



Evaluation of molluscicidal activity of *Anadenanthera colubrina* extracts on adult mollusc and embryos of the species *Biomphalaria glabrata* (Say, 1818)

Avaliação da atividade moluscicida do extrato de *Anadenanthera colubrina* sobre moluscos adultos e embriões da espécie *Biomphalaria glabrata* (Say, 1818)

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Schistosomiasis affects over 200 million people and a matter of concern for public health. One of the methods of combating the disease is through control of the snails of the species *Biomphalaria glabrata*. To evaluate the molluscicidal activity of *Anadenanthera colubrina* bark extract on embryos and adults of the species *B. glabrata* and on *Artemia salina*. The *A. salina* were exposed for 24 h to *A. colubrina* extract (0.125, 0.25, 0.5 and 1.0 mg/mL) diluted in seawater, while *B. glabrata* snails were exposed to the same period the extract (0.125, 0.25, 0.5, 0.6, 0.7 and 1.0 mg/mL) diluted in filtered and dechlorinated water. We observed that the extract at a concentration of 0.125 mg/mL showed low toxicity for *A. salina* used in relation to other concentrations. In the assay with *B. glabrata* snails were observed in adults the concentration of 0.25 mg/mL caused 80% mortality. In embryos, concentrations of 0.125; 0.25 and 0.5 mg/mL did not affect the embryo viability when compared with control group. The results show that the aqueous extract of *A. colubrina* demonstrated potential to be used as a biodegradable molluscicide to combat snails of the species *B. glabrata*.
Keywords: *Anadenanthera colubrina*, *Biomphalaria glabrata*, toxicity

A esquistossomose afeta mais de 200 milhões de pessoas e constitui motivo de preocupação para saúde pública. Um dos métodos de combate a doença é por meio de controle dos caramujos da espécie *Biomphalaria glabrata*. Para avaliação a atividade moluscicida do extrato da casca de *Anadenanthera colubrina* sobre embriões e adultos da espécie *B. glabrata* e sobre *Artemia salina*. As *A. salina* foram expostas durante 24 h ao extrato de *A. colubrina* (0,125; 0,25; 0,5 e 1,0 mg/mL) diluídos em água do mar, ao passo que moluscos *B. glabrata* foram expostos pelo mesmo período ao extrato (0,125; 0,25; 0,5; 0,6; 0,7 e 1,0 mg/mL) diluído em água filtrada e desclorificada. Observamos que o extrato na concentração de 0,125 mg/mL apresentou baixa toxicidade para *A. salina* em relação a outras concentrações utilizadas. No ensaio realizado com *B. glabrata* foi observado nos caramujos adultos que a concentração de 0,25 mg/mL gerou mortalidade de 80%. Nos embriões, as concentrações de 0,125; 0,25 e 0,5 mg/mL não apresentaram alterações na viabilidade embrionária, quando comparado com grupo controle. Os resultados mostram que o extrato aquoso de *A. colubrina* demonstrou potencial para ser utilizado como moluscicida biodegradável no combater aos caramujos adultos da espécie *B. glabrata*.

Palavras-chave: *Anadenanthera colubrina*, *Biomphalaria glabrata*, toxicidade

1. INTRODUCTION

Schistosomiasis is an endemic disease in several tropical countries caused by *Schistosoma mansoni*. This disease is one of the most prevalent parasitic infections observed in tropical countries, and negatively affects the economy and public health [1, 2]. The intermediate hosts of *S. mansoni* are species that belong to the genera *Biomphalaria*, *Bulinus* and *Oncomelania*. The

incidence of schistosomiasis is linked to lack of basic sanitation, as well as, to precarious socioenvironmental condition [3].

According to World Health Organization statistics, schistosomiasis is a disease that affects more than 239 million people around the world [4], distributed in several countries from Asia, America, Caribbean, Mediterranean and Africa [2]. Depending on the region, the *S. mansoni* presents genetic variations that are important for disease epidemiology [5]. Moreover, the schistosomiasis is the second most incident tropical disease and is behind only of malaria [6, 7].

The *B. glabrata* is a mollusk of Planorbidae family, that can be found in several countries in aquatic environments with steady or low flow water, such as lakes, ponds, wells, rivers backwaters, streams, irrigation channels and drainage, or any natural or artificial area waterlogged [8, 9].

Several chemical substances are used in efforts to combat the vector mollusk. It is estimated that approximately 7000 chemical products have already been tested for this purpose, and, actually, the chemical substance niclosamide is the one that is commercially available and indicated by the World Health Organization. Although niclosamide presents high toxicity to molluscs, it is highly toxic to other aquatic organisms [10, 11].

In this context, there is increasing interest in the search of new easily biodegradable substances with molluscicidal activity from plant extracts. In the literature, there are various reports about plants and their active principles, which were studied for their potential molluscicide [12, 13, 14, 15]. It is believed that in Brazil less than 5% of the plants have been studied to determine the phytochemical analysis of secondary metabolites [16], and this makes regional plants of great interest for investigation of new molluscicides.

The *Anadenanthera colubrina*, popularly known as angico, is native to the equatorial regions of South America and can be found in areas with altitudes above 400 m. In Brazil, its distribution was observed in several states belonging to the North, Northeast, Southeast and South regions [17]. It possesses, among bioactive secondary metabolites, phenolic compounds and tannins [18], which are promising molluscicide substances.

2. MATERIALS AND METHODS

2.1. *Anadenanthera colubrina* collection and storage

Samples from the bark of *A. colubrina* were collected in a caatinga region within the Institute of Agronomic Research. The area is located in the city of Caruaru, Agreste microzone of Pernambuco state, Brazil (08°14'18.2" S and 35°54'57.1" W). A specimen was identified and deposited in the herbarium Professor Vasconcelos Sobrinho in Rural Federal University of Pernambuco, under number 48663.

2.2. Extract preparation of *Anadenanthera colubrina*

The samples were dried, pounded into a knife mill and sieved using a 2x2 mm mesh sieve. The crude extract of *A. colubrina* was obtained by macerating 5 g of the bark at 100 mL of 80% methanol. Then, the solution was kept in a dark chamber for 72 h, and, posteriorly, the extract was dried in a rota-evaporator (Fisaton model 803) at reduced pressure.

2.3. Phytochemical analysis of extract of *Anadenanthera colubrina*

Chromatographic analysis was performed on a thin layer to identify the main classes of secondary metabolites (tannins, flavonoids, steroids, terpenoids, sugars), according to the methodology established by Wagner and Bladt [19], in addition, identification of total phenols by the method described in European Pharmacopoeia [20].

2.4. Toxicity assay using brine shrimp (*Artemia salina*)

The crustacean *Artemia salina* is used as a biological model for assessing the toxicity of *A. colubrina* extract to non-target organisms. Thus, the eggs (25 mg) of *A. salina* were placed in a recipient with seawater (pH 8.0; 25–30 °C) for 48 h with aeration until their outbreak. After this, *A. salina* was collected for analysis of viability. The animals were divided into five groups (n=10–11), as follows: one negative control group (containing only sea water, C), one positive control group (niclosamide, NCL) and four groups exposed to bark extract of *A. colubrina* at concentrations of 0.125, 0.25, 0.5 and 1.0 mg/mL for 24 h. The tests for evaluation of mortality and survival were performed in accordance to Santos et al. (2010a) [21] in quadruplicate.

2.5. *Biomphalaria glabrata* molluscs breeding and maintenance

The molluscs of *B. glabrata* species were obtained in São Lourenço da Mata (state of Pernambuco, Brazil). They were bred and kept for generations in the vivarium of Department of Biophysics and Radiobiology of Federal University of Pernambuco. The adult molluscs were maintained in an aerated plastic aquarium (50x23x17 cm) with filtered water (pH 7.0) at 25 °C. They were fed lettuce leaves (*Lactuca sativa*).

2.6. Molluscicidal Activity

For the evaluation of molluscicidal activity, molluscs (n=90) of uniform size (shell diameter between 10–16 mm) were distributed into six groups, as follows: negative control (purified water, C), positive control (NCL) and four groups exposed to *A. Colubrina* filtered extract dissolved in water at concentrations of 0.125, 0.25, 0.5 and 1.0 mg/mL for 24 h. The assay was repeated 5 times, using triplicate in each one.

2.7. Embryotoxicity

The bioassay was performed according to the methodology described by Oliveira-Filho and Paumgartten 2000 [22]. It was collected five samples of intact eggs, each one containing approximately 100 embryos at the blastula stage (15 h after the first cleavage) selected by observing at a stereoscopic microscope (Leica MZ6; Leica Microsystems, Wetzlar, Germany). The samples were placed in Petri plates (90x15 mm) and exposed to *A. colubrina* bark extract (0.125, 0.25, 0.5, 0.6, 0.7 and 1.0 mg/mL), filtered and dechlorinated water (C, negative control) or filtered and dechlorinated water containing 1.0 µg/mL niclosamide (NCL, positive control), for assay performed with adult molluscs. The groups were incubated for 24 h (25 ± 2 °C) and all remained samples in dechlorinated and filtered water were analyzed for 7 days. The following parameters were determined in triplicates using a stereoscopic microscope (Leica KL300; Leica Microsystems, Wetzlar, Germany): mortality, embryonic stage (blastula, gastrula, immature trochophore, trochophore, immature veliger, veliger and hypo stage), the number of embryos to complete development (hatched or having reached the hypo stage) or not fully developed (dead, malformed or delayed normal development).

2.8. Statistical analysis

The statistical analysis of results was performed using the program GraphPad Prism version 5.0 for Windows (GraphPad Prism, San Diego, California, USA) using ANOVA followed by Newman-Keuls test (significance $p < 0.05$). To calculate the lethal concentration needed to kill 50% (LC₅₀), it was performed Probit Regression.

3. RESULTS

3.1. Phytochemical analysis

In Table 1, the result may be observed in the phytochemical analysis for the presence of secondary metabolites. Was performed a thin-layer chromatography to identify the main metabolites present in the extract samples.

Table 1: Shows the presence (+) or absence (-) of the specific compound of extract of *Anadenanthera colubrina*.

Secondary metabolites					
Steroids	Flavonoids	Tannins	Sugars	Terpenoids	Phenols
-	-	+	+	-	+

3.2. Toxicity Assay (environmental toxicity)

The figure 1 shows mortality of *Artemia salina* exposed to the extract from the bark of *A. colubrina*.

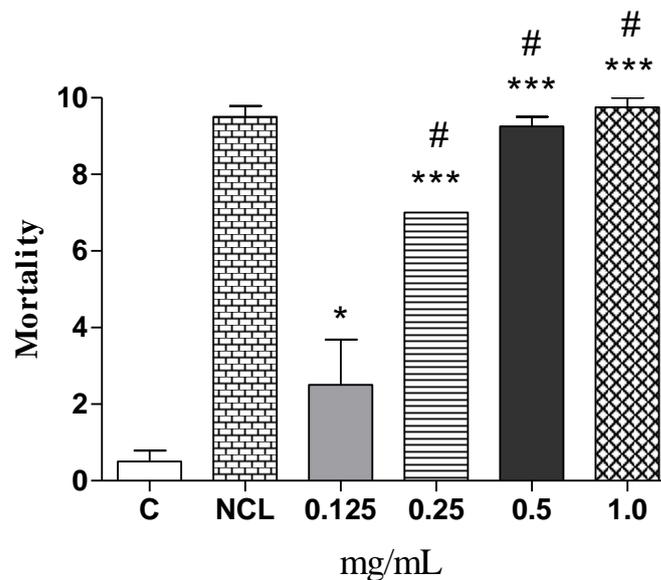


Figure 1: Toxicity assay of *Artemia salina* with the bark extract of *A. colubrina* for 24 h. * and *** vs. C; # vs. 0.125 mg/mL.

Although the *A. colubrina* bark extract increased *Artemia salina* mortality at the concentration of 0.125 mg/mL comparing to control group, it was observed a much greater toxicity at concentrations of 0.25, 0.5 and 1.0 mg/mL. The LC_{50} calculated for *A. salina* exposed to *A. colubrina* extract was 0.212 mg/mL.

3.3. Molluscicide activity

In Table 2 it is presented the mortality data from molluscs exposed to *A. colubrina* extract.

Table 2: Adults molluscs *Biomphalaria glabrata* exposed to *Anadenanthera colubrina* extract for 24 h.

[] mg/mL	Total dead molluscs	Dead molluscs	%	Living molluscs	%
C	15	0	0	15	100
NCL	15	15	100	0	0
0.125	15	4	26.66	11	73.33
0.25*	15	12	80	3	20
0.5*	15	15	100	0	0
1.0*	15	15	100	0	0

The data where $p < 0.05$ demonstrate significant differences between the groups, * vs. C. Where, C - control and NCL - niclosamide.

At concentrations of 0.125 mg/mL, the *A. colubrina* induced mortality frequency of 26.6%, but this value was not significantly different from negative control group. However, at a concentration of 0.25 mg/mL, the mortality induced by the extract (80%) was significantly different compared to negative control group. The molluscs exposed to concentrations of 0.5 and 1.0 mg/mL presented 100% mortality, which is similar to that observed in the group exposed to niclosamide. The result obtained in this bioassay with extract *A. colubrina* originated a lethal concentration needed to kill 50% (LC_{50}) 0.155 mg/mL for adults of *B. glabrata*.

3.4. Embryotoxicity

In Table 3, it can be observed the impact of *A. colubrina* extract on the viability of *B. glabrata* embryos.

Table 3: Embryos *Biomphalaria glabrata* exposed to *Anadenanthera colubrina* extract for 24 h.

[] mg/mL	Total embryos	Unviable embryos	%	Viable embryos	%
C	294	13	4.5	281	95.6
NCL	300	300	100	0	0
0.125	292	5	1.7	287	98.3
0.25	295	6	2	289	98
0.5	300	31	10.4	269	89.6
0.6*	300	140	46.7	160	53.3
0.7*	298	151	50.7	147	49.3
1.0*	312	312	100	0	0

The data where $p < 0.05$ demonstrate significant differences between the groups, * vs. C. Where, C - control and NCL - niclosamide.

The groups exposed to doses of 0.125, 0.25 and 0.5 mg/mL of extract presented similar embryotoxicity to negative control group. On the other side, the embryos exposed to the extract at concentrations of 0.6, 0.7 and 1.0 mg/mL presented mortality of 46.7, 50.7 and 100%, respectively, which were statistically higher than the negative control group. The LC_{50} calculated for embryos exposed to *A. colubrina* extract was 0.692 mg/mL. The figure 2 shows features of viable and non-viable embryos (dead and malformed) after exposure to *A. colubrina* extract.

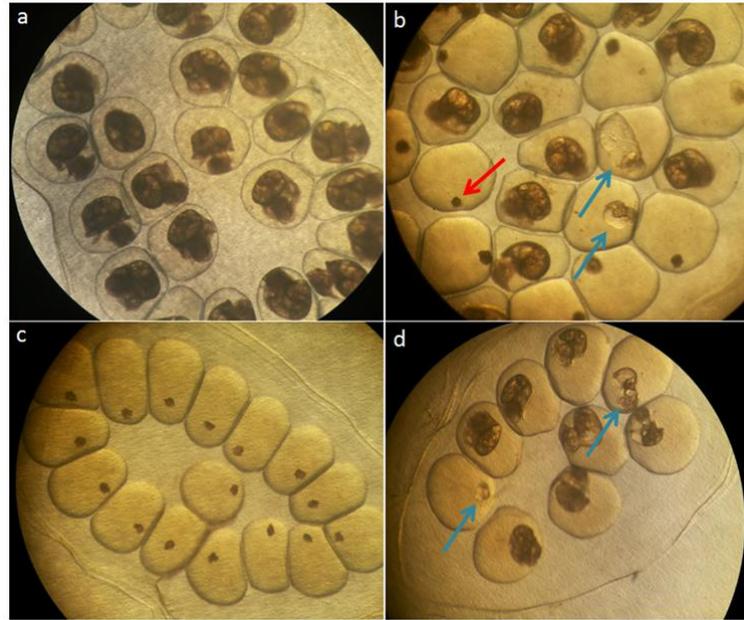


Figure 2: *Biomphalaria glabrata* embryos. a) Normal embryos; b) Dead embryos (red arrow) and malformed embryos (blue arrow); c) Dead embryos; d) Malformed embryos (blue arrow).

4. DISCUSSION

The molluscicides plant, when compared to synthetic ones, present minimal environmental damage, constitute an effective and low-cost method that may help control schistosomiasis. Despite this, only synthetic molluscicides are commercialized [23]. Studies investigating molluscicidal activity in plants had received special attention in the last decades due to the low-cost alternative and less aggressive approach to nature [24, 25]. The World Health Organization sets standard procedures for test several molluscicides and recommends prospection of plants and vegetable products endowed with molluscicide properties that can be used without affecting the equilibrium of the environment [26].

In this work, the environmental toxicity was estimated by *A. salina* sensibility to *A. colubrina* extract. The mortality rate was estimated by calculating LC_{50} , which was 0.212 mg/mL. Albeit, there is no literature data about environmental toxicity of *A. colubrina*, we verify that this extract has low environmental toxicity comparing to other vegetable extracts of plants of the same region, as *Turnera ulmifolia* leaf extract ($LC_{50} = 0.224$ mg/mL) [27]; *curcuma longa* L. extracts ($LC_{50} = 0.319$ mg/mL) and many species of the genus *Eleocharis* (LC_{50} of 0.476 mg/mL) [28].

The use of molluscicides requires the comprehension of the mechanism of action of the substances in molluscs. To achieve this, it is necessary to detail phytochemical profile of the plant, as well as, mollusc physiologic response against the chemical constituents. Based on the increased mortality of *B. glabrata* molluscs induced by *A. colubrina* extract, the mechanisms of action of the extract combating mollusc deserve to be explored.

Experiments *in vitro* evaluated the effects of leaf and stem extracts of *A. colubrina*, *Leucaena leucocephala* and *Mimosa tenuiflora* on the larval unshedding of *Haemonchus contortus*. In these experiments, it was observed that tannins present a key role in this process. The six extracts blocked the larval unshedding when incubated at 0.3 mg/mL for 3 h with addition of sodium hypochlorite. It is suggested that *A. colubrina*, *L. leucocephala* and *M. tenuiflora* can be used in the control of gastrointestinal nematodes, and the tannins may be the main compounds involved in the effects [22].

Studies with saponins present in the extract of *Agave decipiens* [29] and *Yucca desmettiana* [30] was verified that the substances present in the extracts showed molluscicide activity against the *Biomphalaria Alexandrina* snail, where this effect on mollusc can be related to the nature of the

chain, number of residues and sequence sugars present in the molecule structure. However, *A. colubrina* extract was not observed the presence of sugar after the completion of phytochemical analysis.

Research conducted with *Eucalyptus exserta* bark extract was shown that the compound has molluscicidal and cytotoxic activity these being effects relating the presence of terpenoids [31]. In contrast, the extract used in this study did not present terpenoids.

Molluscicidal activity of plants has been being linked to the presence of phenols and tannins [26, 32]. It is suggested that the active principle of *A. colubrina* is attributed to the presence of chemical compounds coming from the tannins and phenols which strengthens the need for further study of these substances present in the plant.

Previously, it had not yet been reported the toxicity of *A. colubrina* on embryos and adults of *B. glabrata*. Though some plant extracts presents molluscicidal activity for adults specimens, they may be inactive for embryos. This behavior could be related to the selectivity of the gelatinous membrane that prevents spawning penetration and action of the substances on embryos [33]. These eggs are covered with perivitelline fluid, synthesized and secreted by the albumen gland and encapsulated by a membrane produced by the *pars contorta*. Therefore, the membranes and the gelatinous mass that surrounds the eggs are effective barriers against the penetration of toxic substances [34, 35] present in the extract of *A. colubrina*. In the present study, high concentrations (between 0.5 and 1.0 mg/mL) of the extract, which could impregnate the spawning surface, caused lethality in embryos in a dose-dependent manner.

5. CONCLUSION

In conclusion, this study points that the aqueous extract of *A. colubrina*, although do not increase mortality of large part of the embryos, decreases their viability and helps the vector combat. On the other side, if demonstrate molluscicidal activity at low concentrations *A. colubrina* extract it could potentially be used as a biodegradable molluscicide to combat adult snails of the species *B. glabrata*.

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