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Original Article

Growth inhibitory properties of *Backhousia myrtifolia* Hook. & Harv. and *Syzygium anisatum* (Vickery) Craven & Biffen extracts against a panel of pathogenic bacteria

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ABSTRACT

Introduction: *B. myrtifolia* and *S. anisatum* are native Australian trees with uses as traditional medicines and bushfoods. Essential oils produced from leaves of these species have reputed antiseptic properties. Despite this, *B. myrtifolia* and *S. anisatum* leaf solvent extractions have not been rigorously examined for antibacterial properties against many pathogens. **Methods:** The antimicrobial activity of aqueous and methanolic leaf extracts of *B. myrtifolia* and *S. anisatum* was investigated by disc diffusion and growth time course assays against a panel of pathogenic bacteria. The growth inhibitory activity was quantified by MIC determination. Toxicity was determined using the *Artemia franciscana* nauplii bioassay. **Results:** Methanolic and aqueous *B. myrtifolia* and *S. anisatum* leaf extracts inhibited the growth of a wide range of bacterial species. Growth of both gram positive and gram negative bacteria was inhibited the *B. myrtifolia* and *S. anisatum* extracts to approximately the same extent. In general, *S. anisatum* extracts were more potent inhibitors of bacterial growth than were the *B. myrtifolia* extracts, and (with some noteable exceptions) the methanolic extracts were generally more potent than the aqueous extracts. The *B. myrtifolia* and *S. anisatum* extracts were particularly potent inhibitors of *P. mirabilis* growth, with MIC values as low as 105 µg/mL (aqueous *S. anisatum* extract). The antibacterial activity of the methanolic *B. myrtifolia* and *S. anisatum* extracts were further investigated by growth time course assays which showed significant growth inhibition in cultures of *E. coli, K.* pneumpniae and *P. mirabilis* within 1 h of exposure. All extracts were determined to be nontoxic in the *Artemia franciscana* nauplii bioassay, indicating their safety for internal use as well as for topical uses. **Conclusions:** The lack of toxicity of the *B. myrtifolia* and *S. anisatum* extracts and their growth inhibitory bioactivity against a panel of pathogenic bacteria partially validate Australian Aboriginal usage of these species as antiseptic agent and indicate their potential in the development of antiseptic agents.

Key words: *Backhousia myrtifolia*, *Syzygium anisatum*, Cinnamon myrtle, Anise myrtle, Australian plants, Antibacterial activity, Medicinal plants.

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INTRODUCTION

The therapeutic potential of the aromatic flora of Australia has been recognised for many thousands of years. The first Australians had well-developed ethnopharmacological systems and understood the therapeutic properties of a wide variety of aromatic Australian plants.[1] More recently, Sir Joseph Banks (the botanist aboard Captain James Cook's voyage of the Endeavour) coined the phrase tea tree' for the endemic Australian plant *Melaleuca alternifolia* due to similarities in use with *Camellia sinensis*, and some of its perceived therapeutic properties.[1] Nowadays, a thriving trade exists, with 'Tea Tree' essential oils marketed globally. Similarly, the therapeutic properties of *Eucalyptus* spp. are well documented.[2,3] Plants of the genus *Eucalyptus* are now recognised for their potent antimicrobial activities and are commonly used as topical antiseptics. More recently, the bacterial growth inhibitory properties of many genera within the family *Myrtaceae* have been documented. In particular, *Callistemon* spp.,[4] *Eugenia* spp.,[1] *Kunzea* spp.,[1,5] *Leptospermum*[1] and *Syzygium* spp.[6-8] have been reported to inhibit the growth of a wide panel of bacteria, including many medicinally important pathogens. Despite this, the antibacterial properties of several *Myrtaceae* are yet to be rigorously examined.

Backhousia myrtifolia Hook. & Harv. (Figure 1a) is a medium tree which is native to subtropical rainforest areas of eastern Australia. The leaves of this plant are dried to produce 'cinnamon myrtle', which is used as an herb and food flavouring.[9] Studies in our laboratory[10] and elsewhere[11,12] have demonstrated the antibacterial activity of leaves of the taxonomically related species *Backhousia citriodora* F. Muell. (commonly known as lemon myrtle). Interestingly, no reports of Aboriginal medicinal use of either *B. citriodora* or *B. myrtifolia* were found in the literature, although the leaves of both species were used in cooking. Most of the studies of *Backhousia* spp. antibacterial potential focus on the essential oil of the leaves.[11,12] In most plants of this genus, the levels and composition of the essential oil terpenoid components receives the most interest. In particular, the monoterpenoid composition of *Backhousia* spp. has been extensively reported. *B. citriodora* essential oils contain more than 90% citral, which comprises a mixture of neral (α-citral; Figure 1c) and geranial (β-citral; Figure 1d).[13] Both neral and geranial have been previously reported to have potent antibacterial activity against a variety of bacteria.[11,14,15] Similarly, the high monoterpenoid composition of *B. myrtifolia* essential oils is known, with methyl eugenol (Figure 1e) and elemicin (Figure 1f) recognised as the major components. The antiseptic properties of these compounds is also well established.[16]

Syzygium anisatum (Vickery) Craven & Biffen (Figure 1b; formerly *Backhousia anisata*; commonly known as anise myrtle) is a related species which has a similar but limited occurrence in eastern Australian subtropical rainforests.[1] As for *Backhousia* spp., *S. anisatum* is used as a bushfood spice and the leaves are distilled for the essential oil, which is rich in anethole (anise camphor; Figure 1g) and chavicol (Figure 1h). Anethole and chavicol have potent bactericidal activity, with minimum bactericidal activities (MBC) as low as 100 µg/mL.[17] Despite this, studies into the antibacterial properties of *S. anisatum* extracts towards many bacterial pathogens are lacking.

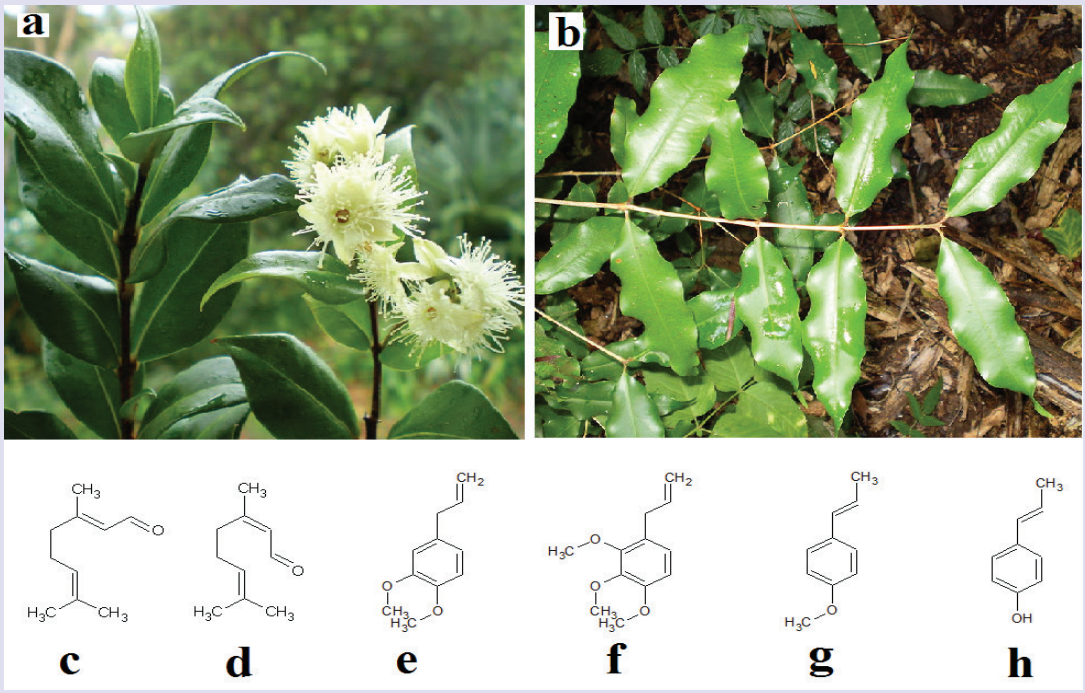

Figure 1: (a) *B. myrtifolia* and (b) *S. anisatum* leaves as well as the chemical structures of the terpenoid components (c) neral (α-citral), (d) geranial (β-citral), (e) methyl eugenol, (e) elemicin, (g) anethole and (h) chavicol.

Most of the studies reporting antibacterial properties for *Backhousia* spp. and *Syzygium* spp. have examined essential oils. The use of essential oils for the testing of antimicrobial activity can be problematic. The relative insolubility of many of the oil components retards their diffusion through agar gels in agar dilution or disc diffusion studies. Many studies have utilised solubilising agents (eg. Tween 80) to aid oil component diffusion, resulting in variable results.[18,19] Solubilising agents appear to increase the susceptibility of some bacteria to antimicrobial agents, decrease the susceptibility of others, whilst having no effect on yet other bacteria. Recent studies have reported the antibacterial activity of methanolic extracts of *B. citriodora* leaves against a panel of bacteria.[10] The current report was undertaken to extend those studies to examine 2 taxonomically related *Myrtaceae* spp. (*B. myrtifolia* and *S. anisatum*) for similar growth inhibitory properties against a panel of pathogenic bacteria.

MATERIALS AND METHODS

Plant collection and extraction

Backhousia myrtifolia Hook. & Harv. and *Syzygium anisatum* (Vickery) Craven & Biffen leaves were obtained from and identified by Philip Cameron, senior botanic officer, Mt Cootha Botanical Gardens, Brisbane, Australia. Leaf samples were dried in a Sunbeam food dehydrator and stored at -30ºC. Prior to use, the leaves were freshly ground to a coarse powder and 1 g quantities were weighed into separate tubes. A volume of 50 mL methanol (Ajax, Australia; AR grade) or sterile deionised water were added to individual tubes and extracted for 24 hrs at 4ºC with gentle shaking. The extracts was 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 4ºC until use.

Qualitative phytochemical studies

Phytochemical analysis of the *B. myrtifolia* and *S. anisatum* leaf extracts for the presence of saponins, phenolic compounds, flavonoids, phytosteroids, triterpenoids, cardiac glycosides, anthraquinones, tannins and alkaloids was conducted by previously described assays.[20-21]

Antibacterial screening

Test microorganisms

All media was supplied by Oxoid Ltd., Australia. Reference strains of *Klebsiella pneumoniae* (ATCC31488), *Escherichia coli* (ATCC 25922) and *Proteus mirabilis* (ATCC21721) were purchased from American Tissue Culture Collection, USA. Clinical isolate microbial strains of *Aeromonas hydrophilia*, *Alcaligenes feacalis*, *Bacillus cereus*, *Citrobacter freundii*, *E. coli*, *K. pneumoniae*, *P. mirabilis*, *Pseudomonas fluorescens*, *Salmonella newport, Serratia marcescens*, *Shigella sonneii*, *Staphylococcus aureus, Staphylococcus epidermidis* and *Streptococcus pyonenes* were obtained from Ms Michelle Mendell and Ms Jane Gifkins, Griffith University. All stock cultures were subcultured and maintained in nutrient broth at 4ºC.

Evaluation of antimicrobial activity

Antimicrobial activity of all plant extracts was determined using a modified disc diffusion assay.[22-24] Briefly, 100 µL of each bacterial culture was grown in 10 mL of fresh nutrient broth until they reached a count of ~10^8 cells/mL. A volume of 100 µL of the bacterial suspension was spread onto nutrient agar plates and extracts were tested for antibacterial activity using 5 mm sterilised filter paper discs. Discs were infused with 10 µL of the plant extracts, allowed to dry and placed onto the inoculated plates. The plates were allowed to stand at 4°C for 2 h before incubation at 30°C for 24 h. The diameters of the inhibition zones were measured to the closest whole millimetre. Each assay was performed in at least triplicate. Mean values (± SEM) are reported in this study. Standard discs of ampicillin (10 µg) were obtained from Oxoid, Australia and were used

Table 1: The mass of dried extracted material, the concentration after resuspension in deionised water and qualitative phytochemical screenings of the aqueous and methanolic *B. myrtifolia* and *S. anisatum* extracts

Plant Species	Extract	Mass of Dried Extract (mg)	Concentration of Resuspended Extract (mg/mL)	Total Phenolics	Water Soluble Phenolics	Water Insoluble Phenolics	Cardiac Glycosides	Saponins	Triterpenes	Phytosteroids	Alkaloids (Mayer Test)	Alkaloids (Wagner Test)	Flavonoids	Tannins	Free Anthraquinones	Combined Anthraquinones
B. myrtifolia	M	253	25.3	+++	+++	+	-	++	++	-	-	-	+	++	-	-
	W	178	17.8	+++	+++	++	-	+	+	-	-	-	+	+	-	-
S. anisatum	M	165	16.5	+++	+++	++	-	+	++	-	-	-	+	++	-	-
	W	118	11.8	+++	+++	++	-	+	++	-	-	-	+	++	-	-

+++ indicates a large response; ++ indicates a moderate response; + indicates a minor response; - indicates no response in the assay. M=methanolic extract; W=aqueous extract.

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.[25,26] Briefly, the plant 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.[27] Briefly, 3 mL of the ATCC reference bacterial cultures (*Klebsiella pneumoniae* (ATCC31488), *Escherichia coli* (ATCC 25922) and *Proteus mirabilis* (ATCC21721)) in nutrient broth were added to 27 mL nutrient broth containing 3 mL of 10 mg/mL methanolic 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 ($K_2Cr_2O_7$) (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.[28-30] Briefly, 400 µL of seawater containing approximately 46 (mean 46.2, n=125, SD 12.5) *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 400 µL seawater negative control was run in triplicate for each plate. All treatments were performed in at least triplicate. The wells were checked at regular intervals and the number of dead counted. The nauplii were considered dead if no movement of the appendages was detected within 10 seconds. After 24 h, all nauplii were sacrificed and counted to determine the total % mortality per well. The LC_{50} with 95% confidence limits for each treatment was determined using probit analysis.

Statistical analysis

Data are expressed as the mean ± SEM of at least three independent experiments. 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 *B. myrtifolia* and *S. anisatum* leaves with methanol or deionised water yielded dried extracts ranging from 118 mg (aqueous *S. anisatum* extract) to 253 mg (methanolic *B. myrtifolia* extract) (Table 1). Higher extraction yields were determined for both the *B. myrtifolia* extracts than for the corresponding *S. anisatum* extracts. 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 all solvents extracted similar classes of phytochemicals. All had high levels of phenolics (high levels of water soluble phenolics, moderate levels of water insoluble phenolics). Generally, all extracts also contained moderate levels of triterpenoids and tannins, as well as low levels of flavonoids. All extracts were generally devoid of all other classes of phytochemicals.

Antimicrobial activity

To determine the growth inhibitory activity of the *B. myrtifolia* and *S. anisatum* extracts against the panel of pathogenic bacteria, aliquots

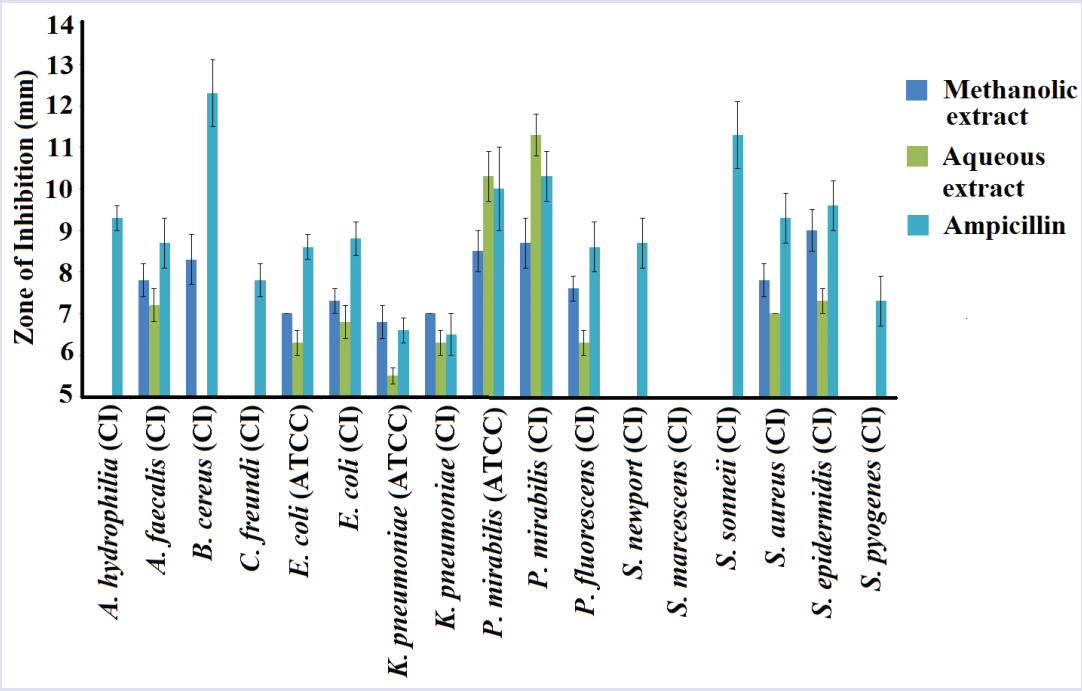

Figure 2: Growth inhibitory activity of aqueous and methanolic *B. myrtifolia* leaf extracts against the clinical isolate (CI) and reference (ATCC) bacterial strains measured as zones of inhibition (mm) ± SEM. Blue bars represent inhibition by methanolic *B. myrtifolia* extracts; green bars represent inhibition by aqueous *B. myrtifolia* extracts; aqua bars represent the inhibition by the ampicillin control. Amp = ampicillin (10 µg) control. All determinations were in at least triplicate and the results are expressed as mean zones of inhibition (mm) ± SEM.

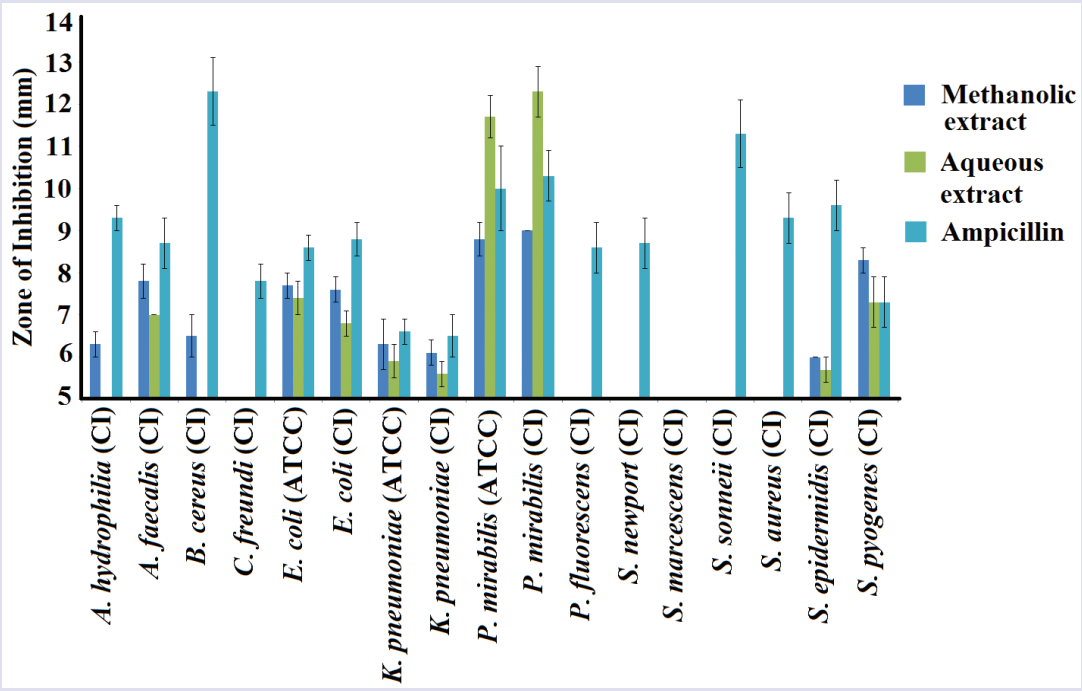

Figure 3: Growth inhibitory activity of aqueous and methanolic *S. anisatum* leaf extracts against the clinical isolate (CI) and reference (ATCC) bacterial strains measured as zones of inhibition (mm) ± SEM. Blue bars represent inhibition by methanolic *S. anisatum* extracts; green bars represent inhibition by aqueous *S. anisatum* extracts; aqua bars represent the inhibition by the ampicillin control. Amp = ampicillin (10 µg) control. All determinations were in at least triplicate and the results are expressed as mean zones of inhibition (mm) ± SEM.

Table 2: Minimum bacterial growth inhibitory concentration (µg/mL) of the methanolic and aqueous *B. myrtifolia* and *S. anisatum* extracts

Bacterial Species	Strain	*B. myrtifolia*		*S. anisatum*	
		M	W	M	W
A. hydrophilia	Clinical isolate	-	-	>10,000	-
A. feacalis	Clinical isolate	5081	5405	1792	3284
B. cereus	Clinical isolate	3700	-	>10,000	-
C. freundii	Clinical isolate	-	-	-	-
E. coli	ATCC:25922	>10,000	>10,000	893	3363
E. coli	Clinical isolate	>10,000	>10,000	1255	3884
K. pneumoniae	ATCC:31488	>10,000	>10,000	4339	>10,000
K. pneumoniae	Clinical isolate	>10,000	>10,000	5150	>10,000
P. mirabilis	ATCC:21721	1183	307	885	289
P. mirabilis	Clinical isolate	837	125	623	105
P. fluorescens	Clinical isolate	4538	>10,000	-	-
S. newport	Clinical isolate	-	-	-	-
S. marcescens	Clinical isolate	-	-	-	-
S. sonneii	Clinical isolate	-	-	-	-
S. aureus	Clinical isolate	2760	3873	-	-
S. epidermidis	Clinical isolate	2322	1755	>10,000	>10,000
S. pyonenes	Clinical isolate	-	-	1684	4190

Numbers indicate the mean MIC and LC_{50} values of triplicate determinations. - indicates no inhibition. M=methanolic extract; W=aqueous extract.

(10 µL) of each extract were screened in the disc diffusion assay. Aqueous and methanolic *B. myrtifolia* extracts inhibited a broad spectrum of the bacterial species screened (Figure 2). The methanolic *B. myrtifolia* extract was a more potent growth inhibitor than the aqueous extract against most bacterial species (as assessed by the sizes of the zones of inhibition). Noteably, the opposite trend was noted for *P. mirabilis* growth inhibition, with the aqueous extracts inhibiting the reference and clinical strains by 10.3 ± 0.6 and 11.3 ± 0.6 mm respectively. This inhibition was particularly noteworthy compared to the inhibition by the ampicillin control (10 µg: inhibition zones of approximately 10 mm) In contrast, the methanolic *B. myrtifolia* extract inhibited *P. mirabilis* growth by 8.5 ± 0.6 mm (reference strain) and 8.6 ± 0.6 mm (clinical isolate strain).

Similar bacterial growth inhibitory trends were noted for the *S. anisatum* extracts (Figure 3). As reported for the *B. myrtifolia* extracts, *P. mirabilis* was more susceptible to growth inhibition by the *S. anisatum* extracts than were the other bacterial species screened. Indeed, inhibition zones of 11.7 ± 0.5 mm and 12.3 ± 0.6 mm were measured for the inhibition of the aqueous extract against the reference and clinical isolate strains of *P. mirabilis* respectively.

Both gram positive and gram negative bacteria were affected by the *B. myrtifolia* and *S. anisatum* extracts. Of the 10 gram negative bacterial strains tested, 5 (50 %) and 4 (40 %) were inhibited by methanolic and aqueous *B. myrtifolia* leaf extracts respectively. *S. anisatum* extracts had a similar profile, inhibiting the same numbers of gram negative bacteria as the corresponding *B. myrtifolia* extracts. In contrast, the methanolic *B. myrtifolia* and *S. anisatum* extracts inhibited 3 of the 4 (75 %) of the gram positive bacterial species tested respectively whilst both of the aqueous extracts inhibited the growth of only 1 of the 4 gram positive species tested (25 %).

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 extracts of both species were potent growth inhibitors of several bacterial species (as judged by MIC; Table 2). *P. mirabilis* was the most susceptible bacteria to the *B. myrtifolia* and *S. anisatum* extracts, with MIC values generally <1000 µg/mL (<10 µg infused into the disc). The aqueous extracts of both species were particularly potent *P. mirabilis* growth inhibitors. MIC values of 307 and 125 µg/mL were determined for aqueous *B. myrtifolia* extract against the reference and clinical strains of the bacterium respectively. The aqueous *S. anisatum* extract was a similarly potent growth inhibitor, with MIC values of 289 (reference *P. mirabilis* strain) and 105 µg/mL (clinical isolate *P. mirabilis* strain). The methanolic extracts were also potent *P. mirabilis* growth inhibitors, albeit with slightly higher MIC values (in the 600-1200 µg/mL range). As *P. mirabilis* infection is a common cause of urinary tract infections and has also been identified as a trigger of rheumatoid arthritis,[31,32] *B. myrtifolia* and *S. anisatum* extracts have potential for the prevention of these diseases in genetically susceptible individuals.

The methanolic *S. anisatum* extract was also a potent *E. coli* growth inhibitor (MIC's of approximately 900-1200 µg/mL against the different bacterial strains). The aqueous and methanolic *S. anisatum* extracts were also moderate inhibitors of *A. feacalis* and *S. pyonenes*, and inhibited *K. pneumoniae* with lower potency. In contrast, aqueous and methanolic *B. myrtifolia* extracts were moderate inhibitors of *S. aureus* and *S. epidermidis* growth. The methanolic *B. myrtifolia* extract (but not the aqueous *B. myrtifolia* extract) was also a moderate inhibitor of *B. cereus* and *P. fluorescens* growth. Low growth inhibition (or no inhibition) was noted for all other extract/bacterium combinations, with MIC values >5000 µg/mL.

Bacterial growth time course assay

The antibacterial activity of the *B. myrtifolia* and *S. anisatum* extracts was further investigated in the reference bacterial strains by bacterial growth time course assays in the presence and absence of the extract.

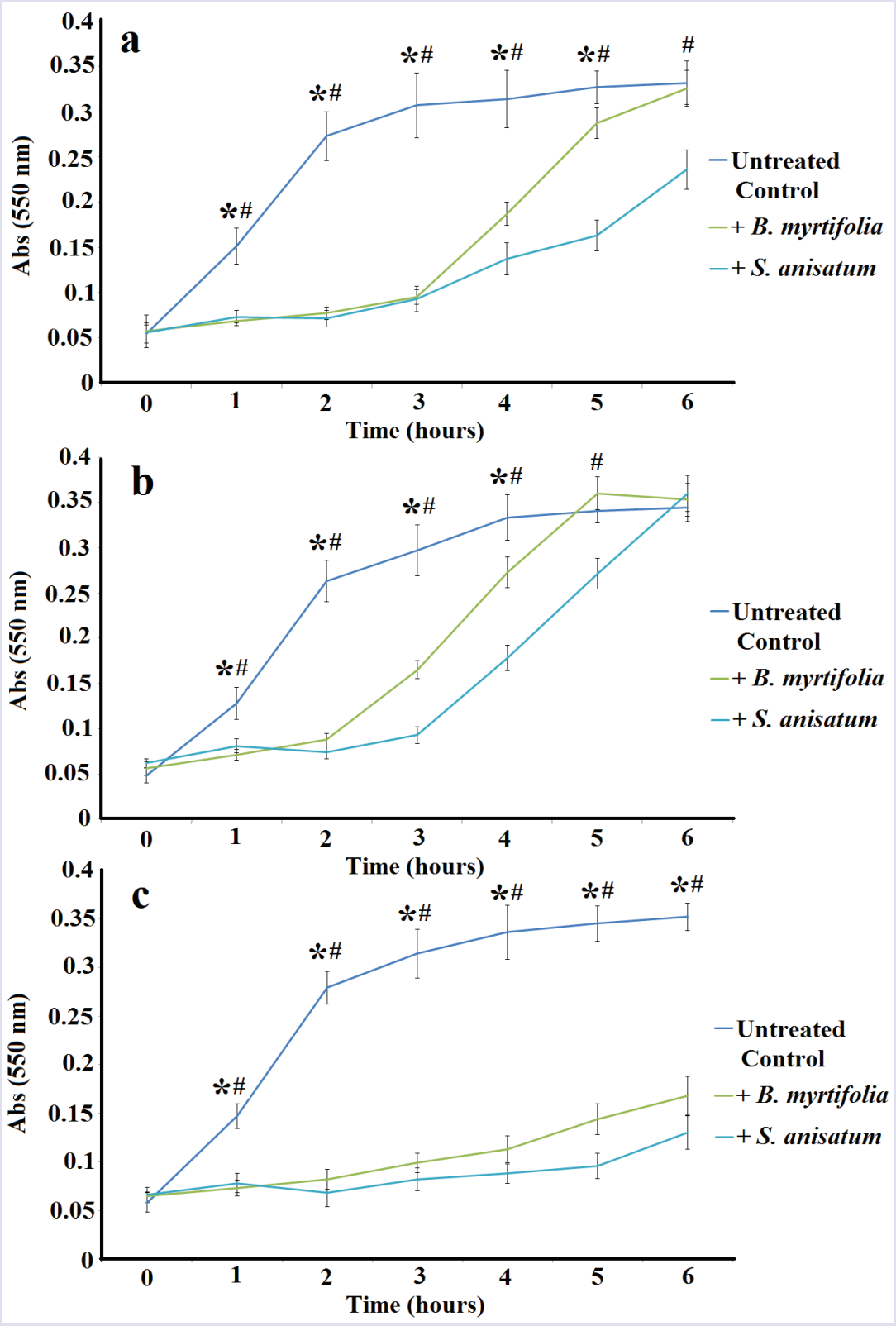

Figure 4: Bacterial growth curves for (a) *E. coli*, (b) *K. pneumoniae*, (c) *P. mirabilis*. All bioassays were performed in at least triplicate and are expressed as mean ± SEM. * = growth results in the presence of methanolic *B. myrtifolia* extract that are significantly different to the untreated control growth ($p<0.01$); # = growth results in the presence of methanolic *S. anisatum* extract that are significantly different to the untreated control growth ($p<0.01$).

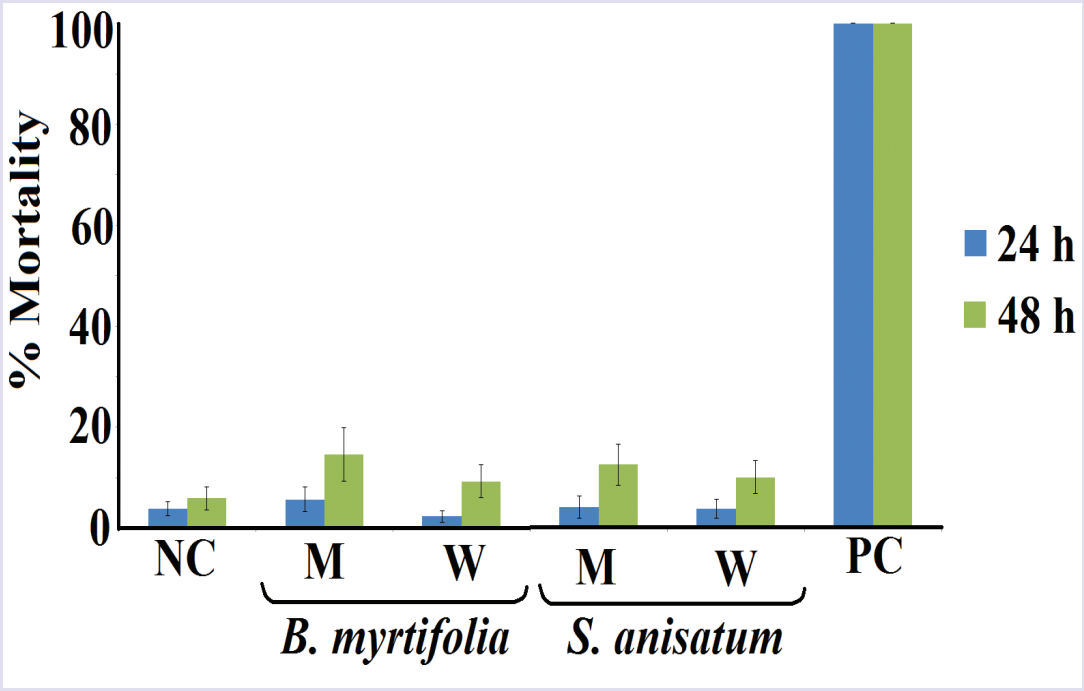

Figure 5: The lethality of the aqueous and methanolic *B. myrtifolia* and *S. anisatum* leaf extracts (2000 µg/mL), potassium dichromate (1000 µg/mL) and a seawater control. M=methanolic extract; W=aqueous extract; NC=negative (seawater) control; PC=positive control (1000 µg/mL potassium dichromate). All bioassays were performed in at least triplicate and are expressed as mean ± SEM.

As the methanolic extracts were generally more potent than the aqueous extracts (except for *P. mirabilis* inhibition where aqueous extracts were more potent), only the effect of the methanolic extracts on bacterial growth time course was examined. The starting concentration of the extract used in these assays was 1000 µg/mL. Methanolic extracts of both *B. myrtifolia* and *S. anisatum* significantly inhibited *E. coli* (Figure 4a), *K. pneumoniae* (Figure 4b) and *P. mirabilis* (Figure 4c) growth within 1 h, indicating a rapid antimicrobial action. Whilst *E. coli* and *K. pneumoniae* growth was inhibited for at least the first 4 hours of the time course, these bacteria were generally able to overcome this inhibition by 6 h, with the recorded turbidity not significantly different to that of the untreated control. This indicates that the growth inhibition of these bacteria was bacteriostatic for the methanolic *B. myrtifolia* and *S. anisatum* extracts at the concentrations tested. In contrast, inhibition of *P. mirabilis* by the methanolic *B. myrtifolia* and *S. anisatum* 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* at the dose tested.

Quantification of toxicity

The toxicity of the *B. myrtifolia* and *S. anisatum* extracts was initially tested in the *Artemia franciscana* nauplii bioassay at a concentration of 2000 µg/mL (Figure 5). All extracts induced low levels of mortality at 24 and 48 h, similar to the % mortality seen for the seawater control. As none of the extracts induced toxicity significantly different from the seawater control, all were deemed to be nontoxic. In contrast, the potassium dichromate positive controls 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 3). For comparison, serial dilutions of potassium dichromate were also tested. All *B. myrtifolia* and *S. anisatum* 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.[29]

Table 3: LC_{50} (95% confidence interval) for *Artemia* nauplii exposed to the aqueous and methanolic *B. myrtifolia* and *S. anisatum* leaf extracts and the reference toxin potassium dichromate

Extract	LC_{50} (µg/mL)	
	24 h	48 h
B. myrtifolia Methanol	4963 ± 87	5016 ± 135
B. myrtifolia Water	4553 ± 74	5340 ± 113
S. anisatum Methanol	5576 ±121	5708 ± 147
S. anisatum Water	4738 ± 107	5120 ± 143
Potassium dichromate	88 ± 5	82 ± 4

Results represent the mean ± SEM of triplicate determinations.

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 synthetic drugs, and the failure of current drug regimens to effectively treat many diseases. This current study reports on the growth inhibitory properties of *B. myrtifolia* and *S. anisatum* leaf extracts against a panel of pathogenic bacteria, and on their toxicity. The gram positive and gram negative bacteria tested in this study demonstrated similar susceptibilities towards the *B. myrtifolia* and *S. anisatum*

extracts, albeit with a slightly higher susceptibility for the gram negative bacteria. Many previous studies with other plant species report a greater susceptibility towards solvent extracts for South American,[33] African[34] and Australian plant extracts.[35,36]

Our study examined the ability of *B. myrtifolia* and *S. anisatum* extracts to inhibit the growth of a panel of medicinally important bacterial pathogens. The aqueous extracts of both species were identified as being particularly potent inhibitors of *P. mirabilis*. MIC values against the clinical *P. mirabilis* isolate strain of 125 and 105 µg/mL were determined for the aqueous *B. myrtifolia* and *S. anisatum* extracts respectively. As *P. mirabilis* can trigger rheumatoid arthritis in genetically susceptible individuals[31,32] these extracts have potential for the development of rheumatoid arthritis inhibitory therapies. The methanolic *S. anisatum* extract also displayed moderate *K. pneumoniae* growth inhibitory properties (MIC values < 5000 µg/mL for both the reference and clinical strains). As *K. pneumoniae* can trigger ankylosing spondylitis in genetically susceptible individuals[37,38] this extract may also be useful in the prevention of ankylosing spondylitis. Furthermore, the methanolic *S. anisatum* extract was a good inhibitor of *S. pyogenes* growth. *S. pyogenes* may cause a myriad of diseases including streptococcal pharyngitis, impetigo and rheumatic heart disease, depending on which tissue it infects.[39,40] Thus, the methanolic *S. anisatum* extract may be useful in the prevention of these diseases.

Aside from inhibition of the bacterial triggers of the autoimmune disease discussed above, the *B. myrtifolia* and *S. anisatum* extracts also were moderate to good inhibitors of several other bacterial pathogens. Both the aqueous and methanolic *S. anisatum* extracts inhibited *E. coli* growth. However, the methanolic extract displayed the greater efficacy, with MICs of 893 and 1255 µg/mL against the reference and clinical isolate strains respectively. Thus, this extract has potential in the treatment of diarrhoea and dysentery caused by enteric *E. coli* infections, as well as urinary tract *E. coli* infections. The aqueous and methanolic *B. myrtifolia* extracts were also moderate to good inhibitors of *S. aureus* and *S. epidermidis* growth (MICs 1750-2750 µg/mL). As both of these bacteria are skin disease pathogens, the *B. myrtifolia* extracts also may have applications as topical treatments of these diseases.

Whilst an investigation of the phytochemistry of the *B. myrtifolia* and *S. anisatum* extracts was beyond the scope of our study, other plants of the family *Myrtaceae* are well known for their high terpenoid contents. In particular, high monoterpenoid contents have been reported for many *Myrtaceae* spp.[1] Monoterpenes have been reported to exert a wide variety of biological effects including antibacterial, antifungal, anti-inflammatory and anti-tumour activities[16] and therefore may contribute to the bacterial growth inhibitory activity of the *B. myrtifolia* and *S. anisatum* extracts reported here. A wide variety of monoterpenoids including camphor, carvone, cineole, borneol, menthone, pinene, terpinene, as well as their derivatives, inhibit the growth of an extensive panel of pathogenic and food spoilage bacteria.[41] Interestingly, several of these monoterpenoids have also been reported to suppress NF-κB signaling (the major regulator of inflammatory diseases).[42-45] This may be particularly relevant for the extracts which inhibited *P. mirabilis* (a bacterial trigger of rheumatoid arthritis)[31,32] and *K. pneumoniae* (a trigger of ankylosing spondylitis).[37,38] The terpene components in these extracts may have a pleuripotent mechanism in blocking the autoimmune inflammatory diseases and relieving its symptoms by acting on both the initiator and downstream inflammatory stages of the disease. Further phytochemical evaluation studies and bioactivity driven isolation of active components is required to further evaluate the mechanism(s) of bacterial growth inhibition.

Another commonality between the inhibitory *B. myrtifolia* and *S. anisatum* extracts was that all contained moderate levels of tannins. Many studies have reported potent growth inhibitory activities for a number of tannin compounds. Gallotannins have been reported to inhibit the growth of a broad spectrum of bacterial species[46] through a variety of mechanisms including binding cell surface molecules including lipotoichoic acid and proline-rich cell surface proteins,[47,48] and by inhibiting glucosyltransferase enzymes.[49] Elligitannins are also highly potent inhibitors of bacterial growth, with MIC values as low as 62.5 µg/mL.[46,48] Ellagitannins have also been reported to function via several antibiotic mechanisms including interaction with cytoplasmic oxidoreductases and by disrupting bacterial cell walls.[46,48] Thus, it is likely that multiple compounds within the *B. myrtifolia* and *S. anisatum* extracts may contribute to the inhibition of *S. pyogenes* growth.

The findings reported here also demonstrate that all of the *B. myrtifolia* and *S. anisatum* extracts were nontoxic towards *Artemia franciscana* nauplii, with LC_{50} values substantially > 1000 µg/mL. Extracts with LC_{50} 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, whilst these studies have demonstrated the potential of the *B. myrtifolia* and *S. anisatum* extracts in the development of future antibiotic chemotherapeutics for the prevention and treatment of urinary tract infections, autoimmune diseases (particularly rheumatoid arthritis and ankylosing spondylitis) and some skin diseases, 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 *B. myrtifolia* and *S. anisatum* extracts as inhibitors of pathogenic bacteria growth. Furthermore, their lack of toxicity indicates than they are safe for internal as well as topical treatment. Further studies aimed at the purification and identification of bioactive components are needed 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; **LC_{50}:** The concentration required to achieve 50% mortality; **MIC:** Minimum inhibitory concentration.

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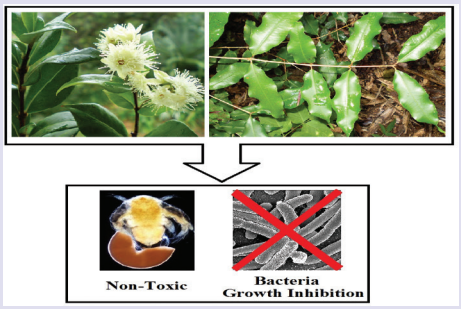
PICTORIAL ABSTRACT



SUMMARY

- *B. myrtifolia* and *S. anisatum* leaf extracts inhibited the growth of a wide range of bacterial species.
- Growth of both gram positive and gram negative bacteria was inhibited to approximately the same extent.
- *B. myrtifolia* and *S. anisatum* extracts were particularly potent inhibitors of *P. mirabilis* growth.
- All extracts were nontoxic in the *Artemia franciscana* nauplii bioassay.

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.