

Tasmannia lanceolata (Poir.) A.C. Sm. Berry Extracts Inhibit the Growth of the Skin Pathogens *Staphylococcus aureus* and *Staphylococcus epidermidis*

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ABSTRACT

Background: *Tasmannia lanceolata* (Poir.) A.C. Sm. pepper berries were used as a nutritious food and natural medicine by the first Australians. This study focuses on the growth inhibitory activity of *T. lanceolata* pepper berry extracts against the bacterial skin pathogens *Staphylococcus aureus* and *Staphylococcus epidermidis*. **Materials and Methods:** *Tasmannia lanceolata* pepper berry powder was extracted with solvents of varying polarity and screened for inhibition of bacterial growth against *S. aureus* and *S. epidermidis*. Inhibition on agar was assessed by disc diffusion techniques, while the minimum inhibitory concentrations (MICs) were also quantified by liquid dilution assays. Extract toxicities were examined using *Artemia nauplii* bioassays and therapeutic indexes (TIs) were calculated as a measure of therapeutic safety. **Results:** All *Tasmannia lanceolata* pepper berry extracts inhibited the growth of *S. aureus* and *S. epidermidis*. The ethyl acetate extract was the best inhibitor of both bacteria, with LD MIC values of 563 and 750 µg/mL against *S. aureus* and *S. epidermidis* respectively. The methanolic (LD MICs of 891 µg/mL against both bacteria) and aqueous extracts (LD MICs of 1031 and 1375 µg/mL against *S. aureus* and *S. epidermidis* respectively) also displayed noteworthy antibacterial activity. All extracts were determined to be non-toxic in the *Artemia nauplii* bioassays. **Conclusion:** Methanolic, aqueous and ethyl acetate *T. lanceolata* pepperberry extracts had noteworthy inhibitory activity against *S. aureus* and *S. epidermidis*. These extracts were also non-toxic in the *Artemia nauplii* bioassay, indicating their potential for topical and oral treatment against infections of these bacteria.

Keywords: Winteraceae, Mountain pepper berry, Tasmanian pepper, Bacterial skin infections, Antioxidant, Australian plants, Traditional medicine.

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INTRODUCTION

Tasmannia lanceolata (Poir.) Sm. (family Winteraceae; commonly known as Tasmanian pepper or mountain pepper berry) is a shrub which is endemic to the woodlands and cool temperate rainforests of Tasmania and the south-eastern region of the Australian mainland.¹ It is a medium to large shrub that varies between 2-5 m in height (Figure 1a). Individual plants are unisexual, having either male or female flowers. The stems, branches and twigs are red in colour. The aromatic leaves (Figure 1b) are lanceolate to narrowly elliptical in shape (4-12 cm long, 0.7-2 cm wide) with a distinctly pale under surface. Small creamy-white unisexual flowers appear during the summer months. These develop into small fleshy black 2 lobed berries (5-8 mm wide; Figure 1c)

during autumn. The berries contain several notable compounds including polygodial (Figure 1d) and piperine (Figure 1e), which give the berries their characteristic peppery aroma and taste.¹

The berries, leaves and bark of this species have historical uses as a food and as a traditional medicine by the first Australians.^{1,2} 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. *Tasmannia lanceolata* is used as flavouring agent by the first Aborigines and more recently by European settlers. The leaves are used as an herb and the berries are used as a spice. The first Australians also used *T. lanceolata* as a therapeutic agent to treat stomach disorders and as an emetic, as well as general usage as a tonic.^{1,2} Reports also exist of the use of *T. lanceolata* by Australian Aborigines for the treatment and cure of skin disorders, venereal diseases, colic, stomach ache and as a quinine substitute.^{1,2} 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



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species, *Drimys wintera* (winter bark)³ to treat scurvy due to its high antioxidant content.^{1,2}

Several studies have examined the antibacterial activity of *T. lanceolata* extracts against a panel of bacteria including *Yersinia enterocolitica*,⁴ *Bacillus anthracis*,⁵ *Clostridium perfringens*,⁶ *Proteus mirabilis*⁷ and a panel of bacterial gastrointestinal pathogens.⁸ *Tasmannia lanceolata* pepper berries and leaves have also been screened for anti-protozoal activity against *Giardia duodenalis* and noteworthy activity has been reported.^{9,10} 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 aimed to investigate the growth inhibitory activity of *T. lanceolata* pepper berry extracts against *Staphylococcus aureus* and *Staphylococcus epidermidis* as a preliminary evaluation of their potential to treat bacterial skin infections.

MATERIALS AND METHODS

Plant material and extraction

The *Tasmannia lanceolata* (Poir.) A.C.Sm. pepper berries 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 (3x20 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 analyses of the *T. lanceolata* pepper berry extracts for the presence of saponins, phenolic compounds, flavonoids, phytosterols, triterpenoids, cardiac glycosides, anthraquinones, tannins and alkaloids were conducted by previously described assays.¹¹

Antibiotics

Pre-loaded ampicillin (10 µg) standard discs were obtained from Oxoid Ltd., Australia and were used as positive controls for the disc diffusion screening studies.

Bacterial cultures

Reference strains of *Staphylococcus aureus* (ATCC 25923) and *Staphylococcus epidermidis* (ATCC 122292) were purchased from American Type Culture Collection, USA and cultured in nutrient broth (Oxoid Ltd., Australia). Streaked nutrient agar (Oxoid Ltd., Australia) plates were tested in parallel to ensure the purity of all bacterial cultures and for sub-culturing. All bacterial cultures were incubated at 37°C for 24 hr and were subcultured and maintained in nutrient broth at 4°C until use.

Evaluation of antibacterial activity on agar

Antibacterial activity screening of the *T. lanceolata* pepper berry extracts on solid agar was achieved using a modified disc diffusion assay method.¹²⁻¹⁴ 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 a negative control for the antibiotics.

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) method¹⁵⁻¹⁸ 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 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.

Artemia nauplii toxicity screening

Potassium dichromate ($K_2Cr_2O_7$) (AR grade, Chem-Supply, Australia) was prepared in deionised water (4 mg/mL) and serially diluted in artificial seawater for use as a reference toxin. Toxicity of the *T. lanceolata* pepper berry extracts, the reference toxin and the conventional antibiotics was assessed using a modified *Artemia franciscana* nauplii lethality assay.¹⁹⁻²¹ The LC_{50} with 95% confidence limits for each treatment was calculated using probit analysis.

Calculation of Therapeutic Index (TI)

Therapeutic indexes (TI) of the *T. lanceolata* pepper berry extracts against the target bacteria were calculated by standard methods²² using the following formula and are included as a measure of the suitability of the extracts for therapeutic usage:

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

Statistical analysis

Data are expressed as the mean \pm 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 a p value < 0.01 considered to be significant.

RESULTS

Liquid extraction yields and qualitative phytochemical screening

Tasmannia lanceolata pepper berry extractions of 1 g of dried and ground berry material using 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 extracts generally extracted similar albeit lower phytochemical profiles as compared to the methanolic and aqueous extracts.

Antibacterial activity

To determine the ability of the crude plant extracts to inhibit the growth of *S. aureus* and *S. epidermidis*, aliquots (10 μ L) of each extract were initially screened using a disc diffusion assay. All of the *T. lanceolata* pepper berry extracts substantially inhibited the growth of *S. aureus* (Figure 2). The methanolic and ethyl acetate extracts were substantially better inhibitors of *S. aureus* growth than was the aqueous extract (as judged by the ZOI size). Notably, the aqueous extract would mainly contain polar compounds, with few mid to low polarity compounds. In contrast, the methanolic and ethyl acetate extracts would also contain mid-polarity compounds in addition to the higher polarity compounds, indicating that mid-polarity compounds may be the main contributors to the anti-*S. aureus* activity reported herein. Similar results were also obtained when the extracts were screened against *S. epidermidis*, with the greatest activity again corresponding to the methanolic and ethyl acetate extracts (Figure 3). 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* pepper berry extracts were further quantified by determining the MIC values (Table 2). The ethyl acetate extract was particularly effective at inhibiting bacterial growth, with LD MIC values of 563 and 750 μ g/

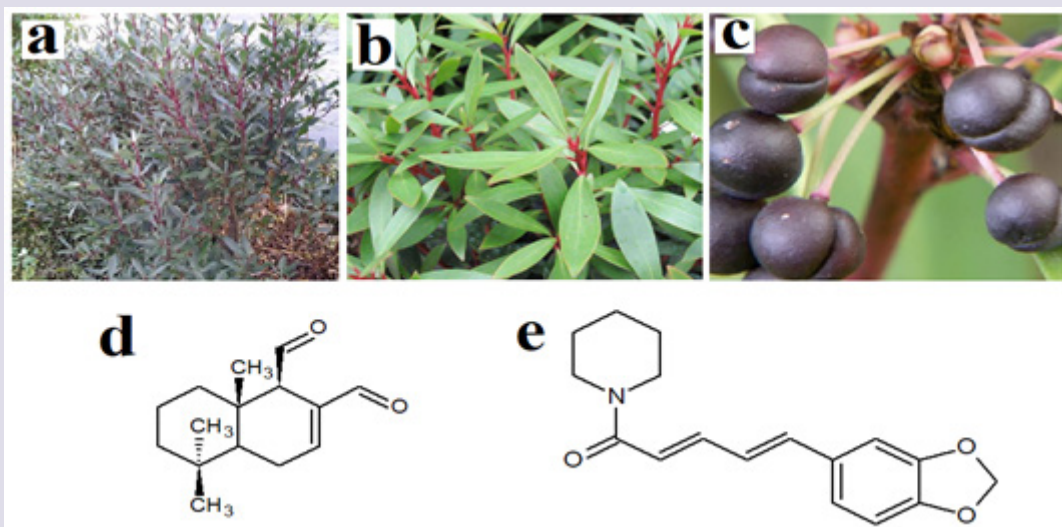


Figure 1: *Tasmannia lanceolata* (a) whole plant, (b) leaves, (c) berries, as well as the phytochemical constituents (d) polygodial and (e) piperine.

Table 1: The mass of dried extracted *T. lanceolata* pepper berry 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	-	-	-
	Draggendorff'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.

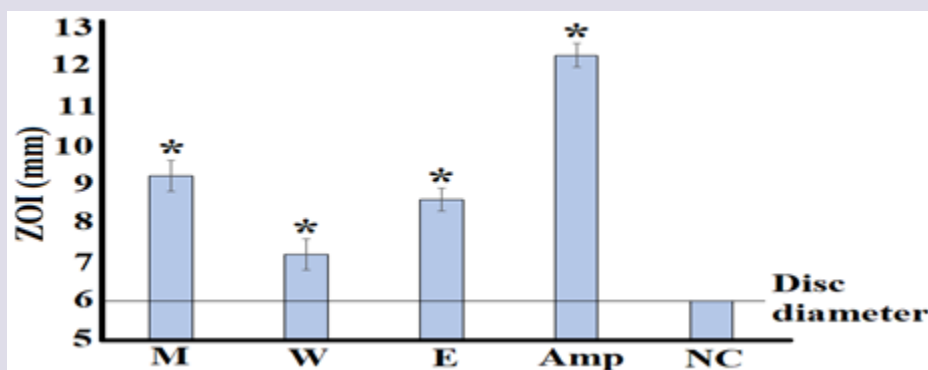


Figure 2: Growth inhibitory activity of the *T. lanceolata* pepper berry extracts against *S. aureus* (ATCC 25923) measured as zones of inhibition (mm). M=methanolic extract; W=aqueous extract; E=ethyl acetate extract; Amp=ampicillin (10 µg); NC=negative control (0.5 % DMSO). Results are expressed as mean zones of inhibition±SEM. * indicates results that are significantly different to the negative control ($p < 0.05$).

mL determined against *S. aureus* and *S. epidermidis* respectively. Noteworthy activity was also measured for the methanolic extract (LD MIC values of 891 µg/mL against both bacteria). In contrast, higher LD MIC values (>1000 µg/mL) were noted for the aqueous extracts against both bacteria, indicating lower (although useful) antibacterial activity. As the aqueous extract would be expected to contain only polar molecules (whereas the methanolic and ethyl acetate extracts would also contain mid-polarity compounds), it is likely that the main antibacterial *T. lanceolata* pepper berry

compounds are mid-polarity compounds, although this remains to be confirmed.

Quantification of toxicity

All extracts were initially screened in the *Artemia* nauplii assay at 2000 µg/mL (Figure 4). Additionally, potassium dichromate was also tested in the bioassay as a reference toxin. The reference toxin was rapid in its onset of mortality, promoting nauplii death within the first 3 hr of exposure, with 100% mortality evident within 5

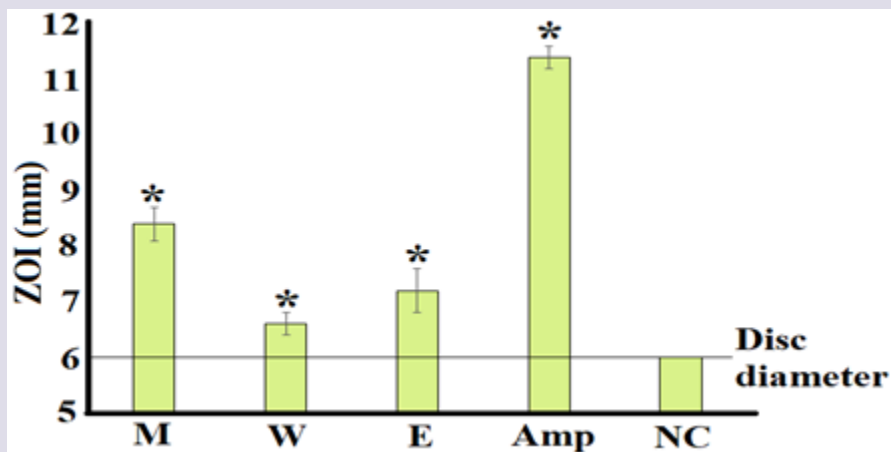


Figure 3: Growth inhibitory activity of the *T. lanceolata* pepper berry extracts against *S. epidermidis* (ATCC 122292) measured as zones of inhibition (mm). M=methanolic extract; W=aqueous extract; E=ethyl acetate extract; Amp=ampicillin (10 µg); NC=negative Control (0.5 % DMSO). Results are expressed as mean zones of inhibition±SEM. * indicates results that are significantly different to the negative control ($p < 0.05$).

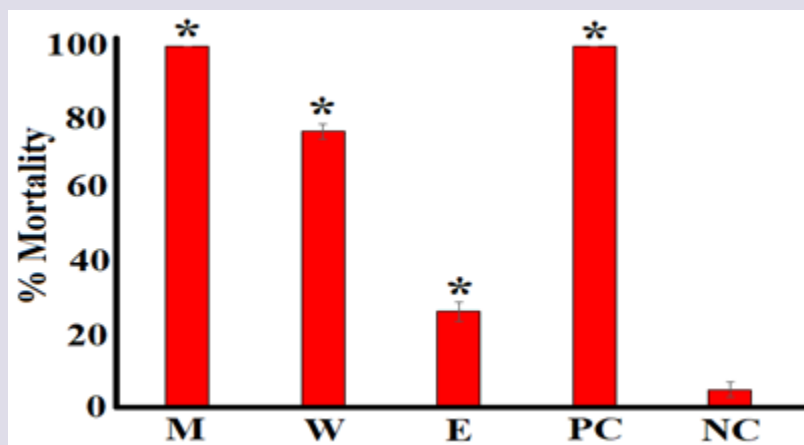


Figure 4: The lethality of the *T. lanceolata* pepperberry extracts (2000 µg/mL), as well as the potassium dichromate (1000 µg/mL) and seawater controls towards *Artemia franciscana* nauplii after 24 hr exposure. M=methanolic extract; W=aqueous extract; E=ethyl acetate extract; PC=potassium dichromate 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. aureus* and *S. epidermidis* growth (µg/mL) of the *T. lanceolata* pepperberry extracts and toxicity in the *Artemia* nauplii bioassay.

Extract	Antibacterial activity MIC (µg/mL)				Toxicity		
	<i>S. aureus</i>		<i>S. epidermidis</i>		ALA (µg/mL)	Therapeutic index (TI)	
	DD	LD	DD	LD		<i>S. aureus</i>	<i>S. epidermidis</i>
Methanolic extract	1215	891	1540	891	1874	2.1	2.1
Aqueous extract	1375	1031	2654	1375	2637	2.56	1.92
Ethyl acetate extract	783	563	984	750	NA	CND	CND

DD: Disc diffusion; LD: Liquid dilution; NA=an IC_{50} values was not achieved as toxicity did not exceed 50 % at any concentration tested; CND=could not determine as LC_{50} could not be calculated. Numbers indicate the mean DD MIC and LD MIC values of triplicate determinations. - indicates no inhibition at any concentration tested. TI (therapeutic index) was calculated using LD MIC.

hr (data not shown). The methanolic and aqueous extracts also induced substantially >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 LC₅₀ values of the *T. lanceolata* pepper berry extracts towards *A. nauplii* are displayed in Table 2. LC₅₀ values substantially >1000 µg/mL were determined for the methanolic and aqueous extracts. As extract with LC₅₀ values >1000 µg/mL towards *Artemia* nauplii have been defined as being non-toxic in this assay,¹⁹ all of the *T. lanceolata* pepper berry extracts were deemed to be non-toxic.

Calculation of therapeutic Index

To further evaluate the suitability of the *T. lanceolata* pepper berry extracts as antibacterial therapeutic agents, 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 LC₅₀ for this extract would be substantially above 3000 µg/mL. Assuming an LC₅₀ substantially >3000 µg/mL, given the LD MIC values of 563 and 750 µg/mL against *S. aureus* and *S. epidermidis*, it is reasonable to assume that the LC₅₀ values would be substantially in excess of 5, indicating the safety of this extract for therapeutic usage against these bacteria. Notably, TI values of 1.9-2.6 were calculated for the methanolic and aqueous extracts, indicating that whilst these extracts would be useful in treating *S. aureus* and *S. epidermidis* infections, 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

Tasmania lanceolata pepper berries 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 pepper berries (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* pepper berry extracts are yet to be examined against many bacterial pathogens, including the skin pathogens *S. aureus* and *S. epidermidis*. 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*

pepper berry extracts against *S. aureus* and *S. epidermidis*. Both bacteria were susceptible to inhibition by all of the *T. lanceolata* pepper berry extracts. The ethyl acetate extract was the best inhibitor of both bacteria, with LD MIC values of 563 and 750 µg/mL against *S. aureus* and *S. epidermidis* respectively. The methanolic (LD MICs of 891 µg/mL against both bacteria) and aqueous extracts (LD MICs of 1031 and 1375 µg/mL against *S. aureus* and *S. epidermidis* respectively) also displayed noteworthy antibacterial activity.

Whilst a thorough examination of the phytochemical composition of the *T. lanceolata* pepper berry extracts was beyond the scope of this 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^{24,25} 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* pepper berry extracts. Some terpenoids have potent broad-spectrum antibacterial activity²⁶ 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.¹ 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. All extracts were nontoxic in the *Artemia* nauplii bioassay. Therefore, it is likely that the *T. lanceolata* pepper berry extracts are reasonably safe for both prophylactic and therapeutic use. However, further toxicity studies using mammalian cell lines are required to confirm the safety of these extracts.

CONCLUSION

The strong growth inhibitory activity of *T. lanceolata* pepper berry extracts against *S. aureus* and *S. epidermidis* and their lack of toxicity highlight their potential for the prevention and treatment of *Staphylococcus* spp. skin disease, as well as other infections of these bacteria. Further studies are required to isolate the compound(s) responsible for this activity and to elucidate the antibacterial mechanisms involved.

ACKNOWLEDGEMENT

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CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

ABBREVIATIONS

DMSO: Dimethyl sulfoxide; **LC₅₀:** The concentration required to achieve 50% mortality; **MIC:** Minimum inhibitory concentration.

SUMMARY

- Methanolic, aqueous and ethyl acetate *T. lanceolata* pepper berry extracts were screened for the ability to block the growth of *S. aureus* and *S. epidermidis*.
- The ethyl acetate *T. lanceolata* pepper berry extract was a particularly good inhibitor of *S. aureus* and *S. epidermidis* growth (LD MICs of 563 and 750 µg/mL respectively).
- The methanolic and aqueous *T. lanceolata* extracts were also noteworthy inhibitors of *S. aureus* and *S. epidermidis* growth, albeit with slightly higher LD MIC values.
- Toxicity of the extracts was evaluated using *Artemia nauplii* bioassays.
- The lack of toxicity of the *T. lanceolata* pepper berry extracts indicates that they are safe for topical or oral use against *S. aureus* and *S. epidermidis* infections.

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