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Natural Products

In-vitro effect of the methanolic extract of *Momordica charantia* on hatching of eggs of *Haemonchus* sp.

Efecto *in-vitro* del extracto metanólico de *Momordica charantia* sobre la eclosión de huevos de *Haemonchus* sp.

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Abstract

Background: Endoparasitism, particularly infections by gastrointestinal nematodes (e.g., *Haemochus* sp.), has been associated with economic losses within sheep production systems in tropical regions. Thus, implementing therapeutic alternatives that are environmentally sustainable is essential for parasite integral control programs.
Objectives: Evaluate *in-vitro* the effect of *Momordica charantia* methanolic extract on the *Haemonchus* sp. eggs' hatching process.
Methods: Nematode eggs were retrieved from experimentally infected sheep and exposed to 10, 20, 40, 80, and 160 mg/mL of methanolic extract of *M. charantia*. Hatching percentages were recorded from five replicates, and CL_{50} and CL_{90} were estimated through Probit regression analysis.
Results: A significant effect on the hatching percentages were observed, from 24.2% up to 84.6% inhibition ($p<0.05$). The LC_{50} and LC_{90} estimated were 52.2 mg/mL (95%CI 37.87-63.22) and 201.45 mg/mL (95%CI 186.01-221.89), respectively. Utilizing a preliminary phytochemical analysis, potential antihelmintic compounds such as alkaloid, triterpenes, and anthracenic glycosides groups were identified in the methanolic extract.
Conclusions: In the *in-vitro* test, the methanolic extract of *M. charantia* was effective in inhibiting the hatching of *Haemonchus sp*., which is important to promote other bio-guided fractionation studies of this plant on different life stages of *H. contortus*, this

being a plant species widely adapted to the conditions of the piedmont (foothills) of Meta, Colombia.
Keywords: haemonchosis, phytotherapy, sheep farming.

Resumen

Introducción: El endoparasitismo, particularmente las infecciones causadas por nematodos gastrointestinales (p. ej., *Haemonchus* sp.), ha sido asociado con pérdidas económicas en los sistemas de producción ovina de los países del trópico. Por lo tanto, es esencial la implementación de alternativas terapéuticas sostenibles para el ambiente con el fin de efectuar programas de control integrado del parásito.

Objetivos: Evaluar el efecto *in-vitro* de los extractos metanólicos de *M. charantia* sobre el proceso de eclosión de los huevos de *Haemonchus* sp.

Métodos: Los huevos derivados de un ovino monoinfestado se expusieron a concentraciones de 10, 20, 40, 80 y 160 mg/mL del extracto metanólico de *M. charantia*. Se registraron los porcentajes de eclosión de cinco réplicas, y la obtención de CL_{50} y CL_{90} se realizó a través de regresión lineal por el método Probit.

Resultados:. Se observó un efecto significativo en los porcentajes de eclosión; una inhibición desde 24.2% hasta 84.6% ($p<0.05$). Finalmente se estimaron CL_{50} y CL_{90} de 52.2 mg/mL (95%IC 37.87-63.22) y 201.45 mg/mL (95%IC 186.01-221.89), respectivamente. Así mismo, utilizando un análisis fitoquímico preliminar; en el extracto metanólico de *M. charantia* se identificaron compuestos antihelmínticos potenciales, tales como alcaloides, triterpenos, y glucósidos antracénicos.

Conclusiones: En las pruebas *in-vitro,* el extracto metanólico de *M. charantia* fue eficaz para inhibir la eclosión de *Haemonchus* sp., lo cual es importante para promover otros estudios bio-guiados de esta planta sobre diferentes estadios de vida de *H. contortus*, siendo esta una especie vegetal ampliamente adaptada a las condiciones delpiedemonte del Meta, Colombia.

Palabras clave: hemoncosis, fitoterapia, ovinocultura.

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Introduction

The infestation by nematodes of the genus *Haemonchus* is a very relevant problem in small ruminant livestock production in tropical countries. This disease causes weight loss, immunosuppression, and gastroenteritis-associated malabsorption syndrome that

affect the animals' welfare and normal development. Else, causing significant economic losses in the sheep production system due to both the effect on product yield and reproduction (1-5).

Gastrointestinal nematodes of the genus *Haemonchus* are hematophagous parasites and are the primary pathogen agent in the ruminants abomasum (6). Adult parasites of the *H. contortus, H. placei,* and *H. similis* the species can consume up to 0.05 mL of blood/day from their host, having a high clinical impact (7). The parasite sexual reproduction takes place in a natural host life cycle. Females are prolific, laying up to 10,000 eggs/day (pre-patent period of 3-5 weeks), which leads to a large number of infective and pre-infective forms of the parasite in the grasslands (eggs and L1, L2, and L3) (8). According to Stromberg and Gasbarre (9), the *Haemonchus* sp. larvae with infective capacity (L3) migrate from feces to forage approximately seven days post-hatch at 23 °C and 65% RH (8, 9). Later, Silva et al. (10) demonstrated that the L_3 vertical migration on the forage is critical in the parasite transmission, allowing its ingestion by the grazing host. This migratory behavior is influenced by climatic conditions such as temperature, relative humidity, sunlight, and rainfall (3, 11-13).

H. contortus and *H. placei* have been reported in Colombian herds since 1983 (14). Researches on the parasite ecology has been conducted in different agroclimatic zones (15-19). In a 4-year study using weaned calves, Parra and Uribe (20) found a high level of pasture infestation with *Haemonchus* sp. larvae associated with periods of high rainfall (April to June, and August to September) in the piedmont (foothills) of the eastern Colombian plains.

Worldwide, this parasitosis control has been implemented since the 19th century, mainly using regular pharmaceutical compounds. Nevertheless, due to these products' unsupervised dosage regimes, the parasitic organisms have developed resistance (1, 21-23). Consequently, FAO recommends developing sustainable pest control strategies in animal production systems (e.g., against gastrointestinal parasites); sustainable from the environmental perspective and long-time effective from the animal health perspective (24). Thus, based on the large numbers of ethnopharmacological reports, the research on medicinal plant extracts for parasite control has become a widely distributed scientific practice that had yield relevant findings for integrated pest management practice (25-28). Moreover, the use of plant extracts can have less impact on the environment due to their short biodegradation time in the soil compared to traditional pharmaceutical compounds (29).

Various types of traditional plants for medical use have been studied to obtain extracts that present potential antiparasitic activity. Hence, the anthelmintic activity of *M. charantia* has been registered in *in-vitro* and *in-vivo* trials and by phytochemical analyses in other latitudes (30-32). *M. charantia* is known as "the bitter melon" and belongs to the *Cucurbitaceae* family (33). The plant grows in tropical areas of Asia, Amazon, East Africa, and the Caribbean. It is cultivated throughout the world as a food source and for medicinal purposes (34).

M. charantia has been used traditionally as a medicinal plant in different countries such as Brazil, China, Cuba, Ghana, Haiti, India, México, Malaysia, New Zealand, Nicaragua, Panamá Perú, and Colombia (35-37). Some reports have already described the exploration of the potential effects of *M. charantia* extracts for the control of *H. contortus* in small ruminants (38-40). In this regard, the anthelmintic capacity of *M. charantia* has been attributed to different secondary metabolites such as the triterpene compound called cucurbitacin B, the non-protein amino acid called cucurbitin (3-amino-pyrrolidine-3-carboxylic acid), many saponins, and various sterols (41, 42). Other compounds such as cucurmosin (ribosome-inactivating protein) found in stem and leaves have also been studied (43).

The objective of this study was to evaluate the *in-vitro* effect of the methanolic extracts of *M. charantia* on the hatching process of *Haemonchus sp.* nematode as a potential therapeutic alternative for integrated parasite management programs in sheep production systems of the piedmont region in Meta, Colombia.

Materials and methods

Methanolic extract preparation

10 Kg of *M. charantia* leaves were collected in the Barcelona farm of Universidad de Los Llanos (Villavicencio, Meta, Colombia) during February (dry season). A sample of the leaf, stem, and fruit-flowering was sent to the Colombian National Herbarium to perform a taxonomic classification. The methanolic extract was made from *M. charantia* leaves, dried in a recirculating air oven at 40 °C for 72 hours. Then, the dry material was pulverized, obtaining a homogeneous sifting. The material was percolated continually until exhaustion with methanol 98% (Merck®, Germany). The percolated liquid was filtered and concentrated at 40°C using a Roto-evaporator (IKA®, Brazil). The resulting extract was reconstituted in 0.5% dimethyl sulfoxide solution (DMSO) (Merck®, Germany) to 10, 20, 40, 80, and 160 mg/mL concentrations for the *in-vitro* tests, following the methodology proposed by Domingues et al. (28).

Egg hatch inhibition test

Following the Michael et al. (45) methodology, eggs of *Haemonchus sp*. were obtained from 20g of feces taken from the rectal ampulla of a monoinfected sheep. Its collection and concentration were performed using different sieves (1 mm, 106 μm, 53 μm, and 25 μm mesh) according to the method published by Bizimenyera et al. (46) based on the original method reported by Coles et al. (47). The effect of the extract in eggs was evaluated according to the guidelines proposed by the WAAVP (World Association for the Advancement of Veterinary Parasitology) and described by Powers et al. (48). Briefly, approximately 100 eggs were exposed (250 μL final volume) to the five treatment levels of the methanolic extract of *M. charantia*, to 25 μg/mL of Albendazole (Sigma®, Germany; positive control), and 0.5% DMSO (negative control) (Table1). Each test was replicated five times; all tests were performed 24 hours at 27°C and RH>80%. After that, a drop of Lugol's Iodine solution (PipingRock®, U.S.A.) was added to stop the hatching process. Larvae (L1) and eggs were counted using an inverted microscope (Leica®, Germany). The hatching inhibition percentage (HI %) was determined for each level of treatment with the following formula: HI % = 100 (P test/P total), where "P test" is the number of eggs and "P total" is the number of eggs plus the count of L1 individuals. This research was approved by the General Director of Research of the Universidad de Los Llanos through the project "Alternativa terapéutica para el control de la haemoncosis con base en el extracto metanólico de *Momordica charantia* en sistemas de producción bovina del piedemonte del departamento del Meta" according to announcement 01-P-2013, which guarantees the humane treatment of the experimental animals used.

Preliminary phytochemical screening

The preliminary phytochemical analysis of *M. charantia* leaves was conducted following the colorimetric methods and thin-layer chromatography methodology proposed by Sanabria (44). These methods permit the detection of alkaloids, steroids or triterpenoids, flavonoids, naphthokines or anthraquinones, tannins, lactones, coumarins, and other cardiotonics.

Statistical analyses

The hatching inhibition values obtained are presented as hatching inhibition percentages and were compared to the negative control group (DMSO 0.5%) using the Chi-square

test for comparing two proportions distributions ($p<0,05$). The LC_{50} and LC_{90} were estimated from egg hatching inhibition results through the Probit regression model analysis (estimate plus 95% confidence interval). The data were organized and analyzed with OpenStat 4.0, version 7.0 statistical program.

Results

The methanolic extracts of *M. charantia* showed satisfactory hatching inhibition results on *Haemonchus* sp eggs (Table 1). Concentrations from 1 to 8% (10 to 80 mg/mL) showed significant differences with the DMSO 0.5% negative control group ($\chi^2 = 0.024$, p-value < 0.05). Likewise, inhibitory action to the hatching of *Haemonchus* sp. eggs in the concentration of 16% (160 mg/mL) of the plant extract was similar to the Albendazole 25µg/mL control group. We also found significant differences in hatching inhibition percentages (p-value < 0.001) to DMSO 0.5%. For this study, according to the Probit regression results, the LC_{50} estimated was 52.18 mg/mL (95%CI: 37.87 - 63.22), and the estimated LC_{90} was 201.45 mg/mL (95%CI: 186.01 - 221.89).

Table 1. Inhibition effect on the hatching of *Haemonchus* sp. exposed to the methanolic extract of *M. charantia*.

Extract concentration - controls	**% Hatching inhibition**
10 mg/mL	38.3% ^{A}
20 mg/mL	24.2% ^{A}
40 mg/mL	34.5% ^{A}
80 mg/mL	50.4%^{A}
160 mg/mL	84.6% ^{B}
Albendazole 25 µg/mL	100%^{B}
DMSO 5 mg/mL	0%

Different superscript letters in the same column indicate significant differences comparing to the level of DMSO 0.5% negative control treatment, Chi-square test. A $p<0.05$, B $p<0.001$.

Table 2, presents the characterization of secondary metabolites groups in the methanolic extract of *M. charantia*, obtained by qualitative techniques of colorimetry and thin-layer chromatography. These techniques allowed us to characterize alkaloids, triterpenes, and anthracenic glucosides.

Table 2. Characterization of the groups of secondary metabolites in the methanolic extracts of *M. charantia*.

Groups of secondary metabolites	Colorimetric test /result	Thin-layer chromatography - Pure samples/result
Alkaloids	Mayer's reagent (-)	Quinidine (+)
	Ammonium reineckate (-)	
	Dragendorf's reagent (+)	
	Valser's reagent (-)	
Flavonoids	Shinoda's reagent (-)	Flavone (-)
Triterpenes	NA	(+)
Anthracenic glucosides	NaOH 5% NH_4 2% (+)	Sacred Cascara (-)
Cardiogenic glucosides	Kedde's reaction (-)	Digitalina (-)
	Keller – Kilian's reaction (-)	

(-): Absence, (+): Presence, NA: not applied.

Discussion

M. charantia has been used in traditional medicine (30-32, 34-37). The phytochemical assets of its extracts have been appraised due to antibacterial (49, 50), anticancer (51, 52), antidiabetic (53, 54), antidepressant (55), antifungal (56, 57), antiviral (58), and anthelmintic (30-32, 59-62) properties, among others. Likewise, nutritional analyses of *M. charantia* indicate that this plant is rich in fiber, calcium, potassium, iron, and vitamins A and C (63). The pulp around the ripe fruit seeds is a good source of carotenoid lycopene (64). Besides, *M. charantia* is an important source of phenolic compounds with high antioxidant and antimutagenic properties (65).

The plant extracts action mechanisms can affect different stages of the *Haemonchus* sp. life cycle, including the free-living phase (pre-parasitic) and either the L_3 infective and parasitic phases. The antihelmintic effect of cucurbits is related to the presence of hydrolases in *M. charantia* capable of binding to the parasite's cuticle, activating a proteinase enzymatic complex that promotes the digestion of the parasite's cuticle, finally causing the death of the parasite (78-80). Regarding the results of our study, the

catalytic activity of the anthracenic glycosides found in *M. charantia* (Table 2) could be responsible for the degradation of the *Haemonchus sp.* eggshell membrane. It may play a central role in the antiparasitic activity (81). However, further research is necessary to elaborate elucidation of the chemical processes involved.

Beloin et al. (82) worked with different extracts of *M. charantia* leaves and found a direct anthelmintic effect of momordicins (saponins) and triterpenes on *Caenorhabditis elegans* (nematode) using a 500 mg/mL lethal concentration. Similar results were reported in a study by Ling et al. (83). They found momordicins in an ethanolic extract of *M. charantia*, and demonstrated the adverse effects on the feeding processes of the L_2 and L_3 stages of *Plutella xylostella* (a pest of ornamental crops). They also reported lethal larval concentrations of momordicin II of 76, 69, and 116.24 mg/mL, respectively. Other effects reported were inhibition of weight gain and reduced larval survival.

Studies by Islam et al. (84) used aqueous methanolic and ethanolic extracts of *M. charantia* leaves, showing effects on the hatching of *Ascaridia galli eggs*. Using a concentration of 4% of the extract, they reported 70% and 67% eggs development adverse effects with the aqueous extract and 65% with the methanolic extract. These results indicated that a higher concentration was needed and support the concentration used in the present study (16%) to achieve similar results to the positive control (Table 1).

Tjokropranoto and Nathania (59) studied the effect of *M. charantia* in adult nematodes. From an *in-vivo* experiment in pigs, they reported that after treatments with an ethanolic extract of *M. charantia* leaves at concentrations of 10%, 20%, and 40%, the average percentage of *Ascaris suum* that were paralyzed or killed was 75%, 83%, and 88% respectively. Likewise, Chastity et al. (60) studied an ethanolic extract of *M. charantia* leaves to estimate the mortality rate on *Ascaris suum.* They found that the adult nematodes mortality time was 10 hours, with the 80% extract concentration compared to the 4 hours required using pyrantel pamoate (positive control). Besides, Shahadat et al. (61) y Alam et al. (62) studied the effect of aqueous extracts of *M. charantia* on *Ascardia galli* affecting broilers. They found very high mortality rates (75% and 95%, respectively) using concentrations up to 100 mg/mL. This high concentration was chosen to induce short-term mortalities. From the studies described, it is inferred that more studies are needed on the effect of *M. charantia* extract in adult and larval stages of *Haemonchus sp.*

We confirmed the presence of terpenes, alkaloids, and anthraquinone glycosides, which are important secondary metabolites attributed to *M. charantia* with antihelmintic potential (Table 2). These compounds have been reported for this purpose by Poolperm and Jiraungkoorskul (30), Morton (43), and Bauri et al. (66). These secondary metabolites can also be found in other medicinal plants with anthelmintic activity: terpenes (67-72), alkaloids (73-75), and glycosides (67, 70, 73, 76). However,

flavonoids, other anthelmintic metabolites described in *M. charantia* were not detected by our techniques (66, 77).
The scientific inferences exposed in this study, similar to others in the detection of secondary metabolites groups of and describing their anthelmintic effects, confirm the potential of *M. charantia* extracts as beneficial compounds to be part of the integrated parasite control programs in livestock production systems in the tropics.

Conclusions

In the *in-vitro* test, the methanolic extract of *M. charantia* effectively inhibited the hatching of *Haemonchus* sp. The presence of secondary metabolites like alkaloids, triterpenes, and anthracene glucosides was detected in the methanolic extract of *M. charantia* (under the given collection period, location, and conditions).
It is necessary to develop a process of bio-guided fractionation of the methanolic extract of *M. charantia* in order further to purify the relevant compounds and evaluate its biological activity on different life stages of *H. contortus*; this being a plant species widely adapted to the conditions of the piedmont (foothills) of Meta, Colombia.

Conflicts of interest

The authors declare no conflict of interest.

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Author contributions

Conceptualization: DAJ; methodology: DAJ and AV; DAJ and AV; writing (original draft preparation) DAJ and AV; Review and editing, DAJ, AV, and LCL. All authors have read and agreed to the published version of the manuscript.