

Full Length Research Paper

Anthelmintic activity of *Artemisia annua* in sheep-model

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Artemisia annua L. is a well-known source of artemisinin, an antimalarial drug. This compound has been used in traditional medicine to treat malaria for many years. The anthelmintic property of *A. annua* has also been attributed to artemisinin, hence the belief that artemisinin could be useful as an alternative anthelmintic to control *Haemonchus contortus* in livestock. The present study focused on evaluating the anthelmintic activity of *A. annua* against *H. contortus* by oral administration on infected sheep in a controlled clinical trial. The trial was conducted using 24 male sheep of the Santa Inês breed, with average weight of 20 kg, artificially infected with *H. contortus* (Embrapa 2010 isolate). The animals were kept in metabolic cages and randomly divided into four homogeneous groups with six animals each, being: (T1) negative control (untreated), (T2) positive control: 10 mg/kg BW of levamisole phosphate, (T3) fed 10% *A. annua* (0.2% BW), (T4) fed 20% *A. annua* (0.4% BW). EPG counts were conducted on days -3, -2, -1, 0 (treatment day), 3, 7, 10, 14, 17, 21, 24 and 28 post-treatment. After extraction of the artemisinin in plant material, it was quantified in triplicate and analyzed by high-performance liquid chromatography with infrared detection. The average efficacy in the positive control treated with levamisole was 89%. Moderate anthelmintic efficacy against *H. contortus* was observed in the groups fed 0.2 and 0.4% BW *A. annua* for 30 days (32.9 and 14.8%, respectively), mainly from day 21 post treatment (47.1 and 25.2%, respectively). At the highest dose, the animals avoided eating due to the compound's bitter taste. It is concluded that *A. annua* presents moderate anthelmintic activity in sheep in both doses. The lowest dose was accepted by animals and seems to have practical use. Oral administration was safe and after further *in vivo* trials could be introduced in organic farms in tropical countries.

Key words: Artemisinin, sesquiterpenoids, gastrointestinal nematodes, control, veterinary ethnopharmacology.

INTRODUCTION

Parasitism by gastrointestinal nematodes is one of the major problems of small ruminants, being responsible for high mortality. Among all nematode species, *Haemonchus contortus* is the main culprit in tropical countries. In Brazil, this blood-sucking abomasal parasite is responsible for large losses to sheep breeders. Small ruminant infections are controlled by using the commercially available chemicals. However, there are various reports of multidrug-resistant in small ruminant nematodes throughout the world (Veríssimo et al., 2012; Chagas et al., 2013; García et al., 2016; Keegan et al., 2016). Unfortunately, the strong selection pressure that has been imposed associated with incorrect frequency and doses are responsible for the anthelmintic inefficacy of the available chemical groups (Soares et al., 2009).

Therefore, other approaches to control gastrointestinal nematodes in small ruminants have been studied. These include *refugia* and targeted selective treatment (Van Wyk and Reynecke, 2011), as well as the evaluation of plants' biological effects for use in phytotherapy (Chagas, 2015). *Artemisia annua* L., Asteraceae family, has been studied because it is a well-known source of the antimalarial drug artemisinin, which has been used in folk medicine for the treatment of malaria for many years (Weathers et al., 2014; Ruan et al., 2016). Studies have reported different biological effects of artemisinin as well as for its derivatives dihydroartemisinin (DHA), artesunate (ART) and artemether (ARTE). These substances have presented anti-inflammatory (Salminen et al., 2008; Stebbings et al., 2016), antioxidant (Gupta et al., 2016), antifungal (Santomauro et al., 2016), antibacterial (Tajehmiri et al., 2014) and anti-HIV (Lubbe et al., 2012) activities. 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).

The anthelmintic property of *A. annua* has been attributed to artemisinin and it is believed that this compound can be useful as an alternative to control *H. contortus* in livestock (Ferreira et al., 2011; Sprenger et al., 2015). Studies of the *Artemisia* genus have shown negative and positive results. Different species have been evaluated against gastrointestinal nematodes *in vivo*: crude ethanol extracts of *A. annua*, *Artemisia absinthium* and their essential oils in gerbils artificially infected with *Haemonchus contortus* (Squires et al., 2011); *Artemisia brevifolia* aqueous extract in sheep naturally infected with mixed gastrointestinal nematodes (Iqbal et al., 2004); *A. absinthium* leaves in alfalfa pellet diet against *H. contortus* in lambs (Valderrábano et al.,

2010); and ethanol extracts of *A. absinthium* in gastrointestinal nematodes of sheep (Tariq et al., 2009).

Interestingly, neither *A. annua* extracts nor artemisinin have been tested in sheep infected with gastrointestinal nematodes. Therefore, in a previous trial by our research group, naturally infected sheep treated with *A. annua* sodium bicarbonate extract (aqueous 0.1% sodium bicarbonate) in a single dose of 2 g/kg BW in 20 mL of soybean oil had non-significant EPG reduction (19%). However, the extract tested *in vivo* contained no artemisinin, but had high antioxidant capacity (Cala et al., 2014). Lack of artemisinin was expected due to the affinity of this substance to ethanolic solvents instead of aqueous. Authors indicated that the anthelmintic activity of the extract, previously detected *in vitro*, was not due to the presence or lack of artemisinin but, may be due to the high content of antioxidant compounds (e.g., flavonoids) measured by the oxygen radical absorbance capacity (ORAC). So, the objective of this study was to establish a controlled clinical trial to investigate the anthelmintic activity of *A. annua*, rich in artemisinin this time, by oral administration in sheep artificially infected with *H. contortus* as shown in graphical abstract.

MATERIALS AND METHODS

Plant material collection

A. annua variety CPQBA-UNICAMP was cultivated in Paulínia, 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 the weeds were controlled manually until the disappearance of the lines between the plants due to their development. The soil in the experimental area was classified as typical clayey eutroferic red latosol. The seedlings were formed in sleeves under screening with 50% interception and cultivated in the field for approximately 4 months from October, with spacing of 0.6 x 1.0 m. Harvesting was done in the state preceding flowering and only the top third of the plant was harvested, resulting in artemisinin content between 1.0 and 1.1%. The leaves were sun-dried with periodic turning, followed by manual separation of the stalks and thick twigs. Thus, the final raw material consisted only of leaves, which were ground into powder.

Quantification of artemisinin by HPLC-RI

Chromatographic analysis was done by high-performance liquid chromatography with a refractive index detector (HPLC/RI) according to a previously described protocol (Celeghini et al., 2009). It was performed with a modular Waters system comprised of a Waters 515 pump, a column oven, a Waters 2414 refraction index detector and an using a LC-CN column (4.6 x 250 mm, 5 µm particle size, Luna Phenomenex, Macclesfield, UK). Separations were made in the isocratic mode, using methanol: water (60:40v/v)

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Table 1. Quantification of artemisinin (ART) from *A. annua* per 100 g of dry weight analyzed by HPLC-RI.

Sample	Weight (mg)	Volume (mL)	Concentration ($\mu\text{g/mL}$)	(%) w/w	Mean	Standard deviation	CV (%)
Sample-A	250.0	5.0	487,841	0.980			
Sample-B	251.0	5.0	481,932	0.960	0.96	0.010	1.03
Sample-C	251.6	5.0	481,649	0.960			

mL/min at a flow rate of 1 mL/min with 20 μL injection volume. The detector and column temperature was 35°C. *A. annua* (250 mg) was extracted using Ultra Turrax mixture for 2 min, 6000 rpm at room temperature (25°C) with three portions of 5 mL methanol, following clean-up procedures (Celeghini et al., 2006).

Artemisinin was quantified by analytical curve. The artemisinin analytical standard (Sigma-Aldrich, Sant Louis - USA) stock solutions (2491 $\mu\text{g/mL}$) were prepared in methanol and successively diluted in the range of 50 to 1250 $\mu\text{g/mL}$, three replicates each in methanol, retention time 7.0 min. All samples were analyzed by HPLC as described above. A graphic correlating area under the curve (AUC) with the respective concentration was plotted and analyzed by linear regression using Empower software (Waters).

Infection of animals

Before the experiment, 24 males of the Santa Inês breed, approximately four months old and with mean live body weight (BW) of 20 kg, were kept indoors in collective stalls, where they were fed with corn silage, concentrated feed, mineral salt and water *ad libitum*. To remove the natural infection by nematodes, the animals received levamisole phosphate (Ripercol® 150F, Fort Dodge, Brazil) at a dose of 10 mg/kg BW (body weight) each 24 h for three consecutive days and albendazole (Valbazen®, Pfizer, Brazil) in a single dose of 10 mg/kg BW on the last day.

After treatment, fecal samples were collected directly from the rectum of each animal to confirm their worm-free status. Then, the sheep were artificially infected with approximately 4,000 larvae of the *H. contortus* isolate Embrapa2010, characterized as resistant to benzimidazole, macrocyclic lactones and imidazothiazoles (Chagas et al., 2013).

The experimental protocols were approved by the Embrapa Pecuária Sudeste (CPPSE - Southeast Livestock Unit of the Brazilian Agricultural Research Corporation) Animal Care and Use Committee. Animals were under the care of a veterinary assistant during the entire experiment.

A. annua evaluation *in vivo*

This *in vivo* study was performed at CPPSE, which is located in the city of São Carlos, São Paulo state. Twenty-eight days after artificial infection, the sheep were weighed and eggs per gram of feces (EPG) were recorded for each animal on days -3, -2 and -1 (Ueno and Gonçalves, 1998). Lambs were allocated into homogeneous experimental groups based on their weight and mean EPG (of the three days). All the rams were placed in metabolic cages to receive their respective treatments (n=6): T1: negative control (untreated), T2: positive control (10 mg/kg BW levamisole phosphate), T3: fed 10% *A. annua* (0.2% BW), T4: fed 20% *A. annua* (0.4% BW). The T3 and T4 animals were given five days to adjust to the diet (1%

day 1, 5% day 2, 5% day 3, 10% day 4, 10% day 5) and then received *A. annua* for 30 consecutive days. Samples of feces were collected individually directly from the rectum to count EPG on days 0, 3, 7, 10, 14, 17, 21, 24 and 28 post-treatment.

Data analysis

The results of EPG were natural log-transformed (\ln^{-1}) and analyzed by a split-plot design, where α_i , β_j and $(\alpha\beta)_{ij}$ represent the whole plot and ω_k , and δ_{jk} represent the split-plot. The sums of squares for the factors were computed for three-way analysis of variance without replication employing the mixed models method by the *T*-test at 95% confidence, using the R program (R Core Team, 2016). The mean EPG values obtained were used to calculate the efficacy against *H. contortus* as follows: % efficacy = mean control – mean treated/mean control x 100.

RESULTS

Phytochemical analysis

In the quantification of artemisinin, the equation curve was $Y = 1.17e+003 X + 4.49e+003$, the correlation coefficient $R^2 = 0.9998 \pm 0.0005$, the detection limit (LOD) = 2.2 $\mu\text{g/mL}$ and, the quantification limit (LOQ) = 7.5 $\mu\text{g/mL}$. The phytochemical analysis showed the presence of artemisinin in a concentration of 0.96% \pm 0.010 or 483.87 $\mu\text{g/mL}$ (Table 1). Each 100 g of dried plant material contained 0.96 g of artemisinin. As each sheep received approximately 40 (0.2% BW) and 80 g (0.4% BW) of *A. annua* per day, it can be estimated that they ingested approximately 384 and 768 mg of artemisinin/day, respectively. The quantification showed high concentration of artemisinin (960 mg/100 g).

In vivo trial

The infections were monitored through the experiment and compared with the positive and negative control as extremes. On day zero, the average EPG counts (non-transformed data) for all treatments were as follows: positive control (T1 = 1625), negative control (T2 = 1625), *A. annua* 0.2% BW (T3 = 1908) and *A. annua* 0.4% BW (T4 = 2083) (Figure 1).

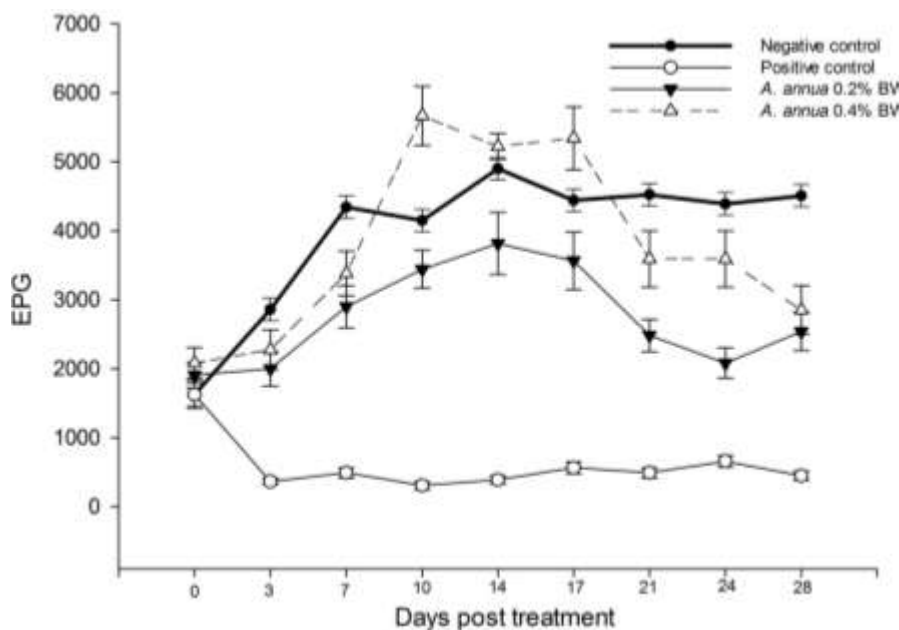


Figure 1. Non-transformed mean EPG in Santa Inês sheep artificially infected with *H. contortus*, in four groups: negative control, positive control, fed with 0.2 and 0.4% BW of *A. annua*.

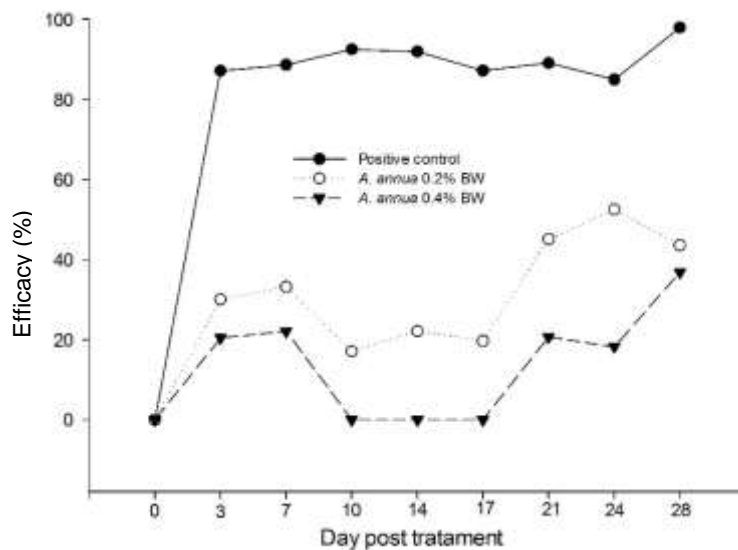
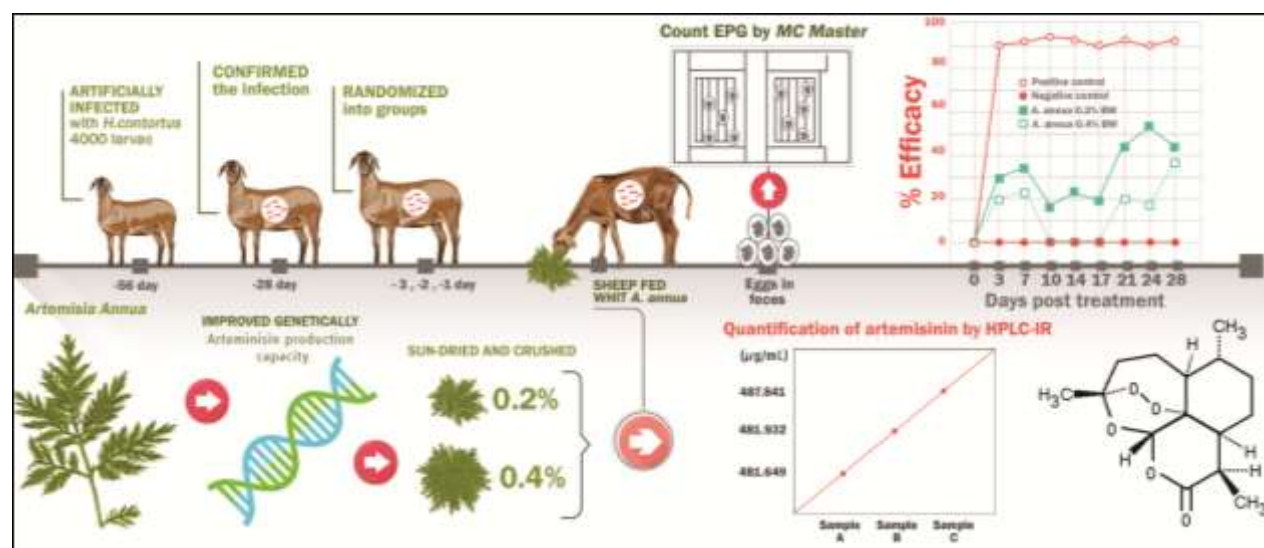


Figure 2. Mean efficacy (%) of the reduction of EPG in Santa Inês sheep artificially infected with *H. contortus*, for all treatments: positive control, fed with 0.2% and 0.4% BW of *A. annua*.

In the negative control, constant EPG from day 7 to 28 post treatment was observed, whereas in the positive control group, levamisole phosphate presented high efficacy (89.0% average) from day 3 (Figure 2). Moderate anthelmintic efficacy against *H. contortus* was observed

in the groups fed 0.2 and 0.4% BW of *A. annua* for 30 days (32.9 and 14.8%, respectively), mainly from day 21 after treatment (47.1 and 25.2%, respectively) (Figure 2). Regarding data analysis of the log-transformed EPG values, no significant EPG reduction for both oral doses



Graphical Abstract

of *A. annua* was observed. However, treatment with levamisole statistically differed in all days (from days 3 to 28) of the negative control and treatments. Toxic reactions were not observed in any animal that ingested *A. annua* daily in both concentrations, although at the highest dose animals did not eat all the plant material because of the bitter taste.

DISCUSSION

Knowledge on physicochemical characteristics of plants has contributed to advances in biotech research, leading to the breeding of commercially important plants. In this study, the oral administration of *A. annua* in infected sheep was evaluated. The quantification of artemisinin was performed by HPLC/IR and artemisinin was detected in a mean concentration of 0.96% or 483.87 $\mu\text{g/mL}$. This denoted that 100 g of dried plant material had 960 mg of artemisinin. Lapkin et al. (2014) investigated the variability of metabolic profiles of *A. annua* grown in different geographical regions and observed differences. According to Charles et al. (1990), there is variation from 0.003 to 0.39% in artemisinin content in leaves of *A. annua* worldwide. The findings in the present study can be explained by *A. annua* genotype used (CPQBA/UNICAMP), produced by genetic breeding and adapted to Brazilian climatic conditions. This content is not normally found.

In the present study, the animals that ate *A. annua* at 0.2 and 0.4% BW received daily 384 and 768 mg of artemisinin, respectively. However, it is noticed that at the highest dose, the plant material was not totally ingested due to the bitter taste, explaining the worse response

than at the lower dose. Adverse reactions were not observed in any animal that ate *A. annua* daily at both concentrations. Keiser et al. (2008) evaluated semi-synthetic artemisinin at dosages of 40 and 80 mg/kg orally and at 40, 80 and 160 mg/kg intramuscularly in sheep. They reported that even the highest dose of 160 mg/kg was well tolerated. In the present study, at least at the lower dose, which was totally ingested by the animals, this plant species and artemisinin content (384 mg) were safe. This is the first report of daily administration of *A. annua* against *Haemonchus* infection.

A. annua produces a wealth of monoterpenes and sesquiterpenes, including the well-known sesquiterpene lactone artemisinin (Ruan et al., 2016). Artemisinin is pointed out as the main compound responsible for the anthelmintic activity of *A. annua*. The mechanisms of action attributed to this metabolite include interference with parasite transport proteins, disruption of parasite mitochondrial function, modulation of host immune function and inhibition of angiogenesis (Golenser et al., 2006). In the present study, at the half dose, the average efficacy levels were 32.9% and 47.1% for 21 days after treatment. Different studies have already proved the anthelmintic effect in sheep with plants of the genus *Artemisia* (family Asteraceae). The crude ethanol extract of *A. absinthium* demonstrated EPG reductions of 90.46% in sheep at 2.0 g/kg BW and 82.85% at 1.0 g/kg BW, both on day 15 post treatment (Tariq et al., 2009). Maximum reduction (67.2%) in EPG was recorded on day 14 post treatment in sheep with mixed gastrointestinal nematode infection treated with *A. brevifolia* crude aqueous extract at 3 g/kg BW (Iqbal et al., 2004). Cala et al. (2014) evaluated *A. annua* sodium bicarbonate extract

(2 g/kg BW) and artemisinin (100 mg/kg BW) in naturally infected sheep. Artemisinin-treated and extract-treated sheep had non-significant EPG reductions of 28 and 19%, respectively, but this extract had no artemisinin. Irum et al. (2015) evaluated *Artemisia vestita* and *Artemisia maritima* methanol extracts on *H. contortus* in artificially infected sheep. The EPG reductions were 87.2 and 84.5%, respectively, at 50 mg/kg, 4 weeks after treatment.

The differences found among those studies are related to plant species, artemisinin content and administration form (extract, plant material) and the relationship with bioavailability and absorption. Studies have shown that artemisinin has poor bioavailability in small ruminants. Ferreira and Gonzalez (2008) studied the stability in bovine rumen fluid and kinetics in goats. Artemisinin recovery rates from rumen culture ranged from 67 to 92% at pH 6.8 and were 95% at pH 3.0. The kinetics data showed that artemisinin was metabolized to dihydroartemisinin by goats, while unabsorbed artemisinin was eliminated in feces. Dihydroartemisinin peaked in the blood (0.7 µg/mL) at 12 h, and decreased to 0.18 µg/mL at 24 h. After 24 h, artemisinin concentration in feces was 2.4 µg/g, indicating artemisinin's poor bioavailability in goats when provided orally and as capsules. Posner et al. (2004) observed high time-dependent first-pass metabolism in the gut and liver, this drug are conjugates such as glucuronides and can be eliminated through phase-II metabolism. Therefore, artemisinin has low water solubility, resulting in poor and erratic absorption upon oral administration, so the artemisinin conjugation reactions and hydrolysis are the principal reasons these derivatives have a short half-life. The authors decided to evaluate the dry plant mixed in the food because this seems to be the most practical way for future use of this technology by organic farmers, since the oral administration of artemisinin did not present good results in other studies.

There is a need to develop new approaches to find potential anthelmintic plants that can be used to formulate commercial products. Natural compounds can also provide more structural diversity than synthetic anthelmintic drugs, to reduce the occurrence of resistance (Irum et al., 2015). In light of these aspects, the results reported here allow us to infer that *A. annua*, administered orally presented moderate anthelmintic activity in sheep at both doses. The lowest dose was accepted by animals and seems to have practical use. The oral administration was safe and therefore the use in organic farms in tropical countries would be a good option after been more accurate *in vivo* trials.

Conflict of Interests

The authors have not declared any conflict of interests.

ACKNOWLEDGEMENT

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