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Research Letter

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 for 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 *Melaleucas* 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

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to make sweet drinks.[13] *Callistemon* species also had roles as traditional bush medicines for Australian Aborigines [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.[14]

A recent report has demonstrated the antibacterial activity of a related *Callistemon* species (*Callistemon rigidus*).[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.[16] 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.

MATERIALS AND METHODS

Plant Collection and Extraction

The extracts investigated in this study have been described previously.[16] 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.

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 and 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.[19,20] 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 *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.

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 using a modified *Artemia franciscana* nauplii lethality assay.[21-23] *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 42 (mean 41.8, n = 164, SD 16.2) 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

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 stongest inhibitory

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

Microbial Species	Mean Zone of Inhibition ± SD (mm)				
	Antibiotic	*C.citrinus* leaf extract	*C.citrinus* flower extract	*C. salignus* leaf extract	*C. salignus* flower extract
Gram negative rods					
Aeromonas hydrophilia	17.3 ± 0.6 (Chl)	6.0 ± 0	6.0 ± 0	-	-
Alcaligenes faecalis	13.3 ± 0.6 (Amp)	20.7 ± 1.2	15.7 ± 3.2	20.3 ± 0.6	28.7 ± 1.2
Citrobacter fruendii	23.0 ± 1.0 (Chl)	-	-	-	-
Enterobacter aerogenes	17.3 ± 0.3 (Chl)	-	-	-	-
Escherichia coli	16.7 ± 0.6 (Amp)	-	-	-	-
Klebsiella pneumoniae	18.3 ± 0.6 (Amp)	-	8.3 ± 0.3	-	-
Pseudomonas aeuroginosa	31.6 ± 0.3 (Cip)	-	-	-	-
Pseudomonas fluorescens	21.0 ± 0 (Chl)	9.6 ± 0.3	15.6 ± 0.3	-	18.3 ± 0.3
Salmonella salford	25.3 ± 0.3 (Amp)	-	12.0 ± 0	-	-
Seratia marescens	25.7 ± 0.6 (Chl)	-	-	-	-
Yersinia enterocolitia	16.3 ± 0.3 (Amp)	-	8.6 ± 0.3	-	17.3 ± 0.3
Gram positive rods					
Bacillus cereus	25.3 ± 0.6 (Chl)	14.6 ± 0.3	17.3 ± 0.3	13.7 ± 1.5	15.6 ± 0.6
Bacillus subtilis	22.7 ± 0.6 (Amp)	19.3 ± 0.3	18.0 ± 1.0	13.6 ± 0.3	17.3 ± 0.3
Gram positive cocci					
Staphylococcus aureus	16.3 ± 0.3 (Amp)	13.6 ± 0.3	15.0 ± 0	17.6 ± 0.3	8.6 ± 0.3
Fungi					
Aspergillus niger	18.0 ± 0 (Cip)	-	-	-	-
Candida albicans	25.7 ± 0.6 (Nys)	-	-	7.6 ± 1.2	-
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).

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. flourescens*, *Y. enterocolitia* 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

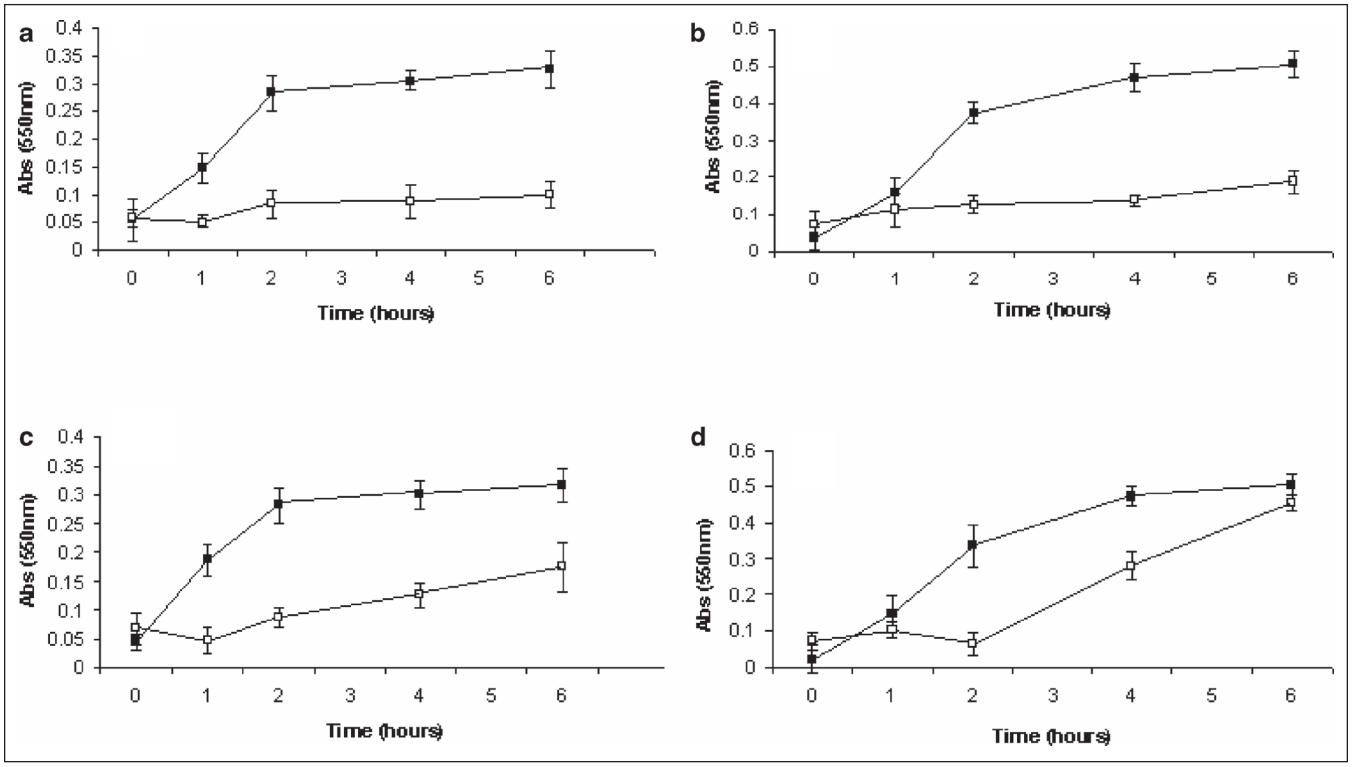

Figure 1: Inhibition of bacterial growth by a methanolic extract of *C. citrinus* leaves against (a) *B. cereus*, (b) *B. subtilis*, (c) *P. fluorescens*, (d) *A. hydrophilia*. For all graphs, □ represent measured bacterial growth values for test cultures (with extract) and ■ represent control bacterial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

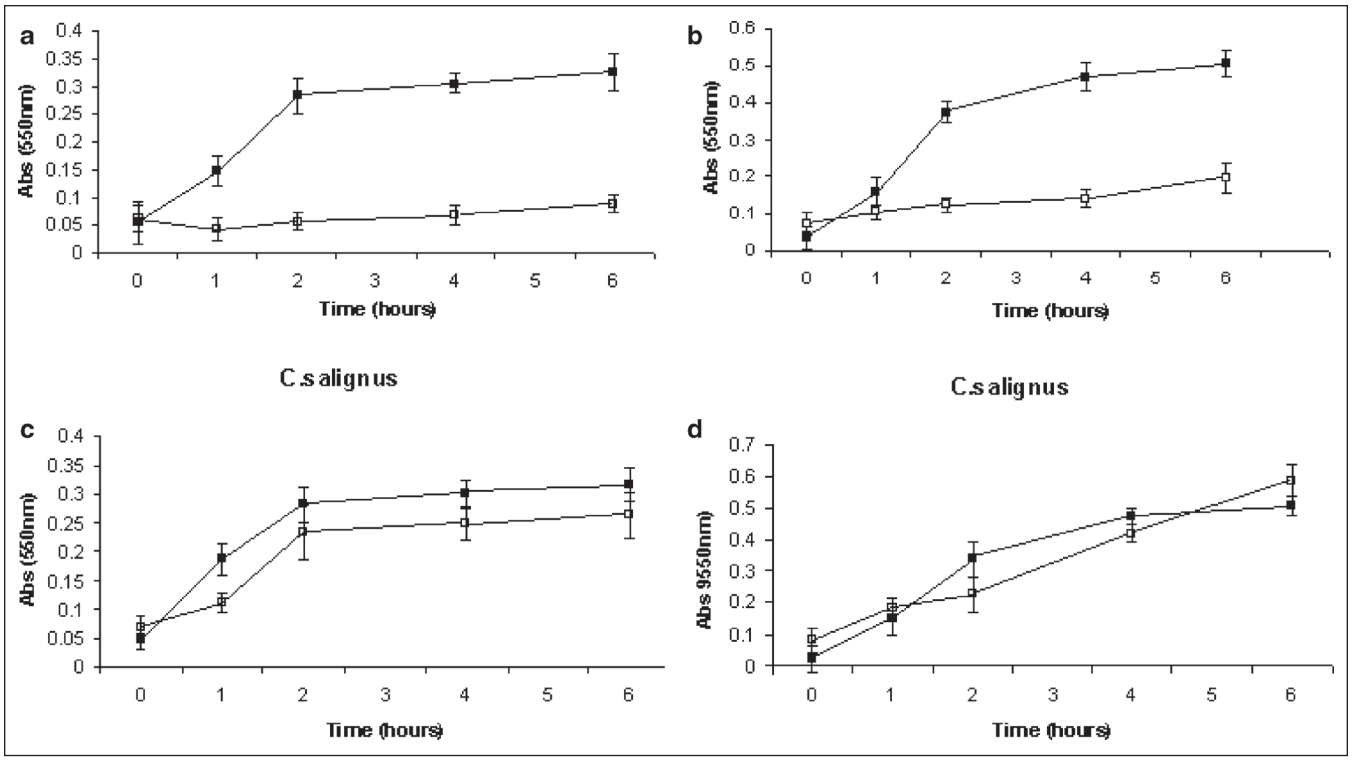

Figure 2: Inhibition of bacterial growth by a methanolic extract of *C. salignus* leaves against (a) *B. cereus*, (b) *B. subtilis*, (c) *P. fluorescens*, (d) *A. hydrophilia*. For all graphs, □ represent measured bacterial growth values for test cultures (with extract) and ■ represent control bacterial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

by bacterial growth time course assays in the presence and absence of the extract. The *C. citrinus* leaf extract significantly inhibited *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. Furthermore, a decrease in optical density was seen for *B. cereus, A. hydrophilia* and *P. fluorescens* treated with *C. citrinus* leaf extract which may indicate bacterial lysis had occurred. Similarly, *C. salignus* leaf extract also significantly inhibited *Bacillus cereus* (Figure 1a) and *Bacillus subtilis* (Figure 1b) growth within 1 h, indicating a rapid antimicrobial action. The *C. salignus* leaf extract did not significantly inhibit the growth of either the *P. fluorescens* (Figure 2c) or *A. hydrophilia* (Figure 1d), in agreement with the disc diffusion assay results.

To examine the toxicity of the Callistemon extracts, they were tested in the *Artemia franciscana* nauplii bioassay at a concentration of 2500 μg/ml (Figure 3). The *C. citrinus* leaf (Figure 3a) and flower (Figure 3b) extracts only induced low levels of mortality, similar to the % mortality seen for the seawater control (Figure 3f) at all time points. Similarly, the *C. salignus* leaf extract (Figure 3c) did not induce mortality above that seen for the seawater control at any time point. The *C. salignus* flower extract (Figure 3d) 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 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 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 LC_{50} 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 LC_{50} 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 LC_{50} value of 1986 ± 210. As LC_{50} 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

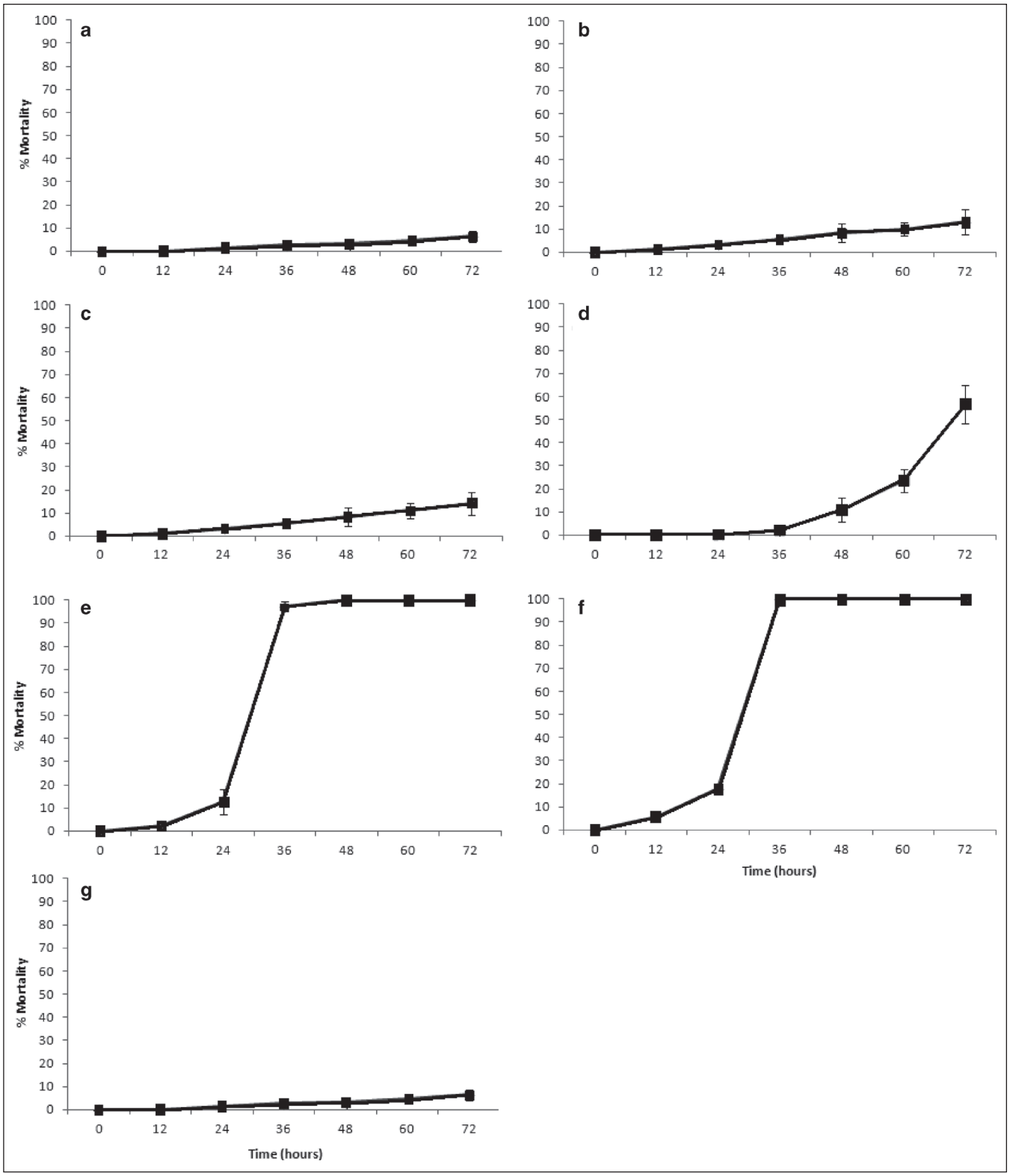

Figure 3: Brine shrimp lethality of (a) *C. citrinus* leaf methanolic extract (2500 µg/ml), (b) *C. citrinus* flower methanolic extract (2500 µg/ml), (c) *C. salignus* leaf methanolic extract (2500 µg/ml), (d) *C. salignus* flower methanolic extract (2500 µg/ml), (e) potassium dichromate (800 µg/ml), (f) Mevinphos (2000 µg/ml) and (g) seawater control. All bioassays were performed in at least triplicate and are expressed as mean ± standard error.

potential for the use of Callistemon extracts as antisceptic 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

Table 2: LC_{50} (95% confidence interval) for brine shrimp nauplii exposed to *C. citrinus* and *C. salignus* extracts, the reference toxins potassium dichromate and Mevinphos and a seawater control

Plant Species	Plant Part Tested	LC50 (µg/ml)		
		24 h	48 h	72 h
C. citrinus	leaves	NA	NA	NA
C. citrinus	flowers	NA	NA	NA
C. salignus	leaves	NA	NA	NA
C. salignus	flowers	NA	NA	1986 ± 210.4
Mevinphos		1418 ± 172	546 ± 45	123 ± 18
Potassium Dichromate		-	82 ± 4	79 ± 5

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

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 ethnobotanical approach to screening plants as potential sources of bioactive substances.[38]

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