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RESEARCH

Evaluation of the ovicidal activity and fasciolicidal activity of the extract of ethyl acetate from *Artemisia ludoviciana* Nutt. spp. *mexicana* and of artemisinin against adult parasites of *Fasciola hepatica*

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Abstract
The objective of this work was to evaluate the effect of the ethyl acetate extract from *A. ludoviciana* (EALM) and artemisinin against adult parasites and eggs of *F. hepatica*. For the ovicidal assay, cell culture plates with 24 wells were used, and 90 to 110 *F. hepatica* eggs were placed in each well. The eggs were exposed to concentrations of 100, 200, 300, 400, and 500 mg/L EALM and incubated for 16 days. Additionally, triclabendazole (TCBZ) was used as a reference drug at concentrations of 10 and 50 mg, and the response of artemisinin at concentrations of 10 and 20 mg was simultaneously assessed. Adult flukes were exposed to concentrations of 125, 250, 375, and 500 mg/L EALM. The results of the ovicidal action of EALM on the eggs showed that concentrations greater than 300 mg/L were significant, with ovicidal percentages greater than 60% observed on day 16 of incubation ($p < 0.05$). The maximum efficiency of EALM on adult flukes was reached 72 h post-exposure at a concentration of 125 mg/L ($p < 0.05$).

Keywords Adult flukes · Artemisinin · Extract · *Fasciola hepatica* · Ovicidal

Introduction

Fasciolosis is among the most serious liver diseases worldwide in the field of veterinary medicine and generates millions of dollars of economic losses (WHO 2020; FAO 2021). In addition, fasciolosis is a disease of serious public health concern, as it is an emerging zoonosis found in 70 countries; approximately 2.4 to 17 million people are infected worldwide and 1 billion more at risk (Sabourin et al. 2018; Fairweather et al. 2020). Over the years, fasciolosis has spread due to the growth of the global livestock industry and climatic factors favorable to bacterial adaptation and a greater geographical distribution of the intermediate host (Dargie 1987; Mas-Coma et al. 2005; Rojo et al. 2012; Rodríguez-Vivas et al. 2017; Sabourin et al. 2018; Alba et al. 2021; Chai and Jung 2022). Fasciolosis is caused by the fluke *Fasciola hepatica*. This parasite utilizes an indirect cycle and is located in the bile ducts of ruminants, swine, equines, rabbits, and humans (Javaregowda and Rani 2017). For decades, the main treatment for fasciolosis has involved chemotherapeutics (Olaechea et al. 2011). Unfortunately, due to the indiscriminate use of these drugs together with the poor prevention and diagnosis and genetic adaptation of the parasite, an increase in anthelmintic resistance to these drugs has occurred in different parts of the world (Moll et al. 2000; Olaechea et al. 2011; Kaplan and Vidyashankar 2012; Rojo et al. 2012; Ortiz et al. 2013; Hanna et al. 2015; Novobilsky et al. 2016; Ceballos et al. 2019; Kamaludeen et al. 2019; Romero et al.

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2019; Fairweather et al. 2020; Kelley et al. 2020). An alternative substance to address this problem is natural products from plants with biological activity, which can be molecules or plant extracts. In Mexico, *Artemisia ludoviciana* Nutt. spp *mexicana* (estafiate), which belongs to the Asteraceae family, is recognized for its curative effect against diseases of the gastrointestinal tract (Andrade 2009; BDMTM/UNAM 2009). Extracts of different polarities obtained from the *A. ludoviciana* have demonstrated efficacy in vitro against young or recently excysted flukes, causing mortality greater than 90% at concentrations ranging from 125 to 500 mg/L (Álvarez et al. 2015; Ezeta et al. 2016). Recently, the effect of ethyl acetate extract from the *A. ludoviciana* on the tegument of recently excysted young flukes was demonstrated. Likewise, artemisinin was identified by HPLC/mass spectrometry as the major compound of the active extract (Ezeta et al. 2020). The objective of this work was to evaluate the effect of the ethyl acetate extract from *A. ludoviciana* and artemisinin against adult parasites and eggs of *F. hepatica*.

Methodology

Vegetal material

Healthy leaves of *A. ludoviciana* Nutt. spp. *mexicana* were collected in the vicinity of the Center for Teaching, Research and Extension in Tropical Livestock (CEIEGT), of the Faculty of Veterinary Medicine and Zootechnics (FMVZ), of the National Autonomous University of Mexico (UNAM), located in Martínez de la Torre—Tlapacoyan, municipality of Tlapacoyan, Veracruz, Mexico (19° 57′ 42″ N, 97° 12′ 39″ W). Taxonomic identification was performed in the herbarium of the Faculty of Higher Studies-Iztacala, UNAM (FESI-UNAM), and voucher number 2156 IZTA was assigned. The plant selection criteria were based on previous reports (Ibarra et al. 2012; Álvarez et al. 2015; Ezeta et al. 2020).

Preparation of crude extract of *A. ludoviciana* Nutt. spp mexicana (EALM)

The leaves were dried at a constant temperature of 60 °C for 3 days and then ground. The ground material was macerated at room temperature with ethyl acetate for one week. The extract was filtered and concentrated to dryness under reduced pressure in a Heidolph© Mod. Laborota 4000 rotary evaporator. Extracts were obtained once per week for 2 months and stored at 4 °C. The ethyl acetate extract obtained from *A. ludoviciana* (EALM) was used to perform the biological tests.

Collection of adult specimens of *Fasciola hepatica* and in vitro tests

F. hepatica adults were obtained directly from infected bovine livers. The collection was performed in the municipal slaughterhouse of Toluca, State of Mexico. The specimens were first obtained and washed with phosphate-buffered saline to remove excess blood and bile and then placed in Roswell Park Memorial Institute (RPMI)-1640 medium at 37 °C to be transported to the Helminth Experimental Chemotherapy Laboratory of the Parasitology Department FMVZ-UNAM. Once in the laboratory, the flukes were washed several times with RPMI-1640 medium and placed in 20-ml tissue culture dishes in a medium created with 50% RPMI 1640 medium and 50% bovine serum. A mixture of antibiotics (100 IU penicillin + 100 mg/ml streptomycin) was added to the medium to prevent bacterial growth. Finally, 4 flukes were placed in each box (ratio of 1 fluke for every 5 ml of medium).

A stock solution was prepared with EALM at a concentration of 500 mg/L, which was previously dissolved in 100 µL of solvent and calibrated with distilled water to form the stock solution, from which dilutions were performed to obtain the corresponding concentrations. Adult flukes were exposed in triplicate to concentrations of 125, 250, 375, and 500 mg/L EALM, incorporating the corresponding controls for each solvent to confirm that the solvent did not affect the parasite; in addition, negative controls without any treatment were used. Additionally, triclabendazole (TCBZ) was used as a reference drug at concentrations of 10 and 50 mg (TCBZ was donated by the Dept. of Biopharmacy of the Faculty of Chemistry, UNAM), and the response of artemisinin (SIGMA- ALDRICH©, 98% purity, Ref. 361593–100 mg) at concentrations of 10 and 20 mg was simultaneously assessed. Once the different groups were exposed, the samples were incubated at 37 °C in a 5% CO_2 atmosphere. (Burden and Hammet 1980; Hegazi et al. 2007; Helmy et al. 2008; Aguayo et al. 2018; Rehman et al. 2020; Sánchez et al. 2020; Guo et al. 2021). The test readings were obtained at 24, 48, and 72 h postexposure. To evaluate the effectiveness of the extract and the drugs on adult parasites, the mobility and mortality of each were considered according to the methodology and motility criteria described by Jeyathilakan et al. (2010). The control groups were observed, washed, and placed daily in a fresh culture medium to maintain their viability. Each experiment was performed in triplicate (De Mello et al. 2023).

All the procedures described were performed under aseptic conditions using a BG© Mod. CFLV-130 laminar flow hood (Álvarez et al. 2015; WHO 2019; Ezeta et al. 2020, Rehman et al. 2020).

Fasciolicide efficacy

The anti-fasciola efficacy was determined by comparing the survival of the treated group in relation to the control group as follows (Wood et al. 1995):

$$\text{Efficacy}(\%) = \frac{\text{Number of live flukes in the control group} - \text{Number of live flukes in the treated group}}{\text{Number of live flukes in the control group}} \times 100$$

Ovicidal activity of EALM

To obtain *F. hepatica* eggs, gallbladders were collected from the livers of sheep affected by fascioliasis in the municipal slaughterhouse of Toluca, State of Mexico. The livers were transported to the laboratory at a temperature of 4 to 8 °C. Once in the laboratory, the bile contents were obtained aseptically and mixed with 400 to 500 ml of distilled water to settle the mixture and eggs for approximately 20 to 30 min. After that period, 2/3 of the volume of the mixture was decanted, and the samples underwent further gauging with distilled water. Then, the samples were left to settle for the same amount of time, which was performed until the liquid was as clear as possible and the eggs could be recovered from the bottom. The eggs were left at 4 °C for 24 h before ovicidal evaluation (Moazeni and Khademolhoseini 2016; Ceballos et al. 2019; Reigate et al. 2021). For the ovicidal assay, NUNC© cell culture boxes with 24 wells were used, and 90 to 110 *F. hepatica* eggs were placed in each well. The eggs were exposed in triplicate to concentrations of 100, 200, 300, 400, and 500 mg/L EALM; control wells with solvent (ethyl acetate) and wells without treatment were used as control controls. Additionally, TCBZ was used at concentrations of 10 and 50 mg, and artemisinin at concentrations of 10 and 20 mg was assessed. Three replicates of each of the concentrations were carried out. Each of the boxes was covered with aluminum foil to protect them from light, and they were incubated for 14 and 16 days at a temperature of 28 °C and 80% humidity. After that period, they were exposed to 2 h of artificial light so that the miracidia would hatch. The ovicidal activity was evaluated according to formulas from the following (Najafi et al. 2017; Knepper et al. 2018; Machado et al. 2020):

$$\text{Eggs hatched}(\%) = \frac{\text{number of eggs hatched}}{\text{total number of eggs}} \times 100$$

$$\text{Ovicidal activity}(\%) = \frac{\%\ \text{eggs hatched in control} - \%\ \text{eggs hatched after drug incubation}}{\%\ \text{eggs hatched in control}} \times 100$$

Analysis of data

The data obtained were analyzed through the analysis of variance (ANOVA) test, Probit analysis, Dunnett's test, and Kruskal−Wallis test with a confidence interval of 95% to determine if there were statistically significant differences between the different treatments using SYSTAT v.12.0 32-bits (Systat Software, Inc. USA, 2008).

Results

EALM yields

Initially, 2.86 kg of green matter was collected, and when dried, 126 g of dry matter was obtained. At the end of the ethyl acetate extraction, a total of 17.85 g of EALM was obtained.

Ovicidal activity

The ovicidal activity was determined according to the number of hatched eggs in the different experimental groups. Regarding the percentages of ovicidal action, significant differences are observed between the reference compounds and EALM concentrations. Of the different concentrations of EALM, concentrations greater than 300 mg/L stand out, as ovicidal percentages greater than 60% were observed on both days of incubation ($p < 0.05$). The complete results of the percentage of ovicidal action of the eggs exposed to TCBZ, artemisinin, and EALM are shown in Table 1 and Fig. 1. The ovicidal effect increased according to the dose−response relationship. The efficacy of EALM increased when the eggs were exposed for 2 more days, which represented a longer extract exposure time.

The observations obtained for the morphology of the eggs exposed to EALM identified changes in relation to the control group are shown in Fig. 2. When the eggs were exposed to 300 mg/L EALM (Fig. 2E), miracidium

Table 1 Percentage of ovicidal action of TCBZ, artemisinin, and EALM, by days of incubation, in the different study groups

Days	Average % hatching in controls	Negative control	Solvent control (EtOAc)	Groups								
				TCBZ		Artemisinin		EALM (mg/L)				
				10 mg	50 mg	10 mg	20 mg	100	200	300	400	500
14	46.5	0	0	3.23	16.13	57.75	65.96	19.13	42.38	67.88	75.69	83.55
16	54.8	0	0	3.89	17.03	59.10	68.23	50.04	51.25	85.99	88.94	93.65

EtOAc ethyl acetate

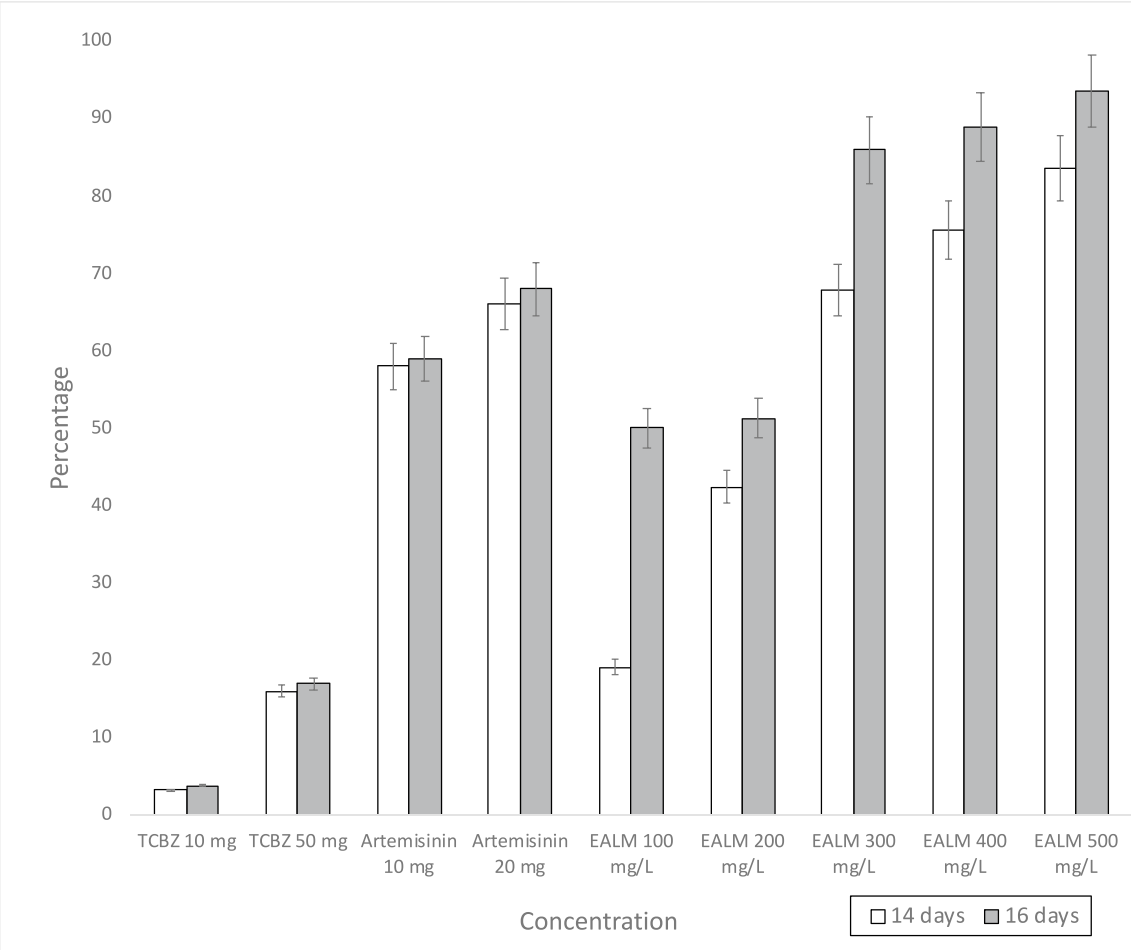

Fig. 1 Percentage of ovicidal action of TCBZ, artemisinin, and EALM, by days of incubation, in the different study groups

formation was observed. However, changes in the internal and external morphology of the egg were detected. Presumably, these changes affected the development and hatching of the miracidia. Eggs exposed to EALM at a concentration of 400 mg/L (Fig. 2F) showed a change in the external egg morphology, especially in one of the egg poles (indicated with the arrow), and a change in the germ cells within the egg started to become noticeable. In an area in the group treated with 500 mg/L EALM (Fig. 2G), the outline of the eggs is not adequately distinguished (indicated with the arrows), and their content has begun to emerge, suggesting that their morphology was damaged. Regarding the eggs treated with artemisinin, eggs without larval development and others with miracidium formation were observed. However, in both cases, small changes could be seen in the egg periphery, suggesting that the compound caused damage and the egg integrity was affected (Fig. 2H and I).

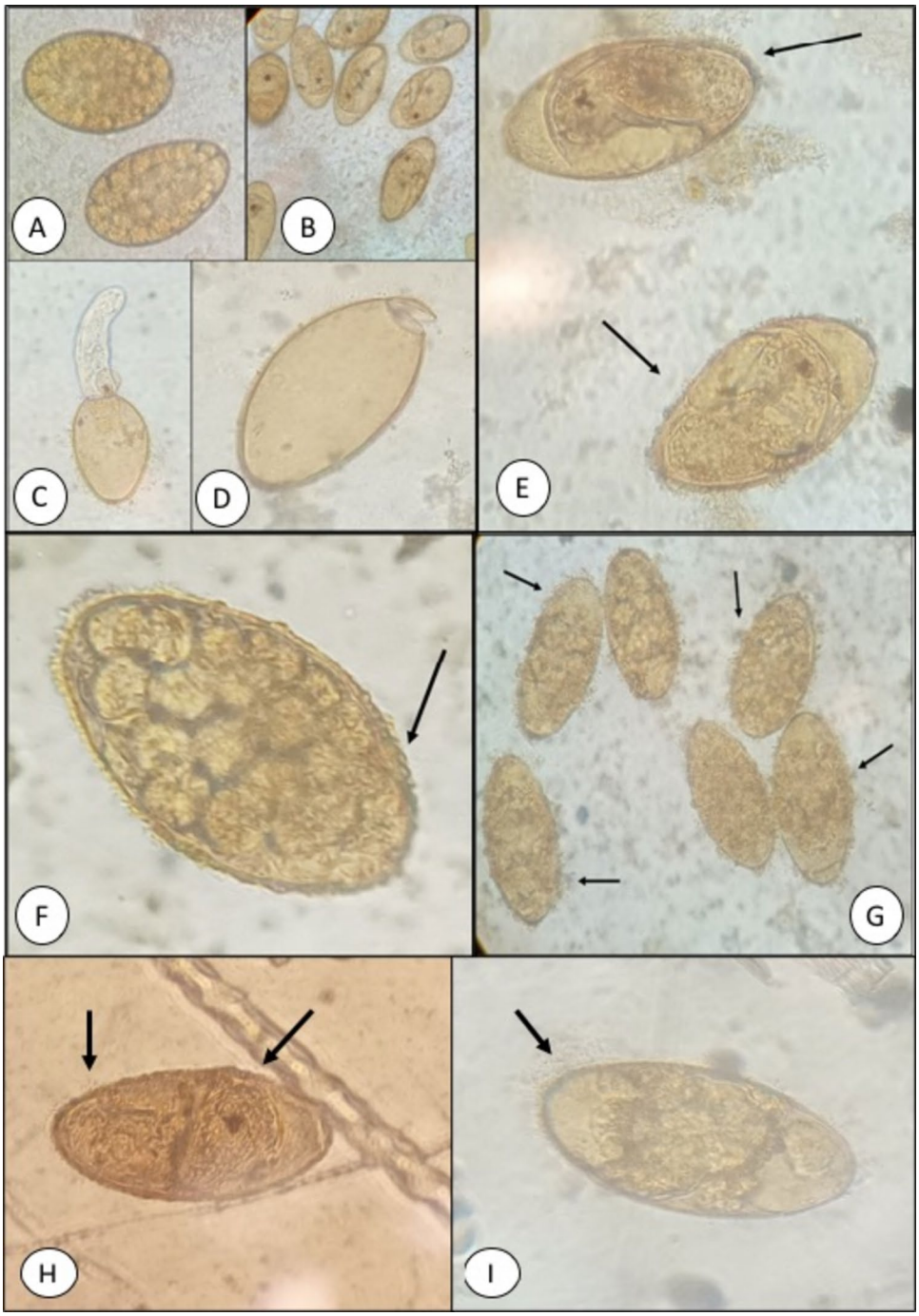

Fig. 2 Observations obtained for the morphology of the eggs of *F. hepatica* exposed to EALM and artemisinin. **A** Eggs before being incubated; **B** Larval eggs after being incubated at 16 days; **C** miracidia emerging from the egg after being subjected to artificial light for 2 h; **D** empty egg after subjection to 2 h of artificial light; **E** egg of *F. hepatica* treated with 300 mg/L EALM; **F** egg of *F. hepatica* treated with 400 mg/L EALM; **G** egg of *F. hepatica* treated with 500 mg/L EALM; **H** egg of *F. hepatica* treated with 10 mg of artemisinin; and **I** egg of *F. hepatica* treated with 20 mg of artemisinin

Fasciolicidal activity in adult flukes

The controls without any treatment did not suffer mortality during the duration of the experiment. In the groups treated with TCBZ and artemisinin, 100% efficacy was observed from the first 24 h at the two respective concentrations ($p < 0.05$). The fasciolicidal efficiencies of EALM at different concentrations showed differences, and 500 mg/L EALM was the only group that began to exhibit 72% efficacy at 24 h postexposure ($p < 0.05$). At 48 h postexposure, 125 mg/L showed 94% efficacy, and 250, 375, and 500 mg/L resulted in 100% efficacy ($p < 0.05$). Finally, at a concentration of 125 mg/L, the maximum efficacy was reached 72 h postexposure ($p < 0.05$) (Fig. 3). In general, an effect was observed in relation to concentration and exposure time. The complete fasciolicidal activities of EALM, TCBZ, and artemisinin against adult parasites are shown in Table 2. Regarding the different exposure times, an increasing efficacy began to be seen in the first 24 h of exposure, with concentrations of 500 mg/L reaching almost 100% efficacy 48 h postexposure ($p < 0.05$).

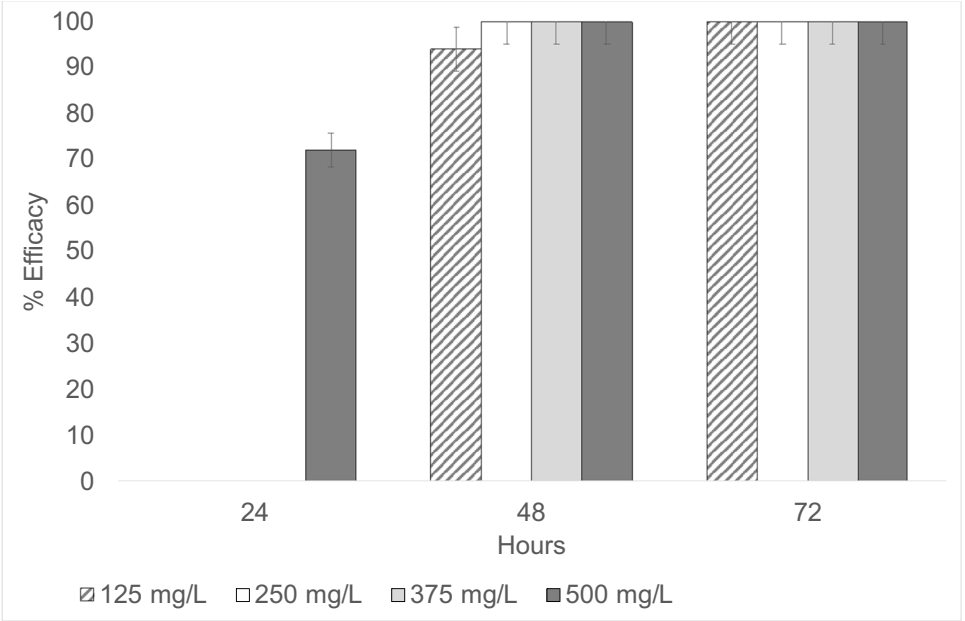

Fig. 3 Evaluation of the different concentrations of EALM at different exposure times ($P < 0.05$)

Table 2 Percentages of fasciolicidal efficacy in *F. hepatica* adults, triclabendazole, artemisinin, and EALM at different concentrations and exposure times

Hours	Concentration							
	TCBZ		Artemisinin		EALM			
	10 mg	20 mg	10 mg	20 mg	125 mg/L	250 mg/L	375 mg/L	500 mg/L
24	100	100	100	100	0	0	0	72 ± 0.092
48	100	100	100	100	94 ± 0.096	100	100	100
72	100	100	100	100	100	100	100	100

TCBZ triclabendazole

± denotes standard deviation

$P < 0.05$

Lethal concentration (LCs) 50, 90, and 99 estimates from EALM

Probit analysis showed that the LC_{50}, LC_{90}, and LC_{99} values for the EALM with fasciolicidal efficacy in *F. hepatica* adults in vitro determined in this study are 80.1 mg/L, 310.9 mg/L, and 362.9 mg/L, respectively.

Discussion

Previously, ovicidal assays of different parasites have been used to evaluate the efficacy of various compounds and plant extracts; recently, they have begun to be evaluated against *F. hepatica* eggs (Tunc et al. 2000; Kamaraj and Rahuman 2011; Moazeni and Khademolhoseini 2016; Moazeni et al. 2017; Najafi et al. 2017; Knepper et al. 2018; Machado et al. 2020). EALM had not been evaluated in this way, and this experiment showed an ovicidal effect on *F. hepatica* eggs. Presumably, there was damage to the eggshell, which may have affected its permeability. However, it is still necessary to determine in greater depth the type of damage that EALM has on the eggs to try to elucidate its mechanism of ovicidal action. In addition, certain drugs have been evaluated against *F. hepatica* eggs, such as ivermectin, artemisinin, and albendazole, all of which show ovicidal action, but the mechanism of action is still not clear (Moazeni et al. 2017). Regarding the evaluation of artemisinin and its ovicidal action, the observed damage was sufficient to alter egg integrity and prevent the development of miracidia and their hatching, although further studies are still necessary to visualize the nature of the damage to *F. hepatica*. In this experiment, although TCBZ was selected as the reference drug, it did not exhibit a considerable ovicidal percentage, in alignment with the results obtained by Álvarez et al. (2009). Additionally, the fasciolicidal effect of EALM shows its efficacy on adult parasites. Previously, its efficacy has been shown in newly

excysted flukes, reaching fasciolicidal efficacy greater than 90% from the first 24 h postexposure (Ezeta et al. 2016, 2020). It is important to emphasize the differences that exist between young and adult flukes. From the time the definitive host is infected until the adult forms reach the liver, approximately 6 to 7 weeks pass. During this time, the changes in its tegument are influenced by the progression through the intestinal mucosa, peritoneum, and liver (Robinson et al. 2022; González et al. 2021). During their migration, young flukes, with the help of their glycocalyx, proteolytic systems, genetic expression, and changes in their energy metabolism, are able to better evade the host's immune response, achieving greater adaptation and survival. All of this results in better development of the tegument, giving the flukes, among other things, better environmental protection (González et al. 2021). This may explain the differences between the fasciolicidal efficiencies of EALM in immature and adult forms of *F. hepatica*, especially at different exposure times where 100% efficacy is reached.

Additionally, the fasciolicidal effect of artemisinin has been demonstrated. Taking into account the previous work of Ezeta et al. (2020), the ethyl acetate extract of *A. ludoviciana* and its fractions point to the presence of artemisinin within them as the probable compound that generates the effect on parasites. Artemisinin is a constant metabolite within the genus *Artemisia* spp. and has been useful in treating *Plasmodium falciparum* infections in humans (Klayman 1985; Keizer and Utzinger 2007; Ferreira et al. 2011). Studies have been carried out with artesunate and artemether, which are semisynthetic derivatives of artemisinin, against *F. hepatica*, and an interruption in spermatogenesis in the adult flukes, affecting their reproductive system, was observed (O'Neill et al. 2009, 2015a, b, 2017). In another experiment, artemether caused extensive damage to the tegument of *F. hepatica* (Keiser and Morson 2008). However, none of these studies have directly evaluated artemisinin. Taking this into account, it is necessary to continue with studies that allow us to see the real damage to adult flukes caused by EALM and artemisinin, the compounds identified within the *A. ludoviciana* extract (Ezeta et al. 2020), to determine the affected area and compare the effect on the tegument of young and adult flukes. This would help in the ongoing effort to propose in vitro EALM as an integral option for the control of fasciolosis, covering different stages of parasite development and providing new insights into strategies against the fluke eggs.

Conclusion

The extract of *A. ludoviciana* obtained from ethyl acetate has an ovicidal effect on *F. hepatica* eggs and fasciolicidal efficacy in adult forms of the parasite.

Author contribution Alonso Ezeta Miranda: term, conceptualization, methodology, validation, formal analysis, investigation, data curation, and writing original draft; José Guillermo Ávila Acevedo: term, conceptualization, methodology, validation, resources, writing review and editing, and supervision; Yolanda Vera Montenegro: term, conceptualization, and resources; Gerardo Francisco Márquez: conceptualization and investigation.

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Data availability All data supporting the findings of this study are available in the document.

Declarations

Ethical approval Not applicable.

Consent to participate The authors give their consent to participate in the editorial process by the journal.

Consent for publication The authors give their consent to publish the research work in the journal.

Competing interests The authors declare no competing interests.

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