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Plants for people:

Laboratory study report

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2010

Contributing author information

Louis Evans, Professor of Aquatic Science at Curtin University, was the overall Project Leader and directly responsible for the research projects conducted at Titjikala and Leonora.

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List of shortened forms

ATCC	American Type Culture Collection
CFU	Colony Forming Unit
CPE	cytopathic effect
EMEM	Eagle Minimum Essential Medium
FCS	foetal calf serum
GC/MS	gas chromatography – mass spectrometry
IMVS	Institute of Medical and Veterinary Sciences (Adelaide, South Australia)
MBC	minimum bactericidal concentration
MEM	minimum essential medium
MIC	minimum inhibitory concentration
MNTD	maximum non-toxic dose
MRSA	Methicillin-resistant Staphylococcus aureus
NCCLS	National Committee for Clinical Laboratory Standards
NOEC	no observed effect concentration
PW FBC	purified water freshly boiled and cooled
RIRDC	Rural Industries Research and Development Corporation
TLC	thin-layer chromatography
v/v	volume of solute/volume of solution
w/v	weight/volume

Executive summary

This is an abridged report of the analysis of plants from the three case study sites of the Plants for People project: Titjikala, Ceduna and Leonora.

Laboratory studies performed

Laboratory studies were conducted on medicinal plants from Titjikala (12 species; 20 samples tested) and Ceduna (Scotdesco) (2 species; 4 samples tested) and on bush food plants from Leonora (7 species tested). Most of the results from the studies on medicinal plants are presented in this report. The remaining results were provided in a confidential report to the DKCRC. The studies on nutritionalanalysis of bush food plants from Leonora are presented in Evans et al. 2010.

Medicinal plant extracts were tested using bioassays and pharmacological, antibiotic, antiviral and anti-tumour test procedures at four different laboratories in Western Australia and South Australia. The results of all but the anti-tumour studies are presented in this abridged report. All plant species investigated in this project were identified by examination of voucher specimens provided to staff of either the Western Australian Herbarium or the Alice Springs Herbarium.

Medicinal plant studies – Titjikala and Ceduna

Specimen collection and processing procedures

Plant samples were collected by Aboriginal project team members on three separate occasions and transported in foam boxes by air or road to a quarantine-approved laboratory at the School of Pharmacy, Curtin University of Technology (Table 1). They were processed at this laboratory and either subjected to chemical screening (essential oil analysis (first batch only); alkaloids, saponins, tannins) or transported to another laboratory for other laboratory testing (Curtin University ecotoxicology laboratory – bioassays; University of South Australia – antibiotic, antifungal, antiviral tests; University of Western Australia – anti-tumour tests).

Table 1: Medicinal plant collection details

Plant species	Collection site	Collection date	Material collected
Acacia tetragonophylla	Titjikala	24–27 July 2004 11 October 2005	Leaves, stems, flowers Root bark
Codonocarpus cotinifolius	Titjikala	11 October 2005	Leaves, stems
Eremophila alternifolia	Scotdesco	6 April 2005	Leaves, stems
Eremophila duttonii	Titjikala	11 October 2005	Leaves, stems
Eremophila freelingii	Titjikala	24–27 July 2004 11 October 2005	Leaves, stems Leaves, stems
Eremophila latrobei	Titjikala	24–27 July 2004	Leaves, stems, flowers
Eremophila longifolia	Titjikala	24–27 July 2004	Leaves, stems
Eremophila sturtii	Titjikala	24–27 July 2004	Leaves, stems, flowers
Euphorbia drummondii	Titjikala	24–27 July 2004	Leaves, stems, flowers, fruit
Euphorbia tannensis	Titjikala	11 October 2005	Leaves, stems
Hakea divaricata	Titjikala	24–27 July 2004 11 October 2005	Bark Bark
Sarcostemma australe	Titjikala	24–27 July 2004	Stems
Scaevola spinescens	Scotdesco	6 April 2005	Leaves, stems

Pharmacological screening results

We screened four *Eremophila* species (*Eremophila freelingii*; *E. latrobei*; *E. longifolia*; *E. sturtii*), *Sarcostemma australe*, *Acacia tetragonophylla* (leaves, stems and flowers), *Hakea divaricata* and *Euphorbia drummondii* for essential oil content. Essential oils were detected in the four *Eremophila* plant species and in the *Euphorbia drummondii* sample. However, the amounts that were present tended to be lower than had been reported in the literature. It was concluded that the long time delay between collection and processing (2–5 days) may have led to a loss of these volatile materials from the plant samples and that no further screening would be conducted.

Plant extracts testing positive for alkaloids, saponins and tannins are shown in Table 2. With the exception of three plant extracts (*Acacia tetragonophylla* (root bark), *Eremophila duttonii* and *Euphorbia tannensis*), all plant extracts tested positive for one or more of the three chemical components. *Eremophila freelingii* tested positive in all three screening tests. *Sarcostemma australe* showed intense precipitation with all three reagents used in the alkaloid test screen, proving strong evidence of the presence of alkaloids in this plant extract. *Sarcostemma australe* also reacted strongly in the saponin screening test, as did the *Euphorbia drummondii* extract. Evidence for the presence of tannins was obtained with all plant extracts except for *Acacia tetragonophylla* (root bark), *Codonocarpus cotinifolius*, *Eremophila duttonii*, *Eremophila longifolia*, *Euphorbia tannensis*, *Sarcostemma australe* and *Scaevola spinescens*.

Table 2: Positive results in pharmacological and bioassay screening tests

Plant species	Pharmacological screening tests			Bioassay screening tests*	
	Alkaloids	Saponins	Tannins	24-hour and/or 48-hour Daphnia tests	Artemia, Daphnia 24-hour and Daphnia 48-hour tests
Acacia tetragonophylla (leaves, stems, flowers)		√	√		
Acacia tetragonophylla (root bark)				√	
Codonocarpus cotinifolius			√	√	
Eremophila alternifolia			√	√	√
Eremophila duttonii				√	√
Eremophila freelingii	√	√	√	√	√
Eremophila latrobei	√		√	√	
Eremophila longifolia			√		
Eremophila sturtii			√	√	√
Euphorbia drummondii		√	√	√	√
Euphorbia tannensis				√	
Hakea divaricata			√	√	√
Sarcostemma australe	√	√			
Scaevola spinescens	√	√		√	

* Positive result indicates LC50 < 450 µg/mL.

On the basis of these chemical screening tests, further studies are recommended on the pharmacological properties of *Eremophila freelingii* and *Sarcostemma australe*.

Bioassay results

Three bioassays were performed on the plant extracts – a brine shrimp (*Artemia*) assay and two *Daphnia* immobility tests, one extending for 24 hours and the other for 48 hours. With the exception of the *Hakea divaricata* plant extract, all extracts which displayed toxicity in the *Artemia* test were more toxic to *Daphnia* with lower 24-hour and 48-hour LC50 values, suggesting that the *Daphnia* test procedure was a more sensitive test for bioactivity than the brine shrimp assay. Using 450 µg/mL as an indicator of samples warranting further investigation (Sam 1993), 12 of the 15 samples had LC50s below the cut-off value for the 24-hour and/or 48-hour *Daphnia* test, the exceptions being *Acacia tetragonophylla* (leaves, stems, flowers), *Eremophila longifolia* and *Sarcostemma australe* (Table 2). Six plant extracts (*Eremophila alternifolia*, *Eremophila duttonii*, *Eremophila freelingii*, *Eremophila sturtii*, *Euphorbia drummondii* and *Hakea divaricata* (outer bark only)) were positive for toxicity in all three screening tests. It was of interest to note that the *Hakea divaricata* outer bark was more toxic than the inner bark extract in all three toxicity tests.

On the basis of these results, further studies are recommended on the bioactivity of *Eremophila alternifolia*, *Eremophila duttonii*, *Eremophila freelingii*, *Eremophila sturtii*, *Euphorbia drummondii* and *Hakea divaricata* (outer bark).

Comparison of screening test results with traditional plant knowledge

A comparison of the results obtained in laboratory screening tests of the 15 plant extracts with the documented accounts of the traditional uses of these plant materials is shown in Table 3. All plant extracts tested positive in either one or both of the suite of pharmacological or bioassay screening tests. This comparison provides clear validation of the accuracy of traditional knowledge with respect to the medicinal properties of the plant extracts examined in this study.

Table 3: Comparison of reported medicinal use and screening test results

Plant species	Titjikala plant name	Reported medicinal use		Positive result in at least one screening test	
		J. Briscoe (pers. comm. 2002–2006)	Barr et al. (1993)	Pharmacological tests	Bioassays*
Acacia tetragonophylla (leaves, stems, flowers)	Wakalpulka, Arlketyerre	Remove warts Skin lesions	Skin lesions Limb fractures Remove warts	Yes	No
Acacia tetragonophylla (root bark)	Wakalpulka, Arlketyerre	Treat arthritis		No	Yes
Codonocarpus cotinifolius	Kaluti	Make people strong	Symptoms of flu Pain relief	Yes	No
Eremophila alternifolia	Irmangka irmangka	Skin conditions Chew for toothache (Note: Mr Briscoe stressed that this was an important medicine)	Colds, fever, internal pain, severe illness	Yes	Yes
Eremophila duttonii	Muntjunpa	Colds (as a rub)	Respiratory infections, fever and chronic malaise	No	Yes
Eremophila freelingii	Aratja	Treat itchy skin	Infected skin lesions, scabies, colds, diarrhoea	Yes	Yes
Eremophila latrobei			Respiratory infections, fever, chronic fatigue	Yes	Yes

Plant species	Titjikala plant name	Reported medicinal use		Positive result in at least one screening test	
		J. Briscoe (pers. comm. 2002–2006)	Barr et al. (1993)	Pharmacological tests	Bioassays*
Eremophila longifolia	Tulypurpa	Treat itchy skin	Skin lesions, scabies, muscle or joint pain, colds	Yes	No
Eremophila sturtii	Watara, munyunpa	Colds – used as a drink	Colds and minor skin lesions	Yes	Yes
Euphorbia drummondii	Mangka-mangka	Skin conditions	Skin conditions	Yes	Yes
Euphorbia tannensis	Ipi-ipi	Skin conditions	Skin conditions	No	Yes
Hakea divaricata	Witjinti	Skin conditions	Skin lesions; obtain powder from heated bark, dust on area and protect with a bandage Sharp lobes of leaves used to treat warts	Yes	Yes
Sarcostemma australe			Skin disorders	Yes	No
Scaevola spinescens		Note: has been reported by Aboriginal people from WA as a cancer cure (P. Kerr, pers. comm. 2004)	(Decoction of roots taken for stomach ache and urinary troubles (Bindon 1996))	Yes	No

* Positive result indicates LC50 < 450 µg/mL.

Antimicrobial and antiviral laboratory test results

Standard strains of four Gram-positive bacteria, three Gram-negative bacteria and two yeasts were used to assess the antimicrobial activity of different plant extracts. None of the plant extracts tested was active against Gram-negative bacteria. *Euphorbia drummondii* was the only extract that was active against the yeasts *Candida parapsilosis* and *C. albicans*. Four *Eremophila* species (*Eremophila alternifolia*; *E. duttonii*; *E. freelingii*; *E. sturtii*), *Acacia tetragonophylla* (leaves and stems) and *Euphorbia drummondii* were positive against at least two different strains of Gram-positive bacteria (Table 4). *Eremophila duttonii* exhibited particularly strong activity. The same plant extracts, with the exception of *Eremophila alternifolia* (which was not tested), were also active against the clinical Methicillin-resistant *Staphylococcus aureus* (MRSA) isolates tested.

Extracts were screened for antiviral activity in a whole-cell assay with human rhinovirus (a frequent causative agent of the common cold), coxsackievirus A21 (a cause of 'summer flu'), herpes simplex virus type 1 (the cause of 'cold sores'). Modest antiviral activity was observed with only one plant extract, *Codonocarpus cotinifolius*, a plant species that was used traditionally to treat influenza (Barr et al. 1993).

On the basis of the antimicrobial tests, further studies are recommended on the *Eremophila* species showing activity against Gram-positive bacteria and the clinical MRSA isolates, in particular *E. duttonii*. Further investigations of the antiviral properties of *Codonocarpus cotinifolius* are also warranted.

Table 4: Antimicrobial and antiviral test results

Plant species	Antimicrobial studies		Antiviral studies
	Active against Gram-positive bacteria	Active against clinical isolates of MRSA	
Acacia tetragonophylla (leaves, stems, flowers)	√	√	
Acacia tetragonophylla (root bark)			
Codonocarpus cotinifolius			√
Eremophila alternifolia	√		
Eremophila duttonii	√	√	
Eremophila freelingii	√	√	
Eremophila latrobei			
Eremophila longifolia			
Eremophila sturtii	√	√	
Euphorbia drummondii	√	√	
Euphorbia tannensis			
Hakea divaricata			
Sarcostemma australe			
Scaevola spinescens			

Bush food plant study – Leonora

Proximate analysis was performed on the fruits, seeds, flowers or tubers of six different bush food plants: *Eremophila latrobei* (native fuchsia), *Pisolithus* sp. (desert puffball), *Portulaca* sp. (pigweed), *Marsdenia australis* (silky pear), *Calandrinia schistorhiza* (bush potato), mulga apples from *Acacia ramulosa* var., and seeds from an unknown plant species (‘Kawun’) that was not identified.

The dry seeds of Kawun had the highest percentage of dry matter (90%). The percentage dry matter in the *Pisolithus* sp. was five times higher than that of leaves of pigweed, *Portulaca* sp., and 2.5 times higher than that of bush potato, *Calandrinia schistorhiza*. The highest protein level was found in the dry seeds of Kawun (30.1%). The desert puffball, *Pisolithus* sp., also had a high protein content (21.5%) and the highest fibre content (14.9%). The bush potato, *Calandrinia schistorhiza*, had the lowest protein level and the second highest carbohydrate content.

1. Background to the project's development

This laboratory study of Australian indigenous plants is a component of the Plants for People project which originated from discussions with Aboriginal elders from the Titjikala community, 130 kilometres south of Alice Springs. The elders observed low intergenerational transference of knowledge of traditional Aboriginal bushcraft, plant and animal uses, and dreaming stories. This knowledge was remaining privy to the older generation, not as a matter of cultural protocol, but because the interests of the prospective students were prioritised towards Western media and entertainment. This traditional knowledge was seen by the elders as being important to retain the community's cultural identity and to develop enterprise opportunities of benefit to the community through tourism, horticulture and education.

The Plants for People project focuses on plant use to scope ways to advance livelihoods, cultural integrity, self-esteem, health and wellbeing within such Aboriginal communities. The project involved Aboriginal communities at three case study sites in arid Australia. The major thrust of the project was to develop best practice approaches for documenting and ensuring the survival of traditional knowledge about plants of cultural significance and to use that knowledge to advance the livelihoods of Aboriginal people.

Specifically, the project sought to develop:

1. insight into best practice approaches for planning and implementing a traditional knowledge documentation, reclamation, and generational transfer program in an Aboriginal community or group
2. insight into preferred business and enterprise development approaches for Australian Aboriginal people, drawing on research findings and on existing knowledge of community and social development theory and practice
3. Aboriginal community participants' knowledge and skills in plant specimen collection, documentation and storage, photography and multimedia storage, and plant propagation and cultivation
4. early-stage plant propagation and cultivation trials and facilities for use in Aboriginal business enterprises
5. identification processes for plant species that have nutritional or medicinal value and potential for cultivar development
6. approaches to inform Aboriginal knowledge on the medicinal uses of plants through laboratory validation and the application of Aboriginal intellectual property rights
7. 'knowledge registers' and support the documentation of protocols to access the knowledge they contain.

The project also sought to gather information on the nutritional value, health benefits, seasonality and safety of selected bush foods for dissemination to participating groups.

The laboratory study is a principal outcome against three of the goals above. In particular, it sought to identify plant species having nutritional or medicinal value, to inform Aboriginal knowledge on the medicinal uses of indigenous plants, and to provide a basis for the dissemination to participating groups of information about the nutritional value, health benefits, seasonality and safety of selected bush foods.

2. Laboratory studies on medicinal plants

2.1 Introduction

A variety of publications on traditional Aboriginal medicinal plants has emerged in recent years. The most well known, even on an international scale, is a volume titled *Traditional Aboriginal Medicines in the Northern Territory of Australia* (Barr et al. 1993), which was compiled through the cooperative efforts of Aboriginal people from Northern Australia and project teams from the Northern Territory Health Service and the Conservation Commission of the Northern Territory. Frequently, these accounts on Aboriginal herbal medicines incorporate basic chemical and pharmacological information and the results of extensive literature searches to validate their traditional use. These screens provide a first impression of a plant's chemical component spectrum and any cytotoxic or antimicrobial activity.

Antimicrobial properties of various plant components, for instance, are well documented, with traditional plant medicines from a variety of areas of the world found to exhibit antibacterial, antifungal and antiviral properties (Cowan 1999). Australian Aboriginal people have used plant medicines for a variety of symptoms indicative of microbial disease, including skin afflictions (such as sores, infected wounds, warts), symptoms of respiratory illness (coughs, nasal congestion, sore throat), ear and eye complaints, gastrointestinal symptoms (such as diarrhoea and abdominal pain), fever and joint pain (Smith 1991, Barr et al. 1993, Latz 1995). Published ethnobotanical literature (Goddard & Kalotas 2002, Smith 1991, Barr et al. 1993, Latz 1995) and work with Aboriginal communities involved in the Plants for People project have indicated that a number of plant species that grow in arid areas of Australia have been used for treating symptoms of microbial disease. Some arid-land plant species have been shown to exhibit antimicrobial activities in vitro that correlate with their traditional uses. These include species of the genera *Callitris* ('native pine'), *Cymbopogon* ('native lemon grass') (Barr et al. 1993), *Eremophila* ('emu bush') (Barr et al. 1993, Palombo & Semple 2002), and *Santalum* (Jones et al. 1995), which have been shown to have antibacterial activity, and *Pterocaulon* ('applebush'), which has been shown to have antiviral activity (Semple et al. 1999).

Effective screening for bioactive compounds in plants can be accomplished by using inexpensive, rapid and reliable bioassays (Atta-ur-Rahman et al. 2001). A number of laboratory assays used for primary testing are based on toxicological methods, which are designed to estimate tolerance of living organisms to acute or chronic exposure to chemicals. Revealed toxicity of the extract may indicate a potential to develop drugs with killing actions towards pathogenic organisms. Recent advances in developments of micro-scale toxicity bioassays have made available test protocols employing microscopic aquatic organisms like brine shrimps, rotifers and water fleas (Persoone & Wells 1987). The use of different organisms in general toxicity bioassays increases their applicability and result reliability. In the brine shrimp bioassay, the salinity of plant water extracts should be elevated at least up to five per cent as this saltwater test species may not tolerate a freshwater environment. Adding salts to plant extracts may cause precipitation and alter their toxicity. In this case, bioassays with freshwater rotifers or *Daphnia* may be applied with the same efficiency.

2.2 Materials and methods

2.2.1 Extraction of plant material

Collecting and handling plant material

Plant samples were collected on two occasions in Titjikala in the Northern Territory (Batch 1 and Batch 2) and on one occasion in Scotdesco in South Australia.

Plant samples collected near Leonora in Western Australia were also collected and analysed. The report of the analysis of plant materials from the Leonora case study site comprises Section 3 of this report.

Designation of plant species is shown in Table 5. The samples were packed in foam boxes and sent to Curtin University of Technology in Perth by air fright or road as soon as possible after collection. In most instances they arrived at the university within two days. To enable essential oil extraction, the first batch of samples was stored in a quarantine-approved facility at 7ºC and extraction of the volatiles was begun as soon as possible, in most instances within one or two days. The samples collected at a later stage (batch 2 and the Scotdesco samples) were dried at 37ºC in a quarantine-approved facility for subsequent Soxhlet extraction.

Table 5: Designation of plant material

ID	Scientific name	Common name	Plant part
Titjikala, Batch 1			
1 (i)	*Eremophila longifolia*	Emu bush	Leaves, stems
2 (i)	*Eremophila latrobei*	Native fuchsia	Leaves, stems, flowers
5 (i)	*Eremophila sturtii*	Turpentine/kerosene bush	Leaves, stems, flowers
8 (i)	*Eremophila freelingii*	Rock fuchsia	Leaves, stems, flowers
10 (i)	*Sarcostemma australe*	Ipi Ipi (Pitjantjatjara)	Stems
11A (i)	*Acacia tetragonophylla*	Dead finish	Leaves, stems
11B (i)	*Acacia tetragonophylla*	Dead finish	Leaves, stems, flowers
12 (i)	*Hakea divaricata*	Fork-leafed corkwood	Bark
15 (i)	*Euphorbia drummondii*	Caustic/milk weed, mat spurge	Leaves, stems, flowers, fruit
Titjikala, Batch 2			
T19	*Eremophila duttonii*		Leaves, stems
T20	*Euphorbia tannensis*		Stem (leaves)
T21	*Eremophila duttonii*		Leaves, stems
T22	*Hakea sp.*		Bark
T24A	*Hakea divaricata*	Fork-leafed corkwood	Inner bark
T24B	*Hakea divaricata*	Fork-leafed corkwood	Outer bark
T25	*Codonocarpus cotinifolius*		Stems, leaves
T26	*Euphorbia tannensis*		Stem (leaves)
T27	*Eremophila freelingii*	Rock fuchsia	Leaves, stems
T28	*Acacia tetragonophylla*	Dead finish	Root bark
Scotdesco, South Australia			
S1.1	*Eremophila alternifolia*	Narrow-leaf fuchsia bush	Leaves, stems
S1.2	*Eremophila alternifolia*	Narrow-leaf fuchsia bush	Leaves, stems
S3	*Scaevola spinescens*	Fan flower, maroon bush	Leaves, stems
S7	*Eremophila alternifolia*	Narrow-leaf fuchsia bush	Leaves, stems

Essential oil extraction

The weight of fresh plant material to be used for essential oil extraction (about one third of the available total quantity of material) was recorded. The material was broken into small pieces and subjected to hydrodistillation for two hours. In the case of plant samples with a high content of essential oils, distinct oil droplets became evident in the aqueous distillate. The oil could thus be directly collected. In cases where no distinct oil was observed in the distillate, the aqueous extract was tested for odour as an indication of the presence of volatile compounds, and then extracted several times with dichloromethane. The combined organic extracts were dried with anhydrous $MgSO_4$, filtered, carefully evaporated at room temperature and stored away from light exposure in suitable vials until analysis.

Essential oil analysis

Obtained essential oil samples were analysed by gas-chromatography (initial temperature 60ºC for two minutes, 10ºC per minute ramp, final temperature 250ºC held for five minutes) and constituting compounds identified by mass spectrometry on a HP 5 Column MS (30-metre length, 0.25 mm diameter, 0.25 µm coating).

Methanol extraction by Soxhlet

Plant samples were stored in paper bags in a quarantine-approved area at 37ºC until completely dry. They were then broken into small pieces using a mortar and pestle or powdered using a hammer mill, before being stored in an air-tight container protected from light. The plant material was extracted with methanol in a suitably sized Soxhlet apparatus for several days until the new cycle's extract appeared colourless. The solvent was then evaporated under reduced pressure using a rotary evaporator and dried to constant weight in a vacuum oven. This methanol extract was then stored in a suitable container protected from light. About three quarters of it were sent off to associated research groups for pharmacological testing, i.e. one for general toxicity screening using the brine shrimp and *Daphnia* acute assays (Curtin University of Technology), another one for cytotoxic screening on cancer cell lines (University of Western Australia) and one for antibacterial and antiviral screening (University of South Australia). The remaining material was used for a range of basic chemical screening tests undertaken at the School of Pharmacy, Curtin University of Technology.

2.2.2 Chemical screening for alkaloids

Procedures for chemical screening for alkaloids were based on methods described in *Traditional Bush Medicines: An Aboriginal Pharmacopoeia* (Aboriginal Communities of the Northern Territory of Australia 1988).

Preliminary spot tests

One millilitre (1 mL) of 2M H_2SO_4 was added to a small amount of methanol plant extract and a few drops of the resulting supernatant were transferred to a spot dish. One drop of Dragendorff's reagent was added and the presence/intensity (ranging from + to +++) and colour of the observed precipitate recorded. The procedure was repeated using Mayer's and Wagner's reagents.

Dragendorff's reagent:	Bismuth nitrate	8.0 g
	Nitric acid (dil.)	20 ml
	Potassium iodide	27.2 g
	PW FBC[1] to	100 ml
Mayer's reagent:	Mercuric chloride	1.36 g
	Potassium iodide	5.0 g
	PW FBC to	100 ml
Wagner's reagent:	Iodine	1.3 g
	Potassium iodide	2.0 g
	PW FBC to	100 ml

1 Purified water freshly boiled and cooled

Spot test after basic extraction step

Samples that tested positive in the above described preliminary spot tests were subjected to a basic extraction step followed by further spot tests to account for the chemical nature of the alkaloid(s) present in the extract: about 2 mL of 2M H_2SO_4 and 1 mL of dichloromethane were added to a small sample of the methanol extract. After shaking, the organic layer was removed and the acidic aqueous solution extracted two more times with 1 mL of dichloromethane. Each time, the obtained organic solution was discarded. The aqueous solution was basified with concentrated ammonia solution and the resulting solution again extracted three times with 1 mL of dichloromethane, this time combining and retaining the obtained organic extracts. Both the combined organic and the alkaline aqueous extracts were then tested for the presence of alkaloids by adding a drop of Dragendorff's, Wagner's or Mayer's reagent respectively to a drop of the test solution. Again, the presence, intensity (+ to +++) and colour of any resulting precipitate was recorded. A positive response in the organic extract indicates the presence of basic alkaloids; a precipitate in the alkaline aqueous solution indicates quaternary alkaloids.

A more specific test was also performed with samples that tested positive in any of the above preliminary spot tests in an attempt to identify tropane-type alkaloids.

Detection of tropane-type alkaloids with Vitali-Morin test

Two drops of fuming HNO_3 (98%) were added to a small amount of methanol plant extract in a spot dish. After evaporation, two drops of a saturated alcoholic KOH solution were added and the resulting colour recorded.

2.2.3 Chemical screening for saponins

Procedures for chemical screening for saponins were based on methods described in Simes et al. (1959) and Cook (1961).

Spot tests

One drop of the aqueous extract obtained in the froth test described below was combined with one drop of concentrated H_2SO_4 in a spot dish. The resulting colour was then recorded.

Similarly, a mixture of equal volumes of concentrated H_2SO_4 and aqueous $FeCl_3$ solution (5%) was prepared and one drop of this solution then combined with one drop of aqueous plant extract in a spot dish. Again, the observed colour was recorded.

The so-called Liebermann-Burchard test was also performed by dissolving a small amount of the methanol plant extract in 1 mL of acetic anhydride and adding 2–3 drops of concentrated H_2SO_4. Green, blue, red, pink or purple colours can be seen as indication of the presence of steroids and triterpenes, common aglycones of saponin-type compounds.

Froth test

Ten millilitres of deionised water at 60ºC were added to 0.1 g of methanol extract in a 25-mL measuring cylinder and the suspension shaken for 30 seconds. The volume of resulting froth and the duration of its persistence were recorded after one, five, 10 and 15 minutes. In cases where only a small amount of methanol extract was available, the procedure was scaled down to 30 mg of extract and 2 mL of water using a 10-mL measuring cylinder.

2.2.4 Preliminary chemical screening for tannins

Procedures for chemical screening for tannins were based on methods given by Mueller-Harvey (2001).

One- and two-dimensional thin-layer chromatography analysis

Methanol plant extracts were analysed by one- and two-dimensional thin-layer chromatography (Mobile Phase 1: butanol : acetic acid : H_2O 60:15:25; Mobile Phase 2: acetic acid : H_2O 2:98) and analysed under UV-light after exposure to ammonia vapours. Furthermore, the obtained plates were also treated with $FeCl_3/K_3Fe(CN)_6$ reagent. To minimise background colour, the plates were soaked in dilute HCl followed by water immediately after spraying. Using this technique, galloyl esters and gallotannins appear as violet fluorescent spots under UV-light, their fluorescence enhanced on fumigation with ammonia vapour. Ellagic acid produces a violet spot that darkens on exposure to ammonia vapour. The spraying reagent detects phenolic groups as blue spots.

2.2.5 Screening tests for bioactivity (toxicity tests)

Extract preparations for toxicity testing

A stock solution of a plant extract with concentration of 1000 µg/mL was prepared by dissolving dry extract in dimethyl sulfoxide (DMSO) and then diluting with water. Other concentrations of the plant extract were produced by diluting the stock solution with water.

The final concentration of DMSO in the stock solution was 10 g/L as recommended by Atta-ur-Rahman et al. (2001). That concentration was determined as NOEC (no observed effect concentration) for immobilisation of *Daphnia magna* exposed to DMSO for 24 and 48 hours (De la Torre et al. 1995). The available data of DMSO toxicity to *Artemia salina* are inconsistent. The 24-hour LC50 reported for A. salina varied from 6.7–90 g/L (Barahona-Gomariz et al. 1994, Calleja & Persoone 1993). Inconsistency in data for DMSO toxicity could result from differences in sensitivity of test organisms and quality of DMSO, which is highly hygroscopic. Preliminary testing of DMSO at the concentration of 10 g/L showed no toxicity to *A. salina* larvae and *D. magna* neonates used as test organisms in our bioassays.

Test animals

Artemia salina

Thirty milligrams of dry cysts of the brine shrimp *Artemia salina* (INVE Ltd., Thailand) were hydrated in a small Petri dish (5-cm ID) filled with 12 mL filtered sea water diluted to salinity of 5%. The cysts in water were exposed to 4000 lux light for one hour at 25°C and incubated for 23 hours in darkness at 25°C. The hatched larvae (instar I) were transferred to fresh diluted sea water in another Petri dish using a plastic micropipette and incubated for an additional 24 hours at 25°C. By the end of this period, the larvae moulted into instar II-III stage and were harvested for use in toxicity tests.

Daphnia magna

Stocks of Daphnia dormant eggs (ephippia) were obtained from the overseas supplier, Microbiotests Inc., Belgium. The content of a vial with ephippia was poured into the micro sieve and rinsed thoroughly with tap water to eliminate all traces of the storage medium. Ephippia were transferred into the hatching Petri dish in 50 mL Standard Freshwater (ISO recipe 6341) pre-aerated by air bubbling. The covered Petri dish was incubated for three days at 21°C under continuous illumination of 6000 lux. After hatching and two hours prior to collecting, the *Daphnia* neonates were fed spirulina powder suspended in the Standard Freshwater.

Toxicity test using Artemia salina *larvae*

The toxicity tests were conducted in 24-well microplates. Each well in the test plate was filled with 1 mL of the solution. To minimise the dilution of the test solutions, transfer of the brine shrimp larvae to the multi-well plate was accomplished in two steps: 1) transfer of the larvae from the Petri dish into

rinsing wells of the plate; 2) transfer of the larvae from the rinsing wells to actual test wells. The animal transfer was conducted under a dissection microscope using a disposable polyethylene pipette. Each test solution was tested with 10 test animals in each of three replicate wells.

The multi-well plates with test animals in test solutions were covered by lids and incubated in darkness at 25°C for 24 hours. At the end of the test, the number of dead animals in each well was counted and recorded. An individual larva was considered dead if its appendages and antennae did not move for 10 seconds of observation.

Toxicity test using Daphnia magna *neonates*

The bioassays were conducted in disposable polycarbonate test plates composed of six rinsing wells and 24 test wells arranged in six rows. Each well of the test plate was filled with 10 mL of test solution. The Petri dish was put on the transparent stage three centimetres above a light table provided with a black strip to enhance the contrast and facilitate neonate catching. Using a disposable plastic micropipette, at least 20 actively swimming neonates were individually transferred into each rinsing well with a minimal amount of water taken with the neonates. The pipette was rinsed after each transfer. After that, five neonates from each rinsing well were transferred into four test wells of each row.

The test plate was covered with a strip of Parafilm and a lid was incubated at 20°C in darkness for 48 hours. After 24 and 48 hours incubation, the *Daphnia* neonates were examined under the microscope in each well. The neonates were considered dead or immobilised if they lay on the bottom and did not resume swimming within 15 seconds of observation. The dead or immobilised neonates were counted in each well and numbers were recorded in a result sheet.

Reference toxicant tests

Potassium dichromate ($K_2Cr_2O_7$) was used as the reference toxicant in the toxicity tests. Test concentrations of potassium dichromate were measured using photometry with Spectroquant® reagents (Merck 1.14758.0001). The LC50 values calculated for *Artemia* and *Daphnia* exposed to potassium dichromate in our reference bioassays are given in Table 6. Mortality in the control and toxicity of the reference toxicant were not significantly different from the acceptable values set by MicroBioTests Inc. for the batch of *D. magna* used in the test. The LC50 of 30 mg/L derived for potassium dichromate in our reference bioassay with *Artemia* was close to the published value of 34 mg/L (Sam 1993).

Table 6: Toxicity indices of potassium dichromate in reference bioassays

Test organism	Toxicity index	Toxicity index value, mg/L	95% Confidence interval, mg/L
Artemia salina, larvae	24 hour LC50	30	26 - 34
Daphnia magna, neonates	24 hour LC50	1.33	1.06 – 1.69
Daphnia magna, neonates	48 hour LC50	0.83	0.66 – 1.04

2.2.6 Antimicrobial testing

Plant extract stock solution preparation

A stock solution (50 mg/mL) of each methanol extract was prepared in DMSO (Ajax Chemicals, New South Wales).

Antibiotics and chemicals

Benzyl penicillin was used as positive control (known inhibitor) for the Gram-positive bacteria; gentamicin sulfate (G-3632, Sigma, St Louis, Missouri) was used as positive control for the Gram-negative bacteria; fluconazole 25µg discs (Sensi-DiscTM 232045, Becton, Dickinson & Company,

USA) were used as a positive control for the yeast assays; and ampicillin (A-9518, Sigma, St Louis, Missouri) was used as positive control for the minimum inhibitory concentration (MIC) experiment. Resazurin sodium salt (R7071) was purchased from Sigma (St Louis, Missouri).

Microorganisms and media

In this study, standard strains of four Gram-positive bacteria, three Gram-negative bacteria and two yeasts were used to assess the antimicrobial activity of different plant extracts. The bacterial strains, obtained from stock cultures preserved at –70°C at the School of Pharmacy and Medical Sciences at the University of South Australia, included *Staphylococcus aureus* ATCC 25923, *Staphylococcus aureus* ATCC 29213, *Streptococcus pyogenes* ATCC 10389, *Streptococcus pneumoniae* ATCC 49619, *Salmonella typhimurium* ATCC 13311, *Pseudomonas aeruginosa* ATCC 27853, and *Escherichia coli* ATCC 25922. The yeasts strains, *Candida albicans* ATCC 90028, and *Candida parapsilosis* ATCC 90018, were provided by Dr David Ellis of the Women's and Children's Hospital, Adelaide, South Australia.

All bacteria were grown on blood agar plates (Colombia agar – CM331, Oxoid, supplemented with 5% sheep blood) at 37°C. The yeasts were grown on Sabouraud Dextrose Agar (CM44, Oxoid). For the agar-well assay, all bacteria with the exception of Streptococcus pyogenes and S. pneumoniae were grown on Mueller-Hinton agar (CM337, Oxoid) at 37°C. Streptococcus pyogenes and S. pneumoniae were grown on Mueller-Hinton agar supplemented with 5% sheep blood at 37°C in the presence of 5% CO_2. The yeasts were assayed on Mueller-Hinton agar supplemented with 0.5 µg/mL methylene blue and 2% (w/v) glucose (NCCLS 2003a). Mueller-Hinton broth was used for the MIC and minimum bactericidal concentration (MBC) experiments for the *Staphylococcus* species while brain heart infusion broth was used for the *Streptococcus* species.

Agar well antimicrobial assay

The agar-well assay of Hufford et al. (1975) as described by Rojas et al. (2003) was used with some slight modifications to determine the antibacterial activity of all plant extracts against both Gram-positive and Gram-negative bacteria.

Twenty millilitres of the appropriate molten agar (45°C) was mixed aseptically with 200 µl of a bacterial suspension (3 x 108 CFU/ml) and poured into sterile Petri dishes. The suspensions were prepared by suspending colonies from overnight cultures in normal saline solution and adjusting the turbidity to that of a 1 McFarland standard. Once the plates were hardened, a sterile 8 mm cork borer was used to make wells on the plates. Twenty microlitres of a 50 mg/mL stock solution of each plant extract was introduced into each well (1 mg extract/well) and the plates were kept in the fridge at 4°C for two hours in order for the extract and DMSO to diffuse into the agar. All plates were incubated overnight at 37°C. Those plates inoculated with *Streptococcus pyogenes* and *S. pneumoniae* were incubated in the presence of 5% carbon dioxide. Antimicrobial activity was recorded if a zone of growth inhibition greater than 8 mm was measured. Any extracts showing activity were tested twice more.

Disc diffusion assay

All the plant extracts were tested against the yeast strains using the disk diffusion method described by the National Committee for Clinical Laboratory Standards' proposed guideline for antifungal disk diffusion susceptibility testing of yeasts (NCCLS 2003a). The plant extracts were tested at a concentration of 1 mg/disk on Whatman 6-mm disks (Whatman International Ltd). Twenty microlitres of a 50 mg/mL stock solution of each plant extract were introduced onto each disc. DMSO and fluconazole controls were included in each experiment. Plates were incubated at 37°C for 24 hours and any zones of inhibition were measured.

Broth microdilution assay

The broth microdilution method described by Mann and Markham (1998) with modifications was used to determine the MIC and MBC of active plant extracts.

A sterile 96-well plate with lid was used for the MIC experiment. Duplicate two-fold serial dilutions of extract (100 µl/well) were prepared in the appropriate broth containing 2% DMSO to produce a concentration range of 4.0–0.0313 mg extract/mL (Staphylococcus assay) or 2.0–0.0153 mg extract/mL (Streptococcus assay). Two-fold dilutions of ampicillin were used as a positive control. One hundred microlitres of a bacterial cell suspension was prepared in the appropriate broth corresponding to 1 x 106 CFU/mL and was added in all wells except for those in columns 10, 11 and 12 which served as saline, extract and media sterility controls, respectively. Controls for bacterial growth without plant extract were also included on each plate. The final concentration of bacteria in the assay was 5 x 105 CFU/mL. The final concentration of extracts was 2.0–0.0156 mg/mL (Staphylococcus assay) or 1.0–0.0078 mg/mL (Streptococcus assay). The prepared dishes were then placed on a shaker for ten minutes before being incubated at 37°C overnight. The *Streptococcus* species were incubated in the presence of 5% CO_2. After incubation, dishes were examined with the naked eye for any growth. The MIC of each extract was determined as the lowest concentration at which no growth was observed in the duplicate wells. Ten microlitres of resazurin solution (0.01%) was then added to the wells. The dish was then placed on the shaker for ten minutes, transferred into the incubator for a further 30 minutes, and assessed visually for any change in colour from blue to pink indicating reduction of the dye due to bacterial growth. The highest dilution (lowest concentration) that remained blue corresponded to the MIC. Experiments were performed in duplicate.

Following determination of the MIC, a 10 µL aliquot was taken from each of the wells of a duplicate plate (not treated with resazurin) at the concentration corresponding to the MIC and those concentrations above the MIC. Each aliquot was mixed with 190 µl of appropriate broth in a sterile 96-well plate. Ten microlitres was also taken from the control wells for no extract treatment, saline control, extract control and media sterility control and each was mixed with 190 µL of the appropriate broth. The samples were incubated under the same conditions as in the MIC experiment; then the presence or absence of bacterial growth was determined. The MBC was the lowest concentration of the extract at which no growth occurred. Controls for media sterility, extract without bacteria and saline controls were checked for the absence of bacterial growth.

Antibacterial testing of active extracts against clinical isolates of multi-drug resistant Staphylococcus aureus (MRSA)

An agar dilution method (NCCLS 2003b) was used with some slight modification to determine activity of the most active extracts against multi-drug resistant clinical isolates of *Staphylococcus aureus*. Clinical isolates of multi-drug resistant *S. aureus* were obtained from Lance Mickan, Infectious Diseases Laboratory, Institute of Medical and Veterinary Sciences (IMVS), Adelaide, South Australia. These isolates had been tested for susceptibility to 15 different antibiotics. Sixty-eight isolates that were resistant to at least three different antibiotics were selected and used in testing of plant extracts that had been shown to have activity against standard strains of Gram-positive bacteria.

Extracts were tested in serial two-fold dilutions over the concentration range of 4–0.0313 mg/mL. Ampicillin was used as a positive control (known inhibitor). *Staphylococcus aureus* ATCC 25923 and *Staphylococcus aureus* ATCC 29213 were used as control strains in the assay.

Isolates were streaked on blood agar plates and incubated at 37°C overnight. Single colonies were emulsified in 0.85% NaCl to obtain a bacterial suspension that corresponded in turbidity to a 0.5 McFarland standard which was used as inoculum for the agar dilution in Mueller-Hinton agar. The

inoculation of the clinical isolates and standard strains was performed using a multi-point inoculator (School of Pharmacy and Medical Sciences). After inoculation, the plates were incubated overnight at 37°C. The lowest extract concentration at which an isolate did not grow was considered as the MIC.

2.2.7 Antiviral testing

Cells and viruses

Extracts were screened for antiviral activity in a whole cell assay with human rhinovirus (a frequent causative agent of the common cold), coxsackievirus A21 (a cause of 'summer flu') and herpes simplex virus type 1 (the cause of 'cold sores').

H1-HeLa cells (ATCC CRL-1958) (human carcinoma, cervix) obtained from the American Type Culture Collection (ATCC), Manassas, Virginia, and Vero cells (African green monkey kidney cells) obtained from the Infectious Diseases Laboratory, IMVS, were tested for the absence of Mycoplasma contamination. H1-HeLa cells were grown in Eagle Minimum Essential Medium (EMEM modified) with Earle's Balanced Salts (JRH Biosciences, Lenexa, Kansas) supplemented with non-essential amino acids for MEM (JRH Biosciences, Lenexa, Kansas), 10% heat-inactivated foetal calf serum (FCS) (JRH Biosciences, Lenexa, Kansas) and 2 mM L-glutamine. Vero cells were grown in Dulbecco's Modified Eagle Medium with 20 mM HEPES buffer and 2 mM L-glutamine (Infectious Diseases Laboratory, IMVS) supplemented with 5% FCS (JRH Biosciences, Lenexa, Kansas).

Human rhinovirus 14 (strain 1059, ATCC VR-284) and Coxsackievirus A21 (ATCC VR-850) were obtained from the ATCC. A reference strain of herpes simplex virus type 1 (SC16) was kindly provided by Bill Winslow, Infectious Diseases Laboratory, IMVS. Human rhinovirus 14 (HRV-14) and Coxsackievirus A21 (CVA21) were propagated in H1-HeLa cells and herpes simplex virus type 1 (HSV-1) in Vero cells. Both CVA21 and HSV-1 were grown in 37°C in a humidified atmosphere of 5% CO2 in air. HRV-14 was grown at 33°C.

Reagents

Known antiviral compounds guanidine HCl and acycloguanosine (acyclovir) were obtained from Sigma, St. Louis, Missouri. Dimethyl sulfoxide (DMSO, sterile, biotechnology grade) was obtained from Sigma, St. Louis, Missouri.

Antiviral testing of plant extracts

Extracts were pre-solubilised in DMSO prior to dilution in cell culture media to give a stock solution of extract. This solution was further serially diluted in cell culture media to give the desired working concentrations. The maximum concentration of all extracts tested in the antiviral assays was 1 mg/mL. A final concentration of no more than 1% v/v DMSO was used in the antiviral and cytotoxicity assay.

Extracts were tested for inhibition of virus-induced cytopathic effect and toxicity to actively growing cells using methods based on those described previously (Semple 2001). Cells were seeded into 96-well microtitre cell culture plates (Sarstedt, Technology Park, South Australia or Iwaki, Japan) at an initial concentration of 1 x 104 cells/well (for H1-HeLa) and 2 x 104 cells/well (for Vero cells) and incubated at 37°C in a humidified atmosphere of 5% CO_2 in air for 4–6 hours to allow cell attachment. One series of triplicate wells was then infected with virus at a multiplicity of infection of approximately 0.01 $TCID_{50}$ units/cell. The second series of wells was mock-infected with media only. Two-fold serial dilutions of plant extracts (with a maximum final concentration of 1 mg/mL) were then added to the two series of triplicate wells to allow simultaneous determination of antiviral and cytotoxic effects. Controls of mock-infected cells without compound treatment and untreated cells infected with virus were included in triplicate on each plate. Controls for the DMSO concentrations in extract samples were

also tested for antiviral and cytotoxic effects. Acyclovir (HSV-1 assay) and guanidine HCl (HRV-14 and CVA21 assay) were tested with each batch of extracts as a positive control (known inhibitor) in the assay.

Plates were incubated in a humidified atmosphere of 5% CO_2 in air at 37°C (HSV-1 and CVA21 assay) or 33°C (HRV-14 assay) until the wells containing untreated cells infected with virus showed complete (100%) cytopathic effect (CPE) by microscopic examination (cell rounding, detachment and complete destruction of the cell monolayer) and the cells in the mock-infected, untreated wells had grown to form a confluent or near-confluent monolayer. Incubation periods were 48 hours for CVA21, 68–72 hours for HSV-1 and 72–84 hours for HRV-14. Plates were scored by microscopic examination for inhibition of CPE and cytotoxicity. The maximum non-toxic dose (MNTD) for the extract was the dilution of the extract at which mock-infected cells showed normal morphology and cell density by microscopic examination when compared to control cells grown without extract.

2.3 Results

2.3.1 Extraction of plant material and essential oils analysis

The results of essential oil (Samples 1(i) to 15(i) only) and methanol Soxhlet extractions are summarised in Table 7.

Table 7: Summary of essential oil and methanol extractions

	Volatile oil extraction			Methanol extraction
Sample*	**Fresh weight (g)**	**Odour**	**Droplets**	**Weight (g)**
1 (i)	109.6	'artichoke'	Evident	155.2
2 (i)	31.97	'artichoke'	Evident	81.3
3 (i)	20.2	'soil'	-	10.5
5 (i)	53.5	'rockmelon'	Evident	124.6
8 (i)	40.4	'lemongrass'	Evident	74.4
10 (i)	93.7	'tea'	-	183.7
11A (i)	21.0	'wet newspaper'	-	41.0
11B (i)	11.3	'coriander'	-	56.6
12 (i)	3.3	'mildew'	-	27.2
15 (i)	30.0	'spinach'	-	84.9
T19	N/A			112.4
T20	N/A			
T21	N/A			103.5
T22	N/A			48.0
T24A	N/A			64.4
T24B	N/A			
T25	N/A			
T26	N/A			
T27	N/A			91.3
T28	N/A			
S1.1	N/A			153.0
S1.2	N/A			129.4
S3	N/A			185.6
S7	N/A			46.7

*For the sample ID allocations, see Table 5.

Samples 3(i), 10(i), 11a(i), 11b(i) and 12(i) did not produce any significant peaks on GC/MS, even when run in very high sample concentration, which indicated the absence of large quantities of volatile compounds. For all other samples, which were hydrodistilled, results of preliminary screening of essential oil composition are presented in Table 8. Sample 1(i) does not appear to contain 'classic' volatile compounds as a computer match for any of its mass spectra was unsuccessful and indicated mainly large molecular weight compounds. Similar molecular weight compounds were found in sample 2(i). In sample 5(i) elemol and β-eudesmol were identified as the two major constituents. In Sample 8(i) three major constituents were identified with very high levels of confidence, but it was impossible to correlate the obtained mass spectrum for the most prominent compound, which accounted for about one third of the entire volatiles in this sample. Sample 15(i) yielded traces of volatile oil, which were successfully analysed by GC/MS. Three of the five most abundant compounds were identified with high levels of confidence as 2-methoxy-4-(2-propenyl) phenol, elemol and β-eudesmol.

Table 8: Essential oil analysis

ID	Scientific name	GC peak area (%)	Constituent
1(i)	*Eremophila longifolia*	19.3	NI (MW 207)
		40.2	NI (MW 401)
		40.5	NI (MW 458)
2(i)	*Eremophila latrobei*	21.5	NI (MW 248)
		58.0	NI (MW 250)
		5.9	NI (MW 231)
		8.7	NI (MW 230)
5(i)	*Eremophila sturtii*	44.6	Elemol
		44.0	β-Eudesmol
		9.1	NI (MW 220)
8(i)	*Eremophila freelingii*	18.1	Elemol
		17.8	(+)-Spathulenol
		18.8	(-)-Globulol
		6.3	NI (MW 193)
		34.0	NI (MW 151)
15(i)	*Euphorbia drummondii*	7.2	2-Methoxy-4-(2-propenyl)phenol
		23.6	Elemol
		7.8	NI (MW 243)
		9.9	NI (MW 401)
		7.1	β-Eudesmol
		30.5	NI (MW 429)
		8.6	NI (MW 503)

NI – not identified

2.3.2 Alkaloid screening tests

Samples 2(i), 3(i), 8(i), 10(i), T27, T28 and S3 were tested positive in the preliminary spot tests with at least one of the three alkaloid spotting reagents. *Eremophila latrobei* (2(i)) and *E. freelingii* (T27) registered a colour change with two of the three reagents. The strongest indication for the presence of alkaloids in these preliminary tests certainly came from the *Sarcostemma australe* (10(i)) which showed intense precipitation with all three reagents (Table 9).

When followed up by pH-guided fractionation spot tests, in those cases confirming the preliminary test results all but one sample registered a colour with one or more of the reagents (Table 10). It appears that most might contain several types of alkaloids, including tertiary bases and quaternary alkaloids. According to the results of the Vitali-Morin test, three of the screened samples appear to contain tropane-type alkaloids, a fairly common class of plant bases (Table 11).

Table 9: Preliminary spot tests

Sample*	Dragendorff's		Mayer's		Wagner's	
	Precipitate colour	Presence/ intensity	Precipitate colour	Presence/ intensity	Precipitate colour	Presence/ intensity
1 (i)	-		-		-	
2 (i)	Orange	+	-		Brown	++
3 (i)	-		-		Orange	+
5 (i)	-		-		-	
8 (i)	-		-		Orange	++
10 (i)	Orange	+++	Green/Cream	++	Brown	+++
11A (i)	-		-		-	
11B (i)	-		-		-	
12 (i)	-		-		-	
15 (i)	-		-		-	
T19	-		-		-	
T20	-		-		-	
T21	-		-		-	
T22	-		-		-	
T24A	-		-		-	
T24B	-		-		-	
T25	-		-		-	
T26	-		-		-	
T27	Orange/Brown	+	-		Orange	+
T28	Orange/Brown	++	-		-	
S1.1			-		-	
S1.2			-		-	
S3	Orange/Brown	++	-		-	
S7			-		-	

(Intensity range from + to +++)

*For the sample ID allocations, see Table 5.

Table 10: Spot test after basic extraction

Sample*	Dragendorff's		Mayer's		Wagner's	
	Aqueous phase	Organic phase	Aqueous phase	Organic phase	Aqueous phase	Organic phase
2 (i)	Orange (++)	Red/orange (++)	Yellow (+)	-	Brown (++)	Brown (+)
3 (i)	Orange (+)	Red/orange (++)	-	-	-	Brown (+)
8 (i)	Orange (++)	Red/orange (+)	-	-	Brown (+)	-
10 (i)	Orange (+)	Orange (+)	-	-	-	Brown (+)
T27	-	-	-	-	-	-
T28	Orange (+)	-	-	-	-	--
S3	Orange (++)	-	-	-	-	-

*For the sample ID allocations, see Table 5.

Table 11: Vitali-Morin test for tropane-type alkaloids

Sample*	Change	Colour
2 (i)	Yes	Dark brown
3 (i)	No	Orange
8 (i)	Yes	Dark brown
10 (i)	Yes	Dark brown
T27	No	Cream
T28	No	Orange
S3	No	Cream

*For the sample ID allocations, see Table 5.

2.3.3 Saponin screening tests

From the screening tests for saponins it appears that some of the samples analysed contain surfactant-type compounds as is indicated by a persistent froth on shaking with water. Particularly, samples 10(i) and 15(i) are characterised by a strong foaming activity (Table 12). When comparing the colours obtained in the preliminary spot tests with the original extract colour, a large number of samples also show a distinct intensification and/or change in colour (Table 13), both indications of the presence of steroids and triterpenes, which are common constituents of saponins.

2.3.4 Tannin screening test

Most samples except for samples 10(i), T19–24, T26 T28 and S3 produced UV-active spots when subjected to one- or two-dimensional thin-layer chromatography (TLC), which also turned blue when sprayed with $FeCl_3/K_3Fe(CN)_6$ reagent, indicating the presence of phenolic groups. In the case of samples 1(i) and 5(i), two distinctly different types of tannin-like compounds were identified whereas sample 11(i) appears to have two chemically related types of tannins, which showed similar behaviour on TLC. A similar conclusion can be drawn from the results obtained for sample 15(i), whereas samples 2(i), 3(i), 8(i) and 12(i) appear to have only one type of astringent compound, in all cases with a similar chemical behaviour.

Table 12: Froth volume (mL) assessed in froth test

Sample*	Time (minutes)					Froth type
	0	1	2	10	15	
1 (i)	trace					
2 (i)	trace					
3 (i)	2 ml	2 ml	1.5 ml	1 ml	1 ml	fine
5 (i)	trace					
8 (i)	1 ml	1 ml	1 ml	1 ml	0.5 ml	coarse
10 (i)	9 ml	1.5 ml	0.5 ml	0.25 ml	trace	fine
11A (i)	3 ml	2.5 ml	1 ml	1 ml	0.5 ml	fine/coarse
11B (i)	2 ml	2 ml	2 ml	1.5 ml	1.5 ml	fine/coarse
12 (i)	trace					
15 (i)	4 ml	1 ml	0.25 ml	trace		fine/coarse
T19	trace					
T20	trace					
T21	trace					
T22	trace					
T24A	trace					
T24B	trace					
T25	trace					
T26	trace					
T27	trace					

Sample*	Time (minutes)					Froth type
	0	1	2	10	15	
T28	trace					
S1.1	trace					
S1.2	trace					
S3	0.5 ml	0.5 ml	0.3 ml	-	-	coarse
S7	trace					

*For the sample ID allocations, see Table 5.

Table 13: Preliminary spot tests

Sample*	Colour with conc. H_2SO_4	Colour with $H_2SO_4/FeCl_3$	Liebermann-Burchard test	Initial colour
1 (i)	green	green	yellow	yellow/green
2 (i)	orange/green	green	brown/yellow	yellow/green
3 (i)	faint orange	faint green	dark brown	faint yellow
5 (i)	yellow	yellow	orange	faint yellow/green
8 (i)	yellow	yellow	yellow/orange	faint yellow
10 (i)	yellow/green	yellow/green	orange	yellow
11A (i)	bright yellow	yellow/green	yellow	faint yellow
11B (i)	bright yellow	yellow/green	yellow	light brown
12 (i)	faint orange	faint yellow	yellow/green	faint yellow
15 (i)	bright yellow	yellow	bright yellow	yellow
T19	yellowish		brown	pale brown
T20			light brown	greenish yellow
T21	yellowish		brown	pale brown
T22			light brown	pale reddish brown
T24A			brown	light cream
T24B			light brown	pale brown
T25		greenish brown	bluish green	light green
T26			greenish brown	light green
T27			greenish brown	greenish brown
T28			greenish brown	orange
S1.1	fluorescent green	greenish brown	greenish brown	pale greenish brown
S1.2	fluorescent green	greenish brown	greenish brown	pale greenish brown
S3	fluorescent green	greenish brown	violet	pale greenish brown
S7	fluorescent green	greenish brown	greenish brown	pale greenish brown

*For the sample ID allocations, see Table 5.

2.3.5 Screening tests for toxic activity

Observed results

Artemia salina

The data on Artemia larvae mortality after 24 hours of exposure to 10, 100 and 1000 µg/mL extract concentrations are given in Table 14. Due to low solubility in water, some extract components formed amorphous or particulate precipitate, which settled down on the bottom or was suspended or floating on the surface. No or little mortality was observed at the highest concentration of extracts 2(i), 3(i), *5(i), 10(i), 11A(i), 11B(i), T25, T26, T28 and S3. The complete Artemia mortality at the highest concentration was observed in extracts 1(i), 5(i), 8(i), 12(i), 15(i), T19 through to T24B, and T27. The same extracts caused zero or negligible mortality at the concentration of 100 µg/mL. Therefore, the more accurate toxicities of these extracts were determined in bioassays with five concentrations

in the range of 100–1000 µg/mL (Table 14). Sample 24B caused 100% mortality at concentration as low as 316 µg/ml, while three samples from South Australia caused 100% mortality at concentration 562 µg/mL.

Table 14: Mortality of Artemia *larvae (%) exposed to plant extract concentrations for 24 hours*

Sample	Extract concentration (µg/ml)								
	10	38	56	100	178	316	562	800	1000
Control	0	0	0	0	0	0	0	nt	0
1(i)	0	nt	nt	0	0	0	96.7	nt	100
2(i)	0	nt	nt	0	nt	nt	nt	nt	3.3
3(i)	0	nt	nt	0	nt	nt	nt	nt	0
5(i)	0	nt	nt	0	0	6.7	83.3	nt	100
*5(i)	0	nt	nt	0	nt	nt	nt	nt	6.7
8(i)	0	nt	nt	3.3	0	40	100	nt	100
10(i)	0	nt	nt	0	nt	nt	nt	nt	0
11A(i)	0	nt	nt	0	nt	nt	nt	nt	0
11B(i)	0	nt	nt	0	nt	nt	nt	nt	0
12(i)	0	nt	nt	0	0	0	0	nt	100
15(i)	3.3	nt	nt	0-3.3	0	0	96.7	nt	100
T19	nt	nt	nt	0	20	83.3	93.3	nt	100
T20	nt	nt	nt	10	13.3	10	13.3	nt	100
T21	nt	nt	nt	6.7	20	83.3	96.7	nt	100
T22	nt	nt	nt	0	0	83.3	100	nt	100
T24A	nt	nt	nt	0	0	3.3	26.7	nt	100
T24B	nt	nt	nt	3.3	86.7	100	100	nt	100
T25	6.7	nt	nt	0	nt	nt	nt	nt	33.3
T26	nt	nt	nt	6.7	10	3.3	0	nt	26.7
T27	nt	nt	nt	3.3	20	93.3	100	nt	100
T28	0	nt	nt	0	nt	nt	nt	nt	6.7
S1.1	nt	nt	0	0	10	86.7	100	nt	nt
S1.2	nt	nt	0	0	23.3	96.7	100	nt	nt
S3	nt	nt	0	0	3.33	3.33	10	0	3.33
S7	nt	0	0	0	13.3	93.3	100	nt	nt

For the sample ID allocations, see Table 5.

Two values in one cell represent results of two independent tests.

nt – not tested

Daphnia magna

All plant extracts except for 12(i) were tested for toxicity to *D. magna* at three concentrations: 10, 100 and 1000 µg/mL (Table 15). *Daphnia* immobility developed in 24 hours in half or less of the tested animals in the highest concentration of extracts 3(i), *5(i), 10(i), 11A(i), 11B(i) and T25. Of these extracts, further immobility development up to 100% at that concentration was observed only in the extract 3(i). The highest concentration caused complete *Daphnia* immobility in extracts 2(i), 5(i), 8(i), 15(i), T19-T22, T24B and T26 through to T28 after the 24-hour exposure, and in extracts 1(i), 3(i) and T24A after the 48-hour exposure.

No partial immobilisation of *Daphnia* was observed after 48-hour exposure in solutions of extracts 1(i), 2(i) and 8(i). More accurate toxicities of these extracts were determined in definitive tests using five concentrations in the range of 100–1000 µg/mL (Table 15).

Table 15: Daphnia *immobilisation in plant-extract solutions in range-finding and definitive tests*

Sample	24-hour immobilisation, %							48-hour immobilisation, %						
	0 µg/mL (control)	10 µg/ml	100 µg/ml	178 µg/ml	316 µg/ml	562 µg/ml	1000 µg/ml	0 µg/mL (control)	10 µg/ml	100 µg/ml	178 µg/ml	316 µg/ml	562 µg/ml	1000 µg/ml
1(i)	0	0	0	5	5	15	75-80	0	0	0	5	10	25	100
2(i)	0	0	0	10	30	75	100	0	0	0-15	30	85	100	100
3(i)	0	0	20	nt	nt	nt	20	0	0	85	nt	nt	nt	100
5(i)	0	0	20	nt	nt	nt	100	0	0	45	nt	nt	nt	100
*5(i)	0	0	0	nt	nt	nt	50	0	0	0	nt	nt	nt	95
8(i)	0	0	0-5	75	100	100	100	0	0	0-55	100	100	100	100
10(i)	0	0	0	nt	nt	nt	0	0	0	5	nt	nt	nt	25
11A(i)	0	0	0	nt	nt	nt	15	0	0	0	nt	nt	nt	30
11B(i)	0	0	0	nt	nt	nt	15	0	0	0	nt	nt	nt	25
12(i)	nt	nt	nt	nt	nt	nt	nt	nt	nt	nt	nt	nt	nt	nt
15(i)	0	15	65	nt	nt	nt	100	0	35	95	nt	nt	nt	100
T19	0	nt	100	100	100	100	100	0	nt	100	100	100	100	100
T20	0	nt	90	95	100	100	100	0	nt	95	100	100	100	100
T21	0	nt	95	100	100	100	100	0	nt	100	100	100	100	100
T22	0	nt	0	5	5	60	100	0	nt	0	5	15	95	100
T24A	0	nt	nt	nt	0	0	95	0	nt	nt	nt	0	35	100
T24B	0	nt	0	0	5	90	100	0	nt	0	0	5	100	100
T25	0	0	5	nt	nt	nt	10	0	5	5	nt	nt	nt	85
T26	0	10	70	nt	nt	nt	100	0	95	100	nt	nt	nt	100
T27	0	nt	50	100	100	100	100	0	nt	100	100	100	100	100
T28	0	0	0	nt	nt	nt	100	0	0	5	nt	nt	nt	100

For the sample ID allocations, see Table 5.

Two values in one cell represent results of two independent tests.

nt – not tested

Samples T19, T20, T21, T26 and T27, which showed high toxicity at 100 µg/ml, were further tested in the lower concentration range (1–81 µg/ml) (Table 16).

Table 16: Daphnia *immobilisation in plant-extract solutions in definitive tests*

Sample	24-hour immobilisation, %						48-hour immobilisation, %					
	0 µg/mL (control)	1 µg/ml	3 µg/ml	9 µg/ml	27 µg/ml	81 µg/ml	0 µg/mL (control)	1 µg/ml	3 µg/ml	9 µg/ml	27 µg/ml	81 µg/ml
T19	0	0	0	15	35	55	0	0	5	15	60	95
T20	0	10	25	70	70	60	0	30	70	80	90	95
T21	0	0	0	30	5	75	0	0	0	35	15	100
T26	0	0	10	30	60	85	0	5	30	75	90	100
T27	0	0	0	0	0	10	0	0	0	0	5	35

For the sample ID allocations, see Table 5.

The data on three *Eremophila alternifolia* samples (S1.1, S1.2 and S7) and one *Scaevola spinescens* sample (S3) are summarised in Table 17.

Table 17: Daphnia *immobilisation (%) in plant-extract solutions in range-finding and definitive tests*

Sample	Exposure, h	Concentration, µg/ml							
		0	10	24	64	100	160	400	1000
S1.1	24	0	0	0	0	80	85	100	100
S1.2	24	0	0	0	0	nt	95	100	nt
S3	24	0	0	nt	nt	5	nt	nt	30
S7	24	0	0	0	5	nt	95	100	nt
S1.1	48	0	0	0	0	80	100	100	100
S1.2	48	0	0	0	20	nt	100	100	nt
S3	48	0	0	nt	nt	20	nt	nt	95
S7	48	0	0	5	15	nt	100	100	nt

For the sample ID allocations, see Table 5.

nt – not tested

Calculated LC50 values

The LC50 values derived by the Spearman-Karber method (Hamilton et al. 1977) for *Artemia* and *Daphnia* bioassays are given in Table 18.

Table 18: Plant extracts LC50 (95% confidence interval) values (µg/ml) calculated using data of three-concentration screening test or five-concentration definitive test (*)

Sample	Plant species Plant parts used for extraction	24-hour *Artemia* bioassay	24-hour *Daphnia* bioassay	48-hour *Daphnia* bioassay
1(i)	*Eremophila longifolia* Leaves, stem	426 (417–434)*	786 (693–892)*	595 (514–690)*
2(i)	*Eremophila latrobei* Leaves, stem, flowers	>1000 (NC)	387 (323–463)*	210 (173–254)*
5(i)	*Eremophila sturtii* Leaves, stem, flowers	441 (404–480)*	225 (126–399)	115 (60–219)
*5(i)	*Eremophila sturtii* Filter-paper residue	>1000 (NC)	1000 (NC)	336 (297–380)
8(i)	*Eremophila freelingii* Leaves, stem, flowers	339 (297–386)*	148 (130–169)*	65 (43–98)*
10(i)	*Sarcostemma australe* Stem	>1000 (NC)	>1000 (NC)	>1000 (NC)
11A(i)	*Acacia tetragonophylla* Leaves, stem	>1000 (NC)	>1000 (NC)	>1000 (NC)
11B(i)	*Acacia tetragonophylla* Leaves, stem, flowers	>1000 (NC)	>1000 (NC)	>1000 (NC)
12(i)	*Hakea divaricata* Bark	750 (NC)*	nd	nd
15(i)	*Euphorbia drummondii* Leaves, stem, flowers, fruits	426 (417–434)*	53 (27–106)	18 (10–33)
T19	*Eremophila duttonii* Leaves, stem	246 (214–281)	38 (26–54)	19 (14–26)
T20	*Euphorbia tannensis* Stem (leaves)	692 (594–804)	10 (5–16)	1.8 (0.6–3.4)
T21	*Eremophila duttonii* Leaves, stem	227 (196–261)	34 (21–51)	26 (15–53)
T22	*Hakea* sp. Bark	261 (241–282)	501 (432–581)	387 (342–436)

Sample	Plant species Plant parts used for extraction	24-hour *Artemia* bioassay	24-hour *Daphnia* bioassay	48-hour *Daphnia* bioassay
T24A	*Hakea divaricata* Inner bark	631 (570–697)	761 (NC)	613 (542–693)
T24B	*Hakea divaricata* Outer bark	143 (129–157)	434 (394–477)	409 (387–433)
T25	*Codonocarpus cotinifolius* Stem, leaves	>1000	>1000	365 (274–486)
T26	*Euphorbia tannensis* Stem (leaves)	>1000	21 (14–32)	5.2 (3.5–7.6)
T27	*Eremophila freelingii* Leaves, stem	212 (188–239)	103 (91–116)	70 (40–93)
T28	*Acacia tetragonophylla* Root bark	>1000	316 (NC)	282 (225–353)
S1.1	*Eremophila alternifolia* Stem, leaves	242 (220–266)	116 (100–134)	101 (64–160)
S1.2	*Eremophila alternifolia* Stem, leaves	211 (191–233)	106 (97–116)	83.7 (70.6–99.2)
S3	*Scaevola spinescens* Stem, leaves	>1000	>1000	226 (139–368)
S7	*Eremophila alternifolia* Stem, leaves	228 (209–249)	101 (89–115)	83.8 (70.2–100)

2.3.6 Antimicrobial activity

Results of antimicrobial screening using agar diffusion assays are shown in Table 19. Most of the *Eremophila* species tested, with the exception of *Eremophila longifolia* and *Eremophila latrobei*, showed some activity against at least two different strains of Gram-positive bacteria. The two extracts of *Eremophila duttonii* (T19 and T21) were the most active against the four Gram-positive organisms. *Acacia tetragonophylla* (11Ai) and *Euphorbia drummondii* (15i) also showed activity against the two *Staphylococcus aureus* strains.

None of the plant extracts tested was active against the Gram-negative organisms. *Euphorbia drummondii* was the only extract that was active against the yeasts *Candida parapsilosis* and *C. albicans*.

The MIC and MBC results for the extracts against standard strains of *Staphylococcus* and *Streptococcus* species are shown in Tables 20 and 21 respectively. In agreement with the results obtained with the agar well diffusion assay, the two extracts of *Eremophila duttonii* had the lowest MICs against the Gram-positive organisms. These extracts were also found to have bactericidal effects against these organisms at concentrations of twice the MIC. Other *Eremophila* extracts tested also had MIC values below 1 mg/mL. The extract of *Euphorbia drummondii* had MICs against the *Staphylococcus* species of 0.25–0.5 mg/mL; however, MICs of this extract against *Streptococcus* species were higher (1 mg/mL).

The extracts of *Eremophila duttonii* also showed good activity against all clinical MRSA isolates tested, with most of the isolates having MICs in the range 0.0625–0.25 mg/mL. Table 22 shows a summary of the MIC ranges for the different plant extracts against the clinical MRSA isolates. The full details of MIC values for each extract tested with each clinical isolate are given in Appendix 1 of this report.

Table 19: Antimicrobial activity of plant extracts determined by agar diffusion assays

		Microorganisms tested								
Sample	**Plant species**	*Staphylococcus aureus* **ATCC-25923**	*Staphylococcus aureus* **ATCC-29213**	*Streptococcus pyogenes* **ATCC-10389**	*Streptococcus pneumoniae* **ATCC-49619**	*E. coli* **ATCC-25922**	*Salmonella typhimurium* **ATCC-13311**	*Pseudomonas aeruginosa* **ATCC-27853**	*Candida albicans* **ATCC-90028**	*Candida parapsilosis* **ATCC-90018**
1(i)	*Eremophila longifolia*	-	-	-	-	-	-	-	-	-
2(i)	*Eremophila latrobei*	-	-	-	-	-	-	-	-	-
5(i)	*Eremophila sturtii*	12	11	10	10	-	-	-	-	-
*5(i)	*Eremophila sturtii* Filter-paper residue	10	10	-	-	-	-	-	-	-
8(i)	*Eremophila freelingii*	13	13	12	13	-	-	-	-	-
10(i)	*Sarcostemma australe*	-	-	-	-	-	-	-	-	-
11A(i)	*Acacia tetragonophylla*	11	11	-	-	-	-	-		-
11B(i)	*Acacia tetragonophylla*	-	-	-	-	-	-	-	-	-
12(i)	*Hakea divaricata*	-	-	-	-	-	-	-	-	-
15(i)	*Euphorbia drummondii*	18	16	-	-	-	-	-	13	13
T19	*Eremophila duttonii*	18	16	12	12	-	-	-	-	-
T20	*Euphorbia tannensis*	-	-	-	-	-	-	-	-	-
T21	*Eremophila duttonii*	18	16	13	12	-	-	-	-	-
T22	*Hakea* sp.	-	-	-	-	-	-	-	-	-
T24A	*Hakea divaricata*	-	-	-	-	-	-	-	-	-
T24B	*Hakea divaricata*	-	-	-	-	-	-	-	-	-
T25	*Codonocarpus cotinifolius*	-	-	-	-	-	-	-	-	-
T26	*Euphorbia tannensis*	-	-	-	-	-	-	-	-	-
T27	*Eremophila freelingii*	14	12	13	12	-	-	-	-	-
T28	*Acacia tetragonophylla*	-	-	-	-	-	-	-	-	-
S1.1	*Eremophila alternifolia*	10	10	9	9	-	-	-	-	-
S3	*Scaevola spinescens*	-	-	-	-	-	-	-	-	-
S7	*Eremophila alternifolia*	10	10-	9-	9-	-	-	-	-	-

Numbers indicate the diameters (in mm) of the zones of growth inhibition around each well. Numbers are the average of triplicate experiments. The negative symbol (–) indicates no activity. The concentration of each extract per well or disk was 1 mg/mL.

Table 20: MIC and MBC values (mg/mL) for active extracts against Staphylococcus *species*

	S. aureus **ATCC 25923**		*S. aureus* **ATCC 29213**	
Sample/Plant species	**MIC**	**MBC**	**MIC**	**MBC**
5(i) *Eremophila sturtii*	0.5	1	1	2
8(i) *Eremophila freelingii*	0.5	2	1	2
11A(i) *Acacia tetragonophylla*	>2	ND	>2	ND
15(i) *Euphorbia drummondii*	0.25	2	0.5	2
T19 *Eremophila duttonii*	0.03125	0.0625	0.0625	0.125
T21 *Eremophila duttonii*	0.03125	0.0625	0.0625	0.125
S1 *Eremophila alternifolia*	0.25	1	0.25	1
S7 *Eremophila alternifolia*	0.125	0.5	0.25	1

ND – not determined

Table 21: MIC and MBC values (mg/mL) for active extracts against Streptococcus *species*

	S. pneumoniae **ATCC 49619**		*S. pyogenes* **ATCC 10389**	
Sample/Plant species	**MIC**	**MBC**	**MIC**	**MBC**
5(i) *Eremophila sturtii*	0.25	0.5	0.25	0.25
8(i) *Eremophila freelingii*	0.25	0.5	0.25	0.25
11A(i) *Acacia tetragonophylla*	>2	ND	>2	ND
15(i) *Euphorbia drummondii*	1	2	1	2
T19 *Eremophila duttonii*	0.0625	0.125	0.0625	0.125
T21 *Eremophila duttonii*	0.0625	0.125	0.0625	0.125
S1 *Eremophila alternifolia*	0.25	0.5	0.25	0.5
S7 *Eremophila alternifolia*	0.25	0.5	0.25	0.5

ND – not determined

Table 22: Summary of MIC results (mg/mL) of active extracts against standard Staphylococcus *species and clinical isolates of MRSA determined by the agar dilution assay*

Sample/Plant species	*S. aureus* **ATCC 25923 MIC**	*S. aureus* **ATCC 29213 MIC**	**68 Clinical MRSA MIC range***
5(i) *Eremophila sturtii*	0.25	0.5	0.125–2
8(i) *Eremophila freelingii*	0.25	0.5	0.125–2
11A(i) *Acacia tetragonophylla*	2	2	1–2
15(i) *Euphorbia drummondii*	0.25	0.5	0.125–0.5
T19 *Eremophila duttonii*	0.125	0.125	0.0625–0.5
T21 *Eremophila duttonii*	0.125	0.125	0.0625–0.5
T27 *Eremophila freelingii*	0.25	0.5	0.125–2
Ampicillin (positive control)	0.00025	0.0005	0.00025–0.064

*MIC range for different clinical isolates of MRSA

2.3.7 Antiviral activity

Testing of plant extracts for activity against herpes simplex virus type 1

Results of the screening of extracts for antiviral activity against HSV-1 are shown in Table 23. All extracts were toxic to cells at the maximum concentration tested of 1 mg/mL. Most extracts required dilution to a concentration of at least approximately 16–60 mg/mL to be non-toxic to Vero cells. One extract (from *Euphorbia drummondii*) required dilution to a concentration of approximately 1 mg/mL. None of the extracts tested produced significant inhibition of HSV cytopathic effect at non-cytotoxic concentrations. The two extracts of *Eremophila alternifolia* (S1.1 and S7) showed a small amount of inhibition (less than 25% inhibition of cytopathic effect) at the maximum non-cytotoxic concentration.

At the maximum concentration of DMSO used in the assay (1% v/v), there was no inhibition of viral cytopathic effect by DMSO. Concentrations of 0.5% v/v DMSO and higher produced some inhibition of Vero cell growth; however, the concentrations of DMSO equivalent to those at or below the MNTD of all the extracts did not affect cell growth.

Testing of plant extracts for antiviral activity against human rhinovirus 14

Results of the screening of extracts for antiviral activity against HRV-14 are shown in Table 24. All extracts were toxic to cells at the maximum concentration tested of 1 mg/mL. Most extracts required dilution to a concentration of approximately 16–60 µg/mL to be non-toxic to H1-HeLa cells. The extract of *Euphorbia drummondii* required dilution to a concentration of approximately 1 µg/mL, and the extract of *E. tannensis* required a dilution to a concentration of approximately 0.5 µg/mL.

The extract of *Codonocarpus cotinifolius* produced modest inhibition (25–50% by visual inspection) of HRV cytopathic effect at non-cytotoxic concentrations. The two extracts of *Eremophila alternifolia* (S1.1 and S7) and one extract of *Eremophila duttonii* (T19) showed a small amount of inhibition of cytopathic effect at the maximum non-cytotoxic concentration. Extracts of *Hakea divaricata* (12(i), T22, T24A and T24B) produced some inhibition of HRV cytopathic effect but only at concentrations above the maximum non-cytotoxic concentrations.

At the maximum concentration of DMSO used in the assay (1% v/v), there was no inhibition of viral cytopathic effect or H1-HeLa cell growth by DMSO.

Table 23: Antiviral screening of plant extracts against herpes simplex virus type 1 grown in Vero cells

Sample	Plant species	MNTD* for Vero cells (µg/mL)	Antiviral activity against HSV1 SC16 **
1 (i)	*Eremophila longifolia*	31.3	-
2 (i)	*Eremophila latrobei*	64.1	-
5 (i)	*Eremophila sturtii*	30.5	-
8 (i)	*Eremophila freelingii*	32.0	-
10 (i)	*Sarcostemma australe*	16.0	-
12 (i)	*Hakea divaricata*	15.6	-
15 (i)	*Euphorbia drummondii*	0.98	-
11A (i)	*Acacia tetragonophylla*	60.9	-
11B (i)	*Acacia tetragonophylla*	32.0	-
T19	*Eremophila duttonii*	15.6	-
T20	*Euphorbia tannensis*	7.8	-
T21	*Eremophila duttonii*	15.6	-
T22	*Hakea* sp.	15.6	-
T24A	*Hakea divaricata*	31.3	-
T24B	*Hakea divaricata*	15.6	-
T25	*Codonocarpus cotinifolius*	125	-
T26	*Euphorbia tannensis*	7.8	-

Sample	Plant species	MNTD* for Vero cells (µg/mL)	Antiviral activity against HSV1 SC16 **
T27	*Eremophila freelingii*	62.5	-
T28	*Acacia tetragonophylla*	15.6	-
S1.1	*Eremophila alternifolia*	31.3	-/+
S7	*Eremophila alternifolia*	31.3	-/+
S3	*Scaevola spinescens*	31.3	-
	Acyclovir (positive control)		above 0.17 µg/mL

* MNTD – maximum non-toxic dose to cells

** Antiviral activity assessed as inhibition of viral cytopathic effect (CPE) compared to untreated controls which showed complete destruction of the cell monolayer: - = no antiviral activity at MNTD; + = approx. 25% inhibition of viral CPE at MNTD; ++ = approx. 50% inhibition of viral CPE at MNTD; +++ = approx. 75% inhibition of viral CPE at MNTD; ++++ = greater than 75% inhibition of viral CPE at MNTD

Table 24: Antiviral screening of plant extracts against human rhinovirus type 14 grown in H1-HeLa cells

Sample	Plant species	MNTD* for H1-HeLa cells (µg/mL)	Antiviral activity against HRV 14 (strain 1059)**
1 (i)	*Eremophila longifolia*	30.5	-
2 (i)	*Eremophila latrobei*	128	-
5 (i)	*Eremophila sturtii*	15.2	-
8 (i)	*Eremophila freelingii*	30.4	-
10 (i)	*Sarcostemma australe*	60.9	-
11A (i)	*Acacia tetragonophylla*	32.0–64.0	-
11B (i)	*Acacia tetragonophylla*	15.6	-
12 (i)	*Hakea divaricata*	30.4	-
15 (i)	*Euphorbia drummondii*	0.98	-
T19	*Eremophila duttonii*	15.6	-/+
T20	*Euphorbia tannensis*	0.49	-
T21	*Eremophila duttonii*	7.8	-
T22	*Hakea* sp.	15.6	-
T24A	*Hakea divaricata*	15.6	-
T24B	*Hakea divaricata*	15.6	-
T25	*Codonocarpus cotinifolius*	125	+/++
T26	*Euphorbia tannensis*	0.49	-
T27	*Eremophila freelingii*	31.3	-
T28	*Acacia tetragonophylla*	125	-
S1.1	*Eremophila alternifolia*	31.3	-/+
S3	*Scaevola spinescens*	31.3	-
S7	*Eremophila alternifolia*	31.3	+
Guanidine HCl (positive control)		1.25 mM	1.25 mM

* MNTD – maximum non-toxic dose to cells

** Antiviral activity assessed as inhibition of viral cytopathic effect (CPE) compared to untreated controls which showed complete destruction of the cell monolayer: - = no antiviral activity at MNTD; + = approx. 25% inhibition of viral CPE at MNTD; ++ = approx. 50% inhibition of viral CPE at MNTD; +++ = approx. 75% inhibition of viral CPE at MNTD; ++++ = greater than 75% inhibition of viral CPE at MNTD

Testing of plant extracts for antiviral activity against coxsackievirus A21

Results of the screening of extracts for antiviral activity against CVA21 are shown in Table 25. All extracts were toxic to cells at the maximum concentration tested of 1 mg/mL. Most extracts required dilution to a concentration of approximately 15–60 mg/mL to be non-toxic to H1-HeLa cells. One extract (from *Euphorbia drummondii*) required dilution to a concentration of approximately 0.5 mg/mL. The extracts of *Euphorbia tannensis* required dilution to approximately 1 mg/mL.

Only the extract of *Codonocarpus cotinifolius* produced a small amount of inhibition of CVA21 CPE at non-cytotoxic concentrations. The extract of *Hakea divaricata* did not produce any inhibition of cytopathic effect with this virus, even at concentrations above the maximum non-cytotoxic concentration.

At the maximum concentration of DMSO used in the assay (1% v/v), there was no inhibition of H1-HeLa cell growth by DMSO. Concentrations of 0.5% v/v DMSO and higher produced some inhibition of coxsackievirus cytopathic effect; however, the concentrations of DMSO equivalent to those at or below the MNTD of all the extracts did not interfere with viral cytopathic effect.

Table 25: Antiviral screening of plant extracts against Coxsackievirus A21 grown in H1-HeLa cells

Sample	Plant species	MNTD* for H1-HeLa cells (µg/mL)	Antiviral activity against coxsackievirus A21**
1 (i)	*Eremophila longifolia*	62.5	-
2 (i)	*Eremophila latrobei*	64.1	-
5 (i)	*Eremophila sturtii*	65.6	-
8 (i)	*Eremophila freelingii*	32.0	-
10 (i)	*Sarcostemma australe*	31.3	-
11A (i)	*Acacia tetragonophylla*	62.5	-
11B (i)	*Acacia tetragonophylla*	62.5	-
12 (i)	*Hakea divaricata*	16.4	-
15 (i)	*Euphorbia drummondii*	0.49	-
T19	*Eremophila duttonii*	15.6	-
T20	*Euphorbia tannensis*	0.98	-
T21	*Eremophila duttonii*	7.8	-
T22	*Hakea* sp.	15.6	-
T24A	*Hakea divaricata*	15.6	-
T24B	*Hakea divaricata*	15.6	-
T25	*Codonocarpus cotinifolius*	62.5	-/+
T26	*Euphorbia tannensis*	0.98	-
T27	*Eremophila freelingii*	62.5	-
T28	*Acacia tetragonophylla*	250	-
S1.1	*Eremophila alternifolia*	31.3	-
S3	*Scaevola spinescens*	62.5	-
S7	*Eremophila alternifolia*	31.3	-
Guanidine HCl (positive control)		1.25 mM	Above 39 µM

* MNTD – maximum non-toxic dose to cells

** Antiviral activity assessed as inhibition of viral cytopathic effect (CPE) compared to untreated controls which showed complete destruction of the cell monolayer: - = no antiviral activity at MNTD; + = approx. 25% inhibition of viral CPE at MNTD

2.4 Discussion

2.4.1 Extraction of plant material and essential oils analysis

In the first batch of samples (1(i) – 15(i)), fresh plant material was hydrodistilled as soon as possible after collection in an attempt to screen for the presence of essential oil and obtain a preliminary profile of volatiles present where applicable. In all but the case of sample 10(i), where a reasonably large quantity of plant material was available, the absence of any detectable amounts of volatile compounds might have been due to the limited quantity of fresh plant material being available for essential oil extraction.

According to the Conservation Commission of the Northern Territory (Barr et al. 1993, p. 233), *Eremophila longifolia* contains a very small quantity of essential oil (0.025%) with α- and β-pinene as well as limonene as the major constituents. No evidence for those particular compounds was found here despite a reasonable amount of fresh material being available for hydrodistillation. This discrepancy might warrant further investigation as it could be the result of seasonal influence or a procedural effect. A similar situation is found for sample 2(i), where the Conservation Commission of the Northern Territory (Barr et al. 1993, p. 227) reports 0.06% of essential oil with α-pinene and guaiol as main constituents. In this case, however, these noticeable differences to findings of this study could be caused by an insufficient amount of fresh plant material being available for extraction of adequate amounts of essential oil for GC/MS analysis. Results for sample 5(i) are very similar to the Conservation Commission of the Northern Territory (Barr et al. 1993, p. 239) where a relatively high amount of essential oil was reported (0.5%) and γ-elemene and β-eudesmol identified as the two major constituents. Findings of 2-methoxy-4-(2-propenyl) phenol, elemol and β-eudesmol in sample 15(i) could not be correlated to the literature as no data were found on the essential oil composition of *Euphorbia drummondii*.

From the preliminary GC/MS analysis, it can be concluded that particularly volatile compounds with a low molecular weight might have been lost during transport and storage in this study. This might account for the noticed discrepancies between findings of this study, where often only large molecular weight compounds with limited volatility and unsatisfactory identification matches were detected, and the literature, which reports mainly 'typical' small molecular weight volatiles.

As a result from the above findings it was concluded that essential oil screening with the amounts available for this project and its inherent transport challenges might not be feasible and the subsequent samples collected were therefore dried and Soxhlet extracted with methanol only.

2.4.2 Alkaloid screening tests

Comparing findings on alkaloids present in the plant extracts with information available in the literature, it is interesting to note that the two *Eremophila* species (*E. latrobei* 2(i) and *E. freelingii* (8(i) and T27) returned negative alkaloid tests in an earlier study of these plants' leaves (Barr et al. 1993, pp. 220, 226). It might therefore be useful to follow up on these results with a more detailed investigation of the plants' various base fractions. The presence of alkaloids detected in this study might be caused by alkaloids being present in the plants' stems and/or flowers as the entire aerial parts of the two species were collected, extracted and analysed in this study, not only the leaves. In line with the Conservation Commission of the Northern Territory (Barr et al. 1993, p. 44) the root bark of *Acacia tetragonophylla* (T28) reacted positive in those preliminary alkaloid spotting tests and it can therefore be argued that the earlier tested sample (11A and B(i)), which contained a range of morphological plant parts, most likely did not respond as a result of low alkaloid concentration. No literature information was available on the presence of alkaloidal compounds in the other two plant samples that tested positive in this screening: *Sarcostemma australe* (10(i)) and *Scaevola spinescens* (S3).

2.4.3 Saponin screening tests

The strong frothing observed with *Sarcostemma australe* is in line with the Conservation Commission of the Northern Territory (Barr et al. 1993, p. 522), although a blue colour was recorded for the Liebermann-Burchard spot test whereas in this study an orange colour was observed, which might, however, reflect a colour change to red in an originally yellow solution. Leaves of *Eremophila freelingii* and aerial parts of *Euphorbia drummondii* are known to contain steroid or triterpene-type compounds (as indicated by a green colour development in the Liebermann-Burchard test) but no surfactant-type compounds (Barr et al. 1993, pp. 220, 282). In this study, a small amount of froth was observed for sample 8(i) and a much stronger reaction was obtained with sample 15(i) indicating the presence of saponins. In the former case, these compounds might again be present in the plant's stem and/or flowers,

which were included in this study's screening tests. In the case of sample 15(i), the presence of saponins might be associated with the plant's flowers and fruits. No screening results for saponins using the froth method were found in the literature for *Acacia tetragonophylla*; however, the presence of steroids and triterpenes as indicated by a positive Liebermann-Burchard test (particularly in the roots but to a smaller extent also in the phyllodes) was noted (Aboriginal Communities of the Northern Territory of Australia 1988, p. 249). In this study, only the aerial parts collected returned positive froth test results (11A and B (i)) as well as colour change in the various spot tests.

2.4.4 Tannin screening test

According to the literature, all screened *Eremophila* species (except *E. exiliflorus*, for which no data were found) contain 2-4% of tannic acid in their leaves (Barr et al. 1993, pp. 220, 227, 232) and *Euphorbia drummondii* (Barr et al. 1993, p. 282) is reported to contain 2% of tannic acid in its aerial parts. No information was available for all other screened species.

It appears that in those instances where information was available in the literature the findings of this study were in agreement, thus validating, to an extent, this preliminary tannin screening program. It might, however, be valuable, particularly for those cases where no previous data was found, to extend the screening program to include semi-quantitative analyses.

2.4.5 Toxicity tests

Although the brine shrimp bioassay is often used for preliminary testing of plant extracts for bioactivity, there is no standard toxicity criteria associated with significant potential bioactivity. Meyer et al. (1982) considered significant toxicity at LC50 values below 1000 µg/mL. Sam (1993) used LC50 values of 450 µg/mL and below as indicators for samples warranting further evaluation and fractionation of the crude extracts. Out of eleven plant extracts tested with the Artemia bioassay in the present study, 15 extracts displayed toxicity with LC50<1000 µg/mL, including 12 extracts with LC50<450 µg/mL.

Those plant extracts (excluding 12(i)) which displayed toxicity to *Artemia* were more toxic to *Daphnia* with lower 24-hour and 48-hour LC50 values. The plant extracts 2(i), 3(i) and T26 were not toxic to *Artemia*, but were toxic to *Daphnia* after 24-hour and/or 48-hour exposure respectively. Plant extracts 10(i), 11A(i) and 11B(i) displayed no significant toxicity either to *Artemia* or *Daphnia*.

Some test solutions displayed instability resulting in various levels of precipitation during the course of bioassays. The differences in test solution appearances in the *Artemia* and *Daphnia* bioassays can be attributed to different salinities of dilution water, temperatures and durations of incubation.

To analyse the relationship between toxicity indices of plant extracts to *Artemia* and *Daphnia*, the LC50 values were ranked and analysed by the non-parametric Spearman rho-correlation statistic. The significant correlation was confirmed for the *Artemia* and *Daphnia* 24-hour LC50 values. There was no significant correlation between the *Artemia* 24-hour LC50 and *Daphnia* 48-hour LC50 values (apparently due to small N), although the *Daphnia* 24-hour and 48-hour LC50 values correlated significantly (Table 26).

Table 26: Correlation matrix for LC50 values of plant extracts (N=24) tested on Artemia *and* Daphnia

Parameters	Artemia 24-hour LC50	Daphnia 24-hour LC50	Daphnia 48-hour LC50
Artemia 24-hour LC50			
Correlation coefficient	1.00		
Significance	0		
Daphnia 24-hour LC50			
Correlation coefficient	0.56	1.00	
Significance	0.005	0	
Daphnia 48-hour LC50			
Correlation coefficient	0.29	0.81	1.00
Significance	0.175	0.000	0

Lellau and Liebezeit (2003) characterised 28 plant extracts by toxicity to *Artemia* larvae and *Daphnia* adults in 24-hour and 48-hour bioassays, respectively, and by inhibition of tumour initiation (ITI) and growth (ITG) in a potato disk assay. We analysed their data using Spearman rho-statistics for correlation after ranking. The plant extract toxicities to *Artemia* and *Daphnia* had the correlation coefficient of 0.73 and correlated with ITI at the coefficients of 0.84 and 0.69, respectively, and with ITG at the coefficients of 0.67 and 0.42, respectively. All correlation coefficients were highly significant ($p<0.0001$). The LC50 values for *Daphnia magna* and *Artemia* were demonstrated to correlate for 36 hydrocarbons (Abernethy et al. 1986). The correlation of toxicities of various plant extracts or hydrocarbons to *Artemia* and *Daphnia* indicates similarity in acute responses of these two planktonic crustaceans (freshwater and saltwater) to organic compounds. The combination of *Artemia* and *Daphnia* bioassays enhances reliability of conclusions about toxicity potentials of tested substances.

Significant toxicity of extracts of *Hakea divaricata*, *Euphorbia drummondii* and four species of *Eremophila* to *Artemia* and *Daphnia* was demonstrated in the present study. These results indicate potent biological activity of these plants and warrant their further examination.

2.4.6 Antimicrobial activity

Extracts of *Eremophila* species – in particular, two extracts of *Eremophila duttonii* – were found to be the most active extracts against Gram-positive organisms in this study. None of the *Eremophila* extracts exhibited antibacterial activity against the Gram-negative bacteria or antifungal activity against the yeast species tested. These findings are in agreement with previous findings that some members of the genus *Eremophila* with medicinal uses exhibit selective antibacterial activity against Gram-positive organisms, with *Eremophila duttonii* showing the strongest activity of the medicinal *Eremophila* species previously tested (Palombo & Semple 2001, Pennacchio et al. 2005). A recently published report has suggested that the antibacterial activity of an ethanolic extract of *E. duttonii* is due to effects on the cytoplasmic membrane of *Staphylococcus aureus* which may lead to increased membrane permeability in the presence of the extract (Tomlinson & Palombo 2005). Although preliminary fractionation of the extract of this species has been described in a previous study (Shah et al. 2004), the active component or components have not been characterised.

An extract of *Euphorbia drummondii* was found to exhibit some antibacterial activity against Gram-positive organisms, with greater activity against *Staphylococcus* species than *Streptococcus* species. This was also the only extract tested that showed any antifungal activity against the *Candida* (yeast) species. The antimicrobial activity of this particular *Euphorbia* species does not appear to have been reported in the mainstream medical literature, although various other *Euphorbia* species have been previously shown to contain antibacterial components (Valente et al. 2004, Cateni et al. 2003).

In conclusion, extracts of some *Eremophila* species collected from study sites at Titjikala in the Northern Territory and Scotdesco in South Australia have exhibited antibacterial activity against Gram-positive organisms. The extract of *Eremophila duttonii* requires further characterisation to determine the active components present. An extract of *Euphorbia drummondii* was also shown to have some activity against Gram-positive bacteria and yeast species.

2.4.7 Antiviral activity

Antiviral screening was conducted on 23 extracts from 14 different plant species collected at Titjikala community (Northern Territory) and Scotdesco (Far West Coast, South Australia).

The only extract showing some modest antiviral activity (around 25–50% inhibition of rhinovirus cytopathic effect by visual inspection) was an extract of the stems and leaves of *Codonocarpus cotinifolius*. The extract also showed a small amount of inhibition of coxsackievirus at non-cytotoxic concentrations. Both rhinovirus and coxsackievirus belong to the same virus family, *Picornaviridae*. This may indicate that the extract has some specific antiviral activity. Further experiments are required

to confirm this. Follow-up experiments will be conducted on this plant extract to determine cytotoxicity and antiviral activity (using a tetrazolium-based assay). Preliminary separation of the extract using solvent-solvent partition will be used to see if there is more clear separation of the cytotoxic activity from the antiviral activity in partitioned fractions. Antiviral activity with this species does not appear to have been reported previously in the literature.

A small amount of inhibition of the cytopathic effect of herpes simplex virus and rhinovirus (25% or less) was seen with extracts of *Eremophila alternifolia* and one extract of *Eremophila duttonii* (rhinovirus only). The cytopathic effect of human rhinovirus was inhibited by the extracts of *Hakea divaricata* only at dilutions above the MNTD, possibly indicating that the inhibition of the virus is only due to cytotoxic effects on the cells. Further investigation of these extracts may be warranted. Submission of these extracts to some preliminary chemical separation may allow separation of the antiviral and cytotoxic effects.

For some extracts, some differences were seen in the maximum non-toxic dose between the different cell lines (Vero and H1-HeLa), and between H1-HeLa cells in the coxsackievirus and rhinovirus assays. The different sensitivity of H1-HeLa cells to some extracts (usually a difference of one two-fold dilution) may result from the different growth conditions (cells are grown at 33ºC in the rhinovirus assay and 37ºC in the coxsackievirus assay) and the different length of exposure to the extract in the two different assays (48 hours for coxsackievirus assay and 72–84 hours for rhinovirus assay).

In conclusion, antiviral activity was detected at modest levels with only one plant extract tested in this study, that of the leaf and stem extract of *Codonocarpus cotinifolius*. Further tests are required to further characterise these effects.

Appendix 1: MIC results of active extracts against clinical MRSA isolates

MIC values for all plant extracts are in mg/mL while those for ampicillin (amp.) are in µg/ml.

	MIC values							
Clinical isolate number	*Eremophila freelingii* **(8i)**	*Eremophila freelingii* **(T27)**	*Eremophila sturtii* **(5i)**	*Euphorbia drummondii* **(15i)**	*Acacia tragonophylla* **(11Ai)**	*Eremophila duttonii* **(T19)**	*Eremophila duttonii* **(T21)**	*Amp.*
MRSA -13	0.125	0.125	0.125	0.125	1	0.0625	0.0625	64
MRSA -14	0.25	0.25	0.25	0.25	2	0.125	0.125	64
MRSA -15	0.125	0.125	0.25	0.25	1	0.0625	0.0625	16
MRSA -16	0.25	0.25	0.25	0.25	2	0.0625	0.0625	16
MRSA -17	0.125	0.125	0.125	0.125	1	0.0625	0.0625	64
MRSA -18	0.25	0.125	0.25	0.25	1	0.125	0.125	32
MRSA -19	0.125	0.125	0.125	0.125	1	0.0625	0.0625	64
MRSA -20	0.125	0.25	0.25	0.25	2	0.0625	0.0625	64
MRSA -22	0.25	0.25	0.25	0.5	2	0.125	0.125	64
MRSA -23	0.25	0.25	0.25	0.5	2	0.125	0.125	16
MRSA -24	0.25	0.25	0.25	0.125	2	0.125	0.125	64
MRSA -61	0.25	0.25	0.5	0.5	2	0.125	0.125	8
MRSA -62	0.25	0.25	0.5	0.5	2	0.125	0.125	32
MRSA -63	0.25	0.25	0.5	0.5	2	0.125	0.125	64
MRSA -64	0.25	0.25	0.5	0.25	2	0.125	0.125	64
MRSA -65	0.25	0.25	0.5	0.5	2	0.125	0.125	16
MRSA -66	0.125	0.125	0.25	0.25	1	0.125	0.125	64
MRSA -67	0.25	0.25	0.5	0.5	2	0.125	0.125	32
MRSA -68	0.5	0.5	0.5	0.5	2	0.25	0.125	64
MRSA -69	0.25	0.25	0.25	0.25	2	0.125	0.125	0.5
MRSA -70	0.5	0.5	0.5	0.25	2	0.25	0.125	32
MRSA -71	0.25	0.25	0.5	0.5	2	0.125	0.125	64
MRSA -72	0.25	0.25	0.5	0.5	2	0.125	0.125	16
MRSA -73	0.25	0.25	0.25	0.25	1	0.125	0.125	64
MRSA -74	0.5	0.5	0.5	0.5	2	0.125	0.125	32
MRSA -75	0.25	0.25	0.5	0.5	2	0.125	0.125	64
MRSA -76	0.25	0.25	0.5	0.5	2	0.125	0.125	64
MRSA -77	0.25	0.25	0.5	0.5	2	0.125	0.125	8
MRSA -78	0.5	0.5	0.5	0.5	2	0.25	0.125	64
MRSA -79	0.5	0.5	0.5	0.5	2	0.125	0.125	32
MRSA -80	0.25	0.25	0.5	0.5	2	0.125	0.125	64

	MIC values							
Clinical isolate number	*Eremophila freelingii* **(8i)**	*Eremophila freelingii* **(T27)**	*Eremophila sturtii* **(5i)**	*Euphorbia drummondii* **(15i)**	*Acacia tragonophylla* **(11Ai)**	*Eremophila duttonii* **(T19)**	*Eremophila duttonii* **(T21)**	*Amp.*
MRSA -81	0.25	0.25	0.5	0.5	2	0.125	0.125	64
MRSA -82	0.25	0.25	0.5	0.5	2	0.125	0.125	2
MRSA -83	0.25	0.25	0.5	0.125	2	0.125	0.125	32
MRSA -84	0.5	0.5	0.5	0.25	2	0.125	0.125	32
MRSA -85	0.25	0.25	0.5	0.5	2	0.125	0.125	32
MRSA -86	0.25	0.25	0.5	0.5	2	0.125	0.125	8
MRSA -87	0.25	0.25	0.5	0.25	2	0.125	0.0625	16
MRSA -88	0.125	0.125	0.5	0.125	1	0.125	0.0625	64
MRSA -89	0.25	0.25	0.5	0.25	2	0.125	0.125	0.5
MRSA -90	0.125	0.125	0.125	0.125	1	0.125	0.125	32
MRSA -91	0.25	0.25	0.5	0.125	2	0.125	0.125	64
MRSA -92	0.25	0.25	0.5	0.5	2	0.125	0.125	64
MRSA -93	0.25	0.25	0.5	0.5	2	0.125	0.125	32
MRSA -94	0.25	0.25	0.5	0.5	2	0.125	0.125	64
MRSA -95	0.25	0.25	0.5	0.5	2	0.125	0.125	64
MRSA -96	0.25	0.25	0.5	0.5	2	0.125	0.0625	0.5
MRSA -97	0.25	0.25	0.5	0.5	2	0.125	0.125	32
MRSA -98	0.25	0.25	0.5	0.25	2	0.125	0.125	64
MRSA -99	0.25	0.25	0.5	0.5	2	0.125	0.125	16
MRSA -101	0.25	0.25	0.5	0.125	2	0.125	0.125	64
MRSA -102	0.125	0.125	0.125	0.125	1	0.0625	0.0625	32
MRSA -103	2	2	2	0.5	2	0.5	0.5	16
MRSA -104	0.5	0.5	0.5	0.5	2	0.25	0.125	2
MRSA -105	0.25	0.25	0.5	0.5	2	0.125	0.125	32
MRSA -106	0.25	0.25	0.5	0.5	2	0.125	0.125	32
MRSA -107	0.25	0.25	0.25	0.125	1	0.125	0.0625	64
MRSA -108	0.25	0.25	0.25	0.25	1	0.125	0.0625	0.5
MRSA -109	0.25	0.25	0.5	0.25	2	0.125	0.125	32
MRSA -110	0.5	0.5	0.5	0.5	2	0.125	0.125	32
MRSA -111	0.25	0.25	0.5	0.5	2	0.125	0.125	64
MRSA -112	0.25	0.25	0.5	0.5	2	0.125	0.125	32

	MIC values							
Clinical isolate number	*Eremophila freelingii* **(8i)**	*Eremophila freelingii* **(T27)**	*Eremophila sturtii* **(5i)**	*Euphorbia drummondii* **(15i)**	*Acacia tragonophylla* **(11Ai)**	*Eremophila duttonii* **(T19)**	*Eremophila duttonii* **(T21)**	*Amp.*
MRSA -113	0.5	0.5	0.5	0.5	2	0.25	0.25	32
MRSA -114	0.125	0.125	0.25	0.125	1	0.125	0.0625	64
MRSA -115	0.25	0.25	0.5	0.5	2	0.125	0.125	2
MRSA -116	0.5	0.5	0.5	0.5	2	0.25	0.125	0.5
MRSA -119	0.25	0.25	0.5	0.5	2	0.125	0.125	32
MRSA -120	0.25	0.25	0.25	0.25	2	0.125	0.125	64
Sa - 25923	0.25	0.25	0.25	0.25	2	0.125	0.125	0.25
Sa - 29213	0.5	0.5	0.5	0.5	2	0.125	0.125	0.5

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