Atividade in vitro da secreção de Lucilia cuprina frente à Leishmania amazonensis, Ttrypanosoma cruzi e linhagens celulares

In vitro activity of larval secretions from Lucilia cuprina against Leishmania amazonensis, Trypanosoma cruzi and cell lines

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RESUMO
As pesquisas com produtos naturais representam um valioso recurso, pois são fonte de compostos biologicamente ativos que podem ter atividade contra uma variedade de patógenos que afetam a saúde humana e animal. Os dípteros da espécie Lucilia cuprina, pertencente à família Calliphoridae, conhecidos como moscas varejeiras, são cosmopolitas e têm importante função na natureza devido ao desenvolvimento de suas larvas em matéria orgânica em decomposição. Alguns estudos chegaram à conclusão de que a produção de enzimas digestivas secretadas pelas larvas são importantes para o estabelecimento e sobrevivência desses insetos no hospedeiro e no ambiente devido a sua secreção ter ação bactericida, antiparasitária e antifúngica e ao hábito alimentar necrobiontófago, esses dipteros têm sido utilizados em bioterapia e pesquisas contra

vários patógenos, entretanto, são poucos os estudos com foco na secreção desta espécie. Portanto, o objetivo deste trabalho foi verificar a atividade da secreção larval in vitro de L. cuprina, extraída de larvas L3 em diferentes concentrações por um período de 24 horas, frente à Leishmania amazonensis, Trypanosoma cruzi, linhagem tumoral de melanoma B16-F10 e HFF-1 de fibroblasto humano. Evidenciou-se uma redução na viabilidade de promastigotas de L. amazonensis nos tratamentos com secreção larval de 0,25%, 1% e 2% (viabilidade reduzida para 43,33%, 60% e 37,33% respectivamente). No ensaio com T. cruzi as concentrações 0,25% e 0,5% de secreção larval foi ativa mostrando diminuição de viabilidade de epimastigotas para 35,28% e 43,07%. A secreção larval mostrou capacidade citotóxica para as células tumorais. A concentração de 2% foi significativa em todos os ensaios, MTT, Vermelho Neutro (NR) e Crystal Violeta (VC), com redução de viabilidade para 79,3%, 85% e 85,6%, respectivamente. O tratamento a 1% mostrou diferença significativa tanto para NR (viabilidade de 74%) como VC (viabilidade de 83,6%). A concentração de 0,5% no NR diminuiu viabilidade para 80,6%. Nos ensaios com linhagem não tumoral a secreção mostrou baixa toxicidade, demonstrando segurança. O ensaio fluorimétrico realizado para quantificação de dsDNA (Picogreen) indicou um aumento de 14% à 25% de DNA extracelular. Os resultados fluorimétricos com o marcador 2,7'-diclorofluoresceína (DCFH-DA) mostraram um aumento de 20% e 22% de espécies reativas nas concentrações de 0,25 e 2%, sendo indicativo de um possível mecanismo de morte celular por radicais livres. Os resultados revelam que a secreção larval de L. cuprina, em diferentes concentrações, demonstrou atividade antiprotozoária e citotóxica na linhagem tumoral.

Palavras-Chave: Díptera, Bioterapia, Calliphoridae, Trypanosomatidae Células Tumorais.

ABSTRACT

Research on natural products presents these valuable resources that are sources of biologically active compounds with activity against a variety of pathogens that affect human and animal health. Dipterans of the species Lucilia cuprina, belonging to the Calliphoridae family, known as blowflies, are cosmopolitan and have an important function in nature due to the development of their larvae in decomposing organic matter. Studies found that the digestive enzymes secreted by the larvae play crucial roles in the establishment and survival of these parasites in the host and environment. These flies have become promising in biotherapy because of the biological activity of their secretions that have bactericidal, antiparasitic and antifungal activities; nevertheless, there are few studies focusing on these secretions. Therefore, the objective of this work was to measure the activity of L. cuprina secretions in vitro, extracted from L3 larvae at various concentrations for 24 hours, against Leishmania amazonensis, Trypanosoma cruzi, and the melanoma tumor line B16-F10 and the human fibroblast line HFF-1. There was a reduction in the viability of L. amazonensis promastigotes in treatments with larval secretions of 0.25%, 1% and 2% (viability reduced to 43.33%, 60.00% and 37.33% respectively). In the assay with T. cruzi, concentrations of 0.25% and 0.5% showed decreased viability of epimastigotes to 35.28% and 43.07%, respectively. Larval secretions showed cytotoxic capacity for tumor cells. The effect of 2% was significant in all assays (MTT, neutral red (NR) and crystal violet (CV), reducing viability to 79.3%, 85% and 85.6%, respectively. At 1%, there was a significant difference for both NR (74%) and CV (83.6%) assays. The concentration of 0.5% in the NR decreased viability to 80.6%. In tests with non-tumor lineages, secretion showed low toxicity, demonstrating safety. The fluorometric test performed for quantification of dsDNA (PicoGreen)

indicated an increases of 14% to 25%. Fluorimetric results with the 2,7'-dichlorofluorescein marker (DCFH-DA) showed increases of 20% and 22% of reactive species at concentrations of 0.25% and 2%, respectively, suggesting the possible mechanism of cell death by free radicals. These results suggest that larval secretions from L. cuprina, at various concentrations, have antiprotozoal and cytotoxic activities in tumor cell lines.

Keywords: Dipterans, Biotherapy, Calliphoridae, Trypanosomatidae, Tumor cells.

1 INTRODUCTION

The search for new therapeutic alternatives to treat parasitic diseases and carcinomas has been the focus of numerous scientific investigations. The discovery and use of drugs for the treatment of various diseases is considered one of the greatest contributions to humanity, allowing the control and cure of various pathologies (Steverding, 2017).

Neglected tropical diseases (NTDs) profoundly impact the world in terms of health, social and economics, and Brazil is an example of a developing country that particularly suffers from the neglect of these diseases; such diseases persist due to a lack of studies (Castro, 2010; Brasil, 2017; Brasil, 2020). American Tegumentary Leishmaniasis and Chagas disease are caused by protozoa of the family Trypanosomatidae of the genera Leishmania and Trypanosoma, respectively (Monteiro, 2017; Silva, 2009; Brasil, 2017; Souza, 2012; Steverding, 2017).

Current therapeutic strategies for leishmaniasis and Chagas disease are restricted (Chatelain and Konar, 2015). For leishmaniasis, treatment relies on pentavalent antimonials, introduced in Brazil in the 40s. These are drugs of choice in many countries, along with Amphotericin B®, considered the second choice for treatment (Vanaerschot et al., 2014; Tunez, 2019). However, the chemotherapy available is limited and the efficacy is far from ideal, as there is no satisfactory drug therapy for all forms that the disease may present (Moreira, 2012; Fonseca, 2016; Furini, 2016; Garbian, 2017).

For Chagas disease, treatment includes Nifurtimox® and Benznidazole®, which are the only agents allowed in Brazil. Although Benznidazole® is the primary drug, and it can produce cures in the acute phase, in the chronic phase, the treatment becomes only palliative, because the trypomastigote forms evolve to amastigotes and, at this stage, the drugs cannot act efficiently (Brasil, 2020; Lozano, 2015; Silva, 2016; Tunez, 2019).

The drugs used to treat these two endemic diseases are highly toxic, with variable efficacy, the emergence of resistance is significant, and the costs of treatment are high. In recent decades, there has been an intense effort by researchers to develop alternatives to the treatment of these parasites, especially exploring natural products (Ramírez et al., 2012; Saadi et al., 2020; Brazão et al., 2020).

Melanoma is a type of malignant tumor with a high metastatic potential that is resistant to conventional anticancer agents. Although it is not the most frequent type of neoplasia, it has a high mortality rate and is a major global health problem (Inca, 2018; Viana, 2018; Xu and Mao, 2016). Generally, drugs used to treat cancer target cells that are multiplying and are not specific to the type of cancer. They are characterized by side-effects, toxicities, high costs and in some cases, resistance and ineffectiveness. Despite constant efforts and significant advances in research and development of new chemotherapeutic agents, the recurrence of disease due to tumor heterogeneity, resistance to chemotherapeutic agents, adverse reactions, toxicity and permanent damage caused by these treatments remain major problems and challenges in the search for cures (Zellmer et al., 2014; Zhao, 2016). Therefore, the search for new therapies that are more efficient, less toxic and safer is extremely important (Alvez, 2012; Perez, 2012; Brasil, 2015).

Lucilia cuprina is a species of the family Calliphoridae that has been used in biotherapy because of its proven biological activity. In Brazil, few studies have been carried out with this commonly found species; therefore, little is known about its constituents and possible activities (Al-saeed and Mahmood, 2012; Teh et al., 2013; Silva, 2013).

The great advantage of using biomolecules, such as larval secretions, is low toxicity, little or no side-effects, as well as characteristics of natural treatment with low production costs. The use of L. cuprina larvae is advantageous, as they are easy to breed, maintain and rapidly multiply in a relatively short time (Arrivillaga et al., 2008; Campo, 2017; Rocha, 2018).

Our research group has been conducting extensive screening of biological, phytochemical and synthetic compounds of insects with antiparasitic potential. Among the promising results is the larval secretions of this dipteran. The objective of the present study was to evaluate the in vitro biological activity of larval secretions of L. cuprina against Leishmania amazonensis, Trypanosoma cruzi and the cell lines B16F10 and HFF-1.

2 MATERIALS AND METHODS

2.1 MAINTENANCE OF THE DIPTERAN COLONY IN THE LABORATORY

The flies were kept in entomological cages (30 cm X 30 cm X 30 cm) at 25 ± 1 ° C under a 12 hours light/dark light cycle and 70% relative humidity in the vivarium. The adult flies were fed with honey and water ad libitum. Bovine liver was made available for oviposition. The larvae were kept in ventilated pots for the evolution of larval stages L1, L2 and L3. Subsequently, the L3 larvae were placed in a container with sand where they evolved to the pupal stage and hatched in adult male and female flies (Cossetin et al., 2018).

2.2 EXTRACTION OF LARVAL SECRETIONS

Before proceeding with secretion collection, the L3 larvae were subjected to antisepsis with rapid washing in running water and 0.5% sodium hypochlorite for 5 minutes followed by rinsing with sterile distilled water. One hundred L3 larvae were used to acquire the secretions, which were incubated with 750 µl of phosphate buffered saline (PBS) for 5 hours at room temperature and protected from light. The larvae were removed from the solution and the material was centrifuged at 1300 g for 7 minutes. Subsequently, the material was filtered through a 0.22-µm filter and the filtered liquid was transferred to sterile Eppendorf tubes (Cruz-saavedra et al., 2016; Sanei-dehkordi et al., 2016).

2.3 PROTOZOA

The L. amazonensis strain (MHOM/BR/77/LTB0016) was purchased from the Protozoology Laboratory belonging to the Department of Microbiology, Immunology and Parasitology (MIP) of the Federal University of Santa Catarina (UFSC), together with that of T. cruzi (cepaY). Both were maintained and cryopreserved at the Veterinary Parasitology Laboratory of the Federal University of Santa Maria (LAPAVET-UFSM).

To test the antiprotozoal activity, it was necessary to culture promastigote forms of L. amazonensis, which were maintained in Schneider® culture medium, supplemented with fetal bovine serum (FBS), human urine, antibiotics; cultivation of T. cruzi epimastigote forms was made in LIT medium. Both protozoa were cultured at 37 ºC and 5% CO_2, specific for their development.

In order to maintain in vitro cultivation, weekly growing was carried out with variations depending on the confluence of the strain, avoiding media saturation and mortality rate according to Romão et al. (2006) and Castro (2010).

2.4 EVALUATION OF THE IN VITRO ACTIVITY OF LARVAL SECRETIONS OF L. CUPRINA IN L. AMAZONENSIS PROMASTIGOTES AND EPIMASTIGOTES OF T. CRUZI

The tests were performed in 96-well plates, with a final concentration of 5 x 10^6 parasites/ml in specific medium for each protozoan, incubated at 37 ºC in 5% CO_2 in the presence of various concentrations (0.25%, 0.5%, 1% and 2%) of larval secretions (POLAT et al., 2012). As negative and positive controls, dimethyl sulfoxide (DMSO) (2%) and Amphotericin B® were used for L. amazonensis and for the tests with T. cruzi, DMSO (2%) and Benznidazole® were used, respectively. After 24 hours of incubation, microscopic counting of active parasites was performed in the Neubauer chamber to verify mortality determined by the method of Brener (1962).

2.5 CELL CULTURE

The melanoma strains B16-F10 and human fibroblast HFF-1 belonging to the Biosciences Laboratory of the Franciscan University (UFN) were acquired from the Rio de Janeiro Cell Bank (BCRJ, Rio de Janeiro, Brazil). The cells were grown in Dulbecco's Modified Eagle Medium® (DMEM), supplemented with 10 % of fetal bovine serum and 1% of antibiotics (penicillin/streptomycin), maintained at a temperature of 37 °C, and 5% CO_2, as recommended by the BCRJ. Before reaching the confluence (70–80% of their saturation density) the cells were subjected to subculture to avoid eventual cell death due to excess cells.

For expansion, the cells were mechanically removed from the surface of the 25 cm^2 bottle with the aid of a cell scraper. Then, the cells with culture medium were transferred to falcon tubes that were centrifuged for 5 minutes at 1300 RPM and the formed pellet was resuspended in culture medium and transferred to a new 75-cm^2 bottle.

To perform the tests, cells were seeded in 96-well microplates containing complete culture medium at a concentration of 1 x 10^4 cells/200 µL (well), in triplicate, incubated at a temperature of 37 °C with 5% CO2. In the positive control, hydrogen peroxide at a concentration of 100 mM (Ourique et al., 2017) was added in the cytotoxicity tests (Park, 2013) in the 2,7'-dichlorofluorescein test. After 24 hours of cell adhesion, the medium was removed and new medium was added with the secretion of L. cuprina in concentrations of 0.1%, 0.25%, 0.5%, 1% and 2% and again incubated in the same conditions for tests 2.5.1; 2.5.2; 2.5.3; 2.5.4 and 2.5.5, performed at the UFN Biosciences Laboratory and described below.

2.5.1 Colorimetric assay (MTT)

The colorimetric test was performed according to Mosmann (1983). After the 24 hours incubation period, 20 µL was added to the wells of the sterile solution containing MTT (5 mg/mL 1x PBS) and the plates were again incubated for another 4 hours. Then, the supernatants were carefully removed from the wells and the cells were resuspended in 200 µL of DMSO. The inhibition of cell growth was detected by measuring the absorbances at 570 nm in an ELISA device.

2.5.2 Neutral red colorimetric assay (NR)

The neutral red (NR) colorimetric assay was performed according to Repetto (2008). After 24 hours of incubation of the plate containing medium with the cells and secretions in the various concentrations under analysis, the supernatants were removed and the dye was added at 0.04 µg/mL for 3 hours. Subsequently, the cells were washed to remove excess dye and then a solution containing 1% acetic acid, 50% ethanol and 49% water was added to disrupt cell structures. The reading was performed on a spectrophotometer with a wavelength of 540 nm.

2.5.3 Crystal violet colorimetric assay (CV)

The CV colorimetric assay was performed according to Feoktistova (2016). To perform violet crystal staining in the 96-well plates, initially the medium containing the cell line and secretions at the various concentrations was discarded and 100 µl of the crystal violet solution (5% crystal violet, 1.7% NaCl, 3% paraformaldehyde in 33.3% ethanol) was added to the wells and maintained for 3 minutes at room temperature. Subsequently, the absorbance was evaluated at 570 nm in a spectrophotometer.

2.5.4 Fluorimetric DNA Quantification Assay using DNA-PicoGreen® reagent

The fluorometric test was carried out according to Ahn et al. (1996). An additional assay was performed from the quantification of the concentration of double-stranded DNA (dsDNA) present or not in the supernatant in the culture plate containing the cell line and the various concentrations of L. cuprina secretions. For this analysis, dsDNA PicoGreen® fluorescent dye was used. After 24 hours of incubation, the culture plate was centrifuged and 10 µL of the supernatant was transferred to a new culture plate. Then, 80 µL of 1x tris-EDTA buffer (Tris HCl 10 mmol/L and EDTA 1 mmol/L pH = 7.5) and 5 µL of the PicoGreen reagent were added. The plate was incubated for 5 minutes at room

temperature (protected from light) and the reading was performed at 520 nm emission and 480 nm wavelengths on a fluorimeter (SpectraMax® i3x - Molecular Devices).

2.5.5 2,7'- Dichlorofluorescein (DCF)

The DCF assay was performed according to Esposti (2002), and after the treatment period, 50 µL of the cell suspension, 65 µL of 10 mmol/L Tris HCl and 10 µL DCFH-DA 10 µM were transferred to new plates, and incubated for 30 minutes, at 37 ºC, with 5% CO_2, in a humidified incubator. After that period, the fluorescence was measured in a spectrofluorometer and the results were expressed as fluorescence intensity of the treatments in relation to the control with cells alone.

Statistical analysis

The analyses were performed using GraphPad Prism. To compare treatments, one-way analysis of variance (ANOVA) was performed, followed by Tukey's post hoc test. Statistically different values were considered $P < 0.05$ *, $P < 0.01$ ** and $P < 0.001$ ***.

3 RESULTS

The data obtained in the evaluation of the in vitro antiprotozoal activity of larval secretion demonstrated its capacity to reduce the viability of L. amazonensis promastigotes in the 24 hours period. The results are shown in Figure 1, expressed as a percentage of viability of promastigotes due to the assessed concentration of larval secretion. The treatments showed a significant difference at concentrations of 0.25%, 1% and 2% of larval secretions, being efficient in reducing the parasite viability to 43.33%, 60.00% and 37.33%, respectively; this was greatest reduction of viability found in the study with L. amazonensis, when compared to the control.

The antiprotozoal activity of larval secretion of L. cuprina demonstrated the ability to reduce the viability of T. cruzi epimastigotes in vitro after 24 hours (Figure 2). The treatments with the lowest concentrations of larval secretions significantly reduced cell viability in relation to control. The lowest concentration, 0.25%, showed the highest activity in this study in general, with a decrease in the viability of epimastigotes to 35.28%, without dose-dependence, as the concentration of 0.5% showed viability of 43.07%.

The in vitro cytotoxicity assays and the viability of the tumor line B16-F10 and non-tumor HFF-1 were evaluated after 24 hours of exposure to different concentrations of the larval secretion of L. cuprina using various methods.

The colorimetric cytotoxicity assays of larval secretion of L. cuprina demonstrated low or no toxicity in non-tumor cells after 24 hours of exposure to the studied concentrations (Figure 3). These results serve as a control for safety in their subsequent use in probable test in vivo since, in the MTT test, the larval secretion did not present a decrease in non-tumor cell viability when compared to the negative control (cells + culture medium) at any studied concentration.

In the NR assay, the concentration of 1% was active; the cell viability was 87.6%, showing low cytotoxicity very far from the positive control H_2O_2 (hydrogen peroxide) which reduced the number of viable cells.

In the CV assay, only concentrations of 0.5% and 2% showed activity, with cell viability of 90% and 88.6%, respectively. There was extremely low cytotoxicity, very close to negative control, while the positive control reduced cell viability to 19%.

From the analysis of cytotoxicity with the tumor line, the larval secretions were active, capable of decreasing cell viability (Figure 4). The concentration of 2% reduced the amount of cells in all assays (MTT, NR and CV) reducing viability to 79.3%, 85% and 85.6%, respectively. There was also possible to notice difference in concentration 1% for both NR and CV, with viability to 74% and 83.6%, respectively. In addition, it is observed that at the concentration of 0.5% in the NR test the cell viability was 80.6%.

The fluorometric tests (Figure 5) were performed with the tumor line B16F10 over a 24 hours period. The dsDNA quantification test showed that it was not only possible to identify the cell's genetic material in the extracellular medium at the concentration of 0.25%, but also the treatments 0.1%, 0.5%, 1% and 2% caused high cytotoxicity and there was an increase in the amount of extracellular DNA of 14%, 18%, 15% and 25%, respectively, when compared to the control. In the test that evaluates oxidative stress, DCFH-DA, the results obtained showed an increase in the production of reactive species at concentrations of 0.25% and 2%. This quantification of the increase in reactive species was 20 and 22%, respectively, which can lead to damage to macromolecules like DNA, evidenced in PicoGreen with a significant increase, suggesting that the tests were complementary.

4 DISCUSSION

In the results of the larval secretion activity against L. amazonensis, we observed a viability of 37.33%, a very high reduction rate, for the highest concentration tested. Like Rottini et al. (2015), who tested the natural product α-bisabolol, also obtained significant results in the same conditions, showing that about 69% of the promastigotes suffered damage to the mitochondrial membrane after treatment. These important morphological changes, indicating damage to the organelle, suggest inhibition of the parasite's metabolic activity through the ability to cross membranes, attributed to the presence of the hydroxyl group in this compound. Therefore, we believe that the antileishmanial power of secretion is due to the inhibition of cellular respiration, which interferes with oxygen metabolism and consequently generates reactive species, damaging DNA molecules.

Another hypothesis to explain the antiprotozoal activity is the inhibition of ergosterol biosynthesis. Reported by several authors as Tunez in 2015, for example, who tested naphthoquinones involved in various types of biological activities related to damage caused by structural changes and the potential of the mitochondrial membrane; the authors demonstrated leishmanicidal, trypanocidal and antitumor activities (Rocha, 2018; Sucre et al., 2017; Tunez, 2015).

The results obtained in relation to the viability of L. amazonensis are satisfactory when compared to a study by Polat et al. (2012) who also evaluated the larval secretion of dipterans. They demonstrated the activity of L. sericata against promastigote forms only with 100% concentration of the secretion directly on the parasite; however, they were unable to verify activity in culture medium. In addition, a study with larval secretion of Calliphoridae, also in the period of 24 hours and against promastigote forms, demonstrated efficacy in low concentrations, with significant viability reduction greater than 50%, in agreement with the findings of our study (Laverde-paz et al., 2018).

A study conducted by Arrivillaga et al. (2008) was a preliminary assessment with Lucilia spp. in cutaneous wounds caused by L. amazonensis in hamsters. They authors reported successful healing of localized lesions after treatment, reducing between 80% and 100% of the lesion area in only 12 h. As the protozoan was completely non-viable, further tests should be carried out to analyze an alternative for the treatment of cutaneous leishmaniasis. The exact mechanism of action of larval secretion in cutaneous leishmaniasis is unknown. It is believed that the presence of proteolytic enzymes liquefies the necrotic tissue, producing ammonia that changes the pH, resulting in unfavorable alkaline conditions for many species (Díaz-roa et al., 2018).

More recently, larval secretions of two species of flies were also tested for cutaneous leishmaniasis. The results showed that both were able to reduce the number of parasites and that Lucilia spp. were significantly more effective than Sarconesiopsis spp., which also had a better effect in reducing the size of ulcers in BALB/c mice, at a concentration of 5%. In that same study, cytotoxicity for the cell line was found at a concentration of 40% of larval secretion with high toxicity, reducing viability to 20%, while the concentration of 5% did not show any toxic effect. The concentrations determined by these researchers are much higher when compared to those of the present study, as we showed activity in much lower concentrations of larval secretion, 0.25%, 1% and 2% (Sanei-dehkordi et al., 2016).

In a study by Cruz-saavedra et al. (2016), dipteran larval secretions were tested before and after the appearance of lesions caused by Leishmania panamensis in hamsters, demonstrating improvement in ulcers, also presenting protective effect on the development of wounds. In addition, the histopathological examination confirmed that the infection revealed the presence of the parasite in all animals in the study except those that received pretreatment with larval secretions. These results demonstrate that larval secretion activity can be used to treat cutaneous leishmaniasis, which is in accordance with our in vitro results.

The action of larval secretions of L. cuprina in the present study showed high toxicity for protozoa and low toxicity for the cells of the mammalian host. This suggests that the secretion is capable of activating the apoptotic process in protozoan cells by alkylating the genetic material and increasing the oxidative stress, by forming reactive species that produce highly electrophilic intermediates that can affect various molecules in the cell (Souza, 2019).

The results obtained in our investigation of larval secretion are promising because bioactive natural products and their derivatives elucidated by Tunez (2019) did not demonstrate an expected trypanocidal capacity in any of the different methods tested in T. cruzi. However, a study with an extract of the roots of Arrabidaea brachypoda demonstrated significant activity in vitro against T. cruzi with treatment for 24 hours, as in our investigation (Rocha et al., 2014).

Another investigation of antiprotozoal activity used the diterpene 12-hydroxy-11,14-diketo-6,8,12-abietatrien-19,20-olide (HABTO) obtained from the aerial parts of Salvia cuspidata on T. cruzi epimastigotes, with inhibitory effect of the parasite at low concentrations and low cellular toxicity in 24 hours trials, as was the case in our study.

They also verified intense vacuolization in the parasite and concluded that the activity of the compound was due to the increase in lipophilicity (Lozano et al., 2015).

Through the analysis of the essential oil of compounds of the Anacardiaceae family, we found that the antiprotozoal activity and cytotoxicity were mediated by increased lysosomal capacity and nitric oxide production. The compounds are able to cross the cytoplasmic membrane, affecting the structure of the various layers of polysaccharides, fatty acids and phospholipids, consequently changing permeability. In addition, the production of nitric oxide, which is extremely reactive, is stimulated by protective cytokines such as interferon gamma, causing damage to parasite DNA (Moreira, 2014; Carvalho et al., 2017; Menezes et al., 2017). In view of this, we believe that the antiprotozoal activity of larval secretions is related to the damage of the cytoplasm by interrupting the metabolic pathways of lipids and proteins, interfering in the potential of mitochondrial membranes, generating an increase in oxidative stress and damage to DNA.

The focus of our investigation is the search for a new anti-trypanosomal agent, with little or no toxicity to host cells, as was the aim of Khraiwesh et al. (2012), who investigated 1,4-naphthoquinone and Nifurtimox® derivatives replaced by imido group; they were able to demonstrate promising activity against trypanosomes, testing more potent agents than Nifurtimox®, through derivations in the structure-activity of the compounds. Some of these compounds were not very cytotoxic to a fibroblast cell line, as was the case for our findings in this work.

These new therapeutic targets appear to have great relevance and may serve as possible solutions for the development of new trypanocides. In short, this reflects the importance of assessing larval secretion in a non-tumoral lineage such as fibroblasts, because we detected little or no toxicity, in line with the evaluation of the activity of the extract of the root of Commelinaceae, which showed important trypanocidal activity, though against fibroblasts it presented moderate cytotoxicity, suggesting that the secretions may be well tolerated by host cells (Estevam et al., 2016).

In this study, in addition to the antiprotozoal activity, we showed the cytotoxic capacity against a tumor lineage, as did Tunez (2019), who performed a preclinical study with 24 gold (Au) complexes and confirmed these products as metallopharmaceuticals with antileishmanial and antitumor activity. All compounds they tested showed potent activity, mainly in a murine melanoma lineage, caused by oxidative stress, generating reactive oxygen species or generating its accumulation by inhibiting the trypanothione

reductase enzyme and causing mitochondrial damage. There were data that corroborate our MTT, NR and CV assays because the larval secretions were active against B16F10. These findings also agree with our fluorimetric assays for quantification of dsDNA and dichlorofluorescein that signal genetic material in the extracellular environment and increases in reactive intracellular species.

Other in vitro studies highlight the effect of destabilizing the lipid bilayer and membrane integrity measured through the fluorescence intensity of the dye that has the ability to penetrate these damaged cells and intercalate with nucleic acid fragments. When diphtheria secretions bind to DNA causing interference, it is suggested that they inhibit intracellular functions, explaining our results expressed in the assay with dsDNA PicoGreen against the tumor line (Díaz-roa et al., 2016; Sousa et al., 2004).

Díaz-Roa et al. (2016) also tested dipteran secretions against non-tumor Vero cells, and the viability was approximately 92%, a result very similar to ours, in which we analyzed safety against a non-tumor fibroblast cell. Viability was 87% in a single concentration of all under analysis in the NR test; in CV, only two concentrations showed cytotoxicity being very close to the negative control, with a 10% and 11% reduction in viability, while the positive control was above 81%. Likewise, we did not show any toxic potential in the MTT assay in any of the concentrations evaluated, which demonstrated safety with 100% viability.

Other studies also show cytotoxicity of natural products against cell lines, such as essential oils of Eucalyptus benthamii, and the results indicate that cytotoxic activity probably involved cell death by apoptosis with cell DNA degradation (Döll-boscardin et al., 2012). The production of reactive species in biological systems generates increased oxidative stress that can lead to irreversible damage to macromolecules, such as DNA. It is noteworthy that, in this study, we showed extracellular DNA, reinforcing the possibility that the larval secretion has antitumor activity inducing cell death by the generation of reactive species.

Sousa et al. (2012) found that lemon balm essential oil (Melissa officinalis) has cytotoxic activity against some cancer cell lines, including B16F10, and that they are potentially effective in altering the enzymatic activity of mitochondria and starting an injury that leads to cell death, corroborating our study. It has also been reported that these natural products can cause damage to the cell membrane, as they cause depolarization and alter fluidity through free radicals.

The great advantage of using biomolecules is evident, such as larval secretion, with low toxicity, little or no side effects, in addition to being a natural treatment with low cost for production. They are also advantageous also because the insect species in use is easy to maintain and multiply in a relatively short time. In view of the above, the use of larval secretions, considering all the effects provided or modulated by it, can reduce parasitemia and the viability of cancer cells.

There are still few studies on the use of larval secretion of L. cuprina; however, we have shown that, even in low concentrations, there was in vitro activity against flagellated protozoa (L. amazonensis and T. cruzi); there was cytotoxic activity in the melanoma tumor line and we also demonstrated safety of its use in normal healthy cells. From these results, we suggest that characterization of larval secretions and testing in vivo are necessary to better elucidate their activity for use against T. cruzi, Leishmania as well as in tumor cells.

Figure 1. Antiprotozoal activity of larval secretion of L. cuprina in vitro against promastigote forms of L. amazonensis. Results expressed as a percentage of the negative control (100%). Reading at 24 hours with a final volume of 180 □l containing 106 promastigotes/µl. Percentage of viability of promastigotes due to the concentration of larval secretion (LS). LS 0.25% (43.33), LS 0.5% (71.06), LS 1% (60.0) and LS 2% (37.33). The data represents mean ± SEM of three independent experiments, analyzed Two-Way ANOVA with post-hoc Tukey test. * p <0.05 (* Control vs LS or L. amazonensis or L. amazonensis + SL group) (#SL vs Control, L. amazonensis or L. amazonensis + SL group).

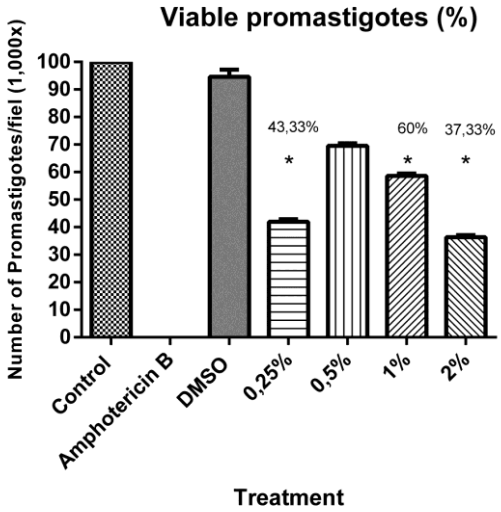


Figure 2. Antiprotozoal activity of larval secretion of L. cuprina in vitro against epimastigote forms of T. cruzi. Results expressed as a percentage of the negative control (100%). Reading in 24 hours with a final volume of 180 □l containing 10^6 epimastigotes/µl. Percentage of viability of epimastigote forms due to the assessed concentration of larval secretion (LS). LS 0.25% (35.28), LS 0.5% (43.07), LS 1% (64.29) and LS 2% (51.38). The data represents mean ± SEM of three independent experiments, analyzed Two-Way ANOVA with post-hoc Tukey test. * p <0.05 (* Control vs LS or T. cruzi or T. cruzi + SL group) (#SL vs Control, T. cruzi or T. cruzi + SL group).

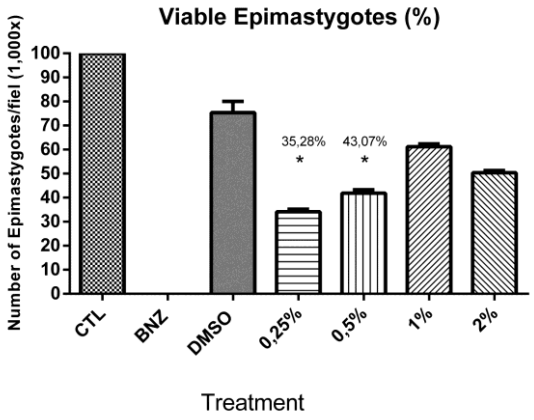


Figure 3. Colorimetric cytotoxicity assays in non-tumor line HFF-1. Results expressed as a percentage of the negative control (100%). Negative control (C-): cells in culture medium ($1x10^4$cells/200 µL/well). Positive control (C+): cells in culture medium and hydrogen peroxide (100 mM). MTT. Neutral red (NR). Crystal violet (CV). Data expressed as mean ± standard deviation (SD). The means followed by the symbology do not differ statistically. The analyzes were performed by one-way variance (ANOVA), followed by the Tukey post-test. Values with p <0.05 were considered statistically significant. Being * p <0.05, ** p <0.01 and *** p <0.001.

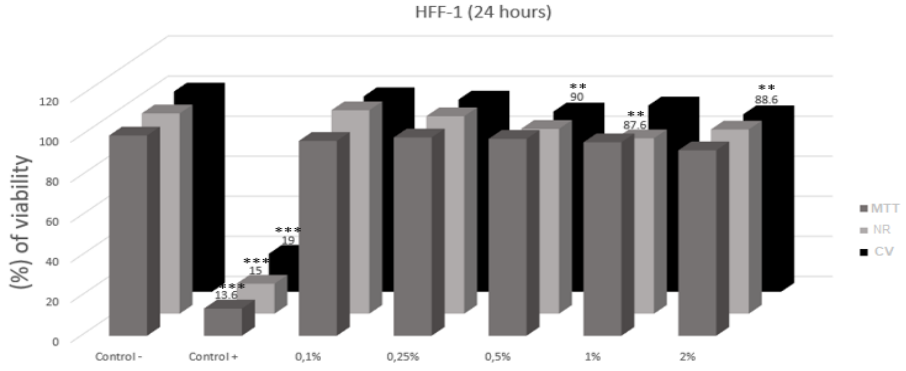


Figure 4. Colorimetric cytotoxicity assays in the murine melanoma tumor line B16-F10. Results expressed as a percentage of the negative control (100%). Negative control (C-): cells in culture medium ($1x10^4$cells/200 µL/well). Positive control (C+): cells in culture medium and hydrogen peroxide (100 mM). MTT. Neutral red (NR). Crystal violet (CV). Data expressed as mean ± standard deviation (SD). The means followed by the symbology do not differ statistically. The analyzes were performed by one-way variance (ANOVA), followed by the Tukey post-test. Values with p <0.05 were considered statistically significant. * p <0.05, ** p <0.01 and *** p <0.001.

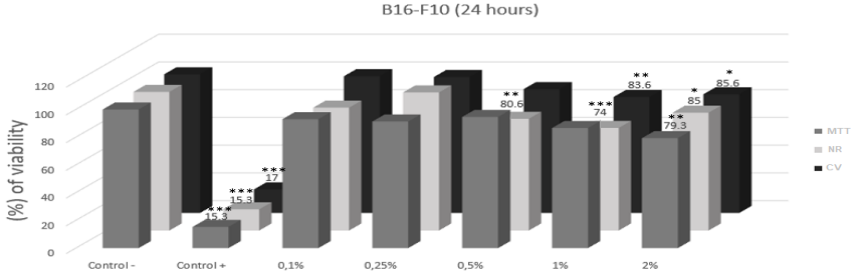
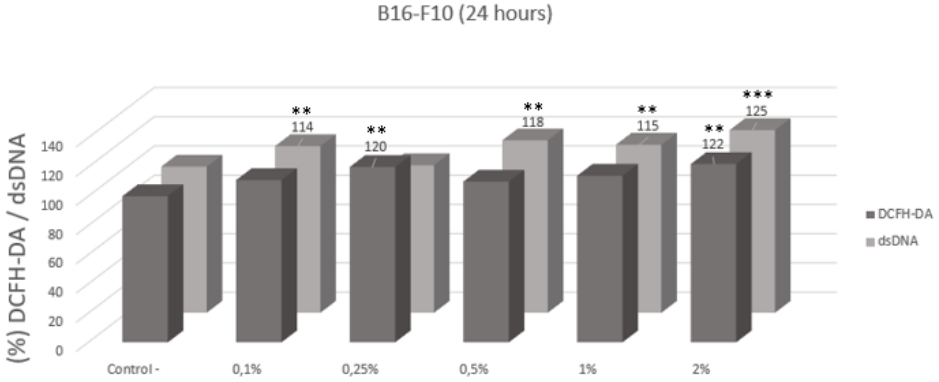


Figure 5. Fluorimetric assays for quantification of dsDNA and dichlorofluorescein in the murine melanoma tumor line B16-F10. Results expressed in fluorescence intensity emitted. Negative control (C-): cells in culture medium (1x10^{4}cells/200µL/well). Positive control (C+): cells in culture medium and hydrogen peroxide (100 mM). PicoGreen dichlorofluorescein. Data expressed as mean ± standard deviation (SD). The means followed by the symbology do not differ statistically. The analyzes were performed by one-way variance (ANOVA), followed by the Tukey post-test. Values with p <0.05 were considered statistically significant. * p <0.05, ** p <0.01 and *** p <0.001.



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