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Research Article

Tasmannia stipitata as a Functional Food/Natural Preservative: Antimicrobial Activity and Toxicity

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ABSTRACT: Introduction: *Tasmannia stipitata* (Dorrigo pepper) is an endemic Australian plant with a history of use by indigenous Australians as a food. It is taxonomically related to *Tasmania lanceolata* which has documented therapeutic properties as well as uses for food flavouring. **Methods:** *T. stipitata* solvent extracts were investigated by disc diffusion assay against a panel of bacteria and fungi. Their MIC values were determined to quantify and compare their efficacies. The ability to inhibit the proliferation of *Giardia duodenalis* was determined by direct cell counts and by using an MTS based cell proliferation assay. Toxicity was determined using the *Artemia franciscana* nauplii bioassay. **Results:** Methanolic, aqueous and ethyl acetate *T. stipitata* leaf and berry extracts displayed antibacterial activity in the disc diffusion assay. The berry methanolic extract had the broadest antibacterial range, inhibiting the growth of all 22 of the 23 bacteria tested (95.7%) and 2 of the 4 fungal species (50%) tested. In comparison, 18 of the bacterial species (81.8%) and 2 of the fungal species (50%) were inhibited by at least 1 of the leaf extracts. The methanol, water and ethyl acetate extracts of both berries and leaves all had similar efficacies and ranges of microbes inhibited. Whilst broad spectrum activity was seen for these extracts, they displayed only moderate to low efficacy (as determined by the zones of inhibition and MIC analyses). All extracts were more effective at inhibiting the growth Gram-negative bacteria than Gram-positive bacteria or fungi. Furthermore, the methanol, water and ethyl acetate extracts of both berry and leaf were potent inhibitors of Giardial proliferation. All *T. stipitata* extracts were non-toxic in the *Artemia fransiscana* bioassay with LC_{50} values greatly in excess of 1000 μg/ml. **Conclusion:** The lack of toxicity of the *T. stipitata* extracts and their moderate broad spectrum inhibitory bioactivity against bacteria, fungi and Giardia indicates their potential as natural food preservatives and as medicinal agents in the treatment and prevention of microbial diseases.

KEYWORDS: Winteraceae, *Tasmannia stipitata*, Dorrigo pepper, antibacterial, food spoilage, food poisoning, functional food, natural preservative.

INTRODUCTION

Members of family Winteraceae have been used for a broad range of dietary and medicinal purposes by a wide variety of ethnic and cultural groupings. The best documented of these is the South American species *Drimys winteri*. The stem and bark of this species has been used as a stimulant and as a tonic in traditional Brazilian medicinal systems.[1] They are also used for the treatment of a wide variety of diseases and medicinal conditions including use as an analgesic, and to treat diarrhoea, inflammation, and ulcers.[1,2] This species also has widespread usage in the treatment of scurvy due to its high antioxidant content.[3] Of the other Winteraceae species, several have a history of ethnobotanical usage, usually for purposes related to their polygodial contents and high levels of antioxidants. Indeed, high levels of the compound polygodial (which gives the family Winteraceae a characteristic peppery flavour) (Figure 1a) and high antioxidant contents are common characteristics of Winteraceae species. Other antioxidant molecules common to Winteraceae species include safrole (Figure 1b), gallic acid (Figure 1c), chlorogenic acid (Figure 1d), quercetin (Figure 1e), rutin (Figure 1f), lutein (Figure 1g), α-tocopherol (vitamin E) (Figure 1 h), vitamin a (Figure 1i) and folic acid (Figure 1j).

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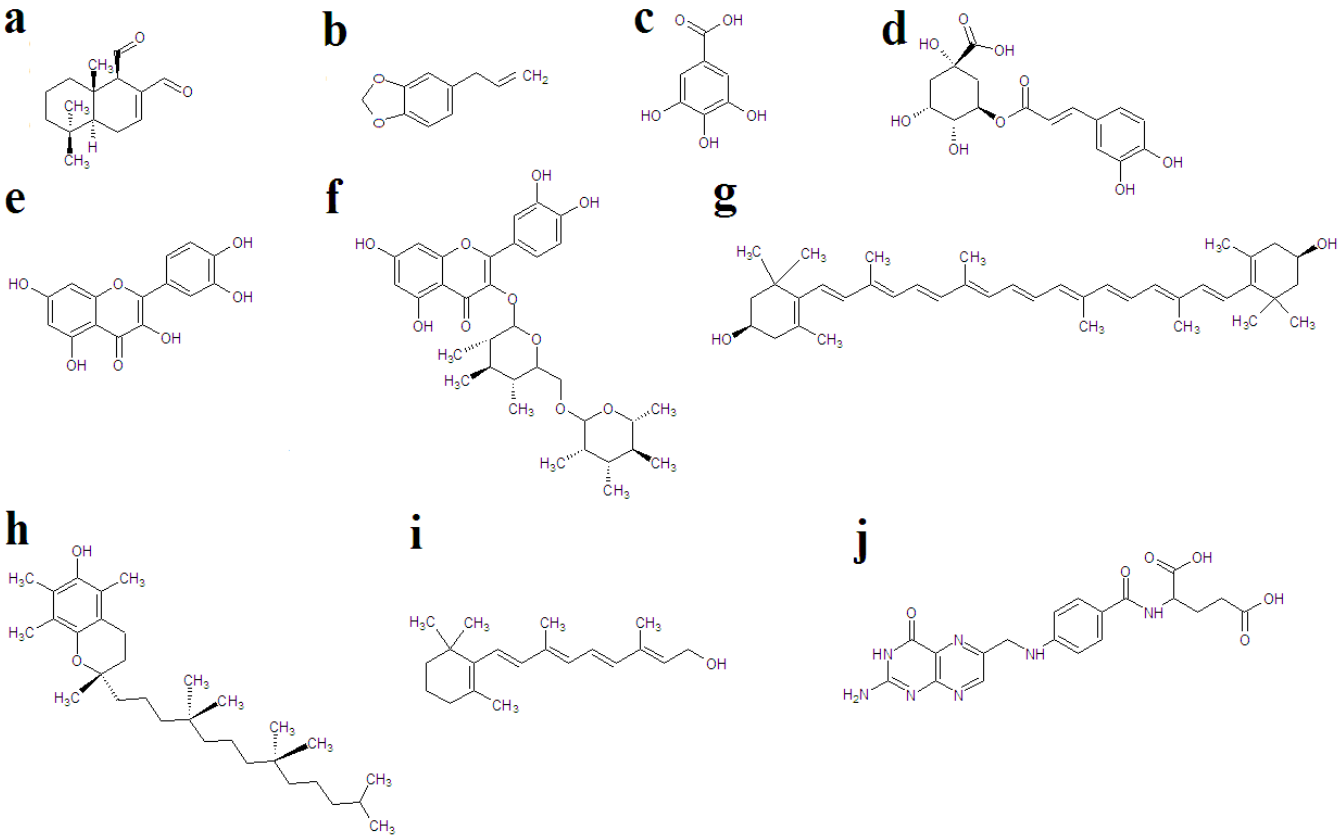

Figure 1. Chemical structures of antioxidant molecules common across Winteraceae species (a) polygodial, (b) safrole, (c) gallic acid, (d) chlorogenic acid, (e) quercetin, (f) rutin, (g) lutein, (h) α-tocopherol (vitamin E), (i) vitamin a, (j) folic acid.

Epidemiological studies have shown that a diet high in antioxidants may have preventative effects against the development of degenerative diseases such as cancer,[4] cardiovascular diseases,[5] neural degeneration,[6] diabeties and obesity.[7] The antioxidant activity of many plants has been associated with their phenolic contents. Many phenolic compounds have been shown to have strong antioxidant activities and may protect cells against oxidative damage by directly scavenging free radicals.[8] Phenolic compounds may also interact directly with receptors or with enzymes involved in cellular signal transduction.[9] Common classes of plant phenolic compounds include flavonoids, tannins and anthrocyanins. The medicinal potential of plants with high antioxidant contents has been receiving much recent attention[10,11] and reports have linked antioxidant levels and redox management with anticancer activity[12]

There has been recent interest in the medicinal and functional food properties of Australian Winteraceae species due to recent reports of their high antioxidant contents.[11] *Tasmannia lanceolata* (Tasmanian pepper, pepper berry) in particular has attracted attention due to reported antioxidant contents of over 120 μmol/g fruit (approximately 3 times the antioxidant content of blueberries, which themselves are considered to have high antioxidant contents). The same study also reported *T. lanceolata* to have even greater leaf antioxidant contents (more than 4 fold higher than those reported for blueberries). Interestingly, ascorbic acid (which makes a significant contribution to the antioxidant content of many fruits) was reported to be below the threshold of detection in this study and therefore would not contribute significantly to the high antioxidant content of *T. lanceolata*. *T. lanceolata* leaves have also been reported to have phenolic antioxidant contents up to 4 times higher than in basil leaves (*Ocimum basilicum*),[13] higher levels than determined for peppermint leaves[14] and similar levels to the phenolic antioxidant contents of maple, silver birch and spruce leaves.[15] The phenolic contents of *T. lanceolata* berries are also high, although these levels are significantly lower (less than 20%) than the leaf phenolic antioxidant levels. The contents are similar to those reported for those reported for *Piper nigrum* (black pepper) and *Lycium barbarum* (Chinese Barbary Wolfberry fruit),[14] but approximately half the level of black sesame and peach kernel.[15]

Further interest has focussed on the antimicrobial properties of high antioxidant foods and thus on their potential as natural preservatives.[16-19] A recent study reported potent, broad spectrum antibacterial and anti-fungal properties for *T. lanceolata* extracts.[20] That study not only indicated the therapeutic potential of *T. lanceolata* against a range of infectious diseases, but also looked at its potential as a natural preservative by specifically studying the growth inhibition of a panel of microbial species specifically associated with food spoilage and food poisoning. 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. 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.[18-23] Thus, high antioxidant foods such as *T. lanceolata* have potential uses as natural preservatives, and as functional foods in the prevention and treatment of food borne diseases.

Whilst *T. lanceolata* has received much recent interest, studies into other closely related Australian species are lacking. *Tasmannia stipitata* (Dorrigo pepper, northern pepperbush) is a shrub which is endemic to the rainforests and temperate woodlands of northern New South Wales and southern Queensland regions of Australia. It is a medium shrub that varies between 2-5 m in height. Individual plants are dioecious, with male and female flowers on separate plants. The aromatic leaves are lanceolate to narrowly elliptical in shape (8-13 cm in length). Fleshy dark blue-black 2 lobed berries (5-8 mm wide) develop in autumn. The berries and the leaves have similar culinary uses and tastes to *T. lanceolata* and are also considered to have high antioxidant and polygodial contents and many of the same therapeutic properties. Despite its ethnobotanical usage and taxonomic relationship to *T. lanceolata*, there is a lack of rigorous scientific studies into the therapeutic properties of *T. stipitata*. The current study was undertaken to test *T. stipitata* leaf and berry 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. stipitata* 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 Source and Extraction

T. stipitata semi-dry berries and dried leaves were purchased from A Taste of the Bush, Australia. The berries were thoroughly dried in a Sunbeam food dehydrator and subsequently the dried plant materials were stored at -30°C. Prior to use, the plant materials were thawed and freshly ground to a coarse powder. Individual 1 g quantities of the ground leaves and berries were weighed into individual tubes and 50 ml of methanol, deionised water or ethyl acetate were added. All solvents were obtained from Ajax and were AR grade. The ground berries and leaves were individually 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. stipitata* 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. Reference strains of *Acinetobacter bayleii* (ATCC33304), *Klebsiella pneumoniae* (ATCC31488), *Proteus mirabilis* (ATCC21721), *Proteus vulgaris* (ATCC21719) and *Pseudomonas aeruginosa* (ATCC39324) were purchased from American Tissue Culture Collection, USA. All other 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 approxi-

mately 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 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) and nystatin (100 µg) were obtained from Oxoid Ltd. and served as positive controls for antibacterial and antifungal activity respectively. 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.[31,32] 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.

Inhibitory Bioactivity Against *Giardia duodenalis* trophozoites

Parasite Culture

The *Giardia duodenalis* S-2 (sheep strain 2) trophozoite strain used in this study were previously supplied by Professor Andre Buret, University of Calgary, Canada. *G. duodenalis* tropozoites were maintained and subcultured anaerobically at 37°C in TYI-S-33 growth media supplemented with 1% bovine bile (Sigma), 10% Serum Supreme (Cambrex Bioproducts) and 200 IU/ml penicillin/200 µg/ml streptomycin (Invitrogen, USA). Confluent mid log phase cultures were passaged every 2 days by chilling the cultures on ice for a minimum of 10 min, followed by vortexing to dislodge the adherent trophozoites from the walls of the culture vessel. Fresh culture media (5 ml) was seeded with approximately 1 x 10^5 trophozoites for each passage.

Evaluation of Anti-Giardial Activity by CellTiter Bioassay

Anti-Giardial activity of the extracts was assessed as in previous studies.[33] Briefly, aliquots of the trophozoite suspension (70 µl) containing approximately 1 x 10^5 trophozoites were added to the wells of a 96 well plate. A volume of 30 µl of the test extracts or the vehicle solvent or culture media (for the negative controls) was added to individual wells and the plates were incubated anaerobically at 37°C for 12 hours in a humidified anaerobic atmosphere. A volume of 20 µl of CellTiter 96® AQueous One Solution Cell Proliferation Assay Reagent (Promega) was subsequently added to each well and the plates were incubated for a further 3 hours. Absorbances were recorded at 490 nm using a Molecular Devices, Spectra Max M3 plate reader. All tests were performed in at least triplicate and triplicate controls were included on each plate. The anti-proliferative activity of each test was calculated as a percentage of the negative control using the following formula:

$$\text{Giardial growth (\% untreated control)} = (A_{ct}/A_{cc}) \times 100$$

A_{ct} is the corrected absorbance for the test extract (calculated by subtracting the absorbance of the test extract in media without cells from the extract/cell/test combination) and A_{cc} is the corrected untreated control (calculated by subtracting the absorbance of the untreated control in media without cells from the untreated/cell/media combination).

Evaluation of Anti-Giardial Activity by Direct Parasite Enumeration

Anti-Giardial activity of the extracts was also assessed by direct enumeration of parasite numbers in the presence or absence of extracts. For each test, aliquots of the trophozoite suspension (70 µl) containing approximately 1 x 10^5 trophozoites were added to the wells of a 96 well plate. A volume of 30 µl of the test extracts or the vehicle solvent or culture media (for the negative controls) was

added to individual wells and the plates were incubated anaerobically at 37°C for 8 hours in a humidified anaerobic atmosphere. Following the 8 h incubation, all tubes were placed on ice for a minimum of 10 min, followed by vortexing to dislodge the adherent trophozoites from the walls of the culture vessel. The suspensions were mounted onto a Neubauer haemocytometer (Weber, UK) and the total trophozoites per ml were determined. The anti-proliferative activity of the test extracts was determined and expressed as a % of the untreated control trophozoites per ml.

Determination of IC50 Values Against Giardial trophozoites

For IC50 determinations, the plant extracts were tested by both methods across a range of concentrations. The assays were 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 IC50 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 43 (mean 43.2, n = 155, SD 14.5) *A. franciscana* nauplii were added to wells of a 48 well plate and immediately used for bioassay. A volume of 400 µl of diluted plant extracts or the reference toxin 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. stipitata* berry and leaf with various solvents yielded dried plant extracts ranging from 114 mg (leaf ethyl acetate extract) to 293 mg (leaf water extract) (Table 1). Deionised water and methanol generally gave relatively high yields of dried extracted material, whilst ethyl acetate extracted lower masses for both the berries and leaves. 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 after resuspension in deionised water and qualitative phytochemical screenings of T. stipitata berry and leaf 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)	Flavanoids	Tannins	Free Anthraquinones	Combined Anthraquinones
Berry	Methanol	279	27.9	+++	+++	++	-	+++	+	-	-	-	+++	-	-	-
	Water	207	20.7	+++	+++	+++	-	++	+	-	-	-	+++	-	-	-
	Ethyl Acetate	170	17	+	+	+	-	+	++	-	-	-	++	-	-	-
Leaf	Methanol	232	23.2	+++	+++	++	-	+++	+	-	-	-	+++	-	-	-
	Water	293	29.3	+++	+++	+++	-	++	+	-	-	-	+++	-	-	-
	Ethyl Acetate	114	11.4	+	+	+	-	-	+	-	-	-	++	-	-	-

+++ 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 moderate to high 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 2). Indeed, the growth of all of the Gram-negative species was inhibited by at least 1 of the *T. stipitata* extracts. The berry ethyl acetate extract displayed the broadest antibiotic specificity, inhib-

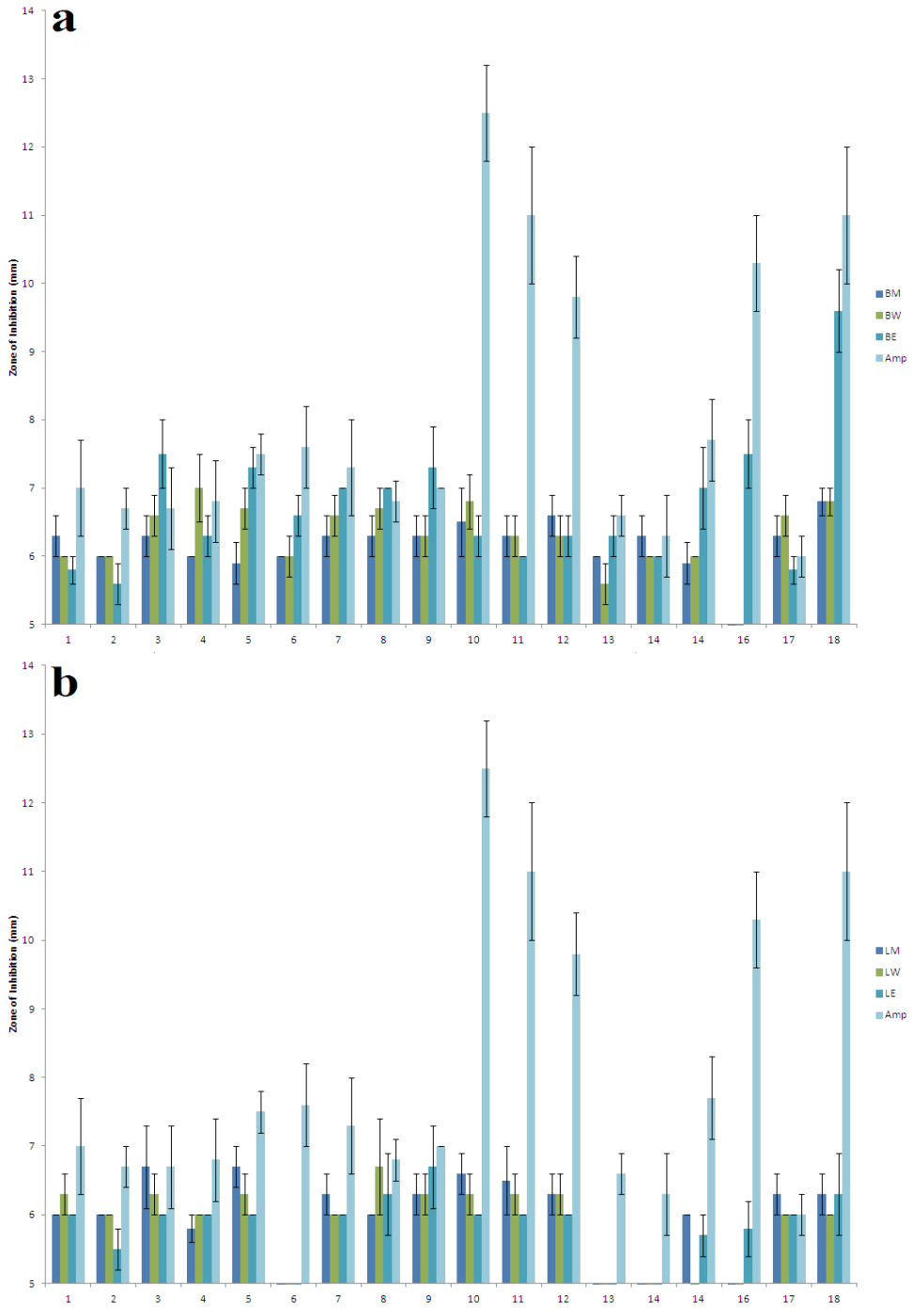

Figure 2. Antibacterial activity of (a) *T. stipitata* berry and (b) *T. stipitata* leaf extracts against Gram-negative bacteria measured as zones of inhibition (mm). BM = *T. stipitata* berry methanolic extract; BW = *T. stipitata* berry water extract; BE = *T. stipitata* berry ethyl acetate extract leaf; LM = *T. stipitata* leaf methanolic extract; LW = *T. stipitata* leaf water extract; LE = *T. stipitata* leaf ethyl acetate extract leaf; 1 = *A. hydrophilia* (clinical isolate); 2 = *A. hydrophilia* (ATCC33304); 3 = *A. faecalis*; 4 = *A. hydrophilia*; 5 = *C. freundii*; 6 = *E. aerogenes*; 7 = *E. coli*; 8 = *K. pneumoniae* (clinical isolate); 9 = *K. pneumoniae* (ATCC31488); 10 = *P. mirabilis* (clinical isolate); 11 = *P. mirabilis* (ATCC21721); 12 = *P. vulgaris* (ATCC21719); 13 = *P. aeruginosa* (clinical isolate); 14 = *P. aeruginosa* ATCC39324); 15 = *P. fluorescens*; 16 = *S. newport*; 17 = *S. marcescens*; 18 = *S. sonnei*; Amp = ampicillin (2 µg) control. Results are expressed as mean zones of inhibition ± SEM.

iting the growth all of the Gram-negative bacteria tested, although the measured zones of inhibition against most were relatively low (below 8 mm for all bacterial species). The most potent growth inhibition was seen for the berry ethyl acetate extracts against *S. sonnei* (inhibition zone of 9.6 ± 0.6 mm). Indeed, the berry ethyl acetate extract was generally more effective than the berry methanol and water extracts against the majority of bacteria that it inhibited, despite extracting much less material. The leaf extracts were generally not as effective as the berry extracts, with 3 bacterial strains (*E. aerogenes, P. aeruginosa* (clinical isolate) and *P. aeruginosa* reference strain (ATCC39324)) growing in the presence of all leaf extracts.

Gram-positive bacterial growth was also inhibited by both berry and leaf extracts, although the range of bacteria was more limited than for Gram-negative bacteria (Figure 3). Four of the 5 Gram-positive bacterial species (80%) were

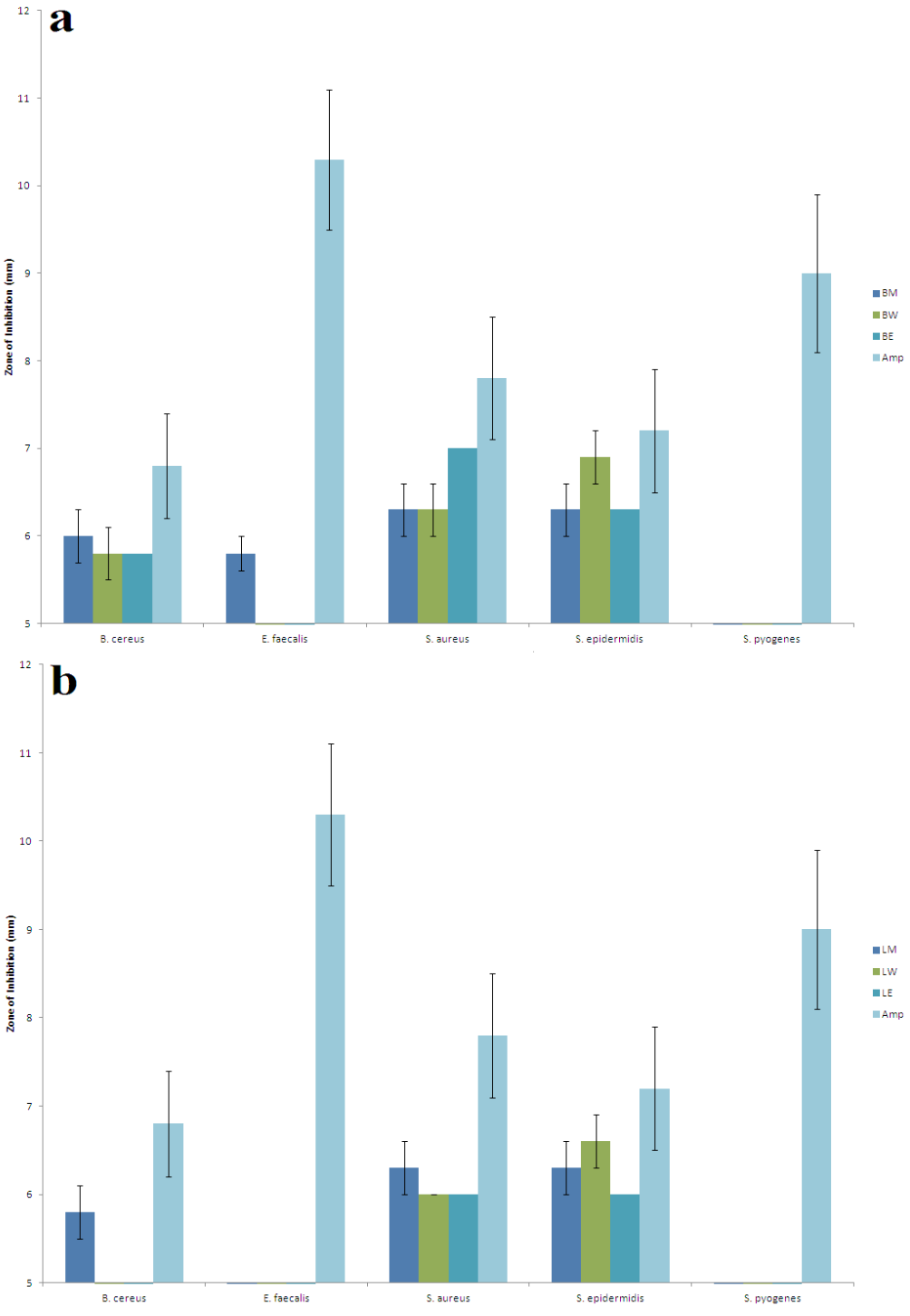

Figure 3. Antibacterial activity of (a) *T. stipitata* berry and (b) *T. stipitata* leaf extracts against Gram-positive bacteria measured as zones of inhibition (mm). BM = *T. stipitata* berry methanolic extract; BW = *T. stipitata* berry water extract; BE = *T. stipitata* berry ethyl acetate extract leaf; LM = *T. stipitata* leaf methanolic extract; LW = *T. stipitata* leaf water extract; LE = *T. stipitata* leaf ethyl acetate extract leaf; Amp = ampicillin (2 µg) control. Results are expressed as mean zones of inhibition ± SEM.

inhibited by at least 1 berry extract compared to 3 bacteria (60%) inhibited by at least 1 leaf extract. *S. aureus* and *S. epidermidis* were the most susceptible of the Gram-positive bacteria, being inhibited by the methanol, water and ethyl acetate of both the berries and leaves. In contrast, *S. pyogenes* growth was not inhibited by any of the *T. stipitata* extracts. The berry was more versatile than the leaf at inhibiting Gram-positive bacterial growth, as determined by the number of susceptible bacteria. It is noteworthy that whilst the *T. stipitata* extracts inhibited the growth of a broad panel of both Gram-positive and Gram-negative bacteria, the relatively small zones of inhibition indicate only low to moderate efficacy.

Fungal growth was less susceptible to the *T. stipitata* extracts than was bacterial growth (as determined by zones of inhibition) (Figure 4). *A. niger* was the most resistant, growing in the presence of all of the extracts tested.

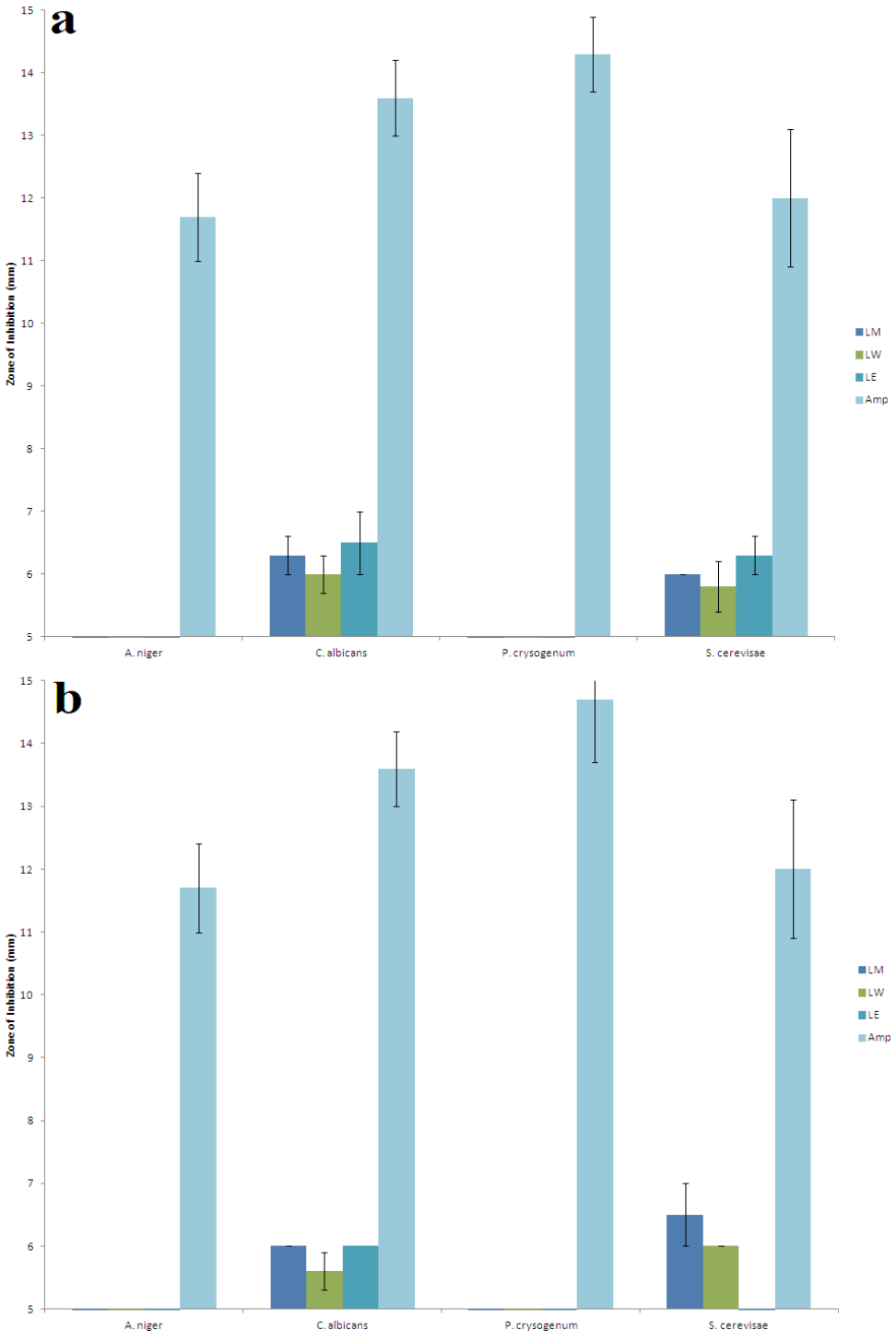

Figure 4. Inhibitory activity of (a) *T. stipitata* berry and (b) *T. stipitata* leaf extracts measured as zones of inhibition (mm) against fungal species. BM = *T. stipitata* berry methanolic extract; BW = *T. stipitata* berry water extract; BE = *T. stipitata* berry ethyl acetate extract leaf; LM = *T. stipitata* leaf methanolic extract; LW = *T. stipitata* leaf water extract; LE = *T. stipitata* leaf ethyl acetate extract leaf; Nys = nystatin (100 µg) control. Results are expressed as mean zones of inhibition ± SEM.

This species was a particularly resistant strain, also growing in the presence of ampicillin (unpublished results). *P. chrysogenum* was also resistant to the *T. stipitata* extracts, although its growth was not inhibited by the ampicillin control (unpublished results). In contrast, *C. albicans* and *S. cerevisiae* were inhibited by 6 (60%) and 5 (50%) of the 10 plant extracts respectively.

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 to moderate concentrations, with MIC values against the susceptible bacterial and fungal species generally less than 2000 μg/ml (< 20 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 87 μg/ml (0.9 μg impregnated into the disc) for the berry water extract. Similarly low *P. mirabilis* MIC values were also seen for several other extracts. Interestingly, whilst the inhibition zone studies indicated low antifungal efficacy, lower MIC values (generally <2000 μg/ml) were

Table 2: Minimum inhibitory concentration (μg/ml) of T. stipitata extracts against susceptible microbial species.

	Microbial Species	Berry			Leaf		
		BM	MW	BE	LM	LW	LE
Gram negative bacteria	***A. baylii***	1163	1584	2238	1374	1486	1995
	***A. baylii* (ATCC33304)**	1192	1640	2481	1423	1545	2218
	A. faecalis	464	387	134	942	1470	2085
	A. hydrophilia	873	447	628	1135	983	824
	C. freundi	1427	1888	1061	855	1227	1320
	E. aerogenes	947	891	583	-	-	-
	E. coli	747	689	552	942	1089	1277
	K. pneumoniae	423	298	169	658	391	327
	***K. pneumoniae* (ATCC31488)**	450	336	124	523	480	401
	P. mirabilis	154	87	268	297	228	375
	***P. mirabilis* (ATCC21721)**	289	163	307	362	247	380
	***P. vulgaris* (ATCC21719)**	177	298	406	429	397	556
	P. aeruginosa	1218	2247	985	-	-	-
	***P. aeruginosa* (ATCC39324)**	1044	1730	1554	-	-	-
	P. fluorescens	1528	1893	687	1735	-	2880
	S. newport	-	-	487	-	-	1369
	S. marcenscens	1218	897	2394	1525	1678	1849
	S. sonnei	271	325	86	873	1559	906
Gram positive bacteria	***B. cereus***	870	1447	1320	1347	-	-
	E. faecalis	1939	-	-	-	-	-
	S. aureus	1165	947	783	1484	2076	1832
	S. epidermidis	1437	527	1280	1983	1166	1550
Fungi	***C. albicans***	980	1427	692	1885	3417	2178
	S. cerevisae	1338	1763	822	783	1127	-

Numbers indicate the mean MIC values of at least triplicate determinations. - indicates no growth inhibition. BM = T. stipitata berry methanolic extract; BW = T. stipitata berry water extract; BE = T. stipitata berry ethyl acetate extract ; LM = T. stipitata leaf methanolic extract; LW = T. stipitata leaf water extract; LE = T. stipitata leaf ethyl acetate extract.

determined for the fungi susceptible to the berry and leaf extracts, indicating their moderate inhibitory activity.

Anti-Giardial Activity

T. stipitata fruit extracts were screened for their ability to inhibit *G. duodenalis* growth by assessing the effects on metabolic rates in an MTS based spectrometric assay (Figure 5a), and by direct counts of *G. duodenalis* trophozoite numbers (Figure 5b). Similar proliferation inhibition levels were determined in both assays. The methanol, water and ethyl acetate extracts displayed significant inhibitory activity. The berry water and leaf methanol extracts were particularly potent, inhibiting 100% of the Giardial growth (compared to the untreated control) when tested undiluted in the MTS spectrometric assay. Similarly, approximately 95% inhibition of proliferation

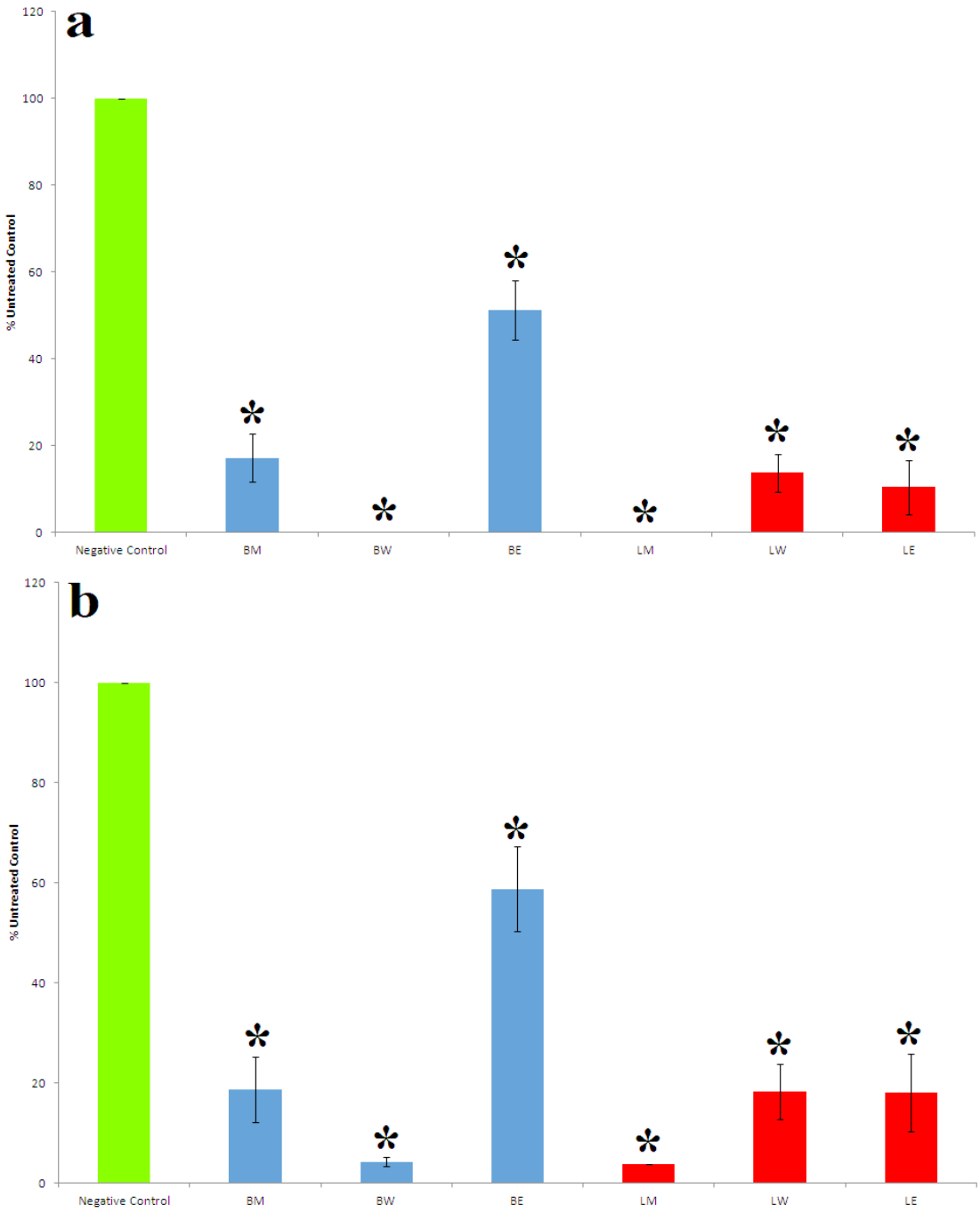

Figure 5. Inhibitory activity of *T. stipitata* fruit extracts against *Giardia duodenalis* trophozoites measured as a percentage the untreated control by (a) MTS based spectrometric assay; (b) direct trophozoite counts. Results are expressed as mean ± SEM of at least triplicate determinations. * indicates results that are significantly different to the untreated control ($p<0.01$).

was determined by the direct enumeration assay method for these extracts. The other methanol, water and ethyl acetate extracts were also very effective at inhibiting trophozoite growth (generally inhibiting more than 80% of the growth of the negative controls in both assays).

T. stipitata extracts were further tested over a range of concentrations to determine the IC50 values (Table 3) for each extract against *G. duodenalis*. Inhibition of trophozoite growth was dose-dependent, with the level of inhibitory activity decreasing at lower concentrations. Interestingly, whilst the level of inhibition decreased for the methanol, water and ethyl acetate extracts in a dose-dependent manner, all concentrations still significantly inhibited *G. duodenalis* trophozoite growth at the lowest concentration tested (100 µg/ml). The berry water extract as well as the leaf methanol and ethyl acetate extracts was particularly good inhibitors of *G. duodenalis* proliferation, with an IC50 ≤ 350 µg/ml. The other extracts, whilst less potent, also displayed good anti-Giardial activity (generally <1000 µg/ml).

Quantification of Toxicity

T. stipitata berry and leaf extracts were initially screened at 2000 µg/ml in the assay (Figure 6). For comparison, the reference toxin potassium dichromate (1000 µg/ml) was

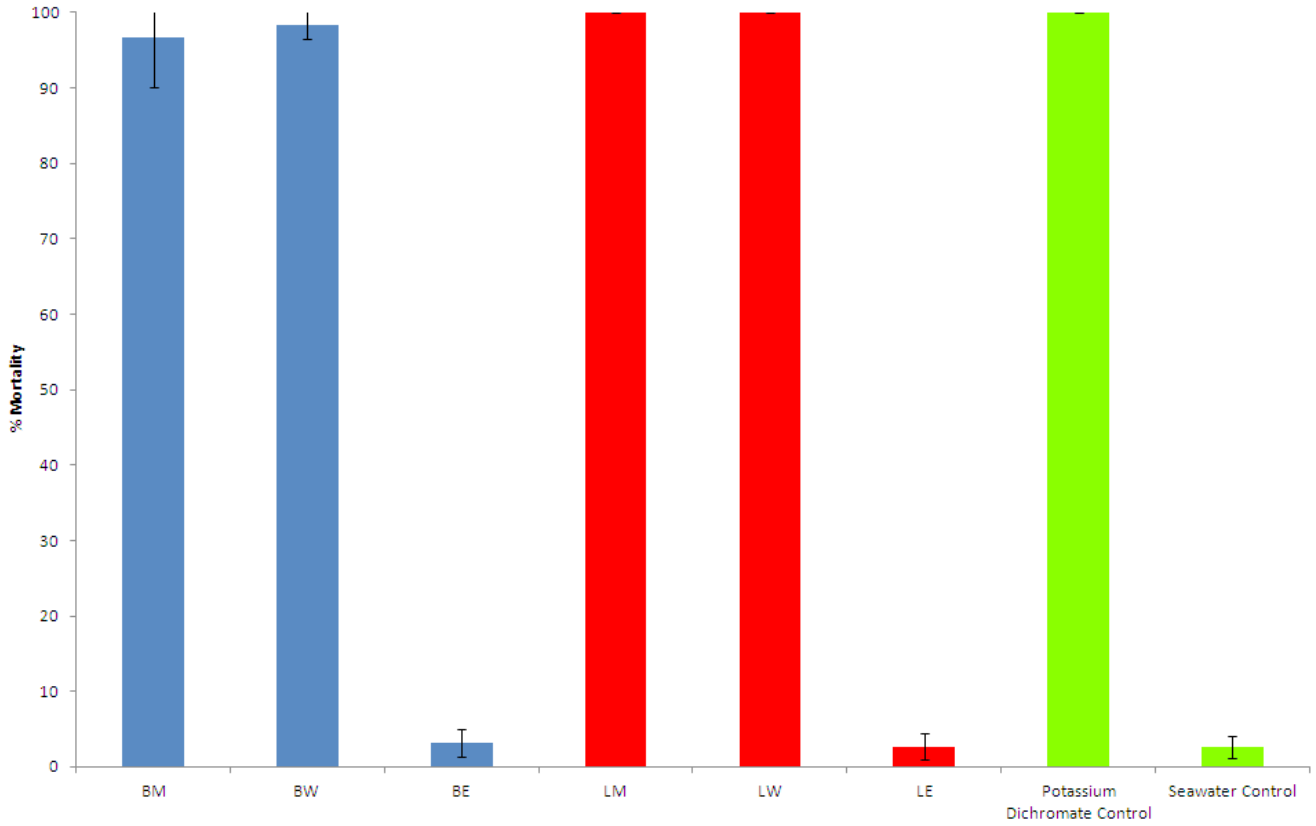

Figure 6. The lethality of *T. stipitata* extracts (2000 µg/mL) and the potassium dichromate control (1000 µg/mL) towards Artemia nauplii following 24 hours exposure. BM = *T. stipitata* berry methanolic extract; BW = *T. stipitata* berry water extract; BE = *T. stipitata* berry ethyl acetate extract leaf; LM = *T. stipitata* leaf methanolic extract; LW = *T. stipitata* leaf water extract; LE = *T. stipitata* leaf ethyl acetate extract leaf. All tests were performed in at least triplicate and the results are expressed as mean ± SEM.

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 4–5 hours (unpublished results). Similarly, the *T. stipitata* methanol and water extracts displayed mortality rates significantly elevated above those of the artificial seawater negative control at 24h.

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 24 hours.

Table 3 shows the LC50 values of the *T. stipitata* extracts towards *A. franciscana*. No LC50 values are reported for the berry and leaf ethyl acetate extract as less than 50% mortality was seen for all concentrations tested. All extracts displayed low toxicity (generally much greater than 1000 µg/ml) following 24 h exposure. Extracts with an LC50 of greater than 1000 µg/ml towards *Artemia* nauplii have been defined as being nontoxic.[38] Therefore all *T.stipitata* extracts are considered to be nontoxic.

Table 3: The IC50 values (µg/ml) of *G. duodenalis* and the LC50 values (µg/ml) for *Artemia franciscana* nauplii exposed to *T. stipitata* leaf and berry extracts.

Extract	LC50 (µg/ml)		IC50 (µg/ml)	
	24 h	48 h	MTS spectrometric Giardial determination	Direct cell counts
***T. stipitata* fruit methanol**	1667	892	789	847
***T. stipitata* fruit water**	1130	731	205	373
***T. stipitata* fruit ethyl acetate**	-	-	1029	1241
***T. stipitata* leaf methanol**	1313	838	128	206
***T. stipitata* leaf water**	1287	744	773	904
***T. stipitata* leaf ethyl acetate**	-	-	350	378
Potassium dichromate	238	52	ND	ND

Numbers indicate the mean LC50 or IC50 values of triplicate determinations. - indicates that LC50 or IC50 values were not obtained as the% mortality did not exceed 50% at any dose tested. ND = not determined.

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. stipitata* berry and leaf extracts, and on their toxicity. The Gram-negative bacteria tested in this study demonstrated greater susceptibilities towards the *T. stipitata* extracts than did the Gram-positive bacteria. This is in contrast to previous studies which have reported a greater susceptibility of Gram-positive bacteria towards solvent extracts for South American,[39] African[40] and Australian plant extracts,[41,42] although other examples of plants having a greater effect on Gram-negative bacteria have also been reported.[17,24]

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.[43] *B. cereus* and *B. subtilis*,[44] *E. coli*,[45] *C. freundii*[46] and *K. pneumoniae*[46] 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* releases factors that stimulate histamine production resulting in gastrointestinal, neurological (palpitations, headaches, itching), cutaneous (hives, rash) and hypertension symptoms.[47] 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.[43] *S. aureus* and *S. epidermidis* were each inhibited by all of the berry and leaf methanol, water and ethyl acetate extracts tested. Most of these extracts displayed moderate activity with MIC values generally < 2000 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. stipitata* methanol, water and ethyl acetate extracts, even at low concentrations (<500 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 *P. mirabilis* inhibition.[48]

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*).[44-47] Therefore, food based antibacterial agents with inhibitory activity against psychrotrophic bacteria are especially useful. All of the psychrotrophic bacteria tested in this study were inhibited by nearly all of the methanol, water and ethyl acetate *T. stipitata* extracts. The *T. stipitata* berry extracts were generally stronger and more versatile inhibitors of the psychrotrophic bacteria associated with spoilage than were the leaf extracts, based on the MIC's and the number of psychrotrophic bacteria inhibited. Indeed, all *T. stipitata* berry extracts blocked the growth of every psychrotrophic bacterial species tested. Furthermore, these extracts generally displayed low MIC values (as low as 387 μg/ml for the berry water extract against *A. faecalis*), 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. stipitata* extracts (with the exception of the leaf ethyl acetate extract) demonstrated moderate to 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. stipitata* 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.[43] Future studies into the effects of *T. stipitata* 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.[43] *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.[43] Other fungi including Candida species can readily grow in high acid, salt or sugar environments so are not controlled by many common preservation methods.[43] Furthermore, some fungal genera (e.g. *Aspergillus*) produce mycotoxins (e.g. *A. flavus* produces aflatoxin) which cause serious food poisoning.[43] 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. stipitata* extracts proved to be moderate inhibitors of *C. albicans* and *S. cerevisiae* growth. 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.

Significant growth inhibition of the food/water borne gastrointestinal parasite *Giardia duodenalis* was also noted for the *T. stipitata* extracts in our study. Giardial infection (giardiasis) is a re-emerging disease which afflicts large numbers of individuals worldwide, with higher incidence in countries with poorer socio-economic conditions, inadequate sanitary conditions, untreated water

supplies and poor diet.[49] Whilst generally not fatal, giardiasis results in debilitating symptoms including bloating, diarrhoea, excess gas, loss of appetite, loose and watery stool, stomach cramps and haematuria. Currently, there are only a narrow range of drugs effective against giardiasis, including quinalones and imidazole derivatives. None of these drugs is ideal as they produce unpleasant side effects including nausea, vertigo, vomiting, diarrhoea and hallucinations.[49,50] Furthermore, increasing reports of the failure of current treatments to address this disease indicates a developing drug resistance of several Giardia species.[50] Our studies demonstrate that *T. stipitata* possesses anti-Giardial activity and thus may also be useful in the prevention and treatment of Giardisis.

Individual extract components responsible for the antimicrobial potential of the plant extracts were not identified in the current study. However, various compounds have been previously identified in extracts and oils of the related species *T. lanceolata*.[51] 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.[51] It is likely that these components may also be present in the *T. stipitata* extracts and may 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. stipitata* extracts.

The findings reported here also demonstrate that none of the *T. stipitata* berry and leaf extracts displayed significant toxicity towards *Artemia franciscana* nauplii. Previously, compounds with an LC50 of greater than 1000 µg/mL towards *Artemia* nauplii have been defined as being nontoxic.[38] None of the extracts tested displayed LC50 values less than 1000 µg/mL. It was therefore determined that all *T. stipitata* extracts examined in this study were nontoxic.

CONCLUSION

In conclusion, the results of this study demonstrate the potential of *T. stipitata* 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. stipitata* towards infective diseases. 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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