Susceptibility of ten *Haemonchus contortus* isolates from different geographical origins towards acetone:water extracts of polyphenol-rich plants. Part 2: Infective L_3 larvae

J.I. Chan-Pérez[a], J.F.J. Torres-Acosta[a,*], C.A. Sandoval-Castro[a], G.S. Castañeda-Ramírez[a], G. Vilarem[c], C. Mathieu[c], H. Hoste[b]

[a] *FMVZ, Universidad Autónoma de Yucatán, Km 15.5 Carretera Mérida-Xmatkuil, Mérida, Yucatán, Mexico*
[b] *INRA UMR 1225 Interactions Hôte Agents Pathogènes, 23 Chemin des Capelles, F31076, Toulouse, France*
[c] *Université de Toulouse, INP-ENSIACET, LCA (Laboratoire de Chimie Agro industrielle), F31030 Toulouse, France*

ARTICLE INFO

Keywords:
Larval exsheathment inhibition assay
Haemonchus contortus
Acacia pennatula
Onobrychis viciifolia
Polyphenols

ABSTRACT

This study explored the variation in susceptibility to acetone:water plant extracts between infective larvae (L_3) of ten *Haemonchus contortus* isolates from different geographical origin. The L_3 of 10 different isolates were exposed either to the acetone:water extract of a temperate plant (*Onobrychis viciifolia*) or a tropical plant (*Acacia pennatula*) and were evaluated with the larval exsheathment inhibition assay (LEIA). The L_3 of each isolate were incubated with different concentrations of each extract (0, 25, 50, 100, 200, 400, 600, 800, 1000 and 1200 μg/mL of phosphate buffered saline (PBS)). After incubation, the exsheathment process of L_3 was induced using a solution with sodium hypochlorite (2%) and sodium chloride (16.5%). The proportion of exsheathed L_3 was determined for each concentration at 0, 20, 40 and 60 min. Effective concentrations 50% (EC_{50}) and the corresponding 95% confidence intervals (95% CI) were calculated for every isolate with both extracts. Moreover, a resistance ratio (RR) was calculated for each extract to compare isolates, using the most susceptible isolate as the respective reference for each extract. To determine the role of polyphenols on the reported effect, a second set of incubations was made for each isolate and each extract, using the extracts at a concentration of 1200 μg/mL PBS with or without polyvinylpolypyrrolidone (PVPP), a polyphenol blocking agent, and controls without extract. The ten different *H. contortus* isolates showed variation in susceptibility for each of the 2 extracts tested ($P < 0.05$). The EC_{50} values for *A. pennatula* extract ranged from 36 to 501 μg/mL (RR: 2.11–13.68). Meanwhile, the EC_{50} values for *O. viciifolia* extract ranged from 128 to 1003 μg/mL (RR: 1.25–7.82). The use of PVPP revealed that polyphenols were responsible for the anthelmintic activity recorded for both extracts. However, tested *H. contortus* isolates suggested that susceptibility to one polyphenol-rich extract did not determine the susceptibility to the other polyphenol rich extract. The latter result indicated that the different *H. contortus* isolates varied in their susceptibility to the polyphenols present in each extract evaluated.

1. Introduction

In recent years, there has been a considerable effort to identify the potential anthelmintic (AH) activity of different plant extracts against gastrointestinal nematodes (GIN) of ruminants (Githiori et al., 2006; Sandoval Castro et al., 2012). Such AH activity against different species of GIN, and their different life stages, has been associated to the plant secondary metabolites contained (Hoste et al., 2016). In this search for secondary metabolites of bioactive plants there has been an effort to promote specific methodologies aiming at standardizing the techniques employed for testing effectiveness of plant extracts (Hoste et al., 2008, 2015). However, such standardization is neither official nor generally accepted. As a result, it is difficult to compare the AH activity obtained with different plant materials and methodologies around the world. Furthermore, there are many sources of variation that have been identified commencing from the type of solvents used in the extraction process (ethanol, acetone, water, *etc*.) (Hernández Villegas et al., 2011), variation in the content of plant secondary metabolites between plants (Novobílsky et al., 2013) due to region of origin, cultivar or season, and the variation between plant parts (leaves, stems, fruits or agroindus trial by products) (Marie Magdeleine et al., 2010; Vargas Magaña et al., 2014b). There are also differences due to the *in vitro* techniques used,

* Corresponding author.
E-mail address: tacosta@correo.uady.mx (J.F.J. Torres-Acosta).

http://dx.doi.org/10.1016/j.vetpar.2017.04.023

Table 1
Names of ten *Haemonchus contortus* isolates used to compare their sensitivity to acetone:water extracts of *Acacia pennatula* and *Onobrychis viciifolia* with the respective institution and country of origin, climate of origin and anthelmintic resistance status.

Isolate	Origin	Climate of origin[a]	Anthelmintic Resistance Status	Reference
FMVZ	Facultad de Medicina Veterinaria y Zootecnia-Universidad Autónoma de Yucatán, Yucatán, México.	Aw (Hot tropical savannah)	Benzimidazole resistant	(Chan-Pérez et al., 2016)
PARAISO	Commercial sheep farm, Yucatán, México.	Aw (Hot tropical savannah)	Benzimidazole resistant	(Ortíz-Ocampo et al., 2016)
CENID	Centro Nacional de Investigación Disciplinaria, INIFAP, SAGARPA, Morelos, México.	Cfb (Marine west coast climate)	Benzimidazole resistant	(Vargas-Magaña et al., 2015)
FESC	Facultad de Estudios Superiores, Cuatitlan, Universidad Nacional Autónoma de México, Cuautitlán, México.	Cwb (Oceanic subtropical highland climate)	Benzimidazole resistant	(Vargas-Magaña et al., 2015)
MOF23	United States Department of Agriculture, Virginia, United States of America	Cfa (Humid subtropical climate)	Moxidectin resistant	(Rajan et al., 2002)
CAVR	Chiswick Avermectin Resistant, Commonwealth Scientific and Industrial Research Organization (CSIRO), Armidale, Australia.	Cfb (Marine west coast climate)	Ivermectin resistance	(Le Jambre et al., 1995)
WHITE RIVER	White River, South Africa.	Cfa (Humid subtropical climate)	Multi-drug resistant	(Le Jambre et al., 1995)
INRA	Institut National de la Recherche Agronomique, Toulouse, France.	Cfb (Marine west coast climate)	AH Susceptible	(Chan-Pérez et al., 2016)
JUAN	Commercial sheep farm, Toulouse, France.	Cfb (Marine west coast climate)	AH Susceptible	(Chan-Pérez et al., 2016)
McMASTER	CSIRO, Armidale, Australia	Cfb (Marine west coast climate)	AH Susceptible	(Le Jambre et al., 1995)

[a] Koppen classification was used to describe climate of origin (CantyMedia, 2017).

where the larval exsheathment inhibition assay (LEIA) or the larval motility inhibition assay (LMIA), both using the infective larvae (L_3), may show different AH activity even when using the same plant extract (Alonso Díaz et al., 2011). Such variation in the AH effect could be more evident when evaluating different life stages (*i.e.* eggs *vs.* L_3). Recent evidence suggests the existence of another source of variation for the AH activity that has not been previously considered: the geographical origin of the parasite isolates. The first authors to propose such variability were Calderón Quintal et al. (2010), using the larval migration inhibition assay (LMIA) on three different *Haemonchus contortus* isolates from Mexico. Such study showed that doses of tropical polyphenol rich plant extracts which were effective against temperate *H. contortus* isolates (Alonso Díaz et al., 2008b), were not effective against an isolate from tropical Mexico (UADY isolate), while that extract seemed to work well against an isolate from temperate Mexico (FESC isolate). To confirm such difference in susceptibility between French (temperate region) and Mexican *H. contortus* isolates from temperate and tropical regions, Vargas Magaña et al. (2014b) used respective LEIA to find that French isolates tended to show higher susceptibility to the plant secondary metabolites of acetone:water extracts from tree leaves and agroindustrial by products. According to the latter results, infective larvae from Mexico had 3.3 times more chances to be declared resistant towards the AH activity of plant extracts compared to French isolates ($P < 0.05$). The apparent low susceptibility of Mexican *H. contortus* infective larvae towards acetone: water extracts of plants could represent a widespread phenomenon not exclusive of Mexican isolates. Recently, Chan Pérez et al. (2016) demonstrated the existence of phenotypic variation in the susceptibility towards two tannin rich acetone:water extracts using the egg hatch assay between ten *H. contortus* isolates from different geographical origins. In the case of eggs, the geographical origin of isolates was not associated with their susceptibility to acetone:water polyphenol rich extracts. The latter set a precedent showing that different *H. contortus* isolates have a constitutive phenotypic tolerance towards plant second ary metabolites from acetone:water extracts. If variability is confirmed for L_3, it could open new insights in the interactions between plant secondary compounds and the parasitic GIN. Therefore, this study explored the variation in the susceptibility to acetone:water plant extracts between infective larvae of *Haemonchus contortus* isolates from 10 different geographic origins. The role of polyphenols on the AH activity of acetone:water extracts from two plants (*Acacia pennatula* and *Onobrychis viciifolia*) was also evaluated.

2. Material and methods

2.1. Production of Acacia pennatula and Onobrychis viciifolia extracts

Acetone:water extract produced with *Acacia pennatula* fresh leaves was provided by the Campus de Ciencias Biológicas y Agropecuarias (CCBA), Universidad Autónoma de Yucatán (UADY), México. The acetone:water extract produced with *Onobrychis viciifolia* foliage was produced at the Unité Mixte de Recherche Interactions Hôtes Agents Pathogènes (UMR/IHAP) of Institut National de la Recherche Agronomique/École Nationale Vétérinaire de Toulouse (INRA/ENVT), France. These extracts were previously used to explore the *in vitro* variation of susceptibility of *H. contortus* eggs towards the two tannin rich extracts (Chan Pérez et al., 2016). The extraction procedure was performed as follows: fresh leaves of the two plants were collected to obtain acetone:water extracts. The *A. pennatula* foliage was collected in November 2011 in Mérida, México (20°52′N, 89°37′W) and the *O. viciifolia* foliage was collected in March 2009, in Realville, France (44°11′N, 1° 48′ W). The foliage of respective plants was ground and 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. The acetone was removed by using a rotovapor. Then, for *A. pennatula* extract, this was filtered (Whatman© No. 50) and washed 4

times with dichloromethane to remove pigments and lipids and the filtrate was lyophilized. In the case of *O. viciifolia*, the material was lyophilized after acetone removal without prior washing with dichlor omethane. To avoid hydration of both extracts, they were kept refrigerated in sealed flasks until used (4 °C) (Alonso Díaz et al., 2008a). A single batch of acetone:water extract of each plant species evaluated was used in the respective larval exsheathment inhibition assays (LEIA) to avoid variation due to extract procedures. The Total phenols (TP) and total tannins (TT) were determined for both extracts using the Folin Ciocalteu method (Prince and Butler, 1977).

2.2. Haemonchus contortus isolates

Ten *H. contortus* isolates from different geographic origins and different commercial AH susceptibility status were tested in order to explore the overall variability in susceptibility to acetone:water plant extracts. The name of isolates, the geographical origins, climate of origin, anthelmintic resistance status for each isolate are provided in Table 1.

2.3. Management of donor animals for the production of Haemonchus contortus larvae

Donor animals risen free of GIN infections from birth were kept in individual pens with raised slatted floors before and during the experiment. To prevent accidental infections with trichostrongylid parasites through the consumption of freshly harvested grass, donor animals were fed a balanced diet based on grass hay and commercial concentrate feed. Water was available *ad libitum*. Before infection with respective *H. contortus* isolates, donor animals received an anthelmintic treatment with benzimidazole (10 mg kg^{-1} for sheep and 20 mg kg^{-1} for goats) and levamisole (7.5 mg kg^{-1} for sheep and 12 mg kg^{-1} for goats) to prevent the establishment of any previous GIN accidental infections. Ten days after the preventive AH treatment, samples of faeces were obtained from all donor animals and faeces were examined with the flotation technique to confirm the absence of GIN eggs prior to the artificial infections.

2.4. Production of H. contortus isolates

Donor goats were infected with 3000 L_3 of each *H. contortus* isolate (MOF23, CAVR, WHITE RIVER, INRA, JUAN and McMASTER). The donor goats were maintained in doors with the respective monospecific infection at the École Nationale Vétérinaire de Toulouse (ENVT), France. Twenty eight days after infections, fresh faeces of donor animals were used in coprocultures to produce infective larvae of each *H. contortus* isolate. The L_3 recovered from coprocultures of donor goats maintained at ENVT, France, were transferred to the Campus de Ciencias Biológicas y Agropecuarias of Universidad Autónoma de Yucatan, Mexico (CCBA UADY).

New donor worm free animals (sheep) were prepared at CCBA UADY as previously described to reproduce the isolates received from France and the *H. contortus* isolates from Mexico (FMVZ, PARAISO, CENID and FESC). Two donor sheep were infected with 3000 L_3 for each *H. contortus* isolate produced. In this manner the L_3 of the ten isolates used in the study had similar age when the respective assays were performed. Furthermore, all the tests were performed at the parasitology laboratory in Mexico under similar conditions. This strategy aimed to reduce the risk of bias due to differences in aspects such as the feeding strategy of donors, species of donors, procedures for the production of L_3, harvest and storage of L_3, laboratory micro environmental conditions or reagents used for the *in vitro* tests.

2.5. Larval exsheathment inhibition assay (LEIA)

Preparation of stock solution (5000 μg/mL) of *A. pennatula* or *O. viciifolia* extracts were made in phosphate buffered saline (PBS, Sigma©) prepared with purified water. The LEIA was conducted following the procedure described by Jackson and Hoste (2010). The PBS was added to plastic tubes (990, 980, 960, 920, 840, 760, 680, 600 and 520 μl of PBS) and the respective volume of stock solutions of *A. pennatula* or *O. viciifolia* extract (10, 20, 40, 80, 160, 240, 320, 400 and 480 μL of stock solution, respectively). One tube was used as negative control contain ing 1000 μl of PBS without extract. Finally, 1000 μL of infective larvae solution (L_3 ~ 1000/mL) was added to each tube to obtain the final extract concentrations (25, 50, 100, 200, 400, 600, 800, 1000 and 1200 μg/mL). Tubes with larvae were incubated for 3 h at 24 °C. After the incubation, the larvae were centrifuged for 3 min at 123*g* and washed 3 times with PBS. Then, four aliquots of larvae solution were placed in eppendorf vials (200 μL each) for each concentration and controls. The process of L_3 exsheathment was artificially induced by contact with a solution of sodium hypochlorite (2%) and sodium chloride (16.5%) diluted at 1/300, 1/400, 1/480, 1/600 or 1/800 in PBS. To choose which dilution of sodium hypochlorite should be used, the optimal exsheathment for each isolate was determined before each assay to ensure that > 95% of exsheathment was found after 60 min in the respective negative controls without extract. The exsheathment of L_3 was observed at 0, 20, 40 and 60 min. The exsheathment process was stopped at each time point by flaming the respective slides that contained the L_3. Then, the numbers of sheathed and ex sheathed larvae were recorded.

To determine the influence of the polyphenols on the process of exsheathment, a second set of exsheathment assays was conducted. Two extract solutions at concentration of 1200 μg/mL were prepared for each plant extract, one of which was incubated with polyvinylpolypyr rolidone (PVPP, Sigma Aldrich©, P6755) a polyphenol blocking agent. The solutions were added with 50 mg PVPP/mL. All the solutions were incubated for 2 h at 24 °C. After incubation, the solutions with PVPP were centrifuged at 1849*g* for 5 min. Then, supernatant was used for testing as described above.

2.5.1. Data analysis

The percentage of exsheathment (E%) was calculated according to the following formula:

$$\%\ E = \frac{\text{number of exsheathed } L_3}{\text{number of exsheathed } L_3 + \text{number of ensheathed } L_3} \times 100$$

Data from the LEIA for each *H. contortus* isolate with acetone:water extract of each plant tested, were used to determine the effective concentration required to inhibit 50% of exsheathment (EC_{50}), with respective 95% confidence intervals (95% CI) using the PoloPlus 1.0 software (LeOra software, 2002). Significant differences between the EC_{50} obtained for the ten isolates for each plant extract were deter mined by comparing the respective 95% CI. Non overlapping 95% CI indicated statistical difference between isolates ($P < 0.05$) (Jeske et al., 2009).

The EC_{50} values of the most susceptible isolate to *A. pennatula* or *O. viciifolia* extracts respectively were used as reference to calculated resistant ratio (RR) using the follow formula (Sangster and Dobson, 2002):

$$RR = \frac{EC_{50} \text{of ``resistant'' isolate}}{EC_{50} \text{of the most susceptible isolate}}$$

For each extract and each isolate tested, respective analyses of variance, using a completely randomized design, were used to deter mine differences in the mean percentage of exsheathment (dependent variable) between treatments: Control (0 μg extract/mL PBS) and the respective extract (1200 μg extract/mL PBS) with and without PVPP, with the respective post hoc analysis using the Fisher's LSD of Statgraphics Centurion XV (2005).

Table 2
Effective concentration of acetone:water *Acacia pennatula* or *Onobrychis viciifolia* extracts (μg/mL) required to achieve 50% inhibition of larval exsheathment (EC_{50}), 95% confidence intervals (95% CI) and the respective resistance ratio (RR) of *Haemonchus contortus* isolates from different geographical regions.

Isolate	*Acacia pennatula* EC_{50} (95% CI)	RR	Isolate	*Onobrychis viciifolia* EC_{50} (95% CI)	RR
FESC (Mexico)	**36.64 (28.52–44.84)**a[a]		WHITE RIVER	**128.24 (113.22–144.03)**a[a]	
McMASTER (Australia)	77.46 (63.68–95.89)b	2.11	MOF23	152.94 (109.15–189.57)ab	1.25
CENID (Mexico)	92.41 (64.53–116.73)bc	2.52	FESC	198.87 (159.66–247.24)bcd	1.55
INRA (France)	105.80 (74.95–152.52)bcd	2.88	FMVZ	214.89 (161.40–263.40)bcd	1.67
WHITE RIVER (South Africa)	108.57 (96.35–133.21)bcd	2.96	JUAN	298.65 (226.58–369.21)cde	2.32
FMVZ (Mexico)	128.93 (113.72–144.72)cd	3.51	INRA	320.99 (277.37–364.12)de	2.50
CAVR (Australia)	158.37 (122.44–245.02)de	4.32	CENID	409.32 (307.36–476.43)e	3.19
PARAISO (Mexico)	216.87 (156.80–304.24)e	5.91	PARAISO	714.29 (515.90–879.36)f	5.56
MOF23 (USA*)	217.46 (164.40–365.55)e	5.93	CAVR	973.60 (854.94–1149.71)f	7.59
JUAN (France)	501.40 (421.98–576.26)f	13.68	McMASTER	1003.39 (840.23–1318)f	7.82

Different letters in the same column indicate a significant difference ($p < 0.05$).
[a] Values in bold were taken as susceptible reference for each extract to calculate respective RR.
* United States of America.

3. Results

3.1. Polyphenol content

Variations in the total polyphenol (TP) and total tannin (TT) contents were observed for both extracts. For the *A. pennatula* extract, TP was 37.26%, while TT content was 31.75%. In the case of the *O. viciifolia* acetone:water extract, the TP and TT were 2.66% and 0.98% respectively.

3.2. Variation of susceptibility between H. contortus exposed to A. pennatula or O. viciifolia extracts

Table 2 shows the EC_{50} with their 95% CI and the respective resistance ratio (RR) obtained for each *H. contortus* isolate exposed to *A. pennatula* acetone:water extract. The EC_{50} values for *A. pennatula* ranged from 36 to 501 μg/mL. According to the EC_{50} and 95% CI, the FESC isolate was selected as the susceptible reference to estimate RR, which ranged from 2.11 to 13.68.

Table 2 also shows the EC_{50} with respective 95% CI and RR for each *H. contortus* isolate exposed to *O. viciifolia* acetone:water extract. The EC_{50} values for *O. viciifolia* ranged from 128 to 1003 μg/mL. According to the EC_{50} and 95% CI, the WHITE RIVER isolate was used as the susceptible reference to estimate RR, which ranged from 1.25 to 7.82.

3.3. Role of polyphenols in the anthelmintic activity

Table 3 shows the exsheathment of *H contortus* L_3 larvae resulting from incubations with *A. pennatula* or *O. viciifolia* extracts at a concentrations of 1200 μg extract/mL PBS incubated with and without PVPP. The *A. pennatula* extract inhibited 100% of the exsheathment in all the isolates tested. For the *O. viciifolia* extract the exsheathment was 100% inhibited for most isolates except for PARAISO, which pre sented > 90% efficacy. In all cases, the incubation of extracts with PVPP removed almost completely the AH activity compared to incuba tions without PVPP ($P < 0.05$), suggesting that the exsheathment inhibition was associated with the polyphenols present in each extract.

4. Discussion

The difference in susceptibility between *H. contortus* isolates ex posed to acetone:water extracts was first suspected when tropical tannin rich plant extracts were tested using isolates from France or México. The first studies performed in France by Alonso Díaz et al. (2008b) showed a clear AH effect at a certain dose level. When the *in vitro* test was performed using the same extracts and doses against *H. contortus* from Yucatán, México, the resulting efficacy was lower (Calderón Quintal et al., 2010). The latter was found using the LMIA, a technique that has shown to be less sensitive than the LEIA (Alonso Díaz et al., 2011). Later on, Vargas Magaña et al. (2014b) used the LEIA to compare *H. contortus* isolates from Mexico and France and their study suggested a lower overall susceptibility towards acetone:water extracts for some Mexican isolates. Confirming variability in the susceptibility between isolates could be an important issue to consider for the design of ruminant's GIN control/management schemes based on plant secondary compounds.

Table 3
Proportion (%) of exsheathed L_3 of *Haemonchus contortus* from different geographic regions resulting from incubations with PBS (Control), *Acacia pennatula* or *Onobrychis viciifolia* extracts at 1200 μg/mL PBS with or without polyvinylpolypyrrolidone (PVPP).

Isolate (Country)	*Acacia pennatula*				*Onobrychis viciifolia*			
	Control PBS	1200 μg/mL	1200 μg/mL + PVPP	S.E.	Control PBS	1200 μg/mL	1200 μg/mL + PVPP	S.E.
FMVZ (MEX)	100.0a	0.0b	92.2c	1.65	100.0a	0.0b	96.3a	1.26
PARAISO (MEX)	89.8a	0.0b	71.2a	9.20	93.3a	9.9b	85.3a	3.77
FESC (MEX)	97.1a	0.0b	98.7a	1.83	98.1a	0.0b	100.0a	1.10
CENID (MEX)	97.0a	0.0b	94.8a	2.13	–	–	–	–
INRA (FRA)	97.1a	0.0b	95.2a	1.36	100.0a	0.0b	98.5a	0.87
JUAN (FRA)	98.3a	0.0b	91.5c	1.03	100.0a	0.0b	92.9a	2.38
CAVR (AUS)	95.6a	0.0b	89.8a	3.18	100.0a	0.0b	95.6c	1.13
McMASTER (AUS)	83.5a	0.0b	83.8a	1.52	100.0a	0.0b	100.0a	0.00
WHITE RIVER (ZAF)	95.5a	0.0b	92.4a	2.49	100.0a	0.0b	100.0a	0.00
MOF23 (USA)	92.4a	0.0b	86.2a	4.00	100.0a	0.0b	93.0c	0.64

Different letters in the same line indicates significant difference ($P < 0.05$) between treatments (control PBS, extract, extract + PVPP) for each of the two respective plant extracts. S.E.: standard error. MEX: Mexico, FRA: France, AUS: Australia, ZAF: South Africa, USA: United States of America.

4.1. Polyphenol content and its role on the anthelmintic effect evaluated

The two acetone:water extracts tested here showed different total phenol and total tannin contents, with the *A. pennatula* extract showing concentrations more than fifteen times higher than the *O. viciifolia* extract. Such different concentration could be attributed to the natural features of the plant materials tested or to differences in the extraction procedure used for both plants, where the *O. viciifolia* extract was not washed with dichlorometane and the *A. pennatula* extract was washed with dichlorometane prior to lyophilization. In spite of such difference in polyphenol content between the two extracts tested, it was possible to confirm that the inhibition of exsheathment was caused by poly phenols in the case of both extracts. The latter was confirmed when the incubation of plant extracts with PVPP removed the AH activity on L_3 of all *H. contortus* isolates ($P < 0.05$) (see Table 3). Once it was confirmed that polyphenols were responsible for the AH activity in both plant extracts, irrespective of plant species or the extraction process, the variability of the ten *H. contortus* isolates was studied.

4.2. Variation of susceptibility between H. contortus exposed to A. pennatula or O. viciifolia extracts

The present study confirmed the existence of differences in suscept ibility towards polyphenol rich extracts between different *H. contortus* isolates (Table 2). The EC_{50} values obtained for each isolate allowed classifying isolates according to their different susceptibility. Thus, one or two isolates were classified as the most susceptible to each plant extract tested and all the other isolates evaluated were classified as resistant towards the extracts tested, showing different RR for each extract. The present results are consistent with evidence of differences in susceptibility obtained with eggs of the same ten *H. contortus* isolates used in the present study (Chan Pérez et al., 2016). That study also used acetone:water extracts of *A. pennatula* and *O. viciifolia* foliage.

It was evident that the degree of susceptibility of different isolates towards the *A. pennatula* extract was clearly different to that found for the *O. viciifolia* extract (Table 2), even when polyphenols were causing the inhibition of exsheathment in the case of both plant extracts. Thus, the different *H. contortus* isolates reacted differently when exposed to the plant secondary compounds present in each extract and resulted in a different ranking order for each extract (Table 2). The different EC_{50} reported for the *H. contortus* isolates exposed either to *A. pennatula* or *O. viciifolia* extracts might be associated to differences in quantity, quality or type of tannins (procyanidin or prodelphinidin) present in both extracts. Difference between extracts could also be explained by a synergism between tannins and other polyphenol compounds (Klongsiriwet et al., 2015) or the structure (oligomeric or polymeric) as both could increase AH activity (Molan et al., 2004; Brunet et al., 2008; Mechineni et al., 2014).

Difference in L_3 susceptibility reported for both extracts allowed to calculate a resistance ratio (RR) for each isolate exposed to each extract (Table 2). The RR should be considered as the relative expression of the phenotypic susceptibility towards polyphenols between different *H. contortus* isolates. No relationship was detected between RR and their geographical origin or their susceptibility towards commercial AHs. Contrary to previous studies, the evidence obtained here do not suggest that the tropical plant extract (*A. pennatula*) was less effective against *H. contortus* from hot tropical areas, or more effective against isolates from Europe, Oceania or temperate areas of America or Africa. Similarly, there was no evidence suggesting that the plant extract of the temperate plant (*O. viciifolia*) was more effective against isolates from tropical zones or less effective against the temperate isolates tested. Thus, it seems that the different RR of each isolate tested was the expression of the natural capacity to tolerate the plant secondary compounds contained in the extracts. Similar tolerance to xenobiotic materials has been observed for bacteria intrinsically resistant to antibiotics (Cox and Wright, 2013).

An important aspect found in this study was that both isolates from Yucatan (FMVZ and PARAISO), obtained from sheep and goats brows ing for many generations in the tropical deciduous forest of Yucatan, did not show the lowest susceptibility towards extracts compared to other isolates. A high RR was expected for Yucatan isolates due to the isolates' constant exposure to ruminant diets containing polyphenol rich plants in the tropical deciduous forest of Yucatan, México (González Pech et al., 2014, 2015). However, the PARAISO isolate was consistently less susceptible to both plant extracts tested. This same isolate has been classified as resistant to other acetone:water plant extracts, including leaves from tropical trees and plant by products (Vargas Magaña et al., 2014a, 2014b). Nevertheless, the current results obtained for the PARAISO isolate are still insufficient to confirm the existence of resistance due to selection pressure from browsing in the tropical forest.

Variation of susceptibility to polyphenolic compounds between isolates of GIN species might have been missed during the early evaluation of plant extracts using *in vitro* studies. It has to be remembered that the plant extract evaluation included the EC_{50} and respective 95% CI only recently. The latter allowed the identify differences in susceptibility for different *H. contortus* isolates and even different life stages. For instance Annonacea leaf extracts (Castañeda Ramírez et al., 2014), and acetone:water extracts from different tropical tree leaves and by products (Vargas Magaña et al., 2014a; Chan Pérez et al., 2016, Ortíz Ocampo et al., 2016Vargas Magaña et al., 2014aVar gas Magaña et al., 2014a; Chan Pérez et al., 2016, Ortíz Ocampo et al., 2016Vargas Magaña et al., 2014aVargas Magaña et al., 2014a; Chan Pérez et al., 2016, Ortíz Ocampo et al., 2016).

At present it is difficult to identify the mechanism by which the L_3 differ in their susceptibility to polyphenol rich extracts. Thus, it is important to explore if such variability is related to differences in the potential of extracts to interfere with specific enzymes related to the exsheathment process (i.e., lipases, aminopeptidases, serine and cy steine proteases) (Page et al., 2014), or differences in the capacity to block or compete with exsheathment factor receptors on surface of the sheath. As it was mentioned before, the polyphenols were identified as the main compounds responsible for the AH activity in the present study. Thus, it is important to confirm if the compounds contained in each extract differ in their capacity to bind to the structural proteins present in the different isolates evaluated. Such mechanism has also been proposed as an explanation for the difference in susceptibility in the egg hatching inhibition of *H. contortus* (Vargas Magaña et al., 2014a) and structural damages in eggs of *Cooperia* spp. (von Son de Fernex et al., 2015). The variation in susceptibility towards plant secondary compounds can help to identify the mechanisms of action of different plant metabolites and their interaction with receptors present in the nematode sheath leading to differences in their exsheathment process.

5. Conclusion

This study showed that *H. contortus* isolates originated from several geographical regions differed in their susceptibility towards the poly phenol rich plant extracts of *A. pennatula* or *O. viciifolia*. Although polyphenols were responsible for the AH activity of both acetone:water extracts tested, susceptibility of different *H. contortus* isolates ranked differently for each type of acetone:water extract. Thus, susceptibility to one polyphenol rich extract did not determine the susceptibility to the other polyphenol rich extract. The latter suggest that the different *H. contortus* isolates varied in their susceptibility to the polyphenols present in each extract evaluated.

Conflict of interest statement

None

Acknowledgments

This work was financed by CONACYT Mexico (Project CB 2013 01/221041) and is a result from collaborative project between France and México (CONACYT PCP No. 229330). J.I. Chan Pérez acknowledges the scholarship from CONACYT to undergo PhD studies. We thank Dr. Alfredo Cuellar Ordáz for the infective larvae of FESC UNAM and Dr. Pedro Mendoza de Gives for the CENID isolate. We equally thank Prof. M. Varady, Institute of Parasitology, Slovakia, for providing some of the *Haemonchus contortus* isolates.

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