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Original Article

Brachychiton rupestris (T.Mitch. ex Lindl.) K. Schum. Extracts Inhibit the Growth of *Streptococcus pyogenes*

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ABSTRACT

Introduction: *Streptococcus pyogenes* is a gram positive pathogenic bacterium which causes a variety of diseases including streptococcal pharyngitis, impetigo and rheumatic heart disease. Many *Brachychiton* spp. have reported uses to treat pathogenic illness and are rich in flavonoids with reported antibacterial activity. Despite this, *B. rupestris* leaf extracts have not previously been examined for bacterial growth inhibitory properties. **Methods:** The ability of *B. rupestris* leaf extracts to inhibit the growth of *S. pyogenes* was investigated by disc diffusion and growth time course assays. The growth inhibitory activity was further quantified by MIC determination. Toxicity was determined using the *Artemia franciscana* nauplii bioassay. **Results:** The methanolic and aqueous *B. rupestris* leaf extracts were potent inhibitors of *S. pyogenes* growth, with MIC values as low as 445µg/mL. The antibacterial activity of the methanolic and aqueous *B. rupestris* leaf extracts were further investigated by growth time course assays that showed significant growth inhibition within 1h of exposure. All extracts were determined to be nontoxic in the *Artemia franciscana* nauplii bioassay, indicating their safety for use in preventing *S. pyogenes* associated diseases caused by these pathogens. **Conclusion:** The lack of toxicity of the *B. rupestris* leaf extracts and their growth inhibitory bioactivity against *S. pyogenes* indicate their potential in the development of new therapies for rheumatic fever, pharyngitis, impetigo and other illnesses caused by this bacterium.

Key words: Malvaceae, Bottle tree, Australian plant, Traditional medicine, Antibacterial, Rheumatic fever, Impetigo.

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INTRODUCTION

Streptococcus pyogenes is a versatile gram-positive bacterial pathogen which can infect many tissues in humans, resulting in different diseases. In nature, *S. pyogenes* is present as part of the natural skin flora of humans and animals which under certain conditions can cause disease.[1,2] Diseases in humans can vary and range from non-life threatening epithelial/throat infections, such as pharyngitis or skin infections including impetigo and scarlet fever, to potentially fatal internal infections such as pneumonia, necrotizing fasciitis, toxic shock syndrome or meningitis.[3-4] Some *Streptococcus* species can also trigger autoimmune rheumatic heart disease.[5-6] 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 from antibiotic overuse,[7] therapies aimed at alleviating the symptoms are often the preferred option unless complications arise. Probing traditional medicines and natural plant resources offers an alternate means of fighting streptococcal diseases through the prevention of bacterial growth.

Brachychiton rupestris (T.Mitch. ex Lindl.) K.Schum. (family Malvaceae; commonly known as Queensland bottle tree, narrow-leaved bottle tree; synonyms *Delabechea rupestris* T.Mitch. ex. Lindl., *Brachychiton delabechei* F.Muell., *Sterculia rupestris* (T.Mitch. ex. Lindl.)) is a large succulent, deciduous tree which grows up to 25 metres high. The tree develops bulbous trunk (Figure 1a) from which its common names are derived. Indeed, trunks up to 3.5m diameter have been measured for some trees. *B. rupestris* is native to tropical and sub-tropical regions in the north-eastern state of Queensland, Australia. The first Australians ate the roots of young plants the tree as a food. Several *Brachychiton* spp., including *B. rupestris*, were also used medically by the Australian Aborigines to treat wounds, sores and eye infections.[8] Laboratory-based studies have confirmed the antimicrobial activity of several *Brachychiton* spp. against a broad panel of microbes,[9] although *B. rupestris* remains poorly studied.

The leaves have been reported to be particularly rich in flavonoids and flavonoid glycosides including kaempferol (Figure 1d), kaempferol 3-*O*-β-D-rutinoside (Figure 1e), kaempferol 3-*O*-(2",6"-dirhamnosyl)-β-glucoside [K 3-*O*-(2"-rhamnosylrutinoside)] (Figure 1f), Quercetin 3'-methyl ether (isorhamnetin) (Figure 1g) and isorhamnetin 3-*O*-β-D-rutinoside (Figure 1h).[9] Interestingly, many flavonoids have potent antibacterial activity[10] and this species may therefore be useful in blocking bacterial infections. Despite this, antibacterial studies of *B. rupestris* extracts are lacking. The current study was undertaken to screen of *B. rupestris* leaf extracts for the ability to inhibit the growth of the gram-positive bacterial pathogen, *S. pyogenes*.

MATERIALS AND METHODS

Plant collection and extraction

Brachychiton rupestris (T. Mitch. ex Lindl.) K.Schum. leaves were obtained from two different sources in geographically diverse regions of Brisbane, Australia. The first set of leaves (referred to as tree 1 or Botanical Gardens tree) were obtained from Philip Cameron, senior botanical officer, Mt Cootha Botanical Gardens, Brisbane, Australia. The other leaves were obtained from a tree in the Logan area, south of Brisbane (gps coordinates (27.6428°S, 153.1135°E). The leaf samples were dried separately in a Sunbeam food dehydrator, ground to a coarse powder and stored at -30°C until use. A volume of 50mL of AR grade methanol (Ajax Fine Chemicals, Australia) or sterile deionised water was added to individual 1g masses of the plant material was extracted for 24 hours at 4°C, with gentle shaking. The extract was filtered through filter paper (Whatman No. 54) under vacuum, followed by lyophilisation. The resultant pellets were weighed to determine the extraction yield and subsequently 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.

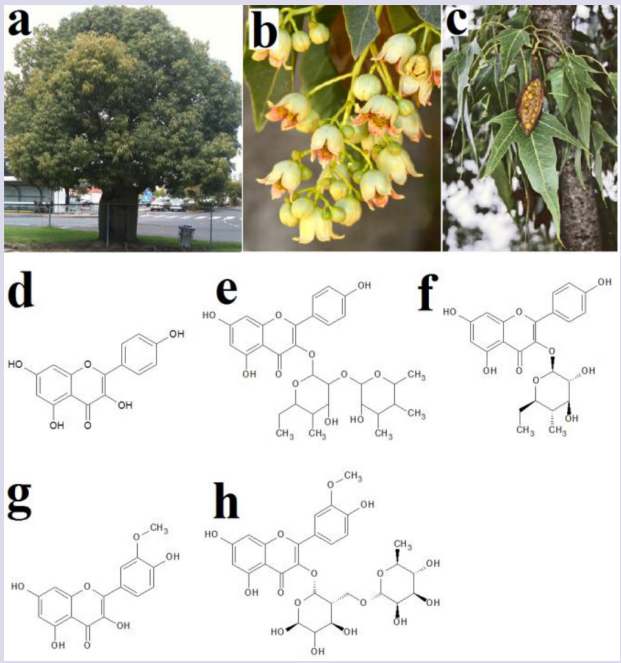

Figure 1: *B. rupestris* (a) whole tree, (b) flowers, (c) leaves and seed pod, as well as the phytochemical compounds (d) kaempferol, (d) kaempferol 3-O-β-D-rutinoside, (e) kaempferol 3-O-(2″,6″-dirhamnosyl)-β-glucoside [K 3-O-(2″-rhamnosylrutinoside)], (f) Quercetin 3′-methyl ether (isorhamnetin) and (g) Isorhamnetin 3-O-β-D-rutinoside.

Qualitative phytochemical studies

Phytochemical analysis of the *B. rupestris* 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 purchased from Oxoid Ltd., Australia. The reference strain of *Streptococcus pyogenes* (ATCC19615) was purchased from American Tissue Culture Collection, USA. The clinically isolated *S. pyogenes* strain was 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 *B. rupestris* leaf extracts was determined using a modified disc diffusion assay.[14-16] Briefly, 100µL of the bacterial suspension in log phase was spread onto nutrient agar plates and the extracts were tested for antibacterial activity using 6mm sterilised filter paper discs. The discs were each infused with 10µL of the individual 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 37°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 *B. rupestris* leaf extracts were diluted in deionised water (1% DMSO) and tested across a range of concentrations. Discs were individually infused with 10µL of each extract, allowed to dry and placed onto the inoculated plates. The assay was completed as outlined above and graphs of the ZOI versus ln 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 *S. pyogenes* (ATCC19615) in nutrient broth were individually added to 27mL nutrient broth containing 3mL of 10mg/mL of the extract to give a final extract concentration of 1000µg/mL in the assay. The tubes were incubated at 37°C with gentle shaking. The optical density was measured hourly at 550nm for a 6h incubation period. Control tubes were incubated under the same conditions but without the extract. All assays were performed in triplicate.

Toxicity screening

Artemia franciscana nauplii toxicity screening

Toxicity was tested using an adapted *Artemia franciscana* nauplii lethality assay.[20-22] Briefly, *A. franciscana* nauplii were incubated in the presence of the extracts, reference toxin (1mg/mL potassium dichromate) or artificial seawater (negative control) at 25±1°C under artificial light. All treatments were performed three times in triplicate (n=9). The number of dead were counted in each well at 24h and 48h. At the completion of the 48h exposure period, the remaining live nauplii were sacrificed and the total number of nauplii in each well were counted and used to calculate the % mortality per well. LC_{50} values were calculated for each treatment using probit analysis.

Statistical analysis

Data are expressed as the mean ± SEM of three independent experiments with internal triplicates (n=9). 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 *B. rupestris* leaves with methanol yielded 348 and 409mg of extracted material for the botanical gardens and suburban trees respectively (Table 1). Water extracted lower yields of 260 and 286mg for the botanical gardens and suburban trees respectively. All extracts were resuspended in 10mL of deionised water (containing 1% DMSO), resulting in an extract concentrations shown in Table 1. Qualitative phytochemical studies showed that all of the extracts contained high levels of phenolics, flavonoids and tannins, as well as moderate levels of saponins. Lower levels of triterpenoids, phytosterols and alkaloids were also detected. Cardiac glycosides and anthraquinones were completely absent or below the detection thresholds for these assays.

Antimicrobial activity

To determine the growth inhibitory activity of the *B. rupestris* leaf extracts against *S. pyogenes*, aliquots (10µL) of each extract were screened in the disc diffusion assay. The *B. rupestris* leaf extracts inhibited the growth of both a *S. pyogenes* reference and clinical isolate strains (Figure 2). The

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

			Botanic Gardens Tree		Suburban Tree	
			Methanolic extract	Aqueous extract	Methanolic extract	Aqueous extract
	Mass of extracted material (mg)		348	260	409	286
	Concentration of resuspended extract (mg/mL)		34.8	26	40.9	28.6
Qualitative Phytochemical Tests	Phenols	Total phenols	+++	+++	+++	+++
		Water soluble phenols	+++	+++	+++	+++
		Insoluble phenols	+++	+++	+++	+++
	Saponins	Froth persistence	+	++	++	++
		Emulsion test	+	++	+	++
	Cardiac glycosides	Keller-Kiliani Test	-	-	-	-
	Triterpenoids	Salkowski Test	++	+	+	+
	Phytosterols	Acetic Anhydride Test	+	+	+	+
	Alkaloids	Meyer's Test	++	+	+	+
		Wagner's Test	+	+	+	+
		Draggendoff's Test	+	+	-	-
	Flavonoids	Kumar Test	+++	+++	+++	+++
	Tannins	Ferric Chloride Terst	+++	+++	+++	+++
		Lead Acetate Test	++	+	++	++
	Anthra- quinones	Free	-	-	-	-
		Combined	-	-	-	-

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

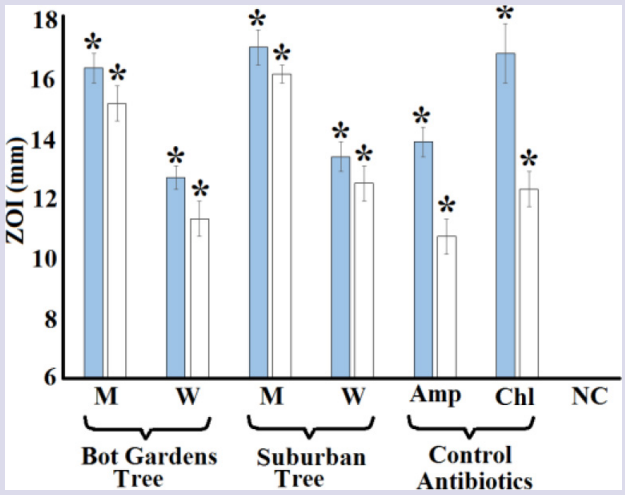

Figure 2: Growth inhibitory activity of *B. rupestris* leaf extracts and reference antibiotics against various bacterial species measured as ZOIs (mm) ± SEM. Shaded bars represent the growth inhibition activity of the *B. rupestris* extracts against the reference *S. pyogenes* strain; open bars represent the growth inhibition activity of *B. rupestris* extracts against clinical strain of *S. pyogenes*. Ampicillin and chloramphenicol standard discs (10µg) were used as positive controls. All assays were completed three times, each with internal triplicates (n=9) and the results are expressed as mean zones of inhibition (mm) ± SEM.

methanolic and aqueous extracts were effective inhibitors of both strains, although the reference strain was generally more susceptible than the clinical isolate (as judged by the size of the ZOI). The potency of the extracts compared well with that of the positive control antibiotics ampicillin and chloramphenicol, each of which were tested at high doses (10µg/disc). Indeed, the methanolic *B. rupestris* extracts produced similar size ZOIs as the chloramphenicol discs and substantially larger ZOIs than ampicillin.

The antimicrobial efficacy was further quantified by determining the MIC value. All extracts were strong inhibitors of *S. pyogenes* growth, with MIC values substantially <1000µg/mL. Indeed, the methanolic extract produced from the leaves of the botanical gardens tree had an MIC value of 445µg/mL against the reference bacterial strain. As *S. pyogenes* can trigger rheumatic fever in genetically susceptible people.[5,6] this extract may be useful for preventing this disease (and other diseases caused by this bacterium).

Bacterial growth time course assay

The antibacterial activity of the *B. rupestris* methanolic (Figure 3a) and aqueous leaf extracts (Figure 3b) was further investigated in the reference strain of *S. pyogenes* by bacterial growth time course assays in the presence and absence of the extracts. The starting concentration of the extract used in these assays was 1000µg/mL. The methanolic *B. rupestris* extract significantly inhibited *S. pyogenes* within 1h of exposure, indicating a rapid antimicrobial action. The absorbance of the *S. pyogenes* culture (and thus the bacterial growth) remained substantially lower than the untreated control for the entire 6h incubation period. Indeed, the turbidity had not significantly increased throughout the 6h growth period, indicating that

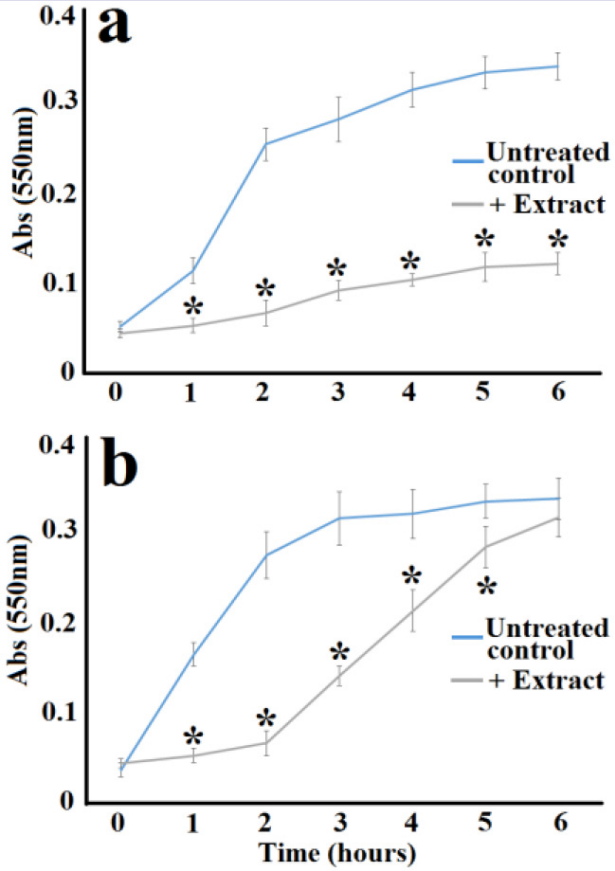

Figure 3: Bacterial growth curves for the *B. rupestris* leaf (a) methanolic and (b) aqueous extracts of the botanical gardens tree against the reference *S. pyogenes* strain. All bioassays were performed three times in triplicate (n=9) and are expressed as mean ± SEM. * = results that are significantly different between the treated and the untreated control growth (P<0.01).

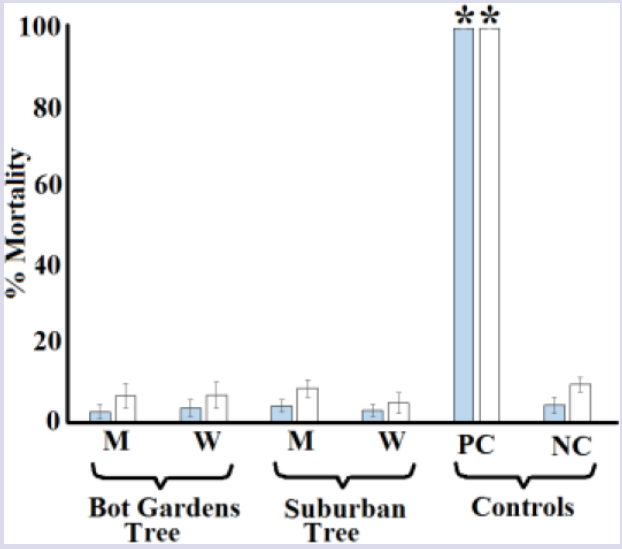

Figure 4: The lethality of the *B. rupestris* leaf extracts, potassium dichromate control (1000µg/mL) and seawater (negative control). Shaded bars represent the mortality induced by the *B. rupestris* extracts following 24h exposure; open bars represent the mortality induced by the B. rupestris extracts following 48h exposure. All bioassays were performed three times in triplicate (n=9) and are expressed as mean ± SEM. * indicates results that are significantly different to the untreated (seawater) control at the equivalent exposure time (P<0.01).

the extracts may be bacteriocidal at the concentrations tested (Figure 3a). The aqueous extract also was rapid in its inhibition of *S. pyogenes* growth, with a significant decrease in bacterial growth noted within the first hour of incubation. However, in contrast to the methanolic extract, the levels of bacterial growth had returned to similar levels to that of the untreated control by the end of the 6h incubation period (as judged by turbidity; Figure 3b). This may indicate that the aqueous *B. rupestris* extract has bacteriostatic effects at the tested concentration, rather than bactericidal effects.

Quantification of toxicity

The toxicity of the *B. rupestris* leaf extracts was initially tested undiluted in the *A. franciscana* nauplii bioassay (Figure 4). The mortality in the presence of all extracts was not significantly different to that of the untreated control at 24h and thus were deemed to be non-toxic. Extracts with 24h LC_{50} values >1000µg/mL have previously been defined as non-toxic.[22] In contrast, the potassium dichromate positive control induced substantial mortality within 4h (results not shown), with 100% mortality induction seen by 24h. The mortality induction remained low for the *B. rupestris* extract at 48h. Indeed, the % mortality induction was substantially <50% for all extracts at all times tested and therefore it was not possible to determine LC_{50} values for any of the *B. rupestris* extracts (Table 2).

DISCUSSION

Streptococcus is a genus of gram-positive coccus bacteria that are associated with a wide variety of diseases including bacteremia, cellulitis, dental caries, endocarditis, impetigo, meningitis, pharyngitis, pneumonia, respiratory infections, rheumatic fever, sepsis, subcutaneous and organ abscesses and urinary tract infections. The *Streptococcus* spp. are grouped according to their haemolytic properties and Lancefield serotyping. Group A streptococcal pharyngitis is an acute infection of the nasopharynx and/or oropharynx and is initiated through infection by *S. pyogenes*.[23] *S. pyogenes* infections are the most common bacterial cause of pharyngitis and are responsible for up to 33% of all diagnosed cases of sore throat in children and up to 10% in adults.[24] Although mostly non-life threatening, Group A streptococcal infections are a significant economic burden. Indeed, recent estimates place the societal cost (both medical and nonmedical) in the United States alone ranging from $224 to $539 million dollars annually.[25] Whilst the bacterium responds well to antibiotic treatment,[24] the increasing risk of drug resistance[7] highlights the need to develop alternatives to fight these and other diseases. Probing plant resources via reference to traditional medicines for previously undiscovered antibacterial products offers an alternative to the traditional drug design and synthesis methods.

Several *Brachychiton* spp., including *B. rupestris*, are documented as treatments for wounds, sores and eye infections in Australian Aboriginal traditional healing systems.[8] Interestingly, all of these conditions may be caused by bacterial pathogens and several studies have reported strong antimicrobial activity for other *Brachychiton* spp.[9] Surprisingly, despite the traditional therapeutic use of *B. rupestris* and the confirmed antibacterial activity of taxonomically similar species, confirmation of

Table 2: Minimum inhibitory concentrations (µg/mL) of the *B. rupestris* leaf extracts against each bacterial strain and LC50 values (µg/mL) against *Artemia* nauplii.

	Organism	Exposure time (h)	MIC or LC50 (ug/mL)			
			Gardens tree		Suburban tree	
			Methanolic extract	Aqueous extract	Methanolic extract	Aqueous extract
Bacterial growth inhibition	*S. pyogenes* (reference strain)	24	445	938	659	1187
	S. pyogenes (clinical strain)	24	750	1145	843	1252
Toxicity	*Artemia* nauplii	24	CND	CND	CND	CND
		48	CND	CND	CND	CND

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.

the antimicrobial properties of *B. rupestris* is lacking. In the current study, we determined that methanolic and aqueous *B. rupestris* extracts were good inhibitors of *S. pyogenes* growth. This growth inhibitory activity was 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),[23-24] 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.[26-28] 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).[27-29] Furthermore, acute *S. pyogenes* infections of subcutaneous tissues can induce the potentially fatal disease necrotizing fasciitis.[28] 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. The efficacy of the *B. rupestris* leaf extracts indicates that they may have potential in the treatment of these illnesses and further investigation is warranted.

Whilst an examination of the phytochemistry of the *B. rupestris* leaf extracts was beyond the scope of our study, a commonality of this genus is their relatively high levels of flavonoid components including kaempferol and quercetin, as well as their glycosides.[9] Flavonoids (and their glycosides) have been reported to inhibit the growth of a broad spectrum of bacterial species.[30-34] One study examined the ability of quercetin and rutin and their corresponding glycosides to inhibit the growth of *Pseudomonas maltophilia* and *Enterobacter cloacae*.[31] That study showed that the quercetin glycosides showed the strongest inhibitory activity of the flavonoids glycosides tested. Many of the other glycosides also inhibited bacterial growth, albeit with lower efficacy. Another study tested the inhibitory activity of a panel of 38 flavonoids against methicillin resistant *Staphylococcus aureus* (MRSA) and reported moderate antibacterial activity for several flavonoids including quercetin and luteolin. Rutin was also shown to have a low MIC against multi-resistant β-lactamase producing *Klebsiella pneumonia*.[32] Another study reported that kaempferol and myricetin are potent inhibitors of *Porphyromonas gingivalis gingivalis*, *Prevotella intermedia*, *Streptococcus mutans* and *Actinomyces viscosus,* each with MIC values of approximately 20µg/mL.[33-34] Thus, flanonoids have potential in the treatment of infective diseases and much more study is required to examine the structure/activity relationships of these compounds, as well as the mechanisms of their action.

It is likely that other phytochemical classes may also contribute to the growth inhibitory properties of these extracts. Our qualitative phytochemical screening studies indicate that alkaloids, polyphenolics, saponins, tannins and terpenes were also present in the *B. rupestris* leaf extracts. Terpenoids have been previously reported to have potent broad spectrum antibacterial activity[34] and therefore may contribute to the inhibitory activity against *S. pyogenes*. Many studies have also reported potent antibacterial activities for a wide variety of tannins.[35] Further phytochemical evaluation studies and bioactivity driven isolation of active components is required to further evaluate the mechanism of *S. pyogenes* growth inhibition.

The findings reported here also demonstrate that all of the extracts tested in our study were nontoxic towards *Artemia franciscana* nauplii, with LC_{50} values substantially >1000µg/mL. Extracts with LC_{50} values >1000µg/mL towards *Artemia* nauplii are defined as being nontoxic.[21] Whilst our preliminary toxicity studies indicate that these extracts may be safe for use as *S. pyogenes* growth inhibitors, studies using human cell lines are required to further evaluate the safety of these extracts.

CONCLUSION

The growth inhibitory activity of the *B. rupestris* leaf extracts against *S. pyogenes* and their lack of toxicity indicate their potential for 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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CONFLICT OF INTEREST

The authors declare no conflicts of interest.

ABBREVIATIONS

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

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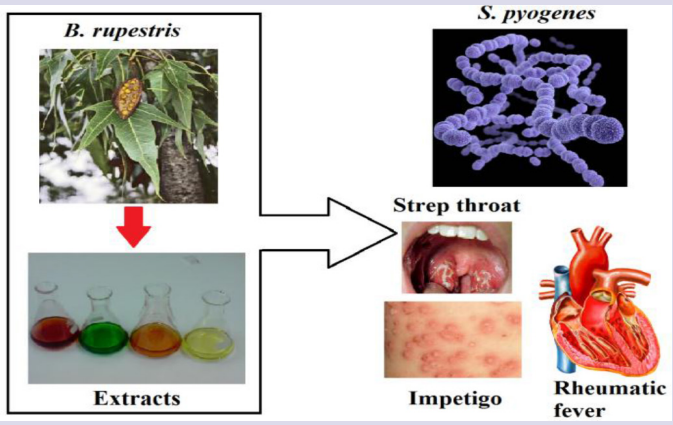
PICTORIAL ABSTRACT



SUMMARY

- *B. rupestris* leaf extract were screened for the ability to block the growth of *S. pyogenes*.
- The antibacterial activity was quantified by determining the MIC values of each extract.
- The growth time course of the most potent extract was examined.
- Toxicity of the *B. rupestris* extracts was determined using the *Artemia* nauplii toxicity bioassay.

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