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Antileishmanial activity and ultrastructural changes of sesquiterpene lactones isolated from *Calea pinnatifida* (Asteraceae)

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ABSTRACT

Bioactivity-guided fractionation of antileishmanial active CH_2Cl_2 phase of MeOH extract from leaves of *Calea pinnatifida* led to isolation of two sesquiterpene lactones calein C (**1**) and calealactone C (**2**), which structures were stablished on the basis of spectroscopic analysis. Compounds **1** and **2** displayed potent activity against *Leishmania amazonensis* promastigotes with EC_{50} of 1.7 and 4.6 μg mL^{-1}, respectively. Compound **2** presented low cytotoxicity for J774 macrophages and displayed activity against amastigote forms of *L. amazonensis* similar to miltefosine with CC_{50} values of 31.73 and 27.18 μg mL^{-1}, respectively. Additionally, compounds **1** and **2** caused ultrastructural changes in promastigotes leading to a loss of their classical structural morphology, as evidenced by electron microscopy. Also compound **2** decreased the mitochondria membrane potential. To the best of our knowledge, this is the first occurrence of **1** and **2** in *C. pinnatifida*. The results obtained highlighted the importance of studying sesquiterpene lactones isolated from *Calea pinnatifida* in terms of antileishmanial activity, in order to understand the mechanism of action of the isolated compounds in promastigotes forms of *L. amazonensis*.

1. Introduction

Calea genus, belonging to the Asteraceae, is composed by approximately 125 species [1]. Some of them have been chemically investigated and sesquiterpene lactones as well as furan sesquiterpenes have been identified [2]. There are several biological activities related to the compounds present in the genus *Calea*, as anti-inflammatory, antiplasmodial, antifungal, antimicrobial and cytotoxic activities, which are credited to the presence of germacranolides [3]. Previous studies have shown many pharmacological properties in species of *Calea:* CH_2Cl_2 extract of *C. uniflora* showed *in vitro* activity against trypomastigote forms of *Trypanosoma cruzi* [4], germacranolides from *C. urticifolia* were described with cytotoxic activity against leukemia U937 cells [5] and germacranolides with antileishmanial activity, including calealactone C, D, E and calein D, isolated from *C. zacatechichi* [6].

C. pinnatifida, popularly known as “aruca” and “cipó-cruz”, has been encountered specially in Brazilian “cerrado” biome [7], and has been used in folk medicine to treat stomachaches, giardiasis and amebiasis. In Brazil this species is commercially available in popular market as EtOH extract of the leaves to treat amoebic dysentery [8]. Phytochemical study of aerial parts of *C. pinnatifida* indicated the occurrence of fatty esters, glycoside of *p*-hydroxybenzoic acid, anisic acid, chromenes, stigmasterol, polyacetylene derivatives and germacranolides [8]. Pharmacological activities of *C. pinnatifida* such as cytotoxicity induced by apoptosis in different tumor cells and inhibitory activity of NF-κB factor have been credited to the presence of germacranolides [9,10]. Additionally, chromenes from *C. pinnatifida* have been reported moderated antileishmanial activity against amastigote forms of *Leishmania amazonensis,* with a range of inhibition intracellular growth of 32.3% and 39.3% [11].

Leishmaniasis is a neglected infectious disease caused by a protozoan of the genus *Leishmania,* which is widely distributed around the World. The vectors responsible to transmit the parasites for animals and

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humans are sandflies belonging to the *Psychodida* family, and *Phlebotomus* genus (Old World) or *Lutzomyia* (New World) [12]. After infection, animals or humans can develop cutaneous or visceral leishmaniosis, which will depend on infecting species of *Leishmania*. The *Leishmania amazonensis* is the etiological agent of localized cutaneous leishmaniosis but some strains of this species are able to cause the most serious clinical form, the anergic diffuse cutaneous leishmaniasis, whose classical treatment is not efficient, besides, some strains belonging to this species are able to visceralize during natural or experimental infections [13–15]. Concerning the treatment, the most important drugs used ones are Glucantime, Pentamidine or Amphotericin B, although nowadays, according to a standardization of the World Health Organization, the first-choice treatment is pentavalent antimonials [16]. All these drugs presented serious side effects to the patients, including renal, heart and liver toxicities that, indeed, limit their use [17].

In continuation to the investigation of antiparasitic compounds from Brazilian flora [18,19] the present study was undertaken to conduce a bioactivity-guided fractionation of extract/partition phases from the leaves of *C. pinnatifida* and determine the effect of isolated compounds against promastigotes and intracellular amastigotes of *Leishmania amazonensis*.

2. Results and discussion

In the present study, hexane, CH_2Cl_2 and EtOAc partition phases from MeOH extract from leaves of *C. pinnatifida* were evaluated to antileishmanial activity against *L. amazonensis*. In this regard, it was verified that CH_2Cl_2 phase was the most active one, displaying EC_{50} of 6.1 ± 0.8 μg mL^{-1} (Table 1). Hexane and EtOAc phases showed a marginal leishmanicidal activity with EC_{50} values of 126.6 ± 6.1 and 41.9 ± 5.4 μg mL^{-1}, respectively. After fractionation over Sephadex LH-20, fraction B displayed higher potential against *L. amazonensis* promastigote forms with EC_{50} of 2.5 ± 0.5 μg mL^{-1} (Table 1).

Bioactive fraction B was purified by CC over silica *flash,* Sephadex LH-20 and reverse phase HPLC, affording two known germacranolides: calein C (**1**) and calealactone C (**2**), as showed in Fig. 1. The structure of calein C was determined based on the spectrometric analyses (NMR and MS) and comparison with those reported in the literature to calein C previously isolated from *C. urticifolia* [20]. Ferreira et al. [8] described an isomer of calein C, arucanolide, with reverse position of the metacrytlate and acetate groups, as the major constituent of C. *pinnatifida*. However for the definition of the relative positions of the acetate and methacrylate groups, 2D NMR spectra (HMBC and HSQC) with a benzene-d_6 were performed, once in the spectrum obtained in $CDCl_3$ the H-8 and H-9 signals appeared with an overlap of these signals which made the definition of the acetate methacrylate positions doubtful. A benzene-d_6 spectrum allowed assigning the signals H-8/C-8 at δ_H 6.03/δ_C 73.8 and H-9/C-9 at δ_H 5.72/δ_C 74.6 in the HSQC spectrum. In addition in HMBC spectrum H-8 and H-9 signals were correlated with each carbonyl: H-9 with δ_C 169.8 (acetate group) and H-8 with δ_C 165.2 (methacrylate group) confirming the positions of the acetate and the methylacrylate groups. Considering that arucanolide was described from the same plant, it was suggested recently the revision of the structure in the work of Ferreira et al. 1980, by our research group [21]. Calealacone C was identified by analysis of their spectral data and comparison with those reported in the literature [5,6]. Also this is the first occurrence of **1** and **2** in *C. pinnatifida*.

Table 1
In vitro antileishmanial activity against promastigote forms of *L. amazonensis* of hexane, CH_2Cl_2 and EtOAc phases, obtained from *Calea pinnatifida,* as well as fractions A – G obtained by fractionation of CH_2Cl_2 phase in Sephadex LH-20.

Samples		EC_{50} (μg mL^{-1})
Partition phases	Hexanes	126.6 ± 6.1
	CH_2Cl_2	6.1 ± 0.8
	EtOAc	41.9 ± 5.4
Fractions from CH_2Cl_2 phase	A	No active
	B	2.5 ± 0.5
	C	15.3 ± 3.4
	D	19.5 ± 2.7
	E	99.7 ± 9.3
	F	37.7 ± 5.9
	G	35.9 ± 6.5

Compounds **1** and **2** were able to eliminate *L. amazonensis* promastigotes with EC_{50} values of 1.73 and 4.61 μg mL^{-1}, respectively. Comparatively, the standard drug miltefosine eliminated *L. amazonensis* with EC_{50} of 5.90 μg mL^{-1}. Concerning cytotoxicity for J774 macrophages, it was verified that **1** was the most cytotoxic compound, presenting a CC_{50} of 1.42 ± 0.07 μg mL^{-1}. On the other hand, compound **2** showed a CC_{50} of 31.73 ± 2.09 μg mL^{-1} and the standard drug displayed CC_{50} values of and 27.80 ± 6.43 μg mL^{-1}, as illustrated in Table 2.

In spite of the strong leishmanicidal effect of compound **1**, this sesquiterpene lactone was also toxic for J774 macrophages indicating a reduced selectivity to tested parasite (SI = 0.83). According to literature data [21] calein C was also able to induce apoptosis in MCF-7 cells and inhibits mitotic progression. Otherwise, compound **2** displayed similar toxicity of that induced by miltefosine (CC_{50} values of 31.73 and 27.18 μg mL^{-1}, respectively) and due to the higher potential of **2** against tested parasite, the selectivity index to compound **2** (6.90) was slightly higher than miltefosine (4.71).

Germacranolides are sesquiterpenoids with potent anti-*Leishmania* activity. Wu et al. [6] identified six different bioactive sesquiterpene lactones from *C. zacatechichi* with EC_{50} ranging from 0.77 to 3.45 μg mL^{-1} against promastigote forms of *L. donovani*. In this case, calealactone C was the compound more active against *L. donovani* (EC_{50} of 0.77 μg mL^{-1}). Also calealactone C showed to be more active against *L. donovani* compared to *L. amazonensis* (EC_{50} of 4.61 μg mL^{-1}). Neurolenin B, C and D, isolated from *Neurolaena lobata*, also displayed activity against *L. (L.) mexicana* and *L. (V.) braziliensis* [22]. In addition, promastigote forms of *L. (L.) mexicana* incubated with mexicanin I, helenalin and dehydroleucodine isolated from plants native to Cuyo (Argentina), were eliminated and irreversible alterations were observed [23]. These compounds displayed low toxicity for Vero, Hela, fibroblast L929, and melanoma B16F10 cell lineages [24]. These data reinforce that calealactone C presents leishmanicidal activity, without causing cell injuries, suggesting selectivity of this compound for parasites.

The activity against amastigote forms of *L. amazonensis* infected macrophages treated with different concentrations (3.0, 6.0 and 12.0 μg mL^{-1}) of compound **2** was evaluated. Compound **2** was also effective against intracellular amastigotes (EC_{50} = 4.24 ± 0.28 μg mL^{-1}), while miltefosine showed an EC_{50} of 10.1 ± 1.12 μg mL^{-1} (Table 2). The cytotoxicity of compound **2** was determined with peritoneal macrophages and showed a CC_{50} value of 31.73 ± 2. Noteworthy Compound **2** demonstrated highest *in vitro* selectivity (SI = 7.21) than standard drug miltefosine (2.75).

The antiamastigote activity of sesquiterpene lactones have previously been reported in the literature. In the case of parthenolide, obtained from *Tanacetum parthenium*, was observed a reduction in the internalization of amastigote forms in 84.7% when *L. amazonensis*-infected J774 macrophages were treated with 5.0 μg mL^{-1} of this compound [25]. The sesquiterpene lactones tirotundin 3-*O*-methyl ether, tagitinin F, 4β,10α-dihydroxy-3-oxo-8β-isobutyryloxyguai-11(13)-en-6α,12-olide, isolated from *Tithonia diversifolia,* were also active against intracellular amastigotes of *L. (V.) braziliensis* [26]. Axenic amastigotes of *L. (L.) donovani* seems to be sensitive to anthecotulide, 4-hydroxyanthecotulide and 4-acetoxyanthecotulide, displaying EC_{50} of 8.18, 3.27 and 12.50 μg mL^{-1}, respectively. Therefore, similarly to other structurally related sesquiterpene lactones, compound **2** was also able to eliminate intracellular amastigotes.

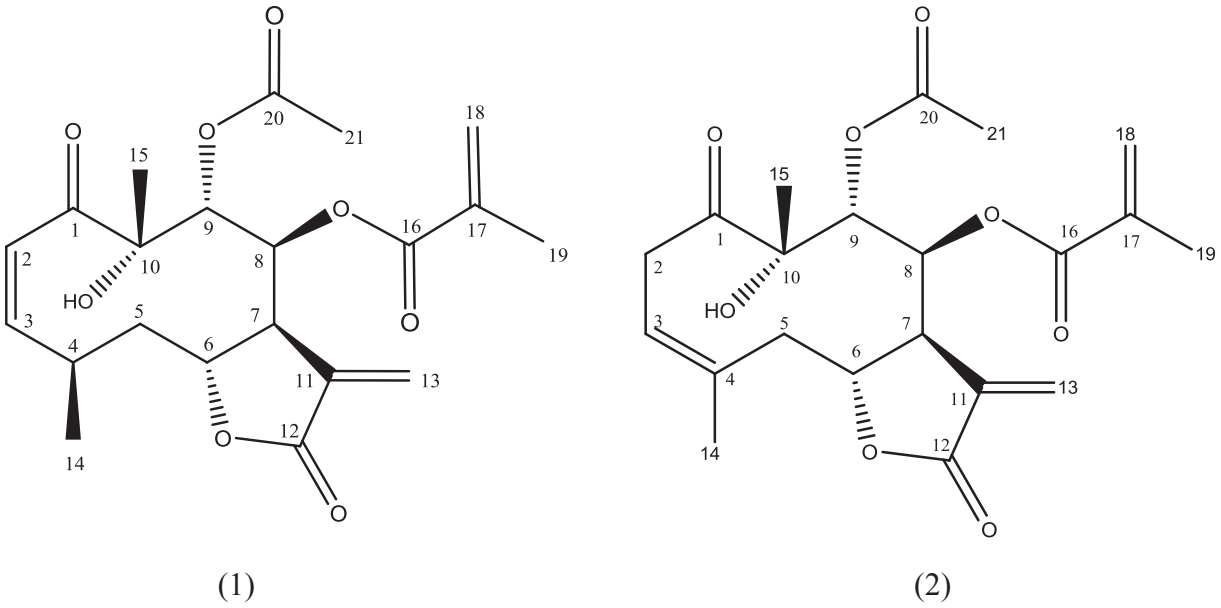

Fig. 1. Structures of sesquiterpene lactones calein C (**1**) and calealactone C (**2**) isolated from *C. pinnatifida.*

Table 2
Calein C (**1**) and calealactone C (**2**) sesquiterpene lactones from *C. pinnatifida* were assayed against *L. amazonensis* promastigotes and amastigotes and their cytotoxic activities on J774 macrophages, and expressed as effective concentration 50% (EC_{50}) for parasites, and cytotoxic concentration 50% (CC_{50}) for macrophages.

Compounds	EC_{50} (μg mL^{-1})^{p*}	CC_{50} (μg mL^{-1})	SI^{p}	EC_{50} (μg mL^{-1})^{a**}	SI^{a}
1	1.73 ± 0.19	1.42 ± 0.07	0.83	NE**	NE
2	4.61 ± 0.23	31.73 ± 2.09	6.90	4.24 ± 0.28	7.21
Miltefosine	5.90 ± 0.61	27.80 ± 6.43	4.71	10.1 ± 1.12	2.75

*p activity in promastigote forms;** activity in intracellular forms; EC_{50} – Effective concentration 50%; CC_{50} – Cytotoxic Concentration 50%; p – promastigotes; a – amastigotes.

In order to evaluate the parasite ultrastructural changes in the tested parasite, promastigote forms in *L. amazonensis* were treated with compounds **1** and **2**. Electron microscopy transmission (Fig. 2A) shows the untreated promastigotes, displaying a fusiform external morphology with preserved cell membrane and cytoplasm. Intact glycosomes, nucleus, mitochondria and kDNA were also verified. Otherwise, parasites treated with EC_{50} of compounds **1** and **2** lost their classical external morphology and became rounded, indicating intracellular disorganization. Although intracellular alterations induced by compounds **1** and **2** were different, suggesting distinctive affinity of compounds to cellular targets, studies demonstrated that promastigotes of *L. (V.) braziliensis* became smaller and with a round shape external morphology after incubation with sesquiterpene lactone-rich preparation, suggesting that this class of molecules have similar targets in cells [27]. Additionally, treatment with compound **1** leads to the formation of large cytoplasmatic vacuoles (Fig. 2B and C), the kinetoplast became swelled (Fig. 2B, inset) and blebs were visualized (Fig. 2C, short arrow). Moreover, pores were detected in the complex mitochondria-kinetoplast (Fig. 2C, black arrow), the parasite nucleus was pyknotic and chromatin seems to be fragmented (Fig. 2B). Therefore, morphological data suggest that parasites treated with compound **1** underwent programmed cell death, since cell shrinkage, membrane-containing vacuoles, mitochondrial blebs and chromatin fragmentation are characteristic of apoptosis or even autophagic process [27]. Structural-related sesquiterpenes lactones, such as parthenolide, mexicanin I, helenalin, and dehydroleucodine also trigger morphological alterations compatible with programmed cell death in *Leishmania*, that include cell shrinkage, formation of intracellular vacuole, mitochondria swelling, blebs and chromatin fragmentation [23,25]. On the other hand, parasites treated with compound **2** showed a more preserved cytoplasm in comparison with that observed after incubation with compound **1**. The nucleus seems to be disorganized and degenerated, as observed by the electron-dense material in nuclear area, as indicated in Fig. 2D. The mitochondrion seems to be fragmented (Fig. 2E). In this group, a tubular structure in mitochondria was observed (Fig. 2D), that possibly followed to the fragmentation of mitochondria (Fig. 2E). The kDNA was also degraded, as observed in Fig. 2E.

Although external morphological alterations were similar in the parasites treated with compounds **1** and **2**, some essential differences can be observed, since compound **2** acts mainly in parasite mitochondria and nucleus, and therefore, cell shrinkage and nuclear degradation can be an effect related to the mitochondria and nucleus degradation. Costunolide, a structurally related sesquiterpene lactone, displayed cytotoxicity for human bladder tumor cells, and completely altered the physiology of mitochondria [28], moreover the sesquiterpene lactones, helenalin, mexicanin, and dehydroleucodine [23] were able to eliminate *L. mexicana* promastigotes and they caused disorganization of parasite nuclei. Similarly to other structurally related sesquiterpene lactones, compound **2** seems to target both mitochondria and nuclei of parasites, affecting *L. amazonensis* viability.

Considering that the ultrastructural analysis of promastigotes treated with compound **2** demonstrated that mitochondria is one of the main organelles damaged during the treatment, a test of mitochondrial membrane potential was performed in order to better understand the physiological changes caused in mitochondria of promastigote forms of *L. amazonensis* treated with this substance. Thus, the effect of compound **2** in the parasite mitochondrial was evaluated using a fluorescence probe (Rhodamine 123). It was possible to observe that compound **2** interfered with parasite mitochondria after 45 min of incubation, and it was kept, at least until 24 h of incubation, when ultraestructural alterations were also observed. Noteworthy, the oligomicyn A decreased the mitochondria membrane potential at 15 min of incubation, as demonstrated in Fig. 3.

Compound **2** demonstrated the same pattern of fluorescence intensity reduction over oligomycin A, a substance that acts as an inhibitor of ATP synthase, preventing the formation of ATP. Therefore, the loss of membrane potential leads to impairment of the physiological

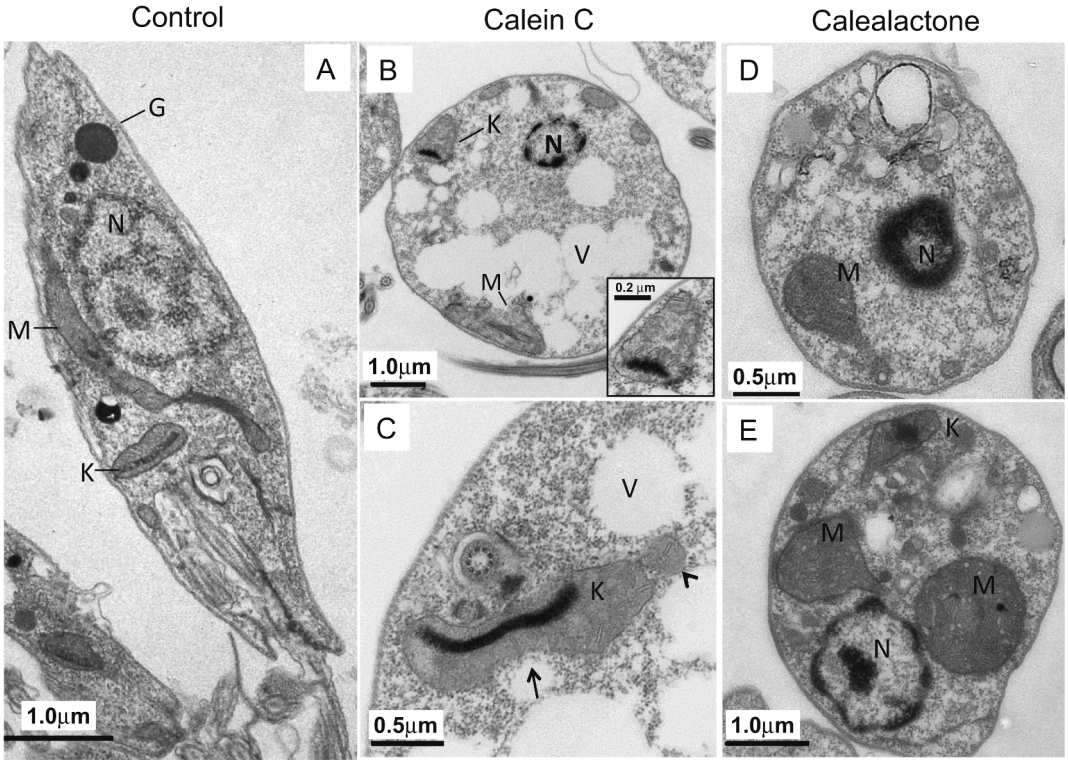

Fig. 2. Promastigote forms of *L. amazonensis* treated with EC_{50} of compounds **1** or **2**, and ultraestructural modification evaluated by transmission electron microscopy. (A) Untreated promastigotes; (B and C) Parasites treated with EC_{50} of compound **1**; (D and E) Parasites treated with EC_{50} of compound **2**. G – glycosome; M – mitochondria; K – kinetoplast; N –nucleus; V – vacuole.

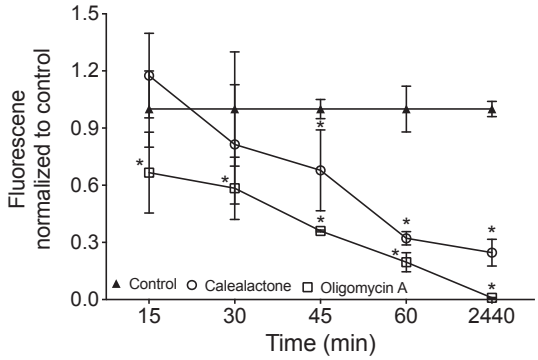

Fig. 3. Promastigote forms of *L. amazonensis* were treated with EC_{50} (12.0 µg mL^{-1}) of compound **2** and the mitochondria membrane potential was evaluated during 15, 30, 45, 60 and 244 min.

functions of the mitochondria, reduction of energy and, consequently, cell death [29]. Thus, to the best of our knowledge, this is the first study showing that calealactone C targets parasite mitochondria, leading to the death. Clearly, other mechanisms can be involved, but according to the ultrastructural and physiologic characteristics this organelle can be the first one to be impacted with the treatment.

3. Conclusion

Brazilian flora represents an unexplored source of potential secondary metabolites which could be promptly used as novel drug leads against neglected diseases. From leaves of *Calea pinnatifida*, two active compounds against leishmaniasis were isolated, the sesquiterpene lactones calein C (1) and calealactone C (2). Also they caused ultrastructural changes in promastigotes of *L. amazonensis* leading to a loss of their classical structural morphology. Thus considering the importance of studying novel and selective drug candidates against protozoans, these compounds could be used as promising tools to future drug design studies.

4. Experimental procedures

4.1. General experimental procedures

^{1}H and ^{13}C NMR uni and bidimensional spectra of compounds were recorded, respectively, at 300 and 75 MHz in a Bruker Ultrashield 300 Advance III spectrometer. $CDCl_3$ (Aldrich) was used as the solvent and as the internal standard. Silica gel *flash* (Merck, 230–400 mesh) and Sephadex LH-20 were used for the column chromatographic separation, while silica gel 60 PF_{254} (Merck) was used for analytical and preparative TLC. HPLC analysis was performed in a Dionex Ultimate 3000 chromatograph, using a Luna Phenomenex RP-18 column (3 µm, 150 × 5 mm) and UV-DAD detector.

4.2. Plant material

Leaves of *Calea pinnatifida* were collected from a single tree in the Atlantic Forest area of São Paulo City, SP, Brazil (coordinates 23 53′08.86″S, 46 40′10.45″W), in October 2012. Botanical identification was performed by Dr. Oriana A. Fávero from Universidade Presbiteriana Mackenzie-SP. A voucher specimen (C.R. Figueiredo 25) has been deposited in SPF Herbarium of Departamento de Botânica from Instituto de Biociências of Universidade de São Paulo.

4.3. Extraction and bioactivity-guided fractionation

Fresh leaves of *C. pinnatifida* (300 g), were dried, grounded and then exhaustively extracted using MeOH at room temperature. After evaporation of the solvent under reduced pressure, the obtained crude extract (10 g) was ressuspended in MeOH:H_2O 2:1. After partition using

hexanes, CH_2Cl_2 and EtOAc, were obtained the three phases which were evaluated to anti-*Leishmania* activities. Bioactive CH_2Cl_2 phase (650 mg) was selected for bioactivity-guided fractionation. This phase was subjected to column chromatography (CC) over Sephadex LH-20 using MeOH as mobile phase to give seven fractions (A – G). Active fraction B (420 mg) was subjected to CC over silica *flash* using increasing amounts of MeOH in CH_2Cl_2 as solvent to afford three fractions (B1 – B3). Fraction B2 (236 mg) was then fractionated by CC over Sephadex LH-20 using MeOH as eluent to afford bioactive fraction B2-2 (215 mg). Part of this fraction (100 mg) was purified over semi-preparative RP-18 HPLC, eluted with ACN:H_2O 4:6 (flow rates 3.6 mL/min, UV 218 nm), to afford calein C **1** (40 mg) and calealactone C **2** (3 mg).

4.4. Evaluation of anti-promastigote effect of compounds *1* and *2*

Promastigote forms of *L. amazonensis* (2×10^7 promastigotes mL^{-1}) were incubated in 96-well culture plate in R10 medium with **1** or **2** in a range of 100–0.76 μg mL^{-1}. The standard drug miltefosine in the range of 0.78–100.00 μg mL^{-1} was used as a positive control. Negative control group was cultivated in medium and DMSO as vehicle solution (never exceeding 1% v/v). The parasites were incubated for 24 h at 25 °C. Then, the plate was washed with 200 μL of sodium chloride 0.9% (w/v) three times with centrifugation at 3000 rpm, 10 min at 4 °C, followed by addition of MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) (5 mg mL^{-1}). Four hours later, 50 μL of 10% sodium dodecyl sulfate (SDS) was added to each well. The plates were further incubated for 18 h and read in ELISA reader at 595 nm. Effective concentration 50% (EC_{50}) was estimated using Graph Pad Prism 5.0 software.

4.5. J774 macrophages culture and cytotoxicity assay

Approximately 2×10^5 J774 macrophages were cultured in RPMI 1640 medium (Life Technologies), supplemented with 10% heat-inactivated fetal bovine serum, 10 μg mL^{-1} of gentamicin, and 1000 UmL^{-1} of penicillin (R10). Compounds **1** and **2**, as well as miltefosine (0.78–100.00 μg mL^{-1}) were added and after 24 h, cell viability was analyzed by MTT method. As negative control, J774 macrophages were cultivated in medium and DMSO as vehicle solution (never exceeding 1% v/v). Cytotoxic concentration 50% (CC_{50}) was estimated using Graph Pad Prism 5.0 software.

4.6. Macrophage infection and treatments

J774 macrophages (2×10^5 macrophage) were cultivated in round cover slips in 24-well plate, followed by infection with *L. amazonensis* amastigotes at a ratio of 10 parasites per macrophage. Plates were incubated at 5% CO_2 at 35 °C. After 24 h of culture, compound **2** (at 3.12, 6.25 and 12.50 μg mL^{-1}) and EC_{50} of miltefosine (10.00 μg mL^{-1}) were added in infected culture. After 24 h round cover slips were dried at room temperature, fixed in MeOH, and stained with Giemsa. The concentration able to decrease in 50% the infection index (EC_{50}) was estimated using the software Graph Pad Prism 5.0 software.

4.7. Ultraestructural alterations

Promastigote forms of *L. amazonensis* (2×10^7 promastigotes mL^{-1}) were incubated in 96-well culture plate in R10 medium with the EC_{50} of **1** and **2** during 24 h, at 25 °C. Control group was cultivated with medium and vehicle solution DMSO (never exceeding 1% v/v). The plate was centrifuged at 3000 rpm, 4 °C, 10 min and washed three times with 200 μL of NaCl 0.9%. The pellets were ressuspended in glutaraldehyde 2% and incubated at 4 °C, during 60 min. Parasites were post-fixed in 1% of OsO_4, and these materials were stained and block staining in 1% aqueous uranyl acetate overnight, dehydrated using EtOH. Then, samples were embedded in a polyester resin, thin sectioned with a LKB ultratome, double-stained by uranyl acetate and lead citrate (Ladd Research Industries), and examined with a JEOL 1010 (Tokyo, Japan) transmission electron microscope (TEM).

4.8. Mitochondrial membrane potential

Promastigote forms of *L. amazonensis* at the density 10^6 promastigote/well were seeded on a 96-well microplate with the EC_{50} of compound **2** (4.6 μg mL^{-1}) and at 15, 30, 45 and 60 min the mitochondrial membrane potential was analyzed using the probe rhodamine 123 (Rho). Oligomycin A was used as an inhibitor of mitochondrial membrane potential at 7.91 μg mL^{-1}. Control parasites were incubated only with medium. After these time-points, parasites were stained with 0.30 μg mL^{-1} of Rho for 15 min in the dark, at 25 °C. Then, parasites were washed three times with 200 μL of PBS and the fluorescence was measured using a fluorimetric microplate reader with excitation and emission wavelengths of 507 and 529 nm, respectively. Blank wells were constituted with PBS plus Rho. Neither parasites nor compound **2** give background in the reactions.

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Conflict of interest

The authors declare no conflict of interest.

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