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# Therapeutic potential of hesperidin methyl chalcone in the experimental treatment of cutaneous leishmaniasis

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## ABSTRACT

Drug repositioning seeks to discover new applications for a drug that has already been approved in the market, resulting in faster and lower cost solutions, generally used for diseases that receive little investment, such as cutaneous leishmaniasis. In this context, hesperidin, commercially approved as Daflon®, is a flavonoid that belongs to the chalcones group, a class that has antileishmanial potential. The present study evaluated the *in vitro* and *in vivo* antileishmanial activity of commercial hesperidin. Hesperidin (9 to 0.56 mg $mL^{-1}$) was tested against promastigote and amastigote forms of four dermotropic species of *Leishmania*, namely *L. (L.) amazonensis, L. (V.) guyanensis, L. (V.) braziliensis* and *L. (V.) naiffi*. For the *in vivo* tests, hamsters were infected in the snout and the lesions were treated with intralesional hesperidin. The treatment effectiveness was assessed by measuring the total volume of the lesion on the snout and determining the parasitic load. The *in vitro* results showed moderate toxicity in murine macrophages, with higher efficacy in *L. (L.) amazonensis* when compared to the other species tested. The *in vivo* results showed that hesperidin was able to gradually reduce the size of lesions by *L. (L.) amazonensis*, although it did not induce clinical and parasitological cure. Thus, hesperidin showed potential in *in vitro* tests against *L. (L.) amazonensis* and further studies with new formulations and experimental treatment schemes should be carried out.

**KEYWORDS:** flavonoids, *Leishmania*, drug replacement, preclinical study

# Potencial terapêutico da hesperidina metil chalcona no tratamento experimental da leishmaniose cutânea

## RESUMO

O reposicionamento de medicamentos busca descobrir novas aplicações para um medicamento já aprovado no mercado, resultando em soluções mais rápidas e de menor custo, geralmente utilizadas para doenças que recebem pouco investimento, como a leishmaniose tegumentar. Nesse contexto, a hesperidina, aprovada comercialmente como Daflon®, tem despertado interesse científico por ser um flavonoide pertencente ao grupo das chalconas, classe que possui potencial antileishmania. O presente estudo avaliou a atividade antileishmania *in vitro* e *in vivo* da hesperidina comercial. A hesperidina (9 a 0,56 mg $mL^{-1}$) foi testada contra as formas promastigota e amastigota de quatro espécies dermotrópicas de *Leishmania*, sendo elas *L. (L.) amazonensis, L. (V.) guyanensis, L. (V.) braziliensis* e *L. (V.) naiffi*. Para os testes, os hamsters foram infectados no focinho e as lesões tratadas com hesperidina intralesional, cuja eficácia foi avaliada através da aferição do volume total da lesão no focinho e a determinação da carga parasitária. Os resultados *in vitro* demonstraram toxicidade moderada em macrófagos murinos e melhor eficácia em *Leishmania (L.) amazonensis* quando comparados às demais espécies testadas. Os resultados *in vivo* mostraram que a hesperidina foi capaz de reduzir gradativamente o tamanho das lesões causadas por *L. (L.) amazonensis*, embora não tenha induzido a cura clínica e parasitológica. Assim, a hesperidina mostrou-se promissora em estudos *in vitro* contra *Leishmania (L.) amazonensis* e novos estudos com novas formulações e esquemas de tratamento experimental devem ser realizados.

**PALAVRAS-CHAVE:** flavonóides, *Leishmania*, substituição de drogas, estudo pré-clínico

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## INTRODUCTION

Cutaneous leishmaniasis (CL) is a tropical, infectious-parasitic and non-contagious disease caused by the protozoa of the genus *Leishmania*. It presents itself as a polymorphic manifestation of the skin and mucosa, and is associated with high morbidity (Blanco and Nascimento-Júnior 2017; Glans *et al*. 2018). In Brazil, there are seven pathological species that cause CL, six of the subgenus *Viannia* and one of the subgenus *Leishmania*, namely *L. (V.) braziliensis* Vianna, 1911, *L. (V.) guyanensis* Floch, 1954, *L. (V.) lainsoni* Silveira, Shaw, Braga & Ishikawa, 1987, *L. (V.) naiffi* Lainson & Shaw, 1989, *L. (V.) shawi* Lainson, Braga & de Souza, 1989, *L. (V.) lindenbergi* Silveira, Ishikawa & de Souza, 2002 and *L. (L.) amazonensis* Lainson & Shaw, 1972 (Teles *et al*. 2016).

As recommended by the Brazilian Ministry of Health, the treatment of the different clinical forms of CL is carried out via the use of five drugs, namely meglumine antimoniate, pentamidine isethionate, amphotericin B or liposomal amphotericin B and the recently included miltefosine (Brasil 2017; SVS 2020), but there are therapeutic limitations due to low efficacy, high toxicity, long and painful treatment, and high financial costs, addition, parasites may be resistant to treatment (Brasil 2017). Given these limitations, the World Health Organization recommends the search for new medicines and treatments for CL (WHO 2021).

One path in the search for new treatments is drug repositioning, which aims to investigate whether a drug that has already been approved and is used clinically has some activity against another disease that is still untreated (Xue *et al*. 2018; Jourdan *et al*. 2020). This strategy has generally been used for diseases that receive little investment, such as CL (Bustamante *et al*. 2019). In this context, some flavonoids have aroused interest in the scientific community. Among them is hesperidin, a substance that belongs to the group of chalcones, which has been approved in clinical therapy and is commercially known as Daflon®, and is used in synergism with diosmin for the treatment of varicose veins and other symptoms related to chronic venous insufficiency (Servier 2019). Hesperidin has been investigated for its anticancer (Pandey *et al*. 2021), antioxidant (Hager-Theodorides *et al*. 2021), antiviral (Attia *et al*. 2021), immunomodulatory (Berkoz *et al*. 2021) activity, and as a treatment for visceral leishmaniasis (Tabrez *et al.* 2021a).

There are no studies in the literature that investigate the repositioning of hesperidin for the treatment of CL. Nonetheless, Tabrez *et al.* (2021a) showed results of hesperidin activity in promastigote ($IC_{50}$ 1.019 mM) and amastigote ($IC_{50}$ 0.285 mM) forms of *Leishmania donovani* (Laveran & Mesnil, 1903) and studied its possible mechanism of action. Hesperidin (at a concentration of 2.0 mM) induced reactive oxygen species in 96.7% of the parasites in a dose-dependent manner and consequent induction of apoptosis-like cell death, together with the possible inhibition of a key ergosterol-biosynthetic enzyme (Tabrez *et al.* 2021a).

In this study, we evaluated the antileishmanial activity of hesperidin *in vitro* and *in vivo* against different species of *Leishmania* that cause CL in Brazil.

## MATERIAL AND METHODS

### Substances

The target molecule under study was hesperidin methyl chalcone, which was purchased commercially (Sigma Aldrich™, São Paulo, Brazil), and meglumine antimoniate (Glucantime®; Sanofi-Aventis, São Paulo, Brazil), which is the standard drug used as the positive control.

### Parasitic mass

We used strains of *Leishmania (Leishmania) amazonensis* (IFLA/BR/1967/PH8), *Leishmania (Viannia) guyanensis* (MHOM/BR/1975/M4147), *Leishmania (Viannia) braziliensis* (MHOM/BR/1975/2904) and *Leishmania (Viannia) naiffi* (MDAS/BR/1979/M5533) maintained and cryopreserved in the Laboratory of Leishmaniasis and Chagas Disease at Instituto Nacional de Pesquisas da Amazônia (INPA), Manaus, Brazil. The strains were initially cultivated in Novy-MacNeal-Nicolle (NNN) medium and the expanded culture in Roswell Park Memorial Institute medium (RPMI 1640 - Sigma Chemical Co. St. Louis, USA), which was supplemented with 10% inactivated fetal bovine serum (iFBS) (LGC Biotecnologia, São Paulo, Brazil) and 50 $\mu g.mL^{-1}$ of gentamicin (Novafarma, Brazil), and incubated at 25 ºC.

### Ethical approval

This study was approved by the ethics committee on the use of animals at INPA for the performance of the *in vitro* and *in vivo* tests (authorization # 029/2020 CEUA/INPA).

### Cytotoxicity assay

Murine peritoneal macrophages were grown in 96-well plates at a concentration of $10^5$ cells $mL^{-1}$ in RPMI medium without the Ph/phenol red indicator (Sigma Chemical Co. St. Louis, USA), supplemented with 10% iFBS and kept in an incubator at 5% $CO_2$ (Form Series II Water Jacket $CO_2$ Incubator, Thermo Scientific, USA) and 37 °C for 24 hours. Cells were treated with different concentrations of hesperidin (9.00, 4.50, 2.25, 1.12 and 0.56 mg $mL^{-1}$) and Glucantime® (8.00, 4.00, 2.00, 1.00 and 0.50 mg $Sb^{5+}$ mL) distributed on each plate in triplicate and five contractions. Wells with untreated cells and wells without cells were used as controls. The hesperidin concentrations followed Gomes *et al.* (2017).

Cell viability was evaluated by the colorimetric method using Alamar Blue® (Sigma Aldrich™, USA) at 24, 48 and 72 hours (one plate per period of time) with the addition of 10 µL of sodium resazurin salt stock solution (4 $mg.mL^{-1}$ in

phosphate-buffered saline solution) in each well. The plates were again incubated for another 12 h at 37 °C, after which they were read using a spectrophotometer (Elx800™, BIO-TEK®, Winooski, Vermont, USA) at 590 nm (Chagas *et al*. 2021).

To evaluate cytotoxic activity, murine peritoneal macrophages were quantified in a Neubauer chamber, adjusted to $10^5$ cells $mL^{-1}$ and then incubated in a 24-well plate (three plates) against hesperidin at pre-established concentrations, kept under study at 37ºC with 5% CO2, in order to evaluate the morphology of the macrophages against the substances under test over a period of 24 to 72 hours.

### Biological assay with promastigote forms

Promastigotes (2 x$10^6$ promastigotes $mL^{-1}$) of the species under study were added to 96-well plates (one plate per species) and exposed to hesperidin and Glucantime® distributed on each plate in triplicate and five concentrations as described for the cytotoxicity assay. Wells with untreated parasites were used as the negative control. Biological activity was determined by quantifying viable promastigotes at 24, 48 and 72 hours in a Neubauer chamber using an optical microscope (Nikon Eclipse E200, Japan) at 400x magnification. Data were expressed as the mean inhibitory concentration ($IC_{50}$) (Chagas *et al*. 2021).

### Biological assay with amastigote forms

Murine peritoneal macrophages at a concentration of $10^4$ cells $mL^{-1}$ were cultured on glass coverslips inserted into 24-well plates (three plates for each species tested) with RPMI culture medium that was supplemented with 10% iFBS and infected with *Leishmania* spp. promastigotes at a concentration of $10^5$ promastigotes $mL^{-1}$, corresponding to the proportion of five promastigotes per macrophage for each species tested and then placed in an oven at 37 ºC with 5% $CO_2$ for two hours. Infected macrophages were treated with different concentrations of hesperidin (9.00, 4.50, 2.25 and 1.12 mg $mL^{-1}$) and Glucantime® (8.00, 4.00, 2.00 and 1 .00 mg $Sb^{5+}$ $mL^{-1}$) distributed on each plate in triplicate and four contractions for 24, 48 and 72 hours at 37 °C in 5% $CO_2$. Infected and untreated cells were used as negative control. Subsequently, the coverslips were fixed and stained every 24 hours using the Rapid Panoptic method (Laborclin®, Paraná, Brazil) and analyzed using light microscopy. The percentage of infected cells was determined by randomly quantifying 100 infected and uninfected cells on each coverslip. Data were also expressed by the mean inhibitory concentration ($IC_{50}$) (Chagas *et al*. 2021).

### *In vivo* biological assay

A total of 108 golden *hamsters* (*Mesocricetus auratus* Waterhouse, 1839), weighing approximately 180 g and aged 90 days, were used in this assay. This animal model was used due to its high susceptibility to dermotropic pathologies and the clinical evolution of the disease to present signs similar to those observed in humans. Furthermore, some of the species analyzed in the study have the capacity to evolve into oronasal infections (mucosal leishmaniasis), a manifestation that is difficult to treat in humans, which is why we chose to evaluate the progression of the disease/treatment in the snout region.

The animals were divided into three groups (A, B and C) of 36 animals. The animals were submitted to infection by inoculation in the snout of 0.1 $mL^{-1}$ of promastigotes ($10^6$ promastigotes $mL^{-1}$) of *L. (V.) guyanensis* (Group A), *L. (L.) amazonensis* (Group B), *L. (V.) braziliensis* (Group C). *Leishmania* (*V.*) *naiffi* does not develop macroscopically visible skin lesions, therefore this species was not used in this assay. Each group was assigned to the following treatments/control (six animals per treatment/control): I) non-infected (GNI); II) infected and untreated (GINF); III) infected and treated intralesionally with saline solution (placebo) (GTP); IV) infected and treated intralesionally with 1.15 mg $Sb^{5+}$ $kg^{-1}$ $day^{-1}$ of Glucantime® (GTG); V) infected and treated intralesionally with 1.15 mg $mL^{-1}$ of hesperidin (GTH1); VI) infected and treated intralesionally with 4 mg $mL^{-1}$ of hesperidin (GTH2).

After the lesion appeared, the respective treatments were applied every 15 days, totaling 45 days of treatment (4 applications), according to the Brazilian Ministry of Health guidelines for intralesional treatment. After 45 days of treatment, the animals were observed (without receiving treatment) for another 15 days, thus totaling a follow-up of 60 days.

During the treatment, the total volume of the lesion was monitored weekly by measuring the snout derived average length, width and height using a digital caliper (Zaas precision®, Brazil - 0.02 mm precision), photo-documentation of the snout for macroscopic morphological analysis of the lesion, and weighing of the animals for observation of weight gain or loss (Comandolli-Wyrepkowki *et al*. 2017). Monitoring was carried out by measuring the snout to monitor, in addition to the lesion, the formation of edema called "tapir nose", characteristic of this pathology.

At the end of the experimental period, all animals were anesthetized (ketamine and xylazine) and euthanized intramuscularly. After euthanasia, biological material was collected (fragments of the lesion, liver, spleen, kidneys and blood), and the organs were weighed on precision scales (Shimadzu®, Brazil – 0.1 mg precision) (Comandolli-Wyrepkowki *et al*. 2017).

### Parasitological and hematological evaluation

The tissue fragments collected from the lesion area of the animals were used to make prints on glass slides, which were later stained using the Rapid Panoptic method. Quantification

of infected cells was determined by counting 25 fields at random under an optical microscope (Nikon Eclipse E200, Japan) at 1,000x magnification.

Lesion fragments and the liver were cultured in NNN medium for 7 days at 25 °C and then the presence or absence of promastigotes was evaluated. Cultures were considered positive when at least one form of the parasite was isolated in the culture medium (Comandolli-Wyrepkowki *et al*. 2017).

Blood smears were made on slides and then stained using the Rapid Panoptic method. The analysis was performed by counting 100 cells per slide with differentiation and quantification of leukocyte cells under an optical microscope with a 400x magnification.

### Statistical analysis

IC50 was obtained through linear regression using the number of living cells. A one-way ANOVA followed by Tukey's test was used to assess the significance of the differences between the groups of the *in vivo* biological assay, at the 5% significance level. All statistical analyses was performed using the GraphPad Prism program version 6.0 for Windows (GraphPad Software, San Diego, CA)

## RESULTS

### Cytotoxicity assay

Hesperidin showed moderate toxicity to murine peritoneal macrophages, with an average cell viability of 75% at the highest concentrations (9.00, 4.5, and 2.25 mg $mL^{-1}$) and 90% at the lower concentrations (1.12 and 0.56 mg $mL^{-1}$) (Figure 1). Cell viability and morphology of cells treated with hesperidin varied little in relation to the control group with untreated cells, with the presence of reduced-sized cells and differences in cell delimitations being observed at the higher concentrations tested.

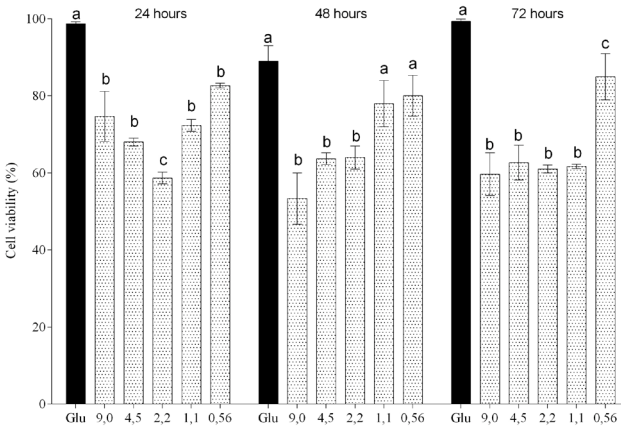


**Figure 1.** Cytotoxic effect of hesperidin (9.00-0.56 mg $mL^{-1}$, dotted bars) and Glucantime® (8 mg $Sb^{5+}$·$mL^{-1}$, black bars) on murine peritoneal macrophages at 24, 48 and 72 hours, evaluated by cell viability. Different letters within periods indicate significant differences according to a Tukey test ($p < 0.05$).

### Biological assay with promastigote forms

Hesperidin showed a lower $IC_{50}$ in *L. (L.) amazonensis* promastigotes at 24 hours ($IC_{50}$ < 0.56 mg), followed by *L. (V.) naiffi* at 72 hours ($IC_{50}$ < 0.56 mg), *L (V.) guyanensis* at 72 hours ($IC_{50}$ 2.93 mg) and *L. (V.) braziliensis* at 48 hours ($IC_{50}$ 3.7 mg) (Table 1).

With *L. (L.) amazonensis,* hesperidin induced a reduction in parasite viability (> 50%), with a significant difference at all concentrations in relation to the controls at 24 hours, and for the concentrations 9.00, 4.50 and 2.25 mg $mL^{-1}$ at 48 and 72 hours (Figure 2a). With *L. (V.) guyanensis,* there was a significant reduction in parasite viability at 72 hours compared to the negative control (Figure 2b). With *L. (V.) braziliensis,* there was a significant reduction in parasite viability at all times when compared to control groups (Figure 2c). With *L. (V.) naiffi,* the reduction in parasite viability occurred at 48 and 72 hours in relation to the negative control (Figure 2d).

### Biological assay with amastigote forms

Hesperidin showed a lower $IC_{50}$ in *L. (L.) amazonensis* amastigotes at 72 h ($IC_{50}$ 3.06 mg), *L. (V.) guyanensis* at 24 hours ($IC_{50}$ 6.29 mg), *L. (V.) naiffi* at 72 hours ($IC_{50}$ 7.17 mg) and *L. (V.) braziliensis* at 48 hours ($IC_{50}$ 7.66 mg) (Table 2).

**Table 1.** Mean inhibitory concentration ($IC_{50}$) of hesperidin and Glucantime® in promastigote forms of *Leishmania* spp. Values are the mean ± standard deviation of three replicates.

| Species | Period (hours) | Hesperidin | Glucantime® |
|---|---|---|---|
| *L. (L.) amazonensis* | 24 | < 0.5 ± 2.0 | 10.1 ± 4.9 |
| | 48 | 1.5 ± 3.2 | 0.56 ± 3.4 |
| | 72 | 2.9 ± 2.2 | 3.3 ± 4.9 |
| *L. (V.) guyanensis* | 24 | 9.2 ± 11.7 | 11.0 ± 9.7 |
| | 48 | 9.1 ± 8.5 | 10.1 ± 12.0 |
| | 72 | 2.9 ± 3.1 | 2.92 ± 32.8 |
| *L. (V.) braziliensis* | 24 | 4.2 ± 8.0 | 11.6 ± 5.8 |
| | 48 | 3.7 ± 12.2 | 6.3 ± 9.5 |
| | 72 | 4.1 ± 12.0 | 5.2 ± 4.9 |
| *L. (V.) naiffi* | 24 | 4.1 ± 7.6 | 5.7 ± 17.0 |
| | 48 | 1.3 ± 8.9 | 3.2 ± 14.3 |
| | 72 | < 0.5 ± 17.0 | 0.3 ± 29.3 |

**Table 2.** Mean inhibitory concentration ($IC_{50}$) of hesperidin and Glucantime® in amastigote forms of *Leishmania* spp. Values are the mean ± standard deviation of three replicates.

| Species | Period (hours) | Hesperidin | Glucantime® |
|---|---|---|---|
| *L (L.) amazonensis* | 24 | 6.2 ± 1.9 | 10.7 ± 2.8 |
| | 48 | 6.7 ± 1.5 | 9.5 ± 1.2 |
| | 72 | 3.0 ± 1.0 | 9.9 ± 1.5 |
| *L. (V.) guyanensis* | 24 | 6.2 ± 2.8 | 9.5 ± 1.7 |
| | 48 | 7.4 ± 1.7 | 3.2 ± 5.1 |
| | 72 | 7.6 ± 2.1 | 4.2 ± 4.9 |
| *L. (V.) braziliensis* | 24 | 8.6 ± 2.8 | > 8 ± 3.5 |
| | 48 | 7.6 ± 2.6 | > 8 ± 2.1 |
| | 72 | 10.4 ± 1.7 | > 8 ± 1.2 |
| *L. (V.) naiffi* | 24 | 12.3 ± 1.7 | 7.4 ± 3.8 |
| | 48 | 9.4 ± 1.7 | 9.2 ± 4.2 |
| | 72 | 7.1 ± 1.4 | 8.9 ± 2.8 |

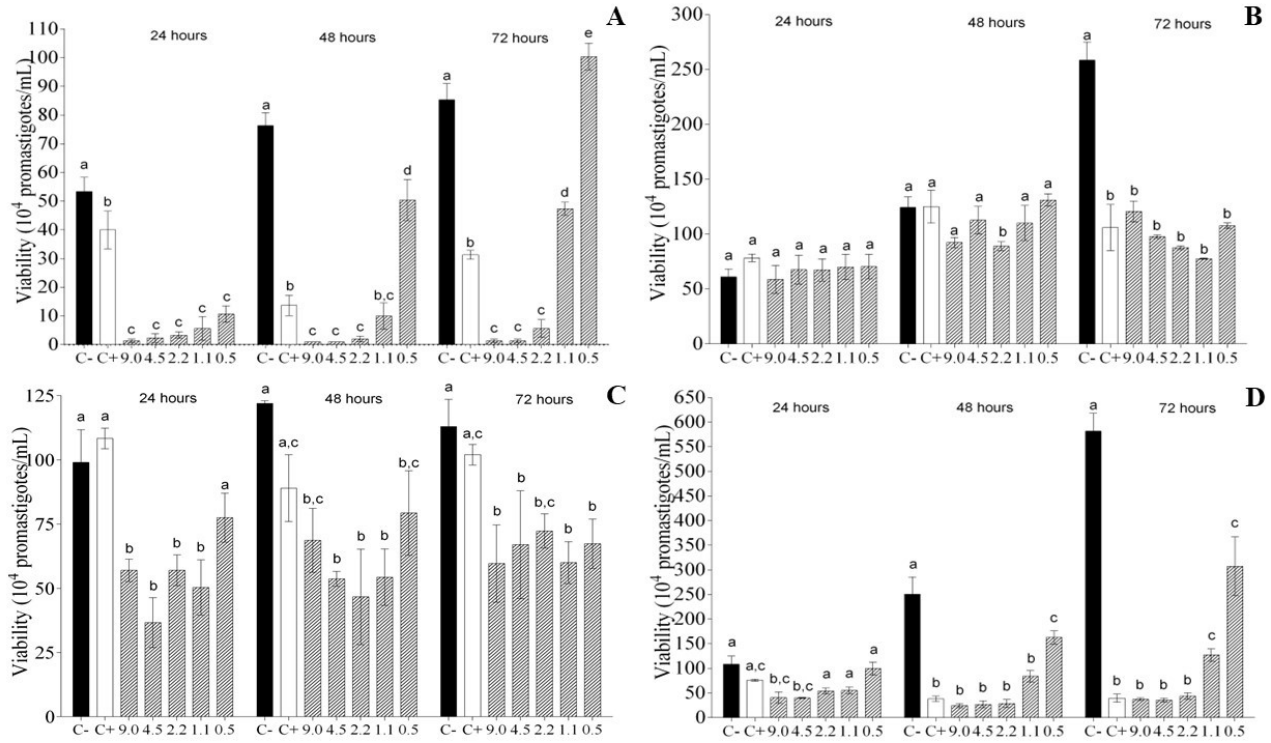


**Figure 2.** Hesperidin activity (9.00-0.56 mg $mL^{-1}$, striped bars) against the promastigote forms of *L. (L.) amazonensis* (A), *L. (V.) guyanensis* (B), *L. (V.) braziliensis* (C), *L. (V.) naiffi* (D) at 24, 48 and 72 hours, compared to the negative control (black bar) and Glucantime® (8 mg $Sb^{5+}mL^{-1}$, positive control, white bar). Columns represent the mean and bars the standard deviation. Different letters within periods indicate significant difference according to a Tukey test ($p < 0.05$).

As observed in the promastigote forms, hesperidin was more effective against *L. (L.) amazonensis*, inducing a reduction of > 50% in the infection rate at 9.00, 4.50 mg $mL^{-1}$ and > 40% at 2, 25 and 1.12 mg $mL^{-1}$ throughout the entire incubation period, but did not difer significantly from Glucantime® (Figure 3a). Similar results were observed against *L. (V.) guyanensis* at 24 and 48 hours, *L. (V.) braziliensis* at all times and *L. (V.) naiffi* at 48 and 72 hours, not differing significantly from Glucantime® (Figure 3b-d).

## *In vivo* biological assay

The hamsters infected with *L. (L.) amazonensis* (GTH1) had a significantly lower weight (mean ± SD = 135.3 ± 5.2 g) than the other groups (172.1 ± 4.6 g). There were no significant differences in weight among groups in *L. (V.) guyanensis* and *L. (V.) braziliensis* assays. In the animals infected with *L. (L.) amazonensis,* there was a continuous reduction in the volume of the snout in GTH1 (56%) and GTH2 (49%), which did not differ significantly from GTG (57%) and GINF.

In animals infected with *L. (V.) guyanensis* and *L. (V.) braziliensis*, a reduction in the lesion volume was observed, on average, until the 45th day of treatment and, after that, an increase was observed again in GTG, GTH1 and GTH2. At the end of the experiment, there was a significant difference for the GNI group ($p < 0.001$); however, there was no significant difference for the GINF group. At the end of treatment animals of the GTH1 group that were infected with *L. (V.) guyanensis* reduced the lesion volume by 41% and the GTH2 group infected with *L. (V.) braziliensis* reduced the lesion volume by 29% (Figure 4).

Animals infected with *L. (L.) amazonensis* presented edema with an apparent lesion. In GINF and GTP, these signs were intensified from the 30th day onwards, which resulted in a larger snout volume and ulcerated lesions at the end of the treatment when compared to the other experimental groups. After the 15th day of treatment, GTH1 and GTH2 progressed to exacerbated ulcerated lesions, followed by the formation of eschars and the consequent reduction of the edema, without clinical cure. GTG showed healing from the 30th day onwards and consequent clinical cure of the animals.

In the groups infected by *L. (V.) guyanensis* and *L. (V.) braziliensis*, the animals presented nodule formation with a small lesion and scabs, expressed in GINF, GTP and GTH1 throughout the experiment period. Only in GTG was there clinical cure of all animals. In GTH2 infected with *L. (V.) braziliensis*, half of the group showed clinical cure (Figure 5).

## Parasitological and hematological evaluation

After treatment parasite viability revealed the presence of viable flagellate parasites in the cultures of the lesion and liver fragments in all groups, and there was no significant difference in the infectivity rate between the treatment groups in all

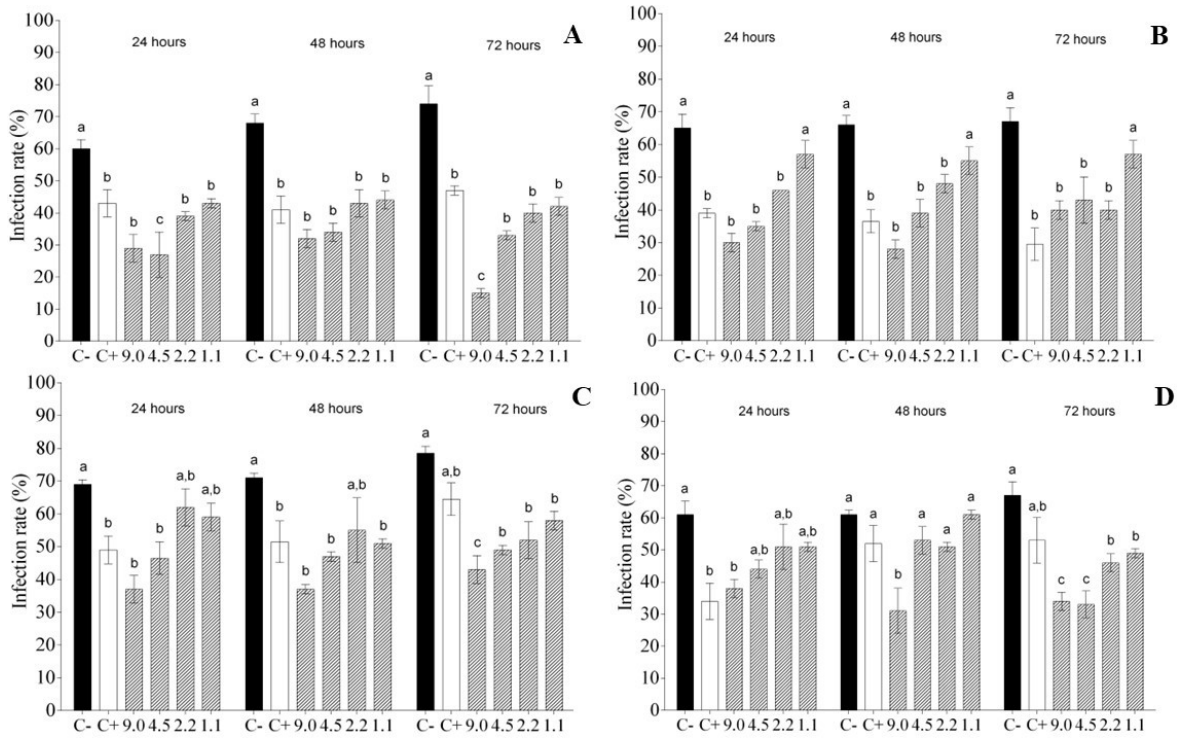


**Figure 3.** Effect of hesperidin (9.00-0.56 mg.mL$^{-1}$, striped bars) on intracellular amastigotes of *L. (L.) amazonensis* (A), *L. (V.) guyanensis* (B), *L. (V.) braziliensis* (C), *L. (V.) naiffi* (D) at 24, 48 and 72 hours after treatment with different concentrations of hesperidin, compared to the negative control (black bars) (infected and untreated macrophages), and Glucantime® (8 mg.$Sb^{5+}$.mL$^{-1}$, white bars). Columns represent the mean and bars the standard deviation. Different letters within periods indicate significant differences according to a Tukey test ($p < 0.05$).

the species tested. Only GTG in *L. (L.) amazonensis* showed a significantly lower macrophage infectivity compared to GTH2 (Figure 6). Organ weight (liver, spleen and kidney) and leukocyte parameters did not differ significantly among the experimental groups in all species tested.

## DISCUSSION

This study is an unprecedented report on the antileishmanial activity of hesperidin in CL. Due to the lack of information in the literature regarding its antileishmanial activity, the results obtained in this study will be presented from the perspective of the antileishmanial activity of hesperidin and other substances of the flavonoid class that have already been documented against a species that causes visceral leishmaniasis (VL).

In our study, hesperidin showed moderate cytotoxicity and cell viability (61.3% to 80%), which is similar to what was observed with flavonoids such as quercetin (50% to 100%) (Caetano *et al*. 2019) and cynaroside (20% to 50 %) (Tabrez *et al*. 2021b), yet FM09h (an amine-linked flavonoid) did not show cytotoxicity to macrophages (Chan *et al*. 2021).

In our antileishmanial activity tests, a dose-dependent response was observed in which the highest concentrations of hesperidin (9 and 4.5 mg mL$^{-1}$) were more effective in inhibiting promastigotes and amastigotes, similarly to the results by Gervazone *et al.* (2018) and Tabrez *et al*. (2021b). However, as different pathogen species respond differently to a drug, only the $IC_{50}$ and infection rate will assess whether the drug under study is showing antileishmanial biological activity or inducing cellular toxicity (Silva 2008).

Against *L. (L.) amazonensis*, hesperidin was able to inhibit promastigote forms (> 75%, $IC_{50}$ < 0.56 mg) and amastigote forms (> 50%, $IC_{50}$ 3.06 mg) after 72 hours, significantly higher compared to Glucantime®. These results corroborate Chan *et al*. (2021) for the flavonoid FM09h against amastigotes (90%) and Gervazone *et al*. (2018) for the flavonoid 2HF against promastigotes (79%, $IC_{50}$ 20.9 µM) and amastigotes (90%, $IC_{50}$ 3.09 mM). Both latter studies used macrophages infected with *L. (L.) amazonensis* and observed the reduction of intracellular amastigotes without destruction of the macrophage.Against *L. (V.) braziliensis*, hesperidin was significantly more effective than Glucantime® only against promastigotes (< 50%, $IC_{50}$ 3.7 mg), while Chan *et al*. (2021) and Caetano *et al*. (2019) describe an efficacy for both evolutionary forms against this species, with a significant difference in relation to the positive control.

Against *L. (V.) guyanensis* and *L. (V.) naiffi,* hesperidin was able to reduce the promastigote forms and the rate of infection by amastigotes, but did not differ significantly from Glucantime®. As far as we know, there are no studies

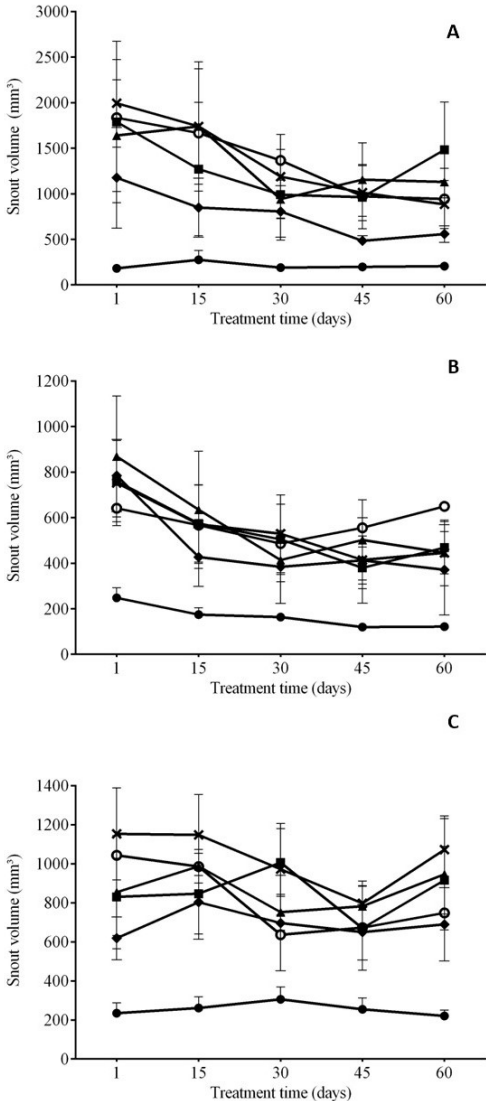


**Figure 4.** Snout volume of golden hamsters infected with *L. (L.) amazonensis* (A), *L. (V.) guyanensis* (B) and *L. (V.) braziliensis* (C). Black circles = uninfected and untreated – GNI (positive control); squares = infected and untreated - GINF (negative control); triangles = infected and treated with placebo - GTP (negative control); diamond = infected and treated with 1.15 mg $Sb^{5+}$ $kg^{-1}$ $day^{-1}$ Glucantime® - GTG (positive control); X = infected and treated with 1.15 mg $mL^{-1}$ hesperidin – GTH1; white circle = infected and treated with 4 mg $mL^{-1}$ hesperidin – GTH2.

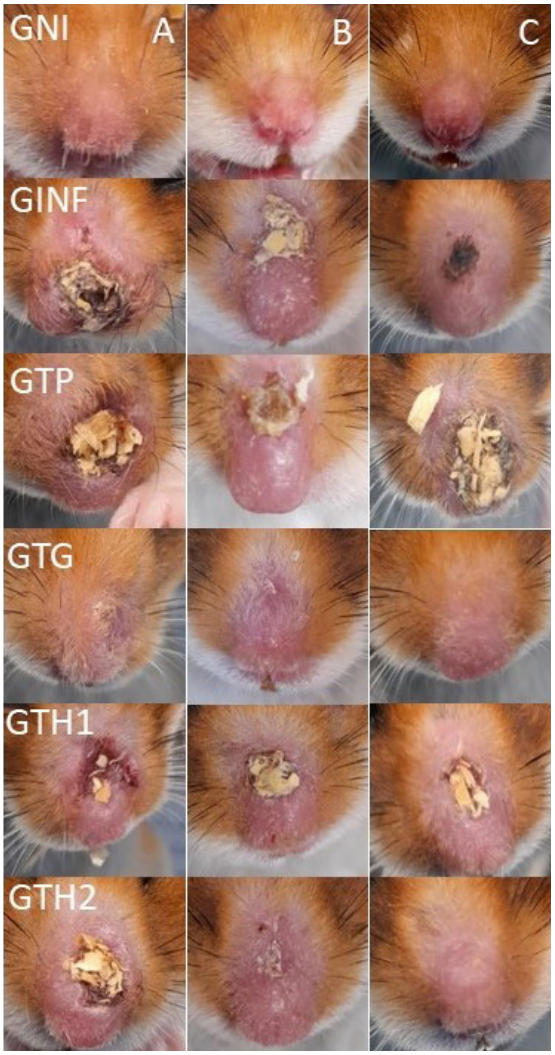


**Figure 5.** Aspect of lesions on the snout of golden hamsters, *Mesocricetus auratus* infected by *L. (L.) amazonensis* (A), *L. (V.) guyanensis* (B) and *L. (V.) braziliensis* (C) at the end of the 60-day experimental period. GNI – uninfected and untreated animal (positive control); GINF – infected, untreated animal (negative control); GTP – infected animal treated with placebo (negative control); GTG – infected animal treated with Glucantime® (1.15 mg/$Sb^{5+}$/kg/day) (positive control); GTH1 – infected animal treated with 1.15 $mg.mL^{-1}$ of hesperidin; GTH2 – infected animal treated with 4 $mg.mL^{-1}$ of hesperidin. The columns represent the different species and the lines represent the treatment groups.s

in the literature that evaluate the antileishmanial activity of a flavonoid for these species, so that this is the first report on antileishmanial activity of a flavonoid against these species.

As expected, the tested *Leishmania* species responded differently to *in vitro* treatment with hesperidin, however it is possible to observe similar response patterns by subgenus. The subgenus *Leishmania* showed higher susceptibility to hesperidin, with significantly higher eficacy than Glucantime® in both evolutionary forms. Against *Viannia*, hesperidin also induced a reduction in both evolutionary forms, but did not differ significantly from Glucantime®, with the exception of *L. (V.) brasiliensis* in promastigote form. The only other report of antileishmanial activity of hesperidin showed efficacy against promastigotes and amastigotes of *L. (L.) donovani* that causes VL (Tabrez *et al.* 2021a). As VL causes a disease with different clinical manifestations from those of CL, it is also expected that it responds differently to a drug. However, these data support the pattern of higher susceptibility of the subgenus *Leishmania* to hesperidin.

The results of the cytotoxicity essay in macrophages suggest that there may have been greater toxicity in terms of the cells being infected. Cells infected by different species of

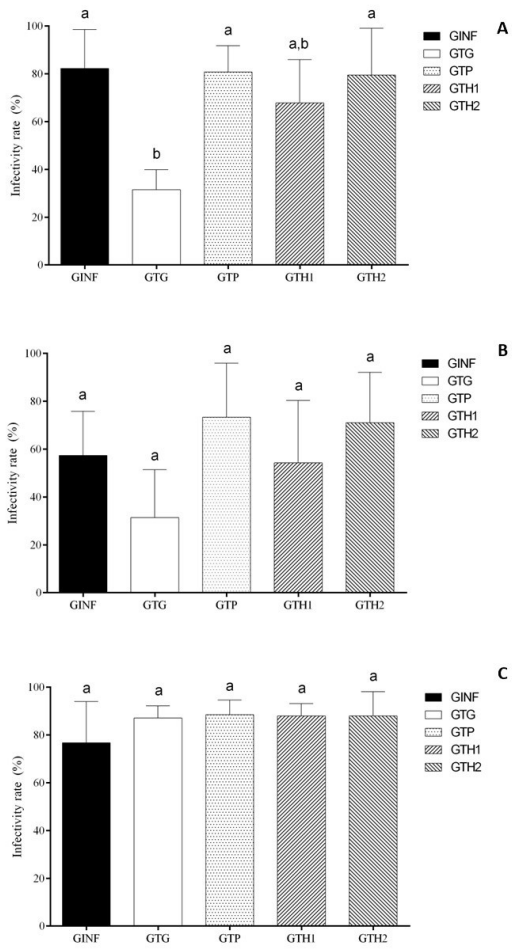


**Figure 6.** Macrophage infectivity rate quantified by printing on slides of lesion fragments from golden hamsters infected with *L. (L.) amazonensis* (A); *L. (V.) guyanensis* (B) and *L. (V.) braziliensis* (C). Infected and untreated animals (GINF); infected animals treated with 1.15 mg $kg_{-1}$ $day_{-1}$ of Glucantime® (GTG); infected animals treated with placebo (GTP); infected animals treated with 1.15 mg $mL^{-1}$ of hesperidin (GTH1); infected animals treated with 4 mg $mL^{-1}$ of hesperidin (GTH2). Each column represents the mean and each bar the standard deviation of three replicates. Different letters within periods indicate significant differences according to a Tukey test ($p < 0.05$).

*Leishmania* are more susceptible to the cytotoxic effects of compounds than non-infected cells (Silva 2008), which may be owed to cell membrane alterations caused by the parasite (Quintana *et al*. 2010). It is thus important that further studies on antileishmanial activity of hesperidin investigate the mechanism of action of this substance in infected and non-infected cells to determine the cytotoxicity of the substance.

Currently, the drugs used in the treatment of CL are associated with numerous adverse side effects, including high toxicity, drug resistance and requires parenteral administration. It is considered a difficult treatment to administer (DNDi 2018) and intralesional treatment is preferable since it allows the direct delivery of drugs to the skin lesions, avoiding or minimizing the adverse effects of systemic therapy, which can lead to a reduction in toxicity (Franco *et al.* 2016; Nassif *et al*. 2017). Our results indicated that the animals treated with hesperidin did not achieve clinical cure (lesion healing) nor parasitological cure (absence of parasites) against *L. (L.) amazonensis* and *L. (V.) guyanensis*. Against *L. (V.) braziliensis,* there was clinical cure in half of the animals treated with the higher dosis of hesperidin, although there was no parasitological cure, while in animals treated with Glucantime® clinical cure was achieved in all cases, but also no parasitological cure. According to the Brazilian Ministry of Health, clinical cure of CL occurs when re-epithelialization of the ulcerated lesions and the total regression of the infiltration and erythema are observed (Brasil 2017), thus hesperidin did not meet the official standard of clinical cure for CL against any of the species tested.

Few studies have evaluated the *in vivo* effect of flavonoids in the search for new treatment alternatives for CL. A promising antileishmanicidal effect against *L. (L.) amazonensis* in mice was observed for 2HF (50 mg $kg^{-1}$ $day^{-1}$ administered orally, with significantly higher efficacy compared to Glucantime®) (Gervazone *et al.* 2018) and FM09h (10 mg $kg^{-1}$ $day^{-1}$ administered intralesionally) (Chan *et al*. 2021). No toxic reactions, nor significant changes in the weight and leukogram of the animals, or in the size, weight and appearance of their organs were observed in the latter studies, suggesting that these substances did not cause apparent toxicity to mice.

It is characteristic of the subgenus *Leishmania* to present a more exacerbated evolution of CL with extensive lesions, causing large ulcerations in some cases, while in the subgenus *Viannia* the presence of nodules with small and controlled lesions is more common (Brasil, 2017). This differential response to infection is reflected in our results, as the hamsters infected with *L. (V.) brasiliensis* were more responsive to treatment, resulting in clinical cure with Glucantime® and partial clinical cure with hespiridin.

There is no validated animal model for *in vivo* tests with *Leishmania*, and consequently a diversity of animal models are used, such as golden hamsters (Chagas *et al*. 2021), C57BL/6 mice (Sampaio *et al*. 2003), BALB mice/C and CBA mice (Reis *et al*. 2006). Hamsters are among the most promising experimental models for the study of leishmaniasis, due to their high susceptibility to dermotropic pathologies and for presenting a clinical evolution with signs similar to those observed in humans (Dea-Ayuela *et al*. 2007).

In the *in vivo* studies, the subgenera of *Leishmania* showed differencial clinical evolution patterns in response to treatment, although different from what was observed in the *in vitro* tests. The large and ulcerated lesions with progressive evolution and little response to treatment in the subgenus *Leishmania* may have occurred due to the difficulty in controlling the size of the lesions, while the small and controlled lesions in the subgenus *Viannia* were comparatively

more responsive to treatment, resulting in clinical cure with Glucantime® against *L. (V.) brasiliensis*.

## CONCLUSIONS

Our study revealed that hesperidin shows promising activity against promastigote and amastigote forms of *L. (L.) amazonensis*. However, in the *in vivo* tests with golden hamsters, the best result with hesperidin was the 50% induced clinical cure of animals infected with *L. (V.) brasiliensis* when treated intralesionally with 4 mg $mL^{-1}$. Further studies should evaluate the effectiveness of hesperidin using new experimental treatment schemes, with emphasis on concentrations of the active ingredient, treatment time, *Leishmania* species involved and routes of administration, while also seeking to elucidate the therapeutic mechanisms involved in the response against infection of the species that cause CL.

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**DATA AVAILABILITY**

The data that support the findings of this study are not publicly available.