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Research Article

Determination of the *in vivo* activity of leaves extract of *Zanthoxylum Chiloperone var. Angustifolium* (*Tembetary hú*) orally and intralesionally administered to BALB/c mice experimentally infected with *Leishmania*

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Abstract

Natural products are becoming increasingly important as an unlimited source for obtaining chemical substances with possible pharmacological potential. Current existing drugs for the treatment of cutaneous leishmaniosis produce major side effects; therefore the search for new drugs is justified. The stem bark of *Zanthoxylum chiloperone var. Angustifolium Engl. (Rutaceae)* is traditionally used in Paraguay for its antiparasitic properties. The leaf extract was evaluated for the first time to determine its leishmanicidal activity in BALB/c mice infected with amastigote forms of *Leishmania amazonensis* (PH8). The mice were treated orally with the extract at three concentrations (100, 50 and 10 mg/mL), intralesional (50 mg/mL), and subcutaneously using glucantime as a control (100 mg/mL). The percentage of decrease in parasite load was measured and with intralesional 50 mg/kg a reduction of 72% occurred, with the reference drug (Glucantime) a reduction of 62% was obtained with the same oral dose a reduction of 50%, while with an oral dose of 10 mg/mL the percentage of reduction was 55%. When the oral dose was increased to 100 mg/mL, the reduction percentage of the parasitic load was only 16%. These results indicated that the leaf extract of *Z. chiloperone var. angustifolium Engl.* at low oral concentrations (50 and 10 mg/mL) had very good activity against *L. amazonensis*, and it was even more efficacious intralesionally at 50 mg/mL but at the oral dose of 100 mg/kg has very reduced antiparasitic activity. This study showed the efficacy of the extract leaves of *Z. chiloperone* in reducing the parasite load in an *in vivo* test, so its use as a potential leishmanicidal could be suggested to develop and evaluate new drugs for the oral treatment of leishmaniosis disease with fewer side effects and lower cost.

More Information

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Introduction

Leishmaniasis is a disease caused by a protozoan of the genus *Leishmania*. It is classified within the group of flagellates and belongs to the *Trypanosomatidae* family. Its complex cycle of transmission includes different species of parasites, reservoirs, and vectors [1-4]. The *Leishmania* genus consists of 20 or more species divided into two subgenera: *Leishmania* and *Viannia* [1,2-6]. *Leishmania* infection is transmitted by the bite of infected sandflies and can affect humans and various animals. The insect that acts as a vector in the American continent belongs to the genus *Lutzomya* and in the Asian, European and African continents to the genus *Phlebotomus* [1,2,4-9].

It is estimated that 2 million new cases occur worldwide each year, 1.5 million cases of cutaneous (CL) or tegumentary (TL) leishmaniasis, and 500.000 cases of visceral leishmaniasis

(VL). It is currently considered one of the most important endemic diseases in the world [1,2,4-9] and has become an important public health problem in Latin America, due to its wide distribution and high prevalence.

It has three pathological forms: diffuse cutaneous (DC), mucocutaneous leishmaniasis (MCL) and CL. CL is the most frequent form and produces painless chronic skin lesions in exposed areas of the body, ranging from small nodules to large ulcers capable of persisting for months or years, leaving scars for life and causing severe disability, although finally, they heal [2-6,10-19].

In our country, the cases of CL have decreased in the last ten years; however, the cases of CML have increased by 24.5% due to inadequately treated CL. In 2014, approximately 130 cases of CL were registered in Paraguay, and now the prevalence of CML is similar to CL, but the occurrence is more dispersed covering the entire national territory [1]. MCL caused by *L. braziliensis* is characteristic of de Eastern Region, mainly in departments such as San Pedro, Caaguazú, Canindeyú, and Alto Paraná [1-3,9-22].

More and more emphasis is being placed on the importance of early diagnosis and adequate treatment of affected people, particularly due to the fact that in recent decades the treatment used in public health has been characterized by the scarcity of therapeutic options with currently used drugs causing great toxicity, affecting the heart, liver, pancreas or nervous system, often leading to partial or total abandonment of the treatment [1-6,17-28].

The use of medicinal plants to treat different pathologies is a practice that has been carried out for a long time. Plants are known for their anthelmintic, antibacterial, and insecticidal activities, and a large number of them are also used for their antiparasitic activities. Some publications have shown the diversity of natural products with antiprotozoal activity, including active molecules against the etiological agents of malaria, leishmaniasis, Chagas disease, and African trypanosomiasis [7,16-30].

In Paraguay, the use of medicinal plants is a custom that has been transmitted from generation to generation until today [1]. Results from studies made using *in vivo* assays have been reported for CL using natural and synthetic products demonstrating an efficacy of 80% to 90% in reducing the parasitic load in the lesions [11-15,17-34]. In the leaf extracts of vegetables of the genus *Zanthoxylum (Rutaceae),* metabolites containing alkaloids are commonly found, among which those of the benzophenanthridine type stand out and are of great interest due to their varied biological activity. Many of them have powerful antitumor, antimalarial, anti-leukemic, antioxidant, anti-HIV, antibacterial, antimicrobial, and anti-fungal activities, among others [6-8,16-19]. Several biological tests have been carried out using extracts and molecules isolated from the bark of wild species of *Z. chiloperone var. Angustifolium Engl.* (popularly known as *Tembetary hú)* in which leishmanicidal, trypanocidal and anti-fungal activities have been demonstrated. The two active components isolated from this species were identified as can thin-6-one and 5-methoxycanthin-6-one. In the trypanocidal biological activity tests from leaf extracts, the alkaloid 5-methoxycanthin-6-one was identified as the main active molecule [15-22,26-34]. Other recent studies have shown that the crude extract of the stem bark and leaves of *Z. chiloperone* has activity against *Trypanosoma cruzi* at 50 mg/Kg weight dose and oral administration for two weeks with a significant parasitemia reduction [14-22]. Due to these promising preliminary results, this plant was chosen for this study.

Carrying out this experimental work is part of the search for new molecules with possible leishmanicidal biological activity, allowing the development and evaluation of new drugs for the treatment of this disease, with fewer adverse effects, less costly, more powerful, more selective, more efficient and used by oral administration in order to contribute to the well-being to the population affected by this disease. For this, the activity of the leaf extract of *Z. chiloperone* against leishmaniasis was evaluated in experimentally infected BALB/c mice using different administration routes.

Materials and methods

Chemicals

The aerial parts of the plant were ground in a blade mill which reduced the material to a fine powder. To obtain the extract, HPLC grade methanol (JT Baker, Center Valley, PA, USA) was used as the extraction solvent. The material was allowed to settle for 24 to 48 hours and subsequently subjected to vacuum filtration with qualitative filter paper. The extract filtrates were concentrated on a rotary vacuum evaporator (Heidolph, 94200, Bio block, Germany). The concentrated extract was then transferred to a frosted flask. It was left uncovered under a hood for 24 hours so the methanol evaporated completely.

Animals

BALB/c mice, raised in the Animal facility of the Health Sciences Research Institute of the Asuncion National University, were 6 to 8 weeks old and had approximately 18 g to 20 g of weight were used in the experimental protocols.

Parasites

For the infection, *L. amazonensis* (IFLA/BR/1967/PH8) was used. The parasites were maintained by passage every six to eight weeks in Golden hamsters (*Mesocricetus auratus*).

In vivo studies

BALB/c mice (n = 36) were inoculated in the right hind footpad with 1.7 x 10^6 amastigotes obtained from donor hamsters. The parasites were delivered in 100 µL of phosphate-buffered saline (PBS). The disease progression was monitored

by the measurement of lesion diameters weekly for up to 7 to 12 weeks [14-19].

Treatment

It was initiated two weeks after parasite inoculation when the infection was well established and lesions were obvious. The BALB/c mice were randomly divided into six groups of six mice each, 975 µL of N-Methylglucamine antimonite (glucantime) were diluted in 15 mL of PBS and administered to one group of mice using a dose of 100 mg/Kg of body weight daily for 15 days by subcutaneous route. The selected compound was administered orally at 10, 50 and 100 mg/Kg of body weight and intralesionally at 50 m/Kg of body weight to four different groups, and the control group only received Phosphate Buffered Saline (PBS). The protocol lasted 5 weeks from the first infection to mice sacrifice; they received 15 days of treatment with the exception of the intralesional route group whose treatment lasted only 7 days.

The animals were sacrificed two weeks after finishing treatment to assess parasitological loads in the infected footpad. Briefly, the lesions of the infected footpad were excised, weighed, and homogenized in a tissue glass grinder and then homogenized in 1 mL of Buffer Saline, and then counted in a Neubauer chamber using a microscope (Olympus) at a magnification of 40x. The number of parasites per gram in the lesion was calculated by the following equation:

Parasite burden = geometric mean of the number of parasites in each duplicate/(number of microscope field counted x weight of lesion x (25000) hemocytometer correction factor) [15-19].

The formulas used to find the concentration and percentages of the parasites [15-19].

– The concentration of Parasites in the Neubauer Chamber

$$\text{Parasites} = \frac{\text{Nº de parasites counted x } 10.000}{\text{Nº of one quadrant}} = \text{Nº parasites / mL}$$

- Percentages of Parasites Suppression;

Average Parasite load of the PBS Control Group _____ 100%

Average Parasite load of the study group _____ x

x=…….. %

Suppression % = 100 - % study group =…. %

Statistical analysis

The mean and standard deviation were calculated by using Microsoft EXCEL software. Comparisons of parasite suppression in the infected footpads of the untreated and drug-treated groups were done by the analysis of variance (ANOVA). Data were considered statistically significant at $p < 0.05$.

Ethical aspects

All procedures involving animals were carried out in accordance with the International Guiding Principles for Biomedical Research using animals, of the Council for International Organizations of Medical Sciences (CIOMS) and Ethical Committee of the IICS (Ref. No. M06/2014). The minimum possible number of animals was used and all necessary precautions were taken to avoid unnecessary suffering. The animals were kept in an environment with controlled temperature and adequate amounts of food and water [35].

Results

In vivo assays

The almost null effect produced by Phosphate Buffered Saline (PBS), the negative control, was demonstrated by the high weight values of the granulomas as well as the parasite counts compared with the effects of the reference drug group, where the weight of the granulomas was lower as well as the count and concentration of parasites (Table 1).

Table 2 shows the percentage of parasite suppression of each extract dose, of glucantime and PBS control group with no parasites suppression. To calculate the percentage, the values of the parasitic load of PBS groups were taken as 100% to find the percentage of the parasitic load of each treated group, then subtracted from 100% to obtain the percentage of suppression.

The values were averaged and then compared with the reference drug. A statistically significant difference was obtained when comparing the concentration of parasites in

Table 1: Granuloma weights and amastigotes counts of the mice. Control groups treated with PBS and Glucantime, respectively.

Sample	Granuloma weight (g) PBS Glucantime	Counting (parasites/16 quad.) PBS Glucantime	Concentration (parasites/mL) PBS Glucantime
R1	0.3486 - 0.0877	3097 - 774	31×10^6 - 8×10^6
R2	0.3677 - 0.2480	3297 - 1836	33×10^6 - 18×10^6
R3	0.4630 - 0.1469	3496 - 734	35×10^6 - 7×10^6
R4	0.2869 - 0.2830	2680 - 1226	27×10^6 - 12×10^6
R5	0.3781 - 0.0788	3034 - 427	30×10^6 - 4×10^6
R6	0.1670 - 0.4276	1078 - 2033	11×10^6 - 20×10^6
Average			28×10^6 - 12×10^6

R1: Mouse 1; R2: Mouse 2; R3: Mouse 3; R4: Mouse 4; R5: Mouse 5; R6: Mouse 6

Table 2: Parasites suppression percentages; in mice groups treated with different concentrations of the leaf extract of *Z. chiloperone* and control groups.

Groups	Product	Concentration	Route	% parasites suppression
Group 1	PBS		Orally	-
Group 2	*Glucantime*	100 mg/Kg	Subcutaneous	62%
Group 3	*Z. chiloperone*	50 mg/Kg	Intralesional	72%
Group 4	*Z. chiloperone*	100 mg/kg	Orally	16%
Group 5	*Z. chiloperone*	10 mg/kg	Orally	55%
Group 6	*Z. chiloperone*	50 mg/Kg	Orally	50%

Data collected from amastigotes counts, performed in a 40 x microscope

the groups treated with the extract under study vs. the control group treated with the reference drug. For the dose of 100 mg/kg orally, an average of 25.6 x 10^6 parasites/mL was obtained and for the oral dose of 50 mg/kg, 15 x 10^6 parasites/mL were found, while for the same intralesional dose an average of 8.7 x 10^6 parasites/mL was obtained. In the positive control (Glucantime) a value of 12 x 10^6 parasites/mL was obtained and in the negative control, 28 x 10^6 parasites/mL was found.

The final percentage of parasites suppression of group 3 with 50 mg/kg by intralesional route was 72%, of the positive control it was 62%, the dose of 100 mg/kg suppressed 16%, the oral dose of 50 mg/kg suppressed 50%, while the dose of 10 mg/kg suppressed 55% and the negative control did not suppress any parasites. All values were corroborated by the ANOVA test, which showed a statistically significant difference between all groups.

Discussion

In relation to the concentrations of the leaves extract of *Z. chiloperone var. Angustifolium Engl.*, it was observed that there was a percentage of parasite suppression up to the concentration of 50 mg/kg. As the concentration of the extract was increased, the percentage of parasite suppression decreased, being the highest concentration at 100 mg/kg. The concentrations of 10 and 50 mg/kg were the ones that presented the lowest weight of the granulomas and the lowest numbers of counted parasites and with a higher percentage of parasite suppression.

Regarding the routes used for the extract under study, which was oral and intralesional, it was observed that the intralesional route with a concentration of 50 mg/kg presented a significant decrease in both the weight of the lesions and the number of parasites compared to the group that received the same concentration but orally [36]. In relation to the percentage of parasite suppression compared to the other groups, this was the one that presented the highest percentage of parasite suppression [17,37,38]. It can be said that there was a probable direct relationship between the weight of the granuloma and the number of parasites, since the higher the weight, the more parasites were found [36-38]. In the mice that received treatment with a dose of 100 mg/Kg, very high concentrations were observed with a maximum of 37.2 x 10^6 parasites/mL Compared to the group that received a dose of 50 mg/kg, a decrease was observed to a minimum concentration of 1.7 x 10^6 parasites/mL In average the 50 mg/kg dose had 15 x 10^6 parasites/mL being very close to the group treated with glucantime, which had a concentration of 12 x 10^6 parasites/mL, while the group treated with the 100 mg/kg dose was away from the reference group with an average concentration of 26 x 10^6 parasites/mL, been closer to the negative control group with a concentration of 28 x 10^6 parasites/mL.

Regarding the antiparasitic activity, it was also possible to see that the number of parasites varied in each mouse in the different groups. This is due to the physiology of the animal, which is not the same in all of them and each mouse reacts differently [36-38]. However, when averaging the counting of parasites, it was possible to clearly observe the concentration that suppressed the most parasites as well as the most effective route of administration.

This data showed that there was no dose-dependent relationship since increasing the dose did not show a greater decrease in the concentration of parasites. This is corroborated by the percentage of parasite suppression since the group with the 100 mg/kg dose showed suppression of only 16% higher than the glucantime group, which had suppression of 62%. In contrast, it was seen that the groups that received the doses of 50 mg/kg and 10 mg/kg suppressed 50% and 55% of the parasites respectively. Regarding routes of administration, the intralesional route showed suppression of 72%, even higher than that of the reference group. These data are related to previous publications, which observed that the root and bark extract of *Z. chiloperone var. Angustifolium Engl* exhibited antiparasitic activity *in vivo* assays against *L. amazonensis* and *T. cruzi* parasites, and in this work, we demonstrate for the first time that the leaf extract also exhibits activity against *L. amazonensis* parasites at a certain concentration and that it even more effective intralesionally [11-13].

Another point to take into account is that the administration of the reference drug is subcutaneous, which is a more direct route of administration than the oral route since the components go more easily into circulation and in the oral route the products in some cases can undergo significant first-pass metabolism before entering the circulation and the extracts were administered orally except for one group that was administered intralesionally (36-38). Taking into account these factors and the complications presented by the oral route, the percentage of suppression of some concentrations tested was very close to that of Glucantime, demonstrating the great activity of the leaf extract of *Z. chiloperone var. Angustifolium Engl.*

Conclusion

The antiparasitic activity of the metabolic extract of *Z. chiloperone var. Angustifolium Engl* was infected in mice with *L. amazonensis* and the results obtained showed that some concentrations used were very effective in eliminating the parasite. Additionally, it was found that a high concentration of 100 mg/kg of that extract had very little or almost no antiparasitic activity. After comparing the results obtained from the extract of *Z. chiloperone var. Angustifolium* at concentrations of 50 y 10 mg/Kg orally with the reference drug glucantime, very similar suppressions of parasites were obtained. When comparing routes of administration between groups and with the reference drug a better result was obtained

in the group treated intralesionally with the concentration of 50 mg/kg, even showing a greater suppression of parasites than the one treated with glucantime. These results indicate that components with antiparasitic activity are also found in the leaves extract.

Limitations and recommendations

The results showed that the extract of *Z. chiloperone var. Angustifolium Engl* has components that have antiprotozoal activity comparable to the reference drug. The extract should be fractionated by liquid-liquid partitioning or other procedures in order to group components of similar polarity. Subsequently, test the fractions and separate the most active ones by chromatographic procedures until obtaining more purified compounds and then evaluate again the activity of these compounds.

The main future research proposal focuses on giving continuity to the study, given the multiple advantages that could be obtained by considering expanding the concentrations to be tested as long as we can count on the necessary quantities of experimental animals. More studies are required to determine the effectiveness of the extract at lower doses than those tested in this study to later adjust to the most appropriate one that has a truly effective antiparasitic activity preferably orally.

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