Growth inhibitory properties of extracts prepared from selected *Leptospermum* and *Melaleuca* species against a panel of pathogenic bacteria

Lindiwe Nomathemba Mpala¹, Getmore Rumbudzai Chikowe¹, Ian Edwin Cock¹,²*¹

¹School of Natural Sciences, Griffith University, 170 Kessels Rd, Nathan, Brisbane, Queensland 4111, AUSTRALIA.
²Environmental Futures Research Institute, Griffith University, 170 Kessels Rd, Nathan, Brisbane, Queensland 4111, AUSTRALIA.

**ABSTRACT**

**Introduction:** *Leptospermum longifolium* (C.T. White & W.D. Francis) S.T. Blake, *Leptospermum petersonii* Bailey and *Melaleuca alternifolia* (Maiden & Betche) Cheel are aromatic native Australian trees with uses as traditional medicines. Essential oils produced from leaves of these species have reputed antiseptic properties against many bacteria. Despite this, *L. longifolium*, *L. petersonii* and *M. alternifolia* leaf solvent extractions have not been rigorously examined for antibacterial properties against many pathogens. **Methods:** The antimicrobial activity of methanolic *L. longifolium*, *L. petersonii* and *M. alternifolia* leaf extracts was investigated by disc diffusion and growth time course assays against a panel of pathogenic bacteria. The growth inhibitory activity was quantified by MIC determination. **Toxicity** was determined using the *Artemia franciscana* nauplii bioassay. **Results:** The methanolic *L. longifolium*, *L. petersonii* and *M. alternifolia* leaf extracts inhibited the growth of a wide range of bacterial species. Growth of both gram positive and gram negative bacteria was inhibited by all extracts. The *L. longifolium* and *L. petersonii* extracts were generally more potent inhibitors of bacterial growth than was the *M. alternifolia* extract against most bacterial species. *A. hydrophila*, *C. freundii*, *P. mirabilis* and *B. cereus* growth was particularly susceptible to the extracts, with MIC values as low as 147 µg/mL (inhibition of *A. hydrophila* growth by the *L. longifolium* extract). The antibacterial activity of the *L. longifolium*, *L. petersonii* and *M. alternifolia* extracts were further investigated by growth time course assays, with significant growth inhibition recorded in all cultures within 1 h of exposure. All extracts were determined to be nontoxic in the *Artemia franciscana* nauplii bioassay, indicating their safety for therapeutic uses. **Conclusions:** The lack of toxicity of the methanolic *L. longifolium*, *L. petersonii* and *M. alternifolia* leaf extracts and their growth inhibitory bioactivity against a panel of pathogenic bacteria partially validate Australian Aboriginal usage of these species as antiseptic agents and indicate their potential in the development of antiseptic agents.

**Key words:** Myrtaceae, *Leptospermum longifolium*, *Leptospermum petersonii*, *Melaleuca alternifolia*, Tea-tree, Australian plants, Antibacterial activity, Medicinal plants.

**Correspondence:** Ian Edwin Cook, School of Natural Sciences, Griffith University, 170 Kessels Rd, Nathan, Brisbane, Queensland 4111, AUSTRALIA.
Phone no: +61 7 3735 7637; Fax: +61 7 3735 5282
E-mail: I.Cock@griffith.edu.au (I. E. Cock)
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**INTRODUCTION**

Plants produce a wide variety of secondary metabolites which provide characteristic pigment, odour and flavour characteristics. In addition, these compounds may also provide the plants with protection against microbial challenge.¹ Traditional plant derived medicines have been used for thousands of years in most parts of the world and with the increase in microbial antibiotic resistance, their use in fighting bacterial pathogens is becoming the focus of intense study.²,³ Whilst much of the research into traditional medicinal plant use has focused on Asian,⁴ African⁵ and South American⁶ plants, the therapeutic potential of the flora of Australia has also been recognised for thousands of years. The first Australians had well developed ethnopharmacological systems and understood the therapeutic properties of a wide variety of aromatic Australian plants.⁷ Despite this, relatively few studies have rigorously examined the antibacterial activity of Australian native plants. However, recently there has been increased study in this field.

The healing properties of Australian plants of the family Myrtaceae have been understood by Australian Aborigines for many thousands of years. The genuses *Leptospermum* and *Melaleuca* are particularly prevalent in traditional Aboriginal healing systems.⁷ More recently, Sir Joseph Banks (the botanist aboard Captain James Cook’s voyage of the Endeavour) coined the phrase ‘tea-tree’ for the endemic Australian plant *Melaleuca alternifolia* due to similarities in use with *Camellia sinensis*, and some of its perceived therapeutic properties.⁷ However, this common name creates some taxonomic confusion, with several *Leptospermum* and *Kunzea* species also collectively known as ‘tea-tree’. Nowadays, a thriving trade exists, with ‘tea tree’ essential oils marketed globally as an antiseptic. The bacterial growth inhibitory properties of many genera within the family Myrtaceae have been documented. In particular, *Callistemon* spp.,⁸ *Eugenia* spp.,⁹ *Kunzea* spp.,¹⁰ *Leptospermum* spp.,¹¹,¹² and *Syzygium* spp.¹³–¹⁵ have been reported to inhibit the growth of a wide panel of bacteria, including many medically important pathogens. It is noteworthy that many of these studies have screened essential oils for antibacterial activity and the growth inhibitory properties of several Myrtaceae extracts are yet to be rigorously examined.

*Leptospermum longifolium* (C.T. White & W.D. Francis) S.T. Blake (Figure 1a) and *Leptospermum petersonii* Bailey (Figure 1b) are large shrubs/small trees which are native to sclerophyll forests and subtropical rainforest areas of eastern Australia. *Melaleuca alternifolia* (Maiden & Betche) Cheel (commonly known as narrow leaved tea-tree, Figure 1c) is a related species which has a similar but limited occurrence in eastern Australian subtropical sclerophyll forests, particularly besides streams and in swampy areas.⁷ The leaves of all 3 species are distilled commercially to produce essential oils rich in geraniol (Figure 1d), neral (Figure 1e), citronellal (Figure 1f), pinene (Figure 1g), sabinenene (Figure 1h), α-terpinene (Figure 1i), limonene (Figure 1j) and 1,8-cineole (Figure 1k).⁷ Most studies into the antibacterial potential of the family Myrtaceae focus on the essential oil of the leaves.⁷,¹⁵,¹⁶ In most *Leptospermum* and
Melaleuca species, the levels and composition of the essential oil terpenoid components receives the most interest. In particular, the monoterpenoid compositions of several species have been extensively reported. \(^7\) Leptospermum spp. essential oils contain especially high levels of citral, which comprises a mixture of geranial (β-citral; Figure 1d) and neral (α-citral; Figure 1e). \(^7,13,14\) Both neral and geranial have been previously reported to have potent antibacterial activity against a variety of bacteria. \(^17,18\) Similarly, a high composition of other monoterpenoids in various Myrtaceae essential oils has been reported, with citronellal (Figure 1f), pinene (Figure 1g), sabine (Figure 1h), α-terpinene (Figure 1i), limonene (Figure 1j) and 1,8-cineole (Figure 1k) often reported as major components. The antiseptic properties of these compounds is well established, with potent bacteriostatic and bactericidal activities reported. \(^2\)

Most of the studies reporting antibacterial properties for Leptospermum spp. and Melaleuca spp. have examined essential oils. However, the use of essential oils for the testing of antimicrobial activity can be problematic. The relative insolubility of many of the oil components retards their diffusion through agar gels in agar dilution or disc diffusion studies. Many studies have utilised solubilising agents (e.g. Tween 80) to aid oil component diffusion, resulting in variable results. \(^20,21\) Solubilising agents appear to increase the susceptibility of some bacteria to antimicrobial agents, decrease the susceptibility of others, whilst having no effect on yet other bacteria. The current study was undertaken to examine 3 taxonomically related Myrtaceae spp. (L. longifolium, L. petersonii and M. alternifolia) for growth inhibitory properties against a panel of pathogenic bacteria.

**MATERIALS AND METHODS**

**Plant collection and extraction**

Leptospermum longifolium (C.T. White & W.D. Francis) S.T. Blake, Leptospermum petersonii Bailey and Melaleuca alternifolia (Maiden & Betches) Chees leaves were obtained from and identified by Philip Cameron, senior botanic officer, Mt Cootha Botanical Gardens, Brisbane, Australia. Leaf samples were dried in a Sunbeam food dehydrator and stored at -30°C. Prior to use, the leaves were freshly ground to a coarse powder and 1 g quantities were weighed into separate tubes. A volume of 50 mL methanol (Ajax, Australia; AR grade) was added to individual tubes and extracted for 24 hrs at 4°C with gentle shaking. The extract was filtered through filter paper (Whatman No. 54) under vacuum, followed by drying by rotary evaporation in an Eppendorf concentrator 5301. The resultant pellets were dissolved in 10 mL sterile deionised water (containing 1% DMSO). The extracts were passed through a 0.22 µm filter (Sartorius) and stored at 4°C until use.

**Qualitative phytochemical studies**

Phytochemical analysis of the L. longifolium, L. petersonii and M. alternifolia leaf extracts for the presence of saponins, phenolic compounds, flavonoids, phytosteroids, triterpenoids, cardiac glycosides, anthraquinones, tannins and alkaloids was conducted by previously described assays. \(^7,22-24\)

**Antibacterial screening**

**Test microorganisms**

All media was supplied by Oxoid Ltd., Australia. Clinical isolate microbial strains of Aeromonas hydrophilia, Alcaligenes faecalis, Bacillus cereus, Citrobacter freundii, Escherichia coli, Klebsiella pneumoniae, Proteus mirabilis, Pseudomonas fluorescens, Salmonella newport, Serratia marcescens, Shigella sonnei, Staphylococcus aureus, Staphylococcus epidermidis and Streptococcus pyogenes were obtained from Ms Michelle Mendell and Ms Jane Gikins, Griffith University. All stock cultures 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 assay. \(^25-27\) Briefly, 100 µL of each bacterial culture was grown in 10 mL of fresh nutrient broth until they reached a count of ~10⁶ cells/mL. A volume of 100 µL of the bacterial suspension was spread onto nutrient agar plates and extracts were tested for antibacterial activity using 5 mm sterilised filter paper discs. Discs were infused with 10 µL of the plant extracts, allowed to dry and placed onto the inoculated plates. The plates were allowed to stand at 4°C for 2 h before incubation at 30°C for 24 h. The diameters of the inhibition zones were measured to the closest whole millimetre. Each assay was performed in at least triplicate. Mean values (± SEM) are reported in this study. Standard discs of ampicillin (10 µg) were obtained from Oxoid, Australia and were used as positive controls to compare antibacterial activity. Filter discs infused with 10 µL of distilled water were used as a negative control.

**Minimum inhibitory concentration (MIC) determination**

The minimum inhibitory concentration (MIC) of each extract against susceptible bacteria was determined as previously described. \(^28,29\) Briefly, the plant extracts were diluted in deionised water and tested across a range of concentrations. Discs were infused with 10 µL of the test dilutions, allowed to dry and placed onto inoculated plates. The assay was completed as outlined above and graphs of the zone of inhibition versus concentration were plotted for each extract. Linear regression was used to determine the MIC values of each extract.

**Bacterial growth time course assay**

Bacterial growth time course studies were performed as previously described. \(^30\) Briefly, 3 mL of the and A. hydrophilia, C. freundi and P. mirabilis bacterial cultures in nutrient broth were added individually to 27 mL nutrient broth containing 3 mL of 10 mg/mL methanolic plant extract to give a final concentration of 1000 µg/mL in the assay. The tubes were incubated at 30°C with gentle shaking. The optical density was measured hourly at 550 nm for a 6 h incubation period. Control tubes were incubated under the same conditions but without the extract. All assays were performed in triplicate.

**Toxicity screening**

**Reference toxin for toxicity screening**

Potassium dichromate (K₂Cr₂O₇) (AR grade, Chem-Supply, Australia) was prepared as a 4 mg/mL solution in distilled water and was serially diluted in artificial seawater for use in the Artemia franciscana nauplii bioassay.

**Artemia franciscana nauplii toxicity screening**

Toxicity was tested using an adapted Artemia franciscana nauplii lethality assay. \(^31-33\) Briefly, 400 µL of seawater containing approximately 54 (mean 54.2, n=75, SD 11.5) A. franciscana nauplii were added to wells of a 48 well plate and immediately used for bioassay. A volume of 400 µL of diluted plant extracts or the reference toxin were transferred to the wells and incubated at 25 ± 1°C under artificial light (1000 Lux). A 400 µL seawater negative control was run in 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 dead if no movement of the appendages was detected within 10 seconds. After 24 h, all nauplii were sacrificed and counted to determine the total % mortality per well. The LC₅₀ₐ with 95% confidence limits for each treatment was determined using probit analysis.
Statistical analysis
Data are expressed as the mean ± SEM of at least three independent experiments. One way ANOVA was used to calculate statistical significance between control and treated groups with a P value < 0.01 considered to be statistically significant.

RESULTS
Liquid extraction yields and qualitative phytochemical screening
Extraction of 1 g of L. longifolium, L. petersonii and M. alternifolia leaves with methanol yielded dried extracts ranging from 93 mg (methanolic M. alternifolia extract) to 130 mg (methanolic L. longifolium extract) (Table 1). The dried extracts were resuspended in 10 mL of deionised water (containing 1% DMSO), resulting in the extract concentrations shown in Table 1. Qualitative phytochemical studies showed that all extracts contained similar classes of phytochemicals. All had high levels of phenolics and tannins. Generally, all extracts also contained moderate levels of flavonoids and moderate to high levels of saponins. Low to moderate levels of triterpenoids, phytosterols and alkaloids were also present in all extracts. The L. longifolium, L. petersonii and M. alternifolia extracts were devoid of all other classes of phytochemicals.

Antimicrobial activity
To determine the growth inhibitory activity of the L. longifolium, L. petersonii and M. alternifolia extracts against the panel of pathogenic bacteria, aliquots (10 µL) of each extract were screened in the disc diffusion assay. All extracts inhibited a broad spectrum of the gram negative bacterial species (Figure 2). Indeed, all extracts inhibited the growth of 5 of the 10 gram negative bacteria screened (50%). The methanolic L. longifolium extract was a potent growth inhibitor against most bacterial species (as assessed by the sizes of the zones of inhibition), with zones of inhibition >10 mm against A. hydrophilia, C. freundi and P. mirabilis. This inhibition was particularly noteworthy compared to the inhibition by the ampicillin (10 µg: inhibition zones of approximately 8.6, 8.3 and 10.3 mm respectively against the same bacterial species) and chloramphenicol controls (2 µg: inhibition zones of approximately 9.7, 9.3 and 8.6 mm respectively against the same bacterial species). The L. longifolium extract was also a good inhibitor of A. faecalis and K. pneumoniae growth, albeit with smaller zones of inhibition (< 10 mm). Similar bacterial growth inhibitory trends were noted for the L. petersonii and M. alternifolia extracts against the gram negative bacterial species (Figure 2). As reported for the L. longifolium extract, the growth of A. faecalis, A. hydrophilia, C. freundi, K. pneumoniae and P. mirabilis was strongly inhibited by the L. petersonii and M. alternifolia extracts.

The growth of gram positive bacteria was also potently inhibited by the L. longifolium, L. petersonii and M. alternifolia extracts (Figure 3). All of the gram positive bacterial species tested were inhibited by each of the extracts. B. cereus and S. aureus were the most susceptible gram positive bacteria (as judged by the zones of inhibition), with zones of inhibition as large as 10.6 mm (L. longifolium and L. petersonii inhibition of S. aureus). Notably, the Leptospermum spp. extracts were substantially more potent inhibitors of gram positive bacterial growth than was the M. alternifolia extract.

The antimicrobial efficacy was further quantified by determining the MIC values for each extract against the microbial species which were determined to be susceptible. All extracts were potent growth inhibitors of several bacterial species (as judged by MIC; Table 2). P. mirabilis was highly susceptible to all of the extracts, with MIC values generally <500 µg/mL (<5 µg infused into the disc). As P. mirabilis infection is a common cause of urinary tract infections and has also been identified as a trigger of rheumatoid arthritis, the L. longifolium, L. petersonii and M. alternifolia leaf extracts have potential for the prevention and treatment of these diseases in genetically susceptible individuals.

The L. longifolium and L. petersonii extracts, but not the M. alternifolia extract, were also potent K. pneumoniae growth inhibitors (MICs of 320 and 687 µg/mL for the L. longifolium and L. petersonii extracts respectively). As K. pneumoniae can trigger ankylosing spondylitis in genetically susceptible individuals, these extracts may also be useful in the prevention and treatment of this disease. Furthermore, all extracts were moderate inhibitors of S. pyogenes growth, with MIC values 1000-1500 µg/mL. S. pyogenes may cause a myriad of diseases including streptococcal pharyngitis, impetigo and rheumatic heart disease, depending on which tissue it infects. Thus, the methanolic L. longifolium, L. petersonii and M. alternifolia leaf extracts may also be useful in the prevention and treatment of these diseases.

Bacterial growth time course assay
The antibacterial activity of the L. longifolium, L. petersonii and M. alternifolia extracts was further investigated in the reference bacterial strains by bacterial growth time course assays in the presence and absence of the extract. As A. hydrophilia, C. freundi, and P. mirabilis were the most susceptible to the L. longifolium, L. petersonii and M. alternifolia extracts, these species were selected for growth time course studies. The starting concentration of the extract used in these assays was 1000 µg/mL. All extracts significantly inhibited A. hydrophilia (Figure 4a), C. freundi (Figure 4b) and P. mirabilis (Figure 4c) growth within 1 h, indicating a rapid antimicrobial action. Furthermore, growth inhibition of all bacterial species by the L. longifolium, L. petersonii and M. alternifolia extracts was still significantly inhibited by the end of the 6 h time course study. This may indicate that these extracts have bactericidal activity against these bacteria at the doses tested.

Quantification of toxicity
The toxicity of the L. longifolium, L. petersonii and M. alternifolia extracts was initially tested in the Artemia franciscana nauplli bioassay at a concentration of 2000 µg/mL (Figure 5). All extracts induced low levels of mortality at 24 and 48 h, similar to the % mortality seen for the seawater control. As none of the extracts induced >50% mortality, all were deemed to be nontoxic. Extracts with an LC50 of greater than 1000 µg/mL towards Artemia nauplii have previously been defined as being nontoxic. In contrast, the potassium dichromate positive controls induced mortality within 4 h (results not shown), with 100% mortality induction seen by 24 h.

DISCUSSION
Plant derived remedies are becoming increasingly sought after in the treatment of a myriad of diseases and disorders due both to their perception of greater safety than synthetic drugs, and the failure of current drug regimens to effectively treat many diseases. Our study examined the activity of methanolic L. longifolium, L. petersonii and M. alternifolia leaf extracts to inhibit the growth of a panel of medicinally important bacterial pathogens, and on their toxicity. The gram positive and gram negative bacteria tested in this study were susceptible to all of
Antibacterial activity of selected Leptospermum spp. and Melaleuca spp.

Table 1: The mass of dried extracted material, the concentration after resuspension in deionised water and qualitative phytochemical screenings of the methanolic L. longifolium, L. petersonii and M. alternifolia extracts

<table>
<thead>
<tr>
<th>Plant Species</th>
<th>Mass of Dried Extract (mg)</th>
<th>Reuspended Extract (mg/mL)</th>
<th>Mass of Dried Extract (mg)</th>
<th>Reuspended Extract (mg/mL)</th>
<th>Total Phenolics</th>
<th>Water Soluble</th>
<th>Water Insoluble</th>
<th>Cardiac Glycosides</th>
<th>Saponins</th>
<th>Froth Persistence</th>
<th>Emulsion test</th>
<th>Salkowski Test</th>
<th>Acriatic Anhydride Test</th>
<th>Mayer's Test</th>
<th>Wagner's Test</th>
<th>Dragendorf's Test</th>
<th>Shiroda Test</th>
<th>Kumar's Test</th>
<th>Ferric Chloride Test</th>
<th>Lead Acetate Test</th>
<th>Combined</th>
<th>Anthraquinones</th>
</tr>
</thead>
<tbody>
<tr>
<td>L. longifolium</td>
<td>130</td>
<td>13</td>
<td>+++</td>
<td>+++</td>
<td>+++</td>
<td>+++</td>
<td>-</td>
<td>+++</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+++</td>
<td>+</td>
<td>+</td>
<td>+++</td>
<td>-</td>
<td>-</td>
<td>Free</td>
<td>Combined</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>L. petersonii</td>
<td>117</td>
<td>11.7</td>
<td>+++</td>
<td>+++</td>
<td>+++</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>-</td>
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<td>+</td>
<td>+</td>
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<td>-</td>
<td>Free</td>
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<td>-</td>
</tr>
<tr>
<td>M. alternifolia</td>
<td>93</td>
<td>9.3</td>
<td>+++</td>
<td>+++</td>
<td>+++</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>-</td>
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<td>-</td>
<td>Free</td>
<td>Combined</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

+++ indicates a large response; ++ indicates a moderate response; + indicates a minor response; - indicates no response in the assay.

Table 2: Minimum bacterial growth inhibitory concentration (µg/mL) of the L. longifolium, L. petersonii and M. alternifolia extracts against susceptible bacterial species

<table>
<thead>
<tr>
<th>Bacterial species</th>
<th>Gram negative</th>
<th>L. longifolium</th>
<th>L. petersonii</th>
<th>M. alternifolia</th>
</tr>
</thead>
<tbody>
<tr>
<td>A. faecalis</td>
<td></td>
<td>1438</td>
<td>849</td>
<td>2600</td>
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<tr>
<td>A. hydrophilia</td>
<td></td>
<td>147</td>
<td>279</td>
<td>705</td>
</tr>
<tr>
<td>C. freundi</td>
<td></td>
<td>619</td>
<td>720</td>
<td>1350</td>
</tr>
<tr>
<td>E. coli</td>
<td></td>
<td>-</td>
<td>-</td>
<td>815</td>
</tr>
<tr>
<td>K. pneumoniae</td>
<td></td>
<td>320</td>
<td>687</td>
<td>-</td>
</tr>
<tr>
<td>P. mirabilis</td>
<td></td>
<td>485</td>
<td>648</td>
<td>449</td>
</tr>
<tr>
<td>P. fluorescens</td>
<td></td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>S. newport</td>
<td></td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>S. marcescens</td>
<td></td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>S. sonnei</td>
<td></td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Gram positive</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>B. cereus</td>
<td></td>
<td>755</td>
<td>284</td>
<td>1500</td>
</tr>
<tr>
<td>S. aureus</td>
<td></td>
<td>428</td>
<td>389</td>
<td>2842</td>
</tr>
<tr>
<td>S. epidermidis</td>
<td></td>
<td>978</td>
<td>846</td>
<td>2978</td>
</tr>
<tr>
<td>S. pyogenes</td>
<td></td>
<td>1230</td>
<td>1084</td>
<td>1486</td>
</tr>
</tbody>
</table>

Numbers indicate the mean MIC values of triplicate determinations. - indicates no inhibition.

The extracts, although a greater susceptibility was noted for gram positive bacterial species. Many previous studies with other plant species have also reported a greater susceptibility of gram positive bacteria towards solvent extracts for South American, African and Australian plant extracts.\(^{36,37}\)

The L. longifolium, L. petersonii and M. alternifolia leaf extracts were particularly potent inhibitors of P. mirabilis (MIC values <500 µg/mL). As P. mirabilis infection is a common cause of urinary tract infections and has also been identified as a trigger of rheumatoid arthritis,\(^{36,37}\) the L. longifolium, L. petersonii and M. alternifolia leaf extracts have potential for the prevention of these diseases in genetically susceptible individuals. The L. longifolium and L. petersonii extracts were also potent K. pneumoniae growth inhibitors. As K. pneumoniae can trigger ankylosing spondylitis in genetically susceptible individuals\(^{40,41}\) this extract may also be useful in the prevention and treatment of this autoimmune disease. Furthermore, all extracts were moderate inhibitors of S. pyogenes growth with MIC values 1000-1500 µg/mL. S. pyogenes may cause a myriad of diseases including streptococcal pharyngitis, impetigo and rheumatic heart disease, depending on which tissue it infects.\(^{42,43}\) Thus, the methanolic L. longifolium, L. petersonii and M. alternifolia leaf extracts may also be useful in the prevention and treatment of these diseases.

Aside from inhibition of the growth of the bacterial triggers of the autoimmune disease discussed above, the L. longifolium, L. petersonii and M. alternifolia leaf extracts were also moderate to good inhibitors of several other bacterial pathogens. All extracts inhibited A. faecalis, A. hydrophilia, C. freundi and B. cereus growth, with MICs generally <1000 µg/mL. Thus, these extracts have potential in the treatment diseases caused by these pathogens (e.g. food poisoning, diarrhoea and dysentery). The L. longifolium and L. petersonii extracts were also good inhibitors of S. aureus and S. epidermidis growth (MICs 400-1000 µg/mL). The M. alternifolia extract was a moderate inhibitor of the growth of these bacteria, with MICs <3000 µg/mL. As both of these bacteria are skin disease pathogens, these extracts also may have applications as topical treatments of these diseases.

Whilst an investigation of the phytochemistry of the methanolic L. longifolium, L. petersonii and M. alternifolia leaf extracts was beyond the scope of our study, previous studies have reported high terpenoid contents for essential oils of these species.\(^{36,37}\) Similarly, other plants of the family Myrtaceae are also well known for their high terpenoid contents.\(^{7}\) Monoterpenes have been reported to exert a wide variety of biological effects including antibacterial, antifungal, anti-inflammatory and antitumour activities\(^{22}\) and therefore may contribute to the bacterial growth inhibitory activity of the L. longifolium, L. petersonii and M. alternifolia extracts reported in our study. A wide variety of monoterpenoids including camphor, carveone, cineole, borneol, menthone, pinene, terpinene, as well as their derivatives, inhibit the growth of an extensive panel of pathogenic and food spoilage bacteria.\(^{44}\) Interestingly, several of these monoterpenoids have also been reported to suppress NF-κB signaling (the major regulator of inflammatory diseases).\(^{45,46}\) This may be particularly relevant for the extracts which inhibited P. mirabilis (a bacterial trigger of rheumatoid arthritis)\(^{36,37}\) and K. pneumoniae (a trigger of ankylosing spondylitis).\(^{40,41}\) The terpene components in...
Antibacterial activity of selected *Leptospermum* spp. and *Melaleuca* spp.

**Figure 1:** (a) *L. longifolium*, (b) *L. petersonii*, (c) *M. alternifolia*, (d) geraniol, (e) neral, (f) citronellal, (g) α-pinene, (h) sabinene, (i) α-terpinene, (j) limonene, (k) 1,8-cineole.

**Figure 2:** Growth inhibitory activity of the *L. longifolium*, *L. petersonii* and *M. alternifolia* leaf extracts against gram negative bacterial species and the ampicillin (10 µg) and chloramphenicol (2 µg) controls. All determinations were performed in at least triplicate and the results are expressed as mean zones of inhibition (mm) ± SEM.
Antibacterial activity of selected Leptospermum spp. and Melaleuca spp.

Figure 3: Growth inhibitory activity of the L. longifolium, L. petersonii and M. alternifolia leaf extracts against gram positive bacterial species and the ampicillin (10 µg) and chloramphenicol (2 µg) controls. All determinations were performed in at least triplicate and the results are expressed as mean zones of inhibition (mm) ± SEM.

these extracts may therefore have a pleuripotent mechanism in blocking the autoimmune inflammatory diseases and relieving its symptoms by acting on both the initiator and downstream inflammatory stages of the disease. Further phytochemical evaluation studies and bioactivity driven isolation of active components is required to further evaluate the mechanism(s) of bacterial growth inhibition.

Another commonality between the inhibitory methanolic L. longifolium, L. petersonii and M. alternifolia leaf extracts was that all contained high levels of tannins (Table 1). Many studies have reported potent growth inhibitory activities for a number of tannin compounds. Gallotannins have been reported to inhibit the growth of a broad spectrum of bacterial species through a variety of mechanisms including binding cell surface molecules including lipotoichoic acid and proline-rich cell surface proteins, and by inhibiting glucosyltransferase enzymes. Ellagitannins are also highly potent inhibitors of bacterial growth, with MIC values as low as 62.5 µg/mL. Ellagitannins have also been reported to function via several antibiotic mechanisms including interaction with cytoplasmic oxidoreductases and by disrupting bacterial cell walls. Thus, it is likely that multiple compounds within the L. longifolium, L. petersonii and M. alternifolia leaf extracts may contribute to the inhibition of bacterial growth.

The findings reported here also demonstrate that the L. longifolium, L. petersonii and M. alternifolia leaf extracts were nontoxic towards Artemia franciscana nauplii, with LC₅₀ values substantially > 1000 µg/mL. Extracts with LC₅₀ values > 1000 µg/mL towards Artemia nauplii are defined as being nontoxic. Whilst our preliminary toxicity studies indicate that these extracts may be safe for therapeutic use, studies using human cell lines are required to further evaluate the safety of these extracts. Furthermore, whilst these studies have demonstrated the potential of the L. longifolium, L. petersonii and M. alternifolia leaf extracts in the development of future antibiotic chemotherapeutics for the prevention and treatment of urinary tract infections, autoimmune diseases (particularly rheumatoid arthritis and ankylosing spondylitis) and some skin diseases, more work is required to isolate the inhibitory components and determine the mechanism(s) of inhibition.

CONCLUSION

The results of this study demonstrate the potential of the L. longifolium, L. petersonii and M. alternifolia leaf extracts as inhibitors of pathogenic bacteria growth. Furthermore, their lack of toxicity indicates than they may be safe for internal as well as topical treatment in the doses tested. Further studies aimed at the purification and identification of bioactive components are needed to examine the mechanisms of action of these agents.

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CONFLICTS OF INTEREST

The authors report no conflicts of interest.

ABBREVIATIONS USED

DMSO: Dimethyl sulfoxide; LC₅₀: The concentration required to achieve 50 % mortality; MIC: minimum inhibitory concentration.
Figure 4: Bacterial growth curves for (a) *A. hydrophila*, (b) *C. freundi*, (c) *P. mirabilis*. All bioassays were performed in at least triplicate and are expressed as mean ± SEM. * = growth results in the presence of *L. longifolium* extract that are significantly different to the untreated control growth ($p<0.01$); # = growth results in the presence of *L. petersonii* extract that are significantly different to the untreated control growth ($p<0.01$); ^ = growth results in the presence of *M. alternifolia* extract that are significantly different to the untreated control growth ($p<0.01$).
Antibacterial activity of selected Leptospermum spp. and Melaleuca spp.

REFERENCES


Figure 5: The lethality of the aqueous and methanolic L. longifolium, L. petersonii and M. alternifolia leaf extracts (2000 µg/mL), potassium dichromate (1000 µg/mL) and a seawater control. Blue bars represent the % mortality following 24 h exposure to the extract/toxin. Green bars represent the % mortality following 48 h exposure to the extract/toxin. NC=negative (seawater) control; PC=positive control (1000 µg/mL potassium dichromate). All bioassays were performed in at least triplicate and are expressed as mean ± SEM.
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PICTORIAL ABSTRACT

- L. longifolium, L. petersonii and M. alternifolia extracts displayed broad spectrum antibacterial activity against gram positive and gram negative bacteria.
- All extracts were potent inhibitors of P. mirabilis growth, with MICs generally <500 µg/mL.
- A. hydrophilia, C. freundii, and B. cereus were also particularly susceptible (MICs as low as 147 µg/mL)
- All L. longifolium, L. petersonii and M. alternifolia extracts were nontoxic.

ABOUT AUTHORS

Ms Lindiwe Mpala completed a BSc at Griffith University in life sciences. Following graduation, she undertook a research project in Dr Ian Cock’s laboratory in the School of Natural Sciences at Griffith University. The project examined the growth inhibitory properties of a variety of Australian native plants against an extensive panel of bacterial pathogens.

Ms Getmore Chikowe completed a BSc at Griffith University in life sciences. Following graduation, she undertook a research project in Dr Ian Cock’s laboratory in the School of Natural Sciences at Griffith University. The project examined the growth inhibitory properties of a variety of Australian native plants against an extensive panel of bacterial pathogens.

Dr Ian Cock leads a research team in the Environmental Futures Research Institute and the School of Natural Sciences at Griffith University, Australia. His research involves bioactivity and phytochemical studies into a variety of plant species of both Australian and international origin, including Aloe vera, South Asian and South American tropical fruits, as well as Australia plants including Scaevola spinescens, Pittosporum phylliraeoides, Terminalia ferdinandiana (Kakadu plum), Australian Acacias, Syzygiums, Petalostigmas and Xanthorrhoea johnsonii (grass trees). This range of projects has resulted in nearly 200 publications in a variety of peer reviewed journals.

SUMMARY

- L. longifolium, L. petersonii and M. alternifolia extracts displayed broad spectrum antibacterial activity against gram positive and gram negative bacteria.
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