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SHORT NOTE [NOTA CORTA]

IN VITRO ANTHELMINTIC EFFECT OF *Acacia gaumeri, Havardia albicans* AND QUEBRACHO TANNIN EXTRACTS ON A MEXICAN STRAIN OF *Haemonchus contortus* L_3 LARVAE

[EFECTO ANTIHELMÍNTICO *IN VITRO* DE LOS EXTRACTOS DE *Acacia gaumeri, Havardia albicans* Y QUEBRACHO SOBRE LARVAS L_3 DE UNA CEPA MEXICANA DE *Haemonchus contortus*]

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SUMMARY

The *in vitro* anthelmintic (AH) effect of *Acacia gaumeri* (AG), *Havardia albicans* (HA) and Quebracho tannin extracts on a Mexican strain of *Haemonchus contortus* L_3 larvae was evaluated. Water/acetone extracts of two tropical plants (AG and HA) and a commercial tannin preparation (*Schinopsis* sp, Quebracho) were screened to evaluate the *in vitro* AH effect using the larval migration inhibition (LMI) assay. The *Haemonchus contortus* L_3 larvae originated from a donor sheep (FES-Cuautitlán, UNAM). The concentration of condensed tannins in plants extract was 53.9 %, 73.1 % and 55.4% for AG, HA and Quebracho respectively. Clear AH effects were obtained with HA and Quebracho extracts with different concentrations (600, 1200, 1800 and 2400 µg/ml). Quebracho extract caused a reduction in larval migration which ranged from 15.5 to 25.9% as compared to PBS. The HA extract showed a stronger inhibition effect (30.0 to 53.1%). The AG extract did not inhibit larval migration at the concentrations used in this trial. These *in vitro* results suggest that HA have potential AH properties similar to those of Quebracho against a Mexican strain of *H. contortus*.

Keywords: *In vitro* anthelmintic effect, larval migration inhibition, *Acacia gaumeri*, *Havardia albicans*, *Schinopsis* sp, *Haemonchus contortus*

RESUMEN

El efecto antihelmíntico *in vitro* de los extractos acetonicos de *Acacia gaumeri* (AG), *Havardia albicans* (HA) y un preparado comercial de taninos (*Schinopsis sp*, Quebracho) sobre larvas L_3 de una cepa Mexicana de *Haemonchus contortus* fueron evaluados utilizando pruebas de inhibición de la migración larvaria (LMI). Las larvas L_3 de *Haemonchus contortus* provenían de un donador ovinos (FES-Cuautitlán, UNAM). El contenido de taninos condensados de los extractos de AG, HA y Quebracho, fue de 53.9 %, 73.1 % y 55.4 % respectivamente. Se observaron efectos AH con los extractos de HA y Quebracho con las diferentes concentaciones evaluadas (600, 1200, 1800 and 2400 µg/ml). El extracto de Quebracho causó una reducción en la migración larvaria que varió de 15.5 a 25.9 % en relación al PBS. Se observó un mayor efecto sobre la inhibición de la motilidad larvaria con extracto de HA (30.0 a 53.1 %). El extracto de AG no inhibió la migración larvaria con las diversas concentraciones evaluadas en este estudio. Los resultados *in vitro* sugieren que el extracto de HA posee potencialmente propiedades AH similares a aquellas del extracto de Quebracho sobre una cepa Mexicana de *H. contortus*.

Palabras clave: Efecto antihelmintico *in vitro*, inhibición de la migración larvaria, *Acacia gaumeri, Havardia albicans, Schinopsis sp., Haemonchus contortus.*

INTRODUCTION

The direct anthelmintic (AH) activity, attributed to phenolic compounds, specifically condensed tannins, is present in certain forages (Niezen *et al.*, 1995, Hoste *et al.*, 2006). The nematocidal activity of tannin extracts has been tested *in vitro* using legume forages or woody plants grown under temperate conditions

(Molan *et al.*, 2003, Paolini *et al.*, 2004). More recently, studies using extracts from tannin rich plants grown under tropical conditions showed an *in vitro* anthelmintic effect against L_3 larvae of *Haemonchus contortus* and *Trichostrongylus colubriformis* from temperate areas (Alonso-Díaz *et al.,* 2008a, 2008b). One of the tannin rich plants tested *in vitro* was tested *in vivo* with goat kids (Brunet et al., 2007).

Several species of the *Acacia* genus are recognized for their high content of condensed tannins (CT) (Bruneton, 2001). The AH effect of some species has been confirmed using *in vitro* (Max *et al.,* 2003; Alonso-Díaz et al., 2008a, 2008b) and *in vivo* tests (Cenci *et al.*, 2007, Kahiya *et al*., 2003). Some species of *Acacia* are present in Yucatan, Mexico. *Acacia gaumeri* is accepted by browsing sheep and goats (Hernández-Orduño *et al.*, 2008) making it a possible candidate for nematode management due to its content of CT (Ayala-Burgos *et al.*, 2006). *Havardia albicans*, another tropical tannin rich plant, has been used in the tannery industry (Arellano-Rodríguez *et al*., 2003). *Schinopsis sp.* (Quebracho) extracts have shown *in vitro* and *in vivo* AH activity against temperate nematodes (Athanasiadou *et al.*, 2001; Max *et al.,* 2005; Paolini *et al.*, 2003a,b). However, the information of the *in vitro* AH effect of tannin rich extracts on strains of nematodes from tropical regions is lacking. The aim of the study was to determine the *in vitro* AH effect of native tropical tanniniferous plant extracts (*A. gaumeri*, *H. albincans*) and Quebracho on *Haemonchus contortus* infective larvae (Mexican strain) to explore their potential use as nutraceutical plants.

MATERIAL AND METHODS

Collection area

The plant material was collected in the deciduous tropical forest of the north of Yucatan, Mexico (20º48' N and 89º42' W) in an area around the Faculty of Veterinary Medicine of the Universidad Autonoma de Yucatan (FMVZ-UADY). This work was carried out on May, 2007. The climate of the area is hot sub-humid tropical with summer rainfalls. Average temperature varies from 26 to 27.8 °C and annual rainfall ranges from 940 to 1100 mm (García, 1988).

Preparation of plant extracts

Fresh leaves of *H. albicans* and *A. gaumeri* were utilized. The leaves (250 g) from each plant were mixed separately with acetone:water (70:30 v/v) containing ascorbic acid (3 g l^{-1}). The mixture was sonicated during 20 minutes (Branson model 5510) and filtered with commercial filter paper (No. 50). Then, the mixture was washed five times with methylene chloride (1:1 ratio) to remove chlorophyll and lipids. Acetone was removed from the extract by vacuum assisted rotovaporation (<60 ˚C; Büchi model B-480). The aqueous fraction was freeze-dried (UL Standard 61010A, Labconco ®). The lyophilized extract was maintained in refrigeration until use (4 ºC).

Condensed tannin determination

To determinate CT content, the lyophilized extracts from *A. gaumeri*, *H. albicans* and Quebracho (Sigma-Aldrich Co. USA) were reconstituted with acetone:water (70:30 v/v). The quantification of CT was carried out with the Butanol HCL method (which measures proanthocyanidins) and was reported as anthocyanidin equivalent (Porter *et al.*, 1986). Also, the Vainilline Assay, which measures flavonoides and condensed tannins, was performed using catequin as standard (Price and Butler, 1977).

Biological activity

The biological activity was determined by a radial diffusion technique. In this technique each molecule of tannin migrates through the gel until it encounters free protein and precipitates (Hagerman, 1987). Agar was prepared with 1% agarose in acetate buffer, 1% bovine haemoglobine (Sigma-Aldrich Co., USA), and 1.2 mg of chloramphenicol (cloramfeni Ofteno; Sophia S.A. de C.V. Mexico) per 100 ml of agar. Agar pH was adjusted to 5.0. Ten ml of agar were placed in Petri dishes (10 cm diameter). On each Petri dish 5 wells were made in the agar (4 mm diameter each). The outer wells were used to place 15 µl of each reconstitute plant extract and Quebracho tannin and the centre well to place Resorcinol (Sigma-Aldrich® Inc. Germany) as standard. Samples were incubated for 24 hours at 25 ˚C. The images of the radial diffusions were digitalized and the precipitation areas were measured with computer software (GNU Image Manipulation Program 2.2). Protein precipitating power was expressed as astringency units relative to the standard. Each extract was assayed with 4 replicates.

Preparation of gastrointestinal nematode larvae

Larvae were cultured from faeces of a sheep with a mono-specific *H. contortus* infection. The *H. contortus* strain used for this trial originated from the Parasitology Laboratory FMVZ, FES-Cuautitlán, UNAM (Cuatitlán, México). Due to its origin (sheep from grazing systems), the strain used had not been previously exposed to the tannin rich plants as those used in this study. Faeces were collected from the donor sheep daily in the morning using plastic containers. Faeces were homogenized and moistened to achieve a crumbly consistency in a plastic container

with an aluminum lid. The faeces were incubated at room temperature for 7 to 8 days. This allowed the eggs of *H. contortus* to hatch and develop to the infective L_3 larvae. At the end of the incubation, the mixture was placed in Baerman's apparatus overnight to harvest the L_3 larvae. The larvae were sieved (250 mm) to remove faecal debris. Then, larvae were stored at 4 ˚C for later use.

Larval migration inhibition bioassay (LMI)

The inhibitory activity of the native tanniniferous plants and Quebracho extracts was evaluated with a larval migration inhibition (LMI) assay *in vitro* (Rabel *et al.*, 1994). This assay measures the percentage of L_3 larvae of *H. contortus* that fails to pass through the sieve relative to the larvae in control wells containing phosphate-buffer solution (PBS). A range of plant extract concentrations (600, 1200, 1800 and 2400 µg/ml diluted on PBS) were used. Four ml of larval solution concentrated at ~1000 L_3/ml were added to tubes containing four ml of either PBS, an anthelmintic control (levamisole 0.4%) or a range of plant extract concentrations for each plant species. All incubations were carried out for three hours at 24 ˚C. Thereafter, the L_3 were added with 4 ml of PBS and centrifuged (3500 rpm during 5 min). The supernatant was removed (4 ml). This procedure was repeated three times. Then, 800 µl of this solution were added to inserts equipped with a 20 µm mesh, positioned in a conical tube with the mesh just above PBS. Four replicates were run for each plant concentration as well for the PBS and the anthelmintic controls. As in other studies, the 20 µm mesh was selected in order to ensure that migration of larvae through the sieves was an active phenomenon. After three hours at room temperature, the inserts were retrieved and L_3 which had actively migrated through the mesh into the PBS below, were counted under a stereomicroscope at magnification 20x, based on a 10% aliquot.

Data calculation and statistical analyses

The larval migration (%) was determined according to the following equation:

$$\%LM = B/A * 100$$

Where *A* is the number of larvae deposited in the sieves in the wells, and *B* is the number of larvae migrating through sieves in wells.

The significance of differences among different treatments and the PBS control in each experiment was assessed in a complete randomized design using GLM (general linear models) procedures.

Larval migration inhibition was determined according to the equation reported by Rabel et al., (1994):

$$\% LMI = ((A-B)/A) * 100$$

Where *A* is the number of larvae migrating through the sieves in negative control wells (PBS), and *B* is the number of larvae migrating through sieves in the respective treatment wells (containing extracts). No statistical analysis was performed for these values.

RESULTS

Condensed tannins in the plant extracts

The results CT and their biological activity for extracts of *A. gaumeri*, *H. albicans* and Quebracho extract are shown in table 1. The highest quantity of CT was found in *H. albicans* and the lowest in *A. gaumeri* (Butanol-HCl and Vanillin assays). Quebracho extract showed the highest biological activity and *A. gaumeri* the lowest.

Larval migration inhibition bioassay

Percentage migration (%LM) in the PBS control group had means of 85.6%, 84.9% and 81.5% for the LMI assays of *A. gaumeri*, *H. albicans* and Quebracho extract respectively. Likewise, in the levamisol group the larval migration was 2.0%, 2.5% and 1.8% for the LMI assays of *A. gaumeri*, *H. albicans* and Quebracho extract respectively. The extracts of *H. albicans* and Quebracho caused a significant reduction on the larval migration compared to the PBS control ($P<0.05$). Both plant extracts caused inhibition of larval migration, relative to the respective PBS controls, at all the concentrations tested. The inhibition of larval migration caused by *H. albicans* extract was -53.1%, -35.6%, -48.7% and -30.0% for the concentrations 2400, 1800, 1200 and 600 µg/ml respectively. The inhibition of larval migration caused by Quebracho extract was -15.5%, -25.6%, -19.8% and -18.4% for the concentrations 2400, 1800, 1200 and 600 µg/ml respectively. The *A. gaumeri* extract did not cause a reduction in the larval migration of *H. contortus* at any concentrations used in this trial ($P>0.05$) (Table 2).

DISCUSSION

The legume forages naturally available in the tropical areas of the world may represent a viable source of nutrients. The value of these plants is not limited to their high protein contents in the fodder, a rich source of nitrogen for ruminants, but also to the potential role of some plant secondary compounds in the production and health of ruminants. The use of tannin rich plants as nutraceuticals has been explored recently.

Table 1. Condensed tannin contents and the biological activity of taniniferous plants extracts.

Plant extract	Condensed tannins (g/100g extract)		Biological activity*
	Butanol- HCl	Vainillin	
Acacia gaumeri	53.91	15.03	6.28
Havardia albicans	73.10	173.21	19.66
Quebracho tannin	55.39	42.90	36.41

* Measured as precipitation per gram of extract relative to resorcinol standard.

Table 2. Percentage of migration of *Haemonchus contortus* infective larvae exposed to different concentrations of tanniniferous plants extracts using the larval migration inhibition (LMI) assay.

Treatments	PBS	2400µg/ml	1800 µg/ml	1200 µg/ml	600 µg/ml
Acacia gaumeri	85.57±13.3	87.0±7.2	77.5±7.3	97.5±4.4	96.0±4.5
Havardia albicans	84.88±1.8	39.8±12.0 *	54.7± 5.3*	43.6± 12.9 *	59.4± 3.9*
Quebracho	81.54± 4.2	68.9±12.0 *	60.4±3.1 *	65.4± 4.4*	66.5± 5.7*

* Values in the respective rows are statistically different to those of the PBS values.

The first step has been to demonstrate that sheep and goats can consume large quantities of tannin rich fodder (Alonso-Díaz *et al.*, 2008c; 2008d). The tannin rich plants used in the present trial have shown to be well accepted/consumed by sheep and goats (Hernandez-Orduño *et al.*, 2008). If these legume fodders are consumed, then the second step is to screen for an *in vitro* AH effect. *In vitro* screening of the AH effect of CT in tannin rich forages is an important step in the identification of potential candidates for future use against GIN in ruminants. *In vitro* tests can help to envisage nutraceutical alternatives since most *in vitro* results tend to be confirmed by *in vivo* results (Niezen *et al.*, 1995, 1998; Molan *et al*., 2000a; Paolini, 2003; Hoste *et al.*, 2008).This two successive steps have been shown using tannins contained in temperate forages (Hoste *et al*., 2006). More recently, an *in vitro* AH effect of tannins contained in tropical plants of Yucatan was demonstrated using infective larvae of *H. contortus* (Alonso-Díaz *et al.*, 2008a) and *Trichostrongylus colubriformis* (Alonso-Díaz *et al.*, 2008b) from temperate countries.

The current trial is the first successful attempt to identify an *in vitro* AH effect of an extract obtained from a tannin rich plant (*H. albicans*) from Yucatan, Mexico, on *H. contortus* L_3 larvae originated from Mexico (FES-Cuautitlan strain, UNAM). The AH results obtained are consistent with the high content of CT found in *H. albicans* and the high biological activity of its extracts. However, an inhibitor of tannins such as PEG or PVPP should be used in future trials to confirm the role of CT on the *in vitro* AH effect of the extract, as it has been performed elsewhere (Alonso-Díaz et al., 2008a, 2008b).
The extract of *A. gaumeri* did not show an *in vitro* AH effect at the concentrations used in the experiment. Although this plant extract had a considerable quantity of CT (according to the butanol-HCl technique), the extract showed a low biological activity. The later may help to explain the lack of an *in vitro* AH effect. However, it must be considered that the lack of *in vitro* AH effect with the LMI assay should not be considered the final result. Alonso-Díaz *et al*. (2008a, 2008b) reported a discrepancy between *in vitro* results obtained with the LMI and the exsheatment assays using *Piscidia piscipula* extracts against *H. contortus* and *T. colubriformis*.

The two native plants chosen for the present trial were selected for their widespread use as browsing plants by goats and sheep. These plants can be consumed by sheep and goats without evidence of toxicity (Hernandez-Orduño *et al*., 2008). Also, these plants are widely distributed in the Yucatan peninsula (Flores-Guido, 2001). In the case of *H. albicans* the choice was also justified because it has been reported as a plant used for tannery in Yucatan (Arellano-Rodríguez *et al.*, 2003), in a similar manner to the Quebracho in other regions of the world. The *H. albicans* and *A. gaumeri* belong to the same botanical family (*Fabaceae*). Plants of this family (*i.e. Peltophorum africanum* Sond.) have been reported as inhibitors of egg hatching and larval development (Bizimenyera *et al.*, 2006). The *Acacia* genus has also been associated with decreased nematode egg excretion (Cenci *et al*., 2007). Similarly, the extracts of *A. pennatula* another tanniniferous plant from Yucatan, Mexico, showed an *in vitro* AH activity against French strains of *H. contortus* and *T. colubriformis* using the LMI assay (Alonzo-Díaz *et al.*, 2008a, 2008b). The *A. pennatula* extract used by those authors contained six times more condensed tannins

and twice as much biological activity than the *A. gaumeri* used in the present study. Therefore, the AH effect of different *Acacia* species should not be generalized to plants of the same genera before a careful evaluation of both secondary compound content and its biological activity as shown by Kahiya *et al*., 2003).

The present study also showed the first *in vitro* AH effect of Quebracho extracts on a Mexican strain of *H. contortus* infective larvae. To our knowledge, this is the first report using the LMI assay to test the AH effect of Quebracho against *H. contortus*. The results of the present study showed a reduction in the migration ability of larvae when compared to those incubated in the PBS control. These are consistent with results obtained with other *H. contortus* strains both, *in vitro* and *in vivo* (Athanasiadou *et al*., 2001; Max *et al.*, 2005; Paolini *et al.*, 2003a,b). However, no direct comparison can be made between experiments because results have been obtained with different techniques/assays.

In the present trial the *H. contortus* L_3 used were younger than two weeks of age. Most LMI tests have been developed with larvae older than one month of age. These age of larvae is used elsewhere (Molan *et al.*, 2000; Paolini *et al.,* 2003a) as they are supposed to be related to infectivity. However, trials performed in Mexico showed that *H. contortus* infective larvae of less than two weeks are clearly infective (Ojeda-Robertos *et al.*, 2006). Further studies need to determine if the *in vitro* AH activity of CT from plant extracts increase their activity against older *H. contortus* larvae as reported for *T. colubriformis* by Molan *et al.* (2000b).

The mechanisms of action of the AH effect of CT from tannin rich plants are still under investigation. The capacity of CT to bind with proteins (Dixon *et al.*, 2005), in particular to proline rich proteins, seems to be associated with the AH effect. Proline rich proteins are also present in the cuticle of parasitic nematodes such as adult *H. contortus* and CT may affect their biology (Hoste *et al.*, 2006). Results of Brunet and Hoste (2006) confirmed that the number of free hydroxyl groups of the CT is implicated in the impairment of exsheathment of *H. contortus* and *T. colubriformis*. The later has been further tested *in vitro* and *in vivo* in Mexico. Firstly, extracts of *Lysiloma latisilliquum*, a tannin rich fodder, showed a clear reduction in the exsheatment *H. contortus* and *T. colubriformis* infective larvae *in vitro*. Then, *in vivo* results obtained with goats fed *L. latisiliquum,* showed a reduction of *H. contortus* and *T. colubriformis* larval establishment (Brunet *et al*., 2007).

CONCLUSION

This experiment demonstrated that *H. albicans* extracts have an AH effect on *H. contortus* infective larvae which originated from the highlands of Mexico. Also, an AH effect of Quebracho extract on *H. contortus* was confirmed using the LMI test. No effect on larval migration was observed for the *A. gaumeri* extract. The role of tannins must be confirmed *in vitro* and *in vivo* before practical application in farm condition.

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