Susceptibility of *Trypanosoma evansi* in the Copaiba Oil: *In Vitro* Test and in Mice Experimentally Infected with the Parasite

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**ABSTRACT**

**Background:** *Trypanosoma evansi* is a flagellate which belongs to the salivate section, commonly observed parasitizing blood of equines, ruminants, pigs, dogs and wild animals in different regions of the world. It causes many losses to farmers due to death of animals and drug spending in endemic areas. The treatment of this disease in Brazil is only performed with diminazene aceturate; however it has been ineffective for many animals. During the last years many studies have been carried out with natural products such as the essential oils. Copaiba oil stands out due some properties described as anti-inflammatory, healing, antiedematogenic, antitumor, parasitic and antibacterial. Therefore, this study aimed to test, *in vitro* and *in vivo*, the susceptibility of *T. evansi* to copaiba oil.

**Materials, Methods & Results:** The oils used in this study were obtained from *Copaifera reticulata* and *Copaifera duckei* trees, commonly found in the Tapajos National Forest. The procedure received authorization of IBAMA due the scientific purposes. This study identified three oils identified as copaiba 4-C (*C. reticulata*), copaiba 5-C (*C. duckei*) and copaiba 8-C (*C. reticulata*). The bioassay was performed in *vitro* using specific culture medium for *T. evansi*, previously described by Baltz, 1985. Copaiba oil was dissolved in dimethyl sulfoxide (DMSO) and tested in three concentrations (0.5, 1.0 and 2.0%) in culture medium containing the parasite. To the control test (without oil) the same volume of DMSO (10 µL) was added. Dimnazene aceturate was also used as a positive control at 0.5% of concentration. The counting of trypanosomes was performed in triplicate in a Neubauer chamber after 1, 3, and 6 hours after the experiment onset. For the tests *in vivo*, mice were infected (*n* = 40) and divided in 5 groups of 8 animals each. Group A consisted of healthy animals and Group B comprised animals infected with *T. evansi* and untreated. The other groups were infected and treated orally with oil of copaiba 4-C (Group C), copaiba 5-C (Group D) and copaiba 8-C (Group E), using a dose of 0.63 mL/kg/day for five consecutive days at intervals of 24 hours. *In vitro* tests with copaiba oil showed a reduction in the number of alive trypanosomes for the three tested concentrations, when compared to the control test after 1 and 3 h, similar to what occurred with testing aceturate. At 6 hours, it was not observed alive parasites in the test groups, differently from the control group which had an increase of trypanosomes compared to the time zero. The trypanocidal activity had a dose-dependent effect. In the *in vivo* experiment, the oil of copaiba administered orally had no curative efficacy for any of the groups; however group D treated with *C. duckei* showed prolonged longevity of the mice when compared to the groups B and C.

**Discussion:** *Trypanosoma cruzi* and *Leishmania amazonensis* have been challenged on their susceptibility to oil of copaiba, with obtention of trypanocidal and leishmanicidal effects, similar process that occurred in this study with *T. evansi*. According to scientific literature the copaiba oil increases the membrane permeability, as well as provides the depolarization of the mitochondrial membrane in parasite cells. A group of mice in this study showed prolonged longevity, showing that the variation of their compositions influence the trypanocidal effect. Based on these results it was concluded that the *T. evansi* may is susceptible to the oil of copaiba. Therefore, it can be natural product used as a new alternative and supplementar activity in the treatment of this protozoan, as have been suggested for leishmaniasis.

**Keywords:** trypanosomosis, *Copaifera*, treatment, trypanocidal activity.
INTRODUCTION

Trypanosoma evansi is a flagellate salivate section that is commonly observed parasitizing the blood of domestic and wild animals, and transmitted mainly by blood-sucking insects [16]. In Brazil, T. evansi affects mainly horses and the prevalence of infection is altered by region [7,8]. It was believed that humans were immune to infection by the parasite [11], but in 2005 was made the first record of this disease in people [10]. In the Pantanal the disease is endemic and causes great losses to farmers due to the death of animals and spending on medication to control the disease curative or a prophylactic trial [16]. The treatment of this disease is made based diminazene aceturate in Brazil, but this drug has been shown to be ineffective for many animals [6].

The copaibeira, common name of the tree of the genus Copaifera, is a very common plant in the Amazon region, subject of many studies, for its medicinal properties are varied, providing many studies, the different species [17]. Among the medicinal properties of copaiba oil have been highlighted as anti-inflammatory activity, healing, antiedematogenic, antitumor, parasitic and bacterial [9,12,15,17,18]. Recent studies have described the action trypanocidal and antileishmanial of different oils of copaiba [9,13,15].

Cases and outbreaks of infection with T. evansi have increased worldwide, as well as cases of ineffectiveness of trypanocidal drugs. Thus, alternative methods such as with plant extracts and essential oils would be a good therapeutic option. Therefore, this study aims to evaluate the susceptibility of Trypanosoma evansi copaiba oil, through of tests in vitro and in vivo.

MATERIALS AND METHODS

Acquisition of copaiba oils

Copaiba oil used for this experiment was obtained from Copaifera reticulata and Copaifera duckei trees, extracted from the Tapajos National Forest, Belterra town, Pará state, in a Federal Conservation Unit. The oil extraction had authorization and approval of IBAMA with scientific purposes (protocol number: 31028-1; issued on 09/09/2011). This study identified three oils; copaiba 4-C (C. reticulata), copaiba 5-C (C. duckei) and copaiba 8-C (C. reticulata). All these oils correspond to the rainy season of 2011.

In vitro tests against T. evansi

• Culture medium: The culture medium of T. evansi was adapted Baltz [2]. For preparation of this medium was used minimal essential medium (MEM) without glutamine (0.376 g), glutamin2 (0.016 g), sodium bicarbonate3 (0.088 g), glucose4 (0.04 g), sodium-free HEPES5 (0.238 g) solution of nonessential amino acids 100 x6 (200 µL), penicillin7 (1596 U/mL), streptomycin8 (100 µg/mL). The ingredients were dissolved and homogenized in 30 mL of water, the pH was adjusted 7.1 with NaOH. Then, was completed with water up to a volume of 42 mL of medium and checked osmolarity (0.30). Next, the culture medium was sterilized by filtration 0.22 µm and stored in the refrigerator. On the day of testing, the volume of 10 mL was separated in falcon tube, which was added 1 µL/mL of of hypoxanthine9, 50 mM (dissolved in NaOH, 0.1 M) and 2 µL/mL of 2-mercaptoethanol10, 1.2 mM. Then, the complete culture medium was equilibrated for two hours in the oven (37°C with 5% CO2).

• Acquisition of trypanosomes: The flagellates were obtained from the laboratorial infection of a rat with T. evansi isolate [4], which was maintained cryopreserved in liquid nitrogen. When the infected rat showed high parasitemia (1.2 x 107 trypanosomes/µL), it was anesthetized with isoflurane and blood collected by cardiac puncture, then stored in EDTA tubes. For separation of trypanosomes, blood (200 µL) was diluted in complete culture medium (200 µL), stored in epperndorf and centrifuged at 400 x g for 10 min. In sequence, the supernatant was removed with the parasites, without cells from blood, allocated in the culture medium and the counting in a Neubauer chamber.

• Bioassays: For the tests in vitro, the culture medium with the parasites was distributed in microplates (270 µL/pool). Then, was added 10 µL copaiba oil (dissolved in DMSO)11 at concentrations of 0.5, 1.0 and 2.0%. For validate this study was used a negative control (diluent added in the case DMSO) and one positive control (commercial product trypanocidal: aceturato of diminazene dilution in DMSO, 0.5%), both the volume of 10 µL. The tests were performed in triplicate. After 1, 3 and 6 h of the beginning of the experiment were performed counting of parasites living in a Neubauer chamber.
In vivo tests against T. evansi

This study used 40 female mice (25 g and two months old) were divided into five groups (A, B, C, D and E) with eight animals each. Group A consisted of healthy animals, not infected. Group B was used as positive control (infected and not treated). The groups C, D and E were infected with an isolate of *T. evansi* [4] by intraperitoneal route with a dose $1.3 \times 10^6$ per animal parasites. Then the animals were treated with three different oils copaiba (identified as copaiba 4-C (group C), copaiba 5-C (Group D) and copaiba 8-C (group E)) at a dose $0.63 \text{ mL/kg/day}$. The treatment duration of 5 days, where the oil diluted in DMSO was given orally by gavage. Treatment began on some day of infection, with a difference of 3 h between procedures. Groups A and B received the same volume of DMSO. The dose of copaiba oil was chosen based on the results of toxic effects of other investigators [1,3].

The experiment lasted 20 days, and this period evaluated daily parasitemia by blood smear, according to the technique described by Da Silva and cols. [5]. Data were collected from prepante period, longevity, parasitemia and mortality of the animals, to determine the effect of treatment with copaiba oil in mice infected with *T. evansi*.

Statistical analysis

The results were submitted to analysis of variance (ANOVA) and Duncan test to verify the accuracy of the data. Statistically different results with $P < 0.05$ were considered.

RESULTS

In vitro tests

Trypanocidal activity dose-dependent the three copaiba oils on trypomastigotes of *T. evansi* were observed (Figure 1). After 1 and 3 h of experiment was found to reduce the number of parasites living in bioassays with different concentrations of oil, similar to what occurred in the bioassay aceturato of diminazine. The same did not occur in the negative control, where verified is even a small increase of parasites. No parasites found in live three concentrations of oil after 6 h. Apparently, oils identified as copaiba 4-C and copaiba 8-C (*C. reticulata*) killed parasites more rapidly than copaiba 5-C (*C. duckei*).

In vivo tests

The results of in vivo tests are shown in Table 1. Twenty-four hours after infection and treatment with oils has been observed in the smear parasite blood of all mice infected. Treatment with the three different oils had no curative effect, because all animals died during the experiment, presented high parasitemia (more than 100 parasites/field at 1000 x). The treatment given to group D (*C. duckei*) increased the longevity of mice when compared to groups B and C.
In *in vitro*, *T. evansi* was susceptible to treatment with copaiba oil, similar to diminazene aceturate that one drug trypanocidal. During counts trypomastigotes, an increase in the permeability of the plasma membrane of the parasite, and this occurred because an increase in volume, followed by lysis of the membrane and death of the protozoan. Researchers report that in vitro, copaiba oil treatment of *Leishmania amazonensis* led to an increase in plasma membrane permeability, and depolarization in the mitochondrial membrane potential in parasite cells [14]. Another study by the same research group reported that epimastigote, trypomastigote and amastigote of *Trypanosoma cruzi* is susceptible to treatment with copaiba oil from different species of copaibea, as well as *C. reticulata*, *C. duckei*, *C. paupera*, *C. martii*, *C. multijuga*, *C. officinalis*, *C. lucens* and *C. cearensis* [9]. According to the study, treatment with copaiba oil cause lipid peroxidation in *T. cruzi*, and thus alters the permeability of the cell membrane and mitochondrial [9].

Based on the results in vitro, an experiment was designed *in vivo*, using mice experimentally infected with *T. evansi* as experimental model. However, the therapeutic protocol used with the copaiba oil had no effective curative for all the groups, but in a group treated (group D - *C. duckei*), an increase in longevity of animals was observed. The oils tested had no quantification of the constituents, which may vary from oil and so have caused this difference in longevity between groups. Research studying the effects of the constituents in the oil copal, as copalic acid, copalic-hydroxy acid, b-caryophyllene, agathic acid, kaurenioic acid, pinifolic acid, acid polyaltic and methyl copalate on *T. cruzi* observed tripanocidal action for all these components [9]. In the case of infection by *T. evansi* in mice, which is usually acute with mortality of rodents between 4-7 days post-infection, and treatment with copaiba oil might be acting as an inhibitor of the immune response, property is described [18]. Then the anti-inflammatory response to treatment with copaiba oil could hamper the control of parasitemia, and thus the action trypanocidal the oil would be insufficient to kill all the trypanosomes in the blood and tissues, and cure the mice. In a new study, seek to increase the doses, similar to what was done to *L. amazonensis*. For according to researchers, mice that received oral treatment with copaiba oil have significant reduction in the average lesion size the paw (site of infection) compared with untreated mice [14].

Based on the results it was concluded that copaiba oil has tripanocidal action against *T. evansi* to cause morphological and ultrastructural changes in parasites, because the permeability of the plasma membrane. Nevertheless, the treatment had no curative efficacy in mice infected with *T. evansi*, however increased longevity in one of the groups. Against *T. evansi*, *in vivo* the oil of the species *C. duckei* had better results compared to *C. reticulata*. Researchers have reported that sazanolidade can influence the constituents of the oil and thus its action trypanocidal [9], however the observed difference in longevity does not apply in this study, because the three oils were collected in the rainy season of 2011. Maybe we could have a greater therapeutic success, if the experimental model used to develop chronic infection, as has been studied in leishmaniasis. This is the first study involving treatment with oil copaiba for *T. evansi*, new projects will be undertaken to try to associate this natural product the chemotherapeutic and thus increase the efficacy of therapeutic protocol.
SOURCES AND MANUFACTURERS
1MEM - Sigma-Aldrich, St. Louis, MO, USA.
2Glutamin - Sigma-Aldrich, St. Louis, MO, USA.
3Sodium bicarbonate - Sigma-Aldrich, St. Louis, MO, USA.
4Glucose - Sigma-Aldrich, St. Louis, MO, USA.
5Sodium-free HEPES - Sigma-Aldrich, St. Louis, MO, USA.
6Solution of nonessential amino acids - Sigma-Aldrich, St. Louis, MO, USA.
7Penicillin - Ariston, São Paulo, SP, Brazil.
8Streptomycin - Ariston, São Paulo, SP, Brazil.
9Hypoxanthine - Sigma-Aldrich, St. Louis, MO, USA.
102-mercaptetanol - Sigma-Aldrich, St. Louis, MO, USA.
11DMSO - Merck Brasil, Rio de Janeiro, RJ, Brazil.
12Ganazeg - Hertape Calier Saúde Animal, Juatuba, MG, Brazil.

REFERENCES

Ethical approval. The procedure was approved by the Animal Welfare Committee of Federal University of Santa Maria, number 65/2012.

Declaration of interest. The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

