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Research Article

Pharmacological Effect of *Krameria cytisoides* on *Trypanosoma cruzi in vitro* and *in vivo Krameria cytisoides* on *Trypanosoma cruzi*

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Abstract

Objective: The know the pharmacological effect of *Krameria cytisoides* extract *in vitro* and *in vivo*, as well as the cytotoxicity of this compound, determined.

Material and Methods: For *in vitro* determination, concentrations of 40, 80 and 160 mg/kg of *K. cytisoides* were used. Cytotoxicity was assessed with VERO cells using concentrations between 10 and 160 µg/L. The pharmacological effect *in vivo* was investigated with five groups males per groups 3 of Balb/c mice. All mice were inoculated with $1x10^3$/ µL trypomastigotes. Three groups were injected with 40, 80 or 160 mg/mL. The fourth group was treated with 100 mg/kg of the benznidazole reference drug, and no treatment was given to the fifth group.

Results: The results demonstrated that the LD_{50} was 69 µg/mL, with an LC_{50} cytotoxicity of 160 µg/mL; thus, no toxic effect was observed. Regarding the *in vivo* results, 61% and 74% flagellates death was observed at the highest concentration of K. cytisoides, and the reference drug benznidazole, respectively.

Discussion and Conclusions: According to the results, the low toxicity and lethality of *K. cytisoides* compared to benznidazole suggests that the former could be used as a drug of choice for Chagas disease.

Keywords: *Krameria Cytisoides; T. Cruzi*; Epimastigotes; Trypomastigotes

Introduction

Trypanosoma cruzi infection is a public health problem in Latin America that affects approximately 8 to 11 million people (Pan American Health Organization 2012) In quantitative estimates made by the PAHO and WHO, 29,500.000 people/year are exposed in endemic areas of Mexico. The calculated prevalence and incidence are 1,028 and 0.007, respectively (Pan American Health Organization) [1,2].

For Chagas disease treatment, two specific drugs against *T. cruzi* are administered during acute infection: nifurtimox and benznidazole. These compounds have important side effects, such as cutaneous type reactions, digestive intolerance, neurological reactions, polyneuritis, headache, dizziness, insomnia, asthenia, migratory arthritis, fever and renal failure. In addition, these two drugs have limitations due to ineffectiveness [3].

Therefore, it is crucial to discover new drugs that achieve a cure with fewer toxic effects, since the two drugs mentioned above are the only currently available compounds for children and adolescents in chronic phases of the disease [4]. Considering the limitations of these treatments against *T. cruzi*, the aim of this study was to evaluate the trypanocide activity of new products, such as *K. cytisoides*, which is of natural origin, and to propose it as a therapeutic alternative for both pediatric and adult patients. *Kra-*

meria cytisoides is a trifoliate, native and endemic plant of San Luis Potosí that can be found throughout the American continent. The foliar part has been used for anti-inflammation, stomach pain and wound healing [5,6]. In this work, we investigated the effect of methanolic extract of *Krameria cytisoides* directed against *T. cruzi* epimastigotes and trypomastigotes. We determined the LD_{50} *in vitro* investigated its pharmacological activity in Balb/c mice *in vivo*, and evaluated cytotoxicity in VERO cells to derive the LC_{50}.

Materials and Methods

Biological species

Epimastigotes

The *T. cruz*i NINOA isolate obtained from an infant with an acute infection from the state of Oaxaca, Mexico was cultured at 37°C in LIT medium [7]. The parasites were harvested in 50 mL Falcon tubes by centrifugation at 1500xg in a Sorvall Biofuge refrigerated centrifuge and washed with 1X PBS (10 mM sodium phosphate and 150 mM sodium chloride).

Trypomastigotes

The NINOA isolate from *T. cruzi* was routinely maintained in triatomines and by serial passages in Balb/c mice. Cardiac puncture was performed to obtain trypomastigotes from blood to infect male Balb/c mice at an intraperitoneal (ip) weight of $2x10^5$ parasites.

Extract from *Krameria cytisoides*

The methanolic extract was obtained as described in the literature [8]. For the *in vitro* study, 0.005g was weighed and dissolved in 1 mL dimethyl sulfoxide (DMSO) ATCCR-4X™ A 1: 5 dilution was made to yield a final concentration of 1000 µg/mL. In the *in vivo* study, the animals were weighed to calculate the required amount of *K. cytisoides* extract needed to reach the doses of 40, 80 and 160 mg/kg. Benznidazole was used as a reference drug using a pill containing 100 mg of the active ingredient, from which the amount needed to administer 100 mg/kg to the mice was calculated.

Biological tests

Calculation of the LC_{50} for $21x10^6$ parasites was performed in log phase to distribute them into 35 vials. Seven groups of five vials each were formed: negative control without *K. cytisoides* (Group 1), reference control for 100 µg/mL benznidazole (Group 2), and experimental groups treated with *K. cytisoides* at concentrations of 10, 20, 40, 80 and 160 µg/mL (Groups 3-7). The vials were incubated at 28°C for 12 hours, after which the parasites were stained with 0.4% trypan blue at a 1:2 ratio at 37°C for 30 minutes. Then 0.1% formalin was added and left at 37°C for 30 minutes. The parasites were counted in a Neubawer chamber and the average of the five determinations was obtained and plotted with a target of 100% viability. The lethality was calculated at 50% according to the obtained LC_{50} graph.

Pharmacological effect in vivo on Balb/c mice

Twelve male mice were divided into four groups of three Balb/c mice infected intraperitoneally with $2x10^5$ *T. cruzi* parasites. The infected mice were maintained in light-dark cycles of 12 x 12 hours with access to food and water when the mice were in the log phase of infection. The samples were weighed to determine the oral administration of *K. cytisoides* extract in the following groups: negative control (Group 1; 5% gum arabic), positive control (Group 2; benznidazole, 100 mg/kg), injection with *K. cytisoides* in two concentrations of 80 and 160 mg/kg of body weight (Groups 3 and 4, respectively). The infection was monitored at 0, 2, 4 and 6 hours, with 100% viability at time zero. The animals were bled by cardiac puncture, and the flagellates were extracted and counted to determine the average live and dead flagellates per mm^3. The viability and mortality of the parasites were plotted at different times of infection according to the graph to determine the LD_{50}.

With these results, the mean and standard deviation were determined. A statistically significant difference was observed between the two concentrations of *K. cytisoides*, using the $p < 0.05$ test and Stata program. The number of viable and dead parasites at different times of infection was plotted.

Cytotoxicity CL_{50}

A 96 well plate was prepared with 100 µL/well of VERO ATCCR CCL81 cells cultured in MEM medium (GIBCO-11965-092) supplemented with 10% ATCCR 30 - 2020 bovine serum. The plate was incubated at 37°C in a partial atmosphere of 5% CO_2. In Column 1, 100 µL of MEM medium was added to 10 µL dimethyl Sulfoxide (DMSO). In Column 2, ATCCR-4X TM was added to the next four lower wells with 100 µL of MEM medium plus 10 µL benznidazole at 100 µg/ml. In Columns 3 - 12, 100 µL of MEM medium was added, and the concentrations of 10, 20, 40, 80 and 160 µg/mL were quadrupled with *K. cytisoides* dissolved in DMSO. The plate was incubated at 37°C in a 5% CO_2 for 24 hours. Next, 50 µL/well MTT solution was added and incubated under the same conditions for 4 hours. Subsequently, 100 µL/well of SDS-HCl was added and incubated at room temperature overnight. The O.D. at 540 nm in an ELISA reader reported the absorbance data as cell viability and the average of all absorbances of each treatment was compared with the cellular control data plus DMSO referred to as 100% cell viability.

Results

Calculation of the LC_{50} in virus The results of *in vitro* viability for determination of the LD_{50} of *T. cruzi* when using different concentrations of *K. cytisoides* are presented in figures 1 and 2. the extract

of *K. cytisoides* had activity against *T. cruzi* NINOA in the epimastigote phase, with trypanocidal effects ranging from 40 µg/mL to 80 µg/mL.

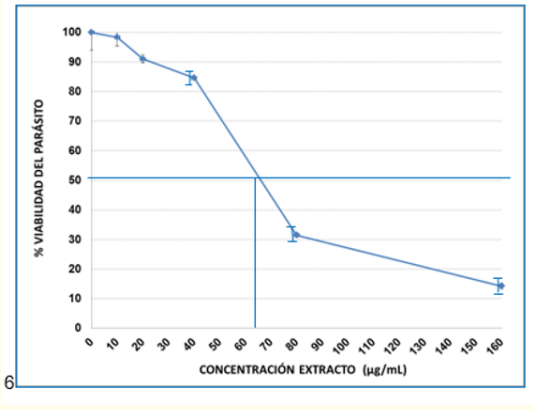

Figure 1: Effect of K. cytisoides on the Viability of T. cruzi isolated NINOA in the Epimastigote phase in vitro. Likewise, the results show the percentage of the parasite sample on a slope between 40 and 80 µg/mL of K. cytisoides, which is shown in Figure 2.

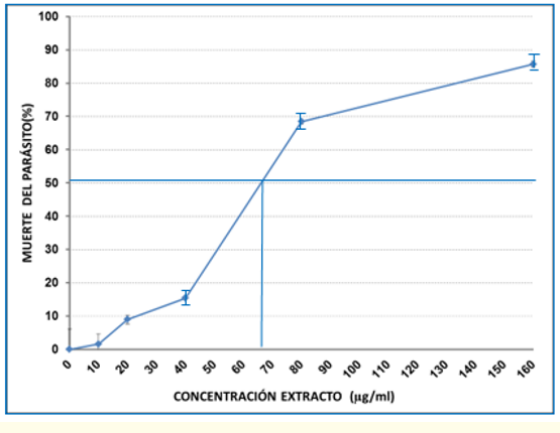

Figure 2: Trypanocidal effect of Kramecin on T. cruzi Isolated NINOA in the Epimastigote phase in vivo.

The in vivo effect of K. cytisoides extract is shown in Figure 3. The lytic effect of flagellates in mice at different treatment times at a concentration of 80 mg/kg, compared to benznidazole at 2 hrs, was shown to be statistically significant. However, when the concentration of K. cytisoides increased to 160mg/kg, the results after 2 hrs or 4 hrs of treatment did not present statistically significant differences with benznidazole, while the lethality was very similar between the two groups. Nonethless, the same concentration of 160 mg/kg showed significant differences with the 6 hr benznidazole treatment group.

Cytotoxicity

The determination of the cytotoxicity of *K. cytisoides* is shown in figure 3 and 4, where the effect of the extract on cell viability is displayed. As the concentration of *K. cytisoides* increased, the percentage of death decreased. We observed that the extract had no cytotoxic effects on the cells and could thus be used as treatment without affecting the infected individual. When comparing the results obtained between kramine and benznidazole, 10 µg/mL had the maximum toxic effect in cells and was more cytotoxic compared to benznidazole.

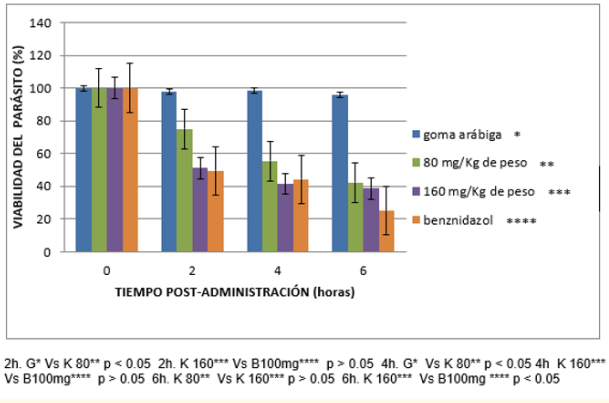

Figure 3: Effect of K. cystisoides on the Viability of T. cruzi in short time frames, in vivo. Within the groups of the trial, the concentration of 160 mg/kg had a percentage of lysis of the parasite that was significantly different to that obtained with benznidazole, as shown in Table 1. The percentages of death that each treatment presented are shown alongside the time duration of exposure.

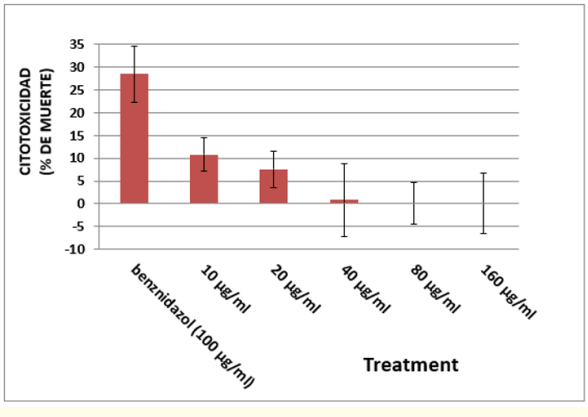

Figure 4: Determination of the Cytotoxicity of K. cytisoides on the VERO Cell Line at concentrations of 10, 20, 40, 80 and 160 µg/mL.

Discussion

Drugs available for the treatment of Chagas disease have adverse effects, such as skin reactions, digestive intolerance, and neurological reactions. Studies are currently being carried out on plants that act against *T. cruzi*, and a variety of genera and species have been reported. In this work, the trypanocidale effect of *K. cytisoides* on the protozoan *T. cruzi* was determined. The results showed that 61% of the parasites died at a *K. cytisoides* concentration of 160 mg/kg, while the lethality was 74% after 6 hrs of treatment with 100mg/kg body weight of benznidazole. This result indicates that, while the lytic effect on *T. cruzi* is important, the low values of cytotoxicity of kramecin render it safe and compliant with requirements for the application for treating American trypanosomiasis. Specifically, the safety of kramecin was determined by values of cytotoxicity in VERO cells and the effectiveness was demonstrated by the *in vitro* results on epimastigotes at 80 µg/mL. Kramecin caused 44% lysis of the parasites at 4 hrs of treatment and benznidazole caused 55% lysis in the same time duration.

As mentioned by Jimenez-Coello., *et al*. [9], in the last twenty years, there has been an increase in research reporting the effect of compounds derived from plants against *T. cruzi*, such as the work by Grael and colleagues in [10]. These reports support the possibility of using methanolic or ethanolic extracts to implement new treatment methods. In the specific case of Polanco-Hernández., *et al*. [11], extracts from 12 different Yucatan plants were tested, and two species had 100% lysing activity at 100 µg/mL against *T. cruzi* NINOA, while another plant had 50% lysis at the same concentration.

Overall, as the concentrations of these extracts decreased, the trypanocidal activity also decreased. A comparison of different reports revealed the trypanocidal action of the *K. cytisoides* extract. The *K. cytisoides* formula is described as a compound formed by a polymer of six monomers in cyclic formation, with each monomer consisting of a cyclic peroxide of five hydroxymethylated groups with ether groups [8]. The anti-inflammatory effect is postulated to be related to the inhibitory activity of phospholipase A2 and cyclo-oxygenase. Although the trypanocidal activities of these two enzymes and how they could participate in the lysis of the parasite remain unclear, *K. cytisoides* has a clear lytic effect on the parasite. This phenomenon was experimentally demonstrated in terms of percentages of lysis, as shown in table 1, where the percentages were 61% and 74% at 6 hours postadministration for *K. cytisoides* and benznidazole, respectively. Since there are no previous studies on *K. cytisoides* in a biological model against *Trypanosoma cruzi*, it is important to discuss the characterization of *K. cytisoides*. If we consider that the extract was obtained from a metal product, according to the work done by Pérez-Gutiérrez., *et al*. [8], it can be seen that the methanolic extracts are composed of lignans, neolignans and norneolignans, which could participate in the lytic activity of the compound. Therefore, a summation effect could exist, which has been seen in other investigations on plants acting against *Trypanosoma cruzi*. These plants display a great variety of genera and species that commonly belong to the classification of angiosperms plants according APG III [12-17] and another report from Sales-Alviano in 2012.

The example that was most similar to the methanolic extract of Kramein was in close order to that Zygophyllales. The order Malpighiales within the family Euphorbiaceae revealed that the methanolic extract of Croton cajucara is a good trypanocidal against epimastigotes and trypomastigotes of *T. cruzi*.

The proposal for improve ing the efficiency of treatments has led to research on the combined use of anti-*T. cruzi* drugs with synergistic effects. Synergy could help reduce the dose of each compound and thus decrease side effects.

Time post-treatment (hours)	**2**	**4**	**6**
Benzimidazol 100 mg/kg	50%	55%	74%
Kramecina 160 mg/kg	48%	58%	61%
Kramecina 80 mg/kg	24%	44%	57%

Table 1: Percentages of death of *T. cruzi* isolated NINOA obtained in the short-term *in vivo* test.

The concentration of *K. cytisoides* at 160 mg/kg was considered the most effective in the short-term *in vivo* test.

Conclusions

A) The pharmacological effect of kramecin tested in the *in vitro* assay was demonstrated to be efficient in reducing parasitemia from 80 µg/mL with 69% lysis. The LC_{50} was also reported. B) For the *in vivo* test, at concentrations of 160 mg/kg *K. cytisoides*, the lysis of the parasite was sufficient to exert antiparasitic effects. Therefore, at 160 mg/kg of extract weight, there is pharmacologi-

cal activity of 61% lethality versus 74% for short-term benznidazole in animal models. C) *Krameria cytisoides* extract is nontoxic between concentrations of 10 to 160 µg/mL in VERO cells with a CC50 greater than 160 µg/µL, thereby rendering it a potential compound for treating trypanosomiasis.

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