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Original Article

An upscaled extraction protocol for *Tasmannia lanceolata* (Poir.) A.C. Sm.: Anti-bacterial, anti-Giardial and anticancer activity

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ABSTRACT

Background: *Tasmannia lanceolata* is an endemic Australian plant with a high anti-oxidant capacity. Liquid solvent extractions of *T. lanceolata* inhibit bacterial growth and block proliferation of several carcinomas and the gastrointestinal parasite *Giardia duodenalis*. Despite these promising therapeutic properties, methods for the rapid extraction of large quantities of *T. lanceolata* are lacking. This study aimed to develop a rapid supercritical extraction method to produce extracts which retain therapeutic propertyes and phytochemistry characteristics. **Materials and Methods:** *T. lanceolata* fruit and leaf were extracted by both solvent maceration extraction and supercritical fluid extraction (SFE). The extracts were tested for the ability to inhibit bacterial and *G. duodenalis* growth. Inhibition of CaCo2 and HeLa cancer cells was evaluated using MTS-based colorimetric cell proliferation assays. Toxicity was evaluated using an *Artemia franciscana* nauplii bioassay and GC-MS headspace analysis was used to evaluate phytochemical similarity between the extracts. **Results:** *T. lanceolata* berry and leaf SFEs displayed strong bacterial growth inhibitory activity against bacterial triggers of autoimmune inflammatory diseases, with efficacies similar to the smaller scale liquid solvent extractions. The growth inhibition of the berry SFE was particularly noteworthy against *P. mirabilis* and *K. pneumoniae*, with MIC values of approximately 160 and 190 µg/mL, respectively. The berry and leaf SFE extracts also had similar antiproliferative potencies against *G. duodenalis* (492 and 375 µg/mL, respectively), CaCo2 (4133 and 3347 µg/mL, respectively) and HeLa carcinomas (2652 and 3497 µg/mL, respectively) to those determined for the corresponding liquid solvent extractions. GC-MS analysis of the berry SFE revealed similar terpenoid components and similar abundances to those in liquid solvent berry extraction. Furthermore, all SFEs were either non-toxic or of only low toxicity in the *Artemia franciscana* toxicity assay. **Conclusion:** The *T. lanceolata* SFE retained the tested therapeutic properties, were non-toxic and had similar phytochemical profiles as smaller scale liquid solvent extractions. Thus, SFE is a viable method of rapidly extracting large masses of *T. lanceolata* plant material to produce quality extracts which retain therapeutic properties.

Key words: Tasmanian pepper, Mountain pepper berry, Supercritical fluid extraction, Antibacterial activity, *Giardia duodenalis*, Anti-proliferative activity, Anti-cancer activity, Polygodial.

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INTRODUCTION

Tasmannia lanceolata (Poir.) A.C. Sm.(family Winteraceae), commonly known as Tasmanian pepper or mountain pepper berry, is a medium sized shrub which is endemic to the woodlands and cool temperate rainforests of Tasmania and the south-eastern region of the Australian mainland. As with many of the other Winteraceae species, *T. lanceolata* berries, leaves and bark have traditional uses as a food and as a medicinal plant.[1] Australian Aborigines used *T. lanceolata* as a therapeutic agent to treat stomach disorders and as an emetic, as well as general usage as a tonic.[2,3] Reports also exist of the use of *T. lanceolata* by the first Australians for the treatment and cure of skin disorders, venereal diseases, colic, stomach ache and as a quinine substitute.[2] Furthermore, recent studies have reported an exceptionally high antioxidant capacity for *T. lanceolata* leaves and berries.[4-6] Indeed, total ferric reducing antioxidant powers (FRAP) and DPPH reduction activities of both the *T. lanceolata* leaf and berry extracts were reported to be substantially higher than those determined for a blueberry control.

Based on its high antioxidant capacity, the medicinal potential of *T. lanceolata* has received much recent attention (Table 1). *T. lanceolata* leaf and berry extracts have been reported to have a wide variety of bioactivities which would confer therapeutic potential. The bacterial growth inhibitory activity of *T. lanceolata* has been particularly well reported. *T. lanceolata* leaf and berry extracts inhibit the growth of a broad panel of bacteria which cause food spoilage and/or cause gastrointestinal distress, diarrhoea and dysentery.[7] That study reported potent growth inhibitory activity, with MIC values generally <1000 µg/mL. Indeed, MIC values as low as 5 µg/mL were determined against some bacterial species. Both the leaf and berry were potent growth inhibitors and the growth of both Gram positive and Gram negative bacteria were inhibited. Furthermore, both mesophilic and psychrotropic bacteria were affected by the *T. lanceolata* extracts, as were spore forming bacteria. The extracts were similarly potent inhibitors of the growth of bacteria associated with skin diseases (*Staphylococcus aureus, Staphylococcus epidermidis, Streptococcus pyogenes*).[7] *T. lanceolata* extracts have even been reported to be effective inhibitors of the growth of the bacteria which cause anthrax (*Bacillus anthricis*)[8] and gas gangrene (*Clostridium perfringens*).[9] Both of these bacteria are endospore formers and are generally considered to be difficult to treat and decontaminate.

The potential of *T. lanceolata* leaf extract in treating inflammation has also been reported.[10] That study reported that exposure of RAW264.7 murine macrophages to *T. lanceolata* leaf extract induces a reduction in levels of pro-inflammatory enzymes, COX-2 and iNOS, and thus a reduction in inflammation. Other recent studies have also highlighted the potential of *T. lanceolata* extracts in inhibiting the initiating events of selected autoimmune inflammatory diseases and thus their potential in the prevention and treatment of rheumatoid arthritis, ankylosing

Table 1: Selected disease models and the drug targets that *T. lanceolata* extracts have been studied against.

Disease	Target/Test System	Plant Product Tested	Comments/Phytocompounds/ Mechanisms	References
Chronic oxidative disease	Various antioxidant enzymes: Oxygen radical scavenging, ferric radical reducing antioxidant power, TEAC evaluation and ABTS reduction	Solvent extractions	Ascorbic acid, anthocyanins, chlorogenic acid, caffeic acid, rutin, quercetin	4-6
Food poisoning, diarrhoea, dysentry	A broad panel of bacterial species associated with food spoilage (including mesotrophs and psyschrotrophs), food poisoning, diarrhoea and dysentery.	Solvent extractions of peppercorn, berry and leaf	Polyphenolic compounds, flavonoids, saponins, terpenoids	7,9
Skin diseases	*Staphylococcus aureus, Staphylococcus epidermidis, Streptococcus pyogenes*	Solvent extractions of peppercorn, berry and leaf	Polyphenolic compounds, flavonoids, saponins, terpenoids	7
Inflammation	RAW264.7 murine macrophages, COX-1, COX-2, iNOS, PGE2	Leaf solvent extractions	Leaf extracts induced a reduction in levels of pro-inflammatory enzymes, COX-2, iNOS.	10
Chronic autoimmune inflammatory diseases (rheumatoid arthritis, rheumatic fever, ankylosing spondylitis, multiple sclerosis)	*Proteus mirabilis* and *Proteus vulgaris* (triggers of rheumatoid arthritis), *Klebsiella pneumoniae* (trigger of ankylosing spondylitis), *Actinitobacterbaylyi* and *Pseudomonas aeruginosa* (triggers of multiple sclerosis), *Streptococcus pyogenes* (trigger of rheumatic fever)	Solvent extractions of peppercorn, berry and leaf	Stilbenes (including piceid and combretastatins) were highlighted.	7, 11-13
Cancer	HepG2 hepatocarcinoma cells, HT-29 colon carcinoma cells, AGS stomach carcinoma cells, BL13 bladder carcinoma cells were analysed for antiproliferative activity in an MTS based assay.	Leaf solvent extractions	Exposure to the high antioxidant extracts induced a significant increase in apoptosis in HepG2 cells and blocked proliferation in all other carcinoma cell lines	15
Giardiasis	*Giardia duodenalis*	Solvent extractions of berry and leaf	Stilbenes including combretastatins, multiple tannins, monoterpenoids, sesquiteriterpenoids (polygodial was highlighted)	14
Anthrax	*Bacillus anthricis*	Solvent extractions of berry and leaf	Multiple tannins, monoterpenoids, sesquiteriterpenoids (polygodial was highlighted), stilbenes including combretastatins	8
Gas gangrene	*Clostridium perfringens*	Solvent extractions of berry and leaf	Polygodial, cineole, linalool, terpineol, carophyllene oxide	9

spondylitis and multiple sclerosis.[7,11-13] Thus, it is likely that *T. lanceolata* extracts have pluripotent effects against inflammation and inflammatory diseases and thus they may be particularly useful therapeutic agents against these diseases.

The inhibitory properties of *T. lanceolata* extracts against the growth of several eukaryotic cells have also been reported. Berry and leaf *T. lanceolata* extractions are potent inhibitors of *Giardia duodenalis* growth[14] and thus have potential in the treatment of giardiasis. Furthermore, leaf extracts have anti-cancer activity against a panel of carcinoma cell lines.[15] That study examined the effect of *T. lanceolata* leaf extract on HepG2 hepatocarcinoma cells, HT-29 colon carcinoma cells, AGS stomach carcinoma cells and BL13 bladder carcinoma cell lines. A significant increase in apoptosis was reported in HepG2 cells, whilst the leaf extract blocked proliferation in all other carcinoma cell lines. Whilst much more work is required to further elucidate the anticancer mechanisms and the bioactive phytocompounds involved, the study did indicate very good anti-cancer potential for *T. lanceolata* leaves.

Several recent reports have examined the phytochemical composition of *T. lanceolata* extracts.[8,9,11] These phytochemical evaluations have utilised LC-MS techniques to examine polar and mid-polarity compounds in leaf and berry extracts.[11] Similarly, the lower polarity compounds have been detected and identified using GC-MS techniques.[8,9] Irrespective of the technique used, a common feature of *T. lanceolata* composition is the high abundance of the sesquiterpenoid polygodial (Figure 1a). Indeed, some studies report polygodial levels as high as may account for nearly 40% of some *T. lanceolata* preparations.[16] Further terpenoids including cineole (Figure 1b), eugenol (Figure 1c), linalool (Figure 1d) and terpineol (Figure 1e) have also been reported in relative abundance in *T. lanceolata* extracts.[8,9,16]

Whilst conventional laboratory extraction has been useful in initial studies for screening *T. lanceolata* berries and leaves for therapeutic properties, upscaling the extraction to produce commercially relevant levels would use large amounts of expensive solvent and be time consuming to remove the solvents by drying. Furthermore, there are very real environmental pollution concerns associated with large scale usage of organic

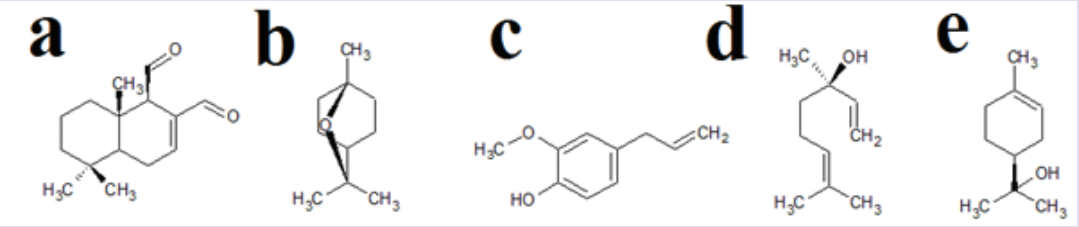

Figure 1: Chemical structures of selected terpenoid molecules identified in *T. lanceolata* berry extracts:(a) polygodial, (b) cineole, (c)eugenol, (d) linalool, (e) terpineol.

solvents. Up-scaled extraction protocols which provide a high yield of *T. lanceolata* phytochemicals are therefore of considerable interest. However, the larger scale extraction procedure would need to produce extracts with similar phytochemical profiles and therapeutic properties as those reported in the laboratory scale pilot studies. Supercritical fluid extraction (SFE) offers a number of advantages compared to conventional liquid extraction for larger scale extract preparation. Significantly less solvent is required, extraction is significantly more rapid than for extraction with an organic solvent and the properties of the supercritical fluid can be readily altered by varying the temperature and pressure, allowing for greater selectivity in extraction. Thus, SFE is ideally suited to the preparation of larger scale extract preparations.

In this study, we describe a preparative scale extraction of *T. lanceolata* berry, leaf and stem material using a SFE method which results in considerably higher extract yields at a reasonable cost. Furthermore, the therapeutic potential of these upscaled extractions was compared to the properties of the laboratory scale extractions by comparing their ability to inhibit the growth of selected bacteria associated with selected autoimmune inflammatory diseases and the growth of the gastrointestinal parasite *Giardia duodenalis*. The anti-cancer activity of *T. lanceolata* extracts was also evaluated against 2 cancer cell lines and the toxicity was determined to evaluate their usefulness as medicinal agents. Furthermore, the phytochemistries of the most promising solvent and supercritical extracts were compared by GC-MS headspace analysis targeting several marker compounds.

MATERIALS AND METHODS

Laboratory scale extraction

T. lanceolata leaf and berry were obtained from Diemen Pepper in Tasmania as a dried product. The dried pieces were subsequently ground into a coarse powder. The powdered plant material was extracted by standardised solvent extraction methods.[7,11] Briefly, an amount of 1 g of powdered plant material was weighed into each of five tubes and five different extracts were prepared by adding 50 mL of methanol, water, ethyl acetate, chloroform, or hexane, respectively. Extraction in each solvent was undertaken for 24 hrs at 4°C with gentle shaking. All solvents were obtained from Ajax, Australia and were AR grade. The extracts were filtered through filter paper (Whatman No. 54) under vacuum, followed by drying by centrifugal evaporation in an Eppendorf concentrator 5301. The resultant dry extract was weighed and redissolved in 10 mL deionised water (containing 1% DMSO).

Sub-critical extraction of *Tasmannia lanceolata* with dimethyl ether (DME).

The ground Tasmanian pepper samples (berry or leaves) were packed into the biomass chamber of the extraction system. The system was sealed and evacuated before the plant material in the biomass chamber was covered with compressed solvent (dimethyl ether). The compressed gas was cycled repeatedly across the plant material for 20 min. Sub-critical DME extraction was carried out at room temperature and at a pressure of 500 MPa. The solvent was recycled and stored in a solvent reservoir. After the compressed gas (the dimethyl ether) had been removed, the material that had been extracted from the plant biomass was collected in a separate vessel.

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.[17-19]

Antibacterial screening

Test microorganisms

All media was supplied by Oxoid Ltd. Australia. Reference strains of *Acinitobacter baylyi* (ATCC33304), *Klebsiella pneumoniae* (ATCC31488), *Proteus mirabilis* (ATCC21721), *Proteus vulgaris* (ATCC21719) and *Pseudomonas aeruginosa* (ATCC39324) were purchased from American Tissue Culture Collection, USA. All other clinical microbial strains were obtained from the School of Natural Sciences teaching laboratory, Griffith University. All stock cultures were subcultured and maintained in nutrient broth at 4°C.

Evaluation of antimicrobial activity

Antimicrobial activity of all plant extracts was determined using a modified disc diffusion method.[20-22] Briefly, 100 µL of the test bacteria were grown in 10 mL of fresh nutrient broth until they reached a count of approximately 10^8 cells/mL as determined by direct microscopic determination. One hundred microliters of microbial suspension was subsequently spread onto the agar plates. The extracts were applied onto 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 hrs before incubation with the test microbial agents. The plates were then incubated at 30°C for 24 hrs and the diameters of the inhibition zones were measured in millimetres. All measurements were to the closest whole millimetre. Each antimicrobial assay was performed in at least triplicate and mean values were determined. Standard discs of ampicillin (10 µg) were obtained from Oxoid Ltd. Australia and served as positive controls. Filter discs impregnated with 10 µL of distilled water were used as negative controls.

Minimum inhibitory concentration (MIC) determination

The minimum inhibitory concentration (MIC) of the *T. lanceolata* extracts were determined by the disc diffusion method across a range of doses.[23] The plant extracts were diluted in deionised water across a

concentration range of 5 mg/mL to 0.1 mg/mL. 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 reference *Giardia duodenalis* trophozoite strain (ATTC203333) used in this study was purchased from American Tissue Culture Collection, USA. *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 1×10^5 trophozoites for each passage.

Evaluation of anti-Giardial activity

Anti-Giardial activity of the *T. lanceolata* extracts was assessed by direct enumeration of parasite numbers in the presence or absence of extract.[14,22] For each test, aliquots of the trophozoite suspension (70 µL) containing approximately 1×10^5 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 hrs 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 IC_{50} values against Giardial trophozoites

For IC_{50} determinations, the plant extracts were tested by the direct enumeration method 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 IC_{50} values.

Screen for anti-cancer bioactivity

Cancer cell lines

The CaCo2 and HeLa carcinoma cell lines used in this study were obtained from American Type Culture Collection (Rockville, USA). The cells were cultured in Roswell Park Memorial Institute (RPMI) 1640 medium (Life Technologies), supplemented with 20 mM HEPES, 10 mM sodium bicarbonate, 50 µg/ml streptomycin, 50 IU/mL penicillin, 2 mM glutamine and 10% foetal calf serum (Life Technologies). The cells were maintained as monolayers in 75 mL flasks at 37°C, 5% CO_2 in a humidified atmosphere until approximately 80% confluent.

Evaluation of cancer cell anti-proliferative activity

Evaluation of the antiproliferative activity of the *T. lanceolata* extracts was as previously described.[4,21] Briefly, 1 mL of trypsin (Sigma) was added to the culture flasks and incubated at 37°C, 5% CO_2 for 15 min to dislodge the cancer cells. The cell suspensions were then transferred to a 10 mL centrifuge tube and sedimented by centrifugation. The supernatant was discarded and the cells were resuspended in 9 mL of fresh media. Aliquots of the resuspended cells (70 µL, containing approximately 5000 cells) were added to the wells of a 96 well plate. A volume of 30 µL of the test extracts or cell media (for the negative control) was added to individual wells and the plates were incubated at 37°C, 5% CO_2 for 12 hrs in a humidified atmosphere. A volume of 20 µL of Cell Titre 96 Aqueous One solution (Promega) was subsequently added to each well and the plates were incubated for a further 3 hrs. 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:

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

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).

Determination of IC_{50} values against CaCo2 and HeLa carcinoma cells

For IC_{50} determinations, the plant extracts were tested by the Cell Titre 96 colourimetric method 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 IC_{50} values.

Toxicity screening

Reference toxins for biological screening

Potassium dichromate ($K_2Cr_2O_7$) (AR grade, Chem-Supply, Australia) was prepared as a 2 mg/mL solution in distilled water and was serially diluted in synthetic seawater for use in the *A. franciscana* nauplii bioassay.

Artemia franciscana nauplii toxicity screening

Toxicity was tested using a modified *Artemia franciscana* nauplii lethality assay.[24-26] Briefly, *A. franciscana* cysts were obtained from North American Brine Shrimp, LLC, USA (harvested from the Great Salt Lake, Utah). Synthetic seawater was prepared using Reef Salt, AZOO Co., USA. Seawater solutions at 34 g/L distilled water were prepared prior to use. An amount of 1g of *A. franciscana* cysts were incubated in 500 mL synthetic seawater under artificial light at 25°C, 2000 Lux with continuous aeration. Hatching commenced within 16-18 h of incubation. Newly hatched *A. franciscana* (nauplii) were used within 10 h of hatching. Nauplii were separated from the shells and remaining cysts and were concentrated to a suitable density by placing an artificial light at one end of their incubation vessel and the nauplii-rich water closest to the light was removed for biological assays. The extracts and positive control were also serially diluted in artificial seawater for LC_{50} determination. A volume of 400 µL of seawater containing approximately 46 (mean 45.8, n=125, SD 12.3) nauplii were added to wells of a 48-well plate and immediately used for bioassay. The plant extracts were diluted to 4 mg/mL in seawater for toxicity testing, resulting in a 2 mg/mL concentration in the bioassay. A volume of 400 µL of diluted plant extract 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 at least 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 moribund if no movement of the appendages was

Table 2: The mass of dried extracted material, the concentration after resuspension in deionised water (mg/mL) and qualitative phytochemical screenings of the *T. lanceolata* extracts.

Plant Species	Extract	Mass of Dried Extract (mg)	Concentration of Resuspended Extract (mg/mL)	Total Phenolics	Water Soluble Phenolics	Water Insoluble Phenolics	Cardiac Glycosides	Saponins	Triterpenes	Phytosteroids	Alkaloids (Mayer Test)	Alkaloids (Wagner Test)	Flavonoids	Tannins	Free Anthraquinones	Combined Anthraquinones
Berry	M	171	17.1	+++	+++	+++	-	++	+	-	-	-	+++	++	-	-
	W	111	11.1	+++	+++	+++	-	-	-	-	-	-	+++	++	-	-
	E	57	5.7	+	+	++	-	+	++	-	-	-	++	+	-	-
	C	87	8.7	-	-	-	-	-	+	-	-	-	+	-	-	-
	H	33	3.3	-	-	-	-	-	+	-	-	-	-	-	-	-
Leaf	M	144	14.4	+++	+++	+++	-	+++	+	-	-	-	+++	++	-	-
	W	134	13.4	+++	+++	+++	-	++	-	-	-	-	+++	++	-	-
	E	47	4.7	+	+	++	-	-	+	-	-	-	++	+	-	-
	C	53	5.3	-	-	-	-	-	-	-	-	-	+	-	-	-
	H	38	3.8	-	-	-	-	-	-	-	-	-	-	-	-	-
Stem	M	85	8.5	+++	++	+	-	+	++	-	-	-	+	++	-	-
	W	57	5.7	+++	++	+	-	++	+	-	-	-	+	++	-	-
	E	32	3.2	++	+	+	-	-	++	-	-	-	+	+	-	-
	C	40	4.0	-	-	-	-	-	+	-	-	-	-	-	-	-
	H	31	3.1	-	-	-	-	-	-	-	-	-	-	-	-	-
Supercritical extracts	B	ND	23.3	+++	+++	++	-	++	+	-	-	-	+++	++	-	-
	L	ND	16.8	+++	+++	++	-	++	+	-	-	-	+++	+++	-	-
	S	ND	10.1	+++	+++	+	-	+	-	-	-	-	++	++	-	-

+++ indicates a large response; ++ indicates a moderate response; + indicates a minor response; - indicates no response in the assay. M=methanolic extract; W=aqueous extract; E=ethyl acetate extract; C=chloroform extract; H=hexane extract; B=berry; L=leaf; S=stem; ND=not determined.

observed within 10 sec. After 48 h, all nauplii were sacrificed and counted to determine the total number per well. The LC_{50} with 95% confidence limits for each treatment was calculated using probit analysis.

Non-targeted GC-MS head space analysis

Separation and quantification of phytochemical components were performed using a Shimadzu GC-2010 plus (USA) linked to a Shimadzu MS TQ8040 (USA) mass selective detector system as previously described.[27] Briefly, the system was equipped with a Shimadzu auto-sampler AOC-5000 plus (USA) fitted with a solid phase micro-extraction fibre (SPME) handling system utilising a Supelco (USA) divinyl benzene/carbowax/polydimethylsiloxane (DVB/CAR/PDMS). Chromatographic separation was accomplished using a 5% pheny l, 95% dimethylpolysiloxane (30 m×0.25 mm id×0.25 µm) capillary column (Restek USA). Helium (99.999%) was employed as a carrier gas at a flow rate of 0.79 mL/min. The injector temperature was set at 230°C. Sampling utilised a

SPME cycle which consisted of an agitation phase at 500 rpm for a period of 5 sec. The fibre was exposed to the sample for 10 min to allow for absorption and then desorbed in the injection port for 1 min at 250°C. The initial column temperature was held at 30°C for 2 min, increased to 140°C for 5 min, then increased to 270°C over a period of 3 mins and held at that temperature for the duration of the analysis. The GC-MS interface was maintained at 200°C with no signal acquired for a min after injection in split-less mode. The mass spectrometer was operated in the electron ionisation mode at 70eV. The analytes were then recorded in total ion count (TIC) mode. The TIC was acquired after a min and for duration of 45 mins utilising a mass range of 45-450 m/z.

Statistical analysis

Data are expressed as the mean ± SEM of at least three independent experiments. One way ANOVA was used to calculate statistical significance between control and treated groups with a P value < 0.01 considered to be statistically significant.

RESULTS

Liquid extraction yields and qualitative phytochemical screening

Extraction of 1 g of dried plant material with various solvents yielded dried plant extracts ranging from approximately 31 mg (*T. lanceolata* stem hexane extract) to 171 mg (*T. lanceolata* berry methanolic extract) (Table 2). Water and methanol gave the highest yields of dried extracted material for all *T. lanceolata* plant materials tested, whilst ethyl acetate, chloroform and hexane extracted substantially lower masses. The dried extracts were resuspended in 10 mL of deionised water (containing 1% DMSO) resulting in the extract concentrations shown in Table 2. In contrast, the supercritical extractions of all *T. lanceolata* plant materials generally yielded higher concentration extracts (Table 2).

Phytochemical studies (Table 2) show that methanol and water extracted the widest range and largest amount of phytochemicals of the laboratory scale extractions. The aqueous and methanolic extracts both showed moderate to high levels of total phenolics (water soluble and insoluble phenolics), flavonoids and tannins. The methanolic extract also contained moderate levels of saponins. Similar classes of phytochemicals were detected in the ethyl acetate extract, albeit generally at lower levels than in the aqueous and methanolic extracts. Most of the phytochemical classes were not evident in the chloroform or hexane extracts. Indeed, these extracts only contained detectable levels of triterpenoids. The supercritical extractions displayed similar phytochemical profiles as the laboratory scale aqueous and methanolic extractions. All of the supercritical extractions contained high levels of total phenolics (particularly water-soluble phenolics), flavonoids and tannins. Moderate levels of saponins and low levels of triterpenoids were also generally detected in the supercritical berry, leaf and stem *T. lanceolata* extracts. Thus, qualitative phytochemical analysis indicates that the larger scale supercritical extractions contain similar classes of phytochemical as evident in the laboratory scale extractions which were prepared in the same way as extractions with previously published therapeutic properties (Table 1).[7,11]

Antimicrobial activity

Aliquots (10 µL) of each extract were tested in the disc diffusion assay against bacterial species associated with the induction of rheumatoid arthritis (*Proteus mirabilis*, Figure 2a; *Proteus vulgaris*, Figure 2b), ankylosing spondylitis (*Klebsiella pneumoniae*, Figure 3), multiple sclerosis (*Acinitobacter baylyi*, Figure 4a; *Pseudomonas aeruginosa*, Figure 4b) and rheumatic fever (*Streptococcus pyogenes*, Figure 5). Consistent with previous studies,[11] the methanolic and aqueous berry and leaf extracts were potent inhibitors of reference and clinical strains of *P. mirabilis* (Figure 2a), with zones of inhibition approximately 8-13.5 mm. The leaf extracts were particularly potent, with zones of inhibition of 12.6 ± 0.3 and 12.0 ± 1.0 mm for the methanolic leaf extract (against the reference and clinical strains, respectively) and 12.8 ± 0.4 and 13.2 ± 0.4 mm for the aqueous leaf extract (against the reference and clinical strains, respectively). The aqueous and methanolic berry and leaf extracts were similarly potent growth inhibitors against *P. vulgaris* (Figure 2b). Zones of inhibition of 8.7 ± 0.4 and 12.8 ± 0.4 mm were recorded for the methanolic berry and leaf extracts, respectively. Similarly, growth inhibition zones of 8.0 and 13.2 ± 0.4 mm were seen for the aqueous berry and leaf extracts, respectively. Whilst the berry and leaf ethyl acetate extracts, as well as the aqueous and methanolic stem extracts also inhibited *Proteus* spp. growth, they had lower efficacy (as judged by the zone of inhibition) than evident for the corresponding methanolic and aqueous extracts.

The supercritical extracts also were good inhibitors of *Proteus* spp. growth. The supercritical berry extract was particularly potent, with zones of inhibition of 11.3 ± 0.6 and 10.6 ± 0.6 mm against *P. mirabilis* (reference and clinical strains, respectively), and 9.6 ± 0.3 mm against *P. vulgaris*. The leaf extract was also a good inhibitor of *P. mirabilis* growth, with inhibition zones of 8.7 ± 0.3 (reference strain) and 8.2 ± 0.4 mm (clinical strain). The supercritical leaf extract displayed slightly better efficacy against *P. vulgaris* with a 9.3 ± 0.6 mm zone of inhibition. Thus, the supercritical berry extract had similar efficacy to the small scale laboratory extraction (as judged by zone of inhibition). In contrast, the supercritical leaf extract was a less potent growth inhibitor compared with the smaller scale solvent extraction. As *Proteus* spp. (particularly *P. mirabilis*) are triggers of rheumatoid arthritis in genetically susceptible people,[11,12] these extracts may be useful in the prevention and treatment of this disease.

A similar activity profile was evident for *K. pneumoniae* growth inhibition (Figure 3). The methanolic leaf extract was the most potent growth inhibitor, with zones of inhibition of 9.6 ± 0.6 (reference strain) and 8.3 ± 0.6 mm (clinical strain). This compares favourably with the ampicillin control (10 µg) which had 9.2 ± 0.4 (reference strain) and 8.7 ± 0.3 mm (clinical strain) zones of inhibition. The aqueous leaf extract was a similarly good *K. pneumoniae* growth inhibitor, with 8.7 ± 0.4 and 8.6 ± 0.3 mm zones of inhibition recorded against the reference and clinical strains, respectively. Whilst displaying smaller zones of inhibition (generally substantially <8.0 mm), the berry (methanolic, aqueous and ethyl acetate extracts) and stem (methanolic and aqueous extracts) also inhibited *K. pneumoniae* growth. Notably, the supercritical berry (8.3 ± 0.3 and 7.5 ± 0.5 mm against the reference and clinical strains) and leaf extracts (8.0 and 7.8 ± 0.4 mm against the reference and clinical strains) had similar potency to the small scale solvent extractions. As *K. pneumoniae* can trigger ankylosing spondylitis in genetically susceptible individuals,[13] these extracts have potential in the prevention and treatment of this disease.

The *T. lanceolata* extracts were also screened for growth inhibitory activity against bacterial triggers of multiple sclerosis (*Acinitobacter baylyi*, Figure 4a; *Pseudomonas aeruginosa*, Figure 4b).[28,29] The methanolic and aqueous leaf extracts were the most potent *A. baylyi* growth inhibitors, with zones of inhibition ranging from 8.2-8.6 mm (compared to 8.6 mm for the 10 µg ampicillin control). Although smaller inhibition zones (*7-7.8* mm) were measured for the methanolic and aqueous berry extracts, these extracts were still deemed to be good *A. baylyi* growth inhibitors. Similarly, the supercritical berry (7.3 ± 0.3 and 7.6 ± 0.3 mm against the reference and clinical strains, respectively) and leaf extracts (7.0 and 7.3 ± 0.3 mm against the reference and clinical strains, respectively) were also good inhibitors of *A. baylyi* growth.

The aqueous and methanolic extracts (berry and leaf) also inhibited *P. aeruginosa* growth (Figure 4b), albeit with relatively small zones of

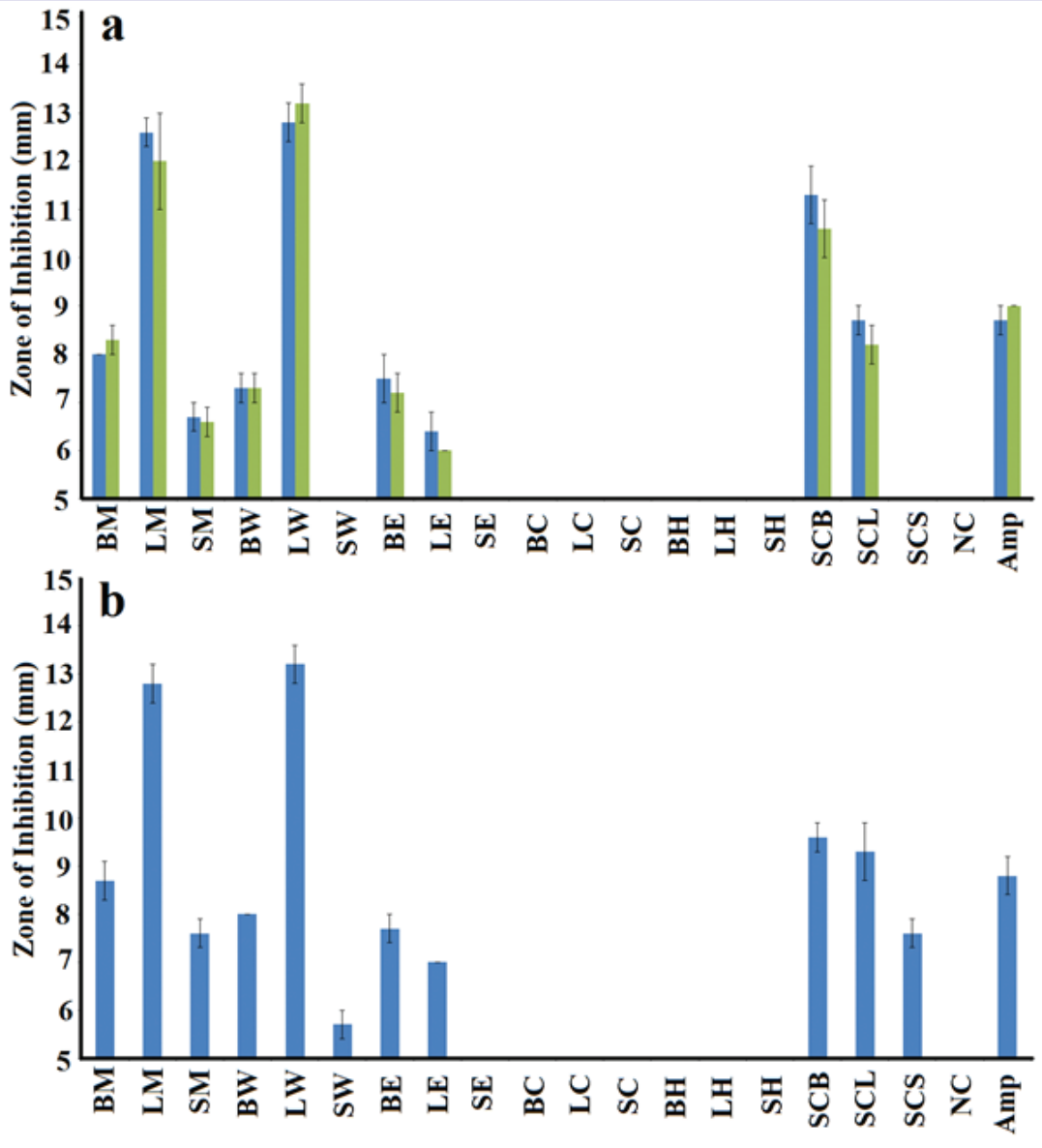

Figure 2: Antibacterial activity of *T. lanceolata* extracts measured as zones of inhibition (mm) against a bacterial trigger of rheumatoid arthritis: (a) *Proteus mirabilis*, (b) *Proteus vulgaris*. Results are expressed as mean ± SEM of at least triplicate determinations. Blue bars represent inhibition of the reference bacterial strain. Green bars represent inhibition of the clinical bacterial strain. BM=methanolic berry extract; LM=methanolic leaf extract; SM=methanolic stem extract; BW=aqueous berry extract; LW=aqueous leaf extract; SW=aqueous stem extract; BE=ethyl acetate berry extract; LE=ethyl acetate leaf extract; SE=ethyl acetate stem extract; BC=berry chloroform extract; LC=leaf chloroform extract; SC=stem chloroform extract; BH=berry hexane extract; LH=leaf hexane extract; SH=stem hexane extract; SCB=supercritical berry extract; SCL=supercritical leaf extract; SCS=supercritical stem extract; NC=negative control; Amp=ampicillin control (10 µg).

inhibition. The strongest inhibition (6.5 ± 0.5 mm) was recorded for the methanolic berry extract against reference *P. aeruginosa* strain. The supercritical berry and leaf extracts had similar potencies, with inhibition zones ≤ 6.3 mm. Whilst this inhibition is relatively poor in comparison the other bacterial species screened, it is noteworthy that both the reference and clinical *P. aeruginosa* strains are antibiotic resistant strains. Indeed, the 10 µg ampicillin control used in our studies only produced 6.2 ± 0.4 and 5.5 ± 0.3 mm zones of inhibition for the reference and clinical strains, respectively. This finding is supported by previous studies which have also reported these strains to be antibiotic resistant.[23,27] Thus, despite the relatively small inhibition zones, the *T. lanceolata* extracts may still be useful for the inhibition of *P. aeruginosa* growth. Therefore, as both *A. baylyi* and *P. aeruginosa* can trigger multiple sclerosis in genetically susceptible individuals,[28,29] these extracts have potential in the prevention and treatment of this disease.

The *T. lanceolata* aqueous and methanolic berry and leaf extracts were also good inhibitors of *Streptococcus pyogenes* growth (Figure 5). The aqueous leaf extract was the most potent growth inhibitor, with an inhibition zone of 10.6 ± 0.4 mm. However, unlike the inhibition reported for the other bacterial species, the berry ethyl acetate was also a very

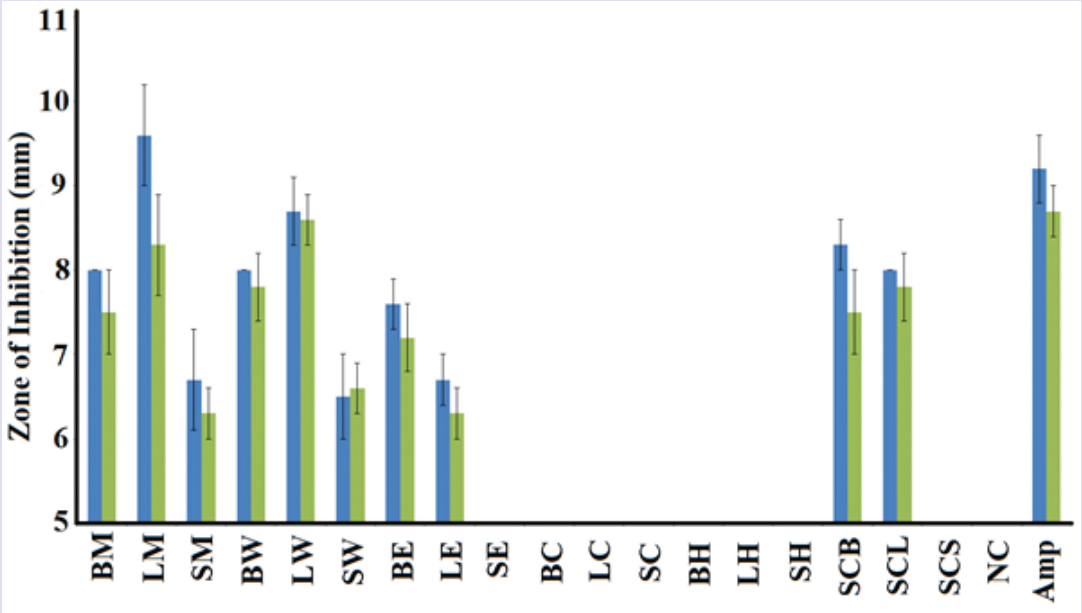

Figure 3: Antibacterial activity of *T. lanceolata* extracts measured as zones of inhibition (mm) against a bacterial trigger of ankylosing spondylitis (*Klebsiella pneumoniae*). Results are expressed as mean ± SEM of at least triplicate determinations. Blue bars represent inhibition of the reference bacterial strain. Green bars represent inhibition of the clinical bacterial strain. BM=methanolic berry extract; LM=methanolic leaf extract; SM=methanolic stem extract; BW=aqueous berry extract; LW=aqueous leaf extract; SW=aqueous stem extract; BE=ethyl acetate berry extract; LE=ethyl acetate leaf extract; SE=ethyl acetate stem extract; BC=berry chloroform extract; LC=leaf chloroform extract; SC=stem chloroform extract; BH=berry hexane extract; LH=leaf hexane extract; SH=stem hexane extract; SCB=supercritical berry extract; SCL=supercritical leaf extract; SCS=supercritical stem extract; NC=negative control; Amp=ampicillin control (10 µg).

good growth inhibitor of *S. pyogenes* (9.8 ± 0.4 mm). The berry methanolic (8.3 ± 0.3 mm) and aqueous extracts (7.6 ± 0.3 mm), as well as the methanolic leaf extract (7.6 ± 0.3 mm), were also good growth inhibitors. In comparison, the supercritical leaf extract displayed potent growth inhibitory activity (9 mm). The supercritical berry extract was also a relatively good *S. pyogenes* growth inhibitor, with a zone of inhibition of 7.3 ± 0.3 mm. *S. pyogenes* has been implicated in a number of diseases including rheumatic fever. Thus, the *T. lanceolata* extracts have potential in the prevention and treatment of these diseases.

The relative level of antimicrobial activity was further evaluated by determining the MIC values (Table 3) for each extract against the bacterial species which were shown to be susceptible by disc diffusion assays. Most of the extracts were effective at inhibiting microbial growth at low concentrations, with MIC values against the bacterial species that they inhibited often substantially <500 µg/mL (<5 µg infused into the disc), indicating the potent antimicrobial activity of these extracts. The methanolic berry extract was a particularly potent bacterial growth inhibitor, with MIC values as low as 15 µg/mL (<0.15 µg infused into the disc) against *P. mirabilis*. This extract was similarly potent against *K. pneumoniae* (MIC values as low as 33 µg/mL; 0.3 µg infused into the disc), *A. baylyi* (MIC values as low as 280 µg/mL; 2.8 µg infused into the disc), *P. aeruginosa* (MIC values as low as 288 µg/mL; 2.9 µg infused into the disc) and *S. pyogenes* (MIC values of 423 µg/mL; 4.2 µg infused into the disc). These MIC values compare favourably with the inhibition of the pure ampicillin standard which was tested using 10 µg per disc. The methanolic leaf, as well as the aqueous and ethyl acetate berry and leaf extracts were also good bacterial growth inhibitors, generally with MIC values <500 µg/mL against most bacterial species.

The supercritical fluid berry and leaf extracts were also effective at inhibiting bacterial growth at low concentrations, with MIC values against the bacterial species that they inhibited often substantially <300 µg/mL (<3 µg infused into the disc). The supercritical berry and leaf extracts were particularly potent against *Proteus* spp. and *K. pneumoniae*. Indeed, the berry and leaf extracts had MIC values of approximately 160 µg/mL (1.6 µg infused into the disc) and 280 µg/mL (2.8 µg infused into the disc), respectively against *P. mirabilis*. In contrast, distinct differences were noted between the berry and leaf supercritical extracts against the other bacterial species. MIC values of approximately 150 µg/mL (1.5 µg infused into the disc) were recorded for the supercritical berry extract against *K. pneumoniae*. In contrast, MIC values of approximately 1500 µg/mL (15 µg infused into the disc) were measured for the corresponding supercritical leaf extract. Whilst this MIC value indicates that this is still a good bacterial growth inhibitor, it is an order of magnitude less potent than the supercritical berry extract. Whilst the supercritical extracts were not as potent at inhibiting the growth of *A. baylyi* (MICs of approximately 1200-2000 µg/mL), *P. aeruginosa* (MICs of approximately 800-2000 µg/mL) and *S. pyogenes* (MICs of approximately 750-1300 µg/mL), the MIC values are still indicative of moderate to potent bacterial growth inhibition. Thus, the supercritical extracts (particularly the berry extract) have potential in the prevention and treatment of diseases associated with these bacteria.

Anti-Giardial activity

T. lanceolata extracts were also screened for their ability to inhibit *Giardia duodenalis* growth (Figure 6). In agreement with previous reports,[14] the methanol and water extracts displayed particularly potent inhibitory activity, each completely blocking *G. duodenalis* proliferation (compared

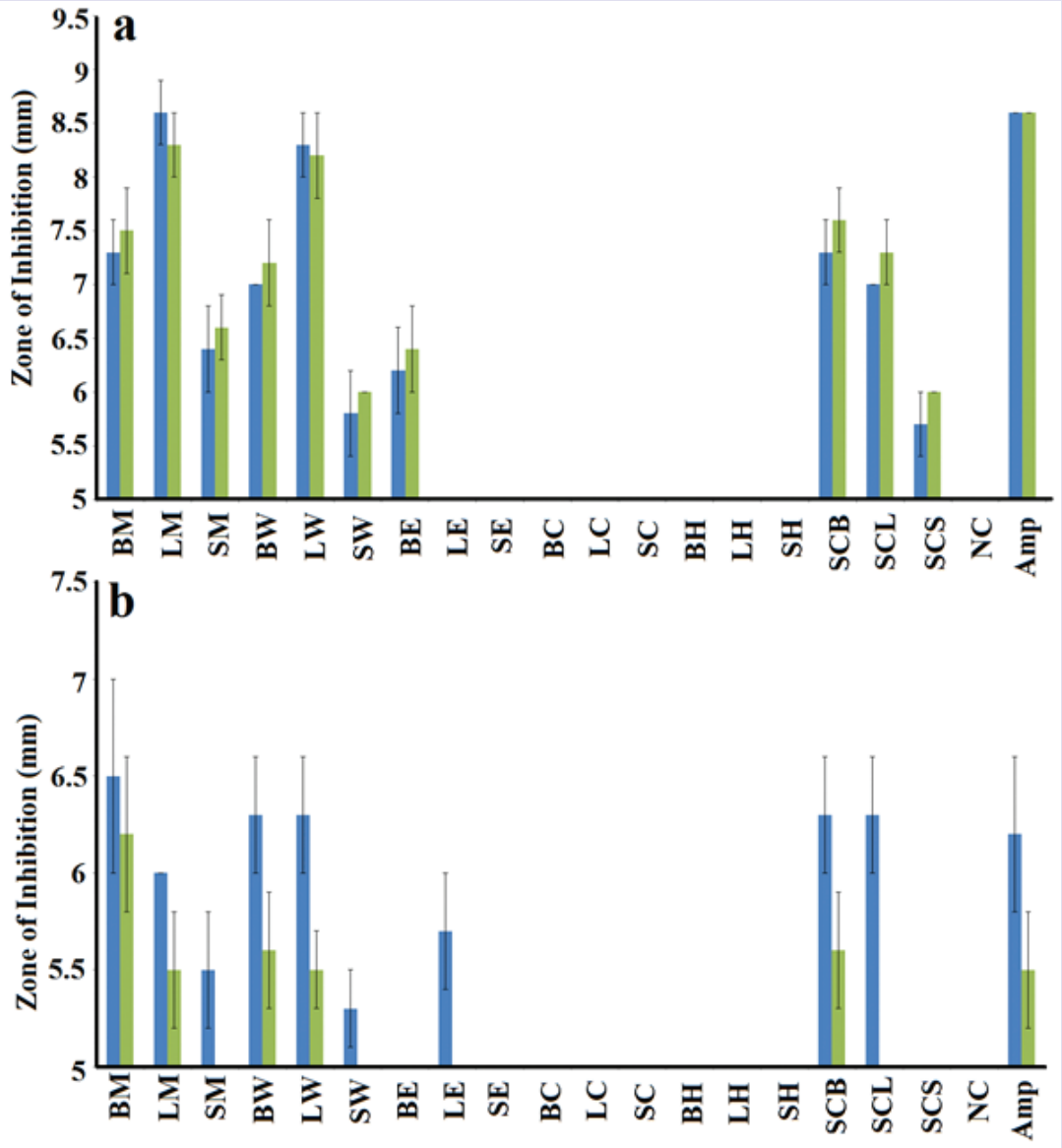

Figure 4: Antibacterial activity of *T. lanceolata* extracts measured as zones of inhibition (mm) against a bacterial trigger of multiple sclerosis: (a) *Acinitobacter baylyi*, (b) *Pseudomonas aeruginosa*. Results are expressed as mean ± SEM of at least triplicate determinations. Blue bars represent inhibition of the reference bacterial strain. Green bars represent inhibition of the clinical bacterial strain. BM=methanolic berry extract; LM=methanolic leaf extract; SM=methanolic stem extract; BW=aqueous berry extract; LW=aqueous leaf extract; SW=aqueous stem extract; BE=ethyl acetate berry extract; LE=ethyl acetate leaf extract; SE=ethyl acetate stem extract; BC=berry chloroform extract; LC=leaf chloroform extract; SC=stem chloroform extract; BH=berry hexane extract; LH=leaf hexane extract; SH=stem hexane extract; SCB=supercritical berry extract; SCL=supercritical leaf extract; SCS=supercritical stem extract; NC=negative control; Amp=ampicillin control (10 µg).

to the untreated control). The ethyl acetate berry (100% inhibition compared to the negative control), leaf (90% inhibition compared to the negative control) and stem extracts (71% inhibition compared to the negative control) were also very effective at inhibiting trophozoite growth. The chloroform and hexane *T. lanceolata* extracts were less effective inhibitors of *G. duodenalis* proliferation. The berry and leaf chloroform extracts inhibited 65% and 35% of the *G. duodenalis* proliferation, respectively (compared to the untreated control). The berry, leaf and stem hexane extracts also significantly inhibited *G. duodenalis* proliferation by approximately 33%, 42% and 14%, respectively. In comparison, all supercritical extracts were potent *G. duodenalis* inhibitors, each totally blocking proliferation.

The *T. lanceolata* extracts were further tested over a range of concentrations to determine the IC_{50} 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. The small scale methanolic extracts were generally the most potent antiproliferative agents, with IC_{50} values of 194 and 175 µg/mL determined for the berry and leaf extracts, respectively. However, the aqueous and ethyl acetate extracts were also potent inhibitors of *G. duodenalis*

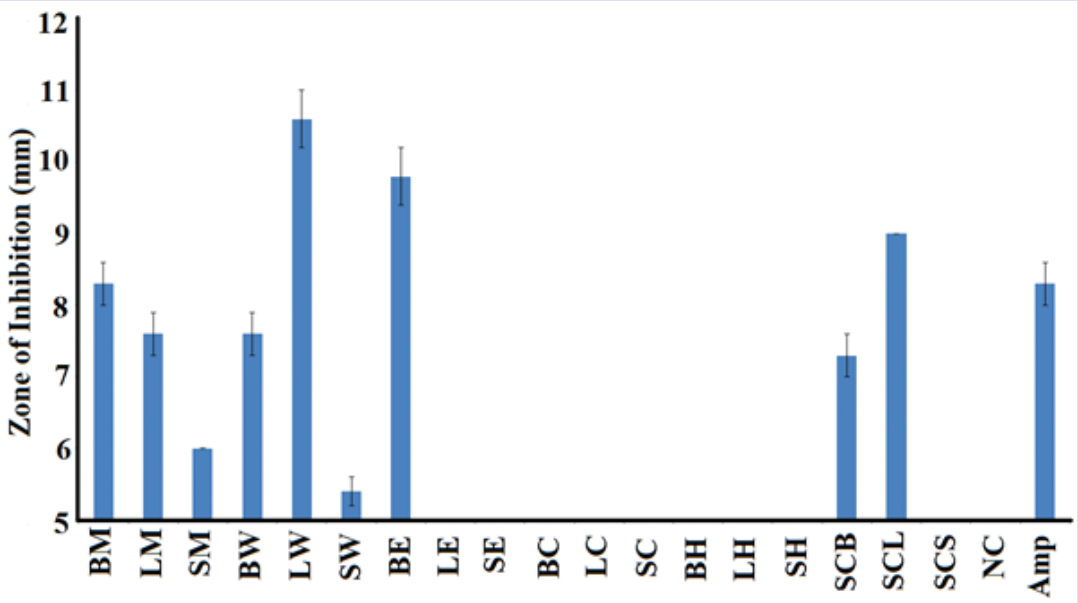

Figure 5: Antibacterial activity of *T. lanceolata* extracts measured as zones of inhibition (mm) against a bacterial trigger of rheumatic fever (*Streptococcus pyogenes*). Results are expressed as mean ± SEM of at least triplicate determinations. BM=methanolic berry extract; LM=methanolic leaf extract; SM=methanolic stem extract; BW=aqueous berry extract; LW=aqueous leaf extract; SW=aqueous stem extract; BE=ethyl acetate berry extract; LE=ethyl acetate leaf extract; SE=ethyl acetate stem extract; BC=berry chloroform extract; LC=leaf chloroform extract; SC=stem chloroform extract; BH=berry hexane extract; LH=leaf hexane extract; SH=stem hexane extract; SCB=supercritical berry extract; SCL=supercritical leaf extract; SCS=supercritical stem extract; NC=negative control; Amp=ampicillin control (10 µg).

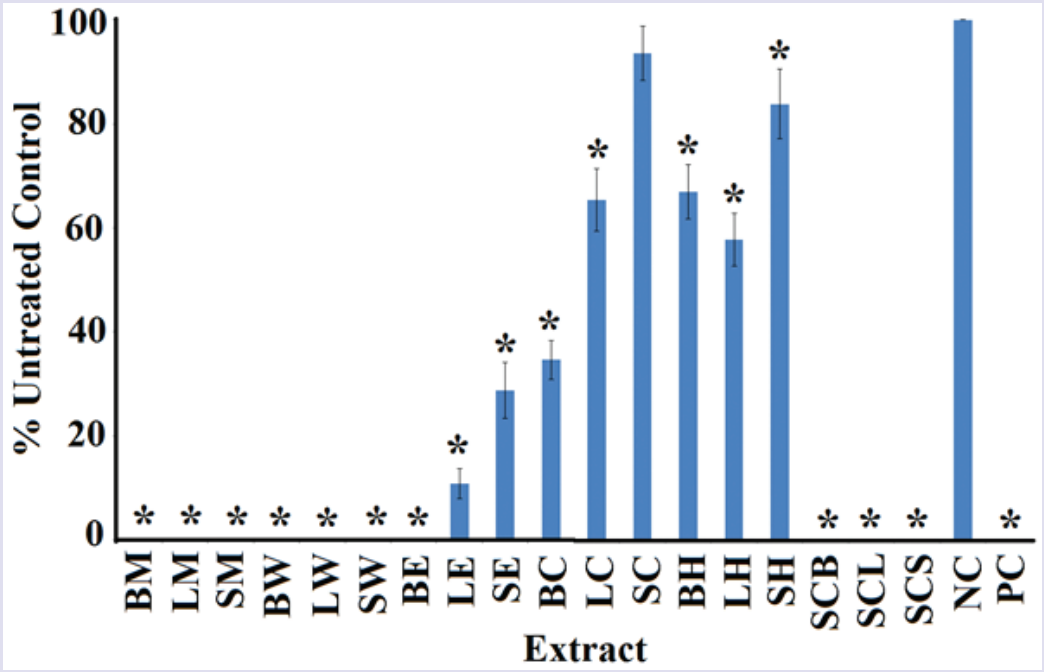

Figure 6: Inhibitory activity of *T. lanceolata* extracts against *Giardia duodenalis* trophozoites measured as a percentage of the untreated control. Results are expressed as mean ± SEM of at least triplicate determinations. BM=methanolic berry extract; LM=methanolic leaf extract; SM=methanolic stem extract; BW=aqueous berry extract; LW=aqueous leaf extract; SW=aqueous stem extract; BE=ethyl acetate berry extract; LE=ethyl acetate leaf extract; SE=ethyl acetate stem extract; BC=berry chloroform extract; LC=leaf chloroform extract; SC=stem chloroform extract; BH=berry hexane extract; LH=leaf hexane extract; SH=stem hexane extract; SCB=supercritical berry extract; SCL=supercritical leaf extract; SCS=supercritical stem extract; NC=negative (seawater) control; PC=metronidazole control. * indicates results that are significantly different to the negative control ($P<0.01$).

proliferation, with IC_{50} values generally <600 µg/mL. The supercritical fluid extractions were similarly potent *G. duodenalis* inhibitors, with IC_{50} values of 492, 375 and 883 µg/mL for the supercritical berry, leaf and stem extracts, respectively.

Inhibition of cancer cell proliferation

The *T. lanceolata* extracts were tested against 2 cancer cell lines (CaCo2 colorectal carcinoma cells, Figure 7; HeLa cervical cancer cells, Figure 8) to determine their ability to inhibit cancer cell growth. The methanol and water extracts displayed potent inhibitory activity against CaCo2 cells, with proliferation inhibited to as low as approximately 1% of the untreated control cell growth for the methanolic berry extract (Figure 7). Indeed, all methanolic and aqueous extracts inhibited the growth by >85% compared with the untreated control. The berry, leaf and stem ethyl acetate extracts were also effective at inhibiting CaCo2 proliferation (to approximately 44, 48 and 89% of untreated cell proliferation respectively). Of the chloroform extracts, only the berry extract significantly inhibited CaCo2 proliferation. Interestingly, the hexane extracts not only did not inhibit *G. duodenalis* proliferation, but a significant stimulation of CaCo2 proliferation was noted. This is consistent with previous studies from our group which have reported similar growth stimulation for these extracts.[4] The supercritical fluid extracts were also potent inhibitors of CaCo2 proliferation. The supercritical berry, leaf and stem extracts inhibited CaCo2 proliferation by 98, 100 and 69%, respectively.

The *T. lanceolata* extracts were also shown to affect the proliferation of HeLa cells (Figure 8). Whilst the extracts were potent inhibitors of HeLa proliferation, the inhibition was generally of lower efficacy than was evident with the CaCo2 cells. The methanol and water extracts were generally the most potent inhibitors of HeLa cells. The methanolic berry, leaf and stem extracts inhibited proliferation by 81, 93 and 66%, respectively. Similarly, the corresponding aqueous extracts inhibited HeLa proliferation by 93, 96 and 78%, respectively compared with the untreated control. The berry, leaf and stem ethyl acetate extracts were also potent inhibitors of HeLa cell proliferation (by approximately 72, 82 and 39%, respectively compared to the untreated cell proliferation). Of the chloroform and hexane extracts, only the leaf extracts significantly inhibited HeLa proliferation (19 and 26% inhibition, respectively compared to untreated control proliferation). Interestingly, the berry chloroform and hexane extracts not only did not inhibit HeLa proliferation, but a significant stimulation of cellular proliferation was noted. The supercritical fluid extracts were also potent inhibitors of HeLa proliferation. The supercritical berry, leaf and stem extracts inhibited HeLa proliferation by 100, 100 and 73%, respectively.

The *T. lanceolata* extracts were further tested over a range of concentrations to determine the IC_{50} values (Table 3) for each extract against CaCo2 and HeLa proliferation. Inhibition of proliferation was dose-dependent for all extracts, with the level of inhibitory activity decreasing at lower concentrations (Table 3). The aqueous *T. lanceolata* leaf extract was a particularly potent anti-proliferative agent, with IC_{50} values of 150 and 230 µg/mL against CaCo2 and HeLa cells, respectively. The IC_{50} values calculated for all other small scale solvent extractions were generally >1000 µg/mL, indicating moderate anti-proliferative activity. Similarly, the IC_{50} values determined for the supercritical fluid extractions (2500-4000 µg/mL) are in a similar range and are also indicative of moderate anti-proliferative activity.

Quantification of toxicity

T. lanceolata extracts were diluted to 4000 µg/mL (to give a bioassay concentration of 2000 µg/mL) in artificial seawater for toxicity testing in the *Artemia* nauplii lethality bioassay. For comparison, the reference toxin potassium dichromate was also tested in the bioassay. Potassium dichromate was rapid in its induction of mortality, with mortality evident within 4 hours of exposure (unpublished results). All of the *T. lanceolata* extracts were slower at inducing mortality, with ≥ 12 hrs needed for mortality induction. Despite the slower onset of mortality, the small scale solvent extractions induced mortality significantly above that of the artificial seawater control (Figure 9). Similarly, the supercritical fluid extractions induced similar mortality levels at 24 h. To further evaluate the toxicity, each extract was tested across a range of concentrations in the *Artemia* nauplii bioassay. Table 3 shows the extract and control toxin concentrations required to achieve 50% mortality (LC_{50}) at various times. As toxicity of crude plant extracts has previously been defined as 24 LC_{50} values <1000 µg/mL,[26] the measured LC_{50} values indicate that none of the small scale *T. lanceolata* solvent extractions were significantly toxic. Similarly, all of the supercritical extractions also had LC_{50} values substantially > 1000 µg/mL and were thus also deemed to be non-toxic.

Non-targeted GC-MS headspace analysis of the *T. lanceolata* extracts

As the methanolic *T. lanceolata* berry extract and the supercritical berry extract generally had the most potent bacterial growth inhibitory efficacy, anti-Giardial activity and carcinoma cell anti-proliferative activity (as determined by MIC and IC_{50}; Table 3), they were deemed the most promising extracts for further phytochemical analysis. Optimised GC-MS parameters were developed and used to examine the phytochemical composition of these extracts. The resultant gas chromatograms are presented in Figures 10a and Figure 10b, respectively. Several marker compounds (polygodial, cineole, eugenol, linalool, terpineol) were selected as a comparison between the extracts as these have previously been reported to be present in relative abundance in *T. lanceolata* berry extracts.[8,9] All of these compounds were detected in both the solvent extract and in the supercritical fluid extract. The major peak in both extracts eluted at approximately 16.4 min. This compound was identified as linalool by database spectral comparison and by co-elution with an authentic standard. The relative abundance of this compound was nearly identical between the 2 extracts. Another major peak (identified as polygodial) was particularly evident in the methanolic extract at 20.9 min (Figure 10a). This compound was also identified in the supercritical berry extract. Whilst polygodial was still a major component in this extract, it is apparent that its relative abundance had decreased in the supercritical extract compared to the smaller scale solvent extract. Instead, the relative abundance of several other terpenoids has increased in the supercritical berry extract compared to the smaller scale methanolic berry extract. A large relative increase is evident for cineole (at 15.1 min). Significant increases are also evident for terpineol (19.1 min) and eugenol (23.8 min). Thus, the overall composition between the 2 extracts appears to be similar although there is variation in the relative abundances of the selected marker compounds.

DISCUSSION

T. lanceolata berry and leaf extracts have been highlighted for their medicinal properties. Recent studies have reported berry extracts to have a high antioxidant capacity,[4] as well as strong antibacterial activity,[7-9,11] anti-inflammatory activity[11] and anti-Giardial activity.[14] The leaves also have high antioxidant contents,[4-6] and are good inhibitors of bacterial growth.[7-9,11] Furthermore, *T. lanceolata* leaf extracts induce a reduction in pro-inflammatory enzymes in an inflammatory cell model[10] and inhibit microbial triggers of several autoimmune inflammatory diseases.[11] Leaf extracts have been reported to inhibit cellular proliferation in several carcinoma cell models, as well as inducing apoptosis in HepG2 cells.[15] Similarly, *T. lanceolata* leaf extracts also block *Giardia duodenalis* proliferation and therefore have potential in the treatment of giardiasis.[14]

Table 3: Minimum bacterial growth inhibitory concentration (µg/mL) of the extracts, *G. duodenalis* and carcinoma anti-proliferative IC_{50} values (µg/mL), and LC_{50} values (µg/mL) in the *Artemia* nauplii bioassay.

		P. mirabilis		*P. vulgaris*	*K. pneumoniae*		*A. baylyi*		*P. aeruginosa*		*S. pyogenes*	*G. duodenalis*	Carcinoma cells		Toxicity
		Reference strain	Clinical strain	Clinical strain	Reference strain	Clinical strain	Reference strain	Clinical strain	Reference strain	Clinical strain	Clinical strain	Clinical strain	CaCo2	HeLa	A. franciscana
Berry	M	15	38	87	87	33	280	406	288	658	423	194	3070	1910	3573
	W	126	203	331	305	198	376	500	145	308	1087	483	1330	1663	2376
	E	57	126	154	254	118	585	812	-	-	347	572	>5000	1755	1584
	C	-	-	-	-	-	-	-	-	-	-	1094	CND	CND	1750
	H	-	-	-	-	-	-	-	-	-	-	CND	CND	CND	4018
Leaf	M	643	576	454	872	785	123	185	246	648	355	175	1030	810	3096
	W	56	103	208	400	12	385	561	307	513	287	576	150	230	2665
	E	265	403	303	916	780	-	-	-	-	-	862	>5000	1800	2283
	C	-	-	-	-	-	-	-	-	-	-	CND	CND	CND	2877
	H	-	-	-	-	-	-	-	-	-	-	CND	CND	CND	3006
Stem	M	1385	1894	1150	2217	920	1208	1558	746	-	1157	754	>5000	>5000	3087
	W	-	-	>5000	3540	1858	1593	1736	820	-	>5000	1355	>5000	>5000	1995
	E	-	-	-	-	-	-	-	-	-	-	1827	CND	CND	1765
	C	-	-	-	-	-	-	-	-	-	-	CND	CND	CND	2380
	H	-	-	-	-	-	-	-	-	-	-	CND	CND	CND	CND
Supercritical extracts	B	178	158	200	188	123	1224	1621	873	1230	761	492	4133	2652	1236
	L	272	291	249	1411	1985	1238	1779	2201	-	1288	375	3005	3497	824
	S	-	-	1035	-	-	>5000	>5000	-	-	-	883	>5000	>5000	1231
Positive Controls	Met	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	18	NT	NT	NT
	Cis	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	8400	15710	NT

Numbers indicate the mean MIC, IC_{50} and LC_{50} values of triplicate determinations. - indicates no inhibition. CND indicates that an IC_{50} or LC_{50} value could not be determined as inhibition or mortality did not exceed 50 % at any concentration tested.

NT=not tested; B=berry; L=leaf; S=stem; M=methanolic extract; W=aqueous extract; E=ethyl acetate extract; C=chloroform extract; H=hexane extract; Met=metronidazole; Cis=cisplatin.

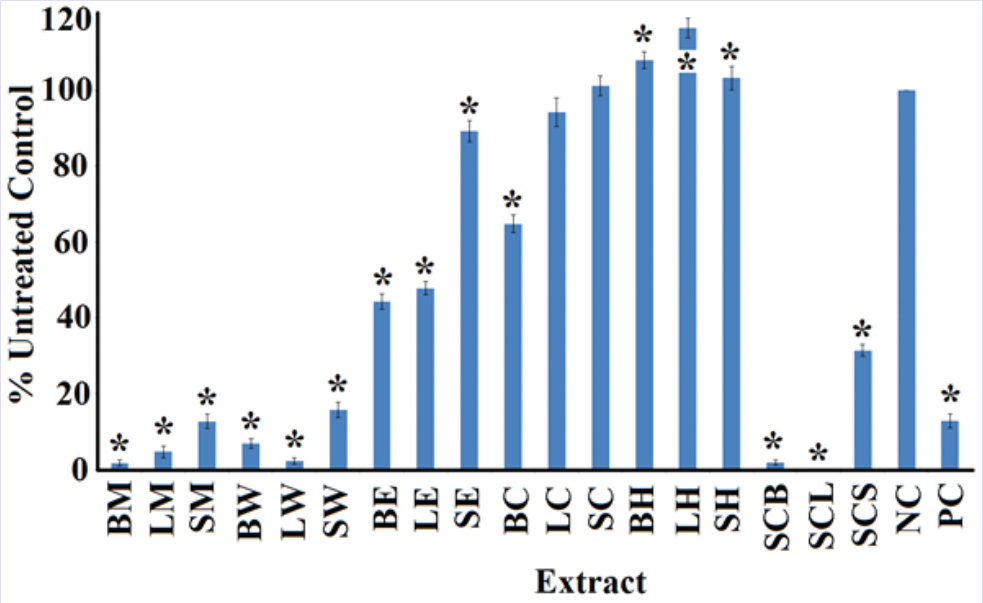

Figure 7: Anti-proliferative activity of *T. lanceolata* extracts and untreated controls against CaCo2 carcinoma cells measured as percentages of the untreated control cells. Results are expressed as mean percentages ± SEM of at least triplicate determinations. BM=methanolic berry extract; LM=methanolic leaf extract; SM=methanolic stem extract; BW=aqueous berry extract; LW=aqueous leaf extract; SW=aqueous stem extract; BE=ethyl acetate berry extract; LE=ethyl acetate leaf extract; SE=ethyl acetate stem extract; BC=berry chloroform extract; LC=leaf chloroform extract; SC=stem chloroform extract; BH=berry hexane extract; LH=leaf hexane extract; SH=stem hexane extract; SCB=supercritical berry extract; SCL=supercritical leaf extract; SCS=supercritical stem extract; NC=negative (seawater) control; PC=cisplatin control (50 mg/mL). * indicates results that are significantly different to the negative control (P<0.01).

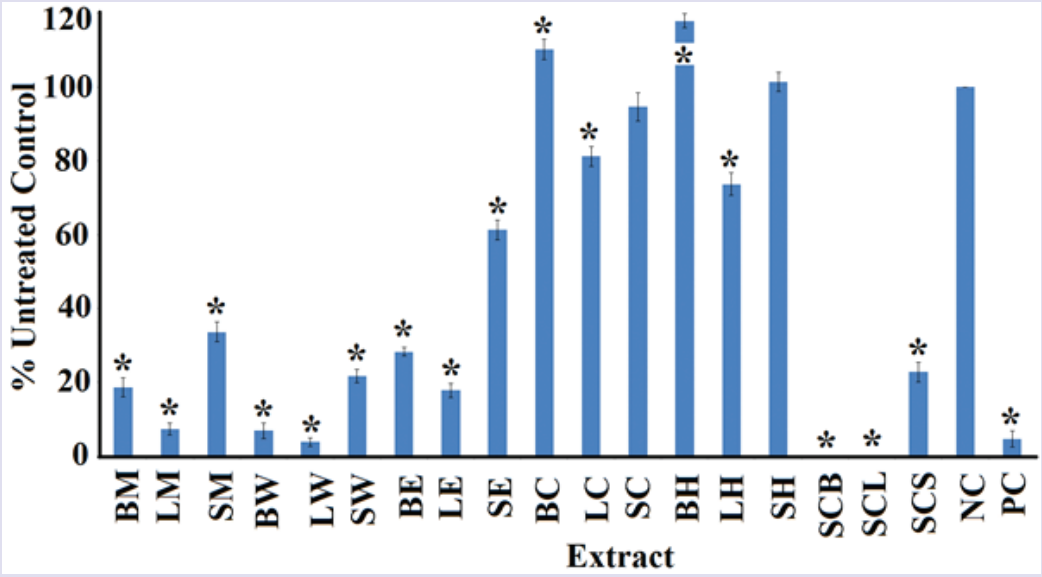

Figure 8: Anti-proliferative activity of *T. lanceolata* extracts and untreated controls against HeLa carcinoma cells measured as percentages of the untreated control cells. Results are expressed as mean percentages ± SEM of at least triplicate determinations. BM=methanolic berry extract; LM=methanolic leaf extract; SM=methanolic stem extract; BW=aqueous berry extract; LW=aqueous leaf extract; SW=aqueous stem extract; BE=ethyl acetate berry extract; LE=ethyl acetate leaf extract; SE=ethyl acetate stem extract; BC=berry chloroform extract; LC=leaf chloroform extract; SC=stem chloroform extract; BH=berry hexane extract; LH=leaf hexane extract; SH=stem hexane extract; SCB=supercritical berry extract; SCL=supercritical leaf extract; SCS=supercritical stem extract; NC=negative (seawater) control; PC=cisplatin control (50 mg/mL). * indicates results that are significantly different to the negative control (P<0.01).

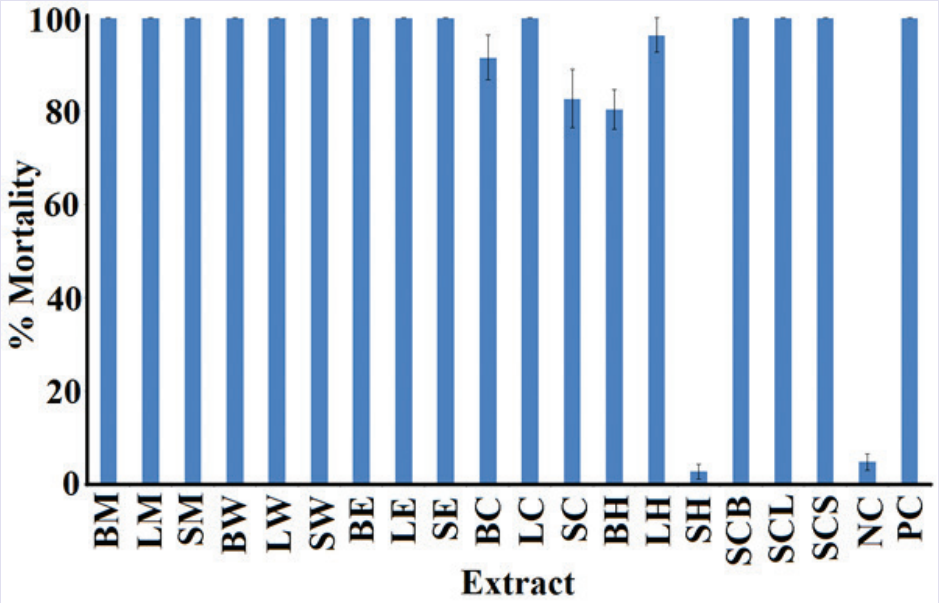

Figure 9: The lethality of *T. lanceolata* extracts and potassium dichromate (1000 µg/mL) and artificial seawater controls towards *Artemia franciscana* nauplii after 24 hrs exposure. Results are expressed as mean ± SEM of at least triplicate determinations. BM=methanolic berry extract; LM=methanolic leaf extract; SM=methanolic stem extract; BW=aqueous berry extract; LW=aqueous leaf extract; SW=aqueous stem extract; BE=ethyl acetate berry extract; LE=ethyl acetate leaf extract; SE=ethyl acetate stem extract; BC= berry chloroform extract; LC=leaf chloroform extract; SC=stem chloroform extract; BH=berry hexane extract; LH=leaf hexane extract; SH=stem hexane extract; SCB=supercritical berry extract; SCL=supercritical leaf extract; SCS=supercritical stem extract; NC=negative (seawater) control; PC=potassium dichromate control.

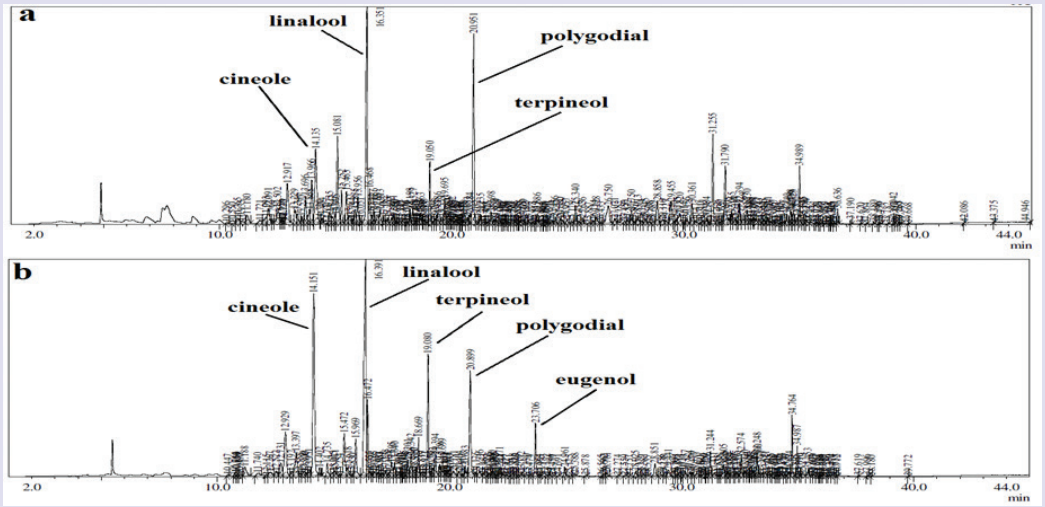

Figure 10: Head space gas chromatogram of 0.5 µL injections of *T. lanceolata* berry (a) methanolic extract and (b) supercritical berry extract. The extracts were dried and resuspended in methanol. Major phytochemical components are identified as a comparison between the extraction methods.

However, it is noteworthy that all of these studies utilised small scale laboratory extraction methodologies to prepare extracts for biological screening. Despite numerous promising reports of therapeutic properties, we were unable to find any studies which report methods to scale up the extraction to commercially relevant levels.

The aim of this study was to develop a quick and simple extraction method which would allow the extraction of substantially increased masses of plant material without significantly affecting the quality of the extract produced. Supercritical fluid extraction (SFE) was selected for the development of an extraction protocol as it has several advantages over other methods:

- Supercritical fluids (SF) have similar solvating powers to liquid organic solvents but higher diffusivities, lower viscosities and lower surface tension.

- Alteration of the temperature and pressure conditions can readily be adjusted, allowing for rapid separation of analytes.
- The polarity of the SF can readily be modified to provide more selective separation.
- The extracts produced by SFE do not have residual solvent present (as may be the case in solvent extraction protocols). This is extremely favourable for extracts with pharmaceutical or food uses.
- Diffusivities are much faster in SFs than in liquid solvents. Therefore SFE is substantially more rapid than liquid solvent extraction. Less time is required to produce large amounts of extract using SFE.
- Substantially less costly solvent is used than for liquid solvent extraction. As well as decreasing processing costs, this results in significant reductions in drying time and substantially less environmental risk due to solvent pollution.

With consideration of these potential benefits, ethanol dimethyl ether based SFE protocol was developed and trialled. As previous studies have reported the therapeutic potential of the berry and leaves, these plant materials were both extracted using our SFE protocol and tested in parallel with extracts produced by liquid solvent extraction. As an extension of these studies, we also extracted *T. lanceolata* stem and twig material by both SFE and liquid solvent extraction techniques. A variety of bioassay systems were selected for bioactivity screening. These tests were chosen to give the broadest possible understanding of the therapeutic properties of the extracts. All extracts were screened for the ability to inhibit the growth of bacterial species that are triggers for autoimmune inflammatory diseases as these would not only provide insight into the ability to block bacterial growth as well as indicating therapeutic options in some inflammatory diseases. The extracts were also screened to for the ability to inhibit the growth of the eukaryotic pathogen *G. duodenalis* and the carcinoma cell lines CaCo2 and HeLa.

A thorough literature search was unable to find any previous studies examining the therapeutic properties of *T. lanceolata* stem and twig material. This is perhaps surprising as the properties of berry, leaf and peppercorn have been extensively reported previously. The stem material is a by-product of current harvesting methods and is generally considered of little value. It was believed that if the stem material was found to have therapeutic properties similar to those reported for the berry and leaf, then this 'waste material' may be repurposed for the production of therapeutic agents. Interestingly, the stem extracts did have notable therapeutic properties. In particular, the methanolic and aqueous stem extracts inhibited the growth of most bacterial species, with MIC values in the range 750-2000 µg/mL. These values indicate moderate to good growth inhibitory activity. Thus the aqueous and methanolic stem extracts have potential in the prevention and treatment of rheumatoid arthritis, ankylosing spondylitis, multiple sclerosis and rheumatic fever. However, despite this promising finding, it is noteworthy that these MIC values indicate that the stem extracts are 1-2 orders of magnitude less potent that the corresponding berry and leaf extracts. For example, the MIC of the methanolic stem extract towards the reference *P. mirabilis* strain (1385 µg/mL) is approximately 100 times less potent than the methanolic berry extract against the same bacterium (15 µg/mL). Good anti-Giardial IC_{50} values were also determined for the methanolic stem extract (754 µg/mL). However, this extract was also a considerably less potent inhibitor of *G. duodenalis* proliferation than the corresponding berry and leaf methanolic extracts (194 and 175 µg/mL, respectively).

Screening the supercritical berry and leaf extracts for therapeutic properties demonstrated that both had potent antibacterial properties with similar efficacies to the smaller scale liquid solvent extractions. The MIC values of the supercritical berry and leaf extracts against most bacterial species were <1000 µg/mL, indicating the potential of these extracts in the prevention and treatment of some autoimmune inflammatory diseases. The MIC values of the supercritical berry extract against *P. mirabilis* (178 and 158 µg/mL against reference and clinical strains, respectively) and *K. pneumoniae* (200 and 188 µg/mL against reference and clinical strains, respectively) indicate that these extracts may be especially useful for the prevention of rheumatoid arthritis and ankylosing spondylitis. The supercritical berry and leaf extracts also had similar anti-proliferative potencies against *G. duodenalis,* CaCo2 and HeLa carcinomas to those determined for the corresponding liquid solvent extractions. Thus, whilst the supercritical extraction method used in these studies resulted in a far greater extraction throughput as well as reduced extraction time and costs, the resultant supercritical extracts retained therapeutic activities at or near the potency of the smaller scale liquid solvent extracts. Furthermore, toxicity screening in the *Artemia* nauplii bioassay indicate that the berry and stem supercritical extracts are non-toxic with LC_{50} values >1000 µg/mL. Whilst an LC_{50} value <1000 µg/mL was determined for the supercritical leaf extract, this value indicates only low toxicity. The *A. franciscana* assay is sensitive to extreme pH ranges and samples with high phenolic acid contents may adversely alter the pH. Thus, it is possible that the apparent toxicity reported in this study is a fallacious result due to the high phenolic acid content of the extracts and these are in fact non-toxic. Thus, SFE can produce substantially greater masses of extracted material with similar therapeutic potential as the smaller liquid solvent extraction techniques.

A comparison of the phytochemical composition of the liquid solvent extracts and the supercritical fluid extracts was used as a further quality control measure. Several important terpenoids were selected for this study on the basis of their known bioactivities relevant to the therapeutic properties tested in this study, and reports of their relevant abundance in *T. lanceolata* extracts.[8,9] The selected terpenoids have been previously reported to have potent broad spectrum antibacterial activity[1] and therefore are likely to contribute to the inhibitory activity reported in this study. Furthermore, these terpenoids have also been reported to suppress NF-κB signalling (the major regulator of inflammatory diseases) and are thus likely to contribute to the anticancer and anti-inflammatory properties of *T. lanceolata.*[30-33] Minor changes in phytochemical abundance were evident between the extracts produced by the different methods. A higher relative abundance of polygodial was evident in the berry methanolic extract produced by liquid solvent extraction compared to the relative abundance in the supercritical berry extract. Conversely, the supercritical berry extract had correspondingly greater abundance of cineole, terpineol and eugenol. The relative abundance of linalool was consistent between the extracts.

Whilst this study provides insight into the phytochemical compositions of the *T. lanceolata* extracts, it is noteworthy that no single technique will detect and identify all compounds responsible for any therapeutic property in an extract. Our study utilised a GC-MS headspace technique to examine the extracts. This technique was chosen as the biomarker compounds are known to be detected using these techniques.[8,9] As detection of volatile, relatively nonpolar compounds is suited to GC-MS headspace analysis, this was an appropriate analytical tool for detecting terpenoids components. However, these extracts are likely to contain many more polar compounds that were not detected in this study. Indeed, several previous studies utilising LC-MS analysis have highlighted the phytochemical complexity of *T. lanceolata* extracts. Thus, whilst extraction by SFE techniques does not appear to have resulted in dramatic changes in phytochemical composition (at least for the volatile lower polarity compounds), further studies using LC-MS analysis are required to ensure that more dramatic differences between the extracts are not apparent with respect to their polar components.

CONCLUSION

The results of this study demonstrate that supercritical fluid extraction is a viable method of extraction of large masses of *T. lanceolata* plant material rapidly and with high yields. Similarly, the supercritical extracts generally retain their therapeutic properties, are non-toxic and have similar profiles and abundances of several therapeutically important terpenoids as smaller scale liquid solvent extractions.

ACKNOWLEDGEMENTS

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CONFLICT OF INTEREST

The authors report no conflicts of interest.

ABBREVIATION USED

DMSO: Dimethyl sulfoxide

IC50: The concentration required to achieve a 50 % reduction in activity

LC50: The concentration required to achieve 50 % mortality

MIC: minimum inhibitory concentration

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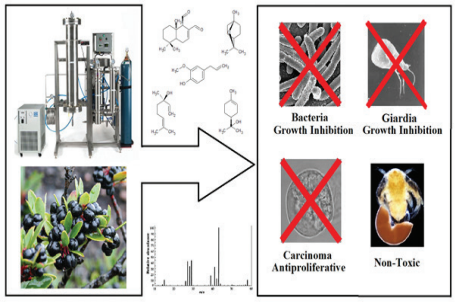
PICTORIAL ABSTRACT



SUMMARY

- *T. lanceolata* berry and leaf SFEs displayed strong bacterial growth inhibitory activity against bacterial triggers of autoimmune inflammatory diseases.
- The SFEs had similar efficacies to the smaller scale liquid solvent extractions.
- The SFE also had similar antiproliferative potencies against *G. duodenalis,* CaCo2 and HeLa carcinomas to the solvent extractions.
- GC-MS analysis revealed similar terpenoid components and similar abundances between the SFEs and the solvent extractions.
- All SFEs were either non-toxic or of only low toxicity in the *Artemia franciscana* toxicity assay.

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Ms Low Vallette is a postgraduate student at School of Biology, Ecole de Biologie Industrielle (EBI), Cergy, France. In 2015, she undertook a research project in Dr Ian Cock's laboratory in the School of Natural Sciences at Griffith University examining the therapeutic properties of a variety of Australian native plants.

Ms Camille Rabadeaux is a postgraduate student at School of Biology, Ecole de Biologie Industrielle (EBI), Cergy, France. In 2015, she undertook a research project in Dr Ian Cock's laboratory in the School of Natural Sciences at Griffith University examining the therapeutic properties of a variety of Australian native plants.

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Dr Ian Cock leads a research team in the Environmental Futures Research Institute and the School of Natural Sciences at Griffith University, Australia. His research involves bioactivity and phytochemical studies into a variety of plant species of both Australian and international origin, including *Aloe vera*, South Asian and South American tropical fruits, as well as Australia plants including *Scaevola spinescens*, *Pittosporum phylliraeoides, Terminalia ferdinandiana* (Kakadu plum), Australian *Acacias*, *Syzygiums*, *Petalostigmas* and *Xanthorrhoea johnsonii* (grass trees). This range of projects has resulted in nearly 200 publications in a variety of peer reviewed journals.