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Original Article

Ixodicidal effect of extracts from *Cordia boissieri*, *Artemisia ludoviciana* and *Litchi chinensis* on *Rhipicephalus* (*Boophilus*) *microplus* (Acari: Ixodidae)

Efeito ixodicida de extratos de Cordia boissieri, Artemisia ludoviciana e Litchi chinensis sobre Rhipicephalus (Boophilus) microplus (Acari: Ixodidae)

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Abstract

The ixodicidal activity of the methanolic extracts of *Artemisia ludoviciana* (Astereceae), *Cordia boissieri* (Boraginaceae) and *Litchi chinensis* (Sapindaceae) against two field populations of *Rhipicephalus* (*Boophilus*) *microplus* from the state of Nuevo Leon (NL) and Veracruz (VER) was evaluated. The extract of *L. chinensis* in the concentration of 150 mg/ml showed efficacies of 100% and 99% against engorged females and mortalities of 98% and 99% against larvae. *C. boissieri* in the same concentration showed efficacies of 71% and 37% against engorged adults and mortalities of 33.04% and 10.33% against larvae and *A. ludoviciana* had efficacies of 94% and 83% in adults and mortalities of 89.39% and 89.21% against larvae in both populations respectively. The enzymatic activity of Acetylcholinesterase (AChE), Carboxylesterase (CaE), Glutathione-S-Transferase (GST) and Alkaline Phosphatase (ALP) was measured in both populations of ticks. As a result, a significant difference between both populations was shown, being the VER population the one that exhibited a higher enzymatic activity ($p \leq 0.05$). It can be concluded that the methanolic extract of the seed of *L. chinensis* shows potential ixodicidal activity and can be used as an alternative source of tick control, however, prior characterization, toxicity and formulation studies are necessary.

Keywords: *R.* (*B.*) *microplus*, synthetic ixodicides, plant extracts, enzymatic activity.

Resumo

No presente trabalho, a atividade ixodicida de extratos metanólicos de *Artemisia ludoviciana* (Astereceae), *Cordia boissieri* (Boraginaceae) e *Litchi chinensis* (Sapindaceae) contra *Rhipicephalus* (*Boophilus*) *microplus* foi avaliada em duas populações de campo nos estados de Nuevo León (NL) y Veracruz (VER). O extrato de *L. chinensis* na concentração de 150 mg/ml, apresentou eficácia de 100% e 99% contra fêmeas ingurgitadas, e taxas de mortalidade de 98% e 99% contra larvas. *C. boissieri*, na mesma concentração, apresentou eficácia de 71% e 37% contra adultas ingurgitados, e taxas de mortalidade de 33,04% e 10,33% contra larvas e *A. ludoviciana* apresentou eficácias de 94% e 83% em adultos e mortalidade de 89,39% e 89,21% contra larvas em ambas as populações respectivamente. Por outro lado, a atividade enzimática da Acetilcolinesterase (AChE), Carboxilesterase (CaE), Glutationa-S-Transferase (GST) e Fosfatase Alcalina (ALP) foi medida em ambas as populações de carrapatos. Como resultado, foi apresentada uma diferença significativa entre as populações, sendo a população VER a que apresentou maior atividade enzimática ($p \leq 0,05$). Assim, pode-se concluir que o extrato metanólico da semente de *L. chinensis* apresenta potencial atividade ixodicida e pode ser utilizado como fonte alternativa de controle de carrapatos, porém estudos prévios de caracterização, toxicidade e formulação são necessários.

Palavras-chave: *R. (B.) microplus*, ixodicidas sintéticos, extratos de plantas, cinética enzimática.

1. Introduction

The tick *Rhipicephalus* (*Boophilus*) *microplus* is an endemic ectoparasite of livestock mainly in tropical and subtropical regions of the world, causing great economic losses due to its direct and indirect effects, amounting to $573.61 million dollars in Mexico (Rodríguez-Vivas et al., 2004, 2017; Barros-Battesti et al., 2006).

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Currently the most widespread method for tick control is the use of synthetic ixodicides, such as pyrethroids (SP), organophosphorus compounds (OP) and amitraz (AM), which have played a fundamental role in the control of *R.* (*B.*) *microplus*. However, due to the extensive and indiscriminate use of these chemical products, this tick species has developed resistance to the main classes of acaricides in different countries (Fernández-Salas et al., 2012; Guerrero and Pruett, 2003).

This resistance has developed mainly due to intrinsic or biological factors related to the tick, such as the production of genetic mutations in the dominant resistance allele and changes in the enzymatic metabolism in tick populations (Guerrero et al., 2001; Foil et al., 2004).

In Mexico, the resistance of ticks to ixodicides is recognized in several states, mainly in the tropics (Rodríguez-Vivas et al., 2007). Nerveless, the synthetic ixodicides used to control the tick are usually toxic to humans and the environment, as well as having residual effect on meat and milk in cattle (Del Puerto et al., 2014).

New therapeutic options are being investigated in order to reduce the use of synthetic ixodicides. In this context, studies show that botanical oils and extracts are proposed as possible control methods for *R.* (*B.*) *microplus* (Adenubi et al., 2016).

Compounds from plant extracts provide a potential alternative to existing ixodicides, based on promising results in controlling ixodicide-susceptible and -resistant ticks (Chagas et al., 2002; Fernándes and Freitas, 2007; Adenubi et al., 2016).

Likewise, studies carried out with *Cordia boissieri* extracts show antibacterial, antifungal and antioxidant activity (Salazar-Aranda et al., 2011). In addition, studies with *Artemisia ludoviciana* showed the presence of antimicrobial, antiparasitic, and antioxidant activity (Zavala-Sánchez et al., 2002; Said Fernández et al., 2005; Lopes-Lutz et al., 2008). Also, studies on *Litchi chinensis* have shown its antioxidant, anticancer, antimicrobial, antiviral and anti-inflammatory activity (Yang et al., 2012; Lin et al., 2015; Wen et al., 2014; Nimmanpipug et al., 2009; Yamanishi et al., 2014). However, none of these plants have reports of ixodicidal activity on the cattle tick. Therefore, the aim of this study was to evaluate the ixodicidal activity of methanolic extracts from leaves of *C. boissieri*, *A. ludoviciana* and seeds of *L. chinensis* as well as synthetic ixodicides in two different populations of *R.* (*B.*) *microplus* engorged females and larvae.

2. Materials and Methods

2.1. Plant material and extraction

The plant material of *C. boissieri* was collected in Higueras, Nuevo León (NL) (25°57'50.9" N and 100°01'15.2" W), *A. ludoviciana* was collected in Guadalupe, (NL) (25°42'07.6" N and 100°11'33.9" W), and *Litchi chinensis* was collected in Veracruz (VER) (22°28'17.09" N and 93°36'39.9" W), in Mexico. The extracts were obtained using a Soxhlet extractor (700ml of methanol / 70g of plant material). The product obtained was evaporated in a rotary evaporator (Heidolph, Laborota, 4003-control, Germany) at 30ºC and under reduced pressure. The extract obtained was dried at 25°C and stored at 4°C (Borges-Argáez et al., 2007).

2.2. Synthetic ixodicides

Three ixodicides were selected based on the most used products in both regions: Ticoff® (Lapisa), active ingredient Cypermethrin which belongs to the pyrethroids, (Concentration: 1.5mL/1L), Asuntol® liquid (Bayer), active ingredient Coumaphos in the Organophosphorus group (OP) (Concentration: 1mL/1L), and Garra Ban MO 29® (Lapisa), which is an association of the active ingredients Chlorpyrifos and Permethrin belonging to the Organophosphorus and Pyrethroid groups (Concentration: 1mL/1L).

2.3. Tick collection

Female *R.* (*B.*) *microplus* ticks were collected from naturally infested cattle in production units in the city of General Bravo, (NL) (25°42'07.6" N and 100°11'33.9" W) and another production unit in Tantoyuca, (VER) (21°21'07.6" N and 98°14" W), both states within the Mexican territory. The collection method was carried out in accordance with the recommendations of the FAO (2004). Two groups of engorged female ticks were made, one used for the adult immersion test (AIT). The second group was incubated at 27±2°C, with a relative humidity of 80-90% (Cen-Aguilar et al., 1998) for two weeks until eggs were laid and the larvae used for the larval package test (LPT), larval immersion test (LIT), and for biochemical characterization of populations by the enzymatic activity of AChE, CaE, GST, and ALP.

2.4. Enzyme extracts

Pools of 100mg of *R. (B) microplus* larvae were formed in 10 volumes (1:10) of distilled water, homogenized in FastPrep®-24 (MP Biomedicals) at 2,500 RPM for 10 minutes at 4°C. The resulting supernatants were used as a source of the enzymes acetylcholinesterase (AChE), carboxylesterase (CaE), Glutathione-S-transferase (GST) and alkaline phosphatase (ALP). The protein concentration of the enzyme extracts was determined using bovine serum albumin (BSA) as a standard (Bradford, 1976). The results were expressed in milligrams of protein per milliliter (mg. ml^{-1}).

2.5. Evaluation of enzyme activity

Enzyme activity was determined according to Ellman et al. (1961) and modified as described by Li et al. (2005). For AChE, the reaction solution contained 0.24mM acetylthiocholine chloride (Sigma-Aldrich) and 0.64mM 5,5′-dithiobis-(2-nitrobenzoic acid) (DTNB) (Sigma-Aldrich), prepared in phosphate buffer and 10µl of the enzymatic extract. For CaE, an assay was performed in which the following was poured in each well: enzymatic extract; 50mM tris-HCl buffer (pH 7.1) and the substrate p-nitrophenylacetate was added at a final concentration of 0.0005 M to start the reaction. For ALP, enzyme extract was added to each well, diethanolamine buffer 1.0M (pH

9.8) with 50mM $MgCl_2$. To start the reaction, the substrate 4-nitrophenyl-phosphate was added. For the evaluation of the enzyme kinetics of the three enzymes, substrate degradation was measured by spectrophotometric readings at 405nm every 2 min for 12 min. For GST, a reaction mix was made with 19.8ml of Dulbecco's phosphate buffered saline (pH 7.2), 200µl of 200mM reduced L-glutathione (G4251 Sigma), and 200 µl of 1-chloro-2,4-dinitrobenzene (CDNB) 100mM (S2569 Sigma). In each well, 10µl of the enzyme extract and 190µl of the reaction mixture were added, the absorbance readings were made at 340nm every 2 min for 12 min, having a total of six readings for each well. Eighteen replicates were performed for each population, three blanks per microplate were included which did not contain enzymes.

2.6. Adult immersion test (AIT)

The AIT described by Drummond et al. (1973) was used to evaluate the synthetic ixodicides, which were diluted in water with the concentrations recommended by the manufacturer, using water as a control and the ixodicidal activity of methanolic extracts (ME) at concentrations of 50, 100 and 150mg/ml, against engorged females of *R. (B.) microplus* which were dissolved in methanol 60%, used as a control. This solvent has been reported to not have a significant effect on tick mortality at this concentration (Chagas et al., 2003). This test was performed in triplicate with a total of 42 groups from the two populations. Each group of ten ticks was dipped for five minutes in each treatment, that is, each diluted ixodicide solution, each concentration of the ME and each control. After immersion, the groups were dried and dorsally taped using double-sided tape, in a previously identified Petri dish. They were incubated at 27±2°C and with a relative humidity of 80-90%, which are ideal conditions for oviposition.

After 14 days of incubation, the mortality rate of adult females was evaluated, and the fertile egg mass of each group was weighed and placed in glass vials and incubated under the same conditions; after 30 days the egg hatching analysis was performed. From these data, the reproductive index (RI) (Equation 1) and the efficiency index (EI%) (Equation 2) of the treatments were determined, using the following formulas, described by Drummond et al. (1973):

RI = Reproductive Index:

$$RI = \left(egg\ weight \times \%\ egg\ hatch \times 20{,}000\right) / \ weight\ of\ engorged\ female \quad (1)$$

EI = Efficacy Index (%):

$$EI = \left(\begin{matrix} RI\ of\ the\ control\ group - \\ RI\ of\ the\ treated\ group \times 100 \end{matrix}\right) / RI\ of\ the\ control\ group \quad (2)$$

2.7. Larval packet test (LPT)

A modified LPT was used to assess (Stone and Haydock, 1962) the effect of the synthetic ixodicides on mortality at the concentration recommended by the manufacturer. The commercial synthetic ixodicides were diluted in two parts of trichloroethylene (Tc) (CTR, MSDS, Mexico) and one part of olive oil (OO) (Sigma-Aldrich) to prepare the treatment solutions with the concentration recommended by the manufacturer. 0.67ml of each solution was added onto 850mm x 750mm Whatman No. 1 filter papers (Whatman International Ltd., Maidstone, England). Control groups were prepared with the acaricide-free Tc-OO solution. The filter papers were allowed to dry for 24 hours at room temperature before testing. Approximately 70-100 larvae were taken from the tubes with a paintbrush and placed into each filter paper which was then folded in half and sealed with foldback clips. The packets were then incubated at 27±2°C with a relative humidity of 80-90%. After 24 h, the packets were opened and the numbers of alive and dead larvae were counted. Three replicates were made for each treatment. Larval mortality was determined by the Abbott's formula (Equation 3) (Abbott, 1925) recommended by FAO (2004):

$$\%\ Mortality = \left(\begin{matrix} \%\ Treatment\ Mortality - \\ \%\ Control\ Mortality \end{matrix}\right) / \left(\begin{matrix} 100 - \% \\ Control\ Mortality \end{matrix}\right) \times 100 \quad (3)$$

2.8 Larval immersion test (LIT)

The LIT technique by Shaw (1966), was modified to evaluate the effect of the ME of the plants. 400 to 500 larvae of 7-14 days of age were exposed to increasing concentrations of each extract using three dilutions (50, 100, and 150mg/ml) and a control (methanol 60%) for 10 minutes under agitation (shaker). After this time, the larvae were placed into filter papers (Whatman No. 1), identified, and closed with foldback clips. Packets were incubated for 24 hours at 27±1.5°C and with a relative humidity of 70–80% and dead larvae were recorded for mortality. These assays were performed by triplicate for every plant extract and concentration. Larval mortality was determined by the Abbott's formula (Abbott, 1925) recommended by the FAO (2004).

2.9. Statistical analysis

The data was analyzed in the SigmaPlot 14.0 software for AIT results, a non-parametric test (Kruskal-Wallis) was used to determine the statistically significant differences between the groups and the mean difference was made with the Bonferroni correction. For LPT and LIT tests, a one-way analysis of variance (ANOVA) was performed and multiple comparisons were made using Tukey's test. All the above was done with a significance level of $p \leq 0.05$ and was considered as statistically significant.

3. Results

3.1 Evaluation of enzyme activity

In the enzymatic activity study of CaE, GST, AChE and ALP (Figure 1), a significant difference was observed between the NL and VER populations, where the population VER showed an increased enzymatic activity.

3.2 in vitro ixodicidal activity

The ME ixodicidal activity of *C. boissieri* and *A. ludoviciana* leaves and *L. chinensis* seeds was evaluated on adult

females and larvae of *R.* (*B.*) *microplus*. The efficacy of the synthetic ixodicides for commercial use and the ME against engorged females of *R.* (*B.*) *microplus* was evaluated by the AIT (Drummond, 1976) measuring mortality, egg mass and inhibition of larval hatching in the populations from NL (Table 1) and VER (Table 2). The NL population showed a statistically significant difference between the control and the three ME in the mortality of engorged females, highlighting the 100% mortality of the *L. chinensis* seed extract at the concentration of 150mg/ml. Regarding the egg mass, only the *L. chinensis* seed extract in the concentrations of 100 and 150mg/ml, presented a statistical significant difference with the control. In the hatching percentage, the three ME presented significant statistical difference with the control. In the VER population, with the *C. boissieri* extract, the mortality of the engorged females and the mass of eggs were not statistical significant different from the control. In the hatching percentage, the extracts of *C. boissieri* and *A. ludoviciana* in the concentration of 50mg/ml did not presented a statistical significant difference with the control. Meanwhile the *L. chinensis* seed extract in the concentration of 150mg/ml had an efficacy of 99%.

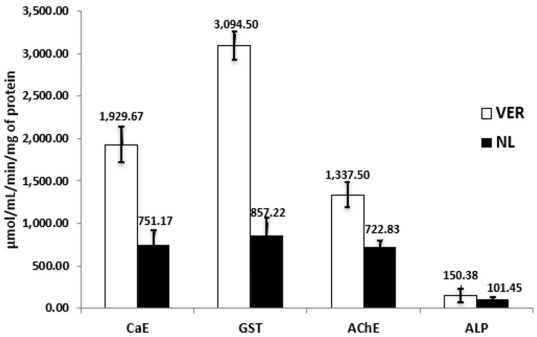

Figure 1. Enzymatic activity of AChE, GST, CaE, and ALP in larvae populations of *R.* (*B.*) *microplus* from the states of NL and VER.

The evaluation of the commercial synthetic ixodicides in the NL population (Table 1) showed a statistical significant difference in the mortality of the engorged females with the coumaphos and chlorpyrifos-permethrin towards the control, however, it did not happen with the cypermethrin. Only the chlorpyrifos-permethrin mixture presented a significant difference with the control regarding the mass of eggs. In hatching percentages, the three ixodicides showed a statistical difference towards the control, highlighting that cypermethrin had low ixodicide activity, compared to the other two products. In the VER population (Table 2), a statistical significant difference was only observed between the control and the coumaphos in the mortality of the females and in the hatching percentage. In relation to the mass of eggs, no ixodicide had a statistical significant difference with the control.

Table 1. Activity of synthetic ixodicides and methanolic extracts against adult ticks and reproductive parameters of *R.* (*B.*) *microplus* in the population of NL.

Nuevo León	Mortality %	Egg mass weight (g)	Egg Hatching %	Efficiency index
Cypermethrin	0.00±0.00^{c}	0.85±0.06^{a}	70.00±42.43^{b}	32.17
Coumaphos	90.00±14.14^{a}	0.08±0.11^{a}	0.00±0.00^{c}	100
Chlorpyrifos + Permethrin	100.00±0.00^{a}	0.00±0.00^{b}	0.00±0.00^{c}	100
Control	0.00±0.00^{c}	0.85±0.15^{a}	100.00±0.00^{a}	-
Cordia boissieri				
50 mg/ml	5.00±7.07^{b}	0.61±0.14^{a}	35.00±42.43^{b}	55.13
100 mg/ml	10.00±0.00^{b}	0.72±0.03^{a}	65.00±7.07^{b}	57.00
150 mg/ml	20.00±28.28^{b}	0.60±0.17^{a}	50.00±14.14^{b}	71.27
Methanol 60%	0.00±0.00^{c}	0.98±0.14^{a}	100.00±0.00^{a}	-
Artemisia ludoviciana				
50 mg/ml	20.00±14.14^{b}	0.45±0.12^{a}	20.00±0.00^{b}	83.38
100 mg/ml	25.00±21.21^{b}	0.56±0.33^{a}	20.00±14.14^{b}	88.50
150 mg/ml	35.00±35.36^{b}	0.55±0.24^{a}	10.00±0.00^{b}	94.90
Methanol 60%	0.00±0.00^{c}	0.98±0.14^{a}	100.00±0.00^{a}	-
Litchi chinensis				
50 mg/ml	75.00±21.21^{a}	0.22±0.17^{a}	12.50±10.61^{b}	96.65
100 mg/ml	90.00±0.00^{a}	0.03±0.04^{b}	30.00±42.43^{b}	98.26
150 mg/ml	100.00±0.00^{a}	0.00±0.00^{b}	0.00±0.00^{c}	100
Methanol 60%	0.00±0.00^{c}	0.98±0.14^{a}	100.00±0.00^{a}	-

Common corresponding letters a-c in a given column indicates no significant differences ($p > 0.05$).

Table 2. Activity of synthetic ixodicides and methanolic extracts against adult ticks and reproductive parameters of *R.* (*B.*) *microplus* in the population of VER.

Veracruz	Mortality %	Egg mass weight (g)	Egg Hatching %	Efficiency index
Cypermethrin	0.00±0.00[b]	0.92±0.01[a]	100.00±0.00[a]	3.56
Coumaphos	45.00±7.07[a]	0.35±0.00[a]	10.00±0.00[c]	96
Chlorpyrifos + Permethrin	5.00±7.07[b]	0.73±0.07[a]	90.00±0.00[a]	29
Control H_2O	0.00±0.00[b]	0.85±0.15[a]	100.00±0.00[a]	-
Cordia boissieri				
50 mg/ml	5.00±7.07[b]	0.98±0.08[a]	85.00±7.07[a]	28.91
100 mg/ml	0.00±0.00[b]	0.79±0.05[a]	90.00±0.00[a]	27.22
150 mg/ml	5.00±7.07[b]	0.77±0.02[a]	80.00±0.00[a]	37.10
Methanol 60%	0.00±0.00[b]	0.94±0.06[a]	100.00±0.00[a]	-
Artemisia ludoviciana				
50 mg/ml	15.00±7.07[a]	0.65±0.10[a]	70.00±14.14[a]	52.77
100 mg/ml	10.00±0.00[a]	0.45±0.06[a]	35.00±7.07[b]	83.57
150 mg/ml	25.00±7.07[a]	0.46±0.06[a]	35.00±7.07[b]	83.82
Methanol 60%	0.00±0.00[b]	0.94±0.06[a]	100.00±0.00[a]	-
Litchi chinensis				
50 mg/ml	25.00±7.07[a]	0.49±0.05[a]	40.00±14.14[b]	79.43
100 mg/ml	25.00±7.07[a]	0.25±0.06[a]	25.00±7.07[b]	93.73
150 mg/ml	60.00±14.14[a]	0.27±0.07[a]	5.00±0.00[c]	99
Methanol 60%	0.00±0.00[b]	0.94±0.16[a]	100.00±0.00[a]	-

Common corresponding letters a-c in a given column indicates no significant differences ($p > 0.05$).

Comparing the commercial synthetic ixodicides and the ME in the NL population (Table 1), it is important to mention that the three concentrations of the extract of *L. chinensis* did not differ significantly in the mortality of engorged females with the coumaphos and the chlorpyrifos + permethrin mixture. In the egg mass, the chlorpyrifos-permethrin mixture did not present statistical difference with the *L. chinensis* extract in the 100 and 150mg/ml concentrations, in the hatching percentage, only the concentration of 150 mg/ml of the *L. chinensis* extract and coumaphos ixodicides and the chlorpyrifos-permethrin association had no significant difference. It is worth mentioning that the ME of *L. chinensis*, the coumaphos ixodicides and the chlorpyrifos-permethrin mixture, obtained an efficacy rate of 100%. The ME of *A. ludoviciana* and *C. boissieri* had higher ixodicidal activity than cypermethrin. In the VER population (Table 2), the ME of *C. boissieri* behaved the same as cypermethrin and the chlorpyrifos-cypermethrin mixture, while the ME of *A. ludoviciana* and *L. chinensis* as the coumaphos in the mortality of the engorged females. Only the concentration of 150 mg/ml of the ME of *L. chinensis* and the coumaphos did not presented a significant difference in the hatching percentage.

In the cattle tick larvae, synthetic ixodicides and ME showed ixodicide activity (Table 3). The cypermethrin had very low mortalities in the VER population and slightly low mortalities in the NL population, coumaphos and the chlorpyrifos-permethrin association presented a mortality of 100% in the NL population, and in the VER population coumaphos maintained an effectiveness with a mortality of 100%. Meanwhile, the ME of *C. boissieri* tested in both populations showed ixodicidal activity, however its efficacy was low. The mortalities in the ME of *A. ludoviciana* at concentrations of 50, 100 and 150mg/ml in both populations, presented similarities, being slightly higher in the NL population. Both populations presented the highest mortality with the concentrations evaluated of the ME of *L. chinensis*.

4. Discussion

Plants have been used for many years in traditional medicine due to their pharmacological effects (Prieto-González et al., 2004; Oliveira et al., 2011; Pio et al., 2019). In recent decades, plant extracts have been used for their antimicrobial activity (Sharma et al., 2017; Souza et al., 2018), and acaricides on ticks (Adenubi et al., 2016). Scientific studies of *Cordia* species have been intensified, which demonstrates the great interest in phytochemical, biological and pharmacological studies (Matias et al., 2015; Debiasi et al; 2021), especially the specie *C. boissieri*, whose capacity has been reported from different extracts with antibacterial, antifungal and antioxidant activity (Salazar-Aranda et al., 2011; Viveros-Valdez et al., 2016).

Table 3. *In vitro* ixodicide activity of synthetic ixodicides and plant extracts in both populations against *R.* (*B.*) *microplus* larvae.

Treatment	Veracruz	Nuevo Leon
Cypermethrin	6.03±1.69^{gh}	97.38±0.99^{abc}
Coumaphos	100±0.00^{a}	100±0.00^{a}
Chlorpyrifos + Permethrin	97.50±0.57^{ab}	100±0.00^{a}
Control H_2O	2.00±0.88^{h}	2.00±0.88^{g}
Cordia boissieri		
50 mg/ml	3.79±1.01^{h}	13.74±1.22^{f}
100 mg/ml	4.87±0.24^{h}	16.08±1.62^{f}
150 mg/ml	10.33±1.53^{g}	33.04±10.07^{e}
Methanol 60%	4.57±1.66^{h}	4.57±1.66^{fg}
Artemisia ludoviciana		
50 mg/ml	73.86±3.00^{f}	87.31±0.74^{d}
100 mg/ml	82.20±2.06^{e}	86.85±0.07^{d}
150 mg/ml	89.21±2.26^{cd}	89.39±0.79^{cd}
Methanol 60%	4.57±1.66^{h}	4.57±1.66^{fg}
Litchi chinensis		
50 mg/ml	85.09±0.68^{de}	88.87±0.00^{cd}
100 mg/ml	94.23±1.13^{bc}	91.58±4.91^{cd}
150 mg/ml	99.73±0.38^{a}	98.44±0.56^{bc}
Methanol 60%	4.57±1.66^{h}	4.57±1.66^{fg}

Common corresponding letters a-h in a given column indicates no significant differences ($p > 0.05$).

The *A. ludoviciana* plant belongs to the Asteraceae family which has a diversity of plant species with pharmacological activities and acaricidal effects (Adenubi et al., 2016), as in the case of *Artemisia absinthium*, whose methanolic extracts in a concentration of 200mg/ml presented 100% of mortality in the tick species *Rhipicephalus sanguineus* (Godara et al., 2014). It has been shown that *A. ludoviciana* has antimicrobial, antiparasitic, and antioxidant activity (Zavala-Sánchez et al., 2002; Said Fernández et al., 2005; Lopes-Lutz et al., 2008). *L. chinensis* has different ethnopharmacological uses (Ibrahim and Mohamed, 2015) such as: antioxidant, anticancer, antimicrobial, antiviral, anti-inflammatory, antidiabetic, hepatoprotective, immunomodulatory and antithrombotic activity (Yang et al., 2012; Lin et al., 2015; Wen et al., 2014; Nimmanpipug et al., 2009; Yamanishi et al., 2014; Chang et al., 2013; Bhoopat et al., 2011; Jing et al., 2014; Sung et al., 2012). Furthermore, overexpression of esterases and Glutathione S-transferases is associated with metabolic detoxification of pesticides (Bellgard et al., 2012). In ticks, these enzymes have been implicated in resistance through the metabolic detoxification mechanism (Bellgard et al., 2012; Nandi et al., 2015; Ghosh et al., 2015; Gupta et al., 2016; Ghosh et al., 2017; Chigure et al., 2018; Fular et al., 2018). Regarding esterases, CaE catalyze the hydrolysis of esters and are classified in the serine hydrolase superfamily, involved in detoxification and playing an important physiological role in lipid metabolism (Ran et al., 2009). AChE is a key enzyme in the nervous system of animals, hydrolyzing the neurotransmitter acetylcholine (Temeyer et al., 2013). Studies on ixodicide-resistant strains of *R. (B.) microplus* have shown that esterases, particularly CaE and AChE, are associated with resistance, implying an increased metabolic detoxification and insensitivity to the action site (Li et al., 2005). Baxter and Barker (2002) demonstrated the relationship between resistance to organophosphorus (OP) and a high AChE activity in Australian tick populations. Likewise, point mutations of CaE and AChE genes have been detected in resistant strains of this species, which are associated with resistance (Hernández et al., 2000). Similarly, in *R.* (*B.*) *microplus*, three esterases, characterized as CaE, were detected based on enzymatic inhibition and a high activity of these enzymes was shown in a resistant strain (Villarino et al., 2003). Regarding the results obtained from the evaluation of the enzymatic activity of the populations (Figure 1), it was observed that the esterases (CaE and AChE) presented a significant difference between the two populations, being the population of VER the one with the highest enzymatic activity. A study carried out by Fular et al. (2020), reports values of different enzymatic activities for CaE, AChE and GTS, in a susceptible and a resistant strain of *R. (B.) microplus*. Taking into account these values, the VER population presented values of CaE enzymatic activity similar to the resistant strain and the population of NL to the susceptible strain. Furthermore, it is known that the CaE activity is related to the presence of resistance to organophosphorus and pyrethroids, since it plays an important role in the metabolic detoxification of pyrethroids. The results of the bioassays align with this as it was observed that in adults (Tables 1 and 2) and in larvae (Table 3), where a high enzyme activity and a low tick mortality was observed in the population of VER regarding AChE, which means higher enzymatic activity in the population of VER. However, a low mortality was not shown with the organophosphorus, this could be due to the fact that the larvae have a resistance to the penetration of the organophosphorus and this allows them to inhibit or delay the penetration of the chemical through their exoskeleton (Alonso-Diaz et al., 2006). GSTs are enzymes that catalyze the conjugation between glutathione and various molecules. They play the most important role in the cellular detoxification mechanism of xenobiotic and endogenous compounds (Agianian et al., 2003). Chemical exposure in arthropods, in this case ticks, is a classic event that selects resistance to pesticides, related to high GST activity (Ketterman et al., 2001; Wei et al., 2001; Freitas et al., 2007). In the results obtained from the GST (Figure 1), a high value was shown in the VER population, similar to that presented in the study by Fular et al. (2020). However, the values in the population of NL are below the value of the resistant strain and higher than the susceptible one. In the population of VER higher values were observed comparing to those of the resistant strain presented by Fular et al. (2020), assuming that the low effectiveness of pyrethroids in the VER population in adults (Table 2) and larvae (Table 3), is due to the significant increase in the enzymatic activity of GST. Alkaline phosphatase (ALP) is a digestive enzyme involved in adsorption and

transport mechanisms through the hydrolysis of phosphate groups (Moss, 1992). An increase in the ALP activity has been associated with the detoxification mechanisms of phosphorus compounds, particularly in insects that become resistant to pesticides (González et al., 2015). ALP is also involved in the glucose and fatty acids transport across the midgut epithelial membrane, as has been observed in *Bombyx mori* (Vlahovic et al., 2009). In insects, few studies have been conducted on the use of ALP as a biomarker, however, Bounias et al. (1996) observed an increase in ALP activity after copper treatment in honey bees. Likewise, Badiou-Bénéteau et al. (2012) evaluated ALP as a biomarker to indicate exposure of the *Apis mellifera* bee to pesticides from the neonicotinoid family, observing changes of enzymatic activity in exposed bees. In ticks, there are no reports of the evaluation of ALP associated with resistance to acaricides. However, the presence of activity by this enzyme was observed, being higher in the population of VER, showing a significant difference compared to NL.

The synthetic ixodicides evaluated on the engorged female ticks presented low efficacy indices in the VER population (Table 2), and in the NL population (Table 1) only cypermethrin presented low activity, while the ME of *C. boissieri, A. ludoviciana* and *L. chinensis* showed ixodicidal activity on adult female ticks, being the last one that presented the highest efficacy rates (Table 1 and 2). In larvae, high mortality was shown with the synthetic ixodicides (Table 3), being the cypermethrin the only that presented a low efficacy in VER and a moderately low efficacy in NL, both efficacies below what is described in the NOM-006-ZOO-1993 (NOM, 1994), which states that the efficacy of ixodicides must be higher than or equal to 98%, which agrees with what was described by Fernández-Salas et al. (2012), where the presence of strains resistant to cypermethrin are reported with a 3% of mortality in discriminating doses in four municipalities of the state of VER. In the same way, Esparza Rentería and Esparza Sevilla (2015) demonstrated the presence of strains slightly resistant to cypermethrin in the state of NL, thus reinforcing the result obtained in this study, demonstrating the increase in resistance to pyrethroids in recent years. Coumaphos and the chlorpyrifos-permethrin mixture showed higher efficacy in the NL population. There are few studies on the diagnosis of resistance in the state of NL; however, Esparza Rentería and Esparza Sevilla (2015) confirms the presence of susceptible strains in the state. Studies carried out by the National Center for Animal Health Verification Services (CENAPA), between 2015 and 2017, do not indicate the presence of resistance to organophosphorus and to the pyrethroid-organophosphorus association in the state of NL (Neri, 2018) as there is a lack of data due to the fact that most of the producers in the state do not send samples for resistance diagnosis, despite the fact that they have significant resistance problems. In the VER population, the efficacy of coumaphos was of 100%, showing that currently this population is not under pressure from this ixodicide, therefore, the effectiveness of the product is still considerable for its application. In addition, the chlorpyrifos-permethrin association was lower, not reaching what was established by NOM-006-ZOO-1993 (NOM, 1994), however, Rodríguez-Vivas et al. (2011), reports that if a mortality of 80-99% is obtained, the ixodicide could be used for six more months and then stop it and using it for at least two years, while other families of ixodicides are used with annual rotation, but if the mortality obtained is very low (<60%), this product can no longer be used since the resistance of *R.* (*B.*) *microplus* towards organophosphorus and pyrethroids is genetically characterized by incomplete dominance, so it is possible to find populations of ticks resistant to these acaricides years after their use has ceased. The results in the NL population, in terms of association, were similar to those obtained by Fernández-Salas et al. (2012), where they report the presence of multiresistant strains in four municipalities of the state of VER. In addition, Rodríguez-Vivas et al. (2007) reports the presence of strains resistant to the different associations of synthetic ixodicides in four states of Mexico, including VER. It should be noted that the populations of *R.* (*B.*) *microplus* in this state are mostly under pressure with these ixodicides, Therefore, normally obtain less efficacy from these, which is also due to the fact that the efficacy of ixodicides varies according to the regions, depending mainly on factors such as; ecological niches, livestock management and use of ixodicides (Jonsson, 1997), thus demonstrating that environmental and operational factors (Denholm and Rowland, 1992), have led to an increase in resistance in the VER state, in addition to the fact that ticks have a different toxicological response, due to the resistance situation in each population (Guerrero et al., 2001; Foil et al., 2004).

The ME of *C. boissieri* evaluated in the two populations (Table 3), showed ixodicidal activity, however, the efficacy in general was low, this may be due to the fact that the extract acts as an inhibitor of AChE, which is the target enzyme of the organophosphorus, where in populations of resistant ticks it is altered, as could be the case of the VER population where the extract presented less efficacy. This biological activity was reported by Marini et al. (2018), demonstrating the *in vitro* ability to inhibit AChE of three species of the *Cordia* genus, being *Cordia megalantha* the species that presented the higher activity, however, confirmatory studies of this mechanism of action are necessary. The efficacy indices and mortality percentages of the ME of *A. ludoviciana* (Table 3) in both populations presented similarities, being slightly higher in the NL population. Godara et al. (2014), describe that the ME of the species *Artemisia absinthium* at a concentration of 200mg/mL, presented a mortality of 100% in the tick species *R. sanguineus*. With this, it can be considered that the increase in the concentration of the *A. ludoviciana* extract, or a fractionation could result in higher mortality. The ME of *L. chinensis* (Table 3) in both populations, presented the highest mortality in the concentration of 150 mg/ml. These results can be compared with plant extracts that currently appear in commercial products such as: *Azadirachta indica* where 8.68mg/ml causes 90% of effectiveness (Avinash et al., 2017), *Cymbopogon citratus* at a concentration of 125mg/ml, causes 98.78% of effectiveness (Chungsamarnyart & Jiwajinda, 1992), *Thymus vulgaris* at a concentration of 20mg/ml, causes 98.1% of effectiveness (Monteiro et al., 2009). The results in this study are not so far from those reported for the *C. citratus* plant, which

leads to work on the process of obtaining and purifying the extract, to obtain greater effectiveness, in the same way to take into account that plants of the same species can vary in the amount of chemical components, due to their inter-specific variations and other factors such as: seasonality, circadian rhythm, development, temperature, ultraviolet radiation, water availability, altitude and atmospheric pollution, among others, changing the production rate of secondary metabolites presenting a different effectiveness (Gobbo-Neto and Lopes, 2007). It is worth mentioning that due to its high acaricidal activity shown in this study, the methanolic extract of the *L. chinensis* seed can be used as an alternative source to control *R.* (*B.*) *microplus* infestations, delaying the development of resistance to ixodicides, on the other hand, this is only the *in vitro* ixodicidal effect; therefore, it is necessary to carry out additional *in vivo* studies (Martins & González, 2007) in order to see if the minimum ixodicidal concentration tested in this study can cause the same activity, due to difficulties related to external environmental conditions (Mulla and Su, 1999).

5. Conclusion

The VER population demonstrated a significant increase in enzymatic activity and presented a lower efficacy to the commercial synthetic ixodicides regarding the NL population, however, the efficacy and mortality rates of the evaluated methanolic extracts presented similarities in both populations. The *L. chinensis* seed extract showed ixodicide potential to be used as an alternative source for the control and treatment of the *R.* (*B.*) *microplus* tick, however, previous studies of phytochemical characterization, purification, toxicity and formulation are necessary.

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