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Lack of impact of dietary inclusion of dried *Artemisia annua* leaves for cattle on infestation by *Rhipicephalus (Boophilus) microplus* ticks

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ABSTRACT

The present study evaluated whether a natural dietary additive, dried *Artemisia annua* leaves, may be useful to control *Rhipicephalus (Boophilus) microplus* on naturally infested cattle. Twenty heifers of the Canchim breed, weighing around 250 kg, were divided into two equally sized experimental groups: 1) control animals and 2) animals receiving 200 g/day of dried *A. annua* leaves for two months. Before treatment began, the animals were homogeneously distributed in control and treatment groups based on their pre-treatment weight and tick infestation level. Counts of engorged female ticks then occurred weekly during the two-month experimental period. We also monitored cattle weight gain and packed cell volume (PCV). Artemisinin (0.96%) was quantified in the plant material by high-performance liquid chromatography with refractive index detector (HPLC-IR). No statistical differences between the control and treatment groups were observed for engorged female counts (log averages of 1.3 ticks and 1.4 ticks per animal, respectively), daily cattle weight gain (0.910 kg and 0.888 kg, respectively) or PCV (33.5% and 33.0%, respectively). We conclude that the oral supplementation of cattle feed with dried *A. annuna* leaves did not control natural infestation of *R. (B.) microplus*. The hypothesis of artemisinin's action on cattle ticks by ingestion through the animals' blood was not confirmed at the evaluated dose.

1. Introduction

The tick *Rhipicephalus (Boophilus) microplus* is widely distributed in cattle-producing countries and causes huge economic impact, estimated at approximately $ 3.24 billion/year in Brazil alone (Grisi et al., 2014). Among cattle ectoparasites, *R. (B.) microplus* is considered the most important disseminator of disease agents, since it can transmit the protozoans *Babesia bovis* and *B. bigemina* and the rickettsial bacterium *Anaplasma marginale*. Severe infestation with *R. (B.) microplus* is controlled by using commercially available chemicals. However, these acaricides are often administered with incorrect frequency and concentration, causing animal toxicity and environmental pollution as well as selection pressure for ectoparasite resistance (Soares et al., 2009). The use of plant extracts to control ticks would bring positive effects such as lower cost, faster degradation in the environment and lower residue levels (Chagas, 2015).

In recent years, several natural dietary additives based on botanical elements have been studied as sustainable alternatives for parasite control, such as *Azadirachta indica*, *Sophora flavescens* and *Artemisia annua* (Youn and Noh, 2001; Allen and Fetterer, 2002; Tipu et al., 2002; Brisibe et al., 2008; Charlie-Silva et al., 2017). *Artemisia annua*, which belongs to the family Asteraceae, contains a sesquiterpene lactone endoperoxide called artemisinin that has been traditionally used for the treatment of malaria (Nascimento et al., 2012). Studies show that artemisinin also has antileishmanial and antitumor activities. Wang et al. (2017) suggest that artemisinin inhibits the expression of the NF-κB reporter gene induced by TNF-α in a dose-dependent manner, inhibiting skin inflammatory responses and hence acting as a therapeutic agent for inflammatory-related diseases. Studies have reported different biological effects not only for artemisinin, but also for its derivatives dihydroartemisinin (DHA), artesunate (ART) and artemether (ARTE). These substances have antifungal (Santomauro et al., 2016), antibacterial (Tajehmiri et al., 2014) and anti-HIV properties (Van der Kooy, 2014). The potential anticancer action has been investigated as well (Tajehmiri et al., 2014; Weathers et al., 2014; Humphreys et al., 2016; Ko et al., 2016).

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Brisibe et al. (2008) investigated dried *A. annua* leaves as a botanical coccidiostat in coccidia-infested chickens. The inclusion of *A. annua* in the diet inhibited the growth of bird parasites similar to the effect of commercial anticoccidial therapy. In addition, there was higher food intake, which resulted in higher weight gain in broilers. Recently, Charlie-Silva et al. (2017) also supplemented sheep with dried *A. annua* leaves and observed a moderate anthelmintic effect against the gastrointestinal nematode *Haemonchus contortus*. This effect was also attributed to artemisinin and it is believed that this compound can be useful as an alternative in the treatment of various diseases (Ferreira et al., 2011; Sprenger et al., 2015). The present study aimed to investigate whether addition of dried *A. annua* leaves to cattle feed may control infestations by cattle ticks.

2. Materials and methods

2.1. Plant material collection

Artemisia annua variety CPQBA-UNICAMP was cultivated in Paulinia, São Paulo state, Brazil (Lat. 22°48′02.38"S, Long. 47°06′43.10"W; altitude 612 m). The plants were spray irrigated and weeds were controlled manually. The soil in the experimental area was classified as typical clayey eutroferric red latosol. The seedlings were formed in sleeves under screening with 50% interception and cultivated in the field for approximately four months, with a spacing of 0.6 × 1.0 m. Harvesting was done in the stage preceding flowering and only the top third of the plant was collected, resulting in an artemisinin content between 1.0 and 1.1%. The leaves were dried for 48 h at 40 °C with air circulation in a greenhouse with periodic turning, followed by manual separation of the stalks and thick twigs. The final raw material consisted only of leaves, which were ground into powder.

2.2. Quantification of artemisinin by HPLC-RI

Chromatographic analysis was performed by high-performance liquid chromatography with a refractive index detector (HPLC/RI) according to a previously described protocol (Celeghini et al., 2009), with a modular Waters system comprised of a Waters 515 pump, a column oven, a Waters 2414 refraction index detector and an LC-CN column (4.6 × 250 mm, 5 µm particle size, Luna Phenomenex, Macclesfield, UK). Separations were carried out in the isocratic mode, using methanol: water (60:40 v/v) mL/min at a flow rate of 1 mL/min with 20 µL injection volume. The detector and column temperature was 35 °C. Dried powder of *A. annua* leaves (250 mg) was subjected to extraction using an Ultra Turrax device for 2 min, at 6000 rpm at room temperature (25 °C) with three portions of 5 mL of methanol, following clean-up procedures (Celeghini et al., 2006).

Artemisinin was quantified by an analytical curve. Stock solutions (2491 µg/mL) of analytical grade artemisinin (Sigma-Aldrich, St. Louis, MO, USA) were prepared in methanol and successively diluted in the range of 50–1250 µg/mL, with three replicates, at retention time of 7.0 min. All samples were analyzed by HPLC as described above. A graph correlating area under the curve (AUC) with the respective concentration was plotted and analyzed by linear regression using the Empower software (Waters).

2.3. Artemisia annua evaluation in vivo

All animal procedures were approved by the Embrapa Pecuária Sudeste Ethics and Animal Experimentation Committee (Protocol 06/2012). This *in vivo* study was performed at the experimental farm of Embrapa Pecuária Sudeste (CPPSE), which is located in the city of São Carlos, São Paulo state. Climatic data (temperature, relative humidity and rainfall) were obtained from the meteorological station of the CPPSE experimental farm. Twenty heifers of the Canchim breed (3/8 zebu x 5/8 Charolais), with body weights of approximately 250 kg and naturally infested by *R. (B.) microplus* ticks, were pre-selected. The animals remained without acaricidal treatment for 40 d before the experiment and then were kept in separate *Panicum maximum* grass pastures sub-divided into 24 pens managed in a rotated system every 3 d (October–December 2011).

The heifers were homogeneously distributed between the two groups (A and B) according to their average weight and the number of engorged females with body length between 4.5 and 8 mm, present on the left side of each animal in the three days prior to treatment (−3, −2, −1) (Wharton and Utech, 1970). In group A (control), the animals were fed a diet composed of 100 g of soybean meal and 300 g of cornmeal per animal in a collective feeder, with water and mineral salt provided *ad libitum*. In group B (treated), heifers received 200 g of dried *A. annua* leaves, 100 g of soybean meal and 300 g of cornmeal per animal in a collective trough, with water and mineral salt provided *ad libitum*. For adaptation, in the three days prior to the experiment, the animals from the treated group ingested 100 g per animal of *A. annua*, then received 200 g during the 60 d of the experiment. Tick counts were performed on the left side of each animal on days 1, 3, 7, 14, 21, 28, 35, 42, 49 and, 56 post-treatment.

Additional parameters were used to monitor the health of the animals during the trial. On days −3, 28 and 56 post-treatment, each animal was weighed and a blood sample was drawn from the jugular vein in a BD Vacutainer® tube containing the anticoagulant EDTA, to determine the packed cell volume (PCV).

Tick count values were transformed into $\log_{10}(n + 1)$ and subjected to analysis of variance in a factorial scheme subdivided in time. For the comparison between means, the *t*-test was adopted at the significance level of 5% ($p \leq 0.05$). Weight and PCV values were analyzed by a split-plot design, with subunits in time.

3. Results

In the quantification of artemisinin, the curve's equation was Y = 18e + 002 X + 8.87e + 003, the correlation coefficient $R2 = 0.999400 \pm 0.0005$, the detection limit (LOD) = 2.2 µg/mL and, the quantification limit (LOQ) = 7.0 µg/mL. The phytochemical analysis showed the presence of artemisinin in a concentration of 0.96% ± 0.010 or 483.87 µg/mL. Therefore, each 100 g of dried plant material contained 0.96 g of artemisinin. As the heifers were offered 200 g of *A. annua* daily, it can be estimated that they ingested approximately 1.9 g of artemisinin per day.

Table 1 reports the averages and standard errors of tick counts (log data were used to run the statistical analysis) on the animals during the experiment. In the statistical analysis, no significant difference was observed between the control and treatment groups for counts of engorged female ticks on the left side of the animals. However, differences were seen among days during the experimental period. Overall, the log-transformed averages were 1.3 ticks per animal in the control group and 1.4 ticks per animal in the treated group.

The weight gain during the experimental period is shown in Fig. 1. No statistical difference was detected between control and treatment groups at $p < 0.05$. The daily weight gain was 0.910 kg/day in the control group and 0.888 kg/day in the treated group. Likewise, no difference ($p > 0.05$) was detected in the PCV values of the control animals compared to the treated animals, with averages of 35.3% and 34.5%, respectively.

4. Discussion

Medicinal plants have a long history as important sources of substances with different chemical structures that can have deleterious activities against parasites. In the present study, the oral administration of *A. annua* in cattle infested naturally with ticks was evaluated. The quantification of artemisinin was performed by HPLC/IR and demonstrated a high concentration (1.9/200 g). This meant that 100 g of dried