Journal of Ethnopharmacology 160 (2015) 32–40

Contents lists available at ScienceDirect

Journal of Ethnopharmacology

journal homepage: www.elsevier.com/locate/jep

Research Paper

Syzygium cumini (L.) Skeels essential oil and its major constituent α-pinene exhibit anti-*Leishmania* activity through immunomodulation *in vitro*

Klinger Antonio da Franca Rodrigues [a,*,1], Layane Valéria Amorim [a], Clarice Noleto Dias [b,1], Denise Fernandes Coutinho Moraes [b], Sabrina Maria Portela Carneiro [a], Fernando Aécio de Amorim Carvalho [a]

[a] Graduate Program in Pharmacology, Medicinal Plants Research Center, Federal University of Piauí, 64049-550 Teresina, PI, Brazil
[b] Laboratory of Pharmacognosy II, Department of Pharmacy, Federal University of Maranhão, 65085-580 São Luís, MA, Brazil

ARTICLE INFO

Article history:
Received 4 September 2014
Received in revised form
11 October 2014
Accepted 13 November 2014
Available online 25 November 2014

Keywords:
Syzygium cumini
α-Pinene
Essential oil
Anti-*Leishmania*
Immunomodulatory activity

ABSTRACT

Ethnopharmacological relevance: *Syzygium cumini* (L.) Skeels (Myrtaceae), commonly known as “jambolão” in Brazil is widely used in folk medicine against leishmaniasis, inflammation, chronic diarrhea, and ulcers. It is one of the most commonly used plants for the treatment of diabetes worldwide. In previous studies, *Syzygium cumini* was shown to possess antihyperlipidemic and anti-allergic properties, and to exhibit good performance as an antimicrobial agent against bacteria, fungi, and protozoa parasites of the genus *Leishmania* and *Trypanosoma*. This study was aimed at evaluating the effects of *S. cumini* essential oil (ScEO) and its major component α-pinene on *Leishmania* (*Leishmania*) *amazonensis*, as well as their cytotoxicity and possible mechanisms of action.

Materials and methods: To evaluate the anti-proliferative effect on *Leishmania*, effects on promastigote and axenic amastigote forms were assessed using tetrazolium salt (MTT) assay. The intramacrophagic amastigotes were exposed to ScEO and α-pinene to determine the survival index. To gain insight into the mechanism of action involved in the effect on the samples, we evaluated the modulation of macrophage activation state by observing structural (phagocytic and lysosomal activities) and cellular (nitric oxide increase) changes. To assess the safety profile of ScEO and α-pinene, murine macrophages and human red blood cells were treated with ScEO and α-pinene and the selectivity index was calculated for each treatment.

Results: α-Pinene was effective against *Leishmania amazonensis* promastigote forms, with a half-maximal inhibitory concentration (IC_{50}) value of 19.7 μg/mL. α-Pinene was more active (IC_{50} values of 16.1 and 15.6 μg/mL against axenic and intracellular amastigotes, respectively) than ScEO (IC_{50} values of 43.9 and 38.1 μg/mL against axenic and intracellular amastigotes, respectively). Our results showed that the anti-*Leishmania* effects were mediated by immunomodulatory activity, as evidenced by the observed increases in both phagocytic and lysosomal activity, and the elevated NO levels. ScEO and α-pinene exhibited low cytotoxicity against murine macrophages and human erythrocytes. The 50% cytotoxicity concentration (CC_{50}) values for the macrophages in the MTT assay were 614.1 and 425.2 μg/mL for ScEO and α-pinene, respectively, while the corresponding half-maximal hemolytic concentration (HC_{50}) values were 874.3 and 233.3 μg/mL.

Conclusions: Taken together, the results demonstrate that ScEO and its major constituent α-pinene have significant anti-*Leishmania* activity, modulated by macrophage activation, with acceptable levels of cytotoxicity

* Corresponding author. Tel.: +55 83 8636 3310.
E-mail addresses: klinger.antonio@gmail.com (K.A.d.F. Rodrigues), layane.valeria@hotmail.com (L.V. Amorim), cla_noleto@yahoo.com.br (C.N. Dias), deniseufma2013@gmail.com (D.F.C. Moraes), sa.portela@hotmail.com (S.M.P. Carneiro), famorim@ufpi.edu.br (F.A.d.A. Carvalho).
[1] Present address: Graduate Program in Bioactive Natural and Synthetic Products, Health Sciences Center, Federal University of Paraíba, 58051-970 João Pessoa, PB, Brazil.

http://dx.doi.org/10.1016/j.jep.2014.11.024
0378-8741/© 2014 Elsevier Ireland Ltd. All rights reserved.

in murine macrophages and human erythrocytes. Further work is warranted, involving more in-depth mechanistic studies and *in vivo* investigations.

© 2014 Elsevier Ireland Ltd. All rights reserved.

1. Introduction

Leishmaniasis comprises a complex of parasitic diseases caused by more than 20 protozoa species of the genus *Leishmania*, Trypanosomatidae family, that affect approximately 12 million people in 88 countries throughout the world (Feasey et al., 2010; Shukla et al., 2011; WHO, 2014). The life cycle of these parasites includes two distinct forms: a motile extracellular promastigote form found in the sand fly vector and a nonflagellated intracellular amastigote form which can be found within the mononuclear phagocytes in the mammalian host (De Almeida et al., 2003; Chappuis et al., 2007). The clinical manifestations are diverse and may range from ulcerative skin lesions to the visceral form, which is the most severe form of leishmaniasis and is associated with generalized infection and high mortality (David and Craft, 2009; Abebe et al., 2012).

The chemotherapy currently available for the treatment of leishmaniasis is far from satisfactory. The first choice treatment was introduced in 1945 and is still based on pentavalent antimonials (Sb^V)—meglumine antimoniate (Glucantime®) and sodium stibogluconate (Pentostam®). The second-line drugs amphotericin B, pentamidine, and paromomycin are used in cases of antimonial resistance. However, these drugs have important limitations, including toxic side effects, high cost, and the need for prolonged treatment (Croft and Coombs, 2003; Dos Santos et al., 2011; Monte Neto et al., 2011; Tiuman et al., 2011). Given these limitations, there is an urgent need for the discovery of new therapies against leishmaniasis.

Different strategies have been attempted to obtain novel compounds effective against *Leishmania* spp. A number of natural products with diverse structural classes showed anti-*Leishmania* properties, including monoterpenes, sesquiterpenes, and other constituents of essential oils, demonstrating that such substances may be promising research options for the development of new drugs (Ueda-Nakamura et al., 2006; Angel et al., 2014).

Syzygium cumini (L.) Skeels (Syn. *Eugenia jambolana* Lam. or *Syzygium jambolana* Dc or *Eugenia cuminii* Druce), commonly known as “jambolão” in Brazil, is an evergreen tree belonging to the family Myrtaceae (Faria et al., 2011). It originated in tropical Asia, specifically in the Indian subcontinent, and is common in regions with tropical and subtropical climate, which can be found in most Brazilian states (Schossler et al., 2004; Baliga et al., 2011). Phytochemical investigations reported that *Syzygium cumini* leaves contain various terpenoids, alkaloids, lignans, and phenolics, including quercetin, myricetin, sitosterol, myricetin, and betulinic acid (Mir et al., 2009; Srivastava and Chandra, 2013). The medicinal uses of *Syzygium cumini* have been recognized in various systems of traditional folk medicine, with *Syzygium cumini* being one of the world's most commonly used plants for the treatment of diabetes (Trojan-Rodrigues et al., 2012; Ayyanar et al., 2013). In addition, this species has been used for treatment of leishmaniasis (Ayyanar and Subash-Babu, 2012), diseases caused by bacteria, fungi and viruses (Maciel et al., 2008), inflammation (Muruganandan et al., 2001), chronic diarrhea (Veigas et al., 2007), and intestinal and genitourinary tract ulcers (Chandrasekaran and Venkatesalu, 2004). Previous studies have demonstrated that *Syzygium cumini* has anti-allergic (Brito et al., 2007), antihyperglycemic (Oliveira et al., 2005), antihyperlipidemic (Ravi et al., 2005), antioxidant (Elangovan et al., 2000), antiviral (Sood et al., 2012), antibacterial (Bag et al., 2012), antifungal, and trypanocidal (Dos Santos et al., 2012) properties.

Recently, we focused our attention on the chemical composition of the essential oil from *Syzygium cumini* (ScEO) and its potential activity against promastigote forms of *Leishmania* (*Leishmania*) *amazonensis*, one of the etiologic agents of American tegumentary leishmaniasis (ATL) (Dias et al., 2013). The most abundant compounds in the essential oil were found to be monoterpenes (87.12%), with α-pinene (31.85%), (*Z*)-β-ocimene (28.98%), and (*E*)-β-ocimene (11.71%) being the major components. ScEO has shown significant anti-*Leishmania* activity against the promastigote forms (IC_{50} values of 60 μg/mL) (Dias et al., 2013). In the present study, we report for the first time the anti-*Leishmania* activity of ScEO and its major constituent α-pinene against amastigote forms of *Leishmania amazonensis*, a life stage that is responsible for the clinical manifestation of leishmaniasis, and propose a possible mechanism of action.

2. Materials and methods

2.1. Chemicals and drugs

Schneider's and RPMI 1640 culture mediums, Griess reagent (1% sulfanilamide in H_3PO_4 10% (v/v) in Milli-Q water), antibiotics penicillin and streptomycin, and other cell culture reagents including fetal bovine serum (FBS), thioglycollate medium, Zymosan, Neutral Red (NR), and tetrazolium salt (3-(4,5-dimethylthiazol-2-yl)2,;5-diphenyltetrazolium bromide; MTT) were purchased from Sigma Chemical (St. Louis, MO, USA). The chemotherapy drug meglumine antimoniate (Glucantime®; 300 mg/mL) was obtained from Aventis Pharma (São Paulo, SP, Brazil). Panoptic staining kit was obtained from Laborclin, PR, Brazil. The reagents glacial acetic acid, ethanol, sodium dodecyl sulfate (SDS), formaldehyde, sodium chloride, calcium acetate, anhydrous sodium sulfate, and dimethylsulfoxide (DMSO, 99% purity) were purchased from Merck Chemical Company (São Paulo, SP, Brazil).

2.2. ScEO and major components

Syzygium cumini leaves were collected and the essential oil was extracted by conventional steam hydrodistillation. Essential oil was characterized using gas chromatography–mass spectrometry (GC–MS), as previously described (Dias et al., 2013). A voucher specimen was deposited (No. 1079/SLS017213) in the Herbarium “Ático Seabra” in São Luiz, Maranhão State, Brazil. α-Pinene ($C_{10}H_{16}$, 98% purity) was purchased from Sigma Chemical (St. Louis, MO, USA). Its structure is shown in Fig. 1.

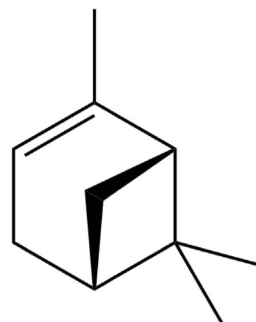

Fig. 1. Chemical structure of α-pinene.

2.3. Leishmania culture conditions

Leishmania (*Leishmania*) *amazonensis* (IFLA/BR/67/PH8) were maintained as amastigotes in several weekly passages in Swiss mice and *in vitro* as promastigotes at 26 °C in supplemented Schneider's medium, pH 7 (10% heat-inactivated fetal bovine serum (FBS), 100 U/mL penicillin and 100 μg/mL streptomycin), as previously described (Carneiro et al., 2012). Extracellular amastigote-like forms were obtained by *in vitro* differentiation of promastigotes of *Leishmania amazonensis* in the stationary growth phase by increasing the temperature to 32 °C and decreasing the pH to 4.6 (Ueda-Nakamura et al., 2006).

2.4. Animals and peritoneal macrophages

Male and female Swiss mice (4–5 weeks old) obtained from section biotherium of the Medicinal Plants Research Center, Health Sciences Center, Federal University of Piauí, (NPPM/CCS/UFPI), Terezina, Brazil, were maintained under controlled temperature (24 ± 1 °C) and light conditions (12-h light/dark cycle). Murine peritoneal macrophages were collected from mice 5 days after intraperitoneal inoculation with 1.5 mL of 3% brewer thioglycollate medium. Animals were treated according to the Guiding Principles (NIH publication #85-23, revised in 1985) for the Care and Use of Animals and all experimental protocols were approved by the Research Ethics Committee (CEEAPI-UFPI/PI no. 001 � 2012).

2.5. Activity against promastigotes and axenic amastigotes of Leishmania amazonensis

Inhibition of parasite growth was assayed using the MTT colorimetric assay, as previously described (Valadares et al., 2011). Briefly, promastigotes or axenic amastigotes in the logarithmic growth phase were cultured in 96-well cell culture plates at 1×10^6 parasites per well in 100 μL of Schneider's medium with increasing concentrations of α-pinene (for wells containing promastigote and amastigote axenic forms) and ScEO (for wells containing axenic amastigotes). The plates were incubated for 48 h in a biological oxygen demand (B.O.D.) incubator at 26 °C for promastigotes and 32 °C for axenic amastigotes. Cell viability was measured by adding MTT (5 mg/mL) and incubating the cells for 4 h prior to the addition of 10% SDS. The absorbances were measured using an ELISA plate reader (Biosystems model ELx800, Curitiba, PR, Brazil) at 540 nm. Glucantime® was used as a positive control.

2.6. Anti-Leishmania activity against intramacrophagic amastigotes

Macrophages were harvested and plated onto culture plates with 24 wells at a concentration of 1×10^6 cells/mL in supplemented RPMI 1640 (10% inactivated FBS, 100 U/mL penicillin, and 100 μg/mL streptomycin), containing sterile round coverslips at 13 mm. Culture plates were incubated at 37 °C and 5% of CO_2 for 3 h for cell adhesion. Adhered macrophages were then incubated with a new medium containing promastigotes (in stationary phase) at a ratio of 10 promastigotes to 1 macrophage at 5% CO_2 and 37 °C for 4 h. The medium was subsequently aspirated to remove non-internalized parasites and the wells were washed with 0.01 M phosphate buffered saline (PBS). The infected culture was incubated with a range of concentrations of ScEO, α-pinene, and Glucantime® at concentrations observed not to be toxic to macrophages for 48 h. After this period, the coverslips were removed and stained with Panoptic staining kit. For each treatment, the number of infected macrophages and the parasite load were counted using optical microscopy (Carneiro et al., 2012).

2.7. Determination of cytotoxicity

2.7.1. Cytotoxic effect on macrophages and the calculation of selective index (SI)

Cytotoxicity evaluation was carried out in 96-well plates using the MTT assay. Approximately 1×10^6 macrophages per well were incubated in 100 μL of supplemented RPMI 1640 medium at 37 °C and 5% CO_2 for 4 h for cell adhesion. Non-adherent cells were removed by washing with RPMI 1640 medium. ScEO and α-pinene were diluted in supplemented RPMI 1640 medium, added at a range of concentrations, and incubated at 37 °C with 5% CO_2 for two days. Following incubation, cytotoxicity was assessed by adding MTT (5 mg/mL). The supernatant was discarded and the formazan crystals were dissolved by addition of 100 μL of DMSO. Finally, absorbance at 550 nm was measured using an ELISA plate reader. Selectivity index of each treatment was calculated by dividing the 50% cytotoxicity concentration (CC_{50}) observed for peritoneal macrophages of Swiss mice by the half-maximal inhibitory concentration (IC_{50}), measured for different forms of *Leishmania*.

2.7.2. Red blood cell lysis assay

The hemolytic activity was evaluated by mixing 80 μL of a 5% suspension of fresh human red blood cells (O^+) in PBS with 20 μL of different concentrations of ScEO or α-pinene and incubating at 37 °C for 1 h. The reaction was slowed by adding 200 μL of PBS and the suspension was centrifuged (1000*g* for 10 min). The supernatant was transferred to a 96-well plate and cell lysis was quantified by spectrophotometrical measurement of absorbance at 540 nm, as previously described (Löfgren et al., 2008). The maximal lysis and blank control were obtained by replacing the extract sample with an equal volume of PBS or distilled water, respectively. The hemolysis percentage was determined using the following equation:

$$\text{Hemolysis }(\%) = \frac{\text{Abs. of sample} - \text{Abs. of blank control}}{\text{Abs. of maximal lysis} - \text{Abs. of blank control}} \times 100$$

2.8. Immunomodulatory activity

2.8.1. Lysosomal activity

The uptake of the cationic dye NR, which is concentrated in the lysosomal compartments of macrophages, was assessed as previously described (Bonatto et al., 2004). Peritoneal macrophages (1×10^6 cells per well) were harvested and plated with samples in serial dilutions at 37 °C and 5% CO_2 for 48 h. Next, cells were treated with 10 μL 2% NR in PBS and incubated for 30 min. The wells were subsequently washed twice with PBS and the internalized NR was solubilized by adding the extraction solution (glacial acetic acid 1% v/v, ethanol 50% v/v dissolved in twice-distilled water). After 30 min on a Kline shaker (model AK 0506), the absorbance at 550 nm was read using the ELISA plate reader.

2.8.2. Phagocytosis assay

Peritoneal macrophages were plated at a concentration of 1×10^6 cells per well and cultured with the samples at 37 °C and 5% CO_2. After 48-h incubation, 10 μL of NR-stained zymosan was added and the cells were incubated at 37 °C for 30 min. Following incubation, 100 mL of Baker's fixative (formaldehyde 4% v/v, sodium chloride 2% w/v, calcium acetate 1% w/v in distilled water) was added to stop the zymosan phagocytosis. After 30 min, the cells were washed with PBS to remove NR and zymosan non-phagocytosed by macrophages. NR was solubilized by adding the extraction solution in the Kline shaker. The absorbance was subsequently measured at 550 nm using the ELISA plate reader (Grando et al., 2009).

2.8.3. Nitric oxide (NO) production

The NO production was estimated by measuring nitrite levels. Macrophages (1×10^6 per well) were incubated at 37 °C with 5% CO_2 for 4 h for cell adhesion. Half of the treatment groups were stimulated with *Leishmania amazonensis* at a 10:1 promastigotes to macrophages ratio for 30 min. The cells were subsequently incubated alone in RPMI 1640 medium (background control) or separately stimulated with either the samples or LPS (2 μg/mL) for 24 h. Following incubation, cell culture supernatant was collected and mixed with an equal volume of Griess reagent. Absorbance was determined at 550 nm using a microplate reader. Nitrite production was extrapolated from a standard curve obtained with a range of concentrations of sodium nitrite (1–150 μM).

2.9. Statistical analysis

The assays were carried out in triplicate and in three independent experiments. Differences between groups were analyzed using one-way ANOVA with Bonferroni post-hoc test using GraphPad Prism® software version 5.0, considering the P value < 0.05 as statistically significant. The IC_{50}, CC_{50}, and half-maximal hemolytic concentration (HC_{50}) values were calculated using probit regression model (SPSS program, version 13.0) and assuming a confidence level of 95% ($P < 0.05$).

3. Results

3.1. Anti-Leishmania activity assay

α-Pinene showed cytotoxic effect on promastigotes of *Leishmania amazonensis*, with cellular death percentages of 93.7%, 83.2%, and 58.4% observed at concentrations of 100, 50, and 25 μg/mL, respectively (Fig. 2A), with IC_{50} calculated at 19.7 μg/mL (Table 1). The effect of ScEO on the promastigotes was previously determined (Dias et al., 2013). The cytotoxic profile of ScEO and its major constituent α-pinene was evaluated by *in vitro* testing against axenic amastigote forms of *Leishmania amazonensis*. By comparing the degree of inhibition following 48 h of exposure, α-pinene was observed to be the most effective tested substance, with 99.08%, 90.8%, and, 78.97% inhibition observed at concentrations of 100, 50, and 25 μg/mL (Fig. 2B), respectively, with an IC_{50} of 16.1 μg/mL (Table 1). At the same concentrations, ScEO showed 98.78%, 65%, and 32.5% inhibition (Fig. 2B), with IC_{50} of 43.9 μg/mL. Low activity of the reference drug Glucantime® was observed on axenic amastigotes following 48 h exposure, with IC_{50} of 624.5 μg/mL.

3.2. Effect of ScEO and α-pinene on macrophage infection

Results of the treatments with ScEO and α-pinene on macrophages infected with *Leishmania amazonensis* are presented in Fig. 3 and Table 1. The values obtained showed that the treatment

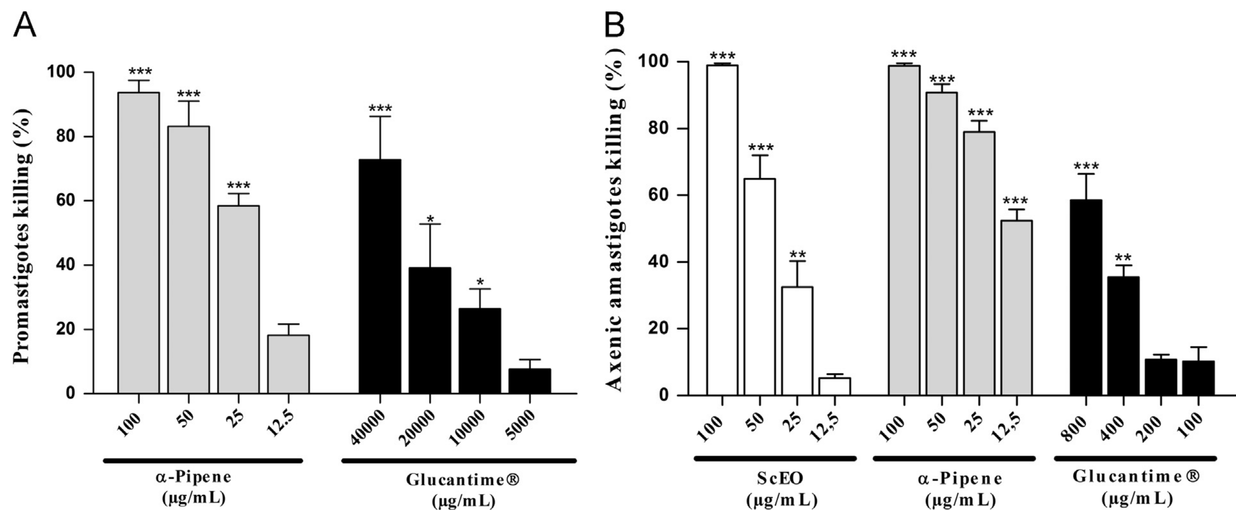

Fig. 2. Anti-*Leishmania* activity against promastigotes (A) and axenic amastigotes (B). Parasites (1×10^6) were exposed to different concentrations for 48 h and cell viability was assessed by tetrazolium salt (MTT). ScEO – *Syzygium cumini* essential oil. *$P < 0.05$ *vs.* control; **$P < 0.01$ *vs.* control; ***$P < 0.001$ *vs.* control.

Table 1
Anti-*Leishmania* activity, cytotoxic effects against mammalian cells, and selectivity index values calculated for *Syzygium cumini* essential oil (ScEO), α-pinene, and Glucantime®.

Compounds	Macrophages	Red blood cell	Promastigotes			Axenic amastigotes			Intramacrophagic amastigotes		
	CC_{50}, μg mL^{-1}	HC_{50}, μg mL^{-1}	IC_{50}, μg mL^{-1}	SI_m	SI_{rb}	IC_{50}, μg mL^{-1}	SI_m	SI_{rb}	IC_{50}, μg mL^{-1}	SI_m	SI_{rb}
ScEO	614.1	874.3	60[a]	10.2	14.5	43,9	13.9	19.9	38.1	16.1	22.9
α-Pinene	425.2	233.6	19.7	21.5	11.8	16.1	26.4	14.5	15.6	27.2	14.9
Glucantime	412.9	139.7	1200[a]	0.3	0.1	624.5	0.6	0.2	167.4	2.4	0.83

SI_m (selectivity index)=CC_{50}/IC_{50}.
SI_{rb} (selectivity index)=HC_{50}/IC_{50}.
[a] Data already published (Dias et al., 2013).

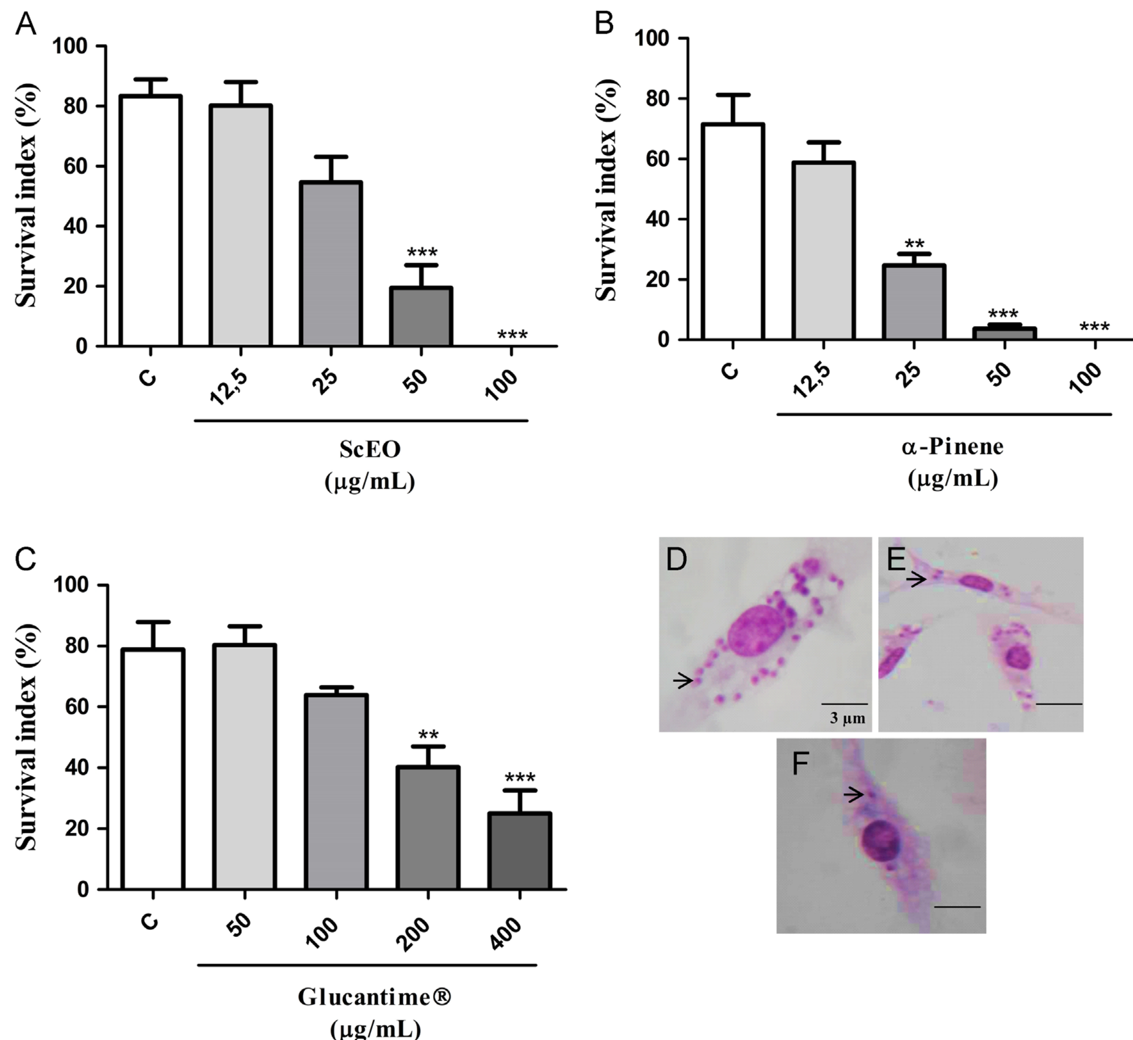

Fig. 3. Effect of *Syzygium cumini* essential oil (ScEO), α-pinene, and the reference drug Glucantime® on the survival index of amastigotes internalized in macrophages after 48 h of exposure. Murine macrophages were infected with promastigote form of *Leishmania amazonensis* and treated at different concentrations of ScEO (A), α-pinene (B), and Glucantime® (C). Detail of non-treated macrophage (D). Detail of macrophage treated with 50 μg/mL of ScEO (E). Detail of macrophage treated with α-pinene at 50 μg/mL (F). Arrows indicate amastigote forms within the macrophages. Results represent the means ± S.E.M. of three experiments performed in triplicate. (*) $P<0.05$ *vs.* control; **$P<0.01$ *vs.* control; ***$P<0.001$ *vs.* control. C=control.

of macrophages with both ScEO and α-pinene diminished the survival rate of amastigotes, with ScEO concentrations of 50 and 100 μg/mL (Fig. 3A and E), and α-pinene concentrations of 25, 50, and 100 μg/mL (Fig. 3B and F) showing an effect. α-Pinene proved to be more effective than ScEO against intramacrophage amastigotes, with IC_{50} of 15.6 μg/mL, compared to IC_{50} of 38.1 μg/mL calculated for ScEO (Table 1). In addition, Glucantime® activity on the intracellular amastigotes was demonstrated, with IC_{50} of 167.4 μg/mL, suggesting that it exhibits higher activity against this stage of the parasite than against the promastigote stage.

3.3. Cytotoxicity determination

The results of the assessments of ScEO and α-pinene cytotoxicity in murine macrophages and human erythrocytes are shown in Fig. 4 and Table 1. ScEO demonstrated minor cytotoxicity against macrophages and erythrocytes, with CC_{50} of 614.1 μg/mL and HC_{50} of 874.3 μg/mL, while α-pinene reduced the viability of both cells with CC_{50} of 425.2 μg/mL and HC_{50} of 233.6 μg/mL (Table 1). However, the safety of extracts was expressed as SI values, which are the ratios between the values of CC_{50} or HC_{50} and the IC_{50} of different models of anti-*Leishmania* activity. The SI represents the number of times that the effect of the substance is more selective for the parasite rather than to the mammalian cells. α-Pinene proved to be the safest for macrophages, with SI of 21.5, 26.4, and 27.2 for promastigotes, axenic amastigotes, and intramacrophage amastigotes, respectively, while ScEO treatments were associated with SI values of 10.2, 13.9, and 16.1 for the same parasite forms (Table 1). On the other hand, ScEO was found to be safer for human blood type O^+ erythrocytes, due to its low observed toxicity against these cells, with SI of 14.5, 19.9, and 22.9 against promastigotes, axenic amastigotes, and intramacrophage amastigotes, respectively. α-Pinene SI was determined to be 11.8, 14.5, and 14.9 in the same cells (Table 1). Glucantime® showed the highest toxicity of the tested substances (CC_{50} 412.9 μg/mL and HC_{50} 139.7 μg/mL), being more toxic to erythrocytes than to parasites (SI under 1) (Table 1).

3.4. Lysosomal activity and phagocytosis test

Activation parameters of macrophages and immunomodulation, such as the increase in lysosomal volume and phagocytosis, were assessed on the basis of the retention of neutral red and phagocytosis of stained zymosan by the macrophages. As shown in Fig. 5A, ScEO promoted a volume increase in the endocytic compartment at concentrations of 50, 100, and 200 μg/mL, while

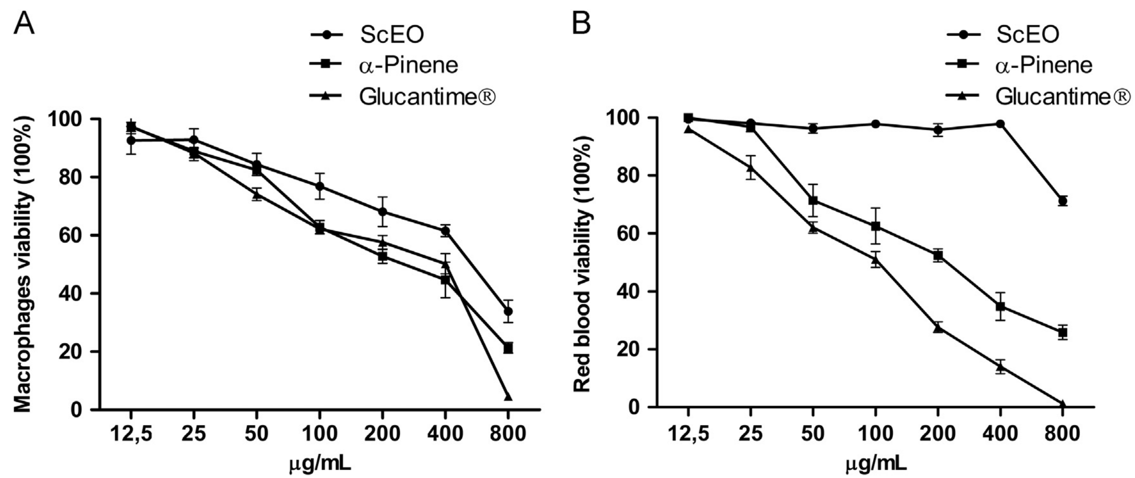

Fig. 4. Cytotoxic effects of *Syzygium cumini* essential oil (ScEO) and *α*-pinene against murine macrophages (A) and human erythrocytes (B). Murine macrophages were incubated for 48 h in the presence of different concentrations. Macrophage viability was measured by tetrazolium salt (MTT) assay. Hemolytic activity was assessed in a 5% suspension of human erythrocytes after 1 h of incubation. Data are expressed as means ± S.E.M. of three experiments performed in triplicate.

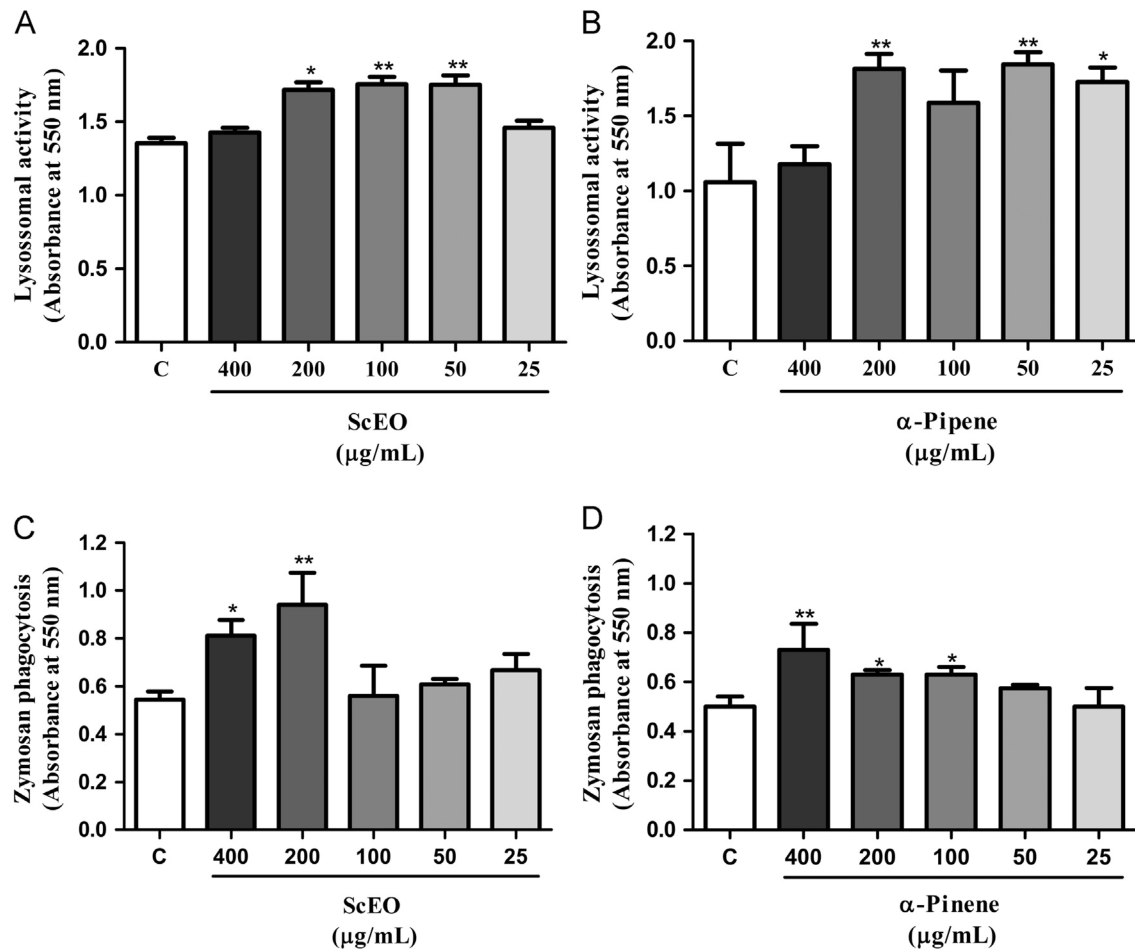

Fig. 5. The influence of *Syzygium cumini* essential oil (ScEO) and *α*-pinene on the lysosomal volume (A) and phagocytic activity (B). Murine macrophages were treated with a range of concentrations for 48 h. Lysosomal activity was analyzed spectrophotometrically by quantifying the increase in neutral red (NR) uptake following solubilization with the extraction solution. The phagocytosis was analyzed by incorporation of NR-stained zymosan, solubilized with the extraction solution. Data are presented as mean ± S.E.M. of three experiments carried out in triplicate. **P* < 0.05 *vs.* control; ***P* < 0.01 *vs.* control. C = control.

α-pinene significantly increased the retention of the neutral red in the secretory vesicles of macrophages at 25, 50, and 200 μg/mL concentrations (Fig. 5B). Similarly, the phagocytic activity of macrophages was augmented by a treatment with ScEO at 200 and 400 μg/mL concentrations (Fig. 5C), and by *α*-pinene at 100, 200, and 400 μg/mL concentrations (Fig. 5D).

3.5. Measurement of nitric oxide (NO) production

The production of nitric oxide (NO), an index of macrophage activation, was quantified by measuring the concentrations of nitrites made by macrophages incubated in the presence or absence of the promastigote forms of *Leishmania amazonensis*. Incubation with ScEO

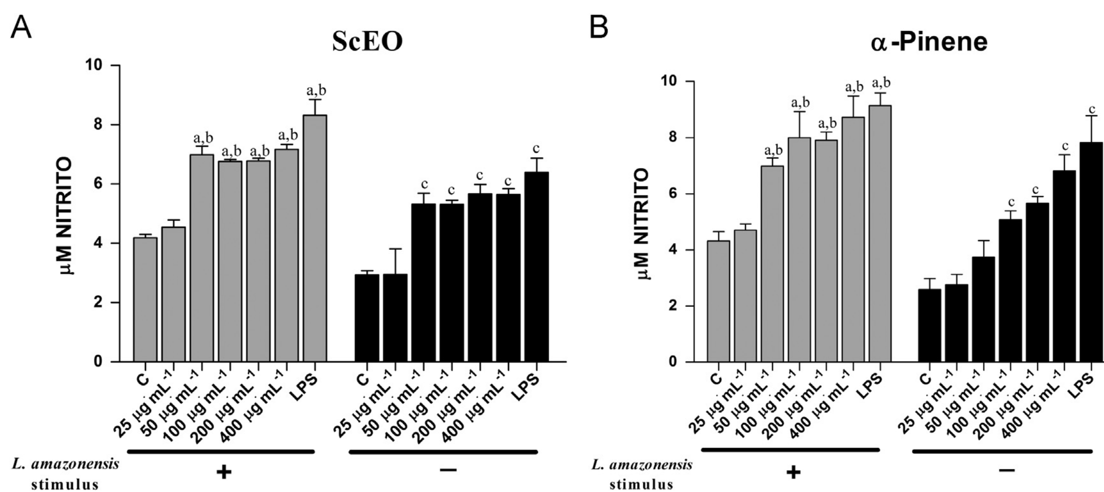

Fig. 6. Production of nitric oxide (NO) by non-stimulated and *Leishmania*-stimulated murine macrophages. Murine macrophages were treated with *Syzygium cumini* essential oil (ScEO) (A) and α-pinene (B) for 24 h. After this period, cell-free culture media were mixed with Griess reagent. LPS – bacterial lipopolysaccharide (2 μg/mL). Data are presented as mean ± S.E.M. of three experiments carried out in triplicate. $^{a}P<0.05$ *vs. Leishmania*-stimulated control; $^{b}P<0.05$ *vs. Leishmania*-unstimulated cells of the same group; $^{c}P<0.05$ *vs. Leishmania*-unstimulated control cells. C = control.

increased NO production at concentrations of 50 to 400 μg/mL not only in *Leishmania*-stimulated macrophages but also in macrophages incubated without *Leishmania* (Fig. 6A). Macrophages treated with α-pinene demonstrated an increase in the concentration of nitrites and, consequently, NO at concentrations between 50 and 400 μg/mL in stimulated macrophages and 100–400 μg/mL in non-stimulated macrophages (Fig. 6B). Bacterial lipopolysaccharide (LPS) was used as a positive control, showing high NO production (Fig. 5).

4. Discussion

In the elucidation of new treatments for leishmaniasis, research evaluating the active compounds present in vegetable species was found to be very promising. Among these compounds, a number of essential oils, complex mixtures of volatile substances, have presented with anti-*Leishmania* activity against different forms of the parasite through *in vitro* and *in vivo* tests. Studies with essential oils of *Ocimum gratissimum* L. (Ueda-Nakamura et al., 2006), *Copaifera cearensis* Huber ex Ducke (Santos et al., 2008), *Chenopodium ambrosioides* L. (Monzote et al., 2006), *Cymbopogon citratus* (D.C.) Stapf (Santin et al., 2009; Machado et al., 2012), and *Lippia sidoides* Cham. (Oliveira et al., 2009) demonstrated the potential of these substances in the search for new anti-*Leishmania* agents. We have previously reported that ScEO showed cytotoxic effects against the promastigote form of *Leishmania amazonensis* (Dias et al., 2013). Here, we report the activity of its major constituent, α-pinene, which was shown to be even more effective than the essential oil against promastigotes. Many major constituents extracted from other essential oils present better anti-*Leishmania* activity when isolated than when applied in the mixture. Thymol (De Medeiros et al., 2011) and linalool (Rosa et al., 2003) are illustrative examples of this difference in efficacy between formulations. The differences between the pharmacological activities of essential oils and their isolated compounds result from mutually antagonizing effects between the secondary compounds which may influence the reabsorption rate of reagents and therefore alter the bioavailability of active compounds (Bakkali et al., 2008).

Essential oils have some specifications regarding their mechanism of anti-*Leishmania* action. Due to the lipophilic character of the components, they easily pass through cytoplasmic membranes, interfering with their composition, comprising polysaccharides, fatty acids, and phospholipids, turning the membranes permeable and promoting cell lysis. In addition to the effect on the membrane, components of the essential oils interfere with the potential of the mitochondrial membranes, causing a release of free radicals that act on the DNA of the parasite, and could thereby lead to apoptosis or necrosis of the cells (Tariku et al., 2010, 2011). The drive towards minimizing the use of animals in laboratories, not only in evaluations of the pharmacologic action but also in studies evaluating the toxicity of novel compounds, has led towards the development of alternative *in vitro* methods that are to be used whenever possible. Experimental models using amastigotes of *Leishmania* are important, since these forms are responsible for the clinical manifestations of the leishmaniasis (Di Giorgio et al., 2005). Axenic amastigote cultures have been developed for use in studies with this stage of the parasite life cycle, facilitating the screening of novel drugs (De Muylder et al., 2011). Furthermore, models with the intracellular form provide the most efficient method for relating the *in vitro* activity of a drug with its effectiveness in the *in vitro* assay (Martinez-Rojano et al., 2008). *Leishmania* amastigote form presents higher sensitivity to the treatment with ScEO, α-pinene, and Glucantime® than the promastigote form. The difference in the anti-*Leishmania* activity may be related to the biochemical and metabolic differences between the two stages (Gupta et al., 2001). Moreover, the higher activity against intramacrophage amastigotes observed with ScEO and α-pinene treatments may indicate the activation of anti-*Leishmania* activities of the macrophages (Kolodziej and Kiderlen, 2005). To exert their microbicide activities, macrophages develop both structural (spreading, phagocytosis, vacuolization, and increased lysosomal volume) and cellular mechanisms (changes in the NO profile, altered cytokine levels, and matrix metalloproteinase secretion) (Bonatto et al., 2004; Bhattacharya et al., 2013). In the present work, we evaluated two structural mechanisms of anti-*Leishmania* activity (lysosomal and phagocytic activities), as well as the cellular mechanism of induction of the NO synthesis through the modulation of the state of activation of macrophages. Our results showed that ScEO and α-pinene increase both parameters, suggesting the involvement of these mechanisms in their anti-*Leishmania* activity. Phagocytosis and lysosomal activity are functions of the innate immunity, important for the control of infections and leading to the degradation of pathogens and presentation of antigens (Harrison et al., 2003; Niedergang and Chavrier, 2004). After pathogen endocytosis, the newly formed

phagosome undergoes fusion with endosomes, followed by fusion with lysosomes to produce a phagolysosome (Niedergang and Chavrier, 2004). The phagolysosome is a compartment filled with acid hydrolases and reactive oxygen species in which most of the degradation of the encompassed content occurs. Phagocytized pathogens are thus killed within the phagolysosome (Lee et al., 2003; Lopes et al., 2006). An important pathway that may be involved in the mechanism of anti-*Leishmania* activity could be the stimulation of NO production in the macrophages. NO was considered for a very long time to be the most effective mechanism involved in the defense against *Leishmania* (Gantt et al., 2001). NO is created following the activation of macrophages by cytokines such as interferon-γ (IFN-γ) and tumor necrosis factor α (TNF-α). Once inside the phagolysosome, NO combines with the superoxide anion to produce peroxynitrite, which is highly reactive and acts as a microbicide (Bogdan and Röllinghoff, 1998; Ueda-Nakamura et al., 2006). Aiming to investigate the safety of ScEO and α-pinene on the mammalian cells and evaluate the selectivity of these substances for the parasite, we evaluated the cytotoxicity of the substances in murine peritoneal macrophages and human erythrocytes. ScEO and α-pinene displayed a good safety profile with both cell types, as shown by the SI relative to the promastigotes, axenic amastigotes, and intramacrophage amastigotes. The high selectivity for the parasite justifies future studies evaluating the anti-*Leishmania* activity of ScEO and α-pinene through *in vivo* models to gain insight into other mechanisms of action that may be involved. In contrast to the evaluated plant extracts, Glucantime® was shown to have SI lower than 1, indicating higher toxicity to macrophages than to *Leishmania*. This result can be explained by the *in vivo* effects of the pentavalent antimonials. Even though this drug class is the first choice for the treatment of cutaneous and visceral leishmaniasis, these drugs cause high toxicity and several side effects (Misra et al., 2010).

5. Conclusions

Based on the results of this study, we can conclude that ScEO and α-pinene are biologically active against both stages of *Leishmania amazonensis*, while being safe for mammalian cells. This study also showed that the anti-*Leishmania* activity is mediated by immunomodulation, as evidenced by the stimulation of NO production and the observed increases in both phagocytic and lysosomal activities. Further work is warranted, involving more in-depth mechanistic studies and *in vivo* investigations.

Acknowledgments

The authors are grateful to Coordenação de Aperfeiçoamento de Pessoal de Nível Superior – CAPES and Conselho Nacional de Desenvolvimento Científico e Tecnológico – CNPq for financial support.

References

Abebe, T., Hailu, A., Woldeyes, M., Mekonen, W., Bilcha, K., Cloke, T., Fry, L., Seich, A.l., Basatena, N.K., Corware, K., Modolell, M., Munder, M., Tacchini-Cottier, F., Müller, I., Kropf, P., 2012. Local increase of arginase activity in lesions of patients with cutaneous leishmaniasis in Ethiopia. PLoS Neglected Tropical Diseases 6, e1684. http://dx.doi.org/10.1371/journal.pntd.0001684.

Angel, G.R., Menon, N., Vimala, B., Nambisan, B., 2014. Essential oil composition of eight starchy *Curcuma* species. Industrial Crops and Products 60, 233–238. http://dx.doi.org/10.1016/j.indcrop.2014.06.028.

Ayyanar, M., Subash-Babu, P., 2012. *Syzygium cumini* (L.) Skeels: a review of its phytochemical constituents and traditional uses. Asian Pacific Journal of Tropical Biomedicine 2, 240–246. http://dx.doi.org/10.1016/S2221-1691(12)60050-1.

Ayyanar, M., Subash-Babu, P., Ignacimuthu, S., 2013. *Syzygium cumini* (L.) Skeels., a novel therapeutic agent for diabetes: folk medicinal and pharmacological evidences. Complementary Therapies in Medicine 21, 232–243. http://dx.doi.org/10.1016/j.ctim.2013.03.004.

Bag, A., Bhattacharyya, S.K., Pal, N.K., Chattopadhyay, R.R., 2012. In vitro antibacterial potential of *Eugenia jambolana* seed extracts against multidrug-resistant human bacterial pathogens. Microbiological Research 167, 352–357. http://dx.doi.org/10.1016/j.micres.2012.02.005.

Bakkali, F., Averbeck, S., Averbeck, D., Idaomar, M., 2008. Biological effects of essential oils – a review. Food and Chemical Toxicology 46, 446–475. http://dx.doi.org/10.1016/j.fct.2007.09.106.

Baliga, M.S., Bhat, H.P., Baliga, B.R.V., Wilson, R., Palatty, P.L., 2011. Phytochemistry, traditional uses and pharmacology of *Eugenia jambolana* Lam. (black plum): a review. Food Research International 44, 1776–1789. http://dx.doi.org/10.1016/j.foodres.2011.02.007.

Bhattacharya, S., Ghosh, P., De, T., Gomes, A., Gomes, A., Dungdung, S.R., 2013. In vivo and in vitro antileishmanial activity of *Bungarus caeruleus* snake venom through alteration of immunomodulatory activity. Experimental Parasitology 135, 126–133. http://dx.doi.org/10.1016/j.exppara.2013.06.006.

Bogdan, C., Röllinghoff, M., 1998. The immune response to *Leishmania*: mechanisms of parasite control and evasion. International Journal for Parasitology 28, 121–134. http://dx.doi.org/10.1016/S0020-7519(97)00169-0.

Bonatto, S.J.R., Folador, A., Aikawa, J., Yamazaki, R.K., Pizatto, N., Oliveira, H.H.P., Vecchi, R., Curi, R., Calder, P.C., Fernandes, L.C., 2004. Lifelong exposure to dietary fish oil alters macrophage responses in Walker 256 tumor-bearing rats. Cellular Immunology 231, 56–62. http://dx.doi.org/10.1016/j.cellimm.2004.12.001.

Brito, F.A., Lima, L.A., Ramos, M.F.S., Nakamura, M.J., Cavalher-Machado, S.C., Siani, A.C., Henriques, M.G.M.O., Sampaio, A.L.F., 2007. Pharmacological study of anti-allergic activity of *Syzygium cumini* (L.) Skeels. Brazilian Journal of Medical and Biological Research 40, 105–115. http://dx.doi.org/10.1590/S0100-879 × 2007000100014.

Carneiro, S.M.P., Carvalho, F.A.A., Santana, L.C.L.R., Sousa, A.P.L., Neto, J.M.M., Chaves, M.H., 2012. The cytotoxic and antileishmanial activity of extracts and fractions of leaves and fruits of *Azadirachta indica* (A Juss.). Biological Research 45, 111–116. http://dx.doi.org/10.4067/S0716-97602012000200002.

Chandrasekaran, M., Venkatesalu, V., 2004. Antibacterial and antifungal activity of *Syzygium jambolanum* seeds. Journal of Ethnopharmacology 91, 105–108. http://dx.doi.org/10.1016/j.jep.2003.12.012.

Chappuis, F., Sundar, S., Hailu, A., Ghalib, H., Rijal, S., Peeling, R.W., Alvar, J., Boelaert, M., 2007. Visceral leishmaniasis: what are the needs for diagnosis, treatment and control? Nature Reviews Microbiology 5, 873–882. http://dx.doi.org/10.1038/nrmicro1748.

Croft, S.L., Coombs, G.H., 2003. Leishmaniasis– current chemotherapy and recent advances in the search for novel drugs. Trends in Parasitology 19, 502–508. http://dx.doi.org/10.1016/j.pt.2003.09.008.

David, C.V., Craft, N., 2009. Cutaneous and mucocutaneous leishmaniasis. Dermatologic Therapy 22, 491–502. http://dx.doi.org/10.1111/j.1529-8019.2009.01272.x.

De Almeida, M.C., Vilhena, V., Barral, A., Barral-Netto, M., 2003. Leishmanial infection: analysis of its first steps. A review. Memórias do Instituto Oswaldo Cruz 98, 861–870.

De Medeiros, M., das, G.F., da Silva, A.C., Citó, A.M., das, G.L., Borges, A.R., de Lima, S.G., Lopes, J.A.D., Figueiredo, R.C.B.Q., 2011. In vitro antileishmanial activity and cytotoxicity of essential oil from *Lippia sidoides* Cham. Parasitology International 60, 237–241. http://dx.doi.org/10.1016/j.parint.2011.03.004.

De Muylder, G., Ang, K.K.H., Chen, S., Arkin, M.R., Engel, J.C., McKerrow, J.H., 2011. A screen against *Leishmania* intracellular amastigotes: comparison to a promastigote screen and identification of a host cell-specific hit. PLoS Neglected Tropical Diseases 5, e1253. http://dx.doi.org/10.1371/journal.pntd.0001253.

Di Giorgio, C., Delmas, F., Akhmedjanova, V., Ollivier, E., Bessonova, I., Riad, E., Timon-David, P., 2005. In vitro antileishmanial activity of diphyllin isolated from *Haplophyllum bucharicum*. Planta Medica 71, 366–369. http://dx.doi.org/10.1055/s-2005-864106.

Dias, C.N., Rodrigues, K.A.F., Carvalho, F.A.A., Carneiro, S.M.P., Maia, J.G.S., Andrade, E.H.a., Moraes, D.F.C., 2013. Molluscicidal and leishmanicidal activity of the leaf essential oil of *Syzygium cumini* (L.) Skeels from Brazil. Chemistry & Biodiversity 10, 1133–1141. http://dx.doi.org/10.1002/cbdv.201200292.

Dos Santos, A.O., Costa, M.A., Ueda-Nakamura, T., Dias-Filho, B.P., da Veiga-Júnior, V.F., de Souza Lima, M.M., Nakamura, C.V., 2011. *Leishmania amazonensis*: effects of oral treatment with copaiba oil in mice. Experimental Parasitology 129, 145–151. http://dx.doi.org/10.1016/j.exppara.2011.06.016.

Dos Santos, K.K.A., Matias, E.F.F., Tintino, S.R., Souza, C.E.S., Braga, M.F.B.M., Guedes, G.M.M., Rolón, M., Vega, C., de Arias, A.R., Costa, J.G.M., Menezes, I.A., Coutinho, H.D.M., 2012. Cytotoxic, trypanocidal, and antifungal activities of Eugenia jambolana L. Journal of Medicinal Food 15, 66–70. http://dx.doi.org/10.1089/jmf.2010.0298.

Elangovan, V., Shohami, E., Gati, I., Kohen, R., 2000. Increased hepatic lipid soluble antioxidant capacity as compared to other organs of streptozotocin-induced diabetic rats: a cyclic voltammetry study. Free Radical Research 32, 125–134. http://dx.doi.org/10.1080/10715760000300131.

Faria, A.F., Marques, M.C., Mercadante, A.Z., 2011. Identification of bioactive compounds from jambolão (*Syzygium cumini*) and antioxidant capacity evaluation in different pH conditions. Food Chemistry 126, 1571–1578. http://dx.doi.org/10.1016/j.foodchem.2010.12.007.

Feasey, N., Wansbrough-Jones, M., Mabey, D.C.W., Solomon, A.W., 2010. Neglected tropical diseases. British Medical Bulletin 93, 179–200. http://dx.doi.org/10.1093/bmb/ldp046.

Gantt, K.R., Goldman, T.L., McCormick, M.L., Miller, M.A., Jeronimo, S.M.B., Nascimento, E.T., Britigan, B.E., Wilson, M.E., 2001. Oxidative responses of human and murine

macrophages during phagocytosis of *Leishmania chagasi*. The Journal of Immunology 167, 893–901. http://dx.doi.org/10.4049/jimmunol.167.2.893.

Grando, F.C.C., Felício, C.A., Twardowschy, A., Paula, F.M., Batista, V.G., Fernandes, L.C., Curi, R., Nishiyama, A., 2009. Modulation of peritoneal macrophage activity by the saturation state of the fatty acid moiety of phosphatidylcholine. Brazilian Journal of Medical and Biological Research 42, 599–605. http://dx.doi.org/10.1590/S0100-879 × 2009005000003.

Gupta, N., Goyal, N., Rastogi, A.K., 2001. In vitro cultivation and characterization of axenic amastigotes of *Leishmania*. Trends in Parasitology 17, 150–153. http://dx.doi.org/10.1016/S1471-4922(00)01811-0.

Harrison, R.E., Bucci, C., Vieira, O.V., Schroer, T.A., Grinstein, S., 2003. Phagosomes fuse with late endosomes and/or lysosomes by extension of membrane protrusions along microtubules: role of Rab7 and RILP. Molecular and Cellular Biology 23, 6494–6506. http://dx.doi.org/10.1128/MCB.23.18.6494-6506.2003.

Kolodziej, H., Kiderlen, A.F., 2005. Antileishmanial activity and immune modulatory effects of tannins and related compounds on *Leishmania* parasitised RAW 264.7 cells. Phytochemistry 66, 2056–2071. http://dx.doi.org/10.1016/j.phytochem.2005.01.011.

Lee, W.L., Harrison, R.E., Grinstein, S., 2003. Phagocytosis by neutrophils. Microbes and Infection 5, 1299–1306. http://dx.doi.org/10.1016/j.micinf.2003.09.014.

Löfgren, S.E., Miletti, L.C., Steindel, M., Bachère, E., Barracco, M.A., 2008. Trypanocidal and leishmanicidal activities of different antimicrobial peptides (AMPs) isolated from aquatic animals. Experimental Parasitology 118, 197–202. http://dx.doi.org/10.1016/j.exppara.2007.07.011.

Lopes, L., Godoy, L.M.F., de Oliveira, C.C., Gabardo, J., Schadeck, R.J.G., de Freitas Buchi, D., 2006. Phagocytosis, endosomal/lysosomal system and other cellular-aspects of macrophage activation by *Canova medication*. Micron 37, 277–287. http://dx.doi.org/10.1016/j.micron.2005.08.005.

Machado, M., Pires, P., Dinis, A.M., Santos-Rosa, M., Alves, V., Salgueiro, L., Cavaleiro, C., Sousa, M.C., 2012. Monoterpenic aldehydes as potential anti-*Leishmania* agents: activity of *Cymbopogon citratus* and citral on *L. infantum*, *L. tropica* and *L. major*. Experimental Parasitology 130, 223–231. http://dx.doi.org/10.1016/j.exppara.2011.12.012.

Maciel, M.C.G., Farias, J.C., Maluf, M.J., Gomes, E.A., Pereira, P.V.S., Aragão-Filho, W.C., Nascimento, F.R.F., 2008. *Syzygium jambolanum* treatment improves survival in lethal sepsis induced in mice. BMC Complementary and Alternative Medicine 8, 57. http://dx.doi.org/10.1186/1472-6882-8-57.

Martinez-Rojano, H., Mancilla-Ramirez, J., Quiñonez-Diaz, L., Galindo-Sevilla, N., 2008. Activity of hydroxyurea against *Leishmania mexicana*. Antimicrobial Agents and Chemotherapy 52, 3642–3657. http://dx.doi.org/10.1128/AAC.00124-08.

Mir, Q.Y., Ali, M., Alam, P., 2009. Lignan derivatives from the stem bark of *Syzygium cumini* (L.) Skeels. Natural Product Research 23, 422–430. http://dx.doi.org/10.1080/14786410802038697.

Misra, P., Sashidhara, K.V., Singh, S.P., Kumar, A., Gupta, R., Chaudhaery, S.S., Gupta, S., Sen, Majumder, H.K., Saxena, A.K., Dube, A., 2010. 16alpha-Hydroxycleroda-3,13 (14)Z-dien-15,16-olide from *Polyalthia longifolia*: a safe and orally active antileishmanial agent. British Journal of Pharmacology 159, 1143–1150. http://dx.doi.org/10.1111/j.1476-5381.2009.00609.x.

Monte Neto, R.L., Sousa, L.M.A., Dias, C.S., Barbosa Filho, J.M., Oliveira, M.R., Figueiredo, R.C.B.Q., 2011. Morphological and physiological changes in *Leishmania* promastigotes induced by yangambin, a lignan obtained from *Ocotea duckei*. Experimental Parasitology 127, 215–221. http://dx.doi.org/10.1016/j.exppara.2010.07.020.

Monzote, L., Montalvo, A.M., Almanonni, S., Scull, R., Miranda, M., Abreu, J., 2006. Activity of the essential oil from *Chenopodium ambrosioides* grown in Cuba against *Leishmania amazonensis*. Chemotherapy 52, 130–136. http://dx.doi.org/10.1159/000092858.

Muruganandan, S., Srinivasan, K., Chandra, S., Tandan, S.K., Lal, J., Raviprakash, V., 2001. Anti-inflammatory activity of *Syzygium cumini* bark. Fitoterapia 72, 369–375.

Niedergang, F., Chavrier, P., 2004. Signaling and membrane dynamics during phagocytosis: many roads lead to the phagoslome. Current Opinion in Cell Biology 16, 422–428. http://dx.doi.org/10.1016/j.ceb.2004.06.006.

Oliveira, A.C.P., Endringer, D.C., Amorim, L.A.S., das Graças, L., Brandão, M., Coelho, M.M., 2005. Effect of the extracts and fractions of *Baccharis trimera* and *Syzygium cumini* on glycaemia of diabetic and non-diabetic mice. Journal of Ethnopharmacology 102, 465–469. http://dx.doi.org/10.1016/j.jep.2005.06.025.

Oliveira, V.C.S., Moura, D.M.S., Lopes, J.A.D., de Andrade, P.P., da Silva, N.H., Figueiredo, R.C.B.Q., 2009. Effects of essential oils from *Cymbopogon citratus* (DC) Stapf., *Lippia sidoides* Cham., and *Ocimum gratissimum* L. on growth and ultrastructure of *Leishmania chagasi* promastigotes. Parasitology Research 104, 1053–1059. http://dx.doi.org/10.1007/s00436-008-1288-6.

Ravi, K., Rajasekaran, S., Subramanian, S., 2005. Antihyperlipidemic effect of *Eugenia jambolana* seed kernel on streptozotocin-induced diabetes in rats. Food and Chemical Toxicology 43, 1433–1439. http://dx.doi.org/10.1016/j.fct.2005.04.004.

Rosa, M.D.S.S., Mendonca-Filho, R.R., Bizzo, H.R., Rodrigues, I.D.A., Soares, R.M.A., Souto-Padron, T., Alviano, C.S., Lopes, A.H.C.S., 2003. Antileishmanial activity of a linalool-rich essential oil from *Croton cajucara*. Antimicrobial Agents and Chemotherapy 47, 1895–1901. http://dx.doi.org/10.1128/AAC.47.6.1895-1901.2003.

Santin, M.R., dos Santos, A.O., Nakamura, C.V., Dias Filho, B.P., Ferreira, I.C.P., Ueda-Nakamura, T., 2009. In vitro activity of the essential oil of *Cymbopogon citratus* and its major component (citral) on *Leishmania amazonensis*. Parasitology Research 105, 1489–1496. http://dx.doi.org/10.1007/s00436-009-1578-7.

Santos, A.O., Ueda-Nakamura, T., Dias Filho, B.P., Veiga Junior, V.F., Pinto, A.C., Nakamura, C.V., 2008. Effect of Brazilian copaiba oils on *Leishmania amazonensis*. Journal of Ethnopharmacology 120, 204–208. http://dx.doi.org/10.1016/j.jep.2008.08.007.

Schossler, D.R.C., Mazzanti, C.M., Da Luz, S.C.A., Filappi, A., Prestes, D., Silveira, A.F., Cecim, M., 2004. *Syzygium cumini* and the regeneration of insulin positive cells from the pancreatic duct. Brazilian Journal of Veterinary Research and Animal Science 41, 236–239. http://dx.doi.org/10.1590/S1413-95962004000400003.

Shukla, A.K., Patra, S., Dubey, V.K., 2011. Deciphering molecular mechanism underlying antileishmanial activity of *Nyctanthes arbortristis*, an Indian medicinal plant. Journal of Ethnopharmacology 134, 996–998. http://dx.doi.org/10.1016/j.jep.2011.01.044.

Sood, R., Swarup, D., Bhatia, S., Kulkarni, D.D., Dey, S., Saini, M., Dubey, S.C., 2012. Antiviral activity of crude extracts of *Eugenia jambolana* Lam. against highly pathogenic avian influenza (H5N1) virus. Indian Journal of Experimental Biology 50, 179–186.

Srivastava, S., Chandra, D., 2013. Pharmacological potentials of *Syzygium cumini*: a review. Journal of the Science of Food and Agriculture 93, 2084–2093. http://dx.doi.org/10.1002/jsfa.6111.

Tariku, Y., Hymete, A., Hailu, A., Rohloff, J., 2010. Essential-oil composition, antileishmanial, and toxicity study of *Artemisia abyssinica* and *Satureja punctata* ssp. punctata from Ethiopia. Chemistry & Biodiversity 7, 1009–1018. http://dx.doi.org/10.1002/cbdv.200900375.

Tariku, Y., Hymete, A., Hailu, A., Rohloff, J., 2011. In vitro evaluation of antileishmanial activity and toxicity of essential oils of *Artemisia absinthium* and *Echinops kebericho*. Chemistry & Biodiversity 8, 614–623. http://dx.doi.org/10.1002/cbdv.201000331.

Tiuman, T.S., Santos, A.O., Ueda-Nakamura, T., Filho, B.P.D., Nakamura, C.V., 2011. Recent advances in leishmaniasis treatment. International Journal of Infectious Diseases 15, e525–e532. http://dx.doi.org/10.1016/j.ijid.2011.03.021.

Trojan-Rodrigues, M., Alves, T.L.S., Soares, G.L.G., Ritter, M.R., 2012. Plants used as antidiabetics in popular medicine in Rio Grande do Sul, southern Brazil. Journal of Ethnopharmacology 139, 155–163. http://dx.doi.org/10.1016/j.jep.2011.10.034.

Ueda-Nakamura, T., Mendonça-Filho, R.R., Morgado-Díaz, J.A., Korehisa Maza, P., Prado Dias Filho, B., Aparício Garcia Cortez, D., Alviano, D.S., Rosa, M., do, S.S., Lopes, A.H.C.S., Alviano, C.S., Nakamura, C.V., 2006. Antileishmanial activity of eugenol-rich essential oil from *Ocimum gratissimum*. Parasitology International 55, 99–105. http://dx.doi.org/10.1016/j.parint.2005.10.006.

Valadares, D.G., Duarte, M.C., Oliveira, J.S., Chávez-Fumagalli, M.A., Martins, V.T., Costa, L.E., Leite, J.P.V., Santoro, M.M., Régis, W.C.B., Tavares, C.A.P., Coelho, E.A.F., 2011. Leishmanicidal activity of the *Agaricus blazei* Murill in different *Leishmania* species. Parasitology International 60, 357–363. http://dx.doi.org/10.1016/j.parint.2011.06.001.

Veigas, J., Narayan, M., Laxman, P., Neelwarne, B., 2007. Chemical nature, stability and bioefficacies of anthocyanins from fruit peel of *Syzygium cumini* Skeels. Food Chemistry 105, 619–627. http://dx.doi.org/10.1016/j.foodchem.2007.04.022.

WHO 2014. Leishmaniasis. Available at: 〈http://www.who.int/mediacentre/factsheets/fs375/en/〉. (accessed 20.08.14.).