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Original Article

Pouteria australis (R.Br.) Baehni Leaf Extracts Lack Antibacterial Activity and are Non-toxic *in vitro*

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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 family are known to possess therapeutic properties. The genus *Pouteria* (family Sapotaceae) has a long history of medicinal usage in many areas of the world. Many species are known to have therapeutic properties, several of which have well established antibacterial bioactivities. **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* methanolic and aqueous extracts were completely ineffective at inhibiting the growth of gram-positive and gram-negative panels of bacteria. The extracts were nontoxic to *Artemia* nauplii following 24 hr exposure. **Conclusion:** Despite the taxonomic relationship with several bioactive *Pouteria* spp., *P. australis* leaf extracts were completely ineffective bacterial growth inhibitors. However, these extracts may have other therapeutic properties and testing against protozoa, fungi, virus and tumour cells is required.

Key words: Sapotaceae, Black apple, Australian plant, Traditional medicine, Medicinal plants, Toxicity.

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INTRODUCTION

Plant medicines have been used by human civilisations since before written records and are still the primary therapeutic option in many regions of the world. For example, Indian Ayuverdic medicine is used by approximately 85% of the Indian population to treat disease.[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* (L.) G.Don) along with the semi-synthetic analogue vindesine, the analgesics morphine and codeine (from *Papaver somniferum* L.), the anticholinogenic drug atropine derived from plants of the family *Solinaceae* (*Atropa belladonna* L., *Datura stramonium* L. and *Mandragora officinarum* L.), the anticancer drug taxol (derived from *Taxus brevifolia* Nutt.) and the cardiac glycoside digoxin (from *Digitalis purpurea* L.).[5]

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 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. Another important selection method is to examine species closely related to plants for which medicinal potential is well established. Many plant secondary metabolites are regarded as family, genus, or species specific and investigation of species closely related to those used as traditional medicines may lead to natural therapeutic discovery.[2]

In recent years, the development of bacterial pathogens that are either extremely (XDR) or totally drug resistant (TDR) to common clinically used antibiotics[6] 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.[7] For a number of reasons reviewed elsewhere,[6] 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.[8,9]

The genus *Pouteria* (family Sapotaceae) consists of approximately 325 species which occur in tropical and subtropical regions globally.[10] Many *Pouteria* spp. produce edible fruit that are important foods in the regions in which they grow. Several *Pouteria* spp. also have long histories of medicinal use in the treatment of a wide variety of medical disorders and conditions. For example, the antinociceptive and antinflammatory properties of *Pouteria ramifloria* (Mart.) Radlk. roots is well established.[11] Several species are also useful as antimicrobial therapies. *Pouteria torta* (Mart.) Radlk. leaf extracts have been reported to inhibit *Staphylococcus aureus, Pseudomonas aeruginosa,*[12] *Caldosporium shaerrospermon* and *Bacillus cereus* growth.[13] Furthermore, *Pouteria pallida* (C.F.Gaertn.) Baehni extracts inhibit a multidrug resistant *S. aureus.*[14] There is substantially less information available about Australian *Pouteria* species and far more work is needed to verify their therapeutic potential.

Pouteria australis (R.Br.) Baehni (synonym *Planchonella australis*; commonly known as black apple, wild plum, black plum, yellow buttonwood) is a medium to tall rainforest tree that is native to north eastern regions of Australia. The thick, leathery leaves (Figure 1a) are simple and measure 8-16cm long by 2-5cm wide. The flowers are axial and develop into 20-65mm long purple/black fruit (Figure 1b) that

contain 2-5 brown seeds (Figure 1c). Whilst we were unable to find records of *P. australis* being used medicinally by the first Australians, the related species *Pouteria queenslandica* P.Royen (synonym *Planchonella queenslandica* (P.Royen) Jessup) was used to treat sore throats and as an antiseptic.[15] Notably, its antibacterial activity has also been verified against an extensive panel of bacteria.[16] Despite this, *P. australis* remains to be tested for antibacterial activity. There is also a lack of information on the phytochemical composition of this species. However, the phytochemistry of related species has been reported and multiple triterpenoids have been identified, including spinasterol, (Figure 1d) 3β, 28-dihydroxy-olean-12-enyl fatty acid esters, (Figure 1e) and betulinic acid fatty acid esters (Figure 1f).[10] Many of these have been reported to have potent antibacterial activity[17,18] and may therefore contribute to the antibacterial properties of some *Pouteria* spp. 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

Pouteria australis (R.Br.) Baehni leaves were obtained from and identified by Philip Cameron, senior botanic officer, Mt Cootha Botanical Gardens, Brisbane, Australia. The leaves were thoroughly washed in deionised water and dehydrated in a Sunbeam food dehydrator within 4 hr of collection. The dried material was ground to a coarse powder. Individual 1g 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 5mL deionised water. The extract was passed through 0.22µm filter (Sarstedt) and stored at 4°C.

Qualitative phytochemical studies

Phytochemical analysis of the *P. australis* leaf extracts for the presence of saponins, phenolic compounds, flavonoids, phytosteroids, triterpenoids, cardiac glycosides, anthraquinones, tannins and alkaloids was conducted by standard assays.[19-21]

Antibacterial screening

Test microorganisms

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 fluorescens*, *Salmonella newport, Serratia marcescens, Shigella sonneii, 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. australis* leaf extracts was determined using a modified disc diffusion assay.[22-24] Briefly, 100µL 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 10µL of the individual plant extract, allowed to dry and placed onto the inoculated plates. The plates were allowed to stand at 4°C for 2hr before incubation at 37°C for 24hr. 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 (10µg) and chloramphenicol (10µg) were obtained from Oxoid, Australia and were used as positive controls to compare antibacterial activity. Filter discs infused with 10µL of distilled water (containing 1% DMSO) were used as a negative control.

Artemia franciscana nauplii toxicity screening

Toxicity was tested using an adapted *Artemia franciscana* nauplii lethality assay.[25-27] Briefly, *A. franciscana* nauplii were incubated in the presence of the extracts, reference toxin (1mg/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 number of dead were counted in each well at 24hr and 48hr. At the completion of the 48hr 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 1g of dried and powdered *P. australis* leaves with methanol and water yielded 296 and 265mg of extracted material respectively (Table 1). The extracts were resuspended in 10mL of deionised water (containing 1% DMSO), resulting in an 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. Lower levels of saponins, triterpenoids and tannins were also detected. Cardiac glycosides, phytosterols, alkaloids and anthraquinones were completely absent or below the detection thresholds for these assays.

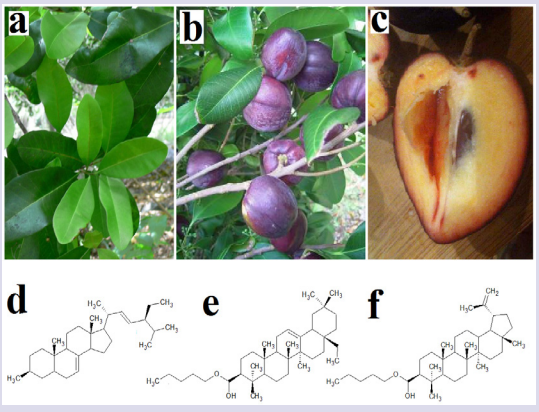

Figure 1: *P. australis* (a) leaves, (b) whole fruit, (c) fruit cut open and the structures of compounds identified in other *Pouteria* spp.: (d) spinasterol, (e) 3β, 28-dihydroxy-olean-12-enyl fatty acid ester, (f) betulinic acid fatty acid ester.

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.

			Methanolic extract	Aqueous extract
	Mass of extracted material (mg)		296	265
	Concentration of resuspended extract (mg/mL)		29.6	26.5
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.

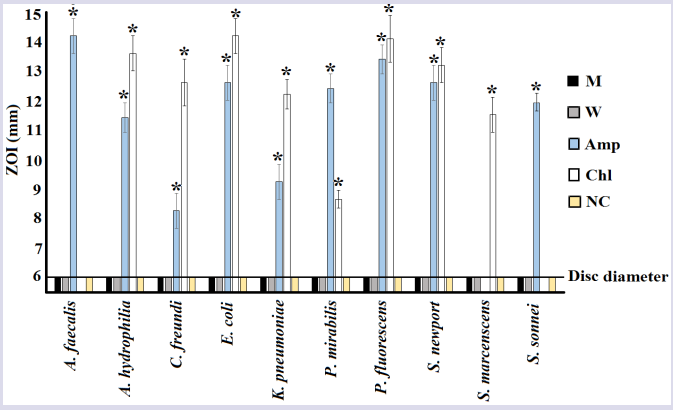

Figure 2: Growth inhibitory activity of *P. australis* leaf extracts and reference antibiotics against gram-negative bacterial species measured as ZOIs (mm) ± SEM. Ampicillin (Amp) and chloramphenicol (Chl) standard discs (10µg) were used as positive controls. M = methanolic extract; W = aqueous extract;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

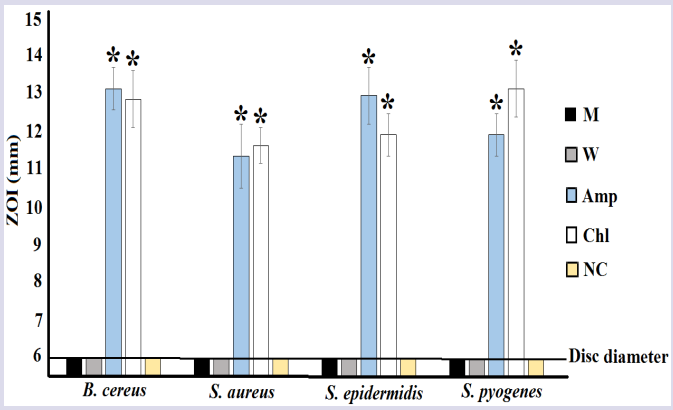

Figure 3: Growth inhibitory activity of *P. australis* leaf extracts and reference antibiotics against gram-positive bacterial species measured as ZOIs (mm) ± SEM. Ampicillin (Amp) and chloramphenicol (Chl) standard discs (10µg) were used as positive controls. M = methanolic extract; W = aqueous extract; 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. australis* leaf extracts, aliquots (10µL) 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 14.3mm (chloramphenicol against *E. coli*). We were

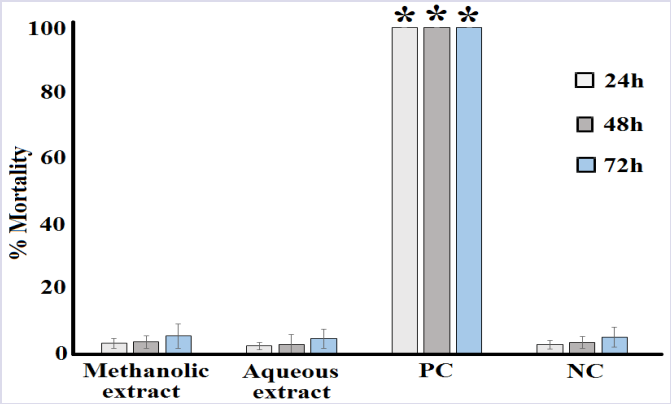

Figure 4: The lethality of the *P. australis* leaf extracts, potassium dichromate control (1000µg/mL) and seawater (negative control) following 24, 48 and 72 hr of exposure. All bioassays were performed three times in triplicate (n=9) and are expressed as mean ± SEM. * indicates results that are significantly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

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 2mg/mL in the *A. franciscana* nauplii bioassay (Figure 4). The mortality in the presence of either extract was not significantly different to that of the untreated control at 24hr and thus both extracts were deemed to be non-toxic. Extracts with 24hr LC_{50} values >1000µg/mL have previously been defined as non-toxic.[26] In contrast, the potassium dichromate positive control induced substantial mortality within 4h (results not shown), with 100% mortality induction seen by 24hr. The mortality increased slightly following exposure to the *P. australe* leaf extracts at 48hr and was further increased following 72hr exposure. However, at all times, the mortality was not significantly different to the negative control.

DISCUSSION

Due to recent increases in bacterial resistance to many antibiotics, the development of new antibiotic chemotherapies is a high priority for medical science.[6,7] A concurrent decrease in the discovery of new antibiotic medicines by conventional strategies has increased interest in evaluating medicinal plants for new antibiotic chemotherapies.[28] Whilst there is a lack of evidence of the use of *P. australis* medicinally, the related Australian species *P. queenslandica* was used medicinally by the first Australians to treat sore throats and as an antiseptic.[15] Furthermore, as a number of other taxonomically related *Prouteria* spp. also have antibacterial activity.[10] Therefore *P. australis* was deemed a viable target for antibacterial screening. Interestingly, the *P. australis* extracts were completely inactive against all gram-positive and gram-negative bacteria tested.

Notably, 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 been widely utilised in other studies.[29-33] 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. For examining nonpolar mixtures, other techniques such as liquid dilution assays may be preferred. As *Pouteria* spp. are known to contain nonpolar terpenoid components, their activity may have been significantly under estimated. Liquid dilution studies may have been better suited to screen the *P. australis* 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 (eg. 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 indicate that the extracts examined were nontoxic (24 hr LC_{50} >1000µg/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.[26,34] However, further studies are required to determine whether this is also true for the *P. australis* leaf extracts examined in these studies.

CONCLUSION

Methanolic and aqueous *P. australis* leaf extracts displayed no antibacterial activity in the disc diffusion assay against a panel of human pathogenic bacteria, despite their close taxonomic relationship with other *Pouteria* spp. with well-known antibacterial properties. The extracts were nontoxic towards *Artemia* nauplii.

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CONFLICT 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.

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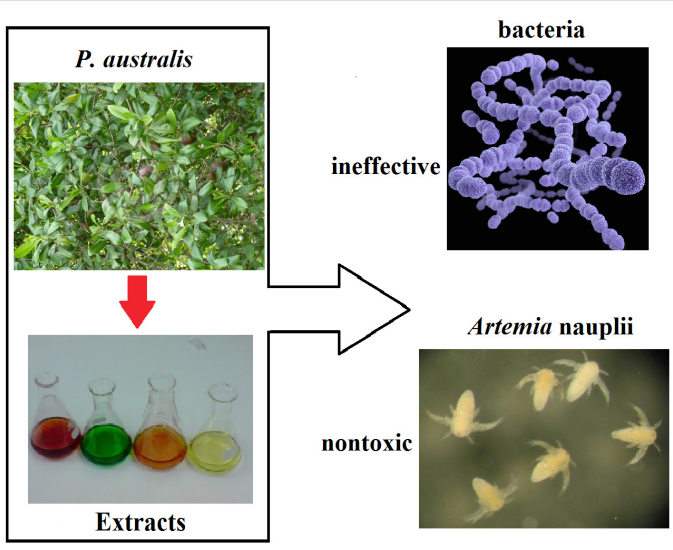
PICTORIAL ABSTRACT



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* extracts was determined using the *Artemia* nauplii toxicity bioassay.
- Both the methanolic and aqueous extracts were nontoxic.

ABOUT AUTHORS

Ms Lindiwe Mpala completed at BSc at Griffith University in life sciences. Following graduation, she undertook a research project in Dr Ian Cock's laboratory in the School of Natural Sciences at Griffith University. The project examined the growth inhibitory properties of a variety of Australian native plants against an extensive panel of bacterial pathogens.

Dr Ian Cock leads a research team in the Environmental Futures Research Institute and the School of Natural Sciences at Griffith University, Australia. His research involves bioactivity and phytochemical studies into a variety of plant species of both Australian and international origin, including Aloe vera, South Asian and South American tropical fruits, as well as Australia plants including Scaevola spinescens, Pittosporum phylliraeoides, Terminalia ferdinandiana (Kakadu plum), Australian Acacias, Syzygiums, Petalostigmas and Xanthorrhoea johnsonii (grass trees). This range of projects has resulted in nearly 200 publications in a variety of peer reviewed journals.

Ms Getmore Chikowe completed at BSc at Griffith University in life sciences. Following graduation, she undertook a research project in Dr Ian Cock's laboratory in the School of Natural Sciences at Griffith University. The project examined the growth inhibitory properties of a variety of Australian native plants against an extensive panel of bacterial pathogens.