

## POMEGRANATE EXTRACTS ON LARVAL INHIBITION OF *Haemonchus* spp AND *Cooperia* spp OBTAINED FROM CATTLE

### EXTRATOS DE ROMÃ SOBRE INIBIÇÃO LARVAL DE *Haemonchus* spp E *Cooperia* spp PROVENIENTES DE BOVINOS

Carolina dos ANJOS<sup>1</sup>; Bruno Toledo SILVA<sup>1</sup>; Luciane HOLSBACK Silveira Fertoni<sup>2</sup>; Camile Sanches SILVA<sup>1</sup>; Leopoldo Sussumu MATSUMOTO<sup>2</sup>; Regildo Márcio Gonçalves da SILVA<sup>3</sup>; Erika Cosendey Toledo de MELLO-PEIXOTO<sup>2\*</sup>.

1. Médico Veterinário do Centro de Ciências Agrárias, Universidade Estadual do Norte do Paraná - UENP, Campus, Bandeirantes, PR, Brasil; 2. Professor, Doutor, Medicina Veterinária e Agronomia do Centro de Ciências Agrárias - UENP, Bandeirantes, PR, Brasil; 3. Doutor, Faculdade de Ciências e Letras de Assis, Laboratório de Fitoterápicos - UNESP, Assis, SP, Brasil. emellopeixoto@uenp.edu.br; regildos@yahoo.com.br

**ABSTRACT:** Consumers increasingly demand natural food and absent of toxic waste and medicated. The aim of this study was to evaluate the aqueous extract of dried and *in natura* stem from pomegranate on the larval inhibition of *Haemonchus* spp. and *Cooperia* spp. obtained from cattle. Dried and *in natura* aqueous extract from pomegranate stem (AEPS), negative control (autoclaved distilled water) and positive control (levamisole hydrochloride 0.05 mg / mL) were evaluated. Four mL of each treatment were used, and the results were evaluated in triplicate by egg counts per gram of feces (EPG), larval culture and counting of the larvae. The mean of the larvae that developed per gram of feces was submitted to logarithmic transformation  $\log_2 (X + 10)$ . The EPG had an average value of 3.620 eggs, identified in larval culture from *Haemonchus* spp. and *Cooperia* spp. Dried AEPS presented, by the reduction of larvae, effectiveness of 92.29% for *Haemonchus* spp. and 96.97% for *Cooperia* spp. *In natura*, this extract showed 80% of efficacy against *Cooperia* larvae, being considered moderately effective; however, for *Haemonchus* spp. this extract was not effective. These results are specially important for organic and agroecological systems of production, that not allow the use of chemical parasiticides. In conclusion, the use of dried AEPS showed potential anthelmintic and may represent important natural therapeutic option.

**KEYWORDS:** Agroecology. Medicinal Plants. *Punica granatum* Linn. Worm.

## INTRODUCTION

Recent surveys point out that Brazil seems to be the largest beef exporter and the sixth largest milk producer (FAO, 2010). However, higher rates of productivity should only be achieved by an efficient parasiticide control.

Gastrointestinal nematode parasitism remains one of the main threats that affect the livestock. These losses are related to poor feed conversion, low weight gain and reduced production of beef or milk and could compromising the animal welfare. These factors provide a strong impetus for the current research on alternative solutions to minimize this problem.

The helminthosis are especially prevalent in development countries, due to the poor practices and inadequate management. The treatment is usually performed using chemically synthesized drugs which have favored the helminth resistance, as well as eliminating residues in the environment (SOUZA et al., 2008; LIMA et al., 2010).

The development of anthelmintic resistance has motivated the search for alternative treatment strategies to parasite control, including the use of

herbal medicines (VIEIRA, 2008). Thus, investigations of natural bioactive compounds represent potential sources to control animal health and food security, reducing the amount and frequency of the use of chemicals products.

Representing a sustainable alternative, the use of medicinal plants, including their antiparasitic properties, have increased as reported in several studies in the field of veterinary medicine (CEZAR et al., 2008; NASCIMENTO et al., 2009; JOSHI et al., 2011; BATATINHA et al., 2011). The search for organic foods has expanded around the world, becoming an important consumer market. This fact has encouraged the farmers, especially in agro-exporters countries, adopting sustainable technologies for productive resources, aiming to accord these consumers. In Brazil, the search for organic products has grown around 10% annually. Consumers are increasingly demanding healthier foods, produced from healthy animals and free of drug residues, from animals reared in systems that promote animal welfare, and which are sustainable and environmentally correct (D'ALMEIDA, 2005; MEDEIROS et al., 2011).

The phytotherapy can be employed as an effective method in the management of gastrointestinal parasites, however studies involving herbal medicine in veterinary medicine still seems to be scarce. The pomegranate, *Punica granatum* Linn. (Punicaceae), is a deciduous shrub or small tree commonly cultivated worldwide in tropical and subtropical climates. The pomegranate tree can be extremely long lived (around 100 years old) and could be found throughout Brazil, although in small amounts. Their leaves are glossy and lance shaped, while the bark turns gray as the tree ages. The edible fruit is a large, globose berry, red-green or violet when ripe. It presents numerous angular seeds, covered by a thick skin, pulpy, with sweet taste that is slightly acidic, with their edible pulp, tasty and refresher (GOMES, 2007).

Healthy benefits of pomegranates have largely been attributed to their antibacterial and anti-inflammatory properties. Their main phytochemicals compounds are alkaloids (pelletierine, isopeletierine, methylpelletierine), tannins, phenolic compounds (anthocyanins, quercetin, phenolic acids), flavonoids, ascorbic acid, conjugated fatty acid and ursolic acid (LANSKY; NEWMANN, 2007). Otero and Hidalgo (2004) reported that tannins may exert direct anti-parasitic properties by reducing the fecundity of female nematodes, or indirectly, by increasing the immune response to protect the dietary protein from rumenal degradation, increasing their availability in the large intestine (KETZIS, 2006). The *in vitro* anthelmintic activity of tannins was characterized by reduced hatch, development, motility of larvae (JOSHI et al., 2011) and adults. *In vivo*, they cause reductions in EPG (MAX et al., 2009; JOSHI et al., 2011.) and parasitic infection (MINHO et al., 2008; MAX et al., 2009). The tannins are not absorbed by the gastrointestinal tract of small ruminants, occurs disposal in feces and it reduces the pasture contamination (MUPEYO et al., 2011).

The tannins anthelmintic efficacy can also be influenced by the developmental stage of the parasite (HOSTE et al., 2006; BRUNET et al., 2008). The tannins affect the larval establishment (L3) and consequently block the infection. When the host is infected with adults, the function of nematode reproductive changes, reducing the EPG and pasture contamination (MARTÍNEZ-ORTÍZ-DE-MONTELLAN et al., 2010; JOSHI et al., 2011). These effects cause significant prospects for the control of nematodes by consumption of tanniniferous plants (OLIVEIRA et al., 2011). Therefore, the pomegranate has gained renewed interest as potential anti-parasitic due to their main

component, the tannins. The great variability observed in relation to the anthelmintic activity of tannins effects in ruminants determines important perspectives for the control of nematodes and drug-resistant populations to most of the anthelmintic drugs available. Thus, the present study aimed to determine the efficacy, *in vitro*, of the aqueous extract of pomegranate's stem bark, on larvae of *Haemonchus* spp. and *Cooperia* spp. from cattle.

## MATERIAL AND METHODS

For preparation of the aqueous extract of pomegranate's stem bark, the plant material was collected on September 2011 at Bandeirantes - PR. Plants were harvested at the end of the morning, with temperatures about 25 °C and 80% of relative humidity.

In the Essential Oils & Biotherapics laboratory from North of Paraná State University, the material was peeled, macerated and packed in craft paper bag. The drying was performed in a forced air incubator at 60 °C until the pomegranate stems reach a constant weight, obtaining the dried extract after 72 hours. Obtaining of the *in natura* extract did not considered this drying process.

To prepare the aqueous extract, 50g of plant material were weighed on an analytical balance and immersed in 300 mL of autoclaved distilled water, thus were obtained the concentration of 12.5% (w/v).

The boiling was proceeded in water bath at 70 °C for 60 minutes and subsequent filtration was made using hydrophilic cotton. The extract was placed in amber sterile bottle and stored frozen in a freezer at -20 °C (JARDINI et al., 2007).

To determine the antioxidant activity (AA%), total phenol and flavonoids, the extracts were referred to the Phytoterapic Laboratory of the Department of Biotechnology, from Paulista State University - UNESP, Assis. After lyophilization, the extracts were diluted at concentrations of 25, 50, 75, 100, 250, 500 and 1000µg/mL and evaluated in triplicate.

The antioxidant activity of the extracts was determined by the donor ability of H<sup>+</sup> to the stable radical DPPH (2, 2-diphenyl-1-picrylhydrazyl), according to the *in vitro* methodology proposed by Blois (1958). The DPPH assay method is based on the reduction of DPPH, a stable free radical, from violet to yellow color. The test is sensitive enough to detect low concentrations of active ingredients (MANIAN et al., 2008), and the result can be viewed by the discoloration degree of the reagent after 30 minutes required for the reaction to reach a

plateau, beyond the low value of  $IC_{50}$ ; in other words, the ability of the extract to inhibit 50% of the stem oxidation (DI MAMBRO; FONSECA, 2005). It was used 1 mL of acetate buffer solution (pH 5.5 and 100 mM), 1.25 mL of ethanol PA, 250 $\mu$ L of DPPH solution (250 $\mu$ M) and 50mL of the samples. The DPPH shows maximum absorbance at 517 nm, which decreases in the presence of  $H^+$  donor molecules, indicated by the violet color changes to yellow. The extract reacts with the DPPH radical in low light environment, and then is subjected to ultraviolet visible spectrophotometer (UV-Vis) at a wavelength of 517 nm (BRAND-WILLIAMS et al., 1995). The calculation of the antioxidant activity was performed according to the formula:  $Antioxidant\ Activity\ (\%) = [(A_{control} - A_{sample})/A_{control}] * 100$ , where  $A_{sample}$  is the absorbance of the samples after 30 minutes and  $A_{control}$  is the absorbance of DPPH; both at 517 nm.

For the total phenols determination, the method used was *Folin-Ciocalteu*, using gallic acid as standard to comparison. To each 0.5 mL of the extract was added 5 mL of distilled water and 0.25 mL of *Folin-Ciocalteu* reagent (molybdate, tungstate and phosphoric acid). After 3 minutes, was added 1 mL of saturated  $Na_2CO_3$  at 10%, and the mixture was stored for one hour. The absorbance was measured at 725nm using a UV-Vis spectrophotometer. The results were expressed as milligrams of gallic acid per gram of extract. Gallic acid is a precursor of several types of phenolic compounds and has a simple structure, being considered the standard substance.

The determination of total flavonoids of the extract was determined by UV-Vis spectrophotometer. Samples were prepared according to the Zhishen et al. (1999) methodology, based on the complexation of flavonoids with  $AlCl_3$ , causing displacement of the absorption bands to longer wavelengths. A 250 $\mu$ L aliquot of the extracts concentrations that was above mentioned was added to 1.25 mL of distilled water and 75 $\mu$ L of a  $NaNO_2$  solution at 5%. After 6 minutes, 150 $\mu$ L solution  $AlCl_3/H_2O$  at 10% was added. After 5 minutes, 0.5 mL of 1M NaOH solution was added, and the total volume completed with 2.5 mL of distilled water. The samples were vortexed and the absorbance measured at 510 nm. The results were expressed in milligrams of rutin per gram of extract. The rutin, as the quercetin, presents the flavonoids basic structure and can be used as an indirect indicator of flavonoids.

Tannins content of the extract was estimated by following the method as described by Fagbemi et al. (2005). The extract (1 mL) was mixed with

Folin- Ciocalteu's reagent (0.5 mL), followed by the addition of saturated  $Na_2CO_3$  solution (1 mL) and distilled water (8 mL). The reaction mixture was allowed to stand for 30 min at room temperature. The supernatant was obtained by centrifugation and absorbance was recorded at 725 nm using UV-Visible Spectrophotometer. Tannins content was calculated as mg tannic acid equivalent from a linear regression equation obtained from a calibration curve ( $R^2 = 0.99304$ ):  $Abs_{725nm} \wedge 0,0696 \wedge [TA](mg)$  where  $[TA](mg)$  is the concentration of tannic acid taken as standard.

The feces were obtained from eight female cross-bred calves, three months old, that were never subjected to anthelmintic treatment before. These animals came from a Dairy farm located at Paraguaçu Paulista/SP. Feces were collected directly from the rectum, stored in plastic bags and transported in cool boxes at approximately 4 °C.

At the Laboratory of Parasitology from UENP, the egg counts per gram of feces (EPG) were performed, according to the methodology of Gordon and Whitlock (1939), and larval culture by the technique described by Roberts and O`Sullivan (1950) modified. It was used 4g of feces, 8g of sterile pine sawdust, and 4 mL of the respective treatments, as the dried aqueous extract from pomegranate stem (AEPS), *in natura* AEPS, Levamisole (0.05 mg/mL) as positive control and autoclaved distilled water (as negative control). The larval culture were performed in triplicate and coded, to the experiment be performed blind. They were incubated for ten days in a Biochemical Oxygen Demand (BOD) micro processed incubator, at the temperature of 28 °C.

The larvae were collected according to the methodology described by Roberts and O'Sullivan (1950), placed in conical centrifuge tube and subjected to cooling. The content obtained from larval culture was measured. Then, counting and identification of the larvae was performed in 50  $\mu$ L of the solution by optical microscopy, following the criteria of Keith (1953). The total number of larvae was calculated based on the total volume of the larvae solution to each treatment (UENO; GONÇALVES, 1998).

To obtain the value of the larvae developed per gram of feces (LDPG), the total number of larvae observed in each treatment was divided by four (assuming that 4g of feces used). The percentage of reduction in LDPG in each extract was determined by reducing the count of larvae in feces (RCLF) compared to the negative control

group by the formula: % efficacy = [1 - (LDPG mean in the treated group/ LDPG mean in the control group)] x 100. The effectiveness was classified as highly effective (> 98%), effective (98-90%), moderately effective (89-80%) and insufficiently effective (<80%), as established by Wood et al. (1995).

For statistical analysis, the LDPG mean was transformed in logarithmic, Log (x+10). It was used a completely randomized design, analysis of variance and Tukey's test at 5% significance level, by GraphPrism 5 program.

**Table 1.** Average developed larvae per gram of feces (LDPG) transformed to Log (X + 10), the dried and *in natura* aqueous extract of pomegranate stem treatments (AEPS), distilled water and levamisole.

TREATMENTS	LDPG <i>Haemonchus</i> (Log.)	LDPG <i>Cooperia</i> (Log.)	LDPG Total of Larvae (Log.)
Dried AEPS	0.20 <sup>b</sup>	1.39 <sup>b,c</sup>	1.41 <sup>b,c</sup>
<i>In natura</i> AEPS	1.21 <sup>a,b</sup>	2.36 <sup>a,c</sup>	2.42 <sup>a,c</sup>
Distilled water	1.31 <sup>a,b</sup>	3.07 <sup>a</sup>	3.09 <sup>a</sup>
Levamisole	0.33 <sup>a,b</sup>	0.81 <sup>b</sup>	0.96 <sup>b</sup>

Means followed by the same lower case letter(s) in the column do not differ significantly between each other at 5% level of probability by the Tukey test.

The effectiveness of the treatments could be analyzed according to the values supported by the Ministry of Agriculture, Livestock and Food Supply, in the recommendations contained in

## RESULTS

At the moment before the treatments, the EPG presents a mean value of 3,620 Strongylid-type eggs, representing massive infection (UENO; GONÇALVES, 1998). After using treatments, the number of LDPG of *Haemonchus* spp and *Cooperia* spp was lower for dried AEPS treatment, compared with the other extracts (Table 1). Regarding the results for the *Haemonchus* spp, any treatment showed difference when compared to the negative control.

Ordinance nº 48, on 12 May 1997. Thus, the dried AEPS was effective for the helminths assessed (Table 2) in this study.

**Table 2.** Percentage reduction of developed larvae per gram of feces (effectiveness) regarding the treatment of dried and *in natura* aqueous extract of pomegranate stem (AEPS), and levamisole.

TREATMENTS	% <i>Haemonchus</i>	% <i>Cooperia</i>	% Total of Larvae
Dried AEPS	92.29	96.97	96.84
<i>In natura</i> AEPS	42.20	80.24	79.19
Levamisole	88.87	99.34	99.05

Concerning to the physico-chemical analyzes, dried AEPS showed antioxidant activity from the concentration of 50 µg/mL, with values corresponding to 2.89%, reaching 68.41% at the concentration of 1000 mg/mL.

Total phenol analysis presented 0.15 mg of gallic acid/g of extract to 100 µg/mL, and 0.37 mg of gallic acid/g of extract for concentration of 1000 µg/mL. For the content of flavonoids, only the concentration of 1000 µg/mL showed values corresponding to 0.06 mg of rutin/g of extract. In relation to the tannins content (mg/g dry weight), the AEPS presented 278,88 mg/g which represented 27,88%.

## DISCUSSION

Turning to the levamisole, the low efficacy could be explained by the parasitic resistance to the pharmacological basis of this drug. According to Sczesny-Moraes et al. (2010), these resistances could be observed in the genera *Haemonchus*, *Trichostrongylus*, *Cooperia* and *Oesophagostomum*, in greater or lesser intensity, being the genera *Haemonchus* the most resistant.

The interest in antioxidants is present, mainly due to the effects of free radicals in the organism, which are produced from the oxidations, essential for aerobic life. Free radicals react with DNA and RNA, proteins and other oxidizable substances, promoting damage that could contribute to the cellular aging (PEREIRA et al., 2009).

Perhaps the presence of other substances alkaloids present in the extracts may have been responsible for the antioxidant activity observed. Noda et al. (2002) evaluated acetone extract of *P. granatum* and pointed the contribution of three anthocyanins (delphinidin, cyanidin and pelargonidine) for the antioxidant activity. They noted that these anthocyanins inactivate the superoxide and inhibit the lipid peroxidation induced by H<sub>2</sub>O<sub>2</sub>.

Phenolic compounds are one of the main groups of molecules that act as antioxidants, being found in pomegranate extracts. The flavonoids, one of the most diverse and widespread phytochemicals groups, are probably the most important natural compounds, possessing a wide spectrum of chemical products and biological activity, including free radical scavenging properties (PEDRIALLI et al., 2010).

Lorenzi and Matos (2002) performed phytochemical analysis of the pomegranate tree, recording the presence of up to 28% of gallic tannins in the stem bark and fruit, and to a lesser extent, in leaves. Hoste et al. (2006), have assimilated the tannins anthelmintic activity to reduce hatching, larval development and motility, and decreased in adults' motility. Additionally, they reported the larval unshathing, which was also observed in this present study.

Corroborating to these results, Mupeyo et al. (2011) also noted the influence of tannins on the larval hatching and development of nematodes. Also considered that, as tannins are not absorbed by the small ruminants' gastrointestinal tract, your elimination occurs by feces and could reduce pasture contamination. According Athanasiadou et al. (2001) the tannins are complexed with abomasum protein, being unavailable to exert abomasal anthelmintic activity. Therefore, further studies must be performed to evaluate the *in vivo*

efficacy in the control of *Haemonchus* spp, characterized by parasitize the abomasum.

One of the problems in the phytoterapics research is the variation of the herbal active principle in plants depending on the season, stage of development, geographical location, collection and preparation, causing considerable changes and compromises the quality and quantity of these ingredients (ROZWALKA et al., 2008). Thus, *in vitro* studies are justified as preliminary essential processes to the study of new natural parasitocidal drugs with potential for clinical application. Natural bioactive substances can be too much important for the control of animal health and food safety, and could help to decrease the amount or frequency of use the chemical products. This aspect is particularly important for agroecological, organic or biological-dynamic production systems, which the use of chemical drugs is considered a limiting factor.

## CONCLUSION

The use of aqueous extract of the pomegranate's stem bark, mainly the dried extract, revealed *in vitro* efficacy in the control of bovine worms showing up a potential anthelmintic to contribute as adjunctive therapy to control this bovine disorder.

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**RESUMO:** Os consumidores têm exigido cada vez mais alimentos naturais e ausentes de resíduos medicamentosos. O objetivo deste estudo foi avaliar o extrato aquoso de caule de romã seco e *in natura*, sobre a inibição larval de *Haemonchus* spp. e *Cooperia* spp. provenientes de bovinos. Foram avaliados extrato aquoso de caule de romã (EACR) seca e *in natura*, controle negativo (água destilada autoclavada) e controle positivo (cloridrato de levamisol 0,05 mg/mL). Foram utilizados quatro mL de cada tratamento, e os resultados foram avaliados em triplicata, por meio de contagem de ovos por grama de fezes (OPG), cultura de larvas e contagem das larvas. A média de larvas desenvolvidas por grama de fezes foi submetida à transformação logarítmica Log. (x+10). A contagem de OPG apresentou valor médio de 3.620 ovos, sendo identificadas na cultura de larvas de *Haemonchus* e *Cooperia*. EACR seca apresentou por meio da redução de larvas, eficácia efetiva de 92,29% para *Haemonchus* e 96,97% para *Cooperia*. *In natura*, este extrato apresentou 80% de eficácia sobre larvas de *Cooperia*, sendo considerado moderadamente efetivo, porém, para *Haemonchus* o mesmo não foi efetivo. Esses resultados são especialmente importantes para os sistemas orgânicos e agroecológicos de produção, que não permitem a utilização de parasiticidas químicos. Concluiu-se que a utilização de EACR seca apresentou potencial anti-helmíntico, podendo representar importante opção terapêutica natural.

**PALAVRAS-CHAVE:** Agroecologia. Plantas Mediciniais. *Punica granatum* Linn. Verminose.

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