

Podocarpus elatus R.Br. Leaf Extracts are Ineffective Inhibitors of the Growth of a Panel of Bacterial Pathogens

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ABSTRACT

Introduction: Closely related plant species often share similar secondary metabolites and bioactivities and are therefore good targets for bioactivity testing when one or more species within a genus are known to possess therapeutic properties. The genus *Podocarpus* has a long history of medicinal usage in many areas of the world. Many species are known to have therapeutic properties, several species of which have well established antibacterial bioactivities. **Materials and Methods:** The ability of *P. elatus* 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:** The *P. elatus* methanolic and aqueous extracts were ineffective at inhibiting the growth of gram-positive and gram-negative panels of bacteria and were nontoxic in the *Artemia* lethality assay following 24 hr exposure. **Conclusion:** Despite its reported therapeutic uses against other diseases and its close taxonomic relationship with several bioactive *Podocarpus* spp. with antibacterial activity, *P. elatus* leaf extracts were ineffective bacterial growth inhibitors. However, these extracts may have other therapeutic properties and testing against protozoa, fungi, virus and tumour cells is required.

Keywords: Podocarpaceae, Illawarra plum, Brown plum, Plum pine, Traditional medicine, Medicinal plants, Toxicity.

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INTRODUCTION

The use of natural plant therapeutics is as old as human civilisation and in many regions of the world is still the primary modality of health care. Ayurvedic medicine in India for example is still commonly practiced, with approximately 85% of Indians using crude plant preparations for the treatment of various diseases and ailments.¹ Even in Western civilisations; plants play an important role in medicine. At least 25% of pharmaceuticals prescribed worldwide are directly obtained from plants with many more drugs being semi-synthetic derivatives of natural plant precursors.²⁻⁴ Examples of medicinally important plant derived compounds include the anti-malarial drug quinine and its derivatives (from *Cinchona* spp.), the antitumour drugs vincristine and vinblastine (from *Catharanthus roseus*) along with the semi-synthetic analogue vindesine, the analgesics morphine and codeine (from *Papaver somniferum*), the anti-cholinergic drug atropine derived from plants of the family *Solanaceae* (*Atropa belladonna*, *Datura stramonium* and *Mandragora officinarum*),

the anticancer drug taxol (derived from *Taxus brevifolia*) and the cardiac glycoside digoxin (from *Digitalis purpurea*).⁵

Despite the potential of plants to provide us with useful pharmaceutical agents, the field is still poorly studied. Only an estimated 5-10% of the approximately 300, 000-500, 000 plant species worldwide have been screened for 1 or more bioactivities.⁵ With so many plant species yet to be tested, it is essential that plant selection processes narrow the field. The main selection criteria currently used is to select plants on the basis of ethnobotanical usage as traditional medicines. Other important selection criteria is to examine plants closely related to plants for which medicinal potential is well established, or plants with known physiochemical properties for which a therapeutic property has previously been reported. Plants with high antioxidant contents are considered particularly promising for the development of new therapies as many antioxidant plant compounds have protective and curative effects. Indeed, plants high antioxidant capacities have been previously reported to have protect and treat cancer.⁶⁻¹⁰ cardiovascular diseases,¹¹ neural degeneration,¹² diabetes and obesity.¹³ Interestingly, multiple recent studies have also reported noteworthy inhibitory activity against multiple bacteria, highlighting the potential of high antioxidant plant extracts as antibacterial chemotherapies.¹⁴⁻²⁰



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In recent years, the development of bacterial pathogens that are either extremely (XDR) or totally drug resistant (TDR) to common clinically used antibiotics²¹ has resulted in the need to develop new 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.²² For a number of reasons reviewed elsewhere,²¹ 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.^{14,23-26}

Podocarpus is a genus of conifers that is widely distributed across areas that comprise the ancient supercontinent Gondwana.

They are particularly prevalent in Africa, South America, India, Australia and the Oceania region. Many species have a history of traditional use to treat fevers, asthma, respiratory diseases, arthritis and inflammation and sexually transmitted infections.²⁷⁻²⁹ Notably, several of these diseases are caused by bacterial pathogens. *Podocarpus elatus* R.Br. (commonly known as Illawarra plum, plum pine, brown pine, daalgaal; synonym *Nageia elata* (Endl.) F. Muell.) is a medium to large evergreen tree (Figure 1a) that is endemic to the east coast regions of Australia. It grows to 30-36 m in height, with a trunk that can reach a diameter of 1.5 m. Its leaves are lanceolate (up to 25cm long by 2 cm wide) (Figure 1b). It produces cones that when fertilised develop into bark blue/black fleshy berry-like fruit (Figure 1c), which have high antioxidant contents.³⁰ Indeed, that study reported that *P. elatus* fruit have radical scavenging activity and total reducing capacities of 8.9 and 215 $\mu\text{mol/g}$ of fresh fruit respectively. Furthermore, similar studies in our group have confirmed the high antioxidant capacity of this fruit.⁹ Whilst our understanding of the phytochemistry of *P. elatus* fruit is currently incomplete, it

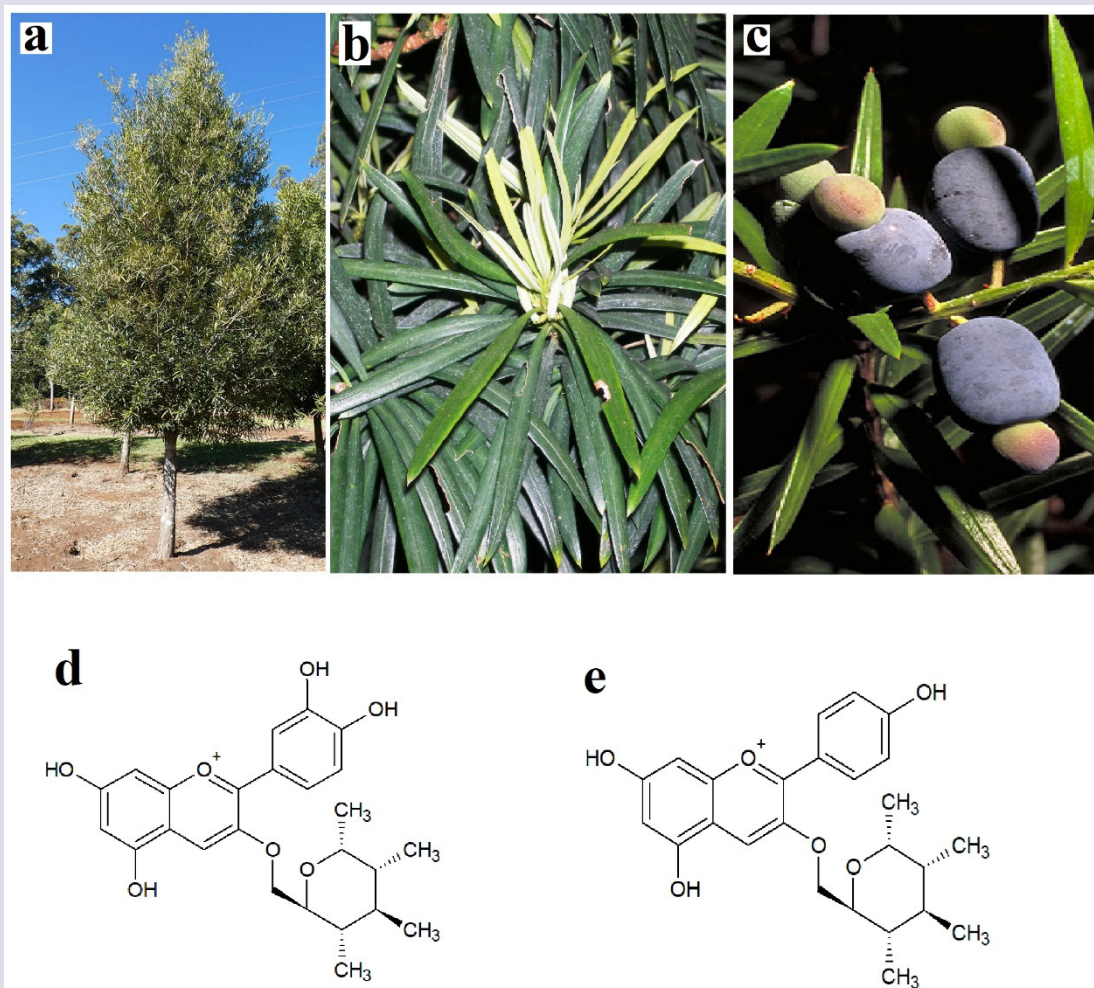


Figure 1: *P. elatus* (a) whole tree, (b) leaves, (c) fruit, and the structures of the anthocyanidin glycosides (d) cyanidin-3-glucoside (e) pelargonidin-3-glucoside.

has been reported to be particularly rich in the anthocyanidins cyanidin-3-glucoside (Figure 1d) pelargonidin-3-glucoside (Figure 1e).³⁰ These molecules both have good antioxidant activity and may provide the fruit with therapeutic properties. Despite its known phytochemistry and the traditional uses of other *Podocarpus* spp. to treat bacterial infections, *P. elatus* has yet to be rigorously examined for the ability to inhibit bacterial growth. This study screened *P. elatus* leaves for the ability to inhibit the growth of a panel of bacterial pathogens of human importance.

MATERIALS AND METHODS

Plant Material

Collection of Plant Material and Extraction

Podocarpus elatus R.Br. leaves were collected from a verified tree in Brisbane, Australia, washed in deionised water and processed within 4 hr of collection. The tree was monitored for 3 months prior to harvesting to ensure that it received no pesticides or fertilisers, and none were used in a 3 m radius of the plant. Voucher specimens of the leaves (GU2018PELa) are stored in the School of Environment and Science, Griffith University. The harvested leaves were dried in a Sunbeam food dehydrator and the dried material was ground to a coarse powder. Individual 1 g masses of the dried plant material was extracted extensively in 50 mL methanol (Ajax Fine Chemicals Australia, 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 10 mL deionised water. The extract was passed through 0.22 µm filter (Sarstedt) and stored at 4°C.

Qualitative Phytochemical Studies

Phytochemical analysis of the *P. elatus* leaf extracts for the presence of saponins, phenolic compounds, flavonoids, phytosteroids, triterpenoids, cardiac glycosides, anthraquinones, tannins and alkaloids was conducted by standard assays.^{15,26,31}

Antibacterial Screening

Test Microorganisms

All media was purchased from Oxoid Ltd., Australia. The reference strains of *E. 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 faecalis*, *Bacillus cereus*, *Citrobacter freundii*, *Pseudomonas fluorescens*, *Salmonella newport*, *Serratia marcescens*, *Shigella sonnei*, *Staphylococcus aureus* and *Staphylococcus epidermidis* strains were obtained from Ms Michelle Mendell and Ms Jane Gifkins, Griffith University. All stock cultures were subcultured and maintained in nutrient broth at 4°C.

Evaluation of Antimicrobial Activity

Antimicrobial activity of the *P. elatus* leaf extracts was determined using a modified disc diffusion assay.³²⁻³⁴ Briefly, 100 µL of the each bacterial suspension in log phase was spread onto individual nutrient agar plates and the extracts were tested for antibacterial 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 (\pm 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 (ALA).^{26,35,36} 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 and 48 hr. At the completion of the 48 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. LC₅₀ 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 1g of dried and powdered *P. elatus* leaves with methanol and water yielded 362 and 286 mg of extracted material respectively (Table 1). The extracts were resuspended in 10 mL of deionised water (containing 1% DMSO), resulting in an extract concentration shown in Table 1. Qualitative phytochemical studies showed that both extracts had similar phytochemical profiles. Both contained high levels of phenolic compounds and flavonoids. Lower levels of saponins, triterpenoids and tannins were also detected. Cardiac glycosides, and alkaloids and anthraquinones were completely absent or below the detection thresholds for these assays.

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

		Methanolic extract	Aqueous extract	
Mass of extracted material (mg)		362	36.2	
Concentration of resuspended extract (mg/mL)		286	28.6	
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	-	-
		Draggendorff'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.

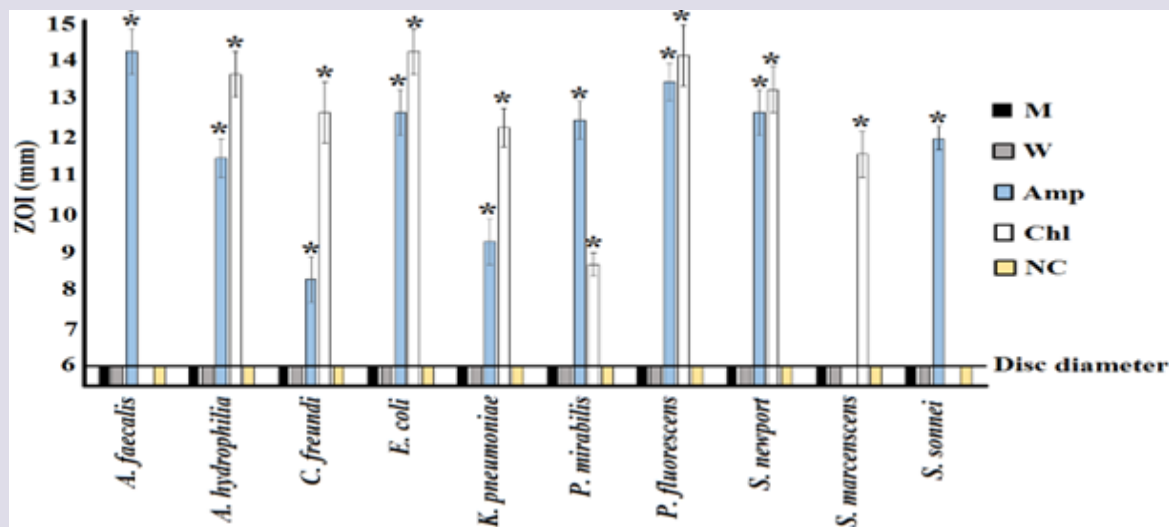


Figure 2: Growth inhibitory activity of *P. elatus* leaf extracts and reference antibiotics against gram-negative bacterial species measured as ZOIs (mm)±SEM. Ampicillin (Amp) and chloramphenicol (Chl) standard discs (10 µg) were used as positive controls. M=Methanolic extract; W=aqueous extract; NC=Negative 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.

Antibacterial activity

To determine the growth inhibitory activity of the *P. elatus* leaf extracts, aliquots (10 µL) of each extract were screened in the disc diffusion assay. The *P. elatus* 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 ZOIs 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.

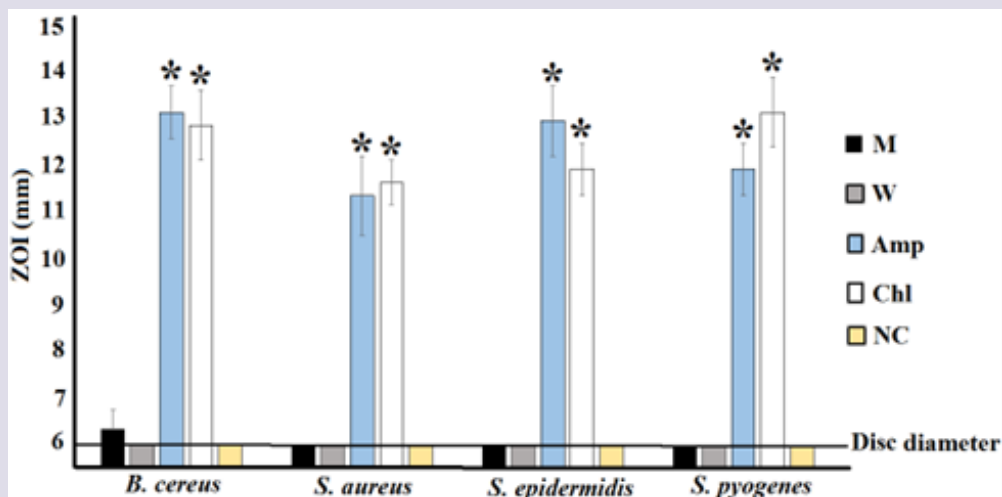


Figure 3: Growth inhibitory activity of *P. elatus* leaf extracts and reference antibiotics against gram-positive bacterial species measured as ZOI (mm)±SEM. Ampicillin (Amp) and chloramphenicol (Chl) standard discs (10 µg) were used as positive controls. M=Methanolic extract; W=aqueous extract; NC=Negative 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.

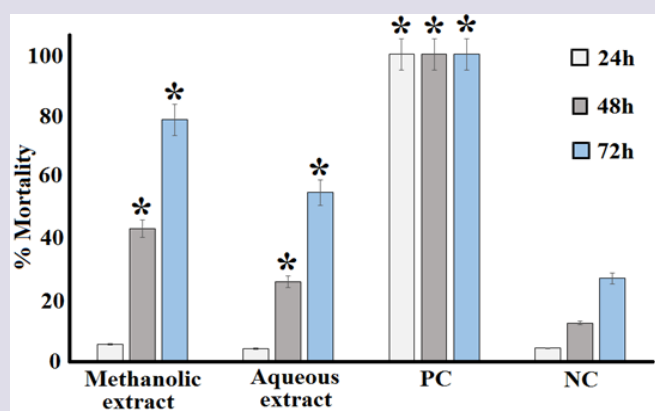


Figure 4: The lethality of the *P. elatus* leaf extracts, potassium dichromate Positive Control (PC) (1000 µg/mL) and seawater (Negative Control: NC) following 24, 48 and 72 hr of exposure. All bioassays were performed three times in triplicate ($n=9$) and are expressed as mean±SEM. *indicates results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

Quantification of toxicity

The toxicity of the *P. elatus* leaf extracts was initially tested at 2 mg/mL in the *A. franciscana* nauplii bioassay (Figure 4). The mortality in the presence of all extracts was not significantly different to that of the untreated control at 24 hr and thus was deemed to be non-toxic. Extracts with 24 hr LC_{50} values >1000 µg/mL have previously been defined as non-toxic.³⁶ 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. elatus*

leaf extracts at 48 hr and was further increased following 72 hr exposure.

DISCUSSION

Due to recent increases in bacterial resistance to many antibiotics, the development of new antibiotic chemotherapies is a high priority for medical science.^{20,21} A concurrent decrease in the discovery of new antibiotic medicines by conventional strategies has increased interest in re-evaluating medicinal plants for new antibiotic chemotherapies.²⁰ As *P. elatus* is taxonomically related to multiple *Podocarpus* spp. that have antibacterial activity,²⁷⁻²⁹ it was deemed a viable target for antibacterial screening. Interestingly, the *P. elatus* extracts were inactive against all gram-positive and gram-negative bacteria tested.

It is noteworthy that 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 methodology 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. For this reason, whilst this is a handy assay for screening aqueous extracts, this technique may not be ideal for nonpolar compounds (e.g. when screening essential oil and their components). For examining nonpolar

mixtures, other techniques such as liquid dilution assays may be preferred. As *Piper* spp. are known to contain nonpolar terpenoid components their activity may have been significantly underestimated. Liquid dilution studies may have been better suited to screen the *Podocarpus elatus* for activity and future studies will use these techniques to re-examine the extracts for antibacterial activity.

Diffusion of molecules within an agar gel is also affected by the size of the molecules. The movement of large, complex phytochemicals (e.g. complex tannins) through agar gels by diffusion would also be retarded and may provide a false idea of the efficacy of an extract. As many tannins have well described antibiotic properties, screening for growth inhibition using agar diffusion techniques may give a distorted view of its inhibitory potential.

The findings reported here also indicate that the extracts examined were non-toxic (24 hr $LC_{50} > 1000 \mu\text{g/mL}$) 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.³⁶ However, further studies are required to determine whether this is also true for the *P. elatus* leaf extracts examined in these studies.

CONCLUSION

Methanolic and aqueous *P. elatus* leaf extracts lacked antibacterial activity in the disc diffusion assay against a panel of human pathogenic bacteria, despite their reported therapeutic properties against other diseases and close taxonomic relationship with *Podocarpus* spp. with established antibacterial properties. The extracts were nontoxic towards *Artemia nauplii*.

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CONFLICT OF INTEREST

The authors declare that there is no conflict 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. elatus* 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. elatus* extracts was determined using the *Artemia nauplii* toxicity bioassay.

• Both the methanolic and aqueous extracts were nontoxic.

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