LEISHMANICIDAL ACTIVITY IN AMASTIGOTES FORMS OF *Leishmania (Leishmania) amazonensis* TREATED WITH LAPACHOL AND B-LAPACHONE

Áquila Carolina Fernandes Herculano Ramos-Milaré[1], Daniele Stéfanie Sara Lopes Lera-Nonose[1], Aline Ávila Brustolin[1], Bruna Gomes Sydor[1], Vanessa Nesi-Reis[1], Expedito Leite Silva[1], Wilker Caetano[1], Izabel Galhardo Demarchi[1]; Thaís Gomes Verzignassi Silveira[1]; Maria Valdrinez Campana Lonardoni[1]

[1]Universidade Estadual de Maringá (UEM), Maringá, Paraná, Brasil.
aquilacarolina.fhr@gmail.com

ABSTRACT

Introduction: the available chemotherapeutic treatments of cutaneous leishmaniasis present many problems, such as several adverse side effects and the development of resistant strains. Natural compounds have been investigated as potential antileishmanial agents. **Objective:** the effects of lapachol and β-lapachone on amastigote forms of *L. (L.) amazonensis* were analyzed in the present study. **Methods:** microscopic counting was performed to evaluate the inhibitory effects of lapachol and β-lapachone on intracellular infection. The results were analyzed using Student's t-test or ANOVA followed by the Tukey at a 95% confidence. **Results:** the compounds significantly inhibited the survival rate of *L. amazonensis* amastigotes compared with untreated cells. **Conclusion:** lapachol and β-lapachone may be promising leishmanicidal agents. Further *in vivo* studies should be conducted to evaluate the anti-*Leishmania* and cytotoxicity activity.

Keywords: Leishmaniasis, *Leishmania amazonensis*, Antileishmanial activity, Cytotoxicity, Naphthoquinones

ATIVIDADE LEISHMANICIDA EM FORMAS AMASTIGOTAS DE *Leishmania (Leishmania) amazonensis* TRATADAS COM LAPACHOL E B-LAPACHONA

RESUMO

Introdução: os tratamentos quimioterápicos disponíveis para leishmaniose cutânea apresentam muitos problemas, como efeitos colaterais adversos e o desenvolvimento de cepas resistentes. Compostos naturais têm sido investigados como potenciais agentes anti-*Leishmania.* **Objetivo:** no presente estudo os efeitos do lapachol e da β-lapachona nas formas amastigotas de *L. (L.) amazonensis* foram analisados. **Métodos:** para avaliar os efeitos inibitórios do lapachol e da β-lapachona na infecção intracelular, foi realizada contagem microscópica. Os resultados foram analisados usando o teste *t* de Student ou ANOVA seguido pelo Tukey seguindo um intervalo de 95% de confiança. **Resultados**: os compostos inibiram significativamente a taxa de sobrevivência de amastigotas de *L. amazonensis* em comparação aos controles não tratados. **Conclusão:** o lapachol e a β-lapachona podem ser promissores agentes leishmanicidas. Estudos *in vivo* devem ser conduzidos para avaliar a atividade anti-*Leishmania* e citotóxica desses compostos.

Palavras-Chave: Leishmanioses, *Leishmania amazonensis,* Atividade anti-*Leishmania,* Citotoxicidade, Naftoquinonas

INTRODUCTION

Leishmaniasis is a noncontagious infectious disease, and this disease has a broad spectrum of clinical forms, ranging from cutaneous and mucocutaneous lesions to visceral lesions. Cutaneous leishmaniasis affects approximately 1 million people worldwide and threatens 350 million people who live in high-risk areas, mainly in developing countries. One of the main species that is responsible for cutaneous leishmaniasis is *Leishmania (Leishmania) amazonensis*, which is also the etiological agent of diffuse cutaneous leishmaniasis, the most severe and destructive clinical form of the disease (MARZOCHI; MARZOCHI, 1994; SILVEIRA et al., 2009; WORLD HEALTH ORGANIZATION, 2010).

Pentavalent antimonial compounds have been used as standard first-line treatment for CL, amphotericin B and pentamidine are second-line drugs. However, the available drugs a parenteral administration for long periods, cause systemic severe side effects, and tend to select resistant parasites and to therapeutic failure (NASSIF et al., 2017; PONTE-SUCRE et al., 2017; REVEIZ et al., 2013). Therefore, it is of most importance to search for effective, less toxic and low-cost drugs and therapeutic strategies for the treatment of leishmaniasis. The potential effects of lapachol and β-lapachone on *L. (L.) amazonensis* have not yet been investigated. In this study, we evaluated the activity of lapachol and β-lapachone on amastigote forms of *L. (L.) amazonensis.*

METHODS

Chemicals: lapachol and β-lapachone were obtained in the chemistry laboratory at Universidade Estadual de Maringá, Paraná, Brazil. Lapachol was extracted from the bark of Purple Ipê (*Tabebuia avellanedae*), and β-lapachone was obtained through acid treatment, leading to yield and a purity > 98 % (BARBOSA; NETO, 2013). In all experiments, the compounds were solubilized in dimethylsulfoxide (DMSO; Sigma Aldrich, St. Louis, MO, USA), then they were diluted in culture medium (199 or RPMI 1640 - Gibco, New York, NY, USA) at the concentrations specified in each experiment, with lower DMSO concentrations than those that present leishmanicidal activity (data not shown).

Parasite strain and culture: *L. (L.) amazonensis* (MHOM/BR/1977/LTB0016) were maintained by inoculating the left footpad of BALB/c mice with $1x10^7$ parasites. After 30 to 40 days, the animals were anesthetized with isoflurane and then euthanized with CO_2 in an appropriate chamber. Popliteal ganglions were removed and fragments incubated in medium 199 (Gibco®, New York, USA), supplemented with 10% of fetal bovine serum (FBS), 20 mM of L-glutamine (Sigma-Aldrich) and antibiotics (100 UI/mL of penicillin and 0.1 mg/mL of streptomycin, both obtained from Sigma-Aldrich) in pH 7.2. The cultures were maintained by periodic subculture at 25ºC (DEMARCHI et al., 2012).

Cytotoxicity assay: The use of animals was approved by the Ethics Committee on the Use of Animals of the State University of Maringá (CEUA/UEM) under the number 3018070817/2017.

A cell suspension (500 µl, 1×10^6 macrophages/mL) obtained from BALB/c mice was distributed on 13-mm-diameter sterile glass coverslips (Glastecnica, São Paulo, SP, Brazil) and placed in 24-well culture plates (TPP, Sigma-Aldrich, Trasadingen, Switzerland) to evaluated the activity of Lapachol and β-lapachone on intracellular amastigotes. The plates were incubated in a 5% CO_2 atmosphere for 2 h at 37°C, followed by washes with sterile PBS to remove nonadherent cells. Adherent macrophages were incubated in RPMI 1640 culture medium with the *L. (L.) amazonensis* (6:1) for 4 h at 37°C. After incubation at 37°C for 24 h in

a 5% CO_2 atmosphere with the compounds, the cells on the coverslips were removed, washed and stained with Fast Panoptic, and fixed on glass slides with Entellan (Merck, Darmstadt, Germany). The number of intracellular amastigotes was determined by counting 200 macrophages in an optical microscope, the infection index was calculated by multiplying the percentage of infected macrophages by the number of parasites per macrophages (NESI-REIS et al., 2018). All of the conditions were tested in duplicate and two independent experiments, non-treated cells were used to control infection.

RESULTS

The lapachol at concentrations of 82.28, 41.14 and 20.57 µg/mL and β-lapachone at concentrations of 3.26, 1.63 and 0.82 µg/mL significantly inhibited the survival rate of *L. amazonensis* amastigotes. The infection index was reduced by approximately at 83.11% (infection index = 45), 57.59% (infection index = 114) and 34.95% (infection index = 175) for lapachol, and 78.49% (infection index = 58), 83.25% (infection index = 45) and 80.22% (infection index = 53) for β-lapachone, respectively (Fig. 1).

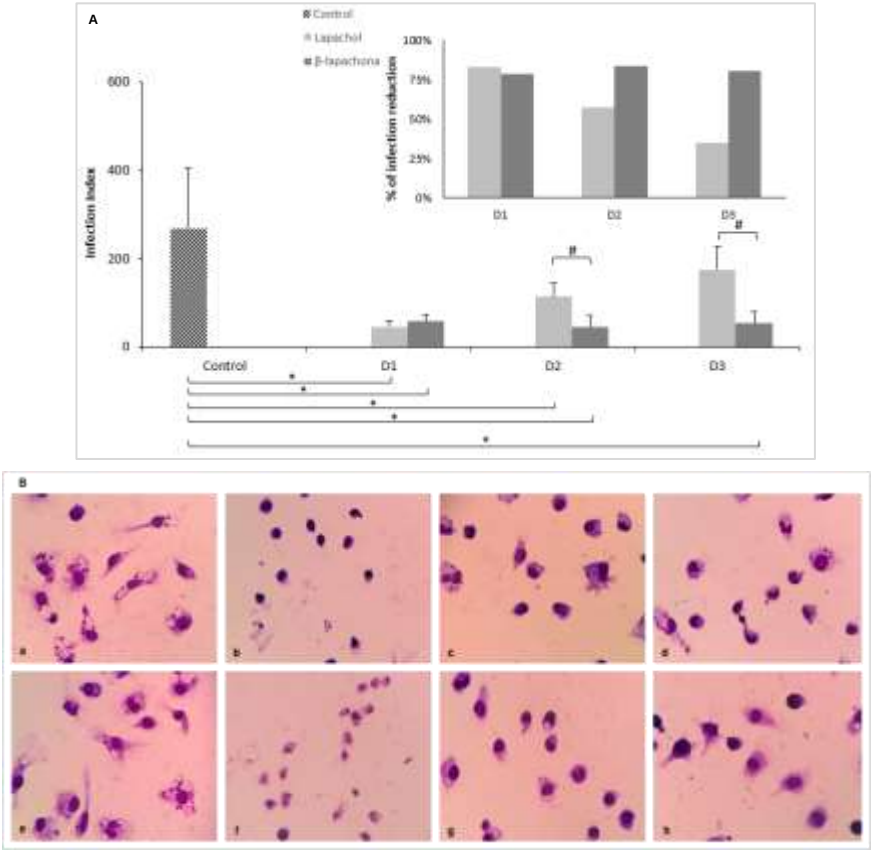

Fig. 1 Effect of lapachol and β-lapachone derived from *Tabebuia avellanedae* on intracellular forms of *Leishmania (Leishmania) amazonensis*. (A) The infection index was obtained by multiplying the percentage of infected macrophages by the average number of parasites per macrophage, and the percentage of infection reduction was obtained by diminishing from the control infection index the sample infection index and dividing this by control infection index. All of the conditions were tested in duplicate and in two independent experiments. The analysis was performed after 24 h of incubation at 37°C in a 5% CO_2 atmosphere. ($^{\#}$) Indicate statistical differences comparing lapachol and β-lapachone using Student's t-test, (*) the compounds in relation to the control (untreated cells) using ANOVA followed by the Tukey (p<0.05). (B) Peritoneal macrophages from BALB/c mice were infected and treated with

compounds. Infected macrophages without treatment (B [a, e]). Infected macrophages treated with lapachol at 82.28, 41.14 and 20.57 µg/mL (B [b-d, respectively]). Infected macrophages treated with β-lapachone at 3.26, 1.63 and 0.82 µg/mL (B [f-h, respectively]). The cells were stained with Fast Panoptic reagent and visualized in an optical microscope (Olympus CX21) at 1000x magnification.

DISCUSSION

The development of drugs resistance, safety, efficacy, and less toxicity has been increasingly required for the treatment of leishmaniasis, and lapachol and β-lapachone derived from *Tabebuia avellanedae* promoted the death of *L. (L.) amazonensis* amastigote. Teixeira et al., 2001, showed that lapachol displayed marked *in vitro* anti-amastigote activity (76–89%) against *L. (L.) braziliensis* at concentrations of 0.0125 to 0.05 mg/mL (TEIXEIRA; ALMEIDA, 2001). The epoxy-α-lapachone is another compound that exhibited activity on *L. (L.) amazonensis* cause measurable effects on promastigote and amastigote forms of the parasite, affecting plasma membrane organization (SOUZA-SILVA et al., 2015).

CONCLUSION

In conclusion, the results showed that lapachol and β-lapachone exhibited activity on amastigote of *L. amazonensis.* The compounds have potential as agents against intracellular microorganisms, such as *Leishmania*, but *in vivo* studies and other cytotoxicity assays must be conducted to ensure the efficacy and safety of the compounds for leishmaniasis treatment.

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