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 CL₅₀ and CL₉₀ 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 LC₅₀ and LC₉₀ 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 CL50 y CL90 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 CL50 y CL90 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 antihelminticos 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 de fraccionamiento bio-guiados de esta planta sobre diferentes estados de vida de H. contortus, siendo esta una especie vegetal ampliamente adaptada a las condiciones del piedemonte del Meta, Colombia.

Palabras clave: hemoncosis, fitoterapia, ovinocultura.

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 L3 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 explored 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 (ribose-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 extract 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 WAAAVP (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 following the 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, naphthoquinones, 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 extract 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*.

<table>
<thead>
<tr>
<th>Extract concentration - controls</th>
<th>% Hatching inhibition</th>
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<tbody>
<tr>
<td>10 mg/mL</td>
<td>38.3% A</td>
</tr>
<tr>
<td>20 mg/mL</td>
<td>24.2% A</td>
</tr>
<tr>
<td>40 mg/mL</td>
<td>34.5% A</td>
</tr>
<tr>
<td>80 mg/mL</td>
<td>50.4% A</td>
</tr>
<tr>
<td>160 mg/mL</td>
<td>84.6% B</td>
</tr>
<tr>
<td>Albendazole 25 µg/mL</td>
<td>100% B</td>
</tr>
<tr>
<td>DMSO 5 mg/mL</td>
<td>0%</td>
</tr>
</tbody>
</table>

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.

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).

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 extract of *M. charantia*.

<table>
<thead>
<tr>
<th>Groups of secondary metabolites</th>
<th>Colorimetric test /result</th>
<th>Thin-layer chromatography - Pure samples/result</th>
</tr>
</thead>
<tbody>
<tr>
<td>Alkaloids</td>
<td>Mayer’s reagent (-)</td>
<td>Quinidine (+)</td>
</tr>
<tr>
<td></td>
<td>Ammonium reineckate (-)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Dragendorf’s reagent (+)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Valser’s reagent (-)</td>
<td></td>
</tr>
<tr>
<td>Flavonoids</td>
<td>Shinoda’s reagent (-)</td>
<td>Flavone (-)</td>
</tr>
<tr>
<td>Triterpenes</td>
<td>NA</td>
<td>(+)</td>
</tr>
<tr>
<td>Anthracenic glucosides</td>
<td>NaOH 5% NH$_4$ 2% (+)</td>
<td>Sacred Cascara (-)</td>
</tr>
<tr>
<td>Cardiogenic glucosides</td>
<td>Kedde’s reaction (-)</td>
<td>Digitalina (-)</td>
</tr>
<tr>
<td></td>
<td>Keller – Kilian’s reaction (-)</td>
<td></td>
</tr>
</tbody>
</table>

found momordicins in an ethanolic extract of M. charantia, and demonstrated the adverse effects on the feeding processes of the L2 and L3 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 Ascaridia 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 anthelmintic 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; 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.

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