Control of leishmaniasis remains a challenge due to the high toxicity of the chemotherapeutic drugs presently available. The ongoing search for better leishmanicidal compounds has brought herbal drugs into the limelight as safe and effective substitutes to conventional therapies which have various drawbacks. The current study was designed to evaluate the antileishmanial effect of Quassia amara against Leishmania amazonensis and Leishmania infantum. Different fractions (Hexane (Q1), dichloromethane (Q2), ethyl acetate (Q3) and butanol (Q4)) obtained from the liquid-liquid partition of the crude Q. amara methanol extract were tested against Leishmania promastigote forms. After 120 h of treatment with Q. amara fractions (1 to 500 µg/ml), the minimal inhibitory concentration (MIC) was determined using resazurin. The most active fraction (Q2) was analyzed using thin layer chromatography (TLC) and high performance liquid chromatography-diode array detector (HPLC-DAD) techniques. The effects of Q2 on parasite ultrastructure were investigated by transmission electron microscopy. In addition, Leishmania-infected macrophages were treated with Q2 in order to evaluate the anti-amastigote effect. Among the fractions tested, Q2 showed the highest activity against L. amazonensis and L. infantum promastigotes MIC values of 62.5 and 31.25 µg/ml, respectively. There were ultrastructural alterations, such as nuclear chromatin condensation, intense vacuolization and autophagic and myelin-like figures in parasites treated with Q2 (62.5 µg/ml). Macrophages previously infected with L. amazonensis and L. infantum promastigotes showed a drastic reduction in the number of parasites recovered in the supernatant after Q2 treatment at MIC values. The TLC and HPLC fingerprints of Q2 showed that alkaloids were the main chemical constituents in this fraction. The results presented herein showed that the alkaloid-rich fraction Q2 is a promising source of antileishmanial agents. Further investigation will be necessary in order to isolate and test the substance(s) responsible for the bioactivity.

Key words: Antileishmanial activity, Quassia genus, alkaloids, macrophage infection, nitric oxide.
INTRODUCTION

Leishmaniasis is a group of illnesses caused by several species of protozoa belonging to the *Leishmania* genus. They are considered endemic in 98 countries and have a broad spectrum of clinical manifestations ranging from disfiguring skin lesions, known as tegumentary leishmaniasis (TL) to the visceral form of the disease, visceral leishmaniasis (VL) or kala-azar, that can lead to death (Hartley et al., 2012; Rebbestad et al., 2012). Brazil possesses the highest incidence of TL and VL with 26,008 and 3,481 cases reported, respectively (Roqueline et al., 2014). The dissemination of the disease may occur by human-to-human transmission (anthroponotic transmission) or from animals to humans (zoonotic transmission). In both cases, parasitic (infective metacyclic promastigote forms) are transmitted during the insect vector hematophagy (Chakravarty and Sundar, 2010). Once inoculated in the dermis-epidermis junction of the vertebrate hosts, infective parasites are phagocytized by macrophages and then initiate the differentiation process to amastigotes, the evolutive form responsible for the disease establishment and progression (De Pablos et al., 2016). Since macrophages play a pivotal role in the infection as host cells, the search for drugs able to elicit an effective antileishmanial response by these cells becomes essential.

Pentavalent antimony compounds and amphotericin B are the main drugs prescribed for the treatment of leishmaniasis. In addition, other drugs can be used such as pentamidine and paromomycin, which are more effective against the tegumentary form of the disease and miltefosine, the orally active drug indicated for VL treatment (Sundar and Singh, 2016; Dorlo et al., 2012; Frézard et al., 2009; Jhingran et al., 2009). However, these drugs present variable efficacies and high toxicity (e.g. cardiotoxicity, hepatotoxicity and nephrotoxicity), which may lead to diverse side effects. The development of parasite drug resistance is an additional problem for the control of leishmaniasis (Sundar and Chakravarty, 2015). In this scenario, several research laboratories around the world have been focused on the prospecting of plant species as a strategy for discovery of more effective substances against leishmaniasis.

*Quassia amara* L. (Simaroubaceae) is a tree species found in tropical regions including Central and South America, where it is popularly known as “pau-tentente”, “amargo”, “grande hombre” or “quassia”. The tree can reach 8 m in height featuring compound leaves, red flower petals and drupes about 1 to 2 cm long. Several extracts obtained from the leaves, bark or wood of *Quassia amara* have traditionally been used as natural remedies for the treatment of digestive and liver disorders, and malaria (López and Pérez, 2008). In fact, the *Quassia* genus is a known source of biologically active extracts against protozoa. The aqueous extract of *Quassia africana* root bark displayed antitrypanosomal, antiplasmodial and antileishmanial activities in concentrations ranging from 0.46 to 5.94 µg/ml (IC50 values) (Musuyu Muganza et al., 2012). Some authors have described the antimalarial activity of *Q. amara* species. Ajaiyeoba et al. (1999) demonstrated that the methanol extract of *Q. amara* leaf displayed strong antimalarial activity in a murine model. Further investigations revealed that quassinoids such as simalikalactone D, simalikalactone E, quassin andneoquassin were responsible for the antimalarial properties (Mishra et al., 2010; Cachet et al., 2009; Bertani et al., 2006). The present work investigates the antileishmanial potential of an alkaloid-rich fraction obtained from *Q. amara*.

MATERIALS AND METHODS

Plant and extraction procedures

*Q. amara* stem bark was commercially obtained in a popular street market located in Aracaju ( Sergipe, Brazil). Samples were authenticated by Dr. Rosana C. Lopes at the RFA Herbarium, Department of Botany, Federal University of Rio de Janeiro (IB/UFRJ), where a voucher specimen is deposited (RFA 40769). The samples (315 g) were extracted for 5 days at room temperature using methanol (1 L). Then, the methanol extract was dried (yield =1.23%), resuspended in 150 ml of distilled water, and subjected to solvent partitioning (1:1) in the following order: hexane, dichloromethane, ethyl acetate and butanol. After the evaporation of the solvents Q1 (yield =20.5%), Q2 (yield =16.6%), Q3 (yield =2.96%) and Q4 (yield =0.87%) fractions were obtained, respectively, and kept in amber flasks at 4°C until biological and chromatographic analysis.

Thin layer chromatography (TLC) analysis

TLC was performed using TLC Silica gel plates (Merck), 0.25 mm; eluent system: 2% methanol/dichloromethane; detection: Dragendorff and sulfuric acid spray reagents. After spraying the plates were visually analyzed.

Q2 fraction analysis by fast high performance liquid chromatography-diode array detector (HPLC-DAD)

Liquid chromatography was performed using a Shimadzu fast HPLC-DAD. The sample was applied using an automatic injector and separated on a Kinetex C18 column, 150 mm x 4.6 mm, 2.6 µm at 35°C. The mobile phase was a gradient of solvent A (H2O with 0.1% of formic acid) and B (acetonitrile) from 3 to 25% of B, 0
to 12 min and from 25 to 75% of B, 12 to 22 min. The acetonitrile used was HPLC grade (Tedia) and the water was purified with a Milli-Q system. Q2 (2.0 mg) was dissolved in 1.0 ml of water and then centrifuged for 10 min prior the analysis. The flow and sample injection volume were 1 ml/min and 2.0 µl, respectively. The chromatogram was acquired at a wavelength of 275 nm.

**Parasites tested**

*Leishmania amazonensis* (IFLA /BR/1967/PH8) and *Leishmania infantum* (MHOM/BR/1974/PP75) promastigotes were donated by the Leishmania Type Culture Collection of Oswaldo Cruz Institute/Fiocruz (Rio de Janeiro/RJ/Brazil). Parasites were axenically cultured in PBHIL medium supplemented with 10% fetal bovine serum (Rodrigues et al., 2010).

**Leishmania inhibitory assay**

In order to evaluate the antileishmanial activity of *Q. amara* extracts, *L. infantum* and *L. amazonensis* promastigote forms were harvest at early stationary phase (96 h), washed twice with PBS (150 mM NaCl; 20 mM phosphate buffer, pH 7.2) and re-suspended in fresh culture medium at a concentrations of 10⁷ parasites/ml. Then, 100 µl of the cellular suspension was added to 96-well microplates, where *Q. amara* fractions had been previously diluted (final concentrations ranging from 0.97 to 500 µg/ml). The microplates were incubated at 28°C for 24, 48 and 120 h. Parasite growth was evaluated daily by direct counting of the cells in a Neubauer chamber. The minimum inhibitory concentration (MIC) was determined using resazurin as the cellular viability indicator (Rolon et al., 2006). Amphotericin B was used as positive control. Alternatively, cultures presenting negative parasite growth were re-incubated in fresh culture media aiming to evaluate a leishmanicidal or leishmanistatic effect.

**Ultrastructure analysis**

*L. amazonensis* promastigote forms were harvested at early stationary phase and treated with Q2 fraction at inhibitory (MIC) and sub-inhibitory (MIC/2) concentrations for 24 h at 28°C. After the incubation period, the parasites were washed twice with PBS and then fixed in 0.9% glutaraldehyde solution (2.5% glutaraldehyde in 0.1 M sodium cacodylate buffer containing 3.5% sucrose, pH 7.4) at 4°C for 60 min. The samples were sent to the Rudolf Barth Platform (IOC/FIOCRUZ/RJ) and processed as previously described (Rodrigues et al., 2013). Image acquisition was made with a JEOL JEM1011 transmission electron microscope.

**Cytotoxic evaluation**

Peritoneal macrophages were obtained as previously described (Rodrigues et al., 2013). The maintenance procedures and euthanasia of animals were approved by the UFRJ Committee of Ethics for the Use of Animals (license 154/13). Macrophages (2.5×10⁵ cells) were allowed to adhere into 96-well microplates for 20 min at 37°C and 5% CO₂ atmosphere. The macrophage cultures were washed with PBS and incubated in the presence of *Q. amara* Q2 fraction at concentrations ranging from 0.97 to 500 µg/ml for 24 and 48 h. The cellular viability of Q2-treated macrophages was evaluated by resazurin reduction assay as previously described (Al-Musayyeb et al., 2012).

**Macrophage infection and nitric oxide production**

The infection assays were performed following the protocol previously described by Passero et al. (2010) with slight modifications. Briefly, peritoneal macrophages (1.0 × 10⁶ cells/ml) were distributed in 96-well microplates. Subsequently, promastigotes forms of *L. amazonensis* or *L. infantum* were added (5 parasites per macrophage). The parasite-macrophage interaction was performed in RPMI 1640 medium supplemented with 10% FBS at 37°C and 5% CO₂ atmosphere for 24 h. After the incubation period, free promastigotes were removed by extensive washing with PBS and then the cultures were treated with Q2 at 31.25 and 15.62 µg/ml, MIC and subMIC, respectively. After 48 h of treatment, culture supernatants were collected for nitric oxide determination (Green et al., 1990). The infected macrophages were washed and re-incubated with PBHIL supplemented with 10% FBS for 72 h at 28°C in order to evaluate the number of promastigotes that recovered. The experiment was performed in triplicate and the number of viable promastigotes was determined by the average of cell counts using a Neubauer chamber.

**Statistical analysis of the data**

The mean and standard error of at least three experiments were determined. Statistical analysis of the differences between mean values obtained for experimental groups was done by means of Student’s t-test. P values of 0.05 or less were considered significant.

**RESULTS**

**Parasite growth inhibition**

Among the four fractions, Q2, Q3 and Q4 displayed antileishmanial activity against *L. amazonensis* and *L. infantum* promastigotes with MIC values ranging from 31.25 to 500 µg/ml (Table 1). Q1 was not active against the promastigotes at the highest concentration tested (MIC > 500 µg/ml). Q2 presented the best results and was able to terminate *L. amazonensis* and *L. infantum* growth at 31.25 and 62.5 µg/ml, respectively. Moreover, Q2 exerted an inhibitory effect on *Leishmania* growth at the sub-inhibitory concentrations (subMIC) of 15.62 and 31.25 µg/ml for *L. amazonensis* and *L. infantum*, respectively (Figure 1A and 1B). Based on these results the researcher decided that all further experiments would be only with the Q2 fraction.

**Ultrastructural alterations**

Ultrastructural alterations in Q2-treated *L. amazonensis* promastigotes were investigated using TEM. Figure 2A and B shows control parasites displaying normal morphology and intracellular organelles. After 24 h exposure to 62.5 µg/ml of Q2 (MIC value), significant alterations were observed in the parasites, such as electron dense granules and myelin-like figures in the cytoplasm (Figure C). In addition, parasites showed mitochondrial dilatation with concentric membranes in the
mitochondrial matrix, nuclear chromatin condensation and the presence of autophagic structures (Figure 2E). Vesicles with cytoplasmic content released from the cell body into the flagellar pocket were also noted (Figure 2D).

Cytotoxicity for peritoneal macrophages

The effect of *Q. amara* Q2 fraction on peritoneal macrophages was investigated (Table 1). Q2 displayed toxic effect at 500 µg/ml (minimal cytotoxic concentration - MCC); allowing its use at effective concentrations against *L. amazonensis* and *L. infantum*.

Anti-amastigote activity

In order to evaluate the effects of Q2 in vitro, peritoneal macrophages were previously infected with *L. amazonensis* or *L. infantum* promastigotes and then treated with Q2 at MIC and subMIC concentrations. After 48 h of infection, Q2 was removed by washing and the cultures were reincubated under proper conditions for promastigote recovery. The results demonstrate that the number of promastigotes recovered in the treated macrophage culture supernatants was very low, regardless of the Q2 concentration tested (Table 2). In addition, the nitric oxide (NO) produced by the macrophages during the infection period was determined. The study showed that only *L. amazonensis* and *L. infantum*-infected macrophages treated at MIC concentration of Q2 produced high amounts of NO when compared to their respective untreated cultures. Indeed, the NO production was about 159 and 183% higher than the untreated cultures, respectively.

Chromatographic analysis of Q2

Alkaloid-rich fraction (Q2) was detected by TLC with...
Table 1. Antileishmanial activity of the four fractions derived from the *Q. amara* methanol extract.

<table>
<thead>
<tr>
<th>Fractions</th>
<th>Minimal inhibitory concentrations (µg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td><em>L. amazonensis</em></td>
</tr>
<tr>
<td></td>
<td>24 h</td>
</tr>
<tr>
<td>Q1</td>
<td>&gt;500.0</td>
</tr>
<tr>
<td>Q2</td>
<td>62.5</td>
</tr>
<tr>
<td>Q3</td>
<td>500.0</td>
</tr>
<tr>
<td>Q4</td>
<td>250.0</td>
</tr>
<tr>
<td>AmphB</td>
<td>1.95</td>
</tr>
</tbody>
</table>

Q1: *Q. amara* hexane fraction; Q2: *Q. amara* dichloromethane fraction; Q3: *Q. amara* ethyl acetate fraction; Q4: *Q. amara* butanol fraction; AmphB: Amphotericin B.

Table 2. Effects of Q2 on *L. amazonensis* and *L. infantum* intracellular amastigotes and nitric oxide production by infected macrophages.

<table>
<thead>
<tr>
<th>Q2 treatment</th>
<th>Promastigote recovery (×10^4 cells/mL)</th>
<th>NO₂ (µM)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td><em>L. amazonensis</em></td>
<td><em>L. infantum</em></td>
</tr>
<tr>
<td>Control</td>
<td>46.0 (±2.55)</td>
<td>33.0 (±6.33)</td>
</tr>
<tr>
<td>MIC</td>
<td>0.01 (±0.47)</td>
<td>0.04 (±0.03)</td>
</tr>
<tr>
<td>SubMIC</td>
<td>0.01 (±0.92)</td>
<td>0.17 (±0.1)</td>
</tr>
</tbody>
</table>

La: *Leishmania amazonensis*; Li: *Leishmania infantum*; SLa: Supernatant of *L. amazonensis*-infected cultures; SLi: Supernatant of *L. infantum*-infected cultures; MIC: minimum inhibitory activity; SubMIC: sub-inhibitory concentration. MIC and subMIC values for *L. amazonensis* were 31.25 and 15.62 µg/ml, respectively. MIC and subMIC values for *L. infantum* were 62.5 and 31.25 µg/ml, respectively.

Figure 2. Photomicrographs of *L. amazonensis* promastigotes treated with Q2 fraction. (A-B) Control. (B) Flagellar pocket and mitochondrion containing the kinetoplast. (C-F) MIC-treated parasites. (C) Nuclear chromatin condensation, electron dense bodies (*) and myelin-like figures (κ). (D) Dilated flagellar pocket containing vacuoles (*) and a multi vesicular structure (κ). (E) Mitochondrial swelling and a vesicle (κ) inside this organelle. (F) Intense vacuolization process and an autophagic structure (*). n, nucleous; m, mitochondrion; k, kinetoplast; f, flagellum; fp, flagellar pocket; L, lipid.
characteristic orange color after revelation with Dragendorff reagent. The spots on TLC plates did not show red coloration when sprayed with sulfuric acid reagent which indicates the probable absence of quassinoids (Polonsky et al., 1980). The chemical fingerprint obtained by the HPLC-DAD analysis confirmed the presence of this class of substances, showing their characteristic UV spectra (Figure 3).

**DISCUSSION**

Plants represent a promising source of medicinal substances that are biologically active against several pathogens, including *Leishmania*. Sarkar et al. (2013) reported that the antileishmanial activity of the petroleum ether, chloroform and methanol extracts obtained from the leaves of *Pleumaria pudica*. In that study, all the extracts had strong activity against the *Leishmania donovani* promastigote forms with IC_{50} values ranging from 2.93 to 3.04 µg/ml. Triterpenoids were the main substances found in the extracts and may be responsible for the bioactivity (Sarkar et al., 2013). The crude methanol extract of *Lantana ukambensis* leaves was also active against *L. donovani* with IC_{50} value at 6.9 µg/ml (Sawadogo et al., 2012a). Tannins, triterpenes, and steroids were previously reported by these authors as the main substances of the extract (Sawadogo et al., 2012b). Bhattacharjee et al. (2009) studied the effect of the quassinoid quassin isolated from *Q. amara* as immunomodulator for controlling the establishment of *L. donovani* infection. The use of *Q. amara* against different infectious diseases (Mans et al., 2016; Bertani et al., 2006; Ajaiyeoba et al., 1999) encouraged us to investigate the antileishmanial potential of this species against *L. amazonensis* and *L. infantum*.

Using the bioguided approach, the study reported that Q2 (dichloromethane fraction of the crude methanol extract) was the most active fraction against both parasites after treatment for 120 h. Table 1 shows that the antileishmanial activity of the extracts, including Q2, is time- and dose-dependent. Other species belonging to the Simaroubaceae family have been described as biologically active against several pathogens in a time and dose dependent manner. Gu et al. (2014) reported that the ethanol extract from *Ailanthus altissima* increased *Psoroptes cuniculi* and *Sarcoptes scabiei* mortality with increasing both extract concentration and post-treatment time. In the present study, MIC values decreased in one fold dilution when parasites were treated for 120 h. Parasite killing was confirmed after reincubation of inhibited cultures in fresh medium and no growth was detected. In addition, Figure 1 shows that *L. amazonensis* promastigote growth was strongly inhibited by Q2 in the first 72 h of treatment even at the sub-inhibitory concentration of 15.62 µg/ml. The complete elimination of the parasites occurred after 96 h of treatment at 31.25 µg/ml. On the other hand, the growth of *L. infantum* promastigotes was inhibited by all Q2 concentrations tested until 96 h of treatment when compared with control cells. However, parasites regained growth after this period except those treated at concentrations above 62.5 µg/ml. In order to determine if Q2 displays leishmanicidal activity, the *L. amazonensis* and *L. infantum* promastigote forms treated with MIC values (31.25 and 62.5 µg/mL, respectively) were reintroduced into fresh culture medium (in the absence of Q2). We observed that those cultures were no longer able to grow, showing strong evidence that Q2 displayed a leishmanicidal effect.

Q2 caused serious damages to the *L. amazonensis* promastigote forms. Mitochondrial swelling, the presence of myelin-like figures and autophagosomal structures on cytoplasm suggested disturbances of the sterol biosynthesis (Figure 2). This study is in line with other study report when using known or novel synthetic ergosterol synthesis inhibitors to treat *Leishmania* (de Macedo-Silva et al., 2015; de Macedo-Silva et al., 2013; Zeiman et al., 2008). Plant-derived products, extracts or isolated substances can inhibit *Leishmania* sterol biosynthesis. Previously, our group reported that a fraction (B2) obtained from the hexane extract of *Arrabidaea chicha* caused similar damages to *L. infantum* promastigotes (Rodrigues et al., 2014).
treatment with 1 to 5 µM tomatidine, a tomato alkaloid, led to the inhibition of Δ24(25)-sterol methyltransferase, an essential enzyme for the ergosterol production (Medina et al., 2012).

Several studies have shown that alkaloids, flavonoids, chalcones, terpenoids, saponins, and phenolic compounds display antileishmanial effect (Sandjo et al., 2016; Singh et al., 2014; Wink, 2012). In the present study, HPLC-DAD was employed to evaluate the phytochemical profile of Q2. This technique is able to define the chemical classes of substances from natural origins based on their characteristic UV spectrum (Escarpa and Gonzalez, 1998; Justesen et al., 1998). The principal substances of Q2 detected by TLC presented orange spots after chemical revelation with Dragendorff reagent and HPLC/DAD demonstrated the UV spectra that indicate alkaloid class (Figure 3). There are few studies on the alkaloid content of Q. amara, which includes indole and canthin-6-one alkaloids (Barbetti et al., 1987; Barbetti et al., 1990; Njar et al., 1993). In fact, the investigation of the pharmacological potential of Q. amara-isolated alkaloids has been neglected, since quassinoids have been described as the main biologically active agents in this species. In this study, Q2 fraction rich in alkaloids displayed minimum cytotoxic concentration against peritoneal macrophages at 500 µg/ml. In addition, Q2 reduced the number of parasites recovered on L. amazonensis- and L. infantum-infected macrophages cultures at MIC concentrations, with a concomitant increase of NO production of about 2.8 and 2.6 fold higher than the controls, respectively (Table 2). Recently, Mans et al. (2016) reported the antileishmanial activity of 25 Surinamese medicinal plants traditionally used to treat cutaneous leishmaniasis, including Q. amara. The authors observed that the Q. amara aqueous extract was able to inhibit L. major NADIMS and L. donovani GEDII promastigotes growth (IC50 values of 51 and 68 µg/ml, respectively). However, the aqueous extract of Q. amara was extremely toxic for THP-1 cells (IC50 < 16 µg/ml). They also observed that the aqueous extract reduced in 50% the number of intracellular amastigotes at 288 µg/ml. These data is controversial since cytotoxic concentrations were used in the infection assay. In the present study, infected macrophages were treated with parasite subinhibitory concentrations, allowing us to evaluate the treatment effect over internalized parasites without macrophage damage. Nevertheless, the study cannot assure that the obtained results were caused by the alkaloid content of Q2. Studies in our laboratory are in progress in order to fractionate Q2 and evaluate the antileishmanial activity of the isolated substances.

**Conclusion**

Our results demonstrated the antileishmanial properties of Q. amara against the etiological agents of cutaneous and visceral leishmaniasis, L. amazonensis and L. infantum, respectively. Despite the quassinoids bioactivity described in the literature, the study showed that an alkaloid-rich fraction (Q2) obtained from Q. amara was the most active fraction against the parasites. This is an interesting finding due the relevance of alkaloids as potential therapeutic agents. The present study highlights Q. amara, a plant widely used in folk medicine, as a source of antileishmanial agents.

**Conflict of interests**

The authors have not declared any conflict of interest.

**ACKNOWLEDGEMENTS**

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