

# *In vitro* activity of *Artemisia annua* L (Asteraceae) extracts against *Rhipicephalus (Boophilus) microplus*

Atividade *in vitro* de extratos de *Artemisia annua* L (Asteraceae) sobre *Rhipicephalus (Boophilus) microplus*

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## Abstract

The activity of plant extracts on parasites may indicate groups of substances that are potentially useful for controlling *Rhipicephalus (Boophilus) microplus*. The aim of the present study was to investigate the *in vitro* action of *Artemisia annua* extracts on this tick. The concentrations of the sesquiterpene lactones artemisinin and deoxyartemisinin present in plant extracts were quantified via high-performance liquid chromatography. Four extracts produced from the concentrated crude extract (CCE) were evaluated on larvae using the impregnated paper method, with readings after 24 hours of incubation. The engorged females were immersed in the CCE and in its four derived extracts for five minutes, with incubation for subsequent analysis of biological parameters. The extracts were not effective on the larvae at the concentrations tested (3.1 to 50 mg.mL<sup>-1</sup>). The CCE showed greater efficacy on engorged females (EC<sub>50</sub> of 130.6 mg.mL<sup>-1</sup> and EC<sub>90</sub> of 302.9 mg.mL<sup>-1</sup>) than did the derived extracts. These results tend to confirm that the action of artemisinin on engorged females of *R. (B.) microplus* is conditional to their blood intake. In this case, *in vitro* methods would be inadequate for effective evaluation of the action of *A. annua* on *R. (B.) microplus*.

**Keywords:** Phytotherapy, artemisinin, tick, control, *R. (B.) microplus*, *Artemisia annua*.

## Resumo

A atividade de extratos vegetais sobre parasitas pode indicar grupos de substâncias de uso potencial no controle de *Rhipicephalus (Boophilus) microplus*. O objetivo do presente estudo foi investigar a ação *in vitro* de extratos de *Artemisia annua* sobre esta espécie. A concentração das lactonas sesquiterpênicas artemisinina e deoxiartemisinina presentes nos extratos vegetais, foi quantificada via cromatografia líquida de alta eficiência. Quatro extratos produzidos a partir do extrato bruto concentrado (EBC) foram avaliados sobre larvas pela metodologia do papel impregnado, com leitura após 24 horas de incubação. As fêmeas ingurgitadas foram imersas por cinco minutos no EBC e nos seus quatro extratos derivados, e incubadas para posterior análise dos parâmetros biológicos. Os extratos não tiveram eficácia sobre as larvas nas concentrações avaliadas (de 3,1 a 50 mg.mL<sup>-1</sup>). O EBC apresentou melhor eficácia sobre as fêmeas ingurgitadas (CE<sub>50</sub> de 130,6 mg.mL<sup>-1</sup> e CE<sub>90</sub> de 302,9 mg.mL<sup>-1</sup>) que os extratos derivados. Esses resultados tendem a confirmar que a ação da artemisinina sobre as fêmeas ingurgitadas de *R. (B.) microplus* estaria condicionada à sua ingestão através do sangue. Nesse caso, os métodos *in vitro* seriam inadequados para a efetiva avaliação da ação de *A. annua* sobre *R. (B.) microplus*.

**Palavras-chave:** Fitoterapia, artemisinina, carrapato, controle, *R. (B.) microplus*, *Artemisia annua*.

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## Introduction

Good results from cattle rearing depend on nutritional, reproductive and health management, among other factors. In health management, parasite control is a major challenge, since rearing implies inevitable parasitic infestation. Brazil has one of the largest cattle herds in the world, estimated at 173.2 million head (ANUALPEC, 2009). *Rhipicephalus (Boophilus) microplus* tick (Canestrini, 1887) is considered largely responsible for economic losses in cattle production. Research has shown that Brazil spends about 800 million dollars a year on acaricides to combat this parasite (FURLONG et al., 2007). However, control using chemicals has become increasingly less tenable for a number of reasons, such as high cost, short time of effectiveness, rapid development of resistance, inadequate management and disregard of the lack of time and possibility of pesticide waste in the environment and products of animal origin (GRISI et al., 2002).

For this reason, searching for new alternatives to conventional acaricides for parasite control has become inevitable. Several specific investigations have been conducted on *R. (B.) microplus*, in which the antiparasitic potential and bioactivity of plant extracts have been evaluated. The essential oil of *Eucalyptus staigeriana* (Myrtaceae) has been tested on larvae and engorged females and was found to cause mortality ranging from 12.5 to 100%. When the oil was turned into an emulsifiable concentrate, efficiency of 100% was obtained at a concentration of 3.9% (CHAGAS et al., 2002). *In vivo* tests have shown that the 0.25% hexane extract of *Melia azedarach* (Meliaceae) reduced the infestation of *R. (B.) microplus* by around 24% (BORGES et al., 2005). The essential oil of *Cymbopogon winterianus* (Poaceae) at 7% concentration inhibited 100% of egg hatchability in treated engorged females, although the compound isolated were not so effective (MARTINS, 2006). The 2.7% alcoholic extract of *Cymbopogon citratus* caused mortality of 43% in the field (HEIMERDINGER et al., 2006). The oil of *Copaifera reticulata* (Caesalpiniaceae) presented an  $EC_{50}$  of 3.5 ppm for larvae (FERNANDES; FREITAS, 2007). The hexane extract of *Hypericum polyanthemum* (Guttiferaceae) killed 100% of the larvae at 6.25 mg.mL<sup>-1</sup> (RIBEIRO et al., 2007).

*Artemisia annua* L. (Asteraceae) (the target of the present study) and other species of this genus contain a sesquiterpene lactone called artemisinin, which has already been proven to have antimalarial activity. The mechanisms of action, clinical characteristics and toxicity of artemisinin have been studied extensively. Agro-technological, pharmacological and chemical studies have also been conducted on other sesquiterpene compounds present in this plant (RODRIGUES et al., 2006). In addition to the rapid action of artemisinin at nanomolar concentrations for controlling *Plasmodium* spp. (CREEK et al., 2005; FERREIRA et al., 2006), the genus *Artemisia* also has an effect on gastrointestinal nematodes, thereby decreasing the number of eggs per gram of feces (EPG) in small ruminants (IDRIS et al., 1982; IQBAL et al., 2004; TARIQ et al., 2009). There have not been any specific studies in the literature examining the action of *A. annua* on the tick *R. (B.) microplus*. Hence, the present study aimed to evaluate the effect of extracts of *A. annua* on *R. (B.) microplus*, and to identify and quantify its constituents, thus providing future possibilities for preparing safe, standardized and commercially available formulations for farmers.

## Material and Methods

### 1. Production of *A. annua* extracts

*A. annua* leaves and twigs genotype Ch × Viet55, which was selected for late flowering and was cultivated in the Multidisciplinary Centre for Chemical, Biological and Agricultural Research (CPQBA) at UNICAMP, was used throughout the present study. To extract the active ingredients, the traditional method was used on a weight of 1 kg of dried and ground plant material. In this process, the plant was subjected to extraction using ethanol (96° GL) as the extractor liquid at room temperature, with the aid of mechanical agitation for 4½ hours (3 × 1½ hours). The extracts were filtered, pooled and concentrated to ⅓ of the total volume, thus providing the so-called concentrated crude extract (CCE), which was subjected to evaporation. From this extract, another four extracts were produced under cold conditions in accordance with Simões et al. (2004), using different solvents for non-sequential extraction in: A) water followed by lyophilization; B) water basified with NaHCO<sub>3</sub> (sodium bicarbonate) at 0.1%; C) ethanol (96° GL), followed by concentration in a rotary evaporator; and D) dichloromethane, followed by concentration in a rotary evaporator.

### 2. Quantification of artemisinin and deoxyartemisinin in the *A. annua* extracts

The extracts were suspended in 5.0 mL volumetric flasks with chromatographic-grade methanol, filtered through 0.45 µm Millipore and analyzed by means of refractive index high-performance liquid chromatography (RI-HPLC). To prepare the mobile phase, 60:40 solvents of H<sub>2</sub>O to MeOH were used, with filtration on 0.45 µm Millipore and sonication under vacuum. To prepare the calibration curve, 65 mg of the extracts was weighed, and this was diluted in 25 mL of HPLC-grade methanol, thereby resulting in a concentration of 2,444 µg.mL<sup>-1</sup>, of analytical standard with 94% purity. Injections in triplicate were made at seven points on the analytical curve, at concentrations from 50 to 1,250 µg.mL<sup>-1</sup>, and the curve was obtained through the Empower software. The chromatograph was used equipped with an ionization detector and a Phenomenex Luna CN 100A° capillary column (250 mm × 4.6 mm × 5 µm), and with volume injection of 20 µL and flow rate of 1 mL/min. This procedure had a sensitivity of 32 °C and internal temperature of 35 °C. The quantification of artemisinin (retention time of 7 minutes) and deoxyartemisinin (6.5 minutes) content in the extracts was done using the methodology validated by Celeghini et al. (2009), from the external standard method.

### 3. Sensitivity of larvae in the impregnated paper test

Engorged females from the colony of *R. (B.) microplus* that is maintained by Embrapa Pecuária Sudeste (CPPSE) were placed in an incubator (± 27 °C and RH > 80%) for larvae production, for use 14 to 21 days after hatching. The extracts were tested at five concentrations: aqueous (A) and sodium bicarbonate (B) extracts

were used at concentrations ranging from 12.5 to 50 mg.mL<sup>-1</sup>; and ethanol (C) and dichloromethane extracts (D) between 3.1 and 12.5 mg.mL<sup>-1</sup>. These concentrations were defined according to the available quantity of each of the extracts and from *in vitro* results with eggs and larvae of gastrointestinal nematodes (CALA, 2010). The ethanol extracts were dissolved in 0.33% Tween 80 and 10% ethanol, with one control consisting of distilled water and another of distilled water, and the other solvents at the same concentrations. All tests were conducted with three replications.

Approximately 100 larvae were placed between 2 × 2 cm filter papers that had newly been impregnated with the extracts, thereby forming a sandwich. This was placed inside a filter paper envelope and sealed. This approach is recommended by the World Food and Agriculture Organization (CHAGAS et al., 2003). The envelopes were kept in an incubator (±27 °C and RH > 80%) and counting of live and dead larvae was performed after 24 hours. To calculate the percentage mortality, the following formulas were used:

$$\text{Mortality (\%)} = \frac{\text{dead larvae} \times 100}{\text{total larvae}} \quad (1)$$

$$\text{Average Mort. (\%)} = \frac{(\text{mort. replication 1}) + (\text{mort. replication 2}) + (\text{mort. replication 3})}{3}$$

#### 4. Sensitivity of engorged females in the immersion test

Engorged females collected from cattle at CPPSE were weighed in order to form homogeneous groups of 10 females, which were immersed for 5 minutes in the plant extracts tested (DRUMMOND et al., 1973). After this period, the females were removed from the solution, dried on paper towels and placed in properly identified Petri dishes bearing double-sided tape. The groups were placed in the incubator (27 °C and RH > 80%) for 18 days and, after the spawning period, the eggs were weighed and transferred to properly labeled syringes, sealed and placed in the incubator. The hatchability was verified visually by comparing the number of remaining eggs with the shells (AMARAL, 1993).

Because the expected effectiveness was not obtained in the larva test, the immersion test on engorged females was set at higher concentrations, but again respecting the availability of each extract. Five concentrations were evaluated: aqueous extracts (A) from 40 to 280 mg.mL<sup>-1</sup>; sodium bicarbonate extracts (B) and dichloromethane extracts (D) from 20 to 220 mg.mL<sup>-1</sup>; and ethanol extracts (C) and CCE extracts from 10 to 140 mg.mL<sup>-1</sup>. Three replicates were performed for each concentration and the solvents 0.33% Tween 80 and 10% ethanol were used for the ethanol extracts. Control groups were prepared with solvents as mentioned above. Toxicity limits had been determined previously (CHAGAS et al., 2003). The percentage efficiency was calculated in accordance with Drummond et al. (1973):

$$\text{Reproductive efficiency index: REI} = \frac{\text{egg weight} \times \% \times 20,000 \text{ hatching}^*}{\text{weight of engorged females}} \quad (2)$$

\*Constant that indicates the number of eggs present in 1 g.

$$\text{Extract effectiveness: EE} = \frac{(\text{ER control} - \text{ER treated})}{\text{ER control}} \times 100 \quad (3)$$

The results were analyzed using the Probit procedure (from SAS) to determine the EC<sub>50</sub> and EC<sub>99</sub>. The data on the five concentrations were compared within each extract using the Tukey test.

## Results

The chemical analyses on the four plant extracts enabled quantification of artemisinin (to which antiparasitic action has been attributed) and deoxyartemisinin, another sesquiterpene lactone that is present in extracts from this species (Table 1). Taking the values found, it was observed that the dichloromethane extract (D) had the highest amounts of artemisinin and deoxyartemisinin, followed by the ethanol extract (C).

All the larvae remained alive after exposure to the extracts at all the concentrations tested. In the immersion test, no significant action was seen at any concentration for the four extracts derived. It was only possible to calculate the EC<sub>50</sub> and EC<sub>90</sub> for the CCE extract (p ≥ 0.05): 130.6 mg.mL<sup>-1</sup> (115.9 to 152.7) and 302.9 mg.mL<sup>-1</sup> (238.6 to 436.0), respectively. The results regarding reproductive efficiency and the effectiveness of the CCE and its four derivative extracts can be seen in Table 2.

## Discussion

From assessment of the records of *A. annua* activity on a variety of parasites, it was decided to evaluate its potential for controlling the tick *R. (B.) microplus*. It was observed in the larva test that the four extracts of *A. annua* had no effectiveness at the concentrations evaluated. There was an expectation of better results with dichloromethane, which contained a large amount of artemisinin (1343.9 g.mL<sup>-1</sup>) because of its high affinity for the solvent, which could explain its more significant biological activity. One factor that may have affected the results was the cracking condition of the CCE; other substances that might have been acting synergistically within the antiparasitic effect were diluted in the subsequent four extracts, thereby resulting in lower effectiveness for the dichloromethane extract than for the CCE.

Doses of *Artemisia herba-alba* from 2 to 30 g were administered orally to goats that had been artificially infected with the gastrointestinal nematode *H. contortus*. There was a reduction in EPG in the abomasum of adults, as well as reductions in histological damage or blood disorders (IDRIS et al., 1982). In sheep that received the crude ethanol and methanol extracts of *Artemisia brevifolia* for 14 days, at doses of 3.0 g.kg<sup>-1</sup> body weight (bw), EPG reductions of 67.2 and 62.1% were detected (IQBAL et al., 2004). Aqueous and ethanol crude extracts of *Artemisia absinthium* were also administered orally to infected sheep, and there was a reduction in EPG: in the aqueous extract, 80.49% was obtained at a dose of 2 g.kg<sup>-1</sup> bw and in the ethanol extract, 90.46% at a dose of 2 g.kg<sup>-1</sup> bw and 82.85% at a dose of 1 g.kg<sup>-1</sup> bw (TARIQ et al., 2009).

Although artemisinin-derived drugs have shown effects on a variety of parasites such as *Fasciola hepatica* and gastrointestinal nematodes in small ruminants. *Plasmodium* spp., *Coccidia* spp., *Babesia* spp., *Leishmania* spp., *Neospora caninum* and *Schistosoma* spp., and have been successfully tested as cancer

**Table 1.** Quantification of the sesquiterpene lactones artemisinin and deoxyartemisinin ( $\mu\text{g}\cdot\text{mL}^{-1}$ ) in aqueous, sodium bicarbonate, ethanol and dichloromethane extracts of *Artemisia annua*, via high-performance liquid chromatography.

Code	Extract	Artemisinin	Deoxyartemisinin
A	Aqueous	108.627	38.068
B	Sodium bicarbonate	93.476	37.532
C	Ethanol	989.985	289.054
D	Dichloromethane	1,343.929	316.838

**Table 2.** Reproductive efficiency index (REI) and extract effectiveness (EE) in engorged females of *Rhipicephalus (Boophilus) microplus*, tested by means of immersion in different concentrations ( $\text{mg}\cdot\text{mL}^{-1}$ ) of *Artemisia annua* extracts: concentrated crude extract (CCE), aqueous, sodium bicarbonate, ethanol and dichloromethane.

Extract	Concentration	REI	EE	*
CCE	10	95	0.0	a
	20	95	0.0	a
	40	91	4.2	a
	80	68	28.4	b
	140	45	52.6	c
Aqueous	40	94	0.7	a
	80	92	2.7	a
	160	86	9.2	a
	220	88	6.8	a
	280	83	12.5	a
Sodium bicarbonate	20	85	10.7	a
	40	78	17.3	a
	80	80	15.7	a
	160	84	11.7	a
	220	75	21.0	a
Ethanol	40	81	14.1	a
	80	65	31.0	a
	160	92	2.8	a
	220	78	17.5	a
	280	76	19.4	a
Dichloromethane	20	88	6.8	a
	40	86	9.0	a
	80	75	18.0	a
	160	82	13.1	a
	220	91	3.5	a

\*Different letters in the same column represent a difference. ( $p \geq 0.5$ ) within the same extract.

treatments (FERREIRA, 2007), they were not shown to be effective on *R. (B.) microplus* in the present study. Derivatives devoid of the peroxide bond, such as deoxyartemisinin, are considered completely inactive on parasites. Thus, the fundamental group that confers activity to artemisinin, and even to synthetic substances, is lead peroxide (KLAYMAN, 1985; MESHNICK et al., 1996). Endoperoxides are classified as blood schizonticidal agents in the case of *Plasmodium* spp. control in malaria (TARANTO et al, 2006).

The mode of action of artemisinin in gastrointestinal nematodes, trematodes and monogeneans has been extensively investigated (KEISER et al., 2006, 2008; EKANEM; BRISIBE, 2010). Such

studies may explain how artemisinin can be applied to control other parasites of veterinary importance, such as *R. (B.) microplus*. Artemisinin is thought to exert its effect by reacting with the heme groups of the hemoglobin molecules digested by parasites, altering the cell structure and its functions through the free radicals derived from artemisinin, and thus affect the growth and reproduction of parasites (WRIGHT; WARHURST, 2002 *apud* FERREIRA; GONZALEZ, 2008). In this manner, it can be claimed that the action of artemisinin occurs in two steps: first, there is the generation of free radical; and then, alkylation of specific proteins that are essential for parasite survival (SILVA, 2006). Indeed, such generation of free radicals depends on iron ions present in the heme group, and this is the major step of the mechanism of action, since the formation of a complex between the heme group and artemisinin causes disruption of the endoperoxide bridge by means of catalytic reduction (LA-SCALEA et al., 2007). However, in addition to the feeding mechanism, which enables breakage of the peroxide bridge, other mechanisms may also be involved, since artemisinin has been shown to have action against the non-hematophagous parasite *Echinostoma caproni* in mice (KEISER; UTZINGER, 2007).

Thus, if the mode of action of artemisinin on the parasite is indirect, i.e. impairing its reproduction, for example, its efficacy on engorged *R. (B.) microplus* would be conditional on its intake through the blood. The same has been said for gastrointestinal nematodes in small ruminants (FERREIRA et al., 2006). If ingestion is the process that activates the main mechanism of action of artemisinin in *R. (B.) microplus*, *in vitro* methods would be inadequate for effective evaluation of *A. annua* in this parasite. Thus, we now intend to conduct *in vivo* tests at CPPSE, in which cattle may receive *A. annua* in food, in order to elucidate this hypothesis.

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