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In vitro antiplasmodial activity of flower extracts from *Combretum leprosum* Mart (mofumbo)

Atividade antiplasmodial in vitro de extratos das flores de Combretum leprosum Mart (mofumbo)

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Abstract

Malaria is the cause of hundreds of thousands of deaths per year, besides putting billions of people at risk of developing disease. When it comes to its therapy, the drugs used currently are losing its efficacy due to increase in the frequency of resistant strains of the parasite, highlighting the importance for the search of new classes of molecules presenting antiplasmodial activity. In the present work, the antiplasmodial activities of five extracts (crude, hexane, chloroform, ethyl acetate and methanol) from the flowers of Combretum leprosum are assessed. The method employed for obtaining the extracts was silica gel column chromatography, and the techniques used for the analysis of antiplasmodial activity (IC_{50}) and citotoxicity (MLD_{50}) were ELISA and MTT respectively, where a selectivity index was calculated after the obtaining of these two values. The extract presenting the highest antiplasmodial activity was the chloroform extract (IC_{50}= 3.97 μg/mL), however, this extract also presented the higher cytotoxicity (MLD_{50}= 18.12 μg/mL), and therefore the extract presenting the best overall activity was the methanolic extract (IS = 18.97). The study demonstrated the plant Combretum leprosum has active substances against P. falciparum and therefore is a potential to be explored in further pharmacological studies with this parasite.

***Keywords**: Malaria; Chemotherapy; Plants*

Resumo

A malária é responsável por milhares de mortes por ano, além de bilhões de pessoas estarem sob o risco de desenvolvê-la. Quanto à sua terapia os fármacos utilizados atualmente estão perdendo a eficácia devido ao aumento na frequência e cepas resistentes do parasita, e esse fato ressalta a importância da busca por novas classes de moléculas com atividade antiplasmodial. O presente estudo objetivou analisar a atividade antiplasmodial de cinco extratos (bruto, hexânico, clorofórmico, acético e metanólico) das flores da planta Combretum leprosum. A metodologia empregada para a obtenção dos extratos foi a cromatografia em silica gel, e as técnicas empregadas para a análise da atividade antiplasmodial (IC50) e citotoxicidade (MDL50), respectivamente, foram ELISA e MTT, sendo calculado um índice de seletividade (IS) após a obtenção dos dois valores. O extrato que apresentou a maior atividade antiplasmodial foi o clorofórmico (IC50= 3,97 μg/mL), contudo, este apresentou alta citotoxicidade (MDL50= 18,12 μg/mL), e foi considerado que o extrato que apresentou o melhor IS foi o do extrato metanólico (IS= 18,97). O estudo demonstrou que a planta Combretum leprosum possui substâncias ativas contra Plasmodium falciparum e, portanto, constitui-se em um potencial a ser explorado em estudos farmacológicos posteriores com este parasita.

***Palavras-chave**: Malária; Quimioterapia; Plantas*

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1 Introdution

Over 3 billion people are estimated to be at risk of being infected with malaria and therefore developing disease, and according to latest estimates, hundreds of thousands of deaths caused by it occurred in each year of this millennium (WHO, 2014). The world region that suffers the heaviest burden is Africa, where the great majority of cases of malaria occur, mainly in children under the age of five (WHO, 2014). The disease is caused by parasitic protozoa of the genus Plasmodium, of which five species are known to infect humans: *P. falciparum, P. vivax, P. malariae, P. ovale, and P. knowlesi* (COX, 2010). The parasite life cycle begins when a female mosquito of *Anopheles* genus bites a human and releases the sporozoites from its salivary glands to the dermis, where they reach the avascular tissue and then a percentage of them migrates and enters the blood vessels, making their way to the liver, where they undergo cellular modifications and perform rounds of asexual replication (ALY; VAUGHAN; KAPPE, 2009).

After leaving the liver, the parasite begins other cycles of asexual replication inside red blood cells, using molecules and cellular structures of the host cells to survive, grow and reproduce, generating thousands of merozoites and causing the characteristic symptoms of the disease (TILLEY; DIXON; KIRK, 2011). When it comes to its chemotherapy, most of the cases of uncomplicated malaria are treated currently by the use of ACT's drugs, although is already known that these drugs are losing its efficacy due to the increase in frequency of resistant strains (VISSER; VUGT; GROBUSCH, 2014), which highlights the importance for the search of new molecules bearing antiplasmodial activity.

Plants have been used during millennia by humanity, and pharmacological studies of medicinal plants yielded useful molecules in the treatment of various diseases, including malaria (GINSBURG; DEHARO, 2011), and despite the great number of existing species of plants, just a small percentage of them have been characterized pharmacologically, making the biodiversity of world's flora a great potential to be explored (DERDA; HADÁS, 2015). Data in literature show that over 1200 species of plants, belonging to 160 families, are used to treat malaria and fever, and on average, a fifth of the patients with malaria in endemic countries use traditional medicines to treat the disease (WILLCOX; BODEKER, 2004).

Combretum leprosum is a scandent shrub or liana plant that can be found in Brazil, Paraguay and Bolivia. Some of the distinctive features of the species are the opposite leaves, 4-winged fruit and panicle inflorescences (LOIOLA et al., 2009). Local populations in the northeast region of Brazil use some of its parts as expectorants, to treat coughs and to staunch blood flow (AGRA et al., 2007; SILVA; BARROS; NETO, 2015). *In this context,* The aim of this study *is to assess* the antiplasmodial potential of *C. leprosum* flower extracts *against P. falciparum in vitro*.

2 Material and Methods

Obtaining and preparation of the extracts

Flowers of *C. leprosum* were collected in Viçosa, State of Ceará, in May 2001. They were properly dried and grinded *and after* dipped in ethanol (99%) for the extraction. The drying of the extract was performed by rota-evaporation and part of it was subjected to a chromatographic column with silica gel as standing phase (SASIDHARAN et al., 2011), whereas the other part constituted the crude extract (CLCrE). The solvents used were hexane, chloroform, ethyl acetate and methanol for the obtaining of the following extracts: Hexane extract (CLHE); Chloroform extract (CLClE); Ethyl Acetate extract (CLEAE) and methanol extract (CLME). For the carrying out of cytotoxic and antiplasmodial tests, extracts were solubilized with ethanol, where all solutions were performed at the moment of the biological experiments.

In vitro Antiplasmodial tests against *P. falciparum*

The chloroquine-resistant strain (W2) was cultured in human red blood cells as established by Trager and Jensen (1976) with modifications, using a protocol previously established and standardized by Laboratório de Malária do Centro de Pesquisas René Rachou (DE ANDRADE-NETO et al., 2004). The predominance of rings in the culture was accomplished by using sorbitol, according to literature (LAMBROS; VANDERBERG, 1979), and the hematocrite and parasitemia adjusted by the addition of red blood cells. Culture of synchronized parasites was distributed in microplates containing culture medium and red blood cells. Before the addition of the parasites, the drugs were added in serial dilutions. For the negative control, no drug was added to the infected red blood cells, and for the positive control, chloroquine was added to the parasite culture. In the ELISA test (NOEDL et al., 2002), two plates were prepared: one of them (the test plate) containing the parasites and drugs, and the other one containing the antibodies. The test plate was incubated for 72 hours, wherein the negative control was withdrawn 24 hours for

being used as background. After 72 hours, the plates were frozen and thawed twice for the lysis of the red blood cells. For the sensibilization of the test plates, the primary antibody was added (MPFM-55A ICLLAB®, EUA). Wells were washed and incubated, and for each well was added 100 µL of the samples of *P. falciparum* hemolyzed culture and at six wells of the plates corresponding to the background was added 100µL of negative controls. The plate was incubated during one hour in moist chamber and washed three times, adding to each well 100µL of the secondary antibody (MPFG55P ICLLAB®, EUA). After incubation in moist chamber, the plate was incubated during 5 to 10 minutes at room temperature, and the reaction was stopped with the addition of sulfuric acid. The reading was performed by a spectrophotometer of microplates (ELISA reader). The inhibition of 50% of the parasite growth (IC_{50}) was determined by dose-response curves, through linear regression, using the program Origin.

Citotoxicity assays *in vitro* on HepG2 cells

Human cell line HepG2 was cultured as recommended (CALVO-CALLE et al., 1994). Cells were cultured in culture bottles, with culture medium (RPMI) and gentamicin. They were maintained in an incubator and its medium was replaced each two days. For the preparation of the plates, they were washed with incomplete medium and treated in tripsin-EDTA and incubated for the detachment of the cells from the culture bottle. After, the complete RPMI medium was added, followed by centrifugation at room temperature. The supernatant was discarded and the sediment resuspended in RPMI. After the preparation of the plates was added MTT (Sigma Aldrich) in the plates (DENIZOT; LANG, 1986). After incubation with MTT, supernatant was withdrawn and the dye at the bottom of the wells diluted with DMSO in a volume of 100µL/well. The microplates were read by an ELISA reader using a filter of 570 nm. The Minimum Lethal Dose of 50% of cells (MLD_{50}) was also determined by the program Origin (OriginLab Corporation, Northampton, MA, EUA).

Selectivity Index

The Selectivity Index (SI) was calculated as a quotient of the MLD_{50} and IC_{50} values. If the SI was lower than 10, the extract was considered toxic; if the value was higher than 10, it was considered non- toxic (WENIGER et al., 2001).

3 Results and Discussion

All of the extracts assayed in this study exhibited antiplasmodial activities to some degree (Table 1). . Despite its high antiplasmodial activity, the chloroform extract (IC_{50} = 3.97 µg/mL) also showed great toxicity for the human cell line HepG2 (MLD_{50} = 18.12 µg/mL) (Table 1). However, the subsequent fractioning of this extract will be needed in order to assess the cytotoxic and antiplasmodial activities of its molecules, so one can verify whether the molecule presenting the highest antiplasmodial activity in this extract is also the main cause for the toxicity observed towards HepG2 cells. The extract that presented the best overall activity was the methanolic extract (SI = 18.97), despite it did not present neither the highest antiplasmodial activity nor the lower citotoxicity (Figure 1)

Table 1 - IC_{50}, MLD_{50} and SI of *C. leprosum* flower extracts

Drug	IC50 (µg/mL)	MLD50 (µg/mL)	SI	RESULT
CLCrE	25.25	246.74	9.77	Inactive/Toxic
CLHE	12.20	168.89	13.84	Active/Non toxic
CLClE	3.97	18.12	4.56	Inactive/Toxic
CLEAE	11.46	123.66	10.79	Active/Non toxic
CLME	5.89	111.74	18.97	Active/Non toxic
CQ	0.04	297	7425	Active/Non toxic

IC50: Concentration causing 50% inhibition, MLD50 : Dose lethal to 50% of the population

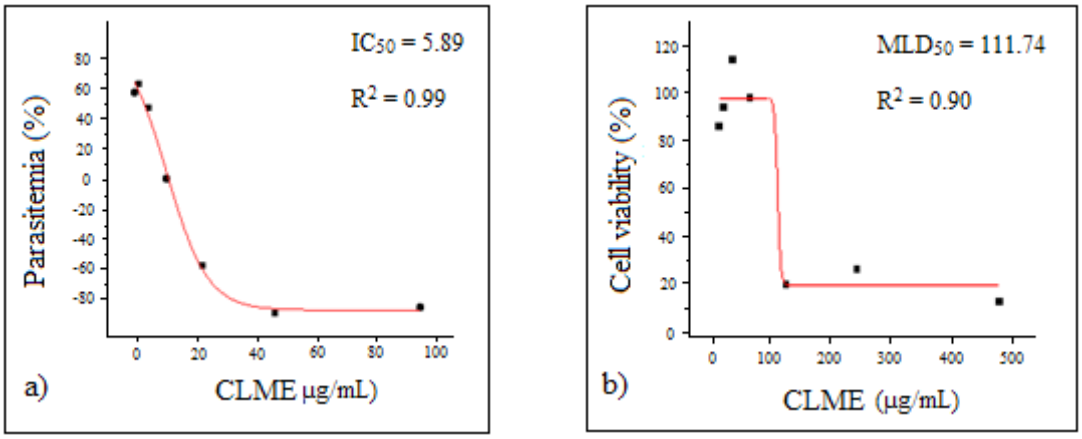

Figure 1 - Dose-response relationship curve of the methanolic extract (the extract presenting the higher SI) of the antiplasmodial (a) and citotoxicity (b) tests, respectively.

Most of the pharmacological studies of *C. leprosum* report activities against non-infectious pathological processes, such as neurodegenerative diseases (FACUNDO et al., 2005), ulceration (NUNES et al., 2009) and snake venom intoxication (FERNANDES et al., 2014). However, in a study performed by Teles and co-workers (2011), where from the fruits of *C. leprosum* were tested the ethanolic extract and a lupane, an antileishmanial activity was reported. The ethanolic extract showed an IC_{50} of 24.8 µg/mL. This result was similar to that of our study, in which the crude extract, also extracted with ethanol, obtained an IC_{50} of 25.70 µg/mL. The lupane exhibited an IC_{50} of 3.3 µg/mL. The ethanolic extract was tested on a 25 µg/mL concentration and the lupane was tested on a 5 µg/mL for citotoxicity. On these concentrations, neither the extract nor the lupane were toxic (TELES et al., 2011). The citotoxicity result of the ethanolic extract for *Leishmania* is also in agreement with our work. Further studies using the lupane against *Leishmania* were carried out for investigation of the activity of a liposomal formulation carrying the substance (BARROS et al., 2013) and for its interference in amastigote replication inside mammal cells, as well as investigation of its action mechanism through bioinformatics tools (TELES et al., 2015). This study, therefore, contributes to the understanding of the antiprotozoal activites of *C. leprosum*.

Species of the genus *Combretum* have already been investigated in respect to its pharmacological activities against non-infectious and infectious diseases, including antiplasmodial activities for some of these species (LIMA et al., 2012; ROY et al., 2014). Furthermore, some of them are used in traditional medicine to treat malaria and fever (TABUTI, 2008; TRAORE et al., 2013; DIARRA et al., 2015; AGBOEKA e al., 2016). The activity of our methanolic extract (IC_{50}= 5.89 µg/mL) was similar to that presented by *C. molle* methanolic extract (IC_{50}= 5.7 µg/mL), which was extracted from its leaves. The citotoxicity value was significantly different (MLD_{50}= 40.3 µg/ml in K562S human monocyte cell line), possibly due to the use of a different cell line (GANSANÉ et al., 2009).

An acetonic eluate, extracted from the bark of the stem of *Combretum molle* showed great activity against erythrocytic forms of *P. falciparum*, presenting an IC_{50} of 8.17 µg/mL. The citotoxicity of this extract in KB cells was 86 µg/mL (MLD_{50}), resulting in an SI of 10.5 (ASRES et al., 2001). In a study done by Moosophon and coworkers, four flavanes isolated from the stems of *Combretum griffithii* showed the following antiplasmodial activities (15.74, 13.04, 9.66, and 14.45 µg/mL of IC_{50}), although it's respective toxicities to cell lines were significantly high. A dichloromethane extract of *C. sericum* leaves resulted in a low IC_{50} (9µg/ml) (MOOSOPHON et al., 2013). Niass and co-workers (2016) tested three medicinal plants, amongst them, Combretum glutinosum, against *P. falciparum*. Five extracts from this plant were assayed (ethyl acetate, IC_{50} = 41 µg/mL ; diethyl ether, IC50 = 65 µg/mL ; chloroform, IC_{50} = 38 µg/mL ; metanol, IC_{50} = 45 µg/mL, and methanol/water, IC_{50} = 35 µg/mL), of which none of them resulted in an IC_{50} lower than 10 µg/mL.

The ethanolic extract of the leaves of *C. molle* presented an IC_{50} value of 25 µg/mL, being very similar to the IC_{50} value of the crude extract of our work (25.25 µg/mL) (TRAORÉ-COULIBALY et al., 2013). Another study performed in 2013 with medicinal plants of Burkina Faso, in which are included *Combretum collinum*, showed very promising results. A chloroform extract obtained from the leaves of this species resulted in an IC_{50} = 0.2 µg/mL, with a SI = 140. An extract containing only alkaloids, extracted with chloroform resulted in an IC_{50} = 0.4 µg/mL and a SI of 113. 6 (SANON et al., 2013). These studies show the genus has a great pharmacological potential to be explored in the antimalarial therapy.

Although the isolation of an active principle was not carried out, it is likely that the molecule presenting the highest antiplasmodial activity is a triterpene, since compounds of this class have several studies reporting its antiplasmodial activities (NOGUEIRA; LOPES, 2011; RAMALHETE et al., 2011; RAMALHETE et al., 2014) and this

chemical class seems to be the most abundant in species of the genus *Combretum* (DAWE et al., 2013) and in the species *C. leprosum* (FACUNDO et al., 1993; FACUNDO et al., 2008). Triterpenes that were isolated from the genus *Combretum* also showed activities against another microorganisms, like fungi (BISOLI et al., 2008), bacteria (ANGEH et al., 2007a; ANGEH et al., 2007b; SONGCA, RAMURAFHI; OLUWAFEMI, 2013), viruses (ASRES; BUCAR, 2005) and other species of parasites (TELES et al., 2011; HAAVIKO et al., 2014).

4 Conclusions

The species *C. leprosum* has active substances against *P. falciparum* W2 strain *in vitro*, and despite the highest antiplasmodial activity was presented by the chloroform extract, it also showed high toxicity towards the human cell line HepG2. However, only the subsequent fractioning of this extract can identify what substances are the causes for each of these effects identified in the present study. *Within this context*, new research is needed in order to improve the knowledge about the activity of the molecules of this plant, such as the isolation and characterization of the active principle and *in vivo* tests with the extracts and/or the isolated substance, aiming the identification of a new molecule with potential to be used in antimalarial therapy.

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