

## Major Article

# Ultrastructural study of morphological changes in *Schistosoma mansoni* after *in vitro* exposure to the monoterpene rotundifolone

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### Abstract

**Introduction:** Schistosomiasis, a parasitic disease caused by trematode flatworms of the genus *Schistosoma*, affects more than 200 million people worldwide, and its control is dependent on a single drug, praziquantel. Here, we report the *in vitro* effect of rotundifolone, a monoterpene isolated from *Mentha x villosa* (Lamiaceae), on *Schistosoma mansoni* adult worms. **Methods:** The *in vitro* effect of rotundifolone on adult *Schistosoma mansoni* was evaluated by analysis of behavior and mortality and through a scanning electron microscopic analysis of ultrastructural changes in the tegument of the worms. **Results:** At concentrations of 3.54 and 7.09 µg/mL<sup>-1</sup> rotundifolone, no worm mortality was observed at any of the sampling intervals. A minor reduction in movement of the tail, suckers, and gynecophoral canal membrane was observed after 96 h of exposure to 7.09 µg/mL<sup>-1</sup> rotundifolone. At 70.96 µg/mL<sup>-1</sup>, a lack of movement was observed from 72h onwards and all worms were deemed dead; similar effects were observed at 48h with 177.4 µg/mL<sup>-1</sup>, and at 24h with 354.8 µg/mL<sup>-1</sup> and 700.96 µg/mL<sup>-1</sup>. Rotundifolone also caused death of all parasites and separation of coupled pairs into individual males and females after 24h at 354.8 µg/mL<sup>-1</sup>. **Conclusions:** The main changes in the tegument induced by the different ROT treatments were: after 24h incubation, bubble lesions spread over the entire body and loss of tubercles occurred in some regions of the ventral region.

**Keywords:** Schistosomicidal activity. *Schistosoma mansoni*. *Mentha x villosa*. Rotundifolone.

### INTRODUCTION

Schistosomiasis is a parasitic disease caused by trematode flatworms of the genus *Schistosoma* that is common in many tropical countries and affects more than 207 million people living in conditions of poor sanitation and/or with less developed social infrastructure<sup>1</sup>. Chemotherapy is the only immediate recourse to minimize the prevalence and incidence of this disease worldwide<sup>2</sup>. At present, praziquantel (PZQ) is the drug of choice for the treatment of all forms of schistosomiasis<sup>3</sup>. However, dependence on a single drug is a concern because of the possible development of resistance in some strains<sup>4,5</sup>. A second drug, oxamniquine (OXA), acts against *Schistosoma mansoni*, which is found in Africa, and against *Schistosoma haematobium* in South America, where it is the only species

of this genus present. OXA is manufactured in Brazil by Pfizer<sup>6</sup>. OXA has a complicated manufacturing process, with large fermentation tanks required for its biological synthesis, resulting in a higher cost than that required for PZQ synthesis. Due to this difficulty, PZQ is generally the only drug utilized at the moment<sup>7</sup>.

Praziquantel is a pirazyl isoquinoline and is effective against all *Schistosoma* species infecting humans<sup>8</sup>; it has been successfully used over the last 20 years as the drug of choice in most areas where the disease is endemic<sup>9-11</sup>. Although PZQ has several advantages, including tolerability, safety, efficacy, and low cost, it does not protect individuals from reinfection and is not active against the immature stages of the worm, such as the schistosomula, pre-adult and juvenile adult stages<sup>12</sup>. Therefore, there is an urgent need to identify new drugs that can combat *S. mansoni*.

Investigative methods such as scanning electron microscopy (SEM) have been employed to study the effects of compounds on the tegument of many helminthes<sup>13</sup>, especially *S. mansoni*<sup>14</sup>. In this context, the search for natural bioactive compounds against

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*S. mansoni* offers a potentially valuable approach<sup>15,16</sup>. Members of the plant family Lamiaceae have been widely studied as a source of secondary metabolites with biological activity<sup>17,18</sup>. Among the species of this family, *Mentha x villosa* Hudson has been used in traditional medicine owing to its antiparasitic properties. It is known popularly as *hortelã-rasteira*, *hortelã comum*, or *hortelã-da-folha-miúda*<sup>19</sup>. A commercial formulation named Giamebil®, which has as its active compound the dry extract from the leaves and stem of *M. x villosa*, has been shown to have amebicidal (*Entamoeba histolytica*) and giardicidal (*Giardia lamblia*) activities<sup>20</sup>. Recent studies have also demonstrated the efficacy of extract from *M. x villosa* against *Trichomonas vaginalis* infection<sup>21</sup>.

Rotundifolone (ROT) is a monoterpene that is present in large amounts in the leaves of *M. x villosa*. This study had greater impact to ROT, because it represents more than 50% (can reach 70%) of the total essential oil from *Mentha x villosa* (MVEO) reported previously<sup>17</sup>. It has the chemical formula C<sub>10</sub>H<sub>14</sub>O<sub>2</sub> (**Figure 1**) and a molecular weight of 166. ROT is an important chemical constituent of the essential oil of many *Mentha* species (*M. rotundifolia*, *M. suaveolens*, *M. spicata*, *M. longifolia*, and *M. x villosa*)<sup>21</sup>. It has been reported to have hypotensive and bradycardic<sup>22</sup>, antimicrobial<sup>23</sup>, spasmolytic<sup>24</sup>, larvicidal<sup>25</sup>, antitumour effects<sup>26</sup> and schistosomicidal activity on adult *S. mansoni*<sup>27</sup>.

Considerable efforts are underway to develop novel drugs for prevention and cure of schistosomiasis. Recent studies by our research group have demonstrated that ROT has *in vitro* schistosomicidal activity<sup>17</sup>. However, there are no studies on the possible ultrastructural changes in adult *S. mansoni* that are induced by treatment with ROT.

## METHODS

### Ethical considerations

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

### Botanical material

Fresh leaves of *M. x villosa* were collected from the Medicinal Plants Garden of the Pharmaceutical Technology Laboratory, Federal University of Paraíba (LTF/UFPB) between April and June 2011. The plants were identified and authenticated by Dr. F. J. Abreu Matos (Laboratory of Natural Products, Federal University of Ceará) and by 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).

### Isolation and identification of rotundifolone

To extract MVEO, 10 kg of leaves were steam-distilled for 8h. The oil obtained (0.1%) was dried over anhydrous sodium sulfate in the usual manner and stored at 4°C<sup>13</sup>. We used a gas chromatograph coupled to a mass spectrometer (Shimadzu QP-5000) under the following analytical conditions: capillary

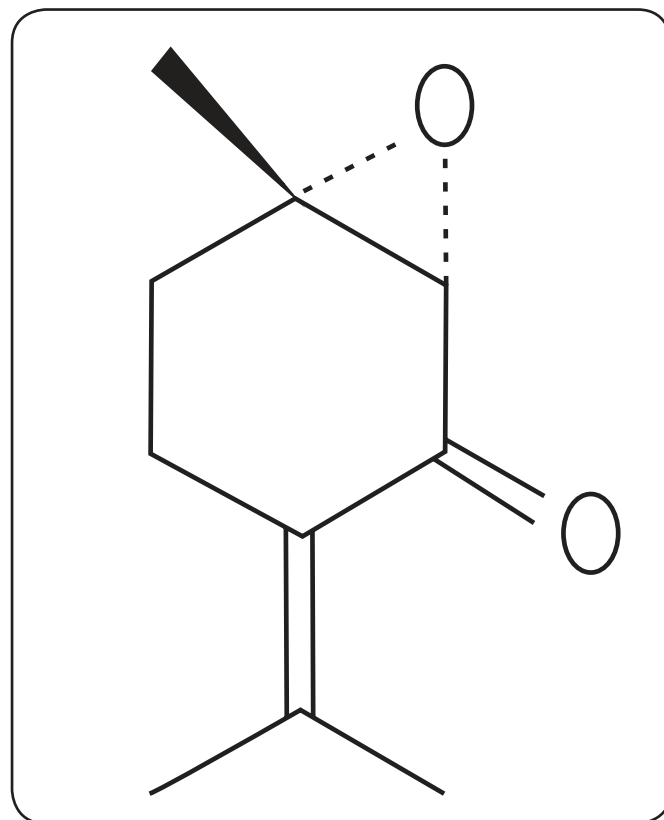


FIGURE 1 - Chemical structure of rotundifolone.

column, OV-5 (30m × 0.25mm × 0.25µm); injector (Ohio Valley Specialty Chemical, Inc.), 240°C; detector, 230°C; electron impact, 70 eV; gasdrag, He; flow, 1.0mL/min<sup>-1</sup>; split, 1/20; program temperature, 60-240°C at 3°C min<sup>-1</sup>; and solution injection volume, 1µL (1µL of essential oil per 1mL of ethyl acetate). The compounds were identified by comparing their mass spectra using the Gas Chromatography Mass Spectrometry (GC-MS) database system (Nist 62 lib.) and the Kovats retention index.

The oil was subjected to preparative layer chromatography (Merck silica gel PF254 plates, 40 × 20cm). The plates were eluted three times using hexane as the solvent. Pure ROT (**Figure 1**) was thereby obtained from *M. x villosa* leaves. The chemical purity of ROT (more than 99.9%) was determined by high-performance liquid chromatography.

### Maintenance and collection of adult *Schistosoma mansoni*

*Schistosoma mansoni* strain BH (Belo Horizonte, Minas Gerais, Brazil) was used in this study. The parasites were maintained in *Biomphalaria glabrata* snails and Swiss strain mice in the laboratory at the Aggeu Magalhães Research Center of the Oswaldo Cruz Foundation. Female Swiss mice weighing 20 ± 5g were used as the definitive host and infected transcutaneously with about 120 cercariae (this is the number of cercariae needed to ensure infection of a single mouse) using

the tail immersion technique. The animals were kept under controlled temperature and light conditions and had ad libitum access to food and water.

At 55 days after infection, adult *S. mansoni* were recovered from the hepatic portal system of infected mice and left for 2 h to adapt to the culture medium, washed in RPMI 1640 medium buffered with HEPES (20mM), pH 7.5, supplemented with penicillin (100IU/ml<sup>-1</sup>), streptomycin (100µg/ml<sup>-1</sup>) and 10% fetal bovine serum (Gibco), and placed in petri dishes containing 2mL of sterile culture medium<sup>17</sup>.

### *In vitro* antischistosomal assay

The *in vitro* assays of antischistosomal treatments were carried out using ROT dissolved in 100% DMSO. Five worms were placed into each well and incubated at 37°C in an atmosphere containing 5% CO<sub>2</sub>. After 2h to adapt to the culture medium, MVEO and ROT (3.5, 7.0, 71, 177.4, and 354.8µg/mL<sup>-1</sup>) were added. As a negative control, adult worms were incubated in RPMI 1640 and RPMI 1640 + 1.6% DMSO. As a positive control, worms were incubated in 0.5µg/mL<sup>-1</sup> PZQ. The worms were monitored every 24h until 120h and changes in motor activity were assessed and the mortality rate was determined<sup>27</sup>. The evaluation of the viability of adult worms was based on the standard procedures for screening schistosomicidal compounds established by the World Health Organization (WHO) Special Program for Research and Training in Tropical Diseases. In this evaluation system, (+++) indicates normal activity, (++) slight loss of movement, with active tail, suckers, and gynecophoral canal membrane, (+) indicates movement of tails and suckers alone, and (-) indicates no movement. The worms were considered to be dead when no movement was identified after 3 min of observation using an inverted microscope<sup>27</sup>.

### Scanning electron microscopy

Scanning electron microscopy was used to evaluate morphological changes in *S. mansoni* worms after the *in vitro* treatments. Worms were incubated for 24h and after their death naturally they were washed in sodium cacodylate buffer (pH = 7.2), fixed in 2.5% glutaraldehyde (pH = 7.4) for 24h, and then fixed in 1% osmium tetroxide for 1h. The samples were dehydrated through an ethanol series, dried in a critical point dryer, and then mounted on stubs and coated with gold using a sputter coater. The specimens were examined using a JEOL-5600LV microscope<sup>27</sup>.

## RESULTS

### *In vitro* effects of rotundifolone on *Schistosoma mansoni* adult worms

At ROT concentrations of 3.5 and 7.0µg mL<sup>-1</sup>, no worm mortality was observed during the course of the experiment. A minor loss of movement of the tail, suckers, and gynecophoral canal membrane was observed after 96h exposure to 7µg/mL<sup>-1</sup> ROT. Worms in the negative control groups (RPMI 1640 and RPMI 1640 + DMSO 1.6%) remained viable throughout the analysis period. In the positive control with PZQ, all parasites

died within 24h. At 71µg mL<sup>-1</sup> ROT, mortality and absence of movement were observed from 72h; similar effects were apparent from 48h exposure to 177.4µg/mL<sup>-1</sup> ROT, and from 24h exposure to 354.8µg mL<sup>-1</sup> and 700.96µg mL<sup>-1</sup> ROT (Table 1).

### Ultrastructural analysis of rotundifolone-induced morphological changes in *Schistosoma mansoni*

Adult worms in the control groups showed no changes in behavior up to 120 h and remained vigorous during this time. Male worms in the control groups had a tegument that was covered with tubercles and tiny projections (spines). The back was long and contained the gynecophoral canal. The area between the oral and ventral suckers did not have any tubercles, spines, or sensory papillae (Figure 2A and Figure 2B). A large number of tubercles with typical spines (Figure 2C) as well as sensory papillae (Figure 2D) was observed.

After treatment with PZQ, all worms were dead by 24h of incubation. Under SEM, adult worms treated with 0.5µg/mL<sup>-1</sup> PZQ showed a spiral-shaped body (Figure 2E). The tegument

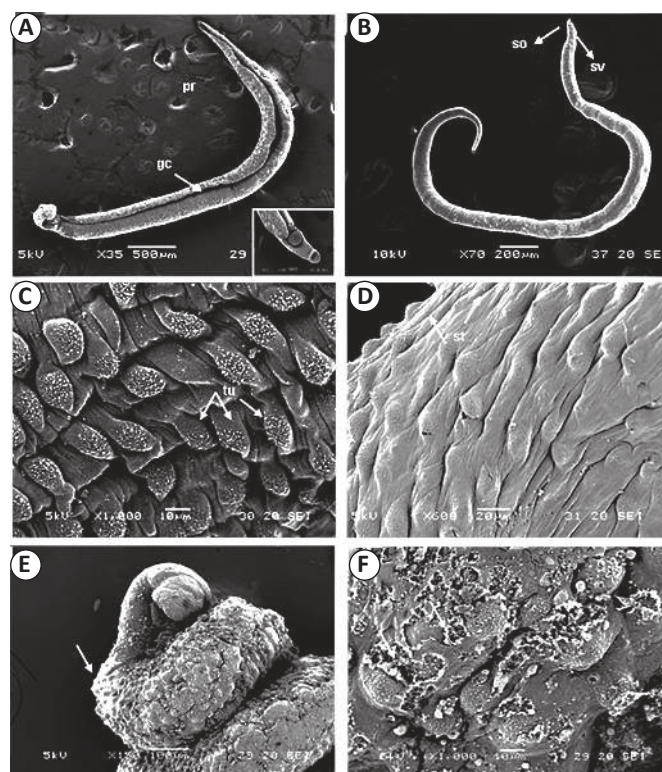


FIGURE 2 - (A-F) Micrographs of adult *Schistosoma mansoni* worms. (A-B) Micrographs of untreated adult worms. The micrograph of an untreated male shows the gynecophoral canal (gc) and the thinner posterior region (pr). (B) The anterior region of a female *Schistosoma mansoni* with an oral sucker (os) and ventral sucker (sv). (C) Tubercles (tu) with spines are present on the tegument of a male. (D) Grooves (arrows) are present in the testa (st). (E-F) Micrographs of adult worms treated with PZQ (0.5µg/mL<sup>-1</sup>): (E) Worm showing a twisted body shape (arrow) and extensive destruction of the tegument. (F) Worm showing severe damage to the seed coat, with loss of spicules and extensive ulceration of the tegument (arrows). PZQ: praziquantel.

**TABLE 1**  
*In vitro* effect of different concentrations of rotundifolone on mortality of adult *Schistosoma mansoni*.

Groups	Incubation period (h)	Number of dead worms (%)	Changes in motor activity
Control	120	-	+++
DMSO 1%	120	-	+++
PZQ	24	100.0	-
0.7µg mL <sup>-1</sup>	120	-	+++
3.54µg/mL <sup>-1</sup>	120	-	+++
7.09µg/mL <sup>-1</sup>	96	-	++
70.96µg/mL <sup>-1</sup>	72	100.0	-
177.4µg/mL <sup>-1</sup>	48	100.0	-
354.8µg/mL <sup>-1</sup>	24	100.0	-
700.96µg/mL <sup>-1</sup>	24	100.0	-

Negative control (RPMI 1640; DMSO + RPMI 1640). Positive control with 0.5µg/mL<sup>-1</sup> PZQ. Rotundifolone: +++ normal activity; ++ slight loss of movement, with activity of the tail, suckers, and gynecophoral canal membrane. **RPMI 1640**: Roswell Park Memorial Institute; **DMSO**: dimethyl sulfoxide; **PZQ**: praziquantel.

showed loss of tubercles and spines and many regions were ulcerated (**Figure 2F**).

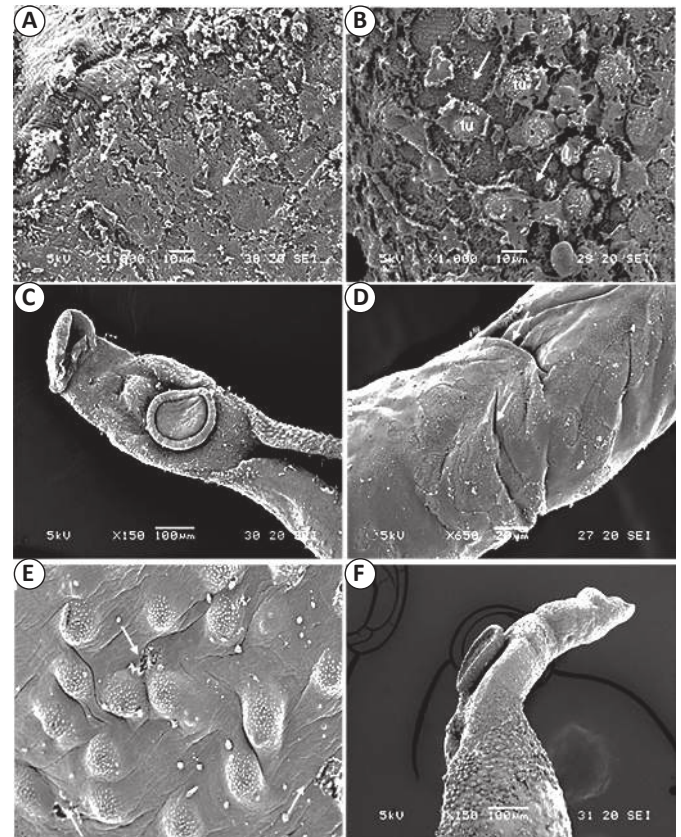
The main changes in the tegument induced by the different ROT treatments were: after 24h incubation at 354.8µg/mL<sup>-1</sup> ROT, bubble lesions spread over the entire body (**Figure 3A**) and loss of tubercles occurred in some regions of the ventral region (**Figure 3B**); after 48h incubation at 177.4µg/mL<sup>-1</sup> ROT, increased rate of death of worms, which showed destruction of the oral sucker and contraction of the ventral sucker (**Figure 3C**). The severity of the lesion in the tegument lesion increased after 72h of ROT treatment (71µg/mL<sup>-1</sup>) ( **Figure 3D**). The lowest ROT concentrations (3.54 and 7.09µg/mL<sup>-1</sup>) did not cause mortality of adult worms after 120h of exposure; however, changes in the tegument of the worms were observed. At 7.09µg/mL<sup>-1</sup> ROT, damage to the tegument was seen at higher magnifications (**Figure 3E**) and in the worms treated with 3.54µg/mL<sup>-1</sup> there was destruction of some tubercles (**Figure 3F**).

**DISCUSSION**

The tegument of *S. mansoni* is an important structure that is involved in both absorption of nutrients and secretion of some products; it also has a role in protecting the organism from the immune response of the host. Therefore, the tegument is a critically important target for antischistosomal drugs<sup>28</sup>. Electron microscopic analyses, including TEM, have documented damage to the tegument of *S. mansoni* caused by exposure to synthetic and natural compounds<sup>14</sup> for various antischistosomal prototype compounds<sup>29</sup>.

Recently, *in vitro* studies for new active compounds against *S. mansoni*<sup>14-16</sup> showed that ROT, a monoterpene found in *Mentha* species, showed promising antischistosomal properties against adult schistosomes<sup>17,27</sup>.

Matos-Rocha et al.<sup>17</sup> determined the composition of MVEO and evaluated its *in vitro* effects on the viability of adult *S. mansoni*. The main constituents of MVEO were identified as ROT (70.96%), limonene (8.75%), trans-caryophyllene (1.46%), and β-pinene (0.81%). In viability tests, neither trans-



**FIGURE 3 - (A-F)** Micrographs of adult *Schistosoma mansoni* treated with different concentrations of ROT. (A) 24h incubation with 700.96µg/mL<sup>-1</sup>, ROT induced disarray in the tegument (arrows); it was not possible to identify tubercles and spikes. (B) Adult *Schistosoma mansoni* after a 24-h incubation with 354.8µg/mL<sup>-1</sup> ROT show minor damage compared to the highest concentration, and it was possible to identify tubercles (tu); between the tubercles, note sloughing of the tegument (arrow). (C) Anterior region of an adult worm after 48h incubation with 177.4µg/mL<sup>-1</sup> ROT. There is clear damage to the oral sucker oral (so) and ventral sucker (sv). (D) After 72h exposure to 71µg/mL<sup>-1</sup> ROT, the worm shows a flattened appearance and has a twisted tegument (arrow) and superficial ulceration (asterisk). (E) Adult worms after 96h in 7.1µg/mL<sup>-1</sup> ROT. Vesicles have formed (arrow). (F) After 120h of incubation with 3.5µg/mL<sup>-1</sup> ROT, no tubercles can be identified. ROT: rotundifolone.

caryophyllene or  $\beta$ -pinene had anti-schistosomicidal activities. Rather, the schistosomicidal activity of MVEO appeared to be due to ROT, its major constituent. When analyzed alone, ROT showed similar activity to MVEO. To date, the possible effects of ROT on the ultrastructure of *S. mansoni* has not been examined.

The results of this study show that all tested concentrations, ROT was able to cause damage to the tegument of *S. mansoni*. These findings corroborate the report of Lima et al.<sup>14</sup> in which they investigated the characteristics of the tegument of adult worms obtained from mice infected with cercariae of *S. mansoni* and subjected to treatment with allicin at a concentration of 20mg/mL<sup>-1</sup>. Lima et al.<sup>14</sup> found a variety of structural changes to the tegument of the worms, including destruction of tubercles, and induction of edema and ulcers. In addition, there were changes to the number and volume of thorns and the tubercles showed a modified appearance. In the present study, there was a marked difference in the morphology of worms treated with PZQ compared with ROT. Macroscopic examination of adult *S. mansoni* exposed to PZQ found that they showed muscle contractions that caused them to stay in a retracted or twisted shape. This behavior was not observed in worms treated with MVEO and its constituents.

Mendonça et al.<sup>30</sup> compared the *in vitro* anthelmintic effect of PZQ against recent *S. mansoni* isolates by estimating relative motility, survival indices, and tegumental surface alterations. They found that the damage caused by PZQ on the integument of adult worms was dose-dependent, and increased with increasing PZQ concentrations.

The mechanism by which ROT exerts its *in vitro* effect on *S. mansoni* is not clear. It has been suggested that, because of the large number of constituents, essential oils may have no specific cellular target<sup>31</sup>. Additionally, as the lipophilic compounds present in essential oils can pass through the cell wall, integument, and cytoplasmic membrane, they may damage the structure of the cellular membranes during their passage, which may cause cellular lysis<sup>32,33</sup>. Regarding their biological properties, some of the activities of essential oils are associated with loss of ions and reduction of membrane potential, as well as collapse of the proton pump and depletion of the ATP pool<sup>34,35</sup>.

Our analyses here demonstrate the effectiveness of ROT as a candidate compound with significant *in vitro* effects against *S. mansoni* adult worms. This monoterpene was able to cause morphological alterations in the tegument of adult schistosomes. Overall, these results are important as there is an urgent need to develop new agents against schistosomes. Because ROT is generally recognized as safe, this compound may have broad antiparasitic applications. We recommend further analysis to better understand the *in vivo* efficacy of this new potential treatment for schistosomiasis.

#### Conflict of interest

The authors declare that there is no conflict of interest.

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