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In vitro anthelmintic activity of *Chenopodium ambrosioides* for control of *Neoechinorhyncus buttnerae* in tambaqui (*Colossoma macropomum*)

Atividade anti-helmíntica *in vitro* de *Chenopodium ambrosioides* para controle de *Neoechinorhyncus buttnerae* no tambaqui (*Colossoma macropomum*)

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ABSTRACT

Neoechinorhyncus buttnerae is among the main causative agents of diseases in tambaqui (*Colossoma macropomum*) and has caused great losses in fish farming. Although important, there is still no effective treatment protocol for acanthocephalosis. Therefore, the aim of this study was to evaluate the *in vitro* anthelmintic activity of *Chenopodium ambrosioides* (mastruz) in the control of *Neoechinorhyncus buttnerae*. Parasites were collected from the intestine of naturally infected animals and randomly distributed in Petri dishes containing the following treatments in triplicate and standardized in 5 mL of RPMI1640 medium: TR1 (RPMI+spray-dried extract of mastruz 10%); TR2 (RPMI+spray-dried extract 5%); positive control (RPMI+levamisole 15 mg/mL); negative control (RPMI). The parasites were kept at room temperature and observed every 15 min, and were considered dead when they remained motionless even after external stimuli. Body deformities were observed in parasites after exposure to treatments (TR1 and TR2), and both treatments with spray-dried extract of mastruz were effective in controlling acanthocephalans in the first 15 min of the experiment. Therefore, mastruz has been shown to be an effective alternative for the control of *N. buttnerae* and has the potential for continuing *in vivo* studies with the aim of developing a therapeutic protocol.
Keywords: Phytotherapy; Helminth; Treatment; Fish; Toxicity.

RESUMO

O *Neoechinorhyncus buttinerae* está entre os principais agentes causadores de enfermidades no tambaqui (*Colossoma macropomum*) e tem ocasionado grande prejuízo na piscicultura. Embora importante, ainda não existe protocolo de tratamento eficaz para a acantocefalose. Por isso, o objetivo deste estudo foi avaliar a atividade anti-helmíntica *in vitro* do *Chenopodium ambrosioides* (mastruz) no controle de *Neoechinorhyncus buttinerae*. Os parasitos foram coletados do intestino de animais naturalmente infectados e distribuídos aleatoriamente em placas de Petri contendo os seguintes tratamentos em triplicata e padronizados em 5 mL de meio RPMI1640: TR1(RPMI+ Extrato seco por aspersão do mastruz 10%); TR2 (RPMI+ Extrato seco por aspersão 5%); CTR positivo (RPMI+Levamisol 15mg/mL); CTR negativo (RPMI). Os parasitos foram mantidos em temperatura ambiente e observados a cada 15 min, sendo considerados mortos quando permaneciam imóveis mesmo após estímulos externos. Foram observadas deformidades corporais nos parasitos após exposição aos tratamentos (TR1 e TR2) e todos os tratamentos com extrato seco por aspersão de mastruz foram eficazes no controle dos acantocéfalos nos primeiros 15

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min de experimento. Portanto, o mastruz demonstrou ser uma alternativa eficaz para controle de *N. buttinerae* tendo potencial para continuidade de estudos *in vivo* para elaboração de protocolo terapêutico.
Palavras- chave: Fitoterápico; Helminto; Tratamento; Peixe; Toxicidade.

INTRODUÇÃO

Acanthocephalosis is a disease caused by the presence of acanthocephalans in different portions of the digestive tract of vertebrates. These parasites have a whitish coloration, are macroscopically visible and have a proboscis (invaginable and full of spines), an important organ of attachment of the parasite in the intestine of the host and one that is responsible for multiple lesions (EIRAS et al., 2006; EIRAS et al., 2010).

Among the species of acanthocephalans of greatest importance currently in fish farming is *Neoechinorhynchus buttnerae* (GOLVAN, 1956), which is found in tambaqui (*Colossoma macropomum* CUVIER, 1818) and its hybrids. With a heteroxenous life cycle, most often involving an ostracode as an intermediate host (Lourenço, Morey, Pereira & Malta, 2018), the presence of this endoparasite can cause mortality in fry, reduction in growth and weight loss, since the animals present hyporexia and cachexia, which has generated losses in all regions of Brazil, especially in the north and northeast of Brazil (JERÔNIMO et al., 2017; OLIVEIRA et al., 2019; SANTOS et al., 2019).

In an attempt to control the presence of this important pathogen in the farming environment, the empirical use of drugs in fish farming has become common since, at the moment, there is no effective treatment and nor even specific approved drugs for the treatment of this helminthiasis in Brazil (CHAGAS, MACIEL and AQUINO-PEREIRA, 2015).

One important alternative is the use of herbal medicines in fish farming, and a number of studies have proved their effectiveness. Since they are natural, they are potentially less toxic, less concentrated, biodegradable, and leave low levels of residues in the animal, in addition to reducing the risk of pathogen resistance, and in some cases, have immunostimulating activity in animals (ABUTBUL et al., 2004; TAVECHIO et al., 2009; ALY and MOHAMED, 2010; FIGUEIREDO et al., 2011).

One of the oldest and most used plants due to its therapeutic and edible properties is *Chenopodium ambrosioides* L., which is distributed almost throughout Brazil. It is also listed in the RENISUS - the Unified Health System's National List of Medicinal Plants of Interest (RENISUS, 2020, DEMBITSKYA et al., 2008; KOKANOVA-NEDIALKOVA et al., 2009; SÉRVIO et al., 2011).

In the literature, this plant species is recorded as having analgesic, anti-inflammatory, anticholinesterase, antioxidant, wound healing, antimicrobial, and fungicidal properties, as well as being used in folk medicine to combat helminths. In its chemical constitution, phenolic

substances, flavonoids, terpenoids and steroids can be highlighted (GOLYNSKI, 2003; BORBA and AMORIM, 2004; CARVALHO et al., 2010; JARDIM, 2006; FARIA et al., 2010; SÉRVIO et al., 2011).

Given the scientific evidence of the antiparasitic activity of mastruz for other animal species, it may be an alternative for the control of parasites in fish; however, the pharmaceutical form to be used and the effective doses and toxicity for both animals and pathogens are still unknown.

Therefore, in order to contribute to research on the therapeutic potential of mastruz in fish farming, the present study aimed to evaluate the *in vitro* anthelmintic activity of mastruz in the control of *Neoechinorhyncus buttnerae* in tambaqui.

MATERIAL AND METHODS

Acquisition and treatment of plant material

The plant material consisted of aerial parts of *C. ambrosioides* (leaves + inflorescences + fruits + seeds) acquired from and identified at EMBRAPA Western Amazon in April 2018.

After acquisition, all material was cleaned and separated (aerial parts of the branches) manually. After separation, the material was placed in an oven with air circulation and renewal at a temperature of 45 ^{o}C for a sufficient period of time to achieve the ideal humidity for storage (below 12%), according to the Brazilian Pharmacopoeia 5th ed. After drying, the aerial parts were ground separately in knife mill with a mesh opening equal to 1 mm.

Experimental design and acquisition of parasites

The experimental design was completely randomized and consisted of 2 treatments and 2 controls in triplicate in RPMI 1640 medium: TR1 (RPMI+spray-dried extract 10%); TR2 (RPMI+spray-dried extract 5%); positive control (RPMI+levamisole 15 mg/mL) and negative control (RPMI). These included parasites removed after dissection of two naturally parasitized fish with an average weight of 275 g and length of 27 cm, which were obtained from a fish farm located in Manacapuru, AM.

After removal of the parasites from the intestine, they were washed in physiological solution at 0.9% NaCl for distribution in Petri dishes containing the tested solutions (10 parasites per plate) for observation under a stereomicroscope (Zeiss®) by different observers at room temperature (25 °C), and the mortality rate and time of death of the parasites were recorded every 15 minutes, according to the methodology described by Fajer-Ávila et al. (2003), with modifications.

Acanthocephalans were considered dead when they remained motionless even after external stimuli. The test was considered complete after 8 hours of observation, which is an

approximate time described in other studies in the literature (SOUZA et al., 2018; OLIVEIRA et al., 2019; SANTOS et al., 2021; SEBASTIÃO et al., 2021).

Preparation of solutions for *in vitro* testing

All solutions were previously prepared in RPMI 1640 medium (Sigma), with the final proportional value of 5 mL of RPMI medium being calculated for each solution.

Preparation of positive control (levamisole) and negative control (RPMI**)**

The positive control was prepared using 5 mL of RPMI 1640 medium containing 15 mg/mL of levamisole obtained from levamisole in the form of 5% oral solution (Ripercoll solution). The negative control was RPMI 1640 medium.

Preparation of treatments with spray-dried extract (SDE 5% and SDE 10%)

The spray-dried extract (SDE) was obtained after drying in a spray dryer (MSD 1.0, Labmaq do Brasil®), an aqueous extractive solution was obtained from the aerial parts of mastruz prepared with a drug:solvent ratio of 10 % (m/v).

The treatment with 5% SDE contained 5 ml of RPMI 1640 medium with 5 mg/mL of spray-dried extract of mastruz and the treatment with 10% SDE was contained 5 ml of RPMI 1640 medium with 10 mg/mL of spray-dried extract of mastruz.

Ethical aspects

This study was authorized by the Ethics Commission on the Use of Animals (CEUA) of the Federal University of Amazonas, under protocol number: 024/2019.

Effectiveness of treatment

The anthelmintic efficacy of each treatment was calculated according to the following modified formula of Wang et al. (2008): AE=[C - T] × 100%/C, where AE is anthelmintic efficacy, C is the average number of live parasites of *N. buttnerae* in the control group, and T is the average number of live parasites in the treatment.

Determination of total phenolic content

The concentration of total phenols was quantified using the method described by Al-Qassabi and Weli (2008) and Singleton and Draper (1965). The analysis was performed in 96-well microplates, and for the preparation of the analytical curve of the standard, 20 µL of the gallic acid standard (stock solution) was added, then 20 µl of methanol (HPLC grade) was added and 20 µL was removed from the first well, with successive dilutions. Subsequently, 150 µL of Folin Ciocalteau 10% solution was added. After 5 minutes, 150 µl of $NaHCO_3$ solution (6%) was added to the medium. The medium was read at a wavelength of 750 nm in a multi-plate absorbance reader (ELX 808 Biotek, Burlington, VT) for 90 minutes.

The presence of phenolic substances in the sample was detected by converting the yellow color of the rational medium to the blue color and the phenolic content of the samples was

expressed as a percentage compared to the gallic acid standard and also as µg equivalent to gallic acid (µgGAE) when compared to consecutive dilution of the standard. The following equation was used: total phenols (%) = (sample absorbance/standard absorbance) x 100.

RESULTS

Total phenolic content of the SDE

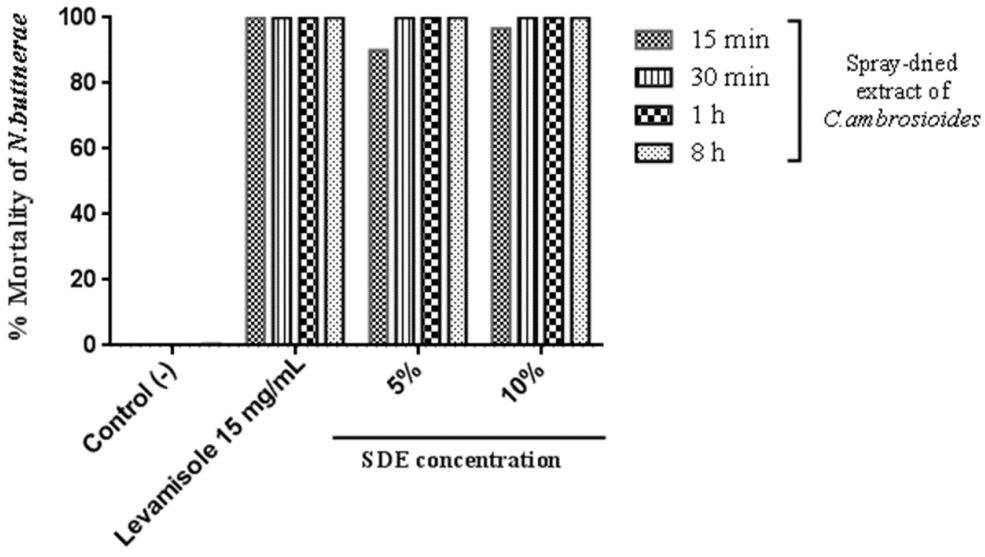
The SDE of *C. ambrosioides* has a good amount of phenol content, and was standardized with a content of 10.45 µg GAE/g of dry extract. This result corroborates with that found in the literature in which the presence of a high content of total phenols in extracts derived from mastruz is cited (JORGE et al., 1986; ALENCAR et al., 2010; SÁ, 2013; SÁ et al., 2015; PEREIRA et al., 2015).

Anthelmintic activity of the SDE of mastruz *in vitro*

Figure 1 shows the mortality of the acanthocephalans in the tested solutions during the 8-hour exposure period of the *in vitro* test. In just 30 min of exposure, 100% of the parasites died in the treatments with the solution containing the dry extract of *C. ambrosioides* (mastruz), thus demonstrating that in concentrations of 5% and 10%, mastruz has anthelmintic activity for the control of *N. buttnerae.*

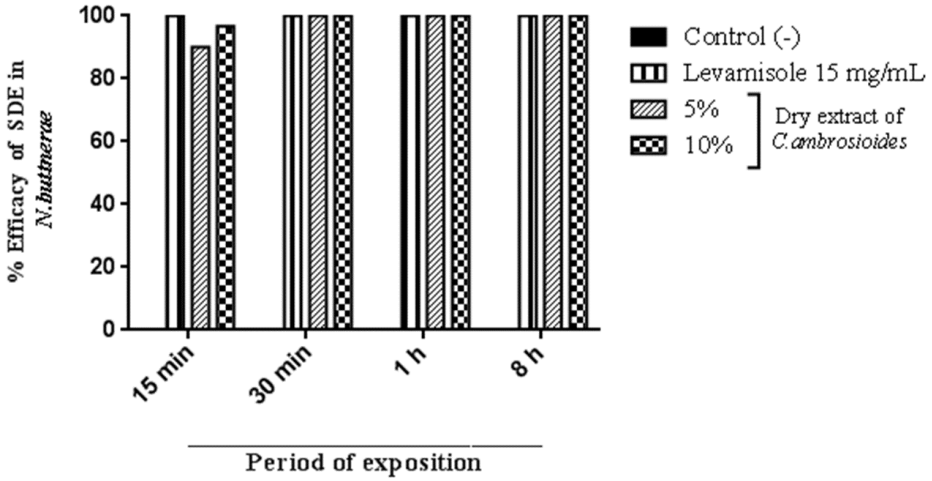
In the positive control (with levamisole) the parasites died in just 15 minutes, while in the negative control (with the RPMI 1640 culture medium), the parasites were still alive after 8 h of experiment.

Figure 1: Mortality Percentage of *Neoechinorhynchus buttnerae* removed from *Colossoma macropomum* and exposed for 8 h to different treatments with mastruz in *in vitro* tests.



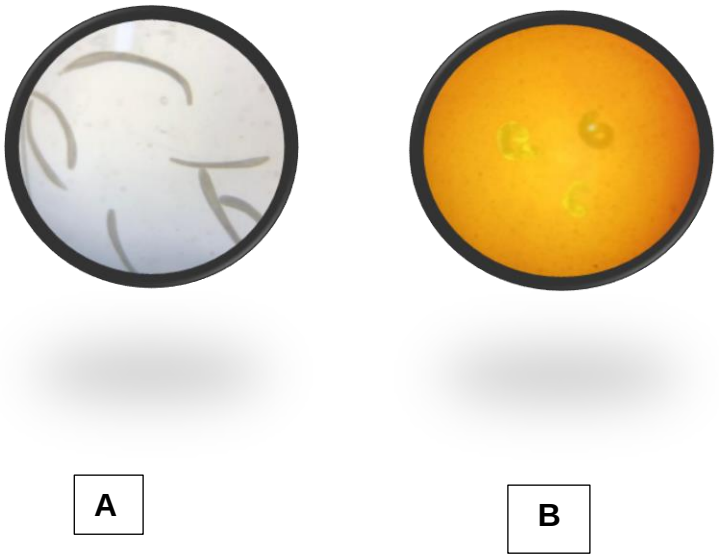
The solutions containing the dry extracts of *C. ambrosioides* demonstrated *in vitro* anthelmintic efficacy of 96.7% and 90% in 10% and 5% of spray-dried extract (SDE), respectively. It is noteworthy that with the increasing concentration, there is a greater antiparasitic activity (Figure 2).

Figure 2: Efficacy of mastruz against *N. buttnerae* in *C. macropomum* after 15 min, 30 min, 1 h and 8 h of exposure.



In the treatment with SDE 10%, it was possible to observe alterations such as deformation of the body (swelling and wrinkling) (Figure 3).

Figure 3: **A:** Live acanthocephalans with white coloration and normal body morphology, as all parasites at the beginning of the experiment. **B:** Dead acanthocephalans in SDE 10% with deformities in the body (swelling and wrinkling in the body).



Fonte: Araújo, J. K. O. 2023.

DISCUSSION

In vitro studies with extracts and essential oils have been widely researched in the world, and use various methodologies according to the expected biological effect (QUEIROZ et al., 2022). In the present study, the objective was to evaluate the *in vitro* anthelmintic activity of mastruz in the control of *N. buttnerae*, since the *in vitro* assay aids the decision regarding the choice of methodologies to be used in the *in vivo* efficacy experiment (PARK et al., 2014).

In Figure 1, it is possible to observe that, when using SDE of mastruz at 5% and SDE of mastruz at 10%, all parasites died within 30 min, which demonstrates that mastruz has anthelmintic activity against *N. buttnerae*. This is similar to the activity of the positive control with levamisole, in which all parasites died within 15 min, though different from the results observed in the negative control where there were still live parasites after 8 h of experiment.

These results differ from those found in the literature regarding the mortality of parasites during the exposure to treatments, as in the study conducted by Oliveira et al. (2019). In this study, the authors tested *in vitro* extracts of banana residues (leaf, stalk and heart) for the control of acanthocephalans in tambaqui, and exposed the parasites to different concentrations. Only at the concentration of 50 mg/mL were the three tested residues effective, and the stalk was the part that presented 100% efficacy after 5 hours of experiment. In 1 hour, the efficacy was 20%, i.e., less time than presented in our study with SDE of mastruz since, within 15 min, at least 90% of the parasites were already dead in treatments (Figure 2).

In another *in vitro* study by Oliveira et al. (2019), of the avermectins tested, only emamectin benzoate, ivermectin, levamisole and praziquantel had the highest efficacy against *N. buttnerae*, and mortalities only began after 6 hours of experiment. This is an anthelmintic activity period much longer than that presented in this study with mastruz, for which, within 1 hour of exposure, 100% of the parasites in the treatments tested, with the exception of the positive control group, had already died.

On the other hand, Taraschewski et al. (1990) tested di-n-butyltin oxide *in vitro* against acanthocephalus *Neoechinorhynchus rutili* and *Echinorhynchus truttae*, but did not obtain satisfactory results, demonstrating that di-n-butyltin oxide, despite having parasiticidal activity in other studies, was not effective in controlling the acanthocephalans tested.

Similarly, mastruz when studied in conjunction with other helminths *in vitro* by Monteiro (2012), who used the aqueous extract of mastruz *in vitro* at concentrations of 0; 0.65; 1.3; 2.6; 3.9 and 5.2 mL/L in a Petri dish containing physiological solution, did not obtain good results in the low concentrations tested, only in the concentration of 2.6 mL/L did the author obtain 100% mortality of *Monogeneas* sp. in 1 h, which is also longer than what was obtained in the present study with mastruz.

The studies with plants for the treatment of helminths that were found in the literature, in the vast majority, involved essential oils or hydroalcoholic extracts, as a result of the disadvantages related to the use of synthetic products in fish farming, and in order to avoid harmful effects and ensure greater food safety (CORRAL et al., 2018, SANTOS et al., 2019, MANJOLO et al., 2019, MONTEIRO, 2020, JESUS and PEREIRA, 2020).

For example, Salaro (2018) evaluated the *in vitro* antiparasitic efficacy of the essential oils of *Mentha piperita, Melaleuca alternifolia, Ocimum basilicum, Thymus vulgaris* and *Allium sativum* against *N. buttnerae*, and only obtained effective results with *T. Vulgaris*, which, among the tested oils, was the most effective against the acanthocephalans.

Santos et al. (2018) tested the *in vitro* efficacy of the essential oils from *Piperaceae* species in the control of *N. buttnerae*, and presented results similar to those of the present study with SDE of *C. ambrosioides*, since there was mortality within just 15 min of exposure in all the treatments tested.

Santos et al. (2021) evaluated the *in vitro* anthelmintic activity of eugenol, thymol and tannic acid against *N. buttnerae*. Among the compounds tested, eugenol was the most efficient and eliminated 100% of the parasites within 15 min of exposure, which is similar to the result obtained in the present study with levamisole in the positive control and close to the length of time obtained with 5% and 10% SDE of mastruz.

In an *in vitro* assay for the control of the acanthocephalan *N. buttnerae*, Sebastião et al. (2021) tested nine concentrations (1.0, 1.5, 2.0, 2.5, 3.0, 3.5, 4.0, 4.5 and 5.0 mg/kg) of Acantex® and a control (without the product). Only at a concentration of 3.0 mg/kg did Acantex® show 100% efficacy in the control of *N. buttnerae* after exposure for 2 hours, which is longer than the result obtained with mastruz in this study.

As for the deformities shown in Figure 3 (wrinkling and swelling), few studies report these alterations in *in vitro* experiments, a condition observed in the SDE 10%. Only in the work of Costa et al. (2018) was this reported when studying a culture medium that was suitable for *N. buttnerae*. Similarly, Benesh and Valtonen (2007), who also report swelling in acanthocephalans, justified that this occurs due to an impaired capacity for osmoregulation that results in the death of the parasite.

It is necessary to use products that are suitable for fish farming and which are obtained via extraction and application methodologies with minimal environmental impact. According to Pavanelli et al. (2008) and Macedo et al. (2013), the administration of products in fish farming should be in moderation, because the knowledge about the action of substances in the environment, the residue in the animal and the concentration that varies between the different species, can cause serious damage; thus, studies that prove the effectiveness and safety in the application are essential.

As such, the SDE of mastruz has been shown to have anthelmintic activity *in vitro* in the control of *N. buttnerae*, contributing to the continuity of studies that intend to evaluate the *in vivo* toxicity and efficacy and determine an appropriate therapeutic protocol for use in fish farming.

CONCLUSION

With the results obtained, mastruz proves to be an excellent alternative in the control of *N. buttnerae*; however, complementary tests of *in vivo* toxicity and efficacy with tambaqui are necessary to determine the appropriate therapeutic protocol for the species.

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