Parasitology

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The *in vitro* antileishmanial activity of essential oil from *Aloysia gratissima* and guaiol, its major sesquiterpene against *Leishmania amazonensis*

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Research Article

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Abstract

Leishmaniases is a tropical disease caused by protozoa of the genus *Leishmania* for which the current treatment is expensive, besides increasing reports of parasite resistance. This study investigated the anti-*Leishmania amazonensis* activity of the essential oil from *Aloysia gratissima* (AgEO) and guaiol, the major sesquiterpene constituent in the oil. Our results showed that AgEO killed promastigotes and intracellular amastigotes at an IC_{50} of 25 and 0·16 $\mu g\ mL^{-1}$, respectively, while guaiol killed amastigotes at an IC_{50} of 0·01 $\mu g\ mL^{-1}$. Both AgEO and guaiol were safe for macrophages up to 100 $\mu g\ mL^{-1}$, as evaluated by the dehydrogenase activity, membrane integrity and phagocytic capacity. AgEO and guaiol did not induce nitrite oxide (NO) in resting macrophages and inhibited the production of NO in lipopolysaccharide-stimulated macrophages. The ultrastructural analysis suggested that AgEO and guaiol act directly on parasites, affecting promastigotes kinetoplast, mitochondrial matrix and plasma membrane. Together, these results pointed out that AgEO and guaiol could be promising candidates to develop anti-*Leishmania* drugs.

Introduction

Leishmaniases are neglected diseases caused by protozoa from the genus *Leishmania,* affecting individuals from 98 countries, with an estimate of 1·1 to 1·7 million cases of leishmaniasis occuring each year (Alvar *et al.* 2012). Leishmaniasis presents a wide-spectrum of clinical manifestations, such as mucocutaneous, cutaneous or visceral disease (Pace, 2014).

Currently, drugs used for the treatment of leishmaniasis rely on pentavalent antimonials, amphotericin B (AmpB), miltefosine, pentamidine and paromomycin, which are limited, highly toxic and expensive. Antimonials are still the first line of treatment choice, but in case of therapeutic failure due to resistance cases as in India or French Guiana, AmpB and pentamidine have been used as alternatives, respectively. Miltefosine, the only oral drug available for visceral leishmaniasis treatment in India, is teratogenic and parasite resistance has also been reported (De Menezes *et al.* 2015; Singh *et al.* 2016). Therefore, more attention has been given to active extracts and molecules isolated from plant species used in folk medicine as possible sources for developing new drugs to control and prevent leishmaniasis (Sen and Chatterjee, 2011; Ullah *et al.* 2016).

Essential oils (EOs) are natural and complex mixtures of compounds obtained from different plants parts, such as flowers, leaves, fruits, roots or seeds, commonly used in folk medicine to treat different types of diseases (Costa, 1994). In general, EOs are constituted of monoterpenes and sesquiterpenes compounds and some are reported to be active against *Leishmania* (Kvist *et al.* 2006; Escobar *et al.* 2010; Medeiros *et al.* 2011; Farias-Junior, *et al.* 2012; Soares *et al.* 2013; Monzote *et al.* 2014; Andrade *et al.* 2016; Ceole *et al.* 2017; Moreira *et al.* 2017; Silva *et al.* 2017).

Many plant species belonging to genus *Lippia* (Verbenaceae) are popularly used in the folk medicine in Northeast of Brazil for treatment of acne, scabies and skin wounds, and as oral antiseptic or liquid soap preparations to prevent and treat fungal infections (Lacoste *et al.* 1996). An antiprotozoal effect has been found in an extensive analysis comprising 19 EOs from *Lippia* species, in which they presented microbicidal effect against *Leishmania chagasi* and *Trypanosoma cruzi* (Escobar *et al.* 2010). The anti-leishmanial activity of *Lippia sidoides* and its two major compounds thymol and carvacol against *Leishmania amazonensis* and *L. chagasi* has also been described (Oliveira *et al.* 2009; Medeiros *et al.* 2011; Farias-Junior *et al.* 2012).

Aloysia gratissima (Gillies and Hook) (*Lippia lycioides* Cham. Steudel) species, known as 'Brazil lavender', are commonly used in the Brazilian folk medicine for the treatment of digestive and respiratory diseases, and their flowers are employed in perfume compositions. Some

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reports have characterized the microbicidal effect of *A. gratissima* against bacteria, yeast and virus (Dellacasa *et al.* 2003; Garcia *et al.* 2003; Santos *et al.* 2013, 2015). Guaiol is a sesquiterpene alcohol found in many medicinal plants, which have been proved to possess antibacterial and antitumor activities (Choudhary *et al.* 2007; Yang *et al.* 2016).

Here we show for the first time the leishmanicidal activity of *Aloysia gratissima* essential oil (AgEO) and of its major sesquiterpene compound guaiol, on promastigotes and amastigotes of *L. amazonensis in vitro*. This *Leishmania* species is found along the Americas and is recognized as the causative agent of a broad spectrum of clinical manifestations that include cutaneous, anergic diffuse cutaneous and even visceral disease (Barral *et al.* 1991; Convit *et al.* 1993; Grimaldi and Tesh, 1993).

Materials and methods

Ethics statement

All experiments using animals were performed in strict accordance with the Brazilian animal protection law of the National Council for the Control of Animal Experimentation (CONCEA, Brazil). Protocols were approved by the Committee for Animal Use of the Universidade Federal Rural do Rio de Janeiro (CEUA) number 22/14

Plant material

Leaves and branches of *Aloysia gratissima* (Gillies & Hook.) Tronc. (syn. *Lippia lycioides* Cham.) were collected in the Manguinhos campus of the Oswaldo Cruz Foundation, Rio de Janeiro located at S 22° 52′46″, W 43°14′52″, 9 m above sea level. A voucher of *A. gratissima* was registered and deposited in the Herbarium of the Rio de Janeiro Botanical Garden Collection under the number RB 327302.

EOs extraction

Fresh aerial parts (leaves and branches, 1020 g) of *Aloysia gratissima* were hydrodistilled (2 L water) using a 12-L round bottom flask coupled to a modified Clevenger apparatus during 6 h, to afford a yellowish clear fluid (0·52% yield on fresh leaves). After separating an aliquot (10 mg) for gas chromatography (GC) analysis, this volatile oil was stored in sealed amber ampoules at −20°C until the biological tests could be performed.

Gas Chromatography (GC) analysis

Analyses were performed on a gas chromatograph (HP 6890N Network GC System) fitted with a 30 m × 0·32 mm × 0·25 μm film thickness HP-5 capillary column and operating in split mode at a ratio of 1:20 (split/splitless injector). Helium was used as carrier gas (flow: 1 mL min^{-1}, inlet pressure: 5·83 psi). The initial oven temperature was 70 °C (held for 5 min). The temperature was then raised to 250 °C at 3 °C min^{-1}. The injection volume was 1 μL of a 3 mg mL^{-1} solution of the oil in CH_2Cl_2. The sample composition was obtained from electronic integration measurements using flame ionization detection (FID, 270 °C) by considering the relative peak. Injections were carried out in triplicate and standard deviations were considered. Samples were also analysed on an HP 6890N GC mass spectrometry (MS) apparatus fitted using the same column and the responses were processed in a Mass Selective Detector (MSD) Productivity ChemStation Software. The chromatographic conditions were the same as above, except for the flow, which was 0·5 mL min^{-1}. The mass analyzer operated at an ion source temperature of 250 °C, electron impact ionization energy of 70 eV and an acquisition mass range from 10 to 400 *m*/*z* (3·66 scan s^{-1}). Individual identification of the constituents was performed by comparison of their GC retention indices determined with reference to a homologous series of normal C_{10}-C_{30} alkanes, and comparison of the fragmentation patterns in the mass spectra with those from the Wiley Library Software 59943B (Adams, 2007).

Parasites

Leishmania amazonensis (WHOM/BR/75/Josefa) promastigotes were cultured at 26 °C in Schneider´s Insect Medium containing 10% fetal calf serum (FCS – Cripion), 10% human urine and gentamicin (40 μg mL^{-1}; Schering–Plough, Rio de Janeiro, Brazil).

Leishmanicidal activity

Promastigote cultures (10^6 mL^{-1}) were treated with different concentrations of the AgEO and its constituent (-)-guaiol [3*R*,6*S*,10*S*)-6,10, α,α-tetramethylbicyclo [5·3·0] dec-1(7)-ene-3-methanol, 97% Sigma-Aldrich] and viable parasites were counted daily in a Neubauer chamber.

Anti-amastigote activity was assayed in infected mouse macrophages as previously described (Ferreira *et al.* 2011; Soares *et al.* 2007). Briefly, thioglycolate-stimulated macrophages from BALB/c mice cultured in RPMI 1640 (Biochrom KG, Germany) containing 10% FCS at 34 °C and 5% CO_2 were infected with promastigotes at a 10:1 ratio for 1 h and cultured for 24 h as described above. Afterwards, the cultures were treated with AgEO or guaiol for 24 h, washed, fixed and Giemsa stained. The infectivity index (% of infected macrophages × average number of amastigotes per macrophage) was determined microscopically by counting at least 200 cells in duplicate cultures.

Assessment of parasite load

The infected macrophages incubated or not for 24 h with AgEO were washed and treated with 0·01% sodium dodecyl sulphate (SDS) for 10 min to allow the release of live amastigotes. The cultures were subsequently fed with 1 mL of Schneider's medium supplemented with 10% FCS and cultured at 27 °C for 2 days. The relative intracellular load of amastigotes was measured by the number of differentiated motile promastigotes (Soares *et al.* 2007, 2010; Ferreira *et al.* 2011).

Macrophage cytotoxic assays

2,3-Bis-(2-methoxy-4-nitro-5-sulfophenyl]-2H-tetrazolium-5-carboxyanilide salt) (XTT) test

The thioglycolate-stimulated macrophages (2 × 10^5 well^{-1}) from BALB/c mice adhered to 24 well plates were treated or not with AgEO or guaiol at the indicated concentrations, followed by incubation at 37 °C and 5% CO_2 for 24 h. Afterwards, these cells were treated with XTT for 3 h as previously described (Soares *et al.* 2007). Sodium azide was used as the positive control. The percentage of viable cells was determined towards the untreated cell cultures (100%).

Phagocytosis assay

The thioglycolate-stimulated macrophages were treated with different concentrations of AgEO at 37 °C and 5% CO_2 for 24 h, washed and incubated with promastigotes at a 10:1 ratio at 34 °C and 5% CO_2 for 1 h. Afterwards, the cultures were washed, fixed, stained with Giemsa and the percentage of phagocytosed parasites was determined microscopically by counting at least 200 cells in triplicates. The results were expressed as the percentage of phagocytosis determined towards the untreated control as described by Soares *et al.* (2007).

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Table 1. Chemical composition of *Aloysia gratissima* essential oil

Constituents	RI	Relative abundance (%)[a]
Monoterpenes		
Sabinene	975	4·33 ± 0·03
Limonene	1029	4·04 ± 0·03
1,8-Cineole	1031	17·64 ± 0·05
Sabinene hydrate <cis>	1070	1·27 ± 0·01
Sabinene hydrate <trans>	1109	0·15 ± 0·01
Borneol	1169	0·53 ± 0·03
Terpinen-4-ol	1177	0·42 ± 0·01
α-Terpineol	1188	0·93 ± 0·02
Total identified monoterpenes		29·3%
Sesquiterpenes		
δ-Elemene	1338	4·97 ± 0·03
α-Copaene	1376	0·28 ± 0·01
β-Bourbonene	1388	0·43 ± 0·01
β-Elemene	1390	2·27 ± 0·02
trans-Caryophyllene	1417	4·99 ± 0·00
β-Copaene	1432	1·27 ± 0·02
γ-Elemene	1434	3·25 ± 0·05
Aromadendrene	1441	2·05 ± 0·02
α-Humulene	1454	1·36 ± 0·01
allo-Aromadendrene	1460	0·39 ± 0·02
γ-Muurolene	1476	0·21 ± 0·03
Germacrene D	1484	6·15 ± 0·01
Bicyclogermacrene	1500	5·61 ± 0·04
Germacrene A	1509	0·34 ± 0·06
Cubenol	1515	0·15 ± 0·02
γ-Cadinene	1513	0·19 ± 0·02
Italicene ether	1537	0·21 ± 0·00
Elemol	1549	2·07 ± 0·02
Germacrene B	1561	2·16 ± 0·04
Spathulenol	1578	2·55 ± 0·02
Caryophyllene oxide	1583	1·97 ± 0·12
Guaiol	1600	10·33 ± 0·03
Humulene epoxide II	1608	0·59 ± 0·01
Rosifoliol	1600	0·83 ± 0·01
1,10-di-epi-Cubenol	1619	0·28 ± 0·02
1-epi-Cubenol	1628	1·12 ± 0·01
Eremoligenol	1631	0·16 ± 0·02
Muurola-410(14)-dien-1β-ol	1631	0·16 ± 0·02
Hinesol	1644	0·46 ± 0·03
Selina-311-dien-6α-ol	1647	0·35 ± 0·02
Torreyol	1646	0·20 ± 0·04
Cubenol	1646	0·22 ± 0·04
β-Eudesmol	1653	0·54 ± 0·03
α-Eudesmol	1653	0·28 ± 0·02
7-epi-α-Eudesmol	1662	0·87 ± 0·05
Bulnesol	1671	4·52 ± 0·05
Guaia-3,10(14)-dien-11-ol	1649	0·15 ± 0·00
Eudesma-4(15)-7-dien-11β-ol	1688	0·19 ± 0·01
Amorpha-4,9-dien-2-ol	1700	0·33 ± 0·01
Nootkatol	1714	1·00 ± 0·04
2α-Hydroxy-amorpha-4,7(11)-diene	1775	0·39 ± 0·03
Total identified sesquiterpenes		66·5%

(*Continued*)

RI, retention index. See Adams (2007).
[a]Values from the relative peak area in the chromatograms.

Trypan blue exclusion test

The thioglycolate-stimulated macrophages were treated with different concentrations of AgEO or guaiol at 37 °C and 5% CO_2 for 24 h, followed by addition of 0·03% Trypan blue. Cell viability was calculated by counting at least 200 cells in triplicates (Ferreira *et al.* 2011).

Nitric oxide (NO) production

The thioglycolate-stimulated macrophages (10^6 cells well^{-1}) were activated or not with 100 ng mL^{-1} of lipopolysaccharide (LPS, *Escherichia coli* O111:B4, Sigma) and/or murine interferon-γ [100 ng mL^{-1}], and treated or not with 1 μg mL^{-1} of AgEO or guaiol. After incubation at 37 °C and 5% CO_2 for 48 h, the nitrite concentration was determined by Griess method. The results are expressed as the mean ± S.E.M. of 3 independent experiments carried out in triplicates.

Promastigotes ultrastructural analysis

Promastigotes were treated at the IC_{50} concentration and with 50 μg mL^{-1} of AgEO, and guaiol was used at 50 μg mL^{-1}, whereas the controls contained 0·2% dimethyl sulfoxide (DMSO). After 48 h, the cells were fixed with 2·5% glutaraldehyde and 4% paraformaldehyde in 0·1 M sodium cacodylate buffer (pH 7·2), 5 mM calcium chloride and 3·7% sucrose. Promastigotes were then washed, post-fixed in 1% OsO_4, 0·8% potassium ferricyanide and 5 mM calcium chloride, and further rinsed in the same buffer. After dehydration in acetone, parasites were embedded in PolyBed812 (Polysciences Inc., Warrington, PA, USA). Ultra-thin sections were collected on 300 mesh copper grids, stained with uranyl acetate and lead citrate, then observed in a Field Electron and Ion (FEI) Morgagni F 268 (Eindhoven, the Netherlands) transmission electron microscope operating at 80 kV.

Statistical analysis

Data were analysed using the *Student's t-test* when comparing two groups or *one-way ANOVA* for more than two groups, using the GraphPad Prism 5.0 software; $P < 0·05$ was considered significant.

Results

Aloysia gratissima leaves essential oil (AgEO) was analyzed by GC-FID and its constituents were characterized by GC-MS

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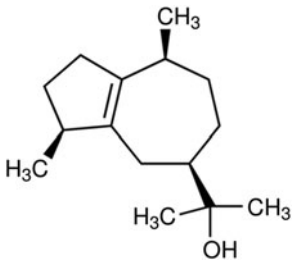

Fig. 1. Chemical structure of guaiol.

(Table 1). The alcohol guaiol (Fig. 1) was identified as the major sesquiterpene in the oil (10·5%).

Initially, we evaluated the AgEO toxicity for *L. amazonensis* assessing promastigotes growth. Our results demonstrated that AgEO affected promastigote growth in a dose-dependent manner. Inhibitions of 31%, 44% and 85% of parasite growth was obtained after 72 h post-treatment with 1, 10 and 100 μg mL^{-1} of AgEO, respectively. The IC_{50} at 48 h and 72 h to compound exposure were 25 and 14 μg mL^{-1}, respectively (Fig. 2).

To evaluate the toxicity of AgEO to host macrophages, these cells were treated with different concentrations of AgEO and the dehydrogenase activity was assessed by the XTT method. AgEO was nontoxic to peritoneal macrophages at the tested concentrations (Fig. 3A). In the same way, AgEO did not affect the membrane integrity, as assayed by the Trypan blue exclusion test (Fig. 3B). Moreover, AgEOs did not inhibit the macrophage phagocytic ability when compared with 1% DMSO vehicle. Interestingly, around 50% increase in the macrophage phagocytic capacity was observed after treatment with AgEO (Fig. 3C).

Amastigotes are responsible for the maintenance of infection in vertebrate hosts. Hence, the effect of AgEO on intramacrophage amastigotes was investigated. Our data showed that AgEO induced a significant decrease in the parasite survival index when compared with untreated control, with inhibition rates of 35·5%, 50% and 61% at 0·01, 0·1 and 1 μg mL^{-1} of AgEO, respectively. The IC_{50} found for amastigotes was 0·16 μg mL^{-1} (Fig. 4A). To further confirm the anti-amastigote effect, cultures were assayed in conditions to allow the AgEO-surviving amastigote to differentiate into promastigotes. We found an 85% reduction in the amastigote intracellular load at 2 μg mL^{-1} of AgEO, similar to the reduction promoted by 1 μg mL^{-1} of amphotericin B (Fig. 4B).

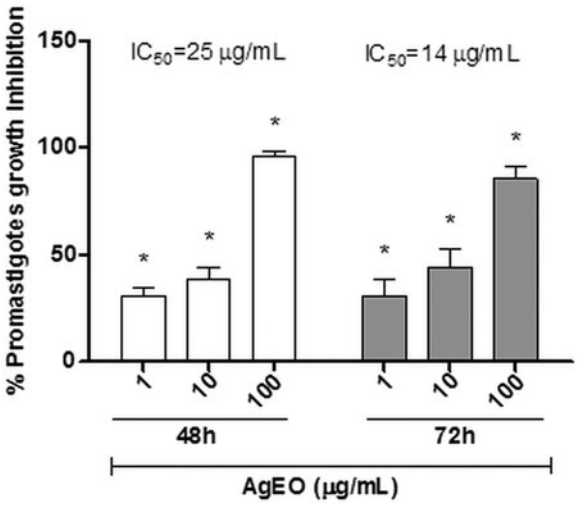

Fig. 2. Inhibition of *Leishmania amazonensis* promastigote proliferation by *Aloysia gratissima* essential oil (*Ag*EO). Parasites (10^6 mL^{-1}) were treated or not with the indicated concentrations of AgEO or 1% DMSO (vehicle control). Anti-promastigote activity was determined by counting viable parasites in a Neubauer chamber at 48 and 72 h post-treatment. Results are expressed as a percentage of growth inhibition + standard error of the mean (S.E.M.) of 3 independent experiments. *P < 0·05 in relation to untreated control.

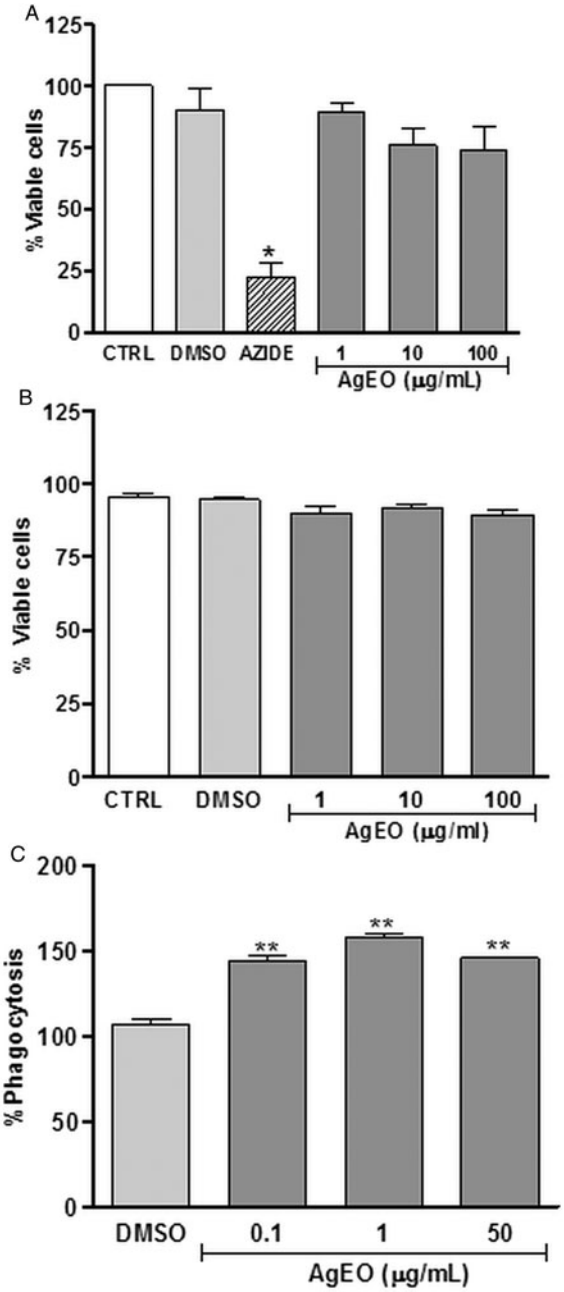

Fig. 3. Toxicity of *Aloysia gratissima essential oil* (*Ag*EO) for murine peritoneal macrophages. (A) Adhered peritoneal macrophages were treated for 24 h with 1% DMSO (vehicle), or AgEO at the indicated concentrations and cell viability was evaluated by the XTT assay. Sodium azide (1%, Azide) was used as positive control. Data are expressed as mean +S.E.M. from three independent experiments in triplicate. *P < 0·05. (B) Macrophages treated as above and the cell viability was evaluated by Trypan Blue exclusion assay. Data are expressed as mean +S.E.M. from three independent experiments in triplicate. (C) Macrophages treated as above were incubated with promastigotes at 10 parasites: 1 macrophage ratio for 1 h, washed, fixed and the phagocytic index calculated in relation to untreated controls (100%). Data are expressed as mean +S.E.M. from three independent experiments in triplicate. **P < 0·01

We also evaluated the ability of AgEO to modulate NO production, since this cellular signaling molecule is known to be highly effective against *Leishmania*. Our results demonstrated that 1 μg mL^{-1} AgEO did not affect NO production in resting macrophages and in lipopolisacaride (LPS)/Interferon-gama (IFN-γ) activated-macrophages as well (Fig. 4C).

The cytotoxicity of guaiol was likewise evaluated, and our results demonstrated its non-toxicity to macrophages up to

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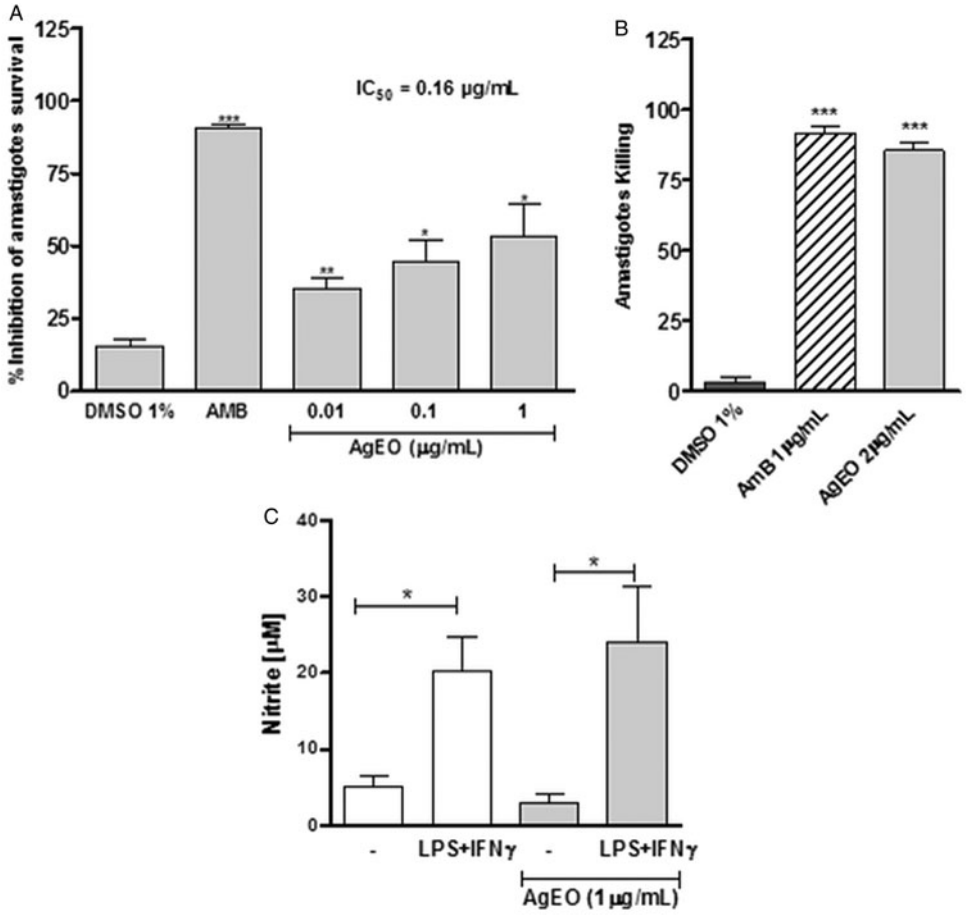

Fig. 4. Anti-*Leishmania amazonensis* effect of *Aloysia gratissima essential oil* (AgEO) on amastigote-infected macrophages. (A) Infected peritoneal macrophages were treated with 1% DMSO (vehicle) or with AgEO at the indicated concentrations and amastigote survival assessed 24 h later. Results from at least four independent experiments done in triplicate are shown as percentage of inhibition of amastigotes survival in relation to vehicle-treated cells ± S.E.M.. $^{*}P < 0{\cdot}05$; $^{***}P < 0{\cdot}001$. (B) Amastigote-infected mouse peritoneal macrophages treated with 2 μg mL^{-1} AgEO and after 24 h Schneider medium was added to the cultures. Parasite survival was assessed after 48 h by counting motile promastigotes. Amphotericin B (AMB 1 μg mL^{-1}), was used as a positive control. Results from three independent experiments done in triplicate are shown as percentage of amastigotes killing in relation to vehicle (DMSO 1%) treated cells +S.E.M.. *** $P < 0{\cdot}001$. (C) Effect of *Aloysia gratissima* essential oil (AgEO) on nitric oxide (NO) production. Murine peritoneal macrophages were activated or not with LPS [100 ng mL^{-1}] and IFN-ɣ [100 ng mL^{-1}] in the presence or not of AgEO at 1 μg mL^{-1}. NO production was evaluated 48 h later by the Griess method. The results are expressed as nitrite [μM] and represent the mean of 4 experiments.$^{*}P < 0{\cdot}05$.

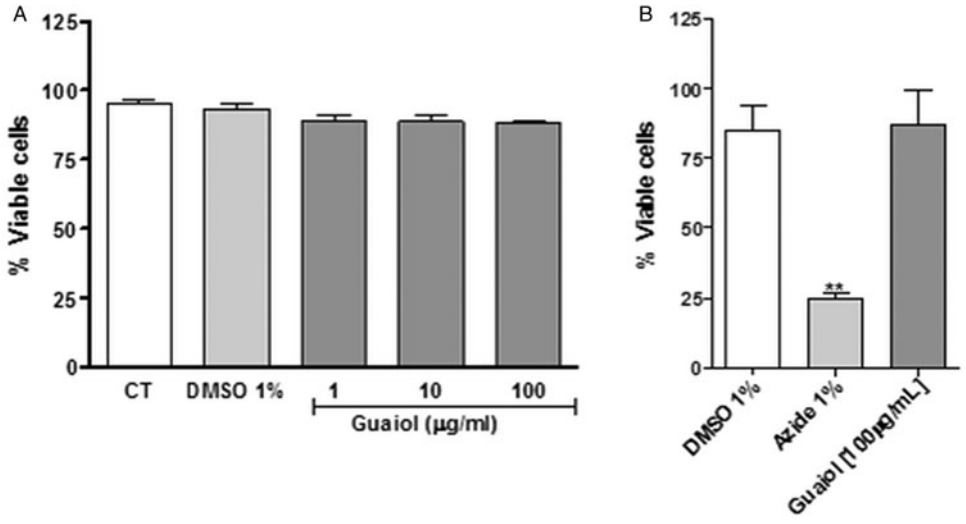

Fig. 5. Toxicity of Guaiol for peritoneal macrophages. (A) Adhered macrophages treated or not with 1, 10 and 100 μg mL^{-1} guaiol, DMSO 1% (vehicle) for 24 h and cell viability was evaluated by the Trypan Blue exclusion assay. Data are expressed as the mean +S.E.M. of three independent experiments in triplicate. (B) Adhered peritoneal macrophages were treated with 100 μg mL^{-1} guaiol, DMSO 1% (vehicle) or 1% sodium azide (Azide) and cell viability was evaluated by XTT assay. Data are expressed as mean +S.E.M. from three independent experiments in triplicate. $^{**}P < 0{\cdot}01$.

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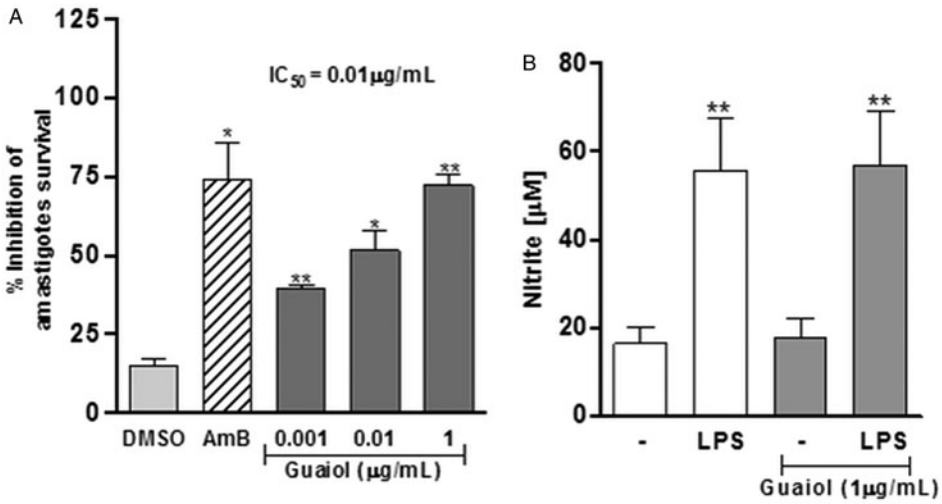

Fig. 6. Anti-amastigote effect of Guaiol. (A). *Leishmania*-infected BALB/c mouse peritoneal macrophages were treated with 1% DMSO (vehicle) and guaiol at the indicated concentrations and amastigote survival assessed 24 h later. Results from three independent experiments done in triplicate are shown as percentage of inhibition of amastigote survival in relation to vehicle-treated cells +S.E.M.. $^{**}P < 0{\cdot}05$. Amphotericin B (AMB 1 μg mL^{-1}), was used as a positive control. (B) Effect of Guaiol on nitric oxide (NO) production. Murine peritoneal macrophages were activated or not with lipopolysaccharide (LPS) [100 ng mL^{-1}] in the presence or not of guaiol at 1 μg mL^{-1}. NO production was evaluated 48 h later by the Griess method. The results are expressed as nitrite [μM] and represent the mean of 4 experiments.$^{***}P < 0{\cdot}001$

100 μg mL^{-1}, as determined by Trypan blue exclusion (Fig. 5A) and XTT (Fig. 5B) assays.

Thereafter, we tested the guaiol activity against amastigotes, finding an inhibition of 39·65, 51·75 and 75·35% of amastigotes survival inside macrophages compared with untreated control at 0·001, 0·01 and 1 μg mL^{-1} of guaiol, respectively (Fig. 6A). Guaiol showed IC_{50} of 0·01 μg mL^{-1} against amastigotes. In addition, the antiamastigote activity observed was independent of NO, since the guaiol treatment of stimulated or unstimulated macrophages, at 1 μg mL^{-1}, did not modulate NO production (Fig. 6B).

The morphological alterations on AgEO and guaiol treated-promastigotes were analyzed by transmission electron microscopy (Fig. 7A–G). Control promastigotes exhibited a dense cytoplasm, where the lipid bodies, the nucleus with the characteristic heterochromatin (Fig. 7A) and the compact array of kDNA filaments and the flagellar pocket containing multiple vesicles can be observed (Fig. 7B). Parasites treated with 25 μg mL^{-1} AgEO showed an increase in the size of lipid bodies causing the rupture of the parasite membrane (Fig. 7C). Promastigotes treated with 50 μg mL^{-1} AgEO and guaiol presented mitochondrial swelling and a remarkable alteration in the kDNA network, with disorganization of the DNA filaments (Fig. 7D). Moreover, lipid bodies presenting irregular edges and electronic densities and budding of small vesicles from the cell body membrane could be observed (Fig. 7E). This treatment induced also an intense swelling of the mitochondrion and cytoplasmic compartments (Fig. 7F) and the appearance of long cell body membrane projections in the edge of the flagellar pocket (Fig. 7G).

Discussion

Our data presented for the first time the leishmanicidal activity of *Aloysia gratissima* (Gillies and Hook) on *L. amazonensis*, a New World species that is the causative agent of cutaneous, diffuse cutaneous and visceral diseases. We demonstrated that AgEO is active against promastigotes by inhibiting their growth in a dose-dependent manner. Moreover, the AgEO effectiveness attained higher levels along the exposition time, given that, after a unique treatment, the IC_{5O} value at 72 h was 1·8 times lower compared with the IC_{50} at 48 h. Importantly, the AgEO was also able to reduce amastigote survival inside infected macrophages *in vitro*. The amastigotes were unable to differentiate into promastigotes after AgEO treatment, as verified by the parasite load assay, thus confirming parasite damage by the EO usage. The IC_{50} value of AgEO for amastigotes was lower than that found for promastigotes, indicating that amastigotes are somehow more sensitive to the oil leishmanicidal effects. This finding is also relevant since amastigotes are responsible for the disease maintenance in the vertebrate hosts.

Microbicidal effects of AgEO have been demonstrated for bacteria, fungi and viruses (Dellacasa *et al.* 2003; Garcia *et al.* 2003; Santos *et al.* 2013). AgEO obtained from leaves and flowers was bactericidal and fungicidal, although EO obtained from flowers was more effective against the Gram-negative *P. aeruginosa*, the Gram-positive *S. pneumonia* and the yeast *C. albicans*. These differences may be attributed to the higher amount of sesquiterpenes present in the flower EO (Santos *et al.* 2013). It has also been reported that AgEO is able to inhibit the growth of Junin virus and inactivate herpes simplex virus type 1 (Garcia *et al.* 2003).

The composition of AgEO obtained by hydrodistillation was characterized by GC-MS. The main constituents are the monoterpene 1,8-cineole (17·6%) and the sesquiterpene alcohol guaiol (10·5%), along with other guaiol isomers (azulene-types structures, 7·3%) and hydrocarbons of the germacrene-types sesquiterpenes (total up to 17%). Additionally, trans-caryophyllene and its oxide (7% total) were also identified in the oil composition. A similar sesquiterpene constitution in the oil of *A. gratissima* leaves and flowers was recently described (Santos *et al.* 2013), although a distinct monoterpene composition was characterized. Other studies identified similar compounds as well as different proportions of guaiol in *A. gratissima* leaf oil such as 2·6% (Santos *et al.* 2013), 11·5% (Santos *et al.* 2015) and 12·5% of guaiol (Trovati *et al.* 2009). These differences can be attributed to diverse environmental conditions, including the type of soil, climate, plant age and the part of the plant used to obtain the oil (Velasco-Negueruela and Pérez-Alonso, 1993).

EOs from diverse plant species as well as isolated mono- and sesquiterpenes have been investigated for their antiprotozoal activity (Piątkowska and Rusiecka-Ziółkowska, 2016; Sharifi-Rad *et al.* 2017). Concerning the AgEO constituents, there are reports on the leishmanicidal activity of *trans*-caryophyllene (Soares *et al.* 2013), whilst the monoterpenes 1,8-cineole and sabinene showed to be biologically barely effective or ineffective at all (Mikus *et al.*

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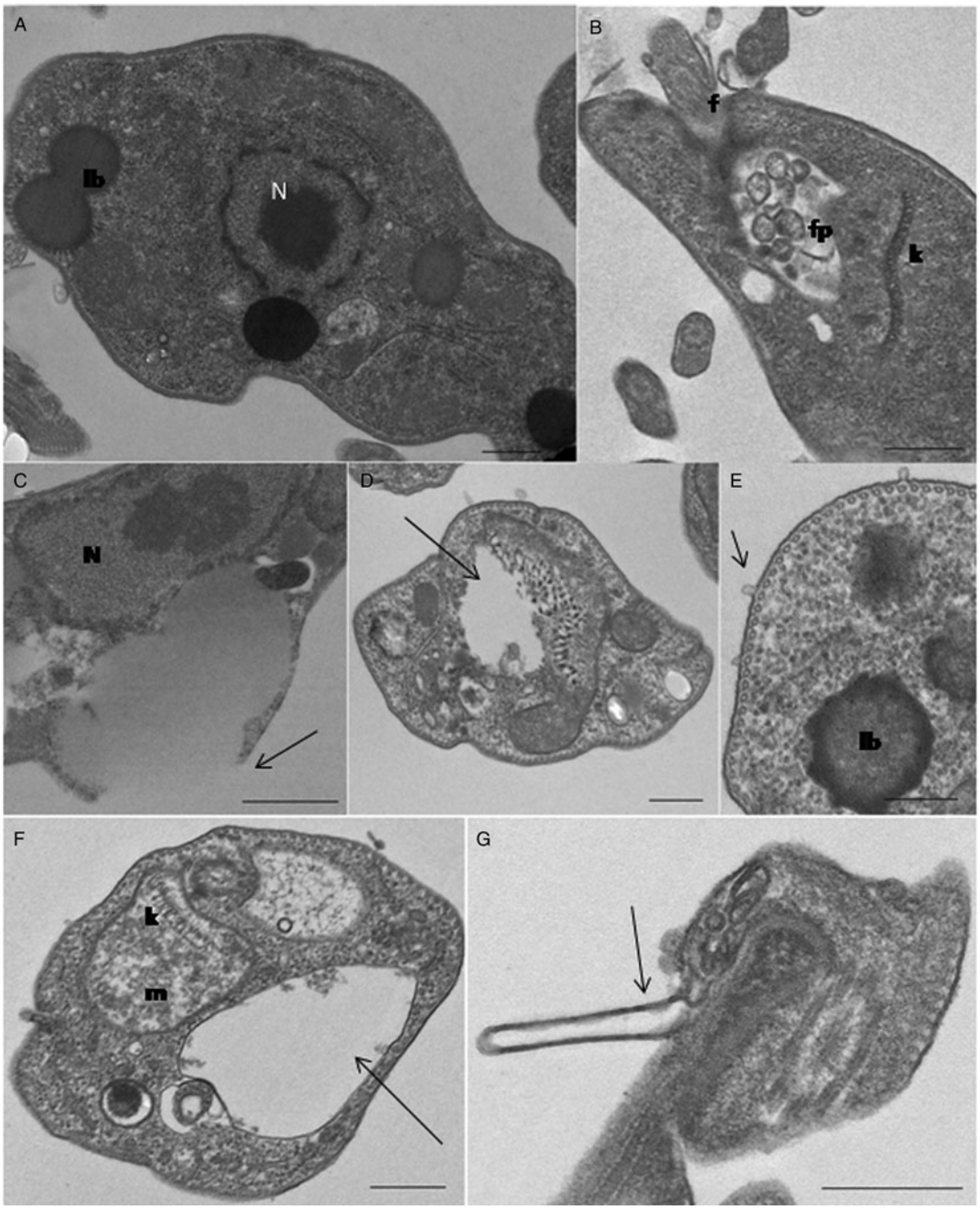

Fig. 7. Ultrastructural effects of AgEO and Guaiol on *Leishmania amazonensis* promastigotes. (A, B) Untreated parasites displayed the normal nuclear morphology (N), kinetoplast (k), lipid bodies (lb) and flagelar pocket (fp); (C-E) AgEO treated parasites with 25 μg mL^{-1} (C) and 50 μg mL^{-1} (D-E) swollen of the lipid bodies with the rupture of the plasma membrane (arrow in C), mitochondrial sewlling, altered kDNA network (arrow in D), the presence of lipid bodies showing irregular edges and the budding of small vesicles from the cell body membrane (small arrow in E); (F-G) Guaiol treated parasites showed the swelling of mitochondria and cytoplasmic compartments (arrow in F) and the presence of large projections of the cell body membrane (arrow in G). f, flagellum. Bars = 0·5 nm.

2000; Leal *et al.* 2013; Camargos *et al.* 2014; Machado *et al.* 2014). EOs obtained from other *Lippia* species, having different compositions, have also been demonstrated to be toxic against *L. amazonensis* and *L. chagasi* promastigotes and amastigote forms (Oliveira *et al.* 2009; Medeiros *et al.* 2011; Farias-Junior *et al.* 2012). Thus, the *L. sidoides* EO resulted in an IC_{50}/48 h of 44·38 μg mL^{-1} on inhibiting *L. amazonensis* promastigotes and an IC_{50}/72 h of 89 μg mL^{-1} on *L. chagasi* promastigotes (Oliveira *et al.* 2009; Medeiros *et al.* 2011). In our study, we found the IC_{50}/48 h around 1·77 times lower for *A. gratisima* on *L. amazonensis* promastigotes, compared with *L. sidoides*.

Recently, a study with EOs from several plants demonstrated their toxicity for *L amazonensis* promastigotes, and guaiol (9·35%; 48·3%) was identified in *Matricaria chamomilla and Bulnesia sarmientoi*, respectively, although pure guaiol was not tested against the parasites (Andrade *et al.* 2016). Once guaiol was the major sesquiterpene constituent identified in our leaf oil extract, we tested the capacity of this component to inhibit amastigotes survival inside infected macrophages, using a commercially available guaiol. The use of a compound already commercially available is cheaper, faster and interesting for future *in vivo* tests to find promising leishmanicidal compound. This is

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one of the recommendation of Drugs for Neglected Diseases initiative (DNDi), in order to facilitate the discovery of novel compounds for future treatment for neglected diseases. Our data established for the first time that guaiol inhibits amastigotes survival with IC_{50} of 0·01 $\mu g\,mL^{-1}$ against amastigotes, suggesting that AgEO effects could be assigned to the major presence of guaiol in the oil constitution, although the effect of other similar sesquiterpenes (e.g. bulnesol and germacrenes) cannot be definitely excluded.

Since the AgEO was toxic to the promastigotes, we then evaluated its toxicity for macrophages and we found that the cells viability was not affected upon the higher concentration tested (100 $\mu g\,mL^{-1}$) when evaluated by the dehydrogenases assay. Moreover, the macrophage membrane integrity was not affected by AgEO treatment at the higher concentrations tested, and the macrophage phagocytic capacity was even increased; together, these results pointed out to the selectivity of AgEO. Similar toxicity properties for host cells were found for guaiol

NO is a mediator involved in the leishmanicidal activity of macrophages and, thus, we analyzed whether AgEO or guaiol could modulate this mechanism. Our results indicated a NO-independent effect of both AgEO and guaiol suggesting a direct effect on the parasite. This possibility is strengthened by the ultrastructural alterations observed in parasites treated by both samples, as altered kinetoplast, loss of mitochondrial matrix, membrane rupture and altered lipid bodies. Chemical studies about microbial guaiol transformation using fermentation techniques with different species of fungi showed that guaiol can be converted in metabolites, which are toxic for several bacterium species (Choudhary *et al.* 2007). Thus, we can speculate that guaiol can be either directly metabolized by the parasites, generating toxic products or by host cells, hence generating leishmanicidal metabolites.

In conclusion, we report for the first time the leishmanicidal effect of EO from *Aloysia gratissima* and of its main sesquiterpene constituent, guaiol, against *Leishmania amazonensis*. Together our results could contribute to the establishment of new antiprotozoal compounds, aiming at treating leishmaniasis.

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Author contributions. MCG, DCS, TSP and RCS performed the experiments; LHP, DCS conceived and designed the experiments; MCG, DCS, EMS, ACS, MFSR and LHP analysed the data; MGMD, ACS, MFSR and TSP contributed with reagents/materials/analysis tools; MCG, DCS, EMS and LHP wrote the paper.

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Conflict of interest. The authors declare no conflict of interest.

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