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,
Geranin D, from *Geranium nivaeum* 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 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 TYI-S-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 (~ 2.7 × 10⁴ 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 × 10⁴ 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 × 10⁴ 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
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: \((L ÷ (L + D)) × 100\) 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 \(\mu g\) 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 \(\mu l\)) 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 \(\mu l/min\). 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.

RESULTS

Strawberry, blackberry, cloudberry and arctic bramble were as effective in killing *Giardia* as the positive control, metronidazole, at 50 \(\mu g\) 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 \(\mu g\) 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. 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 \(\mu g\) GAE per well. Untreated trophozoites and metronidazole (67 \(\mu g\) 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 (\(P ≤ 0.05\)).
effective and caused high levels of killing at 20 μg 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 Sanguin 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

<table>
<thead>
<tr>
<th>Peak no.</th>
<th>Retention time</th>
<th>PDA max.</th>
<th>m/z [M-H]</th>
<th>MS²</th>
<th>Putative identity</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>21.25</td>
<td>275</td>
<td>783, 481, 301</td>
<td>481, 301, 275</td>
<td>Pendunculagin isomer</td>
</tr>
<tr>
<td>2</td>
<td>28.31</td>
<td>275</td>
<td>633, 301, 275</td>
<td>301</td>
<td>Galloyl-HHDP-glucose</td>
</tr>
<tr>
<td>3 Front</td>
<td>40.03</td>
<td>275</td>
<td>1869</td>
<td>1567, 1265, 1235, 1103, 933</td>
<td>Sanguin H6 isomer</td>
</tr>
<tr>
<td>Rear</td>
<td>1717, 933</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>4 Front</td>
<td>41.17</td>
<td>275</td>
<td>1567</td>
<td>1415, 1113, 933, 783</td>
<td>Sanguin H6</td>
</tr>
<tr>
<td>Rear</td>
<td>933</td>
<td></td>
<td></td>
<td>897, 631, 451, 315</td>
<td>without gallic moiety</td>
</tr>
<tr>
<td>5</td>
<td>42.15</td>
<td>275</td>
<td>1401</td>
<td>Multiple</td>
<td>Lambertianin C</td>
</tr>
<tr>
<td>6</td>
<td>43.52</td>
<td>275</td>
<td>1869</td>
<td>1567, 1265, 1235, 1103, 933, 631</td>
<td>Sanguin H6</td>
</tr>
<tr>
<td>7</td>
<td>47.88</td>
<td>275</td>
<td>783</td>
<td>898, 633</td>
<td>Galloyl-bis-HHDP glucose</td>
</tr>
<tr>
<td>8</td>
<td>49.85</td>
<td>370</td>
<td>301</td>
<td>301</td>
<td>Ellagic acid</td>
</tr>
<tr>
<td>9</td>
<td>57.73</td>
<td>275</td>
<td>1085, 783, 301</td>
<td>915, 897, 783, 633, 451</td>
<td>Unknown ellagitannin</td>
</tr>
</tbody>
</table>

identified. The ellagitannin content of the cloudberry extract was estimated at approximately 72.0 ± 1.4% compared to 44.1 ± 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 vitro and in vivo (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 cloudberrys 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, Sanguin H6, survived digestion in the upper gastrointestinal tract.

As well as having a high percentage of ellagitannins, cloudberrys are notable for having substantial levels of unconjugated p-coumaric acid and benzoic...
acids (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, queretin 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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