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An *in vitro* evaluation of the antileishmanial and cytotoxic activity of methyl gallate associated with conventional treatment for cutaneous leishmaniasis

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Cutaneous leishmaniasis (CL) is a non-contagious infectious-parasitic disease caused by protozoa of the genus *Leishmania* sp. It is considered a neglected tropical disease and involves public health issues because it affects socially vulnerable populations. The therapy recommended by the Brazilian Ministry of Health as the standard treatment for CL presents numerous challenges. Therefore, it is necessary to look for alternative treatments that are more effective and safer without any side effects on the patients. The phenolic compound methyl gallate (MTG) is an alternative candidate for study due to its antileishmanial biological activity. Methyl gallate has shown considerable biological activity against *Leishmania amazonensis*. Thus, investigating the potential of this compound in combination with Glucantime® and Pentacarinat® against Leishmania species and evaluating its cytotoxic profile is critical in the treatment and management of CL. The experimental design involved *in vitro* assays with promastigotes of *L. amazonensis* and *L. guyanensis* to evaluate the antileishmanial inhibitory activity of methyl gallate in monotherapy and in combination with conventional treatments, as well as to assess its cytotoxic profile using peritoneal macrophages extracted from BALB/c mice. As a result of the antileishmanial activity test, methyl gallate showed parasite inhibitory activity against *L. amazonensis and L. guyanensis* promastigotes, both in monotherapy and in combination with the conventional CL drugs. Methyl gallate or its combinations did not show any signs of cytotoxicity. These results provide a basis for future studies on this polyphenolic compound as a potential alternative drug for development and adoption in the therapeutic protocol for CL treatment.

Key words: Cutaneous leishmaniasis, neglected tropical diseases, methyl gallate, combination therapy, *in vitro* assays.

INTRODUCTION

Cutaneous leishmaniasis (CL) is an infectious, non-contagious parasitic disease caused by protozoa of the genus *Leishmania* sp. These parasites are obligatorily intracellular and target phagocytic cells of the immune

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system, especially macrophages, for their reproduction (Saar et al., 1998). These biological agents alternate between two well-known developmental stages: amastigotes and promastigotes, depending on the biological phase of the parasite. CL is vectorially transmitted through the blood meal of female sandflies of the genus *Lutzomyia* sp., popularly known as straw mosquitoes (Camargos et al., 2014; PAHO, 2023). This disease is cosmopolitan and more prevalent in underdeveloped and emerging countries. In 2022, approximately 90% of CL cases were distributed in seven countries: Afghanistan, Algeria, Brazil, Iran, Iraq, Pakistan, and the Syrian Arab Republic (WHO, 2022).

Additionally, it is characterized as a neglected tropical disease that involves public health issues, being grouped among the five most relevant endemic infectious-parasitic diseases by the Brazilian Ministry of Health. Its main target is the most vulnerable populations in rural areas without access to adequate health services (Maia-Elkhoury et al., 2021).

The Brazilian Ministry of Health recommends pharmacotherapy for CL using three main types of compounds: meglumine antimoniate (Glucantime®), which acts on the parasite's enzymes and inhibits its oxidative phosphorylation cycle; amphotericin B (Anforicin B®), an antifungal that binds to the sterols present in the parasite's membrane, thus altering its permeability and causing its internal contents to leak out; and pentamidine isethionate (Pentacarinat®), whose most accepted mechanism is mitochondrial topoisomerase inhibition, interfering with the transcription and replication of the parasite's DNA (Gonçalves, 2011; Neves et al., 2011; Brasil, 2017).

Despite being a recommended therapy, these conventional CL drugs have adverse side effects and toxicity that can compromise patients' vital organs. Additionally, in leishmaniasis-endemic countries, there has been an increase in reported cases of parasite resistance to pentavalent antimoniate, making it impossible to provide patients with an effective treatment regimen (Tiwari et al., 2018). Due to these limitations and the adverse and toxic effects of these drugs during the treatment period, along with the invasive routes of administration, it is necessary to undertake serious research for alternative compounds that are more effective, safer, accessible to the vulnerable population, and less painful (Santiago et al., 2021).

One way of improving existing treatments for CL may be to use combination therapies, which have gained prominence as alternatives to the monolithic therapy recommended for leishmaniasis due to the increase in parasite resistance to the existing drugs. The principle of combination therapy aims at the synergistic interaction of drugs that act at different sites, with a lower dosage and a shorter duration of treatment, reducing cost and toxic effects. This approach makes therapy more effective for the population and helps prevent parasite resistance (Sundar and Chakravarty, 2015).

Schubach and Conceição-Fátima (2014) demonstrated satisfactory efficacy with a reduction in the dosage of meglumine antimoniate in the therapeutic regimen and an increase in treatment time, making the drug less toxic for patients. An example of combination therapy is the use of Imiquimod®, a dermatological cream used to treat skin tumors, with Glucantime®, which has shown better efficacy in healing lesions and reducing treatment time (Miranda-Verasteg et al., 2009).

Another potential option in the search for alternative treatments of CL is natural products containing polyphenolic compounds. These secondary metabolites have significant biological potential with pharmacological actions (Passero et al., 2022; Liang et al., 2023). Among the diversity of polyphenolic compounds is methyl gallate, a polyphenol derived from gallic acid, which has a wide range of biological activities such as antioxidant (Huang et al., 2021), antitumor (Lee et al., 2013), antimicrobial (Acharyya et al., 2015), and anti-inflammatory activities (Chae et al., 2010). Dias et al. (2020) highlighted the antileishmanial activity potential of this compound, demonstrating a range of benefits with its use to treat the disease. Therefore, the aim of this study is to investigate the response of the polyphenolic compound methyl gallate in monotherapeutic form and/or combined with conventional CL drugs recommended by the Brazilian Ministry of Health, through *in vitro* assays against the *Leishmania* sp. parasite and its cytotoxicity profile, to identify a possible therapeutic candidate.

METHODOLOGY

Chemical samples

The methyl gallate was acquired from Sigma Aldrich, under the chemical name Methyl 3,4,5-trihydroxybenzoate, and the conventional drugs, meglumine antimoniate (Glucantime®) and pentamidine isethionate (Pentacarinat®) were provided by Tropical Medicine Foundation (FMT-HVD).

Parasite maintenance

The assays used promastigote forms of *Leishmania (Leishmania) amazonensis* (IFLA/BR/1967/PH8) and *Leishmania (Viannia) guyanensis* (MHOM/BR/1975/M4147) which were cryopreserved in the cryobank of the Laboratory of Leishmaniasis and Chagas' Diseases/COSAS/INPA. The RPMI medium supplemented with inactivated fetal bovine serum (IFBS) and 50 $\mu g.mL^{-1}$ of antibiotic (Gentamicin®) was used to grow the promastigote forms, which were incubated at 25ºC in accordance with Jaffe et al. (1984) and then used in the bioassays.

Determination of the antileishmanial activity and 50% inhibitory concentration (IC_{50}) of the chemical samples

The antileishmanial activity of the substances was assessed by inhibiting the growth and mortality of promastigotes. The substances (methyl gallate, meglumine antimoniate and pentamidine isethionate) were diluted in RPMI culture medium without SFBi supplementation, filtered and concentrations of 100 to

0.625 µg mL-$_1$ were used for the test. The negative control was 1% DMSO (Dimethylsulfoxide/Merck). For the bioassay, a 96-well plate with the test samples together with the parasites and the control was incubated in an oven at 25ºC for 24 to 72 h. The bioassays were carried out in triplicate and the average number of live cells was used to calculate the IC_{50}. (Comandolli-Wyrepkowski et al., 2017).

In vitro cytotoxicity tests

Young BALB/C mice from the Central Bioterium of the National Institute for Amazonian Research - INPA were used to obtain the macrophages, and the project was approved under CEUA number 170/2022. The animals were euthanized and then 5 mL of RPMI medium was injected into the peritoneal cavity. The macrophages were collected using a syringe and needle, centrifuged, re-suspended and diluted in complete culture medium to obtain 10^5 cells/mL according to Gordon et al. (1974). The cells were grown in 96-well plates in complete RPMI medium, in an incubator containing 5% CO_2 (Form Series II Water Jacket CO_2 Incubator, Thermo Scientific, USA) at 37ºC, for 24 h).

After 24 h, the cells were incubated in the presence of the chemicals in different concentrations in an oven at 37ºC for 48 and 72 h. The wells without cells were kept blank and the wells with cells but without treatment were kept as controls.

Cell viability was assessed using resazurin sodium salt (Alamar Blue® - Sigma Aldrich™, USA) by adding 10 µL of resazurin sodium salt stock solution (4 mg mL^{-1} in phosphate buffered saline) to each well and the plates were incubated again for a further 12 h at 37ºC. The absorbance rate was read on a spectrophotometer (BioTek®) using a wavelength of 570 nm. Data were normalized according to the formula: % survival = Abs. sample-Abs. blank / Abs. control-Abs. blank × 100 (Ahmed et al., 1994; do Nascimento et al., 2019).

Cell selectivity index (SI)

To determine the selectivity of concentrations with antileishmanial activity against peritoneal macrophages, the selective index (SI) was obtained by the ratio of the cellular CC_{50} divided by the 50% inhibitory concentration (IC_{50}) for promastigotes, during the 72 hours using the following equation:

$$\mathrm{SI} = \frac{\mathrm{CC50\ in\ peritoneal\ macrophages\ (cytotoxicity)}}{\mathrm{IC50\ against}\ \textit{Leishmania}\ \mathrm{spp.}}$$

Statistical analysis

The multiplication of parasite cells was determined by sigmoidal curves using GraphPad Prism 8.0 software, analyzing the respective 95% confidence intervals and linear coefficients.

RESULTS AND DISCUSSION

The treatment of CL is based on the clinical aspects of the disease and the infecting species. Although there is a recommended therapy for this disease, several problems exist with the current drugs, including their high cost and long treatment duration. These factors motivate the search for new treatments that can provide greater benefits, with one alternative being combination therapy. This approach allows for a reduction in drug dosages, consequently reducing their toxic effects and shortening treatment time (Brasil, 2017; Sasidharan andd Saudagar, 2021; Mann et al., 2021; Mathison and Bradley, 2023). The study of combination therapy has already been adopted in pre-clinical trials for CL. In a study by Peixoto et al. (2024), a test with a microemulsion containing epoxy-α-lapachone associated with meglumine antimoniate (Glucantime®) was conducted in Balb/C mice infected with *L. amazonensis*. The results showed a reduction in both the parasite load and the edema of the mice's paw lesions, with a considerable improvement in the toxicity profile compared to monotherapy.

Therefore, results such as these encourage further biological tests to evaluate the synergistic action of substances against *Leishmania* sp., as well as the data obtained in this study. After the *in vitro* tests to determine antileishmanial activity, the monotherapy that showed the most promise against the *L. amazonensis* and *L. guyanensis* species was Pentacarinat® (PTM), which exhibited a high inhibitory level during the 72 h of testing at all concentrations, which were reduced due to the high level of toxicity already described in the literature: 10 µg.mL^{-1} to 0.625 µg.mL^{-1} (Amato, 2006).

This is reflected in the results of the 50% inhibitory index (IC_{50}), which for this substance was <0.6 µg.mL^{-1}, as presented in Table 1. The 50% inhibitory concentration (IC_{50}) was calculated from the determination of antileishmanial activity, and the classification is based on a study by Osorio et al. (2007), where the active concentrations in *in vitro* biological assays are classified as: highly active (IC_{50} < 10 µg.mL^{-1}), active (IC_{50} between 10-50 µg.mL^{-1}), moderately active (IC_{50} between 50 to 100 µg.mL^{-1}), and non-active (IC_{50} >100 µg.mL^{-1}).

Methyl gallate (MTG) also showed promising results in terms of antileishmanial activity, especially at its highest concentrations (100 to 25 µg.mL^{-1}). Based on the IC_{50} results shown in Table 1, MTG can be classified as an active substance for both species. These findings support the antileishmanial activity of this substance, as observed in another study where a topical formulation containing methyl gallate was applied to treat lesions in golden hamsters infected with *L. amazonensis*, demonstrating its ability to reduce parasite load and control lesion progression (Bacha et al., 2023).

Regarding the use of Glucantime® (GLUC) against these two species of *Leishmania*, it did not achieve a satisfactory inhibitory index, showing a considerable increase in live parasites after 72 h, with an IC_{50} of >100 µg.mL^{-1}, classifying it as non-active. This may be due to the fact that meglumine antimoniate is a prodrug, meaning it is administered in an inactive form and requires liver metabolization for complete activation (Burguera et al., 1993). Additionally, several cases of parasite resistance to this drug have been reported, which could explain the increase in live parasites during the trial (Hadighi et al., 2006; Zarean et al., 2015).

The combination of methyl gallate and Pentacarinat® (MTG + PTM) showed a high level of parasite inhibition

Table 1. Cytotoxic and antileishmanial activity in promastigotes of *Leishmani*a spp., and IC_{50} values of chemistry samples. CC_{50} = Cell Concentration of murine macrophages; SI = Selectivity Index.

Sample	CC_{50} (µg.mL^{-1})		IC_{50} (µg.mL^{-1})						SI	
	Murine MØ		*L. amazonensis*			*L. guyanensis*			*L. a*	*L. g*
	48 h	72 h	24 h	48 h	72 h	24 h	48 h	72 h	72 h	72 h
MTG	208	222	32 ± 2	14 ± 2	12 ± 1	54 ± 5	44 ± 4	11 ± 1	19	20
MTG + GLUC	371	>500	50 ± 2	46 ± 5	56 ± 1	>100 ± 6	83 ± 1	25 ± 4	10	20
MTG + PTM	382	322	52 ± 2	15 ± 0.1	<7 ± 0.1	<7 ± 0.1	<7 ± 0.1	<7 ± 0.1	46	46
Glucantime®	>500	>500	>100 ± >10	>100 ± >10	>100 ± 1	84 ± 8.7	>100 ± >10	>100 ± 1	5	5
Pentacarinat®	1	23	<0.6 ± 1	<0.6 ± 0.1	<0.6 ± 0.1	<0.6 ± 0.1	<0.6 ± 0.1	<0.6 ± 0.1	38	38

during the 72-h experimentation against both species, with better performance at concentrations of 110 to 14 µg.mL^{-1}. Table 1 shows the IC_{50} results of this combination during the 72-h test, with a significant reduction in concentration from 52 to <7 µg.mL^{-1} for *L. amazonensis*. For *L. guyanensis*, this combination was classified as highly active during the 72 h of analysis, with an IC_{50} equal to <7 µg.mL^{-1}, since PTM is the drug of first choice for this species, corroborating the highly active classification of its association with MTG (Brasil, 2017).

As for the combination of methyl gallate and Glucantime® (MTG + GLUC), greater inhibition activity was observed at a concentration of 200 µg.mL^{-1} after 72 h of testing. There was a significant improvement in antileishmanial activity compared to GLUC monotherapy. The MTG + GLUC sample was classified as moderately active, with an IC_{50} of 56 µg.mL^{-1}. For *L. guyanensis*, this combination was classified as active, with an IC_{50} of 25 µg.mL^{-1} at 72 h, obtaining a more favorable result than GLUC monotherapy for this species. According to the Tegumentary Leishmaniasis Surveillance Manual, GLUC is not recommended for *L. guyanensis*, which corroborates the benefit of combined therapy (Brasil, 2017).

Regarding the cell cytotoxicity test with primary cultured macrophages, no significant cytotoxic profile was observed in the combined and monotherapeutic treatments, apart from PTM, as shown in graph B of Figure 1. From the calculation of the 50% cytotoxic concentration (CC_{50}), which is the amount of the compound needed to induce cytotoxicity in half of the cells, the CC_{50} for PTM at 72 h was 23%, as shown in Table 1 (Kyriazis et al., 2013). The cytotoxic profile observed for Pentacarinat® has already been described in the literature, such as in the work by Behnia et al. (2021). In this study, an *in vitro* assessment of pentamidine isethionate's effects against *Acanthamoeba* trophozoites and cysts was conducted, which included a cytotoxicity test on Vero cells. The results indicated a dose-dependent decrease in cell viability, demonstrating a higher cytotoxic profile at elevated concentrations.

As stated in this study, methyl gallate (MTG) emerged as a promising candidate for CL treatment due to its ability to inhibit parasite concentrations without exhibiting a cytotoxic profile. This finding is consistent with the results reported by Dias et al. (2020), who conducted cytotoxicity tests of methyl gallate on murine cells and J774 cells. Their results demonstrated no cytotoxic effects on murine cells, although there was a reduction in J774 cell concentration, attributable to its known antitumor action, as previously indicated by Lee et al. (2013).

After correlating the IC_{50} and CC_{50} data, the selectivity index (SI) can be calculated, which measures the magnitude of the effect by comparing the CC_{50} of murine macrophages with the IC_{50} of *Leishmania* spp. parasites. A compound with an SI greater than 1 is considered more selective against *Leishmania* sp. and thus a promising candidate for CL treatment (Makwali et al., 2015).

For the combination of MTG + GLUC, as shown in Table 1, the SI for *L. amazonensis* was 10 times more toxic to the parasite than to the cells, and for *L. guyanensis* it was 20 times more toxic, similar to MTG monotherapy. However, a significant improvement was observed compared to GLUC monotherapy, with an SI 5 times more toxic for both species, highlighting the therapeutic advantage of combination therapy for enhanced parasite selectivity.

Considering the selectivity index, the therapeutic combination of MTG + PTM was found to be the most effective treatment, demonstrating a forty-six-fold greater lethality to the parasite compared to murine cells. Additionally, they exhibited inhibitory concentrations reflecting remarkable activity. This finding is promising, suggesting that in clinical application, it would be feasible to reduce the Pentacarinat® dose, thereby ensuring a more effective and safer treatment for the patient.

Conclusion

The results of the current study demonstrate that the combination of methyl gallate with the drugs recommended by the Brazilian Ministry of Health, Glucantime® and Pentacarinat®, was highly effective against *L. amazonensis* and *L. guyanensis*, while also showing minimal cytotoxicity and maintaining high cell viability. Given that MTG + PTM exhibited a forty-sixfold

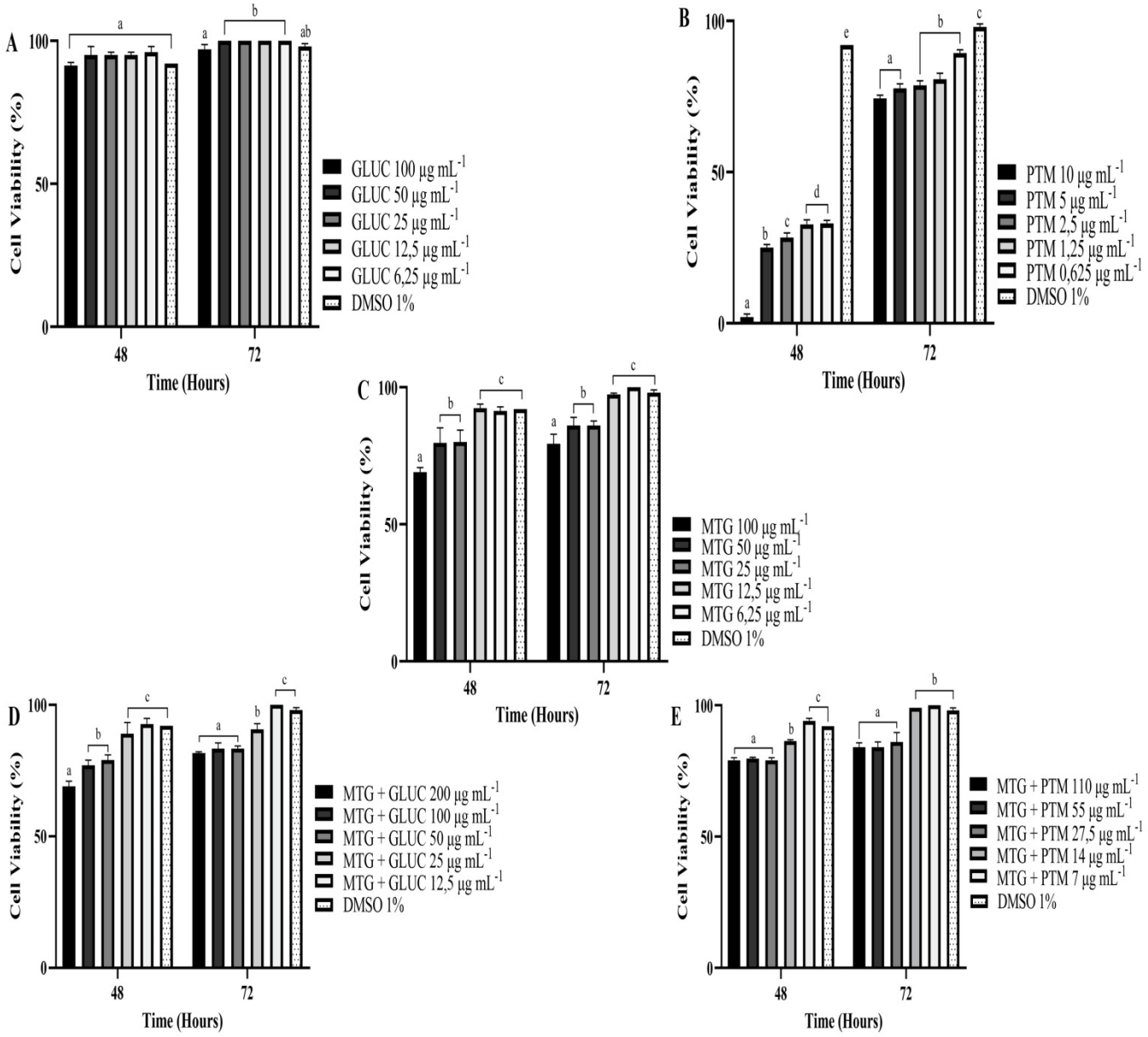

Figure 1. Cytotoxic activity of Glucantime® (A), Pentacarinat® (B), methyl gallate (C), methyl gallate + Glucantime® (D) and methyl gallate + Pentacarinat® (E) on primary cultured macrophages (murine) incubated at 37ºC for 48 and 72 h, assessed by Alamar Blue® colorimetric cell viability**.**

higher cytotoxicity against parasite cells compared to murine cells, it is suggested that further research be conducted, including additional pre-clinical trials and potentially clinical trials, to refine the pharmacotherapeutic protocol for patients diagnosed with cutaneous leishmaniasis.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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