

Anthelmintic activity of *Leucaena leucocephala* pod on gastrointestinal nematodes of sheep (*in vitro*)

Actividad antihelmíntica de la vaina de *Leucaena leucocephala* sobre nematodos gastrointestinales de ovinos (*in vitro*)

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ABSTRACT

Due to the resistance that parasites have acquired to conventional drugs, new strategies are currently being sought to control the cycle of parasites associated with health problems in animal production. The objective of the study was to determine the anthelmintic activity of *Leucaena leucocephala* pod (LLP) hydroalcoholic extract on gastrointestinal nematodes of sheep. A hydroalcoholic extract of LLP was prepared and the concentrations 50, 25, 12.5 and 6.25 mg/mL were evaluated to determine the activity on eggs hatching inhibition of gastrointestinal nematodes in sheep and the mortality on larvae of nematodes (L3). It was determined that the hydroalcoholic extract of LLP at 50 mg / mL inhibited the eggs hatching of nematode (20%) and at 6.25 mg/mL killed 22% of the larvae of nematodes (L3). The hydroalcoholic extract of LLP contains active metabolites that act in inhibition eggs hatching on field nematodes and on infective L3. The hydroalcoholic extract of LLP can be an unconventional alternative for the prevention and control of parasites in small ruminant at field level.

Keywords: Hydroalcoholic extract, *Leucaena leucocephala* pods, anthelmintic, field nematodes.

RESUMEN

Debido a la resistencia que han adquirido a fármacos convencionales, actualmente se buscan estrategias novedosas para controlar el ciclo de los parásitos asociados a problemas sanitarios en animales de producción. El objetivo del estudio fue determinar la actividad antihelmíntica del extracto hidroalcohólico de vaina de *Leucaena leucocephala* (VLL) sobre nematodos gastrointestinales de ovinos. Se preparó un extracto hidroalcohólico de VLL y se evaluaron las concentraciones 50, 25, 12.5 y 6.25 mg/mL, para determinar la actividad sobre la inhibición de la eclosión de los huevos de los nematodos gastrointestinales de ovinos y la mortalidad sobre larvas L3 de los nematodos. Se determinó que el extracto hidroalcohólico de VLL a una concentración de 50 mg/mL inhibe la eclosión de los huevos de los nematodos en un 20% y a 6.25 mg/mL mató al 22% de las larvas L3 de los nematodos. El extracto hidroalcohólico de VLL contiene metabolitos activos que actúan en la inhibición de la eclosión de huevos de nematodos de campo y sobre la larva infectiva L3. El extracto hidroalcohólico de VLL puede ser una alternativa no convencional para la prevención y control de las parasitosis de pequeños ruminantes a nivel de campo.

Palabras clave: Extracto hidroalcohólico, vainas de *Leucaena leucocephala*, antihelmíntico, nematodos de campo.

INTRODUCTION

The sheep breeding is one of the agricultural activities of greater economic importance in the Mexican Republic, due to the acceptance of its meat and by-products by the population; the production system that predominates in this species is the extensive, system in which parasitosis are frequent, because there are species of gastrointestinal nematodes that comply with their biological cycle in pastures ([González et al., 2012](#); [Roeber et al., 2013](#)).

Some of the nematodes that affect the small ruminants with higher prevalence in Mexico are: *Haemonchus contortus*, *Trichostrongylus colubriformis*, *Cooperia curticei* and *Oesophagostomum columbianum* ([González et al., 2012](#)). The parasitosis directly affect the economy of sheep producers, because they cause losses associated with reduced growth rates in young animals, low body conditions, reduced fertility, increased susceptibility to diseases of different origins and increased mortality ([Hernández-Alvarado et al., 2018](#); [Zapata et al., 2016](#)).

Currently control of gastrointestinal nematodes is a challenge in the extensive production systems of small ruminants, because some populations of gastrointestinal nematodes have developed resistance to commercial anthelmintics ([Torres-Costa et al., 2012](#); Muchiut et al., 2018). An alternative control is through the use of secondary metabolites extracted from plants, such as terpenes, alkaloids, saponins, anthraquinones and tannins; it has been shown that some of these compounds are involved in vital functions of the nematodes such as mobility, nutrition and possibly their reproduction ([Hernández-Alvarado et al., 2018](#)).

Leucaena leucocephala is a tropical tree, native to Central America, with medicinal properties; ranging from the control of stomach diseases to contraceptive activity ([Nehdi et al., 2014](#)). Studies conducted by [Ademola and Idowu in 2006](#), determined the nematicidal effect of *Leucaena leucocephala* seeds on the infective larva of *Haemonchus contortus* ([Ademola and Idowu, 2006](#)); regarding this, Hernández and collaborators determined the anthelmintic activity of the hydroalcoholic extract of *Leucaena leucocephala*, on nematodes of small ruminants ([Hernández et al., 2014](#)). The objective of the study was to determine the anthelmintic activity of the hydroalcoholic extract of *Leucaena leucocephala* pod, on some gastrointestinal nematodes of sheep.

MATERIAL AND METHODS

Obtaining plant material

One kilogram of *Leucaena leucocephala* (VLL) pods was collected in the municipality of Soledad de Graciano Sánchez, located at coordinates 100°56 'west longitude and 22°11' north latitude in San Luis Potosí State, Mexico. The pods were collected in the month of July of 2017, during the morning and of at least 10 trees.

Obtaining the extract

The pods of *Leucaena leucocephala* were dried in the shade at room temperature, after the drying process macerated and mixed 240 g of the dried pod with 2000 ml of a hydroalcoholic solution (30% methanol and 70% distilled water); filtration, solvent removal and concentration of secondary metabolites were performed according to the methodology described by Rivero *et al.* ([Rivero-Pérez *et al.*, 2016](#)).

Dilutions of the extract were made with sterile distilled water to obtain concentrations of 50, 25, 12.5 and 6.25 mg/ml, according to the methodology described by Olmedo-Juárez *et al.* ([Olmedo-Juárez *et al.* 2017a](#)); which were evaluated to determine the anthelmintic activity on the inhibition of egg hatching and mortality of larvae of gastrointestinal nematodes *in vitro*.

Inhibition of the hatching of nematode eggs

20 samples were randomly collected, with approximately 30 grams of sheep feces (Suffolk-Hampshire), directly from the rectum of each animal, from a sheep production unit located in the Tepeapulco municipality in the Hidalgo State. The samples were identified and transported at refrigeration temperature, in the laboratory they were carried out the flotation techniques and Mac Master, to identify the presence of gastrointestinal nematodes and the number of eggs per gram of feces, according to the methodology described by Rivero-Pérez in 2018. ([Rivero-Pérez *et al.*, 2018](#)).

Egg recovery from gastrointestinal nematodes

The samples with more than 1000 eggs per gram of feces were mixed vigorously to later take approximately 30 grams of feces that were washed in sieves of 200, 100, 75 and 37 microns; To recover, concentrate and obtain a clean egg solution, the methodology described by von Son-de Fernex and collaborators was followed in 2015 ([von Son-de Fernex *et al.*, 2015](#)). The concentration of clean eggs was determined and adjusted between 150-200 eggs in 50 µl.

Evaluation of the inhibition of egg hatching of nematodes

In a 96-well plate, 50 µl of a previously adjusted solution, plus 50 µl of the extract were placed at the different concentrations (50, 25, 12.5 and 6.25 mg/ml); Ivermectin (5 mg/ml) was used as a positive control and distilled water as a negative control. The plate was incubated at 30 °C for 48 hours; for the plate reading, 10 aliquots of 10 µl were observed in the microscope with the 4x objective of each well and the number of eggs and larvae in each aliquot was determined. To determine the percentage of inhibition of egg hatching, it was used the following formula described by Castillo-Miter *et al.* ([Castillo-Miter., *et al.* 2017](#)):

$$\% \text{ of inhibition of hatching} = \frac{L1}{L1 + \text{Egg}} * 100$$

Where L1 is equal to larva 1.

Evaluation of activity on L3 larvae of nematodes

Recovery of L3 larvae of nematodes

With the remaining stool samples of those animals that presented more than 1000 eggs per gram of feces, a stool culture was performed; which consists of mixing the feces with distilled water and polyurethane foam (1.5 x 1.5x 0.5 cm); to later incubate them for 10 days at room temperature (15 to 20 °C). After the incubation time, it was proceeded to mount the Bearman technique to recover the L3 larvae of gastrointestinal nematodes, which were washed with saturated saline solution, until the greater amount of organic matter was eliminated.

The cleaned larvae were unsheathed in a 3% sodium hypochlorite solution; to remove the remains of sodium hypochlorite, the larvae were washed 3 times with 3 ml of distilled water. The concentration of larvae was determined and adjusted between 150-200 larvae in 50 µl.

Larvicidal evaluation

In a 96-well plate, 50 µl of a previously adjusted solution, plus 50 µl of the extract were placed at the different concentrations (50, 25, 12.5 and 6.25 mg / ml); Ivermectin (5mg / ml) was used as a positive control and distilled water as a negative control. It was incubated at 30 ° C for 48 hours. For the plate reading, 10 aliquots of 10 µl were observed in the microscope with the 4X objective of each well, and the number of live and dead larvae was determined. The data obtained were used to determine the percentage of mortality, and the following formula was used:

$$\% \text{ of mortality} = \frac{\text{Killed larvae} \times 100}{\text{Total larvae}}$$

Statistical analysis

A completely randomized design was used, the data obtained were analyzed by means of an analysis of variance and a comparison of means by Tukey at a confidence level of 95%, the statistical package [SAS V9.0](#).

RESULTS

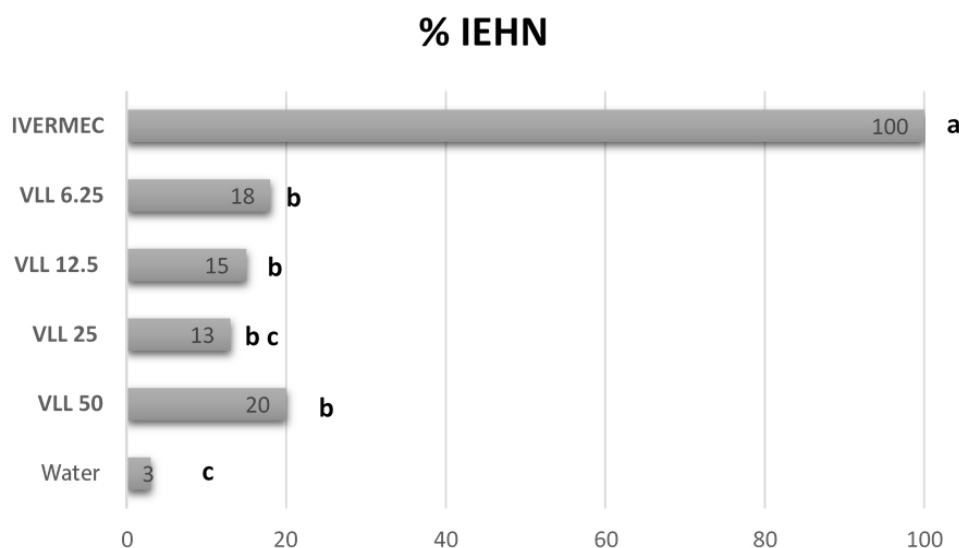
The main gastrointestinal nematodes observed in the samples of the sheep were: *Haemonchus contortus*, *Cooperia spp*, *Ostertagia spp*, *Chabertia spp*, *Moniezia spp* and *Strongyloides spp*; being the most frequent *Haemonchus contortus* and *Cooperia spp*. By means of the Mc Mater technique, an average of 1,795 eggs per gram of feces was determined in the samples collected.

Inhibition of egg hatching of nematodes

A percentage of hatching inhibition of 20, 13, 15, and 18 was determined; for the concentrations evaluated 50, 25 and 12.5, 6.25 mg/ml respectively. According to the statistical analysis, no significant differences were observed between them ($P = 0.0001$), hatching of the eggs was 100% inhibited by Ivermectin, and control with water inhibited hatching by 3%, as observed in the [Figure 1](#).

Evaluation of activity on L3 larvae of nematodes

A percentage of larval mortality of identified gastrointestinal nematodes of 10, 16, 20 and 22 was determined; and for the tested concentrations 50, 25 and 12.5, 6.25 mg/ml respectively. No significant statistical differences were observed ($P = 0.0001$) between concentrations 25 and 12.5, 6.25 mg/ml; the concentration of 50 mg/ml, did not show significant statistical differences with the water control and the concentration of 50 mg/ml, as observed in [Table 1](#). For its part, the Ivermectin at a concentration of 5 mg/ml, killed to 97% of the larvae.



%IEHN; percentage of Inhibition of the hatching of nematode eggs

VLL; pod hydroalcoholic extract *Leucaena leucocephala*

IVERMEC; Ivermectin to 5 mg/mL

Different literals in the bars indicate significant statistical differences ($P \leq 0.05$)

Figure 1. Percentage of inhibition of egg hatching of gastrointestinal nematodes of sheep with the use of hydroalcoholic extract of *Leucaena leucocephala* pod.

Table 1. Percentage of mortality of larvae of gastrointestinal nematodes of sheep with the application of hydroalcoholic extract of *Leucaena leucocephala* pod.

Treatment	Concentration	% Mortality $\pm$ DE
Water	0	5 ^d (± 1.7)
VLL	50 mg/mL	10 ^{cd} (± 1.6)
VLL	25 mg/mL	16 ^{bc} (± 1.5)
VLL	12.5 mg/mL	20 ^b (± 2.0)
VLL	6.25 mg/mL	22 ^b (± 0.9)
Ivermectin	5 mg/mL	98 ^a (± 1.5)

VLL, hydroalcoholic extract of *Leucaena leucocephala* pod

Different literals indicate significant statistical differences ($P \leq 0.05$)

DE; Standard deviation

DISCUSSION

The gastrointestinal nematodes identified in the present study correspond to those identified in the study of the prevalence of gastrointestinal parasites in sheep slaughtered in a Tabasco, Mexico; performed by [González et al., 2012](#), which were associated with economic losses in the production of small ruminants from Colombia ([Zapata et al., 2016](#)).

The arboreal *Leucaena leucocephala*, has been studied due to its pharmacological potential by different researchers, in the case of anthelmintic activity against gastrointestinal nematodes of small ruminants, different results have been obtained; on the other hand Hernández and collaborators evaluated in an *in vivo* study the *Leucaena leucocephala* extract at a dose of 30 ml per day, determining a reduction of 54% in the elimination of eggs and adult parasites in Katahdin-Pelibuey lambs ([Hernández et al., 2014](#)).

However, in the present study the hydroalcoholic extract of the *Leucaena leucocephala* pod at a concentration of 50 mg/ml, inhibits the hatching of 20% of the eggs; results that do not coincide with what was published with [Ademola and Idowu \(Ademola and Idowu, 2006\)](#), who determined that the aqueous extract of *Leucaena leucocephala* seeds does not affect the hatching of *Haemonchus contortus* eggs; coinciding only in that the extract did affect the viability of larvae L3, activity that was dependent on the evaluated concentration. In this regard, at a concentration of 6.25 mg / ml, the best effect against L3 larvae was observed, with a mortality of 22%; surpassing similar studies, who report a larvicidal activity at a concentration of 100 mg / ml of *Acacia cochliacantha* leaf extract, against the nematodes *Haemonchus contortus* (25%), *Cooperia punctata* (8.5%) and *Haemonchus placei* (16%) ([Olmedo-Juárez., et al 2017b](#)).

On the other hand Von Son-de Fermex and collaborators performed an elucidation of the leaves of *Leucaena leucocephala*, managing to isolate and identify a metabolite with activity against *Cooperia spp* at a concentration of 0.06 mg / ml ([von Son-de Fernex et al., 2015](#)). According to the aforementioned, it is suggested to perform an elucidation of the hydroalcoholic pod extract of *Leucaena leucocephala* to isolate and identify the molecule or molecules responsible for the activity, against the inhibition of hatching of eggs and larval mortality against nematodes of field.

Regarding the above, Cala and collaborators mention that it is necessary to search for novel strategies to eliminate and control the cycle of parasites associated with health problems in production species such as sheep, which are accessible in terms of economy, so that they can be used by producers in rural areas with low resources ([Cala et al., 2012](#)).

The evaluation of the anthelmintic resistance was not part of the objective of the present study, however, it was determined that the Ivermectin at a concentration of 5mg / ml, inhibits 100% the hatching of eggs and eliminates 98% of the infecting larvae of the nematodes of field evaluated in the present investigation.

CONCLUSION

The hydroalcoholic extract of *Leucaena leucocephala* pod acts by inhibiting the hatching of eggs of field nematodes and affecting the viability of infective larvae L3. The evaluated extract can be an unconventional alternative for the prevention and control of the parasitosis cycle of small ruminants at the field level.

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