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Original Article

Tasmannia lanceolata (Poir.) A.C.Sm. Pepperberry Extracts Inhibit the Growth of Selected Bacterial Gastrointestinal Pathogens

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ABSTRACT

Background: *Tasmannia lanceolata* (Poir.) A.C.Sm. Pepperberries contain high antioxidant contents and were used by the first Australians for a variety of therapeutic purposes. However, their growth inhibitory activity has yet to be verified against many bacteria. This study aimed to investigate the efficacy of *T. lanceolata* pepperberry extracts against diarrhoea- and dysentery-causing pathogens, as well as against non-pathogenic gastrointestinal bacterial strains. **Materials and Methods:** Powdered *T. lanceolata* pepperberries were extracted with solvents of varying polarity and screened for inhibition of bacterial growth against a panel of gastrointestinal bacteria. Susceptibility was assessed by disc diffusion techniques, whilst the minimum inhibitory concentrations (MICs) were quantified by liquid dilution assays. Extract toxicities was examined using *Artemia* nauplii bioassays and therapeutic indexes (TIs) were calculated as a measure of therapeutic safety. **Results:** *Tasmannia lanceolata* pepperberry extracts did not significantly inhibit the growth of the non-pathogenic/beneficial gut microflora bacteria *A. faecalis* or *E. cloacae*, but displayed noteworthy growth inhibitory activity against pathogenic *A. hydrophila, E. coli, S. sonneii, C. freundii, P. fluorescens, S. newport, S. marcescens* and *B. cereus*. The ethyl acetate extract was a particularly good inhibitor of bacterial growth, with MIC values as low as 375 µg/mL against some bacterial pathogens. All *T. lanceolata* pepperberry extracts were nontoxic in the *Artemia* nauplii assay, indicating their potential for clinical usage. **Conclusion:** The noteworthy inhibitory activity of the *T. lanceolata* pepperberry ethyl acetate extract reported herein indicates that it may be useful in treating bacterial gastrointestinal diseases and further studies are warranted. Furthermore, the *T. lanceolata* pepperberry ethyl acetate extract is nontoxic and does not adversely affect the growth of several bacteria components of the normal human gastrointestinal microbiome, further supporting its clinical use.

Keywords: Winteraceae, Mountain pepperberry, Tasmanian pepper, Disc diffusion, Liquid dilution assays, Food poisoning, Diarrhea.

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INTRODUCTION

According to recent World Health Organization (WHO), approximately half a million children under the age of 5 die each year from diarrhoeal disease.[1] This equates to more than1400 deaths per day. Indeed, diarrhoea is a major cause of childhood mortality, accounting for 8% of all death among children under 5 years of age.[2] Furthermore, the development of multi-drug antibiotic resistance by many bacterial pathogens has rendered existing antibiotic chemotherapies ineffective against diarrhoea-causing pathogens.[1,3-5] There is an urgent need to develop novel chemotherapeutic options to combat bacterial gastrointestinal illnesses. Many plants used in traditional medicinal systems are effective in the treatment of diarrhoea. However, many plants are yet to be screened for the ability to inhibit the growth of bacterial gastrointestinal pathogens. Most of the studies examining the antibacterial properties of medicinal plants have focussed on Asian,[6-8] Middle Eastern[9-11] and African plants,[12-15] as well as plants from the Americas.[16,17] Substantially fewer studies have examined the effects of native Australian plants, although several recent studies have reported noteworthy growth inhibitory activity for several Australian plants with high antioxidant contents against bacterial gastrointestinal pathogens.[18-22]

Tasmannia lanceolata (Poir.) Sm. (family Winteraceae; commonly known as Tasmanian pepper or mountain pepperberry) is a shrub that is endemic to the woodlands and cool temperate rainforests of Tasmania and the south-eastern region of the

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Australian mainland.[23] The berries, leaves and bark of this species have historical uses as a food and as a traditional medicine by the first Australians.[23,24] 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 Australians, 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.[23,24] 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.[23,24] 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)[25] to treat scurvy due to its high antioxidant content.[23,24]

Several studies have examined the antibacterial activity of *T. lanceolata* extracts against a panel of bacteria including *Yersinia enterocolitica*,[26] *Bacillus anthracis*,[27] *Clostridium perfringens*,[28] *Proteus mirabilis*,[29] and a panel of food spoilage bacteria.[30] *Tasmannia lancelata* pepperberries and leaves have also been screened for anti-protozoal activity against *Giardia duodenalis* and noteworthy activity has been reported.[31,32] Despite these earlier studies, *T. lanceolata* extracts have not yet been tested for antibacterial activity against many other bacterial species. This study investigates the growth inhibitory activity of *T. lanceolata* pepperberry extracts against selected gastrointestinal (GI) bacteria including *Aeromonas hydrophila, Alcaligenes faecalis,* B*acillus* cereus*, Citrobacter freundii, Escherichia coli, Pseudomonas fluorescens, Salmonella newport, Shigella* sonneii, which can cause GI diseases in humans. *Alcaligenes faecalis* can cause systemic infections and symptomatic peritonitis which can be fatal if untreated.[33] *Salmonella newport* causes an estimated 1.2 million illnesses, 23,000 hospitalizations, and 450 deaths annually, and *Salmonella* spp. are a serious threat to public health.[34] *Bacillus cereus* produces mucin in the gut, which induces the onset of pathogenesis.[35] *Shigella sonneii* is a major public health concern worldwide, especially in developing countries, with acute intestinal infections requiring a minimum infective dose as low as 10–100 bacterial cells,[36] while *A. hydrophila* can induce intestinal mucosal barrier function damage and inflammation.[37] The genus *Escherichia* consists of facultative anaerobic Gram-negative bacilli that belong to the family Enterobacteriaceae, and although most *E. coli* strains live harmlessly in the colon and seldom cause disease in healthy individuals, a number of pathogenic strains can cause intestinal and extra-intestinal diseases, both in healthy and immunocompromised individuals.[38] In contrast, *Enterococcus faecalis* and *Enterobacter cloacae* are harmless bacterial residents of the gastrointestinal microbiome. They have been included in this study to determine whether *T. lanceolata* pepperberry extracts are capable of inhibiting these non-pathogenic strains, which could ultimately alter the gut microbiome. Thus, the efficacy of *T. lanceolata* pepperberry extracts against some bacterial pathogens that trigger diarrhea, and against two non-pathogenic GI bacteria, were assessed in this study.

MATERIALS AND METHODS

Pepperberry source

Tasmannia lanceolata (Poir.) A.C.Sm. pepperberries 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 stored at -30°C until use.

Preparation of extracts

Individual 1 g quantities of dried and ground were weighed into separate tubes and methanol, deionised water orv ethyl acetate were added to a final volume of 50 mL. All organic solvents were obtained from Ajax Fine Chemicals, Australia and were AR grade. The ground plant materials were individually extracted for 24 hr at 4°C with gentle shaking. The extracts were subsequently filtered through Whatman No. 54 filter paper under vacuum to remove particulate matter and the extracts were dried at 4°C under reduced pressure. The resultant extracts were weighed to determine yield and were then resuspended in 10 mL deionised water (containing 0.5 % DMSO). The suspensions were briefly sonicated (3 x 20 s pulse cycles, at 2 kHz), 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* pepperberry extracts for the presence of saponins, phenolic compounds, flavonoids, phytosterols, triterpenoids, cardiac glycosides, anthraquinones, tannins and alkaloids were conducted by previously described assays.[39]

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. Penicillin-G (potency of 1440-1680 µg/mg), chloramphenicol (≥98 % purity by HPLC), erythromycin (potency ≥850 µg/mg), ciprofloxacin (≥98 % purity by HPLC), and tetracycline (≥95% purity by HPLC) were purchased from Sigma-Aldrich (Australia) and were used as controls for the microplate liquid dilution assay. All antibiotics were prepared in sterile deionised water at stock concentrations of 0.01 mg/mL and stored at 4°C until use.

Bacterial cultures

Reference strains of *Alcaligenes faecalis* (BAA-2365), *Aeromonas hydrophila* (ATCC7966), *Bacillus cereus* (ATCC14579), *Enterobacter cloaceae* (ATCC 7256), *Enterococcus faecalis* (BAA-2365), *Escherichia coli* (ATCC 25922) and *Shigella sonneii* (ATCC 25931) were purchased from the American Type Culture Collection (USA). The *Citrobacter freundi, Pseudomonas fluorescens, Salmonella newport* and *Serratia marcescens* clinical isolate strains screened herein were a gift from Ms Michelle Mendell of the School of Environment and Science teaching laboratory. All bacteria were cultured in nutrient broth (Oxoid Ltd., Australia) at 37°C for 24 hr and were subcultured and maintained in nutrient broth at 4°C until use. Streaked nutrient agar (Oxoid Ltd., Australia) plates were tested in parallel to ensure the purity of all bacterial cultures and for sub-culturing.

Disc diffusion bacterial growth inhibition assays

Antibacterial activity screening of the *T. lanceolata* pepperberry extracts on solid agar was assessed using a modified disc diffusion assay method.[39,40] Briefly, single colonies of the test bacteria isolated from streaked agar plates were grown at 37°C in 40 mL of fresh nutrient broth media until they reached a count of approximately 10^8 cells/mL. The visual turbidity of each culture was adjusted in order to prepare 0.5 McFarland standards. A volume of 100 µL of the individual microbial suspensions were spread onto nutrient agar plates. Extracts (10 µL) were infused onto filter paper discs (6 mm in diameter), with negative control discs containing 10 µL of extract resuspension solvent (0.5% DMSO). Preloaded ampicillin discs (10 µg) were purchased from Oxoid Ltd, Australia and were used as positive controls in the disc diffusion assay, whilst filter discs infused with 10 µL of distilled water were used as a negative control. Discs were allowed to dry and were then applied to inoculated agar. The plates were incubated at 37°C for 24 hr. The zone of inhibition (ZOI) was measured for each disc and was inclusive of the 6 mm diameter of the filter disc.

Minimum inhibitory concentration (MIC) determination

The minimum inhibitory concentration for each extract was determined using a microplate liquid dilution MIC method,[14,40] which is considered to be a highly 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. Briefly, 100 µL of resuspended extract stocks or antibiotics (1 µg) were added to 100 µL of nutrient broth in the top row of 96-well microplates. Positive control lanes containing nutrient broth only, 0.5% DMSO (control for extracts), or deionised (control for antibiotics) were also prepared. Three-fold serial dilutions were prepared in each subsequent row, each containing 100 µL of nutrient broth. Finally, 100 µL of a 1:100 dilution of a 0.5 McFarland bacterial standard were added to each well, and the microplates were incubated at 37°C for 24 hr. p-Iodonitrotetrazolium violet (Sigma-Aldrich, Australia) dissolved in sterile deionised water to produce a 0.2 mg/mL INT solution was prepared and a 40 µL volume of this solution was added into all wells. Microplates 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

An aqueous preparation (4 mg/mL) of potassium dichromate ($K_2Cr_2O_7$) (AR grade, Chem-Supply, Australia) was serially diluted in artificial seawater for use as a reference toxin. Toxicities of the *T. lanceolata* pepperberry extracts, the reference toxin and the conventional antibiotics were assessed using a modified *Artemia franciscana* nauplii lethality assay.[42,43] The LC_{50} with 95% confidence limits for each treatment was calculated using probit analysis.

Evaluation of therapeutic index

The therapeutic indexes (TI) of the *T. lanceolata* pepperberry extracts were calculated 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 ± SEM of three independent experiments, each with internal triplicates (n=9) unless otherwise stated. One way ANOVA was used to calculate statistical significance between control and treated groups, with a P value < 0.01 considered as statistically significant.

RESULTS

Liquid extraction yields and qualitative phytochemical screening

Tasmannia lanceolata pepperberry 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.

Screening for bacterial growth inhibition activity against gram negative bacteria

The Gram-negative bacteria screened in this study (Figure 1 and Figure 2) were selected as most of them are relatively common causes of food poisoning.[43] In contrast, *A. faecalis* (Figure 1a) and *E. cloaceae* (Figure 1c) were included in the study as they are normal/non-pathogenic components of the human gastrointestinal flora. Notably, *E. cloaceae* appeared to be partially resistant to all of the *T. lanceolata* pepperberry extracts, with only relatively small zones of inhibition (ZOIs) measured (7-7.8 mm) (Figure 1c). The effects of treatments for gastrointestinal illnesses on the normal gastrointestinal microflora is an important consideration when using food poisoning and diarrhoeal chemotherapies. Therapies that affect the normal gastrointestinal balance may allow potentially pathogenic bacterial species to compete effectively against beneficial non-pathogenic species, thereby inducing further illness. Thus, the relative low inhibitory activity of the *T. lanceolata* pepperberry extracts against *E. cloaceae* indicates that they may not adversely affect the levels of this bacterium in the gastrointestinal tract. In contrast, this bacterium was sensitive to the ampicillin control antibiotic, with a ZOI of >12 mm, indicating that the assay was functioning correctly.

In contrast, the *T. lanceolata* pepperberry extracts had substantial inhibitory effects on the growth of *A. faecalis* (Figure 1a). This bacterium is also a normal component of the human gastrointestinal microbiome and is generally considered non-pathogenic, although it is an opportunistic bacteria and can induce illness in immunocompromised people.[43] The methanolic extract was a particularly good inhibitor of *A. faecalis* growth, with a ZOIs of 8.3 mm. The stronger inhibitory effects of the *T. lanceolata* pepperberry extracts against this bacterium may impact on their usefulness in the treatment of gastrointestinal diseases and further studies are required to examine the effects in mixed culture systems, and their effects against other non-pathogenic gastrointestinal microflora species. Interestingly, the *A. faecalis* strain examined in this study was highly sensitive to ampicillin (ZOI = 12.6 mm).

Notably, the *T. lanceolata* pepperberry extracts displayed substantial inhibitory activity against all of the pathogenic reference (Figure 1) and clinical isolate strains (Figure 2) gram negative bacteria associated with gastrointestinal illness. *Aeromonas hydrophilia* was included in this study as it is a relatively common cause of food poisoning in humans.[43] This bacterium produces aerolysin (a cytotoxic enterotoxin), as well as several proteins that induce gastroenteritis and diarrhoea. Notably, the methanolic *T. lanceolata* pepperberry extract was a particularly good inhibitor of *A. hydrophilia* growth, with a ZOI

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.

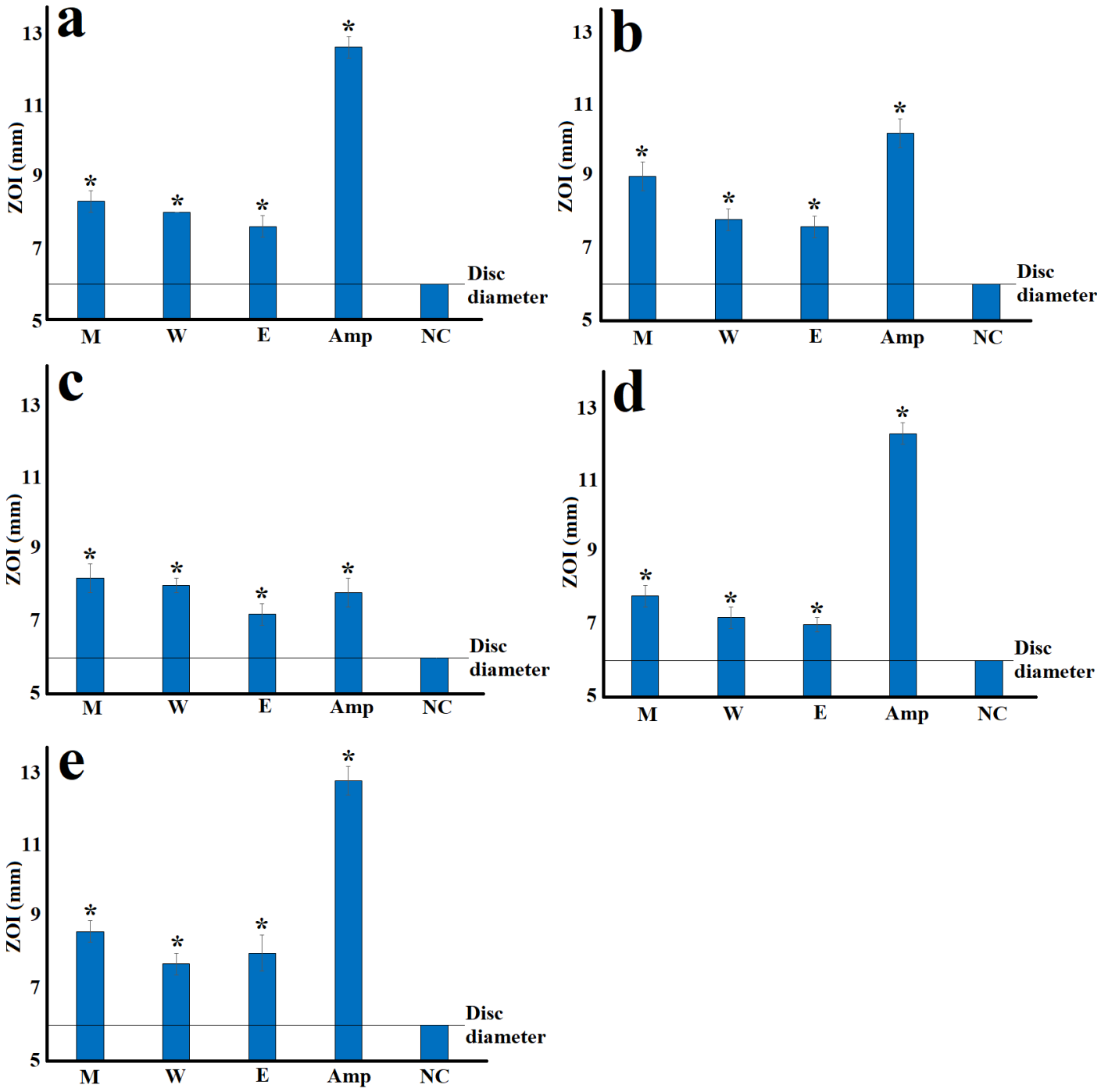

Figure 1: Growth inhibitory activity of the *T. lanceolata* pepperberry extracts against the reference gram negative gastrointestinal bacterial strains (a) *Alcaligenes faecalis* (BAA-2365), (b) *Aeromonas hydrophila* (ATCC7966), (c) *Enterobacter cloaceae* (ATCC 7256), (d) Escherichia coli (ATCC 25922) and (e) *Shigella* sonnei (ATCC 25931) measured as zones of inhibition (mm). M = methanolic extract; W = aqueous extract; E = ethyl acetate extract; Amp = ampicillin (10 µg); NC = negative seawater control (0.5 % DMSO). Results are expressed as mean zones of inhibition ± SEM. * indicates results that are significantly different to the negative control ($p<0.05$).

of 9 mm measured (Figure 1b). The aqueous and ethyl acetate extracts also significantly inhibited *A. hydrophilia* growth, albeit with substantially smaller ZOIs (7.8 and 7.6 mm respectively), indicating weaker inhibitory activity (Figure 1b). Notably, this bacterium was highly susceptible to the ampicillin control (ZOI = 10.2 mm), indicating that the assay was functioning correctly.

The methanolic, aqueous and ethyl acetate *T. lanceolata* pepperberry extracts also inhibited the *E. coli* strain screened in our study, albeit with relatively small ZOIs (Figure 1d). Indeed, ZOIs of 8 mm were measured when each extract was tested against this *E. coli* strain. In contrast, this *E. coli* strains was highly sensitive to the ampicillin control (ZOI = 12.3 mm). *Escherichia coli* is a normal component of the gastrointestinal microflora and

most strains are non-pathogenic. Thus, the relatively low activity of the *T. lanceolata* pepperberry extracts indicates that they may not profoundly affect the gastrointestinal microbial balance adversely. However, some *E. coli* strains are highly virulent and are major causes of food poisoning in humans.[44] Thus, the inhibitory activity indicates that the extracts may have some (limited) potential for treating *E. coli* related food poisoning, and further studies are required to isolate and identify the bioactive extract components.

The gram negative coccus bacterium *Shigella sonneii* was included in this study as it is also a relatively common cause of food poisoning in humans.[43] The methanolic *T. lanceolata* pepperberry extract was a particularly strong inhibitor of *S. sonneii* growth, with a ZOI of 8.6 mm respectively (Figure 1e), highlighting the potential of the methanolic *T. lanceolata* pepperberry extract for the treatment of shigellosis. The aqueous and ethyl acetate extracts also significantly inhibited *S. sonneii* growth, albeit with substantially smaller ZOIs (7.7 and 8.0 mm respectively), indicating lower growth inhibitory activity. It is noteworthy that inhibition in this assay is reliant on the diffusion on molecules in the aqueous agar gel environment, which is dependent upon the polarity of the extract components. As the ethyl acetate extract would contain medium to low polarity compounds, measurement of ZOIs in this assay may underestimate the growth inhibitory activity in liquid environments (such as the gastrointestinal tract), and testing in liquid assays (such as the liquid dilution MIC results presented later in this report) is also required.

The *T. lanceolata* pepperberry extracts also displayed noteworthy activity against the clinical isolate gram negative strains. Whilst *C. freundii* (Figure 2a), *P. fluorescens* (Figure 2b) and *S. marcescens* (Figure 2d) are normal components of the human gastrointestinal microbiome are and generally nonpathogenic, disruptions of the gastrointestinal microbial balance can result in gastrointestinal distress.[44] Therefore, inhibition of these bacteria may be useful when these bacteria are present in elevated levels in the gastrointestinal system. Notably, the methanolic *T. lanceolata* pepperberry extract had noteworthy activity against all of these bacteria, with ZOIs of 8.9, 8.6 and 9.3 mm respectively. In contrast, *Salmonella* spp. are one of the most common causes of food poisoning in humans, particularly from eggs and milk, as

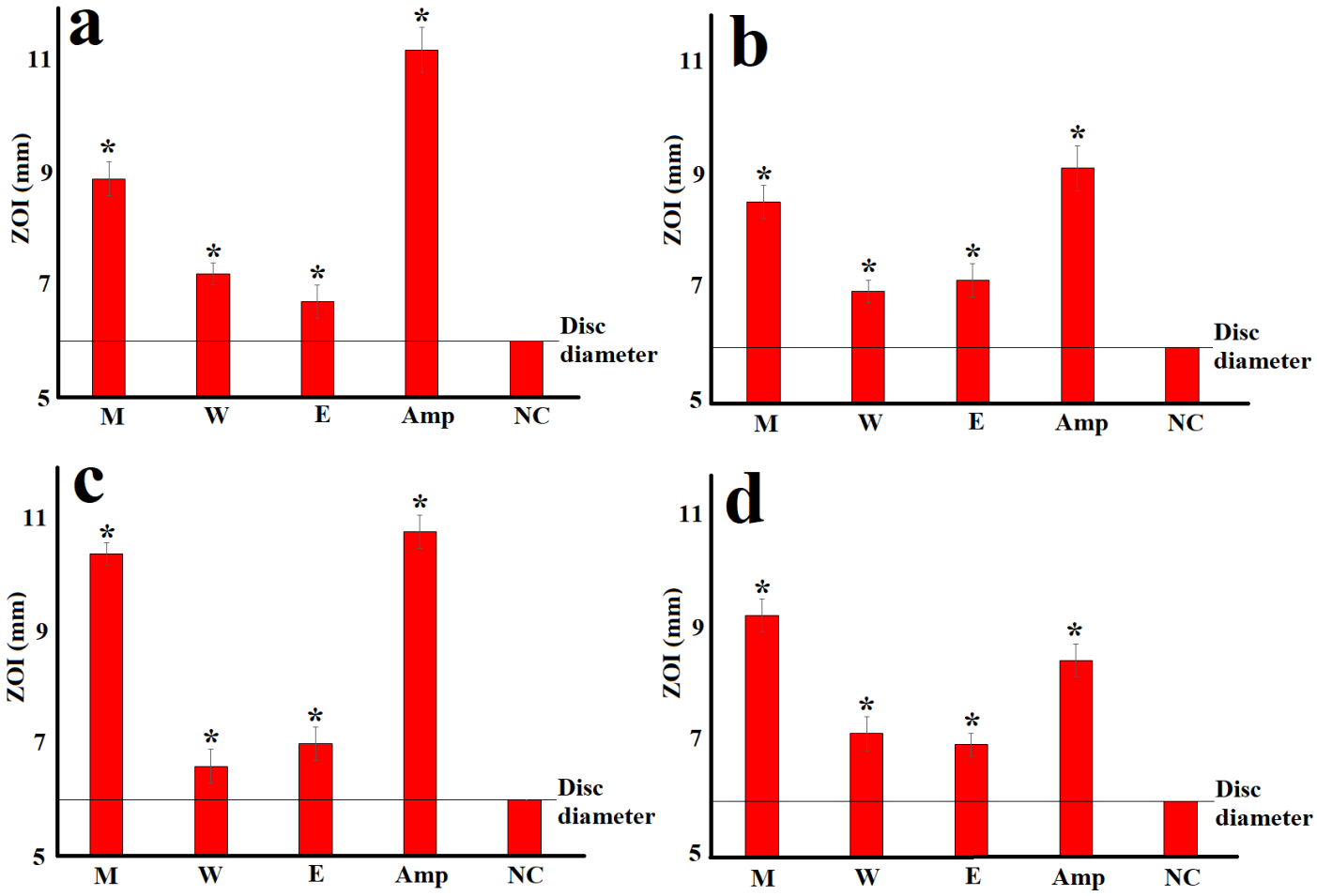

Figure 2: Growth inhibitory activity of the *T. lanceolata* pepperberry extracts against the gram negative clinical isolate strains of gastrointestinal bacteria: (a) *Citrobacter freundii*, (b) *Pseudomonas fluorescens*, (c) *Salmonella newport*, (d) and *Serratia marcescens*, measured as zones of inhibition (mm). M = methanolic extract; W = aqueous extract; E = ethyl acetate extract; Amp = ampicillin (10 µg); NC = negative seawater 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).

Table 2: Liquid dilution MIC values against gastrointestinal bacteria (µg/mL) of the *T. lanceolata* pepperberry extracts, and toxicity (LC_{50}) in the *Artemia* nauplii bioassay.

Test bacteria	Antibacterial activity MIC (µg/mL)			ALA LC_{50} (µg/mL)		Therapeutic index (TI)		
	M	W	E	M	W	M	W	E
Alcaligenes faecalis (BAA-2365)	2375	1375	625	1483	2010	0.62	1.46	CND
Aeromonas hydrophila (ATCC7966)	594	688	375			2.5	2.92	CND
Enterobacter cloaceae (ATCC 7256)	3063	2750	1500			0.49	0.74	CND
Escherichia coli (ATCC 25922)	1188	688	563			1.25	2.92	CND
Shigella sonnei (ATCC 25931)	594	1032	375			2.5	1.95	CND
Citrobacter freundii	1188	2750	1500			1.25	0.74	CND
Pseudomonas fluorescens	891	1375	750			1.66	1.46	CND
Salmonella newport	891	2750	1500			1.66	0.74	CND
Serratia marcescens	2375	2750	750			0.62	0.74	CND
Bacillus cereus (ATCC 14579)	1188	2063	375			1.25	0.97	CND
Enterococcus faecalis (BAA-2365)	2375	2750	1500			0.62	0.74	CND

CM = methanolic extract; W = aqueous extract; E = ethyl acetate extract; CND = could not determine as LC_{50} could not be calculated, but the TI is assumed to be relatively high. Numbers indicate the mean MIC values of triplicate determinations.

well as from incorrectly stored meat or poultry products[43] and therefore their control is important to medical science, as well as to the food industry. Notably, the methanolic *T. lanceolata* pepperberry extract was a strong inhibitor of *S. newport* growth (ZOIs = 10.4 mm; 2c), highlighting its potential in the treatment of salmonellosis. Notably, the *S. newport* strain tested herein was also susceptible to the ampicillin control (ZOI = 10.8 mm), confirming that the assay was functioning correctly.

Inhibition of the growth of gastrointestinal gram positive bacterial pathogens

Bacteria of the genus *Bacillus* can also cause food poisoning and it can be particularly difficult to control their growth in stored food. Whilst refrigeration of food inhibits the growth of many pathogenic bacteria, *Bacillus* spp. are psychrotropic and readily grow at reduced temperatures.[43] Indeed, the retardation of the growth of other bacteria below 5°C, provides a selective advantage for psychrotropic pathogens and *Bacillus*-induced gastrointestinal disease is particularly relevant in refrigerated foods. Additionally, *Bacillus* spp. form heat-resistant endospores, making food processing methods such as pasteurisation relatively ineffective. Therefore, treatments that are effective against *Bacillus* spp. may be particularly useful, both therapeutically and as a method of food preservation. Noteworthy growth inhibitory activity was noted for the methanolic and ethyl acetate *T. lanceolata* pepperberry extracts against *B. cereus* (Figure 3), with ZOIs >8.8 and 9.68 mm respectively. Notably, the extracts were stronger inhibitors of *B. cereus* growth than the ampicillin control was (ZOI = 8 mm). Thus, the *T. lanceolata* pepperberry extracts may be particularly useful against antibiotic-resistant *Bacillus* strains.

Enterococcus faecalis is a commensal bacteria that is a normal component of the gastrointestinal microbiome in humans and is generally non-pathogenic except in immunocompromised individuals.[43] Notably, whilst all of the *T. lanceolata* pepperberry extracts inhibited *E. faecalis* growth, only relatively small ZOIs were measured for all extracts (ZOIS 6.8-7.6 mm). Thus, the relative low inhibitory activity of the *T. lanceolata* pepperberry extracts against *E. faecalis* indicates that they may not adversely affect the levels of this bacterium in the gastrointestinal tract, and therefore would not affect the gastrointestinal microbiome balance. In contrast, this bacterium was sensitive to the ampicillin control antibiotic, with a ZOI of 13.6 mm, indicating that the assay was functioning correctly.

Quantification of minimum inhibitory concentration (MIC)

The antimicrobial activity of the *T. lanceolata* pepperberry extracts was further evaluated by determining the MIC values using liquid dilution MIC assays (Table 2). In this study, MIC values <500 µg/mL are defined as potent antibacterial activity, 500-1000 µg/mL is defined as strong antibacterial activity, 1000—2000 µg/mL as noteworthy activity and MIC values >2000 µg/mL are classed as low antibacterial activity. The *T. lanceolata* pepperberry extracts displayed potent to noteworthy growth inhibitory activity against several bacterial strains. The ethyl acetate *T. lanceolata* pepperberry extracts was the most potent inhibitor of the majority of bacterial pathogens, with MICs as low as 375 µg/mL recorded against *A. hydrophila, S. sonneii* and *B. cereus*. Ethyl acetate is a medium polarity solvent, indicating that some of the antibacterial *T. lanceolata* pepperberry extracts components are of medium polarity, such as flavonoids. The methanolic extract

was also a good inhibitor of many of the gastrointestinal bacterial pathogens. That extract was most potent against *S. sonneii*, with a MIC value of 595 µg/mL recorded. The MIC values of the ethyl acetate and methanolic *T. lanceolata* pepperberry extracts against *S. sonneii* are particularly interesting as *S. sonneii* is a major cause of food poisoning and gastrointestinal illness globally.[36] Thus, the strong-potent activity of the extracts against this bacterium highlights it's potential for treating *S. sonneii*-induced gastrointestinal illness.

All of the *T. lanceolata* pepperberry extracts were also particularly good inhibitors of *A. hydrophilia*, with MIC values of 594, 688 and 375 µg/mL recorded for the methanolic, aqueous and ethyl acetate extracts respectively. *Aeromonas hydrophilia* is a relatively common cause of food poisoning in humans, further highlighting the potential of these extracts to treat diarrhoea.[43] *Salmonella* spp. are one of the most common causes of food poisoning globally and are classified a serious threat to public health.[34] Notably, the methanolic extract was a strong inhibitor of *S. newport* (891 µg/mL), whilst moderate activity was noted for the ethyl acetate extract (1500 µg/mL). In contrast, the aqueous extract produced low inhibitory activity against this bacterium. The aqueous and ethyl acetate *T. lanceolata* pepperberry extracts were also good inhibitors of *E. coli* growth (MIC values of 688 and 563 µg/mL respectively), whilst the methanolic extract also had noteworthy activity against this bacterium (MIC = 1188 µg/mL). As some *E. coli* strains are causes of food poisoning in humans,[43] these findings indicate that the *T. lanceolata* pepperberry extracts may be useful for treating *E. coli* related food poisoning. Strong growth inhibitory activity was also noted for the ethyl acetate extract against *P. fluorescens, S. marcescens* and *B. cereus*, as well as for the methanolic extract against *P. fluorescens.* All other bacterial gastrointestinal pathogens were also susceptible to the *T. lanceolata* pepperberry extracts, although with MIC values indicting only moderate-low activity.

The methanolic and aqueous *T. lanceolata* pepperberry extracts were relatively ineffective against *E. cloaceae,* although moderate activity (MIC = 1500 µg/mL) was recorded against this bacterium. This is a noteworthy finding as *E. cloaceae* is a normal component of the normal human gastrointestinal microbiome.[43] Anti-diarrhoeal chemotherapies should ideally inhibit the growth of pathogenic bacteria, without upsetting the balance of the normal gastrointestinal microbiome. Decreases in the levels of normal and beneficial bacteria may provide selective advantages for pathogenic bacteria to grow by removing competing bacterial species. This indicates that the *T. lanceolata* pepperberry extracts may be particularly useful for treating gastrointestinal disease, without inducing further negative effects, although this remains to be verified *in vivo. Alcaligenes faecalis* was also included in this study as it is also generally considered non-pathogenic (although it can cause gastrointestinal distress in immunocompromised people).[43] Whilst *E. cloaceae* growth was inhibited by all of the *T. lanceolata* pepperberry extracts, the MIC values of the methanolic and aqueous extracts indicate moderate and low activity respectively, thus further verifying the potential of these extracts for the treatment of gastrointestinal diseases clinically. Further studies are required to evaluate the effects of *T. lanceolata* pepperberry extracts against other bacterial components of the gastrointestinal microbiome, and to evaluate the effects *in vivo*.

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 hr (data not shown). 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

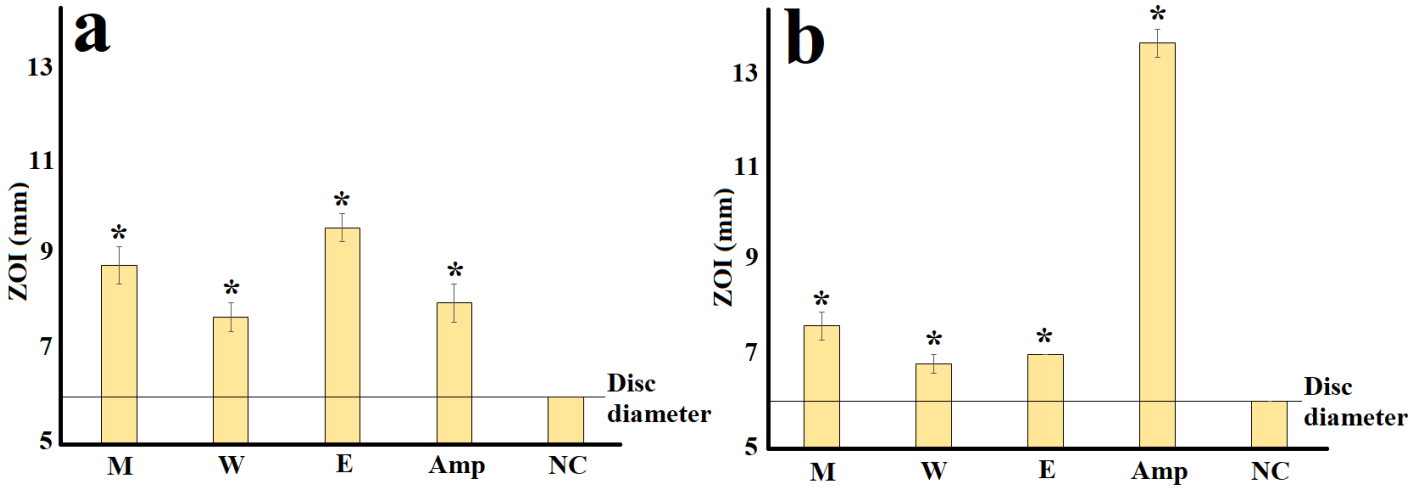

Figure 3: Growth inhibitory activity of the *T. lanceolata* pepperberry extracts against the reference gram positive strains of gastrointestinal bacteria: (a) *Bacillus cereus* (ATCC 14579), (b) *Enterococcus* faecalis (BAA-2365) measured as zones of inhibition (mm). M = methanolic extract; W = aqueous extract; E = ethyl acetate extract; Amp = ampicillin (10 µg); NC = negative seawater 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).

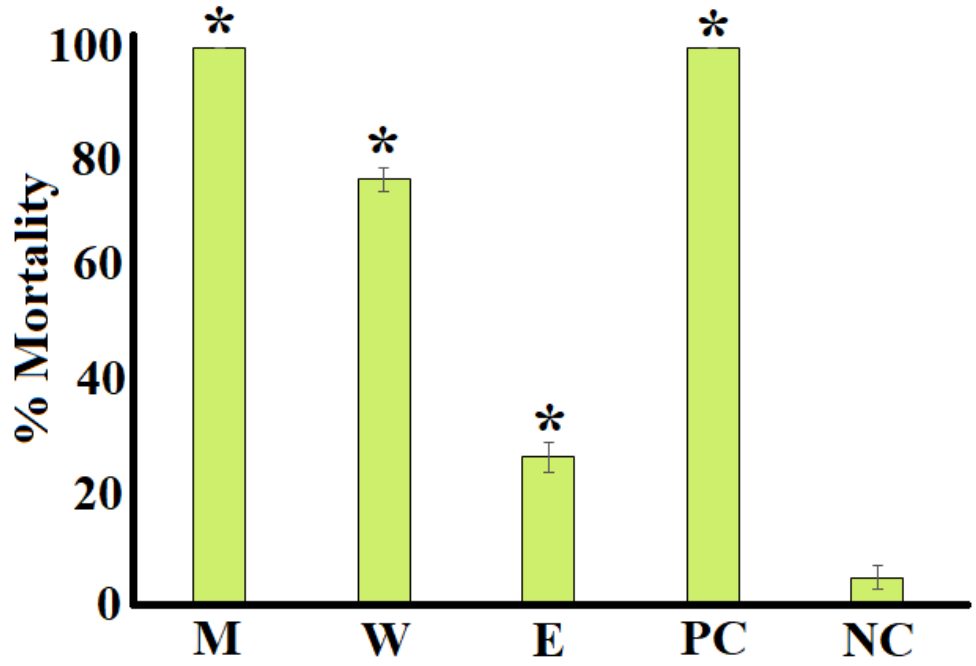

Figure 4: The lethality of the *T. lanceolata* pepperberry extracts (2000 µg/mL), as well as the potassium dichromate (1000 µg/mL) and negative seawater controls towards *Artemia franciscana* nauplii after 24 hr exposure. M = methanolic extract; W = aqueous extract; E = ethyl acetate extract; PC = potassium 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 ($n = 9$).

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 extracts with LC_{50} values >1000 µg/mL towards *Artemia* nauplii have been defined as being non-toxic in this assay,[41,42] all of the *T. lanceolata* pepperberry extracts were deemed to be non-toxic.

Calculation of therapeutic index

To further evaluate the suitability of the *T. lanceolata* pepperberry extracts as therapeutic agents, their therapeutic indexes (TI) were also calculated (Table 2). For this study, TI values ≥2 were considered noteworthy. We were unable to calculate TI values for the ethyl acetate extract against any bacteria as that extract did not produce mortality >50% at the highest concentration tested. However, the nontoxicity of this extract indicates that it would have high TI values, although this remains to be verified. The safety of the methanolic and aqueous extracts against *A. hydrophilia*, and for the aqueous extract only against *E. coli* were particularly promising, with TI values >2.5 against each of those bacteria. Therefore these extracts are considered to be safe to use therapeutically *in vitro*, although further *in vivo* studies are required to confirm the safety of these extracts before they can be adapted for clinical use.

DISCUSSION

Diarrhoea remains a leading killer of children, accounting for 8% of all deaths among children under the age of 5 annually.[44,45] Indeed, more than half a million children in this age group die each year from diarrhoeal disease,[44,45] which equates to over 1400 deaths per day. Furthermore, as bacterial pathogens develop further resistance to conventional antibiotics, medicines are expected to have reduced efficacy against diarrhoeal diseases and corresponding increases in mortality rates are expected.[46-48] There is an urgent need to develop new therapeutic options to combat these illnesses, either through the development of novel drug molecules, or by utilizing new therapy regimens that enable (or re-purpose) previously effective antibiotics that have lost their potency due to the emergence of highly resistant infections.

Plants are a promising source of new therapies to treat gastrointestinal diseases and numerous traditional medicines have been reported to be effective in the treatment of gastrointestinal illness.[49-51] *Tasmannia lanceolata* pepperberries have been used for thousands of years by Australian first nation groups to treat multiple pathogenic illnesses, gastrointestinal distress and diarrhoea.[23,24] However, the antibacterial properties of *T. lanceolata* pepperberry extracts have not been extensively reported and much more work is required. This report aimed to address this gap in the literature by screening *lanceolata* pepperberry extracts against clinically relevant bacterial gastrointestinal pathogens, thereby evaluating their potential for the treatment of gastrointestinal illness in humans.

The bacteria screened in this study were selected because they are either causes of food poisoning and diarrhoea, or are normal components of the human gastrointestinal microbiome. *Bacillus* spp.[43,52] *A. hydrophilia*[43] and *E. coli*[53] produce and secrete enterotoxins and other proteins taht may cause gastroenteritis and diarrheal diseases. Notably, many of these toxins are heat stable and are thus not readily destroyed by heat treatments/pasteurisation. The *T. lanceolata* pepperberry extracts susceptibility profile varied widely between the bacterial species tested. For example, *A. hydrophilia* was highly susceptible to all *T. lanceolata* pepperberry extracts (MIC values 375-688 µg/mL), whilst substantially weaker activity was noted against *C. freundii* and *S. marcescens*. Some trends were evident. In particular, the ethyl acetate was generally the best inhibitor of most of the bacteria tested, whilst the aqueous extract was substantially less effective against most bacteria.

Studies on the antibacterial properties of *T. lanceolata* pepperberry extracts are lacking. Therefore, one aim of this project was to investigate the efficacy of *T. lanceolata* pepperberry extracts against gastrointestinal pathogens that cause diarrhoea. Notably, the *T. lanceolata* pepperberry extracts inhibited the growth of all bacteria screened herein, albeit with varying potencies. The ethyl acetate extract was a particularly good inhibitor of *A. hydrophilia, E. coli, S. sonneii, P. fluorescens, S. marcescens* and *B. cereus*, with MIC values substantially <1000 µg/mL against each of these bacteria. This suggests that ethyl acetate *T. lanceolata* pepperberry extract (in particular) contains components that could be used to treat illnesses that feature these disease-causing bacteria. Importantly, none of the *T. lanceolata* pepperberry extracts substantially affected the growth of the normal gut flora strains tested in this study (*E. cloacae* and *E. faecalis*). This indicates that *T. lanceolata* pepperberry extracts treatment may be used safely without inducing detrimental shifts in the gastrointestinal microbiome that may allow harmful/pathogenic bacteria to flourish.

Whilst a detailed investigation of the phytochemistry of the *T. lanceolata* pepperberry extracts was beyond the scope of this study, the qualitative phytochemical studies highlighted several phytochemical classes that may contribute to the bacterial growth inhibitory activity, and to the combinational effects noted in this study. All *T. lanceolata* pepperberry extracts had relatively high abundances in polyphenolics and flavonoids, as well as lower levels of saponins and tannins. The antibacterial activities are well known for a wide variety of flavonoids.[23,54] Flavonoids inhibit bacterial growth via to a variety of mechanisms, including their ability to complex with extracellular and soluble proteins and as well as bacterial cell walls.[55] Similarly, multiple tannins have broad antibiotic properties via a variety of mechanisms including binding, inactivating and/or precipitating of microbial proteins.[56] Polyphenolic compounds are toxic to microorganisms via non-specific interaction with proteins or by reaction with sulfhydryl groups.[57] Therefore, phytochemical purification and structural analysis studies are required to evaluate the growth inhibitory mechanism(s) and to identify the bioactive extract component(s).

CONCLUSION

The *T. lanceolata* pepperberry extracts (particularly the ethyl acetate extract) inhibit the growth of multiple bacterial gastrointestinal pathogens, without substantially affecting normal gastrointestinal microflora. Additionally, the *T. lanceolata* pepperberry extracts were nontoxic, further indicating their suitability for therapeutic usage. Further studies are required to isolate the inhibitory compounds and identify their antimicrobial mechanisms.

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SUMMARY

- Methanolic, aqueous and ethyl acetate *T. lanceolata* pepperberry extracts were screened for the ability to block the growth of selected gastrointestinal bacteria.
- The ethyl acetate *T. lanceolata* pepperberry extract was a particularly good inhibitor of *A. hydrophilia, S. sonnei*, and *B. cereus,* with MICs of 375 µg/mL.
- The methanolic *T. lanceolata* extracts was also a noteworthy inhibitor of *A. hydrophilia* and *S. sonnei* growth.
- 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 use against bacterial gastrointestinal infections.

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