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RESEARCH ARTICLE

Phytochemical profile and nematicidal activity of a hydroalcoholic extract from Cazahuate flowers (*Ipomoea pauciflora* M. Martens & Galeotti) against *Haemonchus contortus* infective larvae

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ABSTRACT

Haemonchus contortus (*Hc*) is a hematophagous parasite affecting the health and productivity of flocks. The administration of chemical anthelmintic drugs (AH) is the common method of deworming; however, generates resistance in the parasites to AH and it is a public health risk due to drug residues in milk, meat and sub-products. Natural compounds from plants are explored to diminish this parasitosis, improving their health and productivity, without the negative effects of AH. *Ipomoea* genus is a group of climbing plants belonging to the Convulvulaceae family possessing perennial leaves and tuberous roots. Medicinal properties has been attributed to this plant including nutritional agents, emetics, diuretics, diaphoretics, purgatives and pesticides. The objective of this study was assessing the in vitro nematocidal activity of a hydroalcoholic extract (HA-E) obtained from *Ipomoea pauciflora* (Cazahuate) flowers against *Hc* infective larvae (L3) and to identify its phytochemical profile (PhC-P). The assay was carried out using microtiter plates (MTP). Four HA-E concentrations were assessed and Ivermectin and distilled water were used as positive and negative control groups, respectively. Approximately 100 *Hc* L3 were deposited in each well (n=12) and incubated at 25–35°C for 7 days. Data were analyzed using ANOVA and a General Linear Model (GLM) followed by Tukey test ($P<0.05$). The treatments showing a concentration-dependent effect (CDE) were analyzed to identify their 50% and 90% lethal concentrations ($CL_{50, 90}$) via a Probit Analysis. The highest mortality was observed at 50 mg/mL (82.64 ± 0.71%) and the lowest at 6.25 mg/mL (56.46 ± 2.49%), showing a CDE with increasing mortality from 6.25 to 50 mg/mL. The PhC-P revealed the presence of alkaloids, coumarins, flavonoids, tannins and triterpenes/sterols. A HA-E from flowers of *I. pauciflora* will be considered to assess its potential use in the control of haemonchosis in small ruminants.

Keywords: Nematocidal; Cazahuate; *Haemonchus*; phytocompounds.

INTRODUCTION

Gastrointestinal nematodes (GIN) infection is a problem affecting both the health and productivity of small ruminants all over the world (Ortiz-Pineda *et al*., 2020). The frequent administration of synthetically produced chemical anthelmintic drugs (CAD) has been the most common control method. However, this approach has favored the development of anthelmintic resistance (AR), diminishing the efficacy of treatments and resulting in deterioration of animal health (Bosco *et al*., 2020). In this context, AR can be defined as the capability of GIN to survive when exposed to regular doses of CAD administered in animals that are normally lethal for most nematodes (Stewart *et al*., 2020).

Due to its highly pathogenic effect and its important economic consequences the hematophagous nematode *Haemonchus contortus* is one of the most studied species of GIN, particularly in relation to AR and alternative control strategies (OIE, 2022). Strategies for the prevention and control of GIN other than the use of CAD have been explored, including a nutritional strategy based on the use of protein- and metabolizable energy-rich diet that promote the immune self-defense system of the small ruminants (Hoste *et al*., 2016); biological control using natural control of nematodes i.e., nematophagous fungi (Mendoza de Gives, 2022) and the use of plants (Olmedo-Juárez *et al*., 2022) or plant compounds with an antiparasitic effect (Delgado-Núñez *et al*., 2020). Species belonging to the *Ipomoea* genus are considered as sacred plants that are used in religious rituals due to their hallucinogenic properties e.g., *Ipomoea arborescens, I. purpurea* and *I. tricolor.* Other plants like *Ipomoea nil, I. mauritania* and *I. marginata* have been used as remedies in indigenous traditional herbal medicine, and other species e.g., *Ipomoea batatas, I. aquatica, I. dumosa* and *I. purga*, are regular constituents of the diet in Indian communities (Martínez

et al., 2007; Díaz-Pontones, 2009). Regarding the potential use of plants containing secondary metabolites with medicinal properties, members of the Convolvulaceae family e.g., *Ipomoea batatas, I. tricolor, I. pauciflora* and *I. murucoides,* are traditionally used as nutritional agents, emetics, diuretics, diaphoretics, purgatives (Pereda-Miranda & Bah, 2003) and pesticides (Jackson & Peterson, 2000; Vera-Curzio *et al*., 2009). These species have attracted the attention of workers around the world due to the ability of some of their secondary metabolites to cure some disease symptoms e.g., muscle pain, toothache, cough and insect bites (Monroy-Ortíz & Castillo, 2007). The origin of *Ipomoea pauciflora* (also called "Cazahuate o palo bobo") is unknown; however, this species is widespread in southern Mexico, mainly in Michoacán, Morelos, Puebla, Guerrero, Oaxaca and Chiapas, and in Central America it can be found in Guatemala (Dorado-Ruíz *et al*., 2013). This plant is commonly found in warm and semi-warm climates and it has been associated with deciduous tropical woodlands and xerophile shrubs. *I. pauciflora* is a shrubby tree that can reach 8 m high. Its stem can reach up to 25 cm in diameter, its leaves range between 5 and 15 cm in length and from 3 to 8 cm in width (Figure 1A), it possesses inflorescence with 1 to 5 white flowers and its seeds are coated with soft white hairs (Carranza, 2008) (Figure 1B).

To date there are no records about any anthelmintic activity of this plant or its products against GIN in small ruminants. The objectives of this study were to assess the *in vitro* nematicidal activity of a hydroalcoholic extract (HA-E) obtained from *I. pauciflora* flowers against *H. contortus* infective larvae and to identify its phytochemical profile.

MATERIALS AND METHODS

Allocation

Bioassays were performed at the Faculty of Agricultural and Environmental Sciences of the Autonomous University of Guerrero at Iguala de la Independencia Municipality, State of Guerrero, Mexico. The phytochemical profile was analyzed at The National Center of Disciplinary Research in Animal Health and Innocuity, INIFAP-SAGAR-Mɪxico, Jiutepec Municipality, State of Morelos, Mexico.

Plant material

Plant collection was carried out during December 2020 at the flowering stage. Plants were collected at Sabana Grande Village, Tepecoacuilco de Trujano Municipality, Guerrero state, Mexico (18°09'24.2" N and 99°33'09.5" W; altitude 743 m.o.s.l.) (Figure 2). The vegetation in this area is classified as deciduous tropical woodland. Two hundred and fifty grams of Cazahuate flowers were collected and dried in the shade at room temperature (25 to 35°C) for 5 days to obtain a dry weight of 150 g. Plant material was kept in paper bags until use. Flowers were taxonomically identified at the Center of Research in Biodiversity and Conservation (CIByC-UAEM), Voucher Code: 39803, Herbarium of the Autonomous University of the State of Morelos (UAEM).

Biological material

Haemonchus contortus infective larvae

Faecal cultures containing *H. contortus* eggs from an artificially infected lamb were prepared in plastic bowls. The egg-donor animal was treated under strict humanitarian controlled conditions according to principles of animal welfare and the total elimination of unnecessary animal suffering, based on the Good Management Practices policies established at INIFAP. The Norma Oficial Mexicana (Official Mexican Standard) with official rule number NOM-052-ZOO-1995 (http:/www.senasica.gob.mx), as well as the Ley Federal de Sanidad Animal (Federal Law for Animal Health) DOF 07-06-2012 (https://www.gob.mx/cms/uploads/attachment/file/118761/LFSA.pdf) were strictly abided and all the procedures performed in this study were carried out in accordance with the ethical standards outlined by INIFAP.

Faeces were crushed and tap water with polyurethane particles were added and mixed to obtain a homogeneous mass. Faecal cultures were maintained at room temperature (28–35°C) for 7 days. After incubation infective larvae were recovered using the Funnel Baermann technique. Larvae were exposed to a sodium hypochlorite solution at 0.187% concentration for 5 to 10 minutes to induce the unsheathing process. After that, larvae were washed three times with distilled water for 1.5 min at 3500 rpm to eliminate sodium hypochlorite (Delgado-Núñez *et al*., 2020).

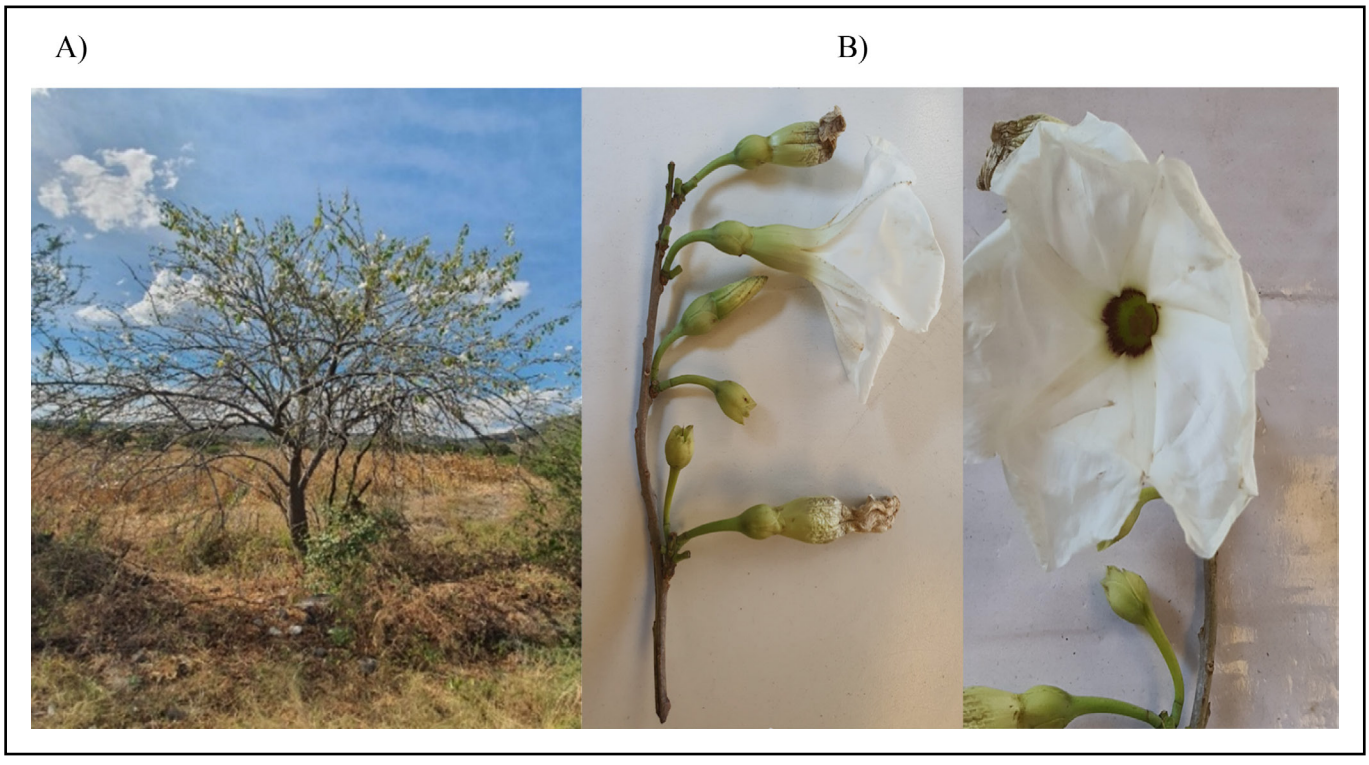

Figure 1. *Ipomoea pauciflora* A) tree and B) flowers, from Sabana Grande community at Tepecuacuilco Municipality, State of Guerrero.

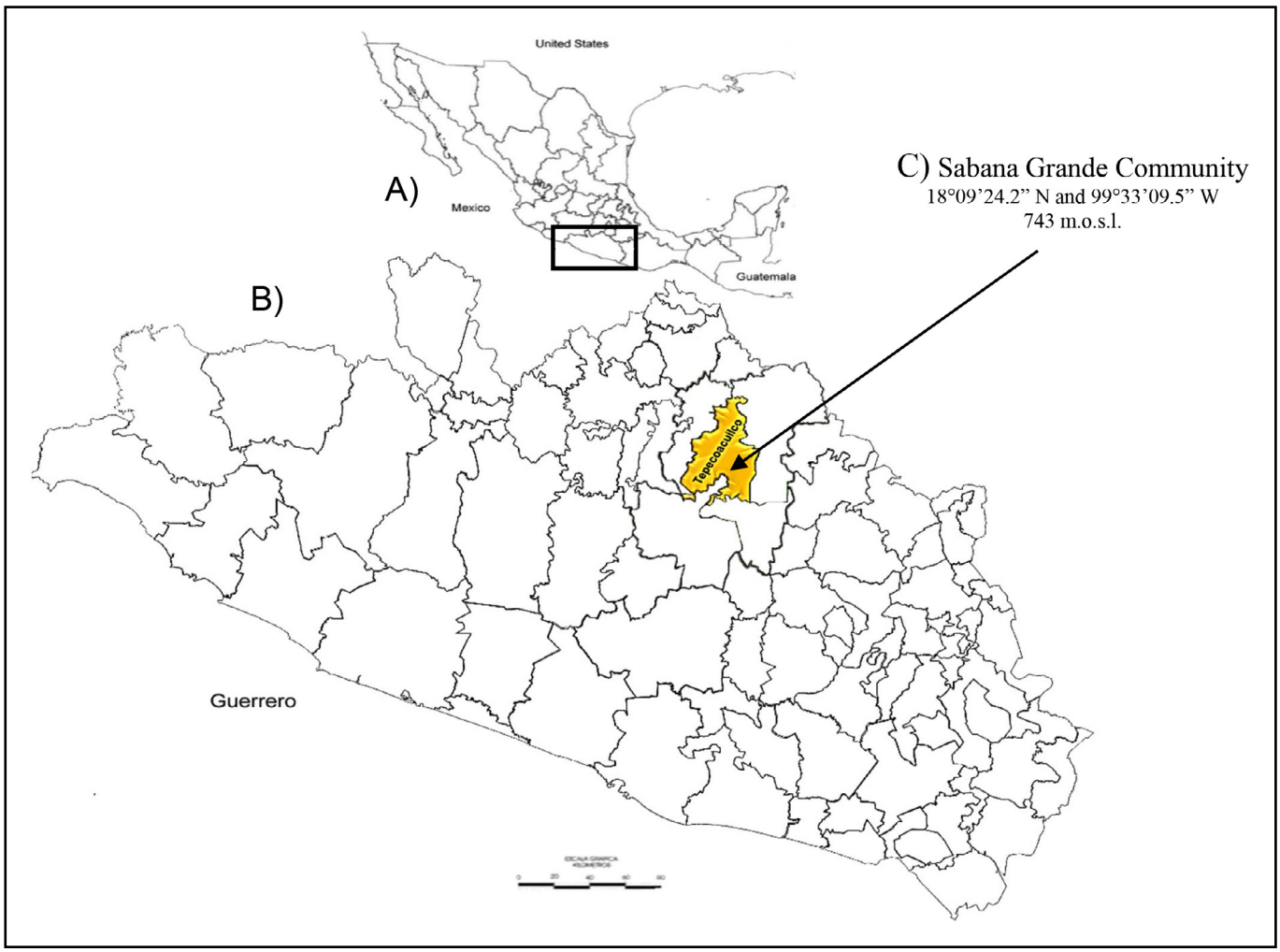

Figure 2. A) Map of the Mexican Republic; B) Map of the State of Guerrero and C) Sabana Grande community, Tepecuacuilco de Trujano, State of Guerrero, Mexico.

Ipomoea pauciflora hydroalcoholic extract (HA-E)

One hundred and fifty grams of dehydrated *I. pauciflora* flowers were used for the extraction process, which began with maceration in 70:30 water-methanol (volume/volume) at a 1:10 (weight/volume) ratio. The extract was stored under lightless conditions for 24 h. Later on, the plant material was filtered through gauze, cotton and Whatman paper No. 4 to eliminate residues and particles of plant material. The elimination of residual solvents was performed by controlling the vacuum and temperature (45–50°C) to avoid any possible degradation of compounds and/or bioactive principles using a B chi R-300 (Switzerland) rotatory evaporator. At the end of the process a semi-solid extract was obtained that was eventually lyophilized and a brown, solid material was obtained. The final extract was kept at 4°C until use for anthelmintic assessment.

Qualitative phytochemical analysis of Ipomoea pauciflora secondary metabolites

The presence of secondary metabolites in the alcoholic extract of *I. pauciflora* flowers was identified by chemical reaction using specific reagents as follows: Dragendorff, Mayer and Wagner for alkaloids; Bornträger for coumarins; Mg^{2+} particles and HCl for flavonoids. The $FeCl_3$, gelatin and saline solution assays were used to determine the presence of tannins. Additionally, the Liebermann-Burchard & Salkowski reaction was used to identify the presence of triterpenes and sterols and finally the foam formation assay was performed to determine the presence of saponins (Olmedo-Juárez *et al*., 2022).

***Haemonchus contortus* (L3) larval mortality assay**

Every assay was performed in a 96-well microtiter plate (n=12). Different concentrations of the extract were used as follows: 6.25, 12.5, 25 and 50 mg/mL. Fifty microliters of each concentration and 50 µL of an aqueous suspension containing 100±20 *H. contortus* (L3) were deposited in each well giving a final volume of 100 µL. Ivermectin at 5 mg/mL and distilled water were used as the positive and negative controls, respectively. This experiment was repeated in triplicate. All plates were incubated at room temperature (25–35°C) for 72 hours. After incubation ten 10-µL aliquots were taken from each well of each treatment and put on slides for observation under a microscope and to count both live and dead larvae. Means of live and dead larvae were recorded and a mortality rate was estimated according to the following formula:

% Mortality= [(live larvae mean) / (live larvae mean + dead larvae mean)] × 100

Microscopic analysis

Aliquots from each treatment including HA-E/larvae interaction as well as negative and positive controls were put on slides and covered with a coverslip in order to visualize any morphological changes in larvae from the different treatments using a light microscope (LEICA DM6 B). Larvae were photographed using 40× magnification. Image analysis was performed using the LAS program Version 4.9 [Build:129] copyright C ©2003-2016 Leica Microsystems Limited (Switzerland).

Statistical analysis

Data for larval mortality percentages were analyzed using ANOVA. A comparison between means of the different treatments was performed using the Tukey test ($P<0.05$). The treatments that showed a concentration-dependent effect were selected to determine their 50% and 90% lethal concentrations (CL_{50} and CL_{90}) via PROBIT analysis.

RESULTS

***Ipomoea pauciflora* hydroalcoholic extract (HA-E) performance**

The HA-E obtained from *I. pauciflora* flowers produced 17.82 g (11.82% yield) of a brown powder.

***Ipomoea pauciflora* secondary compounds**

The results of the qualitative phytochemical analysis of *I. pauciflora* showed the presence of alkaloids, coumarins, flavonoids, tannins and triterpenes/sterols (Table 1).

Larval mortality assay

The results of the larval mortality assay after 72 hours exposure of *H. contortus* infective larvae (L3) to *I. pauciflora* HA-E as well as controls are shown in Table 2. Larval mortalities ranging between 56.46% and 68.24% were recorded at 6.25, 12.5 and 25 mg/mL of HA-E concentrations. Larval mortality was below 1% in negative control and 100% in positive control. In addition, a clear concentration/dependent effect was recorded (*P<0.05*) from 6.25–50 mg/mL (56.46 ± 2.49% to 82.64 ± 0.71%).

The lethal concentrations (50% and 90% mortality) are shown in Figure 3. The lethal minimum inhibitory concentration (LC_{50}) was 5.67 mg (4.28–6.99 mg/mL) and the LC_{90} was 84.38 mg (62.71–128.37 mg/mL).

Table 1. Groups of secondary metabolites identified in *Ipomoea pauciflora* (Cazahuate) flowers using different reagents

Metabolite and reagent	Colorimetric reaction	Results
Alkaloids		
Dragendorff	Turbidity or precipitate	++
Mayer	(red to orange, white to cream	++
Wagner	and brown)	++
Coumarins		
Borntrnger	Yellow fluorescence (U.V)	++
Flavonoids		
Mg^{2+} and HCL	Red, orange and violet colour	++
Tannins		
Ferric chloride ($FeCl_3$)	Hydrolysable tannins (blue)	–
	Condensed tannins (green)	+++
Confirmation		
Solution of gelatine	Precipitate white	+
Gelatine and saline solution	Precipitate white	+
Saline solution	Precipitate white	–
Triterpenes/sterols		
Liebermann-Buchard	Colour blue, blue-green (sterols)	+
Salkowski	Red to purple (triterpene)	+
Saponins		
Water	Foam formation	–

(–) Not detected (+) light positive reaction (++) positive reaction (+++) strong positive reaction.

Table 2. Mortality percentages of *Haemonchus contortus* infective larvae after exposure to different concentration of a *Ipomoea pauciflora* hydroalcoholic extract

Treatments	Means of dead *H. contortus* (L3)/ total number of larvae	Mortality percentages ± SD
I. pauciflora (mg/mL)		
HA-E_{50}	85.75/97.75	82.64 ± 0.71^{b}
HA-E_{25}	70.25/95.5	68.24 ± 1.28^{c}
HA-$E_{12.5}$	60.75/99	61.39 ± 1.50^{d}
HA-$E_{6.25}$	56/100.75	56.46 ± 2.49^{e}
H_2O	1/101.25	0.95 ± 0.79^{f}
Ivermectin (5 mg/mL)	99/99	100 ± 0.0^{a}
Variation Coefficient	2.22	
R^2	0.99	

^{abcdef} = Means into same column with different literal indicate statistic difference (Tukey P<0.05).
SD= Standard Deviation.

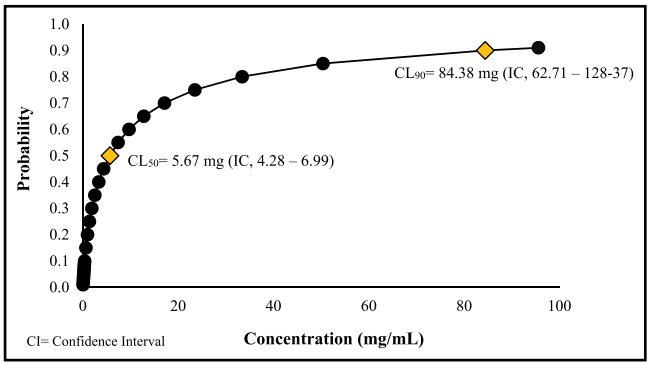

Figure 3. Lethal concentrations to cause 50 and 90% mortalities of *Haemonchus contortus* (L3) after 72 h exposure to *Ipomoea pauciflora* hydroalcoholic extract.

Morphological analysis of larvae by microscopic analysis

A set of microphotographs showing the characteristics of *H. contortus* (L3) in controls and after exposure to HA-E is shown in Figure 4. The appearance of a normal larva was observed in negative control group (Figure 4a). The larvae showed regular active undulatory sinusoidal movement while both external and internal morphological structures were normal. There were no apparent morphological changes in positive control group; however the larvae were motionless and curved, stretched or semi-stretched (Figure 4b). In order to confirm their status as dead larvae, a physical stimulus were applied with a needle on their cuticle to see if they would move or remain motionless. Consequently, 100% of the larvae were designated as dead larvae. Larvae exposed to HA-E had no noticeable external changes; however there were severe internal changes observed mainly with loss of intestinal cell integrity (Figure 4c).

DISCUSSION

In a study performed by Mila-Arango *et al*. (2014) authors reported 10.2% yield using an aqueous extract obtained from the same plant species. This yield is similar to the one we obtained in our study. The percentage of yield depends on the polarity grade. In this context, solvents with medium and low polarity organic solvents result in a lower performance (Soto-García & Rosales-Castro, 2016).

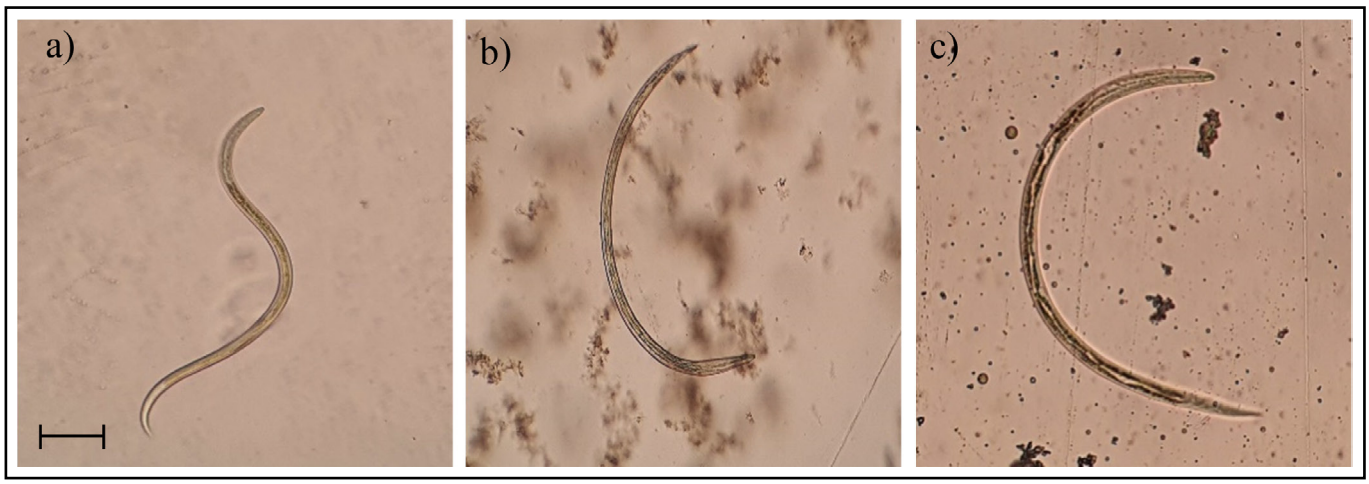

Figure 4. Microphotographs of *Haemonchus contortus* larvae from a) negative control, b) positive control and c) *Ipomoea pauciflora* HA-E exposed groups after 72 hours. Bar scale= 40µM.

The *Ipomoea* spp. contain a wide variety of secondary metabolites with a number of biological activities including insecticidal, herbicidal and medicinal (Valencia-Díaz *et al*., 2021). The *I. pauciflora* HA-E used in this study contained important secondary metabolites i.e., alkaloids, coumarins, flavonoids, a high amount of tannins, a low content of triterpenes/sterols and no saponins. This profile is similar to those reported by Jenett-Siems *et al*. (2004), who explored the chemical compounds of *Astripomoea malvacea* and *Falkia repens*, and by Mila-Arango *et al*. (2014), who worked with *Ipomoea murucoides* and *I. pauciflora* (these plants belong to the Convolvulaceae family). These studies revealed only small differences among the species; the metabolic variations could be due to abiotic factors in the environment including soil properties, water, light, height, temperature, cutting or amputation and also biotic factors such as an attack of pathogenic microorganisms, such as viruses, bacteria, fungi, nematodes and herbivorous organisms (Hartmann, 2007; Agrawal & Weber, 2015). The HA-E from *I. pauciflora* flowers resulted in a relatively high percentage mortality (>82%) of *H. contortus* larvae at 50 mg/mL. Nonetheless; there was more than 56% larval mortality at the lowest concentration (6.25 mg/mL). These findings indicate a clear concentration-dependent effect ($P<0.05$) of the extract on the larvae. Likewise, 50% and 90% lethal concentrations (LC_{50}, LC_{90}) were identified at 5.67 mg (CI, 4.28–6.99) and at 84.38 mg (CI, 62.71–128-37), respectively.

It is important to mention that this is a biological study and may have variations in plants from same species from other sites including other altitudes and latitudes with different environmental conditions and even variations in the susceptibility of other *H. contortus* strains. Nevertheless, to date there is only limited information about the nematicidal activity of plant extracts obtained from *Ipomoea* spp. against the blood-feeding nematode *H. contortus*. This could be considered as an area of opportunity, since chemical anthelmintic drugs synthesized in the laboratory are usually used as a common GIN control by farmers. Therefore, these drugs could be replaced by natural compounds obtained from plants like *I. pauciflora*. In another study of a hydroethanolic extract from leaves of another species, *I. imperati*, Hertzberg *et al*. (2002) reported *in vitro* inhibition of the unsheathing process of *H. contortus* (L3). These authors reported an LC_{50} of 0.22 mg/mL. The fact that this extract inhibited the unsheathing process is important, given that the third stage is a transitional developmental into the parasitic stage of *H. contortus*. If this extract is able to break the life cycle of the parasite at this stage, it could stop the establishment of the parasitosis (Hertzberg *et al*., 2002). Similarly, a methanolic extract obtained from seeds of the plant *Ipomoea hederacea* showed high *in vitro* larvicidal activity against *H. contortus* (L3) with an LC_{50} of 2.07 mg/mL (Zia-Ul-Haq *et al*., 2012). These authors used ivermectin as a control with an LC_{50} at 3.19 mg/mL. These findings indicate that some polar compounds from *Ipomoea* spp. exert high anthelmintic activity against *H. contortus*. In recent years, other biological models have been explored for the response to *Ipomoea* spp. extracts with different polarities (Table 3). For example, a hexane and chloroform extract obtained from *I. pauciflora* seeds resulted in high *in vitro* entomopathogenic activity against the lepidopteran *Spodoptera frugiperda* (Guzmán-Pantoja *et al*., 2010). In addition, methanolic extracts from *I. murucoides* leaves and flowers caused 49.16% and 35.59% mortality in *S. frugiperda* larvae, respectively, at 2 mg/mL (Vera-Curzio *et al*., 2009). Plant organic extracts with a wide range of polarity have been used in a number of anthelmintic bioassays; this chemical separation strategy is very helpful for obtaining a large amount of secondary metabolites with potential use in the control of agricultural pests. On the other hand, plant extracts macerated with water (polar extracts) showed lower nematicidal activity than those extracts obtained using organic agents (Bizimenyera *et al*., 2006). This could be due to water can only extract a few bioactive compounds (Alawa *et al*., 2003). In contrast, medium polarity organic solvents i.e., dichloromethane, ethyl acetate and acetone, and low polarity agents i.e., petroleum ether, hexane and toluene, can separate a wider range of compounds with higher ovicidal and larvicidal activity against *H. contortus* (Adamu *et al*., 2013; Fouche *et al*., 2016). Regarding the results of the microscopy analysis, similar morphological alterations were observed in the larvae exposed to the *I. pauciflora* extract in another study where *H. contortus* infective larvae were exposed to isorhamnetin (a flavonoid compound obtained from *Prosopis laevigata* leaves) and loss of architectural integrity of intestinal cells was observed (Delgado-Núñez *et al*., 2020). Other morphological changes i.e., slimming of either the anterior and posterior parts of the body, were observed when *H. contortus* (L3) were exposed to compounds obtained from *Acacia cochliacantha* HA-E (Olmedo-Juárez *et al*., 2017). It is interesting that larvae exposed to ivermectin showed no apparent morphological changes. This could be because the mode of action of this drug is hyperpolarizing and paralyzes the pharyngeal and somatic muscles, but no structural changes occur (Laing *et al*., 2017). The results obtained in the present study encourage further identification of the secondary metabolites responsible for nematicidal activity.

Table 3. Efficacy records in the nematocidal activity of plants extracts using organic solvents against nematodes of different taxonomic groups

Plant	Part of the plant	Solvent	Blank nematode	Efficacy (EHI)	Author(s)
Artemisia absinthium	Plant shoots	Water	*Meloidogyne incognita*	98.7%	Korayem *et al.,* 1993
Citrullus colosynthis	Plant shoots			87.5%	
Thymus vulgaris	Plant shoots			100%	
Punica granatum	Fruits			100%	
Ricinus communis	Peeled Seeds			87%	
Chelidonium majus	Aerial parts	Methanol	*Toxocara canis*	RM_{50} = 28 µM	Satou *et al.,* 2002
Macleaya cordata	Aereal parts			RM_{50} = 18 µM	
Macleaya cordata	Aereal parts			RM_{50} = 58 µM	
Dichapetalum filicaule	Roots	Petroleum ether	*Necator americanus*	100% EHI	Chama *et al.,* 2015
		Chloroform/acetone		98.7% EHI	
		Methanol		100% EHI	
Ajania nubigena	Aerial parts	Methanol	*Trichuris muris*	CI_{50}= 9.7 µg/mL	Wangchuk *et al.,* 2016
Anacardium occidentale	Barks	Ethanol 70%	*Onchocerca ochengi*	LC_{50}/IC_{50})2.76 µg/mL	Ndjonka *et al.,* 2018
Sophora mollis	Roots and stems	Water	*Meloidogyne incognita*	78% mortality	Ismail *et al.,* 2020
S. mollis	Leaves			88% mortality	
Ephedra intermedia	Roots & aerial parts			80% mortality	
Urtica dioica	Roots & Stems			30% mortality	
	Leaves			80% mortality	
Allium sativum	Bulbs			92% mortality	
Zingiber officinale	Rhizome	Water-Methanol	*Oesophagostomum dentatum*	62% larval migration	Garcɒa-Munguɒa *et al.,* 2021

RM50 = Relative Mobility50; EHI = Egg Hatching Inhibition.

CONCLUSIONS

The results obtained in this study show that a hydroalcoholic extract obtained from *I. pauciflora* flowers possesses important *in vitro* nematicidal activity against *H. contortus*, which is considered one of the most economically important nematodes affecting the livestock industry. Additionally, the phytochemical profile showed the presence of alkaloids, coumarins, flavonoids, tannins and triterpenes/sterols. These compounds could be implicated in the observed nematicidal activity; however, further studies to elucidate the nematicidal molecule through spectroscopic and spectrometric studies must be performed.

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Conflict of Interest

The authors declare no conflicts of interest.

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