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In vitro susceptibility of ten *Haemonchus contortus* isolates from different geographical origins towards acetone:water extracts of two tannin rich plants

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ABSTRACT

The aim of the study was to examine the variation in the *in vitro* susceptibility of ten *Haemonchus contortus* isolates from different geographical origins using respective egg hatch assays (EHA) with acetone:water extracts of two tannin containing plants, chimay (*Acacia pennatula*) and sainfoin (*Onobrychis viciifolia*). Fresh eggs were incubated in PBS with different concentrations of each extract (0, 600, 1200, 2400, 3600, 5000 and 8000 μg/ml PBS). Additional concentrations were tested for *O. viciifolia* (75, 100, 200 and 400 μg/ml PBS). Effective concentrations 50% (EC_{50}), with the corresponding 95% confidence interval (95% CI), were calculated for every isolate with both extracts. Moreover, a resistance ratio (RR) was calculated to compare the isolates, using the most susceptible isolate for each extract as the respective reference. A second set of incubations were made using polyvinylpolypyrrolidone (PVPP) (0, 5000 μg/ml, 5000 μg/ml + PVPP) to determine the influence of polyphenols on the AH effect. The proportion of morulated eggs, eggs with L_1 larvae failing eclosion (%LFE), and emerged larvae were estimated at different extract concentrations. Data of each isolate was used to calculate the effective concentration 50% (EC_{50}) for each extract. The EC_{50} of each isolate was used to determine resistance ratio (RR) for the different isolates. For the 2 extracts, a susceptibility variation in egg hatching was observed for the different *H. contortus* isolates. The EC_{50} values for *A. pennatula* ranged from 2203 to 14106 μg (RR from 2.01 to 6.40). The *O. viciifolia* extract showed higher variability with EC_{50} values ranging from 104 to 4783 μg (RR from 3.66 to 45.74). The main AH effects of the two extracts tested on the ten isolates consisted in blocking the emergence of L_1 larvae (higher% LFE). Additional observations on emerged larvae showed that extract exposure caused alterations in the internal structure, separating the cuticle from the pharynx, bulb and intestinal cells. The use of PVPP revealed that (a) condensed tannins were not the sole plant secondary metabolites responsible for the AH effects, and (b) different *H. contortus* isolates showed variability in the role of tannins either on the ovicidal effect or the %LFE.

1. Introduction

Because of the increasing diffusion of resistance to anthelmintics in gastrointestinal nematodes (GIN), several research groups are examining the role of different plant secondary metabolites (PSMs) with potential anthelmintic (AH) activity against GIN of small ruminants. The PSMs contained in forage plants could be used either as phytotherapeutic materials or fed as nutraceuticals (Hoste et al., 2015). It is well known that the AH activity of plant extracts or nutraceuticals show considerable variability because of differences in the quantity or quality of the bioactive PSMs. The causes behind the variability of the AH activity are currently being studied. The subject is particularly difficult mainly due to the lack of information on the precise PSM molecules and/or combination that provides the observed AH effect on GIN for each plant. Also, it is currently unknown how these PSM interact to enhance or reduce the AH effect. However, even without the precise knowledge of the bioactive compound(s) providing the AH effects, it has been suggested that the variability of the AH activity could be related to factors directly affecting the plant material, including: geographical ori-

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gin, climate (seasonality), phenological stage and cultivar, amongst other factors (Manolaraki, 2011; Novobilský et al., 2013). It is also known that the AH activity varies greatly between different parts of the same plant (roots, bark, leaves, pods, seeds, etc.). There is also variation that can be attributed to the extract and the extract AH assessment: sample preservation method, solvents or solvent combinations used to produce an extract, purification process, bioassay used to test the AH activity, *etc.* However, it is until recently that the variation of the AH effect due to the geographical origin of the parasites was suggested as part of the equation. The work by Calderón-Quintal et al. (2010) showed that *H. contortus* isolates from a hot-tropical area of Mexico needed more acetone:water extract to produce a significant AH effect on the motility of L_3 larvae, compared to isolates of the same parasite from other latitudes (Alonso-Díaz et al., 2008b). Further evidence was generated when Vargas-Magaña (2014) compared the exsheathment process of a range of *H. contortus* isolates from tropical (Mexico) and temperate (France) areas with different acetone:water extracts from foliage and agricultural by-products. The latter suggested again that some Mexican isolates were less susceptible to the bioactive PSM contained in different acetone:water extracts compared to French isolates. The existence of variation in the susceptibility of the different *H. contortus* isolates to the plant secondary compounds contained in different plant crude extracts imply the need to consider the inherent susceptibility of the isolates when evaluating phytotherapeutic or nutraceutical materials. On the other hand, the analysis of such variability could help to identify possible mechanisms of action and even possible mechanisms of resistance to the PSMs.

To the best of our knowledge, there is no information on the possible variability of *H. contortus* eggs to the AH effect of acetone:water extracts. A recent study showed that acetone:water extracts obtained from different plant foliages and agroindustrial by-products affected the egg hatching process of *H. contortus* (Vargas-Magaña et al., 2014). Those different extracts caused mainly a failure of L_1 eclosion but also showed a limited but significant ovicidal effect for some extracts. With such tool, it seemed feasible to explore whether eggs of different *H. contortus* isolates obtained from different parts of the world, and with different status of AH resistance to conventional drugs, show any variability on the AH effect when exposed to acetone:water plant extracts. Thus, the objectives of the present study were (1) to assess *in vitro* susceptibility of eggs of *H. contortus* isolates from different geographical origins to the AH effect of acetone:water extracts of two tannin containing plants and (2) to examine the role of polyphenols on the AH effect shown with the egg hatch assay.

2. Materials and methods

2.1. Extract of chimay (Acacia pennatula) and sainfoin (Onobrychis viciifolia)

Acetone:water extracts of these plants were provided by the Campus de Ciencias Biológicas y Agropecuarias, Universidad Autónoma de Yucatan, Mexico (CCBA-UADY) and the UMR, IHAP, INRA/ENVT, France, respectively. The extraction procedure was as described below: fresh leaves of the two plants were collected to obtain acetone:water extracts. Five hundred grams of each material (*A. pennatula* and *O. viciifolia*) were mixed in a 70:30 solution of acetone–water with ascorbic acid (1 g/L) to prevent the extracts oxidation. The mixture was sonicated for 20 min in a water-bath Acetone was removed by using a rotovapor. For the case of *A. pennatula* extract, it was filtered and washed 4 times with dichloromethane to remove pigments and lipids and the filtrated material was lyophilized. To avoid hydration of both extracts, these were kept under refrigeration in sealed flasks until used (4 °C) (Alonso-Díaz et al., 2008a). For each plant species, a single batch of acetone:water extract was used for the egg hatch assays performed with all the *H. contortus* isolates. By using the same batch of extract for each plant material we intended to avoid any variability due to differences in the composition of the respective extracts. In this way the study focused on the variability due to parasite isolates.

2.2. Haemonchus contortus isolates

Ten *H. contortus* isolates from different origins were tested: FMVZ, PARAISO and CENID (hot tropics, Mexico), FESC (temperate, Mexico), INRA and JUAN (France) (Vargas-Magaña, 2014), MOF23 (USA) (Rajan et al., 2002), CAVR and McMASTER (Australia) and WHITE RIVER (South Africa) (Le Jambre et al., 1995). Infective L_3 larvae of all the different isolates were used to infect respective donor sheep or goats with a single specific isolate. Donor sheep/goats were raised free of nematode infection and were kept in individual pens with concrete floors before and during the course of the experiment. Before the infection with the respective isolates, donors received a preventive anthelmintic treatment with 2 broad-spectrum anthelmintics. Sheep received bezimidazole at 10 mg kg^{-1} and levamisole at 7.5 mg kg^{-1}, while goats received bezimidazole at 20 mg kg^{-1} and levamisole at 12 mg kg^{-1}. Ten days after AH treatment, donor sheep or goats were infected with 3000 L_3 infective larvae of the respective *H. contortus* isolate. To prevent accidental infections with any other trichostrongyle species, the donor sheep or goats were maintained in-doors either at the École Nationale Vétérinaire de Toulouse (ENVT), France, or at the Campus de Ciencias Biológicas y Agropecuarias, Universidad Autónoma de Yucatan, Mexico (CCBA-UADY). Furthermore, animals were fed a balanced diet based on parasite-free cut and carry grass or dry hay and a commercial concentrate feed. Water was available *ad libitum*. Donor animals were used to harvest *H. contortus* eggs that were obtained from fresh fecal samples. Mexican isolates were evaluated in the parasitology laboratory of CCBA-UADY, Mexico and the other isolates were evaluated in the tannins laboratory of INRA-ENVT, France. The same person performed all *in vitro* assays over the course of 5 months in order to reduce a possible variation in the execution of the *in vitro* technique.

2.3. Recovery of Haemonchus contortus eggs

Fresh *H. contortus* eggs were recovered from the faeces of each donor animal. Faeces were collected using new plastic bags for each sample and were processed within 3 h after collection. The technique for the recovery of nematode eggs from the faeces was carried out essentially as described by Coles et al. (1992). In short, approximately 20 g of faeces were macerated with a mortar with 200 ml of water. The suspension was filtered using gauze and the filtrate was placed in 15 ml tubes. The tubes were centrifuged at 943 g for 3 min. The supernatant was removed, leaving sediment. Then, a saturated sugar solution (1.28 density) was added to each tube. The tubes were shaken using a vortex until the sediment was dissolved and the tubes were centrifuged again at 943 g for 3 min. Finally, the surface layer of the solution was recovered with a bacteriological loop. The eggs were concentrated in a single tube with 10 ml of Phosphate Buffered Saline (PBS). Eggs were washed 3 times to eliminate the remaining sugar and re-suspended using PBS. Twelve drops of 20 μl were taken and eggs observed were counted. The number of eggs per milliliter was determined and this suspension was diluted to reach a concentration of 200 eggs per ml for its use in an egg hatch assay.

2.4. Egg hatch assay (EHA)

Preparation of stock solution (20,000 μg/ml) of the *A. pennatula* or the *O. viciifolia* acetone:water extracts was made in PBS with dimethyl sulfoxide (DMSO, D8418 Sigma©) at 4%. A stock solution of thiabendazole (TBZ, T8904 Sigma©) was prepared with DMSO (1000 μg/ml). The EHA was conducted following the procedure suggested by von Samson-Himmelstjerna et al. (2009) and Jackson and Hoste (2010). A multi-well plate (24-wells) was used containing PBS (940, 880, 760, 640, 500 or 200 μl of PBS) and the respective volume of stock solution of *A. pennatula* or *O. viciifolia* extract (60, 120, 240, 360, 500 or 800 μl of extract stock solution). The PBS was used for negative control (800 μl of PBS with DMSO at 4% plus 200 μl of PBS). The TBZ was used for positive control (990 μl of PBS plus 10 μl of TBZ stock solution). Finally, 1000 μl of the *H. contortus* egg suspension (200 eggs/ml) was added to each well to obtain the final of extract concentrations (600, 1200, 2400, 3600, 5000 and 8000 μg/ml PBS). All the extract concentrations and the control wells (positive and negative) were tested with three replicates. The protocol used was the same for all the *H. contortus* isolates evaluated.

Additional concentrations were tested for *O. viciifolia* (75, 100, 200 and 400 μg/ml PBS) for CENID and PARAISO isolates. The multi-well plates were incubated at 24 to 28 °C for 48 h. After the incubation period, two drops of iodine solution was added to each well to kill and dye the eggs and larvae present. Subsequently, the number of eggs that failed to form larvae, the number of eggs that failed to complete their eclosion (L_1 larvae failing eclosion) and the number of larvae emerged in each well were determined. Hatching percentage was calculated as described below. The analyses included only those plates with a hatching-rate of 70% or higher in the negative control wells.

To determine the influence of polyphenols on the egg hatching process, respective extract solutions were incubated with a condensed tannin blocking agent, the polyvinylpolypyrrolidone (PVPP), for 2 h at 24 °C at 50 mg of PVPP per ml of solution. Subsequently, the tubes were centrifuged at 1849 × *g* for 5 min. The supernatant was used for testing at 5000 μg/ml of the respective extracts in the same manner as described above. To discard any influence of PVPP on the egg hatching processes, eggs of all the different isolates tested were incubated with a solution containing only PVPP and PBS.

2.5. Data analyses

The number of eggs, including those that failed to form larvae (morulated eggs) or eggs with larvae which failed to complete their eclosion, together with the number of larvae emerged for every isolate exposed to the different extracts at the concentrations previously described, were used to determine the hatching rate.

The egg hatch rate (%EH) was calculated as:

$$\%EH = \frac{\text{Number of larvae}}{\text{Number of morulated eggs} + \text{number of eggs containing a larva} + \text{number of larvae}} \times 100$$

The percentage of larvae failing to complete their eclosion (%LFE) was calculated as reported by Vargas-Magaña et al. (2014):

$$\%LFE = \frac{\text{Number of eggs containing a larva}}{\text{Number of morulated eggs} + \text{number of eggs containing a larva} + \text{number of larvae}} \times 100$$

The percentage of eggs that failed to form larvae (ovicidal effect, %OE) was calculated as reported by Vargas-Magaña et al. (2014):

$$\%OE = \frac{\text{Number of morulated eggs}}{\text{Number of morulated eggs} + \text{number of eggs containing a larva} + \text{number of larvae}} \times 100$$

The proportion (%) of eggs (morulated + containing larvae) and larvae emerged were used to determine the effective concentration required to inhibit 50% of hatching (EC_{50}) with respective 95% confidence intervals (95% CI) using the PoloPlus 1.0 software (LeOra Software, 2002). Due to the fact that it was not practically feasible to evaluate *A. pennatula* concentrations higher than 8000 μg/ml PBS for MOF23, FESC and PARAISO isolates, the EC_{50} values generated were above the highest tested concentration. Those extrapolated values were considered only as an indication to classify those isolates in terms of their resistance to *A. pennatula* acetone:water extract.

The EC_{50} value of the most susceptible *H. contortus* isolate to both *A. pennatula* and *O. viciifolia* extracts (CENID Mexico) was used as reference to calculate respective resistant ratios (RR) using the following formula (Sangster and Dobson, 2002):

$$RR = \frac{EC_{50}\ \text{resistance isolate}}{EC_{50}\ \text{most sensitive isolate}}$$

Data obtained from the PVPP incubations of both extracts were analyzed with respective GLM to assess differences in the percentage values of eggs showing either ovicidal effect or larvae failing eclosion and larvae emerged between the results of the PBS control, and those obtained for the extract solutions at a concentration of 5000 μg/ml PBS, with and without PVPP.

3. Results

3.1. Variability in the in vitro susceptibility of Haemonchus contortus isolates towards acetone:water extracts

Table 1 shows the effective concentration 50% (EC_{50}) and the resistance ratio (RR) obtained for each *H. contortus* isolate exposed to *A. pennatula* and *O. viciifolia* acetone:water extracts. In general terms, the *A. pennatula* extract showed lower AH activity than the *O. viciifolia* extract.

Eggs from CENID isolate were the most susceptible to both extracts. A variation in the efficacy of extracts against different *H. contortus* isolates was evident. The EC_{50} values for *A. pennatula* ranged from 2203 to 14106 μg. Consequently, wide variation was also observed for the RR (from 2.01 to 6.40). According to the 95% CI, the isolates were classified in three categories that were significantly different ($P < 0.05$): sensitive (CENID), moderate resistant (INRA, McMASTER, FMVZ, CAVR, WHITE RIVER and JUAN) and resistant (MOF23, FESC and PARAISO).

In the case of *O. viciifolia,* the EC_{50} variation ranged from 104 to 4783 μg. Although the extract concentrations were smaller, the RR values ranged more 3.66–45.74. Less *O. viciifolia* extract was needed to reach the EC_{50} for most *H. contortus* eggs, with the sole exception of the McMASTER isolate that seemed to require similar concentrations of *A. pennatula* to reach the 50% reduction of egg hatching. The 95% CI was used to group the different isolates in three mutually exclusive categories: sensitive (CENID and PARAISO), moderate resistant (INRA, JUAN, FESC, WHITE-RIVER, CAVR and FMVZ) and resistant (MOF23 and McMASTER).

Table 1
Effective concentration of acetone:water *Acacia pennatula* and *Onobrychis viciifolia* extracts (μg/ml) required for achieving 50% inhibition of egg hatching (EC_{50}) and the resistance ratio (RR) of isolates of *Haemonchus contortus* from different geographical regions (CENID Mexico being the susceptible reference).

Isolate	EC_{50} (95% CI) *Acacia pennatula*	RR[a]
CENID (Mexico)	2203.29 (1848.92–2540.13)a	
INRA (France)	4429.73 (3821.34–5098.00)b	2.01
McMASTER (Australia)	5129.20 (4276.39–6643.85)b	2.32
FMVZ (Mexico)	5591.44 (4903.41–6430.68)b	2.53
CAVR (Australia)	5874.18 (5581.61–6199.03)b	2.66
WHITE RIVER (South Africa)	6350.27 (5862.31–6931.41)b	2.88
JUAN (France)	6362.46 (5978.65–6803.73)b	2.88
MOF23[b] (USA)	8360.60 (7310.14–10029.73)c	3.79
FESC[b] (Mexico)	12256.90 (9310.75–29200.60)c	5.56
PARAISO[b] (Mexico)	14106.05 (7108.42–104760.32)c	6.40
Isolate	EC_{50} (95% CI) Onobrychis viciifolia	RR[†]
CENID (Mexico)	104.57 (78.58–136.30)a	
PARAISO (Mexico)	383.10 (318.00–447.23)b	3.66
INRA (France)	676.13 (659.51–695.22)c	6.46
JUAN (France)	678.76 (457.88–847.26)bc	6.49
FESC (Mexico)	971.46 (857.20–1093.97)d	9.28
WHITE RIVER (South Africa)	1044.77 (482.05–1609.18)dc	9.99
CAVR (Australia)	1111.58 (694.48–1474.91)dc	10.62
FMVZ (Mexico)	1428.07 (1174.38–1682.01)d	13.65
MOF23 (USA)	2207.39 (1839.09–2531.84)e	21.10
McMASTER (Australia)	4783.65 (3812.31–10944.33)f	45.74

95% CI: 95% confidence intervals.
Different letters in the same column means a significant difference ($P < 0.05$).
[a] RR of each isolate relative to the most sensitive isolate.
[b] Values of EC50 are higher than the highest concentration tested (8000 μg/ml). The values are a projection only used to illustrate that isolates were clearly less susceptible.

3.2. *Role of tannins on the AH effects*

Incubations of the *H. contortus* isolates using PBS with and without PVPP showed that the latter caused no interference on the proportion of morulated eggs, LFE and larvae emerged which allowed a proper assessment of the AH effect of extracts.

Table 2 shows the proportion of *H. contortus* morulated eggs (ovicidal effect), %LFE and larvae emerged resulting from incubations with *A. pennatula* or *O. viciifolia* extracts at a concentration of 5000 μg/ml PBS with and without PVPP. In Table 2 is evident that the ovicidal effect is a minor AH effect compared to the %LFE. However, it is possible to observe that different isolates showed variation in the ovicidal effect and the %LFE. The ovicidal effect recorded for the *A. pennatula* acetone:water extract differed between the *H. contortus* isolates. Only the CENID, INRA and FESC isolates showed ovicidal effect and the addition of PVPP did not remove such AH effect. Furthermore, the PARAISO isolate showed a weak ovicidal activity after incubation with PVPP (Table 2). The ovicidal effect recorded for the *O. viciifolia* extract was more complex. The extract showed ovicidal effect with and without the addition of PVPP for some isolates (CENID, CAVR and FESC). For the WHITE RIVER isolate, the effect was evident with the extract and was enhanced with PVPP incubation. Meanwhile, the ovicidal effect of other isolates (McMASTER, JUAN and MOF23) was only found after incubation with PVPP. The ovicidal effect was removed by PVPP only for the INRA isolate. Lastly, the PARAISO isolate did not show any ovicidal effect with or without PVPP.

Table 2 shows that failure of L_1 larvae eclosion (%LFE) was by far the main AH activity recorded for *A. pennatula* and *O. viciifolia* extracts. The effect was reported in 7 of the 9 isolates tested with PVPP. The incubation with *A. pennatula* extract significantly increased the %LFE of CENID, McMASTER, CAVR, MOF23, FESC, PARAISO and INRA isolates. After the incubation of the extract + PVPP, the %LFE was not completely removed for CENID, MOF23, FESC and PARAISO isolates. The effect on %LFE was removed by PVPP only for the McMASTER and CAVR isolate. The JUAN isolate, showed the effect on %LFE only after incubation with PVPP. The effect of extract on the %LFE for INRA was not removed by PVPP. Finally, WHITE-RIVER isolate did not show any effect on %LFE with or without PVPP.

The incubation with *O. viciifolia* extract increased the %LFE of CENID, CAVR, WHITE-RIVER, MOF23, FESC and PARAISO isolates (Table 2). After the incubation with the extract +PVPP three of isolates (WHITE-RIVER, MOF23 and FESC) reduced the AH activity but remained different to PBS control values ($P < 0.05$), three isolates did not improve the %LFE (CENID, INRA and CAVR) and one isolate (PARAISO) showed a higher %LFE. Finally, two isolates (McMASTER and JUAN) showed an AH effect only after incubation with PVPP.

3.3. *Anthelmintic activity on the emerged larvae*

During the evaluation of the AH effects on *H. contortus* eggs for the *A. pennatula* and *O. viciifolia* extracts several morphological alterations were observed on the larvae emerged from eggs when exposed to acetone:water extracts, compared to PBS controls. Changes were evident even when exposed to the lowest concentration tested with both extracts (600 μg/ml PBS). The emerged larvae from the PBS controls (Fig. 1a) showed normal cuticle, rhabditoid pharynx with bulb and intestinal cells. Meanwhile, larvae exposed to *A. pennatula* or *O. viciifolia* extracts showed a separation between the cuticle and the internal structures including the pharynx, bulb and intestinal cells (Fig. 1b and c). At 5000 μg/ml PBS, the larvae cuticle was clearly swollen and the internal structures (pharynx, bulb and intestine) were not distinguishable (Fig. 2a for *A. pennatula* and Fig. 2c for *O. viciifolia*). Larvae exposed to extracts at 5000 μg/ml PBS + PVPP showed less evident lesions, which were similar to the changes observed at 600 μg/ml PBS (Fig. 2b and d).

4. Discussion

This study produced the first evidence of variation in the susceptibility between different *H. contortus* isolates in the AH effect observed when exposing eggs to acetone:water extracts under *in vitro* conditions. This study confirmed that the main AH effect of acetone:water extracts against *H. contortus* eggs is to block the hatching process of the L_1 larvae already formed inside the egg,

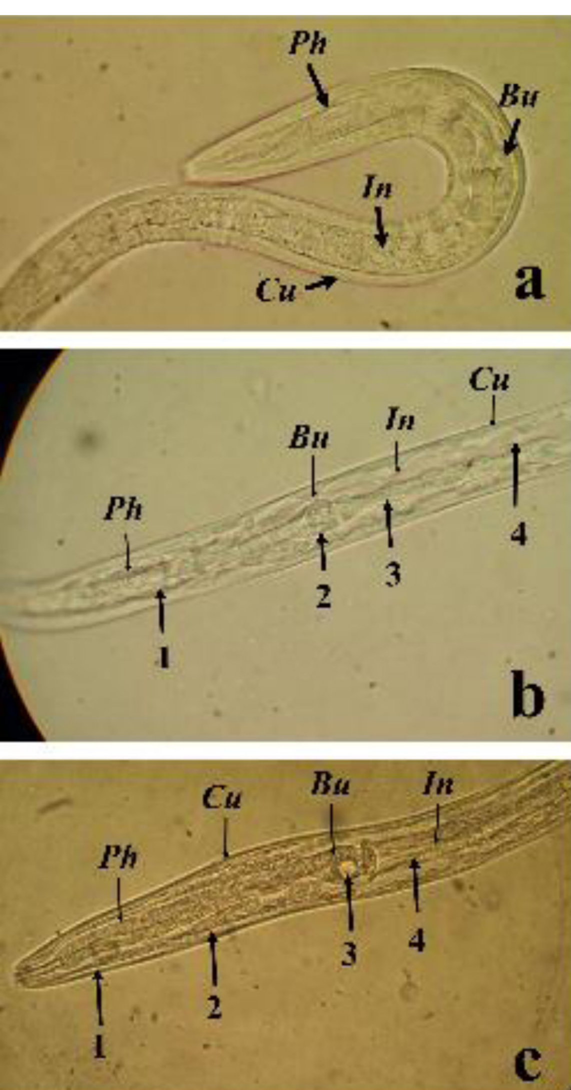

Fig. 1. (a) *Haemonchus contortus* larva recovered in control (PBS) showing the Pharynx (*Ph*), bulb (*Bu*), intestinal cells (*In*) and cuticle (*Cu*). (b) *Haemonchus contortus* larva exposed to *Acacia pennatula* (600 μg/ml PBS). 1. Separation of pharynx and cuticle. 2. Separation of bulb and cuticle. 3. Shrinkage of intestinal cells. 4. Separation of intestine and cuticle. c. *Haemonchus contortus* larva exposed to *Onobrychis viciifolia* (600 μg/ml PBS). 1 and 2. Separation of pharynx and cuticle. 3. Reduction of bulb size. 4. Degradation of intestinal cells and separation from the cuticle.

resulting in a high proportion of larvae failing eclosion (%LFE) as it has been reported for different acetone:water extracts including different plants materials and agroindustrial by-products (Vargas-Magaña et al., 2014). Moreover, larvae emerging from eggs which have been exposed to the tested extracts showed morphological changes suggestive of an AH effect. Until now, the exact mechanism causing a significant %LFE is not totally clear. Different mechanisms for PSM action have been proposed such as: inhibition of enzymes responsible for rupture of the eggshell, binding to structural proteins in the membrane that prevent their degradation, binding to membrane receptors (competition of hatching factors) or co-occurrence (synergy) of all previous mechanism (Hoste et al., 2012; Vargas-Magaña et al., 2014).

The present study tested two acetone:water extracts on fresh eggs from ten different *H. contortus* isolates originated from different parts of the world. Although the acetone:water extracts tested required relatively high concentrations to achieve an EC_{50} on eggs compared to doses required to reach a clear AH effect for the L_3 exsheathment process (Vargas-Magaña, 2014), the present study provides further evidence suggesting variability of susceptibility between different *H. contortus* isolates when exposed to acetone:water extracts. As it was mentioned above, evidence suggesting variability in the susceptibility between different *H. contortus* isolates was first suggested when using the *in vitro* larval motility inhibition assay (Calderón-Quintal et al., 2010) and the larval exsheathment inhibition assay (Vargas-Magaña, 2014). Those studies suggested that L_3 larvae from Mexican isolates were less susceptible than French isolates. In the case of eggs, a lower sensitivity of the Mexican isolates was not strictly evident. Thus, although it was expected that Mexican isolates could be more resistant than isolates from other parts of the world, particularly for the *A. pennatula* extract, it was not necessarily the case. Firstly, one of the Mexican isolates, CENID, showed highest sensitivity ($P < 0.05$) than all the other isolates for both extracts tested. On the other hand, two Mexican isolates were classified as the most resistant when exposed to *A. pennatula* extract (FESC and PARAISO), meanwhile a fourth Mexican isolate was classified amongst the intermediate resistant isolates (FMVZ). For the *O. viciifolia* extract there were two Mexican isolates classified as the most susceptible (CENID and PARAISO), meanwhile, the FESC and FMVZ isolates showed similar levels of susceptibility as other isolates from other parts of the world. Thus, contrary to the work with L_3 larvae, eggs

Table 2
Effect of the addition of PVPP on the proportion of morulated eggs (ME), larvae failing eclosion (LFE) and larvae of *Haemonchus contortus* from different geographical regions resulting from incubations with *Acacia pennatula* and *Onobrychis viciifolia* extracts at a concentration of 5000 μg/ml PBS.

Isolate	Life stage	PBS	*Acacia pennatula*			*Onobrychis viciifolia*		
			5000 μg/ml PBS	5000 μg/ml PBS + PVPP	S.E.	5000 μg/ml PBS	5000 μg/ml PBS + PVPP	S.E.
CENID (Mexico)	ME	0.95a	9.48b	11.13b	0.86	18.31b	10.23b	2.94
	LFE	0.98a	59.43b	41.42c	2.78	81.41b	88.79b	2.84
	Larvae	98.07a	31.08b	47.45c	3.14	0.28b	0.97b	0.40
INRA (France)	ME	2.36a	7.85b	7.52b	0.93	11.56b	7.78ab	2.14
	LFE	9.04a	35.83b	32.20b	1.75	88.07b	91.43b	2.25
	Larvae	88.59a	56.31b	60.27c	1.09	0.36b	0.79b	0.21
McMASTER (Australia)	ME	7.48a	4.15a	9.18a	2.11	8.72ab	17.39b	2.85
	LFE	20.15a	35.50b	33.33ab	3.89	19.16a	68.25b	3.49
	Larvae	72.36a	60.35a	57.48a	5.66	72.11a	14.35b	3.36
CAVR (Australia)	ME	4.81a	4.89a	4.79a	1.40	16.73b	23.21b	3.30
	LFE	8.09a	35.43b	9.90a	2.55	78.75b	68.08b	3.20
	Larvae	87.09a	59.67b	85.29a	3.32	4.51b	8.70b	2.22
WHITE RIVER (South Africa)	ME	1.73a	0.97a	0.79a	0.68	10.25b	24.56c	1.63
	LFE	20.06a	30.56a	21.75a	3.55	86.35b	71.84c	2.04
	Larvae	78.20a	68.47a	77.45a	3.87	3.39b	3.59b	0.90
JUAN (France)	ME	2.95a	4.28a	2.03a	1.04	3.92a	10.87b	1.10
	LFE	14.53a	18.44ab	24.44b	1.90	0.34b	61.91c	2.13
	Larvae	82.51a	77.27ab	73.52b	2.51	95.73b	27.22c	1.85
MOF23 (USA)	ME	23.90a	21.91a	24.43a	1.55	30.76ab	38.42b	2.39
	LFE	4.68a	41.23b	13.28c	2.46	65.66b	56.16c	1.94
	Larvae	71.42a	36.85b	62.28a	3.55	3.56b	5.42b	1.37
FESC (Mexico)	ME	0.82a	8.13b	8.41b	1.52	8.44b	10.78b	0.80
	LFE	1.87a	46.71b	31.41c	2.43	90.47b	76.36c	1.11
	Larvae	97.30a	45.15b	60.17c	2.57	1.08b	12.85c	1.11
PARAISO (Mexico)	ME	2.59a	2.19a	6.51b	1.02	7.33a	2.60a	1.55
	LFE	2.05a	35.89b	25.54c	1.83	81.61b	93.65c	2.16
	Larvae	95.36a	61.90b	67.94b	2.15	11.05b	3.75c	1.88

Different letters in the same row in each category of each extract means a significant difference ($P < 0.05$).
No data were recorded for FMVZ because hatching rate in control wells was below 70%.

from Mexican *H. contortus* isolates were equally susceptible than other isolates from different origins. The eggs of Mexican *H. contortus* isolates may have not develop resistance when compared with other isolates possibly due to a reduced exposure to PSM in the faeces, in terms of quantity or quality, compared to other life stages dwelling in the lumen of the gastrointestinal tract such as L_3, L_4, L_5 or adults.

4.1. Role of tannins on AH effects

In this study, a second set of EHA using the acetone:water extracts with and without PVPP, attempted to identify the possible role of tannins in the AH activity observed. The results of these assays indicate that different *H. contortus* isolates show variability in the role of tannins either on the ovicidal effect or the%LFE. It confirmed earlier evidence suggesting that tannins are not the sole responsible of the AH activity on *H. contortus* eggs as shown by Vargas-Magaña et al. (2014). The new set of *in vitro* assays, in which tannins were removed with PVPP showed the following outcomes:

(a) Addition of PVPP was not associated with a reduction of the ovicidal effect of *A. pennatula* and *O. viciifolia* extracts. Three *H. contortus* isolates (CENID, FESC and INRA) showed ovicidal effect for the *A. pennatula* extract irrespective of the addition of PVPP and a further isolate (PARAISO) showed the ovicidal effect only when PVPP was added. Similarly, the *O. viciifolia* extract showed ovicidal effect irrespective of the addition of PVPP (CENID, CAVR and FESC) and the addition of PVPP enhanced the ovicidal effect for the WHITE-RIVER isolate. In the case of INRA, the addition of PVPP reduced the ovicidal effect. Finally, McMASTER, JUAN and MOF23 only showed an ovicidal effect when incubated with PVPP. In summary, these results suggest that condensed tannins are not the sole PSM involved in the ovicidal effect of both extracts.

(b) Addition of PVPP was not associated with a reduction of the %LFE for *H. contortus* incubated with tested extracts. The different isolates showed variation in the susceptibility that included different scenarios depending on the extract tested. Some isolates reduced the AH activity when incubated with PVPP but they remained different to PBS control values. Other isolates did not show a reduction of the AH effect when incubated with PVPP. Finally, some isolates showed an AH effect only after incubation with PVPP or further increased the AH effect observed with the extracts alone.

4.2. Anthelmintic activity on the larvae emerged

The emerged larvae exposed to both tested extracts showed structural changes that were not observed in the larvae recovered from PBS control assays. The structural changes detected in the larvae were observed even in those emerged larvae exposed to low extract concentrations of *A. pennatula* and *O. viciifolia* (Fig. 1b and c). Although EHA only evaluates the egg hatching, in this study it was possible to observe lesions on the larvae recorded during the counting procedures of each bioassay. The damage observed on the larvae indicated that *A. pennatula* and *O. viciifolia* extracts could be more effective against the larval stages rather than eggs of *H. contortus*. However, to probe that it would be necessary to evaluate those extracts with another *in vitro* assay, such as the Larval Development Assay. Changes observed on the larvae exposed

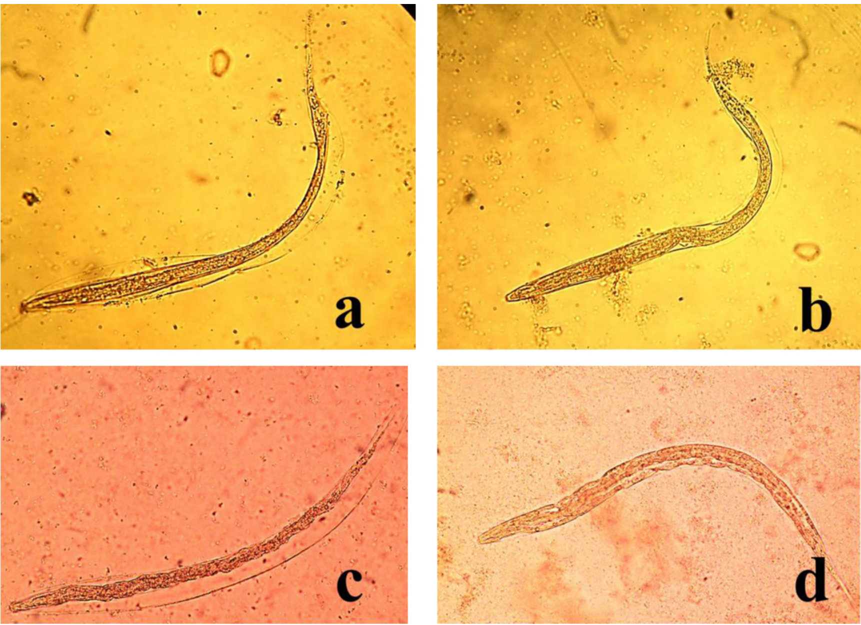

Fig. 2. Effect of the addition of PVPP on the alterations caused by acetone water extracts on *Haemonchus contortus* larvae: (a) *Acacia pennatula* 5000 μg/ml PBS, (b) *A. pennatula* 5000 μg/ml PBS + PVPP, (c) *Onobrychis viciifolia* 5000 μg/ml PBS and (d) *O. viciifolia* 5000 μg/ml PBS + PVPP.

to extracts consisted on the separation of the larval cuticle from the internal structures. Lesions were evident at 40× with a microscope and seemed consistent with those degenerative changes in the cells of the *H. contortus* and *Trichostrongylus colubriformis* L_3 larvae reported by Brunet et al. (2011) using 1200 μg *O. viciifolia* acetone:water extract. Those authors reported ultrastructure changes in the infective larvae exposed to the extract, including local or general dissociation between the cuticle and hypodermis of larvae as well as degradation of muscle fiber layer and striated muscle and lysis of those cells. Although there are no previous reports on the ultrastructure lesions of *H. contortus* L_3 larvae exposed to *A. pennatula* extracts, the damages observed in the present experiment were similar to those of *O. viciifolia*. The use of PVPP showed that tannins were partly involved in the AH activity against larvae because the damage was milder, although not eliminated, in those parasite incubated with extract +PVPP incubation (Fig. 2).

5. Conclusion

Variation in the *in vitro* susceptibility of *H. contortus* eggs towards *A. pennatula* or *O. viciifolia* acetone:water extracts was found amongst different isolates tested although such difference could not be related to their origin. For all isolates, blocking the L_1 larvae from hatching, represented as the %LFE, was the dominant mechanism of action with both extracts tested, while the ovicidal effect was less evident.

The different *H. contortus* isolates showed variability in the role of polyphenols either on the ovicidal effect or the %LFE, and confirmed that condensed tannins were not the sole responsible of the AH activity of acetone:water extracts on *H. contortus* eggs.

Conflict of interest

None.

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