

Antimicrobial Activity of *Allocasuarina littoralis* methanolic leaf extracts

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Abstract

The antimicrobial activity of a methanolic extract of *Allocasuarina littoralis* leaves was investigated by disc diffusion assay against a panel of bacteria and fungi. *A. littoralis* leaf extract inhibited the growth of 8 of the 14 bacteria tested (57%). Gram-positive and Gram-negative bacteria were both inhibited by *A. littoralis* extract although Gram-positive bacteria were more susceptible. 5 of the 11 Gram-negative bacteria (45%) and 100% of the Gram-positive bacteria tested had their growth inhibited by *A. littoralis* extract. *A. littoralis* leaf extract displayed no antifungal activity towards any of the fungi when tested by disc diffusion assay. The antibacterial activity of *A. littoralis* leaf extract was further investigated by growth time course assays which showed significant growth inhibition in cultures of *Bacillus cereus*, *Bacillus subtilis* and *Pseudomonas fluorescens* within 1 h but not of *Aeromonas hydrophila*.

Introduction

Allocasuarina littoralis (black she-oak; previously known as *Casuarina littoralis* and *Casuarina suberosa*), a member of the genus *Allocasuarina* (family *Casuarinaceae*) are native trees to Australia, Southern Asia and the Pacific islands (Yasodha *et al.*, 2004). Some members of the *Casuarinaceae* family (*Casuarina cunninghamiana*, *Casuarina equisetifolia*, *Casuarina glauca*) have also been introduced to other areas such as USA and the Caribbean (Staples *et al.*, 2000) and Southern Africa (Henderson, 1995) where they are considered invasive species.

Members of the *Casuarinaceae* family were important as a food source to the Aboriginal people of Australia prior to European settlement. The Aborigines in the Canberra region are known to have eaten the leaves and young cones of *Allocasuarina verticellata* as a food (Nash, 2004). The *Casuarinaceae* family

also had a role as traditional bush medicines for Australian Aborigines. The bark of *Casuarina equisetifolia* was used as an astringent and to treat diarrhoea, dysentery, headache and fever (Maiden, 1913; Maiden, 1889). On Groote Eylandt in the Northern Territory of Australia, the twigs were soaked in water and used as a mouthwash (Maiden, 1889). No evidence was found of Australian Aborigines, nor any other ethnic group, using *Casuarinaceae* as antimicrobial agents.

A recent report has demonstrated the antibacterial activity of a related *Casuarinaceae* species (*Casuarina equisetifolia*) against a variety of bacterial species (Parekh *et al.*, 2005). Studies within this laboratory (Cock, 2008) have also found antibacterial activity in methanolic extracts of *A. littoralis* leaves 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 (Cock, 2008). Briefly, *Allocasuarina littoralis* leaves were collected from Toohey Forest, Brisbane, Australia and were identified with reference to a taxonomic key to Toohey Forest plants (Coutts and Catterall, 1980). *A. littoralis* leaves were dried in a Sunbeam food dehydrator and the dried material was ground to a coarse powder. 1 g of the powdered leaves 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.

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 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* 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 *A. littoralis* leaf extract was determined using a modified Kirby-Bauer (Bauer *et al.*, 1966) disc diffusion method. Briefly, 100 µl of the test bacteria/fungi were grown in 10 ml of fresh media until they reached a count of approximately 10⁸ cells ml⁻¹ for bacteria, or 10⁵ 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 *A. faecalis*, *A. hydrophilia*, *B. cereus*, *B. subtilis*, *C. freundii*, *K. pneumoniae*, *P. aeuroginosa*, *P. fluorescens*, *S. marcescens*, *Y. enterocolitia*, *C. albicans* and *S. cerevisiae* were incubated at 30 ° C for 24 hours, then the diameters of the inhibition zones were measured in millimetres. Plates inoculated with *E. aerogenes*, *E. coli*, *S. Salford* and *S. aureus* were incubated at 37 ° C for 24 hours, then the diameters of the inhibition zones were measured. *A. 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.

Bacterial Growth Time Course Assay

3 ml of bacterial cultures (*B. cereus*, *B. subtilis*, *A. hydrophilia*, *P. fluorescens*) in nutrient broth were 27 ml nutrient broth containing 0.5 ml *A. littoralis* extract (diluted 1 in 10 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.

Results and Discussion

A. littoralis leaf extract was diluted to a 25 mg/ml concentration. 10 µl of the extract was tested in the disc diffusion assay against 17 microorganisms (table 1). The *A. littoralis* leaf extract inhibited the growth of 8 of the 14 bacteria tested (57%). The antibacterial activity was relatively strong against *A. faecalis*, *P. fluorescens*, *S. Salford* and *S. aureus* (as determined by the diameter of the zone of inhibition).

Table 1: Antibacterial activity of *A. littoralis* extract. Numbers indicate the mean diameters of inhibition of triplicate experiments ± standard deviation. – indicates no growth inhibition. Chl indicates chloramphenicol (10 µg) was used as the positive control. Amp indicates ampicillin (2 µg) was used as the positive control. Cip indicates ciprofloxacin (2.5 µg) was used as the positive control. . Nys indicates nystatin nystatin discs (100 µg) was used as the positive control.

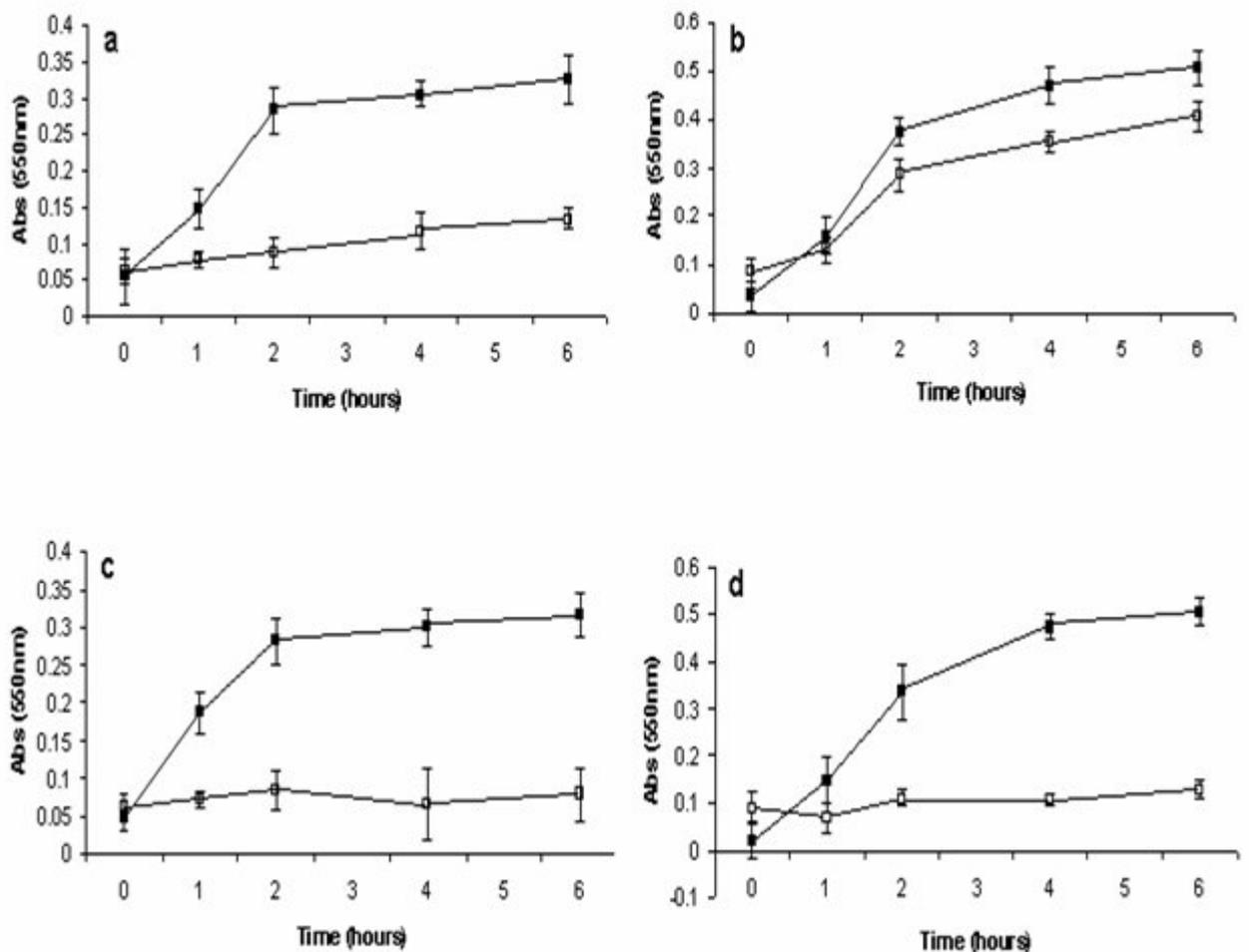
Microbial Species	Mean Zone of Inhibition \pm SD (mm)	
	Antibiotic	<i>A. littoralis</i> extract
Gram negative rods		
<i>Aeromonas hydrophilia</i>	17.3 \pm 0.6 (Chl)	-
<i>Alcaligenes faecalis</i>	13.3 \pm 0.6 (Amp)	16.7 \pm 1.2
<i>Citrobacter freundii</i>	23.0 \pm 1.0 (Chl)	-
<i>Enterobacter aerogenes</i>	17.3 \pm 0.3 (Chl)	-
<i>Escherichia coli</i>	16.7 \pm 0.6 (Amp)	-
<i>Klebsiella pneumoniae</i>	18.3 \pm 0.6 (Amp)	10.0 \pm 1.0
<i>Pseudomonas</i> <i>aeruginosa</i>	31.6 \pm 0.3 (Cip)	-
<i>Pseudomonas</i> <i>fluorescens</i>	21.0 \pm 0 (Chl)	18.0 \pm 0
<i>Salmonella salford</i>	25.3 \pm 0.3 (Amp)	14.0 \pm 0
<i>Serratia marescens</i>	25.7 \pm 0.6 (Chl)	-
<i>Yersinia enterocolitica</i>	16.3 \pm 0.3 (Amp)	10.0 \pm 1.0
Gram positive rods		
<i>Bacillus cereus</i>	25.3 \pm 0.6 (Chl)	9.6 \pm 0.3
<i>Bacillus subtilis</i>	22.7 \pm 0.6 (Amp)	9.3 \pm 0.3
Gram positive cocci		
<i>Staphylococcus aureus</i>	16.3 \pm 0.3 (Amp)	16.6 \pm 0.3
Fungi		
<i>Aspergillus niger</i>	18.0 \pm 0 (Cip)	-
<i>Candida albicans</i>	25.7 \pm 0.6 (Nys)	-
Yeast		
<i>Saccharomyces</i> <i>cerevisiae</i>	21.3 \pm 0.6 (Nys)	-

Both Gram-positive and Gram-negative bacterial growth were inhibited by *A. littoralis* leaf extract although a greater susceptibility of Gram-positive bacteria was apparent. Of the 11 Gram-negative bacteria tested, 5 (45%) were inhibited by *A. littoralis* extract. The extract inhibited the growth of all of Gram-positive bacteria tested. The greater susceptibility of Gram-positive bacteria has been previously reported for South American (Paz *et al.*, 1995), African (Kudi *et al.*, 1999; Vlietinck *et al.*, 1995) and Australian (Palombo and Semple, 2001) plant extracts. Susceptibility differences between Gram-positive and Gram-negative bacteria may be due to cell wall structural differences between these classes of bacteria. The Gram-negative bacterial cell wall outer

membrane appears to act as a barrier to many substances including antibiotics (Tortora *et al.*, 2001). No antifungal activity was seen in the present study, although a limited panel of fungi was tested.

The antibacterial activity of the *A. littoralis* leaf extract was further investigated by bacterial growth time course assays in the presence and absence of the extract. The concentration of the extract used in these assays was 41.7 $\mu\text{g/ml}$. *A. littoralis* leaf extract was able to significantly inhibit *B. cereus* (figure 1a), *P. fluorescens* (figure 1c) and *B. subtilis* (figure 1d) growth within 1 h indicating a rapid antimicrobial action. *Aeromonas hydrophilia* (figure 1b) growth was not inhibited greatly by *A. littoralis* leaf extract, in agreement with previously reported results (Cock, 2008).

Figure 1: Inhibition of bacterial growth by methanolic extract of *A. littoralis* leaves against (a) *B.cereus*, (b) *A. hydrophilia*, (c) *P.fluorescens*, (d) *B.subtilis*. For all graphs, \square represent measured bacterial growth values for test cultures (with extract) and \blacksquare represent control bacterial growth values (no extract). Values are the mean of triplicate determinations.



In summary, these studies and previous studies within this laboratory (Cock, 2008) are the first studies that have demonstrated the antibacterial activity of *A. littoralis* leaf extracts. Both Gram-positive and Gram-negative bacteria were susceptible to *A. littoralis* leaf extract. The broad range of microbial susceptibilities indicates the potential of *A. littoralis* leaf extract as a surface disinfectant as well as for medicinal purposes and possibly as food additives to inhibit 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 the use as antiseptic agents and as a food additive.

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