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ORIGINAL ARTICLE /ARTÍCULO ORIGINAL

FIRST ELECTRON PROBE X-RAY MICROANALYSIS OF THE ELEMENTAL COMPOSITION OF THE LYOPHILIZED LATEX OF *EUPHORBIA MILII* VAR. *HISLOPII* AND ITS IMPACT IN THE MALE *SCHISTOSOMA MANSONI*

LA PRIMERA SONDA DE ELECTRONES DE MICROANÁLISIS DE RAYOS X DE LA COMPOSICIÓN ELEMENTAL DEL PRODUCTO LIOFILIZADO DE LATEX DE *EUPHORBIA MILII* VAR. *HISLOPII* Y SU IMPACTO EN EL MACHO DE *SCHISTOSOMA MANSONI*

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ABSTRACT

The aim of this work was to study the elemental composition of the lyophilized latex obtained from *Euphorbia milii var. hislopii* (N.E.Br.) Ursch & Leandri) and the tegument of adult male of *Schistosoma mansoni* Sambon, 1907 originated from cercariae previously exposed to the latex. The composition and concentrations of 15 elements were characterized using scanning electron microscopy (SEM) and energy dispersive spectroscopy (EDS). The Magnesium, Potassium and Rhodium were found in high *weight percent* in the lyophilized latex. High levels of Sodium, Nitrogen and Magnesium elements were detected respectively in control adult male *S. mansoni*. The cercariae exposition of *S. mansoni* to the *E. milii* latex changed the elemental composition of adult parasite, which showed higher level of Nitrogen instead of Sodium as observed in normal parasites. Furthermore the comparison of elements of the control group and the adult worms from cercariae exposed to the latex showed a significant decrease in Nitrogen and increase in Sodium, Potassium and Magnesium. The present work represents the first analysis of the elemental composition of the lyophilized latex of *E. milii var. hislopii* and its impact in the male *S. mansoni* by Electron Probe X-Ray Microanalysis.

Keywords: Cercariae - Chemical elements - *Euphorbia milii* - Schistosomiasis - Tegument - X-ray microanalysis

RESUMEN

El objetivo de este trabajo fue estudiar la composición elemental del látex liofilizado obtenido de *Euphorbia milii* var. *hislopii* (N.E.Br.) Ursch & Leandri) y el tegumento del macho adulto de *Schistosoma mansoni* Sambon, 1907 originado a partir cercarias previamente expuesta al látex. La composición y las concentraciones de 15 elementos se caracterizaron mediante microscopía electrónica de barrido (SEM) y la espectroscopía de dispersión de energía (EDS). El magnesio, potasio y sodio se encontraron en un alto porcentaje en peso en el látex liofilizado. Altos niveles de los elementos sodio, magnesio y nitrógeno se detectaron, respectivamente, en el control de los machos adultos de *S. mansoni*. La exposición de cercarias de *S. mansoni* al látex de *E. milii* cambió la composición elemental del parásito adulto, que mostró un mayor nivel de nitrógeno en lugar de sodio como se observó en los parásitos normales. Además, se compararon los elementos del grupo de control y de los gusanos adultos de las cercarias expuestas al látex y mostraron una disminución significativa de nitrógeno y un aumento de sodio, potasio y magnesio. El presente trabajo representa el primer análisis de la composición elemental del látex liofilizado de *E. milii* var. *hislopii* y su impacto en el macho de *S. mansoni* por la Sonda de microanálisis de Electrones de Rayos X.

Palabras-clave: cercarias - Elementos químicos - *Euphorbia milii* - Esquistosomiasis - microanálisis de rayos X - Tegumento

INTRODUCTION

On Schistosomiasis control studies, despite the efficacy of chemotherapy in reducing morbidity, a decrease in the transmission of the parasite was not achieved (Bockarie *et al.*, 2013). Among these, many studies using bioactive plant compounds have been undertaken (Yadav & Jagannadham, 2008; Singh *et al.*, 2010; Hamed, 2010; Mello-Silva *et al.*, 2011; Lima *et al*., 2012). In these, *Euphorbia milii* var. *hislopii* has been indicated as the most promising for use in official WHO programs for schistosomiasis control (WHO 2002). The biocidal activity of the latex has been tested for the control of snail's intermediate hosts of different parasites (Vasconcellos & Schall, 1986; Giovanelli *et al.*, 2001; Vasconcellos & Amorim, 2003; Mello-Silva *et al.*, 2010; Crignis *et al.*, 2012).

One of the main practices to evaluate the effect of control actions in *S. mansoni* is to evaluate the tegument of the parasite, because this structure is important to the survival, performing vital functions such as the absorption of organic and inorganic compounds and the evasion of the host immune system (Braschi *et al.*, 2006). In addition, the male worm tegument is especially important because it is also responsible for the development and maturation of female worms (Ribeiro-Paes & Rodrigues, 1997). The tegumental surface of *S. mansoni* exposed to anthelmintic drugs, exhibits deformations such as scaling, erosion, spikes and bubbles (Xiao *et al.*, 2000; Lorsuwannarat *et al.*, 2013).

Despite the extensive accumulated knowledge of tegumental functions and biochemistry, little is known of the inorganic composition of this structure under the pressure of anthelmintic drugs. In recent years, the elemental microanalysis method, based on scanning or transmission electron microscopy/energy dispersive X-ray spectrometry (SEM/EDS or TEM/EDS), has been applied to the detection and semi-quantification of chemical elements across a broad spectrum of physical and biological samples, including parasites (Goldstein *et al.*,

2003; Buron *et al*., 2009; Morais & Malta, 2016).

The aim of this work was study the elemental composition of the lyophilized latex obtained from *Euphorbia milii* var. *hislopii* and the tegument of adult male of *S. mansoni* originated from cercariae previously exposed to the latex.

MATERIALS AND METHODS

Euphorbia milii var. *hislopii* samples

The *E. milii* var. *hislopii* latex was collected in the Ilha do Governador district (22°48´09´´S/43°12´35´´W), Rio de Janeiro, Brazil (Vasconcellos & Amorin, 2003). The latex sample collected was pre-frozen in dry ice and absolute ethanol and subsequently lyophilized at -52°C on 8 $x10^{-1}$ mBar during three cycles of 12 h in a Modulo 4K Freeze Dryer with an acrilic chamber (Edwards High Vacuum Int., UK). The lyophilized pellet obtained was diluted in distilled water and homogenized by sonication for 20 min.

Schistosoma mansoni adult's samples

The *B. glabrata* (Belo Horizonte-lineage BH) used in the experiments had shell diameters between 8-10 mm and they were individually infected with 7±1 miracidia of *S. mansoni* (lineage BH) at the Laboratório de Esquistossomose Experimental (LEE), Instituto Oswaldo Cruz. After thirty days, the snails were individually exposed to light (60-watt incandescent bulb for 1 h) in becker (5ml) with distilled water to look for the release of cercariae. After this period, the snails were removed and the cercariae were separated into two groups.

The first cercarial group was kept in distilled water for one hour. The second group was exposed for the same period (one hour) to a sublethal concentration of the powdered lyophilized latex of *E. milii* (LC_{50}=1.4 mg/L) as described by Schall *et al*. (1992) for *Biomphalaria glabrata* snails.

After this, fifteen female Swiss-Webster mice were infected with exposed cercariae and another fifteen with unexposed cercariae (control group). At 65 days post-infection, part of *S. mansoni* males, both the exposed and control groups, were collected and fixed in 70% ethanol.

The worms were dehydrated in an ethanol series (80%, 90% and three changes of 100%) prior to being critical point dried using CO_2. Powdered lyophilized latex, four specimens of the control group and four of the exposed group of S. mansoni were mounted in stubs, sputter-coated with carbon and observed in a Scanning Electron Microscope Leo 1430VP coupled to a probe X-ray (EDS) at the Núcleo de Microscopia e Microanálise of Centro de Ciências Biológicas e da Saúde, Universidade Federal de Viçosa, Minas Gerais. We selected for SEM-EDS analysis the following elements: N (Nitrogen), Na (Sodium), Mg (Magnesium), Al (Aluminum), K (Potassium), Cr (Chromium), Fe (Iron), Cu (Copper), Rh (Rhodium), Pd (Palladium), Ag (Silver), Cd (Cadmium), Pt (Platinum), Hg (Mercury) and Pb (Lead). The qualitative and semi-quantitative composition of these elements were determined using the X-ray spectrum performed on a manually selected area and took into consideration 100% of the total amount of weight percent of elements measured (wt.%).

The results were expressed for each sample as the mean ± standard deviation and submitted to analysis of variance (ANOVA) and Student's t tests (α = 5%, $p \leq 0,05$) (GraphPad, V.4.00, Prism, GraphPad, vol. 3.02 Prism Inc.).

Ethics

This research was approved by the Animal Ethics Committee of the Oswaldo Cruz

Foundation (CEUA-FIOCRUZ LW-07/13), in accordance with the guidelines of the Brazilian College for Animal Experiments (COBEA).

RESULTS

The ultrastructure of the lyophilized latex varied in the size and shape of the particles. Even after lyophilization, the latex formed pellets, clustered and arranged its particles of different sizes and shapes in the form of a resin (Fig. 1A).

The energy dispersive spectroscopy (SEM-EDS) of the lyophilized latex sample showed the *weight percent* (wt.%) *of* K (45.14), Rh (31.39), Mg (11.45), Hg (2.58), Pt (2.24), Al (2.14), Na (2.03), Pb (1.85) Cu (0.47), Cd (0.42), Cr (0.25) e Fe (0.13). The elements N, Pd and Ag were absent from the sample of lyophilized latex.

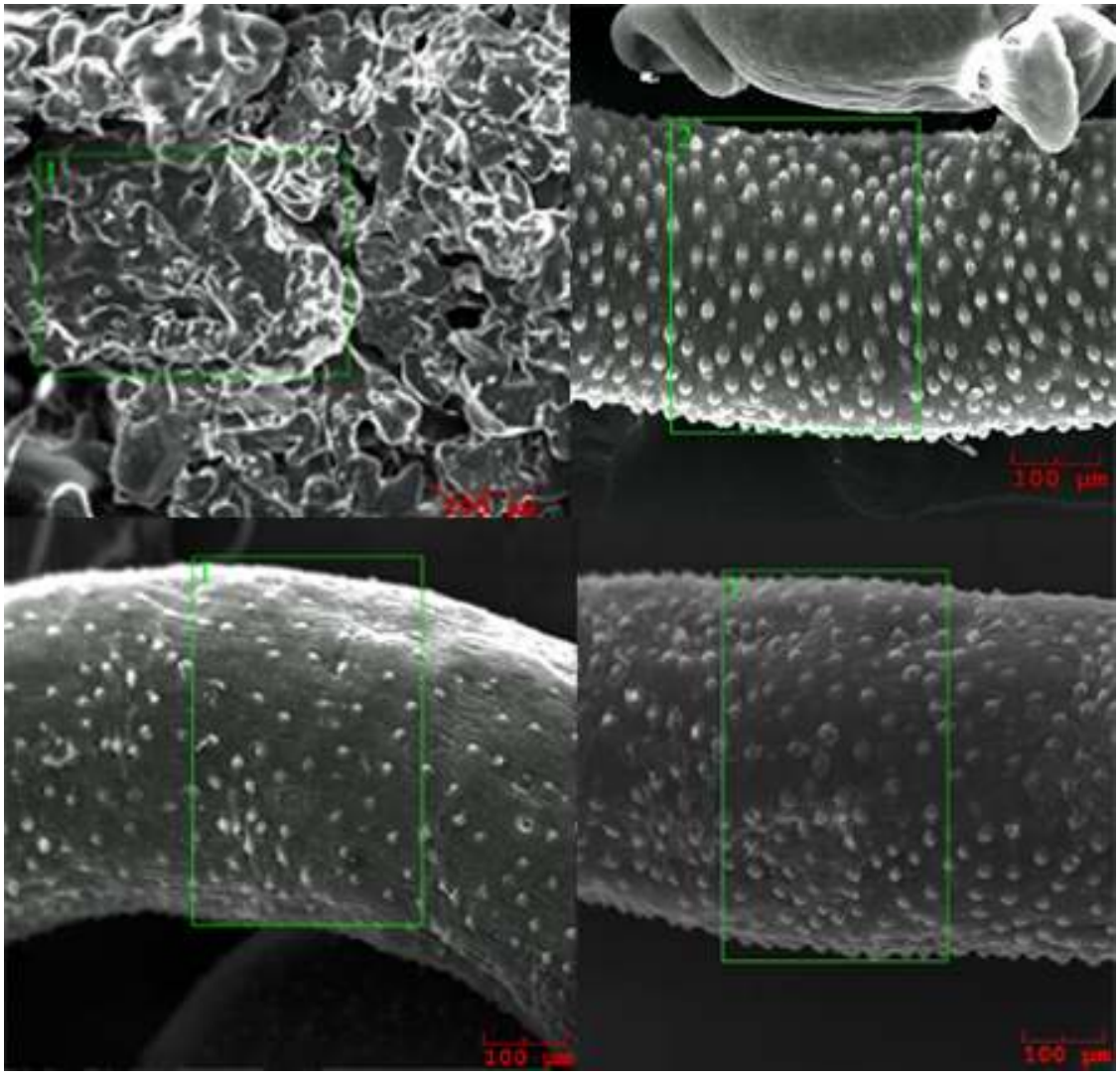

Figure. 1. Structural feature of powered lyophilized latex of *Euphorbia milii* var. *hislopii* and morphology of adult *Schistosoma mansoni* tegument came from cercariae previously exposed to this latex, by *S*canning electron microscopy: (A) Po*wdered lyophilized latex. (B) Control tegument of adult male of S. mansoni. (C-D) Tegument of adult male of S. mansoni* exposed to the latex at the cercarial stage showing intense damage to the external surface.

Table 1. Chemicals concentration on tegument of four adult males of *Schistosoma mansoni*. Control group (C) and group exposed (E) for one hour to 1.4 mg/L *Euphorbia milii var. hislopii*. Data are expressed as mean ± standard deviation (α = 5%. p≤0.05). N (Nitrogen). Na (Sodium). Mg (Magnesium). Al (Aluminum). K (Potassium). Cr (Chromium). Fe (Iron). Cu (Copper). Rh (Rhodium). Pd (Palladium). Ag (Silver). Cd (Cadmium). Pt (Platinum). Hg (Mercury) and Pb (Lead).

Elements	N		Na		Mg		Al		K		Cr		Fe		Cu	
Parasites	C	E	C	E	C	E	C	E	C	E	C	E	C	E	C	E
1	6.9	35.0	23.2	17.0	14.6	10.8	13.5	9.2	1.6	0.8	0.2	0.2	0.9	0.8	13.0	12.2
2	15.2	39.5	20.8	14.9	14.3	11.6	14.4	10.4	1.2	0.5	0.2	0.1	0.6	0.5	12.6	8.8
3	23.4	31.5	20.4	13.3	13.33	8.2	10.0	5.6	0.9	0.9	0.1	0.3	0.8	1.1	17.8	21.4
4	30.1	41.8	17.4	12.7	11.1	9.9	10.3	9.0	0.9	0.6	0.1	0.1	0.7	0.9	11.8	10.7
Mean	18.9±10.0	37.0±4.6	20.4±2.3	14.5±1.9	13.3±1.6	10.1±1.4	12.1±2.2	8.5±2.0	1.1±0.3	0.7±0.1	0.2±0.0	0.2±0.0	0.7±0.1	0.8±0.2	13.8±2.6	13.3±5.5
P –value	0.017		0.008		0.025		0.060		0.037		0.479		0.624		0.878	

Elements	Rh		Pd		Ag		Cd		Pt		Hg		Pb	
Parasites	C	E	C	E	C	E	C	E	C	E	C	E	C	E
1	11.3	3.6	0.6	0.0	0.8	0.000	0.614	0.000	3.423	3.674	3.472	2.680	5.322	3.59
2	9.78	6.3	0.7	0.0	1.1	0.213	0.000	0.000	3.077	1.876	2.845	2.634	2.806	2.09
3	1.3	1.6	0.0	0.0	0.0	0.000	0.000	0.242	3.768	4.794	4.146	5.428	3.606	5.15
4	7.4	5.2	0.0	0.0	0.3	0.108	0.000	0.000	3.137	2.581	3.553	2.783	2.664	3.07
Mean	7.4±4.4	4.2±2.0	0.3±0.4	0.0±0.0	0.5±0.5	0.08±0.1	0.15±0.3	0.06±0.12	3.35±0.3	3.2±1.2	3.5±0.5	3.3±1.3	3.6±1.2	3.4±1.2
P -value	0.227		-		0.091		0.593		0.861		0.872		0.898	

The SEM-EDS analyses of adult males of *S. mansoni* in control and exposed groups were conducted in the mid-dorsal section of the tegument (Figs 1B-1D). Each of the 15 elements analyzed in each worm and their weight as a percentage (wt.%) are presented in Table 1. As determined by EDS, all elements were present in both groups, but Pd was absent in the exposed group. The levels of *alkali metals Na* ($p<0,008$), K ($p<0,037$) and of alkali earth metal Mg ($p<0,025$) w*ere significantly higher in the tegument of the control group* and the non-metal N *was significantly higher in the exposed* group. The percentages of Cr, Fe, Cu, Pt, H and Pb did not vary significantly between the control and the exposed groups.

The ultrastructure of the normal tegument in the mid-dorsal part of males of *S. mansoni* showed a pattern of a well organized distribution of tubercles and spines (Fig. 1B). The tegument of adult parasites originating from cercariae exposed to the latex of *E. milii* var. *hislopii* exhibited pronounced changes in the mid-dorsal surface. The most significant morphological changes observed were (i) a reduction in the number and size of the tubercles and spines, (ii) a disorganized distribution of the tubercles and (iii) damage to the tegument in the form of hollows and wrinkles (Figs 1C-1D).

DISCUSSION

The bioaccumulation of metals by helminths is a well acknowledged phenomenon; this is a process which, as pointed out in the overview by Buron *et al.* (2009), can be influenced by factors such as the nature of the metal itself, host age and motility, and the age, stage of development, sex and site of the parasite. The case, where cercariae of *S. mansoni*, which had been exposed to the latex of *E. milii* var. *hislopii*, produced effects in adult worms, showed that bioaccumulation can pass through different life stages from the environment to the definitive host.

The *E. milii* var. *hislopii* is a widely distributed plant that occurs in almost all habitats and occupies a wide range of climes and soils (Zahra *et al.* 2014). The products of their secondary metabolites have been described as rich in diterpenes, triterpenes, flavonoids, saponins, tannins, alkaloids, esters, ricin and phenols (Mwine & Damme, 2011). These compounds have proved to be useful as anti-plasmodial, anti-malarial and molluscicidal products (Mwine & Damme, 2011). Previous tests with cercariae of *S. mansoni* exposed to this latex showed a slightly toxic effect, but without death of cercariae (De-Carvalho *et al.*, 1998). At present, this is the first description of the chemical elements of the lyophilized latex of *E. milii* var. *hislopii*.

Toxic metals were found in the latex, as Al (2.14%), Pt (2.24%), Hg (2.58%) and Rh (31.39 %). The occurrence of Rh is rare, being reported, in the environment as a discharge of anti-cancer medications or emitted by catalytic converters for automobile (Sures *et al.*, 2003; Singer *et al.*, 2005). In our study, the Rh even in high concentrations in the latex did not accumulate significantly in the exposed group (4.2%) when compared to the control group (7.4%). In the latex the weight percent of Al, Cu (0.47) and Pb (1.85) were low in relation to the control (12.1, 13.8 and 3.6 respectively) and exposed groups (8.5, 13.3 and 3.4). Morais & Malta (2016) also observed Cu, Pb and Al, among other elements, in different digenean metacercariae from fish caught in Manaus polluted streams (Brazil). Although they correlated the presence of these metals with toxic effluents originating from Manaus Industrial District factory sewers, it is interesting to note that Cu, Pb and Al were present in both our control and exposed groups, probably indicating that these elements may occur in different digenean species.

Other elements, such as Cd, Ag, and Pd were found in low concentrations in the tegument of the worms; the latter was only detected in the control group. Even though the biological functions of these elements are understudied, it is known that they are toxic in high concentrations, but essential for life in small quantities (Morley *et al.*, 2002). According to Sigel *et al.* (2009), there is a metallothionein-protein (MT) responsible for cell protection against reactive oxidants, including nitric oxide which has the capacity to bind to heavy metals such as Cu, Cd and Ag. González-Mejia *et al.* (2014) commented that the nitric oxide radical has a biological activity limiting the local inflammation caused by parasites. The reduction of Cd, Al, Rh and Ag in our exposed group in relation to MT protection against schistosomiasis is still to be evaluated.

Shaw & Erasmus (1984) evaluated the tegument of male of *S. mansoni* by electron probe X-Ray microanalysis using eight inorganic compounds. These authors observed high concentrations of K and low concentrations of Na, Fe and Mg, respectively. In our experiment, on the contrary, control group showed highest values of Na and Mg and small values of K and Fe. Shaw & Erasmus (1984) suggested that wt% values can change according to factors such as the strain of the parasite and host, fixation method and sample preparation and the metabolic/physiology *status* of the parasites.

The comparison of elemental composition of surface tegument between the control group of male of *S. mansoni* with the parasites formed from cercariae previously exposed to the latex, showed significant differences with higher levels of Na, Mg and K in the control group. In the exposed group, only N was significantly higher than in control group.

The tegument of *S. mansoni* seems to be primarily dependent on K^+ and Na^+ gradients that are responsible for a partial depolarization of the surface membranes that leads to muscular contractions of the parasite (Smyth & Halton 1983). Comparing the elemental composition present in the latex with both male *S. mansoni* groups, we observed a large amount of K in the lyophilized latex (45.14), a low percentage in control group (1.1) and the exposed group had an even lower one (0.7). With regard to Na present in the latex (2.03) there was a significant difference ($p=0.008$) between the control (20.4) and the exposed groups (14.5). We observed that the exposure of cercariae to the latex did not change the muscle contraction of live adult parasites, in accordance with the proportion of ions of K^+ and Na^+ that remained regular. Therefore, the latex had no influence in the K^+ and Na^+ bomb.

Galaktionov & Dobrovolskij (2003) described that the contents in the excretory vesicle of trematodes also include Calcium (Ca), Mg and Phosphorous (P). The Mg was found in the control (13.3) and exposed groups (10.1) with a significant decrease in the latter. The alteration of Mg levels may be related to osmoregulation and also play an important role in muscle contraction together with Na and K (Galaktionov & Dobrovolskij 2003).

The tegument of *S. mansoni* constantly renews the outer layer of its syncytium, being adapted for ion transport, osmoregulation, absorption and the secretion of substances (Faghiri *et al.*, 2010). Besides, the parasite presents the mimetism capacity to scape to the host's immune system. The changes in wt% of the elemental composition of *S. mansoni* tegument may be involved in the morphological changes observed in tegument of the exposed group such as: reduction of tubercles and spines. Similar results were reported in the male tegument of *S. mansoni* exposed to praziquantel, artemether and the latex of allicin (Liang *et al.*, 2001; Xiao *et al.*, 2002; Lima *et al.*, 2012). These morphological changes may weaken the tegument, interfering with exchanges through the syncytium and thus

affecting the physiology of *S. mansoni*.

We conclude that when the cercariae of *S. mansoni* are exposed to *E. milii* latex, the subsequent adult male presents alterations in morphological and elemental composition of the tegument. It is possible that these changes may indirectly affect the ontogeny of the parasite.

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