

## Research Letter

# Antimicrobial activity of *Leptospermum bracteata* and *Leptospermum juniperium* methanolic extracts

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**ABSTRACT: Introduction:** Australian *Leptospermum* species had roles as traditional bush medicines for Australian Aborigines, including uses as antiseptic agents. Despite this ethnobotanical usage, the antimicrobial properties of *Leptospermum* spp. have not been rigorously studied. **Methods:** The antimicrobial activity of methanolic extracts of *Leptospermum bracteata* and *Leptospermum juniperium* leaves and flowers were investigated by disc diffusion and growth time course assays against a panel of bacteria and fungi. Toxicity was determined using the *Artemia franciscana* nauplii bioassay. **Results:** *L. bracteata* leaf and flower extracts inhibited the growth 36% and 50% of the bacteria tested respectively. Both Gram-positive and Gram-negative bacteria were equally affected by *L. bracteata* leaf extract. Of the 11 Gram-negative bacteria tested, 4 (36%) were inhibited by *L. bracteata* leaf extract compared with 1 of the 3 Gram-positive bacteria tested (33%). The *L. bracteata* flower extract was more selective with Gram-positive bacteria being more susceptible. Of the 14 Gram-negative bacteria tested, 4 were inhibited by *L. bracteata* flower extract (29%) compared with 100% of the Gram-positive bacteria tested. *L. juniperium* extracts were less effective antibacterial agents than were the *L. bracteata* extracts. *L. juniperium* leaf extract inhibited the growth of 36% of the bacteria tested compared to only a single bacteria (7%) being inhibited by the flower extract. Gram-positive bacteria (100% inhibited) were more susceptible to *L. juniperium* leaf extract than were Gram negative bacteria (18% inhibited). No growth inhibitory activity was seen for any *Leptospermum* extract towards any of the fungi tested. The antibacterial activity of *L. bracteata* flower and *L. juniperium* leaf extracts was further investigated by growth time course assays which showed significant growth inhibition in cultures of *Bacillus cereus*, *Bacillus subtilis*, *Pseudomonas fluorescens* and *Aeromonas hydrophila* within 1 h. **Conclusions:** The low toxicity of these *Leptospermum* extracts and their inhibitory bioactivity against a panel of bacteria validate Australian Aboriginal usage of *Leptospermum bracteata* and *Leptospermum juniperium* as antiseptic agents and confirms their medicinal potential.

**KEYWORDS:** *Leptospermum bracteata*, *Leptospermum juniperium*, Australian plants, antibacterial activity, medicinal plants, toxicity

## INTRODUCTION

Bacterial resistance to currently used antibiotics is becoming a concern to public health.<sup>[1]</sup> The development of bacterial super resistant strains is resulting in currently used antibiotic agents failing to end many bacterial infections. For this reason the search is ongoing for new

antimicrobial agents, either by the design and synthesis of new agents, or through the search of natural sources for as yet undiscovered antimicrobial agents.<sup>[2]</sup> The antiseptic qualities of medicinal plants have been long recognised. Recently there has 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.<sup>[3]</sup>

*Leptospermum* (family *Myrtaceae*) is a genus of more than 80 species that are widely distributed in Australia, with a few species also native to New Zealand and Malaysia.<sup>[4]</sup> The antiseptic properties of several *Leptospermum* species is well known.<sup>[5]</sup> Particularly well studied is the antimicrobial properties of *Leptospermum scoparium* (Manuka), a species endemic to New Zealand<sup>[6]</sup> and eastern Australia.<sup>[7]</sup>

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This species has been traditionally used medicinally for many ailments. The leaf vapour was used for colds and coughs, the gum exudate for scalds and burns, aqueous bark and seed extracts for infections and inflammation and the leaves for urinary complaints.<sup>[5,8]</sup> Honey derived from *L. scoparium* is known as a good antibacterial agent.<sup>[9,10]</sup> The medicinal properties of other *Leptospermum* is less well studied although some species were also known to be used by Australian Aborigines as antiseptic agents.<sup>[5]</sup> Reports have also demonstrated the antibacterial and antifungal activity of other Australian species including *Leptospermum petersonii* (lemon scented tea tree)<sup>[11,12]</sup> and *Leptospermum amboinense*.<sup>[13]</sup> Research into the medicinal value of other *Leptospermum* species is less extensive and much still needs to be done to identify their antimicrobial potential.

A recent study has demonstrated the antibacterial activity of methanolic extracts of *Leptospermum bracteata* and *Leptospermum juniperium* against a limited panel of bacteria.<sup>[14]</sup> The current study was undertaken to validate and extend these observations against a wider panel of bacteria and fungi.

## MATERIALS AND METHODS

### Plant collection and extraction

The extracts investigated in this study have been described previously.<sup>[14]</sup> Briefly, *Leptospermum juniperium* (leaves and flowers) were collected from Toohey Forest, Brisbane. *Leptospermum bracteata* (leaves and flowers) were collected from verified trees in Brisbane, Australia. Samples were dried in a Sunbeam food dehydrator and the dried material was ground to a coarse powder. 1 g of each of the powdered samples was extracted extensively in 50 ml methanol (Ajax, AR grade) for 24 hours 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 pellet was dissolved in 15 ml 20 % methanol. The extract was passed through 0.22 µm filter (Sarstedt) and stored at 4 °C.

### Test microorganisms

All media was supplied by Oxoid Ltd. All microbial strains were obtained from Tarita Morais, Griffith University. Stock cultures of *Aeromonas hydrophilia*, *Alcaligenes faecalis*, *Bacillus cereus*, *Bacillus subtilis*, *Citrobacter freundii*, *Enterobacter aerogenes*, *Escherichia coli*, *Klebsiella pneumoniae*, *Pseudomonas aeruginosa*, *Pseudomonas fluorescens*, *Salmonella salford*, *Serratia marcescens*, *Staphylococcus aureus* and *Yersinia enterocolitica* were subcultured and maintained in nutrient broth at 4 °C.

*Aspergillus niger*, *Candida albicans*, and *Saccharomyces cerevisiae* were maintained in Sabouraud media at 4 °C.

### Evaluation of antimicrobial activity

Antimicrobial activity of each plant extract was determined using a modified Kirby-Bauer disc diffusion method.<sup>[15–17]</sup> Briefly, 100 µl of the test bacteria/fungi were grown in 10 ml of fresh media until they reached a count of approximately 10<sup>8</sup> cells/ml for bacteria, or 10<sup>5</sup> cells/ml for fungi. 100 µl of microbial suspension was spread onto agar plates corresponding to the broth in which they were maintained.

The extracts were tested using 6 mm sterilised 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 hours before incubation with the test microbial agents. Plates inoculated with *Alcaligenes faecalis*, *Aeromonas hydrophilia*, *Bacillus cereus*, *Bacillus subtilis*, *Citrobacter freundii*, *Klebsiella pneumoniae*, *Pseudomonas aeruginosa*, *Pseudomonas fluorescens*, *Serratia marcescens*, *Yersinia enterocolitica*, *Candida albicans* and *Saccharomyces cerevisiae* were incubated at 30 °C for 24 hours, then the diameters of the inhibition zones were measured in millimetres. Plates inoculated with *Enterobacter aerogenes*, *Escherichia coli*, *Salmonella Salford* and *Staphylococcus aureus* were incubated at 37 °C for 24 hours, then the diameters of the inhibition zones were measured. *Aspergillus niger* inoculated plates were incubated at 25 °C for 48 hours then the zones of inhibition were measured. All measurements were to the closest whole millimetre. Each antimicrobial assay was performed in at least triplicate. Mean values are reported in this report. Standard discs of ampicillin (2 µg), chloramphenicol (10 µg) or ciprofloxacin (2.5 µg) were obtained from Oxoid Ltd. and served as positive controls for antimicrobial activity. For fungi, nystatin discs (100 µg, Oxoid Ltd.) were also used as a positive control. Filter discs impregnated with 10 µl of distilled water were used as a negative control.

### Bacterial growth time course assay

Bacterial growth time course studies were performed as previously described.<sup>[18–21]</sup> Briefly, 3 ml of bacterial cultures (*Bacillus cereus*, *Bacillus subtilis*, *Aeromonas hydrophilia*, *Pseudomonas fluorescens*) in nutrient broth were added to 27 ml nutrient broth containing 3 ml *Leptospermum bracteata* or *Leptospermum juniperium* extracts (diluted 1 in 100 in sterile deionised water). The tubes were incubated at 30 °C with gentle shaking. The optical density was measured at 550 nm after 0, 1, 2, 4 and 6 h incubations. Control tubes were incubated under the same conditions but without the extract. All assays were performed in triplicate.

## Toxicity screening

### Reference toxins for toxicity screening

Potassium dichromate ( $K_2Cr_2O_7$ ) (AR grade, Chem-Supply, Australia) was prepared as a 1.6 mg/ml solution in deionised water and was serially diluted in artificial seawater for use in the *Artemia franciscana* nauplii bioassay. Mevinphos (2-methoxycarbonyl-1-methylvinyl dimethyl phosphate) was obtained from Sigma-Aldrich as a mixture of cis (76.6%) and trans (23.0%) isomers and prepared as a 4 mg/ml stock in distilled water. The stock was serially diluted in artificial seawater for use in the bioassay.

### *Artemia franciscana* nauplii toxicity screening

Toxicity was tested using a modified *Artemia franciscana* nauplii lethality assay.<sup>[22–24]</sup> *Artemia franciscana* Kellogg 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. 2 g of *A. franciscana* cysts were 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. Seawater (400 µl) containing approximately 43 (mean 42.7, n = 184, SD 19.0) nauplii were added to wells of a 48 well plate and immediately used for bioassay. The plant extracts were diluted to 5 mg/ml in seawater for toxicity testing, resulting in a 2.5 mg/ml concentration in the bioassay. 400 µl of diluted plant extracts and the reference toxins 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 dead if no movement of the appendages was observed within 10 seconds. After 72 h 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.

## RESULTS AND DISCUSSION

*L. bracteata* leaf and flower extracts were diluted to 13 mg/ml and 18 mg/ml respectively. *L. juniperium* leaf and flower extracts were diluted to 16 mg/ml and 6 mg/ml

respectively. 10 µl of each extract was tested in the disc diffusion assay against 17 microorganisms (Table 1). The *L. bracteata* leaf extract inhibited the growth of 5 of the 14 bacteria tested (36%). The antibacterial activity was strongest against *P. fluorescens* and *Y. enterocolitica* (as determined by the diameter of the zone of inhibition). The *L. bracteata* flower extract was a more effective antibacterial agent, inhibiting the growth of 7 of the 14 bacteria tested (50%). The antibacterial activity of the *L. bracteata* flower extract was strongest against *A. faecalis* and *P. fluorescens* (as determined by the diameter of the zone of inhibition).

*L. juniperium* leaf extract inhibited the growth of 5 of the 14 bacteria tested (36%). The antibacterial activity was strongest against *A. faecalis*, *B. subtilise* and *S. aureus* (as determined by the diameter of the zone of inhibition). The *L. juniperium* flower extract was not an effective anti-septic agent, inhibiting the growth of only of a single bacterium (*A. faecalis*) (7%).

Both Gram-positive and Gram-negative bacteria were equally affected by *L. bracteata* leaf extract. Of the 11 Gram-negative bacteria tested, 4 (36%) were inhibited by *L. bracteata* leaf extract. The leaf extract also inhibited the growth of 1 of the 3 Gram-positive bacteria tested (33%). The *L. bracteata* flower extract was more selective with Gram-positive bacteria being more susceptible. Of the 14 Gram-negative bacteria tested, 4 were inhibited by *L. bracteata* flower extract (29%). All of the Gram-positive (100%) bacteria tested were inhibited by *L. bracteata* flower extract.

The greater susceptibility of Gram-positive bacteria has previously also been reported for another *Leptospermum* species (*Leptospermum amboinense*).<sup>[13]</sup> Indeed, the greater susceptibility of Gram-positive bacteria is a common trend, previously reported for South American,<sup>[25]</sup> African,<sup>[26,27]</sup> and Australian<sup>[28]</sup> plant extracts. Results within this laboratory have also confirmed the greater susceptibility of Gram-positive bacteria towards other Australian plant extracts.<sup>[14,16,29–33]</sup> The Gram-negative bacterial cell wall outer membrane is thought to act as a barrier to many substances including antibiotics.<sup>[34]</sup> The uptake of the *L. bracteata* leaf extract antibiotic agents by Gram-negative bacteria is presumably not affected by the cell wall outer membrane. In contrast, other studies have demonstrated that Gram-negative bacteria are more susceptible to plant extracts from other Australian plant species.<sup>[35–37]</sup>

*L. juniperium* leaf extract was also more effective at inhibiting the growth of Gram-positive bacteria than of Gram-negative bacteria. Of the 11 Gram-negative tested, only

**Table 1: Antibacterial activity of *Leptospermum bracteata* and *Leptospermum juniperium* leaf and flower extracts**

Microbial Species	Mean Zone of Inhibition $\pm$ SD (mm)				
	Antibiotic	<i>L. bracteata</i> leaf extract	<i>L. bracteata</i> flower extract	<i>L. juniperium</i> leaf extract	<i>L. juniperium</i> flower extract
<b>Gram negative rods</b>					
<i>Aeromonas hydrophilia</i>	17.3 $\pm$ 0.6 (Chl)	–	–	7.3 $\pm$ 0.6	–
<i>Alcaligenes faecalis</i>	13.3 $\pm$ 0.6 (Amp)	10.7 $\pm$ 1.2	20.7 $\pm$ 1.2	11.7 $\pm$ 1.2	9.3 $\pm$ 0.3
<i>Citrobacter freundii</i>	23.0 $\pm$ 1.0 (Chl)	–	–	–	–
<i>Enterobacter aerogenes</i>	17.3 $\pm$ 0.3 (Chl)	–	–	–	–
<i>Escherichia coli</i>	16.7 $\pm$ 0.6 (Amp)	6.7 $\pm$ 0.6	–	–	–
<i>Klebsiella pneumoniae</i>	18.3 $\pm$ 0.6 (Amp)	–	13.0 $\pm$ 1.0	–	–
<i>Pseudomonas aeruginosa</i>	31.6 $\pm$ 0.3 (Cip)	–	–	–	–
<i>Pseudomonas fluorescens</i>	21.0 $\pm$ 0 (Chl)	15.3 $\pm$ 0.3	21.3 $\pm$ 0.3	–	–
<i>Salmonella salford</i>	25.3 $\pm$ 0.3 (Amp)	–	–	–	–
<i>Serratia marcescens</i>	25.7 $\pm$ 0.6 (Chl)	–	–	–	–
<i>Yersinia enterocolitica</i>	16.3 $\pm$ 0.3 (Amp)	13.3 $\pm$ 0.3	21 $\pm$ 0	–	–
<b>Gram positive rods</b>					
<i>Bacillus cereus</i>	25.3 $\pm$ 0.6 (Chl)	–	11.0 $\pm$ 1.0	7.6 $\pm$ 0.3	–
<i>Bacillus subtilis</i>	22.7 $\pm$ 0.6 (Amp)	–	9.6 $\pm$ 0.3	9.0 $\pm$ 1.0	–
<b>Gram positive cocci</b>					
<i>Staphylococcus aureus</i>	16.3 $\pm$ 0.3 (Amp)	11.3 $\pm$ 0.3	8.3 $\pm$ 0.3	9.7 $\pm$ 1.2	–
<b>Fungi</b>					
<i>Aspergillus niger</i>	18.0 $\pm$ 0 (Cip)	–	–	–	–
<i>Candida albicans</i>	25.7 $\pm$ 0.6 (Nys)	–	–	–	–
<b>Yeast</b>					
<i>Saccharomyces cerevisiae</i>	21.3 $\pm$ 0.6 (Nys)	–	–	–	–

Numbers indicate the mean diameters of inhibition of triplicate experiments  $\pm$  standard deviation. – indicates no growth inhibition. Amp indicates ampicillin (2  $\mu$ g). Chl indicates chloramphenicol (10  $\mu$ g). Cip indicates ciprofloxacin (2.5  $\mu$ g). Nys indicates nystatin (100  $\mu$ g).

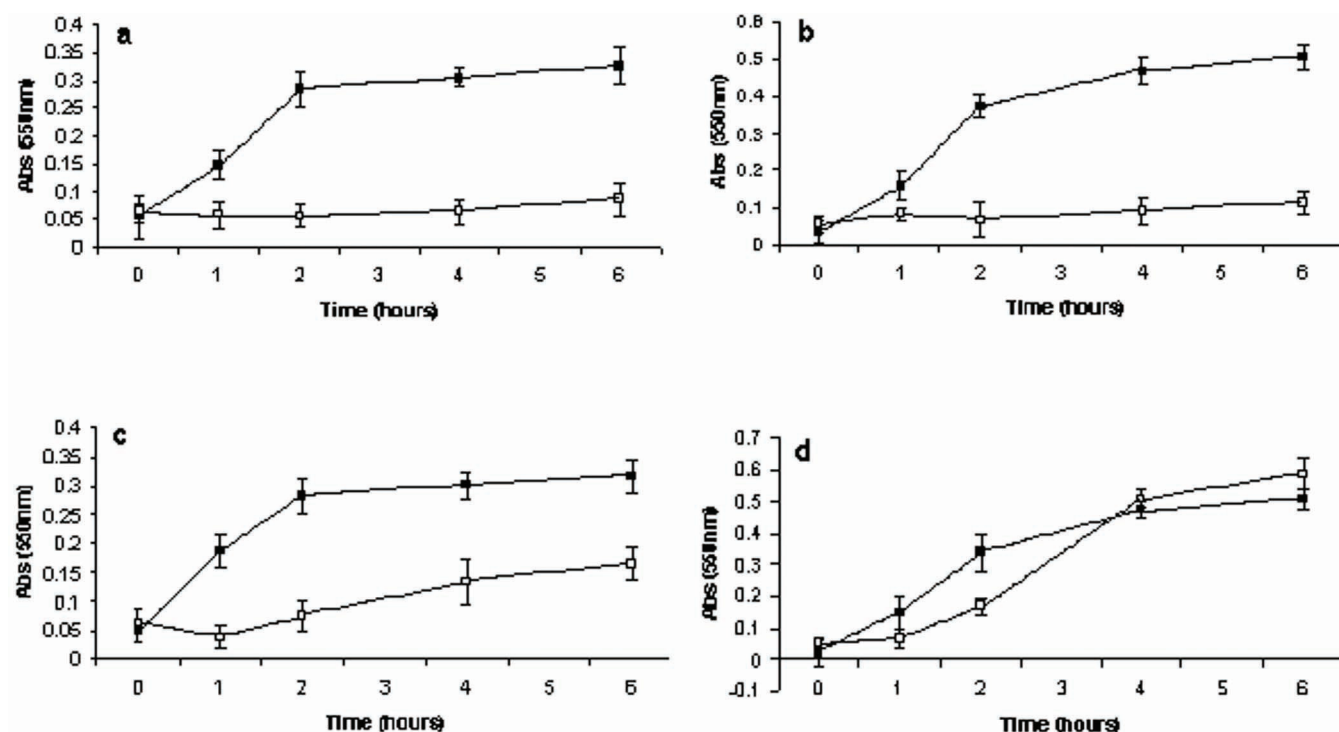
2 (18%) were inhibited compared to 100% inhibition of the Gram-positive bacteria tested. *L. juniperium* flower extract was not an effective antibacterial agent, inhibiting the growth of only 1 of the 11 Gram-negative bacteria tested (9%) and none of the Gram-positive bacteria (0%). None of the *Leptospermum* extracts displayed any antifungal activity.

The antibacterial activity of the *L. bracteata* flower extract was further investigated by bacterial growth time course assays in the presence and absence of extract. The concentration of the extract used in these assays was 18  $\mu$ g/ml. *L. bracteata* flower extract was able to significantly inhibit *Bacillus cereus* (Figure 1a), *Bacillus subtilis* (Figure 1b) and *Pseudomonas fluorescens* (Figure 1c) growth within 1 h indicating a rapid antimicrobial action. *Aeromonas hydrophilia* (Figure 1d) growth was not significantly affected by *L. bracteata* flower extract, in agreement with previously reported results.<sup>[14]</sup> Furthermore, a decrease in optical density was seen for *B. cereus*, *B. subtilis* and *P. fluorescens* treated with *L. bracteata* flower extract which may indicate bacterial lysis had occurred.

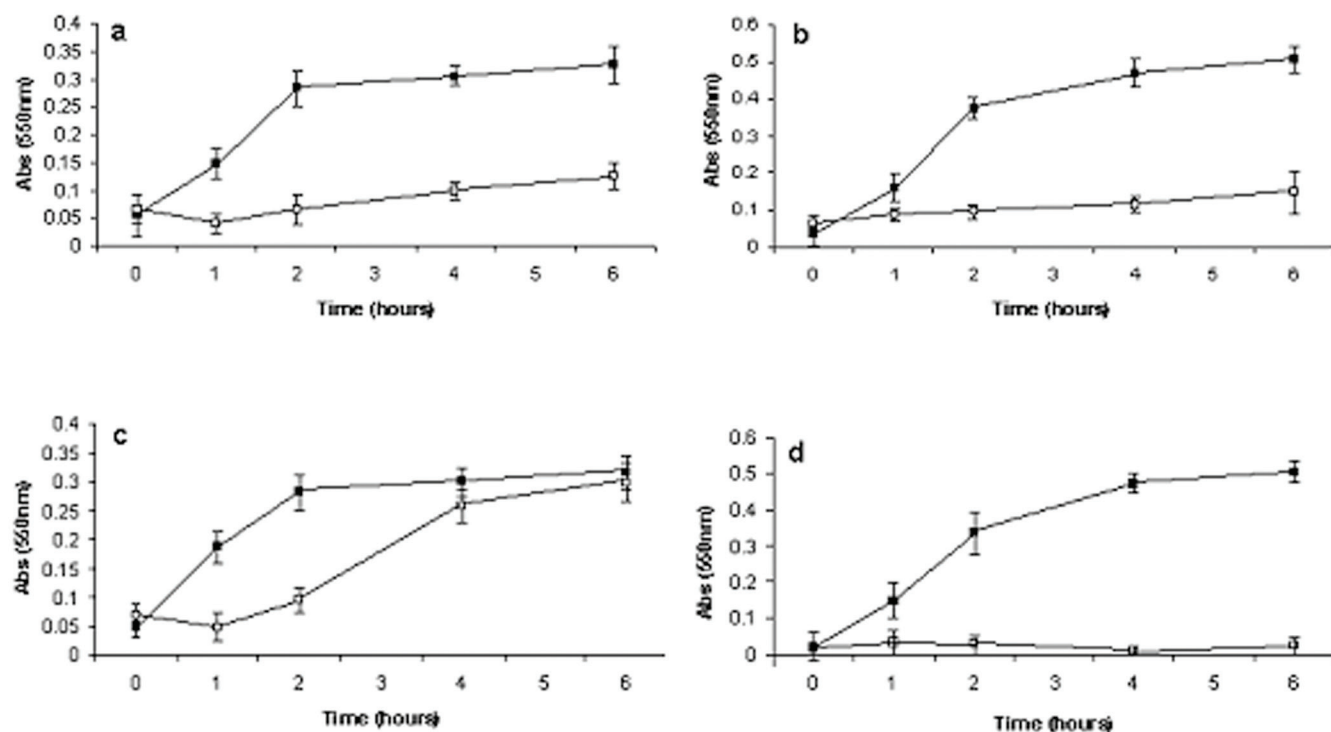
The antibacterial activity of *L. juniperium* leaf extract was also investigated by bacterial growth time course assays in the presence and absence of the extract. The concentration of the extract used in these assays was 16  $\mu$ g/ml. The *L. juniperium* leaf extract was able to significantly inhibit *Bacillus cereus* (Figure 1a), *Bacillus subtilis* (Figure 1b), *Pseudomonas fluorescens* (Figure 1c) and *Aeromonas hydrophilia* (Figure 1d) growth within 1 h indicating a rapid antimicrobial action, in agreement with previously reported results.<sup>[14]</sup> Furthermore, a decrease in optical density was seen for *B. cereus* and *P. fluorescens* treated with *L. juniperium* leaf extract which may indicate bacterial lysis had occurred.

To examine the toxicity of the *Leptospermum* extracts, they were tested in the *Artemia franciscana* nauplii bioassay at a concentration of 2000  $\mu$ g/ml (Figure 3). The *L. bracteata* leaf extract (Figure 3a) only induced low levels of mortality, similar to the % mortality seen for the seawater control (Figure 3f) at all time points. Similarly, the *L. juniperium* leaf (Figure 3c) and flower (Figure 3d) extracts did not induce mortality above that seen for

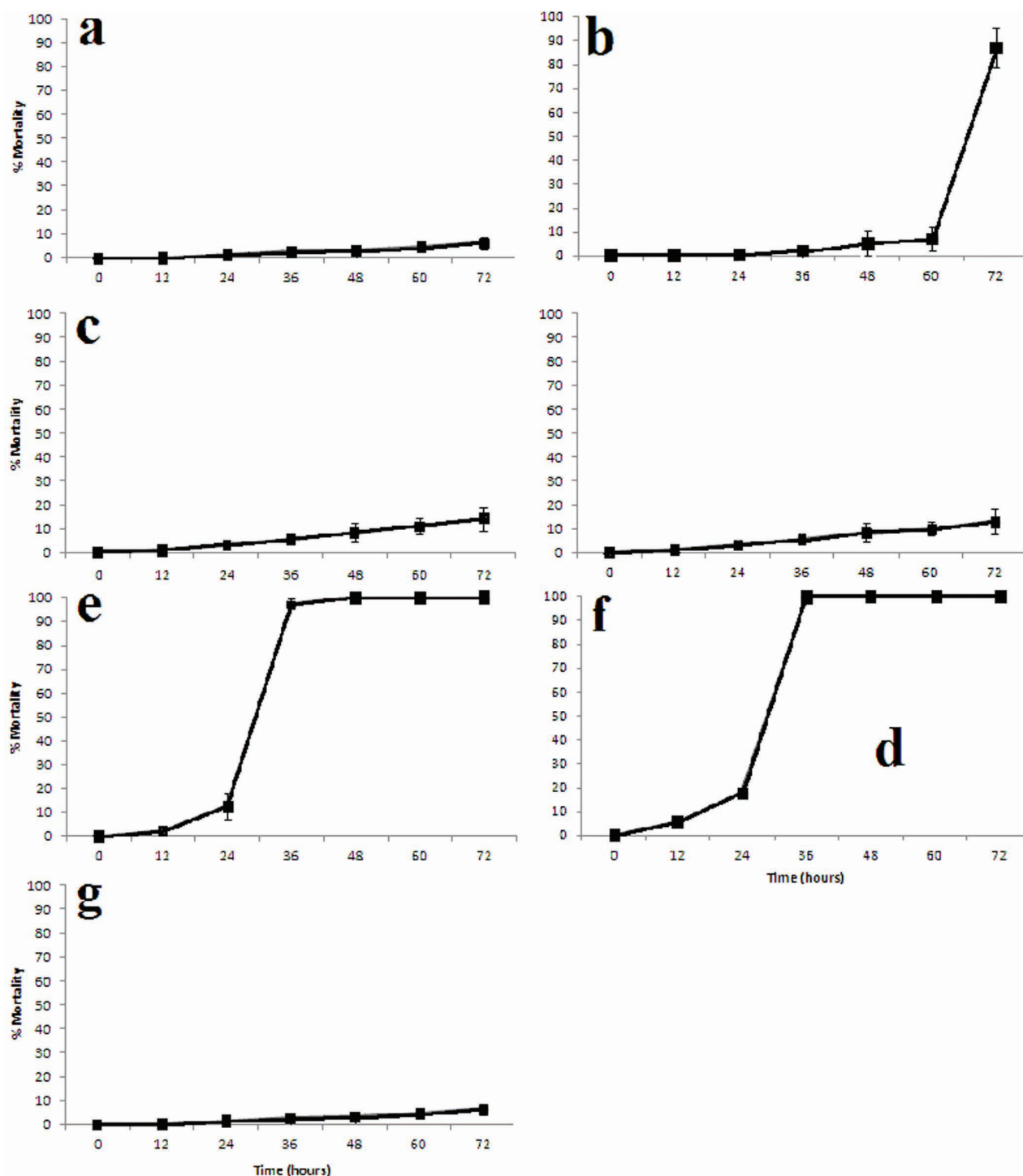




**Figure 1.** Inhibition of bacterial growth by a methanolic extract of *L. bracteata* flowers against (a) *B. cereus*, (b) *B. subtilis*, (c) *P. fluorescens*, (d) *A. hydrophilia*. For all graphs, □ represent measured bacterial growth values for test cultures (with extract) and ■ represent control bacterial growth values (no extract). All bioassays were performed in at least triplicate and are expressed as mean  $\pm$  standard deviation.



**Figure 2.** Inhibition of bacterial growth by a methanolic extract of *L. juniperium* leaves against (a) *B. cereus*, (b) *B. subtilis*, (c) *P. fluorescens*, (d) *A. hydrophilia*. For all graphs, □ represent measured bacterial growth values for test cultures (with extract) and ■ represent control bacterial growth values (no extract). All bioassays were performed in at least triplicate and are expressed as mean  $\pm$  standard deviation.



**Figure 3.** Brine shrimp lethality of (a) *L. bracteata* leaf methanolic extract (2000 µg/ml), (b) *L. bracteata* flower methanolic extract (2000 µg/ml), (c) *L. juniperium* leaf methanolic extract (2000 µg/ml), (d) *L. juniperium* flower methanolic extract (2000 µg/ml), (e) potassium dichromate (800 µg/ml), (f) Mevinphos (2000 µg/ml) and (g) seawater control. All bioassays were performed in at least triplicate and are expressed as mean  $\pm$  standard error.

the seawater control at any time point. The *L. bracteata* flower extract (Figure 3b) induced elevated mortality, although even these results indicate a low level of toxicity, with 72 h exposure needed for >50% mortality induction. In contrast, both positive controls induced

mortality within 24 h, with 100% mortality induction seen by 36 h.

To quantify the toxicity of the extracts,  $LC_{50}$  values were determined by testing across the concentration range

**Table 2: LC<sub>50</sub> (95% confidence interval) for brine shrimp nauplii exposed to *L. bracteata* and *L. juniperium* extracts, the reference toxins potassium dichromate and Mevinphos**

Plant Species	Plant Part Tested	LC <sub>50</sub> (µg/ml)		
		24 h	48 h	72 h
<i>L. bracteata</i>	leaves	NA	NA	NA
<i>L. bracteata</i>	flowers	NA	NA	1247 ± 73
<i>L. juniperium</i>	leaves	NA	NA	NA
<i>L. juniperium</i>	flowers	NA	NA	NA
Mevinphos		1418 ± 172	546 ± 45	123 ± 18
Potassium Dichromate		–	82 ± 4	79 ± 5

NA indicates that LC<sub>50</sub> values were not obtained as ≥50% mortality was not reached for this time point. Results represent the mean ± standard deviation of triplicate determinations.

2000 µg/ml to 10 µg/ml in the *Artemia franciscana* nauplii bioassay (Table 2). For comparison, serial dilutions of potassium dichromate and Mevinphos were also tested. No LC<sub>50</sub> values are reported for the *L. bracteata* leaf extract or the *L. juniperium* leaf and flower extracts at any time tested as no significant increase in mortality above the seawater controls was seen for these extracts at any time tested. This indicates that these extract are non-toxic. The *L. bracteata* flower extract does display low toxicity at 72 h with an LC<sub>50</sub> value of 1247 ± 73. As LC<sub>50</sub> values ≥1000 µg/ml are defined as non-toxic<sup>[38]</sup> the *L. bracteata* flower extract was classed as non-toxic.

In conclusion, these studies and previous studies within this laboratory (Cock, submitted for publication) show that *L. bracteata* and *L. juniperium* extracts contain antibacterial components and support the traditional New Zealand Maori and Australian Aboriginal medicinal use of some *Leptospermum* species to protect against infection by both Gram-positive and Gram-negative bacteria. As many *Leptospermum* species<sup>[9]</sup> and products such as honey<sup>[9,10]</sup> have also been used as a food by New Zealand Maoris and Australian Aborigines, there is also potential for the use of *Leptospermum* additives in other foods to protect against food spoilage. However, further studies are needed before these extracts can be applied to these purposes. In particular, toxicity studies against human cell lines are needed to determine the suitability of these extracts for use as antiseptic agents and as food additives. One study has reported on the relative toxicity of bark extracts from a related species (*L. amboinense*) against HepG2, and two carcinoma cell lines.<sup>[13]</sup> Conversely, the same report also showed this extract to have low toxicity towards brine shrimp. The brine shrimp lethality assay is generally considered to be a good indication of toxicity to mammalian cells.<sup>[38]</sup> No data was found for *L. bracteata* and *L. juniperium* toxicity. Further studies are needed to fully determine the cytotoxicity of these extracts. These results

provide further support the ethnobotanical approach to screening plants as potential sources of bioactive substances.<sup>[40]</sup>

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