International Journal of Antimicrobial Agents 50 (2017) 467–472

Contents lists available at ScienceDirect

International Journal of Antimicrobial Agents

journal homepage: www.elsevier.com/locate/ijantimicag

Antiparasitic activity of nerolidol in a mouse model of schistosomiasis

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ARTICLE INFO

Article history:
Received 29 March 2017
Accepted 17 June 2017

Keywords:
Schistosoma
Schistosomiasis
Antischistosomal compound
Nerolidol
Natural product

ABSTRACT

Schistosomiasis is a major public health problem worldwide, especially in poor communities. Since praziquantel is currently the only drug available to treat schistosomiasis, there is an urgent need to identify new antischistosomal drugs. Nerolidol is a sesquiterpene present as an essential oil in several plants that has been approved by the FDA. This study evaluated the in vivo antischistosomal activity of nerolidol in a mouse model of schistosomiasis infected with either adult or juvenile stages of *Schistosoma mansoni*. A single dose of nerolidol (100, 200 or 400 mg/kg) administered orally to mice infected with adult schistosomes resulted in a reduction in worm burden and egg production. Treatment with the highest nerolidol dose (400 mg/kg) caused significant reduction in a total worm burden of 70.06% ($P < 0.001$). Additionally, the technique of quantitative and qualitative oograms showed that a single 400 mg/kg nerolidol dose achieved an immature egg reduction of 84.6% ($P < 0.001$). In faecal samples, the Kato–Katz method also revealed a reduction of 75.2% in eggs/g at a dose of 400 mg/kg ($P < 0.001$). Furthermore, scanning electron microscopy revealed that nerolidol-mediated worm killing was associated with tegumental damage. In contrast to activity against adult *S. mansoni* infection, oral treatment with nerolidol 400 mg/kg had low efficacy in mice harbouring juvenile schistosomes. Since nerolidol is already in use globally as a food additive and has a proven safety record, evaluation of this natural compound's potential for treatment of schistosomiasis could be entirely cost effective in the near future.

1. Introduction

Schistosomiasis is a major neglected tropical disease caused by blood-dwelling flatworms of the genus *Schistosoma* [1]. It affects more than 200 million people in tropical and subtropical regions of the world, especially in poor communities without access to safe drinking water and adequate sanitation [1,2]. The economic and health effects of schistosomiasis are considerable, and the disease disables more than it kills. In children, schistosomiasis can cause anaemia, stunting and a reduced ability to learn. Chronic schistosomiasis may affect people's ability to work and in some cases can result in death [3]. The current estimate of yearly disability-adjusted life-years (DALYs) for schistosomiasis is 3.4 million [2]. Human infection is due to three main species, namely *Schistosoma mansoni* and *Schistosoma japonicum*, which causes intestinal/hepatic schistosomiasis, and *Schistosoma haematobium*, which results in urinogenital disease [1].

Over the last several decades, chemotherapy using praziquantel (PZQ) has been a widely used strategy for the control and treatment of schistosomiasis. However, the drug does not prevent reinfection and, owing to use for more than three decades, the emergence of PZQ-resistant schistosomes is a constant threat. In addition, there is a critical deficiency in its therapeutic profile as it lacks activity against juvenile parasites [4]. Therefore, the search for a new chemotherapy is crucial to effectively control schistosomiasis in the future.

Natural products, especially from medicinal plants, present a diversity of molecules and have been a reliable source of chemotherapeutic agents, including in anthelmintic drug discovery [5,6]. Artemisinin and chloroquine are examples of plant-derived products with important therapeutic value. In recent years, an increasing number of studies have shown that nerolidol, an aliphatic sesquiterpene alcohol found in essential oils of several plants,

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http://dx.doi.org/10.1016/j.ijantimicag.2017.06.005
0924-8579/

exhibits a variety of biological properties, such as antimicrobial, antitumour, antioxidant, antinociceptive, antiulcer, anti-inflammatory and insecticidal properties (for review see [7]). In addition, nerolidol has demonstrated activity against several species of parasites, including *Plasmodium* [8], *Babesia* [9], *Trypanosoma* [10] and *Leishmania* [11]. It is important to note that nerolidol is used in many food and scented products and has been approved by the US Food and Drug Administration (FDA) [12,13].

Our group previously demonstrated that nerolidol at 31–62 μM possesses schistosomicidal activity against ex vivo *S. mansoni* adult worms [14]. We also showed that nerolidol caused morphological alterations in the tegument of parasites in a concentration-dependent manner. On the other hand, in vivo studies to determine the chemotherapeutic potential of nerolidol in the treatment of schistosomiasis have not yet been described. The present study investigated the in vivo antischistosomal activity of nerolidol administered by the oral route in mice infected with either adult or juvenile stages of *S. mansoni*.

2. Materials and methods

2.1. Animals

BALB/c mice were obtained from the Universidade Estadual de Campinas (Campinas, Brazil). For in vivo studies, 3-week-old BALB/c mice were infected with 80 cercariae of *S. mansoni* (BH strain). All animals were kept under environmentally controlled conditions (temperature 25 °C, humidity 70%) and had access to water (municipal tap water supply) and rodent food ad libitum.

2.2. Ethics statement

Procedures involving animals were carried out in accordance with Brazilian legislation. The protocol for maintenance of the *S. mansoni* life cycle was approved by the local ethics committee on animal experimentation.

2.3. Experimental design

In the first step, to study the dose–response relationship of nerolidol (a mixture of *cis*- and *trans*-nerolidol; Sigma-Aldrich, St Louis, MO) in adult *S. mansoni* infection (patent infection), mice were treated with a single oral dose of nerolidol at 49 days post-infection (p.i.). Nerolidol was mixed in corn oil and was administered to rodents in 100 μL by the oral route. The control group received an equal volume of corn oil only. Mice harbouring a chronic schistosome infection were divided into four experimental groups, with each group consisting of 10 animals treated with a single oral dose as follows: Group I, vehicle-treated control group; Group II, group treated with nerolidol at 400 mg/kg; Group III, group treated with nerolidol at 200 mg/kg; and Group IV, group treated with nerolidol at 100 mg/kg.

Subsequently, on the basis of their in vivo activity against adult schistosomes, nerolidol was tested in mice harbouring juvenile *S. mansoni* (pre-patent infection). In this case, a group of 10 mice was ere treated with a single 400 mg/kg oral dose of nerolidol at 21 days p.i. (pre-patent infection). Ten untreated mice served as controls.

2.4. In vivo drug assessment

At 2 weeks post-treatment, mice were euthanised and surviving schistosomes residing in the mesenteric veins and liver were counted and sexed as previously described [15]. Assessment of therapeutic efficacy was also based on the technique of quantitative and qualitative oograms using a fragment of the ascending colon (10 mm) [16] as well as the Kato–Katz method for quantitative faeces examination [17]. In the oogram pattern, eggs were scored as immature, mature or dead [16].

2.5. Scanning electron microscopy (SEM) studies

To determine whether nerolidol can cause morphological alterations in adult schistosomes recovered from mice, two additional mice were orally treated with 400 mg/kg nerolidol and were dissected at 24 h and 48 h post-treatment [15]. Worms were extracted from the mesenteric veins and liver as described above, were rinsed twice in phosphate-buffered saline and were fixed in 1 mL of 2.5% glutaraldehyde (Merck-Millipore, Cotia, SP, Brazil) for 3–24 h at room temperature. Samples were prepared as previously described [18]. Briefly, specimens were air-dried, were mounted on stubs and were metalised with gold using a Desk II sputter coater (Denton Vacuum LLC, Moorestown, NJ). Samples were then visualised using a JEOL JSM-6460LV high-resolution scanning electron microscopy (JEOL Ltd., Tokyo, Japan) at an accelerating voltage of 20 kV.

2.6. In vitro drug assessment

In vitro drug assessment was performed as previously reported [19–21]. Briefly, juvenile (21-day-old) or adult (49-day-old) schistosomes, freshly recovered from a rodent host, were washed and were placed in a 24-well culture plate (TPP, St Louis, MO) containing RPMI 1640 medium (Vitrocell, Campinas, SP, Brazil) supplemented with 10% fetal bovine serum (FBS), and 200 μg/mL streptomycin and 200 IU/mL penicillin (Vitrocell) at 37 °C in a 5% CO2 atmosphere. Newly transformed schistosomula were obtained by mechanical transformation using a vortex mixer. Parasites were cultivated for 3 h prior to the bioassay in 169 medium (Vitrocell) containing antibiotics and supplemented with 10% FBS at 37 °C in a 5% CO2 atmosphere. For in vitro bioassays, schistosomula were transferred to 24-well culture microplates (TPP) containing ca. 50 parasites/well and were cultured in medium 169. All stages of *S. mansoni* were maintained continuously in medium (with or without drugs) for 72 h at 37 °C in a 5% CO_2 atmosphere. The following drug concentrations were evaluated: 6.25, 12.5, 25, 50, 100, 200 and 400 μM. All worms were monitored using an inverted microscope.

2.7. Statistical analysis

Statistical analyses were performed using GraphPad Prism v.6.0 software (GraphPad Software Inc., La Jolla, CA). Dunnett's test was used to analyse the statistical significance of differences between mean experimental and control values [15]. A P-value of <0.05 was considered significant. The 50% lethal concentration (LC_{50}) was also calculated using sigmoid dose–response curves, along with the 95% confidence interval (CI) [5].

3. Results

First, the dose–response relationship of nerolidol in adult *S. mansoni* infections was evaluated. Groups of 10 mice were treated orally with single doses of nerolidol (100, 200 or 400 mg/kg) at 49 days p.i. Then, on the basis of their in vivo activity against adult schistosomes (patent infection), nerolidol was tested in mice harbouring juvenile *S. mansoni* (pre-patent infection). Furthermore, schistosomula, juveniles and adult *S. mansoni* were each incubated in vitro with nerolidol over a wide concentration range (6.25–400 μM).

3.1. Oral treatment with nerolidol in mice harbouring patent infections significantly reduced worm burden

In mice infected by adult *S. mansoni*, there was a significant reduction in worm burden upon oral treatment with nerolidol ($P < 0.001$). The highest activity was observed with a single dose of nerolidol 400 mg/kg, with a total worm burden reduction of 70.06%. At a dose of 200 mg/kg, the total worm burden reduction was 48.8%. At the lowest dose investigated (100 mg/kg), nerolidol showed no significant effect on worm burden (reduction of 10.98%) compared with infected untreated controls. The total, male and female worm burdens following treatment of *S. mansoni* infections with nerolidol are shown in Fig. 1.

3.2. Oral treatment with nerolidol in mice harbouring patent infections significantly reduced egg production

In addition to the in vivo antischistosomal effects described above, oral treatment with nerolidol reduced egg production. The effect of nerolidol (100, 200 and 400 mg/kg) on egg development stages (oogram) and faecal egg load are shown in Figs 2 and 3.

In the wall of the intestine, eggs at all developmental stages were observed in the treated group, but the frequency of immature eggs was significantly lower compared with infected untreated controls. Administration of single doses of 200 mg/kg and 400 mg/kg achieved reductions in the number of immature eggs of 69.7% and 84.6%, respectively ($P < 0.001$). At a dose of 100 mg/kg, the reduction of immature eggs was 29.89% ($P < 0.05$). The oogram also showed significant increases in the proportion of dead eggs at 200 mg/kg and 400 mg/kg nerolidol ($P < 0.001$), whereas there was no significant reduction in the number of *S. mansoni* eggs with a single dose of 100 mg/kg compared with the control *S. mansoni*-infected mice (Fig. 2).

In faecal samples collected from mice, the Kato–Katz method revealed that nerolidol significantly reduced the number of eggs compared with the infected untreated control group, with reductions of 48.32% ($P < 0.01$) and 75.2% ($P < 0.001$) at doses of 200 mg/kg and 400 mg/kg, respectively (Fig. 3).

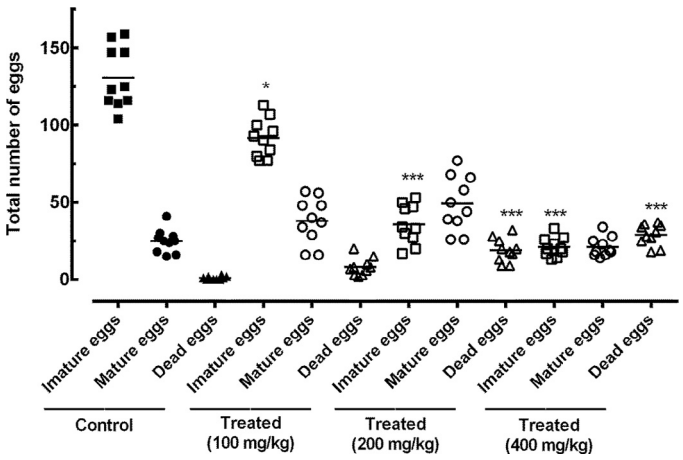

Fig. 2. Effect on egg development stages (oogram) of a single oral dose of nerolidol administered to mice harbouring a 49-day-old adult *Schistosoma mansoni* infection. Points represent data from individual mice that were infected and treated with nerolidol, or infected and untreated (control) mice. Horizontal bars represent median values. $^{*}P < 0.05$; $^{***}P < 0.001$ compared with untreated groups.

3.3. Tegumental changes in adult Schistosoma mansoni harboured in mice treated with nerolidol

SEM examinations of adult *S. mansoni* recovered from infected untreated mice and nerolidol-treated mice (400 mg/kg) in patent infections are presented in Fig. 4. Nerolidol caused morphological alterations in the tegument both of male and female schistosomes, and all worms showed slight to moderate focal damage of the dorsal surface of the tegument. Male worms showed changes in the tubercles, namely swelling and shortening or even loss of the spines on the surface. There were numerous blebs around the tubercles. In female worms, slight to moderate peeling of the dorsal surface was seen.

3.4. Oral treatment with nerolidol in mice harbouring pre-patent infections had low efficacy in reducing worm burden and egg production

Because nerolidol at a dose of 400 mg/kg was the most potent against *S. mansoni* adults (Figs 1–3), the effects of this compound

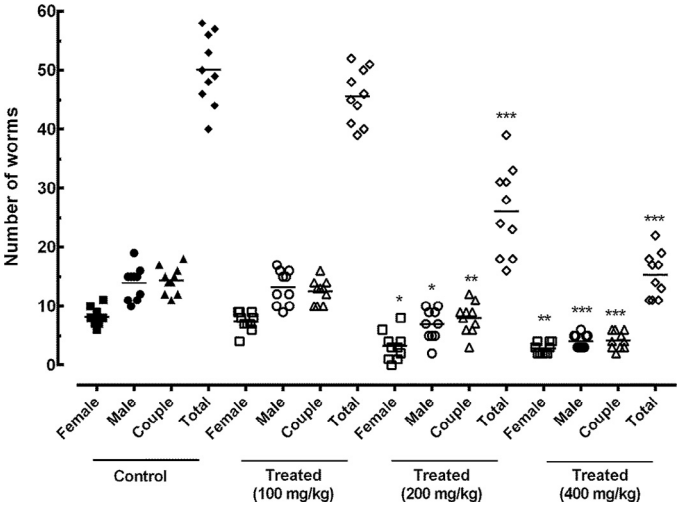

Fig. 1. Effect on worm burden of a single oral dose of nerolidol administered to mice harbouring a 49-day-old adult *Schistosoma mansoni* infection, stratified by sex. Points represent data from individual mice that were infected and treated with nerolidol, or infected and untreated (control) mice. Horizontal bars represent median values. $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$ compared with untreated groups.

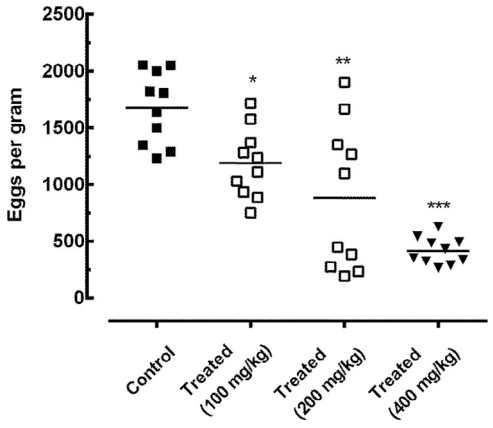

Fig. 3. Effect on stool egg load of a single oral dose of nerolidol administered to mice harbouring a 49-day-old adult *Schistosoma mansoni* infection. Points represent data from individual mice that were infected and treated with nerolidol, or infected and untreated (control) mice. Horizontal bars represent median values. $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$ compared with untreated groups.

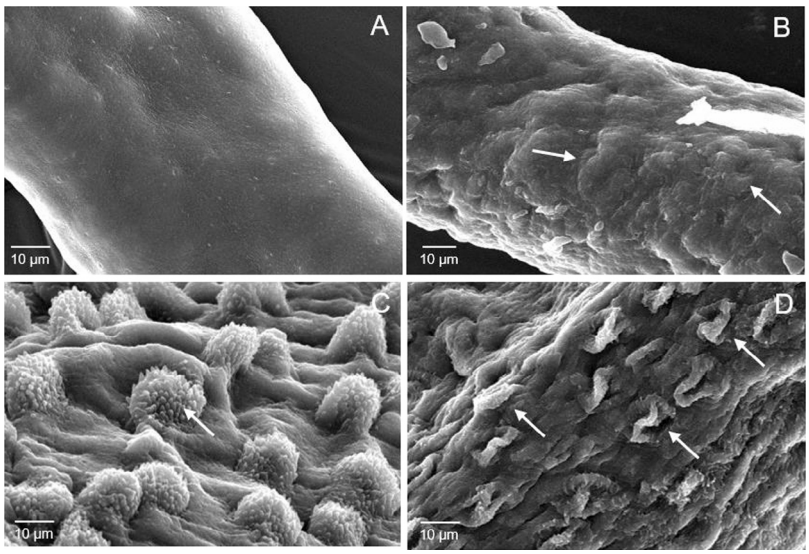

Fig. 4. Scanning electron microscopy (SEM) investigation of adult *Schistosoma mansoni* recovered from mice. Animals harbouring a 49-day-old adult *S. mansoni* infection were treated with a single oral dose of nerolidol 400 mg/kg. Mice were killed at 48 h post-treatment and images were obtained by SEM. (A) Female worms from infected and non-treated animal (control). (B) Female worms from infected animals and treated with nerolidol; tegumental surface showing blebs, swelling and shortening. (C) Male worms from infected and non-treated animal (control); dorsal tegumental surface showing tubercles and spines on the surface. (D) Male worms from infected animals and treated with nerolidol; dorsal tegumental surface showing swelling, shortening and collapse of the tubercles or even loss of the spines on the surface.

on juvenile *S. mansoni* worms were further analysed. However, in contrast to the in vivo activity of nerolidol in mice harbouring adult infections, treatment of juvenile *S. mansoni*-infected mice with a single 400 mg/kg oral dose of nerolidol showed a weak reduction in worm burden and eggs compared with the control group. Indeed, moderate, but non-significant, total worm burden reductions of 30.13% were achieved with nerolidol (Fig. 5A). Moreover, analysis of the oogram pattern showed all developmental stages with a predominance of immature eggs and no significant reduction in immature eggs compared with untreated mice (Fig. 5B). In faecal samples, the Kato–Katz technique also revealed a low egg reduction of 26.66% compared with the control group.

3.5. *Nerolidol exhibited in vitro antischistosomal properties against different stages of* Schistosoma mansoni

The in vitro antischistosomal effects of nerolidol on newly transformed schistosomula, juvenile (21-day-old) and adult (49-day-old) *S. mansoni* were evaluated at different concentrations (6.25–400 µM). In summary, LC_{50} values of 117.08 µM (95% CI 99.07–126.22 µM), 124.62 µM (95% CI 105.49–143.06 µM) and 84.99 µM (95% CI 75.32–108.61 µM) were calculated for nerolidol on schistosomula, juvenile and adult stages, respective.

4. Discussion

Nerolidol is a terpene approved by the FDA as a food-flavouring agent. It is present in the essential oils of several plants and exhibits a variety of biological and pharmacological properties [7]. Based on available pharmacokinetic and toxicological data, nerolidol is considered safe and it has great potential to be used as a new chemical or therapeutic drug [7,12,13]. A previous study demonstrated that 31–62 µM nerolidol leads to killing of ex vivo *S. mansoni* adult worms [14]. In the present study, nerolidol was tested experimentally for the first time in an *S. mansoni*-infected mouse model. Single 100–400 mg/kg oral doses were given to mice 49 days p.i. (patent infection). After that, a single 400 mg/kg oral dose of nerolidol was given to mice 21 days p.i. (pre-patent infection). Worm load, faecal egg load and frequency of egg developmental stages were evaluated and SEM investigations were performed. In addition, different stages of *S. mansoni* (schistosomula, juveniles and adults) were each incubated in vitro using nerolidol over a wide concentration range (6.25–400 µM).

Similar to PZQ, which acts primarily against adult worms [22], oral treatment with nerolidol was more effective against adult *S. mansoni* than the juvenile stage. The dose–response relationship of nerolidol in mice harbouring adult schistosomes revealed a significant gradual decrease in the total number of worms and oviposition. Indeed, the highest dose administered (400 mg/kg) resulted in reductions of 70.06% and 84.6% in worm burden and immature eggs, respectively. At this single oral dose, i.e. 400 mg/kg, other studies that used mice harbouring an adult *S. mansoni* infection, the following total worm burden reductions were observed: 0–85% with antiandrogens [23]; 0–80% with aryl ozonides [24]; ca. 20% with triphenylphosphonium derivatives [25]; 0–83% with bridged 1,2,4,5-tetraoxanes, alphaperoxides and tricyclic monoperoxides [26]; 0–44% with trioxolanes [27]; 12–32% with 3-alkoxy-1,2-dioxolanes [28]; and 0–66% with *N*,*N*′-diarylurea MMV665852 analogues [29].

Interestingly, data from the current in vivo study showed that nerolidol treatment significantly reduced the burden of male worms ($P < 0.001$) compared with female worms ($P < 0.01$). It appears that this compound was slightly more active against male worms, specifically at the dose of 400 mg/kg, unlike other drugs that act preferentially against female schistosomes [15,30] or are equally active against both worm sexes [31], and this result points to a sex-specific interference of the drug with the target or to different drug targets. Similar to the antischistosomal properties of nerolidol, oxamniquine is more active against male than females worms [32]. This phenomenon was not observed when treating juvenile stages of *S. mansoni*.

Schistosomes are parasitic platyhelminths that are dioecious, with the females producing hundreds of eggs per day. Owing to the importance of eggs for the life cycle and for inducing pathogenesis [1], assessment of therapeutic efficacy in *S. mansoni*-infected mice was also based on the technique of quantitative and qualitative oograms as well as the Kato–Katz method for quantitative faecal examination. The oogram is used for obtaining microscopic counts of schistosome eggs, including the different evolutionary stages [27].

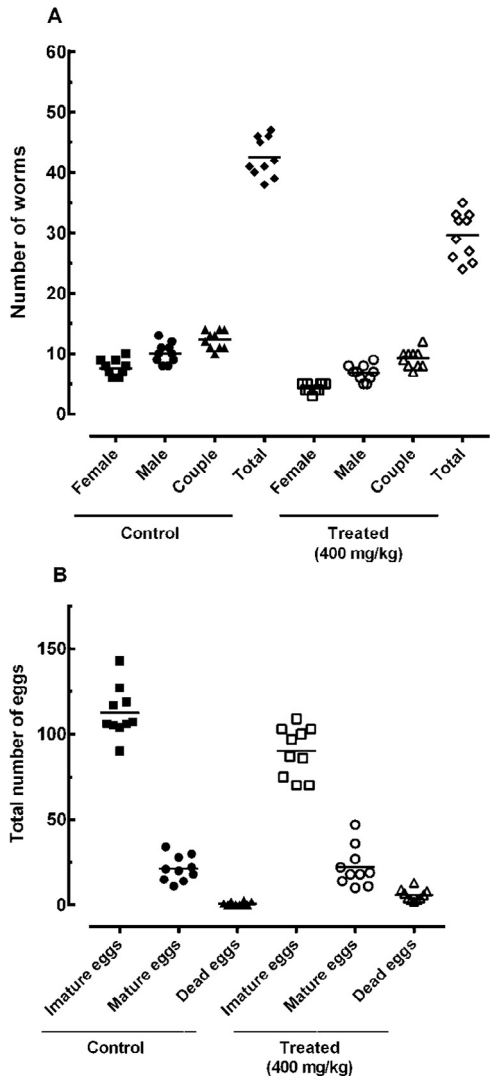

Fig. 5. Effect of a single oral dose of nerolidol 400 mg/kg administered to mice harbouring a 21-day-old adult *Schistosoma mansoni* infection: (A) effect on worm burden, stratified by sex; and (B) effect on egg development stages (oogram). Points represent data from individual mice that were infected and treated with nerolidol, or infected and untreated (control) mice. Horizontal bars represent median values.

The Kato–Katz technique is a cheap and simple method commonly used for detecting schistosome eggs in stool samples [17]. These methods have been frequently used for selecting new drugs (e.g. [15,18]). Regarding the oogram pattern, treatment with nerolidol in the patent period resulted in a dose-dependent impairment of egg development compared with control treatments. For example, treatment with the highest dose of nerolidol (400 mg/kg) resulted in a decrease in immature eggs of 84.6%. This finding could be attributed to a high reduction in the worm burden as a result of treatment with nerolidol and/or inhibition of oviposition by mature worm pairs. Moreover, there was a significant increase in the number of dead eggs noted in mice treated at doses of 200 mg/kg and 400 mg/kg. A significant reduction in total eggs and a decrease in total immature eggs with an increase in dead eggs have been described with other antischistosomal compounds [15,33], including PZQ [34].

The tegument is the major interface between the schistosome and its external environment. In addition to providing protection, the tegument has been a major target for antischistosomal drugs [35–37]. SEM analysis revealed morphological alterations on the tegument of worms, such as disintegration, sloughing and erosion of the surface. The exact mechanism by which nerolidol exerts its effect on *S. mansoni*, especially in the adult stage, is still not clear. However, like other terpenes, nerolidol has high hydrophobicity, allowing it to penetrate across membranes and to interact with intracellular proteins and/or intra-organelle sites [7,38]. It was reported that nerolidol inhibits the in vitro growth of promastigote *Leishmania* species, inhibiting isoprenoid biosynthesis by blocking an early step in the mevalonate pathway [38]. Regarding biological properties of terpenes, some of their activities are associated with their typical lipophiles, loss of ions and reduction of membrane potential, as well as collapse of the proton pump and depletion of the ATP pool [39]. Also, it is known that terpenes are capable of causing morphological changes in the tegument of parasites, and a relationship has been observed between tegumental damage and the death of worms [14,37,40]. Damage to the tegument along the worm's body would have impaired the functioning of the tegument and also destroyed the defence system of the worm, and so it could easily be attacked by the host's immune system [41].

Finally, considering that our earlier in vitro studies demonstrated schistosomicidal activity of nerolidol against adult worms [14], in this study we evaluated the in vitro anthelmintic properties of nerolidol on *S. mansoni* schistosomula and juvenile stages. Nerolidol is more potent on the adult stage (LC_{50} of ca. 85 μM) and has similar activity on schistosomula and juvenile stages (LC_{50} of ca. 120 μM). This feature suggests a different mode of action for nerolidol, but the effect of the compound on different stages of schistosomes in vitro and in vivo remains to be elucidated.

In conclusion, nerolidol is a common food additive approved by the FDA that showed an antischistosomal effect in a murine schistosomiasis model, especially in the period of patent infection. In addition to reducing worm burden, oral treatment with nerolidol resulted in a marked reduction in total egg production, with a decrease in total immature eggs and an increase in dead eggs. Because schistosomiasis control relies on a single drug and there is field evidence for the evolution of drug resistance, there is an urgent need to identify new antischistosomal drugs. Since nerolidol is already in use globally as a food additive and has a safety record, evaluation of the potential of the natural compound for the treatment of schistosomiasis could be entirely cost effective in the near future.

Funding: This work was supported by Fundação de Amparo à Pesquisa do Estado de São Paulo (FAPESP), Brazil [grant 2016/22488-3]. MPS and DBR are recipients of a postgraduate fellowship from Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES), Brazil. The funders had no role in study design, data collection and interpretation, or the decision to submit the work for publication.

Competing interests: None declared.

Ethical approval: Procedures involving animals were carried out in accordance with Brazilian legislation [11790/2008]. The protocol for maintenance of the *S. mansoni* life cycle was approved by the Comissão de Ética no Uso de Animais (CEUA), Brazil [protocol no. 05/2015].

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