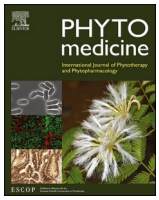
Phytomedicine 135 (2024) 156045

Contents lists available at ScienceDirect

Phytomedicine

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



In vivo antischistosomal efficacy of *Porcelia ponderosa* γ-lactones

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ARTICLE INFO

Keywords:
Porcelia ponderosa
Acetogenins
Anti-*Schistosoma mansoni* activity
In vivo assays

ABSTRACT

Background: Schistosomiasis, caused by the parasitic blood fluke *Schistosoma mansoni*, is a significant global health concern, particularly in tropical and subtropical regions. The available chemotherapeutic drug is restricted to praziquantel with present problems related to efficacy, toxicity and resistance, justifying the search for new drugs. Different natural products, including γ-lactones, have demonstrated anthelmintic activity. Thus, in this study, new γ-lactones from *Porcelia ponderosa* were investigated for their anti-*S. mansoni* effects *in vitro* and *in vivo*.

Purpose: To evaluate the therapeutical potential against *S. mansoni* of the mixture of γ-lactones **1** + **2** obtained from *Porcelia ponderosa* seeds.

Study design and methods: The precipitate formed during the concentration of CH_2Cl_2 extract from seeds of *P. ponderosa* showed to be composed by a mixture of the new γ-lactones **1** + **2** (in a ratio 77:23) which were chemically characterized using NMR and ESI-HRMS. This mixture was evaluated *in vitro* and *in vivo* against *S. mansoni*, using a murine model of schistosomiasis. Additionally, toxicity of the mixture of **1** + **2** (77:23) was determined using mammalian cell lines (*in vitro*) or the model organism *Caenorhabditis elegans* (*in vivo*).

Results: Seeds of *P. ponderosa* afforded a mixture of two unreported γ-lactones, 3-hydroxy-4-methylene-2-(tetracosa-17′*Z*,23′-diene-13′,15′-diynyl)-but-2-enolide (**1**) and 3-hydroxy-4-methylene-2-(tetracos-17′*Z*-ene-13′,15′-diynyl)-but-2-enolide (**2**). Initially, the antischistosomal activity of the mixture of **1** + **2** (77:23) was investigated *in vitro,* and obtained results demonstrate reduced activity against *Schistosoma mansoni* worms (EC_{50} of 83.3 μg/ml) in comparison to positive control praziquantel (EC_{50} of 1.5 μg/ml). However, when tested *in vivo* using oral administration at 400 mg kg^{-1}, the standard dose used in the murine model of schistosomiasis, the mixture of **1** + **2** (77:23) revealed expressive reductions in both worm burden (65.7 %) and egg production (97.2 %), similar of those observed to praziquantel (89.7 % and 91.5 %, respectively). On the other hand, when treated using 200 and 100 mg kg^{-1}, reductions in worm burden (25.7 and 12.4 %) and egg production (33.6 and 13.3 %) were also observed. Importantly, the mixture of **1** + **2** (77:23) exhibited no toxicity using mammalian cell lines (*in vitro*) or *C. elegans* (*in vivo*).

Conclusion: Considering the promising *in vivo* activity of γ-lactones from *P. ponderosa,* the mixture of **1** + **2** (77:23) can be considered as promising candidate for the development of novel antischistosomal therapeutics,

Abbreviations: ATCC, American type culture collection; BHI, brain heart infusion medium; CC_{50}, 50 % cytotoxic concentration; $CDCl_3$, deuterated chloroform; DAD, diode-array detector; DMEM, Dulbecco's modified eagle medium; DMSO, dimethyl sulfoxide; EBR, egg burden reduction; EC_{50}, 50 % effective concentration; EPG, eggs per gram; ESI-HRMS, electrospray ionization – high resolution mass spectrometry; EtOAc, ethyl acetate; EtOH, ethanol; HPLC, high performance liquid chromatography; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; NGM, nematode growth medium; NMR, nuclear magnetic resonance; PBS, phosphate buffered saline; PZQ, praziquantel; RPMI, Roswell Park Memorial Institute; SI, selectivity index; TLC, thin layer chromatography; UV, ultraviolet; WBR, worm burden reduction; WHO, World Health Organization.

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https://doi.org/10.1016/j.phymed.2024.156045
Received 21 June 2024; Received in revised form 27 August 2024; Accepted 11 September 2024
Available online 12 September 2024

underscoring the importance of biodiversity exploration in the search for effective treatments against neglected tropical diseases.

Introduction

Schistosomiasis, caused by the parasitic blood fluke *Schistosoma*, is a significant global health concern, particularly in tropical and subtropical regions. With approximately 250 million people affected worldwide and 10 % of the global population at risk of infection, schistosomiasis imposes a considerable burden on public health (Lo et al., 2022). The primary causative agent, *Schistosoma mansoni*, contributes to morbidity and mortality, primarily through its egg output, leading to severe hepatic and splenic complications (McManus et al., 2018). While current control measures primarily rely on preventive chemotherapy, predominantly using praziquantel, reports of reduced efficacy and the concern of drug resistance pose significant challenges to effective treatment (Deol et al., 2019; Kabuyaya et al., 2018)). Systematic reviews and meta-analyses have revealed that praziquantel achieves a cure rate of approximately 75 % for *S. mansoni* infections (Zwand and Olliaro, 2014 and 2017) underscoring its efficacy limitations. As the WHO aims to eliminate schistosomiasis as a public health problem by 2030 (WHO, 2021) the need for novel therapeutic approaches becomes increasingly apparent (Ferreira et al., 2022).

Natural products, including terpenoids, flavonoids, alkaloids, lignoids, polyketides, and related metabolites present promising avenues for drug discovery against neglected tropical diseases, including schistosomiasis (Azevedo et al., 2023). As part of our continuous work regarding the discovery of potent antiparasitic natural products, in the present study, seeds of *Porcelia ponderosa,* a Brazilian plant species found in the Amazonian rainforest (Murray, 1993), afforded a mixture of two new γ-lactones (compounds **1** and **2** - Fig. 1) which displayed activity against *Schistosoma mansoni* through *in vivo* preclinical assays using a murine model of schistosomiasis. To the best of our knowledge, there were no previous studies conducted with *P. ponderosa* but the identification of different γ-lactones with expressive antiparasitic efficacy including *Trypanosoma cruzi* and *Leishmania (L..) infantum* (Brito et al., 2021; Oliveira et al., 2019) were reported in *P. macrocarpa.* Thus, the main objective of this study was to identify new natural anthelmintic agents to treat schistosomiasis, contributing to the search for alternative treatments for schistosomiasis, crucial for achieving WHO's elimination goals.

Material and methods

General experimental procedures

NMR (^{1}H and ^{13}C) spectra were recorded on a Varian INOVA spectrometer, operating at 500 and 125 MHz, respectively, using $CDCl_3$ or DMSO-d_6 (Sigma-Aldrich) as solvents and internal standard. Chemical shifts are reported in δ units (ppm) and coupling constants (*J*) in Hz. ESI-HRMS spectra were measured on a Bruker Daltonics MicroTOF QII spectrometer, at negative mode. IR spectrum was recorded in a Perkin-Elmer Infrared UATR spectrophotometer. Sephadex LH-20 (GE Healthcare) was used for column chromatography while silica gel F_{254} (Macherey-Nagel - MN) was used for analytical TLC. HPLC analyses were performed on a Thermofisher™ Scientific equipment model UltiMate™ 3000 BioRS System, DAD UVD-170OU using a Phenomenex Kinetex EVO C_{18} (5 μm, 150 mm × 4.6 mm, flow rate: 1.0 ml/min) column. Reagents and solvents were acquired from commercial sources and were used without further purification.

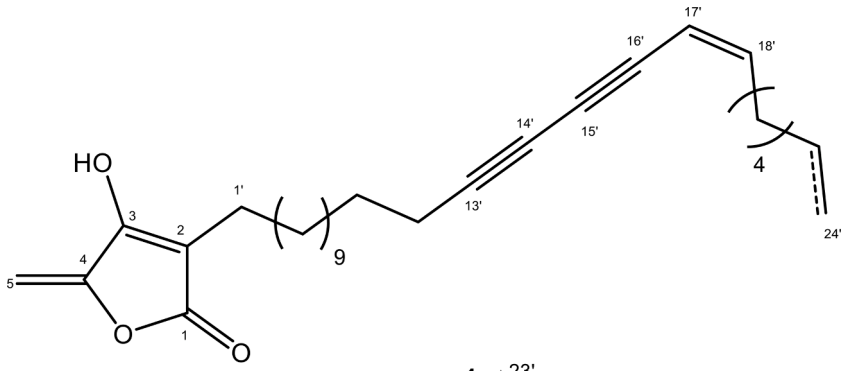
Plant material

Seeds from *Porcelia ponderosa* were collected in the region of Amazon Forest, city of Tarauacá – Acre State, Brazil (8°10′02.1″S and 70°53′01.0″W) in March 2020. The plant material was identified and a voucher has been deposited at the Herbarium of Federal University of Acre, Southern Cross Campus, under the number CFCZS M.C.Souza 791, and received a registration code at SisGen A4123E4.

*Extraction and obtention of compounds **1** + **2***

Dried and milled seeds (480 g) were defatted with hexanes (8 × 400 ml) and sequentially extracted with CH_2Cl_2 (10×450 ml) at room temperature. The combined organic solution was concentrated under reduced pressure to give 9.3 g of CH_2Cl_2 extract and 2.0 g of a precipitate that showed to be composed for a mixture of **1** + **2** (in a ratio 77:23) by HPLC (C_{18} column, CH_3CN:H_2O 85:15 at 45 °C, flow rate 1.0 ml/min, and detection at 210 nm).

*3-Hydroxy-4-methylene-2-(tetracosa-17′Z,23′-diene-13′,15′-diynyl)-but-2-enolide (**1**).* White amorphous solid (77 %, mixture with **2**); R_t (HPLC): 12.8 min; UV (CH_3CN/H_2O) λ_{max} 283, 267, 252 nm; IR ν_{max} 2918, 2848, 1723, 1632, 1607, 1464, 1270, 1253, 1062 cm^{-1}; ^{1}H NMR (500 MHz, $CDCl_3$) δ 6.00 (dt, 10.9, 7.5, H-18′), 5.74 (ddt, 17.1, 10.5, 6.6, H-23′), 5.48 (brd d, 10.9, H-17′) 5,10 (d, 2.9, H-5a), 5.03 (d, 2.9, H-5b),



1 $\Delta^{23'}$

2

Fig. 1. Structures of γ-lactones 1 and 2 obtained from *P. ponderosa* seeds.

5.02 (m, H-24′a), 4.97 (m, H-24′b), 2.41 (q, 7.4, H-19′), 2.30 (t, 7.1, H-12′), 2.24 (t; 7.5, C-1′), 2.14 (q, 7.2, C-22′), 1.52 (m, H-11′), 1.23 (s, H-2′/H-10′ and H-20′ and H-21′); ^{13}C NMR (125 MHz, $CDCl_3$) 171.5 (C-1), 161.7 (C-3), 150.0 (C-4), 146.7 (C-18′), 137.7 (C-23′), 115.3 (C-24′), 108.8 (C-17′), 105.7 (C-2), 91.9 (C-5), 85.3 (C-13′), 78.7 (C-16′), 72.0 (C-15′), 65.2 (C-14′), 33.0 (C-22′), 29.8 (C-19′), 29.1 – 30.0 (C-2′/C-10′, C-20′ and C-21′), 28.1 (C-11′), 21.6 (C-1′), 19.7 (C-12′); ESI-HRMS *m/z* 435.2918 $[M - H]^-$ (calcd for $C_{29}H_{39}O_3$ 435.2899) and 549.2854 $[M + CF_3COO^-]$ (calcd for $C_{31}H_{40}O_5F_3$ 549.2827) and $[2\ M - H]^-$ at 871.5904 (calcd for $C_{58}H_{79}O_6$ 871.5876).

*3-Hydroxy-4-methylene-2-(tetracos-17′Z-ene-13′,15′-diynyl)-but-2-enolide (**2**)*. White amorphous solid (23 %, mixture with **1**); R_t (HPLC): 17.1 min; UV λ_{max} (CH_3CN/H_2O) 283, 267, 252 nm; IR ν_{max} 2918, 2848, 1723, 1632, 1607, 1464, 1270, 1253, 1062 cm^{-1}; ^{1}H NMR (500 MHz, $CDCl_3$) δ 6.00 (dt, 10.8, 7.5, H-18′), 5.48 (brd d, 10.9, H-17′) 5.10 (d, 2.9, H-5a), 5.03 (d, 2.9, H-5b), 2.41 (q, 7.4, H-19′), 2.30 (t, 7.1, H-12′), 2.24 (t; 7.5, C-1′), 2.14 (q, 7.2, C-22′), 1.52 (m, H-11′), 1.23 (s, H-2′/H-10′ and H-20′/H-23′), 0.88 (t, 7.0, H-24′); ^{13}C NMR (125 MHz, $CDCl_3$) 171.5 (C-1), 161.7 (C-3), 150.0 (C-4), 146.7 (C-18′), 108.8 (C-17′), 105.7 (C-2), 91.9 (C-5), 85.3 (C-13′), 78.7 (C-16′), 72.0 (C-15′), 65.2 (C-14′), 29.8 (C-19′), 29.1 – 30.0 (C-2′/C-10′ and C-20′/C-23′), 28.1 (C-11′), 21.6 (C-1′), 19.7 (C-12′), 14.0 (C-24′); ESI-HRMS *m/z* 437.3072 $[M - H]^-$ (calcd for $C_{29}H_{41}O_3$ 437.3056), 551.3008 $[M + CF_3COO^-]$ (calcd for $C_{31}H_{42}O_5F_3$ 551.2984) and $[2\ M - H]^-$ at 875.6201 (calcd for $C_{58}H_{83}O_6$ 875.6189).

Animals and parasites

The life cycle of *S. mansoni* (BH strain) is maintained by routine passage through *Biomphalaria glabrata* snails and Swiss mice at Guarulhos University (UNG, Guarulhos, SP, Brazil). Female Swiss mice, three weeks old, were purchased from Anilab (São Paulo, Brazil). Both snails and mice were kept under environmentally controlled conditions (25 °C and humidity of 50 %), with free access to water and food. Cercariae of *S. mansoni* were obtained from infected intermediate host snails in our laboratories as described previously (Pavani et al., 2023).

In vitro anthelmintic assay

Adult male and female parasites were recovered by perfusion from Swiss mice that had been infected with 100 cercariae, 42 days previously. Next, parasites were placed in RPMI 1640 medium supplemented with 10 % fetal bovine serum, containing 100 μg/ml streptomycin and 100 IU/ml penicillin (Vitrocell, Campinas, SP, Brazil) and were incubated in a 24-well culture plate (Corning, New York, NY, USA) containing one pair of parasites *per* well. The mixture of **1** + **2** (77:23) were dissolved in DMSO and then were tested using serial dilution from 200 μg/ml for determination of their 50 % effective concentration (EC_{50}). Each concentration was tested in triplicate. Parasites were kept for 72 h (37 °C, 5 % CO_2), and their viability was monitored microscopically. Negative control (0.5 % DMSO) and positive control (praziquantel) were included in the bioassays. During the culture time period, parasites were monitored using an inverted light microscope (BEL Engineering INV 100, Monza [MB], Italy) and a stereomicroscope (Leica Microsystems EZ4E, Wetzlar, Germany) (Brito et al., 2022). To evaluate the stability of tested compounds in the used vehicle, the mixture of **1** + **2** (77:23 - 20 mg) was dissolved in DMSO-d_6 (600 μl) and ^{1}H NMR spectra were recorded in a period of 36 h (0 min, 15 min, 30 min, 1 h, 12 h, 24 h, and 36 h).

Cytotoxicity assay

The cytotoxicity assay was conducted using Vero cells obtained from the ATCC. Vero cells were maintained in 25 cm^2 tissue culture flasks (Corning) with DMEM supplemented with 10 % heat-inactivated fetal calf serum and 2 mM l-glutamine. For the experimental procedure, cells were seeded in 96-well plates (Corning) using DMEM supplemented with 10 % heat-inactivated fetal calf serum and 2 mM l-glutamine (Oliveira et al., 2024). After 24 h of cell adhesion at 37 °C and 5 % CO_2, the cells were treated with a concentration range of 100 - 1000 μg/ml of the mixture of **1** + **2** (77:23). Following 72 h of incubation, MTT solution was added to each well, and the plates were further incubated for 3 h. Subsequently, the absorbance of the formazan product was measured at 595 nm using an Epoch Microplate Spectrophotometer (BioTek Instruments, Winooski, VT, USA) (Mengarda et al., 2021; Sessa et al., 2020; Silva et al., 2017). The assay was performed in triplicate and repeated three times. The results were expressed as a percentage of the control, and the CC_{50} values were determined. SI values for tested compounds were calculated by dividing the CC_{50} values obtained on Vero cells by the CC_{50} values determined on *Schistosoma mansoni*.

Toxicity assay using Caenorhabditis elegans

The toxicity assay using the nematode *C. elegans* was conducted following established procedures (Rocha et al., 2023). *C. elegans* (strain N2) was maintained at 22 °C on NGM agar plates seeded with the *Escherichia coli* strain OP50, as *per* standard protocols (Stiernagle, 2006). For the experimental procedure, L4-stage worms were transferred to individual wells of a 96-well plate, with approximately 25 worms per well. Each well contained a solution consisting of 60 % M9 buffer, 10 μg/ml cholesterol, and 40 % BHI medium. The mixture of **1** + **2** (77:23) was added to each well at concentrations ranging from 100 to 1000 μg/ml, with triplicate wells for each concentration. The nematodes were then incubated at 22 °C for 24 h, after which their viability was assessed using a Motic inverted microscope (AE2000, Canada). Worm survival was determined based on mobility and morphology, with rigid, stick-shaped organisms considered dead, and those displaying sinusoidal, worm-like movement classified as alive. The experiments were independently replicated three times to ensure the reproducibility and reliability of the results.

In vivo antiparasitic studies

For *in vivo* efficacy studies, three-week-old female Swiss mice were subcutaneously infected with 80 *S. mansoni* cercariae each. At 42 days post-infection, during the patent infection phase, the mice were divided into three independent experiments and received three different doses (400, 200 and 100 mg kg^{-1}) of the mixture of **1** + **2** (77:23), dissolved in 150 μl of Tween 80 %, 350 μl of PBS, and 500 μl of EtOH, using 11 mice. In each experiment, a control group of three to five infected mice was treated with the vehicle only. Following standard and international protocol, all treated mice were weighed, euthanized, and dissected 56 days postinfection. For measurement of worm burden, schistosomes were collected from the hepatic portal system and mesenteric veins, sexed, and counted. Therapeutic efficacy was also based on the technique of qualitative and quantitative oograms in the intestine, as well as the Kato-Katz method for quantitative fecal examination for additional evaluation of the therapeutic efficacy (Brito et al., 2022). To evaluate the stability of the tested compounds in the used vehicle, the mixture of **1** + **2** (77:23 – 3 mg) was dissolved in a solution composed by Tween 80 % (7.5 μl), PBS (17.5 μl) and EtOH (25.0 μl) and was incubated at 25 °C during 60 min. Sequentially, the solution was extracted with EtOAc (50 μl) and the organic phase was dried over Na_2SO_4. After filtration and evaporation of the solvent under reduced pressure, the pellet was resuspended in CH_3CN (1.0 mg/ml) and analyzed by HPLC (C_{18} column).

Randomization and blinding

For the *in vivo* testing, animals were randomly assigned to experimental groups, and pharmacological treatments were randomly counterbalanced. The euthanasia of the animals within each group was also performed randomly. While the investigators were not blinded to the

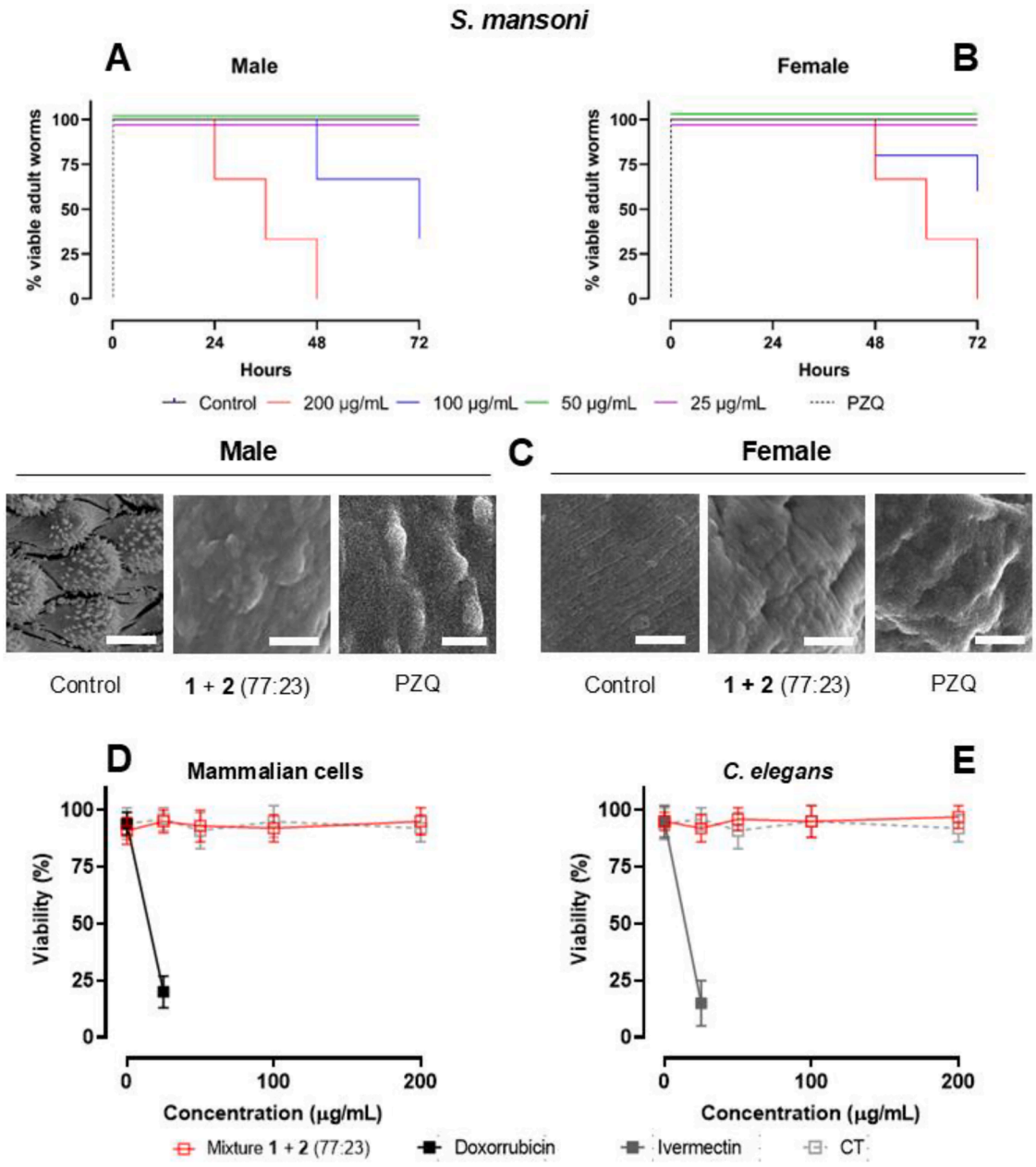


Fig. 2. (A) Viability of *in vitro* adult *Schistosoma mansoni* male worms following exposure to the mixture of **1** + **2** (77:23) as well as PZQ. (B) Viability of *in vitro* adult *Schistosoma mansoni* female worms following exposure to the mixture of **1** + **2** (77:23) as well as PZQ. (C) Scanning electron microscopy investigation of adult male and female *S. mansoni* following incubation with the mixture of **1** + **2** (77:23) as well as PZQ. (D) *In vitro* cytotoxicity assay of Vero cells following exposure to the mixture of **1** + **2** (77:23). Doxorubicin was used as a standard drug and vehicle-treated cells were used as control. (E) *In vivo* cytotoxicity assay using *C. elegans* following exposure to the mixture of **1** + **2** (77:23). Ivermectin was used as standard drug and vehicle-treated cells were used as control.

treatment groups, measures were taken to mitigate bias. Specifically, different individuals conducted the assessments for all parameters, including (i) worm counts, (ii) analysis of egg stages in the intestine (oogram), and (iii) fecal egg counts. Furthermore, data analysis was conducted by two independent investigators who were not involved in the experimental procedures, thus minimizing potential bias in interpretation.

Statistical analysis

Statistical analysis was conducted using GraphPad Prism 8.0 software. The CE_{50} and CC_{50} values were determined from sigmoid dose-response curves. For *in vivo* studies, the Kruskal-Wallis nonparametric test was employed. A significance threshold of $p < .05$ was applied for determine statistical significance.

Ethical statement

Animal studies are reported in compliance with the National Centre for the Replacement and Refinement & Reduction of Animals in Research (NC3Rs) ARRIVE guidelines. All experiments were conducted in conformity with the Brazilian law for Guidelines for Care and Use of Laboratory Animals [Law 11790/2008]. The protocol for experimental design was approved by the *Comissão de Ética no Uso de Animais* (CEUA) at Universidade Guarulhos (São Paulo, Brazil; protocol ID 47/20).

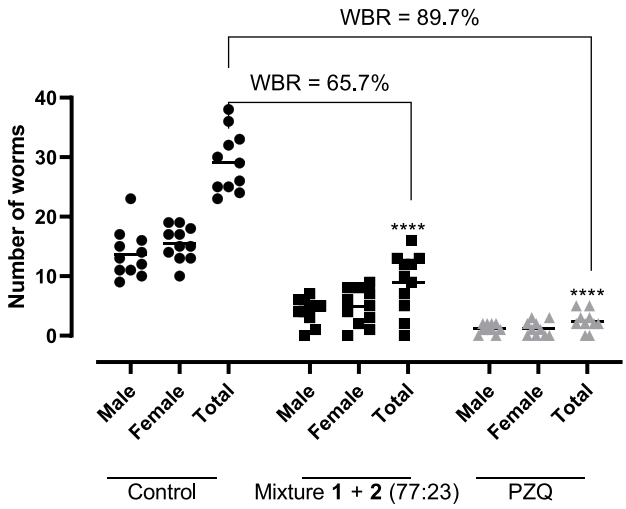


Fig. 3. Effect of the mixture of **1** + **2** (77:23) on the parasite burden of mice infected with *S. mansoni*. The mixture of compounds was administered orally using a single dose (400 mg kg^{-1}, 42 days after infection) to mice harboring adult *S. mansoni*. On day 56 postinfection, all animals were euthanized, and parasite burdens were determined by sex (male and female schistosomes). Each point represents data from individual mice across three independent experiments (11 animals per experimental group), with horizontal bars indicating the median values. Praziquantel (PZQ) was used as a standard drug. ****$p < .0001$.

Results and discussion

The precipitate formed during the concentration of CH_2Cl_2 extract from seeds of *P. ponderosa* appeared to be homogeneous by TLC and showed to be composed of two substances - **1** (R_t = 12.8 min, 77 %) and **2** (R_t = 17.1 min, 23 %) by HPLC analysis. Despite an excellent resolution being detected using a C_{18} analytic column, the purification of compounds **1** and **2** was not possible by semi-preparative HPLC procedures due to the degradation observed during the process of elimination of the solvents used in the chromatographic procedures (CH_3CN/H_2O). Similarly, the use of a normal phase column conducted irreversible adsorption and further decomposition of **1** and **2**. Therefore, chemical characterization and biological assays, including *in vivo* approach, were performed using the mixture of **1** + **2** (77:23) since no degradation process was observed to precipitated obtained from seeds of *P. ponderosa*.

*Chemical characterization of **1** and **2** from seeds of P. ponderosa*

ESI-HRMS (negative mode) spectrum of compound **1** exhibited [M - H]$^-$ and [2 M - H]$^-$ ion peaks at *m/z* 435.2918 and 871.5904, respectively, indicating a molecular formula $C_{29}H_{40}O_3$ with ten unsaturation. Similarly, the spectrum of compound **2** showed two peaks at *m/z* 437.3072 and 875.6201, corresponding to molecular formula $C_{29}H_{42}O_3$, with nine unsaturation. UV spectra of both compounds showed absorptions at λ_{max} 252, 267, and 283 nm, characteristic of a conjugated enediyne system (Bousserouel et al., 2012). ^{1}H NMR spectrum of mixture **1** + **2** showed two coupled doublets at δ 5.03 and δ 5.10 (J = 2.9 Hz), assigned to the geminal hydrogens H-5a and H-5b of a 3-hydroxy-γ-lactone system, similar to those isolated from *P. macrocarpa* (Oliveira et al., 2019). This spectrum showed also signals at δ 5.48 (d, J = 10.9 Hz, H-17′) and 6.00 (dt, J = 10.9 and 7.5 Hz, H-18′), attributed to the hydrogens of the enediyne system in the aliphatic side chain, with *Z* configuration (Bousserouel et al., 2012; Wongsa et al., 2011). Additional peaks at δ 5.74 (ddt, J = 17.1, 10.5 and 6.6 Hz, H-23′) and δ 4.97/5.02 (m, H-24′a and H-24′b) were assigned to a terminal double bond to compound **1**, whereas the triplet at δ 0.88 (J = 7.0 Hz, H-24′) corresponds to the methyl group of the saturated derivative (**2**), corroborating with the results observed in the analysis of mass spectra to each compound. In the ^{13}C NMR spectrum of the mixture of **1** + **2**, the presence of a 3-hydroxy-γ-lactone moiety was confirmed by the signals at δ 171.5 (C-1), 161.7 (C-3), 150.0 (C-4), 105.7 (C-2), and 91.9 (C-5) (Oliveira et al., 2019). The signals corresponding to *sp* carbons of the enediyne system were observed at δ 85.3 (C-13′), 78.7 (C-16′), 72.0 (C-15′), and 65.2 (C-14′) whereas those assigned to the carbons of a conjugated Z double bond were observed at δ 146.7 (C-18′) and 108.8 (C-17′) (Bousserouel et al., 2012; Wongsa et al., 2011). Furthermore, signals of sp^2 carbons for the terminal double bond (compound **1**) were observed at δ 137.7 (C-23′) and 115.3 (C-24′) (**1**), while the signal at δ 14.0 was assigned to methyl (C-24′) for compound **2**. Finally, the

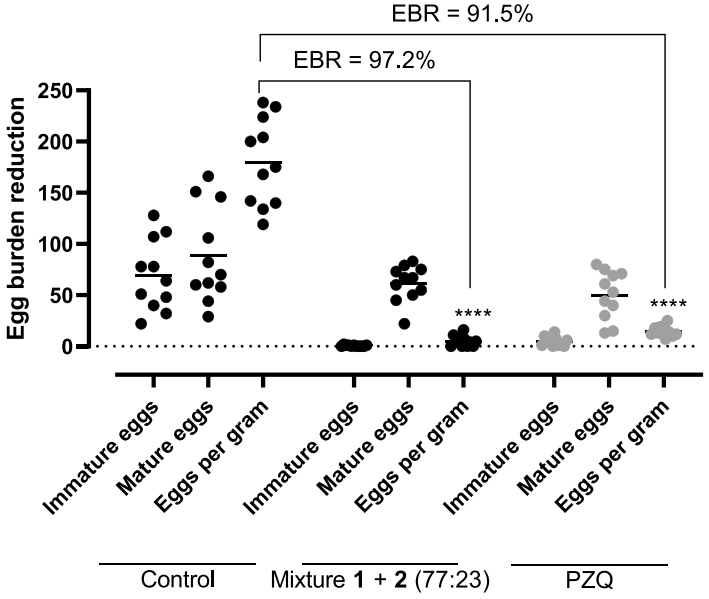


Fig. 4. Effect of the mixture of **1** + **2** (77:23) on the parasite burden of mice infected with *S. mansoni*. The mixture of compounds was administered orally using a single dose (400 mg kg^{-1}, 42 days after infection) to mice harboring adult *S. mansoni*. On day 56 postinfection, all animals were euthanized. Egg burdens were quantified using oogram analysis for intestinal eggs and the Kato-Katz technique for fecal eggs. Each point represents data from individual mice across three independent experiments (11 animals per group), with horizontal bars indicating median values. Praziquantel (PZQ) was used as a standard drug. ****$p < .0001$.

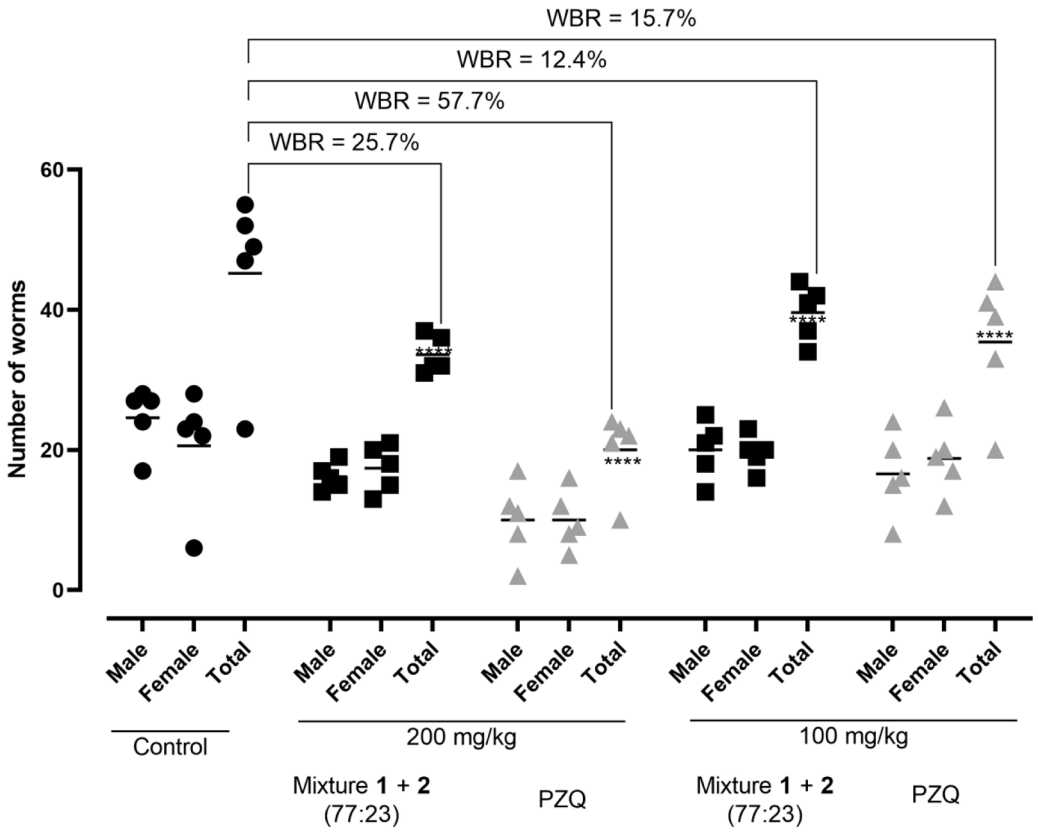


Fig. 5. Effect of the mixture of **1** + **2** (77:23) on the parasite burden of mice infected with *S. mansoni*. The mixture of compounds was administered orally using two different doses (200 and 100 mg kg^{-1}, 42 days after infection) to mice harboring adult *S. mansoni*. On day 56 postinfection, all animals were euthanized, and parasite burdens were determined by sex (male and female schistosomes). Each point represents data from individual mice across three independent experiments (5 animals per experimental group), with horizontal bars indicating the median values. Praziquantel (PZQ) was used as a standard drug. ****$p < .0001$.

positioning of enediyne system was proposed between C-13′ and C-18′ to both compounds **1** and **2** by analysis of the fragmentation profile in the respective MS/MS spectra due to the presence of peaks at *m/z* 277.1837/277.1799 (fragmentation at C-12′) and 137.0250/137.0542 (fragmentation at C-2′) for compounds **1** and **2**, respectively (see supporting information). Therefore, structures of new γ-lactones were characterized as 3-hydroxy-4-methylene-2-(tetracosa-17′*Z*, 23′-diene-13′,15′-diynyl)-but-2-enolide (**1**) and 3-hydroxy-4-methylene-2-(tetracos-17′*Z*-ene-13′,15′-diynyl)-but-2-enolide (**2**).

*In vitro treatment with the mixture of **1** + **2** (77:23) impacts the viability of adult S. mansoni*

To conduct the *in vitro* bioassay, samples containing the mixture of **1** + **2** (77:23) as well as positive control praziquantel were dissolved in DMSO and then were tested using serial dilution from 200 μg/ml for determination of EC_{50} values. As depicted in Fig. 2, control parasites-maintained viability throughout the observation period, while praziquantel induced immediate mortality in all worms. In contrast, the mixture of **1** + **2** (77:23) exhibited mortality in a time and concentration-dependent manner. Notably, at the highest concentration tested (200 μg/ml), all male schistosomes succumbed within 48 h (Fig. 2A), followed by female parasites within 72 h (Fig. 2B). Intriguingly, the treatment initially targeted male worms before affecting females, consistent with findings from previous studies suggesting a sex-specific susceptibility to anthelmintic compounds (Brito et al., 2022; Costa et al., 2023). Calculation of the EC_{50} values after 72 h of incubation afforded 83.3 μg/ml for the mixture of **1** + **2** (77:23), for both male and female parasites, indicating a reduced *in vitro* potency in comparison to positive control praziquantel (EC_{50} of 1.5 μg/ml). A possible decomposition of γ-lactones in DMSO was discarded since no differences were observed in the recorded ^{1}H NMR spectra for the mixture of **1** + **2** (77:23) in a period of 36 h (0 min, 15 min, 30 min, 1 h, 12 h, and 36 h - see supporting information).

However, scanning electron microscopy analysis of *S. mansoni* adult parasites exposed to the mixture of **1** + **2** (77:23) at lethal concentrations (100 and 200 μg/ml) revealed significant morphological changes in the tegumental surfaces of both male and female worms. Control parasites exhibited intact surface structures, while those exposed to the mixture of **1** + **2** (77:23) displayed severe alterations such as swelling, sloughing, and shortening of tubercles. Even at lower concentrations, substantial tegumental disruption was evident, suggesting a dose-dependent effect of tested mixture of **1** + **2** (77:23). These findings are consistent with observations from other studies investigating the effects of various natural compounds such as the alkaloid piplartine (Mengarda et al., 2020) and the lignoid dehydrodieugenol B (Rocha et al., 2023) on the tegument of *S. mansoni*. Thus, morphological alterations induced by enediyne γ-lactones may not only contribute to parasite death but also expose worm antigens to the host immune system, potentially enhancing the efficacy of the treatment (Doenhoff et al., 1987; Roquini et al., 2023).

*Assessment of safety profile in vitro and in vivo of mixture of **1** + **2** (77:23)*

In the present study, a mammalian cell line (Vero) was employed to determine the cytotoxic concentrations required to inhibit 50 % of cell growth (CC_{50}) and the SI value for the mixture of **1** + **2** (77:23). Additionally, an *in vivo* toxicity assay was conducted using the model organism *Caenorhabditis elegans*. Encouragingly, the mixture of **1** + **2**

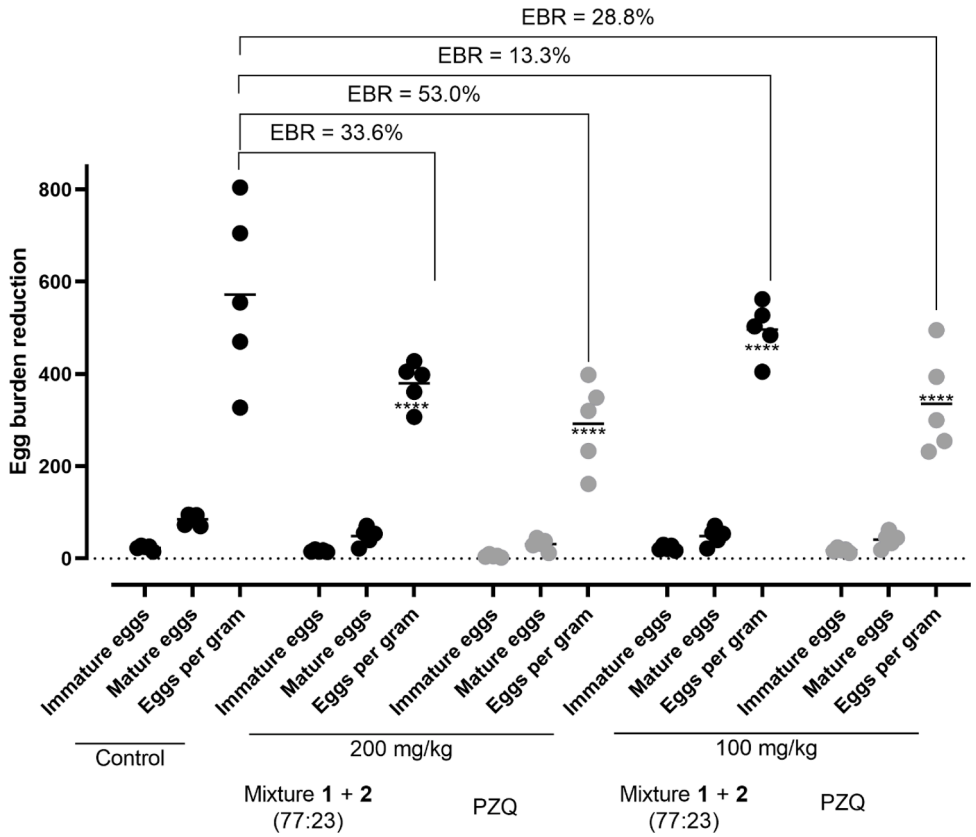


Fig. 6. Effect of the mixture of **1** + **2** (77:23) on the parasite burden of mice infected with *S. mansoni*. The mixture of compounds was administered orally using two different doses (200 and 100 mg kg^{-1}, 42 days after infection) to mice harboring adult *S. mansoni*. On day 56 postinfection, all animals were euthanized. Egg burdens were quantified using oogram analysis for intestinal eggs and the Kato-Katz technique for fecal eggs. Each point represents data from individual mice across three independent experiments (5 animals per group), with horizontal bars indicating median values. Praziquantel (PZQ) was used as a standard drug. ****$p < .0001$.

(77:23) demonstrated no toxicity against Vero cells, with CC_{50} values exceeding 500 μg/ml (Fig. 2D). Consequently, this mixture exhibited a favorable SI value, greater than 6. Moreover, *in vivo* toxicity assessments using *C. elegans* revealed no adverse effects associated with the mixture of **1** + **2** (77:23), as showed on Fig. 2E. These findings are highly promising and underscore the potential of tested mixture as a candidate for further investigation in antischistosomal drug development. The absence of cytotoxicity both *in vitro* and *in vivo* enhances the attractiveness of γ-lactones from *P. ponderosa* as a safe and effective therapeutic agent against schistosomiasis.

*Oral administration of the mixture of **1** + **2** (77:23) at standard dose (400 mg kg^{-1}) used in the murine model of schistosomiasis resulted in a notable reduction in both worm and egg burden*

Despite the reduced *in vitro* anthelmintic activity of γ-lactones from *P. ponderosa*, this mixture displayed a favorable toxicity profile. Thus, the evaluation of the mixture of **1** + **2** (77:23) in a murine infection model, specifically targeting the patent stage of the disease, was performed. Initially, a single oral dose of 400 mg kg^{-1} of tested mixture was administered to the mice following the standard protocol for experimental schistosomiasis in a murine model (Lago et al., 2018; 2019). The primary parameter assessed was the reduction in worm burden. Upon counting and sexing the worms, the data were graphed, illustrating individual values categorized by male, female, and total worms (Fig. 3). The analysis revealed a substantial decrease in total worm burden in animals treated with mixture **1** + **2** (77:23) compared to the control (vehicle-treated animals), achieving a reduction of 65.7 %. This reduction is noteworthy, particularly when compared to the standard drug praziquantel, which typically reduces 90 % of the parasite load. Previous studies demonstrated that different natural products considered actives against *S. mansoni* using an *in vivo* approach (at 400 mg kg^{-1} and same route of administration) displayed lower or similar values of worm burden reduction in comparison to the mixture of **1** + **2** (77:23) from *P. ponderosa*, such as cardamonin - 46 % (Carvalho et al., 2021), licarin A - 50 % (Mengarda et al., 2021), dehydrodieugenol B - 50 % (Rocha et al., 2023), epiisopilosine - 58 % (Guimarães et al., 2018), 15β-senecioyl-oxy-*ent*-kaur-16-en-19-oic acid - 62 % (Sessa et al., 2020), piplartine - 60 % (Mengarda et al., 2020), and nerolidol - 70 % (Silva et al., 2017). Furthermore, the mixture of **1** + **2** (77:23) demonstrated a significant reduction in *S. mansoni* egg production. In a patent infection, the production of parasite eggs plays a pivotal role in inducing an inflammatory response as they migrate from the blood vessels to the intestine or bladder for transmission (McManus et al., 2018). Therefore, reducing egg load is considered a crucial parameter for evaluating the efficacy of anthelmintic drug candidates (Brito et al., 2022). Evaluation of EBR revealed a substantial decrease of 99.21 % in immature eggs in the intestine and 97.26 % in EPG in the feces following oral treatment with the mixture of **1** + **2** (77:23) (Fig. 4). This significant reduction suggests not only a decrease in egg laying by females but also correlates with the observed reduction in worm burden, further highlighting the efficacy of the mixture of **1** + **2** (77:23) administered at 400 mg kg^{-1}, the standard dose used in the murine model of schistosomiasis (Lago et al., 2018; 2019), as a potential anthelmintic agent. Additionally, reductions in worm burden (25.7 and 12.4 %) and egg production (33.6 and 13.3 %) were also observed (Fig. 5 and 6) when the animals were treated using 200 and 100 mg kg^{-1} of the mixture of **1** + **2** (77:23).

Finally, no differences were observed in the HPLC chromatograms registered before and after incubation of mixture of **1** + **2** (77:23) with the vehicle (see supporting information), indicating that both γ-lactones

are stable in the tested solution. This result suggests that, in future studies, the bioactive lactones **1** + **2** can be administered using different delivery systems including nanocarriers such as liposomes, micelles, and nanoemulsions (Solís-Cruz et al., 2021). Altogether, obtained results underscore the potential of enediyne γ-lactones from *P. ponderosa* seeds as promising candidates for the development of novel therapeutic approaches against schistosomiasis.

Conclusion

In conclusion, the results of this study highlight the promising antischistosomal activity of a mixture of two new chemically related γ-lactones **1** and **2**, obtained from seeds of *P. ponderosa* seeds. *In vitro* assays demonstrated mortality of *S. mansoni* worms in a time and concentration-dependent manner, with the mixture of **1** + **2** (77:23) exhibiting reduced effects on both male and female parasites. Otherwise, *in vivo* studies using a murine infection model revealed a substantial reduction in both worm burden and egg production following oral administration of the mixture of **1** + **2** (77:23). Importantly, the tested mixture demonstrated no signs of toxicity in mammalian cell lines or in the model organism *C. elegans*, indicating its favorable safety profile. These findings underscore the potential of *P. ponderosa* seeds as a promising source for the development of novel therapeutic natural products against schistosomiasis, especially γ-lactone derivatives. Further research is warranted to elucidate the underlying mechanisms of action and to assess its efficacy in clinical settings, including the use of nanocarriers and other related delivery systems.

CRediT authorship contribution statement

Dalete Christine S. Souza: Writing – original draft, Visualization, Validation, Methodology, Investigation, Formal analysis, Data curation. **Carlos H. Totini:** Writing – original draft, Validation, Supervision, Methodology, Investigation, Formal analysis, Data curation. **Rayssa A. Cajás:** Validation, Supervision, Investigation, Data curation. **Thainá R. Teixeira:** Visualization, Methodology, Investigation, Formal analysis. **Emerson A. Oliveira:** Visualization, Supervision, Resources, Methodology, Formal analysis. **Maria E. Cirino:** Supervision, Methodology, Investigation, Data curation. **Maria C. Souza:** Writing – original draft, Validation, Resources, Investigation, Formal analysis. **Maria C. Salvadori:** Supervision, Software, Investigation, Funding acquisition, Data curation. **Fernanda S. Teixeira:** Software, Methodology, Investigation, Formal analysis. **Josué de Moraes:** Writing – review & editing, Writing – original draft, Visualization, Supervision, Resources, Project administration, Investigation, Funding acquisition, Formal analysis, Data curation, Conceptualization. **João Henrique G. Lago:** Writing – review & editing, Writing – original draft, Visualization, Supervision, Resources, Project administration, Methodology, Investigation, Funding acquisition, Formal analysis, Data curation, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgments

This work was supported by the Fundação de Amparo à Pesquisa do Estado de São Paulo (FAPESP, grant 2023/08418-6 and 2023/12447-1). J.M. and J.H.G.L. also received the established investigator fellowship from *Conselho Nacional de Desenvolvimento Cientifico e Tecnologico* (CNPq). This study was financed in part by the Coordenação de Aperfeiçoamento de Pessoal de Nivel Superior (CAPES), finance code 001. The funding institutions did not have any role in study design, data collection, data analysis, interpretation, or writing of the report in this study.

Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.phymed.2024.156045.

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