

In vitro anthelmintic activity of aqueous leaf extract of *Annona muricata* L. (Annonaceae) against *Haemonchus contortus* from sheep

L.E. Ferreira^a, P.M.N. Castro^a, A.C.S. Chagas^b, S.C. França^a, R.O. Belebony^{a,*}

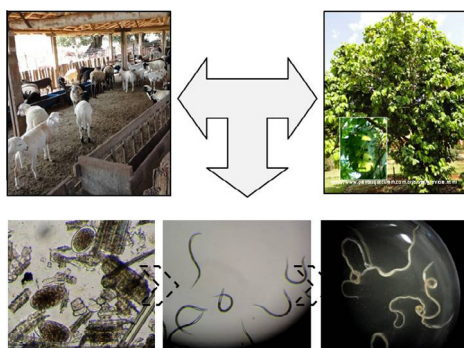
^a Unidade de Biotecnologia, Universidade de Ribeirão Preto (UNAERP), Ribeirão Preto, SP, Brazil

^b Embrapa Pecuária Sudeste (CPPSE), São Carlos, SP, Brazil

HIGHLIGHTS

- Aqueous leaf extract of *Annona muricata* is effective against eggs, L3 larvae and adults of *Haemonchus contortus*.
- Aqueous leaf extract of *A. muricata* is rich in phenolic compounds.
- This work validated the ethnopharmacological potential of *A. muricata*.

GRAPHICAL ABSTRACT



ARTICLE INFO

Article history:

Received 5 July 2012

Received in revised form 19 March 2013

Accepted 25 March 2013

Available online 9 April 2013

Keywords:

A. muricata

Anthelmintics

Gastrointestinal nematodes

H. contortus

Medicinal plants

Phytotherapy

ABSTRACT

Despite the overall progress of sheep farming in Brazil, infections with the gastrointestinal parasite *Haemonchus contortus* represent one of the most important problems in sheep production, aggravated by the increasing resistance of nematodes to traditional anthelmintic drugs caused by inadequate sheep flock management by breeders. Ethnopharmacological data indicate *Annona muricata* as a promising alternative for the control of gastrointestinal nematodes because of its general anthelmintic properties. The aim of this work was to evaluate the *in vitro* anthelmintic effects of *A. muricata* aqueous leaf extract against eggs, infective larvae and adult forms of parasitic nematode *H. contortus*. At higher doses, *A. muricata* extract showed 84.91% and 89.08% of efficacy in egg hatch test (EHT) and larval motility test (LMT), respectively. In the adult worm motility test, worms were completely immobilized within the first 6–8 h of nematode exposition to different dilutions of extract. Phytochemical analysis indicated the presence of phenolic compounds in *A. muricata* aqueous leaf extract that may be responsible for the anthelmintic effects observed. Moreover those results validate the traditional use of *A. muricata* as a natural anthelmintic and then the pharmacological potential of its compounds for future *in vivo* investigations.

© 2013 Elsevier Inc. All rights reserved.

1. Introduction

Sheep farming has been introduced in Brazil by the XVI century. The consolidation of the sheep breeding activity in some Brazilian

states was favored by the diverse geo-climatic regions of the country and, particularly, by the rising interest in sheep-derived products whose sales have markedly increased revenues over the last years (Vieira, 2008). Despite the overall progress, gastrointestinal parasites in small ruminants continue to be one of the most important causes of sub-clinical diseases, poor production and economic losses for the sector (Veríssimo et al., 2012). The most prevalent parasites in Brazilian sheep flocks belong to the Trichostrongylidae family and include *Trichostrongylus colubriformis*, *Oesophagostomum*

* Corresponding author. Address: Unidade de Biotecnologia-UNAERP, Av. Costá-
bile Romano, 2201, Zip Code 14096-900, Ribeirão Preto, SP, Brazil. Fax: +55 16
36037030.

E-mail addresses: rbelebony@unaerp.br, reneusp@yahoo.com (R.O. Belebony).

columbianum, and *Haemonchus contortus*. The last species accounts for about 80% of the parasite burden of infected animals (Arosemena et al., 1999) and is well known for its high pathogenicity (Angulo-Cubillán et al., 2010). The regular use of anthelmintic drugs, in an attempt to control infections caused by those parasites and the inadequate flock management has led to the emergence of resistant nematode species (Prichard, 1994; Akhtar et al., 2000). This problem has been reported in different Brazilian regions (Almeida et al., 2010) and also registered globally (Melo et al., 2009), it causes serious losses in productivity and it is the major limiting factor for the livestock sector.

Considering the traditional use of medicinal plants in developing countries, *in vitro* and *in vivo* studies have been carried out to investigate plant species effectiveness as alternative anthelmintics to control gastrointestinal infections in small ruminants or even as nematocidal in general (Batista et al., 1999; Slomp et al., 2009). *Annona muricata* L. (Annonaceae), popularly known in Brazil as graviola, is a medium-sized fruit tree commonly found in the tropics. *A. muricata* has been widely used in folk medicine as anthelmintic, antipyretic, sedative, antispasmodic, anticonvulsant and as hypotensive agent in humans. Investigations carried out with different species of the genus *Annona* showed that aqueous extracts of *Annona senegalensis* (Alawa et al., 2003; Ndjonka et al., 2011) and methanol extracts and ethyl acetate extracts of *A. squamosa* (Souza et al., 2008; Kamaraj and Abdul Rahuman, 2011) present *in vitro* anthelmintic activity against different nematodes, validating the pharmacological potential of genus. The biological activities of *Annona* extracts have been attributed to the occurrence of annonaceous acetogenins, a class of natural compounds extracted from leaves (Geum-Soog et al., 1998; Wu et al., 1995) and seeds (Chang and Wu, 2001) of species of the Annonaceae family. Souza et al. (2008) demonstrated the antiparasitic activity of these compounds, including against *H. contortus*.

Considering the traditional use of *A. muricata* as an anthelmintic agent against human gastrointestinal parasitism and since previous phytochemical data indicate a high content of antiparasitic acetogenins in the leaves and seeds of plants from *Annona* genus, the aim of this work was to evaluate the *in vitro* anthelmintic effects of *A. muricata* aqueous leaf extract against eggs, infective larvae and adult forms of parasitic nematode *H. contortus*.

2. Materials and methods

2.1. Preparation of the *A. muricata* extract and phytochemical analysis

Leaves of *A. muricata* were collected from plants cultivated at the Terra de Ismael Grange located in the Municipality of Jurucê, São Paulo, Brazil (21°4'12.5" latitude; 47°4'8.0" longitude; altitude of 545 m). Following the traditional preparation for antiparasitic purpose, the crude extract was prepared by the simple infusion method using 100 g of triturated fresh leaves for 1 L ultrapure Milli-Q boiling water. After 30 min of infusion, the whole content was blended in a domestic blender, filtered in a 500 µm sieve and centrifuged (Excelsa Baby I, Fanem) at 2318g for 5 min. The aqueous supernatant was collected and stored at -20 °C until use.

For detection of phenolic compounds, alkaloids or acetogenins in *A. muricata* extract, preliminary phytochemical tests based on visual checking for color modification were performed applying NP/PEG (2-Aminoethyl diphenyl-borinate/Polyethylene glycol), Dragendorff and Kedde reagents on a silica gel 60 coated on Thin Layer Chromatography (TLC) plates (Fluka). Additionally, to obtain a chromatographic profile of the extract, High Performance Liquid Chromatography (HPLC) analyses were performed on a Shimadzu LC10ADvp system equipped with a Supelco LC18 column (Supelco-sil™ RP-18, 250 × 4.6 mm; 5 µm) and the elutes were monitored

by diode array detector SPD10A Shimadzu at 250 nm. A two-solvent gradient system of water: acetic acid 0.1% (A) and methanol (B) was used following a linear gradient from 10% to 66% of (B) until 32 min, returning to 10% (B) in the last 13 min of chromatographic running. The flow rate was 1 mL/min. An UV scan (190–380 nm) (SPDM10A Shimadzu detector) was employed for further investigation of selected chromatographic peaks eluted at first 15 min of chromatographic running.

2.2. *In vitro* tests

Eggs to perform the egg hatch test (EHT) and for culture of infective larvae for larval motility test (LMT) were obtained from fecal samples collected rectally from a monospecifically *H. contortus* infected sheep (Male Santa Inês breed), held in Embrapa Southeast Livestock Unit (CPPSE), São Carlos-SP, Brazil. Gastrointestinal nematode infection was confirmed by egg counting (eggs per gram feces, EPG) as described by Ueno and Gonçalves (1998). All experimental procedures involving the use of animals were previously approved by the Ethics Committee of UNAERP (Protocol 022/09).

2.2.1. Egg hatch test

The EHT followed the method described by Coles et al. (1992) with modifications. Briefly, 20 g feces were collected rectally from the infected animal (>2000 EPG). Next, feces were wet under running water through a series of overlapping sieves (500, 150, 90, and 20 µm). Eggs retained on the last sieve were recovered with supersaturated saline solution by simple flotation. The recovered eggs were washed three times in distilled water by use of centrifugation (Excelsa Baby I, Fanem) at 2318g for 5 min in order to obtain the final aqueous egg suspension. Aliquots of the suspension (100 µL) containing approximately 100 eggs were distributed in microdilution plates. The crude *A. muricata* extract was added to the plate at final dilutions of 50%; 25%; 12.5%; 6.25%; 3.125% (v/v). Levamisole (2 mg/mL) and distilled water were respectively used as positive and negative controls. The entire experiment was carried out for 24 h at 27 °C. The number of eggs and first-stage (L₁) larvae was counted as proposed by the World Association for the Advancement of Veterinary Parasitology (W.A.V.P.) and described in detail by Powers et al. (1982). Results were expressed as % inhibition of eggs hatch as a representation of three independent experiments performed in triplicate.

2.2.2. Larval motility test

For evaluation of the motility of third-stage (L₃) larvae in the presence or absence of increasing dilutions of extract, eggs were initially cultured according to the method of Ueno and Gonçalves (1998) with modifications. Briefly, 20 g of feces with nematode eggs at a concentration of around 2000 EPG were homogenized with sterile wood shavings at a proportion of 1:2 (v/v). The material was moistened daily by spraying distilled water. After incubation for 7 days at room temperature, L₃ were recovered by spontaneous migration using warm water (37 °C). Next, 50 µL suspensions containing 50 L₃ were distributed in microdilution plates and the crude extract of *A. muricata* was added at the same final dilutions as used in the EHT. The plates were incubated for 24 h at 27 °C and the number of motile and non-motile larvae was counted, particularly focusing on the presence or absence of smooth sinusoidal movement, respectively. Positive (Levamisole, 2 mg/mL) and negative (distilled water) controls were included in the experiment. The smooth sinusoidal movement, if present, is additionally stimulated through exposition of larvae to microscopy light or shaking of microdilution plates. Results were expressed as % inhibition of larval motility as a representation of three independent experiments performed in triplicate.

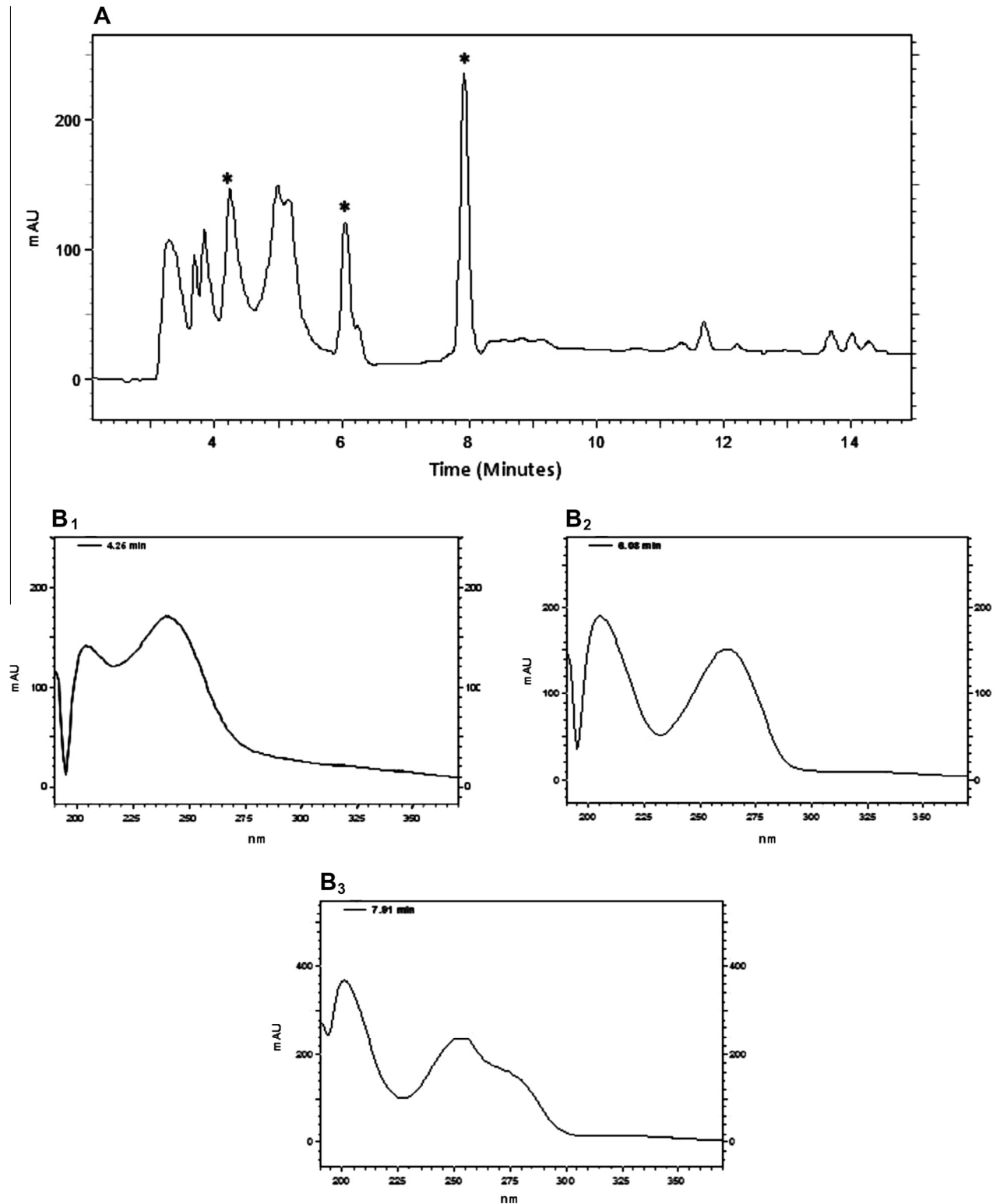


Fig. 1. (A) Chromatographic profile of aqueous leaf extract of *A. muricata* at first 15 min of chromatographic running; (B) UV scan (190–380 nm) for the chromatographic peaks with retention time of 4.26 min (B₁); 6.08 min (B₂) and 7.91 min (B₃) complying with compounds of phenolic nature. (*) Chromatographic peaks analyzed in B₁; B₂ and B₃.

2.2.3. Adult worm motility test

The adult worm motility test was carried out according to the method of Hounzangbe-Adote et al. (2005). *H. contortus* adult worms were directly collected from the abomasum of the mono-specifically *H. contortus* infected sheep used as donor of feces for

EHT and LMT (>2000 EPG) and sacrificed according to the protocol approved by the Ethics Committee. Immediately after animals' death, the abomasum was removed, opened and washed with saline at 37 °C for the collection of adult worms. P of parasites were then exposed to a final volume of 750 µL of crude *A. muricata* ex-

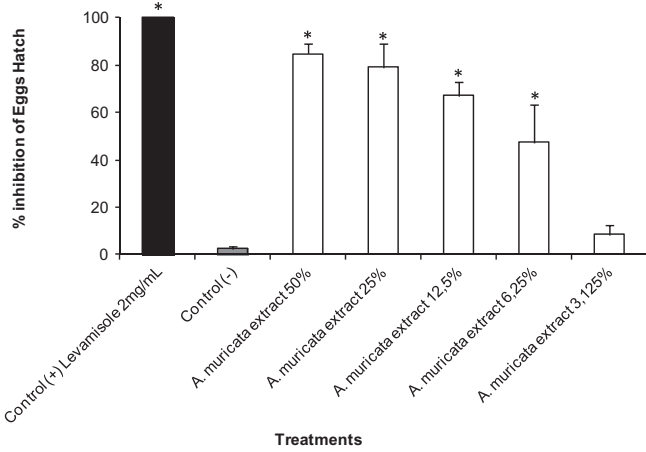


Fig. 2. Inhibitory effect of different dilutions of *A. muricata* extract on egg hatch test against *Haemonchus contortus* from sheep (**p* < 0.05).

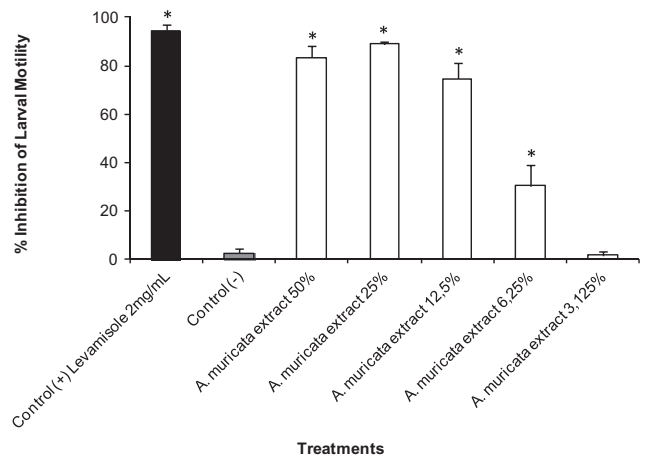


Fig. 3. Inhibitory effect of different dilutions of *A. muricata* extract on larval motility test against *Haemonchus contortus* from sheep (**p* < 0.05).

tract (100%) or at same volume of aqueous dilutions of crude extract (33 and 11%; v/v) using microdilution plates containing 3 adult worms per well/group. Positive (Levamisole, 2 mg/mL) and negative (saline) controls were included in the assay. The plates were kept in an oven at 37 °C and the motility of the parasites was checked at regular intervals of 2 h until worms in the negative control lost their motility, what generally occurred about 14 h after the beginning of the test under our experimental conditions.

Results were expressed as % of motility as a representation of three independent experiments performed in triplicate.

2.3. Statistical analysis

The results were compared by analysis of rates and proportions (z-test) (*p* < 0.05). Statistical analysis was performed using the SigmaStat for Windows Version 3.5.

3. Results

3.1. Phytochemical results

TLC analysis using NP/PEG, Dragendorff and Kedde reagents were negative for both acetogenins and alkaloids but positive for phenolic compounds. A chromatographic profile of aqueous leaf extract of *A. muricata* is focused on the first 15 min of chromatographic running where the most prevalent compounds or groups of compounds of extract were eluted (Fig. 1A). Also, a UV scan for the selected chromatographic peaks with retention time of 4.26, 6.08 and 7.91 min is showed on Fig. 1B, which complies with a chemical profile of phenolic compounds.

3.2. Egg hatch test

The results of the EHT indicated a significant activity of the aqueous extract of *A. muricata* leaves (Fig. 2). The first four dilutions (50%; 25%; 12.5% and 6.25%; v/v) inhibited the hatching of *H. contortus* eggs by 84.91%, 79.12%, 66.97% and 47.42%, respectively. A higher percentage of inhibition (99.94%) was observed for the positive control (Levamisole; 2 mg/mL). The mean hatching inhibition rate for negative control was only 2.38%.

3.3. Larval motility test

The aqueous extract of *A. muricata* leaves at serial dilutions of 50%, 25%, 12.5% and 6.25% inhibited the motility of L₃ by 83.29%, 89.08%, 74.62% and 30.47%, respectively (Fig. 3). The positive control promoted a 94.59% inhibition, whereas the inhibition of motility in the negative control was 2.60%.

3.4. Adult worm motility test

Different concentrations of the aqueous *A. muricata* extract significantly inhibited adult worm motility over the 14 h of exposure in a very faster way compared to the negative control. However, this effect was not dose dependent. In this experiment, different doses of the aqueous *A. muricata* extract completely inhibited adult worm motility in the first 6–8 h of observation. This effect was

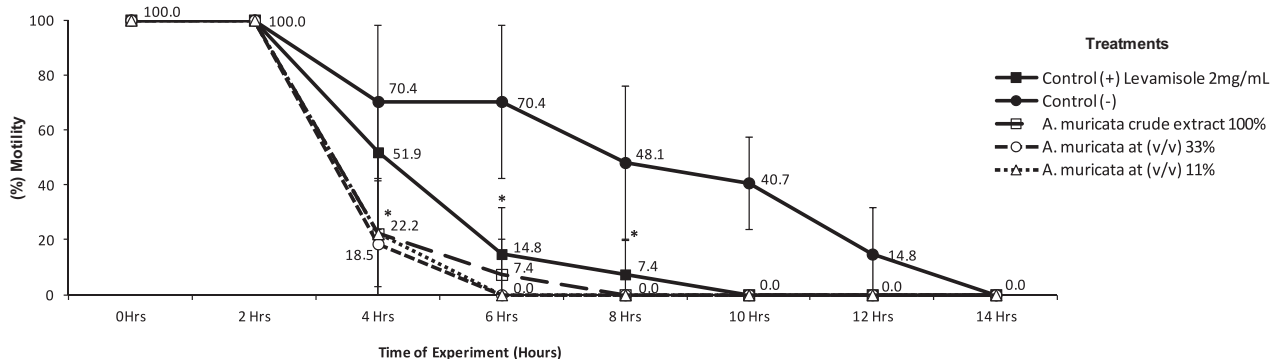


Fig. 4. Inhibitory effect of different dilutions of *A. muricata* extract on adult worm motility test against *Haemonchus contortus* from sheep (**p* < 0.05).

similar to that seen for the positive control but different from that observed for the negative control in which about 70% of the worms were still motile in the first 6 h of the experiment (Fig. 4).

4. Discussion

In vitro tests to evaluate the inhibition of egg hatching (Coles et al., 1992), larval motility (Kotze et al., 2006) and adult worm motility (Hounzangbe-Adote et al., 2005) are widely used in veterinary parasitology to the prospecting of novel anthelmintic agents (Costa et al., 2002; Vasconcelos et al., 2007). The advantage of those assays is that compounds or materials to be tested are in direct contact with the different life-cycle stages of the parasite. So far, *in vitro* screenings of potential anthelmintic agents prior to *in vivo* testing have been shown to be a rational and practical strategy since they save time and money and also minimize the number of animals necessary for the development of a new therapeutic agent. However, compounds or substances that are effective *in vitro* do not necessarily work equally *in vivo*. This kind of divergence in results may be attributed to key factors related to the bio-availability as well as pharmacology of those compounds or substances in the host organism, destruction of active compounds by gut flora metabolism in rumen and different conditions observed *in vitro* versus *in vivo* (Chagas and Vieira, 2007; Peneluc et al., 2009). These considerations should be taken into account when developing new anthelmintic agents.

Powers et al. (1982) designed guidelines for evaluating *in vitro* the anthelmintic efficacy of drugs that have been adopted by the W.A.A.V.P. According to these authors, effective anthelmintic agents should inhibit worm egg hatching and larval motility by more than 90% and when inhibiting 80–90% should be considered moderately effective. Thus, our *in vitro* results obtained with *A. muricata* extract against *H. contortus* eggs and larvae, particularly in the higher dilutions, allow us to classify the tested extract as moderately effective. However, it should be noted that the same extract exhibited an anthelmintic efficiency very close to that observed for positive control in adult worm motility test. Moreover, it is important to remark that the same extract showed a statistically significant anthelmintic activity against the three life-cycle stages of the parasite, i.e., eggs, larvae, and adult parasites. This finding is significant since, theoretically, it reduces the chances of the occurrence of resistance of the parasite when using the extract in clinical practices due to anthelmintic action on different phases of worm development (Hounzangbe-Adote et al., 2005).

The antiparasitic effects of plants of the genus *Annona* and of acetogenins found in species of this genus have been previously reported. In *in vitro* tests performed by Souza et al. (2008) the aqueous extract of *A. squamosa* L. (sugar apple) caused eggs hatching inhibition of 19.4% against gastrointestinal nematodes of cattle. Furthermore, in *in vivo* tests with sheep using the same plant it was observed a 40% reduction of *H. contortus* egg counts (Vieira et al., 1999). Souza et al. (2008), found that the extract and acetogenins isolated from seeds of *A. squamosa*, caused 90% inhibition of *H. contortus* egg hatching. Although the mechanisms of action of anthelmintic plant extracts of the Annonaceae family are still unclear, studies have suggested that the mechanism of inhibition of egg hatching and larval development of different parasites is related to the inhibition of cell division and, consequently, to the formation and development of vital structures of the parasite (Gallardo et al., 1998).

Despite promising indications of the effectiveness of acetogenins as antiparasitics and considering that due to acetogenins chemical nature these compounds may not be effectively extracted from a plant using water as solvent, our results using *A. muricata* aqueous extract could not be associated to the acetogenins, what

would suggest that the anthelmintic effects observed in this work could be related to other types of molecules. Indeed, phytochemical analysis did not reveal any type of acetogenins or even alkaloids in the extract used in our experiments. Conversely, both TLC plates revealed with NP/PEG color reagent for aqueous extract (Data not showed) and the UV scan analysis for the most prevalent chromatographic peaks observed in our reverse phase chromatography (Fig. 1B) indicated the presence of phenolic compounds in the aqueous leaf extract of *A. muricata*. This information may be valuable for further purification of *A. muricata* extract in search for active compound(s). Furthermore, since acetogenins have been associated with neurodegeneration in rats and in humans (Champy et al., 2004) the absence of acetogenins in the plant extract used in this work is a fact somewhat motivating, because it makes of *A. muricata* aqueous extract a safe drug to treat target animals if compared to plant extracts prepared with organic solvents presuming the acetogenin extraction. In fact, the results of clinical, hematological and biochemical analyses showed no significant differences among sheep orally treated with saline and with the ones treated with higher doses of *A. muricata* aqueous extract in preliminary toxicological experiments in acute scale (Data not showed).

Finally, the classical treatment of nematode infections using conventional anthelmintic drugs resulted in about 294 million dollars of veterinary market revenue in 2004 (Molento et al., 2004). The consequences of this situation go beyond the rising costs of livestock management. In addition to the problem of resistance discussed above, there is no clear evidence that synthetic anthelmintics leave no residues in meat that would pose potential public health hazards (Rodrigues et al., 2007). Therefore, the identification of novel promising anthelmintic plant extracts such as *A. muricata* extract may contribute for the development of phytotherapeutic products that could be more cost effective, safer, and more accessible and provide a lower risk of resistance than the conventional therapeutic arsenal currently employed.

Acknowledgments

The authors are grateful to Fapesp and Ouro Fino Saúde Animal for financial supporting and to Bianca W. Berton, Sarazetti I.V. Pereira, Maicon A. Sousa, Jeferson M.M. Macedo, Frank R. Cardoso and Rafael C. Andrade for technical and scientific assistance.

References

- Akhtar, M.S., Iqbal, Z., Khan, M.N., Lateef, M., 2000. Anthelmintic activity of medicinal plants with particular reference to their use in animals in the Indo-Pakistan subcontinent. *Small Ruminants Research* 38, 99–107.
- Almeida, F.A., Garcia, K.C.O.D., Torgerson, P.R., Amarante, A.F.T., 2010. Multiple resistance to anthelmintics by *Haemonchus contortus* and *Trichostrongylus colubriformis* in sheep in Brazil. *Parasitology International* 59, 622–625.
- Alawa, C.B.I., Adamu, A.M., Gefu, J.O., Ajanusi, O.J., Abdu, P.A., Chiezey, N.P., Alawa, J.M., Bowman, D.D., 2003. *In vitro* screening of Nigerina medicinal plants (*Vernonia amygdalina* and *Annona senegalensis*) for anthelmintic activity. *Veterinary Parasitology* 113, 73–81.
- Angulo-Cubillán, F.J., García-Coiradas, L., Alunda, J.M., Cuquerella, M., De La Fuente, C., 2010. Biological characterization and pathogenicity of three *Haemonchus contortus* isolates in primary infections in lambs. *Veterinary Parasitology* 171, 99–105.
- Arosemena, N.A.E., Bevilaqua, C.M.L., Melo, A.C.F.L., Girão, M.D., 1999. Seasonal variations of gastrointestinal nematodes in sheep and goats from semi-arid area in Brazil. *Revista Medicina Veterinária* 150, 873–876.
- Batista, L.M., Bevilaqua, C.M.L., Moraes, S.M., Vieira, L.S., 1999. *In vitro* ovicidal and larvicidal effect of the plants *Spigelia anthelmia* and *Momordica charantia* against the nematode *Haemonchus contortus*. *Ciência Animal* 9, 67–73.
- Chagas, A.C.S., Vieira, L.S., 2007. *Azadirachta indica* (Neem) action in goats gastrointestinal nematodes. *Brazilian Journal of Veterinary Research and Animal Science* 44, 49–55.
- Champy, P., Höglinger, G.U., Féger, J., Gleye, C., Hocquemiller, R., Laurens, A., Guérineau, V., Laprèvote, O., Medja, F., Lombès, A., Michel, P.P., Lannuzel, A., Hirsch, E.C., Ruberg, M., 2004. Annonacin, a lipophilic inhibitor of mitochondrial complex I, induces nigral and striatal neurodegeneration in rats: possible

- relevance fo atypical parkinsonism in Guadeloupe. *Journal of Neurochemistry* 88, 63–69.
- Chang, F.R., Wu, Y.C., 2001. Novel cytotoxic annonaceous acetogenins from *Annona muricata*. *Journal of Natural Products* 64, 925–931.
- Coles, G.C., Bauer, C., Borgsteede, F.H., Geerts, S., Klei, T.R., Taylor, M.A., Waller, P.J., 1992. World association for advancement in veterinary parasitology (W.A.A.V.P.) methods for the detection of anthelmintic resistance in nematodes of veterinary importance. *Veterinary Parasitology* 44, 35–44.
- Costa, C.T.C., Morais, S.M., Bevilaqua, C.M.L., Souza, M.M.C., Leite, F.K.A., 2002. Ovicidal effect of *Mangifera indica* L. seeds extracts on *Haemonchus contortus*. *Brazilian Journal of Veterinary Parasitology* 11, 57–60.
- Gallardo, T., Aragón, R., Tormo, J.R., Blázquez, M.A., Zafra-Polo, M.C., Cortes, D., 1998. Acetogenins from *Annona glabra* seeds. *Phytochemistry* 47, 811–816.
- Geum-Soog, K., Zeng, L., Alali, F., Rogers, L.L., Wu, F., McLaughlin, J.L., Sastrodihardjo, S., 1998. Two new mono-tetrahydrofuran ring acetogenins, annomuricin E and muricapentocin, from the leaves of *Annona muricata*. *Journal of Natural Products* 61, 432–436.
- Houngzangbe-Adote, M.S., Paolini, V., Fouraste, I., Moutairou, K., Hoste, H., 2005. *In vitro* effects of four tropical plants on three life-cycle stages of the parasitic nematode *Haemonchus contortus*. *Research in Veterinary Science* 78, 155–160.
- Kamaraj, C., Abdul Rahuman, A., 2011. Efficacy of anthelmintic properties of medicinal plant extracts against *Haemonchus contortus*. *Research in Veterinary Science* 91, 400–404.
- Kotze, A.C., Le Jambre, L.F., O'Grady, J., 2006. A modified larval migration assay for detection of resistance to macrocyclic lactones in *Haemonchus contortus*, and drug screening with Trichostrongylidae parasites. *Veterinary Parasitology* 137, 294–305.
- Melo, A.C.F.L., Bevilaqua, C.M.L., Reis, I.F., 2009. Anthelmintic resistance to benzimidazole in gastrointestinal nematodes from small ruminants of semi-arid Brazilian northeast. *Ciência Animal* 10, 294–300.
- Molento, M.B., Tasca, C., Gallo, A., Ferreira, M., Bononi, R., Stecca, E., 2004. Método Famacha como parâmetro clínico individual de infecção por *Haemonchus contortus* em pequenos ruminantes. *Ciência Rural* 34, 1139–1145.
- Ndjonka, D., Agyare, C., Lüersen, K., Djafsia, B., Achukwi, D., Nukenine, E.N., Hensel, A., Liebau, E., 2011. *In vitro* activity of Cameroonian and Ghanaian medicinal plants on parasitic (*Onchocerca ochengi*) and free-living (*Caenorhabditis elegans*) nematodes. *Journal of Helminthology* 85, 304–312.
- Peneluc, T., Domingues, L.F., Almeida, G.N., Ayres, M.C., Moreira, E.L., Cruz, A.C., Bittencourt, T.C.B., Almeida, M.A., Batatinha, M.J., 2009. Anthelmintic activity of aqueous extract of *Zanthoxylum rhoifolium* Lam. leaves (Rutaceae). *Revista Brasileira Parasitologia Veterinaria* 18, 43–48.
- Powers, K.G., Wood, I.B., Eckert, J., Gibson, T., Smith, H.J., 1982. World associations of the advancement of veterinary parasitology (W.A.A.V.P.) guidelines for evaluating the efficacy of anthelmintics in ruminants (bovine and ovine). *Veterinary Parasitology* 10, 265–284.
- Prichard, R., 1994. Anthelmintic resistance. *Veterinary Parasitology* 54, 259–268.
- Rodrigues, A.B., Athayde, A.C., Rodrigues, O.G., Silva, W.W., Faria, E.B., 2007. Evaluation of the efficacy of anthelmintics to control gastrointestinal nematodes in goats raised in the state of Paraíba. *Pesquisa Veterinaria Brasileira* 27, 162–166.
- Slomp, L., Pereira, P.S., Zingaretti, S.D., Belebony, R.O., 2009. *In vitro* nematocidal effects of medicinal plants from the Sao Paulo state-Brazil. *Pharmaceutical Biology* 47, 230–235.
- Souza, M.M., Bevilaqua, C.M., Morais, S.M., Costa, C.T., Silva, A.R., Braz-Filho, R., 2008. Anthelmintic acetogenin from *Annona squamosa* L. seeds. *Anais Academia Brasileira Ciências* 80, 271–277.
- Ueno, H., Gonçalves, P.C., 1998. Manual para diagnóstico das helmintoses de ruminantes, 4th ed. Japan International Cooperation Agency, Tokyo.
- Vasconcelos, A.L.C., Bevilaqua, C.M., Morais, S.M., Maciel, M.V., Costa, C.T., Macedo, I.T., Oliveira, L.M., Braga, R.R., Silva, R.A., Vieira, L.S., 2007. Anthelmintic activity of *Croton zehntneri* and *Lippia sidoides* essential oils. *Veterinary Parasitology* 148, 288–294.
- Veríssimo, C.J., Niciura, S.C., Alberto, A.L., Rodrigues, C.F., Barbosa, C.M., Chiebao, D.P., Cardoso, D., da Silva, G.S., Pereira, J.R., Margatho, L.F., da Costa, R.L., Nardon, R.F., Ueno, T.E., Curci, V.C., Molento, M.B., 2012. Multidrug and multispecies resistance in sheep flocks from São Paulo state. *Brazilian Journal of Veterinary Parasitology* 187 (1–2), 209–216.
- Vieira, L.S., 2008. Métodos alternativos de controle de nematóides gastrintestinais em caprinos e ovinos. *Tecnologia & Ciência Agropecuária* 2 (2), 49–56.
- Vieira, L.S., Cavalcante, A.C.R., Pereira, M.F., Dantas, L.B., Ximenes, L.J.F., 1999. Evaluation of anthelmintic efficacy of plants available in Ceará state, northeast Brazil, for the control of goat gastrointestinal nematodes. *Revista Medicina Veterinária* 150, 447–452.
- Wu, F.E., Zeng, L., Gu, Z.M., Zhao, G.X., Zhang, Y., Schwedler, J.T., McLaughlin, J.L., Sastrodihardjo, S., 1995. New bioactive monotetrahydrofuran annonaceous acetogenins, annomuricin C and muricatocin C, from the leaves of *Annona muricata*. *Journal of Natural Products* 58, 909–915.