# The potential of selected Australian medicinal plants with anti-Proteus activity for the treatment and prevention of rheumatoid arthritis

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## ABSTRACT

**Background:** A wide variety of herbal medicines are used in indigenous Australian traditional medicinal systems to treat rheumatoid arthritis (RA) and inflammation. The current study was undertaken to test the ability of a panel of Australian plants with a history of the ethnobotanical usage in the treatment of inflammation for the ability to block the microbial trigger of RA. **Materials and Methods:** One hundred and six extracts from 40 plant species were investigated for the ability to inhibit the growth of the bacterial trigger of RA (Proteus mirabilis). The extracts were tested for toxicity in the Artemia nauplii bioassay. The most potent inhibitor of P. mirabilis growth was further analyzed by reversed-phase high performance liquid chromatography (RP-HPLC) coupled to high accuracy time-of-flight (TOF) mass spectroscopy. **Results:** Sixty-five of the 106 extracts tested (61.3%) inhibited the growth of P. The *Aleurites moluccanus, Datura leichardtii, Eucalyptus major, Leptospermum bracteata, L. juniperium, Macadamia integriflora* nut, *Melaleuca alternifolia, Melaleuca quinquenervia, Petalostigma pubescens, P. triloculorae, P. augustifolium, Scaevola spinescens, Syzygium australe, and Tasmannia lanceolata* extracts were determined to be the most effective inhibitors of *P. mirabilis* growth, with minimum inhibitory concentration (MIC) values generally significantly below 1000 µg/ml. *T. lanceolata* fruit extracts were the most effective *P. mirabilis* growth inhibitors, with a MIC values of 11 and 126 µg/ml for the methanolic and aqueous extracts, respectively. Subsequent analysis of the *T. lanceolata* fruit extracts by RP-HPLC coupled to high-resolution TOF mass spectroscopy failed to detect resveratrol in either *T. lanceolata* fruit extract. However, the resveratrol glycoside piceid and 2 combretastatin stilbenes (A-1 and A-4) were detected in both T. lanceolata fruit extracts. With the exception of the Eucalyptus and Syzygium extracts, all extracts exhibiting Proteus inhibitory activity were also shown to be nontoxic, or of low toxicity in the Artemia nauplii bioassay. **Conclusions:** The low toxicity of these extracts and their inhibitory bioactivity against Proteus spp. indicate their potential in blocking the onset of rheumatoid arthritis.

**Key words:** Australian plants, combretastatin, inflammation, Piceid, *Proteus mirabilis*, resveratrol, rheumatoid arthritis, stilbene

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## INTRODUCTION

Autoimmune inflammatory disorders (AIID's) are a group of debilitating conditions including rheumatoid arthritis (RA), ankylosing spondylitis, lupus and multiple sclerosis, which afflict genetically susceptible individuals. There is no common susceptibility profile for these disorders. RA, for example, is most prevalent in middle-aged to older women, whereas the onset of ankylosing spondylitis occurs most frequently in younger males.[1] There are currently no cures for any of these conditions and current treatment strategies aim to alleviate the symptoms (particularly pain and swelling) via the use of analgesics and anti-inflammatory agents, and/or to modify the disease process through the use of disease-modifying anti-rheumatic drugs (DMARDs). None of these treatments are ideal as prolonged usage of these drugs can result in unwanted side effects and toxicity.[2] There is a need to develop safer, more effective drugs for the treatment of inflammatory diseases which will not only alleviate the symptoms, but which may also cure or prevent the disease.

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Eradication of the cause of an inflammatory disease is an attractive target for drug design as this would not only block/decrease the late-phase inflammatory symptoms, but would also block the immune response and subsequent tissue damage associated with AIID's. While the causes of RA are not comprehensively understood, it is generally accepted that it is an autoimmune disorder which is triggered by specific microbial infections in genetically susceptible individuals (individuals with the MHC class 2 allele HLA-DR4).[3] *Proteus mirabilis* infections have been proposed to trigger RA based on several lines of evidence:

- Elevated serum levels of *P. mirabilis* specific cross-reactive antibodies have frequently been reported in individuals suffering from RA[4-9]
- *P. mirabilis* antibodies from RA patients have cytopathic effects on joint tissue possessing *P. mirabilis* cross-reactive antibodies[10]
- *P. mirabilis* infections have been frequently reported in urine samples from patients with RA[11]
- Sera from rabbits immunized with HLA-DR4 positive lymphocytes bind specifically to *Proteus*[12]
- Amino acid sequence homologies have been identified between the "EQ/KRRAA" motif present in RA HLA-susceptibility antigens and the "ESRRAL" amino acid sequence present in *P. mirabilis* hemolysins[13]
- A further sequence homology between the "LRREI" sequence of type XI collagen (present in joint cartilage) and the "IRRET" motif present in *P. mirabilis* urease enzyme has also been reported.[14]

Based on the evidence linking *Proteus* bacterial infections with pathogenesis, a mechanism of RA disease progression has been proposed [Figure 1]: Gastrointestinal *P. mirabilis* acts as a trigger for RA.[10] Thus, limiting the levels of gastrointestinal *P. mirabilis* (1) would prevent RA initiation and minimize its downstream effects. Gastrointestinal *P. mirabilis* will not initiate the autoimmune events associated with RA unless it is able to interact with the immune system. This most often results from urinary tract infections (2) or when epithelial lesions (or other epithelial interruptions) allow for the production of anti-*P. mirabilis* antibodies.[10,15] Inhibition of the causative agents of gut lesion forming conditions (e.g. Crohn's disease) would also be expected to decrease RA initiation events. Furthermore, prevention and early detection of urinary tract infections (UTIs) (the major pathway for interaction of *P. mirabilis* with the immune system) and/or the colonization of the bladder (2 and 3) would block the onset of RA and lessen downstream effects. Blocking the immune response by blocking the interaction of *P. mirabilis* with immunological cells (4) or by immunomodulation (5) also prevents the production of self-reactive antibodies (6) or the cross-reactivity with

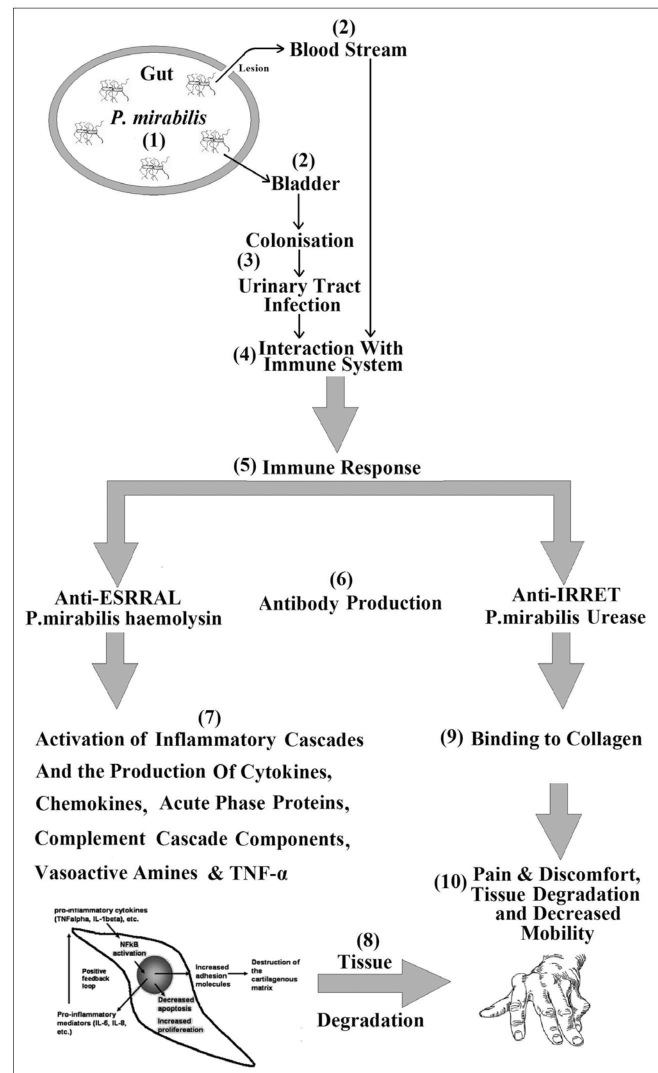


**Figure 1:** A proposed schematic representation of the main events in rheumatoid arthritis (RA) disease etiology and progression. Only major events are shown. Numbers refer to current and/or proposed targets for the prevention and treatment of RA

self-tissue, (9) thereby diminishing the later phase events of RA and thus the disease symptoms (10). However, immunomodulatory therapy should be used with caution as inhibiting the patient's immune capability would also expose the patient to a variety of other infections. Most current RA therapies target the later phase events by (7) blocking the inflammatory cascades or (8 and 10) by decreasing the symptoms of RA (e.g. pain, swelling, heat). While drugs targeting the late events are effective in easing patient discomfort, they still allow the tissue damage (which is associated with the self-reactive antibody action) to occur. Targeting earlier events prior to the induction of the immune response would not only alleviate the symptoms and discomfort of RA, but would also lessen/prevent the joint damage associated with chronic inflammation.

Targeting *P. mirabilis* infections may provide a new therapeutic approach for preventing and treating RA. One strategy is the development of anti-*Proteus* vaccines. While the production of a vaccine may block *Proteus* pathogenesis, it is also a problematic approach as anti-*Proteus* antibodies would also be likely to cross-react with the host connective tissue in susceptible individuals and thus exacerbate the symptoms of RA. If antibodies lacking cross-reactive epitopes are developed in the future, this approach may be effective as it would block susceptible individuals from acquiring a *Proteus* infection, thus effectively blocking RA progression. However, the development and usage of *Proteus* sensitive antibiotics may prove a more effective means of treating the RA bacterial trigger and thus blocking this disease. By destroying the *Proteus* bacteria, this treatment modality would be expected to greatly reduce the impact of the bacteria and the production of anti-*Proteus* antibodies and thus decrease disease progression.

Many antibiotics are already known to inhibit *Proteus* growth and/or have bactericidal effects toward *Proteus* spp. However, the development of super-resistant bacterial strains has resulted in currently used antibiotic agents failing to end many bacterial infections.[16,17] For this reason, the development of new anti-*P. mirabilis* chemotherapeutic agents for the prevention and treatment of RA has received recent attention. Recent studies have examined the anti-*P. mirabilis* activity of conventional antimicrobials such as carbapenems[18] and of complementary and alternative therapies including nano-metallic preparations[19] and traditional South African medicinal plants.[20] A re-examination of traditional medicines for the treatment of inflammation and rheumatic conditions is an attractive prospect as the antiseptic qualities of medicinal plants have been long recognized and recorded. Furthermore, there has recently been a revival of interest in herbal medications due to a perception that there is a lower incidence of adverse reactions to plant preparations compared to synthetic pharmaceuticals.

In this study, a selection of Australian plants was identified which were traditionally used for the treatment of RA as well as other inflammatory conditions [Table 1]. While the ethnobotanical uses of these plants in traditional Aboriginal medicine systems have been recorded, rigorous scientific studies are lacking for many species. We were

**Table 1: The ethnobotanical usage and common names of the plant species tested in this study**

| Plant species | Common name | Part used medicinally | Part used in this study | Medicinal use | References |
|---|---|---|---|---|---|
| *Acacia auriculiformis* | Earleaf acacia, earpod wattle, northern black wattle, tan wattle | Leaves | Leaves | Allergy, antiseptic, rash | [21] |
| *Acacia diasparima* | Hickory wattle, until recently known as *Acacia aulacocarpa* | Leaves | Leaves | Allergy, antiseptic, rash | [21,25,27] |
| *Acacia leptoloba* | Irvine bank wattle | Leaves | Leaves | Allergy, antiseptic, sore eyes, rash | [21] |
| *Aleurites moluccanus* | Candlenut, Indian walnut, varnish tree | Leaves, nut, bark | Nut | Rheumatism, swelling, inflammation | [22] |
| *Alphitonia excelsa* | Red ash, soap tree | Leaves, nut | Leaves | Antimicrobial (especially skin microbes/skin sores), rheumatism, swelling, inflammation | [21,22] |
| *Alpinia caerulea* | Native ginger | Roots, leaves, fruit | Fruit, leaves | Young tips of roots are edible and have similar medicinal properties to *Ziniber officinale* (ginger), especially inflammation, nausea, arthritis, slowing cancer growth | [21,22] |
| *Backhousia citriodora* | Lemon myrtle, lemon scented myrtle, lemon scented ironwood | Leaves | Leaves | Antimicrobial (especially skin microbes/ skin sores), high in essential oils, used for food flavoring | [21,62] |
| *Backhousia myrtiflora* | Cinnamon myrtle, Australian lancewood, carrol ironwood, grey myrtle | Leaves | Leaves | Antimicrobial (especially skin microbes/ skin sores), high in essential oils, used for food flavoring | [21] |
| *Callistemon citrinus* | Crimson bottlebrush | Leaves | Leaves, flowers | Rheumatism, swellings, inflammation, skin disorders, treatment of respiratory infections, antimicrobial | [21,26,27] |
| *Callistemon formosus* | Cliff bottlebrush | Leaves | Leaves | Rheumatism, swellings, inflammation, skin disorders, treatment of respiratory infections, antimicrobial | [21,26,27] |
| *Callistemon salignus* | White bottlebrush, willow bottlebrush | Leaves | Leaves, flowers | Rheumatism, swellings, inflammation, skin disorders, treatment of respiratory infections, antimicrobial | [21,26,27] |

*Contd...*

**Table 1: Contd...**

| Plant species | Common name | Part used medicinally | Part used in this study | Medicinal use | References |
|---|---|---|---|---|---|
| *Cinnamomum oliveri* | Camphorwood, Oliver's sassafrass, black sassafras, cinnamon wood | Bark, leaf, fruit | Leaf | Oils made from the distillation of the bark are used to treat rheumatism, swellings, inflammation, skin disorders, antimicrobial | [21,22] |
| *Davidsonia pruriens* | Davidson's plum, ooray | Fruit, leaves | Fruit, leaves | Medicinal activities unknown-included because of its very high antioxidant activity (high antioxidant content has been linked with prevention of chronic diseases) | [21,23,63] |
| *Duboisia leichhardtii* | Corkwood, poisonous corkwood, Queensland Duboisia, yellow basswood | Leaves, bark | Leaves | Treatment of pain, inflammation, nausea and intestinal problems | [21,22] |
| *Elaeocarpus angustifolius* | Blue quandong, blue fig, blue marble tree | Fruit | Fruit | Medicinal activities unknown-included because of its very high antioxidant activity (high antioxidant content has been linked with prevention of chronic diseases) | [21,23,63] |
| *Eucalyptus baileyana* | Black stringy bark | Bark (gum), leaves | Leaves | Rheumatism, swellings, inflammation, skin disorders, stomach disorders, bactericide (wounds, sores), high leaf terpene content | [21,22,28] |
| *Eucalyptus major* | Queensland grey gum | Bark (gum), leaves | Leaves | Rheumatism, swellings, inflammation, skin disorders, stomach disorders, bactericide (wounds, sores), high leaf terpene content | [21,22,28] |
| *Kunzea flavescens* | Yellow Kunzea | Leaves, fruit | Leaves | Ethnomedicinal usage unknown, but other Kunzea species are known to be used in the treatment of rheumatism, swellings, inflammation, skin disorders and as a general antimicrobial | [21,22] |
| *Leptospermum longifolium* (also known as *Leptospermum madidum*) | Weeping tea tree | Leaf | Leaf | Rheumatism, swellings, inflammation, skin disorders, stomach disorders, bactericide (wounds, sores), high leaf terpene content | [21,22,29] |
| *Leptospermum bracteata* | Unknown | Leaf | Leaf | Rheumatism, swellings, inflammation, skin disorders, stomach disorders, bactericide (wounds, sores), high leaf terpene content | [21,22,29,37] |
| *Leptospermum juniperium* | Prickly tea tree | Leaf | Leaf | Rheumatism, swellings, inflammation, skin disorders, stomach disorders, bactericide (wounds, sores), high leaf terpene content | [21,22,29,37] |
| *Leptospermum petersonii* (formerly known as *Leptospermum citratum*) | Lemon scented tea tree | Leaf | Leaf | Rheumatism, swellings, inflammation, skin disorders, stomach disorders, bactericide (wounds, sores), high leaf terpene content | [21,22,29,37] |
| *Macadamia integriflora* | Macadamia nut, baupe nut, Queensland nut | Leaf, nut | Leaf, nut | Ethnomedical uses unknown although the nut is known to keep well and has been shown to have antimicrobial properties | [64] |
| *Melaleuca alternifolia* | Narrow leafed paperbark, narrow-leafed tea tree | Leaf | leaf | Rheumatism, swellings, inflammation, skin disorders, stomach disorders, bactericide (wounds, sores), STI's, high leaf terpene content | [21,22] |
| *Melaleuca quinquenervia* | Tea tree | Leaf | Leaf | Rheumatism, swellings, inflammation, skin disorders, stomach disorders, bactericide (wounds, sores), STI's, high leaf terpene content | [21,22] |
| *Petalostigma pubescens* | Quinine bush | Fruit, bark, leaf | Fruit, leaf | Inflammation, skin disorders, stomach disorders, bactericide (wounds, sores), asthma | [21,22,34] |
| *Petalostigma triloculorae* | Long leafed bitter bark, long leafed quinine bush | Fruit, bark, leaf | Fruit, leaf | Inflammation, skin disorders, stomach disorders, bactericide (wounds, sores), asthma | [21,22,34] |
| *Pittosporum augustifolium* (previously known as *Pittosporum phylliraeoides*) | Weeping Pittosporum, butterbush, cattle bush, native apricot, gumbi gumbi, cumby cumby | Fruit, wood, leaves | Leaves | Pain, inflammation, cough, cold, skin disorders, reputed to block cancer proliferation | [21,22] |

*Contd...*

**Table 1: Contd...**

| Plant species | Common name | Part used medicinally | Part used in this study | Medicinal use | References |
|---|---|---|---|---|---|
| *Scaevola spinescens* | Maroon bush, currant bush, prickly fan flower | Stem, leaves | Leaves | Antimicrobial (especially skin microbes/skin sores), pain, urinary disorders | [21,22,35] |
| *Syzygium anisatum* | Anise myrtle, aniseed tree, Ringwood | Leaves | Leaves | Antimicrobial (especially skin microbes/skin sores), high in essential oils, used for food flavoring | [21] |
| *Syzygium austral* | Brush cherry, scrub cherry | Leaves, fruit | Leaves, fruit | High in antioxidants, antimicrobial (especially skin microbes/skin sores), high in essential oils, used for food flavoring | [21,27,30,32] |
| *Syzygium forte* | White apple | Leaves, fruit | Leaves | Antimicrobial (especially skin microbes/skin sores), high in essential oils | [21,27,31] |
| *Syzygium francisii* | Giant water gum, rose satin ash | Leaves, fruit | Leaves | Antimicrobial (especially skin microbes/skin sores), high in essential oils | [21,27,31] |
| *Syzygium moorei* | Coolamon, durobby | Leaves, fruit | Leaves | Antimicrobial (especially skin microbes/skin sores), high in essential oils | [21,27,31] |
| *Syzygium puberculum* | Unknown | Leaves, fruit | Leaves | Antimicrobial (especially skin microbes/skin sores), high in essential oils | [21,27,31] |
| *Syzygium wilsonii* | Water gum, plum satin ash | Leaves, fruit | Leaves | Antimicrobial (especially skin microbes/skin sores), high in essential oils | [21,27,31] |
| *Syzygium leuhmannii* | Riberry, cherry satin ash | Leaves, fruit | Leaves, fruit | High in antioxidants, antimicrobial (especially skin microbes/skin sores), high in essential oils, used for food flavoring | [21,27,30,32] |
| *Tasmannia insipida* | Brush pepperbush | Bark, leaves, berries, peppercorns | Leaves | Used as a flavoring agent; medicinal uses unknown-included due to its taxonomic relationship to *T. lanceolata* | [21,65] |
| *Tasmannia lanceolata* | Mountain pepper, pepperberry | Bark, leaves, berries, peppercorns | Leaves, berries, peppercorns | Used as a flavoring agent; scurvy, stomach disorders, antimicrobial, tonic, skin disorders, STI's, quinine substitute | [21,24,65] |
| *Tasmannia stipitata* | Northern pepperbush, Dorrigo pepper | Bark, leaves, berries, peppercorns | Leaves, berries | Used as a flavoring agent; medicinal uses unknown-included due to its taxonomic relationship to *T. lanceolata* | [21,65] |

*T. lanceolata: Tasmannia lanceolata*

unable to find studies examining any anti-inflammatory properties of *Aleurites moluccanus*, *A. excelsa*, *A. caerulea,* and *Duboisia leichhardtii* despite their usage in traditional healing systems.[21,22] As high levels of antioxidants have therapeutic effects against many diseases and medical conditions including inflammation and bacterial diseases, several high antioxidant plants were also included in this study. *Tasmannia* spp. and *Syzygium* spp. in particular, have been shown to have high antioxidant contents,[23] as well as documented medicinal uses.[21,22] Other species with high antioxidant contents (*Davidsonia pruriens, Elaeocarpus angustifolius*) were also included in the study despite a lack of documented ethnobotanical usage or reports of anti-inflammatory activity.

Several of the plant species identified as having anti-inflammatory properties have been studied previously for their antiseptic properties, although we were unable to find studies aimed at testing these plant species against RA. For example, recent studies have shown *Tasmannia lanceolata* to have potent inhibitory activity towards a wide range of bacteria.[24] Similarly, Australian *Acacia* spp.,[25] *Callistemon* spp.,[26,27] *Eucalyptus* spp.,[27,28] *Leptospermum* spp.,[29] *Melaleuca* spp.,[27] *Syzygium* spp.,[21,27,30-32] *P. augustifolia,*[33] and *Petalostigma* spp.[34] have well-documented antibacterial activity against a wide variety of bacteria. *Scaevola spinescens* extracts have not only been shown to have broad-spectrum antimicrobial activity,[35] but also have documented uses in the treatment of UTI's and urinary disorders.[21,22]

Several other plant species examined in this study are closely related taxonomically with species with known anti-inflammatory and antimicrobial activities. While we were unable to find reports of the ethnobotanical usage of *Kunzea flavescens,* extracts prepared from related *Kunzea* species are used in the treatment of rheumatism, swellings and inflammation.[21,22] Other species examined in this study (e.g. *T. insipidia* and *T. stipitata*) have not been extensively studied for their anti-inflammatory or antimicrobial properties and are included in this study due to their taxonomic relationship with *T. lanceolata*. The selected plant species were extracted, and the extracts were tested against *P. mirabilis* to screen their potential to block the microbial triggers of RA.

## MATERIALS AND METHODS

### Ethnobotanical information

Several literature resources [Table 1] were utilized to identify Australian native plants with a history of usage in the treatment of rheumatism and other inflammatory disorders.

### Plant collection and extraction

The majority of the plant species tested in this study were collected from the Mt Cootha Botanical Gardens, Brisbane Australia. Of the other plants, *Backhousia citriodora*, *B. myrtifolia*, *Elaeocarpus augustifolius*, *P. augustifolium*, *Syzygium anisatum, T. insipidia* and *T. stipitata* were provided by individual members of the Qld Bushfoods Association, Australia. *A. auriculiformis*, *A. diasparima*, *D. pruriens*, *K. flavescens*, *P. pubescens* and *P. triloculorae* were collected from Griffith University, Australia. *Macadamia integriflora*, *Callistemon citrinus*, *C. salignus*, *S. australae* and *S. leuhmannii* were collected from suburban gardens on the south side of Brisbane. *T. lanceolata* was supplied by Taste of Australia native food suppliers. *S. spinescens* was supplied by Jeanie Crago of outback Books. All plants were identified by Philip Cameron, senior botanical officer, the Brisbane Botanical Gardens. Voucher specimens were prepared and are stored at the School of Natural Sciences, Griffith University, Australia. All plant materials were air-dried in the shade and ground into a fine powder.

One gram of each dried plant material was weighed into each of two tubes for each plant. Extracts were prepared by adding 50 ml of either AR grade methanol or distilled water to the tubes. Plant material was extracted in each solvent for 24 h at 4°C with gentle shaking. The extracts were filtered through filter paper (Whatman No. 54). The methanol extracts were subsequently allowed to dry at room temperature. The aqueous extracts were frozen at −70°C and dried by lyophilization. The resultant dry extracts were weighed and redissolved in 10 ml deionized water.

### Antibacterial screening

#### *Test microorganisms*

A reference strain of *P. mirabilis* (American Tissue Culture Collection [ATCC] 43071) was obtained from ATCC. The bacteria were subcultured and maintained in nutrient broth at 4°C.

### Evaluation of antimicrobial activity

Antimicrobial activity of all plant extracts was determined using a modified disc diffusion method.[36,37] Briefly, 100 µl of the test bacteria were grown in 10 ml of fresh nutrient broth until they reached a count of approximately $10^8$ cells/Ml. 100 µl of microbial suspension was spread onto nutrient agar plates.

The extracts were tested using 5 mm sterilized filter paper discs. Discs were impregnated with 10 µl of the test sample, allowed to dry and placed onto inoculated plates. The plates were allowed to stand at 4°C for 2 h before incubation with the test microbial agents. The plates were incubated at 30°C for 24 h, then the diameters of the inhibition zones were measured in millimeters. All measurements were to the closest whole millimeter. Each antimicrobial assay was performed in at least triplicate, and mean values were determined. Standard discs of ampicillin (2 µg) and chloramphenicol (10 µg) were obtained from Oxoid Ltd. and served as positive controls for antimicrobial activity. Filter discs impregnated with 10 µl of distilled water were used as negative controls.

### Minimum inhibitory concentration determination

The minimum inhibitory concentrations (MICs) of the plant extracts were determined by the disc diffusion MIC method[33,35] across a range of doses. Briefly, the plant extracts were serially diluted in deionized water across a range of concentrations. Discs were impregnated with 10 µl of the test dilutions, allowed to dry and placed onto inoculated plates. The assay was performed as outlined above, and graphs of the zone of inhibition versus concentration were plotted for each extract. Linear regression was used to calculate the MIC values.

### Toxicity screening

#### *Reference toxin for biological screening*

Potassium dichromate ($K_2Cr_2O_7$) (AR grade, Chem-Supply, Australia) was prepared as a 1.6 mg/ml solution in distilled water and was serially diluted in synthetic seawater for use in the *Artemia franciscana* nauplii bioassay.

#### *Artemia franciscana nauplii toxicity screening*

Toxicity was tested using a modified *A. franciscana* nauplii lethality assay.[38-40] Briefly, *A. franciscana* cysts were obtained from North American Brine Shrimp, LLC, USA (harvested from the Great Salt Lake, Utah). Synthetic seawater was prepared using Reef Salt, AZOO Co., USA. Seawater solutions at 34 g/L distilled water were prepared prior to use. An amount of 2 g of *A. franciscana* cysts was incubated in 1 l synthetic seawater under artificial light at 25°C, 2000 Lux with continuous aeration. Hatching commenced within 16–18 h of incubation. Newly hatched *A. franciscana* (nauplii) were used within 10 h of hatching. Nauplii were separated from the shells and remaining cysts and were concentrated to a suitable density by placing an artificial light at one end of their incubation vessel and the nauplii rich water closest to the light was removed for biological assays. A volume of 400 µl of seawater containing approximately 37 (mean = 37.2, *n* = 132, SD = 11.6) nauplii was added to wells of a 48 well plate and immediately used for bioassay. The plant extracts were

diluted to 4 mg/ml in seawater for toxicity testing, resulting in a 2 mg/ml concentration in the bioassay. A volume of 400 μl of diluted plant extract and the reference toxin were transferred to the wells and incubated at 25 ± 1°C under artificial light (1000 Lux). A negative control (400 μl seawater) was run in at least triplicate for each plate. All treatments were performed in at least triplicate. The wells were checked at regular intervals, and the number of dead counted. The nauplii were considered moribund if no movement of the appendages was observed within 10 s. Following the 24 h and exposure, all nauplii were sacrificed and counted to determine the total number per well. The $LC_{50}$ with 95% confidence limits for each treatment was calculated using probit analysis.

### High performance liquid chromatography-MS/MS analysis

Chromatographic separations were performed using 10 μL injections of sample onto an Agilent 1290 high performance liquid chromatography (HPLC) system fitted with a Zorbax Eclipse plus C18 column (2.1 × 100 mm, 1.8 μm particle size). The mobile phases consisted of (A) ultrapure water and (B) 95:5 acetonitrile/water at a flow rate of 0.7 mL/min. Both mobile phases were modified with 0.1% (v/v) glacial acetic acid for mass spectrometry analysis in positive mode and with 5 mM ammonium acetate for analysis in negative mode. The chromatographic conditions utilized for the study consisted of the first 5 min run isocratically at 5% B, a gradient of (B) from 5% to 100% was applied from 5 min to 30 min, followed by 3 min isocratically at 100%. Mass spectrometry analysis was performed on an Agilent 6530 quadrupole time-of-flight spectrometer fitted with a Jetstream electrospray ionization source in both positive and negative mode.

Data were analyzed using the Masshunter Qualitative analysis software package (Agilent Technologies). Blanks using each of the solvent extraction systems were analyzed using the Find by Molecular Feature algorithm in the software package to generate a compound list of molecules with abundances >10,000 counts. This was then used as an exclusion list to eliminate background contaminant compounds from the analysis of the extracts. Each extract was then analyzed using the same parameters using the find by molecular feature function to generate a putative list of compounds in the extracts. Compound lists were then screened against three accurate mass databases; a database of known plant compounds of therapeutic importance generated specifically for this study (650 compounds); the Metlin metabolomics database (24,768 compounds); and the Forensic Toxicology Database by Agilent Technologies (7,509 compounds). Empirical formula for unidentified compounds was determined using the find formula function in the software package.

### Statistical analysis

Data are expressed as the mean ± standard error of the mean of at least three independent experiments.

## RESULTS

### Liquid extraction yields

Extraction of 1 g of the dried plant materials with methanol and water yielded dried plant extracts ranging from 19.9 mg (*P. pubescens* leaf water extract) to 477 mg (*T. lanceolata* peppercorn methanolic extract) [Table 2]. In general, methanol was more efficient at extracting material from the plant samples than water. Methanol extracted a greater amount of material for 45 of the 53 plant samples tested (84.9%), whereas water extracted the greater amount of material for 6 of the 53 plant samples (11.3%). Two samples extracted approximately equal masses in both water and methanol. A correlation was also noted between the plant part tested and the amount of extracted material. For 12 plant species (*A. caerulea, C. citrinus*, *C. salignus*, *D. pruriens, Leptospermum bracteata*, *L. juniperium*, *M. integriflora*, *P. pubescens*, *P. triloculare*, *S. australe*, *S. leuhmannii* and *T. lanceolata*), multiple plant materials were extracted. For those species where both leaf and flower were tested (*C. citrinus*, *C. salignus*, *L. bracteata*, *L. juniperium*), extraction of the leaf with either water or methanol generally resulted in higher yields of extracted material than from the flower extracts. Conversely, when both leaf and fruit extracts from the same plant were tested (*A. caerulea*, *D. pruriens*, *M. integriflora*, *P. pubescens*, *P. triloculorae*, *S. australe*, *S. leuhmannii* and *T. lanceolata*), extraction of the fruit with either solvent generally resulted in higher extraction yields than the corresponding leaf extracts. *S. australe* extracts were the exception to this trend, with both methanol and water extracting greater masses from the leaves than from the fruit. The dried extracts were resuspended in 10 ml of deionized water resulting in the extract concentrations shown in Table 2.

### Antibacterial activity

As decoctions and tinctures are the main forms in which plants were traditionally used in ethnobotanical medicinal systems, the zones of inhibition of the methanolic and aqueous extracts were tested undiluted to provide an approximate measure of the efficacy of the form in which the traditional medications would be used. Aliquots (10 μl) of each extract were screened against *P. mirabilis* [Figure 2]. *P. mirabilis* was inhibited by 65 of the 106 (61.3%) extracts tested. Similar numbers of methanolic (33 of 53 methanolic extracts; 62.3%) and aqueous extracts (32 of 53 aqueous extracts; 60.4%) inhibited *P. mirabilis* growth.

The most effective inhibitors of *P. mirabilis* growth were the *T. lanceolata* and *Datura leichardtii* extracts based on

**Table 2: Extract yields, MIC (μg/ml) of plant extracts which displayed inhibitory activity in the screening experiment against *P. mirabilis* and 24 h $LC_{50}$ values (μg/ml) of the plant extracts in the Artemia nauplii bioassay**

| | Species/Extract | μg | μg/ml | MIC (μg/ml) | $LC_{50}$ (μg/ml) |
|---|---|---|---|---|---|
| 1M | *Acacia auriculiformis* (L M) | 150 | 15 | 1318.7 | - |
| 1W | *Acacia auriculiformis* (L W) | 67 | 6.7 | 1893.6 | - |
| 2M | *Acacia diasparima* (L M) | 140 | 14 | 2587.6 | - |
| 2W | *Acacia diasparima* (L W) | 85 | 8.5 | 3361.9 | - |
| 3M | *Acacia leptoloba* (L M) | 83 | 8.3 | 4369.7 | - |
| 3W | *Acacia leptoloba* (L W) | 56 | 5.6 | 4663.9 | - |
| 4M | *Aleurites moluccanus* (N M) | 192 | 19.2 | 243 | - |
| 4W | *Aleurites moluccanus* (N W) | 200 | 20 | 270 | - |
| 5M | *Alphitonia excelsa* (L M) | 108 | 10.8 | - | - |
| 5W | *Alphitonia excelsa* (L W) | 73 | 7.3 | - | - |
| 6M | *Alpinia caerulea* (F M) | 88 | 8.8 | 4337.7 | - |
| 6W | *Alpinia caerulea* (F W) | 72 | 7.2 | 5881.5 | - |
| 7M | *Alpinia caerulea* (L M) | 116 | 11.6 | 735.9 | - |
| 7W | *Alpinia caerulea* (L W) | 73 | 7.3 | 1284.2 | - |
| 8M | *Backhousia citriodora* (L M) | 154 | 15.4 | 425 | - |
| 8W | *Backhousia citriodora* (L W) | 65 | 6.5 | 2230 | - |
| 9M | *Backhousia myrtiflora* (L M) | 253 | 25.3 | 3150 | - |
| 9W | *Backhousia myrtiflora* (L W) | 178 | 17.8 | 3378 | - |
| 10M | *Callistemon citrinus* (L M) | 340 | 34 | 1558.9 | - |
| 10W | *Callistemon citrinus* (L W) | 260 | 26 | 2146.3 | - |
| 11M | *Callistemon citrinus* (Fl M) | 370 | 37 | 1767.8 | - |
| 11W | *Callistemon citrinus* (Fl W) | 240 | 24 | 2005.5 | - |
| 12M | *Callistemon formosus* (L M) | 285 | 28.5 | 1400.6 | - |
| 12W | *Callistemon formosus* (L W) | 228 | 22.8 | 1670 | - |
| 13M | *Callistemon salignus* (L M) | 320 | 32 | 2187.8 | - |
| 13W | *Callistemon salignus* (L W) | 270 | 27 | 2576.3 | - |
| 14M | *Callistemon salignus* (Fl M) | 295 | 29.5 | 2487.9 | - |
| 14W | *Callistemon salignus* (Fl W) | 243 | 24.3 | 2778 | - |
| 15M | *Cinnamomum oliveri* (L M) | 215 | 21.5 | 687.9 | 6760 |
| 15W | *Cinnamomum oliveri* (L W) | 60 | 6 | 1463.1 | - |
| 16M | *Davidsonia pruriens* (L M) | 134 | 13.4 | - | - |
| 16W | *Davidsonia pruriens* (L W) | 43 | 4.3 | - | - |
| 17M | *Davidsonia pruriens* (F M) | 188 | 18.8 | 1725.6 | 6443 |
| 17W | *Davidsonia pruriens* (F W) | 104 | 10.4 | 2238.9 | 2883.3 |
| 18M | *Duboisia leichhardtii* (L M) | 184 | 18.4 | 588.5 | - |
| 18W | *Duboisia leichhardtii* (L W) | 112 | 11.2 | 1267.2 | - |
| 19M | *Elaeocarpus angustifolius* (F M) | 34 | 3.4 | - | 8783 |
| 19W | *Elaeocarpus angustifolius* (F W) | 146 | 14.6 | - | 7699 |
| 20M | *Eucalyptus baileyana* (L M) | 218 | 21.8 | 847.3 | 216.7 |
| 20W | *Eucalyptus baileyana* (L W) | 144.8 | 14.5 | 1145.8 | 783.7 |
| 21M | *Eucalyptus major* (L M) | 427.3 | 42.7 | 449.5 | 662.9 |
| 21W | *Eucalyptus major* (L W) | 165.5 | 16.6 | 873.7 | 896.3 |
| 22M | *Kunzea flavescens* (L M) | 312.4 | 31.2 | - | - |
| 22W | *Kunzea flavescens* (L W) | 177.7 | 17.8 | - | - |
| 23M | *Leptospermum bracteata* (L M) | 208 | 20.8 | 387.8 | - |
| 23W | *Leptospermum bracteata* (L W) | 140 | 14 | 742 | - |
| 24M | *Leptospermum bracteata* (Fl M) | 178 | 17.8 | 653.7 | - |
| 24W | *Leptospermum bracteata* (Fl W) | 115 | 11.5 | 1172.5 | - |
| 25M | *Leptospermum juniperium* (L M) | 184 | 18.4 | 484.3 | - |
| 25W | *Leptospermum juniperium* (L W) | 131 | 131 | 876.3 | - |
| 26M | *Leptospermum juniperium* (Fl M) | 122 | 12.2 | 844.9 | - |
| 26W | *Leptospermum juniperium* (Fl W) | 97 | 9.7 | 1367.5 | - |
| 27M | *Leptospermum longifolium* (L M) | 259 | 25.9 | 1454.3 | - |
| 27W | *Leptospermum longifolium* (L W) | 118 | 11.8 | 1663.8 | - |

*Contd..*

**Table 2: Contd...**

| | Species/Extract | µg | µg/ml | MIC (µg/ml) | $LC_{50}$ (µg/ml) |
|---|---|---|---|---|---|
| 28M | *Leptospermum petersonii* (L M) | 233 | 23.3 | 1499 | 2467 |
| 28W | *Leptospermum petersonii* (L W) | 145 | 14.5 | 1887.2 | 3221 |
| 29M | *Macadamia integriflora* (N M) | 382.5 | 38.3 | 15 | - |
| 29W | *Macadamia integriflora* (N W) | 199.1 | 19.9 | 558 | - |
| 30M | *Macadamia integriflora* (L M) | 75 | 7.5 | 2790 | - |
| 30W | *Macadamia integriflora* (L W) | 102.7 | 10.3 | 4378.2 | - |
| 31M | *Melaleuca alternifolia* (L M) | 185 | 18.5 | 684.9 | - |
| 31W | *Melaleuca alternifolia* (L W) | 120 | 12 | 983.1 | - |
| 32M | *Melaleuca quinquenervia* (L M) | 134 | 13.4 | 435.2 | - |
| 32W | *Melaleuca quinquenervia* (L W) | 87 | 8.7 | 673.8 | - |
| 33M | *Petalostigma pubescens* (L M) | 90 | 9 | 287 | - |
| 33W | *Petalostigma pubescens* (L W) | 19.9 | 2 | 1427 | - |
| 34M | *Petalostigma pubescens* (F M) | 154 | 15.4 | 458.6 | - |
| 34W | *Petalostigma pubescens* (F W) | 65 | 6.5 | 783.9 | - |
| 35M | *Petalostigma triloculorae* (L M) | 123 | 12.3 | 320.7 | - |
| 35W | *Petalostigma triloculorae* (L W) | 111 | 11.1 | 1126.5 | - |
| 36M | *Petalostigma triloculorae* (F M) | 273 | 27.3 | 412.7 | - |
| 36W | *Petalostigma triloculorae* (F W) | 87 | 8.7 | 378.6 | - |
| 37M | *Pittosporum augustifolium* (L M) | 64 | 6.4 | 473.4 | - |
| 37W | *Pittosporum augustifolium* (L W) | 111 | 11.1 | 770.4 | - |
| 38M | *Scaevola spinescens* (L M) | 116 | 11.6 | 151.9 | - |
| 38W | *Scaevola spinescens* (L W) | 210 | 21 | 25.4 | - |
| 39M | *Syzygium anisatum* (L M) | 90 | 9 | 1315.2 | - |
| 39W | *Syzygium anisatum* (L W) | 87 | 8.7 | - | - |
| 40M | *Syzygium australe* (L M) | 270 | 27 | 976.3 | 1879.3 |
| 40W | *Syzygium australe* (L W) | 140 | 14 | 1328.4 | 3310 |
| 41M | *Syzygium australe* (F M) | 254 | 25.4 | 769 | 294 |
| 41W | *Syzygium australe* (F W) | 200 | 20 | 636.9 | 244 |
| 42M | *Syzygium forte* (L M) | 54 | 5.4 | - | 392 |
| 42W | *Syzygium forte* (L W) | 32 | 3.2 | - | 762 |
| 43M | *Syzygium francisii* (L M) | 156 | 15.6 | - | - |
| 43W | *Syzygium francisii* (L W) | 128 | 12.8 | - | - |
| 44M | *Syzygium moorei* (L M) | 132 | 13.2 | - | 1312 |
| 44W | *Syzygium moorei* (L W) | 84 | 8.4 | - | 1687 |
| 45M | *Syzygium puberculum* (L M) | 317 | 31.7 | - | - |
| 45W | *Syzygium puberculum* (L W) | 117 | 11.7 | - | - |
| 46M | *Syzygium wilsonii* (L M) | 108 | 10.8 | - | - |
| 46W | *Syzygium wilsonii* (L W) | 43 | 4.3 | - | - |
| 47M | *Syzygium leuhmannii* (L M) | 180 | 18 | - | 449.8 |
| 47W | *Syzygium leuhmannii* (L W) | 75 | 7.5 | - | 813.1 |
| 48M | *Syzygium leuhmannii* (F M) | 427 | 42.7 | 2900 | 414 |
| 48W | *Syzygium leuhmannii* (F W) | 442 | 44.2 | 4700 | 478 |
| 49M | *Tasmannia insipida* (L M) | 118 | 11.8 | 891.4 | 3228.9 |
| 49W | *Tasmannia insipida* (L W) | 103.9 | 10.4 | 1252.6 | 4732.1 |
| 50M | *Tasmannia lanceolata* (L M) | 144 | 14.4 | 643 | 3096 |
| 50W | *Tasmannia lanceolata* (L W) | 134 | 13.4 | 56 | 2665 |
| 51M | *Tasmannia lanceolata* (F M) | 171 | 17.1 | 11 | 3573 |
| 51W | *Tasmannia lanceolata* (F W) | 111 | 11.1 | 126 | 2376 |
| 52M | *Tasmannia lanceolata* (P M) | 295 | 29.5 | 89 | 4159 |
| 52W | *Tasmannia lanceolata* (P W) | 477 | 47.7 | 414 | 3029 |
| 53M | *Tasmannia stipitata* (L M) | 126.4 | 12.6 | 268.3 | 2878.4 |
| 53W | *Tasmannia stipitata* (L W) | 87.6 | 8.8 | 744.5 | 4641 |
| | Potassium dichromate | | | | 92.3 |

Numbers indicate the mean MIC or $LC_{50}$ values of at least triplicate determinations. -: No growth inhibition or mortality; L: Leaf; F: Fruit; Fl: Flower; N: Nut; M: Methanolic extract; W: Water extract; MIC: Minimum inhibitory concentrations; *P. mirabilis: Proteus mirabilis*

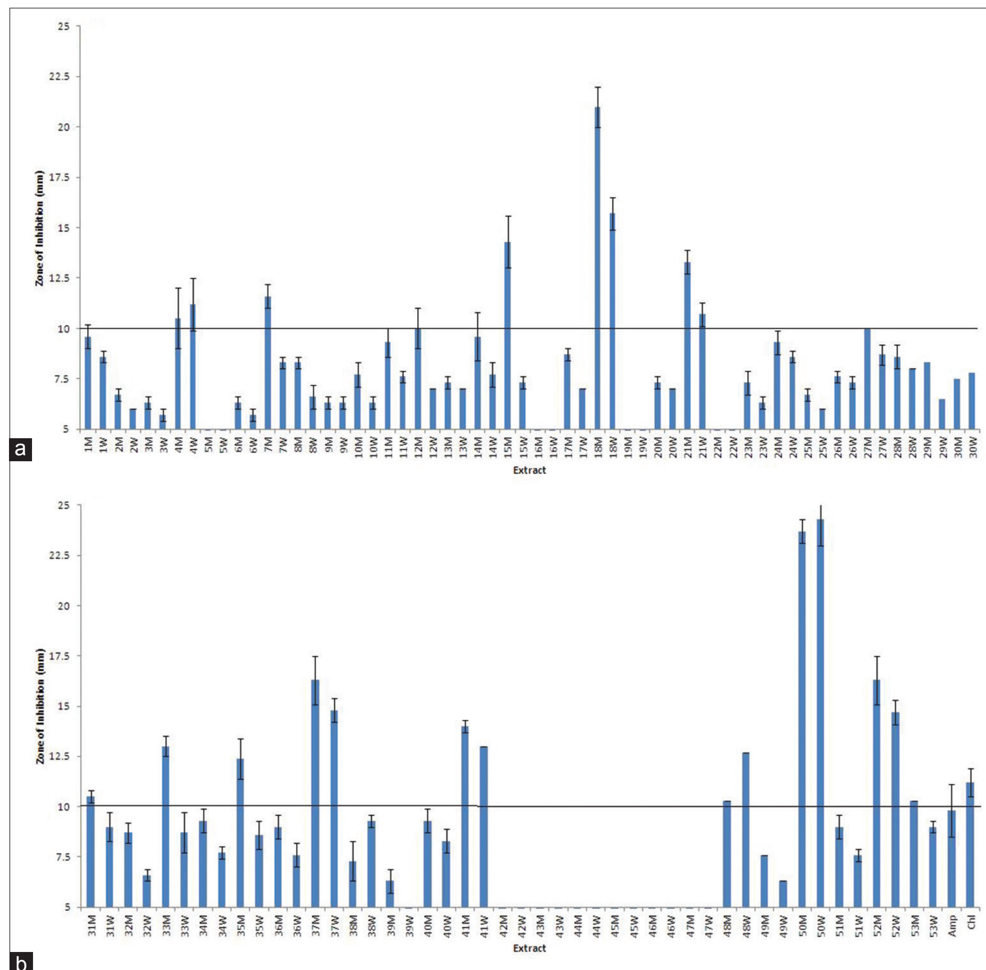


**Figure 2:** Antibacterial activity of plant water and methanolic extracts measured as zones of inhibition (mm) against *Proteus mirabilis*. Inhibition zones are represented as the means of at least triplicate experiments ± standard error of the mean. M and W refer to methanolic and water extracts respectively. (a) 1 = *A. auriculiformis* leaf; 2 = *A. diasparima* leaf; 3 = *A. leptoloba* leaf; 4 = *A. moluccanus* nut; 5 = *A. excelsa* leaf; 6 = *A. caerulea* fruit; 7 = *A. caerulea* leaf; 8 = *B. citriodora* leaf; 9 = *B. myrtiflora* leaf; 10 = *Callistemon citrinus* leaf; 11 = C. *citrinus flower*; 12 = *C. formosus* leaf; 13 = *C. salignus* leaf; 14 = *C. salignus* flower; 15 = *C. oliveri* leaf; 16 = *Davidsonia pruriens* leaf; 17 = *D. pruriens* fruit; 18 = *D. leichhardtii* leaf; 19 = *Elaeocarpus angustifolius* fruit; 20 = *E. baileyana* leaf; 21 = *E. major* leaf; 22 = *Kunzea flavescens* leaf; 23 = *Leptospermum bracteata* leaf; 24 = *L. bracteata* flower; 25 = *L. juniperium* leaf; 26 = *L. juniperium* flower; 27 = *L. longifolium* leaf; 28 = *L. peterson*i leaf; 29 = *Macadamia integriflora* nut; 30 = *M. integriflora* leaf; (b) 31 = *M. alternifolia* leaf; 32 = *M. quinquenervia* leaf; 33 = *Petalostigma pubescens* leaf; 34 = *P. pubescens* fruit; 35 = *P. triloculorae* leaf; 36 = *P. triloculorae* fruit; 37 = *P. augustifolium* leaf; 38 = *Scaevola spinescens* leaf; 39 = *Syzygium anisatum* leaf; 40 = *S. australe* leaf; 41 = *S. australe* fruit; 42 = *S. forte* leaf; 43 = *S. francisii* leaf; 44 = *S. moorei* leaf; 45 = *S. puberculum* leaf; 46 = *S. wilsonii* leaf; 47 = *S. leuhmannii* leaf; 48 = *S. leuhmannii* fruit; 49 = *Tasmannia insipida* leaf; 50 = *T. lanceolata* leaf; 51 = *T. lanceolata* fruit; 52 = *T. lanceolata* peppercorn; 53 = *T. stipitata* leaf; Amp = Ampicillin control (2 μg); Chl = Chloramphenicol control (10 μg)

the zones of inhibition (24.4 mm for the *T. lanceolata* leaf methanolic extract). Indeed, these extracts displayed substantially more potent *P. mirabilis* growth inhibition than either of the control antibiotics (ampicillin and chloramphenicol). The *A. moluccanus* nut, *A. caerulea* leaf, *C. formosus* leaf, *C. oliveri*, *Eucalyptus major*, *L. longifolium*, *M. alternofolia*, *P. pubescens* leaf, *P. triloculare* leaf, *P. augustifolium*, *S. australe* fruit, *S. leuhmannii* fruit, and *T. stipitata* leaf extracts also displayed strong *P. mirabilis* growth inhibition, with zones of inhibition >10 mm and thus were also considered promising anti-*P. mirabilis* extracts.

The relative level of antibacterial activity was further quantified by determining the MIC values for each extract [Table 2]. The *A. moluccanus*, *D. leichardtii, E. major, L. bracteata, L. juniperium, M. integriflora* nut, *M. alternifolia, M. quinquenervia, P. pubescens, P. triloculorae, P. augustifolium, S. spinescens, S. australe,* and *T. lanceolata* extracts were determined to be the most effective inhibitors of *P. mirabilis* growth, with MIC values generally significantly below 1000 μg/ml. *T. lanceolata* fruit extracts were the most potent growth inhibitors, with MICs of 11 and 126 μg/ml for the methanolic and aqueous extracts, respectively. Furthermore, noteworthy was the low MIC values seen for *M. integriflora* nut methanolic extract (15 μg/ml) and the *S. spinescens* leaf water extract (25.4 μg/ml).

### Quantification of toxicity

The plant extracts were serially diluted in artificial seawater for toxicity testing in the *Artemia nauplii* lethality bioassay. For comparison, the reference toxin potassium dichromate was also tested in the bioassay. Figure 3 shows the percentage mortality induced in the *Artemia nauplii* following 24 of exposure. Potassium dichromate (reference toxin) was rapid in its induction of mortality, inducing the onset of mortality within the first 3 h of exposure (results not shown). By 24 h of exposure, potassium dichromate had induced 100% mortality in the *Artemia nauplii*. At 24 h, several extracts induced mortality significantly above that of the seawater control. The *E. baileyana* methanol extract, *S. australe* leaf and fruit methanol and water extracts, *S. leuhmannii* leaf and fruit methanol and water extracts and all extracts of *Tasmannia* spp. induced 100% mortality at 24 h. While appearing less toxic, *C. oliveri* methanol extract, both *D. pruriens* fruit extracts, both *E. angustifolius* extracts, *E. major* methanolic extract and both *S. forte* extracts all induced significant (>50%) mortality in the *Artemia nauplii* bioassay. The levels of mortality for all other extracts following 24 h and exposure was below 50% and thus these extracts were considered nontoxic.

To benchmark the toxicity of the extracts that induced significant mortality for comparison with other toxins, the $LC_{50}$ values of the extracts was determined by testing

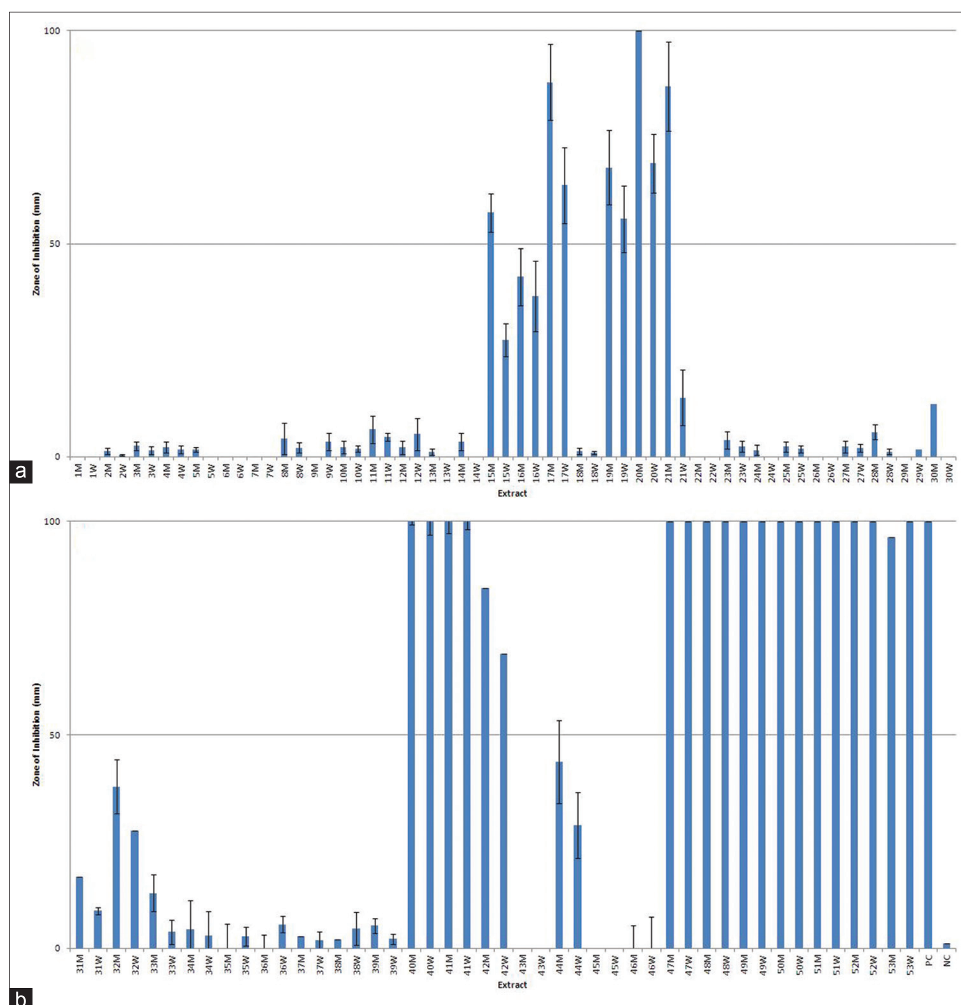


**Figure 3:** The lethality of Australian plant extracts (2000 μg/ml) towards *Artemia franciscana* nauplii after 24 hours exposure. Results are expressed as mean ± SEM of at least triplicate determinations. M and W refer to methanolic and water extracts respectively. (a) 1 = Acacia auriculiformis leaf; 2 = *A. diasparima* leaf; 3 = *A. leptoloba* leaf; 4 = *A. moluccanus* nut; 5 = *A. excelsa* leaf; 6 = *A. caerulea* fruit; 7 = *A. caerulea* leaf; 8 = *Backhousia citriodora* leaf; 9 = *B. myrtiflora* leaf; 10 = *C. citrinus* leaf; 11 = *Callistemon citrinus* flower; 12 = *C. formosus* leaf; 13 = *C. salignus* leaf; 14 = *C. salignus* flower; 15 = *C. oliveri* leaf; 16 = *Davidsonia pruriens* leaf; 17 = *D. pruriens* fruit; 18 = *D. leichhardtii* leaf; 19 = *Elaeocarpus* angustifolius fruit; 20 = *E. baileyana* leaf; 21 = *E. major* leaf; 22 = *Kunzea flavescens* leaf; 23 = *Leptospermum bracteata* leaf; 24 = *L. bracteata* flower; 25 = *L. juniperium* leaf; 26 = *L. juniperium* flower; 27 = *L. longifolium* leaf; 28 = *L. petersoni* leaf; 29 = *Macadamia integriflora* nut; 30 = *M. integriflora* leaf; (b) 31 = *M. alternifolia* leaf; 32 = *M. quinquenervia* leaf; 33 = *Petalostigma pubescens* leaf; 34 = *P. pubescens* fruit; 35 = *P. triloculorae* leaf; 36 = *P. triloculorae* fruit; 37 = *P. augustifolium* leaf; 38 = *Scaevola spinescens* leaf; 39 = *Syzygium anisatum* leaf; 40 = *S. australe* leaf; 41 = *S. australe* fruit; 42 = *S. forte* leaf; 43 = *S. francisii* leaf; 44 = *S. moorei* leaf; 45 = *S. puberculum* leaf; 46 = *S. wilsonii* leaf; 47 = *S. leuhmannii* leaf; 48 = *S. leuhmannii* fruit; 49 = *Tasmannia insipida* leaf; 50 = *T. lanceolata* leaf; 51 = *T. lanceolata* fruit; 52 = *T. lanceolata* peppercorn; 53 = *T. stipitata* leaf. PC = Potassium dichromate positive control (1000 μg/ml); NC = seawater negative control

across the concentration range 2000 μg/ml to 125 μg/ml in the *Artemia nauplii* bioassay. For comparison, potassium dichromate was tested across the same concentration range. Table 2 shows the $LC_{50}$ values of the extracts which had shown toxicity toward *A. franciscana*. While several extracts appeared toxic in the extract mortality screening study [Figure 3], determination of their $LC_{50}$ values demonstrates that most were nontoxic as $LC_{50}$ values of ≥1000 μg/ml have previously been defined as nontoxic in the *Artemia nauplii* bioassay.[41] While the *C. oliveri* methanol extract, both *D. pruriens* fruit extracts, both *E. angustifolius* extracts, *S. australe* leaf extracts and all *Tasmannia* spp. extracts appeared toxic in the screening study [Figure 3], all had $LC_{50}$ values of ≥1000 μg/ml and are therefore considered to be nontoxic. In contrast, all *Eucalyptus* spp. extracts, the *S. australe* fruit extracts and all *S. forte* and *S. leuhmannii* extracts had $LC_{50}$ values ≤1000 μg/ml and were therefore considered to be toxic. While these extracts displayed significant toxicity, potassium dichromate (the positive control) displayed substantially higher toxicity at 24 h (24 h $LC_{50}$ 92.3 μg/ml).

### High performance liquid chromatography-MS/MS analysis of extracts displaying antimicrobial activity

The *Proteus* growth inhibition and MIC studies indicated that *T. lanceolata* fruit extracts had the most promise for further phytochemical characterization. These were examined for the presence of the trans–3,4,5-trihydroxy-trans-stilbene (resveratrol) to examine whether resveratrol may be responsible for the anti-*Proteus* activity observed in these studies. Resveratrol is a potent anti-inflammatory compound produced as a phytoalexin (plant antibiotic) by many diverse plant species including grapes, berries, peanuts[42] as well as some species of pine trees, *Acacias*, Terminalias,[43] orchids, and lilies (Polunin *et al*., 2002).[44]

Optimized HPLC-MS/MS parameters were developed and used to profile the compounds from the methanolic [Figure 4] and aqueous [Figure 5] extractions of *T. lanceolata* fruit. Although the negative ion chromatograms yielded significantly higher signals than those observed for the positive ion (an order of magnitude higher), the negative ion chromatogram had significantly higher background levels, due to ionization of the reference ions in this mode. The *T. lanceolata* fruit methanol extract chromatograms in both positive and negative ion modes [Figure 4] revealed numerous overlapping peaks, particularly in the early and middle stages of the chromatogram corresponding to the elution of polar compounds. Nearly, all of the methanol extract compounds had eluted by 12 min (corresponding to approximately 27% acetonitrile). Indeed, numerous peaks eluted in the first 5 min with 5% acetonitrile. However, a prominent peak

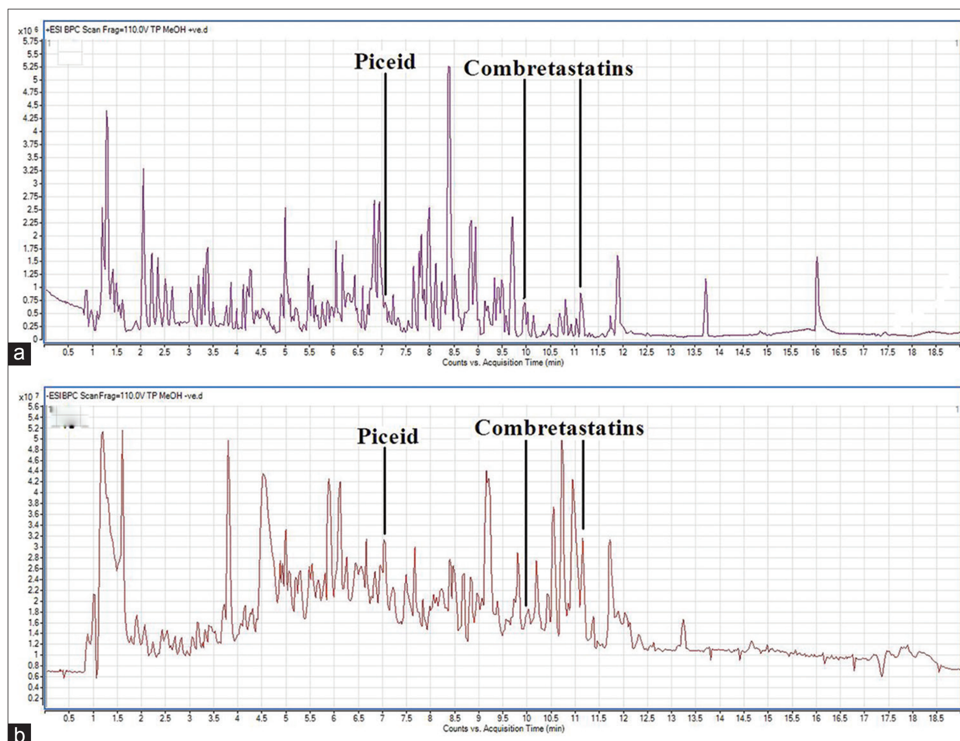


**Figure 4:** Reversed-phase high performance liquid chromatography total peak chromatogram of 10 μl injections of the methanolic extracts of *Tasmannia lanceolata* fruit (a) in positive ionisation mode and (b) in negative ionisation mode. Extracts were dried and resuspended in deionised water. Arrows indicate the stilbene peaks detected in each chromatogram. Chromatography conditions were as described in the methods section

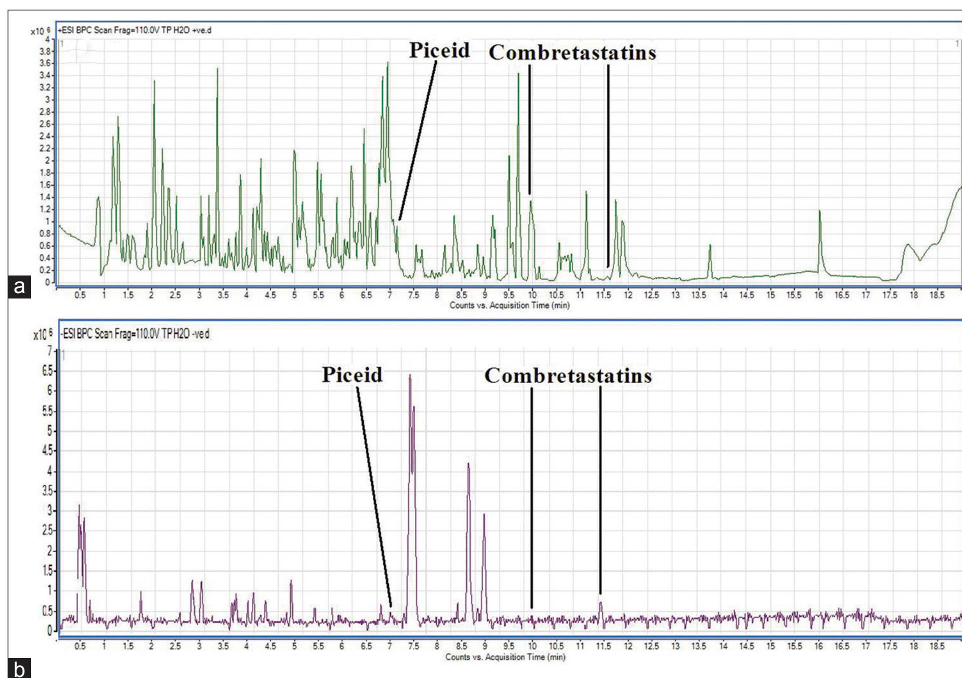


**Figure 5:** Reversed-phase high performance liquid chromatography total peak chromatogram of 10 μl injections of the aqueous extracts of *Tasmannia lanceolata* fruit (a) in positive ionisation mode and (b) in negative ionisation mode. Extracts were dried and resuspended in deionised water. Arrows indicate the stilbene peaks detected in each chromatogram. Chromatography conditions were as described in the methods section

eluting later in the positive ion chromatogram [Figure 4a] at approximately 16 min (corresponding to approximately 44% acetonitrile) indicates the broad spread of polarities of the compounds in this extract. This later eluting peak was not apparent in the *T. lanceolata* fruit methanol extract negative ion chromatogram [Figure 4b].

The *T. lanceolata* fruit water extract chromatograms [Figure 5] also showed large amounts of polar material eluting early in the chromatogram at similar elution volumes as many of the compounds in the methanol extract. The aqueous extracts also had many of the same later eluting peaks seen in the methanolic extracts, including the peak at approximately 16 min. Interestingly, none of the extracts tested displayed peaks corresponding to the molecular mass signal/empirical formula of a pure resveratrol standard in either positive or negative ionisation mode. However, both the methanolic and aqueous *T. lanceolata* fruit extracts displayed small peaks, with molecular weights consistent with picied and combretastatins A-1 and A-4, indicating the presence of only low levels of stilbenes in these extracts. A comparison with the accurate mass databases putatively identified these compounds as Piceid (molecular weight 404.1465 Da), combretastatin A-1 (molecular weight 332.1319 Da) and combretastatin A-4 (molecular weight 316.1287 Da).

An examination of the fragmentation ions in positive mode provided further evidence of the putative identity of these stilbenes in the *T. lanceolata* fruit extracts [Table 3]. Fragmentation of the compound identified as Piceid (identified by comparison to molecular mass databases) yielded peaks corresponding to the molecular ion mass (404.1465 Da) as well as fragments corresponding to 286.1453, 270.0472, 228.1447, 124.1348, and 108.1212 Da. The empirical formulae corresponding to these fragments were determined using the Find by Molecular Feature algorithm in the Masshunter Qualitative analysis software package (Agilent Technologies). The molecular formulae $C_{13}H_{18}O_7$, $C_6H_{12}O_6$, $C_{14}H_{12}O_3$, $C_7H_8O_2$, and $C_7H_8O$ were assigned for the fragments, respectively. Comparison with the molecular mass databases indicated the putative structures of these fragments [Figure 6]. It is likely that fragments b1 and b5 arise from a cleavage of the glucose moiety from the resveratrol moiety of Piceid. Thus, these fragments were putatively identified as resveratrol (228.1447 Da, $C_{14}H_{12}O_3$) and glucose (108.1212 Da, $C_6H_{12}O_6$) respectively. Similarly, fragments b2 (108.1212 Da, $C_7H_8O$) and b4 (124.1348 Da, $C_7H_8O_2$) were assigned to the products arising from the cleavage of resveratrol (b1, 228.1447 Da, $C_{14}H_{12}O_3$). These structural assignments were supported by the presence of fragment b3 ($C_{13}H_{18}O_7$), which would result from glucose (b5) combining with fragment b4.

A similar strategy was used to confirm the structures of the combretastatins. Fragmentation of the compound identified as combretastatin A-1 yielded peaks corresponding to the molecular ion mass (332.1319 Da) as well as fragments corresponding to 182.1872 and 152.1445 Da. The molecular formulae $C_{10}H_{14}O_3$ and $C_8H_8O_3$ were assigned for these

**Table 3: Compound MWs (calculated and measured) and fragment MWs of prominent peaks detected in electron spray ionisation mass spectroscopy. Mass spectral analysis was run in positive ion mode**

| | Piceid | | Combretastatin A-1 | | Combretastatin A-4 | |
|---|---|---|---|---|---|---|
| | MW (Da) | Empirical formula | MW (Da) | Empirical formula | MW (Da) | Empirical formula |
| MW (calculated) | 404.4105 | $C_{21}H_{24}O_8$ | 332.3478 | $C_{18}H_{20}O_6$ | 316.3484 | $C_{18}H_{20}O_5$ |
| MW (measured M$^+$) | 404.1465 | $C_{21}H_{24}O_8$ | 332.1319 | $C_{18}H_{20}O_6$ | 316.1287 | $C_{18}H_{20}O_5$ |
| MW of prominent peaks | 286.1453 | $C_{13}H_{18}O_7$ (b3) | 182.1872 | $C_{10}H_{14}O_3$ (c2) | 182.1788 | $C_{10}H_{14}O_3$ (d1) |
| | 270.0472 | $C_6H_{12}O_6$ (b5) | 152.1445 | $C_8H_8O_3$ (c1) | 138.1463 | $C_8H_{10}O_2$ (d2) |
| | 228.1447 | $C_{14}H_{12}O_3$ (b1) | | | | |
| | 124.1348 | $C_7H_8O_2$ (b4) | | | | |
| | 108.1212 | $C_7H_8O$ (b2) | | | | |

b1, b2, b3, b4, b5, c1, c2, d1, d2 refer to the molecular fragments shown in Figure 6. MW: Molecular weight

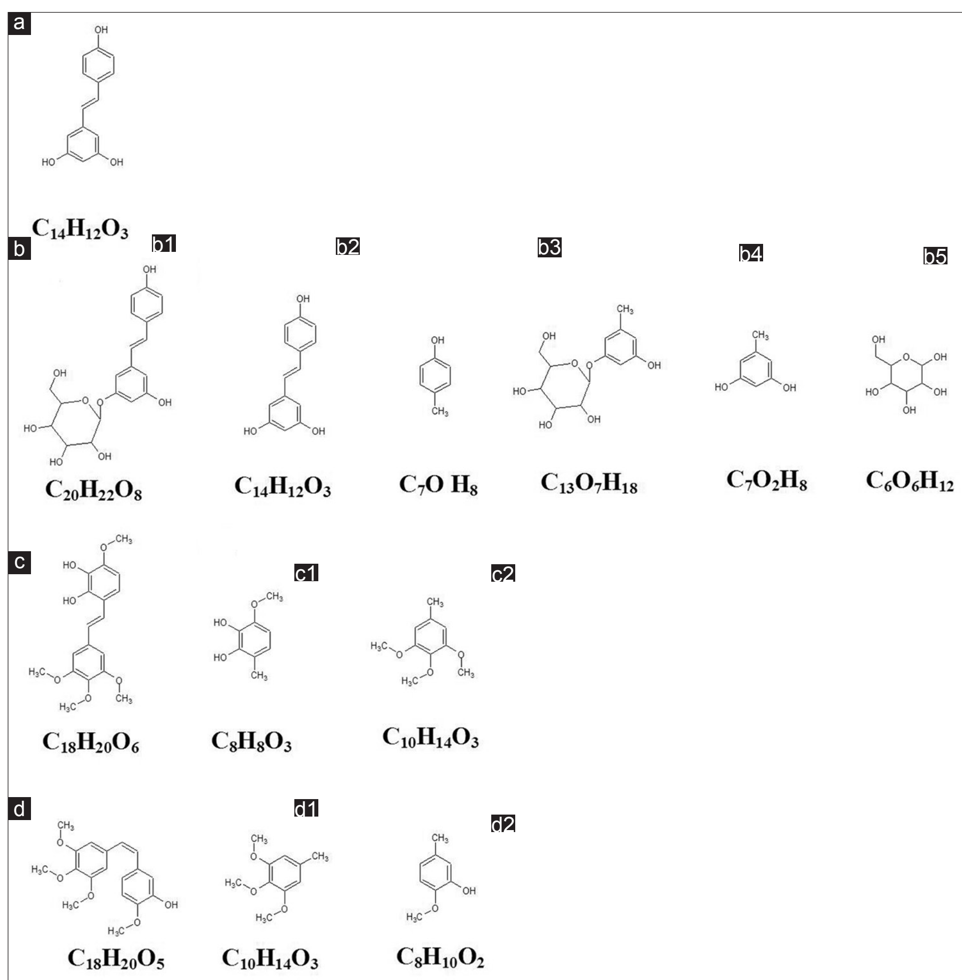


**Figure 6:** Chemical structures of (a) resveratrol and the stilbenes and stilbene glycosides identified in *Tasmannia lanceolata* extracts: (b) piceid, (c) combretastatin A-1, (d) combretastatin A-4. (b1-b5) represent the molecular weight fragments of piceid detected by ESI-MS in positive mode; (c1-c2) represent the molecular weight fragments of combretastatin A-1 detected by ESI-MS in positive mode; (d1-d2) represent the molecular weight fragments of combretastatin A-4 detected by ESI-MS in positive mode

fragments, respectively. Comparison with the molecular mass databases indicated the putative structures of these fragments [Figure 6c1 and 6c2]. These fragments were assigned to the cleavage of combretastatin A-1 (332.1319 Da, $C_{18}H_{20}O_6$) to 182.1872 Da ($C_{10}H_{14}O_3$) and 152.1445 Da ($C_8H_8O_3$) fragments.

The compound putatively identified as combretastatin A-4 [Figure 6d; 316.1287 Da, $C_{18}H_{20}O_5$] also fragmented, giving 182.1788 Da ($C_{10}H_{14}O_3$), providing further evidence of the structural similarity of the compounds putatively identified as combretastatins A-1 and A-4. A further fragment (138.1463 Da) was detected in the fragmentation

of combretastatin A-4. The molecular formula $C_8H_{10}O_2$ was assigned to this fragment by comparison with the molecular mass databases. From a comparison of the proposed fragmentation patterns with the putative molecular ion structure, it is evident that the combretastatin A-4 structure is consistent with these results.

## DISCUSSION

Plant remedies are becoming increasingly sought after in the treatment of a myriad of diseases and disorders due to their perception of greater safety than synthetic drugs, as well as the failure of current drug regimes to effectively treat many diseases. This is especially true for RA. The current treatments utilizing DMARDs to alleviate the symptoms of RA and/or alter the disease progression are not entirely effective and have been associated with numerous adverse effects.[2] Furthermore, many of the current treatments are aimed at treating the symptoms of RA without addressing the underlying causes and disease mechanisms. An understanding of the initiation and progression of RA is important to allow for the development of new drugs to target specific processes and thus more effectively treat the disease.

The role of *P. mirabilis* infection as a trigger of RA is now widely accepted due to a wealth of supporting evidence.[15] A treatment regime targeting the microbial trigger of RA is an attractive prospect as it would be expected to block/decrease production of self-reactive antibodies and thus effectively block disease progression. The results presented here demonstrate the ability of a wide variety of plants traditionally used to treat RA and inflammatory complaints in Australian Aboriginal medicinal systems to inhibit *P. mirabilis* growth when tested in the forms that they would have been used in traditional medicine (decoctions and tinctures). Further quantification by MIC determination demonstrated the efficacy of these extracts as inhibitors of the microbial trigger of RA. The *T. lanceolata* extracts were of particular interest, with MIC's as low as 11 μg/ml (0.11 μg in the disc) for the methanolic fruit extracts. Indeed, these extracts proved to be substantially stronger than the control antibiotics (ampicillin and chloramphenicol) and are thus worthy of further mechanistic studies. The current study was limited to the inhibitory activity toward the microbial trigger of RA. It is possible that many of the plant extracts studied here may also have effects on other inflammatory processes (e.g. cytokine release) and, therefore, may have pluripotent anti-RA mechanisms.

The major anti-inflammatory components of many medicinal plants are known and are already available in a pure form as anti-inflammatory agents. These compounds include a variety of polyphenolic compounds, of which 3,5,4'-trihydroxy-trans-stilbene (resveratrol) has received much recent attention.[45] Most studies examining the role of resveratrol in the treatment of RA concern its ability to act as a potent specific inhibitor of nuclear factor (NF)-κB activation through its induction by tumor necrosis factor alpha and interleukin-1 beta (IL-1 β).[45] Thus, resveratrol treatment is known to block cytokine production and inflammation via its inhibition of NF-κB activation. However, it is possible that resveratrol may also have other anti-rheumatic effects as it also has antibacterial activity against a variety of bacterial species.[46] It was therefore thought likely that it may also function to block the microbial trigger of RA and the plant extracts examined in this study were screened for its presence.

Interestingly, while resveratrol has been reported to have inhibitory activity against other bacterial species, it was unable to inhibit the growth of *P. mirabilis* in our studies, even at high concentrations (unreported results). Furthermore, we were unable to find any reports of *in vitro* anti-*Proteus* growth inhibition in the published literature. Several studies have reported on the ability of resveratrol to inhibit *P. mirabilis* swarming and virulence factor expression *in vivo*, so it is likely that resveratrol does affect *P. mirabilis* colonization and infection of the urinary tract,[47] albeit possibly by mechanisms other than bactericidal or growth inhibition mechanisms. Any extracts that were capable of inhibiting *Proteus* spp. growth and also contained the resveratrol would be likely to have anti-RA activity via several mechanisms (growth inhibition, colonization blocking, cytokine production inhibition) and, therefore, would be particularly effective in treating RA.

High accuracy HPLC-MS/MS analysis of the *T. lanceolata* fruit extracts failed to detect resveratrol in either extract. However, it is possible that resveratrol may still contribute to the anti-inflammatory activities of these extracts. The resveratrol glycoside Piceid (2-[3-hydroxy-5[(E)-2-(4-hydroxyphenyl) ethenyl] phenoxy]-6-(hydroxymethyl) oxane-3,4,5-triol) was detected in both *T. lanceolata* fruit extracts. Piceid may be hydrolysed *in vivo* to remove glucose, thus releasing the resveratrol moiety. Furthermore, while other resveratrol glycosides like polydatin were not detected by matching with the compound databases used in these studies, it is possible that they may still be present in small quantities. Piceid and other glycosylated stilbenes (if present) may also contribute to the anti-inflammatory activity of *T. lanceolata* fruit extracts via mechanisms other than *Proteus* growth inhibition. Several glycosylated stilbenes (including Piceid) have both been shown to block inflammation by decreasing IL-17 production in stimulated human mononuclear cells.[48] Another study[49] determined that the antibacterial

activity of resveratrol is due to a protein tyrosine kinase activity. This same study also reported that resveratrol glycosides do not have the same bacterial inhibitory activity, indicating that free hydroxyl groups on both phenyl groups are required for antibacterial activity.

In addition to Piceid, other stilbenes were also detected in the *T. lanceolata* fruit extracts. Combretastatins A -1 and A -4 were detected in both *T. lanceolata* fruit extracts, although the relatively low peak sizes indicate that both compounds are present in low abundance. Combretastatins are well -known for their potent ability to block cancer cell progression and induce apoptosis by binding intracellular tublin, thereby disrupting microtubule formation.[50] Accounts of direct anti -inflammatory activity of combretastatins are lacking. However, it is believed that they act by a similar mechanism to that of colchicine (N -[(7S) -1,2,3,10 -tetramethoxy -9 -oxo -5,6,7,9 -tetrahydrobenzo[a] heptlen -7 -yl] acetamide) by binding the colchicine binding site on the tubulin peptide and inhibiting polymerization.[51] It is likely that they may have a similar anti -inflammatory activity and mechanism to colchicine.

We were also unable to find accounts of the antibacterial activities of natural combretastatins in the literature. However, recent studies have examined the growth inhibitory activity of several synthetic combretastatin and resveratrol structural analogues.[52] These studies reported potent growth inhibition toward a panel of bacteria including *Staphylococcus aureus*, *Streptococcus pneumoniae*, *Escherichia coli,* and *Neisseria gonorrhoeae*. Especially interesting was the low MIC values of some analogues against *N. gonorrhoeae,* although several other species were also highly susceptible to the modified stilbenes. The same study also reported potent inhibitory effects of the panel of modified stilbenes against the fungal species *Candida albicans* and *Cryptococcus neoformans*.

A number of other inflammatory stilbenes have also previously been reported in other plant species. For example, 2,3,4,5-tetrahydroxystilbene-2-O-β-D-gluco side (TSG) inhibits inflammation by suppressing the induction of pro-inflammatory mediators by reducing NF-κB binding to DNA.[53] The same study detected TSG in numerous herbs used to treat inflammation in Chinese traditional medicine. Furthermore, nine stilbene and stilbene derivatives isolated from the roots of *Cicer* spp. (chickpeas) were shown to inhibit bacterial and fungal growth.[54] While these compounds were not detected in the compound databases used in our studies, it is possible that they may still be present in small quantities.

It is likely that other phytochemical classes may also contribute to the anti-inflammatory properties of these extracts. Alkaloids, anthraquinones, flavonoids, polyphenolics, phytosterols, saponins, tannins, and terpenes have also been linked with anti-bacterial activity in different plant species and thus may be responsible (at least in part) for the anti-anti-*Proteus* activities reported here. Several terpenoids previously reported in *T. lanceolata* fruit extracts have been reported to suppress NF-κB signaling (the major regulator of inflammatory diseases).[55] The monoterpenes limonene[56,57] and α-pinene[58] have been reported to inhibit NF-κB signaling pathways. α-Pinene affects inflammation by inhibiting p65 translocation into the nucleus in *Lipopolysacccharide*-induced NF-κB signaling.[58] Furthermore, many other sesquiterpenes and sesquiterpene lactones also have well-established anti-inflammatory activities.[55] While much work is still needed to characterize the mechanisms of action of these compounds, it appears that NF-κB inhibitory activities may be responsible.

The antimicrobial activity of *Drimys winteri* (a species closely related to *T. lanceolata*) essential oils has been well-documented against a variety of bacterial species, and it has been established that terpenoids contribute to this activity.[59] *Drimys winteri* essential oils contain many of the same monoterpenoid constituents as *T. lanceolata* essential oils (including polygoidal, α-pinene, β-pinene, sabinene, mycrene, terpinene, limonene, and β-phellandrene). That study demonstrated good antibacterial activities for all of these compounds. Further studies have also shown that the monoterpene piperitone reduces the resistance of several strains of Enterobacteriaceae to the antibacterial agent nitrofurantoin.[60] Other studies have reported similar antibacterial activities for the sesquiterpenoids α-cubebene, copaene, and caryophyllene isolated from *Pilgerodendron uviferum*.[61]

This study was limited to an examination of the phytochemistry of the most potent *P. mirabilis* growth inhibitor. Further studies are needed to examine the compounds present in the other highly potent extracts. Specifically, the *A. moluccanus*, *D. leichardtii, E. major, L. bracteata, L. juniperium, M. integriflora* nut, *M. alternifolia, M. quinquenervia, P. pubescens, P. triloculorae, P. augustifolium, S. spinescens* and *S. australe* extracts should also be screened for the presence of stilbenes.

The findings reported here also demonstrate that the majority of plant extracts tested did not display significant toxicity in the *Artemia* nauplii bioassay. Indeed, with the exception of some *Eucalyptus* and *Syzygium* extracts, all other extracts exhibiting *Proteus* inhibitory activity were also shown to be nontoxic, or of low toxicity in the *Artemia nauplii* bioassay. Only extracts from these species displayed $LC_{50}$ values below 1000 μg/ml and

therefore all other plant extracts that inhibit *P. mirabilis* growth are defined as nontoxic as compounds with an $LC_{50}$ of >1000 μg/ml toward *Artemia* nauplii have been previously defined as being nontoxic.[41] However, even the *Eucalyptus* spp. extracts (with LC50 values generally >650 μg/ml) are considered of only moderate toxicity.

## CONCLUSIONS

The results of this study partially validate the usage of these plants in traditional Australian Aboriginal medicinal systems to treat RA and other inflammatory conditions and indicate that the phytochemistry and mechanism of action are worthy of further study. Similarly, it is also of interest to screen these extracts for further activities associated with the treatment of RA (e.g. inhibition of cytokine production) to further evaluate their potential as anti-RA drugs. While the extracts examined in this report are promising as anti-RA agents, caution is needed before these compounds can be applied to medicinal purposes. In particular, further toxicity studies using human cell lines are needed to verify the suitability of these extracts for these purposes.

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