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Article

In vitro anthelmintic evaluation of curcumin against the eggs and larvae of three *Haemonchus contortus* isolates with different susceptibility to ivermectin

Lisandro Alberto Encalada-Mena [a]

Raquel López-Arellano [b]

María Eugenia López-Arellano [c]

María Gabriela Mancilla-Montelongo [d]

Carlos Alfredo Sandoval-Castro [e*]

Agustín Olmedo-Juárez [c]

Juan Felipe de Jesús Torres-Acosta [e]

[a] Universidad Autónoma de Campeche, Facultad de Ciencias Agropecuarias, Campeche, México.

[b] Universidad Nacional Autónoma de México. FES-Cuautitlán, Laboratorio de Ensayos de Desarrollo Farmacéutico, Estado de México, México.

[c] Instituto Nacional de Investigaciones Forestales, Agrícolas y Pecuarias. Centro Nacional de Investigación Disciplinaria en Salud Animal e Inocuidad, Morelos, México.

[d] CONAHCYT-Universidad Autónoma de Yucatán. Facultad de Medicina Veterinaria y Zootecnia, Yucatán, México.

[e] Universidad Autónoma de Yucatán, Facultad de Medicina Veterinaria y Zootecnia, Km 15.5 Carr. Mérida-Xmatkuil, 97315, Mérida, Yucatán, México.

*Corresponding author: carlos.sandoval@correo.uady.mx

Abstract:

Increasing gastrointestinal nematode resistance to conventional anthelmintics (AH) is a growing worldwide problem. Among various sources, the search for alternative AH has focused on plant secondary metabolites such as curcumin. The *in vitro* AH activity of curcumin (CUR) was evaluated against three isolates of the ruminant nematode *Haemonchus contortus* with different susceptibilities to conventional AH. Four *in vitro* tests were run: egg hatching inhibition (EHI), larval migration inhibition (LMI), larval exsheatment inhibition (LEI) and 72 h mortality of unsheathed L_3. Curcumin (CUR) concentration range was 0 - 8.5 µg CUR/mL in the EHI, LMI and mortality tests. In the LEI test it was 0 - 17.3 µg CUR/mL. Concentration-response curves were generated using a log-logistic regression. Experimental design was completely random and results were analyzed with an ANOVA. Curcumin did not exhibit AH activity in the EHI, LMI and mortality tests, but had a significant AH effect in the LEI test. This effect was strongest against the FMVZ-UADY isolate (EC_{50}= 1.9 µg/mL, 95%CI= 1.58-2.31), followed by the Paraíso isolate (EC_{50}= 3.2 µg/mL, 95%CI = 2.69-3.81) and the CENID-SAI, INIFAP isolate (EC_{50}= 7.0 µg/mL; 95%CI= 6.58-7.43). At the evaluated doses, curcumin had an AH effect against exsheatment of *H. contortus* L_3, but no effect on egg hatching, L_3 migration or mortality of exsheathed L_3.

Key words: Polyphenol, Anthelmintic, Larval exsheathed, Polymeric stabilizers.

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Introduction

Gastrointestinal nematodes (GIN) are a major health problem in grazing ruminants[1,2]. Small ruminants with high GIN infections suffer digestive disorders that cause growth retardation or decrease productivity parameters. These parasites can also cause anemia, edema and, in very severe cases, death[3,4]. *Haemonchus contortus* is one of the most important GIN due to its pathogenicity and worldwide distribution in tropical and subtropical regions[5].

Control of *H. contortus* is based on commercial dewormers that can, over time, select for populations with anthelmintic resistance (AR)[6]. Increasing AR is the driving force behind the search for alternative helminth control measures[7]. Plant secondary metabolites, particularly polyphenolic compounds, have an anthelmintic (AH) effect against different *H.*

contortus life stages[8,9]. Turmeric *Curcuma longa* L., a member of the Zingiberaceae family and native to Asia, contains polyphenols. The main polyphenol in *C. longa* extracts is curcumin (CUR) (60-75 %), although smaller proportions of desmethoxycurcumin and bisdesmethoxycurcumin are also present[10,11]. Curcumin has confirmed pharmacological activities such as antioxidant, anti-inflammatory, anticancer, antiviral, antibacterial, and antiparasitic[12-18]. Its antiparasitic activity has been evaluated using extracts produced with solvents of different polarity, and different elements of *C. longa* plants. These extracts' AH activity has been evaluated using different concentrations (mg/mL) against *H. contortus* adults, L_3 and eggs[19-21]. High doses are used because CUR exhibits low hydrosolubility, poor absorption, and rapid degradation, which reduce its bioavailability[10].

Research has focused on increasing CUR solubility by encapsulating secondary metabolites in lipid nanoparticles, nanoemulsions, nanoliposomes, biodegradable polymers and dendrimers, and hydrogels, with the use of casein and cyclodextrins[22,23]. A combination of CUR and polymeric stabilizers such as polyvinylpyrrolidone (PVP) in a solid dispersion (CUR/PVP) has recently been proposed[24,25]. This combination improves CUR solubility and provides low toxicity in cells and tissues[26-28]. Few studies have evaluated the *in vitro* AH activity of *C. longa*, and none mention the CUR metabolite concentrations used in the bioassays; indeed, they assume that any observed AH effect is caused by CUR and other curcuminoids[19-21]. Therefore, the AH activity of CUR against *H. contortus* has yet to be unequivocally demonstrated. The present study objective was to evaluate the *in vitro* AH activity of CUR against three *H. contortus* isolates with different AR status.

Material and methods

Experimental ethics

All experimental animals were handled following applicable laws for germplasm collection (NOM-051-ZOO-1995 and NOM-062-ZOO-1999) (www.gob.mx/senasica). The experimental design and procedures followed the ethical guidelines of the Bioethics Committee of the Faculty of Veterinary Medicine and Zootechny of the Autonomous University of Yucatan (Facultad de Medicina Veterinaria y Zootecnia de la Universidad Autónoma de Yucatán - FMVZ-UADY) (Permit No. CB-CCBA-D-2021-005).

Study area

Production of *H. contortus* in donor animals with monospecific infections, and all *in vitro* bioassays, were performed at the FMVZ-UADY, Xmatkuil, Mérida, Mexico.

Haemonchus contortus isolates

Three *H. contortus* isolates were used in the tests. The AH resistance status of each was determined before their use.

(1) The Paraíso isolate originated from a commercial sheep farm in Umán, Yucatan, Mexico. It is reported to exhibit resistance to ivermectin (IVM; fecal egg count reduction: 64 %), albendazole sulfoxide (ABZ; fecal egg count reduction: 0%), and levamisole (LEV; fecal egg count reduction: 92 %)[29];

(2) The FMVZ-UADY isolate originated from a farm in Merida, Yucatan, Mexico. It has been reported as resistant to ABZ (fecal egg count reduction: 89 %) and LEV (fecal egg count reduction: 87 %)[30], but susceptible to IVM (fecal egg count reduction: 99 %)[29];

(3) The CENID-SAI-INIFAP isolate was provided by the Helminth Department of the National Center for Disciplinary Research in Animal Health and Food Safety (Centro Nacional de Investigación Disciplinaria en Salud Animal e Inocuidad - CENID-SAI) of the National Institute of Forestry, Agriculture and Livestock Research (Instituto Nacional de Investigaciones Forestales Agrícolas y Pecuarias - INIFAP). *In vitro* resistance assays were performed at the FMVZ-UADY. Based on the egg hatch methodology described by Von Samson-Himmelstjerna *et al*[31], this isolate was found susceptible to thiabendazole (TBZ) (EC_{50}= 0.050 μg/mL), and using the larval migration technique[32] it was found susceptible to IVM (EC_{50}= 1.09 μM)[33]. In a separate study using the *in vitro* mortality technique, it was found susceptible to IVM (79.22 % mortality at 11.42 mM)[34].

Donors and collection of monospecific *Haemonchus contortus* isolates

Donor animals were six goats aged 3 to 4 months, 15 kg live weight (LW), raised GIN-free from birth. For each *H. contortus* isolate, two donor animals were infected with 7,000 L_3 *per*

os[35]. At all times, the animals were kept in individual cages with raised floors located inside individual pens with concrete floors at the FMVZ-UADY facilities.

Beginning at 24 d post-infection, fecal samples were collected directly from the rectum of each donor animal in new polyethylene bags[36]. The fecal samples were processed following the McMaster technique to quantify the number of eggs *H. contortus* eggs and quantify them as eggs per gram of feces (EPG)[37]. Each test was performed with 2 g of feces and 28 mL saturated sugar solution (density = 1.28, 50 EPG sensitivity). Third stage larvae (L_3) were obtained from stool cultures maintained in clean plastic jars incubated at 28 °C for 5 to 6 days. The larvae were recovered using the Baermann technique[38], and identified as *Haemonchus* using morphological keys[39].

Haemonchus contortus egg collection

Egg collection was done following the MAFF (1986) procedure[38]. Feces were collected directly from the rectum of donor animals using polyethylene bags. In the laboratory, the feces were placed in a plastic strainer over a porcelain mortar. For every 10 g of feces, 100 mL water was added and the mixture macerated. The feces/water suspension was filtered through a double layer of gauze placed in a funnel, and the liquid recovered in a flask. This liquid was filtered through a 25 μm mesh with a minimal amount of dechlorinated water. Eggs were recovered from the mesh and placed in 50 mL tubes (5810R, Eppendorf, Germany). These were centrifuged at 453 *g* for 5 min, and the supernatant removed. A saturated sugar solution (25 mL, density= 1.28) was added to the sediment, The latter was resuspended in a vortex mixer and the tubes centrifuged again. The surface layer of the solution was recovered with a bacteriological loop and placed in another plastic tube containing water purified by reverse osmosis. This procedure was repeated several times to recover the largest possible number of eggs. Concentration per milliliter was estimated and adjusted until attaining a 200 eggs/mL suspension.

Haemonchus contortus L_3 production

Third stage larvae (L_3) were produced in feces collected every 24 h in polyethylene bags from a mesh placed under each cage. In the laboratory, the feces were washed with running water to remove detritus, and the recovered washed feces were used to perform separate coprocultures for each isolate with a Baermann apparatus[38], and placed in culture bottles with ventilated lids. The bottles were identified with harvest date, isolate name and

concentration (L_3/mL). The larvae were stored under refrigeration (6-10 °C) until used in the *in vitro* migration and exsheathing inhibition tests, and the exsheathed L_3 larvae mortality test.

Curcumin dispersion in polyvinylpyrrolidone (CUR/PVP)

Curcumin has poor solubility[16,40]. Therefore, prior to the *in vitro* studies, an established procedure was applied to improve turmeric E solubility and absorption[25]. Turmeric E (52.28% CUR; Laboratorios Mixim S.A. de C.V., Naucalpan, Mexico) was combined with PVP K30 (Agrimer K-30 Ashland, Columbus, Ohio, U.S.) at a 1:7 ratio. This produced a turmeric E dispersion with a 6.2 % final CUR concentration (CUR/PVP). This procedure was done at the Pharmaceutical Development Testing Laboratory (LEDEFAR), Cuautitlán Higher Education Faculty, National Autonomous University of Mexico (Universidad Nacional Autónoma de México – UNAM).

Preparation of CUR/PVP dispersion stock solutions

For the egg hatching inhibition (EHI), larval migration inhibition (LMI) and 72 h mortality tests of exsheathed L_3, a stock solution was prepared with 32.4 mg CUR/PVP dispersion in 20 mL purified water and stirred with a magnetic bar for 2 h. This suspension was centrifuged (BHG, Germany) at 1.057 *g* for 5 min and the supernatant used in the different bioassay concentrations. For the larval exsheathing inhibition (LEI) test, a stock solution was prepared with 64.8 mg CUR/PVP dispersion in 20 mL purified water.

Curcumin concentration

Because of CUR's low solubility, real CUR content was quantified in the supernatant suspension used in the bioassays. Suspension CUR concentration was measured following methodologies described in Buchi note No. 747[41] and FSSAI[42]. Briefly, the supernatant was analyzed with a UV/VIS spectrophotometer (Lambda 25, Perkin Elmer, Beaconsfield, UK) at 425 nm transmittance. A calibration curve (0 - 6 mg/L) of CUR (Sigma Aldrich® standard cat. C7727, 91% purity) in ethanol was prepared. Pure ethanol (Sigma Aldrich® cat. E7148) was used to correct the background reading. The stock solution prepared with 32.4

mg CUR/PVP was found to contain 11.3 µg CUR/mL, and the stock solution prepared with 64.8 mg CUR/PVP contained 23 µg CUR/mL.

Egg hatching inhibition test

The EHI test was run following Coles *et al*[43]. The egg suspension (200 eggs/mL) was evenly distributed in 24-well plates (1 mL per well). Using the CUR/PVP stock solution containing 11.3 µg/mL CUR, serial dilutions (%) were made to four final concentrations in the wells: 2.3 (20 %), 4.0 (35 %), 5.7 (50 %) and 8.5 (75 %) µg/mL CUR in a 2,000 µL final volume. The positive control was 10 µL Lugol's solution (0.5 % incubated volume), and the negative control was 1,000 µL purified water. The plate was incubated at 28 ºC in a bacteriological oven. After 48 h, egg hatching was stopped by adding 50 µL Lugol's solution per well. A total of two replicates were performed with three repetitions per concentration. The content of each well was counted using McMaster chambers and a compound microscope (10x). In the bottom of the chamber, per sample counts were done of morulated eggs (ME), eggs containing unhatched larvae (UL) and L_1 larvae. The following formula was used:

$$\%\ Inhibition\ hatching = \frac{unhatched\ eggs}{unhatched\ eggs + \ L_1} \times 100$$

Larval migration inhibition

The LMI test was run to evaluate IVM in dimethyl sulfoxide according to Demeler *et al*[32], modified as follows for testing with CUR/PVP. The 11.3 µg CUR/mL concentration of CUR/PVP stock solution was used. A serial dilution (%) was done of the stock solution to produce seven final concentrations in the wells: 0, 0.6 (5 %), 1.1 (10 %), 2.0 (17.5 %), 3.7 (32.75 %), 5.7 (50 %) and 8.5 (75 %) µg CUR/mL. In a 24-well plate, 0 - 500 µL purified water plus 0 - 750 µL stock solution were placed in each well. The positive control was 100 µL (10 %) Lugol's solution, and the negative was 500 µL purified water. In wells with an 8.5 µg CUR/mL final concentration, 250 µL of a larval suspension containing 600 L_3/mL was added, with a 1,000 µL final volume. In the remaining wells, 500 µL of a larval suspension containing 300 L_3/mL was added, with a 1,000 µL final volume. Two replicates and three repetitions were done for each CUR concentration, and the positive and negative controls. The plates were incubated at 28 °C for 24 h. To prepare the migration plates, 500 µL 1.5 %

bacto agar were added to alternating rows, that is, one row with bacto agar and the next empty. A 25 µm mesh was placed in each well containing bacto agar. After incubation in CUR, the L_3 content of the working wells and control wells was transferred onto the mesh. The migration plates were incubated at 28 °C for 24 h to allow the L_3 to migrate through the mesh. After incubation, the mesh was removed, leaving the migrated L_3 larvae in the corresponding wells. Those L_3 that did not migrate and remained in the mesh were transferred to the empty wells in the adjacent rows of the same plate. The mesh was washed with 1,000 µL purified water to recover all L_3 in the corresponding well. One drop Lugol's solution was added to all wells, and the contents of each well poured into a McMaster chamber to count L_3 per well. The number of migrated and non-migrated L_3 in each concentration was counted and percentage of migration calculated using the formula described by Demeler *et al*[32]:

$$\%\ migration\ inhibition = \frac{non\ migrated\ L_3}{migrated\ L_3 + \ non\ migrated\ L_3} \times 100$$

Larval exsheathment inhibition

Inhibition of larval exsheathing (LEI) was quantified following Jackson and Hoste[44]. A suspension containing 1,000 L_3 per mL, and CUR/PVP dispersion stock solution containing 23.0 µg CUR/mL were used. The L_3 were incubated for 3 h at 23 ºC in seven different concentrations, obtained by serial dilution of the stock solution in 15 mL tubes: 0.6 (2.5 %), 2.3 (10 %), 3.5 (15 %), 4.6 (20 %), 8.1 (35 %), 11.5 (50 %) and 17.3 (75 %) µg CUR/mL. Additionally, L_3 were incubated in the respective concentrations of PVP K-30 to rule out any AH activity from the polymer. In the positive control, L_3 were incubated with LEV (120 mg/mL, Laboratorios Aranda S.A. de C.V., Mexico), and in the negative, they were exposed to purified water. The tubes were centrifuged at 453 *g* for 5 min and washed with purified water three times. Larvae exposed to the different treatments were divided into four 200 µL aliquots containing 200 L_3 each. Prior to the LEI test, calibration curves were generated for each isolate by inducing gradual exsheatment in five dilutions of a sodium hypochlorite (2 %) and sodium chloride (16.5 %) solution (1/300, 1/400, 1/480, 1/600 and 1/800) in phosphate buffer solution (PBS, pH 7.4). Exsheatment was monitored every 20 min (0, 20, 40 and 60 min) in 50 µL aliquots (25-50 L_3) using a microscope (10x and 40x). Exsheatment was stopped by flaming the slides covered with coverslips containing the L_3. Four replicates were run for each CUR concentration, and %LEI calculated with the formula:

$$\%\ Inhibition\ of\ exsheathing\ = \frac{L_3\ not\ exsheathed}{L_3\ not\ exsheathed + L_3\ exsheathed} \times 100$$

Mortality in exsheathed L_3

Mortality in exsheathed L_3 caused by CUR was tested following Reyes-Guerrero *et al*[34], with some modifications. When the L_3 of each *H. contortus* isolate were 3 to 15 wk of age, five mortality trials were run using exsheathed L_3 with 0.187 % commercial sodium hypochlorite[45]. Using a CUR/PVP dispersion stock solution containing 11.3 µg/mL CUR, exsheathed L_3 were exposed to five different concentrations: 1.1 (10 %), 2.3 (20 %), 4.0 (35 %), 5.7 (50 %) and 8.5 (75 %) µg CUR/mL. Lugol's solution (10 µL) was used as the positive control and purified water (50 µL) as the negative control. Using 96-well microtiter plates, two replicates and three repetitions were done for each CUR concentration, and the positive and negative controls. In wells with an 8.5 µg/mL CUR final concentration, 25 µL containing 100 L_3 were added to each well. In the remaining wells, 50 µL containing 100 L_3 were added to each well. Final volume in all wells was 100 µL. The plates were incubated at 28 °C for 72 h. After incubation, the live and dead L_3 in each well were counted by collecting the entire well in 10 µL drops that were deposited on slides for counting with an optical microscope (4x). Mortality was calculated as a percentage using the formula:

$$\%\ Mortality = \frac{dead\ L_3}{dead\ L_3\ \ +\ live\ L_3} \times 100$$

Data analysis

The data produced in the assays involving eggs (EHI) or L_3 (LMI, LEI and mortality) were used to generate concentration-response curves in a log-logistic regression with two parameters; the drc extension in the RStudio software was used[46,47]. Each test's EC_{50} and 95% CI were calculated. The percentage data for EHI, LMI, LEI and mortality were analyzed per test with a completely randomized design, using an ANOVA and the GLM function of the mass extension in the RStudio software[42,43]. Two factors were included: the three isolates (Paraíso, FMVZ-UADY and CENID-SAI, INIFAP) and the CUR concentrations in

each test. Comparison of the means was run with a Bonferroni test at a $P<0.05$ significance level.

Before running the ANOVA, a Kolmogorov-Smirnov normality test was run and confirmed that the EHI, LMI and LEI data did not meet the assumption of normality. A Breusch-Pagan heteroscedasticity test found that the same data did not comply with homogeneity of variance. Therefore, the data was transformed with the Box-Cox transformation using the mass extension of the RStudio software[47,48]. Analyses were then done using the transformed values, after confirming the assumptions of normality and homogeneity of variance. However, the results are presented transformed to normal values to facilitate interpretation.

Results

CUR inhibition of egg hatching and L_1 development

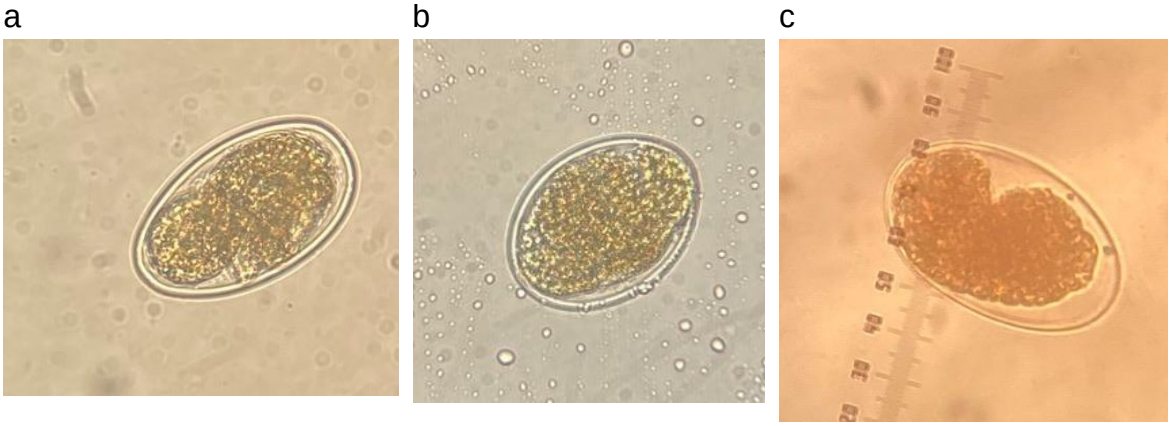
Of the four tested CUR concentrations, only the 5.7 µg/mL CUR achieved 99 % inhibition of hatching in the Paraíso and FMVZ-UADY isolates (Table 1); morulated eggs and larvae were observed (Figure 1 a y b). Inhibition did not decrease significantly for these two isolates even at the highest (8.5 µg CUR/mL) concentration (Table 1). At this same concentration, the L_1 exhibited morphological changes that may be associated with larval damage or non-viability (Figure 2 a and b). In the CENID-SAI, INIFAP isolate, the EHI percentages were low (< 10 %) at all concentrations. The EC_{50} could not be calculated for any of the tested *H. contortus* isolates.

Table 1: Egg hatching inhibition (%, average ± standard deviation) at five curcumin (CUR) concentrations in three *Haemonchus contortus* isolates with different susceptibilities to commercial anthelmintics

Isolate	0 µg CUR/mL	2.3 µg CUR/mL	4 µg CUR/mL	5.7 µg CUR/mL	8.5 µg CUR/mL
Paraíso	5.1 ± 2.5	6.6 ± 3.6	16.4 ± 10.5	100 ± 0 [a]	34.8 ± 50.6
FMVZ-UADY	3.3 ± 0.9	8.3 ± 4.9	7.4 ± 2.4	99.8 ± 0.3 [a]	8.7 ± 3.5
CENID-SAI INIFAP	3.2 ± 1.2	6.5 ± 2.2	7.6 ± 2.1	6.5 ± 1.9 [b]	6.6 ± 1.2

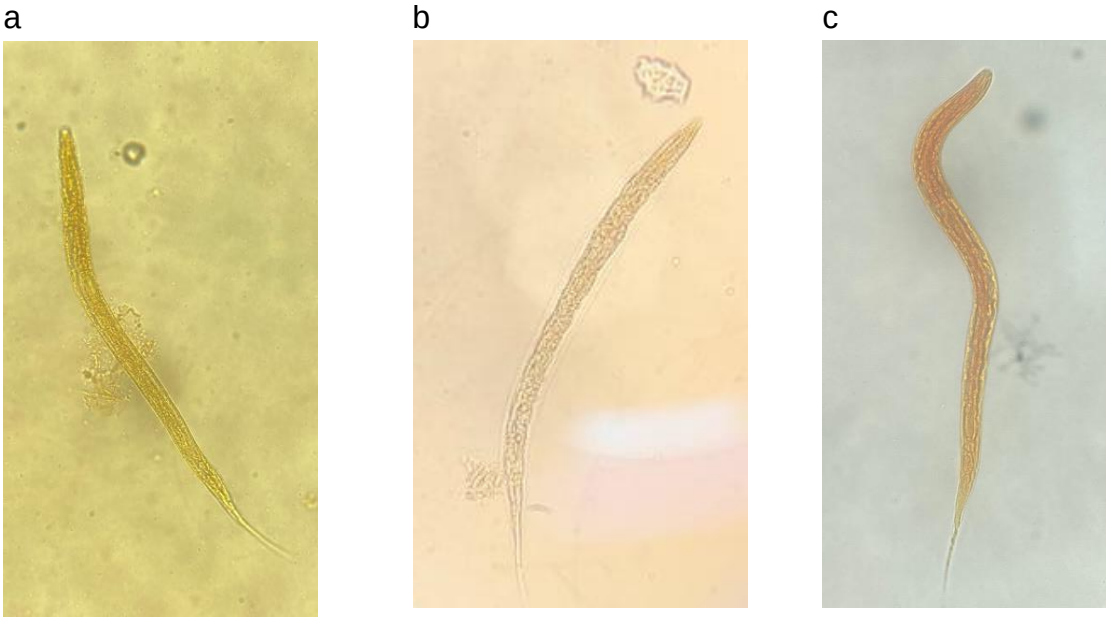
ab Different letter superscripts in the same column indicate significant difference ($P<0.05$).

Figure 1: Eggs of the *Haemonchus contortus* Paraíso isolate (40x) in 5.7 µg/mL CUR concentration in egg hatching inhibition test



(a) Larvated egg, (b) morulated egg, (c) egg from positive control containing thiabendazole.

Figure 2: L_1 larvae of the *Haemonchus contortus* Paraíso isolate (40x) in 8.5 µg/mL CUR concentration in egg hatching inhibition test



(a) Degraded larva, (b) degraded larva with cuticle separated from body, (c) normal L_1 larva in negative control.

CUR inhibition of L_3 migration

Of the six tested concentrations, only the 5.7 µg/mL CUR concentration notably inhibited migration compared to the negative control (P<0.05): 64 % inhibition against the Paraíso isolate and 53 % against the FMVZ-UADY isolate (Table 2). The highest inhibition against the CENID SAI, INIFAP isolate was only 20.3 % in the 8.5 µg/mL CUR concentration, which did not differ from the negative control. As occurred with the EHI results, the EC_{50} could not be calculated for CUR inhibition of migration in the three isolates.

Table 2: L_3 migration inhibition (%, average ± standard deviation) at seven curcumin (CUR) concentrations in three *Haemonchus contortus* isolates with different susceptibilities to commercial anthelmintics

Isolate	0 µg CUR/mL	0.6 µg CUR/mL	1.1 µg CUR/mL	2.0 µg CUR/mL	3.7 µg CUR/mL	5.7 µg CUR/mL	8.5 µg CUR/mL
Paraíso	11.4 ± 3.6	15.2 ± 3.6	18.8 ± 14	13.2 ± 1.8	12.7 ± 3.8	64.1 ± 22.1 [a]	18.1 ± 7.1
FMVZ-UADY	11.1 ± 4.5	19.2 ± 10.5	10.8 ± 3.5	10.8 ± 3.5	12.1 ± 6.8	53 ± 23.1 [a]	18.9 ± 12.9
CENID-SAI INIFAP	8.4 ± 3.1	9.3 ± 7.4	8.4 ± 2.7	8.7 ± 2.4	14.6 ± 4.3	13.6 ± 2.4 [b]	20.3 ± 12.8

[ab] Different letter superscripts in the same column indicate significant difference (P<0.05).

CUR inhibition of exsheatment in L_3

Exsheatment was inhibited in L_3 from 60 to 100 % at 8.1, 11.5 and 17.3 µg CUR/mL. The EC_{50} of each isolate differed from the others since their 95%CI did not overlap. The lowest values were for the FMVZ-UADY isolate (EC_{50}= 1.9 µg/mL, 95%CI = 1.58-2.31), followed by the Paraíso isolate (EC_{50}= 3.2 µg/mL, 95%CI= 2.69-3.81), and CENID-SAI, INIFAP (EC_{50}= 7.0 µg/mL; 95%CI= 6.58-7.43).

CUR-caused mortality in exsheathed L_3

Mortality in exsheathed L_3 of all three isolates was less than 10 % at all the tested CUR concentrations. Therefore, CUR had no apparent effect on mortality in exsheathed L_3 in these isolates.

Discussion

CUR inhibition of egg hatching

All but one of the tested CUR concentrations had no AH effect on hatching of *H. contortus* eggs; indeed, no EC_{50} could be determined for CUR in the three isolates. However, a 99% decrease in hatching was observed with the 5.7 µg CUR/mL concentration in the Paraíso and FMVZ-UADY isolates. Apparently, conditions at this concentration were favorable for solubility of CUR in water and its diffusion through the layers protecting the *H. contortus* egg. This effect was not observed at the highest concentration (8.5 µg CUR/mL). Why this occurred is unclear. It may be due to high solute saturation in the bioassay liquid, or to a hormetic effect. In the latter, a biphasic response occurs in which low doses have no effect, moderate doses cause change, and high doses exhibit little or none of the expected effect (when graphed it has the shape of an inverted "J" or "U")[49]. This phenomenon is a central theme in the biological, adaptive and repair response, and has implications in pharmacology and toxicology[50]. Hormesis is known to occur in different cell types as curcumin concentrations change[51,52]. A *C. longa* methanol:water extract (70:30) is reported to have an AH effect on *H. contortus* eggs, with an EC_{50} of 69.75 µg/mL[20]. In another study, *H. contortus* eggs were affected by a hydroalcoholic (1:9) extract of *C. longa* rhizome (EC_{50} = 100.9 mg/mL), as well as by an aqueous one (EC_{50}= 83.7 mg/mL)[53]. In neither of these two studies was the role of CUR determined in the AH effect of the *C. longa* extracts against *H. contortus* eggs. The solvents used in both studies for compound extraction were of varying polarity and the extracts were not purified for identification of the main compounds. Therefore, any effect of curcumin can only be assumed, since this is the main component in *C. longa* extracts. However, a synergistic effect between different curcuminoids cannot be discounted. For instance, in a study of curcuminoids' effects on *Toxocara canis*, CUR alone exhibited an AH effect on L_2, but this was augmented when CUR was combined with desmethoxycurcumin, bisdesmethoxycurcumin and cyclocurcumin, suggesting that synergism improved its effectiveness[54].

Of note in the present EHI results is that CUR may cause loss of cellular continuity in L_1 at the internal organ level, and these changes may undermine viability in this larval stage. This effect on L_1 has not been reported previously, suggesting the need to use the larval development methodology to evaluate the effects of CUR on larvae at different stages[55].

CUR anthelmintic activity against L_3

In both the LMI and EHI tests, CUR had no AH effect and the EC_{50} could not be calculated for any of the three evaluated isolates. As in the EHI test, the best inhibition of L_3 migration was with the 5.7 µg CUR/mL concentration, though even at this concentration inhibition percentages were only 50 to 60 %. Results in the EHI and LMI tests were inconsistent, possibly due to degradation of CUR. Indeed, the CUR solution in the wells had changed from a light yellow at the beginning of the bioassay to a brown color at the end (48 h)[56,57]. As far as is known, no previous research exists on *in vitro* LMI testing evaluating any *C. longa* extract against *H. contortus* L_3.

The most outstanding result is that the evaluated CUR extracts blocked exsheathment in *H. contortus* L_3. This blockage, which is known to occur in ruminal fluid[58], may prevent L_3 from invading the crypts in the abomasum, and consequently make larvae incapable of transitioning to later stages, including L_4, L_5 and adult[59]. Blockage of exsheathment is associated with the activity of polyphenols in plant extracts from leaves and other elements[8,60]. Curcumin is a polyphenol structurally related to caffeic acid and ferulic acid[61]. Both these acids have been evaluated against *H. contortus* in EHI and LEI bioassays[8]. Ferulic acid has ovicidal activity at 200-400 µg/mL, and in the LEI test caffeic acid exhibited activity at 7.8 µg/mL and ferulic acid at 20.6 µg/mL. In the present results, CUR inhibited L_3 exsheathment at concentrations < 7 µg/mL, lower than polyphenols mentioned above.

The mechanism by which CUR inhibits exsheathment in the three evaluated *H. contortus* isolates is unknown. The effectiveness of CUR's inhibition of exsheathment varied between the three isolates. How effective CUR is at blocking exsheathment may depend on how it interacts with enzymes, proteins, nucleic acids, biomolecules and different receptors types in the sheath of *H. contortus*[62]. A recent metabolomics study reported a panel of metabolites directly responsible for L_3 exsheathment, which were associated with amino acids and with the purine and pyruvate metabolism pathways in *H. contortus*[63]. Further research is needed on the possibility that CUR's effects on one or more of these metabolic pathways is responsible for its inhibition of exsheathment.

The present results confirm that CUR does not affect mortality in exsheathment L_3. The low mortality observed here in exsheathment L_3 (<10 %) coincides with the low mortality (<10 %) reported elsewhere with a *C. longa* extract at 100 µg/mL[20], and the absence of any effect on L_3 mortality with two *C. longa* extracts[53]. These studies contrast with the work of Nasai *et al*[21], in which an ethanolic *C. longa* extract increased L_3 mortality (78 %) at doses of 200 mg/mL. However, a large amount of extract was used in this study, which is unfeasible under *in vivo* conditions. Nonetheless, even though no EC_{50} could be estimated for CUR in

the L_3 mortality assay, the EHI test demonstrated that CUR can cause changes compatible with non-viability of *H. contortus* L_1.

Finally, the results confirm that *H. contortus* isolates from different geographic regions exhibit different *in vitro* susceptibility to natural plant compounds, both in the EHI and LEI tests[64,65]. These previous studies suggest parasites implement an adaptive process to survive in the presence of secondary compounds with AH activity ingested by the ruminant during grazing. In the present study, the isolates had varying responses at the evaluated CUR concentrations in the different *in vitro* bioassays. This was most evident in the LEI tests in which the three isolates' responses differed notably from each other. It is unclear why the CENID-SAI, INIFAP isolate exhibited the lowest sensitivity to the CUR extract concentrations compared to the two isolates from Yucatan.

Conclusions and implications

The evaluated curcumin extract had no *in vitro* anthelmintic activity against egg hatching, L_3 migration or mortality in exsheathed L_3 in any of the evaluated *H. contortus* isolates (FMVZ-UADY, Paraíso, CENID-SAI, INIFAP). However, it did inhibit exsheathment in L_3, with variable responses in each of the isolates.

Conflict of interests

The authors declare no conflicts of interest.

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