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ECOSYSTEMS

Destruction of *Schistosoma mansoni* sporocysts in *Biomphalaria glabrata* after phytochemical exposure

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Abstract: Schistosomiasis is a neglected tropical disease and affects over 200 million people worldwide. The snail *Biomphalaria glabrata* is one of the intermediate hosts of *S. mansoni*. The aim of this work was to verify the action of *Euphorbia milii* var. *hislopii* latex in the hemocytes profile and histopathology of *B. glabrata* infected by *S. mansoni*. Uninfected and infected snails were exposed to sublethal concentration of *E. milii* latex for 24 hours (1.0 mg/L). The survival rate was 88.5% for the uninfected snails and 66.6% for the infected and exposed snails. In the snails infected by *S. mansoni*, the exposure to *E. milii* latex promoted proliferation of hemocytes in the tentacles, mantle, digestive gland and kidney. In the digestive gland and the kidney, granulomatous reactions occurred around the sporocysts and caused their destruction. The number of circulating hemocytes from the group infected and exposed to *E. milii* latex was significantly higher than in the other groups. Three types of hemocytes were found: hyalinocytes, granulocytes and blast-like cells. We conclude that the *E. milii* latex influenced the cellular immune response of the susceptible *B. glabrata* strain to infection by *S. mansoni*, promoting the destruction of parasites.

Key words: *Biomphalaria glabrata*, *Euphorbia milii*, hemocytes, phytochemical, susceptibility.

INTRODUCTION

Biomphalaria glabrata is the main intermediate host of *Schistosoma mansoni* (Colley et al. 2014, Lu et al. 2018), the causative agent of Schistosomiasis *mansoni*, affecting around 200 million people worldwide and causing up to 260,000 deaths per year (WHO 2017, 2019). In Brazil, intestinal schistosomiasis is largely associated with environmental variables and/or social determinants, infecting approximately 8 to 10 million people every year, while 25 million who live in endemic areas are at risk of infection (Scholte et al. 2012).

The interaction between *S. mansoni* and *B. glabrata* starts when the free-living forms (miracidia) infect the snails and become sporocysts, the longer-term asexual parasitic stage. The success or failure of infection is determined by the balance between the snail's internal defense system (IDS) and the infective mechanism of trematode (Mitta et al. 2017). To establish an infection, the miracidia must be recognized as self, so that the carbohydrates present on their tegument bind to the lectins on the surface of the hemocytes or vice versa, and/or free lectins in the hemolymph form bridges with the carbohydrates present on the surface

of the hemocytes and larvae. If the larvae are recognized as non-self, several mechanisms of the snail's humoral and cellular responses work together to neutralize the non-self particles, especially pathogenic microorganisms (Zänker 2010, Pinaud et al. 2019).

The hemocytes are snails' defense cells acting on the cellular response mechanism. They have variable size and enzymatic content composed by at least three cell populations, hyalinocytes (cells without granularity), granulocytes (cells with high granularity) and blast-like cells (smaller size, called young cells or precursor cells of other hemocytes) (Cavalcanti et al. 2012). The role of snail hemocytes in the recognition, killing and elimination of invading pathogens is described in several papers (Pereira et al. 2008, Prokhorova et al. 2018). The humoral responses include synthesis of antimicrobial peptides, cytotoxic molecules, reactive intermediates of oxygen and nitrogen and pathogen recognition receptors (PRR's) (Rowle & Powell 2007, Allienne et al. 2011). Hence, the complex nature of the snail hemocytes defense response to *S. mansoni* larvae, outlined above, makes analysis of snails exposed to molluscicides a vital component of research aimed at elucidating the array of underlying mechanisms of snail-schistosome compatibility to develop field control strategies.

One strategy to control *Biomphalaria* spp. in the field is the use of molluscicides, recommended by the World Health Organization (WHO). The use of natural products is encouraged by the same organization, and in this respect phytochemicals from *Euphorbia milii* var. *hislopii* have been one of the most studied. Many authors have described the effects of *E. milii* latex on the *S. mansoni*/*B. glabrata* system (Mott 1987, WHO 2014). Other studies have shown that the application of *E. milii* promotes changes in physiological stocks and *S. mansoni*/*B. glabrata* compatibility (Mello-Silva et al. 2010, 2011, Lima et al. 2012)

The effects of *E. milii* latex on the parasite life cycle in both schistosomiasis hosts (snails and mammals) have been described. These effects include reduction of the number of miracidia and cercariae in water bodies, helping control schistosomiasis transmission in endemic areas (Augusto et al. 2015). This strategy is inexpensive, eco-friendly, efficient and may help control in association with current anthelmintic therapy.

MATERIALS AND METHODS

Ethics

This study was approved by the animal experimentation ethics committee of Oswaldo Cruz Foundation (CEUA - Fiocruz L016/2015), in accordance with the guidelines of the Brazilian Society of Laboratory Animal Science (COBEA). This study is registered in the National System of Management of Genetic Heritage and Associated Traditional Knowledge – SisGen (no. A9666E5).

Euphorbia milii var. hislopii latex

To obtain the latex of *Euphorbia milii* var. *hislopii,* samples of the plant were collected in the Ilha do Governador district (22° 48´09´´S/ 43°12´35´´W) Rio de Janeiro, Brazil. The latex was collected and diluted as described by Vasconcellos & Amorim (2003). The calculation of the lethal concentration (LC) of the aqueous extract of the latex was carried out by probit analysis (Finney 1971) and the LC_{50} and LC_{90} values were 1.4 mg/L and 2.7 mg/L, respectively. The concentration used in this experiment was less that sublethal CL_{50} (1.0 mg/L).

Maintenance of *Schistosoma mansoni life* cycle

The *Biomphalaria glabrata* snails (Belo Horizonte, BH lineage) were obtained from the

Schistosomiasis Laboratory of Fiocruz (DCB/ENSP/Fiocruz). The specimens were hatched and reared in laboratory conditions according to the Technical Guidelines on Surveillance and Control of Snails of Epidemiological Importance from the Brazilian Ministry of Health (Brasil 2008). Four hundred specimens of *B. glabrata* were used, with shell diameters of 8 to 12 mm. The snails were fed daily ad libitum with fresh lettuce leaves (*Lactuca sativa* L.), except on the day of exposure to *E. milii* var. *hislopii* latex. The temperature remained between 25 and 28°C during the experiment.

The life cycle of *Schistosoma mansoni* (BH strain) was maintained in the Laboratory for Evaluation and Promotion of Environmental Health (LAPSA/IOC/Fiocruz), where all the experiments were performed. *S. mansoni* miracidia were obtained from the experimental infection in Swiss mice according to the technique described by Fernandez & Thiengo (2006). Each snail was infected with 8-10 miracidia of *S. mansoni*. After 35 days of infection, snails were previously exposed to light for 60 minutes and only the positive snails (cercariae shedding) were exposed to *E. milii* latex.

Experimental design

For experimental design, four snail groups with 100 specimens each were used: Group IE- "*S. mansoni*-infected and exposed to *E. milii* latex"; Group E- "uninfected and exposed to *E. milii*"; Group I- "*S. mansoni*-infected and unexposed"; and Group C- (control) "uninfected and unexposed".

The snails of the IE and E groups were exposed to less than sublethal concentration of *E. milii* latex (1.0 mg/L) for 24 hours. Three replicates of the experiment were performed. The lethal concentration experiment and exposure were performed according to Vasconcellos & Amorim (2003), as recommended by the World Health Organization (1983) and Mott (1987).

Ten samples of *B. glabrata* were exposed in beakers 500 mL of the solution, for 24 hours. The beakers were covered with a plastic screen to allow the air in and keep the snails from escaping; the space between the solution and the screen allowed the animals to leave the solution without leaving the container. During this period, the beakers containing the concentration were kept at 25-28°C temperature and the snails were not fed.

After 24 hours of exposure, the mortality of snails was confirmed by the absence of heartbeats through the observation of the pericardial cavity under a stereomicroscope. Thereafter, the survived snails were counted and separated for the following examinations.

Hemocytes count

After 24 hours of *E. milii* latex exposure, the hemolymph of ten snails from each group was collected by cardiac puncture using a 1 mL syringe equipped with a 27.5G × ½'' needle in 1 mL eppendorf tube and kept in an ice bath during collection.

For cell counting, 10 µl of the hemolymph was diluted (1:1) in saline buffer (18 g L^{-1} D-glucose, 12.2 g L^{-1} KCl, 0.6 gL^{-1} NaHCo3, 380 mOsm, pH 7.8) (Whitten et al. 2001). The number of total circulating hemocytes was determined by direct observation in a hemocytometer chamber by phase-contrast optical microscopy (Souza & Andrade 2012). The hemocytes were characterized according to Cavalcanti et al. (2012).

Histopathological examination

The shells of five snails from each group were removed, and the viscera incubated with 10% Millonig formalin for 24 hours. The material was dehydrated with increasing concentrations of

ethanol, cleared with xylol and embedded in liquid paraffin (60 ºC) according to Tolosa et al. (2003). Paraffin inclusion, longitudinal sectioning and hematoxylin-eosin staining were performed as described by Faro et al. (2013).

Statistical analyses

The effects of latex exposure and/or infection were analyzed by one-way ANOVA. Data are reported as mean ± standard error (SE). Differences among groups were considered statistically significant when $P \leq 0.05$. All analyses were carried out with the Prism 5.0 statistical software.

RESULTS

Results showed that there is no mortality in the unexposed groups (control = C and infected = I). While the survival rate of uninfected snails and exposed to latex (E) was 88.5 %, the infected snails with *S. mansoni* and exposed to latex (IE) recorded 66.6% after 24 hours of the exposure to 1.0 mg/L of *E. milii* latex.

The number of circulating hemocytes in the infected and exposed group to 1.0 mg/L of latex (IE) was significantly higher than that of the other groups (C, E and I). There was a significant reduction of circulating hemocytes in the infected and unexposed group (I) compared to the control group (C). In addition, the number of hemocytes in the *S. mansoni* infected group (I) was significantly lower compared to the IE group. No significant differences were observed in the number of circulating hemocytes between the uninfected (E) and control (C) groups (Figure 1).

The percentage of hyalinocytes, granulocytes and other types of hemocytes circulating in the hemolymph (blast-like cells) of *B. glabrata* was measured, after 24 hours of exposure to *E. milii* latex. High levels of hyalinocytes were observed in all tested groups (C = 59.8%, E = 47.5%, I = 45.7%, IE = 41.3%). The granulocytes were the second major hemocytes type observed in the hemolymph (E =28.4%, I =36.8%, IE= 31.7%). Except in the control group, the blast-like cells were the second major hemocytes with 27.5%, followed by 12.5% of granulocytes. There is no significant differences were found among the cell types and the different groups analyzed after 24 hours. We also observed a significant difference between the number of hyalinocytes and blast-like cells in both the control (C) ($P \leq 0.0354$) and infected (I) ($P \leq 0.0354$) groups (Table I).

The histological examination of *B. glabrata* observed the digestive gland of the control group showed tissue integrity; with the mucosa epithelium types (simple and cylindrical), the core in basal position and with hepatic ducts typical of normal tissue and there is no hemocytes proliferation and no tissue damage are present (Figure 2a). On contrary, different changes were observed in the tissues of uninfected and exposed snails (E); such as the presence of hemocytes in the kidney (Figure 2b), digestive gland (Figure 2c) and mantle (Figure 2d). In the mantle region, a large edema

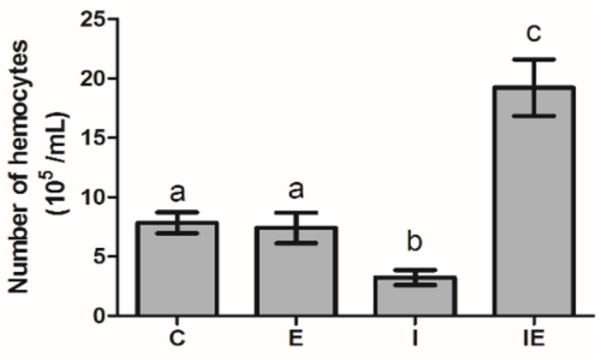

Figure 1. Effect of the E*uphorbia milii* latex exposure on the number of total circulating hemocytes in the uninfected and infected *Biomphalaria glabrata* by *Schistosoma mansoni*. C- Control group; E- Exposed group; I – Infected and unexposed and IE – Infected and exposed. Different letters, significant difference.

Table I. Effect of the E*uphorbia milii* latex exposure on the types of hemocytes in the uninfected and infected *Biomphalaria glabrata* by *Schistosoma mansoni*. Data are mean (percentage).

	C	E	I	IE
Blast-like cells	19 (27.5%)	29.3 (24.0%)	24.0 (17.3%)	34.8 (26.8%)
Granulocytes	8.6 (12.5%)	34.6 (28.4%)	50.8 (36.8%)	41.3 (31.7%)
Hyalinocytes	41.25 (59.8%)	58 (47.5%)	63.2 (45.7%)	53.7(41.3%)

occurred in the basement membrane (it is thin and translucent, which is not common) (Figure 2d), while the digestive epithelial cells became elongated and larger (Figure 2c).

In general, the histological examination of *B. glabrata* exposed to *E. milii* latex revealed tissue changes in both "infected and exposed" (IE) group (Figure 3) and "uninfected and exposed" (E) group (Figure 2). The kidney tissue and mantle region of the "infected and unexposed snails" (group I) showed intense parasitism, hemocytes proliferation without reaction, and no granulomatous formation observed in the tissues as shown in figure (3a).

In the IE group, the exposure to *E. milii* latex in the infected snails stimulated the proliferation of hemocytes in different tissues; tentacle and kidney (Figure 3b), digestive gland (Figure 3c), mantle (Figure 3d), while the number of parasites decreased with slight tissue reaction when compared to group (I).

In details, the kidney tissue and digestive gland of IE group showed parasites reduction with intense cell reaction around the sporocysts and presence of a granulomatous reaction (Figure 3b,c), while the kidney tissue of group (I) was intensely parasitized (Figure 3a), with the presence of both primary and secondary sporocysts. In mantle region of IE group, it was observed tissue destruction, increase of hemocytes number and tissue injury in the sporocysts (Figure 3d).

DISCUSSION

Reports on the changes of snails' cellular and humoral immune responses following exposure to pesticides are rare in literature. It is worth mentioning that this is the first report on the effect of phytochemical products from *E. milii* could change the profile of hemocytes and influence the host's immune response to the parasite.

The *E. milii* latex exposure in *B. glabrata* infected stimulated hemocytes proliferation (circulating and tissue-specific) in the tissue after 24 hours and because of this was observed the presence of a granulomatous structure that caused the death of parasites.

The presence of the product in the water may have affected sporocyst recognition as self and triggering snail response (Augusto et al. 2019). The immunological conditions observed in our study resembled those described in the literature for infection-resistant strains, especially the cell immune response with granuloma formation, which is characteristic of resistant strains (Fried 2016, Augusto et al. 2019). Therefore, our results agree with studies that have shown that several classes of stressors increase hemocyte proliferation in the tissue of *B. glabrata*, such as ferritin, synthetic latex and bacteria (Pinaud et al. 2016).

In the susceptible snails of the infected group (I), the hemocytes presented low number, motility, phagocytic capacity and activation of

new hemocytes, allowing the development of the parasite (Matricon-Gondran & Letorcart 1999, Negrão-Corrêa et al. 2008).

Compatibility between *S. mansoni* and *B. glabrata* is directly related to the incorporation of soluble antigens present in the hemolymph by the primary sporocysts, (Prokhorova et al. 2018, Augusto et al. 2019). Similar reaction was observed in vertebrate hosts, called antigenic mimicry (Augusto et al. 2019). The suppression of the cellular immune response is an adaptative process of the parasite (Prokhorova et al. 2018).

In the present study, hyalinocytes were the most commonly found, followed by granulocytes and blast cells, except in the control group C, where the percentage of hyalinocytes was followed by blast-like cells and granulocytes. These results were in agree with Cavalcanti et al. (2012) who studied the morphology *B. glabrata* hemocytes, and observed that hyalinocytes were the most frequent cell type, followed by granulocytes and blast cells. However, the authors did not report the relation between the type of hemocytes and susceptibility of the parasite. It seems that the hemocytes type does not influence the cellular immune response, but the number of hemocytes does.

In the relation the toxic effect of phytochemicals, several studies have reported on snail tissue damages caused by aqueous plant extracts (Adewunmi & Ogbe 1986, Bode et al. 1996, Pile et al. 1998, Araújo et al. 2002). Using aqueous extract of *E. milii* in *Lymnaea columella*, Pile et al. (1998) observed lesions characterized by degeneration, necrosis and accumulation of liquid in the digestive gland and kidney in specimens submitted to 0.5 mg/L of latex, similar to our findings.

In the present work, it was observed lesions in the digestive gland and mantle, and edema in the epithelium of the kidney. In addition, we also observed dark substances without structures in the digestive gland epithelium, similar to the substances described by Adewunmi & Ogbe (1986). We observed in uninfected and infected *B. glabrata* the same toxic effect in the tissue described by Pile et al. (1998) in *Lymnaea columella*.

In this study, using sublethal concentration of *E. milii* latex, the intermediate host did not die, but the products altered the tissue structure, probably related to toxic effect of this product, which directly interfered in hemocyte profile of the tissues.

The present results revealed that *E. milii* latex was able to trigger the immune response with the proliferation of defense cells. However, when the snail is infected, the triggering of the cellular immune response increases, resulting in the death of the parasite. This product was not capable of killing the parasite forms in this concentration, as already observed by Augusto et al. (2015). However, it influenced the development of the parasite, increases the number of defense cells and directly affectting development of the parasite, causing its death. This product seems to intervene in the like antigenic mimicry of sporocysts, facilitating the recognition of the parasite as non-self. In this way, in this experiment, it seems that *E. milii* latex (1.0 mg/L) acted as a "schistosomostatic" in a manner similar to the action of bacteriostatic products. This latex can be used as indirect therapy and it can be applied in the water streams to influence the cycle both larval stages miracidia and cercariae, which makes this promising natural product for use in endemic areas to control schistosomiasis transmission

The mechanisms of the *B. glabrata* immune response modulation by the exposure to *E. milii* latex are still poorly understood and deserve more investigation. Further studies of synergistic effects between *E. milii* latex exposure and *S. mansoni* infection on the *B. glabrata* immune system are being considered.

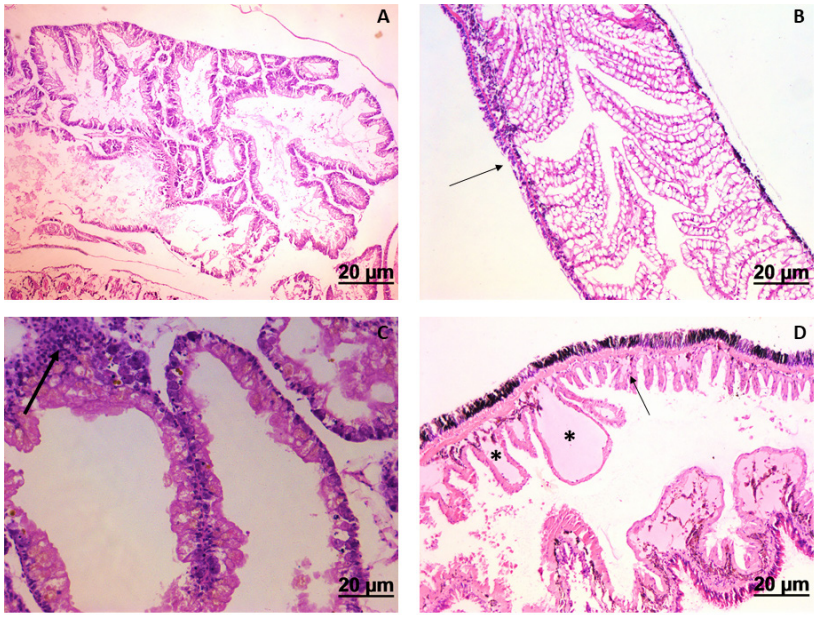

Figure 2. a: Group C "uninfected and unexposed *Biomphalaria glabrata*", digestive gland no changes (magnification 100 ×). b: Group E "uninfected and exposed to *E. milii*", kidney region with presence of hemocytes (arrow) (magnification 100 ×). c: Group E "uninfected and exposed to *E. milii*", digestive gland with more hemocytes present in the tissue (arrow) (magnification 200 ×). d: Group E "uninfected and exposed to *E. milii*", mantle region with edemas (*) and few hemocytes by tissue (arrow) (magnification 100 ×). All sections stained with hematoxylin-eosin.

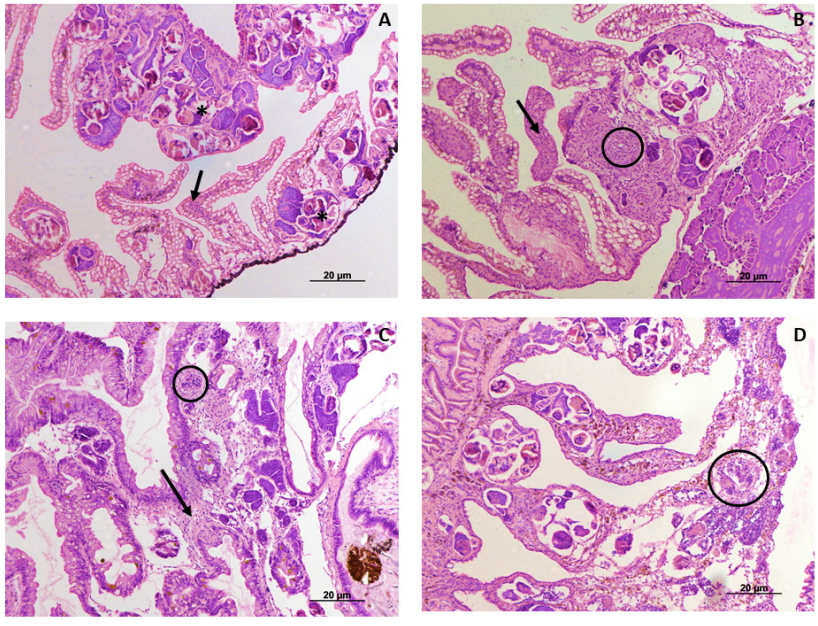

Figure 3. a: Group I "*S. mansoni*-infected and unexposed" kidney and mantle region with presence of sporocysts (*) and tissue hemocytes (arrow) (magnification 100 ×). b: Group IE "*S. mansoni*-infected and exposed to *E. milii* latex", kidney region presence of the dense infiltrate of hemocytes (arrow) and granulomatous type reaction (circle), both around sporocysts. c: Group IE "*S. mansoni*-infected and exposed to *E. milii* latex" digestive gland with the same results of the kidney. d: Group IE "*S. mansoni*-infected and exposed to *E. milii* latex" mantle region, with accumulation of hemocytes, destruction of the parasites (circle). All sections stained with hematoxylin-eosin 100X.

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GF, PSG and CCMS conceived and designed the study. GF, MF and VAC performed the experiments. EM, MJF, SS performed the histological analysis. CCMS revised the manuscript. All authors wrote the paper and approved the final version of the manuscript. This study is part of the Master dissertation of Gabriela Friani at the Postgraduate course in Ciências Veterinárias at Universidade Federal Rural do Rio de Janeiro.