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# Phytochemical profile, *in vitro* activities, and toxicity of optimized *Eugenia uniflora* extracts

[Perfil fitoquímico, actividades *in vitro* y toxicidad de extractos optimizados de *Eugenia uniflora*]

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**Abstract:** In this study, we investigated the influence of mixture design on the chemical profile of *Eugenia uniflora* leaves, evaluating the antioxidant and antimicrobial activities, the toxic and hemolytic potential, with the focus on the improvement of the polyphenol's extraction for incorporation of the extract in semi-solid forms with antifungal action. The chemical analysis was evaluated by UV-Vis and HPLC. The antimicrobial, antioxidant, and hemolytic activities were monitored. The flavonoid content ranged from 2.63-7.98 %w/w and tannins from 5.42-18.29 %w/w. The extract consisted of gallic acid (0.09-1.29%; w/w), ellagic acid (0.09-0.37%; w/w), and myricitrin (0.18-1.20%; w/w). The most successful solvent system with the highest level of active extract was water: ethanol: propylene glycol. The extracts showed fungicidal properties (3.9 µg/mL), high antioxidant activity (IC50: 9.50 µg/mL), and low toxicity. These solvent mixtures can improve the in vitro bioactivities when compared to pure solvents and this result demonstrates the importance of mixture designs as useful tools for creating high-quality herbal products and elucidate the potential of *E. uniflora* glycolic extracts as active herbal pharmaceutical ingredients in topical delivery systems.

**Keywords:** *Eugenia uniflora*; Antimicrobial activity; Hemolytic activity; Toxicity; Mixture design.

**Resumen:** En este estudio investigamos la influencia del diseño de mezclas en el perfil químico de hojas de *Eugenia uniflora*, evaluando las actividades antioxidantes y antimicrobianas, el potencial tóxico y hemolítico, con el foco en la mejora de la extracción de polifenoles para la incorporación del extracto en formas semi-sólidas con acción antifúngica. El análisis químico se evaluó mediante UV-Vis y HPLC. Se monitorizaron las actividades antimicrobianas, antioxidantes y hemolíticas. El contenido de flavonoides osciló entre 2,63 y 7,98% p/p and taninos de 5,42-18,29% p/p. El extracto consistió en ácido gálico (0.09-1.29%; p/p), ácido elágico (0.09-0.37%; p/p) y miricitrina (0.18-1.20%; p/p). El sistema de disolventes más exitoso con el nivel más alto de extracto activo fue agua: etanol: propilenglicol. Los extractos mostraron propiedades fungicidas (3.9 µg/mL), alta actividad antioxidante (IC50: 9.50 µg/mL) y baja toxicidad. Estas mezclas de disolventes pueden mejorar las bioactividades in vitro en comparación con los disolventes puros y este resultado demuestra la importancia de los diseños de mezclas como herramientas útiles para crear productos a base de hierbas de alta calidad y dilucidar el potencial de los extractos glicólicos de *E. uniflora* como ingredientes farmacéuticos a base de hierbas en sistemas de entrega activos.

**Palabras clave:** *Eugenia uniflora*; Actividad antimicrobiana; Actividad hemolítica; Toxicidad; Diseño de mezcla.

## INTRODUCTION

A species native to South America and distributed throughout the Brazilian territory is *Eugenia uniflora* Linn (Myrtaceae), popularly known as "pitangueira", is used in folk medicine to treat various diseases. In the literature, different biological activities have been attributed to this species, such as antioxidant (Filippi *et al*., 2015), antifungal (Silva-Rocha *et al*., 2017), and anti-inflammatory (Falcão *et al*., 2018). The activities already reported for the species has caused increasing interest in the application of *E. uniflora* extracts in cosmetics and pharmaceutical products.

The effectiveness and safety in using herbal products are closely related to the standardization of the plant extracts. Standardization of the extract is an evaluation parameter used for quality assurance during the development and production of herbal medicines (Azadbakht *et al*., 2020). The standardization process involves different approaches, ranging from the pre-treatment of plant material, including the extraction operation (choice of solvents, extraction methods, and operational conditions), to the development and validation of analytical methods suitable for the quantification of phytochemical markers (Khan *et al*., 2019). The use of mixture designs, such as the simplex centroid, plays a pivotal role in improving the extractive efficiency of solvent systems and increasing the reproducibility of the phytochemical and/or bioactive compounds (Clemente *et al*., 2019). Our study used a mixture design to evaluate the extractive performance of solvent systems on the phytochemical profile. We assessed the antioxidant and antimicrobial activities, as well as the toxic and hemolytic potential of extracts from the leaves of *E. uniflora*.

## MATERIAL AND METHODS

### *Herbal material*

*Eugenia uniflora* leaves were collected in Paulista, Pernambuco, Brazil (7°56'19.6"S 34°56'32.5"W). After that, the material was identified at the Agronomic Institute of Pernambuco (IPA) and the corresponding exsiccate was deposited in the Herbarium Dárdano de Andrade Lima (93732). The access to the species was registered in the Sistema Nacional de Gestão do Patrimônio Genético e Conhecimento Tradicional Associado (SisGen) at the number A449575. The material was dried in an air circulation oven (82/480, Lucadema®) at a temperature of 40°C (3 days) until stabilization. After the drying process, the material was crushed in a Willye knife mill (TE-680, Tecnal®) in an intermediate mesh.

### *Crude extracts*

The crude extracts (CEs) were produced according to the methodology proposed by our research group (Bezerra *et al*., 2018; Ramos *et al*., 2017). The material was subjected to turbo-extraction (in 4 cycles of 30 s with 5-minute intervals between each cycle; LAR.2, Metvisa®) in a proportion of 5% (w/v). The liquid extracts (LE) obtained were concentrated (RV10 Basic, IKA®), frozen and, finally, lyophilized (L101, Liotop®) to obtain the CEs.

### *Simplex centroid design and statistical analysis*

Simplex centroid design was applied to evaluate the use of different solvents and their mixtures as factors in the extraction process. The solvents used were water ($H_2O$), ethanol (EtOH), propylene glycol (PROP), its binary and tertiary mixtures. In total, 7 extractive solutions were produced, according to the matrix described in Table No. 1. As response variables, the content of total flavonoids and total tannins was evaluated by UV-Vis spectrophotometry; and, the content of the markers (myricitrin, gallic acid and ellagic acid) by High Performance Liquid Chromatography. The experiments were carried out in triplicate and the results were expressed by the average obtained for each test. The data were studied using least squares multiple regression method to fit the following special cubic model:

$$y = b_1x_1 + b_2x_2 + b_3x_3 + b_{12}x_1x_2 + b_{13}x_1x_3 + b_{23}x_2x_3 + b_{123}x_1x_2x_3 \quad (1)$$

**Where y was the response (total flavonoid content, total tannin content, gallic acid, ellagic acid, and myricitrin contents), and $b_1$ … $b_{123}$ were the regression coefficients**

**Table No. 1**
**Matrix of simplex centroid and results for flavonoid and tannin content by UV-Vis and markers content by HPLC for *E. uniflora* leaves extracts**

| Run | Water | Ethanol | Propylene glycol | TFC (%w/w) | TTC (%w/w) | Gallic acid (%w/w) | Ellagic acid (%w/w) | Myricitrin (%w/w) |
|---|---|---|---|---|---|---|---|---|
| **1** | 1.000 | 0.000 | 0.000 | 4.94 (0.85) | 14.68 (2.52) | 0.092 (4.73) | 0.130 (1.60) | 0.316 (2.45) |
| **2** | 0.000 | 1.000 | 0.000 | 2.63 (1.82) | 5.42 (3.38) | 0.000 (0.00) | 0.145 (2.38) | 0.380 (3.79) |
| **3** | 0.000 | 0.000 | 1.000 | 3.04 (4.39) | 8.61 (1.91) | 0.092 (0.01) | 0.099 (1.16) | 0.186 (2.17) |
| **4** | 0.500 | 0.500 | 0.000 | 7.83 (0.32) | 16.94 (2.85) | 0.134 (1.55) | 0.158 (3.48) | 0.432 (3.40) |
| **5** | 0.500 | 0.000 | 0.500 | 6.78 (1.55) | 16.02 (2.19) | 0.118 (0.97) | 0.113 (0.51) | 0.245 (1.41) |
| **6** | 0.000 | 0.500 | 0.500 | 4.01 (1.96) | 11.90 (0.59) | 0.112 (0.89) | 0.111 (2.38) | 0.233 (4.46) |
| **7** | 0.333 | 0.333 | 0.333 | 7.98 (0.76) | 18.29 (0.63) | 1.293 (0.20) | 0.375 (4.03) | 1.200 (4.47) |

**Where: TFC - Total Flavonoid Content (expressed by rutin); TTC - Total Tannin Content (expresses by gallic acid). All data are expressed by mean (relative standard deviation)**

In the analysis of variance of the data, $p < 0.05$ was considered statistically significant and the mathematical model was evaluated through the lack of fit and the multiple correlation coefficient (Wehrlé *et al*., 1993). Statistical analysis of results and contour plots were generated using the STATISTICA 8.0 software (StatSoft®).

***Spectrophotometry UV-Vis***

***Total flavonoid content***

To determine the total flavonoid content, was used a methodology adapted from Ramos *et al*. (2017), based on complexation with aluminum chloride ($AlCl_3$). From the aliquots of the liquid extracts, dilution of the samples in 50% ethanol in the proportion of 1:5 (v/v) was performed, originating the stock solutions for total flavonoids (SSTF). For the reaction medium, a total volume of 25 mL was considered, of which 5 mL corresponded to the volume of SSTF, 2 mL of methanol solution of 5% $AlCl_3$ (w/v) and 18 mL of 50% ethanol. After a reaction time of 25 min, the solutions were measured in a spectrophotometer (Evolution 60S, Thermo Fisher Scientific®) at a wavelength of 410 nm, using as blank a solution without adding the complexing agent $AlCl_3$. The total flavonoids content was expressed in %m/m of rutin and calculated by the equation:

$$TFC = \frac{A \times DF}{w \times E_{1\,cm}^{1\%}} \qquad (2)$$

Where: TFC – total flavonoid content; A – absorbance of sample; DF – dilution factor; w – weight of herbal material considering loss on drying (g); $E_{1\,cm}^{1\%}$ - specific absorption of the rutin complex (259.4).

***Total tannin content***

The determination of total tannin content was carried out based on the Folin-Ciocalteu method using a validated methodology for the species. Total polyphenols and non-adsorbed polyphenols were assayed, using polyvinylpyrrolidone (PVP) as a complexing agent.

Initially, the stock solutions of extracts for total polyphenols (SSTPC) and non-tanning fraction (SSNTF) were prepared, and the stock solution of the gallic acid standard (SSGA). For the preparation of the SSTPC, aliquots of the liquid extracts were diluted in distilled water in the proportion 1:25 (v/v); from SSTPC, 10 mL were added to 150 mg of PVP and after 30 min under magnetic stirring, the mixture was filtered with the aid of filter paper, to obtain SSNTF; finally, for the preparation of SSGA, gallic acid was dissolved in distilled water resulting in a concentration solution of 1 mg/mL.

Then, different aliquots of the stock solutions were transferred to the reaction media: 0.5 mL SSTPC and SSNTF; and 0.3 mL SSGA. The reaction medium consisted of 10 mL of distilled water, 1 mL of Folin-Ciocalteu and sodium carbonate ($Na_2CO_3$) 10.75% (w/v) to complete the final volume (25 mL) of each sample. After a reaction time of 15 min, the solutions were analyzed by spectrophotometer at a

wavelength adjusted to 780 nm, generating the absorbance values $A_1$, $A_2$ and $A_3$ corresponding to SSTPC, SSNTF and SSGA, respectively. The content of total tannins was determined by the equation:

$$TTC = \frac{DF \times (A_1 - A_2) \times w_1}{w_2 \times A_3} \quad (3)$$

Where: TTC - total tannin content; DF - dilution factor; $A_1$ – absorbance of total polyphenol; $A_2$ - absorbance of non-tanning fraction; $A_3$ - absorbance of standard; $w_1$ - weight of the standard (g); $w_2$ - weight of herbal material considering loss on drying (g).

***High Performance Liquid Chromatography analysis (HPLC)***

Chromatographic analysis of the samples was performed according to a method validated by Bezerra *et al.* (2018) for *E. uniflora*. A Chromatograph (Ultimate 3000, Thermo Fisher Scientific®) coupled to a photodiode array detector (PDA – 3000 (RS), Thermo Fisher Scientific®), column $C_{18}$ (250 mm × 4.6 mm i.d., particle size 5 µm, Dionex®) and $C_{18}$ pre-column (4 mm x 3.9 µm, Phenomenex®). The crude extracts were dissolved in methanol, filtered through 0.45 µm syringe filters (Macherey-Nagel®) and eluted in a mobile phase composed of a mixture of ultrapure water (A) and HPLC grade methanol (B), both acidified at 0.05% (v/v) with trifluoroacetic acid. For elution, a gradient system was used as following: 0-10 min, 10-25% B; 10-15 min, 25-40% B; 15-25 min, 40-70% B; 25-30 min, 75% B; 30-31 min, 75-10% B. The mobile phase flow was 0.8 mL/min at an oven temperature of 22 ± 1°C. Wavelengths of 254, 270 and 340 nm were selected according to the maximum absorption verified by the detector.

A qualitative and quantitative analysis of the samples was performed to identify the chemical markers of *E. uniflora*, and quantification was performed from calibration curves constructed with the reference standards: gallic acid (≥ 98%, Sigma-Aldrich®), acid ellagic (≥ 95%, Sigma-Aldrich®) and myricitrin (≥ 99%, Sigma-Aldrich®). The Chromeleon software (Thermo Fisher Scientific®) was used for data analysis and processing.

***Antimicrobial activity***

The evaluation of antifungal and antibacterial activity was performed by determining the minimum inhibitory (MIC), bactericidal (MBC) and fungicidal (MFC) concentrations of crude *E. uniflora* extracts against fungal strains from *American Type Culture Collection* (ATCC): *Candida albicans* (90028), *C. tropicalis* (750), *C. parapsilosis* (22019), *C. glabrata* (2001) and *C. krusei* (6258); gram-negative bacteria: *Escherichia coli* (UFPEDA224; ATCC 25922), *Salmonella enteriditis* (UFPEDA414), *Pseudomonas aeruginosa* (UFPEDA416; ATCC 27853), *Serratia marcenses* (UFPEDA352) and *Klebsiella pneumoniae* (UFPEDA396); and, gram-positive: *Streptococcus pyogenes* (UFPEDA1023; ATCC 16642), *Staphylococcus saprophyticus* (UFPEDA833), *Staphylococcus aureus* (UFPEDA02; ATCC 25293), *Bacillus megaterium* (UFPEDA462) and *Enterococcus faecalis* (UFPEDA620). The tests were carried out under conditions recommended by the *Clinical and Laboratory Standards Institute* with adaptations for natural products (CLSI, 2012).

For bacteria, Mueller Hinton agar (Merck®) was used in a controlled environment at constant 37°C, during an incubation period of 24 h. In relation to fungal, Sabouraud agar (Merck®) was used, under controlled incubation temperature at 35°C for 48 h. To obtain the inoculum, fungal and bacterial suspensions were prepared in 0.9% saline and adjusted in a spectrophotometer (AJX-1900, Micronal®) to contain approximately $10^6$ CFU/mL (0.5 on the McFarland scale), using as a wavelength of 600 nm for bacteria and 530 nm for fungi.

To determine MIC, the microdilution method (Sabouraud - fungi; Mueller Hinton - bacteria) was used, using a 96-well plate. Serial dilutions of the extracts (10%, dimethylsulfoxide - DMSO) and aqueous solutions of azithromycin, ampicillin, ciprofloxacin, and gentamicin (Pfizer® Inc.) (2 mg/mL), were performed within the concentration range between 1000 and 1.9 µg/mL. The DMSO solution was used as a negative control. The MIC was considered the lowest concentration in which no fungal/bacterial growth was seen, compared to the positive control. After incubating the plates, 20 µL of 0.01% aqueous resazurin solution (Sigma-Aldrich®) was added to the wells to show the inhibition of the growth of microorganisms.

To determine MFC and MBC, an aliquot from each well was transferred to a Petri dish containing Sabouraud agar or Mueller Hinton agar, according to the microorganism. After the incubation period, the plates were observed and the lowest concentration of extract capable of inhibiting the visible growth of the inoculum was determined as MFC/MBC.

***Antioxidant activity***

The antioxidant activity was evaluated *in vitro* using a methodology adapted from Mensor *et al*. (2001), by determining the ability of crude extracts to eliminate DPPH• free radicals (2,2-diphenyl-1-picryl-hydrazil, Sigma-Aldrich®). For this, the solutions of the crude extracts and standard, prepared in ethanol 50% at 1 mg/mL, were diluted in a concentration range of 5-50 µg/mL. The DPPH stock solution was prepared in 0.3 mM ethanol (on the day of analysis and under light). After 30 min of adding the DDPH to the reaction medium, the samples were analyzed in a spectrophotometer at a wavelength of 517 nm, using a mixture of 50% ethanol and DDPH as negative control. The antioxidant activity (AA), expressed as a percentage, was calculated using the equation:

$$AA\ (\%) = 100 - \left[\left(Abs_{sample} - Abs_{blank}\right) \times 100 \div Abs_{control}\right] \quad (4)$$

The concentration capable of sequestering 50% of DPPH ($IC_{50}$) was calculated using the equation of the straight line obtained by the linear range between the concentration points of the extracts/standard, plotted according to the corresponding antioxidant activity. The test was performed in triplicate.

***Artemia salina assay***

The lethality of the extracts was determined against *Artemia salina* Leach (San Francisco Bay Brand, Inc., EUA) according to the methodology described by Meyer *et al.* (1982). Initially, *A. salina* cysts were incubated in natural sea water (adjusted to pH 9.0) under controlled temperature conditions (25-30 °C) to obtain larvae. Then, different concentrations of extract (1-1000 µg/mL) were obtained with saline in tubes, to which 10 viable *A. salina* nauplii were added. After 24 h at room temperature, the number of live larvae was counted, and the lethal concentration ($LC_{50}$) was calculated for each *E. uniflora* extract. The tests were performed in triplicate.

***Hemolytic activity***

The evaluation of hemolytic activity in this study was approved by the Ethics Committee on the Use of Animals at the Federal University of Pernambuco (No. 0077/2019). For the test, blood samples collected from *Swiss Mus musculus* mice (n=3), previously anesthetized, were used. After cardiac puncture, the samples were washed with a solution composed of 0.85% sodium chloride (NaCl) and 10 mM calcium chloride ($CaCl_2$) and were subsequently centrifuged. The supernatant was removed, and the erythrocyte mass was used to obtain a 2% (v/v) cell suspension in saline.

The test was carried out in 96-well microplates using the microdilution method in saline. Serial dilutions of the extracts at 2 mg/mL, previously solubilized in phosphate-saline buffer (PBS), were performed within a concentration range of 2000 to 31.25 µg/mL. Saline solution was used as white, PBS as a negative control and Triton X-100 at 0.1% (v/v) as a positive control (100% hemolysis). After adding the erythrocyte suspension to the wells, the plates were subjected to constant agitation for 1 h and left to stand for the same time at 27 °C. Subsequently, the supernatant was collected for quantification of hemoglobin at a wavelength of 450 nm. The concentration of extract capable of hemolyzing 50% of the erythrocytes ($IC_{50}$) in relation to the positive control was calculated using GraphPad Prism v 5.0 (GraphPad Software, Inc., USA) using a non-linear regression model. The tests were performed in triplicate.

## RESULTS AND DISCUSSION

The presence of different secondary metabolites is widely associated with the biological properties of the herbal species. Several phytocompounds, such as phenolic substances, play an important role and are a source for our discovery of new active ingredients and/or phytotherapeutic agents. These phytotherapeutic agents can reinforce our therapeutic arsenal, especially against resistant microorganisms. Therefore, our research used a simplex centroid mixture design to obtain solvent mixtures containing water, ethanol, and propylene glycol to produce and evaluate different extracts.

***Phytochemical study***

***Statistical analysis***

An analysis of variance (Anova) presented a good fit for analyzing the special cubic model's experimental data. The analyses revealed a good multiple correlation coefficient ($R^2 > 0.99$), high F-value, and $p$-value < 0.05. The calculated $R^2$ (Table No. 2) suggests that more than 99% of the experimental

behavior can be explained by the equations of the mathematical model. In addition, no lack-of-fit was observed, which demonstrates an agreement between the values predicted by the models and the real values, to all the experimental variance to be attributed to random error. Thus, the generated graphics are reliable and adequately describe the data without violating the model's premises (Wehrlé *et al.*, 1993). The regression coefficients of the terms were statistically evaluated using *p*-values and *t*-tests and are shown in Table No. 3. In all analyses a probability value less than 0.05 was considered statistically significant ($p<0.05$).

**Table No. 2**
**Analysis of variance (Anova) for the special cubic model adjusted to experimental data.**

| | Response variables | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | TFC | | TTC | | GA | | EA | | MYR | |
| **Source** | **df** | **SS** | **F** | **SS** | **F** | **SS** | **F** | **SS** | **F** | **SS** | **F** |
| **Model** | 6 | 88.25 | 2348.31* | 403.83 | 760.41* | 3.74 | 32944.18* | 0.174 | 718.63* | 1.968 | 452.12* |
| **Total error** | 14 | 0.09 | - | 1.239 | - | 0.00026 | - | 0.0005 | - | 0.010 | - |
| **Pure error** | 14 | 0.09 | - | 1.239 | - | 0.00026 | - | 0.0005 | - | 0.010 | - |
| **Total adjusted** | 20 | 88.34 | - | 405.07 | - | 3.7464 | - | 0.175 | - | 1.978 | - |
| $R^2$ | - | - | 0.999 | - | 0.996 | - | 0.999 | - | 0.996 | - | 0.994 |
| $R^2$ **(adj)** | - | - | 0.998 | - | 0.995 | - | 0.999 | - | 0.995 | - | 0.992 |

**Where: TFC - Total Flavonoid Content; TTC - Total Tannin Content; GA - Gallic Acid; EA - Ellagic Acid; MYR: Myricitrin. *Significant ($p<0.05$)**

**Table No. 3**
**Statistical analysis for the responses of the special cubic model**

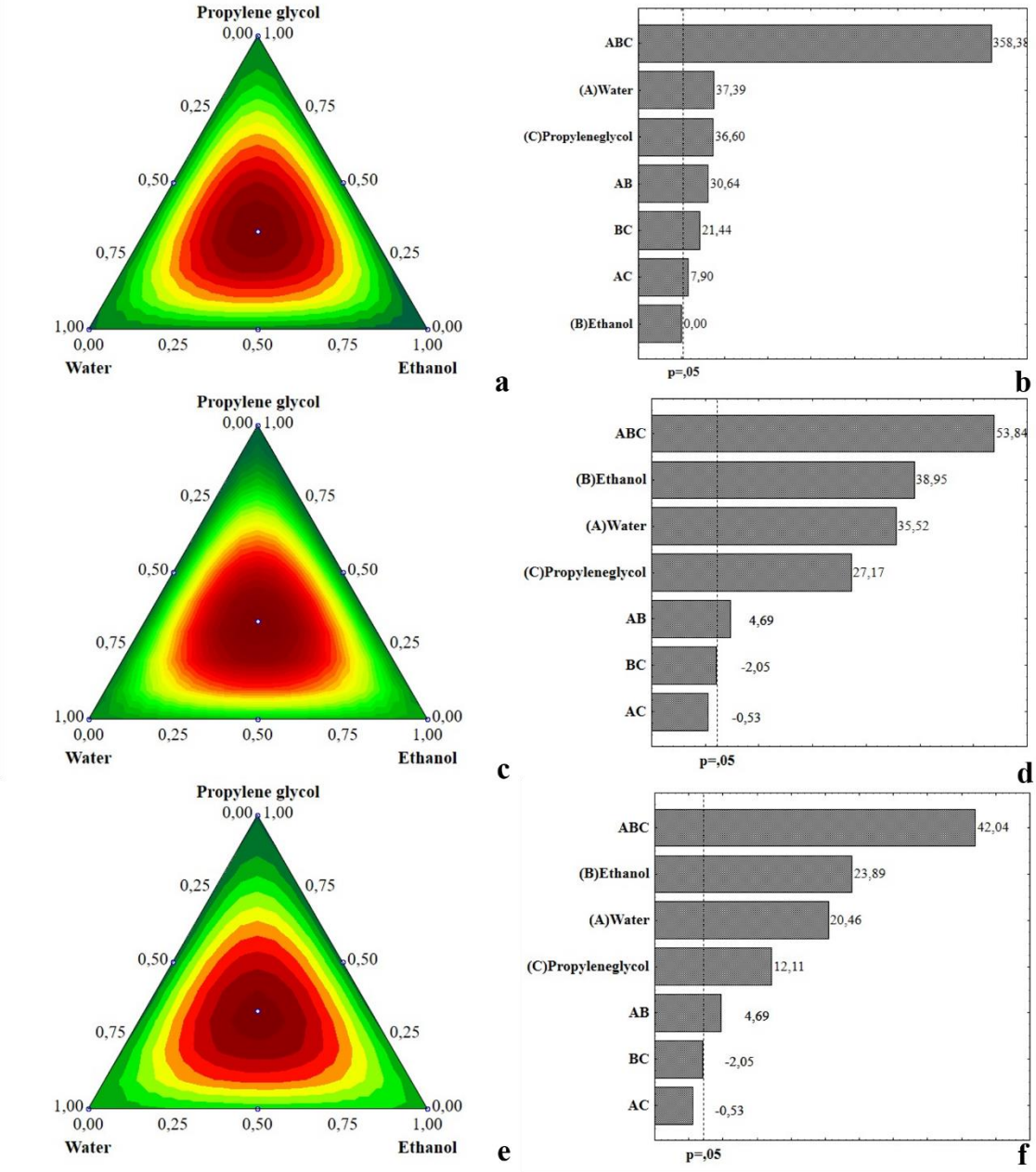
| | TFC | | TTC | | GA | | EA | | MYR | |
|---|---|---|---|---|---|---|---|---|---|---|
| **Terms** | **Coef.** | ***t-test*** | **Coef.** | ***t-test*** | **Coef.** | ***t-test*** | **Coef.** | ***t-test*** | **Coef.** | ***t-test*** |
| $b_1$ | 4.96 | 108.62* | 14.64 | 85.26* | 0.09 | 37.39* | 0.13 | 35.52* | 0.31 | 20.46* |
| $b_2$ | 2.63 | 57.58* | 5.44 | 31.71* | 0.00 | 0.00 | 0.14 | 38.95* | 0.37 | 23.89* |
| $b_3$ | 3.12 | 68.33* | 8.56 | 49.86* | 0.09 | 36.60* | 0.09 | 27.17* | 0.18 | 12.11* |
| $b_{12}$ | 16.19 | 72.33* | 28.20 | 33.52* | 0.37 | 30.64* | 0.08 | 4.69* | 0.35 | 4.69* |
| $b_{13}$ | 11.17 | 49.91* | 17.96 | 21.34* | 0.09 | 7.90* | -0.01 | -0.53 | -0.04 | -0.53 |
| $b_{23}$ | 4.60 | 20.57* | 19.62 | 23.32* | 0.26 | 21.44* | -0.03 | -2.05 | -0.15 | -2.05 |
| $b_{123}$ | 22.14 | 14.05* | 38.01 | 6.42* | 31.04 | 358.38* | 6.82 | 53.84* | 22.54 | 42.04* |

**Where: TFC - Total Flavonoid Content; TTC - Total Tannin Content; GA - Gallic Acid; EA - Ellagic Acid; MYR: Myricitrin. *Significant ($p<0.05$)**

***Total flavonoids (TFC) and total tannin content (TTC) by UV-Vis analysis***

The flavonoids (TFC) content was identified by spectrophotometry in *E. uniflora* leaf extracts and ranged from 2.63 to 7.98% w/w (with results expressed in percentage of rutin). The analysis of the data by Pareto chart and contour plots (Figure No. 1a and Figure No. 1b) showed that pure solvents as well as binary and ternary mixtures, provided a significant positive effect on TFC ($p<0.05$). The most important influence on the flavonoid extraction can be attributed to factor A ($H_2O$). This was followed by the interaction AB ($H_2O$: EtOH). This was closely followed (with a similar magnitude) by factor C (PROP), factor B (EtOH), and the AC interaction ($H_2O$: PROP). The BC and ABC interactions showed less technological importance. However, due to the statistical significance of BC and ABC, they were used to generate the contour plots.

**Figure No. 1**




**Pareto charts and contour plots of the effect of solvents on the total flavonoid (a and b) and total tannin (c and d) content of crude extracts from *E. uniflora* leaves**

The extraction of total tannins (TTC) demonstrated a similar relationship behavior between the solvent systems and TTC in the extracts (Figure No. 1c). The experimental conditions provided extracts containing TTC that varied from 5.42 to 18.29% w/w (expressed in % of gallic acid). However, TTC showed greater dependence on factor A ($H_2O$), followed by factor C (PROP). This was followed by the interaction AB ($H_2O$: EtOH). Thus, the maximum response region undergoes a slight displacement towards factor A ($H_2O$) (Figure No. 1d).

The great influence of water on the extraction of flavonoids and tannins is greater than the solvent mixtures themselves due to the affinity of these compounds for polar solvents (Gasmi *et al*., 2019).

This can be explained by the common occurrence of chemical groups (hydroxyls conjugated to sugars, acids, or alkyl groups) in the structure of these metabolites that increase their interaction with solvents of the same chemical character (Mokrani & Madani, 2016).

Considering the optimized conditions identified by the contour plots, we observed that the best extractive conditions were achieved simultaneously for TFC and TTC using the ternary mixture ABC ($H_2O$: EtOH: PROP; 0.333: 0.333: 0.333). Phenols represent a diverse class of compounds, which allows for great chemical variability (Akbarian *et al*., 2019). Therefore, the use of a solvent system can promote a greater extraction range, since the diversity in the composition of the mixture provides a wider range of polarity, unlike pure solvents. According to Sungpud *et al.* (2019), PROP is a polyol derived from glycerol with a great capacity to solubilize water-insoluble compounds due to the occurrence of hydrophilic and lipophilic regions in its molecular structure. Due to this property of PROP and with the advent of mixtures with other solvents, Sungpud *et al*. (2019) were able to observe an increasing trend in the recovery of total phenols and flavonoids deposited in the cellular matrices. In addition, a possible mechanism that may explain the greater extraction efficiency of the ternary $H_2O$: EtOH: PROP system is due to the relatively small size of the EtOH molecules. The small size promotes the infiltration and swelling of plant tissue, facilitating the extraction of polyphenols and flavonoids by other solvents (PROP and $H_2O$) (Tsai & Lin, 2019).

***HPLC analysis***

In the chromatograms obtained from the different *E. uniflora* extracts (Figure No. 2), it was possible to identify gallic acid (peak 1; Rt = 7.55 min), myricitrin (peak 2; Rt = 22.3 min), and ellagic acid (peak 3, Rt = 23.8 min), corroborating the observation by Bezerra *et al*. (2018). The quantification of these markers in the extracts revealed a content range of 0.092-1.293 % w/w of gallic acid, 0.099-0.375% w/w of ellagic acid, and 0.186-1.200% w/w of myricitrin.

**Figure No. 2**

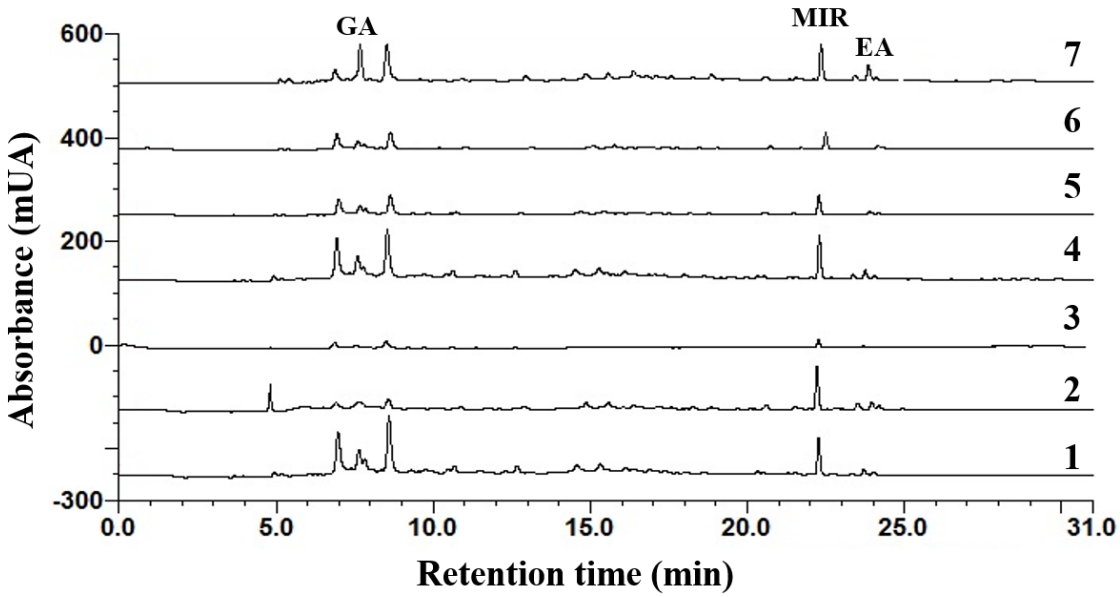



**Figure No. 2**
**Chromatographic profile of crude extracts of *E. uniflora* leaves at 270 nm**
**Where: 1 - Gallic acid; 2 - Myricitrin; 3 - Ellagic acid; $H_2O$ - aqueous extract; EtOH - ethanolic extract; PROP - glycolic extract; $H_2O$: EtOH - hydroethanolic extract; $H_2O$: PROP - extract with the mixture of water and propylene glycol; EtOH: PROP - extract with the mixture of ethanol and propylene glycol; $H_2O$: EtOH: PROP - Extract with the mixture of water, ethanol, and propylene glycol**

An interesting behavior was observed using the analysis of variance. According to the generated Pareto charts (Figures No. 3a, Figure No. 3c, and Figure No. 3e), the content of the tannins (gallic acid and ellagic acid), and the flavonoid (myricitrin) showed a better yield on pure solvents when compared to binary mixtures, a response similar to that obtained by spectrophotometry. Conversely, the ternary mixture ABC ($H_2O$: EtOH: PROP) prevailed, exercising greater technological importance in the extraction of the markers. This synergistic relationship between the components of the mixture was confirmed by the contour plots (Figures No. 3b, Figure No. 3d, and Figure No. 3f). On the contour plots, the maximum levels of extraction are clearly displaced to the region of the mixture containing the three solvents.

For the extraction of ellagic acid and myricitrin (Figures No. 3c and Figure No. 3e), the ABC interaction was the most successful, followed by factors B (EtOH) and A ($H_2O$) in similar degrees. The next important was factor C (PROP) and then the AB ($H_2O$: EtOH) interaction. The extraction of gallic acid (Figure No. 3a) followed the same order, except for factor B. The EtOH extract samples showed undetectable levels of gallic acid (Table No. 1). Thus, the results for this marker showed a greater relationship with the ABC interaction, followed by factors A and C in similar magnitudes, followed by the interactions AB, BC (EtOH: PROP), and AC ($H_2O$: PROP).

**Figure No. 3**

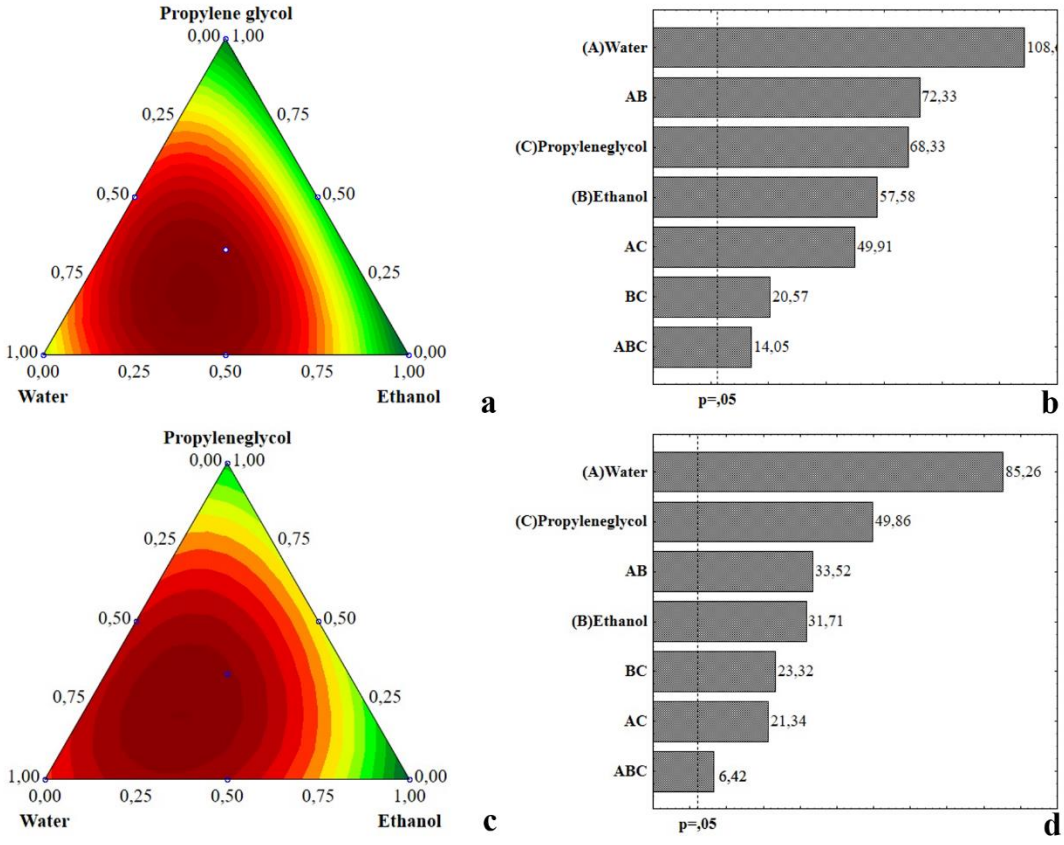



**Figure No. 3**
**Pareto charts and contour plots of the effect of solvents on the content of Gallic acid (*a* and *b*), Ellagic acid (*c* and *d*) and Myricitrin (*e* and *f*) of crude extracts from *E. uniflora* leaves**

The technological importance of pure solvents is evidenced by the results of UV-Vis and demonstrated in the solvent system using HPLC. There is a non-selectivity of pure solvents for bioactive compounds of interest. While $H_2O$ had a greater influence on the recovery of TFC and TTC, the ternary $H_2O$: EtOH: PROP system was more selective in the extraction of the main *E. uniflora* markers (gallic acid, ellagic acid, and myricitrin). Water as an extractor solvent, despite its numerous advantages over organic solvents, is not very selective and produces extracts with undesirable components. Therefore, extractions using more than one solvent provide better separation and isolation of phenolic compounds (Stojiljković *et al*., 2016). Garmus *et al.* (2019) evaluated *E. uniflora* leaf extracts using different solvents and extractive methods; he identified an increase in the extraction yield as a function of the polarity of the solvents, with hydroethanolic extracts showing the greatest yields of phenols and flavonoids. Although hydroethanolic solvents are supported in the literature as efficient extractors of tannins and flavonoids (Nešić *et al*., 2019), we observed that the addition of PROP to this system significantly increased the levels of gallic acid and ellagic acid (tannins) and myricitrin (flavonoid). Corroborating these observations, Tubtimdee & Shotipruk (2011) reached a higher content of total phenolics, gallic acid and ellagic acid in *Terminalia chebula* extracts using solvent systems composed of water-propylene glycol. In addition, PROP is considered safe by the Food and Drug Administration (FDA). PROP is commonly used in pharmaceutical products as topical, oral, and injectable formulations, and as a preservative in food (Lim *et al*., 2014).

The use of a mixtures design constitutes an important tool in the extraction stage because it demonstrates (within the particularities of the herbal matrix) conditions of interaction between solvents that result in greater selectivity of the extraction systems by bioactive metabolites of interest. Thus, it is possible to develop procedures that guarantee the quality and chemical identity of plant species while minimizing the costs of obtaining herbal medicines (Bezerra *et al*., 2020).

***In vitro activities***

***Minimum Inhibitory (MIC) and Minimum Bactericidal/Fungicidal Concentrations (MBC/MFC)***

In general, most of the *E. uniflora* extracts showed excellent activity against both fungal and bacterial strains, with MIC up to 1.9 µg/mL for different microorganisms. The strains (fungal and bacterial) were susceptible to the action of the extracts and showed growth inhibition at the concentration range of 1.9-500 µg/mL (Table No. 4). The only exception was *C. albicans*, which presented little sensitivity to extracts obtained with $H_2O$, EtOH, and $H_2O$:EtOH.

When the MBC and MFC were determined, the bactericidal/fungicidal concentration must be considerably increased when compared to the MICs, (with levels above 1000 µg/mL for most of the microorganisms evaluated) before the extracts exerted any effective control. In contrast, some extracts maintained or slightly changed the MIC concentrations to MBC/MFC. All the extracts evaluated showed fungicidal activity against at least one *Candida* sp. strain, with most showing activity against non-*Candida albicans* strains, and a few were bactericidal. The microorganisms more susceptible to the fungicidal and bactericidal action of *E. uniflora* extracts were *C. parapsilosis*, *C. tropicalis*, *C. krusei*, and *S. enteritidis*, *S. pyogenes*, *S. saprophyticus*, respectively. Among the extracts, $H_2O$:EtOH:PROP is the most important, it was a fungicide in a concentration range of 3.9-125 µg/mL against all the evaluated strains of *Candida*. The second most successful was the fungicidal/bactericidal $H_2O$: EtOH extract (250-500 µg/mL). The least successful was the fungicidal EtOH extract (7.8-125 µg/mL). It can be inferred from the MIC and MBC/MFC values that *E. uniflora* extracts showed predominantly good bacteriostatic action and fungicidal activity that varied from good to moderate (Ayres *et al*., 2008).

It was also possible to determine a clear relationship between the fungicidal/bactericidal performance of the extracts and their chemical profiles. For example, the extracts with the best antimicrobial action $H_2O$:EtOH:PROP and $H_2O$:EtOH presented, in this order, the highest levels of quantified metabolites - TFC (7.98/7.83% w/w), TTC (18.29/16.94% w/w), GA (1.29/0.13% w/w), EA (0.37/0.15% w/w) and MYR (1.20/0.43% w/w). The EtOH extract did have a low content of TFC, TTC, and GA, it showed a certain selectivity to MYR (0.380% w/w) and EA (0.14% w/w), these metabolites may be involved in causing its effective fungicidal performance.

Studies indicate that these constituents can give herbal derivatives antimicrobial action through different mechanisms. For example, in the case of flavonoids, inhibition of nucleic acid synthesis,

cytoplasmic membrane function, and energy metabolism (Kanwal *et al*., 2011). As for tannins, the connection with ergosterol, and the formation of pores in the cell membrane structure may be involved in its antifungal action (Carvalho *et al*., 2018). In addition, *in vitro*/*in vivo* studies also show the potential antifungal activity of gallic acid and ellagic acid (Li *et al*., 2015). Gallic and ellagic acids may act by connecting to the cell membrane as well as through the inhibition of ergosterol biosynthesis, affecting the viability of fungal cells.

**Table No. 4**
**Minimum Inhibitory Concentration (MIC) and Minimum Fungicidal Concentration (MFC) or Minimum Bactericidal Concentration (MBC) results of crude extracts from *E. uniflora* leaves**

| | MIC/MFC or MIC/MBC | | | | | | |
|---|---|---|---|---|---|---|---|
| **Strains** | **$H_2O$** | **EtOH** | **PROP** | **$H_2O$: EtOH** | **$H_2O$: PROP** | **EtOH: PROP** | **$H_2O$: EtOH; PROP** |
| *C. albicans* | 1000/>1000 | 1000/>1000 | 125/>1000 | 1000/>1000 | 500/>1000 | 62.5/>1000 | 125/125 |
| *C. tropicalis* | 125/>1000 | 125/125 | 250/>1000 | 250/250 | 250/250 | 500/500 | 125/125 |
| *C. parapsilosis* | 62.5/500 | 1.9/7.8 | 125/500 | 62.5/500 | 1.9/7.8 | 1.9/7.8 | 62.5/125 |
| *C. glabrata* | 31.2/>1000 | 62.5/>1000 | 125/>1000 | 31.2/>1000 | 31.2/>1000 | 31.2/>1000 | 15.6/15.6 |
| *C. krusei* | 62.5/>1000 | 62.5/62.5 | 62.5/>1000 | 62.5/>1000 | 62.5/>1000 | 62.5/>1000 | 3.9/3.9 |
| *E. coli* | 31.2/>1000 | 31.2/>1000 | 62.5/>1000 | 62.5/>1000 | 62.5/>1000 | 62.5/>1000 | 62.5/>1000 |
| *S. enteriditis* | 62.5/>1000 | 235/1000 | 31.2/500 | 62.5/500 | 62.5/1000 | 62.5/500 | 62.5/1000 |
| *S. saprophyticus* | 1.9/>1000 | 1.9/1000 | 1.9/500 | 1.95/1000 | 125/1000 | 31.2/1000 | 125/1000 |
| *S. pyogenes* | 3.9/>1000 | 3.9/1000 | 3.9/1000 | 3.91/500 | 3.9/>1000 | 3.9/1000 | 3.9/>1000 |
| *S. aureus* | 1.9/>1000 | 1.9/1000 | 1.9/1000 | 1.95/1000 | 1.9/>1000 | 1.9/1000 | 1.9/>1000 |
| *B. megabacterium* | 7.8/>1000 | 16.6/>1000 | 16.6/>1000 | 16.6/>1000 | 16.6/>1000 | 16.6/>1000 | 16.6/>1000 |
| *P. aeruginosa* | 7.8/>1000 | 7.8>1000 | 7.8/1000 | 16.625/1000 | 31.2/>1000 | 31.2/>1000 | 31.2/>1000 |
| *S. marcenses* | 125/>1000 | 125>1000 | 31.2/>1000 | 125/>1000 | 250/>1000 | 62.5/>1000 | 125/>1000 |
| *E. faecalis* | 62.5/>1000 | 125>1000 | 250/>1000 | 62.5/>1000 | 125/>1000 | 125/>1000 | 62.5/>1000 |
| *K. pneumoniae* | 62.5/>1000 | 15.6/1000 | 62.5/1000 | 62.5/1000 | 62.5/>1000 | 62.5/>1000 | 62.5/>1000 |

**Where: $H_2O$ - aqueous extract; EtOH - ethanolic extract; PROP - glycolic extract; $H_2O$: EtOH - hydroethanolic extract; $H_2O$: PROP - extract with the mixture of water and propylene glycol; EtOH: PROP - extract with the mixture of ethanol and propylene glycol; $H_2O$: EtOH: PROP - Extract with the mixture of water, ethanol and propylene glycol**

The cell membrane of Gram-positive bacteria consists of several layers of peptidoglycan functioning perpendicular to a group of teichoic acids. Conversely, the cell membrane of gram-negative bacterial compositions is a single layer of peptidoglycan that is surrounded by a membrane structure called an external membrane containing lipopolysaccharides. Therefore, the cell and membrane of gram-positive bacteria are thicker than gram-negative bacteria. The fat-soluble phenolic portion of *E. uniflora* extracts can penetrate the thick cell membrane of gram-positive strains and cause toxicity to the internal components of the cell. This is related to the absorption capacity of the molecules, which may play an important role in microbial inactivation. Thus, the antibacterial activity is not only affected by the content of the components, but also by the profile of these compounds in the extracts and their absorption capacity (Akbarian *et al*., 2019).

***Antioxidant, Hemolytic and Toxicity properties***

Most of the samples contained similar $IC_{50}$ values within a range of 9.507-16.072 µg/mL, except for the PROP extract, which had the lowest antioxidant capacity. Extracts are considered to have high antioxidant activity when $IC_{50}$ values are below 50 µg/mL (Table No. 5). Considering the chemical composition of *E. uniflora* extracts, the polyphenols

within it have several biological effects and are considered one of the main groups of compounds acting as primary antioxidants or as free radical terminators. The efficiency of phenolic compounds as antioxidants and antioxidants depends on factors such as the number of hydroxyls attached to the aromatic ring, the binding site, and their position in the aromatic ring (Sungpud *et al*., 2019).

**Table No. 5**
**Antioxidant, toxicity, and hemolytic activities of crude extracts from *E. uniflora* leaves**

| Extracts | Antioxidant activity $IC_{50}$ (µg/mL) | Toxicity $LC_{50}$ (µg/mL) | Hemolytic activity $IC_{50}$ (µg/mL) |
|---|---|---|---|
| **$H_2O$** | 9.511 | >1000 | >200 |
| **EtOH** | 11.046 | >1000 | >200 |
| **PROP** | 47.021 | 890.07 | >200 |
| **$H_2O$: EtOH** | 9.507 | >1000 | >200 |
| **$H_2O$: PROP** | 16.072 | >1000 | >200 |
| **EtOH: PROP** | 10.538 | 745.55 | >200 |
| **$H_2O$: EtOH: PROP** | 11.031 | 900.34 | >200 |

**Where: $H_2O$ - aqueous extract; EtOH - ethanolic extract; PROP - glycolic extract; $H_2O$: EtOH - hydroethanolic extract; $H_2O$: PROP - extract with the mixture of water and propylene glycol; EtOH:P ROP - extract with the mixture of ethanol and propylene glycol; $H_2O$: EtOH: PROP - extract with the mixture of water, ethanol, and propylene glycol**

Gallic acid consists of an aromatic ring, three phenolic hydroxyl groups, and a carboxylic acid group. The three hydroxyl groups are linked to the aromatic ring in an ortho position to each other. This structural order is the determining factor for the strong antioxidant capacity of the molecule (Badhani *et al*., 2015). The ellagic acid has four hydroxyl groups and two lactones. The hydroxyl group can increase the antioxidant activity, and the antioxidant efficiency is directly related to the degree of hydroxylation and can decrease in the presence of sugar (Shakeri *et al*., 2018). Finally, for a flavonoid to possess antioxidant power, it must have a 3',4-dihydroxy structure in ring B; 2,3 double bond together with a 4-oxo group on ring C; and the presence of a 3-hydroxyl group in ring C and a 5-hydroxyl group in ring A. Therefore, flavonols (such as myricitrin) that are present in *E. uniflora* leaf extracts, meet these structural requirements (Hwang & Chung, 2018; Jucá *et al*., 2020).

In addition, all extracts were inactive against Swiss mice (*Mus musculus*) erythrocytes ($IC_{50}$ > 200 µg/mL) and were non-toxic ($LC_{50}$ > 1000 µg/mL) or with low toxicity (745.55-890.07 µg/mL) for *Artemia salina* (Costa-Lotufo *et al*., 2005; Déciga-Campos *et al*., 2007). The use of *A. salina* and the hemolysis test is often applied in toxicity tests to ascertain biocompatibility and, above all, the safe use of medicinal plants, extractive derivatives, and fractions. It is considered convenient to use these methods because the test with *A. salina* is fast, simple, and sensitive to toxic substances. The hemolytic activity test on erythrocytes allows us to observe whether the cytotoxicity of the extract is related to membrane damage (Marreiro *et al*., 2014; Merino *et al*., 2015).

## CONCLUSIONS

The results analysis revealed important statistical differences between UV-Vis and HPLC. While the pure solvents expressed a greater effect on TFC and TTC, the ternary solvent mixture system $H_2O$: EtOH: PROP was more selective and efficient in the extraction of the markers GA, EA, and MYR from the leaves of *E. uniflora*. The extracts obtained from the simplex centroid were predominantly bacteriostatic and fungicidal activities. It was possible to verify that the relationship between antimicrobial activity and the chemical profile of the $H_2O$: EtOH: PROP extract had the highest phenolic content and was the most promising for fungicidal activity for all the strains evaluated. In addition, *E. uniflora* extracts exhibited high antioxidant capacity and biocompatibility in the preliminary safety tests. The PROP demonstrated a great contribution to the extraction of compounds of interest and, consequently, in the *in vitro* bioactivities of the extracts. The *E. uniflora* PROP is the safest and best preserved when extracted using $H_2O$: EtOH: PROP

for medicinal preparations, especially in products for topical delivery systems.

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