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Analysis of bioactivities and chemical composition of *Ziziphus joazeiro* Mart. using HPLC–DAD

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ABSTRACT

The aim of this study was to evaluate the chemical profile and antioxidant, antimicrobial and antiparasitic activities of the hydroalcoholic extract of the leaves of *Ziziphus joazeiro* Mart. (HELZJ). The antioxidant DPPH and FRAP assays and chemical profile were determined by colorimetric methods and HPLC/DAD. The antiparasitic, antibiotic and antibiotic-modifying activity were evaluated by microdilution assays. The HPLC–DAD assay showed the presence of mostly tannins and flavonoids, such as caffeic acid and quercetin. The levels of polyphenols and flavonoids were 183.136 mg/g extract and 7.37 mg/g extract, respectively. DPPH and FRAP showed low antioxidant activity for the extract. The antibacterial and antifungal activities were not of clinical relevance, showing MIC > 1024 μg/mL. However, synergism was observed between HELZJ and the antibiotics amikacin and gentamicin, which resulted in decreased bacterial drug resistance. EHFZJ showed low toxicity in fibroblasts in vitro, while antiparasitic results against *Trypnosoma cruzi*, *Leishmania braziliensis* and *Leishmania infantum* were not clinically relevant. Thus, our results indicate that *Z. joazeiro* Mart. (HELZJ) could be a source of plant-derived natural products that could lead to the development of promising new antibiotic compounds for infectious diseases.

1. Introduction

Since the 1970s, the World Health Organization has pointed out the importance of traditional medicine as an alternative resource for discovering new drugs (Pan et al., 2013). The majority of populations in the world have obtained and documented information about medicinal plant species used to treat various illnesses. Even in the poorest regions of the planet in urban centres, medicinal plants can be found in the street fairs, popular markets and residential backyards (Badke, Budó, Alvim, Zanetti, & Heisler, 2012). Despite the enormous development of chemical synthesis, more than 30% of new chemical discoveries and 25% of currently prescribed drugs worldwide have origins in medicinal plants (Rates, 2001). The increasing use and potential financial value of these medicinal plants have brought to light the need for further investigations into their medical and drug applications. Medicinal plants are used as raw material in the synthesis of complex molecules of pharmacological interest, which have stirred the interest of the pharmaceutical industry worldwide (Cavalcante, Neto, De Omena Bomfim, & Flória-Santos, 2013).

Rhamnaceae is a plant family consisting of about 900 species across the planet, where only 23 species occur in South America. The genus *Ziziphus* comprises about 30 species (Heald, 2004), 9 of which are found in Brazil (Forzza & De Janeiro, 2010). The species *Ziziphus joazeiro* is a tree that occurs in the Brazilian semiarid caatinga biome, where it is popularly known as "juazeiro" (Fernandes & Araújo, 2011). From this species, the local population use fruits for food and extracts of leaves, bark, inner bark and roots as traditional medicines for treating fever, bacterial infection, general pain, gingivitis, and respiratory diseases and for topical healing, hepatic and cardiac tonic, diuretic, and other such purposes. Based on this popular application, *Z. joazeiro* has become the target of some pharmacological studies with satisfactory findings related

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to its antifungal (Cruz et al., 2007), gastroprotective (Romão, Costa, Terra, Boriollo, & Soares, 2010) and antimicrobial properties (Silva et al., 2011).

In tropical countries, with hot and humid conditions, there are various parasitic diseases, such as Leishmaniasis and Chagas disease. Side by side with bacterial and fungal infections, *Trypanosoma cruzi* and *Leishmania* infections are considered as an inevitable part of the life for people in endemic regions, and an extremely diverse group of these etiological agents are responsible for millions of cases of deformity, chronic disability and social stigmatization every year (Park, van Leeuwen, & Chochorelou, 2013). Some of these diseases exist in areas with poor sanitation and where insects and other disease-carrying animals thrive and persist in areas where people have little or no means of accessing essential drugs to prevent or treat such infections (Eckert & Kohler, 2014). Accordingly, traditional medicinal resources, especially plants, have emerged to fill this gap and have played a major role in managing infectious disorders (Abe & Ohtani, 2013; Tempone et al., 2008), and in some countries, they contribute significantly to the primary health care of the population (Leonti & Casu, 2013).

Since chemical properties should be the first step to understand some biological findings for natural compounds, we first determined the chemical profile of the hydroalcoholic extract of the leaves of *Z. joazeiro* Mart. (HELZJ) and then investigated its antioxidant activity and anti-parasitic (*T. cruzi* and *Leishmania*) and antibiotic (bacteria and fungi) activities alone or in combination with conventional antibiotics in current use.

2. Materials and methods

2.1. Collection and preparation of the extract

Z. joazeiro Mart. leaves were collected in Chapada do Araripe, Crato city, Ceará, Brazil. The plant material was identified by Dr. Maria Arlene Pessoa da Silva, and a voucher specimen was deposited with the identification number 30.2013 at the Herbarium "Dárdano de Andrade Lima" of Universidade Regional do Cariri (URCA), Crato, Ceará, Brazil. The leaves were dried without being exposed to sunlight and powdered at room temperature. The powdered material was extracted by maceration using a 1:1 ethanol and water mixture for 72 h at room temperature. The extract was then filtered and concentrated under vacuum in a rotary evaporator at 45 °C, 27–30 rpm and 760 mmHg. The remaining solvent was placed in a water bath to evaporate the residual ethanol in a nitrogen atmosphere, and after 24 h, the hydroalcoholic extract was frozen and freeze-dried. The powder was stored in an amber bottle and stored at 4 °C in a refrigerator for later analytical procedures and pharmacological tests.

2.2. Qualitative phytochemical assay

Phytochemical tests were qualitatively performed to determine the presence of phenolic acids, flavonoids, tannins and saponins. The tests were based on colorimetric readings and formation of precipitate as described by Matos (1997).

2.3. Determination of total phenols and flavonoids by colorimetric assay

Total phenols were determined using the Folin–Ciocalteu reagent as described by Roby, Sarhan, Selim, and Khalel (2013) with slight modifications. Samples of 200 μL of extract at different concentrations, in triplicate, were mixed with 1.5 mL of Folin–Ciocalteu reagent and allowed to stand for 5 min at room temperature. Next, 1.5 mL of sodium bicarbonate solution (60 g/L) were added to the mixture and the tubes incubated for 90 min at room temperature. Absorbance was measured at 750 nm using a UV–visible spectrophotometer (Libra S32, Biochrom, France) and total phenols were quantified using a standard curve. The standard curve was prepared with the absorbance readings of various concentrations of gallic acid solutions (25–150 μg/mL in 80% methanol). The equation $y = 0.012x + 0.103$ ($R^2 = 0.984$) was obtained and used to determine the total phenols in the extract in gallic acid equivalents (GAE), mg/g dry extract. All determinations were carried out in triplicate and the results were reported as the mean ± SD.

Total flavonoid content was measured by the alluminum chloride colorimetric assay (Kontogianni et al., 2013) with slight modifications. A 0.1 mL volume of extract or standard solutions of catechin at concentrations of 50, 100, 150, 200, 250 or 300 mg/L was mixed with 0.1 mL aluminium trichloride in ethanol (20 g/L), and diluted with ethanol to 2.5 mL. The absorbance was read at 415 nm after 40 min at room temperature. Control samples or standard solutions were prepared with 0.1 mL plant extract/standard solution and 0.1 mL of ethanol and diluted to 2.5 mL. All determinations were carried out in triplicate. The level of total flavonoids was calculated on the basis of the standard curve of catechin (from 50 to 300 mg/mL – equation $y = 0.033x + 0.056$ ($R^2 = 0.972$) and expressed as mg catechin equivalent per 100 g of extract (mg CA/100 g)

2.4. Quantitation phenolic composition by HPLC–DAD

High performance liquid chromatography (HPLC–DAD) was performed with a Shimadzu Prominence Auto Sampler (SIL-20A) HPLC system (Shimadzu, Kyoto, Japan), equipped with Shimadzu LC-20AT reciprocating pumps connected to a DGU 20A5 degasser with a CBM 20A integrator, SPD-M20A diode array detector and LC solution 1.22 SP1 software. Reverse-phase chromatographic analyses were carried out under gradient conditions using a Phenomenex C18 column (4.6 mm × 250 mm) packed with 5-μm diameter particles; the mobile phase was water containing 1% formic acid (A) and acetonitrile (B), and the gradient was as follows: 13% B up to 10 min and then changed to obtain 20, 30, 50, 60, 70, 20 and 10% B in 20, 30, 40, 50, 60, 70 and 80 min, respectively, according to Boligon et al. (2012), with slight modifications. HELZJ was dissolved in ethanol at 20 mg/mL and analysed for the presence of eleven (gallic acid, chlorogenic acid, caffeic acid, ellagic acid, catechin, epicatechin, quercetin, rutin, isoquercitrin, quercitrin and kaempferol) antioxidant compounds. The identification of these compounds was established by comparing their retention time and UV absorption spectrum with that of commercial standards.

We used a flow rate of run in HPLC of 0.7 μL/min and 40 μL injection volume, and the detection wavelength was 254 nm for gallic acid, 280 nm for catechin and epicatechin, 325 nm for caffeic, chlorogenic and ellagic acids, and 365 nm for quercetin, isoquercitrin, quercitrin, rutin and kaempferol. All samples and the mobile phase were filtered through a 0.45-μm membrane filter (Millipore) and then gassed in a ultrasonic bath prior to use. Reference standards were prepared in HPLC mobile phase in a concentration range of 0.250–0.030 μg/mL for kaempferol, quercetin, quercitrin, isoquercitrin, catechin, epicatechin and rutin, and 0.300–0.030 μg/mL for caffeic, chlorogenic and ellagic acids. The chromatography peaks were confirmed by comparing their retention time with those of reference standards and by DAD spectra (200–500 nm). The limit of detection (LOD) and limit of quantification (LOQ) were calculated based on the standard deviation of the response and the slope using three independent analysis curves. LOD and LOQ were calculated as $3.3\sigma/S$ and $10\sigma/S$, respectively, where σ is the standard deviation of the response and S is the slope of the calibration curve (International Conference on Harmonization (ICH) of Technical

Requirements for the Registration of Pharmaceuticals for Human use & Methodology. ICH Q2(R1). Geneva (Nov., 2005).

2.5. Antioxidant activity: DPPH-radical scavenging and FRAP assays

Antioxidant activity was determined using the DPPH free radical method (Rufino et al., 2007). In a glass tube, 100 μL of HELZJ at different concentrations (250, 125, 50, 25, 10 and 5 μg/mL) or 100 μL of ascorbic acid solution at concentrations of 5, 10, 25 and 50 μM were mixed with 3.9 mL of 0.06 mM DPPH in methanol. This solution was mixed with a stirring rod in a dark room. The negative control contained 100 μL methanol instead of extract or ascorbic acid. The absorbance was measured at 515 nm and repeated every minute until the reading stabilized. The standard curve was determined for DPPH at different concentrations (10, 20, 30, 40, 50 and 60 μM). Methanol was used for the blank. The antioxidant result was expressed as median effective concentration (EC_{50}), obtained by linear regression.

Antioxidant activity was also determined by the FRAP assay, which measures iron-reducing capacity using a spectrophotometric method, as described by Benzie and Strain (1996), with modifications. Briefly, 300 mM acetate buffer at pH 3.6 and 10 mM 2,4,6-tripyridyl-S-triazine (TPTZ) were used in a solution of 40 mM or 20 mM HCl of $FeCl_3{\cdot}6H_2O$. The working solution was prepared by mixing standard acetate buffer, TPTZ solution and $FeCl_3{\cdot}6H_2O$ solution in 10:1:1 proportions. The temperature of the solution was raised to 37 °C before use. The 0.15 mL of HELZJ was added to react with 2.85 mL of FRAP reagent for 30 min in the dark. In this method, the ferric complex (Fe^{+3}-TPTZ) is reduced to the ferrous complex (Fe^{+2}-TPTZ) in the presence of an antioxidant and under acidic conditions. Antioxidant activity was expressed as μmol Fe^{+2}/g extract, using the absorbance obtained for extract and standard ferrous sulphate curve. The reading of the complex was done at 593 nm.

2.6. Determination of antibiotic and antibiotic-modifying activity

Tests for antibacterial activity were performed with the standard bacterial strains *Staphylococcus aureus* ATCC 25923, *Escherichia coli* ATCC 25922 and *Enterobacter aerogenes* and with the multidrug-resistant strains *S. aureus* 10 (SA10), *E. coli* 06 (EC06) and *E. aerogenes* CN64 (EA CN64). In parallel, antifungal activity was also evaluated with the fungal strains *Candida krusei* 01, *Candida tropicalis* 20 and *Candida albicans* 62 and multidrug-resistant strains *C. krusei* LMBM 01 (CK LMBM01), *C. tropicalis* LM 20 (LM20) and *C. albicans* LM 62 (CA LM62). All microbial strains were obtained from the collection of microorganisms in the Microbiology and Molecular Biology Laboratory of URCA. Stocks of the cultures were kept in heart infusion agar (HIA) and stored in a refrigerator.

To evaluate the drug-modifying effect of HELZJ in combination with antibiotics, different classes of antibacterial agents were used (amoxicillin, ciprofloxacin, penicillin G, gentamicin, amikacin and vancomycin). With regard to the influence of HELZJ on antifungal activity, three drugs were used: mebendazole, benzoyl metronidazole and nystatin.

The minimum inhibitory concentration (MIC, μg/mL) was determined in 10% BHI using microdilution assays, where 1024 μg/mL HELZJ was serially diluted twofold and a microbial suspension of 10^5 CFU/mL was added (Javadpour et al., 1996; Oliveira et al., 2006). MIC is defined as the lowest concentration at which there is no growth of microorganisms. For the evaluation of substances for antibiotic-modifying activity, a sub-inhibitory concentration (MIC/8) was determined for bacteria (SA10, EC and EA 06 CN64) and for fungi (LMBM01 CK CT and CA LM62 LM20). The plates were incubated for 24 h at 37 °C and resazurin was used to determine viable bacteria. Retention of blue colour indicates no bacterial growth while red colour indicates bacterial growth. The growth of fungal strains was determined according to turbidity.

To assess drug-modifying activity of HELZJ, antibiotics, starting at 2500 μg/mL, and the antifungal, starting at 5000 μg/mL, were serially diluted twofold to 2.4 μg/mL or 4.8 μg/mL in microplates. Next, 100 μL of 10% BHI with HELZJ and bacterial or fungal agents (10^5 CFU/mL) were added to each well. The final concentration of HELZJ was 128 μg/mL. The plates were incubated at 37 °C and read after 24 h and microbial growth was determined as described above.

2.7. Determination of antiparasitic activity

To evaluate HELZJ for in vitro anti-*T. cruzi* activity, the clone CL-B5 of this protozoan was used (Buckner, Verlinde, La Flamme, & Van Voorhis, 1996). The parasites, transfected with the *E. coli* gene for β-galactosidase (lacZ), were kindly provided by Dr. Buckner of the Gorgas Memorial Institute (Panama). Epimastigote forms were grown at 28 °C in liver infusion tryptose medium – LIT – (Difco, Detroit, MI), supplemented with 10% fetal bovine serum (FBS; Gibco, Carlsbad, CA), penicillin (Ern, S.A., Barcelona, Spain) and streptomycin (ReigJofr and S.A., Barcelona, Spain), as described by Le-Senne, Muelas-Serrano, Fernández-Portillo, Escario, and Gómez-Barrio (2002). Parasites were collected in the exponential growth phase for testing.

The antiepimastigote activity test was carried out in 96-well microplates with exponentially growing cultures as described by Vega, Rolón, Martínez-Fernández, Escario, and Gómez-Barrio (2005). CD39 was inoculated at a concentration of 1×10^5 cells/mL in 200 μL of tryptose broth medium. The plates were incubated with the drugs at concentrations of 100 and 500 μg/mL at 28 °C for 72 h. Afterwards, 50 μL of CPRG substrate solution (chlorophenol red-β-D-galactopyranoside) were added, for a final concentration of 200 μM. The plates were incubated for an additional 6 h at 37 °C and read at 595 nm. The efficiency of each compound was estimated by linear regression analysis, which indicated the concentration of extract needed for 50% maximal effect (CE_{50}).

Cultures of *Leishmania braziliensis* and *Leishmania infantum* were obtained from Instituto de Investigaciones en Ciencias de la Salud, Asunción, Paraguay-IICS and identified by isoenzyme analysis. Tests for inhibition of promastigote forms were performed using a strain of *L. braziliensis* (MHOM/BR/75/M2903) and *L. infantum* (MCAN/ES/92/BCN83), grown at 28 °C for 72 h in Schneider's Drosophila medium supplemented with 20% FBS.

In testing for antiparasitic activity, *Leishmania* promastigotes in cell culture were grown in RMPI medium. The test compounds were dissolved in DMSO and serially diluted twofold in 96-well microplates. The promastigotes were then added at a final concentration of 10^6 cells/mL, and their growth was evaluated after 72 h by direct counting of cells and compared with an untreated control. The efficiency of each compound was estimated by linear regression analysis, which indicated the concentration of extract needed for50% maximal effect (CE_{50}).

2.8. Determination of cytotoxicity

Cytotoxicity tests were done with the fibroblast cell line NCTC-929 cultured in minimal essential medium (MEM; Sigma). The culture medium was supplemented with 10% heat-inactivated FBS, 100 U/mL penicillin G and 100 μg/mL streptomycin. The cultures were maintained at 37 °C in a humid atmosphere with 5% CO_2. Cell viability was assessed by the resazurin reduction assay (Rolón et al., 2006) with cells grown in 96-well microplates at a final concentration of 3×10^4 cells/well. After the culture medium was removed, 20 μL of 2 mM resazurin was added to each well with a final volume of 200 μL. The plates were incubated for 3 h and

resazurin reduction was determined by absorbance readings at 490 and 595 nm. The blank control was subtracted and each concentration determined in triplicate.

2.9. Statistical tests

Statistical analysis was done using the Prism program 5.0. The groups were compared using two-way ANOVA, with the post hoc Tukey test for one-way analysis and post hoc Bonferroni test for two-way analysis. $p < 0.05$ was considered significant. Median effective concentration (EC_{50}) was calculated by linear regression.

3. Results and discussion

3.1. Chemical analysis

In the present study, HELZJ was found to contain saponins, tannins, polyphenols and flavonoids. In particular, the presence of saponins in HELZJ was assessed by the formation of persistent foam after the extract was dissolved in distilled water and the solution stirred vigorously. In fact, the presence of saponins was previously reported in several studies with plants of the species *Z. joazeiro*, where the presence of this secondary metabolite was found in the fruits (Melo, Da Rocha, Dos Santos, Chavasco, & Chavasco, 2012), inner bark (Barbosa Filho, Trigueiro, Cheriyan, & Bhattacharyya, 1985) and leaves (Tripathi et al., 2001). Besides, the high levels of saponins in juazeiro justify its main commercial use in making soap and as a personal hygiene product with detergent properties (Fonseca & Branco, 2014).

Chemical analysis by high performance liquid chromatography (HPLC) of HELZJ revealed the presence of the following compounds: gallic acid (tR = 10.09 min; peak 1), catechin (tR = 16.32 min; peak 2), chlorogenic acid (tR = min 21.17; peak 3), caffeic acid (tR = 24.93 min; 4 peak), ellagic acid (tR = 29.86 min; peak 5), epicatechin (tR = 34.19 min; peak 6), rutin (tR = min 39.05; peak 7), isoquercitrin (tR = 44.89 min; peak 8), quercitrin (tR = 47.61 min; peak 9), quercetin (tR = 51.07 min; peak 10) and kaempferol (60.34 tR = min; peak 11) (Fig. 1). This analysis also revealed the presence of flavonoids, tannins and phenolic acids in extract from *Z. joazeiro* (Table 1).

The chromatographic runs resulted in calibration curves for the following standards: gallic acid, $Y = 14{,}286x + 1395.8$ ($R^2 = 0.9996$); catechin, $Y = 15{,}097x + 1189.3$ ($R^2 = 0.9997$); epicatechin, $Y = 13{,}601x + 1194.5$ ($R^2 = 0.9992$); caffeic acid, $Y = 12{,}758x + 1259.7$ ($R^2 = 0.9996$); chlorogenic acid: $Y = 13{,}461x + 1275.3$ ($R^2 = 0.9992$); ellagic acid: $Y = 13{,}576x + 1346.4$ ($R^2 = 0.9999$); rutin, $Y = 12{,}845 + 1305.7$ ($R^2 = 0.9999$); quercetin, $Y = 13{,}560x + 1192.6$ ($R^2 = 0.9991$); isoquercitrin, $Y = 12{,}873x + 1325.6$ ($R^2 = 0.9998$); quercitrin, $Y = 11{,}870x + 1329.8$ ($R^2 = 0.9993$); and kaempferol, $Y = 14{,}253x + 1238.9$ ($R^2 = 0.9997$).

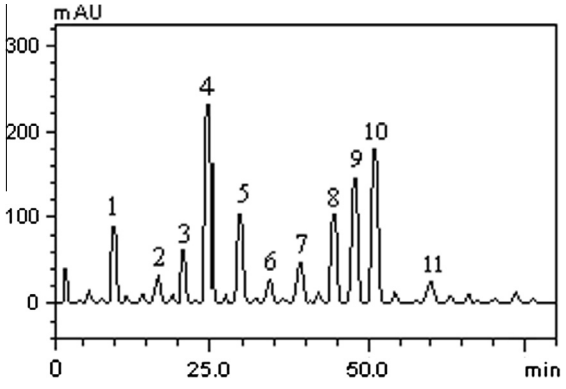

Fig. 1. Representative high performance liquid chromatography profile of the hydroalcoholic extract of *Ziziphus joazeiro* Mart. UV detection at 325 nm. Gallic acid (peak 1), catechin (peak 2), chlorogenic acid (peak 3), caffeic acid (peak 4), ellagic acid (peak 5), epicatechin (peak 6), rutin (peak 7), isoquercitrin (peak 8), quercitine (peak 9), quercetin (peak 10) and kaempferol (peak 11).

Table 1
Phenolic compositions of the extract from *Ziziphus joazeiro* Mart. by HPLC–DAD.

Components	*Ziziphus joazeiro*		LOD	LOQ
	mg/g	%	μg/mL	μg/mL
Gallic acid	13.67 ± 0.01[a]	1.36	0.015	0.049
Catechin	5.28 ± 0.01[b]	0.52	0.032	0.105
Chlorogenic acid	10.45 ± 0.03[c]	1.04	0.009	0.029
Caffeic acid	29.61 ± 0.01[d]	2.96	0.024	0.078
Ellagic acid	15.09 ± 0.02[a]	1.50	0.013	0.042
Epicatechin	4.83 ± 0.01[b]	0.48	0.019	0.060
Rutin	9.72 ± 0.02[e]	0.97	0.027	0.090
Isoquercitrin	15.24 ± 0.03[a]	1.52	0.008	0.026
Quercitine	19.38 ± 0.01[f]	1.93	0.035	0.114
Quercetin	21.30 ± 0.02[g]	2.13	0.019	0.063
Kaempferol	5.17 ± 0.03[b]	0.51	0.026	0.085

Results are expressed as average ± standard deviation (SD) of three determinations. Averages followed by different letters differ by Tukey test at $p < 0.001$. LOD (limit of detection) and LOQ (limit of quantification).

The HPLC results indicated that the extract contained 68.82 mg/g total phenols and 55.57 mg/g flavonoids, while the colorimetric method indicated 183.13 mg/g total phenols and 7.37 mg/g flavonoids. Colorimetric assay apparently overestimated the amount of polyphenols present in HELZJ. According to Bragagnolo (2001), colorimetric methods have a tendency to overestimate the levels of compounds due to the presence of interfering substances that also react with the reagents. However, chromatographic methods, although more expensive, are considered more specific, since they are able to isolate these interferences.

The tannins gallic acid, caffeic acid, ellagic acid, catechin and epicatechin were detected in the Z. *joazeiro* extract. This group of compounds can be found in different parts of the plant such as leaves, bark, inner bark, fruits and seeds. They are rich in phenolic groups and are chemically classified into hydrolysable and condensed (Paes, Diniz, Marinho, & Lima, 2006). Studies on the pharmacological properties of tannins suggest a significant antibacterial action (Loguercio, Battistin, Vargas, Henzel, & Witt, 2005; Oliveira et al., 2006), as well as anti-inflammatory (Norata et al., 2007), antiparasitic (Morais-Braga et al., 2013) and gastroprotective (Júnior et al., 2013; Pellizzon et al., 2012) activities.

The flavonoids (quercetin, isoquercetin, quercitin, kaempferol and rutin) detected here in *Z. joazeiro* have been previously described as having antioxidant properties (Rosa et al., 2010). Among all the phenolic acids identified in HELZJ, caffeic acid showed the highest level, and several studies have demonstrated the antioxidant activity of both gallic and caffeic acids (Sato et al., 2011; Cho, Kim, Ahn, & Je, 2011).

3.2. Antioxidant activity

Since antioxidant activity has been considered a key characteristic for the compounds detected, HELZJ was tested for this activity in this study by the DPPH free radical scavenging and iron reducing assays. We obtained an EC_{50} of 735.72 μg/mL for HELZJ and 40.82 μg/mL for ascorbic acid for the DPPH test, and an EC_{50} of 870 μg/mL for the FRAP test. In a comparative study, Silva et al. (2011) identified an EC_{50} of 461.88 and 1743.05 μg/mL for leaves and bark of *Z. juazeiro*, respectively, and an EC_{50} of 13.95 μg/mL for ascorbic acid, where these values were in line with our finding of lower DPPH scavenging activity in HELZJ when compared to ascorbic acid.

Polyphenols, such as tannin and flavonoids, can be beneficial for human health by exerting various biological effects, including

Table 2
Anti-*Leishmania*, anti-*Trypanosoma* and cytotoxic activities of *Ziziphus joazeiro* Mart. (μg/mL).

Strains	Products	CE_{50} μg/mL
L. braziliensis	Pentamidine	5.69
L. braziliensis	*Z. joazeiro*	>5000
L. infantum	Pentamidine	5.69
L. infantum	*Z. joazeiro*	693.67
T. cruzi	Nifurtimox	20.02
T. cruzi	*Z. joazeiro*	612.06
Fibroblasts N-CTC 929	*Z. joazeiro*	119.34

free-radical scavenging, metal chelation, modulation of enzymatic activity, and alteration of signal transduction pathways (Sousa et al., 2007). The low antioxidant activity can be explained by the presence of natural products with pro-oxidant activities, such as some phenolic compounds detected in HELZJ, e.g., gallic acid and caffeic acid (Martin-Cordero, Jose Leon-Gonzalez, Manuel Calderon-Montano, Burgos-Moron, & Lopez-Lazaro, 2012; Sato et al., 2011; Young-Sook et al., 2011).

3.3. Cytotoxicity and antiparasitic assays

In vitro cytotoxicity was evaluated using cultured NCTC 929 fibroblasts and antiparasitic activity was estimated in cultured mammalian cells and the parasitic agents *T. cruzi*, *L. braziliensis* and *L. infantum*. HELZJ showed an IC_{50} of 119.34 μg/mL in N-CTC 929 fibroblasts, demonstrating a low toxicity. Regarding anti-*T. cruzi* activity using epimastigote forms, the IC_{50} was 612.06 μg/mL, and for the protozoans *L. braziliensis* and *L. infantum*, IC_{50} values were >5000 and 693.67 μg/mL, respectively (Table 2). These results demonstrate that HELZJ has an antiparasitic effect. However, this activity is not considered clinically relevant according to Santos et al. (2012), who believe that only an IC_{50} less than 500 μg/mL can be considered therapeutically relevant.

Some species of the genus *Ziziphus* such as *Ziziphus oenoplia var* and *Ziziphus spina christi* have been identified as possessing antiparasitic activity against *Leishmania* and *T. cruzi* (Osorio, Montoya, & Arango, 2006; Tonkal, Salem, Jamjoom, Altaieb, & Al-Bar, 2005). It is believed that this activity is due to the presence of alkaloids, since these compounds possess trypanocidal activity by inhibiting cellular respiratory pathways (Shahat et al., 2001). Finding new potential compounds against protozoans is of importance because the few currently available drugs used against *T. cruzi* (e.g., benzonidazole) and *Leishmania* (e.g., pentavalent antimonials) are highly toxic to mammalian cells due to the high reactivity of their metabolites (Buckner & Urbina, 2012; Nussbaum, Honek, Cadmus, & Efferth, 2010).

3.4. Antibiotic and antibiotic-modifying activity

Bacteria and fungi are also important targets in the investigation of natural products used in popular medicine. Our data revealed a MIC ⩾ 1024 μg/mL for all bacteria and fungi tested, indicating no evidence of antimicrobial activity. These results are in accordance with other authors who classified the antimicrobial activity of *Z. joazeiro* leaves as mild compared to extracts obtained from other parts of this plant (Melo et al., 2012; Silva et al., 2011). These findings are in line with studies by Melo et al. (2012), who did not identify antifungal activity with *Z. joazeiro* extracts, and Cruz et al. (2007), who concluded that only the extract of the inner bark of *Z. joazeiro* had activity against the fungi *C. albicans*, *Candida guilliermondii*, *Cryptococcus neoformans*, *Trichophyton rubrum* and *Fonsecaea pedrosoi*.

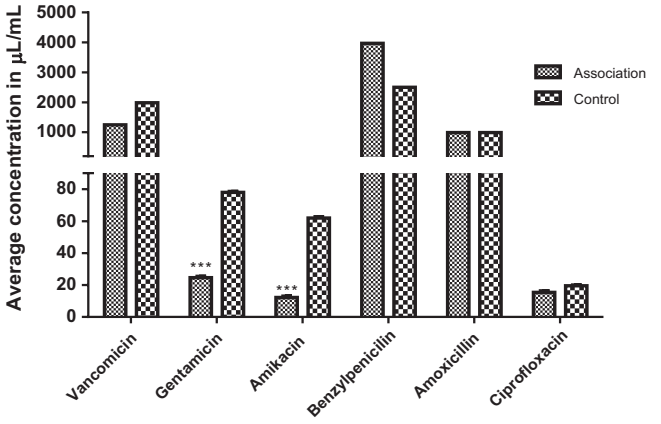

Fig. 2. Representation of the modulatory activity of HELZJ against the multidrug-resistant strains of *Enterobacter aerogenes*. Each well of the microdilution plate contained HELZJ in steady sub-inhibitory concentration of antibiotics in decreasing concentrations. This test was performed in triplicate.

The antibiotic-modifying assay was carried out to evaluate the influence of HELZJ on the action of conventional antibiotics against multidrug-resistant bacterial strains. The results showed that the combination of the antibiotics gentamicin and amikacin with HELZJ reduced the concentration required for inhibition of bacterial growth from 78 to 19 μg/mL and 78 μg/mL to 9.7 μg/mL (Table 3), respectively, displaying synergistic effects.

The synergistic action of antibiotics has already been used for a long time, for example, the combination of β-lactam antibiotics and β-lactamase inhibitors, and it is still a competent alternative strategy against various mechanisms of bacterial resistance. Due to this fact, there is currently a growing search for new natural or synthetic compounds capable of inhibiting at low concentrations the metabolism of various species of multiresistant microorganisms (Silveira, Nome, Gesser, & Terenzi, 2006). Interest in combining natural products and synthetic antibiotics has gained prominence, because natural products mat potentiate the activity of clinically used drugs (Matias et al., 2012; Oliveira et al., 2011). HELZJ has shown a significant modulatory effect, when amikacin and gentamicin were combined with it, resulting in greater toxicity to *E. aerogenes* (Fig. 2). This effect can be explained, in part, by the presence of secondary metabolites that alter the permeability or rupture the cell membrane of microorganisms (Johann, 2012) and that improve antimicrobial action, such as flavonoids (Zuanazzi, 2000).

Table 3
Concentration values of growth inhibition of antibiotics (μg/mL) against *Staphylococcus aureus*, *Escherichia coli* and *Enterobacter aerogenes*.

Antibiotic	*Staphylococcus aureus*		*Escherichia coli*		*Enterobacter aerogenes*	
	Association	Control	Association	Control	Association	Control
Vancomycin	1250	1250	2500	2500	2500	2500
Gentamicin	19.5	19.5	156	156	19**	78
Amikacin	19.5	19.5	156	156	9.7**	78
Benzylpenicillin	>2500	>2500	>2500	>2500	>2500	>2500
Amoxicillin	>2500	>2500	>2500	>2500	1250	1250
Ciprofloxacin	19.5	19.5	78	78	19.5	19.5

** $p < 0.001$ relative to the control by Tukey test.

The antibacterial effect of HELZJ may also be related to the presence of saponins, which feature the ability to form complexes with steroids, proteins and phospholipids in cellular membranes, allowing diverse biological actions, including defense against bacteria (Thakur, Melzig, Fuchs, & Weng, 2011). These secondary metabolites may act on the cell membrane by altering its permeability or causing its complete destruction (Schenkel, Gosmann, & Athayde, 2001; Sparg et al., 2004). However, no modulatory effect was shown with antifungal agents, where this result was corroborated by other studies for this species (Cruz et al., 2007).

4. Conclusion

The extract of the leaves of *Z. joazeiro* Mart. showed the presence of flavonoids, phenols, tannins and saponins, and antioxidant capacity by the DPPH free radical scavenging and FRAP assays, although different patterns were seen when compared to ascorbic acid. There was no clinically relevant antiparasitic activity against *T. cruzi* or *Leishmania*, but the extract showed low cytotoxicity to mammalian cells (fibroblasts). Besides, the extract was synergistic in combination with gentamicin or amikacin against *Enterobacter aerogenes* and in combination with gentamicin against *S. aureus*. The present work demonstrates that the extract of *Z. joazeiro* is a promising natural product in the development of phytomedicines against drug-resistant infections.

Conflict of interest

The authors declare that they have no conflict of interests.

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