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EDGARD FRANCO GOMES

Inhibition of infective larvae exsheathment and egg hatching of the nematode *Haemonchus contortus* with extracts of tannin-rich plants

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Versão revisada de acordo com a Resolução CoPGr 6018 de 2011

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“Shemá Yisrael, HaShem Elokeinu, HaShem ECHAD.” Devarim (“Deuteronômio”) 6:4

“O justo se preocupa com a vida de seus animais, porém mesmo a compaixão do malévolo é cruel” Mishlei (“Provérbios”) 12:10

“Para mim, não é qualquer questão que é digna de ser questionada. Eu questiono apenas lá onde sou questionado. Por pessoas sou questionado.” Franz Rosenzweig

“Eu descobri em mim mesmo desejos os quais nada nesta terra podem satisfazer, a única explicação lógica é que eu fui feito para um outro mundo.” C. S. Lewis

“Com um pingo de mel se apanham mais moscas do que com um barril de fel.” Abraham Lincoln

“Acorde cedo se você quiser a vida ou a terra de outro homem. Nenhuma batalha é vencida deitado numa cama. Nenhuma ovelha terá o lobo preguiçoso.” Provérbio Viking

“Corte sua própria lenha. Assim, ela aquecerá você duas vezes.” Henry Ford

“A maior recompensa para o trabalho do homem não é o que recebe por ele, mas o que se torna por meio dele.” John Ruskin

“O caminho que leva ao que realmente desejamos é longo e difícil, mas só seguindo esse caminho é que vamos alcançar nosso alvo.” M. Splinter

RESUMO

GOMES, E. F. **Inibições do desembainhamento de larvas infectantes e da eclodibilidade do nematoide *Haemonchus contortus* com extratos de plantas taniníferas.** 2013. 66 f. Dissertação (Mestrado). – Centro de Energia Nuclear na Agricultura, Universidade de São Paulo, Piracicaba, 2013.

Objetivou-se com o presente trabalho verificar a bioatividade dos extratos das plantas taniníferas *Acacia mearnsii*, *Myracrodruon urundeuva*, *Caesalpinea bracteosa* e *Leucaena leucocephala* contra os estágios de ovo e de larva L_3 infectante de *Haemonchus contortus*. Para isso, dois ensaios *in vitro* foram realizados: o Teste da inibição da eclodibilidade dos ovos (TIEO) e o teste da Inibição do desembainhamento larvar (TIDL). O TIEO consiste na incubação de ovos recém recuperados das fezes de animais infectados em solução liquida de extrato de planta por 24 horas e posterior diferenciação entre larvas e ovos não eclodidos. As concentrações utilizadas para *A. mearnsii* foram de 50,00, 25,00, 12,50, 6,25, 3,12, 1,56, 0,78 e 0,39 mg/mL; 1,56, 0,78, 0,39, 0,19, 0,09 e 0,04 mg/mL para *M. urundeuva*; 6,25, 3,12, 1,56, 0,78, 0,39 e 0,19 mg/mL para *C. bracteosa*; e 6,25, 3,12 e 1,56 mg/mL para *L. leucocephala*. O TIDL consiste no desembainhamento artificial de larvas infectantes, obtidas através de coprocultura, que passaram por período de incubação de três horas em solução liquida de extratos de plantas nas concentrações 1.200, 600, 300 e 150 µg/mL. As doses letais (DL) 50 e 99 foram calculadas para ambos testes. Um efeito dose-dependente foi encontrado para os dois testes, exceto para a *L. leucocephala* no TIEO, onde não foi possível calcular o valor da DL_{50} e DL_{99} para o respectivo teste nas doses escolhidas. Os resultados da DL_{50} para TIEO foram de 0,18, 0,32 e 7,20 mg/mL e da DL_{99} foram de 4,31, 5,41 e 187,26 mg/mL respectivamente para *M. urundeuva*, *C. bracteosa* e *A. mearnsii*. Para o TIDL, a DL_{50} foi de 0,40, 0,52, 1,24 e 2,24 mg/mL e da DL_{99} foi de 2,37, 2,28, 19,99 e 2,53 x 10^3 mg/mL respectivamente para *M. urundeuva*, *A. mearnsii*, *L. leucocephala* e *C. bracteosa*. Para o TIEO, as duas maiores concentrações de *A. mearnsii* e as três maiores de *C. bracteosa* foram efetivas (mais de 90% de bioatividade); as três maiores concentrações de *M. urundeuva* e a 0,78 mg/mL de *C. bracteosa* foram moderadamente efetivas (entre 80 e 90% de bioatividade); as concentrações de pouca efetividade (entre 60 e 80% de bioatividade) foram a 12,50 mg/mL (*A. mearnsii*), 0,19 mg/mL (*M. urundeuva*), e 0,39 mg/mL (*C. bracteosa*); as demais concentrações foram consideradas ineficientes (menos de 60% de bioatividade). Para o TIDL, apenas a maior concentração de *A. mearnsii* e *M. urundeuva* foram efetivas; a concentração de 600 µg/mL de *M. urundeuva* foi moderadamente efetiva; todas as outras concentrações foram ineficientes. Entretanto, deve-se observar que apesar de ineficientes em bloquear o desembainhamento, algumas concentrações atrasaram significativamente o desembainhamento das larvas, sendo esse um resultado de interesse. Assim, foi observado que os extratos, excetuando o extrato de *L. leucocephala* quanto a eclodibilidade, possuem bioatividade *in vitro* nas doses utilizadas contra a eclosão dos ovos e contra o desembainhamento das larvas de *H. contortus*.

Palavras-chave: Verminose. Resistência anti-helmíntica. *Haemonchus*. Etnoveterinária. *In vitro*.

ABSTRACT

GOMES, E. F. **Inhibition of infective larvae exsheathment and egg hatching of the nematode *Haemonchus contortus* with extracts of tannin-rich plants.** 2013. 66 f. Dissertation (Masters). – Centro de Energia Nuclear na Agricultura, Universidade de São Paulo, Piracicaba, 2013.

The aim of this work was to assess the bioactivity of extracts of the tannin-rich plants *Acacia mearnsii, Myracrodruon urundeuva, Caesalpinea bracteosa* and *Leucaena leucocephala* against egg and infective larvae stages of *Haemonchus contortus*. Two *in vitro* assays were held: an Egg Hatch Assay (EHA) and a Larval Exsheathment Inhibition Assay (LEIA). The EHA consists of the incubation of previously recovered eggs from infected animal's faeces in a solution of plant extract for 24 hours and later differentiation between larvae and non-hatched eggs. The concentrations used were 50.00, 25.00, 12.50, 6.25, 3.12, 1.56, 0.78 and 0.39 mg/mL for *A. mearnsii*; 1.56, 0.78, 0.39, 0.19, 0.09 and 0.04 mg/mL for *M. urundeuva*; 6.25, 3.12, 1.56, 0.78, 0.39 and 019 mg/mL for *C. bracteosa*; and 6.25, 3.12 and 1.56 mg/mL for *L. leucocephala*. The LEIA consists in the artificial exsheathment of infective larvae, obtained by previous coproculture, after a three hour incubation period with plant extract solution in the concentrations 1,200, 600, 300 and 150 µg/mL. The 50 and 99 lethal doses (LD) were calculated for both tests. A dose-dependent effect was found in the two tests, except for *L. leucocephala* in EHA, where it was not possible to calculate DL_{50} and DL_{99} with the chosen doses. The DL_{50} results for EHA were 0.18, 0.32, and 7.20 mg/mL and for DL_{99} were 4.31, 5.41, and 187.26 mg/mL, respectively for *M. urundeuva, C. bracteosa*, and *A. mearnsii*. For LEIA, the DL50 were 0.40, 0.52, 1.24, and 2.24 mg/mL and for DL_{99} these were 2.37, 2.28, 19.99 and 2.53 x 10^3 mg/mL respectively for *M. urundeuva, A. mearnsii, L. leucocephala* and *C. bracteosa*. The two highest concentrations of *A. mearnsii* and the three highest for *C. bracteosa* were effective (more than 90% of bioactivity); the three highest concentrations of *M. urundeuva* and the 0.78 mg/mL level of *C. bracteosa* were moderately effective (between 80 and 90% of bioactivity); the concentrations with low effectiveness (between 60 and 80% of bioactivity) were the 12.50 mg/mL (*A. mearnsii*), 0.19 mg/mL (*M. urundeuva*), and 0.39 mg/mL (*C. bracteosa*); all other concentrations were ineffective (less than 60% of bioactivity). For the LEIA, only the highest dose from *A. mearnsii* and *M. urundeuva* were effective; the 600 µg/mL were moderately effective and all other doses were ineffective. It should be observed that even if the dose is ineffective against exsheathment, some concentrations were able to significantly delay the process. It was observed that the extracts had bioactivity *in vitro* within the chosen doses against the hatchability of eggs and exsheathment of larvae of *H. contortus*, except for *L. leucocephala*, which was not able to block the hatching of eggs.

Key-words: Parasitosis. Anthelmintic resistance. *Haemonchus*. Ethnoveterinary. *In vitro*.

SUMMARY

1. INTRODUCTION

The goat and sheep production are expanding in Brazil, according to data from Brazilian Institute of Geography and Statistics (IBGE, 2011). But gastrointestinal parasites still an ongoing issue against this production, being one of the greatest worldwide problems in ruminants, particularly in sheep and goats. Under dosing, mass treatment, and use of the same drug treatment for long periods might have worked together for the development of a drug resistance in the parasites (LOUVANDINI et al., 2002; PIMENTEL NETO; FONSECA, 2002; WALLER, 2006).

Haemonchus contortus is one of the most common parasites in small ruminants. It is haematophagous, and it is found worldwide. It is well known because of its high pathogenicity. When animals acquire high infections, they become anaemic because of the loss of blood and, because of that, a high loss of nutrients, such as proteins, lipids and minerals. They also may also have oedema because of hypoproteinemia. For a long time these infections have been treated with chemical anthelmintics, which has led to high levels of drug resistance. Because of that resistance, alternative efforts to fight against parasites have been investigated in different fronts, such as pasture rotation, selection for genetic resistant hosts, biologic control of parasites, vaccination, and phytotherapy, among others. The techniques aim at lowering the level of infection in the animals and, consequently, to lower the tax of pasture contamination (BAIN, 1999; JACKSON et al., 2009; TAYLOR; COOP; WALL, 2010).

Regarding phytotherapy, there is a vast amount of Brazilian tannin-rich plants, but the anthelmintic properties of these are mostly unknown. A better way to screen these plants is by doing *in vitro* assays, because they are cheaper, collected rapidly, show repeatability, and they have sensitivity. Testing them first using *in vivo* would be costly and also too slow. Many tests can be used to analyze tannin-rich plants such as the Egg Hatch Assay, Larvae Inhibition Motility Assay, Larval Migration Inhibition Assay, Larval Exsheathment Inhibition Assay, Larval Feeding Inhibition Assay, and others. These tests focus on different stages of the nematode life cycle, and they show that tannin-rich plants do play a role similar to anthelmintics. Once the *in* vitro tests are carried out, the plants that showed bioactivity may be selected and tested in *in vivo* assays (POWERS et al., 1982; BRUNET; HOSTE, 2006; GOMES; BANDEIRA, 2012).

Haemonchus contortus is a nematode specie. The general comprehension of nematodes physiology is important to better understand the *H. contortus* physiology. Even though this work focuses on *H. contortus* hatching and exsheathing, a brief description of nematodes as a group will be given, highlighting the important differences found with *H. contortus* when needed.

2. INTRODUÇÃO

A produção de caprinos e ovinos está em expansão no Brasil, segundo dados do Instituto Nacional de Geografia e Estatística (IBGE, 2011). Entretanto, parasitas gastrintestinais são um problema contra a produção, e são um dos principais problemas na produção mundial de ruminantes, particularmente de ovelhas e cabras. Baixa dosagem, tratamento em massa e uso do mesmo princípio ativo por períodos longos são fatores que juntos contribuíram para o desenvolvimento de resistência anti-helmíntica dos parasitas (LOUVANDINI et al., 2002; PIMENTEL NETO; FONSECA, 2002; WALLER, 2006).

Haemonchus contortus é um dos vermes mais comuns em pequenos ruminantes. É hematófago, encontrado mundialmente e conhecido por conta de sua alta patogenicidade. Quando animais têm altas infecções, eles se tornam anêmicos pela perda de sangue e, por conta desta, também ocorre uma grande perda de nutrientes, como proteínas, lipídeos e minerais, podendo ocasionar edema nos animais, por conta da hipoproteinemia. Por muito tempo essas infecções foram tratadas com uso de moléculas comerciais, o que ocasionou, pelo mal uso das moléculas, uma grande resistência aos medicamentos. Por conta da resistência, tentativas de controle alternativo têm sido buscadas, como a rotação de pastagens, seleção de animais geneticamente resistentes, controle biológico dos parasitas, vacinação, fitoterapia, entre outras. Visa-se através das técnicas diminuir a infecção dos animais e, consequentemente, diminuir a infestação dos pastos (BAIN, 1999; JACKSON et al., 2009; TAYLOR; COOP; WALL, 2010).

Com relação à fitoterapia, existem muitas plantas taniníferas na flora brasileira, mas suas atividades anti-helmínticas são pouco conhecidas. Uma forma viável para selecionar essas plantas é através de testes *in vitro*, porque eles são baratos, são mais rápidos, possuem repetibilidade e sensibilidade. Testar inicialmente *in vivo* pode ser muito caro e demorado. Muitos testes podem ser usados para analisar plantas taniníferas tais como o Teste da Inibição da Eclodibilidade dos Ovos (TIEO), o Teste da Inibição da Motilidade Larvar, o Teste da Inibição da Migração Larvar, o Teste da Inibição do Desembainhamento Larvar (TIDL), o Teste da Inibição da Alimentação Larvar, entre outros. Esses testes focam em diferentes estágios do ciclo de vida do nematoide e através deles são mostrados o papel das plantas taniníferas no combate aos helmintos. Depois de selecionar as plantas nos testes *in vitro*, as plantas que apresentaram bioatividade são selecionadas e testadas *in vivo* (POWERS et al., 1982; BRUNET; HOSTE, 2006; GOMES; BANDEIRA, 2012).

Haemonchus contortus é uma espécie de nematoide. Para a compreensão da fisiologia do *H.* contortus, faz-se necessária a compreensão da fisiologia dos nematoides como uma grupo. O foco desse trabalho é a eclodibilidade e o desembainhamento de *H. contortus*, entretanto, uma breve descrição dos nematoides será apresentada, destacando o que é especifico para o *H. contortus* quando necessário.

3. REVIEW OF THE LITERATURE

3.1. Developmental stages of nematode larvae and feeding behaviour

An egg and four juvenile stages precede the maturity of nematodes, i.e., the stages of development are first stage larvae (L_1), second stage larvae (L_2), third stage larvae (L_3), fourth stage larvae (L_4), and fifth stage, or adult, larvae (L_5). Each stage has its own morphology and feeding behavior, to ensure survival of the species. It is important to notice that even the nematode's eggs show remarkable adaptations. The egg shell is complex, having layers with different permeability. They have an elliptical shape and have between 20 to 50 µm in width and 50 to 100 µm in length. *Haemonchus contortus* eggs are 44 µm in width and 74 µm length. Female length ranges from 300 µm (*Bunonema* and *Paratylenchus*) to 8.4 m (*Placentonema gigantissima*, a whales' parasite). The nematode's egg has basically three layers: the inner one is a thin membrane; in the middle there is a thick layer of chitin, and an outer protein layer. The inner layer is responsible for the relatively impermeability of the egg shell to polar compounds. The middle layer is not present at one or both ends of the egg in many species, giving origin to an operculum. The last layer is secreted by the wall of the uterus of the female (LEE, 1965; GAUGLER; BILGRAMI, 2004; TAYLOR; COOP; WALL, 2010).

Before hatching, environmental conditions such as temperature and moisture must be optimal. Also, there is a partial control of hatching from the unhatched larva, since it starts to attack the inner layer of the egg shell. It is known that the egg shell becomes permeable to water, possibly because of that attack. The egg hatches only when the first stage larva is fully formed for most parasitic nematodes (LEE, 1965; TAYLOR; COOP; WALL, 2010).

Moulting is the process in which the larva loses its cuticle and becomes the following stage's larva (Figure 1). The cuticle itself is a complex structure: it has three main layers, with collagen in each one, and an epicuticle. The L_5 stage does not moult, but its cuticle keeps growing as the animal grows. In *H. contortus*, the L_1 and L_2 larval stage are bacterial-feeders, and they feed from the bacteria from the faeces of the host. The cuticle of the L_3 larvae is a reminiscent of the L_2's cuticle. The L_3 larva is fully encased in the L_2's sheath, with all anal, oral, and excretory pore orifices being completely closed. So, the L_3 larva does not feed. The stimulus for the beginning of the L_3's exsheathment, in other words, the process of losing its sheath, is the contact of the larvae with a solution of lower or higher pH. In an *in vivo* situation, that solution may have soluble carbonic dioxide gas, undissociated carbonic acid, or

hydrochloric acid. With the beginning of the stimulus, the larvae respond with secretion of the moulting fluid, and this fluid makes the cuticle detach from the larvae. The fluid is produced close to the excretory pore, at the anterior end of the nematode, and distributed in a circular pattern. Commonly, the cuticle detaches first of the anterior portion of the larvae like a cap, and the larvae swims outside the cuticle. Part of the inner layer of the sheath is dissolved and reabsorbed and the outer layer sheds. For *H. contortus* no enzyme from the host is necessary for exsheathment. It all depends on the worm's enzymes (LEE, 1965; ANDERSON, 1992; RUPPERT; BARNES, 1996).

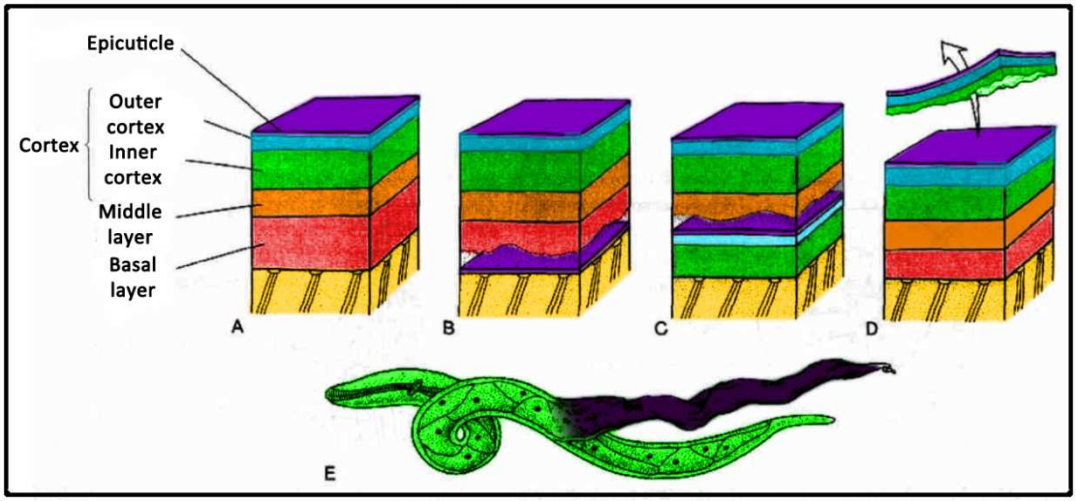

Figure 1 - The details vary from species to species, but the moulting process occurs basically in this sequence. (A) Intact cuticle. (B) Secretion of new epicuticle and digestion of the basal layer. (C) Secretion of the new outer and inner cortex, partial digestion of the old middle layer. (D) Secretion of the new basal layer to complete the new cuticle. (D) and (E) Discarding the old cuticle. Adapted from Ruppert and Barnes (1996)

According to Lee (1965), in *in vivo* conditions, the L_3 larvae need 15 minutes of pH lowering stimuli to start the exsheathment, but the whole process may take up to three hours. In an *in vivo* experiment with cannulated sheep, Brunet et al. (2007) found that approximately 80% of larvae were exsheathed after 160 minutes, with rumen average pH of 5.72. *Haemonchus contortus* larvae need a high concentration of H_2CO_3 in order to start the exsheathment. That may explain why *H. contortus* has such a narrow host range, because these conditions are hard to be found in species other than ruminants.

Parasites have specific dietary sources. Many adaptations have been occurring in order for this to happen. There were some changes in feed behavior and in the response to the environmental conditions. Morphologic changes in the cephalic region of the worms have developed, allowing these animals to feed in different fonts and ways. For example, their pharyngeal glands produce a secretion that is mixed with the food while it is passing through the pharynx, and it is believed that this secretion is for helping in the digestion process; plant nematodes have a stylet; bacterial-feeding nematodes from the digestive tract of invertebrates have specific structures in the cephalic region; others feed from the food that the host has fed, as seen in Ascaridoidea (LEE, 1965; GAUGLER; BILGRAMI, 2004).

The oral cavity of the parasites has a huge morphologic and function variation and also varies to the presence of teeth. Small denticles may exist to break down food or to retain it. These teeth can happen in different formats, the simplest being a single solid cuticular protuberance of the stoma in the inner oral cavity. In this last case, the protuberance breaks down food, and liberates small portions of it, so it can enter the oral cavity by lip or pharyngeal suction. Nematodes that parasite plants usually pierce the cell wall and sucks the content through its hollow stylet. The tooth of adult nematodes that lives in the digestive tract of vertebrates is not just for feeding, they also help the nematode to be fixed in the sites they live (for example, the *Ancylostoma*). Others, such as *H. contortus*, developed a lancet to cause tissue lesions from where the parasites are fed (LEE, 1965; GAUGLER; BILGRAMI, 2004).

There is a phenomenon of sequestration of the L_3 larvae when the population of adult nematodes is high, in *H. contortus.* If the population is high, the immunological system is being overstimulated, making harder for new larvae to fixate. So, the L_3 larvae enter the gastric mucosa and stay there until the adult population becomes senescent and dies. When this happens, the immunological system will be less stimulated and the fixation will be easier. So, the L_3 larvae restart their development, by becoming L_4 larvae, later adult larvae, and fixing in the abomasum. Apparently, there is a relation between the number of adult nematodes fixed and the number of larvae sequestrated. Another situation of sequestration may happen is in severe drought periods or in temperate places, i.e., the larvae are sequestrated during autumn and restart the development during spring. By the end of autumn, the larvae stay in this situation of sequestration. In spring, the larvae become adults, when the environment outside the animal is supportive to egg development. It has also been observed a correlation between parasites egg laying and the period when sheep are lambing. It has been observed that there is an increase in egg laying by nematodes in the peripartum and lactation periods. It is known that there is an interrelation between the egg laying of nematode females

and the levels of prolactin produced by the host. So, as the levels of prolactin go higher because of peripartum and lactation period, the nematode's egg production also increases. The young animals are more susceptible to infection, since their immunological systems are less developed (ANDERSON, 1992; WEISCHER; BROWN, 2001).

3.1.1. Habitat and ethology of nematodes

Nematodes are distributed in vastly different environments. Free living forms can be found in land, fresh water, and salt water. They can be found in deserts, Polar Regions, and in the tropics as well as in high mountains and the depths of the ocean. Some of them are present in places of extreme heat, such as hot springs of water, which can reach 53 °C. This characteristic of the nematodes can be seen in a quote of Mr. Nathan A. Cobb, a famous nematologist: "In short, if all the matter in the universe except the nematodes were swept away, our world would still be dimly recognizable, and if, as disembodied spirits, we could then investigate it, we should find its mountains, hills, vales, rivers, lakes, and oceans represented by a film of nematodes. The location of towns would be decipherable, since for every massing of human beings there would be a corresponding massing of certain nematodes. Trees would still stand in ghostly rows representing our streets and highways. The location of the various plants and animals would still be decipherable, and, had we sufficient knowledge, in many cases even their species could be determined by an examination of their erstwhile nematode parasites" (RUPPERT; BARNES, 1996, p. 283, our translation).

But different environments demand different nematode behavior. So, parasites with riskier behavior – those who have to migrate, invade, infect the host, for example – usually have larger females, which are capable of laying a greater amount of eggs. *Haemonchus contortus* has a behavior of negative geotropism in the last free living stage, i.e., L_3. After living as bacterial-feeders (L_1 and L_2 stages), the L_3 larvae migrate through a water film to the top of the grass leaves, to make it easier to be eaten by the host (LEE, 1965; GAUGLER; BILGRAMI, 2004).

3.2. Parasitism and related issues

Parasitism is a type of interaction overspread in the Animal Kingdom. For example, all classes from the Chordates are parasitized. Particularly, animals from salt and fresh water, and those from land, wild or domestic, are attacked by parasite nematodes. These nematodes can be so harmful to the host that it may be killed (LEE, 1965; WEISCHER; BROWN, 2001).

Sheep may be affected by different species of nematode parasites, such as *H. contortus* (abomasum), *Teladorsagia circumcicta* (abomasum), *Trichostrongylus colubriformis* (duodenum), *Strongyloides papillosus* (small intestine), *Oesophagostomum venulosum* (large intestine), among others. The adult forms of *H. contortus* have between 10 to 20 mm of length for males and 18 to 30 mm of length for females. This parasite has a cephalic lancet, in order to punch the internal wall of the abomasum and, by doing so, to suck blood from the host (FORTES, 1987; WEISCHER; BROWN, 2001; TAYLOR; COOP; WALL, 2010).

Parasitosis may cause different type of issue to the host, depending on the tract it is fixed, depending on the host, etc. For example, in the case of *H. contortus*, each adult worm sucks from the host 0.05 mL of blood/day. So, an animal infected with a population of 5,000 adult larvae loses 250 mL of blood/day. If nothing is done, the animal would go under a severe anemia, hypoalbuminemia due to great loss of plasma proteins (which leads to oedema), vitamins, minerals, etc. If there are bad feeding conditions, the animal would die quickly. It is known that, usually, adult parasites are found in only one organ of the gastrointestinal tract of the host, i.e., adult *H. contortus* is found exclusively in the abomasum (LEE, 1965; WEISCHER; BROWN, 2001; TAYLOR; COOP; WALL, 2010).

3.2.1. Trichostrongylodea family

The Trichostrongylodea family is the largest of the bursade nematodes. Most are parasites of land vertebrates, such as bats, rats, ruminants, marsupials and monotremes. They are rarely found in amphibians, reptiles and birds. The parasites of this family, one being *H. contortus*, are usually found in the gastrointestinal tract of their hosts. Some can be found parasitizing bronchi, biliary ducts, nasal cavity, and mammal glands. The hosts get infected by ingesting the infective larva, i.e., L_3 larvae (LEE, 1965; ANDERSON, 1992).

3.2.2. Parasitism origin

The studying of the origin of parasitism is a complex subject, since it's hard to find fossil record, at least for parasites. This happens because parasites are very small animals with few mineral-inorganic structures, making it hard to be preserved as a fossil (ROTH, 1980; WEISCHER; BROWN, 2001).

Some authors postulate that, initially, parasites held a mutualism relation with the hosts, i.e., maybe at an old period in the history of life, the nowadays parasites were not in this type of relation with their hosts. They were in mutualism or commensalism interactions, and were probably bacterial-feeders. Nowadays, those types of interactions still exist, as seen in the microbiota in the gastrointestinal tract of ruminants and non-ruminants or in the microbiota in the roots of many plants. It can be postulated by the fact that parasites usually have very specific sites, in very specific hosts. Hardly, for example, *H. contortus* would be able to parasitize non-ruminant or plant species. So, maybe there was a change in the relations of the parasite-host by loss of genetic variation or Horizontal Gene Transfer (HGT). By HGT, genes from other species, such as bacteria and fungi, would be able to pass to new species, like nematodes. It has been seen that plant parasitic nematode's cellulase genes was gained by HGT from different microbial donors, when phylogenetic reconstruction was held (ROTH, 1980; WEISCHER; BROWN, 2001; MAYER et al., 2011).

A characteristic favorable to animal and plant parasitism is the appearance of teeth-like structures. Before the development of such structures, the parasites were bacterial-feeders. For example, the parasite *Rhabditis strongyloides* have juvenile stages that penetrate in the skin of small rodents and become inactive until the host dies. After it dies, the larvae feed on bacteria. Also, in the case of animal hosts, the negative geotropism behavior was a great advance leading to parasitism and the presence of epidermal syncytium was an important feature to allow animals to live in stressful osmotic environments, such salt water and inside host's gastrointestinal tract. Other evidence is found in some fungi-feeders nematodes. Many species of the genera *Cryptaphelenchus* have a commensal relation with beetles that dwells in tree's bark. When there's deterioration of the fungi, for example, in the case of drought, the worms start to produce juvenile L_3 larvae, and these get fixed to adult beetles and by that they are transported to new trees to start a new cycle, establishing a commensalism relation. This way is only commensalism. But it is known that some species in this genera started to invade the beetles, developing parasitism. They get installed in the beetle's excretory tract until the exsheath to the L_4 stage, and after that they become fungi-

feeders again. Other nematode's species, such as *Parasitaphelenchus*, have relations between beetles that range from commensalism to parasitism, depending on the phase of development of the nematode. *Parasitaphelenchus* L_1 to L_3 larvae are fungi-feeders. The L3 larvae penetrates in the body of beetles, moult to L_4, and start to live inside its hemocele. When the beetle reaches the reproductive phase, the L4 nematodes are expelled, and moult to L5; the nematode's males ones lives just until the copulation and the females a little bit more (RUPPERT; BARNES, 1996; WEISCHER; BROWN, 2001).

In relation to parasitism in plants, there is some similarity to the cases of invertebrate parasitism cited above. The worms would have been initially bacterial or fungi-feeders, and after they developed anatomical structures, like the stylet and an alimentary tube, to parasitize plants. The stylet has the function to make a small aperture in the plant's roots. By the aid of the alimentary tube, the larvae suction the liquid content of the cytoplasm, like drinking straws sucking beverages. It's remarkable that the worm seals that alimentary tube after it ends its feeding, probably to avoid secondary infection to the plant. Examples of these worms are the ones from the orders *Dorylaimida* and *Triplonchida*. Another parasitizing method was developed by the worms from the *Aphelenchida* and *Tylenchida* orders. There are basically three levels of parasitism, with increasing degree of invasion of the root tissue: ectoparasite form, semi-ectoparasite form and endoparasite form. The ectoparasite forms are mobile and they feed from root hairs and rizoderme cells, and these turn necrotic and later the parasites must continually seek new sites of infection. In the semi-ectoparasitism, like in *Cacopauruspestis*, the individuals are semi-sessiles, with larvae that possess long stylet, being able to collect nutrients from the deepest plant's tissue. In the last stage, endoparasitism, the females are motionless while the males and juveniles are ectoparasites. A species that behaves like that is *Tylenchulus semipenetrans*, which parasitizes citric plants (ROTH, 1980; WEISCHER; BROWN, 2001).

Nematodes from the *Athelencoides* genera present different levels of parasitism: free-living form, ectoparasites most of the time, ecto-endoparasites, endoparasites most of time, and endoparasites. There is a gradual change in the behavior of the parasites from the same genera, from the free-living form to the completely parasite. It might happen because of genetic degeneration. Loss of genetic information and HGT might have contributed to the changes in the relations of mutualism and commensalism (ROTH, 1980; WEISCHER; BROWN, 2001; MAYER et al., 2011).

3.2.3. Possible benefits of parasitism

Parasitism, in some cases, may bring some benefits to the host, but in general they are not as perceptible as the damage. In the Persian Gulf, a research was held with sharks that were in an area contaminated by lead and cadmium. Nine sharks were fished and it was noticed that these were hosting two parasite species, *Anthobothrium* sp. and *Paraorigmatobothrium* sp., both cestodes. They have collected some samples from the sharks' tissues (gonads, intestine, muscle and liver) and some samples from the cestodes for heavy metal concentration. They realized that the parasites had about 278 and 332 times higher cadmium and lead concentration, respectively, for *Anthobothrium* sp, and 455 and 438 times higher concentration respectively for cadmium and lead in *Paraorigmatobothrium* sp. if compared to the shark's muscle tissue (ROTH, 1980; MALEK et al., 2007).

In an experiment with *Pseudacrisregilla* (known as the Pacific Tree Frog), researchers found that in the presence of higher parasite's diversity, the hard effects of the most virulent ones was kind of diluted by the competition with less virulent species. The opposite also happened, if the diversity of parasite is lower, the hosts are more affected by the presence of one more virulent, since there is no competition (JOHNSON; HOVERMAN, 2012).

One interesting characteristic of nematodes found in the soil or in the roots of plants is that they play an important role in the mineralization and in the addition of nitrogenous compounds to the soil. Also, there is a microbiota associated with the worms, and they also mineralize the soil. So, plants that grow in these conditions have a higher growth rate than others that do not (GAUGLER; BILGRAMI, 2004).

3.2.4. Animal's ethological defenses against parasites and pathogens

Animals have developed ways to remove or avoid parasites or pathogens. These ethological defenses include behaviors such as movements to remove ectoparasites, licking, ingestion of medicinal herbs to eliminate worms, fumigation of the nests to expel flies, quarantine (animal style), and immunization (animal style). These behaviors are used sometimes as therapeutic or prophylactic measure. Studies on animal self-medication could be a good way for discovering new medicines, both for humans and animals (HART, 2011; DE DE ROODE; LEFÈVRE; HUNTER, 2013).

One interesting behavior is present in grazing animals. Ruminants and horses have been known to avoid grazing on places close to recent dropped faeces. Carnivores do not defecate in their den. They exit the den to defecate and urinate, in order to avoid contact with the excrements. But the newborns are not so mobile. The female may practice coprophagy quickly after one defecates. The female is not affected by parasites, since she is ingesting eggs, not the infective hatched form. Licking may be another way to avoid diseases. It has been shown that dog's saliva is antibiotic, been effective against *Streptococcus canis* and *Escherichia coli*. Female dogs lick their nipples, in a kind of bactericidal wash against common disease-causing microorganisms, just before the newborn start lactating. Male rats lick compulsively their penis after copulation, in order to avoid sexual transmitted diseases (EZENWA, 2004; HART, 2011).

Some animals grab materials from the environment in order to fumigate their nest. For example, it is has been noted that house sparrows and finches usually grab cigarette butts to their nests. By increasing the levels of nicotine there, the mite infestation is lowered. California dusky-footed wood rats bring leaves of bay to their nest, apparently as a mean of control against an overabundance of fleas. Starlings also bring leaves to their nest when young starlings are present. In this case it is believed that the leaves should retard the hatching of louse eggs and have an antibacterial effect (HART, 2011; DE ROODE; LEFÈVRE; HUNTER, 2013).

Animals are able to make an animal style self-immunization and self-medication. For example, for more than just protecting the mates, nesting area and food resources, territorial defenses may play a role on a type of quarantine, protecting a specific den against a conspecific animal by peripheralizating it, since it may carry strange pathogens with it. Another kind of quarantine not very accounted is the cannibalism of newborns. A sick newborn may be a reservoir of pathogens against its siblings. So, by detecting ill signs like hypothermia and inactivity, the female may cannibalize this animal. This behavior is observed in dogs, cats, and rats. Peripheralizating of a stranger may also play a role on immunization. Both the conspecific and the other animals will experience small samples of foreign pathogens, because of the enforced space barrier. This immunization occurs by faecal droppings and share of same water source. So, the foreign animal and the native animals will experience each other pathogens slowly, being both sides immune to the new pathogens, in case of the stranger is allowed in the group. Another way of slow immunization is seen in mothers that brings prey to the young ones. It surely does help making a bridge between weaning and hunting, but also presents new, yet local, pathogens to the animals. About self-

medication, some animals graze on specific medicinal herbs in order to eliminate parasites in two ways, physically or chemically. It is well noted that dogs, cats, and chimpanzees would eat whole rough leaves to maintain low worm loads, most times in a prophylactic way. Sometimes, the leaves are seen associated with expelled parasites. Other studies show that small-ruminants may graze on tannin-rich forages if they are available, when the infection is present. This grazing of tannin-rich forages declines with the decline of the worm burdens in the animals. So, it's a type of self-medication by the animals. Animals must benefit by the availability of tannin-rich fodder on its pasture, because they would self-medicate (LISONBEE et al., 2009; HART, 2011; JUHNKE et al., 2012).

3.3. Ethnoveterinary

Ethnoveterinary can be categorized as an oral tradition, which has been passed down generation to generation, mostly in places where the access to veterinary services is absent or expensive (TOYANG et al., 2007). Most of the techniques use medicinal herbs, which are easy to find. Plants utilized in the ethnoveterinarian's treatment of livestock against helminthes are rich in secondary metabolites from plants, most of them being tannins, and it's known that tannins play a role as an anthelmintic. An advantage of using tannin-rich plants is that they leave no residual in meat, as chemical anthelmintic drugs would so (BAHUAUD et al., 2006; HOSTE et al., 2006; BRUNET et al., 2007).

3.3.1. Tannins

Tannins are secondary compounds found in plants. They play a role in the plant's secondary metabolism, acting against herbivory and diseases, and also by mimetizing hormones. They are polymers of phenolic monomers, arranged differently from plant to plant. That is why different plant's extracts have different responses in *in vitro* and *in vivo* assays. There are a very wide array of phenolic compounds in plants, from the lignin in the wall of the plant´s cells to the anthocyanidins (flower pigments). Tannins are polyphenolic compounds with well-defined characteristics: they are able to interact and precipitate macromolecules (for example, proteins and enzymes) such as the ones found in the animal skin. Also, it is soluble in water, acetone, and alcohols (BRUNETON, 1999; HAGERMAN, 2002a; BRUNET; HOSTE, 2006).

Tannins are divided basically in two groups, Condensed Tannins (CT) and Hydrolysable Tannins (HT). The CT are polymers formed by the condensation of monomers of flavans. The HT are polymers formed by gallic and ellagic acid monomers and carbohydrate (usually glucose). It is important to note that each plant has its own monomer pattern, producing its own tannin molecule. That might explain why different plants have different anthelmintic results against nematodes (BRUNETON, 1999; HAGERMAN, 2002b; BRUNET; HOSTE, 2006; HAGERMAN, 2010).

Initially, tannin-rich plants were used by mankind in leather tanning process due to its characteristic to bind and alter the spatial arrangement of proteins. Nowadays, it is being used for ethnoveterinary practices, with interesting results. Also, since consumers are these days more concerned about drug residues and animal green production, tannins may play an important role in preventing or treating animals against nematodes in green animal production farms. It also may be used as antimicrobial substance since inhibitory effects against *Staphylococcus aureus* and *Escherichia coli* were found (BAHUAUD et al., 2006; LONE et al., 2013).

3.3.1.1. Leather tanning mechanisms

Knowing the mechanisms of leather production is important to help us to understand how tannins may work against nematodes, because the principles are similar, at least hypothetically. The process aims at making the hides of animals a material heat-resistant, with greater durability and also malleable, which are important characteristics of leather (BRUNETON, 1999).

Briefly, the skin is divided in three layers, the epidermis, dermis, and subcutaneous. About one third of the dermis is formed by collagen. Collagen is a triple helix protein rich in glycine, alanine, arginine, proline, and hydroxyproline amino acids, and it is known that tannins have great attraction to these last two amino acids. Collagen has also a loose arrangement, giving it great motility. The tanning process changes the arrangement of the molecules of collagen, making it rot proof by making new hydrogen and covalent bonds between the collagen fibers, making it stick together. Briefly, it changes the molecular arrangement, giving it new characteristics, different from the ones it had to have before, physiologically speaking (HOSTE et al., 2006; MANN; MCMILLAN, 2013).

3.3.1.2. Tannin's role against nematodes

Tannins play a role acting in different stages of the nematode life cycle. It can get fixed to the proteins (or enzymes) from the egg shell or from the sheath, preventing the hatching and moulting processes, by preventing the enzyme activity or its conjugation to targeted proteins; it can agglutinate to the proline and hydroxyproline amino acids which are found in high concentrations in the oral and vaginal region of the nematodes, preventing the worms from feeding and reproducing. Another way tannins may work is by delaying the exsheathment of the infective larvae. It was found that less than 40% of larvae exsheathed after 160 minutes in cannulated animals fed with 100% diet of the tannin-rich plant sainfoin. Tannins also may increase the amount of bypass protein for the animal, giving it a greater protein supply for its immunological system. In a recent report, Martínez-Ortíz-de-Montellano et al. (2013) showed the action of contact of adult females *H. contortus*' larvae with tanniferous fodders, both *in vitro* and *in vivo*. It was shown that there was aggregations to the larva's cephalic region, anus, vulva and alterations in the cuticle, such as the appearance of longitudinal and transversal folds, thickening, and wrinkling for the *in vitro* test, and changes in the cuticle, such as the folds, wrinkling, and thickening for the *in vivo* assay. This changes may help understand the results found in *in vivo* experiments, like Faecal Egg Count (FEC) reduction, either by damage to the reproductive tract and by damage in the cephalic region which would led to malnutrition, and smaller nematode burdens (BAHUAUD et al., 2006; HOSTE et al., 2006; BRUNET et al., 2007; LISONBEE et al., 2009; MANOLARAKI et al., 2010; HOSTE et al., 2012).

3.3.2. Plants

The extracts utilized in the present work were from *Acacia mearnsii, Myracrodruon urundeuva, Caesalpinea bracteosa* and *Leucaena leucocephala*.

Acacia mearnsii, from the Mimosoideae family, is a leguminous tree native to Australia and is commonly known as Acácia Negra in Brazil. It has been utilized in nitrogen fixation, in the recovery of degraded soil, as firewood, and also as a tannin source, being those usually used in the leather industry (CENTRO DE INTELIGÊNCIA EM FLORESTAS, 2013). Also, some research has been held in *in vivo* and *in vitro* assays. The results from these

authors showed that the plants might have bioactive compounds against nematodes (ALONSO-DÍAZ et al., 2011; CENCI et al., 2007).

Caesalpinea bracteosa, from the Caesalpinoideae family, is widely found in the Brazilian Caatinga. Its common name in Brazil is Catingueira. It is a small slow growing tree, and its wood is often used as flooring, its leaves as fodder for ruminants, and many parts of the plant for ethnomedicine. It was the most cited plant in different population groups in different types of treatment, from the cure of abdominal pain to the cancer treatment (CENTRO NORDESTINO DE INFORMAÇÕES SOBRE PLANTAS, 2013; GOMES; BANDEIRA, 2012).

Myracrodruon urundeuva (which has been classified before as *Astroniam urundeuva*), from the Anacardiaceae family, is present both in Brazilian Caatinga and Cerrado, being commonly known as Aroeira in Brazil. It has 17% of tannins in the bark, therefore considered one of the most resistant woods against putrefaction from Brazilian flora (CARVALHO, 2003). All the tissues of the plant have abundant level of tannins. Those are responsible for the protection of plants against herbivores and microorganisms. They have a healing, antiseptic, antioxidant, and anti-inflammatory actions. The plant has great use in Ethnomedicine in the treatment of gynecological issues, abdominal pain, diarrhea, and conjunctivitis (GOMES; BANDEIRA, 2012).

Leucaena leucocephala, from the Mimosaceae family, is one of the most used leguminous trees as fodders and its common name is Leucena in Brazil. Also, it is used as wood, firewood, green fertilizer, shade, human food, and erosion control. The *L. leucocephala* extract had a fungicidal effect against colonies of the fungus *Leucoagaricus gongulophorus* (SOUZA et al., 2012). In an *in vitro* assay, Alonso-Diaz et al. (2008b) showed that the *L. leucocephala* extract had a dose-dependent effect on the migration of *H. contortus* L_3 larvae.

3.4. In vitro tests

The way compounds will interact with the animal can be assessed through *in vivo* tests. But, in many places, these type of testing may be too expensive, too slow or have many ethics related issues. So, *in vitro* testing may be a good, fast, less laborious, and more economical manner of screening plants of ethnoveterinary potential use. But one must bear in mind that *in vitro* tests may overestimate or underestimate what would happen if tested in the animal, since *in vitro* tests do not respond to the complexity found in animal's physiology. An ideal *in vitro* test is one that has low cost, high sensibility, rapid collection of results and

repeatability. So, one can select plant samples that have showed effectiveness in *in vitro* and test it later in *in vivo* (POWERS et al., 1982; JACKSON; HOSTE, 2010). Also, standard protocols should be followed by different researchers, so the data from the assays may be comparable. In order to determine such standards, Powers et al. (1982) published a guideline to evaluate anthelmintic activity. These authors postulate that groups should have at least three doses of treatment. This guideline was adopted by the World Association for the Advancement of Veterinary Parasitology (WAAVP).

The Brazilian flora has a vast amount of tannin-rich plants that may have use for ethnoveterinary purposes. Quantifying and qualifying the anthelmintic activity of those plants may be valuable, but it may be too expensive to do it first with *in vivo* methods. *In vitro* testing may be a better alternative. After *in vitro* screening, plants that showed great anthelmintic activity would be selected and tested *in vivo* (GOMES; BANDEIRA, 2012; SILVA et al., 2012; FERREIRA et al., 2013).

The Egg Hatch Assay (EHA) is an *in vitro* test that focuses on the egg stage and its hatching. The egg solution are kept in contact with different pre-tested concentrations of plant extracts for 24 hours with suitable conditions for egg hatching. The readings after 24 hours are done in an inverted microscope and it's possible to differentiate the eggs from the larvae. The tannins interact with the collagen from the egg shell, and it may change the morphological structure, making it not able to hatch. Thinking about the nematode cycle, eggs would have contact with tannins in the intestines of the animal and also in its faeces. So, it would not be able to hatch, or hatching taxes would be at least lowered, making it to drop the contamination of the pastures with L_3 larvae, and further to drop the infection taxes of the animals (JACKSON; HOSTE, 2010; FERREIRA et al., 2013). Some examples of reports with different plants tested in *in vitro* are found in Table 1. These reports may be an example of the importance of the EHA in the screening of potential new anthelmintic material.

The Larval Exsheathment Inhibition Assay (LEIA) is an *in vitro* assay that focus on the ecdysis process between the L_3 and L_4 larvae, also known as exsheathment. In the test, an artificially exsheathment stimuli is given to L_3 larvae previously exposed to tannin-rich extracts for three hours. Tannins would interact with the collagen of the cuticle and alter that in a way it wouldn't be able to exsheath. In the *in vivo* process, if an animal is being fed with a tannin-rich fodder, the tax of exsheathment will probably drop, depending on the tannin molecules from the fodder. If the nematodes are not or just some of them are capable to exsheath, the infection will not be established or it will be established, but at lower taxes, which would be still a benefit for the animals (BRUNET et al., 2007; JACKSON; HOSTE,

2010). Some examples of reports in which were tested plant extracts or essential oils against the exsheathment of L_3 larvae are found in Table 1. This reports may show the importance of the LEIA for the screening of tanniferous plants.

Table 1 – Examples of reports that used *in vitro* assays for the screening of plants

Author	Year	Assay	Plant
ADAMU; NAIDOO; ELOFF	2013	EHA	*Brachylaena discolor*, *Melia azedarach*, *Cyathea dregei*, *Milletia grandis*, *Indigofera frutescens*, *Apodytes dimidiate*, *Heteromorpha trifoliata*, *Maesa lanceolata*, *Leucosidae sericea*, *Zanthoxylum capense*, *Clerodendrum glabrum*, and *Strychnos mitis* extracts
ALONSO-DÍAZ et al.	2008a	LEIA	*Acacia pennatula*, *Leucaena leucocephala*, *Lysiloma latisiliquum and Piscidia piscipula* extracts
ALONSO-DÍAZ et al.	2008b	LEIA	*Acacia pennatula*, *Leucaena leucocephala*, *Lysiloma latisiliquum and Piscidia piscipula* extracts
ALONSO-DÍAZ et al.	2011	LEIA	*Acacia gaumeri*, *Brosimum alicastrum*, *Havadia albicans*, *and Leucaena leucocephala* extracts
BAHUAUD et al.	2006	LEIA	*Castanea sativa*, *Erica Erigena*, *Pinus sylvestres*, and *Sarothammus scoparius* extracts
BRUNET et al.	2007	LEIA	*Onobrychis viciifolia* extract
DE OLIVEIRA et al.	2011	EHA	*Myracrodruon urundeuva* extract
DE OLIVEIRA et al.	2011	LEIA	*Myracrodruon urundeuva* extract
DOMINGUES et al.	2013	EHA	*Ananas comosus* industrial residue
FERREIRA et al.	2013	EHA	*Annona muricata* extract
KATIKI	2011	EHA	*Cymbopogon schoenanthus*, *Mentha piperita*, and *Cymbopogon martinii* essential oils
KATIKI	2011	LEIA	*Cymbopogon schoenanthus*, *Mentha piperita*, and *Cymbopogon martinii* essential oils
OLIVEIRA et al.	2011	LEIA	*Anadenanthera colubrine*, *Leucaena leucocephala*, *and Mimosa tenuiflora* extracts
PESSOA el al.	2002	EHA	*Ocimum gratissimum* extract

3.5. Objectives

The objectives of the present work were to determine the bioactivity of the plant extracts of *A. mearnsii*, *M. urundeuva*, *C. bracteosa*, and *L. leucocephala* against the hatching and the exsheathment of the nematode *H. contortus*.

4. MATERIAL AND METHODS

4.1. Plant samples

Myracrodruon urundeuva, *C. bracteosa*, and *L. leucocephala* were harvested from the semi-arid region of the Brazilian state of Pernambuco (latitude 8 ° 4 'S and longitude 34 ° 53' W) in four different counties, namely Floresta, Serra Talhada, Itacuruba and Petrolândia. This region presents two distinct annual periods based in the rainfall rates. The rainfall period lasts from three to four months, between February and May; the drought period lasts from eight to nine months, between June and January. It is worth noting that this drought period may last up to 18 months, because rainfall may be irregular and torrential.

The plants were collected from four different sites in each collection area. In each site, material from five different plants from the same species were collected, emulating the animal's grazing behavior. Three kilograms of leaves and branches were collected from each plant. The fresh material was dried in shade for 96 hours inside cardboard trays. After that, the samples were stored in paper bags and were transported to Center for Nuclear Energy in Agriculture, in Piracicaba, São Paulo, Brazil, where it were reduced to 1,0 mm powder by a Wiley grinder. The powdered material where store inside plastic flasks in a light-free place at room temperature. The plants utilized in the experiment were *M. urundeuva*, *L. leucocephala*, *A. mearnsii*, and *C. bracteosa*. The *A. mearnsii* plant material was obtained commercially from SETA S/A EXTRATIVA TANINO DE ACÁCIA.

4.2. Laboratory facilities

The extracting process and the EHA were held in the Bromatology Laboratory of the Instituto de Zootecnia, in Nova Odessa, São Paulo, Brazil, between June 2012 and April 2013. The LEIA was held in the Animal Nutrition Laboratory of the Center for Nuclear Energy in Agriculture, in Piracicaba, São Paulo, Brazil, between April 2013 and June 2013.

4.3. Bioactive extracts

For the *in vitro* tests, the tannin content from the plants was extracted following the methodology of Barrau et al. (2005). The fresh plant were dried and ground fine, using sieves

of 1 mm drill. Five grams of this material were placed inside a beaker that was later covered with a tinfoil for protection against the sunlight. This material were extracted with 50 mL of a 70:30acetone: distilled water solution for one hour in the magnetic stirrer (Figure 2).The acetone causes cell lyses, letting the molecules of chlorophyll, lipids, and tannins free in the solution (BARBIERI et al., 2010). After this phase, the material was filtrated in a number two filter paper with the aid of a Buchner funnel, a vacuum pump, and a kitasato. The solid residues were discarded and the liquid solution were placed in the BÜCHI HB 140 rotary-evaporator until the acetone evaporated (Figure 3). The water in the rotary-evaporator were at 35 °C. After the acetone evaporated, the resulting solution was placed inside a separating funnel and washed three times in dichloromethane, with 50 mL of dichloromethane each time. Since the dichloromethane is a non-polar solution and more dense (1.325 g/mL at 25 °C) than the water, and the tannins being polar molecules, the tannins are kept in the water phase, and lipids and chlorophyll (non-polar molecules) are discarded with the dichloromethane (SIGMA-ALDRICH, 2012; FAO, 2009). After this washing phase, the extracts were placed in bottles, frozen and then lyophilized in a Labconco - Freeze Dryer 5 lyophilizer (LABCONCO CORPORATION, Kansas City CAT. N. 75050). The *A. mearnsii* extract was bought from SETA S/A EXTRATIVA TANINO DE ACÁCIA. These lyophilized and commercial plant extracts were evaluated in EHA and in LEIA.

Figure 2 - Beaker covered with tinfoil on the magnetic stirrer

Figure 3 - BÜCHI HB 140 rotary-evaporator

The *M. urundeuva* extract were brittle after lyophilization, and through time became and remained a powder. The *C. bracteosa* and *L. leucocephala* were also brittle at first, but within few days, *C. bracteosa*'s became a syrup and *L. leucocephala's* became sticky. They appeared to be highly hygroscopic.

After lyophilization, total phenols (TP), total tannins (TT), and condensed tannins (CT) were determined according to Makkar et al. (2000). Two hundred mg from *C. bracteosa* and *L. leucocephala* and 100 mg from *A. mearnsii* and *M. urundeuva* were taken and placed inside a glass flask. Ten mL of aqueous acetone (70%) were added to each flask and a 20 minutes ultrasonic bath were held at room temperature. The contents were transferred to assay tubes and centrifuged at 3,000 RMP at 4 °C for 10 minutes. The supernatant was collected. From this supernatant all analyses were done.

For TP measurements, suitable aliquots from the supernatant were taken and placed inside assay tubes. The volume was made up to 0.5 mL with distilled water; 0.25 mL of Follin-Ciocalteu 1N, and 1.25 mL of 20% sodium carbonate solution were added. The tubes were agitated and keep in dark for 40 minutes. After that, the absorbance were recorded at 725 nm in a spectrophotometer (DU-64 Beckman). The amounts were expressed in gram equivalent of tannic acid per kilogram of dry matter.

Total tannins measurement was achieved by the binding of polyvinyl pyrrolidone (PVPP) (Sigma P-6755) to the tannin molecules. One hundred mg were weighted and placed in assay tubes, and 1.00 mL of distilled water and 1.00 mL of the supernatant were added and the tubes were agitated. The assay tubes were centrifuged (at 4 °C 3,000 RPM for 10 minutes) and the supernatant from this last solution was taken, and it contained only simple phenols (SP), since the tannins precipitated with the PVPP. The SP solution was read in the spectrophotometer with the same conditions as the last one. The SP measurement were subtracted from the TP, and the result was the TT. Total tannins are also expressed in gram equivalent of tannic acid per kilogram of dry matter.

For the measurement of CT, 0.50 mL of the diluted extract solution were taken and placed inside assay tubes and 3.00 mL of the butanol-HCL reagent (95:5 n-butanol:HCl 37%) and 0.10 mL of ferric reagent (2.00 g of ferric ammonium sulfate diluted in 100 mL of HCl 2N) were added. Tubes were agitated and covered with glass marbles. After, they were placed inside a boiling water bath for 60 minutes. The tubes were cooled after this period and readings were performed in the spectrophotometer, with readings at 550 nm of absorbance. Condensed tannins are expressed in in gram equivalent of leucocyanidin/kg dry matter.

4.4. Egg Hatch Assay

The EHA was done following the methodology from Jackson and Hoste (2010).

4.4.1. Egg recovery

For the recovery of eggs, two animals were kept indoors following the recommendations from “Animal Welfare Approved Standards for Sheep” (MELLOR; HEMSWORTH, 2005) and were mono-infected with *Haemonchus contortus.*

About 15 g of feces were taken directly from the rectum of these sheep, according to Bizimenyera et al. (2006). These feces were mixed to distilled water at 40 °C and a Fecal Aqueous Solution (FAS) was obtained. The FAS solution was passed through a 1.0 mm sieve, separating the solid portion – which was discarded – from the liquid portion (FAS) – which was kept in a bucket. After that, the FAS were passed through a 106 µm sieve, discarding again the solid portion. The remaining FAS were passed through a 53 µm sieve similarly to the previous times and then through a 25 µm sieve and the eggs were kept in this last sieve’s mesh.

The FAS was placed in assay tubes, and then centrifuged for three minutes at 3,000 RPM. After the centrifugation, the upper phase was discarded and it was added saturated saline solution to the assay tubes and a new centrifugation under the same conditions was done. The upper phase was collected in the 25 µm sieve and abundantly rinsed with distilled water. Two new centrifugations process were carried out under the same conditions: after the first, the upper phase was discarded and saturated saline solution was added; after the second, the upper phase was kept in the 25 µm sieve and rinsed thoroughly distilled with water. This last solution will be called Egg Solution (ES).

The ES was placed in a beaker. The concentration of eggs/mL was calculated by reading of five fractions of 20 µL each in an optical microscope, at a magnification of 10x (Figure 4). After that, the mean of number of eggs was calculated as eggs/mL.

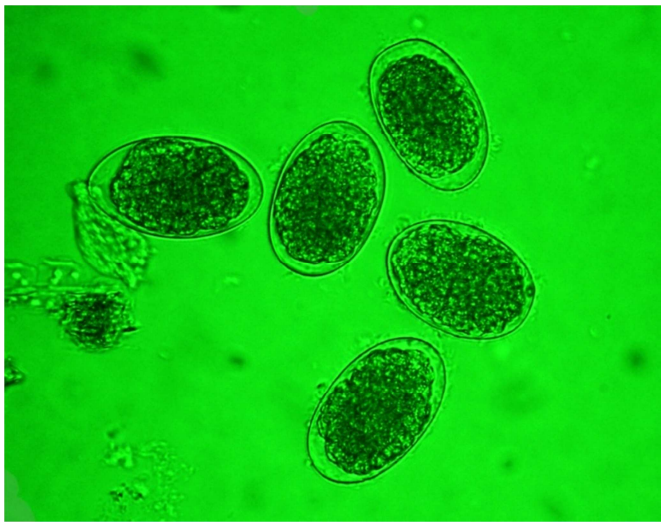

Figure 4 - *Haemonchus contortus* eggs

4.4.2. Preparation of concentrations of bioactive extracts for EHA

The concentrations used in the test were obtained through a pre-test, intending that the higher dose was the Lethal Dose 99% (LD_{99}) and the lower dose was the Lethal Dose 0% (LD_0). So, the concentrations used for *A. mearnsii* were 50, 25, 12.5, 6.25, 3.12, 1.56, 0.78, and 0.39 mg/mL; for *M. urundeuva*, 1.56, 0.78, 0.39, 0.19, 0.09, and 0.04 mg/mL; and for *C. bracteosa*, 6.25, 3.12, 1.56, 0.78, 0.39, and 0.19 mg/mL. It was not possible to find the DL_{50} and DL_{99} for *L. leucocephala* through the pre-test, because the chosen concentrations were not even reaching the DL_{50}; if higher concentrations were chosen, the reading on the microscope

were not viable, since it was extremely dark to differentiate the eggs or larvae from the extract solution. So, for this extract, three concentrations were chosen, namely 6.25, 3.12, and 1.56 mg/mL. Thirty repetitions were done for each concentration, for each extract.

4.4.3. Preparation of the 24-well plates

Twenty-four-well plates were prepared for each extract, with six wells for each concentration at each reading round (Figure 5). An EHA's stock solution (SS_{EHA}) of concentration two times higher the maximum concentration wanted for each extract was prepared, in other words, 3.12 mg/mL, 12.5 mg/mL, 100 mg/mL, and 12.5 mg/mL for *M. urundeuva*, *L. leucocephala*, *A. mearnsii*, and *C. bracteosa*, respectively. Methanol was used to help on the dilution of extracts, on the proportion methanol: SS_{EHA} of 1:50. Dilutions of this SS_{EHA} were done to obtain all other wanted concentrations for each extract. Two hundred fifty microliters of solution with equal to twice higher the real concentration required for that well. Then, 250 µL of an ES with 400 eggs/mL concentration was added to each plate well. By doing that, each well reached the desired concentration. In each well there was around 100 eggs and 500 µL of total solution. A negative and a positive control groups were also prepared. The negative control consisted just of adding 250 µL of distilled water instead of SS_{EHA}. For the positive control a 0.78 mg/mL Thiabendazole solution were used. After that, the well-plates were placed inside an incubator at 27 °C for 24 hours.

Figure 5 - Twenty-four-well plates

4.4.4. Microscope readings for EHA

The readings were performed with the aid of an inverted microscope, at magnitude 10x. The eggs were counted apart from the larvae, and this data was tabulated in files for further statistical analysis.

4.5. Larval Exsheathment Inhibition Assay

The LEIA was done following the methodology from Jackson and Hoste (2010).

4.5.1. Obtainment of larvae

The infective larvae (L_3) of *H. contortus* were obtained through coproculture process, from feces of mono-infected sheep donors, according to Roberts and O'Sullivan (1950). Between ten to 15 grams of feces were collected directly from the rectum of the donors. These feces were moistened with distilled water and homogenized with vermiculite in a 50:50 proportion, in way it became a firm pasty material. This material was then placed inside glass bottles and covered with a plastic film. The bottles were kept away from sunlight and stood at room temperature (20 °C) for one week of incubation.

After seven days, each bottle was fulfilled with distilled water up to the rim, and a Petri dish was placed on the bottle, to seal it. This set was turned upside down, allowing no or just a little air inside it. The empty space of the Petri dish was filled with distilled water. After six hours the distilled water from the Petri dish was collected inside assay tubes, called the Larvae Solution (LS). The concentration in larvae/mL was calculated by reading five fractions of 20 µL each with the aid of an optical microscope, at magnitude 10x.

4.5.2. Preparation of concentrations of bioactive extracts for LEIA

The concentrations for the LEIA test were chosen based on the standard concentrations used in assays from papers on the same field of research. The concentration used for all four plant extracts was 1.200, 600, 300, and 150 µg/mL and also a negative control, that was prepared just with distilled water. Thirty repetitions for each concentration were carried out (ALONSO-DÍAZ et al., 2008a; 2008b; MANOLARAKI et al., 2010).

4.5.3. Preparation of the assay tubes

In order to initiate the assay, plant extract was weighted in a precision balance in the proportion of 2,400 µg/mL. Then, in order to help to dilute the plant extract, methanol was added in the proportion of methanol:stock solution of 1:50. The volume of distilled water was re-calculated and this new volume was added to the assay tube. This solution is now called LEIA's stock solution (SS_{LEIA}) and it had 2,400 µg/mL.

Five assay tubes were taken and identified (Figure 6-A). It was added 1 mL of SS_{LEIA} in the first two tubes (Figure 6-B). Then, in one of them, 1 mL of distilled water was added, dropping the initial concentration in half (Figure 6-C). One mL from this last tube was taken and added in a third tube (Figure 6-D). One mL of distilled water was added to this third tube, reducing the concentration, from 1,200 mg/mL to 600 µg/mL (Figure 6-E). This same procedure was done as many times necessary to acquire all needed concentrations, as can be seen in Figure 6-F to 6-H. One mL of distilled water was added to the tube destined to be the negative control group (Figure 5-I). One milliliter of 1,000 L_3/mL LS was added to each tube, making the concentrations drop in half again, reaching the final desired concentrations (Figure 6-J). It is important to notice that each tube has two mL of solution. After this, the assay tubes were placed inside an incubator at 23°C for 3 hours, each shaken every hour.

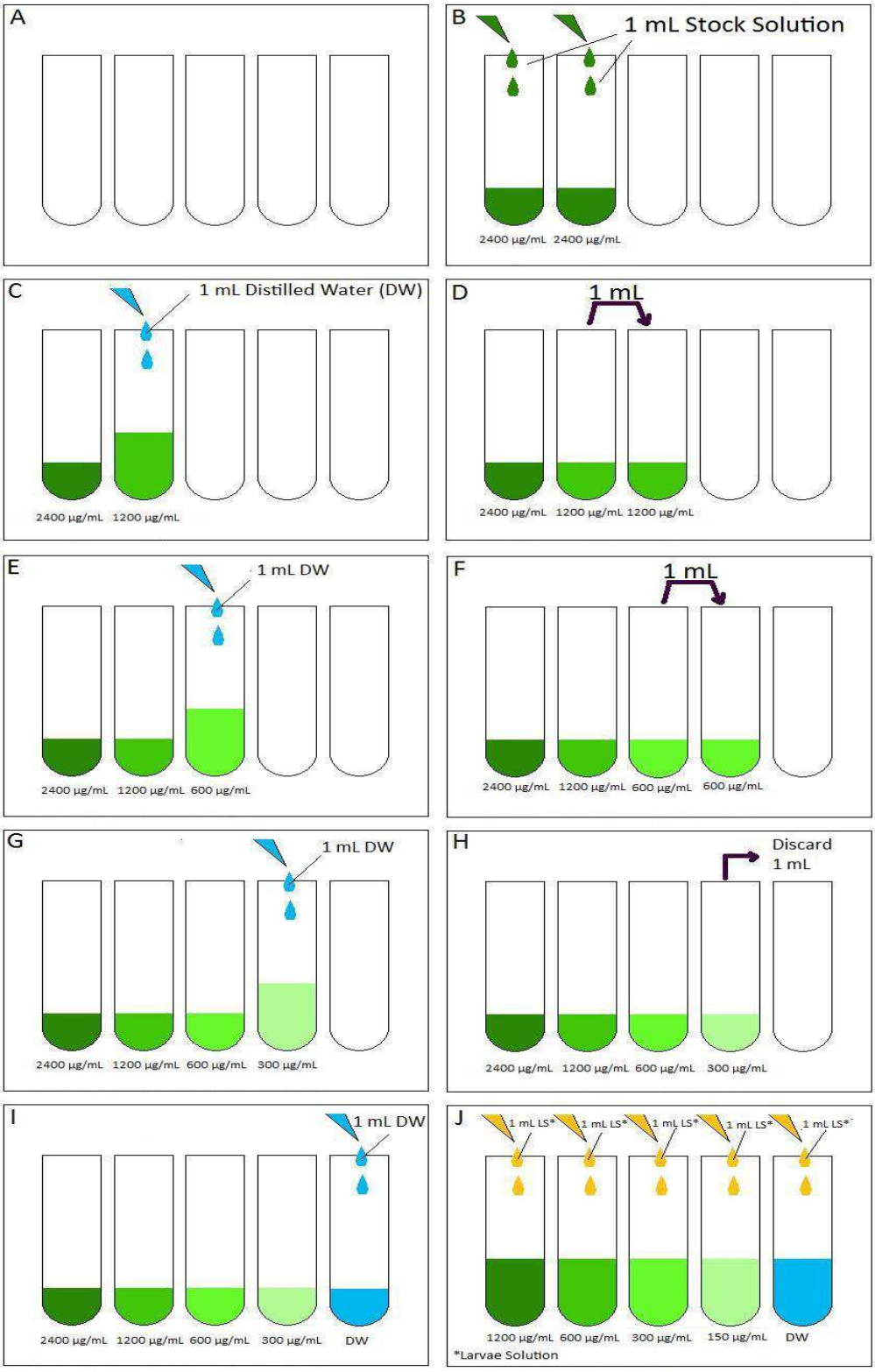

Figure 6 - Scheme for the dilution of the SS_{LEIA}. (A) Five empty tubes. (B) Adding one mL of SS_{LEIA} in each first two tubes. (C) Adding one mL of distilled water in the second tube. (D) Taking one mL of solution from the second tube and adding it to the third tube. (E) Adding one mL of distilled water to the third tube. (F) Taking one mL of solution from the third tube and adding it to the fourth tube. (G) Adding one mL of distilled water in the fourth tube. (H) Discarding one mL of solution from the fourth tube. (I) Adding one mL of distilled water in the last tube. (J) Adding one mL of LS in each tube

After the three hours incubation, centrifugation and washing of the solution from each tube was done (Figure 7). The settings of each centrifugation were 3.000 RPM during three minutes at room temperature. Each washing procedure were done by taking one mL of solution from each tube after the centrifugation and adding one mL of distilled water in each tube, except after the last centrifugation, because at this time no distilled water was added to the tubes. There was a total of four centrifugations intercalated with washings. The remaining volume of each tube was one mL after all centrifugations.

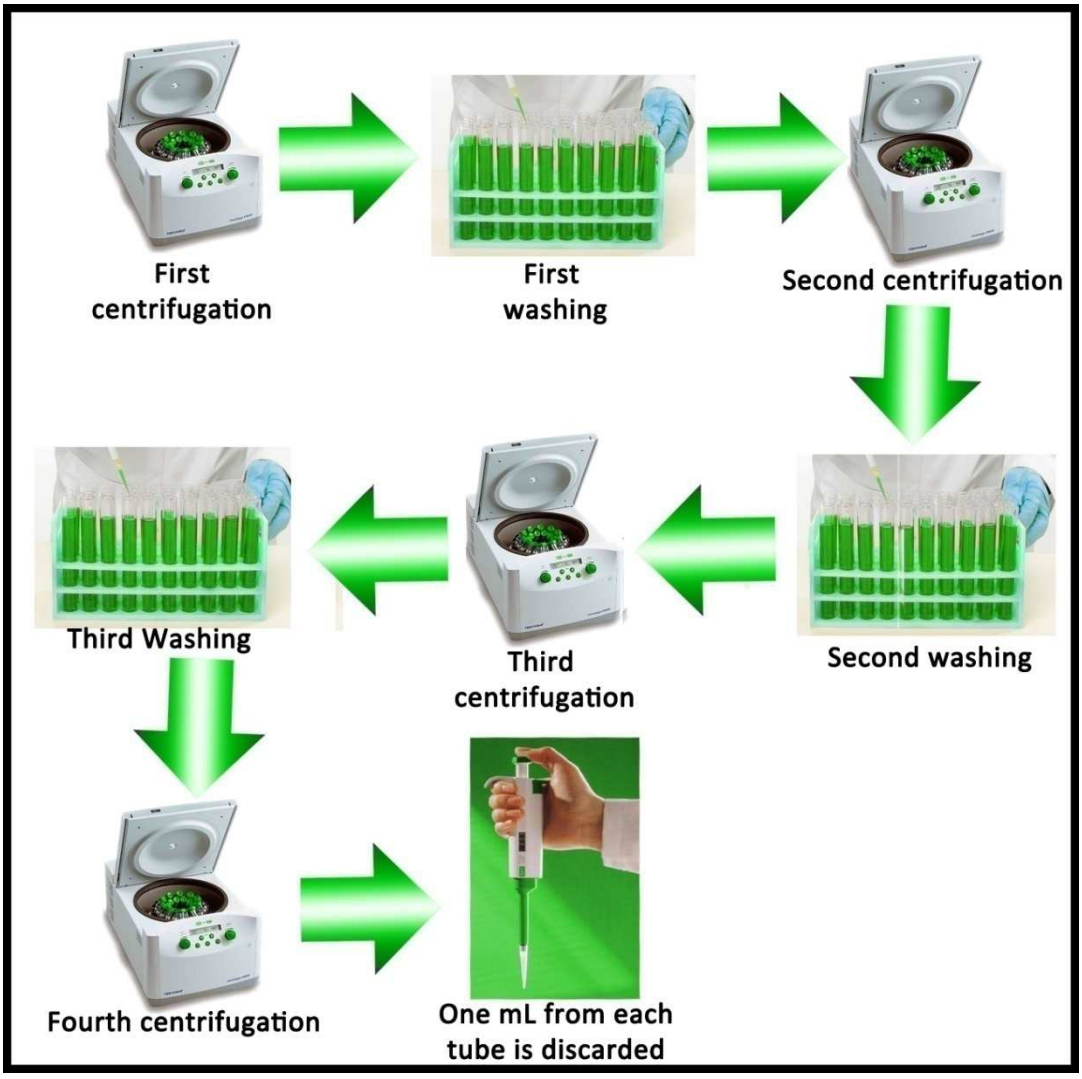

Figure 7 - Scheme of centrifugation and washing of the samples after incubation. The settings of all centrifugations were 3.000 RPM for three minutes at room temperature. The washings consisted of discarding one mL of the solution from each tube and adding one mL of distilled water in each tube, except for the fourth washing that consisted only in discarding one mL of solution from each tube

Four aliquots of 200 µL were taken from each tube and placed inside pre-identified microtubes. There was four microtubes for each concentration of the assay and for the negative control group. Each microtube consisted of a repetition of the assay.

4.5.4. Microscope readings for LEIA

In order to start the assay, 12 glass slides were organized on a plastic tray. These glass slides were separated in four different time-groups, namely Starting Time, 20 Minutes, 40 Minutes and 60 Minutes, and each group had three slides. An aliquot of 30 µL were taken from each microtube and placed on the glass slide, and so the remaining volume of the microtubes was 170 µL. Each slide from the ST group was taken, and the larvae on it were killed by heat stress, with the aid of a candle (Figure 8). The ashes from the bottom of the slide that appeared after the heat stress were cleaned with toilet-paper. One hundred seventy µL of Exsheathment Solution (ES) were added in each microtube. This ES consisted in 2.0% sodium hypochlorite solution and distilled water in the proportion 1:74 (pH 11.02) and its purpose was to emulate the physiological conditions of the gastric apparatus of the ruminants, to stimulate the larvae to exsheath. The one hour period for the assay started at the time the ES solution was added. The readings on the microscope where done at the Starting Time, 20 minutes, 40 minutes, and 60 minutes.

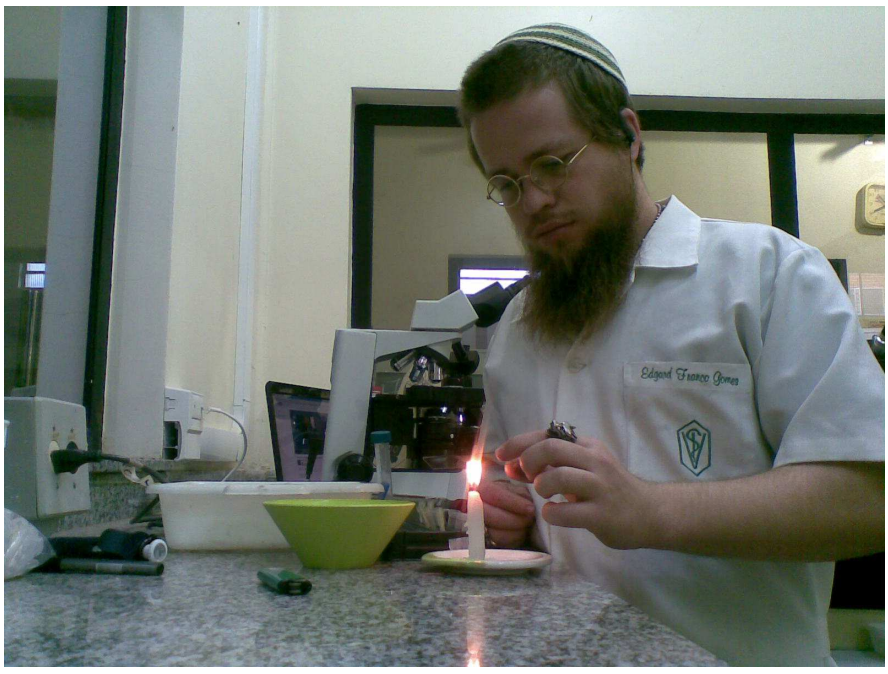

Figure 8 – Killing the larvae by heat stress with the aid of a candle

The readings for the other time-groups were done the same way of the first one: 30 µL of each microtube where taken followed by heat stress to kill the larvae. The material from the slides was read in the optical microscope, at 10x magnitude. The L_3 larvae were differentiated in ensheathed and in exsheathed, and the data was tabulated in files for later statistical analysis.

4.6. Statistical analysis

The SAS® Probit procedure was used to determine the dose effect and to calculate the lethal dose (LD) of each plant extract in both *in vitro* tests. It was performed by fitting regression using normal and logistic distribution. The parameters estimative of these equations were obtained by the maximum likelihood method. Contrasts between doses and negative control were carried out within the GLM procedure.

5. RESULTS

Concerning the yield of the plant extracts, *M. urundeuva* had 15.36%, *C. bracteosa* had 5.79%, and *L. leucocephala* had 7.58% of yield (Table 2). The calculation of yield was not done for *A. mearnsii* because it was obtained commercially. Regarding the condensed tannins (CT), *A. mearnsii* had approximately 32.25%, *M. urundeuva* had approximately 7.27%, *C. bracteosa* had approximately 1.27%, and *L. leucocephala* had approximately 16.74%. These results are found in Table 3.

Table 2 - Yield of the plant extracts

	Dry Plant Material (g)	Bioactive Extract (g)	Yield (%)
M. urundeuva	300.00	46.10	15.36
C. bracteosa	591.00	34.24	5.79
L. leucocephala	360.00	27.31	7.58

Table 3 – Tannin analysis of the extracts

Plant extract	Total phenols*	Total tannins*	Condensed tannins**
A. mearnsii	751.73	700.49	322.5
M. urundeuva	766.61	618.5	72.72
C. bracteosa	434.34	407.68	12.71
L. leucocephala	238.01	177.69	167.49

*Amounts expressed in gram equivalent of tannic acid/kg dry matter.

**Amounts expressed in gram equivalent of leucocyanidin/kg dry matter.

The results for EHA are found in Table 4, Table 5, and Figure 9. Between 90 and 95% of the eggs from the negative controls were hatched after the 24 hours incubation period. From the positive controls, approximately less than 5% of the eggs have hatched in all EHAs after the incubation period. The lowest LD_{50} was found for the *M. urundeuva* extract (0.18 mg/mL) and the highest for *A. mearnsii* (7.20 mg/mL). The same pattern was found for the LD_{99}, the lowest one for *M. urundeuva* extract (4.31 mg/mL) and the highest for *A. mearnsii* extract (187.26 mg/mL). It was not possible to calculate the LD_{50} or LD_{99} value for *L. leucocephala* extracts within the chosen doses.

Table 4 - LD_{50} (mg/mL) and fiducial limits of *A. mearnsii*, *M. urundeuva* extract, *C. bracteosa*, and *L. leucocephala* extracts in Egg Hatch Assay (EHA)

	EHA (mg/mL)			
	A. mearnsii	*M. urundeuva*	*C. bracteosa*	*L. leucocephala*
DL_{50}	7.20 (6.96 - 7.44)	0.18 (0.17 - 0.18)	0.32 (0.31 - 0.33)	*
DL_{99}	187.26 (141.70 - 258.20)	4.31 (3.90 - 4.78)	5.41 (4.98 - 5.93)	*

* It was not possible to calculate this value with the chosen concentrations

Table 5 – Mean egg eclosion (%) of *Haemonchus contortus* eggs. Eggs were previously exposed during 24 hours to different concentrations (mg/mL) of tanniniferous plants extracts

	Concentrations (mg/mL)	Plant extract			
		A. mearnsii	*M. urundeuva*	*C. bracteosa*	*L. leucocephala*
Mean egg eclosion (%)	Negative control	90.53	95.84	93.31	93.05
	0.04	-	96.93	-	-
	0.09	-	63.21**	-	-
	0.19	-	28.44**	87.18*	-
	0.39	91.91	14.40**	23.26**	-
	0.78	90.40	15.07**	10.69**	-
	1.56	89.73	11.69**	6.21**	97.14
	3.12	81.68**	-	6.15**	94.20
	6.25	66.70**	-	4.48**	96.69
	12.50	34.17**	-	-	-
	25.00	5.28**	-	-	-
	50.00	1.46**	-	-	-

- Dosage not calculated for this extract

* Significantly different compared to the respective negative control (P<0.01)

** Significantly different compared to the respective negative control (P<0.0001)

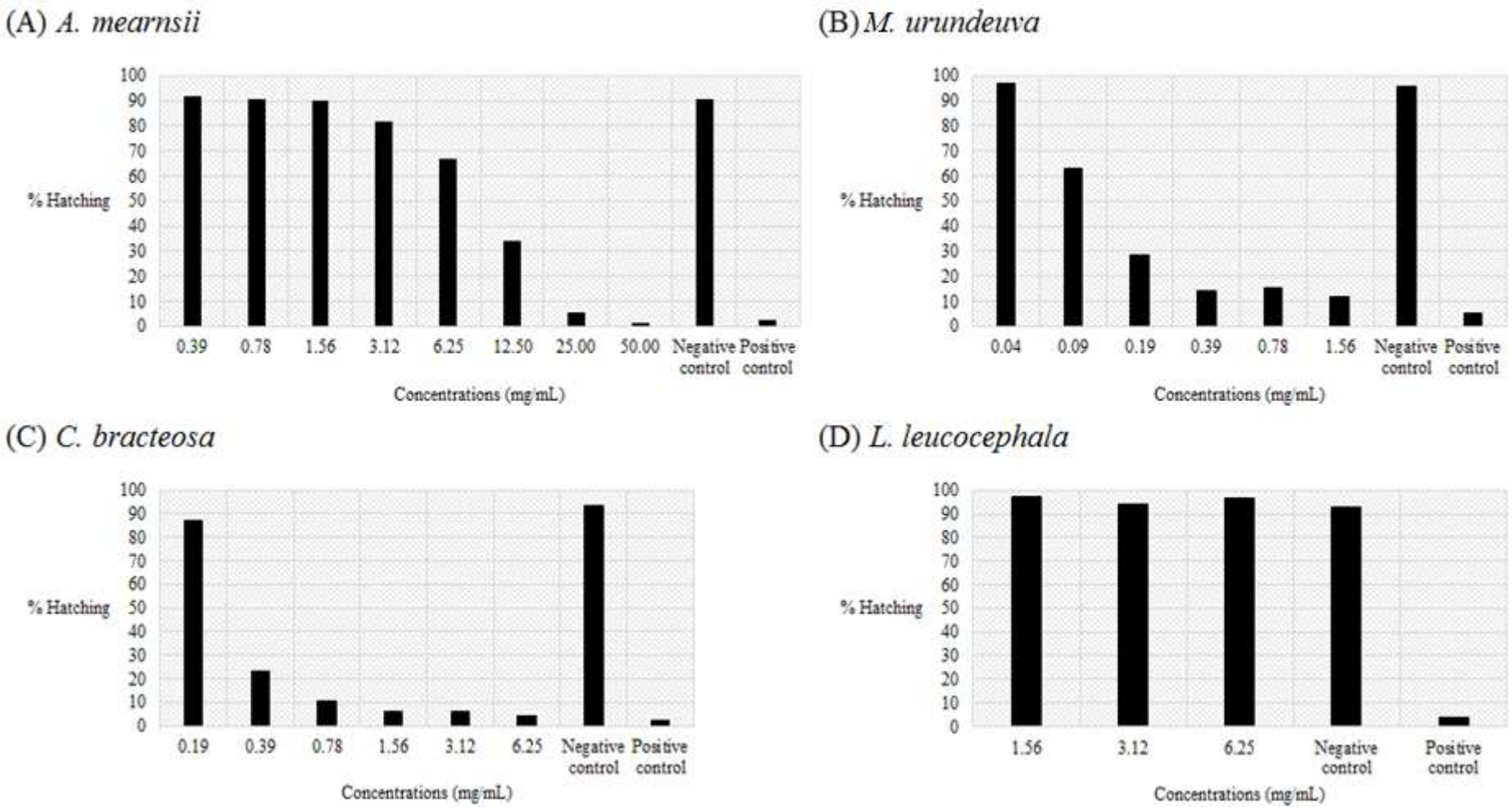

Figure 9 – Effects of 24 hours contact of *H. contortus* eggs with extracts of *A. mearnsii* (A), *M. urundeuva* (B), *C. bracteosa* (C), and *L. leucocephala* (D), on the process of hatching. The LD_{50} was 7.20, 0.18, and 0.32, respectively for *A. mearnsii*, *M. urundeuva*, and *C. bracteosa*. It was not possible to calculate this value for *L. leucocephala* with the chosen concentrations

The results for LEIA are found in Table 6, Table 7, and Figure 10. Between 95 and 99% of the L_3 larvae from the negative controls exsheathed after the contact with the ES. The lowest LD_{50} value found for LEIA was from *M. urundeuva* (0.40 mg/mL) and the highest for *C. bracteosa* (2.24 mg/mL). A slightly different pattern was found for the LD_{99}: *A. mearnsii* had the lowest value (2.28 mg/mL) and *C. bracteosa* the highest (2,538.27 mg/mL).

Table 6 - LD_{99} (mg/mL) and fiducial limits of *A. mearnsii*, *M. urundeuva* extract, *C. bracteosa*, and *L. leucocephala* extracts in Larval Exsheathment Inhibition Assay (LEIA) against *H. contortus*

	LEIA (mg/mL)			
	A. mearnsii	*M. urundeuva*	*C. bracteosa*	*L. leucocephala*
DL_{50}	0.52 (0.49 - 0.54)	0.40 (0.38 - 0.42)	2.24 (1.68 - 3.43)	1.24 (1.14 - 1.38)
DL_{99}	2.28 (2.01 - 2.64)	2.37 (2.03 - 2.86)	2.53×10^3 (5.81×10^2 - 2.39×10^4)	19.99 (14.08 - 31.05)

Table 7 – Mean larval exsheathment (%) of *Haemonchus contortus* infective L3 larvae after one hour incubation with Exsheathment Solution. Larvae were previously exposed during 3 hours to different concentrations (µg/mL) of tanniniferous plants extracts

Plant extract	Mean larval exsheathment (%)				
	Negative control	150	300	600	1,200
A. mearnsii	99.52	97.11	84.56**	46.02***	3.91***
M. urundeuva	99.84	88.61	76.74***	29.85***	1.78***
C. bracteosa	95.96	86.16	79.85*	74.21**	64.71***
L. leucocephala	97.78	94.95	90.64	85.65*	54.59***

* Significantly different compared to the respective negative control (P<0.05)
** Significantly different compared to the respective negative control (P<0.01)
*** Significantly different compared to the respective negative control (P<0.0001)

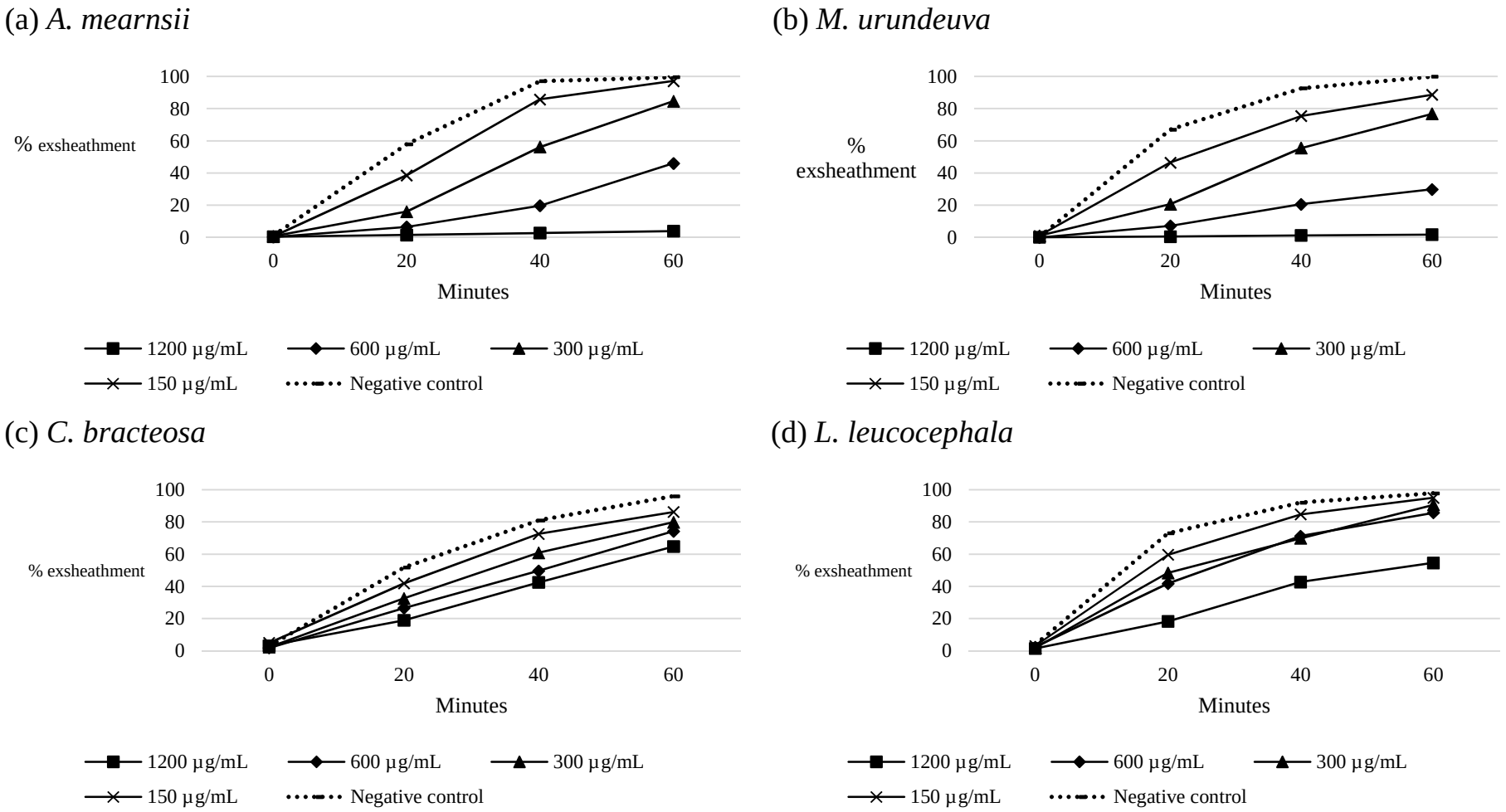

Figure 10 - Effects of three hours contact of *H. contortus* L_3 larvae with extracts of *A. mearnsii* (A), *M. urundeuva* (B), *C. bracteosa* (C), and *L. leucocephala* (D), on the process of artificial exsheathment after addition of Exsheathment Solution. The LD_{50} was 0.52, 0.40, 2.24, and 1.24 mg/mL respectively for *A. mearnsii*, *M. urundeuva*, *C. bracteosa*, and *L. leucocephala.*

6. DISCUSSION

The extract's bioactivity may rely on the CT content quality or quantity from each plant extract. The extract with the highest percentage of CT was *A.* mearnsii (approximately 32.25%), followed by *L. leucocephala* (approximately 16.74%), *M. urundeuva* (approximately 7.27%), and *C. bracteosa* (approximately 1.27%). A dose-dependent inhibition of hatching was found for all extracts, except *L. leucocephala*, within the chosen doses. A dose-dependent effect for the LEIA was also found, either by completely inhibiting the exsheathment or delaying it, these results were more perceptible for *A. mearnsii*, *M. urundeuva*, and *L. leucocephala* and less for *C. bracteosa*.

No mortality was found in the negative control groups with the use of methanol as a solvent. At first, we have tried to dilute the extracts with distilled water, but this was not satisfactory. Later, we tried with Tween 80, but the solution became a foam, preventing the differentiation of the eggs from the larvae. So we tried with a methanol:stock solution (1:50) and it diluted the extracts without killing the eggs or the larvae from both assays.

Most reports use the DL_{50} and DL_{99} to interpret the results from assays (ALONSO-DÍAZ et al., 2011; KATIKI, 2011; ADAMU; NAIDOO; ELOFF, 2013; DOMINGUES et al., 2013). The same pattern from the DL_{50} concentrations has been found for the DL_{99} in the EHA. *Myracrodruon urundeuva* had the lowest concentration for both DL_{50} and DL_{99} (0.18 and 4.31 mg/mL, respectively), followed by *C. bracteosa* (0.32 and 5.41 mg/mL, respectively), and *A. mearnsii* (7.20 and 187.26 mg/mL, respectively). The extracts with the lowest content of CTs were those with the lowest DL_{50} and DL_{99} concentrations, and that may lead to two different assumptions: or the CTs from them have an elevated bioactivity, or the CTs are not the molecules involved in these results. If the CT molecules of *M. urundeuva* have a better bioactivity than the others, this may rely on the kind of monomers from that tannins. According to Brunet and Hoste (2006), the amount of free hydroxyl groups in the monomers of CTs is a key factor, i.e., monomers with more free hydroxyls were more potent inhibitors of the exsheathment of L_3; unfortunately, no test to characterize these monomers was held. Silva et al. (2012) points another characteristic of *M. urundeuva*: it has also a rich fraction content of chalcones, and these molecules also have presented bioactivity, such as molluscicidal and antimicrobial, in the assays performed. Unfortunately, we cannot confirm the role of tannins in present studies, since no test showed the presence of tannin binding compounds, such as PVPP, in the experimental design. Alonso-Díaz et al. (2011) points that it

would be essential to detect if it is the action of tannins or other plant's secondary metabolites. Yet, De Oliveira et al. (2011) used PVPP to check the role of tannins in an experiment with *M. urundeuva* extracts and they found that the bioactivity of the extract was lost after the addition of PVPP. All five highest concentrations from *A. mearnsii*, *M. urundeuva*, and *C. bracteosa* had significantly differed from the negative control ($P<0.0001$). For *L. leucocephala*, no tested concentration had a significant difference from the negative control.

In the LEIA, a different pattern has been found for the DL_{50} and DL_{99}, with the change being between the orders of *M. urundeuva* and *A. mearnsii*. For the DL_{50}, *M. urundeuva* had the lowest concentrations (0.40 mg/mL), followed by *A. mearnsii* (0.52 mg/mL), *L. leucephala* (1.24 mg/mL), and *C. bratecteosa* (2.24 mg/mL). For the DL_{99}, *A. mearnsii* had the lowest concentration (2.28 mg/mL), followed by *M. urundeuva* (2.37 mg/mL), *L. leucocephala* (19.99 mg/mL), and *C. bracteosa* (2,530 mg/mL). The CT content from *A. mearnsii* and *C. bracteosa* were the highest ones and their DL_{50} and DL_{99} may be linked to this content.

Basically, three different categories of results were found for LEIA after 60 minutes: a significant delay in exsheathment may be also an interesting result (BAHUAUD et al., 2006). In the first category, nearly total significant ($P<0.0001$) inhibition was achieved for the 1,200 µg/mL concentration; in the second, an evident delay was found ($P<0.0001$); in the third, there was nearly no significant inhibition of exsheathment if compared to the negative control. Only *A. mearnsii* and *M. urundeuva* achieved nearly total inhibition at 1,200 µg/mL ($P<0.0001$). A delay was found for *A. mearnsii* (600 µg/mL), *M. urundeuva* (600 µg/mL), *C. bracteosa* (1,200 µg/mL) and *L. leucocephala* (1,200 µg/mL) at 60 minutes ($P<0.0001$); *Caesalpinea bracteosa* 600 µg/mL ($P<0.01$) and 300 µg/mL ($P<0.05$) concentrations also had a delay if compared to the negative control ($P<0.01$). The other concentrations did not inhibited the exsheathment of the larvae. Bahuaud et al. (2006) point that a delay in the process may be also an important result. They found that *E. eurigena* and *P. sylvestres* significantly delayed the exsheathment of *H. contortus* after 60 minutes of LEIA. According to Lee (1965), if the process of exsheathment is delayed, the taxes of infection will be dropped, since *H. contortus* has a specific time-frame (around three hours) to exsheath and achieve infection of the host.

Another way of classifying the results from these tests is using the guideline adopted by the WAAVP (POWERS et al., 1982; NERY; DUARTE; MARTINS, 2009; FERREIRA et al., 2013). It has been postulated that a highly effective response from the *in vitro* tests are

those in which are found more than 90% inhibition. If the results ranged between 80 and 90%, the concentration of the extract has moderate effectiveness; from 60 to 80%, low effectiveness. If the results are less than 60%, that dose is ineffective. So, in the EHA, the highly effective concentrations were 50.00 and 25.00 mg/mL (*A. mearnsii*) and 6.25, 3.12, and 1.56 mg/mL (*C. bracteosa*). The moderately effective concentrations were 1.56, 0.78, and 0.39 mg/mL (*M. urundeuva*) and 0.78 mg/mL (*C. bracteosa*). Those with low effectiveness were 12.50 mg/mL (*A. mearnsii*), 0.19 mg/mL (*M. urundeuva*), and 0.39 mg/mL (*C. bracteosa*). All the other concentrations were ineffective according to W.A.A.V.P. guideline (POWERS et al., 1984). About the LEIA, only the 1,200 µg/mL concentration from *A. mearnsii* and *M. urundeuva* was highly effective, no concentration was moderately effective, the 600 µg/mL concentration from *M. urundeuva* had low effectiveness and all other concentrations from all plant extracts were ineffectiveby the standards of the above cited guideline.

The doses in which *M. urundeuva* and *C. bracteosa* extracts showed effectiveness against the hatching were lesser if compared to the effective doses from the extracts others reports. For example, Pessoa et al. (2002) found 100% inhibition of hatching with 5 mg/mL of *Ocimum gratissimum*. De Oliveira et al. (2011) found 97.73% of inhibition for *M. urundeuva* with the dose of 1.25 mg/mL, a dose slightly slower if compared to the one found by us, so we confirm the findings from this author; also, they used PVPP in the assay, in order to confirm the role of CT for this plant. It was found that with the addition of PVPP, the anthelmintic efficacy was lost. Adamu, Naidoo and Eloff (2013) found 100% inhibition with 12.50 mg/mL for *Brachylaena discolor*, *Melia azedarach*, *Cyathea dregei*, *Milletia grandis*, and *Indigofera frutescens*, 100% with 6.25 mg/mL of *Apodytes dimidiate*, 100% with 3.13 mg/mL for *Heteromorpha trifoliata*, *Maesa lanceolata*, and *Leucosidae sericea*, 95% with 12.50 mg/mL of *Zanthoxylum capense*, and 90% with 12.50 mg/mL of *Clerodendrum glabrum* and *Strychnos mitis*. Ferreira et al. (2013) found that *Annona muricata* inhibited 84.91% of hatching at 0.5 g/mL. The effective dose we found for *A. mearnsii* was higher if compared to most of the cited reports. That can emphasize that the quality of the biological molecule may be different between extracts, at least for *M. urundeuva*. *Caesalpinea bracteosa* extract showed low effectiveness in the LEIA and high effectiveness in the EHA; it may imply that the bioactive molecules from it are not CTs, or at least not them alone. Other molecules classes may have a role in this case.

Other reports showed similar or better results with other plants, using lower doses, after 60 minutes of LEIA test, against *H. contortus*. Alonso-Díaz et al. (2011), testing *Acacia*

gaumeri, *Brosimum alicastrum*, *Havadia albicans*, and *L. leucocephala* at 600 µg/mL concentration, found, respectively, 93.50%, 86.00%, 98.10%, and 78.90% of inhibition of exsheathment. Bahuaud et al. (2006) found approximately 100.00%, 70.00%, 42.00%, and 18.00% inhibition, respectively with *Castanea sativa*, *Erica Erigena*, *Pinus sylvestres*, and *Sarothammus scoparius* extracts at 600 µg/mL concentration. Brunet el al. (2007) found for *Onobrychis viciifolia* extract, 50.00% and 90.00% inhibition, respectively for the doses of 600 and 1,200 µg/mL. Alonso-Díaz et al. (2008a) found inhibitions of 51.00%, 53.60%, 49.10% and 63.80% respectively for *Acacia pennatula, L. leucocephala*, *Lysiloma latisiliquum* and *Piscidia piscipula* at 1,200 µg/mL. Oliveira et al. (2011), testing the leaf's and stem's extract of *Anadenanthera colubrine*, *L. leucocephala*, and *Mimosa tenuiflora*, found that all of them inhibited completely the exsheathment of *H. contortus* at the dose of 300 µg/mL. De Oliveira et al. (2011) found a 100.00% inhibition of exsheathment with 300 µg;mL of *M. urundeuva*; this dose was lesser if compared with ours. The results that we found for *L. leucocephala* were not those found in the literature. This may rely on the quality of the fresh leaves, or the period of the year it was harvested.

Differences between the results from the two assays were expected to happen. The egg shell makes the eggs less sensible to the bioactivity of the extracts than the L3 larvae. This may rely on its structure, because it has a harder barrier if compared with the double sheath from the L3 larvae. It is important to notice that the doses from *A. mearnsii* that showed effective bioactivity in the EHA were around 200 times higher than those chosen used in LEIA. For *M. urundeuva*, no dose reached the highly effective class from the guideline, but three of them were moderately effective, i.e., had an efficacy between 80 and 90%, being the lowest one the 0.39 mg/mL; for LEIA, the highest dose (1,200 µg/mL) was classified with highest effectiveness (> 90%). Two things can be inferred from this: again, the eggshell makes the eggs less sensible to the biomolecules, which would make necessary the use of higher doses, and that the quality of CT found in *M. urundeuva* may be more bioactive than the ones from others extracts, or maybe it is due to the presence of chalcones.

The variability in the results, for example for the LEIA's DL_{99} dose from *C. bracteosa*, may have happened because of the number of repetitions. Usually, for EHA only six repetitions are done; for LEIA, only four. We have done 30 repetitions for each assay for each extract. The DL_{50} may have varied day to day. Amarante et al. (1997), Álvarez-Sánchez et al. (2005), and Katiki (2011) also found similar day to day variation in the DL_{50} in *in vitro* assays, including EHA and LEIA among them.

Some authors showed the efficacy of *A. mearnsii* extract in *in vivo* trials. Cenci et al. (2007), giving 18 g of *A. mearnsii* extract per week for the treated group, found a general reduction for both FEC and worm burden (WD) after slaughter in lambs naturally infected with *H. contortus*, *Trichostrongylus colubriformis*, *Oesophagostomum columbianum*, *Cooperia sp*., *Strongyloides papillosus*, *Trichuris globulosa*, and *Moniezia expansa*. Minho et al. (2008) gave 1.6 g/kg of live weight of *A. mearnsii* extract to the treated group of lambs in lambs naturally infected with *H. contortus* and *T. colubriformis*, and also found a reduction in FEC and WD. These are examples of what type of assays may be performed in order to test the plant extracts in *in vivo* experiments; also, they show that tanninferous plants may play a role against parasitic infections.

7. CONCLUSIONS

Anthelmintic activity was found for the extracts, being the *M. urundeuva* the one that showed bioactivity at small doses for both EHA and LEIA. The extract from *A. mearnsii* showed activity at lower doses for LEIA, but for EHA just in higher doses. *Caesalpinea bracteosa* showed better activity for the EHA and *L. leucocephala* for the LEIA. More research is needed to test the toxicity from the plants and then an *in vivo* trial, to see how the polymers from the plants will work when interacting with the animals.

The use of *in vitro* assays may be a good alternative before testing *in vivo*, because through that it is possible to screen potential anthelmintics plants rapidly and economically if compared to the *in vivo* assays.

However, it is hard to produce the purified plant extract, because it demands time, labor, dangerousness (for the working with dichloromethane), and the yield is too low. Also, to obtain the fresh eggs and larvae is too laborious. These are maybe the hardest parts about using *in vitro* assays.

An interesting anthelmintic effect was found for all extracts for at least one of the assays, highlighting the *M. urundeuva* and *A. mearnsii* extracts for both assays. An advantage of using these plants are that they have a potential use as forage, mostly at the North and Northeast regions of Brazil, and also among small producers. Nevertheless, for nutraceuticals use of the plants, toxicity and bioactivity *in vivo* assays must be done. Another option is the use of the extracts as phytotherapy; for that, tests for calculation of dosages must be realized.

8. CONCLUSÕES

Foi encontrada atividade anti-helmíntica para os extratos, de forma que *M. urundeuva* foi a que apresentou biotividade com menores doses tanto para o TIEO quanto para o TIDL. O extrato de *A. mearnsii* mostrou atividade em menores doses para o TIDL, mas em doses mais altas para o TIEO. *Caesalpinea bracteosa* mostrou melhor atividade para o TIEO e *L. leucocephala* para o TIDL. É necessário a realização de mais pesquisas, visando testar a toxicidade das plantas e posteriormente testes *in vivo*, visando-se conhecer como os polímeros das plantas irão interagir com os animais.

O uso de testes *in vitro*, em um primeiro momento, é uma boa alternativa ao uso de testes *in vivo*, no sentido da seleção de plantas com potencial anti-helmíntico mais rápida e economicamente do que a opção de um teste *in vivo* inicialmente.

Entretanto, uma grande dificuldade é a produção dos extratos purificados, pois demanda tempo, labor, possui periculosidade (por se trabalhar com diclorometano) e o rendimento dos extratos é muito baixo. Além das dificuldades em relação a preparação dos extratos, a obtenção de ovos e larvas é bastante trabalhosa. Esses são provavelmente os pontos mais difíceis em se usar testes *in vitro*.

Foi encontrado efeito anti-helmíntico interessante para todos os extratos utilizados pelo menos em um dos testes, destacando-se os extratos de *M. urundeuva* e de *A. mearnsii* para ambos os testes. Uma vantagem em relação a essas plantas é o seu potencial uso forrageiro, principalmente nas regiões Norte e Nordeste do Brasil, e também entre pequenos produtores. Entretanto, para o uso nutracêutico das plantas, testes de toxicidade e de bioatividade das plantas *in vivo* devem ser realizados. Uma possível opção é o uso dos extratos purificados como fitoterápicos; para isso, testes devem ser realizados para o cálculo das dosagens devem ser realizados.

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