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Chemical composition, antiparasitic and cytotoxic activities of aqueous extracts of *Ziziphus joazeiro* Mart.

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ABSTRACT

Objective: To compare the *in vitro* antiparasitic activity of aqueous extracts from *Ziziphus joazeiro* leaves and stem bark against *Trypanosoma cruzi*, *Leishmania braziliensis*, and *Leishmania infantum*, as well as to evaluate its cytotoxicity in mammalian cells, in addition to identifying the chemical composition of the extracts.

Methods: *Ziziphus joazeiro* leaf and stem bark aqueous extracts were prepared by cold extraction maceration and subjected to ultra-efficient liquid chromatography coupled to a quadrupole/time of flight system. The susceptibility assays used *Trypanosoma cruzi* CL-B5 strains and promastigote forms of *Leishmania braziliensis* and *Leishmania infantum* for antiparasitic activity of the extracts. Moreover, mammalian fibroblasts NCTC clone 929 were used for cytotoxicity analysis.

Results: Terpenoid compounds, flavonoids and phenolic acid were identified in extracts. The stem bark aqueous extracts presented more significant results in terms of antiparasitic activity compared with the leaf aqueous extracts, especially against *Leishmania braziliensis* and *Leishmania infantum* promastigote forms with an IC_{50} < 500 μg/mL. The cytotoxicity evaluation showed moderate toxicity of the stem bark aqueous extracts, which is relevant information for the rational use of this plant part since it is widely used by the population.

Conclusions: These preliminary results may contribute to the formulation of new therapeutic agents against this group of neglected diseases, so further investigations are required to delineate the mechanisms of action mainly of the aqueous extract of stem bark of *Ziziphus joazeiro*.

1. Introduction

Infectious diseases termed as neglected diseases are caused by parasitic or infectious agents which mainly affect populations living in conditions of poverty and social inequality. The World Health Organization (WHO) emphasizes that housing, food, poor sanitation and lack of health care are the main causes of these diseases[1,2]. Leishmaniasis, malaria, dengue, Chagas disease, leprosy and tuberculosis can be highlighted as examples of neglected diseases[3].

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Chagas disease is a parasitic infection caused by the flagellate protozoan *Trypanosoma cruzi* (*T. cruzi*) and transmitted through vectors. Chagas disease is a systemic and chronic disease considered to be an endemic tropical disease in 21 countries in Latin America where its main transmission mechanism occurs through triatomine vectors. Secondary transmission mechanisms such as transfusion (through blood transfusion or infected organ transplantation), congenital (mother-child transmission, if the woman has the disease) and oral (through ingestion of foods infected with insect feces) transmission exist. During the *T. cruzi* biological cycle, three evolutionary forms (trypomastigote, epimastigote and amastigote) can be observed[4,5]. According to the WHO, there are approximately 8 million people infected, with 56 thousand new cases per year and 12 thousand annual deaths[6].

Leishmaniasis is characterized as a group of diseases caused by protozoa from more than 20 *Leishmania* species where the parasites have two life cycle stages: promastigote and amastigote. Transmission of these parasites is achieved through a phlebotomine vector[7,8]. There are three main types of leishmaniasis: cutaneous, which causes ulcers on exposed body parts (face, arms and legs); visceral, one of the most severe forms, is characterized by fever, weight loss, spleen and liver enlargment, followed by anemia; and mucocutaneous, where the lesions cause destruction of the nose, mouth, throat and surrounding mucous tissue membranes[9,10]. The WHO noted that in 2014 more than 90% of new cases occurred in six countries: Brazil, Ethiopia, India, Somalia, South Sudan and Sudan[11].

Neglected diseases suffer from a therapeutic disadvantage as they do not attract the interest of pharmaceutical industries[12]. Chagas' disease treatment is achieved by only two drugs, nifurtimox and benzonidazole. For leishmaniasis, pentavalent antimonials, amphotericin B and pentamidine are used. In general, these drugs have a high parasite resistance index, often requiring high administration doses, which generate considerable toxicity[13,14]. Thus, the importance of finding new compounds which act as chemotherapeutic agents for the treatment of these diseases is evident.

Moreover, natural products derived from various plant parts can be used as agents for the treatment of infections, mainly due to the bioactive potential of secondary metabolites present in their composition[15]. *Ziziphus joazeiro* (*Z. joazeiro*) Mart. (Rhamnaceae) is a tree species widely used in ethnomedicine with several proven biological activities such as antifungal, antibacterial, antioxidant, antipyretic, anti-inflammatory and astringent activity[16,17].

The objective of this work was to evaluate the *in vitro* antiparasitic activity of the *Z. joazeiro* aqueous extracts derived from its leaves and stem bark against *T. cruzi*, *Leishmania braziliensis* (*L. braziliensis*), and *Leishmania infantum* (*L. infantum*), as well as its cytotoxic potential in mammalian cells, in addition to identifying the chemical composition of the extracts through ultra-efficient liquid chromatography coupled to quadrupole/time of flight system (UPLC-MS-ESI-QTOF).

2. Materials and methods

2.1. Collection area and plant material

The leaves and stem bark were collected from eight *Z. joazeiro* Mart. specimens located in the Sítio Ipueiras, in the rural area of the Brejo Santo municipality, south of Ceará, Brazil, at the foot of the Chapada do Araripe (geographical coordinates, south latitude and west longitude of Greenwich: 1: 442 m, 07°28'54.4"S/39°01'47.2"W; 2: 431 m, 07°28'53.3"S/39°01'46.1"W; 3: 436 m, 07°28'50.5"S/39°01'57.6"W; 4: 440 m, 07°28'42.8"S/39°02'10.2"W; 5: 447 m, 07°28'48.5"S/39°02'12.0"W; 6: 441 m, 07°28'51.4"S/39°02'16.0"W; 7: 439 m, 07°28'54.6"S/39°02'07.6"W; 8: 436 m, 07°28'58.6"S/39°01'48.8"W). The voucher material was deposited in the Herbarium Dárdano de Andrade Lima of the Regional University of Cariri - URCA under n° 13.346 and identified as *Z. joazeiro* Mart. The collection took place in the month of February 2017, from 7:30 to 9:00 in the morning. The plant material was sent to the laboratory, cleaned and weighed.

2.2. Extract preparation

The aqueous extracts from the leaves and stem bark (AEL and AEB) of *Z. joazeiro* were prepared by cold extraction maceration[18]. Fresh leaves were cut to increase their surface area, while the stem barks were dried at room temperature and ground in a mechanical mill. Subsequently, both were added in distilled sterile water and maintained in a container protected from light and air. After 72 h, the extracts were filtered, frozen and taken to a lyophilizer (-60 ℃) producing a crude extract of 39.9 g and 111.58 g, respectively.

2.3. Compound identification through UPLC-MS-ESI-QTOF

The analysis was performed on a Waters ACQUITY UPLC system to the Q-TOF Premier mass spectrometer (Waters MS Technologies, Manchester, UK) with electrospray ionization interface (ESI) in negative ionization mode. Chromatographs were run on a Waters Acquity UPLC BEH column (150 mm × 2.1 mm, 1.7 μm), fixed temperature of 40 ℃, mobile water phases with 0.1% formic acid (A) and acetonitrile, 1% formic acid (B), gradient ranging from 2% to 95% B (15 min), flow 0.4 mL/min and injection volume 5 μL. The negative ESI mode was acquired in the range of 110-1 180 Da, fixed source temperature at 120 ℃, desolvation temperature 350 ℃, desolvatation gas flow of 500 L/h, extraction cone 0.5 V, voltage capillary of 2.6 kV. Leucine-enkephalin was used as a lock mass. The acquisition mode was MS^E. MS data were collected for *m/z* values in the range of 110-1 180 Da with a scan time of 0.1 over an analysis time of 19 min. The accurate mass and molecular formula assignments were obtained with the MassLynx 4.1 software (Waters MS Technologies).

2.4. Cell lines

The *T. cruzi* assays used CL-B5 parasite strains (clone CL-B5) transfected with the β-galactosidase gene of *Escherichia coli* (LacZ). Epimastigote forms were cultured in infusions of liver tryptase with 10% fetal bovine serum, penicillin and streptomycin at 28 ℃, being harvested during the exponential growth phase[19]. For the leishmanicidal activity, promastigotes of *L. braziliensis* and *L. infantum* grown at 26 ℃ were used in Schneider's medium, supplemented with 10% fetal bovine serum, 2% normal human urine plus penicillin and streptomycin. In the NCTC clone 929 mammalian fibroblast cytotoxicity test, they were cultured in RPMI 1640 medium (Sigma) supplemented with 10% fetal bovine serum, penicillin and streptomycin. Cells in the pre-confluence phase were harvested with trypsin, maintained at 37 ℃ in a humidified atmosphere of 5% CO_2.

2.5. In vitro trypanocide and leishmanicidal assays

The trypanocidal assay was performed on 96-well microdilution plates with cultures that did not reach the stationary phase[20]. Epimastigotes were seen (1×10^5 cells/well) on 200 mL of RPMI médium and incubated with the products at 28 ℃ for 72 h. Subsequently, 50 μL of chlorophenol red-β-D-galactopyranoside solution was added, incubated at 37 ℃ for an additional 6 h and then read at 595 nm in a spectrophotometer. Nifurtimox was used as reference drug. Each concentration was tested in triplicate. The percentage of inhibition (%AE) was calculated.

The leishmanicidal assay was based on the method developed by Mikus and Steverding[21] with modifications. Promastigotes (2.5×10^5 parasites/well) were grown in 96-well plastic plates. The extracts were dissolved in dimethyl sulfoxide (DMSO), and different dilutions of up to 200 mL of final volume were added. After 48 h at 26 ℃, 20 μL of resazurin solution was added and the oxidation-reduction was quantified at 570 to 595 nm. Each concentration was tested in triplicate. In each test, Metronidazole, the reference drug, was used as a control. The antipromastigote percentages (%AP) were calculated.

2.6. Cytotoxicity assays

The method for assessing cell viability was colorimetric, with resarzurine[22]. Fibroblasts NCTC 929 were seeded (5×10^4 cells/well) in 96-well microdilution plates with 100 μL RPMI 1640 medium for 24 h at 37 ℃ in 5% CO_2 atmosphere. The medium was replaced by different concentrations of the extracts in 200 μL of medium and then incubated for another 24 h. Growth controls were also included. Subsequently, a 20 μL volume of 2 mM resazurin solution was added and the plates incubated for 3 h to evaluate cell viability. Each concentration was tested in triplicate. The cytotoxicity of each compound was estimated by calculating the percentage of cytotoxicity (%C).

2.7. Statistical analysis

The IC_{50} of the results about cytotoxic effect, anti-leishmania and anti-trypanosoma activities were calculated using the software GraphPad Prism 7.0, applying a sigmoidal regression curve of dose-response.

3. Results

3.1. Compounds identified in the aqueous extracts of the leaves and stem bark of Z. joazeiro Mart.

The chromatographic analysis of the *Z. joazeiro* Mart. extracts was determined by UPLC-MS-ESI-QTOF in the negative mode. The results obtained show 12 compounds present in AEB, with the identification of 7 saponin derivatives which belong to the terpenoid classes (Table 1).

Twenty-four compounds were found in AEL, with 12 compounds being identified (Table 1), including four terpenoids, seven flavonoids and one phenolic acid.

3.2. Antipromastigote and antiepimastigote activities of the extracts of Z. joazeiro Mart.

Table 2 presents the antipromastigote and antiepimastigote results of the *Z. joazeiro* extracts, in addition to the standard drug results. AEB presented more significant results for its antiparasitic activity in comparison to the AEL, especially against the *L. braziliensis* and *L. infantum* promastigote forms.

The AEL presented antiparasitic activity against promastigotes and epimastigotes, with an $IC_{50} > 500$ μg/mL, however, without clinical relevance.

The IC_{50} values of AEL for antipromastigote activities were 1 241 μg/mL (*L. braziliensis*) and 33 770 μg/mL (*L. infantum*) and for antiepimastigote activity, the IC_{50} value was 76 640 μg/mL.

The AEB was the most efficient extract against the promastigote forms with an 85.71% inhibition for *L. braziliensis* and 68.44% for *L. infantum*, at 1 000 μg/mL concentration. The IC_{50} values for antipromastigote activities were 327.4 μg/mL (*L. braziliensis*) and 405.2 μg/mL (*L. infantum*) and for antiepimastigote activity, the IC_{50} value was 1 080 μg/mL.

3.3. Cytotoxicities of the extracts of Z. joazeiro Mart.

The cytotoxicity of AEL was not observed. The AEB showed moderate toxicity, with an IC_{50} value for cytotoxicity of 333.9 μg/mL, and inhibition of 61.87% fibroblasts at the concentration of 250 μg/mL.

Table 1. Identification of compounds by UPLC-QTOF in aqueous extract of stem bark (AEB) and leaves (AEL) of *Z. joazeiro*.

Samples	Quantitative	Classes	Compound
AEB	7	Terpenoids	Saponin derivatives
	5	None	None
AEL	4	Terpenoids	Saponin derivatives
	7	Flavonoids	C-flavone glycosides
			Myricetin-*O*-glucoside
			Rutin
			Quercetin-*O*-hexoside
			Quecetin- robnoside
			Ramnazin-3-*O*-rutinoside
			Ramnazin-hexoside
	1	Phenolic acid	Dihydroxybenzoic acid pentoside
	12	None	None

Table 2. Antipromastigote and antiepimastigote activities of the extracts of *Z. joazeiro* Mart (mean± SD)(%).

Samples (μg/mL)	%AP (L.b)	%AP (L.i)	%AE
AEL			
1 000	51.11±2.33	3.88±0.86	1.72±0.80
500	23.51±0.43	0.00±0.22	0.00±0.31
250	13.05±0.75	0.00±0.31	0.00±0.61
AEB			
1 000	85.71±0.70	68.44±0.42	43.00±4.74
500	82.16±0.47	67.89±0.20	26.71±0.14
250	42.92±0.78	51.83±0.43	25.78±0.64
Metronidazole			
2.0	100.0	100.0	-
1.0	97.9	97.9	-
Nifurtimox			
1.0	-	-	54.9
0.5	-	-	45.6

AP: antipromastigote percentage; AE: antiepimastigote; L.b: *L. braziliensis*; L.i: *L. infantum*. AEL: aqueous extracts of leaves of *Z. joazeiro*; AEB: aqueous extracts of stem bark of *Z. joazeiro*.

4. Discussion

The *Ziziphus* genus is known for having a presence of alkaloids and polysaccharides, in addition to a significant number of flavonoids, tannins and saponins in its composition[23,24].

Regarding antiparasitic activity, in a study carried out by Brito *et al*[25] using a *Z. joazeiro* leaf hydroethanolic extract, no relevant antiparasitic activity was observed against *T. cruzi* (IC_{50}: 612.06l μg/mL), *L. braziliensis* (IC_{50}: > 5 000 μg/mL) and *L. infantum* (IC_{50}: 693.67 μg/mL), with the extract exhibiting a low cytotoxic activity.

Gomes *et al*[26] evaluated the antimalarial activity of aqueous extract from *Z. joazeiro* stem bark, observing complete nematode egg inhibition, *Haemonchus* spp., where the IC_{50} was determined at 1.9 μg/mL of the extract.

In this study, the AEL did not present relevant antiparasitic activity. However, some constituents of the flavonoid class, such as myricetin-*O*-glucoside, quercetin-*O*-hexoside and quecetin-robnoside, which are derived from myricetin and quercetin, have been observed in its composition. When isolated from plant extracts, myricetin and quercetin were effective in reducing *T. cruzi* strains, in addition to presenting low toxicity to mammalian cells[27]. In this way, it is believed that the constituents present in complexes do not have the effect that their isolated form has.

The cytotoxicity presented by the AEB may be related to the presence of saponins in its chemical composition. Several studies report high cytotoxicity for many saponins[28-30].

Saponins are molecules produced by the plant's secondary metabolism which act primarily as a chemical defense system against herbivores, as well as fungal and bacterial infections[31,32]. Despite reports on its toxicity, these molecules are responsible for a wide variety of biological activities such as molluscicidal, antiparasitic[33], anti-inflammatory, cytotoxic, anti-platelet and anti-diabetic activities[23,26,29,34].

In conclusion, comparative evaluation of the antiparasitic activity of the *Z. joazeiro* aqueous extracts demonstrated the efficacy of the AEB against promastigote forms and an antiparasitic action without clinical relevance of the AEL. The cytotoxicity from the AEB extract observed is of relevant information for the rational use of this plant part since it is widely used by the population. UPLC-QTOF analysis revealed compounds which can be used as a basis for further biological studies against this group of neglected diseases.

Conflict of interest statement

The authors declare that there is no conflict of interest.

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