ARTIGO ORIGINAL

ACTIVITY OF *Stryphnodendron polyphyllum*, A PLANT FROM THE BRAZILIAN SAVANNAH, AGAINST HEMOCYTES OF *Biomphalaria glabrata*, AN INTERMEDIATE HOST OF *Schistosoma mansoni*

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ABSTRACT

The snail *Biomphalaria glabrata* acts as an intermediate host to *Schistosoma mansoni*, an endemic parasite in several countries. *B. glabrata* hemocytes are related to its defense against infection by trematodes, including *S. mansoni.* In the present paper, the activity of molluscicidal substances such as the tannic acid extracts of *Stryphnodendron polyphyllum*, a plant from the Brazilian Savannah, on the morphology and number of *B. glabrata* hemocytes was evaluated. The bark and leaf extracts of *S. polyphyllum* were diluted in dechlorinated water and groups of snails were exposed to 25 and 50 $mg.L^{-1}$ concentrations of the extracts, as well as, to tannic acid during 24h. Subsequently, hemolymph was removed from the pericardic region. Hemocyte subpopulations were detected and classified as small (5.0 – 6.9 µm), medium (7.0 – 8.9µm), large (9.0 – 12.0µm) and giant (over 12µm), the latter being a novel classification. The extracts stimulated an increase in the number of hemocyte cells in the hemolymph. This is understood as a defense mechanism against toxic substances such as tannic acid, present in high levels in both tested extracts. Hemocytes showed vacuoles in the cytoplasm due to the presence of such substances, which are signs of cellular death due to apoptosis. We conclude that the extracts are highly effective against *B. glabrata*, recommending further biological impact studies aiming its use as a natural molluscicide.

KEY WORDS: *Biomphalaria glabrata. Schistosoma mansoni. Stryphnodendron polyphyllum.* Molluscicide. Hemocytes.

INTRODUCTION

Schistosomiasis is a worldwide endemic disease, being an important cause of death by infectious diseases in developing countries. It is related to the lack of basic sanitation and low quality of life (22). The snail of the genus *Biomphalaria* serves as

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an intermediate host of schistosomiasis and plays a crucial part in the transmission of this parasitosis, which means that in a certain location where there are snail specimens and a single patient discharging viable eggs, this is enough to establish a new infection site (18, 19). The importance of snail control is, therefore, significant.

A number of Brazilian plants with molluscicidal activity have been registered. Various research groups emphasized the use of natural extracts in combating the snail hosts (9, 3, 14, 23). An endless source of plants with this potential is to be found in the savannah, a biome characteristic of the Brazilian Mid-West (2). Many of these plants have high levels of tannin, which provides the plants with the defense against herbivores as well as limited growth of pathogenic microorganisms. The Brazilian savannah plant *Stryphnodendron polyphyllum*, one of the plants known as "barbatimão", is widespread in this biome, and is prescribed for diarrhea, gynecological problems and wound healing in folk medicine (21).

Hemocytes in the circulating hemolymph and tissues are the snail's physiological response to environmental alterations and infection (4, 14). They are considered the snail's defense cells, being similar to human granulocytes in the presence of phagocytosis granules, conferring resistance to gamma radiation and phagocytary ability related to the regulation by cyclic adenosine monophosphate (cAMP). This process allows for the identification of a non-specific defense system directly related to the snail's susceptibility and resistance to infection by trematodes such as *S. mansoni* (6, 7, 10, 12, 13). The presence of plasmatic factors, calcium and lecithins also influence the phagocytary ability of hemocytes of susceptible and resistant snails to *S. mansoni* infection (5).

In the snail *B. glabrata,* the hemocytes have varied morphology corresponding to different physiological conditions and their transitions. In quiescent hemocytes, the cellular cortex is narrow. The organelles are spread around the cytoplasm in circulating and sedentary cells. In stress-activated hemocytes the cortical region is enlarged by the polymerization of actin and the organelles are around the nucleus. Fixed phagocytes are components of connective tissue and the presence of various lysosomes with phosphatase and peroxidases activity indicate high phagocytic ability (16, 20, 25).

The literature indicates that the knowledge about hemolymphatic cells and their relation to the *Biomphalaria – Schistosoma* system has been much improved. The aim of this study is, thus, to expand these investigations and describe the effect of extracts of the Brazilian Savannah plant *S. polyphyllum* on the morphology and quantification of hemolymph hemocytes of *B. glabrata*.

MATERIAL AND METHODS

Snails maintenance

Snails of the species *B. glabrata* were maintained in 10-l aquaria filled with dechlorinated water at an average temperature of 28° C. The tanks were cleaned

twice a week and the snails fed on lettuce and snail food (agar, calcium carbonate, oatmeal) *ad libitum* (24).

Bioassays

The bark and leaf extracts of *Stryphnodendron polyphyllum* were diluted in dechlorinated water. Three groups of assays with a total of 15 specimens were carried out. In group 1 (control group), the snails were placed in dechlorinated water without the extract. In group 2 the snails were exposed to crude extracts of *S. polyphyllum* at concentrations of 25 and 50 $mg.L^{-1}$. This group was divided into two subgroups. Thus, snails of subgroups A and B were exposed to bark and leaf extracts, respectively. In group 3, the snails were exposed to tannic acid also at 25 and 50 $mg.L^{-1}$ concentrations. This substance was used separately aiming at observing differences in its performance when concentrated or mixed to other substances of the *S. polyphyllum* extract. The bioassays lasted 24 hours and the snails were fed as usual.

Hemolymph removal

Mollusk shells were cleaned with 70% alcohol and distilled water and then dried with absorbent paper to remove the hemolymph. Under a stereoscope microscope and with the aid of a surgical scalpel, a small hole was made in the pericardic region aiming at the hemolymph flow. This resulted in filling the central opening of the shell and increasing the hemolymph flow as the snail pulled into its shell. The hemolymph was immediately removed and placed in a microtube kept on ice (5, 6, 15). As soon as the average volume of hemolymph/snail was determined, a sample of 30μL of the hemolymph of each group was stained with 5μL of Tripan Blue-vital dye, allowing for the determination of the relative proportion of live and dead cells with the aid of a Neubauer chamber (15). Hemolymph smears were then made and stained with Giemsa solution for hemocyte analysis and identification of granules in the cytoplasm.

Morphological analysis

The hemolymphatic smears allowed for the observation of hemocyte subpopulations, which were distinguished by cell diameter. They were examined with a computerized image analyzing system (Axiovision 3.1 – Zeiss), which determines the diameter measurements (μm). For each measurement, the range is given in parentheses. A modified version of the classification of Matricon-Gondran and Letocart (15) identified the hemocytes into the following categories: small (with approximately 5.0 – 6.9 μm in diameter), medium (with approximately 7.0 – 8.9 μm in diameter), large (with approximately 9.0 – 12 μmin diameter) (15) and giant (with more than 12μm in diameter). Each bioassay was repeated three times.

Statistical analysis

The sizes were expressed in mean or median according to the normality test by Kolmogorov-Smirnov. In the statistical analysis the χ^2 test was used to relate hemocyte survival to the presence of the natural extract and the ANOVA test followed by the Tukey test were used to compare concentrations of snail hemocytes under normal and test conditions. The statistic significant differences were considered when $p<0.05$.

RESULTS

The hemocytes from the control group presented a regular rounded/oval shape, peripheric basophilic nucleus and large acidophilic cytoplasm. These color characteristics were present in hemocytes of all groups. The following cell types were detected in all subpopulations: small cells with median diameter of 6.00 μm; medium cells with an average size of 8.00 μm (figure 1A, 1B) and large cells with median diameter of 10.36 μm. The giant cells showed a median diameter of 12.54 μm (table 1).

Table 1. Median (minimum-maximum) diameter of hemocyte subpopulations of *Biomphalaria glabrata* submitted to different concentrations of *Stryphnodendron polyphyllum* extracts and to tannic acid.

Group (n=15)		Small cells (μm)	Medium cells (μm)	Large cells (μm)	Giant cells (μm)
Group 1[1]		6.00 (2.50-6.96)	8.00 (7.08-8.96)	10.36 (9.03-11.80)	12.54 (12.02-16.56)
Group 2A[2]	25 mg.L^{-1}	5.80 (2.40-6.71)	7.55 (7.00-8.30)	10.52 (9.30-11.87)	15.46 (12.47-21.09)
	50 mg.L^{-1}	5.87 (3.52-6.86)	8.25 (7.03-.8.78)	10.44 (9.03-12.00)	17.08 (12.16-35.55)
Group 2B[3]	25 mg.L^{-1}	6.38 (3.23-6.91)	7.46 (7.00-8.81)	10.12 (9.08-11.70)	16.01 (12.21-30.31)
	50 mg.L^{-1}	5.90 (5.30-6.70)	7.80 (7.20-8.93)	9.29 (9.02-11.21)	13.87 (12.04-21.20)
Group 3[4]	25 mg.L^{-1}	5.66 (4.78-6.84)	8.26 (7.09-8.96)	9.34 (9.10-11.46)	15.35 (15.14-15.56)
	50 mg.L^{-1}	5.80 (2.70-6.60)	8.00 (1.20-8.50)	9.85 (9.80-9.90)	13.50 (12.70-18.40)

[1] Snails exposed to dechlorinated water (control group); [2] Snails exposed to bark extract of *S. polyphyllum*; [3] Snails exposed to leaves extract of *S. polyphyllum*; [4] Snails exposed to tannic acid.

The average volumes of hemolymph as well as the average number of hemocytes/μL according to group are described in table 2. There were no statistical differences between the volumes from the snails exposed to the extracts, to tannic acid and from the control group.

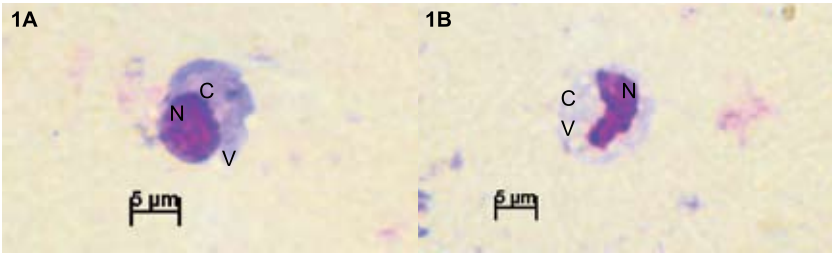

Figure 1. Hemolymph cells from *Biomphalaria glabrata.* 1A, 1B. Medium sized hemocytes, group 1 (control group), Giemsa. N – peripheric basophilic nucleus; C – acidophilic cytoplasm; V – vacuoles.

Table 2. Volume of *Biomphalaria glabrata* hemolymph and hemocyte count per µl according to the group analyzed.

Group (n=15)		Average volume of hemolymph pool (µL)	Average volume of hemolymph/snail (µL)	Average hemocytes/µL
Group 1[1]		762.5	152.5	4,503
Group 2A[2]	25 mg.L^{-1}	566.67	113.33	4,357
	50 mg.L^{-1}	560	112	5,380
Group 2B[3]	25 mg.L^{-1}	616.67	123.33	3,557
	50 mg.L^{-1}	723.33	144.67	5,097
Group 3[4]	25 mg.L^{-1}	486.67	97.33	4,573
	50 mg.L^{-1}	510	102	6,210

[1] Snails exposed to dechlorinated water (control group); [2] Snails exposed to bark extract of *S. polyphyllum*; [3] Snails exposed to leaves extract of *S. polyphyllum*; [4] Snails exposed to tannic acid.

The mortality rates of hemocytes exposed to different concentrations of *S. polyphyllum* extracts and tannic acid are shown in figure 4. The mortality rate was higher in snails from group 2A at 25 mg.L^{-1} concentration.

In group 2A (25 mg.L^{-1} concentration) the median diameter of the small cells was 5.80 µm, while of medium-sized cells was 7.55 µm, of large cells was 10.52 µm (figure 2A, 2B) and of giant cells 15.46 µm (table 1). In hemocytes from snails from group 2A (50 mg.L^{-1} concentration) the median diameter noted for the small cells was 5.87 µm, for medium cells was 8.25 µm (figure 3A, 3B), for large cells was 10.44 µm and for giant cells was 17.08 µm (figure 3; table 1).

Among snails from group 2B (at 25 mg.L^{-1} concentration) the median diameter of the small cells was 6.38 µm, of medium cells was 7.46 µm, of large cells was 10.12 µm, and of giant cells was 16.01 µm (table 1). In the hemocytes from snails of group 2B, at 50 mg.L^{-1} concentration, the median diameter of small cells was 5,90 µm, of medium cells 7.80 µm, large cells 9.29 µm and giant cells 13.87 µm (table 1).

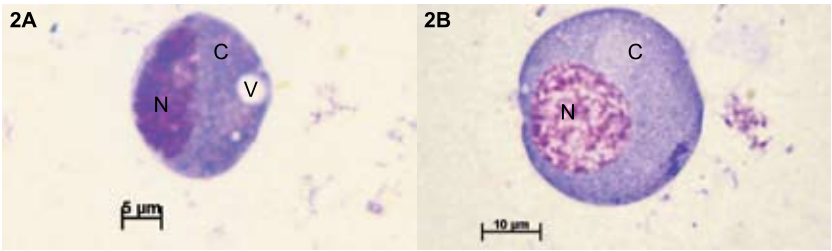

Figure 2. Hemolymph cells from *Biomphalaria glabrata* exposed to *Stryphnodendron polyphyllum* extracts, group 2A at 25 mg.L^{-1} concentration. 2A. Large sized hemocyte; and 2B. Giant hemocyte. Giemsa. N – peripheric basophilic nucleus; C – acidophilic cytoplasm; V – vacuoles.

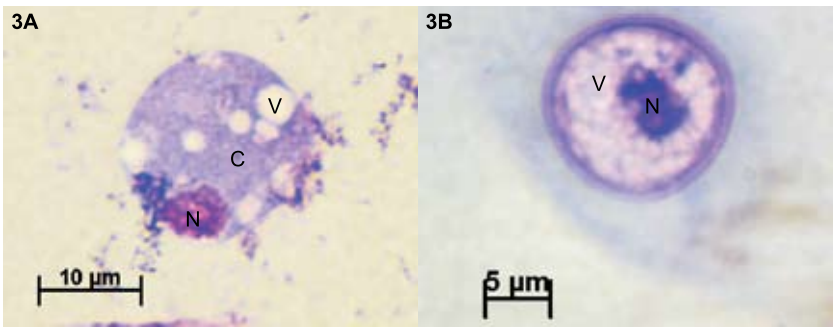

Figure 3. Hemolymph cells from *Biomphalaria glabrata.* 3A. Giant hemocyte, group 2B, 50 mg.L^{-1} concentration. Giemsa. 3B. Small sized hemocyte, group 3, 50 mg.L^{-1} concentration. Giemsa. N – peripheric basophilic nucleus; C – acidophilic cytoplasm; V – vacuoles.

In the hemocytes from snails exposed to tannic acid (group 3), at 25 mg.L^{-1} concentration, the median diameter of small cells was 5.66 µm, medium cells 8.26 µm, large cells 9.34 µm and giant cells 15.35 µm (table 1). In the 50 mg.L^{-1} concentration, the median diameter of the small cells was 5.80 µm, of medium cells was 8.00 µm, of large cells was 9.85 µm, and of giant cells was 13.50 µm (table 1).

Hemocyte cytoplasm with no granules and a nucleus with one to two nucleoles was noted in all groups. In groups 2 and 3 the presence of cytoplasmatic vacuoles and free nuclei were observed from ruptured cells, probably due to the excessive number of vacuoles in the cytoplasm.

The survival of the hemocytes was significantly correlated to the concentration of *S. polyphyllum* extracts (χ^2, $p<0,05$). There was a significant difference between the concentrations of hemocytes/µl of hemolymph in the different groups (ANOVA, $p<0,05$). There was no significant difference in the

diameter of the four subpopulations of hemocytes found in the hemolymph of the groups studied (ANOVA, p>0,05). A significant difference was found when comparing the diameters of small, medium, large and giant cells within the groups tested (ANOVA, p<0,05).

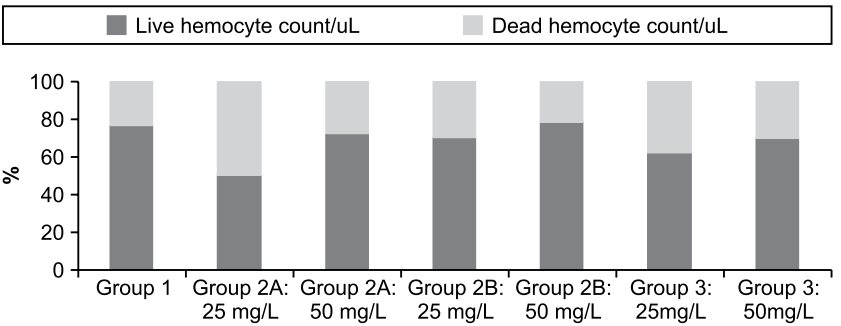

Figure 4. Mortality rate of hemocytes from *Biomphalaria glabrata* exposed to *Stryphnodendron polyphyllum* extracts and to tannic acid. Group 1: Snails exposed to dechlorinated water (control group); Group 2A: Snails exposed to bark extract of *S. polyphyllum*; Group 2B: Snails exposed to leaves extract of *S. polyphyllum*; Group 3: Snails exposed to tannic acid.

DISCUSSION

The analysis of the defense system of *B. glabrata* is significant to the understanding of the parasite-host relationship, particularly regarding *S. mansoni*. This study verified the morphometry and concentration of *B. glabrata* snail hemocytes exposed to plant extracts, considering that no similar literature references were found.

Hemocyte classification has not been standardized in the literature until now. In the present study, it was possible to note the same basophilic and acidophilic characteristics in all hemocyte subpopulations similarly to the ones described by Delgado *et al* (11). These authors (11) also made a differential counting of the hemocyte subpopulations, but the classification was based on color affinity of these cells. Yet, the subpopulations were classified according to the cell diameter as described by Matricon-Gondran and Letocart (15). Thus, small, medium, large, and giant cell subpopulations were observed. The last category was introduced for the first time. As regards the diameters of small, medium, large, and giant cells in the same group, a significant difference was noted, where diameters can vary from 2.40μm to 31.31μm.

In the present study, the number of hemocyte per μL of hemolymph was similar to that previously found by Delgado *et al* (11). Thus it reached a maximum

number of 6,210/µl whereas in the latter study the *B. glabrata* snail hemocyte count reached 6,450/µl. This could be explained as a reaction to the presence of toxic substances in the water, which could induce an immune response from the snail.

In preliminary studies the extracts from *S. polyphyllum* bark and leaves were used in mortality tests of *B. glabrata.* Thus, snails exposed to 50 $mg.L^{-1}$ concentration resulted in a mortality rate of 70% after 24h (8). Alcanfor (1) analyzed the histology of the digestive gland of *B. glabrata* snails exposed to bark and leaf extracts of *S. polyphyllum*. The vacuoles found are consistent with those observed in the hemocytes exposed to the same plant extracts in this study.

The concentrations of *B. glabrata* hemocytes significantly varied at the concentrations of the plant extracts. More specifically, the increase in hemocyte concentration is directly related to the presence and concentration of the extracts as well as its mortality rate, seen in figure 4 and table 2. This might be explained by the phagocytary and internal defense activity inherent to hemocytes (21, 6).

The tannic acid present in the bark and leaf extracts of *S. polyphyllum* is considered the active principle in natural molluscicides made out of tannin plants (2, 21). Indeed, greater concentrations of hemocytes in group 3 (table 2) in both concentrations were noted. Mendes *et al.* (17) tested another tannin plant, *S. barbatiman*¸ on *B. glabrata* snails and detected greater mortality of adults when exposed to 100 $mg.L^{-1}$ concentrations. This finding shows that tannic acid alone seems to affect the snail's defense system more strongly than when naturally present in the plant extract.

To sum up, hemocytes were classified into four groups due to their diameter, taking into account the fact that morphology is affected by the concentration and quality of the extracts found in the environment. Further studies on the morphology of hemocytes exposed to tannin extracts are particularly encouraged. They may reveal interesting aspects of the mollusk-parasite relationship as well as alternative molluscicides against schistosomiasis snail hosts. Due to the enormous Brazilian biodiversity, highly efficient and low cost products can be discovered, encouraging its conservation. Natural products are best assimilated by the environment causing lower impact on other co-habiting species in sites populated by infected snails.

RESUMO

Atividade de *Stryphnodendron polyphyllum,* uma planta do cerrado do Brasil, contra hemócitos de *Biomphalaria glabrata,* um hospedeiro intermediário de *Schistosoma mansoni.*

O caramujo *Biomphalaria glabrata* atua como hospedeiro intermediário de *Schistosoma mansoni*, um parasito endêmico em vários países. Os hemócitos de *B. glabrata* estão relacionados à sua defesa contra infecções por trematódeos como *S. mansoni*. No presente artigo, avaliou-se pela primeira vez o efeito de substancias

moluscicidas como o extrato tânico de *Stryphnodendron polyphyllum*, uma planta do cerrado brasileiro, sobre a morfologia e número de hemócitos de *B. glabrata.* Os extratos da casca e das folhas de *S. polyphyllum* foram diluídos em água desclorada. Os grupos de caramujos foram expostos a concentrações de 25 e 50 $mg.L^{-1}$ dos extratos e de ácido tânico por um período de 24h. E, posteriormente, a hemolinfa foi retirada da região pericárdica. As subpopulações de hemócitos foram detectadas e classificadas como pequenas (5,0 – 6,9 μm), de tamanho médio (7,0 – 8,9μm), grandes (9,0 – 12,0μm) e gigantes (mais que 12μm), sendo a última ainda não descrita na literatura. Os extratos estimularam um aumento no número de hemócitos na hemolinfa, o que é entendido como um mecanismo de defesa contra substancias tóxicas como o ácido tânicos, presente em altos níveis em ambos os extratos testados. Os hemócitos apresentaram vacúolos no citoplasma devido à presença de tais substancias indicando sinais de morte celular por apoptose. Concluímos que os extratos são altamente eficazes contra *B. glabrata* e recomendamos maiores estudos para seu uso como moluscicida natural.

DESCRITORES: *Biomphalaria glabrata. Schistosoma mansoni. Stryphnodendron polyphyllum.* Moluscicida. Hemócitos.

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