, J., Houghton, P., Mensah, A., Hylands, P., Westbrook, J. and Dunn, M. (2000). "Determination of the potential wound-healing effects of *Buddleja globosa* and *B. davidii* leaves using an *in-vitro* human dermal fibroblast assay." *Journal of Pharmacy and Pharmacology* **52**(S9): 308-308.

Scalbert, A. (1991). "Antimicrobial properties of tannins." *Phytochemistry* **30**(12): 3875-3883.

Schmidt, C., Fronza, M., Goettert, M., Geller, F., Luik, S., Flores, E., Bittencourt, C., Zanetti, G., Heinzmann, B. and Laufer, S. (2009). "Biological studies on Brazilian plants used in wound healing." *Journal of Ethnopharmacology* **122**(3): 523-532.

Semple, S., Nobbs, S. F., Pyke, S. M., Reynolds, G. D. and Flower, R. L. P. (1999). "Antiviral flavonoid from *Pterocaulon sphacelatum*, an Australian Aboriginal medicine." *Journal of Ethnopharmacology* **68**(1-3): 283-288.

Semple, S. J., Pyke, S. M., Reynolds, G. D. and Flower, R. L. P. (2001). "*In vitro* antiviral activity of the anthraquinone chrysophanic acid against poliovirus." *Antiviral Research* **49**(3): 169-178.

Semple, S. J., Reynolds, G. D., O'Leary, M. C. and Flower, R. L. P. (1998). "Screening of Australian medicinal plants for antiviral activity." *Journal of Ethnopharmacology* **60**(2): 163-172.

Sen, C. K. (2003). "The general case for redox control of wound repair." *Wound Repair and Regeneration* **11**(6): 431-438.

Sen, C. K., Khanna, S., Gordillo, G., Bagchi, D., Bagchi, M. and Roy, S. (2006). "Oxygen, Oxidants, and Antioxidants in Wound Healing - An Emerging Paradigm." *Annals of the New York Academy of Sciences* **957**(1): 239-249.

Senatore, F., Soria, E. U., Soria, R. U., Porta, G. D. and De Feo, V. (1998). "Essential oils from two Peruvian Satureja species." *Flavour and Fragrance Journal* **13**(4): 1-4.

Serrano, J., Puupponen Pimiä, R., Dauer, A., Aura, A. M. and Saura Calixto, F. (2009). "Tannins: current knowledge of food sources, intake, bioavailability and biological effects." *Molecular Nutrition & Food Research* **53**(2): 310-329.

Shah, A., Cross, R. F. and Palombo, E. A. (2004). "Identification of the antibacterial component of an ethanolic extract of the Australian medicinal plant, *Eremophila duttonii*." *Phytotherapy Research* **18**(8): 615-618.

Short, P. S., Albrecht, D. E., Cowie, I. D., Lewis, D. L. and Stuckey, B. (2011). *Checklist of the Vascular Plants of the Northern Territory*, Northern Territory Herbarium, Department of Natural Resources, Environment, The Arts and Sport.

Smith, J. E. (2004). An Ethnopharmacological Investigation into the Antimicrobial Activity of *Eremophila* Species. *School of Biological, Biomedical and Molecular Sciences*. Armidale, NSW, University of New England. **B.Sc. :** 120.

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.

Smith, N. (1993). *Ngarinyman Ethnobotany: Aboriginal Plant Use from the Victoria River Area, Northern Australia*. Northern Territory Botanical Bulletin no. 16. Darwin, Conservation Commission of the Northern Territory.

Smith, N. M. and Wightman, G. M. (1989). *Ethnobotanical Notes from Belyuen, Northern Territory, Australia*. Northern Territory Botanical Bulletin no. 10. Darwin, Conservation Commission of the Northern Territory.

Smyth, T., Ramachandran, V., Brooks, P. and Smyth, W. (2009). "A study of the antibacterial activities of selected Australian medicinal plants." *Journal of Pharmacognosy and Phytotherapy* **1**(6): 82-86.

Steenkamp, V., Mathivha, E., Gouws, M. and Van Rensburg, C. (2004). "Studies on antibacterial, antioxidant and fibroblast growth stimulation of wound healing remedies from South Africa." *Journal of Ethnopharmacology* **95**(2-3): 353-357.

Stevenson, P. C., Simmonds, M. S. J., Sampson, J., Houghton, P. J. and Grice, P. (2002). "Wound healing activity of acylated iridoid glycosides from *Scrophularia nodosa*." *Phytotherapy Research* **16**(1): 33-35.

Stoddart, D. M. (1990). *The scented ape: the biology and culture of human odour*. Cambridge, Cambridge University Press.

Sullivan, T. P., Eaglstein, W. H., Davis, S. C. and Mertz, P. (2001). "The pig as a model for human wound healing." *Wound Repair and Regeneration* **9**(2): 66-76.

Surveswaran, S., Cai, Y. Z., Corke, H. and Sun, M. (2007). "Systematic evaluation of natural phenolic antioxidants from 133 Indian medicinal plants." *Food Chemistry* **102**(3): 938-953.

Sweeney, A. P., Wyllie, S. G., Shalliker, R. A. and Markham, J. (2001). "Xanthine oxidase inhibitory activity of selected Australian native plants." *Journal of Ethnopharmacology* **75**(2-3): 273-277.

Tak, J.-H., Kim, H.-K., Lee, S.-H. and Ahn, Y.-J. (2006). "Acaricidal activities of paenol and benzoic acid from Paeonia suffruticosa root bark and monoterpenoids against Tyrophagus putrescentiae (Acari: Acaridae)." *Pest Management Science* **62**: 551-557.

Tetko, I. V., Tanchuk, V. Y., Kasheva, T. N. and Villa, A. E. P. (2001). "Estimation of aqueous solubility of chemical compounds using E-state indices." *Journal of Chemical Information and Computer Sciences* **41**(6): 1488-1493.

Thompson, D. (2008). *ORAC Antioxidant testing: Efficiency gains through improved curve fitting.* ((Poster Presentation)). Royal Australian Chemical Institute NSW Natural Product Chemistry Group Annual Symposium 2008, Southern Cross University, Lismore, October 2008.

Tippett, L. M. and Massy-Westropp, R. A. (1993). "Serrulatane diterpenes from *Eremophila duttonii*." *Phytochemistry* **33**(2): 417-421.

Tomlinson, S. and Palombo, E. A. (2005). "Characterisation of antibacterial Australian medicinal plant extracts by investigation of the mechanism of action and the effect of interfering substances." *Journal of Basic Microbiology* **45**(5): 363-370.

Trombetta, D., Castelli, F., Sarpietro, M. G., Venuti, V., Cristani, M., Daniele, C., Saija, A., Mazzanti, G. and Bisignano, G. (2005). "Mechanisms of antibacterial action of three monoterpenes." *Antimicrobial Agents and Chemotherapy* **49**(6): 2474-2478.

Trotter, R. T. and Logan, M. H. (1986). Informant consensus: A new approach for identifying potentially effective medicinal plants. *Plants in Indigenous Medicine and Diet, Biobehavioral Approaches*. N. L. Etkin. Bedford Hills, NY, Redgrave Publishing Co.**:** 91-112.

Varga, J., Diaz-Perez, A., Rosenbloom, J. and Jimenez, S. A. (1987). "PGE_2 causes a coordinate decrease in the steady state levels of fibronectin and types I and III procollagen mRNAs in normal human dermal fibroblasts." *Biochemical and Biophysical Research Communications* **147**(3): 1282-1288.

Vasilev, K., Cook, J. and Griesser, H. J. (2009). "Antibacterial surfaces for biomedical devices." *Expert Review of Medical Devices* **6**(5): 553-567.

Verpoorte, R. (2000). "Pharmacognosy in the new millennium: leadfinding and biotechnology." *Journal of Pharmacy and Pharmacology* **52**(3): 253-262.

Wagner, H. and Bladt, S. (1996). *Plant drug analysis*. Dordrecht, Springer.

Webb, L. J. (1969). "The Use of Plant Medicines and Poisons by Australian Aborigines." *Mankind* **7**(2): 137-146.

Whitney, E. N., Rolfes, S. R., Crowe, T., Cameron-Smith, D. and Walsh, A. (2010). *Understanding Nutrition: Australian and New Zealand Edition*. South Melbourne, Cengage Learning.

Wickens, K. and Pennacchio, M. (2002). "A search for novel biologically active compounds in the phyllodes of *Acacia* species." *Conservation Science Western Australia* **4**(3): 139-144.

Wightman, G., Roberts, J. G. and Williams, L. (1992). *Mangarrayi Ethnobotany: Aboriginal Plant Use from the Elsey Area, Northern Australia*. Northern Territory Botanical Bulletin no. 15. Darwin, Conservation Commision of the Northern Territory.

Wightman, G. M. (2006). *Mangroves of the Northern Territory, Australia Identification and Traditional use*. Northern Territory Botanical Bulletin no. 31. Darwin, Department of Natural Resources, Environment and the Arts.

Wightman, G. M., Dixon, D., Williams, L. and Dalywaters, I. (1992). *Mudburra Ethnobotany: Aboriginal Plant Use from Kulumindini (Elliott), Northern Australia*. Northern Territory Botanical Bulletin no. 14. Darwin, Conservation Commission of the Northern Territory.

Wightman, G. M., Jackson, D. M. and Williams, L. L. V. (1991). *Alawa Ethnobotany: Aboriginal Plant Use from Minyerri, Northern Australia*. Northern Territory Botanical Bulletin no. 11. Darwin, Conservation Commission of the Northern Territory.

Wightman, G. M., Kalabidi, G. J., Dood, T. N. N., Frith, R. N. D., Jiwijiwij, M. N., Oscar, J. N. N., Wave Hill, R. J. W., Holt, S., Limbunya, J. J. and Wadrill, V. N. (1994). *Gurindji Ethnobotany: Aboriginal Plant Use from Daguragu, Northern Australia*. Northern Territory Botanical Bulletin no. 18. Darwin, Conservation Commision of the Northern Territory.

Wightman, G. M. and Smith, N. M. (1989). *Ethnobotany, Vegetation and Floristics of Milingimbi, Northern Australia*. Northern Territory Botanical Bulletin no. 6. Darwin, Conservation Commission of the Northern Territory.

Williams, A. C. and Barry, B. W. (2004). "Penetration enhancers." *Advanced Drug Delivery Reviews* **56**(5): 603-618.

Wohlmuth, H. and Brooks, L. O. (2007). *Contemporary phytotherapy in Australia: An ethnobotanical survey.* (Poster Presentation). 6th International Conference on Phytotherapeutics, Canberra, September 2007.

Woolfe, M. L., Chaplin, M. F. and Otchere, G. (1977). "Studies on the mucilages extracted from okra fruits (*Hibiscus esculentus* L.) and baobab leaves (*Adansonia digitata* L.)." *Journal of the Science of Food and Agriculture* **28**(6): 519-529.

Wynjorrotj, P., Flora, S., Brown, N., Jatbula, P., Galmur, J., Katherine, M., Merlan, F. and Wightman, G. (2005). *Jawoyn Plants and Animals*. Northern Territory Botanical Bulletin no. 29. Darwin, Conservation Commission of the Northern Territory.

Yunupingu, B., Marika-Yunupingu, L., Marika, D., Marika, B., Marika, B., Marika, R. and Wightman, G. (1994). *Rirratjingu Ethnobotany: Aboriginal Plant Use from Yirrkala, Arnhem Land, Australia*. Northern Territory Botanical Bulletin no. 21. Darwin, Conservation Commission of the Northern Territory.

Zheng, W. and Wang, S. Y. (2001). "Antioxidant activity and phenolic compounds in selected herbs." *Journal of Agricultural and Food Chemistry* **49**(11): 5165-5170.

Zola, N. and Gott, B. (1992). *Koorie Plants, Koorie People: Traditional Aboriginal Food, Fibre and Healing Plants of Victoria*. Melbourne, Koorie Heritage Trust.

Appendix I: Survey of Ethnobotanical literature (supplementary data)

Table I.1 Regression residual analysis of Northern Territory medicinal plants

	Number of species per family					
Family	CVPNT	NTMP	*Predicted NTMP*	*Residuals*	*Standard Residuals*	Ranking
MYRTACEAE Juss., nom. cons.	181	37	10.5	26.5	8.0	1
FABACEAE Lindl., nom. cons.	619	48	35.2	12.8	3.9	2
PROTEACEAE Juss., nom. cons.	54	14	3.4	10.6	3.2	3
SCROPHULARIACEAE Juss.	39	11	2.5	8.5	2.6	4
SOLANACEAE Juss.	62	12	3.8	8.2	2.5	5
LAMIACEAE	74	10	4.5	5.5	1.7	6
COMBRETACEAE R.Br., nom. cons.	23	6	1.6	4.4	1.3	7
SANTALACEAE R.Br., nom. cons.	10	5	0.9	4.1	1.2	8
LORANTHACEAE Juss., nom. cons.	33	6	2.2	3.8	1.2	9
MORACEAE Gaudich., nom. cons.	23	5	1.6	3.4	1.0	10
AMARYLLIDACEAE J.St., nom. cons.	6	4	0.7	3.3	1.0	11
APOCYNACEAE Juss.	59	6	3.7	2.3	0.7	12
PICRODENDRACEAE Small, nom. cons.	7	3	0.7	2.3	0.7	13
RHIZOPHORACEAE Pers., nom. cons.	11	3	0.9	2.1	0.6	14
RHAMNACEAE Juss., nom. cons.	13	3	1.1	1.9	0.6	15
MELIACEAE Juss.	15	3	1.2	1.8	0.6	16
ASPARAGACEAE Juss., nom. cons.	20	3	1.5	1.5	0.5	17
CUCURBITACEAE Juss., nom. cons.	20	3	1.5	1.5	0.5	18
CANNABACEAE Martinov	3	2	0.5	1.5	0.5	19
EUPHORBIACEAE Juss., nom. cons.	75	6	4.6	1.4	0.4	20
PANDANACEAE R.Br., nom. cons.	5	2	0.6	1.4	0.4	21
MENISPERMACEAE Juss., nom. cons.	7	2	0.7	1.3	0.4	22
NYMPHAEACEAE Salisb., nom. cons.	7	2	0.7	1.3	0.4	23
CAPPARACEAE Juss.	10	2	0.9	1.1	0.3	24
LAURACEAE Juss., nom. cons.	10	2	0.9	1.1	0.3	25
SAPINDACEAE Juss.	28	3	1.9	1.1	0.3	26
CELASTRACEAE R.Br., nom. cons.	14	2	1.1	0.9	0.3	27
CAMPANULACEAE Juss.	16	2	1.2	0.8	0.2	28
BURSERACEAE Kunth	1	1	0.4	0.6	0.2	29
FLAGELLARIACEAE Dumort., nom. cons.	1	1	0.4	0.6	0.2	30
NELUMBONACEAE A. Rich., nom. cons.	1	1	0.4	0.6	0.2	31
PHILYDRACEAE Link, nom. cons.	1	1	0.4	0.6	0.2	32
HERNANDIACEAE Blume, nom. cons.	2	1	0.4	0.6	0.2	33
OPILIACEAE Valeton, nom. cons.	2	1	0.4	0.6	0.2	34
PIPERACEAE Giseke, nom. cons.	2	1	0.4	0.6	0.2	35
SURIANACEAE Arn., nom. cons.	2	1	0.4	0.6	0.2	36
TACCACEAE Dumort., nom. cons.	2	1	0.4	0.6	0.2	37
LYTHRACEAE J.St.-Hil., nom. cons.	20	2	1.5	0.5	0.2	38
BIXACEAE Kunth.	3	1	0.5	0.5	0.2	39
CASUARINACEAE R.Br., nom. cons.	3	1	0.5	0.5	0.2	40
DIOSCOREACEAE R.Br., nom. cons.	3	1	0.5	0.5	0.2	41
GYROSTEMONACEAE A.Juss.	3	1	0.5	0.5	0.2	42

CVPLNT: Checklist of the Vascular Plants of the Northern Territory; NTMP: Northern Territory Medicinal plants

Table I.1 (Continued from previous page)

	Number of species per family					
Family	CVPNT	NTMP	*Predicted NTMP*	*Residuals*	*Standard Residuals*	Ranking
LECYTHIDACEAE A.Rich.	3	1	0.5	0.5	0.2	43
MYRISTICACEAE R.Br., nom. cons.	3	1	0.5	0.5	0.2	44
PASSIFLORACEAE Juss. ex Roussel, nom. cons.	4	1	0.6	0.4	0.1	45
BIGNONIACEAE Juss.	5	1	0.6	0.4	0.1	46
ORCHIDACEAE Juss., nom. cons.	41	3	2.6	0.4	0.1	47
EBENACEAE Guerke	6	1	0.7	0.3	0.1	48
ANACARDIACEAE R.Br.	8	1	0.8	0.2	0.1	49
HAEMODORACEAE R.Br., nom. cons.	8	1	0.8	0.2	0.1	50
PLANTAGINACEAE Juss.	26	2	1.8	0.2	0.1	51
VITACEAE Juss., nom. cons.	9	1	0.8	0.2	0.0	52
MENYANTHACEAE Dumort.	12	1	1.0	0.0	0.0	53
THYMELAEACEAE Juss.	15	1	1.2	-0.2	-0.1	54
CLEOMACEAE Bercht. & J.Presl.	16	1	1.2	-0.2	-0.1	55
BORAGINACEAE Juss.	71	4	4.3	-0.3	-0.1	56
ARECACEAE Bercht. & J.Presl, nom. cons.	18	1	1.3	-0.3	-0.1	57
AQUIFOLIACEAE Bercht. & J.Presl	1	0	0.4	-0.4	-0.1	58
BATACEAE Mart. ex Perleb	1	0	0.4	-0.4	-0.1	59
BORYACEAE M.W.Chase, Rudall & Conran	1	0	0.4	-0.4	-0.1	60
CABOMBACEAE Rich. ex A. Rich., nom. cons.	1	0	0.4	-0.4	-0.1	61
CERATOPHYLLACEAE Gray, nom. cons.	1	0	0.4	-0.4	-0.1	62
DICHAPETALACEAE Baill., nom. cons.	1	0	0.4	-0.4	-0.1	63
HANGUANACEAE Airy Shaw	1	0	0.4	-0.4	-0.1	64
HYDROLEACEAE R.Br. ex Edwards	1	0	0.4	-0.4	-0.1	65
HYPERICACEAE Juss., nom. cons.	1	0	0.4	-0.4	-0.1	66
IRIDACEAE Juss., nom. cons.	1	0	0.4	-0.4	-0.1	67
LIMEACEAE Shipunov ex Reveal	1	0	0.4	-0.4	-0.1	68
MARTYNIACEAE Horan.	1	0	0.4	-0.4	-0.1	69
MUNTINGIACEAE C.Bayer *et al.*	1	0	0.4	-0.4	-0.1	70
NEURADACEAE Kostel.	1	0	0.4	-0.4	-0.1	71
NITRARIACEAE Lindl.	1	0	0.4	-0.4	-0.1	72
PENTAPHYLACACEAE Engl.	1	0	0.4	-0.4	-0.1	73
PODOSTEMACEAE Rich. ex Kunth, nom. cons.	1	0	0.4	-0.4	-0.1	74
PUTRANJIVACEAE Meisn.	1	0	0.4	-0.4	-0.1	75
SALICACEAE Mirb., nom. cons.	1	0	0.4	-0.4	-0.1	76
SMILACACEAE Vent., nom. cons.	1	0	0.4	-0.4	-0.1	77
SPHENOCLEACEAE Baskerv.	1	0	0.4	-0.4	-0.1	78
TAMARICACEAE Link, nom. cons.	1	0	0.4	-0.4	-0.1	79
XANTHORRHOEACEAE Dumort., nom. cons.	1	0	0.4	-0.4	-0.1	80
ZINGIBERACEAE Martinov, nom. cons.	1	0	0.4	-0.4	-0.1	81
CHRYSOBALANACEAE	2	0	0.4	-0.4	-0.1	82
ERYTHROXYLACEAE Kunth, nom. cons.	2	0	0.4	-0.4	-0.1	83
PLUMBAGINACEAE Juss., nom. cons.	2	0	0.4	-0.4	-0.1	84

CVPLNT: Checklist of the Vascular Plants of the Northern Territory; NTMP: Northern Territory Medicinal plants

Table I.1 (Continued from previous page)

	Number of species per family					
Family	CVPNT	NTMP	*Predicted NTMP*	*Residuals*	*Standard Residuals*	Ranking
RANUNCULACEAE Juss., nom. cons.	2	0	0.4	-0.4	-0.1	85
RUPPIACEAE Horan., nom. cons.	2	0	0.4	-0.4	-0.1	86
SIMAROUBACEAE DC.	2	0	0.4	-0.4	-0.1	87
STEMONACEAE Caruel, nom. cons.	2	0	0.4	-0.4	-0.1	88
TYPHACEAE Juss., nom. cons.	2	0	0.4	-0.4	-0.1	89
VIOLACEAE Batsch, nom. cons.	2	0	0.4	-0.4	-0.1	90
ALISMATACEAE Vent., nom. cons	3	0	0.5	-0.5	-0.2	91
APONOGETONACEAE Planch., nom. cons.	3	0	0.5	-0.5	-0.2	92
ARISTOLOCHIACEAE Juss., nom. cons.	3	0	0.5	-0.5	-0.2	93
ASPHODELACEAE Juss.	3	0	0.5	-0.5	-0.2	94
BURMANNIACEAE Blume, nom. cons.	3	0	0.5	-0.5	-0.2	95
COLCHICACEAE DC., nom. cons.	3	0	0.5	-0.5	-0.2	96
CRASSULACEAE J.St.-Hil., nom. cons.	3	0	0.5	-0.5	-0.2	97
ERICACEAE Juss.	3	0	0.5	-0.5	-0.2	98
HYDATELLACEAE U.Hamann	3	0	0.5	-0.5	-0.2	99
HYPOXIDACEAE R.Br., nom. cons.	3	0	0.5	-0.5	-0.2	100
PEDALIACEAE R.Br.	3	0	0.5	-0.5	-0.2	101
RESTIONACEAE R.Br., nom. cons.	3	0	0.5	-0.5	-0.2	102
MALVACEAE Juss.	234	13	13.5	-0.5	-0.2	103
BYBLIDACEAE Domin	4	0	0.6	-0.6	-0.2	104
CLUSIACEAE Lindl., nom. cons.	4	0	0.6	-0.6	-0.2	105
POTAMOGETONACEAE Bercht. & J.Presl, nom. cons.	4	0	0.6	-0.6	-0.2	106
SAPOTACEAE Juss.	4	0	0.6	-0.6	-0.2	107
CYMODOCEACEAE Vines, nom. cons.	5	0	0.6	-0.6	-0.2	108
ELAEOCARPACEAE Juss., nom. cons.	5	0	0.6	-0.6	-0.2	109
JUNCAGINACEAE Rich., nom. cons.	5	0	0.6	-0.6	-0.2	110
OLACACEAE Juss. ex R.Br., nom. cons.	5	0	0.6	-0.6	-0.2	111
OLEACEAE Hoffsgg. & Link	5	0	0.6	-0.6	-0.2	112
OXALIDACEAE R.Br., nom. cons.	5	0	0.6	-0.6	-0.2	113
PITTOSPORACEAE R.Br.	5	0	0.6	-0.6	-0.2	114
PONTEDERIACEAE Kunth, nom. cons.	5	0	0.6	-0.6	-0.2	115
ARACEAE Juss., nom. cons.	23	1	1.6	-0.6	-0.2	116
CACTACEAE Juss., nom. cons.	6	0	0.7	-0.7	-0.2	117
JUNCACEAE Juss., nom. cons.	6	0	0.7	-0.7	-0.2	118
ONAGRACEAE Juss., nom. cons.	6	0	0.7	-0.7	-0.2	119
XYRIDACEAE C.Agardh, nom. cons.	6	0	0.7	-0.7	-0.2	120
CENTROLEPIDACEAE Endl., nom. cons.	7	0	0.7	-0.7	-0.2	121
MELASTOMATACEAE Juss., nom. cons.	7	0	0.7	-0.7	-0.2	122
MOLLUGINACEAE Bartl., nom. cons.	7	0	0.7	-0.7	-0.2	123
PAPAVERACEAE Juss., nom. cons.	7	0	0.7	-0.7	-0.2	124
FRANKENIACEAE Desv., nom. cons.	8	0	0.8	-0.8	-0.2	125
URTICACEAE Juss., nom. cons.	8	0	0.8	-0.8	-0.2	126

CVPLNT: Checklist of the Vascular Plants of the Northern Territory; NTMP: Northern Territory Medicinal plants

Table I.1 (Continued from previous page)

	Number of species per family					
Family	CVPNT	NTMP	*Predicted NTMP*	*Residuals*	*Standard Residuals*	Ranking
ELATINACEAE Dumort., nom. cons.	9	0	0.8	-0.8	-0.3	127
PRIMULACEAE Batsch ex Borkh.	9	0	0.8	-0.8	-0.3	128
APIACEAE Lindl.	10	0	0.9	-0.9	-0.3	129
VERBENACEAE J.St-Hil.	10	0	0.9	-0.9	-0.3	130
NYCTAGINACEAE Juss., nom. cons.	12	0	1.0	-1.0	-0.3	131
PHRYMACEAE Schauer	12	0	1.0	-1.0	-0.3	132
ANNONACEAE Juss., nom. cons.	13	0	1.1	-1.1	-0.3	133
HEMEROCALLIDACEAE R.Br.	13	0	1.1	-1.1	-0.3	134
OROBANCHACEAE Vent.	14	0	1.1	-1.1	-0.3	135
DROSERACEAE Salisb., nom. cons.	16	0	1.2	-1.2	-0.4	136
HALORAGACEAE R.Br., nom. cons.	16	0	1.2	-1.2	-0.4	137
RUTACEAE Juss.	34	1	2.2	-1.2	-0.4	138
POLYGONACEAE Juss., nom. cons.	17	0	1.3	-1.3	-0.4	139
ACANTHACEAE Juss.	35	1	2.3	-1.3	-0.4	140
LOGANIACEAE R.Br. ex Mart.	35	1	2.3	-1.3	-0.4	141
AIZOACEAE Martinov nom. cons.	19	0	1.4	-1.4	-0.4	142
BRASSICACEAE	37	1	2.4	-1.4	-0.4	143
COMMELINACEAE Mirb., nom. cons.	20	0	1.5	-1.5	-0.4	144
ARALIACEAE Juss.	21	0	1.5	-1.5	-0.5	145
ERIOCAULACEAE Martinov, nom. cons.	22	0	1.6	-1.6	-0.5	146
HYDROCHARITACEAE Juss., nom. cons.	22	0	1.6	-1.6	-0.5	147
CARYOPHYLLACEAE Juss., nom. cons.	23	0	1.6	-1.6	-0.5	148
PHYLLANTHACEAE Martinov, nom. cons. prop.	42	1	2.7	-1.7	-0.5	149
ZYGOPHYLLACEAE R.Br., nom. cons.	29	0	2.0	-2.0	-0.6	150
RUBIACEAE Juss.	139	6	8.2	-2.2	-0.7	151
LINDERNIACEAE Borsch & al.	33	0	2.2	-2.2	-0.7	152
LENTIBULARIACEAE Rich.	37	0	2.4	-2.4	-0.7	153
POLYGALACEAE Hoffmanns. & Link, nom. cons.	37	0	2.4	-2.4	-0.7	154
PORTULACACEAE Juss., nom. cons.	38	0	2.5	-2.5	-0.7	155
STYLIDIACEAE R.Br.	40	0	2.6	-2.6	-0.8	156
CONVOLVULACEAE Juss.	77	2	4.7	-2.7	-0.8	157
DILLENIACEAE Salisb., nom. cons.	54	0	3.4	-3.4	-1.0	158
ASTERACEAE Martinov	269	12	15.5	-3.5	-1.1	159
GOODENIACEAE R.Br.	112	2	6.6	-4.6	-1.4	160
CYPERACEAE Juss., nom. cons.	285	6	16.4	-10.4	-3.1	161
AMARANTHACEAE Juss., nom. cons.	218	2	12.6	-10.6	-3.2	162
POACEAE Barnhart, nom. cons.	559	20	31.8	-11.8	-3.6	163

CVPLNT: Checklist of the Vascular Plants of the Northern Territory; NTMP: Northern Territory Medicinal plants

Table I.2 Regression residual analysis of medicinal plants of the dermatological complaints category

	Number of species per family					
Family	AMS	DCC	Predicted DCC	Residuals	Standard Residuals	Ranking
SCROPHULARIACEAE Juss.	11	9	5.8	3.2	2.5	1
EUPHORBIACEAE Juss., nom. cons.	13	10	6.8	3.2	2.5	2
AMARYLLIDACEAE J.St., nom. cons.	5	5	2.7		1.8	3
FABACEAE Lindl., nom. cons.	57	32	29.7	2.3	1.8	4
COMBRETACEAE R.Br., nom. cons.	6	5	3.2	1.8	1.4	5
RUBIACEAE Juss.	6	5	3.2	1.8	1.4	6
MALVACEAE Juss.	16	10	8.4	1.6	1.2	7
PROTEACEAE Juss., nom. cons.	16	10	8.4	1.6	1.2	8
ORCHIDACEAE Juss., nom. cons.	3	3	1.6	1.4	1.1	9
RHIZOPHORACEAE Pers., nom. cons.	3	3	1.6	1.4	1.1	10
MORACEAE Gaudich., nom. cons.	5	4	2.7	1.3	1.0	11
PLANTAGINACEAE Juss.	5	4	2.7	1.3	1.0	12
ARACEAE Juss., nom. cons.	2	2	1.1	0.9	0.7	13
CANNABACEAE Martinov	2	2	1.1	0.9	0.7	14
CUPRESSACEAE Gray, nom. cons.	2	2	1.1	0.9	0.7	15
LAURACEAE Juss., nom. cons.	2	2	1.1	0.9	0.7	16
MENISPERMACEAE Juss., nom. cons.	2	2	1.1	0.9	0.7	17
PANDANACEAE R.Br., nom. cons.	2	2	1.1	0.9	0.7	18
PHYLLANTHACEAE Martinov, nom. cons. prop.	2	2	1.1	0.9	0.7	19
APOCYNACEAE Juss.	10	6	5.3	0.7	0.6	20
ANACARDIACEAE R.Br.	1	1	0.6	0.4	0.3	21
BIGNONIACEAE Juss.	1	1	0.6	0.4	0.3	22
BIXACEAE Kunth.	1	1	0.6	0.4	0.3	23
BURSERACEAE Kunth	1	1	0.6	0.4	0.3	24
CARICACEAE Dum.	1	1	0.6	0.4	0.3	25
CASUARINACEAE R.Br., nom. cons.	1	1	0.6	0.4	0.3	26
CLEOMACEAE Bercht. & J.Presl.	1	1	0.6	0.4	0.3	27
EBENACEAE Guerke	1	1	0.6	0.4	0.3	28
HEMEROCALLIDACEAE R.Br.	1	1	0.6	0.4	0.3	29
HERNANDIACEAE Blume, nom. cons.	1	1	0.6	0.4	0.3	30
LECYTHIDACEAE A.Rich.	1	1	0.6	0.4	0.3	31
LOGANIACEAE R.Br. ex Mart.	1	1	0.6	0.4	0.3	32
PASSIFLORACEAE Juss. ex Roussel, nom. cons.	1	1	0.6	0.4	0.3	33
PHILYDRACEAE Link, nom. cons.	1	1	0.6	0.4	0.3	34
PHYTOLACCACEAE R. Br.	1	1	0.6	0.4	0.3	35
PITTOSPORACEAE R.Br.	1	1	0.6	0.4	0.3	36
VITACEAE Juss., nom. cons.	1	1	0.6	0.4	0.3	37
ASPARAGACEAE Juss., nom. cons.	3	2	1.6	0.4	0.3	38
CAPPARACEAE Juss.	3	2	1.6	0.4	0.3	39
MELIACEAE Juss.	3	2	1.6	0.4	0.3	40
PICRODENDRACEAE Small, nom. cons.	3	2	1.6	0.4	0.3	41
RHAMNACEAE Juss., nom. cons.	3	2	1.6	0.4	0.3	42

AMS: Australian medicinal species; DCC: Dermatological Complaints Category

Table I.2 (Continued from previous page)

	Number of species per family					
Family	AMS	DCC	Predicted DCC	Residuals	Standard Residuals	Ranking
ACANTHACEAE Juss.	2	1	1.1	-0.1	-0.1	43
CONVOLVULACEAE Juss.	2	1	1.1	-0.1	-0.1	44
HAEMODORACEAE R.Br., nom. cons.	2	1	1.1	-0.1	-0.1	45
LYTHRACEAE J.St.-Hil., nom. cons.	2	1	1.1	-0.1	-0.1	46
AMARANTHACEAE Juss., nom. cons.	4	2	2.1	-0.1	-0.1	47
BORAGINACEAE Juss.	4	2	2.1	-0.1	-0.1	48
SANTALACEAE R.Br., nom. cons.	8	4	4.2	-0.2	-0.2	49
ANNONACEAE Juss., nom. cons.	1	0	0.6	-0.6	-0.5	50
ARALIACEAE Juss.	1	0	0.6	-0.6	-0.5	51
ARECACEAE Bercht. & J.Presl, nom. cons.	1	0	0.6	-0.6	-0.5	52
BRASSICACEAE	1	0	0.6	-0.6	-0.5	53
DENNSTAEDTIACEAE Lotsy.	1	0	0.6	-0.6	-0.5	54
DIOSCOREACEAE R.Br., nom. cons.	1	0	0.6	-0.6	-0.5	55
FLAGELLARIACEAE Dumort., nom. cons.	1	0	0.6	-0.6	-0.5	56
GENTIANACEAE Juss.	1	0	0.6	-0.6	-0.5	57
GERANIACEAE Juss., nom. cons.	1	0	0.6	-0.6	-0.5	58
GYROSTEMONACEAE A.Juss.	1	0	0.6	-0.6	-0.5	59
MENYANTHACEAE Dumort.	1	0	0.6	-0.6	-0.5	60
MUSACEAE Juss.	1	0	0.6	-0.6	-0.5	61
MYRISTICACEAE R.Br., nom. cons.	1	0	0.6	-0.6	-0.5	62
NELUMBONACEAE A. Rich., nom. cons.	1	0	0.6	-0.6	-0.5	63
OPILIACEAE Valeton, nom. cons.	1	0	0.6	-0.6	-0.5	64
ROSACEAE Juss.	1	0	0.6	-0.6	-0.5	65
SMILACACEAE Vent., nom. cons.	1	0	0.6	-0.6	-0.5	66
SURIANACEAE Arn., nom. cons.	1	0	0.6	-0.6	-0.5	67
TACCACEAE Dumort., nom. cons.	1	0	0.6	-0.6	-0.5	68
THYMELAEACEAE Juss.	1	0	0.6	-0.6	-0.5	69
URTICACEAE Juss., nom. cons.	1	0	0.6	-0.6	-0.5	70
CELASTRACEAE R.Br., nom. cons.	3	1	1.6	-0.6	-0.5	71
RUTACEAE Juss.	3	1	1.6	-0.6	-0.5	72
CYPERACEAE Juss., nom. cons.	7	3	3.7	-0.7	-0.5	73
CAMPANULACEAE Juss.	2	0	1.1	-1.1	-0.9	74
NYMPHAEACEAE Salisb., nom. cons.	2	0	1.1	-1.1	-0.9	75
CUCURBITACEAE Juss., nom. cons.	4	1	2.1	-1.1	-0.9	76
GOODENIACEAE R.Br.	4	1	2.1	-1.1	-0.9	77
MYRTACEAE Juss., nom. cons.	43	21	22.4	-1.4	-1.1	78
SAPINDACEAE Juss.	7	2	3.7	-1.7	-1.3	79
LAMIACEAE	15	6	7.9	-1.9	-1.4	80
ASTERACEAE Martinov	13	4	6.8	-2.8	-2.2	81
POACEAE Barnhart, nom. cons.	21	8	11.0	-3.0	-2.3	82
LORANTHACEAE Juss., nom. cons.	6	0	3.2	-3.2	-2.5	83
SOLANACEAE Juss.	16	3	8.4	-5.4	-4.2	84

AMS: Australian medicinal species; DCC: Dermatological Complaints Category

Table I.3 Use categories and mentioned in Australian ethnobotanical literature and CAR

Use category / Use subcategory / Use as reported in literature	Number of species in use category (n_T)	Number of use reports for category (n_{UR})	Cultural Agreement Ratio (CAR)
Dermal complaints	**211**	**450**	**0.53**
'Sores' (dermal lesions)	**135**	**246**	**0.45**
'Sores'	131	235	0.44
"Open sores"	7	7	0.00
Rectal sores/abscesses	2	2	0.00
"Persistent or intractable sores"	1	2	1.00
Sores on genitals	1	1	NA
Sores/lesions of lips	1	1	NA
Wounds	**66**	**112**	**0.41**
Wounds and cuts	57	91	0.38
Ulcerations	10	25	0.63
Wound dressing	10	12	0.18
"Infected cuts"	9	10	0.11
Bleb vesicles (Blisters)	4	7	0.50
Dressing for compound fracture	3	3	0.00
Lavage of compound fracture wound	2	2	0.00
Dressing for ritual scarification wounds	2	2	0.00
Circumcision wounds	1	1	NA
(Oral): "Heals wounds"	1	1	NA
Burns	**13**	**18**	**0.29**
Burns	11	15	0.29
Burn dressing	2	2	0.00
Sunburn	2	2	0.00
Dermal 'infections'	**32**	**59**	**0.47**
"Infected skin lesions"	13	30	0.59
"Infected skin conditions"	7	12	0.45
"Infected sores"	3	6	0.60
Localised skin infections	4	5	0.25
"Various skin infections"	2	2	0.00
"Weeping sores"	1	1	NA
"Encrusted sores"	1	1	NA
"Septic inflammation and abscess"	1	1	NA

Table I.3 (Continued from previous page)

Use category / Use Subcategory / Use as reported in literature	Number of species in use category (n_T)	Number of use reports for category (n_{UR})	Cultural Agreement Ratio (CAR)
Fungal dermatophytes	**20**	**34**	**0.42**
Fungal dermatophytes	20	34	0.42
Leprosy sores	**15**	**25**	**0.42**
Leprosy sores	15	25	0.42
Boils	**30**	**52**	**0.43**
Boils	30	52	0.43
Scabies	**51**	**80**	**0.37**
Scabies	51	80	0.37
Dermal inflammation/ irritation	**49**	**72**	**0.32**
Anti-pruritic	17	21	0.20
Dermal rash	14	15	0.07
"Itchy skin conditions"	6	12	0.55
Dermal rash/infected or inflamed skin	5	7	0.33
Anti-pruritic "prickly heat"	4	5	0.25
Eczema	3	4	0.33
'Pustular rash'	1	4	1.00
General dermal irritation	3	3	0.00
"Itchy scaly skin"	1	3	1.00
Anti-pruritic for processionary caterpillar itch	2	2	0.00
"Itchy patches"	1	2	1.00
Dermal rash prophylaxis	1	1	NA
Skin allergies	1	1	NA
Anti-pruritic (bites and stings)	1	1	NA
Dermal unspecified	**39**	**42**	**0.07**
General dermal complaints	18	19	0.06
Various skin disorders	17	17	0.00
Dermal unspecified	6	6	0.00
Dermal 'other'	**12**	**16**	**0.27**
Mechanical debriding agent	3	6	0.60
Sunburn prophylaxis	3	3	0.00
"Cancer sores"	2	3	0.50
Moisturiser	1	1	NA

Table I.3 (Continued from previous page)

Use category / Use Subcategory / Use as reported in literature	Number of species in use category (n_T)	Number of use reports for category (n_{UR})	Cultural Agreement Ratio (CAR)
"Counterirritant"	1	1	NA
Lancet for draining infected boils	1	1	NA
Applied to "sun cancer"	1	1	NA
Dermal viral lesions	**14**	**23**	**0.41**
Herpes virus lesions	8	15	0.50
(Wash); for measles	3	3	0.00
Anti -pruritic "chicken pox"	2	3	0.50
Herpes virus lesions mouth	1	1	NA
(Oral): Herpes virus	1	1	NA
(Topical): Small pox	1	1	NA
Soap plants	**10**	**26**	**0.64**
Soap/detergent	9	22	0.62
Cleaning of skin	3	5	0.50
Removal of warts and tumours	**6**	**10**	**0.44**
Wart removal	5	7	0.33
(Mechanical): Wart removal	3	3	0.00
Haemostasis	**6**	**8**	**0.29**
Haemostasis	2	4	0.67
Post partum bleeding	3	3	0.00
(Smoking): Post partum bleeding	1	1	NA
Haemorrhoids (Bleeding)	1	1	NA
Ophthalmic complaints	**36**	**54**	**0.34**
"Sore eyes"	23	25	0.08
Unspecified eye complaints	8	8	0.00
"Sore, inflamed or infected eyes"	4	7	0.50
Infected and inflamed eyes	4	5	0.25
Eye drops or wash	4	5	0.25
Infected eyes	2	2	0.00
Inflamed skin around eyes	2	2	0.00
Inflamed eyes	1	1	NA
(Vapour): Sore eyes	1	1	NA

Table I.3 (Continued from previous page)

Use category / Use Subcategory / Use as reported in literature	Number of species in use category (n_T)	Number of use reports for category (n_{UR})	Cultural Agreement Ratio (CAR)
Bites and stings	**15**	**25**	**0.42**
(Topical): Marine and insect stings	2	8	0.86
Snakebite	5	6	0.20
Insect stings	4	5	0.25
Marine stings	4	4	0.00
Stingray sting	3	3	0.00
"Bites and stings"	2	2	0.00
Stonefish sting	2	2	0.00
Dental/mouth complaints	**30**	**49**	**0.40**
Mouth/buccal complaints	**14**	**21**	**0.35**
Sores/lesions of mouth	6	9	0.38
"Sore mouth"	4	4	0.00
Oral candidiasis	2	3	0.50
(Topical): infections of mouth	1	3	1.00
Infant oral candidiasis	2	2	0.00
(Mouthwash): Oral infections	1	2	1.00
(Mouthwash): "Sore mouth"	1	1	NA
Irritation of mouth (infants)	1	1	NA
Mouth	1	1	NA
Gingivitis	1	1	NA
Dental	**21**	**35**	**0.41**
Toothache mouthwash	8	13	0.42
Toothache (direct application to tooth or cavity)	7	13	0.50
Toothache	10	11	0.10
Ear/nose/throat complaints	**28**	**49**	**0.44**
Throat	**23**	**44**	**0.49**
Sore throat	21	42	0.51
(Oral): 'To clean out the throat'	2	2	0.00
(Oral): Laryngitis	1	1	NA
Aural	**5**	**5**	**0.00**
Painful ears	2	2	0.00
Infected or sore ears	2	2	0.00
Earache	1	1	NA

Table I.3 (Continued from previous page)

Use category / Use Subcategory / Use as reported in literature	Number of species in use category (n_T)	Number of use reports for category (n_{UR})	Cultural Agreement Ratio (CAR)
Respiratory complaints	**133**	**297**	**0.55**
Respiratory flu like illness	**102**	**215**	**0.53**
(Wash): Respiratory illness	59	112	0.48
(Oral): Respiratory illness	51	82	0.38
(Topical):Respiratory illness	11	17	0.38
(Inhalation): respiratory flu like illness	10	15	0.36
Leaves used as aromatic pillow in resp. illness	5	12	0.64
(Wash): Fever, pain and malaise associated with colds and flu	5	9	0.50
(Smoke inhalation): Colds and flu	6	6	0.00
(Topical) Ointment or crushed leaves rubbed on body during resp. Illness	4	4	0.00
(Smoking): Colds and flu	2	2	0.00
Respiratory illness	2	2	0.00
(Buccal): Respiratory illness	1	2	1.00
(Topical): Febrile respiratory illness	1	1	NA
Lower respiratory tract complaints	**37**	**51**	**0.28**
(Inhalation): Lower respiratory tract complaints	18	22	0.19
(Oral): Chest infection	8	9	0.13
(Topical):Bronchitis	2	6	0.80
(Topical): Chest infection	4	5	0.25
(Oral): Bronchitis	3	4	0.33
(Oral): Tuberculosis	2	3	0.50
(Oral): 'Illness of chest/lung'	2	2	0.00
(Topical): Pleurisy and bronchitis	1	1	NA
(Topical): 'Illness of chest/lung'	1	1	NA
(Buccal): Chest infection	1	1	NA
(Topical) Tuberculosis	1	1	NA
Congestion of chest lung	**4**	**4**	**0.00**
(Oral): Lung decongestant	3	3	0.00
(Topical): Decongestant (Chest)	1	1	NA
Nasal congestion/sinusitis	**17**	**25**	**0.33**
(Inhalation): Nasal congestion/sinusitis	8	10	0.22
(Inhalation): Sinusitis	4	7	0.50

Table I.3 (Continued from previous page)

Use category / Use Subcategory / Use as reported in literature	Number of species in use category (n_T)	Number of use reports for category (n_{UR})	Cultural Agreement Ratio (CAR)
Nasal decongestant	6	6	0.00
(Inhalation): "Head cold"	3	3	0.00
(Oral): Nasal congestion	1	2	1.00
Congestion (respiratory tract unspecified)	**44**	**65**	**0.33**
(Inhalation): Decongestant	25	35	0.29
(Wash): Decongestant	18	21	0.15
(Oral): Decongestant	15	17	0.13
(Smoke inhalation): Congestion caused by respiratory infections	1	1	NA
Asthma/bronchospasm	**5**	**5**	**0.00**
(Oral): Asthma	2	2	0.00
(Inhalation): Vapour/smoke inhaled for asthma/bronchospasm and respiratory difficulties	2	2	0.00
(Wash): Asthma/bronchospasm and respiratory difficulties	1	1	NA
(Oral): 'Breathlessness'	1	1	NA
Cough	**41**	**69**	**0.38**
(Oral): Cough	24	37	0.36
Cough	14	17	0.19
(Oral): Expectorant	6	11	0.50
(Topical): Cough	3	4	0.33
Pain and headache associated with respiratory flu like illness	**8**	**13**	**0.42**
(Topical): Aches and pains associated with resp. illness	4	7	0.50
(Topical): Muscle cramp associated with respiratory illness	2	2	0.00
(Topical): Headache associated with severe cold	1	2	1.00
(Topical): "Chest pain associated with severe cold/flu"	1	2	1.00
(Topical): Application sinusitis/nasal congestion associated headache	1	1	NA
Applied to ear in head cold	1	1	NA
Fever	**25**	**41**	**0.40**
(Topical): Fever	16	24	0.35
(Oral): Fever	6	13	0.58
(Smoking): Fever	2	3	0.50
(Topical): Fever in children	1	1	NA

Table I.3 (Continued from previous page)

Use category / Use Subcategory / Use as reported in literature	Number of species in use category (n_T)	Number of use reports for category (n_{UR})	Cultural Agreement Ratio (CAR)
(Oral): Post-partum fever	1	1	NA
(Topical): "Cooling"	1	1	NA
Bone fractures and disorders	**9**	**9**	**0.00**
(Dressing): Bone fracture	3	3	0.00
(Topical): Bone fracture	2	2	0.00
Bruising or bone fracture	2	2	0.00
(Topical): Bone disorders	1	1	NA
(Oral): Bone disorders	1	1	NA
Pain and inflammation	**108**	**175**	**0.39**
Joint disorders and pain	**25**	**33**	**0.25**
Joint pain	11	14	0.23
(Topical): Rheumatism	9	10	0.11
Swelling and pain in joints	3	4	0.33
Rheumatism	2	2	0.00
(Topical): Aching or swollen joints back neck ribs	1	2	1.00
(Topical): Joint dislocation	1	1	NA
(Topical): "Applied to sprained or jarred limbs"	1	1	NA
Skeleto-muscular pain	**34**	**48**	**0.30**
(Topical): Muscle pain	10	12	0.18
(Topical): Muscle cramps	5	10	0.56
Backache	3	6	0.60
(Vapour/heat): Backache	4	4	0.00
"Bruises sprains and muscular ache	3	4	0.33
"Liniment"	3	3	0.00
(Topical): Application "aching bones and muscles	2	2	0.00
(Topical): "Pain in chest"	2	2	0.00
Joint/muscle pain caused by trauma	2	2	0.00
Aching and fatigued muscles	2	2	0.00
(Topical): Backache arthritis and painful swellings	1	2	1.00
(Oral): Back/abdominal pain	1	2	1.00
(Topical): General weakness (muscular)	1	2	1.00
(Topical): Chronic internal pain	1	1	NA
"Pain in chest""	1	1	NA

Table I.3 (Continued from previous page)

Use category / Use Subcategory / Use as reported in literature	Number of species in use category (n_T)	Number of use reports for category (n_{UR})	Cultural Agreement Ratio (CAR)
Pain: General/unspecified	**43**	**54**	**0.21**
(Topical): Aches and pains	25	29	0.14
(Oral): General pain	7	7	0.00
(Smoke/vapour): Pain	7	7	0.00
(Topical): Pain and inflammation	4	7	0.50
(Oral): Aches and pains	4	5	0.25
(Wash): Generalised pain	4	5	0.25
Swelling/oedema	**15**	**18**	**0.18**
Swelling/bruising	11	12	0.09
Swelling/pain legs/knees	4	5	0.25
(Oral): Oedema	1	1	NA
Headache	**46**	**70**	**0.33**
(Topical): Headache	38	51	0.26
(Oral): Headache	10	18	0.47
Headache	4	4	0.00
(Inhalation): Headache	3	3	0.00
(Oral): Headache and fatigue	2	2	0.00
Urinary tract complaints and venereal disease	**10**	**14**	**0.31**
Venereal disease	5	8	0.43
Pain on urination	1	3	1.00
Diuretic (mechanical)	1	1	NA
Topical: venereal disease	1	1	NA
(Oral): Venereal disease	1	1	NA
"Genital diseases"	1	1	NA
"Kidney diseases"	1	1	NA
(Topical): "Kidney diseases"	1	1	NA
GI Complaints	**66**	**105**	**0.38**
Diarrhoea/dysentery	**43**	**68**	**0.37**
(Oral): Diarrhoea	17	28	0.41
(Oral): Diarrhoea/Gastrointestinal distress	16	22	0.29
(Wash): Diarrhoea	7	8	0.14
(Oral): Diarrhoea/vomiting	3	3	0.00

Table I.3 (Continued from previous page)

Use category Use Subcategory Use as reported in literature	Number of species in use category (n_T)	Number of use reports for category (n_{UR})	Cultural Agreement Ratio (CAR)
(Wash): Diarrhoea/vomiting	3	3	0.00
(Oral): Diarrhoea in infants/children	2	2	0.00
(Smoking): Diarrhoea/Gastrointestinal distress (smoking)	2	2	0.00
"Dysentery"	2	2	0.00
Diarrhoea (Unspecified application)	1	1	NA
Leaves rubbed on hands to prevent diarrhoea	1	1	NA
(Wash): To relieve pain associated with diarrhoea	1	1	NA
Pain associated with GI conditions	**10**	**14**	**0.31**
(Oral): Stomach ache	4	4	0.00
Relieve pain associated with diarrhoea	1	3	1.00
(Topical): Abdominal pain	2	2	0.00
(Wash): Abdominal pain	2	2	0.00
(Oral): Abdominal pain	2	2	0.00
Stomach ache topical	1	1	NA
(Oral): Pain in alimentary tract	1	1	NA
GI Complaints (nausea and general GI distress)	**9**	**11**	**0.20**
(Oral): Gastrointestinal distress	4	4	0.00
Nausea/vomiting	3	3	0.00
(Oral): Nausea/vomiting	1	1	NA
(Inhalation): Vapour inhaled for nausea	1	1	NA
(Wash): Gastrointestinal distress	1	1	NA
General GI disturbance + nausea	1	1	NA
GI complaints: (General/unspecified)	**14**	**15**	**0.50**
Laxative	4	4	0.00
(Oral): Constipation	4	4	0.00
GI complaints unspecified	2	3	0.50
(Oral): Chronic ailments of chest/GI tract	1	1	NA
"Alimentary ulcers"	1	1	NA
(Oral): Aperient	1	1	NA
(Oral): Purgative	1	1	NA
(Oral): GI parasites	1	1	NA

Table I.3 (Continued from previous page)

Use category / Use Subcategory / Use as reported in literature	Number of species in use category (n_T)	Number of use reports for category (n_{UR})	Cultural Agreement Ratio (CAR)
Indigestion	**5**	**5**	**0.00**
Indigestion	3	3	0.00
"Aid to digestion"	1	1	NA
Antacid type treatment	1	1	NA
Reproductive	**37**	**49**	**0.25**
Neonatal smoking	**20**	**27**	**0.27**
(Smoking): Neonatal smoking	15	19	0.22
(Smoking): Neonate and mother	5	6	0.20
Post-natal tonic (neonate and mother)	2	2	0.00
Lactation/Mammary	**14**	**17**	**0.19**
Lactation inducer	7	10	0.33
Improve lactation with mechanical irritation of nipple	2	2	0.00
(Smoking): Lactation inducer	1	1	NA
(Inhalation): smoke to stop lactation	1	1	NA
Lactation modifier	1	1	NA
Improve lactation	1	1	NA
Breast enlargement	1	1	NA
Mammary swelling	1	1	NA
Abortifacients and contraceptives	**6**	**6**	**0.00**
Contraceptive	4	4	0.00
(Oral): Abortifacient	1	1	NA
(Oral): Contraceptive	1	1	NA
Neurological	**54**	**79**	**0.32**
Neurological	**9**	**9**	**0.00**
Sedative: soporific	2	2	0.00
(Smoking): Diaphoretic smoking ("nervous conditions")	2	2	0.00
Depression	2	2	0.00
Topical for contusion	1	1	NA
(Smoking): Sedative - paediatric	1	1	NA
Mental illness sedative	1	1	NA

(Table I.3) Continued from previous page)

Use category / Use Subcategory / Use as reported in literature	Number of species in use category (n_T)	Number of use reports for category (n_{UR})	Cultural Agreement Ratio (CAR)
Narcotics'/psychoactive	**45**	**70**	**0.36**
Tobacco substitute	12	22	0.48
(Buccal): "Narcotic quid"	9	9	0.00
(Buccal): Used as chewing tobacco	7	9	0.25
(Buccal): Pituri substitute	8	8	0.00
(Buccal): 'Pituri'	7	7	0.00
Mixed with chewing tobacco to increase potency (not including species used to produce alkaline ash)	5	5	0.00
"Narcotic"	2	4	0.67
'Narcotic' (alcohol like effect)	2	2	0.00
Used to induce drowsiness	2	2	0.00
"Used as intoxicant"	1	1	NA
"Stupefying drink"	1	1	NA
(Oral): 'Narcotic' drink	1	1	NA
Mixed with pituri to increase potency (not including species used to produce alkaline ash)	1	1	NA
Tonics	**28**	**31**	**0.10**
(Wash): For "weakness and fatigue"	4	6	0.40
(Oral): Tonic	5	5	0.00
(Wash): For "chronic internal ailments"	2	2	0.00
(Wash): Tonic	2	2	0.00
(Topical): Leaves placed on babies knees hip and spine to help walk quickly and to make strong and healthy	2	2	0.00
(Smoking): Prepare babies for travel	2	2	0.00
(Topical): General tonic to prevent illness	1	1	NA
(Poultice): Convalescence after prolonged illness	1	1	NA
(Smoking): Convalescence after prolonged illness	1	1	NA
"Tonic for blood and heart trouble"	1	1	NA
"Blood tonic"	1	1	NA
(Oral): "Tonic for blood and nerves"	1	1	NA
"Refreshing tonic"	1	1	NA
(Oral): Tonic in minor illness (colds, low fever, GI illness)	1	1	NA
(Oral): Tonic for debility	1	1	NA

Table I.3 (Continued from previous page)

Use category / Use Subcategory / Use as reported in literature	Number of species in use category (n_T)	Number of use reports for category (n_{UR})	Cultural Agreement Ratio (CAR)
(Topical): Tonic for young children	1	1	NA
(Oral): Drunk to treat "weak heart"	1	1	NA
To assist infants who are "slow to begin walking"	1	1	NA
(Wash): "Refreshment"	1	1	NA
"Feeling of cleanliness"	1	1	NA
Miscellaneous	**64**	**84**	**0.24**
'Contemporary' diseases	**11**	**11**	**0.00**
"Diabetes"	3	3	0.00
(Wash): For mumps	2	2	0.00
"Cancer cure"	2	2	0.00
"Lung cancer"	1	1	NA
Reputed to successfully treat type II diabetes	1	1	NA
Reputed to cure cancer	1	1	NA
"Heart disease"	1	1	NA
(Topical): Breast Cancer	1	1	NA
General malaise and non specific illness	**34**	**45**	**0.25**
(Wash): General complaints	12	13	0.08
(Topical):General malaise	10	13	0.25
Leaves used to make bed for ill babies	4	5	0.25
(Smoking): General illness	3	4	0.33
"Used for various complaints"	3	3	0.00
(Oral): General malaise	2	2	0.00
(Wash): For chronic malaise	1	2	1.00
(Wash): "Fever and malaise"	1	1	NA
(Oral): Fever and headache associated with infections	1	1	NA
(Wash): Fever and headache associated with infections	1	1	NA
(Wash): Palliative treatment in severe illness	1	1	NA
General medicine for infants	1	1	NA
(Topical): "Any severe illness"	1	1	NA
(Topical): 'Wasting diseases'	1	1	NA

Table I.3 (Continued from previous page)

Use category / Use Subcategory / Use as reported in literature	Number of species in use category (n_T)	Number of use reports for category (n_{UR})	Cultural Agreement Ratio (CAR)
Miscellaneous medicinal uses	**10**	**11**	**0.10**
Tea substitute/"Drunk as tea"	3	4	0.33
Nasal irritant	2	2	0.00
Thirst	2	2	0.00
"Powder"	1	1	NA
Used to ally hunger	1	1	NA
Cosmetic	**14**	**18**	**0.24**
Strong irritant used to burn in scarification markings	2	3	0.50
Prevention of hair growth	2	3	0.50
Hair lotion	2	2	0.00
"Acupuncture" with sharp new growth for weight loss and reduction	2	2	0.00
Hair restorer	1	2	1.00
Hair shampoo/conditioner	1	2	1.00
Scarification	1	1	NA
Promote greying of hair	1	1	NA
To "make skin shine" (cosmetic)	1	1	NA
Skin darkening	1	1	NA

Appendix II: Notes on traditional aqueous preparations and HPLC voucher chromatograms

Table II.1 Collection localities of plant specimens used in the study

Herbarium Collectors number	Species	Family	Date collected	Coordinates (DMS lat. S, long. E)	Alt (m)	EPE (m)	Locality
JES030	*Acacia auriculiformis*	Fabaceae	22/08/2007	12°26'43.8"S, 130°52'38.4"E	46	5	Charles Darwin NP, Darwin, NT
JES049	*Acacia holosericea*	Fabaceae	26/08/2007	14°21'44.1"S, 132°26'07.3"E	130	4	Jodetluk community, Katherine area (in bank of creek bed) , NT
JES034	*Avicennia marina*	Acanthaceae	23/08/2007	12°20'41.4"S, 130°53'06.1"E	14	3	Sandy Ck. Outlet Casurina nature reserve, Darwin area, NT
JES042	*Bauhinia cunninghamii*	Fabaceae	25/08/2007	14°29'30.8"S, 132°15'17.4"E	106	6	Katherine River, Katherine, NT
JES048	*Buchanania obovata*	Anacardiaceae	26/08/2007	14°21'44.1"S, 132°26'07.1"E	133	4	Jodetluk community, Katherine area, NT
JES066	*Buchanania obovata*	Anacardiaceae	28/08/2007	12°28'47.9"S, 130°59'19.5"E	30	4	Palmerston area, NT
JES041	*Callitris intratropica*	Cupressaceae	25/08/2007	14°29'27.0"S, 132°15'20.5"E	121	3	Katherine R., Katherine, NT
JES035	*Camptostemon schultzii*	Bombaceae	23/08/2007	12°20'42.8"S, 130°53'05.4"E	12	3	Sandy Creek outlet Casurina nature reserve, Darwin area, NT
JES059	*Cochlospermum fraseri*	Bixaceae	26/08/2007	14°10'39.3"S, 132°06'27.3"E	131	4	Edith Falls area 60 km N of Katherine, NT
JES043	*Crinum angustifolium*	Amaryllidaceae	25/08/2007	14°21'44.4"S, 132°26'06.8"E	129	4	SE Bank of creek bed, Jodetluk community, Katherine area, NT
JES058	*Cymbopogon ambiguus*	Poaceae	26/08/2007	14°21'45.3"S, 132°26'03.6"E	131	5	Jodetluk community, Katherine area, NT
JES021	*Eremophila alternifolia*	Myoporaceae	29/08/2003	31°01'39.4"S, 141°35'43.8"E	200	20	Menindee rd, 17 km SE from Broken hill, NSW
JES023	*Eremophila alternifolia*	Myoporaceae	29/08/2003	31°01'39.4"S, 141°35'43.8"E	200	20	Menindee rd, 17 km SE from Broken hill, NSW
SM010	*Eremophila duttonii*	Myoporaceae	30/11/2004	31°14'31.3"S,142°16'52.4"E	261	11	Mutawintji NP, S of landing strip near hut, NSW
SM011	*Eremophila duttonii*	Myoporaceae	30/11/2004	31°14'31.8"S,142°16'53.6"E	203	14	Mutawintji NP, S of landing strip near hut, NSW
SM006	*Eremophila latrobei*	Myoporaceae	30/11/2004	31°17'15.8"S,142°16'53.3"E	226	11	Mutawintji NP cuesta escarpment face near to park entrance, W of visitors centre, NSW
SM007	*Eremophila latrobei*	Myoporaceae	30/11/2004	31°17'15.8"S,142°16'53.3"E	226	11	Mutawintji NP cuesta escarpment face near to park entrance, W of visitors centre, NSW
SM008	*Eremophila latrobei*	Myoporaceae	30/11/2004	31°17'15.8"S,142°16'53.3"E	226	11	Mutawintji NP cuesta escarpment face near to park entrance, W of visitors centre, NSW
JES019	*Eremophila longifolia*	Myoporaceae	28/08/2003	31°16'33.0"S, 142°18'07.1"E	216	11	Mutawintji NP, E side of Homestead ck. Gorge 5 m below crest, NSW
SM003	*Eremophila longifolia*	Myoporaceae	29/11/2004	31°16'32.7"S, 142°18'07.7"E	275	12	Mutawintji NP, immediately E of bore servicing homestead, NSW
SM004	*Eremophila longifolia*	Myoporaceae	29/11/2004	31°16'45.1"S, 142°18'07.3"E	219	14	Mutawintji NP, Homestead gorge, NSW
SM005	*Eremophila longifolia*	Myoporaceae	29/11/2004	31°16'40.2"S, 142°18'07.4"E	209	20	Mutawintji NP, NSW
SM012	*Eremophila longifolia*	Myoporaceae	30/11/2004	31°17'30.0"S, 142°17'59.7"E	216	22	Mutawintji NP, 200 m, 300 degrees bearing from shearers qauters, NSW
SM016	*Eremophila longifolia*	Myoporaceae	01/12/2004	31°43'51.2"S, 142°30'24.9"E	187	10	Barrier Hwy. ca. 100 km W of Wilcannia, NSW
SM017	*Eremophila longifolia*	Myoporaceae	01/12/2004	31°43'31.1"S, 142°40'53.1"E	227	18	Barrier Hwy. ca. 100 km W of Wilcannia, NSW
SM018	*Eremophila longifolia*	Myoporaceae	01/12/2004	31°41'47.7"S, 143°44'19.7"E	194	9	Barrier Hwy. ca. 50 km E of Wilcannia, NSW
JES001	*Eremophila longifolia*	Myoporaceae	23/02/2003	32°06'06.2"S, 144°11'42.0"E	92	20	Ivanhoe/Cobar region, NSW
SM019	*Eremophila longifolia*	Myoporaceae	01/12/2004	31°32'37.5"S, 145°17'42.3"E	199	12	Barrier Hwy., ca. 50 km W of Cobar, NSW
SM001	*Eremophila sturtii*	Myoporaceae	28/11/2004	31°32'26.5"S,141°39'41.9"E	287	9	Near creek bed at turn off to Mutawintji NP from Tibooburra rd. (Barrier Range) NSW
JES040	*Erythrophleum chlorostachys*	Fabaceae	25/08/2007	14°29'27.2"S, 132°15'20.4"E	121	4	Katherine R., Katherine, NT
JES025	*Eucalyptus tetrodonta*	Myrtaceae	22/08/2007	12°26'39.8"S, 130°52'33.8"E	28	5	Charles Darwin NP, Darwin, NT
JES037	*Flagellaria indica*	Flagellariaceae	25/08/2007	14°29'29.5"S, 132°15'08.7"E	93	8	Low level Nature park, Katherine, NT
JES032	*Hibiscus tiliaceus*	Malvaceae	23/08/2007	12°20'00.1"S, 130°53'40.4"E	11	4	Lee point foredunes, Darwin area, NT
JES031	*Ipomoea pes-caprae*	Convolvulaceae	23/08/2007	12°20'01.1"S, 130°53'40.4"E	2	4	Lee point foredunes, Darwin area, NT
JES060	*Morinda citrifolia*	Rubiaceae	27/08/2007	12°22'34.1"S, 130°51'45.2"E	14	4	Foreshore, Rapid Creek outlet, Darwin area, NT
JES027	*Pandanus spiralis*	Pandanaceae	22/08/2007	12°26'36.4"S, 130°52'32.6"E	28	8	Charles Darwin NP, Darwin, NT
JES063	*Scaevola sericea*	Goodeniaceae	27/08/2007	12°24'56.2"S, 130°49'50.2"E	18	4	East point reserve, Darwin area, NT

Table II.2 Plant parts, preparation methods, summary of data and sources for traditional preparations examined in the study

Label code	Species	Parts used	Extraction/preparation method	Mass of parts extracted (gm)	Volume of solvent (mL)	Time extracted (hr:min)	Final volume (mL)	pH	Concentration (mg/mL)	Yield (% $^{w}/_{w}$)	Source
JES030LD	*Acacia auriculiformis*	Leaves	Decoction (leaves crushed by hand)	40.00	500	0:15	390	5.21	8.0	7.8	Barr *et al.* (1988)
JES049LD	*Acacia holosericea*	Leaves	Decoction (leaves crushed by hand)	40.00	500	0:15	390	5.39	8.7	8.5	Barr *et al.* (1988)
JES034LTD	*Avicennia marina*	Leaves	Decoction	75.00	500	0:15	410	5.73	14.4	7.9	Barr *et al.* (1988)
JES042TD	*Bauhinia cunninghamii*	Root	Decoction (outer bark removed and root	20.00	500	0:15	380	5.35	3.8	7.2	Barr *et al.* (1988)
JES066TD	*Buchanania obovata*	New growth terminal stems	Decoction (outer bark removed and stem	17.00	500	0:15	380	4.30	4.2	9.5	Barr *et al.* (1988)
JES066SE	*Buchanania obovata*	New growth terminal stems	Infusion (outer bark removed and stem	70.00	500	3:00	500	4.15	9.7	6.9	Levitt and Lyon (1981)
JES048BD	*Buchanania obovata*	Inner bark	Decoction	120.00	400	0:15	330	4.99	20.4	5.6	Levitt and Lyon (1981)
JES041TD	*Callitris intratropica*	Inner bark	Decoction	120.00	500	0:15	340	4.43	13.8	3.9	Barr *et al.* (1988)
JES035SE	*Camptostemon schultzii*	Timber	Warm Infusion (60°C) (charred pieces of timber)	50.00	400	0:20	350	9.71	6.2	4.3	Barr *et al.* (1988)
JES059EJ	*Cochlospermum fraseri*	Fruit pulp and seeds	Expressed juice	91.33	NA	NA	23	4.97	95.2	2.4	Barr *et al.* (1988)
JES043TS	*Crinum angustifolium*	Bulb	Cold infusion (20°C) (Bulb macerated)	135.00	400	24:00	400	3.90	18.8	5.6	Barr *et al.* (1988)
JES043BD	*Crinum angustifolium*	Bulb	Decoction (bulb macerated)	135.00	400	0:15	335	5.47	18.0	4.5	Barr *et al.* (1988)
JES058AD	*Cymbopogon ambiguus*	Whole plant	Decoction (plant cut into 1-2 cm sections)	30.00	500	0:15	360	5.12	3.6	4.3	Barr *et al.* (1988)

Table II.2 (Continued from previous page)

Label code	Species	Parts used	Extraction/preparation method	Mass of parts extracted (g)	Volume of solvent (mL)	Time extracted (hr:min)	Final volume (mL)	pH	Concentration (mg/mL)	Yield (% $^{w}/_{w}$)	Source
JES023TD	*Eremophila alternifolia*	Leaves	Decoction	50.00	1000	0:15	820	4.91	5.1	8.3	Barr *et al.* (1988)
JES021TD	*Eremophila alternifolia*	Leaves	Decoction	50.00	1000	0:15	840	4.66	5.0	8.4	Barr *et al.* (1988)
SM010TD	*Eremophila duttonii*	Leaves	Decoction	67.00	1000	0:15	750	4.53	9.7	10.9	Barr *et al.* (1988)
SM011TD	*Eremophila duttonii*	Leaves	Decoction	70.00	1000	0:15	765	4.53	6.2	6.8	Barr *et al.* (1988)
SM007TD	*Eremophila latrobei*	Leaves	Decoction	40.00	1000	0:15	780	3.90	5.4	10.5	Barr *et al.* (1988); Latz (1995)
SM008TD	*Eremophila latrobei*	Leaves	Decoction	40.00	1000	0:15	800	3.90	6.1	12.3	Barr *et al.* (1988); Latz (1995)
SM003TD	*Eremophila longifolia* (IC)	Leaves	Decoction	50.00	1000	0:15	780	4.82	7.7	12.1	Barr *et al.* (1988)
SM018TD	*Eremophila longifolia* (KC)	Leaves	Decoction	50.00	1000	0:15	740	4.95	8.1	12.0	Barr *et al.* (1988)
SM019TD	*Eremophila longifolia* (BC)	Leaves	Decoction	50.00	1000	0:15	780	4.49	8.3	12.9	Barr *et al.* (1988)
SM001TD	*Eremophila sturtii*	Leaves	Decoction	40.00	1000	0:15	820	4.49	9.3	19.1	Barr *et al.* (1988)
JES040LTD	*Erythrophleum chlorostachys*	Leaves and Inner bark	Decoction	31.00	500	0:15	385	4.75	4.7	5.8	Barr *et al.* (1988)
JES040BTD	*Erythrophleum chlorostachys*	Inner bark	Decoction	40.00	500	0:15	410	4.50	10.1	10.3	Barr *et al.* (1988)

IC: Isomenthone chemotype; KC: Karahanaenone chemotype; BC: Borneol chemotype

Table II.2 (Continued from previous page)

Label code	Species	Parts used	Extraction/preparation method	Mass of parts extracted (g)	Volume of solvent (mL)	Time extracted (hr:min)	Final volume (mL)	pH	Concentration (mg/mL)	Yield (% $^{w}/_{w}$)	Source
JES028LTD	*Eucalyptus tetrodonta*	Leaves	Decoction (leaves crushed by hand)	20.00	500	0:15	400	4.18	5.4	10.8	Barr *et al.* (1988)
JES028BTD	*Eucalyptus tetrodonta*	Inner bark	Decoction	10.00	500	0:15	400	3.54	1.2	4.7	Barr *et al.* (1988)
JES037SE	*Flagellaria indica*	Stems	Warm Infusion (60°C) (crushed with mortar and pestle)	95.00	500	0:15	465	5.43	7.1	3.5	Levitt and Lyon (1981)
JES037EJ	*Flagellaria indica*	Terminal nodes	Expressed juice	9.65	NA	NA	4	5.17	118.0	4.9	Levitt and Lyon (1981)
JES032BHE	*Hibiscus tiliaceus*	Stems	Warm infusion (60°C) (outer bark removed and stem shredded)	70.00	200	3:00	200	5.77	9.5	2.7	Barr *et al.* (1988)
JES032SE	*Hibiscus tiliaceus*	Stems	Cold infusion (20°C) (outer bark removed)	38.50	500	20:00	440	5.97	3.3	3.8	Levitt and Lyon (1981)
JES031SE	*Ipomoea pes-caprae*	Leaves	Cold infusion (20°C) (10 leaves)	19.75	500	20:00	486	5.80	0.8	2.1	Barr *et al.* (1988)
JES31HSE	*Ipomoea pes-caprae*	Leaves	Warm infusion (60°C) (10 leaves)	19.75	500	20:00	410	6.20	1.5	3.2	Barr *et al.* (1988)
JES060EJ	*Morinda citrifolia*	Fruit pulp	Expressed juice (outer layer of fruit removed)	150.00	NA	NA	46	4.50	51.1	1.6	Levitt and Lyon (1981)
JES027LBD	*Pandanus spiralis*	Leaf base	Decoction	100.00	500	0:15	375	5.09	13.6	5.1	Barr *et al.* (1988)
JES027EJP	*Pandanus spiralis*	Leaf tips	Expressed juice diluted with 100 mL H_2O	100.00	100	NA	182	5.45	8.3	1.5	Barr *et al.* (1988)
JES063EJ	*Scaevola sericea*	Fruit	Expressed juice	75.00	NA	NA	18	5.11	75.8	1.8	Barr *et al.* (1988)

7.1.5.1 *Acacia auriculiformis* A.Cunn. ex Benth. (Fabaceae) leaf decoction

The decoction of *A. auriculiformis* leaves (extract JES030LD) was prepared with material from specimen JES030 (Collected in the vicinity of Charles Darwin national park, Darwin, NT). Preparation was based on descriptions from Barr *et al.* (1988). Barr *et al.* (1988) describes use of a decoction of leaves (one handful, crushed and boiled in water), for the treatment of dermal sores. To prepare the extract, an approximate mass for the required quantity of leaves was estimated (40 gm). Leaves were crushed by hand (in aluminium foil) and extracted for 15 minutes in 500 mL boiling deionised water. The resulting extract which was a pale green-yellow colour, was decanted and cooled and final volume estimated at 390 mL. The extract was then filter sterilised and transferred into containers for storage and lyophilisation. 200 mL of the extract was lyophilised which appeared as a beige-yellow powder. Extract characteristics were as follows: pH, 5.21, concentration: 8.0 mg · mL^{-1}, yield: 7.8% $^{w}/_{w}$.

7.1.5.2 *Acacia holosericea* A.Cunn. ex G.Don (Fabaceae) leaf decoction

The decoction of *A. holosericea* leaves (extract JES049LD) was prepared with material from specimen JES049 (Collected from the bank of Maude creek, Jodetluk community, NT). Preparation was based on descriptions from Barr *et al.* (1988). Barr *et al.* (1988) describes the same method of preparation and traditional use of decoctions of this species as that given for *A. auriculiformis*. For extraction, the procedure for extraction of *A. auriculiformis* as given above (7.1.5.1) was followed. Approximate mass for the required quantity of leaves was estimated also at 40 gm. The resulting extract which was a pale green-yellow colour, was decanted and cooled and final volume estimated at 390 mL. The extract was then filter sterilised and transferred into containers for storage and lyophilisation. 200 mL of the extract

was lyophilised which appeared as a beige-white powder. Extract characteristics were as follows: pH, 5.39, concentration: 8.7 mg · mL^{-1}, yield: 8.5% $^{w}/_{w}$.

7.1.5.3 *Avicennia marina* (Forssk.) Vierh. (Acanthaceae) leaf decoction

A. marina leaf decoction (extract JES034TD) was prepared with material from specimen JES034 (Collected in the vicinity of Sandy Creek outlet, Darwin, NT). Preparation was based on descriptions by Barr *et al.* (1988). 75 gm of the leaves were extracted by boiling in 500 mL of deionised water for 15 minutes. The resulting extract (coloured a dark green) was decanted and cooled and the final volume measured (410 mL). A 200 mL sample was frozen and lyophilised. The lyophilised extract appeared as a green powder. Extract characteristics were as follows: pH 5.73, concentration: 14.4 mg · mL^{-1}, yield: 7.9% $^{w}/_{w}$.

7.1.5.4 *Bauhinia cunninghamii* (Benth.) Benth. (Fabaceae) leaf decoction

The decoction of *B. cunninghamii* root (extract JES042TD) was prepared using material from specimen JES042 (Collected from riparian zone of the Katherine river, Katherine, NT). Extract preparation was based on descriptions given by Barr *et al.* (1988) where use of decoctions of root tissue for the treatment of dermal sores and *Scabies* infestation is described (in this source *B. cunninghamii* is referred to with the nomenclatural synonym: *Lysiphyllum cunninghamii* (Benth.) de Wit.). Barr *et al.* (1988) describes traditional extraction of a 30 cm section of root, from which outer bark has been removed and which has been crushed by "pounding well". To prepare the extract a 30 cm section of root was cut and outer bark peeled away using a knife. The orange coloured root was then pounded flat using a mortar and pestle. The pounded root (20 gm) was then extracted for 15 minutes in 500 mL boiling deionised water. The resulting extract, which was a burnt-orange colour, was decanted and cooled and final volume determined (380 mL) before filter sterilising and transferring into containers for storage and lyophilisation. 200 mL of the extract was lyophilised which appeared as a burnt-

orange powder. Extract characteristics were as follows: pH, 5.35, concentration: 3.8 mg · mL^{-1}, yield: 7.2% $^{w}/_{w}$.

7.1.5.5 *Buchanania obovata* Engl. (Anacardiaceae) inner bark decoction and terminal stem decoction and infusion

Three traditional extracts were prepared using material from *B. obovata* These included a decoction of inner trunk bark from a mature specimen, a decoction of terminal recent growth stems from a juvenile plant and a cold infusion extract of terminal recent growth stems. The decoction of trunk inner bark (extract JES048BD) of was based on descriptions of the use of boiled aqueous extracts of trunk inner bark for treatment of wounds given by Levitt and Lyon (1981). To prepare the extract 120 gm of inner trunk bark from a tall mature tree (specimen JES048, collected from Jodetluk community north of Katherine, NT) was extracted by boiling in 400 mL of deionised water for 15 minutes. The resulting extract was decanted, cooled and filter sterilised. Final extract volume was 330 mL, 200 mL of which was frozen and lyophilised. Extract colour was pale yellow-brown and the lyophilised extract appeared as a pale yellow-brown powder. Extract characteristics were as follows: pH 4.99, concentration: 20.4 mg · mL^{-1}, yield: 5.6% $^{w}/_{w}$.

Decoction and cold infusion extracts of *B. obovata* terminal recent growth stems was prepared from specimen JES66 (collected in Palmerston area, NT), and was based on descriptions given by Barr *et al.* (1988) of the use of either type of preparation for treatment of dermal sores and cuts, as an eye lotion or a mouthwash for toothache, and similar descriptions of a cold infusion (quantity not specified) by Levitt and Lyon (1981) for the treatment of ophthalmic complaints. Barr *et al.* (1988) describes preparation of the treatment by soaking or boiling a 15-20 cm section of terminal stem which has been crushed after removal of the hard outer bark. To prepare the decoction a 20 cm section of recent growth

stem (17 gm) was stripped of outer bark to reveal the green, watery inner bark and pale sapwood. The stem was then crushed using a mortar and pestle and extracted for 15 min in 500 mL of boiling, deionised water. The resulting extract was decanted, cooled and filter sterilised. Final extract volume was 380 mL, 200 mL of which was frozen and lyophilised. Extract colour was pale yellow-green and the lyophilised extract appeared as a pale yellow-beige powder. Extract characteristics were as follows: pH 4.30, concentration: 4.2 mg · mL^{-1}, yield: 9.5% $^{w}/_{w}$.

For the cold infusion of inner bark and sapwood of terminal recent growth stems as described by Levitt and Lyon (1981) (extract JES066SE), a larger quantity (70 gm) of terminal stems were stripped of outer bark, pounded using a mortar and pestle and extracted in 500 mL of deionised water at room temperature over a period of 3 hr. The resulting extract was decanted and filter sterilised. Final extract volume 500 mL, 300 mL of which was frozen and lyophilised. Extract colour was pale yellow-green and the lyophilised extract appeared as a pale yellow-beige powder. Extract characteristics were as follows: pH 4.15, concentration: 9.7 mg · mL^{-1}, yield: 6.9% $^{w}/_{w}$.

7.1.5.6 *Callitris intratropica* R.T.Baker & H.G.Sm. (Cupressaceae) inner bark decoction

C. intratropica inner bark decoction (extract JES041TD) was prepared following a description by Barr *et al.* (1988), of the use of a decoction of a handful of inner bark in a few cups of water for treatment of sores and cuts. The extract was prepared using material from specimen JES040 (Collected in the vicinity of the Katherine river, Katherine, NT). 120 gm of inner bark pieces (ca. 3-4 cm^2, obtained by shaving from exposed trunk with machete blade) were extracted by boiling in 500 mL of deionised water for 15 minutes. The resulting extract was decanted, cooled and filter sterilised. Final extract volume was 340 mL, 150 mL of

which was frozen and lyophilised. Extract colour was dark red-brown and the lyophilised extract appeared as a red-brown powder. Extract characteristics were as follows: pH 4.43, concentration: 13.8 mg · mL^{-1}, yield: 3.9% $^{w}/_{w}$.

7.1.5.7 *Camptostemon schultzii* Mast. (Bombacaceae) charred wood infusion

C. schultzii infusion of charred wood (extract JES035TD) was prepared with material from specimen JES035 (Collected in the vicinity of Sandy Creek outlet, Darwin, NT) based on descriptions from Wightman (2006) and Barr *et al.* (1988) of treatments for dermal sores, *Scabies* and fungal dermatophytic infections prepared by infusing the wood from *C. schultzii*, which has been incompletely charred in a fire, in seawater. Several pieces of wood, each ca. 2 cm^3, were charred over a Bunsen flame and left to smoulder briefly in a crucible. Care was taken to ensure wood was not completely burned to ash or charcoal (inspection of fragments revealed ca. 3-4 mm surface depth of charcoal). 50 gm of the charred fragments were infused for 20 minutes in deionised water heated to 60° C. The resulting extract was decanted, cooled and filter sterilised. Final extract volume was 350 mL, 150 mL of which was frozen and lyophilised. Extract colour was dark grey-brown and the lyophilised extract appeared as a grey powder. Extract characteristics were as follows: pH 9.71, concentration: 6.2 mg · mL^{-1}, yield: 4.3% $^{w}/_{w}$.

7.1.5.8 *Cochlospermum fraseri* Planch. (Bixaceae) expressed juice of fruit pulp

C. fraseri expressed juice of fruit (extract JES059EJ) was prepared with material from specimen JES059 (Collected in the vicinity of Edith Falls, ca. 90 km north of Katherine, NT). The extract was prepared based on descriptions by Barr *et al.* (1988) of the use of juice from seed/flesh and direct application of seeds and flesh of unripe fruit to treat boils. Unripe fruit was split open and seeds and flesh scraped out. 91.33 gm of seeds and flesh were packed into the body of a 100 mL syringe mounted on a ratcheting clamp. Juice was extracted by

applying pressure to the plunger until no more liquid could be expressed. 23 mL of a bright orange juice was yielded 10 mL of which was frozen and lyophilised. The lyophilised extract appeared as a bright orange, hygroscopic powder. Extract characteristics were as follows: pH 4.79, concentration: 95.2 mg · mL^{-1}, yield: 2.4% $^{w}/_{w}$.

7.1.5.9 *Crinum angustifolium* R.Br. (Amaryllidaceae) aqueous bulb extract

Traditional extracts of *C. angustifolium* were prepared with material from specimen JES043 (collected from Jodetluk community north of Katherine, NT). Two separate extracts were prepared including a 24 hr room temperature infusion of macerated bulb material based on traditional preparation described by Barr *et al.* (1988), and a boiled decoction of the macerated bulb material as also described by Barr *et al.* (1988) and Wynjorrotj *et al.* (2005) (both preparations used in the traditional treatment of infected and inflamed skin, leprotic lesions and major wounds). For the 24 hr infusion (extract JES043TS) One half of the bulb (135 gm) was macerated in a Waring blender and added to 400 mL of sterile deionised water. The mixture was allowed to extract for 24 hr and the resulting colourless solution decanted and filter sterilised. 200 mL was frozen and lyophilised. The lyophilised extract appeared as a colourless crystaline powder. Extract characteristics were as follows: pH 3.90, concentration: 18.8 mg · mL^{-1}, yield: 5.6% $^{w}/_{w}$.

For the boiled aqueous decoction (extract JES043BD) 135g of the remaining half of the bulb was macerated in a Waring blender and added to 400 mL of sterile deionised water. The mixture was boiled for 15 minutes, and the resulting colourless solution decanted, cooled and filter sterilised. 200 mL was frozen and lyophilised. The resulting lyophilised extract also appeared as a colourless crystaline powder. Extract characteristics were as follows: pH 5.47, concentration: 18.0 mg · mL^{-1}, yield: 4.5% $^{w}/_{w}$.

7.1.5.10 *Cymbopogon ambiguus* (Hack.) A.Camus (Poaceae) whole plant decoction

The decoction of *C. ambiguus* (extract JES058TD) was prepared with material from specimen JES058 (Collected in the vicinity of Jodetluk community, NT). Preparation was based on descriptions of traditional use of decoctions for the treatment of dermal sores and *Scabies* infestation from Barr *et al.* (1988) and Ms Jenny Brown of the Jodetluk community (NT). Barr *et al.* (1988) describes preparation of a decoction of leaves whereas Ms Brown advised that the whole plant (aerial and subterranean parts) was typically used. All descriptions of traditional medicinal use of this plant and related *Cymbopogon* spp. stipulate that only young, green plants should be used. At the time of collection however (late August - early dry season) all *Cymbopogon* sp. had matured and were dry. The extract was prepared using all parts of a single plant. Roots and subterranean parts were washed in water to remove soil. Plant was cut into 1-2 cm lengths (total mass: 30 gm) and extracted for 15 minutes in 500 mL boiling, deionised water. The resulting extract which was a pale yellow-straw colour, was decanted and cooled and final volume determined at 360 mL. The extract was then filter sterilised and transferred into containers for storage and lyophilisation. 180 mL of the extract was lyophilised which appeared as a yellow-straw coloured powder. Extract characteristics were as follows: pH, 5.12, concentration: 3.6 mg · mL^{-1}, yield: 4.3% $^{w}/_{w}$.

7.1.5.11 *Eremophila alternifolia* R.Br. (Myoporaceae) aqueous leaf decoctions

Traditional extracts of *E. alternifolia* were prepared as outlined by Barr *et al.* (1988) where decoction of a small handful of dried leaves is described. Material used was from specimens JES021 and JES023 (Collected from locality 20 km south of Broken Hill, near Menindee NSW). Leaves attached to branches were dried over a number of days in paper bags placed in a fume extraction cabinet. Plant material was weighed daily to monitor moisture loss and was considered sufficiently dry when no further loss of mass could be observed (occurring

between three and four days). To prepare extracts JES21TD and JES23TD (leaf decoctions), an approximate mass for the required quantity of leaves was estimated at 25 gm. 50 gm of dried leaves and twigs were extracted in 1000 mL of boiling deionised water (corresponding to traditional preparation of ca. 25 gm in 500 mL water) for 15 minutes. The resulting extracts which were a pale straw colour, were decanted, cooled to 20° C and final volume measured (820 mL for JES23TD and 840 mL for JES21TD). The extracts were then filter sterilised and transferred into containers for storage and lyophilisation. A 500 mL sample of each filtered extract was frozen and lyophilised. The resulting lyophilised extract was a pale straw-yellow powder. pH of filtered extracts was 4.91 (JES23TD) and 4.66 (JES21TD). Concentration and extract yields were determined at 5.1 mg · mL^{-1} and 8.3% $^{w}/_{w}$ respectively for JES23TD and 5.0 mg · mL^{-1} and 8.4% $^{w}/_{w}$ for JES21TD.

7.1.5.12 ***Eremophila duttonii*** **F.Muell. (Myoporaceae) aqueous leaf decoctions**

Traditional extracts of *E. duttonii* were prepared as outlined by Barr *et al.* (1988) where a decoction of two handfuls of leaves is described as a traditional treatment for dermal sores, wounds, or as a lotion for sore eyes and/or a treatment for pharyngitis. Material from specimens SM010 and SM011 (collected from Mutawintji national park, NSW) was used to prepare extracts (SM010TD and SM011TD). An approximate mass for the required quantity of leaves ("two handfuls") was estimated at ca. 67 gm. Fresh leaves and twigs from each specimen were extracted in 1000 mL of boiling deionised water for 15 minutes. The resulting extracts which were a dark brown colour (described by Barr *et al.* (1988) as dark green) were decanted and cooled and the final volume measured (750 mL for SM10TD and 765 mL for SM11TD). Extracts were then filter sterilised and transferred into containers for storage and lyophilisation. A 500 mL sample of each filtered extract was frozen and lyophilised, the resulting lyophilised extract appearing as a dark brown powder in both cases. The pH of filtered extracts was estimated at 4.60 (SM010TD) and 4.58 (SM011TD). Concentration and

extract yields were estimated at 6.2 mg · mL^{-1} and 6.8% $^{w}/_{w}$ respectively for SM011TD and 9.7 mg · mL^{-1} and 10.9% $^{w}/_{w}$ for SM010TD.

7.1.5.13 *Eremophila latrobei* F.Muell. (Myoporaceae) aqueous leaf decoctions

Material used was from specimens SM007 and SM008 (collected from Mutawintji national park, NSW). Latz (1995) has described traditional use of *E. latrobei* leaf decoctions for treatment of *Scabies* infestations without giving specific description of preparation. For the extracts prepared here, a quantity of leaves and twigs approximating one handful (40 gm) was extracted in 1000 mL of water. Fresh leaves and twigs were separated from branches and extracted in 1000 mL of boiling deionised water for 15 minutes. The resulting extracts which were a pale straw colour were decanted and cooled and the final volume measured (780 mL for SM007TD and 800 mL for SM008TD). Extracts were then filter sterilised and transferred into containers for storage and lyophilisation. A 500 mL sample of each filtered extract was frozen and lyophilised, the resulting lyophilised extract appearing as a straw-yellow coloured powder in both cases. Other characteristics were as follows: SM007TD: pH 3.90, concentration: 5.4 mg · mL^{-1}, yield: 10.5% $^{w}/_{w}$; SM008TD: pH 3.90, concentration: 6.1 mg · mL^{-1}, yield: 12.3% $^{w}/_{w}$.

7.1.5.14 *Eremophila longifolia* (R.Br.) F.Muell. (Myoporaceae) aqueous leaf decoctions

Three separate extracts were prepared from material belonging to specimens of *E. longifolia* representative of each of the three distinct essential oil chemotypes identified during the course of the study. Material used included that from specimen SM003 (isomenthone chemotype: collected from Mutawintji national park, NSW), SM018 (karahanaenone chemotype: collected from the Murray-Darling depression region, NSW) and SM019 (Borneol chemotype: collected from the Cobar peneplain, NSW). Traditional preparation followed that described by Barr *et al.* (1988) (decoction of one handful of leaves, used in

treatment of dermal sores, *Scabies* infestation, minor wounds and boils). Fresh leaves and twigs were separated from branches and an approximate mass for the required quantity was estimated for extraction in 1000 mL of water (50 gm). Fresh leaves and twigs were extracted in 1000 mL of boiling deionised water for 15 minutes. The extract was then decanted and cooled. Final volumes after extraction were 780 mL (SM003TD), 740 mL (SM018TD) and 780 mL (SM019TD). Extracts were filter sterilised and transferred into containers for storage and lyophilisation. A 500 mL sample of each filtered extract was frozen and lyophilised. *E. longifolia* extracts were predominantly coloured green with a distinct bluish tint seen in Extracts SM018TD and SM019TD. This bluish tint was retained in the lyophilised extracts (pale bluish-grey-green powders) and was most pronounced in the extract of SM019. Characteristics of the extracts were as follows: SM003TD: pH 4.82, concentration: 7.7 $mg \cdot mL^{-1}$, yield: 12.1% $^{w}/_{w}$; SM018TD: pH 4.95, concentration: 8.1 $mg \cdot mL^{-1}$, yield: 12.0% $^{w}/_{w}$; SM019TD: pH 4.49, concentration: 8.3 $mg \cdot mL^{-1}$, yield: 12.9% $^{w}/_{w}$.

7.1.5.15 *Eremophila sturtii* R.Br. (Myoporaceae) aqueous leaf decoctions

Material used was from specimen SM001 (collected from Mutawintji national park, NSW). Traditional preparation followed that described by Barr *et al.* (1988) for preparations derived from *Eremophila freelingii* which is used interchangeably with *E. sturtii* by some groups (15 minute decoction of two handfuls of leaves in ca. 1000 mL water used for treatment of minor wounds, "open sores" and *Scabies* infestation). Fresh leaves and twigs were separated from branches and an approximate mass for the required quantity was estimated for extraction in 1000 mL of water (40 gm, based on approximate mass of "two handfuls"). Fresh leaves and twigs were extracted in 1000 mL of boiling deionised water for 15 minutes. The extract was decanted and cooled and final volume recorded (820 mL). The extract (dark green coloured) was filter sterilised and transferred into containers for storage and a 500 mL sample frozen

and lyophilised. Lyophilised extract appeared as a green powder. Extract characteristics were determined as follows: pH 4.82, concentration: 9.3 mg · mL^{-1}, yield: 19.1% $^{w}/_{w}$.

7.1.5.16 *Erythrophleum chlorostachys* (F.Muell.) Baill. (Fabaceae) aqueous leaf and inner bark decoctions

Traditional extracts of *E. chlorostachys* were prepared using material from specimen JES040 (Collected in the vicinity of the Katherine river, Katherine, NT). Two separate extracts were prepared based on based on traditional preparations described by Barr *et al.* (1988). These included a decoction of one handful of leaves with a 5 cm^2 piece of inner bark and a decoction of inner bark material only (used for treatment of dermal sores and minor wounds). The decoction of leaves and inner bark (extract JES040LTD) was prepared as follows: Mass for "one handful" of leaves was estimated at approximately 25 gm. Mass of a 5 cm^2 piece of inner bark was estimated at approximately 6 gm. Leaves and bark were extracted with 500 mL of boiling, deionised water for 15 minutes. The extract was decanted and cooled and final volume recorded (385 mL). The extract (dark green-brown coloured) was filter sterilised and transferred into containers for storage and a 200 mL sample frozen and lyophilised. Lyophilised extract appeared as a green-brown powder. Extract characteristics were determined as follows: pH 4.75, concentration: 4.7 mg · mL^{-1}, yield: 5.8% $^{w}/_{w}$.

Typical quantities for traditional preparation of inner bark decoctions were not specified by Barr *et al.* (1988). For this extract (extract JES040LTD), 40 gm of inner bark pieces (ca. 3-4 cm^2, obtained by shaving from exposed trunk with a blade) were used. Inner bark extracted with 500 mL of boiling, sterile deionised for 15 minutes. The extract was decanted and cooled and final volume recorded (410 mL). The extract (dark red-brown colour) was filter sterilised and transferred into containers for storage and a 200 mL sample frozen and

lyophilised. Lyophilised extract appeared as a dark red-brown powder. Extract characteristics were determined as follows: pH 4.50, concentration: 10.1 mg · mL^{-1}, yield: 10.3% $^{w}/_{w}$.

7.1.5.17 **Eucalyptus tetrodonta F.Muell. (Myrtaceae) juvenile terminal leaf and inner bark decoctions**

Traditional extracts of *E. tetrodonta* were prepared using material from specimen JES028 (Collected in the vicinity of Charles Darwin national park, Darwin, NT). Two separate extracts were prepared based on traditional preparations described by Barr *et al.* (1988). These included a decoction of crushed, terminal recent growth leaves (used as treatment for dermal sores and minor wounds - "to cure infection and promote healing"), and a decoction of a piece of inner bark material from young plant or suckering regrowth (used as treatment for infected dermal sores and minor wounds and as a mouthwash for pain or sores in the mouth and on the tongue). The decoction of terminal leaves (extract JES028LTD) was prepared as follows: Mass for the "one handful" of leaves required was estimated at approximately 20 gm. Leaves were crushed by hand (in aluminium foil) and extracted in 500 mL of water for 15 minutes. The extract was decanted and cooled and final volume recorded (400 mL). The extract (red-brown colour) was filter sterilised and transferred into containers for storage and a 200 mL sample frozen and lyophilised. Lyophilised extract appeared as a red-brown powder. Extract characteristics were as follows: pH 4.18, concentration: 5.4 mg · mL^{-1}, yield: 10.8% $^{w}/_{w}$.

The decoction of inner bark (extract JES028BTD) was prepared as follows: Mass for the 7 X 20 cm piece of thin inner bark required was estimated at approximately 10 gm. Inner bark was extracted in 500 mL of boiling deionised water for 15 minutes. The extract was decanted and cooled and final volume recorded (400 mL). The extract (pale brown coloured) was filter sterilised and transferred into containers for storage and a 200 mL sample frozen and

lyophilised. Lyophilised extract appeared as a pale-brown powder. Extract characteristics were as follows: pH 3.54, concentration: 1.2 mg · mL^{-1}, yield: 4.7% $^{w}/_{w}$. Barr *et al.* (1988) describes both preparations as exhibiting a deep purple colouring. Although material used was as specified (reddish terminal leaves and inner bark from young suckering growth) neither preparation was seen to develop this particular colouration during extraction.

7.1.5.18 *Flagellaria indica* L. (Flagellariaceae) expressed juice of terminal nodes and warm infusion of stems

Traditional extracts of *F. indica* were prepared using material from specimen JES037 (Collected from the banks of the Katherine river, Katherine, NT). Two separate extracts were prepared based on traditional preparations described by Levitt and Lyon (1981). These included the expressed juice from terminal nodes and a warm infusion of crushed stems (both used for the treatment of ocular complaints). The expressed juice of terminal nodes (extract JES037EJ) was prepared as follows: Tapered, terminal nodes (9.65 gm total) were separated by hand from stems. These were packed into the body of a 10 mL syringe mounted on a ratcheting clamp. Juice was extracted by applying pressure to the plunger with the clamp until no more liquid could be expressed. 4 mL of a pale yellow liquid was yielded. The liquid was filter sterilised and transferred into containers for storage and lyophilisation. A 1 mL sample was frozen and lyophilised; the lyophilised extract appeared as a brown powder. Extract characteristics were as follows: pH 5.17, concentration: 118.0 mg · mL^{-1}, yield: 4.9% $^{w}/_{w}$.

The warm infusion of stems (extract JES037SE) was prepared by and extracting 95 gm of stems which had been crushed using a mortar and pestle, for 15 minutes in 500 mL deionised water which had been heated to 60° C. The resulting extract which was a pale green-yellow colour, was decanted and cooled and final volume estimated at 465 mL. The extract was then

filter sterilised and transferred into containers for storage and lyophilisation. A 200 mL sample of the extract was lyophilised which appeared as a pale green powder. Extract characteristics were as follows: pH, 5.43, concentration: 7.1 mg · mL^{-1}, yield: 3.5% $^{w}/_{w}$.

7.1.5.19 ***Hibiscus tiliaceus*** **L. (Malvaceae)**

Traditional extracts of *H. tiliaceus* were prepared using material from specimen JES032 (Collected from foreshore area in the vicinity of Casuarina nature reserve, Darwin, NT). Two separate extracts were prepared based on based on traditional preparations described by Barr *et al.* (1988) and Levitt and Lyon (1981). Barr *et al.* (1988) describes preparation of a warmed infusion of inner stem bark for the treatment of boils. Barr *et al.* (1988) additionally describes extraction of juice from inner bark (prepared by removing a strip of outer and inner bark and squeezing) which is also used to treat boils on the skin. This was attempted with the specimens collected however the inner bark was too dry to yield sufficient fluid so the alternative method of extracting inner bark was followed. Levitt and Lyon (1981) describe preparation of a similar warmed infusion of inner stem bark and sapwood for the treatment of wounds and overnight, cold infusion of stem inner bark for treatment of boils. Both sources also describe the use of the bark itself as a dressing for wounds where the inner bark contacts the wound (outer bark is fibrous and flexible and long strips may be cut from stem and trunk). To prepare the heated infusion (extract JES32BHSE) outer bark was removed from a 20 cm section of stem (70 gm total) which was shredded and extracted for 3 hr in 200 mL of water which had been heated to 60° C. The resulting extract was decanted, cooled and final volume determined (200 mL). The extract (a pale green-brown slightly viscous liquid) was then filter sterilised and transferred to containers for storage and lyophilisation. A 25 mL sample was lyophilised forming a hygroscopic brown powder. Extract characteristics were as follows: pH 5.77, concentration: 9.5 mg · mL^{-1}, yield: 2.7% $^{w}/_{w}$.

To prepare the overnight cold infusion of stem inner bark (extract JES32HSE) the outer bark was removed from terminal recent growth stems (ca. 1 cm diameter). Stems (35.8 gm) were extracted for 20 hr in 500 mL of deionised water. The resulting extract was decanted and final volume determined (490 mL). The extract (a pale green-brown lubricious and slightly viscous liquid) was then filter sterilised and transferred to containers for storage and lyophilisation. A 25 mL sample was lyophilised forming a hygroscopic brown powder. Extract characteristics were as follows: pH 5.97, concentration: 3.3 mg · mL^{-1}, yield: 3.8% $^{w}/_{w}$.

7.1.5.20 ***Ipomoea pes-caprae*** **(L.) R.Br. (Convolvulaceae) heated and cold infusions**

Traditional extracts of *I. pes-caprae* were prepared using material from specimen JES031 (Collected from beach fore-dunes in the vicinity of Casuarina nature reserve, Darwin, NT). Two separate extracts were prepared based on based on a traditional preparation described by Barr *et al.* (1988) for treatment for *Scabies* infestation where ten leaves are extracted in approximately 500 mL of water. As water temperature for this preparation was not specified by Barr *et al.* (1988), two extracts were prepared, one a cold infusion in room temperature water (ca. 20°C) and the other a warm infusion (water heated to 60°C). To prepare the cold infusion (extract JES31SE) 10 leaves (19.75 gm) were extracted overnight (20 hr) in 500 mL of water. The resulting extract was decanted and filter sterilised. The extract produced was a pale green-brown colour and final volume was 486 mL. A 250 mL sample was frozen and lyophilised. The lyophilised extract appeared as a dark-brown hygroscopic powder. Extract characteristics were as follows: pH 5.80, concentration: 0.8 mg · mL^{-1}, yield: 2.1% $^{w}/_{w}$.

To prepare the heated infusion (extract JES31HSE) 10 leaves were selected and one of the leaves trimmed with scissors so that total mass was 19.75 gm (for consistency with cold infused extract). The 19.75 gm of leaves were extracted in 500 mL of water which had been

heated to 60° C. The leaves were left to extract for 20 hr. The resulting extract was decanted and filter sterilised. The extract produced was a pale green-brown colour and final volume was 410 mL. A 250 mL sample was frozen and lyophilised. The lyophilised extract appeared as a dark-brown hygroscopic powder. Extract characteristics were as follows: pH 6.20, concentration: 1.5 mg · mL^{-1}, yield: 3.2% $^{w}/_{w}$.

7.1.5.21 *Morinda citrifolia* L. (Rubiaceae) expressed juice of fruit

M. citrifolia expressed juice of fruit (extract JES060EJ) was prepared with material from specimen JES060 (Collected in the vicinity of the Rapid creek outlet, NT). The extract was prepared based on descriptions of the use of the flesh of green, unripened fruit by Levitt and Lyon (1981) as a topical treatment for minor wounds. Barr *et al.* (1988) additionally reports the ripe fruit to be eaten as a treatment for sore throat. 150 gm flesh was collected from two green, unripened fruits and packed into the body of a 100 mL syringe mounted on a ratcheting clamp. Juice was extracted by applying pressure to the plunger until no more liquid could be expressed. 46 mL of a dark brown juice was yielded 3 mL of which was frozen and lyophilised. The lyophilised extract appeared as a dark brown hygroscopic powder. Extract characteristics were as follows: pH 4.50, concentration: 51.1 mg · mL^{-1}, yield: 1.6% $^{w}/_{w}$.

7.1.5.22 *Pandanus spiralis* R.Br. (Pandanaceae) leaf base decoction and diluted expressed juice of leaf tips

Traditional extracts of *P. spiralis* were prepared using material from specimen JES027 (Collected in the vicinity of Charles Darwin national park, Darwin, NT). Two separate extracts were prepared based on based on traditional preparations described by Barr *et al.* (1988) where use of a preparation of decoction of leaf bases is described as treatment for red and infected eyes along with preparations of diluted juice squeezed from inner flesh of leaf tips for treatment of sore throat and mouth. Barr *et al.* (1988) additionally describes use of the

leaf base as a dressing for sores on the skin along with the practice of eating the leaf base as a treatment for sore throats and pain in the mouth.. To prepare the decoction of leaf bases (extract JES27LBD) 100 gm of leaf base was extracted for 15 minutes in 500 mL of boiling deionised water. The resulting extract was decanted, cooled and final volume determined (375 mL). The extract appeared as a pale, almost colourless beige liquid which was then filter sterilised and transferred to containers for storage and lyophilisation. A 200 mL sample was lyophilised forming a hygroscopic brown powder. Extract characteristics were as follows: pH 5.77, concentration: 13.6 mg · mL^{-1}, yield: 5.1% $^{w}/_{w}$.

To prepare the dilute juice of leaf tips (extract JES27EJP) 100g the white flesh of leaf tips was collected by scraping from outer epithelial layers and packed into the body of a a 100 mL syringe mounted on a ratcheting clamp. Juice was extracted by applying pressure to the plunger until no more liquid could be expressed. 82 mL of a pale, brownish liquid was yielded to which 100 mL of deionised water was added. The mixture was filter sterilised and transferred to containers for storage and lyophilisation. A 60 mL sample was lyophilised forming a hygroscopic brown powder. Extract characteristics were as follows: pH 5.45, concentration: 8.3 mg · mL^{-1}, yield (of undiluted juice): 3.3% $^{w}/_{w}$.

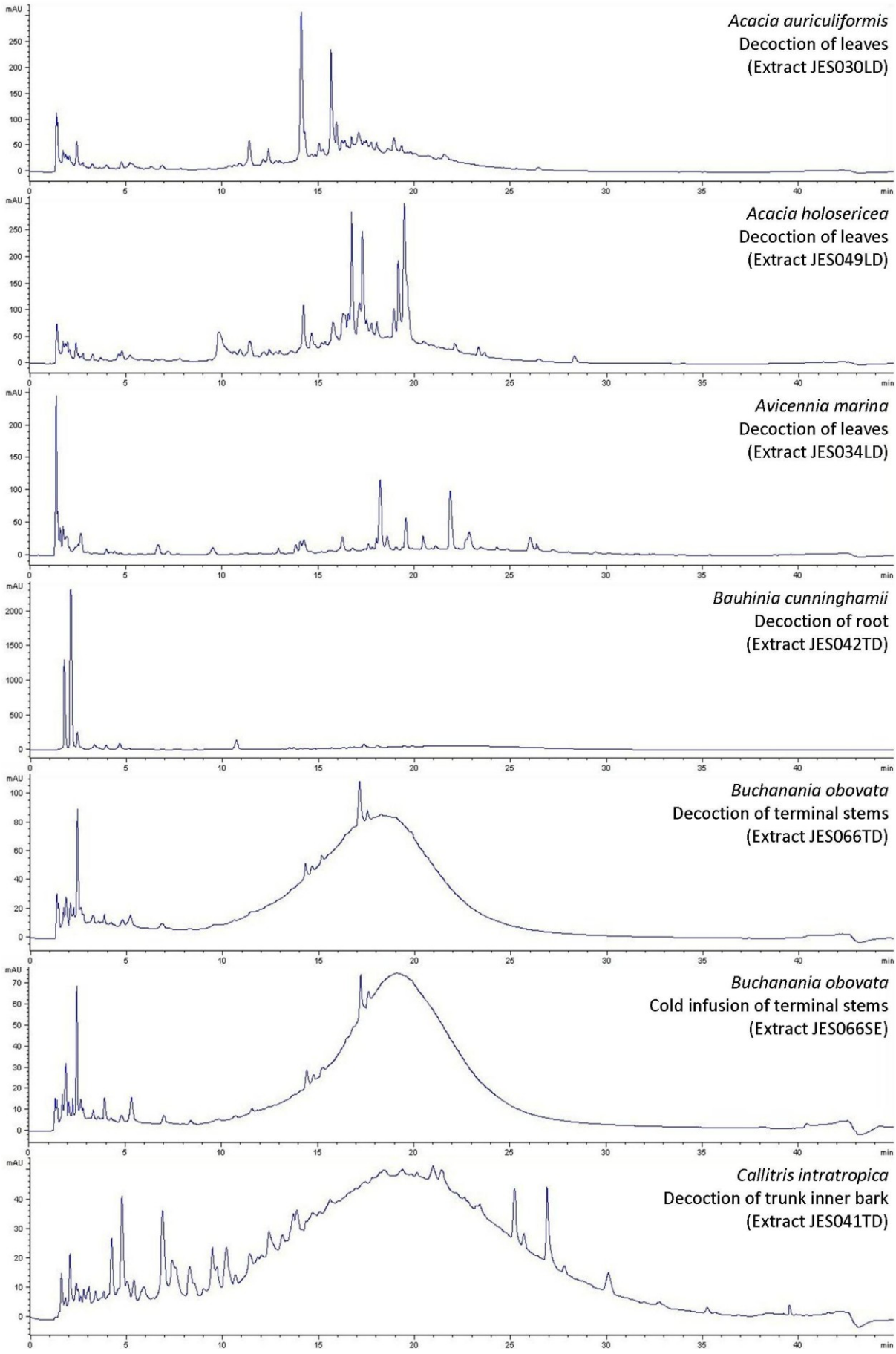
7.1.5.23 *Scaevola sericea* Vahl. (Goodeniaceae) expressed juice of fruit

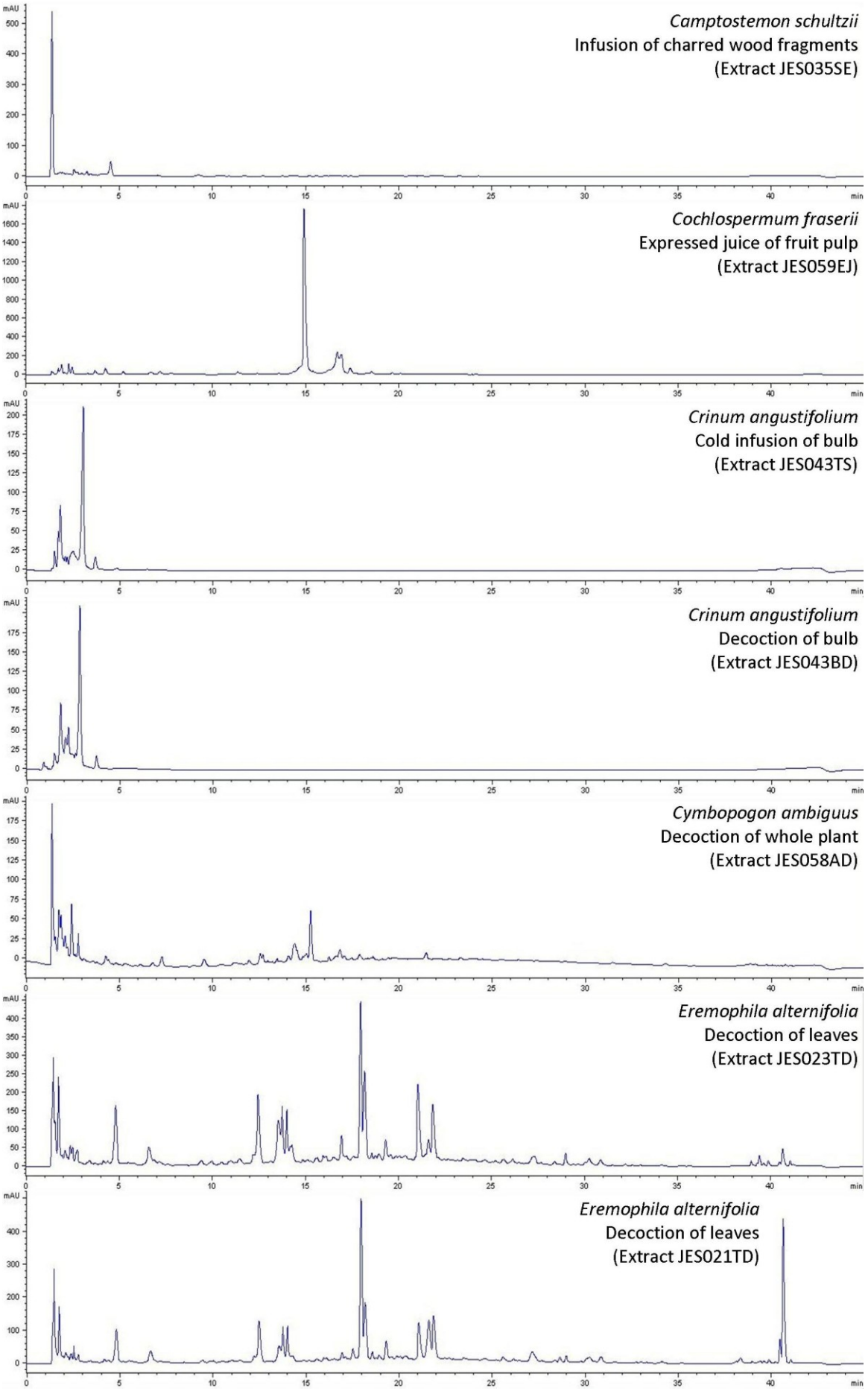
Expressed juice of *S. sericea* fruit was prepared using material from specimen JES063 (Collected within the vicinity of East point reserve, Darwin, NT). The extract was prepared based on descriptions of the use of juice from ripened fruit by Barr *et al.* (1988) to treat redness, soreness and infection of the eye. Ripe fruit (75 gm) was selected and packed into the body of a 100 mL syringe mounted on a ratcheting clamp. Juice was extracted by applying pressure to the plunger until no more liquid could be expressed. 18 mL of a colourless juice was yielded 10 mL of which was frozen and lyophilised. The lyophilised

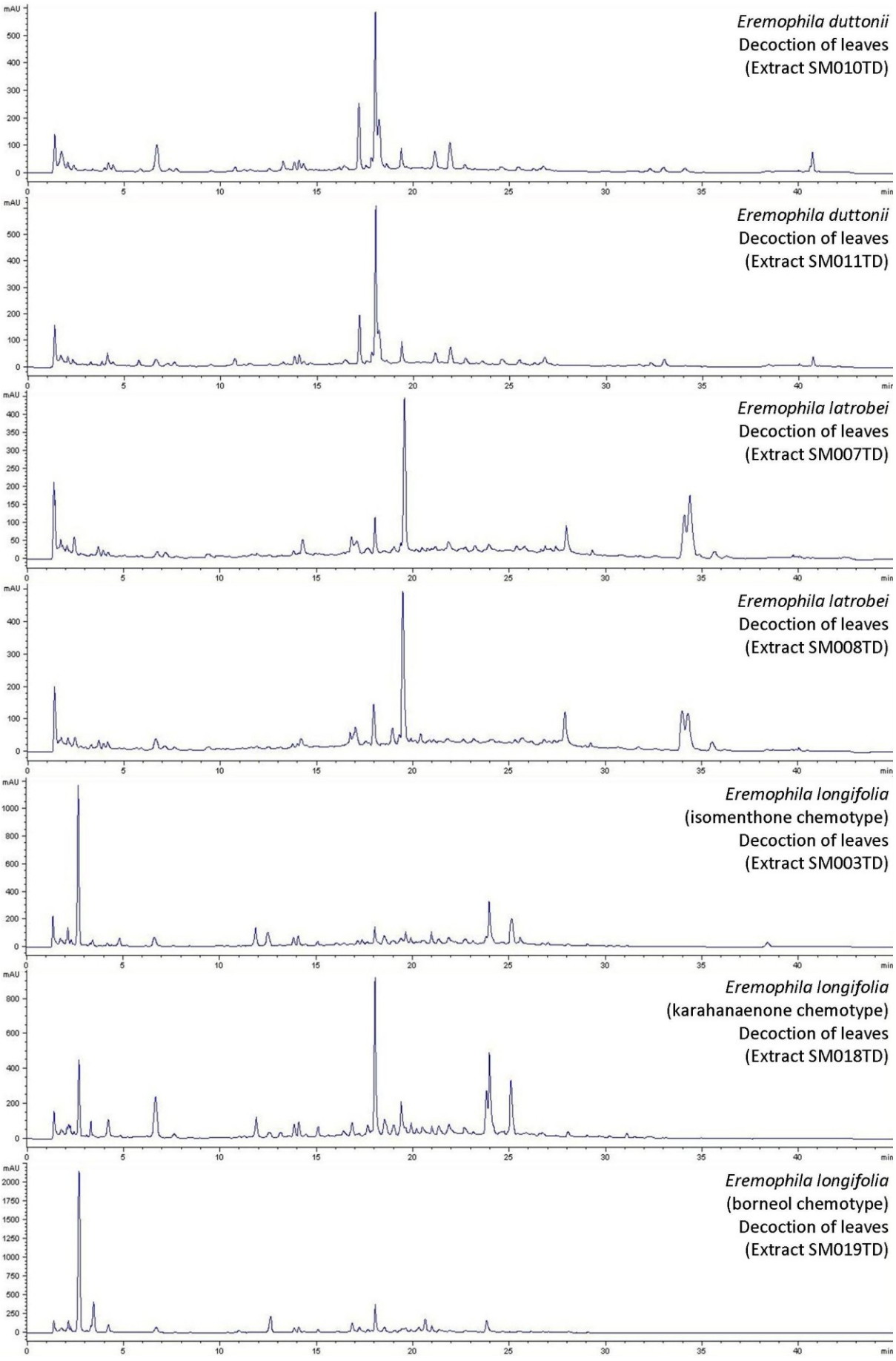
extract appeared as a brown powder. Extract characteristics were as follows: pH 5.11, concentration: 75.8 mg · mL^{-1}, yield: 1.8% $^{w}/_{w}$.

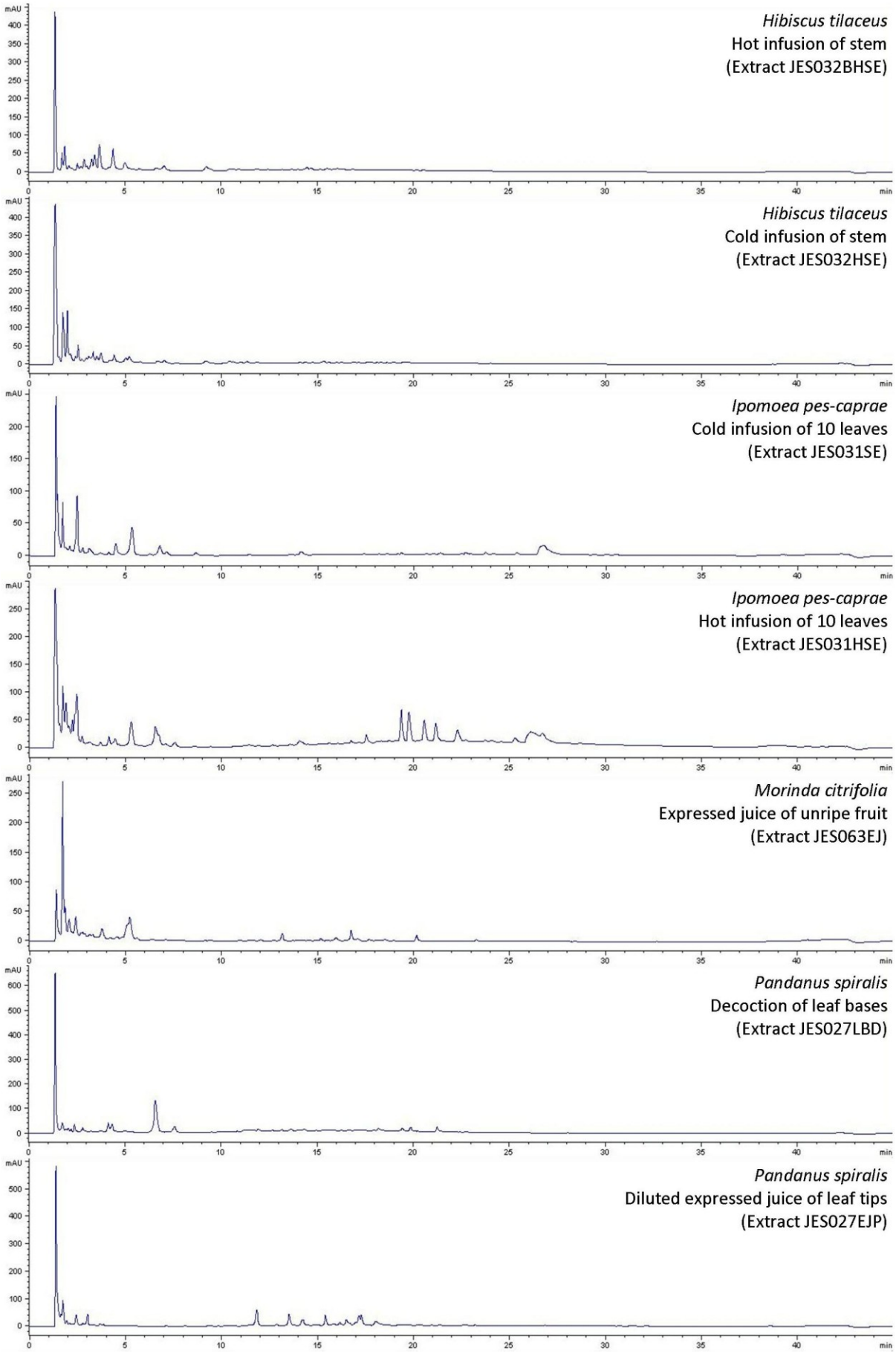
7.1.5.24 **Traditional preparation HPLC chromatogram vouchers**

HPLC Chromatogram vouchers for samples of the traditional preparations are presented below. Chromatograms shows absorbance at λ254 nm as extracted from spectra collected using the photodiode array detector. Methods followed to generate the chromatographic vouchers are outlined in Chapter 2 (2.3).


Acacia auriculiformis
Decoction of leaves
(Extract JES030LD)
Acacia holosericea
Decoction of leaves
(Extract JES049LD)
Avicennia marina
Decoction of leaves
(Extract JES034LD)
Bauhinia cunninghamii
Decoction of root
(Extract JES042TD)
Buchanania obovata
Decoction of terminal stems
(Extract JES066TD)
Buchanania obovata
Cold infusion of terminal stems
(Extract JES066SE)
Callitris intratropica
Decoction of trunk inner bark
(Extract JES041TD)
mAU
min


Camptostemon schultzii
Infusion of charred wood fragments
(Extract JES035SE)
Cochlospermum fraserii
Expressed juice of fruit pulp
(Extract JES059EJ)
Crinum angustifolium
Cold infusion of bulb
(Extract JES043TS)
Crinum angustifolium
Decoction of bulb
(Extract JES043BD)
Cymbopogon ambiguus
Decoction of whole plant
(Extract JES058AD)
Eremophila alternifolia
Decoction of leaves
(Extract JES023TD)
Eremophila alternifolia
Decoction of leaves
(Extract JES021TD)
mAU
min


Eremophila duttonii
Decoction of leaves
(Extract SM010TD)
Eremophila duttonii
Decoction of leaves
(Extract SM011TD)
Eremophila latrobei
Decoction of leaves
(Extract SM007TD)
Eremophila latrobei
Decoction of leaves
(Extract SM008TD)
Eremophila longifolia
(isomenthone chemotype)
Decoction of leaves
(Extract SM003TD)
Eremophila longifolia
(karahanaenone chemotype)
Decoction of leaves
(Extract SM018TD)
Eremophila longifolia
(borneol chemotype)
Decoction of leaves
(Extract SM019TD)
mAU
min


Hibiscus tilaceus
Hot infusion of stem
(Extract JES032BHSE)
Hibiscus tilaceus
Cold infusion of stem
(Extract JES032HSE)
Ipomoea pes-caprae
Cold infusion of 10 leaves
(Extract JES031SE)
Ipomoea pes-caprae
Hot infusion of 10 leaves
(Extract JES031HSE)
Morinda citrifolia
Expressed juice of unripe fruit
(Extract JES063EJ)
Pandanus spiralis
Decoction of leaf bases
(Extract JES027LBD)
Pandanus spiralis
Diluted expressed juice of leaf tips
(Extract JES027EJP)
mAU
min

Appendix III. Column chromatography fractions, miscellaneous information and publications

Table III.1 Stepped gradient elution mobile phase composition and mass of fractions from separation of *E. duttonii* hexane and DCM extracts with low pressure column chromatography

Fractions	Hexane (%)	DCM (%)	Et-OAc (%)	V_e (mL)	SM011 E. duttonii 1' hexane extract fraction mass (mg)	SM011 E. duttonii 2' DCM extract fraction mass (mg)
1	100	0	-	50		16.72
2	100	0	-	100	*99.24	55.00
3	100	0	-	150		82.18
4	100	0	-	200		89.76
5	100	0	-	250	*8.56	33.74
6	100	0	-	300		35.31
7	95	5	-	350		47.07
8	95	5	-	400	4.93	383.61
9	95	5	-	450	22.2	674.15
10	90	10	-	500	135.17	838.50
11	90	10	-	550	123.73	216.90
12	90	10	-	600	86.29	112.90
13	70	30	-	650	98.71	142.29
14	70	30	-	700	275.07	178.90
15	70	30	-	750	291.69	213.89
16	50	50	-	800	336.16	180.85
17	50	50	-	850	159.38	647.05
18	50	50	-	900	88.59	296.41
19	30	70	-	950	69.46	75.03
20	30	70	-	1000	69.25	55.48
21	30	70	-	1050	62.44	36.35
22	10	90	-	1100	61.16	38.14
23	10	90	-	1150	56.95	36.46
24	10	90	-	1200	136.90	30.25
25		100	-	1250	124.65	30.37
26		100	-	1300	531.16	19.89
27		100	-	1350	622.95	21.30
28		95	5	1400	412.58	17.98
29		95	5	1450	118.96	18.27
30		95	5	1500	81.95	18.94
31		90	10	1550	250.99	14.57
32		90	10	1600	33.90	13.85
33		90	10	1650		31.15
34		70	30	1700		58.74
35		70	30	1750		122.57
36		70	30	1800		38.74
37		50	50	1850	*37.37	59.27
38		50	50	1900		35.95
39		50	50	1950		42.01
40		30	70	2000		45.33
41		30	70	2050		30.42
42		30	70	2100		38.10
43		10	90	2150	17.41	20.86
44		10	90	2200	32.73	40.73
45		10	90	2250	33.33	19.26
46		-	100	2300	25.03	14.66
47		-	100	2350	41.02	3.618

V_e: Elution volume; *: Combined fractions

Table III.2 Concentration/quantity of traditional aqueous preparations used in antimicrobial assays

Label code	Species	Description	Concentration of traditional preparation (mg · mL^{-1})	Range of well concentrations for MIC/MBC assays (mg · mL^{-1})	Quantity of extract per disc in disc diffusion assays (µg)
JES030LD	*Acacia auriculiformis*	Decoction of leaves	8.0	8.00 - 0.25	48
JES049LD	*Acacia holosericea*	Decoction of leaves	8.7	16.00 - 0.50	52
JES034LTD	*Avicennia marina*	Decoction of leaves	14.4	16.00 - 0.50	86
JES042TD	*Bauhinia cunninghamii*	Decoction of root	3.8	4.00 -0.125	23
JES066TD	*Buchanania obovata*	Decoction of terminal stems	4.2	8.00 - 0.25	25
JES066SE	*Buchanania obovata*	Cold infusion of terminal stems	9.7	16.00 - 0.50	58
JES048BD*‡	*Buchanania obovata*	Decoction of inner trunk bark	20.4	32.00 – 0.016‡	122
JES041TD	*Callitris intratropica*	Decoction of inner trunk bark	13.8	16.00 - 0.50	83
JES035SE	*Camptostemon schultzii*	Warm infusion of charred wood	6.2	n.d.	37
JES059EJ	*Cochlospermum fraseri*	Expressed juice of fruit pulp	95.2	128.00 – 0.006‡	571
JES043TS	*Crinum angustifolium*	Cold infusion of bulb	18.8	n.d.	113
JES043BD	*Crinum angustifolium*	Decoction of bulb	18.0	n.d.	108
JES058AD	*Cymbopogon ambiguus*	Decoction of whole plant	3.6	n.d.	21
JES023TD*	*Eremophila alternifolia*	Decoction of leaves	5.1	8.00 - 0.25	31
JES021TD*	*Eremophila alternifolia*	Decoction of leaves	5.0	8.00 - 0.25	30
SM010TD	*Eremophila duttonii*	Decoction of leaves	9.7	16.00 - 0.50	58
SM011TD	*Eremophila duttonii*	Decoction of leaves	6.2	8.00 - 0.25	37
SM7TD	*Eremophila latrobei*	Decoction of leaves	5.4	n.d.	32
SM8TD	*Eremophila latrobei*	Decoction of leaves	6.1	n.d.	37
SM003TD	*Eremophila longifolia* (IC)†	Decoction of leaves	7.7	16.00 - 0.50	46
SM018TD*	*Eremophila longifolia* (KC)†	Decoction of leaves	8.1	16.00 - 0.50	49
SM019TD	*Eremophila longifolia* (BC)†	Decoction of leaves	8.3	16.00 - 0.50	50
SM001TD*	*Eremophila sturtii*	Decoction of leaves	9.3	8.00 - 0.25	56
JES040LTD	*Erythrophleum chlorostachys*	Leaf/inner trunk bark decoction	4.7	8.00 - 0.25	28
JES040BTD*‡	*Erythrophleum chlorostachys*	Trunk inner bark decoction	10.1	16.00 - 0.008‡	61
JES028LTD*‡	*Eucalyptus tetrodonta*	Terminal leaf decoction	5.4	8.00 - 0.004‡	32
JES028BTD	*Eucalyptus tetrodonta*	Decoction of trunk inner bark	1.2	2.00 - 0.063	7
JES037SE	*Flagellaria indica*	Warm infusion of stems	7.1	8.00 - 0.25	43
JES037EJ	*Flagellaria indica*	Expressed juice of terminal nodes	118.0	n.d.	708
JES032BHSE	*Hibiscus tiliaceus*	Warm infusion of stem inner bark	9.5	n.d.	57
JES032SE	*Hibiscus tiliaceus*	Cold infusion of stem inner bark	3.3	4.00 -0.125	20
JES031SE	*Ipomoea pes-caprae*	Cold infusion of leaves	0.8	n.d.	5
JES31HSE	*Ipomoea pes-caprae*	Cold infusion of leaves	1.5	n.d.	9
JES060EJ	*Morinda citrifolia*	Expressed juice of unripe fruit	51.1	64.00 - 2.00	307
JES027LBD*	*Pandanus spiralis*	Decoction of leaf bases	13.6	16.00 - 0.50	82
JES027EJP*	*Pandanus spiralis*	Expressed juice of leaf tips diluted	8.3	8.00 - 0.25	50
JES063EJ	*Scaevola sericea*	Expressed juice of fruit	75.8	128.00 - 4.00	455

*: 2.5% v/v DMSO added to facilitate solubilisation of stock solution; ‡: MIC below lowest dilutions used in initial experiments (experiments repeated with serial dilution proceeding from lowest concentration where inhibition of growth was previously recorded); †: (*Eremophila longifolia* chemotypes), IC: Isomenthone chemotype; KC: Karahanaenone chemotype; BC: Borneol chemotype

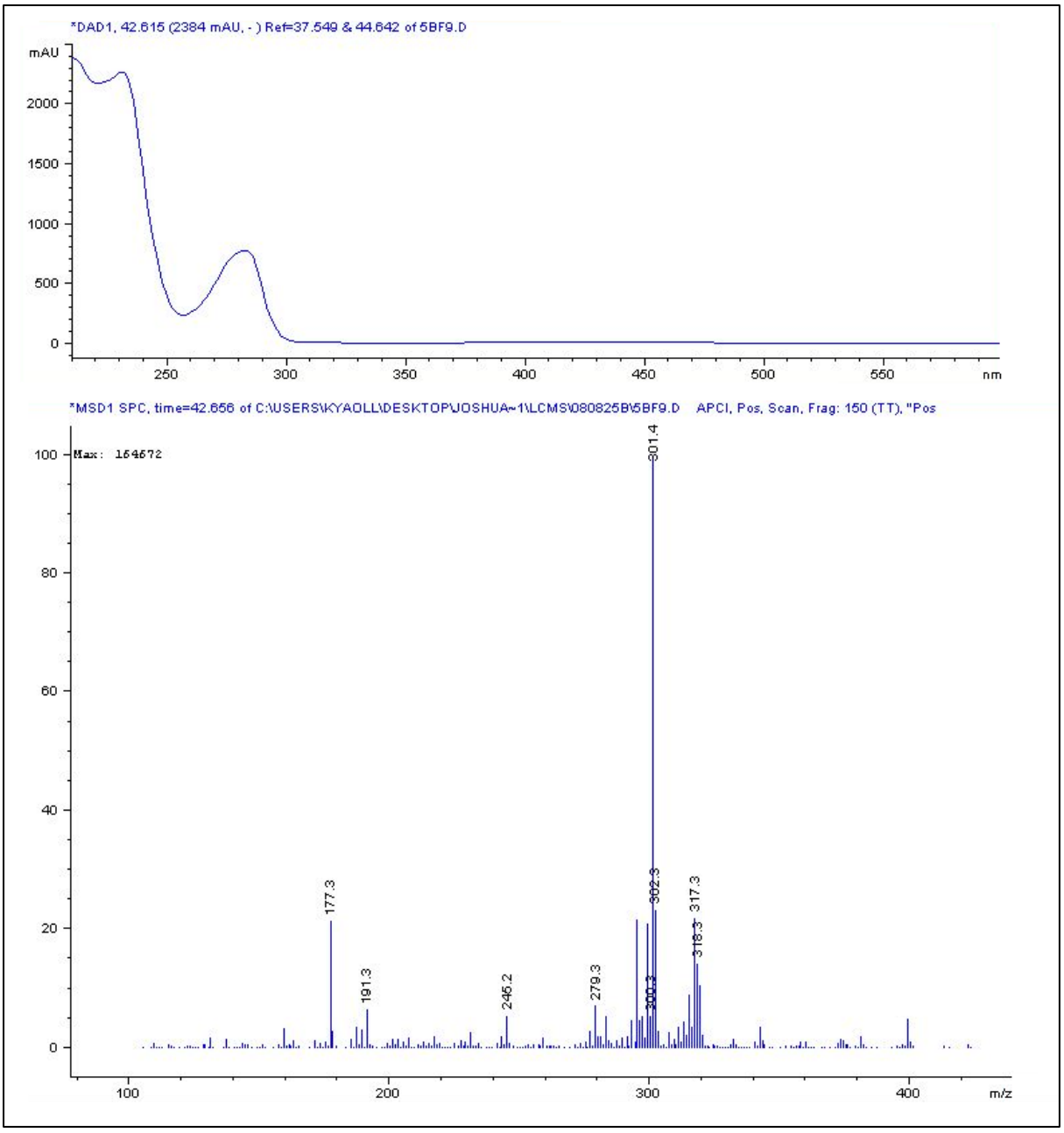


Figure III.1 UV/Visible absorption spectrum and mass spectrum (fragmentation pattern at 150 eV positive ACPI) of serrulat-14-en-7,8,20-triol (1) (separation of *E. duttonii* 2'DCM extract column chromatography fraction #9).

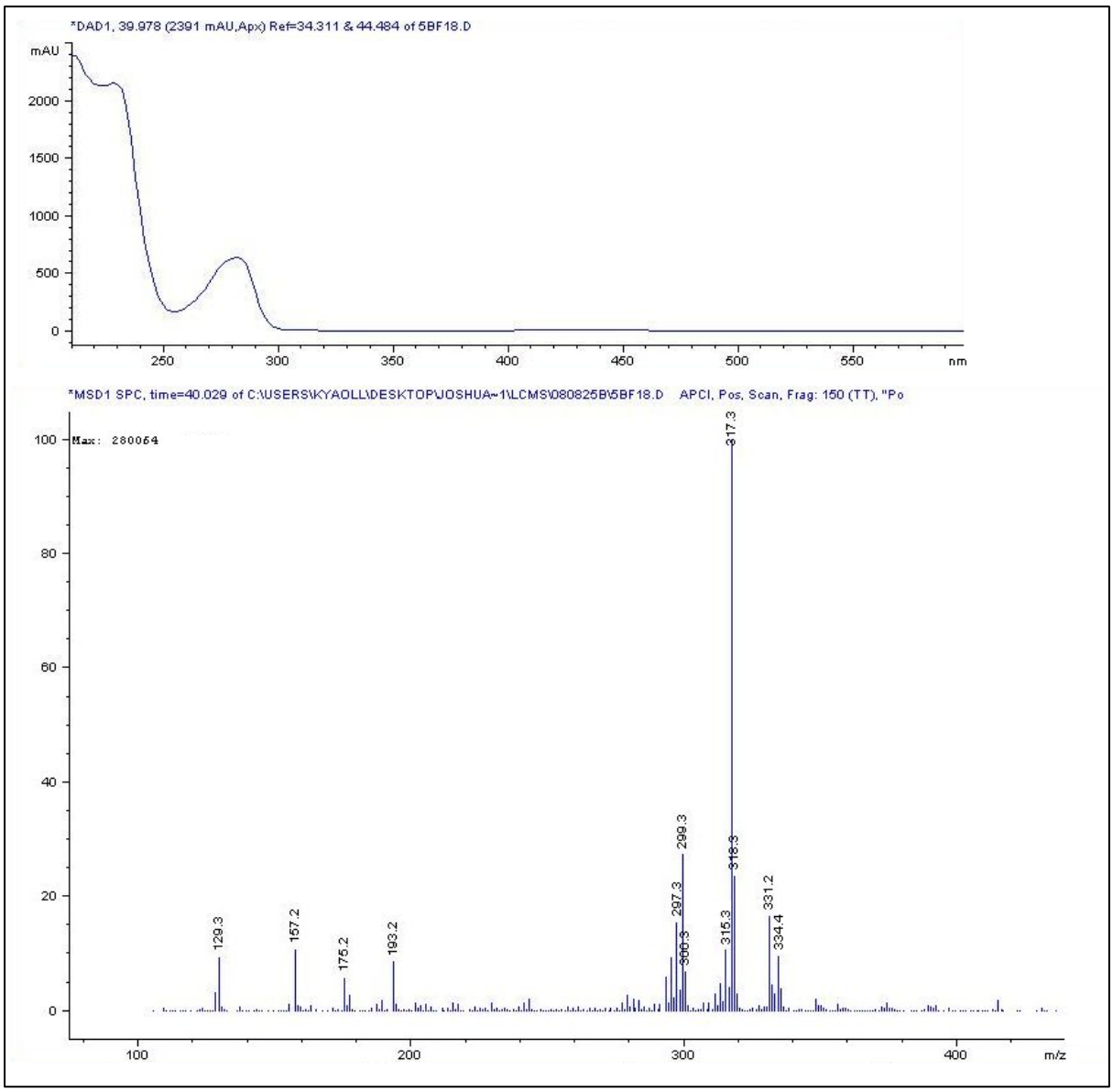
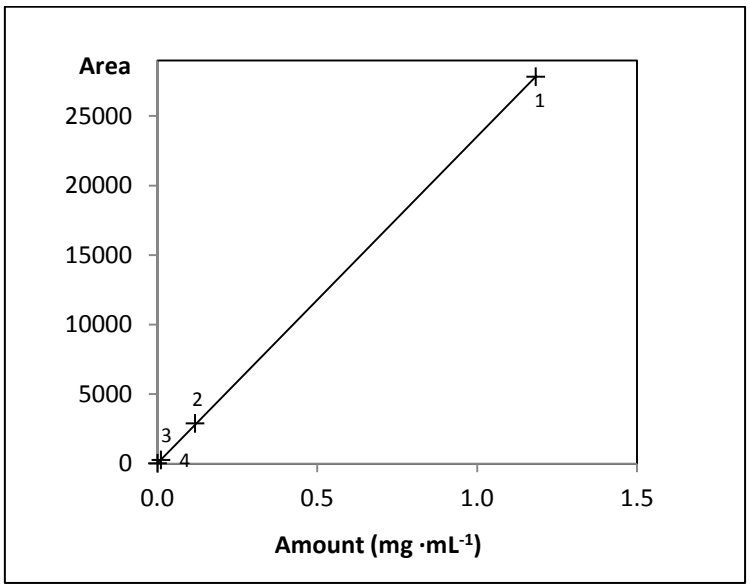


Figure III.2 UV/Visible absorption spectrum and mass spectrum (fragmentation pattern at 150 eV positive ACPI) of serrulat-14-en-3,7,8,20-tetraol (2) (separation of *E. duttonii* 2'DCM extract column chromatography fraction #18).



Sample	Level	Amount (mg·mL^{-1})	Area	Amount/Area
serrulat-14-en-3,7,8,20-tetraol	4	1.184 X 10^{-3}	32.733	3.61711E-05
serrulat-14-en-3,7,8,20-tetraol	3	1.184 X 10^{-2}	271.912	4.35435E-05
serrulat-14-en-3,7,8,20-tetraol	2	1.184 X 10^{-1}	2901.335	4.08088E-05
serrulat-14-en-3,7,8,20-tetraol	1	1.184	27821.710	4.25567E-05
Correlation:	0.99999			
Residual standard deviation:	61.15993			
Formula:	y = mx + b			
m:	23482.23			
b:	27.72016			
x:	Amount (mg·mL^{-1})			
y:	Area			

Figure III.3 Calibration curve and data for quantitative assay of serrulat-14-en-3,7,8,20-tetraol by HPLC

7.1.5.25 **Operating licences for collection of plant specimens:**

NSW National Parks and Wildlife Services NSW, General Licence/Authority:

(Number GLE/E 327)

Northern Territory Parks and Wildlife Commission: Permit to take Wildlife for Commercial Purposes:

(Number 26758)

Jarwoyn Association Aboriginal Corporation: Permit to enter Jarwoyn, Eva Valley, Beswick for sampling and collecting plant material

(Issued 24/08/2007) by Wes Miller