Anais da Academia Brasileira de Ciências (2016) 88(3 Suppl.): 1851-1861
(Annals of the Brazilian Academy of Sciences)
Printed version ISSN 0001-3765 / Online version ISSN 1678-2690
http://dx.doi.org/10.1590/0001-3765201620150131
www.scielo.br/aabc

Inhibitory effects of *Zanthoxylum rhoifolium* Lam. (Rutaceae) against the infection and infectivity of macrophages by *Leishmania amazonensis*

BERNARDO MELO NETO[1], JOSEANA M.S.R. LEITÃO[1], LUCIANO G.C. OLIVEIRA[1], SÉRGIO E.M. SANTOS[1], SABRINA M.P. CARNEIRO[1], KLINGER A.F. RODRIGUES[1], MARIANA H. CHAVES[2], DANIEL D.R. ARCANJO[1,3] and FERNANDO A.A. CARVALHO[1,4]

[1]Núcleo de Pesquisas em Plantas Medicinais, Universidade Federal do Piauí, Campus Ministro Petrônio Portella, SG-15, Ininga, 64049-550 Teresina, PI, Brasil
[2]Departamento de Química, Universidade Federal do Piauí, Campus Ministro Petrônio Portella, SG-02, Ininga, 64049-550 Teresina, PI, Brasil
[3]Departamento de Biofísica e Fisiologia, Universidade Federal do Piauí, Campus Ministro Petrônio Portella, SG-08/09, Ininga, 64049-550 Teresina, PI, Brasil
[4]Departamento de Bioquímica e Farmacologia, Universidade Federal do Piauí, Campus Ministro Petrônio Portella, SG-08, Ininga, 64049-550 Teresina, PI, Brasil

Manuscript received on February 22, 2015; accepted for publication on March 1, 2016

ABSTRACT

Zanthoxylum rhoifolium Lam. (Rutaceae) has been traditionally used in the treatment of microbial infections and parasitic diseases. In the present study, the antileishmanial effect induced by the ethanol extract of stem barks from *Z. rhoifolium* (ZR-EEtOH) and its n-hexane fraction (ZR-FHEX) on infection and infectivity of murine macrophages by promastigote forms of *Leishmania amazonensis* were investigated. In different set of experiments, macrophages or promastigotes were pretreated with ZR-EEtOH or ZR-FHEX at non-lethal concentrations for 24 hours, and then macrophages were submitted to infection by promastigotes. Moreover, their effects on activation of macrophages, as well as on the DNA content, size and number of promastigotes by flow cytometry were also evaluated. The infection rate and the number of internalized amastigote forms were markedly decreased after pretreatment of macrophages or promastigotes when compared with non-treated cells. The increase in phagocytic capability and nitrite content was also observed. Furthermore, the decrease of DNA content, size and number of promastigotes was also observed. In conclusion, ZR-EEtOH and ZR-FHEX promoted a markedly significant antileishmanial effect and reduction of infection of macrophages, probably underlying defense mechanisms activation in macrophages. These findings reinforce the potential application of *Z. rhoifolium* in the treatment of leishmaniasis.

Key words: *Leishmania,* Macrophages*,* Nitric oxide, Promastigote, Phagocytic activity, *Zanthoxylum.*

INTRODUCTION

The leishmaniasis is a complex of diseases caused by digenetic protozoa from *Leishmania* genus which affects around two million people per year and is also considered an endemic disease in 88 countries. Furthermore, around 350 million of people are under the risk throughout the world (Monzote et al. 2007, World Health Organization 2010). The treatment of this disease is based on drugs which possess a sort of drawbacks, as follows: only parenteral administration is possible,

Correspondence to: Daniel Dias Rufino Arcanjo
E-mail: daniel.arcanjo@ufpi.edu.br

higher toxicity, high costs, long-lasting therapy and therapeutic spectrum restricted to only specific clinical forms of the disease (Chappuis et al. 2007, Lindoso et al. 2012).Traditionally, medicinal plants have been widely used in traditional medical practices to treat parasitic diseases and currently have received special attention concerning the search for new therapies against leishmaniasis. Therefore, several plants and their respective isolated compounds, such as chalcones, lignans, alkaloids, flavonoids, terpenes, saponins and quinones, have been studied for the treatment of leishmaniasis and have shown to be promising antileishmanial agents (Kayser et al. 2001, Sen and Chatterjee 2011, Singh et al. 2014).

Zanthoxylum rhoifolium Lam. (Rutaceae) is a widespread plant that occurs in South America, especially in Brazilian rainforest, in the states of Minas Gerais, Rio de Janeiro, Piauí and Ceará (Freitas et al. 2011, Gonzaga et al. 2003, Pereira et al. 2010). This species is traditionally used in the treatment and prevention of malaria. In Bolivia, the bark and leaves of *Z. rhoifolium* are used as antipyretic. In Peru, it is used due to its digestive and tonic properties. Besides, *Z. rhoifolium* is popularly used in Brazil for treatment of inflammation, microbial infection, cancer and malaria (Jullian et al. 2006, Da Silva et al. 2007). Furthermore, a sort of secondary metabolites, mainly lignans, alkaloids, terpenoids and flavonoids, have been found in this species. Likewise, studies have shown that these alkaloids exhibit antimicrobial properties (Gonzaga et al. 2003, Tavares et al. 2014).

Previous reports have demonstrated antinociceptive (Pereira et al. 2010), gastroprotective (Freitas et al. 2011) and antihypertensive effects (Ferreira-Filho et al. 2013) for *Z. rhoifolium* in different rodents experimental models. Interestingly, a preliminary study reports the leishmanicidal effect for *Z. rhoifolium* against promastigote forms of *Leishmania amazonensis* (Moura-Costa et al. 2012). However, its effects on the ability of *L. amazonensis* to infect macrophages as well as its effects against internalized amastigote forms of *L. amazonensis* remain still unknown. Therefore, the antileishmanial effects of *Z. rhoifolium* against infection and infectivity of macrophages by *L. amazonensis*, as well as the possible underlying mechanisms, were evaluated in this study.

MATERIALS AND METHODS

Botanical Source, Extracts and Fractioning

Stem barks from *Z. rhoifolium* Lam. (Rutaceae) were collected in January 2005 at Pedro II city, Piauí state, Brazil. The specimens were trees around 10 m of height and their stems were around 29.5 cm of diameter. The pluviometric index was around 1150.2 mm and the predominant soil was dystrophic red-yellow latosol (oxisol). The botanical identification was performed by Profa. Dra. Roseli Farias Melo de Barros. The voucher specimen (TEPB 13870) was deposited at Graziela Barroso Herbarium of Federal University of Piauí (UFPI). Stem barks of *Z. rhoifolium* (1.0 kg) were dried, powdered and then extracted exhaustively at room temperature with ethanol. The solvent was removed by rotaevaporation, yielding the ethanol extract (ZR-EEtOH 85.0 g; 8.5%, w/w). Then, the ZR-EEtOH (50 g) was fractioned in n-hexane, yielding ZR-FHEX (7.0 g; 14.0%, w/w). Both ZR-EEtOH and ZR-FHEX were lyophilized. For the experimental protocols, they were dissolved in dimethylsulfoxide (DMSO) to a maximum concentration of 0.5%.

From ZR-FHEX, the pentacyclic triterpenoid lupeol was isolated by fractionation in a silica gel column, with gradient elution from n-hexane-ethyl acetate (95:5), providing a solid material which was crystallized with methanol and indentified as lupeol (1.1 g; 12.5%, w/w) by determination of its structure by NMR analysis (Pereira et al. 2010).

Animals

Male or female Swiss mice (20-25 g, 6 weeks) were used for obtaining peritoneal macrophages.

All procedures involving animal experimentation were according to the Brazilian Law no. 1,000 of 2012, and previously approved by the Animal Experimentation Ethical Committee from the Federal University of Piauí, Brazil (0022/2010).

Parasites

The *Leishmania amazonensis* strain (IFLA/BR/67/PH8) was maintained in biochemical oxygen demand (BOD) at 26 °C and cultivated in a Schneider's medium (Sigma Chemical, USA) supplemented with 10% inactivated fetal calf serum-FCS (Sigma Chemical, USA), penicillin (100 U/mL) and streptomycin (100 µg/mL).

Evaluation of *Z. rhoifolium* Against *L. amazonensis* Promastigotes

Promastigote forms of *L. amazonensis* (1×10^6/well) were plated in 96-well microplates (TPP, Switzerland) containing Schneider's medium. Then, ZR-EEtOH or ZR-FHEX (400 to 3.13 µg/mL) was added in triplicate. Schneider's medium with 0.5% DMSO was used as the negative control group. After 24, 48 and 72 hours of incubation at 26 °C, the number of viable promastigotes from each well was counted in a Neubauer chamber (Carneiro et al. 2012, Rodrigues et al. 2013).

Assessment of Cytotoxic Activity of *Z. rhoifolium*

Thioglycollate (3%; 1.5 mL) was administered in the peritoneal cavity of Swiss mice. After 72 hours, the macrophages were elicited by washing the abdominal cavity with 8 mL of sterile phosphate buffered saline (PBS) at pH 7.4 and 4 °C. The aspirate was centrifuged at 4 °C and 184 G for 10 minutes, resuspended in RPMI 1640 medium and the cells plated in 24-well plates (1×10^5 cells/500 µL of RPMI 1640 medium). Afterwards, the medium was replaced by RPMI 1640 supplemented with 10% FCS, penicillin and streptomycin, and then ZR-EEtOH or ZR-FHEX (100 to 3.12 µg/mL) were incubated for 48 hours at 5% CO_2 and 37 ºC. The cell viability was based on capability of cells to reduce MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazoliumbromide) to formazan. After 4 h, the absorbances were readed at 550 nm (Reilly et al. 1998, Carneiro et al. 2012).

Effect of *Z. rhoifolium* on Infection by *L. amazonensis* Promastigotes after Pretreatment of Macrophages

Macrophages were cultured as described above. After cell adhesion, the medium was replaced by RPMI 1640 supplemented with 10% FCS, penicillin and streptomycin. Then, ZR-EEtOH (354, 88.6, and 22.1 µg/mL) or ZR-FHEX (80, 20, and 5 µg/mL) was added. After 30 minutes, the medium was replaced again and then 2×10^6 infective promastigotes were added per well. After 3 hours, the coverslips were removed and stained with Giemsa. For each coverslip, 100 cells were assessed, and the numbers of infected macrophages and internalized amastigotes per macrophage were counted as described by Soares et al. (2007). Three independent experiments were carried out in triplicate for each concentration.

Effect of *Z. rhoifolium* on the Infective Capability of *L. amazonensis* promastigotes

The macrophages were elicited as described above, and then plated at 2×10^5 per well in sterile 96-well plates with RPMI 1640 containing sterile 13 mm round coverslips. After cell adhesion, the medium was replaced by RPMI 1640 supplemented with 10% FCS, penicillin and streptomycin. Then, promastigotes were incubated at 26 ºC with ZR-EEtOH or ZR-FHEX at their respective 24-h half mean inhibitory concentrations (1/2 IC_{50}) for 30 minutes. Then, the promastigotes were centrifuged (367 G, 4 °C) for 10 minutes, resuspended in RPMI 1640 and plated at ratio of 10 promastigotes per macrophage. After incubation at 37 °C and 5% CO_2 for 3 hours, the coverslips were removed and stained with Giemsa. For each coverslip, 100 cells were assessed, and the numbers of infected macrophages

and internalized amastigotes per macrophage were counted as described by Soares et al. (2007). Three independent experiments were carried out in triplicate for each concentration. Association Indexes (AI) were obtained by the mean number of internalized amastigotes multiplied by the percentage of infected macrophages (Rosa et al. 2003).

Determination of Phagocytic Capability and Lysosomal Volume

Macrophages were obtained and plated (2×10^5 per well) as previously described. After cell adhesion, the medium was replaced by RPMI 1640 supplemented with 10% FCS, penicillin and streptomycin. Then, ZR-EEtOH or ZR-FHEX (100 to 3.12 µg/mL) were incubated for 48 hours at 5% CO_2, 37 ºC. For determination of phagocytic capability, 10 µL of stained zymosan solution (neutral red solution 0.3 mL and zymosan not opsonized 0.02 g in PBS 3 mL) was used. Besides, for determination of lysossomal volume, 10 µL of 2.0% neutral red solution in DMSO was used. Both solutions were separately incubated for 30 min at 5% CO_2, 37 °C. The supernatants were discarded and 100 mL of an extractive aqueous solution composed by 96% glacial acetic acid (1%, v/v) and ethanol (50%, v/v) was added for 30 minutes. The absorbances were readed at 550 nm (Bonatto et al. 2004).

Determination of Nitrite Content

Macrophages were obtained and plated (2×10^5 per well) in RPMI 1640 medium with ZR-EEtOH or ZR-FHEX (100 to 3.12 µg/mL) for 30 minutes (5% CO_2, 37 ºC). Then, the medium was replaced by RPMI 1640 containing promastigotes in stationary growth phase at ratio of 10 promastigotes per macrophage (10:1) and incubated for 24 hours (5% CO_2, 37 °C). Afterwards, the nitrite content was measured in the supernatant after addition of Griess' Reagent (1:1) to the medium. The absorbances were measured at a 550 nm, and the concentrations of nitrite was calculated from a $NaNO_2$ (1.0 – 150 µM) standard curve (Genestra et al. 2003).

Extraction, Purification and Quantification of DNA from *L. amazonensis* after Pretreatment with *Z. rhoifolium*

Promastigote forms of *L. amazonensis* at logarithmic growth phase (1×10^5) were pretreated with ZR-EEtOH or ZR-FHEX at concentrations of 7.96, 15.92 and 31.84 µg/mL for 72h. Afterwards, the DNA from parasites was extracted by using the QIAamp® DNA MiniKit (QIAGEN, Venlo, Netherlands). Briefly, the parasites were centrifuged at 184 G, the pellets were resuspended with 200 µL of PBS, 20 µL of proteinase K, 4 µL of RNAse and 200 µL of AL Buffer were added and incubated at 56 ºC for 30 min. Then, 200 µL of ethanol (96%) was added, the samples were transfered to a 2.0 mL column and centrifugated (6.000 G for 1 minute). Afterwards, a sequence of buffers was sequentially added and followed by centrifugation, as follows: 500 µL of AW1 buffer, 6,000 G for 1 minute; 500 µL of AW2 buffer, 20,000 G for 4 minutes; and 500 µL of AE buffer, 6.000 G for 1 minute (manufacturer's protocol). Then, the DNA samples were quantified by a NanoDrop 2000 UV-Vis spectrophotometer (Thermo Scientific, Wilmington, DE, USA).

Moreover, the DNA samples from *L. amazonensis* pretreated with ZR-EEtOH or ZR-FHEX were also submitted to enzymatic digestion by Eco RI e Hind III enzymes (Sigma-Aldrich, St. Louis, MO, USA). Briefly, 2 µL of buffer and 6 µL of samples were incubated in a MasterCycler® gradient thermal cycler at 37 ºC for 120 minutes (Eppendorf AG, Hamburg, Germany). These preparations were submitted to an inactivation process at 65 ºC for 20 minutes, followed by a running in gel electrophoresis in 1.5% agarose gel (25 V, 300 mA) for 120 minutes.

Morphology and Quantification of *L. amazonensis* by Flow Cytometry after Pretreatment with *Z. rhoifolium*

According to the previous section, promastigote forms of *L. amazonensis* were evaluated by growth

and viability after pretreatment with ZR-EEtOH or ZR-FHEX for 24h, 48h and 72h. Afterwards, the viable cells previously treated at concentration of 7.96 mg/mL were submitted to flow cytometry analysis by BD-FACSCanto® II cell analyzer (BD Company, Franklin Lakes, NJ, USA). A total of 10,000 events were observed in the region that corresponded to the parasites.

Statistical Analysis

The IC_{50} (inhibitory concentration for 50% of cells) CC_{50} (cytotoxic concentration for 50% of cells) were calculated by probit analysis (software SPSS® 13.0), and the selectivity index (SI) were obtained by the ratio between cytotoxicity (CC_{50}) of host cells/IC_{50} of promastigotes and amastigotes in macrophages. For the flow cytometry, the graphs were plotted by using the software BD CellQuest software v 6.1.3.The others analyzes, presented results are the mean ± SEM and were analyzed using a one-way analysis of variance, followed by Bonferroni's test for multiple comparisons, for which is $p<0.05$ was considered significant.

RESULTS AND DISCUSSION

The major finding of this study is the ethanol extract of stem barks from *Z. rhoifolium* (ZR-EEtOH) and its n-hexane fraction (ZR-FHEX) presented an effective inhibitory effect of infection of macrophages by *L. amazonensis*. The ZR-EEtOH fractioning provided the non-polar fraction ZR-FHEX which possesses around 4.0-fold higher antileishmanial activity than ZR-EEtOH against promastigote forms of *L. amazonensis* (Table I). Interestingly, Moura-Costa et al. (2012) has previously demonstrated the leishmanicidal effect of an aqueous and two hydroalcoholic extracts from *Z. rhoifolium* stem barks. The addition of ethanol instead of solely water in the extractive process not only enhances the leishmanicidal effect of *Z. rhoifolium*, but also significantly decreased the cytotoxicity against VERO cells. These findings reinforce evidences of the non-polar fractions as source of compounds and biomarkers for *Z. rhoifolium*-induced antileishmanial effect.

In this sense, terpenes are non-polar compounds with recognized antileishmanial property (Arruda et al. 2005, Cechinel-Filho and Yunes 1998). Mechanisms underlying terpenes-induced antileishmanial effect have been associated to inhibition of protease activity, lipid synthesis, cell cycle or indirectly by modulating macrophage activation (Soares et al. 2012). Terpenes such as nerolidol, an oxygenated sesquiterpene, effectively inhibit isoprenoid biosynthesis, such as dolichol, ergosterol, and ubiquinone in promastigotes (Arruda et al. 2005, Rodrigues et al. 2013). The triterpene dihydrobetulinic acid induces apoptosis-like cell death of *L. donovani* by targeting DNA topoisomerases (both I and II) and preventing DNA cleavage (Alakurtti et al. 2010). Additionally, the diterpene dolabelladienetriol purified from the marine algae

TABLE I
Mean inhibitory concentration (IC_{50}) and mean cytotoxic concentration (CC_{50}) of ethanol extract of stem barks from *Z. rhoifolium* (ZR-EEtOH) and its hexanic fraction (ZR-FHEX) against promastigote forms of *L. amazonensis*.

Treatment	IC_{50} (µg/mL)			CC_{50} (µg/mL)	SI
	24 h	48 h	72 h	48 h	48 h
ZR-EEtOH	88.58 (50.9 – 166.7)[a]	16.41 (8.8 – 28.6)[a]	9.57 (4.3 – 18.3)[a]	>400	>24.37
ZR-FHEX	19.24 (11.4 – 31.6)[a]	13.66 (7.7 – 23.2)[a]	7.96 (4.2 – 13.9)[a]	>400	>29.28

[a]Confidence intervals.

Dictyotapfaffii decreases the infection of macrophages by *Leishmania* spp., even in the presence of factors that exacerbate parasite growth, such as IL-10 and TGF-β (Soares et al. 2012).

Moreover, the presence of triterpenes in *Z. rhoifolium* was previously confirmed by qualitative tests (Freitas et al. 2011), as well as by the isolation and identification of lupeol as the major constituent of ZR-FHEX, confirmed by ^{1}H and ^{13}C NMR spectroscopic analyses (Pereira et al. 2010). This compound exhibited numerous biological activities including anti-inflammatory, anti-cancerous, cardioprotective, hepatoprotective, anti-microbial and antiprotozoal properties. Considered as a promising antiprotozoal agent, lupeol has demonstrated a significant inhibitory effect against parasites which cause malaria, trypanosomiasis or leishmaniasis (Siddique and Saleem 2011). Accordingly, lupeol demonstrated significant antileishmanial effect against promastigotes of *L. braziliensis*, *L. amazonensis* and *L. donovani* at IC_{90} of 100 µg/mL (Fournet et al. 1992). Interestingly, the effect of lupeol obtained from ZR-FHEX against promastigote forms was effective with IC_{50} of 13.61 µg/mL at 72 h (data not shown), which might indicates lupeol as a biologically active biomarker of *Z. rhoifolium*.

The cytotoxicity was evaluated by determination of mean cytotoxic concentration (CC_{50}) against murine peritoneal macrophages by MTT test. The cytotoxicity against macrophages and promastigotes were compared by determination of the selectivity index (SI) (CC_{50}/IC_{50}) (Table I), and the results demonstrate toxicological selectivity of ZR-EEtOH and ZR-FHEX to promastigotes rather than macrophages, a valuable characteristic of effective drugs for the treatment of leishmaniasis. Accordingly, Moura-Costa et al. (2012) has demonstrated low cytotoxicity for *Z. rhoifolium* hydroalcoholic extract in VERO cells, despite of its marked antileishmanial activity, reinforcing the higher selectivity of ZR-EEtOH and ZR-FHEX to the parasites. Interestingly, lupeol-rich extracts and fractions has demonstrated a marked antileishmanial effect and low cytotoxicity. A previous study reported that the hexanic fraction of Brazilian brown propolis possess lupeol-high content and a marked antileishmanial activity against promastigote and amastigote forms of *L. amazonensis*, as well as low cytotoxicity when compared with the ethanol extract (Santana et al. 2014).

Considering these evidences, the effect of the pretreatment of the macrophages with the ZR-EEtOH or ZR-FHEX on the resistance to infection by *L. amazonensis* was also investigated. Three hours after induction of infective process, a significant reduction of infection and number of intracellular amastigote forms was observed in ZR-EEtOH- or ZR-FHEX-pretreated macrophages (Fig. 1). Moreover, the pretreatment of promastigotes with the ZR-EEtOH or ZR-FHEX at concentrations of half the IC_{50} at 24 h promoted a significant reduction in the number of internalized amastigotes per macrophage allied to the reduction of the percentage of infected macrophages. The best result was obtained for ZR-FHEX at a concentration around 4.4-fold lower than ZR-EEtOH (Fig. 2).

The activation of macrophages is involved in the control of intracellular infection by parasites from *Leishmania* genus (Ghazanfari et al. 2006). Accordingly, many antimicrobial enzymatic systems are activated during phagocytosis in order to digest pathogens and/or phagocytized substances, and they probably underlying the increase of cell resistance against *Leishmania* (Toledo et al. 2009). In this work, a significant increase of phagocytosis activity of zymosan particles was observed in ZR-EEtOH- or ZR-FHEX-pretreated macrophages (Fig. 3a and 3b). Besides, no significant alterations were observed in the determination the lysosomal volume (data not shown). These evidences demonstrate the activation of phagocytic cells probably underlies the *Z. rhoifolium*-induced antileishmanial effect.

The synthesis and release of nitric oxide (NO) is considered the most effective mechanism under-

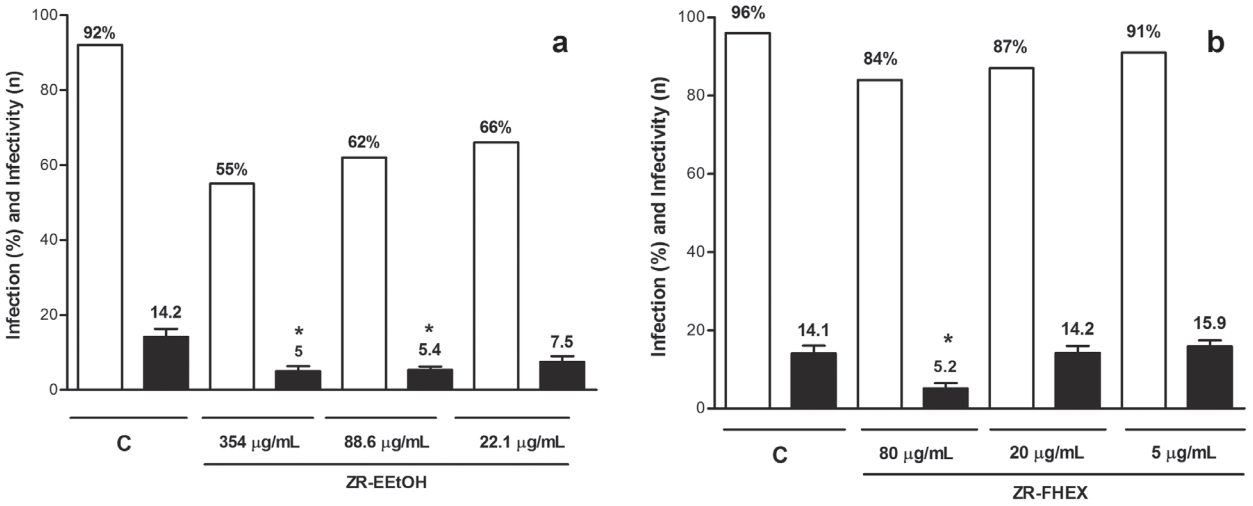

Figure 1 - Effect of pretreatment of macrophages with ZR-EEtOH or ZR-FHEX, expressed by percentage of macrophages infection (□) and average number of amastigotes per infected macrophage (■). Values are expressed as mean ± s.e.m. One-way ANOVA followed by Bonferroni's post-test; *p<0.05 compared with control.

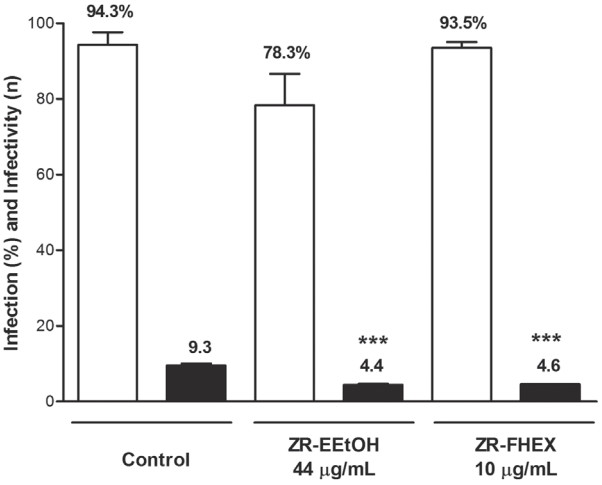

Figure 2 - Effect of pretreatment of promastigote forms of *L. amazonensis* with ZR-EEtOH (44 μg/mL) or ZR-FHEX (10 μg/mL), expressed by percentage of infection of macrophages (□) and average number of amastigotes per infected macrophage (■). Values are expressed as mean ± s.e.m. One-way ANOVA followed by Bonferroni's post-test; ***p<0.001 compared with control.

lying the cell defense of macrophages against parasites from *Leishmania* genus (Green et al. 1990, Bogdan and Röllinghoff 1998). The parasite is able to survive inside macrophages due to its ability of inhibit the expression or activity of inducible nitric oxide synthase (iNOS), the production of cytokines involved in the regulation of iNOS and the NO synthesis by glycosylinositol phospholipids on the surface of amastigotes, as well as stimulate the production of transforming growth factor TGF-β (Balestieri et al. 2002, Barral-Netto et al. 1992, Proudfoot et al. 1995). In this work, a slight increase of nitrite production was observed after the pretreatment of macrophages with the ZR-EEtOH or ZR-FHEX (Fig. 3c and 3d). Interestingly, ZR-EEtOH and ZR-FHEX promoted a marked increase of nitrite production in macrophages previously infected with promastigotes (data not shown). These findings suggest the induction the Th1 immune response triggered by NO signaling pathway the as a possible underlying mechanism in ZR-EEtOH- or ZR-FHEX-induced antileishmanial effect (Bogdan and Röllinghoff 1998).

In order to evaluate the DNA damage as a possible mechanism underlying antileishmanial effect of *Z. rhoifolium* against *L. amazonensis*, promastigote forms were pretreated with ZR-EEtOH or ZR-FHEX at concentrations of 7.96, 15.92 and 31.84 μg/mL for 24 h. Then, a marked concentration-dependent decrease of DNA content was observed after pretreatment with ZR-EEtOH or ZR-FHEX, showing a higher efficacy for ZR-FHEX (Fig. 4). Furthermore, after submitting the extracted DNA

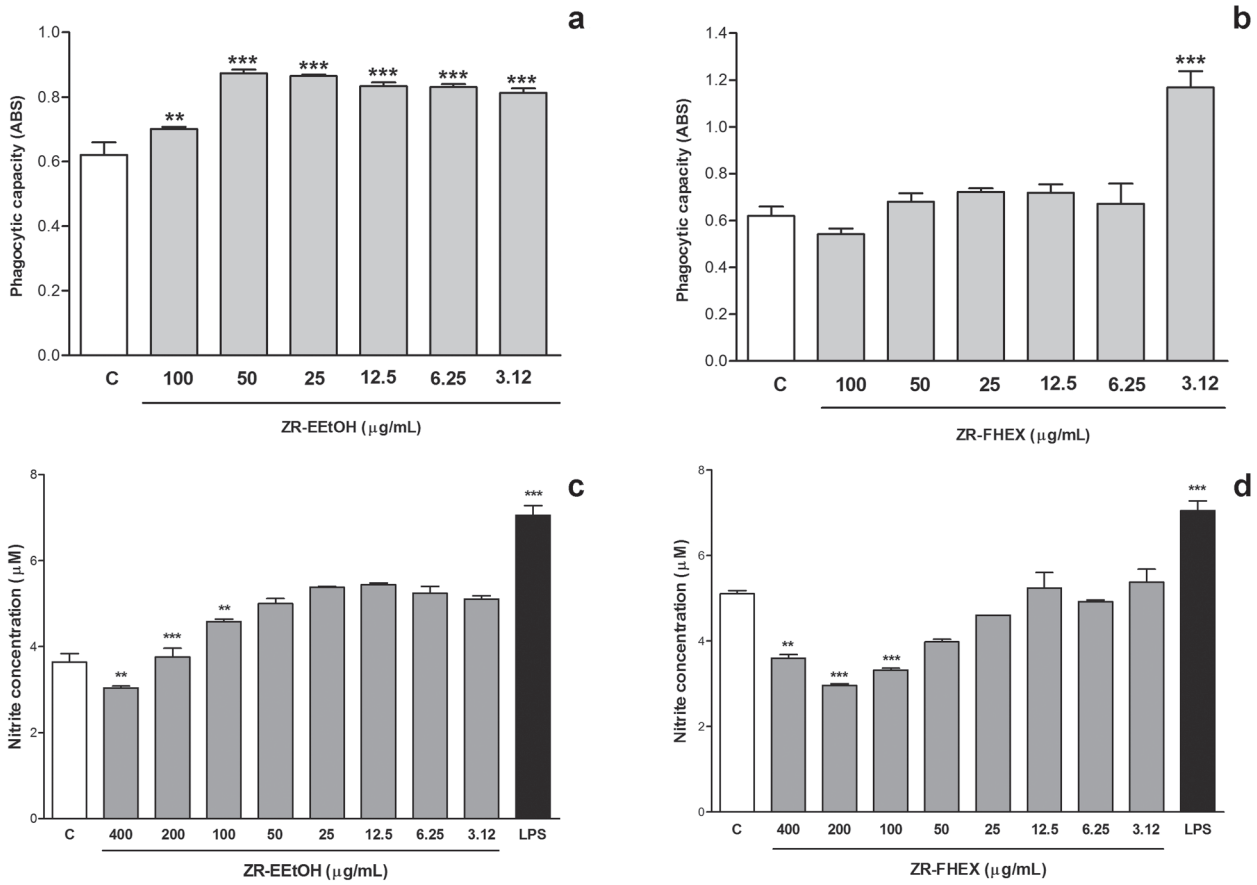

Figure 3 - Phagocytosis of zymosan particles (**a** and **b**) and Colorimetric measurement of nitrite (**c** and **d**) produced by macrophages pretreated with ZR-EEtOH or ZR-FHEX. Values are expressed as mean absorbance ± s.e.m at 550 nm. One-way ANOVA followed by Bonferroni's post-test; **p<0.01, ***p<0.001 compared with control. LPS: lipopolysaccharide from *Escherichia coli*.

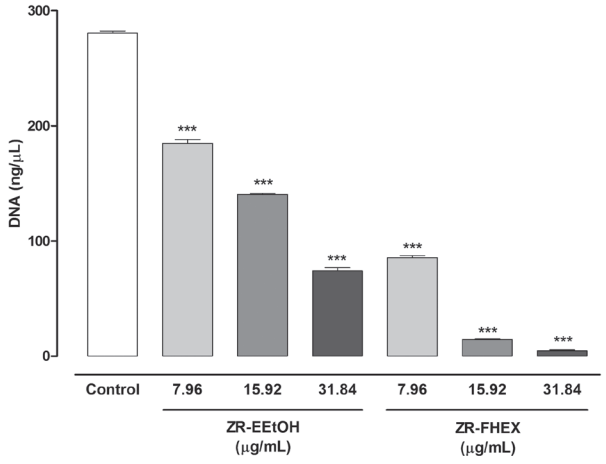

Figure 4 - Quantification of DNA extracted from *Leishmania amazonensis* after pretreatment with ZR-EEtOH or ZR-FHEX for 72 h. Values are expressed as mean ± s.e.m. One-way ANOVA followed by Bonferroni's post-test; ***p<0.001 compared with control.

to enzymatic digestion in the presence of restriction endonucleases enzymes Eco RI and Hind III, differences between DNA-related bands were not observed when compared with control (data not shown). These results indicate that ZR-EEtOH or ZR-FHEX was not able to change possible sites of action of these endonucleases and then induce mutations in the *Leishmania* genome.

Then, considering marked decreases in DNA content after exposure to ZR-EEtOH and ZR-FHEX were observed at higher concentrations (15.92 and 31.84 μg/mL), ZR-EEtOH- or ZR-FHEX-pretreated promastigote forms of *L. amazonensis* at concentration of 7.96 μg/mL were submitted to morphological and quantitative analyses by flow cytometry. Scatterplots were detected by the

voltage pulse as a function of the sum of the pulse heights in 10,000 cells for the control group ZR-EEtOH- or ZR-FHEX-pretreated promastigotes, and results were expressed as amplitude (FSC-A) versus area (FSC-W). A decrease in cell size of *L. amazonensis* promastigote forms was observed after pretreatment with ZR-EEtOH or ZR-FHEX when compared with control group (Fig. 5). Besides, the density of the cell culture was analyzed by the frequency of population and the average of emitted fluorescence, and a decrease of size and number of parasites population, as shown in Fig. 5. Furthermore, the numbers of events were counted for control and each concentration, and a marked reduction was observed in 90.31% and 90.38% for ZR-EEtOH and ZR-FHEX, respectively (Fig. 5). These findings might suggest induction of cell death or inhibition of parasites growth as a possible mechanism underlying ZR-EEtOH- or ZR-FHEX-induced antileishmanial effect.Hence, ZR-EEtOH- and ZR-FHEX-induced antileishmanial effect involves the decrease of the infection and the infectivity rates of macrophages by *L. amazonensis*. The NO release and the macrophages activation is suggested as probable underlying mechanisms, as well as the inhibition of parasites growth. Moreover, the ZR-FHEX is more effective than ZR-EEtOH considering its better antileishmanial effect and decrease of DNA content of promastigotes. Therefore, further investigation regarding the promising treatment of leishmaniasis by *Z. rhoifolium* and further underlying mechanisms is markedly reinforced.

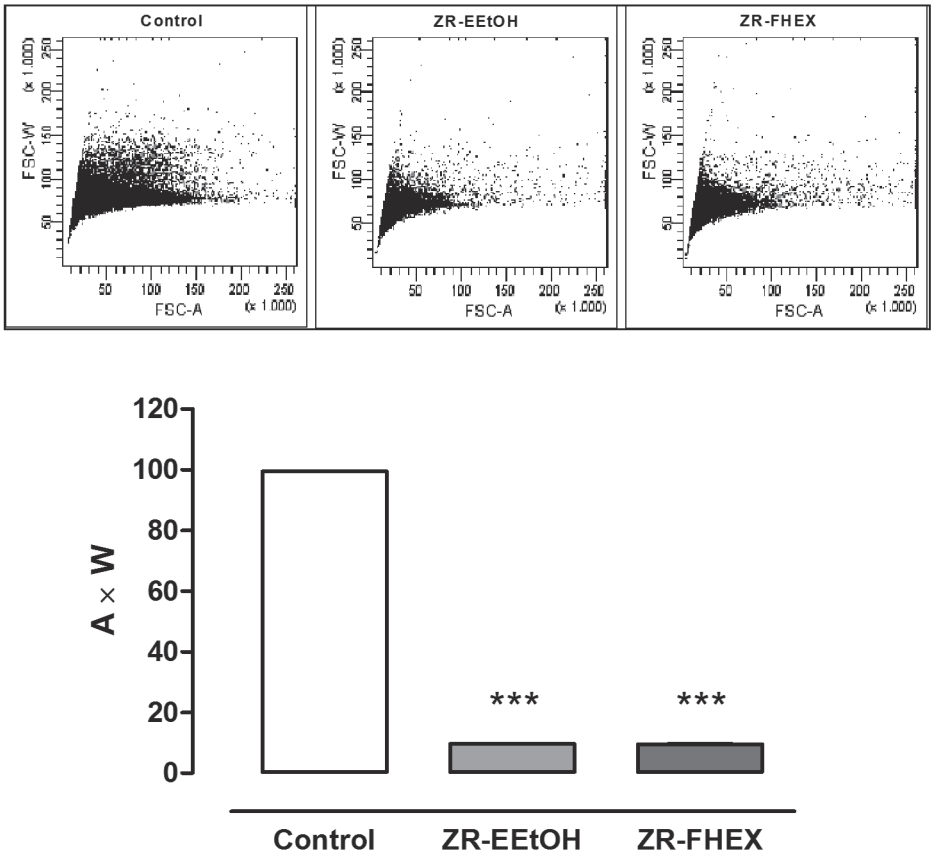

Figure 5 - Percentual of number of events of promastigote forms of *L. amazonensis* after pretreatment with ZR-EEtOH or ZR-FHEX at concentration of 7.96 µg/mL by determination of size (FSC-A) and density (FSC-W) ratio (A × W) by intracellular flow analysis (FACS Diva v.6.1.3). One-way ANOVA followed by Bonferroni's post-test; ***p<0.001 compared with control.

ACKNOWLEDGMENTS

Universidade Federal do Piauí (UFPI), Fundação de Amparo à Pesquisa do Estado do Piauí (FAPEPI), Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES) and Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq) for financial support.

RESUMO

Zanthoxylum rhoifolium Lam. (Rutaceae) tem sido utilizada tradicionalmente no tratamento de infecções microbianas e doenças parasitárias. No presente estudo, o efeito antileishmania induzido pelo extrato etanólico de cascas do caule de *Z. rhoifolium* (ZR-EEtOH) e sua fração hexânica (ZR-FHEX) sobre a infecção e infectividade de macrófagos por formas promastigotas de *Leishmania amazonensis* foram investigados. Em diferentes experimentos, macrófagos ou promastigotas foram pré-tratadas com ZR-EEtOH ou ZR-FHEX em concentrações não-letais por 24 horas, e então os macrófagos foram submetidos à infecção pelas promastigotas. Além disso, seus efeitos sobre a ativação de macrófagos, assim como concentração de DNA, tamanho e número de promastigotas por citometria de fluxo também foram avaliados. A taxa de infecção e o número de formas amastigotas internalizadas foram consideravelmente reduzidos após os pré-tratamentos de macrófagos ou de promastigotas quando comparados com células não tratadas. O aumento na capacidade fagocítica e nas concentrações de nitrito também foi observado. Por sua vez, a redução na concentração de DNA, tamanho e número de promastigotas também foram observados. Em conclusão, ZR-EEtOH e ZR-FHEX promoveram um significativo efeito antileishmania e redução na infecção de macrófagos, provavelmente envolvendo ativação de mecanismos de defesa em macrófagos. Estes achados reforçam a potencial aplicação de *Z. rhoifolium* no tratamento da leishmaniose.

Palavras-chave: *Leishmania,* Macrófagos, Óxido Nítrico, Promastigotas, Atividade fagocítica, *Zanthoxylum.*

REFERENCES

ALAKURTTI S, HEISKA T, KIRIAZIS A, SACERDOTI-SIERRA N, JAFFE CL AND YLI-KAUHALUOMA J. 2010. Synthesis and anti-leishmanial activity of heterocyclic betulin derivatives. Bioorg Med Chem 18: 1573-1582.

ARRUDA DC, D'ALEXANDRI FL, KATZIN AM AND ULIANA SR. 2005. Antileishmanial activity of the terpene nerolidol. Antimicrob Agents Chemother 49: 1679-1687.

BALESTIERI FM, QUEIROZ AR, SCAVONE C, COSTA VM, BARRAL-NETTO M AND ABRAHAMSOHN A. 2002. *Leishmania (L) amazonensis*-induced inhibition of nitric oxide synthesis in host macrophages. Microbes Infect 4: 23-29.

BARRAL-NETTO M, BARRAL A, BROWNELL CE, SKEIKY YA, ELLINGSWORTH LR, TWARDZIK DR AND REED SG. 1992. Transforming growth factor-beta in leishmania infection: a parasite escape mechanism. Science 257: 545-548.

BOGDAN C AND RÖLLINGHOFF M. 1998. The immune response to Leishmania: mechanisms of parasite control and evasion. Int J Parasitol 28: 121-134.

BONATTO SJ, FOLADOR A, AIKAWA J, YAMAZAKI RK, PIZATTO N, OLIVEIRA HH, VECCHI R, CURI R, CALDER PC AND FERNANDES LC. 2004. Lifelong exposure to dietary fish oil alters macrophage responses in Walker 256 tumor-bearing rats. Cell Immunol 231: 56-62.

CARNEIRO SMP, CARVALHO FAA, SANTANA LCLR, SOUSA APL, MOITA-NETO JM AND CHAVES MH. 2012. The cytotoxic and antileishmanial activity of extracts and fractions of leaves and fruits of *Azadirachta indica*. Biol Res 45: 111-116.

CECHINEL-FILHO Y AND YUNES RA. 1998. Estratégias para obtenção de compostos farmacologicamente ativos a partir de plantas medicinais. Conceitos sobre modificação estrutural para otimização da atividade. Quím Nova 21: 99-105.

CHAPPUIS F, SUNDAR S, HAILU A, GHALIB H, RIJAL S, PEELING RW, ALVAR J AND BOELAERT M. 2007. Visceral leishmaniasis: what are the needs for diagnosis, treatment and control? Nat Rev Microbiol 5: 873-882.

DA SILVA SL, FIGUEIREDO PMS AND YANO T. 2007. Chemotherapeutic potential of the volatile oils from *Zanthoxylum rhoifolium* Lam. leaves. Eur J Pharmacol 576: 180-188.

FERREIRA-FILHO ES, ARCANJO DDR, MOURA LHP, SILVA-FILHO JC, PAULINO ET, RIBEIRO EAN, CHAVES MH, OLIVEIRA RCM AND OLIVEIRA AP. 2013. Antihypertensive and vasorelaxant effects of ethanol extract of stem barks from *Zanthoxylum rhoifolium* Lam. in rats. Indian J Exp Biol 51: 661-669.

FOURNET A, ANGELO A, MUÑOZ V, ROBLOT F, HOCQUEMILLER R AND CAVÉ A. 1992. Biological and chemical studies of *Pera benensis*, a Bolivian plant used in folk medicine as a treatment of cutaneous leishmaniasis. J Ethnopharmacol 37: 159-164.

FREITAS FFBP ET AL. 2011. Gastroprotective activity of *Zanthoxylum rhoifolium* Lam. in animal models. J Ethnopharmacol 137: 700-708.

GENESTRA M, ECHEVARRIA A, CYSNE-FINKELSTEIN L, VIGNÓLIO-ALVES L AND LEON LL. 2003. Effect of amidine derivatives on nitric oxide production by *Leishmania amazonensis* promastigotes and axenic amastigotes. Nitric Oxide 8: 1-6.

GHAZANFARI T, HASSAN ZM AND KHAMESIPOUR A. 2006. Enhancement of peritoneal macrophage phagocytic activity against *Leishmania major* by garlic (*Allium sativum*) treatment. J Ethnopharmacol 103: 333-337.

GONZAGA WA, WEBER AD, GIACOMELLI SR, DALCOL II, HOELZEL SCS AND MOREL AF. 2003. Antibacterial Alkaloids from *Zanthoxylum rhoifolium*. Planta Med 69: 371-374.

GREEN SJ, MELTZER JR MS, HIBBS JB AND NACY CA. 1990. Activated macrophages destroy intracellular *Leishmania major* amastigotes by an L-arginine-dependent killing mechanism. J Immunol 144: 278-283.

JULLIAN V, BOURDY G, GEORGES S, MAUREL S AND SAUVIAN M. 2006. Validation of use a traditional antimalarial remedy from French Guiana *Zanthoxylum rhoifolium* Lam. J Ethnopharmacol 106: 348-352.

KAYSER O, KIDERLEN AF, BERTELS S AND SIEMS K. 2001. Antileishmanial activities of aphidicolin and its semisynthetic derivatives. Antimicrob Agents Chemother 45: 288-292.

LINDOSO JAL, COSTA JML AND GOTO ITQH. 2012. Review of the current treatments for leishmaniases. Res Rep Trop Med 3: 69-77.

MONZOTE L, MONTALVO AM, SCULL R, MIRANDA M AND JUAN A. 2007. Combined Effect of the Essential Oil from *Chenopodium ambrosioides* and Antileishmanial Drugs on Promastigotes of *Leishmania amazonensis*. Rev Inst Med Trop São Paulo 49: 257-260.

MOURA-COSTA GF, NOCCHI SR, CEOLE LF, MELLO JCP, NAKAMURA CV, DIAS-FILHO BP, TEMPONI LG AND UEDA-NAKAMURA T. 2012. Antimicrobial activity of plants used as medicinals on an indigenous reserve in Rio das Cobras, Paraná, Brazil. J Ethnopharmacol 143: 631-638.

PEREIRA SS, LOPES LS, MARQUES RB, FIGUEIREDO KA, COSTA DA, CHAVES MH AND ALMEIDA FRC. 2010. Antinociceptive effect of *Zanthoxylum rhoifolium* Lam. (Rutaceae) in models of acute pain in rodents. J Ethnopharmacol 2: 227-231.

PROUDFOOT L, O'DONNELL CA AND LIEW FY. 1995. Glycoinositolphospholipids of *Leishmania major* inhibit nitric oxide synthesis and reduce leishmanicidal activity in murine macrophages. Eur J Immunol 25: 745-750.

REILLY TP, BELLEVUE FH, WOSTER PM AND SVENSSON CK. 1998. Comparison of the *in vitro* cytotoxicity of hydroxylamine metabolites of sulfamethoxazole and dapsone. Biochem Pharmacol 55: 803-810.

RODRIGUES KAF, AMORIM LV, OLIVEIRA JMG, DIAS CN, MORAES DFC, MAIA JGS, CARNEIRO SMP AND CARVALHO FAA. 2013. *Eugenia uniflora* L. essential oil as a potential anti-*Leishmania* agent: effects on *Leishmania amazonensis* and possible mechanisms of action. Evid Based Complement Alternat Med 2013: 10.

ROSA MSS, MENDONÇA-FILHO RR, BIZZO HR, RODRIGUES IA, SOARES RMA, SOUTO-PADRÓN T, ALVIANO CS AND LOPES AHCS. 2003. Antileishmanial activity of a linalool-rich essential oil from *Croton cajucara*. Antimicrob Agents Chemother 47: 1895-1901.

SANTANA LCLR, CARNEIRO SMP, CALAND-NETO LB, ARCANJO DDR, MOITA-NETO JM, CITÓ AMGL AND CARVALHO FAA. 2014. Brazilian brown propolis elicits antileishmanial effect against promastigote and amastigote forms of *Leishmania amazonensis*. Nat Prod Res 28: 340-343.

SEN R AND CHATTERJEE M. 2011. Plant derived therapeutics for the treatment of Leishmaniasis. Phytomedicine 18: 1056-1069.

SIDDIQUE HR AND SALEEM M. 2011. Beneficial health effects of lupeol triterpene: A review of preclinical studies. Life Sci 88: 285-293.

SINGH N, MISHRA BB, BAJPAI S, SINGH RK AND TIWARI VK. 2014. Natural Product Based Leads To Fight Against Leishmaniasis. Bioorg Med Chem 22: 18-45.

SOARES DC, CALEGARI-SILVA TC, LOPES UG, TEIXEIRA VL, DE PALMER PAIXÃO IC, CIRNE-SANTOS C, BOU-HABIB DC AND SARAIVA EM. 2012. Dolabelladienetriol, a compound from *Dictyotapfaffii* algae, inhibits the infection by *Leishmania amazonensis*. PLoS Negl Trop Dis 6: E1787.

SOARES DC, PEREIRA CG, MEIRELES AA AND SARAIVA EA. 2007. Leishmanicidal activity of a supercritical fluid fraction obtained from *Tabernaemontana catharinensis*. Parasitol Int 56: 135-139.

TAVARES LC, ZANON G, WEBER AD, NETO AT, MOSTARDEIRO CP, CRUZ IBM, OLIVEIRA RM, ILHA V, DALCOL II AND MOREL AF. 2014. Structure-activity relationship of benzophenanthridine alkaloids from *Zanthoxylum rhoifolium* having antimicrobial Activity. PLoS One 9: e97000.

TOLEDO KA, SCHWARTZ C, OLIVEIRA AF, CONRADO MCAV, BERNARDES ES, FERNANDES LC, ROQUE-BARREIRA MC, PEREIRA-DA-SILVA G AND MORENO NA. 2009. Neutrophil activation induced by Artin M: Release of inflammatory mediators and enhancement of effector functions. Immunol Lett 123: 14-20.

WORLD HEALTH ORGANIZATION. 2010. Neglected tropical diseases in the world today: Leishmaniasis. In: First WHO report on neglected tropical diseases: working to overcome the global impact of neglected tropical diseases, Geneva, p. 91-96. http://whqlibdoc.who.int/publications/2010/9789241564090_eng.pdf. Acessed 16 July 2014.