Antimicrobial activity of Acacia aulacocarpa and Acacia complanta methanolic extracts

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Research Letter

Antimicrobial Activity of *Acacia aulacocarpa* and *Acacia complanta* Methanolic Extracts

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ABSTRACT: **Introduction:** Australian Acacia species also had a role as traditional bush medicines for Australian Aborigines, including uses as antiseptic agents. **Methods:** The antimicrobial activity of methanolic extracts of *Acacia aulacocarpa* leaves and *Acacia complanta* leaves and flowers were investigated by disc diffusion assay against a panel of bacteria and fungi. Toxicity was determined using the *Artemia franciscana* nauplii bioassay. **Results:** *A. aulacocarpa* leaf extract inhibited the growth of 6 of the 14 bacteria tested (43%). Gram-positive and Gram-negative bacteria were both inhibited by *A. aulacocarpa* leaf extract. 4 of 11 Gram-negative (36%) and 2 of 3 Gram-positive bacteria (67%) had their growth inhibited by *A. aulacocarpa* extract. *A. aulacocarpa* leaf extract displayed no antifungal activity towards any of the fungi tested. The antibacterial activity of *A. aulacocarpa* and *A. complanta* leaf extracts were further investigated by growth time course assays which showed significant growth inhibition in cultures of *Bacillus cereus*, *Aeromonas hydrophilia* and *Pseudomonas fluorescens* within 1 h but not of *Bacillus subtilis*. *A. complanta* flower extract displayed limited antibacterial activity, inhibiting the growth of only a single bacterium (*Bacillus subtilis*) (7%) and displayed no antifungal activity towards any of the fungi tested. *A. complanta* leaf extract was unable to inhibit the growth of any of the bacteria tested but displayed antifungal activity against a nystatin resistant strain of *Aspergillus niger*. It did not affect *Candida albicans* or *Saccharomyces cerevisiae* growth. All extracts displayed low toxicity in the *Artemia franciscana* bioassay. **Conclusions:** The low toxicity of these Acacia extracts and their inhibitory bioactivity against bacteria validate Australian Aboriginal usage of *A. aulacocarpa* and *A. complanta* as antiseptic agents and confirms their medicinal potential.

KEY WORDS: *Acacia aulacocarpa, Acacia complanta*, Australian plants, antibacterial activity, medicinal plants, toxicity

INTRODUCTION

The Acacia genus (family Fabaceae, subfamily Mimosaceae) consists of over 1200 species, more than 700 of which are indigenous to Australia.[1] Other species are spread throughout tropical to warm temperate regions of Africa, India and the Americas. Acacias have also been introduced into other countries for ornamental and economic purposes. Most Acacia species produce quality wood and some are also valuable sources of proteins, tannins, gum, perfumes, paint, ink and flavouring agents.[2, 3] For Australian Aborigines, Acacia seed formed an important part of their diet, providing an easily obtainable, high energy food[4, 5] that could easily be ground to a flour, mixed with water and eaten either raw or cooked to produce a type of unleavened bread. Other parts of some Acacia species are also eaten. Several species exude a sugary gum from wounds to the stem and branches[2, 4] whilst others are hosts for edible grubs often referred to as witchetty grubs by non-Aboriginal Australians.[6]

Australian Acacia species also had a role as traditional bush medicines for Australian Aborigines. Several species have been reported to be used to prepare antimicrobial washes and lotions by Aborigines.[7, 8] Unfortunately most of our understanding of the antimicrobial potential of Australian Acacia species is anecdotal with few species being rigorously studied. One South American Acacia species (*A. aroma*) has been shown to demonstrate antibacterial activity against both Gram-positive and Gram-negative bacteria.[2] Amongst the Australian Acacia species studied, *A. kempeana*, *A. tetragonophylla*,[9] *A. linarioides*, *A. brachystachya*, *A. lineate*, *A. trineura* and *A. olliquinervia*[1] have been reported to have to have antibacterial activity.

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A recent study[10] has demonstrated the antibacterial activity of methanolic extracts of *A. aulacocarpa* and *A. complanta* extracts 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, 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.[10] Briefly, *Acacia aulacocarpa* (leaves), *Acacia complanta* (leaves and flowers) were collected from Toohey Forest, Brisbane, Australia and were identified with reference to a taxonomic key to Toohey Forest plants.[11] 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 resulting in a 14 mg/ml *A. aulacocarpa* leaf extract and 15 mg/ml and 25 mg/ml extracts of *A. complanta* leaf and flower extracts respectively. The extracts 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 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 disc diffusion method previously described.[12] 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 extract was 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 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

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 *Acacia aulacocarpa* and *Acacia complanta* 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 the *Artemia franciscana* nauplii lethality assay.[13] 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 54 (mean 54.5, n = 108, SD 15.9) 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.[14]

RESULTS AND DISCUSSION

Acacia aulacocarpa leaf extract was diluted to a 14 mg/ml concentration and *A. complanta* leaves and flowers were diluted to 15 mg/ml and 25 mg/ml respectively. 10 µl of each extract was tested in the disc diffusion assay against 17 microorganisms (Table 1). The *A. aulacocarpa* leaf extract inhibited the growth of 6 of the 14 bacteria tested (43%). The antibacterial activity was strongest against *S. aureus* and *A. faecalis* (as determined by the diameter of the zone of inhibition) compared to the inhibition of the antibiotic controls. Neither *A. complanta* leaf nor flower extracts were particularly effective at inhibiting bacterial growth. The flower extract inhibited the growth of only of a single bacterium (*Bacillus subtilis*) (7%) whilst the leaf extract showed no antibacterial activity against any of the bacteria tested.

Both Gram-positive and Gram-negative bacteria were affected by *A. aulacocarpa* leaf extract although Gram-positive bacteria were more susceptible. Of the 11 Gram-negative bacteria tested, 4 (36%) were inhibited by *A. aulacocarpa* extract. The extract also inhibited the growth of 2 of the 3 Gram-positive bacteria tested (67%). The ability of *A. aulacocarpa* 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 Acacia extracts from other species.[1] This study also reported the susceptibility of both Gram-positive and Gram-negative bacteria towards various Acacia species extracts. However, the greater susceptibility of Gram-positive bacteria is in agreement with previously reported results for South American,[15] African[16, 17] and Australian[9]

Table 1: Antibacterial activity of *Acacia aulacocarpa* and *Acacia complanta* extracts.

Microbial Species	Mean Zone of Inhibition ± SD (mm)			
	Antibiotic	*A. aulacocarpa* leaf extract	*A. complanta* leaf extract	*A. complanta* flower extract
Gram negative rods				
Aeromonas hydrophilia	17.3 ± 0.6 (Chl)	7.7 ± 0.6	–	–
Alcaligenes faecalis	13.3 ± 0.6 (Amp)	11.3 ± 0.6	–	–
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)	–	–	–
Pseudomonas aeuroginosa	31.6 ± 0.3 (Cip)	–	–	–
Pseudomonas fluorescens	21.0 ± 0 (Chl)	10.6 ± 0.3	–	–
Salmonella salford	25.3 ± 0.3 (Amp)	–	–	–
Seratia marescens	25.7 ± 0.6 (Chl)	–	–	–
Yersinia enterocolitia	16.3 ± 0.3 (Amp)	8.6 ± 0.3	–	–
Gram positive rods				
Bacillus cereus	25.3 ± 0.6 (Chl)	9.0 ± 1.0	–	–
Bacillus subtilis	22.7 ± 0.6 (Amp)	–	–	8.0 ± 0
Gram positive cocci				
Staphylococcus aureus	16.3 ± 0.3 (Amp)	13.3 ± 1.2	–	–
Fungi				
Aspergillus niger	18.0 ± 0 (Cip)	–	8.6 ± 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).

plant extracts. Results within this laboratory[18-21] 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.[22] The uptake of the *A. aulacocarpa* extract antibiotic agents by Gram-negative bacteria is presumably not affected by the cell wall outer membrane.

Of the Acacia extracts tested, only *A. complanta* leaf extract demonstrated 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 any *Acacia* extract.

The antibacterial activity of the *A. aulacocarpa* leaf extract was further investigated by bacterial growth time course assays in the presence and absence of the extract Figure 1). The concentration of the extract used in these assays was 12.7 μg/ml. *A. aulacocarpa* leaf extract was able to significantly inhibit *Bacillus cereus* (Figure 1a), *Aeromonas hydrophilia* (Figure 1c) and *Pseudomonas fluorescens* (Figure 1d) growth within 1 h indicating a rapid antimicrobial action. *Bacillus subtilis* (Figure 1b) growth was unaffected by *A. aulacocarpa* leaf extract, in agreement with previously reported results.[10] Furthermore, a decrease in optical density was seen for *B. cereus, A. hydrophilia* and *P. fluorescens* treated with *A. aulacocarpa* leaf extract which may indicate bacterial lysis had occurred.

A. complanta leaves were also investigated by bacterial growth time course assays in the presence and absence of the extract (Figure 2). *A. complanta* leaf extract was able inhibit *Bacillus cereus* (Figure 2a), albeit only temoparily. Between 1-4 hours of incubation the optical density was significantly decreased in the presence of *A. complanta* leaf extract compared to in the control (absence of extract). This result was surprising as bacterial inhibition studies using disc diffusion had not shown any growth inhibition for *A. complanta* 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 *A. complanta* leaf extract slows initial bacterial growth, the bacteria can overcome this effect with a longer incubation time. Growth of *B. subtilis* (Figure 2b), *P. fluorescens* (Figure 2c) *A. hydrophilia* (Figure 2d) was essentially unaffected by the presence of *A. complanta* leaf extract.

To examine the toxicity of the extracts, the LC_{50} values were determined by testing across the concentration range 5000 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.

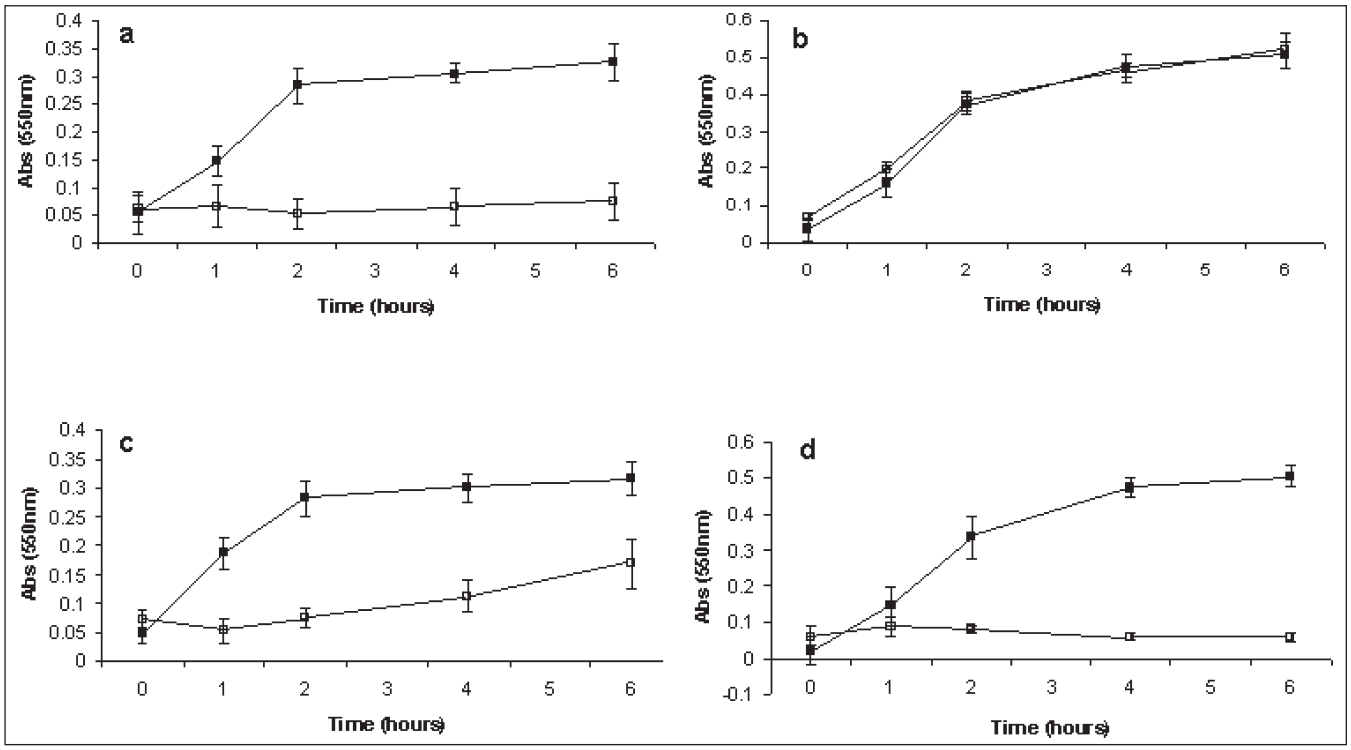

Figure 1: Inhibition of bacterial growth by methanolic extract of *A. aulocarpa* leaves against (a) *B.cereus*, (b) *B.subtilis*, (c) *P.fluorescens*, (d) *A. hydrophilia*. For all graphs, □ represent measured bacterial growth values for test cultures (with extract) and ■ represent control bacterial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

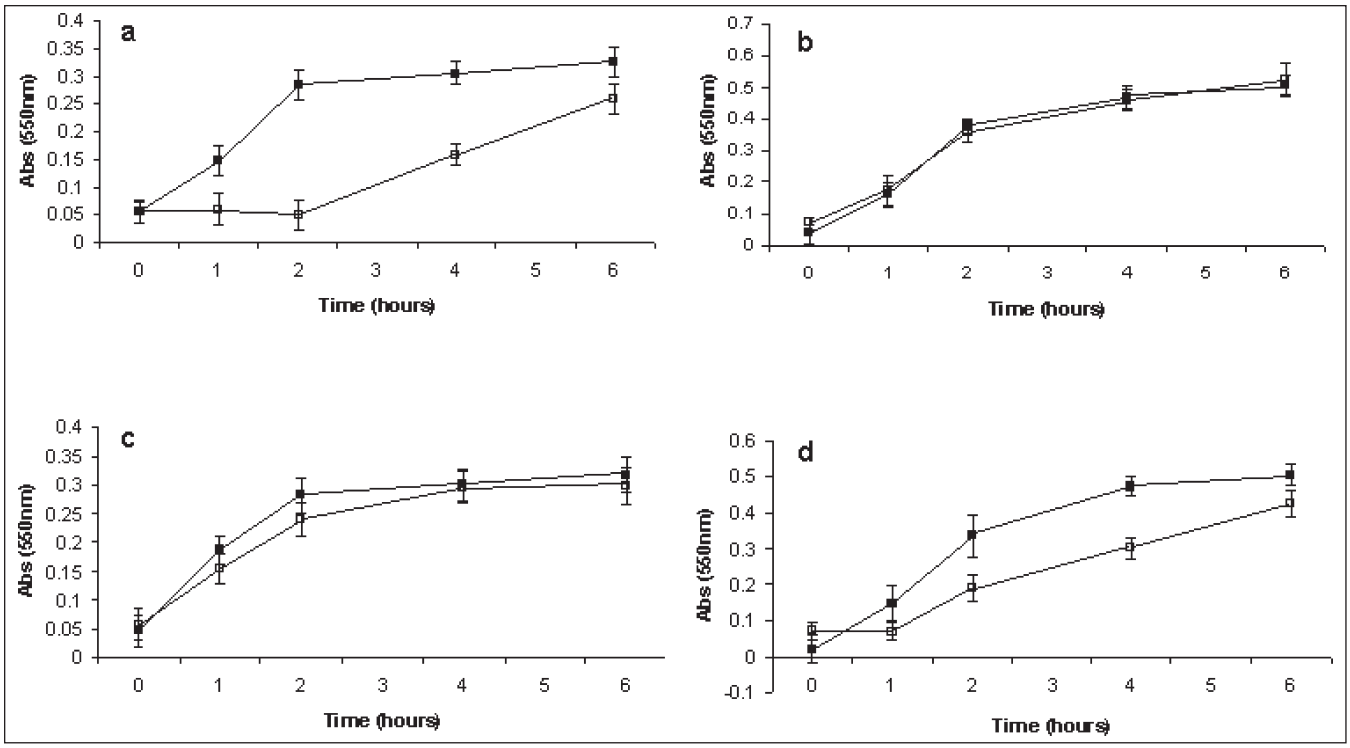

Figure 2: Inhibition of bacterial growth by methanolic extract of *A. complanta* leaves against (a) *B.cereus*, (b) *B.subtilis*, (c) *P.fluorescens*, (d) *A. hydrophilia*. For all graphs, □ represent measured bacterial growth values for test cultures (with extract) and ■ represent control bacterial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

Table 2: Minimum inhibitory concentrations (µg/ml) of *A. aulacocarpa* and *A. complanta* extracts against susceptible bacteria.

		LC_{50} (µg/ml)		
Plant Species	**Plant Part Tested**	**24 h**	**48 h**	**72 h**
Acacia aulacocarpa	leaves	NA	NA	NA
Acacia complanta	leaves	NA	NA	1927 ± 212
Acacia complanta	flowers	1195 ± 92	795 ± 80	785 ± 71
Mevinphos		1418 ± 172	546 ± 45	123 ± 18
Potassium Dichromate		-	82 ± 4	79 ± 5

Numbers indicate the mean MIC values of at least triplicate determinations. NA indicates no result achieved as the % mortality did not reach 50% for any dose at any time point.

No LC_{50} values are reported for the *A. aulacocarpa* 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 *A. complanta* leaf extract at 24 and 48h. As LC_{50} values ≥ 1000 µg/ml are defined as non-toxic[23] this indicates that the *A. complanta* leaf extract is non-toxic. The *A. complanta* flower extract does display low toxicity at 48 and 72h.

In summary, these studies and previous studies within this laboratory[10] show that *A. aulacocarpa* leaf extracts contain antibacterial components and support the traditional Australian Aborigine medicinal use of some Acacias to protect against infection by both Gram-positive and Gram-negative bacteria. As many Acacia species have also provided a food source for Australian Aborigines for thousands of years,[5] there is also potential for the use of Acacia 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 are needed to determine the suitability of these extracts for use as antiseptic agents and as a food additive. One study has reported low toxicity of *A. aulacocarpa* bark extracts against HepG2, and two carcinoma cell lines.[24] The same report also showed that a bark extract from another Acacia species (*A. melanoxylon*) had high toxicity to human 5637 primary bladder carcinoma cells but low toxicity towards all other cells tested. No data

was found for *A. aulacocarpa* leaf extracts or for *A. complanta*. 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.[25]

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