

Aleurites moluccanus (L.) Willd. Extracts Inhibit the Growth of Bacterial Triggers of Selected Autoimmune Inflammatory Diseases

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ABSTRACT

Introduction: *Aleurites moluccanus* (L.) Willd. is a large tree with a wide global distribution. All parts of the tree have been used medicinally and the nut is consumed in a variety of cuisines. Despite this, *A. moluccanus* nut extracts have not been rigorously examined growth inhibitory properties against many bacteria, including the bacterial triggers of autoimmune inflammatory diseases. **Methods:** The antimicrobial activity of *A. moluccanus* nut solvent extractions 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. Toxicity was determined using the *Artemia franciscana* nauplii bioassay. **Results:** Methanolic and aqueous *A. moluccanus* nut solvent extracts were potent inhibitors of all of the bacterial triggers of autoimmune diseases screened in this study. The methanolic extract displayed the most potent bacterial growth inhibitory activity. It was particularly potent against the bacterial triggers of rheumatoid arthritis (MICs of 438 and 215 µg/mL against reference and clinical *Proteus mirabilis* strains; MIC of 187 µg/mL against *Proteus vulgaris*). The methanolic extract was also a good inhibitor of *K. pneumoniae* and *S. pyogenes* growth with MICs < 1000 µg/mL. The aqueous and ethyl acetate extracts were also potent bacterial growth inhibitors, albeit with slightly higher MIC values. The antibacterial activity of the methanolic and aqueous *A. moluccanus* nut extracts were further in-

vestigated by growth time course assays which showed significant growth inhibition in cultures of *P. mirabilis*, *K. pneumoniae* and *S. pyogenes* within 1 h of exposure. All extracts were determined to be nontoxic in the *Artemia franciscana* nauplii bioassay, indicating their safety for prophylactic use in preventing these autoimmune inflammatory diseases. **Conclusions:** The lack of toxicity of the *A. moluccanus* nut extracts and their growth inhibitory bioactivity against the bacterial triggers of rheumatoid arthritis, ankylosing spondylitis and rheumatic heart disease indicate their potential in the development of new therapies targeting the onset of these diseases.

Key words: *Euphorbiaceae*, Candlenut, Rheumatoid Arthritis, Ankylosing Spondylitis, Rheumatic Heart Disease, Antibacterial Activity, Medicinal Plants.

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INTRODUCTION

Autoimmune inflammatory disorders including rheumatoid arthritis, ankylosing spondylitis and rheumatic heart disease are a group of debilitating conditions which may afflict genetically susceptible individuals. There are no cures for any of these conditions. 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 usage of these drugs is often accompanied by unwanted side effects and toxicity.¹ There is a need to develop safer, more effective treatments for these conditions 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.

Although the causes of the autoimmune inflammatory disorders are not well understood, it is generally accepted that they are triggered in susceptible individuals by specific microbial infections. Recent 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.²⁻⁷ The major microbial trigger of rheumatoid arthritis has been identified as *Proteus mirabilis*,²⁻⁴ a normal part of the human gastrointestinal flora. Similarly, *Klebsiella pneumoniae* has been shown to initiate ankylosing spondylitis^{2,5-7} and *Acinetobacter baylyi* and *Pseudomonas aeruginosa* have been linked with the onset of multiple sclerosis.⁸ *Borrelia burgdorferi* is linked with Lyme disease.⁹ Whilst microbial triggers have also been postulated for lupus, the specific causative agents are yet to be identified. Members of the Enterobacteriaceae family are associated with Graves' disease and Kawasaki syndrome and *Mycoplas-*

ma pneumoniae is associated with several demyelinating diseases.¹⁰ 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 also 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. Much of the recent attention has focussed on the therapeutic potential of fruits and berries with high antioxidant contents. The bacterial growth inhibitory properties of many fruit have been particularly well reported.¹¹⁻¹⁵ Indeed, these studies have highlighted several high antioxidant fruits as having particular interest, with MIC values for the crude extracts similar to those of conventional antibiotics. Several of the same high antioxidant fruits also have potent anti-Giardial^{16,17} and anticancer activities.¹⁸

Despite the relative wealth of information on the medicinal and functional food properties of edible fruits and berries, there have been relatively few studies examining the therapeutic potential of edible nuts. Recent studies have reported the therapeutic potential of *Macadamia integriflora* Maiden & Betche nuts¹⁹ as well as leaves and flowers^{20,21} against a broad panel of bacteria. Similarly, a recent study screened almond, cashew, hazelnut and walnut for medicinal properties and reported growth inhibitory activity for several nut extracts against bacterial triggers of several autoimmune diseases.²² Despite this, few other edible nuts

have been rigorously tested for the ability to inhibit bacterial growth.

Aleurites moluccanus (L.) Willd. (family Euphorbiaceae; commonly known as candlenut, candleberry, Indian walnut, varnish tree) is a large tree which is widely distributed globally. Indeed, its native range is not certain as the tree has been naturalised widely and is now found worldwide. The trees grow 15-25 m tall and have pale green ovate leaves (Figure 1a). White to cream flowers (Figure 1b) develop into globose drupes (Figure 1c), which each contain 2 or 3 seeds (Figure 1d). Most parts of the tree have ethnobotanical uses, although the seed is most extensively used in a variety of cuisines, particularly in Asia.²³ The bark is used to treat diarrhoea and dysentery, boiled leaves are used as poultices for headaches, fevers, ulcers and to treat joint inflammation, whilst the flowers and sap are used to treat candidiasis.²³ A recent study screened *A. moluccanus* bark and nut extracts for bacterial and fungal growth inhibitory activity.²⁴ That study reported potent inhibition of *Staphylococcus aureus* and *Candida albicans* growth for the bark extracts. Interestingly, the nut extracts were devoid of bacterial activity. However, that study only screened against 2 bacterial and 1 fungal species and studies examining the growth inhibitory properties of *A. moluccanus* nut extracts against other microbial species are lacking.

Several interesting phytochemical components have been identified in *A. moluccanus* nut extracts and essential oils, further supporting their therapeutic potential. The lipid components linolenic acid (Figure 1e), eicosanoic acid methyl ester (Figure 1f) and γ -tocopherol (Figure 1g) have been reported to be in abundance in *A. moluccanus* nuts.²⁵ Interestingly, medium chain unsaturated fatty acids such as linolenic acid and eicosanoic acid have both antibacterial and anti-inflammatory activities.²⁶ Despite these studies, examination of the antibacterial and anti-inflammatory properties and phytochemistry of *A. moluccanus* nuts is rudimentary. The current report was undertaken to screen *A. moluccanus* nut extracts for growth inhibitory properties against bacterial triggers of selected autoimmune inflammatory diseases.

MATERIALS AND METHODS

Plant collection and extraction

Vacuum sealed organic whole raw organic candle nuts were obtained from Woolworth's supermarkets, Australia. The nuts were processed

in a Sunbeam food dehydrator to ensure that they were thoroughly dehydrated and the nut pieces were subsequently ground into a coarse powder. The powdered nut was extracted by standardised methods^{27,28} Briefly, an amount of 1 g of dried nut powder was weighed into each of five tubes and five different extracts were prepared by individually adding 50 mL of methanol, water, ethyl acetate, chloroform, or hexane respectively. All solvents were obtained from Ajax Chemicals, Australia and were AR grade. The powdered nuts were individually extracted in each solvent for 24 hours at 4 °C with gentle shaking. The extracts were subsequently filtered through filter paper (Whatman No. 54) under vacuum, followed by drying by rotary evaporation in an Eppendorf concentrator 5301. The dry extract was weighed and redissolved in 10 mL deionised water (containing 1 % DMSO). The extracts were passed through 0.22 μ m filter (Sarstedt) and stored at 4 °C until use.

Qualitative phytochemical studies

Phytochemical analysis of the *A. moluccanus* nut extracts for the presence of saponins, phenolic compounds, flavonoids, phytosteroids, triterpenoids, cardiac glycosides, anthraquinones, tannins and alkaloids was conducted by previously described assays.²⁹⁻³¹

Antibacterial screening

Test microorganisms

All media was supplied by Oxoid Ltd. Australia. Reference strains of *Klebsiella pneumoniae* (ATCC31488), *Proteus mirabilis* (ATCC21721) and *Proteus vulgaris* (ATCC21719) 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.³²⁻³⁴ Briefly, 100 μ L of each bacterial culture was grown in 10 mL of fresh nutrient broth until they reached a count of $\sim 10^8$ cells/mL. A volume of 100 μ L 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 μ L 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 (\pm SEM) are reported in this study. Standard discs of ampicillin (10 μ g) and chloramphenicol (2 μ g) were obtained from Oxoid, Australia and were used as positive controls to compare antibacterial activity. Filter discs infused with 10 μ L 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.^{35,36} Briefly, the *A. moluccanus* nut extracts were diluted in deionised water and tested across a range of concentrations. Discs were infused with 10 μ L 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.

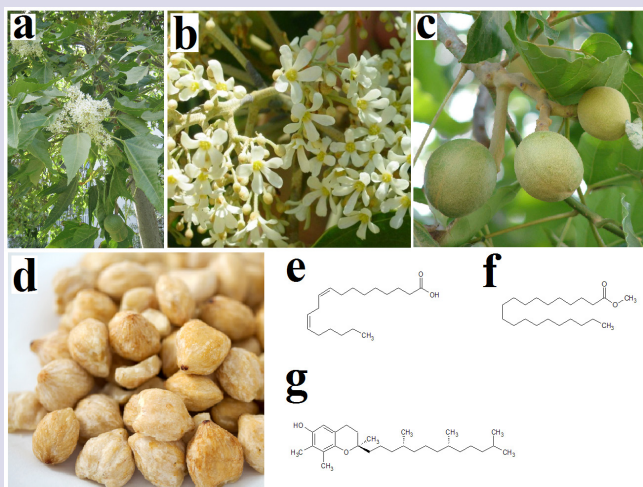


Figure 1: *A. moluccanus* (a) leaves, (b) flowers, (c) fruit, (d) nuts, (e) linolenic acid, (f) eicosanoic acid methyl ester and (g) γ -tocopherol.

Bacterial growth time course assay

Bacterial growth time course studies were performed as previously described.³⁷ Briefly, 3 mL of *Proteus mirabilis* (ATCC21721), *Klebsiella pneumoniae* (ATCC31488) and *Streptococcus pyogenes* (clinical strain) in nutrient broth were added to 27 mL nutrient broth containing 3 mL of 10 mg/mL methanolic and 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.

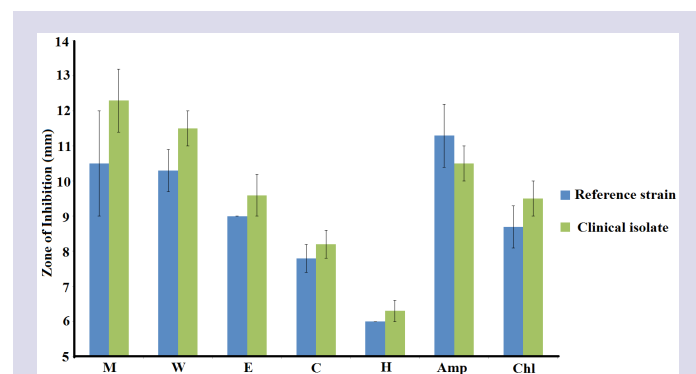


Figure 2: Growth inhibitory activity of *A. moluccanus* nut extracts against the reference (ATCC21721) and clinical *P. mirabilis* strains measured as zones of inhibition (mm) ± SEM. Blue bars represent the reference strain (ATCC21721); Green bars represent the clinical isolate strain; M = methanolic extract; W = aqueous extract; E = ethyl acetate extract; C = chloroform extract; H = hexane extract; Amp = ampicillin (10 µg) control; Chl = the chloramphenicol control (2 µg). All determinations were in at least triplicate and the results are expressed as mean zones of inhibition (mm) ± SEM.

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.³⁸⁻⁴⁰ Briefly, 400 µL of seawater containing approximately 64 (mean 63.8, n = 125, SD 12.7) *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 ± 1°C under artificial light (1000 Lux). A

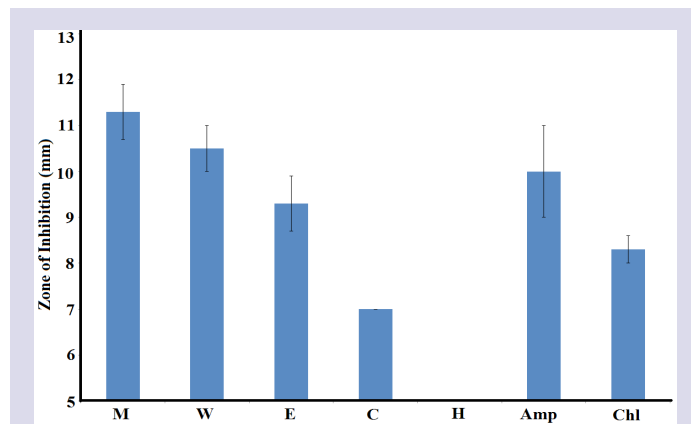


Figure 3: Growth inhibitory activity of *A. moluccanus* nut extracts against the reference (ATCC21719) *P. vulgaris* strain measured as zones of inhibition (mm) ± SEM. M = methanolic extract; W = aqueous extract; E = ethyl acetate extract; C = chloroform extract; H = hexane extract; Amp = ampicillin (10 µg) control; Chl = the chloramphenicol control (2 µg). All determinations were in at least triplicate and the results are expressed as mean zones of inhibition (mm) ± SEM.

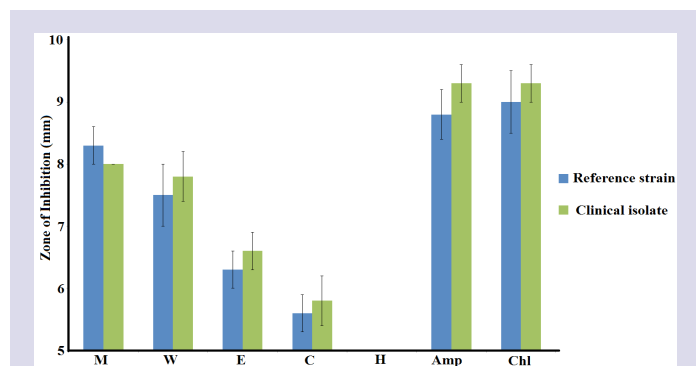


Figure 4: Growth inhibitory activity of *A. moluccanus* nut extracts against the reference (ATCC31488) and clinical *K. pneumoniae* strains measured as zones of inhibition (mm) ± SEM. Blue bars represent the reference strain (ATCC21721); Green bars represent the clinical isolate strain; M = methanolic extract; W = aqueous extract; E = ethyl acetate extract; C = chloroform extract; H = hexane extract; Amp = ampicillin (10 µg) control; Chl = the chloramphenicol control (2 µg). All determinations were in at least triplicate and the results are expressed as mean zones of inhibition (mm) ± SEM.

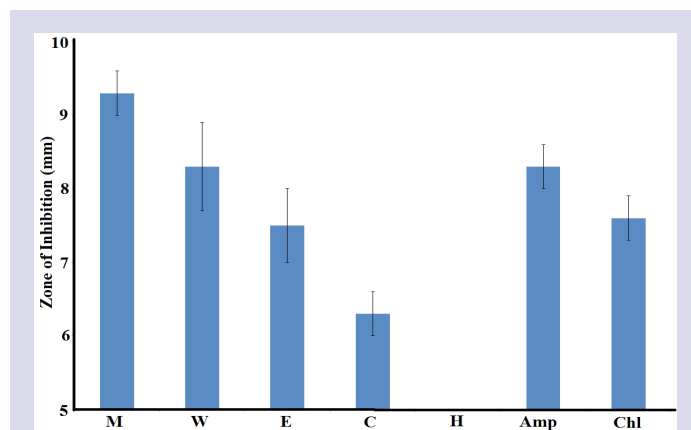


Figure 5: Growth inhibitory activity of *A. moluccanus* nut extracts against the clinical *S. pyogenes* strain measured as zones of inhibition (mm) ± SEM. M = methanolic extract; W = aqueous extract; E = ethyl acetate extract; C = chloroform extract; H = hexane extract; Amp = ampicillin (10 µg) control; Chl = the chloramphenicol control (2 µg). All determinations were in at least triplicate and the results are expressed as mean zones of inhibition (mm) ± SEM.

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

Extract	Mass of Dried Extract (mg)	Resuspended Extract (mg/mL)	Phytochemical Screenings																
			Total Phenolics	Phenols Water Soluble Water Insoluble	Cardiac Glycosides Keller-Kiliani Test	Saponins Froth Persistence Emulsion test	Triterpenes Salkowski Test	Phytosteroids Acetic Anhydride Test	Alkaloids Meyers Test Wagners Test Draggendorffs Test	Flavonoids Shinoda Test Kumar test	Tannins Ferric Chloride Test Lead Acetate Test	Anthraquinones Free Combined							
Methanol	81	8.1	+++	+++	+	-	+	+	+	-	+	+	+	-	-	+	+	-	-
Water	63	6.3	+++	++	-	-	++	+	-	-	+	+	+	+	-	+	+	-	-
Ethyl Acetate	55	5.5	+	+	-	-	-	-	-	-	+	+	+	-	-	-	-	-	-
Chloroform	164	16.4	-	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-
Hexane	58	5.8	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-

+++ 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 *A. mollucanus* nut extracts against each bacterial species and LC_{50} values ($\mu\text{g/mL}$) against *Artemia* nauplii.

	Bacteria	Strain or exposure time	M	W	E	C	H
Antibacterial Inhibition (MIC)		ATCC21721	438	527	287	1448	1873
	<i>P. mirabilis</i>	Clinical isolate	215	410	150	1252	1820
	<i>P. vulgaris</i>	ATCC21719	187	253	675	1558	-
		ATCC31488	734	993	895	1847	-
	<i>K. pneumoniae</i>	Clinical isolate	945	916	826	1635	-
Toxicity	<i>S. pyogenes</i>	Clinical isolate	833	1080	1146	2183	-
	LC_{50} ($\mu\text{g/mL}$)	24 h	CND	CND	CND	CND	CND
		48 h	1440	1854	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.

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 \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 *A. mollucanas* nut with solvents of varying polarity yielded dried extracts ranging from 53 mg (ethyl acetate extract) to 164 mg (chloroform extract) (Table 1). The methanolic extract (81 mg) also yielded a relatively high level of extracted materials. All other extracts were in the range 55–63 mg 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 as well as low to moderate levels of saponins, and alkaloids. 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 *A. mollucanans* nut extracts against the panel of pathogenic bacteria, aliquots (10 µL) of each extract were screened in the disc diffusion assay. All *A. mollucanans* nut extracts inhibited *P. mirabilis* growth (Figure 2). The methanolic and aqueous *A. mollucanans* nut extracts were particularly potent growth inhibitors, each recording zones of inhibition >10 mm against both the reference and clinical strains of the bacterium. Indeed, the methanolic *A. mollucanans* nut extract inhibited *P. mirabilis* growth by 10.5 ± 1.5 mm (reference strain) and 12.3 ± 0.9 mm (clinical isolate strain). The aqueous extract was similarly potent, inhibiting the reference and clinical strains by 10.3 ± 0.6 and 11.5 ± 0.5 mm respectively. This inhibition was particularly noteworthy compared to the inhibition by the ampicillin (10 µg; inhibition zones of 11.3 ± 0.9 mm and 10.5 ± 0.5 mm against the reference and clinical strains respectively) and chloramphenicol controls (2 µg; inhibition zones of 8.7 ± 0.6 mm and 9.5 ± 0.5 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 methanolic and aqueous extracts.

The *A. mollucanans* nut 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 11.3 ± 0.6 mm and 10.5 ± 0.5 mm respectively. Notably, the inhibition by the methanolic and aqueous extracts were substantially more potent than that of the ampicillin and chloramphenicol controls (10.0 ± 1.0 mm and 8.3 ± 0.3 mm respectively). 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 major microbial trigger of rheumatoid arthritis,²⁻⁴ our results indicate the potential of the *A. mollucanans* nut extracts in preventing and treating that disease. The *A. mollucanans* nut extracts also inhibited *K. pneumoniae* growth (Figure 4), although with smaller zones of inhibition. As for *Proteus* spp. inhibition (Figures 2 and 3), the methanolic extract was the most potent bacterial growth inhibitor, with zones of inhibition of 8.3 ± 0.3 mm and 8.0 mm against the reference and clinical *K. pneumoniae* strains respectively. The aqueous extract was also a good growth inhibitor with inhibition zones of 7.5 ± 0.5 mm and 7.8 ± 0.4 mm against the reference and clinical *K. pneumoniae* strains respectively. The ethyl acetate, and chloroform extracts induced only small inhibition zones indicative of only low growth inhibitory activity and the hexane extract was completely devoid of activity. As *K. pneumoniae* been shown to initiate ankylosing spondylitis,²⁻⁵⁻⁷ our results indicate the potential of the *A. mollucanans* nut extracts in the prevention and treatment of that disease.

The methanolic *A. mollucanans* nut extract was also a potent inhibitor of *S. pyogenes*, with a zone of inhibition of 9.3 ± 0.3 mm (Figure 5). Notably, the inhibition by the methanolic extract was substantially more

potent than that of the ampicillin and chloramphenicol controls (8.3 ± 0.3 mm and 7.6 ± 0.3 mm respectively). The aqueous and ethyl acetate extracts were also good bacterial growth inhibitors, with zones of inhibition of 8.3 ± 0.6 mm and 7.5 ± 0.5 mm respectively. *S. pyogenes* can cause a variety of diseases including streptococcal pharyngitis, impetigo and rheumatic heart disease,^{42,43} depending on which tissue it infects. Thus, our results indicate the potential of the *A. mollucanans* nut extracts in preventing and treating rheumatic heart disease, as well as these other diseases.

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, aqueous and ethyl acetate *A. mollucanans* nut extracts were potent or good growth inhibitors of all of bacterial triggers of autoimmune diseases (as judged by MIC; Table 2). *Proteus* spp. were particularly susceptible to the *A. mollucanans* nut extracts, with MIC values generally <500 µg/mL (<5 µg infused into the disc). Indeed, an MIC of 187 µg/mL (<2 µg infused into the disc) was determined for the methanolic extract against *P. vulgaris* growth. The inhibition of all bacterial species (especially by the methanolic, aqueous and ethyl acetate extracts) further supports the potential of the *A. mollucanans* nut extracts for the prevention of these autoimmune diseases in genetically susceptible individuals.

Bacterial growth time course assay

The antibacterial activity of the *A. mollucanans* nut extracts was further investigated in *P. mirabilis* (Figure 6a), *K. pneumoniae* (Figure 6b) and *S. pyogenes* (Figure 6c) by bacterial growth time course assays in the presence and absence of the extract. Only the effect of the methanolic and aqueous extracts on the bacterial growth time course were evaluated as these extracts were generally the most potent inhibitors of bacterial growth. The starting concentration of the extract used in these assays was 1000 µg/mL. The methanolic *A. mollucanans* nut extract significantly inhibited *P. mirabilis* (Figure 6a), *K. pneumoniae* (Figure 6b) and *S. pyogenes* (Figure 6c) growth within 1 h, indicating a rapid antimicrobial action. Whilst *S. pyogenes* growth was inhibited by both the methanolic and aqueous extracts for at least the first 4 hours of the time course, the bacteria were generally able to overcome this inhibition by 6h, with the recorded turbidity not significantly different to that of the untreated control (Figure 6c). This indicates that the growth inhibition of these bacteria was bacteriostatic for these extracts at the concentrations tested. In contrast, inhibition of *P. mirabilis* (Figure 6a) and *K. pneumoniae* (Figure 6b) by the methanolic and aqueous *A. mollucanans* nut extracts was substantially more profound, 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* (Figure 6a) and *K. pneumoniae* (Figure 6b) at the dose tested. Indeed, the turbidity at 6 h was not greatly increased from the starting turbidity for either bacteria.

Quantification of toxicity

The toxicity of the *A. mollucanans* nut extracts was initially tested in the *Artemia franciscana* nauplii bioassay at a concentration of 2000 µg/mL (Figure 7). All extracts induced low levels of mortality at 24 h, similar to the % mortality seen for the seawater control. By 48 h, the methanolic, aqueous and chloroform extracts had begun to induce mortality significantly higher than that in the untreated control. As only the methanolic and aqueous extract 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

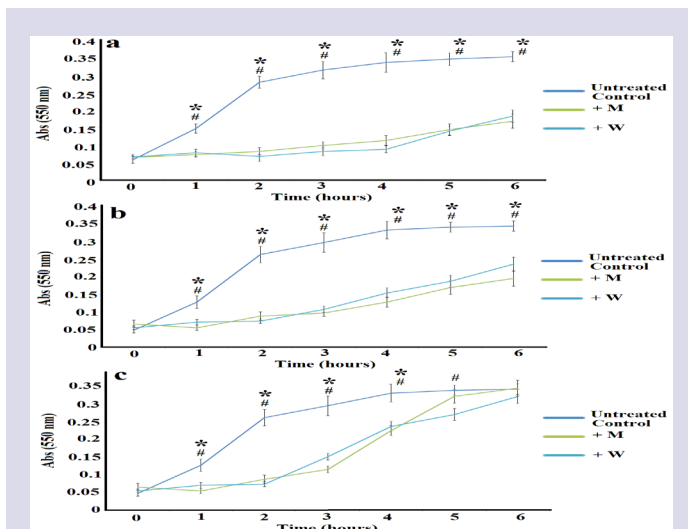


Figure 6: Bacterial growth curves for the methanolic and aqueous *A. moluccanus* extracts against (a) *P. mirabilis* (ATCC21721), (b) *K. pneumoniae* (ATCC31488) and (c) *S. pyogenes* (clinical isolate). All bioassays were performed in at least triplicate and are expressed as mean \pm SEM. * = results 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 ($p < 0.01$).

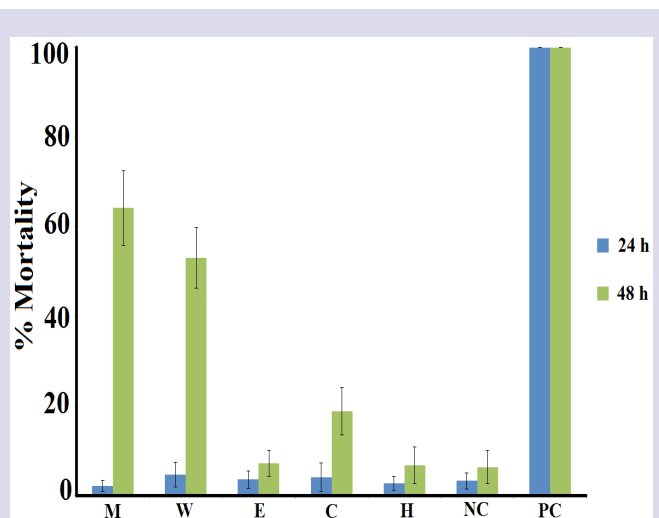


Figure 7: The lethality of the *A. moluccanus* nut extracts (2000 µg/mL), potassium dichromate (1000 µg/mL) and a seawater control. Blue bars represent the % mortality following 24 h exposure; green bars represent the % mortality following 48 h exposure; M = methanolic extract; W = aqueous extract; E = ethyl acetate extract; C = chloroform extract; H = hexane extract; NC = negative (seawater) control; PC = positive control (1000 µg/mL potassium dichromate). All bioassays were performed in at least triplicate and are expressed as mean \pm SEM.

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 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.⁴⁰

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 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 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.⁴³ 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 *A. moluccanus* nut extracts to block microbial triggers of 3 autoimmune inflammatory disorders (*Proteus* spp.: rheumatoid arthritis; *K. pneumoniae*: ankylosing spondylitis; *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 all bacterial autoimmune disease triggers, and were usually more potent growth inhibitors than were the ampicillin and chloramphenicol antibacterial controls.

A high antioxidant capacity has been postulated as being responsible for the medicinal properties of many plants.²⁶⁻⁴⁴ In particular, antioxidants

have been linked to antibacterial, antifungal and antiviral activities, as well as anticancer properties.²⁶⁻⁴⁴ However, other studies have indicated that antioxidants may protect cells from oxidative stress and thus protect against cell death.^{45,46} Whilst those studies examined the effects of antioxidants on eukaryotic cells, it is possible that antioxidants may have a similar protective effect against bacterial cell death. Interestingly, a previous study has reported only low antioxidant capacities for *A. moluccanus* nut essential oils.²⁵ This correlates well with previous studies which have linked low antioxidant content with antibacterial potency in extracts produced from different plant species.^{47,48} Those studies also indicated that there was a trend for lower growth inhibitory efficacy for the high antioxidant extracts and indeed, some high antioxidant extracts instead appeared to induce bacterial growth. The antioxidant capacity of the *A. moluccanus* nut solvent extracts were not determined in our study, although it is likely that they would be consistent with the essential oil study,²⁵ although further studies are required to confirm this.

Whilst a detailed investigation of the phytochemistry of the *A. moluccanus* nut extracts was beyond the scope of our study, qualitative screening studies were used to determine the classes of compounds present. Our qualitative phytochemical screening studies indicate that polyphenolics, saponins and alkaloids were present in the most potent *A. moluccanus* nut extracts. Studies have been linked polyphenolic compounds and alkaloids with anti-bacterial activity in different plant species²⁶ 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 signalling.²⁶ 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.

Although these studies have demonstrated the potential of the *A.*

mollucanus nut extracts to prevent and treat autoimmune disease, much more work is required. This study has only tested these extracts against microbial triggers of 3 autoimmune diseases (rheumatoid arthritis, ankylosing spondylitis and rheumatic heart disease). The microbial triggers for several other autoimmune inflammatory disorders are also known. *Acinetobacter baylyi* and *Pseudomonas aeruginosa* have been linked with the onset of multiple sclerosis.⁸ *Borrelia burgdorferi* is linked with Lyme disease⁹ and *Mycoplasma pneumoniae* is associated with several demyelinating diseases.¹⁰ 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.

The findings reported here also demonstrate that all of the *A. mollucanus* nut extracts were nontoxic towards *Artemia franciscana* nauplii, with LC₅₀ values substantially > 1000 µg/mL. Extracts with LC₅₀ values > 1000 µg/mL towards *Artemia* nauplii are defined as being nontoxic.⁴⁰ 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, whilst these studies have demonstrated the potential of the *A. mollucanus* nut extracts in the development of future antibiotic chemotherapeutics for the prevention and treatment of autoimmune diseases (particularly rheumatoid arthritis, ankylosing spondylitis and rheumatic heart disease), 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 *A. mollucanus* nut as inhibitors of the growth of bacterial species associated with the onset of rheumatoid arthritis, ankylosing spondylitis and rheumatic heart disease. 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.

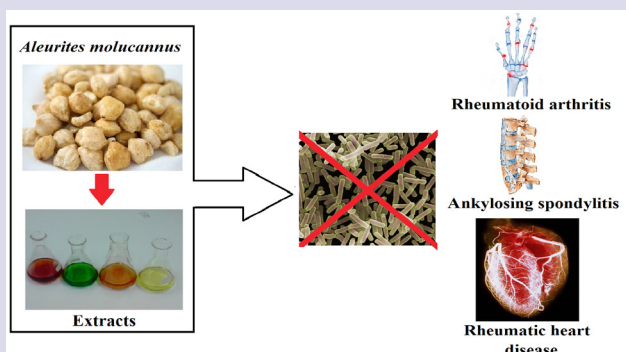
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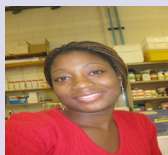
PICTORIAL ABSTRACT



SUMMARY

- *C. olivei* leaf extracts displayed broad spectrum antibacterial activity against gram positive and gram negative bacteria.
- Methanolic, aqueous and ethyl acetate extracts were potent inhibitors of *P. mirabilis* growth (MIC values as low as 127 µg/mL).
- *E. coli*, *K. pneumoniae* and *B. cereus* were also particularly susceptible (MICs substantially <1000 µg/mL).
- All *C. olivei* leaf extracts were nontoxic in the *Artemia* nauplii bioassay.

ABOUT AUTHORS



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



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 AUSTRALIAN 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.