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Chapter 8

Tegument of *Schistosoma mansoni* as a Therapeutic Target

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Additional information is available at the end of the chapter

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1. Introduction

Schistosomiasis is a parasitic disease with great social impact, being regarded as a relevant public health issue in 76 countries in Africa, Asia, and South and Central Americas [1, 2]. It is one of the main water-borne parasitic diseases in the world and it continues to be a major cause of morbidity and mortality, disabling and killing thousands of people every year. Considering that, both public health bodies and pharmaceutical companies need to more diligent regarding that issue [3, 4].

The parasite that causes schistosomiasis mansoni is the *Schistosoma mansoni,* an intravascular digenetic trematode from the family Schistosomatidae. In Brazil, where only that particular schistosome can be found, there are 25 million people living in endemic areas, from which 4 to 6 million are infected, which makes the country the most affected by intestinal schistosomiasis in all Americas. Popularly known as barriga-d'água (water belly) in Brazil, the disease is transmitted by planorbides from genus *Biomphalaria* [5, 6].

The transmission occurs when an infected definitive host eliminates viable eggs of the parasite through stool, getting in contact with bodies of fresh water and contaminating them. Therefore, the disease is directly related to fast urban growth and lack of resources such as safe water supply and adequate sewage system in peri-urban areas [7]. The pathology of the disease is characterized by having two phases, acute and chronic, which are dependent on the life stage of the parasite, as shown in Figure 1.

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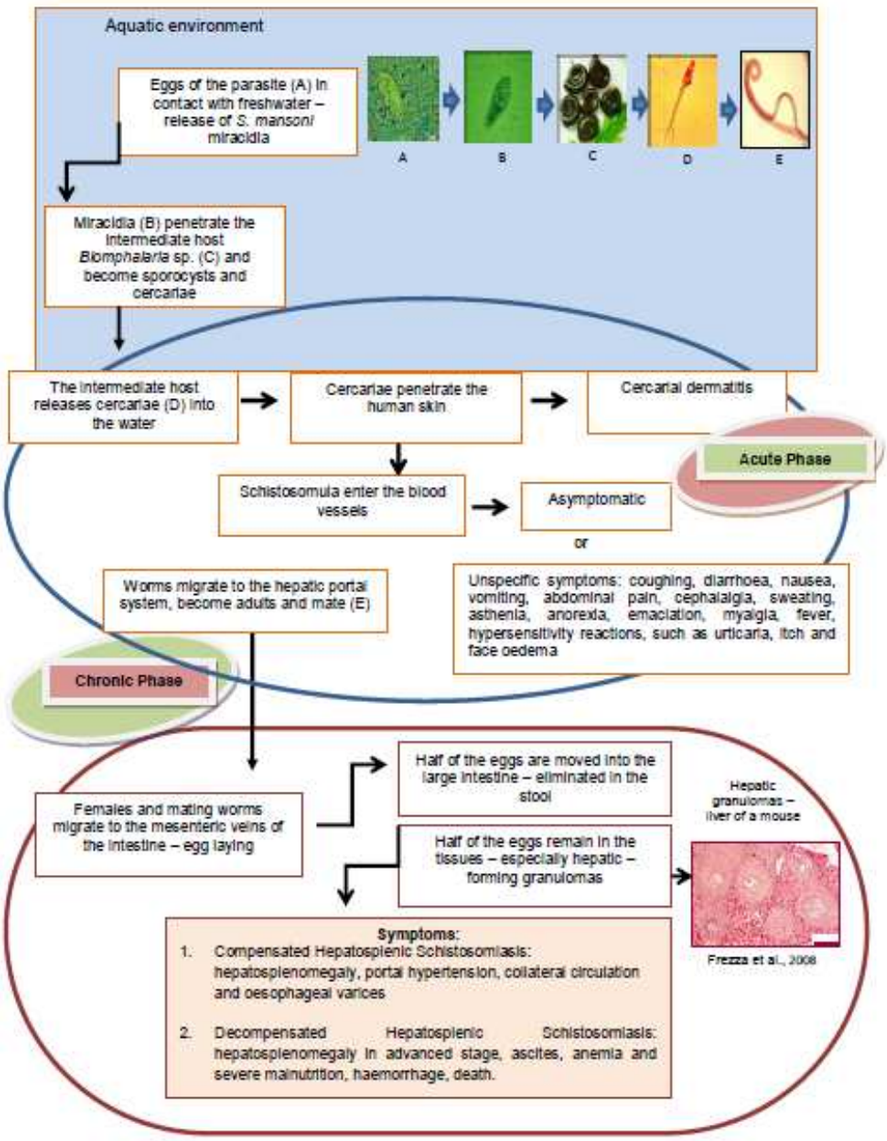

Figure 1. Cycle and pathology of schistosomiasis mansoni.

According to [8], environmental degradation is a determining factor to the dissemination of the disease, even more than poverty and underdevelopment. The authors also consider that some factors contribute to both development and maintenance of schistosomiasis mansoni breeding sites, such as subsistence cultivation; perennial cultivation; flooded areas; meanders and natural channels; springs and taps; environments likely to be polluted by human waste; fish-breeding ponds; ponds or watercourses used for sport fishing, washing of utensils, and bathing; sand deposits on river banks with no vegetation; large debris and garbage deposits and activities along watercourse banks. As a result, the authors believe that the distribution of the disease is not as random as it seems, and that the localities with concentra-

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tion of cases are associated with human activities that interfere with both landscape structure and human behavior, so there is a relationship between land occupation and health decline. In reference [9], the authors have recently confirmed this hypothesis by reporting the rise of the disease in the state of Bahia (Brazil), particularly in the town of Lauro de Freitas, which full ongoing economic growth is attracting intense human migration, which results in disordered urban occupation and environmental disturbances, increasing the risk of expanding the endemic area. Because of that, schistosomiasis mansoni is considered one of the most critical health issues in Brazil, occurring in 19 states (out of 26 states and one Capitol) [9, 10].

2. Therapeutics of schistosomiasis mansoni: Searching for new alternatives

Several intervention measures can be taken to reduce the morbidity of the disease, as well as, to prevent or interrupt the transmission of the parasite from the mollusk intermediate host to humans. Such measures include chemotherapy, environmental sanitation, mollusk control, safe water supplies, and environmental education.

Chemotherapy provides a double benefit: it reduces both the morbidity caused by the presence of adult worms in the human host and the number of eggs eliminated to the environment [11].

There are only two drugs available for the treatment of schistosomiasis mansoni, i.e., oxamniquine and praziquantel. However, since the former has side effects on the human organism (mutagenic and carcinogenic effects, as well as effects on the central nervous system), its production and commercialization are controlled and reduced. Therefore, praziquantel has been practically the only drug available for that treatment since the 1970s [12-14]. To exacerbate the situation, cases of tolerance and resistance of *S. mansoni* to the treatment with both drugs have been recently reported, which raises the need to develop new drugs and forms of controlling the disease [15-17].

In this context, research with medicinal plants becomes a viable alternative, especially in countries with large biodiversity and rich cultural and ethnic diversity, like Brazil, because of the resulting accumulation of local traditional knowledge, which is passed from generation to generation and includes the use and management of medicinal plants as home remedies [18]. Furthermore, there are about 100,000 catalogued plant species in Brazil, and their active ingredients are mostly unknown, as only 8% of those species were studied regarding their chemical composition and therapeutic properties [19]. In recent years, the scientific community has been conducting *in vitro* and *in vivo* tests to examine a variety of essential oils, extracts and isolated compounds from different species with respect to their schistosomicidal potential.

In the last few years, our research group carried out *in vitro* and *in vivo* tests using three species of Brazilian medicinal plants – *Baccharis trimera* (Less) DC., *Cordia verbenacea* DC

and *Phyllanthus amarus* – on adult *S. mansoni* worms. The three species are widely used in Brazilian folk medicine in the forms of infusion and tea on account of their anti-inflammatory properties.

Amongst the several criteria analyzed by our research group to evaluate the therapeutic efficiency of the tested plants, the morphological changes in the tegument of the worms (both males and females) were considered essential, due to the fact that literature reports the damage caused to that structure as cardinal in causing the parasite's death, and studies for the development of new schistosomicidal drugs have been setting it as a target [20].

3. The importance of the tegument of *Schistosoma mansoni*

The tegument of *S. mansoni* plays a key role in its protection against the action of the host's immune system, as it is renovated every six hours [20]. In addition to that, it is capable of absorbing nutrients and molecules and synthesizing some proteins [21-24]. That structure is also extremely important for the success of the infection and the survival of the worm in the host [25-27]. For all those reasons, it has been vastly studied since the end of the 1960s.

3.1. The analysis evolution of the tegument of *S. mansoni*: From optical to electron microscopy

Before microscopy techniques were available for the evaluation of the worm ultrastructure, little was known about the importance of the tegument of *S. mansoni*. The first detailed studies on that structure were carried out in the 1940s, when Gönnert [28], using light microscopy, described the differences between *S. mansoni* males and females, including the fact that males have more and larger thorns.

With the emergence of transmission electron microscopy (TEM) and scanning electron microscopy (SEM) there was a revolution regarding the 'appearance' of the worm and the description of its sexual dimorphism. By using electron beams that either pass through (in the case of TEM) or scan the specimen under analysis (in the case of SEM), electron microscopy, which was developed in the 1930s, had a significantly higher resolving power than optical microscopy, allowing for a detailed observation of samples.

According to [29, 30], *S. mansoni* was the first digenetic trematode to be examined under electron microscopy. For that reason, the ultrastructure of that helminth has been more often studied than the ultrastructure of any other digenetic trematode. By the end of the 1960s and beginning of the 1970s, electron microscopy was used in studies on schistosomiasis mansoni to confirm details of the parasite's tegument and thus allowed for interpretation of the functions of that structure.

In 1968, Hockley [31] described the surface of the worm using SEM, pointing out, for instance, the presence of thorns in the more internal portion of the oral sucker in both sexes, and the fact that the ventral sucker (acetabulum) is longer and more conspicuous in males. Still according to that author, the genital pore was detected in both sexes in the form of an

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opening placed posteriorly to the ventral sucker. The author also reported the presence of tubercles on the dorsal surface of male worms starting from the posterior portion of the ventral sucker, both sides being covered by thorns. The number of such tubercles starts to decrease from the posterior lateral edges of the dorsal surface.

Furthermore, Hockley [31] observed that in the areas between the tubercles the parasite's surface is rugged, with several grooves, as well as a few isolated thorns. On the male worm lateral edge, which bends to form the gynaecophoric canal, the author noticed the presence of large thorns whose function is to capture the female in the canal. Finally, he noticed that females also presents more thorns at the final portion of their surface, but in less quantity than males. Senft and Gibler [32] termed such thorns sensory papillae.

Using TEM, Hockley and McLaren [33] concluded that the surface of *S. mansoni* consists of two opposite lipid bilayers very close to each other and having the form of a cell membrane. Since the tegument does not have lateral membranes, its cytoplasm extends as a continuous unity, or syncytium, around the body of the worm. According to the authors, that syncytial complex is the main route of nutrient absorption (glucose, amino acids, among others), metabolite excretion (lactic acid and others) and protection against attacks by the host's immune system, whereupon it is a crucial target of drugs with schistosomicidal activity.

From the 1980s on, after the establishment of the therapeutics of schistosomiasis mansoni, TEM and especially SEM were also used in an attempt to clarify the action mechanisms of the drugs used in the treatment for the disease, i.e., oxamniquine and praziquantel.

Becker et al. [34], by means of SEM and TEM, realized that worms subjected to oxamniquine showed tegumental vacuolization. Using SEM, Kohn et al. [35] noticed that the drug was producing changes in the structure of the worm on tegumental, muscular and parenchymal levels, causing bubble-like lesions. Magalhães-Filho et al. [36] also noticed vacuolization, destruction of tubercles in male worms, and surface erosion. Recently, praziquantel has been subjected to further studies because it still is the most used drug in the treatment of all types of schistosomiasis.

Using SEM and TEM, Shaw and Erasmus [37] observed extensive damage to the structure of *S. mansoni* specimens subjected to praziquantel, including vacuolization of the tegument and subtegument of females, and destruction of the tegument and musculature. In males, in addition to vacuolization and destruction of the parenchymal tissue, mainly in the dorsal region, loss of cytoplasm, and structural damages to the musculature could be observed. It was noticed that male worms showed more damage in the tegument than females.

Actually, the action mechanism of praziquantel on *S. mansoni* has yet to be fully understood. On the other hand, there is no doubt that studies using SEM and TEM were important in the search to solve that puzzle.

The morphological changes that praziquantel causes to the tegument and in the sarcoplasmic membranes of the parasite are thought to be followed by an increase of antigen exposure on its surface. The antigens are identified and connected with the host's immune response required to complement the activity of the drug [38, 39]. Therefore, praziquantel is believed to interact with the host's immune system to kill the parasite. The last effect of the drug is the rupture of the surface of the worm, leading to its death [40].

In view of the fact that certain strains of the parasite have proved to be tolerant and resistant to the treatment with oxamniquine and praziquantel, it has become necessary to test new drugs. Electron microscopy has been used since the 1990s to know if such drugs are active against the worm by observing the damages caused to its tegument. Many studies using SEM have shown that drugs active against *S. mansoni* are responsible for severe damage to its tegument.

Some drugs, which had their schistosomicidal activity studied, have proved capable of producing tegumental changes. Albuquerque et al. [41] noticed that imidazole derivatives causes damage to the oral sucker in males, in addition to reducing and disorganizing the tubercles. In females the authors noticed erosion and peeling of the tegument, rupture of the surface membrane, and the complete disappearing of sensory structures.

Manneck et al. [42] observed that mefloquine causes higher degree of changes to the tegument of schistosomula and adult females, including peeling and bubble formation. Tests with that drug have also been carried out with other *Schistosoma* sp. (e.g., *S. japonicum*, which causes intestinal schistosomiasis), and these were the effects described: peeling in males and females, fusion of thorns in males, collapse of the sensory papillae, and erosion on the suckers [43].

In assays carried out with arachidonic acid, El Ridi et al. [44] showed extensive changes in the aspect of tubercles in males, including reduction in size and loss of thorns. The suckers also underwent changes such as oedemas and loss of thorns.

Other authors used SEM to attest the activity of natural compounds on the tegument of the worm. Shuhua et al. [24] reported that artemether, a derivative compound of artemisinin, which is extracted from *Artemisia annua,* causes damage to the tegument of both males and females, including peeling, which was more intense in females – not surprisingly, as artemisinin itself is most effective against females [45]. Oliveira et al. [46] noticed extensive peeling on the tegument of male and female worms, destruction of tubercles, thorns and sensory papillae, and changes in the suckers (oral and ventral) after *in vitro* exposure to essential oil of *Baccharis trimera* over an incubation period of 24 hours.

3.2. *S. mansoni* tegument analysis method

Nowadays, other techniques are being used in an attempt to evaluate the activity of candidate drugs on the tegument of the worm. One of them is confocal laser scanning microscopy (CLSM), which, like SEM, provides three-dimensional images. The confocal microscope was developed in 1950, but it only became popular for analysis of biological samples in the 1970s [47-50].

Moraes et al. [27-51] used confocal laser scanning microscopy to analyze the activity of piplartine (isolated from *Piper tuberculatum*) on the tegument of *S. mansoni* adults and schistosomula, and reported reduction in the quantity of tubercles in males and damage to the surface membrane of schistosomula. Moraes [51] proposed the use of both CLSM and SEM to perform a quantitative analysis (by counting the tubercles in a specific area) of the damage caused to the tegument of worms used in drug testing.

Another way to evaluate tegument damage caused by candidate drugs is to use an inverted optical microscope during *in vitro* assays. Magalhães et al. [52] and Moraes et al. [51], for example,

reported damage to the tegument of *S. mansoni* specimens subjected to *in vitro* assays with *Dryopteris* sp. and *Piper tuberculatum*, respectively, considering them either moderate or severe.

Therefore, there are other methods besides electron microscopy to evaluate the activity of a drug or candidate drug on the tegument of *S. mansoni*. Such methods can be classified as qualitative (i.e., it is only possible to notice changes, not to measure them), quantitative (changes can be quantified, i.e., it is possible to count and compare, for instance, the number of tubercles in male worms subjected to the tested sample with the number found in the control group), and qualitative-quantitative (in this case, changes can be both visualized and quantified). Table 1 presents that classification.

Method	Classification	Advantages	Disadvantages	References
Inverted Optical Microscope (used during *in vitro* tests)	Qualitative	- No special preparation is required to visualize the sample -Damages to the tegument can be visualized live, whilst the i*n vitro* experiment is carried out -It is possible to analyze a higher range in less time	-Analysis criteria are subjective: damages must be evaluated subjectively, e.g., they are reported as intense, intermediate, moderate, or even absent	[51, 52,53-57]
Confocal Laser Scanning Fluorescence Microscopy	Quantitative	- Allows damages to be quantified through three-dimensional images, especially on the tegument of male worms, where the number of changed or damaged tubercles is counted -Provides images of great contrast even with weakly fluorescent specimens -Three-dimensional images with a resolving power of 0.1-0.2 µm, providing many details of the surface of the worm	-Sample has to be prepared - Expensive - Complex to operate -High-intensity laser light - Chemical labeling	[51]
Transmission Electron Microscopy	Qualitative	- Allows for the analysis not only of damages caused to the tegument of the worm but also of changes in its musculature and internal organs - Excellent resolution (< 0,2 nm)	- Sample preparation is a delicate and difficult work - Tegumental damages cannot be statistically quantified - Expensive	[43, 58, 59]
Scanning Electron Microscopy	Qualitative/ Quantitative	- Three-dimensional images with high angular resolution (~10 nm), providing many details of the surface of the worm - Like in confocal laser scanning fluorescence microscopy, damages to the tegument can be quantified - High-quality three-dimensional images, ideal to analyse the texture, topography and surface of the worm	- Sample has to be prepared - Expensive	[24, 46,58]

Table 1. Methods to analyze damage to the tegument of *S. mansoni* specimens subjected to either *in vitro* or *in vivo* tests *for* evaluation of candidate drugs*:* classification. Sources: [60-63].

Our research group has evaluated the activity of different fractions of *B. trimera, C. verbenacea* and *P. amarus* on the tegument of adult *S. mansoni* males and females using SEM. We understand that this method provides important data regarding tegumentary changes because its high angular resolution provides high-quality images that allow us to analyse the parasite's surface in detail.

4. Methodologies used in scanning electron microscopy for studies of *S. mansoni*

On account of their naturally hydrated condition, biological samples are relatively complex to process, and only hard objects (e.g., seeds) can be observed through SEM with minimum preliminary treatment. Therefore, the preparation of a biological sample for SEM includes various stages [60, 64].

From the first observations using SEM to the present ones, it can be noticed that the methodology for *S. mansoni* worms preparation has significantly varied from one author to the other. Nevertheless, the steps for preparing the worms have always been respected: fixation, washing to remove the excess of fixatives, post-fixation in osmium tetroxide, dehydration at growing concentrations of ethanol, critical point drying, mounting on aluminum stubs, gold sputtering, and observation under a scanning electron microscope. Table 2 shows differences in the methodologies for preparing *S. mansoni* specimens for SEM analysis.

Although some methodologies for preparation of *S. mansoni* samples for SEM use phosphate buffer, some authors use sodium cacodylate buffer. Considering that, an experiment was carried out in order to evaluate possible differences between the use of such biological buffers. A protocol was used, and only the biological buffer was changed:

1. Fixation in Karnovsky (2.5% glutaraldehyde and 4% paraformaldehyde) with buffering pH between 7.0 and 7.3 (adjusted with 0.2 M HCL) with 0.1 M sodium cacodylate buffer solution or 0.1 M phosphate during 48 hours; 2. Washing in 0.1 M sodium cacodylate buffer or 0.1 M phosphate for one hour, changing the solution every 15 minutes; 3. Post-fixation in 1% osmium tetroxide for one hour; 4. Washing in 0.1 M sodium cacodylate buffer or 0.1 M phosphate for 30 minutes, changing the solution every 10 minutes; 5. Dehydration at growing concentrations of ethanol (50% and 70% during 30 minutes, changing the solution after 15 minutes; 90% and 100% during 30 minutes, changing the solution every 10 minutes); 6. Drying of worms in a critical point dryer; 7. Mounting of the samples on aluminum stubs; 8. Gold Sputtering; 9. Observation under a scanning electron microscope

Figures 2 to 7 present images obtained with different buffers. It could be noticed that the samples subjected to phosphate buffer showed inferior fixation as compared to sodium cacodylate, since the worms became malleable and, especially males, seemed to dehydrate. For that reason, sodium cacodylate buffer was used in the subsequent assays. However, it could also be noticed that the use of Karnovsky solution in fixation, as well as the long period in which the samples stayed therein, rendered the worms stiff, friable and hard to manipulate.

The use of 2.5% glutaraldehyde as fixative for a shorter incubation period (24 hours) was, thus, adopted by our group.

Figure 8 shows the standardized protocol that was applied to all SEM assays with the studied plant species, i.e., *B. trimera, C. verbenacea* and *P. amarus*. The samples were mounted on aluminum stubs, placed in a Balzers critical point dryer, model CPD 030, and a Bal-Tec/Balzers sputtering system (Sputter Coater), model SCD 50, and then were analyzed under a Jeol scanning electron microscope, model JSM 5800LV.

Methodology	Type	Advantages	Disadvantages	References
Pre-fixatives	Glutaraldehyde (AG)	- In addition to not causing protein coagulation, AG is incorporated into tissues (additive fixative), promoting good structural fixation - Maintains good fixation quality up to 30 days if kept at -20°C	- When kept in alkaline medium at 4°C, there can be polymerisation, which reduces the osmolarity of the solution	[65-67]
	Formalin	- Low cost	- Methanol concentration varies from 11% to 16%, extracting most of cytosol and cell membrane, providing bad fixation conditions	[68,69]
	Karnovsky (2.5% glutaraldehyde and 4% paraformaldehyde)	- Paraformaldehyde penetrates 5 times faster than AG, but, because its fixation power is lower, it promotes a weaker initial stabilization, which will then be complemented by AG. As a result, the solution will have more osmolarity	- Combined use of two toxic substances	[70-72]
Buffer solution	Sodium cacodylate	- Absence of phosphate ions that can interfere with cytochemical studies - Preservation of the activity of certain enzymes and resistance to contamination, as it contains arsenic	- Highly toxic, partially for having arsenic in its composition - Its disposal demands special care	[65, 67, 70,71]
	Phosphate	- It is more physiological than sodium cacodylate buffer, not having toxicity	- Can produce artefacts in the form of electron-dense particles	[24,41]

Table 2. Methodological differences for the preparation of *S. mansoni* specimens for SEM. Sources: [73, 74].

Sodium Cacodylate Group – mated worms

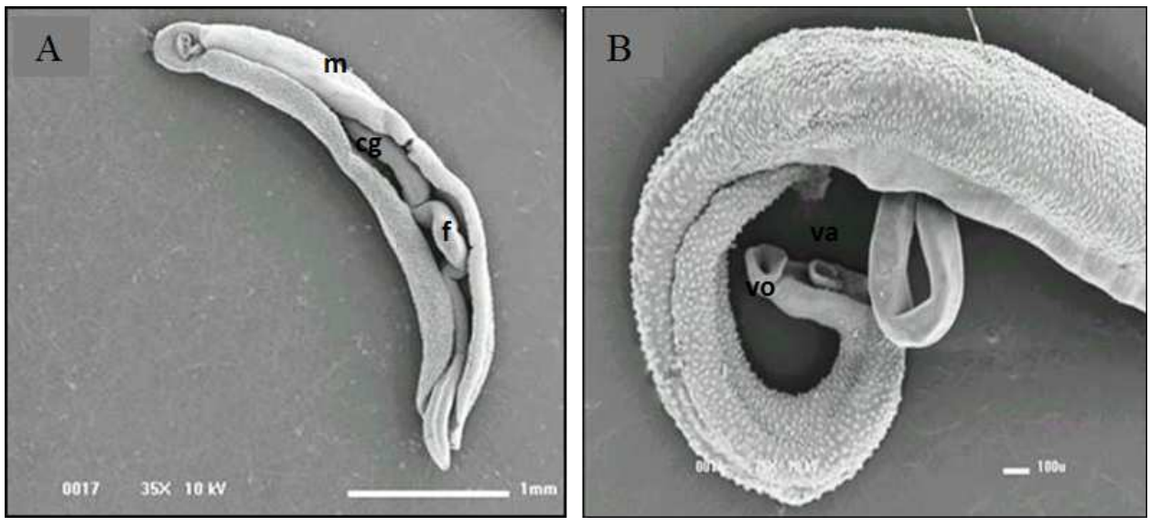

Figure 2. Scanning electron microscopy of adult *S. mansoni* using sodium cacodylate buffer. A-B – mated *S. mansoni* worms; m: male worm; f: female worm; cg: gynaecophoric canal, vo: oral sucker, Va: ventral sucker.

Sodium Cacodylate Group – Tegument

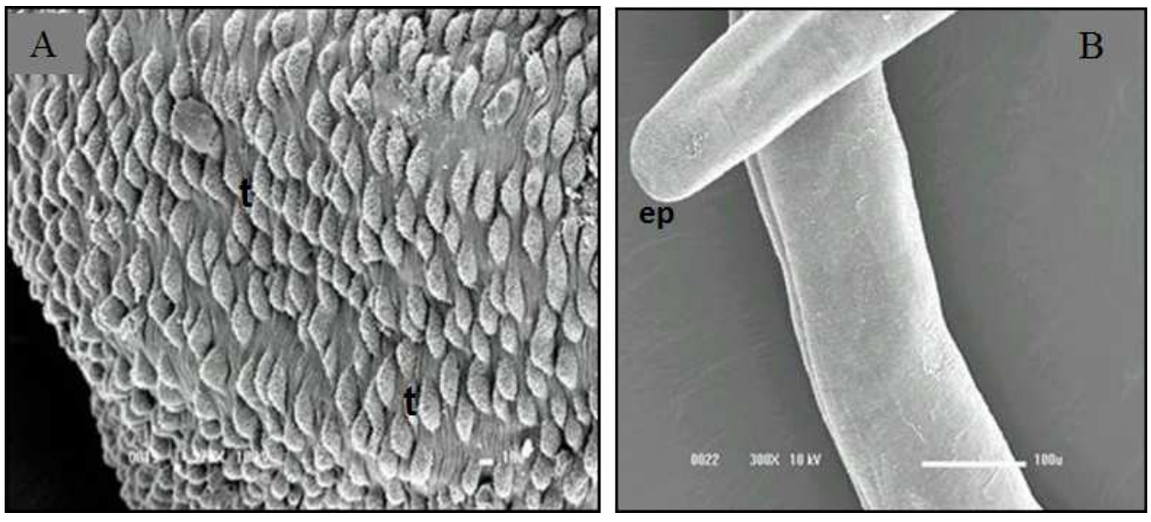

Figure 3. Scanning electron microscopy of the tegument of adult *S. mansoni* worms using sodium cacodylate buffer. **A** – tegument of male worm; **B** – tegument of female worm. **t**. tubercles. **ep** – excretory pore.

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Sodium Cacodylate Group – Suckers

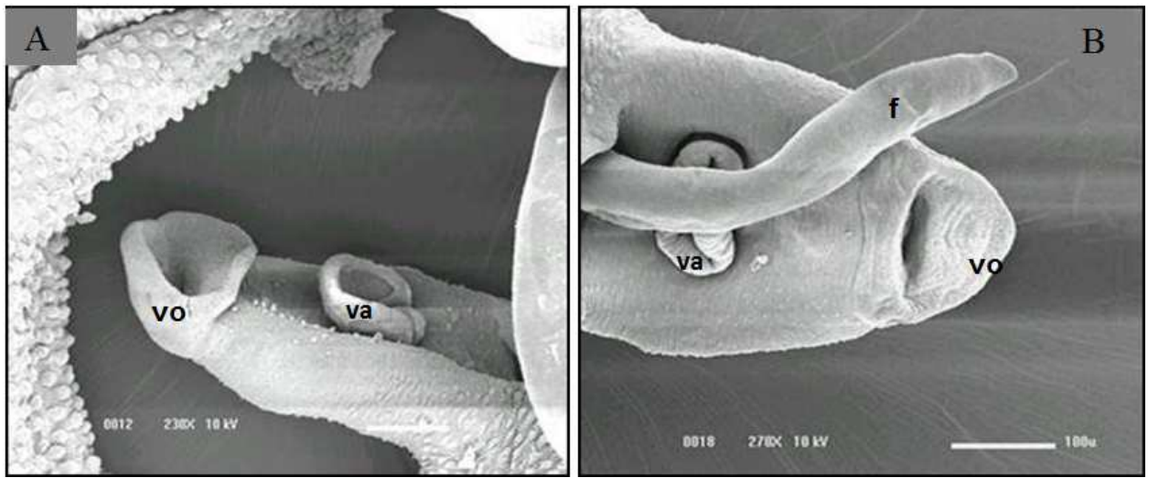

Figure 4. Scanning electron microscopy of *S. mansoni* male worm suckers using sodium cacodylate buffer. vo. oral sucker; va. ventral sucker; f. female worm.

Phosphate Group – Mated Worms

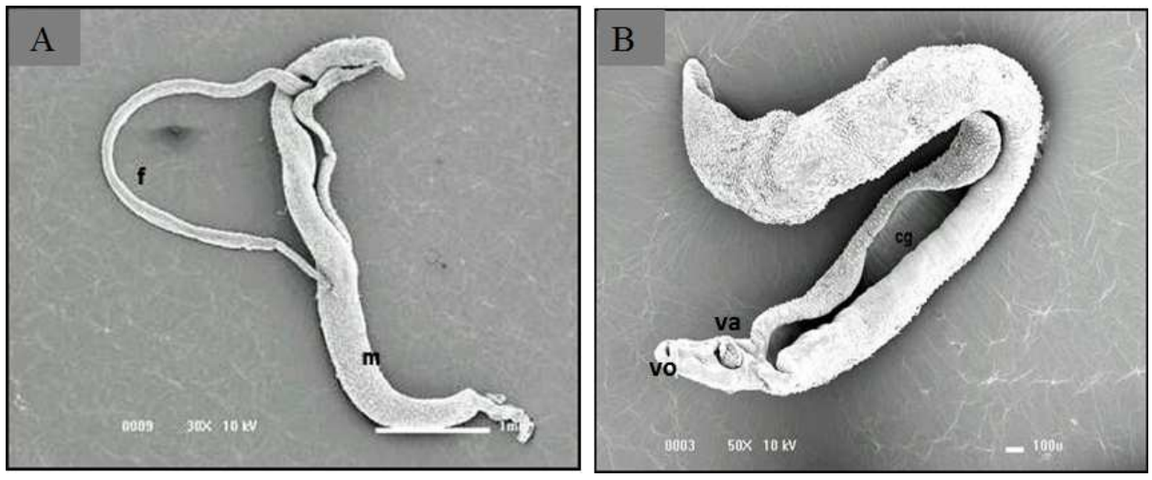

Figure 5. SEM of adult *S. mansoni* worms using sodium phosphate buffer. A- male worm; **f-** female worm; B- male worm; **vo-** oral sucker; **va**- ventral sucker. **cg**- gynaecophoric canal.

Phosphate Group – Tegument

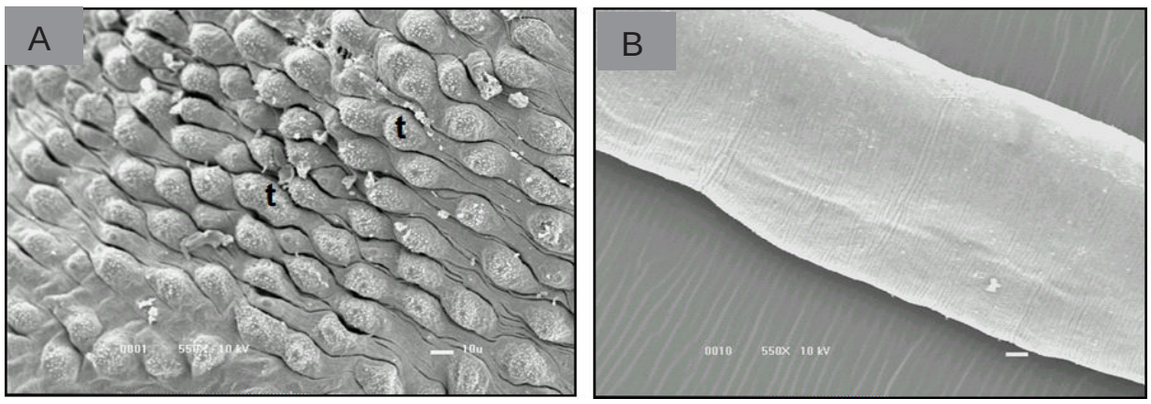

Figure 6. SEM of the tegument of *adult S. mansoni* worms using sodium phosphate buffer. A – tegument of male worm; B – tegument of female worm. **t**: tubercles.

Phosphate Group – Suckers

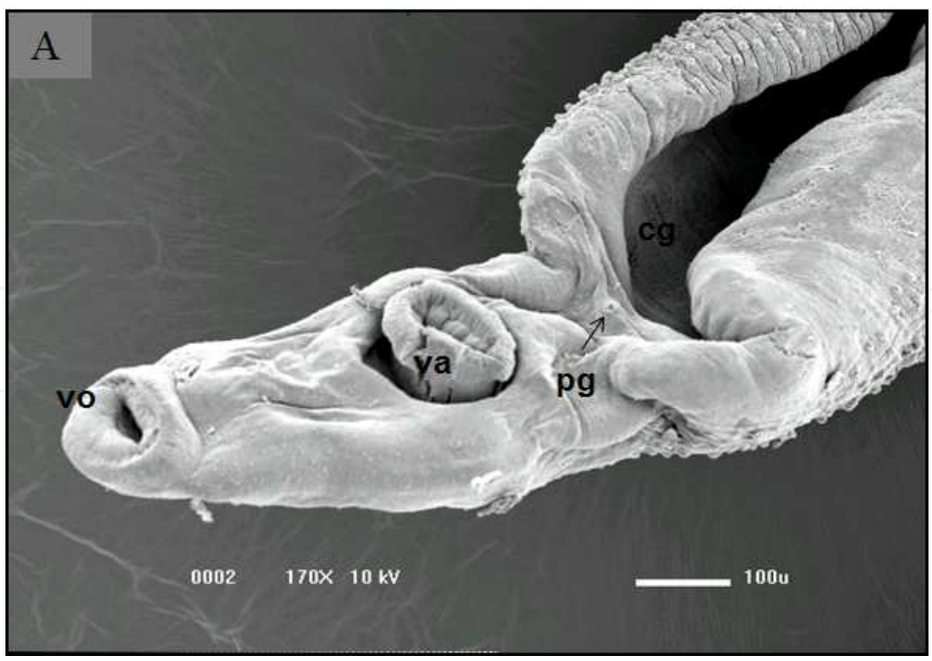

Figure 7. SEM of adult *S. mansoni* worms suckers using sodium phosphate buffer. **A**- male worm, genital pore highlighted (**pg**).

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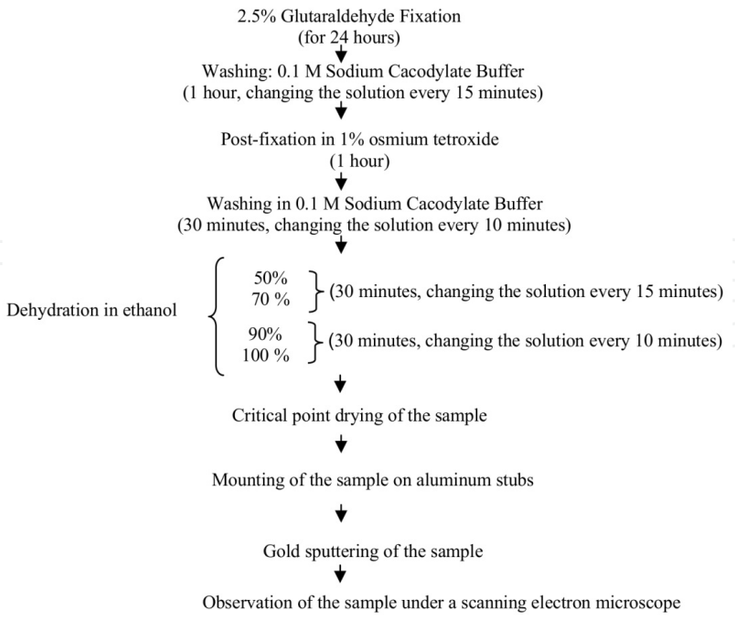

Figure 8. Protocol used by our research group to prepare *S. mansoni* specimens for scanning electron microscopy.

5. Study of the activity of medicinal plants on the tegument of *S. mansoni: Baccharis trimera* (Less) DC, *Cordia verbenacea* DC and *Phyllanthus amarus*

The species *B. trimera, C. verbenacea* and *P. amarus*, whose activity on the tegument of *S. mansoni* was evaluated, are widely used in Brazilian folk medicine. The studied plants were obtained from the Experimental Field of the Chemical, Biological and Agricultural Pluridisciplinary Research Center (CPQBA), Paulínia (22º45'40" S – 47º09'15" W), São Paulo, Brazil. The following fractions were tested, all of them coming from the aerial parts (flowers and/or inflorescences).

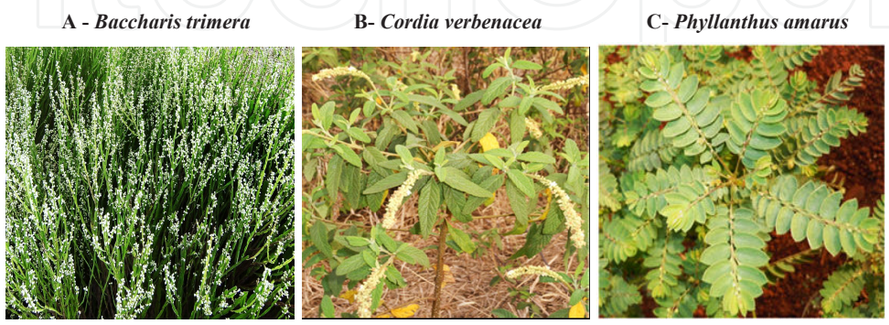

Figure 9. *B. trimera, C. verbenacea* and *P. amarus* specimens. Source: CPQBA, Unicamp, 2011.

- *Baccharis trimera* (Less) DC

The species *B. trimera* (Figure 9-A), known in Brazil as "*carqueja-amarga*", belongs to the family Asteraceae and is used in folk medicine for the treatment of many diseases, in particular hepatic ones. The plant allegedly has tonic, mouth-healing, antipyretic, analgesic, anti-diabetic, and anti-inflammatory properties [75-81]. It is native to the South and Southeast regions of Brazil, also being found in Argentina, Bolivia, Paraguay and Uruguay [82].

B. trimera was used in *in vitro* assays in which mating worms were kept in RPMI-1640 medium with penicillin/streptomycin, incubated in a controlled environment (5% CO_2 and 37°C) [53], and exposed to a fraction hexane fraction, obtained from the fractionation of dichloromethane extract, in lethal concentration of 130 µg/mL for 24 hours. After that period, the worms were prepared for SEM, also according to the protocol shown in Figure 8.

The hexane fraction of *B. trimera* caused changes to the tegument of both males and females and on the oral and ventral suckers. The tegumental peeling was particularly worth noting (Figures 10 to 12).

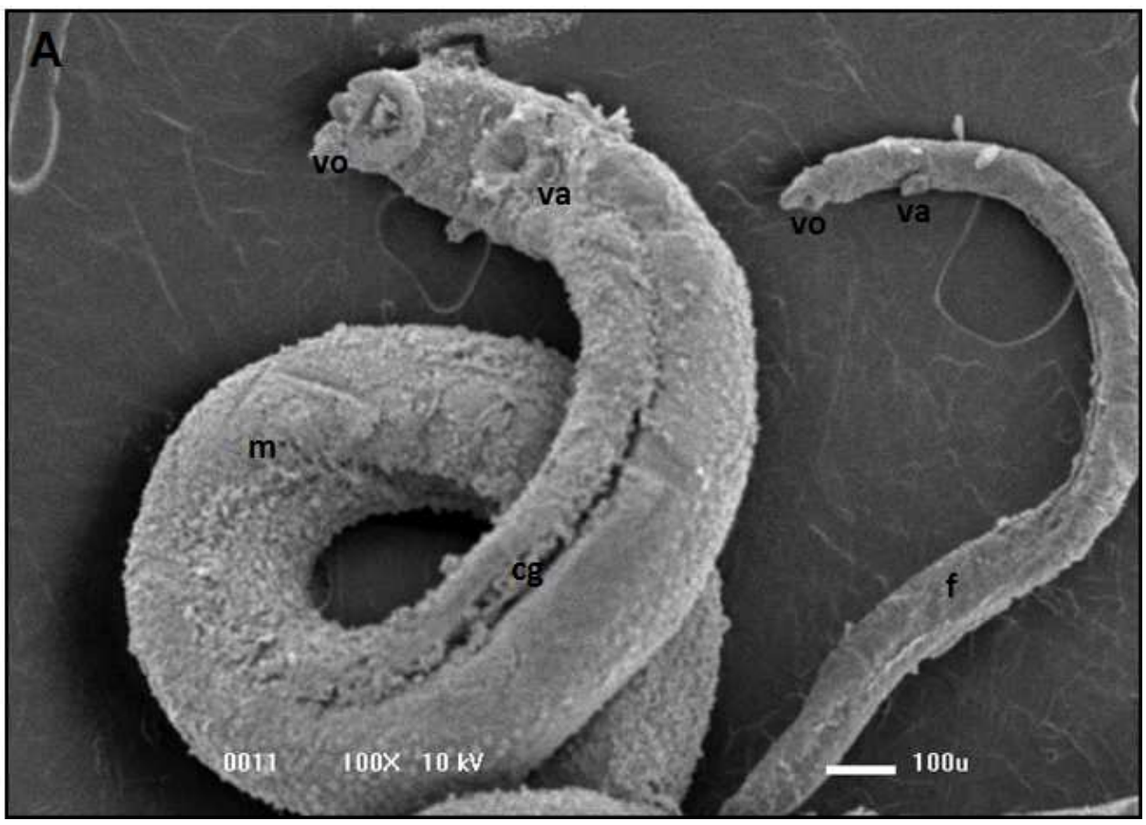

Figure 10. SEM of a *S. mansoni* adult couple after *in vitro* exposure to hexane fraction obtained from the crude dichloromethane extract at the lethal concentration of 130 µg/mL, over an incubation period of 24 hours. **m**- male worm showing changes in its oral **(vo)** and ventral **(va)** suckers, as well as destruction of its tegument; **f –** female worm with tegumental peeling on its body surface.

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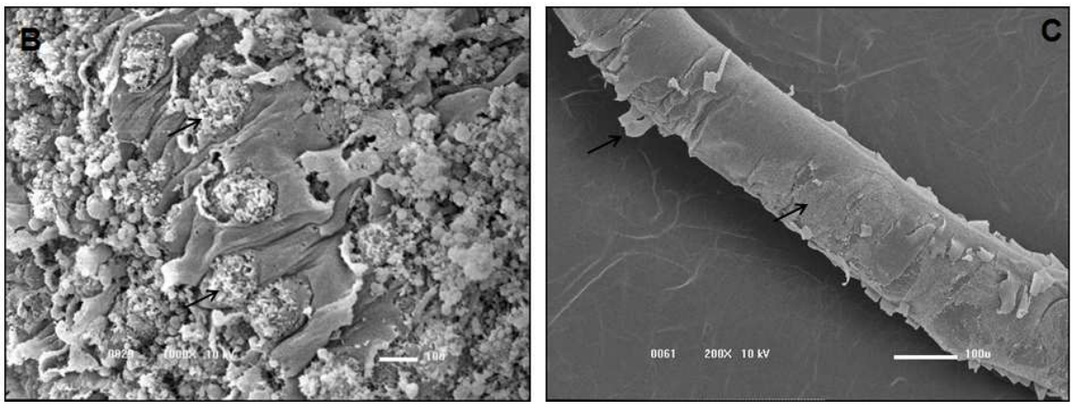

Figure 11. SEM of adult *S. mansoni* specimens after *in vitro* exposure to the hexane fraction obtained from the crude dichloromethane extract at the lethal concentration of 130 µg/mL, over an incubation period of 24 hours. **B.** Male worm, destruction of tubercles and thorns on its tegumental surface. **C.** Female worm with extensive tegumental peeling on its dorsal surface.

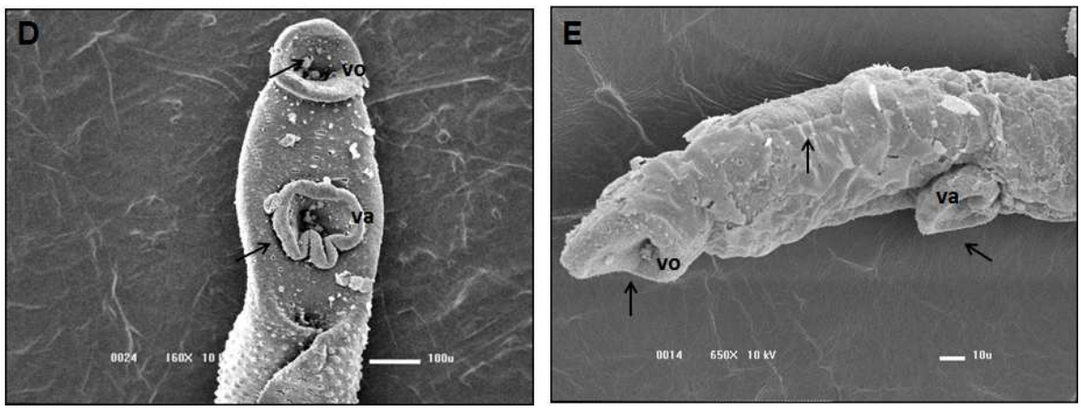

Figure 12. SEM of adult *S. mansoni* specimens after *in vitro* exposure to the hexane fraction obtained from the crude dichloromethane extract at the lethal concentration of 130 µg/mL, over an incubation period of 24 hours. **D.** Male worm with changes in the oral (**vo**) and ventral (**va**) suckers. **E.** Female worm with changes in the suckers and tegumental wrinkling and erosion.

- *Cordia verbenacea* DC

C. verbenacea (Figure 9-B), also referred to as *C. salicina, C. curassavica, C. cylindristachia, Lithocardium fresenii, L. salicinum and L. verbaceum*, is popularly known as *erva-baleeira* or salicina in Brazil. It belongs to the family Boraginaceae and is widely distributed along the Brazilian coast, being found mainly in the littoral zone extending from São Paulo to Santa Catarina [83]. Species of the genus *Cordia* are present in tropical and subtropical areas of Asia, Southern Africa, Australia, Guyana, and South America [84]. Several compounds are found in their aerial parts, including tanins, flavonoids, mucilage, and essential oils. Such parts, along with leaves and inflorescences, have been used in folk medicine in the form of infusions and alcohol extracts because of their antiulcer, antimicrobial and antirheumatic activities, and

tonic, analgesic and anti-inflammatory properties [83,85,86]. In view of the variety of chemical groups found in extracts of *C. verbenacea* and their alleged biological properties, that plant is an important material for pharmaceutical research [87].

C. verbenacea were used in *in vivo* assays with mice Balb/c (*Mus musculus*), infected with 70 cercariae of *S. mansoni* (BH strain) by tail immersion [98], and kept in an isolated environment. Forty-five days following infection, the animals were treated orally by esophageal intubation with 300 mg/kg, administered in a single dose, of fraction 3, obtained from the fractionation of the organic fraction, originated from the ethanol extract [99], with a specific concentration of the tested fraction. Fifteen days after treatment, the animals were euthanized by cervical dislocation. The worms were collected by perfusion of the hepatic portal system [100], and washed in 0.9% NaCl solution and subjected to the protocol shown in Figure 8 in order to be analyzed by scanning electron microscopy.

Fraction 3 of *C. verbenacea* caused an erosion on the tegument of both male and female worms. Formation of vesicles and adhesion of host's cells to the surface of worms (Figure 13) were also observed. No damages to the sucker were found.

- *Phyllanthus amarus*

The plants belonging to the genus *Phyllanthus* are widely distributed in most of the tropical and subtropical countries (in both hemispheres) and include between 550 and 750 species. It is believed that around 200 species of that genus are distributed in the Americas, chiefly in the Caribbean and in Brazil [88, 89]. In Brazil, the plants of that genus are popularly known as stone-breaker ("*quebra-pedra*" in Brazilian Portuguese) because they are recognized by their diuretic properties in Brazilian and other countries' folk medicine, being used in the treatment for kidney and bladder disorders. In addition to helping the elimination of kidney stones, they combat intestinal infections, diabetes and hepatitis B [88-90]. The interest in plants of the genus *Phyllanthus* has been considerably increasing, especially for the species *P. amarus* (family Euphorbiaceae) (Figure 9-C), which is scientifically one of the most studied, many of its compounds having already been isolated and chemically identified. *P. amarus* has a long history of usage in folk medicine because of its rich medicinal effects, being reported to possess potent hepatoprotective [91, 92], anti-inflammatory, analgesic [93-94], hypoglycaemic [95], antiplasmodial (against *Plasmodium berghei*) [96], and antioxidant [97] properties.

P. amarus were used in *in vivo* assays with mice Balb/c (*Mus musculus*), infected with 70 cercariae of *S. mansoni* (BH strain) by tail immersion [98], and kept in an isolated environment. Forty-five days following infection, the animals were treated orally by esophageal intubation [99], with the butanolic fraction 2 in the concentration of the 100 mg/kg for three days. Fifteen days after treatment, the animals were euthanized by cervical dislocation. The worms were collected by perfusion of the hepatic portal system [100], washed in 0.9% NaCl solution and subjected to the protocol shown in Figure 8 in order to be analyzed by scanning electron microscopy.

The butanolic fraction 2 of *P. amarus* caused damage to the male worms' tegument, including perforations, changes in the tubercles, peeling, and formation of vesicles and protuberances. Contraction and swelling were noticed in the region around the suckers (Figure 14). No damage were found in the tegument of females. This fraction did not cause the separa-

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tion of coupled worms, thus female worms remained in the gynaecophoric canal protected from the action of the butanolic fraction 2.

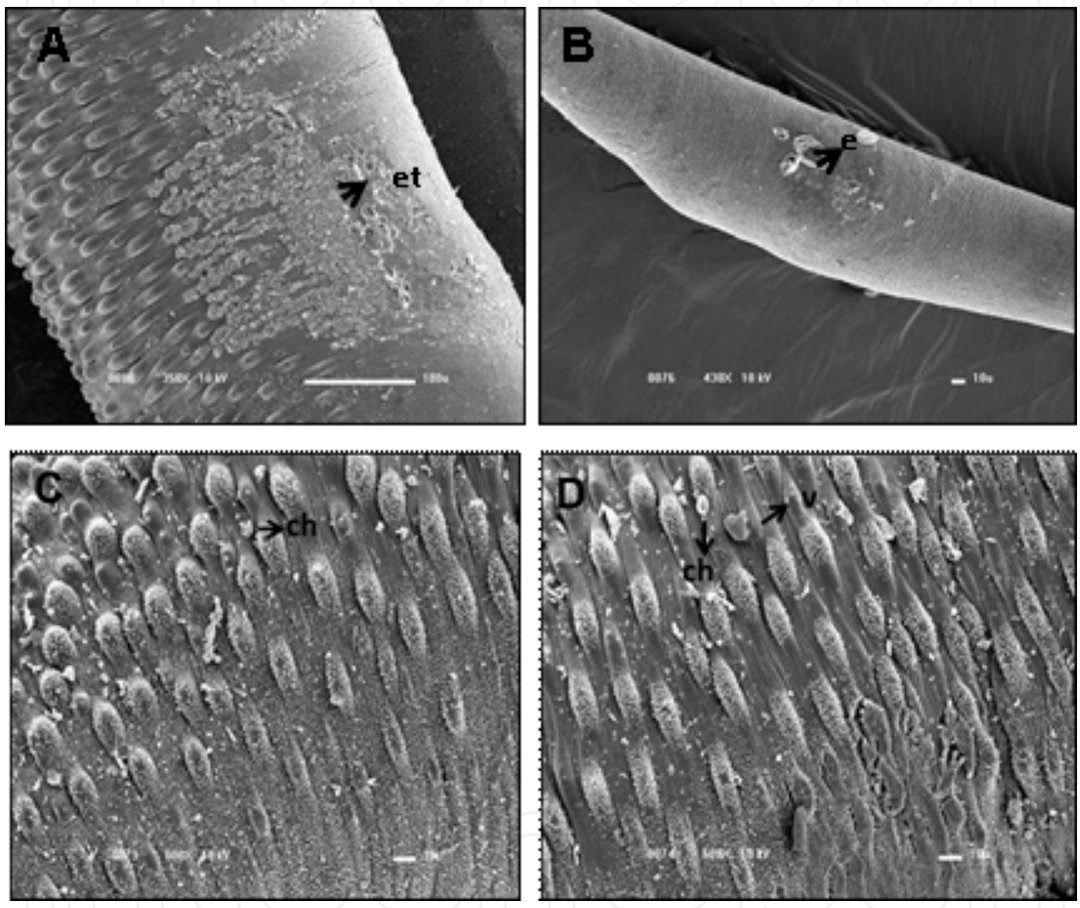

Figure 13. SEM of adult *S. mansoni* specimens after *in vivo* assay with fraction 3 obtained from the organic fraction of *C. verbenacea* at the concentration of 300 mg/kg. **A-B.** Male and female worms, respectively, showing peeling of the tegument. **C-D.** Male worms showing adhesion of host's cells to its tegument and formation of vesicles. **et** – erosion of the tegument; **ch** – host's cells adhered to the surface of *S. mansoni*; **l** – host's leukocytes; **dt** – destruction of the tegument; **v** – vesicle.

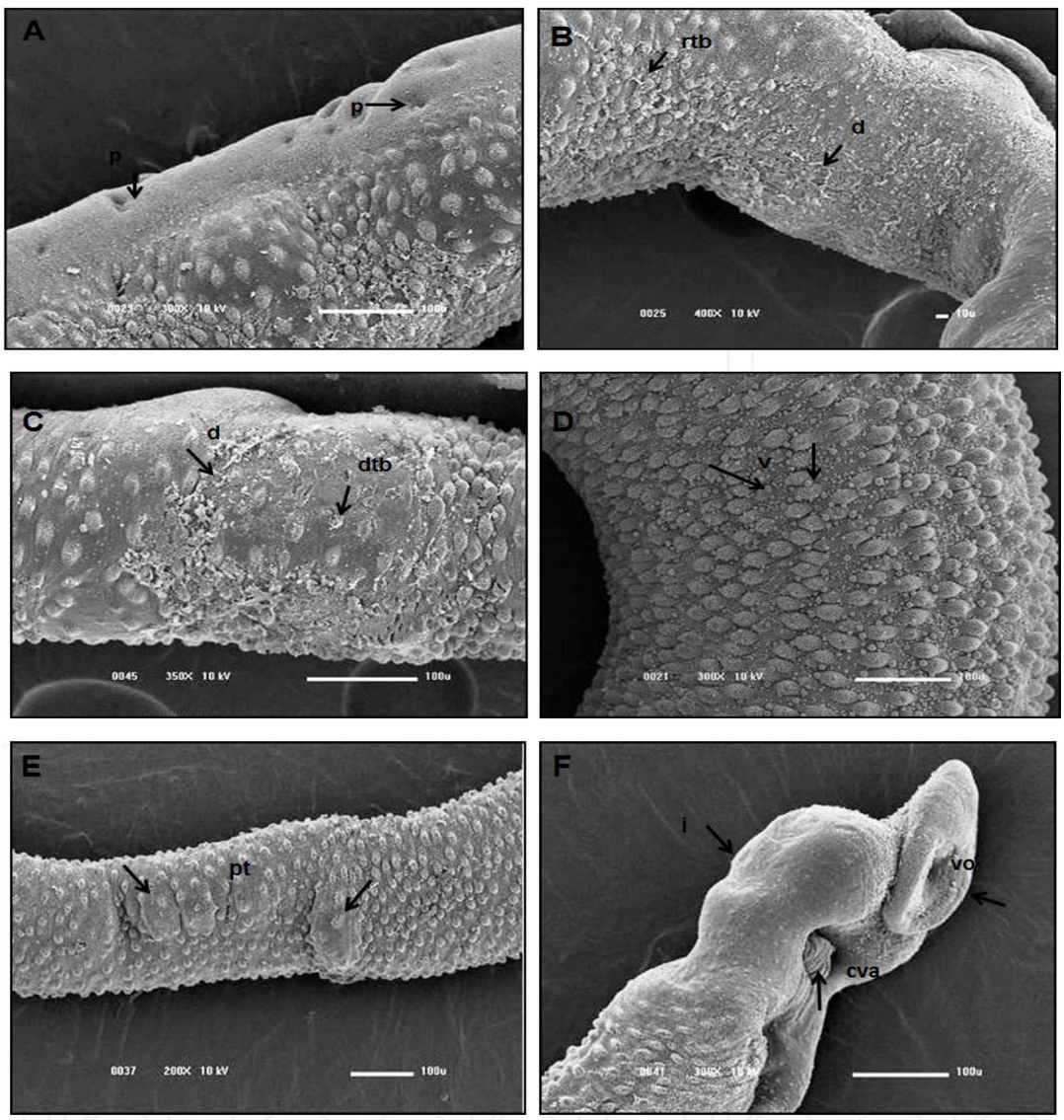

Figure 14. SEM of adult *S. mansoni* specimens after *in vivo* assay with butane fraction 2 from butane extract at the concentration of 100 mg/kg/3 consecutive days. **A.** Area of the gynaecophoric canal showing perforations (**p**). **B.** Rupture of tubercles (**rtb**) and peeling (**d**) on the tegument of a male worm. **C.** Peeling (**d**) and destruction of tubercles (**dtb**). **D.** Formation of several vesicles (**v**). **E.** Formation of protuberances (**pt**) on the tegument. **F.** Contraction of the ventral sucker (**cvv**) and swelling (**i**) of the region around the suckers.

6. Conclusions

- The combined use of 0.1 M sodium cacodylate buffer and 2.5% glutaraldehyde and the reduction in fixation time provided more distinct images with no artefacts, in contrary to the combination of Karnovsky solution with 0.1 M phosphate buffer.

- The tested fractions of *B. trimera, C. verbenacea* and *P. amarus* caused tegumental changes in adult *S. mansoni* specimens, including peeling or erosion of the surface membrane, formation of vesicles, destruction of tubercles, and modifications in the suckers. It is worth noting that such changes were more intense in male worms.
- Since the tegument of *S. mansoni* is a major chemotherapeutic target, we can infer that the fractions of *B. trimera, C. verbenacea* and *P. amarus* have a promising schistosomicidal activity, more studies being needed in order to isolate and identify their compounds active against the worm and to understand their mechanism of action on the tegument.

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