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Effect of *Leucaena leucocephala* leaves hydroalcoholic extract over the hatching of *Haemonchus contortus in vitro*

Efecto del extracto hidroalcohólico de hojas de *Leucaena leucocephala* sobre la eclosión de *Haemonchus contortus in vitro*

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ABSTRACT

Haemonchus contortus (HC) is a gastrointestinal nematode of worldwide importance due to the economic losses it causes directly and indirectly in sheep production. Plant extracts rich in secondary metabolites have been proposed as an alternative method of gastrointestinal nematode control in sheep and goats, as they fulfill their biological function and a sustainable environmental accompaniment. The objective of the present experiment was to determine the effect of the hydroalcoholic extract of *Leucaena leucocephala* leaves (HELL) on the hatching of *Haemonchus contortus in vitro.* The extract was obtained by the maceration technique and was subjected to qualitative phytochemical characterization, in which instaurations, phenolic oxydryls, lactones, flavonols, floratanins, coumarins, saponins and aromatic compounds were identified. Concentrations of 100, 90, 80, 70, 60 and 50 mg/mL of the extract were evaluated on the inhibition of egg hatching (IEH) of HC. The hydroalcoholic extract of *L. leucocephala* showed 71% IEH at 100 mg/mL, an EC_{50} and EC_{90} of 52.22 and 302 mg/mL, respectively. The hydroalcoholic extract of *L. leucocephala* leaves contains bioactive compounds (phenolics, terpenes and flavonoids) that inhibit HC egg hatching with dose-dependent effect. Therefore, HELL could be considered as an alternative control on this nematode, however, it is necessary to determine the specific metabolite that confers the activity, determine its toxicity and perform *in vivo* trials.

Keywords: *Leucaena leucocephala*, hydroalcoholic extract, ovicidal activity, *Haemonchus contortus.*

RESUMEN

Haemonchus contortus (HC) es un nematodo gastrointestinal de importancia mundial debido a las pérdidas económicas que causa de forma directa e indirecta en la producción ovina. Los extractos de plantas ricos en metabolitos secundarios se han propuesto como un método alternativo de control de nematodos gastrointestinales en ovinos y caprinos, ya que cumplen con su función biológica y un acompañamiento ambiental sustentable. El objetivo del presente experimento fue determinar el efecto del extracto hidroalcohólico de hojas de *Leucaena leucocephala* (EHLL) sobre la eclosión de *Haemonchus contortus in vitro*. El extracto se obtuvo mediante la técnica de maceración y fue sometido a una caracterización fitoquímica cualitativa, en la cual se identificaron instauraciones, oxidrilos fenólicos, lactonas, flavonoles, florataninos, cumarinas, saponinas y compuestos aromáticos. Se evaluaron concentraciones de 100, 90, 80, 70, 60 y 50 mg/mL del extracto sobre la inhibición de la eclosión de huevo (IEH) de *Haemonchus contortus*. El extracto hidroalcohólico de *L. leucocephala* mostró 71% de IEH a 100 mg/mL, una CE_{50} y CE_{90} de 52.22 y 302 mg/mL respectivamente. El extracto hidroalcohólico de hojas de *Leucaena leucocephala* contiene compuestos bioactivos (fenólicos, terpenos y flavonoides) que inhiben la eclosión de huevos de HC con efecto dosis dependiente. Por lo tanto, el EHLL podría ser considerado como una alternativa de control sobre este nematodo, sin embargo, es necesario determinar el metabolito especifico que confiere la actividad, determinar su toxicidad y realizar ensayos *in vivo*.

Palabras clave: *Leucaena leucocephala*, extracto hidroalcohólico, actividad ovicida, *Haemonchus contortus*

INTRODUCTION

The production of small ruminants in the world is limited by agro-meteorological, economic and sanitary factors, associated with the presence of microorganisms that cause infectious diseases. Parasitic diseases are a health problem, which mainly affects extensive small ruminant production systems; causing direct economic losses such as animal death, and indirect losses due to the decrease in productive and reproductive parameters (Pérez-Pérez *et al.,* 2014).

Hemonchosis is a frequent parasitic disease in small ruminants, the etiological agent is HC. HC is classified as a hematophagous nematode with tropism for the abomasum, where it attaches to feed. This parasite is associated with a picture of anemia and gastritis, which depending on the number of parasites and severity of clinical manifestations can cause death of adult and neonatal sheep (Kumarasingha *et al.,* 2016).

The control of parasitosis is based on the administration of anthelmintics of synthetic chemical origin, including benzimidazole derivatives, macrocyclic lactones, aminocetonitrile monepantel derivatives and imidazothiazoles (Geary *et al.,* 2015). In this regard, the use of commercial anthelmintics once had promising results for livestock; however, irrational and inappropriate use has generated the development of nematode populations with resistance to anthelmintics; in addition, their ecological impact has been reported, by affecting non-target populations and in terms of food safety, due to the presence of residues of these molecules in products and by-products of animal origin (Zajíčková *et al.,* 2020).

Due to the aforementioned problems, several strategies have been implemented in Integrated Parasite Control (IPC); in this way, the aim is to reduce drug use of chemical origin to mitigate the negative impact on the environment, in addition to offering producers control alternatives that allow them to reduce production costs (Pérez-Pérez *et al.,* 2014).

With this approach, the use of plant extracts and their metabolites have been proposed as one of the most promising alternative methods to prevent and control diseases associated with the presence of some nematodes in ruminants, given the different biological activities that have been reported in plant extracts, including anthelmintic activity (Kotze & Prichard, 2016).

The anthelmintic potential of plants has been associated with their content of secondary metabolites, such as terpenes (25,000 types), alkaloids (12,000 types) and phenolic compounds (8,000 types); of which diverse and possible mechanisms of action have been described according to their chemical structure and the bonds that could be formed with the structural molecules of the parasite egg or larva (Zajíčková *et al.,* 2020).

The mechanism of action of these secondary metabolites is unclear; however it has been hypothesized that they could cause from paralysis and/or larval mortality, interfere with larval development, inhibit egg hatching and interfere with female fertilization (Heeger *et al.,* 2017).

Several results have been obtained with plant extracts evidencing the anthelmintic potential that secondary metabolites possess against some nematodes of importance in ruminants, both *in vitro* and *in vivo* models. Under this premise, studies have been conducted with hydroalcoholic extracts obtained from the fruit and leaves of *Caesalpinia coriaria*, which inhibit the hatching of *H. contortus* and *H. placei* at 25 mg/mL as reported by (Rojo-Rubio *et al.,* 2019).

On the other hand, there is evidence of the evaluation performed by (Castillo-Mitre *et al.,* 2017) who evaluated the hydroalcoholic extract of *Acacia cochliacantha* leaves and reported the inhibition of 98 to 100 % the hatching of *Haemonchus contortus* eggs at concentrations of 0.62 to 1.56 mg/mL respectively; activity that was associated by the authors to the presence of caffeic and coumaric acid derivatives present in the extract.

In the same sense, (Von Son-de Fernex *et al.,* 2015) determined the ovicidal effect of the extract, obtained from *Leucaena leucocephala* leaves, on *Cooperia* spp. and identified quercetin and caffeic acid as the compounds responsible for this effect. With this, they suggest the possibility of a similar effect on other species of nematodes such as *Haemonchus contortus*.

In this regard, (Hernandez *et al.,* 2014) conducted an *in vivo* trial in growing lambs, which were administered 30 mL of *Leucaena leucocephala* extract for 63 days, observing a decrease in the parasite load of 54%; however, authors did not determine the mechanism of action of this effect.

Accordingly, the present study aimed to determine the effect of the hydroalcoholic extract of *Leucaena leucocephala* leaves on the hatching of *Haemonchus contortus in vitro*.

MATERIAL AND METHODS

Plant Material

Leucaena leucocephala leaves were collected in Tejupilco municipality, State of Mexico (18°54′N 100°09′W). Trees were on average 5 years old and 4 m tall and diameter at breast height (DBH) of 23 cm, collecting only the plant material from the canopy. For plant identification, the herbarium of the National Autonomous University of Mexico (UNAM) was consulted and the samples were verified as *Leucaena leucocephala* (Lam.) de Wit (IBUNAM:MEXU:1362417).

Extract preparation

Leucaena leucocephala leaves were dried in the shade at room temperature. 1000 g were macerated in 800 mL of a hydroalcoholic solution (30% methanol and 70% water) and allowed to stand for 72 hours. After the resting time, the extract was filtered using filter paper (Whatman® Qualitative Filter Paper, Grade 1) and then solvents were eliminated under reduced pressure in a rotary evaporator (Büchi-Brand), obtaining a yield of 10 grams. The above was performed according to the methodology described by (González-Alamilla *et al.,* 2019). A stock solution was prepared at a concentration of 400 mg/mL of distilled water, a solution from which the different concentrations were prepared as described by (Marie-Magdeleine *et al.,* 2010). Concentrations evaluated were: 100, 90, 80, 70, 60 and 50 mg/mL.

Qualitative phytochemical characterization of the extract

The qualitative characterization of the extract was performed as described by (González-Alamilla *et al.,* 2019; Morales-Ubaldo *et al.,* 2020). Tests used to determine the presence or absence of the active compounds were: $KMnO_4$ test (for unsaturations), $FeCl_3$ (for phenolic oxydryls, plant tannins), Liebermann-Burchard (for sterols and triterpenes), Salkowski (for sterols), coumarin test, Baljet (for sesquiterpenlactones), H_2SO_4 (for flavonoids), Shinoda (for flavonoids), Dragendorff (for alkaloids), tannin test, floratanin, steroids, shaking and bicarbonate (for saponins). For each test, 500 mg of the extract were used.

Egg Hatch Inhibition Test (EHI)

To obtain *Haemonchus contortus* eggs, a Hampshire breed sheep (3 months old and weighing 37 kg live weight) clinically healthy and free of gastrointestinal nematodes, was infested with L3 of HC strain INIFAP (350 larvae/kg/PV). The donor sheep were kept under standard care, welfare and unnecessary suffering regulations, according to the current legislation of the Mexican Official Standard NOM-062-ZOO-1999. Twenty-one days after infestation, fecal samples were collected and eggs per gram of feces (EPG) were quantified using the Mac Master technique described by (Rivero-Perez *et al.,* 2018).
For the recovery of HC eggs, the methodology described by (von Son-de Fernex *et al.,* 2015) was used, 30 grams of feces were washed with distilled water on 200, 100, 75 and 37 µm sieves. The material retained on the 37 µm sieve was washed with 6 mL of saturated saline and centrifuged at 3,000 rpm/ 3 min. To obtain eggs free of organic matter and saline, the sediment was washed 3 times with distilled water, until a clean egg solution was obtained for bioassay set-up. In a 96-well plate, 50 µL of clean egg solution (distilled water) were added with approximately 150 eggs plus 50 µL of each concentration to be evaluated; with four replicates, 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 h in a humid chamber; after incubation, the number of unhatched eggs and L1 larvae per well was quantified. Finally, the percentage of hatching inhibition (PHI) was calculated (Bizimenyera *et al.,* 2006).

Statistical analysis

Data were analyzed by ANOVA and a Tukey mean comparison at 95% confidence level. The effective concentrations that inhibited 50% (EC_{50}) and 90% (EC_{90}) of HC egg hatching were calculated using a PROBIT analysis in the SAS 9.0 statistical package.

RESULTS AND DISCUSSION

Qualitative phytochemical characterization indicated the presence of unsaturations (reduction to manganese dioxide), which are associated with compounds with ethylene bonds or compounds with two hydroxyl groups on adjacent carbons. A positive result was observed for phenolic oxydryls, which indicate the presence of hormones, pigments or essential oils. The presence of coumarins from the cycloaddition of siccaemic acid was also determined, as well as lactones, product of the union of an alcohol with a carboxylic acid in the same molecule. The positive reaction in the Salkowski test, indicative of the presence of sterols and triterpenes. The presence of flavonols and flavonones was also determined (Azcón-Bieto & Talón, 2003).

The results concerning the tests for chalcones, quinones, Shinoda, sesquiterpenlactones, agitation and bicarbonate, Lieberman-Buchard, tannins and steroids, were negative. The absence of these compounds can be associated with the plant's own characteristics, environmental conditions, such as temperature, soil type, solar radiation and humidity; as well as factors of the extraction process, such as the polarity of the solvent with which they are extracted, among others (Zajíčková *et al.,* 2020).

The characterization of *Leucaena leucocephala* extract by gas chromatography-mass spectrometry was reported by (Salem *et al.,* 2011), in which they indicated that this forage plant has forty-four different secondary metabolites; of which the following chemical compounds stand out: 2(H)-benzofuranone-a-tetrahydro-4,4,7a-trimethyl (a volatile terpene: 23.1%) and pentadecanoic acid-14-methyl-methyl ester (a branched monomethyl acid: 8.2%), among others.

In this same study, it was reported that the alcoholic extract of *Leucaena leucocephala* is mainly constituted by oxygenated hydrocarbons of compounds C10 to C60, and contains predominantly phenolic hydrocarbons.

In the same context, (Von Son-de Fernex *et al.,* 2015) identified three phytochemicals in a biodirected fractionation of the hydroalcoholic extract of *Leucaena leucocephala*, these compounds were quercetin (82.21%), caffeic acid (13.42%) and scopolamine (4.37%).

Although the extract of *L. leucocephala* is rich in phenolic hydrocarbons, which as mentioned above can eventually generate the loss of egg viability, the participation of other types of metabolites, such as saponins and flavonoids, is not ruled out, generating a synergistic effect, since in plants and metabolites work under a joint dynamic, similar to the immune system, by conferring protection against bacteria, viruses, parasites and even predators. This is due to the formation of molecules that allow their survival, or simply by converging at some point in the same metabolic pathway (Azcón-Bieto & Talón, 2003).

In most plants, the presence of secondary metabolites is distributed according to the parts that make up a plant (leaves, flower or fruit, stem and root). Accordingly, the presence of metabolites may present slight variations, since each of the parts that conform it is exposed to different stress factors, and therefore generate the necessary metabolites that confer protection and thus biological activities (Olguín-Rojas *et al.,* 2019).

Different concentrations of the hydroalcoholic extract of *Leucaena leucocephala* were evaluated on the inhibition of HC egg hatching. As can be seen in Table 1, there are significant statistical differences between treatments ($p<0.05$). The hydroalcoholic extract of *Leucaena leucocephala* leaves presented its best percentage of egg hatching inhibition at 100 mg/mL (71%), the concentrations of 90, 80 and 70 mg/mL did not show significant statistical differences among them ($p>0.05$), with 67, 65 and 62% inhibition of egg hatching respectively. The lowest percentages were obtained at concentrations of 60 and

50 mg/mL, which shows a dose-dependent behavior, where the lower the concentration of the extract, the lower the percentage of hatching inhibition.

Table 1. Percentage inhibition of *Haemonchus contortus* egg hatching, using hydroalcoholic extract of *Leucaena leucocephala* leaves

Concentration (mg/mL)	%IEH ± S.E
Distilled water	4 ± 0.22 [e]
Ivermectin	100 [a]
HELL 100	71 ± 0.94 [b]
HELL 90	67 ± 0.63 [c]
HELL 80	65 ± 0.51 [c]
HELL 70	62 ± 0.54 [c]
HELL 60	56 ± 0.83 [d]
HELL 50	48 ± 0.89 [d]
P value	0.001

HELL, hydroalcoholic extract of *Leucaena leucocephala* leaves; IEH, inhibition of egg hatching; S.E, Standard error; different literals within the same column indicate significant statistical differences ($p < 0.05$)

Some research has reported favorable effects of a wide variety of extracts; however, they have not been able to determine the mechanisms by which the loss of egg viability occurs in nematodes.

In this regard, (Hernandez *et al.,* 2014) evaluated the hydroalcoholic extract of *Leucaena leucocephala* and observed a reduction in parasite load of 54% in growing lambs; however, the mechanism of action on the parasites in question was not determined. It has been reported that some compounds present in plant extracts possess metabolites such as saponins and tannins that interfere on fecundity in females, thus decreasing the parasite load in egg count (Zajíčková *et al.,* 2020). Given that background, results obtained in the present investigation could suggest that the decrease in parasite load in the above-mentioned study could be associated with the inhibition of egg hatching, induced by the hydroalcoholic extract of *Leucaena leucocephala* (71% at 100 mg/mL), and reported in the present investigation.

As mentioned, there are direct effects that suggest the ability of tannins to bind to intestinal mucosal proteins and cuticle glycoproteins, or structural proteins of eggs, interfering with development and growth and inhibiting hatching by loss of viability (Hoste *et al.,* 2005). From the ethnobotanical approach, the use of control alternatives based on plant extracts involve a variety of pharmacologically active compounds, especially tannins, flavonoids and phenylpropanoids, which are attributed biological activities such as anti-inflammatory, antioxidant and anthelmintic (Mravčáková *et al.,* 2019). Regarding the applications with anthelmintic activity of plant extracts, the proposed mechanisms are mentioned that may present a variation according to the content of the metabolites present or isolated, depending on the type and polarity of solvents used (Zajíčková *et al.*, 2020).

The ability of tannins to form complexes with glycoproteins can generate the inactivation of enzymes important for egg development, as well as to initiate with the hatching process. In this sense, (Rojo-Rubio *et al.*, 2019) determined that at a concentration of 25 mg/mL of hydroalcoholic extract of *Caesalpinia coriaria* leaves inhibits 95% of HC egg hatching, activity that was associated with the tannin content in *Caesalpinia coriaria* leaves (200 g/kg dry matter). According to the above, it has been documented that tannins can negatively affect the hatching of eggs of this nematode by binding to the egg cuticle, which is rich in glycoproteins, altering the development of the embryo and eventually the hatching process.

The evaluation of different extracts of *Leucaena leucocephala*, such as extracts of pod and aerial parts of the plant, has been reported, as reported by (Rivero-Perez *et al.,* 2019) who determined that the hydroalcoholic extract of *Leucaena leucocephala* pod inhibits the hatching of nematode eggs by 20% at a concentration of 50 mg/mL, a lower percentage than that obtained in the present investigation at the same concentration of 50 mg/mL (48%). In such a way, that it is inferred that the variation of pharmacologically active metabolites is a function of plant parts (Hernandez-Alvarado *et al.,* 2018).

In the study of (Von Son-de Fernex *et al.,* 2015), the effect of the hydroalcoholic extract of *Leucaena leucocephala* leaves on the inhibition of *Cooperia* spp. egg hatching was evaluated and an inhibition 90.49 ± 2.8% was observed; these were analyzed by scanning electron microscopy, observing irregular external damage with small projections and ruptures in the lateral walls of the egg membrane, noting direct damage of the extract fraction against the structural components of the *Cooperia* spp. egg, suggesting a similar behavior for the hydroalcoholic extract of *Leucaena leucocephala* evaluated in the present study.

Regarding effective concentrations (Carvalho *et al.*, 2012) found that ethyl acetate extract, ethanol and essential oils of *Piper tuberculatum*, *Lippia sidoides*, *Mentha piperita*, *Hura crepitans* and *Capara guianensis* presented an EC_{50} on HC hatching inhibition at 0.031, 0.04, 0.037, 2.16 and 2.03 mg/mL respectively. The results of the present investigation indicate an EC_{50} of 52.22 mg/mL and an EC_{90} of 302.8 mg/mL, in comparison, the concentrations are higher; however, this may be associated to the use of different plants and solvents in the extraction process of secondary metabolites, see Figure 1.

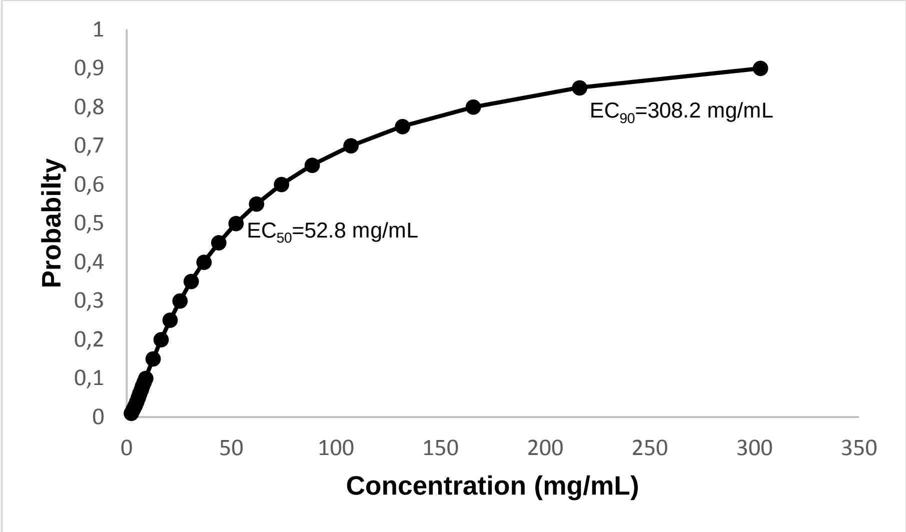

Figura 1. Effective concentrations EC_{50} and EC_{90} of the hydroalcoholic extract of *Leucaena leucocephala* in the hatching test of *Haemonchus contortus* eggs

CONCLUSION

The hydroalcoholic extract of *Leucaena leucocephala* leaves contains groups of secondary metabolites that inhibit HC egg hatching *in vitro* with a dose-dependent effect; however, it is necessary to identify the specific metabolite(s) that confer the activity, determine their toxicity and perform in vivo assays.

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CONFLICT OF INTEREST

All authors declare that they have no conflicts of interest.

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Errata Erratum

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