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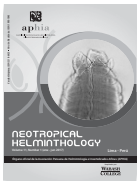

ORIGINAL ARTICLE / ARTÍCULO ORIGINAL

DEVELOPMENT, DIFFERENTIAL PROTEIN EXPRESSIONS AND GENE NETWORK ON *SCHISTOSOMA MANSONI* SUBMITTED TO *EUPHORBIA MILII* LATEX ON CERCARIAL STAGE

DESARROLLO, EXPRESIÓN DIFERENCIAL DE PROTEÍNAS Y REDES GÉNICAS EN *SCHISTOSOMA MANSONI* EXPUESTOS A *EUPHORBIA MILII* LATEX EN ESTADO DE CERCARIA

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ABSTRACT

We tested a new approach to describe how a sublethal dose of the *Euphorbia milii* Des Moul. latex can affect cercariae, inducing changes in the adult *Schistosoma mansoni* Sambon 1907. We analyzed the effects on biology and used proteomic tools to identify the expression of different proteins. The treatment reduced the development of adults (males, 74.1%; females, 76.3%) and decreased the number of eggs released in 89.9%. The protein profile between adult males of *S. mansoni* from the control and exposed groups detected 1,020 spots, 26 were differently expressed and 10 were identified and validated. The proteins downregulated after exposition included actin, 14-3-3 epsilon and aldehyde dehydrogenase. The hsp70 was the only protein that was upregulated. The changes in the proteomic profile of adult males of *S. mansoni* may be related to a muscular fragility of the body. This work provides new insight for schistosomiasis control using a natural, water-soluble product at sublethal concentrations for mollusks. This approach is inexpensive, ecological and efficient as an indirect therapy for schistosomiasis.

Keywords: Schistosomiasis – molluscicide – *Euphorbia milii* – parasitic disease control – proteomic – gene network

RESUMEN

Hemos probado un nuevo enfoque para describir cómo una dosis subletal del látex de *Euphorbia milii* Des Moul. puede afectar a las cercarias, induciendo cambios en el *Schistosoma mansoni* Sambon 1907 adulto. Se analizaron los efectos sobre la biología y se utilizaron herramientas proteómicas para identificar la expresión de diferentes proteínas. El tratamiento redujo el desarrollo de adultos (machos 74,1%, hembras 76,3%) y disminuyó el número de huevos liberados en 89,9%. El perfil proteico entre machos adultos de *S. mansoni* de los grupos control y expuestos detectó 1.020 *spots*, 26 se expresaron de forma diferente y 10 fueron identificados y validados. Las proteínas menos abundante después de la exposición incluyeron actina, 14-3-3 epsilon y aldehído deshidrogenasa. La hsp70 fue la única proteína que se más abundante. Los cambios en el perfil proteómico de los machos adultos de *S. mansoni* pueden estar relacionados con una fragilidad muscular del cuerpo. Este trabajo proporciona una nueva visión para el control de la esquistosomiasis utilizando un producto natural, soluble en agua a concentraciones subletales para moluscos. Este enfoque es barato, ecológico y eficiente como terapia indirecta para la esquistosomiasis.

Palabras-clave: Esquistosomiasis – molusquicida – *Euphorbia milii* – control de enfermedades parasitarias – proteoma – redes génicas

INTRODUCTION

Digenetic trematodes have complex life cycles characterized by a series of striking morphological and biochemical transitions between the aquatic environment and molluscan and mammalian hosts (Evans & miller, 1987; Jolly *et al*., 2007). In the aquatic environment, *Schistosoma mansoni* Sambon, 1907 takes the form of two free-swimming larvae – miracidia and cercariae – in a relatively short-lived 'transitional' stage into freshwater. They must swim to find their hosts before energy sources are exhausted. However, many soluble compounds and different forms of pollution can disrupt their interaction with the next host. The acquisition of soluble macromolecules through the tegument or by ingestion has been reported to induce changes in the genome expression of cercariae of *S. mansoni,* which affect its growth and development in its definitive host (Lenzi *et al.*, 2008; Thornhill *et al*., 2009). Therefore, the use of the latex of *Euphorbia milii* (N.E.Br.) (Ursch & Leandri, 1955) in the control of schistosomiasis could also elicit changes in the gene expression of *S. mansoni.*

The success of the host-parasite relationship depends, among other factors, on the expression, interaction and modulation of proteins for the co-existence of both organisms (Johnston *et al*., 2009). Upregulated genes identified in the cercariae transcriptome are necessary to sustain its swimming behavior and infection of the definitive host (Jolly *et al*., 2007). These genes encode proteins related to the energy metabolism, such as NADH dehydrogenase, subunits of cytochrome C and its homologues, ATP/ADP carriers and motility genes such as actin and fibrillin. In adult parasites, the transcriptomes show the intense expression of genes linked to the escape of the host immune system and motility, to acquire and metabolize a variety of nutrients, including nutrient transporters, such as the glucose transporter (Jolly *et al*., 2007). Roquis *et al.* (2015) showed the influence of environmental factors on changes in gene expression on schistosomula and its effects on adult worms. The effect of these factors can be mitotically heritable and have consequences in the ecology of adult parasites, making them a therapeutic target.

Among the research topics recommended by the WHO (2002) is the development of effective and practical measures for micro-focal transmission, including the use of molluscicides. The latex of *Euphorbia milii* var. *hislopii* Des Moul., tested under laboratory and field conditions, meets these recommendations due to its potential as a natural molluscicide (Lima *et al*., 2012; Augusto *et al*., 2015). De-Carvalho *et al*. (1988) tested high concentrations of *E. milii* latex (10-100 $mg \cdot L^{-1}$ for 2

h) against *S. mansoni* cercariae, and no changes were observed in cercarial survival or the percentage of penetration into the skin of mice.

Therefore, the aim of the current study was to describe how a sublethal dose of the *E. milii* latex can affect cercariae, inducing changes in the adult *S. mansoni,* using proteomic tools to identify the different proteins expressed. Additionally, the development of worms was evaluated using the parasite rate and the number of eggs released.

MATERIALS AND METHODS

Latex - The *E. milii* var. *hislopii* latex was collected at Ilha do Governador district (22°48′09′′S/43°12′35′′W), Rio de Janeiro, Brazil. The collected latex sample was pre-frozen in dry ice and absolute ethanol and subsequently lyophilized at -52 °C on 8 x10^{-1} mBar for three 12-h cycles in a Modulyo 4K Freeze Dryer with an acrylic chamber (Edwards High Vacuum Int., UK). The lyophilized pellet obtained with this process was diluted in distilled water and homogenized by sonication for 20 min. The sublethal doses of the powdered lyophilized latex of *E. milii* used to exposed cercariae were equivalent to the mean, LC_{50}=1.4 mg·L^{-1}, described by Schall *et al.* (1992) for *Biomphalaria glabrata* (Say, 18118).

Animals - The cercariae of *S. mansoni* (LE strain) were collected and separated into two groups. The first group was exposed to a solution of *E. milii* lyophilized latex in distilled water (1.4 mg·L^{-1}) for one hour. The second (control) cercarial group was kept in distilled water for the same time period. Then, 30 female Swiss-Webster mice were infected with 150 exposed cercariae per mouse, and another 30 mice were infected with 150 normal cercariae per mouse, all using percutaneous inoculation. Water and food were given *ad libitum*. At 65 days post-infection, the adult parasites were collected for proteomics analysis.

Parasitological analysis - Three analyses of the number of eggs per gram of stool (EPG) by Kato-Katz were performed in each experimental group. Feces were collected in a one-hour period between 10:00 and 12:00 a.m. For egg counts, three slides were assessed per group, which was repeated once a week for three weeks.

Preparation of a total extract of S. mansoni and two-dimensional electrophoresis - The protein extracts were obtained by the sonication of approximately 15 adult male and 20 adult female parasites per group in extraction solution (7 M urea, 2 M thiourea, 4% CHAPS). Three replicates for each sex and each group were performed.

The total protein concentration was determined using the Bradford method (Bradford, 1976), and the samples were stored at -80 °C until use. The protein concentration from female adult worms was not sufficient to allow for two-dimensional electrophoresis analysis. From male adult worms, the protein concentration allowed separate aliquots containing 150 µg per sample that were diluted to a final volume of 125 µL in Destreak solution (GE Healthcare, Sweden) and 2% IPG buffer (pH 3–10) (GE Healthcare). Seven-centimeter strips (Immobiline, GE Healthcare, Sweden) with an immobilized pH gradient in the range of 3–10 were rehydrated with the protein extract for 20 h using IPGBox (GE Healthcare, Sweden).

Isoelectric focusing was initiated immediately after rehydration. Isoelectric focusing was performed with an automated system (Ettan IPGphor III, GE Healthcare, Sweden) at 20 °C with a constant current of 50 µA/strip and a total of 11.7 kVh following a four-step program: 300 V for 15 hours; linear gradient to 1000 V for 300 vh; linear gradient to 5000 V for 4000 vh and 5000 V for 2000 vh. After isoelectric focusing, the strips were reduced in equilibration buffer (6 M urea, 0.075 M Tris HCl (pH 8.8), 29.3% glycerol, 2% SDS, and 0.002% bromophenol blue) containing 2% dithiothreitol (DTT) for 60 min and then alkylated for 60 min in equilibration buffer containing 5% iodoacetamide.

For the second dimension, the strips were placed on a 12.5% polyacrylamide gel in a Mini Protean Cell system (Bio-Rad, USA). Electrophoresis was performed at a constant 80 V for 2:40 h. The gels were stained with Coomassie Blue G-250 solution for 72 hours under stirring and scanned by ImageScanner III (GE Healthcare, Sweden) using Labscan software (GE Healthcare, Sweden). The spots were quantitatively analyzed using the

ImageMaster 2D Platinum 7.5 software (GE Healthcare, Sweden) and the abundance of each protein spot was calculated by the percentage volume (vol%) with the following parameters: smoothness greater than 2, saliency greater than 50, and area greater than 50. Only those spots that were significantly different at a significance level of $p< 0.05$ based on one-way ANOVA analysis (assuming equal variance) and a ratio above 1.5 were selected for the next step.

In-gel tryptic digestion and mass spectrometry - The significantly different spots were manually excised, treated with washing solution (50% acetonitrile and 0.0025 M ammonium bicarbonate), and dehydrated in 100% acetonitrile in a vacuum centrifuge at room temperature. The proteins were subsequently subjected to reduction (65 mM DTT) and alkylation (100 mM iodoacetamide).

The samples were digested at 37 °C overnight with proteomic-grade trypsin from the porcine pancreas (SIGMA, USA) in 40 mM ammonium bicarbonate and 10% acetonitrile (final concentration: 25 ng/μL). Tryptic peptides were extracted from the gel solution with 50% acetonitrile in 5% formic acid. The extracted peptides were transferred to a sterile tube, dried in a vacuum centrifuge, and resuspended in a solution of 50% acetonitrile and 0.1% trifluoroacetic acid.

One-microliter aliquots of each sample were applied to a steel plate at a 1:1 ratio with a α-cyano-4-hydroxycinnamic acid matrix (Bruker Daltonics, Germany). After crystallization, the plate was inserted into the mass spectrometer for analysis. The first search for proteins was performed by PMF (peptide mass fingerprint) homology in comparison with the NCBI [National Center for Biotechnology Information (NCBInr)] public database with the aid of the online version of Mascot (Matrix Science, London, UK). The taxonomic parameter for the search was restricted to Schistosomatidae considering up to two lost cleavage sites, an error of 0.1 Da in peptide identification, cysteine carbamidomethylation as a fixed modification and methionine oxidation as a variable modification. A second search for proteins was performed with all MS spectra obtained in the m/z range of 800–4000 kDa using a MALDI-TOF-TOF spectrometer (Bruker Daltonics, Germany). The spectra were analyzed using FlexAnalysis 3.3 software (Bruker Daltonics, Germany) for the determination of peaks.

The list of peptide and fragment mass values generated by the mass spectrometer for each spot were submitted to a MS/ MS ion search using Mascot Daemon version 2.4.0 software (Matrix Science, London, UK) to search in the Schistosomatidae Protein DataBase from the UniProt DataBase (download in 03/27/2015 with 40,657 proteins). The search parameters were set as follows: up to one missed cleavage site; 0.15 Da of error for the identification of peptides; carbamidomethylation of cysteine as a fixed modification and the oxidation of methionine as a variable modification.

Scaffold software 4.4.1.1 (Proteome Software, USA) was used to visualize and statistically validate the MS/MS results based in the peptides and proteins identified by Mascot Daemon. The peptide identifications were accepted if they exhibited probability values greater than 90% by the *Peptide Prophet* algorithm (Keller *et al*., 2002), and the protein identifications were accepted if they exhibited probability values greater than 90% by the *Protein Prophet* algorithm (Nesvizhskii *et al*., 2003). Peaks software 7.0 (Bioinformatics Solutions Inc., Canada) was used for the *de novo* sequencing of the peptides (Ma *et al*., 2003), and the identified proteins were statistically and manually validated. To determine the biological processes in which the identified molecules were associated, the Gene Ontology (GO) databases through the UniProt, the KEGG Pathway and Schistodb were used.

Gene network analysis - The STRING 10.0 database (Franceschini *et al*., 2013) was also used to analyze the protein-protein interaction in each group, as it is one of the largest databases of known and predicted protein-protein interactions according to Emily *et al.* (2009). The interactions included direct (physical) and indirect (functional) associations derived from four sources: genomic context, high throughput, co-expression and previous knowledge. The inference on STRING is predicted by the confidence score, and we focused only on highest-confidence interactions (i.e., interactions with a score larger than 0.9) in our results. The identified proteins were grouped into

biological processes and molecular functions according to their ontology. The peptide sequences were submitted to the UniProt database.

Statistical analysis - The results were expressed as the mean number of parasites per mouse and were submitted to Student's t test ($\alpha = 5\%$) performed using the R program (R Development Core Team, 2012). The graphics were constructed using GraphPad Prism software (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 striking effects observed in the development of *S. mansoni* due to the exposition of cercariae to a low dose of the *E. milii* latex included a significant reduction of: (i) the parasite rate (number of adult parasites) and (ii) the number of eggs released in the feces. In the exposed group, the number of adult parasites recovered decreased by 74.1% for males and 76.3% for females and the quantity of eggs released was reduced in 89.9% (Table 1).

The quantity of protein extracted from females (0.75 $\mu g \cdot \mu l^{-1}$ per replicate) was not sufficient for proteomic analysis. In the males (4.7 $\mu g \cdot \mu l^{-1}$ per replicate) with two-dimensional electrophoresis we detected a total of 1,020 spots with 26 differentially expressed between the control and exposed groups. Of these, only 10 spots were detected by the MALDI TOF-TOF spectrometer and subsequently identified by PMF and/or MASCOT to be statistically validated by the Scaffold software and manually validated by Peaks (Table 2).

The most significant cellular components of the 10 valid peptide sequences in the control and exposed groups were: myofibril (p-value ≥0.00018), contractile fiber (p-value ≥0.00018), actin

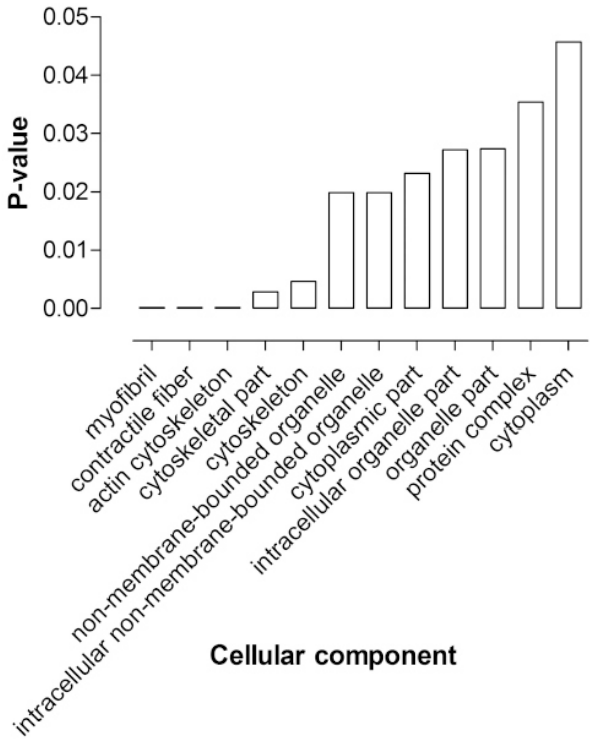
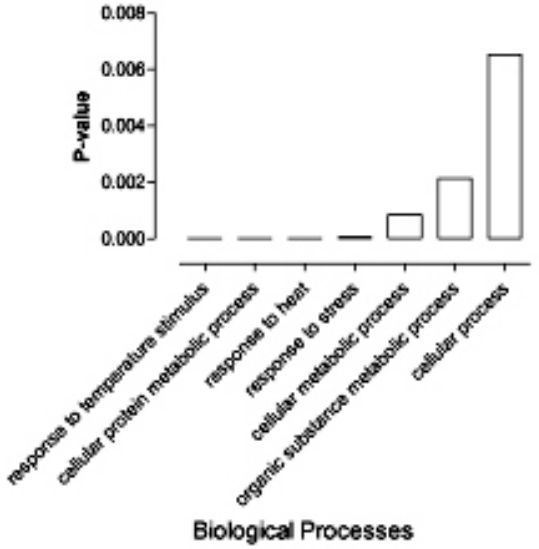
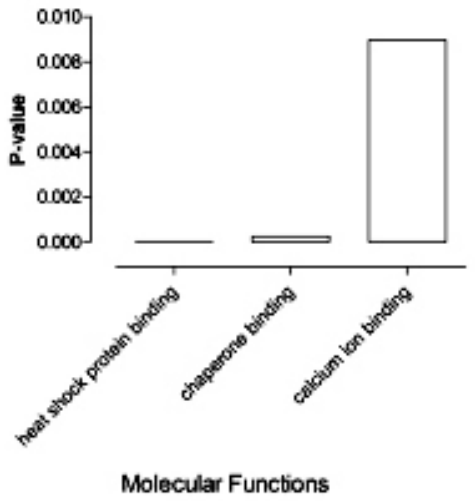
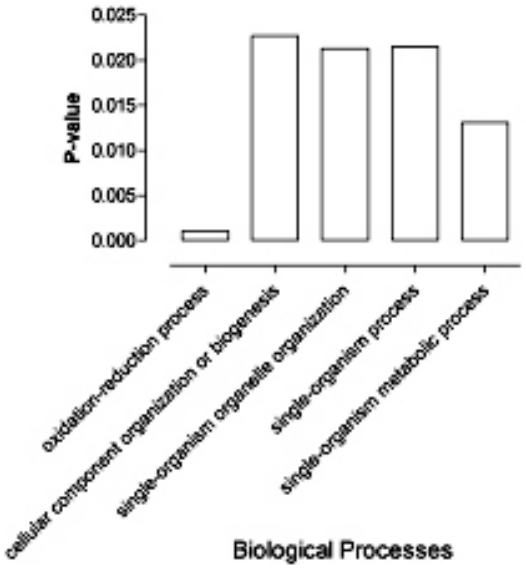


Figure 1. Gene ontology analysis using the String database. Analysis of the cellular components of ten valid peptide sequences from control adult males of *Schistosoma mansoni* and adult males originating from cercariae exposed to *Euphorbia millii* latex.







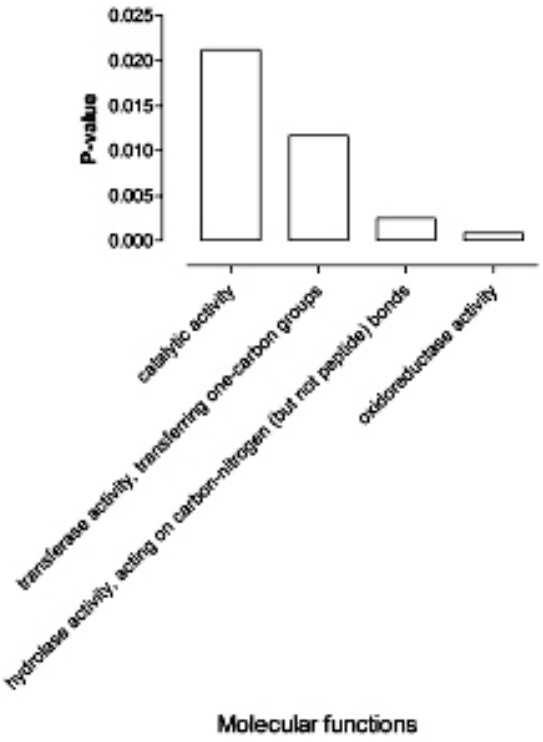


Figure 2. Upregulation of biological processes in adult male *Schistosoma mansoni* from cercariae exposed to latex; **Figure 3**. Upregulation molecular function in adult male *Schistosoma mansoni* from cercariae exposed to latex**; Figure 4.** Downregulation of biological processes in adult male *S. mansoni* from cercariae exposed to latex; **Figure 5.** Downregulation of molecular functions in adult male *S. mansoni* from cercariae exposed to latex.

cytoskeleton (p-value ≥0.00017), cytoskeletal portion (p-value ≥0.0028), cytoskeleton (p-value ≥0.0046), non-membrane-bounded organelle (p-value ≥0.01), intracellular non-membrane-bounded organelle (p-value ≥0.01), cytoplasmic portion (p-value ≥0.02), intracellular portion (p-value ≥0.02), organelle portion (p-value ≥0.02), protein complex (p-value ≥0.03) and cytoplasm (p-value ≥0.04) (Fig. 1). The differential analysis showed that five spots were not expressed (not seen, not altered in expression) in the exposed group (actin-R5, troponin-R8, tropomyosin-R10, paramyosin-R11 and paramyosin-R15). The four proteins that appeared downregulated in the exposed *vs*. control group were actin-R4 (-1.55) and R7 (-4.04), 14-3-3 epsilon-R23 (-1.73) and aldehyde dehydrogenase-R25 (-1.86). The spot protein upregulated in the exposed *vs.* control group was the heat shock protein hsp70 (R16 + 1.71) (Table 2).

The input of the latex on cercariae changed the distribution of protein abundance in adult male *S. mansoni* samples. The phytochemical compounds of the latex were responsible for the upregulation of the peptide sequence involved in three molecular functions: Heat shock protein binding (p-value ≥0.000017), chaperone binding (p-value ≥0.00025), and calcium ion binding (p-value ≥0.0089) (Figure 2). These sequences are involved in many biological processes in the adult parasites, including the cellular protein metabolism process (p-value ≥0.000017), response to heat (p-value ≥0.000017), response to a temperature stimulus (p-value ≥0.000017), response to stress (p-value ≥0.000044), cellular metabolism (p-value ≥0.00085), organic substance metabolism (p-value ≥0.0021) and cellular process (p-value ≥0.0065) (Fig. 3). However, on *S. mansoni* from cercariae exposed to the latex, four molecular functions were downregulated: oxidoreductase activity (p-value ≥0.00089), hydrolase activity (p-value ≥0.0025), transferase activity (p-value ≥0.011) and catalytic activity (p-value ≥0.021) (Fig. 4). After exposition, significant biological processes included the oxidation-reduction process (p-value ≥0.001), cellular component organization or biogenesis (p-value ≥0.02), single-organism organelle organization (p-value ≥0.02), single-organism processes (p-value ≥0.02) and single-organism metabolism (p-value ≥0.01) (Fig. 5).

Analyzing the upregulated and downregulated proteins reported in Table 2 all together by STRING, Smp-198290 (paramyosin), Smp_046590 (actin), Smp_179810 (troponin t), Smp_044010.2_mRNA (tropomyosin-1) and the multifunctional protein hsp70 appear all forming a closed interactome with a clustering coefficient = 0.81 and PPI enrichment p-value of 0.000206. Two proteins were unconnected: Smp_050390 (aldehyde dehydrogenase) and Smp_034840.2 (14-3-3 epsilon) (Fig. 6).

Table 1. Effect of exposure to 1.4 mg·L^{-1} of *Euphorbia milii* latex on the survival rate of definitive host, male and female worms recovered and total *Schistosoma mansoni* recovered in Swiss Webster infected in the laboratory. Group 1 = cercariae kept in distilled water for a period of one hour and Group 2 = cercariae exposed to 1.4 mg·L^{-1} of *E. milli* latex during the same period. *Different letters* indicate *significant differences* between the *means* (α = 5%).

	Survival rate of definitive host	OPG	Male Parasites	Female Parasites	Total Parasites
Group 1	62%	708±28.8^{a}	76.6±5.1^{a}	89.8±6.1^{a}	166.6±11.3^{a}
Group 2	85%	71.4±42.2^{b}	19.8±7.6^{b}	21.2±2.1^{b}	41.1±4^{b}

Table 2. Differentially expressed proteins of adult males of *Schistosoma mansoni* and parasites originated from cercariae exposed to the latex of *Euphorbia milii* var. *hilopii*. Proteins were separated by two-dimensional electrophoresis and identified by Maldi TOF-TOF.

Spot number	Protein	Fold Changes	Anova	*Schistosoma mansoni* Gene names	PMF Peptide sequence	Mascot Peptide sequence	Mascot Score	Protein Sequence Coverage	Mw (Da)	IP
R04	Actin	-1.55	0.0001	Smp_046590	-.MCVPK.H -.MCVPK.H + Oxidation (M) K.HTHWKTER.R R.ISEYVSK.C R.ISEYVSKCLR.L R.LKGTTALNSAISR.H	K.SYELPDGQVITIGNER.F	46	67%	41992	5.30
R05	Actin	*	*	Smp_046590	-.QGVMVGMGQK.D + 2 Oxidation (M) K.DSYVGDEAQSK.R R.VAPEEHPVLLTEAPLNPK.A R.TTGIVLDSGDGVTHTVPIYEGY ALPHAILR.L R.LDLAGR.D K.SYELPDGQVITIGNER.F	R.AVFPSIVGRPR.H K.SYELPDGQVITIGNER.F	55	33%	45865	5.90
R07	Actin	-4.04	0.0044	Smp_046590	Non-identified	R.VAPEEHPVLLTEAPLNPK.A K.SYELPDGQVITIGNER.F	203	9%	41992	5.03
R08	Troponin t, invertebrate	*	*	Smp_179810	Non-identified	R.TVFTGANYAEEYQR.I	64	4%	37472	5.89
R10	Tropomyosin	*	*	Smp_044010.2	Non-identified	K.ALEISEQESAQREESYEETIR.D	70	7%	97815	4.5
R11	Paramyosin	*	*	Smp_021920.1	M.NHDTESHVK.I R.HAADLGFQVDALSERLDEAGG STTQTQELLK.R K.SKFER.E K.TEEFEEMKR.K R.ITELEDTAERER.L K.LTLEIKDLQSEIESLSLENSELIR .R R.RVDELTIEVNTLTSQNSQLESEN LR.L	R.VKDLETFLDEER.R R.VSELTIQVNTLTNDKR.R R.EITVKLEEAEAFATR.E	49	5%	113247	5.4

Spot number	Protein	Fold Changes	Anova	*Schistosoma mansoni* Gene names	PMF Peptide sequence	Mascot Peptide sequence	Mascot Score	Protein Sequence Coverage	Mw (Da)	IP
					R.ENRQMNDQVK.E + Oxidation (M) R.KSTTR.T K.RYESNIADLEIQLDTANK.A R.VKDLETFLDEER.R R.LQLANEIEEIRSTLENLER.L R.VSELTIQVNTLTNDKR.R R.EITVKLEEAEAFATR.E R.KFER.Q K.MKAYK.R + Oxidation (M)					
R15	Paramyosin	*	*	Smp_021920.1	Non-identified	K.DLQSEIESLSLENSELIR.R	34	2%	111600	5.5
R16	Heat shock protein 70	1.71	0.0236	Smp_106930	Non-identified	R.ARFEELNADLFR.G R.FEELNADLFR.G	52	1%	80012	5.9
R23	14-3-3 epsilon	-1.73	0.0304	Smp_034840.2	Non-identified	R.VFSAVEQTEGNR.G K.FREVLESELDR.V K.DILELIDKYLIK.S R.YMAEFSVDPQR.K + Oxidation (M) K.AYQEASEIAATQLFPTHPIR.L	198	26%	32042	5.0
R25	Aldehyde dehydrogenase	-1.86	0.0506	Smp_050390	R.IRQLEDELESTETR.L R.QLEDELESTETR.L R.TFADEER.I R.TFADEERINQLEEQLK.E R.INQLEEQLK.E R.INQLEEQLKESTFMAEDADR.K + Oxidation (M) K.ESTFMAEDADR.K + Oxidation (M) K.YDEAARK.L K.LAITEVELER.A K.ITELEEELR.I K.SLEISEQEAAQR.E K.SLEISEQEAAQREEAYEENIR.D R.EEAYEENIR.D R.EEAYEENIRDLTER.L R.LVNTLQADADRLEDELVTEK.E	K.FREVLESELDR.V K.TVESALGDVFFAAQTTR.Y	30	3%	54412	5.76

Note: The symbol "*" indicates expression only in control group. MW = molecular weight IP = isoelectric point.

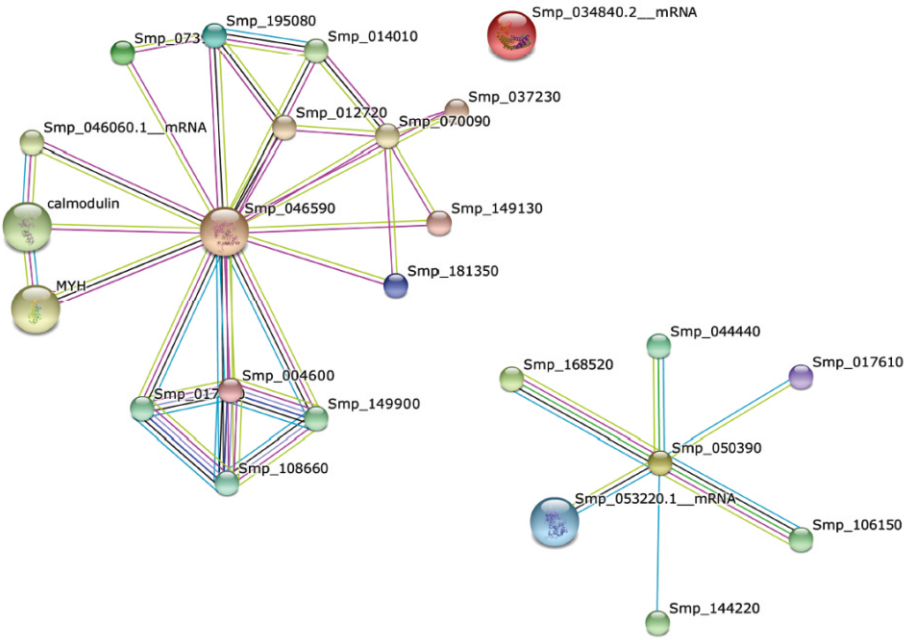


Figure 6. Protein Network interaction for up and downregulated proteins of *Schistosoma mansoni* using the STRING database.

DISCUSSION

It is important to draw attention to the influence of water-soluble compounds in the development of a parasite's life cycle. The input of soluble macromolecules or environmental factors (chemical pollutants, dietary components, temperature changes and other external stresses) in the free larval stages of the parasite could be responsible for changes in genome expression (long-lasting effects on gene expression and chromatin), which modify the phenotype of the worm and thus affect the parasite's biology in ways that could be heritable over the parasite's life span (Thornhil *et al*., 2009; Feil & Fraga, 2012; Roquis *et al*., 2015).

De-Carvalho *et al.* (1988) tested high concentrations of *E. milii* latex against *S. mansoni* cercariae (10-100 mg·L^{-1} for 2 h), and no changes were observed in cercarial survival or viability. However, our results indicated a reduction of parasite development of 74.5% in males and 76.5% in females. These results were similar to the results of Bakry & Mohamed (2011), who used *E. milii* latex at a concentration 73.6% higher than the concentration used in the present study. In our study, the exposition of cercariae to a low concentration (1.4 mg·L^{-1} for 1 h) produced striking effects in the adult worms, with a reduction of the parasite rate, number of eggs released and changes in the male tegument.

The exposition of cercariae to the latex changed the biology of the parasites showing their effectiveness as a control strategy influencing the transmission (reduction of adult worms and the number of eggs released). The proteomic analysis of adult males of *S. mansoni* from exposed cercariae showed protein targets that produced remarkable changes, including the upregulation of three molecular functions (heat shock protein binding, chaperone binding and calcium ion binding) and the downregulation of another four molecular functions (oxidoreductase activity, hydrolase activity, transferase activity and catalytic activity). These data confirm the influence of latex on the development and survival of the parasites.

To support this evidence, 26 differentially expressed proteins in both experimental groups were detected with two-dimensional electrophoresis. It is interesting that five spots were not detected in the exposed group (actin-R5, troponin-R8, tropomyosin-R10, paramyosin-R11, and paramyosin-R15). The actin gene has a strong association with the surface membrane and is very important for the dynamic activity of parasites and other organisms (Braschi *et al*., 2006). This protein is the most abundant protein found in eukaryotic cells and is involved in many biological processes, such as ATP and nucleotide binding, the regulation of the actin cytoskeleton, tissue remodeling and apoptosis (Gourlay & Ayscough, 2005; Watanabe *et al*., 2015). The proteome of *S. mansoni*'s surface presents molecular motor activity, and the layer immediately beneath the plasma membrane is largely composed of actin homologues in a macromolecular complex involved in the shuttling of vesicles to the surface plasma membrane (Braschi *et al*., 2006).

There are several actin isoforms expressed in schistosomes involved in various types of parasite motility (Abbas & Cain, 1987; Abbas *et al.*, 1997). In the present study, actin spots R4 and R7 were downregulated, mostly R7 (-4.04) that was the more downregulated spot seen in this work. Actin spot R5 was not seen in *E. milii*-exposed *vs.* unexposed cercariae (Table 2). In the future, it would be interesting to identify the molecular type of the expression-altered actins. These results clearly indicate that actin is an important target for the *E. milli* phytochemical activity, remembering that *S. mansoni* surface spines are "crystals" of actin (Cohen *et al*., 1982). Furthermore, it has been reported that actin is a target for Praziquantel (Tallima & El Ridi, 2007), the actual drug of choice for schistosomiasis treatment. Actin is also a circulating parasite antigen (together with other muscular proteins) in human *S. mansoni* infections, and it is able to induce an immunoprotective response in experimental animals (Sulbarán *et al*., 2013; Ludolf *et al*., 2014). All this information might provide an explanation for the reduction in the number of adult male worms found in animals infected with *E. milii*-exposed cercariae *vs.* unexposed cercariae. Actin downregulation would also affect its interaction with the troponin/tropomyosin complex, troponin being an actin-binding motor protein.

Troponin T, an invertebrate protein, and paramyosin were exclusive to the control group. The genes Smp_179810 (troponin T) and Smp_0198290 (paramyosin) are involved in the actin cytoskeleton and muscle system and are important targets for the mobility of *S. mansoni*. High levels of expression of paramyosin are also observed in adult male *Schistosoma japonicum* (Gobert *et al*., 2009). The functions of paramyosin in schistosomes are well documented as a structural component of smooth muscle fibers and an immunomodulator of the host immune response through its binding to the immunoglobulin Fc region and its inhibition of complement activation (Loukas *et al*., 2001). Previous studies on the multiple functions of paramyosin showed that it may be a potential vaccine candidate, as it has been suggested to play an important role in immune stimulation, while schistosome paramyosin is postulated to play a role in immune evasion (Jones *et al*., 2004).

Some proteins were downregulated after exposition, such as actin, 14-3-3 epsilon and aldehyde dehydrogenase. The decrease of the 14-3-3 epsilon protein can reveal a decrease in the cell's life cycle on exposed parasites because its proteins are involved in the cell cycle and the PI3K-Akt signaling pathway (Protasio *et al*., 2012). The PI3K-Akt signaling pathway mediates survival signals in a wide range of neuronal cell types and can be involved with the metabolic pathways that regulate cell survival (Brunet *et al*., 2001). The downregulation of aldehyde dehydrogenase reinforces the hypothesis that the parasite likely produces a molecular compensatory mechanism against the stress caused by the latex, as this protein is important in the signaling of stress in glycolysis, pyruvate and arginine metabolism and fatty acid degradation. The changes in the proteomic profile of adult *S. mansoni* from cercariae exposed to the latex (exposed group) were significant, and an increase of heat shock 70 kDa protein (hsp70), a major immunogen in *S. mansoni* infections, was detected. Many phases of *Schistosoma*'s life cycle are specific to increased levels of hsp70 mRNA, and it is more commonly expressed in cercaria-schistosomula transformation and heat-shocked adult parasites (Neumann *et al*., 1993). These data reflect a compensatory mechanism against the host-parasite relationship caused by latex.

One spot was identified for tropomyosin (Smp_044010.2). The sequence was not detected in adult male parasites after exposition, and the absence and/or low expression of this protein (and homologues) may be dangerous for the survival of *S. mansoni* because it interferes with the constant renewal of the tegument, immune modulation with the host, osmoregulation, the absorption and secretion of substances, and reproduction (Faghiri *et al*., 2010).

The protein network significantly indicated that the analyzed proteins have more interactions among themselves than what would be expected for a random set of proteins of similar size, drawn from the genome at p - value = 0.000206. Such an enrichment indicated that the proteins are at least partially biologically connected, as a group, according to STRING definition. The unconnected proteins probably form interactomes with proteins in different pathways, not established in the present study. Smp_050390 (aldehyde dehydrogenase), although multifunctional, is probably mainly involved in oxido-reduction metabolic process in *S. mansoni*.

The present work may also provide new insight on targets for the development of new interventions for future control of schistosomiasis. The changes observed in the proteomic profile of adult *S. mansoni* males occurred by the exposition of cercariae to the *E. milii* var. *hislopii* latex, which induced the significant muscular fragility of the body. We could consider that it may prevent males from maintaining females inside with consequent reduction of the parasite load. The proteins that were not expressed or that were downregulated affected the muscle contraction function in exposed parasites, probably interfering with its intrahost migration.

In conclusion, this study suggests a route for the control of schistosomiasis in endemic areas using natural water-soluble products in sublethal concentrations for mollusks, which reduces the parasite rate in definitive hosts. This indirect therapy is inexpensive, ecological and efficient.

CONFLICT OF INTEREST

The authors declare no conflicts of interest.

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