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IN VITRO ANTIPROTOZOAL ACTIVITY OF CRUDE EXTRACT AND LEAF PARTITIONS OF *Diplopterys pubipetala* (MALPIGHIACEAE)

ATIVIDADE ANTIPROTOZOÁRIA IN VITRO DO EXTRATO BRUTO E PARTIÇÕES DE FOLHAS DE Diplopterys pubipetala (MALPIGHIACEAE)

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ABSTRACT

The *in vitro* antiprotozoal activity of the hydroethanolic extract and its partitions (hexane, dichloromethane, ethyl acetate, and methanol) from *Diplopterys pubipetala* (Malpighiaceae) leaves was evaluated against the protozoa *Trypanosoma cruzi*, *Leishmania amazonensis*, and *Plasmodium falciparum*. The samples were obtained from the collection of young, healthy leaves and subjected to the preparation of the hydroethanolic extract and subsequent partitioning, following previously established methodologies. In the biological assays, all samples showed less than 30% inhibition of protozoan activity, failing to meet the minimum criteria for the characterization of relevant biological activity. These results are consistent with the literature, which also reports low antiprotozoal activity in Malpighiaceae species, although some have demonstrated phytotherapeutic potentials related to antioxidant and anti-inflammatory activities in recent studies. It is concluded that further studies are required to investigate natural compounds as promising alternatives in the development of treatments for neglected diseases caused by protozoa.

Keywords: Biological assays; *Diplopterys pubipetala*; Protozoa.

RESUMO

Avaliou-se a atividade antiprotozoária in vitro do extrato hidroetanólico e das partições (hexano, diclorometano, acetato de etila e metanol) de folhas de Diplopterys pubipetala (Malpighiaceae) frente aos protozoários Trypanosoma cruzi, Leishmania amazonensis e Plasmodium falciparum. As amostras foram obtidas a partir da coleta de folhas jovens e saudáveis e submetidas à preparação do extrato hidroetanólico e subsequente partição, seguindo metodologias previamente estabelecidas. Nos ensaios biológicos, todas as amostras apresentaram inibição da atividade dos protozoários inferior a 30%, não atingindo os critérios mínimos para a caracterização de atividade biológica relevante. Esses resultados estão alinhados com a literatura, que também relata baixa atividade antiprotozoária em espécies de Malpighiaceae, embora algumas tenham

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demonstrado potenciais fitoterapêuticos relacionados às atividades antioxidantes e anti-inflamatórias em pesquisas recentes. Conclui-se que estudos adicionais são necessários para a investigação de compostos naturais como alternativas promissoras no desenvolvimento de tratamentos para doenças negligenciadas causadas por protozoários.

Palavras-chave: *Ensaios biológicos; Diplopterys pubipetala; Protozoários.*

INTRODUCTION

Nature exhibits enormous chemical variety, where complex structures that can be reproduced in the laboratory. Many of the drugs sold are products of synthetic modifications or total syntheses of naturally occurring substances. The pharmaceutical industry over the last two centuries has been used chemical substances originating from nature, especially those isolated from plants, which serve as base models for the development of new molecules and also as active ingredients in themselves, with a large representation in the pharmaceutical market (Noor *et al*., 2022; Bora *et al*., 2023).

In the Cerrado Biome, for example, the Malpighiaceae family is found, which has many species with great therapeutic potential that have already been investigated, such as antimicrobial, allelopathic, antioxidant and antifungal activities (Frias *et al*., 2012; Santos *et al*., 2020; Abbas *et al*., 2022; Sacramento *et al*., 2025). The species *Diplopterys pubipetala*, popularly known as "marvaquero", "cipó preto" (Gates, 1982) or "tucunacá" (Nagamine-Pinheiro *et al*., 2021) has still been little explored in relation to its biological activities.

The serious context of neglected tropical diseases (NTDs) fosters the search for new bioactive substances. The drugs available for NTDs still have restricted use due to lack of efficacy, high cost, difficulty of use or toxicity that they establishes, difficulties of accessing and continuing treatments (Neto *et al*., 2022). It is necessary to conduct research on the phytochemical constituents and their therapeutic potential, as well as to elucidate the mechanisms of action involved and identify the active principles responsible for the biological activities (Vitale *et al*., 2022; Roy *et al*., 2022).

Much of the therapeutic action of plants is related to the biologically active secondary compounds they produce, such as polyphenols, flavonoids and phenolic acids, terpenoids, saponins and others capable of producing beneficial effects on the environment and humans (Li *et al*., 2020; Twaij and Hasan, 2022). In this study, the goal was to evaluate the *in vitro* antiprotozoal activity (*Trypanosoma cruzi*, *Leishmania amazonensis*, *Plasmodium falciparum*) of the hydroethanolic extract and partitions (hexane, dichloromethane, ethyl acetate and methanol) of *D. pubipetala* leaves.

MATERIAL AND METHODS

PLANT MATERIAL

They were collected young and undamaged leaves of *Diplopterys pubipetala* (Malpighiaceae) in the district of Nova Esperança, municipality of Montes Claros (MG), in December 2021 (16º36'07.0"S 43º55'12.7"W; https://maps.app.goo.gl/4NibPT6woJB29KK56), the plant was deposited in the Herbarium Montes Claros (MCMG) under voucher 4033 and the study was registered in the National Genetic Heritage Management System (SisGen) under number A822A14. It was prepared the hydroethanolic extract (Alvarenga *et al.*, 2015). Liquid-liquid extraction occurred by resuspending the extract in hydroethanolic solvent (7:3) and the partitions occurred in the sequence hexane, dichloromethane, ethyl acetate and methanol, with equal volumes (1000 mL; 2×500 mL), and they were subsequently dried in an oven at 38°C with air circulation (Andreo and Jorge, 2006).

Biological tests were performed *in vitro* with the crude extract and all the mentioned partitions.

ANTI-*Trypanosoma cruzi* ACTIVITY

It was prepared a stock solution (20 $mg.mL^{-1}$) in dimethyl sulfoxide (DMSO). Transfer of 1 µL of each sample (20 µg) to the wells of microtiter plates; the solvent was removed in a vacuum centrifuge and kept at -20°C. The assay was based on cells of the L929 lineage with the Tulahuen strain of *Trypanosoma cruzi* transfected with β-galactosidase (Buckner *et al.*, 1996 and modified by Romanha *et al.*, 2010).

The results were expressed as the percentage of growth inhibition. The active compounds were tested in decreasing concentrations to determine the IC_{50} on the amastigote and trypomastigote forms of the parasite. Quadruplicates were performed on the same plate and the experiments were repeated at least once. The positive control was Benznidazole (Roche) at its IC_{50} (1.0 $\mu g.mL^{-1}$ = 3.8 µM).

Cell viability was expressed as the percentage of difference in reduction between treated and untreated cells. The IC_{50} and CC_{50} values were determined in duplicate by linear interpolation using an Excel spreadsheet, and the selectivity index (SI) was calculated by the ratio of CC_{50} on L929 cells/ IC_{50} on *T. cruzi.* An SI value > 10 is an indicator of good selectivity.

ANTI-*Leishmania amazonensis* ACTIVITY

Amastigote and promastigote forms of *L. amazonensis* (strain IFLA/BR/196/PH-8) were obtained from lesions of experimentally infected hamsters and cultured in Schineider's medium, being transformed into axenic amastigote forms to perform the tests (Calhahan *et al.*, 1997).

The assay was performed in duplicate. 96-well microculture plates were used. 90 µL of culture at a concentration of 1x108 parasites.mL^{-1} and 10 µL of the different samples were added to each test well; all controls were duly performed, Amphotericin B (0.2 µg.mL^{-1}) was the reference substance.

It was performed incubation using the dye 3-4,5-dimethyl-thiazol-2-yl-2,5-diphenyltetrazolium bromide (MTT) (Sereno and Lemesre, 1997). The results were expressed as percentage of death after 72 h of incubation and were calculated from the optical absorbance measurements read at λ 570 nm.

ANTI-*Plasmodium falciparum* ACTIVITY

Parasites of the W2 lineage (chloroquine resistant) were cultured in human red blood cells using the Trager and Jensen method (1976) (Andrade-Neto *et al.*, 2007), synchronization occurred by the sorbitol method (Lambros and Vanderberg, 1979).

The tests were performed in triplicate, the extract concentrations ranged from 3.125 µg.mL^{-1} to 200 µg.mL^{-1}. Schizonts were incubated with SYBR Green. *P. falciparum* cultures with 0.5% parasitemia and 2% hematocrit were distributed in 96-well microplates. Each well received the test-compound at different concentrations, and chloroquine was used as a positive control

The incubation, centrifugation and waste disposal protocols, and monitoring were performed with saline and buffer solution (Smilkestein *et al.*, 2004; Siqueira *et al.*, 2012). The viability of the parasites was assessed by inhibition curves that relate the concentration of the compounds with the inhibition of parasite replication. The IC_{50} was calculated using the Origin software (version 5.0), with non-linear regression.

To investigate the toxicity of the compounds, monkey kidney cells (BGM lineage) were used. After 24 h of incubation, cell viability was verified using the neutral red assay (Borenfreund *et al.*, 1988; Aguiar *et al.*, 2012).

The CC_{50} was determined for each compound, and the Selectivity Index (SI) was calculated by the ratio between the CC_{50} and the IC_{50} (Bézivin *et al.*, 2003).

RESULTS

Negative values indicate absence of activity and possible increase in the viability of the parasites in relation to the control group, in the anti-*Trypanosoma* assay, with crude hydroethanolic extract of *D. pubipetala* leaves and the partitions.

The results are expressed in Table 1 below:

Table 1- Result of the anti-Trypanosoma biological assay.

Sample	Reply 1	Reply 2	Average	SD	CV(%)	Result 1 (%)	Result 2 (%)
Crude Extract	0,9	0,9	0,9	0,0	1,7	18,8	-27,6
Hexane Partition	0,6	0,9	0,8	0,2	24,1	-38,5	-17,1
Dichloromethane Partition	0,9	1,0	0,9	0,0	5,3	-37,6	-30,0
Ethyl acetate partition	0,9	0,9	0,9	0,0	1,9	-50	-20,2
Methanol Partition	0,9	0,9	0,9	0,0	2,7	-50	-19,3

Source: Authors

Benznidazole -Bz (1 µg. mL^{-1}) positive control
DMSO solubilizer (1%)
SD= standard deviation
CV= coefficient of variation 8%

In the test to verify the viability of amastigote forms of *L. amazonensis*, in the crude hydro-ethanolic extract of *D. pubipetala* leaves and the partitions, the results obtained for the inhibition percentage were lower than 30%, as shown in Table 2, thus, this screening did not reach the values necessary to consider such extracts as subject to further investigation.

Table 2- Results of anti-*Leishmania* biological tests.

Sample	Reply 1	Reply 2	Average	CV(%)	AMB	DMSO	CP	Result (%)
Crude extract	0,783	0,783	0,783	1	0,114	1,071	1,075	27
Hexane partition	0,99	0,99	0,99	0	0,114	1,071	1,075	8
Dichloromethane partition	0,974	0,974	0,974	0	0,114	1,071	1,075	9
Ethyl acetate partition	0,962	0,96	0,961	0	0,114	1,071	1,075	11
Methanol partition	1,053	1,053	1,053	0	0,114	1,071	1,075	2

Source: Authors.

AMB: amphotericin B (positive control, 89% inhibition)
DMSO: negative control
CV (%)= coefficient of variation
CP= parasite control (mean of absorbance of the control group)

In the anti-*Plasmodium falciparum* assay, the crude hydroethanolic extract of *D. pubipetala* leaves and the partitions occured significant activity (IS > 10) only for the dichloromethane partition, in the first assay, but this activity was not reproducible in a new evaluation, a criterion adopted in the methodology (Bézivin *et al.* 2003) and expressed in Table 3.

Table 3- Results of anti-*Plasmodium* biological tests.

Sample	CC_{50} (µg.mL^{-1})	IC_{50} (µg.mL^{-1})	Atividade (Ensaio SYBR)	IS
Crude extract	≥200	40,2 ± 3,6	No	NA
Hexane partition	≥200	≥50	No	NA
Dichloromethane partition	99,2 ± 8,4	9,3 ±1,14	Yes	11
Ethyl acetate partition	≥200	≥50	No	NA
Methanol partition	≥200	≥50	No	NA

Source: Authors.

Cytotoxicity assessed by the neutral red incorporation assay in monkey kidney cells of the BGM lineage
IS = Selectivity Index = MDL50/IC50; values > 10 are considered non-toxic
NA = not applicable

DISCUSSION

One of the biggest global health problems, which mainly affects poorer countries in Africa, Asia and Latin America, are infections caused by protozoan species such as *Trypanosoma*, *Plasmodium* and *Leishmania*, which significantly cause morbidity and mortality (Khater *et al.*, 2017). The drugs available for the treatment of NTDs are restricted to certain stages of the disease, toxicity often causes difficulty in continuing of use, and added to this is the issue of high cost (Jackson *et al.*, 2020; Neto *et al.*, 2022).

The selection of methods for verifying biological activities *in vitro* allows that the necessary precursor observations to be made in order to select, among crude plant products, those with potentially useful properties for other chemical applications and pharmacological studies (Luize *et al.*, 2005; Kabera *et al.*, 2014).

The results regarding the inactivity of the crude extract and the leaf partitions of *D. pubipetala* in relation to anti-*T. cruzi*, anti-*L. amazonensis* and anti-*P. falciparum* activities are consistent with other results obtained in the literature for Malpighiaceae.

The ethanol extract and leaf partitions of *Malpighia glabra* were evaluated to understand the anti-*T. cruzi* activity profile, and all were considered inactive since the infection reduction percentages were less than 90%, more specifically less than 11% (Peres *et al.* 2021).

In a review work on traditional use, phytochemicals and therapeutic potential in plant extracts from twenty different genera of Malpighiaceae, totaling more than thirty species, only *Byrsonima coccolobifolia* showed significant inhibitory activity for *L. amazonensis*, possibly attributed to the presence of flavonoids (de Sousa *et al.*, 2014; Abbas *et al.*, 2022).

In another study that involved essential oils extracted from the roots of *Banisteriopsis campestris*, data were obtained suggesting selectivity of the sample against *L. amazonensis*, and the cytotoxic effect itself (Rocha *et al.*, 2018).

The alkaloids harmine and harmaline, present in Ayahuasca tea produced from the *Banisteriopsis caapi* plant, have been reported to have significant inhibitory activity against species of the genus *Plasmodium* (Frias *et al*., 2012).

Other species of Malpighiaceae have been tested in the laboratory: *Byrsonima crassifolia*, *Byrsonima verbascifolia*, *Byrsonima crassa* with demonstration of antimalarial activity or used as febrifuges in Central America (Milliken, 1997).

Antiplasmodial (antimalarial) activity has been reported for only two species of Malpighiaceae, most notably for *Tristellateia madagascariensis.* Traditional use of this species involves decoction of the leaves, which is administered orally to alleviate the symptoms of malaria, while inhalation of its vapors has been used to reduce fever (Randrianarivelojosia *et al*., 2003).

Among more recent studies, there is in vivo research, with the species *Stigmaphyllon ovatum*, which produced chemosuppressive activity against malaria, it was found that it consists of several metabolites such as terpenoids, steroids, phenolics and alkaloids, it is estimated that when the antimalarial agent is isolated it could become a precursor or medicine for this disease (Iyekowa *et al*., 2021).

The absence of antiprotozoal activity observed for the plant extract and partitions of *D. pubipetala*, against the protozoa *Trypanosoma cruzi*, *Leishmania amazonensis* and *Plasmodium falciparum*, under the conditions proposed in this study, can be attributed to different factors such as the insufficient concentration of active compounds present in the sample, as well as the absence or little synergy between the constituents for there to be a biological response in the assay (Radulovc *et al*., 2013). Furthermore, it is possible that the extract does not contain metabolites with specific antiprotozoal activity for the species investigated (Bessa *et al*., 2013; Vásquez-Ocmína *et al*., 2018). More specifically, regarding the difference between the antiplasmodial assays, another factor such as the change in the stability of the metabolites may be the cause of the undetected activity.

CONCLUSIONS

Given the challenges related to the treatment of diseases caused by *Trypanosoma*, *Leishmania* and *Plasmodium* that cause morbidity and mortality in much of the world, it is necessary to continue searching for substances that have relevant biological activity in relation to these microorganisms.

For the *in vitro* tests performed with the crude extract and partitions of *D. pubipetala*, no biological activity was observed in the models tested, within the concentrations used. However, considering the limitations of currently available treatments for these parasitic diseases, often characterized by high toxicity, limited efficacy and restrictions on prolonged use, future studies related to the therapeutic potential of medicinal plants should be encouraged, as well as detailed phytochemical investigation of dichloromethane partitioning and in vivo tests with purified fractions. Fractionation-oriented bioactivity studies may reveal pharmacological properties not yet identified.

The development of new drugs from natural sources remains a promising alternative to overcome the challenges posed by conventional therapies, promoting safer and more effective treatments for neglected diseases.

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