ORIGINAL ARTICLE

IN VITRO ANTHELMINTIC EFFECT OF AQUEOUS EXTRACT OF *CROTON BLANCHETIANA* LEAF ON SHEEP ENDOPARASITES

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Highlights: (1) Control of gastrointestinal endoparasites using plants. (2) The use of plant products brings environmental and economic benefits. (3) The aqueous extract is more than 80% effective in inhibiting gastrointestinal parasites.

PRE-PROOF
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IN VITRO ANTHELMINTIC EFFECT OF AQUEOUS EXTRACT OF *CROTON BLANCHETIANA* LEAF ON SHEEP ENDOPARASITES

ABSTRACT

The development of small ruminants has been hampered by infections related to gastrointestinal endoparasites. This problem arose due to the incorrect use of anthelmintics and inadequate management practices, which led to the emergence of parasite resistance. Given this, herbal medicine offers an alternative approach to parasite control. Among the various options, *Croton blanchetianus* stands out for its bioactive compounds and demonstrates antibacterial, antifungal, ectoparasiticidal and anthelmintic properties. Thus, the objective of this study was to evaluate the in vitro ovicidal activity of the aqueous extract derived from *Croton blanchetianus* leaves. For this, fecal samples were collected directly from the rectal ampulla of naturally infected animals, without administration of antiparasitics, for a period of 90 days. When the egg count per gram of feces exceeded 2,000 eggs, these eggs were collected for hatchability testing. Simultaneously, phytochemical analyzes were carried out on the leaves and toxicity tests on *Artemia salina*. The results revealed that the aqueous extract, at concentrations of 5 %, 2.5 %, 1.25 % and 0.625 %, presented hatching inhibition rates of 81.8 %, 70.0 %, 47.8 % and 40.4 %, respectively. Phytochemical analysis confirmed the presence of secondary metabolites in the extract. Furthermore, the toxicity test demonstrated that the *C. blanchetianus* extract was non-toxic to *Artemia salina* at all concentrations tested. Consequently, it can be concluded that the aqueous extract had inhibitory effects on egg hatching due to the presence of several secondary metabolites, thus increasing its potential as an anthelmintic treatment against gastrointestinal parasites in sheep.

Keywords: sheep farming. small ruminants. parasites and phytotherapy.

INTRODUCTION

In Brazil, sheep farming is regarded as one of the primary economic activities, providing income and sustenance for breeders while also serving as a profitable venture and contributing to the food supply of farming families.[1] The expansion of sheep farming across Brazil, particularly in the Northeast region, which accounts for 70.6% of the country's sheep production,[2] has led to a diversification in commercialization. Previously centered around wool and skin production, the industry has now expanded to include the marketing of meat, milk, and related products.

However, the presence of gastrointestinal endoparasites in sheep herds can hinder their development, leading to weight loss, anemia, and a decline in productivity and reproductive potential. Poor sanitary management practices further contribute to the development of parasitic resistance.[3] Consequently, sheep affected by gastrointestinal infections exhibit symptoms that render them unproductive.[4]

Chemical drugs are widely utilized for the treatment of parasites,[5] but their excessive and improper use contributes to the emergence of parasitic resistance. This, in turn, leads to increased expenses for maintaining the animals, elevated mortality rates, and decreased herd productivity.[6] Consequently, producers face significant economic losses.[7]

Therefore, phytotherapy has been embraced as a means of controlling diseases caused by gastrointestinal parasites. Various formulations,[8] including extracts and essential oils, which contain bioactive substances, have been approved for use.[9] Since these compounds are derived from natural sources, they often possess low toxicity,[10] allowing for their utilization in order to reduce the presence of chemical contaminants in the environment.[11]

Vegetable-derived compounds have demonstrated efficacy in controlling microorganisms. Medicinal plants, known and validated by popular usage, have exhibited antibacterial, antifungal, ectoparasitic, and anthelmintic properties.[12]

In this context, the species *Croton blanchetianus*, commonly known as the "quince tree," deserves particular attention. It belongs to the Euphorbiaceae family.[13] This plant species is endemic to Brazil, with a greater presence in the Northeast region.[14] This genus is also known for having a wide production of secondary metabolites, therefore it has been studied for scientific proof of its biological activities.[15] Leaves and bark of this plant are still used today for therapeutic purposes; in folk medicine, quince has traditionally been used to treat problems such as swelling, uterine bleeding, hemoptysis, stomach pain, vomiting and diarrhea.[16] Due to its remarkable potential, quince has been the subject of frequent studies focusing on the biological activity of its essential oils and extracts.[17] However, it is worth highlighting the significant lack of research on the potential of C. blanchetianus against gastrointestinal parasites in sheep.

Hence, this study aimed to assess the ovicidal activity and toxicity of the aqueous extract of *C. blanchetianus*, as well as analyze the phytochemical composition of its leaves. The ultimate goal was to establish a viable and effective strategy for the control of gastrointestinal nematodes, benefiting both the environment and society.

MATERIAL AND METHOD

Ethical and Legal Procedures

The research followed all ethical and legal precepts with approval from the Ethics Committee on the Use of Animals n0 14/2021 and registration in the National System for the Management of Genetic Heritage and Associated Traditional Knowledge n0 A137FA3. The plant sample was deposited in the Herbarium Dárdano de Andrade-Lima, at the Center for Biological Sciences and Health, Universidade Federal Rural do Semi-Árido – UFERSA with exsiccate nº 15132, proving the described species.

Description of the Study Area

The study was conducted in the city of Mossoró located in the western region of the state of Rio Grande do Norte, inserted in the Northeast, with a territorial dimension of 2,099,334 Km². The municipality is located between the geographic coordinates 5º 11’ 15’’ South latitude and 37º 20’ 39’’ West longitude and at an average altitude of 16 meters above sea level. The predominant climate is semi-arid, with two climatic phases: a dry one between June and January, and a rainy one, from February to May. The relative humidity of the air is around 70% and the average annual temperature is 27.4 °C.

Obtaining Plant Material

The leaves of *C. blanchetianus* were collected in the city of Mossoró-Rio Grande do Norte in the morning, then taken to the Laboratory of Biotechnology Applied to Infectious-Parasitic Diseases of the Universidade Federal Rural do Semi-Árido (UFERSA), where the production of the aqueous extract.

Obtaining the Aqueous Extract from the Leaves of *Croton blanchetianus*

The leaves were cleaned and dried at room temperature. After eight days of drying, they were crushed and sieved until obtaining a fine powder that was easy to dissolve. From the vegetable flour obtained, it was subjected to solubilization in a proportion of 5% (weight/volume), where 30 grams of the vegetable were diluted in 600 mL of distilled water. The mixture was left under constant magnetic stirring for 16 hours at 20°C. After the extraction time, the material was filtered through gauze and subjected to centrifugation (8000 rpm, 20 min at 4 °C) to remove insoluble material. The supernatant material was called crude extract (EB). Finally, dilutions in distilled water were prepared from the crude extract, at concentrations of 2.5% and 1.25%, 0.625% .[18]

Phytochemical Determination of the Aqueous Extract

The aqueous extract of *C. blanchetianus* leaves was submitted to phytochemical characterization.[19] Metabolic classes were identified from observations of precipitation, colorimetric and fluorescence reactions, indicating their presence or absence. Reactions were carried out to identify coumarins (Fluorescence Test), phenols (Ferric Chloride Reaction), flavonoids (Cyanidin or Shinoda Reaction), anthraquinones (Borträger), steroid nuclei (Liebermann-Burchard Test), triterpene nuclei (Test Salkowski test), saponins (Foam test – vigorous stirring), condensed tannins and hydrolysable tannins (Stiasny test), free tannins (Gelatin precipitation test).

Collection of Fecal Samples

The animals were randomly selected, regardless of breed or sex, belonging to the herds that presented the end of the residual period of 90 days without treatment with synthetic chemicals.[20] After the selection of the animals, fecal samples were collected directly from the rectal ampoula, to carry out the counting of eggs per gram of feces (OPG) by weighing 4 g of feces according to the McMaster technique, where fecal samples were separated with OPG equal to or more than 2000 eggs.[21]

Egg Hatch Recovery and Test (EHT)

The recovery of nematode eggs was carried out according to the methodology of Hubert and Kerboeuf,[22] with fecal samples diluted in water and passed through a sequence of sieves containing a weight of 0.15; 0.10; 0.036 and 0.02 mm for egg retention. Subsequently, a centrifugation (4,000 rpm; 5 minutes) of the recovered liquid was performed. The sediment from the first centrifugation was resuspended in concentrated saline solution and subjected to a second centrifugation (4,000 rpm; 5 minutes) to obtain the supernatant containing the eggs.

The egg hatch test (EHT) was performed according to the Coles methodology;[23] an average of 100 eggs/well were added in 24-well plates, using five wells for each treatment. The negative control containing eggs incubated in aqueous solution; the positive control containing eggs submitted to chemical treatment with Thiabendazole (32 µl/mL); the experimental group containing the eggs incubated in the crude aqueous extract with concentrations of 5%, 2.5%, 1.25% and 0.625%.

The 24-well plates were incubated in BOD (Biochemical Oxygen Demand) for 48 hours at a temperature of 27 °C and controlled humidity, with subsequent addition of Lugol and counting eggs and larvae in each well, using an inverted microscope.

Toxicity of the Aqueous Extract in *Artemia salina*

The experimental assay to evaluate the toxicity of the essential oil from *C. blanchetianus* was conducted using 24-well plates.[24] *Artemia salina* cysts were hatched in filtered saline solution with a concentration of 40 g/L (water with sea salt) and a pH between 8-9. For each liter of water, 100 mg of A. salina cysts were added. The hatching process took place at room temperature, under 100 W illumination, with constant aeration, for 48 hours. Ten hatched nauplii were then separated and transferred to 24-well plates containing 100 µl of A. salina culture solution and 400 µl of essential oil concentrations from *C. blanchetianus* leaves (10%, 5%, 2.5%, 1.25%). The bioassay was performed in triplicate, with the plant extract tested three times, resulting in a total of nine assays per concentration. The negative control was conducted by adding the culture solution to the wells. Subsequently, the plates were incubated for 24 hours. After the specified time period, the brine shrimps were examined, and the number of live and dead nauplii was recorded. Nauplii showing movement when observed near the light source were considered alive. Tests in which the control group exhibited mortality rates of less than or equal to 10% of the population were considered valid.

Statistical Analysis

The data obtained was organized and recorded in an Excel spreadsheet. The hatching percentage of eggs for each experimental group was calculated using the formula: (number of eggs / (number of eggs + number of L1)) x 100. The Shapiro-Wilk test was applied to assess the normality of the data, and significant differences between the analyzed groups were examined using the Kruskal-Wallis test, followed by the Bonferroni test. A significance level of $p < 0.05$ was adopted for the tests. The 50% lethal concentration (LC50) in the *A. salina* toxicity assay will be determined through non-linear regression analysis using the R 4.1.3 software for data analysis.

RESULTS

Inhibition of Egg Hatching

The results of the Egg Hatch Test (EHT) using the aqueous extract of *C. blanchetianus* with concentrations of 5 %, 2.5 %, 1.25 % and 0.625 %. The positive control using the chemical drug Thiabendazole (32 µl/mL) inhibited hatching by 74.4 %, and the negative control (0.15 M NaCl) showed no significant inhibition in relation to egg hatching (10%). The inhibition rates of the aqueous extract of *C. blanchetianus* were higher at concentrations of 5 % (82 %) and 2.5 % (70 %), which, on the other hand, were not statistically different from what we observed in the positive control. However, the results shown for the concentrations of 1.25 % and 0.625 % did not present a statistically significant difference when compared to the results obtained for the negative control (Figure 1).

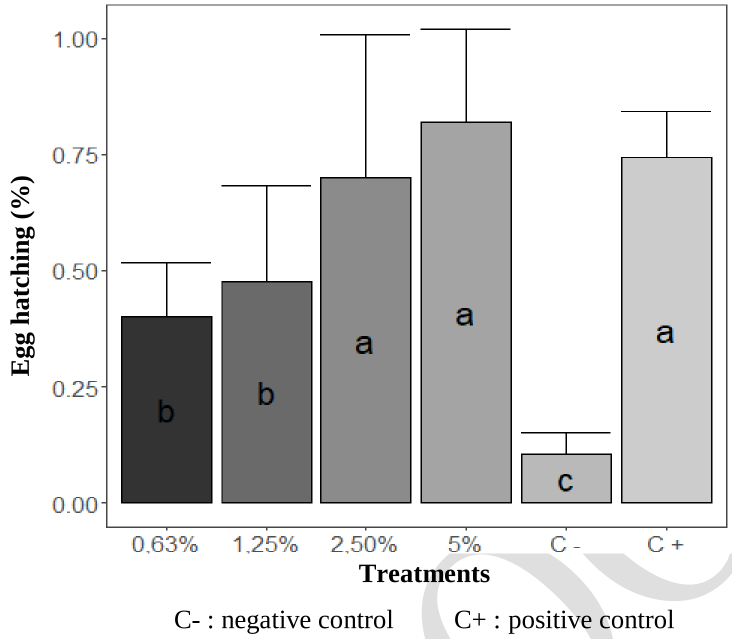

Figure 1. Effect of aqueous extract of C. blanchetianus leaves on the hatching of sheep nematode eggs according to concentrations of 5%, 2.5%, 1.25% and 0.625%. *Mean values followed by the same letter do not differ statistically from each other. (Kruskal-Wallis, $p \leq 0.05$) (Source: Prepared by the authors).

Phytochemical Analysis

As for the analysis of the chemical composition of the aqueous extract of *C. blanchetianus* leaves, the presence of phenols (Ferric Chloride Reaction), flavonoids (Cyanidin or Shinoda Reaction), steroid nuclei (Liebermann-Burchard Test), saponins (Foam Test – vigorous shaking), alkaloids (characterization reaction – precipitation) condensed tannins and hydrolyzable tannins (Stiashny Test), free tannins (Gelatin Precipitation Test).

Acute Toxicity

Results of the toxicity test with *A.salina*. The aqueous extract of *C. blanchetianus* leaves showed an lethal concentration LC50 of 350,907 ul/mL and an R2 of 0.02, demonstrating that the concentrations tested did not present toxicity in the bioindicator used (Figure 2).

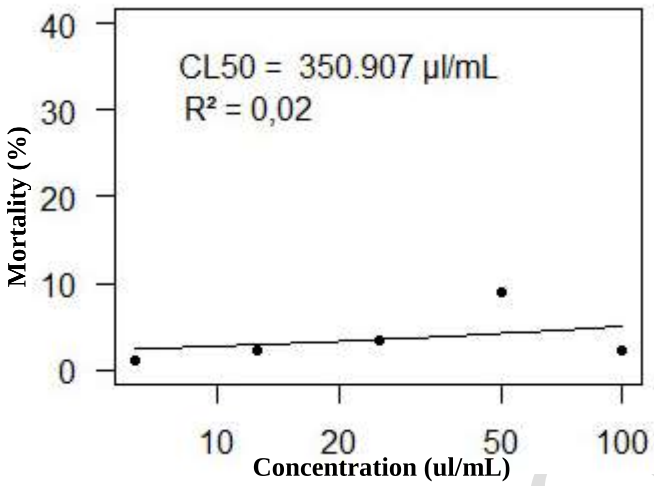

Figure 2. Representation of the toxicity test of the concentrations used of the aqueous extract of the leaves of C. blanchetianus on A. salina (Source: Prepared by the authors).

DISCUSSION

The aqueous extract of C. blanchetianus demonstrated effectiveness in inhibiting egg hatching at concentrations of 5% and 2.5%. However, the percentage of inhibition observed in the positive control was 74.40%, indicating potential evidence of anthelmintic resistance.[25]

According to Coles,[23] an OPG (eggs per gram) count below 90% is indicative of suspected parasitic resistance. Parasites possess various biological and genetic characteristics that contribute to the development of resistance against chemical drugs. Factors such as a high reproduction rate, short life cycles, and a large population size provide helminths with genetic diversity.[6, 26]

Considering the challenge of parasitic resistance, plants used for medicinal purposes can be seen as an economically viable alternative for the control and treatment of gastrointestinal parasites, in addition to demonstrating the potential as an anthelmintic in small ruminants, without dependence on the preparation process.[27] Thus, he states that anthelmintic activity has been reported from plant extracts used in gastrointestinal parasites.[28] Studies carried out with extracts of plants such as *Carica papaya* and *Musa paradisiaca* and larvae of parasites such as *Haemonchus contortus*.[28] Research developed with essential oil of *Cuminum cyminum* proved the effectiveness in the hatching of eggs and in the larval development of parasites.[29] Tests also with aqueous extract of leaves of the *Solanum lycocarpum* plant demonstrated the efficiency of its use through the modified larval hatchability technique, enabling a reduction in the hatching of gastrointestinal parasite eggs.[30]

The ovicidal activity of the extract may be related to the constituents found in the leaves of *C. blanchetianus*, called secondary metabolites: phenols, alkaloids, saponins, flavonoids, steroid terpenes, general tannins and condensed tannins. The secondary metabolites present in plants with anthelmintic property have shown promising results in the fight against gastrointestinal nematodes.[31]

Phenols are commonly found in plants and are known to accumulate in all parts of plants, such as: roots, stems, leaves, fruits and flowers.[32] Due to their chemical diversity, phenols perform several functions such as coupling with digestive proteins to inactivate important enzymes in this process.[33] According to a study carried out, phenols interfere with the parasite's energy production mechanism, as it uncouples oxidative phosphorylation, compromising proteins that remain on the surface of nematode cells, causing the parasite's death.[34]

Tannins and flavonoids are other phenolic compounds present in the extract of *C. blanchetianus* leaves. Tannins are considered natural sources and are widely distributed in stems, leaves and inflorescences of plants, being generally divided into condensed and water-soluble tannins.[35,36] They have antiparasitic action that may be related to their ability to interact and form complexes with the proteins of the parasites, impairing the absorption of nutrients, directly affecting motility and reproduction and larval development and egg hatching.

Flavonoids have shown positive effects related to the health of animals, as they have antioxidant activity and the ability to interact with enzymes present in parasites, causing changes in their energetic pathways of essential metabolic substances for energy absorption. [37]

Alkaloids form the second largest family of secondary metabolites and can be found in various animals, microorganisms and plants.[30] As a biological activity, alkaloids can act in the nervous system, as a neurotransmitter, acting as a sedative, analgesic and muscle relaxant, causing loss of motility and stretching of the parasite, inhibiting its movement. [38]

Terpenes are present in products of plant origin such as extracts and essential oils, so they act by attracting pollinators. They also have insecticidal, antimicrobial, anti-inflammatory, among others.[39]

Saponin was also identified in the aqueous extract, which is an important secondary metabolite of plants, considered a natural compound with anthelmintic action. The biological effects of saponin have been related to its ability to form complexes with the membrane of the parasite, altering its permeability and promoting the entry and exit of molecules, resulting in the formation of vacuoles in the cytoplasm of cells, causing the disintegration of the worm's envelope, impairing its development or causing its death.

The toxicological effect of the concentrations of the aqueous extract of *C. blanchetianus* leaves was determined through the Average Lethal Concentration (LC50). The relationship between toxicity and average lethal concentration presented by plant extracts on *Artemia salina* is from the LC50 values, above 1000 μg/mL are considered non-toxic, LC50 greater than 500 μg/mL presents a low toxicity, LC50 is moderate with values between 100 to 500 μg/mL and toxic lower than 100 μg/mL. Thus, the toxicity test result shows the LC50 value equal to 350,907 μg/mL. Thus, we can see a low level of effect on mortality with the concentrations tested, with a maximum mortality of 40%. In this context, the aqueous extract of *C. blanchetianus* leaves was considered non-toxic in the *in vitro* test, as it presented an LC50 value greater than 1000 μg/mL.[40] These results may be due to the diversity of aspects among individuals of the same species, known as polymorphism, such as temperature, humidity, solar radiation and genetic variability.[6]

Therefore, this study revealed that *C. blanchetianus* is a species that presents interesting and beneficial characteristics when it comes to the formulation of plant-based products against gastrointestinal nematodes. The aqueous extract showed an in vitro ovicidal effect at all concentrations tested and did not show toxicity. In addition, it is a great alternative to replace conventional methods, as it is a viable strategy for use due to its wide availability in semiarid regions, low cultivation cost and ease of preparation. Its drought resistance and low fertilization requirements reduce operational costs, while its easy adaptation makes it ideal for sheep feeding. Therefore, *C. blanchetianus* has several promising secondary metabolites with proven efficacy.

CONCLUSION

The aqueous extract of *C. blanchetianus* leaves was shown to be able to inhibit the hatching of eggs of gastrointestinal parasites in sheep. Making the plant an alternative source for the control of gastrointestinal helminths. The use of plant-based medicines can reduce cases related to parasite resistance, as well as environmental impacts. Thus, we affirm that the results obtained offer a promising basis for future *in vivo* research, which is relevant to validate the practical applicability of herbal medicines in the parasite management of sheep.

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IN VITRO ANTHELMINTIC EFFECT OF AQUEOUS EXTRACT OF *CROTON BLANCHETIANA* LEAF ON SHEEP ENDOPARASITES

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