ULTRASTRUCTURAL CHANGES IN *Schistosoma mansoni* MALE WORMS AFTER *in vitro* INCUBATION WITH THE ESSENTIAL OIL OF *Mentha x villosa* Huds

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SUMMARY

Introduction: The essential oil *Mentha x villosa* (MVEO) has a wide range of actions, including antibacterial, antifungal, antiprotalzal and schistosomicidal actions. The present study aimed to investigate the ultrastructural changes of MVEO on the tegument of adult *Schistosoma mansoni*. Materials and Methods: Different concentrations of MVEO were tested on *S. mansoni* adult worms *in vitro*. Ultrastructural changes on the tegument of these adult worms were evaluated using scanning electron microscopy (SEM) and transmission electron microscopy (TEM). Results: The MVEO caused the death of all worms at 500 μg mL⁻¹ after 24 h. After 24 h of 500 μg mL⁻¹ MVEO treatment, bubble lesions were observed over the entire body of worms and they presented loss of tubercules in some regions of the ventral portion. In the evaluation by TEM, *S. mansoni* adult worms treated with MVEO, 500 μg mL⁻¹, presented changes in the tegument and vacuoles in the syncyntial matrix region. Glycogen granules close to the muscle fibers were visible. Conclusion: The ability of MVEO to cause extensive ultrastructural damage to *S. mansoni* adult worms correlates with its schistosomicidal effects and confirms earlier findings with *S. mansoni*.

KEYWORDS: Schistosomicidal activity; *Schistosoma mansoni*; *Mentha x villosa*.

INTRODUCTION

Schistosomiasis is a neglected disease widespread worldwide and poses a major public health problem. It is caused by parasitic trematode flatworms of the *Schistosoma* genus; moreover, *S. mansoni* is the only species found in Brazil².

The treatment of schistosomiasis is based on the use of praziquantel (PZQ); however, this drug seems ineffective against juvenile stages of *S. mansoni* and its extensive use in mass treatment of populations in schistosomiasis risk areas have favored the emergence of refractory strains of *S. mansoni* to conventional treatment with PZQ³.

Therefore, the search for new drugs that can act against *S. mansoni* becomes relevant, and tools such as scanning electron microscopy (SEM) and transmission electron microscopy (TEM) have been employed to study the effects of compounds on the tegument of many helminths, especially *S. mansoni*. In this context, the search for natural bioactive compounds against *S. mansoni* becomes an interesting alternative⁴.

*Mentha x villosa* Hudson (Lamiaceae) has been used in traditional medicine due to its antiparasitic activity. It is known popularly as “hortelã-rasteira”, “hortelã comum”, or “hortelã-da-folha-miúda”⁵. Giamebil⁶ is a commercial formulation presenting amebicidal (*Entamoeba histolytica*) and giardicidal (*Giardia lamblia*) activities, having as its active ingredient the dry extract from the leaves and stem of *M. x villosa*. Recent studies have also demonstrated the efficacy of *M. x villosa* against *Trichomonas vaginalis*.

Essential oils (EOs) and extracts of aromatic plants have been recognized for many years as a great source of pharmaceutical agents and food additives⁶. Some studies show different biological effects caused by the *M. x villosa* essential oil (MVEO): antimicrobial⁶, hypotensive and bradycardic⁷, cardiovascular⁸, larvicidal⁹, antinociceptive⁶, cytotoxic, antitumor ¹⁰ and schistosomicidal activities ¹¹.

Recent studies developed by our research group have demonstrated the *in vitro* schistosomicidal activity of MVEO¹². However, there are no studies showing ultrastructural changes in *S. mansoni* adult worms after incubation with MVEO.

The aim of this study was to evaluate the ultrastructural changes in *S. mansoni* male worms after *in vitro* incubation with MVEO; the results shown here are supported by TEM and SEM.

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MATERIALS AND METHODS

**Ethics statement:** All experiments involving the use of experimental animals were performed in accordance to the ethical standards of the Fundação Oswaldo Cruz and were approved by the Animal Experimentation Ethics Committee (No. 06/2010).

**Botanical material:** Fresh leaves of the species *M. x villosa* were used. They were gathered from the Medicinal Plants Garden of the Laboratório de Tecnologia Farmacêutica, Universidade Federal da Paraíba between April and June 2011. They were identified and authenticated by Dr. F. J. Abreu Matos (Laboratório de Produtos Naturais, Universidade Federal do Ceará) and Dr. Raymond Harley of the Royal Botanic Gardens, Kew, England. A voucher specimen was deposited in the Prisco Bezerra Herbarium of the Federal University of Ceará (N. 14996).

**Preparation of samples:** To extract MVEO, 10 kg of leaves were steam-distilled for 8 h. The oil obtained (0.1%) was dried over anhydrous sodium sulfate in the usual manner and stored at 4 °C. We used a gas chromatograph coupled to a mass spectrometer (Shimadzu QP-5000) under the following analytical conditions: capillary column, OV-5 (30m × 0.25 mm × 0.25 μm); injector (Ohio Valley Specialty Chemical, Inc.), 240 °C; detector, 230 °C; electron impact, 70 eV; gas drag, He; flow, 1.0 mL/min; split, 1/20; program temperature, 60 °C – 240 °C at 3 °C/min; and solution injection volume, 1 μL (1 μL of essential oil per 1 mL of ethyl acetate). The compounds were identified by comparing their mass spectra using the GC-MS database system (Nist 62 lib.) and the Kovats retention index. The compounds were dissolved in 100% dimethyl sulfoxide (DMSO)\(^\text{19}\).

Praziquantel was commercially available through Sigma-Aldrich (Sigma chemical, St Louis, MO, USA) with purity of 99.9%.

**Obtaining and maintenance of *S. mansoni* adult worms:** The BH *S. mansoni* strain (Belo Horizonte, Minas Gerais, Brazil) was used throughout this study. This strain was maintained in * Biomphalaria glabrata* snails and Swiss Webster mice in a laboratory at the Centro de Pesquisas Aggeu Magalhães of Fundação Oswaldo Cruz. Female Swiss Webster mice weighing 20 ± 5 grams were used as the definitive host, and were infected transectaneously with about 120 cercariae of the BH strain, as previously described\(^\text{14}\), using the tail immersion technique. The animals were exposed for 1 h to the cercariae and they were subsequently kept under controlled temperature and light conditions. Furthermore, they had access to food and water ad libitum\(^\text{19}\).

After fifty-five days of infection, *S. mansoni* adult worms were recovered from the mice by perfusion, washed in RPMI 1640 medium buffered with HEPES (20 mM), pH 7.5, supplemented with penicillin (100 IU mL\(^{-1}\)), streptomycin (100 μg mL\(^{-1}\)), and 10% fetal bovine serum (Gibco), and placed in petri dishes containing 2 mL of sterile culture medium\(^\text{20}\).

**In vitro studies of *S. mansoni* adult worms:** To assess the damage to the tegument, adult worms of *S. mansoni* were recovered from the hepatic portal system of the infected mice and left for a period of 2 h to adapt to the culture medium. MVEO isolate and compound was added in varying concentrations: a) MVEO (5, 10, 100, 250, and 500 μg mL\(^{-1}\)). Then, the worms were incubated at 37 °C in an atmosphere containing 5% CO\(_2\)\(^\text{18}\).

As controls, *S. mansoni* adult worms were incubated in the presence of 1.6% DMSO in RPMI 1640 (negative control) or exposed to 0.5 μg mL\(^{-1}\) PZQ (positive control). All experiments were performed with three replicates. The final volume in each well was 2 mL. The parasites were collected and monitored for routine processing with SEM and TEM at 24, 48, 72, 96, and 120 h intervals. The worms were considered dead when there was no motion detected after 3 minutes of observation. SEM and TEM were used as tools to evaluate the morphological changes in *S. mansoni* adult worms after *in vitro* exposure.

**Transmission Electron Microscopy (TEM):** *S. mansoni* adult worms in each group were fixed (2.5% glutaraldehyde in sodium cacodylate buffer 0.1 M, pH 7.4). After fixation, they were washed with sodium cacodylate buffer 0.1 M, pH 7.4, and postfixed with 1% osmium tetroxide (OsO\(_4\)), in the same buffer, for 2 h in the dark. Then, samples were washed, counterstained block with 5% uranyl acetate in water. Dehydration was performed in a series of increasing acetone (30, 50, 70, 90 and 3 x 100%) for 30 min, each at room temperature and followed by embedding in Embed 812/Araldite resin (Electron Microscopy Sciences, Hartfield, PA) at 70 °C for 48 h. Semi-thin sections were stained with toluidine blue for morphological observation, while ultrathin sections were subsequently contrasted in uranyl acetate for 1 h and lead citrate for 10 min, and observations done in TEM (TEM 100CXII JEOL).

**Scanning Electron Microscopy (SEM):** The worms were incubated for 24 h and, after their death, they were washed with sodium cacodylate buffer (pH = 7.2), fixed with 2.5% glutaraldehyde (pH = 7.4) during 24 h, and then fixed with 1% osmium tetroxide for 1 h. The samples were dehydrated by an increasing amount of ethanol solution, dried in a critical point dryer, and then mounted on stubs and coated with gold using a sputter coater. The material was examined under a JEOL - 5600 LV microscope.

**RESULTS AND DISCUSSION**

The tegument of *S. mansoni* is an important structure for its survival since it is involved in nutrient absorption, secretion of metabolites, osmotic balance, and parasitic defense against the host immune system; this structure is an important target for drug action\(^\text{21}\). Some studies have documented damages to the tegument of *S. mansoni* caused by synthetic\(^\text{22},\text{24}\) and natural\(^\text{25},\text{26}\) antischistosomal compounds.

Ultrastructural analysis was performed on male worms for two reasons: females are frequently in contact with the host microenvironment and studies in the literature have shown that soft tissue alterations are more pronounced in males than in female worms\(^\text{27}\).

**Ultrastructural analysis of MVEO-induced surface damage in *S. mansoni***

**Scanning Electron Microscopy (SEM):** Control groups were not affected for up to 5 days of observation and all worms exhibited vigorous activity. It can be seen that male worms of *S. mansoni* in the control group presented the tegument covered with tubercules and tiny projections (spines). The back was long and contained the gynecophoral canal (gc). The area between the oral and ventral suckers did not have...
any tubercles (tu), spines (sp) or sensory papillae (Fig. 1A-1B). The presence of a large number of tubercles with typical spines (Fig. 1C) as well as sensory papillae (st) (Fig. 1D) was observed.

In assessing the viability of the worms treated with PZQ, it was observed the death of all worms after 24h of incubation. Using SEM, it was identified that the S. mansoni adult worms treated with PZQ (0.5 μg mL⁻¹) showed spiraled body (Fig. 1E). In the tegument there was destruction of tubercules and spines, and many regions with ulceration (Fig. 1F).

After 24 h of MVEO (500 μg mL⁻¹) treatment, bubble lesions were spread over the entire body of the worms (Fig. 2A), and the worms showed loss of tubercules in some regions of the ventral portion (Fig. 2B). After 48 h of incubation at 250 μg mL⁻¹, death of worms was observed, destroyed the oral sucker had been destroyed and the ventral sucker contracted (Fig. 2C). Tegument lesion severity increased after 72 h of MVEO treatment (100 μg mL⁻¹), which caused the basal membrane to become unprotected (Fig. 2D). Lower concentrations (5 and 10 μg mL⁻¹) were unable to cause mortality of S. mansoni adult worms after 120 h of exposure; however, changes in the tegument of the worms were recorded. At a concentration of 10 μg mL⁻¹, tegument erosion can be visualized at higher magnification (Fig. 2E) and in the worms treated with 5 μg mL⁻¹ there was destruction of some tubercules (Fig. 2F).

Transmission Electron Microscopy (TEM): The ultrastructural evaluation of S. mansoni adult worms by TEM revealed the presence of spines, characteristical matrix syncytial, and circular and longitudinal muscles in the subtegumentary region of the worms (Fig. 3A). The TEM analysis of S. mansoni adult worms treated with PZQ showed many changes like vacuoles in tuber, presence of vesicles in the syncytial matrix, and mesenchymal vacuolization (Fig. 3B). In the evaluation by TEM, the S. mansoni adult worms treated with MVEO (500 μg mL⁻¹) presented changes in the tegument and presence...
of vacuoles in the syncytial matrix region. It was visible the presence of glycogen granules close to the muscle fibers (Fig. 4). Essential oils are highly enriched with compounds termed terpenoids that possess several biological properties such as schistosomicidal activity\textsuperscript{21-25,26-30}. In recent years, a number of studies have been developed through in vitro screening using essential oils, extracts, and bioactive compounds from medicinal plants\textsuperscript{27,29-32} to identify a leading substance that can be used in preclinical trials for the treatment of experimental schistosomiasis\textsuperscript{31-36}. These findings were identified by LORSUWANNARAT et al.\textsuperscript{37} when testing plumbagin (100 μg mL\textsuperscript{-1}) in \textit{S. mansoni} adult worms. EISSA et al.\textsuperscript{38} paid attention to this same finding when evaluating the effect of miltefosine (10 μg mL\textsuperscript{-1}) on \textit{S. mansoni} adult worms; however, the authors made observations after 120 h of incubation. LIMA et al.\textsuperscript{39}, when assessing the effects of allicin on the tegument of \textit{S. mansoni}, describe the occurrence of ulceration on the parasite tegument after 120 minutes of incubation with 20 μg mL\textsuperscript{-1} of MVEO. Previously, BERTÃO et al.\textsuperscript{40} also evaluated the effects of miltefosine on \textit{S. mansoni} adult worms by testing 200 μM and found the same results; however, they used only 12 h of incubation.

In the present study, after 48 h of incubation with 250 μg mL\textsuperscript{-1} of MVEO, morphological changes in the oral sucker and ventral suckers of \textit{S. mansoni} adult worms were observed. Comparable to our findings, ALBUQUERQUE et al.\textsuperscript{22} describe similar changes by treating \textit{S. mansoni} with a thioxo-imidazolidine, determined that after less than 1 h, adult \textit{S. mansoni} worms incubated with 20 μg mL\textsuperscript{-1} of MVEO showed damaged tegument and exposed musculature in some worms. Generally, there was a marked difference between the morphology of worms treated with PZQ compared with MVEO, and compounds used individually. In the macroscopic examination, the \textit{S. mansoni} adult worms, when exposed to PZQ, presented muscle contractions causing them to stay retracted or twisted. However, this behavior was not observed in the worms treated with MVEO or their constituents.

The adult worms incubated for 24 h with MVEO (500 μg mL\textsuperscript{-1}) showed damaged tegument and exposed musculature in some worms. Generally, there was a marked difference between the morphology of worms treated with PZQ compared with MVEO, and compounds used individually. In the macroscopic examination, the \textit{S. mansoni} adult worms, when exposed to PZQ, presented muscle contractions causing them to stay retracted or twisted. However, this behavior was not observed in the worms treated with MVEO or their constituents.

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CONCLUSIONS

The ability of MVEO to cause extensive ultrastructural damage to
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