Nerolidol, the main constituent of *Piper aduncum* essential oil, has anti-*Leishmania braziliensis* activity

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(*Received 23 November 2016; revised 20 March 2017; accepted 20 March 2017; first published online 9 May 2017*)

SUMMARY

Leishmania (*Viannia*) *braziliensis* is a protozoan that causes mucocutaneous leishmaniasis, which is an infectious disease that affects more than 12 million people worldwide. The available treatment is limited, has side-effects or is inefficient. In a search for alternative compounds of natural origin, we tested the microbicidal activity of *Piper aduncum* essential oil (PaEO) on this parasite. Our data showed that PaEO had an inhibitory effect on the growth of *L. braziliensis* promastigotes with an IC_{50}/24 h=77·9 μg mL^{-1}. The main constituent (nerolidol: 25·22%) presented a similar inhibitory effect (IC_{50}/24 h = 74·3 μg mL^{-1}). Ultrastructural observation of nerolidol-treated parasites by scanning and transmission electron microscopies revealed cell shrinkage and morphological alterations in the mitochondrion, nuclear chromatin and flagellar pocket. Flow cytometry analysis showed a reduction in the cell size, loss of mitochondrial membrane potential, phosphatidylserine exposure and DNA degradation, which when associated with the morphological changes indicated that nerolidol induced incidental cell death in the *L. braziliensis* promastigotes. The results presented here indicate that nerolidol derivatives are promising compounds for further evaluation against *Leishmania* parasites.

Key words: cell death, essential oil, *Leishmania braziliensis*, nerolidol, *Piper aduncum*.

INTRODUCTION

Mucocutaneous leishmaniasis is an infectious disease caused by diverse flagellate kinetoplastids of the genus *Leishmania*, which are transmitted by *Lutzomyia* phlebotomines (Brasil, 2009). No vaccine is available against human leishmaniasis, and actions to combat the disease are restricted to vector control (Srivastava *et al*. 2016). HIV and *Leishmania* co-infection occurs in 35 countries and increases the lethality of the disease due to the difficulty of clinical management and treatment (Lindoso *et al*. 2016). More than 12 million people are infected worldwide, and 350 million people live in risk areas. In the Americas, 743 970 cases were registered between 2001 and 2013, with an annual average of 57 228 cases (OPAS/OMS, 2015). *Leishmania* (*Viannia*) *braziliensis* is related to this type of infection, with a geographical distribution that extends from Central America to northern Argentina (Basano and Camargo, 2004).

The first-line treatment for mucocutaneous leishmaniasis in Brazil is pentavalent antimonials, and the second choice drugs are amphotericin B and pentamidine. However, all of these drugs exhibit considerable toxicity. Pentavalent antimonials have many adverse reactions that often lead to discontinuation of treatment (OPS, 2013). Therefore, the search for alternative effective compounds is relevant. Natural products appear as a source for treating neglected parasitic diseases (Kayser *et al*. 2003; Ndjonka *et al*. 2013; de Moraes, 2015) and important information for the therapeutic use of plants can be gathered from popular knowledge (Anthony *et al*. 2005). The antiparasitic drugs artemisinin and chloroquine are examples of plant-derived products, and amphotericin B and ivermectin are important antiparasitics isolated from *Streptomyces* microorganisms (de Moraes, 2015).

Different pepper species (*Piper* spp., Piperaceae) are widely used in folk medicine, and their biological activity is well documented. For instance, extracts of diverse peppers (*P. claussenianum, P. nigrum, P. longum* and *P. aduncum*) inhibit tumour growth *in vitro* and *in vivo* and even the incidence of metastasis (Sunila and Kuttan, 2004; Majdalawieh and Carr, 2010; Marques *et al*. 2013; Arroyo-Acevedo *et al*. 2015). Therefore, testing the ability of a pepper species to inhibit the growth of trypanosomatid protozoa appears to be a reasonable proposition.

Piper aduncum (matico) is a perennial bush or small tree with spear-shaped leaves that is native to southern Mexico, the Caribbean and most tropical countries in South America. Indigenous groups in the Americas, such as the Lancadones, make tea

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doi:10.1017/S0031182017000452

from its leaves and roots to treat a wide variety of medical conditions, such as diarrhoea, dysentery, nausea, ulcers and genitourinary tract infections (Cook, 2016). *Piper aduncum* essential oil (PaEO) has the potential to be used as insect repellent (Mamood *et al*. 2017) and antihelminthic against the nematode *Haemonchus contortus* (Gaínza *et al*. 2016). Ethanolic extracts of *P. aduncum* leaves showed considerable antifungal and antibacterial activities *in vitro* (Okunade *et al*. 1997). Additionally, these extracts were active as acaricidal agents against *Tetranychus urticae* (Araújo *et al*. 2012), repellents against *Aedes aegypti* and *Aedes albopictus* (Misni *et al*. 2011), and reduced inflammatory interleukins in dendritic cells (Thao *et al*. 2016), which demonstrated the wide range of biological effects of this plant species.

Essential oils (EOs) of different species of the genus *Piper* (*P. aduncum* var. *ossamum, P. cernuum, P. claussenianum, P. demeraranum, P. duckei, P. heptaphylum* and *P. hispidum*) have demonstrated activity against *Leishmania amazonensis* and *L. infantum* (Marques *et al*. 2010; Carmo *et al*. 2012; Capello *et al*. 2015; Houël *et al*. 2015; Gutiérrez *et al*. 2016). Therefore, studies on the effect of EOs against *Leishmania* are promising. However, few studies have investigated the interaction between EOs and *L. braziliensis*, which is one of the main causative agents of mucocutaneous leishmaniasis in Brazil (Jones *et al*. 1987), primarily due to the difficulty in maintaining this parasite *in vitro*.

Therefore, given the variety of biological activities of *P. aduncum* and the need for new alternative compounds to inhibit parasite growth, we tested the effect of the EO of *P. aduncum* and its major components (nerolidol and linalool) against *L. braziliensis*. Our data showed that nerolidol had leishmanicidal activity. Ultrastructural observations and flow cytometry analysis indicated that nerolidol induced incidental cell death in the *L. braziliensis* promastigotes.

MATERIALS AND METHODS

Chemicals

Linalool, nerolidol, amphotericin B (250 μg mL^{-1}), gentamicin (50 mg mL^{-1}), PMA (phorbol-12-myristate-13-acetate), Schneider's Insect Medium, DAPI (4′,6-diamidino-2-phenylindole), MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide), CCCP (carbonyl cyanide 3-clorophenylhydrazone), Nonidet P 40 substitute (NP-40) and DMSO (dimethyl sulphoxide) were purchased from Sigma-Aldrich (St. Louis, MO, USA). RPMI 1640 medium, fetal bovine serum, propidium iodide, rhodamine 123, RNase A DNase-free and the Alexa Fluor-488 Annexin/Dead Cell Apoptosis Kit were purchased from Thermo Fisher Scientific (Carlbad, CA, USA).

Essential oil

Piper aduncum leaves were collected on March 2013 in the morning, at the Medicinal Plants Garden of the Federal University of Lavras, Minas Gerais (UFLA), Brazil. The essential oil (PaEO) was obtained at the Chemistry Department of UFLA by Clevenger distillation. The chemical characterization was performed by GC–MS, which demonstrated that nerolidol (25·22%) and linalool (13·42%) were the major components (Villamizar *et al*. 2017). For the assays, PaEO, linalool and nerolidol were diluted to 100 mg mL^{-1} in DMSO and kept at 4 °C protected from the light (Escobar *et al*. 2010).

THP-1 cells

THP-1 cells (ATCC TIB-202) were kept at 37 °C in a humidified incubator with a 5% CO_2 atmosphere, in RPMI 1640 medium supplemented with 10% fetal bovine serum (FBS) and 50 μg mL^{-1} of gentamicin. The cell cultures were maintained by weekly subculture when the growth reached 1×10^6 cells mL^{-1}.

Parasites

Leishmania braziliensis (strain MHOM/BR/75/M2903) promastigotes were maintained at 26 °C in Schneider's medium supplemented with 10% FBS and 2% male child urine with two passages per week. For the biological assays with intracellular amastigotes, differentiated THP-1 cells were infected (ratio of 10 parasites per host cell) with promastigotes from 5-day-old cultures.

Cytotoxicity assays

THP-1 cells were seeded into 96-well plates at a density of 5×10^4 cells/well in RPMI 1640 medium containing 200 nM PMA. The plates were incubated for 96 h at 37 °C in a humidified incubator with a 5% CO_2 atmosphere to induce differentiation into adherent macrophages (Donega *et al*. 2014). Then, the cells were incubated for 24 h with 15·625–500 μg mL^{-1} of the compounds, and the cell viability was evaluated by the MTT colorimetric assay. The optical density (O.D.) was read in a BioTek EL800 reader (Biotek, Winooski, VT, USA). Wells with no treatment (negative control) were used for comparison (100% cell viability). The mean cell inhibition (CI) values were obtained from three experiments performed in triplicate according to the equation CI=100−((O.D treatment/O.D. negative control)×100). Dose *x* response curves were used to estimate the CC_{50}/24 h, which was the toxic concentrations for 50% of the culture after 24 h (Escobar *et al*. 2010).

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Alternatively, linalool, nerolidol and amphotericin B were evaluated for their haemolytic potential. For this analysis, type O^+ peripheral blood was collected from a healthy donor, defibrinated for 10 min under stirring using glass beads and washed three times in glucose saline solution (0·85% NaCl and 5% glucose). The red blood cells were resuspended at 3% in the glucose saline solution and seeded into 96-well plates containing different concentrations of each compound tested. The plates were incubated for 3 h at 37 °C, and the absorbance of the supernatant was read at 550 nm in a BioTek EL800 reader. The percentage of haemolysis was correlated to the O.D. values of the positive control (red blood cell solution plus 1% Triton X-100), which was considered 100% haemolysis (Izumi *et al*. 2012).

Activity against promastigotes

Four-day-old *L. braziliensis* promastigotes were adjusted to a density of $4{\cdot}5 \times 10^7$ cells mL^{-1}, and 100 μL $well^{-1}$ of this suspension was seeded into 96-well plates. Different dilutions of PaEO, linalool and nerolidol (final concentration 1–500 $\mu g\ mL^{-1}$) were added to each well (100 μL $well^{-1}$). Amphotericin B was used as a positive control at concentrations ranging from 0·062 to 2 $\mu g\ mL^{-1}$. Parasite viability was assessed by MTT staining after incubation with the drugs for 24 h. The mean cellular inhibition values were obtained from three experiments performed in triplicate, and dose *x* response curves were used to estimate the inhibitory concentration for 50% of the parasites (IC_{50}/24 h). The statistical analysis was performed with GraphPad Prism 5 software (San Diego, CA, USA) by applying two-way ANOVA and the Bonferroni *post hoc* test. A P value <0·05 was considered significant.

Activity against intracellular amastigotes

Differentiated THP-1 cells were seeded into 96-well plates at a density of 5×10^4 cells/well and incubated for 4 h with 5-day-old promastigotes at a proportion of 10 parasites per cell. Then, the cells were washed and incubated for another 48 h. The medium was replaced, and the monolayers were incubated with nerolidol (1·95–62·5 $\mu g\ mL^{-1}$) or amphotericin B (0·062–1 $\mu g\ mL^{-1}$) for 24 h (Donega *et al*. 2014). The cells were fixed with absolute methanol and stained with DAPI. The inhibitory activity was assessed by counting the number of intracellular amastigotes in 10 images randomly captured from 10 microscopic fields (20*x* objective). The values were expressed as the per cent inhibition (%I) calculated as: $\%I=100-((T\times100)/C)$, where T is the average of all treated intracellular amastigotes and C is the average of the total intracellular amastigotes in the negative control (Guru *et al*. 1989; Lakshmi *et al*. 2007).

Electron microscopy

For scanning electron microscopy (SEM), promastigotes were incubated with the IC_{50}/24 h or 2×IC_{50}/24 h of nerolidol or amphotericin B, fixed with 2·5% glutaraldehyde in 0·1 M sodium cacodylate buffer, washed and adhered to coverslips coated with 0·1% poly-L-lysine. The cells were post-fixed with 1% osmium tetroxide, dehydrated in an ascending acetone series, submitted to critical point drying, coated with a 20-nm-thick gold layer, and observed under a JEOL JSM 6010 PLUS-LA (Akishima, Tokyo, Japan) scanning electron microscope.

For transmission electron microscopy (TEM), after treatment with the IC_{50}/24 h or $2 \times IC_{50}$/24 h of nerolidol or amphotericin B, the promastigotes were fixed with 2·5% glutaraldehyde in 0·1 M sodium cacodylate buffer, washed and post-fixed for 1 h with 1% osmium tetroxide. Then, the samples were dehydrated in increasing concentrations of acetone and embedded in Poly/Bed 812 resin for 72 h at 60 °C. Ultrathin sections were contrasted for 30 min with uranyl acetate and for 2 min with lead citrate. The ultrathin sections were observed in a JEOL JEM-1400Plus transmission electron microscope at 80 kV.

Flow cytometry

For each type of analysis, 1×10^6 *L. braziliensis* promastigotes were pre-treated for 24 h with nerolidol or amphotericin B (IC_{50} or $2 \times IC_{50}$), and then labelled according to the different assays. In all cases, 10 000 events were read in an FACSCanto II flow cytometer (Becton–Dickinson, San Jose, CA, USA) and the data were analysed using the FlowJo software v10 (Treestar Software, Ashland, OR, USA). The experiments were performed in triplicate. The statistical analysis was performed in the GraphPad Prism 5 software with one-way or two-way ANOVAs and the *post hoc* Bonferroni test, with $P < 0{\cdot}05$ considered significant.

To estimate the mitochondrial membrane potential (ψm), promastigotes were collected, washed with PBS and incubated for 15 min at 26 °C with 5 $\mu g\ mL^{-1}$ of rhodamine 123 (Rh123) (Petit, 1992). Changes in Rh123 fluorescence emission were quantified by the variation index $VI=[(MT-MC)/MC]$, where MT is median fluorescence of the treated parasites and MC is median fluorescence of the control. Negative VI values indicated mitochondrial membrane depolarization (Mendes *et al*. 2016).

To estimate the cell size, promastigotes were collected, resuspended in 1 mL of PBS and immediately analysed in the cytometer to determine the size and granularity (FSC×SSC) (Jiménez-Ruiz *et al*. 2010). Histograms were generated, and the FSC-H parameter was considered representative of the cell size (Rodrigues *et al*. 2014).

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Table 1. Cytotoxicity (CC_{50}/24 h) and leishmanicidal (IC_{50}/24 h) activity of the *Piper aduncum* essential oil (PaEO), linalool and nerolidol on the *Leishmania braziliensis* promastigote (pro) and amastigote (ama) forms compared to the reference drug amphotericin B (Amph-B). All data are in $\mu g\ mL^{-1}$.

	Macrophage CC_{50}	Red blood cell HC_{50}	Promastigote IC_{50}	$SI_{M\phi}$ pro	SI_{RBC} pro	Amastigote IC_{50}	$SI_{M\phi}$ ama
PaEO	87·14	>500	77·9	1·12	>6·42	ND	–
Nerolidol	104·55	1500	74·3	1·41	20·19	47·5	2·20
Linalool	>500	7281·25	430	>1·16	16·93	ND	–
Amph-B	>32	96·67	0·24	>133·3	402·79	0·5	>64

CC_{50}, cytotoxic concentration for 50% of macrophage cultures after 24 h; HC_{50}, hemolysing concentration for 50% of red blood cells after 24 h; IC_{50}, inhibitory concentration for 50% of parasites after 24 h; $SI_{M\phi}$, selectivity index for macrophages; SI_{RBC}, selectivity index for red blood cells; ND, not done.

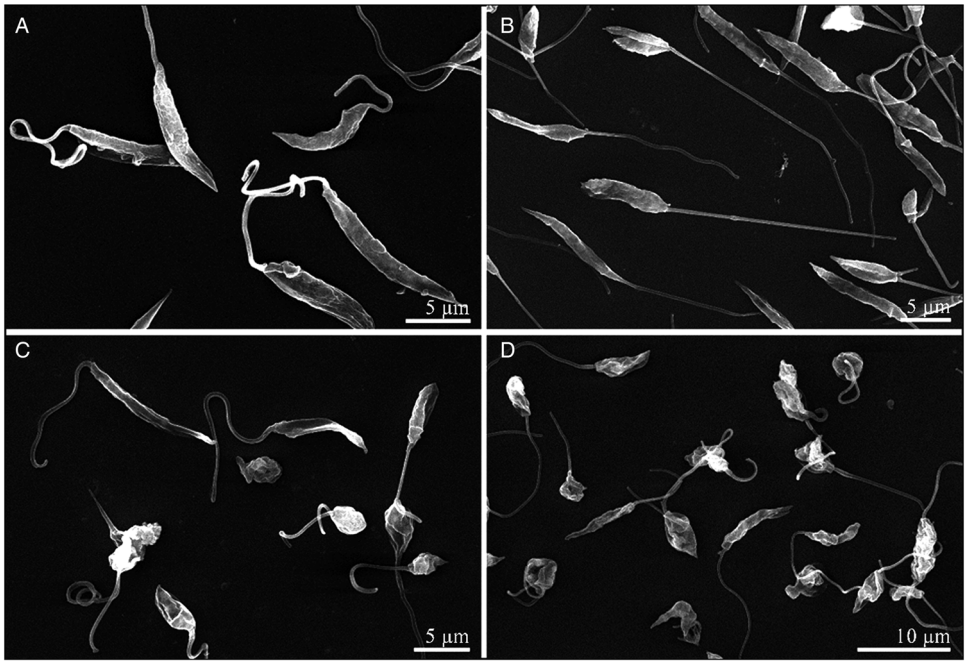

Fig. 1. SEM of *L. braziliensis* promastigotes. (A) Untreated control; (B) Treatment with IC_{50}/24 h of amphotericin B; (C) Treatment with IC_{50}/24 h of nerolidol; (D) Treatment with $2 \times IC_{50}$/24 h of nerolidol. Although no morphological alteration was noted after incubation with amphotericin B, nerolidol treatment caused shrinkage and roundness of the parasite cell body.

For the cell cycle analysis, the promastigotes were resuspended in 500 μL of PBS, to which was added the same volume of permeabilizing solution and DNA staining solution (3·4 mM Tris–HCl, pH 7·4, 0·1% NP-40, 700 U L^{-1} of RNase A-DNase free, 10 mM NaCl and 75 μM propidium iodide). The fluorescence reading was taken after incubation for 10 min at room temperature. The data analysis was performed from a specific gate that excluded doublets. The Dean–Jett–Fox algorithm was used to estimate the percentage of cells in the G_0/G_1, S and G_2/M phases (Kessler *et al*. 2013). In apoptotic cells, DNA fragmentation was perceived as a sub-peak formation in cell fluorescence at the G_0/G_1 stage (Khademvatan *et al*. 2011).

To measure cell membrane integrity and phosphatidylserine (PS) exposure, promastigotes were resuspended in annexin-binding buffer (140 mM NaCl, 5 mM $CaCl_2$ and 10 mM HEPES-Na, pH 7·4) and labelled with Alexa Fluor 488-conjugated Annexin-V and 100 $\mu g\ mL^{-1}$ of propidium iodide (PI). A 0·25% concentration of saponin was used as the positive control (Lazarin-Bidóia *et al*. 2016). In apoptotic cells, PS is exposed and can be detected by its reactivity with Annexin-V. Annexin-V (PS exposure) labelling alone is interpreted as evidence of apoptosis, whereas PI labelling is perceived as necrosis (i.e., loss of membrane permeability/damage to the plasma membrane). Double-labelled cells (Annexin-V and PI) are viewed as cells with a lack of distinction between late apoptosis and necrosis (Islamuddin *et al*. 2014; Chouhan *et al*. 2015; Masic *et al*. 2015; Awasthi *et al*. 2016; Corpas-López *et al*. 2016). Apoptotic cells are also

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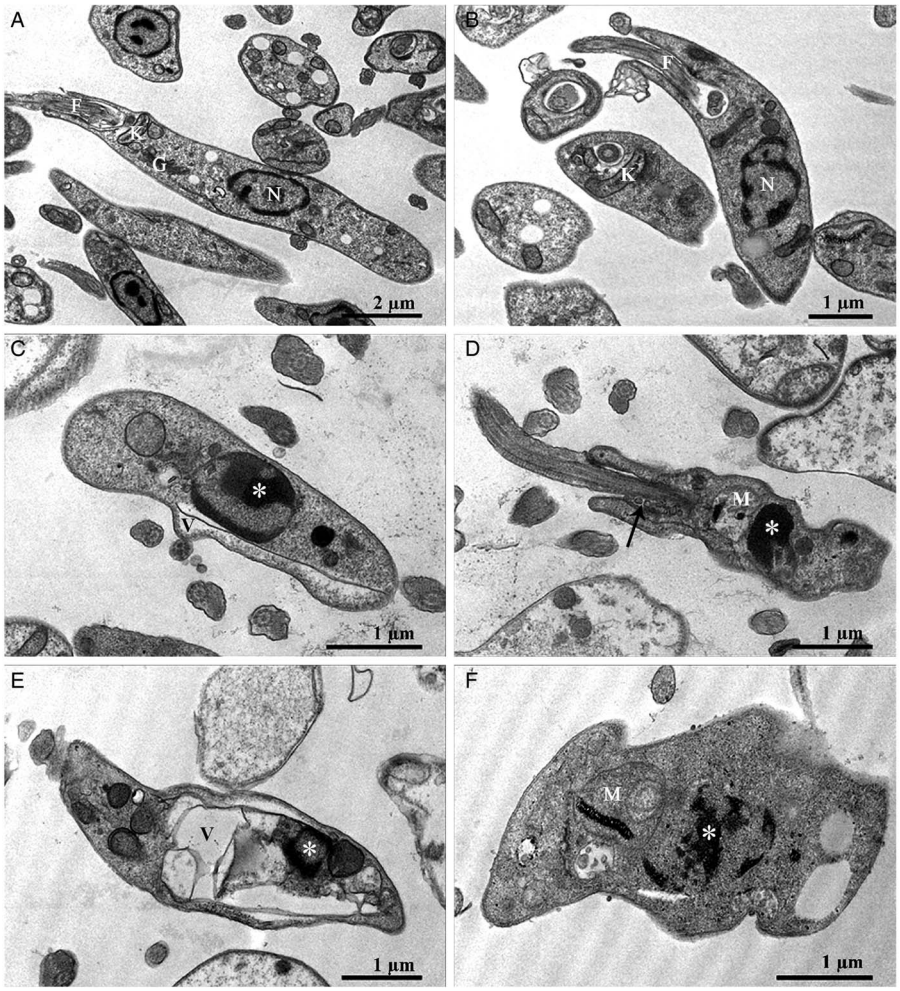

Fig. 2. Transmission electron microscopy of *L. braziliensis* promastigotes. (A) Untreated control; (B) IC_{50}/24 h of amphotericin B, showing no morphological alterations; (C–F) Incubation with IC_{50}/24 h (C, D) or 2 × IC_{50}/24 h (E, F) of nerolidol. Note cytoplasmic vacuolization (V), chromatin condensation (*), vesicles at the flagellar pocket (arrow) and mitochondrial swelling and disorganization (M). F: flagellum; G: Golgi complex; N: nucleus.

recognized by positive labelling with Annexin-V regardless of the positive or negative PI status (Jimenez *et al*. 2008).

RESULTS

EOs are mixtures of saturated and unsaturated hydrocarbons, alcohols, aldehydes, esters, ethers, ketones, terpenes and phenolic oxides that can produce characteristic odours (Ali *et al*. 2015). Linalool and nerolidol are sesquiterpenes that are present as major components in the EO of the *P. aduncum* pepper analysed here. First, we assessed the inhibitory effect of PaEO, nerolidol and linalool on the growth of *L. braziliensis* promastigotes after 24 h of treatment. The IC_{50}/24 h value was determined by the colorimetric MTT method (Supplementary Fig. 1), which is an easy, inexpensive method that is suitable for drug screening (Fumarola *et al*. 2004). The activity profiles of the PaEO and nerolidol on the *L. braziliensis* promastigotes were similar, with comparable IC_{50}/24 h values (77·9 and 74·3 $\mu g\ mL^{-1}$, respectively). In contrast, linalool demonstrated little activity (IC_{50}/24 h = 430 $\mu g\ mL^{-1}$). Determination of the per cent inhibition of intracellular amastigote growth after incubation with nerolidol or amphotericin B (Supplementary Fig. 2) showed that the IC_{50}/24 h value for nerolidol against amastigotes was 47·5 $\mu g\ mL^{-1}$ and for amphotericin B was 500 $ng\ mL^{-1}$ (Table 1).

After the anti-leishmanial potential of PaEO and nerolidol was confirmed *in vitro*, next we assessed the toxic concentration in a mammalian cell line (macrophage differentiated THP-1 cells). Linalool had lower cytotoxicity (CC_{50}/24 h > 500 $\mu g\ mL^{-1}$) than PaEO or nerolidol (87·14 and 104·55 $\mu g\ mL^{-1}$, respectively) (Supplementary Fig. 3). We estimated the Selectivity Index, which expresses the drug-specific selectivity of parasites rather than mammalian cells. *Leishmania braziliensis* intracellular amastigotes were more susceptible ($SI_{M\phi}$ = 2·20) than promastigotes ($SI_{M\phi}$ = 1·41) when incubated with nerolidol. Additionally, the toxicity of

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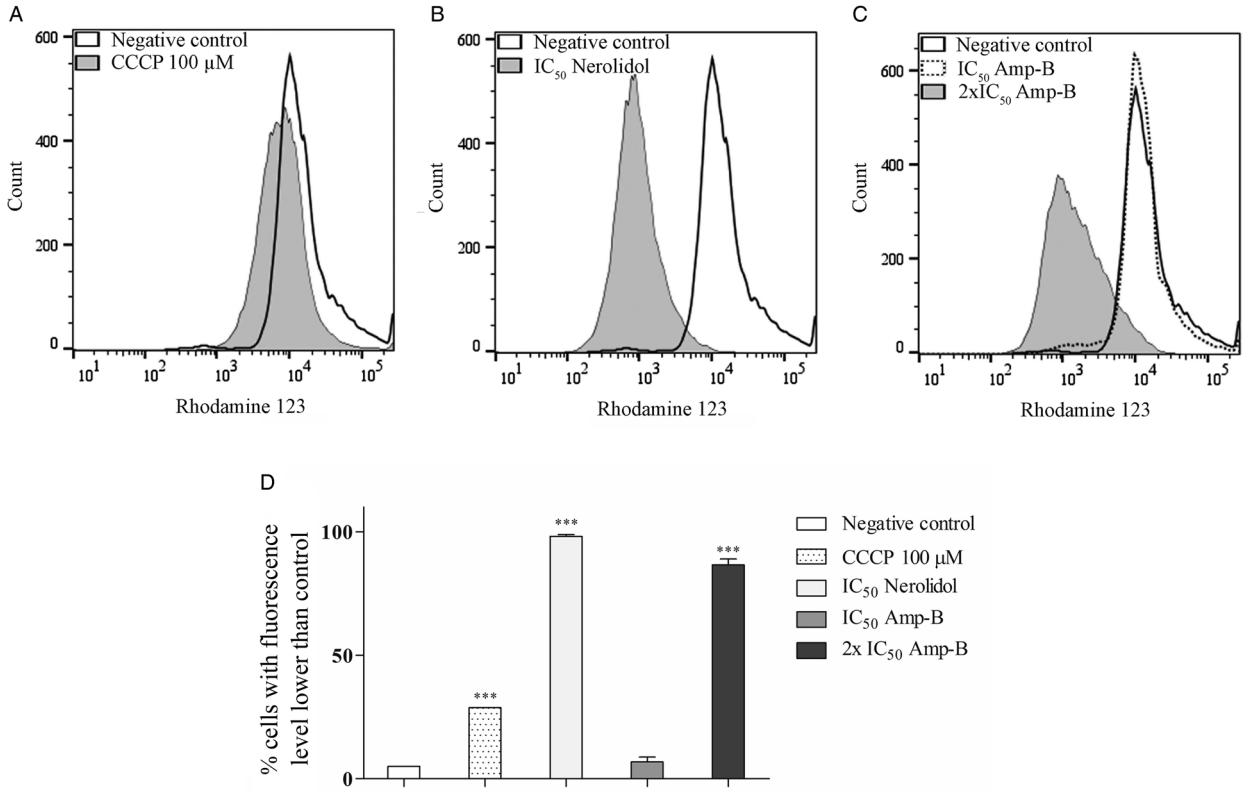

Fig. 3. Mitochondrial membrane potential (ψm) of *L. braziliensis* promastigotes incubated for 24 h with the IC_{50} of nerolidol and the IC_{50} or $2 \times IC_{50}$ of amphotericin B. Graphs in white correspond to the untreated (negative) control. (A) Positive control – 100 μM CCCP; (B) IC_{50} of nerolidol; (C) IC_{50} of amphotericin B and $2 \times IC_{50}$ of amphotericin B; (D) Percentage of parasites with Rh123 fluorescence level lower than the negative control. $*P < 0{\cdot}05$; $**P < 0{\cdot}01$; $***P < 0{\cdot}001$.

Table 2. Mean and standard deviation of the variation index (VI) to assess the mitochondrial membrane potential of promastigotes labelled with rhodamine 123 (Rh123).

	Mean Rh123 fluorescence	VI
Negative control	12093 ± 106	0
IC_{50}/24 h Nerolidol	787 ± 91	−0·93492
IC_{50}/24 h Amp-B	11238·33 ± 111	−0·07067
$2 \times IC_{50}$/24 h Amp-B	1316·67 ± 33	−0·89112
CCCP	7367	−0·3908

Amph-B, amphotericin B; CCCP, positive control.

nerolidol was investigated on human red blood cells (RBCs). The selectivity index (SI) showed that nerolidol was more selective for RBCs (SI = 20·19) than for macrophages or promastigotes (Table 1).

Promastigotes were treated with nerolidol (IC_{50}/24 h or $2 \times IC_{50}$/24 h) and then processed for scanning (SEM, Fig. 1) and transmission (TEM, Fig. 2) electron microscopy. Severe morphological changes were observed by SEM, including reduction, shrinkage and roundness in the parasite cell body (Fig. 1). With respect to the ultrastructure (TEM), mitochondrial damage was noted, with mitochondrial swelling and disorganization, in addition to the presence of internal vesicles and concentric membranes in the organelle (Fig. 2D, F). Other alterations were frequent, such as the occurrence of cytoplasmic vacuolization (Fig. 2C, E), vesicles at the flagellar pocket (Fig. 2D), and chromatin condensation (Fig. 2C–F). Little or no difference was noted by SEM and TEM after treatment with the IC_{50}/24 h of amphotericin B (Figs 1B and 2B) compared with the untreated control (Figs 1A and 2A). Because nerolidol was responsible for ultrastructural damage, its activity was further investigated by flow cytometry. The presence of multiple mitochondrial morphological alterations led us to hypothesize that the mitochondrial membrane potential was altered, thereby contributing to cell death.

Incubation with Rh123 after nerolidol treatment (IC_{50}/24 h and $2 \times IC_{50}$/24 h) led to a reduction in fluorescence of nearly 100% in the promastigotes (Fig. 3B). Both treatments gave similar results and confirmed the mitochondrial damage observed by TEM. Parasites incubated with amphotericin B at the IC_{50}/24 h value presented variation in the mitochondrial membrane potential in only 7% of the cells, which was not significantly different from the negative control. In contrast, higher amphotericin B concentrations ($2 \times IC_{50}$/24 h) induced a

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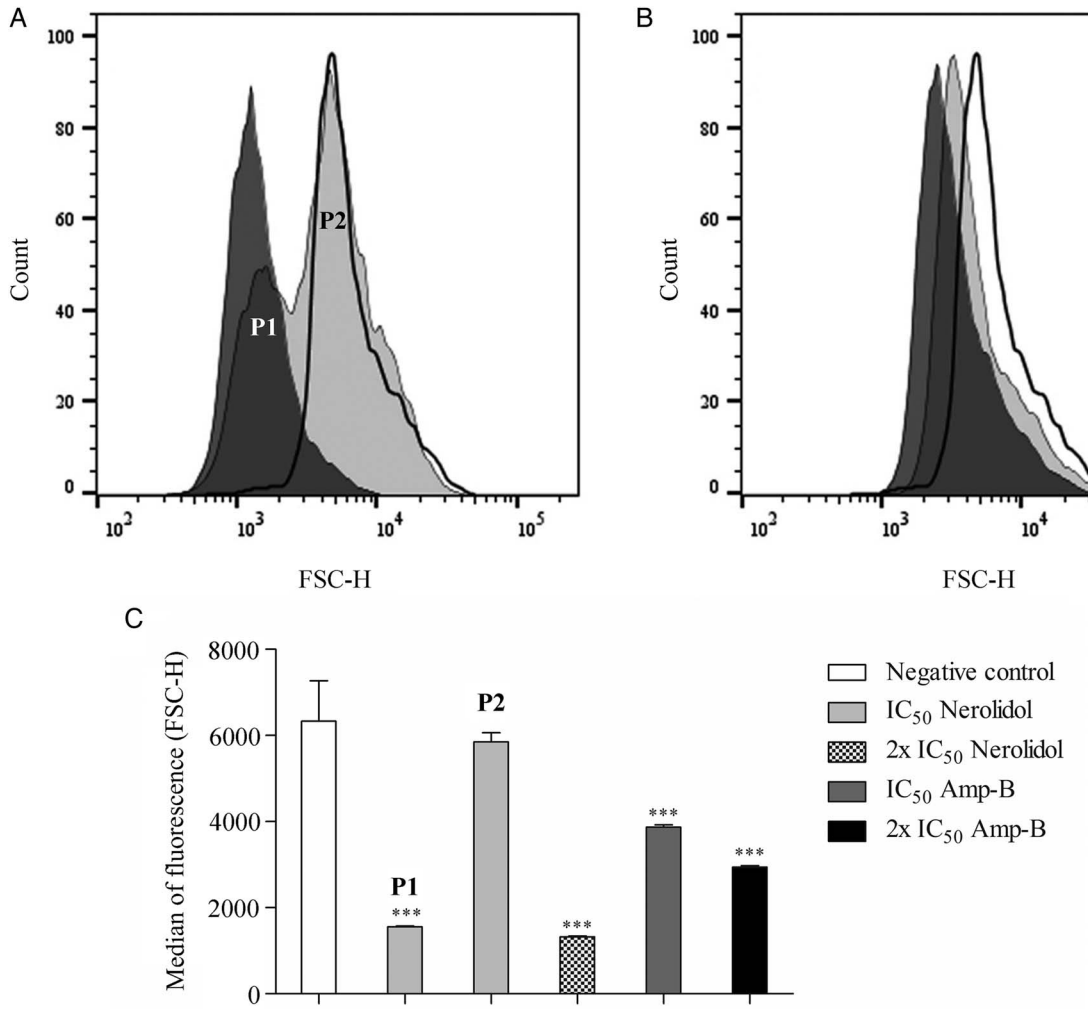

Fig. 4. Cell size of *L. braziliensis* promastigotes treated with nerolidol (A) or amphotericin B (B). P1 and P2 represent two distinct populations formed after treatment with the IC_{50} of nerolidol. In both histograms, the IC_{50} is represented by the light grey filled curve and the $2 \times IC_{50}$ with the dark grey filled curve. The thick and unfilled line curve represents the negative control. (C) Median autofluorescence related to the cell size (FSC-H). $*P < 0{\cdot}05$; $**P < 0{\cdot}01$; $***P < 0{\cdot}001$.

reduction in fluorescence in 80% of the cells (Fig. 3C). Treatment with 100 μM CCCP (positive control, Fig. 3A) reduced the Rh123 signal in 30% of the cells. The VI calculations showed that nerolidol treatment led to mitochondrial membrane depolarization (Table 2).

The cell size of promastigotes incubated with the IC_{50}/24 h value of nerolidol was evaluated by flow cytometry by taking into account the FSC-H parameter (Fig. 4). This experiment confirmed the SEM results, with observations of roundness and diminished parasite cell bodies. Two subpopulations were formed after this treatment. The first subpopulation (P1) comprised 47·78% of the cells and displayed smaller sizes compared with the control parasites. Another subpopulation (P2) included 49·85% of the promastigotes and featured a median cell size that did not significantly differ from the negative control (Fig. 4C), indicating that no changes in cell size occurred in this subpopulation. Treatment with the $2 \times IC_{50}$ of nerolidol resulted in a unique population with almost 100% of the cells smaller in size than the control cells. Parasites treated with amphotericin B exhibited a reduction in volume in a dose-dependent manner that significantly differed from the negative control.

Cell cycle analysis after treatment with the IC_{50}/24 h of nerolidol and PI labelling showed the formation of a G_0/G_1 sub-peak by 30·36% of the cells, which was in contrast to the 0·53% of the population that formed this peak in the negative control cells (Fig. 5). Consequently, fewer cells were in the remaining steps of the cell cycle. No peaks were observed when the $2 \times IC_{50}$ of nerolidol was used, suggesting intense DNA degradation (data not shown). Treatment with amphotericin B did not result in G_0/G_1 sub-peak development. However, a significant increase was observed in the number of cells at the G_0/G_1 phase, which consequently reduced the cells in the S and G_2/M phases (Fig. 5).

To confirm cell death induction by nerolidol in *L. braziliensis*, the cells were double-labelled with

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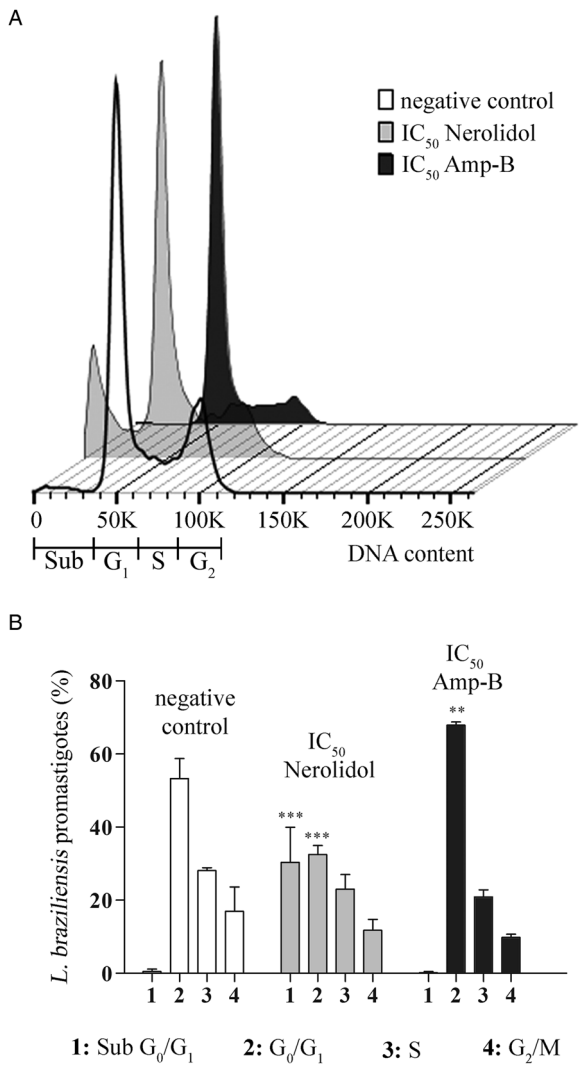

Fig. 5. (A) Histogram of DNA content in the different cell cycle stages (sub G0/G1, G0/G1, S and G2/M) in *L. braziliensis* promastigotes treated with IC_{50} of nerolidol or amphotericin B (Amp-B). Sub: sub G0/G1; G1: G0/G1; G2: G2/M. (B) Percentage of cells at the different cell cycle stages. $^{*}P < 0{\cdot}05$; $^{**}P < 0{\cdot}01$; $^{***}P < 0{\cdot}001$.

Annexin-V and PI. Our results showed that 82·2% of the parasites treated with the IC_{50}/24 h of nerolidol displayed positive labelling for both Annexin-V and PI (Fig. 6B). Nevertheless, 4·64% of the cells were positive exclusively for PI, which did not differ significantly from the negative control (1·35% of cells positive for PI). Amphotericin B treatment at the IC_{50}/24 h concentration (Fig. 6C) did not result in significant differences in Annexin-V/PI staining compared to the negative control. However, 35% of the cells were double-labelled only after 2 × IC_{50} treatment (Fig. 6D).

DISCUSSION

Our data show that PaEO was effective against *L. braziliensis* promastigotes. The result (IC_{50}/24 h = 77·9 μg mL^{-1}) was similar to that obtained for the *P. demeraranum* and *P. duckei* EOs on *L. amazonensis* promastigotes, with IC_{50}/24 h values of 86 and 46 μg mL^{-1}, respectively (Carmo *et al.* 2012). Several EOs have already been tested against different *Leishmania* spp. (Supplementary Table S1), most assays being performed for 72 h, which leads to lower IC_{50} values (Misra *et al.* 2009; Monzote *et al.* 2010; Esperandim *et al.* 2013; Bernuci *et al.* 2016). We carried out tests for 24 h to minimize the effects of nutrient starvation and toxicity due to occasional parasite lysis. Our data confirmed the leishmanicidal activity of *Piper* spp. EOs.

Nerolidol, the main constituent of PaEO (Villamizar *et al.* 2017), showed anti *L. braziliensis* activity (IC_{50}/24 h = 74·3 μg mL^{-1}). This finding validates the antimicrobial potential of compounds obtained from a natural source (Anthony *et al.* 2005). More research is needed to clarify the mechanism of action of the active compounds derived from plants, which may to lead the development of potent anti-leishmanial drugs in the near future (Ullah *et al.* 2016).

The nerolidol activity was previously tested against *L. amazonensis*, *L. braziliensis* and *L. chagasi* promastigotes, resulting in IC_{50}/24 h values of 85, 74 and 75 μM, respectively (Arruda *et al.* 2005). For *L. braziliensis*, the SI for mammalian cells was 1·69, which was similar to the result obtained in our research (SI = 1·41). Nerolidol showed decreased toxicity against erythrocytes (SI = 20·19). One possible explanation is the absence of mitochondria in red blood cells, which suggests that nerolidol acts on intracellular targets rather than via inducing cell membrane disruption.

Low density compounds, such as EOs or small hydrophobic molecules (e.g., nerolidol, which is a sesquiterpene alcohol) can easily cross the plasma membrane to reach intracellular targets (Anthony *et al.* 2005). Terpenes do not affect the membrane protein dynamics; instead, their activity is mostly restricted to the lipidic membrane components, thus altering the membrane fluidity (Camargos *et al.* 2014). Together, these data suggest that nerolidol may damage the parasite membrane, thereby increasing its permeability while inducing cell death mechanisms consistent with an apoptosis-like process in *Leishmania*.

An apoptosis-like process has been suggested as a cell death mechanism in *Leishmania* following drug treatment. For instance, eupomatenoid-5, which is derived from *Piper regnellii*, leads to mitochondrial membrane depolarization, PS exposure and cell size reduction (Garcia *et al.* 2013). The leishmanicidal activity of racemoside-A was evidenced by morphological alterations, such as cell shrinkage, ovoid cells, absence of flagella and chromatin condensation. These effects were attributed to cell death induction detected via Annexin-V, PI and the loss of mitochondrial membrane potential, which culminated in DNA fragmentation and permanency of the

https://doi.org/10.1017/S0031182017000452 Published online by Cambridge University Press

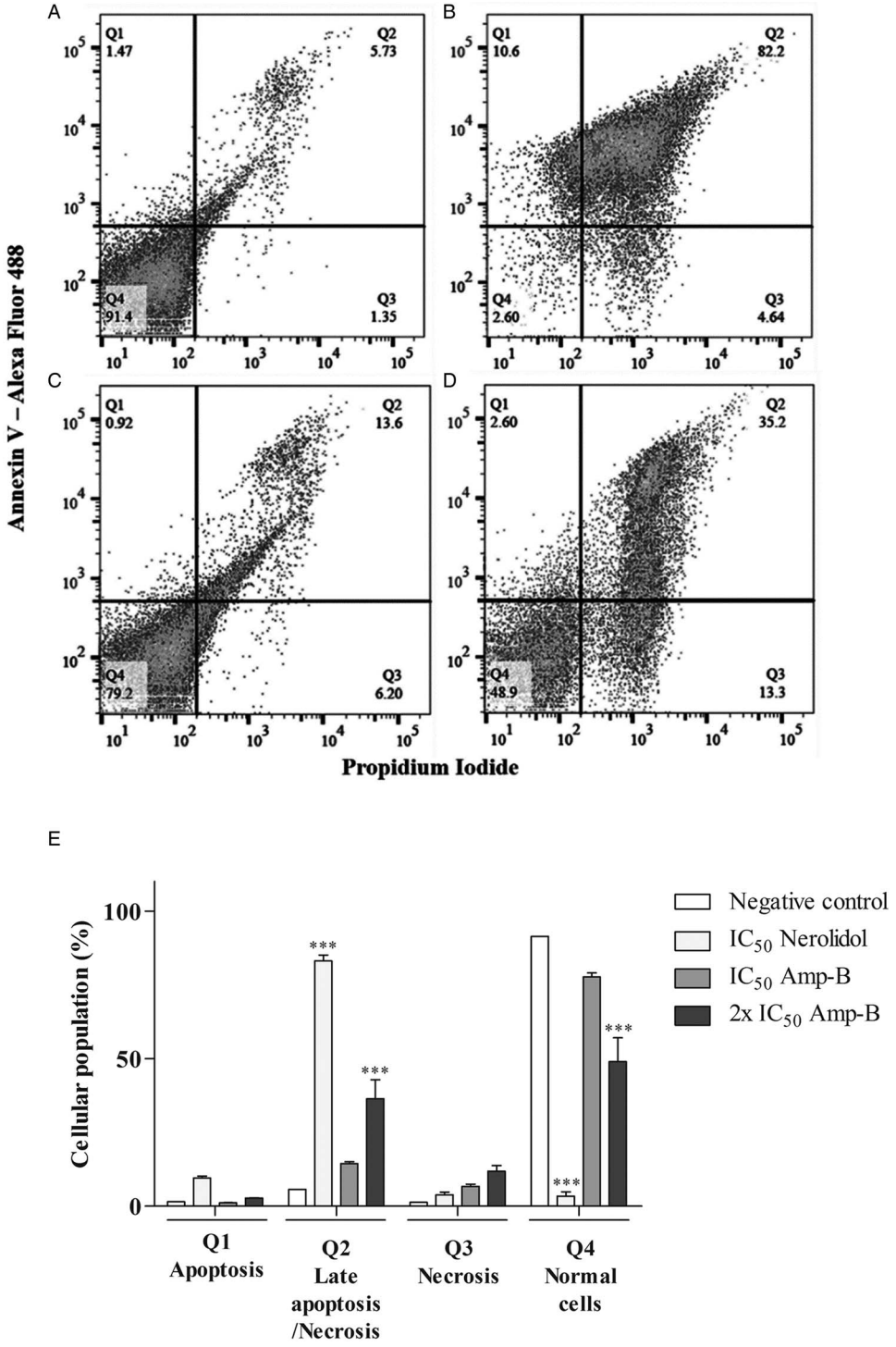

Fig. 6. Assessment of the membrane permeability and phosphatidylserine exposure of *L. braziliensis* promastigotes. (A) negative control; (B) IC_{50}/24 h of nerolidol; (C) IC_{50}/24 h of amphotericin B; (D) 2 × IC_{50}/24 h of amphotericin B; (E) cell population present in each quadrant (Q1: apoptosis; Q2: late apoptosis/necrosis; Q3: necrosis and Q4: live cells). $^{*}P < 0{\cdot}05$; $^{**}P < 0{\cdot}01$; $^{***}P < 0{\cdot}001$.

cells in the sub-G_0/G_1 phase (Dutta *et al*. 2007). DNA fragmentation into multiple 200 bp pieces was observed in *L. amazonensis* promastigotes treated with 40 μM miltefosine for 24 h. Together with PS exposure and the permanence of the cells in the sub-G_0/G_1 phase, this result was interpreted as programmed cell death (Marinho *et al*. 2011).

We found several morphological evidences of an apoptosis-like process in nerolidol-treated *L. braziliensis*, such as mitochondrial membrane depolarization, PS exposure and cell size reduction (cell shrinkage). These evidences indicate that treatment with nerolidol would lead to *L. braziliensis* cell death through an apoptosis-like process, a

https://doi.org/10.1017/S0031182017000452 Published online by Cambridge University Press

nomenclature used in several studies on *Leishmania* cell death (e.g.: Jiménez-Ruiz *et al*. 2010; Gannavaram and Debrabant, 2012; Islamuddin *et al*. 2014; Awasthi *et al*. 2016; Shadab *et al*. 2017). Our morphological data do not indicate necrosis, since oncosis (cell swelling) and plasma membrane rupture were not observed.

Proto *et al*. (2013) reviewed the evidences for unregulated/regulated cell death pathways in parasitic protozoa and proposed that unregulated cell death in these organisms can be classified into just two primary types: necrosis or incidental cell death. According to these authors, regulated necrosis and (regulated) extrinsic apoptosis are precluded in parasitic protozoa owing to the absence of established death receptors and caspases. Trypanosomatids have metacaspases, which have significant structural similarities with caspases, but are not caspases and cannot be considered as mediators of a similar regulated cell death (Proto *et al*. 2013). Therefore, according to Proto *et al*. (2013), in absence of robust biochemical evidence on mechanism, parasitic protozoan caspase independent/metacaspase dependent death should be classified not as apoptosis, but as incidental cell death. In the absence of biochemical evidences, we then conclude that nerolidol-induced incidental cell death in *Leishmania braziliensis*.

The biochemical events leading to cell death may provide key novel therapeutic targets that could be exploited in the design of specific chemotherapeutic agents for pathogenic trypanosomatids (Welburn *et al*. 2006). *Leishmania* cells contain a single mitochondrion, similar to other kinetoplastids. Therefore, this organelle appears to be an interesting chemotherapeutic target, with characteristics that are distinguishable from those of mammalian cells (Inacio *et al*. 2012).

In a *L. donovani* study, parasites treated with pentostam or amphotericin B presented evidence of cell death, with discontinuation in one DNA strand, DNA fragmentation, diminished mitochondrial membrane potential and increased cell permeability (Lee *et al*. 2002). The mechanism of action of amphotericin against *Leishmania* spp. can be explained by its affinity for the sterols present at the parasite membrane. Transmembrane channels are formed following interactions with the membrane, leading to cation loss and cell death (Brajtburg and Bolard, 1996). Thus, nerolidol and amphotericin B appear to play roles in both parasite membrane permeability and cell death.

Currently, only two methods are available to treat and control leishmaniasis: rational use of drugs and vector insect control. A limited number of drugs are available, and these drugs are expensive, have side-effects and are inefficient (Srivastava *et al*. 2016). A study in Latin America to evaluate differences in responses to pentavalent antimonial treatment showed that the cure afforded by the use of this drug differs between distinct *Leishmania* species (Arevalo *et al*. 2007). Therefore, the search for alternative effective compounds from natural sources is relevant. Terpenes are powerful skin permeation promoters (El-Kattan *et al*. 2001; Camargos *et al*. 2014). The sesquiterpene nerolidol showed an anti-*L. braziliensis* effect as described here. Nerolidol has low toxic potential *in vivo* (Lapczynski *et al*. 2008) and presents anti-tumorigenic activity in female rats (Arroyo-Acevedo *et al*. 2015). Obtaining nerolidol derivatives with better SI can be the source for promising candidates in the search for new compounds to kill *Leishmania* parasites.

SUPPLEMENTARY MATERIAL

The supplementary material for this article can be found at http://dx.doi.org/10.1017/S0031182017000452.

ACKNOWLEDGEMENTS

The authors thank the Program for Technological Development in Tools for Health-PDTIS FIOCRUZ for the use of its facilities (Platforms RPT08L – Flow Cytometry-PR and RPT07C – Confocal and Electron Microscopy-PR).

FINANCIAL SUPPORT

This work was supported by Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq), Fundação de Amparo à Pesquisa do Estado de Minas Gerais (FAPEMIG) and Fundação Oswaldo Cruz (Fiocruz).

REFERENCES

Ali, B., Al-Wabel, N. A., Shams, S., Ahamad, A., Khan, S. A. and Anwar, F. (2015). Essential oils used in aromatherapy: a systemic review. *Asian Pacific Journal of Tropical Biomedicine* **5**, 601–611.

Anthony, J. P., Fyfe, L. and Smith, H. (2005). Plant active components - a resource for antiparasitic agents? *Trends in Parasitology* **21**, 462–468.

Araújo, M. J., Câmara, C. A., Born, F. S., Moraes, M. M. and Badji, C. A. (2012). Acaricidal activity and repellency of essential oil from *Piper aduncum* and its components against *Tetranychus urticae*. *Experimental & Applied Acarology* **57**, 139–155.

Arevalo, J., Ramirez, L., Adaui, V., Zimic, M., Tulliano, G., Miranda-Verástegui, C., Lazo, M., Loayza-Muro, R., De Doncker, S., Maurer, A., Chappuis, F., Dujardin, J. C., Llanos-Cuentas, A. and Llanos-Cuentas, A. (2007). Influence of *Leishmania* (*Viannia*) species on the response to antimonial treatment in patients with American tegumentary leishmaniasis. *Journal of Infectious Diseases* **195**, 1846–1851.

Arroyo-Acevedo, J., Chávez-Asmat, R. J., Anampa-Guzmán, A., Donaires, R. and Ráez-Gonzáles, J. (2015). Protective effect of *Piper aduncum* capsule on DMBA-induced breast cancer in rats. *Breast Cancer: Basic and Clinical Research* **9**, 41–48.

Arruda, D. C., D'Alexandri, F. L., Katzin, A. M. and Uliana, S. R. (2005). Antileishmanial activity of the terpene nerolidol. *Antimicrobial Agents and Chemotherapy* **49**, 1679–1687.

Awasthi, B. P., Kathuria, M., Pant, G., Kumari, N. and Mitra, K. (2016). Plumbagin, a plant-derived naphthoquinone metabolite induces mitochondria mediated apoptosis-like cell death in *Leishmania donovani*: an ultrastructural and physiological study. *Apoptosis* **21**, 941–953.

Basano, S. A. and Camargo, L. M. A. (2004). Leishmaniose tegumentar americana: histórico, epidemiologia e perspectivas de controle. *Revista Brasileira de Epidemiologia* **7**, 328–337.

Bernuci, K. Z., Iwanaga, C. C., Fernandez-Andrade, C. M. M., Lorenzetti, F. B., Torres-Santos, E. C., Faiões, V. S., Gonçalves, J. E., Amaral, W., Deschamps, C., Scodro, R. B. L., Cardoso, R. F., Baldin, V. P. and Cortez, D. A. G. (2016). Evaluation of chemical composition and antileishmanial and antituberculosis activities of essential oils of *Piper* species. *Molecules* **21**, 1698.

Brajtburg, J. and Bolard, J. (1996). Carrier effects on biological activity of amphotericin B. *Clinical Microbiology Reviews* **9**, 512–531.

Brasil. Ministério da Saúde (2009). *Guia de Vigilância Epidemiológica*, 7th Edn. Ministério da Saúde, Brasília DF, Brazil, 816 p.

Camargos, H. S., Moreira, R. A., Mendanha, S. A., Fernandes, K. S., Dorta, M. L. and Alonso, A. (2014). Terpenes increase the lipid dynamics in the *Leishmania* plasma membrane at concentrations similar to their IC_{50} values. *PLoS ONE* **9**, e104429.

Capello, T. M., Martins, E. G., Farias, C. F., Figueiredo, C. R., Matsuo, A. L., Passero, L. F., Oliveira-Silva, D., Sartorelli, P. and Lago, J. H. (2015). Chemical composition and *in vitro* cytotoxic and antileishmanial activities of extract and essential oil from leaves of *Piper cernuum*. *Natural Product Communications* **10**, 285–288.

Carmo, D. F. M., Amaral, A. C., Machado, G. M., Leon, L. L. and Silva, J. R. (2012). Chemical and biological analyses of the essential oils and main constituents of *Piper* species. *Molecules* **17**, 1819–1829.

Chouhan, G., Islamuddin, M., Want, M. Y., Ozbak, H. A., Hemeg, H. A., Sahal, D. and Afrin, F. (2015). Leishmanicidal activity of *Piper nigrum* bioactive fractions is interceded via apoptosis *in vitro* and substantiated by Th1 immunostimulatory potential *in vivo*. *Frontiers in Microbiology* **6**, 1368.

Cook, S. (2016). *The Forest of the Lacandon Maya: An Ethnobotanical Guide*. Springer, New York, USA.

Corpas-López, V., Merino-Espinosa, G., Díaz-Sáez, V., Morillas-Márquez, F., Navarro-Moll, M. C. and Martín-Sánchez, J. (2016). The sesquiterpene (-)-α-bisabolol is active against the causative agents of Old World cutaneous leishmaniasis through the induction of mitochondrial-dependent apoptosis. *Apoptosis* **21**, 1071–1081.

de Moraes, J. (2015). Natural products with antischistosomal activity. *Future Medicinal Chemistry* **7**, 801–820.

Donega, M. A., Mello, S. C., Moraes, R. M., Jain, S. K., Tekwani, B. L. and Cantrell, C. L. (2014). Pharmacological activities of cilantro's aliphatic aldehydes against *Leishmania donovani*. *Planta Medica* **80**, 1706–1711.

Dutta, A., Ghoshal, A., Mandal, D., Mondal, N. B., Banerjee, S., Sahu, N. P. and Mandal, C. (2007). Racemoside A, an anti-leishmanial, water-soluble, natural steroidal saponin, induces programmed cell death in *Leishmania donovani*. *Journal of Medical Microbiology* **56**, 1196–1204.

El-Kattan, A. F., Asbill, C. S., Kim, N. and Michniak, B. B. (2001). The effects of terpene enhancers on the percutaneous permeation of drugs with different lipophilicities. *International Journal of Pharmaceutics* **215**, 229–240.

Escobar, P., Milena Leal, S., Herrera, L. V., Martinez, J. R. and Stashenko, E. (2010). Chemical composition and antiprotozoal activities of Colombian *Lippia* spp essential oils and their major components. *Memórias do Instituto Oswaldo Cruz* **105**, 184–190.

Esperandim, V. R., Ferreira, D. S., Rezende, K. C. S., Magalhães, L. G., Souza, J. M., Pauletti, P. M., Januário, A. H., Laurentz, R. S., Bastos, J. K., Símaro, G. V., Cunha, W. R. and Andrade e Silva, M. L. (2013). *In vitro* antiparasitic activity and chemical composition of the essential oil obtained from the fruits of *Piper cubeba*. *Planta Medica* **79**, 1653–1655.

Fumarola, L., Spinelli, R. and Brandonisio, O. (2004). *In vitro* assays for evaluation of drug activity against *Leishmania* spp. *Research in Microbiology* **155**, 224–230.

Gaínza, Y. A., Fantatto, R. R., Chaves, F. C. M., Bizzo, H. R., Esteves, S. N. and Chagas, A. C. S. (2016). *Piper aduncum* against *Haemonchus contortus* isolates: cross resistance and the research of natural bioactive compounds. *Brazilian Journal of Veterinary Parasitology* **25**, 383–393.

Gannavaram, S. and Debrabant, A. (2012). Programmed cell death in *Leishmania*: biochemical evidence and role in parasite infectivity. *Frontiers in Cellular and Infection Microbiology* **2**, 1–9.

Garcia, F. P., Lazarin-Bidóia, D., Ueda-Nakamura, T., Silva, S. O. and Nakamura, C. V. (2013). Eupomatenoid-5 isolated from leaves of *Piper regnellii* induces apoptosis in *Leishmania amazonensis*. *Evidence-Based Complementary and Alternative Medicine* **2013**, 940531.

Guru, P. Y., Agrawal, A. K., Singha, U. K., Singhal, A. and Gupta, C. M. (1989). Drug targeting in *Leishmania donovani* infections using tuftsin-bearing liposomes as drug vehicles. *FEBS Letters* **245**, 204–208.

Gutiérrez, Y., Montes, R., Scull, R., Sánchez, A., Cos, P., Monzote, L. and Setzer, W. N. (2016). Chemodiversity associated with cytotoxicity and antimicrobial activity of *Piper aduncum* var. *ossanum*. *Chemistry & Biodiversersity* **13**, 1715–1719.

Houël, E., Gonzalez, G., Bessière, J. M., Odonne, G., Eparvier, V., Deharo, E. and Stien, D. (2015). Therapeutic switching: from antidermatophytic essential oils to new leishmanicidal products. *Memórias do Instituto Oswaldo Cruz* **110**, 106–113.

Islamuddin, M., Sahal, D. and Afrin, F. (2014). Apoptosis-like death in *Leishmania donovani* promastigotes induced by eugenol-rich oil of *Syzygium aromaticum*. *Journal of Medical Microbiology* **63**, 74–85.

Inacio, J. D., Canto-Cavalheiro, M. M., Menna-Barreto, R. F. and Almeida-Amaral, E. E. (2012). Mitochondrial damage contribute to epigallocatechin-3-gallate induced death in *Leishmania amazonensis*. *Experimental Parasitology* **132**, 151–155.

Izumi, E., Ueda-Nakamura, T., Veiga, V. F., Pinto, A. C. and Nakamura, C. V. (2012). Terpenes from *Copaifera* demonstrated in vitro antiparasitic and synergic activity. *Journal of Medicinal Chemistry* **55**, 2994–3001.

Jimenez, V., Paredes, R., Sosa, M. A. and Galanti, N. (2008). Natural programmed cell death in *T. cruzi* epimastigotes maintained in axenic cultures. *Journal of Cellular Biochemistry* **105**, 688–698.

Jiménez-Ruiz, A., Alzate, J. F., Macleod, E. T., Lüder, C. G., Fasel, N. and Hurd, H. (2010). Apoptotic markers in protozoan parasites. *Parasites & Vectors* **3**, 104.

Jones, T. C., Johnson, W. D., Barretto, A. C., Lago, E., Badaro, R., Cerf, B., Reed, S. G., Netto, E. M., Tada, M. S., Franca, T. F., Wiese, K., Golightly, L., Fikrig, E., Costa, J. M. L., Cuba, C. C. and Marsden, P. D. (1987). Epidemiology of American cutaneous leishmaniasis due to *Leishmania braziliensis braziliensis*. *The Journal of Infectious Diseases* **156**, 73–83.

Kayser, O., Kiderlen, A. F. and Croft, S. L. (2003). Natural products as antiparasitic drugs. *Parasitology Research* **90**(Suppl. 2), S55–S62.

Kessler, R. L., Gradia, D. F., Rampazzo, R. C. P., Lourenço, É., Fidêncio, N. J., Manhaes, L., Probst, C. M., Ávila, A. R. and Fragoso, S. P. (2013). Stage-regulated GFP expression in *Trypanosoma cruzi*: applications from host–parasite interactions to drug screening. *PLOS ONE* **8**, e67441.

Khademvatan, S., Gharavi, M. J. and Saki, J. (2011). Miltefosine induces metacaspase and PARP genes expression in *Leishmania infantum*. *The Brazilian Journal of Infectious Diseases* **15**, 442–448.

Lakshmi, V., Pandey, K., Kapil, A., Singh, N., Samant, M. and Dube, A. (2007). *In vitro* and *in vivo* leishmanicidal activity of *Dysoxylum binectariferum* and its fractions against *Leishmania donovani*. *Phytomedicine* **14**, 36–42.

Lapczynski, A., Bhatia, S. P., Letizia, C. S. and Api, A. M. (2008). Fragrance material review on nerolidol (isomer unspecified). *Food and Chemical Toxicology* **46**(Suppl.), S247–S250.

Lazarin-Bidóia, D., Desoti, V. C., Martins, S. C., Ribeiro, F. M., Ud Din, Z., Rodrigues-Filho, E., Ueda-Nakamura, T., Nakamura, C. V. and Silva, S. O. (2016). Dibenzylideneacetones are potent trypanocidal compounds that affect the *Trypanosoma cruzi* redox system. *Antimicrobial Agents and Chemotherapy* **60**, 890–903.

Lee, N., Bertholet, S., Debrabant, A., Muller, J., Duncan, R. and Nakhasi, H. L. (2002). Programmed cell death in the unicellular protozoan parasite *Leishmania*. *Cell Death and Differentiation* **9**, 53–64.

Lindoso, J. A., Cunha, M. A., Queiroz, I. T. and Moreira, C. H. (2016). Leishmaniasis-HIV coinfection: current challenges. *HIV/AIDS: Research and Palliative Care* **8**, 147–156.

Majdalawieh, A. F. and Carr, R. I. (2010). *In vitro* investigation of the potential immunomodulatory and anti-cancer activities of black pepper (*Piper nigrum*) and cardamom (*Elettaria cardamomum*). *Journal of Medicinal Food* **13**, 371–381.

Mamood, S. N., Hidayatulfathi, O., Budin, S. B., Rohi, G. A. and Zulfakar, M. H. (2017). The formulation of the essential oil of *Piper aduncum* Linnaeus (Piperales: Piperaceae) increases its efficacy as an insect repellent. *Bulletin of Entomological Research* **107**, 49–57.

Marinho, F. A., Gonçalves, K. C. S., Oliveira, S. S., Oliveira, A. C. S. C., Bellio, M., d'Avila-Levy, C. M., Santos, A. L. S. and Branquinha, M. H. (2011). Miltefosine induces programmed cell death in *Leishmania amazonensis* promastigotes. *Memórias do Instituto Oswaldo Cruz* **106**, 507–509.

Marques, A. M., Barreto, A. L., Batista, E. M., Curvelo, J. A., Velozo, L. S., Moreira, D. L., Guimarães, E. F., Soares, R. M. and Kaplan, M. A. (2010). Chemistry and biological activity of essential oils from *Piper claussenianum* (Piperaceae). *Natural Product Communications* **5**, 1837–1840.

Marques, A. M., Paiva, R. A., Fonseca, L. M., Capella, M. A. M., Guimarães, E. F. and Kaplan, M. A. C. (2013). Preliminary anticancer potency evaluation and phytochemical investigation of methanol extract of *Piper claussenianum* (Miq.) C. DC. *Journal of Applied Pharmaceutical Science* **3**, 13–18.

Masic, A., Hernandez, A. M. V., Hazra, S., Glaser, J., Holzgrabe, U., Hazra, B. and Schurigt, U. (2015). Cinnamic acid bornyl ester derivatives from *Valeriana wallichii* exhibit antileishmanial *in vivo* activity in *Leishmania major*-infected BALB/c mice. *PLoS ONE* **10**, e0142386.

Mendes, E. A., Desoti, V. C., Silva, S. O., Ueda-Nakamura, T., Dias Filho, B. P., Yamada-Ogatta, S. F., Sarragiotto, M. H. and Nakamura, C. V. (2016). C5 induces different cell death pathways in promastigotes of *Leishmania amazonensis*. *Chemico-Biological Interactions* **256**, 16–24.

Misni, N., Othman, H. and Sulaiman, S. (2011). The effect of *Piper aduncum* Linn. (Family: Piperaceae) essential oil as aerosol spray against *Aedes aegypti* (L.) and *Aedes albopictus* Skuse. *Tropical Biomedicine* **28**, 249–258.

Misra, P., Kumar, A., Khare, P., Gupta, S., Kumar, N. and Dube, A. (2009). Pro-apoptotic effect of the landrace Bangla Mahoba of *Piper betle* on *Leishmania donovani* may be due to the high content of eugenol. *Journal of Medical Microbiology* **58**, 1058–1066.

Monzote, L., García, M., Montalvo, A. M., Scull, R. and Miranda, M. (2010). Chemistry, cytotoxicity and antileishmanial activity of the essential oil from *Piper auritum*. *Memórias do Instituto Oswaldo Cruz* **105**, 168–173.

Ndjonka, D., Rapado, L. N., Silber, A. M., Liebau, E. and Wrenger, C. (2013). Natural products as a source for treating neglected parasitic diseases. *International Journal of Molecular Sciences* **14**, 3395–3439.

Okunade, A. H., Charles, D., Clark, A. M. and Lentz, D. (1997). Antimicrobial properties of the constituents of *Piper aduncum*. *Phytotherapy Research* **11**, 142–144.

Organização Pan-Americana da Saúde / Organização Mundial da Saúde (2015). *Informe Epidemiológico das Américas*. Informe Leishmanioses No. 3. Organização Pan-Americana da Saúde/Organização Mundial da Saúde, Washington, DC, USA.

Organización Panamericana de la Salud (2013). *Leishmaniasis en las Américas: recomendaciones para el tratamiento*. Organización Panamericana de la Salud, Washington, DC, USA.

Petit, P. X. (1992). Flow cytometric analysis of rhodamine 123 fluorescence during modulation of the membrane potential in plant mitochondria. *Plant Physiology* **98**, 279–286.

Proto, W. R., Coombs, G. H. and Mottram, J. C. (2013). Cell death in parasitic protozoa: regulated or incidental? *Nature Reviews Microbiology* **11**, 58–66.

Rodrigues, J. H., Ueda-Nakamura, T., Corrêa, A. G., Sangi, D. P. and Nakamura, C. V. (2014). A quinoxaline derivative as a potent chemotherapeutic agent, alone or in combination with benznidazole, against *Trypanosoma cruzi*. *PLoS ONE* **9**, e85706.

Shadab, M., Jha, B., Asad, M., Deepthi, M., Kamran, M. and Ali, N. (2017). Apoptosis-like cell death in *Leishmania donovani* treated with KalsomeTM 10, a new liposomal amphotericin B. *PLoS ONE* **12**, e0171306.

Srivastava, S., Shankar, P., Mishra, J. and Singh, S. (2016). Possibilities and challenges for developing a successful vaccine for leishmaniasis. *Parasites & Vector* **9**, 277.

Sunila, E. S. and Kuttan, G. (2004). Immunomodulatory and antitumor activity of *Piper longum* Linn. and piperine. *Journal of Ethnopharmacology* **90**, 339–346.

Thao, N. P., Luyen, B. T., Widowati, W., Fauziah, N., Maesaroh, M., Herlina, T., Manzoor, Z., Ali, I., Koh, Y. S. and Kim, Y. H. (2016). Anti-inflammatory flavonoid C-glycosides from *Piper aduncum* leaves. *Planta Medica* **82**, 1475–1481.

Ullah, N., Nadhman, A., Siddiq, S., Mehwish, S., Islam, A., Jafri, L. and Hamayun, M. (2016). Plants as antileishmanial agents: current scenario. *Phytotherapy Research* **30**, 1905–1925.

Villamizar, L. H., Cardoso, M. G., Andrade, J., Teixeira, M. L. and Soares, M. J. (2017). Linalool, a *Piper aduncum* essential oil component, has selective activity against *Trypanosoma cruzi* trypomastigote forms at 4 °C. *Memórias do Instituto Oswaldo Cruz* **112**, 131–139.

Welburn, S. C., Macleod, E., Figarella, K. and Duzensko, M. (2006). Programmed cell death in African trypanosomes. *Parasitology* **132**, S7–S18.