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The leishmanicidal activity of a cyclopentenedione derivative isolated from the roots of a native Amazonian pepper (*Piper carniconnectivum*)

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Abstract: The Piper species chemistry has been widely investigated and the phytochemical analyses have led to the isolation of a number of active compounds like alkaloids, terpenes and flavones among others. The aim of this study was to evaluate the leishmanicidal activity of 2-[1-hydroxy-3-phenyl-(*Z*,2*E*)-2-propenylidene]-4-methyl-4-cyclopentene-1,3-dione (DCPC), a cyclopentenedione derivative isolated from the roots of *Piper carniconnectivum* C. DC., Piperaceae. Leishmanicidal activity against *Leishmania amazonensis* promastigotes was assessed, and the risk to host cell was assessed by measuring the cytotoxicity to peritoneal macrophages from BALB/c mice *in vitro. L. amazonensis* promastigotes and host macrophages were cultured in the presence of 100, 50, 25, 12.5 and 6 μg/mL of the cyclopentenedione derivative for up to 96 h. At the end of this period, the inhibitory concentrations (IC50) were compared with those from untreated cultures. The IC50 for promastigotes was 4.4 μg/mL after 96 h of treatment with the derivative. The 50% cytotoxic concentration (CC50) against murine peritoneal macrophages was 129 μg/mL. These results indicate that DCPC is a promising molecule for the development of leishmanicidal drugs.

Introduction

Leishmaniasis is a group of parasitic diseases that affects two million people each year and is endemic in 98 countries and territories (WHO, 2010). Brazil is among the seven countries with the highest rates of cutaneous leishmaniasis and is one of the five countries in which almost all cases of visceral leishmaniasis occur (Singh et al., 2006; Gramiccia & Gradoni, 2005).

Depending on the species of *Leishmania*, the disease can manifest as cutaneous leishmaniasis, mucocutaneous leishmaniasis or diffuse cutaneous leishmaniasis, which constitutes American Cutaneous Leishmaniasis (ACL). The most severe form of the disease, visceral leishmaniasis (VL), affects the lymphatic system and may lead to death if untreated (WHO, 2010; Herwaldt, 1999; Lucas et al., 1998).

The chemotherapeutics available for treating this disease are based on pentavalent antimony compounds, which have severe side effects in the heart, kidney, pancreas and liver, as well as reported cases of resistance (Oliveira et al., 2011; Croft & Yardley, 2002). In cases where there are contraindications or resistance to antimony compounds, amphotericin B or pentamidine are used. However, the use of these alternatives is restricted due to their high toxicity and adverse side effects (Lima et al., 2007; Croft & Combs, 2003; Amato et al., 2000).

In the search for new alternatives for the treatment of leishmaniasis, previous studies have focused on natural compounds extracted from animals and plants, which are effective against *Leishmania* and have reduced toxicity for human hosts (Ferreira et al., 2010; Calderon et al., 2009).

The genus *Piper*, which belongs to the Piperaceae family, is widespread in tropical and

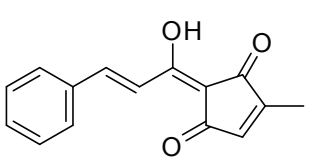
subtropical regions around the world. Many *Piper* species are used for curative purposes by different cultures (Bezerra et al., 2007). The history of the genus *Piper*, described by Parmar et al. (1997), describes the use of this species for the treatment of certain diseases in several different populations. In the Amazon region, several *Piper* species are used in phytotherapy. For example, *P. maginatum* is used for menstrual pains, snake bites and as a tonic for the liver and spleen, and *P. tuberculatum* is used as a sedative, an antidote against snake bites and to combat sexually transmitted diseases and urinary tract infections (Agra et al., 2007, 2008; Rodrigues et al., 2009). Leishmanicidal activity was recently reported from a cinnamic acid derivative (3-(3,4,5-trimethoxyphenyl) isolated from the fruit of *P. tuberculatum* plants (Ferreira et al., 2010). Dihydrochalcones isolated from *P. elongates* have also shown leishmanicidal activity (Hermoso et al., 2003). Phytochemical studies of the leaves and roots of *P. carniconnectivum* C. DC., Piperaceae, led to the discovery of volatile substances, including flavonoids and cyclopentenedione derivatives (Facundo et al., 2004, 2006).

The present study evaluated the leishmanicidal activity of the cyclopentenedione derivative 2-[1-hydroxy-3-phenyl-(*Z*,2*E*)-2-propenylidene]-4-methyl-4-cyclopentene-1,3-dione (**1**) (DCPC), isolated from roots of *P. carniconnectivum* (Facundo et al., 2004), against extracellular *Leishmania amazonensis* promastigotes. We also analyzed the cytotoxic activity of the compound in the peritoneal macrophages of BALB/c mice.

Materials and Methods

Plant material

The cyclopentenedione derivative DCPC (2-[1-hydroxy-3-phenyl-(*Z*,2*E*)-2-propenylidene]-4-methyl-4-cyclopentene-1,3-dione) (**1**) was isolated and identified from the roots of *P. carniconnectivum* C. DC., Piperaceae, following the methods of Facundo et al. (2004). Roots of *Piper carniconnectivum* were collected in Porto Velho, Rondônia, Brazil. The botanical identification (exsiccate number 211.718) was confirmed by Instituto Nacional de Pesquisas da Amazônia (INPA), Manaus, Brazil.



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In vitro *leishmanicidal activity*

The leishmanicidal activity of the DCPC was evaluated by assessing the growth inhibition of promastigotes of *Leishmania amazonensis* PH8 reference strain (IFLA/BR/67/PH8). For these experiments, parasites ($5x10^5$/mL) in the early stationary phase of growth were incubated at 24 ºC for up to 96 h with concentrations of 100, 50, 25, 12.5 and 6 µg/mL of DCPC (dissolved in 0.3% ethanol). Parasite viability was determined daily by counting under an optical microscope with a magnification of 400x, using the dye erythrosine B (0.04% in phosphate buffered saline). The total number of living promastigotes in each sample was compared with the number of promastigotes in an untreated control. Pentamidine was used as reference drug. Each concentration was analyzed in duplicate and experiments were repeated three times.

In vitro *cytotoxicity*

To analyze the viability of the host cells after treatment with DCPC, we used the trypan blue exclusion test. Tests were performed on peritoneal macrophages obtained from BALB/c mice from the Instituto de Pesquisas em Patologias Tropicais-IPEPATRO. The study was approved by the Animal Use Ethics Committee of IPEPATRO (registration No. 006/2008).

The BALB/c mice were injected intraperitoneally with 2 mL of a sterile thioglycollate solution (3%), following the method of Gordon et al. (1974). After four days, the animals were euthanized, and 5 mL of cold RPMI-1640 medium was injected into the peritoneal cavity; after a slight massage the medium was subsequently recovered. The cells obtained were washed and counted in a Neubauer hemocytometer. The peritoneal macrophages ($1x10^5$/mL) were treated with 100, 50, 25, 12.5 and 6 µg/mL of DCPC and maintained for up to 96 h at 37 ºC and 5% CO_2. As a control, macrophages were incubated without DCPC in RPMI-1640 and 0.3% ethanol.

The amount of cytotoxicity was evaluated after 24, 48 and 96 h of DCPC treatment. Cytotoxicity was assessed by adding 10 µL of a trypan blue solution (0.1%) to the cells, and the number of live and dead cells was counted from a sample of 100 cells under an optical microscope with 400x magnification. Cells stained blue were considered dead and live cells were considered to be birefringent.

Statistical analyses

Analysis of variance (ANOVA) followed by the NewMan-Keuls test was used to analyze the results ($\alpha \leq 0.05$).

Results and discussion

The cyclopentenediones belong to a relatively recently recognized class of substances that are composed of several compounds with a limited occurrence in nature (Li et al., 2006; Facundo & Braz-Filho, 2004). The activity and rarity of these compounds have generated great interest in understanding their biological activity and in the development of synthetic analogs. In this study, the leishmanicidal activity of DCPC, a cyclopentenedione derivative isolated from the roots of *Piper carniconnectivum* C. DC., Piperaceae, was evaluated.

The fungicidal activity of Coruscanone A, which is derived from the cyclopentenedione 2-methoxymethylenecyclopent-4-ene-1,3-dione, has been evaluated against *Candida albicans* and *Cryptococcus neoformans*. The activity of coruscanone A, which is isolated from the Peruvian pepper plant *Piper coruscan*, was comparable to or greater than that of amphotericin B. Coruscanone A also has a potent effect on fungal strains that are resistant to fluconazol, and it is therefore considered an alternative for the treatment of high risk immunocompromised patients and may also be of use as template for a new class of synthetic antifungal agents (Li et al., 2006; Babu et al., 2006). However, there have been no studies demonstrating an activity of cyclopentenediones against protozoa. Thus, the present study is the first to present the results of biological analysis of the activity of cyclopentenedione derivatives against *Leishmania amazonensis*.

In Brasil, *Leishmania* (*leishmania*) *amazonensis* is distributed mainly in the Amazon region, in areas of primary and secondary forests (States of Amazonas, Pará, Rondonia and southwest of Maranhão) (Coelho et al., 2011). It is one of the causative agents of localized cutaneous leishmaniasis and the only species responsible for diffuse cutaneous leishmaniasis (DCL). This species can also cause mucocutaneous or visceral leishmaniasis (Barral et al., 1991; Abreu-silva et al., 2003; Tolezano et al., 2007). The diffuse cutaneous leishmaniasis (DCL) is a rare form of cutaneous leishmaniasis that typically begins as localized and not ulcerated papules or nodules. In the DCL, amastigotes disseminate in the skin and produce nodules in several parts of the body such as the face and extremities (Morrison et al., 2009; Silveira et al., 2004; Barral et al., 1991). Although rare, DCL is an incapacitating disease and there is no satisfactory response to treatment (Zerpa et al., 2007). Since the treatment of most clinical forms of leishmaniasis is usually unsatisfactory and the drugs used are highly toxic, it is necessary to find novel compounds with potential leishmanicidal activity.

Facundo & Braz-Filho (2004) suggested that DCPC was derived as a by-product of mevalonic acid and the shikimic pathway. This result was subsequently confirmed by Dias et al. (2005), who studied the potential relevance of synthetic cyclopentenedione derivatives to biological science, using the molecule 2-[1-hydroxy-3phenyl-(*Z*,2*E*)-2-propenylidene]-4-methyl-4-cyclopentene-1,3-dione isolated from *Piper carniconnectivum* as a model.

Piper carniconnectivum, which is commonly known as "long pepper", is native to Amazon in the northern Brazilian (Yuncker, 1972). A number of compounds have been isolated from this plant including a flavonol ("galangin"), a phenylpropanoid (2-metoxi-4,5-metilenodioxipropiofenona), a coumarin known as xanthyletin, three cyclopentenediones and the following four flavonoids: 5-hydroxy-7-methoxy-6-methylflavanone, 5-hydroxy-7-methoxy-8-methylflavanone, 5-hydroxy-7-methoxy-6,8-dimethylflavanone and 2'-hydroxy-4',6'-dymethoxy-3',5'-dimethylchalcone (Facundo et al., 2004; Facundo & Braz-Filho, 2004; Facundo et al., 2006).

We assessed the *in vitro* leishmanicidal activity of DCPC against *Leishmania amazonensis*. The DCPC showed a leishmanicidal activity against the promastigotes of *L. amazonensis*, completely inhibiting the replication of the parasites at a concentration of 100 µg/mL. A concentration of 50 µg/mL inhibited 99.7%, while at concentrations of 25, 12.5 and 6 µg/mL the levels of inhibition were 98%, 71.7% and 64.4%, respectively (Figure 1). The inhibitory concentrations were calculated to be 4.4 µg/mL (IC50) and 18 µg/mL (IC90). The reference drug Pentamidine completely inhibited the growth of the promastigotes in the first 24 h at a concentration of 100 µg/mL, whereas 0.3% ethanol had no effect on parasite growth.

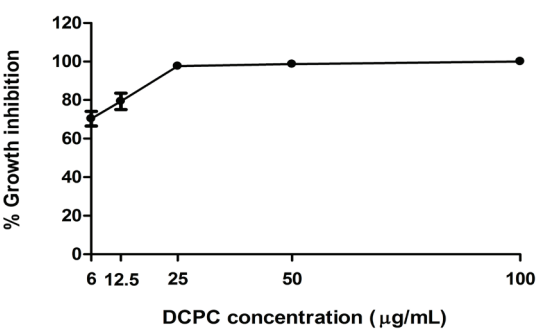

Figure 1. The effect of DCPC on the growth of *Leishmania amazonensis* promastigotes cultured for 96 h at concentrations of 6, 12.5, 25, 50 and 100 µg/mL. Assays were performed in duplicate and represent the average of three independent experiments.

The pentamidine is one of drugs for treatment of leishmaniasis and it is used in cutaneous, diffuse-cutaneous and mucocutaneous leishmaniasis and in visceral leishmaniasis cases resistant to antimonials (Soto-Mancipe et al., 1993). In Brasil, it is the second treatment option for leishmaniasis, but is the first-line

drug in other countries (Nacher et al., 2001; Lai A Fat et al., 2002; Van der Meide et al., 2009). Like pentavalent antimonials, pentamidine is also highly toxic and has many side effects such as nausea, vomiting, dizziness, headache, hypotension, transient hyperglycemia and hypoglycemia (Neves et al., 2011).

Because of the possibility that active compounds that have therapeutic potential against *L. amazonensis* may be toxic to the host cells (Nakamura et al., 2006), we examined the effect of the DCPC on the viability of peritoneal macrophages from BALB/c mice at the same concentrations of DCPC that were used in the promastigote activity assays. The viability of the macrophages at concentrations of 50, 25, 12.5 and 6 µg/mL was above 80% (Figure 2). The 50% cytotoxic concentration (CC50) in macrophages treated with DCPC was 129 µg/mL.

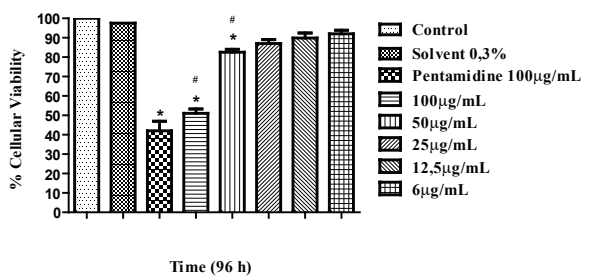

Figure 2. The effect of DCPC on the viability of peritoneal macrophages *in vitro* after 96 h of treatment. The percentage of viable macrophages was evaluated by trypan blue exclusion. Values represent three independent experiments performed in duplicate. Statistically significant differences (p<0.05) relative to the untreated controls are indicated by (*), and statistically significant differences relative to the solvent are indicated by the symbol (#).

The cytotoxicity was evaluated using a selectivity index (SI), which is the ratio of the host cell CC50 to the promastigote IC50. According to the SI, DCPC is 29.3 times less toxic to macrophages than to *L. amazonensis* promastigotes (Table 1). A substance is considered to have promising therapeutic (*e.g.*, leishmanicidal) activity when the resulting SI is greater than 10; however, our results do not eliminate the possibility of adverse side effects *in vivo* (Cardona, 2006).

The leishmaniasis treatment might be affected by the sensitivity of the causative *Leishmania* species, gender and age of patients, body localization of lesions, immunological status of the host, treatment schedule and genetic background of parasites (Croft et al., 2002; Decuypere et al., 2012). The conventional treatment is only partially effective against diseases caused by *L. braziliensis* and *L. amazonensis* (Croft et al., 2002; Silveira et al., 2004). Moreover, resistance to pentavalent antimonials has been identified for *L. (L.) donovani* in India and Nepal. In India, 40-60% of VL patients did not respond to pentavalent antimonial treatment and 25% were unresponsiveness to pentamidine (Mishra et al., 1992; Jha, 2006). Up to now, the evidence that treatment failure in Brazil is due to parasite primary resistance was not found (Zauli-Nascimento et al., 2010; Monte-Neto et al., 2011).

Table 1. Selectivity index (SI) determined from the CC50 values in murine (BALB/c) peritoneal macrophages and from the IC50 in *Leishmania amazonensis* promastigotes.

	Macrophage CC50	Promastigotes IC50	SI
DCPC	129	4.4	29.3

CC50: Cytotoxic concentration for 50% of macrophages; IC50: Concentration at which the number of promastigotes is halved; SI: Selectivity index (CC50 (peritoneal macrophages)/IC50 promastigotes).

With the limited supply of drugs used to treat leishmaniasis, the demand for new antileishmanial drugs has intensified. With the increased resistance to pentavalent antimonials, and the second generation drugs (Croft et al., 2006; Decuypere et al., 2012), DCPC may be a tool for the synthesis of new leishmanicidal drugs. The potential of DCPC for treating human leishmaniasis needs further evaluation, to investigate its action on different Leishmania strains, on intracellular amastigotes and/or in animal models.

In conclusion, the cyclopentenedione derivative DCPC, isolated from the roots of *Piper carniconnectivum,* inhibited the growth of *L. amazonensis* promastigotes with an IC50 of 4.4 µg/mL without affecting macrophage viability at concentrations up to 50 µg/mL. These results indicate, for the first time, that DCPC is a promising molecule for studies investigating the development of leishmanicidal drugs.

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