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Anthelmintic efficacy of cranberry vine extracts on ovine *Haemonchus contortus*

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Abstract

The discovery that plant secondary compounds, including proanthocyanidins (PAC), suppress gastrointestinal nematode (GIN) infection has provided promise for alternative methods of GIN control in small ruminants. This investigation is the first to examine the anthelmintic potential of cranberry vine (CV) against the GIN *Haemonchus contortus*. The purpose of this study was to explore the anti-parasitic activity of CV in the form of a specific organic proanthocyanidin extract (CV-PAC) and an aqueous extract (CV-AqE) containing PAC and other compounds. *In vitro* egg hatching, first (L1) and third (L3) stage larval and adult worm motility and L3 exsheathment were evaluated after a 24-hour incubation with CV products. In addition, CV treated worms were observed via scanning electron microscopy, and a preliminary investigation of the efficacy of CV powder against an experimental infection of *H. contortus* was conducted. The *in vivo* effect on an experimental infection was determined by administering 21.1 g CV powder to lambs ($n = 9$ per group) for three consecutive days, and collecting fecal egg count data for four weeks post-treatment. The effect of CV-PAC on egg hatching, L3 motility and exsheathment was limited. However, a substantial effect was observed on motility of post-hatch L1 (EC_{50} 0.3 mg PAC/mL) and adults (EC_{50} 0.2 mg PAC/mL). The CV-AqE showed more effect on egg hatching (EC_{50} 5.3 mg/mL containing 0.6 mg PAC/mL) as well as impacting motility of L1 (EC_{50} 1.5 mg/mL with 0.2 mg PAC/mL) and adults (EC_{50} 3.4 mg/mL with 0.4 mg PAC/mL), but like CV-PAC, did not substantially effect L3 motility or exsheathment. Scanning electron microscopy revealed an accumulation of aggregate on the cuticle around the buccal area of adult worms incubated in CV-

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AqE and CV-PAC. In the preliminary *in vivo* study, there was a significant effect of treatment over time ($p = 0.04$), although differences in individual weeks were not significant. In summary, both extracts inhibited motility of L1 and adult worms. The higher efficacy of CV-AqE than CV-PAC at levels that contained the same concentrations of PAC tested alone, suggest that other secondary compounds in the CV-AqE contribute to the observed effects on the parasites. This first study of the *in vitro* and *in vivo* effects of CV suggests that this readily available plant product may have utility in integrated control of *H. contortus* and support the need for additional testing to provide further information.

Keywords

egg hatch; larval motility; larval exsheathment; scanning electron microscopy

1. Introduction

Gastrointestinal nematodes (GIN) are a major health concern for small ruminant producers, causing anemia, poor body condition, diarrhea, and death. The barber pole worm (*Haemonchus contortus*) is the most pathogenic GIN species infecting sheep and goats worldwide (Verissimo et al., 2012). With the growth of parasite resistance to commercial dewormers (Jackson and Coop, 2000), alternative methods have been investigated for their efficacy in GIN control. Plants rich in condensed tannins and other secondary compounds have demonstrated anthelmintic efficacy against different species of GIN (Hoste et al., 2006; Werne et al., 2013; Costa-Júnior et al., 2014) and can be utilized in integrated control programs against GIN.

A number of secondary plant compounds have been investigated for their anthelmintic potential. Condensed tannins, also called proanthocyanidins (PAC), have received the most study. Several PAC-containing forages, such as sericea lespedeza (*Lespedeza cuneata*) (Lange et al., 2006; Shaik et al., 2006; Terrill et al., 2009; Acharya et al., 2015), birdsfoot trefoil (*Lotus corniculatus*) (Heckendorn et al., 2007), sainfoin (*Onobrychis viciifolia*) (Heckendorn et al., 2007; Desrues et al., 2016), and sulla (*Hedysarum coronarium*) (Niezen et al., 1998) have been found to show activity against GIN. *In vitro* testing, using plant derived PAC organic extracts (extraction performed using organic solvents; i.e. acetone), has demonstrated inhibition of *H. contortus* egg hatch (Vargas-Magaña et al., 2014) and *Teladorsagia circumcincta* larval development (Molan and Faraj, 2010). Additionally, investigators have attributed anti-parasitic activity of plants against eggs, larvae, and adult worms to a variety of water-soluble secondary compounds such as alkaloids, saponins (Adamu et al., 2010), and phenolic compounds (Ferreira et al., 2013), as well as proanthocyanidins and hydrolyzable tannins. *In vitro* work using crude aqueous extracts as well as organic extracts of plants have all shown anthelmintic efficacy against small ruminant GIN species (Hoste et al., 2006).

The American cranberry (*Vaccinium macrocarpon*), native to the United States, is grown predominantly in Wisconsin, Massachusetts, New Jersey, Oregon and Washington (Polashock et al., 2014). Cranberries contain an abundance of PAC that has been shown to

support mammalian urinary tract health through its bacterial anti-adhesion activity (Howell et al., 2005). Not just the fruit, but also the cranberry vines (stems and leaves) contain high levels of PAC (Ferlemi and Lamari, 2016). The annual pruning of cranberry bogs, as the vines come out of winter dormancy, provides a readily available and inexpensive source of vine. The presence of PAC in cranberry vines (CV), coupled with the abundant availability of this crop in the United States, suggests that cranberry vine may show some anthelmintic activity against GIN.

This study utilizes *in vitro* and *in vivo* methods to investigate the anthelmintic efficacy of cranberry vines against various life stages of *H. contortus*. The specific objectives of this study are to: 1) test the anthelmintic efficacy of cranberry vine purified PAC extract (CV-PAC) as well as a cranberry vine crude aqueous extract (CV-AqE) *in vitro* against *H. contortus* egg hatching, larval motility and exsheathment, and adult worm motility; 2) describe changes in adult following incubation with CV-PAC and CV-AqE via scanning electron microscopy; and 3) test CV powder against an experimental infection of *H. contortus* in lambs.

2. Materials and methods

2.1. Preparation and analysis of cranberry vine powder and extracts

2.1.1. Phytochemical analyses—Analyses on the cranberry vine powder (CV) and cranberry vine powder aqueous extract (CV-AqE) used in this study were performed by the Reed Research Group at the University of Wisconsin-Madison. Phytochemical analyses included the 4-(dimethylamino)cinnamaldehyde (DMAC) method (Krueger et al., 2016; Feliciano et al., 2012a) and the matrix assisted laser desorption/ionization-time of flight mass spectra (MALDI-TOF MS) method (Krueger et al., 2013; Feliciano et al., 2012b). The concentration of PAC within the CV powder and within the CV-AqE extract was determined by the DMAC method, followed by butanol-HCl analysis, using a cranberry proanthocyanidin (c-PAC) standard to reflect the structural heterogeneity of cranberry vine PAC. The CV powder (used to make the CV-PAC and CV-AqE extracts, and used in the *in vivo* trial) contained 108 mg PAC/g powder (c-PAC equivalents). The structures of the CV-PAC and CV-AqE extracts were determined by MALDI-TOF mass spectra, while high performance liquid chromatography (HPLC) (de Pascual-Teresa et al., 1998) was used to confirm the presence of other secondary compounds present in the extracts.

2.1.2. Cranberry vine powder (CV)—Cranberry vines (stems and leaves) of ‘Early Black’ cultivar were collected at Rutgers Marucci Center for Blueberry and Cranberry Research, Chatsworth, NJ, USA, in the spring of 2013. To reduce any possible impact of high temperature on plant secondary compounds, the cranberry vines were washed with water in a plastic tub, rinsed, and spread out to dry on trays at 65-70°C in enclosed heating cabinets for 16 hours. The vines were then ground in a blender before testing the bioactivity of the powder.

2.1.3. Cranberry vine powder proanthocyanidin extract (CV-PAC)—Cranberry vine proanthocyanidins (CV-PAC) were extracted from the dried cranberry vine powder at Rutgers Marucci Center for Blueberry and Cranberry Research, Chatsworth, NJ, USA.

Proanthocyanidin extract was isolated from the CV using solid-phase chromatography according to the established method for PAC isolation (Howell et al., 2005). Reverse phase (C18) followed by adsorption chromatography (Sephadex LH-20) was used to extract and isolate the total PAC. The recovered PAC was stored at room temperature, in the dark. Electrospray mass spectrometry and MALDI-TOF mass spectrometry were utilized to confirm the presence of A-type linkages (Howell et al., 2005).

2.1.4. Cranberry vine powder aqueous extract (CV-AqE)—Stock solution of CV-AqE (40 mg/mL) was prepared for use in the egg hatch and first stage (L1) larval motility assay by measuring 1.2 g of CV powder into a 50 mL polypropylene tube and adding room temperature (RT) tap water up to a volume of 30 mL. Cranberry vine was soaked in the dark at RT for 24 hours. Solid plant matter was centrifuged ($200 \times g$ for 5 minutes) to the bottom of the tube and the supernatant was collected and subsequently used the same day in the *in vitro* assays. A stock solution of 50 mg powder/mL was prepared in the same way for use in the adult motility assay. Representative samples of each stock solution were stored at -20°C for phytochemical analysis.

2.2. Parasitology parameters

Eggs of *H. contortus* were collected from experimentally infected lambs at Virginia Tech and University of Rhode Island (L3 identification >99% *H. contortus*). The *H. contortus* isolate used in this study was maintained by one of the authors (A. Zajac) from a closed flock with minimal drug use. This isolate is effectively treated with the anthelmintics used in this experiment. Briefly, lambs were orally dewormed with levamisole hydrochloride (8.8 mg/kg BW, Prohibit®, AgriLabs, St. Joseph, MO) and ivermectin (0.2 mg/kg BW, Ivomec®, Merial Inc., Duluth, GA) to reduce fecal egg counts (FEC) to <50 eggs per gram (epg) and then orally administered 10,000 *H. contortus* L3 seven days post-anthelmintic treatment. Motility of L1, L3, and adult *H. contortus* was determined by observing motility for five seconds and recording samples as either motile or non-motile (Skantar et al., 2005; Katiki et al., 2013). Blood samples were collected weekly via jugular venipuncture and packed cell volume (PCV) was determined by micro-hematocrit centrifugation for three minutes at $35,720 \times g$. Rectal fecal samples were collected weekly for fecal egg count determination according to the modified McMaster technique with a detection limit of 50 epg (Zajac and Conboy, 2012).

2.3. In vitro assays

2.3.1. Egg hatch and L1 motility assay—The anthelmintic efficacy of CV-PAC and CV-AqE on hatching of *H. contortus* eggs was determined using previously published procedures (Assis et al., 2003; Marie-Magdeleine et al., 2009). Stock solutions in water of CV-AqE (40 mg/mL) and CV-PAC (20 mg/mL), prepared fresh prior to each assay, were serially diluted with tap water. Concentrations used were based on previous studies using PAC extracts at concentrations of 1.2–25 mg/mL (Alonso-Díaz et al., 2008; Brunet et al., 2008; Kamaraj et al., 2011). Feces were collected rectally from donor lambs experimentally infected with *H. contortus*. Eggs were recovered by running fresh feces in water through a series of sieves (1 mm, 355 μm , 150 μm , 38 μm , 25 μm). Eggs retained on the last two sieves were recovered using flotation with a concentrated sodium nitrate solution (Fecasol®,

Vetoquinol U.S.A., Inc., Fort Worth, Texas) and collected on glass cover slips and then rinsed with water. Eggs (100 eggs/well, in 100 μ L water) were placed into 24-well flat-bottomed microtitre plates. Thiabendazole (TBZ, Thermo Fisher Scientific Inc., Waltham, MA, USA) in dimethyl sulfoxide (0.5 μ g/mL) (DMSO, Fisher BioReagents™, Thermo Fisher Scientific Inc., Waltham, MA, USA) and water were used as positive and negative controls respectively. Thiabendazole in DMSO (10 μ L) was added to positive control wells and DMSO (10 μ L) was added to all other wells. Water was added to each well increasing the volume to 1 mL. Serial dilutions of CV-AqE, CV-PAC, or water were added (1 mL) to each well for a total volume of 2 mL. Final concentrations in wells were 10, 5, 2.5, 1.2, 0.6, 0.3 mg/mL for CV-PAC and 20, 10, 5, 2.5, 1.2, 0.6, 0.3 mg/mL for CV-AqE. Five replicates were run for each set of dilutions and controls. The eggs were incubated at 26°C for 24 hours and determined to be hatched or not hatched. Results were expressed as percent hatch inhibition. The larvae that hatched from the eggs were quantified and determined to be motile or non-motile. Results were expressed as percent motility inhibition of eggs hatched.

2.3.2. Exsheathment and L3 motility assay—The effect of CV-PAC and CV-AqE on the survival and exsheathment of L3 larvae *in vitro* was determined. Fresh dilutions from the stock solutions of CV-AqE (40 mg/mL) and the CV-PAC (20 mg/mL) were made for each assay and serially diluted using water. Sheathed larvae were incubated in CV-PAC (10, 5, 2.5, 1.2, 0.6, 0.3 mg/mL), CV-AqE (20, 10, 5, 2.5, 1.2, 0.6, 0.3 mg/mL) or a water control for 24 hours at 37°C. The sheathed L3 (2,000) were added to Earle's Balanced Salt Solution (EBSS, Sigma-Aldrich®, Inc., Natick, MA, USA) up to a volume of 1 mL in a 15 mL polypropylene tube. Water control (1 mL) or treatment (1 mL) was added for a total volume of 2 mL. After 24-hour incubation, percent motility inhibition was determined and exsheathment was induced following established procedures (Conder and Johnson, 1996) using carbon dioxide. After the 18-hour exsheathment incubation, percent larval motility inhibition was determined based on observing motility of 100 larvae/tube for 5 seconds and motile larvae were determined exsheathed or ensheathed. Results were expressed as percent motility inhibition of all larvae (including exsheathed and ensheathed) and percent motile larvae exsheathed.

2.3.3. Adult worm motility assay—The anthelmintic efficacy of CV-PAC and CV-AqE on adult *H. contortus* was determined using previously published *in vitro* procedures (Kotze and McClure, 2001). Stock solutions of CV-AqE (50 mg/mL) and the CV-PAC (2.4 mg/mL) were made and serially diluted using water. The adult *H. contortus* worms were collected at a commercial abattoir from the abomasa of two naturally infected goats. Female *H. contortus* were collected from the folds of the abomasum within 3 hours of slaughter, and washed in RPMI-1640 (GIBCO® RPMI 1640 Medium, Thermo Fisher Scientific Inc., Waltham, MA, USA). Immediately following this wash, each worm was placed for 1-2 hours into holding media, containing 2 mL RPMI-1640 supplemented with 0.8% glucose (Dextrose (D-glucose) Anhydrous (Granular Powder/Cerified ACS), Fisher Chemical™, Thermo Fisher Scientific Inc., Waltham, MA, USA), 2.5 μ g/mL amphotericin B (Sigma-Aldrich®, Inc., Natick, MA, USA), 100 U/mL penicillin and 100 μ g/mL streptomycin (Corning™ cellgro™ Penicillin-Streptomycin Solution, Corning Life Sciences, Tewksbury, MA, USA) and 10 mM N-[2-hydroxyethyl]piperazine-N-[4-butanesulfonic acid] (HEPES)

buffer (HEPES Buffer, Fisher Chemical™, Thermo Fisher Scientific Inc., Waltham, MA, USA) at 38°C, 5% CO₂. After the holding period, adult female worms were individually placed in CV-PAC (1.2, 0.6, 0.3 mg/mL; $n = 40$ worms/concentration), CV-AqE (25, 12.5, 6.2, 3.1 mg/mL; $n = 10$ worms/concentration), RPMI-1640 control ($n = 10$ worms), or TBZ (0.05 mg/mL; $n = 10$ worms) control. The CV-PAC assay was run prior to the CV-AqE assay; therefore, a larger number of worms were collected. Fewer worms were used in subsequent assays due to low variability between worms. Incubation media for all treatments and controls contained RPMI-1640 supplemented with 0.8% glucose, 10 mmol HEPES, 0.25 µg/mL amphotericin B, 10 U/mL penicillin, and 10 µg/mL streptomycin. The worms were incubated at 38°C, 5% CO₂ up to 48 hours.

Motility of the adult worms, based on observing motility for five seconds, was monitored at 24-hour intervals. Worms were determined motile or non-motile (Skantar et al., 2005; Katiki et al., 2013) and results were expressed as percent motility inhibition.

2.3.4. Scanning electron microscopy (SEM) processing of adult worms—Adult *H. contortus* were collected at a commercial abattoir from the abomasa of experimentally infected lambs. Worms (10 per treatment or control) were incubated in CV-PAC (12 mg/mL; 12 or 18 hours), CV-AqE (3.1 or 6.2 mg/mL; 18 hours) or RPMI control (12 or 18 hours). After incubation in CV-AqE, CV-PAC, or RPMI control, all harvested worms (4 worms per treatment or control) were confirmed live and individually preserved in 2% glutaraldehyde solution in phosphate buffer (0.1 M, pH = 7.4) (Glutaraldehyde Solution, Electron Microscopy Sciences, Hatfield, PA, USA) and refrigerated at 4°C until analysis. Harvested worms were assumed to be representative and examined by SEM. Worms were processed for SEM at the Morphology Service Laboratory at Virginia-Maryland College of Veterinary Medicine, Virginia Tech. Fixed worms were washed in 0.1 M Na cacodylate buffer and post-fixed with 2% OsO₄ in 0.1 M Na cacodylate buffer for one hour. Worms were washed again in Na cacodylate buffer and dehydrated in graded ethanol series (15%, 30%, 50%, 70%, 95%, 100%). Worms were then critical point dried (Ladd Critical Point Dryer, Ladd Research Industries, Inc., Williston, Vermont, USA) and sputter coated with gold (SPI-Module Sputter Coater, Structure Probe, Inc., West Chester, Pennsylvania, USA). Immediately following gold coating, worms were observed with a scanning electron microscope (Carl-Zeiss, EVO, Scanning Electron Microscope, Jena, Germany) at the anterior end and the vulva area.

2.4. In vivo study in lambs

The objective of the preliminary *in vivo* study in lambs was to determine whether oral administration of CV, drenched for three consecutive days at a PAC rumen concentration similar to the EC₅₀ against adult worms observed during the *in vitro* studies, would have an acute effect on an established *H. contortus* infection.

2.4.1. Study subjects—This study was conducted with the approval of the Institutional Animal Care and Use Committee (IACUC) of the University of Rhode Island. The lambs used for this study were Dorset and Dorset/Hampshire crossbred lambs born and housed at the University of Rhode Island Peckham Farm, located in Kingston, RI, USA. Spring born

lambs were weaned and kept on pasture until approximately five months of age, with a group average FEC of 550 ± 157 trichostrongyle epg. Five weeks prior to the start of the study, lambs were brought indoors and remained in indoor housing for the entirety of the study. Lambs were group fed 0.91 kg/lamb/day of a commercial sheep pellet and hay diet and allowed free access to water and trace mineralized salt. Lambs were dewormed orally with levamisole and ivermectin as previously described. Fecal egg counts were <50 epg post-anthelmintic treatment.

2.4.2. Study Design—Seven days post-anthelmintic treatment, eighteen lambs (11 female, 7 male) were orally administered 10,000 infective *H. contortus* L3 larvae each. Approximately 35 days after infection, the lambs were assigned to one of two groups balanced for sex and FEC. Following the protocol of Athanasiadou et al. (2000) using quebracho tannin, one group received cranberry vine powder (21.1 g of dried CV/day for 3 consecutive days, $n=9$ lambs) and one group was the control group (no CV, $n=9$ lambs). On day zero, CV lambs were orally administered a slurry of 21.1 g of dried CV suspended in 60 mL water using a 60 mL syringe. It was estimated that administration of 21.1 g/day of CV (corresponding to 2.3 g/day of CV-PAC (21.1 g CV $\times$ 108 mg PAC/g powder) to each lamb would be equivalent to 0.3 mg/mL PAC in the rumen (assuming 8 L of rumen capacity) per day, assuming the concentration would move through the gastrointestinal tract at this estimated 0.3 mg PAC/mL stomach contents. This estimated concentration only describes the initial drenching in an estimated 8 L of stomach contents, as it might have been further diluted when the lambs were fed hay and grain. Fecal egg count and packed cell volume were monitored at day zero and weekly for four weeks after the administration of CV.

2.5. Statistical Analysis

Egg hatch, L1 and L3 motility, exsheathment, and adult worm motility data were analyzed using an analysis of variance (ANOVA) and means separated with Dunnett's t test using the GLM Procedure in SAS (SAS Institute Inc., Cary, NC). Treatment means were compared with significance defined as $p \leq 0.05$. The concentration of extracts required to prevent 50% (EC₅₀) and 90% (EC₉₀) of egg hatching were calculated using PROBIT procedure in SAS, using a confidence interval of 95%. All effective concentrations greater than the actual concentrations tested in the assays were extrapolated values through the PROBIT procedure. Fecal egg count data and packed cell volume data from the *in vivo* lamb study were analyzed using the GLIMMIX Procedure in SAS. This model included terms Treatment, Week, and Treatment*Week. A stepdown Dunnett adjustment for multiple comparisons test was also done for simple effect comparisons of treatment*week least square means by week and by treatment. Fecal egg count and packed cell volume data were analyzed from day zero of the treatment through the following four weeks post-treatment. Significance of least square means was defined as $p \leq 0.05$.

3. Results

3.1. Phytochemical analyses

The ground CV used to make the CV-AqE contained 108 mg PAC/g CV (c-PAC equivalents). The 40 mg/mL stock solution of CV-AqE extract was analyzed using the

DMAC method and contained 4.8 mg PAC/mL extract. The CV-AqE extract under MALDI-TOF MS revealed that the CV-AqE was similar to the CV-PAC, in that they both showed PAC with A-type linkages and similar polymer distributions. However, after HPLC analysis, in addition to PAC, hydroxycinnamic acids and flavonols were detected in the CV-AqE extract (data not shown).

3.2. Effect of CV-PAC and CV-AqE on egg hatching and L1 motility, L3 motility and exsheathment, and adult worm motility

The mean percent of egg hatch inhibition, larval motility inhibition, exsheathment inhibition and adult worm motility inhibition for CV-PAC and CV-AqE are shown in Table 1 and Table 2 respectively. The results of the PROBIT analysis determining effective concentrations of CV-PAC and CV-AqE, that inhibited 50% (EC₅₀) and 90% (EC₉₀) of egg hatching, L1 and L3 motility, exsheathment, and adult worm motility are shown in Table 3. The effectiveness of CV-AqE and CV-PAC differed when tested against the different life stages of *H. contortus* *in vitro*.

The extracts varied in their effect on egg hatching. The CV-PAC demonstrated very little anthelmintic efficacy against egg hatching with the EC₅₀ and EC₉₀ of CV-PAC calculated at 26.6 mg/mL and 618 mg/mL respectively. The aqueous extract appeared to be more effective, with the EC₅₀ and EC₉₀ of the aqueous extract at 5.3 mg/mL (containing 0.6 mg PAC/mL) and 19.8 mg/mL (containing 2.4 mg PAC/mL) respectively.

However, for those eggs that did hatch the effect against the first stage larvae was more similar for the two extracts (Table 3). The EC₅₀ and EC₉₀ of CV-PAC were 0.3 mg/mL and 1.6 mg/mL respectively, while the EC₅₀ and EC₉₀ of CV-AqE were 1.5 mg/mL (containing 0.2 mg PAC/mL) and 3.0 mg/mL (containing 0.4 mg PAC/mL) respectively (Table 3).

In the L3 motility assay the EC₅₀ and EC₉₀ of CV-PAC was 4.0 mg/mL and 52.3 mg/mL respectively (Table 3), while the EC₅₀ and EC₉₀ of CV-AqE was 137 g/mL (containing 16 g PAC/mL) and 460 kg/mL (containing 55 kg PAC/mL) respectively. For the larval exsheathment, the EC₅₀ and EC₉₀ of CV-PAC was 4.0 mg/mL and 17.8 mg/mL respectively, while the EC₅₀ and EC₉₀ of CV-AqE was 11 g/mL (containing 1.3 g PAC/mL) and 10 kg/mL (containing 1.3 kg PAC/mL) respectively.

The CV-AqE had a more rapid impact on adult worm motility with a significant effect detected after 24-hour incubation. The EC₅₀ and EC₉₀ of CV-AqE were 3.4 mg/mL (containing 0.4 mg PAC/mL) and 15.1 mg/mL (containing 1.8 mg PAC/mL) respectively. Inhibition of motility was not observed at 24 hours in adult worms incubated in CV-PAC, therefore incubation of the worms was continued until 48 hours at which time the EC₅₀ and EC₉₀ of CV-PAC was 0.2 mg/mL and 0.8 mg/mL respectively (Table 3).

3.3. Observation of adult worms under scanning electron microscopy (SEM)

Adult worms incubated in both CV-AqE and CV-PAC observed using SEM had accumulated aggregate on the cuticle around the buccal area (Fig. 1). This aggregate appeared on all adult worms incubated in CV-PAC (12 mg/mL) ranging from light to heavy with the heaviest accumulation shown in Fig. 1. D, E, F.

All but one of the four worms incubated in CV-AqE (6.2 and 3.1 mg/mL) had heavy accumulation of aggregate amassed on the cuticle and around the buccal area (Fig. 1. G, H, I). The remaining worm showed moderate accumulation (3.1 mg/mL CV-AqE, images not shown). Control worms incubated in RPMI (Fig. 1. A, B, C) were free of aggregate accumulation and smoother overall than worms incubated in CV-PAC or CV-AqE. All the RPMI control worms (incubated for 12 or 18 hours) were similar (Fig. 1. A, B, C). A closer view of the cuticle of worms from both treatments revealed a buildup of aggregate in the annular furrows of the cuticle (Fig. 1. F and I) that was not present in RPMI control worms (Fig. 1. C). The normal longitudinal cuticular ridges observed on the RPMI control worms (Fig. 1. C) were well structured compared to the longitudinal ridges observed on the treated worms (Fig. 1. F and I) that were less pronounced.

3.4. Effect of CV on an experimental infection of *H. contortus* in lambs

The FEC (mean $\pm$ SEM) of the control and treatment groups are shown in Fig. 2. There was an effect of treatment over time ($p = 0.04$), but significance fell out when comparing treatments at each week. There was a slight suppression in the CV group's average FEC in the first two weeks post-treatment, but this suppression did not persist at week three. There was no effect of treatment ($p = 0.15$) or time ($p = 0.55$) on PCV (mean $\pm$ SEM of all animals for the whole study, data not shown).

4. Discussion

This study evaluated the potential anti-parasitic efficacy of cranberry vine against *H. contortus* by assaying egg hatch, larval motility (L1, L3) and exsheathment, and adult motility *in vitro*, as well as visualizing the effect of CV-PAC and CV-AqE on adult worms via scanning electron microscopy, and testing three days of oral treatment with CV powder against an established experimental infection of *H. contortus* in lambs.

Neither extract significantly reduced egg hatching, L3 motility or exsheathment at concentrations similar to those seen with extracts of other plants with recognized anthelmintic activity *in vitro*. The CV-PAC was not efficacious against egg hatch compared to previous reports of egg hatch inhibition of PAC at concentrations < 1 mg/mL PAC (Molan et al., 2002; Molan and Faraj, 2010). However, for those eggs that hatched, the extracts did significantly reduce motility at much lower concentrations, comparable to a previous report of PAC causing L1 mortality (Molan and Faraj, 2010). The higher efficacy of CV-AqE against egg hatching and L1 motility compared to CV-PAC, at the same concentrations as PAC, potentially indicate that there are other secondary compounds in CV-AqE contributing to the anthelmintic efficacy. These results suggest that any anthelmintic compounds in the extracts may not be able to penetrate the egg shell (Riou et al., 2005), extra layer of cuticle of exsheathed third stage larvae, or sheath of ensheathed third stage larvae. This in contrast to other PAC containing forages which do affect both egg hatching and L3 motility and exsheathment, again indicate that PAC of cranberry may play a reduced role in anthelmintic effects compared to some other plant species. Results may also differ from previous studies due to variation in PAC structure of plant species, as the CV-PAC used in this study had A-

type linkages, unlike most plant tannins, which are comprised of the more common B-type linkage (Howell et al., 2005).

Neither CV-PAC nor CV-AqE were efficacious against larval exsheathment, unlike other studies of exsheathment showing inhibition at concentrations of 1.2 mg/mL PAC (Brunet et al., 2007; Alonso-Díaz et al., 2008). The method of exsheathment in those studies utilized bleach (Brunet et al., 2007; Alonso-Díaz et al., 2008). In this study, we used the CO₂ method, which led to higher levels of establishment in the host compared to larvae treated with bleach (Conder and Johnson, 1996). If the CO₂ method increased larval viability, it may have made larvae more resistant to the impact of the cranberry products.

The CV-PAC and CV-AqE differed in their anthelmintic efficacy against adult worm motility. Inhibition was observed in CV-PAC 24 hours later than for worms incubated in CV-AqE (in comparable PAC concentrations), leading again to the possibility that secondary compounds, other than PAC, could be contributing to the greater anthelmintic activity observed in the crude CV-AqE extract. Supporting this hypothesis, hydroxycinnamic acids and flavonols were detected in the CV-AqE extract, in addition to PAC. Hydroxycinnamic acids and flavonols are commonly found in high concentrations in cranberry leaves and have been associated with antioxidant activity of cranberry leaves (Ferlemi and Lamari, 2016). Flavonols have previously shown anthelmintic efficacy against *H. contortus* and the combination of flavonols and condensed tannins exhibited synergistic anthelmintic effects (Klongsiriwet et al., 2015).

Other authors have investigated the impact of aqueous plant extracts against *H. contortus* (Adamu et al., 2010; Ferreira et al., 2013) and attributed anti-parasitic activity to alkaloids, saponins (Adamu et al., 2010), and phenolic compounds (Ferreira et al., 2013). These aqueous extracts can also contain PAC, although the protocol of preparing extracts with boiling water used in some studies (Ferreira et al., 2013) could cause the compounds to complex (Reed, 1995). To preserve PAC, we used room temperature water to prepare the extract. A CV-AqE extract was included in this study because of its biological relevance to what the animals would ingest in an *in vivo* trial when consuming the ground cranberry vine.

The mechanism(s) of PAC and other secondary plant compounds on *H. contortus* are unknown and may vary with the parasite, its stage of development, and the biochemical characteristics of the plant species (Hoste et al., 2006). The outermost layer of the cuticle, the epicuticle, is lipid-rich and covered by a glycoprotein-rich surface coat which may bind PAC, resulting in the accumulation of aggregate (Page and Johnstone, 2007; Hoste et al., 2006) that may affect worm motility and normal feeding (Martínez-Ortíz-de-Montellano et al., 2013). The observations made with scanning electron microscopy in this study revealed an accumulation of aggregate around the buccal zone and covering most of the cuticular surface coat, specifically in the annular furrows. Similar SEM changes have been observed in adult *H. contortus* worms after *in vitro* exposure to PAC-rich (sainfoin and tzialam) extracts (Martínez-Ortíz-de-Montellano et al., 2013). Alternatively, it is possible that a lack of mobility, caused by degradation of muscle cells, as observed in transmission EM by Brunet et al. (2011), led to aggregate accumulation. While Martínez-Ortíz-de-Montellano et

al. (2013) observed changes in the female vulva *in vitro*, the vulva areas that were viewed in this study of CV did not differ between treatment and control worms.

According to Powers et al. (1982) and the W.A.A.V.P. guidelines (Wood et al., 1995) for evaluating *in vitro* anthelmintic efficacy of drugs, a product inhibiting egg hatch and larval motility should be considered an effective anthelmintic agent with 90% efficacy and considered moderately effective with 80-90% efficacy (Powers et al., 1982). Based on the EC₉₀ of CV-PAC and using motility as an estimate of viability, efficacy would be observed against L1 larvae at < 2 mg/mL and against adult worms at a concentration of 0.8 mg/mL. Based on the EC₉₀ of CV-AqE, efficacy would be observed against egg hatching at concentrations < 20 mg/mL (containing < 2.4 mg PAC/mL), against L1 larvae at a concentration of 3 mg/mL (containing 0.4 mg PAC/mL), and against adults at a concentration of 15 mg/mL (containing 1.8 mg PAC/mL). These concentrations are not dissimilar from *in vitro* activity of other plants that have also shown anthelmintic activity *in vitro* (Brunet et al., 2007; Molan and Faraj, 2010; Martínez-Ortíz-de-Montellano et al., 2013).

To determine if the efficacy observed *in vitro* could be confirmed *in vivo*, the effect of CV on an established experimental *H. contortus* infection in lambs was tested using a feeding regime that was estimated to administer of a concentration of 0.3 mg/mL PAC per day in the rumen, approximating the effective concentration observed *in vitro* against adult worms. Athanasiadou et al. (2000) used PAC-containing quebracho in a similar dosing regimen and observed a reduction in FEC after three consecutive days of dosing that was not reduced further by extending the treatment period to seven days. Although there was no difference in FEC between groups from week to week, there was a significant difference over time, reflecting a difference in the pattern of fecal egg counts. For the first two weeks after treatment, the FEC of the CV treatment group was slightly suppressed. Investigators who fed goats sericea lespedeza (SL) hay for seven weeks, saw a significant reduction in FEC in as little as one week following initiation of feeding (Shaik et al., 2006). The slight suppression in FEC over the first two weeks of our trial could be due to a mild suppressive effect of the cranberry vine. In a study feeding SL to lambs for 49 days (Lange et al., 2006), a notable increase in FEC was observed after SLfeeding was stopped. Possibly the damaged worms in our study would have had the chance to recover from the effects of the three-day treatment by week three when an increase in fecal egg count occurred. Unfortunately, a dose response could not be evaluated in our *in vivo* trial because of the limited amount of CV powder that was available. Nor could we investigate the impact of longer duration of exposure. Other investigators, who also fed SL hay to goats, fed for six weeks and did not see a significant reduction in FEC until day 42 of the trial (Terrill et al., 2009). Although the percentage of total PAC within SL and CV was comparable (Terrill et al., 2009), much less CV was fed and SL PAC could be structurally different from CV-PAC. Additionally, it should be noted that the results of this study could have been impacted by the effects of immunity on the course of infection since the lambs used in this study had been previously exposed to GIN on pasture.

5. Conclusions

This study provided *in vitro* evidence of anthelmintic activity of CV-PAC and CV-AqE extracts against L1 and adult worm motility and some activity of CV-AqE against egg hatching. Testing with CV-AqE supported the hypothesis that there may be other compounds in addition to PAC contributing to the observed anti-parasitic activity. Morphological changes of worms exposed to CV-AqE and CV-PAC *in vitro* also upported these findings. The *in vivo* study provided some indication of anti-parasitic effect of CV. Studies investigating dose response and effect of feeding duration of CV are needed in order to fully explore the potential use of CV as a feed supplement for small ruminant GIN control. While CV may not have a highly lethal effect on stages of *H. contortus*, even suppressive effects of this inexpensive and readily available plant could be of value in an integrated control program.

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Highlights

- Various life stages of *H. contortus* affected by CV extracts in vitro.
- Accumulation of aggregate observed on adult worms exposed to CV.
- Need for further studies of effects of feeding CV to *H. contortus* infected lambs.

Figure 1.

Effect of cranberry vine proanthocyanidin (CV-PAC) and cranberry vine aqueous extract (CV-AqE) on the adult *Haemonchus contortus* worm through scanning electron microscopy. Scanning electron microscopy of the cephalic end of adult *H. contortus* maintained in RPMI control (A, B, C; 12 hour), CV-PAC (D, E, F; 12 hour, 12 mg/mL CV-PAC), or CV-AqE (G, H, I; 18 hour, 3.1 mg/mL CV-AqE). Each row depicts one worm from each treatment, representative of the heaviest accumulation observed. Aggregates of CV-PAC and CV-AqE can be detected around the buccal area (E and H) as well as on cuticle of the body (F and I). The cuticle of worms that were incubated in RPMI control was compared to worms incubated in CV-PAC and worms incubated in CV-AqE. Accumulation of both CV-PAC and CV-AqE was observed on worms that were incubated in cranberry extracts.

Figure 2.

Effect of cranberry vines (CV) on an experimental infection of *Haemonchus contortus* in lambs. Lambs were experimentally infected with *H. contortus* and infection matured for approximately 35 days. Lambs in treatment group (CV n; $n = 9$) were orally drenched once daily for 3 consecutive days with 21.1 grams of CV and lambs in control group were not dosed (Control o; $n = 9$). Fecal egg counts (eggs per gram) were monitored for 4 weeks post-treatment. Treatment $\times$ week, $p = 0.04$, Mean $\pm$ SEM.

Table 1

Effect of cranberry vine proanthocyanidin extract (CV-PAC) on *Haemonchus contortus* egg hatch and L1 motility post-hatch, L3 motility and exsheathment, and adult worm motility.

Concentration mg PAC/mL	Egg Hatch ¹	L1 Motility ¹	L3 Motility ²	Exsheathment ²	Adult Motility (48 hrs) ³
	Percent Inhibition (%)				
TBZ	100 ± 0 ^{**}	—	—	—	94 ± 3*
0.0	9 ± 1	3 ± 1	11 ± 3	2 ± 1	7 ± 3
0.3	7 ± 1	49 ± 2 ^{**}	24 ± 4	1 ± 1	77 ± 5*
0.6	7 ± 1	77 ± 2 ^{**}	18 ± 5	5 ± 2	91 ± 1*
1.2	10 ± 2	79 ± 4 ^{**}	14 ± 3	8 ± 4	97 ± 3*
2.5	9 ± 1	98 ± 1 ^{**}	34 ± 5 ^{**}	23 ± 9	—
5.0	22 ± 2 ^{**}	98 ± 1 ^{**}	41 ± 4 ^{**}	59 ± 9 ^{**}	—
10.0	39 ± 2 ^{**}	98 ± 1 ^{**}	88 ± 2 ^{**}	83 ± 9 ^{**}	—

¹ Eggs ($n = 100$ eggs/well $\times 5$ wells per treatment) were exposed to varying concentrations of CV-PAC, water control (0.0 mg/mL), or thiabendazole (TBZ, 0.5 µg/mL) control for 24 hours and determined hatched or not hatched (percent egg hatch inhibition). The eggs that hatched into L1 larvae were determined motile or non-motile (L1 motility inhibition).

² Larvae (L3) were incubated in varying concentrations of CV-PAC or water control (0.0 mg/mL) for 24 hours. After the 24-hour incubation, larvae were determined motile or non-motile (L3 motility inhibition), and then exposed to CO₂ bubbling to initiate exsheathment and incubated for 18 hours. All motile larvae were determined sheathed or exsheathed ($n = 100$ per concentration per treatment) (percent exsheathment inhibition).

³ Worms were incubated in varying concentrations of CV-PAC ($n = 40$ per concentration), RPMI control (0.0 mg/mL, $n = 10$ per concentration), or thiabendazole (TBZ, 0.05 mg/mL) control for 48 hours. Adult worms were determined motile or non-motile based on observing motility for five seconds (adult motility inhibition). All values are mean $\pm$ SEM.

– Percentage unavailable; not tested

^{*} p 0.05 versus negative control (0.0 mg/mL) within column.

^{**} p 0.001 versus negative control (0.0 mg/mL) within column.

Effect of cranberry vine aqueous extract (CV-AqE) on *Haemonchus contortus* egg hatching and L1 motility post-hatch, L3 motility and exsheathment, and adult worm motility.

Table 2

Concentration	Egg Hatch ¹	L1 Motility ¹	L3 Motility ²	Exsheathment ²	Adult Motility (24 hrs) ³
mg Powder/mL	mg PAC/mL	Percent Inhibition (%)			
TBZ	–	100 ± 0 ^{**}	–	–	94 ± 3 [*]
0.0	0.0	5 ± 1	5 ± 1	3 ± 3	10 ± 10
0.3	0.04	7 ± 1	5 ± 1	4 ± 2	1 ± 1
0.6	0.08	9 ± 2	6 ± 1	0 ± 0	1 ± 2
1.3	0.15	7 ± 1	16 ± 1 ^{**}	6 ± 2	8 ± 2
2.5	0.3	8 ± 1	59 ± 1 ^{**}	7 ± 2	15 ± 4
3.1	0.38	–	–	–	60 ± 20
5.0	0.6	29 ± 5 ^{**}	98 ± 1 ^{**}	2 ± 1	9 ± 3
6.3	0.74	–	–	–	50 ± 10
10.0	1.19	77 ± 4 ^{**}	97 ± 2 ^{**}	6 ± 12	11 ± 4
12.5	1.5	–	–	–	90 ± 10 [*]
20.0	2.38	100 ± 0 ^{**}	100 ± 0 ^{**}	11 ± 3	4 ± 2
25.0	3.0	–	–	–	100 ± 0 [*]

¹Eggs ($n = 100$ eggs/well $\times 5$ wells per treatment) were exposed to varying concentrations of CV-AqE, water control (0.0 mg/mL), or thiabendazole (TBZ, 0.5 μ g/mL) control for 24 hours and determined hatched or not hatched (percent egg hatch inhibition). The eggs that hatched into L1 larvae were determined motile or non-motile (L1 motility inhibition).

²Larvae (L3) were incubated in varying concentrations of CV-AqE or water control (0 mg/mL) for 24 hours. After the 24-hour incubation, larvae were determined motile or non-motile (L3 motility inhibition), and then exposed to CO₂ bubbling to initiate exsheathment and incubated for 18 hours. All motile larvae were determined ensheathed or exsheathed ($n = 100$ per concentration per treatment) (percent exsheathment inhibition).

³Worms were incubated in varying concentrations of CV-AqE ($n = 10$ per concentration), RPMI control (0.0 mg/mL, $n = 10$ per concentration), or thiabendazole (TBZ, 0.05 mg/mL) control for 24 hours. Adult worms were determined motile or non-motile based on observing motility for five seconds (adult motility inhibition). All values are mean $\pm$ SEM.

– Percentage unavailable; not tested.

p 0.05 versus negative control (0.0 mg/mL) within column.

^{**} p 0.001 versus negative control (0.0 mg/mL) within column.

Table 3

Effective concentration that inhibited 50% (EC₅₀) and 90% (EC₉₀) (mg/mL) and 95% confidence interval (95% CI) of CV-PAC and CV-AqE in *Haemonchus contortus* egg hatch inhibition, L1 and L3 motility inhibition, exsheathment inhibition, and adult worm motility inhibition assays.

	Egg Hatch ¹	L1 Motility ²	L3 Motility ³	Exsheathment ⁴	Adult Motility ⁵
CV-PAC EC₅₀					
PAC/mL	26.6 mg ⁺	0.3 mg	4.0 mg	4.0 mg	0.2 mg
(95% CI)	(20.9 – 35.8)	(0.27 – 0.33)	(3.5 – 4.8)	(3.2 – 5.2)	(0.1 – 0.3)
CV-PAC EC₉₀					
PAC/mL	618 mg ⁺	1.6 mg	52.3 mg	17.8 mg	0.8 mg
(95% CI)	(360 – 1E3)	(1.4 – 1.8)	(35.8 – 84.6)	(11.8 – 32.9)	(0.6 – 1.9)
CV-AqE EC₅₀					
powder/mL	5.3 mg	1.5 mg	137 g ⁺	11 g ⁺	3.4 mg
(95% CI)	(5.0 – 5.7)	(1.5 – 1.6)	(2 – 3E12)	(0.4 – 5E9)	(0.2 – 5.8)
PAC/mL	0.6 mg	0.2 mg	16 g ⁺	1.3 g ⁺	0.4 mg
(95% CI)	(0.6 – 0.7)	(0.17 – 0.19)	(0.2 – 3E11)	(0.0 – 6E8)	(0.0 – 0.7)
CV-AqE EC₉₀					
powder/mL	19.8 mg	3.0 mg	460 kg ⁺	10 kg ⁺	15.1 mg
(95% CI)	(17.7 – 22.3)	(2.8 – 3.2)	(0.2 – 8E20)	(0.0 – 2E17)	(8 – 1E3)
PAC/mL	2.4 mg	0.4 mg	55 kg ⁺	1.3 kg ⁺	1.8 mg
(95% CI)	(2.1 – 2.7)	(0.3 – 0.4)	(0.0 – 1E20)	(0.0 – 2E16)	(1.0 – 1E2)

⁺ All values higher than actual assay concentrations tested have been extrapolated through the PROBIT procedure in SAS.