Antileishmanial activity of nerolidol-rich essential oil from *Piper claussenianum*

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Article

Abstract: Leishmaniasis is one of the most neglected tropical diseases, representing a group of parasitic diseases worldwide spread, occurring in 88 tropical and subtropical countries. Approximately 350 million people live in areas of active transmission of leishmaniasis, with about 1-2 million estimated new cases occurring every year. More than 90% of the cutaneous cases appear in developing countries. Efforts to improve the therapeutic arsenal against leishmaniasis have led to the search for new and cheap range of drugs. In this study, the nerolidol-rich essential oil from *Piper claussenianum* (Miq.) C. DC., Piperaceae, was assayed on arginase activity of *Leishmania amazonensis*. The effect of this essential oil on arginase activity levels showed an enzyme inhibition of 62.2%. This result stimulates the scientific interest about the potential value of this plant species on neglected diseases as potential new natural product source of pharmacological interest for the treatment of leishmaniasis.

Introduction

Leishmaniasis is one of the most neglected tropical diseases, due to few tools available for controlling and also the lack of clear criteria for the disease management. Leishmaniasis represents a group of parasitic diseases worldwide spread, occurring in 88 tropical and subtropical countries. Approximately 350 million people live in areas of active transmission of *Leishmania*, as many as 12 million people are believed to be currently infected, with about 1-2 million estimated new cases occurring every year throughout Africa, Asia, Europe, and the Americas directly affected by leishmaniasis. More than 90% of the cutaneous cases appear in developing countries (Desjeux et al., 2004; WHO 2007). This illness can be promoted by more than twenty species of trypanosomatids from the genus *Leishmania*. The parasite is transmitted by insect vectors of the genera *Lutzomyia* and *Phlebotomus* (Diptera: Psychodidae: Phlebotominae), and is able to affect animals and humans (Ashford et al., 2000). The cutaneous leishmaniasis (CL) has afflicted humankind for centuries, and represent an infection characterized by a heteroxenic cycle affecting mainly skin or mucous membranes and is distinguished by the presence of ulcerative lesions. CL may cause the impairment and destruction of mucosal

and cutaneous structures, frequently producing facial mutilation (Murray et al., 2004). Since the discovery of the first class of drugs able to treat leishmaniasis (*i.e.*, pentavalent antimonials), the search for substances with antileishmanial activity, without toxic effects, and able to overcome the emergence of drug resistant strains still remains a current goal (Santos et al., 2008). Efforts to improve therapeutic arsenal against leishmaniasis have led to the search for new and cheap range of drugs. The searching for new drugs with antileishmanial activities, including natural product compounds, have been undertaken worldwide, although problems with the side effects of the chemotherapies currently used have not yet been solved (Santin et al., 2009). Natural products have been widely employed in folk medicine to treat infectious diseases, mainly in communities with inadequate conditions of health and sanitation. Many studies have shown plants of many regions of the world containing compounds with anti-protozoal activity. It has been reported that various essential oils (EO) show inhibitory action against diverse human parasites related to neglected tropical diseases such as *L. amazonensis* (Marques et al., 2010; Silva et al., 2009; Ueda-Nakamura et al., 2006).

In this work a nerolidol-rich essential oil from leaves of *Piper claussenianum* (Miq.) C. DC., Piperaceae,

was tested against *L. amazonensis* enzymatic activity of arginase, in order to understand the growth inhibition mechanism of this EO over this specific parasite, which has been previously reported elsewhere (Marques et al., 2010).

Materials and Methods

Plant material and essential oil extraction

Leaves (100 g) of *Piper claussenianum* (Miq.) C. DC., Piperaceae, were collected in Castelo-ES in January 2010. The botanical vouchers were identified by Dr. Elsie Franklin Guimarães and kept at the Herbarium (HB) of the Rio de Janeiro Botanical Garden (JBRJ), registered under number RB 489043. The fresh and dried plant materials were submitted to hydrodistillation for 2 h in a modified Clevenger-type apparatus. The obtained essential oils (EO) were dried over anhydrous sodium sulphate, yielding 1% w/v in both studied specimens.

GC-FID analysis

The gas chromatography analyses were carried out on a GC 2010 Shimadzu with a ZB-1MS fused silica capillary column (30 m x 0.25 mm x 0.25 μm film thickness). The operating temperatures used were as follows: injector 260 ºC, detector 290 ºC and column oven 60 °C up to 290 ºC (10 °C/min). Hydrogen at 1.0 mL min-1 was used as a carrier gas. The percentages of the compounds were obtained by GC-FID analysis.

GC-MS analysis

Qualitative analyses were carried out on a GC-QP2010 PLUS Shimadzu with a ZB-5MS fused silica capillary column (30 m x 0.25 mm x 0.25 μm film thickness) under the experimental conditions reported for GC-FID analysis. The essential oil components were identified by comparing their retention indices and mass spectra to published data and computer matching with WILEY 275 and the National Institute of Standards and Technology (NIST 3.0) libraries provided by a computer-controlled GC-MS system. The results were also confirmed by comparing the compounds' elution order with their relative retention indices reported in the literature (Adams, 2001). The retention indices were calculated for all the volatile constituents using the retention data of linear *n*-alkanes C_8-C_{24}.

Nuclear magnetic resonance spectroscopy

The EO obtained was analysed by ^{1}H and ^{13}C NMR and recorded on a Brüker DRX 400 spectrometer. The chemical shifts were determined in $CDCl_3$, using TMS as the internal standard. The spectrometric data for the ^{1}H NMR spectra are organised in agreement with the convention: chemical shift (number of protons and multiplicity), and the data for ^{13}C NMR spectra are organised by chemical shift. The signals of the NMR analyses were compared to the literature. (Marques et al., 2010).

Biological assays

Parasite

One *L. amazonensis* strain (MHOM/BR/75/Josefa) originally isolated from a human CL, kindly provided by Dr Elvira Saraiva (UFRJ, Brazil) was used in this study. Infective promastigote forms were maintained by weekly transfers in 25-cm^2 culture flasks with Schneider's insect medium (SIM) (Sigma Aldrich® St. Louis/ USA), pH 7.2, supplemented with 10% fetal bovine serum (FBS) and Gentamicine (80 μg/mL) at 26 °C. By this method, a five passage strain was used in all experiments carried out.

Protozoal arginase activity

Arginase enzyme activity was measured as previously described by Kropf et al. (2005), with slight modifications. Promastigotes were incubated for 72 h at 26 °C in presence or absence of the *P. claussenianum* leaves essential oil IC50 (Marques et al., 2010). Inhibition concentrations previously tested (Marques et al., 2010) was used in all experiments, since the EO stock solution was obtained by a single isolation and extraction. After incubation, 10^8 cells were mixed with 100 μL of 0.1% Triton X-100 and underwent five cycles of 3 min in the vortex and 1 min on ice bath. Thereafter, 100 μL of 25 mM Tris-HCl pH 8.3 were added to the sample. For arginase activity measurements, 10 μL of 10 mM $MnCl_2$ were added to each 100 μL of the lysate. The arginase was then activated by heating the sample for 10 min at 56 °C, and arginine hydrolysis was conducted by incubated the sample with 100 μL of 0.5 M L-arginine, pH 9.7 at 37 °C for 20 min. The reaction was stopped by adding 800 μL of H_2SO_4 (96%), H_3PO_4 (85%) and H_2O (1/3/7, v/v/v) to the sample and mixing with 40 μL of 4% of α-isonitrosopropiophenone (dissolved in ethanol). After heating (95 °C for 30 min), urea concentration was determined by spectrometry at 540 nm wavelenght.

Interaction *Leishmania*-mammal host cells

To assess the influence of IC50 *P. claussenianum* leaves essential oil on parasite host cell interactions, parasites were treated or not for 1 h with the EO, centrifuged at 4500 rpm, at 4 °C for 3 min, washed twice

in 10 mM PBS pH 7.2 and ressuspended in cold sterile Dulbecco's modified Eagle's medium (DMEM) (GIBCO®) to adjust the inoculum to 5 x 10⁶ cells. Coverslips adhered macrophages, obtained from BALB/c mice peritoneal washing, were incubated with the parasites, in a ratio of 10 parasites per host cell, in 300 µL of DMEM supplemented with 2% fetal calf serum at 37 °C 5% CO_2 for an hour. After interaction, the material was fixed in methanol for 5 min, washed and stained with Giemsa 36% for 40 min. Thereafter, coverslips were dehydrated in acetone and xylene, in concentrations 90:10, 70:30, 30:70 and 0:100 respectively, and stuck to a blade 26 x 76 with resin Entellan®.

Nitrite determination

Nitrite is a stable oxidation product of nitric oxide (NO), that can be measured spectrophotometricaly by adding Griess reagent (0.1% sulfanilamide and 0.1% N-1 naphtylethylenediamine in 2.5% phosphoric acid) at the same volume of infected macrophages cultures supernatants. After 20 min at room temperature and in the dark, the absorbance of the chromophore was measured at 540 nm wavelenght. Nitrite concentrations were estimated by comparison with a standard curve prepared with sodium nitrite in DMEM supplemented with 10% fetal bovine serum (v/v). Positive control of NO production, were macrophages treated with INFγ (50 ng/mL) or treated with INFγ and the EO.

Cytotoxicity assay

To evaluate the possible essential oil's toxicity to mammal cells, a cytotoxicity assay was performed using L929 fibroblast cells (mouse) and Raw cells (mouse macrophages). The cells were grown in Eagle's minimum essential medium (Eagle-MEM) supplemented with 10% (v/v) fetal bovine serum, glutamine (0.03 mg/mL), garamycin (50 µg/mL), fungizone (amphotericin B) (2.5 mg/mL), $NaHCO_3$ (0.25%) and HEPES (10 mM) (Schmidt, 1979). Cell cultures were prepared in 96-well microtiter plates (Falcon Plastics, Oxnard, CA, USA) and incubated at 37 °C in a 5% CO_2 atmosphere.

Cytotoxicity was evaluated by the neutral red dye-uptake method (Neyndorff et al., 1990). To determine the cytotoxicity of the *P. claussenianum* essential oil extracted from leaves, several concentrations of each sample (range from 40 to 0.56 mg/mL) were placed in contact with confluent L929 fibroblast cell monolayers and incubated at 37 °C in a 5% CO_2 atmosphere for 24 h. After incubation, cells were incubated in the presence of 0.01% neutral red solution for 3 h at 37 °C in a 5% CO_2 atmosphere. Then, the medium was removed and the cells were fixed with 4% formalin in phosphate-buffered saline, pH 7.2. The dye incorporated by the viable cells

was eluted using a mixture of methanol: acetic acid: water (50:1:49), and the dye uptake was determined by measuring the optical density (OD) of the eluate at 490 nm in an automatic spectrophotometer (ELx800TM-Bio-TeK Instruments, Inc.). The 50% cytotoxic concentration (CC50) was defined as the concentration that caused a 50% reduction in dye uptake.

All experiments were performed in triplicate, and the statistical analysis of the differences between mean values obtained was done by Student's t test. *p* values of 0.05 or less were considered significant.

Results and Discussion

The essential oils obtained by hydrodistillation from leaves of *P. claussenianum* in fresh and in dried material yielded about 1% (w/v) to both cases. Results for the qualitative and quantitative analysis of the essential oils are shown in Table 1 for the leaf volatile constituents. In total, sixteen different compounds were identified, ranging from 92.48% and 97.72% of the oils for fresh and dried leaves respectively. The oils showed a rich sesquiterpene content. Sesquiterpenes were identified as the main volatile fraction of the leaves, being Nerolidol the major constituent, ranging of up to 81.0% in EO obtained by hydrodistillation from fresh and dried samples. The structure of nerolidol was confirmed by GC-MS, ¹H NMR and ¹³C NMR spectra (Marques et al., 2010). The oil from leaves of *P. claussenianum* was characterized by their high content in (E)-nerolidol (81.41; 83.29%) as well as linalool (5.20; 2.15%), γ-muurolene (1.09; 3.22%), (*E*)-caryophyllene (0.64; 1.40%) and γ-elemene (0.54; 0.78%) fresh and dried material, respectively. The high percentage of nerolidol in EO from leaves of *P. claussenianum* allowed us to perform biological assays against *L. amazonensis* to understand the mechanism of growth inhibition of this parasite in cutaneous leishmaniasis. The fresh leaf EO with 81.41% of nerolidol was used to perform the biological assays on arginase inhibition.

Arginase is a dependent manganese metalloenzyme able to hydrolyze L-arginine to L-ornithine and urea, and its activity has been related to the synthesis of nitric oxide (NO), and therefore associated to cytotoxic process and immunological mechanisms (Kanyo et al., 1996; Da Silva et al, 2002). The arginase activity in the Tripanossomatidae family has been shown in some specific genre, and it has been used for identification and classification purposes. The genus *Leishmania* has a detectable arginase activity which is considered to have a role in the production of ornithine reported by Da Silva et al. (2002) and therefore essential for the growth of the parasite, since previous studies revealed that arginase null protozoans are unable to generate polyamines (Vincendeau et al.,

2003; Roberts et al., 2004).

The effect of the leaf essential oil of *P. claussenianum* showed 62.17% inhibition in the levels of arginase activity (Figure 1). Regarding to the *Leishmania*-macrophage interaction mechanisms, when parasites were pretreated for one hour with the IC50 of the EO from leaves of *P. claussenium*, the infected macrophages percentage was reduced in 42.7% (*p*=0,01). When the EO was used on infected macrophages the infected macrophages percentage was reduced in 31.25% (*p*=0,03) (Figure 2). The production of NO from infected macrophages treated for 24 h with the IC50 of the EO was measured by the nitrite concentration in the culture supernatants, using Griess reagent. In this case there was an increase of 17.2% in NO production, an increase greater than that observed when infected cells were treated with INF-γ. Interestingly an increase of 20,5% in NO production could be seen when EO and INF-γ were used together (Figure 3).

Table 1. Identified compounds in the essential oil from leaves of *P. claussenianum*.

Compounds	[a]RI[Lit]	[b]RI	Fresh H.D %	Dry H.D %	Identification
α-pinene	939	936	0.17	-	RI, GCMS
β-pinene	979	976	0.18	0.15	RI, GCMS
β-myrcene	991	987	-	0.18	RI, GCMS
(*Z*)-β-ocimene	1037	1033	0.62	-	RI, GCMS
(*E*)-β-ocimene	1050	1043	0.85	-	RI, GCMS
cis-linalool oxide	1087	1083	0.57	-	RI, GCMS
linalool	1097	1101	5.20	2.15	RI, GCMS
γ-elemene	1338	1340	0.12	1.79	RI, GCMS
β-elemene	1391	1387	0.54	0.78	RI, GCMS
(*E*)-caryophyllene	1419	1415	0.64	1.40	RI, GCMS
α-humulene	1455	1451	0.59	1.09	RI, GCMS
γ-muurolene	1480	1477	1.09	3.22	RI, GCMS
α-selinene	1498	1497	0.35	-	RI, GCMS
δ-cadinene	1523	1520	0.38	0.76	RI, GCMS
(*E*)-nerolidol*	1563	1563	81.41	83.29	RI, GCMS
α-eudesmol	1654	1654	0.50	2.91	RI, GCMS
% Peak sum of identified compounds			92.48	97.72	

[a]RI Lit: Literature Retention Indices; [b]RI: Experimental Retention Indices; H.D: Hydrodistillation; *The structure of (*E*)-nerolidol was also confirmed by NMR and Internal Standard FID analysis.

The effect of essential oil of *P. claussenianum* on arginase activity levels showed a strong and significant inhibition of the enzyme activity. The ability to survive and multiply within macrophages is a particular feature of several microorganisms including *Trypanossoma cruzi* and *Leishmania*. In order to sustain a chronic infection, parasites must subvert macrophage-accessory cell activities and ablate the development of protective immunity

(Alexander et al., 2002). The increase in NO production by macrophages infected and treated with the EO suggests that it may be considered a useful strategy in controlling the infection by inhibiting parasite arginase.

The most important mechanism for the killing of *Leishmania* and for the control of leishmaniasis is the production of nitric oxide by macrophages (Holzmuller et al., 2006). These immune cells can control *Leishmania* infection when Th1 response is mounted and pro-inflammatory cytokines are released. This process leads to the induction of inducible nitric oxide sinthase (iNOS) and NO production. Arginine, a substrate of NO production, is modulated by both Th1 and Th2 responses in a manner that Th1 response increases conversion of arginine to NO, whereas the Th2 response promotes arginase induction (Wanasen & Soong, 2008).

The inhibition of the enzymatic activity may be able to modulate cellular infection in *Leishmania major* and *Leishmania infantum* infected macrophages, while the increasing parasite arginase activity by IL-4 induction promotes *Leishmania* growth (Iniesta & Gomes-Nieto, 2001). Futhermore, Iniesta and colleagues in 2005 demonstrated that *L. major* infection leads to arginase I induction in macrophages, and that host arginase I induction supports *Leishmania* growth.

Previous studies have demonstrated that the arginase activity in *Leishmania* spp. is able to modulate the nitric oxide synthase activity (Boucher et al., 1999; Wanasen & Soong, 2008). Besides, studies using models of visceral leishmaniasis by *L. donovani* showed that the parasite is able to modulate the activity of arginase in spleen cells of infected animals, increasing the enzyme expression and favoring the maintenance of infection (Osorio et al., 2008). Strikingly, the active role played by *Leishmania* arginase in diverting arginine away from the iNOS pathway was demonstrated by the increased host NO response to *L. mexicana* knockout for arginase infection in mice. The *L. mexicana* knockout for arginase infection led to an enhanced Th1 response associated to an INF-γ upregulation as well. Taken together, these responses led to a significant growth attenuation of the parasite in mice (Gaur et al., 2007). Considering the importance of the cellular signaling pathways involving the host cells and parasites of the *Leishmania* genus there are few studies in the literature connecting the importance of the parasite arginase inhibition and macrophage NO production. Therefore, arginase-specific inhibitors may be considered a potential alternative among new substances with pharmacological interest for the treatment of leishmaniasis.

The interference in *Leishmania* - mammalian host cell interaction suggests that the essential oil of *P. claussenianum* may action on early steps of this process of interaction. Therefore this EO may be able to modulate the virulence factors not yet identified, or to block specific
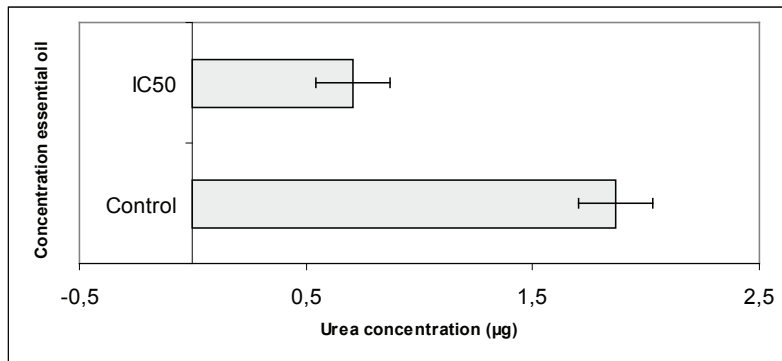
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Figure 1. Essential oil from leaves of *P. claussenianum* in arginase activity of promastigotes of *L. amazonensis.* The cells were incubated in the absence or presence of IC50 for 72 h at 26 °C. The enzyme activity was measured by the concentration of urea in the system (*p*=0.03).
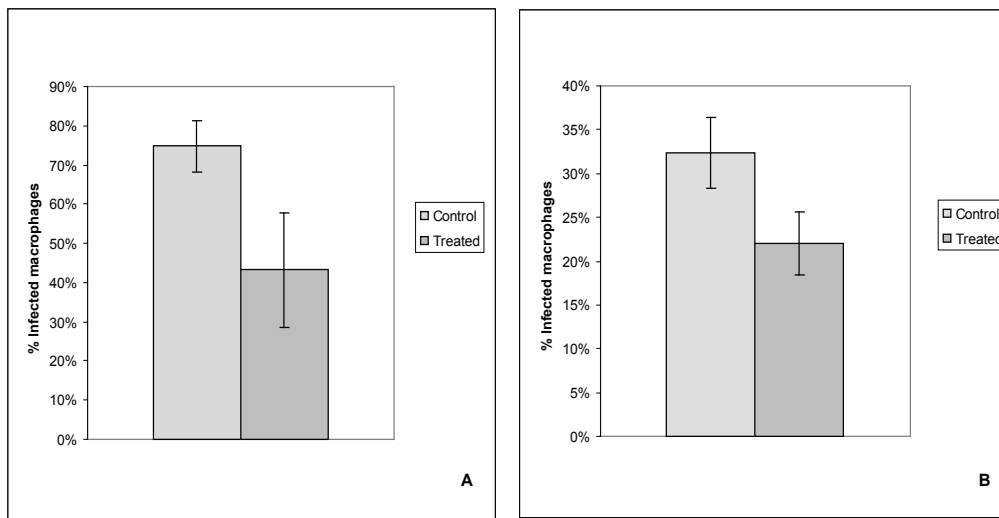

Figure 2. A. Percentage of macrophages infected with *L. amazonensis* pretreated or not with the IC50 of essential oil from leaves of *P. claussenianum* after one hour of interaction. Reduction of 42.7% infection (*p*=0.01). B. Percentage of macrophages infected with *L. amazonensis* treated or not with the IC50 of essential oil from leaves of *P. claussenianum* for 24 h. Reduction of 31.25% of infection (*p*=0.03).
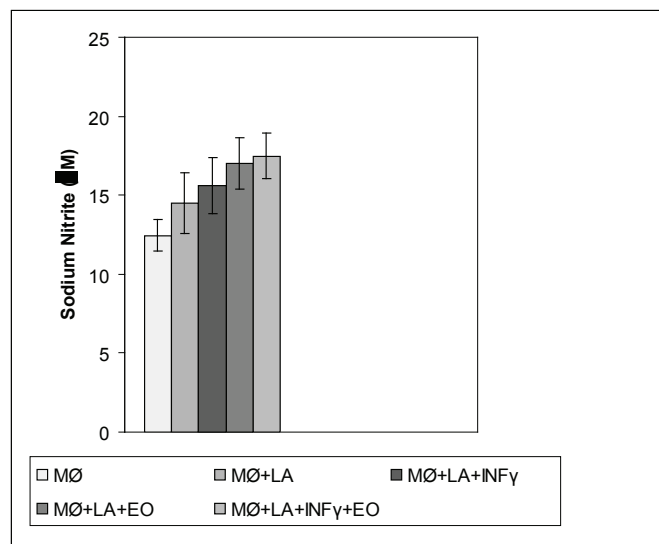

Figure 3. Nitrite dosages of culture supernatants of macrophages (MØ) infected with *L. amazonensis* and treated with the IC50 of the EO for 24 h. As controls were used uninfected and infected MØ treated with INF-γ.

receptors. This experiment was carried out with sub-inhibitory concentration of the EO in order to avoid compromising the viability of parasites. Santos et al. (2009) observed similar results when *L. amazonensis* promastigotes were pre-treated with protease inhibitors for HIV.

The citotoxicity assay revealed that the EO extracted from leaves of *P. claussenianum* has no toxicity effect to fibroblasts nor macrophages cell lines in any concentration tested (ranging from 40 to 0.56 mg/mL). Macrophages and fibroblasts generate from mice were challenged with the essential oil extracted from the leaves of *P. claussenianum* (range 40 to 0.56 mg/mL), whose major component is (*E*)-nerolidol, demonstrating no cytotoxic effect on either cell lines in any concentration. This finding agrees with literature data, in fact toxic effects were not observed in vivo after nerolidol intraperitonial or topic administration in mouse (Arruda at al., 2005). In vivo studies have demonstrated that animals treated with nerolidol in concentrations up to 200 mg/kg showed no clinical signs or morbidity, and in addition, peripheral blood cells and liver taken from these animals showed no DNA damage (Pículo et al., 2010). Thus, the absence of citotoxicity in high concentrations may be considered a relevant issue pointing to the possible administration of this compound as an alternative for the management of leishmaniasis, since it is able to control protozoa growth, as well as modulate some crucial virulence factors, such as the arginase activity and the interaction mechanisms between parasites and mammal host cells.

Conclusion

The essential oil extracted from the leaves of *P. claussenianum* is a natural product rich in (*E*)-nerolidol that has a surprising biological activity, showing efficient growth inhibition of promastigote forms of *L. amazonensis*, and a significant inhibition on its arginase activity as well as an important enhancement in the NO levels in infected macrophages induced by this EO. Moreover, this essential oil is able to interfere with the parasite-host cell interaction, reducing the percentage of infected cells. Nevertheless, no cytotoxic effect was evidenced against any of the mammalian cells lines tested. The results presented in this study points to the *P. claussenianum* leaves essential oil as an important source in the searching for new alternatives for the treatment and management of cutaneous leishmaniasis.

Acknowledgments

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