

**Antiparasitary potential and cytotoxic effect of *Spondias tuberosa* Arruda
(Anacardiaceae)**

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Received: 08/28/2020 | Reviewed: 09/07/2020 | Accept: 09/10/2020 | Published: 09/11/2020

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Abstract

The objective of this work was to evaluate the antiparasitic potential and cytotoxic effect of extracts by the leaves and roots of *S. tuberosa*. The results show that the extracts of *S. tuberosa* have low antipromastigote activity against strains of *L. braziliensis*, since there was no action at concentrations $\leq 500 \mu\text{g/mL}$. While for *L. infantum* there was a significant action of the hydroalcoholic extract of the roots against promastigote forms, since there was $29.33 \pm 1.94\%$ of mortality for the treatment of $1000 \mu\text{g/mL}$. The same extract showed antiepipromastigote action against *T. cruzi* at a concentration $\geq 1000 \mu\text{g/mL}$. Despite the low

antiparasitic activities, it is possible to observe that the extracts of *S. tuberosa* have no cytotoxic action, except the hydroalcoholic extract of the roots. In this extract there was a total of $27.85 \pm 2.41\%$ of cytotoxicity against fibroblastic cells, in the highest concentration evaluated (1000 $\mu\text{g/mL}$).

Keywords: Umbu; Antiprotozoan; Cytotoxicity; Kinetoplastide.

Resumo

O objetivo deste trabalho foi avaliar o potencial antiparasitário e o efeito citotóxico de extratos de folhas e raízes de *S. tuberosa*. Os resultados mostram que os extratos de *S. tuberosa* apresentam baixa atividade antipromastigota contra cepas de *L. braziliensis*, uma vez que não houve ação em concentrações $\leq 500 \mu\text{g/mL}$. Já para *L. infantum* houve uma ação significativa do extrato hidroalcoólico das raízes contra as formas promastigotas, já que houve $29,33 \pm 1,94\%$ de mortalidade para o tratamento de 1000 $\mu\text{g/mL}$. O mesmo extrato apresentou ação antiepimastigota contra *T. cruzi* na concentração $\geq 1000 \mu\text{g/mL}$. Apesar da baixa atividade antiparasitária, é possível observar que os extratos de *S. tuberosa* não apresentam ação citotóxica, exceto o extrato hidroalcoólico das raízes. Neste extrato houve um total de $27,85 \pm 2,41\%$ de citotoxicidade contra células fibroblásticas, na maior concentração avaliada (1000 $\mu\text{g/mL}$).

Palavras-chave: Umbu; Antiprotozoário; Citotoxicidade; kinetoplastida.

Resumen

El objetivo de este trabajo fue evaluar el potencial antiparasitario y efecto citotóxico de extractos de hojas y raíces de *S. tuberosa*. Los resultados muestran que los extractos de *S. tuberosa* tienen baja actividad antipromastigota contra cepas de *L. braziliensis*, ya que no hubo acción a concentraciones $\leq 500 \mu\text{g/mL}$. Mientras que para *L. infantum* hubo una acción significativa del extracto hidroalcohólico de las raíces contra las formas de promastigote, ya que hubo $29.33 \pm 1.94\%$ de mortalidad para el tratamiento de 1000 $\mu\text{g} / \text{mL}$. El mismo extracto mostró acción antiepimastigota contra *T. cruzi* a una concentración $\geq 1000 \mu\text{g/mL}$. A pesar de las bajas actividades antiparasitarias, es posible observar que los extractos de *S. tuberosa* no tienen acción citotóxica, excepto el extracto hidroalcohólico de las raíces. En este extracto hubo un total de $27.85 \pm 2.41\%$ de citotoxicidad contra células fibroblásticas, en la concentración más alta evaluada (1000 $\mu\text{g/mL}$).

Palabras clave: Umbu; Antiprotozoario; Citotoxicidad; Kinetoplastida.

1. Introduction

In Brazil, leishmaniasis and American trypanosomiasis are the most common examples of neglected diseases. This group of diseases is present in over 148 countries, and is characterized by affecting mainly underdeveloped and developing countries, so that they contribute to inequality and social exclusion of those affected (Menezes et al., 2019; Hotez, 2008; Who, 2018). As these diseases are not so present in developed countries, they do not represent a risk to the public health of such places, consequently these nations stopped investing in research aimed at the treatment of these pathologies. Thus, there is a therapeutic disadvantage for neglected diseases, that is, due to their absence in developed countries, the pharmaceutical industries have no interest in the search and formulation of new drugs because they sell little and reach a poor and economically excluded population (Pedra et al., 2011; Santos et al., 2017). In addition, the control of such diseases has an impact on the economy due to the high investments in medicines and patient care, as well as in the control of disease vectors (Bezerra et al., 2019).

Of the two parasitic infections highlighted above, leishmaniasis is considered zoonotic, being caused by protozoa of more than 20 species of the genus *Leishmania* and transmitted by females of sand flies of the genus *Lutzomyia* and *Phlebotomus* during blood meal. The disease is classified into three types: cutaneous, which causes ulcers on exposed parts of the body; mucocutaneous, in which protozoa cause lesions that cause destruction of the mouth, nose, throat and surrounding mucous membranes; and finally visceral, which is the most severe form, which causes fever, weight loss, enlargement of the spleen and liver, followed by anemia (Chappuis et al., 2007). This disease is endemic in 88 countries around the world, occurring mainly in Bangladesh, India and Nepal, with an annual record of 1 million to 1.5 million cases (Chappuis et al., 2007; Sousa & Day, 2011). While for the Brazilian territory in a period of 25 years (1980-2005), around 60 thousand cases were recorded (Maia-Elkhoury et al., 2008).

As for American trypanosomiasis, it is known in Brazil as “Chagas disease”, and it is an infection caused by flagellated protozoa of the species *Trypanosoma cruzi* being transmitted mainly by female triatomines belonging to the genus *Triatoma*, *Panstrongylus* and *Rhodnius* during blood meal. In addition to this form of transmission, strains are transmitted through blood, vertical, oral and accidental routes (Coura, 2015; Machado et al., 2018). It is estimated that around 8 million people are chronically infected by the protozoan with an annual rate of 12 thousand deaths, and in Brazil the highest prevalence occurs in the

North and Northeast regions, with around 5,000 annual deaths (Andrade et al., 2019; Martins-Melo et al., 2014; Pedra et al., 2011).

Such diseases are treated using chemotherapeutic agents, and for leishmaniasis, pentavalent antimonials, amphotericin B and pentamidine are used. However, such drugs are used in high doses to obtain the desired effects, thus, causing toxicity in the host organism, in addition to selecting resistant parasites (Andrade et al., 2019). For Chagas disease, nifurtimoxe Benzonidazole is used to treat illness. However, nifurtimox acts in the formation of O₂, which leads to oxidation of the parasite's membranes. As a consequence, the host's tissues can also be damaged since they are also eukaryotes, which explains the occurrence of several side effects such as insomnia, hyporexia, vomiting and epigastric pain, in view of this, its use was interrupted in some countries of South America (Pedra et al., 2011; Rassi et al., 2002). The second drug, Benzonidazole, has some restrictions in the treatment of disease and has low efficacy during the chronic phase of the disease and high rates of treatment interruption due to subsequent side effects. Such a drug acts by inhibiting the synthesis of proteins and RNA in extracellular and intracellular forms present in the parasitized host (Cruz et al., 2016; Ferreira, 1990; Machado et al., 2018; Pedra et al., 2011).

In view of such unwanted effects, it is necessary to search for new compounds with antiparasitic actions, such as natural products. These can be used as agents in the treatment of infections due to the variety of compounds from secondary metabolism (Costa et al., 2016; Bezerra et al., 2019). Natural products can be found in several ecosystems, such as the Caatinga, a seasonally dry tropical forest located in Northeast Brazil, in which several native species with antiparasitic potential have been reported, such as *Tarenaya longicarpa* Soares Neto & Roalson (Bezerra et al., 2019), *Ziziphus joazeiro* Mart. (Andrade et al., 2019), *Ximenia americana* L. (Menezes et al., 2019), *Luehea paniculata* Mart. & Zucc. (Calixto-Junior et al., 2016).

A native species to the Caatinga and used medicinally for the treatment of infections, digestive disorders, diarrhea, diabetes, kidney infection and throat disorders is *Spondias tuberosa*, Arruda (Anacardiaceae) (Albuquerque et al., 2007; Siqueira et al., 2016). Such a species is popularly known as “imbu”, “umbuzeiro”, “umbu” and in popular medicine several vegetative and reproductive parts are used, which are barks, fruits, roots, resin and leaves (Lins-Neto et al., 2016; Siqueira et al., 2016). Thus, the hypothesis is raised that extracts of the species may present actions against the parasitic strains that cause leishmaniasis and American trypanosomiasis and, due to their popular use, have low toxicity.

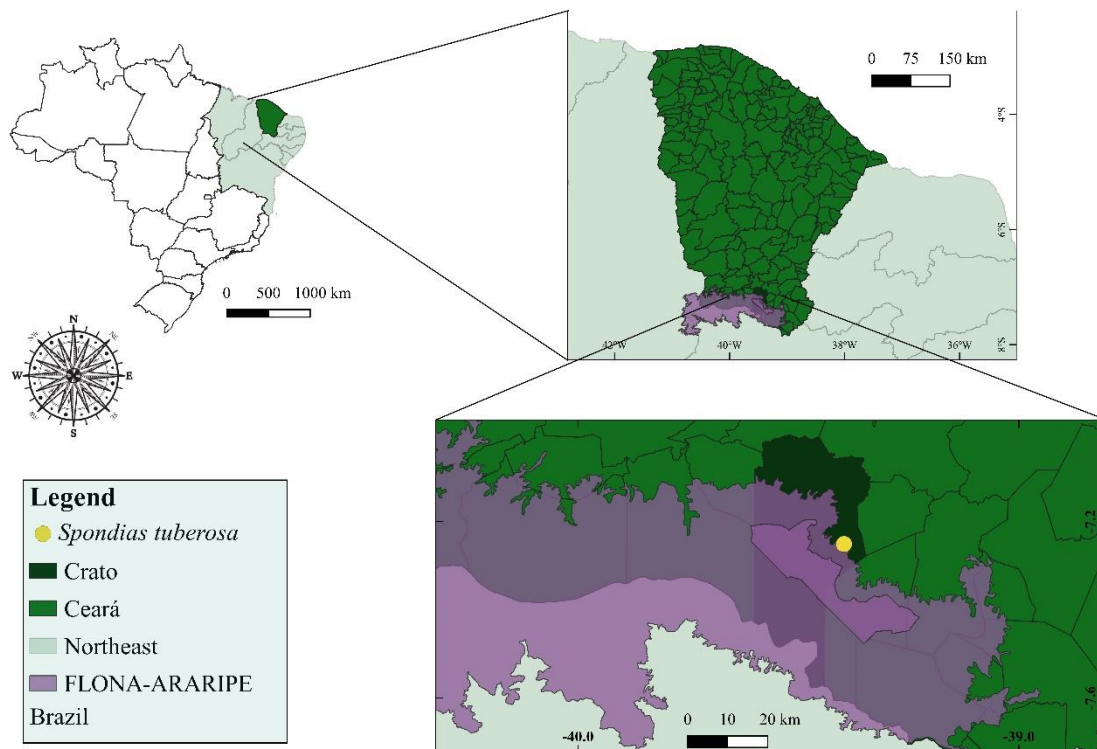
So, in view of the low efficacy of synthetic drugs and using the ethnopharmacological approach, this work aims to investigate whether aqueous extracts and tinctures of the leaves and roots of *S. tuberosa* have an effect against the agents that cause leishmaniasis and American trypanosomiasis, as well as the absence of toxicity.

2. Materials and Methods

2.1 Botanical material gathering

The leaves and roots of *S. tuberosa* were collected in June 2018 under the consent of the Biodiversity Information and Authorization System - SISBIO with the number 64293-1, Lameiro community (07°15'03.1" south latitude and 39°23'48.3" West longitude of Greenwich), in the municipality of Crato, southern Ceará, Brazil, harvested from 7 individuals, from 8:00 am to 9:30 am (Figure 1). An example of collected material was deposited at the Herbarium Caririense Dárdano de Andrade Lima (HCDAL) of the Regional University of Cariri - URCA with the receipt of #13,728, being confirmed by Professor M.^a Ana Cleide Alcântara Morais Mendonça.

Figure 1 - Location of the species *Spondias tuberosa* in the municipality of Crato in the state of Ceará, Brazil.



Source: Almeida-Bezerra (2020).

2.2 Preparation of extracts

To prepare the extracts, young and healthy leaves and roots were used. Aqueous extract was obtained by the infusion process, in which initially the leaves and roots were crushed to increase their contact surface. After that, 1 L of distilled water at 100 °C was added to each 132.2 g of vegetable part, the container was then closed and kept at rest for 15 minutes, for later filtration. While the hydroalcoholic extract (tincture) was prepared by maceration in 70% ethanol in a proportion of 500 g of fresh leaves and 400 g of dried roots for each 2.652 L of ethanol (70%). The mixture was stored in a dark place for 72 h (Matos, 2002).

The drying of the extracts was carried out using the spray drying technique (spray drying) using the Mini-spraydryer MSDi 1.0 equipment (Labmaq do Brasil), using a 1.2 mm spray nozzle, under the following operational conditions: a) control flow rate: 500 mL/h; b) inlet temperature: 130±2°C; c) outlet temperature: 74±2 °C; d) atomization air flow: 45 L/min; e) blower flow: 1.95 m³/min. The spray drying process consists of changing a product that is

in a liquid to a solid state in powder form, through its passage in a heated medium, in a continuous operation (Masters, 1991).

2.3 Antileishmania activity

For leishmanicidal evaluation of extracts of *S. tuberosa*, the promastigote forms of *Leishmania braziliensis* (MHOM/BR/75/M2903) and *Leishmania infantum* (MCAN/ES/92/BCN83) were used. Which were grown in Schneider's *Drosophila* medium, supplemented with 20% SFB at 22 °C.

The biological evaluation was performed according to Mikus & Steverding (2000), where the promastigote cell cultures (2.5×10^5 parasites/well) were grown in microdilution plates. The extracts were diluted in dimethyl sulfoxide (DMSO) in different concentrations (250 - 1000 $\mu\text{g/mL}$). Different dilutions of the substances up to 200 mL of the final volume were also performed. After the 48 h incubation at 26 °C, 20 μL of resazurin solution were added for the oxidoreductive reactions to be quantified in a spectrophotometer with wavelengths from 570 to 595 nm. The tests were performed in triplicate, and the LC_{50} was calculated, the concentration responsible for causing the lethality of 50% of the promastigote forms and the antipromastigote percentages (% AP) were calculated according to the formula:

$$\%AP = \frac{AE - AEB}{AC - ACB} \times 100$$

In which, AE = absorbance of the experimental group; AEB = compound blank; AC = absorbance control group; ACB = culture medium blank.

2.4 Antitripanosome activity

Epimastigote forms of *Trypanosoma cruzi* (Clone CL-B5) were used to evaluate the trypanocidal effects of *S. tuberosa* (Buckner et al., 1996). The epimastigotes forms that were stably transfected with the gene for *Escherichia coli* β -galactosidase (lacZ), were provided by Dr. F. Buckner through the Memorial Gorgas Institute (Panama). The parasites were grown at 28 °C in LIT (Difco, Detroit, MI), supplemented with 10% SFB (Gibco, Carlsbad, CA),

penicillin (Ern, SA, Barcelona, Spain) and streptomycin (ReigJofr´e SA, Barcelona, Spain) (Le Senne et al. 2002).

For the tests, microdilution plates were used with cultures that did not reach the stationary phase (Vega et al., 2005). *T. cruzi* epimastigotes were seeded at 1×10^5 /mL in 200 μ L and the plates were incubated with extracts (250 - 1000 μ g/mL) at 28 °C for 72 h, with 50 μ L of similar CPRG solution added to give the final concentration of 200 μ M. The plates were incubated at 37 °C for an additional 6 h and then read on a spectrophotometer at 595 nm. The LC_{50} was calculated and the percentage of anti-epimastigote (%AE) was calculated using the same formula as the percentalan tipromastigote (% AP).

2.5 Cytotoxic Activity

To evaluate the cytotoxic effects of the extracts of *S. tuberosa*, cell alignment of mammalian fibroblasts NCTC clone 929 was used. The culture medium (Roswell Park Memorial Institue - RPMI) that these cells were cultured was supplemented with 10% fetal bovine serum (SFB) inactivated by heat of 56 °C for 30 minutes, penicillin G (100 U/mL) and streptomycin (100 mg/mL). Cell culture media were maintained at a temperature of 37 °C with a humidified atmosphere of 5% CO_2 .

The cytotoxic evaluation of extracts against fibroblasts was performed by the colorimetric method of R´olon et al., (2006), using resazurin as a developer. In which, NCTC 929 clone cells were seeded (3×10^4) in flat-bottom microdilution plates (96 wells), along with 100 μ L of RPMI in each well. Cell cultivation took place at night at a temperature of 37 °C and an atmosphere of 5% CO_2 . After that, the medium was replaced and extracts (250 - 1000 μ g/mL) were added in 200 μ L of the medium for 24 h. Growth controls were also included. After the incubation period, 20 μ L of a resazurin solution (2 mM) was added to each well. After 3 h the reduction in resazurin was determined by measuring the wavelength absorbance at 490 and 595 nm in a microplate reader. The tests were performed in triplicate. The LC_{50} was analyzed and for the percentage of cytotoxicity of the extracts of *S. tuberosa* the formula was used: in which, %C, corresponds to the percentage of cytotoxicity of natural products, A_{570} and A_{595} represent the values of optical density media at 570 and 595 nm. And the values of 80,586 and 117,216 are the molar extinction coefficients for resazurin.

$$\%C = \frac{A_{570} \times 117,216 - A_{595} \times 80,586 \text{ (Test sample)}}{A_{570} \times 117,216 - A_{595} \times 80,586 \text{ (Control)}} \times 100$$

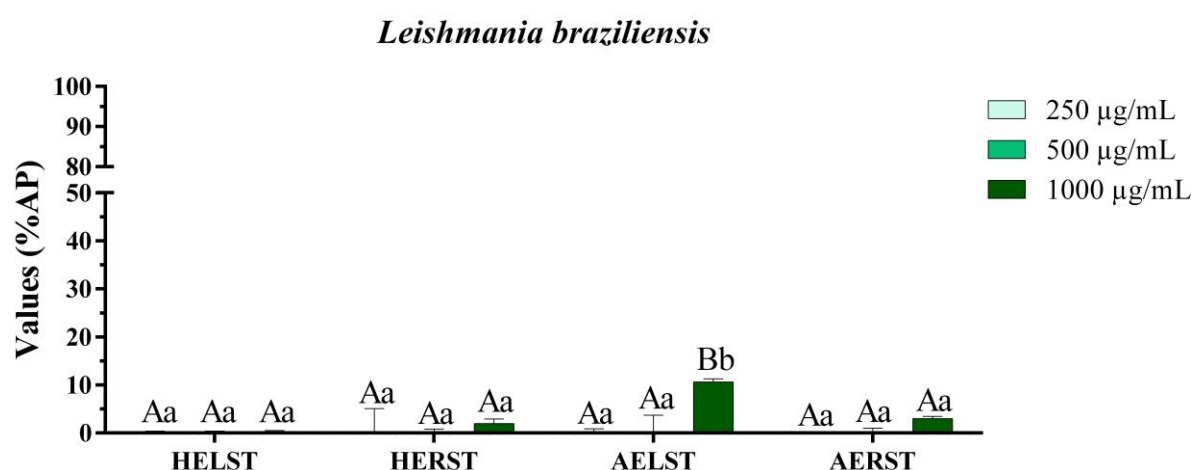
2.6 Statistical analysis

The averages with their respective standard deviations of the antiparasitic and cytotoxic activities of the extracts of *S. tuberosa* were calculated. Afterwards the results were investigated by bidirectional analysis of variance (Anova - Two-way) (Concentration x Extract), followed by the Tukey test at 95% reliability. All analyzes were performed using the GraphPadPrism 6.0 software.

3. Results and Discussion

According to Figure 2, it was demonstrated that the extracts of *S. tuberosa* have a low antipromastigote activity against strains of *L. braziliensis*, since there was no action at concentrations $\leq 500 \mu\text{g/mL}$. Furthermore, regarding the highest concentration evaluated (1000 $\mu\text{g/mL}$), there was no activity for the hydroalcoholic extract of the leaves (HELST), while there was low activity for the other extracts, as in the case of the aqueous extract of the leaves (AELST), in which the natural product caused $10.5 \pm 0.71\%$ of mortality against *L. braziliensis* strains.

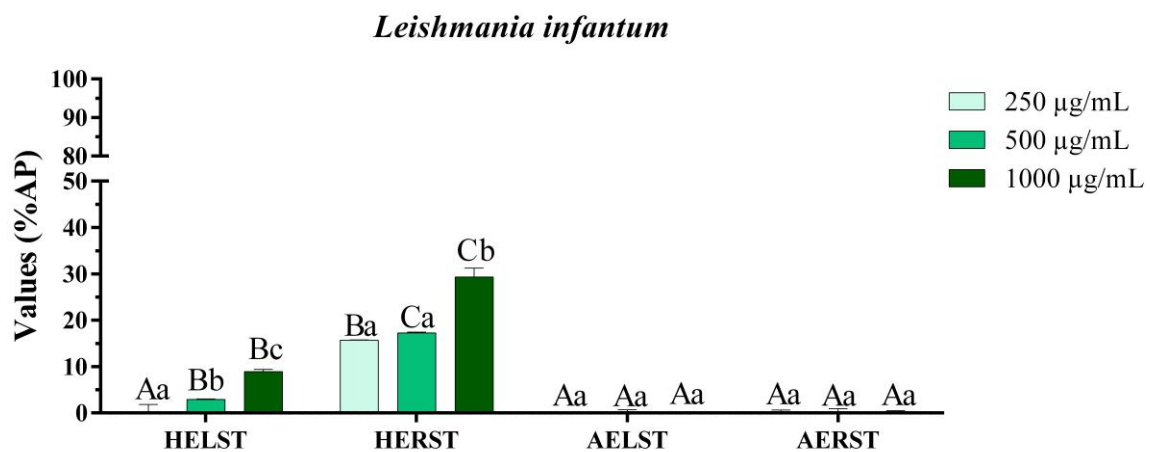
Figure 2 - Percentage of antipromastigote activity (% AP) of hydroalcoholic extract of leaves (HELST) and roots (HERST) and aqueous extract of leaves (AELST) and roots (AERST) of *Spondias tuberosas* against *Leishmania braziliensis* strains.



Different letters show significance by the Tukey test ($p < 0.05$). Lower case letters compare different concentrations for the same extracts and upper case letters compare different extracts of *S. tuberosa* for the same concentrations (Anova-Two-way). Average \pm standard deviation. Source: The Author.

Regarding the antipromastigote action of *S. tuberosa* against strains of *L. infantum*, it was found that there was biological action only for hydroalcoholic extracts (Figure 3). The hydroalcoholic extract of the leaves (HELST), despite having activity in concentrations ≥ 500 $\mu\text{g/mL}$, demonstrated low leishmanicidal potential. However, there was a significant action of the hydroalcoholic extract of the roots against the promastigote forms of *L. infantum*, since there was $29.33 \pm 1.94\%$ of mortality for the treatment of 1000 $\mu\text{g/mL}$.

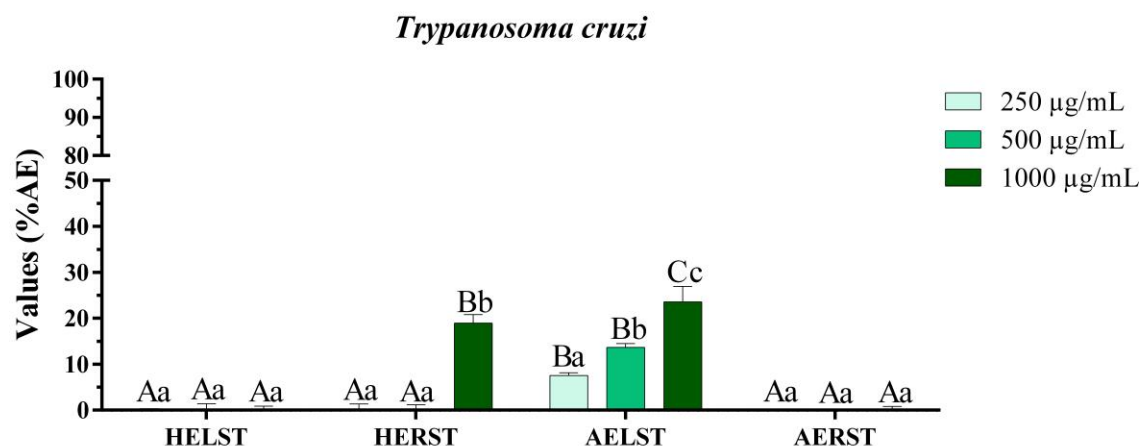
Figure 3 - Percentage of antipromastigote activity (% AP) of hydroalcoholic extract of leaves (HELST) and roots (HERST) and aqueous extract of leaves (AELST) and roots (AERST) of *Spondias tuberosas* against *Leishmania infantum* strains.



Different letters show significance by the Tukey test ($p < 0.05$). Lower case letters compare different concentrations for the same extracts and upper case letters compare different extracts of *S. tuberosa* for the same concentrations (Anova-Two-way). Average \pm standard deviation. Source: The Author.

The extracts of the leaves and roots of *S. tuberosa* showed low antiparasitic activity against the epimastigote strains of *T. cruzi* as seen in Figure 4. The hydroalcoholic extract of the roots showed antiepipastigote action in concentration ≥ 1000 $\mu\text{g/mL}$. The aqueous extract of the leaves, on the other hand, showed activity in concentrations ≥ 250 $\mu\text{g/mL}$, with its highest concentration resulting in $23.53 \pm 3.39\%$ of mortality of the protozoa.

Figure 4 - Percentage of antiepipimastigote activity (% AP) of the hydroalcoholic extract of the leaves (HELST) and roots (HERST) and aqueous extract of the leaves (AELST) and roots (AERST) of *Spondias tuberosas* against the strains of *Trypanosoma cruzi*.



Different letters show significance by the Tukey test ($p < 0.05$). Lower case letters compare different concentrations for the same extracts and upper case letters compare different extracts of *S. tuberosa* for the same concentrations (Anova-Two-way). Average \pm standard deviation. Source: The Author.

According to Pedra (2011), the World Health Organization has determined some parameters to be followed for the formulation of a drug in order to treat a disease. They are: (1) Parasitological cure for acute and chronic cases; (2) Effect in one or a few doses; (3) Low cost; (4) Absence or low side effects to the sick and no teratogenic effects; (5) No need for hospitalization for treatment; and (6) No induction of organism resistance to the drug. Thus, we refute the hypothesis established in this study according to parameter 2, since there was low antiparasitic activity in the extracts of *S. tuberosa*. Because there was no inhibition of more than 50% of the parasites at concentrations $\leq 500 \mu\text{g/mL}$ (Rosas et al., 2007; Vandesmet et al., 2017).

However, despite this low activity, the results of this study are prosperous, because even though they did not eliminate the parasites, the extracts from the leaves and roots of *S. tuberosa* were able to reduce their quantity. This is advantageous for populations that do not have access to other treatments, as the reduction of parasitic strains in the body ends up improving the effectiveness of the immune response and reducing the progression of the disease (Marin-Neto et al., 2009).

Some authors report that species of the Anacardiaceae family have therapeutic potential against parasites, being *Pseudospondias microcarpa* (A.Rich.) Engl., *Annickia chlorantha* (Oliv.) Setten & Maas, *Isolona hexaloba* (Pierre) Engl. & Diels, *Greenwayodendron suaveolens* (Engl. & Diels) Verdc., *Annickia kummeriae* (Engl. & Diels)

Setten & Maas (Et-Touys et al., 2017), *Anacardium occidentale* L. (Bastos et al., 2019), *Ozoroa sphaerocarpa* R. Fern. & A. Fern. (Mokoka et al., 2013) and *Spondias mombin* L. (Traore et al., 2014). In this latest study, the researchers demonstrated that the aqueous extract of the stem bark has activity against strains of *T. cruzi* (LC₅₀ 35.8 µg/mL), *Trypanosoma brucei brucei* (CL₅₀ 2.3 µg/mL) and *Plasmodium falciparum* (LC₅₀ 59.5 µg/mL).

Despite the low antiparasitic activities, it is possible to observe that the extracts of *S. tuberosa* have no cytotoxic action, except the hydroalcoholic extract of the roots (HERST) (Table 01). In this treatment, there was a total of 27.85 ± 2.41% of cytotoxicity against murine fibroblasts in the highest concentration evaluated, in addition, the action is observed in a lower concentration (500 µg/mL).

Table 1 - Percentage of cytotoxicity (%) of extracts from the leaves and roots of *Spondias tuberosa* against murine fibroblasts.

Concentration (µg/mL)	Extract			
	HELST	HERST	AELST	AERST
500	0,0 ± 2,36	22,35 ± 0,66	0,0 ± 0,65	0,0 ± 0,47
1000	0,0 ± 4,06	27,85 ± 2,41	0,03 ± 3,39	0,0 ± 3,39

Legend: Hydroalcoholic extract of leaves (HELST) and roots (HERST) and aqueous extract of leaves (AELST) and roots (AERST) of *Spondias tuberosa*. Average ± standard deviation. Source: The Author.

Extracts from the leaves of *S. tuberosa* were not able to cause toxicity, however Guedes et al. (2020), demonstrated that these organs have compounds with high toxicity, since the hexane extract of the leaves showed a high toxicity against fibroblasts. This is explained by the type of solvent used in the research.

The cytotoxic actions of the hydroalcoholic extract of the roots of *S. tuberosa* may be related to the chemical constituents present in such organ. The action may be the activity of the major compounds or the synergistic action (Bezerra et al., 2019). Among the secondary metabolites present in the extract, highlights (±)-Naringenin (Santos et al., 2019), which is a cytotoxic flavanone against fibroblasts (Stompor et al., 2017), corroborating the results of this work.

Although the study showing some results, it is worth mentioning that the study is *in vitro*, studies using animals (*in vivo*) must be carried out in order to obtain the necessary doses

with the antiparasitic action, so that the research can then be submitted to a clinical study using people and obeying all ethical standards.

4. Conclusion

The aqueous extracts and tinctures of the leaves and roots of *Spondias tuberosa* have low efficacy against strains of *Leishmania braziliensis*, *Leishmania infantum* and *Trypanosoma cruzi* in concentrations of clinical relevance. In addition, the hydroalcoholic extract of the roots shows cytotoxicity against fibroblast-type cells.

This work opens new possibilities for the identification of compounds with antiparasitic action, so that more studies should be carried out aiming at the separation and purification of the bioactive constituents. Subsequently, *in vivo* and clinical studies should be performed.

Acknowledgments

All authors would like to thank the Scientific Research Development Center - CEDIC, for the development of the experiments. The authors would like to thank the funding agencies Coordination for the Improvement of Higher Education Personnel - CAPES, the Ceará Foundation for Support for Scientific and Technological Development - FUNCAP and the National Council for Scientific and Technological Development - CNPq.

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