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Original Article

Phragmites australis (Cav.) Trin. Ex Steud. Leaf Extracts Lack Anti-bacterial Activity and are Non-toxic *in vitro*

Getmore Rumbudzai Chikowe[1], Lindiwe Nomathemba Mpala[1], Ian Edwin Cock[1,2,*]

[1]School of Environment and Science, Nathan Campus, Griffith University, 170 Kessels Rd, Nathan, Brisbane, Queensland, AUSTRALIA.
[2]Environmental Futures Research Institute, Griffith University, 170 Kessels Rd, Nathan, Brisbane, Queensland, AUSTRALIA.

ABSTRACT

Introduction: The recent development of extensively antibiotic resistant bacteria has necessitated the search for novel anti-bacterial compounds. An examination of aromatic plants and traditional medicines is an attractive option for drug discovery. *Phragmites australis* (Cav.) Trin. Ex Steud. Reed is native to many regions globally, including Australia. It has yet to be tested for antibacterial activity. **Materials and Methods:** The ability of *P. australis* leaf extracts to inhibit the growth of a panel of bacterial pathogens was investigated by disc diffusion assay. Toxicity was examined using the *Artemia franciscana* nauplii bioassay. **Results:** *P. australis* leaf methanolic and aqueous extracts were completely ineffective at inhibiting the growth of gram-positive and gram-negative panels of bacteria. The extracts were nontoxic in the *Artemia* nauplii bioassay following 24 hr exposure. **Conclusion:** *P. australis* leaf extracts were completely ineffective bacterial growth inhibitors. However, these extracts may have other therapeutic properties and testing against protozoa, virus and tumour cells is required.

Keywords: Poaceae, Common reed, Antibacterial activity, Antibiotic resistant bacteria, Medicinal plants, Toxicity.

Correspondence:
Dr. Ian Edwin Cock
[1]School of Environment and Science, Nathan Campus, Griffith University, 170 Kessels Rd, Nathan, Brisbane-4111, Queensland, AUSTRALIA.
[2]Environmental Futures Research Institute, Griffith University, 170 Kessels Rd, Nathan, Brisbane, Queensland, AUSTRALIA.
Email: I.Cock@griffith.edu.au

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INTRODUCTION

Traditional plant derived medicines have been used in most parts of the world for a variety of therapeutic purposes, including fighting microbial disease. Indeed, the ability of plant extracts to block the growth of pathogenic bacteria has become a focus of substantial recent study.[1-5] Much of the research into traditional medicinal plant use has focused on Asian,[6-8] African,[9-11] Middle Eastern[12-14] and South American[15] plants. However, despite the potential of plants to provide us with useful pharmaceutical agents, the field is still relatively poorly studied. Only an estimated 5-10% of the approximately 300,000-500,000 plant species worldwide have been screened for one or more bioactivities.[9]

The development of new antibiotic therapies is particularly urgent. The recent establishment of bacterial pathogens that are either extremely (XDR) or totally resistant (TDR) to common clinically used antibiotics[16] has resulted in the need to develop new and effective antibiotic chemotherapies. There are now limited therapeutic options for many diseases caused by bacterial pathogens and the situation is expected to worsen in the future as bacteria exchange resistance genes. Indeed, the development of alternative antibacterial treatment modalities has become crucial and is considered by the World Health Organisation (WHO) to be one of the most serious challenges facing medical science.[17] For reasons reviewed elsewhere,[16] it is unlikely that the previous methods of antibiotic discovery/development will be as successful in the future and new treatment modalities are urgently required. Traditional medicines and herbal remedies have great potential for antimicrobial drug development and there has recently been a substantial increase in interest in this field.[3,18-31]

Phragmites australis (Cav.) Trin. Ex Steud. (synonym *Arundo phragmites* L., *Phragmites berlandieri* E. Fourn.; commonly known as common reed) is a rhizomatous perennial plant that grows to 6 m tall in aquatic environments (especially in alkaline habitats; Figure 1a), although it can also grow in damp soil. It has long leaves (up to 50 cm long by 3 cm wide; Figure 1b) (Figure 1a). The inflorescence (Figure 1c) is green to purple/brown in colour and up to 30 cm long. *P. australis* is rich in flavonoid glycosides, including cyanidin-3-O-(6”-O-succinyl-β-glucopyranoside) (Figure 1d).[32] We were unable to find records of medicinal use of this species in traditional Aboriginal healing systems. However, the first Australians did not have a written record of their traditional knowledge. Instead, they passed their traditions from one generation to the next by oral communication and it is likely that much of this knowledge has been lost as they adopted westernised medicine systems. Furthermore, in comparison to other regions of the world, relatively few studies have rigorously

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examined the anti-bacterial activity of Australian native plants, although there has recently been a substantial increase in interest in this field.[3,18-31] This study was undertaken to screen *P. australis* leaf extracts for the ability to inhibit the growth of a panel of gram-positive and gram-negative bacterial pathogens.

MATERIALS AND METHODS

Plant material

Collection of plant material and extraction

Phragmites australis (Cav.) Trin. Ex Steud. leaves were obtained from and identified by Philip Cameron, senior botanic officer, Mt Cootha Botanical Gardens, Brisbane, Australia. The harvested leaves were washed in deionised water and processed within 4 hr of collection. The leaves were dried in a Sunbeam food dehydrator and the dried material was ground to a coarse powder. An individual 1 g masses of the dried plant material was extracted extensively in 50 mL methanol (Ajax, AR grade) or deionised water for 24 hr at 4°C with gentle shaking. The extract was filtered through filter paper (Whatman No. 54) under vacuum followed by drying by rotary evaporation. The resultant pellet was dissolved in 5 mL deionised water (containing 1% DMSO). The extract was passed through 0.22 µm filter (Sarstedt) and stored at 4°C.

Qualitative phytochemical studies

Phytochemical analyses of the *A. latifolia* leaf extracts for the presence of saponins, phenolic compounds, flavonoids, phytosteroids, triterpenoids, cardiac glycosides, anthraquinones, tannins and alkaloids were conducted by standard assays.[33-35]

Anti-bacterial screening

Test micro-organisms

All media was purchased from Oxoid Ltd., Australia. The reference strains of *Escherichia coli* (ATCC157293), *Klebsiella pneumoniae* (ATCC31488), *Proteus mirabilis* (ATCC21721) and *Streptococcus pyogenes* (ATCC19615) were purchased from American Tissue Culture Collection (ATCC), USA. Clinical isolate microbial strains of *Aeromonas hydrophilia, Alcaligenes feacalis, Bacillus cereus, Citrobacter freundii, Pseudomonas aeruginosa, Pseudomonas fluorescens, Salmonella newport, Serratia marcescens, Staphylococcus aureus, Staphylococcus epidermidis* and *Yersinia entercolitica* strains were obtained from

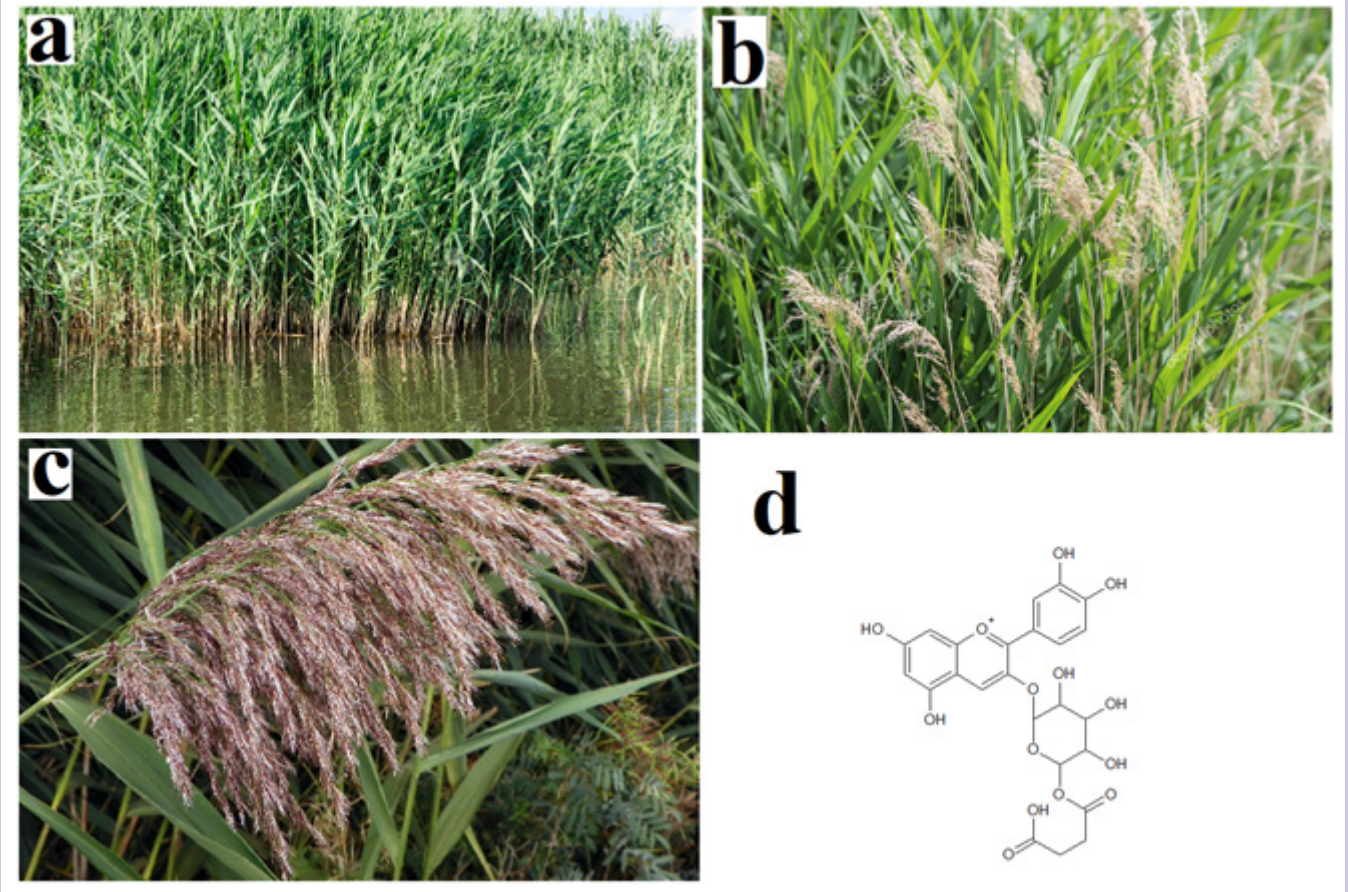

Figure 1: *P. australis* (a) growth habitat, (b) whole plant, (c) flower and (d) the flavonoid glucoside component cyanidin-3-O-(6″-O-succinyl-β-gl ucopyranoside).

Ms Michelle Mendell and Ms Jane Gifkins, Griffith University. All bacterial stock cultures were subcultured and maintained in nutrient broth at 4°C.

Evaluation of anti-microbial activity

Anti-microbial activity of the *A. latifolia* leaf extracts was determined using a modified disc diffusion assay.[3,32,36,37] Briefly, 100 µL of each microbial suspension in log phase was spread onto individual nutrient agar plates and the extracts were tested for antimicrobial activity using 6mm sterilised filter paper discs. The discs were each infused with 10 µL of the individual plant extract, allowed to dry and placed onto the inoculated plates. The plates were allowed to stand at 4°C for 2 hr before incubation at 37°C for 24 hr. The diameters of the Zones of Inhibition (ZOIs) were measured to the closest whole millimetre. Each assay was performed three times in triplicate (n=9). Mean values (±SEM) are reported in this study. Standard discs of ampicillin (10 µg) and chloramphenicol (10 µg) were obtained from Oxoid, Australia and were used as positive controls to compare antibacterial activity. Filter discs infused with 10 µL of distilled water were used as a negative control.

Artemia franciscana nauplii toxicity screening

Toxicity was tested using an adapted *Artemia franciscana* nauplii lethality assay.[38-40] Briefly, *A. franciscana* nauplii were incubated in the presence of the extracts, reference toxin (1 mg/mL potassium dichromate) or artificial seawater (negative control) at 25±1°C under artificial light. All treatments were performed three times in triplicate (n=9). The numbers of dead were counted in each well at 24 hr, 48 hr and 72 hr. At the completion of the 72 hr exposure period, the remaining live nauplii were sacrificed and the total number of nauplii in each well were counted and used to calculate the % mortality per well. LC_{50} values were calculated for each treatment using probit analysis.

Statistical analysis

Data are expressed as the mean±SEM of three independent experiments with internal triplicates (n=9). One-way ANOVA was used to calculate statistical significance between control and treated groups, with a p value <0.01 considered to be statistically significant.

RESULTS

Liquid extraction yields and qualitative phytochemical screening

Extraction of 1 g of dried and powdered *P. australis* leaf with methanol and water yielded 346 and 237 mg of extracted material respectively (Table 1). Methanol was a better extractant, resulting in higher yield of extracted material compared to water. The extracts were resuspended in 10 mL of deionised water (containing 1% DMSO), resulting in the extract concentrations shown in Table 1. Qualitative phytochemical studies showed that both extracts had similar phytochemical profiles. Both contained high levels of phenolic compounds and flavonoids, as well as moderate levels of saponins and tannins and low levels of triterpenoids, phytosterols and alkaloids.

Anti-microbial activity

To determine the growth inhibitory activity of the *P. australis* leaf extracts, aliquots (10 µL) of each extract were screened in the disc diffusion assay. The *P. australis* leaf extracts were ineffective at inhibiting the growth of all gram negative (Figure 2) and gram positive (Figure 3) bacterial species tested. In contrast, both positive control antibiotics (ampicillin and chloramphenicol) were effective growth inhibitors, with ZOI's of up to 14.3 mm (chloramphenicol against *E. coli*). We were therefore unable to determine the MIC values for any extract as they were completely ineffective at all concentrations tested.

Quantification of Toxicity

The toxicity of the *P. australis* leaf extracts was initially tested at 1000µg/mL in the *A. franciscana* nauplii bioassay (Figure 4). The mortality in the presence of all extracts was substantially <50% at 24 hr when 1000 µg/mL concentrations were tested. Thus, the methanolic and water extracts were deemed to be non-toxic. Extracts with 24 hr LC_{50} values >1000 µg/mL have previously been defined as non-toxic.[37-39] In contrast, the potassium dichromate positive control induced substantial mortality within 4 hr (results not shown), with 100% mortality induction seen by 24 hr. The mortality increased following exposure to the *P. australis* leaf extracts at 48 hr and was further increased following 72 hr exposure.

The findings reported here also indicate that the extracts examined were non-toxic (24 hr LC_{50}>1000 µg/mL) in the *Artemia* nauplii bioassay. Whilst toxicity was assessed in this study with the test organism *A. franciscana*, toxicity towards *A. franciscana* has previously been shown to correlate well with toxicity towards human cells for many toxins.[37-39] However, further studies are required to determine whether this is also true for the *P. australis* leaf extracts examined in these studies.

DISCUSSION

The development of new antibiotic chemotherapies is a high priority for medical science due to the recent development of large numbers of antibiotic resistant bacteria strains.[16,17] A parallel decrease in the discovery of new antibiotic medicines by conventional strategies has increased interest in re-evaluating medicinal plants for new antibiotic chemotherapies.[16] Whilst we were unable to find reports of the traditional use of *P. australis* medicinally, the first Australians did not have a written record of their traditional knowledge and instead passed their traditions from one generation to the next by oral communication. It

Table 1: The mass of dried extracted material, the concentration after resuspension in deionised water and qualitative phytochemical screenings of the *P. australis* leaf extracts.

			Leaf	
			Methanolic extract	Aqueous extract
Mass of extracted material (mg)			346	237
Concentration of resuspended extract (mg/mL)			34.6	23.7
Qualitative Phytochemical Tests	Phenols	Total phenols	+++	+++
		Water soluble phenols	+++	+++
		Insoluble phenols	+	+
	Saponins	Froth persistence	++	+++
		Emulsion test	+	+
	Cardiac glycosides	Keller-Kiliani Test	-	-
	Triterpenoids	Salkowski Test	+	+
	Phytosterols	Acetic Anhydride Test	++	+
	Alkaloids	Meyer's Test	+	-
		Wagner's Test	+	-
		Draggendoff's Test	+	-
	Flavonoids	Kumar Test	+++	+++
	Tannins	Ferric Chloride Test	++	+
		Lead Acetate Test	++	+
	Anthraquinones	Free	-	-
		Combined	-	-

+++ indicates a large response; ++ indicates a moderate response; + indicates a minor response; - indicates no response in the assay.

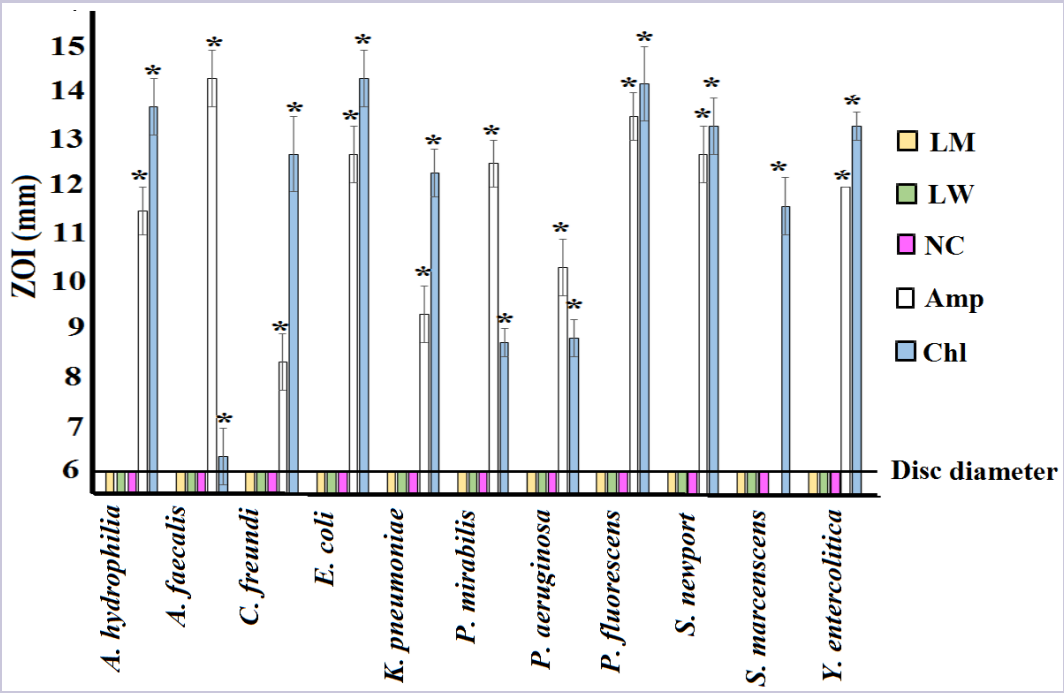

Figure 2: Growth inhibitory activity of *P. australis* leaf extracts and reference antibiotics against gram-negative bacterial species measured as ZOIs (mm)±SEM. L=leaf; M=methanolic extract; W=aqueous extract; Amp=ampicillin (10 µg); Chl=chloramphenicol (10 µg); NC=negative control. All assays were completed three times, each with internal triplicates (n=9) and the results are expressed as mean zones of inhibition (mm)±SEM. *=results significantly different to the negative control (p<0.05).

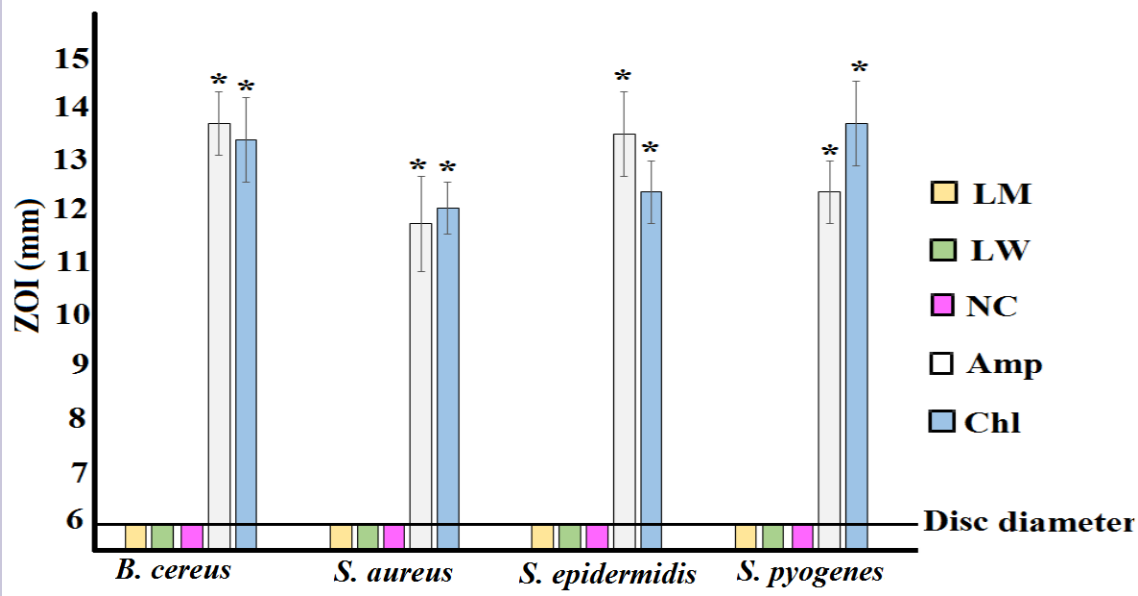

Figure 3: Growth inhibitory activity of *P. australis* leaf extracts and reference antibiotics against gram-positive bacterial species measured as ZOIs (mm)±SEM. L=leaf; W=aqueous extract; Amp=ampicillin (10 µg); Chl=chloramphenicol (10 µg); NC=negative control. All assays were completed three times, each with internal triplicates (n=9) and the results are expressed as mean zones of inhibition (mm)±SEM. *=results significantly different to the negative control (p<0.05).

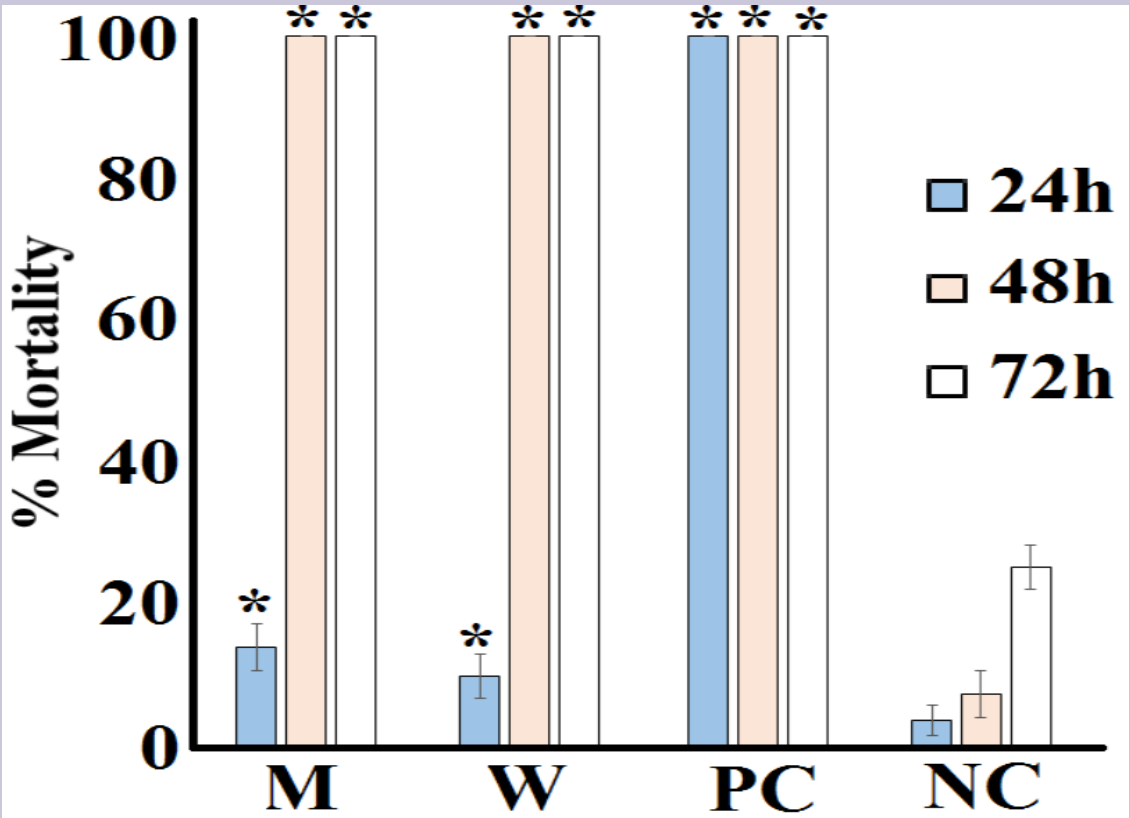

Figure 4: The lethality of the *P. australis* leaf extracts, potassium dichromate control (PC; 1000 µg/mL) and seawater Negative Control (NC) following 24, 48 and 72 hr of exposure. M=methamolic extract; W=aqueous extract. All bioassays were performed three times in triplicate (n=9) and are expressed as mean±SEM. * indicates results that are significantly different to the untreated (seawater) control at the equivalent exposure time (p<0.01).

therefore is likely that much of their traditional knowledge has been lost as they adopted westernised medicine systems. It is therefore possible that some first Australian groups may have had ethnobotanical uses for this species that are no longer widely known, and screening of this species for antibacterial activity is warranted. Notably, the *P. australis* extracts completely lacked inhibitory activity against all bacterial species tested.

A single assay technique was used to screen for antibacterial activity in this study. We chose to use the disc diffusion assay as it is a rapid method and it has previously been widely utilised in other studies. Therefore, comparisons between studies are relatively simple. However, as the disc diffusion method is reliant on the diffusion of a molecule through the aqueous environment of an agar gel, this assay may be affected by the solubility of the extract compounds in the aqueous environment. Polar compounds that are highly soluble in water would be expected to diffuse easily in the gel, whereas less soluble compounds would not diffuse as readily and thus be concentrated around the disc. Diffusion of molecules within an agar gel is also affected by the size of the molecules. The movement of large, complex phytochemicals (e.g., tannins, triterpenoids) through agar gels by diffusion would also be retarded and may provide a false idea of the efficacy of an extract. As many tannins and triterpenoids have well described antibiotic properties, screening for growth inhibition using agar diffusion techniques may give a fallacious view of its inhibitory potential. For this reason, whilst this is a handy assay for screening aqueous extracts, this technique may not be ideal for nonpolar compounds. For examining nonpolar mixtures, other techniques such as liquid dilution assays may be preferred. Liquid dilution studies may have been better suited to screen *P. australis* leaf extracts for activity and future studies will use these techniques to re-examine the extracts for antibacterial activity.

CONCLUSION

Methanolic and aqueous *P. australis* leaf extracts displayed no antibacterial activity in the disc diffusion assay. The extracts were nontoxic towards *Artemia* nauplii.

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CONFLICTS OF INTEREST

The authors declare no conflicts of interest.

ABBREVIATIONS

DMSO: Dimethyl sulfoxide; **LC_{50}:** The concentration required to achieve 50% mortality; **MIC:** Minimum inhibitory concentration; **ZOI:** Zone of inhibition.

SUMMARY

- *P. australis* leaf extracts were screened for the ability to block the growth of a panel of human bacterial pathogens.
- No inhibitory activity was evident against any of the bacterial species tested.
- Toxicity of the *P. australis* leaf extracts was determined using the *Artemia* nauplii toxicity bioassay.
- Both the methanolic and aqueous extracts were nontoxic.

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