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Psilostachyin C: a natural compound with trypanocidal activity

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ABSTRACT

In this study, the antiprotozoal activity of the sesquiterpene lactone psilostachyin C was investigated. This natural compound was isolated from *Ambrosia scabra* by bioassay-guided fractionation and was identified by spectroscopic techniques. Psilostachyin C exerted in vitro trypanocidal activity against *Trypanosoma cruzi* epimastigotes, trypomastigotes and amastigotes, with 50% inhibitory concentration (IC_{50}) values of 0.6, 3.5 and 0.9 μg/mL, respectively, and displayed less cytotoxicity against mammalian cells, with a 50% cytotoxic concentration (CC_{50}) of 87.5 μg/mL. Interestingly, this compound induced ultrastructural alterations, as seen by transmission electron microscopy, in which vacuolisation and a structural appearance resembling multivesicular bodies were observed even at a concentration as low as 0.2 μg/mL. In an in vivo assay, a significant reduction in the number of circulating parasites was found in *T. cruzi*-infected mice treated with psilostachyin C for 5 days compared with untreated mice ($7.4 \pm 1.2 \times 10^5$ parasites/mL vs. $12.8 \pm 2.0 \times 10^5$ parasites/mL) at the peak of parasitaemia. According to these results, psilostachyin C may be considered a promising template for the design of novel trypanocidal agents. In addition, psilostachyin C inhibited the growth of *Leishmania mexicana* and *Leishmania amazonensis* promastigotes (IC_{50} = 1.2 μg/mL and 1.5 μg/mL, respectively).

1. Introduction

Chagas disease, caused by the protozoan parasite *Trypanosoma cruzi*, is endemic in Latin America. Approximately 16–18 million people are infected in the Americas and ca. 100 million people are at risk of contracting the disease [1]. Prophylactic and therapeutic vaccines have been pursued but sterilising immunity has not yet been achieved [2,3]. Leishmaniasis is a group of infections caused by *Leishmania* spp. Annually, 1.5–2 million people around the world are infected by the parasites and 350 million are at risk of contracting the disease [4]. Chemotherapy for the treatment of these parasitoses, which are frequently found to co-infect patients in endemic areas [5–7], has limited efficacy and is not innocuous, mainly due to resistance phenomena and adverse effects. Consequently, new drugs are needed.

In previous work, we reported the in vitro trypanocidal activity of several Argentine medicinal plant species [8]. We have isolated two bioactive sesquiterpene lactones (STLs) from *Ambrosia tenuifolia* presenting in vitro activity against *T. cruzi* epimastigotes and *Leishmania* spp. promastigotes, one of which exerted a significant in vivo trypanocidal effect [9].

Ambrosia scabra Hook. & Arn. (Asteraceae) is a closely related species popularly known as 'ajenjo del campo' and traditionally used against intermittent fevers and worm infections [8,10]. Here we report the trypanocidal and leishmanicidal activities of the STL psilostachyin C isolated from *A. scabra* by bioassay-guided fractionation. In addition, the ultrastructural changes that this compound produced in *T. cruzi* epimastigotes were evaluated.

STLs are C-15 terpenoid compounds and represent an important and biogenetically homogeneous group of secondary metabolites present in higher plants [11]. They display great diversity and an enormously broad spectrum of biological activities, including antiprotozoal activity [12–15]. The discovery of artemisinin (an antimalarial STL isolated from the Chinese herb *Artemisia annua)* has been a major breakthrough in the field of parasitic diseases and has prompted the investigation of these kinds of compounds. In particular, psilostachyin C is a dilactone of the ambrosanolide type that was first isolated from *Ambrosia psilostachya* [16] and

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subsequently from other *Ambrosia* spp. [17]. It has been demonstrated to have molluscicidal activity [18] and inhibitory activity on the G_2 DNA damage checkpoint [19]. However, this is the first time that this compound has been found in *A. scabra* and the first report of its trypanocidal and leishmanicidal activities.

2. Methods

2.1. Plant material

Ambrosia scabra was collected in Buenos Aires, Argentina, in 2007 and was identified by Dr G. Giberti (Museo de Farmacobotánica, Facultad de Farmacia y Bioquímica, Universidad de Buenos Aires, Buenos Aires, Argentina). A voucher specimen (BAF 650) was deposited at the Herbarium of the Museo de Farmacobotánica.

2.2. Bioassay-guided fractionation

Extraction of the aerial parts of *A. scabra* (500 g) was done by maceration with dichloromethane:methanol (1:1) at room temperature. The organic extract was subjected to open column chromatography over silica gel 60 and was eluted successively with cyclohexane, cyclohexane:ethyl acetate (1:1), ethyl acetate and methanol to give 23 fractions of 500 mL each. According to their profile in thin-layer chromatography, these fractions were combined into five final fractions (F_{1AS}–F_{5AS}) and were subsequently tested for trypanocidal activity against *T. cruzi* epimastigotes. Fraction F_{5AS} was chromatographed on a silica gel column eluted with a cyclohexane:CH_2Cl_2 gradient (100:0 to 0:100), CH_2Cl_2:ethyl acetate gradient (100:0 to 0:100) and 100% methanol to obtain 150 fractions ($F_{5AS\ 1–150}$) of 10 mL each. Of these fractions, $F_{5AS\ (75–77)}$ essentially contained one pure compound that crystallised from ethyl acetate.

2.3. Spectrometric analyses

The isolated compound was identified by proton nuclear magnetic resonance (^{1}H NMR) and carbon NMR (^{13}C NMR) (Inova NMR spectrometer; Varian, Palo Alto, CA) (500 MHz in $CDCl_3$), heteronuclear single quantum correlation (HSQC), heteronuclear multiple bond correlation (HMBC), correlated spectroscopy (COSY), electron impact-mass spectrometry (EI-MS) (Agilent 5973) and infrared spectroscopy (Bruker FT-IR IFS25).

2.4. Cell cultures

Trypanosoma cruzi epimastigotes (RA strain) were grown in biphasic medium. *Leishmania mexicana* promastigotes (MNYC/BZ/62/M strain) and *Leishmania amazonensis* promastigotes (IFLA/BR67/PH8 strain) were grown in liver infusion tryptose (LIT) medium. *Trypanosoma cruzi* and *Leishmania* spp. cultures were routinely maintained by weekly passage at 28 °C and 26 °C, respectively.

Trypanosoma cruzi bloodstream trypomastigotes were obtained from infected CF1 mice by cardiac puncture at the peak of parasitaemia on Day 15 post infection. Trypomastigotes were routinely maintained by infecting 21-day-old CF1 mice.

2.5. Animals

Inbred male CF1 and female C3H/HeN mice were nursed at the Departamento de Microbiología, Facultad de Medicina (Universidad de Buenos Aires). Mice were housed in groups of five per cage. Mice were kept in a conventional room at 24 ± 1 °C with free access to a standard commercial diet and water ad libitum under a 12 h light/12 h dark cycle. All procedures were approved by the Ethics Review Board of the Instituto de Estudios de la Inmunidad Humoral (IDEHU-CONICET) and were conducted in accordance with the guidelines established by the National Research Council [20].

2.6. In vitro evaluation of antiprotozoal activity

Growth inhibition of *T. cruzi* epimastigotes as well as *L. mexicana* and *L. amazonensis* promastigotes was evaluated by a [^{3}H] thymidine uptake assay according to Sülsen et al. [21]. Fractions F_{1AS}–F_{5AS} were tested at 10 μg/mL and 100 μg/mL, and the pure compound and fraction F_{5AS} were tested at concentrations ranging from 0.3 μg/mL to 100 μg/mL. Cell density was adjusted to 1.5×10^6 parasites/mL and cells were cultivated in the presence of each fraction or the pure compound for 72 h or 120 h. Benznidazole (1.3–20.8 μg/mL) (Roche, Rio de Janeiro, Brazil) and amphotericin B (0.025–0.8 μg/mL) (ICN, Costa Mesa, CA) were used as controls for *T. cruzi* and *Leishmania* spp. growth inhibition, respectively. Percentage inhibition was calculated as 100–{[(cpm of treated parasites)/(cpm of untreated parasites)] × 100}, and 50% inhibitory concentration (IC_{50}) values were estimated by the Alexander method [22].

To determine whether the parasites could recover after treatment, *T. cruzi* epimastigotes were incubated with the isolated compound (0.2–2.5 μg/mL) for 24 h. Parasites were then centrifuged at 6000 rpm for 10 min, washed once with phosphate-buffered saline (PBS) (NaCl 0.15 M, NaH_2PO_4 0.02 M, NaOH 0.017 M, pH 7.2) and were incubated in fresh medium for 6 days.

The pure compound was also tested on bloodstream trypomastigotes as previously described [9]. Parasite concentration was adjusted to 1.5×10^6 parasites/mL by diluting mouse blood containing trypomastigotes in complete LIT medium. Parasites were seeded (150 μL/well) in duplicate in a 96-well microplate and 2 μL of the compound (0.1–100 μg/mL) or control drug (benznidazole) (0.4–900 μg/mL) was added per well. Plates were incubated for 24 h and the remaining live parasites were counted in a haemocytometer. Percentage inhibition was calculated as 100–{[(live parasites in wells after compound treatment)/(live parasites in untreated wells)] × 100}.

To evaluate the effect of the isolated compound on intracellular forms of *T. cruzi*, 96-well plates were seeded with murine peritoneal macrophages at 5×10^3 per well in 100 μL of culture medium and were incubated for 2 h at 37 °C in a 5% CO_2 atmosphere. Cells were washed and infected with transfected blood trypomastigotes expressing β-galactosidase [23] at a parasite:cell ratio of 10:1. After 2 h of co-culture, plates were washed twice with PBS to remove unbound parasites and the pure compound was added at 0.01–10 μg/mL per well in 150 μL of fresh complete RPMI medium without phenol red (Gibco, Rockville, MD). Controls included infected non-treated cells (100% infection control) and uninfected cells (0% infection control). The assay was developed by addition of chlorophenolred-β-D-galactopyranoside (CPRG) (100 μM) and 1% Nonidet P40, 48 h later. Plates were incubated for 4–6 h at 37 °C. Wells with galactosidase activity turned the media from yellow to red and this reaction was quantified at 570 nm in a microplate reader (Bio-Rad Laboratories, Hercules, CA). Percentage inhibition was calculated as 100–{[(absorbance of treated infected cells)/(absorbance of untreated infected cells)] × 100} and the IC_{50} value was estimated.

2.7. Cytotoxicity assay

Murine peritoneal macrophages were assayed for determination of cell viability by the MTT method. Cells (5×10^5) were settled at a final volume of 150 μL in a flat-bottom 96-well microtitre plate and were cultured at 37 °C in a 5% CO_2 atmosphere

in the absence or presence of increasing concentrations of the pure compound (1–100 μg/mL). After 48 h, 3-(4,5-dimethylthiazol-2yl)-2,5-diphenyltetrazolium bromide (MTT) was added at a final concentration of 1.5 mg/mL. Plates were incubated for 2 h at 37 °C. The purple formazan crystals were completely dissolved by adding 150 μL of ethanol and the absorbance was detected at 570 nm in a microplate reader. Results were calculated as the ratio between optical density in the presence and absence of the compound multiplied by 100. The selectivity index (SI) was calculated as the 50% cytotoxic concentration (CC_{50}) divided by the IC_{50} of the compound for *T. cruzi* trypomastigotes and amastigotes.

2.8. Effect of the compound in the presence of glutathione

Trypanosoma cruzi epimastigotes (2×10^6 parasites/mL) were treated with 1 μg/mL of the pure compound alone or in the presence of 2 mM of the reducing agent glutathione (GSH). Controls were performed with LIT medium alone or with the addition of GSH. Parasite concentration was determined at 24, 48 and 72 h by counting the cells in a Neubauer haemocytometer.

2.9. In vivo assays

Groups of five female C3H/HeN mice (6–8 weeks old; weight 23.8 ± 2.6 g) were infected with 5×10^3 bloodstream *T. cruzi* trypomastigotes by intraperitoneal injection [24–27]. Mice were treated daily with either 1 mg/kg body weight/day of the pure compound or benznidazole for five consecutive days (Days 5–10 post infection). Parasitaemia was individually monitored following red cell lysis by direct microscopic counting of parasites in 5 μL of blood using a haemocytometer. Mice mortality was recorded daily and the results were expressed as percentage of surviving animals [9].

In addition, three groups of uninfected mice were treated as described above in order to evaluate possible toxicity of the compound. On Day 13 post treatment, serum samples were collected by bleeding mice from the tail vein. Serum activities of alanine aminotransferase (ALT) and lactate dehydrogenase (LDH) were determined as markers of hepatic damage. Assays were carried out by ultraviolet spectrophotometry following the kit manufacturer's specifications (Wiener Lab, Buenos Aires, Argentina).

2.10. Transmission electron microscopy

Trypanosoma cruzi epimastigotes were treated with 0.2, 1.0 or 2.5 μg/mL of the purified compound for 24 h. Parasites were fixed with 3% glutaraldehyde and were subsequently washed three times with PBS and post-fixed with 2% osmium tetroxide (OsO_4) overnight. After washing twice in PBS, cells were stained with 1% uranyl acetate [27]. Samples were dehydrated sequentially in ethanol and acetone and were embedded in Epon 812. Ultrathin sections were examined in a Siemens Elmiskop I microscope.

2.11. Statistical analysis

Statistical analysis was carried out using GraphPad Prism 3.0 software (GraphPad Software Inc., San Diego, CA) using one-way analysis of variance (ANOVA). The log-rank test was used for survival curves. All comparisons were referred to the control group. *P*-values of <0.05 were considered significant.

3. Results

3.1. Bioassay-guided fractionation and structure elucidation

Bioassay-guided fractionation of the active organic extract of *A. scabra* yielded five fractions (F_{1AS}–F_{5AS}) that were evaluated for in vitro trypanocidal activity against *T. cruzi* epimastigotes by a [^{3}H] thymidine uptake assay. Fraction F_{5AS} was the most active, inhibiting epimastigote growth by 94.4 ± 0.4% at 10 μg/mL at 72 h (Table 1). An IC_{50} value of 4.5 μg/mL was determined by further testing F_{5AS} at concentrations ranging from 0.3 μg/mL to 100 μg/mL (Supplementary Fig. 1). Purification of this fraction by chromatographic techniques yielded a STL that was identified by spectroscopic methods (Supplementary Fig. 2) as psilostachyin C [4,5-seco-11(13)-pseudoguaien-4,5:12,6-diolide] (Fig. 1). The high purity of psilostachyin C (>95%) was confirmed by high-performance liquid chromatography (HPLC).

Table 1
Effect of fractions F_{1AS}–F_{5AS} from *Ambrosia scabra* on the growth of *Trypanosoma cruzi* epimastigotes.

Fraction	% Growth inhibition (mean ± S.E.M.)			
	72 h		120 h	
	100 μg/mL	10 μg/mL	100 μg/Ml	10 μg/mL
F_{1AS}	13.5 ± 0.6	0.3 ± 0.8	33.5 ± 2.4	17.2 ± 8.7
F_{2AS}	5.9 ± 1.8	4.0 ± 5.6	7.2 ± 4.9	0.5 ± 3.7
F_{3AS}	59.3 ± 1.4	9.4 ± 3.9	63.8 ± 0.1	41.9 ± 4.0
F_{4AS}	69.6 ± 1.9	3.1 ± 1.5	68.9 ± 3.0	13.8 ± 1.4
F_{5AS}	97.8 ± 0.4	94.4 ± 0.4	96.6 ± 0.2	51.4 ± 0.9

S.E.M., standard error of the mean.

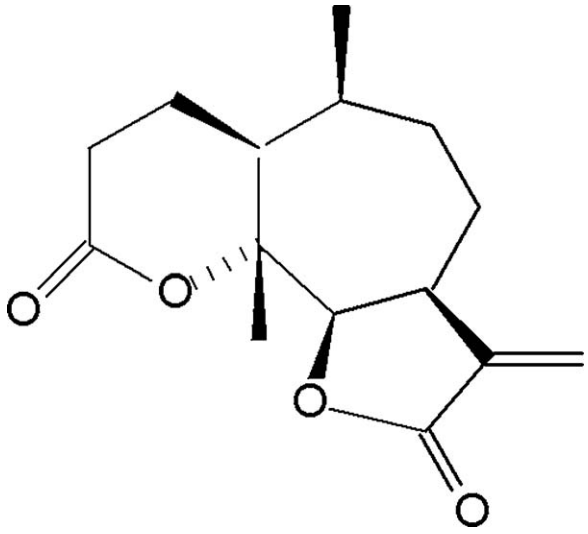

Fig. 1. Chemical structure of psilostachyin C isolated from *Ambrosia scabra*.

3.2. In vitro antiprotozoal activity

The in vitro activity of psilostachyin C against *T. cruzi* epimastigotes is shown in Fig. 2A. Percentage growth inhibitions of 86.7 ± 1.6% (72 h) and 81.2 ± 3.5% (120 h) were observed at 10 μg/mL. IC_{50} values on epimastigotes were 0.6 μg/mL and 0.8 μg/mL after 72 h and 120 h of incubation, respectively. After 24 h of treatment with 2.5 μg/mL of the compound, the parasites could not recover their replication rate (Fig. 2B). Moreover, psilostachyin C showed trypanocidal activity against trypomastigotes, with an IC_{50} value of 3.5 μg/mL (Fig. 3).

To evaluate properly the ability of psilostachyin C to inhibit the intracellular amastigote forms of *T. cruzi*, peritoneal macrophages were infected with transfected blood trypomastigotes expressing β-galactosidase (kindly provided by Frederick S. Buckner) [28] and the activity of the enzyme was quantified after cell disruption. Fig. 4 shows a concentration-dependent inhibition of parasite growth, with an IC_{50} value of 0.9 μg/mL.

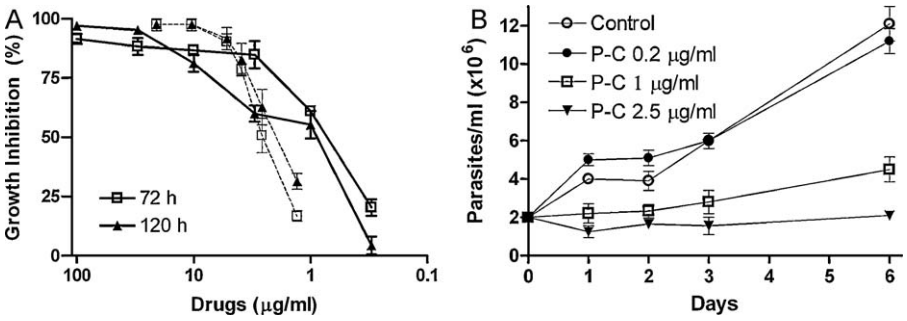

Fig. 2. Effect of psilostachyin C on the growth of *Trypanosoma cruzi* epimastigotes. (A) Inhibition of parasite growth by psilostachyin C. Parasites were incubated in triplicate in the presence of 0.3–100 μg/mL of the compound (solid line) or with 1.3–20.8 μg/mL benznidazole (dotted line). Parasites were cultured for 72 and 120 h, with the addition of [^{3}H] thymidine for the last 16 h. (B) Residual effect of psilostachyin C (P-C) on the growth of *T. cruzi* epimastigotes. Parasites were incubated in the absence or presence of 0.2–2.5 μg/mL psilostachyin C for 24 h. The medium was replaced with fresh medium without the compound and the parasites were allowed to grow for 1, 2, 3 or 6 days. Symbols represent the mean ± standard error of the mean from three independent experiments.

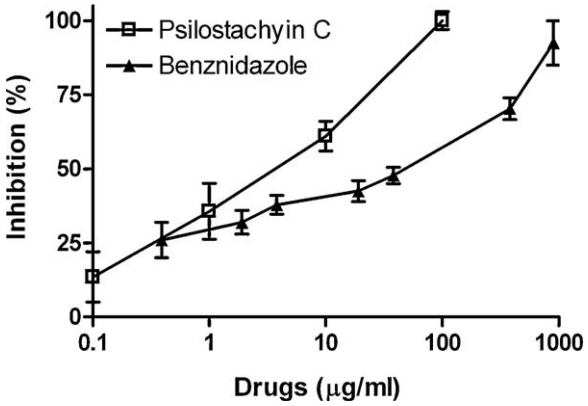

Fig. 3. Effect of psilostachyin C on *Trypanosoma cruzi* trypomastigotes. Bloodstream trypomastigotes were cultured in duplicate in the presence of 0.1–100 μg/mL of the compound or 0.4–900 μg/mL benznidazole. Cultures were done in 96-well plates employing 1.5×10^6 parasites/mL over 24 h and the remaining live parasites were counted in a Neubauer chamber. Symbols represent the mean ± standard error of the mean.

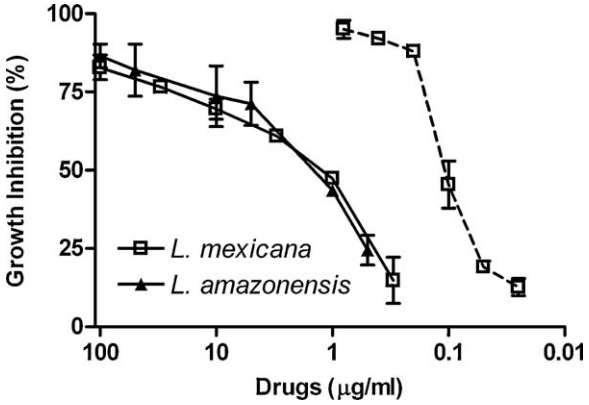

Fig. 5. Effect of psilostachyin C on the growth of *Leishmania mexicana* and *Leishmania amazonensis* promastigotes. Parasites were cultured in triplicate in the presence of 0.3–100 μg/mL of the compound or 0.025–0.8 μg/mL amphotericin B. Parasites were cultured for 72 h with the addition of [^{3}H] thymidine for the last 16 h. Values represent the mean ± standard error of the mean.

When psilostachyin C was tested against two species of *Leishmania* promastigotes, similar inhibitory effects were observed. After 72 h treatment, IC_{50} values were 1.2 μg/mL and 1.5 μg/mL for *L. mexicana* and for *L. amazonensis*, respectively (Fig. 5).

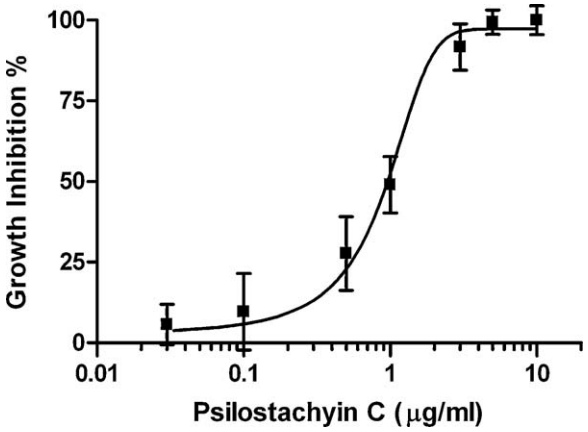

Fig. 4. Effect of psilostachyin C on *Trypanosoma cruzi* amastigotes. Peritoneal macrophages (5×10^3 per well in 100 μL of culture medium) were infected with transfected trypomastigotes expressing β-galactosidase (10:1 parasite:cell ratio). After washing the unbound parasites, psilostachyin C was added at concentrations ranging from 0.01 μg/mL to 10 μg/mL. Two days post infection, Nonidet P40 and chlorophenolred-β-D-galactopyranoside (CPRG) were added and galactosidase activity was measured at an absorbance of 570 nm. Values represent the mean ± standard error of the mean.

3.3. Effect of psilostachyin C on Trypanosoma cruzi epimastigotes in the presence of glutathione

An important increase in the number of epimastigotes was observed when parasites were incubated simultaneously with GSH and psilostachyin C compared with those treated with psilostachyin C alone ($6.8 \pm 0.3 \times 10^6$ parasites/mL vs. $2.5 \pm 0.4 \times 10^6$ parasites/mL) (Fig. 6). However, the number of parasites was significantly lower than that observed in controls ($9.5 \pm 2.0 \times 10^6$ parasites/mL), indicating that the trypanocidal activity of psilostachyin C was attenuated by the reducing agent GSH.

3.4. Cytotoxicity assay

The in vitro cytotoxic effect of psilostachyin C on peritoneal macrophages was evaluated by the MTT method and was expressed as cell viability percentage. The results are shown in Fig. 7. When cells were treated with psilostachyin C, the CC_{50} was 87.5 μg/mL, indicating that the selectivity of psilostachyin C for *T. cruzi* trypomastigotes (SI = 25.0) and amastigotes (SI = 97.2) is greater than that for mammalian cells.

3.5. In vivo assays

Untreated mice infected with *T. cruzi* trypomastigotes displayed high levels of parasitaemia (Fig. 8A) and presented 100% mortality on Day 22 post infection (Fig. 8B). On the other

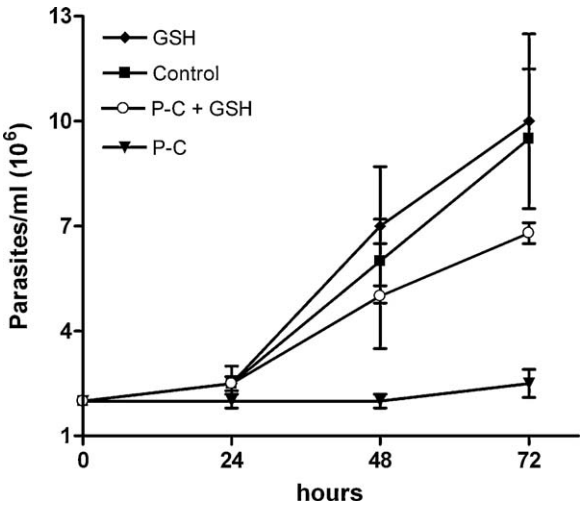

Fig. 6. Effect of psilostachyin C (P-C) on the growth of *Trypanosoma cruzi* epimastigotes in the presence of glutathione (GSH). Parasites were incubated in the presence of 1 μg/mL P-C or 1 μg/mL P-C + 2 mM GSH for 24, 48 or 72 h. Values represent the mean ± standard deviation of three experiments.

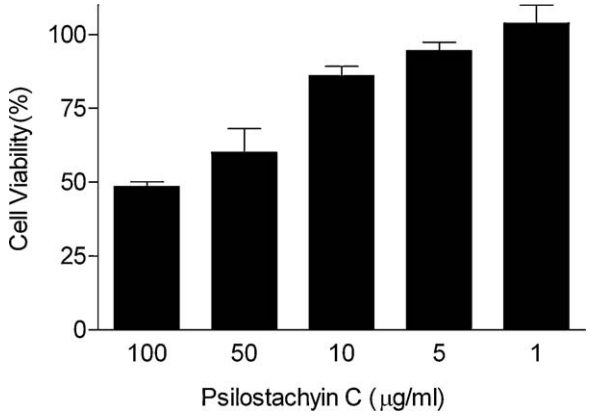

Fig. 7. Effect of psilostachyin C on peritoneal macrophages. Cells were cultured for 48 h in the presence of different concentrations (1–100 μg/mL) of psilostachyin C. Cell viability was determined by the MTT method and was expressed as the ratio between viable cells in the presence and absence of the compound multiplied by 100. Bars represent the mean ± standard error of the mean of three experiments carried out in duplicate.

hand, animals treated with psilostachyin C presented lower levels of circulating parasites and began to die on Day 20, with a survival rate of 20%. Considering the parasitaemia curve throughout the acute phase of infection, calculated as the area under the parasitaemia curve (AUC) [26], psilostachyin C-treated animals presented a two-fold reduction in the number of parasites compared with untreated animals (6.0×10^6 and 1.3×10^7, respectively). More importantly, on Day 16, psilostachyin C-treated mice presented a significant reduction in parasitaemia with respect to untreated mice ($7.4 \pm 1.2 \times 10^5$ parasites/mL vs. $12.8 \pm 2.0 \times 10^5$ parasites/mL; $P < 0.05$). Interestingly, the number of circulating parasites in benznidazole-treated and psilostachyin C-treated animals was not significantly different (AUC 6.0×10^6 and 7.9×10^6, respectively), but all animals treated with benznidazole died by Day 30 after infection whilst 20% of psilostachyin C-treated mice survived by the same time (Fig. 8).

To test the possible toxicity of psilostachyin C, uninfected mice were treated with this compound, benznidazole and PBS for 5 days and were monitored for signs of disease or mortality during 30 days. On Day 13, ALT and LDH enzymes were determined in sera. No differences between psilostachyin C-treated mice compared with PBS control were recorded (ALT 6.2 ± 0.9 IU/L vs. 6.2 ± 1.0 IU/L; and LDH 906 ± 241 IU/L vs. 922 ± 250 IU/L). The tested compound appeared to be well tolerated by the animals. No evident side effects could be observed during the experiment and no death was observed in the 30-day period, indicating that psilostachyin C is not toxic and that the death of the infected animals was due to the parasites.

3.6. Transmission electron microscopy

At a concentration of 2.5 μg/mL, psilostachyin C altered the ultrastructure of *T. cruzi* epimastigotes, inducing cytoplasmic vacuolisation (Fig. 9D). In addition, the compound promoted the appearance of membranous structures resembling cytoplasmic multivesicular bodies. The appearance of multilamellar structures was also observed. Although some parasites exhibited redistribution of nuclear chromatin, the compound did not induce cellular or nuclear morphological alterations. Interestingly, some parasites (ca. 10%) exhibited abnormalities such as the presence of more than two flagella and two kinetoplasts (Fig. 9B and C), suggesting a possible effect of the compound on cytokinesis.

4. Discussion

We have previously reported that the organic extract of *A. scabra* showed significant in vitro trypanocidal activity, inhibiting the growth of *T. cruzi* epimastigotes [8]. Hence, this extract was selected for further study to isolate and identify the active compound(s) responsible for this activity. For this purpose, bioassay-guided fractionation was carried out by conventional chromatographic techniques. Amongst the tested fractions, F_{5AS} showed the highest in vitro inhibitory effect on *T. cruzi* epimastigotes (IC_{50} = 4.5 μg/mL)

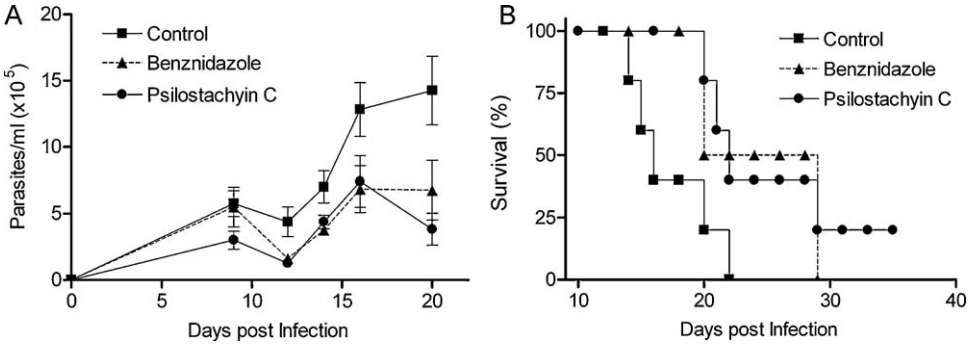

Fig. 8. (A) Parasitaemia levels and (B) survival curves during the acute infection period. C3H/HeN mice were infected with 5×10^3 *Trypanosoma cruzi* bloodstream trypomastigotes and were treated with psilostachyin C or benznidazole from Days 5–10 post infection. Parasitaemia was determined by counting the number of trypomastigotes in 5 μL of fresh blood collected from the tail vein. Data represent the mean ± standard error of the mean. Mortality was recorded every day. Results presented are representative of three independent experiments.

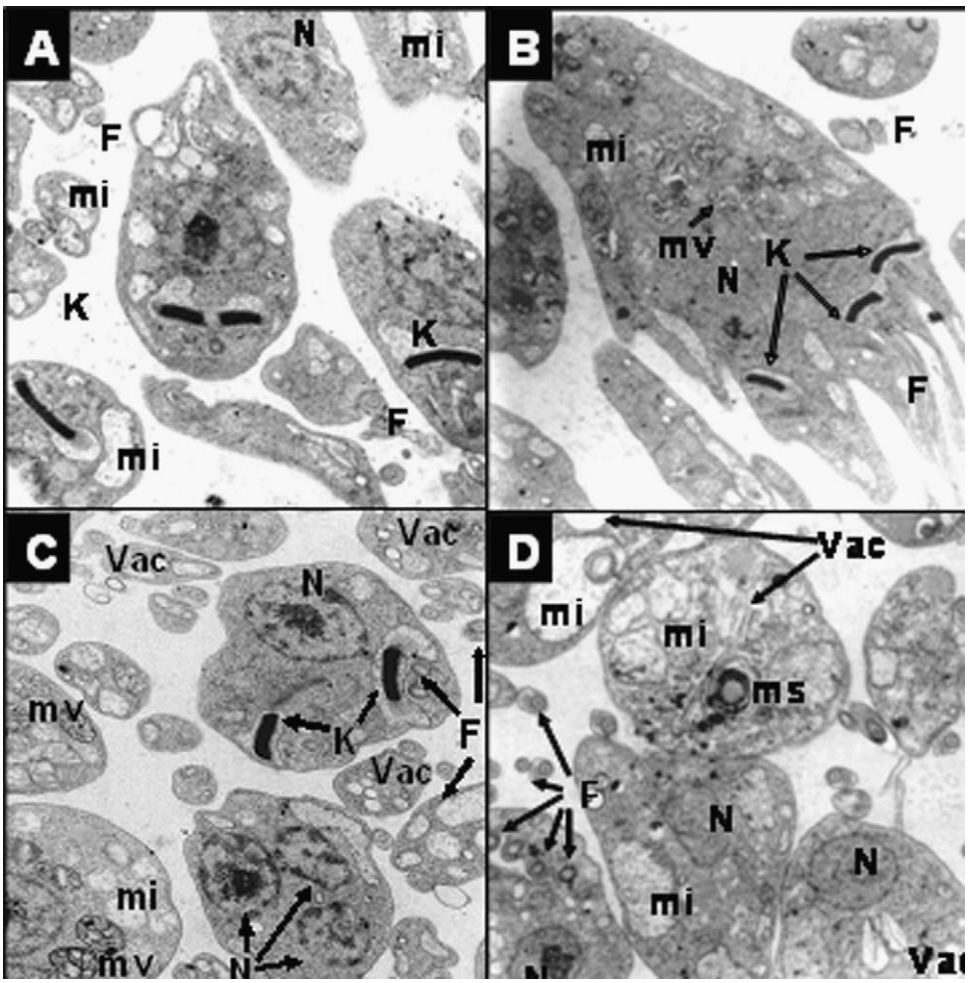

Fig. 9. Ultrastructural effects of psilostachyin C on *Trypanosoma cruzi* epimastigotes. Parasites were incubated with (A) Diamond medium alone or with (B) 0.5 μg/mL psilostachyin C, (C) 1.0 μg/mL psilostachyin C or (D) 2.5 μg/mL psilostachyin C. N, nucleus; K, kinetoplast; F, flagellum; Vac, vacuoles; mi, mitochondria; ms, multilamellar structures; mv, multivesicular bodies. Magnification ×2500.

and was selected for further purification, leading to the isolation and identification of the STL psilostachyin C (Fig. 1).

When psilostachyin C was tested against *T. cruzi* epimastigotes, the compound exerted marked in vitro activity (IC_{50} = 0.6 μg/mL) (Fig. 2A), showing that a progressive increase in trypanocidal activity was gained during the purification process. Moreover, no recovery of the parasite replication rate was observed after removal of the pure compound (2.5 μg/mL) from the medium (Fig. 2B). In addition, when psilostachyin C was assayed on the mammalian stages of *T. cruzi*, it efficiently inhibited both its infective form (the trypomastigote) and the replicative intracellular amastigotes, with IC_{50} values of 3.5 μg/mL and 0.9 μg/mL, respectively (Figs. 3 and 4). Psilostachyin C also exerted in vitro leishmanicidal activity against *L. mexicana* and *L. amazonensis* promastigotes, with IC_{50} values of 1.2 μg/mL and 1.5 μg/mL, respectively (Fig. 5).

Most STLs contain a common functional structure of α-methylene-γ-lactone, which is highly reactive with thiol groups [29]. We have therefore investigated whether this functional structure is responsible for the trypanocidal activity of psilostachyin C. As shown in Fig. 6, the presence of GSH in the culture media attenuated the trypanocidal effect of the compound, indicating that the α-methylene-γ-lactone moiety is not uniquely responsible for the observed activity [11].

To determine the specificity of the trypanocidal activity, an in vitro cytotoxicity test on mammalian cells was carried out. When peritoneal macrophages were treated with psilostachyin C, the CC_{50} value was >100 μg/mL (3 h; data not shown). At 48 h, the CC_{50} value was 87.5 μg/mL.

The SI was calculated to compare the toxicity to mammalian cells and the activity against *T. cruzi* trypomastigotes and amastigotes. The values obtained were 25.0 and 97.2, respectively, indicating that the selectivity of this compound for parasites is greater than for mammalian cells.

In view of these results, psilostachyin C was also evaluated in vivo in a murine model. In this assay, the compound induced a significant decrease in parasitaemia compared with untreated mice (6×10^6 vs. 13×10^6) and proved to be as effective as benznidazole. Although treatments were administered for only 5 days, the reduction in parasitaemia was observed throughout the evaluation period and was reflected in the significant increase in survival time of the animals.

Recently, many STLs have shown interesting in vitro activity against different protozoa, including *T. cruzi*. Nevertheless, to the best of our knowledge, no in vivo studies have been carried out with these compounds, with the exception of the one previously reported by our group [9].

Electron microscopy has proven to be a reliable and useful tool to study morphological alterations and target organelles in the investigation of new drugs for Chagas disease. In this study, it was observed that psilostachyin C causes ultrastructural alterations in *T. cruzi* epimastigotes, such as cytoplasm vacuolisation, the appearance of multilamellar structures, and abnormalities such as the

presence of multiple kinetoplasts and flagella. However, these multiple structures were not accompanied by an increase in the number of nuclei, indicating that the compound could act in a stage between kinetoplast segregation and nuclear division.

Recently, it has been shown that the flagellum begins to replicate during the G2 stage, and later the nucleus and kinetoplast segregate almost simultaneously during the *T. cruzi* cell cycle [30]. The new flagellum emerges from the flagellar pocket and remains short until the kinetoplast segregates and mitosis occurs. However, the new flagellum reaches its final size during cytokinesis. The presence of multiple flagella and kinetoplasts induced by the compound could be related to the inability of the parasite to synchronise replication of these structures with nuclear division. Synthesis of parasite proteins or factors that synchronise these stages of the cycle might be affected by the compound. Detailed molecular studies will be needed to explain this phenomenon better. The compound studied herein did not cause mitochondrial swelling as observed with other compounds that block parasite metabolism [31–33], indicating that it may act by other mechanisms. Moreover, the appearance of multilamellar structures could be due to an autophagic process induced by the isolated compound [34].

According to Lee and Schneider [35], STLs of the ambrosanolide type are one of the promising scaffolds for the discovery or design of new drugs, since these kinds of compounds are not present in current trade drugs.

In conclusion, psilostachyin C showed both in vitro and in vivo trypanocidal activity. Although the mechanism of action of this compound remains to be determined, it could be suggested that it might act as a cytokinesis inhibitor, as an oxidative stress inductor, consequently or independently producing some ultrastructural changes in the parasite. These results make psilostachyin C a promising template for the design of novel trypanocidal agents. Quantitative structure–trypanocidal activity relationship studies amongst members of the STLs are currently being undertaken in our laboratories.

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Competing interests: None declared.

Ethical approval: All procedures with animals were approved by the Ethics Review Board of the Instituto de Estudios de la Inmunidad Humoral (IDEHU-CONICET) and were conducted in accordance with the guidelines established by the National Research Council '*Guide for the Care and Use of Laboratory Animals*'.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.ijantimicag.2011.02.003.

References

[1] World Health Organization. Control of Chagas disease. Second report of the WHO Expert Committee. Geneva, Switzerland: WHO; 2002. Technical Report Series No. 905.

[2] Cazorla SI, Frank FM, Malchiodi EL. Vaccination approaches against *Trypanosoma cruzi* infection. Expert Rev Vaccines 2009;8:921–35.

[3] Cazorla SI, Frank FM, Becker PD, Arnaiz M, Mirkin GA, Corral RS, et al. Redirection of the immune response to the functional catalytic domain of the cystein proteinase cruzipain improves protective immunity against *Trypanosoma cruzi* infection. J Infect Dis 2010;202:136–44.

[4] World Health Organization. Neglected tropical diseases: hidden successes, emerging opportunities. Geneva, Switzerland: WHO; 2009. WHO/HTM/NTD/2009.2.

[5] Chiaramonte MG, Zwirner NW, Caropresi SL, Taranto NJ, Malchiodi EL. *Trypanosoma cruzi* and *Leishmania* spp. human mixed infection. Am J Trop Med Hyg 1996;54:271–3.

[6] Chiaramonte MG, Frank FM, Furer GM, Taranto NJ, Margni RA, Malchiodi EL. Polymerase chain reaction reveals *Trypanosoma cruzi* infection suspected by serology in cutaneous and mucocutaneous leishmaniasis patients. Acta Trop 1999;72:295–308.

[7] Frank FM, Fernández MM, Taranto NJ, Cajal SP, Margni RA, Castro E, et al. Characterization of human infection by *Leishmania* spp. in the Northwest of Argentina: immune response, double infection with *Trypanosoma cruzi* and species of *Leishmania* involved. Parasitology 2003;169:31–9.

[8] Sülsen VP, Güida C, Coussio J, Paveto C, Muschietti L, Martino V. In vitro evaluation of trypanocidal activity in plants used in Argentine traditional medicine. Parasitol Res 2006;98:370–4.

[9] Sülsen VP, Frank FM, Cazorla SI, Anesini C, Malchiodi EL, Freixa B, et al. Trypanocidal and leishmanicidal activities of sesquiterpene lactones from *Ambrosia tenuifolia* Sprengel (Asteraceae). Antimicrob Agents Chemother 2008;52:2415–9.

[10] Muschietti LV, Sülsen VP, Martino VS. Trypanocidal and leishmanicidal activities of South American medicinal plants. In: Martino VS, Muschietti LV, editors. South American medicinal plants as a potential source of bioactive compounds. Kerala, India: Transworld Research; 2008. p. 149–80.

[11] Schmidt TJ. Structure–activity relationships of sesquiterpene lactones. In: Atta-ur-Rahman, editor. Studies in natural products chemistry. Amsterdam, The Netherlands: Elsevier; 2006. p. 309–92.

[12] Picman AK. Biological activities of sesquiterpene lactones. Biochem Syst Ecol 1986;14:255–81.

[13] Luna-Herrera J, Costa MC, González HG, Rodrigues AI, Castillo PC. Synergistic antimycobacterial activities of sesquiterpene lactones from *Laurus* spp. J Antimicrob Chemother 2007;59:548–52.

[14] Karioti A, Skaltsa H, Linden A, Perozzo R, Brun R, Tasdemir D. Anthecularin: a novel sesquiterpene lactone from *Anthemis auriculata* with antiprotozoal activity. J Org Chem 2007;72:8103–6.

[15] Pillay P, Vleggaar R, Maharaj VJ, Smith PJ, Lategan CA. Isolation and identification of antiplasmodial sesquiterpene lactones from *Oncosiphon piluliferum*. J Ethnopharmacol 2007;112:71–6.

[16] Kagan HB, Miller HE, Renold W, Lakshmikantham MV, Tether LR, Herz W. The structure of psilostachyin C, a new sesquiterpene dilactone from *Ambrosia psilostachya* DC. J Org Chem 1966;3:1629–32.

[17] Oberti J, Silva G, Sosa V, Kulanthaivel P, Herz W. Ambrosanolides and secoambrosanolides from *Ambrosia tenuifolia*. Phytochemistry 1986;25:1355–8.

[18] Wang PH, Xu J, Wu MY. Chemical constituents of ragweed (*Ambrosia artemisiifolia* L.). Zhongguo Zhong Yao Za Zhi 1993;18:164–6, 191 [in Chinese].

[19] Sturgeon CM, Craig K, Brown C, Rundle NT, Andersen RJ, Roberge M. Modulation of the G2 cell cycle checkpoint by sesquiterpene lactones psilostachyins A and C isolated from the common ragweed *Ambrosia artemisiifolia*. Planta Med 2005;71:938–43.

[20] National Research Council. Guide for the care and use of laboratory animals. Washington, DC: National Academy Press; 1996.

[21] Sülsen VP, Cazorla SI, Frank FM, Redko FC, Anesini CA, Coussio JD, et al. Trypanocidal and leishmanicidal activities of flavonoids from Argentine medicinal plants. Am J Trop Med Hyg 2007;77:654–9.

[22] Alexander B, Browse DJ, Reading SJ, Benjamin IS. A simple and accurate mathematical method for calculation of the EC_{50}. J Pharmacol Toxicol Methods 1999;41:55–8.

[23] Buckner FS, Verlinde CL, La Flamme AC, Van Voorhis WC. Efficient technique for screening drugs for activity against *Trypanosoma cruzi* using parasites expressing β-galactosidase. Antimicrob Agents Chemother 1996;40:2592–7.

[24] Frank FM, Petray PB, Cazorla SI, Muñoz MC, Corral RS, Malchiodi EL. Use of a purified *Trypanosoma cruzi* antigen and CpG oligodeoxynucleotides for immunoprotection against a lethal challenge with trypomastigotes. Vaccine 2003;22:77–86.

[25] Cazorla SI, Becker PD, Frank FM, Ebensen T, Sartori MJ, Corral RS, et al. Oral vaccination with *Salmonella enterica* as a cruzipain-DNA delivery system confers protective immunity against *Trypanosoma cruzi*. Infect Immun 2008;76:324–33.

[26] Cazorla SI, Frank FM, Becker P, Corral RS, Guzman CA, Malchiodi EL. Prime-boost immunization with cruzipain co-administered with MALP-2 triggers a protective immune response able to decrease parasite burden and tissue injury in an experimental *Trypanosoma cruzi* infection model. Vaccine 2008;26:1999–2009.

[27] Brengio SD, Belmonte SA, Guerreiro E, Giordano OS, Pietrobon EO, Sosa MA. The sesquiterpene lactone dehydroleucodine (DhL) affects the growth of cultured epimastigotes of *Trypanosoma cruzi*. J Parasitol 2000;86:407–12.

[28] Kraus JM, Verlinde CL, Karimi M, Lepesheva GI, Gelb MH, Buckner FS. Rational modification of a candidate cancer drug for use against Chagas disease. J Med Chem 2009;52:1639–47 [Erratum in: J Med Chem 2009;52:4549; J Med Chem 2009;52:4979].

[29] Schmidt TJ, Nour AM, Khalid SA, Kaiser M, Brun R. Quantitative structure–antiprotozoal activity relationships of sesquiterpene lactones. Molecules 2009;14:2062–76.
[30] Elias MC, da Cunha JPC, de Faria FP, Mortara RA, Freymüller E, Schenkman S. Morphological events during the *Trypanosoma cruzi* cell cycle. Protist 2007;158:147–57.
[31] Lazardi K, Urbina JA, de Souza W. Ultrastructural alterations induced by two ergosterol biosynthesis inhibitors, ketoconazole and terbinafine, on epimastigotes and amastigotes of *Trypanosoma* (*Schizotrypanum*) *cruzi*. Antimicrob Agents Chemother 1990;34:2097–105.
[32] Izumi E, Morello LG, Ueda-Nakamura T, Yamada-Ogatta SF, Filho BP, Cortez DA, et al. *Trypanosoma cruzi*: antiprotozoal activity of parthenolide obtained from *Tanacetum parthenium* (L.) Schultz Bip. (Asteraceae, Compositae) against epimastigote and amastigote forms. Exp Parasitol 2008;118:324–30.
[33] Sülsen V, Barrera P, Muschietti L, Martino V, Sosa M. Antiproliferative effect and ultrastructural alterations induced by psilostachyin on *Trypanosoma cruzi*. Molecules 2010;15:545–53.
[34] Menna-Barreto RFS, Salomão K, Dantas AP, Santa-Rita RM, Soares MJ, Barbosa HS, et al. Different cell death pathways induced by drugs in *Trypanosoma cruzi*: an ultrastructural study. Micron 2009;40:157–68.
[35] Lee ML, Schneider G. Scaffold architecture and pharmacophoric properties of natural products and trade drugs: application in the design of natural product-based combinatorial libraries. J Comb Chem 2001;3:284–9.