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Anthelmintic effect of *Pterogyne nitens* (Fabaceae) on eggs and larvae of *Haemonchus contortus*: Analyses of structure-activity relationships based on phenolic compounds

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ABSTRACT

Due to high prevalence and large pathogenicity, *Haemonchus contortus* is the main gastrointestinal nematode in tropical and subtropical regions. This species is responsible for severe economic losses to sheep and goat breeders in Brazil. The control of this parasite is currently compromised, mainly, due to anthelmintic resistance. In the search for natural anthelmintic alternatives, *Pterogyne nitens*, a native Brazilian tree with potential ethnopharmacological activity, has been identified. The aim of this study was to evaluate the anthelmintic activity of ethanolic extracts and phenolic compounds from *P. nitens*, as well as two commercial flavonoids (chrysin and morin), to derive the chemical structure and anthelmintic activity. The ovicidal and larvicidal activity of ethanolic extracts from leaves (EEL) and fruits (EEFR), as well as natural compounds from *P. nitens* on *H. contortus* were evaluated through egg hatch assay (EHA) and larval development assay (LDA). The results showed that all extracts, especially the phenolic compounds were active in the EHA and LDA. The egg hatch inhibitory effects of EEL (EC_{50} = 316 μg/mL) were more potent than EEFR (EC_{50} = 512 μg/mL). However, larval development inhibitory effects of EEL (EC_{50} = 47 μg/mL) and EEFR (EC_{50} = 35 μg/mL) were similar. Among the compounds, the flavones (sorbifolin, pedalitin, and chrysin) did not have inhibitory effects on egg hatching but presented some activity against larval development of *H. contortus*. In contrast, the flavonols (quercetin, rutin, and morin) showed high activity in the EHA but were inactive in the LDA. The addition of at hydroxyl group and rutinose group to the flavonoid structure increased the ovicidal and larvicidal activity, respectively. The phenolic acids showed potent anthelmintic activity: caffeic acid, ferulic acid, and gallic acid had the highest anthelmintic effects, presenting EC_{50} values of 1.48, 0.56, and 4.93 μg/mL in the EHA; and 31, 22, and 33 μg/mL in the LDA, respectively. These results suggest that *P. nitens* might be a source of effective alternative compounds to control *H. contortus*.

1. Introduction

Haemonchus contortus is one of the main gastrointestinal nematodes that parasitizes the abomasum of sheep and goats in tropical and subtropical regions (Amarante et al., 2015). Compared with other nematodes, *H. contortus* is a highly pathogenic hematophagous species that afflicts small ruminants. The females are highly prolific laying from 5,000–10,000 eggs per day (Romero and Boero, 2001). Haemonchosis can result in large economic losses by causing appetite depression, damages to gastric function, and alterations in total protein content, energy, and mineral metabolism of livestock (Zarlenga et al., 2016). When animals have high parasitic loads, they present anemia and submandibular edema, and high mortality can occur in small ruminant flocks (Amarante et al., 2015; Besier et al., 2016).

For more than 50 years, the control of gastrointestinal nematodes, including *H. contortus* has relied on the use of synthetic anthelmintics. However, this mode of control based on chemical molecules has several disadvantages, such as the risk of environmental impact of the drug

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metabolites, because of residues and the increasing development and diffusion of resistant populations of parasites (Wolstenholme et al., 2004). Moreover, a decrease in animal production occurs as a consequence of the reduced efficacy of anthelmintics (Sangster et al., 2018).

Studies to find alternative strategies for the control of nematodes have focused on various options, including selective/rational use of anthelmintics, use of vaccines, genetic selection of sheep and goats for resistance to infection, and prevention of the build-up of infective larvae in pastures by grazing strategies (Torres-Acosta and Hoste, 2008; Charlier et al., 2018). Another strategy is to explore the anthelmintic properties of plants containing bioactive compounds such as secondary metabolites. Within this field, the bulk of studies have been focused on temperate and tropical plants containing phenolic compounds, including condensed tannins and/or flavonoids, either being integrated into the grazing rotation (Robertson et al., 1995), used as nutraceuticals (Hoste et al., 2015) or drenching with tannin extracts to interfere with the gastrointestinal nematode biology (Minho et al., 2008; Lima et al., 2019).

In this context, *Pterogyne nitens* Tulasne (Fabaceae) has been identified as a possible resource to exploit in Brazil and other tropical countries based on both ethnoveterinary and phytochemical aspects. *P. nitens* is popularly called “bálsamo”, “cocal”, “amendoim-bravo”, “madeira-nova” or “yvi-raró” in Brazil, and is the only member of the *Pterogyne* genus within the Fabaceae family (Lorenzi, 2002). Studies in Guarani indigenous communities of Argentina (Crivos et al., 2007) and Bolivia (Bourdy et al., 2004) indicated the potential ethnopharmacological and botanical uses of this tree.

In addition, *P. nitens* contains a variety of bioactive metabolites that have demonstrated biological activities, such as antiproliferative effects against melanoma cells (Regasini et al., 2007), inhibition of myeloperoxidase (Fernandes et al., 2008; Regasini et al., 2008b), radical scavenging properties and antioxidant activities (Okumura et al., 2012; Vellosa et al., 2015), antifungal activity (Lima et al., 2016), antiviral activity (Shimizu et al., 2017), multiresistant antibacterial activity (Coqueiro et al., 2014), and cytotoxic and antitumor activity (Satake et al., 2015; Tajima et al., 2015; De Oliveira et al., 2018).

The first objective of the present study was to investigate the potential anthelmintic effects of ethanolic extracts from different parts of *P. nitens* as well as of purified phenolic compounds based on two *in vitro* assays. Our second objective was to analyze the structure-activity relationships between the different natural phenolic compounds, which was to evaluate the importance of possible changes in the hydroxyl numbers and positions in the anthelmintic activity among the compounds tested. For this purpose, two commercial flavonoids (chrysin and morin) were included in the assays.

2. Material and methods

2.1. Plant materials

Fresh leaves and dried fruits of *P. nitens* were collected at the campus of the Institute of Biosciences, Humanities and Exact Sciences of São Paulo State University (UNESP), São José do Rio Preto, SP, Brazil (20°47′02.4″S 49°21′36.0″W), in July 2014. A voucher specimen (HISA 10,291) was deposited in the Ilha Solteira Herbarium (HISA) at the Faculty of Engineering of UNESP in Ilha Solteira, SP. Brazilian biodiversity access number A85B7D5 was registered with National System for Management of Genetic Heritage and Associated Traditional Knowledge.

2.2. Preparation of extracts

The shade-dried leaves (630 g) and fruits (200 g) were ground in a knife mill. The obtained powder was first macerated with hexane (1 L × 3) for 48 h, to remove the apolar compounds. Then the vegetal material was separated from the extraction solution by simple filtration through filter paper and the filtrate was dried with rotary evaporator. Subsequently, the non-extracted residue was macerated with ethanol (1 L × 3) for 48 h and the same procedure was repeated. Two types of extracts were obtained; ethanolic extract from leaves (EEL, 64 g) and ethanolic extract from fruits (EEFR, 20 g).

2.3. Natural compounds

Flavonoids (flavone, flavonol, and catechin derivative) and phenolic acids were isolated and identified, using chemical procedures reported previously. The flavone derivatives sorbifolin and pedalitin were isolated from leaves (Shimizu et al., 2017), while the flavonol derivatives quercetin and rutin were obtained from fruits (Regasini et al., 2007, 2008b). The flavon-3-ol derivative ourateacatechin and phenolic acids such as caffeic acid, ferulic acid and gallic acid were isolated from flowers (Regasini et al., 2008a). Commercial samples of chrysin (C80105) and morin (M4008) were purchased from Merck® (Fig. 1).

2.4. In vitro anthelmintic assays

2.4.1. Haemonchus contortus isolate

The susceptible *H. contortus* isolate Echevarria1991 (Echevarria et al., 1991) was used for monospecific infection and two lambs were inoculated orally with approximately 4000 third-stage larvae (L_3). Twenty-eight days after the L_3 administration, the infection was confirmed by counting the number of eggs per gram (EPG). Animals with a count over 1500 were considered parasitological competent as feces donors for the *in vitro* assays.

2.4.2. Recovery and preparation of eggs

Egg recovery, egg hatch assay (EHA) and larval development assay (LDA) were performed according to the protocol described by Chagas et al. (2011) with minor modifications. Eggs were recovered from 100 g of fresh feces by mixing with 500 mL of distilled water. The suspension was filtered through 100 μm and 25 μm mesh sieves. Eggs were washed from the 25 μm sieve and centrifuged at 3000 rpm for 5 min to form pellets. The supernatant was removed and a saturated NaCl solution was added to the pellet and centrifuged at 3000 rpm for 5 min. Floating eggs were collected using a 25 μm sieve and washed with phosphate-buffered saline (PBS, 0.1 M phosphate, 0.05 M NaCl, pH 7.2). Eggs were separated, quantified, and used within 1 h for the EHA and LDA.

2.4.3. Egg hatch assay (EHA)

One hundred eggs were added to each well of a 24-well microplate. PBS and thiabendazole (concentrations between 0.024–50 μg/mL; Merck®) were used as negative and positive controls, respectively. Ethanolic extract solutions were evaluated at concentrations of 24, 48, 97, 195, 390, 780, 1560, 3120, 6250, 12,500 and 25,000 μg/mL, flavonoid solutions at 62.5, 125, 250, 500, 1000 and 2000 μg/mL and phenolic acid solutions at 0.19, 0.39, 0.78, 1.56, 3.12, 6.25, 12.5, 15.6, 31.2, 62.5 and 125 μg/mL. Treatments, positive and negative controls were tested in six repetitions, using six wells for each treatment (*i.e.*, approximately 600 eggs). Plates were sealed with PVC film and incubated at 27 °C with relative humidity ≥ 80 % for 24 h. Then eggs and L_1 larvae were counted with an inverted microscope to calculate egg hatch inhibition.

2.4.4. Larval development assay (LDA)

Approximately 100 eggs were added to each well of a 24-well microplate with PBS, *Escherichia coli* (Strain B lyophilized) nutritive medium, and 0.5 mg/mL of amphotericin B (Merck®), reaching a total volume of 250 μL. Plates were incubated for 24 h at 27 °C and ≥ 80 % relative humidity to obtain L_1 larvae when the solutions were added. PBS and ivermectin (concentrations between 0.005–10 μg/mL; Merck®) were used as negative and positive controls, respectively. Ethanolic extract were evaluated at concentrations of 2.4, 4.8, 9.5, 19, 39, 78, 156,

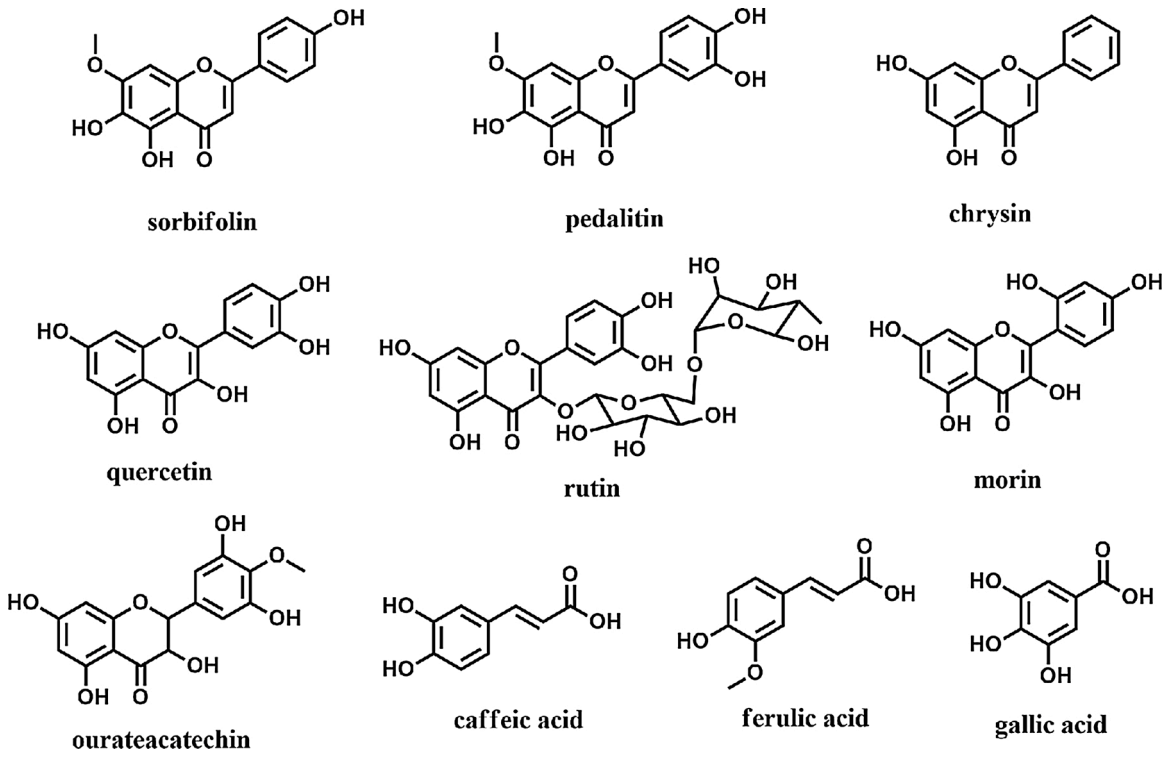

Fig. 1. Structure of sorbifolin, pedalitin, quercetin, rutin, ourateacatechin, caffeic acid, ferulic acid, and gallic acid (obtained from *Pterogyne nitens*), as well as flavonoids chrysin and morin (supplied by Merck®).

312, 625, 1250, and 2500 μg/mL, and flavonoids and simple phenolic compounds solutions at 7.8, 15.6, 31.2, 62.5, 125, 250, 500 and 1000 μg/mL. Treatments, positive and negative controls were tested in six repetitions, using six wells for each treatment and approximately 600 eggs in all. Plates were incubated for 7 days, and each well was analyzed with an inverted microscope to count all L_3 and undeveloped larvae to estimate larval development inhibition.

2.4.5. Statistical analyses

The percent of inhibition of egg hatch (EHA) was calculated by the following equation: % inhibition = A/ (A + B) × 100, where A is the number of unhatched eggs and B is the number of L_1 larvae. The percent of inhibition of larval development (LDA) was calculated using the following equation: % inhibition = (A/B) × 100, where A is the number of $L_1 + L_2$ larvae and B is the total number of larvae ($L_1 + L_2 + L_3$).

Data are presented as the mean percentage ± standard error (SEM). One-way ANOVA followed by the Tukey test ($p \leq 0.05$) was used to assess statistical significance depending on two factors: treatments and concentrations. In order to provide another parameter for comparison between the tested extracts and natural compounds, the concentrations at which occurred 50 % egg hatching inhibition and larval development inhibition (EC_{50}) of the parasites were also calculated. Analyses were performed by SPSS IBM Statistics® v. 20.

3. Results

3.1. Egg hatch assay (EHA)

From the EHA data, the EC_{50} values were calculated for extracts and phenolic compounds from *P. nitens* as well as for commercial flavonoids (Table 1). The ethanolic extracts showed similar EC_{50} values of their effectiveness at inhibiting the egg hatching. Fig. 2A shows that the proportion of egg hatching decreased with increase of the concentrations of ethanolic extracts. The EEL and EEFR presented statistical difference ($p \leq 0.05$) from the 12,500 μg/mL concentration down. The EEL presented a significant reduction of 40 % in hatching at 390 μg/mL, while EEFR exceeded 50 % egg hatch inhibition at the same concentration.

Table 1
EC_{50} (μg/mL) and confidence intervals (95 % CI) of extracts and phenolic compounds from *Pterogyne nitens* in egg hatch assay (EHA) and larval development assay (LDA) against *Haemonchus contortus*.

Extract/phenolic compound		EHA (μg/mL)	LDA (μg/mL)
Extracts	EEL	316 (287–348)	47 (31–63)
	EEFR	512 (456–573)	35 (24–48)
Flavones	sorbifolin	≥ 3000	18 (16–19)
	pedalitin	≥ 3000	83 (76–90)
	chrysin*	≥ 3000	58 (54–63)
Flavonols	quercetin	880 (819–950)	231 (206–259)
	rutin	1260 (1106–1461)	104 (93–115)
	morin*	663 (591–739)	448 (418–481)
Flavan-3-ol	ourateacatechin	≥ 3000	989 (852–1192)
Phenolic acids	caffeic acid	1.48 (1.34–1.64)	31 (29–33)
	ferulic acid	0.56 (0.50–0.63)	22 (21–24)
	gallic acid	4.93 (4.47–5.43)	33 (30–36)
Positive control	Thiabendazole/ Ivermectin	0.01 (0.01–0.01)	0.13 (0.12–0.14)

EEL = ethanolic extracts from leaves; EEFR = ethanolic extracts from fruits; Thiabendazole = positive control in EHA; Ivermectin = positive control in LDA;
* Flavonoids supplied by Merck®.

Because none of the flavones (sorbifolin, pedalitin, and chrysin), and flavan-3-ol (ourateacatechin) were able to inhibit 50 % of the eggs from hatching at the highest concentration, their EC_{50} values were considered ≥ 3000 μg/mL (Table 1). At the highest concentration tested for flavones, the percentage of egg hatch inhibition was statistically equal to the negative control ($p \geq 0.05$). At 2000 μg/mL they presented less than 5% egg hatch inhibition, so their EC_{50} and egg hatch inhibition curve were not calculated.

In contrast, the flavonols (quercetin, rutin, and morin) presented

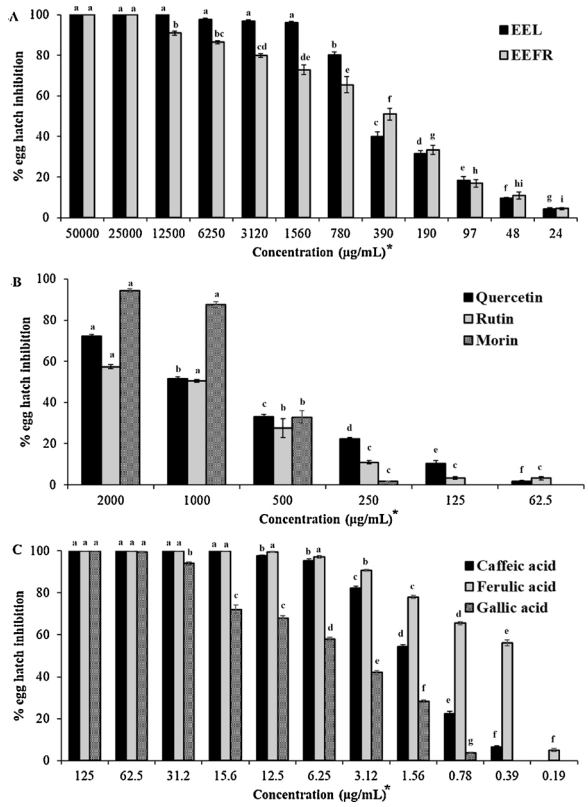

Fig. 2. *In vitro* effect of (2A) ethanolic extracts of leaves (EEL) and fruits (EEFR), (2B) flavonols (quercetin, rutin and morin), (2C) phenolic acids (caffeic acid, ferulic acid and gallic acid) from *Pterogyne nitens* on the egg hatching of *Haemonchus contortus*. Different letters in the columns represent statistical difference ($p \leq 0.5$).

better performance in the EHA. Morin presented the lowest EC_{50} value (663 µg/mL), followed by quercetin (880 µg/mL). Rutin had an EC_{50} value half as effective as morin (1260 µg/mL) (Table 1). Fig. 2B shows the inhibition percentage at different concentrations calculated for the flavonols. At 500 µg/mL concentration, the percentage inhibition values were statistically similar (33 %, 27 %, and 32 % for quercetin, rutin, and morin, respectively).

Based on the EC_{50} values and high inhibition percentages at low concentrations, the phenolic acids (caffeic, ferulic, and gallic acid) were identified as the most potent bioactive compounds. Ferulic acid had an EC_{50} (0.56 µg/mL) three times more potent than caffeic acid (1.48 µg/mL) against egg hatching. Gallic acid was the least effective among the phenolic acids tested (4.93 µg/mL) (Table 1). At 15.6 µg/mL concentration, caffeic and ferulic acid showed 100 % egg hatching inhibition, while gallic acid caused 70 % inhibition ($p \leq 0.05$) (Fig. 2C). Moreover, our results showed that the phenolic acids were able to inhibit the larval hatching at 500 µg/mL or cause larval mortality at 6.25 µg/mL. Hatched larvae appeared sluggish and were often dead at high concentrations of compounds, indicating that they had died during or after incubation but before subsequent observation by microscopy. In the negative control wells of the EHA, at least 98 % of the eggs hatched.

3.2. Larval development assay (LDA)

In relation to the LDA results, the EC_{50} values were calculated (Table 1). The ethanolic extracts showed EC_{50} values indicating a potent effect to inhibit the larval development process. The EEFR ($EC_{50} = 35$ µg/mL) were slightly more active than EEL ($EC_{50} = 47$ µg/mL). The ethanolic extracts presented a concentration-dependent response in the LDA. In the negative control wells, more than 95 % of the larvae developed to L_3. There was a non-significant difference between the anthelmintic activity of EEL and EEFR ($p \geq 0.05$) until the concentration of 78 µg/mL. At the lower concentrations, the proportion of larvae developed from L_1 to L_3 decreased with the increase of concentrations of ethanolic extracts (Fig. 3A). Comparison of the microscopic images of *H. contortus* larvae after incubation for 7 days from the negative control (PBS) and from EEL and EEFR treatment showed that both extracts were able to impact the larval structure at 2500 µg/mL. The undeveloped larvae appeared sluggish and were often dead on the extract's incubation plate, indicating that they might have died during or after seven days of incubation but before subsequent observation by microscopy.

Among the flavones, the most effective compound was sorbifolin ($EC_{50} = 18$ µg/mL). The other two flavones tested were less effective ($EC_{50} = 58$ µg/mL for chrysin and $EC_{50} = 83$ µg/mL for pedalitin) (Table 1). Moreover, sorbifolin was significantly more effective than the other flavones, with 90 % inhibition of larval development at 125 µg/mL ($p \leq 0.05$). Pedalitin and chrysin had the same response at 500 µg/mL (Fig. 3B).

Overall, the flavonols showed lower EC_{50} values than the flavones. Rutin ($EC_{50} = 104$ µg/mL) presented a larvicidal activity twice as effective as quercetin ($EC_{50} = 231$ µg/mL) and four times more than morin ($EC_{50} = 448$ µg/mL). Ourateacatechin was the least effective flavonoid, with high EC_{50} (989 µg/mL) (Table 1). Morin caused 90 % inhibition of larval development at 1000 µg/mL while quercetin and rutin caused 100 % inhibition at the same concentration. At the highest concentrations (1000 µg/mL) tested, ourateacatechin was able to inhibit only 50 % of larval development (Fig. 3C).

The phenolic acids showed similar larvicidal activity. Ferulic acid presented EC_{50} value (22 µg/mL) below that of caffeic acid (31 µg/mL) and gallic acid (33 µg/mL) (Table 1). Fig. 3D shows that at 250 µg/mL, the proportion of larval developed was similar for all phenolic acids (99 %, 100 %, and 95 % for caffeic, ferulic, and gallic, respectively) ($p \geq 0.05$). The percentage of larval development inhibition decreased with the declining concentrations of phenolic acids. In the negative control wells of the LDA, at least 95 % of the larvae developed to L_3.

4. Discussion

The main aim of this study was to investigate the effects of ethanolic extracts and phenolic compounds from *P. nitens* on the *in vitro* egg hatching and larval development of the most prevalent gastrointestinal nematode in tropical and subtropical regions, *Haemonchus contortus*. In addition, we analyzed the structure-activity relationships between the different phenolic compounds tested. The results showed that extracts and phenolic compounds from *P. nitens*, as well as the commercial flavonoids, can disrupt the life cycle of *H. contortus* by preventing the eggs from hatching and/or by preventing L_1 larvae from developing to the infective L_3 stage, which corroborates the traditional use of *P. nitens* as an antiparasitic plant.

In the EHA, both extracts showed potent ovicidal action. The EEFR exhibited slightly less activity than EEL. Thus, these results encouraged us to investigate which compounds of the ethanolic extracts might be responsible for their anthelmintic activity. Likewise, the flavonoids isolated from both extracts had less activity than the crude extracts. However, the effect of flavonols (quercetin and rutin) isolated from EEFR at the 2000 µg/mL concentration cannot be ruled out, since these compounds in the extracts might have acted singly or in synergy for anthelmintic action.

The addition, a hydroxyl group in flavonol structures significantly increased the activity when compared with the flavones. There was a correlation between the number of hydroxy groups in the C-ring and inhibitory activity on egg hatching: the flavones (sorbifolin, pedalitin and chrysin) were less active than the flavonols (quercetin, rutin and morin). Another study reported that the crude ethanolic extract from the aerial parts of *Artemisia campestris*, which contain predominantly

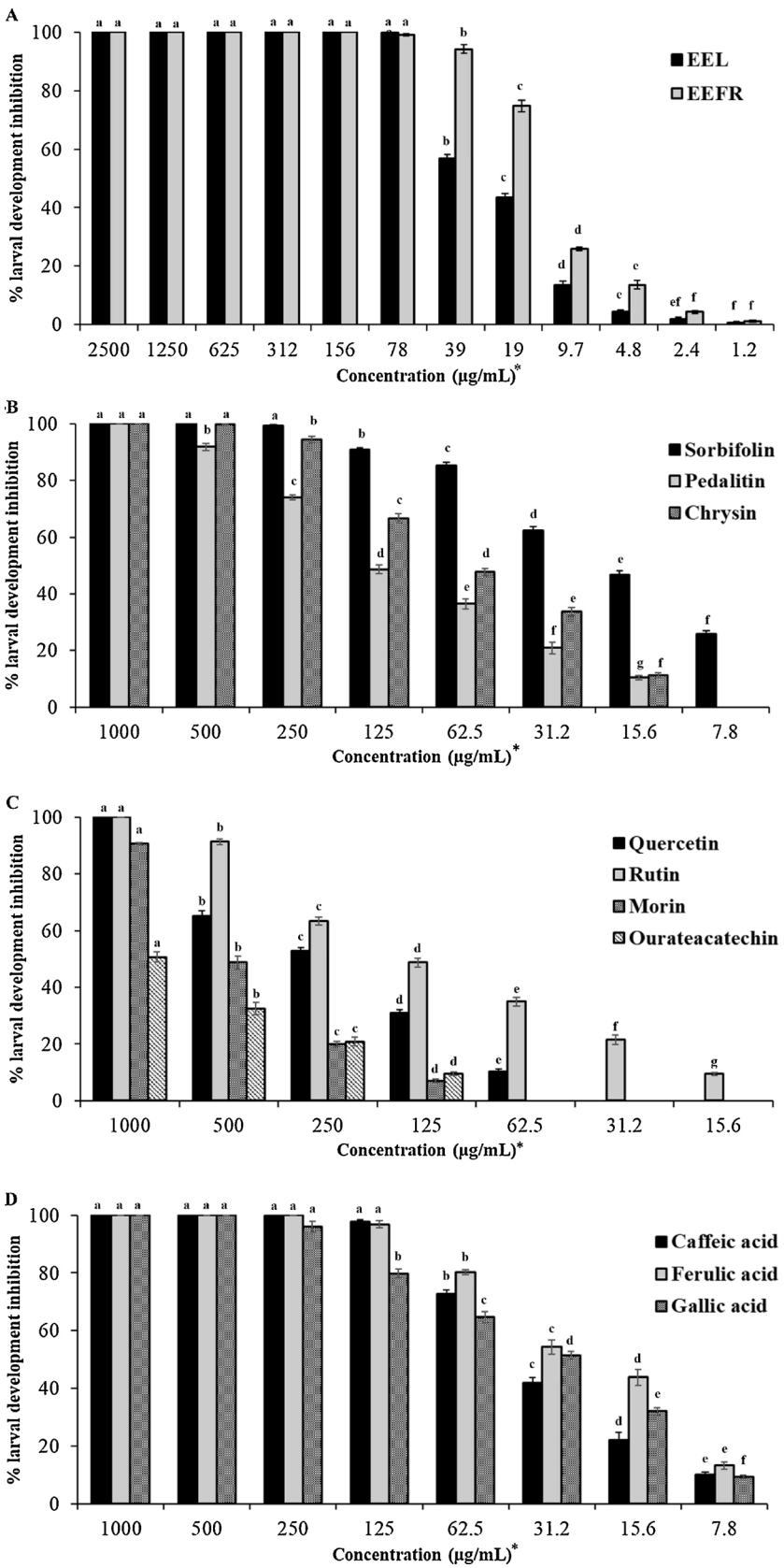

Fig. 3. *In vitro* effect of (3A) ethanolic extracts of leaves (EEL) and fruits (EEFR) from *Pterogyne nitens*, (3B) flavones (sorbifolin and pedalitin) from *P. nitens* and commercial flavone (chrysin), (3C) flavonols (quercetin and rutin), flavan-3-ol (ourateacatechin) from *P. nitens* and commercial flavonol (morin), and (3D) phenolic acids (caffeic acid, ferulic acid, and gallic acid) from *P. nitens* on the larval development of *Haemonchus contortus*. Different letters in the columns represent statistical difference ($p \leq 0.5$).

derivatives of quercetin and apigenin, was able to inhibit egg hatching and caused death and paralysis in adults of *H. contortus* (Makkar et al., 2007). The same trend was observed for ourateacatechin, which has a hydroxyl group in its C-ring. However, it was statistically equal to PBS in the EHA at a concentration of 1000 μg/mL. Other investigations have demonstrated that the tri-hydroxylation of the B-ring of condensed tannin monomers, such as catechin and flavan-3-ols, had enhanced the effect in comparison with the non-substituted. Molan et al. (2003) observed this situation in egg hatching, larval development, and larval migration of *Trichostrongylus colubriformis*. Brunet and Hoste (2006) also detected it in the L_3 exsheathment process of *H. contortus* and *T. colubriformis*.

Escareño-Díaz et al. (2019) evaluated the *in vitro* anthelmintic activities of caffeic acid, coumarin, quercetin, rutin, and their combination against egg hatching and L_3 larval exsheathment of *Cooperia punctata*. They observed that quercetin and rutin did not demonstrate bioactivities on the eggs and larvae. However, when evaluated in combination with caffeic acid and coumarin an effective synergic interaction against the egg hatching was observed, which could suggest a linear correlation between lower molecular weight and higher ovicidal activity, since the EC_{50} values were higher for the smaller molecules (caffeic acid and coumarin).

Similar behavior was observed for the phenolic acids (caffeic acid, ferulic acid and gallic acid) from *P. nitens*, which showed potent anthelmintic activity against the egg hatching. Probably their low molecular weight was an important factor for their passive diffusion, by which the compounds are able to penetrate the nematode eggshell, which is one of the most resistant biological structures and is impermeable to most substances, with the exception of gases and lipid solvents (Arthur and Sanborn, 1969). The penetration of compounds and the effect of temperature on the permeability of the eggshell suggests that the lipid layer provides the main permeability barrier (Wharton, 1980).

In the LDA, the ethanolic extracts were able to inhibit efficiently the larval development at different concentrations. Moreover, at 2500 μg/mL there were larval structure differences between the extracts and PBS, which presented small bubbles in their body under microscopic observation.

On the other hand, among the phenolic compounds, there appeared to be no structure-activity correlation, only concentration dependence. Phenolic acids and sorbifolin caused complete inhibition of larval development at 500–1000 μg/mL while ourateacatechin inhibited less than 50 % at the same concentrations. The flavonols and their glucose derivatives were less active than flavones in the LDA. In the respect, Ayers et al. (2008) isolated methoxylated flavones from a *Struthiola argentea* methanolic extract, which were effective against larval development of *H. contortus*.

The larvicidal activity of flavonols was considered weak, comparing quercetin with its isomer morin, which was less active against the larval development of *H. contortus*. This suggests that the displacement of the hydroxyl group from position 3' to 2' (ring B) modified the anthelmintic activity. The replacement by a sugar subunit (rutinose) in the quercetin structure increase the larvicidal activity of rutin twofold.

Some authors have proposed that the interactions of the phenolic compounds with the surface proteins of larvae by hydrogen bonds and/or hydrophobic interactions (Hagerman et al., 1998; Poncet-Legrand et al., 2006) can explain their eff ;ects on the development process (Kahn and Diaz-Hernandez, 1999; Molan et al., 2003), since they are able to complex with collagen of the nematode cuticle (Bravo, 1998; Jerónimo et al., 2016). An important fact observed was that the egg hatching was not disturbed by the phenolic compounds as much as the larval development. This may be due to the impermeability of the eggshell, as discussed earlier, as well as the duration of the assay. In the EHA the nematode eggs are exposed for only 24 h while in the LDA the L_1 and L_2 larvae are exposed to tested compounds for 7 days. Moreover, in the LDA the larvae are in the feeding stage of their life cycle so they may ingest the compounds.

Either extracts or phenolic compounds are able to achieve the complete inhibition of egg hatching and larval development (Molan et al., 2002). It is important to note that only the benzimidazole class can act at low concentrations as ovicidal, inhibiting the embryogenesis of gastrointestinal nematode eggs (Egerton, 1969). However, the nematode resistance problem to this anthelmintic group has been reported since the 1970s (Coles and Simpkin, 1977) as well as to all other chemical classes (Salgado and Santos, 2016; Abongwa et al., 2017). Therefore, it became necessary to identify anthelmintic methods, such as the use of medicinal plants and their extracts and compounds.

This is the first demonstration that *P. nitens* extracts and compounds are able to inhibit *H. contortus* stages, so they might represent an interesting source for integrated control. However, effective concentrations, synergistic effects, enhanced bioavailability, cumulative effects, or simply the addictive properties of the constituents must be defined previously (Williamson, 2001). Also, absorption by the digestive tract and elimination in the feces (% in the feces in relation to the supplied orally) need to be clarified.

5. Conclusion

In conclusion, our results showed that ethanolic extracts and phenolic compounds from *P. nitens* have potent anthelmintic effects against egg hatching and larval development of *H. contortus*. The ovicidal and larvicidal activity of flavonoids can increase with substitutions in the chemical structure, such as the addition of the hydroxyl group and the rutinose group, respectively. In addition, the phenolic acids showed potent anthelmintic activity, which suggests a promising alternative to control the free-living stages. Further studies will be needed to explore these interactions at the molecular level, as well as *in vivo* experiments to explore the therapeutic efficacy of *P. nitens* to control parasites in small ruminants.

Ethical approval

All procedures were approved by the Embrapa Pecuária Sudeste Ethics Committee on Animal Experimentation (process no. 04/2017), and are in accordance with national and international principles and guidelines for animal experimentation adopted by the Brazilian College of Experimentation (CONCEA).

CRediT authorship contribution statement

Caroline Sprengel Lima: Conceptualization, Investigation, Data curation, Formal analysis, Writing - original draft, Writing - review & editing. **Matheus Henrique Pereira:** Investigation, Data curation, Writing - original draft. **Yousmel Alemán Gainza:** Methodology, Formal analysis, Writing - original draft. **Hervé Hoste:** Validation, Writing - original draft, Writing - review & editing. **Luís Octavio Regasini:** Conceptualization, Supervision, Writing - original draft, Writing - review & editing, Project administration. **Ana Carolina de Souza Chagas:** Supervision, Validation, Funding acquisition, Writing - review & editing, Project administration.

Declaration of Competing Interest

The authors report no declarations of interest.

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