Antimicrobial Activity of Syzygium australe and Syzygium leuhmannii Leaf Methanolic Extracts

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INTRODUCTION

Traditional medicinal plants have been used to treat bacterial infections in many parts of the world for centuries.[1,3] The use of commercially available antibiotics has revolutionised the treatment of microbial infection. Unfortunately, their indiscriminate use 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.

Syzygium is a genus of flowering plants within the family Myrtaceae. Plants of this genus are widespread, occurring in tropical and subtropical regions of South-East Asia, Australia and Africa.[9] Many Syzygium (eg. Syzygium jambos) species produce edible fruit. In the commercially most important species Syzygium aromaticum (clove), the unopened flower bud is used as a spice. This plant also has uses in traditional medicine due to its anaesthetic properties.[10] The antibacterial activity of Syzygium aromaticum is also well known. Numerous studies have reported on the antibacterial[11] and antifungal[12] activities of oils and extracts from this plant. Other Syzygium species from South East Asia (Syzygium jambos)[13] and India (Syzygium cumini) have also been shown to have antimicrobial activity. Much of our knowledge about the antimicrobial activities of Australian Syzygium species is anecdotal although Australian Aborigines are known to use some species as medicinal agents.[11]

ABSTRACT: Introduction: Many species of Syzygium are known to have antiseptic activity. Australian Syzygium species had roles as traditional bush medicines for Australian Aborigines although their antiseptic potential has not been rigorously studied. Methods: The antimicrobial activity of methanolic extracts of Syzygium australe and Syzygium leuhmannii leaves was investigated by disc diffusion assay and growth time course assays against a panel of bacteria and fungi. Toxicity was determined using the Artemia franciscana nauplii bioassay. Results: S. australe leaf extract inhibited the growth of 10 of the 14 bacteria tested (71%). Gram-positive and Gram-negative bacteria were equally susceptible. 8 of the 11 Gram-negative bacteria (73%) and 2 of the 3 Gram-positive bacteria (67%) tested had their growth inhibited by S. australe leaf extract. The extract also displayed antifungal activity against a nystatin resistant strain of A. niger but did not affect C. albicans or S. cerevisiae growth. In comparison, S. leuhmannii leaf extract did not inhibit the growth of any of the microbial agents tested in the disc diffusion assay. The antibacterial activity of S. australe leaf extract was further investigated by growth time course assays which showed significant growth inhibition in cultures of A. hydrophilia, B. cereus, and P. fluorescens within 1 h but not of B. subtilis. S. leuhmannii also inhibited the growth of P. fluorescens and to a lesser extent, A. hydrophilia in the time course assay. Both Syzygium extracts displayed low toxicity in the Artemia franciscana bioassay. Conclusions: The low toxicity of these Syzygium extracts and the inhibitory bioactivity of S. australe against the bacterial panel validate Australian Aboriginal usage of S. australe leaves as antiseptic agents and confirms their medicinal potential.

KEY WORDS: Syzygium australe, Syzygium leuhmannii, Australian plants, antibacterial activity, medicinal plants, toxicity
A recent study has highlighted the therapeutic potential of fruit from the Australian species Syzygium leuhammadii (Riberry) and Syzygium australe (Bush Cherry) due to their extremely high antioxidant levels. Studies within this laboratory have found antibacterial activity in methanolic leaf extracts from Syzygium australe and Syzygium leuhammadii against a limited panel of bacteria. 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. Briefly, Syzygium australe and Syzygium leuhammadii leaves were collected from verified trees in the suburbs of 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 filter paper (Whatman No. 54) under vacuum followed by drying by rotary evaporation in an Eppendorf concentrator.

**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 fecalis*, *Bacillus cereus*, *Bacillus subtilis*, *Citrobacter freundii*, *Enterobacter aerogenes*, *Escherichia coli*, *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.

**Bacterial Growth Time Course Assay**

Bacterial growth time course studies were performed as previously described. Briefly, 3 ml of bacterial cultures (B. cereus, B. subtilis, A. hydrophilia, P. fluorescens) in nutrient broth were added to 27 ml nutrient broth containing 3 ml *S. austral* or *S. leuhammadii* 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 inoculated under the same conditions but without the extract. All assays were performed in triplicate.

**Toxicity Screening**

**Reference Toxins for Toxicity Screening**

Potassium dichromate (K₂Cr₂O₇) (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-methoxybenzyl-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. Briefly, *Artemia franciscana* Kellogg shrimp 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
RESULTS AND DISCUSSION

*S. australe* and *S. leuhmannii* leaf extracts were diluted to 27 mg/ml and 8 mg/ml respectively. 10 µl of the extracts were tested in the disc diffusion assay against 17 microorganisms (Table 1). The *S. australe* leaf extract inhibited the growth of 10 of the 14 bacteria tested (71%). The antibacterial activity was strongest against *A. faecalis*, *K. pneumoniae* and *P. fluorescens* (as determined by the diameter of the zone of inhibition). *S. leuhmannii* failed to inhibit the growth of any of the microbial species tested.

Both Gram-positive and Gram-negative bacterial growth was equally inhibited by *S. australe* leaf extract. Of the 11 Gram-negative bacteria tested, 8 (73%) were inhibited by *S. australe* extract. The extract also inhibited the growth of 2 of the 3 Gram-positive bacteria tested (67%). This is in agreement with previous reports of the antibacterial activity of other *Syzygium* species. *Syzygium cumini*, *Syzygium travancoricum*,[23] *Syzygium aromaticum*[11] and *Syzygium jambos*[13] have been previously shown to have broad antimicrobial activity against both Gram-positive and Gram-negative bacteria. Other studies have shown Gram-negative bacteria to be more susceptible to some Australian plant extracts.[24-26] However, these results are in contrast to previous inhibition

<table>
<thead>
<tr>
<th>Microbial Species</th>
<th>Antibiotic</th>
<th>Mean Zone of Inhibition ± SD (mm)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td><em>S. australe</em> leaf extract</td>
</tr>
<tr>
<td><strong>Gram negative rods</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Aeromonas hydrophilia</td>
<td>17.3 ± 0.6 (Chl)</td>
<td>8.0 ± 0</td>
</tr>
<tr>
<td>Alcaligenes faecalis</td>
<td>13.3 ± 0.6 (Amp)</td>
<td>10.3 ± 0.6</td>
</tr>
<tr>
<td>Citrobacter freundii</td>
<td>23.0 ± 1.0 (Chl)</td>
<td>–</td>
</tr>
<tr>
<td>Enterobacter aerogenes</td>
<td>17.3 ± 0.3 (Chl)</td>
<td>7.3 ± 0.3</td>
</tr>
<tr>
<td>Escherichia coli</td>
<td>16.7 ± 0.6 (Amp)</td>
<td>–</td>
</tr>
<tr>
<td>Klebsiella pneumoniae</td>
<td>18.3 ± 0.6 (Amp)</td>
<td>9.3 ± 1.2</td>
</tr>
<tr>
<td>Pseudomonas aeruginosa</td>
<td>31.6 ± 0.3 (Cip)</td>
<td>7.0 ± 0</td>
</tr>
<tr>
<td>Pseudomonas fluorescens</td>
<td>21.0 ± 0.0 (Chl)</td>
<td>10.0 ± 0</td>
</tr>
<tr>
<td>Salmonella salmod</td>
<td>25.3 ± 0.3 (Amp)</td>
<td>–</td>
</tr>
<tr>
<td>Seratia mesences</td>
<td>25.7 ± 0.6 (Chl)</td>
<td>8.3 ± 0.3</td>
</tr>
<tr>
<td>Yersinia enterocolitica</td>
<td>16.3 ± 0.3 (Amp)</td>
<td>8.3 ± 0.3</td>
</tr>
<tr>
<td><strong>Gram positive rods</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Bacillus cereus</td>
<td>25.3 ± 0.6 (Chl)</td>
<td>8.3 ± 0.3</td>
</tr>
<tr>
<td>Bacillus subtilis</td>
<td>22.7 ± 0.6 (Amp)</td>
<td>–</td>
</tr>
<tr>
<td><strong>Gram positive cocci</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Staphylococcus aureus</td>
<td>16.3 ± 0.3 (Amp)</td>
<td>7.0 ± 0</td>
</tr>
<tr>
<td><strong>Fungi</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Aspergillus niger</td>
<td>18.0 ± 0 (Cip)</td>
<td>8.3 ± 0.3</td>
</tr>
<tr>
<td>Candida albicans</td>
<td>25.7 ± 0.6 (Nys)</td>
<td>–</td>
</tr>
<tr>
<td><strong>Yeast</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Saccharomyces cerevisae</td>
<td>21.3 ± 0.6 (Nys)</td>
<td>–</td>
</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 (20 µg). Cip indicates ciprofloxacin (2.5 µg). Nys indicates nystatin (200 µg). – indicates no growth inhibition.
results reported for many other plant extracts. A greater susceptibility of Gram-positive bacteria has been previously reported for South American,\(^{27}\) African\(^{5,28}\) and several Australian\(^{29}\) plant extracts. Results within this laboratory\(^{16,30-33}\) have also confirmed the greater susceptibility of Gram-positive bacteria towards other Australian plant extracts. The Gram-negative bacterial cell wall outer membrane is thought to act as a barrier to many substances including antibiotics.\(^{16}\) The uptake of the *S. australae* extract antibiotic agent by Gram-negative bacteria is presumably not affected by the cell wall outer membrane.

The *S. australae* extract also demonstrated limited antifungal activity. This extract inhibited the growth of a nystatin resistant strain of *A. niger* but was unable to inhibit *C. albicans* growth. This is an important result as this strain of *A. niger* was resistant to all other antimicrobial agents tested except ciprofloxacin. The only yeast tested in these studies (*S. cerevisiae*) was not inhibited by either of the Syzygium extracts. *S. leuhmannii* extract did not inhibit the growth of any of the microbial species tested.

The antibacterial activity of the *S. australae* extract was further investigated by bacterial growth time course assays in the presence and absence of the extract. *S. australae* extract was able to significantly inhibit *B. cereus* (Figure 1a), *A. hydrophilia* (Figure 1c) and *P. fluorescens* (Figure 1d) growth within 1 h, indicating a rapid antimicrobial action. *Bacillus subtilis* (Figure 1b) was not inhibited in this study, in agreement with previous reports.\(^{16}\) Furthermore, a decrease in optical density was seen for *P. fluorescens* and *A. hydrophilia* exposed to *S. australae* extract, perhaps indicating bacterial lysis had occurred.

Antibacterial activity was also investigated by bacterial growth time course assays in the presence and absence of *S. leuhmannii* extract (Figure 2). *S. leuhmannii* leaf extract was able inhibit *P. fluorescens* (Figure 2c), albeit only temporarily. This result was surprising as bacterial inhibition studies using disc diffusion had not shown any growth inhibition for *S. leuhmannii* leaf extract against any bacterial species. However, it must be noted that the disc diffusion assay requires an incubation time of 24 hours compared to the 6 hour incubation time of these growth course assays. It appears that whilst the *S. leuhmannii* leaf extract slows initial bacterial growth, the bacteria can overcome this effect with a longer incubation time. Indeed, whilst inhibition of *P. fluorescens* growth was evident in the first 4 h of exposure, the bacteria appear to have overcome this inhibitory effect by 6 h. Growth of *A. hydrophilia* (Figure 2d) also displayed partial inhibition whilst the growth of *B. cereus* and *B. subtilis* were essentially unaffected by the presence of *S. leuhmannii* leaf extract.

![Figure 1: Inhibition of bacterial growth by *S. australae* leaf methanolic extract 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.](image-url)
To examine the toxicity of the Syzygium extracts, they initially tested in the *Artemia franciscana* nauplii bioassay at a concentration of 1000 µg/ml (Figure 3). The *S. australae* leaf extract only induced low levels of mortality (Figure 3a), similar to the % mortality seen for the seawater control (Figure 3e). The *S. leuhmannii* extract (Figure 3b) induced toxicity above that of the seawater control, although even these results indicate a low level of toxicity. Only low levels of mortality were seen by 48 h, 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 further investigate the toxicity of these extracts, LC50 values were determined by testing across the concentration range 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 LC50 values are reported for the *S. australae* leaf extract at any time point as no significant increase in mortality above the seawater controls was seen for these extracts at any time tested, indicating that this extract is non-toxic. Similarly, no LC50 values are reported for the *S. leuhmannii* leaf extract at 24 and 48h. The *S. leuhmannii* extract does display low toxicity at 72h with an LC50 value of 878 ± 63. As LC50 values ≥1000 µg/ml are defined as non-toxic,[35] this indicates that the *S. leuhmannii* leaf extract is of low toxicity.

In conclusion, the findings of this study have demonstrated the susceptibilities of a broad range of microbes to *S. australae* leaf extract. Both Gram-positive and Gram-negative bacteria were equally susceptible to *S. australae* leaf extract. The broad range of microbial susceptibilities indicates the potential of *S. australae* leaf extract as a surface disinfectant as well as for medicinal purposes and as food additives to inhibit spoilage. However, further studies are needed before these extracts can be applied to these purposes. In particular, further toxicity studies using 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 low toxicity of other Syzygium species (*S. endophoium*, *S. johnsonii*, *S. papyraceum*, *S. weia*) against HepG2, and two carcinoma cell lines.[88] The same study reported more complex toxicity results for *S. wilsonii* extracts. *S. wilsonii* extracts also displayed low toxicity towards HepG2 and a breast carcinoma cell line but showed substantial toxicity towards a bladder carcinoma cell line.

**ACKNOWLEDGEMENTS**

Financial support for this work was provided by the School of Biomolecular and Physical Sciences, Griffith University, Australia.
Table 2: LC₅₀ (95 % confidence interval) for A. franciscana nauplii exposed to S. australe and S. leuhmanii extracts, the reference toxins potassium dichromate and Mevinphos and a seawater control

<table>
<thead>
<tr>
<th>Plant Species</th>
<th>Plant Part Tested</th>
<th>24 h</th>
<th>48 h</th>
<th>72 h</th>
</tr>
</thead>
<tbody>
<tr>
<td>S. australe</td>
<td>leaves</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>S. leuhmanii</td>
<td>leaves</td>
<td>NA</td>
<td>NA</td>
<td>878 ± 63</td>
</tr>
<tr>
<td>Potassium dichromate</td>
<td></td>
<td>143 ± 18</td>
<td>82 ± 4</td>
<td>79 ± 5</td>
</tr>
<tr>
<td>Mevinphos</td>
<td></td>
<td>1418 ± 172</td>
<td>546 ± 45</td>
<td>123 ± 18</td>
</tr>
<tr>
<td>Seawater control</td>
<td></td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
</tr>
</tbody>
</table>

NA indicates that LC₅₀ values were not obtained as ≥50 % mortality was not reached for this time point. Results represent the mean ± standard deviation of triplicate determinations.

REFERENCES


