

## IN VITRO OVICIDAL AND LARVICIDAL ACTIVITY OF *PSIDIUM CATTLEIANUM* SABINE LEAVES AGAINST GASTROINTESTINAL NEMATODES OF SHEEP<sup>1</sup>

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**ABSTRACT:** Gastrointestinal nematodes can affect sheep productivity via severe weight loss, anemia and death. Infection control is generally performed with synthetic anthelmintic compounds; however, indiscriminate use of these drugs can stimulate the emergence of resistant nematodes. Thus, alternatives to the use of synthetic anthelmintic compounds have been proposed, such as the inclusion of medicinal plants in animal feed. The objective of this study was evaluate the anthelmintic activity of the leaves of the medicinal plant *Psidium cattleianum* Sabine (Araçá) through its hydroalcoholic extract, and using *in vitro* assays with the eggs and larvae of gastrointestinal nematodes obtained from naturally infected donor sheep. The extract exhibited good *in vitro* ovicidal and larvicidal activity, with IC<sub>50</sub> values of 0.55 mg mL<sup>-1</sup> for egg hatching inhibition, 0.20 mg mL<sup>-1</sup> for larval development inhibition and an efficiency greater than 80% in the inhibition of larval migration at all concentrations evaluated (IC<sub>50</sub> < 0.19 mg/mL). Phytochemical analysis detected higher concentrations of saponins, flavonoids, cardiac glycosides, anthraquinones and tannins in the extract. Our results demonstrated that the *Psidium cattleianum* Sabine's leaves exhibit *in vitro* anthelmintic activity, which suggests that in addition to its other medicinal properties, this plant can help control gastrointestinal nematodes in sheep. Future *in vivo* assays should be performed to confirm antiparasitic efficacy.

**Key words:** Araçá, Phytotherapy, *Haemonchus contortus*

## ATIVIDADE OVICIDA E LARVICIDA IN VITRO DAS FOLHAS DE *PSIDIUM CATTLEIANUM* SABINE CONTRA NEMATÓDEOS GASTRINTESTINAIS DE OVINOS

**RESUMO:** Os nematódeos gastrintestinais podem afetar a produtividade dos ovinos por meio de perda de peso severa, anemia e morte. O controle de infecção é geralmente realizado com compostos anti-helmínticos sintéticos; entretanto, o uso indiscriminado dessas drogas pode estimular o surgimento de nematódeos resistentes. Assim, alternativas ao uso de compostos anti-helmínticos sintéticos têm sido propostas, como a inclusão de plantas medicinais na alimentação animal. O objetivo deste trabalho foi avaliar a atividade anti-helmíntica das folhas da planta medicinal *Psidium cattleianum* Sabine (Araçá) através de seu extrato hidroalcoólico, e utilizar ensaios *in vitro* com os ovos e larvas de nematódeos obtidos de ovelhas doadoras naturalmente infectadas. O extrato exibiu boa atividade ovicida e larvicida *in vitro*, com CI<sub>50</sub> de 0,55 mg mL<sup>-1</sup> para inibição da eclosão de ovos, 0,20 mg mL<sup>-1</sup> para inibição do desenvolvimento larval e eficiência superior a 80% na inibição da migração larval em todas as concentrações avaliadas (CI<sub>50</sub> < 0,19 mg mL<sup>-1</sup>). A análise fitoquímica detectou maiores concentrações de saponinas, flavonoides, glicosídeos cardíacos, antraquinonas e taninos no extrato. Nossos resultados demonstraram que o extrato das folhas de *Psidium cattleianum* Sabine exibe atividade anti-helmíntica *in vitro*, o que sugere que, além de outras propriedades medicinais, esta planta pode ajudar no controle de nematódeos gastrintestinais em ovinos. Futuros ensaios *in vivo* devem ser realizados para confirmar a eficácia antiparasitária.

**Palavras-chave:** Araçá, Fitoterapia, *Haemonchus contortus*

## INTRODUCTION

One of the main problems in sheep production is infection caused by gastrointestinal nematodes (GINs) such as *Haemonchus contortus*, which can cause anemia, weight loss, and the death of animals (PLOEGER et al., 2016). Sheep vermifugation is essentially chemical, using drugs belonging to the chemical groups of benzimidazoles, imidothiazoles and avermectins (ABONGWA et al., 2017). However, indiscriminate and non-epidemiological use of these drugs may stimulate anthelmintic resistance, rendering it even more difficult to control parasitosis (TORRES-ACOSTA et al., 2012; AGUERRE et al., 2018).

An alternative that has attained some attention is the inclusion of medicinal plants in the diet of animals. Several researchers have tested the plants used in folk medicine, evaluating their efficacy and safety through *in vitro* assays (FÉBOLI et al., 2016; MENGISTU et al., 2017; OLIVEIRA et al., 2017) at various stages of parasites' lives and *in vivo* with naturally infected and artificially infected sheep (KANOJIYA et al., 2015; KATIKI et al., 2017; BARONE et al., 2018). The anthelmintic properties of the plants are generally related to the presence of phytoconstituents such as tannins, flavonoids and saponins, which are present in larger amounts than other components. Some authors report that the activity observed in these plants may be related to the existence of synergism between tannins and saponins (HERNÁNDEZ-VILLEGAS et al., 2011; MARIE-MAGDELEINE et al., 2014; MENGISTU et al., 2017).

Tannins cause the coagulation of proteins, conducting to the death of parasites, however, the presence of other phytoconstituents can also facilitating the action of the tannins as the saponins and flavonoids (KATIKI et al., 2013; WILLIAMS et al., 2014). Saponins cause destabilization in the cell membranes and increased cell permeability easing the action of tannins on intra-cellular proteins of the parasite (D'ADDABBO et al., 2011). Flavonoids are modulators of P-glycoprotein and improve the anthelmintic potential of condensed tannin *in vitro* (KLONGSIRIWET et al., 2015). Moreover, other phytochemicals in lower concentrations in plant can also act by different mechanisms, contributing to the observed efficacy.

Some evaluated plants have been cited in ethnoveterinary medicine or are used in feeding small ruminants such as *Opuntia ficus* indicates that besides being cited in ethnoveterinary medicine is also used in sheep feed in Brazil and displays anti-inflammatory, analgesic, antioxidant and antiviral activities. Féboli et al. (2016) evaluated *in vitro* the extracts of the cladodes and fruits of *O. ficus* against eggs and larvae of *H. contortus*. According to the authors, both cladodes and fruits have significant anthelmintic activity *in vitro*, suggesting that, beyond its nutritional potential, this plant can also be an ally for parasite control in sheep. The authors also report that the anthelmintic activity of *O. ficus* is related to its tannin content, but this class of compounds is not the only one responsive to the observed activity.

Oliveira et al. (2017) evaluated extracts of *Turnera ulmifolia* L. (leaves and roots), *Parkia platycephala* Benth. (leaves and seeds) and *Dimorphandra gardneriana* Tul. (leaves and bark) *in vitro* assays against *H. contortus*. These plants have been cited in ethnoveterinary studies and are selected naturally by goats in the "cerrado" (Brazilian savanna). The author observed clearly *in vitro* anthelmintic activities for all extracts against *H. contortus* at different stages and indicated the potential use of these species as a promising alternative approach to control helminthic infections of small ruminants.

Cortes-Morales et al. (2019) evaluated the *in vitro* ovicidal activity of *Baccharis conferta* Kunth a wild plant native to Mexico that is used as fodder for farm animals and in the traditional Mexican medicine for the treatment of various diseases. Authors evaluates the aerial parts crude methanolic extract of plant, fractions obtained from HPLC and majoritary compound from more active fraction. The results showed that the flavonoids, isokaempferide displayed powerful ovicidal effects, proving to be a potential alternative for the development of a phytodrug for the control of haemonchosis.

Medicinal plants that are rich in phenolic compounds and other bioactives metabolites represent appropriate subjects for studies evaluating anthelmintic properties, such as *Psidium cattleianum* Sabine (PCS), which comes from the guava family and whose leaves exhibit antiparasitic activity (DEBIAGE et al., 2016). This plant is common in Brazil, being found in the Atlantic Forest (and especially in

dense Rainforest, Sandbank and the Southern Plateau), as well as in several other countries. PCS displays anti-inflammatory, analgesic and antioxidant activities, which are also related to the presence of phenolic compounds and saponins (ALVARENGA et al., 2013). Although numerous researchers have acknowledged that its phytochemical composition is rich in tannins and bioactive compounds, no reports exist in the literature regarding its anthelmintic activity. Therefore, the aim of this study was to evaluate the activities of PCS hydro alcoholic extract from *in vitro* assays with the eggs and larvae of gastrointestinal nematodes obtained from naturally infected sheep.

## MATERIAL AND METHODS

*Psidium cattleianum* Sabine leaves used in this study were collected in Glaucilândia (Minas Gerais, Brazil) in October 2016 and a voucher specimen was deposited in the State University of Montes Claros herbarium under number 3533. The leaves were collected in the morning, washed with water, naturally dried in the shade for 96h, powdered and stored at -10 °C. Hydro alcoholic extract from PCS leaves was prepared through the addition of 100 g of powdered leaves in 1 L ethanol aqueous (7:3). This mixture was kept at room temperature under magnetic stirring for five days. Following this period, it was filtered and the solvent was removed in a rotaevaporator. The PCS extract (PCSE) obtained was frozen until use. For *in vitro* assays, 1000 mg of PCSE were dissolved in 0.2 mL of DMSO and 4.8 mL of water providing a final concentration of 200 mg mL<sup>-1</sup>. This concentration, by dilution with water, was used to obtain the others concentrations used in the assays, so that the maximum concentration of DMSO was of 0.5%. DMSO at 0.5 % was used as negative control (ALVARENGA et al., 2013).

Qualitative phytochemical assays were performed from dry PCS leaves in according to Harbone (1998) to detect the presence of flavonoids, alkaloids, anthraquinones, saponins, cardiotoxic glycosides and tannins. The assays were based on visual observation of colour changes or the formation of precipitates after adding specific reagents. Alkaloids: 1 g of pulverized sample was mixed with 10 mL of 1% HCl, warmed and filtered. The extract obtained was divided in four tubes for reaction

with Dragendorff's, Bouchardat, Bertrand and Meyer reagents (6 drops). The precipitation indicates the presence of alkaloids. Flavonoids: 1 g of sample was heated in 10 mL of 70% ethanol and filtered. 2 mL of the filtrate were mixed with fragment of magnesium metallic and 1mL of HCl concentrated. The formation of rose coloration indicates the presence flavonoid. Saponins: 1 g of plant material was heated with water (10 mL) to boiling. After cooling, the extract was filtered. The resulting liquid was stirred vigorously in a tightly sealed test tube for 15 seconds. Persistent foaming for more than 15 minutes indicates the presence of saponins. Cardiotoxic glycosides: 1 g of sample was mixed with 2 mL of anhydride acetic and followed by 2 mL of H<sub>2</sub>SO<sub>4</sub>. The formation of a green solution indicates the presence of Cardiotoxic glycosides. Anthraquinones: 1 g of sample was heated in 8 mL of 25 % ethanol for 1 minute at 100 °C and filtered. The filtered was mixed with 4 mL of 5% H<sub>2</sub>SO<sub>4</sub> and heated gently for 5 minutes. The result mixture was extracted with 5 mL of ethyl ether. The phases were separated and to aqueous phase was added 5 mL of 5% NH<sub>4</sub>OH and kept for 30 minutes at rest. The rose color indicate the presence of anthraquinones. Tannins: 1 g of sample was mixed with 5 mL of distilled water followed by 3 mL of 2% FeCl<sub>3</sub>. The formation of a dark green color indicates the presence of condensable tannin, dark blue color indicates the presence of hydrolysable tannin and black color indicates the presence of both (HARBONE, 1998).

For *in vitro* assays, the parasites were obtained from feces of sheep naturally infected with gastrointestinal nematodes (95% *H. contortus* and 5% *Trichostrongylus* spp.). Animal use for recovery of the eggs was approved by the Animal use and Ethics Committee, of the Faculty of Engineering, UNESP/Ilha Solteira, according to international standards of animal use in research; clearance certificate number: 12/2013/CEUA. Fecal samples were directly collected from the rectum of the donor animal. The eggs were recovered by using a sequence of sieves, according to the method described by Coles et al. (1992) with some modifications. The fecal samples were mixed with water (40°C) and filtered through 1000 µm, 150 µm, 63 µm, and 25 µm sieves. The eggs were retained in the last sieve, which was washed with distilled water.

The retained eggs were transferred to Falcon tubes (50 mL) and centrifuged with water at 2054 g for 5 min. The supernatant was removed and a saturated NaCl solution was added to re-suspend the precipitated eggs. Following centrifugation under the same conditions, the supernatant was transferred to 25  $\mu$ m sieves, and the eggs were washed with distilled water once again and transferred to another tube. The eggs concentration was estimated through counting the number of eggs in 25  $\mu$ L aliquots and subsequently adjusted to 100 eggs 100  $\mu$ L<sup>-1</sup>.

For egg hatching assay, 100 eggs contained in 100  $\mu$ L of distilled water were added to each well of a 24 well microplate with distilled water, with a total volume of 200  $\mu$ L in each well. Having added the eggs, each well was observed under an inverted microscope to confirm the exact number of eggs in each. All concentrations of PCSE and fractions (0.39, 0.78, 1.56, 3.12, 6.25, 12.5, 25.0 and 50.0 mg mL<sup>-1</sup>), DMSO at 0.5% (negative control), and albendazole at 25  $\mu$ g mL<sup>-1</sup> (positive control) were tested in five repetitions. The plate was then sealed with PVC film and incubated at 28 °C and  $\geq$  80% relative humidity for 48 h. Following this period, the number of eggs and L1 per well was counted under an inverted microscope. The percentage of egg hatching inhibition was determined relative to the number of eggs tested. In order to confirm the action of tannins on the observed anthelmintic effect, the concentration of the extract with the greatest ovicidal effect was incubated with 50 mg mL<sup>-1</sup> polyvinyl polypyrrolidone (PVPP) as described by Alonso-Díaz et al. (2008). The results with and without PVPP at the same concentration of the PCSE were compared.

The larvicidal activity was evaluated through the inhibition of larval development assays (LDA) and inhibition of larval migration assays (LMA). For LDA, 100 eggs were added to each well of a 24-well microplate with distilled water, with a total volume of 100  $\mu$ L in each well. The plates were incubated for 48 h at 28 °C and  $\geq$  80% relative humidity in order to obtain L1, before being counted under an inverted microscope. Following this period, 90  $\mu$ L of nutritive media (consisting of 1 g of yeast in 90 mL of normal saline and 10 mL of Earle's balanced salt), 10  $\mu$ L of amphotericin (25  $\mu$ g mL<sup>-1</sup>) and 200  $\mu$ L of PCSE were added to each well of the 24-well microplate. All concentrations

of PCSE (0.39, 0.78, 1.56, 3.12, 6.25, 12.5, 25.0, and 50.0 mg mL<sup>-1</sup>), water (negative control 1), DMSO at 0.5% (negative control 2) and ivermectin at 10  $\mu$ g mL<sup>-1</sup> (positive control) were tested in five repetitions (FÉBOLI et al., 2016). The plates were further incubated for five days, amounting to a total of seven days. The numbers of L1 and L3 in each well were counted under an inverted microscope. The percentage of larval development inhibition was determined relative to the number of L1 obtained in the initial 48 hours of the test.

In the LMA, the L3 were obtained by the faecal culture of a naturally infected donor sheep. Eggs reached the L3 stage after 8 days and were then collected by sedimentation using Baermann devices. One thousand live L3 (500  $\mu$ L) were added to centrifuge tubes containing PCSE solution (0.39, 0.78, 1.56, 3.12, 6.25, 12.5, 25, and 50.0 mg mL<sup>-1</sup>) for testing (1000  $\mu$ L) or commercial anthelmintic control (levamisole at 1.25 mg mL<sup>-1</sup>). The negative control (PBS, pH 7.2) was added to the tube to obtain a final volume of 2000  $\mu$ L. All of the incubations were carried out at 28 °C for 3 h. Thereafter, the L3 in each tube was washed with PBS and centrifuged (2054g) three times. The tubes were capped with a 25- $\mu$ m sieve and placed on a Petri dish containing water. After 3 h of incubation at room temperature, the number of larvae that migrated through the mesh during the 3h period was counted at 40X magnification by means of the 15% aliquot technique (RABEL et al., 1994). The percentage of larval migration inhibition was determined relative to the number of larvae tested.

The mean percentages of *in vitro* assays were analyzed by ANOVA and compared via the Dunnet test (5%). The final concentrations of the PCSE and in each well of the microplate or tubes were used to determine IC<sub>50</sub>. The IC<sub>50</sub> values (at a concentration yielding a response of 50%) were calculated by means of nonlinear regression analysis conducted with the aid of the GraphPad Prism version 5.0.

## RESULTS AND DISCUSSION

The phytochemical result realized in our study detected flavonoids and tannins in higher proportions beyond of saponin, cardiotonic glycosides and anthraquinones in lower proportions in the PCSE (Table 1). This phytochemical prospection accords with

**Table 1:** Result of phytochemical prospection of as *Psidium cattleianum* Sabine leaves.

Compound	Detection
Alkaloids Mayer	-
Alkaloids Bouchardat	-
Alkaloids Dragendorf	-
Alkaloids Bertrand	-
Flavonoids	+
Saponins	+
Tannins hydrolysable	+
Tannins condensable	+
Cardiotonic glycosides	+
Anthraquinones	+

-: Absence; +: Presence.

the results published in previous literature (ALVARENGA et al., 2013). Although many studies with other plants have been documented, our study is the first to analyze and test PCS for its anthelmintic activity

The results of *in vitro* assays showed that PCSE display anthelmintic activity against eggs and larvae of *H. contortus* (Table 2). The PCSE significantly ( $P < 0.05$ ) inhibited egg hatching in a dose-dependent manner, with an  $IC_{50}$  value of  $0.55 \text{ mg mL}^{-1}$ . The use of PVPP together with PCSE in a final concentration at  $25 \text{ mg mL}^{-1}$  reduced PCSE egg hatch inhibition by 10%. These results indicating that

other phytochemicals, beyond tannins, also contribute to the ovidal activity displayed by the extract. Similar results were obtained for Oliveira et al. (2017) to the evaluate the extract from *T. ulmifolia* leaves which display  $IC_{50} = 0.430 \text{ mg mL}^{-1}$  for ETH. The author also concluded that the tannins are not the only metabolites secondary involved in the antiparasitic effects of the extract. In other study, Santos et al. (2017) evaluated extracts and fractions from *Digitaria insularis* leaves against eggs of *H. contortus* and obtained  $IC_{50}$  values ranging from  $0.27 \text{ mg mL}^{-1}$  to  $0.96 \text{ mg mL}^{-1}$ , however the nematocidal activity of this plant was attributed to the presence of flavones.

Anthelmintic activity of the plants generally is related to the presence of tannins, which cause the coagulation of proteins and thus induce parasites' death (KATIKI et al., 2013). However, the presence of other phytoconstituents can also facilitating the action of the tannins as the saponins (OLIVEIRA et al., 2017). Indeed, saponins cause destabilization in cell membranes and stimulate increased cell permeability, easing the action of tannins on the intra-cellular proteins of the parasite (D'ADDABBO et al., 2011). Others studies have revealed the existence of synergistic anthelmintic activity effects between tannins and other plant secondary metabolites (MARIE-MAGDELEINE et al., 2009; KLONGSIRIWET et al., 2015). However, these interactions are complex and may increase or decrease the AH

**Table 2.** Percentages of *in vitro* inhibition of eggs hatching (EHA), inhibition of larval development (LDA), and inhibition of larval migration (MLA) (mean  $\pm$  SD) of sheep gastrointestinal nematodes (*95% Haemonchus contortus*) by the hydroalcoholic extract from *Psidium Cattleianum* Sabine leaves (PCSE).

Concentration (mg mL <sup>-1</sup> )	EHA (%)	LDA (%)	MLA (%)
25	100.00 $\pm$ 0 <sup>a</sup>	100.00 $\pm$ 0 <sup>a</sup>	100.00 $\pm$ 0 <sup>a</sup>
12.5	99.40 $\pm$ 0.23 <sup>a</sup>	90.7 $\pm$ 1.2 <sup>b</sup>	100.00 $\pm$ 0 <sup>a</sup>
6.25	98.60 $\pm$ 1.39 <sup>a</sup>	85.92 $\pm$ 8.0 <sup>b</sup>	100.00 $\pm$ 0 <sup>a</sup>
3.12	81.30 $\pm$ 2.20 <sup>b</sup>	75.83 $\pm$ 4.3 <sup>c</sup>	98.66 $\pm$ 0.57 <sup>b</sup>
1.56	78.32 $\pm$ 1.23 <sup>b</sup>	61.51 $\pm$ 8.7 <sup>d</sup>	98.22 $\pm$ 0.70 <sup>b</sup>
0.78	56.20 $\pm$ 2.41 <sup>c</sup>	62.60 $\pm$ 5.2 <sup>d</sup>	98.00 $\pm$ 0.40 <sup>b</sup>
0.39	45.30 $\pm$ 3.12 <sup>d</sup>	55.70 $\pm$ 2.7 <sup>d</sup>	94.51 $\pm$ 1.20 <sup>c</sup>
0.19	22.10 $\pm$ 0.67 <sup>e</sup>	49.60 $\pm$ 4.8 <sup>e</sup>	80.25 $\pm$ 2.31 <sup>d</sup>
Positive control	99.45 $\pm$ 0.63 <sup>a</sup>	100.00 $\pm$ 0 <sup>a</sup>	100.00 $\pm$ 0 <sup>a</sup>
Negative control	4.62 $\pm$ 3.29 <sup>f</sup>	4.25 $\pm$ 0.95 <sup>d</sup>	0.00 $\pm$ 0 <sup>e</sup>

\* Different small letters in the same column indicate significant differences ( $p < 0.05$ ).

effects against *H. contortus* eggs (VARGAS-MAGANA et al., 2014).

In relation to larvicidal activity the PCSE exhibited promising results in LDA, blocking development L1 to L3 even at the lowest evaluated concentration with  $IC_{50} = 0.20 \text{ mg mL}^{-1}$  (Table 2) acting in a dose-dependent manner ( $P < 0.05$ ). The larval motility was significantly affected in L3 after three hours of exposure to PCSE in the LMA. Following this period, 100% inhibition of larval migration was observed in concentrations greater than  $3.12 \text{ mg mL}^{-1}$ . In the concentration of  $0.19 \text{ mg mL}^{-1}$ , 80.25% inhibition of larval migration was observed (Table 2). In the concentration of  $0.19 \text{ mg mL}^{-1}$  was observed 80.25% inhibition of larval migration, indicating that the  $IC_{50}$  value is lower than the lowest concentration evaluated. The results demonstrated that PCSE exhibited similar ovicidal and larvicidal activity to that of numerous other plants described in the literature (RAJESWARI, 2014; WILLIAMS et al., 2014; FEBOLI et al., 2016; OLIVEIRA et al., 2017). However, the PCSE has the advantage of being studied in terms of cytotoxicity and phytochemical composition (DESOTI et al., 2011; HO et al., 2012; ALVARENGA et al., 2013; FALEIROS et al., 2016).

## CONCLUSION

In conclusion, PCSE showed ovicidal and larvicidal activity against eggs and larvae of nematodes obtained from naturally infected sheep. Our results provide support for a future *in vivo* study in order to evaluate whether the plant after metabolization by the animal still maintains the same anthelmintic properties as the *in vitro* tests and may be indicated as an alternative in the control of gastrointestinal nematodes of sheep

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