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Original Article

Piper novae-hollandiae Miq. 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 genus are known to possess therapeutic properties. The genus *Piper* has a long history of medicinal usage in many areas of the world. Many *Piper* spp. are known to have therapeutic properties and several have antibacterial bioactivities. **Methods:** The ability of *P. novae-hollandiae* 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. novae-hollandiae* methanolic and aqueous extracts were completely ineffective at inhibiting the growth of panels of gram-positive and gram-negative bacteria. The extracts were nontoxic or of low toxicity to *Artemia* nauplii following 24 h exposure. **Conclusion:** Despite the close taxonomic relationship with several bioactive *Piper* spp. and its therapeutic use by first Australians, *P. novae-hollandiae* 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 warranted.

Key words: *Piperaceae*, Giant pepper vine, Australian plant, 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. Ayuverdic 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 and many more drugs are semi-synthetic derivatives of natural product precursors.[2-4] Examples of medicinally important plant derived compounds include the anti-malarial drugs quinine (from *Cinchona* spp.) and artemesinin (from *Artemisia annua* L.) and their derivatives; the anti-tumour 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 plants closely related to species 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 of novel pharmaceuticals.[2]

In recent years, the development of either extremely (XDR) or totally drug resistant (TDR) bacterial pathogens[6] has resulted in the need to develop new antibiotic chemotherapies. There are now limited therapeutic options for many diseases caused by bacteria 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 currently 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 *Piper* (Family Piperaceae) consists of approximately 1000 species, many with global distribution.[10] Many *Piper* spp. are used as spices for their pleasant flavour characteristics, with *Piper nigrum* L. (black pepper) being the best known for this purpose. The raw unripe *P. nigrum* fruit yields green pepper, the unripe cooked fruit gives black pepper, whilst the ripe fruit is used as white pepper. Several *Piper* spp. also have long histories of medicinal use in the treatment of a wide variety of medical disorders and conditions. The genus *Piper* may be particularly useful as antibacterial therapies. Indeed, the use of *Piper* spp. extracts in ancient Asian ethnopharmacological practices has been well documented and *P. nigrum* is a component of many traditional medicines still commonly in use.[10] *Piper guineense* Schumach. has similar therapeutic uses. Substantially less information is known about the Australian species *Piper novae-hollandiae* Miq., although its medicinal uses by the first Australians have been recorded.[11,12] Whilst it is mainly known for its tonic properties, it was also used in the treatment of some bacterial diseases, including STIs.[11]

P. novae-hollandiae (commonly known as giant pepper vine) is a vigorous climbing vine that grows attached to trees (Figure 1a) in rainforest regions in eastern Australia. *P. novae-hollandiae* has glossy green ovate leaves (up to 12cm by 9cm) (Figure 1b). Small cream coloured flowers form in April to August and develop into small oval shaped red drupes

(Figure 1c). There is a lack of information on the phytochemical composition of this species. However, the phytochemistry of other species has been reported[13] and is characterised by relatively high levels of mono- and sesquiterpenoids, including piperitone, (Figure 1d), myrcene (Figure 1e), α-pinene (Figure 1f), caryophyllene (Figure 1g), caryophyllene oxide (Figure 1h), β-selinene (Figure 1i) and viridiflorol (Figure 1j). Many of these have been reported to have potent antibacterial activity[14] and may therefore contribute to the antibacterial properties of other *Piper* spp. This study was undertaken to screen of *P. novae-hollandiae* leaf extracts for the ability to inhibit the growth of a panel of gram-positive and gram-negative bacterial pathogens.

MATERIALS AND METHODS

Collection of Plant Material and Extraction

Piper novae-hollandiae Miq. Leaves were harvested from wild plants in Toohey Forest Australia, washed in deionised water and processed within 4 hrs of collection. The leaves were dried in a Sunbeam food dehydrator and the dried material was ground to a coarse powder. Individual 1g masses of the dried plant material was extracted extensively in 50mL methanol (Ajax, AR grade) or deionised water for 24 hrs 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. novae-hollandiae* leaf extracts for the presence of saponins, phenolic compounds, flavonoids, phytosteroids, triterpenoids, cardiac glycosides, anthraquinones, tannins and alkaloids was conducted by standard assays.[15-17]

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 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. novae-hollandiae* leaf extracts was determined using a modified disc diffusion assay.[18-20] 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 2h before incubation at 37°C for 24h. 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 were used as a negative control.

Artemia franciscana Nauplii Toxicity Screening

Toxicity was tested using an adapted *Artemia franciscana* nauplii lethality assay.[21-23] 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 24h and 48h. At the completion of the 48h exposure period, the remaining live nauplii were sacrificed and the total number of nauplii in each well were counted and used to calculate the percentage 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. novae-hollandiae* 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 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, phytosterols, alkaloids and anthraquinones were completely absent or below the detection thresholds for these assays.

Antibacterial Activity

To determine the growth inhibitory activity of the *P. novae-hollandiae* leaf extracts, aliquots (10µL) of each extract were screened in the disc diffusion assay. The *P. novae-hollandiae* 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 therefore unable to determine MIC values for the extracts against any bacteria as they were completely ineffective at all concentrations tested.

Quantification of Toxicity

The toxicity of the *P. novae-hollandiae* leaf extracts was initially tested at 2mg/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 24h and thus were deemed to be non-toxic. Extracts with 24h LC_{50} values >1000µg/mL have previously been defined as non-toxic.[23] In contrast, the potassium dichromate positive control induced substantial mortality within 4h (results not shown), with 100% mortality induction seen by 24h. The mortality increased following exposure to the *P. novae-hollandiae* leaf extracts at 48h and was further increased following 72h 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.[6,7] A concurrent decrease in the discovery of new

Table 1: The Mass of Dried Extracted Material, the Concentration After Resuspension in Deionised Water and Qualitative Phytochemical Screenings of the *P. novae-hollandiae* Leaf Extracts.

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

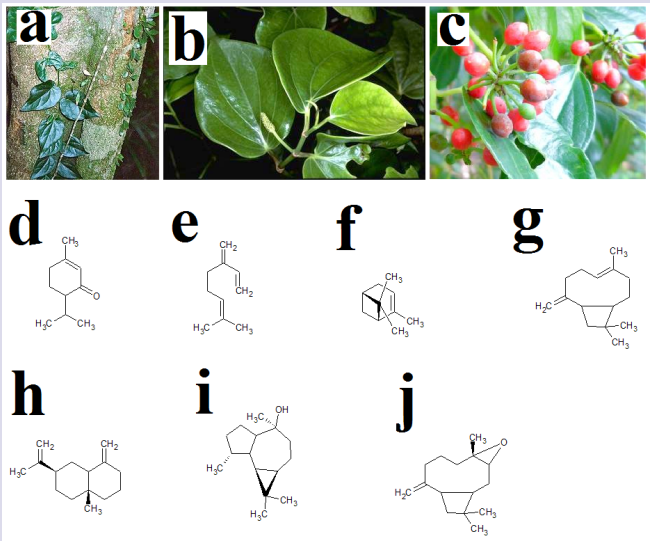

Figure 1: *P. novae-hollandiae* (a) plant attached to a tree, (b) leaves, (c) fruit and the structures of common terpenoids in other *Piper* spp.: (d) piperitone, (e) myrcene, (f) α-pinene, (g) caryophyllene, (h) caryophyllene oxide, (i) β-selinene and (j) viridiflorol.

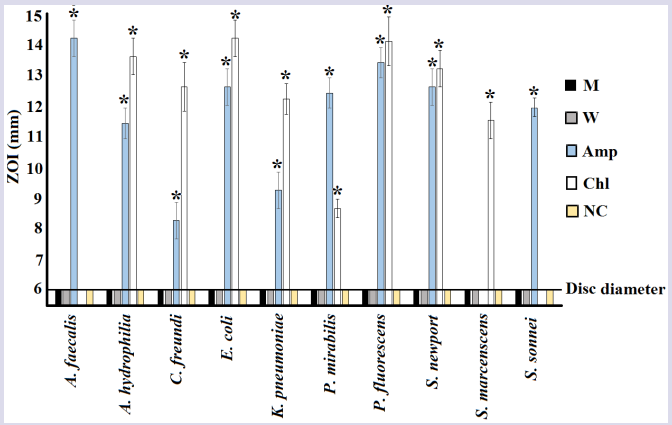

Figure 2: Growth Inhibitory activity of *P. novae-hollandiae* leaf extracts and reference antibiotics against gram-negative bacterial species measured as ZOIs (mm) ± SEM. Ampicillin (Amp) and chloramphenicol (Chl) standard discs (10µg) were used as positive controls. M = methanolic extract; W = aqueous 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. * indicates results that are significantly different to the untreated control (P<0.01).

antibiotic medicines by conventional strategies has increased interest in re-evaluating medicinal plants for new antibiotic chemotherapies.[24] *P. novae-hollandiae* was used by the first Australians to treat a number of diseases, some of which are caused by bacterial pathogens. Furthermore, as *P. novae-hollandiae* is taxonomically related to other species including *P. nigrum* and *P. guineense* that have antibacterial activity, it was deemed a viable target for antibacterial screening. Surprisingly, the *P. novae-hollandiae* extracts were completely 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

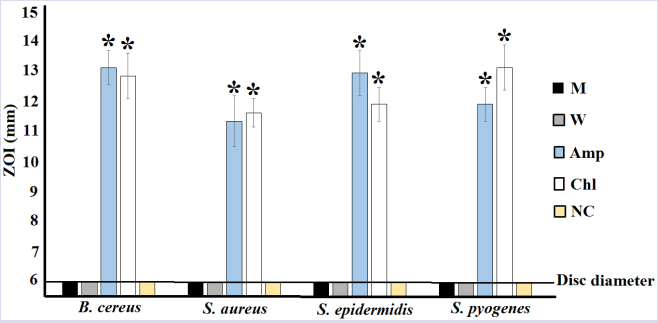

Figure 3: Growth inhibitory activity of *P. novae-hollandiae* leaf extracts and reference antibiotics against gram-positive bacterial species measured as ZOIs (mm) ± SEM. Ampicillin (Amp) and chloramphenicol (Chl) standard discs (10µg) were used as positive controls. M = methanolic extract; W = aqueous 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. * indicates results that are significantly different to the untreated control (*P*<0.01).

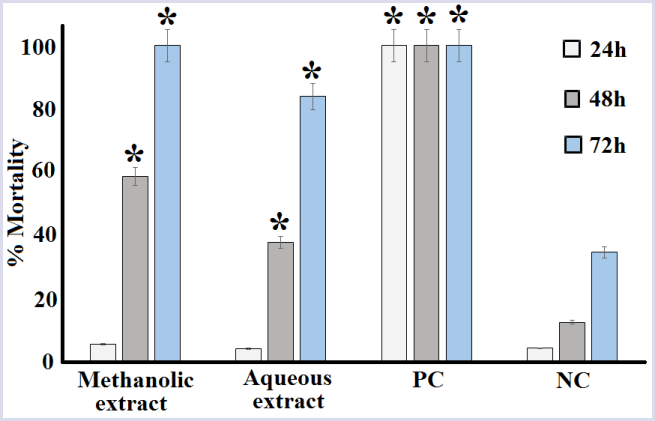

Figure 4: The lethality of the *P. novae-hollandiae* leaf extracts, potassium dichromate control (1000µg/mL) and seawater (negative control) following 24, 48 and 72 hrs of exposaure. All bioassays were performed three times in triplicate (n=9) and are expressed as mean ± SEM. * indicates results that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

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 relatively high levels of nonpolar terpenoid components, their activity may have been significantly under estimated in our study. Liquid dilution studies may have been better suited to screen the *P. novae-hollandiae* 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 hampered 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 have given a distorted view of the inhibitory potential of the extracts.

The findings reported here also indicate that the extracts examined were non-toxic (24h 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.[23] However, further studies are required to determine whether this is also true for the *P. novae-hollandiae* leaf extracts examined in these studies.

CONCLUSION

Methanolic and aqueous *P. novae-hollandiae* 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 *Piper* spp. with well known antibacterial properties. The extracts were non-toxic towards *Artemia* nauplii.

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CONFLICT OF INTEREST

The authors declare 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.

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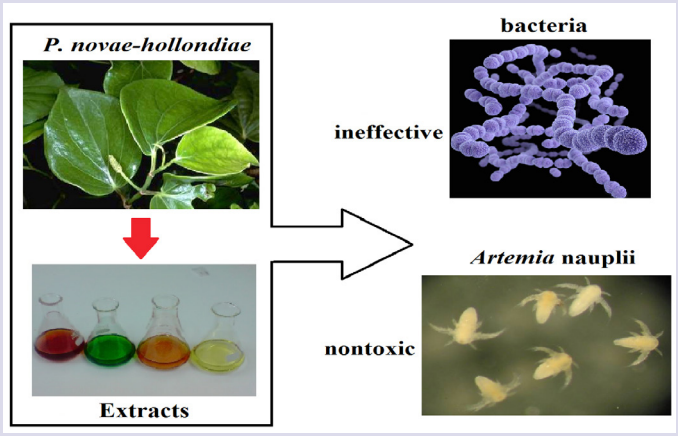
PICTORIAL ABSTRACT



SUMMARY

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

ABOUT AUTHORS

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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.

Lindiwe Nomathemba Mpala: completed 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.