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# METHODS OF MACROPHAGES ACTIVATION AND THEIR MODULATION FOR THE PROSPECTION OF NEW ANTILEISHMANIA DRUGS: A REVIEW

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## Abstract

Leishmaniasis are a group of parasitic zoonoses provoked by protozoa from *Leishmania* genus and belonging to the group of neglected tropical diseases. The search and development for new drugs is necessary not only to investigate the activity against only the parasite, but also to investigate the possible synergistic effect of new drugs with the immune response of the host. In the present review, macrophages are pointed out as potential targets of the investigation of new antileishmanial drugs, and some methodologies in order to assess their activation as response to *Leishmania*-infected cells are presented. Macrophages are an important role in the cellular immune response, since they are cells from mononuclear phagocytic system, the first line of defense of the host, against parasites from *Leishmania* genus. Phagocytic capacity, lysosomal activity, increase of nitric oxide and intracellular calcium levels are parameters regarding assessment of macrophages activation which allow them to be more hostile in order to solve the infection and lead the patient to cure. In this context, we bring 19 substances already investigated and that activate macrophages, what makes them promising in the antileishmanial treatment. Therefore, assessment of macrophages activation, are important tools for discovery of immunomodulatory compounds which have potential to act in synergism with host immune response. Such compounds might be promising as monotherapy in the treatment of leishmaniasis, as well as being used as adjuvants in vaccines and/or in combination with conventional drugs.

**Keywords:** Immunomodulation. Intracellular Calcium. Leishmaniasis. Natural Products. Nitric Oxide. Phagocytosis.

## 1. Introduction

Protozoal infections are a worldwide public health problem, especially in underdeveloped or developing countries, corresponding to 14% of the world population at risk of infection (Kondrashin et al. 2011; Waldron et al. 2011). Leishmaniasis is a neglected tropical disease included among protozoal

infections, where 12 to 15 million people are infected worldwide with 1,5 to 2 million new cases occurring every year, being endemic in 98 countries and spreading on at least 5 continents (WHO 2019)

The infectious potential of leishmaniasis is due not only to the socio-economic context, but also to the high dissemination of the vector at urban areas (Harhay et al. 2011), and the ability of the parasite to harbor and multiply in the hostile interior of cells highly specialized in destroying intracellular pathogens, such as cells of the mononuclear phagocytic system, like the macrophages (Dumas et al. 1997; Naderer and Mcconville 2008; Roy et al. 2014; Islamuddin et al. 2015).

The biological cycle of this parasite, begins when the female of the insect vector of the Phlebotominae Family performs its blood repast, where promastigotes, the flagellate forms of the parasite, are present. During repast, the female vomits the metacyclic promastigotes which immediately are phagocytosed by the macrophages present on the skin of the vertebrate host. Then, the promastigotes become aflagellate, herein denominated as amastigotes (Harhay et al. 2011). In order to survive inside host cells and then spread into host organ, mechanisms of immune silencing and evasion are triggered in the presence of parasites in order to escape from oxidative stress induced by positive hydrolases, lysosomal attack inside macrophage vacuoles, and intracellular calcium which facilitates alterations of the cellular cytoskeleton. Moreover, calcium acts as a microbicidal molecule and a fundamental tool in depolarization of F-actin, a protein which invades the vacuoles and destroys the parasite (Dumas et al. 1997; Dolai et al. 2009).

The mechanisms underlying survival of intracellular amastigotes within macrophages and establish parasitaemia are based on a strong predominance of a cellular immune response triggered by T-helper type 2 (Th-2) lymphocytes, since the restoration of Th-1-type cellular immunity is essential for disease control (Gurunathan et al. 2000; Islamuddin et al. 2015). Firstly, we need to understand how activation of the immune response triggered by Th lymphocytes occurs. Into vertebrate host, when a pathogen is phagocytosed by a professional antigen presenting cell (APC), it is attacked by lysosomal enzymes, promoting oxidative stress, and positive hydrolases to be processed and presented as antigens by molecules of the major histocompatibility complex (MHC) class II (Gill et al. 2003; Klein et al. 2009). Along with the antigen being presented, host cells initiate the expression of cytokines. These two events are sufficient for the activation of Th lymphocytes (Luckheeram et al. 2012). Cytokines, such as interleukins 4 and 10 (IL-4 and IL-10), activate the Th-2 immune response, where Th lymphocytes recruit and activate B lymphocytes to produce immunoglobulins (Ig). In addition, INF-γ, TNF-α and IL-1β activate a Th-1 type immune response, where the Th cells starts recruiting more cells of the mononuclear phagocytic system (MPS), enabling them to increase the expression of nitric oxide (NO), increase the activity lysosomal, phagocytic capacity and $Ca^{2+}$ in order to destroy intracellular pathogens (Figure. 1) (Valadares et al. 2011; Roy et al. 2014; Islamuddin et al. 2015).

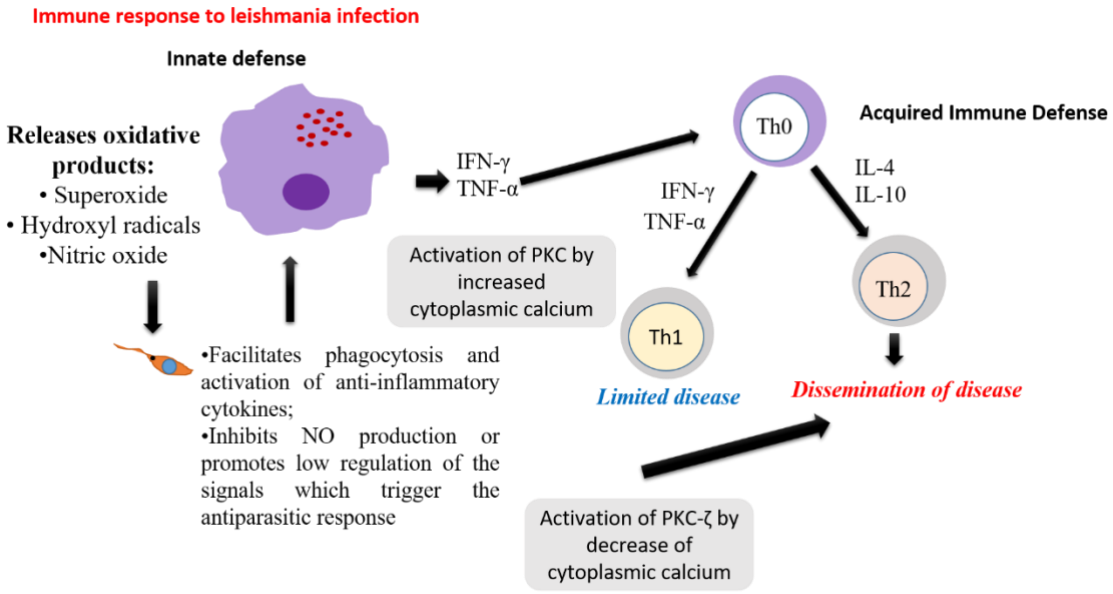


**Figure 1.** Immune response to leishmania infection. During The promastigotes forms of leishmania become amastigotes in the hostile interior of macrophages. Then, in order to survive and promote parasitemia, amastigotes inhibit NO production or promote low regulation of the signals that trigger the antiparasitic

response, thus establishing Th-2 type cellular response. In contrast, the cellular immune response of the Th-1 profile and restoration of the cellular immunity is fundamental for the control of the disease, especially when it involves the mechanisms of activation of macrophages, making them able to control the infection. Such mechanisms involve overregulation of lysosomal activation, phagocytic capacity, increased NO production and intracellular calcium. Adapted from (Bogdan et al. 1996)

Since traditional antileishmanial drugs are considered old-fashioned, limited, high costly and with a range of adverse effects, the search for new drugs with less toxic effects is important (Ashford 2000; Alizadeh et al. 2008). Such drugs must also act in synergism with the host immune response, making the Th-1 profile cellular response prevail so that memory T cells can confer definitive cure and protection against the possible relapses of the disease that usually occur in endemic areas, where wide exposure of hosts to the vector and etiological agent occurs (De Medeiros et al. 2011; Valadares et al. 2011; Carneiro et al. 2012; Rodrigues et al. 2013; Rodrigues et al. 2015; Souza et al. 2017; Alves et al. 2017).

In this sense, considering the immunopathology of leishmaniasis and the key role of the activation of immune cells, especially macrophages, as pharmacological targets to control parasitemia, the objective of this study was to explore the role of the activation of these cells and their modulation through protocols for assessment of immunomodulatory mechanisms for the study and/or discovery of possible new drugs. Furthermore, these protocols are discussed with the effect of previously reported immunomodulatory drugs with antileishmanial properties.

## 2. Material and Methods

### Obtainment of macrophages

Macrophages used in assays for immunomodulation drugs can be obtained from immortalized cell lines such as RAW 264.7 (Kolodziej and Kiderlen 2005) or obtained from peritoneal lavage of BALB/c mice (Alves et al. 2017; de Castro Oliveira et al. 2017; Souza et al. 2017).

In order to obtain murine peritoneal macrophages, animals aging between 4 and 6 weeks must be firstly euthanized under approval of the ethics committee responsible for each research center. After euthanasia, the animals are placed on a flat surface in dorsal decubitus. Right after, an incision is made in the abdomen so that the skin of the ventral region is removed, and the peritoneum is exposed. In total, 8 mL of isotonic phosphate buffered saline solution (PBS, in mM: NaCl, 145; $Na_2HPO_4$, 9.0; $Na_2HPO_4$, 1.0; pH 7,4) is intraperitoneally administered using a sterile needle and syringe. A light massage should be performed in this place in order to obtain the maximum number of cells. After removal of the cells, centrifugation is continued at 3000 rpm, at 4 °C for 10 minutes. After centrifugation, the supernatant should be discarded, and the pellet resuspended in 1.0 ml of RPMI 1640 medium supplemented with 10% fetal bovine serum (FBS) and penicillin-streptomycin 10,000 IU/10 mg. Then the cell counting using Trypan blue dye and a Neubauer chamber (Sigma-Aldrich, St. Louis, EUA) is performed. For each protocol, $2\times10^5$ cells per well are placed in 96-well cell culture dishes and incubated at 37 °C and 5% $CO_2$ (Alves et al. 2017; de Castro Oliveira et al. 2017).

### Preparation of solutions

The stock solution of neutral red dye (Sigma-Aldrich, St. Louis, EUA) is obtained by the solubilization of 2.0 mg of the dye in 1 mL dimethylsulfoxide (DMSO). The extraction solution used consists of glacial acetic acid 96% (1% v/v) and ethanol P.A. (50% v/v) dissolved in bidistilled water. The zimosan (Sigma-Aldrich, St. Louis, EUA) dye used for the phagocytic capacity assays is obtained by diluting 0.3 mL of the stock solution of neutral red and 0.02 g of zymosan in 3 mL of PBS. The Baker's fixative solution consists of formaldehyde 4% v/v, 2% w/v sodium chloride and 1% w/v calcium acetate in distilled water (Grando et al. 2009).

### Evaluation of phagocytic capacity

Macrophages are plated and pre-incubated at 37 °C and 5% $CO_2$ for 4 h according to item 2.1. After this period, the supernatant is removed to discard non-adherent cells from the plate and then the test drug

is added using serial concentrations ranging from 6.25 to 800 µg/mL (Alves et al. 2017). Then, 100 µL of Baker's fixative solution is added to paralyze the phagocytosis process. After 30 minutes, the plate is washed with 0.9% saline solution to remove non-phagocytized zymosan and neutral red. The supernatant is removed, and then 100 µl of extraction solution is added and lightly stirred for 10 minutes. Absorbances should be measured at 550 nm (Figure 2).

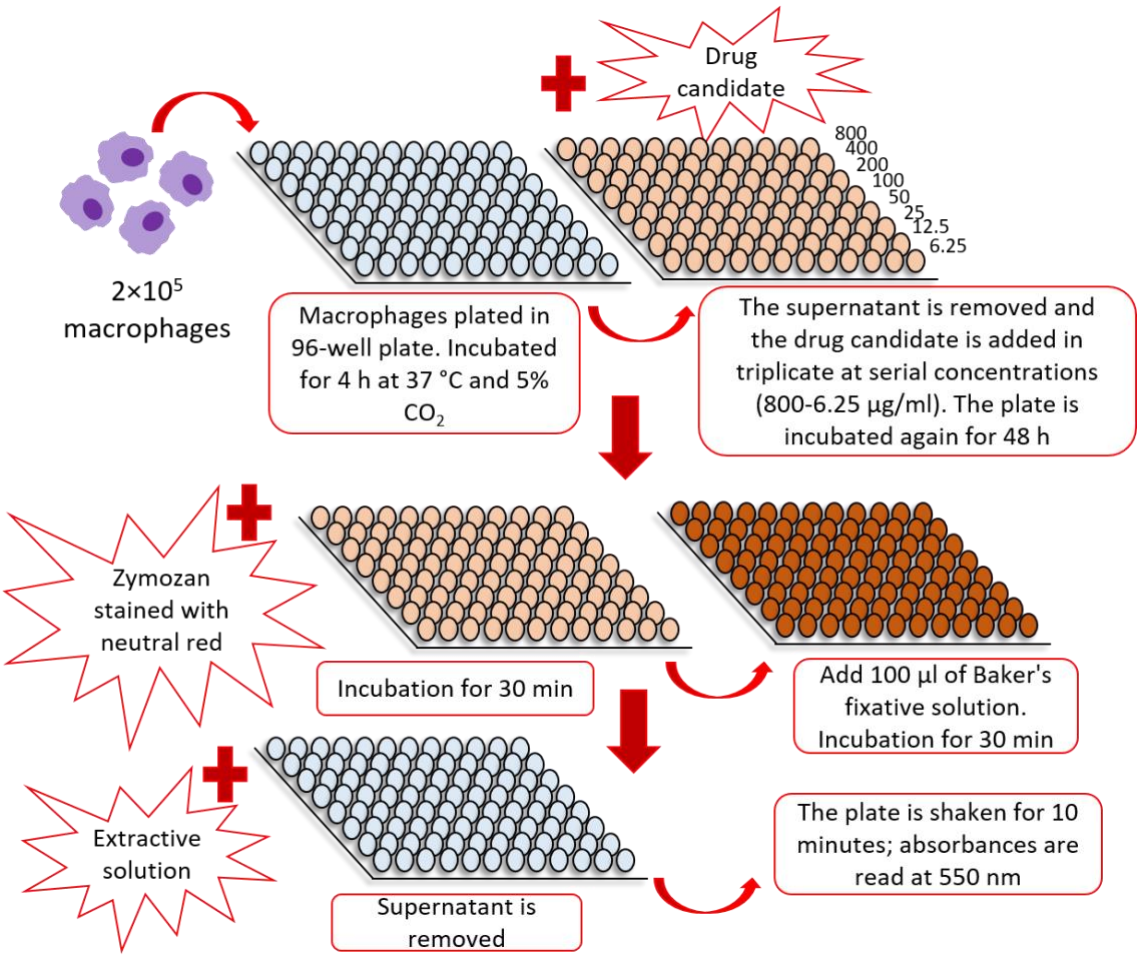


**Figure. 2.** Phagocytosis of zimozan particles by macrophages submitted to treatment by test drugs. The macrophages are incubated for 48 h at 37 °C in a 5% $CO_2$, in the absence and presence of different test drug concentrations, and thereafter, 10 µL of stained zymosan is added. The phagocytosis of zymosan particles is determined colorimetrically after solubilization with extraction solution.

**Evaluation of lysosomal activity**

Macrophages are plated and incubated at 37 °C and 5% $CO_2$ for 4 h according to item 2.1. After this time, the supernatant is removed in order to remove the non-adherent cells, and then the test drug is added at serial concentrations (e.g. 6.25 – 800 µg/mL) (Alves et al. 2017). After 48 h incubation at 37 °C and 5% $CO_2$, 10 µL of 2.0% neutral red solution in DMSO (w/v) is added and incubated for 30 min at 37 °C. After this procedure, the supernatant is removed, 100 µL of extraction solution is added, and then after 30 min the plate is submitted to slight agitation for 10 minutes. Absorbances are measured at 550 nm (Figure 3).

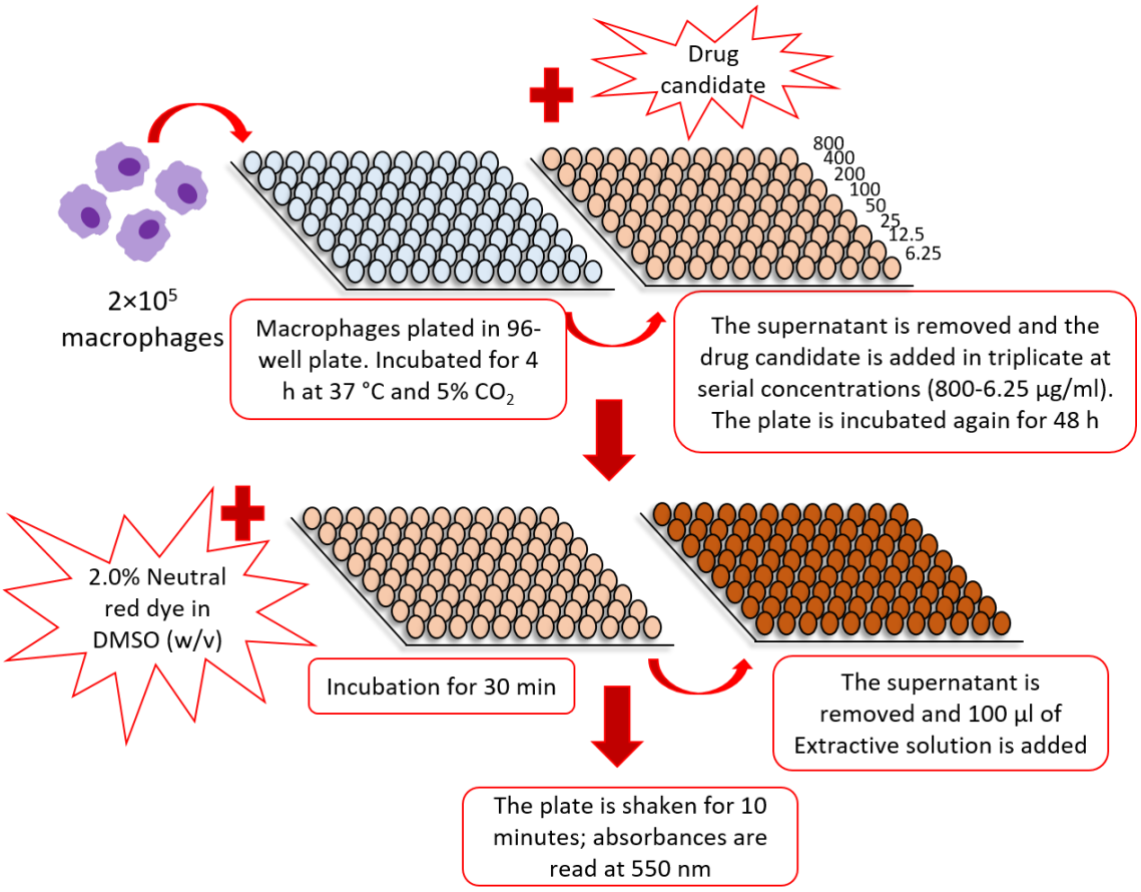


**Figure 3.** Lysosomal activity of macrophages submitted to treatment by test drugs. The macrophages are incubated for 48 h at 37 °C and 5% $CO_2$ in the absence or presence of different test drug concentrations, and thereafter, 10 µL of 2.0% neutral red is added. The neutral red stored in the secretory vesicles is solubilized with extraction solution and determined colorimetrically.

**Evaluation of nitrite production**

Macrophages are plated and incubated at 37 °C and 5% $CO_2$ for 4 h according to item 2.1. After this time the supernatant is removed to remove non-adherent cells from the plate and then the test drug is added at serial concentrations (6.25 – 800 µg/mL) (Alves et al. 2017). After a 24h-incubation period, the cell culture supernatants are transferred to another 96-well plate for measurement of nitrite content (Figure 4). The standard curve for nitrite dosing is prepared with sodium nitrite in Milli-Q® water at varying concentrations from 1 to 150 µM diluted in RPMI 1640 medium. Equal parts of the samples or solutions are prepared in order to obtain the standard curve with the same volume of the Griess reagent (5 % phosphoric acid containing 0.1% N-1-napthylethylenediamine dihydrochloride, 1% sulfanilamide) and the absorbances are read on the plate reader at 550 nm (Soares et al. 2007; Grando et al. 2009; Alves et al. 2017).

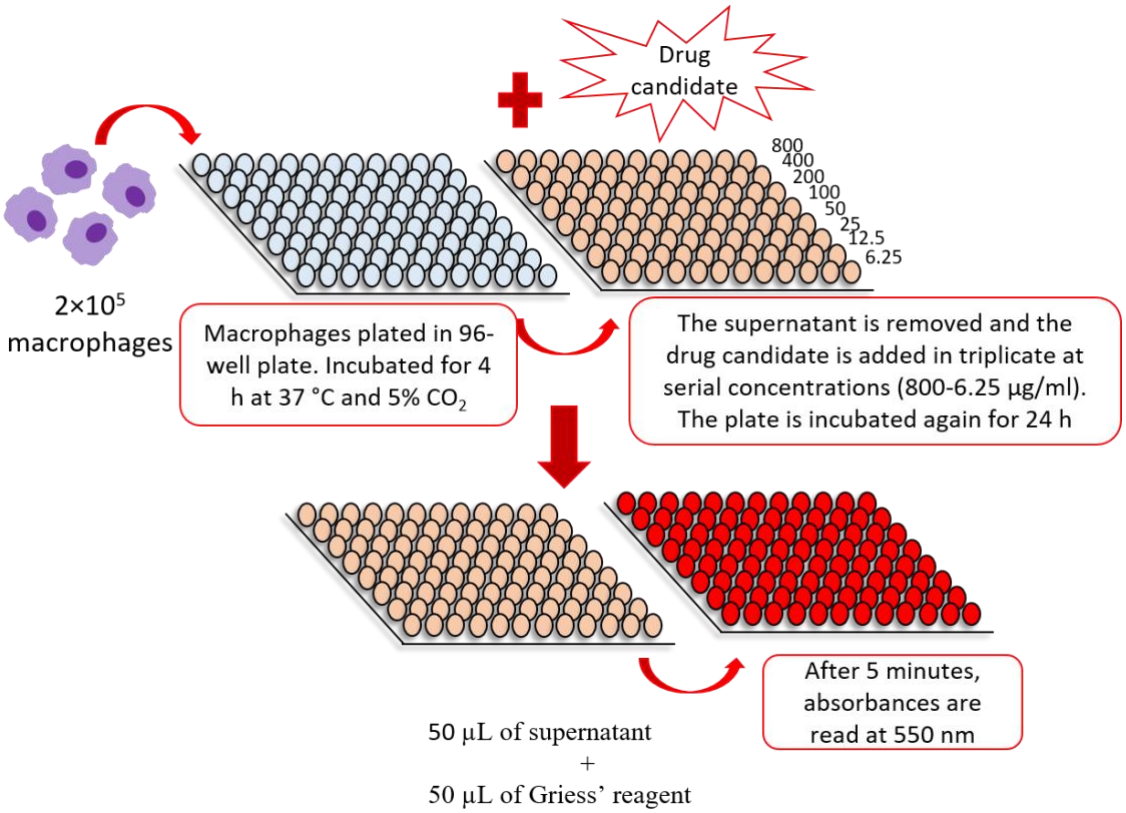


**Figure 4.** Evaluation of nitrite produced by macrophages treated with test drugs. The macrophages are incubated for 24 h at 37 °C in a 5% $CO_2$ in the absence and presence of different concentrations of test drugs, and thereafter, equal parts of Griess reagent and the supernatant are added. Nitric oxide is measured from the concentration of nitrites produced by treated macrophages.

**Quantification of intracellular calcium in macrophages**

Macrophages are plated and incubated at 37 °C and 5% $CO_2$ for 4 h according to item 2.1 in coverglass dishes with 35 mm diameter. After this time, the supernatant is discarded to remove the non-adherent cells on the plate and then the drug test is added at serial concentrations (e.g., 6.25 – 800 µg/mL) (Alves et al. 2017). 300 µL of RPMI medium containing a calcium sensitive fluorophore (e.g., Fura-2 or Fluo-3) at 10 µM is added and incubated for additional 30 min. Cells must be washed twice with sterile PBS in order to remove the excess from fluorophore and 1 mL of HBSS buffer added to each plate for experimentation. Calcium imaging is performed using epifluorescence or confocal microscope, equipped with 340/380 nm (excitation) and 525 nm (emission) filters, coupled to a high-speed CCD camera and compatible software (Figure 5). The cytosolic calcium concentration ($Ca^{2+}_i$) in each macrophage, selected as ROIs, can be estimated by applying the equation $[Ca^{2+}_i]=K_d \cdot (F-F_{min})/(F_{max}-F)$, where $K_d$ is the dissociation constant of the used fluorophore, F is the variable fluorescence intensity, $F_{max}$ and $F_{min}$ are, respectively, the maximum and minimum fluorescence obtained in the presence of ionomycin (5 µM) or EGTA (20 mM) (Alves et al. 2017).

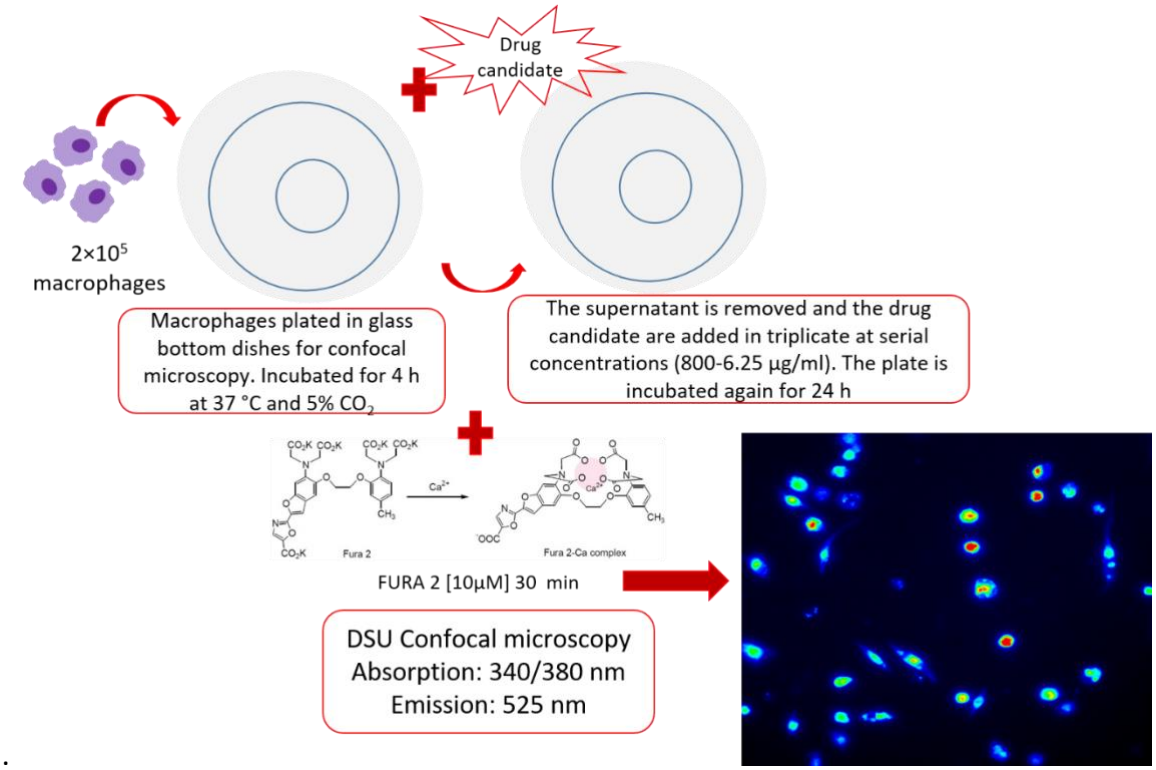


**Figure 5.** Quantification of intracellular calcium in macrophages submitted to stimuli by test drugs. Analysis performed using Fura-2/AM (10 µM) on confocal epifluorescence microscopy (DSU Olympus, Japan).

Another relevant factor is the depolymerization of F-actin, when there is significant presence of intracellular calcium in cells with internalized pathogens, Phagocytosis by neutrophils, macrophages and other professional phagocytes requires rapid remodeling of actin. Disaggregation of F-actin, phagocytosis and maturation of perifagosomal phagocytoma are calcium-dependent processes in macrophages when interacting with pathogens to resolve infections (Tejle et al. 2002).

## 3. Results and Discussion

Macrophages present several functions, such as phagocytosis, tumor cytotoxicity, cytokine secretion, and antigen presentation. They represent a line of defense against pathogens and tumor cells by recognizing and destroying (Young and Hardy 1995; Peters et al. 1996; Klimp et al. 2002). Characteristically, activated macrophages demonstrate increased adhesion and spreading capability, stimulation of DNA synthesis, increased intracellular calcium, modification of cytokine secretion, increased levels of lysosomal enzymes, increased microbicidal/tumoricidal activity by increased NO and reactive oxygen species (ROS), and increased membrane *ruffles* which improve the performance of functions such as locomotion and phagocytosis (Cleary et al. 1999). Activated macrophages which are capable of solve infections by intracellular pathogens as effect of immunomodulatory drugs are able to alter their morphology, becoming fusiform, due to their adhesion and spreading (Figure 6), aiming to increase their total area and facilitate phagocytosis of pathogens (Alves et al. 2017).

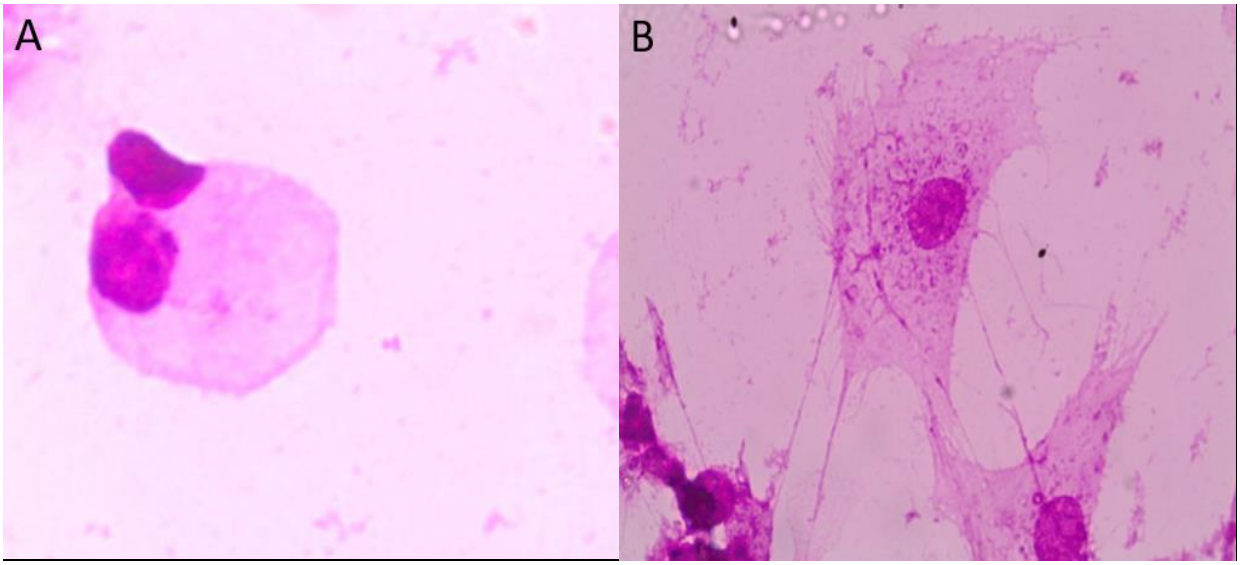


**Figure 6.** A – macrophage without stimulus by drug-activation; B – scattered after the stimulation by testing drugs derived from natural products. The spreading is due to phagocytic capacity activation.

Phagocytosis and the lysosomal system are critical for the functions of macrophages, where internalization, processing and presentation of antigens derived from pathogens to specific immune system cells occur in order to protect the host. After endocytosis of the pathogen, the newly created phagosome undergoes sequential fusion events with endosomes and then with lysosomes to produce a phagolysosome (Niedergang and Chavrier 2004). The phagolysosome is a compartment filled with acidic hydrolases and ROS where most of degradation of internalized content occurs. Drugs that act by activating macrophages in this target, are able to induce these cells to destroy internalized pathogens in their phagolysosomes, besides helping with up-regulation during antigen presentation (Greenberg and Grinstein 2002; Lee et al. 2003; Niedergang and Chavrier 2004).

During antileishmania activity, an important signaling pathway which might be involved is the production of NO by macrophages. Such mechanism has been considered as the most effective involved in host defense against leishmania. Within the phagolysosome, NO combines with superoxide anion to produce peroxynitrite, which is highly reactive and microbicidal (Bogdan and Rollinghoff 1998; Valadares et al. 2011). The parasite survives within the macrophage by the ability to inhibit the expression of inducible nitric oxide synthase (iNOS) enzyme or inhibition of the production of cytokines involved in its regulation, inhibition of NO synthesis by glycosylinositol, amastigote surface phospholipids or stimulus to the production of TGF-β growth transforming factor (Park et al. 2005). Drugs able to activate the NO pathway act by activating macrophages by cytokines such as interferon-γ (IFN-γ) and tumor necrosis factor α (TNF-α), which increase the production of nitric oxide synthase, an enzyme that catalyzes L-arginine to generate NO and L-citrulline (Liew et al. 1990). Once internalized into phagolysosome, NO reacts with superoxide to produce peroxide nitrite, which is highly reactive and acts as a microbicide (Bogdan and Rollinghoff 1998; Ueda-Nakamura et al. 2006).

The macrophage-internalized leishmanias are able to trigger mechanisms of calcium efflux in macrophages. Such mechanisms are referred to the increase of expression of two proteins: PMCA4, which is responsible for the calcium efflux to extracellular environment, and SERCA3, which is responsible for the storage of calcium into endoplasmic reticulum. This mechanism is in continuous operation for cellular homeostasis, but at presence of internalized amastigotes, the expression of PMCA4 and SERCA3 is markedly increased, promoting the increase of calcium efflux to the extracellular medium and its storage into endoplasmic reticulum. Moreover, when activated, the increase cytokines such as TNF-α and IL-4 occur, which upon encountering the lymphocyte with the antigen, MHC-II APC molecules activate T-lymphocytes (Th-0), differentiating them in $T_{CD4+}$ lymphocytes that in turn begin to produce and release INF-γ for activation of Th-1 cellular immune response (Roy et al. 2014; Islamuddin et al. 2015). Once the expression of PMCA4 and SERCA3 is increased by the presence of the parasite, the decrease of intracellular calcium occurs. Under small amount or absence of calcium, another signaling pathway is activated, the not so well known protein kinase zeta (PKζ); this signaling pathway promotes the expression of cytokines such as IL-10,

which activates T-lymphocytes in Th-2 immune response, thus activating B lymphocytes to produce antibodies (Roy et al. 2014). This Th-2 response is responsible for "masking" and promoting the pathogenesis of leishmaniasis in infected hosts (Islamuddin et al. 2015). The action of new drugs on the promotion of intracellular calcium augmentation pathway is directly related to this signaling pathway (Alves et al. 2017).

Table 1 shows a variety of drugs derived from natural, plant or synthetic products which have the ability to activate macrophages based on the parameters assessed using those approaches. Of the various substances that demonstrated this activity, we can highlight the substances that are derived from natural products, for example. Gallic acid and ellagic acid activated macrophages in all parameters demonstrated in this work (Alves et al. 2017), then, other isolated substances such as 2,3 dihydrobenzofuran and alpha-pinene, activated macrophages in increasing their phagocytic capacity, lysosomal activity, and nitric oxide (Rodrigues et al. 2015; De Castro Oliveira et al. 2017). These and the other molecules presented in table 1, have shown to be promising for the development of new antileishmania drugs.

**Table 1.** The drugs with ability to activate macrophages.

| Plant/drug | Activity | | | | Reference |
|---|---|---|---|---|---|
| | Phagocytic(1) | Lysosomic(2) | NO(3) | $Ca^{++}$(4) | |
| *Agaricus blazei* | - | - | X | - | (Valadares et al. 2011; Valadares et al. 2012) |
| *Allium sativum* | - | - | X | - | (Ghazanfari et al. 2000; Ghazanfari et al. 2006) |
| Artemisinin | - | - | X | - | (Islamuddin et al. 2015) |
| α-pinene | X | X | X | - | (Rodrigues et al. 2015) |
| Crotoxin | X | - | X | - | (Farias et al. 2017) |
| Ellagic acid | X | X | X | X | (Alves et al. 2017) |
| *Eugenia uniflora* | X | - | - | - | (Rodrigues et al. 2013) |
| Gallic acid | X | X | X | X | (Alves et al. 2017) |
| *Limonia strumguyonianum* | - | X | X | - | (Krifa et al. 2013) |
| Lupeol | X | X | - | - | (Souza et al. 2017) |
| *Myracrodruon urundeuva* | X | - | - | - | (Carvalho et al. 2017) |
| *Pelargonium sidoides* DC | - | - | X | - | (Kayser et al. 2001) |
| *Platonia insignis* | - | X | - | - | (Souza et al. 2017) |
| Proanthocyanidins and analogues thereof | - | - | X | - | (Kolodziej et al. 2001) |
| Quassin isolated from *Quassia amara* | - | - | X | - | (Bhattachardjee et al. 2009) |
| *Syzygium cumini* | X | X | X | - | (Rodrigues et al. 2015) |
| Tannins and polyphenols | - | - | X | - | (Kolodziej and Kiderlen 2005) |
| *Zanthoxylum rhoifolium* | X | - | | X | (Melo et al. 2016) |
| 2,3-dihydrobenzofurano | X | X | X | - | (de Castro Oliveira et al. 2017) |

[1]Increase of phagocytic capability; [2]Increase of lysosomal volume; [3]Production of nitric oxide; e [4]Increase of intracellular calcium.

The fact that leishmaniasis is an immunopathology (Paranaiba et al. 2017) with limited options of conventional treatment, costly and with a range of adverse effects, (Ashford 2000; Alizadeh et al. 2008) the search for new drugs with antileishmania action must also be linked to the ability to act in synergism with the immune response of the host by activation of macrophages. Moreover, these issues are also linked to reduction of parasite load in the investigation of the percentage of infected macrophages and the survival rate of macrophages-internalized amastigote forms. In many prospective studies of both antileishmania and macrophage-activating drugs, the value of mean inhibitory concentration ($IC_{50}$) for parasite is lower than $IC_{50}$ for host cells. Therefore, the selectivity index (IS) is higher when compared to the intrinsic activity on promastigote forms and macrophages-internalized amastigote forms. This fact is due to the macrophages-activating properties of these molecules, making them capable of solve infection of host cells. Thus, activated macrophages represent the main sign of establishment of cellular response to counteract the parasite, control parasitemia, and solve the infection (Alves et al. 2017).

## 4. Conclusions

Therefore, assessment of macrophages activation, such as phagocytic capacity, lysosomal activity, nitrite production and intracellular calcium, are important tools for discovery of immunomodulatory compounds which have potential to act in synergism with host immune response. Such compounds might be promising as monotherapy in the treatment of leishmaniasis, as well as being used as adjuvants in vaccines and/or in combination with conventional drugs.

**Authors' Contributions:** ALVES, M.M.M.: concenption and design, acquisition of data, analysis and interpretation of data, drafting the article; ARCANJO, D.D.R.: critical review of important intellectual content; CARVALHO, R.C.V.; AMORIM, L.V.; SANTOS, I.L.; SANTOS, R.R.L.; FIGUEIREDO, K.A.; FIGUEIREDO, J.F.S.; SOBRINHO-JÚNIOR, E.P.C.; CRUZ, L.P.L.; SOUSA, V.C.V.; SANTOS, L.P.: acquisition of data, analysis and interpretation of data; GONÇALVES, J.C.R.; CARNEIRO, S.M.P.: analysis and interpretation of data; MENDONÇA, I.L.; CARVALHO, F.A.A.: critical review of important intellectual content. All authors have read and approved the final version of the manuscript.

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**Ethics Approval:** Not applicable.

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**References**

ALIZADEH, B.H., et al. Leishmanicidal evaluation of novel synthetic chromenes. *Archiv der Pharmazie (Weinheim).* 2008, **341**(12), 787-793. https://doi.org/10.1002/ardp.200800128

ALVES, M.M.M., et al. Gallic and ellagic acids: two natural immunomodulator compounds solve infection of macrophages by Leishmania major. *Naunyn Schmiedebergs Archives of Pharmacology*. 2017, **390**(9), 893-903. https://doi.org/10.1007/s00210-017-1387-y

ASHFORD, R.W. The leishmaniases as emerging and reemerging zoonoses. *International Journal for Parasitol*ogy. 2000, **30**(12-13) 1269-1281. https://doi.org/10.1016/s0020-7519(00)00136-3

BHATTACHARJEE, S., et al. Quassin alters the immunological patterns of murine macrophages through generation of nitric oxide to exert antileishmanial activity. *Journal Antimicrobial Chemotherapy*. 2009, **63**(2), 317-324. https://doi.org/10.1093/jac/dkn479

BOGDAN, C., et al. Invasion, control and persistence of Leishmania parasites. *Current Opinion in Immunology.* 1996, **8**(4), 517-525. https://doi.org/10.1016/s0952-7915(96)80040-9

BOGDAN, C. and ROLLINGHOFF, M. The immune response to Leishmania: mechanisms of parasite control and evasion. *International Journal for Parasitology*. 1998, **28**(1), 121-134. https://doi.org/10.1016/s0020-7519(97)00169-0

CARNEIRO, S.M.., et al. The cytotoxic and antileishmanial activity of extracts and fractions of leaves and fruits of Azadirachta indica (A Juss.). *Biological Research*. 2012, **45**(2), 111-116. https://doi.org/10.4067/S0716-97602012000200002

CARVALHO, C.E.S., et al. Anti-Leishmania activity of essential oil of Myracrodruon urundeuva (Engl.) Fr. All.: Composition, cytotoxity and possible mechanisms of action. *Experimental Parasitology*. 2017, **175**, 59-67. https://doi.org/10.1016/j.exppara.2017.02.012

CLEARY, J.A., KELLY, G.E. and HUSBAND, A.J. The effect of molecular weight and beta-1,6-linkages on priming of macrophage function in mice by (1,3)-beta-D-glucan. *Immunology and Cell Biology*. 1999, **77**(5), 395-403. https://doi.org/10.1046/j.1440-1711.1999.00848.x

DE CASTRO OLIVEIRA, L.G.C., et al. In Vitro Effects of the Neolignan 2,3-Dihydrobenzofuran Against Leishmania Amazonensis. *Basic & Clinical Pharmacology & Toxicology*. 2017, **120(**1), 52-58. https://doi.org/10.1111/bcpt.12639

DE MEDEIROS, M.D., et al. In vitro antileishmanial activity and cytotoxicity of essential oil from Lippia sidoides Cham. *Parasitology International*. 2011, **60**(3), 237-241. https://doi.org/10.1016/j.parint.2011.03.004

DOLAI, S., et al. Overexpression of mitochondrial Leishmania major ascorbate peroxidase enhances tolerance to oxidative stress-induced programmed cell death and protein damage. *Eukaryot Cell*. 2009, **8(**11), 1721-1731. https://doi.org/10.1128/EC.00198-09

DUMAS, C., et al. Disruption of the trypanothione reductase gene of Leishmania decreases its ability to survive oxidative stress in macrophages. *EMBO Journal*. 1997, **16**(10), 2590-2598. https://doi.org/10.1093/emboj/16.10.2590

FARIAS, L.H.S., et al. Crotoxin stimulates an M1 activation profile in murine macrophages during Leishmania amazonensis infection. *Parasitology*. 2017, **144**(11), 1458-1467. https://doi.org/10.1017/S0031182017000944

GHAZANFARI, T., et al. Garlic induces a shift in cytokine pattern in Leishmania major-infected BALB/c mice. *Scandinavian Journal of Immunology*. 2000, **52**(5). https://doi.org/10.1046/j.1365-3083.2000.00803.x

GHAZANFARI, T., HASSAN, Z.M. and KHAMESIPOUR, A. Enhancement of peritoneal macrophage phagocytic activity against Leishmania major by garlic (Allium sativum) treatment. *Journal of Ethnopharmacolpgy*. 2006, **103**(3), 333-337. https://doi.org/10.1016/j.jep.2005.08.026

GILL, J., et al. Thymic generation and regeneration. *Immunological Reviews*. 2003, **195**, 28-50. https://doi.org/10.1034/j.1600-065x.2003.00077.x

GRANDO, F.C., et al. Modulation of peritoneal macrophage activity by the saturation state of the fatty acid moiety of phosphatidylcholine. *Brazilian Journal of Medical and Biological Research*. 2009, **42**(7), 599-605. https://doi.org/10.1590/s0100-879x2009005000003

GREENBERG, S. and GRINSTEIN, S. Phagocytosis and innate immunity. *Current Opinion in Immunology*. 2002, **14**(1), 136-145. https://doi.org/10.1016/s0952-7915(01)00309-0

GURUNATHAN, S., et al. Requirements for the maintenance of Th1 immunity in vivo following DNA vaccination: a potential immunoregulatory role for CD8+ T cells. *Journal of Immunology*. 2000, **165**(2), 915-924. https://doi.org/10.4049/jimmunol.165.2.915

HARHAY, M.O., et al. Urban parasitology: visceral leishmaniasis in Brazil. *Trends in Parasitology*. 2011, **27**(9), 403-409. https://doi.org/10.1016/j.pt.2011.04.001

ISLAMUDDIN, M., et al. Th1-biased immunomodulation and therapeutic potential of Artemisia annua in murine visceral leishmaniasis. *PLoS Neglected Tropical Diseases*. 2015, **9(**1), e3321. https://doi.org/10.1371/journal.pntd.0003321

KAYSER, O., KOLODZIEJ, H., and KIDERLEN, A.F. Immunomodulatory principles of Pelargonium sidoides. *Phytotherapy Research*. 2001, **15**(2), 122-126. https://doi.org/10.1002/ptr.785

KLEIN, L., et al. Antigen presentation in the thymus for positive selection and central tolerance induction. *Nature Reviews Immunology.* 2009, **9**(12), 833-844. https://doi.org/10.1038/nri2669

KLIMP, A.H., et al. A potential role of macrophage activation in the treatment of cancer. *Critical Reviews in Oncology/Hematology*. 2002, **44**(2), 143-161. https://doi.org/10.1016/s1040-8428(01)00203-7

KOLODZIEJ, H., et al. Proanthocyanidins and related compounds: antileishmanial activity and modulatory effects on nitric oxide and tumor necrosis factor-alpha-release in the murine macrophage-like cell line RAW 264.7. *Biological and Pharmaceutical Bulletin.* 2001, **24**(9), 1016-1021. https://doi.org/10.1248/bpb.24.1016

KOLODZIEJ, H. and KIDERLEN, A.F. Antileishmanial activity and immune modulatory effects of tannins and related compounds on Leishmania parasitised RAW 264.7 cells. *Phytochemistry*. 2005, **66**(17), 2056-2071. https://doi.org/10.1016/j.phytochem.2005.01.011

KONDRASHIN, A.V., BARANOVA, A.M., MOROZOVA, L.F. and STEPANOVA, E.V. [Global trends in malaria control. Progress and topical tasks in malaria control programs]. *Meditsnskaya Parazitologiya I Parazitarye Bolezni*. 2011, **4**, 3-8.

KRIFA, M., et al. Immunomodulatory and cellular anti-oxidant activities of an aqueous extract of Limoniastrum guyonianum gall. *Journal of* Ethnopharmacol. 2013, **146**(1), 243-249. https://doi.org/10.1016/j.jep.2012.12.038

LEE, W.L., HARRISON, R.E. and GRINSTEIN, S. Phagocytosis by neutrophils. *Microbes and Infection*. 2003, **5**(14), 1299-1306. https://doi.org/10.1016/j.micinf.2003.09.014

LIEW, F.Y., et al. Macrophage killing of Leishmania parasite in vivo is mediated by nitric oxide from L-arginine. *The Journal of Immunology*. 1990, **144**(12), 4794-4797.

LUCKHEERAM, R.V., ZHOU, R., VERMA, A.D. and XIA, B. CD4(+)T cells: differentiation and functions. *Clinical and Developmental Immunology*. 2012, **2012**, 925135. https://doi.org/10.1155/2012/925135

MELO, B.N., et al. Inhibitory effects of Zanthoxylum rhoifolium Lam. (Rutaceae) against the infection and infectivity of macrophages by Leishmania amazonensis. *Anais da Academia Brasileira de Ciências*. 2016, **88**(3) Suppl, 1851-1861. https://doi.org/10.1590/0001-3765201620150131

NADERER, T. and MCCONVILLE, M.J. The Leishmania-macrophage interaction: a metabolic perspective. *Cell Microbiology*. 2008, **10**(2), 301-308. https://doi.org/10.1111/j.1462-5822.2007.01096.x

NIEDERGANG, F. and CHAVRIER, P. Signaling and membrane dynamics during phagocytosis: many roads lead to the phagos(R)ome. *Current Opinion in Cell Biology*. 2004, **16**(4), 422-428. https://doi.org/10.1016/j.ceb.2004.06.006

PARANAIBA, L., et al. An overview on Leishmania (Mundinia) enriettii: biology, immunopathology, LRV and extracellular vesicles during the host-parasite interaction. *Parasitology*. 2017, **145**(10), 1265-1273. https://doi.org/10.1017/S0031182017001810

PETERS, J.H., et al. Dendritic cells: from ontogenetic orphans to myelomonocytic descendants. *Immunology Today*. 1996, **17**(6), 273-278. https://doi.org/10.1016/0167-5699(96)80544-5

RODRIGUES, K.A.F., et al. Eugenia uniflora L. Essential Oil as a Potential Anti-Leishmania Agent: Effects on Leishmania amazonensis and Possible Mechanisms of Action. *Evidence Based Complementary Alternative Medicine*. 2013, **2013**, 279726. https://doi.org/10.1155/2013/279726

RODRIGUES, K.A.F., et al. Syzygium cumini (L.) Skeels essential oil and its major constituent alpha-pinene exhibit anti-Leishmania activity through immunomodulation in vitro. *Journal of Ethnopharmacology*. 2015, **160**, 32-40. https://doi.org/10.1016/j.jep.2014.11.024

ROY, N., et al. Regulation of PKC mediated signaling by calcium during visceral leishmaniasis. *PLoS One*. 2014, **9**(10), e110843. https://doi.org/10.1371/journal.pone.0110843

SOARES, D.C., et al. Leishmanicidal activity of a supercritical fluid fraction obtained from Tabernaemontana catharinensis. *Parasitology International*. 2007, **56**(2), 135-139. https://doi.org/10.1016/j.parint.2007.01.004

SOUZA, A.C., et al. Platonia insignis Mart., a Brazilian Amazonian Plant: The Stem Barks Extract and Its Main Constituent Lupeol Exert Antileishmanial Effects Involving Macrophages Activation. *Evidence Based Complementary Alternative Medicine.* 2017, **2017**, 3126458. https://doi.org/10.1155/2017/3126458

TEJLE, K., MAGNUSSON, K.E. and RASMUSSON, B. Phagocytosis and phagosome maturation are regulated by calcium in J774 macrophages interacting with unopsonized prey. *Bioscience Reports*. 2002, **22**(5-6), 529-540. https://doi.org/10.1023/a:1022025903688

UEDA-NAKAMURA, T., et al. Antileishmanial activity of Eugenol-rich essential oil from Ocimum gratissimum. *Parasitology International*. 2006, **55**(2), 99-105.

VALADARES, D.G., et al. Leishmanicidal activity of the Agaricus blazei Murill in different Leishmania species. *Parasitology International*. 2011, **60**(4), 357-363. https://doi.org/10.1016/j.parint.2011.06.001

VALADARES, D.G., et al. Therapeutic efficacy induced by the oral administration of Agaricus blazei Murill against Leishmania amazonensis. *Parasitolical Research*. 2021, **111**(4), 1807-1816. https://doi.org/10.1007/s00436-012-3028-1

WALDRON, L.S., et al. Molecular epidemiology and spatial distribution of a waterborne cryptosporidiosis outbreak in Australia. *Applied and Environmental Microbiology*. 2011, **77**(21), 7766-7771. https://doi.org/10.1128/AEM.00616-11

WHO. *Leishmaniasis*. Geneva: World Health Organization, 2019. Available from: http://www.who.int/mediacentre/factsheets/fs375/en/

YOUNG, H.A. and HARDY, K.J. Role of interferon-gamma in immune cell regulation. *Journal of Leukocyte Biology.* 1995, **58**(4), 373-381.

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