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Original Article

Inhibition of *Streptococcus pyogenes* growth by native Australian plants: New approaches towards the management of impetigo, pharyngitis and rheumatic heart disease

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ABSTRACT

Introduction: *Streptococcus pyogenes* can cause a variety of diseases including streptococcal pharyngitis, impetigo and rheumatic heart disease, dependant on which tissue it infects. Many Australian plants have documented therapeutic properties as general antiseptics, but are yet to be tested for the ability to inhibit *S. pyogenes* growth. **Methods:** Solvent extracts were prepared using Australian plants with documented ethnobotanical usage to treat bacterial infections, or published antibacterial activity. The extracts were investigated by disc diffusion assay for the ability to inhibit the growth of a clinical strain of *S. pyogenes*. Their MIC values were determined to quantify and compare their efficacies. Toxicity was determined using the *Artemia franciscana* nauplii bioassay. **Results:** *S. pyogenes* growth was inhibited by 24 of the 34 extracts tested. The *Eucalyptus* spp. extracts were particularly potent. MIC values of 341 and 88 µg/mL were determined for the aqueous and methanolic *E. baileyana* extracts respectively. Similarly, MIC values of 134 and 53 µg/mL were determined for the aqueous and methanolic *E. major* extracts respectively. The methanolic wattle seed extract, aqueous and methanolic lemon aspen extracts, aqueous native thyme extract, methanolic river mint extract and the methanolic native basil extract were similarly potent growth inhibitors, with MIC values ≤1000 µg/mL. Several other extracts (methanolic native tamarind, bush tomato, desert lime, native thyme, native sage and the *T. stipitata* leaf extracts, as well as the aqueous river mint, native basil, *T. stipitata* leaf extracts) displayed moderate growth inhibitory activity (MIC=1000-5000 µg/mL). All other extracts were either low potency *S. pyogenes* growth inhibitors or were devoid of inhibitory activity. The *E. baileyana* and *E. major* methanolic extracts, as well as the *E. baileyana* aqueous extract induced significant mortality in the *Artemia fransiscana* bioassay, with LC_{50} values substantially <1000 µg/mL. All other extracts were nontoxic, with LC_{50} values >1000 µg/mL. **Conclusion:** The potent growth inhibitory bioactivity of the *Eucalyptus* spp., lemon aspen, wattle seed, native basil and river mint extracts against *S. pyogenes* demonstrates their potential for the treatment and prevention of *S. pyogenes* induced disease. However, the toxicity of the *Eucalyptus* spp. extracts may limit their use to topical treatments for pharyngitis and impetigo. As the lemon aspen, wattle seed, native basil and river mint extracts were nontoxic, they may also have wider uses in treating systemic illnesses such as rheumatic fever, rheumatic heart disease and cellulitis.

Key words: *Eucalyptus*, Antioxidant, Lemon aspen, Pharyngitis, Impetigo, Rheumatic heart disease, Antibacterial activity.

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INTRODUCTION

Streptococcus pyogenes is a gram-positive, facultative anaerobe and the etiological agent of a number of diseases in humans. In nature, the bacterium is present as part of the natural skin flora of humans and animals which under certain conditions can cause disease.[1] Associated diseases vary in symptoms and severity, ranging from superficial skin infections (impetigo and ecthyma) to rheumatic fever.[2,3] Typically, streptococcal infections are localised on the epidermis or nasopharynx and/or oropharynx and are treated with antibiotics as required. However, due to the self-limiting nature of these infections and the increasing risk of antibiotic resistance unless complications arise monitoring and symptom treatment is often the preferred option. The probing of natural plant resources offers an alternate means of fighting streptococcal diseases through the prevention of bacterial growth.[4]

Several Australian plant species were selected for *S. pyogenes* growth inhibitory activity screening based on their usage in traditional medicine systems[5] and/or their reported antibacterial activities[6-9] (Table 1). Plants of the genus *Eucalyptus* are particularly well known for their antiseptic properties due to their high 1, 8-cineole contents.[5,10-12] The first Australians crushed the leaves and inhaled the volatiles to treat coughs and colds.[5,10] Fresh leaves or decoctions prepared from the leaves were also used as wound antiseptics and to treat skin and throat infections. Furthermore, *in vitro* studies have demonstrated the growth inhibitory properties of *Eucalyptus baileyana* and *Eucalyptus major* extracts towards a panel of bacterial species, indicating their therapeutic potential in treating pathogenic diseases.[11,12] Indeed, essential oils prepared from *Eucalyptus* spp. leaves remain a popular antiseptic agent, not only in Australia, but are also commonly sold in pharmacies internationally.

Whilst the ethnobotanical uses of many Australian plants in traditional Aboriginal medicine systems have been recorded, rigourous scientific studies are lacking for many species.[5,10] Recent studies have reported potent broad spectrum bacterial growth inhibitory activity for *S. spinescens* extracts.[13] Despite this, *S. spinescens* is yet to be evaluated for *S. pyogenes* growth inhibitory activity. *Tasmannia lanceolata*[14] and *Tasmannia stipitata* (family Winteraceae) extracts have potent broad spectrum antimicrobial activity for *in vitro*.[6] Furthermore, *T. lanceolata*[15] and *T. stipitata*[6] have also been reported to inhibit the proliferation of the gastrointestinal protozoal parasite *Giardia duodenalis*.[6,15] We were unable to find similar studies examining the therapeutic potential of *T. insipidia*.

Recent studies have reported exceptionally high antioxidant content of the fruits of several Australian plant species.[8,9,16] In particular, these

studies reported the fruit of *Kunzea pomifera* F. Muell. (muntries) and *Podocarpus elatus* R. Br. (Illawarra plum) to have similar antioxidant capacities to blueberries (which are themselves considered to have a high antioxidant capacity). *Acronychia acidula* F. Muell. (lemon aspen), *Citrus glauca* (Lindl.) Burkill (desert lime) and *Solanum aviculare* G. Forst. (bush tomato) have also been reported to have high antioxidant capacities.[9,16] It has been postulated that the high antioxidant contents of some Australian native fruits may provide them with therapeutic effects.[14,16-20] Similarly, a number of Australian culinary herbs including *Prostanthera rotundifolia* R. Br. (native thyme), *Prostanthera incise* R. Br. (native sage), and *Mentha australis* R. Br. (rivermint) have been reported to have high antioxidant capacities and potent growth inhibitory activity against bacteria associated with the induction of several autoimmune inflammatory diseases.[20] Despite this relative wealth of information documenting antibacterial Australian plants, many are yet to be tested for the ability to inhibit *S. pyogenes* growth. The current study examines the growth inhibitory activity of extracts of selected Australian plants against *S. pyogenes,* and thus their potential in the prevention and treatment of streptococcal pharyngitis, impetigo, rheumatic fever and rheumatic heart disease.

MATERIALS AND METHODS

Plant source and extraction

Acronychia acidula F. Muell. (lemon aspen), *Podocarpus elatus* R. Br. (Illawarra plum), *Kunzea pomifera* F. Muell. (muntries), *Diploglottis australis* Hook. f. (native tamarind), *Acacia vivtoriae* Benth. (wattle seed), *Citrus glauca* (Lindl.) Burkill (desert lime), *Solanum aviculare* G. Forst. (bush tomato), *Prostanthera incise* R. Br. (native sage), *Prostanthera rotundifolia* R. Br. (native thyme), *Ocimum tenuiflorum* L. (native basil) and *Mentha australis* R. Br. (river mint) were obtained from Taste of Australia Bush Food, Australia. Air dried *Tasmannia insipidia* R. Br. leaves and *Tasmannia stipitata* (Vick.) A. C. Smith leaves and berries were supplied and verified by the Queensland Bush foods Association, Australia. *Scaevola spinescens* R. Br. was supplied by Jeannie Crago of Outback Books Australia (a commercial supplier of *S. spinescens* tea) as a pre-dried and course milled whole plant material. *Eucalyptus baileyana* F. Muell. And *Eucalyptus major* (Maiden) Blakely plant materials were collected from Toohey Forest, Brisbane and were identified with reference to a taxonomic key to Toohey Forest plants.[24] Voucher samples of all plant specimens are stored in the School of Natural Sciences, Griffith University (Australia). The plant materials were comprehensively dried in a Sunbeam food dehydrator and the dried plant materials were stored at -30°C. Prior to use, the plant materials were thawed and freshly ground to a coarse powder. Individual 1 g quantities of the ground plant material were weighed into separate tubes and 50 mL of water or methanol were added. All solvents were obtained from Ajax and were AR grade. The ground plant materials were individually extracted in each solvent for 24 h 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 resultant dry extract was weighed and redissolved in 10 mL deionised water (containing 1% DMSO).

Qualitative phytochemical studies

Phytochemical analysis of the extracts for the presence of triterpenoids, cardiac glycosides, flavonoids, saponins, phenolic compounds, phytosteroids, anthraquinones, tannins and alkaloids was conducted by previously described assays.[25-27]

Antibacterial screening

Clinical Streptococcus pyogenes strain

The clinical isolate strain of *Streptococcus pyogenes* used in this study was supplied by Ms. Michelle Mendell of the School of Natural Sciences Griffith University, Australia. All growth studies were performed using nutrient agar (Oxoid Ltd., Australia) under aerobic conditions. Incubation was at 30°C and the stock culture was 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.[28,29] Briefly, 100 µL of *S. Pyogenes* culture was grown in 10 mL of fresh nutrient broth until they reached a count of ~10^8 cells/mL. A volume of 100 µL of the bacterial suspension was spread onto nutrient agar plates and extracts were tested for antibacterial activity using 5 mm sterilised filter paper discs. Discs were infused with 10 µL of the plant extracts, allowed to dry and placed onto the inoculated plates. The plates were allowed to stand at 4°C for 2 h before incubation at 30°C for 24 h. The diameters of the inhibition zones were measured to the closest whole millimetre. Each assay was performed in at least triplicate. Mean values (± SEM) are reported in this study. Standard discs of ampicillin (10 µg) were obtained from Oxoid, Australia and were used as positive controls to compare antibacterial activity. Filter discs infused with 10 µL of distilled water were used as a negative control.

Minimum inhibitory concentration (MIC) determination

The minimum inhibitory concentration (MIC) of each extract against *S. pyogenes* was determined as previously described.[28,30] 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.

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 as previously described.[31-33] Briefly, 400 µL of seawater containing approximately 43 (mean 43.2, n=155, SD 14.5) *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 sec. 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.

Table 1: The medicinal usage, common names and known constituents of the native Australian plant species tested in this study

Plant Species	Part Used in This Study	Common Name/s	Traditional Medicinal Uses and Known Therapeutic Properties	Known Constituents	References
***Acacia vivtoriae* Benth.**	seed	wattle	The seed was ground into a flour and used to make a bread. The seed has reported antibacterial and anticancer properties. Other Acacia spp. were used to treat allergies, rash and as an antiseptic.	Unknown, although other Acacia spp. contain high levels of tannins, terpenoids and saponins	5, 10
***Acronychia acidula* F. Muell.**	fruit	lemon aspen	The fruit has a high antioxidant capacity and was mainly used as a nutritious food. The fruit has reported antibacterial and anticancer properties.	unknown although the related species Acronychia baueri has been reported to contain alkaloids	10
***Citrus glauca* (Lindl.) Burkill**	fruit	desert lime	The fruit has a high antioxidant capacity and was mainly used as a nutritious food. The fruit has reported antibacterial and anticancer properties.	Unknown	16, 19, 20
***Diploglottis australis* Hook. f.**	fruit	Australian native tamarind	The fruit has a high antioxidant capacity and was mainly used as a nutritious food. The fruit has reported antibacterial and anticancer properties.	Unknown	16, 19, 20
***Eucalyptus baileyana* F. Muell.**	leaf	Bailey's stringybark	Used to treat rheumatism, swelling, inflammation, skin disorders stomach disorders, bactericide (wounds, sores)	high terpene content	5, 10, 12
***Eucalyptus major* (Maiden) Blakely**	leaf	grey gum	Used to treat rheumatism, swelling, inflammation, skin disorders stomach disorders, bactericide (wounds, sores)	high terpene content	5, 10, 12
***Kunzea pomifera* F. Muell.**	fruit	muntries	The fruit has a high antioxidant capacity and was mainly used as a nutritious food. The fruit has reported antibacterial and anticancer properties.	ellagitannins (including ellagic acid), gallotannins, terpenoids, purine analogues	5, 10, 21
***Mentha australis* R. Br.**	leaf	river mint	The leaf has a high antioxidant capacity and was mainly used as a nutritious food. The leaf has reported antibacterial and anticancer properties.	high in essential oils; constituents have not been fully established.	10
***Ocimum tenuiflorum* L.**	leaf	Australian native basil	The leaf has a high antioxidant capacity and was mainly used as a nutritious food. The leaf has reported antibacterial and anticancer properties.	unknown although the related species Ocimum sanctum is rich in polyphenolic compounds including eugenol	19
***Podocarpus elatus* R. Br.**	fruit	Illawarra plum	The fruit has a high antioxidant capacity and was mainly used as a nutritious food. The fruit has reported antibacterial and anticancer properties.	unknown	16, 19, 20
***Prostanthera incisa* R. Br.**	leaf	Australian native sage	The leaf has a high antioxidant capacity and was mainly used as a nutritious food. The leaf has reported antibacterial and anticancer properties.	high in essential oils; constituents have not been fully established.	10
***Prostanthera rotundifolia* R. Br.**	leaf	Australian native thyme	The leaf has a high antioxidant capacity and was mainly used as a nutritious food. The leaf has reported antibacterial and anticancer properties.	high in essential oils, particularly 1,8-cineole	10
***Scaevola spinescens* R. Br.**	leaf	currant bush, maroon bush, prickly fan flower	Used as an antiseptic (especially for skin disorders/sores), cancer, pain and urinary disorders. Antiviral properties have also recently been reported.	pentacyclic triterpenoids including lupeol, taraxerol, myricadiol), coumarins (including ammarin, nodakenetin), scaevoloside	1, 10,13, 21, 22

***Solanum aviculare* G. Forst.**	fruit	bush tomato, kangaroo apple	The fruit has a high antioxidant capacity and was mainly used as a nutritious food. The fruit has reported antibacterial and anticancer properties.	unknown	16, 19, 20
***Tasmannia insipidia* R. Br.**	leaves	brush pepperbush	Used mainly as a flavouring agent. No known traditional medicinal uses however recent studies report antibacterial activity	phytochemistry of other *Tasmannia* spp. reports high levels of terpenoids (especially sesquiterpenoids). Stilbenes are also present.	6, 8, 14, 23
***Tasmannia stipitata* (Vick.) A.C. Smith**	leaves and berries	Dorrigo pepper, Northern pepperbush	Used mainly as a flavouring agent. No known traditional medicinal uses however recent studies report antibacterial activity	phytochemistry of other *Tasmannia* spp. reports high levels of terpenoids (especially sesquiterpenoids). Stilbenes are also present.	6, 8, 14, 23

Table 2: The mass of dried extracted material, the concentration after resuspension in deionised water, qualitative phytochemical screenings and antioxidant capacities of the Australian plant extracts.

Extract	Mass of Dried Extract (mg)	Concentration of Resuspended Extract (µg/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
NTW	52	5.2	++	-	+++	-	-	-	-	-	-	+	-	++	-
NTM	107	10.7	+++	-	+++	-	++	-	-	-	-	+	-	-	-
BTW	79	7.9	+++	++	+++	-	+++	++	-	-	++	+++	++	-	-
BTM	313	31.3	+++	-	+++	-	-	++	-	-	++	++	-	-	-
DLW	182	18.2	+++	++	++	+	-	++	-	-	++	+++	-	-	-
DLM	247	24.7	+	-	-	+	++	++	-	-	-	+++	-	-	-
MW	350	35	+++	+++	+++	-	+++	++	-	-	-	+++	++	-	-
MM	524	52.4	+++	+++	-	-	+++	++	-	-	-	+++	++	-	-
WSW	120	12	++	++	+	++	-	++	-	-	-	+++	-	-	-
WSM	88	8.8	++	+++	++	-	++	++	-	-	++	+++	-	-	-
LAW	162	16.2	+++	-	-	+	+++	++	-	-	-	+++	-	-	-
LAM	360	36	+++	-	-	+	-	++	-	-	-	+++	-	-	-
THW	52	5.2	+++	+++	+++	++	+++	++	-	-	++	+++	++	++	-
THM	171	17.1	+++	+++	++	++	+++	++	-	-	++	+++	++	-	-
IPW	195	19.5	+++	++	+++	-	+++	++	-	-	-	++	++	++	++
IPM	314	31.4	+++	+++	+++	-	+++	++	-	-	-	++	++	+	++
NSW	25	2.5	+++	+++	++	++	+++	++	-	-	-	+++	++	-	-
NSM	109	10.9	+++	+++	+++	++	+++	++	-	-	+	++	++	-	-
RMW	30	3	++	++	++	++	-	++	-	-	-	+++	-	-	-
RMM	120	12	+++	+++	+++	++	+++	++	-	-	++	+++	++	+	-
NBW	108	10.8	+++	+	++	++	+++	++	-	-	+	++	+	-	-
NBM	192	19.2	+++	+++	+++	-	+++	++	-	-	++	+++	++	-	-
EBLW	125	12.5	+++	+++	+	-	+	-	-	-	-	++	+	-	-
EBLM	143	14.3	+++	+++	+	-	+	-	-	-	-	+	++	-	-

EMLW	222	22.2	+++	+++	+	-	+	-	-	-	-	+++	+	-	-
EMLM	280	28	+++	+++	+	-	+	-	-	-	-	++	++	-	-
SSLW	210	21	+++	++	-	-	+	-	-	-	-	++	+++	-	-
SSLM	116	11.6	+++	++	-	-	+	-	-	+	-	++	++	-	-
TILW	184	18.4	+++	++	+	-	+	+	-	-	-	++	-	-	-
TILM	221	22.1	+++	++	+	-	++	+	-	-	-	++	-	-	-
TSLW	232	23.2	+++	+++	+++	-	++	+	-	-	-	+++	-	-	-
TSLM	293	29.3	+++	+++	++	-	+++	+	-	-	-	+++	-	-	-
TSBW	207	20.7	+++	+++	+++	-	++	+	-	-	-	+++	-	-	-
TSBM	279	27.9	+++	+++	+++	-	++	+	-	-	-	+++	-	-	-

+++ indicates a large response; ++ indicates a moderate response; + indicates a minor response; - indicates no response in the assay. NTW=aqueous native tamarind extract; NTM=methanolic native tamarind extract; BTW=aqueous bush tomato extract; BTM=methanolic bush tomato extract; DLW=aqueous desert lime extract; DLM=methanolic desert lime extract; MW=aqueous muntries extract; MM=methanolic muntries extract; WSW=aqueous wattle seed extract; WSM=methanolic wattle seed extract; LAW=aqueous lemon aspen extract; LAM=methanolic lemon aspen extract; THW=aqueous native thyme extract; THM=methanolic native thyme extract; IPW=aqueous Illawarra plum extract; IPM=methanolic Illawarra plum extract; NSW=aqueous native sage extract; NSM=methanolic native sage extract; RMW=aqueous river mint extract; RMM=methanolic river mint extract; NBW=aqueous native basil extract; NBM=methanolic native basil extract; EBLW=aqueous *E. baileyana* leaf extract; EBLM=methanolic *E. baileyana* leaf extract; EMLW=aqueous *E. major* leaf extract; EMLM=methanolic *E. major* leaf extract; SSLW=aqueous *S. spinescens* leaf extract; SSLM=methanolic *S. spinescens* leaf extract; TILW=aqueous *T. insipidia* leaf extract; TILM=methanolic *T. insipidia* leaf extract; TSLW=aqueous *T. stipitata* leaf extract; TSLM=methanolic *T. stipitata* leaf extract; TSBW=aqueous *T. stipitata* berry extract; TSBM=methanolic *T. stipitata* berry extract.

RESULTS

Liquid extraction yields and qualitative phytochemical screening

Extraction of 1 g of the native Australian plant materials with the solvents yielded dried plant extracts ranging from 25 mg (aqueous native sage extract) to 524 mg (methanolic muntries extract) (Table 2). Methanol was a better extractant than water, with substantially higher extraction for most plant materials. However, this trend was not observed in the wattle seed and *S. spinescens* extracts, which had substantially higher yields in the aqueous extracts compared to the methanolic extracts.

An extensive range of phytochemicals was detected inboth the methanolic and aqueous extracts for all plant species tested by qualitative phytochemical screening (Table 2). Both solvents typically extracted high levels of phenolics (both water soluble and water insoluble phenolics) for all plant materials. Additionally, all extracts generally contained high levels of flavonoids and moderate to high levels of saponins. Low to moderate levelsof triperenoids were present in most extracts, with the exception of the native thyme, *Eucalyptus* spp. and *S. spinescens* extracts. Similarly, cardiac glycosides, alkaloids and tannins were detected in some, but not all of the extracts. Furthermore, when these classes of compound were detected, they were generally only in low to moderate abundance. All extracts were generally devoid of detectable levels of phytosteroids and anthraquinones.

Antimicrobial activity

To assess the inhibitory activity of the crude plant extracts against *S. pyogenes*, 10 µL aliquots of each extract were screened using a disc diffusion assay. The bacterial growth was inhibited by 24 of the 34 extracts tested (~71%) (Figure 1). The methanolic *E. baileyana* and *E. major* extracts were the most potent inhibitors of *S. pyogenes* growth (as judged by zones of inhibition), with inhibition zones of 12.3 ± 0.6 and 13.3 ± 0.6 mm respectively. This compares favourably with the ampicillin control, which had an inhibitory zone of 12.0 ± 1.0 mm. Whilst less potent than the corresponding methanolic extracts, the aqueous *E. baileyana* and *E. major* extracts were also good *S. pyogenes* growth inhibitors, with10 and 8.3 ± 0.3 mm inhibitory zones respectively.

The antimicrobial efficacy was further quantified by determining the MIC values (Table 3). Several extracts were potent inhibitors of *S. pyogenes* growth, with MIC values <1000 µg/mL (<10 µg infused into the disc). The *Eucalyptus* spp. extracts were particularly potent. Indeed, MIC values of 341 (3.4 µg infused into the disc) and 88 µg/mL (8.8 µg infused into the disc) were determined for the aqueous and methanolic *E. baileyana* extracts respectively. Similarly, MIC values of 134 (1.3 µg infused into the disc) and 53 µg/mL (5.3 µg infused into the disc) were determined for the aqueous and methanolic *E. major* extracts respectively. This compares well with the ampicillin control, which was tested at 10 µg infused into the disc. The methanolic wattle seed extract, aqueous and methanolic lemon aspen extracts, aqueous native thyme extract, methanolic river mint extract and the methanolic native basil extract were similarly potent growth inhibitors, with MIC values ≤1000 µg/mL. MIC values indicative of moderate inhibitory activity (1000-5000 µg/mL) were determined for many of the other extracts (methanolic native tamarind, bush tomato, desert lime, native thyme, native basil, *T. stipitata* leaf extracts, as well as the aqueous wattle seed, river mint, native basil, *T. stipitata* leaf extracts). All other extracts were either low potency *S. pyogenes* growth inhibitors (MIC >5000 µg/mL) or were devoid of inhibitory activity.

Quantification of toxicity

All extracts were initially screened at 2000 µg/mL in the assay (Figure 2). For comparison, the reference toxin potassium dichromate (1000 µg/mL) was also assessed in the bioassay. The potassium dichromate reference toxin was rapid in its onset, inducing nauplii death within the first 3 h of exposure, with 100% mortality evident in the subsequent 4-5 h (unpublished results). The majority of the Australian plant extracts also induced significant *Artemia* nauplii toxicity, with ≥ 50% mortality rates at 24 h. Indeed, only the aqueous desert lime, muntries, native thyme, native sage, river mint and native basil extracts, as well as both *S. spinescens* extracts, induced <50% mortality following 24 h exposure. Thus, only these extracts were deemed nontoxic, whilst all others were deemed toxic.

To further evaluate the effect of toxin concentration on the induction of mortality, the extracts were serially diluted in artificial seawater to testacross a range of concentrations in the *Artemia* nauplii bioassay. Table

Table 3: Minimum inhibitory concentration (µg/mL) of the plant extracts against *S. pyogenes* and LC_{50} values (µg/mL) in the *Artemia nauplii* bioassay

Extract	MIC	LC_{50}
NTW	-	1862
NTM	1194	1595
BTW	7900	5372
BTM	2240	3467
DLW	>10,000	3875
DLM	4200	-
MW	>10,000	-
MM	-	1965
WSW	2000	6254
WSM	867	5763
LAW	607	1872
LAM	597	1500
THW	1000	-
THM	1891	3358
IPW	-	1956
IPM	>10,000	1664
NSW	-	1831
NSM	1278	4015
RMW	1300	-
RMM	537	2658
NBW	1256	2480
NBM	627	7185
EBLW	341	897
EBLM	88	455
EMLW	134	1146
EMLM	53	793
SSLW	-	-
SSLM	-	-
TILW	-	1687
TILM	-	1463
TSLW	1867	1487
TSLM	1265	1813
TSBW	-	1530
TSBM	-	-
PD	ND	186
SW	ND	-

Numbers indicate the mean MIC and LC50 values of triplicate determinations. - indicates no inhibition. NTW=aqueous native tamarind extract; NTM=methanolicnative tamarind extract; BTW=aqueous bush tomato extract; BTM=methanolic bush tomato extract; DLW=aqueous desert lime extract; DLM=methanolic desert lime extract; MW=aqueous muntries extract; MM=methanolic muntries extract; WSW=aqueous wattle seed extract; WSM=methanolic wattle seed extract; LAW=aqueous lemon aspen extract; LAM=methanolic lemon aspen extract; THW=aqueous native thyme extract; THM=methanolic native thyme extract; IPW=aqueous Illawarra plum extract; IPM=methanolic Illawarra plum extract; NSW=aqueous native sage extract; NSM=methanolic native sage extract; RMW=aqueous river mint extract; RMM=methanolic river mint extract; NBW=aqueous native basil extract; NBM=methanolic native basil extract; EBLW=aqueous *E. baileyana* leaf extract; EBLM=methanolic *E. baileyana* leaf extract; EMLW=aqueous *E. major* leaf extract; EMLM=methanolic *E. major* leaf extract; SSLW=aqueous *S. spinescens* leaf extract; SSLM=methanolic *S. spinescens* leaf extract; TILW=aqueous *T. insipidia* leaf extract; TILM=methanolic *T. insipidia* leaf extract; TSLW=aqueous *T. stipitata* leaf extract; TSLM=methanolic *T. stipitata* leaf extract; TSBW=aqueous *T. stipitata* berry extract; TSBM=methanolic *T. stipitata* berry extract. PD=potassium dichromate control; SW=seawater control. ND=the indicated test was not performed.

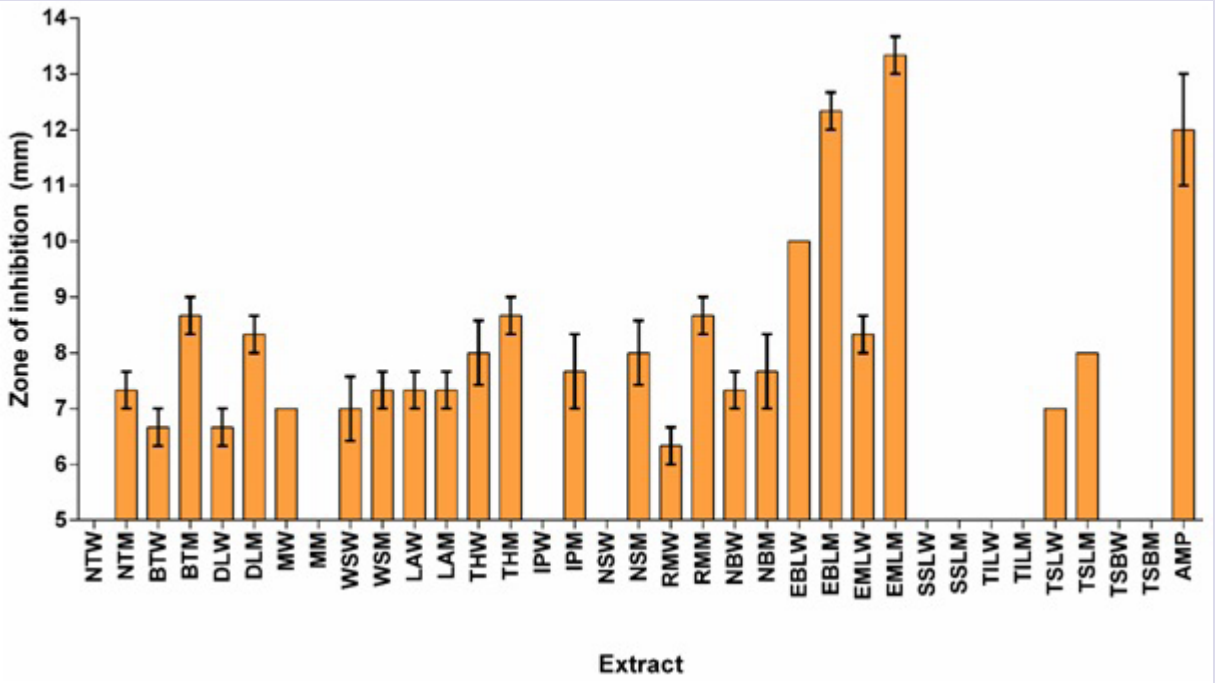

Figure 1: Growth inhibitory activity of Australian plant extracts against the *S. pyogenesclinical* isolate measured as zones of inhibition (mm). NTW=aqueous native tamarind extract; NTM=methanolic native tamarind extract; BTW=aqueous bush tomato extract; BTM=methanolic bush tomato extract; DLW=aqueous desert lime extract; DLM=methanolic desert lime extract; MW=aqueous muntries extract; MM=methanolic muntries extract; WSW=aqueous wattle seed extract; WSM=methanolic wattle seed extract; LAW=aqueous lemon aspen extract; LAM=methanolic lemon aspen extract; THW=aqueous native thyme extract; THM=methanolic native thyme extract; IPW=aqueous Illawarra plum extract; IPM=methanolic Illawarra plum extract; NSW=aqueous native sage extract; NSM=methanolic native sage extract; RMW=aqueous river mint extract; RMM=methanolic river mint extract; NBW=aqueous native basil extract; NBM=methanolic native basil extract; EBLW=aqueous *E. baileyana* leaf extract; EBLM=methanolic *E. baileyana* leaf extract; EMLW=aqueous *E. major* leaf extract; EMLM=methanolic *E. major* leaf extract; SSLW=aqueous *S. spinescens* leaf extract; SSLM=methanolic *S. spinescens* leaf extract; TILW=aqueous *T. insipidia* leaf extract; TILM=methanolic *T. insipidia* leaf extract; TSLW=aqueous *T. stipitata* leaf extract; TSLM=methanolic *T. stipitata* leaf extract; TSBW=aqueous *T. stipitata* berry extract; TSBM=methanolic *T. stipitata* berry extract; AMP=ampicillin (10 µg) control. Results are expressed as mean zones of inhibition (mm) ± SEM.

3 shows the LC_{50} values of the extracts towards *A. franciscana*. No LC_{50} values are reported for the aqueous desert lime, aqueous muntries, native thyme, native sage, river mint and native basil extracts, as well as both *S. spinescens* extractsas <50% mortality was seen across all concentrations tested. With the exception of the aqueous *E. major* leaf extract, all *Eucalyptus* spp. extracts generally had LC_{50} values <1000 µg/mL. All other extracts yielded LC_{50} values substantially >1000 µg/mL following 24 h exposure. As extracts with LC_{50} values of >1000 µg/mL towards *Artemia* nauplii are deemed to be nontoxic,[33] all extracts except the aqueous and methanolic *E. baileyana* extracts and the methanolic *E. major* leaf extract were deemed to be nontoxic.

DISCUSSION

Previous studies have reported potent bacterial growth inhibitory activity for all of the native Australian plant species screened in our study against a variety of pathogenic bacterial species.[6,11-13,23] Extracts prepared from Australian fruits and culinary herbs,[16,19,20] *S. spinescens* leaf extracts[13] and *Tasmannia* spp. leaf and berry extracts[6,8,23] have previously been reported to have inhibitory activity against extensive panels of pathogenic bacteria. With the exception of the *S. spinescens*, *T. insipida* and the *T. stipatata* berry extracts, all species screened in our study inhibited the growth of *S. pyogenes*. In contrast, the bacterial growth inhibitory properties of the *Eucalyptus* spp. have been reported against a narrower range of pathogenic bacteria.[11,12]

The *Eucalyptus* spp. extracts displayed the most potent *S. pyogenes* growth inhibitory activity of the extracts tested in our study. Indeed, an MIC of 53 µg/mL was determined for the methanolic *E. major* leaf extract. However, despite being the most promising *S. pyogenes* growth inhibitory extracts, the methanolic and aqueous *E. baileyana* and methanolic *E. major* leaf extracts displayed substantial toxicity, with LC_{50} values as low as 455 µg/mL (methanolic *E. baileyana* leaf extract). Extracts with LC_{50} values < 1000 µg/ml towards *Artemia* nauplii are defined as being toxic,[33] which may impact on their therapeutic potential. As the LC_{50} values are within the therapeutic ranges that would be required for *S. pyogenes* growth inhibition (determined by MIC), studies using human cell lines are required to further evaluate the safety of these extracts. However, even if the *Eucalyptus* spp. extracts are subsequently deemed unsafe for ingestion, they may still be useful *S. pyogenes* growth inhibitory agents. Impetigo is a cutaneous skin disease often resulting from *S. pyogenes* infection.[34,35] Topical application of the extracts may prove effective in treating this form of the disease. Streptococcal pharyngitis is caused by *Streptococcus* spp. infectionson the pharynx surface.[34] Therefore, gargling with solutions containing *Eucalyptus* spp. extracts may prove effective in treating this disease. However, streptococcal induced rheumatic fe-

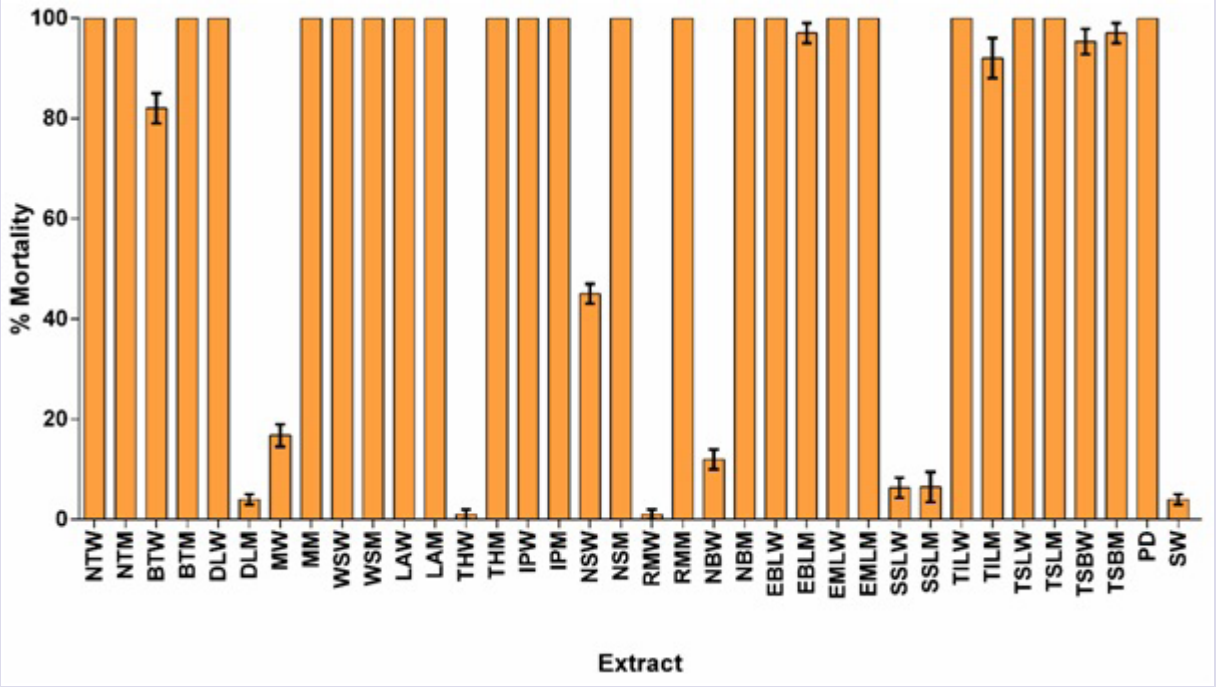

Figure 2: The lethality of the Australian plant extracts (2000 µg/mL) and the potassium dichromate (1000 µg/mL) and seawater controls towards *Artemia franciscana* nauplii after 24 h exposure.
NTW=aqueous native tamarind extract; NTM=methanolic native tamarind extract; BTW=aqueous bush tomato extract; BTM=methanolic bush tomato extract; DLW=aqueous desert lime extract; DLM=methanolic desert lime extract; MW=aqueous muntries extract; MM=methanolic muntries extract; WSW=aqueous wattle seed extract; WSM=methanolic wattle seed extract; LAW=aqueous lemon aspen extract; LAM=methanolic lemon aspen extract; THW=aqueous native thyme extract; THM=methanolic native thyme extract; IPW=aqueous Illawarra plum extract; IPM=methanolic Illawarra plum extract; NSW=aqueous native sage extract; NSM=methanolic native sage extract; RMW=aqueous river mint extract; RMM=methanolic river mint extract; NBW=aqueous native basil extract; NBM=methanolic native basil extract; EBLW=aqueous *E. baileyana* leaf extract; EBLM=methanolic *E. baileyana* leaf extract; EMLW=aqueous *E. major* leaf extract; EMLM=methanolic *E. major* leaf extract; SSLW=aqueous *S. spinescens* leaf extract; SSLM=methanolic *S. spinescens* leaf extract; TILW=aqueous *T. insipidia* leaf extract; TILM=methanolic *T. insipidia* leaf extract; TSLW=aqueous *T. stipitata* leaf extract; TSLM=methanolic *T. stipitata* leaf extract; TSBW=aqueous *T. stipitata* berry extract; TSBM=methanolic *T. stipitata* berry extract; AMP=ampicillin (10 µg) control. Results are expressed as mean zones of inhibition (mm) ± SEM.

ver and rheumatic heart disease result from systemic infections, mainly affecting joint and cardiac tissue. Treatment of these diseases requires ingestion of antibiotics and anti-inflammatory drugs. The toxic nature of the *Eucalyptus* spp. extracts may preclude their use in the treatment of these diseases and instead limit them to topical applications.

Whilst an investigation of the phytochemistry of the *Eucalyptus* spp. extracts was beyond the scope of our study, plants of the genus *Eucalyptus* are well known for their high terpenoid contents. In particular, high 1, 8-cineole contents was reported for several *Eucalyptus* spp.[5,10] Potent bacterial growth inhibitory activity has been reported for 1, 8-cineole against a panel of pathogenic bacteria.[36] Another study reported MIC values for 1, 8-cineole against *Staphylococcus aureus*, *Pseudomonas aeruginosa* and *Escherichia coli* of between 16 and 256 µg/mL.[37] That study did not screen 1, 8-cineole against *S. pyogenes*. *Eucalyptus* spp. are also rich in a variety of other mono-and sesquiterpenoids.[5,10] Some of these terpenoids have been previously reported to have potent broad spectrum antibacterial activity[14] and therefore may contribute to the *S. pyogenes* inhibitory activity.

Another commonality between the inhibitory *Eucalyptus* spp. extracts was that all contained relatively high levels of flavonoids and tannins. Many studies have reported potent growth inhibitory activities for a wide variety of flavonoids against extensive bacterial panels.[38] Similarly, a number of tannin compounds have bacterial growth inhibitory activity. Gallotannins have been reported to inhibit the growth of a broad spectrum of bacterial species[39] through a variety of mechanisms including binding cell surface molecules including lipotoichoic acid and proline-rich cell surface proteins,[40,41] and by inhibiting glucosyltransferase enzymes.[42] Elligitannins are also highly potent inhibitors of bacterial growth, with MIC values as low as 62.5 µg/mL.[39,41] Ellagitannins have also been reported to function via several antibiotic mechanisms including interaction with cytoplasmic oxidoreductases and by disrupting bacterial cell walls.[39,41] Thus, it is likely that multiple compounds within the *Eucalyptus* spp. extracts are contributing to the growth inhibition of *S. pyogenes*.

Lemon aspen extracts, as well as the methanolic wattle seed, native basil and river mint extracts, were also potent *S. pyogenes* growth inhibitors, with MIC values substantially <1000 µg/mL. In contrast with the *Eucalyptus* spp. extracts, these extracts were nontoxic (LC_{50} values substantially >1000 µg/mL). Thus, the therapeutic usage of these extracts need not be limited to external uses. Whilst the *Eucalyptus* spp. extracts may have greater efficacy in topical application, lemon aspen and wattle seed extracts would be more acceptable for systemic streptococcal induced rheumatic fever and rheumatic heart disease. A previous study has reported LC-MS evaluations of lemon aspen extracts prepared in the same

way as those screened in our study.[16] A number of interesting phytocompounds with antibacterial activity were detected. The identification of gingerol, rutin, luteolin, dihydrokaempferol, ellagic acid (and methylated ellagic acid derivatives) and chlorogenic acid was particularly noteworthy. Whether these compounds contribute to the *S. pyogenes* growth inhibitory activity reported here is yet to be determined.

The *S. pyogenes* growth inhibitory activity reported here is particularly noteworthy for the development of future antibiotic chemotherapeutics. Aside from the obvious antibiotic applications to directly treat localised throat (pharyngitis) and skin infections (impetigo),[34,35] a number of substantially more serious illnesses are caused by acute and chronic *S. pyogenes* infections and may also benefit from treatment with these extracts. When *S. pyogenes* invades and colonises deeper tissue it can lead to erysipelas and cellulitis, conditions characterised by localised red, swollen and painful areas, and often by fever and lethargy.[43-45] If not promptly treated, bacterium can spread to other areas via the bloodstream which may result in serious tissue damage and autoimmune diseases such as glomerulonephritis (inflammation of the glomeruli in the kidneys), lymphedema (inflammation of lymph nodes), septic arthritis and rheumatic fever (inflammation of cardiac tissue).[44-46]. Furthermore, acute *S. pyogenes* infections of subcutaneous tissues can induce the potentially fatal disease necrotizing fasciitis.[46] These conditions are not only highly debilitating, but may also be life threatening and new, more effective treatment regimens could potentially prolong and increase the quality of life as well as reducing the burden on the health system.

CONCLUSION

The results of this study demonstrate the potential of *Eucalyptus* spp., lemon aspen, wattle seed, native basil and river mint extracts to block the growth of *S. pyogenes*. The toxicity of the *Eucalyptus* spp. extracts may limit their clinical usage to topical applications. However, the nontoxicity of the lemon aspen fruit, wattle seed, native sage and river mint extracts indicates their potential in the treatment of all manifestations of streptococcal disease, including systemic treatment. Further studies aimed at the purification of the bioactive components are needed to examine the mechanisms of action of these agents.

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CONFLICTS OF INTEREST

The authors report no conflicts of interest.

ABBREVIATIONS USED

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

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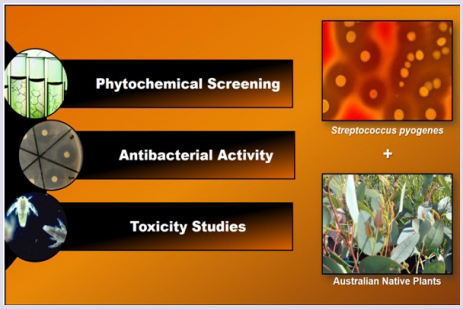
PICTORIAL ABSTRACT



SUMMARY

- *E. major* extracts were particularly potent *S. pyogenes* growth inhibitors, with MIC values of 134 and 53 µg/mL respectively.
- Aqueous and methanolic *E. baileyana* extracts were also potent inhibitors of *S. pyogenes* growth with MIC values of 341 and 88 µg/mL respectively.
- The lemon aspen extracts as well as the methanolic wattle seed, native basil and river mint extracts were also potent growth inhibitors with MIC values substantially <1000 µg/mL.
- All extracts except the *Eucalyptus* spp. Extracts were nontoxic in the *Artemia* nauplii assay.

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Mr Cameron Lee: Completed his Bachelor of Science (BSc) in 2015 and is currently concluding his honours year. His research involves the investigation of thermophilic anaerobes that utilize toxic metals in anaerobic respiration (including uranium and arsenic). He has extensive experience in anaerobic cultivation/isolation and in numerous analytical techniques associated with heavy metal analysis.

Dr Wright: Received his PhD in 2014, for his work investigating the manganese reduction and oxidation characteristics of environmental bacteria. He is currently a postdoctoral researcher at Griffith University, Australia, where he is working on several projects both in the areas of geomicrobiology and pharmacognosy. His present research interests are the use of bacteriogenic manganese oxides in the bioremediation of metal-contaminated sites as well as the use of Australian native plants in the treatment and prevention of various pathogenic bacteria.

Megan Arnold: Is currently undertaking her PhD in Tropical Parasitology at Griffith University's Eskitis Institute for Drug Discovery with a focus on the identification and development of novel chemoprophylactic agents for malaria. Her other research interests include investigating Australian high antioxidant plants for their antibacterial capabilities.

Dr Anthony Greene: Is a senior lecturer and researcher at Griffith University, Brisbane Australia. He obtained his PhD in Microbiology from the University of New South Wales and focuses on extreme environments, Bioremediation and Geomicrobiology. His specific interests include the microbial ecology of thermophilic, saline and alkaliphilic environments and the mechanisms and industrial potential of extremophilic bacteria contained therein.

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.