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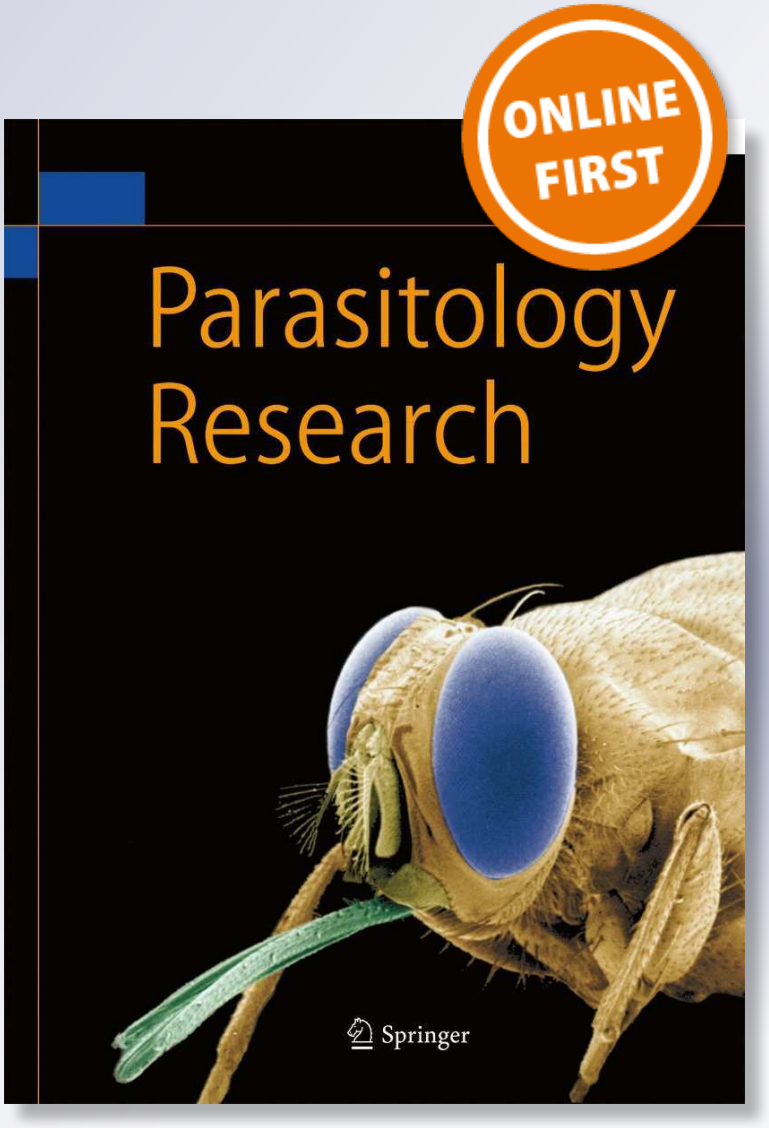

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ORIGINAL PAPER

Terminalia ferdinandiana extracts as inhibitors of *Giardia duodenalis* proliferation: a new treatment for giardiasis

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Abstract Giardisis is a debilitating disease caused by gastrointestinal parasites of the genus Giardia. High-antioxidant *T. ferdinandiana* fruit extracts were investigated for the ability to block *Giardia duodenalis* growth. Methanolic and aqueous extracts had the most potent growth inhibitory activity (IC50 values of approximately 700 and 140 μg/ml, respectively). Ethyl acetate and chloroform extracts also inhibited *G. duodenalis* growth, albeit with lower potency. The hexane extract was completely devoid of *G. duodenalis* growth inhibitory activity. All extracts were nontoxic in the *Artemia fransiscana* bioassay. Nontargeted HPLC-quadrupole time-of-flight (QTOF) mass spectroscopy (with screening against three compound databases) putatively identified 17 compounds in all of the inhibitory extracts but not in the inactive hexane extract. The low toxicity of the *Terminalia ferdinandiana* fruit extracts and their potent *G. duodenalis* growth inhibitory bioactivity indicate their potential as medicinal agents in the treatment and prevention of this disease.

Keywords Gastrointestinal parasite · Complementary and alternative therapies · Kakadu plum · Antioxidant · Antigiardial activity · Antiprotozoal activity

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Introduction

A re-examination of traditional herbal medicines for the treatment of giardiasis is an attractive prospect as the antiseptic qualities of medicinal plants have been long recognised and recorded. There has recently been a revival of interest in herbal medications due to a perception that there is a lower incidence of adverse reactions to plant preparations compared to synthetic pharmaceuticals. Furthermore, the use of complex mixtures such as whole foods or extracts would minimise the risk of developing antibiotic resistant strains of enteric bacteria. Whilst ongoing prophylactic treatment with a single antibiotic would certainly result in resistant bacterial strains, the use of functional foods or plant extracts with potent antibacterial activity would be expected to contain several antibiotic compounds which would be likely to function via several different mechanisms. There is a growing trend in the animal husbandry industry to switch to the usage of crude plant extracts rather than using pure antibiotic compounds for this reason. A literature search has been unable to find any reports of any bacterial species developing resistance to crude plant extracts.

Terminalia ferdinandiana (kakadu plum) is an endemic Australia plant which has received recent interest due to its reported high antioxidant content (Konczak et al. 2010; Netzel et al. 2007). The extremely high levels of ascorbic acid in kakadu plum are particularly noteworthy, with levels reported as high as 6 % of the recorded wet weight. This is approximately 900 times higher (g/g) than the ascorbic acid content in blueberries (which were used as a standard). As a further comparison, oranges and grapefruit (which are considered good sources of ascorbic acid) only contain approximately 0.007 % wet weight (0.5 % dry weight) (Johnson 2003). Due to its high vitamin C levels, the primary use of *T. ferdinandiana* fruit is currently for production of vitamin C in health food, cosmetic

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and pharmaceutical industries. However, *T. ferdinandiana* fruit also contains many other compounds which also contribute to its high antioxidant activity (Konczak et al. 2010; Netzel et al. 2007). While many of these compounds are yet to be identified, *T. ferdinandiana* fruit is known to contain benzoic acids, flavanols or flavanones (Konczak et al. 2010). *T. ferdinandiana* fruit is a good source of gallic acid and ellagic acid (Cunningham et al. 2009; Cherikoff and Kowalski 2008), which demonstrate strong antioxidant activity in vitro (Yilmaz and Toledo 2004). Lipophilic *T. ferdinandiana* fruit extracts are also rich in lutein (a carotenoid antioxidant compound associated with eye health) and with vitamin E and vitamin E analogues (Konczak et al. 2010). Hesperitin as well as the glycosides kaempferol, luteolin and quercetin are some of the other antioxidants present in *T. ferdinandiana* fruit (Konczak et al. 2010).

It has been postulated that the exceptionally high antioxidant content of *T. ferdinandiana* fruit may provide therapeutic effects for this plant (Mohanty and Cock 2012). Indeed, studies within our laboratory have reported potent inhibition of bacterial growth by kakadu plum fruit (Cock and Mohanty 2011) and leaf extracts (Courtney et al. 2015). However, despite the documented ability of kakadu plum to inhibit prokaryotic cell growth, similar studies against eukaryotic infective agents are lacking. In contrast, antiprotozoal activities have been reported for several other Terminalia species. *Terminalia mollis* (Maregesi et al., 2010), *Terminalia catappa* (Mudi & Muhammad, 2009), *Terminalia chebula* and *Terminalia bellerica* (Pinmai et al., 2010) have been reported to have activity against *Plasmodium falciparum. Terminalia avicennoides* (Alayande et al., 2011) and *Tereminalia ivorensis* (Agbedahunsi et al., 2006) have been reported to have inhibitory activity towards *Trypanosomiasis brucei* (the parasite responsible for sleeping sickness). *Terminalia bellerica* has also demonstrated trypanocidal activity against the related organism *Trypanosma evansi* (Shaba et al., 2009a, b) although no reports were found of its activity against *Trypanosomiasis brucei*. Based on its high antioxidant content and the inhibitory activity of other Terminalia species against other protozoan pathogens, the current study was undertaken to examine the ability of high antioxidant kakadu plum fruit extracts to inhibit *Giardia duodenalis* proliferation in vitro.

Materials and methods

T. ferdinandiana fruit pulp samples

T. ferdinandiana fruit pulp was supplied and verified/quality assured by David Boehme of Wild Harvest, Northern Territory, Australia. The pulp was frozen for transport and stored at −10 °C until processed. A voucher specimen (KP2014GD) is stored at Griffith University.

Preparation of extracts

T. ferdinandiana fruit pulp was thawed at room temperature and dried in a Sunbeam food dehydrator. The dried pulp material was subsequently ground to a coarse powder. A mass of 1 g of ground dried pulp was extracted extensively in 50 ml of either methanol, deionised water, ethyl acetate, chloroform or hexane for 24 h at 4 °C with gentle shaking. All solvents were supplied by Ajax and were AR grade. The extracts were filtered through filter paper (Whatman No. 54). The solvent extracts were air dried at room temperature. The aqueous extract was lyophilised by rotary evaporation in an Eppendorf concentrator 5301. The resultant pellets were dissolved in 10 ml deionised water (containing 0.5 % DMSO). The extract was passed through 0.22 μm filter (Sarstedt) and stored at 4 °C.

Qualitative phytochemical studies

Phytochemical analysis of the *T. ferdinandiana* extracts for the presence of saponins, phenolic compounds, flavonoids, polysteroids, triterpenoids, cardiac glycosides, anthraquinones, tannins and alkaloids was conducted by previously described assays (Arkhipov et al. 2014; Kalt and Cock 2014).

Antioxidant capacity

The antioxidant capacity of each sample was assessed using the 2,2-diphenyl-1-picrylhydrazyl (DPPH) free radical scavenging method (Jamieson et al. 2014; Winnett et al. 2014) with modifications. Briefly, DPPH solution was prepared fresh each day as a 400 μM solution by dissolving DPPH (Sigma) in AR grade methanol (Ajax, Australia). The initial absorbance of the DPPH solution was measured at 515 nm using a Spectra Max M3 plate reader and did not change significantly throughout the assay period. A 2 ml aliquot of each extract was evaporated, and the residue resuspended in 2 ml of methanol. Each extract was added to a 96-well plate in amounts of 5, 10, 25, 50 and 75 μl in triplicate. Methanol was added to each well to give a volume of 225 μl. A volume of 75 μl of the fresh DPPH solution was added to each well for a total reaction volume of 300 μl. A blank of each extract concentration, methanol solvent and DPPH was also performed in triplicate. Ascorbic acid was prepared fresh and examined across the range 0–25 μg per well as a reference, and the absorbances were recorded at 515 nm. All tests were performed in triplicate, and triplicate controls were included on each plate. The antioxidant capacity based on DPPH free radical scavenging ability was determined for each extract and expressed as microgram ascorbic acid equivalents per gram of original plant material extracted.

Inhibitory bioactivity against *Giardia duodenalis* trophozoites

Parasite culture

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

Evaluation of antigiardial activity by direct parasite enumeration

Antigiardial activity of the extracts was assessed by direct enumeration of parasite numbers in the presence or absence of extracts (Hart et al. 2014). For each test, aliquots of the trophozoite suspension (70 μl) containing approximately 1×10^5 trophozoites were added to the wells of a 96-well plate. A volume of 30 μl of the test extracts or the vehicle solvent or culture media (for the negative controls) was added to individual wells, and the plates were incubated anaerobically at 37 °C for 8 h in a humidified anaerobic atmosphere. Following the 8 h incubation, all tubes were placed on ice for a minimum of 10 min, followed by vortexing to dislodge the adherent trophozoites from the walls of the culture vessel. The suspensions were mounted onto a Neubauer haemocytometer (Weber, UK), and the total trophozoites per millilitre were determined. The antiproliferative activity of the test extracts was determined and expressed as a % of the untreated control trophozoites per millilitre.

Determination of IC50 values against Giardia trophozoites

For IC50 determinations, the plant extracts were tested by the direct enumeration method across a range of concentrations. The assays were performed as outlined above, and graphs of the zone of inhibition versus concentration were plotted for each extract. Linear regression was used to calculate the IC50 values.

Toxicity screening

Reference toxin for toxicity screening

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

Artemia franciscana nauplii toxicity screening

Toxicity was tested using a modified *Artemia franciscana* nauplii lethality assay (Arkhipov et al. 2014; Kalt and Cock 2014). 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 lx). 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 s. 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.

HPLC-MS/MS 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 to 30 min, followed by 3 min isocratically at 100 %. Mass spectrometry analysis was performed on an Agilent 6530 quadrapole time-of-flight spectrometer fitted with a Jetstream electrospray ionisation source in both positive and negative mode.

Data were 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 (800 compounds); the Metlin metabolomics database (24, 768 compounds) and the Forensic Toxicology Database by Agilent Technologies (7509 compounds). Empirical formula for unidentified compounds was determined using the Find Formula function in the software package.

Statistical analysis

Data were 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. ferdinandiana* fruit with various solvents yielded dried plant extracts ranging from 30 mg (ethyl acetate extract) to 483 mg (water extract) (Table 1). Deionised water and methanol gave relatively high yields of dried extracted material, whilst all other solvents extracted lower masses. The dried extracts were resuspended in 10 ml of deionised water 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, as well as moderate to high levels of tannins. Saponins were also present in low to moderate levels. Triterpenes and alkaloids were also present in low levels in the methanol extract. The ethyl acetate extract also had moderate levels of phenolics, flavonoids and triterpenes as well as low levels of saponins. Low levels of phenolics were detected in the chloroform extract, whilst no phytochemical class was present in detectable levels in the hexane extract.

Antioxidant capacity

Antioxidant capacity (expressed as ascorbic acid equivalence) for the *T. ferdinandiana* fruit extracts are shown in Table 1. The antioxidant capacity ranged from a low of 1 mg ascorbic acid equivalence per gram of dried plant material extracted (hexane extract) to a high of 660 mg ascorbic acid equivalence per gram of dried plant material extracted (methanol extract). Whilst significantly lower than the methanol extract, the aqueous extract also had a high antioxidant capacity with 264 mg ascorbic acid equivalence per gram of dried plant material extracted.

Inhibition of *Giardia duodenalis* proliferation

T. ferdinandiana fruit extracts were screened for their ability to inhibit *G. duodenalis* growth (Fig. 1). The methanol, water, ethyl acetate and chloroform extracts displayed significant inhibitory activity. The hexane extracts was completely ineffective as an inhibitor of proliferation, with no significant difference to the untreated control levels. The methanolic and aqueous extracts were particularly potent, each inhibiting 100 % of the giardial growth (compared to the untreated control). The ethyl acetate and chloroform extracts also significantly inhibited trophozoite growth, albeit with a much lower efficacy than was evident for the methanolic and aqueous extracts (to approximately 76 and 71 % of the growth of the negative controls respectively).

The inhibitory *T. ferdinandiana* extracts were further tested over a range of concentrations to determine the IC50 values (Table 2) for each extract against *G. duodenalis*. Inhibition of trophozoite growth was dose-dependent, with the level of inhibitory activity decreasing at lower concentrations. The water extract was a particularly good inhibitor of *G. duodenalis* proliferation, with an IC50 of 143 μg/ml. The methanol extract, whilst less potent, also displayed good antigiardial activity (at approximately 704 μg/ml). We were unable to determine IC50 values for the ethyl acetate and chloroform extracts as the levels of inhibition did not exceed 50 % at any concentration tested. However, it is noteworthy that the ethyl acetate extract was at a low concentration (3 mg/ml, which equates to 900 μg/ml tested in the assay).

Quantification of toxicity

T. ferdinandia fruit extracts were initially screened at 2000 μg/ml in the assay (Fig. 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 of mortality, inducing mortality within the first 3 h of exposure, and 100 % mortality was evident following 4–5 h (unpublished results). The methanol and water extracts also induced significant mortality following 24 h exposure, indicating that they were toxic at the concentration tested. The ethyl acetate, chloroform and hexane extracts did not induce mortality significantly different to the seawater control and were therefore 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 test across a range of concentrations in the Artemia nauplii bioassay at 24 h. Table 2 shows the LC50 values of the *T. ferdianadiana* fruit extracts towards *A. franciscana*. No LC50 values are reported for the ethyl

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

		M	W	E	C	H
	Mass of extract (mg)	359	483	30	62	18
	Concentration of extract (mg/ml)	35.9	48.3	3	6.2	1.8
Phenolics	Total phenolics	+++	+++	++	+	−
	Water-soluble phenolics	+++	+++	++	−	−
	Water-insoluble phenolics	+++	+++	+	−	−
	Cardiac glycosides	−	−	−	−	−
	Saponins	++	+	+	−	−
	Triterpenes	+	−	++	−	−
	Polysteroids	−	−	−	−	−
Alkaloids	Meyer test	+	−	−	−	−
	Wagner test	+	−	−	−	−
	Flavonoids	+++	+++	++	−	−
	Tannins	++	++	−	−	−
Anthraquinones	Free	−	−	−	−	−
	Combined	−	−	−	−	−
	Antioxidant capacity	660	264	39	7	1

+++ indicates a large response, ++ indicates a moderate response, + indicates a minor response, − indicates no response in the assay. *AA* ascorbic acid. Antioxidant capacity determined by DPPH reduction (expressed as milligram AA equivalence per gram plant material extracted)

acetate, chloroform and hexane extracts as less than 50 % mortality was seen for all concentrations tested. Extracts with an LC50 greater than 1000 μg/ml towards *Artemia* nauplii have been defined as being nontoxic in this assay (Cock and Ruebhart 2009). As none of the extracts had a LC50< 1000 μg/ml, all were considered nontoxic.

HPLC-MS/MS analysis

As the methanolic, aqueous, ethyl acetate and chloroform extracts all displayed antigiardial activity, yet the hexane extract did not, they were further examined by high-accuracy HPLC-mass spectroscopy (MS) quadrupole time-of-flight (QTOF)

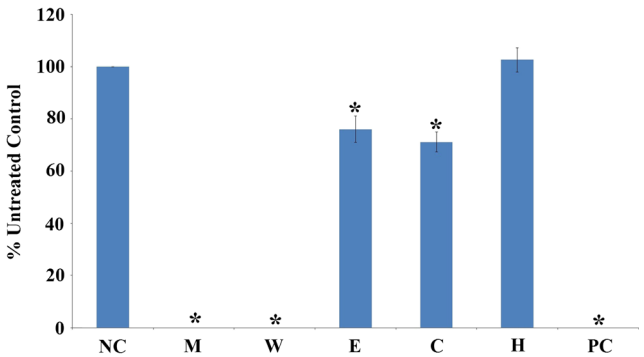

Fig. 1 Inhibitory activity of *T. ferdinandiana* fruit extracts against *Giardia duodenalis* trophozoites measured as a percentage of the untreated control. *M* methanolic extract, *W* water extract, *E* ethyl acetate extract, *C* chloroform extract, *H* hexane extract, *PC* metronidazole control (50 μg/ml), *NC* negative control. Results are expressed as mean ±SEM of at least triplicate determinations. * indicates results that are significantly different to the untreated control ($p<0.01$)

(Arkhipov et al. 2014). The individual extract compound profiles were subsequently compared to identify any compounds that were common across these extracts, yet not present in the hexane extract (which did not display any antigiardial activity) as this has previously been shown to be an effective method of narrowing the focus of compounds responsible for a bioactivity (Cock and Matthews 2015). The resultant total compound positive ion chromatograms are presented in Fig. 3a–e.

The *T. ferdinandiana* methanolic (Fig. 3a) and aqueous extract (Fig. 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, much 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 min indicates the broad spread of polarities of the compounds in this extract. The ethyl acetate, chloroform and hexane extracts are characterised by much fewer peaks than evident in the methanolic and aqueous extracts and a shift towards elution at high acetonitrile percentages. In particular, a prominent peak is present in the ethyl acetate, chloroform and hexane extracts (as well as the methanolic extract) at approximately 15.5–16 min.

Qualitative mass spectral analysis of *T. ferdinandiana* fruit extracts

In total, 1116 unique mass signals were noted across the five *T. ferdinandiana* fruit extracts (results not shown). Of these,

Table 2 The *T. ferdinandiana* fruit extract concentrations which inhibit 50 % of *G. duodenalis* growth (IC50) (μg/ml) or induce 50 % mortality in the Artemia nauplii bioassay (LC50 values) (μg/ml)

	Methanol	Water	Ethyl acetate	Chloroform	Hexane
IC50 (μg/ml) against *G. duodenalis*	704	143	CND	CND	–
LC50 (μg/ml) in Artemia nauplii assay	2115	2080	–	–	–

Numbers indicate the mean MIC and LC50 values of triplicate determinations. *CND* could not be determined as the % inhibition did not reach 50 % at any concentration tested, − indicates no inhibition

only 19 mass signals were present in all of the methanolic, aqueous, ethyl acetate and chloroform extracts but not in the hexane extract. Putative empirical formulas were achieved for all of these compounds. Of the 19 unique molecular mass signals detected across these extracts, 17 compounds (89 %) were putatively identified by comparison to the Metlin metabolomics, forensic toxicology (Agilent) and phytochemical (developed in this laboratory) databases (Table 3). Their structures are shown in Fig. 4.

Discussion

Giardisis is a major cause of infectious diarrhoea in humans and livestock worldwide. There are currently a limited range of drugs available for chemotherapeutic treatment of this disease, with the majority of these used only following clinical diagnosis and generally not for prophylaxis. These drugs are ineffective against some of the life stages of the pathogenic protozoa, have unpleasant and unwanted side effects and may have limited availability in developing countries. Frequent reports of drug toxicity, treatment failure and parasite resistance also highlight the importance to develop new chemotherapeutic treatments with greater efficacy and less severe side effects.

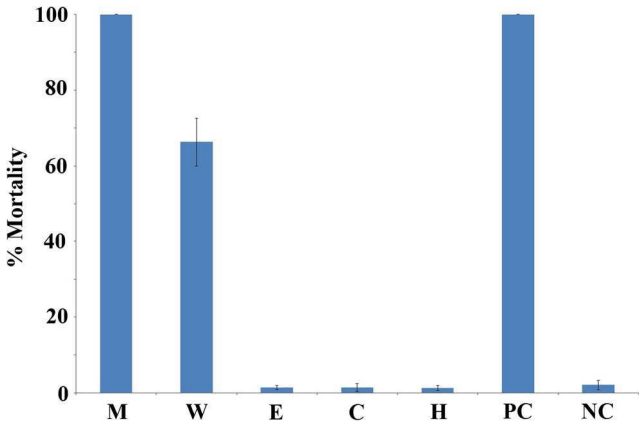

Fig. 2 The lethality of *T. ferdinandiana* fruit extracts (1000 μg/ml) and potassium dichromate control (1000 μg/ml) towards *Artemia franciscana* nauplii after 24 h exposure. *M* methanolic extract, *W* water extract, *E* ethyl acetate extract, *C* chloroform extract, *H* hexane extract, *PC* potassium dichromate control (1000 μg/ml), *NC* negative (seawater) control. Results are expressed as mean±SEM of at least triplicate determinations

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 (Rayan et al. 2005). Our studies demonstrate that *T. ferdinadniana* fruit extracts also possess significant *G. duodenalis* growth inhibitory activity. IC50 values of approximately 700 and 140 μg/ml are reported for the methanolic and aqueous fruit 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. ferdianadiana* fruit extracts were nontoxic in the Artemia nauplii bioassay, further demonstrating their suitability for chemotherapeutic treatment and prophylactic prevention of giardiasis.

The phytochemical composition of all *T. ferdianadiana* fruit extracts was determined and compared to identify compounds common between extracts with *G. duodenalis* growth inhibitory activity but not present in extracts lacking this activity. Similar metabolomic 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 antiviral activity of *Scaevola spinescens*, a comparison of the metabolomic profiles of solvents of varying polarities was able to highlight two compounds from 239 detected mass signals as possibly contributing to this activity (Cock and Matthews 2015). Of these two compounds, one had been previously been reported to have antiviral activity, validating this approach.

High-accuracy QTOF HPLC-MS was used to examine the metabolomic profiles of the various *T. ferdianadiana* fruit extracts in our study. Liquid chromatography-MS (LC-MS) is a good choice for the analysis 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

Fig. 3 Positive ion RP-HPLC total compound chromatogram (TCC) of ▶ 2 μl injections of *T. ferdinandiana* fruit **a** methanolic extract, **b** aqueous extract, **c** ethyl acetate extract, **d** chloroform extract, **e** hexane extract

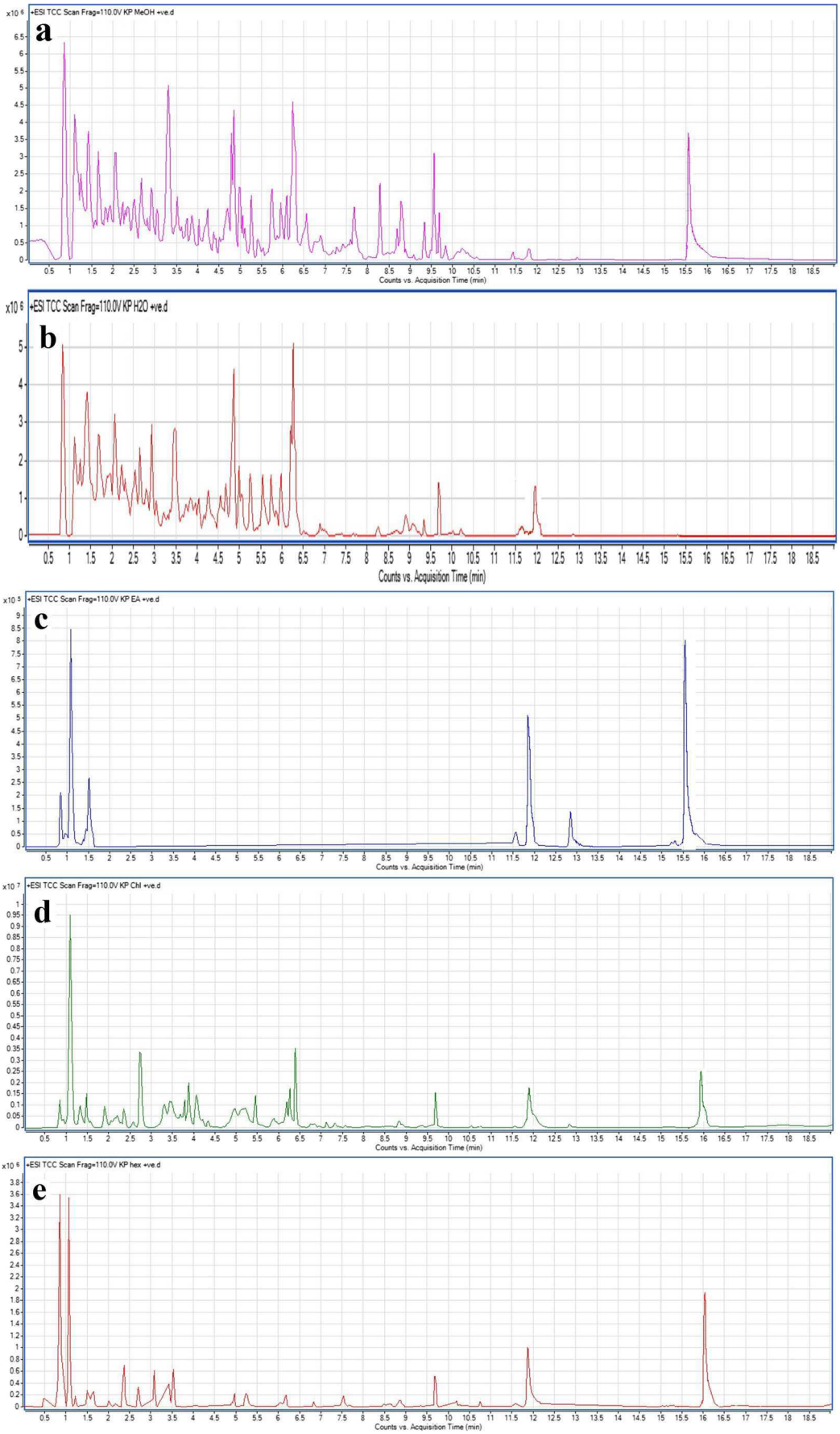
a
+ESI TCC Scan Frag=110.0V KP MeOH +ve.d
b
+ESI TCC Scan Frag=110.0V KP H2O +ve.d
c
+ESI TCC Scan Frag=110.0V KP EA +ve.d
d
+ESI TCC Scan Frag=110.0V KP Chl +ve.d
e
+ESI TCC Scan Frag=110.0V KP hex +ve.d
Counts vs. Acquisition Time (min)

Table 3 Putative identification of compounds in the *T. ferdinandiana* fruit extracts

Name	Formula	Mass	RT	Structure
Purine	C5 H4 N4	120.044	1.502	Fig. 4a
Trihhdroxybutanoic acid (gallic acid)	C4 H8 O5	136.037	1.372	Fig. 4b
Methoxycarbonyloxymethyl methyl carbonate	C5 H8 O6	164.032	1.45	Fig. 4c
Ribonolactone	C5 H8 O5	148.037	1.159	Fig. 4d
Apionic acid	C5 H10 O6	166.048	1.371	Fig. 4e
(1S,5R)-4-oxo-6,8-dioxabicyclo[3.2.1]oct-2-ene-2	C7 H6 O5	170.021	2.419	Fig. 4f
Carboxylic acid	C6 H8 O6	176.03	1.372	Fig. 4g
Ascorbic acid	C6 H10 O6	178.048	1.158	Fig. 4h
Gluconolactone	C7 H12 O6	192.063	1.379	Fig. 4i
Quinic acid glucuronic acid	C6 H10 O7	194.043	1.34	Fig. 4j
Glucoheptonic acid-1,4-lactone	C7 H12 O7	208.058	1.237	Fig. 4k
Eujavanoic acid	C14 H22 O3	238.156	11.034	Fig. 4l
5-(4-Hydroxy-2,5-dimethylphenoxy)-2,2-dimethylpentanoic acid (gemfibrozil M1)	C15 H22 O4	266.152	9.171	Fig. 4m
p-Hydroxytiaprofenic acid	C14 H12 O4 S	276.046	1.358	Fig. 4n
2,3-Dihydroxyphenyl B-D-glucopyranosiduronic acid	C12 H14 O9	302.063	3.243	Fig. 4o
	C11 H14 O10	306.057	2.414	–
Ferulic acid dehydrodimer	C19 H18 O6	342.116	1.075	Fig. 4p
Chebulic acid	C14 H12 O11	356.039	1.301	Fig. 4q
	C32 H55 N5 O5	589.42	15.311	–

Only compounds detected in the all of the methanolic, aqueous, ethyl acetate and chloroform extracts by high-accuracy QTOF LC-MS are shown. Compounds also detected in the hexane extract are not listed

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 identified in all extracts displaying antigiardial activity that were also absent in the inactive hexane extract. Purine (Fig. 4a) was putatively identified in all of the inhibitory extracts. Numerous studies have reported that *Giardia lamblia* are unable to synthesise their own purine or pyrimidine nucleotides (Baum et al. 1989; Jarroll et al. 1989). Instead, *G. lamblia* are reliant on salvage pathways to supply them with nucleotides for nucleic acid synthesis. These studies also reported that *G. lamblia* are incapable of interconversion between purine nucleotides. Furthermore, purine analogues have been reported to inhibit the growth of *G. lamblia* and have been proposed as potential chemotherapeutic agents to treat giardisis (Berens and Marr 1986). It is therefore possible that the *G. duodenalis* examined in our studies may incorporate the purine analogue identified in the inhibitory extracts into their nucleic acids during replication, causing DNA mismatches and blocking giardial proliferation.

The inhibitory extracts also contained a relative abundance of the gallotannin components gallic acid (Fig. 4b) and chebulic acid (Fig. 4q). Gallotannins have been reported to inhibit the growth of a broad spectrum of microbial species (Buzzini et al. 2008) through a variety of mechanisms including binding cell surface molecules including lipotoichoic acid and proline-rich cell surface proteins (Wolinsky and Sote 1984; Hogg and Embery 1982) and by inhibiting glucosyltransferase enzymes (Wu-Yuan et al. 1988). 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 (Tapia-Perez et al. 2003). Furthermore, tannins extracted from other plant species inhibit the growth of other protozoa including *Schistosoma mansoni* (the parasite responsible for schistosoma) (Abozeid et al. 2012).

Of the remaining compounds putatively identified in all inhibitory extracts, the majority of these contain lactone moieties. These include ribonolactone (Fig. 4d), (1S,5R)-4-oxo-6,8-dioxabicyclo[3.2.1]oct-2-ene-2-carboxylic acid (Fig. 4f), ascorbic acid (Fig. 4g), gluconolactone (Fig. 4h), glucuronic acid (Fig. 4j), glucohepatonic acid-1,4-lactone (Fig. 4k), p-hydroxytiaprofenic acid (Fig. 4n) and 2,3-dihydroxy phenyl B-D-glucopyranosiduronic acid (Fig. 4o). This is a noteworthy finding as many of the current chemotherapeutic drugs used to treat giardiasis are lactone-containing compounds, particularly lactone-substituted nitroimidazoles (e.g. metronidazole, secnidazole, tinidazole, ornidazole and albendazole). It has been suggested that compounds containing a lactone moiety may block the giardial lipid deacylation/reacylation

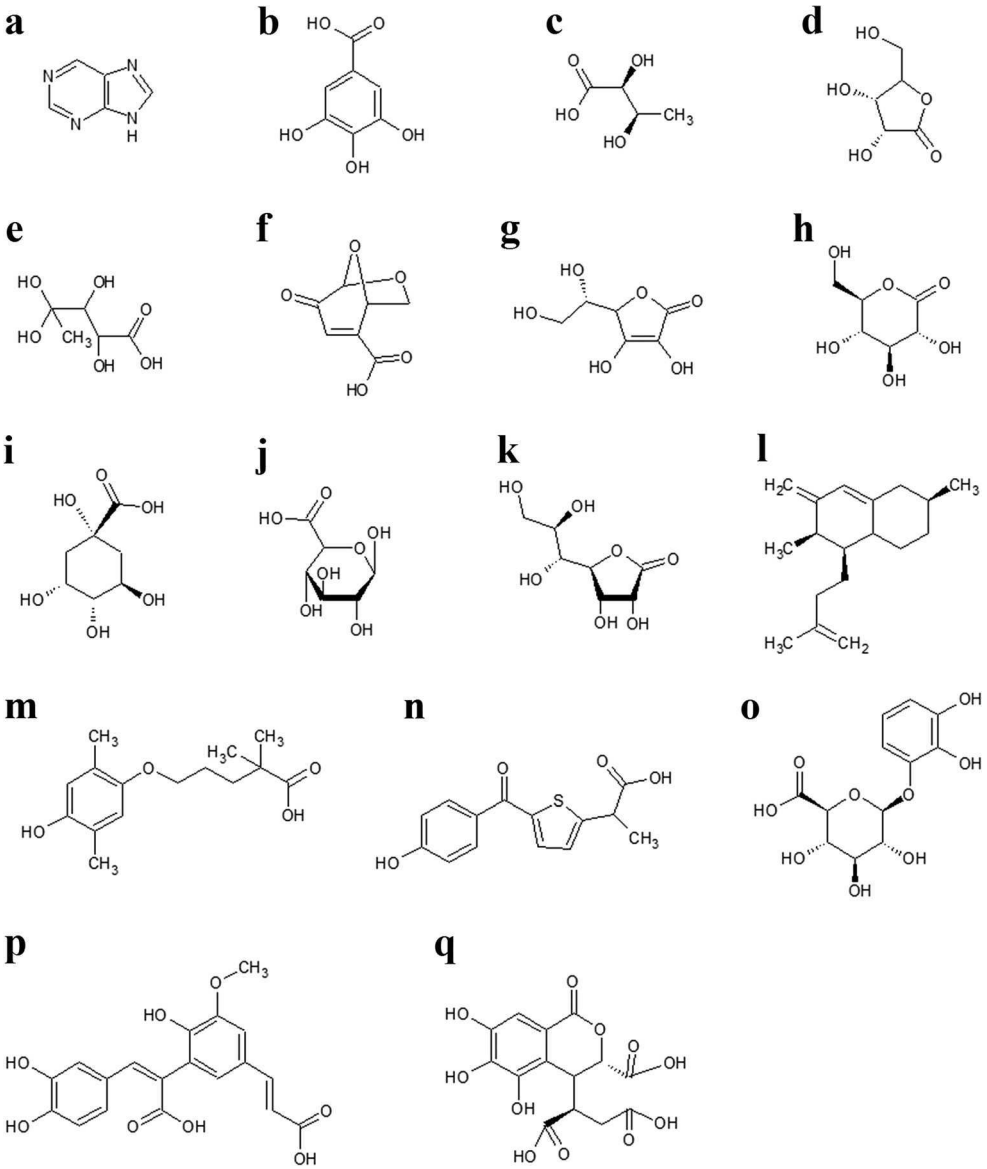

Fig. 4 Chemical structures of *T. ferdinandiana* fruit compounds detected in the methanolic, aqueous, ethyl acetate and chloroform extracts but not in the hexane extract: **a** purine; **b** gallic acid; **c** methoxycarbonyloxymethyl methylcarbonate; **d** ribonolactone; **e** apionic acid; **f** (1S,5R)-4-oxo-6,8-dioxabicyclo[3.2.1]oct-2-ene-2-carboxylic acid; **g** ascorbic acid; **h** gluconolactone; **i** quinic acid; **j** glucuronic acid; **k** glucohepatonic acid-1,4-lactone; **l** eujavonic acid; **m** 5-(4-hydroxy-2,5-dimethylphenoxy)-2,2-dimethylpentanoic acid (gemfibrozil M1); **n** p-hydroxytiaprofenic acid; **o** 2,3-dihydroxyphenyl B-D-glucopyranosiduronic acid; **p** ferulic acid dehydrodimer; and **q** chebulic acid

pathways (Das et al. 2001). 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 (Das et al. 2001). 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 (Fig. 4i) was also identified in all antigiardial *T. ferdianadiana* fruit extracts. Recent studies have reported that substituted quinic acid compounds block leucyl-transfer RNA (tRNA) synthase activity in *G. lamblia* (Zhang et al., 2012). 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 nonsubstituted quinic acid having the same activity. However, if subsequent testing confirms this activity, it is possible that quinic acid may also contribute to the antigiardial activity of the *T. ferdianadiana* fruit 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. ferdianadiana* fruit extracts. However, it is possible that these may also contribute to the antigiardial activity reported here.

Conclusions

The lack of toxicity and potent *G. duodenalis* growth inhibitory activity of the *T. ferdiandiana* fruit extracts demonstrate their potential as therapeutic agents for the treatment of giardisis.

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Conflict of interest The authors declare no competing financial interest.

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