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THE ASSESSMENT OF THE SEASONAL PHYTOCHEMICAL COMPONENTS, ANTIMICROBIAL AND TOXICITY ACTIVITY OF HYDROALCOHOLIC LEAF EXTRACTS OF E. FLORIDA USED IN BRAZILIAN FOLK MEDICINE

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Abstract: Eugenia florida DC., Myrtaceae, is used in folk medicine for gastrointestinal diseases, although its biological activities have rarely been studied. The goal of this study is to assess the seasonal phytochemical components, antimicrobial activity, and toxicity effects of hydroalcoholic leaf extracts of E. florida (EEF) to provide a scientific basis for its use. Phytochemical screening and Thin Layer Chromatography (TLC) profiling of EEF confirmed the presence of various phytochemicals, such as flavonoids, triterpenoids, saponins, and steroids. Total phenolic content (TPC) was assessed using the Folin-Ciocalteu method to evaluate the presence and levels of phenolic compounds in each sample. The summer EEF exhibited the highest TPC value, 34.4 mg GAE/g, and showed good DPPH (2,2-diphenyl-1-picrylhydrazyl) radical scavenging activity, with an average inhibitory concentration (EC50) of 16.03 mg/mL. The antimicrobial activity of EEF was evaluated against twelve bacterial species and three yeasts using the agar dilution and minimum inhibitory concentration (MIC) methods. In vitro antimicrobial activity demonstrated moderate inhibitory effects on Gram-positive bacteria such as Staphylococcus epidermidis and S. aureus (MIC 0.25 mg/mL). Mutagenic and toxicity activities were evaluated by the Ames mutagenicity assay in Salmonella typhimurium and the Artemia salina test. No mutagenic or toxic effects were observed in EEF. TLC analysis of triterpenoids revealed nine spots, with betulinic acid (BA) as the major compound, likely responsible for the biological activities described. BA was isolated by column chromatography and identified by spectroscopy and chromatographic methods (IR, NMR, and mass spectrometry). The presence of BA in the crude extract was quantified using high-performance liquid chromatography (HPLC). It can be concluded that E. florida leaves could be used in the treatment of bacterial gastroenteritis infections.

Keywords: *Eugenia florida*, phytochemical screening, antimicrobial activity, absence of mutagenicity, DPPH-Radical scavenging, NMR.

INTRODUCTION

According to the World Health Organization, approximately 80% of the global population utilizes medicinal and/or herbal plants for the treatment or prevention of various health conditions, with usage rates in developing countries like Brazil potentially reaching as high as 95% (KHAN AND AHMAD, 2019). However, the biological activities and toxicological effects of many of the plants commonly used in traditional and folk medicine have yet to be rigorously validated through scientific research (YUAN *et al.,* 2016; MOREIRA *et al.,* 2014).

In Brazil, the population frequently turns to folk medicine for their health needs, addressing physical, mental, and spiritual concerns. A significant portion of the population lacks access to conventional pharmacological treatments, which leads to a reliance on folk remedies. This ecological awareness often fosters a misleading belief that "all natural products are harmless" (HEYDARI *et al.,* l2022; MELRO *et al.,* 2019; KHAN and AHMAD, 2019). However, some herbal remedies can be inherently toxic due to their constituents and may provoke adverse reactions when misused.

Indeed, many individuals use herbal medicines without adequate scientific understanding and proper guidance. Herbal medicines are derived from plants in various forms, including teas, decoctions, tinctures, syrups, powders, and others. This diversity in preparation methods underscores the necessity for comprehensive studies to ensure the safety and efficacy of these traditional remedies.

It is noteworthy that Eugenia species possess numerous biological and therapeutic properties, such as anti-inflammatory, antidiarrheal, antibacterial, cytotoxic, antioxidant, antitumoral, trypanocidal, antiviral, hypoglycemic, and insulin-stimulating effects (CARNEIRO *et al.,* 2019; MOURA *et al.,* 2018; LIMA *et al.,* 2011; BENFATTI *et al.,* 2010; CONSOLINI and SARUBBIO, 2002).

While some species, such as *Eugenia uniflora* and *Eugenia caryophyllata*, have been extensively studied, there is a notable lack of research on the phytochemical composition and pharmacological properties of *Eugenia florida* DC. This gap in research presents an opportunity for further investigation to elucidate the potential therapeutic benefits and biological activities of this species (BASTOS *et al.,* 2019; SANTOS *et al.,* l2018; QUEIROZ *et al.,* 2015).

Among the wide diversity of medicinal plants popularly prepared, the 'garrafada' has been as one of the most popular in Brazil. This preparation consists in grounded single or mixed species left to infusion in wine or in cachaça, which, is a Brazilian alcoholic beverage with 38-48% alcoholic strength (PASSOS *et al.,* 2018). In fact, the 'garrafada' of *Eugenia* genus leaves have been used in the region northern of Brazil by ingestion for gastrointestinal disorders such as diarrheas (MOURA *et al.,* 2018). The local knowledge of this preparation has been passed on from generation to generation. In this method, the active constituents of the herbal materials are usually processed together with others which cause undesirable effects (OGBONNA *et al.,* 2012). Keeping this in view, this study aims to evaluate the chemical composition, *in vitro* antimicrobial activities, toxicity effects and free radical scavenging capacities of hydroalcoholic extract of *E. florida* leaves.

MATERIALS AND METHODS

PLANT MATERIAL

Healthy leaves of *E. florida* trees were collected once per season during 2016-2017 from Rio de Janeiro Botanical Garden (*Jardim Botânico do Rio de Janeiro*-JBRJ, located at -22°58'2.45"S 43°13'24.67"W and about sea level). The voucher of the specimen was deposited in Herbarium of JBRJ (RB 336.681) and in Herbarium of Green Pharmacy of Farmanguinhos, Fiocruz (FFAR – 443).

PREPARATION OF THE EXTRACTS

The leaves collected at each season were kept at room temperature for 48 hours in a cool dry place followed by drying in an oven at 40°C ± 2°C. The dried leaves were pulverized and 5g of powder material was submitted to extraction by maceration in 100 ml of 50%v/v water:ethanol solution allowed to stand for a period of at least 3 days. The yield in the average 840 mg (16.8%) of the hydroalcoholic (50%, V/V) extract of *E. florida* leaves (EEF), after filtration by Whatman no.1 filter paper and drying rotary evaporator. The EEF was submitted to chemical and biological assays.

QUALITATIVE PHYTOCHEMICAL SCREENING

For phytochemical characterization of major secondary metabolites by Thin-Layer Chromatography (TLC), extracts were applied on Kieselgel GF254 plates. Two different solvent systems were used hexane/ethyl acetate and ethyl acetate/methanol as eluents at volume ratios of 1:4, 1:1 and 4:1 were performed in duplicate. Spots were visualized by UV irradiation (254 nm) before and after spraying of anisaldehyde sulfuric, ceric sulfate, natural products polyethylene glycol reagent (NP/PEG, 1% diphenylboryloxyethylamine in methanol + 5% polyethylene glycol-4000 in

ethanol), 3% Ferric (III) Chloride in methanol/water and sulfuric vanillin reagents to ascertain about the presence of main chemical groups of secondary metabolites. Besides, the presence of steroids, triterpenoids, saponins, flavonoids, tannins, and alkaloids were all checked out using selective reagents through prescribed methods: Liebermann Burchard, Foam Test, Shinoda, Ferric Chloride Test, and Dragendorff (COSTA *et al*, 2010; RATHORE *et al*, 2012).

TOTAL POLYPHENOL CONTENT BY FOLLIN CIOCALTEU

The Folin-Ciocalteu assay (Zhang *et al*, 2006) was performed in well of 96-well microplates to determine the total phenolic content (TPC) of the hydroalcoholic EEF (FERNANDES *et al*, 2017). On 96 well plate 20 µL of crude extracts and 300 µg/mL gallic acid (GA) calibration solutions (3.12, 6.25, 12.5, 25.0, 50.0, and 75.0 µg/mL) were independently added in triplicate to 100 µL of Folin-Ciocalteu reagent (0.2 N) and incubated at room temperature for 5 min, followed by the addition of 80 µL of 7.5% (w/v) sodium carbonate. The mixture was allowed to stand in the dark at room temperature for a further 120 min, and then measurements of absorbance were performed at 760 nm using the SpectraMax M5 Microplate Reader. The total phenolic content was calculated from the calibration curve which was built from response of six different work solution 3.12, 6.25, 12.5, 25.0, 50.0 and 75.0 µg/mL in methanol of gallic acid. The results were analysed by linear regression using the least squares method to calculate the coefficient of correlation ($y = 0.0047x + 0.026$, $R^2 = 0.9994$).

FREE RADICAL SCAVENGING

In free radical scavenging capacity by DPPH method was performed in 96 well plates (FERNANDES *et al*, 2017). Methanolic solution of the extracts and of trolox (positive control) were prepared at 2, 4, 10, 20, 30 and 50 µg/ml from dilution of stock methanolic solutions at 1 mg/mL. 100 µL was mixed in triplicate with same volume of a recently prepared methanolic solution of DPPH. The mixture was kept at room temperature in the dark for 30 minutes before absorbance reading at 517 nm.

ANTIMICROBIAL ACTIVITY

(1) Micro-organisms and growth conditions

Strains of twelve pathogenic bacteria and three yeasts strains applied in this assay were preserved and activated at the Department of Microbiology of DCB/ENSP/FIOCRUZ (Rio de Janeiro, Brazil): *Escherichia coli* ATCC 25922 and ATCC 135218, *Pseudomonas aeruginosa* ATCC 27853, *Enterococcus faecalis* ATCC 29212 and ATCC 51299, *Klebsiella pneumoniae* ATCC 700603, *Proteus mirabilis* ATCC 7002, *Proteus vulgaris* ATCC 8427, *Staphylococcus aureus* ATCC 25913 and ATCC 29213, *Staphylococcus epidermidis* ATCC 12228, *Streptococcus pyogenes* ATCC 19615, *Candida parapsilosis* ATCC 22019, *Candida glabrata* ATCC 2350, *Candida albicans* ATCC 10231. All bacteria were suspended in Brain Heart Infusion broth (BHI) (Difco, Detroit, USA) and the yeasts in Sabouraud dextrose broth (SDB, Difco). Then, the turbidity was adjusted with 0.5 McFarland standards units to equivalent to 10^8 colony-forming units per milliliter (CFU/ml) for bacteria and 1.5 $x10^6$ CFU/mL for fungal strains. They were flood–inoculated onto the surface of BHI and of Sabouraud Dextrose Agar (SDA, Difco). The assays were performed for each microorganism.

(2) Determination of Minimal Inhibitory Concentration (MIC)

Agar Dilution Methodology

The agar dilution method was used as a screening test for the antimicrobial activity of an extract, where the desired amount of extract is incorporated into the culture medium. The extract was mixed with Mueller-Hinton agar so that the concentration of the extract was 3 mg/mL, 2 mg/mL, 1 mg/mL, 0.5 mg/mL, and 0.25 mg/mL, respectively (COSTA *et al.,* 2010). Each extract, from each day, was performed in triplicate, resulting in nine assays. In each 60 mm plate, it was possible to test four strains with 10μL spots. The incubation was carried out in a bacteriological incubator at approximately 35°C for 24 and 48 hours. The lowest dilution where no growth was observed for the strain was defined as the Minimum Inhibitory Concentration (MIC).

Mueller Hinton Methodology

The MIC were determined in triplicate using the Mueller Hinton broth microdilution assay in 96 well-plates according to NCCLS (2012). The bacterial strains grown on nutrient agar at 35±2ºC for 18 to 24 h were suspended in a saline solution (0.9% w/v) to a turbidity of 0.5 McFarland standards. The bacterial suspensions were diluted to inoculate 96 well-plates containing serial dilutions of extracts ranging from 0.06 to 2.0 mg/mL. The final volume in the wells was 100 µl. Ciprofloxacine as an antibacterial and amphotericin B as antifungal positive (2 µg/plate) and negative saline growth controls were performed. The plates were incubated at 37ºC for 24 h. The lowest concentration capable of inhibiting microbial growth was considered the minimal inhibitory concentration (MIC).

ECOTOXICITY TEST BY *A. SALINA* LEACH

The *Artemia salina* toxicity test was performed by adapting the methodology of Meyer *et al.* (1982), in which a saline solution (30 g/L^{-1}) with pH between 8.0 and 9.0 adjusted by 0.1 mol/L NaOH solution was used for hatching *A. salina* eggs and to prepare the other dilutions. After stabilization, 3 g of *A. salina* cysts were allowed to hatch for 48 hours, with constant aeration at 25°C.

Six different concentrations of EEF (500 to 3500 µg / mL) were tested in triplicate by placing 4.0 mL 0.9% saline, 200 µL *A. salina* suspension (contained 10 shrimp larvae) and 50 µL of the extract dilution. The tubes were maintained for 24 hours at 28ºC under fluorescent lamp, then dead and alive animals were counted. The 50% lethal dose (LD50) calculation toxicity was considered low 500 µg/mL, moderate for LD50 between 100 to 500 µg/mL and very high when the LD50 < 100 µg/mL (NGUTA and MBARIA, 2013).

AMES MUTAGENICITY TEST

Ames test was performed to determine the punctual mutagenic potential of EEF on *S. enterica serovar Typhimurium* strains TA97, TA98, TA100, TA102 e TA104 (Table 1). The strains were incubated at 37°C for 16 h in Luria broth (LB) agar with ampicillin (25 µg/mL) and tetracycline (2 µg/mL) until it reached the stationary phase (1-2 x 10^9 cel/mL). 100 µL of each bacterial suspension and 100 µL of extract dilution were added to 500 µL of sodium phosphate buffer (0.2 M, pH 7.4). In tubes were then incubated at 37°C for 20 min. Two mL of surface agar containing histidine and biotin (10%) solution at 45°C was added to the test tube and the final mixture was poured onto a Vogel-Bonner Agar petri dish. After solidification of the agar dispersion the plates were incubated at 37°C for 72 h (MARON and AMES, 1983). To determine spon-

Strain	Mutation[1]		Resistance[2]		Requirement[3]		Spontaneus Reversion/plate[4]	
	Rfa	*uvrB*	Ampi^R	Tetra^R	His	Bio	[-]	[+]
TA97	+	+	+	-	+	+	80-215	>400
TA98	+	+	+	-	+	+	20-55	>100
TA100	+	+	+	-	+	+	100-240	>500
TA102	+	-	+	+	+	-	100-400	>800
TA104	+	+	+	-	+	+	150-500	>1000

Table 1. Main phenotype of *S. enterica serovar Typhimurium* strains.

[1]The mutation are detected in rfa and uvrB genes; [2]Ampi^R - Ampicillin resistance and Tetra^R - Tetracycline resistance; [3] histidine (His) and biotin (Bio) requirement and [4]spontaneous reversion rates ([-]) and induced ([+]); phenotype positive (+); phenotype negative (-).

taneous and induced reversion activity.The His+ revertant colony-forming units (CFUs) were counted manually. A sample was deemed positive or mutagenic when the number of revertant observed in the assay was at least twice the number of spontaneous revertant, as seen in the negative control. This resulted in a mutagenicity index (MI) of greater than or equal to two (MI ≥ 2).

IDENTIFICATION AND CHARACTERIZATION OF BETULINIC ACID (BA)

The EEF was analyzed by HPLC-UV-DAD using the method (slightly modified) described by Taralkar and Chattopadhayay (2012). Analysis HPLC was performed in triplicate using a Sulpelcosil-LC 18 column (250mmx4.6mm; 5μm particle size) on LC 20ADXR system consisted of an autosampler, and SPD-M20A diode array detector. The gradient mobile programming was carried with acetonitrile and water acidified with TFA (0.05% v/v) at flow rate of 1.0mL/min^{-1} in methanol. The retention times and diode array spectra (205 nm) were used to identification betulinic acid (BA). Quantitation of BA determination was carried out by an external standard of BA (Sigma Aldrich) method based on peak areas. Analytes were detected by Electrospray Ionisation Mass Spectrometry (ESI-MS) on a quadruple time-of-flight (Q-TOF) operated in negative ion mode.

The melting point was measured using digital equipment (model MQAPF-302) with a heating rate of 5°C/*min*.

The Fourier Transform Infrared (FTIR) spectrum was recorded on a Thermo Scientific Nicolet 6700 FTIR spectrometer, using the Attenuated Total Reflectance (ATR) accessory with Germanium crystal and Deuterated Triglycine Sulfate (DTGS KBr) standard detector. Spectral data were collected in the range of 4000 to 525 cm^{-1}, with 32 scans and 4 cm^{-1} resolution.

^{1}H and ^{13}C nuclear magnetic resonance (NMR) spectra of BA were recorded on a spectrometer, model Avance 500 (Bruker, Bremen, Germany), 11.5 T, operating at the Larmor frequency at 500 MHz for ^{1}H and 125 MHz for ^{13}C. The spectra were acquired in chloroform-*d* at 22.0 ± 0.1 °C using 30 pulses (12.5 µs for ^{1}H and 7.0 µs for ^{13}C) and a 5 mm switchable probe. ^{1}H NMR spectra were acquired by 1024 scans with a relaxation delay of 2.0 s, 16 K data points, 8278.1 Hz spectral width using a digital resolution of 0.30 Hz. The ^{13}C spectra were acquired by 386,440 scans with 23,980.8 Hz spectral width using a digital resolution of 1.0 Hz and 32 K data points. The chemical shifts (δ) were reported in ppm, using tetramethylsilane (TMS) as the reference and internal standard. ^{1}H-^{1}H correlation spectroscopy (COSY), ^{1}H-^{13}C heteronuclear single quantum correlation (HSQC) and, heteronuclear multiple-bond correla-

tion (HMBC) were conducted using standard Bruker software. Gradient selections, zero filling and/or linear predictions were used in all 2D techniques.

RESULTS AND DISCUSSION

PHYTOCHEMICAL SCREENING

The classes detected in the phytochemical screening of EEF leaves are summarized in Table 2. The results indicated that flavonoids, triterpenoids, and saponins tested positive using Shinoda's test, Liebermann-Burchard reagent, and the foam test, respectively, across all seasons for EEF leaf samples. However, the Liebermann-Burchard test revealed the presence of steroids only in EEF leaf samples collected during the summer, suggesting that this class is present at low levels. Among the tested classes, tannins were not detected, which may be attributed to the sensitivity of the assay. These findings are consistent with existing literature, which has identified these classes of secondary metabolites, particularly flavonoids and saponins, in summer samples (BASTOS *et al*., 2016; BASTOS *et al*.,2019).

Previous of the Eugenia genus have indicated high levels of phenolic compounds, including flavonoids, phenolic acids, tannins, saponins, and terpenoids (CARNEIRO *et al*., 2019).

The phytochemical screening of the EEF extracts using thin-layer chromatography (TLC) confirmed the presence of subclasses of tannins, flavonoids, saponins, and several triterpenoids, as indicated by specific coloration reactions (Table 3). Alkaloids were not detected in the TLC assay. Notably, some spots corresponding to triterpenes in the extracts exhibited the same Rf values as those of the betulinic acid (BA), oleanolic acid (OA), and ursolic acid (UA) reference standards. The TLC assay proved sensitive for identifying tannins in the samples, further corroborating the phytochemical investigation of Eugenia species.

Environmental factors such as precipitation, temperature, and nutrient deficiency are primary causes of abiotic stress, which can influence not only the chemical composition but also the plant's anatomy (DONATO and MORRETES, 2009). The authors noted that the phytochemical composition depends on the maturity of the follicles. For instance, various triterpenes, particularly betulinic acid, have been observed in higher concentrations in mature leaves or those exposed to sunlight (GIL *et al*., 2012), characteristics that apply to the specimens under study.

TPC ANALYSIS

The total phenolic content (TPC) of the leaf extracts from summer and winter demonstrated seasonality dependence (FERNANDES *et al.,* 2017). The results indicated that the total phenolic content is significantly higher in the summer extract (34.4 mg GAE/g) compared to the winter extract (23.6 mg GAE/g), with statistical significance noted ($p<0.05$, t-test) (Table 4). The TPC obtained in this study is comparable to that found in hydroalcoholic extracts (70% V/V) of E. florida leaves collected during the summer, which reported a TPC of 25.8 mg GAE/g (BASTOS *et al.,* 2016).

In another prior study, a crude ethanol extract from air-dried leaves of *E. florida* reached a higher TPC of 12.7 mg quercetin-equivalent (QE)/g. Flavonols and dihydroflavonols, both in glycosylated and aglycone forms, were identified in flavonoid-rich fractions of this material through LC-MS analysis (SANTOS *et al.,* 2018). Consequently, the higher TPC observed in the extracts of the present study, obtained using a hydroalcoholic solvent, can be attributed to the amphiphilic nature of the solvent, which is likely to extract a greater quantity of phenolic compounds from the same organ of Eugenia species.

Compounds	Test performed	Season*			
		Summer	Autumn	Winter	Springer
Alkaloids	Dragendorff's	-	-	-	-
Tannins	$FeCl_3$	-	-	-	-
Flavonoids	Shinoda	+	+	+	+
Triterpenoids	Liebermann Burchard	+	+	+	+
Saponins	Foam	+	+	+	+
Steroids	Liebermann Burchard	+	-	-	-

Table 2. Qualitative phytochemical screening of hydroalcoholic (50%v/v) of EEF leaves.

* Sign indicates the presence (+) or not detected (-) of the chemical constituents.

Phytochemical Class	Reference compound	Solvent system	Detecting reagent	Rf value	Color spot[1]
Alkaloid	Pilocarpine	toluene:EtOAc: Diethylamine (7:2:1, v/v/v)	Dragendorff	nd	nd
Taninn	Geraniline and Catechin	EtOAc:AcOH:HCOOH:H_2O (100:11:11:27, v/v/v/v)	$FeCl_3$, 1%, MeOH; Vanillin sulfuric and heating		Orange (1)
Flavonoid and phenolic acid	Tylirosid and Rutine	EtOAc:HCOOH:AcOH:H_2O (100:11:11:27, v/v/v/v)	NP/PEG, UV light (365 nm)	0.58, 0.65	Orange (3), yellow(1)
Triterpenoid	Betulinic acid, ursolic acid, oleanolic acid	n-hexane:EtOAc (8:2, v/v)	*Anisaldehyde–sulfuric* acid reagent and heating		Violet (9)
Steroid	Cholesterol	n-hexane:EtOAc (8:2, v/v)	Anisaldehyde–sulfuric acid reagent and heating		purple

Table 3. Phytochemical screening by thin layer chromatography of EEF (50% v/v) leaves collected in all seasons.
[1] In parenthesis number of spots observable. nd, not detected. EtOAc, ethyl acetate; AcOH, acetic acid; HCCOH, formic acid.

FREE RADICAL SCAVENING ACTIVITY

Species Eugenia genus, such as *E. pyriformis, E. bimarginata*, and *E. dysenteric*, are recognized for their high levels of phenolic compounds as well as significant antioxidant activity (TAKAO *et al.,* 2015; DAZA *et al.,* 2016). In this context, the antioxidant activity of EEF was evaluated based on its ability to scavenge free radicals using the DPPH (2,2-diphenyl-1-picrylhydrazyl) assay, a widely used method to estimate the scavenging potential of natural products.

The hydroalcoholic extracts of EEF from both winter and summer seasons exhibited an effective concentration (EC50) for free radical scavenging in a 100 mM DPPH solution. The observed values indicated high antioxidant activity, achieving scavenging effects that were at least 2 to 3-fold greater than that of the reference standard, Trolox (EC50 6.6 ± 0.2 µg/mL). The EC50 values for the winter extract were noted at 17.0 ± 0.2 µg/mL, while the summer extract demonstrated a comparable EC50 value of 16.0 ± 0.2 µg/mL.

These results are slightly higher than those reported by Bastos (BASTOS *et al.,* 2019), which obtained an EC50 of 12.77 µg/mL for a hydroalcoholic extract (70% V/V) of leaves from the same species.

ANTIMICROBIAL ACTIVITY

The antimicrobial activity against twelve bacteria and three yeast strains was screened using the microdiluition assay. The highest sensitivity (MIC 0.25 mg/ml) was obtained on the Gram-positive bacteria, *Staphylococcus epidermidis* and *S. aureus* followed on the others gram positive bacteria at MIC 0.5 and 1.0 mg/ml (Table 4). Other species of the genus *Eugenia* L., such as *E. umbelliflora* and *E. uniflora*, also presented low activity against

Gram negative bacteria with MICs greater than 1 mg / mL (DE SOUZA *et al.*, 2018). The maceration time of 7, 14, 21 days and seasonality were also evaluated did not show significantly change bioactivity against the bacterial and yeast strains tested.

These results justify the traditional knowledge of activity of *E. florida* leaves by folk medicine as therapeutic agents in gastrointestinal diseases due to bacterial strains, like *S. aureus*.

ARTEMIA SALINA TEST

To check the toxicity potential, six concentrations of EEF (500-3500 μg/mL) were applied in the *Artemia salina* test.

After 24 hours in treatment with the extract, the average of lethality of each extract concentration the triplicates was performed with the larvae of *Artemia salina*, and the result obtained indicated the LD50 of 2500, which corresponds to five times the low toxicity limit proposed by the same author, which demonstrates a positive result for medical use, since the toxicity is considered low when the lethal dose 50% (LD50) is greater than 500 μg / mL.

AMES TEST

The spontaneous and induced reversion responses of five S. Typhimurium strains in the Ames test, applied to hydroalcoholic extracts of EEF, are summarized in Table 5. The mutagenicity indexes for all tested concentrations across all strains were less than 2.0, indicating that this extract did not induce point mutagenicity.

No other studies have been identified in the literature that performed the Ames test on the species *E. florida*, suggesting the novelty of this research. Additionally, previous research on medicinal plants indicates that few species have been evaluated for their mutagenic potential. One such study utilized the Ames methodology on *Qualea parviflora*, a medicinal plant commonly used for its antiseptic and anti-inflammatory properties, which reported negative results for mutagenicity of its extract (MAZZOLIN *et al.,* 2010).

However, it is important to note that we cannot conclude that all plants within the same genus share the same mutagenic potential. For instance, *Qualea multiflora* and *Qualea grandiforma* exhibited mutagenic activity when evaluated using the Ames test (ARAÚJO *et al.,* 2016). The comparison between these two studies illustrates the variability in mutagenic potential among different species within the same genus, emphasizing the necessity for comprehensive safety evaluations of each plant.

ISOLATION AND CHARACTERIZATION OF BETULINIC ACID (BA)

The TLC screening, utilizing sulfuric anisaldehyde spray and UV irradiation (at both 254 nm and 365 nm), revealed nine distinctive spots, indicating that betulinic acid (BA) was the predominant compound in the crude extract. Consequently, isolating and characterizing BA became our primary objective.

The raw extracts were analyzed using HPLC-UV-DAD, employing a slightly modified methodology based on the work of Taralkar and Chattopadhayay (2012). The gradient mobile phase consisted of acetonitrile and water, which was acidified with trifluoroacetic acid (TFA) at a concentration of 0.05% v/v. The flow rate was maintained at 1.0 mL/min. Quantitative determination of BA was performed using the external standard method, which is based on the peak areas observed in the chromatograms.

The results indicated that the highest concentration of BA (5.5%) was found in leaves collected during the summer, while the lowest concentration (1.9%) was observed in samples collected during the winter.

Through the gradient of $CHCl_3$:MeOH from 99.5:0.5 to 0:100 using column chromatography, an amorphous white powder was successfully isolated .

Micro-organisms	Minimum Inhibitory Concentration (mg/ml)	
Gram-negative	Agar dilution assays	microdilution
Escherichia coli (ATCC 25922)	>3 (>3, >3, >3)	2 (2,2,2)
Escherichia coli (ATCC 135218)	>3 (>3, >3, >3)	2 (2,2,2)
Pseudomonas aeruginosa (ATCC 27853)	>3 (>3, >3, >3)	2 (2,2,2)
Proteus vulgaris (ATCC 8427)	2 (2,2,2)	2 (2,2,2)
Proteus mirabilis (ATCC 7002)	2 (2,1,2)	2 (2,2,2)
Klebsiella pneumoniae (700603)	3 (3,3,3)	2 (2,2,2)
Gram-positive		
Staphylococcus epidermidis (ATCC 12228)	1.0 (1.0, 0.5, 1.0)	0.25 (0.25, 0.25, 0.25)
Staphylococcus aureus (ATCC 29213)	0.5 (0.5, 0.5, 0.5)	0.5 (0.5, 0.5, 0.5)
Staphylococcus aureus (ATCC 25923)	0.5 (0.5, 0.5, 0.5)	0.25 (0.25, 0.25, 0.25)
Enterococcus faecalis (ATCC 29212)	1.0 (1.0, 1.0, 1.0)	1.0 (1.0, 1.0, 1.0)
Enterococcus faecalis (ATCC 51299)	2.0 (2.0, 2.0, 1.0)	1.0 (1.0, 1.0, 1.0)
Streptococcus pyogenes (ATCC 19615)	1.0 (1.0, 1.0, 1.0)	0.5 (0.5, 0.5, 0.5)
Yeast		
Candida parapsilosis (ATCC 22019)	2.0 (2.0, 2.0, 2.0)	2.0 (2.0, 2.0, 1.0)
Candida glabrata (ATCC 2350)	2.0 (2.0, 2.0, 2.0)	2.0 (2.0, 2.0, 2.0)
Candida albicans (ATCC 10231)	2.0 (2.0, 2.0, 2.0)	1.0 (1.0, 1.0, 1.0)

Table 4. Minimum Inhibitory Concentration of hydroalcoholic EEF leaves.

Concentration in µg/ml	TA97	TA98	TA100	TA102	TA104
0.0	63 ± 0 (1.0)	24 ± 2 (1.0)	122 ± 16 (1.0)	526 ± 60 (1.0)	509 ± 50 (1.0)
0.1	95 ± 7 (1.5)	21 ± 2 (0.9)	123 ± 8 (1.0)	576 ± 4 (1.1)	542 ± 33 (1.1)
1	108 ± 0 (1.7)	21 ± 2 (0.9)	124 ± 4 (1.0)	516 ± 20 (1.0)	539 ± 4 (1.1)
10	Cytotoxic	Cytotoxic	128 ± 4 (1.0)	513 ± 22 (1.0)	557 ± 25 (1.1)
100	-	-	128 ± 1 (1.0)	586 ± 33 (1.1)	602 ± 22 (1.2)
1000	-	-	131 ± 14 (1.1)	602 ± 39 (1.1)	749 ± 22 (1.5)
PC	155 ± 30	110 ± 6	1568 ± 85	4000 ± 380	1858 ± 160

Table 5. Reversion per plate and mutagenic index (MI) of hydroalcoholic *EEF* of summer extract in *Salmonella typhimurium* strains

Mean values ± Standard Devition (Mutagenic Indexes) of Histidine + colonies reversitant strains of *Salmonella enterica* serovar Typhimurium. SD: standard deviation; PC: positive controls; Positive controls: N-4-nitroquinoline oxide (1.0 µg/plate) for TA97 and TA98; sodium azide (1.0 µg) for TA100; mitomycin C (0.5 µg) for TA102; methylmethane sulfonate (250.0 µg) for TA104.

The uncorrected melting point of the isolated compound was found to be 274-275°C. High-resolution ESI-MS mass spectrometry in the negative mode revealed a mass peak corresponding to the structural ion [M-H] at 455, which is consistent with the expected molecular weight of the compound. This mass spectrum data supports the identification of the compound and aligns with its anticipated properties based on the isolated characteristics.

Further analysis using IR and NMR spectroscopy will provide additional structural elucidation and confirmation of the compound's identity (Figure 2).Parte superior do formulário

The BA FTIR spectrum showed a weak band at 3452 and 3560 cm^{-1} due to ν_{O-H} from COOH group. The medium band at 2939 and 2870 cm^{-1} were assigned as ν_{CH3}. The band at 1682 cm^{-1} was attributed to the $\nu_{C=O}$ stretching frequency and the medium band at 883 cm^{-1} to $R_2C=CH_2$.

The structure of BA was confirmed by 1 and 2D NMR spectra. The ^{13}CNMR and DEPT 135 spectra (125 MHz) in $CDCl_3$ showed resonances for 6 methyl groups, 6 methines, 11 methylene, and 7 quaternary carbons.

The chemical shifts of protons were assigned through analysis of 2D spectra HSQC and HMBC. The ^{1}H NMR spectrum of the isolated triterpene revealed the presence of six methyl singlets at δ_H 0.75 (H-24), 0.82 (H-25), 0.94 (H-26), 0.97 (H-23), 0.98 (H-27) and δ_H 1.69 (H-30) ppm an allylic methyl group. It also showed signals due to terminal methylene protons (H-29) at δ_H 4.74 (d, J = 2.4 Hz, 1H) and 4.61 (td, J = 2.8, 1.4 Hz, 1H). The signals observed at δ_H 3.19 (dd, J = 11.2, 5.0 Hz, 1H) and 2.99 (ddd, J = 11.4, 9.9, 4.4 Hz, 1H) are assigned to H-3 and H-19, respectively (Figure 3). While the signals at δ_H 2.20 (ddd, J = 12.8, 11.5, 3.7 Hz, 1H) was attributed to H-13 and δ_H 2.27 (dt, J = 3.0, 13.0 Hz, 1H) to H-16. The ^{1}H chemical shifts at range 1.1-1.7 ppm were strongly overlapped in according to A-E rings of lupan skeleton.

The proposed assignment to identify the BA was confirmed by DEPT 135 and HSQC ($^{1}J_{C,H}$) spectra. The quaternary carbons C-4 (d_C 38.9), C-8 (d_C 40.7), C-10 (d_C 37.2), C-14 (d_C 42.4), C-17 (d_C 56.3), C-20 (d_C 150.4) and C-28 (d_C 180.4) were assigned from DEPT 135 and with adjacent protons in HMBC spectra (Figure 4). The latter, in HMBC spectrum, the methyl hydrogens at δ_H 0.75 (H-24) and 0.97 ppm (H-23) showed long range correlation to C-4 at 38.9 ppm ($^{2}J_{C,H}$). The H-25 signal at δ_H 0.82 was correlated to C-10 at d_C 37.2 ($^{2}J_{C,H}$). H-26 (δ_H 0.94) is coupled to C-8 at d_C 40.7 ($^{3}J_{C,H}$) and H-27 (δ_H 0.98) showed long range coupling with C-8 ($^{3}J_{C,H}$) and C-14 at 42.4 ppm ($^{2}J_{C,H}$). The H-16 at δ_H 2.26 is coupled to signal at 56.3 ppm, assigned as C-17. Furthermore, there is a clear coupling of hydrogens H-29b (δ_H 4.74, d, J = 2.4 Hz, 1H), H-19 (δ_H 2.99, ddd, J = 11.4, 9.9, 4.4 Hz, 1H) and H-30 at 1.69 (s, 3H) ppm to signal at 150.4 ppm (C-20). Finally, the C-28 signal at 180.4 ppm is a carbonyl that showed cross coupling with H-16 ($^{3}J_{C,H}$) and a signal around 1.97 ppm assigned as H-22.

CONCLUSION

These the phytochemical screening of the hydroalcoholic (50% v/v) extract from *E. florida* leaves are consistent with the literature surrounding the Eugenia genus. The chemical profile was examined using phytochemical screening and TLC, which identified common constituents in the leaves, including flavonoids, saponins, triterpenoids, and steroids. No significant variation in the phytochemical profiles was observed.

The summer leaf extract displayed a high total phenolic content but demonstrated lower antioxidant capacity as measured by the free radical scavenging activity using the DPPH IC50 method compared to the winter extract. This suggests that while the total phenolic content was elevated in summer leaves, the efficacy in scavenging free radicals was reduced.

Additionally, the EEF exhibited strong antibacterial potential, effectively inhibiting the growth of gram-positive bacteria such as *Staphylococcus aureus* and *S. epidermidis*, with moderate activity against *Enterococcus faecalis* and *Streptococcus pyogenes*. These results support the traditional use of *E. florida* in folk medicine for treating gastrointestinal diseases.

Furthermore, our results regarding the mutagenic and toxicological effects suggest that EEF could be employed safely and effectively by the Brazilian population.

The isolated triterpene was purified using chromatographic techniques, and the characterization based on IR, MS, and both 1D and 2D NMR data, along with comparisons to existing literature, allowed for the identification of the triterpene as betulinic acid.

DISCLOSURE STATEMENT

The authors have no conflicts of interest to declare

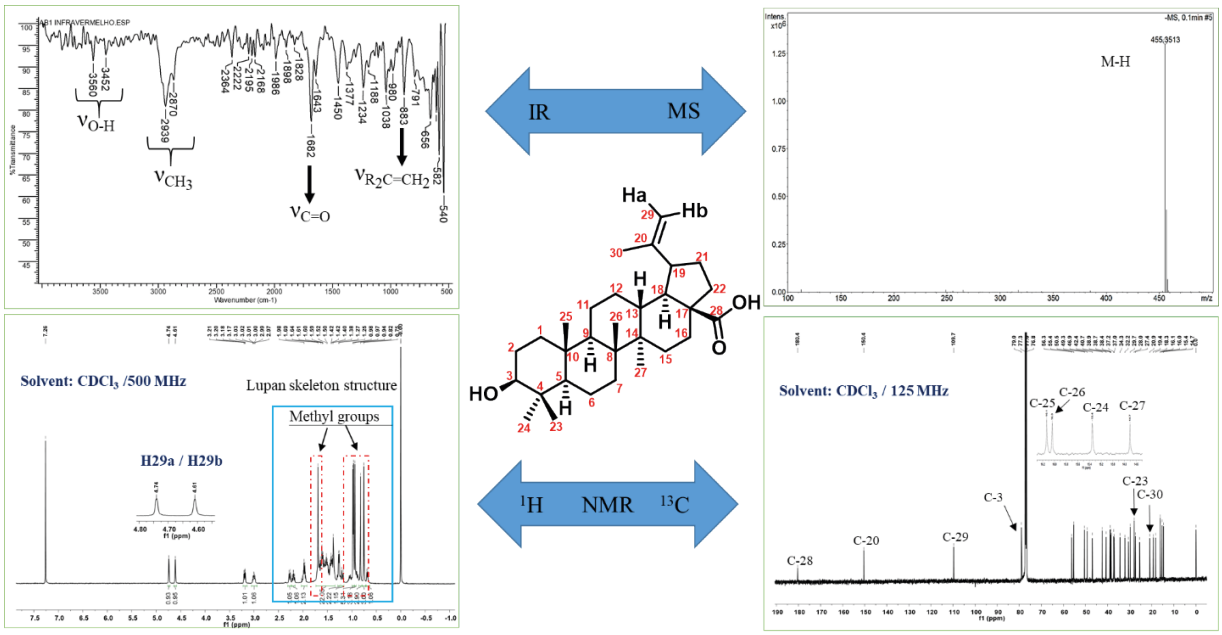

Figure 2. Characterization spectroscopic of betulinic acid isolated from EEF by IR, MS, ^{1}H and ^{13}C NMR.

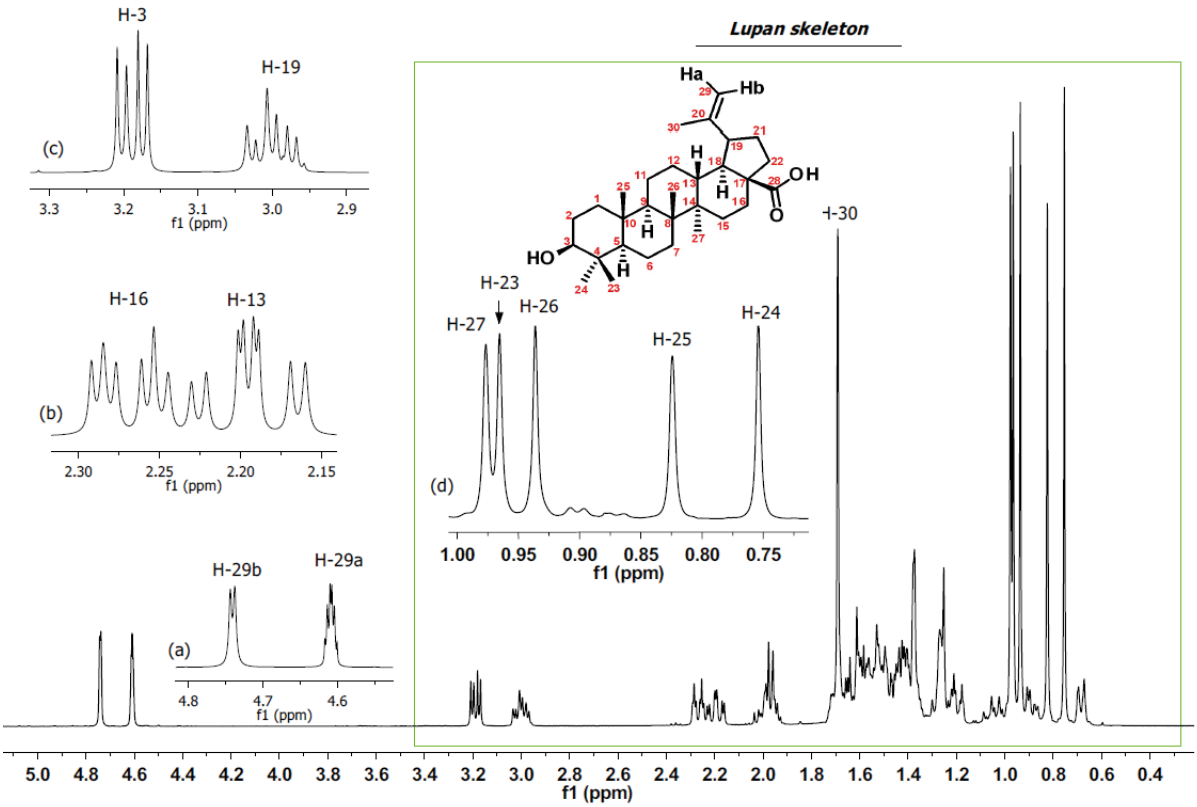

Figure 3. ^{1}H NMR spectrum of the isolated triterpene from EEF (BA). Insets (a), (b) and (c) are expansions with assignments indicated; the unassigned lines are in accordance with a lupan skeleton.

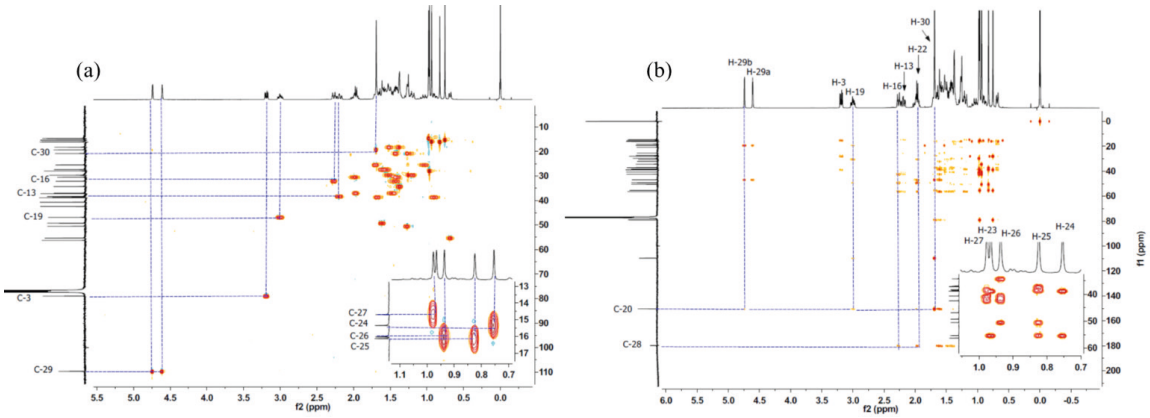

Figure 4. Main correlation observed in 2D NMR data assignment of the isolated compound from EEF (500 MHz, $CDCl_3$): (a) HSQC ($^1J_{C,H}$) and (b) HMBC ($^{2,3}J_{C,H}$) spectra.

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