Antimicrobial activity of *Callistemon citrinus* and *Callistemon salignus* methanolic extracts

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ABSTRACT: **Introduction:** Australian Callistemon species had a role as traditional bush medicine for Australian Aborigines, including use as an antiseptic agent. Despite this ethnobotanical usage, the antimicrobial properties of Callistemon spp. have not been rigorously studied. **Methods:** The antimicrobial activity of methanolic extracts of *Callistemon citrinus* and *Callistemon salignus* 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:** *C. citrinus* leaf extracts inhibited the growth of 43% and flower extracts inhibited the growth of 64% of the bacteria tested, respectively. Gram-positive bacteria (100% inhibited) were more susceptible to *C. citrinus* extracts than were Gram-negative bacteria (27% inhibited by leaf extracts; 55% inhibited by flower extracts). In comparison, *C. salignus* leaf extract inhibited the growth of 29% of the bacteria tested compared with 43% inhibited for the flower extract. Gram-positive bacteria (100% inhibited) were more susceptible to *C. salignus* leaf extract than were Gram-negative bacteria (9% inhibited). Similar results (27% Gram-negative bacteria inhibited and 100% Gram-positive bacterial inhibition) were also seen for *C. salignus* flower extract. Very little antifungal activity was seen with any extract with only *C. albicans* being inhibited by *C. salignus* leaf extract. The antibacterial activity of the *C. citrinus* and *C. salignus* flower extracts was further investigated by growth time course assays. These extracts showed significant growth inhibition activity in cultures of *Bacillus cereus*, *Aeromonas hydrophilia*, *Pseudomonas fluorescens* and *Bacillus subtilis* within 1 hour. All extracts displayed low toxicity in the *Artemia franciscana* nauplii bioassay. **Conclusions:** The low toxicity of these Callistemon extracts and their inhibitory bioactivity against a panel of bacteria validates Australian Aboriginal usage of *Callistemon citrinus* and *Callistemon salignus* as antiseptic agents and confirms their medicinal potential.

KEY WORDS: *Callistemon citrinus*, *Callistemon salignus*, Australian plants, antibacterial activity, medicinal plants, toxicity

INTRODUCTION

Traditional medicinal plants have been used to treat bacterial infections in many parts of the world for centuries.$^{[1,2]}$ The use of commercially available antibiotics has revolutionised the treatment of microbial infection. Unfortunately, their indiscriminate usage has resulted in multiple drug resistances towards many antibiotics$^{[3]}$ and an increase in the search for antimicrobial agents from natural sources.$^{[4]}$ Some studies focusing on the investigation of traditional African,$^{[5,6]}$ Caribbean,$^{[7]}$ and Indian$^{[8]}$ medicinal plants have identified new sources of therapeutic agents. Plant-derived antimicrobial agents are a largely untapped resource with enormous medical potential and much more investigation is needed in this area.

The genus *Callistemon* (family Myrtaceae) consists of 34 species endemic to Australia. Some species have also been introduced to other areas such as USA$^{[9]}$ and Africa$^{[10]}$ where they are often considered to be invasive species. They are closely related to *Melaleuca* which have similar leaf and flower morphology.$^{[11,12]}$ *Callistemons* are commonly referred to as ‘bottlebrushes’ due to the appearance of their flowers. They occur naturally in temperate regions of Australia, particularly on the east and south-west coasts.

*Callistemon* flowers were used as a food source by Australian Aborigines. The flowers were sucked for their nectar or used...
to make sweet drinks.\textsuperscript{[13]} Callistemon species also had roles as traditional bush medicines for Australian Aboriginals.\textsuperscript{[1,14]} The leaves were used to cure respiratory tract infections. Unfortunately most of our understanding of the antimicrobial potential of Australian Callistemon species is anecdotal with few species being properly studied. It has been postulated that terpenes in the leaves may be responsible for the efficacy of Callistemons in traditional treatments.\textsuperscript{[14]} A recent report has demonstrated the antibacterial activity of a related Callistemon species (Callistemon rigidus).\textsuperscript{[15]} Studies within this laboratory have also found antibacterial activity in methanolic extracts of Callistemon citrinus and Callistemon salignus leaves and flowers against a limited panel of bacteria.\textsuperscript{[14]} The current study was undertaken to validate and extend these observations against a wider panel of bacteria and fungi, and to assess the toxicity of the extracts and thus to assess their medicinal potential.

\section*{MATERIALS AND METHODS}

\subsection*{Plant Collection and Extraction}

The extracts investigated in this study have been described previously.\textsuperscript{[14]} Briefly, Callistemon citrinus (leaves and flowers) and Callistemon salignus (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 until use.

\subsection*{Test Microorganisms}

All media was supplied by Oxoid Ltd. All microbial strains were obtained from Tarita Morais, Griffith University. Stock cultures of Aeromonas hydrophila, 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, Candida albicans and Saccharomyces cerevisiae were maintained in Sabouraud media at 4 °C.

\subsection*{Evaluation of Antimicrobial Activity}

Antimicrobial activity of each plant extract and was determined using a modified Kirby-Bauer disc diffusion method.\textsuperscript{[17,18]} Briefly, 100 µl of the test bacteria/fungi were grown in 10 ml of fresh media until they reached a count of approximately 10^8 cells ml^{-1} for bacteria, or 10^5 cells ml^{-1} 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 hydrophila, 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 used as a positive control. Filter discs impregnated with 10 µl of distilled water were used as a negative control.

\subsection*{Bacterial Growth Time Course Assay}

Bacterial growth time course studies were performed as previously described.\textsuperscript{[19,20]} Briefly, 3 ml of bacterial cultures (Bacillus cereus, Bacillus subtilis, Aeromonas hydrophila, Pseudomonas fluorescens) in nutrient broth were added to 27 ml nutrient broth containing 3 ml C. citrinus or C. salignus flower 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.

\subsection*{Toxicity Screening}

\textbf{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 distilled 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.
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**RESULTS AND DISCUSSION**

*C. citrinus* leaf and flower extracts were each diluted to 37 mg/ml. *C. salignus* leaf and flower extracts were each diluted to 35 mg/ml. 10 µl of each extract was tested in the disc diffusion assay against 17 microorganisms (Table 1). The *C. citrinus* leaf extract inhibited the growth of 6 of the 14 bacteria tested (43%). The antibacterial activity was strongest against *A. faecalis* and *B. subtilis* (as determined by the diameter of the zone of inhibition). *C. citrinus* flower extract inhibited the growth of 9 of the 14 bacteria tested (64%) with the strongest antibacterial activity against *B. cereus* and *B. subtilis*.

The *C. salignus* leaf extract inhibited the growth of 4 of the 14 bacteria tested (29%) with the strongest inhibitory activity being against *B. cereus*. The *C. salignus* flower extract inhibited the growth of 3 of the 14 bacteria tested (21%) with the strongest antibacterial activity against *A. hydrophilia*.

### Table 1: Antibacterial activity of *C. citrinus* and *C. salignus* leaf and flower extracts

<table>
<thead>
<tr>
<th>Microbial Species</th>
<th>Antibiotic</th>
<th><em>C. citrinus</em> leaf extract</th>
<th><em>C. citrinus</em> flower extract</th>
<th><em>C. salignus</em> leaf extract</th>
<th><em>C. salignus</em> flower extract</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Gram negative rods</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td><em>Aeromonas hydrophilia</em></td>
<td>17.3 ± 0.6 (Chl)</td>
<td>6.0 ± 0</td>
<td>6.0 ± 0</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td><em>Alcaligenes faecalis</em></td>
<td>13.3 ± 0.6 (Amp)</td>
<td>20.7 ± 1.2</td>
<td>15.7 ± 3.2</td>
<td>20.3 ± 0.6</td>
<td>28.7 ± 1.2</td>
</tr>
<tr>
<td><em>Citrobacter freundii</em></td>
<td>23.0 ± 1.0 (Chl)</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td><em>Enterobacter aerogenes</em></td>
<td>17.3 ± 0.3 (Chl)</td>
<td>-</td>
<td>-</td>
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<tr>
<td><em>Escherichia coli</em></td>
<td>16.7 ± 0.6 (Amp)</td>
<td>-</td>
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<tr>
<td><em>Klebsiella pneumoniae</em></td>
<td>18.3 ± 0.6 (Amp)</td>
<td>-</td>
<td>8.3 ± 0.3</td>
<td>-</td>
<td>-</td>
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<tr>
<td><em>Pseudomonas aeruginosa</em></td>
<td>31.6 ± 0.3 (Cip)</td>
<td>-</td>
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<tr>
<td><em>Pseudomonas fluorescens</em></td>
<td>21.0 ± 0.0 (Chl)</td>
<td>9.6 ± 0.3</td>
<td>15.6 ± 0.3</td>
<td>-</td>
<td>18.3 ± 0.3</td>
</tr>
<tr>
<td><em>Salmonella salford</em></td>
<td>25.3 ± 0.3 (Amp)</td>
<td>12.0 ± 0.0</td>
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<td>-</td>
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<tr>
<td><em>Seratia marescens</em></td>
<td>25.7 ± 0.6 (Chl)</td>
<td>-</td>
<td>8.6 ± 0.3</td>
<td>-</td>
<td>17.3 ± 0.3</td>
</tr>
<tr>
<td><em>Yersinia enterocolitica</em></td>
<td>16.3 ± 0.3 (Amp)</td>
<td>-</td>
<td>8.6 ± 0.3</td>
<td>-</td>
<td>17.3 ± 0.3</td>
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<tr>
<td><strong>Gram positive rods</strong></td>
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<tr>
<td><em>Bacillus cereus</em></td>
<td>25.3 ± 0.6 (Chl)</td>
<td>14.6 ± 0.3</td>
<td>17.3 ± 0.3</td>
<td>13.7 ± 1.5</td>
<td>15.6 ± 0.6</td>
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<tr>
<td><em>Bacillus subtilis</em></td>
<td>22.7 ± 0.6 (Amp)</td>
<td>19.3 ± 0.3</td>
<td>18.0 ± 1.0</td>
<td>13.6 ± 0.3</td>
<td>17.3 ± 0.3</td>
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<tr>
<td><strong>Gram positive cocci</strong></td>
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<tr>
<td><em>Staphylococcus aureus</em></td>
<td>16.3 ± 0.3 (Amp)</td>
<td>13.6 ± 0.3</td>
<td>15.0 ± 0</td>
<td>17.6 ± 0.3</td>
<td>8.6 ± 0.3</td>
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<tr>
<td><strong>Fungi</strong></td>
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<td><em>Aspergillus niger</em></td>
<td>18.0 ± 0 (Cip)</td>
<td>-</td>
<td>-</td>
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<td>-</td>
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<tr>
<td><em>Candida albicans</em></td>
<td>25.7 ± 0.6 (Nys)</td>
<td>-</td>
<td>7.6 ± 1.2</td>
<td>-</td>
<td>-</td>
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<tr>
<td><strong>Yeast</strong></td>
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<tr>
<td><em>Saccharomyces cerevisiae</em></td>
<td>21.3 ± 0.6 (Nys)</td>
<td>-</td>
<td>-</td>
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</tr>
</tbody>
</table>

Numbers indicate the mean diameters of inhibition of triplicate experiments ± standard deviation. – indicates no growth inhibition. Amp indicates ampicillin (2 µg). Chl indicates chloramphenicol (10 µg). Cip indicates ciprofloxacin (2.5 µg). Nys indicates nystatin (100 µg).
effect seen against *A. faecalis*. *C. salignus* flower extract inhibited the growth of 6 of the 14 bacteria tested (43%). The antibacterial activity was strongest against *A. faecalis*, *P. fluorescens*, *Y. enterocolitica* and *B. subtilis* (as determined by the diameter of the zone of inhibition). Indeed, *C. salignus* flower extract was a significantly more effective inhibitor of *A. faecalis* growth than was the ampicillin control.

Both Gram-positive and Gram-negative bacteria were inhibited by *C. citrinus* although Gram-positive bacteria were more susceptible. Of the 11 Gram-negative bacteria tested, 3 (27%) were inhibited by *C. citrinus* leaf extract. The leaf extract inhibited the growth of all of Gram-positive bacteria tested (100%). Likewise, Gram-positive bacteria were more susceptible to *C. citrinus* flower extract than was Gram-negative bacteria. Of the 11 Gram-negative bacteria tested, 6 were inhibited by *C. citrinus* flower extract (55%). 100% of the Gram-positive bacteria tested were inhibited by *C. citrinus* flower extract.

A similar selectivity was seen for *C. salignus* extracts. *C. salignus* leaf extract inhibited the growth of 1 of the 11 Gram-negative bacteria tested (9%) compared to 100% of the Gram-positive bacteria. *C. salignus* flower extract inhibited the growth of 3 of the 11 Gram-negative bacteria tested (27%) and 100% of the Gram-positive bacteria. The ability of *C. citrinus* and *C. salignus* extracts to inhibit the growth of both Gram-positive and Gram-negative bacteria is in agreement with a previous report of the antibacterial activity of a different species of Callistemon (*C. rigidus*).[15]

The greater susceptibility of Gram-positive bacteria is in agreement with previously reported results for South American,[24] African[25,26] and Australian[27] plant extracts. Results within this laboratory have also confirmed the greater susceptibility of Gram-positive bacteria towards other Australian plant extracts.[17,18,28-32] The Gram-negative bacterial cell wall outer membrane is thought to act as a barrier to many substances including antibiotics.[33] The uptake of the *Callistemon* extracts antibiotic agents by Gram-negative bacteria is presumably affected by the cell wall outer membrane. In contrast, other studies have demonstrated that Gram-negative bacteria are more susceptible to plant extracts from different Australian plant species.[34-36]

Of the *Callistemon* extracts tested only *C. salignus* leaf extract demonstrated any antifungal activity. This extract inhibited the growth of *C. albicans* but was unable to inhibit *A. niger* growth. The only yeast tested in these studies (*S. cerevisiae*), was not inhibited by any of the *Callistemon* extracts.

The antibacterial activity of the *C. citrinus* (Figure 1) and *C. salignus* (Figure 2) leaf extracts were further investigated.

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**Figure 1**: Inhibition of bacterial growth by a methanolic extract of *C. citrinus* 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 ± standard deviation.
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mortality within 24 h, with 100% mortality induction seen by 36 h.

To further investigate the toxicity of these extracts, LC50 values were determined by testing across the concentration range 2500 µg/ml to 10 µg/ml in the A. franciscana nauplii bioassay (Table 2). For comparison, serial dilutions of potassium dichromate and Mevinphos were also tested. No LC50 values are reported for the C. citrinus leaf or flower extracts at any time point 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. Similarly, no LC50 values are reported for the C. salignus leaf extract at any time point and for the flower extract at 24 and 48h. The C. salignus flower extract does display low toxicity at 72h with an LC50 value of 1986 ± 210. As LC50 values ≥ 1000 µg/ml are defined as non-toxic[37] this indicates that the C. salignus flower extracts is also of non-toxicity.

In conclusion, these studies and previous studies within this laboratory[16] show that C. citrinus and C. salignus leaf and flower extracts contain antibacterial components and support the traditional Australian Aborigine medicinal use of Callistemon spp. to protect against infection by both Gram-positive and Gram-negative bacteria. As many Callistemon species have been used as a food source[13] and bush medicine[14] by Australian Aborigines for thousands of years, there is

Figure 2: Inhibition of bacterial growth by a methanolic extract of C. salignus leaves against (a) B. cereus, (b) B. subtilis, (c) P. fluorescens, (d) A. hydrophila. 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 ± standard deviation.
potential for the use of Callistemon extracts as antiseptic agents and as food additives to protect against spoilage. However, further studies are needed before these extracts can be applied to these purposes. In particular, further toxicity studies are needed to determine the suitability of these extracts for use as antiseptic agents and as a food additive. No studies...
of Callistemon toxicity towards human cells was found in the literature. Further studies are needed to fully determine the cytotoxicity of these extracts. These results provide further support the ethno-botanical approach to screening plants as potential sources of bioactive substances.[38]

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REFERENCES