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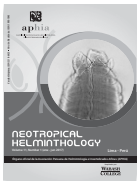

ORIGINAL ARTICLE / ARTÍCULO ORIGINAL

IN VITRO EVALUATION OF OVICIDAL ACTIVITY OF PLANT EXTRACTS AGAINST *HAEMONCHUS CONTORTUS* (TRICHOSTRONGYLIDAE)

EVALUACIÓN *IN VITRO* DE LA ACTIVIDAD OVICIDA DE EXTRACTOS DE PLANTAS CONTRA *HAEMONCHUS CONTORTUS* (TRICHOSTRONGYLIDAE)

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ABSTRACT

Sheep industry is a branch in livestock that has been growing over the years. However, the control of gastrointestinal parasites is considered one of the greatest barriers of this practice. In this sense, *Haemonchus contortus* (Rudolphi, 1803) is a hematophagus parasite which can cause severe anemia, development of mumps, and death. It is known that the inappropriate use of allopathic medicines has promoted the emergence of resistance by this parasite. Thus, the present study aimed to evaluate the ovicidal activity of plant extracts against *H. contortus*. *Haemonchus contortus* eggs were obtained from fecal samples of naturally infected sheep, and purified by modification of Sheather's method. The purified eggs were submitted to six alcoholic plant extracts, at concentrations of 12.5 mg / mL, 25 mg / mL and 50 mg / mL. The plants which have been evaluated were *Allamanda cathartica* L., *Musa* sp, *Nerium oleander* L., *Mirabilis jalapa* L., *Carica papaya* L. and *Brugmansia suaveolens* (Humb. & Bonpl. ex Willd.) Bercht. After 168 h, 50 uL aliquots were analyzed for the presence of eggs turned into blastomere, embryo, or larvae. It was observed that the alcoholic extracts of *N. oleander* and *M. jalapa* were the most promising, since it prevented the outbreak of larvae in all tested concentrations, thus presenting a high potential for composite products intended for grazing decontamination.

Keywords: Environmental decontamination – Haemonchiasis – Plant extracts

RESUMEN

La industria ovina es una rama en el ganado que ha estado creciendo a través de los años, sin embargo, el control de los parásitos gastrointestinales se considera una de las mayores barreras de esta práctica. En este sentido, *Haemonchus contortus* (Rudolphi, 1803) es um parásito hematófago que puede causar anemia severa, el desarrollo de paperas, y la muerte. Se sabe que el uso inapropiado de medicamentos alopáticos ha promovido la aparición de resistencia por parte de este parásito. Así, el presente estudio tuvo como objetivo evaluar la actividad ovicida de extractos de plantas contra *H. contortus*. Los huevos de *H. contortus* se obtuvieron a partir de muestras fecales de ovejas naturalmente infectadas y se purificaron mediante la modificación del método de Sheather. Los huevos purificados se sometieron a seis extractos alcohólicos, a concentraciones de 12,5 mg / mL, 25 mg / mL y 50 mg / mL. Las plantas evaluadas fueron *Allamanda cathartica* L., *Musa* sp, *Nerium oleander* L., *Mirabilis jalapa* L., *Carica papaya* L. y *Brugmansia suaveolens* (Humb. & Bonpl. ex Willd.) Bercht. Después de 168 h, se analizaron alıcuotas de 50 µL para determinar la presencia de huevos transformados en blastómero, embriones o larvas. Se observó que los extractos alcohólicos de *N. oleander* y *M. jalapa* fueron los más prometedores, ya que evitó el brote de larvas en todas las concentraciones ensayadas, presentando así un alto potencial para productos compuestos destinados a la descontaminación de pastoreo.

Palabras clave: descontaminación ambiental – Extractos vegetales – Hemonquiasis

INTRODUCTION

The sheep industry is a branch in livestock that has been growing over the years due to its high profits and low cost of management, however, control of gastrointestinal parasites has been considered one of the greatest barriers of sheep breeding because they cause significant losses in the herd income (Silva *et al*., 2011).

There are several species of gastrointestinal nematodes of sheep, but the most remarkable species is *Haemonchus contortus* (Rudolphi, 1803), because in addition to high frequency of infection, it is the most serious, since this is the only Strongylidae that feeds directly from blood which can result in hypoproteinemia, anemia with development of submandibular swelling, weakness or fatigue after exercise and diarrhea, often leading to death, particularly to young animals (Getachew *et al*., 2007; Prasad *et al*., 2008).

The strongylids parasites have two stages in its cycle: free-living stage and parasitic phase. In free-living phase, under appropriate conditions of oxygen, humidity and temperature, the eggs present in stool form a larva inside the egg in 24 h. This larvae hatch and needs microorganisms to feed. This is L1 larvae. If the environmental conditions remain favorable, between 5 and 10 days, the larvae will suffer cuticle L1 changes, and evolve to L2, and finally L3, which is the infective form (O'Connor *et al*., 2006).

The frequent and inappropriate use of allopathic medicines generates intense selection pressure, leading to a selective advantage for tolerant parasites, allowing them to multiply in future generations, resulting in decreased efficacy of allopathic medicines and promoting the emergence of resistance to them (Sawleha *et al*., 2010; Gilleard, 2013).

In this sense it is important to develop research aimed at the search for new elimination alternatives of this parasite, either through the treatment of animals, whether through the pasture decontamination. Thus, the aim of the present study was to evaluate the ovicidal activity of plant extracts known to have antiparasitic activity against *H. contortus*.

MATERIAL AND METHODS

The plant selection criteria was based on scientific evidence established in the literature, according to which these extracts have presented ovicidal activity and larvicide against nematodes as *Ancylostoma* sp. (Santos *et al*., 2013) and *H. contortus* (Oliveira *et al*., 2010; Pereira *et al*., 2013). For confirmation of botanical identity, vouchers were deposited in the herbarium SPF - University of São Paulo, whose respective numbers are quoted after the scientific name for each species studied. Six plant species were selected to carry out this research: *Allamanda cathartica* L. (Apocynaceae) – G. Akisue 041, *Musa* sp – (Musaceae) G. Akisue 061, *Nerium oleander* L. (Apocynaceae) – G. Akisue 040, *Carica papaya* L. (Caricaceae) – G. Akisue 062, *Mirabilis jalapa* L. (Nyctaginaceae) – G. Akisue 045 and *Brugmansia suaveolens* (Solanacae) Willd. - G. Akisue 030.

To obtain the extracts of *A. cathartica*, *N. oleander*, *M. jalapa* and *B. suaveolens*, leaves of each plant were processed. However, seeds were used of *C. papaya* and the flower of *Musa* were used for obtaining extracts of these vegetables.

The collected plant material was processed in the laboratory of Pharmacognosy (LAFAPLAM), Faculty de Pindamonhangaba. After drying in an oven at 45 ° C and pulverization using an electric coffee grinder (Cuisinart®), alcoholic extracts were prepared using ethanol (absolute alcohol PA) as liquid extractor, by Soxhlet method, according to the norms of the Brazilian Pharmacopoeia (Farmacopeia Brasileira, 2010). The obtained extract was concentrated by rotary evaporation apparatus for evaporation of the solvent to obtain crude extract, which, in turn, was diluted in distilled water to obtain different concentrations to be analyzed.

The experiments were performed at the Laboratory of Parasitology (LAPAM) from the Faculty de Pindamonhangaba, in which were evaluated the ovicidal activity of the above statements, which were tested on three different dilutions (50 mg / mL, 25 mg / mL and 12.5 mg / mL).

To perform the ovicidal test, *H. contortus* eggs were obtained from fecal samples collected directly from the rectum of naturally infected sheep of Santa Inês breed, and purified by modifying the Sheather's method (flotation at 1,600 rpm / min saturated sucrose d = 1.2 g / mL) (Jurasek *et al.*, 2010), followed by collection of 2 mL of the supernatant and repeated washings with distilled water (by centrifugation at 1600 rpm / 1 min).

The ovicidal tests were performed with standard amounts of eggs immersed in the evaluated dilutions and the activity was determined by observing the proportion of viable eggs after 24, 48, 72 and 168 (7 days) h of exposure.

The results were statistically assessed (level of significance = 5%) using nonparametric method (Kruskal-Wallis) according to the normality of the results obtained, followed by Dunn's test, respectively. For that we used the BioEstat 5.0 software.

RESULTS

In the present research was considered effective only extracts that inhibited 100% of egg hatching. After 48 h of experimentation, it was observed 90% of egg hatching in the control group and, among the total extracts tested, the most effective were those obtained from *M. jalap*a and *N. oleander*, since they inhibited 100% off egg hatching at concentrations of 25 mg/ mL and 50 mg/ mL as shown in table 1.

Similarly, after 72 h, the two aforementioned plant extracts remained the same percentage of egg hatching inhibition in concentrations of 25mg/ mL and 50mg/ mL and remained effective in this regard, including 168 h after exposure. It should be noted, however, that among the two most effective vegetables, *N. oleander* also presented ovicidal activity at a concentration of 12.5 mg/ mL at all time intervals in which the assessments were made, as shown in table 2.

Among the other extracts evaluated, the one obtained from *A. cathartica* showed to be effective in inhibiting egg hatching only at a concentration of 50mg/ mL thus being less promising than the two

mentioned above. Moreover, the extract that showed less effectiveness was the one obtained from *C. papaya*, particularly after 72 and 168 h of treatment, at concentrations of 12.5 mg and 25 mg, since there was a high survival of larvae which was statistically similar to that observed in the control group ($p > 0.05$).

Table 1. Ovicidal activity of alcoholics extracts after 24 and 48 h of exposure, against *H. contortus*. BE: blastomered egg; EL: egg with larvae and L: larvae.

	24 h									48 h								
	12.5mg/mL			25mg/mL			50mg/mL			12.5mg/mL			25mg/mL			50mg/mL		
	BE	EL	L	BE	EL	L	BE	EL	L	BE	EL	L	BE	EL	L	BE	EL	L
A. cathartica	2	18	0	0	20	0	1	19	0	0	19	1	3	15	2	0	20	0*
B. suaveolens	6	14	0	7	13	0	0	20	0	2	17	1	3	13	4	4	13	3
C. papaya	9	11	0	4	16	0	5	15	0	3	5	12	5	5	10	3	17	0*
M. jalapa	3	17	0	1	19	0	0	20	0	3	16	1	1	19	0*	7	13	0*
Musa sp.	1	19	0	1	19	0	1	19	0	15	2	3	14	3	3	12	4	4
N. oleander	2	18	0	2	18	0	6	14	0	6	14	0	7	13	0*	5	15	0*
Control	3	17	0	3	17	0	3	17	0	0	2	18	0	2	18	0	2	18

* significant difference ($p<0,05$) compared to other groups. Similarly, after 72 h, the two aforementioned plant extracts remained the same.

Table 2. Ovicidal activity of alcoholics extracts after 72 and 168 h of exposure, against *H. contortus*. BE: blastomered egg; EL: egg with larvae and L: larvae.

	72 h									168 h								
	12.5mg/mL			25mg/mL			50mg/mL			12.5mg/mL			25mg/mL			50mg/mL		
	BE	EL	L	BE	EL	L	BE	EL	L	BE	EL	L	BE	EL	L	BE	EL	L
A. cathartica	0	19	1	6	12	2	5	15	0	0	19	1	2	13	5	4	16	0
B. suaveolens	2	12	6	3	11	6	2	10	8	3	7	10	5	4	11	5	8	7
C. papaya	0	0	20[a]	0	0	20[a]	3	12	5	0	4	16[a]	0	4	16[a]	0	16	4
M. jalapa	3	16	1	3	17	0	7	13	0	7	13	0	5	15	0	6	14	0
Musa sp.	1	19	0	1	19	0	1	19	0	0	10	10	2	5	13	1	19	0
N. oleander	3	17	0	7	13	0	4	16	0	7	13	0	12	8	0	11	9	0
Control	0	3	17[a]	0	3	17[a]	0	3	17	1	0	19[a]	1	0	19[a]	1	0	19

a- Similar letters imply a non-significant difference ($p>0.05$).

DISCUSSION

The results presented above agree in part with those obtained by Santos *et al*. (2013) which evaluated the activity of alcoholic extracts of 10 different vegetables against Ancylostomidae larvae, and of these, four of them showed more promising, to know *N.oleander*, *M. jalapa*, *A. cathartica* and *B. suaveolens*, except the last, which showed no ovicidal activity in this research.

It is noteworthy that, in the study by Santos *et al*. (2013), all the extracts had not presented ovicidal activity, which possibly justified the need for evaluation of larvicidal activity by these authors. On the other hand, in our study, the most promising extracts have already demonstrated effectiveness

in the inhibition of eggs hatching, thus showing a greater potential to control hemonchosis over hookworm.

This evidence also shows that the eggs of *H. contortus* are more susceptible to the action of plant extracts analyzed, compared to eggs of hookworm, thus posing a result of significant importance, since, as previously mentioned, one of the greatest barriers of sheep breeding is related to resistance that this parasite presents with regard to allopathic drugs (Sawleha *et al*., 2010).

Rout *et al.* (2014) demonstrated antimicrobial and antioxidant activity of *N. oleander* and also hypothesized that such activities are due to the presence of active principles such as flavovonoids, terpenes, alkaloids and saponins.

The remarkable biological activity of *N. oleander* also has already been demonstrated by other researchers (Al-Jubouri & Al-Khan, 2008; El-Akhal *et al*., 2015). Al-Jubouri & Al-Khan (2008) evaluated the inhibitory activity of *N. oleander* extract against the in vitro growth of *Leishmania tropica* promastigotes and observed that 2.5 mg / mL of leaf extract was able to inhibit 71.9% of promastigote growth for 96 hours of culture. According to the aforementioned authors, such inhibition may be due to the presence of steroidal glycosides, such as oleandrin and nirin.

El-Akhal *et al*. (2015) evaluated the larvicidal activity of this plant against larvae of *Culex pipiens* L., the transmitting agent of the virus that causes West Nile infection. These authors demonstrated a toxic effect of the hydroethanolic extract with 100% mortality in the minimum concentration of 160mg / mL.

According to these authors, the larvicidal activity of the hydroethanolic extract of *N. oleander* may be due to the major components, among them flavonoids, sterols, terpenes, tripterpenes and coumarins. In addition, *N. oleander* leaves contain a fairly toxic mixture of cardiotoxic glycosides, among which oleandrin, oleandrigenine, digoxin, digitonin, digitoxigenin, nerizoside, neritaloside and odoroside, which may be poisonous to humans, animals, fish, birds and some insects. However, phytochemical studies with regard to active constituents and their biological activities necessarily need to be delineated (El-Akhal *et al*., 2015).

In turn, *M. jalapa* is a plant that has been used for centuries to treat various disorders (Rozina, 2016), but there is still little known about its antiparasitic property. Zachariah *et al*. (2012) evaluated the antiparasitic activity of methanolic extract of *M. jalapa* against *Pheretima phostuma* (Vailliant, 1868) , which is a geohelminth that has been widely used for the initial evaluation of anthelmintic activity *in vitro*, due to its anatomical and physiological similarity with human parasite nematodes. These authors observed that the methanolic extract of *M. jalapa* was able to cause paralysis and death of adult worms of *P. phostuma*, being considered potentially useful as anthelmintic.

According with Zachariah *et al.* (2012) the methanolic extracts of *M. jalapa* were potent as anthelmintic probably because of flavonoids, glycosides and tannins in dose-dependent manner giving shortest time of paralysis and death with 80% w/v concentration.

The fact that three of the extracts evaluated have not proven effective, does not eliminate the possibility that these may be promising, since despite not having presented ovicidal activity, they could cause significant mortality when tested for larvicidal activity, highlighting in this sense the importance of future studies aiming to evaluate this property. For example, despite not having demonstrated ovicidal activity in this research, *Musa* sp. was efficient against *H. contortus* in the concentration of 75mg / mL, with efficacy of 98.5 to 100%, in the inhibition of larval development, in another study conducted by Oliveira *et al*. (2010).

On the other hand, the fact that a plant extract not show significant activity *in vitro*, does not preclude the possibility that it may be potentially useful for the control of hemonchosis *in vivo*. In this sense, with regard to species of *Carica* gender, the expressionless ovicidal activity of the ethanol extract of *C. papaya* seeds found in this study shows that theoretically it would not be useful for grazing decontamination, however Pereira *et al*. (2013) showed that administration of this same extract to naturally infected goats, was able to reduce the number of OPG (eggs per gram of feces)

at 49, 23% and 72%, respectively, thus being potentially useful to use as antiparasitic or in addition to allopathic therapy (Vieira, 2008).

It can be concluded that alcoholic extracts of *A. cathartica*, *M. jalapa* and *N. oleander* are of a high potential for use as grazing decontamination, to control hemoncose in the sheep herd, given the property of inhibiting egg hatch of this parasite in vitro, but further research needs to be designed to check this property on a larger scale and to determine possible environmental impacts of direct use in soil using ecotoxicity tests, for example.

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