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Research Letter

Antimicrobial Activity of *Syzygium australe* and *Syzygium leuhmannii* Leaf Methanolic Extracts

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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 assay 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

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 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 *S. 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 lineare* and *Syzygium cumini*)[14] 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.[1]

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A recent study has highlighted the therapeutic potential of fruit from the Australian species *Syzygium leuhmannii* (Riberry) and *Syzygium australe* (Bush Cherry) due to their extremely high antioxidant levels.[15] Studies within this laboratory has found antibacterial activity in methanolic leaf extracts from *Syzygium australe* and *Syzygium leuhmannii* against a limited panel of bacteria.[16] 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.[16] Briefly, *Syzygium australe* and *Syzygium leuhmannii* 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 5301. The resultant pellet was dissolved in 15 ml 20 % methanol. The extracts were passed through 0.22 µm filters (Sarstedt) and stored at 4 °C until use.

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 feacalis*, *Bacillus cereus*, *Bacillus subtilis*, *Citrobacter freundii*, *Enterobacter aerogenes*, *Escherichia coli*, *Klebsiella pneumoniae*, *Pseudomonas aeuroginosa*, *Pseudomonas fluorescens*, *Salmonella salford*, *Serratia marcescens*, *Staphylococcus aureus* and *Yersinia enterocolitia* 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.[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 feacalis*, *Aeromonas hydrophilia*, *Bacillus cereus*, *Bacillus subtilis*, *Citrobacter freundii*, *Klebsiella pneumoniae*, *Pseudomonas aeuroginosa, Pseudomonas fluorescens*, *Serratia marcescens*, *Yersinia enterocolitia*, *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 ciprafloxicin (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.

Bacterial Growth Time Course Assay

Bacterial growth time course studies were performed as previously described.[19] 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. australe* or *S. leuhmannii* 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 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.

Artemia franciscana Nauplii Toxicity Screening

Toxicity was tested using a modified *Artemia franciscana* nauplii lethality assay.[20-22] Briefly, *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 46 (mean 46.2, n = 93, SD 12.3) nauplii were added to wells of a 48 well plate and immediately used for bioassay. The plant extracts were diluted to 2 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

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 1: Antibacterial activity of *S. australe* and *S. leuhmannii* extracts. Numbers indicate the mean diameters of inhibition of triplicate experiments ± standard deviation

Microbial Species	Mean Zone of Inhibition ± SD (mm)		
	Antibiotic	*S. australe* leaf extract	*S. leuhamannii* leaf extract
Gram negative rods			
Aeromonas hydrophilia	17.3 ± 0.6 (Chl)	8.0 ± 0	–
Alcaligenes faecalis	13.3 ± 0.6 (Amp)	10.3 ± 0.6	–
Citrobacter fruendii	23.0 ± 1.0 (Chl)	–	–
Enterobacter aerogenes	17.3 ± 0.3 (Chl)	7.3 ± 0.3	–
Escherichia coli	16.7 ± 0.6 (Amp)	–	–
Klebsiella pneumoniae	18.3 ± 0.6 (Amp)	9.3 ± 1.2	–
Pseudomonas aeuroginosa	31.6 ± 0.3 (Cip)	7.0 ± 0	–
Pseudomonas fluorescens	21.0 ± 0 (Chl)	10.0 ± 0	–
Salmonella salford	25.3 ± 0.3 (Amp)	–	–
Seratia marescens	25.7 ± 0.6 (Chl)	8.3 ± 0.3	–
Yersinia enterocolitia	16.3 ± 0.3 (Amp)	8.3 ± 0.3	–
Gram positive rods			
Bacillus cereus	25.3 ± 0.6 (Chl)	8.3 ± 0.3	–
Bacillus subtilis	22.7 ± 0.6 (Amp)	–	–
Gram positive cocci			
Staphylococcus aureus	16.3 ± 0.3 (Amp)	7.0 ± 0	–
Fungi			
Aspergillus niger	18.0 ± 0 (Cip)	8.3 ± 0.3	–
Candida albicans	25.7 ± 0.6 (Nys)	–	–
Yeast			
Saccharomyces cerevisiae	21.3 ± 0.6 (Nys)	–	–

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).
– 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.[34] The uptake of the *S. australe* extract antibiotic agent by Gram-negative bacteria is presumably not affected by the cell wall outer membrane.

The *S. australe* 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. australe* extract was further investigated by bacterial growth time course assays in the presence and absence of the extract. *S. australe* 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.s hydrophilia* exposed to *S. australe* 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 temoparily. 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.

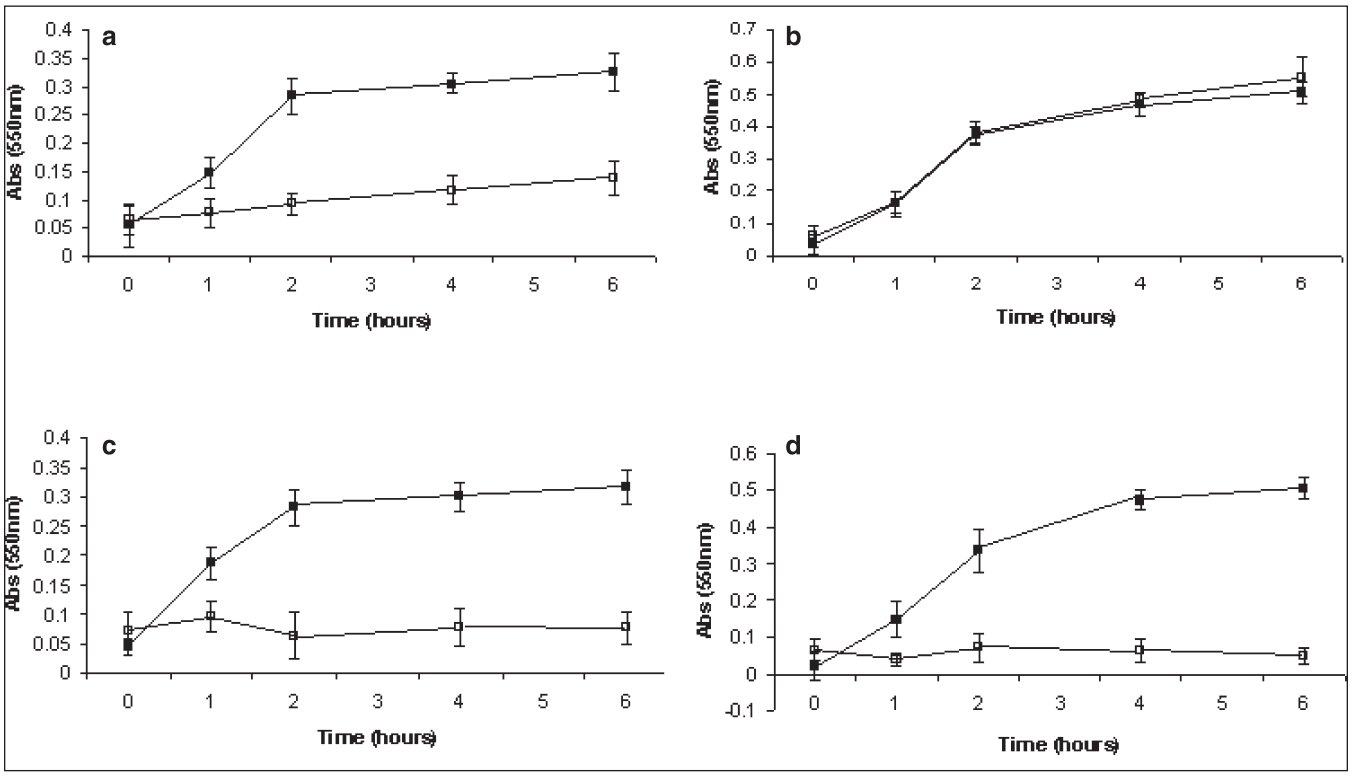

Figure 1: Inhibition of bacterial growth by *S. australae* leaf methanolic extract against (a) *B. cereus*, (b) *B. subtilis*, (c) *P. fluorescens*, (d) *A. hydrophilia*. For all graphs, □ represent measured bacterial growth values for test cultures (with extract) and ■ represent control bacterial growth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

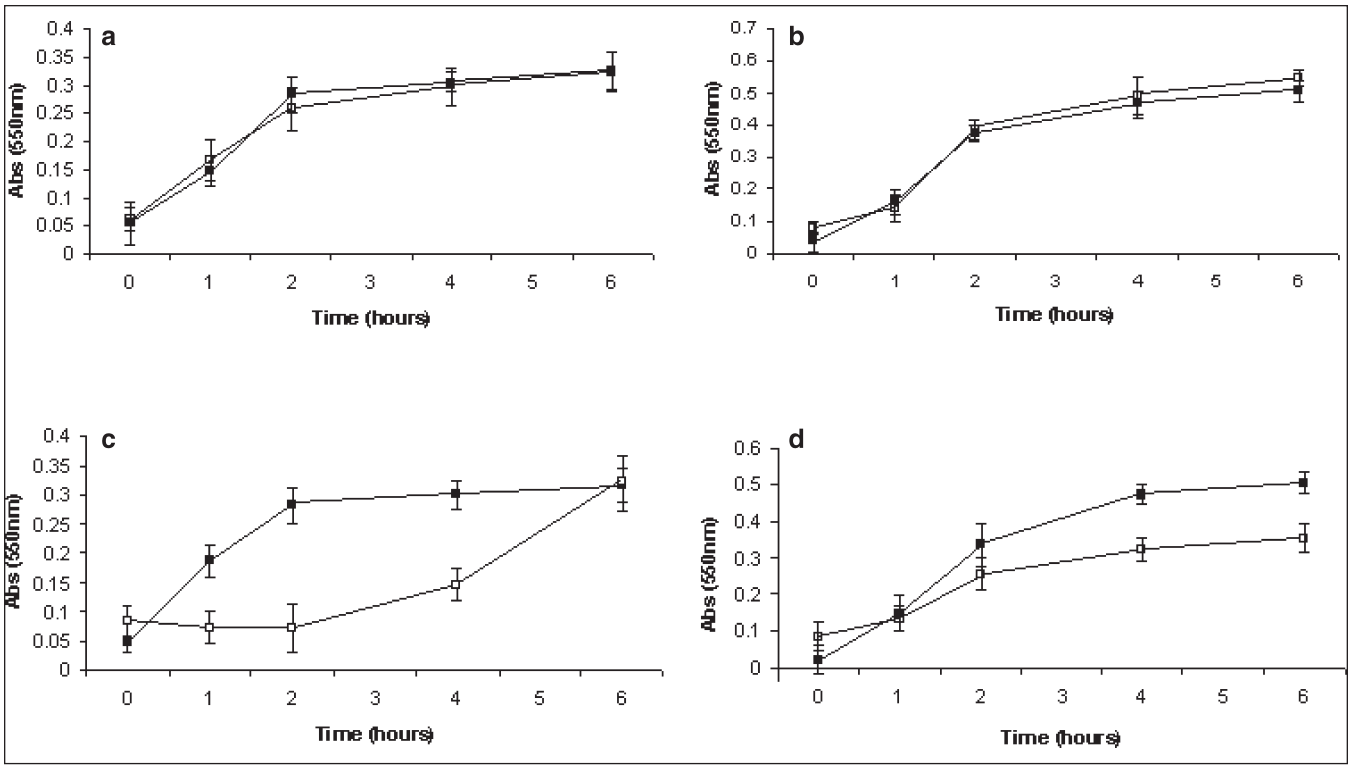

Figure 2: Inhibition of bacterial growth by *S. leuhmannii* leaf methanolic extract against (a) *B. cereus*, (b) *B. subtilis*, (c) *P. fluorescens*, (d) *A. hydrophilia*. For all graphs, □ represent measured bacterial growth values for test cultures (with extract) and ■ represent control bacterial growth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

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

To further investigate the toxicity of these extracts, LC_{50} 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 LC_{50} 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 LC_{50} 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 LC_{50} value of 878 ± 63. As LC_{50} 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. australe* leaf extract. Both Gram-positive and Gram-negative bacteria were equally susceptible to *S. australe* leaf extract. The broad range of microbial susceptibilities indicates the potential of *S. australe* 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. wesa*) against HepG2, and two carcinoma cell lines.[36] 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.

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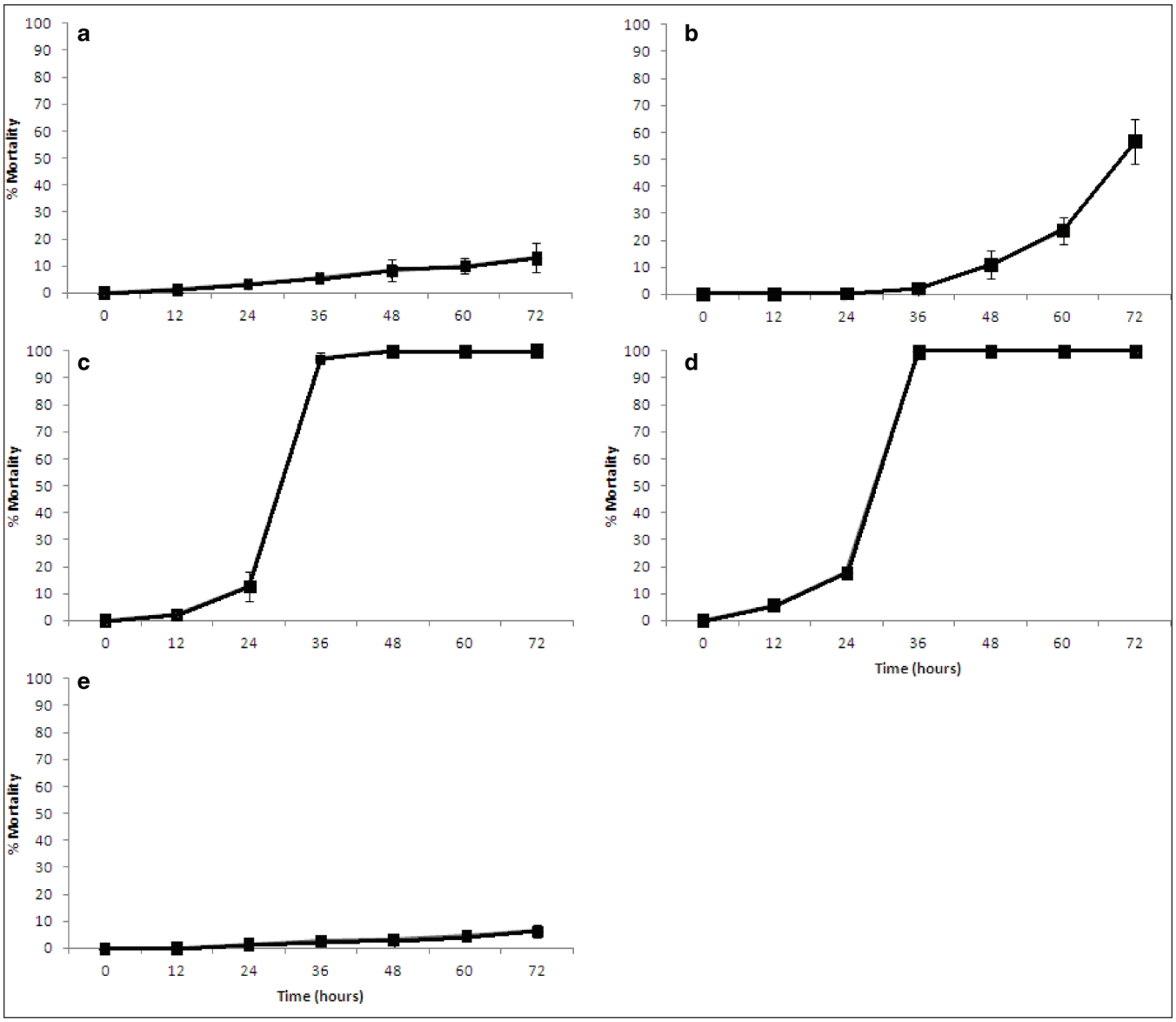

Figure 3: Brine shrimp lethality of (a) *S. australae* leaf methanolic extract (1000 µg/ml), (b) *S. leuhmannii* leaf methanolic extract (1000 µg/ml), (c) potassium dichromate (800 µg/ml), (d) Mevinphos (2000 µg/ml) and (e) seawater control. All bioassays were performed in at least triplicate and are expressed as mean ± standard error.

Table 2: LC_{50} (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

Plant Species	Plant Part Tested	LC_{50} (µg/ml)		
		24 h	48 h	72 h
S. australe	leaves	NA	NA	NA
S. leuhmanii	leaves	NA	NA	878 ± 63
Potassium dichromate		143 ± 18	82 ± 4	79 ± 5
Mevinphos		1418 ± 172	546 ± 45	123 ± 18
Seawater control		NA	NA	NA

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.

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