

Gallic and ellagic acids: two natural immunomodulator compounds solve infection of macrophages by *Leishmania major*

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Abstract Leishmaniasis is a complex of parasitic protozoan diseases caused by more than 20 different species of parasites from *Leishmania* genus. Conventional treatments are high costly, and promote a sort of side effects. Besides, protozoan resistance to treatments has been reported. Natural products have been investigated as a source of new therapeutic alternatives, not only acting directly against the parasite but also being able to synergistically act on the host immune system in order to control parasitemia. Gallic acid (GA) and ellagic acid (EA) are plant-derived phenolic compounds which are able to induce antiinflammatory, gastroprotective, and anticarcinogenic activities. Therefore, the antileishmania, cytotoxic, and immunomodulatory activities of GA and EA were evaluated in this study. Both GA and EA were able to inhibit the growth of *Leishmania major* promastigotes (effective concentration (EC₅₀) values 16.4 and 9.8 µg/mL, respectively). The cytotoxicity against BALB/c murine macrophages for GA and EA was also assessed (CC₅₀ values 126.6 and 23.8 µg/mL, respectively). Interestingly, GA and EA also significantly reduced the infection and infectivity of macrophages infected by

L. major (EC₅₀ values 5.0 and 0.9 µg/mL, respectively), with selectivity index higher than 20. Furthermore, both GA and EA induced high immunomodulatory activity evidenced by the increase of phagocytic capability, lysosomal volume, nitrite release, and intracellular calcium [Ca²⁺_i] in macrophages. Further investigations are reinforced in order to evaluate the therapeutic effects of GA and EA in in vivo experimental infection model of leishmaniasis.

Keywords Antileishmania activity · Cytotoxicity · Fura-2-AM · Immunomodulation · Natural products · Tannins

Introduction

Protozoal infections are considered a worldwide public health problem, especially in underdeveloped countries, where approximately 14% of the population are considered at risk of infection (Kondrashin et al. 2011; Waldron et al. 2011). In this context, leishmaniasis is considered by the World Health Organization (WHO) as one of the six major infectious diseases with a high incidence and capacity to produce deformities. This protozoan infection disease affects more than 12 million people, with up to 3 million new cases reported worldwide (Mitropoulos et al. 2010). Leishmaniasis is caused by more than 20 species from *Leishmania* genus. Among the clinical manifestations of leishmaniasis (cutaneous, mucocutaneous, and visceral), the cutaneous form is the most disseminated and responsible for causing physical deformities in patients. The species *Leishmania major* is responsible for the highest number of cases in the Old World, being endemic in African regions, such as the north of Sahara and Arabian Peninsula (Kaye

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and Scott 2011; Mitropoulos et al. 2010; Nozais 2003; Reithinger and Coleman 2007).

Leishmaniasis is widely distributed, and reaches the Middle East, Africa, and part of the Americas. The infection occurs by the blood meal of females of sand flies, which live in forest areas, caves, rodent dens, and also in the domiciliary area (Reithinger and Coleman 2007; Reithinger et al. 2007). The parasite is digenetic and presents two distinct morphologies: a flagellate form, the promastigote, infecting and present in the insect vector; and another non-flagellated form called amastigote, which is present in the vertebrate host (Madalosso et al. 2012; Montalvo et al. 2012). The parasite harbors and multiplies in the interior of mononuclear phagocytic cells of the vertebrate host, acting by immune evasive mechanisms, such as reducing iNOS (inducible nitric oxide synthase) activity, as well as increasing the Th₂ response, which induces the cell signaling of antiinflammatory cytokines (e.g., IL4, IL10), leading to parasitemia. On the other hand, when a Th₁ response occurs, the overexpression of IFN- γ and TNF- α promotes healing or protection (Islamuddin et al. 2015; Roy et al. 2014a).

Conventional treatments for leishmaniasis are limited, unsafe, and have a range of side effects, as well as contribute to the resistance of the parasite to the treatment. For example, the amphotericin B is widely used as the main drug in the treatment of leishmaniasis, but major side effects are reported, such as cardiotoxicity and nephrotoxicity, besides the high costs (Alizadeh et al. 2008; Ashford 2000). Therefore, studies focused on the development of safer, more effective, and affordable treatment for patients diagnosed with leishmaniasis are necessary.

A sort of natural products obtained from plants has been considered promising candidates due their antileishmania activity. They have cytotoxic potential against the parasite, as well as they are able to trigger immunomodulatory mechanisms involving the activation of iNOS and signaling the participation of cytokines responsible for the Th₁ response, which increases the phagocytic capability and lysosomal volume in macrophages (Islamuddin et al. 2015; Rodrigues et al. 2015). In this context, tannins are classified as phenolic substances derived from the secondary metabolism of plants, where the most part is related to glucose metabolism by different biochemical reactions, such as shikimate or acetate pathway (de Jesus et al. 2012).

Among some important phenolic compounds, the gallic acid and their dimers, such as digallic acid, hexahydroxydiphenic acid, and ellagic acid, are characterized as naturally occurring phenolic lactones in the form of hydrolysable tannins called ellagitannins, commonly found in *Anacardium occidentale* L., *Myracrodruon urundeuva* (Allemão), *Anogeissus leiocarpus* (DC.) Guill. & Perr., *Quercus infectoria* Olivier, and

Stryphnodendron obovatum Benth. (Kheirandish et al. 2016; Murakami et al. 1991; Ribeiro et al. 2015; Shuaibu et al. 2008; Vattem et al. 2005).

Gallic acid (GA) has demonstrated several pharmacological properties such as antioxidant, antiinflammatory (Yang et al. 2015), antimicrobial (Sarjit et al. 2015), antimutagenic, and anticancer (Lee et al. 2003; Lu et al. 2010; Paolini et al. 2015). Besides, the most important biological activities induced by ellagic acid (EA) involve the anticancer, hepatoprotective, DNA topoisomerase inhibitor (Aggarwal and Shishodia 2006; Cortazar et al. 2007; Vattem et al. 2005), antioxidant (Devipriya et al. 2007a, b), antiinflammatory (Papoutsis et al. 2008; Yuce et al. 2007), and gastroprotective (Iino et al. 2001). The antileishmanial activity of GA and EA on promastigote forms has been already reported (Kayser et al. 2003; Kolodziej and Kiderlen 2005; Ribeiro et al. 2015). Interestingly, the increase of NO and cytokines in *L. major*-infected RAW 264.7 cells was previously reported for GA (Radtke et al. 2004). However, the antileishmanial effect of EA against macrophage-internalized amastigote forms of *L. major* as well as its immunomodulatory activity resulting in solving the parasited macrophages still remain unclear.

The lack of safe and effective conventional treatments against leishmaniasis has been arising the search for new therapeutic alternatives, focused not only in the cytotoxic effect against the parasite but also in the ability to synergistically modulate the immune response of the host (Islamuddin et al. 2015; Roatt et al. 2014). In this context, the antileishmania, cytotoxic, and immunomodulatory potential of gallic and ellagic acids were explored in this work.

Material and methods

Chemicals

Dimethyl sulfoxide (DMSO: 99%; PubChem CID: 679), Panoptic staining (PubChem CID: 13735) was purchased from Merck Chemical Company (Germany). The Schneider's medium (PubChem CID: 2723893, RPMI medium (PubChem CID: 1640), fetal bovine serum (FBS; PubChem CID: 86289556), MTT (3-(4,5-dimethylthiazol-2-yl)2,5-diphenyltetrazolium bromide; PubChem CID: 64965), resazurin (PubChem CID: 11077), GA (PubChem CID: 370; Fig. 1a), EA (PubChem CID: 5281855; Fig. 1b), Fura-2/AM (PubChem CID: 24894734), and the antibiotics penicillin and streptomycin (PubChem CID 71311919) were purchased from Sigma-Aldrich (St. Louis, MO, USA). The antibiotic amphotericin B (90% Anf B) was purchased from Cristália (São Paulo, SP, Brazil). The GA and EA were diluted in DMSO at a concentration of 80 mg/mL for the experiments.

Parasites and animals

The *L. major* strain (MHOM/IL/80/Friendlin) was obtained from the Medicinal Plants Research Center of Federal University of Piauí. Parasites were grown in supplemented Schneider's medium (10% heat-inactivated FBS, 100 U/mL penicillin, and 100 µg/mL streptomycin at 26 °C) (Cameiro et al. 2012; Valadares et al. 2011).

Murine macrophages were collected from the peritoneal cavities of male and female BALB/c mice (4–5 weeks old; Medicinal Plants Research Center, UFPI, Brazil), and cultivated in RPMI 1640 medium (10% heat-inactivated FBS, 100 U/mL penicillin, and 100 µg/mL streptomycin at 37 °C and 5% CO₂). Red blood cells were obtained by centrifugation of sheep blood (9 months old). All protocols were approved by the Animal Research Ethics Committee (CEEA-PI no. 053/2015).

Investigation of activity against promastigote forms of *Leishmania major*

Promastigote forms of *L. major* in the late log phase (1×10^6 leishmania/100 µL of medium) were plated in 96-well culture plates containing supplemented Schneider's medium. Then, GA and EA (6.25, 12.5, 25, 50, and 100 µg/mL) were added, and the plates were incubated during 48 h in a BOD (biochemical oxygen demand) incubator at temperature of 26 °C. Remaining 6 h to the end of this period, 20 µL of resazurin (1×10^{-3} mol/L) was added. Afterwards, the absorbances were read in a BioTek microplate reader (model ELx800) at a wavelength of 550 nm. The results were expressed as inhibition of parasite growth (%).

Amphotericin B (Amph B) was used as positive control at concentrations of 0.32, 0.75, 1.25, 2.5, and 5.0 µg/mL. The negative control was the Schneider's medium with promastigotes (1×10^6 cells/well). The cell viability was considered as 100% for the parasite. The blank was read for each concentration and control in order to avoid interference of absorbance of medium of other compounds.

Determination of cytotoxicity for GA and EA

Cytotoxic effect on macrophages and the calculation of selective index

Cytotoxicity evaluation was carried out in 96-well plates using the MTT assay (de Medeiros et al. 2011; Gonçalves et al. 2016). Macrophages (2×10^5 per well) were incubated in 100 µL of supplemented RPMI 1640 medium at 37 °C and 5% CO₂ for 4 h. Non-adherent cells were removed by washing with RPMI 1640 medium. Then, GA and EA were diluted in supplemented RPMI 1640 medium, and added at concentrations of 6.25, 12.5, 25, 50, and 100 µg/mL, followed by incubation at 37 °C with

5% CO₂ for 2 days. The cytotoxicity of Amph B was assessed at concentration of 0.2 µg/mL. Afterwards, 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 a BioTek (ELx800) plate reader. Selectivity index of each treatment was calculated by the ratio between the mean cytotoxic concentration (CC₅₀) for BALB/c peritoneal macrophages and the mean effective concentration (EC₅₀) for promastigote forms of *L. major*.

Red blood cell lysis assay

The hemolytic activity was evaluated by incubating 80 µL of 5.0% fresh sheep red blood cells suspension in PBS with 20 µL of different concentrations of GA, EA, or Amph B, as described previously. The samples were incubated at 37 °C for 1 h. The reaction was slowed by adding 200 µL of PBS, and then the suspension was centrifuged at 1000×g for 10 min). The supernatant was transferred to a 96-well plate, and cell lysis was measured at 540 nm, as previously described. The blank control and maximal lysis (positive control) were obtained by replacing the substance sample with in equal volume of PBS or distilled water, respectively (Lofgren et al. 2008).

Investigation of GA- and EA-induced activity on macrophages infected by *Leishmania major*

Macrophages (2×10^5 cells/mL) were harvested in 24-well plates containing sterile round coverslips at 13 mm and supplemented RPMI 1640 medium (10% inactivated FBS, 100 U/mL penicillin, and 100 µg/mL streptomycin). Culture plates were incubated at 37 °C and 5% of CO₂ for 3 h. Adhered macrophages were then incubated with a new medium containing axenic amastigotes at a ratio of 10 amastigotes per 1 macrophage at 5% CO₂ and 37 °C for 4 h. The medium was subsequently aspirated in order to remove non-internalized parasites, and the wells were washed with 0.01 M phosphate buffered saline (PBS). The infected macrophages were then incubated with GA at 15, 30, and 60 µg/mL (non-toxic concentrations on host cells); EA at 1.75, 3.5, and 7 µg/mL (non-toxic concentrations on host cells); or Amph B at 0.2 µg/mL. 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 (survival index, obtained by counting the number of parasites in 100 macrophages) were counted using optical microscopy (Cameiro et al. 2012). Furthermore, the selectivity indexes of GA, EA, and Amph B were determined by the ratio of the mean CC₅₀ against macrophages to the mean EC₅₀ against macrophage-internalized amastigote forms of *L. major*.

Evaluation of parameters related to macrophage activation

Lysosomal activity

Macrophages (2×10^5 /well) were incubated with GA (15, 30, and 60 $\mu\text{g/mL}$), EA (1.7, 3.5, and 7 $\mu\text{g/mL}$), or Amph B (0.2 $\mu\text{g/mL}$) in a 96-well plate at 37 °C and 5% de CO_2 . After 48 h, 10 μL of neutral red stock solution were added for 30 min. Then, the supernatant was discarded, the wells were washed with 0.9% saline at 37 °C, and 100 μL of extractive solution were added in order to solubilize the neutral red present within the lysosomal secretory vesicles. After 30 min on a Kline shaker, the absorbances were read in a BioTek (ELx800) plate reader at 550 nm (Bonatto et al. 2004).

Phagocytic capability

Macrophages (2×10^5 /well) were incubated with GA (15, 30, and 60 $\mu\text{g/mL}$), EA (1.7, 3.5, and 7 $\mu\text{g/mL}$), or Amph B (0.2 $\mu\text{g/mL}$) in a 96-well plate for 48 h at 37 °C and 5% de CO_2 . After 48 h, 10 μL of zymosan-stained NR solution was added for 30 min. Next, the phagocytic process was interrupted adding 100 μL of Baker's fixative solution during 30 min. Then, the wells were washed with 0.9% saline, and 100 μL of extractive solution were added. After solubilization in a Kline shaker, the absorbances were read in a BioTek (ELx800) plate reader at 550 nm (Grando et al. 2009).

Nitrite measurement

Non-infected macrophages or infected by *L. major* were obtained as described previously, and then incubated with GA (15, 30, and 60 $\mu\text{g/mL}$), EA (1.7, 3.5, and 7 $\mu\text{g/mL}$), or Amph B (0.2 $\mu\text{g/mL}$) at 37 °C and 5% de CO_2 for 24 h. The lipopolysaccharide (LPS) from *E. coli* (2 $\mu\text{g/mL}$) was used as positive control. The standard curve was prepared with sodium nitrite in RPMI medium at varying concentrations of 1, 5, 10, 25, 50, 75, 100, and 150 μM diluted in RPMI 1640 medium. After 24 h, the supernatants were transferred, and then incubated with equal parts of Griess reagent. Thereafter, the absorbances were read in a BioTek (ELx800) plate reader at 550 nm (Soares et al. 2007).

Quantification of cytoplasmatic calcium levels (Ca^{2+}) by confocal microscopy

Macrophages infected or non-infected by *L. major* as described previously were incubated with GA (30 $\mu\text{g/mL}$), EA (7 $\mu\text{g/mL}$) for 48 h at 5% CO_2 at 37 °C. Amph B (0.2 $\mu\text{g/mL}$) and DMSO (60 $\mu\text{g/mL}$) were used as positive and negative controls, respectively. Then, the supernatant was removed, and macrophages were loaded with Fura-2/AM (10 μM) dye for 30 min

(Goncalves et al. 2013). Right after, cells were washed twice with sterile PBS, and imaged in a DSU confocal fluorescence microscope (IX81, Olympus, Japan) coupled to a CCD camera (Hamamatsu, Japan), using 340/380-nm filters. ROIs were created around infected and non-infected macrophages, and the relative fluorescence emitted at 525 nm was quantified by proper software (Cell®, Olympus, Japan) and expressed as arbitrary units of absorbance (a.u.).

Statistical analyses

All assays were performed in triplicate in three independent experiments. The mean EC_{50} and mean CC_{50} with confidence limits of 95% were determined by regression of probits using the software SPSS 13.0. The selectivity index was calculated as the ratio between CC_{50} and EC_{50} . One-way analysis of variance ANOVA followed by the Bonferroni test was performed using the GraphPad Prism version 5.0 program. The statistical significance was considered when $p < 0.05$.

Results

Anti-*Leishmania* activity assay

GA and EA demonstrated concentration-dependent antileishmanial activity on promastigote forms of *L. major*. GA inhibited around 80% of the growth of *L. major* promastigote forms at the concentration of 100 $\mu\text{g/mL}$, whereas EA promoted maximal effect at 100 $\mu\text{g/mL}$. All tested concentrations were able to inhibit the promastigote growth. Amph B exhibited 90% of the growth inhibition of promastigote forms at 2.5 $\mu\text{g/mL}$. The EC_{50} values obtained against promastigote forms are listed in Table 1.

Citotoxicity assessment

The GA- and EA-induced cytotoxic effects against BALB/c murine peritoneal macrophages and sheep red blood erythrocytes are demonstrated in Fig. 2. The GA demonstrated significant cytotoxicity against macrophages by MTT test, starting from the 50 $\mu\text{g/mL}$, with CC_{50} value of 126.6 $\mu\text{g/mL}$, whereas EA

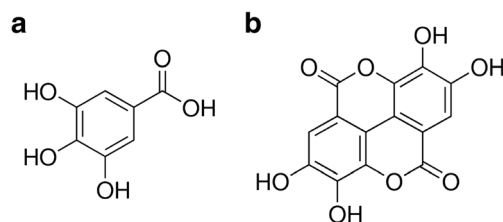


Fig. 1 Molecular structures of the phenolic compounds gallic acid (a) and ellagic acid (b). Gallic acid (GA) has molecular weight of 170.12 g/mol; ellagic acid (EA) is classified as a GA dimer, and has molecular weight of 302.197 g/mol

Table 1 Anti-*Leishmania* activity and cytotoxic effects against mammalian cells for gallic acid (GA), ellagic acid (EA), and amphotericin B (Amph B)

Compounds	Macrophages	Red blood cells	Promastigotes	Intramacrophagic amastigotes	
	CC ₅₀ (μg/mL)	CH ₅₀ (μg/mL)	EC ₅₀ (μg/mL)	EC ₅₀ (μg/mL)	SI _m ^b
GA	126.556	NT ^a	16.408	5.042	25
EA	23.811	NT ^a	9.812	0.928	26
Amph B	8.750	NT ^a	1.742	0.20	43.75

^a Non-toxic at tested concentrations^b Selectivity index for intramacrophagic amastigotes (CC₅₀/EC₅₀)

reduced the viability of macrophages starting from 6.3 μg/mL, resulting in CC₅₀ value of 23.8 μg/mL. The Amph B presents high cytotoxicity against murine macrophages, with CC₅₀ of 8.750 μg/mL. GA, EA, and Amph B did not induced any toxicity on sheep erythrocytes (Table 1).

Effects of GA and EA against infection of macrophages by *L. major*

GA and EA were able to reduce the number of infected macrophages and survival index (Fig. 3). The concentration-dependent decrease of infected cells treated with GA reduced parasitism to approximately 44, 37, and 25% when incubated at concentrations of 15, 30, and 60 μg/mL, respectively. Likewise, the parasitism observed after treatment with EA at concentrations of 1.75, 3.5, and 7 μg/mL were 44, 38, and 30%, respectively (Fig. 4). By the way, Amph B at 0.2 μg/mL decreased the number of infected macrophages to approximately 39%, whereas the negative control was not able to affect the infection of macrophages by *L. major*.

The assessment of survival index showed the negative control group (vehicle) with around seven amastigotes/macrophage, and the positive control group (0.2 μg/mL Amph B) with two amastigotes/macrophage. In this study, after treatment with GA at concentrations of 15, 30, and 60 μg/mL, a concentration-dependent reduction of the amount of amastigotes per macrophages by 2.0, 1.1, and 0.5, respectively, was observed. Besides, EA at concentrations of 1.8, 3.0, and 7.0 μg/mL reduced survival index to 1.8, 1.6, and 1.4 amastigotes/macrophages, respectively (Fig. 4). The EC₅₀ values for survival index of amastigotes on macrophages by *L. major* were calculated for GA and EA, and the values of 5.0 and 0.9 μg/mL were determined, respectively. Interestingly, GA and EA demonstrated higher selectivity to parasites than mammalian cells, resulting in the values of 25 and 26, respectively (Table 1).

Determination of lysosomal activity and phagocytic capability

The GA- or and EA-induced macrophage activation were assessed based on the retention of neutral red and zymosan

particles by macrophages, suggesting the possible immunomodulatory effects on the lysosomal activity and phagocytic capability, respectively. In this work, GA was able to increase the lysosomal activity in macrophages at 15 μg/mL (Fig. 5a). Otherwise, EA was not able to induce any alteration (Fig. 5b). Besides, GA and EA were able to increase the phagocytic capability in all tested concentrations.

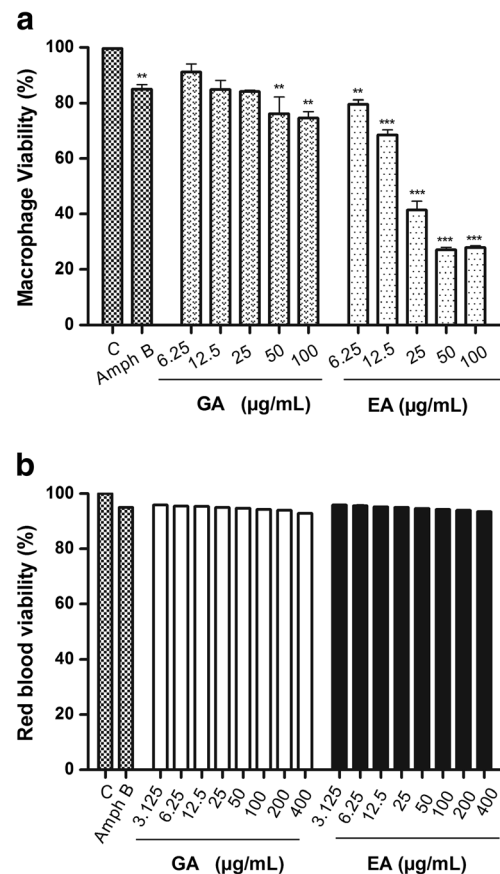


Fig. 2 Cytotoxic effects of GA and EA against BALB/c murine peritoneal macrophages (a) and sheep red blood cells (b). Macrophages and red blood cells were incubated with GA, EA, or Amph B for 48 h. The macrophage viability was evaluated using tetrazolium salt (MTT) test. Data are presented as mean ± SEM of three experiments performed in triplicate **p* < 0.05; ***p* < 0.01; ****p* < 0.001 when compared with control (C) or Amph B

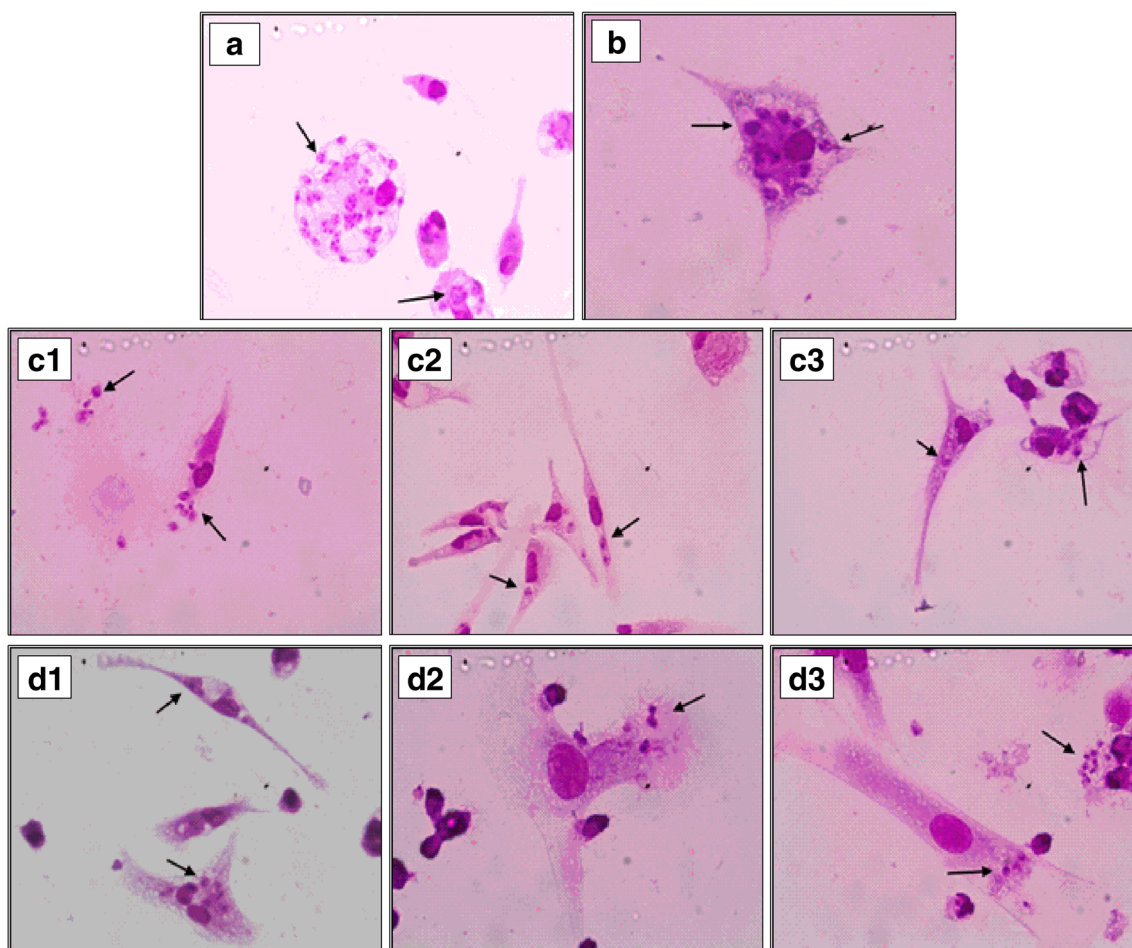


Fig. 3 Macrophages experimentally infected by *Leishmania major* (a). Amph B was used as a positive control at the concentration of 0.2 µg/mL (b). For GA treatment, concentrations of 15 (c.1), 30 (c.2), and 60 µg/mL

(c.3) were used. The EA was evaluated at concentrations of 1.75 (d.1), 3.5 (d.2), and 7.0 µg/mL (d.3). The arrows indicate macrophage-internalized amastigote forms of *L. major*

Measurement of nitrite production

The nitrite production was measured after incubation of GA or EA with non-infected or infected by *L. major*. The GA increased significantly the production of nitrite in non-infected macrophages only at concentration of 60 µg/mL, whereas in the macrophages infected by *L. major*, the nitrite production synthesis occurred in all tested concentrations (Fig. 6a). Besides, non-infected macrophages treated with EA did not show any increase in nitrite levels. Interestingly, a significant increase of nitrite production was observed in macrophages infected by *L. major* at all tested concentrations (Fig. 6b). The LPS was used as a positive control, demonstrating significant nitrite production when compared with the control group.

Cytoplasmic calcium level (Ca^{2+}_i) in murine macrophages

The cytoplasmic levels of Ca^{2+} (Ca^{2+}_i) were determined in the presence of GA, EA, or Amph B in non-infected and infected macrophages by *L. major* (Fig. 7). A significant increase of 130 a.u. in the relative fluorescence was observed for GA

(30 µg/mL) in both infected and non-infected macrophages. A similar increase was also observed in infected macrophages treated with 7 µg/mL of EA, which increased the relative fluorescence to 120 and 150 (a.u.) for non-infected and infected macrophages, respectively. The negative control (DMSO 60 µg/mL) and Amph B (0.2 µg/mL) did not cause any alteration on [Ca^{2+}_i].

Discussion

Studies focused on natural products and their potential application for new treatments for leishmaniasis have been quite promising. The search for new therapeutic alternatives is based on the discovery of the bioactive molecules commonly derived from plants. In this sense, the plant-derived polyphenols have demonstrated marked antileishmania activity against different species and forms of the parasite in both in vitro and in vivo studies. Among these compounds, stilbenoids, phenylpropanoids, flavonoids, and quinones have been reported (de Jesus et al. 2012). Ogungbe et al.

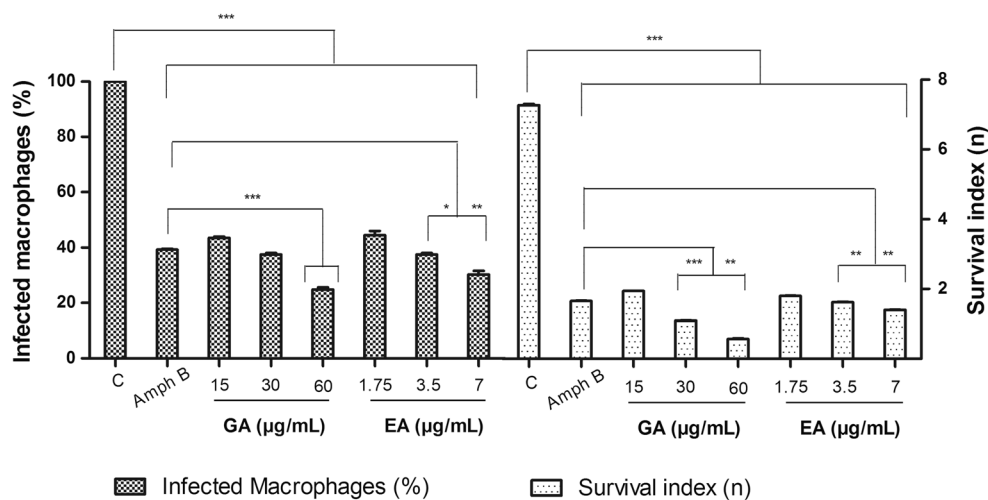


Fig. 4 Effects of GA, EA and amphotericin B on infected macrophages and survival index of BALB/c murine macrophages infected with *Leishmania major*. Cells were treated with GA, EA or Amph B for

48 h. Data are presented as mean \pm SEM of three experiments performed in triplicate. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$ when compared with control (C)

(2014) have described around 352 phenolic compounds with in silico antileishmanial activity towards protein targets, including 10 aurones, 6 cannabinoids, 34 chalcones, 20 chromenes, 52 coumarins, 92 flavonoids, 41 isoflavonoids, 52 lignans, 25 quinones, 8 stilbenoids, 9 xanthenes, and 3 different phenolic compounds. Thus, there is a wide range of substances probably acting sole or synergistically, and then studies regarding the antileishmanial potential of this new molecules are reinforced.

In this study, the antileishmania and cytotoxic potential of gallic and ellagic acids, as well as the immunomodulatory mechanisms related to macrophage activation, were investigated. Both GA and EA demonstrated a high potential as growth inhibitors of promastigote forms of *L. major*, corroborating with studies of antileishmania activity induced by phenolic compounds (Ogungbe et al. 2014; Rizk et al. 2014). Similar EC_{50} values to GA and EA were previously reported to *Stryphnodendron obovatum leiocarpus* (DC.) Guill. & Perr. and *Anogeissus leiocarpus* Benth (Ribeiro et al. 2015; Shuaibu et al. 2008).

The urge to obtain novel molecules with antileishmania activity with higher selectivity towards the parasite, and thus less toxic to the host cells, is urgent. Thus, toxicological investigation of potential therapeutic alternatives against leishmaniasis is markedly important, since the parasite is an obligate intracellular organism (de Medeiros et al. 2011; Islamuddin et al. 2015). The selectivity index (SI) represents how much a treatment is more toxic to the parasite rather than the mammalian host cells. For macrophage-internalized amastigotes, the SI of drug candidates is recommended to be close or greater than 20 (Nwaka and Hudson 2006). In this study, both GA and EA showed significant cytotoxicity against these cells, resulting in a CC_{50} value of 126.6 $\mu\text{g/mL}$ by GA and 23.8 $\mu\text{g/mL}$ by EA. Interestingly, the ratios of

CC_{50} on macrophage cells to EC_{50} against macrophage-internalized amastigotes for both compounds indicate selectivity indexes higher than 20 (Table 1). Besides, GA and EA did not promote hemolytic effect on sheep erythrocytes. Sheep, human (O^+ -type blood), and rabbit erythrocytes have been widely used to assess damage of cell membranes, since they lack high expression levels of proteins or glycoproteins, that could be activated by the new drugs, which might result false positive hemolytic activity (Carneiro et al. 2012).

The experimental model based on macrophage-internalized amastigotes forms of *Leishmania* spp. is considered the in vitro model that adequately resembles the infection that occurred in the host cells (Carneiro et al. 2012). Therefore, compounds able to reduce the survival index of parasitized cells by *Leishmania* spp. are quite promising and relevant to be investigated using in vivo experimental models. In this study, GA and EA were highly efficient in reducing the survival index of murine macrophages by *L. major* in a concentration-dependent manner. Microscopy data show a large agglomeration of amastigotes around the parasitophorous vacuoles in the control group, due to the structural integrity and accumulation of F-actin and myosin Va in the peripheral region, a mechanism established by the parasite to counter cellular defenses, being essential for the establishment of leishmanial infection (Azevedo et al. 2012; Lodge and Descoteaux 2005; Roy et al. 2014a, b). In the groups treated with GA or EA, the parasitophorous vacuoles present a “smog” aspect due to the activation of phagolysosomes, probably due to the depolymerization of F-actin against the evasive mechanisms of the parasite, and then solving the infection (Lodge and Descoteaux 2005). In our study, no alterations were observed in cells treated with Amph B, probably due to its leishmanicidal activity is related to the inhibition of the ergosterol synthesis in the parasite

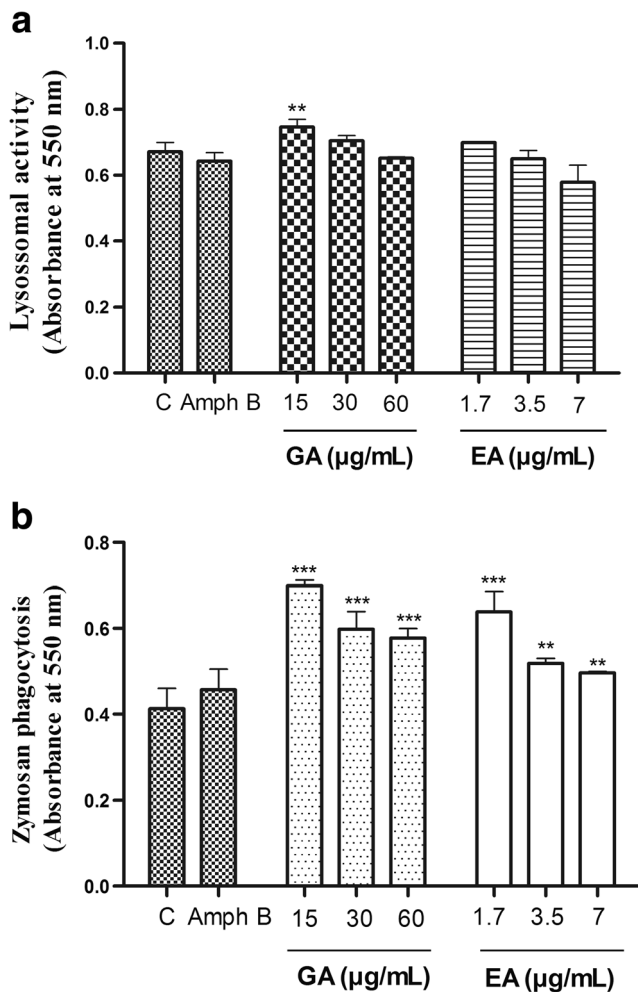


Fig. 5 Effects of GA, EA, and Amph B on lysosomal activity (a) and phagocytic capability (b). Murine peritoneal macrophages were treated at ranging concentrations for 48 h. Lysosomal activity and phagocytic capacity were assessed by quantification of neutral red (NR). Phagocytic capability was assessed by the incorporation of zymosan to NR, solubilized by the extraction solution. Data are presented as mean \pm SEM of three experiments performed in triplicate. * p < 0.05; ** p < 0.01; *** p < 0.001 when compared with control (C)

membrane (Lachaud et al. 2009; Sundar et al. 2007). According to Kolodziej and Kiderlen (2005) and de Macedo-Silva et al. (2011), this aspect observed after treatment with GA or EA is because these cells were activated and capable of solving the infection, not only because of the ability of the compounds to act against the parasite but also other signaling pathways which make the intracellular environment more hostile to the parasite are triggered. Natural products derived from plants are characteristic in reducing the infection and infectivity of macrophages affected by *Leishmania*, which corroborates to this study (Carneiro et al. 2012; de Medeiros et al. 2011; Dias et al. 2013; dos Santos et al. 2012; Rodrigues et al. 2013, 2015).

In this study, parameters of macrophage activation able to induce microbicidal activity, such as phagocytic capacity,

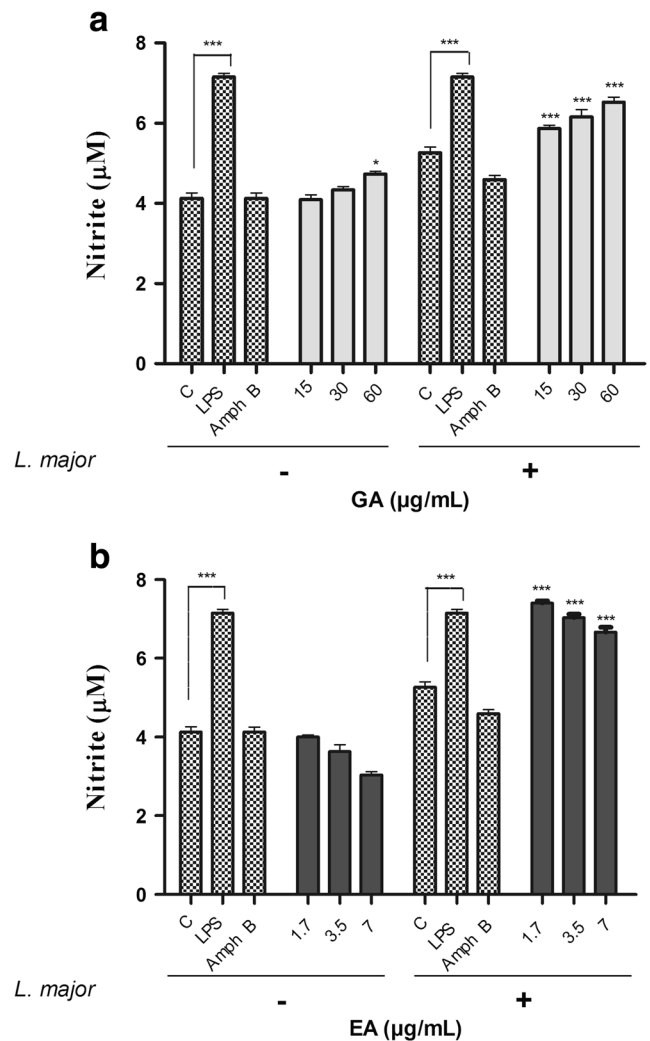


Fig. 6 Nitrite measurement in infected or non-infected BALB/c murine peritoneal macrophages treated with GA (a) or EA (b) and Amph B for 24 h. The culture supernatant was mixed in equal parts with the Griess reagent. LPS (lipopolysaccharide from *Escherichia coli*; 2 µg/mL) was used as positive control. Data are presented as mean \pm SEM of three experiments performed in triplicate. p < 0.05 when compared with non-infected (-) or infected (+) macrophages from control group; p < 0.05 when compared with non-infected (-) or infected (+) macrophages from the LPS group HNS71387

lysosomal activity, induction of NO synthesis, and quantification of cytoplasmic calcium, were evaluated. They acting synergistically in order to control *Leishmania* infections. The dichotomy between the Th₁- and the Th₂-type cellular immune response in hosts affected by the disease leads to the investigation of novel therapeutic alternatives which possess activity not only targeted to the parasite but also induce immunomodulatory activity in order to prevail immune response of the Th₁-type host (Islamuddin et al. 2015). The substances evaluated in this study (GA and EA) induced immunomodulatory effects (Kolodziej and Kiderlen 2005). Phagocytosis and lysosomal activity are functions activated in the innate immune response, important for the control of infections, leading to

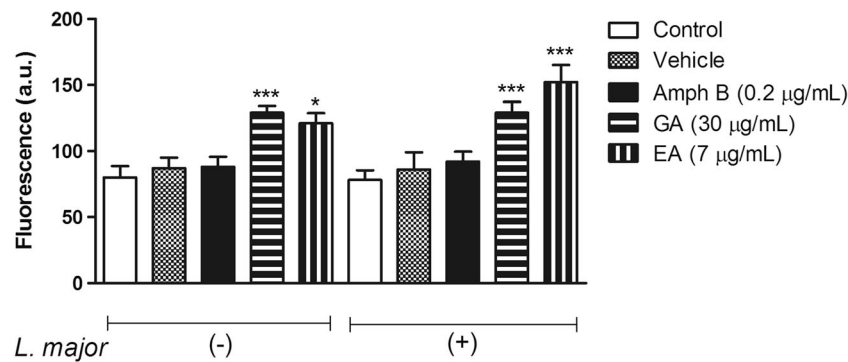


Fig. 7 Quantification of cytoplasmic calcium in BALB/c murine macrophages in the absence (-) or presence (+) of internalized *Leishmania major* amastigote forms treated with vehicle (DMSO 60 µg/mL), Amph B, GA, or EA. The culture supernatant was removed, and RPMI containing Fura-2/AM (10 µM) was added. The coverslips were evaluated by

confocal microscopy using the 340/380-nm filters. Data are presented as mean ± SEM of three images of each coverslip captured by microscopy. The experiment was performed in triplicate * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$ when compared with non-infected (-) or infected (+) macrophages

parasitic degradation and presentation of antigens by MHC class II to CD4⁺ T cells (Niedergang and Chavrier 2004). After the endocytosis of the parasite, the formation of phagosomes from the endosomes occurs, followed by the fusion with lysosomes, then producing the phagolysosomes (Niedergang and Chavrier 2004).

The phagolysosome is a compartment filled with acid hydrolases and reactive oxygen species, and where degradation of pathogens occurs. Thus, pathogens are destroyed within the phagolysosome (Lee et al. 2003; Lopes et al. 2006). One important and highly pathway underlying the mechanism of antileishmania activity is the induction of NO production in macrophages, the most effective mechanism being involved in the defense against *Leishmania* (Gantt et al. 2001). The NO is synthesized after activation of macrophages by cytokines acting in synergism, such as interferon- γ (IFN- γ) and tumor necrosis factor alpha (TNF- α), which increases the expression of inducible nitric oxide synthase (iNOS), an enzyme which catalyzes L-arginine to generate NO and citrulline (Liew et al. 1990). Once inside the phagolysosome, NO combines with superoxide to produce peroxide nitrite, which is highly reactive and acts as microbicidal agent (Bogdan and Rollinghoff 1998; Ueda-Nakamura et al. 2006). Interestingly, the NO pathway induced by EA in macrophages was probably co-stimulated after infection with *L. major*, thus being a high immunomodulatory capacity only in the presence of the pathogen, without altering the cell physiology. These observations corroborate with previous reports for natural products and polyphenols (Islamuddin et al. 2015; Kheirandish et al. 2016; Ogungbe et al. 2014; Rodrigues et al. 2015).

Another interesting factor is the cytoplasmic calcium levels in the host cell. As an important microbicidal agent, intracellular calcium promotes the activation of the classical protein kinase C (PKC) pathway, which activates pro-inflammatory cytokines, such as TNF- α and IFN- γ , in order to activate the cellular Th₁ immune response, and then promoting the recruitment of cells from mononuclear phagocytic

system in order to control the infection (Islamuddin et al. 2015; Roy et al. 2014a). The action of new drugs on the increase of intracellular calcium is directly related to this signaling pathway. In this study, GA and EA were able to increase cytoplasmic calcium levels, possibly promoting this activation. Furthermore, the depolymerization of F-actin from the peripheral region is another calcium-dependent mechanism. Phagocytosis by neutrophils, macrophages, and other professional phagocytes requires rapid remodeling of actin. The disaggregation of periphagosomal F-actin, phagocytosis, and phagocytosome maturation are calcium-dependent processes in macrophages when they interact with pathogens in order to solve infections (Tejle et al. 2002).

Radtke et al. (2004), Kolodziej and Kiderlen (2005), and Yadav et al. (2012) have described the immunomodulatory activity of phenolic compounds, including GA, capable of promoting increased NO synthesis, as well as increasing the expression of genes, resulting from the production of pro-inflammatory cytokines, such as TNF- α and IFN- γ . Besides, miltefosine is one of the conventional drugs widely used in Africa and Europe as the only one to be administered orally to combat leishmaniasis. Its mechanism of action is described by Verma and Dey (2004) as immunomodulatory activity through the IP3/PLC/PKC pathway. In this sense, evidences that GA and EA might act by similar mechanisms can be hypothesized.

In conclusion, GA and EA induced marked activity against promastigote forms of *L. major*, reducing the number of infected macrophages and survival index of *L. major* internalized amastigotes in parasitized macrophages, as well as induced macrophage activation by increase of phagocytic capability, lysosomal volume, NO synthesis, and cytoplasmic calcium release. Therefore, GA and EA are promising molecules for studies regarding the treatment of leishmaniasis. Further investigations are reinforced in order to evaluate the therapeutic effects of GA and EA in in vivo experimental infection model of leishmaniasis.

Compliance with ethical standards All protocols were approved by the Animal Research Ethics Committee from Federal University of Piauí, Brazil (CEEA-PI no. 053/2015).

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