



***Artemisia cina* 30 CH homeopathic treatment against *Haemonchus contortus***



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**Abstract:**

The anthelmintic resistance problem is widely recognized in sheep production. Therefore, new methods of control against gastrointestinal nematodes (GIN) need to be integrated. The aim of this work was to assess the toxicity of *A. cina* 30 CH as a homeopathic product against *Haemonchus contortus* in *in vitro* and *in vivo* assays. *A. cina* 30 CH was obtained from a commercial laboratory, and confirmation of artemisinin as a key ingredient was performed with mass spectrophotometry. The *A. cina* 30 CH and the artemisinin pure reagent were used for the inhibition of egg hatching (IEH) and for the inhibition of larval migration of *H.*

*contortus* L<sub>3</sub> (ILM). In addition, three groups of 10 naturally infected lambs with GIN were treated with *A. cina* 30 CH and albendazole, and 10 were used as control. The parasitic infection was monitored at 0, 7, 14 and 28 d posttreatment (PT) to determine the number of eggs per gram (epg) and FAMACHA index. The *in vitro* data showed 100 % IEH and 64.7 % ILM by *A. cina* 30 CH, and nonlethal activity was observed with the artemisinin pure reagent. The toxicity of *A. cina* 30 CH against *H. contortus* in infected lambs was observed after 7 d of infection. Administration of the *A. cina* 30 CH yielded a 69 % reduction in the epg at 28 d PT, similar to the albendazole ( $P < 0.05$ ). In conclusion, *A. cina* 30 CH had the ability to IEH and ILM of *H. contortus* in *in vitro* assays and reduced the number of eggs of *H. contortus*, which is the primary parasitic nematode in grazing lambs, thereby reducing infection.

**Key words:** *Artemisia cina* 30 CH, Artemisinin, *Haemonchus*, Lambs.

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## Introduction

Gastrointestinal nematodes (GIN), primarily *H. contortus*, which is the most prevalent nematode in tropical regions, are among the primary pathogens that reduce animal production<sup>(1)</sup>. For a long time, anthelmintic drugs have been used as the main traditional method of control, and only one is on the market<sup>(2)</sup>. However, the inadequate use of this drug has caused worldwide resistance problems in various ruminant species<sup>(3,4)</sup>. The high prevalence and the fast dispersion of anthelmintic resistance have increased in domestic ruminants, which show resistance to multiple anthelmintic drugs in certain regions<sup>(5)</sup>. In Mexico, diverse reporting on GIN has occurred, and other strategies of control are under study<sup>(6)</sup>.

The use of different methods of control have been denoted Parasite Integral Control (PIC)<sup>(7)</sup>. The strategy of most studies is to focus on the control of highly pathogenic nematodes, such as *H. contortus* and *Teladorsagia*, in small ruminants because of their blood-feeding habits. Paddock rotation, selection of resistant breeds, biological control (*i.e.*, nematophagous fungi and predatory nematodes)<sup>(8)</sup>, selective deworming, vaccines and derivatives of the traditional herbology (*i.e.*, homeopathic products) are considered in the PIC<sup>(9)</sup>. However, more studies

are needed of alternative methods to reduce the epg and the adult nematodes during husbandry procedures<sup>(10)</sup>.

Homeopathic compounds are substances of different origins, such as vegetable or mineral, that have therapeutic effects. The homeopathic products are prepared following the instructions of the Homeopathic Pharmacopeia<sup>(10,11)</sup>. For instance, the homeopathic products obtained from plants are acquired as ethanolic extracts (generally), and they are diluted in 99 parts alcohols until the desired concentration (decimal and centesimal) below the Avogadro number  $6.02214 \times 10^{23}$  is reached. In this way, the homeopathic drugs are obtained with low inversion and easy extraction and represent a safe method of control<sup>(12)</sup>. Recently, several reports regarding the possible use of homeopathic products with a nematicidal effect have provided a new opportunity to integrate *A. cina* as a novel method of control. *A. cina* is a plant that belongs to the Asteraceae family and contains artemisinin as the active metabolite<sup>(13)</sup>. This plant has shown anthelmintic and antimalarial properties<sup>(14)</sup>. For instance, *A. cina* appears to have a potential therapeutic effect against parasites, but further study is required to determine if *A. cina* can be used as a homeopathic or natural product as a possible anthelmintic against GIN. *A. cina* took is conformed by the 30 centesimal hannemaniana (CH) concentration as reported by the Mexican Homeopathic Pharmacopoeia (concentration:  $10^{-60}$ M), which is suggested to be administered in ruminants. The aim of this study was to determine the antiparasitic efficacy of a homeopathic product based on *A. cina* 30 CH in *in vitro* and *in vivo* assays against a natural infection of small ruminants with GIN.

## Material and methods

### Locality

The analysis by mass spectrometry was carried out in laboratory 5 of Unidad de Investigación Multidisciplinaria, and the *in vitro* analysis carried out in laboratory 3 and 5 of the Unidad de Investigación Multidisciplinaria of Facultad de Estudios Superiores Cuautitlán (FESC), UNAM, in Cuautitlan Municipality, State of México and the *in vivo* assay on a farm in Mixquiahuala Municipality, Hidalgo State at 2,100 m asl with a semi-dry climate, an annual temperature of 16.6° C and rainfall of 500 mm<sup>(15)</sup>.

## Identification of artemisinin in *A. cina* 30 CH

The artemisinin molecules were identified from *A. cina* 30 CH commercial products (Millenium Lab, México). Ultra-performance liquid chromatography with mass spectrometry (UPLC/MS) was used with a reversed-phase column in positive mode. All samples were performed according to the following conditions: 70 cone velocity, Sm (Mn 2\*0.75) and UPLC/MS reading from 200 to 300 m/z laboratory 5 of Unidad de Investigación Multidisciplinaria. Concentration of *A. cina* 30CH was  $10^{-60}$ M.

## Parasites

Faeces positive for parasitic nematode eggs were collected from a donor lamb previously infected with 5,000 eggs of *H. contortus* L<sub>3</sub>, a strain isolated and maintained in the FESC, UNAM. The quantitative McMaster technique was used to determine the number of epg, and coproculture techniques were performed to collect *H. contortus* L<sub>3</sub> at 21 d post-infection (PI). Larvae were kept at -20 °C until used (the larvae recovered from the larval culture were cryopreserved in glycerol; for bioassays, the larvae were thawed at room temperature, and 95 % motility was verified).

## Bioassays

Two different *in vitro* assays were performed to determine the inhibition of egg hatching (IEH) and the inhibition of larval migration (ILM)<sup>(16)</sup>. All techniques were performed using 100 eggs or infective stages of larvae (L<sub>3</sub>) of *H. contortus*. For each assay, three replicates were prepared, and five treatments were applied as follows: 1) 20 µL *A. cina* 30 CH ( $10^{-60}$ M); 2) 100 µL of distilled water (DW, control); 3) 50 mg/mL albendazole (ABZ, control) (Sigma-Aldrich, St Louis, Missouri, USA), solubilized with 0.1 mg/mL dimethyl sulfoxide (DMSO); 4) 20 µL of ethanol; and 5) 1 mg/mL artemisinin (Sigma-Aldrich, St Louis, Missouri, USA).

The IEH was performed in ELISA plates that were incubated at 28 °C for 48 h. The IEH reading was conducted using Lugol's iodine solution, which was added to each well after incubation. The total volume of each ELISA well was read to count the number of active *H. contortus* L<sub>1</sub> and IEH per well with a microscope under 10× magnification (Olympus, model CK-2, Japan).

The ILM received similar treatment as the IEH, except that the ABZ was replaced by levamisole (300 mg/mL). Larvae were also read using Lugol's solution after incubation. The total volume per well was read to determine the ILM.

### ***In vivo* assays**

#### **Lambs**

Thirty Suffolk breed lambs, 16 males and 14 females, 3 mo of age and at 20 d post-weaning, were naturally infected with GIN. All lambs were kept in semi-stabled conditions, grazing in paddocks during the day and kept inside at night. Lambs were fed commercial concentrate and water *ad libitum*. No anthelmintic treatment was applied to any lamb before the present study. All lambs were positive for GIN eggs, which was confirmed by McMaster and coproculture techniques.

#### **Experimental design**

Prior to the treatments, the lambs were randomized into three groups of 10 each with the support of the Statgraphics Centurion XV. Treatments were designed as follows: group A received 1 mL per 5 kg of body weight (BW) by oral route of *A. cina* 30 CH (Millenium Laboratories, México) as a single dose, concentration of *A. cina* 30CH was  $10^{-60}$ M. (1); group B was orally treated with ABZ at 7.5 mg/kg of BW; and group C, without treatment, was used as the control. Faecal and haematological samples were collected at 1 d pre-treatment (0 day) and at 7, 14 and 28 d post-treatment (PT). In addition, eye mucosa colour was observing using the FAMACHA index.

#### **Statistical analysis**

The means of *H. contortus* eggs and larvae L<sub>3</sub> were compared between treatments and control groups using ANOVA analysis, complementary with Tukey's test to identify the differences between treatments, using Statgraphics Centurion XV software. The number of epg was transformed to  $\log_{10} \text{epg} + 10$  to stabilize the variance, and the least significant difference (LSD) test was applied using Statgraphics Centurion XV software with a completely

randomized design that considered repeated measurements over time and treatment. Differences with  $P < 0.05$  were considered to be significant.

## Ethics note

The management of the lambs was performed according to the Guideline of the Institutional Committee for the Care and Use of Experimental Animals of the Facultad de Estudios Superiores Cuautitlán-UNAM (CICUAE- FESC- UNAM) and authorized under Protocol No. DC- 2014-14.

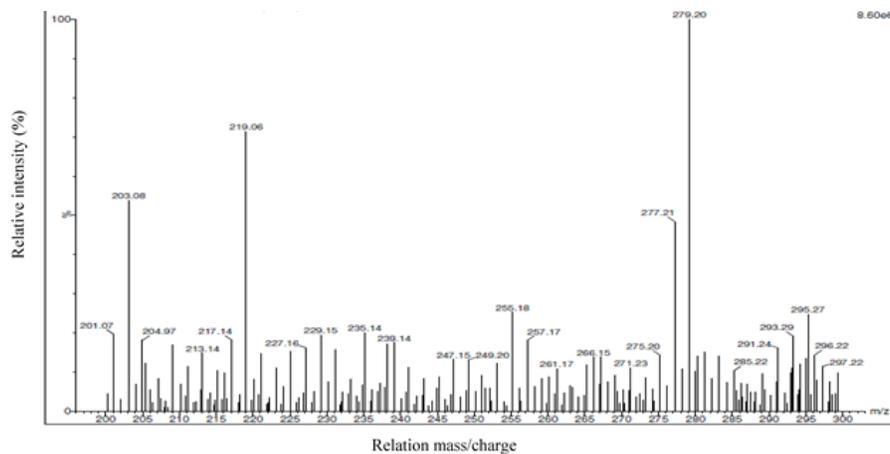
## Results

### Identification of artemisinin in *A. cina* 30 CH

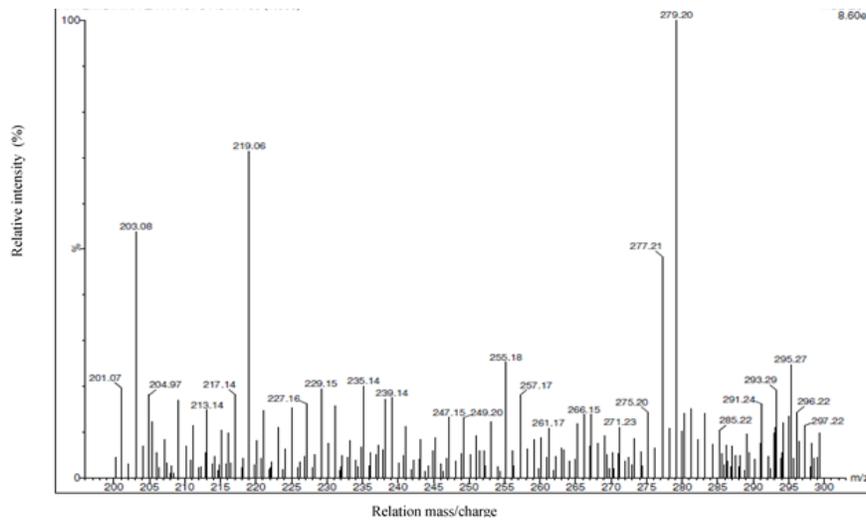
The mass spectrophotometry analysis showed artemisinin molecules in the *A. cina* 30 CH commercial products used in the present study. The *A. cina* 30 CH chromatographic analysis was performed to compare the profile of the commercial products to the artemisinin pure reagent. Figure 1, a-b showed artemisinin molecules corresponding to *A. cina* 30 CH and the artemisinin pure reagent with 279.20 m/z.

**Figure 1a-b:** Mass spectrophotometry analysis of pure reagent (a) and *A. cina* 30 CH (b), showing similarities between 283 and 290 m/z

a



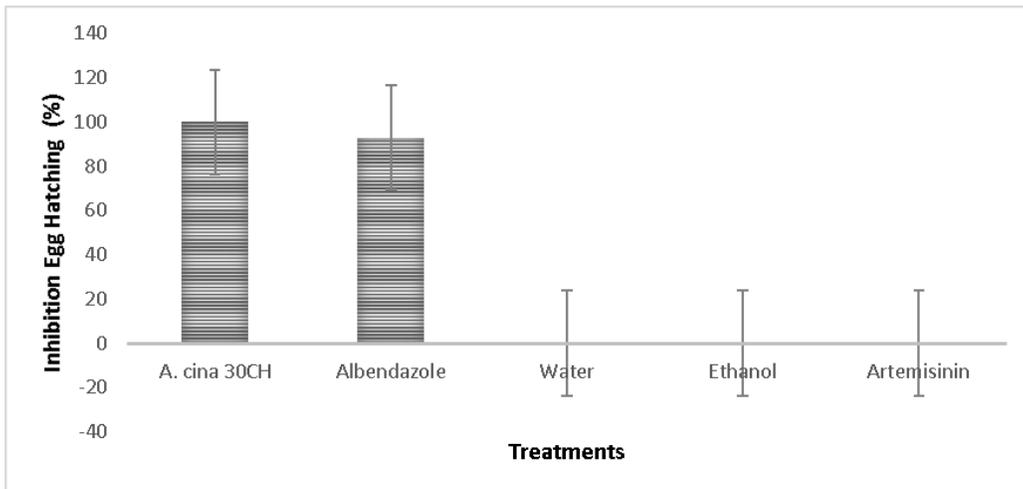
b



***In vitro* assays**

**IEH.** Data obtained from the evaluation of *A. cina* 30 CH showed 100 % IEH of *H. contortus* *A. cina* after 48 h; this result was followed by the ABZ treatment, with 93 %. In contrast, no IEH of *H. contortus* was observed with the 80 % ethanol, the artemisinin pure reagent and the DW treatments did not show effect (Figure 2).

**Figure 2:** Inhibition of egg hatch assays against *Haemonchus contortus* eggs exposed with *Artemisia cina* 30 CH and albendazole treatments and controls (artemisinin, water and methanol)



**ILM.** The *A. cina* 30 CH showed 65.7 % inhibition of migration of the *H. contortus* infective larvae. Different results were observed with the artemisinin pure reagent used at 0.1 and 1 mg/mL with DW and ethanol, with all treated groups showing 100 % larval migration, indicating that no inhibition was observed in the control groups. The levamisole used as the anthelmintic showed lethal efficacy of 100 % against larvae; therefore, no migration was observed (Table 1).

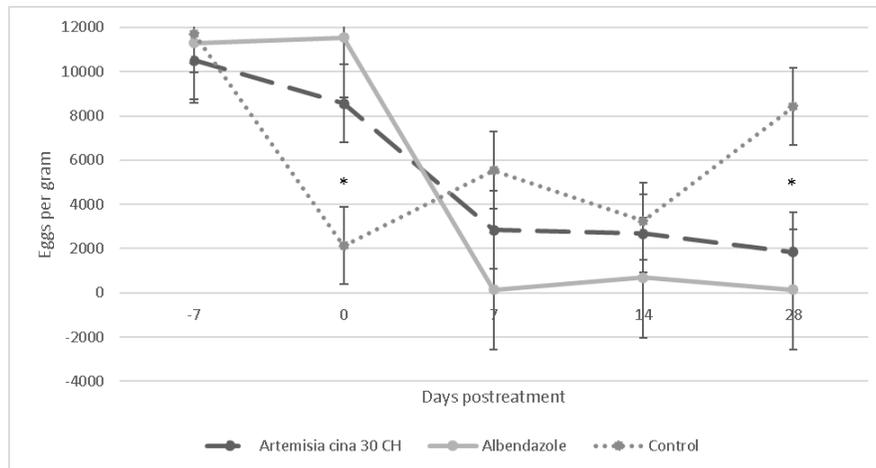
**Table 1:** Percentage of inhibition of larval migration (ILM) against L<sub>3</sub> *Haemonchus contortus* (X±SE)

Treatments	Migration (%)
<i>Artemisia cina</i> 30 CH	35.0 ± 8.1
Levamisole (300 mg/mL)	0
Water	92.0 ± 12.4
Ethanol 80%	86.6 ± 11.5

### *Artemisia cina* nematicide efficacy

Natural infection with GIN on grazing lambs showed two main GIN species: *H. contortus* (75%) and *T. circumcineta* (25 %). Infected lambs for all groups showed a mean of approximately 2,000 epg prior to treatment (d- 0). Through the following periods, significant differences in the reduction in epg were observed at 7 and 14 d PT ( $P<0.05$ ) (Figure 3). In addition, significant differences were also observed between groups A (*A. cina* 30 CH) and B (ABZ) in comparison to group C (control,  $P<0.05$ ) at 7 d.

**Figure 3:** Egg per gram observed at -7, 0, 7, 14 and 28 d posttreatment of lambs infected naturally with gastrointestinal nematodes \*( $P<0.05$ )



**FAMACHA Index Card.** For all groups, the FAMACHA card index was determined to be 3.0 to 5.0 in the *in vivo* study. The FAMACHA values were variable for all groups. Important differences were observed at 14 and 28 d PT for the *A. cina* 30 CH and ABZ treatments (Table 2).

**Table 2:** FAMACHA index of lambs naturally infected with gastrointestinal nematodes and receiving *Artemisia cina* 30 CH or albendazole

Treatment	Days post-treatment				p
	0	7	14	28	
<i>Artemisia cina</i> 30 CH	3.0 ± 0.13 <sup>a</sup>	4.0 ± 0.44 <sup>aA</sup>	3.0 ± 0.52 <sup>bB</sup>	2.0 ± 0.31 <sup>bA</sup>	0.44
Albendazole	5.0 ± 0.26 <sup>a</sup>	3.0 ± 0.21 <sup>bB</sup>	2.0 ± 0.30 <sup>cA</sup>	1.0 ± 0.22 <sup>cA</sup>	0.19
Control	3.0 ± 0.25 <sup>b</sup>	2.0 ± 0.29 <sup>aB</sup>	1.0 ± 0.20 <sup>aA</sup>	3.0 ± 0.71 <sup>bB</sup>	0.62

Equal lower case letters have no statistical significance and different lower case letters have statistical difference within the group ( $P < 0.05$ ). Equal capital letters have no statistical significance and different capital letters have statistical difference between groups ( $P < 0.05$ ).

## Discussion

*A. cina* has chemical compounds, such as the terpenoids, that provide insecticidal activity against reproductive capacity and cause antioxidative stress in the pathogens<sup>(17,18,19)</sup>. In recent years, important results identified artemisinin as having a possible anthelmintic effect<sup>(20)</sup>. For instance, Akkari *et al*<sup>(21)</sup>, reported a lethal dose (LD) of *Artemisia campestris* of 0.8 mg/mL against *H. contortus* when using an ethanolic extract. In the present study, *A. cina* 30 CH showed 100 % of *H. contortus* IEH. In addition, *A. cina* 30 CH showed efficacy for decreasing the larval migration, and these results were like those reported by others<sup>(22)</sup>. Bashtar *et al.*<sup>(22)</sup> described the ethanolic extract of *A. cina* with efficacy against the cestode *Moniezia*. In addition, the present study had 64.7 % ILM of *H. contortus* using *A. cina* 30 CH. It was reported a reduction in the larval rate in rats infected with the nematode *Trichinella spiralis* when the rats were treated with *A. cina* 30 CH, *Podophyllum* 0 and Santoninun 30 CH (homeopathic products) in 68.14 %, 84.10 % and 81.20 % respectively<sup>(23)</sup>. Conversely, artemisinin pure reagent was used as control, and no inhibition of egg or larval migration was observed. These results suggest that the absence of activity might have been produced by the chemical conformation of *A. cina* 30 CH, and a solvent that used compounds with hydrogens and phenyl rings, thereby enabling a fast change in conformation.

Regarding the natural infection with GIN and the *A. cina* 30 CH treatments applied after 2 wk, the ABZ and *A. cina* 30 CH showed significant differences ( $P < 0.05$ ) in reductions in the

number of epg. These findings were like those reported by Bashtar *et al*<sup>(22)</sup>, who found a reduction of proglottids of *Moniezia* sp. in animals treated with *A. cina*. However, more studies are required to confirm the possible anthelmintic effect of *A. cina* 30 CH against nematode stages using artemisinin from native plants.

Treatments with *A. cina* 30 CH and ABZ against natural infection improved the FAMACHA values caused by the blood-feeding habit of *H. contortus* at 7 and 14 d after administration ( $P<0.05$ ). Similar results were found by Cala *et al*<sup>(1)</sup>, with artemisinin supported as a possible nematicide metabolite after infection. Demeler *et al*<sup>(24)</sup> showed the anaemia caused by *H. contortus* infection in lambs treated with ABZ showed nematicidal efficacy. A review carried out by Kerboeuf *et al*<sup>(25)</sup> suggests the activity of flavonoids on the structure and cell target is similar to the antioxidant effect caused by artemisinin. Although the *A. cina* anthelmintic mechanism of action is unknown, determination of this mechanism is needed for its application to nematodes infecting hosts. It was demonstrated the stability of artemisinin in the rumen, which was detectable in blood samples at 33 mg of artemisinin/kg of body weight<sup>(21)</sup>. The study of *A. cina* 30 CH showed participation of the drug as an anthelmintic, and it should be considered as a possible method for use in the control of parasitic nematodes.

## Conclusions and implications

The *A. cina* 30 CH had anthelmintic efficacy against *H. contortus* egg hatching during natural infection. The FAMACHA index suggest reduction of nematode activity after treatment with *A. cina* 30 CH and ABZ. In addition, this product demonstrated inhibition of egg hatching and larval migration, which indicates its possible anthelmintic effect. To optimize the use of this homeopathic compound, the mechanism of its action must be determined.

## Acknowledgments

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## Conflict of interest statement

None of the authors declare a conflict of interest.

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