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Original Article

# *Eugenia uniflora*: a promising natural alternative against multidrug-resistant bacteria

## *Eugenia uniflora*: uma alternativa natural promissora contra bactérias multirresistentes

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**Abstract**

This work aimed to evaluate the chemical composition, antioxidant and antimicrobial activities from crude extract and fractions from leaves of *Eugenia uniflora* Linn. The crude extract was obtained by turbo extraction and their fractions by partitioning. Chromatographic analysis were performed, and the antioxidant capacity was verified by two methods (DPPH$^{\bullet}$ and ABTS$^{\bullet+}$). The Minimal Inhibitory/Bactericidal Concentration were conducted against twenty-two bacteria, selecting five strains susceptible to extract/fractions and resistant to the antibiotics tested. Ampicillin, azithromycin, ciprofloxacin, and gentamicin were associated with Ethyl Acetate Fraction (EAF) against multidrug-resistant strains in modulatory and *checkerboard* tests. The chromatographic data showed gallic acid, ellagic acid, and myricitrin in crude extract, with enrichment in the EAF. The electron transfer activity demonstrated in the antioxidant tests is related to the presence of flavonoids. The Gram-positive strains were more susceptible to EAF, and their action spectra were improved by association, comprising Gram-negative bacilli. Synergisms were observed to ciprofloxacin and gentamicin against *Pseudomonas aeruginosa* colistin-resistant. The results demonstrate that the extract and enriched fraction obtained from the leaves of *E. uniflora* act as a promising natural alternative against multidrug-resistant bacteria.

**Keywords:** antibacterial, antioxidant, chromatography, fraction, modulatory activity.

**Resumo**

Este trabalho teve como objetivo avaliar a composição química, as atividades antioxidantes e antimicrobianas do extrato bruto e frações de folhas de *Eugenia uniflora* Linn. O extrato bruto foi obtido por turbólise e suas frações por partição. Foram realizadas análises cromatográficas e a capacidade antioxidante foi verificada por dois métodos (DPPH$^{\bullet}$ e ABTS$^{\bullet+}$). A Concentração Inibitória/Bactericida Mínima foi realizada contra vinte e duas bactérias, selecionando cinco cepas suscetíveis a extração/frações e resistentes aos antibióticos testados. Ampicilina, azitromicina, ciprofloxacina e gentamicina foram associados à Fração Acetato de Etila (FAE) contra cepas multirresistentes em testes modulatórios e de *checkboard*. Os dados cromatográficos mostraram ácido gálico, ácido elágico e miricitrina em extrato bruto, com enriquecimento na FAE. A atividade de transferência de elétrons demonstrada nos testes antioxidantes está relacionada com a presença de flavonoides. As cepas de Gram-positivas foram mais suscetíveis à FAE, e seus espectros de ação foram melhorados por associação, compreendendo bacilos Gram-negativos. Foram observados sinergismos de ciprofloxacina e gentamicina contra *Pseudomonas aeruginosa* resistente à colistina. Os resultados demonstram que o extrato e a fração enriquecida obtida das folhas de *E. uniflora* atuam como uma alternativa natural promissora contra bactérias multirresistentes.

**Palavras-chave:** antibacteriano, antioxidante, cromatografia, fração, atividade modulatória.

## 1. Introduction

The exacerbated and inappropriate use of antibiotics has contributed to the increase in resistance to these drugs in recent decades, and consequently has increased health care costs, in addition to cases of morbidity and mortality (Gupta and Birdi, 2017; Kheirollahi et al., 2019). In 2021, the World Health Organization (WHO, 2021), documented that the leading cause of death in the world is associated with increasing antibiotic resistance. The indiscriminate and inappropriate use of antibiotics has caused worrying increases in resistance rates in some gram-positive bacteria such as *Staphylococcus aureus* and gram-negative bacteria such as *Escherichia coli*, *Klebsiella pneumoniae,* and *Salmonella* spp. mainly in developing countries (Santos et al., 2022).

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Moreover, oxidative stress is also related to the development of several chronic diseases, associated with inflammatory processes in the elderly population. In this sense, antioxidant therapy, with the use of polyphenols can contribute to the reduction of damage caused by oxidative stress (Forman and Zhang, 2021).

The secondary metabolites, present in plant species, present structural and chemical diversity that reflects in the numerous biological activities reported for medicinal plants. For some time, the use of medicinal plants has been an alternative to alleviate symptoms and treat various diseases, based on the evaluation of the pharmacological potential associated with the safety of use of some species (Abdelghani et al., 2021; Dahmer et al., 2019).

Among the species considered promising is *Eugenia uniflora* Linn (Myrtaceae), popularly known as "pitangueira", whose studies about its chemical composition have demonstrated the presence of phenolic compounds (Bezerra et al., 2018; Melo Candeia et al., 2022; Ramos et al., 2017; Sobeh et al., 2019), proving the need to increase investigations on the structure and biological activities related to these metabolites. Polyphenols, especially flavonoids and tannins, are considered the group of secondary metabolisms whose activities reported in the literature are significant (Kumar et al., 2017; Yamaguchi, 2022). Such metabolites have received special attention, based on the numerous reports of their antioxidant, and antimicrobial properties, including against multidrug-resistant Gram-positive and Gram-negative bacteria (Khadraoui et al., 2022; Yamaguchi, 2022; Yuan et al., 2021).

In this context, the present study aimed to evaluate the chemical composition, and *in vitro* antioxidant, antimicrobial and modulatory activities of crude extract and fractions of *E. uniflora* leaves.

## 2. Material and Methods

### *2.1. Herbal material*

The herbal material of *E. uniflora* (leaves) was collected at 8:00 am, in Recife, Pernambuco, Brazil (7°59'45.9 "S 34°54'33.3" W). A voucher was deposited in the Dárdano de Andrade Lima Herbarium (Agronomic Institute of Pernambuco-IPA) after identification of material by Dra. Rita de Cássia Pereira, under number 91672. The access to the species was registered in the *Sistema Nacional de Gestão do Patrimônio Genético e Conhecimento Tradicional Associado* (SisGen) at the number AFF07B4. The leaves were dried (7 days, 40 °C), in circulating air oven (Luca-82-480, Lucadema®) and then ground in knife mill (Willye-type, TE-680, Tecnal®).

### *2.2. Crude extract and fractions*

The liquid extract was obtained by turbo extraction [20 min; 10% (w/v); acetone: water (7:3, v/v)] and was concentrated under reduced pressure (RV10 Basic, IKA®), frozen, and lyophilized (L101, Liotop®), to obtain the crude extract (CE). The CE was reconstituted in distilled water (1:10, w/v) and partitioned with hexane (1:1, v/v). The aqueous residue was partitioned with ethyl acetate (1:1, v/v). The residues were concentrated, frozen, and lyophilized to obtain the hexane (HF), ethyl acetate (EAF), and aqueous (AqF) fractions (Ramos et al., 2017).

### *2.3. Fingerprint by thin-layer chromatography*

Silica gel plate 60-$F_{254}$ (10-12 µm, Macherey-Nagel®) was used with semi-automated equipment (Linomat V, Camag®) controlled by WinCats® software (Camag®). 30 µL of methanolic solutions of crude extract and fractions (3 mg/mL) and 15 µL of the standard solution (1 mg/mL) were applied in bands of 5 mm width and 5 mm space between them. The standard solution (1 mg/mL) was composed of gallic acid, ellagic acid, and myricitrin (Sigma-Aldrich®). The chromatograms were developed in a twin trough vertical glass chamber (10 cm x 10 cm, Camag®) after saturation (30 min) with the mobile phase ethyl acetate: methanol: water (50:6.75:5, v/v/v).

### *2.4. Fingerprint and quantification by High-Performance Liquid Chromatography (HPLC)*

The analysis was conducted in an HPLC Ultimate 3000, equipped with a diode array detector, a binary pump, a degasser, and an autosampler with a 20 µL loop (ThermoFisher Scientific®). The chromatographic separation was performed using a $C_{18}$ column (250 mm × 4.6 mm, 5 µm, Dionex®) equipped with a pre-column (4 mm x 3.9 µm, Phenomenex®) at a temperature of 23 ± 2 °C. Ultrapure water (A) and methanol HPLC grade (B), both acidified with trifluoroacetic acid (0.05%), were used as the mobile phase by elution gradient as follows: 10–25% B (10 min), 25-40% B (5 min), 40-75% B (10 min), 75% B (5 min) and 75-10% B (1 min) with flow 0.8 mL/min (Bezerra et al., 2018). Wavelengths of 254, 270, and 340 nm were used for detection, according to the maximum absorption measured by the detector. Myricitrin, gallic acid, and ellagic acid (Sigma-Aldrich®) were used as standards. The software Chromeleon (ThermoFisher Scientific®) was used for data analysis and processing.

### *2.5. Total flavonoid content by spectrophotometric analysis*

The total flavonoid content, by direct dilution and acid hydrolysis, was performed following the methodology described by (Ramos et al., 2017) and was expressed as g% of rutin or quercetin.

### *2.6. Antioxidant activity*

The *in vitro* evaluation of the antioxidant activity of crude extract and fractions to scavenging the free radical DPPH· (2,2-diphenyl-1-picrylhydrazyl, Sigma-Aldrich®) was performed by (Mensor et al., 2001) method, with adaptations. The percentage of antioxidant activity (AA) was calculated by the equation: AA (%) = 100 – ($A_{sample}$ x 100)/$A_{control}$). The concentration providing 50% inhibition of DPPH· absorbance ($IC_{50}$) was calculated graphically using a calibration curve in the linear range by exponential regression of the plotted points of the extract concentration *versus* the corresponding scavenging effect. The reducing capacity of the $ABTS^{+}$ (2,2-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) was performed as described by (Re et al., 1999).

The results were expressed as Trolox Equivalent Antioxidant Capacity (TEAC) values, which is µmol trolox/g of CE or enriched fractions.

### *2.7. Antimicrobial activity*

The antibacterial activity of crude extract and fractions was performed against twenty-two microorganisms, available by the Military Hospital of Recife (MHR) and Antibiotic Department Collection (UFPEDA, Federal University of Pernambuco) including *American Type Culture Collection* (ATCC) and multidrug-resistant (MDR) strains. The protocols were registered at the Ethics Committee of the Federal University of Pernambuco, Brazil (number 99925418.8.0000.5208) and approved (number 3.012.185). The suppliers previously described and granted the biochemical identification and resistance phenotype. The strains were cultivated in Mueller-Hinton agar (MHA) (Merck®) overnight at 37 °C. The inoculums were calibrated by suspension of colonies in 0.9% (v/v) saline solution, adjusting in a spectrophotometer (AJX-1900, Micronal®) at 600 nm.

#### *2.7.1. Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC)*

The susceptibility assay was determined by broth microdilution, according to the *Clinical and Laboratory Standards Institute* (CLSI, 2012), with some adaptations for natural products. The samples were solubilized in dimethylsulfoxide: water (1:9, v/v) to a 2 mg/mL concentration. The calibrated inoculum was diluted in 0.9% saline (1:50) and Mueller-Hinton broth (Merck®) (1:20) (final concentration = $10^5$ CFU/mL). The minimal inhibitory concentration (MIC) was determined on 96-well culture plates. In wells of line A, 100 µL of the crude extract or fractions was added; serial dilutions were performed from line A to line H to obtain concentrations ranging between 1000-7.8 µg/mL. In the end, the inoculum (100 µL) was added to all wells. The sterility control, negative control, and standards used were the culture medium, the inoculum, and aqueous solutions (2 mg/mL) of azithromycin, ampicillin, ciprofloxacin, and gentamicin (Pfizer® Inc.), respectively. The plates were incubated at 37 °C (24 h), and the presence or absence of growth was observed. The MIC was considered the smallest concentration of the crude extract or fractions capable of visibly reducing the bacterial growth by 100%, compared to the positive control. To determine MBC, aliquots of 5 µL of each well of the MIC experiment were transposed into Petri plates with MH agar and incubated for 24 h at 37 °C. The MBC corresponded to the lowest concentration of the crude extract or fractions capable of preventing the visible growth of the strains.

### *2.8. Modulatory assay*

The strains considered resistant to the antibiotics tested and simultaneously susceptible to the EAF were selected. The previous protocol established the MIC values used in this experiment. EAF solution (2 mg/mL) prepared in DMSO-water (1:9, v/v) and aqueous solutions (2 mg/mL) of antibiotics azithromycin, ampicillin, ciprofloxacin, and gentamicin (Pfizer® Inc.) were used. The inoculum was diluted in Mueller-Hinton broth (Merck®) until a final concentration of $10^5$ CFU/mL per well. In Eppendorf, an aliquot of 1.5 mL of the inoculum and a volume corresponding to 1/4 of the MIC of EAF were added to reach 1/8 of the EAF MIC per well. An aliquot of 100 µL of this mixture was added horizontally into wells. At well number 1 was added 100 µL of the antibiotic solution and serial dilutions were performed until the $12^{th}$ well to obtain concentrations between 1000-0.5 µg/mL. As sterility and negative control, respectively, culture medium and inoculum were used. The plates were incubated at 37 °C for 24 h, and 0.02% aqueous resazurin solution (20 µL) was added to each well, incubating for 3 hours at 35 °C to verify the presence or absence of growth. The modulatory effect was interpreted as antagonistic, synergistic, or inert.

### *2.9. Checkerboard microdilution assay*

The synergic associations obtained by the modulatory test were evaluated by the *checkerboard* test. The EAF solution (2 mg/mL) was associated with the same antibiotics and strains previously used. The solutions were prepared in Mueller-Hinton broth to obtain subinhibitory concentrations between 1/2-1/64 for the MIC value of the EAF and between 1/2-1/512 for the MIC of the synthetic antibiotic. A 50 µL aliquot of EAF solution was added in vertical orientation and 50 µL of the antibiotic solution in a horizontal orientation in a 96-well microplate. After that, 100 µL of the standard inoculum at 106 CFU/mL was added to each well and the microplate was incubated for 24 h at 35 °C. Subsequently, 20 µL of 0.02% aqueous resazurin was added to the microplates and incubated for 3 h at 35 °C to check for the presence or absence of growth. To evaluate the combinations, the Fraction Inhibitory Concentration Index (FICI) was calculated and the results were interpreted as FICI ≤ 0.5 synergism; 0.5 < FICI < 4 no interaction; and FICI > 4 antagonism (Wei et al., 2011).

### *2.10. Statistical analysis*

The assays were performed in triplicate and as independent experiments. The experimental results were expressed as mean ± standard deviation and were processed using Software Excel (Microsoft® Office 365, 14.0 version, 2016).

## 3. Results

The results of yield, total flavonoid content by UV-Vis, and the markers by HPLC-DAD are summarized in Table 1. The ethyl acetate partitioning increased the flavonoid content observed in the spectrophotometric analysis (CE = 10.88 ± 0.004 g%; EAF = 28.00 ± 0.005 g%), which was evidenced by the TLC and confirmed by HPLC analyses (CE = 0.88 ± 0.001; EAF = 4.58 ± 0.02, as myricitrin) (Figure 1). According to the TLC analysis of *E. uniflora* derivatives, was observed the presence of gallic acid (deep blue band; Rf = 0.72); ellagic acid (greenish band; Rf = 0.33); and myricitrin (yellow/orange band; Rf = 0.50). Concerning the HPLC analysis, some components were identified: gallic acid ($t_R$ = 8.25 min), myricitrin ($t_R$ = 24.52 min) and ellagic acid ($t_R$ = 24.52 min).

Figure 2 shows a DPPH· radical scavenging activity by crude extracts and fractions from leaves of *E. uniflora*. The lowest $IC_{50}$ value, corresponding to the highest scavenging activity of DPPH· radical, was obtained for *E. uniflora* EAF (1.46 µg/mL, $R^2$ = 0.9908).

The results of the ABTS test were 76.74 ± 0.003, 65.05 ± 0.001, 78.45 ± 0.001, and 64.06 ± 0.004, respectively, for CE, HF, EAF, and AqF expressed in TEAC. The equation of the Trolox calibration curve was $y = 0.0003x - 0.6403$, with good correlation coefficient ($R^2$ = 0.9993). In concordance with the DPPH· results, *E. uniflora* EAF exhibited the highest (78.45 µM TE/g extract) TEAC values.

The antibacterial activity of the crude extract of *E. uniflora* (acetone: water, 7:3) and its fractions was evaluated against twenty-two multidrug-resistant pathogenic bacteria, distributed in ten genders, including nosocomial species. Values of MIC/MBC are described in Table 2 (Gram-positive) and Table 3 (Gram-negative), as well as the bacterial resistance phenotype.

Gram-positive strains showed to be more sensitive to *E. uniflora* extract and fractions. Among the Gram-negative *E. coli*, *K. pneumoniae* and *P. aeruginosa* showed low activity for most of the extracts and fractions evaluated (MIC ≥ 1000 µg/mL). The smallest MIC values in this study (7.81 µg/mL) were obtained for *E. uniflora* EAF against *S. agalactiae* and *S. epidermidis*.

The strains *E. coli* MHR 044UC, *K. pneumoniae* MHR 098UC, *P. aeruginosa* MHR 169AT, *S. enteritidis* UFPEDA414, and *S. epidermidis* MHR 073HC were selected for the modulatory assay, utilizing EAF concentrations equivalent to 1/8 of each MIC value. The strains' selection was based on their susceptibility to EAF and resistance to these antibiotics and the results are described in Table 4.

In this evaluation using subinhibitory concentrations for the EAF, five out of ten combinations were synergistic, three antagonists and two inerts with conventional antibiotics and different profiles. Among the drugs tested, ciprofloxacin was the most potentiated, followed by gentamicin and ampicillin. Azithromycin stands out as the antibiotic with the negative modulation against the Gram-positive cocci. Regarding the colistin-resistant phenotype of *P. aeruginosa* MHR169TA, the EAF did not show appreciable antibacterial activity (MIC/MBC = 500/1000 µg/mL). However, when the EAF was incorporated into the growth medium, a reduction in the MIC for ciprofloxacin was observed.

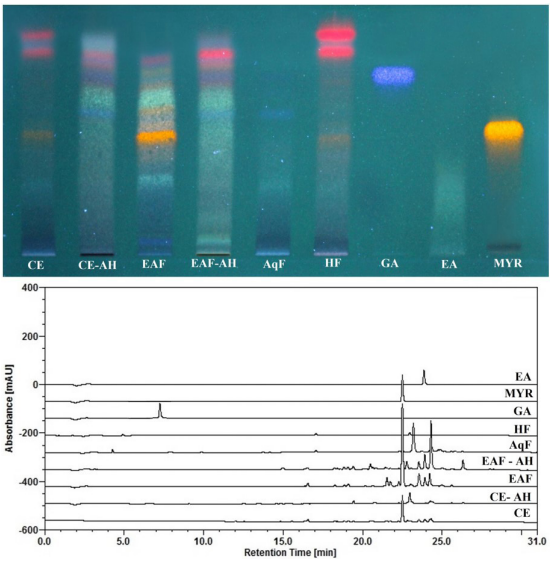


**Figure 1.** TLC and HPLC profiles obtained for crude extracts and fractions from leaves of *E. uniflora*. CE, Crude Extract; CE-AH, Crude Extract After Hydrolysis; EAF, Ethyl Acetate Fraction; EAF-AH, Ethyl Acetate Fraction After Hydrolysis; AqF, Aqueous Fraction; HF, Hexanic Fraction; GA, Gallic Acid; EA, Ellagic Acid; MYR, Myricitrin.

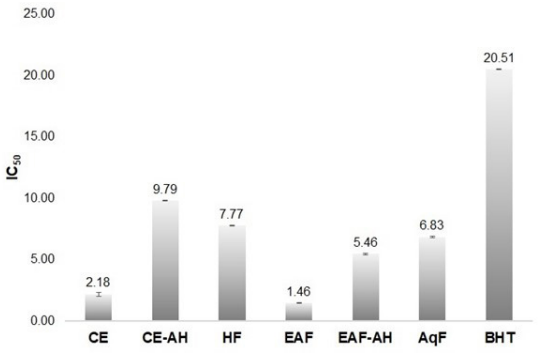


**Figure 2.** DPPH scavenging (%) by *E. uniflora* crude extracts and fractions. CE, Crude Extract; CE-AH, Crude Extract After Hydrolysis; EAF, Ethyl Acetate Fraction; EAF-AH, Ethyl Acetate Fraction After Hydrolysis; AqF, Aqueous Fraction; HF, Hexanic Fraction; BHT: butylated hydroxytoluene

**Table 1.** Yields, total flavonoid and markers content by HPLC of crude extract and fractions from leaves of *E. uniflora*.

| Samples | Yield (%, w/w) | TFC (UV-Vis) (%; w/w) | | | HPLC | | |
|---|---|---|---|---|---|---|---|
| | | DD | | AH | Ellagic acid | Gallic acid | Myricitrin |
| | | Q | R | Q | | | |
| **CE** | 21.80 ± 1.52 | 4.71 ± 0.004 | 10.88 ± 0.004 | 3.021 ± 0.02 | 0.26 ± 0.002 | 0.80 ± 0.01 | 0.88 ± 0.001 |
| **EAF** | 13.83 ± 0.07 | 12.13 ± 0.01 | 28.00 ± 0.005 | 11.93 ± 0.007 | 0.63 ± 0.02 | 1.59 ± 0.01 | 4.58 ± 0.02 |
| **HF** | 24.45 ± 0.08 | 5.11 ± 0.03 | 11.81 ± 0.01 | 4.78 ± 0.02 | 0.27 ± 0.001 | 0.19 ± 0.02 | 1.12 ± 0.001 |
| **AqF** | 80.59 ± 0.51 | 2.63 ± 0.01 | 6.07 ± 0.004 | 1.77 ± 0.01 | 0.12 ± 0.002 | ND | 0.24 ± 0.003 |

All results were expressed as mean ± standard deviation. Total Flavonoid Content (TFC) were expressed in % of Rutin (R) or Quercetin (Q). CE: Crude extract; EAF: Ethyl acetate fraction; HF: Hexanic fraction; AqF: Aqueous fraction; DD: Direct dilution; AH: Acid hydrolysis; ND: Non detected.

**Table 2.** Minimal Inhibitory Concentration (MIC) and Minimal Bactericidal Concentration (MBC) of *E. uniflora* crude extract, fractions and antimicrobial agents against Gram-positive strains.

| Strains | Origin | MIC/ MBC (μg/mL) | | | | | | | | Resistance Phenotype |
|---|---|---|---|---|---|---|---|---|---|---|
| | | CE | HF | EAF | AqF | AMP | AZI | CIP | GEN | |
| *Bacillus megaterium* UFPEDA462 | ND | 1000/ >1000 | */ ND | 500/ 1000 | 1000/ >1000 | 128/ 256 | 32/ 64 | 64/ 128 | 16/ 32 | AMP; ERI; CIP; GEN; TET; CAZ; OXA; CXM, CTX |
| *Enterococcus faecalis* UFPEDA620 | Hemoculture | 125/ 250 | 250/ 500 | 15.62/ 31.25 | 125/ 250 | 256/ 512 | 128/ 256 | 256/ 512 | 128/ 256 | AMP; CAZ; CIP; CTX; CXM; TET |
| *Streptococcus agalactiae* MHR059VS | Vaginal secretion | 125/ 250 | 250/ 500 | 7.81/ 15.62 | 62.5/ 125 | 0.12/ 0.24 | 0.12/ 0.24 | 0.12/ 0.24 | 0.12/ 0.24 | None antibiotic |
| *S. pyogenes* UFPEDA1023 [B] | ATCC 16642 | 250/ 500 | 250/ 500 | 125/ 250 | 250/ 500 | 3.12/ 6.24 | 3.12/ 6.24 | 3.12/ 6.24 | 3.12/ 6.24 | Control Strain |
| *Staphylococcus aureus* UFPEDA02 [A] | ATCC 25293 | 125/ 250 | 250/ 500 | 62.5/ 125 | 125/ 250 | 3.12/ 6.24 | 0.39/ 0.78 | 3.12/ 6.24 | 0.39/ 0.78 | Control Strain |
| *S. epidermidis* MHR073HC [B] | Hemoculture | 500/ 1000 | 1000/ >1000 | 7.81/ 15.62 | 250/ 500 | 512/ 1024 | 128/ 256 | 128/ 256 | 64/ 128 | AMP; AZI; ERI; PEN; PTZ |
| *S. saprophyticus* UFPEDA833 | Uroculture | 500/ 1000 | 1000/ >1000 | 125/ 250 | 500/ 1000 | 32/ 64 | 0.39/ 0.78 | 16/ 32 | 16/ 32 | AMP; TET |

ATCC: American Type Culture Collection; CE: crude extract; HF: Hexanic fraction; EAF: Ethyl acetate fraction; AqF: Aqueous fraction.AMP: ampicillin; AZI: azithromycin; CAZ: ceftazidime; CIP: ciprofloxacin; CTX: cefotaxime; CXM: cefuroxime; ERI: erythromycin; OXA: oxacillin; PEN: penicillin; PTZ: piperacillin tazobactam; TET: tetracycline; ND: not determined. *no inhibition. [A] MSSA: methicillin sensitive *S. aureus*. [B] multidrug-resistant.

**Table 3.** Minimal Inhibitory Concentration (MIC) and Minimal Bactericidal Concentration (MFC) of *E. uniflora* crude extract, fractions and antimicrobial agents against Gram-negative strains.

| Strains | Origin | MIC/ MBC (μg/mL) | | | | | | | Resistance Phenotype |
|---|---|---|---|---|---|---|---|---|---|
| | | CE | HF | EAF | AqF | AMP | CIP | GEN | |
| *Enterobacter aerogenes* MHR062UC | Uroculture | 62.5/ 125 | 250/ 500 | 62.5/ 125 | 125/ 250 | 64/ 128 | 32/ 64 | 32/ 64 | AMC; AMP; CEF; CIP; LEV; NIT; SXT |
| *Escherichia coli* MHR001UC | Uroculture | 1000/ >1000 | */ ND | 125/ 250 | 1000/ >1000 | 32/ 64 | 32/ 64 | 32/ 64 | AMP; CEF, CIP, LEV, SXT |
| *E. coli* MHR044UC [C] | Uroculture | 250/ 500 | 500/ 1000 | 125/ 250 | 500/ 1000 | > 1024/ ND | 256/ 128 | 128/ 64 | AMC; AMP; CAZ; CEF; CIP; FEP; LEV; SXT |
| *E. coli* UFPEDA224 | ATCC 25922 | 125/ 250 | 500/ 1000 | 15.62/ 31.25 | 62.5/ 125 | 0.39/ 0.78 | 0.39/ 0.78 | 0.39/ 0.78 | Control Strain |
| *Klebsiella pneumoniae* MHR180PC [D] | Catheter tip | 1000/ >1000 | */ ND | 500/ 1000 | */ ND | 1024/ >1024 | 512/ 256 | 512/ 256 | AMC; AMP; CAZ; CEF; CFO; CIP; CRO; CST; CXM; ERT; FEP; GEN; IMI; LEV; NIT; PTZ; SXT |
| *K. pneumoniae* MHR098UC [D] | Uroculture | 1000/ >1000 | 1000/ >1000 | 125/ 250 | 250/ 500 | 512/ 256 | 256/ 512 | 256/ 512 | AMC; AMP; CAZ; CEF; CFO; CIP; CRO; CXM; ERT; GEN; LEV; NIT; PTZ; SXT |

ATCC: American Type Culture Collection; CE: crude extract; HF: Hexanic fraction; EAF: Ethyl acetate fraction; AqF: Aqueous fraction; AMC: amoxicillin-clavulanate, AMI: amikacin; AMP: ampicillin; AZT: aztreonam; CAZ: ceftazidime; CEF: cefalotin; CFO: cefoxitin; CIP: ciprofloxacin; CRO: ceftriaxone; CTX: cefotaxime; CST: colistin; CXM: cefuroxime; ERI: erythromycin; ERT: ertapenem; FEP: cefepime; GEN: gentamicin; IMI: imipenem; LEV: levofloxacin; NIT: nitrofurantoin; PTZ: piperacillin tazobactam; SXT: trimethoprim-sulfamethoxazole; TET: tetracycline; ND: not determined. * no inhibition. - not tested. [B] multidrug-resistant. [C] extended-spectrum β-lactamase-producer (ESBL). [D] carbapenemase-producer (KPC).

**Table 3.** Continued...

| Strains | Origin | MIC/ MBC (μg/mL) | | | | | | | Resistance Phenotype |
|---|---|---|---|---|---|---|---|---|---|
| | | CE | HF | EAF | AqF | AMP | CIP | GEN | |
| *K. pneumoniae* MHR180UC [D] | Uroculture | 1000/ >1000 | */ ND | 250/ 500 | */ ND | 1024/ >1024 | 512/ 256 | 512/ 256 | AMC; AMP; CAZ; CEF; CFO; CIP; CRO; CST; CXM; ERT; FEP; GEN; IMI; LEV; NIT; PTZ; SXT |
| *K. pneumoniae* UFPEDA396 | ND | 1000/ >1000 | */ ND | 500/ 1000 | */ ND | 128/ 256 | 32/ 64 | 32/ 64 | AMP; CAZ CEF; CIP; CTX; CXM, TET |
| *Pseudomonas aeruginosa* MHR173SN | Nasal secretion | 500/ 1000 | 1000/ >1000 | 250/ 500 | 250/ 500 | - | 0.39/ 0.78 | 0.39/ 0.78 | None antibiotic |
| *P. aeruginosa* MHR169TA [B] | Tracheal Aspirated | 500/ 1000 | */ ND | 500/ 1000 | 1000/ >1000 | - | >1024/ ND | >1024/ ND | AMI; AZT; CAZ; CIP; FEP; GEN; LEV; PTZ |
| *P. aeruginosa* MHR001UC [B] | Uroculture | 500/ 1000 | 500/ 1000 | 250/ 500 | 250/ 500 | - | 512/ 256 | 512/ 256 | AMI; AZT; FEP; CAZ; CIP; LEV; PTZ |
| *P. aeruginosa* UFPEDA416 | ATCC 27853 | 500/ 1000 | 1000/ >1000 | 250/ 500 | 250/ 500 | - | 0.39/ 0.78 | 0.39/ 0.78 | Control Strain |
| *Salmonella enteritidis* UFPEDA414 | Skin ulcer | 1000/ >1000 | */ ND | 500/ 1000 | */ ND | 64/ 128 | 64/ 128 | 64/ 128 | AMP; CAZ, CEF; CIP; CTX; CXM |
| *Serratia marcescens* MHR172TA | Tracheal Aspirated | 31.25/ 62.5 | 62.5/ 125 | 15.62/ 31.25 | 31.25/ 62.5 | 128/ 256 | 64/ 128 | 64/ 128 | AMC; AMI; AMP; CAZ; CEF; CFO; CIP; CRO; CXM; FEP; LEV; NIT; PTZ; SXT |
| *S. marcescens* UFPEDA352 | ND | 31.25/ 62.5 | 31.25/ 62.5 | 7.81/ 7.81 | 15.62/ 31.25 | 32/ 64 | 16/ 32 | 16/ 32 | AMP; CIP; CEF; CTX; CXM; ERI; GEN |

ATCC: American Type Culture Collection; CE: crude extract; HF: Hexanic fraction; EAF: Ethyl acetate fraction; AqF: Aqueous fraction; AMC: amoxicillin-clavulanate, AMI: amikacin; AMP: ampicillin; AZT: aztreonam; CAZ: ceftazidime; CEF: cefalotin; CFO: cefoxitin; CIP: ciprofloxacin; CRO: ceftriaxone; CTX: cefotaxime; CST: colistin; CXM: cefuroxime; ERI: erythromycin; ERT: ertapenem; FEP: cefepime; GEN: gentamicin; IMI: imipenem; LEV: levofloxacin; NIT: nitrofurantoin; PTZ: piperacillin tazobactam; SXT: trimethoprim-sulfamethoxazole; TET: tetracycline; ND: not determined. * no inhibition. - not tested. [B] multidrug-resistant. [C] extended-spectrum β-lactamase-producer (ESBL). [D] carbapenemase-producer (KPC).

**Table 4.** Minimal Inhibitory Concentration of antibiotics against multidrug-resistant strains, in the absence and presence of *E. uniflora* ethyl acetate fraction at subinhibitory concentrations (1/8 MIC).

| Strain | Individual MIC (μg/mL) | | Combined MIC associated (μg/mL) | % MIC reduced | Outcome |
|---|---|---|---|---|---|
| *S. epidermidis* MHR073HC | AMP | 512 | 125 | 75.6 | synergism |
| | AZI | 128 | * | - | antagonism |
| *E. coli* MHR044UC | CIP | 256 | 250 | 0 | inert |
| | GEN | 128 | * | - | antagonism |
| *K. pneumoniae* MHR098UC | CIP | 256 | 62.5 | 75.6 | synergism |
| | GEN | 256 | 250 | 0 | inert |
| *P. aeruginosa* MHR169TA | CIP | >1024 | 31.2 | >100 | synergism |
| | GEN | >1024 | 500 | 51.2 | synergism |
| *S. enteritidis* UFPEDA414 | AMP | 64 | * | - | antagonism |
| | GEN | 64 | 31.2 | 51.3 | synergism |

MIC: minimal inhibitory concentration; AMP: ampicillin; AZI: azithromycin; CIP: ciprofloxacin; GEN: gentamicin. * no inhibition. - no calculated.

**Table 5.** Checkboard test of *E. uniflora* ethtyl acetate fraction with antimicrobial agents against multidrug-resistant strains.

| Strain | ATB MIC (μg/mL) | | EAF MIC (μg/mL) | Combined MIC - ATB/EAF (μg/mL) | Individual FICI ATB/EAF | FICI (ATB + EAF) |
|---|---|---|---|---|---|---|
| *S. epidermidis* MHR073HC | AMP | 512 | 7.81 | 0.98/ 0.98 | 0.002/ 0.125 | 0.127 |
| *K. pneumoniae* MHR098UC | CIP | 256 | 125 | 15.62/ 7.81 | 0.061/ 0.063 | 0.124 |
| *P. aeruginosa* MHR169TA | CIP | > 1024 | 500 | 15.62/ 7.81 | 0.015/ 0.016 | 0.031 |
| | GEN | > 1024 | 500 | 31.25/ 15.62 | 0.031/ 0.031 | 0.062 |
| *S. enteritidis* UFPEDA414 | GEN | 64 | 500 | 15.62/ 31.25 | 0.244/ 0.063 | 0.307 |

ATB: antibiotic; EAF: ethyl acetate fraction; MIC: minimal inhibitory concentration; FICI: Fraction Inhibitory Concentration Index; AMP: ampicillin; CIP: ciprofloxacin; GEN: gentamicin.

Despite the non-modulation for the combination of EAF-gentamicin for *K. pneumoniae* carbapenemase-producer (KPC), the EAF reduced the fluoroquinolone MIC.

The synergistic associations considered promising were tested to a *Checkerboard* model. The Fractional Inhibitory Concentration Index (FICI) was calculated (Table 5) to verify the EAF behavior through subinhibitory concentration, once this interpretation is not possible in the modulation assay. The subinhibitory concentrations of *E. uniflora* (FICI ≤ 0.5) enhanced the antimicrobial activity of the agents, as well as reduced the EAF individual MIC. Thus, for the bacterial strains tested, the combination of EAF and antibiotics, demonstrated a synergistic effect.

## 4. Discussion

The extraction of bioactive compounds from the plant matrix depends on factors such as the chemical structure of the compounds and the solvents that will be used (Chaves et al., 2020; Lefebvre et al., 2021). Considering the chemical composition of E. uniflora leaves, polyphenols are the metabolites of interest. The extraction of polyphenols is favored by the solubility of these compounds in polar organic solvents, such as ethanol, methanol, acetone and, and the use of these solvents mixed with water increased the extraction of tannins and flavonoids (Zuorro et al., 2019), results also successfully demonstrated by our group for the species (Bezerra et al., 2018, 2020; Ramos et al., 2017). Therefore, obtaining the acetone: water (7:3) extract from *E. uniflora* leaves favored the extraction of polyphenols. Additionally, the partition process evidenced, promoted the removal of apolar compounds with the use of hexane, and the enrichment of the ethyl acetate fraction in phenolic compounds (Chaves et al., 2020; Lefebvre et al., 2021).

Regarding the partition process, the enrichment of EAF stands out, whose chromatographic band of myricitrin was more intense (Lefebvre et al., 2021). The acid hydrolysis process was also performed successfully, evidenced by the absence of yellow-orange bands (TLC) and peaks (HPLC) in the CE-AH (corresponding to flavonoids), whereas the AqF was free of myricitrin. Enrichment of gallic acid and ellagic acid was also evidenced, mainly by HPLC. The data are corroborated by previous results of TLC and HPLC analysis (Bezerra et al., 2018; Melo Candeia et al., 2022).

Flavonoids have structural diversity associated with their C-ring, yet the double bonds of the aromatic rings allow these compounds to absorb UV light. Therefore, spectra observed by UV-Vis spectrophotometry are associated with the corresponding class of flavonoid (flavone, flavanone, flavonol, etc.) (Desmet et al., 2021; Sisa et al., 2010). When substitution of some groups occurs, different spectra are originated, which, when adding the reagent aluminum chloride, can result in bathochromic shifts or hypsochromic shifts (considering the number of radical hydroxyl, methoxyl, methyl, or sugars in the molecule) (Petry et al., 2001; Ramos et al., 2017). Therefore, UV/Vis spectrophotometric analysis indicates that flavonoids of the O-glycosylated flavone type and their respective aglycone are present in the extract, and fractions of *E. uniflora* (evidenced by the bathochromic shift by direct dilution, and hypsochromic shift after acid hydrolysis) (Ramos et al., 2017).

The crude extract and ethyl acetate fraction provided significant amounts of flavonoids and other polyphenols, which were enriched by the partitioning process to improve the antioxidant and antibacterial properties. The antioxidant assay was conducted to investigate the biological potential of crude extract and fractions rich in polyphenols, which occurs mainly due to their redox property, which allows them to act as scavengers of reactive oxygen species (ROS). For evaluation, several assays can be performed, among the most sensitive, fast, and with higher radical stability are DPPH and ABTS. The assays present different mechanisms and the reactivity of phenolic compounds against radicals is dependent on the chemical structure of the molecule (Munteanu and Apetrei, 2021).

The DPPH neutralization assay is based on the presence of hydrogen or electron-donating compounds, such as flavonoids, that can neutralize the DPPH$^{\cdot}$ radical, making it a stable molecule (Munteanu and Apetrei, 2021). The evaluation of antioxidant activity by the DPPH$^{\cdot}$ method showed variation between extract and fractions, which may be associated with differences in the profile of phenolic compounds. A two-step supercritical ethanolic extraction found a slightly higher $IC_{50}$ value of 5.0 μg/mL for *E. uniflora* (Martinez-Correa et al., 2011). Furthermore, the $IC_{50}$ for *E. uniflora* EAF was upper than that found by (da Cunha et al., 2016), where the ethanolic extract from leaves presented only 20% of DPPH$^{\cdot}$ removal

at a concentration of 240 µg/mL. Highlights, the $IC_{50}$ of EAF was fourteen times lower than the BHT value (20.51 µg/mL, $R^2 = 0.9955$). The hexanic fraction showed $IC_{50}$ values highest than the corresponding CE. These results are in concordance with the enrichment promoted by partition.

Generally, the structural configuration of flavonoids shows good antioxidant activity, and the results obtained suggest that an electron transfer mechanism occurs, since the samples react immediately even at low concentrations (Marchi et al., 2022). The DPPH radical reacts less strongly with phenolic acids and sugars. To clarify some of these possible effects, the antioxidant activity of the crude extract and ethyl acetate fraction after acid hydrolysis was also evaluated. Even after the hydrolysis process, the $IC_{50}$ values remain low, inferring the presence of free aglycone and O-glycosylated derivatives in CE-AH and EAF-AH (Platzer et al., 2021). Furthermore, the number of OH groups on the aromatic ring, present in structures such as gallic acid and ellagic acid present in CE and EAF, also exerts an influence on the antioxidant activity of *E. uniflora* (Platzer et al., 2021).

The crude extract, hexane fraction, and ethyl acetate fraction showed a smaller decline in ABTS between the concentrations of 100-250 µg/mL, with a more pronounced reaction between 500-1000 µg/mL, indicating that an electron transfer mechanism occurs and that the polyphenols may have bulky side groups or multiple rings that are preventing diffusion and orientation toward $ABTS^+$. ABTS is oxidized by oxidants to its cation radical $ABTS^+$, so the extract and the analyzed fractions may also act partially by slower transfer of the hydrogen atom to this radical. Considering the majority presence of flavonoids in the CE and EAF of *E. uniflora*, the most plausible reaction mechanism for flavonoids may be the hydrogen atom transfer pathway (Messaadia et al., 2020). Additionally, the presence of low molecular weight phenolic compounds provides fast initial rates and shows a linear increase at lower concentrations (Platzer et al., 2021).

The lowest MIC values in this study (7.81 µg/mL) were obtained for EAF against the Gram-positive clinical isolates *S. agalactiae* and *S. epidermidis*. Regarding the *E. faecalis* strain, the MIC found for EAF 15-times was lower than the MIC for ciprofloxacin, confirming the inhibition potential of the compounds present in the fraction. When we observed low MBC values not so much higher than the MIC values, still the plant sample presents an interesting antibacterial activity (Siddeeqh et al., 2016). At lower concentrations, the extract and fractions are considered bacteriostatic, while at higher concentrations they are bactericidal. For the samples that showed MIC values higher than 1000 µg/mL (total bacterial growth), the MBC was not determined.

Extracts from *E. uniflora* leaves have already been evaluated against yeasts, including non-*Candida albicans Candida* (Souza et al., 2018) and *Candida albicans* (Sardi et al., 2017). Some studies attribute the *E. uniflora* antimicrobial activities to their essential oils rather than their polyphenols (Santos et al., 2018; Falcao et al., 2018; Figueiredo et al., 2019; Pereira et al., 2017). The MIC values of *E. uniflora* evaluated in this study presented values between 125-1000 µg/mL for CE and 7.81-500 µg/mL for EAF, showing that the antibacterial activity may be mediated via phenolic compounds.

Considering the structure/activity relationship, compounds such as flavonols damage the bacterial cytoplasmic membrane by generating hydrogen peroxide (Kanner, 2020); and hydrolyzable tannins are related to the inhibition of nucleic acid synthesis through inhibition of dihydrofolate reductase and/or topoisomerase (Makarewicz et al., 2021). Flavones also can inhibit energy metabolism, through the inhibition of bacterial ATP synthase (Neupane et al., 2019).

Supported by the susceptibility profile obtained from the ethyl acetate fraction, the evaluation of the interference of this fraction on resistance to conventional antibiotics was performed. The antibiotics were selected considering the different spectrums and mechanisms of action, and the emergence and spread of very virulent strains, which increase susceptibility to infections. Thus, the modulation assay with ciprofloxacin is important because infections caused by *K. pneumoniae*, which have several antibiotic resistance genes and several associated virulence factors, are increasingly reported in hospitalized patients worldwide (Lam et al., 2021; Nakamura-Silva et al., 2022), ciprofloxacin (fluoroquinolone) and ampicillin (beta-lactam) resistance are evidenced in this strain (Nakamura-Silva et al., 2022).

An unfavorable effect was verified to *S. enteritidis* MDR, where the EAF did not decrease the ampicillin MIC and presented antagonism, it is because *Salmonella enteritidis* MDR is resistant to traditional first-line antibiotics such as ampicillin and also already has resistance to fluoroquinolones and broad-spectrum cephalosporins (La et al., 2021). This may occur due to the presence of the enzyme β-lactamase, which breaks the amide bond and deactivates ampicillin, which can block bacterial cell wall synthesis since it covalently binds to the binding proteins, involved in the terminal steps of peptidoglycan synthesis, the main component of the bacteria cell wall (Rosa et al., 2021).

Flavonoids, the major components of EAF from *E. uniflora*, have received great interest because they can deprive substrate and rupture membranes of bacterial strains. The Gram-negative strain *P. aeruginosa* has a difference in the content of cell wall peptidoglycans, leading to a unique composition, which makes its intrinsic resistance to most antibiotics (Tao et al., 2022). These results suggest that EAF associated with treatment with ciprofloxacin or gentamicin may damage the cell membrane of *P. aeruginosa*. This occurs due to the low permeability of the outer membrane of the bacterial strain. Thus, the association of EAF and antibiotics increased the cell membrane sensitivity of bacteria resistant to these drugs, thus increasing the inhibitory effect of the antibiotics. In addition, some evidence suggests that flavonoids and tannins inhibit the growth of *P. aeruginosa* through interaction with efflux pumps (Villanueva et al., 2023).

The checkboard method evaluates the synergism between two or more drug combinations, reflecting the inhibition of bacterial growth based on the obtained MIC results, using an equation to calculate the fractional inhibitory concentration index (FICI). The resulting FICI value is interpreted as synergism (≤ 0.5), additive (> 0.5-1.0), indifference (> 1 - ≤ 4), or antagonism (> 4) (Fadwa et al., 2021; Omokhua et al., 2019).

Combinations of conventional antibiotics with extracts/enriched fractions/isolated compounds have been reported as biological/pharmacological intensifiers, simultaneously minimizing the toxic side effects of synthetic drugs

(Caesar and Cech, 2019; Wagner and Ulrich-Merzenich, 2009). The checkboard method demonstrated that the EAF *E. uniflora*-antibiotic combination allowed us to observe an even greater reduction of the MIC of ciprofloxacin and gentamicin for multidrug-resistant *P. aeruginosa*, now involving micrograms instead of milligrams, suggesting acting synergistic action by several mechanisms, highlighting the FICI obtained for these two antibiotics. In this sense, the antibiotic potentiation promoted by EAF can be inferred in the destabilization of the bacterial membrane structure, consequently altering its permeability, facilitating the penetration of the different classes of drugs into the cell wall, as mentioned previously.

Currently, the high consumption of antimicrobials has occasioned selection and spread of resistance mechanisms, including specific amino acid substitutions in quinolone-targets (DNA gyrase and topoisomerase IV), alterations in the outer membrane proteins or efflux pumps overexpression (Chu et al., 2019; Martínez-Trejo et al., 2022). Concerning the aminoglycosides, the RNA plays a key role in the binding ribosome-drug (Ying et al., 2019). Regarding the gentamicin side effects, such as permanent hearing loss (Hodiamont et al., 2022; Nguyen and Jeyakumar, 2019) and the less favorable outcomes of monotherapy (Phe et al., 2019). Considering the above, synergistic combinations would represent an alternative, which may reduce the effective dosage of conventional antibiotics and other limitations.

## 5. Conclusions

The ethyl acetate fraction showed better in vitro performance compared to the crude extract and other fractions (AqF and HF). In addition, it contributed to the increased phenolic content observed in all characterization analyses. The identification and quantification of the main bioactive compounds provided an important correlation with the antioxidant activity. Considering the antibacterial activity, the efficacy was improved after partitioning and the increased concentrations of flavonoids in the EAF played a key role against the bacterial strains. Moreover, the combination of EAF and conventional antibiotics provided a synergistic effect, increasing the antibacterial spectrum and including Gram-negative strains. The additive effect was most pronounced combined with ciprofloxacin and gentamicin against *P. aeruginosa*. Several mechanisms could be explaining the observed synergistic effect, such as flavonoid-membrane interaction and/or inhibition of gene expression, which enhance the cellular uptake of antimicrobials to reach their intracellular targets. However, further studies are needed to clarify the mechanism of the promising synergistic activity observed between the fraction and conventional antibiotics.

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