Parasitol Res (2014) 113:2345–2353
DOI 10.1007/s00436-014-3891-z

ORIGINAL PAPER

Anthelmintic activity of *Artemisia annua* L. extracts in vitro and the effect of an aqueous extract and artemisinin in sheep naturally infected with gastrointestinal nematodes

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Received: 8 August 2013 / Accepted: 9 April 2014 / Published online: 7 May 2014

Abstract There is no effective natural alternative control for gastrointestinal nematodes (GIN) of small ruminants, with *Haemonchus contortus* being the most economically important GIN. Despite frequent reports of multidrug-resistant GIN, there is no new commercial anthelmintic to substitute failing ones. Although trematocidal activity of artemisinin analogs has been reported in sheep, neither artemisinin nor its plant source (*Artemisia annua*) has been evaluated for anthelmintic activity in ruminants. This study evaluated the anthelmintic activity of *A. annua* crude extracts in vitro and compared the most effective extract with artemisinin in sheep naturally infected with *H. contortus*. *A. annua* leaves extracted with water, aqueous 0.1 % sodium bicarbonate, dichloromethane, and ethanol were evaluated in vitro by the egg hatch test (EHT) and with the bicarbonate extract only for the larval development test (LDT) using *H. contortus*. The *A. annua* water, sodium bicarbonate (SBE), ethanol, and dichloromethane extracts tested in vitro contained 0.3, 0.6, 4.4, and 9.8 % of artemisinin, respectively. The sodium bicarbonate extract resulted in the lowest LC_{99} in the EHT (1.27 μg/mL) and in a LC_{99} of 23.8 μg/mL in the LDT. Following in vitro results, the SBE (2 g/kg body weight (BW)) and artemisinin (100 mg/kg BW) were evaluated as a single oral dose in naturally infected Santa Inês sheep. Speciation from stool cultures established that 84–91 % of GIN were *H. contortus*, 8.4–15.6 % were *Trichostrongylus* sp., and 0.3–0.7 % were *Oesophagostomum* sp. Packed-cell volume and eggs per gram (EPG) of feces were used to test treatment efficacy. The SBE tested in vivo contained no artemisinin, but had a high antioxidant capacity of 2,295 μmol of Trolox equivalents/g. Sheep dosed with artemisinin had maximum feces concentrations 24 h after treatment (126.5 μg/g artemisinin), which sharply decreased at 36 h. By day 15, only levamisole-treated sheep had a significant decrease of 97 % in EPG. Artemisinin-treated and SBE-treated sheep had nonsignificant EPG reductions of 28 and 19 %, respectively, while sheep in infected/untreated group had an average EPG increase of 95 %. Sheep treated with artemisinin and *A. annua* SBE maintained blood hematocrits throughout the experiment, while untreated/infected controls had a significant reduction in hematocrit. This is the first time oral dose of artemisinin and an aqueous extract of *A. annua* are evaluated as anthelmintic in sheep. Although oral dose of artemisinin and SBE, at single doses, were ineffective natural anthelmintics, artemisinin analogs with better bioavailability than artemisinin should be tested in vivo, through different routes and in multiple doses. The maintenance of hematocrit provided by artemisinin and *A. annua* extract and the high antioxidant capacity of the latter suggest

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that they could be combined with commercial anthelmintics to improve the well-being of infected animals and to evaluate potential synergism.

Keywords Anthelmintic plants · Phytotherapy · Crude plant extracts · Metabolism · *Haemonchus contortus*

Introduction

Heavy reliance on drenching, farmer resistance to new technologies, few extension agents to teach FAMACHA, and increasing multidrug-resistant ruminant nematodes worldwide are some of the challenges in the control of gastrointestinal nematodes (GIN) in small ruminant systems (van Wyk et al. 1997; van Wyk and Reynecke 2011). Also, the lack of new anthelmintics, the high cost of existent ones, and the possibility of their chemical residues in animal products demand alternative, feasible, and environmentally innocuous control methods that meet consumers' demands and fit the needs of farmers (Chagas et al. 2007, 2008; Ademola and Eloff 2010).

Plants of the genus *Artemisia* (family Asteraceae) are rich sources of sesquiterpenes and other bioactive secondary metabolites, and some species have been used as alternative anthelmintics in livestock (Ferreira 2009; Rahmann and Seip 2007; Valderrábano et al. 2010). *Artemisia annua* produces over 3 tons of dried leaves/ha and grows in a variety of soils and climates. The leaves are the commercial source of artemisinin used to manufacture drugs, such as artemether, arteether, and artesunate, which are effective against chloroquine-resistant malaria (Bhakuni et al. 2001; Ferreira et al. 2005). The leaves are high in crude protein, potassium, and bioactive metabolites including antioxidants (Brisibe et al. 2009; Ferreira et al. 2010). When fed to *Haemonchus*-infected goats at one third of their daily diet for 5 days, leaves had no toxicity and had also no effect against *Haemonchus* (Hart et al. 2008).

In vitro, artemether (at 10–30 μg/mL) caused severe tegument lesions in adult *Fasciola gigantica* 24 h after treatment. Artemether effect on tegument was comparable to triclabendazole, and both affected immune modulation, osmoregulation, and nutrient absorption of *F. gigantica* (Shalaby et al. 2009). In sheep infected with *Fasciola hepatica*, a single dose of 40 mg/kg (i.v.) artesunate reduced egg count and worm burden in 69 and 77 %, respectively, while artemether (i.m.) at 40 mg/kg reduced fecal egg count (eggs per gram, EPG) and worm burden by 97.6 and 91.9 %, respectively (Keiser et al. 2010).

Artemisinin had good stability in buffered rumen fluid and was detected in the blood and feces of goats dosed with 33 mg artemisinin/kg body weight (BW) (Ferreira and Gonzalez 2008). However, neither artemisinin (200 mg/kg BW for 5 days or 400 mg/kg BW single dose), the crude ethanolic extracts of *A. annua*, *Artemisia absinthium* (both at 600 mg/kg BW), nor their essential oils had any anthelmintic activity in gerbils (*Meriones unguiculatus*) artificially infected with *Haemonchus contortus* (Squires et al. 2011).

Artemisia brevifolia aqueous extracts at 3.0 g/kg BW reduced fecal EPG by 67 % in sheep naturally infected with mixed GIN 14 days post infection (Iqbal et al. 2004), while *A. absinthium* leaves, included as 20 % in alfalfa pellet diet throughout infection and treatment periods, significantly reduced worm burden by 49 % and EPG by 73 % (Valderrábano et al. 2010). Also, ethanolic extracts of *A. absinthium* at 1.0 and 2.0 g/kg BW (both single doses) significantly reduced EPG by 82.8 and 90.46 %, respectively, in sheep 15 days after treatment (Tariq et al. 2009). However, neither *A. annua* extracts nor artemisinin has been tested in sheep infected with GIN. The aims of this study were to evaluate the effect of *A. annua* crude extracts in vitro and to compare the most effective extract from the in vitro study with artemisinin, both in single oral doses, in Santa Inês sheep naturally infected with GIN.

Material and methods

Experimental site and climatic conditions

The experiments were performed at the Southeast Livestock Research Center (Pecuária Sudeste) of the Brazilian Agricultural Research Corporation (Embrapa), which is located in the city of São Carlos, São Paulo State. During the in vivo experiment, according to the meteorological station of the research unit, the average temperature was 20.7 °C, the relative humidity was 75 %, and the average precipitation was 718.5 mm/month.

Preparation of *A. annua* extracts

Crude A. annua extract preparation

A. annua extracts were prepared, as described below, by the Centro Pluridisciplinar de Pesquisas Químicas, Biológicas e Agrícolas at the State University of Campinas (Unicamp), Sao Paulo, Brazil, for in vitro testing, and by the Embrapa Southeast Livestock research unit (CPPSE), for test in naturally infected sheep.

Plants were harvested in March 2008, sun-dried in the field for 4 h, and then dried to a constant weight in a gas-powered drier set at 35 °C for 5 days. Four extracts were produced using different solvents in a nonsequential extraction, according to Simões et al. (2004). In all procedures, the ratio of leaf dry matter to solvent was 1:20 and extracted as follows: (1) aqueous extract: water at 90 °C added to ground *A. annua* leaves, cooled to room temperature (RT), filtered, frozen, and

lyophilized; (2) sodium bicarbonate extract: water containing 0.1 % sodium bicarbonate at 90 °C, cooled to RT, frozen, and lyophilized; (3) ethanol extract: dried and ground *A. annua* leaves (1 kg) was extracted with 20 L (5 L—three times—plus a final wash with 5 L) of ethanol 96° GL at room temperature, with mechanical agitation for 1.5 h (all the extracts were filtered through four-folded muslin cloth and evaporated to dryness using a rotary evaporator at 40 °C, according to Rodrigues et al. (2006)); and (4) dichloromethane extract: dichloromethane at room temperature was used following the same methodology used for the ethanol extract.

Sodium bicarbonate extract of A. annua produced by Embrapa

A. annua was cultivated in 2009 at Unicamp, harvested, dried, and sent to Embrapa for extraction. Ground leaves and side stems (300 g) were immersed in 5 L of aqueous sodium bicarbonate (0.1 %), warmed to 70 °C, agitated for 10 min, and left at room temperature for 24 h. The solution was filtered through a muslin cloth (four layers), lyophilized and stored at 4 °C, and protected from light, until used (Tariq et al. 2009). Bioactive extract components were analyzed at the Appalachian Farming Systems Research Center, USDA-ARS (closed in 2011).

Quantification of artemisinin and related compounds in *A. annua* extracts

High-performance liquid chromatography with infrared detection (HPLC-IR) analysis of the Unicamp A. annua extracts

Samples of the extracts were quantified for artemisinin and its related compounds by suspending the extract in methanol, filtering, and analyzing by HPLC-IR. Standards of artemisinin and deoxyartemisinin were prepared as stock solutions in methanol (2.6 mg/mL). A standard curve used to quantify the samples of these two substances was built with standards that ranged from 50 to 1,250 μg/mL, using the software Empower (Celeghini et al. 2009).

HPLC with photodiode array detection (HPLC-UV) analysis of the Embrapa sodium bicarbonate extract

This extract was analyzed for artemisinin, deoxyartemisinin, dihydroartemisinic acid, and artemisinic acid by HPLC-UV according to Ferreira and Gonzalez (2009), with a small modification. A 100-mg sample of the extract was added with 10 mL of methanol and dissolved in an ultrasonic bath for 30 min at 38 °C, and an aliquot was used for HPLC-UV analysis.

HPLC with mass spectrometry (HPLC-MS) quantification of artemisinin and deoxyartemisinin from the Embrapa sodium bicarbonate extract and from feces of treated sheep

Due to close elution of artemisinin and deoxyartemisinin in our HPLC-UV system, quantification of these compounds was confirmed by HPLC-MS, according to a previous method (Ferreira and Gonzalez 2008), with some modifications, as follows: The mobile phase consisted of (A) a mixture of 0.1 % acetic acid/methanol/acetonitrile (38.0:46.5:15.5, *v*/*v*) and (B) acetonitrile under isocratic conditions (100 % A and 0 % B) for 8 min at a flow rate of 0.75 mL min^{-1}; then, from 8 to 12 min, the flow rate was increased to 1 min mL^{-1} and the concentration of B was increased to 50 %. These conditions were held for 6 min to rinse the column. The column was reequilibrated for 3 min with 100 % A at 0.75 mL min^{-1} before the next injection. Separations were performed on a 50×4.6-mm Gemini 3 μm C18 110Å column (Phenomenex, Torrance, CA) at room temperature. Quantification for artemisinin and deoxyartemisinin was performed using selected ion monitoring of *m*/*z* 283.1 and 267.1, respectively. Calibration curves for artemisinin and deoxyartemisinin were generated with standards ranging from 20 to 2,000 μg mL^{-1} with R^2=0.998 and from 2.5 to 500 μg mL^{-1} with R^2=0.999. The limit of quantification (S/N=10) for artemisinin and deoxyartemisinin was 20 and 2.5 ng mL^{-1}, respectively. Retention times were 4.3 and 4.4 min, respectively.

Oxygen radical absorbance capacity (ORAC) test

The Embrapa lyophilized sodium bicarbonate extract (100 mg) was added with 10 mL of acetone/water/acetic acid (70:29.5:0.5), sonicated at 37 °C, and centrifuged at 1,100×*g* for 7 min twice. The supernatants were combined, and the volume was completed to 25 mL with the acetone/water/acetic acid solution. The samples were diluted 200 times in phosphate buffer and transferred to a 48-well plate for ORAC analysis according to the protocol described by Prior et al. (2003). Antioxidant capacity was expressed as micromoles of Trolox equivalents (TE) per gram.

HPLC-UV analysis of artemisinin commercial capsules

Artemisinin capsules (Nutricology, Allergy Research Group) used in the in vivo experiment were quantified for artemisinin by HPLC-UV according to a previous method (Ferreira and Gonzalez 2009).

Extraction of artemisinin and deoxyartemisinin from feces

Feces were dried in an oven at 60 °C for 48 h, ground, and weighed. Approximately 500 mg of each sample was extracted twice with 50 mL of petroleum ether by refluxing for 1 h.

Extracts were combined and taken to a fume hood for total evaporation. Feces extract were reconstituted in acetonitrile, filtered, and set aside for HPLC-MS analysis (Ferreira and Gonzalez 2008). Three fecal samples were redried to estimate the substances in grams per 100 g (percent) based on dry matter.

In vitro tests

Determination of dose response for the sodium bicarbonate extracts

The concentrations of the plant extracts tested on the egg hatch test (EHT) increased by the power of 2, with a total of nine concentrations ranging from 0.625 to 160 μg/mL. For the larval development test (LDT), the concentrations ranged from 0.625 to 20 μg/mL. There were six replicates per concentration in each test. The lowest concentration for the bicabornate extracts was determined by egg hatchability and development results similar to the control, while the highest concentration was based on the turbidity of the solution that still allowed for reading of results. For the ethanol and dichloromethane extracts, establishing a dose response was not possible because all concentrations tested resulted in 100 % inhibition of egg hatching.

Egg hatch test (EHT)

Feces were collected from animals naturally infected with GIN (95 % *H. contortus* and 5 % *Trichostrongylus* sp.), and egg recovery was performed using a series of sieves (Coles et al. 1992). Then approximately 100 eggs were placed in the wells of a 24-well plate, and six concentrations were used with six replicates each. In laboratory tests, *Haemonchus* tolerated Tween 80 at 3 %, but Tween 20 at the same concentration was toxic. Thus, plant extracts dissolved with distilled water containing 3 % Tween 80® were added to the wells. Solvent control was also 3 % Tween 80 in water, and negative control was distilled water. Plates were labeled and conditioned for 24 h in an incubator (27 °C, RH >80 %). Percent efficacy or inhibition of egg hatching (IEH%) was calculated per well: IEH% = [eggs / (eggs + L_1]×100, where eggs=number of unhatched eggs and L_1=number of eggs hatched or larval forms (L_1).

Larval development test (LDT)

Approximately 100 eggs were added into each well that also received 80 μL of the *Escherichia coli* nutritive medium (strain W, ATCC 9637), and the volume was brought to 200 μL with distilled water (Hubert and Kerboeuf 1992). Plates remained in the incubator (27 °C, RH >80 %) for 24 h. After this period, plant extracts dissolved in 1 % dimethyl sulfoxide (DMSO) were added. Solvent control was 1 % DMSO in distilled water, and negative control was distilled water. After 6 days in the incubator, L_1, L_2, and L_3 larvae were counted in each well and the percent efficacy or inhibition of development (IED%) was calculated as follows: IED% =[L_1/(L_1+L_2+L_3)]×100, where L_1 and L_2=number of larvae which failed to develop and L_3=number of developed larvae.

In vivo tests

All experimental protocols were approved by the CPPSE Animal Care and Use Committee. Animals were under the care of a veterinary medical assistant during all 15 days of the experiment. None died or were in poor physical condition, and all of them received anthelmintic treatment at the end.

Santa Inês sheep, maintained on CPPSE naturally infested pastures throughout the year and naturally infected with GIN, were treated with the Embrapa sodium bicarbonate extract and with artemisinin capsules. Infection was confirmed by EPG counts (Ueno and Gonçalves 1998). Animals (24), averaging 7 months old and weighing 24 kg, were divided into four groups (G1–G4) of six animals of both sexes. Animals were confined in separate pens and subjected to a 12-h fast before treatment. Oral doses were as follows: G1—infected/untreated control: 20 mL commercial soybean oil; G2—infected/treated control: 1 mL/10 kg BW levamisole hydrochloride (Ripercol L® 7.5 %, Fort Dodge); G3—*A. annua* sodium bicarbonate (Embrapa) extract: single dose of 2 g/kg BW in 20 mL of soybean oil; and G4—artemisinin (Allergy Research Group®): single dose of 100 mg/kg BW in 20 mL of soybean oil.

Within the animal groups, animals were distributed according to their EPG count on day −7, when groups G1, G2, and G4 presented a respective average EPG of 3,933, 4,433, and 4,583. However, animals in group G3 had a lighter average weight than the other groups and had an average EPG= 14,000. These lighter animals were used to test the *A. annua* extract due to the low availability of the extract. Individual fecal samples were collected daily from day 0 (D0, feces collected immediately before treatment) to day 15 (D15) for fecal egg counts (FEC) and to calculate the percentage of EPG reduction. On alternate days, coproculture was done per group (Ueno and Gonçalves 1998). On days 0, 7, and 14 posttreatment, blood samples were collected to determine the hematocrit. On hours 0, 12, 24, and 36 posttreatment, individual fecal samples were collected for artemisinin and deoxyartemisinin quantification.

Statistical analysis

For the in vitro tests, calculation of the lethal concentrations of the extracts was performed by fitting the probit linear

regression using normal distribution and the generalized linear model used for binary data (logistic regression), with estimates of the parameters of these equations obtained by the maximum likelihood method. The procedure used was the SAS® Probit with the independent variables (dose) transformed using the natural logarithm base (log dose). The fit of the models (goodness of fit) was assessed using the Pearson chi-squared test and maximum likelihood ratio ($P<0.001$).

For the in vivo test, results of EPG were log-transformed using natural logarithm ($\ln x+25$) and analyzed by ANOVA using the GLM procedure (SAS 2003). The effects of group (treatment), period (day), and their interactions were evaluated. Covariate analyses considered the initial weight (lower in the *A. annua* extract group) and EPG on day −7 (D−7) for all treatment groups. The statistical model also considered the removal of an outlier from the *A. annua* extract group (with EPG >40,000) to reduce the initial EPG difference between this group and the others. The log transformation normalized the residue and reduced the model coefficient of variation from 38 to 7.5 %. The hematocrit data were not log-transformed, but were also submitted to a variance analyses by GLM using a split-plot design in time [Y=group+animal (group)+day+day×group+error (b)]. The Tukey test was used to compare treatment means (including coproculture data) at a significance level of 5 %.

Results

The dichloromethane extract had the highest concentration of artemisinin (9.8 %), followed by the ethanol extract (4.4 %). The sodium bicarbonate extract produced by Unicamp had 0.6 % artemisinin, while that produced by Embrapa had 0.91 % of deoxyartemisinin (biologically inactive), but no artemisinin (Table 1). The antioxidant capacity of the Embrapa extract was 2,295 μmol TE/g (RSD=9.2 %, n=8).

Table 1 Quantification of artemisinin (ART), deoxyartemisinin (DOART), dihydroartemisinic acid (DHAA), and artemisinic acid (AA) by HPLC-IR and HPLC-UV in grams per 100 grams of dry weight, of *A. annua* extracts made by Unicamp and Embrapa

Extract type	ART (%)	DOART (%)	DHAA (%)	AA (%)
Water	0.28	0.10	ND	ND
$NaHCO_3$ (0.1 %)[a]	0.60	0.14	ND	ND
$NaHCO_3$ (0.1 %)[b]	0.0	0.91	0.10	0.02
Ethanolic	4.43	1.30	ND	ND
Dichloromethane	9.79	2.30	ND	ND

ND not determined

[a] Prepared at Unicamp, São Paulo, Brazil

[b] Prepared at Embrapa CPPSE, São Carlos, Brazil

The artemisinin capsules contained 100 % (±5 %) of the content (100 mg artemisinin/capsule) claimed in the label, according to HPLC-UV analysis (Ferreira and Gonzalez 2008).

In the in vitro EHT, inhibition of egg hatchability increased significantly with the concentration of the *A. annua* extract dose. The ethanol and dichloromethane extracts provided 100 % inhibition in the EHT in all concentrations tested (from 0.625 to 160 μg/mL) and LC_{50} and LC_{99} could not be calculated. The *A. annua* extract with the lowest effect was the water extract, with CL_{50}=21.7 μg/mL and CL_{99}=66.49 μg/mL for the EHT. Both Unicamp and Embrapa bicarbonate extracts had similarly low values for the LC_{99} (1.50 and 1.27 μg/mL, respectively) (Table 2). Regarding the LDT, only the sodium bicarbonate extract produced by Embrapa was tested and resulted in LC_{50}=1.67 μg/mL and in LC_{99}= 23.8 μg/mL.

Regarding in vivo results, statistical analysis done with repeated measures, covariate analysis, and in log-transformed (NL) EPG data showed that there was a significant effect for treatment or group ($P<0.0001$), for day ($P<0.0001$) and for the interaction group ×day ($P<0.0001$). For clinical analysis, there was no statistical difference among hematocrit means at D0 for control, levamisole, and artemisinin groups, but the hematocrit mean for the *A. annua* extract group was significantly lower than that of other treatments at D0. This latter group included smaller animals with an average EPG higher than that of the other groups (Fig. 1, Table 4). Although low, the hematocrit for this group remained significantly unchanged until the last day (D15) of the experiment (Table 3). Only the group treated with levamisole had a statistical increase in hematocrit from D0 to D15.

Concerning the treatment efficacy, levamisole-treated animals (positive control) had a highly significant decrease (97.1 % efficacy) in EPG on D15, while control (infected/untreated) animals had an increase (not significant after log transformation) in EPG of 95.5 %. Although the EPG of animals treated with artemisinin and *A. annua* extract was reduced by 27.8 and 19.3 %, respectively, both reductions were not significant between D0 and D15 (Table 4).

Table 2 *A. annua* extract dose (micrograms per milliliter) that inhibited 99 % of egg hatching (LC_{99}) of gastrointestinal nematodes of sheep

Solvent used	LC_{99}
Water	66.49
$NaHCO_3$ (0.1 %, aqueous)[a]	1.50
$NaHCO_3$ (0.1 %, aqueous)[b]	1.27

[a] Prepared at Unicamp

[b] Prepared at Embrapa

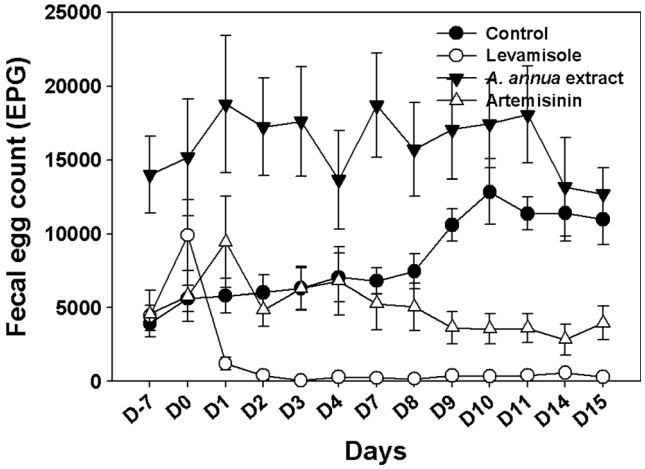

Fig. 1 Mean and standard error bars of nontransformed eggs per gram (EPG) from a week before treatment (day −7) to day 15 (D15) of all treatment groups: infected/untreated (control), levamisole, *A. annua* extract, and artemisinin

Based on coprocultures performed in alternate days from D0 to D15, there was no significant difference in the composition of nematode species infecting the animals of different groups (Tukey's test, $P \geq 0.05$), with *H. contortus* being the most prevalent nematode, followed by *Trichostrongylus* and then *Oesophagostomum*, in the following respective percentages per group: 91.3, 8.4, and 0.3 % (control); 84.3, 15.6, and 0.14 % (levamisole); 89.9, 9.4, and 0.71 % (*A. annua* extract); and 89.4, 10.2, and 0.44 % (artemisinin). Feces samples of sheep treated with artemisinin ranged widely in artemisinin concentration within treatment with averages of 80.1, 126.5, and 49.0 μg artemisinin/g of feces at 12, 24, and 36 h, respectively, while deoxyartemisinin was only present in trace amounts (Table 5). No artemisinin and only traces of deoxyartemisinin were found in feces of sheep treated with the extract (results not shown).

Discussion

In vitro, the similar LC_{99} values of both bicarbonate plant extracts made by Embrapa (1.27 μg/mL) and Unicamp (1.5 μg/mL) indicate that their anthelmintic activity was not due to the presence or lack or artemisinin but, may be, due to the high content of antioxidant compounds (e.g., flavonoids) measured by the oxygen radical absorbance capacity (ORAC) test (ORAC=2,295 μmol TE/g). Flavonoids have been reported previously as having anthelmintic activity (Kerboeuf et al. 2008). The LC_{99} values of both extracts against *Haemonchus* were much lower than those reported for *A. brevifolia* extracts (aqueous and methanolic extracts both at 25 mg/mL), also against *Haemonchus* in vitro (Iqbal et al. 2004).

In vivo, however, 2.0 g/kg BW of extract (or 100 mg of artemisinin/kg BW) at a single dose did cause a significant decrease in EPG, as did levamisole. Our sheep groups had average EPGs from 5,000 to 15,000, while sheep that responded positively to similar *A. brevifolia* treatments had an average EPG of less than 1,000 (Iqbal et al. 2004). These results suggest that a severe GIN infection cannot be treated with single extract doses. As an example, a significant reduction in both worm burden and EPG was achieved in sheep artificially infected with *Haemonchus* and fed with *A. absinthium* from the infection day to day 30 of treatment, when compared to animals fed with other forages (Valderrábano et al. 2010). However, animals still had an average EPG of around 10,000 after 30 days of treatment. Artemisinin-treated animals had D15 hematocrit values similar to levels at D0, while infected/untreated (control) animals had a significant decrease in hematocrit. We believe that, although not significant, the small EPG reduction of 28 % caused by artemisinin at D15 could account for the maintenance in hematocrit values, while the 95 % increase in EPG of control animals could account for the significant reduction in hematocrit levels of infected animals at D15 (Tables 3 and 4).

Regarding the presence of artemisinin in sheep feces, it indicates that artemisinin was partly absorbed by sheep, with fecal levels peaking at 24 h (126.5 μg/g) and then reducing to 49 μg/g 36 h after oral intake. Sheep treated with the extract had no artemisinin in feces (data not shown) as the extract was devoid of artemisinin. A previous experiment with goats showed that dihydroartemisinin, the main active metabolite of artemisinin, was quantified in the blood of goats given with

Table 3 Multiple comparisons of sheep blood hematocrit of control (infected/untreated) and treated groups with levamisole (5 mg/kg BW), *A. annua* extract (2 g/kg BW), and artemisinin (100 mg/kg BW) on the day of the treatment (D0), on day 7 (D7), and on day 15 (D15) posttreatment

Day	Treatments/means			
	Control	Levamisole	*A. annua* extract	Artemisinin
D0	28.3±4.6Aa	25.8±3.4Ab	16.8±3.3Ba	26.1±4.9Aa
D7	25.0±3.7Aab	26.3±2.8Ab	15.5±3.1Ca	21.3±7.0Bb
D15	22.8±3.2Bb	30.1±2.7Aa	14.5±3.5Ca	25.4±3.3Ba

Means followed by the same capital letter across treatments (lines), or small letters within treatment (columns), are not statistically different by Tukey's test ($P \geq 0.05$)

Table 4 Least square mean eggs per gram (EPG), standard error (SE) on the left, and log (NL)-transformed EPG (right), treatment efficacy, and *P* values on reduction of EPG from a week before treatment (D-7) to day 15 (D15) on sheep (n=6) treated with levamisole (5 mg/kg BW), *A. annua* extract (2 g/kg BW), and artemisinin (100 mg/kg BW)

Treatment groups	Average EPG±SE			Average natural log EPG±SE*			Efficacy (%)	*P* value
	D −7	D0	D15	D −7	D0	D15		
Control	3,933±1,372	5,617±1,372	10,983±1,372	8.248±0.259Bb	8.565±0.259ABab	9.240±0.259Aa	−95.5	0.10
Levamisole	4,433±1,372	9,900±1,372	283±1,372	8.337±0.259Ab	8.954±0.259Aa	5.271±0.259Bc	97.1	0.0001
A. annua extract	14,576±16,24	15,757±1,624	12,717±1,372	9.534±0.306Aa	9.540±0.306Aa	9.407±0.259Aa	19.3	0.52
Artemisinin	4,583±1,372	5,783±1,372	4,173±1,565	8.201±0.259Ab	8.417±0.259Aab	7.723±0.295Ab	27.8	0.098

Means followed by the same capital letter across days (lines), or small letters within treatment (column), are not statistically different (t Test, ANOVA with P>0.01)

an oral dose of artemisinin of 28–33 mg/kg BW. Dihydroartemisinin peaked in goat's plasma (approximately 0.7 μg/mL) between 8 and 12 h after oral dosing, with levels decreasing to 0.18 μg/mL 24 h after treatment. Nonabsorbed artemisinin was also found in goat feces (2.4 μg/g) both at 12 and 24 h after dosing (Ferreira and Gonzalez 2008). In the present study, and considering that artemisinin dose per kilogram BW of sheep was about 3.3 times higher than the doses provided to goats in a previous study (Ferreira and Gonzalez 2008), the average artemisinin concentrations (calculated from HPLC-MS analyses of fecal artemisinin) were 1.7 to 4.2 times higher in sheep feces than the ones reported from goat feces. The lower concentration in goat feces suggests that artemisinin has a higher metabolism in goats than in sheep. However, the differential metabolism of artemisinin in goats and sheep has not been evaluated in a single study as it has been done for ivermectin (González Canga et al. 2009).

Artemisinin has been reported to work in the (1) suppression of genes involved in the production of cytokines and other pro-inflammation factors (also triggered by GIN) such as NF-kB and on (2) the animal's immune function (Salminen et al. 2008). Although artemisinin has been beneficial to chickens infected with *Eimeria* spp. (protozoan) and to mice infected with *Schistosoma* spp. (trematode), beneficial effects of secondary plant metabolites in ruminants infected with *H. contortus* (nematode) have been typically hard to obtain with single doses and when animals have high EPG counts.

Also, the poor bioavailability of oral dose of artemisinin suggests that more bioavailable artemisinin derivatives (e.g., artemether, arteether, and artesunate) and different routes of administration (e.g., i.v. and i.m.) must be used to avoid first-pass metabolism in the liver. Despite its anthelmintic effects, the absorption and efficacy of ivermectin varies with the route of administration, formulation, animal species, body condition, age, and physiological status (González Canga et al. 2009), and pharmacokinetic studies in sheep revealed that 60–90 %of ivermectin was eliminated in feces after intraruminal administration (Steel 1993) or regardless of administration route (González Canga et al. 2009).

The potential benefits of feeding animals with bioactive phytochemicals include nutritional effects, anti-inflammatory effects (García-Lafuente et al. 2009; Salminen et al. 2008), immune system modulatory activity (Bin-Hafeez et al. 2003), lower concentration of commercial anthelmintic residues on the environment and in animal products, and anthelmintic activity (Ferreira 2009; Tariq et al. 2009; Valderrábano et al. 2010). However, we believe that plant products, unlike commercial anthelmintics, should be tested in multiple doses. This approach could improve results. Also, results obtained with artificially infected animals with low EPG should be interpreted accordingly. Anthelmintic plant benefits would be better explored by adding them to a daily diet, preceding and during high-infection periods, or by using them in combination with commercial anthelmintics, to evaluate their possible synergistic effects. A plant flavonoid (quercetin), but not avermectin, significantly increased the absorption of moxidectin in lambs (Dupuy et al. 2003). However, in the past 10 years, the combination of plant extracts with commercial anthelmintics has not been studied.

Since its isolation from *A. annua* 43 years ago, artemisinin and its analogs (over 30 years of use) have been safely used (alone or combined with other drugs) against human malaria

Table 5 Concentration, average, and standard errors (SE) of artemisinin and deoxyartemisinin found in feces of individual sheep dosed with 100 mg artemisinin/kg BW, and collected before (0 h), 12 h, 24 h, and 36 h after dosing. Sample for animal 3, at 36 h, was missing from the set

Animal	Artemisinin (μg/g)				Deoxyartemisinin (μg/g)			
	0 h	12 h	24 h	36 h	0 h	12 h	24 h	36 h
1	0	344.6	190.6	55.8	0	6.2	3.6	1.4
2	0	25.2	125.9	75.6	0	3.1	3.4	2.4
3	0	1.6	179.0	–	0	2.0	8.9	–
4	0	6.4	152.3	63.3	0	0.3	3.9	2.2
5	0	0.2	7.1	16.3	0	0.2	0.9	0.7
6	0	102.8	104.2	33.8	0	3.4	3.6	0.9
Average	0	80.1	126.5	49.0	0	2.5	4.0	1.5
SE	0	55.2	27.3	9.7	0	0.94	1.1	0.3

and schistosomiasis. Recent reports also indicate the potential use of artemisinin drugs to treat protozoal infections in livestock. However, the effect of artemisinin and its analogs against GIN in small ruminants has yet to be shown. In our study, artemisinin at a single oral dose provided some beneficial effects on sheep naturally infected with GIN, but only levamisole successfully lowered EPG in infected sheep. Artemisinin does not have the same antiparasitic effect as dihydroartemisinin (the active metabolite of all artemisinin analogs used against malaria and trematodes in humans). Artemisinin analogs work better when combined with other drugs to treat malaria (e.g., with lumefantrine in Coartem®) and to prevent early drug resistance reported for monotherapies. Regarding *A. annua* extracts, these might provide better results if used in multiple doses during high-infection periods and if obtained with solvents other than aqueous sodium bicarbonate.

Acknowledgments This work received financial support from the Brazilian Agricultural Research Corporation (Embrapa) and scholarships and work grants from FAPESP and CNPq. The authors are highly thankful to MSc. Rodrigo Giglioti from Unesp, Dr. Glyn M. Figueira from the University of Campinas, and Dr. Moacir R. Forim from the University of São Carlos for their assistance in this study. Acknowledgements are due to Dr. John Phillips (USDA-ARS) and Dr. Aida Jimenez (University of Charleston, Beckley-WV) for the advice with the statistical analysis and to Mr. Barry Harter, Robert Arnold, and Mrs. J. Diane Snuffer (USDA-ARS) for their help with the chromatographic analyses and figures.

Conflict of interest The authors declare no conflict of interest. The mention of proprietary brands and names is solely for the convenience of the reader and does not imply endorsement of the authors or of the USDA over similar products.

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