Phytochemical Analysis of *Tasmannia lanceolata* Extracts and Inhibition of *Giardia duodenalis* Proliferation

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ABSTRACT

Background: Giardiasis is a debilitating disease caused by gastrointestinal parasites of the genus *Giardia*. *Tasmannia lanceolata* (Tasmanian pepper berry) has a high anti-oxidant capacity and has documented therapeutic properties for a variety of pathogenic diseases. **Materials and methods:** Solvent extracts of *T. lanceolata* berry and leaf were investigated for the ability to block *G. duodenalis* growth. The IC_{50} values of the extracts which displayed inhibitory activity were determined to quantify and compare their efficacies. Toxicity was determined using the *Artemia franciscana* nauplii bioassay. Active extracts were analysed by non-targeted HPLC-QTOF mass spectroscopy (with screening against 3 compound databases) for the identification and characterisation of individual components in crude plant extracts. **Results:** Methanolic, aqueous and ethyl acetate *T. lanceolata* berry and leaf extracts displayed potent *G. duodenalis* growth inhibitory activity. The methanolic extracts were the most potent growth inhibitors with IC_{50} values of approximately 180 µg/ml and 420 µg/ml for the berry and leaf methanolic extracts respectively. The aqueous, ethyl acetate, chloroform and hexane extracts also inhibited *G. duodenalis* growth, albeit with lower potency. HPLC-QTOF mass spectroscopy analysis of the extracts identified 45 compounds which were present in all *T. lanceolata* berry extracts. Forty of these were putatively identified by screening against 3 compound databases. All *T. lanceolata* berry and leaf extracts were non-toxic in the *Artemia fransiscana* bioassay. **Conclusion:** The low toxicity of the *T. lanceolata* extracts and their potent *G. duodenalis* growth inhibitory bioactivity indicates their potential as medicinal agents in the treatment and prevention of this disease.

Key words: Anti-Giardial activity, Anti-oxidant, Complementary, Alternative therapies, Gastrointestinal parasite, Giardisis, Tasmanian pepper.

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INTRODUCTION

Giardia duodenalis is a protozoal, intestinal parasite that is a common aetiological agent of infectious diarrhoea in humans and is especially associated with childhood morbidity, worldwide. According to World Health Organisation surveillance data, *G. duodenalis* (syn. *G. intestinalis*, *G. lamblia*) is one of the most neglected protozoal gastrointestinal diseases in humans with over 280 million cases of giardiasis reported worldwide per annum.[1,2] Acute infection, which is known as giardiasis, is endemic to many countries, particularly those with poor socio-economic conditions, inadequate sanitation, untreated water supplies and poor dietary status.[3] Giardiasis has also been recorded in many other animal species, including domestic pets (cats and dogs).[4] From a zoonotic perspective, there is notable concern that *G. Duodenalis* infections in companion animals, livestock and wild fauna may constitute sources for transmission to humans.[4]

One important issue for public health, land-use and water management professionals is the possible development of a reservoir of *Giardia* infection within the environment. Evidence suggests that this reservoir of infection has the potential to intermittently re-infect community drinking water supplies. Therefore, effective long-term management schemes to meet and maintain water quality standards ultimately depend on a comprehensive understanding of the sources of water contamination, reliable screening methods, and the ability to provide a variety of appropriate and alternative prevention and treatment strategies.[5]

Chemotherapeutic intervention presently offers a limited range of drugs and these are usually only employed after clinical diagnosis. Moreover, these drugs are ineffective against the infectious cysts, can produce unpleasant side effects, and are expensive with limited availability in developing countries. Frequent reports of drug toxicity, treatment failure and parasite drug resistance have, in some instances, also resulted in an increasing reluctance to over-prescribe synthetic anti-microbials. Despite some promising studies, there are no definitive chemoprophylactic, preventative treatments or vaccine for human giardiasis to date. Chemotherapeutic intervention for acutely infected individuals and chronic human and animal carriers has been recommended as a preventative strategy to limit infection transmission and to reduce endemic resurgence.However, only a narrow range of drugs are available. Such drugs include quinacrine, furazolidone, paromycin, the benzimidazoles and the nitroimidazoles.[6]

Metronidazole is currently still the drug of choice, although this is only effective against the trophozoite stage of the parasite, whilst the viable infectious cysts continue to be excreted into the environment. Furthermore, metronidazole and most other anti-giardial drugs produce unpleasant side effects, such as nausea, vertigo, anorexia, vomiting, diarrhoea, and hallucinations.[7,8] Drug toxicity with metronidazole therapy has also been widely reported.[9] The benzimidazoles (albendazole, mebendazole) seem to have lower incidences of adverse side effects compared to other anti-giardial drugs, but these drugs are relatively expensive and not as readily available in many developing countries where giardiasis is most prevalent.[6,9] There have also been increasing reports of treatment failure using the nitroimidazoles and benzimidazoles, and drug resistance by the organism is now a major rising concern.[6,7] Interestingly, from a social perspective, there also appears to be an increasing reluctance towards the use (or over-use) of synthetic anti-microbial drugs in disease treatment and prevention.[10] In more recent years, the introduction of new

and more effective agents has improved the overall chemotherapy for a number of parasitic diseases. However, the pharmaceutical industry is still plagued by numerous problems, including the ongoing development of resistance by infectious organisms to therapeutic agents and the limited repertoire of compounds available.

Therefore, one of the greatest challenges for public, rural, and medical health professionals involved in the control of giardiasis and other parasitic diseases is finding novel, safe, cost-effective drug alternatives. Recent studies have begun to re-examine the use of complementary and alternative therapies including plant extracts used in traditional healing systems,[11] essential oils,[12,13] and functional foods such as saturated fatty acid rich coconut milk[14] to treat giardiasis.

Tasmannia lanceolata (commonly known as Tasmanian pepper or mountain pepper) is a shrub which is endemic to the woodlands and cool temperate rainforests of Tasmania and the south-eastern region of the Australian mainland.[15] It is a medium to large shrub that varies between 2-5 m in height. Individual plants are unisexual, having either male or female flowers. The stems, branches and twigs are red in colour. The aromatic leaves are lanceolate to narrowly elliptical in shape (4-12 cm long, 0.7-2 cm wide) with a distinctly pale under surface. Small creamy-white unisexual flowers appear during the summer months. These develop into small fleshy black 2 lobed berries (5-8 mm wide) during autumn.

The berries, leaves and bark of this species have historical uses as a food and as a medicinal plant.[16] When the berry is air dried it forms a small, hard peppercorn which is suitable for milling or crushing. The berry has a pleasant spicy flavour and sharp aroma. *T. lanceolata* was used as flavouring agent by Australian Aborigines and more recently by European settlers. Historically, the leaves have been used as a herb and the berries have been used as a spice. Australian Aborigines also used *T. lanceolata* as a therapeutic agent to treat stomach disorders and as an emetic, as well as general usage as a tonic.[16] Reports also exist of the use of *T. lanceolata* by Australian Aborigines for the treatment and cure of skin disorders, venereal diseases, colic, stomach ache and as a quinine substitute.[16] Later, European colonists also recognized the therapeutic potential of *T. lanceolata* and the bark was used as a common substitute for other herbal remedies (including those derived from the related South American Winteraceae species, *Drimys wintera* (winter bark)[17] to treat scurvy due to its high anti-oxidant content.[16,18]

Despite its ethnobotanical usage, until recently there have been limited rigorous scientific studies into the therapeutic properties of *T. lanceolata*. Recent studies have demonstrated the high anti-oxidant content of *T. lanceolata* fruit and leaves.[18] It has been postulated that this high anti-oxidant content may provide therapeutic effects for this plant.[15] Indeed, studies within our laboratory have reported potent inhibition of bacterial growth by *T. lanceolata* berries, leaves and peppercorns.[19] However, despite the documented ability of *T. lanceolata* to inhibit prokaryotic cell growth, similar studies against eukaryotic infective agents are lacking. The current study was undertaken to test *T. lanceolata* leaf and berry extracts for the ability to inhibit the growth of the gastrointestinal parasite *G. duodenalis*.

MATERIALS AND METHODS

T. lanceolata samples

Dried *T. lanceolata* berry (without seed) and leaf materials were purchased from GoWild Harvest, Australia. The material was stored at -30ºC until use. The plant materials were thawed and freshly ground to a coarse powder prior to extraction. Individual 1 g quantities of the ground plant materials were prepared by weighing each plant part into each of 5 tubes and adding 50 ml of methanol, water, ethyl acetate, chloroform or hexane respectively. All solvents were obtained from Ajax 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 *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.[20,21]

Inhibitory bioactivity against *Giardia duodenalis* trophozoites
Parasite culture

The *Giardia duodenalis* S-2 (sheep strain 2) trophozoite strain used in this study were previously supplied by Professor Andre Buret, University of Calgary, Canada. *G. duodenalis* tropozoites were maintained and subcultured anaerobically at 37ºC 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 by direct parasite enumeration

Anti-Giardial activity of the extracts was also assessed by direct enumeration of parasite numbers in the presence or absence of extracts.[20] 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), or metronidazole (for the positive control) was added to individual wells and the plates were incubated anaerobically at 37ºC for 6 h in a humidified anaerobic atmosphere. Following the 6 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 and trophozoite viability were determined. All assays were performed in triplicate. 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 across a range of concentrations. The assays were performed as outlined above and graphs of the % of untreated Giardial growth versus concentration were plotted for each extract. Linear regression was used to calculate the IC$_{50}$ values.

Toxicity screening
Reference toxin for toxicity screening

Potassium dichromate ($K_2Cr_2O_7$) (AR grade, Chem-Supply, Australia) was prepared as a 1.6 mg/ml solution in distilled water and was serially diluted in artificial seawater for use in the *Artemia franciscana* nauplii bioassay.

Artemia franciscana nauplii toxicity screening

Toxicity was tested using a modified *Artemia franciscana* nauplii lethality assay.[22,23] Briefly, 400 µl of seawater containing approximately 46 (mean 46.8, n=148, SD 13.6) *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 sec. After 72 h all nauplii were sacrificed and counted to determine the total % mortality per well. The LC$_{50}$ with 95% confidence limits for each treatment was calculated using probit analysis.

Non-biased HPLC-MS quadrapole time-of-flight QTOF analysis

Chromatographic separations were performed using 2 µL injections of sample onto an Agilent 1290 HPLC system fitted with a Zorbax Eclipse plus C18 column (2.1×100 mm, 1.8 µm particle size). The mobile phases consisted of (A) ultrapure water and (B) 95:5 acetonitrile/water at a flow rate of 0.7 mL/min. Both mobile phases were modified with 0.1% (v/v) glacial acetic acid for mass spectrometry analysis in positive mode and with 5 mM ammonium acetate for analysis in negative mode. The chromatographic conditions utilised for the study consisted of the first 5 min run isocratically at 5% B, a gradient of (B) from 5% to 100% was applied from 5 min to 30 min, followed by 3 min isocratically at 100%. Mass spectrometry analysis was performed on an Agilent 6530 quadrapole time-of-flight (QTOF) spectrometer fitted with a Jetstream electrospray ionisation source in both positive and negative mode.

Data was analysed using the Masshunter Qualitative analysis software package (Agilent Technologies). Blanks using each of the solvent extraction systems were analysed using the Find by Molecular Feature algorithm in the software package to generate a compound list of molecules with abundances greater than 10,000 counts. This was then used as an exclusion list to eliminate background contaminant compounds from the analysis of the extracts. Each extract was then analysed using the same parameters using the Find by Molecular Feature function to generate a putative list of compounds in the extracts. Compound lists were then screened against three accurate mass databases; a database of known plant compounds of therapeutic importance generated specifically for this study (650 compounds); the Metlin metabolomics database (24,768 compounds); and the Forensic Toxicology Database by Agilent Technologies (7,509 compounds). Empirical formula for unidentified compounds was determined using the Find Formula function in the software package.

Statistical analysis

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

RESULTS

Liquid extraction yields and qualitative phytochemical screening

Extraction of 1 g of dried *T. lanceolata* berry or leaf with various solvents yielded dried plant extracts ranging from 9 mg (leaf hexane extract) to 171 mg (berry methanolic extract) (Table 1). Deionised water and methanol gave relatively high yields of dried extracted material for both berry and leaf, whilst all other solvents 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 1.

Qualitative phytochemical studies (Table 1) showed that methanol and water extracted the widest range of phytochemicals. Both showed high levels of phenolics (both water soluble and insoluble phenolics) and flavonoids for both berry and leaf. Moderate to high levels of saponins were also seen in the methanolic and aqueous extracts, with higher levels

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

	Extract	Mass of Dried Extract (mg)	Resuspended Extract Concentration (mg/ml)	Total Phenolics	Water Soluble	Water Insoluble	Cardiac Glycosides	Saponins	Triterpenes	Polysteroids	Alkaloids (Meyer test)	Alkaloids (Wagners test)	Flavanoids	Tannins	Free Anthraquinones	Combined Anthraquinones
Berry	Methanol	171	17.1	+++	+++	+++	-	++	+	-	-	-	+++	-	-	-
	Water	111	11.1	+++	+++	+++	-	-	-	-	-	-	+++	-	-	-
	Ethyl Acetate	56.7	5.7	+	+	++	-	+	++	-	-	-	++	-	-	-
	Chloroform	47.2	4.7	+	+	+	-	-	-	-	-	-	-	-	-	-
	Hexane	11	1.1	-	-	-	-	-	-	-	-	-	-	-	-	-
Leaf	Methanol	144	14.4	+++	+++	+++	-	+++	+	-	-	-	+++	-	-	-
	Water	134	13.4	+++	+++	+++	-	++	-	-	-	-	+++	-	-	-
	Ethyl Acetate	17	1.7	+	+	++	-	-	+	-	-	-	++	-	-	-
	Chloroform	37	3.7	+	-	+	-	-	-	-	-	-	-	-	-	-
	Hexane	9	0.9	-	-	-	-	-	-	-	-	-	-	-	-	-

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

Table 2: The *T. lanceolata* berry and leaf extract concentrations which inhibit 50 % of *G. duodenalis* growth (IC$_{50}$) (μg/mL) or induce 50 % mortality in the Artemia nauplii bioassay (LC$_{50}$ values) (μg/mL)

	Berry					Leaf				
	Methanol	Water	Ethyl Acetate	Chloroform	Hexane	Methanol	Water	Ethyl Acetate	Chloroform	Hexane
IC$_{50}$ (μg/mL) against *G. duodenalis*	183.7	467.9	553.7	1180	CND	421.6	630.8	803	CND	CND
LC$_{50}$ (μg/mL) in *Artemia* nauplii assay	3496	2140	-	-	-	-	-	-	-	-

Numbers indicate the mean IC$_{50}$ and LC$_{50}$ values of triplicate determinations. CND = could not be determined as the % inhibition did not reach 50 % at any concentration tested; -indicates that an LC$_{50}$ could not be determined as the % mortality did not exceed 50% at any concentration tested.

Table 3: Putative identification of compounds in the *T. lanceolata* berry extracts

Putative Identification	Empirical Formula	Molecular Mass	Retention Time (mins)	Detection Mode	
				Positive	Negative
	$C_3 H_9 N S_2$	123.0174	0.842	X	
7-hydroxy-5-heptynoic acid	$C_7 H_{10} O_3$	142.0619	0.842	X	
	$C_6 H_{14} O_4$	150.0892	2.351	X	
glucose	$C_6 H_{12} O_6$	180.0633	1.713	X	X
quinic acid	$C_7 H_{12} O_6$	192.0629	1.775		X
Butibufen	$C_{14} H_{20} O_2$	220.1462	10.933		X
Cyclohexyl(1-hydroxycyclopentyl)acetic acid	$C_{13} H_{22} O_3$	226.1566	10.074	X	X
polygodial	$C_{15} H_{22} O_2$	234.1628	7.768	X	X
capsidiol	$C_{15} H_{24} O_2$	236.1779	5.949	X	
Thiram	$C_6 H_{12} N_2 S_4$	239.9893	0.906	X	X
3,3'-(2-Oxo-1,1-cyclohexanediyl)dipropanoic acid	$C_{12} H_{18} O_5$	242.1136	1.917	X	
salutarisolide	$C_{15} H_{20} O_3$	248.1416	7.969	X	X
CITRININ	$C_{13} H_{14} O_5$	250.0842	4.728		X
Desmethylmianserin	$C_{17} H_{18} N_2$	250.1502	8.396		X
3-(1-adamantyl)-2-oxopropyl acetate	$C_{15} H_{22} O_3$	250.1534	9.061	X	X
PUNCTAPORIN B	$C_{15} H_{24} O_3$	252.1719	8.524	X	X
Gamma-Glu-Leu	$C_{11} H_{20} N_2 O_5$	260.1391	10.558	X	
abscisic acid	$C_{15} H_{20} O_4$	264.1357	8.958	X	X
5-(4-hydroxy-2,5-dimethylphenoxy)-2,2-dimethyl-Pentanoic acid (Gemfibrozil M1)	$C_{15} H_{22} O_4$	266.1497	9.192	X	X
8-Butyl-5,6-dihydrobenzo[f]quinazoline-1,3-diamine	$C_{16} H_{20} N_4$	268.1666	8.663	X	X
	$C_{15} H_{27} N O_3$	269.1996	8.51	X	
Lauroylsarcosine	$C_{15} H_{29} N O_3$	271.2151	7.396	X	
cyclandelate	$C_{17} H_{24} O_3$	276.1629	7.865	X	
Benzenepropanoic acid, 4-[2-hydroxy-3-[(1-methylethyl)amino]propoxy]- (Esmolol acid)	$C_{15} H_{23} N O_4$	281.1633	9.717	X	
Gallic acid, 2-ethylhexyl ester isomer 2	$C_{15} H_{22} O_5$	282.1461	7.459		X
Gallic acid, 2-ethylhexyl ester isomer 3	$C_{15} H_{22} O_5$	282.1462	5.305		X
Dihydroartemisinin	$C_{15} H_{24} O_5$	284.1605	8.31	X	X
N-(2,3-Dimethylphenyl)-1,3,5-triazaspiro[5.5]undeca-1,4-diene-2,4-diamine	$C_{16} H_{23} N_5$	285.1948	8.842	X	
Glu Phe	$C_{14} H_{18} N_2 O_5$	294.1216	11.7	X	
gingerol	$C_{17} H_{26} O_4$	294.1807	6.376	X	
Vomitoxin	$C_{15} H_{20} O_6$	296.1259	11.018	X	X

Table 3 Continue

	$C_{17} H_{31} N O_3$	297.2311	8.515	X	
benzeneacetic acid, 4-phenyl-, 4-methylphenyl ester	$C_{21} H_{18} O_2$	302.1283	9.661		X
Matricin	$C_{17} H_{22} O_5$	306.1463	7.97	X	X
Nadolol	$C_{17} H_{27} N O_4$	309.1947	9.716	X	
Glu Tyr	$C_{14} H_{18} N_2 O_6$	310.1166	10.717	X	
N-(2-Furylmethyl)-2-(4-methyl-1-piperazinyl)-4-quinazolinamine	$C_{18} H_{21} N_5 O$	323.1735	8.386	X	
Idebenone Metabolite (QS-8)	$C_{17} H_{24} O_6$	324.1577	6.332	X	X
Tetranor-PGEM	$C_{16} H_{22} O_7$	326.1367	4.007	X	X
9S,10S,11R-trihydroxy-12Z-octadecenoic acid	$C_{18} H_{34} O_5$	330.2407	8.811	X	X
combretastastin A-1	$C_{18} H_{20} O_6$	332.1319	11.166	X	X
dipivefrin	$C_{19} H_{29} N O_5$	351.2041	10.921	X	
Fetoxilate	$C_{36} H_{36} N_2 O_3$	544.2783	4.912	X	
KHAYASIN	$C_{32} H_{42} O_8$	554.2857	9.178		X

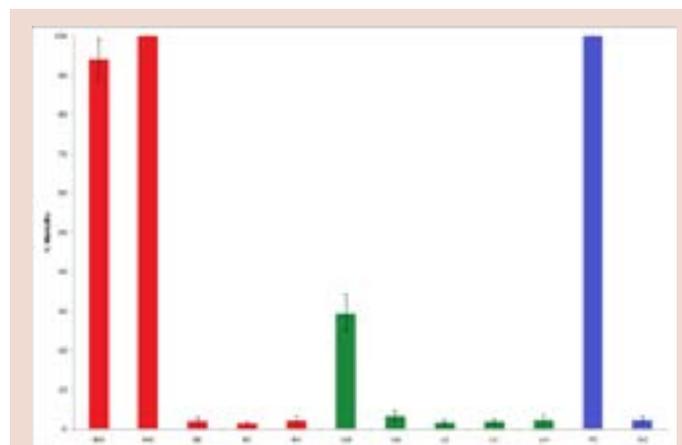Only compounds detected in all berry extracts by high accuracy QTOF LC-MS are shown.
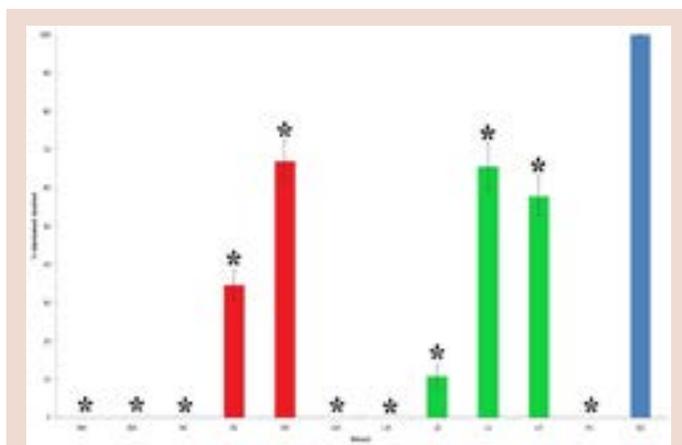
Figure 1: Inhibitory activity of *T. lanceolata* berry and leaf extracts against *Giardia duodenalis* trophozoites measured as a percentage the untreated control. B=berry; L=leaf; M=methanolic extract; W=water extract; E=ethyl acetate extract; C=chloroform extract; H=hexane extract; PC=metronidazole control (50 µg/ml); NC=negative control. Results are expressed as mean ± SEM of at least triplicate determinations. * indicates results that are significantly different (p<0.01) to the untreated control (NC).

Figure 2: The lethality of *T. lanceolata* berry (red) and leaf (green) extracts (1000 µg/ml) and potassium dichromate control (1000 µg/ml) towards *Artemia franciscana* nauplii after 24 h exposure. BM=berry methanolic extract; BW=berry water extract; BE=berry ethyl acetate extract; BC=berry chloroform extract; BH=berry hexane extract; LM=leaf methanolic extract; LW=leaf water extract; LE=leaf ethyl acetate extract; LC=leaf chloroform extract; LH=leaf hexane extract; PC=potassium dichromate control; NC=negative (seawater) control. Results are expressed as mean ± SEM of at least triplicate determinations.

evident in the leaf extracts than in the berry extracts. Low to moderate levels of triterpenes were also detected in the methanolic (but not in the aqueous extracts) of both berry and leaf. No other phytochemical classes were detected in the methanolic and aqueous extracts. The ethyl acetate extracts also had low to moderate levels of phenolics and triterpenes. Low levels of phenolics were detected in the chloroform extract whilst no phytochemical class was present in detectable levels in the hexane extract.

Inhibition of *Giardia duodenalis* proliferation

T. lanceolata berry and leaf extracts were screened for their ability to inhibit *Giardia duodenalis* growth (Figure 1). All extracts displayed significant inhibitory activity. The methanolic and aqueous extracts of both berry and leaf, and the berry ethyl acetate extract, were particularly potent, each inhibiting 100% of the Giardial growth (compared to the untreated control). Furthermore, these extracts were extremely rapid in producing their inhibitory activity, with 100% inhibition seen in less than 5 min of exposure (unpublished results). The leaf ethyl acetate extract also significantly inhibited trophozoite growth (to approximately 11% of the growth of the negative controls).

The inhibitory *T. lanceolata* extracts were further tested over a range of concentrations to determine the IC_{50} values (Table 2) for each extract against *G. duodenalis*. The methanolic berry extract was a particularly good inhibitor of *G. duodenalis* proliferation, with an IC_{50} of 183.7 µg/ml. The aqueous and ethyl acetate berry extracts also displayed good anti-giardial activity (approximately 470 and 550 µg/ml respectively). The leaf methanolic, aqueous and ethyl acetate extracts were also potent inhibitors of *G. duodenalis* proliferation, with IC_{50} values of approximately 420, 630 and 800 µg/mL respectively. We were unable to determine IC_{50} values for the berry hexane extract or for the leaf chloroform and hexane extracts as the levels of inhibition did not exceed 50% at any concentration tested.

Quantification of toxicity

T. lanceolata berry and leaf extracts were initially screened at 2000 µg/ml in the assay (Figure 2). For comparison, the reference toxin potassium

Figure 3: Positive ion RP-HPLC base peak chromatograms (BPC) of 2 μl injections of *T. lanceolata* berry (a) methanolic, (b) aqueous, (c) ethyl acetate, (d) chloroform and (e) hexane extracts.

dichromate (1000 μg/ml) was also tested in the bioassay. The potassium dichromate reference toxin was rapid in its onset of mortality, inducing mortality within the first 3 h of exposure and 100% mortality was evident following 4-5 h (unpublished results). The berry methanolic and aqueous extracts also induced significant mortality following 24 h exposure, indicating that they were toxic at the concentration tested. The berry ethyl acetate, chloroform and hexane extracts did not induce mortality significantly different to the seawater control and were therefore deemed to be nontoxic. The leaf extracts generally induced lower levels of mortality in the *Artemia* nauplii than did the corresponding berry extracts. Indeed, the methanolic extract was the only leaf extract to induce mortality significantly above that of the seawater control, albeit with a mortality substantially below 50%. Thus all of the leaf extracts were deemed to be nontoxic.

To further quantify the effect of toxin concentration on the induction of mortality, the extracts were serially diluted in artificial seawater to testacross a range of concentrations in the Artemia nauplii bioassay at 24 h. Table 2 shows the LC_{50} values of the *T. lanceolata* berry and leaf extracts towards *A. franciscana*. No LC_{50} values are reported for any of the leaf extracts or the berry ethyl acetate, chloroform and hexane extracts as less than 50% mortality was seen for all concentrations tested. Extracts with an LC_{50} greater than 1000 μg/ml towards *Artemia* nauplii have been defined as being nontoxic in this assay.[24] As none of the extracts had a LC_{50}<1000 μg/ml, all were considered nontoxic.

HPLC-MS/MS analysis

As the *T. lanceolata* berry extracts all displayed anti-Giardial activity, they were further examined by high accuracy HPLC-MS QTOF. The individual extract compound profiles were subsequently compared to identify any compounds that were common across these extracts as this has previously been shown to be an effective method of narrowing the focus of compounds responsible for a bioactivity[21] The resultant total compound positive ion chromatograms are presented in Figure 3a-3e.

The *T. lanceolata* berry methanolic (Figure 3a) and aqueous extract (Figure 3b) positive ion base peak chromatogram revealed multiple overlapping peaks, particularly in the early stages of the chromatogram corresponding to the elution of polar compounds. Most of the extracted compounds had eluted in the first 10 min, corresponding to 5-25% acetonitrile. Indeed, many of the peaks eluted in the first 5 min during the isocratic stage of the chromatogram (5% acetonitrile). However, the presence of several prominent peaks between 10 and 16.5 min indicates the broad spread of polarities of the compounds in this extract. The ethyl acetate (Figure 3c), chloroform (Figure 3d) and hexane extracts (Figure 3e) are characterised by fewer peaks than evident in the methanolic and aqueous extracts, and a shift towards a great percentage of the compounds eluting at higher acetonitrile %'s.

Qualitative mass spectral analysis of *T. lanceolata* berry extracts

In total, 998 unique mass signals were noted across the five *T. lanceolata* berry extracts (results not shown). Of these, 45 mass signals were present in all of the berry extracts which had anti-Giardial activity. Putative empirical formulas were achieved for all of these compounds. Of the 45 unique molecular mass signals detected across these extracts, 40 compounds (89%) were putatively identified by comparison to the Metlin metabolomics, forensic toxicology (Agilent) and phytochemicals (developed in this laboratory) databases (Table 3). Their structures are shown in Figure 4.

DISCUSSION

Recent studies have highlighted the potential of plant medicines and have demonstrated that some plant components are very effective inhibitors of *G. duodenalis* growth, with similar potency to the gold standard drug metronidazole.[14] Our studies demonstrates that *T. lanceolata* berry and leaf extracts also possess significant *G. duodenalis* growth inhibitory activity. The berry extracts were particularly potent, with IC_{50} values of approximately 180 and 470 μg/mL for the methanolic and aqueous extracts respectively. Also noteworthy was the rapid action of these extracts, with both the methanolic and aqueous extracts blocking 100% of *G. duodenalis* growth within 5 min of exposure. Furthermore, all *T. lanceolata* extracts were nontoxic in the *Artemia* nauplii bioassay, further demonstrating their suitability for chemotherapeutic treatment and prophylactic prevention of giardiasis.

Whilst all *T. lanceolata* extracts tested demonstrated Giardial antiproliferative activity, the berry extracts displayed higher potency and were therefore chosen for non-biased metabolomic comparison studies. The phytochemical composition of all *T. lanceolata* berry extracts was determined and compared to identify compounds common between extracts with *G. duodenalis* growth inhibitory activity. Similar metabolomics comparison studies have previously been used very successfully to narrow the focus of phytochemicals and allow for the identification of bioactive components in extracts from other plant species. In a recent study examining the anti-viral activity of *Scaevola spinescens*, a comparison of the metabolomic profiles of solvents of varying polarities was able to highlight 2 compounds from 239 detected mass signals as possibly
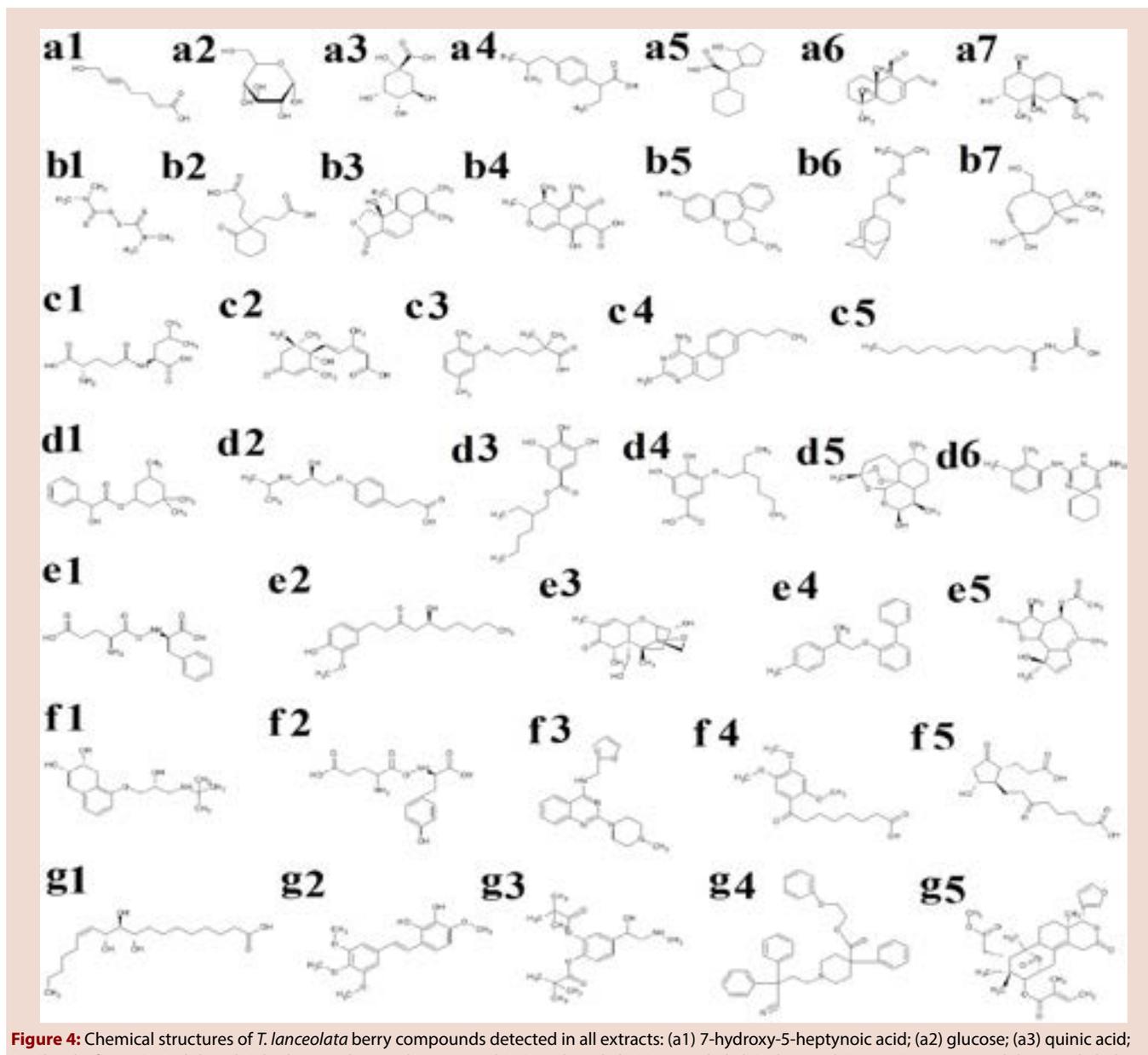
Figure 4: Chemical structures of *T. lanceolata* berry compounds detected in all extracts: (a1) 7-hydroxy-5-heptynoic acid; (a2) glucose; (a3) quinic acid; (a4) butibufen; (a5) Cyclohexyl (1-hydroxycyclopentyl)acetic acid; (a6) polygodial; (a7) capsidiol; (b1) thiram; (b2) 3,3'-(2-Oxo-1,1-cyclohexanediyl) dipropanoic acid, (b3) salutarisolide, (b4) citrinin; (b5) desmethylmianserin; (b6) 3-(1-adamantyl)-2-oxopropyl acetate; (b7) punctaporin B; (c1) gamma-Glu-Leu; (c2) abscisic acid; (c3) gemfibrozil M1; (c4) 8-Butyl-5,6-dihydrobenzo[f]quinazoline-1,3-diamine; (c5) lauroylsarcosine; (d1) cyclandelate; (d2) esmolol acid; (d3) gallic acid, ethylhexyl ester isomer 1; (d4) gallic acid, ethylhexyl ester isomer 2; (d5) dihydroartemisinin; (d6) N-(2,3-dimethylphenyl)-1,3,5-triazaspiro[5,5]undeca-1,4-diene-2,4-diamine; (e1) GluPhe; (e2) gingerol; (e3) vomitoxin; (e4) benzene acetic acid, 4-phenyl-, 4-methylphenyl ester; (e5) matricin; (f1) nadolol; (f2) Glu Tyr; (f3) N-(2-furylmethyl)-2-(4-methyl-1-piperazinyl)-4-quinazolinamine; (f4) idebenone metabolite (QS-8); (f5) tetranor-PGEM; (g1) 9S,10S,11R-trihydroxy-12Z-octadecenoic acid; (g2) combretastastin A-1; (g3) dipivefrin; (g4) fetoxilate; (g5) khayasin.

contributing to this activity.[25] Of these 2 compounds, 1 had been previously been reported to have anti-viral activity, validating this approach.

High accuracy QTOF HPLC-MS was used to examine the metabolomics profiles of the various *T. lanceolata* berry extracts in our study. Liquid chromatography–mass spectroscopy (LC-MS) is a good choice for the analysis of compounds of a wide variety of compounds, particularly those of medium and high polarity. Coupling LC with high mass accuracy spectroscopy techniques using both mild ionisation and electrospray ionisation (ESI) can generate large amounts of useful information for compound identification and metabolomic analysis. Using these methods, molecular ions can be detected and their empirical formulas accurately

determined and compared to databases. Furthermore, coupling this with ESI analysis also allows for the detection and characterisation of characteristic fragments, allowing for rapid identification of unknown compounds in a crude extract.

A number of interesting compounds were putatively identified in all *T. Lanceolata* berry extracts. Interestingly, the drimane-type sesquiterpene dialdehyde polygodial (Figure 4a6) was detected in all berry extracts. Several previous reports have reported polygodial as a major *T. lanceolata* component responsible for the flavour and aroma characteristics of this species.[15] Indeed, it has been reported that polygoidal may account for nearly 40% of commercial *T. lanceolata* essential oil components.[15] Many

studies have reported the therapeutic properties of this compound, including its anti-bacterial,[26] anti-fungal,[27-29] anti-hyperalgesia,[30] anti-inflammatory, anti-allergic and vasorelaxation activities.[31] Several studies have also reported protozoal growth inhibitory activity for polygodial and its derivatives. Potent growth inhibition was reported for polygodial isolated from *Drimysbra siliensis* against *Leishmania* spp. and *Trypanoso macruzi,* with minimum inhibitory polygodial concentrations as low as 2 µg/mL.[32] The same study also reported the induction of significant intracellular structural changes including mitochondrial swelling, loss of microtubule organisation and membrane damage following exposure to polygodial. Interestingly, these changes were extremely rapid, being apparent in<15 min (compared to >240 min for furazolidone which was used as a control drug in that study). This is consistent with our study, which showed significant Giardial death in less than 5 min. Another study also reported potent anti-Leishmanicidal activity for several polygodial derivatives as well as anti-malarial activity against *Plasmodium falciparum*.[33] We were unable to find similar reports of Giardial growth inhibitory activity for polygoidal. However, the inhibitory activity against other protozoal parasites indicates that polygodial may be responsible, at least in part, for the Giardial growth inhibition reported here.

Other structurally similar sesquiterpenoids were also putatively identified in the *T. lanceolata* extracts. Of particular interest, salutarolide (Figure 4b3) was present in all inhibitory *T. lanceolata* berry extracts. Salutarolide has been linked with anti-parasitic activity in multiple plants. In particular, plants of the genus *Warburgia* are known to have significant levels of salutarolide[34] and several *Warburgia* spp. have been reported to have anti-parasitic activities. *Warburgi augandensis* hexane extracts inhibit *Leishmania major* and *Leishmania donovani invitro*, with IC50 values of<10 µg/mL.[35] Similarly, *Warburgia salutaris* aqueous and methanolic extracts were reported to have anti-leishmanial activity, albeit with a higher IC_{50} values (>1 mg/mL). *W. salutaris* has also been reported to have anthelmintic activity against *Haemonchus contortus*.[36]

All extracts also contained a relative abundance of different isomeric forms of the gallotannin components ethyl hexyl gallic acid esters (Figure 4d3 and Figure 4d4). Gallotannins have been reported to inhibit the growth of a broad spectrum of microbial species[37] through a variety of mechanisms including binding cell surface molecules including lipotoichoic acid and proline-rich cell surface proteins,[38,39] and by inhibiting glucosyltransferase enzymes[40] Whilst we were unable to find similar studies reporting inhibitory effects of tannins on *Giardia* spp. growth, a number of studies have reported high tannin contents in a variety of plants used in traditional medicine to treat giardiasis.[41] Furthermore, tannins extracted from other plant species inhibit the growth of other protozoa including *Schistoso mamansoni* (the parasite responsible for schistosoma).[42]

The stilbene combretastatin A1 (Figure 4g2) was also putatively identified in all *T. lanceolata* berry extracts. Combretastatins are well known for their potent ability to block cancer cell progression and induce apoptosis by binding intracellular tublin, thereby disrupting microtubule formation.[43] Whilst we were unable to find reports of the anti-Giardial activity of combretastatin A1, it acts in a similar to that of colchicine (N-[(7S)-1,2,3,10-tetramethoxy-9-oxo-5,6,7,9-tetrahydrobenzo[a]heptlen-7-yl] acetamide) by binding the colchicines binding site in tubulin.[44] Thus, it is likely that combretastatin A1 may have anti-proliferative activity by blocking mitosis and thus blocking Giardial replication.

Several of the remaining compounds putatively identified in all inhibitory extracts contain lactone moieties. These include dihydroartemisin (Figure 4d5), vomitoxin (Figure 4e3), matricin (Figure 4e5) andkhayasin (Figure 4g5). This is aninteresting finding as many of the current chemotherapeutic drugs used to treat giardiasis are lactone containing compounds, particularly lactone substituted nitroimidazoles

(eg. metronidazole, secnidazole, tinidazole, ornidazole and albendazole). It has been suggested that compounds containing a lactone moiety may block the Giardial lipid deacylation/reacylation pathways.[45] As *Giardia* spp. are unable to synthesise lipids by de novo pathways, they use host gastrointestinal precursor lipids for the synthesis of membrane and cellular lipids by deacylation/reacylation reactions.[45] Thus, it is likely that lactone containing compounds may contribute to the inhibition of *G. duodenalis* growth by the blockage of lipid synthesis and metabolism pathways.

Quinic acid (Figure 4a3) was also identified in all *T. lanceolata* berry extracts. Recent studies have reported that substituted quinic acid compounds block leucyl-tRNA synthase activity in *G. duodenalis*.[46] Aminoacyl-tRNA synthases are essential for translation of the genetic code by attaching the correct amino acid to each tRNA. Thus, blockage of leucyl-tRNA synthase activity would result in ineffective Leu-tRNA production and thus the inhibition of protein synthesis. We were unable to find reports of non-substituted quinic acid having the same activity. However, if subsequent testing confirms this activity, it is possible that quinic acid may also contribute to the anti-giradial activity *T. lanceolata* berry extracts. We were unable to find reports of *Giardia* spp. growth inhibitory activity for any of the other compounds putatively identified in the inhibitory *T. lanceolata* berry extracts. However, it is possible that these may also contribute to the anti-giardial activity reported here.

CONCLUSION

The lack of toxicity and effective *G. duodenalis* growth inhibitory activity of the *T. lanceolata* berry and leaf extracts demonstrate their potential as therapeutic agents for the treatment of giardisis.

ACKNOWLEDGEMENTS

CONFLICT OF INTEREST

The authors have no conflicts of interest.

ABBREVIATION USED

DMSO: Dimethyl sulfoxide; **IC_{50}:** The concentration required to achieve 50% of the maximal effect; **LC_{50}:** The concentration required to achieve 50% mortality.

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PICTORIAL ABSTRACT
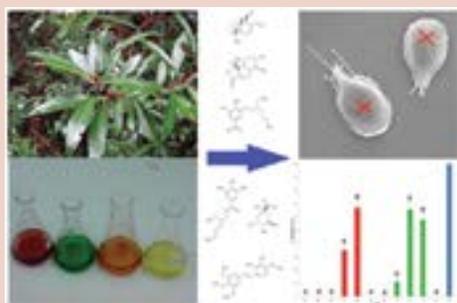
SUMMARY

• Tasmanian pepper (*T. lanceolata*) berry and leaf extracts are potent inhibitors of Giardia growth.

• All Tasmanian pepper extracts were nontoxic in the *Artemia fransiscana* bioassay.

• HPLC-QTOF MS analysis putatively identified 40 compounds in all inhibitory extracts.

• Based on the compound identifications, a pleuripotent anti-giardisis mechanism is proposed.

ABOUT AUTHOR

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 scientific publications in a variety of peer reviewed journals.