https://doi.org/10.22319/rmcp.v15i3.6468

Technical note

Fatty acids and terpenes from the methanolic extract of *Artemisia cina* as possible compounds responsible for the ovicidal effect on *Haemonchus contortus*

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Abstract:

Haemonchus contortus is a hematophagous nematode with a high reproduction rate, considered to be the main problem in grazing small ruminants. Therefore, treatment

alternatives based on the use of plant extracts are sought. This study aimed to evaluate the ovicidal activity of *Artemisia cina* against the parasite *Haemonchus contortus* and to chemically characterize the extract with the highest biological activity through gas chromatography coupled with mass spectrometry (GC-MS). The extracts to be evaluated were obtained through the maceration technique using methanol, ethyl acetate, and n-hexane. The extracts were taken to total dryness and challenged against *H. contortus* eggs using the egg-hatching inhibition technique described by the World Association for the Advancement of Veterinary Parasitology (WAAVP). The methanolic extract (ME) showed 100 % ovicidal activity at a concentration of 4.25 mg/ml, being the most active at a low concentration; therefore, it was characterized by GC-MS. ME mainly contains fatty acids and terpenes; among them are hexadecanoic acid and 2-[4-methyl-6-(2,6,6-trimethylcyclohex-1-enyl)hexa-1,3,5-trienyl][cyclohex]-1-en-carboxyaldehyde. The characterized compounds have shown previously reported anthelmintic activity so that ovicidal activity may be associated with them. In conclusion, the methanolic extract of *A. cina* had a higher ovicidal activity at low concentrations; this is probably due to the presence of fatty acids and terpenes.

Keywords: *Artemisia cina*, *Haemonchus contortus*, Egg hatching, Anthelmintic.

Received: 18/05/2023

Accepted: 02/10/2023

Haemonchus contortus infection is one of the biggest challenges faced by sheep and goat production worldwide. This nematode parasite is highly virulent and has a great economic impact due to the loss of production and the need to control the infection[(1)].

Resistance to commonly used anthelmintics has been a growing problem in the fight against *H. contortus*[(2)]. Although options for sustainable management, such as those contemplated by integrated parasite control, are a strong alternative, more studies are still required to validate the methods and contribute to reducing resistance or protecting molecules present on the market[(3)].

The host's immune response to the presence of *H. contortus* is a crucial factor in counteracting the infection so that individuals in the herd can be classified as resistant, resilient, or susceptible animals. Resistance is associated with a zero parasite load and a low impact on the productive parameters of the herd[(4)]. However, susceptible individuals, who are sometimes fewer in number but who are severely affected by nematodiasis damage, require frequent treatments, inducing problems due to anthelmintic resistance. Therefore, it is necessary to identify new molecules with antiparasitic potential[(4)].

The identification of new molecules and compounds that have activity against parasites is an area in constant evolution. Some plants have been shown to have anthelmintic properties, including effects against *H. contortus*[5]. Currently, antiparasitic control must focus on sustainable strategies that have the least possible impact on the environment, in addition to reducing pressure on nematode strains in constant mutation and selection to genes associated with resistance. Efforts should be directed towards searching for compounds that can cause damage to the parasite, reducing its populations, or even reversing the overexpression of genes responsible for anthelmintic resistance[6].

The genus *Artemisia* contains different species with proven anthelmintic activity, including *A. cina*, which has been used in traditional medicine as an antiparasitic along with plants of the same species, and they have excellent effects on intracellular parasites, nematodes, or even cestodes[7].

The genus *Artemisia* biosynthesizes different secondary metabolites such as sesquiterpenes, diterpenes, sterols, phenoxychromes, phenylpropanes, flavonoids, coumarins, isoprenylcoumarin, caffeoylquinic acid, acetylenes, and lignans that are responsible for anthelmintic activity[8]. Among the molecules with reported antiparasitic activity are artemisinin, santonin, norisoguaicin, and 3'-demethoxy-6-O-demethylisoguaiacin[9-11]. Other authors, such as Sakipova *et al*[10], have reported the presence of artemisinin and santonin[10]. *Artemisia cina* has been shown to be a plant with a high anthelmintic potential for nematodes and cestodes of veterinary importance[8,12]. This study aimed to evaluate the ovicidal activity of *Artemisia cina* against the parasite *Haemonchus contortus* and to propose the structures of the major volatile molecules of the extract with the highest anthelmintic activity through gas chromatography coupled with mass spectrometry.

Plant material: The previously dried and ground aerial parts of *Artemisia cina* in the pre-flowering state (40 to 60 cm in height) were provided by Hunab® laboratories, Mexico, who produce the plant commercially under the following conditions: humidity of 24.6 %, pH 8.7, and salinity of 1.6 %. The plant with voucher number No. 11967 was identified as *Artemisia cina* by Dr. Alejandro Torres-Montúfar from the herbarium of FES Cuautitlán, Cuautitlán, State of Mexico.

Obtaining the plant extract: Samples of approximately 1 kg of plant material were used to perform the solvent extraction by maceration for 72 h at room temperature, using methanol, ethyl acetate, and n-hexane to obtain extracts of high, medium, and low polarity, respectively. After the maceration time, it was filtered using gauze, cotton, and filter paper (Whatman® #4). The resulting filtrate was concentrated at reduced pressure at 40 °C and 100 rpm in a DLAB RE-100 Pro® rotary evaporator. The resulting extract was vacuum-dried and stored in a desiccator at reduced pressure until use.

Thin layer chromatographic (TLC) analysis: Merck® aluminum TLC plates were used with the following conditions: silica gel 60 F254. The mobile phase used to perform the elution of the extracts was 5:5 *n*-hexane:ethyl acetate. For each lane, 15 µl of a solution of 16 mg/ml of each extract and the reference was applied; therefore, a higher intensity of the bands corresponds to a higher concentration. The reference was the *n*-hexane extract reported by Higuera Piedrahita *et al*[21], of which the anthelmintic activity of *A. cina* on *Haemonchus contortus* eggs is reported. The chromatography plates were checked at two wavelengths (254 and 365 nm) before being developed with ceric sulfate. The retention factor (R_F) was calculated with the following equation:

$$Rf = \frac{\text{Solute distance traveled}}{\text{Solvent distance traveled}} \qquad (1)$$

Egg hatching inhibition (EHI): The eggs of *Haemonchus contortus* were obtained from the strain isolated and kept in the FES Cuautitlán. The EHI was performed in 96-well ELISA plates; the protocol used was the one reported by Coles *et al*[13], where 100 eggs were used per well with four replications; the eggs exposed to the treatments were incubated in a wet chamber for 48 h before reading. The EHI reading was performed using an iodine-lugol solution, which was added to each well after incubation. The number of unhatched eggs (dead and larval) and larvae 1 were counted to determine the percentage of egg hatching inhibition using a microscope with 10 X magnification (Olympus, model CK-2, Japan®). Ivermectin (5 mg/ml) was used as a positive control, and water as a negative control. The photographs were taken on the 40X lens using an HK-10 CMOS camera and the ISCapture V3.6.6 software.

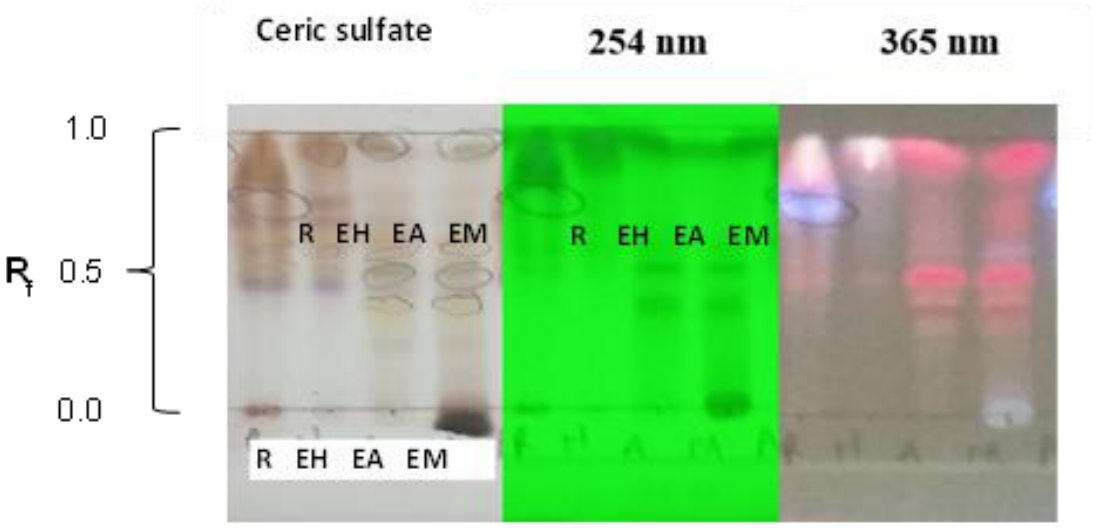
Gas chromatography coupled with mass spectrometry (GC-MS): The volatile components present in the crude extract with the highest activity in inhibiting the hatching of *H. contortus* of eggs were analyzed by GC-MS using an Agilent Technologies HP 6890 gas chromatograph coupled with an MSD 5973 quadrupole mass detector (HP Agilent) and an HP-5MS capillary column (length: 30 m; inner diameter: 0.25 mm; film thickness: 0.25 µM). A constant flow of helium as a carrier gas was adjusted to the column at 1 mL/min. The inlet temperature was set at 250 °C, while the furnace temperature was initially kept at 40 °C for 1 min and increased to 280 °C at intervals of 10 °C/min. The mass spectrometer was used in positive electron impact mode with an ionization energy of 70 eV. The detection was performed in selective ion monitoring mode. The signals were identified and quantified using target ions. The compounds were identified by comparing their mass spectra with the NIST library version 1.7a. The relative percentages were determined by integrating the signals using the GC Chem Station software, version C.00.01. The composition was reported as a percentage of the total signal area.

Statistical analysis: Three replications were performed in duplicate for each extract. The LC_{50} and LC_{90} were calculated through a PROBIT analysis using the SAS 9.0 software. The mean and its standard error were obtained for each extract; a Tukey's multiple comparison of means was performed at 95 % confidence using the Statgraphics program.

Extraction by polarity of *Artemisia cina* extracts allowed the following yield percentages to be obtained: methanol extract (ME) had a yield percentage of 4.1 %, ethyl acetate (EA) 3.86 %, and *n*-hexane (EH) extract 1.09 %. The ME was the one that presented the highest yield, followed by EA and EH.

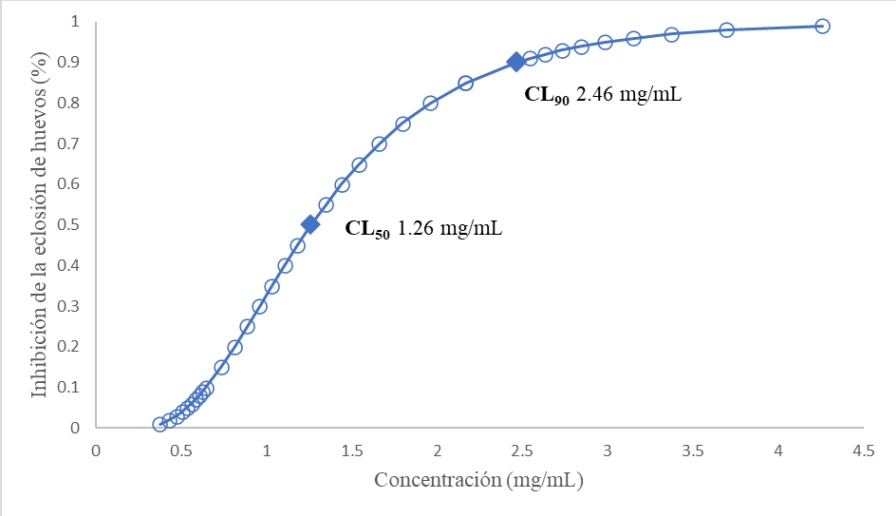
In the comparison of the chemical profile of the different extracts through thin layer chromatography (TLC), an *n*-hexane:ethyl acetate (5:5) system was used, which allowed the separation of a bigger number of bands than other systems. In this system, it was possible to observe the difference in the chemical profile of each of the extracts, where EH presents the highest concentration of compounds between the retention factors (R_F) 0.5 and 1.0, EA between 0.4 and 0.7, and ME in 0.0. According to the R_F and intensity of the bands, the compounds present in EH are mainly of low polarity, those of the EA are of medium polarity, and those of the ME are of higher polarity compared to the other extracts (Figure 1). The resulting extracts were obtained employing a simple maceration using a different plant material for each solvent, avoiding exhaustive extractions.

Figure 1: Reference thin-layer chromatography (R), extracts of *n*-hexane (EH), ethyl acetate (EA), and methanol (ME) and Mobile phase:*n*-hexane: 5:5 ethyl acetate, developer: ceric sulfate



Once the difference in the chemical composition of the three extracts was observed, the inhibition of *H. contortus* eggs hatching was evaluated. A dose-response relationship was observed (Figure 2) in the EHI, which allowed the use of the Probit analysis to calculate the LC_{50} and LC_{90} of the three extracts.

Figure 2: Lethal concentrations LC_{50} and LC_{90} required to inhibit hatching of *H. contortus* eggs after 48 h incubation with an *Artemisia cina* methanolic extract determined by PROBIT analysis



The ME had the highest EHI (LC_{50} 1.26 mg/ml and LC_{90} 2.46 mg/ml), > followed by EA (LC_{50} 2.42 mg/ml and LC_{90} 3.80 mg/ml) and > EH (LC_{50} 3.08 mg/ml and LC_{90} 3.84 mg/ml). In other words, a greater effect of EHI was observed as the polarity of the extracts increased (Table 1).

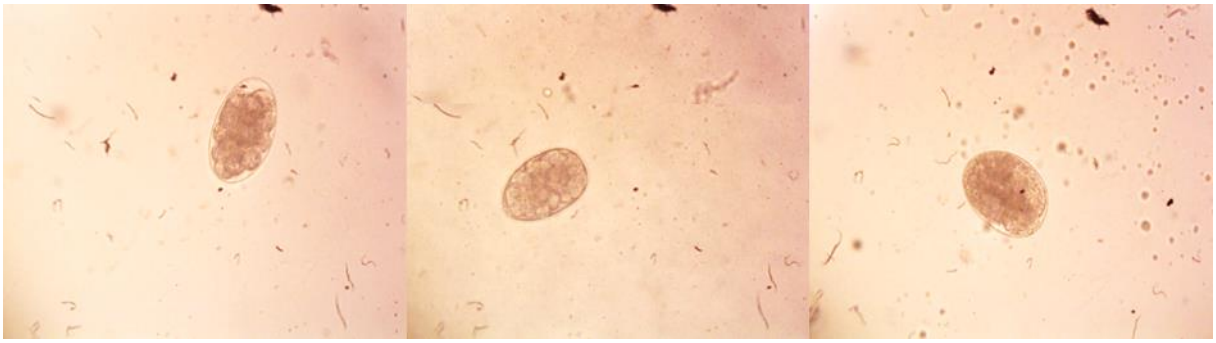
Table 1: Percentage of inhibition of hatching of *Haemonchus contortus* eggs exposed to *n*-hexane, ethyl acetate, and methanolic extracts of *Artemisia cina*

Treatment	LC_{50} (mg/ml)	LC_{90} (mg/ml)
n- hexane	3.08 (2.96 – 3.18)^{a}	3.84 (3.70 – 4.07)^{a}
Ethyl acetate	2.42 (2.27 – 2.55)^{b}	3.80 (3.64 – 4.10)^{a}
Methanol	1.26 (1.18 – 1.34)^{c}	2.46 (2.32 – 2.66)^{b}

^{ab} Equal letters indicate no significant difference between groups. Duncan $\alpha<0.05$.

Photographs were taken of the eggs observed under the microscope at 40X subjected to ME, and hatching inhibition was observed in the treatment with ivermectin and larval eggs in the treatment with methanolic extract (Figure 3). Figure a shows a morulated egg exposed to distilled water without damage before 48 h of exposure to the treatments. It should be noted that at 48 h, eggs exposed to distilled water developed into larvae 1.

Figure 3: *Haemonchus contortus* eggs observed at 40X under different conditions: a) negative control with water; b) positive control of ivermectin (5 mg/ml); c) methanolic extract of *Artemisia cina* at 2.46 mg/ml after 48 h of exposure



a) **b)** **c)**

The ME showed the highest inhibition of egg hatching at lower concentrations compared to the other extracts. Therefore, the main volatile compounds of the ME were determined through GC-MS, and the structure of the primary compounds was proposed according to the fragmentation pattern, which were compared with the NIST library. Considering the above, about 15 different volatile compounds are proposed, of which three are fatty acids, and 12 are terpenes (Table 2).

Table 2: Volatile compounds present in the methanolic extract of *Artemisia cina*

Compound	Retention time (min)	Name	Molecular weight (m/z)	% of area	Type of compound
(1)	9.20	4H-Pyran-4-one, 2,3-dihydro- 3,5 dihydroxy-6-methyl.	144	8.376	Hemiterpene
(2)	11.85	Dihydro aromadendrene	202	1.519	Bicyclic sesquiterpene

(3)	12.85	Caryophyllene	204	0.891	Bicyclic sesquiterpene
(4)	14.91	Caryophyllene oxide	220	9.601	Bicyclic sesquiterpene
(5)	16.80	Spathulenol	220	5.256	Bicyclic sesquiterpene
(6)	17.54	(-) Spathulenol	220	8.552	Bicyclic sesquiterpene
(7)	10.80	Platambin	238	2.794	Bicyclic sesquiterpene
(8)	18.94	Hexadecanoic acid	256	19.185	Saturated fatty acid
(9)	20.241	Phytol	296	3.691	Linear diterpene
(10)	20.55	9-12 Octadecanoic acid (Z,Z)	290	8.463	Unsaturated fatty acid
(11)	20.63	9-12-15 Octadecatrienoic acid methyl ester (Z,Z,Z)	292	9.736	Unsaturated fatty acid
(12)	21.390	Azulene [6,5-b] furan -2,5-dione, decahydro-4a,8 dimethyl-3-methylene, [3aR-(3aα, 4aβ, 7aα, 8β, 9aα)]	248	1.497	Sesquiterpene lactone
(13)	23.07	2-[4-methyl-6-(2,6,6-trimethylcyclohex-1-enyl) hexa-1,3,5-trienyl][cyclohex-1-en-carboxyaldehyde]	280	13.677	Bicyclic diterpene

(14)	23.42	Spiro [7H-cyclohepta[b]furan 7,2'(5H')-furan]-2,5'(3H)-dione, octahydro-8-hydroxy-6,8-dimethyl-3-methylene, [3aS-(3aα, 6β, 7α, 8α,8aα)]	280	2.772	Sesquiterpene lactone
(15)	25.77	Azulene [6,5-b] furan -2,5-dione, decahydro-4a,8 dimethyl-3-methylene, [3aR-(3aα, 4aβ, 7aα, 8β, 9aα)]	248	3.989	Sesquiterpene lactone

In general, the volatile compounds of ME are mainly terpenes and some fatty acids, with sesquiterpenes being the most chemically diverse. Figure 4 shows seven compounds that, according to the percentage of the area under the curve of the total compounds (≥ 8 %), could be considered as the main. According to the fragmentation pattern of the seven major volatile compounds of the *Artemisia cina* ME, the proposed structures are shown in Table 3.

Figure 4: GC-MS chromatogram of the chemical compounds present in the methanolic extract of *Artemisia cina*

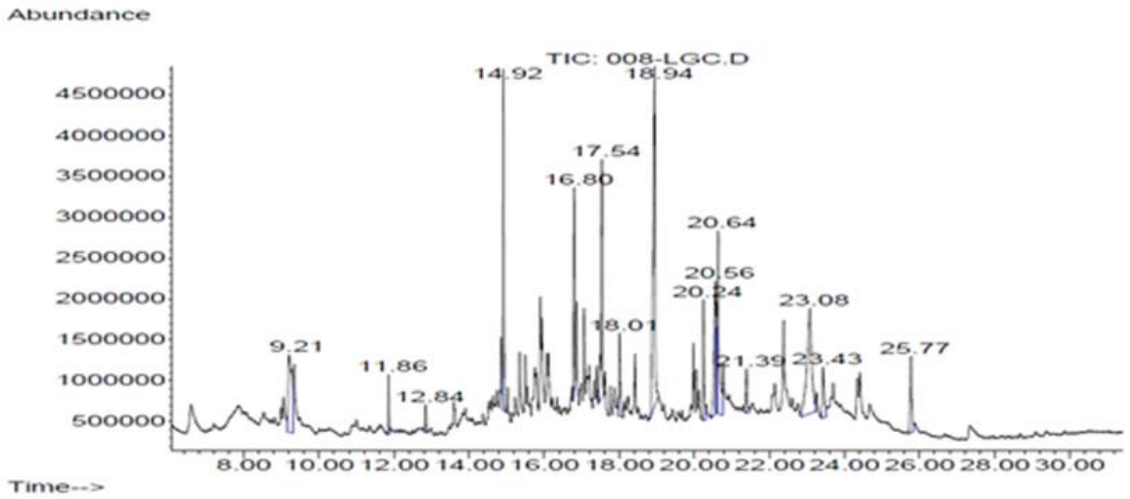
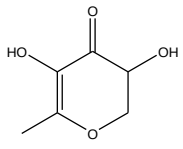
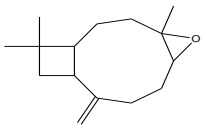
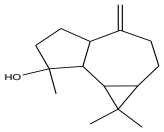
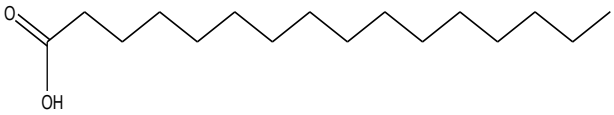
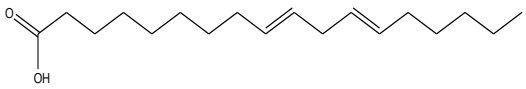
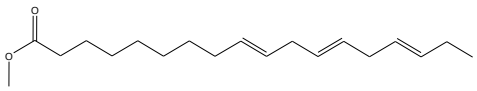
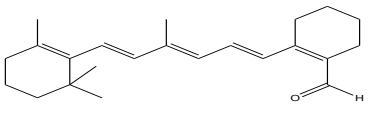


Table 3: Major volatile compounds of the methanolic extract of *Artemisia cina* determined through GC-MS

Compound	RT	Proposed structure
(1)	9.20	
(4)	14.92	
(6)	17.54	
(8)	18.94	
(10)	20.55	
(11)	20.63	
(13)	23.07	

RT= retention time (min).

According to Table 2, the possible major volatile compounds are terpenes and fatty acids. Of the terpenes, the following are present: compound (**1**) a hemiterpene, (**4**) a bicyclic sesquiterpene, (**6**) a tricyclic sesquiterpene, and (**13**) a bicyclic diterpene. Of the fatty acids: (**8**) a saturated fatty acid, (**10**) an unsaturated fatty acid, and (**11**) an unsaturated and esterified fatty acid. Hexadecanoic acid (**8**) is the most abundant in ME, followed by 2-[4-methyl-6-(2,6,6-trimethylcyclohex-1-enyl)hexa-1,3,5-trienyl][cyclohex-1-en-carboxyaldehyde (**13**).

The egg-hatching inhibition (EHI) of ME can be attributed to the presence of saturated and unsaturated fatty acids, such as hexadecanoic acid, which is the most abundant in the *Artemisia cina* ME. Pineda-Alegría *et al*[14] evaluated pentadecanoic acid $CH_3(CH_2)_{13}COOH$), hexadecanoic acid $CH_3(CH_2)_{14}COOH$) (**8**), and stearic acid ($CH_3(CH_2)_{16}COOH$) and found an increase in the EHI by increasing the number of carbons of unsaturated fatty acids, with the most active being palmitic and stearic acids at a dose of 20 mg/ml, where they obtained 100 % EHI of *H. contortus*. Considering that the ME presented LC_{100} in the EHI of 4.25 mg/ml and that one of its primary compounds is hexadecanoic acid, it could be thought that there is a synergism with the other chemical compounds present in ME. Due to the nature of these fatty acids, they could be the potential compounds with ovicidal activity of ME.

The presence of secondary metabolites in plants is a consequence of their interaction with the surrounding environment. These interactions are typical of the biotic and abiotic factors of the place where the plant is located. Regarding the phytochemical profile of the ME of *A. cina* used in this study and the concentration, it is a response to the controlled conditions of the crop since the plant material was obtained from a greenhouse[15].

The ME of *A. cina* has a high content of terpenes, which have been reported with a high ovicidal activity against gastroenteric nematodes of ruminants. These terpenes are caryophyllene oxide (**3**) and spathulenol (**5**), which are part of the main compounds (15.4 % and 5.1 %, respectively) of the essential oil of *Achyrocline satureioides*[16], which have an EC_{50} of 10.42 mg/ml in the EHI on *H. contortus*, compared to the EC_{50} of 1.42 mg/ml in the EHI of the *Artemisia cina* ME, in which (**3**) and (**5**) are present in 9.60 % and 5.25 %.

Considering the above, it is hypothesized that fatty acids and terpenes have different mechanisms of action and could be working together, thus generating a pharmacodynamic interaction[15], in this case, a synergism. Synergism occurs when the effect or response of the mixture is greater than the sum of the combination of the drugs alone[15]. Although it is not common to find drug interactions between chemical compounds, it is desirable to find synergisms between them, as they could be the basis for implementing a drug combination therapy, which could reduce the side effects that usually occur in drug monotherapy[17], which could be an excellent alternative to the use of anthelmintics due to the resistance that currently

exists. A particularity of secondary metabolites is that they are multitarget due to the presence of different functional groups[18]. This synergistic effect should be tested in future studies.

Although terpenes and fatty acids are typical for the genus *Artemisia*, only the presence of santonin, pectolinarigenin[10], 3'-demethoxy-6-O-demethylisoguaiacin, norisoguaicin[19], artemisinin and derivatives[20] has been reported in the *A. cina* plant. Therefore, this work reports the presence of three fatty acids and twelve terpenes other than artemisinin in *A. cina*, of which there is no report. The anthelmintic activity of *A. cina* has been attributed mainly to the *n*-hexane extract[19-21]; for the specific case of the EHI, it was found that the activity increases as the polarity of the extracts increases, thus opening a new perspective to design a phytomedicine with anthelmintic effect.

All the evaluated extracts of *Artemisia cina* showed inhibitory activity of *Haemonchus contortus* egg hatching, with the methanolic extract (ME) being the one that presented the highest activity. ME contains 15 different volatile compounds, of which three are fatty acids and 12 terpenes. Hexadecanoic acid and 2-[4-methyl-6-(2,6,6-trimethylcyclohex-1-enyl)hexa-1,3,5-trienyl][cyclohex-1-en-carboxyaldehydehyde] are the major compounds, which are presumed to be responsible for the ovicidal activity.

Acknowledgments

This work is part of Dr. Luis David Arango de la Pava's postdoctoral stay funded by the General Directorate of Academic Personnel Affairs (DGAPA, for its acronym in Spanish) of the National Autonomous University of Mexico under the direction of Dr. Raquel López-Arellano and Dr. Rosa Isabel Higuera Piedrahita.

Financing

PAPIIT IA204822 Project called "Evaluation of the toxic effect of the *n*-hexane extract of *Artemisia cina* and cinaguaiacin on the biochemical parameters in blood and anatomopathological alterations in Wistar rats after oral administration" of the National Autonomous University of Mexico.

Conflict of interest

The authors declare no conflict of interest.

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