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Yellow mealworm beetle (*Tenebrio molitor*) larvae as an alternative model for antileishmanial drug evaluation

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ABSTRACT

Leishmaniasis is zoonotic disease caused by parasites of the genus *Leishmania*. Available treatments are limited and are associated with a range of adverse effects. The search for potential new drugs involves both *in vitro* and *in vivo* assays. Rodents are primarily employed as experimental models for *in vivo* assays. However, this practice raises ethical concerns, including issues related to environmental impact and animal welfare. Therefore, various alternative methods have emerged to avoid or reduce the use of mammals in laboratories for preclinical trials. The aim of this study was to evaluate *Leishmania amazonensis* infection in yellow mealworm (*Tenebrio molitor*) larvae. *T. molitor* larvae were infected with promastigotes (1×10^5; 1×10^6; and 1×10^7) and assessed through survival curves, degree of melanization, parasitic load, cell proliferation, and oxidative stress levels measured by reduced Glutathione (GSH) and nitrite levels. *Leishmania* promastigotes which invaded *T. motilor* plasmatocytes transformed into intracellular amastigotes. Ten percent of larval death was observed after 24 hours in larvae that received 1×10^5 and 1×10^6 promastigotes and 20 % mortality was found for those that received 1×10^7. The parasitic load revealed approximately 1750 parasites/larva infected with the highest concentration. Furthermore, the larvae showed a cellular response pattern similar to that seen in vertebrate host infections, with increased cell proliferation, activation of plasmatocytes, and elevated GSH and nitrite levels. This is the first study to establish *T. molitor* larvae as an alternative model for investigating *Leishmania* pathogenesis in invertebrates, proposing its use in preclinical trials for exploring potential new drugs to combat leishmaniasis.

1. Introduction

Leishmaniasis is classified as a parasitic zoonosis, caused by more than 20 different species in the *Leishmania* genus and belonging to the group of Neglected Tropical Diseases (Weld et al., 2022). Widely distributed, the disease threatents about 350 million people in more than 90 countries around the globe, with high prevalence in some part of the Americas, Asia, Africa, and the Middle East, and specifically in countries such as Brazil, China, Ethiopia, India, Israel, Kenya, Somalia, Sudan, and Yemen (Saini et al., 2022). Canine leishmaniasis is a major zoonotic disease endemic in more than 80 countries in the world. Dogs are the main animal reservoir for human visceral leishmaniosis and the disease is usually fatal if not treated in people (Morales-Yuste et al., 2022).

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Transmission occurs during the blood meal of female sandflies, which infect vertebrate hosts by regurgitating promastigote forms of the parasite into the skin. These parasites are then phagocytosed by cells of the mononuclear phagocytic system, where they transform into amastigotes. Clinical manifestations can be cutaneous, mucocutaneous, or visceral (Burza et al., 2018). Since the previous century, the general treatment of leishmaniasis has remained the same, with pentavalent antimonials and miltefosine being the first-line drugs, and amphotericin B, pentamidine, and paromomycin as second-line options (Chakravarty and Sundar, 2019; Pradhan et al., 2022). The drugs used to treat canine and human leishmaniasis do not cause complete parasite clearance. These drugs are not only limited and expensive but also cause numerous adverse reactions with increasing reports of parasite resistance (Roatt et al., 2014).

Studies on antileishmanial activity aimed at discovering and developing new drugs and/or therapeutic alternatives involve both *in vitro* and *in vivo* assays. In preclinical *in vivo* trials, murine models, such as BALB/c mice, are typically used. These animals are infected and treated with potential new substances to evaluate their therapeutic effects and toxicity (de Moraes Alves et al., 2020; Islamuddin et al., 2015). However, ethical concerns arise from such practices, including environmental impact and animal welfare, and they inevitably cause pain and suffering to the experimental animals. Therefore, excessive use of laboratory animals has raised ethical issues and prompted legal debates since the first rules about animals' usage in Ireland in 1635, the British Cruelty to Animals Act in 1876, and the Brazilian animal protection rules in 1924 and 1934. The most worldwide actions culminated in the Universal Declaration of Animal Rights published in 1978 (Lorena Ramos et al., 2024). Consequently, various alternative methods have emerged to reduce or eliminate the use of mammals in preclinical testing, focusing on improved time and labor efficiency, cost-effectiveness, and animal welfare (Ferreira et al., 2024)

Tenebrio molitor (Coleoptera, Tenebrionidae) is known for its larvae having good nutritional value, with valuable protein and lipid content. It is omnivorous and feeds on products of animal and vegetable origin. It is considered a "flour pest" and is distributed worldwide (Azzi et al., 2024). Its use as an alternative to mammalian animal models include advantages such as short life cycle, ease of handling, relaitively simple anatomy, as well as physilogical and immune functions that have similarity to those found in vertebrates, however, presenting fewer concerns and ethical implications compared to studies using laboratory mammals (Lorena Ramos et al., 2024). Numerous studies have identified *T. molitor*, *Zophobas morio* (superworm), and *Galleria mellonella* (wax moth) larvae as effective models for assessing toxicity, antimicrobial, antifungal, and antioxidant activities (Campelo et al., 2024; de Souza et al., 2015; Ribeiro et al., 2023; Sousa et al., 2023). It has also been shown in an *in vitro* study that hematocyte cells taken from the hemolymph of *G. mellonella* are capable of phagocytizing the promastigote forms of *Leishmania* and this, in turn, transforming into amastigotes within these cells (Tomiotto-Pellissier et al., 2016). However, the potential of insect larvae as *in vivo* models for the study of *Leishmania* parasite infections has remained unexplored.

In this context, the study aimed to evaluate the infection of *T. molitor* larvae by *Leishmania amazonensis*, as well as their survival, melanization patterns, and cellular response.

2. Materials and methods

2.1. Parasite

Promastigote forms of *L. amazonensis* (IFLA/BR/67/PH8) were cultured in Schneider's medium (Sigma, USA), supplemented with 10 % fetal bovine serum (FBS) (Sigma, USA) and penicillin-streptomycin 10.000 IU/10 mg (Sigma, USA) at 26°C in a biological oxygen demand (B.O.D.) incubator. The cultures were maintained at the Antileishmanial Activity Laboratory, Medicinal Plants Research Center in the Federal University of Piauí.

2.2. Tenebrio molitor

T. molitor larvae were obtained from the Physiology Laboratory of the Department of Veterinary Morphophysiology, Federal University of Piauí (UFPI), Teresina-PI, Brazil. (CCA/UFPI). Their life cycle includes four stages: egg, larva, pupa and beetle, completing the entire cycle in approximately four to five months. The beetles were kept in closed plastic boxes measuring 60 × 40 x 80 cm, fed *ad libitum* with wheat bran and vegetable, including fresh potato pieces distributed every week as a vegetable water source. They were maintained in an isolated room at a temperature of 25°C ± 2°C, relative humidity of 70 ± 5 %, and total darkness except during handling, with optimal ventilation. (Azzi et al., 2024). After hatching, each female beetle lays 200–500 eggs on the substrate. The larvae hatch from the eggs when they are 60-day-old, they reach approximately 20–25 mm in size, and an average weight of 150 mg (Sönmez, 2021). Larvae which were light in color, uniform in size and with unspotted tegument coloration were selected for the experiments, confirming the uniformity of the animals used in this study.

2.3. Infection of the larvae

To assess the infection of *T. molitor* larvae by *L. amazonensis*, 10 larvae were used for each tested inoculum concentration, in duplicate. The promastigote forms in the stationary growth phase were administered at a volume of 10 μL per larva, containing 1×10^5, 1×10^6 and 1×10^7 *Leishmania* promastigores using a Hamilton syringe (701 N, 26-gauge, 100 μL capacity). The application site was in the hemocoel region on the second or third visible sternite beneath the legs, on the ventral or dorsal portion. The larvae were incubated at 33 °C in Petri dishes containing a growth diet, and the number of dead larvae was evaluated after 24 and 48 hours. Larval death was confirmed through visual inspection of each larva, as well by determination of the presence of melanization and/or responses to physical stimuli following a gentle touch (de Souza et al., 2015). Dimethyl sulfoxide (DMSO, Sigma, USA) was used as a postivie control to induce toxicity in the larvae and to promote melanization through toxicity, GSH, and nitrite. For negative control, the larvae were injected with 10 μL saline solution, at the same application site mentioned above (Ribeiro et al., 2023).

2.4. Hemolymph collection and homogenate preparation

After the 48-hour period described above, the larvae were cooled in a cold chamber at 4°C for 3 minutes. Then, a small puncture was made in their cuticle using a 29-gauge needle in the hemocoel region, between the second and third visible sternites below the legs on the dorsal side. Hemolymph was allowed to extravasate (approximately 3 μL), and a short smear was prepared on a microscopy slide. The slides were then stained with Panoptic staining®, purchased from Merck Chemical Company (Germany).and examined under an optical microscope at 1000x magnification.

Subsequently, the larvae were frozen at −20°C for 1 hour to induce death. Each larva was then macerated in a mortar with 2 mL of phosphate-buffered saline (PBS) at pH 7.4. The resulting material (homogenate) was transferred into microtubes (2 mL) and centrifuged at 3.000 rpm for 10 minutes at 4°C, and the supernatant was collected. This supernatant was then used for subsequent analyses of pigmentation and measurements of GSH and nitrite levels.

2.5. Pigmentation evaluation assay

Ten μL of the homogenate samples were mixed in 190 μL phosphate buffer (pH 7.4) and plated in 96-well culture plate, purchased from Kasv (Brazil). The plates were then subjected to gentle shaking for 10 minutes to ensure complete homogenization. Following this, the absorbance was

measured in a microplate spectrophotometer (model BioTek ELx800; Vermont USA) at 550 nm.

2.6. Parasite load evaluation

The parasite load was assessed through direct counting of parasites internalized in plasmatocytes in the hemolymph smear, as previously described and expressed as Leishman-Donovan Units (LDU) (Islamuddin et al., 2015). That was calculated as the number of parasites per 100 hematocytic cells which was multiplied by the larva's weight in milligrams.

2.7. Cellular evaluation of infected larvae

To characterize the basic cellular morphology, adherent hemocytes were stained using Panoptic staining® as previously described. The identification of morphological cell types was based on counting 100 cells, assessing differences in size, morphology, and staining affinity (Vommaro et al., 2021). Images were captured using an Olympus SC30 (Japan) camera attached to the microscope.

2.8. Evaluation of the induction of reduced glutathione (GSH)

The buffer solution used to prepare larval homogenate for GSH evaluation was 0.02 M ethylenediaminetetraacetic acid (EDTA), with a ratio of 100 mg sample per 1 mL buffer. The supernatant from the second homogenate centrifugation (as previously described) was collected and diluted with an equal volume of 10 % trichloroacetic acid (TCA) solution. The mixture was stirred for 30 seconds and then centrifuged at 3000 rpm for 15 minutes. A portion of the supernatant from this centrifugation was mixed with two volumes of Tris buffer (0.4 M EDTA, buffered to pH 8.9). For the spectrophotometic reading, 50 μL of 0.01 M 5,5′-dithiobis-(2-nitrobenzoic acid) (DTNB) was added to each 3 mL of solution. The spectrophotometer was set to a wavelength of 412 nm and zeroed with a blank solution (2 mL of distilled water, 4 mL of Tris buffer, and 100 μL of 0.01 M DTNB). The sample was then read, and the concentrations of reduced sulfhydryl groups, in μM, were calculated by multiplying the absorbance obtained by a factor 317.8 (Habeeb, 1972).

2.9. Nitrite evaluation

Nitrite concentration, which is the final product of nitric oxide production, was determined using the Griess method (Green et al., 1982). Prior to the assay, tissue samples were deproteinized by adding zinc sulfate solution. The buffer solution used for preparing homogenate was phosphate buffer (PBS - pH 7.4), with a ratio of 50 mg of tissue per 1 mL of buffer. The preparation and collection of the supernatant of the homogenate was followed by deproteinization. One hundred and eighty μL of 0.15 M zinc sulfate (ZnSO4), 3 μL of 10 M sodium hydroxide (NaOH), and 300 μL of the sample were added and incubated in a microtube on ice for 15 minutes. The mixture was then centrifuged at 14.600 rpm for 5 minutes to obtain the deproteinized supernatant. For the assay, 100 μL of this deproteinized supernatant was added in duplicate to a 96-well microplate, along with 100 μL of Griess reagent, and kept for 10 minutes. Absorbance was measured at 560 nm.

2.10. Statistical analysis

Survival analyses were determined using the log-rank test, and survival curves were generated using the Kaplan-Meier method. The significance level in all conducted assays was $p < 0.05$ * , utilizing one-way ANOVA followed by the Bonferroni post-hoc test. All experiments were conducted in duplicate, and the results were expressed as mean ± standard desviation.

3. Results

The infection of *T. molitor* larvae by *L. amazonensis* caused death in a concentration-dependent manner. A 10 % mortality rate was observed in larvae infected with 1×10^5 and 1×10^6 promastigotes within the first 24 hours of infection, and it remained constant after 48 hours. In larvae infected with 1×10^7 promastigotes, the mortality rate was 20 % within the first 24 hours and remained constant after 48 hours. Larvae injected with DMSO, exhibited a mortality rate of approximately 50 % after 24 hours, which further increased by 30 % after 48 hours, resulting in an overall mortality rate of 80 %, as shown in the survival curve in Fig. 1.

In addition to mortality, melanization was also observed after the 48-hour incubation period following the inoculation of *Leishmania* promastigotes. All larvae exhibited concentration-dependent melanization, with a statistically significant difference ($P < 0.05$) only in the group that received an inoculum of 1×10^7 *Leishmania* promastigotes (Fig. 2).

When evaluating the hemolymph of the larvae through a smear stained by the Quick Panoptic stain, the presence of *L. amazonensis* amastigote forms internalized in the plasmatocytes of the larvae was observed, confirming the successful transformation of invading promastigotes into intracellular amastigotes, similar to such conversion seen in vertebrate host cells (Fig. 3). An interaction between these parasites and the larvae's defense cells was observed 6 hours after the inoculation of promastigotes when phagocytosis of parasites was evident (Fig. 3 A and B). These plasmocyte phagocytic cells are small in size before phagocytosis occurs. When the parasites are in contact with plasmocytes and become phagocytized, these cells are activated and grow in size. Within 24-72 hours from infection it was already possible to identify the amastigotes forms of *Leishmania* internalized in plasmatocytes, which enlarged due to activation (Fig. 3C, D and E).

The percentage of larvae infected with *L. amazonensis* followed a concentration-dependent pattern of inoculated promastigotes. The group that received 1×10^5 promastigotes showed 5 % infection rate while the group that received 1×10^6 promastigotes showed an infection rate of 75 %. In the group that received 1×10^7 promastigotes, all larvae were infected, resulting in an infection rate of 100 % (Fig. 4A).

In addition, the percentage of infected plasmatocytes and the number of internalized amastigotes were determined by direct detection of amastigote forms. Regarding the number of infected plasmatocytes, *L. amazonensis* amastigotes were found in all groups. The number of infected plasmatocytes was dependent on the amount of *Leishmania* promastigotes in the inoculum. The group of larvae that received the 1×10^7 concentration had a significantly higher ($P < 0.0001$) average of infected plasmatocytes compared to the other groups, which was 15 % (Fig. 4B).

A similar pattern was observed in the assessment of the parasitic load. The group that received an inoculum of 1×10^5 *L. amazonensis* promatigotes had an average number of 300 LDU amastigotes/larvae. The group inoculated with 1×10^6 had an average of 400 LDU, and finally the group that received the highest concentration, 1×10^7, had an average of 1725 LDU/larvae (Fig. 4C). Additionally, the hemocyte types found in the larvae were also observed. The cell types identified were granulocytes and plasmatocytes. Activated plasmatocytes and cell proliferation through mitosis were also quantified (Fig. 5).

There was no significant difference in the number of granulocytes and plasmatocytes in all larvae infected with different concentrations of *Leishmania* when compared to negative control larvae. However, there was a statistically significant difference ($P < 0.01$) in the number of activated plasmatocytes and cellular mitosis in the group infected with 1×10^7 promastigotes. The number of activated plasmatocytes was about 10 per larva, while no activated plasmatocytes were identified in the negative control group, indicating that plasmatocyte activation occurred due to the presence of the parasite. Similarly, cell proliferation through mitotic division occurred more frequently in the group infected with 1×10^7promastigotes ($P < 0.001$). In this group, an average of 5 mitoses per infected larva was observed, while no dividing cells were

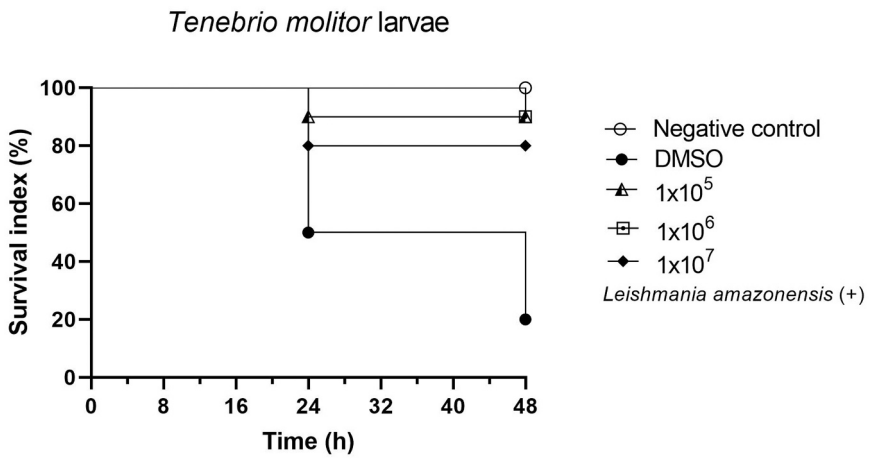

Fig. 1. Kaplan-Meier survival curve of *Tenebrio molitor* larvae infected with different concentrations of *Leishmania amazonensis*. The larvae were incubated for 48 hours post-infection. The negative control group was injected with saline solution, while the positive control group was injected with DMSO.

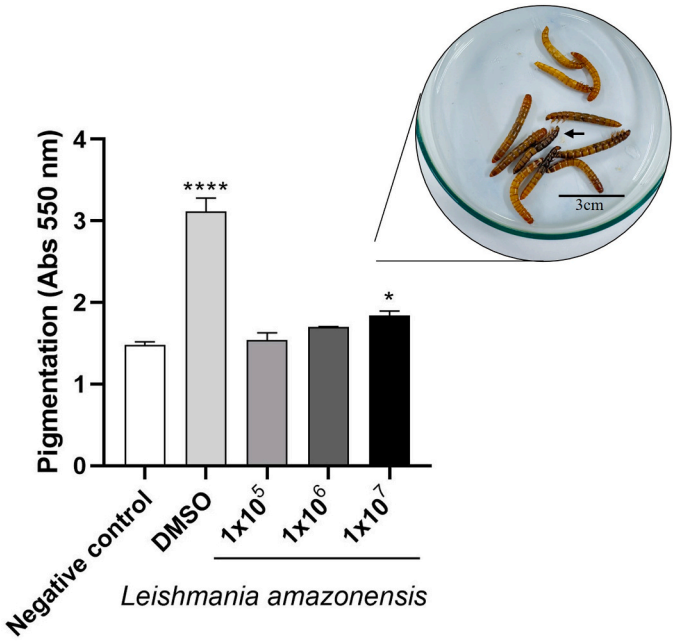

Fig. 2. Evaluation of pigmentation in *Tenebrio molitor* larvae infected with different concentrations of *Leishmania amazonensis*. The larvae showed dark color (black arrow) after inoculation of the parasites. Pigmentation was assessed in the homogenate obtained from larval maceration in absorvance 550 nm. Statistical significance $P < 0.05$ * ; $P < 0.0001$ * ** *.

detected in the negative control group (Fig. 6).

Leishmania infection in mealworm larvae also caused an increase in reduced glutathione levels in the group inoculated with 1×10^7 promastigotes, compared to the negative control larvae group, indicating pathogen-mediated stress (Fig. 7A). In this group, the amount of glutathione was approximately 500 μM/mg, while the negative control larvae group expressed GSH levels of 256 μM/mg (Fig. 7A).

Nitrite, which is the end product of nitric oxide production, an indicator of phagocytic cell activation, was quantified by measuring nitrite concentrations in μMol/mg, by adding (v/v) Griess reagent to the homogenate of larvae infected with different concentrations of *Leishmania*. There was a statstically significant ($P < 0.05$ %) concentration-dependent increase in nitrite, in the group infected with 1×10^7 parasites, where it more than doubled compared to the negative control larvae group (Fig. 7B).

4. Discussion

Many species of vertebrates are susceptible to *Leishmania* spp. infection, including humans, dogs, cats, horses, cattle, foxes, domestic rodents, and also wild mammals, certain avain spp., and reptiles (Baneth and Solano-Gallego, 2022; Batista et al., 2023; Matheus et al., 2021; Mhadhbi and Sassi, 2020). Some of these hosts serve as important reservoirs for this disease, which is spreading worldwide and has become an emerging zoonosis and a unique health problem in many regions (Montaner-Angoiti and Llobat, 2023). This study is the first to describe the experimental infection of *T. molitor* invertebrate larvae with a *Leishmania* sp., expanding the possibilities for studying the pathogenesis of the disease and implementing an alternative model for pre-clinical trials of new drugs to combat this disease.

The use of invertebrates as experimental models in scientific research has a long history. This large and diverse group has facilitated important discoveries that have often led to the creation of entirely new areas of research because working with simple invertebrate organisms such as *Drosophila* and *Aplysia* is convinient and more accepted ethically (Søvik and Barron, 2013). The use of beetles from the Tenebrionidae family, such as *T. molitor* larvae, has extended ethically-accepted studies of acute toxicity, antimicrobial and antiparasitic activity and even their use as a food source for humans and animals. A major advantage of these experimental models is that they are inexpensive and easy to cultivate and do not raise major ethical concerns (Brai et al., 2023; Campelo et al., 2024; Díaz-Navarro et al., 2021; Musembi et al., 2024)

The *T. molitor* larvae used in this study responded to infection by *L. amazonensis*, showing evidence of toxicity, as demonstrated by evaluating the infection kinetics using increasing concentrations of the inoculum that promoted larval death. Other invertebrate models, such as *G. mellonella* (wax moth) and *Nauphoeta cinerea* (speckled cockroach), have also been used to assess the virulence of microorganisms like bacteria and fungi (Muñoz et al., 2020; Rossato et al., 2019; Silva et al., 2018). The presence of xenobiotics (*L. amazonensis*) in the larvae triggered an immune response, described as melanization or brown pigmentation. Melanization is an enzymatic process that plays an important role in the humoral immune response, as well as contributing to wound healing and preventing hemolymph loss (Eleftherianos et al., 2021). Melanization occurs through the deposition of melanin during the nodulation/encapsulation of pathogens, which contributes to the physical isolation of pathogenic microorganisms and prevents their spread within the host. This process is triggered after the recognition of Pathogen-Associated Molecular Patterns (PAMPs) of phagocytic hematocyte cells, especially plasmacytes. Defense cells eliminate pathogens through phagocytosis, melanization, and lysis (Eleftherianos et al.,

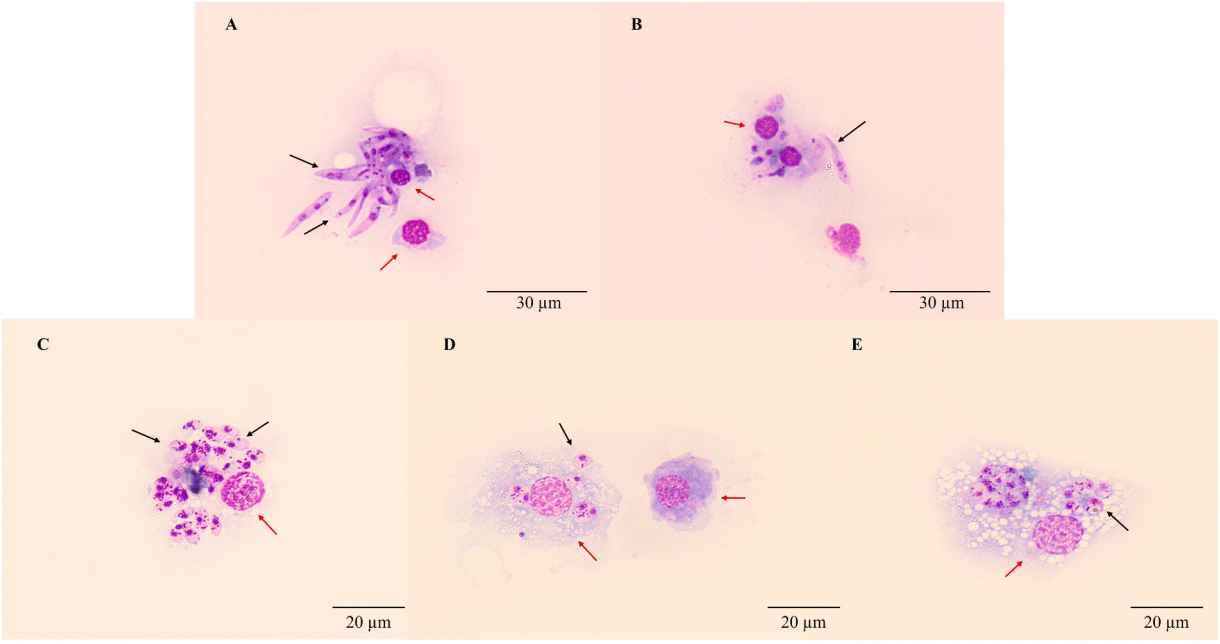

Fig. 3. Plasmatocytes of *Tenebrio molitor* infected with *Leishmania amazonensis*. The amastigote forms were identified by direct examination under optical microscopy (100x objective), evaluating the larval hemolymph in a smear stained by the quick Panoptic kit (Laborclin). The interaction between the promastigote forms of *L. amazonensis* (black arrows) and the larval plasmatocytes (red arrow), and the phagocytosis process can be observed in images A and B. Images C, D and E show internalized amastigotes (black arrow) in plasma cells (red arrow).

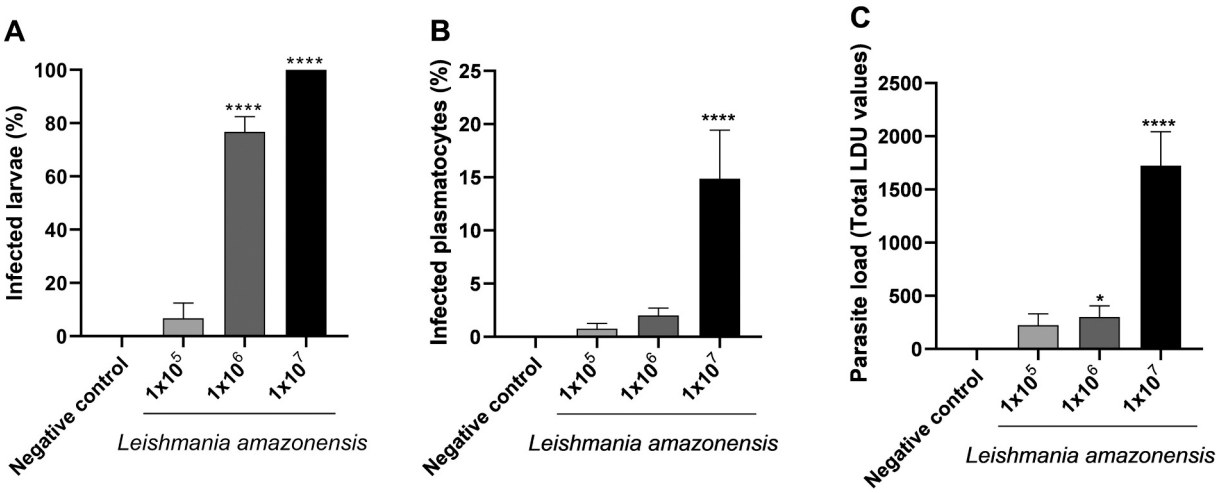

Fig. 4. *Tenebrio molitor* infection by *Leishmania amazonensis*. A - Percentage of *T. molitor* larvae infection by different concentrations of *L. amazonensis*. The infection was assessed by detecting amastigote forms in the hemolymph smears of each inoculated larva. B - Percentage of plasmatocytes in *T. molitor* larvae infected with different concentrations of *L. amazonensis.* Plasmatocytes were counted directly by microscopic visualization (100x objective) by differential counting of 100 cells in different fields. C - Average number of *L. amazonensis* amastigotes in infected *T. molitor* larvae. The graph represents the total average LDU/larva. Parasites were counted by observing 1000 cells per slide multiplied by the average weight of the larvae (150 mg), viewed under a light microscope (1000x objective). $P < 0.05$ * ; $P < 0.0001$ * ** *.

2021; Nakatogawa et al., 2009). In this study, all *T. molitor* larvae inoculated with *L. amazonensis* showed melanization, demonstrating pathogen recognition and the activation of the immune response in an attempt to overcome the infection.

One of the most important cellular defense reactions in insects is phagocytosis, an evolutionarily conserved cellular process between vertebrates and invertebrates to destroy pathogens (Hillyer, 2016). The infection of larvae was confirmed by the presence of amastigote forms internalized in the host's defense cells. This demonstrates the ability of these cells to recognize and phagocytize the pathogen, and surprisingly, the parasite's ability to transform to its next life stage within this "host" organism. The transformation of promastigote forms into amastigotes occurred within these cells, as it does when vertebrate hosts are infected, suggesting the pathogenic capacity of this *Leishmania* sp. in a non-natural host. Other authors have also reported the infectious capacity of *Leishmania* in the cells of another insect, the wax moth *G. mellonella.* When hemocytes of *G. Mellonella* were infected *in vitro* with promastigote forms of *L.* (*Viannia*) *brasiliensis*, it was observed that transformation into amastigotes occurred within these cells (Tomiotto-Pellissier et al., 2016). In this study, infection kinetics were performed with different inoculum (1×10^5, 1×10^6 and 1×10^7 *Leishmania* promastigotes). The fact that no infection was observed when

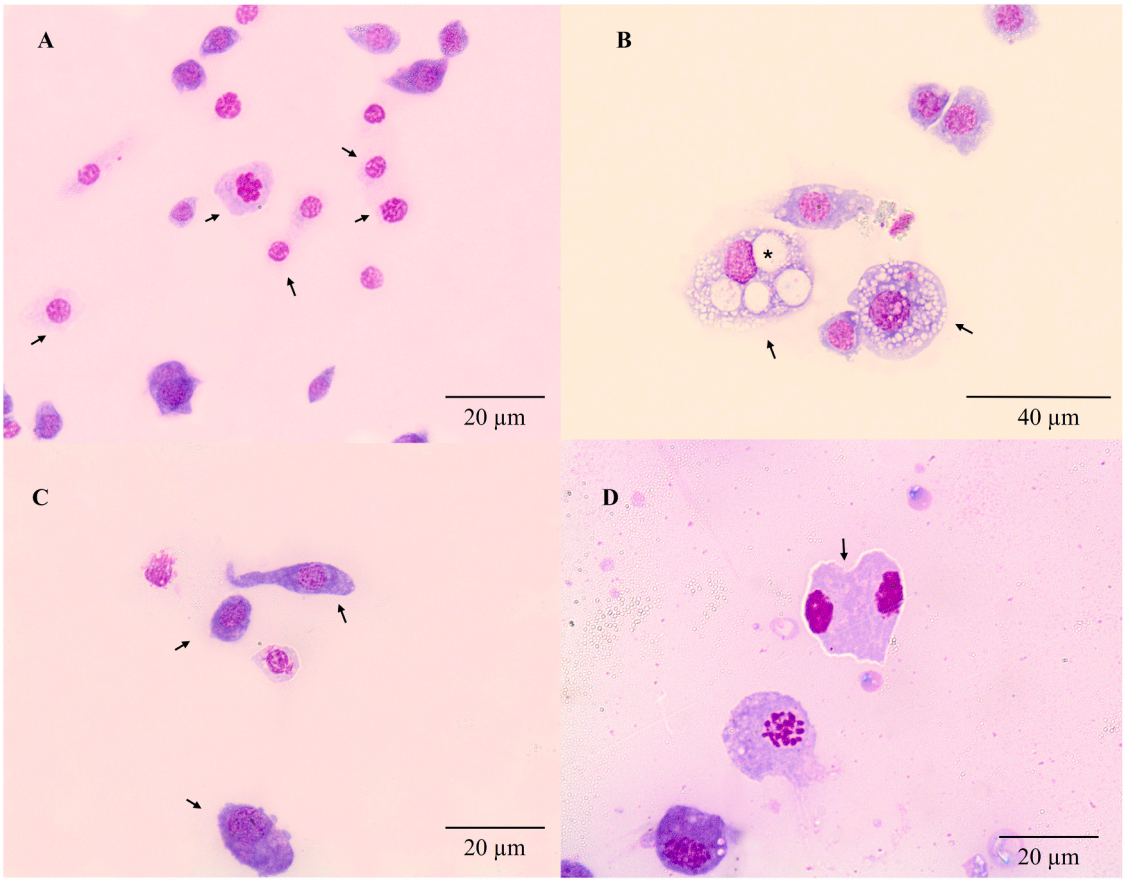

Fig. 5. Identification of cell types found in *Tenebrio molitor* larvae. Plasmatocytes (A), activated plasmatocytes (B), granulocytes (C), and plasmatocytes in mitosis (D) were identified. The symbol (*) in figure B, indicates the presence of parasitophorous vacuoles in the plasmatocytes, confirming their activation. Identification was performed through direct observation (100x objective) of hemolymph smear stained with the quick Panoptic kit (Laborclin).

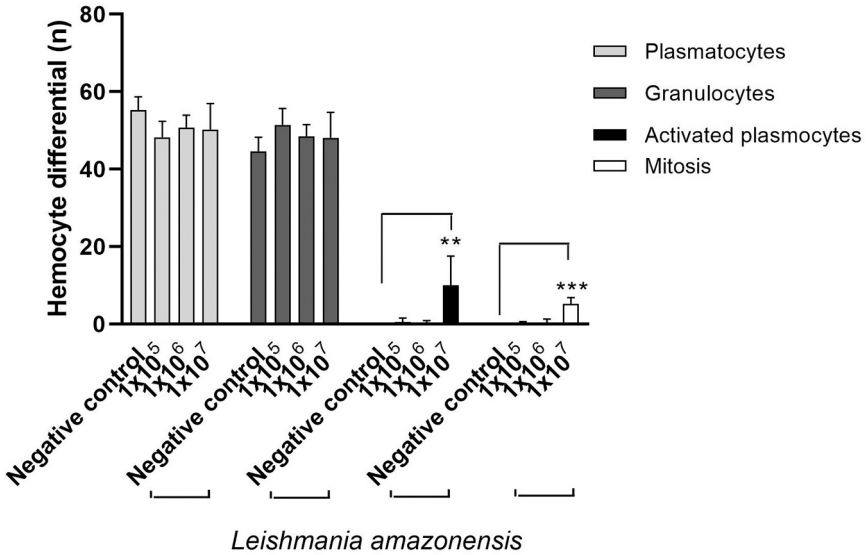

Fig. 6. Differential hemocyte of negative control and infected *Tenebrio molitor* larvae with different numbers of *Leishmania amazonensis*. The cells were identified through direct observation using an optical microscope (1000x objective) of hemolymph smears stained using quick Panoptic kit $P < 0.05$ * ; $P < 0.01$ * *; $P < 0.001$ * ** .

using 1×10^5 promastigotes, may be related to the activity of natural insect substances such as peptides with antimicrobial capabilities or some other innate immune response mechanism that may have been able to suppress the lower grade inoculum infection (Petronio Petronio et al., 2022),

The *T. Molitor* larvae infected with 1×10^7 promastigotes showed a considerable propagation and development of parasitic load (an average of 1750 parasites/larva) suitable for the implementation of this

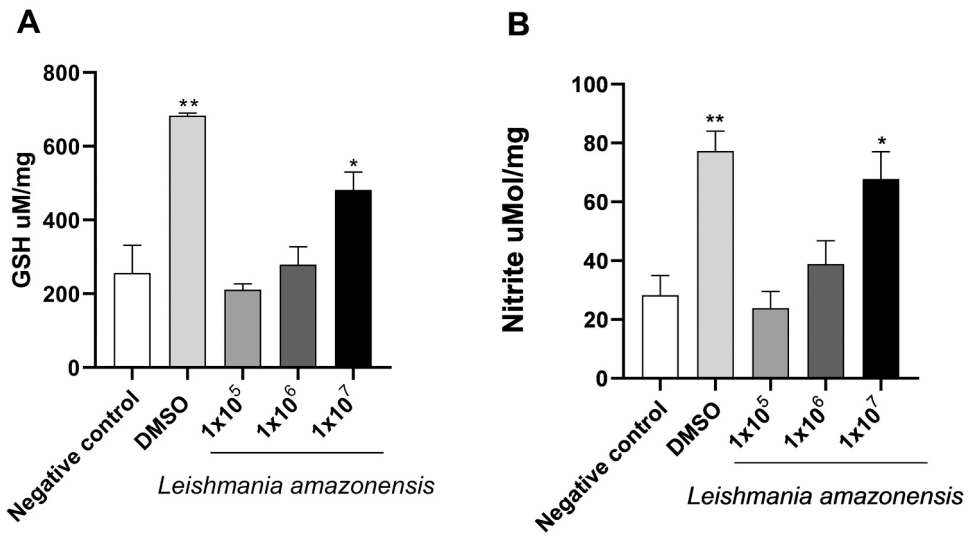

Fig. 7. Quantification of reduced glutathione (GSH) (A) and nitrite (B) in the homogenate of negative and *Leishmania amazonensis* infected larvae. GSH was quantified in the homogenate of the larvae from each group. The reduced sulfhydryl groups were calculated by multiplying the absorbance obtained in the assay by a factor of 317.8. For quantification of nitrite, the homogenate was mixed in equal parts with the Griess reagent. Results are expressed as mean ± SEM, $P < 0.05$ * ; $P < 0.01$ * *.

procedue for use as an alternative model of infection. The use of *Tenebrio* larvae as an experimental infection model could reduce the time needed to evaluate treatment and generate results, as shown in studies evaluating antifungal activity using *G. mellonella* as a model, where experimental treatment can begin 30 minutes after inoculation of the pathogen (Silva et al., 2018). In BALB/c mice infected with *Leishmania*, the duration of experiments and maintenance of infected animals kept until disease develops and results are obtained can take up to 70 days (de Moraes Alves et al., 2020; Wabwoba et al., 2010). *Tenebrio molitor* larvae can be used for antileishmanial drug evaluation studies to obtain faster results, so that only promising compounds can be later tested in conventional animal models. However, the use of this alternative model is aimed at preclinical trials to contemplate the 3 R's policy (Reduction, Refinement and Replacement) in relation to conventional animal models. Substances with antileishmanial activity can be tested in the *in vivo* model of *T. molitor* larvae and those which are most promising may continue to be tested in a conventional model such as the BALB/c mice model (Brai et al., 2023; Vommaro et al., 2021).

The limitations of this model include the lack of a lymph system, antibody production, and target organs such as the bone marrow and spleen, which *Leishmania* organisms react with in the vertebrate host, and the limited time which the model can sustain *Leishmania* infection. However, this model demonstrates the transformation of the promastigte stage of the parasite into intracellular amastigotes in phagocytic cells, and can demonstate the activity of drugs in several pharmaceutical formulations on the intracellular amastigote, which is the life stage of *Leishmania* responsible for dissemination within the vertebrate host and the induction of the pathological impacts of this infection. Another advantage in relation to *T. molitor* model in comparison to the BALB/c infection model is its lower cost and its rapid conclusion, since the progression of infection in this model is fast compared to that of animal infection in conventional models.

The *T. molitor* model also allows infection of a large number of individual larvae at the same time instead of having to infect multiple animals. The breeding and production of *Tenebrio* larvae do not require large facilities, sophisticated infrastructure and intensive labor or technical capacity. On the other hand, the maintenance of laboraotry mice requires more expensive facilities with specialized care in breeding, management and disease control (Brai et al., 2023). Furthermore, as *T. molitor* is an invertebrate, studies involving it usually do not require approval by an ethics committee (Lorena Ramos et al., 2024).

In this study, it was demonstrated that in addition to observing amastigote forms of the parasite within the host cells, there was also an increase in cell proliferation (cells in mitosis) and alterations in the development of hemocyte morphology, particularly in the plasmatocytes, which are the primary phagocytic cells of *T. molitor*. The plasmatocytes of the infected larvae showed typical characteristics of activation, such as 'spreading' and 'smudging,' similar to what occurs in macrophages of BALB/c mice infected by *Leishmania* sp. in *in vitro* studies (Alves et al., 2017). This morphological response of the hemocytes to pathogen challenges may be related to an enhanced activation capacity and an attempt to combat the infection, which has also been observed in experimental infections of *T. molitor* inoculated with *Staphylococcus aureus* (Urbański et al., 2018).

The presence of *Leishmania* in the larvae also triggered an oxidative stress, leading to an increase in GSH and nitrite levels. In insects, the function of the fat body is similar to the liver function in vertebrates, and the reduced GSH biomarker plays a role in stabilizing reactive oxygen species (ROS) molecules to prevent macromolecular damage caused by free radicals (Jones, 2008). In this case, elevated GSH levels were induced by the presence of *Leishmania*, which is consistent with the elevated nitrite levels also observed. In leishmaniasis, an important signaling pathway that may be involved in disease control is the production of NO by phagocytic cells (Bogdan and Riillinghoff, 1998). This mechanism is considered to be one of the most effective in the host's defense against *Leishmania* infection. Within the phagolysosome, NO combines with superoxide anions to produce peroxynitrite, which is highly reactive and microbicidal (Bogdan and Riillinghoff, 1998). These defense mechanisms found in the new model are essential for investigating potential new antileishmanial drugs, and therefore may help in the search for therapeutic alternatives for *Leishmania* spp. treatment focusing not only on the activity of the parasite, but also on the ability of the host molecules to act synergistically with the immune system to control infection and promote healing (Alves et al., 2021).

In fact, the alternative *Leishmania* infection model proposed in this study can reduce, and in some cases replace the use of conventional models for antileishmanial drug assessment. Despite being an invertebrate organism and different in physiological terms to mammalian models, *T. molitor* larvae show similarity to vertebrates. Some of these bioequivalences include the larval fat body which has some functional similarities to the mammalian liver, as it carries out biotransformation and antioxidant processes. Other examples are the larvae's Malpighian

tubules, which perform functions similar to the kidneys, and blood cells (hemocytes) which play a similar role to white blood cells of mammals (phagocytosis, participation in coagulation, melanization and cytokine production). Therefore, drug molecules can be initially screened in this alternative model (Brai et al., 2023). This study addressed the parasite's ability to be infected by *Leishmania* sp. and the ability of this infection to progress from the promastigote stage to intacellular amasitgotes and the larva's response to this infection. We demonstrated here the importance of larval survival, melanization, cell proliferation and production of antioxidant enzymes. Studies supported by this information will be able to evaluate the ability of drugs to modulate these parameters and eliminate infection or reduce the parasite load in this model, selecting the most promising molecules in additional studies employing conventional models.

5. Conclusions

This is the first study to establish the *T. molitor* model to investigate the pathogenesis of *Leishmania* in this host. *T. molitor* larvae were susceptible to infection *by L. amazonensis*, when infected with 1×10^7 promastigotes, and this pattern is reproducible for studies to prospect new antileishmanial drugs. The parameters which can be evaluated include survival rate, melanization, parasite load, cell proliferation and levels of enzymes associated with the oxidative stress. *T. molitor* defense cells are capable of recognizing and phagocytizing the parasite's promastigotes, which then transform into amastigotes within the larval plasmatocytes, leading to changes in the morphology of the host cells, immune and toxic responses. This study validates the use of this new model in preclinical trials, thus contributing to the reduction of ethical conflicts associated with the use of vertebrates in such studies.

CRediT authorship contribution statement

da Rocha Sousa Leonardo: Investigation. **de Almeira José Otávio Carvalho Sena:** Investigation. **Tavares Neto Jose Moreira:** Investigation. **Acha Boris Timah:** Investigation. **Alves Michel Muálem de Moraes:** Writing – review & editing, Writing – original draft, Project administration, Methodology, Investigation, Funding acquisition, Formal analysis, Data curation, Conceptualization. **de Amorim Carvalho Fernando Aécio:** Investigation. **da Franca Rodrigues Klinger Antonio:** Writing – review & editing, Investigation. **Arcanjo Daniel Dias Rufino:** Investigation. **dos Anjos Bianca Soriano:** Investigation. **Neta Pastora Pereira Lima:** Investigation. **de Souza Leal Paulline Paiva Mendes:** Investigation. **de Almeida Passos Victoria Hannah Araújo:** Investigation. **Baneth Gad:** Investigation.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

References

Alves, M.M., de, M., Arcanjo, D.D.R., de Carvalho, R.C.V., Amorim, L.V., Dos Santos, I.L., Santos, R.R.L., Figueiredo, K.A., Figueiredo, J.F.S., Sobrinho-Júnior, E.P.C., da Cruz, L.P.L., Sousa, V.C., Dos Santos, L.P., Gonçalves, J.C.R., Carneiro, S.M.P., de Mendonça, I.L., Carvalho, F.A., de, A., 2021. Methods of macrophages activation and their modulation for the prospection of new antileishmania drugs: a review. Biosci. J. 37, e37077. https://doi.org/10.14393/BJ-V37N0A2021-53770.

Alves, M.M.M., Brito, L.M., Souza, A.C., Queiroz, B.C.S.H., de Carvalho, T.P., Batista, J. F., Oliveira, J.S.S.M., de Mendonça, I.L., Lira, S.R.S., Chaves, M.H., Gonçalves, J.C. R., Carneiro, S.M.P., Arcanjo, D.D.R., Carvalho, F.A.A., 2017. Gallic and ellagic acids: two natural immunomodulator compounds solve infection of macrophages by *Leishmania major*. Naunyn Schmiede Arch. Pharm. 390, 893–903. https://doi.org/10.1007/s00210-017-1387-y.

Azzi, M., Elkadaoui, S., Zim, J., Desbrieres, J., El Hachimi, Y., Tolaimate, A., 2024. *Tenebrio molitor* breeding rejects as a high source of pure chitin and chitosan: Role of the processes, influence of the life cycle stages and comparison with *Hermetia illucens*. Int J. Biol. Macromol. 277, 134475. https://doi.org/10.1016/j.ijbiomac.2024.134475.

Baneth, G., Solano-Gallego, L., 2022. Leishmaniasis. Vet. Clin. North Am. - Small Anim. Pract. 6, 1359–1375. https://doi.org/10.1016/j.cvsm.2022.06.012.

Batista, J.F., Neto, F., das, C.R.M., Lopes, K.S.P., do, P., Sousa, C.M.G., Alcântara, D.S., Baêta, S., de, A.F., Alves, M.M., de, M., de Mendonça, I.L., 2023. Clinical, hematological, biochemical, and histopathological evaluations in domestic cats (*Felis catus*) infected by *Leishmania infantum*. Rev. Bras. De. Parasitol. Vet. 32 (3), e004723. https://doi.org/10.1590/S1984-29612023037.

Bogdan, C., Riillinghoff, M., 1998. The immune response to *Leishmania*: mechanisms of parasite control and evasion. Int. J. Parasitol. 28 (1), 121–134. https://doi.org/10.1016/S0020-7519(97)00169-0.

Brai, A., Poggialini, F., Vagaggini, C., Pasqualini, C., Simoni, S., Francardi, V., Dreassi, E., 2023. *Tenebrio molitor* as a Simple and Cheap Preclinical Pharmacokinetic and Toxicity Model. Int J. Mol. Sci. 24 (3), 2296. https://doi.org/10.3390/ijms24032296.

Burza, S., Croft, S.L., Boelaert, M., 2018. Leishmaniasis. Lancet 392, 951–970. https://doi.org/10.1016/S0140-6736(18)31204-2.

Campelo, J.E.S., Nascimento, M.O., do, Carvalho, A.L.M., Santos, H.S.P., Almeida, J.O.C. S., de, Alves, M.M., de, M., Arcanjo, D.D.R., Tavares Neto, J.M., Muratori, M.C.S., Costa, A.P.R., 2024. Evaluation of the acute toxicity of ellagic acid and gallic acid incorporated in Poloxamer407® gel, in *Zophobas morio* larvae. Toxicol. Vitr. 95, 105727. https://doi.org/10.1016/j.tiv.2023.105727.

Chakravarty, J., Sundar, S., 2019. Current and emerging medications for the treatment of leishmaniasis. Expert Opin. Pharm. 20 (10), 1251–1265. https://doi.org/10.1080/14656566.2019.1609940.

de Moraes Alves, M.M., Arcanjo, D.D.R., Figueiredo, K.A., de Sousa Macêdo Oliveira, J. S., Viana, F.J.C., de Sousa Coelho, E., Lopes, G.L.N., Gonçalves, J.C.R., Carvalho, A.L. M., dos Santos Rizzo, M., Chaves, M.H., de Mendonça, I.L., de Amorim Carvalho, F. A., 2020. Gallic and ellagic acids are Promising adjuvants to conventional amphotericin B or the treatment of cutaneous leishmaniasis. Antimicrob. Agents Chemother. 64 (12), e00807–e00820. https://doi.org/10.1128/AAC.00807-20.

de Souza, P.C., Morey, A.T., Castanheira, G.M., Bocate, K.P., Panagio, L.A., Ito, F.A., Furlaneto, M.C., Yamada-Ogatta, S.F., Costa, I.N., Mora-Montes, H.M., Almeida, R.S., 2015. *Tenebrio molitor* (Coleoptera: Tenebrionidae) as an alternative host to study fungal infections. J. Microbiol Methods 118, 182–186. https://doi.org/10.1016/j.mimet.2015.10.004.

Díaz-Navarro, M., Bolívar, P., Andrés, M.F., Teresa Gómez-Muñoz, M., Martínez-Díaz, R. A., Valcárcel, F., García-París, M., Bautista, L.M., González-Coloma, A., 2021. Antiparasitic effects of potentially toxic beetles (Tenebrionidae and Meloidae) from steppe zones. 13 (7), 489. ⟨https://doi.org/10.3390/toxins⟩.

Eleftherianos, I., Heryanto, C., Bassal, T., Zhang, W., Tettamanti, G., Mohamed, A., 2021. Haemocyte-mediated immunity in insects: Cells, processes and associated components in the fight against pathogens and parasites. Immunology 164 (3), 401–432. https://doi.org/10.1111/imm.13390.

Ferreira, P.M.P., Ramos, C.L.S., Filho, J.I.A.B., Conceição, M.L.P., Almeida, M.L., do Nascimento Rodrigues, D.C., Porto, J.C.S., de Castro e Sousa, J.M., Peron, A.P., 2024. Laboratory and physiological aspects of substitute metazoan models for *in vivo* pharmacotoxicological analysis. Naunyn Schmiede Arch. Pharm. 398 (2), 1315–1339. https://doi.org/10.1007/s00210-024-03437-5.

Green, L.C., Wagner, D.A., Glogowski, J., Skipper, P.L., Wishnok, J.S., Tannenbaum, S.R., 1982. Analysis of nitrate, nitrite, and [15N]nitrate in biological fluids. Anal. Biochem 126 (1), 131–139. https://doi.org/10.1016/0003-2697(82)90118-x.

Habeeb, A.F.S.A., 1972. Reaction of protein sulfhydryl groups with Ellman's reagent. Methods Enzym. 25, 457–464. https://doi.org/10.1016/S0076-6879(72)25041-8.

Hillyer, J.F., 2016. Insect immunology and hematopoiesis. Dev. Comp. Immunol. 58, 102–118. https://doi.org/10.1016/j.dci.2015.12.006.

Islamuddin, M., Chouhan, G., Farooque, A., Dwarakanath, B.S., Sahal, D., Afrin, F., 2015. Th1-biased immunomodulation and therapeutic potential of *Artemisia annua* in murine visceral leishmaniasis. PLoS Negl. Trop. Dis. 9 (1), e3321. https://doi.org/10.1371/journal.pntd.0003321.

Jones, D.P., 2008. Radical-free biology of oxidative stress. Am. J. Physiol. Cell Physiol. 295 (4), 849–868. https://doi.org/10.1152/ajpcell.00283.2008.

Lorena Ramos, C.S., Ivo Beserra Filho, J.A., Menezes, D.P., Michel Ferreira, P.P., 2024. A brief history of the animals' scientific research, international rules and Brazilian regulations. Acad. Bras. Cienc. 96, e20231406. https://doi.org/10.1590/0001-3765202420231406.

Matheus, L.M.D., Duarte, P.O., Ferreira, E. de C., 2021. First detection of *Leishmania* of the subgenus *Viannia* in alipiopsitta xanthops, endemic bird of South America. Biosci. J. 37, e37014. https://doi.org/10.14393/BJ-V37N0A2021-48024.

Mhadhbi, M., Sassi, A., 2020. Infection of the equine population by *Leishmania* parasites. Equine Vet. J. 52 (1), 28–33. https://doi.org/10.1111/evj.13178.

Montaner-Angoiti, E., Llobat, L., 2023. Is leishmaniasis the new emerging zoonosis in the world? Vet. Res Commun. 47, 1777–1799. https://doi.org/10.1007/s11259-023-10171-5.

Morales-Yuste, M., Martín-Sánchez, J., Corpas-Lopez, V., 2022. Canine Leishmaniasis: Update on epidemiology, diagnosis, treatment, and prevention. Vet. Sci. 9 (8), 387. https://doi.org/10.3390/vetsci9080387.

Muñoz, J.E., Ramirez, L.M., Dias, L. dos S., Rivas, L.A., Ramos, L.S., Santos, A.L.S., Taborda, C.P., Parra-Giraldo, C.M., 2020. Pathogenicity levels of colombian strains of *Candida auris* and Brazilian strains of *Candida haemulonii* species complex in both murine and *Galleria mellonella* experimental models. J. Fungi 6, 1–14. https://doi.org/10.3390/jof6030104.

Musembi, J.P., Owino, E.A., Oyieke, F.A., Tanga, C.M., Beesigamukama, D., Subramanian, S., Cheseto, X., Egonyu, J.P., 2024. Efficient agri-food waste valorization using mealworm (Coleoptera: Tenebrionidae) into nutrient-rich biomass

for food and feed. J. Econ. Entomol. 117, 1242–1253. https://doi.org/10.1093/jee/toae035.

Nakatogawa, S. ichi, Oda, Y., Kamiya, M., Kamijima, T., Aizawa, T., Clark, K.D., Demura, M., Kawano, K., Strand, M.R., Hayakawa, Y., 2009. A novel peptide mediates aggregation and migration of hemocytes from an insect. Curr. Biol. 19, 779–785. https://doi.org/10.1016/j.cub.2009.03.050.

Petronio Petronio, G., Pietrangelo, L., Cutuli, M.A., Magnifico, I., Venditti, N., Guarnieri, A., Abate, G.A., Yewhalaw, D., Davinelli, S., Di Marco, R., 2022. Emerging evidence on *Tenebrio molitor* immunity: a focus on gene expression involved in microbial infection for host-pathogen interaction studies. Microorganisms 10 (10), 1983. https://doi.org/10.3390/microorganisms10101983.

Pradhan, S., Schwartz, R.A., Patil, A., Grabbe, S., Goldust, M., 2022. Treatment options for leishmaniasis. Clin. Exp. Dermatol. 47 (3), 516–521. https://doi.org/10.1111/ced.14919.

Ribeiro, I.M.M., de Sousa, V.C., Melo, E.C.S., Carvalho, R. de C.V. de, Santos, M. de S. dos, Neto, J.A. de O.N., Melo, D.S. de, Teixeira, L.S. de A., Citó, A.M. das G.L., Moura, A.K.S., Arcanjo, D.D.R., Carvalho, F.A. de A., Alves, M.M. de M., Mendonça, I.L. de, 2023. Antileishmania and immunomodulatory potential of cashew nut shell liquid and cardanol. Toxicol. Vitr. 87, 105524. https://doi.org/10.1016/j.tiv.2022.105524.

Roatt, B.M., Aguiar-Soares, R.D., de, O., Coura-Vital, W., Ker, H.G., Moreira, N., das, D., Vitoriano-Souza, J., Giunchetti, R.C., Carneiro, C.M., Reis, A.B., 2014. Immunotherapy and immunochemotherapy in visceral leishmaniasis: promising treatments for this neglected disease. Front Immunol. 5, 272. https://doi.org/10.3389/fimmu.2014.00272.

Rossato, J.M., Moresco, T.R., Uczay, J., da Rocha, J.B.T., 2019. *Staphylococcus aureus*-induced sepsis in the lobster cockroach *Nauphoeta cinerea*. Comp. Immunol. Microbiol Infect. Dis. 66, 101343. https://doi.org/10.1016/j.cimid.2019.101343.

Saini, I., Joshi, J., Kaur, S., 2022. Unwelcome prevalence of leishmaniasis with several other infectious diseases. Int Immunopharmacol. 110, 109059. https://doi.org/10.1016/J.INTIMP.2022.109059.

Silva, L.N., Campos-Silva, R., Ramos, L.S., Trentin, D.S., Macedo, A.J., Branquinha, M.H., Santos, A.L.S., 2018. Virulence of *Candida haemulonii* complex in *Galleria mellonella* and efficacy of classical antifungal drugs: a comparative study with other clinically relevant non-albicans Candida species. FEMS Yeast Res 18 (7). https://doi.org/10.1093/femsyr/foy082.

Sousa, J.P.A., de, Sousa, J.M.S., de, Rodrigues, R.R.L., Nunes, T.A., de, L., Machado, Y.A. A., Araujo, A.C., de, da Silva, I.G.M., Barros-Cordeiro, K.B., Báo, S.N., Alves, M.M., de, M., Mendonça-Junior, F.J.B., Rodrigues, K.A., da, F., 2023. Antileishmanial activity of 2-amino-thiophene derivative SB-200. Int Immunopharmacol. 123, 110750. https://doi.org/10.1016/j.intimp.2023.110750.

Sönmez, E., 2021. Effects of cold storage on the developmental biology of *Tenebrio molitor* L., 1758 (Coleoptera: Tenebrionidae) at different larval stages. Turk. Entomoloji Derg. 45, 269–278 https://doi.org/10.16970/ ENTOTED.855011.

Søvik, E., Barron, A.B., 2013. Invertebrate models in addiction research. Brain Behav. Evol. 82, 153–165. https://doi.org/10.1159/000355506.

Tomiotto-Pellissier, F., Cataneo, A.H.D., Orsini, T.M., Thomazelli, A.P.F. dos S., Dalevedo, G.A., de Oliveira, A.G., Panagio, L.A., Costa, I.N., Conchon-Costa, I., Pavanelli, W.R., Almeida, R.S., 2016. *Galleria mellonella* hemocytes: a novel phagocytic assay for *Leishmania* (*Viannia*) *braziliensis*. J. Microbiol Methods 131, 45–50. https://doi.org/10.1016/J.MIMET.2016.10.001.

Urbański, A., Adamski, Z., Rosiński, G., 2018. Developmental changes in haemocyte morphology in response to *Staphylococcus aureus* and latex beads in the beetle *Tenebrio molitor* L. Micron 104, 8–20. https://doi.org/10.1016/j.micron.2017.10.005.

Vommaro, M.L., Kurtz, J., Giglio, A., 2021. Morphological characterisation of haemocytes in the mealworm beetle *Tenebrio molitor* (Coleoptera, tenebrionidae). Insects 12 (5), 423. https://doi.org/10.3390/insects12050423.

Wabwoba, B.W., Anjili, C.O., Ngeiywa, M.M., Ngure, P.K., Kigondu, E.M., Ingonga, J., Makwali, J., 2010. Experimental chemotherapy with *Allium sativum* (Liliaceae) methanolic extract in rodents infected with *Leishmania major* and *Leishmania donovani*. J. Vector Borne Dis. 47 (3), 160–167.

Weld, E.D., Waitt, C., Barnes, K., Garcia Bournissen, F., 2022. Twice neglected? neglected diseases in neglected populations. Br. J. Clin. Pharm. 88 (2), 367–373. https://doi.org/10.1111/bcp.15148.