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MICROBIOLOGY

Anti-*Ehrlichia* properties of the dichloromethane fraction of *Ageratum conyzoides* associated with doxycycline: In vitro study

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ABSTRACT: *The increasing number of cases of canine ehrlichiosis caused by Ehrlichia canis in hospitals and veterinary clinics has demonstrated the need for a new drug protocol for this disease. Doxycycline is used to treat ehrlichiosis, but the resistance of the microorganism to this treatment protocol, as well as the various side effects to the animals, has become a concern. Several studies have shown a positive interaction with extracts of plants and drugs, which allow for the reduction of the concentration necessary to produce the desired effect, minimizing adverse effects. This study determined the efficiency of the combination of the dichloromethane (DCM) fraction of Ageratum conyzoides L. with anti-Ehrlichia activity and doxycycline by using the checkerboard assay. Plant material was collected in São Luís, northeastern Brazil, followed by extraction in MeOH: H_2O (8:2) and partitioning of the DCM fraction. After determining the Minimum Inhibitory Concentration (MIC) of the fraction under study against DH82 cells infected with Ehrlichia canis, it was combined with doxycycline to derive the Fractional Inhibitory Concentration Index (CIF Index). A reduction of 5.83 times the doxycycline minimum inhibitory concentration was observed, showing that this fraction of A. conyzoides composed predominantly by the class of lignans, identified by mass spectrometry notably intensified the activity of doxycycline against E. canis, resulting in a synergistic effect.*
Key words: *Ehrlichia canis; alternative therapies; tetracycline; "catinga-de-bode"; DH82 cells.*

Propriedades anti-*Ehrlichia* da fração diclorometânica de *Ageratum conyzoides* associada com doxiciclina: estudo *in vitro*

RESUMO: *O crescente número de casos de erliquiose canina por Ehrlichia canis em hospitais e clínicas veterinárias tem demonstrado a necessidade de um novo protocolo de medicamentos para essa doença. A doxiciclina é usada para tratar a erliquiose, mas a resistência do microrganismo a esse protocolo de tratamento, bem como os diversos efeitos colaterais para os animais, tornou-se uma preocupação. Vários estudos têm demonstrado interação positiva com extratos de plantas e fármacos, que permitem a redução da concentração necessária para produzir o efeito desejado, minimizando os efeitos adversos. Este estudo determinou a eficiência da combinação da fração diclorometânica (DCM) de Ageratum conyzoides L. com atividade anti-Ehrlichia canis associada com doxiciclina por meio do ensaio de Checkerboard. O material vegetal foi coletado em São Luís, Maranhão, nordeste do Brasil, seguido pela extração em MeOH:H_2O (8:2) e partição da fração diclorometânica. Após a determinação da Concentração Inibitória Mínima (CIM) da fração em estudo frente às células DH82 infectadas com Ehrlichia canis, a mesma foi combinada com a doxiciclina para derivação do Índice de Concentração Inibitória da Fração (Índice CIF). Observou-se uma redução de 5,83 vezes a concentração inibitória mínima da doxiciclina mostrando que esta fração de A. conyzoides, composta predominantemente por lignanas identificadas por espectrometria de massas, notavelmente intensificou a atividade desse fármaco contra E. canis, resultando em um efeito sinérgico.*
Palavras-chave: *Ehrlichia canis; terapia alternativa; tetraciclina; "catinga-de-bode"; células DH82.*

INTRODUCTION

Ehrlichia spp. are obligate intracellular bacterial pathogens that parasitize hematopoietic cells (leukocytes). This rickettsial organism infects a wide range of mammalian hosts, including dogs, cats, cattle, horses, and humans (BOGIĆEVIĆ et al., 2017). Canine monocytic ehrlichiosis (CME) is caused by the bacterium *Ehrlichia canis*, and the ixodid tick *Rhipicephalus sanguineus* (common name: brown dog tick) is its main vector (DAGNONE et al., 2001). The disease may be acute, subclinical, or chronic.

In the acute phase of the disease, clinical signs include depression, lethargy, anorexia,

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pyrexia, lymphadenomegaly and splenomegaly, and weight loss. Affected animals may exhibit bleeding, especially petechiae and ecchymoses on the skin and mucous membranes and occasional epistaxis (WANER & HARRUS 2000; SOUSA et al., 2010). The subclinical phase of the disease consists of persistent thrombocytopenia, leukopenia, and anemia in the absence of clinical signs (VARELA, 2003; SOUSA et al., 2010). The chronic phase of CME may be severe and present as a fatal hemorrhagic syndrome due to bone marrow failure (MYLONAKIS et al., 2003).

The drug of choice for the treatment of the disease in all phases is doxycycline for 28 d (TILLEY et al., 2003). The most frequent clinical signs observed in patients treated with doxycycline are digestive, including nausea, vomiting, diarrhea, abdominal pain, gastritis, and enterocolitis (SAUCEDO, 2003; WORKOWKI, 2002; ZIMMERMAN, 2000; PEREIRA-MAIA et al., 2010). This drug can cross the placental barrier; therefore, it is contraindicated during pregnancy.

According to GONZÁLEZ-LAMOTHE et al. (2009), the products of secondary metabolism accumulated by plants can act as "potentiators of antibacterial activity," favoring the activity of antibiotics whose action is limited by multidrug resistance mechanisms developed by microorganisms. They may also act as "virulence attenuators," adapting the host's immune system response to infection.

Ageratum conyzoides L. is present in the vast majority of the Brazilian states. The common names for this plant are "mentrasto" and "catinga-de-bode." It occurs mainly in anthropic areas and plantations (KISSMANN & GROTH, 1999). This ethnomedicinal plant has anti-inflammatory, analgesic, antidiarrheal, and antimicrobial properties (CIMANGA et al., 2014; PRAJAPATI et al., 2014). Its anti-*Leishmania* activity has been reported (TEIXEIRA et al., 2014). The compounds found in *A. conyzoides* (terpenes, monoterpenes, sesquiterpenes, quinolones, phenolic compounds, and flavonoids) have antimicrobial activity, which has been well described in the literature. Methanolic extracts have already been shown to be effective against *Escherichia coli, Klebsiella oxytoca, Proteus mirabilis, Salmonella thyphimurium, Staphylococcus aureus*, and *Shigella flexneri* (SAMY et al., 2013).

Studies have also confirmed the efficacy of dichloromethane (DCM) extracts against *S. aureus* and *Escherichia coli* (KANYANGA et al., 2014) in addition to inhibiting the replication of intracellular microorganisms (SINGH et al., 2016). This study determined the efficiency of the combination of the DCM fraction of *A. conyzoides* with anti-*Ehrlichia* activity and doxycycline using the checkerboard assay.

MATERIALS AND METHODS

Plant material

A. conyzoides was cultivated and collected at the Berta Langes de Morretes Medicinal Herb Garden of the Federal University of Maranhão (UFMA), municipality of São Luís, State of Maranhão (MA), northeast Brazil (2°33′13.5″S, 44°18′20.8″W) in July 2017 (rainy season), according to the method published in the Brazilian Pharmacopeia (ANVISA, 2010). The plant was herborized and identified, and a sample (voucher specimen, MAR 9099) was deposited at the Herbarium of Maranhão (MAR), located at the Federal University of Maranhão, Brazil. The plant was collected in agreement with the Brazilian laws concerning the protection of biodiversity (SISGEN n° ADBBA07).

Extraction

The aerial parts (1.7 kg) *A. conyzoides* was collected in the early morning hours. Samples were dried at room temperature (25 °C) for a 7 d period. An infrared moisture analyzer (GEHAKA IV 2500) was used to measure plant moisture and calculate yield. Next, the dry aerial parts of *A. conyzoides* were crushed in a mechanical turbolizer. We used exhaustive percolation in H_2O–MeOH (2:8) as the extraction method in this study. Percolation was conducted at room temperature (25 °C) and protected from light. The extractor liquid was completed every 24 h, corresponding to a total of 72 h by the time the plant material was exhausted. The extract were evaporated to dryness under vacuum at approximately 40 °C. Next, the specimen was ultra-frozen at -80 °C to be lyophilized and obtain the dry extract.

From the hydromethanolic extract of the aerial parts of *Ageratum conyzoides* L., hexane, dichloromethane and aqueous fractions were obtained with solvents of increasing polarity (hexane and dichloromethane) by the partitioning process.

The 26 g aliquot of the extract was weighed in a 100 mL beaker and diluted in an 80% methanol solution, which was ultrasound (Ultracleaner®) for 10 min (29 ºC ± 1 ºC) to facilitate dissolution. The sample was resuspended with MeOH: H_2O (8:2) transferred to a separating funnel and partitioned three times with an equal volume (50 mL) of hexane. Right after obtaining the hexane fraction, the rest of

the sample was also fractionated three times with an equal volume (50 mL) of dichloromethane, thus obtaining the fraction of interest and the remainder was considered the aqueous polar fraction. The extract and the concentrated fractions were ultra-frozen to -80 ºC and lyophilized to obtain the dry material.

Analysis of A. conyzoides extract by high-performance liquid chromatography (HPLC) with ultraviolet (UV) detection

After cleaning the extract, the sample was analyzed by HPLC using a Shimadzu® chromatograph (Shimadzu Corp., Kyoto, Japan) consisting of a solvent injection module with a binary pump and UV-Vis detector (SPA-10A). The column used was a Luna 5 µm C18 100 A (150 µm × 4.6 µm). The solvents used were elution solvent A (water + 0.01% formic acid) and elution solvent B (methanol + 0.01% formic acid). Samples were eluted according to the following exploratory gradients: 5% to 100% B in 60 min and 100% to 100% in 60 to 70 min. The flow rate was 1 mL/min, and the column temperature was 20 °C. The injection volume of the sample was 20 µL. Data were collected and processed using LC Solution software (Shimadzu). The baseline separation for the major components of the sample was obtained in a 70 min chromatographic evaluation at 254 nm.

Characterization of components of A. conyzoides by liquid chromatography-mass spectrometry (LC-MS) and flow injection analysis-electrospray ionization-ion trap MS (FIA-ESI-IT/MS)

Characterization of the compounds extracted from *A. conyzoides* was conducted at the Institute of Biosciences, São Paulo State University (UNESP), São Vicente, SP, southeastern Brazil. The samples were infused directly into a mass spectrometer with an IT linear analyzer (Thermo Scientific LTQ XL) equipped with ESI in negative mode (Thermo, San Jose, CA, USA). A stainless-steel capillary tube at 280 °C, spray voltage of 5.00 kV, capillary voltage of -90 V, and -100 V tube lenses at a flow rate of 5 µL/min were used in this procedure. Samples were infused into the mass spectrometer from the HPLC system, in which samples were analyzed online by ESI-MS in negative mode with an associated UV detector. The mass spectra data were obtained using the same Fleet LCQ mass spectrometer from Thermo Scientific® with direct insertion of the sample device via continuous FIA. Samples were ionized using an ESI source. Fragmentations were obtained in multiple stages (MS^n) in an IT-type interface. The negative mode was used for the generation and analysis of all spectra. The experimental conditions were as follows: capillary voltage of -35 V, spray voltage of -5000 V, capillary temperature 350 °C, carrier gas N_2, and flow 60 (arbitrary units). The track acquisition was in a mass range of *m/z* 100–2000 with two or more sweep events performed simultaneously in the spectrum. Compounds were identified by comparing the data from literature and fragmentation patterns.

Culture of E. canis DH82 cells

The *E. canis* strain was obtained from the 35th passage of *E. canis* of the Cuiabá #1 isolate, which belongs to the collection (library) of Rickettsia and *Ehrlichia* from the Laboratory of Virology and Rickettsioses of the School of Veterinary Medicine of the Federal University of Mato Grosso (FMVZ/UFMT), Cuiabá, MT, central-west Brazil. This rickettsial strain was multiplied in DH82 cell monolayers (ATCC number: CRL-10389) and was maintained at 37 °C and 5% CO_2.

Access was registered under the number A9463BB in the National System of Management of Genetic Heritage and Associated Traditional Knowledge according to Art. 41 of Decree Nº. 8772/2016 of the Ministry of the Environment in Brazil.

DH82 cells (Canine Histiocyte: ATCC in CRL-10389) were grown in Dulbecco's Modified Eagle's (DMEM) medium (Sigma Chemical Co., St. Louis, MO, USA) with 5% fetal calf serum (HyClone Laboratories, Logan, Utah, USA) in a culture bottle of 25 cm^2 at 37 °C and 5% CO_2, as recommended by AGUIAR et al. (2007). The rate of *E. canis* infection was determined by screening Diff-Quik stained cell monolayer smears (Laborclin, Pinhais, PR, Brazil) under a light microscope.

When an infection rate of 70% was detected, the cells were resuspended in the same medium and the cell suspension was centrifuged at 4,000 × *g* for 5 min. The experiments were run in 24-well culture plates at 37 °C and 5% CO_2. The infection rate was standardized at 3,000 cells per well and 70% infected cells (AGUIAR et al., 2007).

In another experiment, *E. canis* suspensions were standardized to 800 cells/well with a 70% infection rate. Solutions of the tested products were used in concentrations determined from their respective minimum inhibitory concentrations (MICs). The protocol used to determine the antimicrobial effect of the DCM fraction was adapted from ROLAIN et al. (1998, 2002). The MIC of the DCM fraction of *A. conyzoides* was 200 µg mL^{-1} (as determined in a previous study) and 1 µg mL^{-1}

(according to the manufacturer's label and confirmed in preliminary tests). Initially, 200 µL of the medium was added to the wells of the sterile microplate. Then, 50 µL of each test product, in serial dilutions, was arranged in an orderly manner: vertically, from top to bottom, the MIC of the DCM fraction of *A. conyzoides* decreased, and horizontally from the right of the synthetic drug.

The results showed that in each well, there was a unique combination of concentrations between the two substances (DCM fraction and drug). After 24 h of insertion into the medium, glass slides were prepared from the pellets of each well and stained with the Quick Panticosteal Kit (Laborclin, Pinhais, PR, BR) to evaluate the infection rate. The rate was obtained by counting 100 DH82 cells and identifying them as parasitized (presence of morulae) or not parasitized.

The combined effect of doxycycline (conventional treatment) and DCM fraction of *A. conyzoides* was determined using the checkerboard dilution technique for derivation of the fraction inhibitory concentration index (NIGHTINGALE et al., 2007).

The synergism, antagonism, or additive effect of each of the combinations was evaluated by calculating the value of the combination index (CI), according to Chou (1976, 2006):

$$CI = \frac{d1}{d1x} + \frac{d2}{d2x} \quad (1)$$

where *d1* and *d2* are the concentrations of doxycycline (1) and DCM fraction (2), respectively, responsible for the x effect in combination, and d1x and d2x are the concentrations of 1 and 2, respectively, responsible for the same effect individually. If CI < 1, the treatments have a synergistic effect; if CI > 1, the effect is antagonistic, and if CI = 1, the effect is additive. A standardized isobologram was created by plotting standard concentrations $\frac{d1}{d1x}$ in 1 and $\frac{d2}{d2x}$ in 2 in axes y and x, respectively, where the denominators represented the respective concentration of 1 and 2 alone, reducing the antibacterial load to x%, and the numerators represent the respective concentrations of 1 and 2, reducing the bacterial load to x% in combination. The standard concentrations were calculated as follows:

$$\frac{D1}{D1x} = \frac{D1}{Dm1 \cdot \left(\frac{fa}{1-fa}\right)^{\frac{1}{m1}}} \quad (2)$$

where Dm1 is the IC_{50} of 1 *in vitro*, fa is the fraction affected (or [effect%]/100), and m1 is the slope of linear regressions from median effect graphs using the function,

$$\log\left(\frac{fa}{1-fa}\right) = f(log(D1x)) \quad (3)$$

The IC effect graph representing CI as a function of the associated antibacterial effect was also plotted, as was the log (CRI) plot–an effect that represents the log of the concentration reduction index (CRI) as a function of the antibacterial effect associated. CRI is the ratio of the concentration of a treatment resulting in an x effect alone (d1x), with the concentration of the same treatment resulting in an x effect in combination (d1):

$$DRI = \frac{D1x}{D1} \quad (4)$$

Anti-Ehrlichia assay

The assays were performed for determining the IC_{50} of the different treatments against *E. canis* and were determined from the test concentrations of 25 µg mL^{-1}, 50 µg mL^{-1}, 100 µg mL^{-1}, 200 µg mL^{-1}, 300 µg mL^{-1}, 400 µg mL^{-1}, and 500 µg mL^{-1} in cell monolayers DH82 infected with *E. canis* at a 70% infection rate, cell quantities were standardized at 3,000 cells/well in 24-well plates. Assays were performed in triplicate, where the treatment control used spun doxycycline 1 µg mL^{-1}, according to the package insert, and as a control of bacterial culture, there were wells treated only with distilled water. The protocol used to determine the antimicrobial effect of the test treatments was an adaptation of that of ROLAIN et al. (1998, 2002).

Cell viability analyses were performed using the trypan blue assay (Trypan blue exclusion test of cell viability) (Sigma-Aldrich, St. Louis, MO) according to the protocol and guidelines provided by BARILE (1994).

Statistical analysis

The experimental design used in all biological assays in this study was completely randomized. The mean of each treatment was compared to the respective control. Data were initially transformed to log (X), normalized, and then nonlinear regression was calculated to obtain IC_{50} (50% inhibition concentration) using Graph-Pad Prism 7.0 software (Graph-Pad Inc., San Diego, CA, USA). The effects of interactions between the evaluated treatments and doxycycline were evaluated by multiple drug combination analyses using CompuSyn® software.

RESULTS

This is the first study to calculate the anti-*Ehrlichia* potential of *A. conyzoides* after 18 h and 36 h of treatment with the botanic extract (Fig 1). In triplicate assays, the IC_{50} of the proposed treatment was 200 µg mL^{-1}.

We assessed the viability of the DH82 cells against different concentrations of the tested treatment. They were not toxic at the highest concentration of 500 µg mL^{-1} (Figure 2) when compared with the control group, which was formed by DH82 cells treated with ultrapure distilled water for a 24 h period.

Figure 3 shows the chromatogram of the DCM extract of *A. conyzoides* with the UV absorption profiles of the five chromatographic peaks, as evidenced by the high resolution of overlapping peaks in the reverse phase (Table 1).

Hydroxy methyl coumarin was detected by using m/z 175, syringaresinol m/z 417, deriveds from syringaresinol m/z 403, m/z 431, m/z 387, respectively. Chemically identification of the structure of the compounds present was analyzed by mass spectrometry. Representative structures and fragmentation patters of Hydroxy methyl coumarin (Figure 4 A); syringaresinol (Figure 4 B), derived from syringaresinol (Figure 4 C), derived from syringaresinol (Figure 4 D) derived from syringaresinol (Figure 4 E). Data is representative of at least three independent experiments.

Lignans were the major class of the DCM fraction of *A. conyzoides* collected in São Luís, MA, northeastern Brazil. This class of compounds was first identified in this specimen.

After verifying the association of the efficacy of the DCM fraction of *A. conyzoides*, we performed the association of this with doxycycline, which was reduced 5.83 times relative to the IC_{50} from doxycycline, showing that this fraction of *A. conyzoides* notably enhanced the activity of doxycycline against *E. canis*, resulting in a synergistic effect (Table 2).

The analysis of the isolated points in relation to the additivity line considers that the values below the additivity line are synergistic and the values above are antagonistic. Thus, IC_{50} values below 1 are considered synergistic and above 1 are antagonistic, whereas those equal to 1 are indifferent or additive (Table 2).

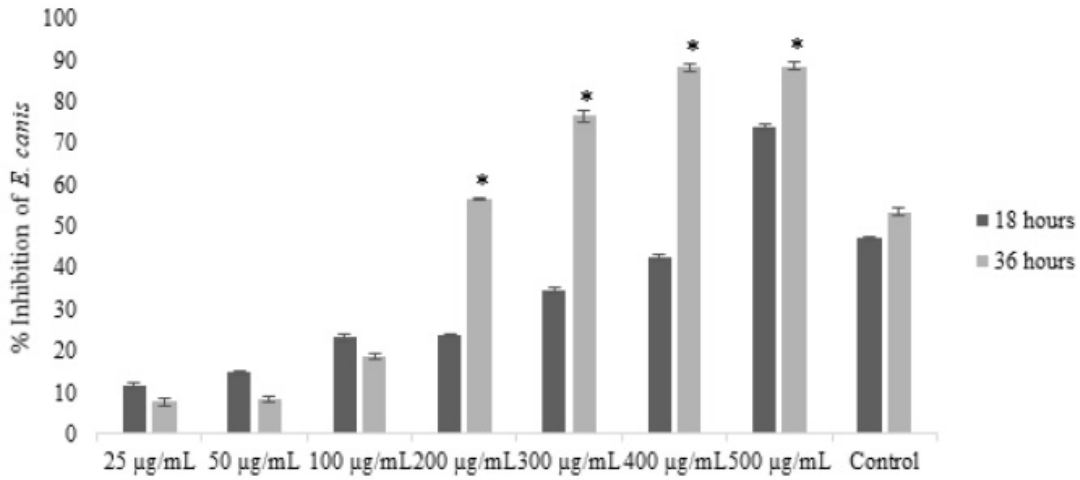

Figure 1 - Percentage of inhibition of Ehrlichia canis in DH82 cells after 18h and 36h of treatment with the A. conyzoides dichloromethane (DCM) fraction and with doxycycline (control group) at different concentrations. Each row represents mean and standard deviation of three independent assays in which *P<0.05 demonstrated that there was no statistically significant difference in relation to the control group according to the Tukey-test with a 95% confidence index.

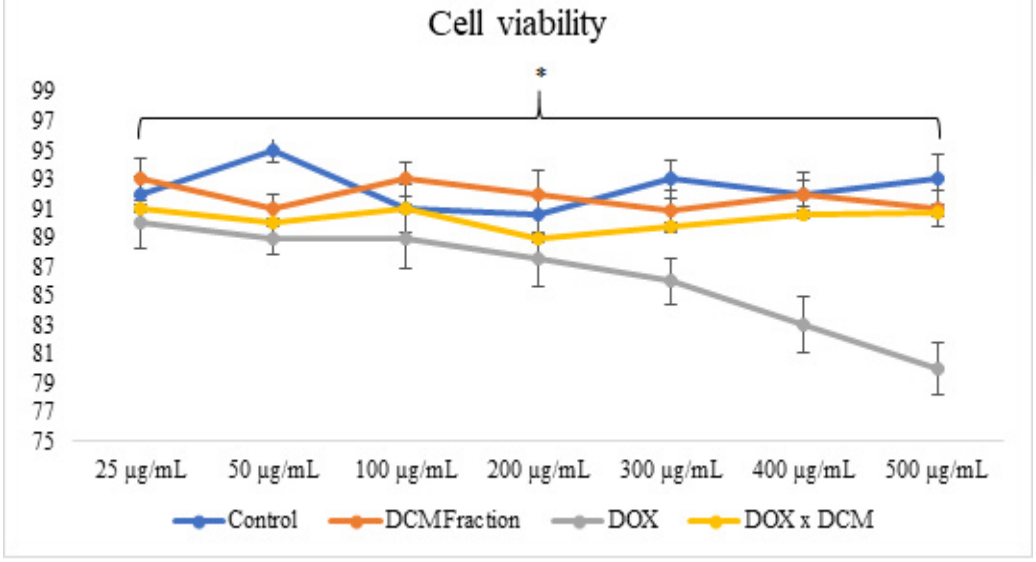

Figure 2 - Each row represents mean and standard deviation of three independent assays. Where *P<0.05 demonstrated that there was no statistically significant difference in relation to the control group according to the unpaired Student's t-test with a 95% confidence index.

The confirmation of the synergistic effects of the *A. conyzoides* DCM fraction was obtained by constructing the isobologram type graphs using the Chou and Talalay method to design the hypothesis and evaluate the various combinations (CHOU & TALALAY, 1984; CHOU, 2010).

The treatment was tested in constant proportions of equipotent concentrations, ranging from 0.25 to 1 times the respective IC_{50} value that was determined for each treatment (Figure 5A). Slope m was also determined from the linear regression of the median effect of plots (Equation (3)), as they reflected the sigmoidicity of the concentration–response curves and were used to calculate the normalized concentrations and treatment reduction indices (Figure 5B).

Standard isobologram is a graphical method for visualizing synergistic combinations with respect to concentrations. Because d1 is the concentration of treatment 1 responsible for an x effect in combination, and d1x is the concentration of treatment 1 responsible for an x effect per se, a normalized concentration d1/d1x, calculated using Equation (2) tends toward zero as smaller concentrations of the treatments in combination are required to achieve an x effect.

As shown in Figure 4C, almost all data points are located in the region where combinations have a synergistic effect, suggesting that both products obtained from *A. conyzoides* act in synergy with doxycycline in the *in vitro* treatment of DH82 cells infected with *E. canis*. The CI effect of the graph also allows the visualization of the combined effects, based on the CI combination index calculated using Equation (1). The CI value is represented by a function of the antibacterial effect associated with each combination, and when the CI value < 1, > 1, and = 1 the effects are synergistic, antagonistic, and additive, respectively (Figure 5D).

Similar to Figure 5C, the combination sites less than 1 indicate a synergistic effect between the DCM fraction of *A. conyzoides* and doxycycline. The fact that the compounds show synergism means that their concentration in combination produces an effect that is more potent than when the treatments are used individually at a similar or greater concentration. For this synergistic property, the inhibitors can be evaluated by calculating the drug CRI (Equation 4) for each treatment of each combination and is plotted with the log (CRI) (Figures 4E).

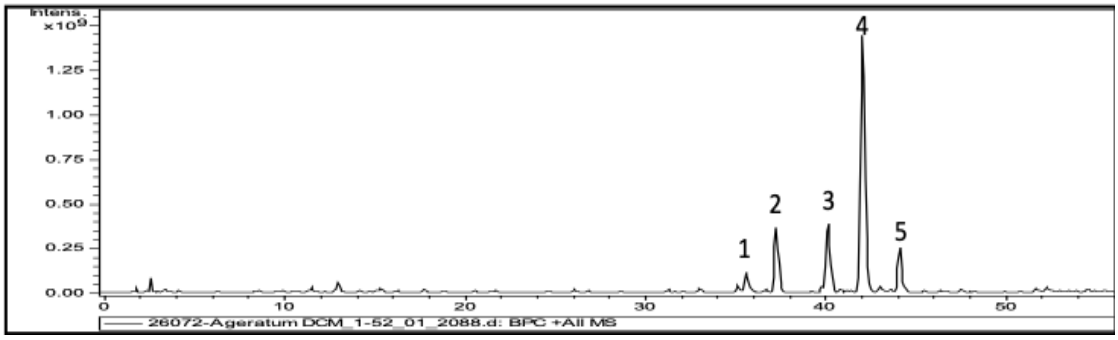

Figure 3 - HPLC-UV chromatogram (254 nm) of A. conyzoides dichloromethane (DCM) fraction.

In our case, the DCM fraction of *A. conyzoides* and doxycycline make up combinations that inhibit *E. canis* infection *in vitro*, and the effect becomes more potent as the CRI increases. Although, this result is expected, it does not translate into synergy. CRI is calculated for individual drugs in a given combination effect.

DISCUSSION

To the best of our knowledge, this is the first study to report the anti-*Ehrlichia* activity of the botanical extract of *A. conyzoides*. Recently, a scientific paper on the efficacy of *A. conyzoides* against *E. canis* was published, but the product used by the researchers was the essential oil (ROSÁRIO et al., 2019).

The DCM fraction of *A. conyzoides* showed remarkable anti-*Ehrlichia* activity at a concentration of 200 µg mL^{-1} (Figure 1). The botanical extract of *A. conyzoides* is active against intracellular microorganisms, such as *Trypanosoma brucei rhodesiense*, *Leishmania donovani* (NOUR et al., 2010), and *Plasmodium falciparum* (OWUOR et al., 2012). Additionally, the efficacy of *A. conyzoides* extract against *L. infantum* (JOSHI et al., 2016) and *L. amazonensis* (TEIXEIRA et al., 2014) has been demonstrated.

The chemical characterization of the DCM fraction of *A. conyzoides* obtained in this study showed that almost all components were secondary metabolites of the lignan class (Figure 3). According to the literature, lignans have remarkable biological activities, including anti-*Leishmania* (ROYO et al., 2003) and trypanocidal activities, which results in mitochondrial dysfunction and oxidative damage and can trigger destructive effects on the biological molecules of these microorganisms, leading to death (BERNARDES et al., 2006; IZUMI et al., 2011; LUIZE et al., 2006; PELIZZARO-ROCHA et al., 2011).

Table 1- Identification of compounds in *Ageratum conyzoides* dichloromethane fraction by LC-ESI-IT/MS.

Peaks	$[M-H]^{-}$ [a]	Ms^{n} [b]	Proposed compound
1	175	159; 145; 132; 115	Hydroxy methyl coumarin
2	417	402;387;372;356;328;300	Syringaresinol
3	403	390; 345	Derived from syringaresinol 1
4	431	415; 400;369	Derived from syringaresinol 2
5	387	372; 356; 341; 313; 285	Derived from syringaresinol 3

[a] Desprotonation
[b] Multiple-stage fragmentations

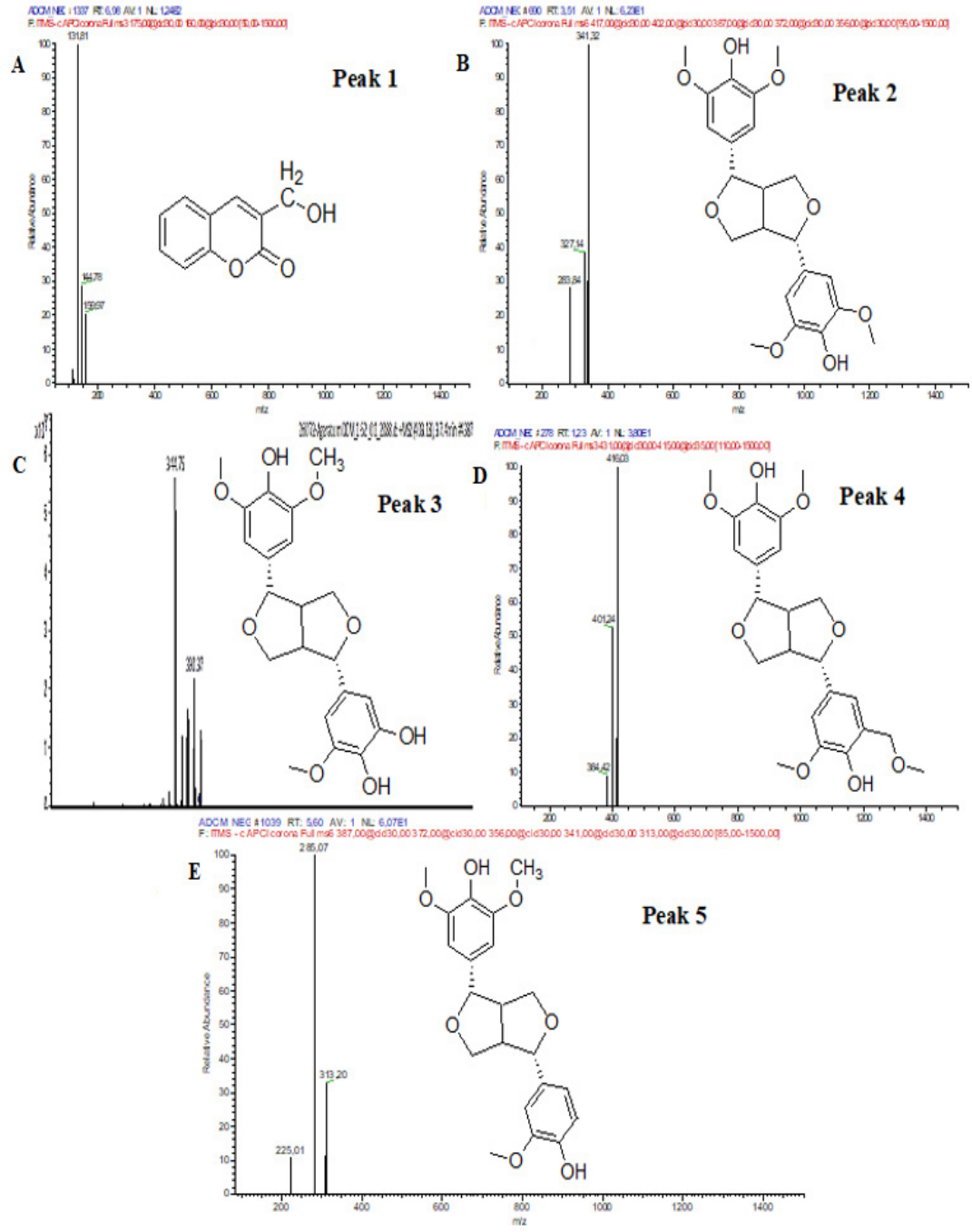

Figure 4 - First-order mass spectrum of compounds from A. conyzoides dichloromethane (DCM) fraction.

The semi-synthetic derivatives of the lignans, including (-) - quinoline, (-) - *O*-benzyl cubebin, and (-) - (*N,N*-dimethylaminoethyl) cubebine also showed antiprotozoal activity against amastigote stages of *T. cruzi* with IC_{50} values of 0.7, 5.7, and 4.7 µm, respectively, after 24 h of incubation (SOUZA et al., 2005). Several studies have shown that lignan (-) - hinoquinine is effective in reducing chromosomal damage induced by doxorubicin because it has an antioxidant effect on the mitochondria of the

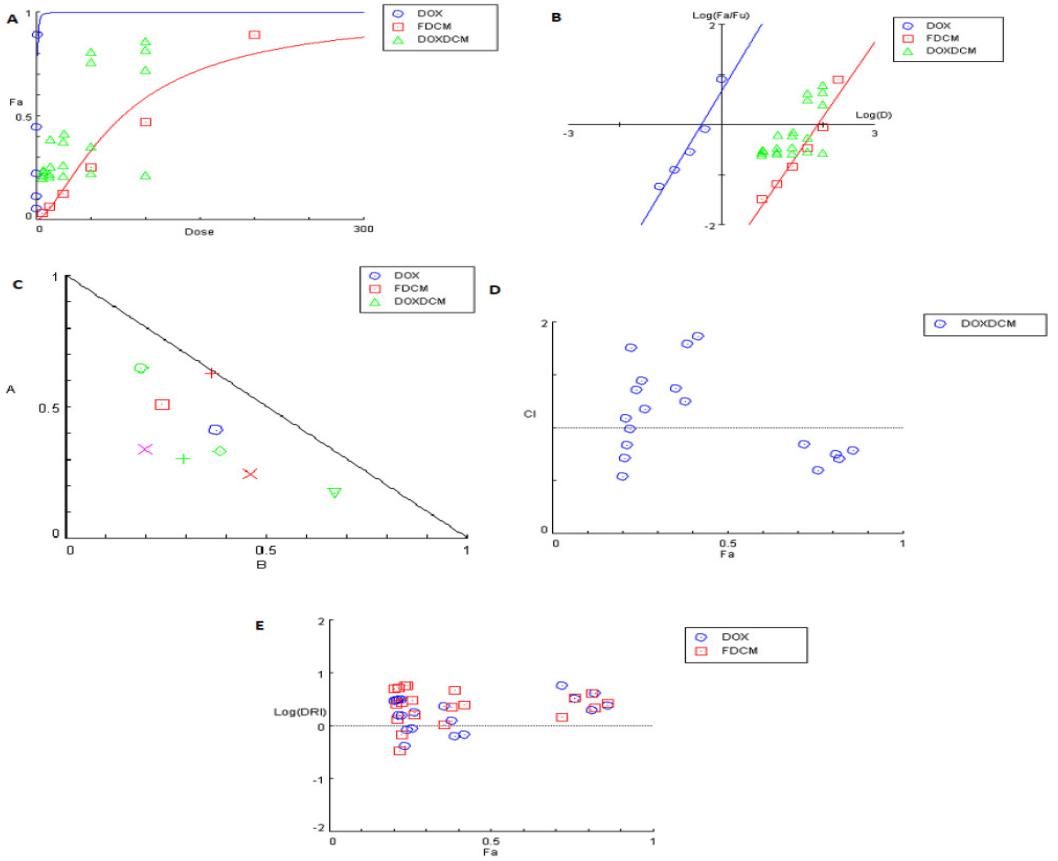

Figure 5 - Evaluation of the synergism of doxycycline in combination with dichloromethanic fraction (DCM) of A. conyzoides. Data sets in blue, red and green correspond to doxycycline, DCM fraction, combinations, respectively. (a) Concentration-response curves of each individual treatment. Doxycycline was evaluated twice, for each of the combinations with DCM fraction. The antibacterial effect was determined by measurement of foci size. (b) Median-effect representation of the concentracion-response curves for each individual compound, using Equation (3). m is the linear regression slope, fa is the "fraction affected", or (% effect) ÷ 100. (c) Normalized isobologram that represents, for each combination, the normalized concentration of each treatment individually required to reach the observed effect in combination (Equation (2)). d1 is the concentration of doxycycline in combination required to achieve x% of inhibition, while Dx1 is the concentration of treatment alone required to achieve x% of inhibition. Data points in zone correspond to combinations with synergistic, additive or antagonistic effects, respectively. (d) CI-effect plot representing the combination index CI, calculated using Equation (1), of each combination as a function of their associated antibacterial effect. The zone a is the same as the ones described in (c). (e) log (CRI)-effect plot representing the drug reduction index (CRI) of compounds as a function of their antibacterial effect in combination. The CRI is calculated for each drug in each combination according to Equation (4) and represents the dilution factor required for a drug to reach the same level of inhibition individually compared with it when in combination. The results are representative of 3 independent experiments.

parasite (SARAIVA et al. 2007; IZUMI et al., 2011). Syringaresinol is a compound reported in the botanical extract (Table 1) and has antitrypanosomal and anti-*Leishmania* activity (COSTA et al., 2018) in addition to its antibacterial activity (ALAMZEB et al., 2013).

To date, the mechanism of action of plant lignans against *E. canis* is unknown. These substances (polyphenols) inhibit the formation of microtubules, preventing cell division (WINK, 2015), because the microtubules are responsible for

Table 2 - Proportion, effect, combination index (CI), minimum concentration and concentration reduction index (CRI) of the associations between doxycycline and the DCM fraction of *Ageratum conyzoides* L.

Associated treatment	Proportion	Efect	C.I	[DOX] (µg mL^{-1})	[DCM] (µg mL^{-1})	CRI DOX	CRI DCM
DOX + FDCM	$^{1}/_{8}$ DOX + $^{1}/_{2}$ FDCM	0.72	0.84	0.125	100	5.83	1.49

the organization of the mitotic spindle. *Ehrlichia* replicates by binary fission and subsequently forms elemental corpuscles, which are seen as pleomorphic inclusions (initial corpuscles) inside the leukocytes (ALMOSNY et al., 2002).

Mechanisms, such as decreasing the intracellular level of the drug, altering the target of the drug, or overexpressing molecules involved in repairing drug damage are adaptations of the microorganism that result in resistance (CROFT et al., 2006).

Therefore, interactions between plant extracts and medications may result in beneficial effects that would not be obtained with the individual use of the products, such as the reduction of adverse and secondary effects, delay of the emergence of multiresistant microorganisms, and possibility of reducing the concentration of the drug (SEHN et al., 2003).

Several studies have already shown the therapeutic potential of *A. conyzoides* against intracellular microorganisms (NWEZE & OBIWULU, 2009; NOUR et al., 2010, OWUOR et al., 2012, CHINWE et al., 2010, MELO et al., 2011; SHAILAJAN et al., 2013; TEIXEIRA et al., 2014; PONÉ et al., 2011; JOSHI et al., 2016).

AHMED et al. (2010) demonstrated the synergistic effect of the combination of tetracycline with the ethanolic extract of leaves and stems of *S. persica* against *S. aureus*. CHATTERJEE et al. (2009) confirmed the synergistic interaction of the ethanolic extract of the *Vangueria spinosa* leaf with the antibiotics doxycycline and ofloxacin against *S. aureus*, *E. coli*, and *K. pneumoniae*.

In this study, we observed a significant modulating action of the DCM fraction of *A. conyzoides* extract when associated with doxycycline, resulting in an increase in the antimicrobial potential of both. GIBSON et al. (2004) stated that plants can be considered as resistance modulators or as acting synergistically with antimicrobials through a mechanism not yet elucidated.

OMOTOSO & ENZE (2019) analyzed that treatment with methanolic extracts of *A. conyzoides* L. can decrease the proliferation of liver tissue, and only exposure to higher doses can help to neutralize this antiproliferative influence. This proliferative inhibition or antiproliferative activity of *A. conyzoides* L. has been reported in a study by ADEBAYO et al. (2010). The expression of Ki-67 that occurs in all phases of the cell cycle (except G0) and P53, in this study, is responsible for the regulation of DNA repair and apoptosis. This association may be associated with the mechanism of action of doxycycline according to OMAR et al. (2011) and GOMES et al. (2017).

A. conyzoides also induced a significant S-phase arrest in HeLa cells of human cervical adenoma (LIN et al., 2020). Thus, leaving a line of study to explore the synergistic effect from the interaction of *A. conyzoides* with doxycycline.

CONCLUSION

The DCM fraction of *A. conyzoides* collected from São Luís, MA, northeastern Brazil, has a promising lignan composition with remarkable anti-*Ehrlichia canis* activity and a synergistic effect in the treatment associated with doxycycline being a potential alternative treatment for one of the most important diseases in companion animals.

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DECLARTION OF CONFLICT OF INTERESTS

The authors declare no conflict of interest. The founding sponsors had no role in the design of the study; in the collection, analyses, or interpretation of data; in the writing of the manuscript, and in the decision to publish the results.

AUTHORS' CONTRIBUTIONS

All authors shared in the design of the study, the collection, analysis, and interpretation of data, and the writing of the manuscript.

REFERENCES

ADEBAYO, A.H. et al. Atividade anticâncer e antirradical de eliminação de *Ageratum conyzoides* L. (*Asteraceae*). **Pharmacogn Mag.** 6: 62-6, 2010. DOI: 10.4103 / 0973-1296.59968.

AGUIAR, D.M. et al. Diagnóstico sorológico de erliquiose canina com antígeno brasileiro de *Ehrlichia canis*. **Cienc Rural** 37(3): 796- 802, 2007. Available from: <http://www.scielo.br/pdf/cr/v37n3/a30v37n3.pdf>. Accessed: 10 sept. 2020.

AHMED, Z. et al. Synergistic effect of *Salvadora persica* extracts, tetracycline and penicillin against *Staphylococcus aureus*. **African J. Basic Appl. Sci.** 2, 25-29, 2010. Available from: <https://www.researchgate.net/publication/269394917>. Accessed: 10 sept. 2020.

ALAMZEB, M. et al. Bioassay guided isolation and characterization of anti-microbial and anti-trypanosomal agents from *Berberis glaucocarpa* Stapf. **Afr J Pharm Pharmacol** 7 (29): 2065-2071, 2013. doi:10.5897/AJPP2013.3444.

ALMOSNY, N.R.P.; MASSARD, C.L. Erliquiose. In: Hemoparasitoses em Pequenos Animais Domésticos e como Zoonoses. **Almosny NRP,** Rio de Janeiro, RJ, 2002.

ANVISA. (2010) Agência Nacional de Vigilância Sanitária. Farmacopeia Brasileira. Brasília, Brasil.

BARILE, FA. **In vitro cytotoxicology.** New York, USA, 1994.

BERNARDES, L.S.C. et al. Synthesis and trypanocidal activity of 1,4-bis-(3,4,5-trimethoxy-phenyl)-1,4-butanediol and 1,4-bis-(3,4-dimethoxyphenyl)-1,4-butanediol. **Bioorg. Med Chem** 14(21): 7075-7082, 2006. doi:10.1016/j.bmc.2006.07.006.

BOGIĆEVIĆ, N. et al. Seroprevalence of *Ehrlichia canis* infection in stray dogs from Serbia. **Mac Vet Rev** 40 (1): 37-42, 2017. Available from: <https://www.macvetrev.mk/2017-1/macvetrev-2016-0096.pdf>. Accessed: 10 sept. 2020.

CIMANGA, R.K. et al. Antibacterial and antifungal screening of extracts from six medicinal plants collected in Kinshasa-Democratic Republic of Congo against clinical isolate pathogens. **J Pharmacognosy and Phytother.** 6 (3): 24-32, 2014. doi: 10.5897/JPP2013.0263.

COSTA, R.S. et al. In vitro antileishmanial and antitrypanosomal activity of compounds isolated from the roots of *Zanthoxylum tingoassuiba* **Rev. bras. farmacogn** 28(5): 551-558, 2018. doi:10.1016/j.bjp.2018.04.013.

CHATTERJEE, S.K. et al. *In vitro* synergistic effect of doxycycline & with ethanolic leaf extract of *Vangueria spinosa* pathogenic bacteria. I**ndian J Med Res.** 130, 475-8, 2009. PMID: 19942754.

CHINWE, UV. et al.Antimalarial activity of *Ageratum conyzoides* in combination with chloroquine and artesunate. **Asian Pacific Journal of Tropical Medicine.** 943-947, 2010.

CHOU, T.C. Derivation and properties of Michaelis-Menten type and Hill type equations for reference ligands. **J. Theor. Biol.** 59, 253–276, 1976. Available from: <https://doi.org/10.1016/0065-2571(84)90007-4>. Accessed: 10 sept. 2020.

CHOU, T.C.; TALALAY, P. Quantitative analysis of dose-effect relationships: the combined effects of multiple drugs or enzyme inhibitors. **Adv Enzyme Regul.** 22: 27-55, 1984. Available from: <https://dx.doi.org/10.1016/0065-2571(84)90007-4>. Accessed: 10 sept. 2020.

CHOU, T.C. Theoretical basis, experimental design, and computerized simulation of synergism and antagonism in drug combination studies. **Pharmacol. Rev.** 58, 621–68,1 2006. Available from: <https://doi.org/10.1124/pr.58.3.10>. Accessed: 10 sept. 2020.

CHOU, T.C. Drug combination studies and their synergy quantification using the Chou-Talalay method. **Cancer Res.** 70: 440-446, 2010. Available from: <https://doi.org/10.1158/00085472.CAN-09-1947>. Accessed: 10 sept. 2020.

CROFT, S.L. et al. Drug resistance in leishmaniasis. **Clinical Microbiology Reviews,** Washington. 19, 111-126, 2006. doi: 10.1128/CMR.19.1.111-126.2006.

DAGNONE, A.S. et al. Erliquiose nos animais e no homem. **Semina: Ci. Agrárias,** Londrina 22 (2): 191-201, 2001. Available from: <http://www.uel.br/revistas/uel/index.php/semagrarias/article/viewFile/2053/1762>. Accessed: 10 sept. 2020.

GOMES, J.R. et al. **Doxycycline reduces the expression and activity of matrix metalloproteinase-2 in the periodontal ligament of the rat incisor without altering the eruption process.** 52(3):353-359, 2017. doi: 10.1111/jre.12398.

GIBSON, G.R. et al. Dietary modulation of the human colonic microbiota: updating the concept of prebiotics. **Nutr. Res. Rev.** 17, 259–275, 2004. doi: 10.1079/NRR200479.

GONZÁLEZ-LAMOTHE, R. et al. Plant Antimicrobial Agents and Their Effects on Plant and Human Pathogens. **Int J Mol Sci** 10(8): 3400–3419, 2009. doi: 10.3390/ijms10083400.

IZUMI, E. et al. Natural products and Chagas' disease: a review of plant compounds studied for activity against *Trypanosoma cruzi*. **Nat Prod Rep** 28(4): 809-823, 2011. doi:10.1039/c0np00069h.

JOSHI, B. et al. In vitro antileishmanial and antimalarial activity of selected plants of Nepal. **J Intercult Ethnopharmacol** 5(4): 383–389, 2016. doi:10.5455/jice.20160728031236.

KANYANGA, R.C. et al. Antibacterial and antifungal screening of extracts from six medicinal plants collected in Kinshasa-Democratic Republic of Congo against clinical isolate pathogens. **J Pharmacognosy Phytother** 6(3): 24-32, 2014. doi: 10.5897/JPP2013.0263.

KISSMANN, K.G.; GROTH. D. **Plantas infestantes e nocivas,** 2ª ed., São Paulo: BASF A.S, 1999.

LIN, Z. Flavonoids in *Ageratum conyzoides* L. Exert Potent Antitumor Effects on Human Cervical Adenocarcinoma HeLa Cells *In Vitro* and *In Vivo.* **Biomed Res Int.** 2020 May 4;2020:2696350. doi: 10.1155/2020/2696350.

LUIZE, P.S.L. et al. Activity of neolignans isolated from *Piper regnellii* (MIQ.) C. DC. var. *pallescens* (C. DC.) YUNCK against *Trypanosoma cruzi*. **Biol Pharm Bull** 29 (10): 2126-2130, 2006. doi:10.1248/bpb.29.2126.

MELO, N.I. et al. Schistosomicidal Activity of the Essential Oil of *Ageratum conyzoides* L. (Asteraceae) against Adult *Schistosoma mansoni* Worms. **Molecules.** 16, 762-773, 2011. DOI: 10.3390 / molecules16010762.

MYLONAKIS, M.E. et al. Evaluation of citology in the diagnosis of acute canine monocytic ehrlichiosis (*Ehrlichia canis*): a comparison between five methods. **Vet Microbiol** 91 (2-3): 197- 204, 2003. doi:10.1016/ S03781135(02)00298-5.

NIGHTINGALE, C.H. et al. **Antimicrobial pharmacodynamics in theory and clinical practice,** vol 2nd ed. Boca Raton, FL: Taylor & Francis Group, LLC, 2007, 536p.

NWEZE, N.E.; OBIWULU, I.S. Anticoccidial effects of *Ageratum conyzoides.* **Journal of Ethnopharmacology.** 122, 6-9, 2009. doi: 10.1016/j.jep.2008.11.014.

NOUR, A.M.M. et al. The antiprotozoal activity of methylated flavonoids from *Ageratum conyzoides* L. **J Ethnopharmacol** 129(1): 127–130, 2010. doi:10.1016/j.jep.2010.02.015.

OMAR, N.F. et al. MT1-MMP expression in the odontogenic region of rat incisors undergoing interrupted eruption. **J Mol Hist.** 42:505-511, 2011. DOI 10.1007 / s10735-011-9356-0.

OMOTOSO, D.R.; EZE, G.I. Antiproliferative potential of methanolic extracts of *Ageratum conyzoides* linnaeus via downregulation of ki-67 and upregulation of p53 protein expression in hepatic tissue of rats. **Niger J Exp Clin Biosci** 7(1): 35-40, 2019. DOI: 10.4103 / njecp.njecp_21_19.

OWUOR, B.O. et al. In vitro antiplasmodial activity of selected Luo and Kuria medicinal plants. **J Ethnopharmacol** 144(3): 779–781, 2012. doi:10.1016/j.jep.2012.09.045.

PELIZZARO-ROCHA, K.J. et al. Trypanocidal action of eupomatenoid-5 is related to mitochondrion dysfunction and oxidative damage in *Trypanosoma cruzi*. **Microb Infect** 13(12-13): 1018-1024, 2011. doi: 10.1016/j. micinf.2011.05.011.

PEREIRA-MAIA, E.C. et al. Tetracyclines and glycylcyclines: an overview. **Quím. Nova** 33(3), 2010. Available from: <https:// doi.org/10.1590/S0100-40422010000300038>. Accessed: 10 sept. 2020.

PRAJAPATI, R. et al. Formulation development, standardization and antimicrobial activity of *Ageratum conyzoides* extracts and their formulation. **Int J Pharm Pharm** Sci 6 (2): 369-374, 2014. Available from: <http://connection.ebscohost.com/c/ articles/95530798/formulation-development-standardization-antimicrobial-activity-ageratum-conyzoides-extracts-their formulation>. Accessed: 10 sept. 2020.

PONÉ, J.W. et al. The *in vitro* effects of aqueous and ethanolic extracts of the leaves of *Ageratum conyzoides* (*Asteraceae*) on Three Life Cycle Stages of the Parasitic Nematode *Heligmosomoides bakeri* (Nematoda: Heligmosomatidae). **Veterinary Medicine International.** Article ID 140293, 2011. Available from: <http://dx.doi. org/10.4061/2011/140293>. Accessed: 10 sept. 2020.

ROLAIN, J.M. et al. In vitro susceptibilities of 27 rickettsiae to 13 antimicrobials. **Antimicrob Agents Chemother** 42:1537–1541, 1998. Available from: <https://doi.org/10.1128/AAC.42.7.1537>. Accessed: 10 sept. 2020.

ROLAIN, J.M. et al. Evaluation of antibiotic susceptibilities of three rickettsial species including Rickettsia felis by a quantitative PCR DNA assay. **Antimicrob Agents Chemother** 46:2747–2751, 2002. Available from: <https ://doi.org/10.1128/AAC.46.9.2747-2751.2002>. Accessed: 10 sept. 2020.

ROSÁRIO, C.J.R.M. et al. Anti-*Ehrlichia* properties of the essential oil of *Ageratum conyzoides* L. and its interaction with doxycycline. **AMB Express** Apr 29; 9(1):58, 2019. doi: 10.1186/ s13568-019-0780-y.

ROYO, V.A. et al. Biologiacal activity evaluation of dibenzilbutirolactones lignans derivates against *Leishmania brasiliensis*. **Rev bras farmacogn** 13 (2): 18-21, 2003. Available from: <http://dx.doi.org/10.1590/S0102695X2003000400007>. Accessed: 10 sept. 2020.

SAMY, R.P. et al. Evaluation of aromatic plants and compounds used to fight multidrug resistant infections. **Evid Based Complement Evid Based Complement Alternat Med Medicine.** Article ID 525613, 17 pages, 2013. Available from: <http://dx.doi.org/10.1155/2013/525613>. Accessed: 10 sept. 2020.

SARAIVA, J. et al. In vitro and in vivo activity of lignan lactones derivatives against *Trypanosoma cruzi*, Parasitol Res 100 (4): 791-795, 2007. doi: 10.1007/s00436-006-0327-4.

SAUCEDO, R. **Tetraciclinas. Farmacocinética.** Universidad de Granada, 2003. Available from: <http://www.ugr.es/~morillas/ temas/tetracic/sld003.htm>. Accessed: 10 sept. 2020.

SEHN, R. et al. Interações medicamentosas potenciais em prescrições de pacientes hospitalizados. **Infarma.** 15, 9-10, 2003.

SHAILAJAN, S. et al. Evaluation of quality and efficacy of an ethnomedicinal plant *Ageratum conyzoides* L. in the management of pediculosis. **Journal of Young Pharmacists.** 5, 139-143, 2013. doi: 10.1016/j.jyp.2013.10.005.

SINGH, B.R. et al. Antimicrobial activity of Methanolic Extract and Ether Extract of *Ageratum conyzoides*. **Pharm Anal Acta.** 7: 471, 2016. doi: 10.4172/2153-2435.1000471.

SOUSA, V.R.F. et al. Avaliação clínica e molecular de cães com erliquiose. **Ciênc rural** 40 (6): 1309-1313, 2010.

SOUZA, V.A. et al. Trypanocidal activity of (-)-cubebin derivatives against free amastigote forms of *Trypanosoma cruzi*. **Bioorg Med Chem Lett** 15(2): 303-307, 2005. doi:10.1016/j.bmcl.2004.10.079.

TEIXEIRA, T.L. et al. Potential therapeutic use of herbal extracts in trypanosomiasis. **Pathog Glob Health** 108(1): 30-36, 2014. doi :10.1179/2047773213Y.0000000120.

TILLEY, L.P. et al. **Consulta veterinária em 5 minutos.** 2. ed. Barueri: Manole, 2003.

VARELA, A.S. Tick-borne Ehrlichiae and Rickettsiae of dogs. In: Bowman DD. **Companion and exotic animal parasitology.** 2003. Available from: <https://pdfs.semanticscholar.org/8676/9120ddd537819275b105f7fbf1650e5689c0.pdf>. Accessed: 10 sept. 2020.

WANER, T.; HARRUS, S. Canine monocytic ehrlichiosis (CME). In: _____. **Recent advances in canine infectious disease.** 13 apr 2000. Available from: <https://pdfs.semanticscholar.org/b19e/15c84a975bac851dfce84c3d4c13ed74737f.pdf>. Accessed: 10 sept. 2020.

WINK, M. Modes of Action of Herbal Medicines and Plant Secondary Metabolites. **Medicines** 2, 251-286, 2015. doi:10.3390/medicines2030251.

WORKOWKI, K.A.A. Sexually transmittes diseases treatment Guidelines. **MMWR Morb Mortal Wkly** Rep. 51 (RR-6):1, 2002. Available from: <https://www.cdc.gov/mmwr/pdf/rr/rr6403.pdf>. Accessed: 10 sept. 2020.

ZIMMERMAN, H.J. (2000). Drug-induced liver disease. **Clin Liver Dis.** 4(1): 73-96, 2000.