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In vitro activity of aryl-thiazole derivatives against Schistosoma mansoni schistosomula and adult worms

--Manuscript Draft--

Manuscript Number:	PONE-D-19-21297
Article Type:	Research Article
Full Title:	In vitro activity of aryl-thiazole derivatives against Schistosoma mansoni schistosomula and adult worms
Short Title:	Activity evaluation of thiazole derivatives on Schistosoma mansoni
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Keywords:	Schistosoma, Schistosoma mansoni, aryl-thiazole, thiazole, in vitro drug treatment, parasites viability, parasites egg deposition
Abstract:	Background: Schistosomiasis is a chronic and debilitating disease caused by a trematode of the genus Schistosoma affecting over 200 million people worldwide. Current strategy for treatment and control of schistosomiasis is the use of Praziquantel, the only drug recommended by the World Health Organization. Development of new drugs is therefore of great importance. Thiazoles are regarded as privileged structures with a broad spectrum of activities and are potential sources of new drug prototypes, since they can act through interactions with DNA and inhibition of DNA synthesis. Methodology/Principal findings: In this context, we report the synthesis of a series of thiazole derivatives and the in vitro schistosomicidal testing 8 molecules (NJ03-08; NJ11-12) containing these structures. Parameters such as motility and mortality, ~~egg laying~~, ~~pairing~~ and parasite viability by ATP quantification, which were influenced by these compounds, were evaluated during the assays. Scanning ~~e~~lectron ~~m~~icroscopy (SEM) was utilized for evaluation of morphological changes in the tegument. Adult worms and schistosomula were treated in vitro with different concentrations (6.25 to 50 μM) of the thiazoles for up to 3 and 5 days, respectively. Adult worms after three days with 50 μM NJ05, NJ07 or NJ05 + NJ07 showed decreased motility to 30–50 % compared to controls. Compound NJ05 was more effective than NJ07, and viability after three days was reduced to 25 % in parasites treated with 50 μM NJ05, compared with a viability reduction to 40 % with 50 μM NJ07. SEM analysis showed ~~severe~~ alterations in adult worms with formation of ~~bulges and~~ blisters throughout the dorsal region of parasites treated with NJ05 and NJ07. Oviposition was ~~extremely~~ affected by treatment with the NJ series compounds; at concentrations of 25 μM and 50 μM, oviposition reached almost zero with NJ05, NJ07 and NJ05 + NJ07 already at day one. Tested genes involved in egg biosynthesis were all confirmed by qPCR as downregulated in females treated with 25 μM NJ05 for 2 days, with a significant reduction in expression of p14, Tyrosinase 2, p48 and fs800. Conclusions/Significance: According to the present study, thiazole derivatives have schistosomicidal activities and may be part of a possible new arsenal of compounds against schistosomiasis.
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In vitro activity of aryl-thiazole derivatives against *Schistosoma mansoni* schistosomula and adult worms

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Short title: Activity evaluation of thiazole derivatives on *Schistosoma mansoni*

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Abstract:

Background:

Schistosomiasis is a chronic and debilitating disease caused by a trematode of the genus *Schistosoma* affecting over 200 million people worldwide. Current strategy for treatment and control of schistosomiasis is the use of Praziquantel, the only drug recommended by the World Health Organization. Development of new drugs is therefore of great importance. Thiazoles are regarded as privileged structures with a broad spectrum of activities and are potential sources of new drug prototypes, since they can act through interactions with DNA and inhibition of DNA synthesis.

Methodology/Principal findings:

In this context, we report the synthesis of a series of thiazole derivatives and the *in vitro* schistosomicidal testing 8 molecules (NJ03-08; NJ11-12) containing these structures. Parameters such as motility and mortality, egg laying, pairing and parasite viability by ATP quantification, which were influenced by these compounds, were evaluated during the assays. Scanning electron microscopy (SEM) was utilized for evaluation of morphological changes in the tegument. Adult worms and schistosomula were treated *in vitro* with different concentrations (6.25 to 50 μM) of the thiazoles for up to 3 and 5 days, respectively. Adult worms after three days with 50 μM NJ05, NJ07 or NJ05 + NJ07 showed decreased motility to 30–50 % compared to controls. Compound NJ05 was more effective than NJ07, and viability after three days was reduced to 25 % in parasites treated with 50 μM NJ05, compared with a viability reduction to 40 % with 50 μM NJ07. SEM analysis showed severe alterations in adult worms with formation of bulges and blisters throughout the dorsal region of parasites treated with NJ05 and NJ07. Oviposition was extremely affected by treatment with the NJ series compounds; at concentrations of 25 μM and 50 μM, oviposition reached almost zero with NJ05, NJ07 and NJ05 + NJ07 already at day one. Tested genes involved in egg biosynthesis were all confirmed by qPCR as downregulated in females treated with 25 μM NJ05 for 2 days, with a significant reduction in expression of p14, Tyrosinase 2, p48 and fs800.

Conclusions/Significance:

According to the present study, thiazole derivatives have schistosomicidal activities and may be part of a possible new arsenal of compounds against schistosomiasis.

Keywords: Schistosoma, *Schistosoma mansoni*, aryl-thiazole, thiazole, *in vitro* drug treatment, parasites viability, parasites egg deposition

Author Summary

Schistosomiasis is a neglected tropical disease considered a serious public health problem caused by Schistosoma worms, praziquantel (PZQ) is the only drug used to treat humans, but selection of resistant parasites after prolonged treatment in a population has been reported. Faced with this panorama and the severity of the disease, the search for new drug candidates with schistosomicidal activity is pressing. A class of aryl-thiazole derivatives has gained attention because of its promising experimental anti-schistosomal activity against schistosomula and adult worms of *Schistosoma mansoni*. In this context, our research group synthesized a series of eight new thiazole derivatives (NJ03-08; NJ11-12) as candidate schistosomicidal agents. Molecules NJ05 and NJ07 stood out, leading to a higher mortality of worms, with a significant decline in motility, inhibition of pairing and oviposition and causing significant ultrastructural alterations such as surface peeling and bubbles formation, with destruction of the tegument in both male and female worms. Compounds NJ05 and

NJ07 together showed promising activity against early-developing larval forms. In females treated with NJ05, a marked decrease was observed in the expression of genes related to egg formation and cell differentiation. In conclusion, the aryl-thiazoles act as a molecular framework that has antischistosomal activity, being a promising target for developing novel drugs against schistosomiasis.

INTRODUCTION

Schistosomiasis is recognized as a serious public health problem in the world [1, 2]. According to the World Health Organization (WHO), it is estimated that more than 206 million people worldwide are affected by the disease and that more than 700 million people live in areas where the disease is endemic [3]. Schistosomiasis is an infection caused by a trematode of the genus Schistosoma and occupies, after malaria, the second position in the world among parasitic infections [4, 5]. This chronic disease is prevalent in tropical and subtropical regions, mainly in regions that lack basic sanitation, allowing the proliferation of contaminating agents, as well as the continuity of people being infected [2, 3, 6]. In addition, the absence of campaigns that promote health education to the population, as well as the lack of dissemination of epidemiological data, favor the continuity of the disease [6, 7].

The current treatment for Schistosomiasis is centered around praziquantel, an acylated quinoline-pyrazine derivative, that is able to act against the main strains of *S. mansoni* that infect humans and has been used for >30 years [8]. However, the exclusive use of praziquantel has been accompanied with reports in the literature that some patients have been resistant to pharmacological treatment [9-11]. Besides, praziquantel has no effect on immature schistosomes and cannot prevent reinfection or alter the schistosome life cycle [12, 13]. If it is considered that there is no effective alternative for the control of schistosomiasis, it is evident that the risk of developing resistance to praziquantel will increase in the coming decades, being a concern for the medical community. In view of this scenario, research priorities for facing schistosomiasis include the development and test of new drugs, test combinations of praziquantel with new drugs as well as monitoring the spread of praziquantel drug resistance [1].

In view of the need to obtain alternatives for the treatment of schistosomiasis and other neglected diseases, efforts have been made within the medicinal chemistry, using various planning techniques to obtain substances with improved structural characteristics and with less toxicity [14, 15] . These attempts comprehend praziquantel chemical structure modification by synthesis and evaluation of its analogues, rational design of new pharmacophores and discovery of new active compounds from screening programs on a large scale [16].

A class of derivatives that has gained attention because of its promising experimental anti-schistosomal activity is the one bearing the thiazole nucleus, which is effective against both immature and adult *Schistosoma mansoni* worms [17]. The thiazole moiety plays an important role in the medicinal chemistry field since it serves as scaffold for new drug synthesis, being already used for example as antimicrobial (sulfathiazole), anthelmintic and fungicide (thiabenzadole) and schistosomicide (niridazole) compounds [18-20]. Niridazole (Ambilhar®) started being used for treatment in 1964, but patients had little tolerance to this drug with serious side effects due to the presence of nitroaromatic chemical groups that act as parasitophores (their presence is essential to kill the worm) and also cause damage to host DNA by formation of adducts that induce mutagenesis [16]. However, advances in the development of medicinal chemistry tools have recently allowed the synthesis of new thiazole derivatives with anti-schistosomal activity. As an example, Santiago, de Oliveira (21) synthetized a set of molecules whose structures have a hydrazine and/or thiazole nucleus as a common group, and different pharmacophores including thiosemicarbazone, phthalyl thiosemicarbazone, phthalyl thiazole, and phthalyl thiazolidinone, and among them the compounds comprised of thiazole and phthalimide were the ones that exhibited the highest activity against worms, with a significant decline in motility, pairing and oviposition, as well as a mortality rate of 100% after 144 h of treatment.

Recently, Reddy, Kumar (22) described the synthesis of a library of hydrazinyl-thiazoles via a three-component reaction of various aldehydes/ketones with thiosemicarbazide and different phenacyl bromides, but the authors did not evaluate any biological potential of the derivatives. Based on the potentially promising features of these new compounds, here we selected eight aryl-thiazole derivatives and examined their efficacy in terms of (i) schistosome survival, (ii) egg output (oviposition), (iii) motility, (iv) couples pairing, (v) ultrastructural alterations in the tegument of *S. mansoni* as determined by scanning electron microscopy (SEM), and (v) expression of *S. mansoni* developmental genes.

MATERIALS AND METHODS

Chemistry

Synthesis of the compounds bioassayed in this study was previously described [22-24]. Production of aryl-thiazole derivatives was carried out at the Laboratory of Chemistry and Therapeutic Innovation, at the Federal University of Pernambuco. Synthesis was carried out in two steps (Fig 1).

Fig 1. Route of synthesis to obtain the aryl-thiazole derivatives (NJ series). In the compounds, R is hydrogen, 4-chloro, 4-bromo or 4-nitro. For 2,4-dimethoxy-aryl thiazole (right side route) R=Cl for NJ03; R=NO_2 for NJ06; R=Br for NJ08 and R=H for NJ12. For 3,4,5-trimethoxy-aryl thiazole (left side route) R=Cl for NJ04; R=NO_2 for NJ05; R=Br for NJ07 and R=H for NJ11.

In the first step, the thiosemicarbazide reacted in equimolar amounts with 2,4-dimethoxy-benzaldehyde (right side route) or 3,4,5-trimethoxy-benzaldehyde (left side route) through a condensation reaction in the presence of hydrochloric acid as the reaction catalyst to obtain a thiosemicarbazone (TSC), which served as an intermediary for thiazole synthesis following its acquisition. This second step was carried out in the presence of different 2-chloro or 2-bromoacetophenones (unsubstituted, 4-chloro-phenyl, 4-bromo-phenyl, 4-nitro-phenyl) under basic conditions (excess sodium acetate) and reflux [25].

Reactions were monitored with analytical thin-layer chromatography in silica gel 60 F254 plates and visualized under UV light (254 nm). Melting points were determined on a Quimis 340 capillary melting point apparatus and were not corrected. IR spectra were measured on Bruker IFS-66 IR spectrophotometer (Bruker, Germany) using KBr pellets. NMR spectra were recorded on Bruker AMX-300 MHz (300 MHz for ^{1}H and 75 MHz for ^{13}C) instruments by using tetramethylsilane as an internal standard. The spectroscopic data of the synthesized compounds (NJ03 to NJ08, NJ11 and NJ12) are in accordance with the structures proposed in Fig 1 and with the spectra published by Reddy, Kumar (22).

Ethics statement

The experimental protocols were in accordance with the Ethical Principles in Animal Research adopted by the Brazilian College of Animal Experimentation (COBEA) and the protocol/experiments have been approved by the Ethics Committee for Animal Experimentation of Institute Butantan (CEUA Nº 1777050816).

Maintenance of parasite life-cycle

The BH strain of *Schistosoma mansoni* (Belo Horizonte, Brazil) was maintained in the intermediate snail host *Biomphalaria glabrata* and the golden hamster (*Mesocricetus auratus*) was used as definitive host. Female hamsters aged 3-4 weeks, freshly weaned, weighing 50-60 g, were housed in cages (30x20x13cm) containing a sterile bed of wood shavings. A standard diet (Nuvilab CR-1 Irradiada, Quimtia S/A, Paraná, Brazil) and water were made available *ad libitum*. The room temperature was kept at 22 ± 2°C and a 12:12 hour light–dark cycle was maintained. Hamsters were infected by exposure to a *S. mansoni* cercariae suspension containing approximately 200-250 cercariae using the ring technique [26]. After 49 days of infection, the *S. mansoni* adult worms were recovered by perfusion of the hepatic portal system [27]. Cercariae were released from infected snails and mechanically transformed to obtain schistosomula *in vitro* [28].

Treatment of adult worms with NJ series compounds

Schistosomula and adult worms were treated with different concentrations of compounds from the NJ series in culture medium specific to each stage as indicated below (from a stock solution of 20 mM NJ series compound in dimethyl sulfoxide – (DMSO), and with the equivalent amount of DMSO in the control assays. Newly transformed schistosomula (NTS) were maintained for 2 h in M169 (Vitrocell) medium supplemented with 2% fetal bovine serum (FBS) (Vitrocell), 1 µM serotonin, 0.5 µM hypoxanthine,1 µM hydrocortisone, 0.2 µM triiodothyronine, penicillin/streptomycin, amphotericin, gentamicin (Vitrocell) at 37°C and 5% CO_2 [29]. Only after 2h incubation in culture medium the NJ series treatment was initiated. Paired adult worms freshly perfused from infected hamsters (see above) were maintained in culture in RPMI medium (Gibco) supplemented with 10% fetal bovine serum (FBS) (Vitrocell), penicillin/streptomycin, amphotericin,

gentamicin (Vitrocell) at 37°C and 5% CO_2 for two hours prior to the beginning of treatment with NJ series compound in DMSO or with 0.1% DMSO alone (control), which were added to the culture.

Motility assay

To evaluate the general condition of adult worms, including motility and mortality rate an inverted microscope was used. Parasites were observed after 1, 2 and 3 days of treatment with different concentrations of NJ series compounds or vehicle dimethyl sulfoxide (DMSO 0.1%). Motility and survival of worms were assessed according to the criteria scored in a viability scale of 0–3 [30]. The scoring system was as follows: 3, complete body movement; 1.5, partial body movement or immobile but alive; and 0, dead. Treatment was considered lethal whenever no worm movement was detected when observed for 2 min. Oviposition and ~~pairing status~~ were also observed and eggs were counted.

Viability assay

Viability of schistosomula and *S. mansoni* adult worms after treatment was determined by a cytotoxicity assay based on the CellTiter-Glo® Luminescent Cell Viability Assay kit (G7570, Promega, Madison, Wisconsin, EUA) [31]. The assay determines the amount of ATP present in freshly lysed adults or in intact schistosomula; the assay signals the presence of metabolically active cells.

In addition, viability of schistosomula was evaluated by the presence or absence of dead parasites, through staining with propidium iodide (PI) [32]. Schistosomula were equally distributed in 96-well microtiter plates, incubated with the different concentrations of NJ05 + NJ07 compounds indicated in the figure or the corresponding DMSO vehicle (control), and 2 μg/mL propidium iodide (PI) (Sigma-Aldrich) were added at 2 days of drug exposure. The parasites were immediately observed with light microscopy at 10 x magnification using a Nikon Eclipse fluorescent inverted microscope. Under fluorescence microscopy, schistosomula death was scored by a red fluorescence signal (572 nm emission microscope filter) [32, 33]. The number of biological replicates that were assayed, as well as the number of parasites that were counted per replicate, is stated in the legends to the figures.

Scanning electron microscopy (SEM)

Adult worms collected by perfusion were immediately transferred to supplemented RPMI medium; parasites were distributed in 6-well plates (adults: 10 paired worm couples per well) with medium. The worms were kept in culture (oven at 37 °C and 5 % CO_2) for 2 h for adaptation, and then NJ series compounds or the equivalent amount of 0.1% DMSO vehicle were added.

~~Ultrastructural analysis was performed with scanning electron microscopy.~~ Adult worms incubated at different concentrations of NJ series compounds or with 0.1% DMSO vehicle for 1 and 2 days were fixed with modified Karnovsky reagent (1% paraformaldehyde, 2.5 % glutaraldehyde, 1 mM calcium chloride in 1 M sodium cacodylate buffer, pH 7.4) and after the fixing stage the material was washed with sodium cacodylate buffer (0.1 mol / L, pH 7.2) and post-fixed with 1 % osmium tetroxide (w / v) for 1 h.

Samples were dehydrated with increasing concentrations of ethanol and then dried with liquid CO_2 in a critical-point dryer machine (model Leica EM CPD030, Leica Microsystems, Illinois, USA). Treated specimens were mounted on aluminum microscopy stubs and coated with gold particles using an ion-sputtering apparatus (model Leica EM SCD050, Leica Microsystems, Illinois, USA) [34]. Specimens were then observed and photographed using an electron microscope (FEI QUANTA 250, *Thermo Fisher Scientific, Oregon,* USA).

RT-qPCR validation of *S. mansoni* developmental genes

For quantitative RT-PCR, complementary DNAs were obtained by reverse transcription (RT) of 50 ng adult worms total RNA using SuperScript IV Reverse Transcriptase (Invitrogen) and random hexamer primers in a 20 µL volume, according to the manufacturer's recommendations. The resulting cDNA was diluted 8-fold in water and qPCR amplification was done with 2.5 µL of diluted cDNA in a total volume of 10 µL using SYBR Green Master Mix (Life Technologies) and specific primer pairs (Table S1). Tested genes were described in previous publications from the literature [35-40] or selected here based on their predicted function, and primers were designed with the use of Primer3 online software. The Light cycle 480 II (Roche) qPCR was used. Results were analyzed by comparative Ct method and the statistical significance was calculated with the two-sided t-test.

To find adequate normalizer genes for qPCR, we looked for evidence of genes with non-detectable changes in expression upon NJ series compounds treatment; ten genes were selected based on literature reports [35-40] and tested by qPCR in the adult female worms for the lack of effect of NJ series compounds on their levels of expression. The two least affected genes were Mitochondrial 28s ribosomal protein s14 (Smp_090920) and PI3K regulatory subunit 4 (Smp_123610). In all cases, the geometric mean expression of these two genes was used as normalizer for calculating the expression levels of genes of interest.

RESULTS

Phenotypic effects of NJ series compounds on *S. mansoni* schistosomula

Eight dimethoxy-aryl thiazole derivatives (Fig 1) were tested for schistosomicidal properties against the schistosomula early forms of the parasite. For this, schistosomula mechanically transformed from cercariae were pre-incubated for 2 h in culture medium, each compound indicated in Fig 2A and Figure S1 was subsequently added, and their effect on schistosomula viability was evaluated every day for a period of five days by measuring the concentration of ATP in the metabolically active cells of the parasite. Different concentrations of each compound from 6.25 to 50 μM were tested, and out of the eight compounds that were assayed (Fig 2A and S1 Text, Fig A), two had a more prominent effect in reducing schistosomula viability, namely NJ05 and NJ07 (Fig 2A). These compounds after five days of exposure decreased the parasites' viability by approximately 40 % (Fig 2A). In order to test for a possible synergistic effect, the best two compounds were used together for schistosomula treatment. Within the first day of treatment with NJ05 + NJ07, across all concentrations tested (12.5, 25 and 50 μM) there was a dose-dependent decrease of 60 to 80% in parasite viability (Fig 2B), indicating a clear synergy between the two compounds. The phenotype was more prominent after five days of treatment, with viability reduced to zero (Fig 2B). The effect on schistosomula viability over 5 days of exposure to each of the other six NJ series compounds is shown in S1 Text Fig A (see Supporting Information); a less marked 10 to 40 % reduction in parasites viability was observed.

Fig 2. Synergistic effect of NJ05 + NJ07 on schistosomula viability. (A and B) ATP quantitation using a luminescent assay to assess schistosomula viability under NJ series compounds exposure. Schistosomula (100-120/well) were incubated with the indicated concentrations of NJ05, NJ07, NJ05 + NJ07 or with vehicle (0.1% DMSO) for up to 5 days. Viability was expressed as % luminescence values relative to the control (DMSO). Mean ± SEM from three replicate experiments. The two-way ANOVA test was used to calculate the statistical significance (*$p < 0.05$; **$p < 0.01$; ***$p < 0.001$). For clarity purposes, we show only the highest p-value obtained from the two-way ANOVA test for each time point on all different concentrations tested.

(C) Quantitation of schistosomula viability using propidium iodide staining; schistosomula were treated for two days with NJ05 + NJ07 at the different concentrations indicated. Percentage of viable schistosomula (non-stained with propidium iodide) is shown. For each condition tested, about 400 schistosomula were used, divided into four biological replicates. Mean ± SEM from four replicate experiments. **(D)** Schistosomula treated with the indicated concentrations of NJ05 + NJ07 or with vehicle (0.1% DMSO) for 2 days were visualized by staining with propidium iodide (marker of dead cells; 572 nm emission filter microscope). For each concentration (indicated at the top), the upper panel shows a light microscopy image and the bottom panel shows the image of the same field with differential fluorescence detection of PI-positive parasites. *Bar= 100 µm.*

Propidium iodide (PI) was used as an alternate method to quantitate viability of schistosomula after a 2-days incubation in the presence of control vehicle (0.1% DMSO) or of 6.25 to 50 µM of the best two compounds together (NJ05 + NJ07) (Fig 2C and 2D). Under fluorescence microscopy, dead schistosomula were detected by a red fluorescence signal from PI (536 nm emission microscope filter) (Fig 2D, lower panels). Total number of schistosomula present in the field was detected and counted under light optical microscopy at each NJ05 + NJ07 concentration (Fig. 2D, upper panels), and the percentage viability was computed and plotted as shown in Fig 2C; mortality was 100 % with 25 and 50 µM NJ05 + NJ07. Incubation with control vehicle (0.1 % DMSO) for 2 days showed no reduction in parasite viability (Fig 2B and Fig 2D, first upper and lower panels).

Effects of NJ series compounds on *S. mansoni* adult worms' viability and motility

In order to evaluate the schistosomicidal activity of all eight compounds on adult worms we measured oviposition, ~~male-female pairing status,~~ worms' viability, motility, and alterations in the tegument. Compounds were tested at a concentration range of 12.5 to 50 µM and observed every day for a period of 3 days.

Again, compounds NJ05, NJ07 and NJ05 + NJ07 exhibited the highest schistosomicidal activities among the eight tested, showing a concentration-dependent significant decrease in

viability of the adult worms, as evaluated by ATP quantitation (Fig 3A – 3C). Compound NJ05 was more effective than NJ07, and viability after three days was reduced to 25 % in parasites treated with 50 μM NJ05 (Fig 3A), compared with a viability reduction to 40 % with 50 μM NJ07 (Fig 3B). No synergistic effect was detected when the two compounds were tested together (NJ05 + NJ07), as the viability was reduced to approximately 25 % after three days of exposure to 50 μM NJ05 + NJ07 (Fig 3C), similar to the reduction caused by NJ05 only. The effect of other tested compounds is shown in S1 Text Fig B (see Supporting Information); after three days of treatment with compounds NJ03, NJ04 and NJ08 (at 50 μM concentration), a reduction in viability to only 75 % was observed.

Fig 3. *In vitro* effects of NJ05, NJ07 and NJ05 + NJ07 on the viability and motility of *Schistosoma mansoni* adult worms. (A - C) Viability was estimated by the total amount of ATP available in the parasites, using a luminescent assay. Pairs of adult worms were treated for 2 days with NJ05, NJ07 and NJ05 + NJ07 at the different concentrations indicated or with vehicle (0.1% DMSO). Viability was expressed as percentage luminescence values relative to the control (DMSO). Mean ± SEM from three replicate experiments, each with 10 worm pairs. **(D - F)** Percentage of relative motility of adult worms treated with different concentrations of NJ05, NJ07 and NJ05 + NJ07 and controls (0.1 % DMSO) at different times of exposure from 1 to 3 days. Mean ± SEM of three experiments, each with 10 worm pairs. *$p < 0.05$, **$p < 0.01$ and *** $p < 0.001$ compared with controls. For clarity purposes, we show only the highest p-value obtained from the two-way ANOVA test for each time point on all different concentrations tested.

S. mansoni adult worms exhibited decreased motility when exposed to NJ05, NJ07 and NJ05 + NJ07, and this was dependent upon concentration and incubation time (Fig 3D – 3F). After three days with 50 μM NJ05, NJ07 or NJ05 + NJ07 motility decreased to 30 – 50 % compared with controls (Fig 3D – 3F). Impairment of the peristaltic movement and reduced ability of the suckers to adhere to the bottom of the culture plates were observed. Among the other compounds of the NJ series tested we can highlight the 50 % decrease in motility of adult worms exposed to 50 μM NJ03 or NJ08 after three days of exposure (S1 Text Fig C; see Supporting Information).

Effects of NJ series compounds on *S. mansoni* adult worm couples ~~pairing and female~~ oviposition

Compound NJ05 showed a more pronounced effect than NJ07 on worm couples ~~pairing~~ (Fig 4A and 4B). Thus, couples exposed for one day to 25 μM or 50 μM NJ05 already exhibited no pairing (Fig 4A), whereas at day one with 25 μM NJ07, couple pairing was not reduced and with 50 μM NJ07, pairing was reduced to approximately 60 % compared with control (Fig 4B). Combination of NJ05 + NJ07 was not effective in enhancing couples unpairing (Fig 4C) when compared with NJ05 alone. Among the other compounds of the NJ series reported in S1 Text Fig D (see Supporting Information), we can highlight the unpairing of approximately 75 % - 90 % of adult worm couples after three days exposure to 50 μM NJ03, NJ04, NJ08 or NJ11.

Fig 4. *In vitro* effects of NJ05, NJ07 and NJ05 + NJ07 on the ~~pairing~~ and oviposition of *~~Schistosoma~~ mansoni* adult worms. (A-C) Monitoring the pairing of control couples and of couples treated with different concentrations of NJ05, NJ07 and NJ05 + NJ07 at different times of exposure from 1 to 3 days. Mean ± SEM of three experiments, each with 10 worm pairs. **(D-F)** Number of eggs released by females incubated with NJ05, NJ07 and NJ05 + NJ07 at different concentrations or with vehicle (0.1 % DMSO) at different times of exposure from 1 to 3 days. *$p < 0.05$, **$p < 0.01$ and *** $p < 0.001$. For clarity purposes, we show only the highest p-value obtained from the two-way ANOVA test for each time point on all different concentrations tested.

In addition, oviposition was ~~extremely~~ affected by treatment with the NJ series compounds, especially NJ05, NJ07 and NJ05 + NJ07 (Fig 4D – 4F). Of note, reduction in oviposition was independent from couple unpairing. Thus, at concentrations of 25 μM and 50 μM, oviposition reached almost zero with NJ05, NJ07 and NJ05 + NJ07 already at day one (Fig 4D – 4F), even when unpairing was incomplete or not present such as with NJ07 (compare Fig 4B and 4E). Results for reduction in oviposition of the other tested compounds are described in S1 Text Fig E (see Supporting Information); little effect was observed in oviposition with these other compounds.

Scanning electron microscopy of ~~treated~~ adult worms

Through ~~s~~canning ~~e~~lectron ~~m~~icroscopy (SEM) it was possible to detect phenotypic alterations between control and treated adult worms. Control adult male schistosomes that were not exposed to any drugs are depicted in Fig 5 (panels 1 – 4) and Fig 6 (panels 1 – 4), showing a normal topography of the surface membrane of anterior and dorsal regions, respectively. Oral and ventral suckers can be clearly visualized (Fig 5, panels 2 – 4). In the anterior portion of the body, the gynecophoral canal, a longitudinal fold of the middle and posterior body that houses the female for the purpose of mating and reproduction, can be identified (Fig 5, panel 2). In Fig 6, panels 1 to 4, the medial and posterior parts of schistosomes are shown, respectively. In these regions, a large number of tubercles with typical spines and ciliated papillae was observed (Fig 6, panels 2 and 3).

Fig 5. Micrographs showing alterations on the ultrastructure of the anterior region of *~~Schistosoma~~ mansoni* adult male worms exposed to NJ05 or NJ07. ~~Scanning electron microscopy~~ of worms exposed for 2 days to 25 μM NJ05 (panels 5 – 8), 25 μM NJ07 (panels 9 – 12) or vehicle (0.1% DMSO) (panels 1 – 4). Note that the size scale bar is shown within the black thin line below each image; **panels 1 and 2**: Control worms (Bar = 500 μm; 100 μm) presenting the fully paired couple; **panels 3 and 4**: Anterior region of control male presenting normal oral and ventral suckers tegument (Bar = 50 μm; 30 μm); **panels 5 and 6**: Anterior region of adult male worm treated with 25 μM NJ05 (Bar = 500 μm; 100 μm); **panels 7 and 8**: Male oral and ventral suckers presenting tegument peeling (Bar = 50 μm); **panels 9 and 10:** Anterior region of male adult worm treated with 25 μM NJ07 (Bar = 500 μm; 100 μm); **panels 11 and 12**: Male oral and ventral suckers presenting tegument without structural alterations (Bar = 50 μm). *f*: *female; os*: *oral sucker; vs*: *ventral sucker; gc: gynecophoral canal*; *la*: *lesion area; ~~cp: ciliated papillae.~~*

Fig 6. Scanning electron micrographs of dorsal and posterior regions of *~~Schistosoma~~ mansoni* adult male worm exposed to NJ05 or NJ07. Worms were exposed for 2 days to 25 μM NJ05 (panels 5 – 8), 25 μM NJ07 (panels 9 – 12) or vehicle (0.1% DMSO) (panels 1 – 4). **Panels 1 to 4**: Medial (panels 1 – 3) and posterior (panel 4) regions of control male worm presenting normal tegument (Bar = 50 μm; 20 μm; 5 μm; 50 μm). Note that the size scale bar is shown within the black thin line below each image. **Panels 5 to 7**: Enlarged view of dorsal region of adult male worm

treated with NJ05 showing tegument lesions and loss of tubercles (Bar = 50 μm; 20 μm; 5 μm; 50 μm); **panel 8**: Posterior region of male adult worm treated with NJ05 showing lesion areas (Bar = 50 μm); **panels 9 to 11**: Dorsal region of male adult worm treated with NJ07 showing bubbles throughout (Bar = 30 μm; 20 μm; 5 μm; 50 μm); **panel 12**: Posterior region of male adult worm treated with NJ07 (Bar = 50 μm). ~~*cp: ciliated papillae*~~*; b: blebs; tb*: *tubercles; la*: *lesion area*.

SEM revealed detailed surface membrane ultrastructural damage on the anterior (Fig 5) and dorsal (Fig 6) regions of adult male worms caused by *in vitro* exposure for 2 days to 25 μM NJ05 (Fig 5 and 6, panels 5 – 8) and NJ07 (Fig 5 and 6, panels 9 – 12) compared with controls (0.1% DMSO) (Figs 5, and 6, panels 1 – 4). Thus, incubation of adult male *S. mansoni* worms with NJ05 resulted in severe tegument destruction of the anterior region (Fig 5, panels 5 – 8). In the ventral anterior region, focal lesions were observed in the oral and ventral suckers (Fig 5, panels 7 and 8). In the anterior portion of the body exposed to NJ07 the tegument showed no alterations (Fig 5, panels 9 – 12), and exhibited numerous ciliated papillae similar to the control (Fig 5, panels 1 – 4).

Severe damage to the dorsal surface of adult male schistosomes was induced by incubation for 2 days with NJ05 (Fig 6, panels 5 – 8) or NJ07 (Fig 6, panels 9 – 12). With NJ05, deterioration of the dorsal tegument was observed at higher magnification (Fig 6, panels 5 – 7), with the presence of lesion areas and loss of numerous tubercles and spines that covered the whole body of the parasite. In the posterior region, focal lesions along the body became evident (Fig 6, panel 8). Countless bubbles and loss of ciliated papillae were observed in the dorsal region of male worm exposed to NJ07 (Fig 6, panels 9 – 11); tegument at the male worm posterior region had no structural alterations (Fig 6, panel 12). Similar structural changes were already detected in adult male worms exposed for only 24 h to NJ05 or NJ07, with a more pronounced effect of NJ05 (S1 Text Fig F, see Supporting Information).

Control females incubated with vehicle (DMSO 0.1%) showed a normal surface (Fig 7, panels 1– 4). In females treated with 25 μM NJ05 for 2 days, severe damage to the dorsal tegument was visualized (Fig 7, panels 6 – 8), exhibiting extensive sloughing that exposed

subtegumental tissues. Presence of blisters can be seen throughout the extension of the dorsal region of female exposed to 25 μM NJ07 for 2 days (Fig 7, panels 10 – 12).

Fig 7. Damaged dorsal surface of *S. mansoni* adult female worms exposed to NJ05 or NJ07. Female worms were exposed for 2 days to 25 μM NJ05 (panels 5 – 8), 25 μM NJ07 (panels 9 – 12) or to vehicle (DMSO 0.1%) (panels 1 – 4) and were observed by scanning electron microscopy; note that the size scale bar is shown within the black thin line below each image. **panel 1:** Low magnification image of female control worm (Bar = 500 μm). **panels 2 to 4:** Images of female control worms dorsal region, with normal structural appearance (Bar = 40 μm; 10 μm and 4 μm); **panel 5:** Low magnification image of female worm exposed to NJ05 (Bar = 500 μm); **panels 6 to 8:** Middle dorsal region of female presenting apparent surface damage with peeling of the tegument membrane (Bar = 50 μm; 10 μm and 5 μm); **panel 9:** Low magnification image of female worm exposed to NJ07 (Bar = 500 μm); **panels 10 to 12:** Dorsal region of female worm presenting bubbles along the tegument (Bar = 30 μm; 10 μm and 4 μm). *p: peeling; la: lesion area; b: blebs.*

Assessment by quantitative PCR of the expression of genes involved with vitellaria and female reproduction

A set of six genes that are involved in egg formation, and participate in cell differentiation was selected for assessment by RT-qPCR of their possible change in expression caused by NJ series compounds. Tested genes involved in egg biosynthesis were all confirmed by qPCR as downregulated in females treated with 25 μM NJ05 for 2 days (Fig 8), with a significant reduction in expression of p14, Tyrosinase 2, p48 and fs800. Two other genes tested by qPCR showed a tendency to be less expressed in treated females (Fig 8), although exhibiting non-statistically-significant changes; they were the Egg Shell Protein (ESP) gene, and the nanos gene, which is involved in vitelline cell differentiation [41]. It is evident that NJ05 had a direct effect on the expression level of selected genes that are involved with eggshell formation and egg development in *S. mansoni* females.

The effect of NJ07 or NJ05 + NJ07 on the expression of the same set of six genes was also tested; worms were treated for 2 days with 25 μM NJ07 or NJ05 + NJ07; the only gene detected by RT-qPCR as significantly 2.8 – 2.9 X downregulated in females under both treatments was nanos, the gene related to cell differentiation (S1 Text Fig G, see Supporting Information). Although oviposition was impaired in both treatment conditions (see Fig 4E and 4F), there was no significant change in expression caused by both treatments among the five tested genes related to eggshell formation and egg development (S1 Text Fig G, see Supporting Information).

Fig 8. Vitellaria and female reproduction gene expression inhibition in adult females upon NJ05 treatment. Adult couples were treated with NJ05 (25 μM) or with vehicle DMSO (0.1 %) for 2 days. The parasites were stored in RNAlater for further processing. All parasite couples were separated and only the females had their RNA extracted followed by cDNA synthesis. The genes measured by qPCR were Smp_131110 (p14), Smp_000430 (Egg Shell Protein (ESP)), Smp_013540 (Tyrosinase 2 (Tyr 2)), Smp_014610 (p48), Smp_000290 (fs800), Smp_055740 (Nanos 2). The geometric mean from two reference genes was used for normalization with the DCT method (Smp_090920 and Smp_123610). The plotted data is retrieved from DDCT analyses in which the DMSO-treated sample is the control. Significant fold-change in gene expression between DMSO and NJ05 treatment is shown by the numbers inside the brackets. Mean ± SEM of four replicates is shown. Student unpaired parametric two-sided t-test was used and statistically significant differences are represented by the asterisks. *p≤0.05; **p≤0.01.

DISCUSSION

Chemotherapy is the only immediate recourse for minimizing the prevalence of schistosomiasis, and currently it involves predominately the administration of a single drug, praziquantel (PZQ). There is an important limitation of the therapeutic profile of PZQ, as it lacks activity against larval developing stages of the parasite; therefore, retreatment is necessary to kill those parasites that have since matured [21, 42]. The present study was performed to evaluate the *in vitro* schistosomicidal effect of the aryl-thiazole derivatives both on adults and ~~on the larval developing stages of the parasite.~~ The following schistosomicidal parameters were analyzed: viability, motility, oviposition, mortality and morphological changes, which are indicators commonly

used for evaluating the schistosomicidal effect of new chemical entities [43, 44]. In light of the parasite biology and according to the evaluation of these parameters, schistosomicidal activity can be effective essentially in three different ways: causing schistosomula death, thus preventing host infection, inhibiting oviposition that could lead to a decrease in the pathological phenotype of schistosomiasis (hepato- and splenomegaly), and causing adult worm's death [44, 45]. All three of the above schistosomicidal effects were present in our *in vitro* assays with NJ compounds, suggesting that further exploration of this class of aryl-thiazole derivatives is warranted.

The aryl-thiazole compounds evaluated in this study are differentiated, in principle, by the substitution pattern of the phenyl ring directly attached to the hydrazine portion (2,4-dimethoxy-phenyl and 3,4,5-trimethoxy-phenyl). Trimethoxylated derivatives (NJ04, NJ05, NJ07 and NJ11) showed a better profile in reducing the viability of *S. mansoni* schistosomula and adult worms. The main electronic change on the molecule caused by tri-substitution is an increase of the electronic density, and this effect significantly increased the schistosomicidal activity, also demonstrated by the synergic effect of compounds NJ05 + NJ07 which differ only by 4-nitro and 4-bromo substituents, respectively. The results corroborate with studies involving other parasites such as *Plasmodium falciparum* [46], *Leishmania braziliensis* [47] and *Trypanosoma cruzi* [48], evidencing the importance of methoxy group trisubstituted aromatic nuclei for antiparasitic activity.

Additionally, the two aryl-thiazole series are discriminated by the presence of different substituents of the phenyl ring attached to the thiazole nucleus (hydrogen, 4-chloro, 4-bromo, 4-nitro). The best compounds evaluated in this study have 4-nitro (NJ05) and 4-bromo (NJ07). Notably, such substitutions reflect an increase in antiparasitic activity as described in the literature. Papadopoulou, Bloomer (49) described the antitrypanosomal activity of 5-nitro-2-aminothiazole and demonstrated activity against *T. cruzi* amastigotes at nM concentrations and was about 4-fold more potent than BNZ (benznidazole-reference drug). Generally, the biological activity of nitro compounds involves the biotransformation of the nitro group, releasing intermediates that disrupt the redox process. Some of those intermediates can attack enzymes, membranes and DNA, providing the basis for their mode of action, including those reported against parasites [50]. NJ compounds might have caused oxidative stress and the consequent biological activity observed here. It is worthwhile to highlight that direct amino acid nitrosylation of SmTGR (Thioredoxin-

Glutathione Reductase) enzyme has been reported for recombinant *S. mansoni* TGR after reaction with furoxan [51], demonstrating the potential of compounds bearing nitro moieties since the SmTGR enzyme is essential for parasite survival and has been the subject of intense studies for identification of potential schistosomicidal agents [51].

The insertion of halogen atoms, in turn, on the compound structure in the discovery phase of new drugs can lead to increased potency and selectivity of the pharmacological target by affecting the pKa, modulating the conformation, increasing hydrophobic interactions between the ligand and the target, increasing lipophilicity and favoring permeation in biological membranes [52, 53]. The increase in cellular permeability of the parasite membrane is an important feature for the action of a target drug; for example, PZQ is supposed to act on the voltage-gated calcium channels of schistosomes [54], but has also been shown to alter membrane fluidity in model membranes [55] and in the *S. mansoni* tegument [56], thus possibly increasing calcium permeability. Permeation in biological membranes may have been fundamental for the good activity presented by NJ07. The phenotypic effects observed over the course of NJ05 treatment on *S. mansoni* couples included reduction in motility, impairment of peristaltic movement and of the ability of their suction suckers to adhere to the bottom of the culture plates, thus generating weak contractions and subsequent paralysis. This may be a consequence of the perturbation in the influx of extracellular calcium, which in eukaryotic cells is a signal for motor events, besides playing an important role in the promotion of apoptosis [57-59].

Changes in the general conditions of worms, including adult worms' motility and tegument damage visualized through high resolution scanning electron microscopy were observed after two days treatment with 25 μM NJ05 + NJ07. Motility assay and cytotoxicity evaluation by ATP quantification revealed an increase in the number of dead parasites that was concentration and time dependent following NJ05 + NJ07 treatment. Maximum death of adult worms occurred after three days of treatment at a concentration of 25 or 50 μM NJ05 + NJ07. Noteworthy, in schistosomula 100 % death happened in the first two days (25 or 50 μM NJ05 + NJ07). The larvae presented lack of movement, granularity and shape alterations, showing that NJ05 + NJ07 combination was effective against younger forms of the parasite. This is of great importance, since

the drugs of choice for treatment of schistosomiasis act on adult worms, having a small action on schistosomula [60].

Detailed microscopic observation showed that NJ05 and NJ07 were capable, separately, of inducing morphological alterations in the outer membrane of all adult worms tested in this work. We found extensive damage to the tegument in both male and female worms after 2 days of exposure of NJ05 and NJ07. Male adult worms of *S. mansoni* treated with NJ05 showed lesions of tegument and loss of tubercles, the females, in turn, demonstrated severe damage on the tegument surface with the presence of blisters and large peeling of the tegument. With NJ07 the dorsal region of male and female adult worms showed bubbles in all their extension. Alterations in the surface ultrastructure of schistosome worms have been investigated by a number of authors in order to evaluate anti-schistosomal drugs [61-66] . Here, the alterations caused by NJ05 and NJ07 indicate that trimethoxy substituent and the presence of NO_2 and Br substituent favor the damage on male and female adult worms.

The compounds tested here appear to influence adult females' oviposition, mainly NJ05 and NJ05 + NJ07, since no eggs at all were found in the culture medium after treatment. Eggs obtained from couples treated with NJ07 were small and not viable. In females treated with NJ05, the expression of genes related to egg biosynthesis including tyrosinase (SmTyr2) [67], eggshell precursors such as p14 [68] and p48 [69] was found to be decreased in relation to the control.

Also, genes related to cell differentiation and development of the germinative line of metazoans such as Nanos were found to have decreased expression in relation to the control in adult females treated with NJ05, NJ07 and NJ05 + NJ07. On platyhelminths, Nanos is generally found to be expressed in the anterior portion of the ovary [41], where the oogonias are located [70], and it plays an essential role in the proper development, regeneration and maintenance of germ cells in several organisms [71-73].

Overall, the results indicate that thiazoles, especially those trimethoxylated derivatives are antiparasitic agents. These compounds showed substantial schistosomicidal properties against schistosomula and adult *S. mansoni* worms, with a significant reduction in viability, motility, pairing, survival rate, oviposition, severe alterations in the tegument and mortality of worms, and further biological studies are warranted to clarify the mechanisms of schistosomicidal action. It is important

to emphasize that NJ05 + NJ07 had an intense activity against the young forms of Schistosoma, a characteristic not encountered in the drugs of choice for treatment of schistosomiasis such as PZQ and oxamniquine [8, 74]. The present study revealed NJ05 to possess the most effective schistosomicidal properties, suggesting its use as an aryl-thiazole prototype for the development of new schistosomicidal compounds.

Acknowledgments

We thank Beatriz Mauricio and Dr. Carlos Jared, Instituto Butantan, Sao Paulo, for access to the scanning electron microscopy facilities in their laboratories. We acknowledge Dr. Eliana Nakano and Patricia Aoki Miyasato, Instituto Butantan, Sao Paulo, and Dr. Pedro Luiz Silva Pinto, Instituto Adolfo Lutz, Sao Paulo, for maintaining the parasite life cycle.

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Supporting Information Legends

Fig A. ATP quantitation using a luminescent assay to assess schistosomula survival under NJ series compounds exposure. Schistosomula (100-120/well) were incubated with the indicated concentrations of NJ03, NJ04, NJ06, NJ08, NJ11 or NJ12 or with control vehicle (0.1% DMSO) for up to 5 days. Viability is expressed as % luminescence values relative to the control (DMSO). Mean ± SEM from three replicate experiments. *p < 0.05 (two-way ANOVA). For clarity purposes, we show only the highest p-value obtained from the two-way ANOVA test for each time point on all different concentrations tested.

Fig B. Effect of NJ series compounds on the viability of *Schistosoma mansoni* adult worms. Viability was estimated by the total amount of ATP available in the parasites, using a luminescent assay. Pairs of adult worms were treated for 1 to 3 days with NJ03, NJ04, NJ06, NJ08, NJ12 and NJ12 at the different concentrations indicated or with vehicle (0.1% DMSO). Viability was expressed as % luminescence values relative to the control (0.1% DMSO). Mean ± SEM from three replicate experiments, each with 10 worm pairs. *p < 0.05 compared with control. For clarity purposes, we show only the highest p-value obtained from the two-way ANOVA test for each time point on all different concentrations tested

Fig C. Effect of NJ series compounds on the motility of *Schistosoma mansoni* adult worms. Percentage of relative motility of adult worms treated with different concentrations of NJ03, NJ04, NJ06, NJ08, NJ12 and NJ12 and controls (0.1 % DMSO) at different times of exposure from 1 to 3 days. Mean ± SEM of three experiments, each with 10 worm pairs. *p < 0.05, **p < 0.01 compared with control. For clarity purposes, we show only the highest p-value obtained from the two-way ANOVA test for each time point on all different concentrations tested.

Fig D. *In vitro* effect of NJ series compounds on pairing of *Schistosoma mansoni* adult worm couples. Monitoring the pairing status of control couples and of treated couples exposed to different concentrations of NJ03, NJ04, NJ06, NJ08, NJ11 and NJ12 at different times of exposure from 1 to 3 days. Mean ± SEM of three experiments, each with 10 worm pairs. *p < 0.05 and **p <

0.01 compared with control. For clarity purposes, we show only the highest p-value obtained from the two-way ANOVA test for each time point on all different concentrations tested.

Fig E. *In vitro* effects of NJ series compounds on oviposition of *Schistosoma mansoni* adult worms. Number of eggs released by females incubated with NJ03, NJ04, NJ06, NJ08, NJ11 and NJ12 at different concentrations or with vehicle (0.1 % DMSO) at different times of exposure from 1 to 3 days. Mean ± SEM of three experiments, each with 10 worm pairs. *p < 0.05 and **p < 0.01 compared with control. For clarity purposes, we show only the highest p-value obtained from the two-way ANOVA test for each time point on all different concentrations tested.

Fig F. Structural damage was observed by scanning electron microscopy of male worms already at 24 h exposure to NJ05 or NJ07. Worms were exposed for 24 h to 25 μM NJ05, 25 μM NJ07 or to vehicle (0.1% DMSO). **panels 1-4**: Control worms (Bar = 500 μm; 100 μm; 50 μm; 50 μm) presenting the fully paired couple (panel 2) and anterior region of the male presenting normal oral and ventral suckers tegument (panels 3 and 4); **panels 5-8**: Anterior region of adult male worm treated with NJ05 with oral and ventral suckers presenting tegument peeling (Bar = 500 μm; 100 μm; 50 μm; 50 μm); **panels 9-12**: Anterior region of adult male worm treated with NJ07 with oral and ventral suckers presenting tegument without structural alterations (Bar = 400 μm; 100 μm; 50 μm; 40 μm); **panels 13-16**: Medial (panels 13 – 15) and posterior (panel 16) regions of control male worm presenting normal tegument structures (Bar = 50 μm; 20 μm; 5 μm; 50 μm); **panels 17-20:** Enlarged view of dorsal region of adult worm treated with NJ05 (panels 17-19) showing tegument lesions and loss of tubercles and posterior region of male adult worm showing lesion areas (Bar = 50 μm; 20 μm; 5 μm; 50 μm); **panels 21-24:** Dorsal and posterior regions of adult worm treated with NJ07 showing bubbles along their extension (Bar = 30 μm; 20 μm; 5 μm; 50 μm). *f*: *female; os*: *oral sucker; vs*: *ventral sucker; gc: gynecophoral canal*; *la*: *lesion area* ; *cp: ciliated papillae; b: blebs; tb*: *tubercles*. (note that the size scale bar is shown within the black thin line below each image).

Fig G: Vitellaria and female reproduction gene expression in adult females upon NJ07 or NJ05+NJ07 treatment. Adult couples were treated with NJ07 (25 μM), NJ05+NJ07 (25 μM) or DMSO (0.1 %) for 2 days. The parasites were stored in RNAlater for further processing. All parasite couples were separated and only the females had their RNA extracted followed by cDNA synthesis. The genes measured by qPCR were Smp_131110 (p14), Smp_000430 (Egg Shell Protein (ESP)), Smp_013540 (Tyrosinase 2 (Tyr 2)), Smp_014610 (p48), Smp_000290 (fs800), Smp_055740 (Nanos 2). The geometric mean from two reference genes was used for normalization with the DCT method (Smp_090920 and Smp_123610). The plotted data is retrieved from DDCT analyses in which DMSO sample is the control. Significant fold-change in gene expression between DMSO and NJ07 or NJ05+NJ07 treatment is shown by the numbers inside the brackets. Student unpaired parametric two-sided t-test was used and statistically significant differences are represented by the asterisks. *p≤0.05; **p≤0.01.

S1 Table. List of primers used in qPCR

Figure 1

Click here to access/download;Figure;Figure 1.tif

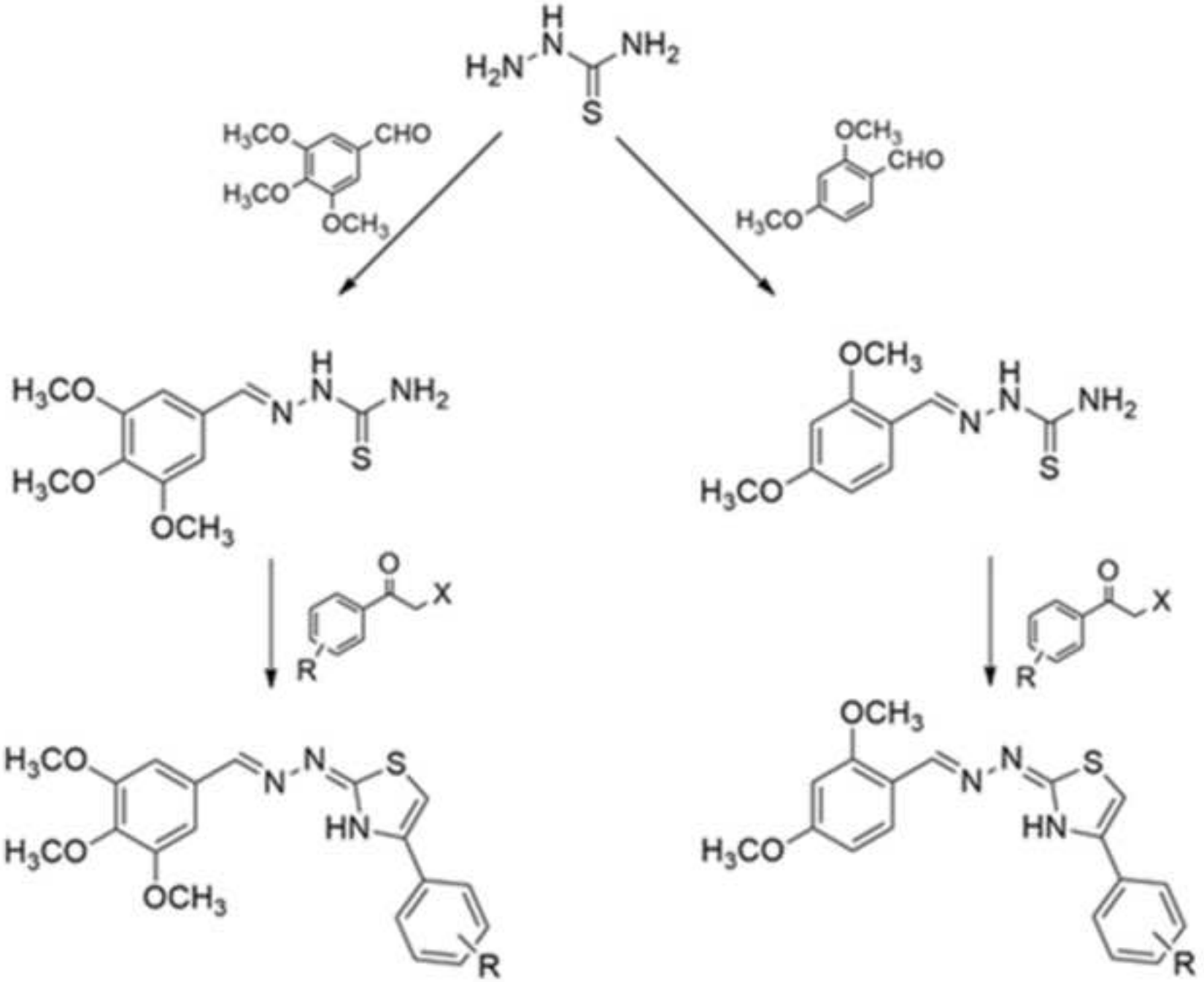

Figure 2

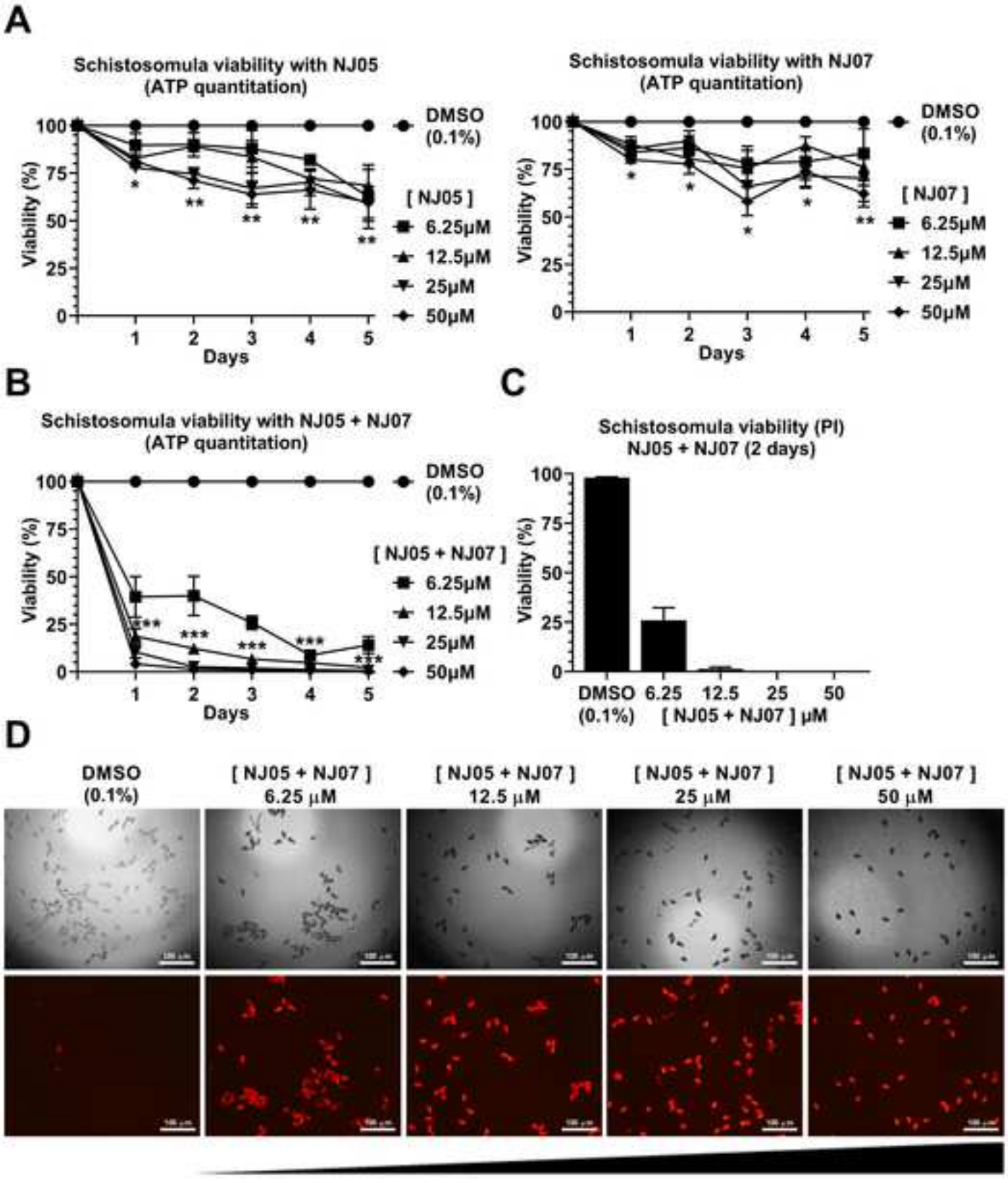

Figure 3

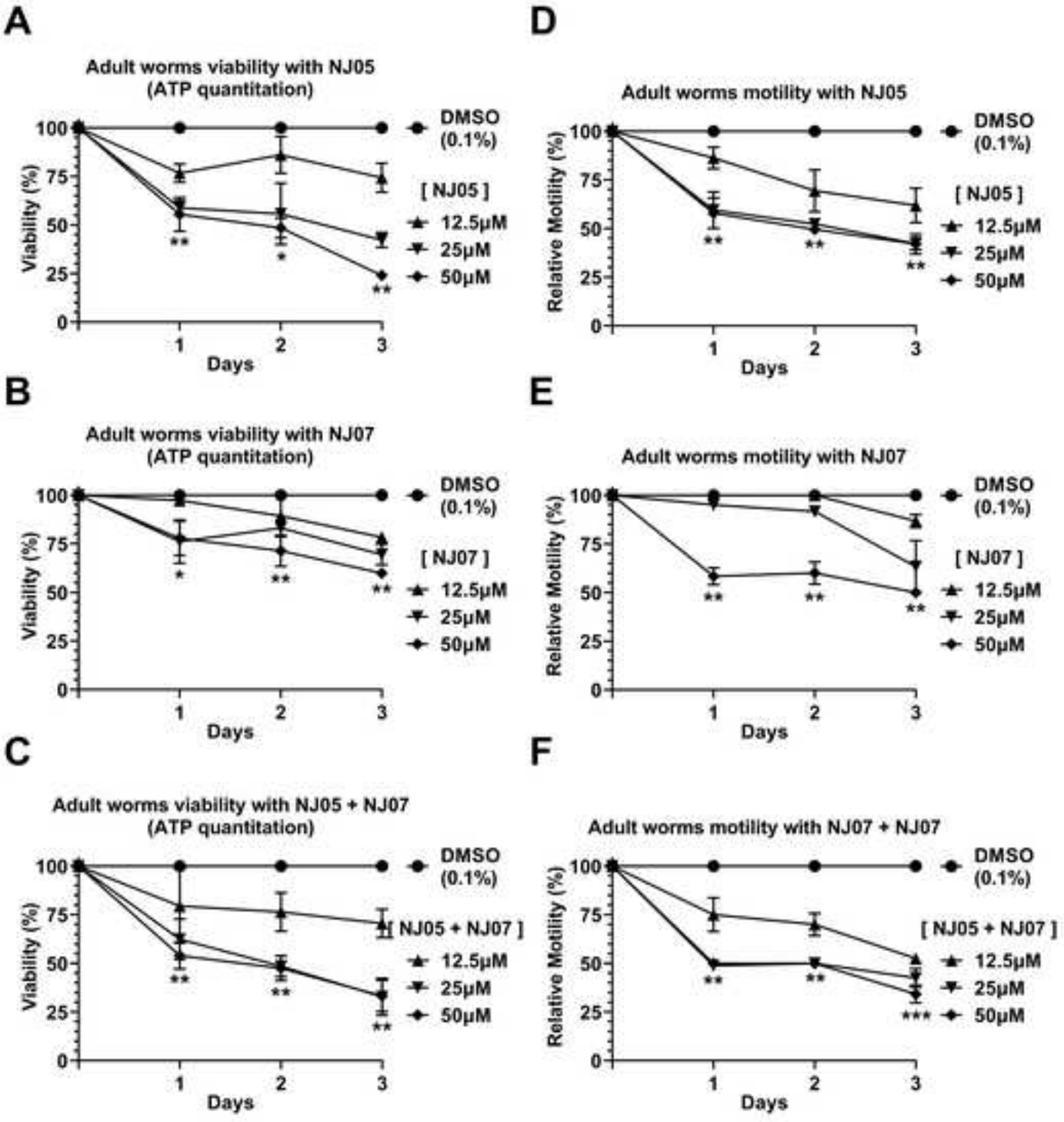

Figure 4

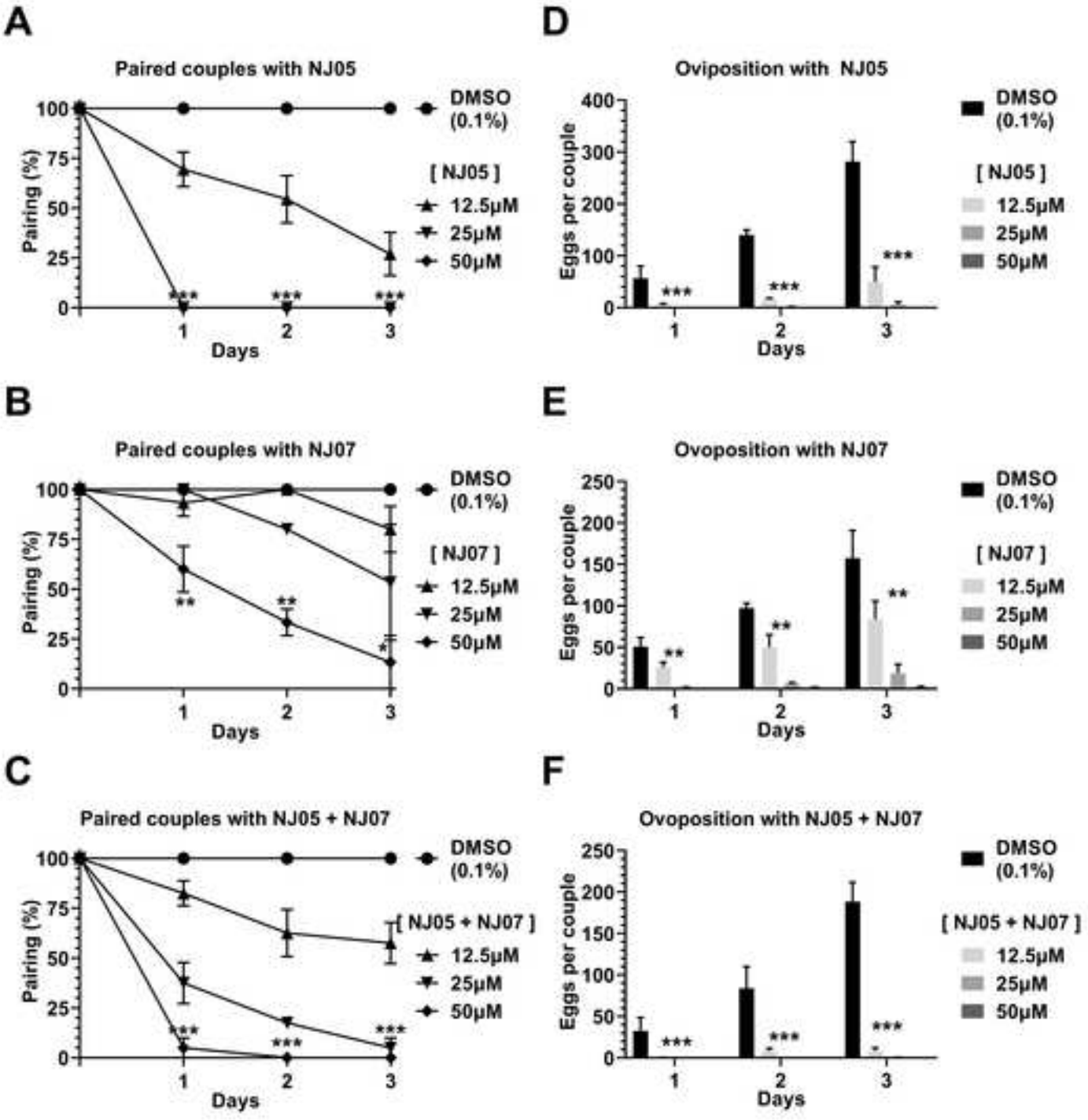

Figure 5

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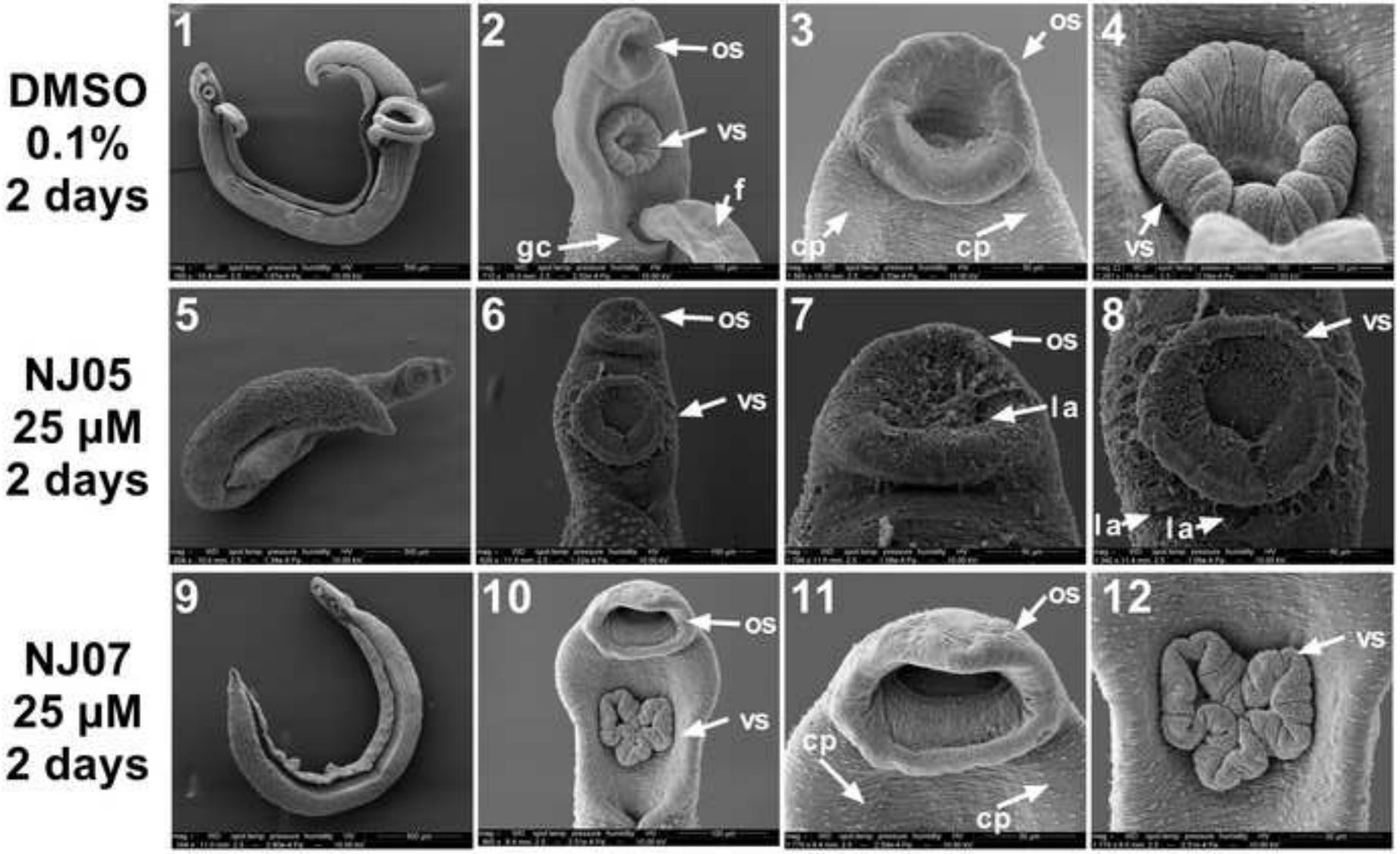


Figure 6

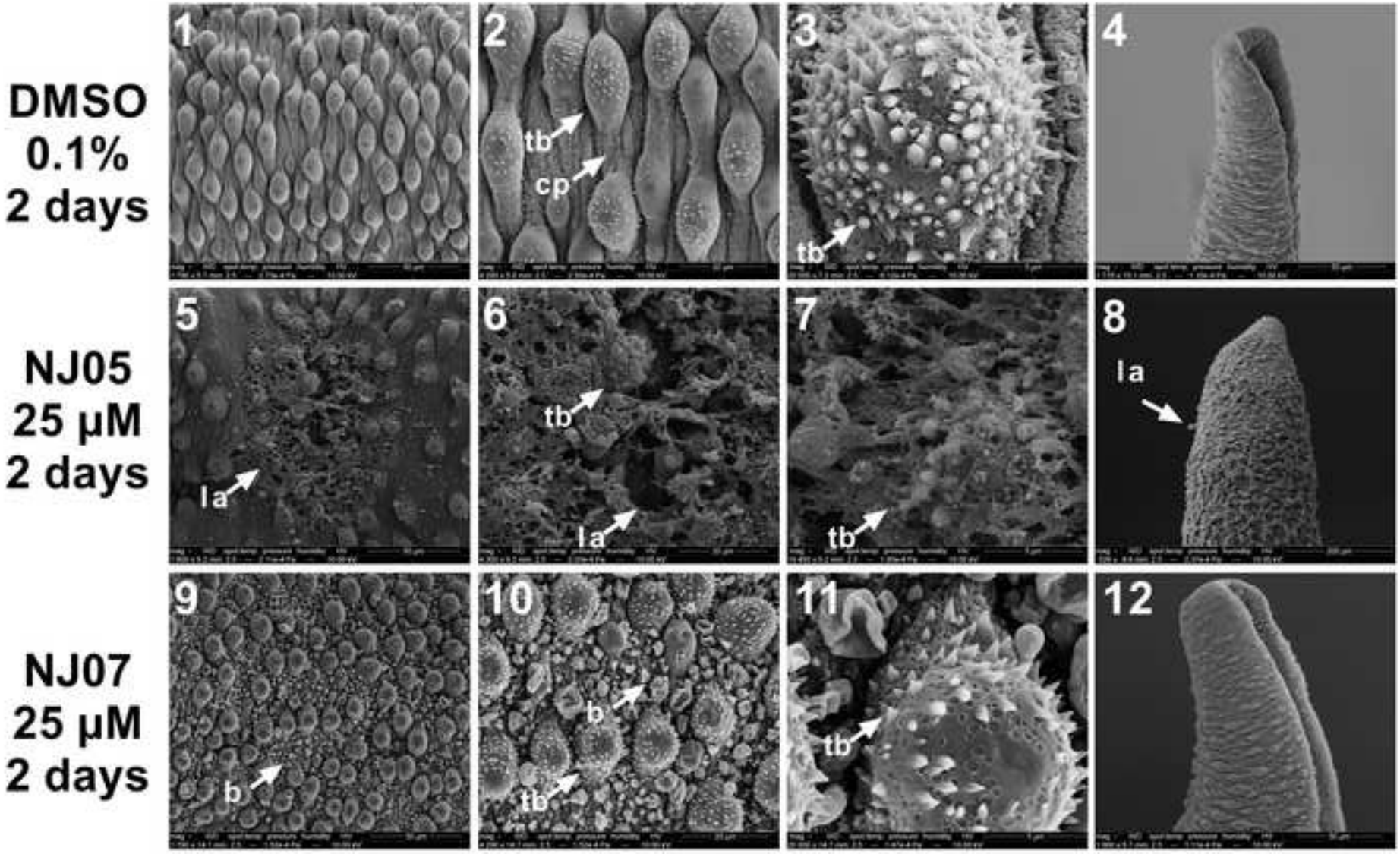


Figure 7

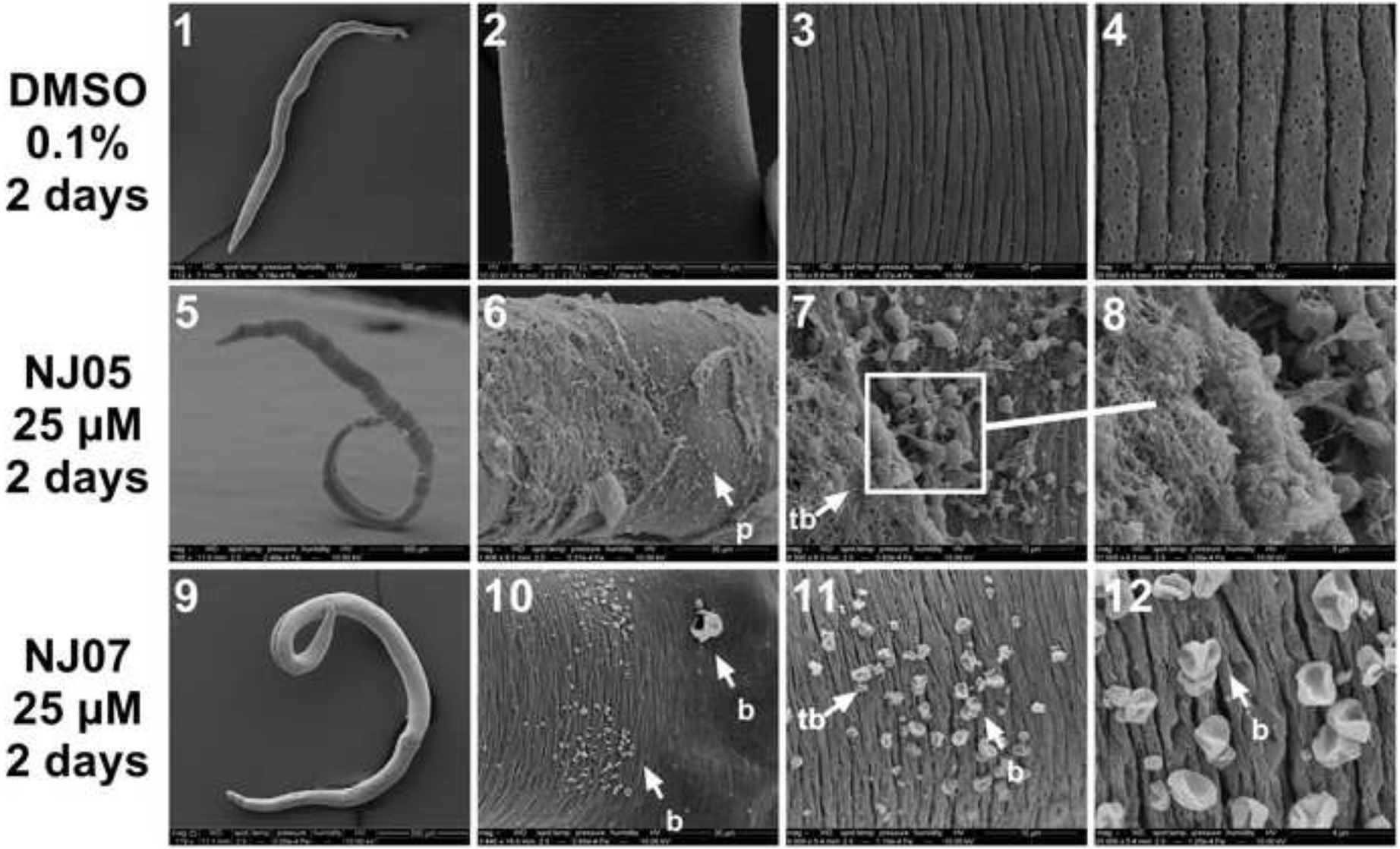
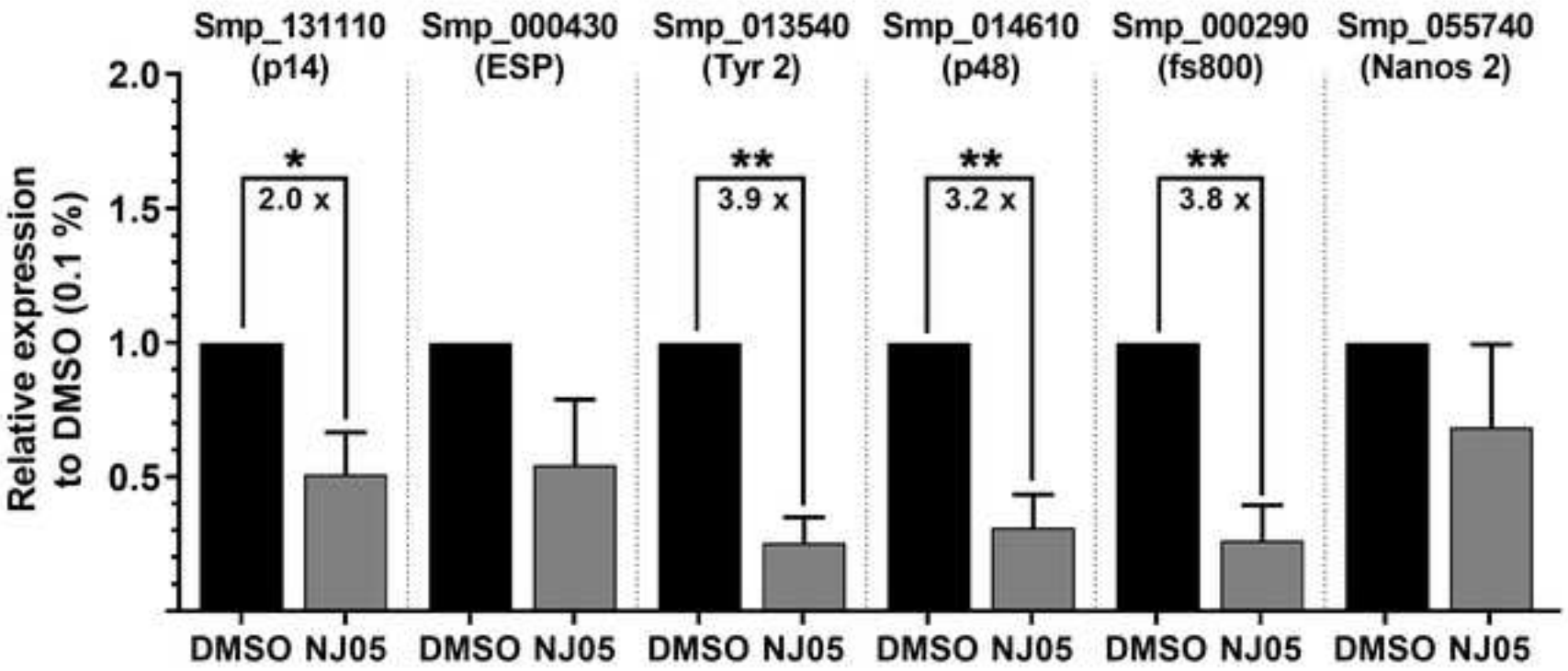


Figure 8



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Supporting Information

S1_Table. List of primers used in qPCR.xlsx

S1 text Supporting information