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Research paper

Anthelmintic-like activity of polyphenolic compounds and their interactions against the cattle nematode *Cooperia punctata*

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ABSTRACT

Polyphenolic compounds (PCs) have been proposed as one of the most bioactive group of secondary metabolites occurring in nature and have been associated to anthelmintic (AH)-like activity of plants against cattle nematodes. However, little is known regarding their synergetic / antagonistic interactions. This study assessed the *in vitro* AH-like activity of commercial PCs: quercetin, caffeic acid, rutin and coumarin, and their combinations against the egg hatching and larval exsheathment of *Cooperia punctata*; one of the most prevalent nematodes affecting grazing cattle in tropical regions. The molecules selected for the *in vitro* analysis were identified as bioactive phytochemicals of plants through bio-guided fractionation in previous studies. To estimate mean effective concentrations (EC_{50}) five increasing concentrations were used for both Egg hatching inhibition assay (EHIA) and larval exsheathment inhibition assay (LEIA) (0.6–9.8 mg mL^{-1} and 0.15–2.4 mg mL^{-1}, respectively). From the four molecules, only rutin did not affect egg hatching; while quercetin, showed no bioactivity against eggs or larvae ($P > 0.766$ and $P > 0.621$, respectively). Best-fit EC_{50} estimated through the EHIA was considered for PCs classification as bioactive (coumarin and caffeic acid) and non-bioactive (quercetin and rutin). Phytochemical interactions were subsequently assessed combining bioactive:non-bioactive PCs (8:2 ratio), and the nature of their interaction was classified using the fractional inhibitory concentration index (FIC_{index}). Combinations had a highly synergistic interaction against larval exsheathment ($FIC_{index} < 0.5$) except for coumarin:rutin against egg hatching ($FIC_{index} > 0.5$). Quercetin and rutin acted as PCs AH-like activity enhancers, reducing EC_{50} of bioactive molecules in a range of 43%–64% and 68%–83% for EHIA and LEIA, respectively. A linear relationship between low molecular weight of molecules and ovicidal activity was observed; where, molecules with lower molecular weight displayed better-fit EC_{50} for ovicidal activity. Furthermore, coumarin and caffeic acid bioactivity against free-living stages of *C. punctata* makes them suitable candidates as markers for anthelmintic-like activity in bioactive forages. Combinations used through this investigation showed a potent anthelmintic-like activity against free-living forms of *C. punctata*, representing a first step towards the identification of promising alternatives for nematode control.

1. Introduction

Gastrointestinal nematodes (GIN) represent one of the major health and production threats of cattle under grazing conditions. *Cooperia punctata* has been described as one of the most prevalent and pathogenic *Cooperia* species of cattle in tropical and subtropical regions (Ramünke et al., 2018) affecting cattle performance and metabolism (Stromberg et al., 2012; Louvandini et al., 2009). Controlling gastrointestinal nematodosis in cattle has become a major challenge due to the emergence of anthelmintic resistance (Alonso-Díaz et al., 2015; Sutherland and Leathwick, 2011). However, several investigations report the use of bioactive plants and its secondary metabolites as a potential alternative for GIN control (Hoste et al., 2015). Among plant secondary metabolites groups, the polyphenolic compounds (PCs) have been reported as one of the most bioactive regarding anthelmintic (AH)-like activity (Engström et al., 2016a,b, 2015; Klongsiriwet et al., 2015; Novobilsky et al., 2011; Alonso-Díaz et al., 2008; Brunet and Hoste, 2006). Although, the mechanism of action of PMS is yet to be

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elucidated; structural differences among PCs such as the degree of hydroxylation and molecular weight have been addressed as two of the main possible characteristics of PCs which allows them to exert or enhance AH-like activity, either by penetrating or binding to parasites structural proteins or other structures with biological importance in parasites; which, when affected, may impair their viability (Spiegler et al., 2017; Engström et al., 2016a,b, Klongsiriwet et al., 2015; Vargas-Magaña et al., 2014).

Several studies have isolated and identified PCs from plant extracts affecting different biological processes of GIN and have proposed a possible synergistic / antagonistic behavior when present in a mixture (Engström et al., 2016a,b; von Son-de Fernex et al., 2015; Brunet and Hoste, 2006). However, there is scarce information regarding the bioactivity mechanism of the pure compounds and its interactions. The PCs assessed through this study (quercetin, caffeic acid, rutin, and coumarin) were selected from previous reports of bio-guided isolation / purification from tropical plants with AH-like activity; some of which, were purified as single molecules or isolated in an 8:2 ratio mixture (von Son-de Fernex et al., 2015, 2017). The objective of this study was to assess the AH-like activity of PCs (quercetin, caffeic acid, rutin and coumarin) and their interactions against free-living stages of the cattle nematode *C. punctata*; which could lead to new AH drugs development and / or to find patterns of activity and consider certain molecules as bioactivity markers with AH-like activity within tropical plants.

2. Materials and methods

2.1. Polyphenolic compounds

Chemical PCs: Quercetin ($C_{15}H_{10}O_7$ •$2H_2O$), Caffeic acid ($C_9H_8O_4$), Rutin ($C_{27}H_{30}O_{16}$•xH_2O) and Coumarin ($C_9H_6O_2$), with Chemical Abstracts Service (CAS) registry numbers® 117-39-5, 331-39-5, 153-18-4, and 91-64-5, respectively; were obtained from Sigma (St. Louis, MO) (Fig. 1).

2.2. Biological material

2.2.1. Egg and larval recovery

Eggs and infective larvae (L_3) were obtained from a donor calf with a monospecific infection of *Cooperia punctata* (isolate *C. p.* CEIEGT-FMVZ-UNAM strain, Mexico; von Son-de Fernex et al., 2017). The calf was housed indoors on a concrete floor, provided with hay, commercial concentrate and free access to water (complying with the Internal Committee for Care and Use of Experimental Animals of the National Autonomous University of Mexico [CICUAE-UNAM] regulations). For egg recovery, feces were collected daily using harnesses and polyurethane collection bags; samples were stored and processed at a temperature of 23.4 ± 0.2 °C (Mean ± SE). The egg recovery process was standardized for completion in 1.25 ± 0.08 h (Mean ± SE) and was performed as described by von Son-de Fernex et al. (2015).

Cooperia punctata third stage larvae (L_3) were obtained from the same donor calf. Feces were collected daily and cultured for seven days, after which, larvae were recovered with Corticelli-Lai methodology (Corticelli and Lai, 1963). Finally, larvae were stored at 4 °C for two months before use. Final concentration of 1000 *C. punctata* L_3 mL^{-1} was achieved either by concentrating the larval inoculum through centrifugation, or by diluting with distilled water (DW).

2.3. Evaluation of pure polyphenolic compounds

2.3.1. Egg hatching inhibition assay

Approximately 100 eggs / 200 µl of egg suspension were pipetted into each well of a 24 well culture plate, and 200 µl of increasing concentrations (0.6, 1.2, 2.4, 4.8 and 9.6 mg mL^{-1}) of the corresponding PCs were placed in each test well. Polyphenolic compounds were diluted as follows: ethanol 2.5% (caffeic acid and coumarin) and dimethyl sulfoxide (DMSO) 2.5% (quercetin and rutin); same solvents were use as negative controls. Thiabendazole 99% (Sigma ®) was used as a positive control at a concentration of 10%. Control well content was also 200 µl. Four replicates were run for each concentration, PCs and control. The plates were incubated at 27.71 ± 0.13 °C (Mean ± SE) for 48 h. A drop of Lugol's iodine solution was added to each well to stop further hatching and all the unhatched eggs and larvae (dead or alive) in each well were counted (Coles et al., 1992).

2.3.2. Larval exsheathment inhibition assay

Four thousand ensheathed *C. punctata* were separately incubated for each of PCs at increasing concentrations of 150, 300, 600, 1200 and 2400 µg mL^{-1} of distilled water (DW), for 3 h at 21 °C. Polyphenolic compounds were diluted as follows: ethanol 2.5% (caffeic acid and coumarin) and dimethyl sulfoxide (DMSO) 2.5% (quercetin and rutin); same solvents were use as negative controls. After incubation, L_3 were washed and centrifuged (908 x *g*) three times in DW (pH 7.2) as described by Alonso-Díaz et al. (2008). Larvae were then subjected to an artificial exsheathment process by contact with a sodium hypochlorite solution (75 µL domestic bleach with 112 µL of 6% sodium hypochlorite, diluted in 15.813 mL of distilled water) modified from Katiki et al. (2013). This was performed to obtain 100% larval exsheathment after 60 min in control groups. The kinetics of the larval exsheathment process was monitored by microscopic observation (40x). Exsheathed and unsheathed larvae were counted at 0, 10, 20, 30, 40, 50 and 60 min. At each time interval, L_3 were killed with a drop of Lugol's

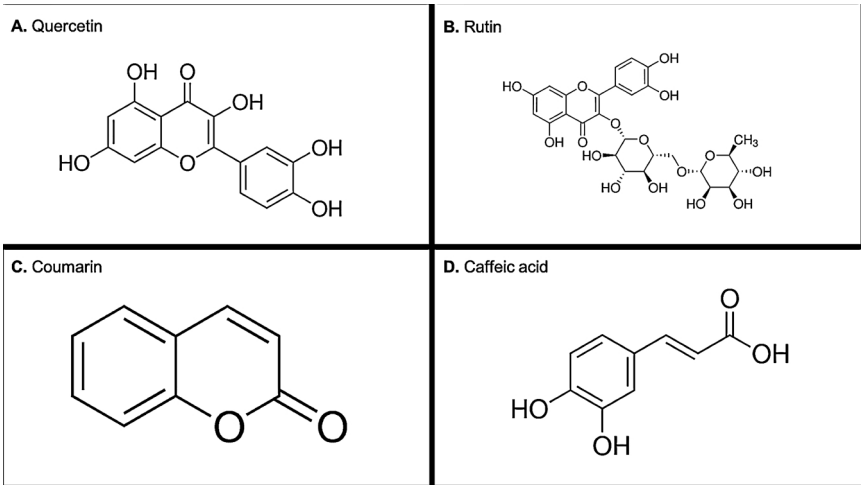

Fig. 1. Chemical structure of the polyphenolic compounds.

iodine solution and examined immediately. Six replicates were run for each concentration, PCs and controls, to look for possible changes in the proportion of exsheathed larvae over time (Alonso-Díaz et al., 2008).

2.4. Evaluation of polyphenolic compounds interactions against free-living stages of C. punctata

Due to the differences of sensitivity of the assays employed through this investigation, the results obtained through the EHIA (see sec. 2.3.1.) were used to classify PCs as anthelmintic-like bioactive molecules (coumarin and caffeic acid) and non-bioactive (quercetin and rutin). Combinations were performed using a bioactive PCs and a non-bioactive one (bioactive:non-bioactive), in an 8:2 ratio, respectively. The anthelmintic-like activity of the PCs combinations was assessed through the bioassays EHIA and LEIA. Bioactive molecules were tested at a final concentration of 9.6 mg mL^{-1} and 1.2 mg mL^{-1} for EHIA and LEIA, respectively.

2.5. Statistical analysis

A general linear model (GLM) was used to assess a dose-dependent behavior of each PCs in all bioassays using the computer program GraphPad Prism® V. 6.1. The percentage of egg hatching inhibition was calculated as: inhibition (%) = 100(1 - Pt / Pc), where Pt is the number of eggs hatched in treatment group, and Pc is the respective number in water or DMSO control groups (Bizimenyera et al., 2006). To fit the dose-response data by non-linear regression (EHIA and LEIA), a four-parameter logistic equation with a variable slope was used using the computer program GraphPad Prism® V. 6.1. All analyses were performed after transforming the data into logarithms (X = logX) and constraining the bottom and top values to 0 and 100, respectively. Finally, the EC_{50}, 95% confidence limits and R^2 values were also calculated. To assess the relationship between the molecular weight of the pure PCs and the EC_{50} obtained for each compound through each assay, a linear regression test was run using the computer program GraphPad Prism® V. 6.1.

The phytochemical interactions were determined on basis of the fractional inhibitory concentration index (FIC_{index}). For which, EC_{50} obtained for each polyphenolic compound was transformed to fractional inhibitory concentration (FIC) as reported by Sanhueza et al., 2017:

$$FICA = \frac{\text{EC50 of compound A in the presence of B}}{\text{EC50 of compound A individually}}$$

$$FICB = \frac{\text{EC50 of compound B in the presence of A}}{\text{EC50 of compound B individually}}$$

To asses if interactions were highly synergistic, synergistic, additive, without interaction or antagonistic, the fractional inhibitory concentration index (FIC_{index}) was calculated as: $FIC_{index} = FIC_A + FIC_B$. Interpretation of FIC_{index} was performed as follow: highly synergistic (FICindex < 0.5); synergistic (FICindex < 1); additive (FICindex = 1); no effect (1 < FICindex < 2) and antagonistic (FICindex > 2) (Mor et al., 2015).

3. Results

3.1. Anthelmintic-like activity of pure polyphenolic compounds

3.1.1. Egg hatching inhibition assay

Mean egg hatching (± SE) of *C. punctata* in negative control ranged from 90 to 100% and the egg hatching was fully inhibited with the positive control. Egg hatching showed a dose-dependent behavior only when exposed to coumarin and caffeic acid ($P < 0.035$ and $P < 0.001$, respectively), the other PCs were considered non-bioactive for AH-like activity due to the low inhibition achieved. Best-fit EC_{50} were 0.811 mg mL^{-1} and 1.157 mg mL^{-1} for coumarin and caffeic acid, respectively (Table 1). Highly significant relationship was observed between the molecular weight and the EC_{50} estimated for the ovicidal activity of pure compounds (R^2: 0.9415, CI 95%, $P < 0.029$) (Fig. 2).

Table 1
Mean effective concentrations (EC_{50}), 95% confidence limits (CL) and coefficients of determination (R^2) of pure polyphenolic compounds (PCs) and their interactions against *C. punctata* egg hatching.

Molecules	EC_{50} (mg mL^{-1})	SE	95 % confidence limits		R^2
			Lower	Upper	
Quercetin	133.8	0.518	10.15	1765	0.564
Coumarin	0.811	0.016	0.748	0.879	0.984
Rutin	8486	0.459	864.7	83282	0.849
Caffeic acid	1.157	0.023	1.029	1.300	0.966
INTERACTIONS (8:2)					
Coumarin : Quercetin	0.348	0.001	0.345	0.351	0.999
Coumarin : Rutin	0.462	0.007	0.445	0.480	0.994
Caffeic acid : Quercetin	0.413	0.084	0.271	0.628	0.667
Caffeic acid : Rutin	0.578	0.07	0.408	0.819	0.814

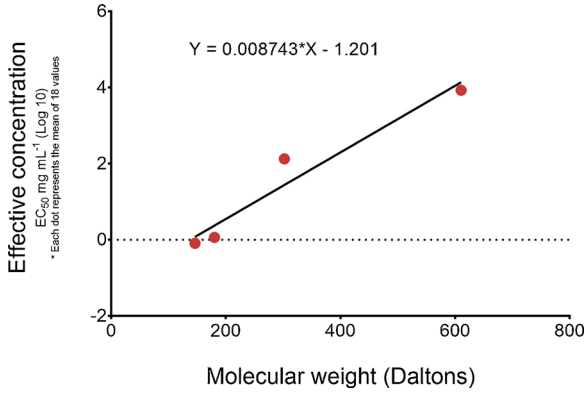

Fig. 2. Linear relationship observed between the molecular weight of pure polyphenolic compounds (PCs) with their mean effective concentration (EC_{50}) against *C. punctata* egg hatching. (Df = 2).

3.1.2. Larval exsheathment inhibition assay

The exsheathment kinetics of *C. punctata* L_3 was similar in all control groups with 100% of exsheathment obtained after a 60 min exposure to the artificial exsheathment fluid. With the exception of quercetin ($P > 0.621$), the PCs fully inhibited the exsheathment process of *C. punctata* after a 3 h incubation period of L_3 to the highest concentration (2400 µg mL^{-1}), showing dose-dependent behaviors. Effective concentrations required for 50% of exsheathment inhibition (EC_{50}) are presented in Table 2. Non-significant relationship was

Table 2
Mean effective concentrations (EC_{50}), 95% confidence limits (CL) and coefficients of determination (R^2) of pure polyphenolic compounds (PCs) and their interactions against *C. punctata* exsheathment process.

Molecules	EC_{50} (mg mL^{-1})	SE	95 % confidence limits		R^2
			Lower	Upper	
Quercetin	ND	ND	ND	ND	ND
Coumarin	0.744	0.033	0.624	0.889	0.952
Rutin	0.651	0.054	0.486	0.872	0.909
Caffeic acid	0.897	0.020	0.807	0.999	0.985
INTERACTIONS (8:2)					
Coumarin : Quercetin	0.231	0.014	0.213	0.249	0.991
Coumarin : Rutin	0.169	0.061	0.123	0.234	0.843
Caffeic acid : Quercetin	0.229	0.029	0.196	0.268	0.947
Caffeic acid : Rutin	0.146	0.052	0.110	0.192	0.889

*ND: Not determined.

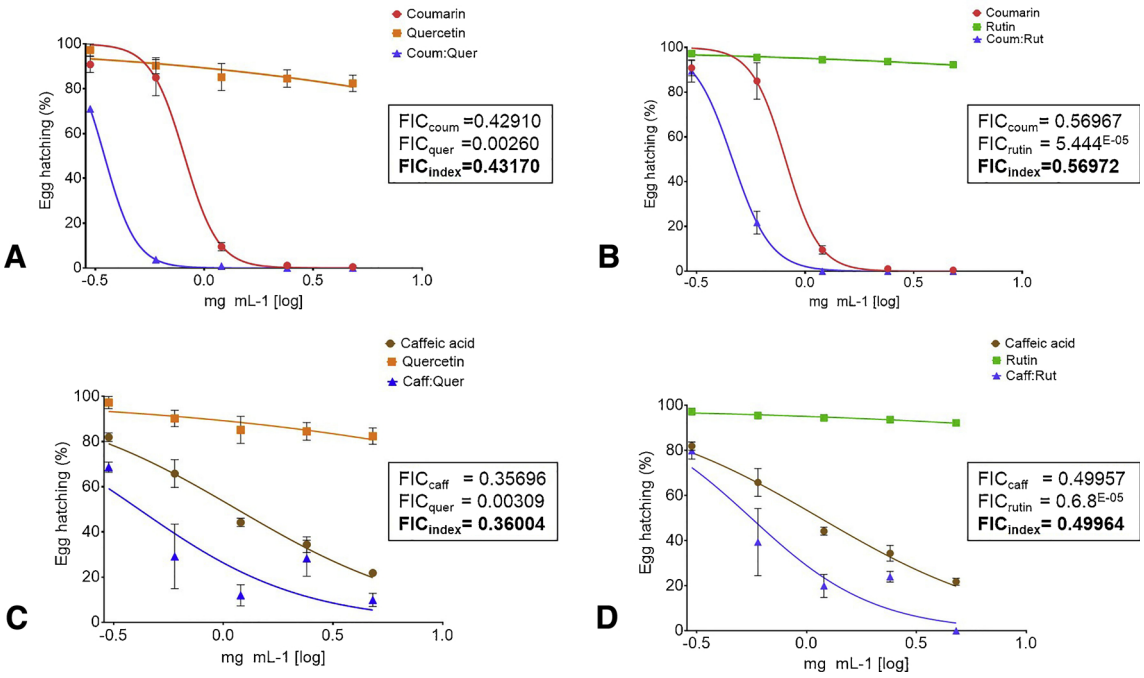

Fig. 3. Fractional inhibitory concentration index (FIC_{index}) values obtained with the polyphenolic compounds (PCs) combinations against *C. punctata* egg hatching. (A. Ovicidal activity of coumarin, quercetin and their interaction; B. Ovicidal activity of coumarin, rutin and their interaction; C. Ovicidal activity of caffeic acid, quercetin and their interaction; D. Ovicidal activity of caffeic acid, rutin and their interaction).

observed between the molecular weight and the anti-exsheathment activity of compounds ($P > 0.749$).

3.2. Anthelmintic-like activity of polyphenolic compound interactions

3.2.1. Effect of polyphenolic compound interactions against C. punctata egg hatching

Quercetin showed a synergistic interaction with both caffeic acid and coumarin decreasing in a twofold the EC_{50} of pure molecules (Table 1). Although rutin also decreased EC_{50} of pure molecules down to a 43% and 49% for coumarin and caffeic acid, respectively. The FIC_{index} values obtained for each combination are presented in Fig. 3. Three out of the four combinations assessed showed a highly synergistic interaction. Coumarin combinations showed an ovicidal activity inhibiting larval development within the egg (Fig. 4. A–I). On the other hand, caffeic acid mainly affected larval hatching (Fig. 5 A–C), thus, when combined with quercetin a trend on impairing larval development was also observed (Fig. 5 D–F).

3.2.2. Effect of polyphenolic compound interactions against C. punctata larval exsheathment

Combination of bioactive molecules with quercetin and rutin

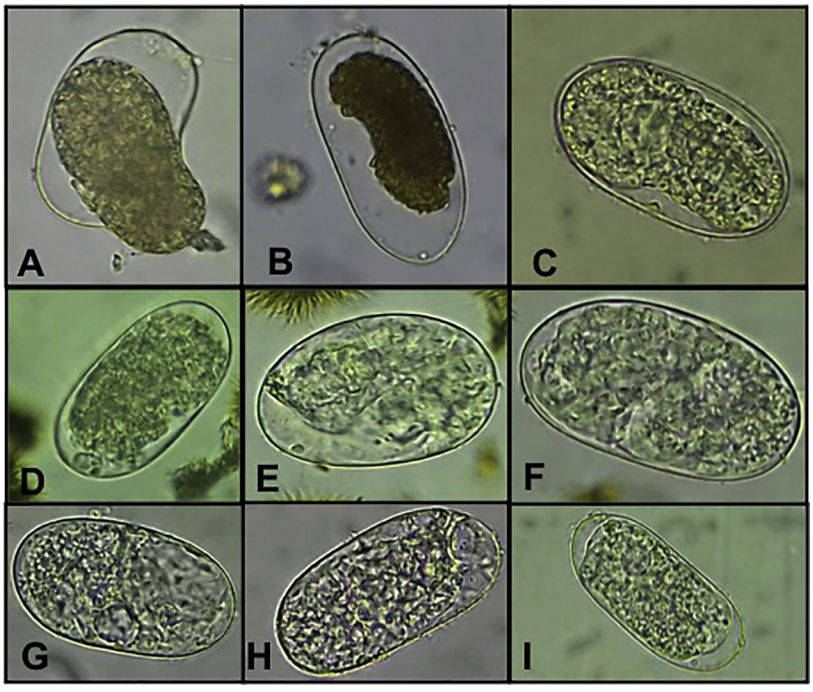

Fig. 4. Bioactivity of coumarin and its combination with quercetin and rutin over *C. punctata* egg stage after 48 h incubation. (A–C. Undeveloped *C. punctata* eggs incubated with coumarin; D–F. Arrested development of eggs incubated in the combination of coumarin and quercetin; G–I. Eggs showing an arrested development of larvae and crater shaped lesions in the embryo after incubated in the combination of coumarin and rutin).

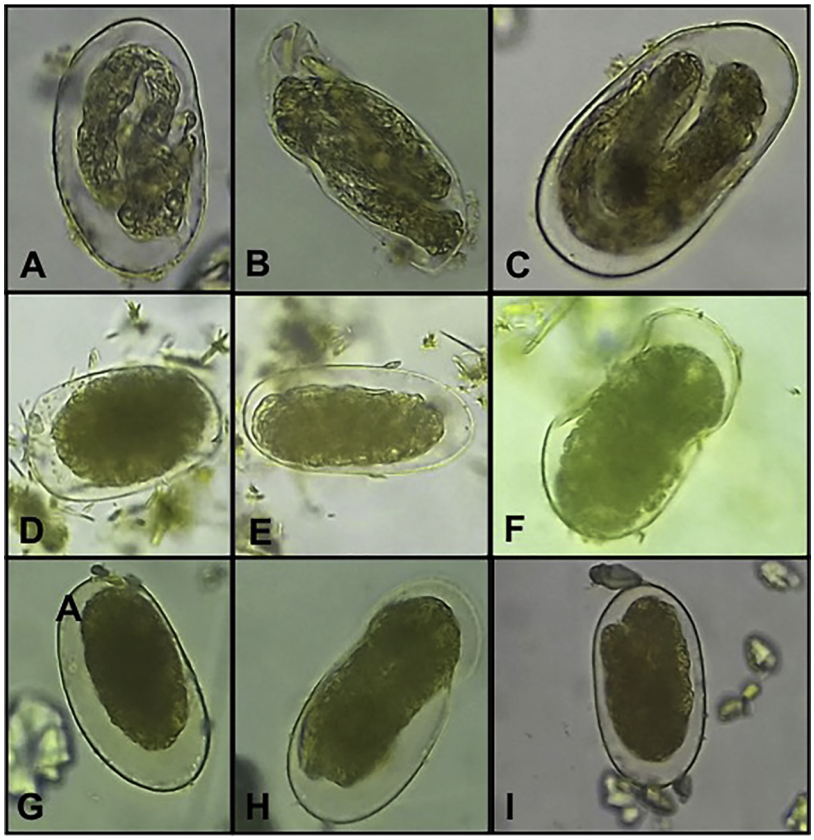

Fig. 5. Bioactivity of caffeic acid and its combination with quercetin and rutin over *C. punctata* egg stage after 48 h incubation. (A–C. *Cooperia punctata* death first-stage larvae within the egg, and eggshell structural damage after incubation with caffeic acid; D–F. Arrested development of larvae within the eggs and eggshell structural damage after incubation with the combination of caffeic acid and quercetin; G–I. Eggs with embryo arrested development and / or death first-stage larvae after incubated in the combination of caffeic acid and rutin).

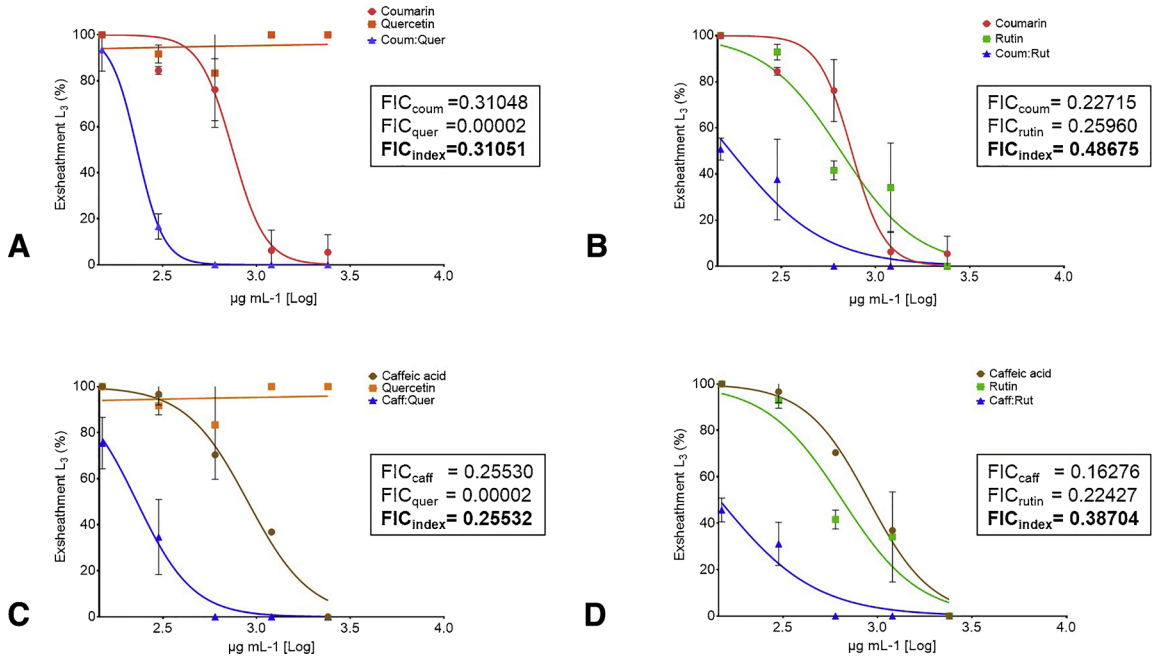

Fig. 6. Fractional inhibitory concentration index (FIC_{index}) values obtained with the polyphenolic compound combinations against *C. punctata* exsheathment process. (A. Anti-exsheathment activity of coumarin, quercetin and their interaction; B. Anti-exsheathment activity of coumarin, rutin and their interaction; C. Anti-exsheathment activity of caffeic acid, quercetin and their interaction; D. Anti-exsheathment activity of caffeic acid, rutin and their interaction).

decreased the EC_{50} obtained for pure compounds. Coumarin alone showed an EC_{50} of 0.744 ± 0.033 mg mL^{-1} which was lowered down to 0.231 ± 0.014 mg mL^{-1} and 0.169 ± 0.061 mg mL^{-1} when combined with quercetin and rutin, respectively (Table 2). Likewise, EC_{50} of caffeic acid was 0.897 ± 0.020 mg mL^{-1} and decreased to 0.229 ± 0.0029 mg mL^{-1} and 0.146 ± 0.052 mg mL^{-1} when combined with quercetin and rutin, respectively. Both quercetin and rutin had a highly synergistic interaction with the bioactive PCs. The FIC_{index} values are presented in Fig. 6.

4. Discussion

Although gastrointestinal nematodosis in cattle are not considered life threatening, GIN infection severely affects animal welfare and the farmer economy (Rodriguez-Vivas et al., 2017). For the past 5 decades, helminth control has relied on the use of broad-spectrum chemicals (benzimidazoles, Imidazotiazoles and macrocyclic lactones); however, the alarming emergence of anthelmintic resistance in cattle nematodes has become a major threat for efficient control strategies (Alonso-Díaz et al., 2015). The study of bioactive plants as a novel approach for helminth control has strongly emerged in the past few years due to their potential use as nutraceuticals and because plants are considered a source for development of novel compounds / products (Cassano et al., 2017). Understanding interaction among PCs and parasites could also help categorize polyphenol markers for the selection of plants with potential anthelmintic-like activity (Engström et al., 2016a,b).

Although polyphenols AH-like mechanism remains unknown, two major hypotheses have been proposed: 1) bioactivity has been directly associated to the degree of hydroxylation and 2) to the molecular weight (Spiegler et al., 2017; Engström et al., 2016a,b, Vargas-Magaña et al., 2014). Our findings seem consistent and in partial agreement with both hypotheses, as the compounds considered bioactive (with ovicidal activity) through this investigation (coumarin and caffeic acid) had the lowest molecular weight among the PCs tested (146.145 Daltons and 180.159 Da, respectively). A highly significant linear relationship between low molecular weight of compounds and ovicidal activity was observed, where, molecular weight higher than 180 Da significantly increased de EC_{50} for ovicidal activity. Such findings are in partial agreement with authors stating that the molecular size of ovicidal compounds should not be bigger than 400–500 Da (Vargas-Magaña et al., 2014), because the less-fit EC_{50} obtained through this investigation were those of quercetin and rutin, which have molecular weights of 302.238 Da and 610.521 Da, respectively. The latter also differs from authors reporting an optimal molecular weight of compounds in a range of 700–2000 Da to affect egg hatching (Engström et al., 2016a,b). Even though our results are inconsistent with previous reports it should be considered that, the nature of the phytochemicals, the nematode species and developmental stages assessed might play an important role on phytochemical bioactivity; possibly related to differences in protein composition of the nematodes external structures. As authors have described for cuticle hydroxyproline / proline ratio between *H. contortus* and other nematode species (Spiegler et al., 2017; Engström et al., 2016a,b).

On the other hand, hydroxylation has been proposed as major factor involved in AH-like activity of PCs due to their capacity to bind with collagen-like proteins, cuticlins and other non-structural proteins of nematodes (Fetterer and Rhoads, 1993), which causes a blockage of the cuticle with the environment resulting in asphyxia and / or cellular toxicity (Spiegler et al., 2017). Thus, through this investigation it was observed, as described by Brunet and Hoste (2006), that hydroxylated molecules have the ability to block the exsheathment process of L_3; however, they have low or no bioactivity against egg hatching of *C. punctata*. Furthermore, hydroxylated molecules assessed through this project proved to be better bioactivity enhancers than AH-like molecules; traits that might be correlated with hydroxyl group's interaction with cell membranes. As it has been found in recent studies, there is a high association between the number of hydroxyl groups present in a molecule and their penetration or adhesion capability (Nakammura et al., 2018).

Interaction among bioactive molecules has been widely studied for antibiotic development, and four types of responses have been reported: strongly synergistic, synergistic, additive, indifferent and antagonistic (Mor et al., 2015). The FIC_{index} has been used to categorize the type of interaction between two or more molecules (Sanhueza et al., 2017); however, the model has been used for antibacterial or antifungal products; and to our knowledge, this is the first attempt to standardize FIC_{index} for secondary plant metabolites with AH-like activity against cattle nematodes. Prior microbiological drug assessment studies have considered both FIC_{index} and the decrease in population of 2-log to define the synergistic interaction of agents (Pei et al., 2009). However, regarding GIN control, it has been reported that due to anthelmintic resistance emergence, not even commercial anthelmintics achieve a 2-log reduction ($\geq$ 99%) (Ramos et al., 2016). Thus, considering the results obtained through this investigation and the classification proposed by Mor et al. (2015), a strongly synergistic activity of PCs could be considered after a 49% reduction of the EC_{50} displayed by the most bioactive molecule alone.

The synergistic interactions observed for quercetin and rutin when combined with coumarin and caffeic acid, could be correlated to their capacity to inhibit specific membrane proteins related with drug resistance mechanisms such as cytochrome and P-glycoproteins (Yang et al., 2014; Mandery et al., 2010); allowing the smaller polyphenols to fully exert their ovicidal potential. Such findings are consistent with previous studies reporting quercetin as a P-glycoprotein (PgP) modulator which has the capacity to enhance anthelmintic activity of both ivermectin and caffeic acid (von Son-de Fernex et al., 2015; Heckler et al., 2014). On the other hand, rutin showed non-ovicidal activity but did impair third stage larvae exsheathment process; which is consistent with previous reports of rutin acting as anthelmintic against roundworms (Dubey et al., 2013). Exsheathment blockage induced by tannins has been directly associated to its capacity to bind with nematodes cuticle proteins (Brunet and Hoste, 2006). Mechanism which might also be related to rutin bioactivity; as the antibacterial, antiviral and anti-inflammatory properties of rutin have been linked to its ability to scavenge free radicals and to bind with structural protein of viruses (Gullon et al., 2017; Selway, 1986). Furthermore, recent studies have reported rutin to enhance or complement antibacterial activity of other flavonoids (Gullon et al., 2017; Ganeshpurkar and Saluja, 2017); which concur with the synergistic activity observed through this project when combined with both coumarin and caffeic acid.

Most of the molecules assessed for AH-like activity and their synergistic interactions include flavonoids and condensed tannin monomers (flavan-3-ols) (Klongsiriwet et al., 2015; Brunet and Hoste, 2006). However, through this investigation, none of the molecules assessed are directly involved in condensed tannins; which might explain some of the bioactivity differences and the nature of the interactions observed. Besides the molecular weight and polymerization of the molecule, one of the main structural differences between condensed tannin monomers (flavan-3-ols) and flavonols (quercetin and rutin) is that the latter group has a 2,3-double bond and a C4 carbonil in ring C, which are absent in the monomers involved in condensed tannins (Tsimogiannis and Oreopoulou, 2019). Authors have reported that such structural moiety differences have an important role on synergistic / antagonistic interactions between flavonoids (Wang et al., 2018). Furthermore, previous studies assessing flavonoid and catechin interactions, have reported that the 2,3-double bond of flavonols enhances trolox equivalent antioxidant capacity (TEAC) and induces a higher anti-viral / bacterial activity (Wang et al., 2018; Minatel et al., 2017). On the other hand, coumarin and caffeic acid do not share the structural moieties of flavonols or flavan-3-ols; however, it has been reported that both PCs inhibit cell proliferation and inhibit different enzymes such as acetylcholinesterase, matrix metalloproteinases among others (Anwar et al., 2012; Mirunalini and Krishnaveni, 2011; Qiang, 2011). In concurrence with our observations, it has been suggested that flavonols bioactivity might be related to their cell signaling modulation capability (Tsao, 2010) opposing to the direct effect affecting parasites morphology proposed for tannins (Hoste et al., 2012).

Although the differences observed in the synergistic interaction of PCs remains unclear, the results suggest that molecular weight, hydroxylation and structure arrangement are the main characteristics involved in PCs interactions and AH-like activity (Jakobek, 2015).

Although much more needs to be understood, it could be suggested

that lower molecular weight of PCs improves ovicidal activity, while hydroxylation enhances anti-exsheathment activity. After assessing different plant extracts bioactivity and analyzing their coumarin and caffeic acid content, those molecules might be considered as secondary metabolite markers to detect AH-like activity of plants. Furthermore, one of the possible limitations of phytochemical drugs used under field conditions is that most of the natural compounds assessed require high concentrations to exert their anthelmintic activity, while phytochemical yields from the plant are very low. Thus, the identification and use of new AH-like molecules like coumarin and caffeic acid, and bioactivity enhancers such as quercetin and rutin could represent an alternative for new drug formulation against resistant nematodes, which could be tested under field conditions at lower concentrations. The combination of polyphenols assessed through this investigation showed their capacity to affect free-living stages *C. punctata*. Further studies targeting adult stages of the nematode, potential toxicity and pharmacokinetics are suggested prior to assess the presented formulations at *in vivo* field trials.

5. Conclusions

Coumarin and caffeic acid showed high ovicidal and anti-exsheathment activity against *C. punctata*; on the other hand, the flavonols (quercetin and rutin) showed low or no anthelmintic activity whatsoever. Thus, when combined with the bioactive molecules, both flavonols acted as bioactivity enhancers reducing effective concentrations in a range of 43%–83%. The ovicidal and anti-exsheathment activity observed with both coumarin and caffeic acid suggests them as possible bioactivity markers for AH-like activity in plants.

Author contributions

1 Escareño-Díaz S: Student performed the different assays of the project.
2 Alonso-Díaz MA: Contributed with the founding acquisition, resources and revision of the final manuscript.
3 Mendoza de Gives P: Contributed with resources and review of the final manuscript.
4 Castillo-Gallegos E: Contributed with resources and review of data analysis and final manuscript.
5 von Son-de Fernex E: Founding acquisition, project administration, design of the study and conceptualization, methodology, supervision, data analysis, original draft writing and editing of the manuscript.

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Declaration of Competing Interest

None.

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