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Original Article

Tasmannia lanceolata (Poir.) A.C.Sm. Pepperberry Extracts Inhibit the Growth of the Pharyngitis Causing Pathogen *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, depending on which tissue it infects. *Tasmannia lanceolata* (Poir.) A.C.Sm. pepperberries were used therapeutically by the first Australians, including for the treatmenbt of bacterial infections. This study focuses on the growth inhibitory activity of *T. lanceolata* pepperberry extracts against *S. pyogenes*. **Materials and Methods:** Solvent extracts were prepared from *Tasmannia lanceolata* pepperberries using solvents of varying polarity. The extracts were investigated by disc diffusion assay for the ability to inhibit the growth of *S. pyogenes*. MIC values were subsequently quantified by both solid-phase and liquid dilution MIC assays. Toxicity was determined using the *Artemia franciscana* nauplii bioassay. **Results:** The methanolic, aqueous and ethyl acetate *T. lanceolata* pepperberry extracts displayed noteworthy antibacterial activity in the disc diffusion and liquid dilution bioassays against *S. pyogenes*. The methanolic extract was particularly potent, with an LD MIC value of 782 µg/mL recorded. The aqueous and ethyl acetate *T. lanceolata* pepperberry extracts also displayed noteworthy growth inhibitory activity of *S. pyogenes*, albeit with substantially higher LD MIC values (1375 and 1125 µg/mL respectively). All extracts were determined to be non-toxic in the *Artemia* nauplii bioassays, with LC_{50} values substantially >1000 µg/mL. **Conclusion:** The noteworthy growth inhibitory bioactivity of the methanolic, aqueous and ethyl acetate *T. lanceolata* pepperberry extracts against *S. pyogenes* demonstrates their potential for the treatment and prevention of pharyngitis, impetigo and rheumatic heart disease. All extracts were nontoxic indicating their safety for therapeutic use.

Keywords: Winteraceae, Australian Plants, Mountain Pepperberry, Tasmanian Pepper, Pharyngitis, Impetigo, Rheumatic Heart Disease, Antibacterial Activity.

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INTRODUCTION

The genus *Streptococcus* comprises over 50 species of gram-positive, non-sporulating cocci-shaped bacteria. Found in a diverse range of environments, or as part of the natural human microflora, *Streptococcus* spp. are primarily facultatively anaerobic, although some are obligate anaerobes.[1] Many species within the genus are pathogenic and responsible for an extensive variety of diseases. Pathogens within the genus can infect ruminants, humans or cause disease in both people and animals.[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]

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 *Streptococcus pyogenes*.[6] *Streptococcus 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.[7] Although mostly non-life threatening, group A streptococcal infections are a significant economic burden. Indeed, the societal cost (both medical and non-medical) in the United States alone was recently estimated to be between $224 and $539 million dollars annually.[8] While the bacterium responds well to antibiotic treatment,[7] the increasing risk of drug resistance highlights the need to develop alternatives to fight these and other diseases. Probing natural plant resources for previously

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undiscovered anti-bacterial products offers an alternative to the traditional drug design and synthesis.

Tasmannia lanceolata (Poir.) Sm. (family Winteraceae; commonly known as Tasmanian pepper or mountain pepperberry; Figure 1a) is a medium to large shrub that is endemic to the woodlands and cool temperate rainforests of Tasmania and the south-eastern region of the Australian mainland.[9] The pepperberries contain several notable compounds including polygodial (Figure 1b) and piperine (Figure 1c), which give the berries their characteristic peppery aroma and taste.[9] The berries, leaves and bark of this species were used as a food flavouring and as a traditional medicine by the first Australians.[9,10] When the berry is air dried it forms a small, hard peppercorn, which is suitable for milling or crushing. The berry has a pleasant spicy flavour and sharp aroma. Amongst its therapeutic uses, the first Australians used *T. lanceolata* to treat stomach disorders and as an emetic.[9,10] The first Australians also used *T. lanceolata* for the treatment of skin disorders, venereal diseases, colic, stomach ache and as a quinine substitute.[9,10] European colonists subsequently recognized the therapeutic potential of *T. lanceolata* and the bark has been used as a substitute for other herbal remedies (including those derived from the related South American Winteraceae species, *Drimys wintera* (winter bark)[11] to treat scurvy due to its high antioxidant content.[9,10]

Several studies have examined the antibacterial activity of *T. lanceolata* extracts against a panel of bacteria including *Yersinia enterocolitica*,[12] *Bacillus anthracis*,[13] *Clostridium perfringens*,[14] *Proteus mirabilis*,[15] and against a panel of bacterial gastrointestinal pathogens.[16] *Tasmannia lancelata* pepperberries and leaves have also been screened for anti-protozoal activity against *Giardia duodenalis* and noteworthy activity has been reported.[17,18] Despite these earlier studies, *T. lanceolata* extracts have not yet been tested for antibacterial activity against many other bacterial species. In particular, the ability of the extracts to inhibit the growth of skin bacteria, including *Staphylococcus* spp., have been largely ignored. This study investigated the growth inhibitory activity of *T. lanceolata* pepperberry extracts against *Streptococcus pyogenes* as a preliminary evaluation of their potential to treat infections of this bacterium.

MATERIALS AND METHODS

Plant source and extraction

The *Tasmannia lanceolata* (Poir.) A.C.Sm. The pepperberries used in this study were purchased from GoWild Harvest (Australia) as semi-dried berries. The berries were further dehydrated in a Sunbeam food dehydrator until a constant mass was obtained upon repeated measurements. A voucher specimen (GUTPGW-2015-BE) is stored at the School of Environment and Science, Griffith University, Australia. The dried berries were ground and individual 1 g quantities of the material were weighed into separate tubes and 50 mL of methanol, deionised water or ethyl acetate were added. All solvents were obtained from Ajax Fine Chemicals, Australia and were AR grade. The ground plant materials were individually extracted in each solvent for 24 hr at 40°C with gentle shaking. The extracts were subsequently filtered through Whatman No. 54 filter paper under vacuum, and dried at 40°C. The resultant dried extracts were weighed and resuspended in 10 mL deionised water (containing 0.5% DMSO). The suspensions were briefly sonicated (3 x 20 s pulse cycles, at 20 kHz) and then sterilised by filtration through a 0.2 µm membranes and stored at 4°C until required for further analysis.

Qualitative phytochemical studies

Phytochemical analysis of the extracts for the presence of triterpenoids, tannins, saponins, phytosteroids, phenolic compounds, flavonoids, cardiac glycosides, anthraquinones, and alkaloids was performed as previously described.[19-21]

Antibacterial screening

All media was supplied by Oxoid Ltd., Australia. A reference strain of *Streptococcus pyogenes* (ATCC 12384) was purchased from the American Type Culture Collection and used in this study. All growth studies were performed using nutrient agar (Oxoid Ltd., Australia) under aerobic conditions. Incubation was at 37°C and the stock culture was subcultured and maintained in nutrient broth at 4°C.

Evaluation of antibacterial activity on agar

Antibacterial activity screening of the *T. lanceolata* pepperberry extracts on solid agar was achieved using a modified disc diffusion assay method.[22-24] Extracts (10 µL) were infused onto Whatman #1 filter discs (6 mm in diameter), and were tested in parallel with negative control discs containing 10 µL of extract solvent (0.5% DMSO). Preloaded ampicillin discs (10 µg) were used as positive controls on each plate to compare antibacterial activity, while filter discs infused with 10 µL of distilled water were used as negative controls.

Minimum inhibitory concentration (MIC) determination

The minimum inhibitory concentration for each extract was determined using a disc diffusion (as described above) across a range of concentrations and Ln linear regression was used to calculate the disc diffusion MIC (DD MIC). The microplate liquid dilution MIC (LD MIC) method[25-28] was also used as a measure of antibacterial strength as it is generally considered the most sensitive bacterial growth inhibitory assay. Furthermore, as microplate liquid dilution MIC assays are perhaps the most commonly used method of quantifying bacterial growth inhibition efficacy, use of this method allows for comparisons with other studies. All microplates were incubated at 37°C for 24 hr following addition of the test samples. *p*-Iodonitrotetrazolium violet (INT) was obtained from Sigma-Aldrich (Australia) and

dissolved in sterile deionised water to produce a 0.2 mg/mL INT solution. A 40 µL volume of this solution was added into all wells and the plates were incubated for a further 6 hr at 24-30°C. Following incubation, the MIC was visually determined as the lowest dose at which colour development was inhibited.

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.[29-31] Briefly, 400 µL of seawater containing ~47 (mean 46.8, *n*=120, SEM 11.7) *A. franciscana* nauplii were added to wells of a 48 well plate and immediately used for bioassay. A volume of 400 µL of diluted plant extracts, reference toxin or seawater control were transferred to the wells and incubated at 25±1°C under artificial light (1000 Lux). A negative control (400 µL seawater) was run in triplicate for each plate. All treatments were performed three time, each with internal triplicates (*n*=9). The wells were checked at regular intervals and the number of dead counted. The nauplii were deemed dead if no movement of the appendages was detected within 10 sec. Following 24 hr exposure, all nauplii were sacrificed by acidification of the seawater and counted to determine the total % mortality per well. The LC_{50} with 95% confidence limits for each treatment was assessed using probit analysis.

Calculation of therapeutic index (TI)

Therapeutic indexes (TI) of the *T. lanceolate* pepperberry extracts against the target bacteria were calculated by standard methods[32] using the following formula, and are included as a measure of their suitability of the extracts for therapeutic usage:

$$\text{Therapeutic index} = (\text{ALA } LC_{50})/(\text{MIC})$$

Statistical analysis

Data are expressed as the Mean±SEM of three independent experiments, each with internal triplicates (*n*=9). One-way ANOVA was used to calculate differences between the control and treated groups, with $p<0.01$ considered to be significant.

RESULTS

Liquid extraction yields and qualitative phytochemical screening

Extraction of 1 g of the dried *Tasmannia lanceolata* pepperberries with various solvents yielded dried plant extracts ranging from 57 mg to 187 mg (Table 1). Aqueous and methanolic extracts provided significantly greater yields of extracted material relative to the ethyl acetate extract, which gave a relatively low yield. The dried extracts were resuspended in 10 mL of deionised water (containing 0.5% DMSO), resulting in the concentrations presented in Table 1. Qualitative phytochemical studies showed that methanol and water extracted the greatest amount and widest range of phytochemicals (Table 1). These solvents extracted high levels of phenolic compounds and flavonoids, as well as moderate levels of saponins and lower levels of tannins. The ethyl acetate

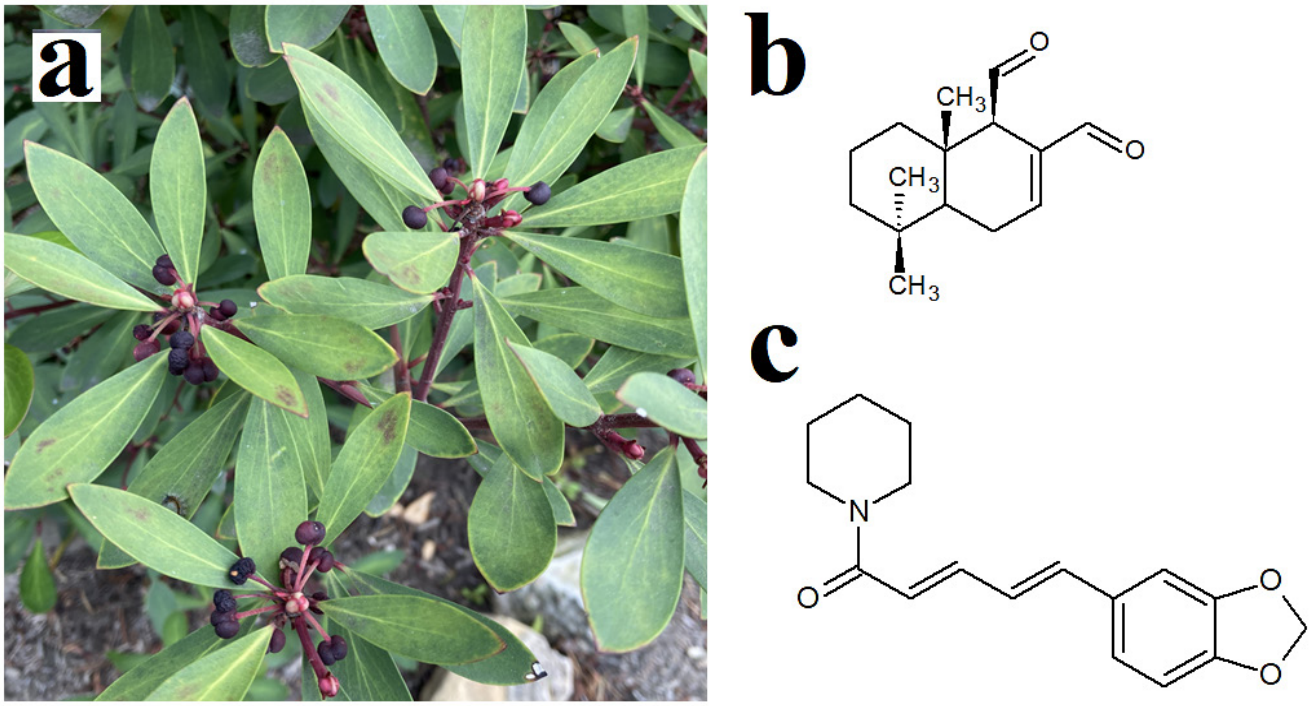

Figure 1: (a) *Tasmannia lanceolata*, as well as the phytochemical constituents (b) polygodial and (c) piperine.

Table 1: The mass of dried extracted *T. lanceolata* pepperberry material, the concentration after resuspension in deionised water (0.5% DMSO) and qualitative phytochemical screenings.

		Methanolic extract	Aqueous extract	Ethyl acetate extract
Mass of extracted material (mg)		187	111	57
Concentration of resuspended extract (mg/mL)		19	11	6
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 Test	+	++	-
	Lead Acetate Test	+	+	-
Anthraquinones	Free	-	-	-
	Combined	-	-	-

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

extracts generally extracted similar, albeit lower, phytochemical profiles compared to the methanolic and aqueous extracts.

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. All of the *T. lanceolata* pepperberry extracts inhibited the growth of *S. aureus* significantly (Figure 2). The methanolic extract was a substantially better inhibitor of *S. aureus* growth than were the aqueous and ethyl acetate extracts (as judged by the ZOI size). Notably, in the initial screening studies, the extracts were tested undiluted as an approximation of how they would be used as traditional medicines. Therefore, the ethyl acetate extract was tested at a substantially lower concentration than the other extracts and its activity may therefore have been substantially better than the screening studies indicated. Therefore, MIC values were also determined so that the potency of the extracts could be compared.

Quantification of minimum inhibitory concentration (MIC)

The antimicrobial efficacies of the *T. lanceolata* pepperberry extracts were further quantified by determining the MIC values (Table 2). The methanolic extract was particularly effective at inhibiting *S. pyogenes* growth, with an LD MIC values of 782 µg/mL. Noteworthy activity was also measured for the aqueous and ethyl acetate extract (LD MIC values of 1375 and 1125 µg/mL respectively).

Quantification of toxicity

All extracts were initially screened at 2000 µg/mL in the assay (Figure 3). 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 hr of exposure and 100% mortality evident in the subsequent 4-5 hr (unpublished results). The methanolic and aqueous extracts also induced substiantially >50% mortality following 24 hr exposure, whilst the ethyl acetate extract induced 26.5% mortality and was therefore deemed to be non-toxic. To further quantify the effects of toxin concentration on the initiation of mortality, the methanolic and aqueous extracts were serially diluted in artificial seawater to test across a range of concentrations in the *Artemia* nauplii bioassay. The 24 hr LC_{50} values of the *T. lanceolata* pepperberry extracts towards *A. nauplii* are displayed in Table 2. LC_{50} values substantially >1000 µg/mL were determined for the methanolic and aqueous extracts. As extract with LC_{50} values >1000 µg/mL towards *Artemia* nauplii have been defined as being non-toxic in this assay,[31] all of the *T. lanceolata* pepperberry extracts were deemed to be non-toxic.

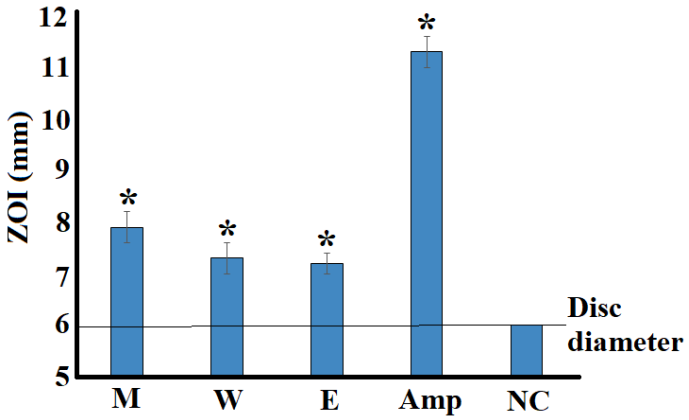

Figure 2: Growth inhibitory activity of the *T. lanceolata* pepperberry extracts against *S. pyogenes* (ATCC 12384) measured as zones of inhibition (mm). M=methanolic extract; W=aqueous extract; E=ethyl acetate extract; Amp=ampicillin (10 µg); NC=negative control (0.5% DMSO). Results are expressed as mean zones of inhibition±SEM. * indicates results that are significantly different to the negative control (p<0.05).

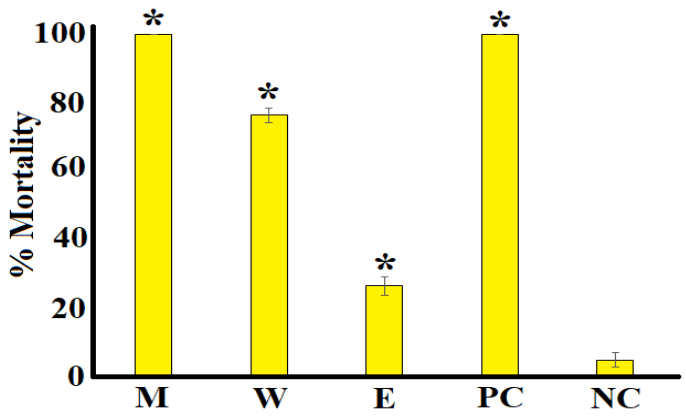

Figure 3: The lethality of the *T. lanceolata* pepperberry extracts (2000 µg/mL) and the potassium dichromate (1000 µg/mL) and seawater controls towards *Artemia franciscana* nauplii after 24 hr exposure. M=methanolic extract; W=aqueous extract; E=ethyl acetate extract; PC=potassium dichromate control; NC=negative (seawater) control. * indicates results that are significantly different to the negative control (p<0.05). Results are expressed as mean % mortality±SEM (n=9).

Table 2: Disc diffusion and liquid dilution MICs against *S. pyogenes* growth (µg/mL) of the *T. lanceolata* pepperberry extracts and LC_{50} values (µg/mL) in the *Artemia* nauplii bioassay.

Test	MIC (µg/mL)		Toxicity	
	DD	LD	ALA (µg/mL)	Therapeutic index (TI)
Methanolic extract	1193	782	1874	2.4
Aqueous extract	1958	1375	2637	1.92
Ethyl acetate extract	1590	1125	NA	CND
Positive control	NA*	0.625*	56#	NT

DD disc diffusion; LD liquid dilution; *=ampicillin was used as the positive control; #=potassium dichromate was used as the positive control; NA=values were not obtained as toxicity did not exceed 50% at any concentration tested; CND=could not determine as a LC_{50} was not obtained; NT=could not be calculated as only one dose was tested, or different positive controls were used across different assays. Numbers indicate the mean values of triplicate determinations. - indicates no inhibition at any concentration tested. TI (therapeutic index) was calculated using LD MIC.

Calculation of therapeutic Index

To further evaluate the suitability of the *T. lanceolata* pepperberry extracts as antibacterial therapeutic agents to treat *S. pyogenes* infections, their therapeutic indexes (TI) were calculated (Table 2). For this study, TI values ≥2 were considered noteworthy. TI values could not be calculated for the ethyl acetate extracts as the % mortality did not exceed 50% at any concentration tested. However, as the ethyl acetate extract was tested at 3000 µg/mL in the *Artemia* nauplii bioassay (with only ~25% mortality noted), the LC_{50} for this extract would be substantially above 3000 µg/mL. Assuming an LC_{50} substantially >3000 µg/mL and given the LD MIC values of 1125 µg/mL, it is reasonable to assume that the LC_{50} value would be substantially in excess of 3, indicating the safety of this extract for therapeutic usage against these bacteria. Notably, TI values of 1.9-2.4 were calculated for the methanolic and aqueous extracts, indicating that whilst these extracts would be useful in treating *S. pyogenes* infections, although caution is recommended to avoid toxicity. Further *in vivo* studies are required to evaluate the safety of these extracts before they can be safely adapted for clinical use.

DISCUSSION

Tasmannia lanceolata pepperberries were used by the first Australians as a nutritious food and general tonic, as well as for their therapeutic properties.[1,2] Recent studies have also reported noteworthy antibacterial activity of extracts prepared from the pepperberries (and also the leaves) against a variety of bacterial species, including *Yersinia enterocolitica*,[4] *Bacillus anthracis*,[5] *Clostridium perfringens*,[6] *Proteus mirabilis*,[7] and a panel of bacterial gastrointestinal pathogens.[8] However, the antibacterial properties of *T. lanceolata* pepperberry extracts are yet to be examined against many bacterial pathogens, including *S. pyogenes*. Due to increasing rates of bacterial resistance to conventional antibiotics, the discovery of new antibiotics is a priority for medical science.[23] Therefore, this study screened the growth inhibitory activity of *T. lanceolata* pepperberry extracts against *S. pyogenes*. Notably, *S. pyogenes* was susceptible to inhibition by all of the *T. lanceolata* pepperberry extracts, although the methanolic extract was a substantially better inhibitor of bacterial growth, with LD MIC value of 782 µg/mL, compared to LD MIC values of 1375 and 1125 µg/mL for the aqueous and ethyl acetate extracts respectively.

The growth inhibitory activity of the *T. lanceolata* pepperberry extracts against *S. pyogenes* is promising for the development of future antibiotic chemotherapeutics. Aside from the obvious antibiotic applications to directly treat localised throat (pharyngitis) and skin infections (impetigo),[6,7] 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.[2-4] If not promptly treated, the 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).[3-5] Furthermore, acute *S. pyogenes* infections of subcutaneous tissues can induce the potentially fatal disease necrotizing fasciitis.[4] These conditions are not only highly debilitating, but may also be life threatening. 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 *T. lanceolata* pepperberry 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 *T. lanceolata* pepperberry extracts was beyond the scope of our study, moderate to high levels of flavonoids were detected in the extracts that were found to exert antibacterial effects. Flavonoids have previously been reported to have good growth inhibitory activity against a broad panel of pathogenic bacteria[33,34] and it is likely that they may contribute to the potent bacterial growth inhibitory activity observed in our study. Other phytochemical classes may also contribute to this activity. Saponins and triterpenes were also detected in the *T. lanceolata* pepperberry extracts. Some terpenoids have potent broad spectrum antibacterial activity[35] and it is therefore likely that they may contribute to the inhibition of the bacteria examined in our study. Several noteworthy compounds have also been identified in previous studies, including a variety of terpenes (including 1, 8-cineole, terpinen-4-ol, α-pinene and β-pinene), flavonoids (including quercetin and rutin), other phenolics (including coumaric acid and caffeic acid) and hydrocarbons.[9] It is likely that these components may also contribute to the antibiotic properties of the extracts tested in this study. Further evaluation of the phytochemical composition and isolation of the active components is required to more rigorously assess the mechanism of bacterial growth inhibition. The findings reported herein also demonstrate that all of the *T. lanceolata* pepperberry extracts tested in our study were nontoxic towards *Artemia franciscana* nauplii, with LC_{50} values substantially >1000 µg/mL as extracts with LC_{50} values >1000 µg/mL towards *Artemia* nauplii are defined as being nontoxic.[31] 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 noteworthy growth inhibitory activity of *T. lanceolata* pepperberry extracts against *S. pyogenes* and their lack of toxicity highlight their potential for the prevention and treatment of the diseases caused by infections of this bacterium. Further studies are required to isolate the compound(s) responsible for this activity and to elucidate the antibacterial mechanisms involved.

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ABBREVIATIONS

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

CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

SUMMARY

- Methanolic, aqueous and ethyl acetate *Tasmannia lanceolata* pepperberry extracts were screened for inhibitory activity against *Streptococcus pyogenes*.
- The potency of the extracts was quantified by determination of MICs in both solid-phase and liquid-phase assays.
- Toxicity of the extracts was evaluated using *Artemia* nauplii bioassays.
- The lack of toxicity of the *T. lanceolata* pepperberry extracts indicates that they are safe for topical or oral use against *S. pyogenes* infections.

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