

Duboisia leichhardtii (F.Muell.) Extracts Inhibit The Growth of Bacterial Triggers of Selected Autoimmune Inflammatory Diseases

Ian Edwin Cock^{1,2}

ABSTRACT

Introduction: *Duboisia leichhardtii* F.Muell. is a medium to large tree which is native to subtropical regions of eastern Australia. *Duboisia* spp. contain a number of psychoactive tropane and pyrrolidine alkaloids with reported antibacterial activity. Despite this, *D. leichhardtii* leaf extracts have not been rigorously examined for growth inhibitory properties against many bacteria, including the bacterial triggers of autoimmune inflammatory diseases. **Methods:** The antimicrobial activity of *D. leichhardtii* leaf solvent extracts was investigated by disc diffusion and growth time course assays against a panel of bacterial triggers of autoimmune diseases. The growth inhibitory activity was further quantified by MIC determination and growth time course assays. Toxicity was determined using the *Artemia franciscana* nauplii bioassay. **Results:** Methanolic and aqueous *D. leichhardtii* leaf solvent extracts were potent inhibitors of the bacterial triggers of rheumatoid arthritis and ankylosing spondylitis. The methanolic extract displayed the most potent bacterial growth inhibitory activity. It was particularly potent against *P. mirabilis* (MICs of 85 and 116 µg/mL against reference and clinical strains respectively) and *P. vulgaris* (MIC of 187 µg/mL). The methanolic extract was also a good inhibitor of *K. pneumoniae* growth (MICs of 143 and 118 µg/mL against reference and clinical strains respectively). The aqueous and ethyl acetate extracts were also potent bacterial growth inhibitors, albeit with higher MIC values. The antibacterial activity of the methanolic and aqueous *D. leichhardtii* leaf extracts were further investigated by growth time course assays which showed significant growth inhibition in cultures of *P. mirabilis* and *K. pneumoniae* within 1 h of exposure. All extracts were determined to be nontoxic in the *Artemia franciscana* nauplii bioassay, indicating their safety for use in preventing these autoimmune inflammatory diseases. **Conclusions:** The lack of toxicity of the *D. leichhardtii* leaf extracts and their growth inhibitory bioactivity against the bacterial triggers of rheumatoid arthritis and ankylosing spondylitis indicate their potential in the development of new therapies targeting the onset of these diseases.

Key words: Solanaceae, Corkwood, Rheumatoid Arthritis, Ankylosing Spondylitis, Multiple Sclerosis, Rheumatic Heart Disease, Scopolamine, Hyoscyamine.

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INTRODUCTION

Autoimmune inflammatory disorders (e.g. rheumatoid arthritis, ankylosing spondylitis, multiple sclerosis and rheumatic heart disease) are a group of debilitating conditions which may afflict genetically susceptible individuals. There are no cures for any of these diseases. Instead, current treatment strategies 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 drug therapy is often accompanied by unwanted side effects and toxicity.¹ There is a need to develop safer, more effective treatments for these conditions which 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 disorders are triggered in susceptible individuals by specific microbial infections. Recent sero-typing studies have identified the microbial triggers of some of these conditions.²⁻⁷ A microbial trigger of rheumatoid arthritis has been identified as *Proteus mirabilis*.²⁻⁴ Similarly, *Klebsiella pneumoniae* has been shown to initiate ankylosing spondylitis.^{2,5-7} *Acinetobacter baylyi* and *Pseudomonas aeruginosa* have been linked with the onset of multiple sclerosis.⁸ *Borrelia burgdorferi* is linked with Lyme disease.⁹ *Streptococcus pyogenes* can induce rheumatic heart disease in genetically susceptible individuals.^{10,11} Members of the Enterobacteriaceae family are associated with Graves' disease and Kawasaki syndrome and *Mycoplasma pneumoniae* is associated with several demyelinating diseases.¹² The development of

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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 autoimmune inflammatory conditions is an attractive prospect as the antiseptic and anti-inflammatory qualities of medicinal plants have been long recognised. 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. *Duboisia leichhardtii* (F.Muell.) (synonym: *Anthocercis leichhardtii* (F.Muell.); commonly known as corkwood, poisoness corkwood, yellow basswood, Queensland *Duboisia*) is a medium to large tree of the family Solonaceae. It grows in subtropical regions of eastern Australia, particularly in South East Queensland and Northern New South Wales. The tree grows to 12 m tall and has thick, corky bark from which its common name is derived. The leaves are narrowly elliptical (Figure 1a; 5–10 cm long). White flowers (Figure 1b) are produced and develop into globose berries (5 mm in diameter). The use of several *Duboisia* spp. in traditional Australian bush medicine to treat gastrointestinal maladies and eye disease has been documented^{13–15}. Furthermore, the leaves, flowers and twigs of several *Duboisia* spp. may have been used by the first Australians as sedatives and hypnotics,^{13–15} although conclusive documentation is lacking.

The phytochemistry of *D. leichhardtii* has not been extensively reported. Indeed, most of the interest in *Duboisia* spp. has focussed on the tropane alkaloids scopolamine (Figure 1e), hyoscyamine (Figure 1c) and norhyoscyamine (Figure 1d), and the pyrrolidine alkaloids (Figure 1f) nicotine and nornicotine (Figure 1g). These compounds are common to all *Duboisia* spp. (although the relative levels differ between species/locations).¹⁵ These alkaloids have attracted interest as all have potent psychoactive properties via acetylcholine receptor antagonism. Thus, they are indicated as sedatives and hypnotics (or stimulants at low doses).¹⁵ However, these alkaloids are also toxic, so should be used with caution. Interestingly, potent antibacterial activity has also been reported for some of these compounds. Scopolamine is a potent inhibitor of the growth of multiple bacteria, with MIC values as low as 2 µg/mL against some bacteria.¹⁶ Similarly, nicotine inhibits the growth of *E. coli*, *P. aeruginosa* and *S. faecalis*.¹⁷ However, MIC values were not determined in that study, making it impossible to compare the efficacy with other studies. Despite these previous studies, examination of the antibacterial properties and phytochemistry of *D. leichhardtii* leaves is rudimentary. The current report was undertaken to screen *D. leichhardtii* leaf extracts for growth inhibitory properties against bacterial triggers of selected autoimmune inflammatory diseases.

MATERIALS AND METHODS.

Plant collection and extraction.

Duboisia leichhardtii (F.Muell.) leaves were obtained from Philip Cameron, senior botanical officer, Mt Cootha Botanical Gardens, Brisbane, Australia. The leaf samples were dried in a Sunbeam food dehydrator and stored at -30 °C. Prior to use, the dried leaves were freshly ground to a coarse powder and 1 g quantities were weighed into separate tubes. A volume of 50 mL methanol, sterile deionised water, ethyl acetate, chloroform or hexane was added to individual tubes and extracted for 24 hours at 4 °C with gentle shaking. All solvents were obtained from Ajax, Australia and were AR grade. The extracts were filtered through filter paper (Whatman No. 54) under vacuum, followed by drying by rotary evaporation in an Eppendorf concentrator 5301. The resultant pellets were dissolved in 10 mL sterile deionised water (containing 1 % DMSO). The extracts were passed through 0.22 µm filter (Sarstedt) and stored at 4 °C until use.

Qualitative phytochemical studies.

Phytochemical analysis of the *D. leichhardtii* leaf extracts for the presence of saponins, phenolic compounds, flavonoids, phytosteroids, triterpenoids, cardiac glycosides, anthraquinones, tannins and alkaloids was conducted by standard assays.^{18–20}

Antibacterial screening

Test micro organisms.

All media was supplied by Oxoid Ltd. Australia. Reference strains of *Acinetobacter baylyi* (ATCC33304), *Klebsiella pneumoniae* (ATCC31488), *Proteus mirabilis* (ATCC21721), *Proteus vulgaris* (ATCC21719), and *Pseudomonas aeruginosa* (ATCC39324) were purchased from American Tissue Culture Collection, USA. All 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 the *D. leichhardtii* extracts was determined using a modified disc diffusion assay.^{21–23} Briefly, 100 µL of each bacterial culture was grown in 10 mL of fresh nutrient broth until they reached a count of ~10⁸ cells/mL. A volume of 100 µL of the bacterial suspension was spread onto nutrient agar plates and the 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.^{24–25} Briefly, the *D. leichhardtii* leaf extracts were 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.

Bacterial growth time course assay.

Bacterial growth time course studies were performed as previously described.²⁶ Briefly, 3 mL of *Proteus mirabilis* (ATCC21721) and *Klebsiella pneumoniae* (ATCC31488) in nutrient broth were individually added to 27 mL nutrient broth containing 3 mL of 10 mg/mL methanolic or aqueous plant extract to give a final concentration of 1000 µg/mL in the assay. The tubes were incubated at 30 °C with gentle shaking. The optical density was measured hourly at 550 nm for a 6 h incubation period. Control tubes were incubated under the same conditions but without the extract. All assays were performed in triplicate.

Toxicity screening

Reference toxin for toxicity screening.

Potassium dichromate (K₂Cr₂O₇) (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.^{27–29} Briefly, 400 μ L of seawater containing approximately 55 (mean 55.2, $n = 125$, SD 14.5) *A. franciscana* nauplii were added to wells of a 48 well plate and immediately used for bioassay. A volume of 400 μ L of diluted plant extracts or the reference toxin were transferred to the wells and incubated at $25 \pm 1^\circ\text{C}$ under artificial light (1000 Lux). A 400 μ L 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 \pm SEM of at least three independent experiments. 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 1 g of dried and powdered *D. leichhardtii* leaves with solvents of varying polarity yielded dried extracts ranging from 137 mg (hexane extract) to 253 mg (methanol extract) (Table 1). The aqueous (225 mg) and chloroform extracts (186 mg) also yielded relatively high levels of extracted material. The dried extracts were resuspended in 10 mL of deionised water (containing 1% DMSO), resulting in the extract concentrations shown in Table 1. Qualitative phytochemical studies showed that the higher polarity methanol and water solvents extracted the greatest diversity and highest levels of phytochemicals. Both contained high levels of phenolics and flavonoids, as well as moderate levels of alkaloids and tannins. The ethyl acetate extract contained similar phytochemical classes, albeit generally at substantially lower levels. Interestingly, despite extracting relatively large amounts of material, the chloroform and hexane extracts were generally devoid of all classes of phytochemicals screened. Due to their nonpolar nature, these extracts would be expected to contain high levels of lipids, hydrocarbons etc. As our qualitative phytochemical studies did not screen for these compounds, they were not detected and other techniques are required to further examine the nature of these nonpolar components.

Antimicrobial activity.

To determine the growth inhibitory activity of the *D. leichhardtii* leaf extracts against the bacterial triggers of the selected autoimmune inflammatory diseases, aliquots (10 μ L) of each extract were screened in the disc diffusion assay. All *D. leichhardtii* leaf extracts inhibited *P. mirabilis* growth (Figure 2). The methanolic and aqueous *D. leichhardtii* leaf extracts were particularly potent growth inhibitors, each recording zones of inhibition >12 mm against both the reference and clinical strains of the bacterium. Indeed, the methanolic *D. leichhardtii* leaf extract inhibited *P. mirabilis* growth by 21 ± 1 mm (reference strain) and 20.6 ± 1.2 mm (clinical isolate strain). The aqueous extract was also potent, inhibiting the reference and clinical strains by 13.3 ± 1.5 and 12.9 ± 1.2 mm respectively. This inhibition was particularly noteworthy compared to the inhibition by the ampicillin control (10 μ g: inhibition zones of 10.3 ± 0.6 mm and 10.6 ± 0.6 mm against the reference and clinical strains respectively). The ethyl acetate, chloroform and hexane extracts also

inhibited the growth of both *P. mirabilis* strains, albeit generally with substantially smaller inhibition zones than were recorded for the methanolic and aqueous extracts.

The *D. leichhardtii* leaf extracts were similarly potent inhibitors of *P. vulgaris* (Figure 3). As for *P. mirabilis*, the methanolic and aqueous extracts were the most potent bacterial growth inhibitors, with zones of inhibition of 18.4 ± 1.4 mm and 11.6 ± 1.2 mm respectively. Notably, inhibition by the methanolic extract was substantially more potent than the inhibition by the ampicillin control (11.8 ± 0.6 mm). The ethyl acetate, and chloroform extracts also inhibited the growth of *P. vulgaris*, albeit with substantially smaller inhibition zones. The hexane extract was completely devoid of inhibitory activity. As *Proteus* spp. (particularly *P. mirabilis*) have been identified as a microbial trigger of rheumatoid arthritis,^{2–4} our results indicate the potential of the *D. leichhardtii* leaf extracts in preventing and treating that disease.

The *D. leichhardtii* leaf extracts were also potent inhibitors of *K. pneumoniae* growth (Figure 4). As reported for *Proteus* spp. inhibition (Figures 2 and 3), the methanolic extract was the most potent bacterial growth inhibitor, with zones of inhibition of 18 ± 1 mm and 18.6 ± 0.9 mm against the reference and clinical *K. pneumoniae* strains respectively. The aqueous extract was also a good growth inhibitor with inhibition zones of 16.3 ± 1.2 mm and 17 mm against the reference and clinical *K. pneumoniae* strains respectively. The ethyl acetate, and chloroform extracts induced smaller inhibition zones, although these were still indicative of moderate to good growth inhibitory activity. By comparison, the hexane extract displayed only low inhibitory activity. As *K. pneumoniae* has been shown to initiate ankylosing spondylitis,^{2,5–7} our results indicate the potential of the *D. leichhardtii* leaf extracts in the prevention and treatment of that disease.

The methanolic and aqueous *D. leichhardtii* leaf extracts also inhibited *A. baylyi* growth, albeit with substantially smaller zones of inhibition (approximately 7.5 and 6.3 mm for the methanolic and aqueous extracts respectively against both reference and clinical strains: Figure 5). Furthermore, none of the *D. leichhardtii* extracts inhibited the growth of either the reference or clinical *P. aeruginosa* strains (Figure 6). Interestingly, the low level of growth inhibition of *P. aeruginosa* by the ampicillin control (6.3 ± 0.3 mm and 6.4 ± 0.4 mm for the reference and clinical strains respectively) indicates that both of these strains were antibiotic resistant. Thus, the *D. leichhardtii* extracts may still prove to inhibit the growth of other, less resistant *P. aeruginosa* strains and further studies are required to test this. However, based on our results, as all *D. leichhardtii* extracts had either low efficacy or no activity against these microbial triggers of multiple sclerosis (*A. baylyi* and *P. aeruginosa*),⁸ it is unlikely that they would be effective in the prevention and treatment of multiple sclerosis.

The methanolic and aqueous *D. leichhardtii* leaf extracts also inhibited *S. pyogenes* growth, albeit with zones of inhibition indicative of only low potency (6.8 ± 0.6 and 5.8 ± 0.4 mm respectively: Figure 7). The ethyl acetate, chloroform and hexane extracts were completely devoid of *S. pyogenes* growth inhibitory activity. *S. pyogenes* can cause a variety of diseases including streptococcal pharyngitis, impetigo and rheumatic heart disease,^{10,11} depending on which tissue it infects. Thus, our results indicate that the methanolic and aqueous *D. leichhardtii* leaf extracts may have some limited potential in preventing and treating rheumatic heart disease, although with only relatively low efficacy. Isolation studies are required to determine whether isolated anti-*S. pyogenes* components, which may be in only low abundance in the crude extract, have clinically relevant efficacy in a pure form.

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 methanolic and aqueous *D. leichhardtii*

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

Extract	Mass of Dried Extract (mg)	Resuspended Extract (mg/mL)	Phenols										Cardiac Glycosides			Saponins		Triterpenes		Phytosteroids		Alkaloids		Flavonoids		Tannins		Anthraquinones	
			Total Phenolics	Water Soluble	Water Insoluble	Keller-Kiliani Test	Froth Persistence	Emulsion test	Salkowski Test	Acetic Anhydride Test	Meyers Test	Wagners Test	Dragendoffs Test	Shinoda Test	Kumar test	Ferric Chloride Test	Lead Acetate Test	Free	Combined										
Methanol	253	25.3	+++	+++	+++	-	+	+	++	++	++	+	+	+++	+++	++	++	+	-	+	+	++	++	++	++	++	++	+	-
Water	225	22.5	+++	+++	++	-	++	+	+	+	++	+	+	+++	++	++	+	+	-	+	+	++	++	++	++	++	++	+	-
Ethyl Acetate	148	14.8	+	+	+	-	-	-	+	-	+	-	-	+	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Chloroform	186	18.6	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Hexane	137	13.7	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-

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

Table 2: Minimum inhibitory concentrations ($\mu\text{g/mL}$) of the *D. leichhardtii* leaf extracts against each bacterial species and LC_{50} values ($\mu\text{g/mL}$) against *Artemia nauplii*.

Antibacterial Inhibition (MIC)	Bacteria	Strain or exposure time		M	W	E	C	H
	<i>P. mirabilis</i>	ATCC21721		85	427	987	3784	4845
		Clinical isolate		116	510	1105	3925	5025
	<i>P. vulgaris</i>	ATCC21719		187	653	1057	3485	-
	<i>K. pneumoniae</i>	ATCC31488		143	490	1257	4074	4992
		Clinical isolate		118	448	983	3953	4686
	<i>baylyi</i>	ATCC33304		1850	3266	-	-	-
		Clinical isolate		2028	3584	-	-	-
	<i>P. aeruginosa</i>	ATCC39324		-	-	-	-	-
		Clinical isolate		-	-	-	-	-
	<i>S. pyogenes</i>	Clinical isolate		1853	2218	-	-	-
		24 h		1340	1490	CND	CND	CND
	LC_{50} ($\mu\text{g/mL}$)	48 h		755	1146	CND	CND	CND

Numbers indicate the mean MIC or LC_{50} values of at least triplicate determinations. - indicates no growth inhibition or toxicity. M = methanolic extract; W = aqueous extract; E = ethyl acetate extract; C = chloroform extract; H = hexane extract. CND indicates that an LC_{50} could not be determined as the mortality did not exceed 50% at any concentration tested.

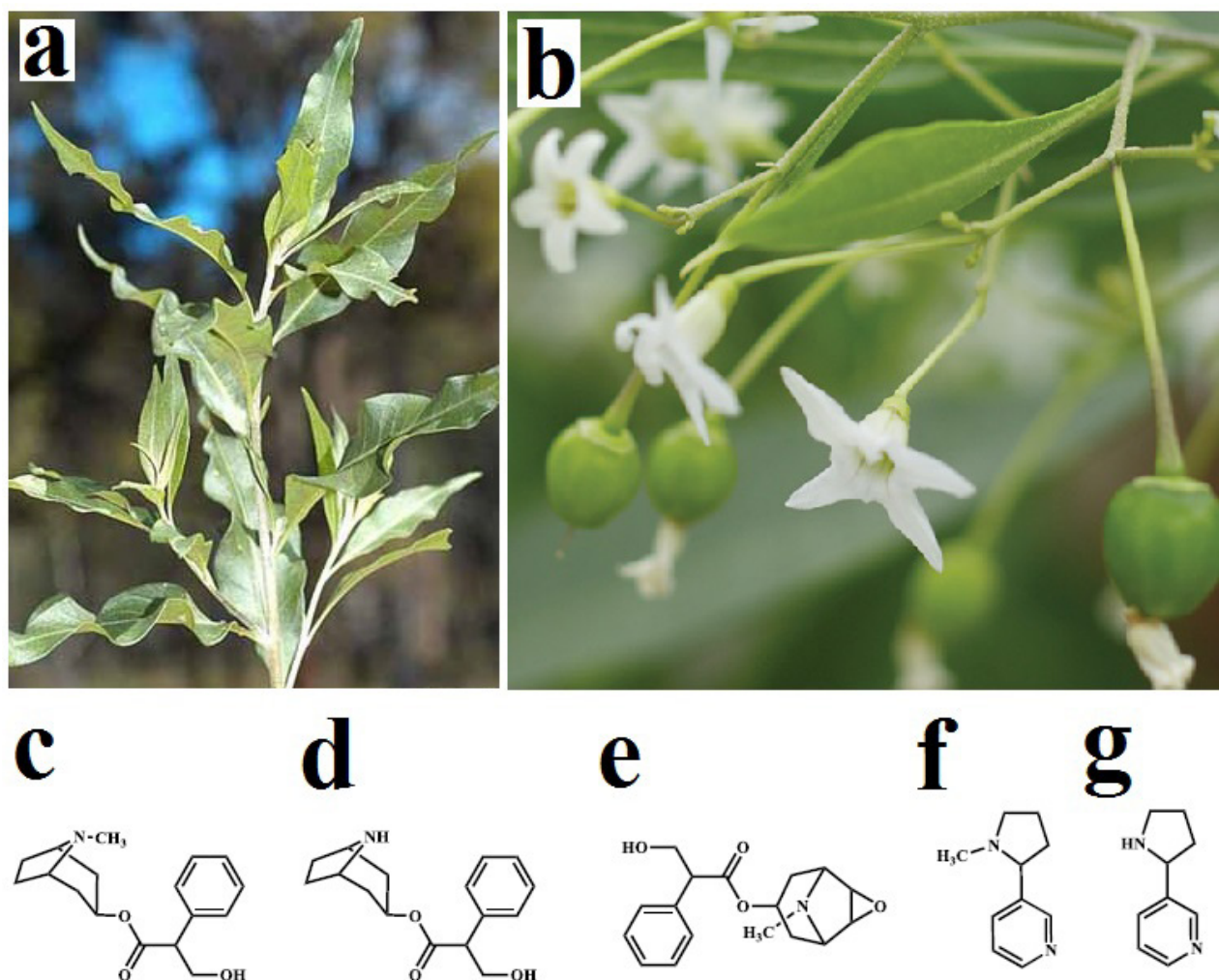


Figure 1: *D. leichhardtii* (a) leaves, (b) flowers and fruit, as well as the phytochemical compounds (c) hyoscyamine, (d) norhyoscyamine, (e) scopolamine, (f) nicotine and (g) nornicotine.

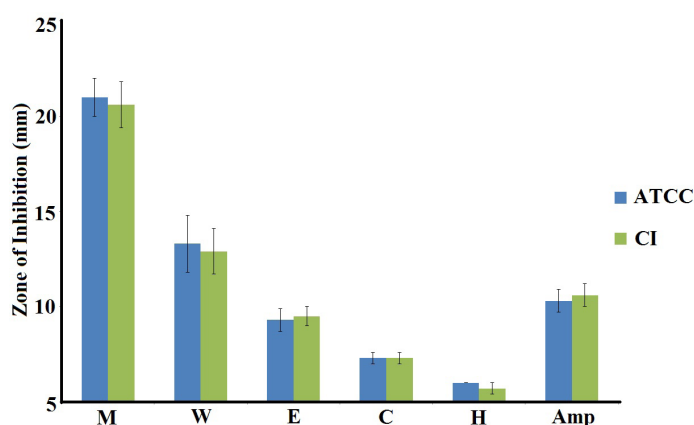


Figure 2: Growth inhibitory activity of *D. leichhardtii* leaf extracts against the reference (ATCC21721) and clinical *P. mirabilis* strains measured as zones of inhibition (mm) \pm SEM. Blue bars represent the reference strain (ATCC21721); Green bars represent the clinical isolate strain; M = methanolic extract; W = aqueous extract; E = ethyl acetate extract; C = chloroform extract; H = hexane extract; Amp = ampicillin (10 μ g) control; ATCC represents the reference *P. mirabilis* strain (ATCC21721); CI represents the clinical isolate strain. All determinations were in triplicate and the results are expressed as mean zones of inhibition (mm) \pm SEM.

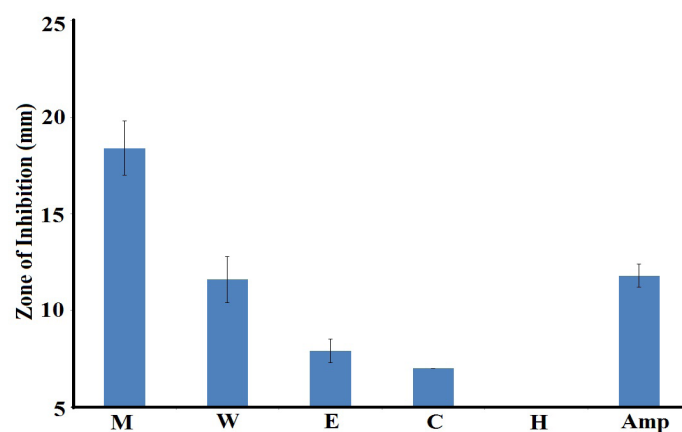


Figure 3: Growth inhibitory activity of *D. leichhardtii* leaf extracts against the reference (ATCC21719) *P. vulgaris* strain as zones of inhibition (mm) \pm SEM. M = methanolic extract; W = aqueous extract; E = ethyl acetate extract; C = chloroform extract; H = hexane extract; Amp = ampicillin (10 μ g) control. All determinations were in triplicate and the results are expressed as mean zones of inhibition (mm) \pm SEM.

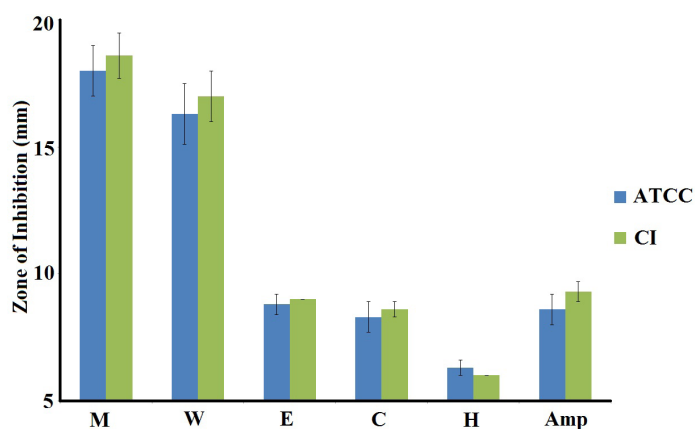


Figure 4: Growth inhibitory activity of the *D. leichhardtii* leaf extracts against the reference (ATCC31488) and clinical *K. pneumoniae* strains measured as zones of inhibition (mm) \pm SEM. Blue bars represent the reference strain (ATCC21721); Green bars represent the clinical isolate strain; M = methanolic extract; W = aqueous extract; E = ethyl acetate extract; C = chloroform extract; H = hexane extract; Amp = ampicillin (10 μ g) control. All determinations were in triplicate and the results are expressed as mean zones of inhibition (mm) \pm SEM.

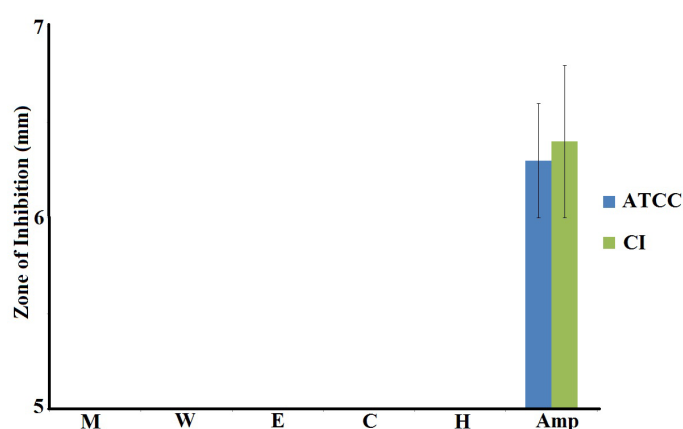


Figure 6: Growth inhibitory activity of the *D. leichhardtii* leaf extracts against reference (ATCC39324) and clinical *P. aeruginosa* strains measured as zones of inhibition (mm) \pm SEM. Blue bars represent the reference strain (ATCC39324); Green bars represent the clinical isolate strain; M = methanolic extract; W = aqueous extract; E = ethyl acetate extract; C = chloroform extract; H = hexane extract; Amp = ampicillin (10 μ g) control. All determinations were in triplicate and the results are expressed as mean zones of inhibition (mm) \pm SEM.

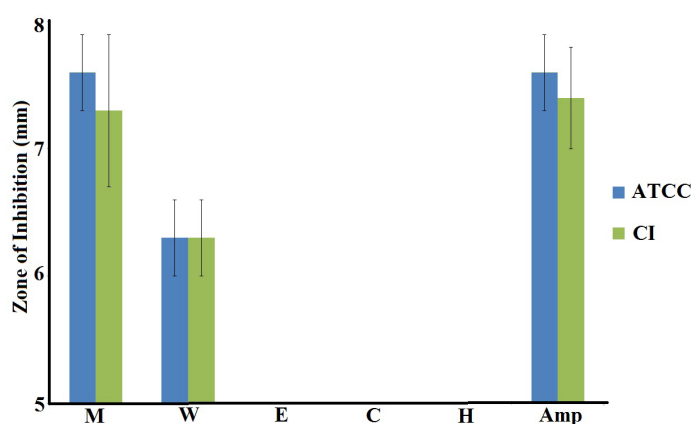


Figure 5: Growth inhibitory activity of the *D. leichhardtii* leaf extracts against reference (ATCC33304) and clinical *A. baylyi* strains measured as zones of inhibition (mm) \pm SEM. Blue bars represent the reference strain (ATCC33304); Green bars represent the clinical isolate strain; M = methanolic extract; W = aqueous extract; E = ethyl acetate extract; C = chloroform extract; H = hexane extract; Amp = ampicillin (10 μ g) control. All determinations were in triplicate and the results are expressed as mean zones of inhibition (mm) \pm SEM.

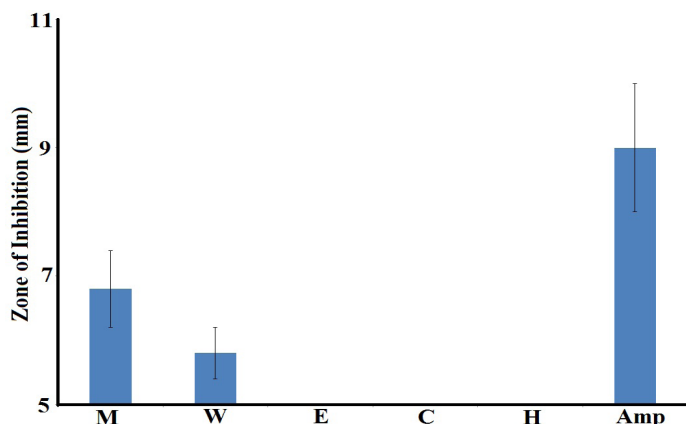


Figure 7: Growth inhibitory activity of the *D. leichhardtii* leaf extracts against the clinical *S. pyogenes* strain measured as zones of inhibition (mm) \pm SEM. M = methanolic extract; W = aqueous extract; E = ethyl acetate extract; C = chloroform extract; H = hexane extract; Amp = ampicillin (10 μ g) control. All determinations were in triplicate and the results are expressed as mean zones of inhibition (mm) \pm SEM.

leaf extracts inhibited all of the bacterial triggers of autoimmune diseases screened except *P. aeruginosa*. *Proteus* spp. and *K. pneumoniae* were particularly susceptible to the methanolic and aqueous *D. leichhardtii* leaf extracts, with MIC values generally <500 μ g/mL (<5 μ g infused into the disc). Indeed, an MIC of 85 μ g/mL (<1 μ g infused into the disc) was determined for the methanolic extract against the reference *P. mirabilis* strain. These MIC determinations further support the potential of the methanolic and aqueous *D. leichhardtii* leaf extracts for the prevention of rheumatoid arthritis and ankylosing spondylitis in genetically susceptible individuals.

Bacterial growth time course assay.

The antibacterial activity of the *D. leichhardtii* leaf extracts was further investigated in *P. mirabilis* (Figure 8a) and *K. pneumoniae* (Figure 8b)

by bacterial growth time course assays in the presence and absence of either the methanolic or aqueous extract. These were the only extracts examined by bacterial growth time course studies as these extracts were generally the most potent inhibitors of bacterial growth. The starting concentration of the extract used in these assays was 1000 μ g/mL. The methanolic *D. leichhardtii* leaf extract significantly inhibited *P. mirabilis* (Figure 8a) and *K. pneumoniae* (Figure 8b) growth within 1 h, indicating a rapid antimicrobial action. Potent inhibition of *P. mirabilis* (Figure 8a) and *K. pneumoniae* (Figure 8b) by the methanolic and aqueous *D. leichhardtii* leaf extracts was noted, with growth still significantly inhibited by the end of the 6 h time course study. This may indicate that these extracts have bactericidal activity against *P. mirabilis* and *K. pneumoniae* at the dose tested. Indeed, the turbidity at 6 h was not greatly increased from the starting turbidity for either bacteria.

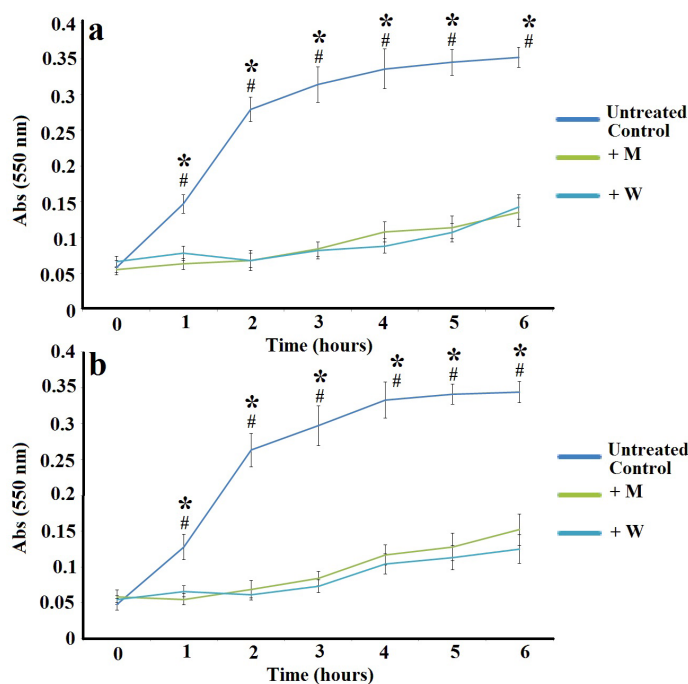


Figure 8: Bacterial growth curves for the methanolic and aqueous *D. leichhardtii* extracts against (a) *P. mirabilis* (ATCC21721) and (b) *K. pneumoniae* (ATCC31488). All bioassays were performed in at least triplicate and are expressed as mean \pm SEM. * = results in the presence of the methanolic extract that are significantly different between the treated and the untreated control growth ($p < 0.01$); # = results in the presence of the aqueous extract that are significantly different between the treated and the untreated control growth ($p < 0.01$).

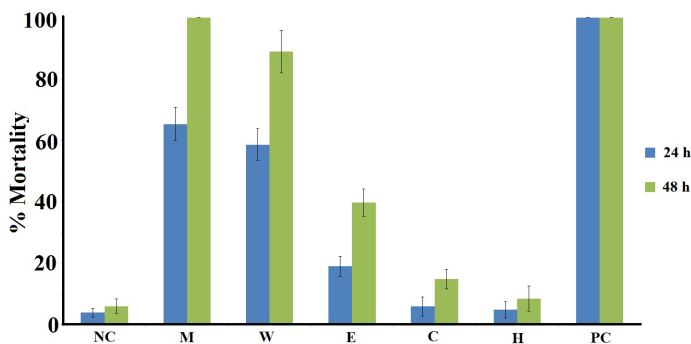


Figure 9: The lethality of the *D. leichhardtii* leaf extracts (2000 $\mu\text{g/mL}$), potassium dichromate (1000 $\mu\text{g/mL}$) and a seawater control. Blue bars represent the % mortality following 24 h exposure; green bars represent the % mortality following 48 h exposure; M = methanolic extract; W = aqueous extract; E = ethyl acetate extract; C = chloroform extract; H = hexane extract; NC = negative (seawater) control; PC = positive control (1000 $\mu\text{g/mL}$ potassium dichromate). All bioassays were performed in at least triplicate and are expressed as mean \pm SEM.

Quantification of toxicity.

The toxicity of the *D. leichhardtii* leaf extracts was initially tested in the *Artemia franciscana* nauplii bioassay at a concentration of 2000 $\mu\text{g/mL}$ (Figure 9). The methanolic and aqueous extracts induced >50% mortality at 24 h at 2000 $\mu\text{g/mL}$ in the screening study and thus were deemed to be toxic. All of the other extracts induced low levels of mortality at 24 h, similar to the % mortality seen for the seawater control. By 48 h, the mortality induction had increased for all of the *D. leichhardtii* leaf

extracts. However, as the methanolic and aqueous extracts were still the only extracts which induced >50 % toxicity at 48 h, all other extracts were deemed to be nontoxic. In contrast, the potassium dichromate positive control induced mortality within 4 h (results not shown), with 100 % mortality induction seen by 24 h.

To further quantify the effect of toxin concentration on the induction of mortality, the extracts were serially diluted in artificial seawater to test across a range of concentrations in the *Artemia* nauplii bioassay (Table 2). For comparison, serial dilutions of potassium dichromate were also tested. All extracts were determined to be nontoxic, with LC_{50} values >1000 $\mu\text{g/mL}$ following 24 h exposure. Extracts with an LC_{50} > 1000 $\mu\text{g/mL}$ towards *Artemia* nauplii have previously been defined as being nontoxic.²⁹

DISCUSSION

Plant derived 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 many synthetic drugs, and the failure of current drug regimens to effectively treat many diseases. This is especially true for chronic disorders such as the autoimmune inflammatory diseases. The current treatments which utilise 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.¹ Furthermore, many of the current treatments are aimed at treating the symptoms without addressing the underlying causes and pathogenic mechanisms. 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. The studies reported here examined the ability of *D. leichhardtii* leaf extracts to block microbial triggers of 4 autoimmune inflammatory disorders (*Proteus* spp.: rheumatoid arthritis; *K. pneumoniae*: ankylosing spondylitis; *A. baylyi* and *P. aeruginosa*: multiple sclerosis; *S. pyogenes*: rheumatic heart disease). Several extracts were identified as potent inhibitors of these bacteria. Particularly noteworthy, the methanolic and aqueous extracts were identified as potent inhibitors of the growth of the bacterial triggers of rheumatoid arthritis and ankylosing spondylitis, and were generally more potent growth inhibitors than were the ampicillin antibacterial controls.

Whilst a detailed investigation of the phytochemistry of the *D. leichhardtii* leaf extracts was beyond the scope of our study, qualitative screening studies were used to determine the classes of compounds present. These studies indicated that polyphenolics, flavonoids, tannins and alkaloids were present in the most potent *D. leichhardtii* leaf extracts. Studies have linked polyphenolic compounds and alkaloids with anti-bacterial activity in different plant species³⁰ and thus these compounds may be responsible (at least in part) for the bacterial growth inhibitory activities reported here. Interestingly, several of these phytochemical classes have also been reported to suppress NF- κ B signaling.³⁰ Thus, these extracts may have pleuripotent effects against autoimmune inflammatory diseases, with therapeutic properties against the trigger events as well as the later inflammatory responses. Further phytochemical evaluation studies and bioactivity driven isolation of the active components is required to further evaluate the mechanism of bacterial growth inhibition.

Previous studies have also confirmed the presence of significant alkaloid components in *Duboisia* spp. extracts. In particular, the tropane alkaloids scopolamine, hyoscyamine and norhyoscyamine, as well as the pyrrolidine alkaloids nicotine and nornicotine have been detected in relatively high levels in many *Duboisia* spp. (although the relative levels differ between species/locations).¹⁵ The tropane alkaloids scopolamine, hyoscyamine and norhyoscyamine have been particularly well studied

as they have potent psychoactive properties via acetylcholine receptor antagonism. Thus, they are indicated as sedatives and hypnotics (or stimulants at low doses).¹⁵ The pyrrolidine alkaloids nicotine and nornicotine also act via the acetylcholine receptor, although they are agonists and function as stimulants.¹⁵ Interestingly, potent antibacterial activity has been reported for scopolamine (MIC values as low as 2 µg/mL against multiple bacteria).¹⁶ Similarly, nicotine inhibits the growth of *E. coli*, *P. aeruginosa* and *S. faecalis*.¹⁷ However, it is noteworthy that all of these alkaloids have been reported to have substantial toxicity, which may limit the usable safe doses. Thus, *D. leichhardtii* leaf extracts and essential oils should be used with caution.

Although our study demonstrated the potential of the *D. leichhardtii* leaf extracts to prevent and treat autoimmune disease, much more work is required. This study has only tested these extracts against microbial triggers of 4 autoimmune diseases (rheumatoid arthritis, ankylosing spondylitis, multiple sclerosis and rheumatic heart disease). The microbial triggers for several other autoimmune inflammatory disorders are also known. *Borrelia burgdorferi* is linked with Lyme disease⁹ and *Mycoplasma pneumoniae* is associated with several demyelinating diseases.¹² It would be interesting to extend our studies to also screen for the ability of the extracts to block these microbial triggers of autoimmune diseases.

Despite the presence of the tropane and pyrrolidine alkaloids in *Duboisia* spp. extracts,¹⁵ our studies determined that all of the *D. leichhardtii* leaf extracts were nontoxic towards *Artemia franciscana* nauplii, with LC₅₀ values > 1000 µg/mL. Extracts with LC₅₀ values > 1000 µg/mL towards *Artemia* nauplii are defined as being nontoxic.²⁹ Whilst our preliminary toxicity studies indicate that these extracts may be safe for therapeutic use, studies using human cell lines are required to further evaluate the safety of these extracts. Furthermore, this study only screened for toxicity defined as mortality. Due to the intoxicating properties of the *Duboisia* spp. alkaloids, it may also be necessary to test the extracts for other markers of toxicity. Whilst these studies have demonstrated the potential of the *D. leichhardtii* leaf extracts in the development of future antibiotic chemotherapeutics for the prevention and treatment of autoimmune diseases (particularly rheumatoid arthritis and ankylosing spondylitis), more work is required to isolate the inhibitory components and determine the mechanism of inhibition.

CONCLUSIONS

The results of this study demonstrate the potential of the *D. leichhardtii* leaf extracts as inhibitors of the growth of bacterial species associated with the onset of some autoimmune inflammatory diseases (particularly rheumatoid arthritis and ankylosing spondylitis). Furthermore, their lack of toxicity indicates that they are safe for internal as well as topical treatment.

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CONFLICTS OF INTEREST

The authors report no conflicts of interest.

ABBREVIATIONS

DMSO: Dimethyl sulfoxide

LC₅₀: The concentration required to achieve 50 % mortality

MIC: minimum inhibitory concentration

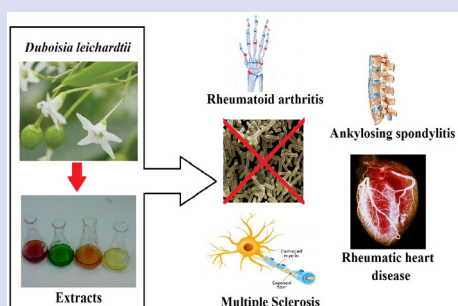
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PICTORIAL ABSTRACT



SUMMARY

- *D. leichhardtii* leaf extracts were potent inhibitors of the bacterial triggers of rheumatoid arthritis (*Proteus* spp.) and ankylosing spondylitis (*K. pneumoniae*).
- The methanolic extract was a particularly potent inhibitor of *Proteus* spp. growth, with MICs <200 µg/mL against all strains.
- The methanolic extract was also a good inhibitor of *K. pneumoniae* growth with MICs < 150 µg/mL.
- The aqueous and ethyl acetate extracts were also potent bacterial growth inhibitors, albeit with slightly higher MIC values.
- All *D. leichhardtii* leaf extracts were nontoxic.

ABOUT AUTHORS



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.