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Cassia fistula extract as alternative treatment against gastrointestinal nematodes of sheep

Cassia fistula como tratamiento alternativo contra nematodos gastrointestinales de ovino

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ABSTRACT

Gastrointestinal nematodes are an important issue in sheep production, these nematodes have developed resistance to traditional drugs, so it is necessary to search new functional and economic therapeutic alternatives; such as the use of plant extracts that have anthelmintic activity, *Cassia fistula* is a Leguminosae, due to its content of phenolic compounds, that can exhibit this activity, the aim of the present experiment was to determine the anthelmintic activity of hydroalcoholic extract from pods of *Cassia fistula* (LO) in vitro, using field nematodes in egg and larval stages to determine the inhibition percentage of eggs hatching and larval mortality. A hydroalcoholic extract of *Cassia fistula* pods was elaborated, stool samples of 20 sheep were collected, the feces were subjected to a parasitoscopic analysis to determine the presence of nematodes and eggs per gram of feces, they were used to obtain eggs and larvae, once obtained, they were challenged with LO (50, 25, 12.5 and 6.26 mg / mL) after the incubation time had elapsed, the percentages of eggs hatching inhibition and larval mortality close to 30% were determined. This suggests that LO has anthelmintic potential on gastrointestinal nematodes of sheep.

Keywords: *Cassia fistula*, gastrointestinal nematodes, antihelmintic activity.

RESUMEN

Las parasitosis causadas por nematodos gastrointestinales son un problema muy relevante en la producción ovina, observándose el desarrollo de resistencia de los nematodos a los fármacos tradicionales, es necesario buscar alternativas terapéuticas funcionales y económicas; como el uso de extractos de plantas que presentan actividad antihelmíntica, *Cassia fistula* es una leguminosa que por su contenido de compuestos fenólicos puede presentar esta actividad, el objetivo del presente experimento fue determinar in vitro la actividad antihelmíntica del extracto hidroalcóholico de vainas de *Cassia fistula* (LO), utilizando nematodos de campo en fase de huevo y larva para determinar porcentaje de inhibición de la eclosión y mortalidad larvaria. Se elaboró un extracto hidroalcóholico de vainas de *Cassia fistula*, se colectaron muestras de heces de 20 ovinos, las cuales fueron sometidas a un análisis coproparasitoscópico para determinar la presencia de nematodos y huevos por gramo de heces, las heces fueron utilizadas para la obtención de huevos y larvas, una vez obtenidos fueron desafiados con LO (50, 25, 12.5 y 6.26 mg/mL). Transcurrido el tiempo de incubación se determinaron porcentajes de inhibición de la eclosión y mortalidad larvaria cercanos al 30%. Lo cual sugiere que LO tiene potencial antihelmíntico sobre nematodos gastrointestinales de ovino.

Palabras clave: *Cassia fistula*, nematodos gastrointestinales, actividad antihelmíntica.

INTRODUCTION

Worldwide, sheep production systems face serious health problems that limit the productivity of animals and generate significant economic losses in the food production chain (Rios-de Alvarez *et al*., 2012; Roeber, Jex and Gasser, 2013; Kumarasingha *et al*., 2016). It has been estimated that the annual cost of treatment against some parasites is more than 103 million dollars (Peter and Chandrawathani, 2005).

In the production of small ruminants the gastrointestinal nematodes with the highest prevalence are *Cooperia sp, Teladorsagia (Ostertagia) circumcincta, Trichostrongylus sp, Oesophagostomum sp* and *Haemonchus contortus*; The latter is the most pathogenic nematode and produces the highest mortality and morbidity rates (Cala *et al*., 2012, Zapata, Velásquez, Herrera, Ríos, and Polanco, 2016). Depending on the parasitic load, age and physiological state, affected animals may present anorexia, diarrhea, weight loss, anemia and edema; signology associated with *Heamonchus contortus* is a blood-sucking parasite. All these alterations result in weight loss, decreased fertility, wool production, milk, and in very severe cases the death of the animal (Cala *et al*., 2012; Carvalho *et al*., 2012; Roeber *et al*., 2013).

Traditionally anthelminthic drugs have been used for the treatment and prevention of Hemoncosis in small ruminants; However, the widespread and indiscriminate administration of these products has resulted in the generation of resistance by the parasite; reported for the first time in 1964 (Carvalho *et al*., 2012). Due to the problems of resistance to commercial anthelmintics, new functional and economic strategies for the control of nematodes are constantly being sought (Cala *et al*., 2012; Carvalho *et al*., 2012), such as: implementing adequate nutrition programs, selection of resistant animals, the integral management of pastures, the use of nematophagous fungi and new anthelminthic compounds derived from plants (Katiki, Chagas, Bizzo, Ferreira and Amarante, 2011); since there is evidence that plants possess bioactive compounds such as terpenes phenolic compounds, glycosides and alkaloids with proven anthelmintic activity (Eguale, Tadesse, and Giday, 2011, Kamaraj and Rahuman, 2011, Zabre *et al*., 2017, Hernández-Alvarado *et al*., 2018).

Cassia fistula is an ornamental legume distributed in countries such as India, Brazil, Mexico, among others (Zhao *et al*., 2013, Antonisamy, Agastian, Kang, Kim and Kim, 2017); its flowers, fruits, seeds and leaves have been used in traditional medicine for the treatment of: diarrhea, gastritis, fever, skin diseases, leprosy, ringworm, fungal infections of the skin, abdominal pain, jaundice and nausea; besides being attributed to healing, hypoglycemic, anti-inflammatory, hepatoprotective, antimicrobial and anthelminthic properties (Zhao *et al*., 2013, Antonisamy *et al*., 2017, Srividhya, Hridya, Shanthi, & Ramanathan, 2017). Based on this information, the present experiment has been proposed with the objective of determining

the anthelmintic activity of the hydroalcoholic extract of *Cassia fistula* pods on *in vitro* gastrointestinal nematodes of sheep.

MATERIAL AND METHODS

Location

The present research was carried out in the Institute of Agricultural Sciences of the Autonomous University of Hidalgo State, in the laboratory of Bacteriology of the Academic Area of Veterinary Medicine and Zootechnics; located in the municipality of Tulancingo de Bravo, Hidalgo, Mexico.

Obtaining extracts

One kilogram of Golden Rain pods (*Cassia fistula*) was collected, which were dried in the shade at room temperature; subsequently, 250 g of the dried pod were macerated and mixed with 1500 ml of hydroalcoholic solution (30% methanol and 70% distilled water), allowing to stand for 72 hours. After this time, the filtration proceeded to separate the solid material from the liquid; finally it was concentrated with a rotoevaporator (Büchi R-300, Switzerland), to eliminate the solvent and concentrate the secondary metabolites.

Subsequently, the extract was diluted with sterile distilled water, to obtain concentrations of 200, 100, 50, 25, 12.5 and 6.26 mg/ml, for the evaluation of the extract on the inhibition of egg hatching and on larval mortality of *in vitro* gastrointestinal nematodes.

Evaluation of the activity of the extract on the inhibition of egg hatching of gastrointestinal nematodes

For this evaluation, 20 stool samples of feces were collected from Sulffork-Hampshire sheep, from a sheep production unit located in Tepeapulco municipality in Hidalgo State, directly from the rectum of each animal, which were identified and transported at refrigeration temperature. to the bacteriology laboratory; where the flotation techniques and Mac Master were performed, to determine the presence of gastrointestinal nematodes and the number of eggs per gram of feces present in them, according to the methodology described by Rivero-Pérez *et al*. (Rivero-Pérez *et al*. ., 2018).

Egg recovery from gastrointestinal nematodes

Once the flotation and Mac Master techniques were performed, those samples that presented more than 1000 eggs per gram of feces, were vigorously mixed, to then take approximately 30 g of feces that were washed in 200,100, 75 and 37 micron sieves. To concentrate the eggs in the last sieve, the concentrate was recovered and added to a 15 ml falcon tube, in which previously 6 ml of saturated saline solution was added; then they were centrifuged at 3000 rpm/3 minutes, obtaining the egg concentrate. This concentrate was added to another tube with distilled water, re-centrifuging 3 times, to remove the excess of saline and organic matter; until obtaining a solution with clean egg (von Son-de Fernex *et al*., 2015).

Once the solution with the clean egg was obtained, the egg concentration was determined in 50 µl (counting the number of eggs in 10 aliquots of 5 µl under an optical microscope); it should be noted that in order to perform the bioassay, a concentration of 150-200 eggs in 50 µl is required.

Evaluation of the extract on the inhibition of egg hatching of gastrointestinal nematodes

In a 96-well plate, 50 µl of a solution of distilled water with 150 eggs/well was placed and 50 µl of each extract was added to the different concentrations (200, 100, 50, 25 and 12.5 mg/ml), to achieve a final volume of 100 µl/well; 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 in a humid chamber. After the incubation period, the plate was read, observing 10 aliquots of 10 µl in the microscope, with the objective 4x and determining the number of eggs and larvae in each aliquot. This information was used to determine the percentage of inhibition of egg hatching by the following formula:

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

Where L1 is equal to larva 1.

Evaluation of the activity of Cassia fistula extract on larvae of gastrointestinal nematodes

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

Once the larvae were recovered, they were drained, mixing 2 ml of a 3% sodium hypochlorite solution (to encourage larvae drainage), with 3 ml of the larval suspension for one minute (during this time it was stirred in the vortex and it was verified under the microscope that the larvae lost their sheath). After this time, the larvae were washed 3 times with 3 ml of distilled water, to remove the remains of sodium hypochlorite. Subsequently, the amount of larvae was determined in 50 µl of distilled water (counting the amount of larvae in 10 aliquots of 5 µl under an optical microscope). It should be noted that in order to carry out the bioassay a concentration of 150-200 larvae in 50 µl is required.

Evaluation of the hydroalcoholic extract of *Cassia fistula* on larvae of gastrointestinal nematodes

Once the unwound larvae were obtained, 50 µl of a distilled water solution with 150-200 larvae was placed in a 96-well plate, then 50 del of each extract was added to the different

concentrations (200, 100, 50, 25 and 12.5 mg/ml) with four repetitions each, using as a positive control Ivermectin (5mg/ml) and distilled water as a negative control; the plate was incubated in a humid chamber at 30 °C for 72 hours. After this time the plate was read, placing 10 aliquots of 10 µl to differentiate, as well as counting live and dead larvae (alive in motion and dead without mobility), under the optical microscope Motic brand with the 4x objective. The data obtained were used to determine the percentage of mortality, using the following equation:

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

Statistical analysis

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.

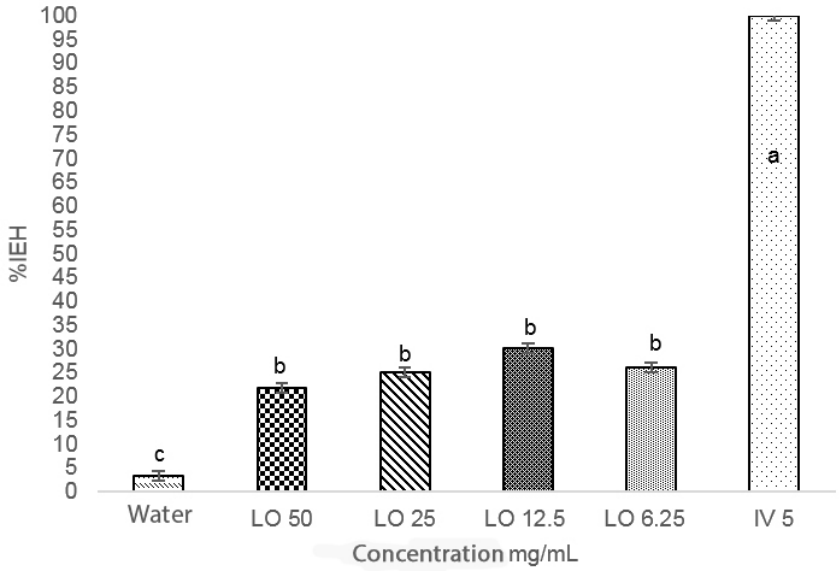
RESULTS

The presence of *Haemonchus contortus, Cooperia* spp., Ostertagia spp, Chavertia ovina, Moniezia and Strongyloides spp, eggs was determined in 85% of the samples collected.

By means of the Mc Master technique, an average of 1795 eggs per gram of feces was determined from the total of the samples collected; which were used for the evaluation of the activity of the hydroalcoholic extract of Lluvia de Oro, on the inhibition of hatching and larval mortality in field nematodes.

When performing the hatching inhibition test, it was observed that the concentrations evaluated 50, 25 and 12.5, 6.25 mg/ml, have very similar percentages of hatching (21.95, 25.03, 30.14 and 26.24%, respectively), without present significant statistical differences among them, but with respect to positive and negative controls (P = 0.0001); in comparison with Ivermectin that produced a 100% inhibition of the hatching of the gastrointestinal nematodes present. Apparently the hydroalcoholic extract of Lluvia de Oro is not very functional, however it must be remembered that it is an extract, not a pure compound like the positive control (Ivermectin). On the other hand, when comparing the % of IEH of the extract to its different concentrations with the negative control, it is observed that there are significant statistical differences, which leads us to establish that although the effect produced by the extract does not go beyond 30 %, if it is a considerable effect (Figure 1).

On the other hand, when evaluating the same extract on the larval mortality of gastrointestinal nematodes, it is observed that the effect is maintained with percentages of mortality very close to the percentages of inhibition of hatching, without significant statistical differences between the extract concentrations evaluated but with respect to the positive control and the negative control (P = 0.0001), between the evaluated concentrations 50, 25, 12.5 and 6.25 mg / ml (Table 1). As expected Ivermectin maintained their activity producing mortality percentages of 98.23%.



% IEH; percentage of egg hatching inhibition
THE; hydroalcoholic extract of Cassia fistula
IV5; Ivermectin at 5 mg / mL
Different literals in the bars indicate significant statistical differences (P≤0.05)

Figure 1. Percentage of inhibition of egg hatching of gastrointestinal nematodes of sheep with the use of hydroalcoholic extract of *Cassia fistula.*

Table 1. Percentage of mortality ± from larvae of gastrointestinal nematodes of sheep with the application of hydroalcoholic extract of *Cassia fistula*.

TREATMENT	CONCENTRATION	% MORTALITY
Water	0	$2,73^{c}$ ± 0.9
LO	50 mg/mL	$29,65^{b}$ ± 2.0
LO	25 mg/mL	$27,12^{b}$ ± 1.9
LO	12.5 mg/mL	$25,81^{b}$ ± 1.6
LO	6.25 mg/mL	$25,67^{b}$ ± 1.2
Ivermectin	5 mg/mL	98.23^{a} ± 1.5

LO; hydroalcoholic extract of Cassia fistula

Different literals in the rows indicate significant statistical differences (P≤0.05)

DISCUSSION

When carrying out the coproparasitoscopic analysis of the stool samples collected, the presence of *Haemonchus contortus, Cooperia sp, Ostertagia sp, Chavertia sp* and *Strongyloides sp*; results coincide with those reported by Zapata and collaborators; who determined that these genera are the most abundant in semiconfinement systems in sheep. It was also found that they are found in amounts exceeding 1700 eggs per gram of feces, that is, they have a high load; this situation may be due to the fact that there is no history of deworming in the production unit; in addition, they are sheep that go out to graze, which

increases the risk of being infected with these parasites (Roeber *et al*., 2013; Zapata *et al*., 2016).

The evaluation of the hydroalcoholic extract of *Cassia fistula* pods, on the inhibition of egg hatching of the nematodes obtained after stool washing; allowed to determine that LO inhibits the hatching of eggs between 21 and 30 % at the different concentrations evaluated (50, 25 and 12.5, 6.25 mg / ml). This effect could be mainly due to the content of phenols, flavonoids, tannins, alkaloids, proanthocyanidins, terpenes and glycosides; identified in the seeds, pulp and pods; which have been identified in other plants that have demonstrated inhibitory activity of the hatching of eggs and gastrointestinal nematodes (Duraipandiyan, Ignacimuthu and Gabriel Paulraj, 2011, Katiki *et al*., 2011, Cala *et al*., 2012, Carvalho *et al*., 2012, Rajagopal, Premaletha, Kiron and Sreejith, 2013, Hernández-Alvarado *et al*., 2018).

The possible mechanism of action by which the secondary metabolites present in the plants inhibit the hatching of nematode eggs, consists of inhibiting or delaying the growth and maturation of the parasites; reducing or suppressing their appetite and altering their reproductive capacity (Carvalho *et al*., 2012).

On the other hand, the evaluation of the extract on the mortality percentage larvae of gastrointestinal nematodes; revealed that the hydroalcoholic extract produces percentages of larval mortality between 25 and 29%. When comparing these results with those obtained by Duraipandiyan and collaborators in 2011, who evaluated ethyl acetate extract obtained from flowers of Cassia fistula; determined percentages of larval mortality on *Spodoptera litura* of 67.5% and of 36.25% on *Helicoverpa armigera*, at 1000 ppm. However, these results cannot be totally comparable with the present experiment, since we worked with ethyl acetate extract obtained from *Cassia fistula* flowers, which present secondary metabolites (glycosides, fistulan, kaempferol, etc.) to different concentrations found in the seeds, pulp and pod of the same plant, and with nematodes that affect plants; not with gastrointestinal nematodes of animals (Duraipandiyan *et al*., 2011; Rajagopal *et al*., 2013).

In a study conducted with methanolic extract of fruit pulp and seeds, to evaluate the effect of this extract on the mortality of larvae of *Pthiatima posthuma*, it was observed that the extract besides producing the death of the larvae, also present an effect of inhibition of motility; however, the percentages of mortality observed are not established, only the time in which inhibition of motility is observed (17 to 82 minutes) and mortality (24 to 118 minutes); as well as the correlation that exists between the dose and the time in which both effects occur (Irshad, Man and Rizvi, 2010).

It has been proposed that the secondary metabolites of plants have various mechanisms, with which they inhibit motility or kill parasites in their larval phase, such as the affinity of phenolic compounds for the glycoproteins (proline) of the parasite cuticle which they unite, inhibit their motility, feeding, reproduction and finally produce their death, or the

membranolitic action of the saponins; among others (Irshad *et al*., 2010; Hernández-Alvarado *et al*., 2018).

CONCLUSION

It is concluded that the hydroalcoholic extract of *Cassia fistula* pods presents antihelminthic activity against gastrointestinal nematodes of sheep; both by inhibiting egg hatching, and by causing larval mortality; so it could be a natural and effective alternative for the control and treatment of nematodes in small ruminants**.**

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