Differential effectiveness of berry polyphenols as anti-giardial agents

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SUMMARY

Following previous work on the anti-giardial effect of blueberry polyphenols, a range of polyphenol-rich extracts from berries and other fruits was screened for their ability to kill *Giardia duodenalis*, an intestinal parasite of humans. Polyphenol-rich extracts were prepared from berries using solid-phase extraction and applied to trophozoites of *Giardia duodenalis* grown *in vitro*. All berry extracts caused inhibition at 166 μg gallic acid equivalents (GAE)/ml phenol content but extracts from strawberry, arctic bramble, blackberry and cloudberry were as effective as the currently used drug, metronidazole, causing complete trophozoite mortality *in vitro*. Cloudberry extracts were found to be the most effective causing effectively complete trophozoite mortality at 66 μg GAE/ml. The polyphenol composition of the more effective berry extracts suggested that the presence of ellagitannins could be an important factor. However, the potency of cloudberry could be related to high ellagitannin content but also to the presence of substantial amounts of unconjugated *p*-coumaric acid and benzoic acid. These *in vitro* effects occur at concentrations easily achievable in the gut after berry ingestion and we discuss the likelihood that berry extracts could be effective anti-giardial agents *in vivo*.

Key words: berry, polyphenol, *Giardia duodenalis*, ellagitannin, trophozoite, mortality.

INTRODUCTION

Giardia duodenalis is a flagellated protozoan parasite that colonizes and reproduces in the human intestine, causing giardiasis, of which the most common symptom is serious diarrhoea. Effective drug treatment for *G. duodenalis* is available, using orally administered metronidazole, but there is evidence of resistance (e.g. Harris *et al*. 2001; Upcroft *et al*. 2006). In addition, side effects such as nausea and headaches combined with an unpalatable metallic taste reduce patient compliance. In poorer countries where giardiasis is endemic, this condition affects both child development and mortality and chemotherapy may be minimally effective due to continual re-infection from the environment and therefore novel drugs and treatments are sought (Rossignol, 2010).

Plants and plant products have been used as traditional remedies for various ailments in numerous countries, with little or no side effects (Jones, 1996). Wild blueberries (*Vaccinium myrtillus*) and wild strawberries (*Fragaria vesca*) have been historically used to treat 'fluxes' (or florid diarrhoea) in the UK (see Anthony *et al*. 2007). The true causes of such diarrhoea/fluxes cannot be confirmed but certainly could be caused by enteropathogens (Cox, 2002) such as *Giardia* and *Cryptosporidium*.

The anti-microbial properties of plant products have been recognized for numerous years, and recently there has been renewed interest in berries and their phenolic constituents such as anthocyanins and ellagitannins (e.g. Nohynek *et al*. 2006). Polyphenol-rich berry extracts inhibited the growth of enteropathogenic bacteria such as *Salmonella*, *Escherichia*, *Staphylococcus*, *Helicobacter*, *Bacillus*, *Clostridium* and *Campylobacter* species, and were bacteriostatic for 3 *Staphylococcus aureus* strains (Puupponen-Pimia *et al*. 2005).

Trophozoites from infective cysts infect the duodenum, attaching onto enterocytes via a combination of hydrodynamic forces and their ventral 'sucking' disc and through lectins specific for D-glucosyl and D-mannose residues (Inge *et al*. 1988). Berry extracts may inhibit the adhesion and colonization of *Giardia* to human enterocytes. Proanthocyanidins found in cranberry juice (*Vaccinium macrocarpon*, and in other *Vaccinium* species) have potent bacterial anti-adhesion activity (Howell *et al*. 2005) and the proanthocyanidin,

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Geranin D, from *Geranium niveum* has potent anti-giardial activity (Calzada *et al*. 2001) Plant products have been demonstrated to possess anti-protozoan effects, but relatively little is known. Garlic extract inhibits *G. duodenalis* trophozoite growth *in vitro* (Lun *et al*. 1994) and treatment with garlic extract can reduce clinical signs and symptoms more rapidly than metronidazole, the current drug of choice (Soffar and Mokhtar, 1991). Whole garlic extract co-cultured with trophozoites causes internalization of flagella and ventral disc fragmentation (Harris *et al*. 2000; Anthony *et al*. 2005). Isoflavones, especially formononetin from *Dalbergia frutescens*, have potent anti-giardial activity *in vitro* (Khan *et al*. 2000) with a possible mode of action being the detachment of *Giardia* trophozoites *in vitro* and *ex vivo* through the inhibition of flagellar motility (Lauwaet *et al*. 2010).

Previous work investigated a pressed extract of blueberries, a commercial drink made from this extract and a polyphenolic-rich extract for their ability to influence *G. duodenalis* trophozoite viability *in vitro* and confirmed the effectiveness of the polyphenolic components (Anthony *et al*. 2007). This study examines the effects of polyphenol-rich extracts from a range of berry species with disparate composition on *Giardia* viability *in vitro*.

MATERIALS AND METHODS

Extraction of berries

Blackcurrants (*Ribes nigrum* L. variety 8982-6) were obtained from Bradenham Hall, Norfolk, UK and blueberries (*Vaccinium myrtillus* L. variety Berkeley) were grown at SCRI. Cloudberries (*Rubus chamaemorus* L.), arctic bramble (*Rubus stellatus* × *R. arcticus*), lingonberries (*Vaccinium vitis-idaea*), sea buckthorn (*Hippophae rhamnoides*) and rowan berries (*Sorbus aucuparia* L. c.v. Sahharnaja) were obtained from Dr Harri Kokko, University of Kuopio. Pomegranates (*Punica granatum* L.) were purchased from a local supermarket. Strawberries (*Fragaria ananassa* variety Elsanta) were obtained from local farmers.

The berries were extracted by the protocol outlined previously (McDougall *et al*. 2009) with minor changes. Briefly, the berries were homogenized in an equal volume to weight of 0.2% (v/v) acetic acid in 50% acetonitrile/ultra-pure water (UPW) in a Waring blender (5 times for 15 sec on full power). The extract was filtered through tripled muslin then centrifuged at 2800 *g* for 10 min at 4 °C to remove suspended polysaccharides.

The extracts were each applied to separate C18 solid-phase extraction (SPE) units (Strata C18-E, GIGA units, 10 g capacity Phenomenex Ltd, Macclesfield, UK) pre-washed in 0.2% (v/v) formic acid in acetonitrile then pre-equilibrated in 0.2% (v/v) formic acid in UPW. The unbound material, which contained the free sugars, organic acids and vitamin C, was collected. The units were washed with 3 column volumes of UPW then the polyphenol-enriched bound extracts eluted with 0.1% (v/v) formic acid in acetonitrile.

Phenol content was measured using a modified Folin–Ciocalteu method (Deighton *et al*. 2000) and quantified as gallic acid equivalents (GAE). Anthocyanin content was measured using the 2-wavelength method outlined previously (Deighton *et al*. 2000). The C18-bound extracts were evaporated to dryness in a Speed-Vac (Thermo-Finnegan Ltd) as required.

Culture of G. duodenalis *trophozoites*

Trophozoites of the BVM strain of *G. duodenalis* were maintained axenically in flat-sided 110 mm × 16 mm culture tubes at 37 °C, in a modified TYI-S-33 medium (Anthony *et al*. 2007) supplemented with 10% (v/v) heat-inactivated foetal bovine serum. Subculturing was performed routinely at 72–96 h intervals. After 72 h of culture, trophozoites were harvested by chilling culture vessels in iced water for 20 min. For addition to *G. duodenalis* cultures, aliquots of dried berry polyphenol extracts were reconstituted in TYIS-33 at a concentration of 2 mg ml^{-1} then passed through a sterile 0.22 μm filter (Sartorius).

Experiments were performed in sterile, 96-well microtitre plates covered with plate-sealer film and lids. To 100 μl of trophozoite culture ($\sim 2.7 \times 10^4$ trophozoites) 200 μl of berry extract diluted in TYI-S-33 was added to give suitable final concentrations. The microtitre plates were sealed and incubated for 24 h at 37 °C, after which the trophozoites were enumerated. The positive control consisted of 2.7×10^4 trophozoites in 300 μl of TYI-S-33 containing 67 μg ml^{-1} metronidazole (found to be the minimal inhibitory concentration within this system that killed 100% of trophozoites) and the negative controls consisted of 2.7×10^4 trophozoites in 300 μl of TYI-S-33 in the absence of berry extracts or metronidazole. All assays were carried out in triplicate.

Trophozoite enumeration was performed using an improved Neubauer haemocytometer and trophozoite viability determined by Trypan blue inclusion/exclusion. Briefly, trophozoites were harvested from wells by chilling the plate on iced water for 20 min and the contents of triplicate wells were combined into 1.5 ml microcentrifuge tubes, and then centrifuged (10 sec, bench top centrifuge 5410, Eppendorf GmbH, Germany). The supernatant was aspirated to waste and the pellet resuspended in 100 μl of TYI-S-33 and 100 μl of 0.4% Trypan blue solution (Gibco, UK) and mixed thoroughly. After mixing, 10 μl were dispensed into both chambers of an
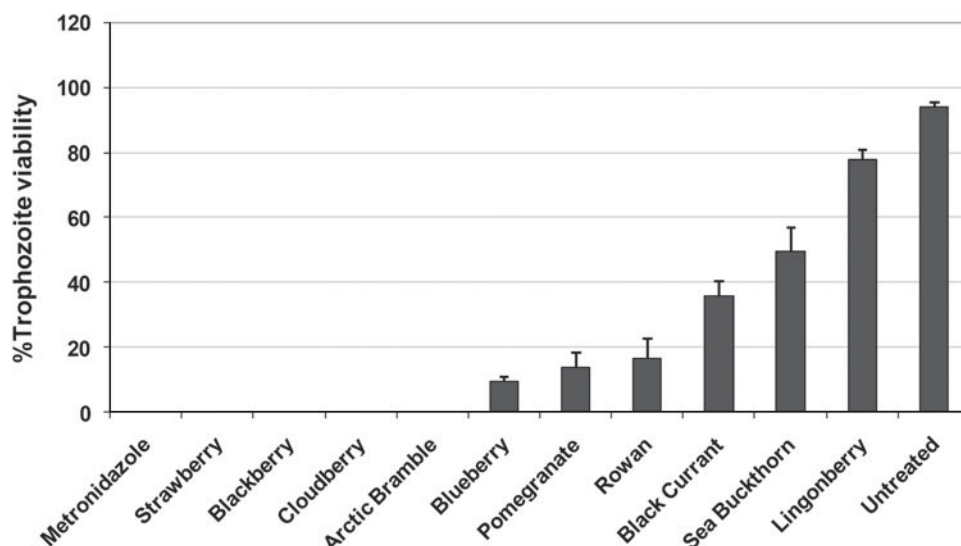
Fig. 1. Effect of berry extracts on *Giardia* viability. Trophozoites (2.7×10^4 trophozoites per well) were incubated for 24 h in the presence or absence of berry polyphenol extracts at 50 μg GAE per well. Untreated trophozoites and metronidazole (67 μg ml^{-1}) treated trophozoites were used as controls. All values are averages of triplicate experiments ± standard error. All berry extracts significantly reduced the survival of *G. duodenalis* trophozoites when compared to untreated trophozoites ($P \leqslant 0.05$).

improved Neubauer haemocytometer, allowed to settle (30–60 sec) then assessed.

Trophozoite viability was determined under bright-field microscopy at a total magnification of ×400 using the following formula: $(L \div (L + D)) \times 100$ where L is the number of live trophozoites (unstained) and D the number of dead trophozoites (stained with Trypan blue) with 3 counts of 100–200 trophozoites being taken and repeated twice.

Liquid Chromatography Mass Spectrometry (LC-MS) analysis

Samples containing 20 μg phenols (GAE) were analysed on an LCQ-Deca system, comprising Surveyor auto-sampler, pump and photo-diode array detector (PDAD) and a ThermoFinnigan mass spectrometer iontrap as described previously (McDougall *et al.* 2009). The PDAD scanned discrete channels at 280 nm, 365 nm and 520 nm. The samples (20 μl) were applied to a C-18 column (Synergi Hydro C18 with polar-end capping, 4.6 mm × 150 mm, Phenomenex Ltd, UK) and eluted over a gradient of 5% acetonitrile (0.5% formic acid) to 30% acetonitrile (0.5% formic acid) over 60 min at a rate of 400 μl/min. The LCQ-Deca LC–MS was fitted with an ESI (electrospray ionization) interface and analysed the samples in positive and negative ion mode. There were 2 scan events; full scan analysis followed by data dependent MS/MS of most intense ions using collision energies (source voltage) of 45%. The capillary temperature was set at 250 °C, with sheath gas at 60 psi and auxiliary gas at 15 psi. The MS was tuned against solutions of ellagic acid (negative mode) and cyanidin-3-*O*-glucoside (positive mode). Only negative mode data are shown.

Putative identifications of polyphenols are based on previous work in cloudberry (McDougall *et al.* 2008, 2009) and previous literature (Mullen *et al.* 2003; Hager *et al.* 2008; Gasperotti *et al.* 2010). Benzoic acid, *p*-coumaric acid and ellagic acid were employed as standards to confirm the identity of peaks. Peaks identified as ellagitannins were quantified using the MS system (Xcalibur) software and their PDA peak areas summed. This figure was divided by the total peak area of all polyphenol peaks to give an estimate of relative ellagitannin content. This estimate was carried out on 3 replicate injections of the arctic bramble and the cloudberry samples. This comparison was facilitated as the same ellagitannin peaks were largely present in cloudberry and arctic bramble.

RESULTS

Strawberry, blackberry, cloudberry and arctic bramble were as effective in killing *Giardia* as the positive control, metronidazole, at 50 μg GAE/well (Fig. 1). These effects were not due to changes in pH as the polyphenol-enriched extracts were effectively devoid of organic acids and did not alter the pH of the medium. There was no correlation between anthocyanin content and effectiveness as both cloudberry (< 0.1%) and blackberry (58.0%) anthocyanin content; (see Supplementary data, Table S1, Online version only) caused complete trophozoite killing at 50 μg GAE well^{-1}.

The most effective berry extracts were re-assayed at lower concentrations to assess dose-response effects (Fig. 2). This revealed an order of effectiveness of blueberry < strawberry < blackberry = arctic bramble < cloudberry. Cloudberry extracts were most
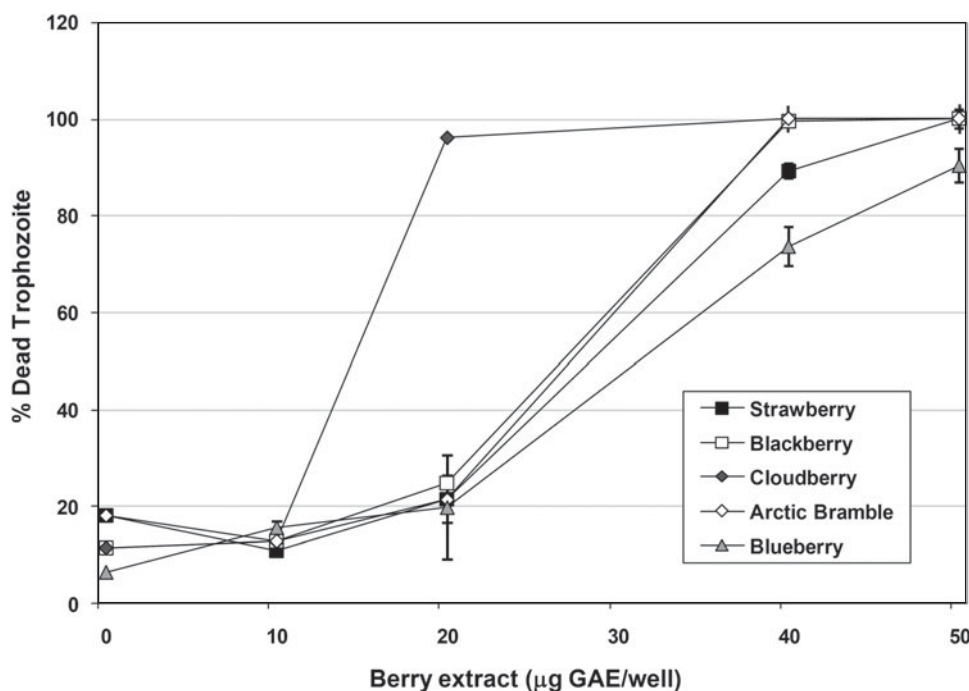
Fig. 2. Dose effects of selected berry extracts on *Giardia* viability. Trophozoites (2.7×10^4 trophozoites per well) were incubated for 24 h in the presence or absence of berry polyphenol extracts at various concentrations. Untreated trophozoites and metronidazole ($67 \, \mu g \, ml^{-1}$) treated trophozoites were used as controls. Control metronidazole treatments caused 100% trophozoite mortality with a replication error averaging at 2.6% and all berry polyphenol extracts caused a dose-dependent reduction in trophozoite viability.
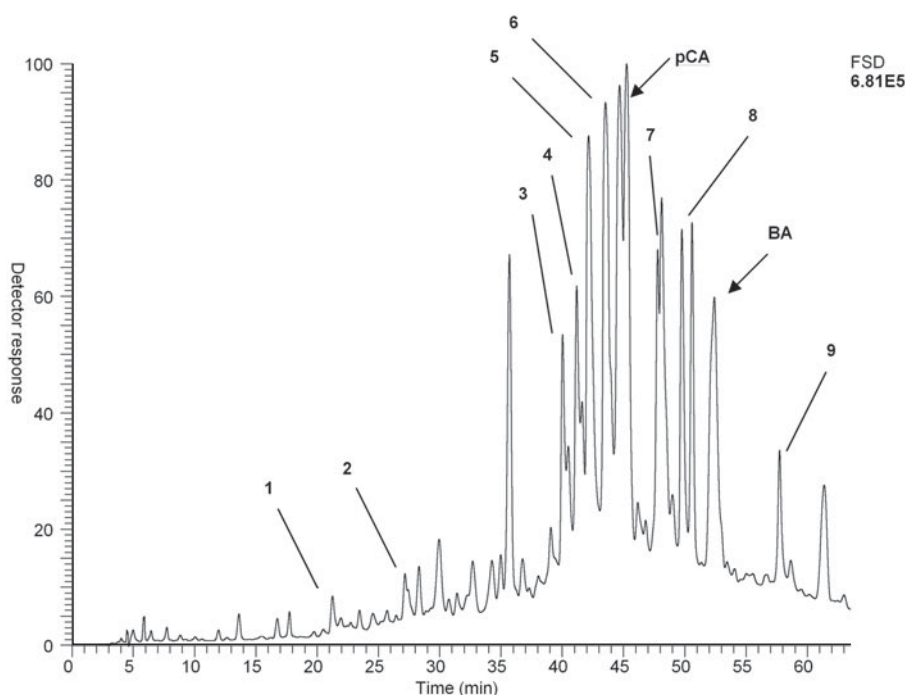

Fig. 3. LC-MS trace of cloudberry extract. All peak assignments relate to Table 1. pCA, *p*-coumaric acid and BA, benzoic acid. The figure in the top right corner is the full-scale deflection value for the PDA.

effective and caused high levels of killing at $20 \, \mu g$ GAE well^{-1} when the other extracts had lost activity.

LC-MS analysis (Fig. 3) confirmed that ellagitannins were the predominant polyphenolic components in the cloudberry extracts (Table 1) but also identified significant amounts of benzoic acid and

p-coumaric acid. As an example, the mass spectral properties leading to the identification of peak 6 as Sanguiin H6 are discussed in more detail in supplementary data (Supplementary Fig. S1, Online version only). Smaller amounts of flavonol derivatives (e.g. quercetin-*3-O*-glucuronide) could also be

Table 1. *Putative identification of ellagitannin peaks in cloudberry extract*

(All MS data are from negative mode ionization. The major signal is given in bold. Putative identifications are based on previous work on cloudberry (McDougall *et al.* 2008, 2009) and previous literature (Mullen *et al.* 2003; Gasperotti *et al.* 2010; Hager *et al.* 2008). More detailed information about the identification of peak 6 as sanguiin H6 is shown in the Supplementary data (Fig. S1), Online version only.)

Peak no.	Retention time	PDA max.	m/z [M-H]	MS^2	Putative identity
1	21.25	275	**783**, 481, 301	481, **301**, 275	Pendunculagin isomer
2	28.31	275	**633**, 301, 275	**301**	Galloyl-HHDP-glucose
3 Front	40.03		**1869**	**1567**, 1265, 1235, 1103, 933	Sanguiin H6 isomer
		275			
Rear			**1717**, 933		Sanguiin H-6
				1415, 1113, 933, 783	without gallic moiety
4 Front	41.17		**1567**	**1265**, 1103, 935, 801, 631	Sanguiin H10
		275			
Rear			**933**	897, 631, **451**, 315	Unknown ellagitannin
5	42.15	275	**1401**	Multiple	Lambertinian C
6	43.52	275	**1869**	**1567**, 1265, 1235, 1103, 933, 631	Sanguiin H6
7	47.88	275	**935**	**898**, 633	Galloyl-bis-HHDP glucose
8	49.85	370	**301**	301	Ellagic acid
9	57.73	275	**1085**, 783, 301	915, **897**, 783, 633, 451	Unknown ellagitannin

identified. The ellagitannin content of the cloudberry extract was estimated at approximately $72.0 \pm 1.4\%$ compared to $44.1 \pm 0.8\%$ for the less effective extract from the related arctic bramble. Although this value is only an estimate (calculated from a ratio of the peak area due to ellagitannins and ellagic acid divided by the total peak area of all identified phenolic components), it certainly reflects the higher content of non-ellagitannin contents such as anthocyanins in arctic bramble (see Supplementary data, Table S1).

Indeed, the high ellagitannin content of cloudberry has been noted previously in this laboratory (McDougall *et al.* 2008) and in previous work (Kähkönen *et al.* 2001; Kopenen *et al.* 2007). For example, Kähkönen *et al.* (2001) estimated the ET content in different cloudberry samples that ranged from 65 to 88% of total phenol content. However, the ellagitannin content of arctic bramble has not been published previously.

DISCUSSION

Berry extracts have long been known to interfere with human microbial pathogens. One of the most widely known is the inhibitory effect of proanthocyanidins from cranberry (Howell *et al.* 2005), and lingonberry (Kontiokari *et al.* 2001), on bacterial urinary tract infections. Berry extracts rich in ellagitannins have also been noted as being particularly effective against human pathogenic micro-organisms (Heinonen *et al.* 2007) with cloudberry being the most effective against the widest range of bacteria and yeasts (Nohynek *et al.* 2006). This builds on work that outlined anti-microbial activity of ellagitannins from other plant sources (Latte and Kolodziej, 2000; Asres *et al.* 2001; Machado *et al.* 2002; Parashar *et al.* 2009, and reviewed by Yoshida *et al.* 2009).

Indeed, ellagitannin-rich extracts and purified ellagitannins have been reported to have effects on human protozoan parasites such as *Trypanosoma* (Shuaibu *et al.* 2008), *Plasmodium* species (Dell'Agli *et al.* 2009) and *Leishmania* species (Kolodziej and Kiderlen, 2005). Ellagic acid, which can be formed by ellagitannin degradation, has also been shown to be effective against *Plasmodium* species *in vivo* and *in vitro* (Soh *et al.* 2009). Ellagic acid was also identified as an active anti-giardial agent in *Rubus coriifolius* (Alanis *et al.* 2003). In addition, other studies have shown toxicity of ellagitannins against the nematode, *C. elegans* (Yamasaki *et al.* 2002).

Here we have shown that the polyphenol-rich extracts of strawberry, blackberry, cloudberry and arctic bramble were as effective as metronidazole in the *in vitro* killing of *G. duodenalis* trophozoites and that cloudberry proved to be the most effective. The minimum concentration of cloudberry phenolics found to be effective against *Giardia* trophozoites in this study was between 10 and 20 μg GAE per well (which equates to 33–66 μg ml^{-1}). Considering that cloudberries can provide around 150 mg GAE/100 g fruit (approx. 1500 μg ml^{-1}: Kähkönen *et al.* 2001), the amount of phenolic material entering the small intestine could easily reach levels shown to be effective *in vitro*, even allowing for partial degradation in the stomach (Clifford and Scalbert, 2000). In studies with ileostomy volunteers, Gonzalez-Barrio *et al.* (2010) have shown that a large proportion of ingested raspberry polyphenols were recovered in ileal fluid and, moreover, about a quarter of the main ellagitannin component, Sanguiin H6, survived digestion in the upper gastrointestinal tract.

As well as having a high percentage of ellagitannins, cloudberries are notable for having substantial levels of unconjugated *p*-coumaric acid and benzoic

acid (Maatta-Riihinen *et al.* 2004). *p*-Coumaric acid has been shown to have a weak effect on *Giardia* trophozoites (Calzada and Alanis, 2007) and the coumaric acid derivative (melilotoside) was identified as an active anti-giardial agent from *Teloxys graveolens* (Calzada *et al.* 2003). The presence of *p*-coumaric and benzoic acid may potentiate the effects on *Giardia* caused by the ellagitannins but perhaps by a different and synergistic mechanism (Fyfe *et al.* 1998; Puupponen-Pimiä *et al.* 2001). In addition, quercetin derivatives, also identified in cloudberry, may also contribute to anti-giardial activity (Amaral *et al.* 2006; Calzada and Alanis 2007).

In conclusion, this study has confirmed that berry polyphenols can influence *Giardia* survival *in vitro* and suggests that ellagitannins are most effective. Tannin-rich preparations may also have efficacious effects on diarrhoeal symptoms (Palombo, 2006) often associated with *Giardia* infection. Unlike many plant sources of anti-giardial agents (e.g. Amaral *et al.* 2006), berries are a natural and palatable foodstuff and therefore have few issues with toxicity, side effects or acceptance. However, further work is required to uncover whether these *in vitro* effects described here can be transferred to the *in vivo* situation and contribute to giardiasis treatment.

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