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Research Article

The potential of *tasmannia lanceolata* as a natural preservative and medicinal agent: antimicrobial activity and toxicity

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ABSTRACT: **Introduction:** *Tasmannia lanceolata* is an endemic Australian plant with a history of use by indigenous Australians as a food and as a medicinal agent. **Methods:** *T. lanceolata* solvent extracts were investigated by disc diffusion assay against a panel of bacteria and fungi and their MIC values were determined to quantify and compare their efficacies. Toxicity was determined using the *Artemia franciscana* nauplii bioassay. **Results:** All *T. lanceolata* extracts displayed antibacterial activity in the disc diffusion assay. The berry methanolic extract had the broadest antibacterial range, inhibiting the growth of all 18 of the bacteria tested (100%). The berry water and ethyl acetate, extracts were also good antibacterial agents inhibited the growth of 17 (94.4%) and 15 (83.3%) of the 18 bacteria tested respectively. Strong inhibitory activity was detected with MIC values as low as 4.8 μg/ml against some bacteria, although many of the measured MIC's were several orders of magnitude higher than this. All extracts were equally effective at inhibiting the growth of both Gram-negative bacteria and Gram-positive bacteria. In contrast, only the *T. lanceolata* peppercorn extracts were effective as antifungal agents (albeit with limited antifungal ranges), inhibiting the growth of 2 of the 4 fungal species tested each (50%). All *T. lanceolata* extracts were non-toxic in the *Artemia fransiscana* bioassay with LC_{50} values greatly in excess of 1000 μg/ml. **Conclusions:** The lack of toxicity of the *T. lanceolata* extracts and their potent broad spectrum inhibitory bioactivity against bacteria and fungi indicates their potential as natural food preservatives and as medicinal agents in the treatment and prevention of microbial diseases.

KEYWORDS: Winteraceae, *Tasmannia lanceolata*, Tasmanian pepper, Australian plants, antibacterial, medicinal plants, food spoilage, food poisoning

INTRODUCTION

Food loss through spoilage is a major global problem. Spoilage can render food unpalatable and/or increase the risks of diseases and food poisoning and can be caused by a variety of physiochemical causes and biological agents. Of the non-microbial causes of food spoilage, oxidation resulting in rancidity is a major issue. Lipid rancidity is a common non-microbial change which may occur during the processing, distribution, storage and preparation of foods.[1] High fat content foods are subject to rancidity via two different processes. Hydrolytic rancidity is caused by the hydrolysis of triglycerides to release free fatty acids (such as short chain butyric acid) which gives foods such as rancid butter their characteristic odour. Oxidative rancidity occurs when the polyunsaturated and monounsaturated components of foods react with oxygen to form peroxides, which subsequently decompose to form ketones, aldehydes and other volatile compounds.[2] Foods with high levels of pro-oxidants (e.g. transition metals, heme containing proteins etc) and/or polyunsaturated fatty acids are particularly susceptible to oxidative rancidity.[3] Oxidative rancidity can be slowed by the use of synthetic antioxidants in combination with citric or phosphoric acid; these

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combinations have proved substantially more effective than single antioxidants in slowing food oxidation.[2] More recently, the potential of high antioxidant natural food has been recognised for retarding oxidative food rancidity.[2] There is potential for high antioxidant herbs to be incorporated into fresh and processed foods to retard rancidity and improve shelf life and safety.

Of more serious concern to the food production industry is microbial induced food spoilage and food poisoning. Incidences of food-borne illnesses were estimated at 76 million cases annually in the USA alone in a 1999 study, with at least 5000 deaths annually directly attributed to food poisoning.[4] This is an important area of concern and there is much effort to develop improved preservation strategies. Methods aimed at inhibiting microbial growth must effectively control initial populations, regrowth of post-processing microbial survivors and contaminant induced populations. This may be achieved by and number of methodologies including alteration of temperature (heating, chilling), pH, water activity (fermentation or dehydration) or oxygen availability (canning, shrink wrap, reduced oxygen packaging, high pressures), irradiation or by chemical preservation.[5]

Currently, the major method of controlling food-borne microbes and thereby reducing spoilage and food toxin production is by the addition of chemical preservatives during the food production process. Commonly used chemical food preservatives for the inhibition of food microflora growth include butylhydroxyanisol (BHA), butylated hydroxytoluene (BHT), calcium propionate, nitrates, nitrites, sulphur dioxide (SO_2) and sulfites (SO_3).[5] The effectiveness of these chemical preservatives is dependent on the type of microbial flora and the physical and chemical characteristics of the food.[5, 6] Of concern, the safety of many of the chemical preservatives used in food has yet to be determined and in some cases these preservatives have been linked with serious health problems. Studies have indicated that chemical preservatives may cause respiratory problems,[7] aggravate attention deficit hyperactivity disorder (ADHD)[8] and cause anaphylactic shock in susceptible individuals.[7]

Due to greater consumer awareness and the negative perceptions of artificial preservatives, consumers are increasingly avoiding foods containing preservatives of chemical origin. Natural antimicrobial alternatives are increasingly being sought to increase the shelf life and safety of processed foods.[9] Plant extracts and oils are candidates for antimicrobial agents that would be more acceptable to consumers due to their natural origin and consumer perception of safety. In addition, many plants have well established antimicrobial activity and several plant species have already been identified for their potential as natural preservatives.[10–14]

Tasmannia lanceolata (commonly known as Tasmanian pepper or mountain pepper) is shrub which is endemic to the woodlands and cool temperate rainforests of Tasmania and the south-eastern region of the Australian mainland.[15] It is a medium to large shrub that varies between 2–5 m in height. Individual plants are unisexual, having either male or female flowers. The stems, branches and twigs are red in colour. The aromatic leaves 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) during autumn.

The berries, leaves and bark of this species have historical uses as a food and as a medicinal plant.[16] 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. *T. lanceolata* was used as flavouring agent by Australian Aborigines and more recently by European settlers. Historically, the leaves have been used as a herb and the berries have been used as a spice. Australian Aborigines also used *T. lanceolata* as a therapeutic agent to treat stomach disorders and as an emetic, as well as general usage as a tonic.[16] 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.[16] Later, European colonists also recognized the therapeutic potential of *T. lanceolata* and the bark was used as a common substitute for other herbal remedies (including those derived from the related South American Winteraceae species, *Drimys wintera* (winter bark))[17] to treat scurvy due to its high antioxidant activity.[16,18]

Despite its ethnobotanical usage, until recently there have been limited rigorous scientific studies into the therapeutic properties of *T. lanceolata*. Recent studies have demonstrated the high antioxidant content of *T. lanceolata* berries.[18] As antioxidants have uses in retarding food rancidity and in inhibiting the growth of food-borne bacteria, *T. lanceolata* may have potential in blocking food spoilage and inhibiting food poisoning. Epidemiological studies have shown that a diet rich in antioxidants is associated with a decreased incidence of chronic diseases.[19] High antioxidant levels have also been shown to act as a preventative against the development of degenerative diseases such as cancer,[20] cardiovascular diseases,[21] neural degeneration,[22] diabetes and obesity.[23] Despite this, the

antimicrobial properties related to food spoilage and food poisoning have not been extensively studied for *T. lanceolata* The current study was undertaken to test *T. lanceolata* leaf, berry and peppercorn extracts for the ability to inhibit microbial growth/contamination against a variety of bacteria involved in food spoilage and/or food poisoning. Through examining the antibacterial capability of the *T. lanceolata* extracts, we aim to assess their potential as additives to foods to retard spoilage and to potentially reduce food poisoning in processed foods.

MATERIALS AND METHODS

Plant collection and extraction

T. lanceolata semi-dry berry (without seed) and dried leaf material was purchased from GoWild Harvest, Australia. Peppercorns (dried berry and seed) were obtained from Diem Pepper, Australia. The material was stored at −30°C until use. The plant materials were thawed and freshly ground to a coarse powder prior to extraction. Individual 1 g quantities of the ground plant materials were prepared by weighing each plant part into each of three tubes and adding 50 ml of methanol, water or ethyl acetate respectively. All solvents were obtained from Ajax and were AR grade. The berry, leaf and whole peppercorn material was extracted in each solvent for 24 hours at 4°C with gentle shaking. The extracts were 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.

Qualitative phytochemical studies

Phytochemical analysis of the *T. lanceolata* extracts for the presence of saponins, phenolic compounds, flavonoids, polysteroids, triterpenoids, cardiac glycosides, anthraquinones, tannins and alkaloids was conducted by previously described assays.[24–26]

Antibacterial screening

Test microorganisms

All media was supplied by Oxoid Ltd. Microbial strains were obtained from Tarita Morais, Griffith University. Stock cultures of *Acinetobacter baylyi*, *Aeromonas hydrophila*, *Alcaligenes feacalis*, *Bacillus cereus*, *Citrobacter freundii*, *Enterobacter aerogenes*, *Enterococcus faecalis*, *Escherichia coli*, *Klebsiella pneumoniae*, *Proteus mirabilis*, *Pseudomonas aeuroginosa*, *Pseudomonas fluorescens*, *Salmonella newport*, *Serratia marcescens*, *Shigella sonnei*, *Staphylococcus aureus*, *Staphylococcus epidermidis* and *Streptococcus pyogenes* were subcultured and maintained in nutrient broth at 4°C. *Aspergillus niger*, *Candida albicans*, *Penicillium chrysogenum* and *Saccharomyces cerevisiae* were maintained in Sabouraud media at 4°C.

Evaluation of antimicrobial activity

Antimicrobial activity of all plant extracts was determined using a modified disc diffusion assay.[27–30] Briefly, 100 µl of the test bacteria were grown in 10 ml of fresh nutrient broth media until they reached a count of approximately 10^8 cells/ml. An amount of 100 µl of bacterial suspension was spread onto nutrient agar plates. For fungal species, 100 µl of the test species was grown in 10 ml of fresh Sabouraud media until they reached a count of approximately 10^6 cells/ml. A volume of 100 µl of bacterial suspension was spread onto Sabouraud agar plates.

The extracts were tested for antibacterial activity using 5 mm sterilised filter paper discs. Discs were impregnated with 10 µl (100 µg) of the test sample, allowed to dry and placed onto inoculated plates. The plates were allowed to stand at 4°C for 2 hours before incubation with the test microbial agents. Plates inoculated with the bacterial species *Alcaligenes feacalis*, *Aeromonas hydrophilia*, *Bacillus cereus*, *Citrobacter freundii*, *Klebsiella pneumoniae*, *Proteus mirabilis, Pseudomonas aeuroginosa*, *Pseudomonas fluorescens* and *Serratia marcescens* and the fungal species *Candida albicans*, *Penicillium crygogenum* and *Saccharomyces cerevisiae* were incubated at 30°C for 24 hours, then the diameters of the inhibition zones were measured in millimetres. Plates inoculated with *Acinetobacter baylyi, Enterobacter aerogenes*, *Enterococcus faecalis*, *Escherichia coli*, *Salmonella newport*, *Shigella sonnei*, *Staphylococcus aureus*, *Staphylococcus epidermidis* and *Streptococcus pyogenes* were incubated at 37°C for 24 hours, then the diameters of the inhibition zones were measured. Plates inoculated with *Aspergillus niger* were incubated at 25°C for 48 hours, then the diameters of the inhibition zones were measured. All measurements were to the closest whole millimetre. Each antimicrobial assay was performed in at least triplicate. Mean values (± SEM) are reported in this study. Standard discs of ampicillin (2 µg) were obtained from Oxoid Ltd. and served as positive controls for antimicrobial activity. Filter discs impregnated with 10 µl of distilled water were used as a negative control.

Minimum inhibitory concentration (MIC) determination

The minimum inhibitory concentration (MIC) of the extracts were determined as previously described.[31-33] Briefly, the plant extracts were diluted in deionised water and tested across a range of concentrations. Discs were impregnated with 10 µl of the test dilutions, allowed to dry and placed onto inoculated plates. The assay was performed as outlined above and graphs of the zone

of inhibition versus concentration were plotted for each extract. Linear regression was used to calculate the MIC values.

Toxicity screening

Reference toxin for toxicity screening

Potassium dichromate ($K_2Cr_2O_7$) (AR grade, Chem-Supply, Australia) was prepared as a 1.6 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 a modified *Artemia franciscana* nauplii lethality assay.[34-37] Briefly, 400 µl of seawater containing approximately 48 (mean 47.8, n = 125, SD 17.4) *A. franciscana* nauplii were added to wells of a 48 well plate and immediately used for bioassay. 400 µl of diluted plant extracts and the reference toxins 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 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 observed within 10 seconds. After 72 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 calculated using probit analysis.

Statistical analysis

Data are expressed as the mean ± SEM of at least three independent experiments.

RESULTS

Liquid extraction yields and qualitative phytochemical screening

Extraction of 1 g of dried *T. lancelotata* berry, leaf and peppercorn with various solvents yielded dried plant extracts ranging from 17 mg to 477 mg (Table 1). The highest amount of extracted material was obtained from the peppercorn (295 mg and 477 mg for the methanol and water peppercorn extracts respectively). The berry and leaf extractions generally gave lower yields. Deionised water and methanol generally gave relatively high yields of dried extracted material whilst ethyl acetate extracted the lowest mass for all plant parts tested. The dried extracts were resuspended in 10 ml of deionised water resulting in the extract concentrations shown in Table 1.

Table 1: The mass of dried extracted material, the concentration of extracts after resuspension in deionised water and qualitative phytochemical screenings of *T. lancelotata berry*, leaf and peppercorn extractions

	Extract	Mass of Dried Extract (mg)	Resuspended Extract Concentration (mg/ml)	Total Phenolics	Water Soluble	Water Insoluble	Cardiac Glycosides	Saponins	Triterpenes	Polysteroids	Alkaloids (Meyer test)	Alkaloids (Wagners test)	Flavonoids	Tannins	Free Anthraquinones	Combined Anthraquinones
Berry	Methanol	171	17.1	+++	+++	+++	–-	++	+	–	–	–	+++	–	–	–
	Water	111	11.1	+++	+++	+++	–	–	–	–	–	–	+++	–	–	–
	Ethyl Acetate	56.7	5.7	+	+	++	–	+	++	–	–	–	++	–	–	–
Leaf	Methanol	144	14.4	+++	+++	+++	–	+++	+	–	–	–	+++	–	–	–
	Water	134	13.4	+++	+++	+++	–	++	–	–	–	–	+++	–	–	–
	Ethyl Acetate	17	1.7	+	+	++	–	–	+	–	–	–	++	–	–	–
Peppercorn	Methanol	295	29.5	+++	++	+	–	+	+	–	–	–	+	–	–	–
	Water	477	47.7	+++	++	+	–	++	–	–	–	–	+	–	–	–
	Ethyl Acetate	94	9.4	++	+	+	–	–	++	–	–	–	+	–	–	–

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

Qualitative phytochemical studies (Table 1) showed that methanol and water extracted the widest range of phytochemicals. Both showed high levels of phenolics (both water soluble and insoluble phenolics) and flavonoids, as well as low to moderate levels of saponins. The ethyl acetate extracts had low to moderate levels of phenolics, triterpenes and flavonoids. Low levels of saponins were also reported for the berry ethyl acetate extract. Neither tannins nor alkaloids were detected in any of the extracts tested.

Antimicrobial activity

To determine the antimicrobial activity of the crude plant extracts, aliquots (10 µl) of each extract were tested in the disc diffusion assay against a panel of bacteria and fungi associated with food spoilage and food poisoning. Gram negative bacterial growth was inhibited by a broad range of the tested plant extracts (Figure 1). *E. faecalis* and *K. pneumoniae* were the most susceptible to growth inhibition, each being inhibited by all of the extracts tested (100%). *P. mirabilis*, *P. aeruginosa* and *S. marcescens* were also highly susceptible, each being inhibited by 8 of the 9 extracts tested (88.9%). *A. bayli* growth was inhibited by 7 extracts (77.8%), whilst *A. hydrophila*, *E. aerogenes* and *E. coli* were inhibited by 6 extracts each (66.7%). *C. freundii*, *S. sonnei*, *S. newpor*t and *P. fluorescens* were inhibited by 5 (55.6%), 5 (55.6%), 4 (44.4%) and 3 (33.3%) extracts respectively. The berry methanolic extract displayed the broadest antibiotic specificity, inhibiting the growth all of the Gram-negative bacteria tested, although the measured zones of inhibition against most were not high (below 9 mm). The most potent growth inhibition was seen for the peppercorn methanol, water and ethyl acetate extracts against *E. coli*, and by the leaf methanolic and water extracts against *P. mirabilis,* each with ≥ 12 mm inhibition zones. Indeed, the leaf methanol and water extracts were significantly more effective than the positive control ampicillin in inhibiting *P. mirabilis* growth.

Gram-positive bacterial growth was also inhibited by a broad range of plant extracts. *S. aureus* and *S. epidermidis* were the most susceptible of the Gram-positive bacteria, being inhibited by 8 of the 9 extracts tested each (88.9%). In contrast, *B. cereus* and *S. pyogenes* growth was inhibited

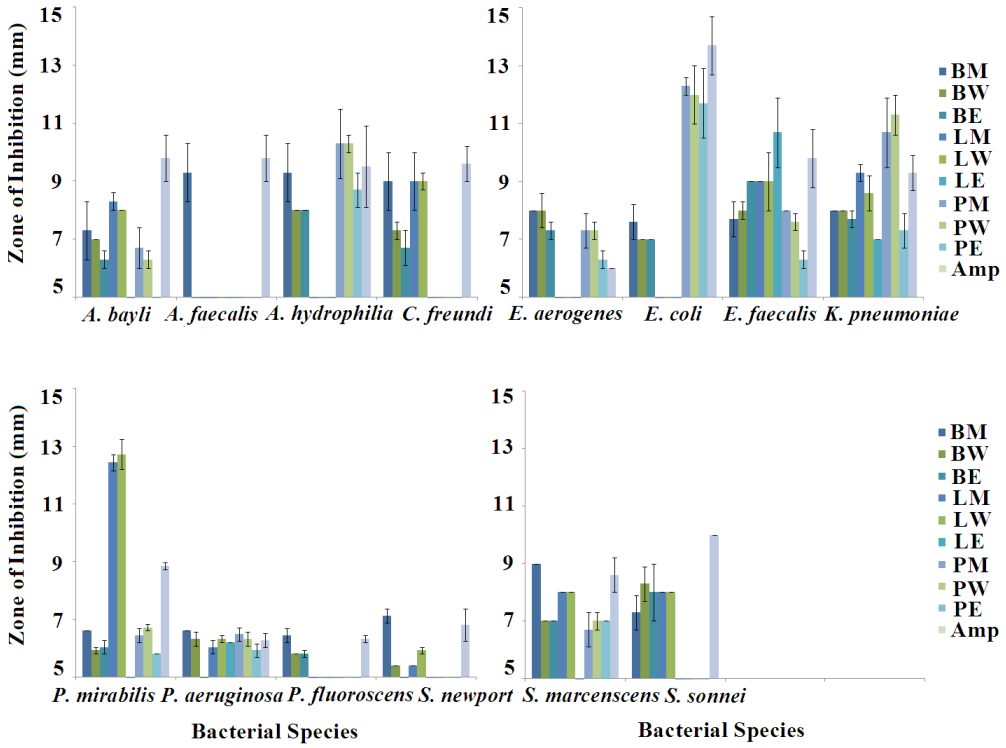

Figure 1. Antibacterial activity of *T. lanceolata* berry, leaf and peppercorn extracts measured as zones of inhibition (mm) against Gram-negative bacteria. BM = *T. lanceolata* berry methanolic extract; BW = *T. lanceolata* berry water extract; BE = *T. lanceolata* berry ethyl acetate extract leaf; LM = *T. lanceolata* leaf methanolic extract; LW = *T. lanceolata* leaf water extract; LE = *T. lanceolata* leaf ethyl acetate extract leaf; PM = *T. lanceolata* peppercorn methanolic extract; PW = *T. lanceolata* peppercorn water extract; LE = *T. lanceolata* peppercorn ethyl acetate extract leaf. Amp = ampicillin (2 µg) control. Results are expressed as mean ± SEM.

by 5 (55.6%) and 4 (44.4%) of the extracts respectively. The berry was the most versatile plant part as determined by the number of susceptible bacteria. Indeed, the berry extracts inhibited all of the Gram-positive bacterial species tested. The leaf methanolic and water extracts were also effective growth inhibitors, inhibiting all of the Gram-positive bacteria. In contrast, the leaf ethyl acetate extract did not inhibit the growth of any Gram-positive bacteria. Screening of the antibacterial efficacy of peppercorn extracts provided mixed results, with all peppercorn extracts inhibiting *S. aureus* and *S. epidermidis*. However, none of the peppercorn extracts inhibited either the growth of *B. cereus* or *S. pyogenes*.

Fungal growth was less susceptible to the *T. lanceolata* extracts than was bacterial growth (as determined by zones of inhibition). *A. niger* was particularly resistant, not being inhibited by any of the extracts tested. This species was a particularly resistant strain, also growing in the presence of the control antibiotic (ampicillin). *P. chrysogenum* was also resistant to the *T. lanceolata* extracts, although its growth was inhibited by the ampicillin control. In contrast, *C. albicans* and *S. cerevisiae* were each inhibited by 3 (33.3%) of the 9 plant extracts. Only the peppercorn extracts were effective inhibitors against these fungi. The peppercorn methanolic extract being particularly effective against *S. cerevisiae* with a zone of inhibition of approximately 13 mm.

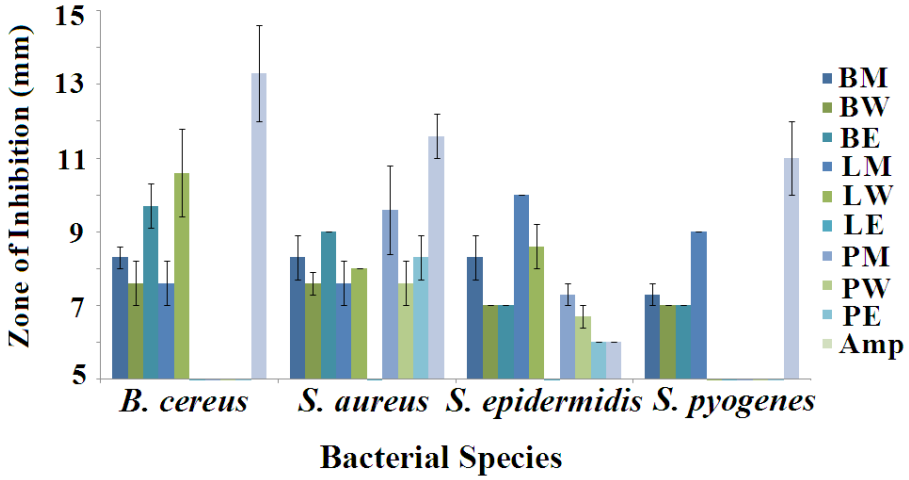

Figure 2. Antibacterial activity of *T. lanceolata* berry, leaf and peppercorn extracts measured as zones of inhibition (mm) against Gram-positive bacteria. BM = *T. lanceolata* berry methanolic extract; BW = *T. lanceolata* berry water extract; BE = *T. lanceolata* berry ethyl acetate extract leaf; LM = *T. lanceolata* leaf methanolic extract; LW = *T. lanceolata* leaf water extract; LE = *T. lanceolata* leaf ethyl acetate extract leaf; PM = *T. lanceolata* peppercorn methanolic extract; PW = *T. lanceolata* peppercorn water extract; PE = *T. lanceolata* peppercorn ethyl acetate extract leaf. Amp = ampicillin (2 μg) control. Results are expressed as mean ± SEM.

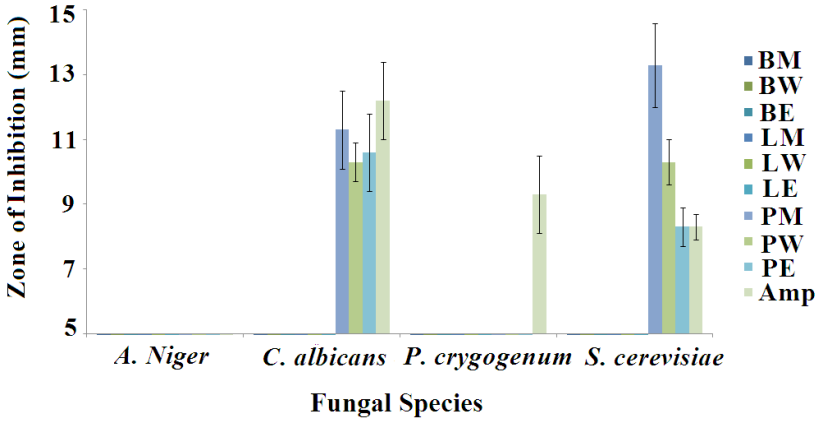

Figure 3. Inhibitory activity of *T. lanceolata* berry, leaf and peppercorn extracts measured as zones of inhibition (mm) against fungal species. BM = *T. lanceolata* berry methanolic extract; BW = *T. lanceolata* berry water extract; BE = *T. lanceolata* berry ethyl acetate extract leaf; LM = *T. lanceolata* leaf methanolic extract; LW = *T. lanceolata* leaf water extract; LE = *T. lanceolata* leaf ethyl acetate extract leaf; PM = *T. lanceolata* peppercorn methanolic extract; PW = *T. lanceolata* peppercorn water extract; PE = *T. lanceolata* peppercorn ethyl acetate extract leaf. Amp = ampicillin (2 μg) control. Results are expressed as mean ± SEM.

The antimicrobial efficacy was further quantified by determining the MIC values for each extract against the microbial species which were determined to be susceptible (Table 2). Most of the extracts were effective at inhibiting microbial growth at low concentrations with MIC values against the susceptible bacterial and fungal species generally less than 1000 μg/ml (< 10 μg impregnated in the disc), indicating the potential of these extracts in controlling food spoilage and inhibiting food poisoning. The MIC values determined against *P. mirabilis* were particularly interesting, with values as low as 4.8 μg/ml (0.5 μg impregnated into the disc) for the berry ethyl acetate extract. Similarly low *P. mirabilis* MIC values were also seen for several other extracts. Interestingly, whilst the inhibition zone studies indicated low antifungal efficacy, low MIC values (approximately 200–300 μg/ml) were determined for the susceptible fungi to the peppercorn extracts, indicating their potent inhibitory activity.

Quantification of toxicity

T. lanceolata berry, leaf and peppercorn extracts were initially screened undiluted as an approximation of the concentration that would be added to food as a preservative. For comparison, the reference toxins potassium dichromate (1000 μg/ml) was also tested in the bioassay. Figure 4 shows the% mortality induced by each extract and by the control toxin following 24 hours exposure. The potassium dichromate reference toxin was rapid in its onset of mortality, inducing mortality within the first 3 hours of exposure and 100% mortality was evident following

Table 2: Minimum inhibitory concentrations (μg/ml) of *T. lanceolata* extracts against susceptible bacteria

	Berry			Leaf			Peppercorn		
	BM	BW	BE	LM	LW	LE	PM	PW	PE
Gram negative rods									
A. baylyi	406	500	812	185	561	–	558	736	–
A. faecalis	932	–	–	–	–	–	–	–	–
A. hydrophila	692	575	575	–	–	–	291	705	264
C. freundii	783	587	1372	525	1521	–	–	–	–
E. aerogenes	501	575	783	–	–	–	724	986	–
E. coli	500	557	484	–	–	–	1347	310	75.7
E. faecalis	525	575	626	485	570	68	387	734	1547
K.pneumoniae	32.9	198	118	785	11.9	780	92.4	858	44.4
P. mirabilis	15	126	4.8	643	55.6	265	89.9	414	114
P. aeruginosa	658	308	–	648	513	561	887	415	1124
P. fluorescens	457	876	500	–	–	–	738	1287	905
S. newport	722	7675	–	7200	4187	–	–	–	–
S. marcescens	387	1638	743	425	566	–	61	869	263
S. sonnei	587	308	575	561	487	–	–	–	–
Gram positive rods									
B. cereus	102	500	93	955	669	–	351	855	320
Gram positive cocci									
S. aureus	898	871	77	847	333	–	543	823	262
S. epidermidis	874	1103	919	946	734	–	351	933	487
S. pyogenes	423	1087	847	355	–	–	–	–	–
Fungi									
C. albicans	–	–	–	–	–	–	201	236	213
S. cerevisiae	–	–	–	–	–	–	316	239	180

Numbers indicate the mean MIC values of at least triplicate determinations. – indicates no growth inhibition. BM = *T. lanceolata* berry methanolic extract; BW = *T. lanceolata* berry water extract; BE = *T. lanceolata* berry ethyl acetate extract leaf; LM = *T. lanceolata* leaf methanolic extract; LW = *T. lanceolata* leaf water extract; LE = *T. lanceolata* leaf ethyl acetate extract leaf; PM = *T. lanceolata* peppercorn methanolic extract; PW = *T. lanceolata* peppercorn water extract; PE = *T. lanceolata* peppercorn ethyl acetate extract leaf.

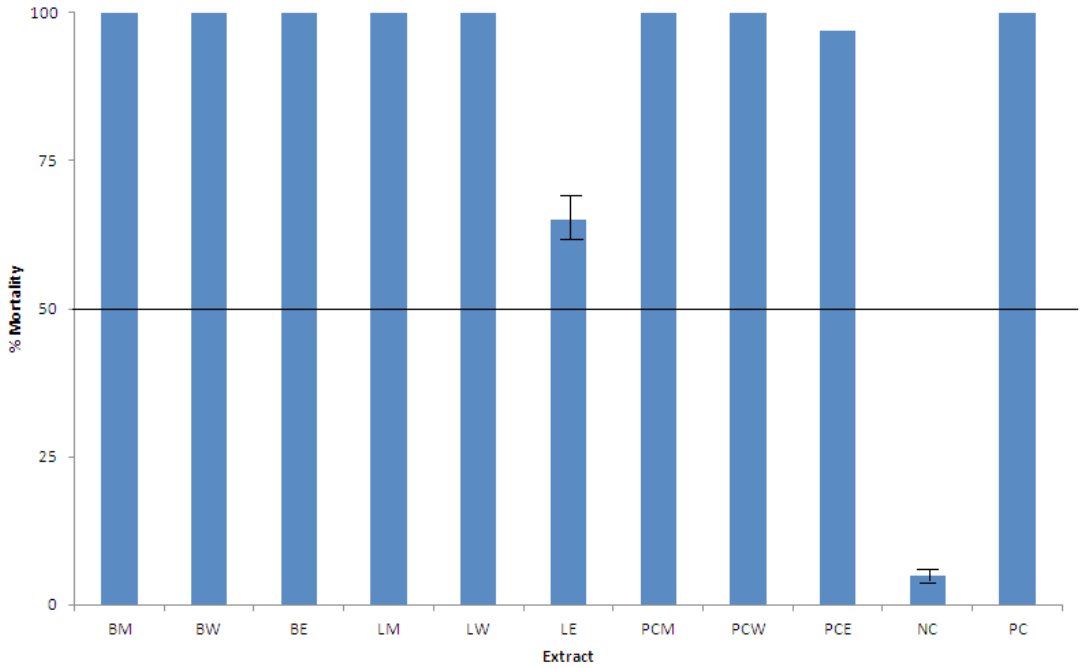

Figure 4. The lethality of *T. lanceolata* extracts and potassium dichromate control (1000 µg/mL) towards Artemia nauplii following 24 hours exposure. BM = *T. lanceolata* berry methanolic extract; BW = *T. lanceolata* berry water extract; BE = *T. lanceolata* berry ethyl acetate extract leaf; LM = *T. lanceolata* leaf methanolic extract; LW = *T. lanceolata* leaf water extract; LE = *T. lanceolata* leaf ethyl acetate extract leaf; PCM = *T. lanceolata* peppercorn methanolic extract; PCW = *T. lanceolata* peppercorn water extract; PCE = *T. lanceolata* peppercorn ethyl acetate extract leaf. Amp = ampicillin (2 µg) control. All tests were performed in at least triplicate and the results are expressed as mean ± SEM.

4–5 hours (unpublished results). Similarly, all of *T. lanceolata* extracts displayed mortality rates significantly elevated above those of the artificial seawater negative control at 24 h. However, it is noteworthy that all of these extracts were tested at high concentrations (Table 1).

To further quantify the effect of toxin concentration on the induction of mortality, the extracts were serially diluted in artificial seawater to test across a range of concentrations in the Artemia nauplii bioassay at 6, 24 and 48 hours. Table 3 shows the LC_{50} values of the *T. lanceolata* extracts towards *A. franciscana*. No LC_{50} values are reported for the berry and leaf ethyl acetate extracts at 6 h as less than 50% mortality was seen for all concentrations tested at these time points. All extracts displayed low toxicity (generally much greater than 1000 µg/ml) at all times tested. Extracts with an LC_{50} of greater than 1000 µg/ml towards *Artemia* nauplii have been defined as being nontoxic.[38] Therefore all *T. lanceolata* extracts are considered to be nontoxic.

DISCUSSION

There is increasing consumer demand to find alternatives for chemical based artificial preservatives as consumers become more aware of the potential for chemical induced health problems. Edible plants could potentially provide a source of inhibitory substances for food-borne pathogens and bacteria associated with food spoilage. This study reports on the antimicrobial activities of *T. lanceolata* berry, leaf and peppercorn extracts, and on their toxicity. The Gram-positive and Gram-negative bacteria tested in this study demonstrated similar susceptibilities towards the *T. lanceolata* extracts. Previous studies with other plant species generally report a greater susceptibility of Gram-positive bacteria towards solvent extracts for South American,[39] African[40] and Australian plant extracts,[41, 42] although examples of plants having a greater effect on Gram-negative bacteria have also been reported.[24, 25]

The bacteria examined in this study were chosen because they are all important in food spoilage and/or food poisoning/intoxication. Staphylococcus spp. (especially *S. aureus*) is one of the most common sources of food borne diseases worldwide.[5] *B. cereus* and *B. subtilis*,[43] *E. coli*,[44] *C. freundii*[45] and *K. pneumoniae*[45] all produce toxins and other proteins that induce gastroenteritis and diarrheal diseases. Many of these toxins are heat stable and are not destroyed by heat treatments/pasteurisation. Therefore, control of these bacteria in food is particularly important. Similarly, *P. mirabilis*

Table 3: LC_{50} (95% confidence interval) for *Artemia franciscana* nauplii exposed to the *T. lanceolata* extracts which displayed toxicity

	LC_{50} (µg/ml)		
Extract	**6 h**	**24 h**	**48 h**
Berry methanol extract	9510	3573	2479
Berry water extract	2868	2376	1755
Berry EA extract	–	3132	3089
Leaf methanol extract	5134	3096	2322
Leaf water extract	3224	2665	2602
Leaf EA extract	–	2766	2525
Peppercorn methanol extract	7884	4159	3880
Peppercorn water extract	3923	3029	2846
Peppercorn EA extract	–	3098	2988

– Indicates that an LC_{50} was not obtained as the mortality did not reach 50% for any concentration tested. EA = ethyl acetate.

releases factors that stimulate histamine production resulting in gastrointestinal, neurological (palpitations, headaches, itching), cutaneous (hives, rash) and hypertension symptoms.[46] Whilst storage of food at refrigerated temperatures inhibits the growth of many of these pathogenic bacteria, the inclusion of antibacterial food components would further enhance food safety.

Of the pathogenic/toxic bacteria tested in this study, Staphylococcus species are generally considered to be the most common source of food poisoning worldwide.[5] *S. aureus* and *S. epidermidis* were inhibited by 8 (88.9%) of the 9 plant extracts tested. Most of the extracts capable of inhibiting Staphylococcus growth displayed potent activity with MIC values generally < 1000 µg/ml. *T. lanceolata* berry ethyl acetate extract was of special interest with an MIC against *S. aureus* of 77 µg/ml, although several other plant extracts are also of interest with low MIC values (< 500 µg/ml). All other pathogenic bacteria were inhibited by at least 1 of the extracts. Of the bacteria associated with food poisoning, *A. faecalis*, *K. pneumoniae* and *P. mirabilis* were particularly susceptible, each being inhibited by all of the *T. lanceolata* extracts, even at concentrations as low as 4.8 µg/ml. The potent anti-Proteus activity has further therapeutic implications as *Proteus mirabilis* has been shown to be a trigger of rheumatoid arthritis (RA) and several plant species have already been highlighted as inhibitors of RA via *Proteus mirabilis* inhibition.[47]

Also particularly interesting was the ability of the extracts to inhibit the growth of psychrotrophic bacteria. Many foods are stored below 5°C in refrigerators to retard bacterial growth. These foods are expected to have long shelf lives, in some cases up to 50 days or more. Between processing and consumption, foods may become temperature abused to 10°C or higher, allowing psychrotrophic bacteria (e.g. *A. faecalis*, *A. hydrophilia*, *B. cereus* and *P. fluorescens*) to cause spoilage. Some pathogenic bacteria are also psychrotrophic (e.g. *B. cereus* and some strains of *C. freundii*, *E. coli* and *K. pneumoniae*).[43–46] Therefore, food based antibacterial agents with inhibitory activity against psychrotrophic bacteria are especially useful. The majority of psychrotrophic bacteria tested (with the exception of *A. faecalis*) were inhibited by at least 5 (55.6%) of the *T. lanceolata* extracts. The *T. lanceolata* berry extracts were the strongest and most versatile inhibitors of the psychrotrophic bacteria associated with spoilage based on the number of MIC's and the number of psychrotrophic c bacteria inhibited. Indeed, the *T. lanceolata* berry methanolic extract blocked the growth of every psychrotrophic bacterial species tested. Furthermore, this extract generally displayed low MIC values (as low as 15 µg/ml against *P. mirabilis*), indicating that it may be especially useful.

Also noteworthy was the ability of many of the extracts to limit the growth of spore forming bacteria. Heat treatment/pasteurisation is commonly used as a method of destroying food bacteria prior to processing and storage. However, when a bacterium produces heat resistant spores (as *B. cereus* does) heat treatment may kill the bacteria present, only to have further *B. cereus* growth occurring from spores. As *B. cereus* is also psychrotrophic, it is especially difficult to control. All *T. lanceolata* extracts (with the exception of the leaf ethyl acetate extract) demonstrated good inhibitory activity against *B. cereus* (as seen from the MICs). Therefore their incorporation into prepared/processed foods may be a valuable method of controlling *B. cereus* induced food spoilage and food poisoning.

The current study focussed on the effect of *T. lanceolata* extracts on aerobic bacteria. However, the anaerobic spore forming bacteria *Clostridium botulinium* is of greater concern to the food industry due to its degree of incidence and the severity of the symptoms seen with botulism poisoning.[4] Future studies into the effects of *T. lanceolata* extracts on anaerobes, including *C. botulinium* are warranted to further evaluate their usefulness as food preservatives.

Fungi are often able to grow in conditions in which many bacteria cannot grow (e.g. low pH, low moisture content, high osmotic pressure). Therefore, preservatives which have very good antibacterial activity may not have the same effects on fungi, allowing fungi involved in food

spoilage/pathogenesis to grow, even in the presence of these antibacterial substances. It is also necessary to assess the inhibitory activity of potential preservatives against fungal species associated with food spoilage and the induction of food poisoning. Aspergillus species (e.g. *A. niger*) successfully grow in low moisture containing foods (e.g. grains, nuts) causing spoilage.[5] *A. niger* also grow prolifically in higher moisture environments and are associated with causing spoilage of fruit and vegetables as well as jams and preserves, cured meats etc.[5] Other fungi including Candida species can readily grow in high acid, salt or sugar environments so are not controlled by many common preservation methods.[5] Furthermore, some fungal genera (e.g. *Aspergillus*) produce mycotoxins (e.g. *A. flavus* produces aflatoxin) which cause serious food poisoning.[5] Therefore, the ability to control the growth of moulds and fungi is important for inhibiting food spoilage and food-borne diseases.

Whilst the fungal species tested in this study were not as susceptible to the plant extracts as were the bacterial species tested (as determined by the number of extracts capable of inhibiting their growth), several *T. lanceolata* extracts proved to be good inhibitors of *C. albicans* and *S. cerevisiae* growth. In particular, the peppercorn methanolic, aqueous extracts and ethyl acetate extracts were all particularly good antifungal agents (as determined by zones of inhibition and MIC's). These were also effective extracts at inhibiting the growth of food spoilage and food poisoning bacteria, indicating their broad range potential against both bacterial and fungal food spoilage and pathogenic species.

Individual extract components responsible for the antimicrobial potential of the plant extracts were not identified in the current study. Various compounds have been previously identified in *T. lanceolata* extracts and essential oils.[15] These compounds include 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. Many of these compounds have also been isolated from other plant species and have been shown to have potent antimicrobial activity.[15] It is likely that these components may also be responsible for the antibiotic properties of the extracts tested in this study. Further studies are required to identify which phytochemical(s) is/are responsible for the antimicrobial bioactivities seen for the *T. lanceolata* extracts.

The findings reported here also demonstrate that none of the *T. lanceolata* berry, leaf and peppercorn extracts displayed significant toxicity towards *Artemia franciscana* nauplii. Previously, compounds with an LC_{50} of greater than 1000 μg/mL towards *Artemia* nauplii have been defined as being nontoxic.[38] None of the extracts tested displayed LC_{50} values less than 1000 μg/mL. It was therefore determined that all *T. lanceolata* extracts examined in this study were nontoxic.

CONCLUSIONS

In conclusion, the results of this study demonstrate the potential of *T. lanceolata* to block bacterial and fungal food spoilage and microbial induced food poisoning. Furthermore, the broad spectrum antimicrobial activity and the low MICs indicate the therapeutic potential of *T. lanceolata* to infective disease. Further evaluation of the antibacterial and antifungal properties of these extracts against a more extensive panel of microbial agents is warranted. Likewise, purification and identification of the bioactive components is needed to examine the mechanisms of action of these agents.

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