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LC–MS characterization, anti-kinetoplastide and cytotoxic activities of natural products from *Eugenia jambolana* Lam. and *Eugenia uniflora*

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ABSTRACT

Objective: To evaluate the trypanocidal, leishmanicidal and cytotoxic activity of *Eugenia jambolana (E. jambolana)* and *Eugenia uniflora (E. uniflora)* extracts and fractions.
Methods: The products were characterized by LC–MS. Antiparasitic assays were performed and cytotoxicity was evaluated in fibroblastos. *In vitro* assays were performed using spectrophotometric evaluation. All assays were performed in thrice.
Results: The results showed that the extracts and the tannic fraction from *E. jambolana* inhibited 100% of the epimastigote lines. The ethanolic extract was the most efficient in all concentrations tested against the three parasite strains. In the cytotoxicity assay the flavonoid fraction showed low toxicity. All *E. uniflora* samples showed cytotoxicity at the highest concentration tested, but the extract showed no toxic effect on the fibroblasts at the lowest concentration. The flavonoid and tannic fractions were more efficient against *Leishmania braziliensis* promastigotes compared to the extract. However, the extracts and the tannic fraction were more effective against *Leishmania infantum* strains. The effect on epimastigote cells was observed at all concentrations tested, with all *E. uniflora* samples. However, the samples were more effective at the highest concentration, where there was inhibition in 100% of the *Trypanosoma cruzi* strains.
Conclusions: The species *E. jambolana* and *E. uniflora* presented antiparasitic activity against all tested parasite strains, indicating that these species can serve as an alternative therapy as they were efficient in the tests performed. The *E. uniflora* extract and the *E. jambolana* flavonoid fraction presented a low cytotoxicity, opening the floor for new biological studies.

1. Introduction

Parasitic diseases are of high incidence in developing countries. Among these, therapeutics of leishmaniasis and trypanosomiasis have been a great challenge because these diseases did not arouse the interest of the pharmaceutical industries, while existing drugs for their treatments present high toxicity [1,2]. According to the World Health Organization (WHO), trypanosomiasis and leishmaniasis are among the infectious-parasitic diseases that are most concerned by the public health agencies for their frequency, therapeutic difficulties, deformities and sequelae that they can cause [3].

Leishmaniasis comprises a complex of parasitic diseases caused by more than 20 protozoa species of the *Leishmania*

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genus, which affect approximately 12 million people in 88 countries around the world [4–6]. Trypanosomiasis is another common parasitic disease in the world caused by *Trypanosoma cruzi* (*T. cruzi*) and represents one of the most serious endemic diseases in Brazil [7].

In the case of Chagas disease, two relevant drugs stand-out, namely nifurtimox and benzonidazole. However, the action of these drugs is influenced by the duration of treatment, age, geographical distribution of patients, among other factors [8]. Chemotherapy currently available for the treatment of leishmaniasis is far from satisfactory. The first-choice treatment was introduced in 1945 and is still based on pentavalent antimonials (Sb^{V})-meglumine antimonate (Glucantime®) and sodium stibogluconate (Pentostam®). Second-line drugs such as amphotericin B, pentamidine and paromomycin are used in cases of antimonial resistance. However, these drugs have toxic side effects, high costs and need extensive treatments [9–12].

Different strategies have been tried to obtain new compounds effective against *Leishmania* spp., thus a number of natural products have already been investigated. Some substances possessing several structural classes have anti-*Leishmania* properties, including monoterpenes, sesquiterpenes and other essential oil constituents, demonstrating that such substances may be promising development options for new medicines [13,14].

The species *Eugenia uniflora* (*E. uniflora*) L., known as 'pitanga', is a plant native to South America [15,16], which presents several biological activities, such as antimicrobial, trypanocidal and leishmaniasis activity [15–18]. *Eugenia jambolana* (*E. jambolana*) Lam., known as 'jambolão' in Brazil, comes from India [19] and presents several biological activities, such as antioxidants, anti-inflammatory, antimicrobial, antidiarrheal, gastroprotective and antiulcerogenic activity [20]. The objective of this study was to verify the leishmanicidal, trypanocidal and cytotoxic activity of the *E. jambolana* Lam. and *E. uniflora* L. ethanolic extracts, as well as their tannic and flavonoid fractions.

2. Materials and methods

2.1. Collection area and plant material

The collection was carried out in the Botanical Garden of Medicinal Plants, the Laboratory of Research of Natural Products-LPPN, Regional University of Cariri-URCA (coordinates: 07°16′ 59.8″ latitude S. and 39° 44′ 46.7″ W; 07° 14′ 18.1″ latitude S. and 39° 24′ 52.9″ W; 07° 14′ 28.0″ latitude S. and 39° 24′ 56.7″ W). The leaves were collected and transported to the Laboratory of Microbiology and Molecular Biology, Regional University of Cariri-URCA. The study was carried out using young and healthy *E. jambolana* Lam. and *E. uniflora* L. leaves. Samples were produced and deposited in Herbarium Dárdano de Andrade Lima of this University under nº 3106 and 3107, identified as *E. uniflora* and *E. jambolana*. Samples were collected in September, October and November, during 7:30 am to 9:00 am. The vegetable material was transported to the laboratory, where it was subjected to qualitative sorting and cleaning, and then weighed and stored for a short period under refrigeration.

2.2. Preparation of extracts and fractions

2.2.1. Ethanol extracts

E. jambolana L. and *E. uniflora* ethanol extracts (EEEJ and EEEU) were prepared using 1 832 g and 1 711 g of previously crushed leaves respectively to increase contact surface. Ethanol 100% was added, then extracts were remained at room temperature and protected from air and light for 96 h [21]. Afterwards the macerate was filtered and taken to a rotary evaporator (Q-344B-Quimis-Brazil) at 40 rpm and 60 °C in a water bath, yielding crude extracts of 124.7 g and 198.2 g respectively.

2.2.2. Flavonoid fractions of E. jambolana L (FFEJ) and E. uniflora (FFEU)

Ten grams (10 g) of each extract were dissolved in 100 mL of hexane, remained in an ultrasonic bath for 10 min and then centrifuged (3 000 rpm) for 10 min. The supernatant was separated, and the residue was added to 100 mL of hexane and processed two more times as described above. One hundred milliliters (100 mL) of chloroform were added to the residue obtained at the end, with an ultrasonic bath for 10 min, and the same procedures were repeated for 3 times. Fifty milliliters (50 mL) of ethyl acetate were added to the residue, and brought to the ultrasonic bath. Then the fraction was dry in the rotary evaporator, and obtained material was determined as the flavonoid fraction [22].

2.2.3. Tannic fractions of E. jambolana L (TFEJ) and E. uniflora (TFEU)

Twenty grams (20 g) of each extract were mixed with 50 mL of the acetone/water (7: 3) mixture, stirred in an ultrasonic bath for 40 min at 5 °C. Following this period, the material was filtered through a common funnel with filter paper, concentrated and evaporated the acetone in a rotary evaporator to get the aqueous phase. Then the aqueous phase was partitioned with petroleum ether (3 times with 50 mL), distilled in a rotary evaporator to ensure complete evaporation of petroleum ether, lyophilized from the aqueous phase to give the tannic fraction [23].

2.3. Identification of compounds by liquid chromatography coupled to mass spectrometry (LC–MS)

The solutions were selected and individually analyzed by LC–MS, an octadecylsilane column (250.0 mm × 4.6 mm, 5 μm, Luna® C18, Phenomenex®) was used as the stationary phase. And mobile phase was composed of 2 solvents: Solvent A-0.1% formic acid in ultrapure water and solvent B-0.1% formic acid in methanol (HPLC grade) with flow rate of 1.0 mL/min, gradient according to Table 1. The stationary phase was maintained at 30 °C, injected 20 μL to the samples (1 mg/mL) in the LC-DAD-MS monitor analyzer of 190 to 400 nm and 50 to 1 000 *m/z*. Analysis was carried out using a Shimadzu® LC-20 equipped with a LC-20ADVP model pump system, DGU-20A degasser, PDA detector model SPD-20AVP, CTO-20ASVP model oven, SIL-20ADVP model automatic injector and SCL-20AVP model controller. Coupled to a ESI-IT mass spectrometer (AmaZon SL, Bruker Daltonics®),

Table 1
Gradient of mobile phase used in the qualitative determination of *E. jambolana* and *E. uniflora* samples.

Time (min)	Solvent A (%)	Solvent B (%)
0	95	5
60	5	95
70	0	100
75	0	100
80	95	5
90	95	5

equipped with an electrospray ionization source operating in the analyzer mode, and by trapping negative ions to divide the HPLC eluent, a flow rate of 0.2 mL/min was introduced at source. The parameters of the mass spectrometer used were: capillary voltage with 3.5 kV; desolvation temperature at 320 ℃; gas flow of 10 L/min; pressure of 60 PSI, using nitrogen as drying gas and misting [24–34].

2.4. Cell lines used

For *in vitro* studies of *T. cruzi*, the clone CL-B5 was used according to the method described by Le Senne *et al.* [35] and Buckner *et al.* [36]. Parasites were stably transfected with the *Escherichia coli* b-galactosidase gene (lacZ). The cultivation, growth and maintenance of epimastigotes and promatigotes were performed according Roldos *et al.* [37]. The viability of these strains was assessed according to Roldos *et al.* [37], using resazurin as a colorimetric method.

2.5. In vitro tripanocide, leishmanicide and cytotoxic assay

The tripanocide assay was performed in 96-well microplates with cultures that had not reached the stationary phase [38]. The leishmanicide and cytotoxic assay were performed according the methods described by Roldos *et al.* [37].

2.6. Data analysis

All data of anti-kinetoplastidae and cytotoxic effect were expressed as the media of a triplicate measure with mean ± standard deviation.

3. Results

3.1. Phytochemical characterization by LC–MS of the extract and fractions of E. jambolana and E. uniflora

From the chromatographic analysis of the samples described in Table 2, it was possible to suggest the presence of several compounds from the flavonols class, with quercetin and myricetin found in greater abundance, by means of the similarity between ultraviolet absorption spectrum, charge mass ratio and fragmentation (MS^2) with data described in the literature and the GNPS (Global Natural Products Social Molecular Networking) database.

Table 2
Total chromatographic analysis by LC–MS of the extract and fractions of *E. jambolana* and *E. uniflora*.

Retention time (min)	[M−H] experimental (*m/z*)	MS^2[M−H] Experimental (*m/z*)	Suggested compound
5.0–5.1	190.93	126.90/172.88	Quinic acid
5.3	481.05	300.89/274.87	Quercetin derivate
8.7	168.91	124.88	Gallic acid
12.6	288.98	204.93/244.99	Catechin/epicatechin
12.7	288.95	204.93/244.99	Catechin/epicatechin
14.0	577.17	407.00/425.01	Procianidine
18.3	449.06	316.95/431.04	Glycosylated Myricetin derivate
19.3	449.02	316.95	Glycosylated Myricetin derivate
21.2	463.06	300.97	Quercetin derivate
21.3	615.12	300.98/463.03	Glycosylated Quercetin derivate
22.4	463.09	178.91/315.95	Myricetin-Ramnoside
23.4	463.06	300.97	Quercetin derivate
24.1	447.08	300.97	Quercetin derivate
24.4	615.13	316.94/463.03	Glycosylated Myricetin derivate
24.5	433.03	300.96	Quercetin-pentoside
24.7–24.8	300.89	–	Ellagic acid
25.1–25.2	433.06	300.95	Quercetin-pentoside
25.5	316.94	150.90/178.93/ 248.84	Myricetin
25.6–25.7	447.08	300.97	Quercetin derivate
25.9–26.0	585.08	433.02/300.95	Quercetin derivate
27.0	461.07	314.94	Glycosylated Isorhamnetin derivate
27.3	27.3	380.95/419.08	Quercetin derivate
29.3	300.93	150.91/178.88/ 272.99	Quercetin
29.7	301.00	178.89/272.92	Quercetin

3.2. Trypanocide, leishmanicidal and cytotoxic activities of the extract and fractions of E. jambolana and E. uniflora

Table 3 showed the antileishmania, cytotoxic and trypanocidal activity of the *E. jambolana* Lam. extract and fractions. The extract and fractions presented low toxicity, with less than 50% toxicity at the highest concentrations. Regarding the evaluation of the antiparasitic activity, all the products were effective in the concentration of 1000 μg/mL, showing a mortality of 64.04%–100% in epimastigotes and the EEEJ was the most efficient against promastigote forms, with mortality varying between 72.03% and 92.16% in the maximal concentration.

In the assays performed, the *E. uniflora* ethanolic extract, as well as the flavonoid and tannic fractions, demonstrated a low toxicity with a fibroblast mortality rate of less than 50% at the concentration of 1000 μg/mL. As for the evaluation of the antiparasitic activity, all the products were effective in the concentration of 1000 μg/mL, presenting a mortality of 100% against epimastigotes. However, all the products were poorly effective against the promastigote forms tested (Table 4).

4. Discussion

The flavonoids content showed the most representative concentration in the extracts and in the flavonoid fractions

Table 3
Antileishmanial, trypanocide and cytotoxic activities of the extracts and fractions from *E. jambolana* Lam.

Sample	μg/mL	% AP (*L. brasiliensis*)	% AP (*L. infantum*)	% AE	% C
EEEJ	1 000.0	72.03 ± 0.57	92.16 ± 0.49	64.04 ± 1.86	41.84 ± 0.10
	500.0	67.65 ± 1.11	54.52 ± 1.00	53.50 ± 0.21	35.09 ± 0.31
FFEJ	1 000.0	27.50 ± 0.89	8.70 ± 0.49	100.00 ± 0.23	38.87 ± 0.73
	500.0	30.32 ± 0.08	0.00 ± 1.31	24.84 ± 1.13	3.26 ± 0.13
TFEJ	1 000.0	44.46 ± 0.71	0.00 ± 2.93	100.00 ± 0.49	36.52 ± 0.64
	500.0	32.45 ± 1.91	0.00 ± 1.86	29.00 ± 1.13	37.73 ± 0.66
Metronidazole	2.0	100.0	100.0	–	–
	1.0	97.9	97.9	–	–
Nifurtimox	1.0	–	–	54.9	–
	0.5	–	–	45.6	–

EEEJ: Ethanol extract of *E. jambolana*; FFEJ: Flavonoid fraction of *E. jambolana*; TFEJ: Tannic fraction of *E. jambolana*; % AP: Percentual of killed promastigote forms; %AE: Percentual of killed epimastigote forms; %C: Percentual of killed fibroblasts NCTC 929. Data are expressed as mean ± SD.

Table 4
Antileishmanial, trypanocide and cytotoxic activities of the extracts and fractions from *E. uniflora* L.

Amostras	μg/mL	% AP (*L. brasiliensis*)	% AP (*L. infantum*)	% AE	% C
EEEU	1 000.0	10.66 ± 0.78	32.62 ± 0.42	100.00 ± 0.47	20.70 ± 1.13
	500.0	4.52 ± 0.28	22.03 ± 0.47	40.13 ± 0.00	0.00 ± 0.32
FFEU	1 000.0	28.12 ± 0.28	0.00 ± 0.05	100.00 ± 0.06	36.01 ± 0.49
	500.0	20.24 ± 0.64	0.00 ± 0.07	37.00 ± 0.07	32.54 ± 0.35
TFEU	1 000.0	42.28 ± 0.09	48.33 ± 0.93	100.00 ± 0.23	44.06 ± 0.80
	500.0	31.09 ± 0.28	17.74 ± 2.21	74.10 ± 0.35	45.71 ± 0.16
Metronidazole	2.0	100.0	100.0	–	–
	1.0	97.9	97.9	–	–
Nifurtimox	1.0	–	–	54.9	–
	0.5	–	–	45.6	–

EEEJ: Ethanol extract of *E. jambolana*; FFEJ: Flavonoid fraction of *E. jambolana*; TFEJ: Tannic fraction of *E. jambolana*; % AP: Percentual of killed promastigote forms; %AE: Percentual of killed epimastigote forms; %C: Percentual of killed fibroblasts NCTC 929. Data are expressed as mean ± SD.

evaluated. These compounds are secondary metabolites of plant origin recognized for several biological activities [39]. In previous studies by Sobral-Souza *et al.* [40], *E. jambolana* species also presented an expressive concentration of flavonoids in its composition. The presence of several chemical constituents in the *Eugenia* sp. samples, such as phenolic acids, ellagic acid, quinic acid were observed, and flavonoids including catechins, procyanidins, quercetin and myricetin in the extract and the fractions were found, through the chemical analysis performed.

From the results, it was evidenced that quercetin and myricetin are the predominant compounds in the analyzed samples. In previous studies it was proved that leaves from *E. jambolana* and *E. uniflora* present different flavonoids and terpenoids as well as β-sitosterol, betulínic acid, crategolico acid, *n*-heptacosane, *n*-nonacosane, n-hentriacontane, n-octacosanol, n-triacontanol, quercetin, myricetin, myricitrin and the glycosylated flavonoid myricetin 3-O-(4″-acetyl)-α-L-rhamnopyranoside [41,42].

Quercetin and other flavonoid derivatives are active components by oral administration in cutaneous infections and visceral leishmaniasis produced *in vivo* [43,44]. Recent studies show that quercetin, quercitrin and isoquercitrin are potent inhibitors of *Leishmania amazonensis* arginase (ARG-L) [45]. In a study of 105 natural compounds, the leishmanicidal activity of the flavonoids fisetin, quercetin, luteolin and 7,8-dihydroxyflavone showed high potency against *Leishmania donovani* amastigotes [46], in addition, these four compounds also showed potential as ARG-L inhibitors [47].

In a study by Quitino *et al.* [48], flavonoid dimers presented trypomastigote activity. In addition, these compounds reduced the blood parasitemia of mice infected with *T. cruzi*. In preliminary results from Silva *et al.* [49], biological assays indicated that chalcones 2′,4′-dihydroxy-3′,5′-dimethyl-6′-methoxychalcone showed potent activity against *T. cruzi* parasites (IC_{50} 13.12 μM) and *Leishmania amazonenses* (100% inhibition at 50 μM), and 2′,6′-dihydroxy-5′-methyl-4′-methoxychalcone was efficient against the protozoan *T. cruzi* (IC_{50} 35.95 μM).

The results showed that the *E. jambolana* extract and the tannic fraction were effective in the inhibition of the epimastigote lineage tested. In a study conducted by Santos *et al.* [50], the *E. jambolana* ethanolic extract inhibited 100% of cells at a concentration of 100 μg/mL, corroborating with this study and showing the effectiveness of the species. The tannic fraction showed no action against the promastigote form of *Leishmania infantum* at the two concentrations tested. EEUJ showed activity against the three parasite strains compared to the flavonoid and tannic fractions, showing a percentage inhibition ≥50% in all tested concentrations. These data are important, since inhibition at this level with a concentration ≤500 μg/mL is considered clinically relevant [51]. The results show that only in the lowest concentration tested did the flavonoid fraction present with low cytotoxicity. In studies carried out by Santos *et al.* [50], the *E. jambolana* extract showed moderate toxicity, according to Roldos *et al.* [37].

All *E. uniflora* samples presented moderate cytotoxicity at the highest concentration tested, but the extract showed no toxic effect to the fibroblasts at the lowest concentration. This result corroborated with another study by Santos *et al.* [52] indicating the *E. uniflora* extract showed a toxicity between 8% and 0%, which is considered low and clinically ineffective [37]. The flavonoid and tannic fractions were more efficient against *Leishmania braziliensis* promastigotes when compared to the extract. However, the extracts and the tannic fraction were more effective against the *Leishmania infantum* strains. The effect on epimastigote cells was observed at all concentrations tested, and *E. uniflora* samples were more effective at the highest concentration, where there was inhibition in 100% of *T. cruzi* strains. Santos *et al.* [52] showed in his study that *E. uniflora* extract had a low toxicity (8% at 100 μg/mL, and this toxicity was reduced to 0% at a concentration of 10 μg/mL).

Other plant species such as *Lygodium venustum* Sw., *Mentha arvensis*, *Annona squamosa* and others have already been studied in relation to their anti-*Trypanosoma* and anti-*Leishmania* activities [53–55]. Some flavonoids isolated from the *E. jambolana* and *E. uniflora* species, such as quercetin, myricetin and catechins, may be the main phytochemicals responsible for the leishmanicidal and trypanocidal activities [17,50,52], thus making them promising species for other biological assays.

The *E. jambolana* and *E. uniflora* species have demonstrated a wide variety of flavonoid compounds associated with antiparasitic activity against promastigotes and epimastigotes, as a low or moderate toxicity against mammalian fibroblasts. Thus, these products can be a starting point for new *in vivo* trials for the possible development of new complementary and alternative therapies against these diseases which are considered and classified as neglected diseases by the World Health Organization.

Conflict of interest statement

The authors declares that they have no conflict of interest.

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