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Original Article

GC-MS analysis of *Tasmannia lanceolata* Extracts which Inhibit the Growth of the Pathogenic Bacterium *Clostridium perfringens*

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ABSTRACT

Introduction: *Clostridium perfringens* is the etiological agent of clostridial myonecrosis and enteritis necroticans. Infections result in exotoxin production, tissue necrosis and unless promptly treated, often result in death. **Methods:** *Tasmannia lanceolata* extracts were investigated for *C. perfringens* growth inhibitory activity by disc diffusion analysis and MIC determination. Toxicity was evaluated by *Artemia* nauplii bioassay and the most potent extracts were phytochemically evaluated by GC-MS headspace analysis. **Results:** All *T. lanceolata* berry and leaf extracts displayed potent *C. perfringens* growth inhibition. The berry extracts were more potent growth inhibitors than the corresponding leaf extracts, although the leaf extracts were also potent growth inhibitors. The berry aqueous, methanolic and ethyl acetate extracts were particularly potent growth inhibitors, with MIC values of 654, 65 and 329 µg/mL respectively. *T. lanceolata* leaf also displayed good efficacy, with an MIC of 839, 1255 and 625 µg/mL for the aqueous, methanolic and ethyl acetate extracts respectively. All extracts were nontoxic in the *Artemia franciscana* bioassay, with LC_{50} values substantially > 1000 µg/mL. Non-biased GC-MS analysis of the aqueous, methanolic and ethyl acetate berry extracts revealed the presence of high relative levels of a diversity of terpenoids. **Conclusions:** The lack of toxicity of the *T. lanceolata* extracts and their potent growth inhibitory bioactivity against *C. perfringens* indicates their potential as medicinal agents in the treatment and prevention of clostridial myonecrosis and enteritis necroticans. GC-MS metabolomic profiling studies indicate that these extracts contained a diversity of terpenoids, with monoterpenoids being particularly abundant.

Key words: Winteraceae, *Tasmannia lanceolata*, Myonecrosis, Enteritis necroticans, Gas gangrene.

INTRODUCTION

Clostridium perfringens is an endospore-forming Gram-positive, obligate anaerobic bacterium that is found in a variety of habitats, including soils, sewage or as a naturally occurring inhabitant of the intestinal microflora of humans.[1] It is classed as an opportunistic pathogen and is an etiological agent of clostridial gastroenteritis as well as several other diseases including myonecrosis.[2] The species *C. perfringens* is divided into five type strains (A-E), each capable of producing exotoxins that are linked to different illnesses of varying severity. These can range from mild food-poisoning to the potentially fatal clostridial myonecrosis (gas gangrene).[3]

Clostridial myonecrosis is a rapidly progressive infection of the soft tissue. The disease is characterised by the necrosis of local muscle and surrounding tissue and can lead to shock and ultimately death, even when promptly treated.[1] Although several *Clostridium* spp. can cause gas gangrene, *C. perfringens* is the primary bacterium responsible for the disease (along with *C. bifermentans*, C. *fallax*, *C. histolyticum*, *C. novyi*, *C. septicum* and *C. tertium*), accounting for up to 80% of all recorded cases.[4] The primary methods of treatment are through prompt antibiotic therapy and surgery, however combining these with hyperbaric oxygen can slow the spread of gas gangrene in patients. The strictly anaerobic nature of *C. perfringens* means that when exposed to oxygen, cellular replication and exotoxin production can be inhibited. However, this method of treatment is bacteriostatic and thus must be combined with antibiotic chemotherapy.[5] Traditional antibiotic therapies offer an effective means of treatment; however, the probing of natural compounds offers an innovative means of treatment and lowers the inherent risk of antibiotic resistance of *C. perfringens*.

Tasmannia lanceolata (Poir.) A.C.Sm (commonly referred to as Tasmanian pepper or mountain pepper berry) is a shrub endemic to the woodlands and cool temperate rainforests of Tasmania and the south-eastern region of the Australian mainland.[6] It

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is a medium to large shrub that varies between 2-5 m in height. Individual plants are unisexual, having either male or female flowers. The stems, branches and twigs are red in colour. The aromatic leaves are lanceolate to narrowly elliptical in shape (4-12 cm long, 0.7-2 cm wide) with a distinctly pale under surface. Small creamy-white unisexual flowers appear during the summer months. These develop into small fleshy black 2 lobed berries (5-8 mm wide) during autumn.

The berries, leaves and bark of this species have historical uses as a food and as a medicinal plant.[6, 7] When the berry is air dried, it forms a small, hard peppercorn which is suitable for milling or crushing. The berry has a pleasant spicy flavour and sharp aroma. *T. lanceolata* was used as flavouring agent by Australian Aborigines and more recently by European settlers. Historically, the leaves have been used as an herb and the berries have been used as a spice. Australian Aborigines also used *T. lanceolata* as a therapeutic agent to treat stomach disorders and as an emetic, as well as general usage as a tonic.[7] *T. lanceolata* was also used traditionally for the treatment and cure of skin disorders, venereal diseases, colic, stomach aches and as a quinine substitute.[7] Later, European colonists also recognized the therapeutic potential of *T. lanceolata* and the bark was used as a common substitute for other herbal remedies (including those derived from the related South American Winteraceae species, *Drimys wintera* (winter bark)[7] to treat scurvy due to its high anti-anti-oxidant content.[7, 8]

Despite its ethnobotanical usage, there have been limited rigorous scientific studies into the therapeutic properties of *T. lanceolata*. Recent studies have demonstrated the high antioxidant content of *T. lanceolata* fruit and leaves.[8] It has been postulated that this high anti-oxidant content may provide therapeutic effects for this plant.[7] Indeed, studies within our laboratory have reported potent inhibition of bacterial growth by *T. lanceolata* berries, leaves and peppercorns against panels of pathogenic and food spoilage bacteria.[9] *T. lanceolata* extracts can also inhibit the growth of a bacterial trigger of rheumatoid arthritis (*P. mirabilis*).[10]

However, despite the documented ability of *T. lanceolata* to inhibit the growth of many bacterial species, to our knowledge there have been no similar studies against *Clostridium perfringens*. The current study was undertaken to test *T. lanceolata* berry and leaf extracts for the ability to inhibit the growth of this pathogen.

MATERIALS AND METHODS

T. lanceolata samples and extraction

Dried *Tasmannia lanceolata* (Poir.) A.C.Sm berry (without seed) and leaf materials were obtained from Go Wild Harvest, Australia. The material was thoroughly dried using a Sunbeam food dehydrator and stored at -30ºC until use. The dried plant materials were thawed and freshly ground to a coarse powder prior to extraction. Individual 1 g quantities were extracted by weighing each powdered plant part into each of 5 tubes and adding 50 mL of methanol, water, ethyl acetate, chloroform or hexane respectively. All solvents were obtained from Ajax, Australia and were AR grade. The berry and leaf material was extracted in each solvent for 24 h at 4ºC 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 extracts were weighed and redissolved in 10 mL deionised water (containing 1% DMSO).

Qualitative phytochemical studies

Phytochemical analysis of the extracts for the presence of alkaloids, anthraquinones, cardiac glycosides, flavonoids, phenolic compounds, phytosterols, saponins tannins and triterpenoids were conducted by previously described assays.[11, 12]

Antibacterial screening

Clinical Clostridium perfringens screening

A clinical strain of *Clostridium perfringens* was supplied by Ms. Jane Gifkins (Griffith University) and was originally isolated and verified by John Bates (Queensland Department of Health). Cultures were grown and maintained using a thioglycolate liquid media (Oxoid Ltd., Australia). All growth studies were performed using nutrient agar (Oxoid Ltd., Australia) under induced anaerobic conditions using anaerobic jars and AnaeroGen™ 3.5L atmospheric generation systems (Thermo Scientific) as previously described.[13] Incubation was at 30 ºC and the stock culture was subcultured and maintained in thioglycolate liquid media at 4 ºC.

Evaluation of antimicrobial activity

Antimicrobial activity of all plant extracts was determined using a modified disc diffusion assay.[14, 15] Briefly, 100 µL of the test bacteria were grown in 10 mL of fresh nutrient broth media until they reached a count of ~10^8 cells/mL. A volume of 100 µL of bacterial suspension was spread onto nutrient agar plates and extracts were tested for antibacterial activity using 5 mm sterilised filter paper discs. Discs were infused 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 h before incubation at 30 °C for 24 h. The diameters of the inhibition zones were measured in millimetres. All measurements were to the closest whole millimetre. Each assay was performed in at least triplicate. Mean values (± SEM) are reported in this study. Standard discs of penicillin (2 µg) and ampicillin (10 µg) were obtained from Oxoid Ltd., Australia and used as positive controls for antibacterial activity. Filter discs infused with 10 µL of distilled water were used as a negative control.

Minimum inhibitory concentration (MIC) determination

The minimum inhibitory concentrations (MIC) of the extracts was determined as previously described.[16, 17] Briefly, the plant extracts were diluted in deionised water and tested across a range of concentrations. Discs were infused with 10 µL of the extract 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. Linear regression was used to calculate the MIC values.

Toxicity screening

Reference toxin for toxicity screening

Potassium dichromate ($K_2Cr_2O_7$) (AR grade, Chem-Supply, Australia) was prepared as a 4 mg/mL solution in distilled water and 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.[18, 19] 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 24 h all nauplii were sacrificed and counted to determine the total % mortality per well. The LC_{50} with 95% confidence limits 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.[20] 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% phenyl, 95% dimethylpolysiloxane (30 m x 0.25 mm id x 0.25 um) 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 70 eV. 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 is expressed as the mean ± SEM of at least three independent experiments.

RESULTS

Extraction of 1 g of the *T. lanceolata* plant materials with various solvents yielded dried plant extracts ranging from 17 mg (*T. lanceolata* leaf ethyl acetate extract) to 171 mg (methanolic *T. lanceolata* fruit extract) (Table 1). Aqueous and methanolic extracts generally gave higher yields of dried extracted material compared to ethyl acetate extracts. The dried extracts were resuspended in 10 mL of deionised water (containing 1 % DMSO) resulting in the extract concentrations shown in Table 1.

Qualitative phytochemical studies showed little difference between the aqueous and methanolic extracts, however there were notable differences between these extracts and the ethyl acetate extracts. High levels of phenolics (both water soluble and insoluble) were extracted in the aqueous and methanolic samples. There were substantially lower levels detected in the corresponding ethyl acetate extracts. Similarly, there was a lower level of flavonoids detected in the ethyl acetate extracts than the corresponding aqueous and methanolic extracts. Triterpenes were detected in both methanolic and ethyl acetate extracts although they were absent in the aqueous extracts.

To measure the inhibitory activity of the crude plant extracts against *C. perfringens*, 10 µL aliquots of each extract were screened using a disc diffusion assay. The bacterial growth was inhibited by all of the 6 extracts tested (Figure 1). The methanolic berry extract was the most potent inhibitor of growth, with inhibition zones of 16.3 ± 0.3 mm. This compares favourably with the penicillin and ampicillin controls, which had inhibitory zones of 12.3 ± 0.3 mm and 13.0 ± 1.0 mm respectively. The aqueous and methanolic extracts showed greater zones of inhibition than the ethyl acetate extracts, with inhibitory zones ≥ 11 mm.

The antimicrobial efficacy was further quantified by determining the MIC values (Table 2). All the extracts were determined to be potent inhibitors

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

Extract	Mass of Dried Extract (mg)	Concentration of Resuspended Extract (mg/mL)	Total Phenolics	Water Soluble Phenolics	Water Insoluble Phenolics	Cardiac Glycosides	Saponins	Triterpenes	Phytosterols	Alkaloids (Mayer Test)	Alkaloids (Wagner Test)	Flavonoids	Tannins	Free Anthraquinones	Combined Anthraquinones
BW	111	11.1	+++	+++	+++	–	–	–	–	–	–	+++	–	–	–
BM	171	17.1	+++	+++	+++	–	++	+	–	–	–	+++	–	–	–
BE	56.7	5.7	+	+	++	–	+	++	–	–	–	++	–	–	–
LW	134	13.4	+++	+++	+++	–	++	–	–	–	–	+++	–	–	–
LM	144	14.4	+++	+++	+++	–	+++	+	–	–	–	+++	–	–	–
LE	17	1.7	+	+	++	–	–	+	–	–	–	++	–	–	–

+++ indicates a large response; ++ indicates a moderate response; + indicates a minor response; - indicates no response in the assay. BW = aqueous *T. lanceolata* berry extract; BM = methanolic *T. lanceolata* berry extract; BE = ethyl acetate *T. lanceolata* berry extract; LW = aqueous *T. lanceolata* leaf extract; LM = methanolic *T. lanceolata* leaf extract; LE = ethyl acetate *T. lanceolata* leaf extract.

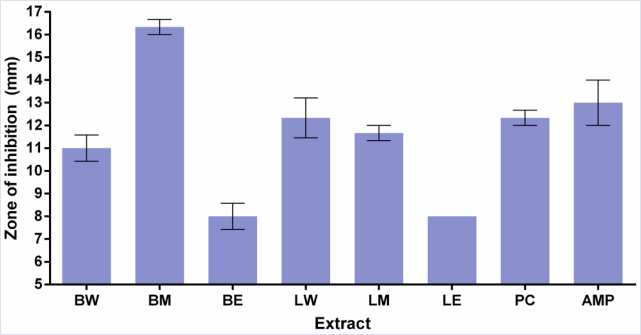

Figure 1: Growth inhibitory activity of *T. lanceolata* extracts against the *C. perfringens* clinical isolate measured as zones of inhibition (mm). BW = aqueous *T. lanceolata* berry extract; BM = methanolic *T. lanceolata* berry extract; BE = ethyl acetate *T. lanceolata* berry extract; LW = aqueous *T. lanceolata* leaf extract; LM = methanolic *T. lanceolata* leaf extract; LE = ethyl acetate *T. lanceolata* leaf extract; PC = penicillin (2 µg); AMP = ampicillin (10 µg). Results are expressed as mean zones of inhibition ± SEM.

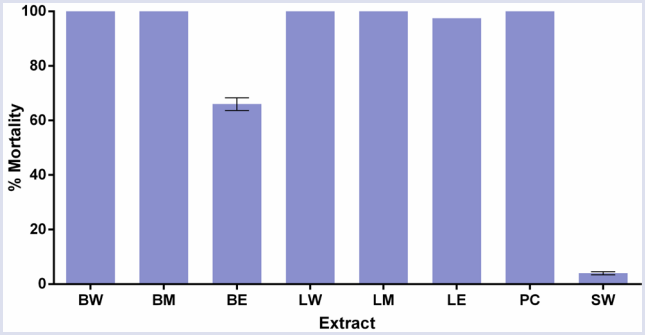

Figure 2: The lethality of the *T. lanceolata* extracts (2000 µg/mL) and the potassium dichromate control (1000 µg/mL) towards *Artemia franciscana* nauplii after 24 h exposure. BW = aqueous *T. lanceolata* berry extract; BM = methanolic *T. lanceolata* berry extract; BE = ethyl acetate *T. lanceolata* berry extract; LW = aqueous *T. lanceolata* leaf extract; LM = methanolic *T. lanceolata* leaf extract; LE = ethyl acetate *T. lanceolata* leaf extract; PC = potassium dichromate control; SW = artificial seawater control. Results are expressed as mean % mortality ± SEM.

Table 2: Minimum inhibitory concentration (µg/mL) of the plant extracts and LC_{50} values (µg/mL) in the *Artemia* nauplii bioassay.

Extract	MIC	LC_{50}
BW	654	2.376
BM	65	3.573
BE	329	3.132
LW	839	2.665
LM	1.255	3.096
LE	625	1.766
PC	ND	186
SW	ND	-

Numbers indicate the mean MIC and LC_{50} values of triplicate determinations. - indicates no inhibition. BW = aqueous *T. lanceolata* berry extract; BM = methanolic *T. lanceolata* berry extract; BE = ethyl acetate *T. lanceolata* berry extract; LW= aqueous *T. lanceolata* leaf extract; LM = methanolic *T. lanceolata* leaf extract; LE = ethyl acetate *T. lanceolata* leaf extract; PC = potassium dichromate control; SW = artificial seawater negative control; ND = the indicated test was not performed.

of *C. perfringens* growth, with MIC <1000 µg/mL for all extracts except the aqueous leaf extract. Even that extract had a relatively low MIC (1255 µg/mL), indicating antibacterial efficacy. The *T. lanceolata* berry extracts were more potent inhibitors than were the corresponding leaf extracts. Indeed, a MIC of 65 µg/mL was determined for the methanolic *T. lanceolata* berry extract. This is particularly noteworthy as it equates to a mass of less than 0.7 µg infused into the disc (compared with 2 and 10 µg for the penicillin and ampicillin controls respectively).

All extracts were initially screened at 2000 µg/mL in the assay (Figure 2). For comparison, the reference toxin potassium dichromate (1000 µg/mL) was also tested in the bioassay. The potassium dichromate reference toxin was rapid in its onset, promoting nauplii death within the first 3 h of exposure with 100 % mortality evident following 4-5 h (unpublished results). Similarly, all the *T. lanceolata* extracts displayed significant mortality rates following 24 h exposure (>50%).

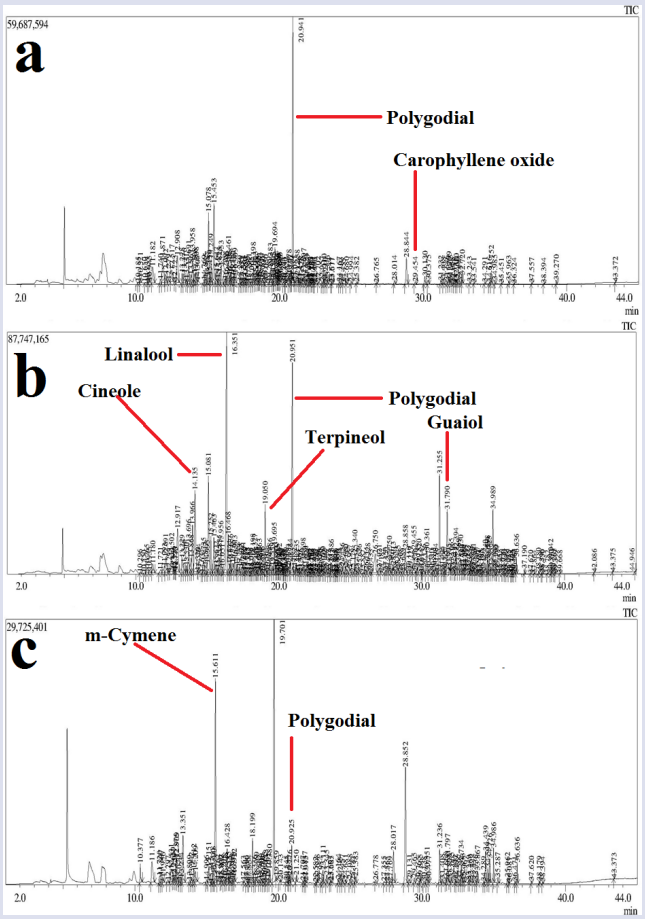

Figure 3: Head space gas chromatograms of 0.5 µL injections of *T. lanceolata* berry (a) aqueous, (b) methanolic and (c) ethyl acetate extracts. The extracts were dried and resuspended in methanol. Chromatography conditions were as described in the methods section.

Table 3: Qualitative GC-MS headspace analysis of the *T. lanceolata* berry extracts, elucidation of empirical formulas and putative identification of each compound.

Molecular Mass	Molecular Formula	R.Time	Name	M	W	E
110	$C_6H_6O_2$	10.19	Acetylfuran		+	
151	$C_8H_9NO_2$	10.375	Methyl N-hydroxybenzenecarboximidoate			+
128	C_8H1_6O	10.55	2,4,4-trimethyl-cyclopentanol	+		
130	$C_7H_{14}O_2$	10.765	Methyl caproate	+		
130	$C_8H_{18}O$	10.892	4-Methyl-4-heptanol		+	
170	$C_{12}H_{26}$	10.935	3,8-Dimethyldecane	+	+	
128	$C_8H_{16}O$	11.18	4-Methyl-2-heptanone	+	+	+
126	C_9H_{18}	11.721	1,1,3,4-Tetramethylcyclopentane	+	+	+
156	$C_{10}H_{20}O$	11.805	2,2,5,5-Tetramethyl-3-hexanone			+
110	$C_6H_6O_2$	11.87	2-Formyl-5-methylfuran		+	
120	C_9H_{12}	11.993	p-Ethylmethylbenzene	+		
120	C_9H_{12}	12.12	m-Ethyltoluene			
156	$C_{10}H_{20}O$	12.299	Decanal	+		+
154	$C_{10}H_{18}O$	12.28	2,2,6-Trimethyl-6-vinyltetrahydropyran		+	
114	C_8H_{18}	12.502	2,4-Dimethylhexane	+	+	
186	$C_9H_{14}O_4$	12.535	alpha.-D-Xylo-Hex-5-enofuranose, 5,6-dideoxy-1,2-O-(1-methylethylidene)-			+
284	$C_{16}H_{28}O_4$	12.626	Succinic acid, 2-methylpent-3-yl trans-he	+		+
126	$C_8H_{14}O$	12.72	6-Methyl-5-hepten-2-one	+	+	+
146	$C_7H_{14}O_3$	12.773	Methyl 2-hydroxy-4-methylpentanoate	+		
170	$C_{12}H_{26}$	13.229	Dodecane	+	+	+
128	$C_8H_{16}O$	13.235	Octanal			
142	$C_{10}H_{22}$	13.53	3,3,5-Trimethylheptane	+	+	
170	$C_{12}H_{26}$	13.696	3,4,5,6-Tetramethyloctane	+	+	
120	C_9H_{12}	13.868	Cumene	+	+	+
140	$C_9H_{16}O$	13.94	Ethanone, 1-(2,2-dimethylcyclopentyl)-			+
142	$C_{10}H_{22}$	13.966	3,3-Dimethyloctane	+	+	
154	$C_{10}H_{18}O$	14.135	Cineole	+	+	
140	$C_9H_{16}O$	14.21	1,1,3-Trimethyl-2-cyclohexanone		+	+
136	$C_{10}H_{16}$	14.404	alpha.-Pinene	+		
136	$C_{10}H_{16}$	14.735	.beta.-Ocimene	+		
244	$C_{13}H_{24}O_4$	14.847	Oxalic acid, isohexyl neopentyl ester	+	+	
278	$C_{14}H_{30}O_3S$	14.995	Sulfurous acid, nonyl pentyl ester	+	+	
114	C_8H_{18}	15.081	3,3-Dimethylhexane	+	+	
120	C_8H_8O	15.15	Bicyclo[4.2.0]octa-1,3,5-trien-7-ol			+
242	$C_{13}H_{22}O_4$	15.463	Ethyl 2-(5-methyl-5-vinyltetrahydrofuran-2-yl)propan-2-yl carbonate	+	+	
212	$C_{14}H_{12}O_2$	15.615	m-Toluic acid, phenyl ester		+	+
134	$C_{10}H_{14}$	15.622	m-Cymene	+		
200	$C_{13}H_{28}O$	15.744	11-Methyldodecanol	+	+	
134	$C_{10}H_{14}$	15.835	1,2-Dimethyl-4-ethylbenzene	+	+	+
170	$C_{10}H_{18}O_2$	15.956	Linalool oxide	+	+	+
172	$C_{10}H_{20}O_2$	16.051	3-(Hydroxymethyl)-2-nonanone	+		
228	$C_{13}H_{24}O_3$	16.23	Carbonic acid, nonyl prop-1-en-2-yl ester		+	

Continued...

Table 3: Cont'd.

Molecular Mass	Molecular Formula	R.Time	Name	M	W	E
154	$C_{10}H_{18}O$	16.351	Linalool	+	+	+
170	$C_{12}H_{26}$	16.468	4-Methylundecane	+	+	+
140	$C_{10}H_{20}$	16.544	1-Ethyl-1,4-dimethylcyclohexane	+	+	
156	$C_{11}H_{24}$	16.636	6-Ethyl-2-methyloctane	+		
134	$C_{10}H_{14}$	16.786	3,7,7-Trimethyl-1,3,5-cycloheptatriene	+	+	
134	$C_{10}H_{14}$	16.913	p-Cymene	+	+	+
156	$C_{10}H_{20}O$	17.233	1-Methyl-4-(1-methylethyl)cyclohexanol	+	+	
156	$C_{10}H_{20}O$	17.402	Neoisocarquejanol	+		
152	$C_{10}H_{16}O$	17.491	4(10)-Thujen-3-ol	+	+	
184	$C_{11}H_{20}O_2$	17.56	2,2,6,6-Tetramethyl-3,5-heptanedione	+	+	+
166	$C_{12}H_{22}$	18.036	1-Hexyl-1-cyclohexene	+		
154	$C_{10}H_{18}O$	18.327	cis-2-Norbornanol	+		+
144	$C_8H_{16}O_2$	18.47	Octanoic acid			+
170	$C_{10}H_{10}O_2$	18.565	trans-Linalool 3,7-oxide	+		
168	$C_{12}H_{24}$	18.663	4,6,8-Trimethyl-1-nonene	+	+	
130	$C_8H_{18}O$	18.913	2,5-Dimethyl-2-hexanol	+	+	
154	$C_{10}H_{18}O$	19.05	.alpha.-Terpineol	+	+	+
152	$C_{10}H_{16}O$	19.283	Myrtenol	+	+	
156	$C_{10}H_{20}O$	19.559	5-Methyl-3-propyl-2-hexanone	+	+	
134	$C_9H_{10}O$	19.695	2-methyl-2-phenyl-oxirane	+	+	+
156	$C_{11}H_{24}$	19.803	2,3,7-Trimethyloctane	+	+	
184	$C_{13}H_{28}$	20.032	4,8-dimethyl-undecane	+	+	
196	$C_{12}H_{20}O_2$	20.155	Nerol acetate	+		
184	$C_{13}H_{28}$	20.445	3,3,5-trimethyl-decane	+	+	
252	$C_{17}H_{32}O$	20.675	13-Heptadecyn-1-ol		+	
296	$C_{20}H_{40}O$	20.683	Phytol	+		
232	$C_{15}H_{20}O_2$	20.784	2,2,3,3-Tetramethylcyclopropanecarboxylic acid, 4-methylphenyl ester	+	+	
234	$C_{15}H_{22}O_2$	20.951	polygodial	+	+	+
184	$C_{13}H_{28}$	21.082	4-methyl-dodecane	+	+	
158	$C_9H_{18}O_2$	21.235	Nonanoic acid	+	+	+
114	C_8H_{18}	21.698	3,3-Dimethylhexane	+	+	
188	$C_{10}H_{20}O_3$	21.83	Butyl butoxyacetate	+	+	+
198	$C_{14}H_{30}$	22.083	4,6-Dimethyldodecane	+	+	
150	$C_{10}H_{14}O$	22.165	(2E,3Z)-2-Ethylidene-6-methyl-3,5-heptadienal	+	+	
222	$C_{16}H_{30}$	22.408	(2-Cyclohexyl-1-methylpropyl)cyclohexane	+	+	
140	$C_9H_{16}O$	22.708	2,3,4,5-Tetramethylcyclopent-2-en-1-ol	+	+	+
194	$C_{12}H_{18}O_2$	22.876	2-Pinen-10-ol, acetate	+		
212	$C_{15}H_{32}$	22.955	2,6,11-Trimethyldodecane	+	+	+
158	$C_{10}H_{22}O$	23.215	2-Propyl-1-heptanol		+	
168	$C_{12}H_{24}$	23.473	4-Methyl-1-undecene	+		
286	$C_{16}H_{30}O_4$	23.619	2,2,4-Trimethyl-1,3-pentanediol diisobutyrate $$ Propanoic acid, 2-methyl-, 2,2-dimethyl-1-(1-methylethyl)-1,3-propanediyl ester	+	+	

Continued...

Table 3: Cont'd.

Molecular Mass	Molecular Formula	R.Time	Name	M	W	E
164	$C_{10}H_{12}O_2$	23.686	p-Eugenol	+		+
180	$C_{10}H_{12}O_3$	24.01	beta.-Phenyllactic acid methyl ester	+		
216	$C_{12}H_{24}O_3$	24.17	Propanoic acid, 2-methyl-, 3-hydroxy-2,2,4-trimethylpentyl ester	+	+	+
241	$C_{13}H_{23}NO_3$	24.305	4-t-Butyl-2-(1-methyl-2-nitroethyl)cyclohexanone		+	+
204	$C_{15}H_{24}$	24.344	.alpha.-Cubebene	+		
180	$C_{12}H_{20}O$	24.456	4a,5-Dimethyloctahydro-2(1H)-naphthalenone	+		+
210	$C_{14}H_{26}O$	24.68	(6Z)-3,7-Dimethyl-6,11-dodecadien-1-ol	+	+	+
204	$C_{15}H_{24}$	24.773	Cyclohexane, 1-ethenyl-1-methyl-2,4-bis(1-methylethenyl)-, [1S-(1.alpha.,2.beta.,4.beta.)]-	+		
204	$C_{15}H_{24}$	25.34	(-)-Aristolene	+		
226	$C_{14}H_{26}O_2$	25.385	2,4,7,9-Tetramethyl-5-decyne-4,7-diol			+
204	$C_{15}H_{24}$	25.636	Caryophyllene	+		
202	$C_{15}H_{22}$	25.887	(3R,4aS,8aS)-8a-Methyl-5-methylene-3-(prop-1-en-2-yl)-1,2,3,4,4a,5,6,8a-octahydronaphthalene	+		
204	$C_{15}H_{24}$	26.114	Zingiberene	+		
204	$C_{15}H_{24}$	26.228	.alpha.-Guaiene	+		
220	$C_{15}H_{24}O$	26.75	(1R,2R,4S,6S,7S,8S)-8-Isopropyl-1-methyl-3-methylenetricyclo[4.4.0.02,7]decan-4-ol	+		
166	$C_{11}H_{18}O$	26.78	Homomyrtenol			+
204	$C_{15}H_{24}$	27.021	Aromandendrene	+		
204	$C_{15}H_{24}$	27.45	Guaia-6,9-diene	+		
166	$C_{10}H_{14}O_2$	27.572	Cyclopentaneacetaldehyde, 2-formyl-3-methyl-.alpha.-methylene-	+		+
202	$C_{15}H_{22}$	27.75	Curcumene	+		+
154	$C_{10}H_{18}O$	28.013	Isogeraniol	+	+	+
220	$C_{15}H_{24}O$	28.174	(2S,3S,6S)-6-Isopropyl-3-methyl-2-(prop-1-en-2-yl)-3-vinylcyclohexanone	+		
204	$C_{15}H_{24}$	28.508	4,5-di-epi-aristolochene	+		
202	$C_{15}H_{22}$	28.689	(3R,4aS,8aS)-8a-Methyl-5-methylene-3-(prop-1-en-2-yl)-1,2,3,4,4a,5,6,8a-octahydronaphthalene	+		
206	$C_{14}H_{22}O$	28.858	3,5-Di-tert-butylphenol	+	+	+
166	$C_{10}H_{14}O_2$	29.455	2-(1-Formylvinyl)-5-methylcyclopentanecarbaldehyde	+	+	
220	$C_{15}H_{24}O$	29.465	Caryophyllene oxide	+	+	+
334	$C_{22}H_{38}O_2$	30.136	Undec-10-ynoic acid, undec-2-en-1-yl ester	+	+	+
222	$C_{15}H_{26}O$	30.361	Cubenol	+	+	
264	$C_{17}H_{28}O_2$	30.811	Nerolidyl acetate	+		
222	$C_{15}H_{26}O$	30.984	Ledol	+		
302	$C_{20}H_{30}O_2$	31.235	cis-5,8,11,14,17-Eicosapentaenoic acid		+	+
220	$C_{15}H_{24}O$	31.255	1H-Cycloprop[e]azulen-7-ol, decahydro-1,1,7-trimethyl-4-methylene-, [1ar-(1a.alpha.,4a.alpha.,7.beta.,7a.beta.,7b.alpha.)]-	+		
328	$C_{22}H_{32}O_2$	31.405	cis-4,7,10,13,16,19-Docosahexanoic acid		+	+
220	$C_{15}H_{24}O$	31.425	2-((2R,4aR,8aS)-4a-Methyl-8-methylenedecahydronaphthalen-2-yl)prop-2-en-1-ol	+		
208	$C_{15}H_{28}$	31.657	Selinan	+		+
222	$C_{15}H_{26}O$	31.79	Guaiol	+		
360	$C_{24}H_{40}O_2$	31.905	Undec-10-ynoic acid, tridec-2-yn-1-yl ester	+	+	
252	$C_{17}H_{32}O$	32.313	14-Methyl-8-hexadecyn-1-ol	+	+	+

Continued...

Table 3: Cont'd.

Molecular Mass	Molecular Formula	R.Time	Name	M	W	E
204	$C_{15}H_{24}$	32.394	Guaia-6,9-diene	+	+	+
220	$C_{15}H_{24}O$	32.569	Dehydroxy-isocalamendiol	+	+	
222	$C_{15}H_{26}O$	32.595	Guai-1(10)-en-11-ol			+
220	$C_{15}H_{24}O$	32.73	(-)-Spathulenol	+	+	+
222	$C_{15}H_{26}O$	32.91	1-Naphthalenol, 1,2,3,4,4a,7,8,8a-octahy	+		
222	$C_{15}H_{26}O$	33.007	2-Naphthalenemethanol, decahydro-.alpha.,.alpha.,4a-trimethyl-8-methylene-, [2R-(2.alpha.,4a.alpha.,8a.beta.)]-	+		
240	$C_{15}H_{28}O_2$	33.075	Cryptomeridiol	+		
152	$C_{10}H_{16}O$	33.243	2,4,6-Trimethyl-3-cyclohexene-1-carboxaldehyde	+	+	
204	$C_{15}H_{24}$	33.355	1H-3a,7-Methanoazulene, 2,3,6,7,8,8a-hexahydro-1,4,9,9-tetramethyl-, (1.alpha.,3a.alpha.,7.alpha.,8a.beta.)-	+		
168	$C_{11}H_{20}O$	33.867	(2,2,6-Trimethyl-bicyclo[4.1.0]hept-1-yl)-methanol	+		+
220	$C_{15}H_{24}O$	34.293	Caryophyllene oxide	+	+	+
204	$C_{15}H_{24}$	34.44	Longicyclene	+		+
222	$C_{15}H_{26}O$	34.598	1H-Cycloprop[e]azulen-4-ol, decahydro-1,1,4,7-tetramethyl-, [1ar-(1a.alpha.,4.alpha.,4a.beta.,7.alpha.,7a.beta.,7b.alpha.)]-	+		+
238	$C_{15}H_{26}O_2$	34.75	Isocalamenediol		+	+
220	$C_{15}H_{24}O$	34.857	Longifolenealdehyde	+		
218	$C_{15}H_{22}O$	34.925	,6,6-Trimethyl-2-(3-methylbuta-1,3-dienyl)-3-oxatricyclo[5.1.0.0(2,4)]octane			+
290	$C_{20}H_{34}O$	34.989	Copalol	+	+	
222	$C_{15}H_{26}O$	35.145	Drim-7-en-11-ol	+		+
124	C_9H_{16}	35.274	(2E,4E)-6,6-Dimethyl-2,4-heptadiene	+		
152	$C_{10}H_{16}O$	35.724	trans-Verbenol	+		
222	$C_{15}H_{26}O$	35.849	Drimenol	+		+
270	$C_{17}H_{34}O_2$	35.92	Isopropyl myristate			
180	$C_{12}H_{20}O$	35.961	2,2,6,8-Tetramethyl-7-oxatricyclo[6.1.0.0(1,6)]nonane	+	+	
206	$C_{15}H_{26}$	36.268	Tricyclo[4.3.0.0(7,9)]nonane, 2,2,5,5,8,8-hexamethyl-, (1.alpha.,6.beta.,7.alpha.,9.alpha.)-	+		
278	$C_{16}H_{22}O_4$	36.47	Diisobutyl phthalate			+
206	$C_{14}H_{22}O$	36.636	Norpatchoulenol	+		+
270	$C_{17}H_{34}O_2$	37.19	Methyl 14-methylpentadecanoate	+		
334	$C_{20}H_{30}O_4$	37.62	Butyl octyl phthalate	+		+
152	$C_{10}H_{16}O$	38.18	cis-Chrysanthenol	+		+
206	$C_{15}H_{26}$	38.396	Patchoulane	+	+	
280	$C_{18}H_{32}O_2$	39.042	cis,cis-Linoleic acid	+		
296	$C_{19}H_{36}O_2$	39.101	Methyl elaidate	+		
234	$C_{15}H_{22}O_2$	39.265	Drinenin	+	+	
284	$C_{18}H_{36}O_2$	39.349	Methyl isoheptadecanoate	+		

+ indicates the presence of the listed compound in the indicated extract.

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. Table 2 shows the LC_{50} values of the extracts towards *A. franciscana*. LC_{50} values >1000 µg/mL towards *Artemia* nauplii have been defined as being non-toxic.[21] Based on these results, all extracts tested were deemed nontoxic.

Optimised GC-MS parameters were developed and used to examine the *T. lanceolata* berry extracts. The resultant gas chromatograms are presented in Figure 3. Numerous overlapping peaks were evident in the aqueous berry extract chromatogram (Figure 3a). A total of 79 peaks were detected in the aqueous *T. lanceolata* berry extract, with the peak eluting at 20.9 min being the most prominent. Comparison with a phytochemical library putatively identified this peak as the sesquiterpenoid polygodial (Table 3). Numerous overlapping peaks were also evident throughout the chromatogram, with a broad range of retention times between 10-40 min. The presence of peaks throughout the chromatogram

attest to the wide range of compounds of widely varying polarity extracted with water.

The methanolic berry extract GC-MS chromatogram (Figure 3b) was more complex than the aqueous extract chromatogram. Indeed, a total of 129 peaks were detected in this chromatogram, with major peaks at approximately 14.1, 15.1, 16.4, 19.1, 21.0, 31.3, 31.8 and 35.0 min. As for the aqueous extract, polygodial (eluting at approximately 21 min) was a major component. In addition, a further major peak was evident in the methanolic extract at approximately 16.5 min. A comparison the phytochemical database putatively identified this peak as linalool (Table 3). This compound was also present in the aqueous extract, albeit at a much lower level. Numerous overlapping peaks were also evident throughout the chromatogram, many at retention times corresponding to peaks in the aqueous extract. This indicates that methanol and water extracted many similar components, although many of the lower polarity compounds appear to be more effectively extracted into methanol than water Fewer peaks were evident in the ethyl acetate extract chromatogram (Figure 3c). Indeed, only 61 unique mass signals were detected in the ethyl acetate extract. As for the other extracts, polygodial was detected in the ethyl acetate berry extract, albeit in substantially lower levels. The ethyl acetate chromatogram also had a major peak present at 19.7 min, which was putatively identified as 2-methyl-2-phenyl-oxirane. Whilst this compound was also detected in both the aqueous and methanolic extracts it was only present in relative abundance in the ethyl acetate extract. Multiple other peaks were also noted in the ethyl acetate extract, many corresponding to peaks in the aqueous and methanolic extracts. However, several peaks in this extract were at different retention times than seen for the more polar methanolic and aqueous extracts.

In total, 156 unique mass signals were noted for the *T. lanceolata* berry extracts by GC-MS headspace analysis (Table 3). Empirical formulas and putative identifications were achieved for these compounds by comparison against a GC-MS mass and spectral database. A noteable feature of the GC-MS analyses is the diversity of terpenoids compounds noted in the *T. lanceolata* extracts. The monoterpenoids linalool oxide (16 min), linalool (16.4 min), cymene (16.9 min), α-terpineol (19.1 min) and isogeraniol (28 min) as well as the sesquiterpenoids polygodial (21 min), caryophyllene oxide (29.5 and 34.3 min) guania-6,9-diene (32.4 min), spathulenol (32.7 min) were present in all extracts. However, multiple other terpenoids were present in some but not all extracts. The sesquiterpenoid polygodial was present in all extracts (at approximately 21 min) as either the major peak, or one of the largest peaks. A further two sesquiterpenoids were also putatively identified as isomers of caryophyllene oxide. The GC-MS headspace analysis was unable to distinguish which of these isomers corresponded to each peak. One isomer was assumed to be caryophyllene oxide whilst the other compound was assumed to be the structural isomer humulene. Several aliphatic keto-compounds were also detected. Of these, 4-methyl-2-heptanone (11.2 min), 1,1,3,3-tetramethylcyclopentane (11.7 min), 6-methyl-5-hepten-2-one (12.7 min) and 2,2,6,6-tetramethyl-3,5-heptanedione (17.6 min) were present in all extracts. Similarly, the aliphatic compounds dodecane (13.2 min), 4-methylunadecane (16.5 min), nonanoic acid (21.2 min), butyl butoxyacetate (21.8 min), 2,6,11-trimethyldodecane (23 min), (6Z)-3,7-dimethyl-6,11-dodecadien-1-ol (24.7 min), undec-10-ynoic acid-undec-2-en-1-yl ester (30.1 min) and the aliphatic ester propanoic acid, 2-methyl-, 3-hydroxy-2,2,4-trimethylpentyl ester (24.2 min) were also present in all *T. lanceolata* berry extracts. Similarly, the benzene derivatives cumene (13.9 min), 1,2-dimethyl-4-ethylbenzene (15.8 min), 2-methyl-2-phenyl-oxirane (20 min) and 3,5-di-tert-butylphenol (28.9 min), were also present in all the berry extracts.

DISCUSSION

Whilst all the *T. lanceolata* extracts screened displayed potent *C. perfringens* growth inhibitory activity, the berry extracts generally had greater efficacy than the corresponding leaf extracts. Therefore, the berry extracts were further analysed to determine their phytochemical composition. An examination of the GC-MS headspace metabolomics profile analysis of the aqueous, methanolic, and ethyl acetate berry extracts highlights several interesting compounds. An obvious feature was the number and diversity of terpenoids present in all extracts. The monoterpoid α-terpineol, as well as the sequiterterpenoids polygodial and caryophyllene oxide, was prevalent in all *T. lanceolata* berry extracts. Indeed, polygodial was the major compound detected in the methanolic and aqueous extracts (based on peak area). This agrees with previous studies which frequently cite polygodial as a major component in *T. lanceolata* berries (6). Indeed, some studies have reported that polygodial may account for nearly 40 % of commercial *T. lanceolata* essential oil components.[22]

The bacterial growth inhibitory activity of polygodial has been reported in several studies. Polygodial isolated from *Warburgia salutaris* was reported to be a potent inhibitor of the growth of a panel of bacteria.[23] Indeed, MIC's of 12.5 µg/mL were reported for polygodial against *Staphylococcus aureus* and *Bacillus subtilis* in that study. Whilst less potent, the same study also reported good growth inhibition for polygodial against *Staphylococcus epidermidis, Micrococcus luteus, Escherichia coli* and *Klebsiella pneumoniae*, with MIC values generally ≤100 µg/mL. Other studies have reported little or no antibacterial activity for polygodial against limited panels of bacteria, although many of these studies only tested at relatively low concentrations (100 µg/mL)).[24] In contrast, more recent studies have demonstrated good bactericidal activity against both Gram-positive and Gram-negative bacteria.[25] Antifungal efficacy and mechanistic studies of polygodial have been more definitive, with several publications highlighting its potent fungicidal activity.[24,26,27] Polygoidal appears to exert its antifungal activity by several mechanisms. It non-specifically disrupts/denatures fungal integral membrane proteins by functioning as a non-ionic surfactant.[25] It also readily reacts with amino acids (especially cysteine and aromatic amino acids), resulting in further denaturation. As an additional antifungal mechanism, polygoidal may permeate cells by diffusing across the cell membrane. Once inside the cell, polygoidal interacts with various intracellular components and affects metabolic processes. It is possible that polygodial also interacts with bacterial cells in a similar way.

The monoterpene α-terpineol was also a common component across all *T. lanceolata* extracts. It is generally believed that monoterpenoids have the most potent broad spectrum bacterial inhibitory activity of all terpenoids compounds, and that this activity is closely linked to their lipophilic character.[28] A variety of monoterpenoids including α-terpineol have been shown to have potent antibacterial activity against a panel of Gram-positive and Gram-negative bacteria.[28,29] The small, hydrophobic nature of monoterpenoids allow them to insert into cytosolic membranes, altering the fluidity and permeability of the membrane, thereby changing the conformation and function of membrane proteins. This consequently interrupts crucial cellular processes including the respiratory chain. Furthermore, the cytoplasmic membrane comprises a cellular barrier to protons and larger ions.[30] Interestingly, bacteria respond to monoterpenoid exposure by modulating membrane fluidity.[31] Despite this, the specific antimicrobial mechanisms of monoterpenoids are not yet fully understood.

Interestingly, the non-specific growth inhibitory mechanism of monoterpenoids is inherently difficult for bacteria to counteract/develop resistance to. Indeed, to the best of our knowledge, no bacteria have yet

developed resistance to any monoterpenoid. Furthermore, as monoterpenoids are generally used in mixtures (extracts, essential oils), it is likely that several growth inhibitory compounds, perhaps with multiple growth inhibitory mechanisms, are used concurrently. This not only enhances the antibacterial efficacy, but also greatly decreases the likelihood of bacteria developing resistance.

A number of other terpenoids were also detected in the *T. lanceolata* berry extracts by GC-MS headspace analysis. Monoterpenoids were particularly prevalent, with 2,2,6-trimethyl-6-vinyltetrahydropyran (geraniol, Figure 4a), cineole (Figure 4b), α-pinene (Figure 4c), β-ocimene (Figure 4d), cymene (Figure 4e), linalool oxide (Figure 4f), linalool (Figure 4g), dihydro-γ-terpineol (Figure 4h), 4(10)-thujen-3-ol (sabinol) (Figure 4i), trans-linalool-3,7-oxide (Figure 4j), myrtenol (Figure 4k), p-eugenol (Figure 4l), homomyrtenol (Figure 4m), aromandendrene (Figure 4n) and isogeraniol (Figure 4o) detected in the *T. lanceolata* berry extracts. The bacterial growth inhibitory properties of many of these monoterpenoids have been extensively documented. One study reported noteworthy antibacterial activity for multiple monoterpenoids including α-terpineol, geraniol, cineole, α-pinene, linalool, sabinene and eugenol against an extended panel of bacteria.[32] Both Gram-positive and Gram-negative bacteria were susceptible, although the susceptibility varied widely between bacterial species. Unfortunately, that study did not report MIC values, making it difficult to compare efficacies with other compounds in other studies. However, the study was particularly interesting as the authors correlated the inhibitory activity of the monoterpenoids with their structures:

- Generally, terpenoids with a phenolic structure are more highly active than non-phenolic terpenoids.
- The presence of a hydroxyl group further enhances the potency of the terpenoids.
- The position of the hydroxyl group also influences the growth inhibitory potency of the terpenoids.
- Alkyl substitutions reduce the surface tension, altering polarity and subsequently altering bacterial selectivity.
- The addition of an acetate moiety further enhances antibacterial efficacy.
- When aldehyde or alcohol moieties are present, the bactericidal activity is increased substantially, possibly via protein degradation, solvating or dehydrating effects.

Similarly, other studies have also reported good antibacterial activity for other monoterpenoids. Eugenol causes bacterial cell wall degradation.[33] Cymene induces swelling of the cytoplasmic membrane, resulting in bacterial death.[34] Carvone dissipates the transmembrane pH gradient and cell potential, thus disrupting general metabolic function.[35] Cinnamaldehyde is a potent inhibitor of *E. coli* and *Salmonella typhimurium* growth.[36] It is believed that the cinnamaldehyde carbonyl group has affinity for bacterial decarboxylases, preventing their function.[37]

Several sesquiterpenoids including α-cubenene (Figure 5a), caryophyllene (Figure 5b), zingiberene (Figure 5c), α-guaiene (Figure 5d), cubenol (Figure 5e), ledol (Figure 5f), spathulenol (Figure 5g), guaiol (Figure 5h), guai-1(10)-en-11-ol (Figure 5i), longicyclene (Figure 5j), isocalamenediol (Figure 5k), longifolenealdehyde (Figure 5l) were also present in the *T. lanceolata* berry extracts. The potent antibacterial activity of sesquiterpenoid lactones isolated from red algae (*Laurencia* spp.) has been reported, with MIC values 10-40 μg/mL against *Chromobacterium violaceum*, *Erwinia* spp., two *Proteus* species. and two *Vibrio* species.[38] Whilst less potent, good growth inhibitory activity has also been reported for selina-4,11(13)-dien-3-on-12-oic acid isolated from *Varthemia iphionoides* against *Bacillus cereus*, *B. subtilis*, *E. coli, Micrococcus luteus, Salmonella enteritides* and *S. aureus.*[39] However, the sesquiterpenoids are generally considered to be less potent bacterial growth inhibitors than the monoterpenoids.[38] Instead, the sesquiterpenoids appear to exert their antibacterial activities through interactions with other phytochemicals. The sesquiterpenoids nerolidol, farnesol, bisabolol and apritone have been reported to enhance the susceptibility of bacteria to other antibiotic compounds, possibly by increasing the permeability of the bacterial membrane to those compounds.[40] Thus, as well as having moderate growth inhibitory activity themselves, it is likely that the *T. lanceolata* sesquiterpenoids enhance the activity of the other antibacterial compounds in the extracts. Whilst reports of antibacterial activity are lacking for many of the other sesquiterpenoids, some have been identified in bacterial growth inhibitory plant extracts.[41-45]

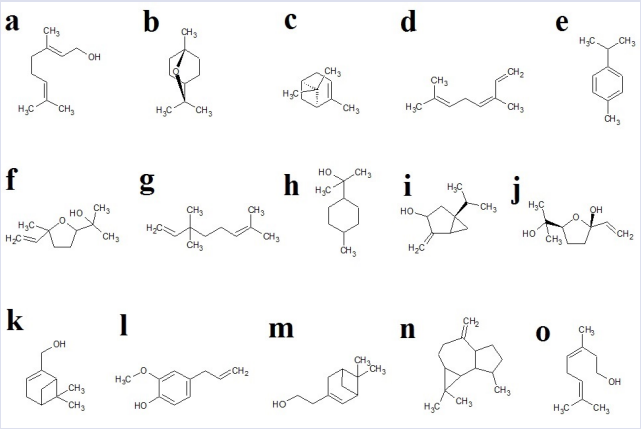

Figure 4: Monoterpenoid components which were detected in the *T. lanceolata* berry extracts by GC-MS headspace analysis: (a) geraniol, (b), cineole (c), α-pinene, (d) β-ocimene, (e) p-cymene, (f) linalool oxide, (g) linalool, (h) dihydro-γ-terpineol, (i) sabinol, (j) trans-linalool-3,7-oxide, (k) myrtenol, (l) p-eugenol, (m) homomyrtenol, (n) aromandendrene and (o) isogeraniol.

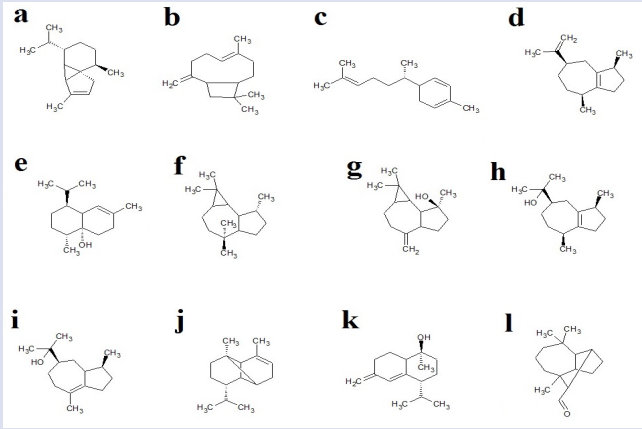

Figure 5: Sesquiterpenoid components detected in the *T. lanceolata* berry extracts by GC-MS headspace analysis: (a) α-cubenene, (b) caryophyllene, (c) zingiberene, (d) α-guaiene, (e) cubenol, (f) ledol, (g) spathulenol, (h) guaiol, (i) guai-1(10)-en-11-ol, (j) longicyclene, (k) isocalamenediol and (l) longifolenealdehyde.

Our study demonstrates that *T. lanceolata* berry extracts contain a variety of different compounds which may contribute to the *C. perfringens* growth inhibitory activity. Furthermore, a comparison between the metabolomics profiles of the extracts has highlighted several compounds of interest. However, it is unlikely that any single molecule is solely responsible for the *T. lanceolata* berry *C. perfringens* growth inhibitory activity. Instead, it is more likely that several compounds contribute to this activity. Furthermore, it is possible that synergistic interactions between the various bioactive components may be potentiating the growth inhibitory activity of the individual components, increasing their efficacy. At the very least, the presence of numerous molecules with growth inhibitory activity indicates that these extracts are likely to function by pluripotent pathways. Further studies are warranted to test the activity of the phytochemical compounds, both individually and in combinations.

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CONFLICTS OF INTEREST

The authors declare that they have no conflict of interest.

ABBREVIATION USED

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

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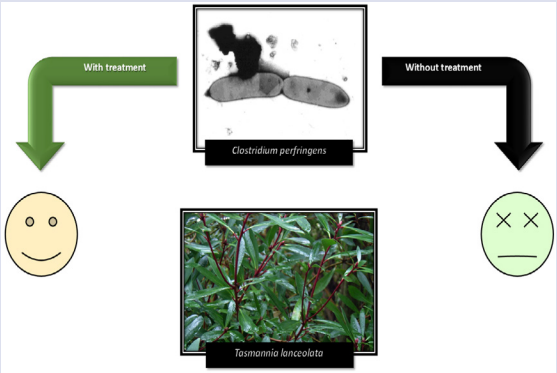
GRAPHICAL ABSTRACT



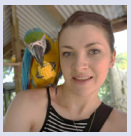
HIGHLIGHTS OF PAPER

- *T. lanceolata* berry and leaf extracts were potent inhibitors of *C. perfringens* growth (MIC values generally <1000 µg/mL).
- *T. lanceolata* berry extracts were particularly potent with MICs of 654, 65 and 329 µg/mL for the aqueous, methanolic and ethyl acetate extracts respectively.
- The aqueous, methanolic and ethyl acetate leaf extracts were also good growth inhibitors, with MICs of 839, 1255 and 625 µg/mL respectively.
- All *T. lanceolata* extracts were non-toxic in the *Artemia* nauplii assay.
- GC-MS metabolomic profiling of the berry extracts highlighted a diversity of terpenoids compounds.

AUTHOR PROFILE

Dr. Mitchell Henry Wright is a postdoctoral researcher at Oregon Health & Science University in Portland, Oregon (USA) where he works on investigating Mn(III) transformations in aquatic systems. Specifically, his research focuses on manganese oxidation/reduction by bacteria and how these organisms influence the geochemical cycling of the metal. His previous postdoctoral posting involved investigating the potential of Australian native plants in the treatment and prevention ofvarious pathogenic bacteria. This has resulted in several publications between both disciplines.

Mr. Cameron Jay Lee completed his Bachelor of Science with Honours in 2016 and is currently beginning his PhD. His research involves the investigation of thermophilic anaerobes that utilize toxic metals in anaerobic respiration (including uranium and arsenic). He has extensive experience in anaerobic cultivation/isolation andin numerous analytical techniques associated with heavy metal analysis.



Ms. Megan Sarah Jean Arnold is currently undertaking her PhD in Tropical Parasitology at Griffith University's EskitisInstitute for Drug Discovery with a focus on the identification and development of novel chemoprophylactic agents for malaria. Her other research interests include investigating Australian high antioxidant plants for theirantibacterial capabilities.

Dr. Anthony Carlson Greene is a senior lecturer and researcher at Griffith University, Brisbane Australia. He obtained his PhD in Microbiology from the University of New South Wales and focuses on extreme environments, Bioremediation and Geomicrobiology. His specific interests include the microbial ecology of thermophilic, saline and alkaliphilic environments and the mechanisms and industrial potential of extremophilic bacteria contained therein.

Dr Ian Edwin 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 *Scaevolaspinescens, Pittosporum phylliraeoides, Terminalia ferdinandiana* (Kakadu plum), Australian Acacias, Syzygiums, Petalostigmas and *Xanthorrhoea johnsonii*(grass trees). This range of projects has resulted in nearly 200 scientific publications in a variety of international peer reviewed journals.

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