Original Article

Pseudowintera colorata (Raoul) Dandy Hydro-Alcohol Leaf Extract Inhibits Bacterial Triggers of Some Autoimmune Inflammatory Diseases

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ABSTRACT

Introduction: *Pseudowintera colorata* (Raoul) Dandy is an evergreen shrub which is endemic to New Zealand. Decoctions, infusions and essential oils produced from the leaves were used traditionally to treat a variety of bacterial diseases. Despite this, *P. colorata* leaf extracts have not been rigorously examined for inhibitory activity against bacterial triggers of autoimmune inflammatory diseases. **Methods:** A *P. colorata* hydro-alcohol leaf extract was analysed for antioxidant capacity by the DPPH free radical scavenging assay. Growth inhibitory activities against bacterial species associated with initiating rheumatoid arthritis, ankylosing spondylitis, multiple sclerosis and rheumatic fever were determined by disc diffusion assay and quantified by MIC determination. Toxicity was determined by *Artemia franciscana* bioassay. **Results:** The *P. colorata* hydro-alcohol leaf extract displayed good DPPH radical scavenging activity, with an antioxidant capacity of 52 µg/mL ascorbic acid equivalency. The extract inhibited the growth of all of the bacterial triggers of autoimmune inflammatory diseases tested. It was a particularly potent inhibitor of *A. baylyi*, with MIC values as low as 427 µg/mL against the clinical strain. *P. mirabilis* and *P. vulgaris* were also highly susceptible to the *P. colorata* hydro-alcohol leaf extract, with MIC values of approximately 800-900 µg/mL against all strains. The extract was also a moderate growth inhibitor (MIC >1000 µg/mL) of all other bacterial strains tested. The extract was determined to be nontoxic in the *Artemia franciscana* nauplii bioassay with 24 h LC$_{50}$ values substantially >1000 µg/mL, indicating its safety for therapeutic use. **Conclusion:** The lack of toxicity of the *P. colorata* leaf extracts and their growth inhibitory bioactivity against a panel of bacterial triggers of autoimmune inflammatory diseases indicate their potential in the prevention and treatment of these diseases in genetically susceptible individuals.

Key words: Winteraceae, Horopito, Pepperwood, Autoimmune Inflammatory Disease, Rheumatoid Arthritis, Ankylosing Spondylitis.

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INTRODUCTION

Winteraceae is a family of flowering plants consisting of approximately 90 species of trees and shrubs divided into 5 genera (*Drimys, Pseudowintera, Takhtajania, Tasmannia* and *Zygogynum*).[1] The Winteraceae have developed as almost exclusively southern hemisphere plants, originating from precursor species on the Gondwanna super continent. Their current distribution ranges from the cool climate regions of the southern Australia and New Zealand through to the temperate and tropical regions of Borneo, Madagascar, Molucca, New Caledonia, Papua new Guinea, the Philippines and Southern and Central America, with the majority concentrated in Australasia and Malanesia.[2] The Winteraceae are characterized as woody evergreen plants with vesselless xylem and plicate carpels.[1,2] They generally have leaves without stipules. The leaves, which are almost always glabrous, have entire margins and are spirally arranged. Flowers are terminal, are generally condensed and can be either bisexual or unisexual. The fruit forms as a fleshy berry with a hard seed. Many Winteraceae species are fragrant and are often used to produce essential oils. *Zygogynum* is the largest Winteraceae genus with approximately 50 species.[1-3] Until recently, *Belliolum, Bubbia* and *Exospermum* were classified as distinct genera. Although most botanists now classify these as subgroupings of the *Zygogynum* genus. *Tasmannia* is the next largest genus with approximately 30 species.[4] *Drimys* consists of 6 species, *Pseudowintera* of 3 species, and *Takhtajania* consists of a single species.[4]

Members of family Winteraceae have been used for a broad range of dietary and medicinal purposes by a wide variety of ethnic and cultural groupings. The best documented of these is the South American species *Drimys winteri*. The stem and bark of this species has been used as a stimulant and as a tonic in traditional Brazilian medicinal systems.[5] They are also used for a wide variety of medicinal purposes including as an analgesic, as well as to treat diarrhoea, inflammation, and ulcers.[5] This species also has widespread usage in the treatment of scurvey due to its high antioxidant content.[6] Of the other Winteraceae species, several have a history of ethnobotanical usage, usually for purposes related to their high antioxidant contents and as a flavourant.

Pseudowintera colorata (Raoul) Dandy (horopito) is one of only 3 species within the genus *Pseudowintea* (the others are *P. axillaris* (J.R. Forst. & G. Forst.) Dandy and *P traversii* (Buchanan) Dandy). All *Pseudowintera* spp. are endemic to New Zealand. *P. colorata* is commonly known as mountain horopito or New Zealand pepperwood due to the hot, peppery flavour of its leaves which are yellow/green with red blotches (Figure 1a). It grows as a shrub or small tree (to 2.5 m high) with a multi-branched stem. The leaves are alternate with long pinnate lobes. The flowers appear in the warmer summer months and develop into fleshy red lobed berries (Figure 1b) during autumn. As with many of the other Winteraceae species, *P. colorata* leaves and berries have historical uses as food and as a medicinal plant. The leaves are particularly useful as a flavorant. Traditionally, the leaves are dried and ground into a powder which is used as a substitute for black pepper. All parts of the plant are reputed to have a

high antioxidant capacity and are rich in ascorbic acid.[6]

The New Zealand Maori people have used *P. colorata* medicinally to treat a variety of diseases including sexually transmitted infections, ringworm, chaffed skin and other skin diseases.[7] It is particularly effective against *Candida albicans* infections and is reputed to aid wound healing and to have antibacterial, anti-inflammatory anti-allergic and insecticide effects. Early European settlers in New Zealand also used *P. colorata* medicinally. The leaves were chewed or prepared as an infusion as a painkiller, as well as for the treatment of diarrhoea.[7] Furthermore, due to its high antioxidant capacity, *P. colorata* has potential for the treatment of multiple other chronic diseases. Oxidative stress is associated with many human diseases including cancer, chronic inflammation, atherosclerosis and Alzheimer's disease.[8] Individuals with elevated dietary intakes of non-enzymatic antioxidants such as vitamins A, C and E are less likely to suffer from some diseases including cancer and chronic inflammation.[9] Furthermore, several studies have demonstrated bacterial growth inhibitory and anti-inflammatory activities for several culinary plants with high antioxidant capacities, and have linked the bioactivities to their free radical scavenging activities.[10-14] As with many other Winteraceae, *P. colorata* leaves are particularly rich in the sesquiterpenoid polygodial (Figure 1c), which is largely responsible for its characteristic hot peppery flavour. This compound is also believed to be responsible for many of the medicinal properties of this species, as well as other members of the family Winteraceae.[6] The leaves are also a good source of other terpenoids including piperitone (Figure 1d).

Autoimmune inflammatory disorders (e.g. rheumatoid arthritis, ankylosing spondylitis, multiple sclerosis and rheumatic fever) are a group of debilitating conditions which afflict genetically susceptible individuals. There are no cures for any of these conditions and current treatment strategies instead aim to alleviate the symptoms (particularly pain, swelling and inflammation) with analgesics and anti-inflammatory agents and/or to modify the disease process through the use of disease modifying drugs. None of these treatments is ideal as prolonged usage of these drugs is often accompanied by unwanted side effects and toxicity.[15] There is a need to develop safe, more effective treatments which will not only alleviate the symptoms, but may also cure or prevent the disease. A greater understanding of the onset and progression of these disorders should greatly assist in more relevant drug discovery and development.

Autoimmune inflammatory disorders are triggered in susceptible individuals by specific microbial infections. Serotyping studies have identified several of the bacterial triggers of some of these conditions and the bacterial antigens responsible for the induction of an immune response (Table 1). A major microbial trigger of rheumatoid arthritis has been identified as *P. mirabilis*,[16] a normal part of the human gastrointestinal flora. Similarly, *K. pneumoniae* has been shown to initiate ankylosing spondylitis[17] and *A. baylyi* and *P. aeruginosa* have been linked with the onset of multiple sclerosis.[18] *Borelia burgforferi* is linked with Lyme disease.[19] Whilst microbial triggers have also been postulated for lupus, the specific causative agents are yet to be identified. Similarly, members of the Enterobacteriaceae family are associated with Graves' disease and Kawasaki syndrome and *Mycoplasma pneumoniae* is associated with several demyelinating diseases.[20] The development of antibiotic agents targeted at the specific bacterial triggers of autoimmune inflammatory disorders would enable afflicted individuals to target these microbes and thus prevent the onset of the disease and reduce the severity of the symptoms once the disease has progressed.

A re-examination of traditional medicines for the treatment of inflammation and rheumatic conditions is an attractive prospect as the antiseptic qualities of medicinal plants have been long recognised and recorded. Furthermore, there has recently been a revival of interest in herbal medications due to a perception that there is a lower incidence of adverse reactions to plant preparations compared to synthetic pharmaceuticals. Antimicrobial plant extracts with high antioxidant contents are particularly attractive as they may treat the symptoms of inflammation as well as blocking the microbial trigger and thus may have pluripotent effects. The current study was undertaken to examine the growth inhibitory activity of a *P. colorata* hydro-alcohol extract against bacterial species associated with the onset of four autoimmune diseases.

MATERIALS AND METHODS

Pseudowintera colorata extract

Pseudowintera colorata (Raoul) Dandy hydro-alcohol leaf extract was obtained from Optimal RX, Australia as a 45 % ethanol extract. A 10 mL volume of the extract was dried by rotary evaporation in an Eppendorf concentrator 5301 and subsequently dissolved in 10 mL sterile deionised water (containing 1 % DMSO). The extract was passed through 0.22 μm filter (Sarstedt) and stored at 4 °C until use.

Qualitative phytochemical studies

Phytochemical analysis of the *P. colorata* hydro-alcohol leaf extract for the presence of saponins, phenolic compounds, flavonoids, phytosteroids, triterpenoids, cardiac glycosides, anthraquinones, tannins and alkaloids was conducted by previously described assays.[23;24;25]

Antioxidant capacity determination

The antioxidant capacity of each sample was assessed using the DPPH free radical scavenging method.[26] with modifications. Briefly, DPPH solution was prepared fresh each day as a 400 μM solution by dissolving DPPH (Sigma) in AR grade methanol (Ajax, Australia). The initial absorbance of the DPPH solution was measured at 515 nm using a Molecular Devices, Spectra Max M3 plate reader and did not change significantly throughout the assay period. A 2 mL aliquot of each extract was evaporated and the residue was resuspended in 2 mL of methanol. Each extract was added to a 96-well plate in amounts of 5, 10, 25, 50, 75 μL in triplicate. Methanol was added to each well to give a volume of 225 μL. A volume of 75 μL of the fresh DPPH solution was added to each
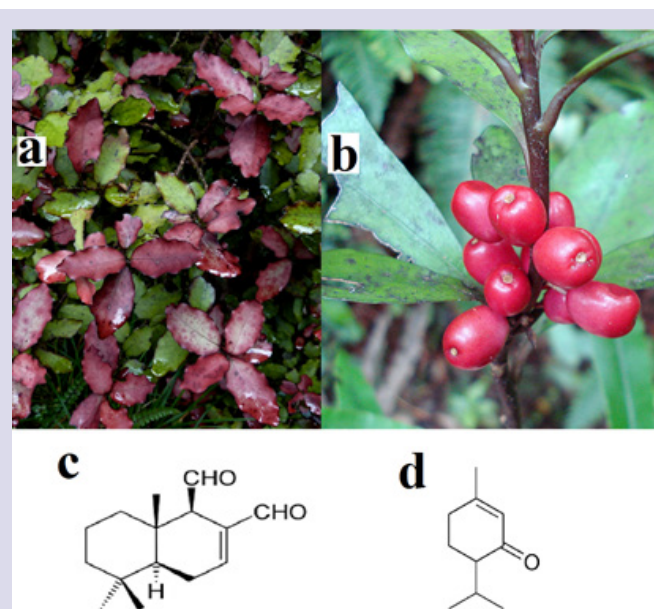
Figure 1: *P. colorata* (a) leaves and (b) fruit, as well as the chemical structures of (c) polygodial and, (d) piperitone.

Table 1: The bacterial triggers of some autoimmune inflammatory diseases examined in this study as well as the bacterial antigen and host susceptibility antigen sequences.

Disease	Bacterial Trigger	Bacterial Antigen	Bacterial Sequence	Host Antigen	Host Sequence	References
Rheumatoid arthritis	*Proteus mirabilis* and possibly also other *Proteus* spp.	haemolysin	ESRRAL	MHC class 2 allele HLA-DR4	EQ/KRRAA	[16-22]
		urease	IRRET	type XI collagen	LRREI	[15-21]
Ankylosing spondylitis	*Klebsiella pneumoniae*	nitrogenase reductase enzyme	QTDRED	MHC class 1 allele HLA-B27	QTDRED	[16]
		pullulanase	DRDE	MHC class 1 allele HLA-B27	DRED	[16]
		pullulanase	GxP	types I, III and IV collagen	GxP	[16]
Multiple sclerosis	*Pseudomonas aeriginosa*	ϒ-CMLD	TRHAYG	Myelin-neuronal antigen MBP	SRFSYG	[17]
	Acinetobacter spp.	4-CMLD	SRFAYG	Myelin-neuronal antigen MBP	SRFSYG	[17]
		3-OACT-A	LTRAGK	Myelin-neuronal antigen MOG	LYRDGK	[17]
		Acinteobacter regulatory protein	*KKVEEI	Neurofilament-M protein	*KKVEEI	[17-19]

MOG = myelin oligodendrocyte glycoprotein; MBP = myelin basic protein; 4-CMLD = 4-carboxy-muconolactone decarboxylase; 3-OACT-A = 3-oxoadipate CoA-transferase; ϒ-CMLD = ϒ-carboxy-muconolactone decarboxylase. * indicates the sequence likely to be responsible for crossreactivity although this is yet to be confirmed.

well to give a total reaction volume of 300 μL. A blank of each extract concentration, methanol solvent, and DPPH was also performed in triplicate. Ascorbic acid was prepared fresh and examined across the range 0-25 μg per well as a reference and the absorbance were recorded at 515 nm. All tests were performed in triplicate and triplicate controls were included on each plate. The antioxidant capacity based on DPPH free radical scavenging ability was determined for each extract and expressed as μg ascorbic acid equivalents per mL of extract.

Antibacterial screening

Test microorganisms

All media was supplied by Oxoid Ltd. Reference strains of *Proteus mirabilis* (ATCC21721), *Proteus vulgaris* (ATCC21719), *Klebsiella pneumoniae* (ATCC31488), *Acinitobacter baylyi* (ATCC33304) and *Pseudomonas aeruginosa* (ATCC39324) were purchased from American Tissue Culture Collection, USA. All other clinical microbial strains were obtained from the School of Natural Sciences teaching laboratory, Griffith University. All stock cultures were subcultured and maintained in nutrient broth at 4 °C.

Evaluation of antimicrobial activity

Antimicrobial activity of all plant extracts was determined using a modified disc diffusion assay.[27-29] Briefly, 100 μL of each bacterial culture was grown in 10 mL of fresh nutrient broth until they reached a count of ~10^8 cells/mL. A volume of 100 μL of the bacterial suspension was spread onto nutrient agar plates and extracts were tested for antibacterial activity using 5 mm sterilised filter paper discs. Discs were infused with 10

μL of the plant extracts, allowed to dry and placed onto the inoculated plates. The plates were allowed to stand at 4°C for 2 h before incubation at 30°C for 24 h. The diameters of the inhibition zones were measured to the closest whole millimetre. Each assay was performed in at least triplicate. Mean values (± SEM) are reported in this study. Standard discs of ampicillin (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.

Minimum inhibitory concentration (MIC) determination

The minimum inhibitory concentration (MIC) of each extract against susceptible bacteria was determined as previously described.[30,31] Briefly, the *P. colorata* hydro-alcohol leaf extract was diluted in deionised water and tested across a range of concentrations. Discs were infused with 10 μL of the test dilutions, allowed to dry and placed onto inoculated plates. The assay was completed as outlined above and graphs of the zone of inhibition versus concentration were plotted for each extract. Linear regression was used to determine the MIC values of each extract.

Toxicity screening

Reference toxin for toxicity screening

Potassium dichromate ($K_2Cr_2O_7$) (AR grade, Chem-Supply, Australia) was prepared as a 4 mg/mL solution in distilled water and was serially diluted in artificial seawater for use in the *Artemia franciscana* nauplii bioassay.

Artemia franciscana nauplii toxicity screening

Toxicity was tested using an adapted *Artemia franciscana* nauplii lethality assay.[32-34] Briefly, 400 µL of seawater containing approximately 49 (mean 48.8, n = 75, SD 10.7) *A. franciscana* nauplii were added to wells of a 48 well plate and immediately used for bioassay. A volume of 400 µL of diluted plant extracts or the reference toxin were transferred to the wells and incubated at 25 ± 1ºC under artificial light (1000 Lux). A 400 µL seawater negative control was run in 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 dead if no movement of the appendages was detected within 10 seconds. After 24 h, all nauplii were sacrificed and counted to determine the total % mortality per well. The LC_{50} with 95% confidence limits for each treatment was determined using probit analysis.

Statistical analysis

Data are expressed as the mean ± SEM of at least three independent experiments.

RESULTS

Qualitative phytochemical screening and antioxidant capacity determination

A 10 mL volume of the 45% *P. colorata* hydro-alcohol leaf extract was dried by rotary evaporation in an Eppendorf concentrator 5301. The resultant dry extract was subsequently redissolved in 10 mL deionised water (containing 1% DMSO). Qualitative phytochemical studies of the *P. colorata* leaf extract showed high levels of phenolics, saponins and flavonoids, and low levels of triterpenoids and tannins (Table 2). An antioxidant capacity (expressed as ascorbic acid equivalence) of 52 µg ascorbic acid equivalence per mL of *P. colorata* hydro-alcohol leaf extract was also calculated.

Antimicrobial activity

To determine the growth inhibitory activity of the *P. colorata* hydro-alcohol leaf extract against the panel of pathogenic bacteria, 10 µL of the extract was screened in the disc diffusion assay. Both reference and clinical strains of *Proteus mirabilis* were inhibited by the *P. colorata* hydro-alcohol leaf extract (Figure 2). However, inhibition was weak (as judged by the zones of inhibition), with inhibition zones of 7.9 ± .3 mm and 8.2

± 0.4 mm measured for the reference and clinical strains respectively. It is noteworthy that these *P. mirabilis* strains were also relatively resistant to the ampicillin control (inhibition zone of 8.3 ± 0.3 mm). This is consistent with the susceptibility profiles of these bacterial strains previously reported in other studies.[10-12] In contrast, both *P. mirabilis* strains were highly susceptible to chloramphenicol (10 µg: inhibition zones of 11.3 ± 0.6 mm and 12.0 ± 0.5 mm for the reference and clinical strains respectively). Similarly, *P. vulgaris* growth was also susceptible to inhibition by the *P. colorata* leaf hydro-alcohol extract (Figure 3). Indeed, an 8.3 ± 0.3mm zone of inhibition was measured for *P. vulgaris* growth. This indicates that *P. vulgaris* is more susceptible to the extract than *P. mirabilis* was. This inhibition also compares well with the ampicillin control (inhibition zone of 7.9 ± 0.3 mm) but is substantially less potent than the chloramphenicol control (13.7 ± 0.6 mm).

The growth of both reference and clinical *K. pneumoniae* strains was inhibited by the *P. colorata* leaf hydro-alcohol extract (Figure 4), albeit with smaller zones of inhibition compared to the growth inhibition of the *Proteus* spp. Interestingly, both *K. pneumoniae* strains were completely resistant to ampicillin and had only relatively small zones of inhibition when tested against chloramphenicol, indicating that both *K. pneumoniae* strains were antibiotic resistant strains.

In contrast, potent *A. baylyi* growth inhibition was noted for the *P. colorata* leaf hydro-alcohol extract (Figure 5 zones of inhibition of 9.6 ± 0.3 mm and 9.0 ± 0.5 mm against the reference and clinical strains respectively). This is a particularly significant result as the ampicillin control only inhibited *A. baylyi* growth with low efficacy, indicating that the *A. baylyi* strain tested in our study was ampicillin resistant. Indeed, previous studies from our group have also shown the low susceptibility of these *A. baylyi* strains to a number of conventional antibiotics.[10,11] In contrast, both *A. baylyi* strains were highly susceptibile to chloramphenicol (12.6 ± 0.3 mm and 11.8 ± 0.4 mm against the reference and clinical strains respectively). In contrast, only relatively weak *P. aeruginosa* growth inhibition was noted for the *P. colorata* leaf hydro-alcohol extracts (Figure 6 zone of inhibition <7 mm for both strains). However, this compares well to the ampicillin (6.4 ± 0.3 mm for the reference strain) and chloramphenicol controls (7.8 ± 0.4 mm and 7.6 ± 0.3 mm for the reference and clinical strains respectively).

Table 2: Qualitative phytochemical screenings and antioxidant capacities of the *P. colorata* hydro-alcohol leaf extract.

DPPH antioxidant activity	Total Phenolics	Water Soluble Phenolics	Water Insoluble Phenolics	Cardiac Glycosides	Saponins	Triterpenes	Phyto steroids	Alkaloids (Mayer Test)	Alkaloids (Wagner Test)	Flavonoids	Tannins	Free Anthraquinones	Combined Anthraquinones
52	+++	++	++	-	++	+	-	-	-	++	+	-	-

+++ indicates a large response; ++ indicates a moderate response; + indicates a minor response; - indicates no response in the assay. Antioxidant capacity was determined by DPPH reduction and is expressed as µg ascorbic acid equivalence per mL hydro-alcohol extract.
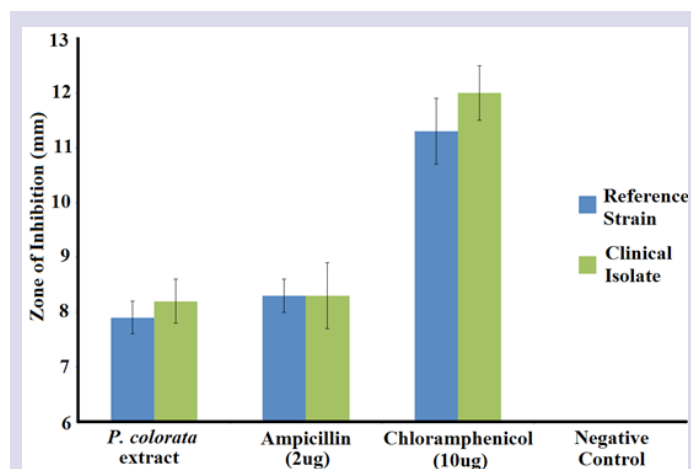
Figure 2: Growth inhibitory activity of *P. colorata* leaf hydro-alcohol extract and the ampicillin (2 µg) and chloramphenicol (10 µg) controls against *P. mirabilis* measured as zones of inhibition (mm). The blue bars represent the inhibitory activity against the reference strain (ATCC:21721) and the green bars represent the zones of inhibition against the clinical strain. All determinations were in at least triplicate and the results are expressed as mean zones of inhibition (mm) ± SEM.

The *P. colorata* leaf hydro-alcohol extract also inhibited *S. pyogenes* growth (Figure 7 zone of inhibition = 7.8 ± 0.4 mm). This is a significant result as the ampicillin control only inhibited *S. pyogenes* growth with low efficacy (6.6 ± 0.3 mm), indicating that the *S. pyogenes* strain tested in our study was ampicillin resistant. This supports previous studies which have also reported low susceptibility of this *S. pyogenes* strains to several conventional antibiotics.[10-12] In contrast, *S. pyogenes* was highly susceptible to chloramphenicol, with an inhibition zone of 10.2 ± 0.4 mm)

The antimicrobial efficacy was further quantified by determining the MIC values for each extract against the microbial species which were determined to be susceptible. The *P. colorata* leaf hydro-alcohol extract was a potent growth inhibitor of several bacterial species/strains (as judged by MIC; Table 3). *A. baylyi* was the most susceptible bacteria to the *P. colorata* leaf hydro-alcohol extract, with MIC values as low as 427 µg/mL (approximately 4 µg infused into the disc) recorded against this bacteria. The extract was also a moderate inhibitor of both *P. aeruginosa* strains, with MIC's <2500 µg/mL. As both *A. baylyi* and *P. aeruginosa* are bacterial triggers of multiple sclerosis in genetically susceptible people, the *P. colorata* leaf hydro-alcohol extract has potential in the prevention and treatment of multiple sclerosis

The MIC values determined for the *P. colorata* leaf hydro-alcohol extract against the reference and clinical *P. mirabilis* strains also indicate potent growth inhibition (933 and 784 µg/mL respectively). The extract was a similarly potent inhibitor of *P. vulgaris* growth, with an MIC of 805 µg/mL. *Proteus* spp. infections (particularly *P. mirabilis* infections) are common causes of urinary tract infections and have also been identified as triggers of rheumatoid arthritis.[16,17] Therefore, the *P. colorata* leaf hydro-alcohol extract has potential for the prevention and treatment of these diseases in genetically susceptible individuals. In contrast, MICs >1000 µg/mL were determined for all other bacterial strains, indicating only moderate growth inhibition for these bacteria.

Quantification of toxicity

The toxicity of the *P. colorata* leaf hydro-alcohol extract was initially tested in the *Artemia franciscana* nauplii bioassay at a concentration of 2000 µg/mL (Figure 8). For comparison, the reference toxin potassium

dichromate (1000 µg/mL) was also tested in the bioassay. The potassium dichromate reference toxin was rapid in its onset of mortality, inducing nauplii death within the first 3 hours of exposure and 100 % mortality was evident following 4-5 hours (results not shown). Whilst the extract also induced 100 % mortality, a greater period of exposure was required to achieve 100 % mortality. Indeed, less than 10 % mortality was noted following 12 hours exposure and 24 hours was required to kill all of the nauplii (unpublished results). The *P. colorata* leaf hydro-alcohol extract therefore was deemed to be toxic (based on the screening study mortality) and the toxicity was further evaluated to determine the LC$_{50}$.

To further quantify the effect of toxin concentration on the induction of mortality, the extract was serially diluted in artificial seawater to test across a range of concentrations in the *Artemia* nauplii bioassay (Table 3). For comparison, serial dilutions of potassium dichromate were also tested. The extract was determined to be nontoxic, with LC$_{50}$ values substantially greater than 1000 µg/mL following 24 h exposure. Extracts with an LC$_{50}$ of greater than 1000 µg/mL towards *Artemia* nauplii have previously been defined as being nontoxic.[34]

DISCUSSION

Plant remedies are becoming increasingly sought after in the treatment of a myriad of diseases and disorders due both to their perception of greater safety than synthetic drugs and the failure of current drug regimens to effectively treat many diseases. This is especially true for the autoimmune inflammatory diseases. The current treatments utilising disease modifying anti-rheumatic drugs (DMARDs) to alleviate the symptoms of these diseases and/or alter the disease progression are not entirely effective and have been associated with numerous adverse effects.[15] Furthermore, many of the current treatments aimed to treat the symptoms without addressing the underlying causes and pathogenic mechanisms. Therefore, whilst these treatments may alleviate pain, redness, swelling etc., they do not address the tissue degeneration which occurs as a consequence of the disease progression. A better understanding of the mechanisms for initiation and progression of the autoimmune inflammatory diseases is important for developing new drugs to target specific processes and thus more effectively treat autoimmune inflammatory diseases.
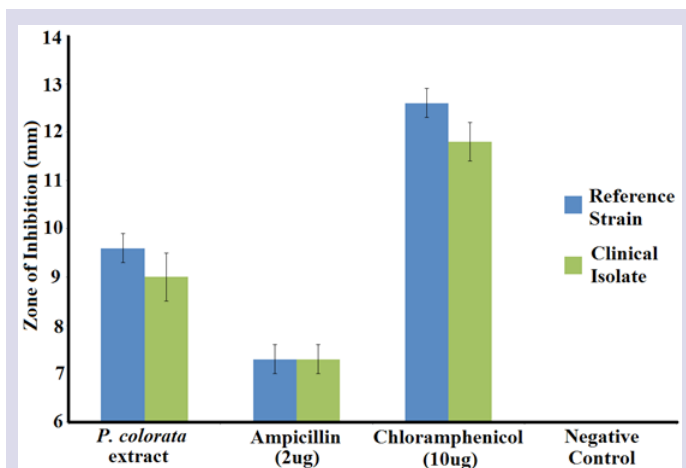
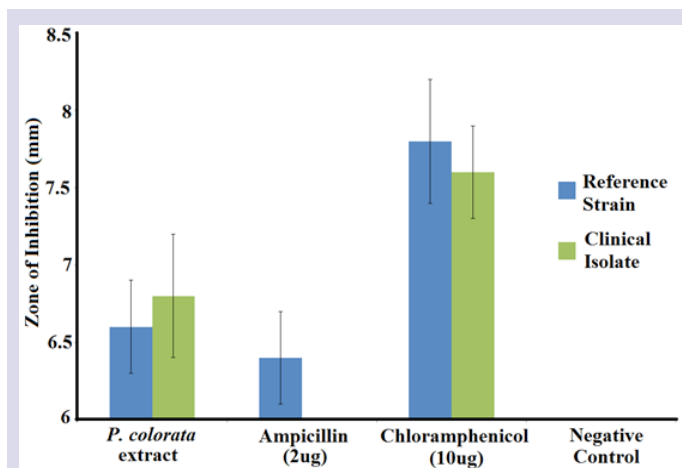Our study examined the ability of a commercial *P. colorata* leaf hydro-
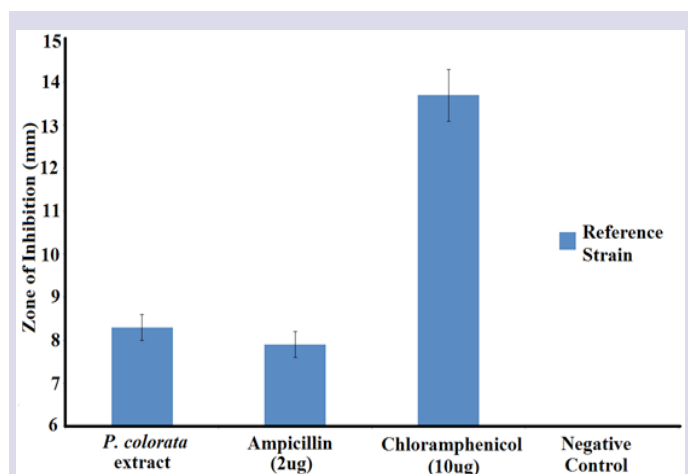
Figure 3: Growth inhibitory activity of *P. colorata* leaf hydro-alcohol extract and the ampicillin (2 µg) and chloramphenicol (10 µg) controls against *P. vulgaris* (ATCC:21719) measured as zones of inhibition (mm). All determinations were in at least triplicate and the results are expressed as mean zones of inhibition (mm) ± SEM.
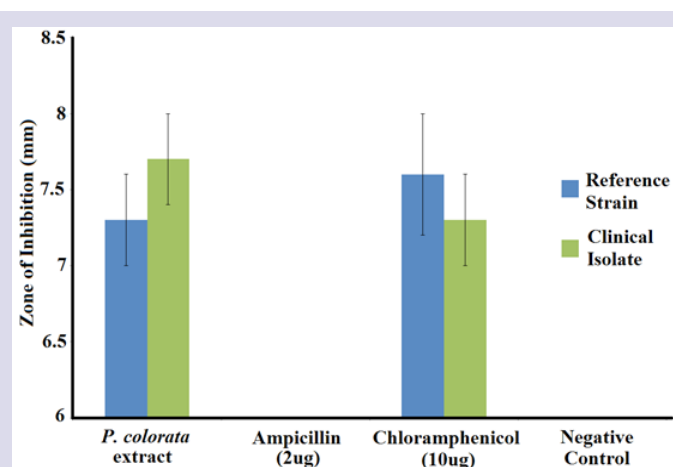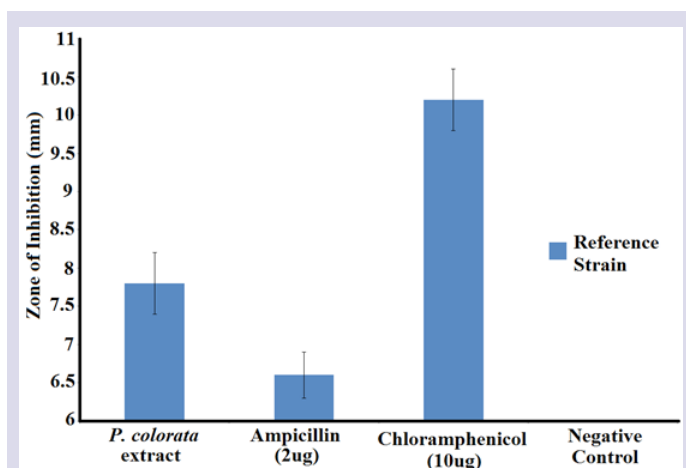

Figure 4: Growth inhibitory activity of *P. colorata* leaf hydro-alcohol extract and the ampicillin (2 µg) and chloramphenicol (10 µg) controls against *K. pneumoniae* measured as zones of inhibition (mm). The blue bars represent the inhibitory activity against the reference strain (ATCC:31488) and the green bars represent the zones of inhibition against the clinical strain. All determinations were in at least triplicate and the results are expressed as mean zones of inhibition (mm) ± SEM.

Figure 5: Growth inhibitory activity of *P. colorata* leaf hydro-alcohol extract and the ampicillin (2 µg) and chloramphenicol (10 µg) controls against *A. baylyi* measured as zones of inhibition (mm). The blue bars represent the inhibitory activity against the reference strain (ATCC:33304) and the green bars represent the zones of inhibition against the clinical strain. All determinations were in at least triplicate and the results are expressed as mean zones of inhibition (mm) ± SEM.

Figure 6: Growth inhibitory activity of *P. colorata* leaf hydro-alcohol extract and the ampicillin (2 µg) and chloramphenicol (10 µg) controls against *P. aeruginosa* measured as zones of inhibition (mm). The blue bars represent the inhibitory activity against the reference strain (ATCC:39324) and the green bars represent the zones of inhibition against the clinical strain. All determinations were in at least triplicate and the results are expressed as mean zones of inhibition (mm) ± SEM.

Figure 7: Growth inhibitory activity of *P. colorata* leaf hydro-alcohol extract and the ampicillin (2 µg) and chloramphenicol (10 µg) controls against *S. pyogenes* (clinical isolate) measured as zones of inhibition (mm). All determinations were in at least triplicate and the results are expressed as mean zones of inhibition (mm) ± SEM.

Table 3: Minimum bacterial growth inhibitory concentration (µg/mL) of the *P. colorata* hydro-alcohol and LC_{50} values (µg/mL) in the Artemia nauplii bioassay.

Bacterial Species	Strain	MIC (µg/mL)	LC_{50} (µg/mL)	
			24 h	48 h
P. mirabilis	ATCC:21721	933	NT	NT
	Clinical isolate	784	NT	NT
P. vulgaris	ATCC:21719	805	NT	NT
K. pneumoniae	ATCC:213488	1570	NT	NT
	Clinical isolate	1358	NT	NT
A. baylyi	ATCC:33304	635	NT	NT
	Clinical isolate	427	NT	NT
P. aeruginosa	ATCC:39324	2366	NT	NT
	Clinical isolate	2180	NT	NT
S. pyogenes	Clinical isolate	1446	NT	NT
A. franciscana	nauplii	NT	1365	1147

NT = not tested

alcohol extract to inhibit the growth of some bacterial triggers of autoimmune inflammatory diseases. The extract was a particularly potent inhibitor of *P. mirabilis*, *P. vulgaris* and *A. baylyi*, with MIC values substantially <1000 µg/mL. As *P. mirabilis* (and possibly *P. vulgaris*) can trigger rheumatoid arthritis in genetically susceptible individuals.[16,17] these extracts have potential for the development of rheumatoid arthritis inhibitory therapies. Similarly, as *A. baylyi* is a trigger of multiple sclerosis, the *P. colorata* leaf hydro-alcohol extract also has potential in prevention and treatment of this disease. *P. aeruginosa* may also trigger multiple sclerosis in some people. Interestingly, the extract was also inhibited the growth of this bacterium (albeit with substantially lower potency), further attesting to its therapeutic properties in people genetically sus-

ceptible to multiple sclerosis. Furthermore, the extract also displayed moderate *K. pneumoniae* and *S. pyogenes* growth inhibitory properties, As *K. pneumoniae* and *S. pyogenes* can trigger ankylosing spondylitis and rheumatic fever respectively in genetically susceptible individuals,[16] the *P. colorata* leaf hydro-alcohol extract may also be useful in the prevention and treatment of these diseases.

Whilst a detailed investigation of the phytochemistry of the *P. colorata* leaf hydro-alcohol extract was beyond the scope of our study, qualitative screening studies were used to determine the classes of compounds present. The extract contained relatively high levels of total phenolics, flavonoids and saponins. Lower levels of tannins and triterpenoids were also detected. It is likely that these and other phytochemical classes may
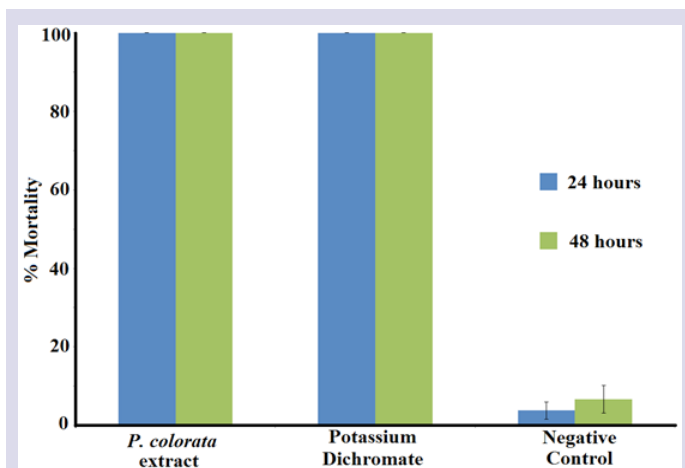
Figure 8: The lethality of *P. colorata* leaf hydro-alcohol extract (2000 µg/mL), potassium dichromate (1000 µg/mL) and a seawater control. Blue bars represent the % mortality following 24 h exposure to the extract/toxin. Green bars represent the % mortality following 48 h exposure to the extract/toxin. All bioassays were performed in at least triplicate and are expressed as mean ± SEM.

contribute to the growth inhibitory properties of these extracts. Many studies have reported potent antibacterial activities for a wide variety of these compounds.[6] Further phytochemical evaluation studies and bio-activity driven isolation of active components is required to evaluate the mechanism of bacterial growth inhibition.

The findings reported here also demonstrate that the *P. colorata* leaf hydro-alcohol extract was nontoxic towards *Artemia franciscana* nauplii, with 24h and 48h LC_{50} values of 1365 and 1147 µg/mL respectively. Extracts with LC_{50} values > 1000 µg/mL towards *Artemia* nauplii are defined as being nontoxic.[34] Whilst our preliminary toxicity studies indicate that the *P. colorata* leaf extract may be safe for therapeutic use, studies using human cell lines are required to further evaluate the safety of these extracts. Furthermore, whilst these studies have demonstrated the potential of the *P. colorata* leaf hydro-alcohol extract in the development of future antibiotic chemotherapeutics for the prevention and treatment of autoimmune diseases, more work is required to isolate the inhibitory components and to determine the mechanism of inhibition.

CONCLUSION

The results of this study demonstrate the potential of the *P. colorata* leaf hydro-alcohol extract for the prevention and treatment of rheumatoid arthritis, ankylosing spondylitis, multiple sclerosis and rheumatic fever in genetically susceptible people. Furthermore, the lack of toxicity indicates than the extract is safe for therapeutic use. Further studies aimed at the purification and identification of bioactive components are required to examine the mechanisms of action of these agents.

ACKNOWLEDGEMENT

CONFLICTS OF INTEREST

The authors report no conflicts of interest.

ABBREVIATIONS

DMSO: Dimethyl sulfoxide; LC_{50}: The concentration required to achieve 50 % mortality; MIC: minimum inhibitory concentration

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GRAPHICAL ABSTRACT
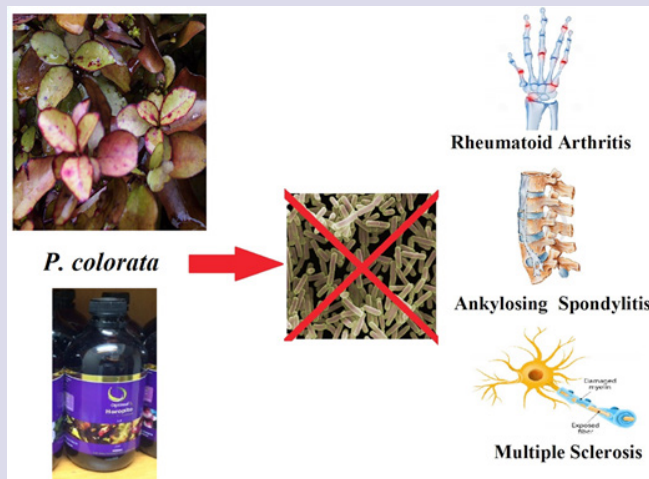
SUMMARY

- *P. colorata* leaf extract was a potent inhibitor of *A. baylyi*, *P. mirabilis* and *P. vulgaris* growth, with MIC values as low as 427 µg/mL.
- The extract was also a moderate inhibitor of *K. pneumoniae*, *P. aeruginosa* and *S. pyogenes* growth (MIC values 1500-2500 µg/mL).
- The *P. colorata* leaf extract was nontoxic in the *Artemia* nauplii bioassay.
- The *P. colorata* leaf extract has potential in the prevention and treatment of rheumatoid arthritis, ankylosing spondylitis, multiple sclerosis and rheumatic fever.

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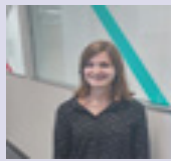
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