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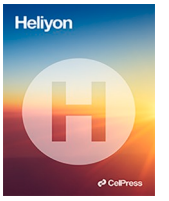

Research article

Influence of seasonality and habitat on chemical composition, cytotoxicity and antimicrobial properties of the *Libidibia ferrea*

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A B S T R A C T

Libidibia ferrea Mart, belonging to the Fabacee family, is a medicinal plant known for its biological properties and production of phenolic compounds. Previous studies reveal the biological activity of its phenolic constituents, making it very promising for the development of new medicines. Seasonality and geographic distribution of species can modify the production of secondary metabolites in Fabaceae species in terms of the preferentially activated metabolic pathways and, consequently, interfere with the medicinal properties of these species. Studying the influence of seasonality on the production of phenolic constituents is essential to establish conditions for "cultivation," species collection, standardization, production, and safety in traditional medicine. This unprecedented study proposed to evaluate the influence of seasonal variations and habitat on the production of phenolic compounds and biological properties of the ethanolic extracts of the stem bark from *L. ferrea*, whose specimens were collected from the Caatinga and the Atlantic Forest, biomes of Brazil. Antimicrobial activity was determined by broth microdilution. Cytotoxicity was evaluated through a colorimetric assay using MTT. ABTS and DPPH radical reduction methods estimated antioxidant capacities. Folin-Ciocalteu and $AlCl_3$ spectrophotometric methods quantified total phenolics and flavonoids, respectively. In turn, radial diffusion quantified tannin content. PCA score plot and HCA dendogram were obtained by multivariate analysis of ^1H NMR data. The cytotoxicity against C6 glioma cells was observed only for Atlantic Forest extracts (EC_{50} = 0.13–0.5 mg mL^{-1}). These extracts also showed selectivity against Gram-positive bacteria *Bacillus subtilis* (ATCC 6633) [MICs 500–2000 μg mL^{-1}], *B. cereus* CCT 0096) [MIC = 250 μg mL^{-1}], *Staphylococcus aureus* (ATCC 6538) [MICs = 250–500 μg mL^{-1}], *S. epidermidis* (ATCC 12228) [62.5–1000 μg mL^{-1}], mainly to *Staphylococcus* sp. Caatinga extracts showed higher production of flavonoids and antioxidants in the summer [7.36 ± 0.19 μg QE mg^{-1} extract; IC_{50ABTS} = 4.86 ± 0.05 μg mL^{-1}], spring [5.96 ± 0.10 μg QE mg^{-1} extract; IC_{50ABTS} = 5.96 ± 0.08 μg mL^{-1}], winter [4.89 ± 0.25 μg QE mg^{-1} extract; IC_{50ABTS} = 6.72 ± 0.08 μg mL^{-1}]. Regarding habitat, two discriminating compound patterns in the studied biomes were revealed by NMR. The results indicated that the Caatinga biome offers better conditions for activating the production of phenolics [336.34 ± 18.1 μgGAE mg^{-1} extract], tannins [328.38 ± 30.19 μgTAE mg^{-1} extract] in the summer and flavonoids in

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winter, spring, and summer. The extracts that showed the best antioxidant activities were also those from the Caatinga. In turn, extracts from the Atlantic Forest are more promising for discovering antibacterial compounds against *Staphylococcus* sp and cytotoxic for C6 glioma cells. These findings corroborated the traditional use of *L. ferrea* bark powder for treating skin wounds and suggest the cytotoxic potential of these extracts for glioblastoma cell lines.

1. Introduction

The growing demand for medicinal plants, mainly in developing countries, is due to their therapeutic properties. However, the variation in the production of active metabolites, often attributed to the climatic differences among the biomes, geographic distribution of specimens of a given plant species, and seasons, can affect the quality and safety of using these species [1–5]. These abiotic factors can induce the expression of specific genes of a given metabolic pathway to the detriment of others and induce the biosynthesis of a specific class of metabolite, and thus, affect the medicinal efficacy of these species [2–5]. The influence of seasonality on medicinal plants' chemical composition and biological activity can contribute information on the best conditions for producing active ingredients, whether for commercial cultivation purposes or to guarantee quality in use by traditional communities [6,7].

Phenolic compounds such as phenolic acids, flavonoids, lignans, hydrolyzable and condensed tannins, among others, are secondary metabolites of medicinal plants, which present broad biological effects, such as anticancer, anti-infectious, antimicrobial, and prevention of cardiovascular disorders and aging [8–12]. These compounds are also necessary for plant adaptation to changes in their natural habitat and for their defense in situations of environmental stress, such as incidence of solar radiation, availability of nutrients in the soil, water supply, temperature, humidity, herbivory, and pathogen attacks, among others [13–17].

Libidibia ferrea (Mart. Ex Tul.) L. P. Queiroz var. ferrea is a tree that can reach up to 20 m in height, with woody fruits and yellow flowers; it is a Brazilian native species belonging to the Caesalpinia genus, Fabaceae family, commonly known as "pau-ferro" or "jucá [9,18,19]. *L. ferrea* makes up the list of medicinal plants in the Brazilian National List of Medicinal Plants of Interest for the Unified Health System (RENISUS) [20]. This species has a high potential to be explored in terms of chemical and biological profiles due to its use in traditional medicine, mainly by communities of Brazilian Caatinga, for the treatment of pain, asthma, cough, flu, inflammation [21], enterocolitis, diarrhea [22–24], wound healing [24] and cancer prevention [23,25]. Previous phytochemical studies on *L. ferrea* show that phenolic compounds are the main secondary metabolites responsible for their biological properties [18,26], as C-glycosylated flavonoids (flavone) [27], and hydrolyzable tannins, mainly derived from gallic acid, which were isolated from the ethanolic from the stem bark [28–30]. Its extracts obtained from leaves stems, and fruits have pharmacological properties such as hypoglycemic, antidiabetic [30,31], anti-inflammatory, anti-ulcer, analgesic [26,32,33], anticancer, antioxidant [23,34,35], antifungal and antibacterial [21,24,26,36,37].

Alcoholic extracts from *L. ferrea* fruits showed growth inhibition of oral pathogens such as *Candida albicans*, *Streptococcus mutans*, *S. salivarius*, *S. oralis*, and *Lactobacillus casei* [24, 37]; cyclohexane and chloroform extracts from the leaves were active against *Bacillus subtilis*, *Staphylococcus aureus*, *Escherichia coli*, and *Pseudomonas aeruginosa* [9]; aqueous extracts of the stem bark inhibited *S. aureus*, *S. epidermidis*, *Enterococcus faecalis*, Methicillin-resistant *S. aureus* (MRSA), *Salmonella enteritidis, Shigella flexneri, E. coli*, and *Klebsiella pneumoniae* [22].

The antibacterial and antifungal activities of *L. ferrea* extracts against strains that can cause skin infections, among others, such as *Staphylococcus* sp, *Bacillus* sp, *Pseudomonas* sp, and *Candida* sp [38–43], reveal the antimicrobial potential of this species and reinforce the traditional use of stem bark powder for healing skin wounds [24]. These wounds are often associated with secondary microbial infectious processes [44]. Therefore, antimicrobial prospecting for this species can contribute to the discovery of new antimicrobial agents, which is urgent due to the emergence of antimicrobial resistance, a severe public health problem [45,46]. Additionally, the study of how seasonal changes can alter the antimicrobial effect against microorganisms of these genera can contribute to the harvesting of parts of the plant in periods where the highest production yield of its active components occurs and, thus, guarantee the safety of its use in traditional medicine [5].

The crude alcohol from the fruit showed inhibition of cell proliferation in the HT-29 tumor line of human colorectal cancer [23]. The ethanolic extract of the leaf showed cytotoxicity for human cancer lines of the liver (HepG2), breast (MCF-7), colon (HCT-116), larynx (Hep2), and prostate (PC3). These studies highlight the anti-cancer potential of *L. ferrea* [47]. However, there are still no reports on the evaluation of the cytotoxicity of *L. ferrea* extracts on C6 glioma cell, even given the severity of the glioma, which is an aggressive brain tumor, which, depending on the grade, can lead to the death of the patient with treatment within two years. Due to the resistance of Glioma cells to chemotherapeutic agents, there is an urgent need to explore the cytotoxicity of natural products against this target. One of the most used models for these tests is with C6 glioblastoma cells administered to Wistar rats [48–50].

Specimens of *L. ferrea* are distributed in Brazilian biomes such as Caatinga, Atlantic Forest, and Cerrado [9,18]. However, *L. ferrea* occurs mainly in the Northeastern Caatinga, a biome exclusive to Brazil, and occupies an area equivalent to 11 % (~844,453 km2) of the national territory [10,51,52]. This semi-arid region is characterized by low humidity and irregular rainfall, with long periods of rainfall shortage, which can reach up to about nine months, directly influencing the region's water availability, with an average annual rainfall of 800 mm. Irregular rains are concentrated between summer and autumn. Furthermore, it has shallow soils and a relative humidity of 50 %. The winter and spring seasons are relatively dry, and the periods of the greatest humidity are observed in summer and autumn. However, occasional torrential monsoons may occur in a few months of the year [53–57].

Species of *L. ferrea* are also distributed in the Atlantic Forest biome, a global biodiversity hotspot rich in biodiversity and species endemism. However, anthropogenic action has constantly threatened its conservation [58,59]. This biome is characterized by different

climates, with regions marked by the humid subtropical climate in the South, others by the tropical climate, and some that still occur very close to the Northeastern semi-arid Caatinga. Due to their proximity to the sea, these regions are subject to significant amounts of rain and strong winds. Generally, rains are concentrated at the end of summer and winter, emphasizing autumn, with little significant thermal differences in monthly averages in summer and winter [51,60].

The Caatinga and the Atlantic Forest are biomes with very different climate and vegetation characteristics; therefore, we hypothesized that the difference in the biome of origin would cause differences in the production of their secondary metabolites. Thus, through the geographical distribution of the medicinal plant *L. ferrea* in several Brazilian biomes, mainly Caatinga and Atlantic Forest and, because of the demand for research on seasonality and secondary metabolism in native species in Brazil, new studies in this field of knowledge must be developed [9].

Herein, the influence of seasonal variations in the contents of total phenolics and flavonoids, by Folin-Ciocalteu and aluminum chloride colorimetric assays, respectively, along with tannins content, by radial diffusion method, was evaluated in the ethanolic extracts of the stem bark of *L. ferrea* specimens collected in the Caatinga and the Atlantic Forest, Brazilian biomes, and also compared their metabolic profiles through multivariate analysis by hydrogen nuclear magnetic resonance. Furthermore, it was studied how seasonality interfered with the biological effects of these extracts, such as cytotoxic against the C6 glioma cell, by cell viability test with MMT; antioxidant activities by DPPH and ABTS assays and antibacterial and antifungal properties, by broth microdilution. In this study, the analytical methods chosen for the quantification of metabolites were the classic and widely used ones [61–63], as well as those used in antimicrobial [64,65] and cytotoxicity [66,67] assays.

2. Materials and methods

2.1. Chemicals

Quercetin (≥95 %), tannic acid, 2,2-diphenyl-1-picrylhydrazyl (DPPH), 2,2′-azino-bis-3-ethylbenzothiazoline-6-sulfonic acid (ABTS), (+) - 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid - Trolox (≥97 %), type I agarose and anhydrous potassium persulfate ($K_2S_2O_8$), dimethyl sulfoxide (DMSO) were acquired from Sigma-Aldrich (Aldrich Brasil Ltda., Brazil). L-ascorbic acid (≥99.0 %), ethanol PA (99.5 %), and anhydrous sodium carbonate Na_2CO_3 (≥99.5) were obtained from Synth (LABSYNTH, Brazil), while ultrapure monohydrate gallic acid and aluminum chloride (III) (≥99.5 %) from Vetec (VETEC-Sigma-Aldrich, Brazil). The Folin-Ciocalteu reagent and bovine serum albumin–BSA were acquired from Merck (Merck, USA). Glacial acetic acid PA and sodium hydroxide were obtained from Dinâmica (Dinâmica, Brazil. Nutrient broth, malt extract, yeast extract, potato extract, and agar were purchased from Acumedia (Acumendia, USA). DMSO-d_6 and TMSP-d4 (2,2,3,3-d4-3- sodium trimethylsilylpropionate) were purchased from Cambridge Isotope Laboratories, Inc. (USA). The reagents used in antimicrobial susceptibility tests were ciclopirox olamine [(LOPROX®), 10 mg g^{-1}, Sanofi-Aventis, Brazil)], DMSO [(≥99.0), Sigma-Aldrich (Aldrich Brasil Ltda., Brazil], DMEM Mix F12, penicillin G, gentamicin, amphotericin B, glucose, L-glutamine, and fetal from Gibco (Thermo Fisher Scientific, Brazil).

2.2. Plant material

Stem bark fragments from *L. ferrea* were collected in the four seasons of the year: winter, spring, summer, and autumn in the two Brazilian biomes: Atlantic Forest [municipality of Jussari, Bahia State, Brazil (15° 11′ 29″ S and 39° 29′ 43″ W), in the periods of July and December 2018 and March and June 2019] and Caatinga [São Francisco sub-middle region, in the municipality of Juazeiro, Bahia State, Brazil (09° 24′ 42″ S and 40° 29′ 55″ W), in July and November 2018 and April and June 2019]. The vouchers of the specimens were registered at Herbarium RADAMBRASIL (HBS), Jardim Botânico, Salvador, Bahia, Brazil, under the numbers 61849 and 61850 for the specimens collected in Juazeiro and Jussari, respectively (Additional information is in the supplementary material (Table S1).

2.3. Preparation of extracts

Previous studies of *L. ferrea* indicated that ethanolic extracts were antimicrobial and cytotoxic, thus justifying the choice of these extracts as extracting solvent [23,24,37,47]. Plant material fragments from *L. ferrea* stem bark were ground and dried, separately subjected to maceration with ethanol PA (99.5 %), for 72 h, with three replications [68]. The obtained alcoholic solutions were distilled under reduced pressure (580 mmHg) for the obtention of the ethanolic extract of the stem bark, which was coded according to the biome and season of each collection, as Caatinga biome: Winter (WIC); Spring (SPC); Summer (SUC) and Autumn (AUC), and Atlantic Forest biome: Winter (WIF); Spring (SPF); Summer (SUF) and Autumn (AUF). The yields of these extracts and data regarding the behavior of rain and temperature over the months in Juazeiro and Jussari–Bahia, Brazil, where the two *L. ferrea* specimens were collected, are shown in Table S1 (Supplementary data).

2.4. Multivariate analysis of the extracts by ^1H NMR

Extract sample (20 mg) solutions were prepared in 600.0 μL of DMSO-d_6, containing 0.02 % TMSP-d4 (2,2,3,3-d4-3-sodium trimethylsilylpropionate) and, subsequently, they were transferred to NMR tubes (5 mm). The NMR experiments were performed on an Agilent (Varian) Inova 500 spectrometer (Agilent Technologies, Inc., Santa Clara, USA) operating at 11.7 T, equipped with a 5-mm inverse detection with field gradient on the actively shielded z-axis. In the triplicate acquisition of ^1H NMR spectra, the spectral window (SW) was adjusted to the range from −1 to 11 ppm, with 32768 points. Sixty-four acquisitions (Number of Scans - NS) were

taken with a 7.0 μs pulse (PW), 5.5 s acquisition time (AQ), waiting time between each acquisition of 2.0 s (D1), and receiver gain factor of 34 (RG). The temperature was controlled to 300K, and TMSP-d4 was used as an internal standard (δ 0.0). The spectra were processed using the ACD/Labs Release software (version 12.01), applying exponential multiplication of FIDs by a factor of 0.3 Hz and Fourier transform of 32 k points. The phase correction was performed manually, and the baseline correction was applied throughout the spectral range. All processed ^{1}H NMR spectra were converted to a matrix that was built with the aid of the Origin software, version 8.0. The chemometric treatment was performed with the aid of the Pirouette® software, version 4.5 (InfoMetrix Inc., Bothell, Washington, USA). The parameters and the selection of variables were optimized from the exploratory analysis of data from the ^{1}H NMR spectra to obtain better results from PCA and HCA. Regarding the parameters, normalization (norm one) and autoscaling were used, excluding the solvent regions, such as residual water from suppression, DMSO-d_6, and TMSP-d4, spectral noise, and regions of the spectra that did not contribute to the discrimination result.

2.5. Quantification of tannins, total phenolics and flavonoid content

Determination of total phenolics: The methodology for the measurement of total phenolics (TP) was according to the method described by Ma et al. (2020) [69], with few modifications [70]; 20 μL of a methanolic solution of the ethanolic extract from the stem bark (1 mg mL^{-1}), 20 μL of an aqueous solution of the Folin-Ciocalteu reagent (10 % v v^{-1}) and 60 μL of sodium carbonate solution (7.5 %, m v^{-1}) were mixed, in a 96-well microplate, protected from light. The volume of each well was completed to 300 μL with distilled water. Subsequently, the mixture was incubated for 20 min, at room temperature, protected from light. The calibration curve was similarly constructed with gallic acid in the concentration range of 1.0–8.0 μg mL^{-1} and, for each point of the curve, an aliquot of the methanolic gallic acid solution (0.2 mg mL^{-1}), 20 μL of the Folin-Ciocalteu reagent (10 % v v^{-1}) and 60 μL of sodium carbonate solution (7.5 %, m v^{-1}) was mixed in a 96-well microplate, with volume adjusted to 300 μL with distilled water. Methanol was used as a blank. The 96-well plate was incubated with the standards for 20 min at room temperature protected from light. After this period, the absorbances were measured at 760 nm, using a UV/VIS microplate spectrophotometer (LMR-96-8, Loccus, Brazil). The experiment was carried out in triplicate, and the total phenolic content (TP) was expressed as mean [μg of gallic acid equivalent (GAE)/mg of dry extract] ± standard deviation of the triplicates.

Determination of flavonoid content: The methodology for measuring the total flavonoid content (FC) was according to Obeng et al. (2020) [71], with few modifications [70]. For wells of the 96-well microplate, 60 μL of the methanolic solution of the plant extract (2 mg mL^{-1}) and 6 μL of the methanolic $AlCl_3$ solution (2 % m v^{-1}) were transferred, with the volume adjusted to 300 μL, with distilled water. The microplate was incubated for 30 min and protected from light. The calibration curve was built with the 1.5–12.0 μg mL^{-1} concentration range of the quercetin standard. The absorbance readings (sample, standard, and blank) were taken after the 30-min incubation period, at 414 nm, on a UV/VIS microplate spectrophotometer (LMR-96-8, Loccus, Brazil). The experiment was carried out in triplicate. The results of flavonoid content (FC) were expressed as mean [μg of quercetin equivalent (QE)/mg of dry extract] ± SD of the triplicates.

Quantification of tannins: The tannin content (TC) was quantified by the radial diffusion method, according to Maier et al. (2017) [72], with few modifications [70]. A buffer solution was prepared with acetic acid (0.5 μmol L^{-1}) and ascorbic acid (60 μmol L^{-1}), with the addition of NaOH solution (2 mol L^{-1}) until pH = 5.0. The solid medium was prepared by mixing this buffer solution with type I Agarose, under occasional heating and stirring, until complete solubilization. After cooling this mixture to 45 °C, 0.10 g of bovine serum albumin - BSA was added, under stirring; 0.9 mL aliquots of this medium were distributed in Petri dishes (9 cm in diameter). These plates were sealed with PVC film and stored under refrigeration at 4 °C for 72 h. After this period, in each plate, 7-mm wells were made under the solid medium, to which 20 μL of a 20 mg mL^{-1} solution of 50 % plant extract (v v^{-1}) in methanol was added. The calibration curve was prepared using a tannic acid standard (2.5–50 mg mL^{-1}). The halo diameters of the extracts and standard samples were measured after 72 h of incubation at 30 °C. The experiment was performed in triplicate. The blank was 50 % methanol (v v^{-1}).

2.6. Biological activities of extracts

2.6.1. Cytotoxicity in C6 glioma cells

All extracts were tested for cytotoxic activity against the C6 glioma cell lines obtained from the Cell Bank of the Neurochemistry and Cell Biology Laboratory (LabNq), following the methodology used by Lemes et al. (2019) [73]. The extracts were dissolved in dimethyl sulfoxide (DMSO, Sigma, St. Louis, MO) at 14.3 mg mL^{-1} and filtered under pressure. The sample was dissolved in DMEM culture medium (Cultilab, SP, Brazil), supplemented with 100 IU mL^{-1} penicillin G, 100 μg mL^{-1} of streptomycin, 7.0 mM glucose, 2.0 mM L-glutamine, 0.011 g L^{-1} pyruvate and 10 % (v v^{-1}) fetal calf serum (Gibco, Grand Island, NY, USA). For cell viability, C6 glioma cells were seeded in 96-well plates at a density of 2.4 x 104 cells cm^{-2} and treated with the tested concentrations of the extracts: winter [0.7; 0.4; 0.2; 0.14; 0.07; 0.04; 0.02; 0.014; 0.007; 0.004 mg mL^{-1}]; for the summer and spring seasons [1; 0.6; 0.3; 0.2; 0.1; 0.06; 0.03; 0.02; 0.01; 0.006 mg mL^{-1}]. Cells grown in culture medium with 0.5 % DMSO (v v^{-1}) or with the medium alone were used as a control. The cells were treated for 48 h in a humidified atmosphere with 5 % CO_2 at 37 °C. After the treatment, 100 μL^{-1} of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide was added to 1.0 mg mL^{-1} to each well and the cells were incubated for 2 h at 37 °C. The media was removed, and the cells were lysed by adding sodium dodecyl sulfate, 20 % (w v^{-1}), and 50 % dimethylformamide (v v^{-1}). Absorbance was measured in a plate reader (THERMO PLATER, Brazil) at 595 nm. Cell viability was determined as the percentage of absorbance in relation to untreated controls. EC_{50} was determined by graphically representing the percentage of cell viability as a function of the log of the concentrations of the tested extracts. The equation generated in the GraphPad Prism software was used to

calculate the EC_{50}.

2.6.2. Antimicrobial assay

The microorganisms used in the tests were *Bacillus subtilis* (ATCC 6633), *B. cereus* (CCT 0096), *Staphylococcus aureus* (ATCC 6538), *S. epidermidis* (ATCC 12228), *Escherichia coli* (ATCC 94863), *Pseudomonas aeruginosa* (CCT 0090), *Salmonella choleraesuis* (ATCC 14028), *Candida albicans* (ATCC 18804), and *C. glabrata* (CCT0728), all acquired in the Tropical Cultures Collection (CCT)/André Tosello Foundation. The cultures were maintained in a solid medium: bacteria on nutrient agar and yeasts in malt extract. The bacteria and fungi cultures were incubated for 24 h (36 °C) and 72 h (26 °C), respectively. The antimicrobial activity of the extracts was evaluated by the broth microdilution method to determine the minimum inhibitory concentration (MIC), according to CLSI (2018) [74] technical standards, with minor modifications [75]. The extracts were considered active when they inhibited microbial growth at concentrations lower than the highest concentration tested (2000 μg mL^{-1}). The compounds benzylpenicillin (100 μg mL^{-1}), gentamicin (100 μg mL^{-1}), ciclopirox olamine (400 μg mL^{-1}), and amphotericin B (250 μg mL^{-1}) were used as positive controls. In the 96-well plate, from those that showed no turbidity, when reading the broth microdilution assay, 10 μL were inoculated in nutrient agar for bacteria and malt extract for yeast in Petri dishes. Subsequently, the plates were incubated at appropriate temperatures and periods for each microorganism. After incubation, the lowest concentration tested, in which there was no visible microbial growth, the minimum bactericidal concentration (MBC) and minimum fungicidal concentration (MFC) were determined [70].

2.6.3. Antioxidant activities

2.6.3.1. DPPH assay. The DPPH assay was performed according to the Mercado-Mercado et al. (2020) [76], with modifications [70]; 280 μL of the DPPH stock solution (80 μmoL L^{-1}) and 20 μL of the extract solution were added to a 96-well plate. Extract concentrations in the range from 10 to 1.67 μg mL^{-1} were used to plot the IC_{50} values. The plates were incubated at room temperature in the dark for 30 min, and the absorbances were obtained at 515 nm. The calibration curve was constructed by adding 280 μL of the DPPH radical solution (80 μmoL L^{-1}) and aliquots of the trolox standard solution (0.2 mg mL^{-1}), completing the volume of 300 μL with methanol for the construction of the calibration curve in the concentration range of 1.0–6.0 μg mL^{-1}. After 30 min of incubation, the absorbance was measured at 515 nm in a UV/VIS microplate spectrophotometer (LMR-96-8, Loccus, Brazil). The experiment was carried out in triplicate.

2.6.3.2. ABTS assay. The antioxidant capacity by the ABTS test was determined according to the method of Le Grandois et al. (2017) [77]. The cation of the ABTS radical (ABTS•+) was formed by reacting ABTS solutions (7 m mol L^{-1}) with potassium persulfate (2.45 mmol L^{-1}). This reagent was stored and protected from light at room temperature for 16 h. Subsequently, the ABTS•+ solution was diluted with methanol until it reached an absorbance in the range of 0.8–1, at 734 nm, in a UV/VIS microplate spectrophotometer (LMR-96-8, Loccus, Brazil); 280 μL of the stock solution of the cationic radical ABTS•+ and 20 μL of the extract solution were added to the 96-well plate. Concentrations of 10–1.67 μg mL^{-1} of the plant extracts were used to plot the IC_{50} values. The calibration curve in the range of 1.0–10 μg mL^{-1} was constructed by mixing 280 μL of the radical solution and different aliquots of the stock solution of the trolox standard (0.5 mg mL^{-1}) plus methanol to complete the volume of the wells (300 μL). After 20 min of incubation, the absorbance was measured at 734 nm by a UV/VIS microplate spectrophotometer (LMR-96-8, Loccus, Brazil). All experiments were carried out in triplicate.

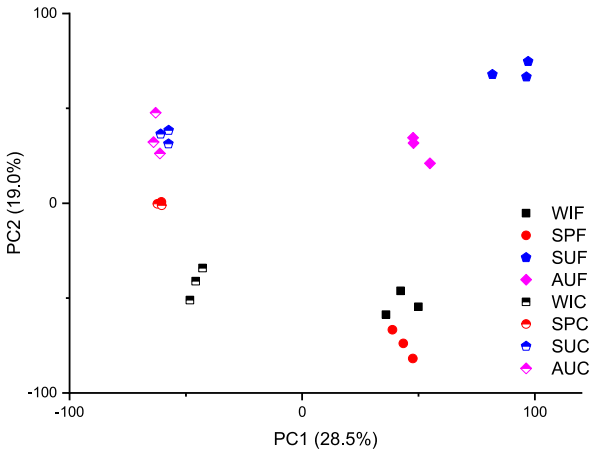

Fig. 1. PCA score plot (PC1 × PC2) for the ^1H NMR spectra of the extracts (47.5 % variance). The grouping of the samples represents the similarity relationship of the composition of extracts [Ethanolic extracts from the stem bark of the biomes: **Caatinga**: Winter (WIC); Spring (SPC); Summer (SUC) and Autumn (AUC) and the **Atlantic Forest**: Winter (WIF); Spring (SPF); Summer (SUF) and Autumn (AUF)].

2.6.3.3. Statistical analysis. Statistical analysis was performed using IBM Statistical Package for the Social Sciences Statistics® (International Business Machines - IBM) and Microsoft® Excel 2010 programs (Microsoft). Analysis of variance was used to identify statistically significant differences between the samples ($P < 0.05$), followed by Tukey's multiple comparison tests. The results are presented as the mean of replicates ± standard deviations. Paired correlations between different response variables were also conducted to confirm the existing correlations.

3. Results and discussion

The principal component analysis (PCA) of the ^{1}H NMR of L. ferrea stem extracts, which were obtained in all seasons of the year in each biome (Caatinga e Forest Atlantic), suggested the metabolic variations with two main discriminating grouping patterns concerning geographical origin, where the negative PC1 scores refer to those of the Caatinga and the positive ones to those of the Atlantic Forest (Fig. 1). Extracts from the Caatinga showed differences in chemical composition when compared to those from the Atlantic Forest in the same seasons, as indicated by the distances between the points on the PCA plot (Fig. 1). Furthermore, ^{1}H NMR spectra of ethanolic extracts indicated that extracts from the Caatinga (Figs. S1A, S1B, S1C and S1D, Supplementary Data) showed more intense signals in the region of aromatic hydrogens. At the same time, those from the Atlantic Forest were more intense in the region of aliphatic hydrogens (Figs. S2A, S2B, S2C, and S2D, Supplementary Data). These results were also confirmed by the dendrogram for hierarchical cluster analysis (HCA) (Fig. 2).

In both biomes, the metabolic variances between the extracts in the four seasons are more significant for the Atlantic Forest than for the Caatinga, with greater dispersion of the triplicates of the extracts. The seasons in the Atlantic Forest are well defined and the climatic variations associated with seasonal changes can justify the dispersions of the triplicates in the extracts. In the Caatinga, the seasons are not well defined, with rainy and dry periods; thus, the slight variations observed for this biome directly influence the lower dispersion in the chemical composition of the extracts. In the sample group from the Caatinga, the groupings in the dendrogram show similarity only between those of Autumn/Spring, while for the Atlantic Forest, groupings exist between the extracts of Autumn/Summer and Spring/Winter (Figs. 1 and 2).

Additionally, these results arise from the different environmental conditions of each biome studied. The climates of the Atlantic Forest and Caatinga biomes are humid tropical and semi-arid, respectively. Therefore, the Atlantic Forest has an annual rainfall of around 1500 mm, with precipitation well distributed throughout the year, with rainier periods at the end of summer, autumn and winter; while the Caatinga biome is characterized by scarce rainfall, with irregular distribution throughout the year, high temperatures, with annual precipitation of around 250–700 mm, more concentrated in late spring, summer and autumn. Consequently, relative humidity is low in the Caatinga [51,78,79].

All ethanolic stem extracts from *L. ferrea* obtained in the four seasons, winter (WI), spring (SP), summer (SU), and autumn (AU), in the Caatinga and Atlantic Forest, showed the respective average percentage values of total phenolic, flavonoid and tannins contents [(32.69 ± 2.16 %; 326.93 ± 21.62 μg mg^{-1} of gallic acid equivalent, GAE); (0.59 ± 0,02 %; 5.97 ± 0.25 μg mg^{-1} of quercetin equivalent, QE) and (29.92 ± 2.91 %; 299.20 ± 29.11) μg mg^{-1} tannic acid equivalent, TAE)] (Table 1), following values obtained by Araújo et al. (2014) [22] and Comandolli-Wyrepkowski et al. (2017) [80], who estimated the polyphenolic content of stem extracts

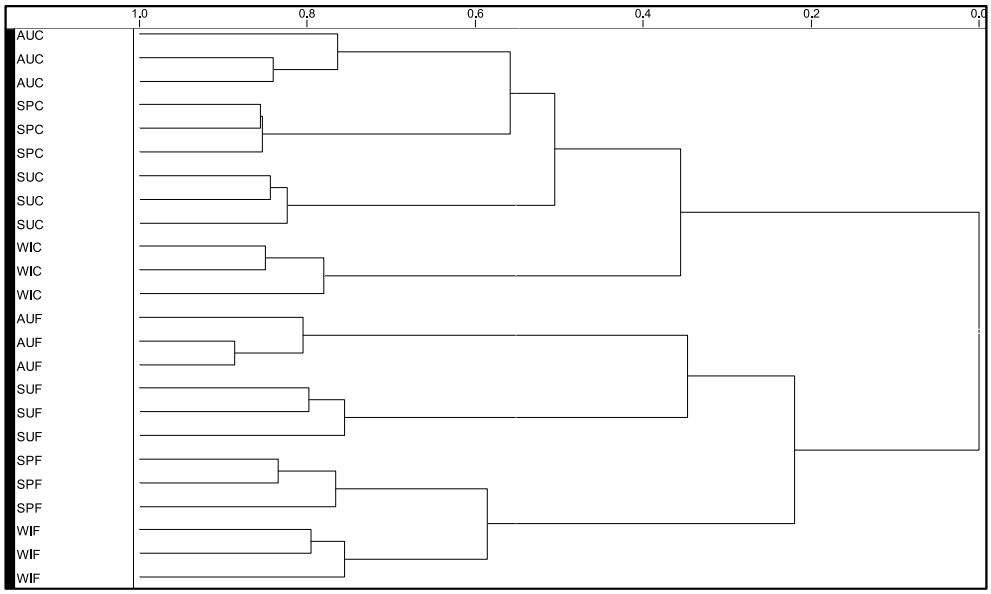

Fig. 2. HCA dendrogram obtained from ^{1}H NMR spectra representing the similarity relationship of the composition of extracts (similarity index of 0.55) [Ethanolic extracts from the stem bark of the biomes: **Caatinga**: Winter (WIC); Spring (SPC); Summer (SUC) and Autumn (AUC) and the **Atlantic Forest**: Winter (WIF); Spring (SPF); Summer (SUF) and Autumn (AUF)].

Table 1
Total phenolic, flavonoid and tannin contents and antioxidant activity of the ethanolic extracts from *L. ferrea* stem bark.

Extracts[a]	Contents (μg mg^{-1} ± SD)			Antioxidant activity (IC_{50}, μg mL^{-1} ± SD)	
	Phenolics	Flavonoids	Tannins	DPPH	ABTS
WIC	328.82 ± 33.81 $^{(aA)}$	4.89 ± 0.25 $^{(aC)}$	308.25 ± 62.85 $^{(aA)}$	7.93 ± 0.10 $^{(aA)}$	6.72 ± 0.08 $^{(bA)}$
SPC	327.95 ± 10.53 $^{(aA)}$	5.96 ± 0.10 $^{(aB)}$	326.37 ± 27.23 $^{(aA)}$	7.60 ± 0.08 $^{(aB)}$	5.96 ± 0.08 $^{(bC)}$
SUC	336.34 ± 18.1 $^{(aA)}$	7.36 ± 0.19 $^{(aA)}$	328.38 ± 30.19 $^{(aA)}$	7.58 ± 0.08 $^{(bB)}$	4.86 ± 0.05 $^{(bD)}$
AUC	332.00 ± 12.8 $^{(aA)}$	7.75 ± 0.31 $^{(bA)}$	278.06 ± 17.43 $^{(aA)}$	7.23 ± 0.09 $^{(bC)}$	7.34 ± 0.07 $^{(bA)}$
WIF	342.71 ± 16.27 $^{(aA)}$	4.23 ± 0.11 $^{(bC)}$	328.38 ± 30.19 $^{(aA)}$	8.08 ± 0.09 $^{(aB)}$	9.41 ± 0.11 $^{(aC)}$
SPF	350.24 ± 20.86 $^{(aA)}$	3.15 ± 0.11 $^{(bD)}$	338.45 ± 17.43 $^{(aA)}$	6.82 ± 0.08 $^{(bD)}$	9.47 ± 0.11 $^{(aC)}$
SUF	271.5 ± 31.4 $^{(bB)}$	5.70 ± 0.38 $^{(bB)}$	217.67 ± 17.43 $^{(bB)}$	9.17 ± 0.10 $^{(aA)}$	12.66 ± 0.12 $^{(aA)}$
AUF	301.9 ± 29.18 $^{(aAB)}$	8.75 ± 0.55 $^{(aA)}$	268.06 ± 30.19 $^{(aAB)}$	7.64 ± 0.09 $^{(aC)}$	9.99 ± 0.11 $^{(aB)}$
Mean value	326.93 ± 21.62	5.97 ± 0.25	299.20 ± 29.11		
Trolox				4.34 ± 0.05	6.03 ± 0.06

Different letters in parentheses indicate significant differences between samples by Tukey's HSD ($P < 0.05$). Lower case letters: Comparison of biome samples at the same stations. Capital letters: Comparison of samples in each biome.

[a] Ethanolic extracts from the stem bark of the biomes: **Caatinga**: Winter (WIC); Spring (SPC); Summer (SUC) and Autumn (AUC) and the **Atlantic Forest**: Winter (WIF); Spring (SPF); Summer (SUF) and Autumn (AUF). μg mg^{-1} of GAE: micrograms of gallic acid equivalent per milligram of dry extract; (μg mg^{-1} QE: micrograms of quercetin equivalent per milligram of dry extract) and μg mg^{-1} of TAE: micrograms of tannic acid equivalent per milligram of dry extract).

aqueous and methanol extracts stem of the 30.47 % and 32.21 %, respectively.

Between both biomes, the total levels of phenolics were significantly different only in the summer [336.34 ± 18.10 μg mg^{-1} GAE (Caatinga) and 271.50 ± 31.4 μg mg^{-1} GAE (Atlantic Forest)] (Table 1), according to the Tukey test at 98 % confidence level. It was also only in the summer that the content of tannins [(328.38 ± 30.19 μg mg^{-1} TAE (Caatinga) and (217.67 ± 17.43 μg mg^{-1} TAE (Atlantic Forest)] (Table 1) presented significant differences between the considered biomes, also according to the Tukey test at 98 % confidence level. Metabolites derived from phenolics, such as gallic, elastic, and caffeic acids, among others, have already been identified in the stem of *L. ferrea* [36].

In the summer, abiotic stresses in the Caatinga are more significant than in the Atlantic Forest, with higher temperatures and a lower rainfall rate, which influence the metabolism of the phenylpropanoid pathway, increasing the production of phenolic compounds in this season to protect the plant against high solar radiation [81]. Additionally, when a species is subjected to abiotic or biotic stress, several genes are activated or deactivated, resulting in changes in the levels of various metabolites [11,78,79,81].

The highest levels of flavonoids in the two biomes were obtained in autumn extracts [Caatinga: AUC (7.75 ± 0.31 μg mg^{-1} QE) and Atlantic Forest: AUF (8.75 ± 0.55 μg mg-1 QE)]. However, these outcomes had no significant differences at 98 % confidence level (Tukey test). On the other hand, the results indicated that the specimens' geographic distribution influenced the biosynthesis of flavonoids during the winter, spring, and summer. In these seasons and according to the Tukey test (at 98 % confidence level), all Caatinga extracts showed higher levels of flavonoids, especially in summer [7.36 ± 0.19 μg mg^{-1} QE], followed by winter and spring, than those of the Atlantic Forest (Table 1). These results follow previous studies that show the activation of flavonoid biosynthesis when plant species are exposed to high UV radiation, long periods of drought, and low precipitation [82–85]. In this sense, the stress effects that reach the Caatinga also favor metabolic pathways for producing flavonoids. Although extracts from the Atlantic Forest had lower flavonoid contents than those from Caatinga, significant differences in levels of flavonoids were noted in all seasons studied, with

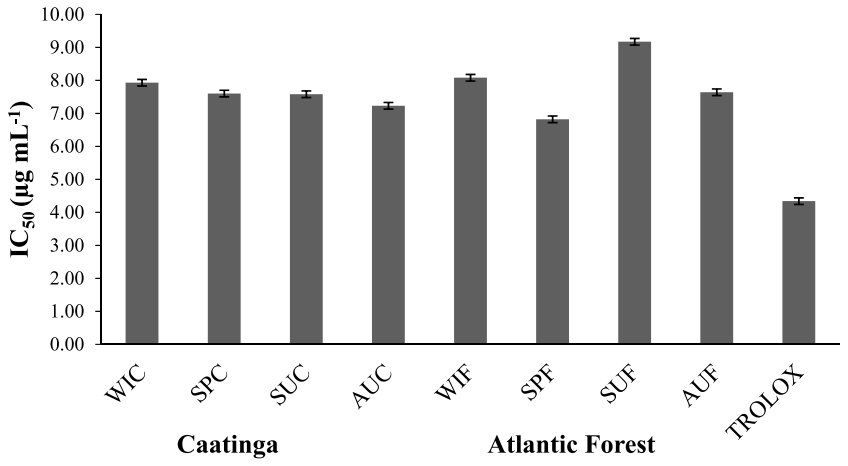

Fig. 3. Antioxidant activity of the ethanolic extracts from *L. ferrea* stem bark, by DPPH assay, [**Caatinga**: Winter (WIC); Spring (SPC); Summer (SUC) and Autumn (AUC) and the **Atlantic Forest**: Winter (WIF); Spring (SPF); Summer (SUF) and Autumn (AUF)].

higher values in autumn and summer. In a previous study, flavonoids have already been isolated from the stem bark of *L. ferrea*, such as vitexin, orientin, isorientin, and isovitexin, among others [27].

The correlations between the values of phenolics, flavonoids and total tannins, in the different extracts, were provided by Pearson's correlation coefficients. In these samples, the levels of total phenolic are not correlated with that of flavonoids, as already observed ($R^2 = 0.1434$) (Fig. S3A, Supplementary Data). However, there was a greater correlation between the total phenolic content and tannins ($R^2 = 0.8617$) (Fig. S3B, Supplementary Data). In turn, a much more adequate correlation between these two levels was obtained by removing the sample of autumn extract in the Caatinga ($R^2 = 0.967$) (Fig. S3C, Supplementary Data). Previous studies indicate the prevalence in the biosynthetic route for the production of tannins, in relation to that of flavonoids, in this species [29,30,36].

The antioxidant activity of *L. ferrea* stem bark extracts was expressed at a concentration capable of inhibiting DPPH and ABTS radicals by 50 % (IC_{50}) (Table 1, Fig. 3 and Fig. 4). The IC_{50} values of the stem extracts for the DPPH radical varied significantly in the studied biomes throughout all seasons except for winter extracts (Caatinga). The extracts with the most significant antioxidant effect were those from autumn [7.23 ± 0.09 μg mL^{-1}] (Caatinga) and spring [6.82 ± 0.08 μg mL^{-1}] (Atlantic Forest), however, these extracts showed lower antioxidant capacities than the standard (IC_{50} troloxDPPH $= 4.34 \pm 0.05$ μg mL^{-1}).

In the ABTS free radical scavenging method, the IC_{50} values of the extracts in all seasons in the studied biomes showed significant differences, with the strongest antioxidant activity obtained for those from Caatinga, with an IC_{50} value range of 4.86 at 7.64 μg mL^{-1}, compared to the Atlantic Forest. The most antioxidant extracts were summer (IC_{50} of 4.86 ± 0.05 μg mL^{-1}) in the Caatinga and winter (IC_{50} of 9.41 ± 0.11 μg mL^{-1}) in the Atlantic Forest. The results of antioxidant activity by DPPH of all stem bark extracts in both biomes are correlated with the total levels of phenolics ($R^2 = 0.6077$) (Fig. S4D, Supplementary Data) and tannins ($R^2 = 0.4515$) (Fig. S4E, Supplementary Data). However, there is an increase in the correlation with the phenolic content ($R^2 = 0.9202$) (Fig. S4F, Supplementary Data) when the winter and autumn extracts are removed in the Atlantic Forest. Similar behavior is observed for antioxidant activity and total tannin content when those of autumn, Caatinga, and winter, Atlantic Forest, are excluded from the correlation analysis ($R_2 = 0.7563$) (Fig. S4G). The antioxidant effect of the extracts, by ABTS, showed little correlation with the levels of phenolics ($R^2 = 0.3795$) (Fig. S5H, Supplementary Data) and tannins ($R^2 = 0.4261$) (Fig. S5I, Supplementary Data).

The cytotoxicity of all extracts was evaluated in the C6 glioma cells of rats. The EC_{50} values were obtained for extracts that showed cytotoxic effects in a preliminary screening. Of all the extracts screened, only those from the Atlantic Forest (winter, spring, and summer) were cytotoxic. The extracts collected in the Atlantic Forest showed selectivity concerning cytotoxicity against C6 glioma cells, with satisfactory results for those of winter, spring, and summer, with EC_{50} values of 0.13, 0.23 and 0.5 mg mL^{-1}, respectively (temozolomide = EC_{50} of 560 μmol L^{-1}) (Fig. 5). The selectivity of the extracts from the Atlantic Forest agrees with the study of metabolic variation through the analysis of PCA and HCA, which showed two distinct patterns of secondary metabolites in the two biomes. The cytotoxic effect is directly linked to the chemical composition of the extracts. Although polyphenolic compounds are considered responsible for the biological activities of *L. ferrea*, the cytotoxicity in C6 glioma cells of extracts from the Atlantic Forest could not be related tothe presence of these compounds. This statement is valid because the extracts from the Caatinga were those with the highest phenolic, tannins and flavonoids contents, and were not cytotoxic to C6 Glioma. Other classes of metabolites, such as terpenes, have already been identified in the stem of *L. ferrea* [36]. Compounds from this class have shown cytotoxicity against glioma cells [86,87].

The screening of ethanolic extracts from *L. ferrea* stem for cytotoxicity in C6 glioma cells was evaluated for the first time in this study. However, extracts from different parts of *L. ferrea* have already been tested in other cancer cell lines [23,25,47]. The aqueous ethanol extract of *L. ferrea* leaves showed a cytotoxic effect against the HepG2 (liver), Hep2 (larynx), MCF-7 (breast), and HCT-116

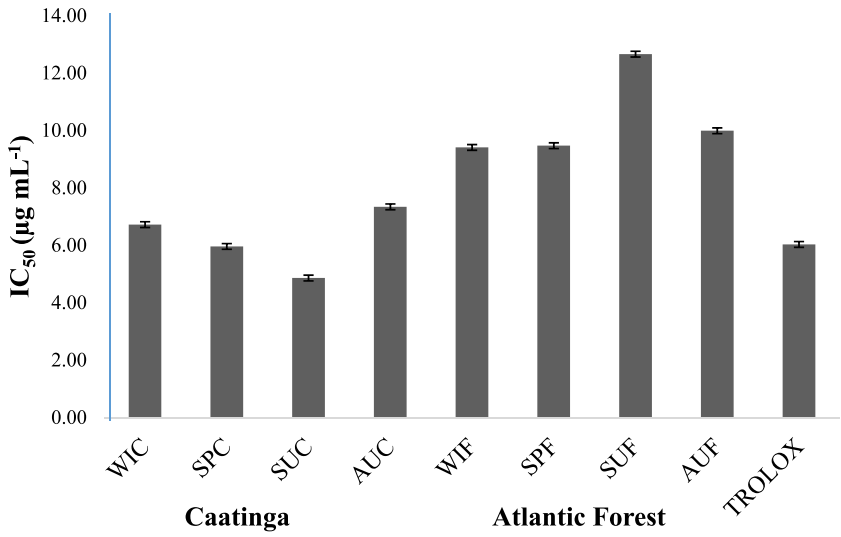

Fig. 4. Antioxidant activity of the ethanolic extracts from *L. ferrea* stem bark, by ABTS assay, [**Caatinga**: Winter (WIC); Spring (SPC); Summer (SUC) and Autumn (AUC) and the **Atlantic Forest**: Winter (WIF); Spring (SPF); Summer (SUF) and Autumn (AUF)].

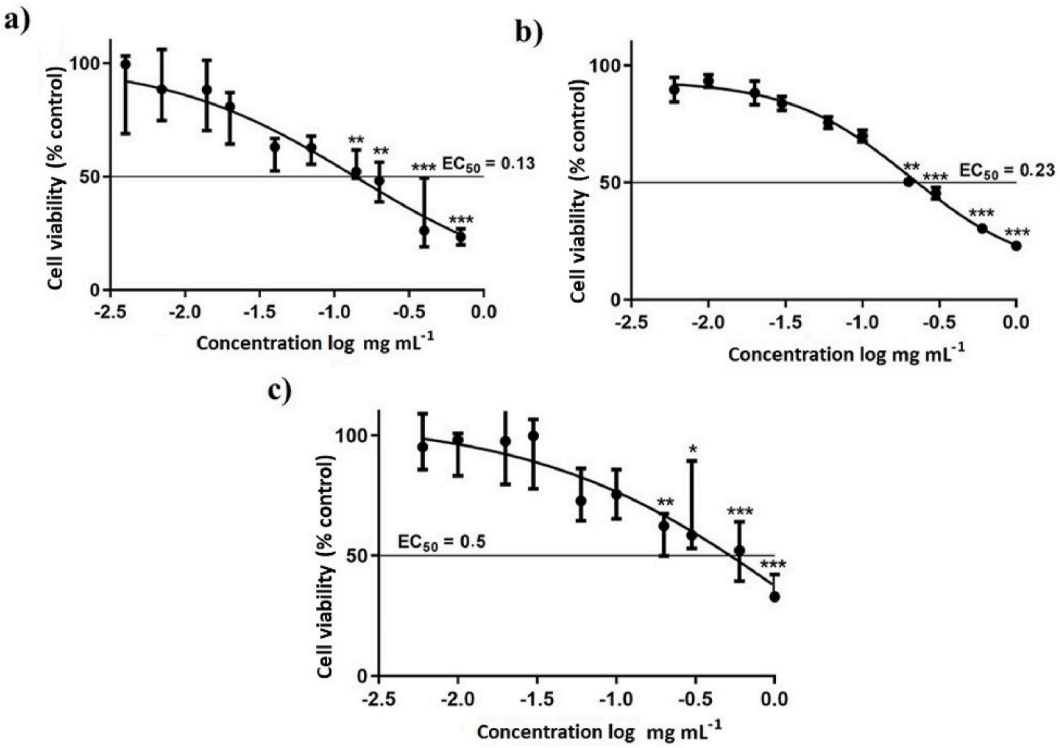

Fig. 5. Cell viability curve of the C6 glioma cells after treatment with *L. ferrea* stem extracts from the Atlantic Forest, obtained in winter (a); spring (b) and summer (c).

(colon) cell lines, with IC_{50} values of 19.3 μg mL^{-1}, 20 μg mL^{-1}, 21.8 μg mL^{-1}, 24.47 μg mL^{-1}, respectively [47]. Crude extracts of the fruits of *L. ferrea*, obtained in ethanol 60 % v v^{-1} and 80 % v v^{-1}, showed inhibition of proliferation in HT-29 cell line (human colorectal), respectively, of 50 % (dose 25 mg mL^{-1}) and 43.7 % (dose of 12.5 mg mL^{-1}) [23]. Although these previous studies corroborate the anticancer potential of *L. ferrea* extracts, the results cannot be compared with those obtained in the present study because the extracts evaluated in the previous studies are from different parts of the plant and different cell lines used.

Regarding the antimicrobial activity, among the gram-positive bacteria, *Bacillus cereus* and *Staphylococcus aureus* were sensitive to all stem extracts tested (Table 2). However, the extracts obtained from the Atlantic Forest were selective for *S. epidermidis*, with higher effect bactericidal effect from winter (MIC = 125.0 μg mL^{-1}; MBC = 500.0 μg mL^{-1}) and spring (MIC = 62.5 μg mL^{-1}; MBC = 1000 μg mL^{-1}) (Table 2). Regarding Gram-negative bacteria, only extracts from the Caatinga biome showed an effect against *Escherichia coli* and *Pseudomonas aeruginosa*. Of these, the one from the summer season was more bacteriostatic and bactericidal against *P. aeruginosa* (MIC = 500 μg mL^{-1}; MBC = 2000 μg mL^{-1}) (Table 2). The extracts most enriched in total phenolics and tannins were also the most active for Gram-positive bacteria in both biomes.

Extracts in cyclohexane, chloroform, ethyl acetate, and methanol from the leaves of *L. ferrea* (collected in the State of Pernambuco, Brazil) were active against Gram positive bacteria, such as *S. aureus* and *B. subtilis*. Aqueous extract of *L. ferrea* fruits (Pernambuco, Brazil) showed an effect against *S. aureus* [18]. Ethanolic extract of the fruit and seed of *L. ferrea* (collected in the State of Amazonas, Brazil) were antibacterial against *Streptococcus mutans* [28]. Ethanolic extract from the leaves of *L. ferrea* (collected in the State of Ceará, Brazil) and ethanolic extract from the inner bark of *L. ferrea* (Pernambuco, Brazil) did not inhibit *S. aureus* [36]. Although these previous studies report the antibacterial activities of *L. ferrea* extracts, these cannot be compared with our results due to differences in the geographic distribution of the specimens and the part of the plant selected for antimicrobial studies. Previous studies of extracts from different parts of *L. ferrea* suggest that phenolic compounds, such as hydrolyzable tannins, are responsible for the antimicrobial effects of these species' extracts [10,18,28,37].

Although the present study has generated substantial results, it is limited to just one year of study. Therefore, sampling over a more extended period, over a few years, would confirm whether the results obtained are part of a trend.

4. Conclusions

The habitat of *L. ferrea* species influenced the biosynthesis of phenolics, tannins, and flavonoids, as well as the antioxidant activity and cytotoxicity in C6 glioma cells. Caatinga extracts had the highest phenolics, tannins, and flavonoids levels and the best antioxidant effects. Given the therapeutic potential of *L. ferrea*, which has generally been attributed to polyphenolics, the results indicated that Caatinga extracts would offer more outstanding quality and safety in use and preparation of pharmaceutical products for treating

Table 2
Antimicrobial activity of *L. ferrea* stem extracts.

[a]Extracts	MIC/MBC and MFC (μg mL^{-1})													
	Gram-positive								Gram-negative				Fungi	
	Sa		*Sep*		*Bs*		*Bc*		*Pa*		*Ec*		*Ca*	
	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC	MIC		MIC	MFC
WIC	500	1000			1000	1000	500	–	500	–	1000		2000	–
SPC	500	1000			2000	–	250	–	500	–	1000		–	
SUC	500	1000			500	2000	250	–	500	2000	1000		–	
AUC	500	1000			1000	–	250	–	500	–	1000			
WIF	250	500	125	500	500	500	250	–						
SPF	250	500	62.5	1000	500	500	250	–						
SUF	250	1000	1000	2000	2000	2000	250	–						
AUF	500	500	1000	2000	2000	–	250	–						
Benzylpenicillin	0.04	>25	2.2	>25	0.08	0.16	25	25						
Gentamicin									1.25	>5	0.63	>5		
Ciclopirox olamine													12.5	>25

[a] Ethanolic extracts from the stem bark of the biomes: **Caatinga**: Winter (WIC); Spring (SPC); Summer (SUC) and Autumn (AUC) and the **Atlantic Forest**: Winter (WIF); Spring (SPF); Summer (SUF) and Autumn (AUF). *Sa- Staphylococcus aureus; Sep- Staphylococcus epidermidis; Bs-Bacillus subtilis; Bc- Bacillus cereus; Ec- Escherichia coli; Pa- Pseudomonas aeruginosa; Ca- Candida albicans*. Initial concentration: benzylpenicillin - 25 μg mL^{-1}; gentamicin - 5 μg mL^{-1}; ciclopirox olamine - 25 μg mL^{-1}; - no effect on the highest concentration tested (2000 μg mL^{-1}).

diseases. Extracts from the Atlantic Forest showed selectivity for Gram positive bacteria. In addition to geographical distribution, the results suggest that the summer offers the best conditions to activate the biosynthesis of phenolics and tannins. In turn, autumn, followed by summer, were the seasons in which flavonoids were most produced in both biomes. The antibacterial effects of stem extracts in both biomes, especially against the bacteria *S. aureus*, *S. epidermidis*, and *P. aeruginosa*, corroborated the traditional use of *L. ferrea* bark powder for treating skin wounds.

Data availability statement

This manuscript and its supplementary material contain all the results obtained from our work. Therefore, there was no data sharing in any available public repository. However, we may make any data available upon request.

CRediT authorship contribution statement

Aiane Nascimento Santana: Methodology. **Júlia Oliveira Tanajura Mendes:** Methodology. **Madson de Godoi Pereira:** Conceptualization. **Yasmin Almeida Alvarenga:** Methodology. **Elisangela Fabiana Boffo:** Investigation, Formal analysis. **Florisvaldo da Silva Ramos:** Methodology. **Ramon Santos El-Bachá:** Resources, Investigation, Conceptualization. **Floricéa M. Araújo:** Supervision, Conceptualization. **Suzimone de Jesus Correia:** Investigation, Formal analysis, Conceptualization. **Maria Herbênia Lima Cruz Santos:** Data curation. **Caline Gomes Ferraz:** Resources, Formal analysis. **Paulo R. Ribeiro de Jesus:** Supervision, Resources, Investigation, Conceptualization. **Lourdes C. de Souza Neta:** Writing – review & editing, Methodology, Investigation, Formal analysis, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.heliyon.2024.e30632.

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