

Melodorum leichhardtii (F.Muell.) Benth. Extracts Inhibit the Growth of Klebsiella pneumoniae

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ABSTRACT

Introduction: *Melodorum leichhardtii* F.Muell. Benth. is a rainforest vine which is native to north-eastern rainforest regions of Australia. *Melodorum* spp. are rich in a variety of terpenoids with reported antibacterial activity. Despite this, *M. leichhardtii* leaf extracts have not previously been examined for bacterial growth inhibitory properties. **Methods:** The antimicrobial activity of a methanolic *M. leichhardtii* leaf extract was investigated by disc diffusion and growth time course assays against a panel of bacterial pathogens. The growth inhibitory activity was further quantified by MIC determination. Toxicity was determined using the *Artemia franciscana* nauplii bioassay. **Results:** The methanolic *M. leichhardtii* leaf extract was a potent inhibitor of *Klebsiella pneumoniae* (MICs of 388µg/mL). The extract was also a low-moderate inhibitor of *B. cereus* growth (MICs 3678µg/mL) but was completely devoid of inhibitory activity against all other bacterial species tested. The antibacterial activity of the methanolic *M. leichhardtii* leaf extract was further investigated by growth time course assays that showed significant growth inhibition in cultures of *K. pneumoniae* and *B. cereus* 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 diseases caused by these pathogens. **Conclusion:** The lack of toxicity of the *M. leichhardtii* leaf extracts and their growth inhibitory bioactivity against *K. pneumoniae* and *B. cereus* indicate their potential in the development of new therapies targeting these bacteria.

Key words: Annonaceae, Zig-zag vine, Australian plant, Traditional medicine, Antibacterial, Ankylosing spondylitis, Eudesmol.

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INTRODUCTION

Plants produce a wide variety of secondary compounds, which provide them characteristic pigment, odour and flavour characteristics, may also give them antimicrobial properties.¹ Traditional plant derived medicines have been used in most parts of the world for a variety of therapeutic purposes, including fighting microbial disease. Indeed, the ability of plant extracts to block the growth of pathogenic bacteria has become a focus of recent study.²⁻³ Much of the research into traditional medicinal plant use has focused on Asian,⁴ African⁵ and South American⁶ plants. However, the therapeutic potential of the flora of Australia has also received recent attention. The first Australians had well-developed medicinal systems and understood the therapeutic properties of a wide variety of Australian plants and how to use them effectively.⁷ Despite this, relatively few studies have rigorously examined the antibacterial activity of Australian native plants, although there has recently been a substantial increase in interest in this field.

Melodorum leichhardtii (F.Muell.) Benth. (family Annonaceae; commonly known as zig-zag vine, acid drop vine, merangara, wild banana; synonyms *Rauwenhoffia leichhardtii* (F.Muell.) Diels, *Fissistigma leichhardtii* (F.Muell.) Merr., *Unona Leichardtii* F.Muell.) is a vigorous climbing shrub which is native to rainforest regions of north eastern Australia. The vine has dark green alternate leaves which are lanceolate, with entire margins and grow 5-20cm long by 2-7cm wide (Figure 1a). Orange/brown flowers form opposite the leaves (Figure 1b) and develop into fleshy orange peanut shaped fruit (Figure 1c). The fruit are edible and have a pleasant acidic flavour. The first Australians consumed these fruit fresh off the forest floor. The stems and bark of this species was used treat some tumours and have been shown to be effective at inhibiting the proliferation of some cell lines.⁸

The leaves have been reported to be particularly rich in sesquiterpenoids, including α -eudesmol (Figure 1d), β -eudesmol (Figure 1e), β -carophyllene (Figure 1f) and bicyclogermacrene (Figure 1g), as well as the monoter-

penoid α -pinene (Figure 1h).⁹ Interestingly, many of these terpenoids have potent antibacterial activity¹⁰ and this species may therefore also be useful in blocking bacterial infections. Despite this, antibacterial studies of *M. leichhardtii* leaves are lacking. The current study was undertaken to screen of *M. leichhardtii* leaf extracts for growth inhibitory properties against a panel of pathogenic bacteria.

MATERIALS AND METHODS

Plant collection and extraction

Melodorum leichhardtii (F.Muell.) Benth. 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 1g quantities were weighed into separate tubes. A volume of 50mL methanol (Ajax Fine Chemicals, Australia) was added and the plant material was extracted for 24 hrs at 4°C with gentle shaking. The extract was filtered through filter paper (Whatman No. 54) under vacuum, followed by drying by rotary evaporation in an Eppendorf concentrator 5301. The resultant pellet was dissolved in 10mL sterile deionised water (containing 1% DMSO). The extracts were passed through 0.22µm filter (Sarstedt) and stored at 4°C until use.

Qualitative phytochemical studies

Phytochemical analysis of the *M. leichhardtii* leaf extracts for the presence of saponins, phenolic compounds, flavonoids, phytosteroids, triterpenoids, cardiac glycosides, anthraquinones, tannins and alkaloids was conducted by standard assays.¹¹⁻¹³

Antibacterial screening

Test microorganisms

All media was supplied by Oxoid Ltd., Australia. The reference strain of *Klebsiella pneumoniae* (ATCC31488) was purchased from American

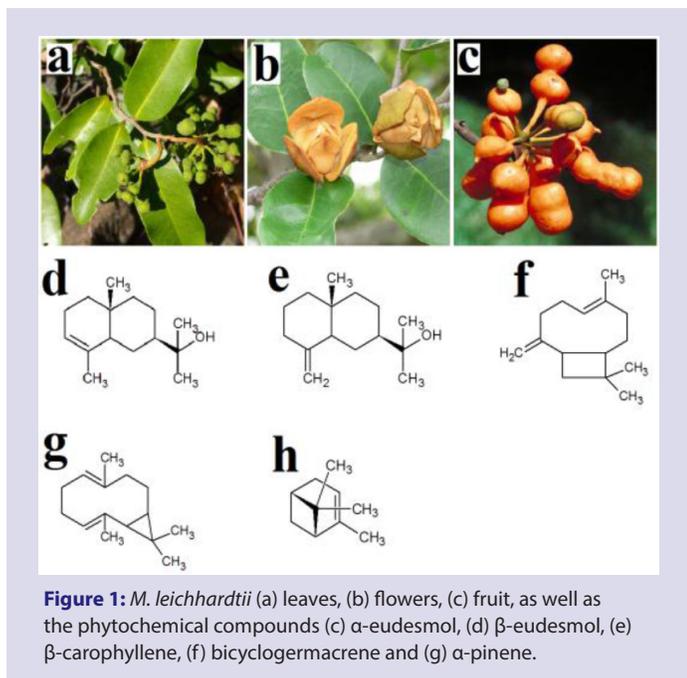


Figure 1: *M. leichhardtii* (a) leaves, (b) flowers, (c) fruit, as well as the phytochemical compounds (d) α-eudesmol, (e) β-eudesmol, (f) β-carophyllene, (g) α-pinene.

Tissue Culture Collection, USA. All other bacterial species were clinically isolated strains and 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 *M. leichhardtii* extracts was determined using a modified disc diffusion assay.¹⁴⁻¹⁶ Briefly, 100µL of each bacterial culture was grown in 10mL of fresh nutrient broth until they reached a count of ~10⁸ cells/mL. A volume of 100µL of the bacterial suspension was spread onto nutrient agar plates and the extracts were tested for antibacterial activity using 5mm sterilised filter paper discs. Discs were infused with 10µL of the plant extract, 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 zones of inhibition (ZOIs) were measured to the closest whole millimetre. Each assay was performed three times in triplicate (n=9). Mean values (± SEM) are reported in this study. Standard discs of ampicillin (10µg) and chloramphenicol (10µg) were obtained from Oxoid, Australia and were used as positive controls to compare antibacterial activity. Filter discs infused with 10µL of distilled water were used as a negative control.

Minimum inhibitory concentration (MIC) determination

The minimum inhibitory concentration (MIC) of each extract against susceptible bacteria was determined as previously described.¹⁷⁻¹⁸ Briefly, the methanolic *M. leichhardtii* leaf extract was diluted in deionised water and tested across a range of concentrations. Discs were infused with 10µL of the test dilutions, allowed to dry and placed onto the 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.¹⁹ Briefly, 3mL of *Bacillus cereus* (clinical isolate) and *Klebsiella pneumoniae* (ATCC31488) in nutrient broth were individually added to 27mL nutrient broth containing 3mL of 10mg/mL of the extract to give a

final concentration of 1000µg/mL in the assay. The tubes were incubated at 30°C with gentle shaking. The optical density was measured hourly at 550nm 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 4mg/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.²⁰⁻²² Briefly, 400µL of seawater containing approximately 48 (mean 47.6, n = 125, SD 13.2) *A. franciscana* nauplii were added to wells of a 48 well plate and immediately used for bioassay. A volume of 400µL 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 400µL 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 LC₅₀ 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. One way ANOVA was used to calculate statistical significance between control and treated groups with a *P* value < 0.01 considered to be statistically significant.

RESULTS

Liquid extraction yields and qualitative phytochemical screening

Extraction of 1g of dried and powdered *M. leichhardtii* leaves with methanol yielded 135mg of dried extract (Table 1). The extract was resuspended in 10mL of deionised water (containing 1% DMSO), resulting in an extract concentration of 13.5mg/mL. Qualitative phytochemical studies showed that the extract contained high levels of phenolics, phytosterols and flavonoids, as well as moderate levels of tannins and triterpenoids. Lower levels of alkaloids were also detected in the extract whilst cardiac glycosides and saponins were completely absent or below the detection thresholds for these assays.

Antimicrobial activity

To determine the growth inhibitory activity of the *M. leichhardtii* leaf extract against a panel of human bacterial pathogens, aliquots (10µL) of each extract were screened in the disc diffusion assay. The *M. leichhardtii* leaf extract only inhibited the growth of *K. pneumoniae* and *B. cereus* and was completely ineffective against all other bacterial species (Figure 2). The inhibition of *K. pneumoniae* was particularly noteworthy, with a zone of inhibition of 21 ± 1.8mm determined for this bacterium. The potency of this extract compared well with that of the positive control antibiotics ampicillin and chloramphenicol, each of which were tested at high doses (10µg/disc). Indeed, the *M. leichhardtii* extract produced similar sizes zones of inhibition (ZOIs) as the chloramphenicol discs (21.3 ± 1.3mm) and substantially larger than the ampicillin ZOI (10.3 ± 0.6mm).

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

Extract	Mass of Dried Extract (mg)	Resuspended Extract (mg/mL)	Total Phenolics	Phenols	Cardiac Glycosides	Saponins	Triterpenes	Phytosterols	Alkaloids	Flavanoids	Tannins	Anthraquinones							
	135	13.5	Water Soluble	Water Insoluble	Keller-Kiliani Test	Froth Persistence	Emulsion test	Salkowski Test	Acetic Anhydride Test	Meyers Test	Wagners Test	Draggendorffs Test	Shinoda Test	Kumar test	Ferric Chloride Test	Lead Acetate Test	Free	Combined	
			+++	+++	+++	-	-	-	++	+++	++	+	+	++	+++	++	+	+	-

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

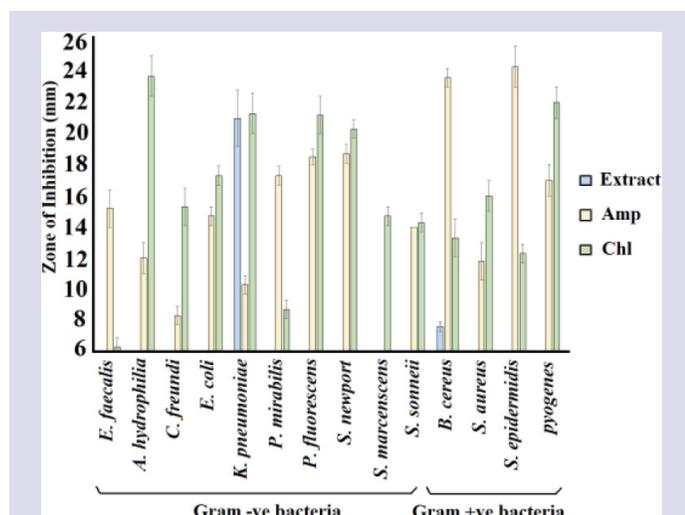


Figure 2: Growth inhibitory activity of *M. leichhardtii* leaf extracts and reference antibiotics against various bacterial species measured as zones of inhibition (mm) \pm SEM.

Blue bars represent the growth inhibition activity of the *M. leichhardtii* extract; Yellow bars represent the growth inhibition activity of ampicillin standard discs (10 μ g); Green bars represent the growth inhibition activity of chloramphenicol standard discs (10 μ g); Amp = ampicillin; Chl = chloramphenicol. All assays were completed three times, each with internal triplicates (n=9) and the results are expressed as mean zones of inhibition (mm) \pm SEM.

The antimicrobial efficacy was further quantified by determining the MIC value. *K. pneumoniae* was particularly susceptible to the methanolic *M. leichhardtii* leaf extract with an MIC value of 388 μ g/mL (<4 μ g infused into the disc). As *K. pneumoniae* can trigger ankylosing spondylitis in genetically susceptible people,²³⁻²⁵ this extract may be useful for preventing this disease (and other diseases caused by this bacterium). The extract also showed moderate growth inhibitory activity towards *B. cereus*, with an MIC of 3678 μ g/mL (~37 μ g infused into the disc).

Bacterial growth time course assay

The antibacterial activity of the *M. leichhardtii* leaf extracts was further investigated in *B. cereus* (Figure 3a) and *K. pneumoniae* (Figure 3b) by bacterial growth time course assays in the presence and absence of the methanolic *M. leichhardtii* extract. The starting concentration of the extract used in these assays was 1000 μ g/mL. The *M. leichhardtii* extract significantly inhibited *B. cereus* (Figure 3a) and *K. pneumoniae* (Figure 3b) growth within 1 h, indicating a rapid antimicrobial action. The absorbance of the *B. cereus* culture (and thus the bacterial growth) had returned to similar levels to that of the untreated control by the end of the 6 hr incubation period. This may indicate that the *M. leichhardtii* extract has bacteriostatic effects on *B. cereus*. In contrast, the growth of *K. pneumoniae* (Figure 3b) by the methanolic *M. leichhardtii* leaf extracts was still significantly inhibited by the end of the 6 h time course study. Indeed, the turbidity had not significantly increased throughout the 6 hr growth period, indicating the extract may be bacteriocidal towards *K. pneumoniae* at the dose tested.

Quantification of toxicity

The toxicity of the *M. leichhardtii* extracts was initially tested undiluted in the *Artemia franciscana* nauplii bioassay (Figure 4). The mortality in the presence of the extract was not significantly different to that of the untreated control at 24 h and thus were deemed to be non-toxic. Extracts with 24 hr LC₅₀ values >1000 μ g/mL have previously been defined as non-toxic.²² In contrast, the potassium dichromate positive control induced substantial mortality within 4 h (results not shown), with 100 % mortality induction seen by 24 h. By 48 h, the mortality induction had also increased for the *M. leichhardtii* extract. However, the % mortality was still substantially <1000 μ g/mL.

DISCUSSION

Plant derived remedies are 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 some current drug regimens to effectively treat disease. Our study reports on the growth inhibitory properties of a methanolic *M. leichhardtii* leaf extract against

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

	Bacteria	Exposure time (h)	MIC or LC_{50} ($\mu\text{g/mL}$)
Bacterial growth inhibition	<i>K. pneumoniae</i>	24	388
	<i>B. cereus</i>	24	3678
Toxicity		24	CND
	LC_{50} ($\mu\text{g/mL}$)	48	CND
		72	946

Numbers indicate the mean MIC or LC_{50} values of three independent experiments in triplicate (n=9). CND indicates that an LC_{50} could not be determined as the mortality did not exceed 50% at any concentration tested.

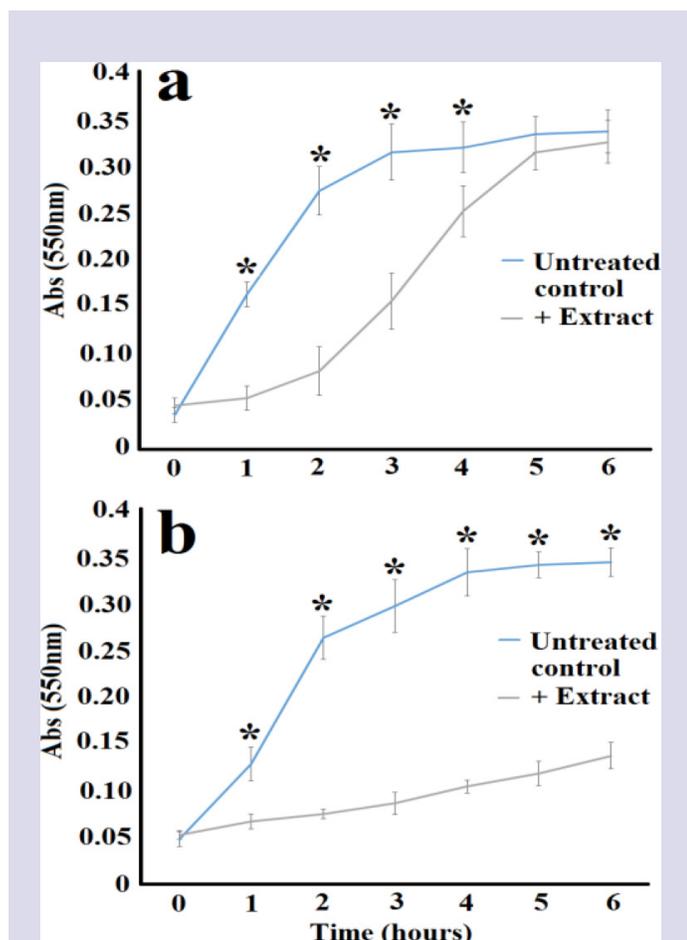


Figure 3: Bacterial growth curves for the *M. leichhardtii* extract against (a) *B. cereus* (clinical isolate) and (b) *K. pneumoniae* (ATCC31488). All bioassays were performed three times in triplicate (n=9) and are expressed as mean \pm SEM. * = results that are significantly different between the treated and the untreated control growth ($P < 0.01$).

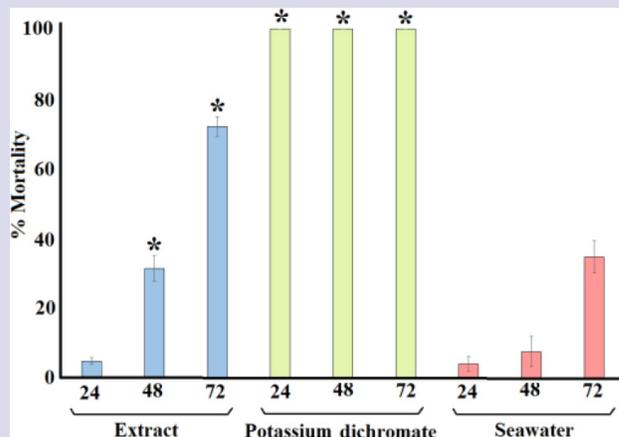


Figure 4: The lethality of the *M. leichhardtii* leaf extracts, potassium dichromate control (1000 $\mu\text{g/mL}$) and seawater (negative control). Blue bars represent the % mortality following exposure to the extract; green bars represent the % mortality following exposure to potassium dichromate; red bars represent the % mortality following exposure to artificial seawater. 24, 48 and 72 refers to the time of exposure in hrs. All bioassays were performed three times in triplicate (n=9) and are expressed as mean \pm SEM. * indicates results that are significantly different to the untreated (seawater) control at the equivalent exposure time ($P < 0.01$).

a panel of pathogenic bacteria and on its toxicity. The extract was a particularly potent inhibitors of *K. pneumoniae* growth, with an MIC value of 388 $\mu\text{g/mL}$. As *K. pneumoniae* can trigger ankylosing spondylitis in genetically susceptible individuals,²³⁻²⁵ this extract may be useful in the prevention and treatment of the autoimmune disease. Whilst not as potent, the *M. leichhardtii* extract was also a moderate inhibitors of *B. cereus* growth (MICs 3678 $\mu\text{g/mL}$). Therefore, the *M. leichhardtii* extract also has therapeutic potential in the treatment of food poisoning, diarrhoea and dysentery. None of the other bacterial species tested were inhibited by the *M. leichhardtii* extract, indicating that compounds in the extracts function via specific mechanisms, rather than via general membrane perturbations.

Whilst a detailed investigation of the phytochemistry of the *M. leichhardtii* leaf extract was beyond the scope of our study, qualitative screening studies were used to determine the classes of compounds present. Notably, the extract contained relatively high levels of total phenolics, flavonoids, tannins, triterpenoids and phytosterols. It is likely that these and other phytochemical classes may contribute to the growth inhibitory properties of the *M. leichhardtii* extract. Our qualitative phytochemical screening studies also indicated that alkaloids were also present in the extract, albeit in low relative abundance. Many studies have reported potent antibacterial activities for a wide variety of these compounds.¹⁰ Further phytochemical evaluation studies and bioactivity driven isolation of active components is required to further evaluate the mechanism of bacterial growth inhibition.

The findings reported here also demonstrate that the *M. leichhardtii* leaf extract was non-toxic towards *Artemia franciscana* nauplii, with LC_{50} values substantially $> 1000\mu\text{g/mL}$. Extracts with LC_{50} values $> 1000\mu\text{g/mL}$ towards *Artemia* nauplii are defined as being nontoxic.²² 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 *M. leichhardtii* leaf extracts in the

development of future antibiotic chemotherapeutics (particularly for the prevention and of ankylosing spondylitis in genetically susceptible people, as well as other disease caused by *K. pneumoniae* infections), more work is required to isolate the inhibitory components and determine the mechanism of inhibition. Whilst these studies have demonstrated the potential of the methanolic *M. leichhardtii* leaf extract in the development of future antibiotic chemotherapeutics, more work is required to isolate the inhibitory components and determine the mechanism of inhibition.

CONCLUSION

The results of this study demonstrate the potential of the *M. leichhardtii* leaf extracts as an inhibitor of the growth of some pathogenic bacteria (especially *K. pneumoniae*). Furthermore, their lack of toxicity indicates that they are safe for internal as well as topical treatment. Further studies aimed at the purification and identification of bioactive components are required to examine the mechanisms of action of these agents.

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CONFLICT 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; **ZOI:** Zone of inhibition.

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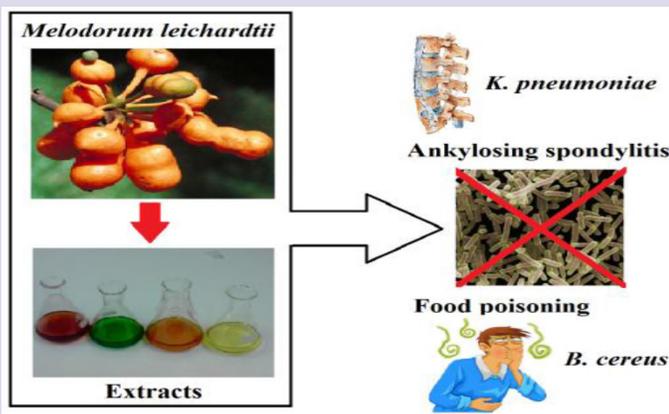
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SUMMARY

- M. leichhardtii* methanolic leaf extract displayed antibacterial activity against *B. cereus* and *K. pneumoniae*.
- The extract was a particularly potent inhibitor of *K. pneumoniae* growth (MIC 388 µg/mL).
- B. cereus* was also susceptible to the extract, albeit with substantially higher MIC values (3678 µg/mL).
- The methanolic *M. leichhardtii* leaf extract was nontoxic in the *Artemia* nauplii bioassay.

PICTORIAL ABSTRACT



ABOUT AUTHORS



Author profile (Getmore Rumbudzai Chikowe):

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.

Author profile (Lindiwe Nomathemba Mpala):

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



Author profile (Dr Ian Cock): 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.