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Fasciola hepatica and Rumen Flukes - *In Vitro* Evaluation of Main Commercial Anthelmintics*

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ABSTRACT

***Background*:** Trematode infections are of great importance as they affect the health of many species of mammals as cattle, sheep and goat. *Fasciola hepatica* represents the main trematode zoonosis and risks to human and cattle and paramphistomosis is one emerging parasitic diseases of ruminants widely distributed in the world. The economic expenses are incurred by the use of ineffective anthelmintics for trematode control. Besides to faecal egg count reduction test (FECRT) to determine the anthelmintic efficacy, can be used *in vitro* assays, by this the aim of the study was to determine the lethal doses (LD) with hatching egg test (EHT) of the main commercial anthelmintics used for the control of trematodes in cattle. ***Materials, Methods & Results*:** Liver and rumen were examined from cattle slaughtered in Tabasco, Chiapas and Campeche states from Mexico. *F. hepatica* eggs were recovered from gallbladder and rumen fluke eggs collected from adult parasites in saline solution. Subsequently, the hatching egg assays were performed placing 100 trematode eggs in distilled water in each one of 96 wells of polystyrene plates. After making the appropriate dilutions, several concentrations of commercial anthelmintics were evaluated, ranging from 0.04 to 80.63 mM for triclabendazole + 0.046 to 96.87 mM febendazole (TC+FBZ), from 0.04 to 91 mM for rafoxanide (RAFOX), from 0.02 to 43.74 mM for closantel (CLOS), from 0.036 to 76.18 mM for clorsulon + 0.002 to 3.31 mM ivermectin (CLORS+ IVM) and from 0.163 to 334.47 mM for nitroxynil (NITROX). A control group (water) was included in each plate. Lethal doses were obtained using the Probit procedure and analysis of the means with a one-way statistical design. Most drugs used against rumen fluke eggs presented a high LD_{50} and therefore were ineffective to cause egg mortality, such was the case of RAFOX that presented LD_{50} from 4,580 to 10,790 µg/mL (7 to 17 mM). CLOS presented the lowest LD_{50} (80 µg/mL or 0.12 mM) on rumen fluke eggs. TC+FBZ was found to be effective drug against the development of *F. hepatica* eggs in many samples. In the same way NITROX showed a low LD_{50} (37 to 63 µg/mL or 0.13 to 0.22 mM), but RAFOX presented a highest LD_{50} (1,450 µg/mL or 2.32 mM). ***Discussion*:** The present study focused on screen the ovicidal activity and determining in vitro lethal doses 50 of main commercial anthelmintics used to control *F. hepatica* and rumen fluke as rapid tests in a tropical region from Mexico. The FECRT is the main method to detect effectiveness of anthelmintic and other method is the coproantigen reduction test (CRT) by ELISA. Both tests require many infected animals depending the number of treatments and by this the egg hatch assay (EHA) represent a complementary diagnosis of effectiveness of anthelmintic products to compare between regions and even between farms, because few animals are required from the farm to collect trematode eggs, and it is possible to know the effectiveness against various anthelmintics at the same time. Efficacy studies on trematodes using egg hatching tests are scarce, although they have the advantage that they can be applied to both *F. hepatica* and rumen fluke. TC+FBZ was one of the most effective products in inhibiting the development of *F. hepatica* eggs. However, RAFOX showed low effectiveness against trematode eggs, with very high lethal doses. These results agree with a study that show low efficacy against the development of *Paramphistomum cervi* eggs and with the FECRT test reductions of 75% and 80.58% were obtained, in times from 7 to 84 days after treatment with RAFOX. NITROX and CLORS were drugs that had good efficacy on the development of *F. hepatica* eggs. A differential response between liver and rumen fluke was observed. The anthelmintics used against rumen fluke eggs show low ovicidal activity and in *Fasciola hepatica* TC+FBZ show the best activity.

Keywords: trematodes, cattle, effectiveness, anthelmintics, ovicidal activity, egg hatching.

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INTRODUCTION

Trematodes are parasites of great importance that affect domestic animals of economic interest. *Fasciola hepatica* is the most important species because cause a zoonotic disease that results in loss over US$3 billion annually [9]. In cattle liver fluke has the highest prevalence and greatest damage to productivity [8]. However, rumen fluke has regained importance in several countries in Europe [7]. The presence of trematodes of veterinary importance is determined by the abundance of intermediate hosts, snails, which are responsible of dissemination of infective phases [18]. The economic losses are due to the expenses incurred by the use of anthelmintics for their trematode control. Another cause is by seized of liver when infections with *F. hepatica* in cattle are present [23]. In the case of rumen fluke, damage to the animal occurs when the metacercaria breaks loose and migrates from the small intestine to the rumen. Several countries such as United Kingdom [4], Spain [14], Portugal [2] Sweden [16], Indonesia [29], Iran [20], Nigeria [32], Peru [26] and Mexico [24] indicate high prevalence of trematodes in liver and rumen in cattle.

To avoid damage in health and reduce the prevalence of trematodes, remove the use of several anthelmintics have been used [25,34] as main method of control. The faecal egg count reduction and coproantigen tests [22] are used to determine effectiveness. However, they are expensive and time consuming. Other method to determine the anthelmintic efficacy is the eggs hatch test [11]. Therefore, the aim of this study was to determine the lethal doses of the main commercial anthelmintics used for the control of trematodes in cattle.

MATERIALS AND METHODS

Location

Three slaughterhouses were visited in Southeastern Mexico, belonging to the municipalities of Jalapa, in Tabasco state, located at 17° 75' N and 92° 84' W; Juarez in Chiapas state, located at 17° 41' N and 93° 13' W; and Escárcega, in Campeche state, located at 18° 60' N and 90° 77' W. A total of 14 samplings were carried out, during the months of January to October 2021.

Trematode collection

After slaughter and dressing of the cattle, the liver and rumen were inspected. Adult specimens of *Fasciola hepatica* and rumen flukes were collected from parasitized animals. The rumen flukes were collected in groups of 50 specimens in 15 mL conical tubes containing 10 mL of running water. Immediately, the trematodes were washed again with running water to remove rumen debris. Subsequently, the paramphistomid eggs were obtained by leaving the parasites in the conical tubes for 5 h at room temperature (27°C) in 0.9% physiological saline solution.

Eggs from *F. hepatica* were obtained from the bile collected from animals that tested positive for this parasite, after searching in the liver and finding adults. The bile was stored in a 50 mL flask and the eggs were later recovered in the laboratory.

Recovery and quantification of trematode eggs

The trematode eggs were recovered after washing with running water in a 37 μm sieve[1]. The recovered eggs were transferred to 15 mL conical tubes with 10 mL of sterile water and the egg count was carried out in 20 subsamples of 10 μL aliquots after vortexing. The average of the 20 aliquots was extrapolated to the total volume. The final concentration was diluted to one egg per microlitre. To eliminate bacteria from the solution, one drop of streptomycin sulphate (10 mg/L)[2] and benzylpenicillin (10,000 units/L)[2] were added to the tube for later *in vitro* tests [13].

In vitro *test to determine the anthelmintic efficacy*

To evaluate the effectiveness of anthelmintics the hatching egg tests were carried out using the established technique in *Fasciola hepatica* [6,11], with some modifications. Briefly, 96-well polystyrene plates (NUNC, Maxisorb Invitrogen)[2] were used. In each well, 100 μL of sterilised water with 100 trematode eggs were placed. In addition, 100 μL of an anthelmintic was added to the first row, which was serially diluted in the 12 wells of the row (1:1 in each well). A different anthelmintic was placed in each row and the same procedure was carried out, so the following drugs were tested: triclabendazole+febendazole (TC+FBZ) [Saguaymic plus triclabendazole 10% + fenbendazole 10%;][3], at a concentration of 14 to 29,000 μg/mL (0.04 to 80.63 mM) for triclabendazole + (0.046 to 96.87 mM) febendazole; rafoxanide (RAFOX) [Rafoxcur® 200 mg/mL][4], at a concentration of 27.8 to 57,000 μg/mL (0.04 to 91 mM); closantel (CLOS) [Closantel 10%; Closiver ADE+B12®][5], at a concentration of 14 to 29,000 μg/mL (0.02 to 43.74 mM); clorsulon

(CLORS+IVM) [Ivermitrem® Ivermectin 10 mg + Clorsulon 100 mg][6], at a concentration of 14 to 29,000 µg/mL (0.036 to 76.18 mM) + 1.4 to 2,900 µg/mL (0.002 to 3.31 mM) ivermectin; and nitroxinil (NITROX) [Nitroxinil 34%; Nitromic™][3] at a concentration of 47.4 to 97,000 µg/mL (0.163 to 334.47 mM). The control group consisted of 100 eggs contained in distilled water without anthelmintic. Additionally, 250 µL of distilled water was added to each well of the plate.

The polystyrene plates were placed in a dark incubator at room temperature (26°C) for a period of 14 days. After that, the plates were exposed to daylight for a period of 12 h, in order to stimulate the hatching of trematodes eggs. Subsequently, the content of each well was examined under an optical microscope (Olympus CX-21, 10X)[7] and the number of eggs at each stage of development was recorded.

The eggs were classified as non-viable (dead, empty, not embryonated or in cell division without movement after 15 days of incubation) or viable (eyespot, hatched or larvae) according to Fairweather [11]. To test the variability of samples, the highest number of repetitions were made in each anthelmintic used (Table 1).

Table 1. Number of *in vitro* assays evaluating each anthelmintic by study region to obtain the lethal dose against fluke eggs.

Trematode	Anthelmintic	Jalapa - Tabasco	Juárez - Chiapas	Escárcega - Campeche	Total
Paramphistomids	Triclabendazole+febendazole	7	15	6	28
	Rafoxanide	6	16	5	27
	Nitroxinil	7	14	6	27
	Closantel	4	12	6	22
	Clorsulon+ivermectin	5	14	6	25
Fasciola hepatica	Triclabendazole+febendazole	6	5	0	11
	Rafoxanide	6	4	0	10
	Nitroxinil	6	4	0	10
	Closantel	6	1	0	7
	Clorsulon+ivermectin	5	2	0	7

Statistical analysis

From the proportion of viable and non-viable eggs from the *in vitro* assays, the median lethal dose (LD_{50}), the 95 lethal dose (LD_{95}) and the maximum lethal dose (LD_{99}) were calculated using the logistic regression model (Probit) of the SAS program [30]:

$$Pr(Response) = C + (1-C)\, F(x'\beta) = C + (1-C)\, \Phi(b0 + b1 \times \log10(Doses))$$

Where: Pr: is the probability of a response. β: is a vector of estimated parameters. F: is a cumulative distribution function (Normal). X: is a vector of explanatory variables. C: is the natural response rate (proportion of individuals responding to zero dose). Φ: the normal cumulative distribution function.

To obtain the average lethal doses, a statistical analysis was carried out for each anthelmintic used (TC+FBZ, RAFOX, NITROX, CLOS and CLORS+IVM), in order to show the effect of the sampling site (Tabasco, Campeche and Chiapas) and determine the variability of the LD_{50} and LD_{95}. For this, the general lineal method (GLM) procedure of SAS was used, in addition, the separation of means was carried out with the LS means procedure [30].

RESULTS

Hatching of rumen fluke eggs

Most of the anthelmintics used against rumen fluke eggs showed higher LD_{50} (Table 2) than showed in *F. hepatica* (Table 3). RAFOX required very high concentration (3,350-10,790 µg/mL or 5.3 to 17.2 mM) to achieve 50% egg mortality in rumen fluke. This product presented high variability and differences among LD95 values in the different study locations. In Tabasco higher lethal doses (107,750 µg/mL or 172.1 mM) than Campeche and Chiapas (4,380 and 6,070 µg/mL

or 6.99 and 9.7 mM, respectively) was found. NITROX and TC+FBZ presented high LD_{50}, and very high LD_{99} in some of the sampling sites. Similarly, CLORS+IVM show high doses reported in mg/mL and mM (Table 2).

RAFOX showed the highest variability in the control of paramphistomid eggs, and the lethal doses obtained were greater than the other anthelmintics used (Figure 1).

The lethal doses obtained in all the sampling sites allowed determination of the variability lethal doses for the main products used against rumen fluke eggs. The LD_{50} was high for all products with narrow confidence intervals (CI) [Table 3]. In the LD_{95} of NITROX and CLORS+IVM, a wider CI was observed. RAFOX was an inefficient product and presented highly variable lethal doses in its replicates.

Hatching of Fasciola hepatica *eggs*

The mean lethal doses of the different anthelmintics analysed on *F. hepatica* eggs showed that TC+FBZ was 100% effective in all doses, regardless of the site (Table 4). NITROX also presented some 100% effective repetitions, with means of 37 µg/mL or 0.13 mM in Tabasco. The LD_{50} of all products was low, but not for LD_{95} and LD_{99}, which were high specifically for RAFOX and CLORS+IVM in Tabasco, in addition, RAFOX and NITROX in Chiapas. The cattle from Campeche did not present *F. hepatica* during the sampling time. Therefore, *in vitro* tests were not performed for that site.

The mean lethal dose obtained in *F. hepatica* egg hatching was low in 4 of the anthelmintics used in the study (TC+FBZ, NITROX, CLORS+IVM, CLOS), while RAFOX showed the highest values (Table 5) of LD_{50} and LD_{95}.

Table 2. Average lethal doses of main anthelmintic products used against rumen fluke by cattle origin in Southeastern Mexico.

Anthelmintic	N	Tabasco (mg/mL)			Chiapas (mg/mL)			Campeche (mg/mL)		
		LD_{50}	LD_{95}	LD_{99}	LD_{50}	LD_{95}	LD_{99}	LD_{50}	LD_{95}	LD_{99}
Triclabendazole+febendazole	23	0.34 (0.9)	14.05 (39.1)	170.60 (474.3)	0.23 (0.64)	2.14 (5.95)	13.00 (36.1)	0.35 (0.9)	19.01 (52.8)	113.60 (315.9)
Rafoxanide	20	10.79 (17.2)	107.75[a] (172.1)	293.49[a] (468.8)	4.58 (7.3)	6.07[b] (9.7)	6.86[b] (10.9)	3.35 (5.35)	4.38[b] (6.99)	4.96[b] (7.92)
Nitroxinil	13	0.11 (0.38)	4.38 (15.1)	26.24 (90.5)	0.65 (2.24)	22.55 (77.8)	152.30 (525.2)	0.09 (0.31)	0.35 (1.2)	2.93 (10.1)
Closantel	19	0.21 (0.32)	2.72 (4.1)	8.42 (12.7)	0.04 (0.06)	0.11 (0.17)	0.19 (0.29)	0.10 (0.15)	0.69 (1.0)	1.78 (2.7)
Clorsulon +ivermectin	23	0.63 (1.7)	8.99 (23.6)	34.96 (91.8)	1.39 (3.6)	3.25 (8.5)	18.79 (49.4)	0.91 (2.39)	11.58 (30.4)	48.83 (128.3)

[abc]Means with different literal within a row at each lethal dose are significantly different ($P \leq 0.05$); N: total of assays; LD: Lethal doses. In brackets lethal doses in millimolar (mM).

Table 3. General means of the lethal doses with their confidence intervals in the main anthelmintic products used against rumen fluke from cattle.

Anthelmintic	LD_{50} , mg/mL (mM)			LD_{95} , mg/mL(mM)	
	N	Mean	LCI-UCI	Mean	LCI-UCI
Triclabendazole+febendazole	28	0.32 (0.9)	0.14-0.50 (0.4-1.4)	0.73 (2.0)	0.44-1.03 (1.2-2.9)
Rafoxanide	23	3.30 (5.3)	2.00-4.53 (3.2-7.2)	19.5 (31.1)	0.00-39.9 (0-63.7)
Nitroxinil	13	0.27(0.9)	0.05-0.49 (0.2-1.7)	5.87 (20.2)	0.99-10.75 (3.4-37.1)
Clorsulon	25	1.07 (2.81)	0.60-1.54 (1.6-4.0)	6.75 (17.7)	2.26-11.25 (5.9-29.6)
Closantel +ivermectin	22	0.08 (0.1)	0.02-0.14 (0.03-0.2)	2.06 (3.1)	0.28-3.84 (0.4-5.8)

LCI: lower confidence interval; UCI: Upper confidence interval at 95%; LD_{50}: Lethal doses at 50%; LD_{95}: Lethal doses at 95%; N: total assays. In brackets lethal doses in millimolar (mM).

Table 4. Average lethal doses of the main anthelmintic products against *Fasciola hepatica* in Southeastern Mexico.

Anthelmintic	N	Tabasco (mg/mL)			Chiapas (mg/mL)		
		LD_{50}	LD_{95}	LD_{99}	LD_{50}	LD_{95}	LD_{99}
Triclabendazole +febendazole	9	*	*	*	*	*	*
Rafoxanide	10	0.42 (0.7)	17.03 (27.2)	221.80 (350.3)	2.91 (4.6)	12.09 (19.3)	27.30 (43.6)
Nitroxinil	5	0.037 (0.13)	0.06 (0.2)	0.10 (0.3)	0.06 (0.2)	32.03 (110.4)	459.60 (1584.8)
Clorsulon +ivermectin	6	0.31 (0.8)	3.73 (9.8)	47.23 (124.1)	-	-	-

*100% mortality at all doses; N: total assays; LD_{50}: Lethal doses at 50%; LD_{95}: Lethal doses at 95%. In brackets lethal doses in millimolar (mM).

Table 5. General means of the lethal doses with their confidence intervals of the main anthelmintic products used against *Fasciola hepatica*.

Anthelmintic		LD_{50}, mg/mL (mM)		LD_{95}, mg/mL (mM)	
	N	Mean	LCI-UCI	Mean	LCI-UCI
Triclabendazole +febendazole	5	0.0016 (0.004)	0.0015-0.0017 (0.0042-0.0047)	0.007 (0.02)	0.005-0.009 (0.014-0.025)
Rafoxanide	10	1.41 (2.3)	0.00-2.90 (0.0-4.6)	5.79 (9.24)	0.46-11.13 (0.7-17.8)
Nitroxinil	5	0.026 (0.09)	0.009-0.042 (0.03-0.14)	0.074 (0.25)	0.049-0.098 (0.17-0.34)
Clorsulon	7	0.26 (0.68)	0.068-0.45 (0.18-1.18)	0.47 (1.2)	0.083-0.865 (0.21-2.27)
Closantel +ivermectin	6	0.0026 (0.004)	0.0016-0.0068 (0.002-0.01)	0.0062 (0.009)	0.0019-0.014 (0.003-0.02)

LCI: lower confidence interval; UCI: Upper confidence interval at 95%; LD_{50}: Lethal doses at 50%; LD_{95}: Lethal doses at 95%. In brackets lethal doses in millimolar (mM).

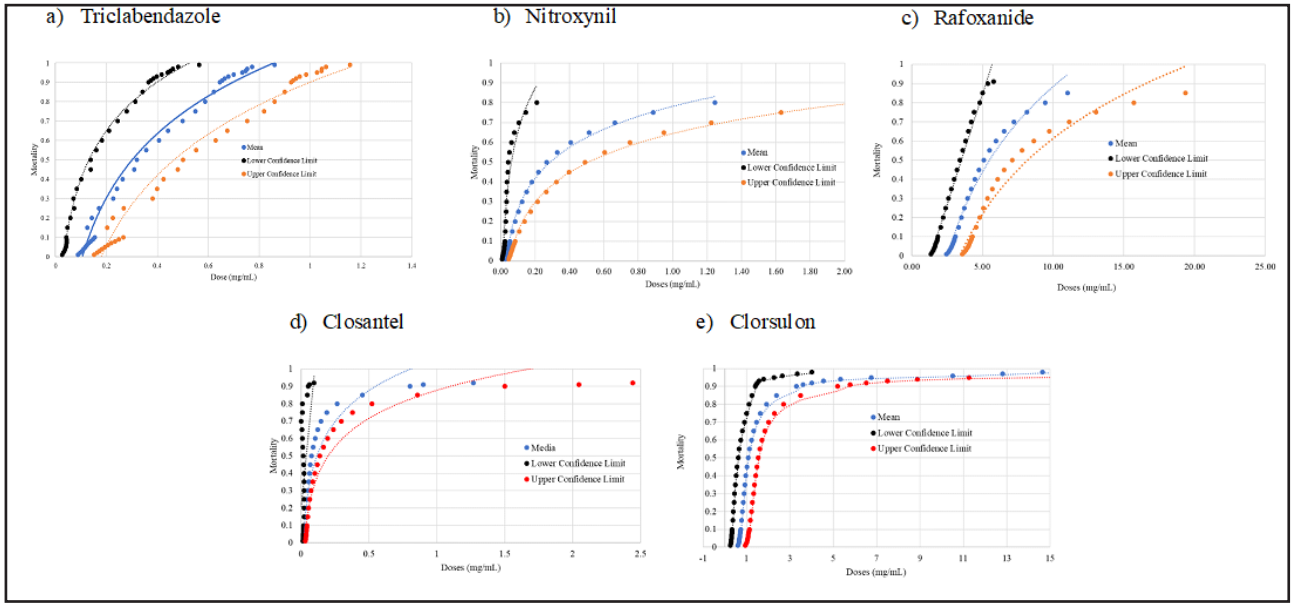

Figure 1. Mean lethal doses and confidence intervals of main anthelmintics on paramphistomid eggs from cattle raised in Southeast Mexico.

DISCUSSION

The present study focused on determining *in vitro* lethal doses to serve as references for later studies on the effectiveness of the main drugs used against *Fasciola hepatica* and rumen fluke, as rapid tests. Other methods to know the effectiveness of anthelmintic are FECRT and coproantigen reduction test (CRT) by ELISA, that require many pretreated animals [5]. Therefore, Fairweather [11] proposed the egg hatch test (EHA) for the diagnosis of resistance to triclabendazole in *F. hepatica*. Additionally, this proposal allows determination of the efficacy of anthelmintic products in trematode eggs *in vitro* [13]. Furthermore, it is an easy diagnostic method, and by collecting eggs from a few animals from the farm, it is possible to know the effectiveness against various anthelmintics, while *in vivo* tests are costly due to the number of repetitions required and the handling of the cattle. On the other hand, studies on trematodes using egg hatching tests are scarce.

In southeastern Mexico *F. hepatica* and rumen fluke presented high prevalence and mixed infections has been observed in many farms. The high humidity and temperature conditions, typical of the hot climates that exist in the region, contributed to the abundance of both trematode species. Ojeda-Robertos [24] found a high prevalence of *F. hepatica* and rumen fluke in the same area (Jalapa, Tabasco, Mexico) during the rainy season. Therefore, it is important to consider the microclimate of each region in the prevalence of trematodes [28]. Considering that the prevalence of *Paramphistomum cervi* ranges between 3.33-96.67% in Tabasco [27] and between 0-61% for flukes (*F. hepatica* and rumen fluke) in some areas of Tabasco and Chiapas, Mexico [12], the importance of this study lies in proposing the lethal doses that allow knowing the effectiveness of the products used in the control of both trematodes.

TC+FBZ was one of the most effective products in inhibiting the development of *F. hepatica* eggs. The high effectiveness was possibly related to the mixture of the 2 anthelmintics belonging to benzimidazoles and the infrequent use of this product in the region. However, in some studies, resistance to TC has been indicated. TC has been the main drug used in controlling the immature and mature stages of *F. hepatica* in many regions of the world [5]. The mechanism of action, by intervening in the formation of microtubules, affects the reproductive system of the parasite, inhibiting the production of eggs of the adult trematode [33]. However, Alvarez [1] point out that TC+FBZ does not have ovicidal activity against *F. hepatica* eggs, despite being evaluated in susceptible eggs. Similarly, it is one of the drugs with the greatest increase in resistance in Europe, including its combinations with other products [10], which has also been indicated by Kamaludeen [19], who determined a widespread resistance in the north-west of England and Wales using FECRT tests, with values of 0% in effectiveness. However, in another *in vivo* study, efficacy against *F. hepatica* was found to be between 46-69% for the same area as the present study [17], but ovicidal activity was found in the *in vitro* tests. The efficacy of TC+FBZ is possibly due to the fact that the egg samples came from animals that had not previously been exposed to this drug, since cattle deworming in the region makes use of other drugs such as ivermectin, which has surely allowed TC to continue being effective in the study region. In the case of rumen fluke, the efficacy of TC+FBZ was low as shown by the high concentrations of lethal doses obtained. This possibly because this is not a specific drug against rumen fluke, and in Mexico there is still no authorised anthelmintic for the control of this group of trematodes [3].

The RAFOX showed low effectiveness against both *F. hepatica* and rumen fluke eggs, with very high lethal doses in cattle from Tabasco. These results agree with the low efficacy against the development of *P. cervi* eggs from other study [13]. It also coincides with the low efficacy obtained in the FECRT test, with reductions of 75% and 80.58%, in times from 7 to 84 days after treatment with RAFOX [31]. In contrast, CLOS showed good efficacy in inhibiting the development of paramphistomid eggs, despite belonging to the group of salicylanilides to which RAFOX also belongs [21]. Therefore, CLOS represents an alternative to be rotated with other products, even when there is resistance to TC and RAFOX [15]. However, there are already reports of CLOS resistance in *F. hepatica*, even in its combination with ivermectin [22].

NITROX and CLORS+IVM were drugs with good efficacy to stop the development of *F. hepatica* eggs. NITROX belongs to the group of halogenated phenols, with great effect against adults of this fluke [25]. In addition, it has the capacity to inhibit the development of eggs [13], as observed in some samples that

presented 100% of mortality, although in Juárez some lethal doses were high, which implies some degree of resistance. This may be because of the constant use of the commercial product in the treatment of internal parasites.

Some drugs presented a great variability in the results, with repetitions highly effective and others samples were ineffective in the same product. The specific anthelmintic management of the cattle is probably the most logical explanation since the clinical history of the treatments was not known. The results of lethal doses obtained from *in vitro* tests allow us to identify the ovicidal activity of the products. Therefore, the high variability of products when compared to different strains of trematode eggs, gives a broad panorama of efficacy in different regions and not only at the level of the farm.

CONCLUSIONS

The high lethal doses from egg hatching tests of the 5 main commercial anthelmintic used in the southeast of Mexico for the control rumen fluke indicate the ineffectiveness in these species.

In *Fasciola hepatica* high effectiveness of triclabendazole + febendazole and Nitroxinil were observed and low lethal doses determined so these products are ovicidal. While the only product without ovicidal capacity was rafoxanide, which obtained a very high lethal dose.

The great variability in the efficacy of anthelmintics as observed in the wide confidence intervals of lethal doses to control trematode eggs hatching indicates that it is necessary to carry out more *in vitro* and *in vivo* studies to determine the degree of anthelmintic resistance that exists in the region because producers are accustomed to deworming livestock frequently without knowing how effective anthelmintics are in controlling flukes.

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***Ethical approval*.** The procedures were carried out in accordance with the Official Mexican Standard 051-ZOO-1995 for humanitarian treatment of mobilized animals.

***Declaration of interest*.** The authors report no conflicts of interest and are alone responsible for the content and writing of the paper.

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