

## Research Article

# An Examination of the Medicinal Potential of *Pittosporum phylliraeoides*: Toxicity, Antibacterial and Antifungal Activities

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**ABSTRACT: Introduction:** *Pittosporum phylliraeoides* is an endemic Australian plant historically used as a medicinal agent by indigenous Australians. *P. phylliraeoides* solvent extracts were tested for antibacterial and antifungal activities and toxicity *in vitro*. **Results:** All extracts displayed antibacterial activity in the disc diffusion assay. The methanol and hexane extracts demonstrated the broadest specificity, inhibiting the growth of 4 of the 14 bacteria tested (28.6%). The water, ethyl acetate, and chloroform extracts inhibited the growth of 2 (14.3%), 3 (21.4%), and 3 (21.4%) of the 14 bacteria tested respectively. *P. phylliraeoides* methanolic extract was also effective as an antifungal agent, inhibiting the growth of a nystatin resistant strain of *Aspergillus niger*. It did not affect the growth of *Candida albicans*. All extracts were more effective at inhibiting the growth of Gram-negative bacteria than Gram-positive bacteria. Indeed, only the methanol and hexane extracts were capable of inhibiting the growth of any of the Gram-positive bacteria, inhibiting the growth of only 1 of the 4 (25%) Gram-positive bacteria tested each. All *P. phylliraeoides* extracts displayed low toxicity in the *Artemia franciscana* bioassay. The only significant increase in mortality above that of the control was seen for the ethyl acetate, chloroform and hexane extracts, although even these extracts displayed low toxicity, inducing less than 50% mortality at 72 h. **Conclusions:** The low toxicity of the *P. phylliraeoides* extracts and their inhibitory bioactivity against bacteria and fungi validate Australian Aboriginal usage of *P. phylliraeoides* and indicates its medicinal potential.

**KEYWORDS:** *Pittosporum phylliraeoides*, Gumbi Gumbi, Australian plants, antibacterial, medicinal plants

## INTRODUCTION

*Pittosporum phylliraeoides* (Family *Pittosporaceae*) (commonly known as weeping Pittosporum, cattlebush, Gumbi Gumbi, Gumby Gumby, Cumbi Cumbi and native apricot) is an endemic Australian tree/shrub that grows to approximately 8 m tall and is distributed throughout the drier areas of the Australian continent. It has pendulous branchlets with alternate, narrow tapered leaves, often with a hooked point. In the winter and spring, the tree develops small yellow flowers which may either be solitary or occur as clusters in leaf forks. The flowers develop to small (6-20 mm) oval

yellow skinned fruit which ripen in late summer/early autumn, opening into two valves with several seeds inside. Australian Aborigines used *P. phylliraeoides* as a medical plant to treat a variety of conditions.<sup>[1-5]</sup> An infusion of the leaves, seeds, fruit pulp or wood was used to treat bruises, muscle ache, sprains and cramps. *P. phylliraeoides* infusions were drunk to treat coughs and colds as well as to induce lactation. A decoction of fruit was used both externally and by ingestion to treat eczema and pruritus.

Despite its range of traditional medicinal uses, the phytochemistry and therapeutic potential of *P. phylliraeoides* has not been extensively studied. One study examined 40 different Australian plants for antiviral bioactivities.<sup>[6]</sup> This study found that *P. phylliraeoides* leaf extracts were capable of inhibiting greater than 25% of Ross River virus (RRV) induced cytopathicity. This demonstrated the antiviral potential of *P. phylliraeoides* and provided support for the

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traditional Aboriginal use of *P. phylliraeoides* infusions to treat viral diseases including colds and coughs. The same study found *P. phylliraeoides* leaf extracts ineffective against poliovirus or cytomegalovirus (CMV) induced cytopathicity, although different cell lines were used for all three assays. The authors questioned whether the lack of antiviral activity towards poliovirus and RRV in their study was due to variations in the way the extract components behaved in the different cell lines, or whether it suggested that plant components interfered with a step in the replication cycle which is specific to RRV.

*P. phylliraeoides* also had uses in the treatment of various cancers by Aborigines.<sup>[1-5]</sup> This ethnopharmacological knowledge was traditionally passed on by word of mouth, instead of by written record, and unfortunately much of our understanding of Aboriginal medicine has been lost with Aboriginal society merging into mainstream Australian society. Recent anecdotal accounts have also credited *P. phylliraeoides* with anticancer activity,<sup>[7]</sup> although these have yet to be verified by rigorous scientific examination. A recent scientific study examined the anticancer potential of *P. phylliraeoides* leaf extracts.<sup>[8]</sup> The extracts were shown to have moderate cytotoxic activity towards A427 lung cancer cells, however, as these were preliminary studies only the efficacy of these extracts requires verification. Anticancer activity has also been detected in other related *Pittosporum* species from Madagascar,<sup>[9]</sup> South Africa,<sup>[10]</sup> New Caledonia<sup>[11]</sup> and Asia.<sup>[12]</sup>

*P. phylliraeoides* is reported to contain a number of pentacyclic triterpenoid saponogenins.<sup>[13]</sup> In particular, phillyregenin (a dihydroxylactone), R1-barrigenol, 27 - desoxyphillyrigenin (3fl - hydroxytaraxastan - 28, 20fl - olide), 23 - hydroxyphillyrigenin (3fl, 23, 27- trihydroxytaraxastan - 28, 20fl - olide), dihydropriverogenin A, 16 - desoxybarringtogenol C and barringtogenol C, were isolated from *P. phylliraeoides* and identified in this study. Similar pentacyclic triterpenoids isolated from *Alchornea latifolia* have been linked with cytotoxic activity towards Hep-G2 and A-431 human cancer cell lines and are potent inhibitors of topoisomerase II.<sup>[14]</sup> Pentacyclic triterpenoids have also been associated with antitumour,<sup>[15]</sup> anti-HIV<sup>[16]</sup> and antioxidant bioactivities.<sup>[17]</sup> Additionally, pentacyclic triterpenoids from *Laggera pterodonta* have shown antiviral activity against herpes viruses.<sup>[18]</sup> Studies have also demonstrated the antibacterial activity of pentacyclic triterpenoids from a variety of plants.<sup>[19-21]</sup>

Surprisingly, the antiseptic properties of *P. phylliraeoides* remain largely unstudied. The antibacterial and antifungal properties of other Australian plants are well known. For instance, species belonging to the genera *Eucalyptus*,<sup>[22-25]</sup> *Callistemon*,<sup>[26]</sup> *Leptospermum*,<sup>[14,27-29]</sup> *Melaleuca*<sup>[30,31]</sup> and *Syzygium*<sup>[27,32-35]</sup> are known to have antimicrobial activities. The current study reports on the antibacterial, and

antifungal properties of *P. phylliraeoides* extracts as well as examining their toxicity to determine their potential as antibiotic agents.

## MATERIALS AND METHODS

### Plant collection and extraction

*P. phylliraeoides* plant material was provided by Philip Higson of the Queensland Bush Foods Association as pre-dried and coarse milled whole plant material. The material was stored at -30 °C until use.

1 g of plant material was weighed into each of 5 tubes and 5 different extracts were prepared by adding 50 ml of methanol, water, ethyl acetate, chloroform, or hexane respectively. All solvents were obtained from Ajax and were AR grade. Leaf material was extracted in each solvent for 24 h at 4 °C with gentle shaking. The extracts were filtered through filter paper (Whatman No. 54) under vacuum followed by drying by rotary evaporation in an Eppendorf concentrator 5301. The resultant dry extract was weighed and redissolved in 10 ml deionised water.

### Qualitative phytochemical studies

Phytochemical analysis of *P. phylliraeoides* extracts were conducted by modified versions of previously described assays.<sup>[25,36,37]</sup> The modified assays are briefly outlined below.

#### Saponins

1 ml of pure extract was added to 1 ml deionised water and shaken vigorously for 30s. The tubes were allowed to stand for 15 min and the presence or absence of persistent frothing was noted. Persistent frothing indicated the presence of saponins.

#### Phenolic compounds

Phenolic compounds were detected using a modified version of the Folin-Ciocalteu procedure.<sup>[37]</sup> 200 µl of crude extract was added to 2 ml of 3% aqueous sodium carbonate, followed by the addition of 200 µl Folin-Ciocalteu reagent. The mixture was allowed to stand for 30 min at room temperature. The formation of blue/gray colour indicated the presence of phenolic groups.

#### Water soluble phenol test

2 drops of 1% ferric chloride were added to 500 µl of each extract. A red colour change indicated presence of water soluble phenols.

#### Water insoluble phenol test

500 µl of dichloromethane, 3 drops of 1% ferric chloride and 1 drop of pyridine were added to 500 µl of each extract and mixed. The presence of insoluble phenols was indicated by a colour change.

### Flavonoids

Flavonoids were detected using a modified Kumar test.<sup>[36]</sup> 100 µl of aqueous sodium hydroxide was added to 1 ml of each extract. The development of an intense yellow colour indicated the presence of flavonoids. 100 µl of concentrated HCl was added to the solution. Reversion to the original colour confirmed the presence of flavonoids.

### Polysteroids

Polysteroids were detected using a modified version of the Leiberman-Buchard test.<sup>[36]</sup> Three drops of acetic anhydride was added to 500 µl of crude extract followed by the addition of a few drops concentrated sulphuric acid. The solution was allowed to sit at room temperature for 5 min. Formation of a blue/green colour indicated the presence of polysteroids.

### Triterpenoids

Triterpenoids were detected using a modified version of the Salkowski test.<sup>[25]</sup> 1 ml of extract was slowly added to 400 µl chloroform, followed by careful addition of 400 µl concentrated sulphuric acid. Formation of a red/brown/purple colour at the interface indicated the presence of triterpenoids.

### Cardiac glycosides

Cardiac glycosides were detected using a modified version of the Keller Kiliani test.<sup>[37]</sup> 500 µl of extract was added to 500 µl glacial acetic acid. A few drops of 1% aqueous iron chloride and concentrated sulphuric acid were then carefully added. The presence of a red/brown ring of the interface or the formation of a green/blue colour throughout the solution indicated the presence of cardiac glycosides.

### Anthraquinones

Anthraquinones were detected using modified versions of the Kumar and Ajaiyeoba tests.<sup>[36,37]</sup> The modified Kumar test involved addition of a few drops of concentrated sulphuric acid to 500 µl pure extract, followed by the careful addition of 500 µl of ammonia. A rose pink colour indicated the presence of free anthraquinones. For the Ajaiyeoba test, 450 µl of crude extract was added to 50 µl concentrated HCl and allowed to stand at room temperature for several minutes. 500 µl chloroform was then carefully added. The formation of a rose pink colour indicated the presence of combined anthraquinones.

### Tannins

Tannins were detected using a modified version of the Ferric chloride test.<sup>[36]</sup> Two drops of 1% aqueous ferric chloride reagent were added to 500 µl of crude extract. The mixture was observed for the formation of blue, blue-black, green or green-black colouration which indicated the presence of tannins.

### Alkaloids

Two methods were used to test for the presence of alkaloids:

#### Mayers reagent test

200 µl of pure extract was treated with a few drops of aqueous solution of hydrochloric acid and 500 µl Mayer's reagent. Formation of a white precipitate indicated the presence of alkaloids.

Mayer's reagent: Mercuric chloride (1.358 g) was dissolved in 60 ml deionised water. Potassium Iodide (5.0 g) was dissolved in 10 ml deionised water. The mercuric chloride and potassium iodide solutions were mixed and made up to 100 ml with deionised water.

#### Wagners reagent test

200 µl of each extract was treated with a few drops of an aqueous solution of hydrochloric acid and 500 µl Wagner's reagent. A reddish-brown flocculent precipitate indicated the presence of alkaloid.

Wagner's reagent: 1.27 g iodine and 2 g potassium iodide were dissolved in 5 ml deionised water and made up to final volume 100 ml with deionised water.

### Antimicrobial screening

#### Test microorganisms

All microbial strains were obtained from Michelle Mendell and Tarita Morais, Griffith University, Australia. Stock cultures of *Aeromonas hydrophila*, *Alcaligenes faecalis*, *Bacillus cereus*, *Citrobacter freundii*, *Escherichia coli*, *Klebsiella pneumoniae*, *Proteus mirabilis*, *Pseudomonas fluorescens*, *Salmonella newport*, *Serratia marcescens*, *Shigella sonnei*, *Staphylococcus aureus*, *Staphylococcus epidermidis* and *Streptococcus pyogenes* were subcultured and maintained in nutrient broth at 4 °C. Stock cultures of *Aspergillus niger* and *Candida albicans* were subcultured and maintained in Sabouraud media at 4 °C.

#### Evaluation of antimicrobial activity

Antimicrobial activity of all plant extracts was determined using a modified Kirby-Bauer disc diffusion method.<sup>[38]</sup> Briefly, 100 µl of the test bacteria/fungi were grown in 10 ml of the appropriate fresh broth until they reached a count of approximately 10<sup>8</sup> cells/ml of bacteria or 10<sup>5</sup> cells/ml for fungi (as determined by direct microscopic determination). One hundred microliters of microbial suspension was spread onto agar plates corresponding to the broth in which they were maintained.

The extracts were tested using 5 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 h before incubation with the test microbial agents. Plates inoculated with *A. faecalis*, *A. hydrophila*, *B. cereus*, *C. freundii*, *K. pneumoniae*,

*P. mirabilis*, *P. fluorescens*, *S. marcescens*, and *C. albicans* were incubated at 30 °C for 24 h, the diameters of the inhibition zones were then measured in mm. Plates inoculated with *E. coli*, *S. Newport*, *S. sonnei*, *S. aureus*, *S. epidermidis* and *S. pyogenes* were incubated at 37 °C for 24 h, the diameters of the inhibition zones were then measured. *A. niger* inoculated plates were incubated at 25 °C for 48 h, the zones of inhibition were then measured. All measurements were to the closest whole mm. Each antimicrobial assay was performed in at least triplicate. Mean values are reported in this study. Standard discs of ampicillin (2 µg), chloramphenicol (10 µg) and nystatin (100 µg) were obtained from Oxoid Ltd. and served as positive controls for antimicrobial activity. Filter discs impregnated with 10 µl of distilled water or 10 µl of 10% methanol were used as negative controls.

#### **Minimum inhibitory concentration (MIC) determination**

The minimum inhibitory concentration (MIC) of the *P. phylliraeoides* extracts were determined by the disc diffusion method across a range of doses. The plant extracts were diluted in deionised water across a concentration range of 5 mg/ml to 0.1 mg/ml. Discs were impregnated with 10 µl of the test dilutions, allowed to dry and placed onto inoculated plates. The assay was performed as outlined above and graphs of the zone of inhibition versus concentration were plotted for each extract. Linear regression was used to calculate the MIC values.

#### **Toxicity screening**

##### **Reference toxins for biological 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 synthetic 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 *A. franciscana* nauplii lethality assay developed for the screening of active plant constituents with the following modifications.<sup>[39]</sup> *A. franciscana* 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. 400 µl of seawater containing approximately 42 (mean 41.6,  $n = 150$ , SD 17.8) nauplii were added to wells of a 48 well plate and immediately used for bioassay. The plant extracts were diluted to 4 mg/ml in seawater for toxicity testing, resulting in a 2 mg/ml concentration in the bioassay (except where specified). 400 µl of diluted plant extract and the reference toxins were transferred to the wells and incubated at  $25 \pm 1$  °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 moribund if no movement of the appendages was observed within 10 s. 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.<sup>[40]</sup>

#### **Statistical analysis**

Data are expressed as the mean  $\pm$  SD of at least three independent experiments. The Paired *T*-Test was used to calculate statistical significance between control and treated groups with a *P* value < 0.05 considered to statistically significant.

## **RESULTS**

#### **Liquid extraction yields and qualitative phytochemical screening**

Extraction of 1 g of dried plant material with various solvents yielded dried plant extracts ranging from 20.6 mg to 120.1 mg (Table 1). Deionised water and chloroform both gave relatively high yields of dried extracted material (111.2 and 120.1 mg respectively) whilst ethyl acetate extracted the lowest mass (20.6 mg). The dried extracts were resuspended in 10 ml of deionised water resulting in the extract concentrations shown in Table 1.

Qualitative phytochemical studies (Table 2) show that methanol and water extracted the widest range of phytochemicals. Both showed moderate to high levels of phenolics (both water soluble and insoluble phenolics), saponins, triterpenoids and flavanoids. The only difference detected between the methanol and water extracts was the presence of low levels of tannins in the water extract. The ethyl acetate and chloroform extracts had low levels of flavanoids whilst the ethyl acetate extract also had low levels of saponins. However, only a low response was seen for each of these solvents in these tests. None of the classes of phytochemicals tested for were detected in the hexane extract.

### Antimicrobial activity

10 µl of each extract was tested in the disc diffusion assay against 14 bacteria (Table 3). All extracts displayed antibacterial activity, as evidenced by the inhibition of growth for 2-4 species of the tested bacterial panel. The methanolic and hexane extracts displayed the broadest antibiotic specificity, inhibiting the growth of 4 of the 14 bacteria tested (28.6%). The methanolic extract was particularly potent against *P. mirabilis* as determined from the zone of inhibition (16.3 ± 0.3 mm). Indeed, the methanol extract was more effective than the ampicillin and chloramphenicol controls in inhibiting *P. mirabilis* growth. The methanol and hexane extracts were the only extracts capable of inhibiting the growth of any Gram-positive bacteria, each inhibiting 1 of the 4 bacteria tested (25%). *P. phylliraeoides* ethyl acetate and chloroform extracts each inhibited the growth of 3 of the 14 bacteria tested (21.4%). The water extract had the narrowest antibacterial specificity, inhibiting the growth of only 2 of the 14 bacteria tested (14.3%). Whilst displaying a narrow range of specificity,

the water extract displayed potent antibacterial activity against *P. mirabilis* as determined from the zone of inhibition (16.3 ± 0.3 mm).

*P. phylliraeoides* methanol extract also demonstrated limited antifungal activity. The extract inhibited the growth of a nystatin resistant strain of *A. niger* but was ineffective against *C. albicans*. Although the inhibition of *A. niger* was not particularly strong (as determined by the zone of inhibition), this does identify the *P. phylliraeoides* methanol extract as a possible antifungal agent to combat otherwise resistant strains of *A. niger*. No other extract inhibited the growth of either of the fungi tested.

The relative level of antibacterial activity was further evaluated by determining the MIC values for each extract against the bacterial species which were determined to be susceptible. MIC values were evaluated in the current studies by disc diffusion across a range of concentrations. This has previously been determined to be a valid method of MIC determination as MIC values determined by disc diffusion correlate well with those determined by broth dilution assays.<sup>[41]</sup>

The methanol extract was particularly effective at inhibiting the growth of *S. pyogenes*, with growth inhibition seen at concentrations as low as approximately 8 µg/ml. The methanolic extracts MIC values were also below 1000 µg/ml for *mirabilis* and *S. marcescens*. Furthermore, the methanol extract was the only sample tested that displayed antifungal activity, inhibiting the growth of *A. niger* at concentrations as low as approximately 40 µg/ml. This is a significant

**Table 1: The mass of dried material extracted with the various solvents and the concentration after resuspension in deionised water**

Solvent	Mass of Dried Extract (mg)	Resuspended Extract Concentration (mg/ml)
Methanol	64.0	6.4
Deionised Water	111.2	11.1
Ethyl Acetate	20.6	2.1
Chloroform	120.1	12.0
Hexane	70.0	7.0

**Table 2: Qualitative phytochemical screenings of solvent extractions**

Extract	Phenols		Cardiac Glycosides	Saponins	Triterpenes	Phytosteroids	Alkaloids		Flavanoids	Tannins	Anthraquinones			
	Total Phenolics	Water Soluble					Water Insoluble	Keller-Kiliani Test			Froth Persistence	Salkowski Test	Acetic Anhydride Test	Meyers Test
Methanol	++	+	+	-	++	++	-	-	-	++	++	-	-	-
Water	+++	+	+++	-	+++	++	-	-	-	+++	+++	+	-	-
Ethyl Acetate	-	-	-	-	+	-	-	-	-	+	+	-	-	-
Chloroform	-	-	-	-	-	-	-	-	-	+	+	-	-	-
Hexane	-	-	-	-	-	-	-	-	-	-	-	-	-	-

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

**Table 3: Antimicrobial activity of *P. phylliraeoides* solvent extracts measured as zones of inhibition (mm)**

	Methanol extract	Water extract	Ethyl acetate extract	Chloroform extract	Hexane extract	Ampicillin	Chloramphenicol	Nystatin
<b>Gram negative rods</b>								
<i>A. faecalis</i>	–	–	–	–	–	9.8 ± 0.8	6.2 ± 0.6	NT
<i>A. hydrophilia</i>	–	–	7.3 ± 0.6	–	–	9.5 ± 1.4	18.0 ± 0.8	NT
<i>C. freundii</i>	–	–	–	–	–	9.8 ± 0.8	25.3 ± 1.5	NT
<i>E. coli</i>	6.5 ± 0.0	–	–	6.0 ± 0.0	–	10.7 ± 1.8	20.0 ± 0.8	NT
<i>K. pneumoniae</i>	–	–	–	–	9.0 ± 0.0	9.8 ± 1.2	24.0 ± 0.7	NT
<i>P. mirabilis</i>	16.3 ± 0.3	14.7 ± 0.3	9.3 ± 0.6	–	9.2 ± 0.6	14.6 ± 0.7	8.2 ± 0.6	NT
<i>P. fluorescens</i>	–	–	–	–	–	13.7 ± 1.8	16.8 ± 1.5	NT
<i>S. newport</i>	–	–	–	–	–	9.5 ± 1.4	15.2 ± 1.2	NT
<i>S. marcescens</i>	7.5 ± 0.5	8.7 ± 0.4	8.0 ± 0.5	7.7 ± 0.3	8.3 ± 0.3	–	14.9 ± 1.6	NT
<i>S. sonnei</i>	–	–	–	7.7 ± 0.3	–	12.4 ± 0.8	18.3 ± 1.3	NT
<b>Gram positive rods</b>								
<i>B. cereus</i>	–	–	–	–	–	16.8 ± 1.2	12.8 ± 0.9	NT
<b>Gram positive cocci</b>								
<i>S. aureus</i>	–	–	–	–	7.3 ± 0.6	14.3 ± 1.5	18.8 ± 0.6	NT
<i>S. epidermidis</i>	–	–	–	–	–	32.2 ± 1.3	13.0 ± 1.0	NT
<i>S. pyogenes</i>	6.7 ± 0.3	–	–	–	–	14.0 ± 1.3	10.3 ± 0.7	NT
<b>Fungi</b>								
<i>A. niger</i>	6.6 ± 0.3	–	–	–	–	NT	NT	–
<i>C. albicans</i>	–	–	–	–	–	NT	NT	25.0 ± 1.8

Numbers indicate the mean diameters (mm) of inhibition of at least triplicate experiments ± standard deviation. – indicates no growth inhibition. NT indicates not tested. Ampicillin (2 µg), chloramphenicol (10 µg) and nystatin (100 µg) were used as positive controls. Deionised water was included as a negative control.

**Table 4: Minimum inhibitory concentrations (µg/ml) of *P. phylliraeoides* extracts against susceptible microbes**

	MIC (µg/ml)								
	<i>A. hydrophilia</i>	<i>E. coli</i>	<i>K. pneumoniae</i>	<i>P. mirabilis</i>	<i>S. marcescens</i>	<i>S. sonnei</i>	<i>S. aureus</i>	<i>S. pyogenes</i>	<i>A. niger</i>
Methanol extract	–	1369.2	–	473.4	737.3	–	–	7.8	40.4
Water extract	–	–	–	770.4	1544.5	–	–	–	–
Ethyl acetate extract	506.7	–	–	665.2	216.1	–	–	–	–
Chloroform extract	–	2518.7	–	–	1314.5	32.2	–	–	–
Hexane extract	–	–	307.9	1203.1	784.1	–	5557.6	–	–

Numbers indicate the mean MIC values of at least triplicate determinations. – indicates no growth inhibition.

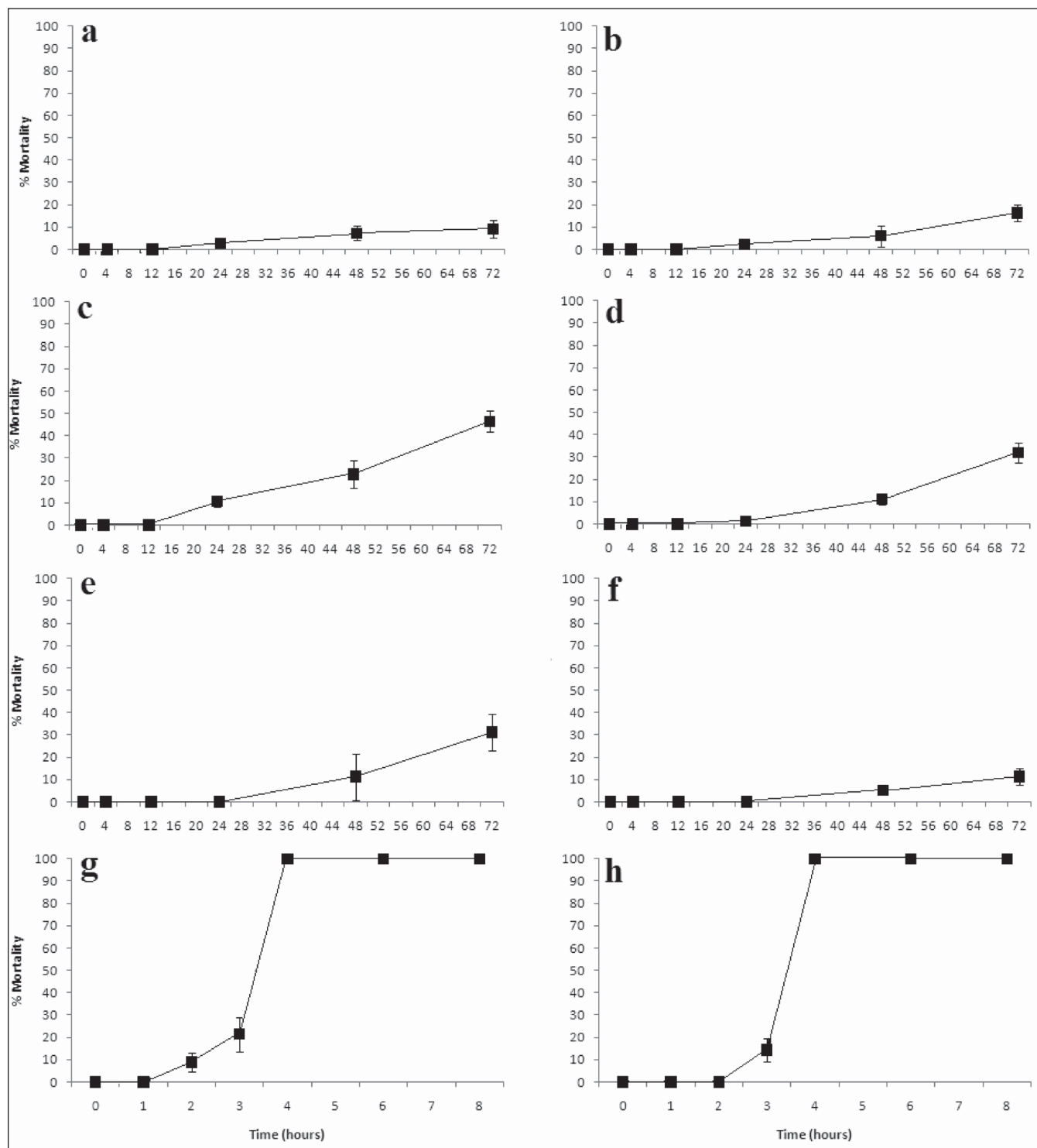
result as the strain of *A. niger* tested was a resistant strain which was not inhibited by the nystatin control. The hexane extract also displayed potent antibacterial activity with MIC values below 1000 µg/ml for two bacteria (*K. pneumoniae* and *S. marcescens*). The ethyl acetate and chloroform extracts both inhibited the growth of three bacteria, although the ethyl acetate extract exhibited the more potent bioactivity,

with MIC values less than 1000 µg/ml for all the bacterial species whose growth it inhibited. However, the potent growth inhibition of the chloroform extract towards *S. sonnei* (32.2 µg/ml) is noteworthy. The water extract inhibited the growth of only two bacterial species (*P. mirabilis* and *S. marcescens*), although MIC values below 1000 µg/ml were seen for only a single species (*P. mirabilis*).

### Quantification of toxicity

*P. phylliraeoides* methanol, water, chloroform and hexane extracts were diluted to a concentration of 4000 µg/ml in artificial seawater for toxicity testing, resulting in 2000 µg/ml concentrations in the *Artemia* nauplii lethality bioassay. The less concentrated ethyl acetate extract was diluted to

2000µg/ml in artificial seawater for toxicity testing, resulting in 1000 µg/ml concentrations in the *Artemia* nauplii lethality bioassay. For comparison, the reference toxins potassium dichromate (1000 µg/ml) and Mevinphos (2000 µg/ml) were also tested in the bioassay. Figure 1 shows the % mortality induced by each extract and by the controls at



**Figure 1:** Brine shrimp lethality of (a) *P. phylliraeoides* methanol extract (2000 µg/ml), (b) *P. phylliraeoides* water extract (2000 µg/ml), (c) *P. phylliraeoides* ethyl acetate extract (1000 µg/ml), (d) *P. phylliraeoides* chloroform extract (2000 µg/ml), (e) *P. phylliraeoides* hexane extract (2000 µg/ml), (f) artificial seawater negative control, (c) potassium dichromate (1000 µg/ml), (d) Mevinphos (2000 µg/ml). All bioassays were performed in at least triplicate and are expressed as mean ± standard deviation.

various times. The potassium dichromate (Figure 1g) and Mevinphos (Figure 1h) reference toxins were rapid in their onset of mortality. Both reference toxins induced mortality within the first 3 hours of exposure and 100% mortality was evident following 4-5 hours. In contrast, all of the *P. phylliraeoides* extracts (Figures 1a-e) displayed mortality rates only slightly elevated above those of the artificial seawater negative control (Figure 1f) at 24, 48 and 72 h. At no time point tested did the mortality induction by any *P. phylliraeoides* extract reach 50%, even at the relatively high doses tested (2000 µg/ml for methanol, water, chloroform and hexane extracts; 1000 µg/ml for ethyl acetate extract) so it was not possible to accurately determine an LC<sub>50</sub> for any extract. Furthermore, as toxicity has previously been defined for plant extracts as the induction of ≥ 50% mortality at concentrations ≤ 1000 µg/ml,<sup>[39]</sup> all *P. phylliraeoides* extracts are considered to be of low toxicity.

## DISCUSSION

The current study reports on the antibacterial and antifungal activities of various *P. phylliraeoides* extracts, and on their toxicity. The ability of *P. phylliraeoides* extracts to inhibit the growth of Gram-negative, and to a lesser extent Gram-positive bacteria, is in agreement with previous reports of the antibacterial activity of other Australian native plants that have a history of medicinal usage by Australian Aborigines. The antiseptic properties of *Eucalyptus*,<sup>[22-25]</sup> *Leptospermum*,<sup>[14,27-29]</sup> and *Melaleuca*<sup>[30,31]</sup> species have been extensively studied and shown to inhibit the growth of a wide variety of both Gram-positive and Gram-negative bacteria.

The current study shows Gram-negative bacteria to be more susceptible to *P. phylliraeoides* extracts than Gram-positive bacteria, although this may be due to the small sample of Gram-positive bacteria tested. Indeed, only the methanolic and hexane extracts were capable of inhibiting any Gram-positive bacteria, each inhibiting the growth of a single bacterium of the 4 Gram-positive bacteria tested (25%). The greater susceptibility of Gram-negative bacteria observed in this study is in contrast to previous studies which have reported a greater susceptibility of Gram-positive bacteria towards solvent extracts for South American,<sup>[42]</sup> African<sup>[43,44]</sup> and Australian<sup>[45]</sup> plant extracts. Results within this laboratory have also confirmed the greater susceptibility of Gram-positive bacteria towards other many other Australian plant extracts,<sup>[27]</sup> although examples of nonpolar extracts from Australian plants having a greater effect on Gram-negative bacteria have also been reported.<sup>[46]</sup>

Individual *P. phylliraeoides* extract components responsible for the antibacterial potential of the solvent extracts were

not identified in the current study. Previous reports have identified various bioactive components of other Australian medicinal plants (*Eucalyptus*,<sup>[47]</sup> *Leptospermum*,<sup>[48]</sup> *Melaleuca*<sup>[49]</sup>). These plants all contain terpenes including 1, 8-cineole, terpinen-4-ol, α-pinene and β-pinene. Both 1, 8-cineole and terpinen-4-ol have antimicrobial activity.<sup>[50,51]</sup> Recent studies have also reported on the antibacterial activities of *Callistemon*<sup>[26]</sup> and *Syzygium*<sup>[20,32-34]</sup> species. It has been postulated that terpene components may also be responsible for the antiseptic properties of these plants.<sup>[52]</sup> *P. phylliraeoides* leaves contain a number of pentacyclic triterpenoid saponogenins including phillyregenin (a dihydroxylactone), R1-barrigenol, 27 - desoxyphillyrigenin (3fl - hydroxytaraxastan - 28, 20fl - olide), 23 - hydroxyphillyrigenin (3fl, 23, 27- trihydroxytaraxastan - 28, 20fl - olide), dihydropriverogenin A, 16 - desoxybarringtogenol C and barringtogenol C.<sup>[13]</sup> Whilst the phytochemistry of the *P. phylliraeoides* extracts investigated in the current study was not extensively examined, phenolic compounds, triterpenoids, saponins, flavanoids and tannins were detected by qualitative assays. Further studies are required to determine which of these classes of phytochemical is responsible for the recorded bioactivities and to further identify the individual bioactive compounds.

The findings reported here also demonstrate that none of the *P. phylliraeoides* extracts displayed significant toxicity towards *A. franciscana*. None of the extracts tested induced mortality above 50%. Whilst the ethyl acetate, chloroform and hexane extracts induced mortality approaching 50%, this was not until 72 h. As most toxicity studies using *Artemia* nauplii usually report 24 h and/or 48 h LC<sub>50</sub> values, the reporting of mortality at 72 h may not be required as it does not provide data for cross study comparisons. Furthermore, even at 72 h the mortality induction by all extracts was below 50%. The extracts in the current study were all tested at concentrations of 2000 µg/ml in the bioassay (with the exception of the ethyl acetate extract which was tested at 1000 µg/ml). Previously, compounds with an LC<sub>50</sub> of greater than 1000 µg/ml towards *Artemia* nauplii have been defined as being non-toxic.<sup>[39]</sup> It was therefore determined that all *P. phylliraeoides* extracts were non-toxic towards *Artemia* nauplii.

In conclusion, the results of this study partially validate the traditional Australian Aboriginal usage of *P. phylliraeoides* to treat bacterial and fungal diseases and indicate that *P. phylliraeoides* is worthy of further study. Further evaluation of the antibacterial and antifungal properties of these extracts against a more extensive panel of microbial agents is warranted. Likewise, purification and identification of the bioactive components is needed to examine the mechanisms of action of these agents. Whilst the extracts examined in this report are promising as antibacterial and antifungal agents, caution is needed before these



compounds can be applied to medicinal purposes. In particular, further toxicity studies using human cell lines are needed to verify the suitability of these extracts for these purposes.

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