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Original Article

Investigation of phytochemical composition and bioactivity evaluation of extracts from *Myrsine umbellata* Mart.

Investigação da composição fitoquímica e avaliação da bioatividade de extratos de *Myrsine umbellata* Mart.

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Abstract

The objective of the study was to carry out phytochemical prospection through colorimetric tests to determine the groups of secondary metabolites and also to determine the total content of phenolic compounds (TPC) present in plant extracts methanol (ME), ethyl acetate (EAE), hexane (HE) and dichloromethane (DE) from the leaves of *Myrsine umbellata*, as well as to investigate the antimicrobial activity against twelve standard ATCC strains by the broth microdilution technique; the antioxidant potential by the DPPH method and the ABTS method and the antibiofilm potential on the biofilm biomass of standard bacteria by the crystal violet technique and tetrazolium salt reduction (MTT) assay. Phytochemical prospection detected the presence of saponins, steroids, alkaloids, anthocyanins, anthocyanidins, flavonoids, and tannins. The results of the quantitative phytochemical estimation revealed a higher content of total phenolics in DE (280.24 ± 0.037 µM GAE g ext. $^{-1}$) followed by ME (159.01 ± 0.031 µM GAE g ext. $^{-1}$). The ME showed the best biological activities when compared to the other extracts tested. We observed antimicrobial activity against Gram-positive *Staphylococcus epidermidis* strain (MIC 3.12 and MBC 6.25), antioxidant percentage of 92.58% against the DPPH radical and 420.31 µM Trolox g ext. $^{-1}$ against the ABTS radical, finally showed antibiofilm action against Gram-positive strain *Staphylococcus aureus*, with eradication of the biomass in 92.58%. The results suggest that EM from *M. umbellata* represents an alternative source of plant bioactives for the development of natura products.

Keywords: capororoca, biological activities, biofilm, bioactive compounds.

Resumo

O objetivo do estudo foi realizar a prospecção fitoquímica através de testes colorimétricos para determinar os grupos de metabólitos secundários e também determinar o teor total dos compostos fenólicos (TPC) presente nos extratos vegetais metanol (EM), acetato de etila (EAE), hexano (EH) e diclorometano (ED) das folhas de *Myrsine umbellata*, bem como investigar a atividade antimicrobiana contra doze cepas padrão ATCC pela técnica de microdiluição em caldo; o potencial antioxidante pelo método DPPH e pelo método ABTS e o potencial antibiofilme sobre a biomassa padrão de biofilmes bacterianos pela técnica do cristal violeta e ensaio de redução do sal de tetrazólio (MTT). A prospecção fitoquímica detectou a presença de saponinas, esteróides, alcaloides, antocianinas, antocianidinas, flavonoides e taninos. Os resultados da estimativa fitoquímica quantitativa revelaram maior teor de fenólicos totais em ED (175,15 ± 0,037 µM GAE g ext. $^{-1}$) seguido de EM (159,02 ± 0,031 µM GAE g ext. $^{-1}$). O EM apresentou as melhores atividades biológicas quando comparado aos demais extratos testados, observamos atividade antimicrobiana contra cepa Gram-positiva *S. epidermidis* (MIC 3.12 e MBC 6.25), percentual antioxidante de 92,58% frente ao radical DPPH e 420,44 mg Trolox g ext. $^{-1}$ frente ao radical ABTS, por fim apresentou ação antibiofilme contra cepa Gram-positiva *S. aureus*, com erradicação da biomassa em 92,58%. Os resultados sugerem que o EM de *M. umbellata* representa uma fonte alternativa de bioativos vegetais para o desenvolvimento de produtos em natura.

Palavras-chave: capororoca, atividades biológicas, biofilme, compostos bioativos.

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1. Introduction

Brazil is one of the countries with the greatest diversity of plant species on the planet, which is why plants have been researched as an alternative to health problems, especially due to their active principles. Thus, the Brazilian industry has sought safer options for pathogen control, pointing out as promising the natural antimicrobials, such as plant extracts. Studies have already demonstrated their efficiency in controlling resistant microorganisms, since they are natural products with probably less toxicity. In addition, they represent a less harmful way for the consumer, who will be free from antibiotic overdoses and possible health damage (Bona et al., 2010; Silva et al., 2016).

The inappropriate application of synthetic antimicrobials affects the fields of human and veterinary medicine, as high dosages of antimicrobials/antifungals are used. This leads to the selection of resistant pathogens when trying to combat infections caused by microorganisms. This resistance causes genetic changes, which modify the different biochemical mechanisms that prevent the action of drugs. Therefore, it is important to search for new antimicrobial substances, aiming for a more efficient, economical, and less harmful option to the environment (Arantes et al., 2016).

Due to these problems and the increasing concern of the population with the use of synthetic products, there is a demand in studies regarding biological activities. In addition, the use of plant products has been widely studied for antioxidant purposes, since they may have compounds that control excess free radicals in the cell. Therefore, plant extracts become strong candidates for studies related to this biological activity (Pupo and Gallo, 2007).

Furthermore, plant extracts have been studied and used for biofilm control. Some bacterial cells can form an extracellular polymer matrix composed of nucleic acids, proteins, and polysaccharides, called biofilms. Biofilms manifest unique developmental properties and phenotypic traits that favor protection from the external environment and provide better adhesion to surfaces. Thus, they reduce susceptibility to commercialized antimicrobials, becoming more resistant in environments with adverse conditions (Flach et al., 2005; Pasternak, 2009). The presence of biofilms causes surface deterioration and/or proliferation of pathogenic microorganisms, leading to damage in industry when present on equipment, and in health by increasing the mortality rate due to diseases caused by the resistance of bacteria to antimicrobials and disinfectants. Therefore, plant extracts can be a promising source to combat multi-resistant microorganisms (Pasternak, 2009; Xavier et al., 2003; Frozi et al., 2017).

Research on plant extracts from native Brazilian plants shows that the genus *Myrsine* (has as synonym *Rapanea*), belonging to the family Primulaceae, comprises about 300 species distributed pantropically, with 26 being recorded in Brazil (Ricketson and Pipoly, 1997; Freitas and Carrijo, 2014). The species of this genus have antifungal potential: *R. melanophloeos* (L.) Mez (Ohtani and Hostettmann, 1992), *M. umbellata* Mart. and *M. coriacea* (Sw.) R.br. ex Roem & Schult. (Miranda et al., 2015), antibacterial: *R. parviflora* (A.dc.) Mez (Suffredini et al., 2007) and *Rapanea* sp. (Montovani et al., 2009), anti-inflammatory: *M. guianensis* (Aubl.) Kuntze (Ospina et al., 2001), antioxidant: *M. guianensis* (Ospina et al., 2001) and *M. coriacea* (Miranda et al., 2015), and insecticidal: *M. umbellata* (Githiori et al., 2002).

The species chosen in this research, *M. umbellata* Mart., is a native Brazilian tree, popularly known as capororoca. It is widely distributed from Pernambuco to Rio Grande do Sul. In folk medicine its barks are used for diuretic treatment, urinary tract infections, liver problems and as a remedy for the treatment of leprosy (leprosy) (Farias et al., 1993; Vilela et al., 1993; Lorenzi, 2009; Nappo et al., 2004). There are few studies on the biological activities of its leaves. In the literature it has been reported that the ethyl acetate extract has antifungal potential, and the dichloromethane extract has insecticidal activity against *Spodoptera frugiperda* larvae (Miranda et al., 2015).

Given the above, studies with plant bioactives are of great value as an alternative to health problems resulting from bacterial resistance and the use of synthetic products. Conducting research with plant extracts using native plant species for therapeutic purposes promotes scientific validation and valorization of the regional flora, being essential to emphasize their conservation.

The present study aims to perform the phytochemical characterization, evaluate the antimicrobial, antioxidant and antibiofilm activities of plant extracts from the leaves of *Myrsine umbellata* species against standard ATCC strains of medical importance.

2. Materials and Methods

2.1. Collection and identification of M. umbellata Mart. leaves

M. umbellata leaves were collected in the Paulo Gorski Ecological Park, located in Cascavel, Paraná, Brazil (24º57'51.61'S and 53º26'14.80'W) between the months of August 2019 and March 2020, in the morning, in the early hours of the sunny day and with temperature around 20ºC, humid subtropical climate, between late winter and early autumn, phenological state of the vegetative plant and collection time around one hour. The plant was identified by Lázaro Henrique Soares de Moraes Conceição, exsiccate was delivered to the Herbarium of the State University of Western Paraná (UNOP) for botanical identification and registration UNOP 10731.

2.2. Obtaining the plant extracts

M. umbellata leaves were dried in an air oven at 40º C for 48 to 72 hours, and then ground in a knife mill (Willey-type) to a grain size of 0.42 mm. Plant extracts were prepared according to the methodology proposed by (Pandini et al., 2015; Santana et al., 2021), with modifications. The dried plant material (10 g) was extracted with different solvents (100 mL): methanol P.A. (ME), ethyl acetate P.A. (EAE), hexane P.A. (HE), and dichloromethane P.A. (DE). These liquid preparations were kept on a rotary shaker at 220 rpm for 24 hours. After this period, the solutions were filtered using Whatman No.1 filter paper and then centrifuged at 5000 rpm for 15 minutes.

Then, the supernatant was collected and submitted to rotary evaporation for total elimination of the solvents, obtaining the crude organic extracts at the end of the process. The extracts were stored under light in a freezer at 4º C. The yield of the plant extracts was calculated by the expression: Percentage (%) = (extract mass (g) /dried and ground vegetable mass (g)) x 100.

2.3. Phytochemical screening

The phytochemical tests of the different plant extracts of *M. umbellata* were performed according to the methodology described by (Matos, 1997; Silva Neto et al., 2021), with modifications. These tests were based on colorimetric visualization and/or precipitate formation after the addition of specific reagents. The classes of secondary metabolites identified were alkaloids using Dragendorff reagent; saponins from reaction with distilled water and hydrochloric acid P.A.; steroids and triterpenoids by Liebermann-Burchard reaction; anthocyanins, anthocyanidins, aurones, chalcones, flavanonols, flavones, flavonols and xanthones (flavonoids) from pH changes in the medium; tannins by reaction with ferric chloride and coumarins by fluorescence reaction with potassium hydroxide.

2.4. Quantitative analysis of the total content of phenolic compounds in plant extracts from leaves of M. umbellate

The total content of phenolic compounds in the extracts was performed according to the Folin-Ciocalteu method, described by Slinkard and Singleton (1997), modified (Bandeira et al., 2022), preparing a test solution of each tested extract (EE, EM and EA), containing 0.0250 g of extract diluted in 1 mL of methanol.

The total phenolic content (TPC) test was determined using the Folin-Ciocalteu reagent. In summary, 1 mL of the mother solution was mixed with 2.5 mL of 10% methanolic solution of the Folin-Ciocalteu reagent (w/v). The mixture was allowed to stand for 5 minutes at room temperature and then 2 mL of aqueous Na_2CO_3 solution (75 g.L $^{-1}$) were added. The mixture was homogenized using a vortex, then incubated for 10 minutes at 50°C with intermittent agitation and subjected to an ice-water bath. The absorbance of the samples was measured at 750 nm against the blank. A gallic acid solution (1 mg.mL^{-1}) was used as a standard with different dilutions for the calibration curve. The TPC was expressed as micromols of gallic acid equivalents (GAE) per gram of dry extract according to the calibration curve obtained through a linear regression between the absorbance and the concentration of gallic acid, y = 0.0065x + 0, 0166 (R^2 = 0.9995), where y indicates the absorbance of the sample and x indicates the concentration of gallic acid equivalents in the sample, in mg.L^{-1}. The amount of TPC was calculated as µM of gallic acid equivalents in micromols per gram of extract (µM GAE g ext. $^{-1}$) and calculated as mean value ± standard deviation (SD) (n = 3).

2.5. Antimicrobial activity – MIC/MBC

2.5.1. Microorganisms used

The experimental assays with plant extracts were performed in the Microbiology and Biotechnology Laboratory (LAMIBI) of the Western Paraná State University (UNIOESTE), Cascavel Campus. For the assay, different strains were used from the *American Type Culture Collection* (ATCC) and the *Cefar Culture Collection Diagnostic* (CCCD), encompassing seven Gram negative strains: *Escherichia coli* (ATCC 25922), *Salmonella enterica* Enteritidis (ATCC 13076), *Salmonella enterica* Typhimurium (ATCC 14028), *Salmonella enterica* Abaetetuba (ATCC 35640), *Pseudomonas aeruginosa* (ATCC 27853), *Proteus Mirabilis* (ATCC 25933) and *Klebsiella pneumoniae* (ATCC 13883); and four Gram positive: *Staphylococcus aureus* (ATCC 25923), *Enterococcus faecalis* (ATCC 19433), *Staphylococcus epidermidis* (ATCC 12228) and *Bacillus subtilis* (CCD-04); one yeast *Candida albicans* (ATCC 10231) (Scur et al., 2014; Pandini et al., 2018).

For the assays, the microorganisms were recovered in enrichment broth with Brain Heart Infusion (BHI) and incubated for 24 hours at 36± 1ºC. After this period, the strains were striated in Muller Hinton Agar (MH) and incubated at 36± 1ºC for 24 hours. To standardize the inoculum the strains were diluted in saline solution (0.85%), resulting in the final concentration of 1x10^5 CFU. mL^{-1} for bacteria and 1x10^6 CFU.mL^{-1} for *C. albicans* yeasts. The inoculum was compared by turbidity with the reading pattern 0.5 on the scale McFarland and the absorbance of suspension spectrophotometer (A600 = 0.3) (Scur et al., 2014).

2.5.2. Minimum Inhibitory Concentration (MIC)

The assays were performed according to the broth microdilution methodology described by Weber et al. (2014) and CLSI (2015) with modifications. All plant extracts of *M. umbellata* were solubilized in methanol P. A., obtaining a stock solution (400 mg. mL $^{-1}$). In 96-well microdilution plates (Vasconcelos et al., 2021), 150 µl of Muller Hinton broth (MH) per well were dispensed from the second column for bacterial strains and MH broth per well for bacteria and broth of the Roswell Park Memorial Institute (RPMI) for yeast *C. albicans*. In the first column, 300 µl of the solution plant extract/methanol/MH were added in each well, and then serial dilutions were performed obtaining concentrations ranging from 200 to 0.09 mg. mL $^{-1}$. At the end, 10 µl of inoculum was added in each well and the plates were incubated at 36 ± 1º C for 18-24 hours. For the positive control, the commercial antibiotic Gentamicin (200 mg. mL^{-1}) and the commercial antifungal Nystatin (200 mg. mL^{-1}) were used. As a negative control, the inoculum was added to the MH (bacteria) and RPMI (yeast), without the presence of the extract to verify the viability of the tested microorganism. A sterility control of the extracts solubilized in methanol P.A. and a control of P.A. methanol diluent was also performed to verify for interference in the assay. Trifeniltetrazole chloride (TTC) from 20 µl to 0.5% (35 ± 1ºC for 15 min.) was used as a colorimetric indicator of microbial metabolism, in the presence of the red color was considered negative evidence of inhibition effect of plant extracts and essential oil. Three experiments of triplicate MIC were carried out, being possible to determine the lowest concentration of EA, EE and OE capable of inhibiting microbial growth.

2.5.3. Minimum Bactericidal Concentration (MBC) and Minimum Fungicidal Concentration (MFC)

For the trial, the methodology of (Weber et al., 2014) was performed, with modifications. Prior to the addition of 0.5% TTC to determine the MIC, a 2 µl aliquot was removed from each well of the microdilution plate and sown onto the surface of MH agar. The plates were incubated at 36 ± 1º C for 18-24 hours. The assay was performed in triplicate, and to determine the MBC/ MFC it was observed if there was microbial growth in the MHA, allowing to verify the lowest concentration of plant extracts capable of causing the death of the bacteria/fungus tested.

2.6. Antioxidant activity

2.6.1. Determination of antioxidant activity by the free radical scavenging method 2,2-diphenyl-1-picryl-hydrazyl (DPPH)

The antioxidant activity of plant extracts was performed according to the 2,2-diphenyl-1-picril-hydrazil (DPPH) free radical reduction method, proposed by (Rufino et al., 2007; Pandini et al., 2018), with modifications. Initially, a calibration curve (0, 10, 20, 30, 40, 50, 60 µM DPPH) was made to obtain the concentration of DPPH in the medium after the reaction with the plant extracts, determining the equation y = 0.114x -0.0973 (R^2 = 0.99), where y is the concentration of DPPH and absorbance. The plant extracts were then solubilized in methanol P. A., obtaining concentrations ranging from 0.1 to 15 mg. mL $^{-1}$. A 0.1 mL aliquot of these plant extracts was added to 3.9 mL of DPPH methanolic solution (60 µM) and homogenized in a tube shaker. As negative control was used 1 mL of methanol P. A. added to 3.9 mL of DPPH, and as a positive control was used the synthetic antioxidant BHT (butyl hydroxy-toluene) at different concentrations (0.10 to 1.0 mg. mL^{-1}).

The tests were performed in spectrophotometer at 515 nm at 1-minute reading intervals until absorbance stabilization. All concentrations were defined from the pre-test to determine antioxidant activity in the range of approximately 7% to 97% DPPH free radical sequestration. As a blank, methanol was used to calibrate the spectrophotometer. The percentage of free radical sequestration (AA%) was expressed by the Equation 1:

$$AA\% = \frac{(A_0 - A_1)}{A_1} \text{x} 100 \quad (1)$$

Where A_0 is the absorbance of the negative control and A_1 is the absorbance of the sample. To calculate the IC_{50} (amount of antioxidant substance necessary to reduce the initial concentration of DPPH by 50%), the concentrations of plant extracts and BHT were used to obtain the equation of the line with R^2 greater than 0.80, and thus found the IC_{50} value, from the linear regression. The tests were performed in triplicate and expressed as mean ± standard deviation. The IC_{50} results were analyzed using ANOVA with Tukey's test ($p<0.05$), using XLSTAT 2014 statistical program. Then, the extracts were calculated by the equation AAI = AA% / IC_{50}, where AA% is the percentage of free radical sequestration and IC_{50} (amount of antioxidant substance necessary to reduce the initial concentration of DPPH by 50%), and classified according to the AAI: Antioxidant Activity Index into weak (AAI <0.5), moderate (AAI between 0.5 and 1.0), strong (AAI between 1.0 and 2.0) and very strong (AAI> 2.0), according to the methodology proposed in (Scherer and Godoy, 2009).

2.6.2. Determination of antioxidant activity by capturing 2,2'- azino-bis (3-ethylbenzo-thiazoline-6-sulfonic acid (ABTS+) free radical

The antioxidant activity of plant extracts was performed according to the 2,2'-azino-bis (3-ethylbenzo-thiazoline-6-sulfonic acid (ABTS+) radical reduction method proposed by Rufino et al. (2007), Miao et al. (2018) and Li et al. (2021), with modifications.

A standard calibration curve was performed with Trolox, where a stock solution of Trolox was prepared at a concentration of 2000 µM.L^{-1}, weighing 0.0010g of Trolox for 2 mL of ethanol, and then serial dilutions were performed at concentrations of 200, 133.33, 88.89, 59.26, 35.51, 26.34, 17.56, 11.71 µM.L^{-1}. In a dark environment, an aliquot of 30 µL of each Trolox solution was transferred to test tubes, mixed with 3.0 mL of the ABTS+ radical solution, homogenized in a tube shaker. Afterwards, the reading was performed (750 nm) after 6 minutes of mixing. As a blank, 200 µL of ethane was used to calibrate the spectrophotometer. Trolox concentrations were plotted (µM) on the X axis and the respective absorbances on the Y axis and the equation of the line y = -0.0033x -0.6792 (R^2 = 0.96) was determined. From the equation of the line, the absorbance referring to 1,000 µM of Trolox was calculated, according to Equation 1: y= - ax + b, where x corresponds to 1,000 µM of Trolox, and y is the absorbance corresponding to 1,000 µM from Trolox.

ABTS+ was prepared by mixing 7 µM.$L^{-1}$of ABTS stock solution (in deionized water) with 2.45 µM.L^{-1} potassium persulfate (in deionized water). The mixture was allowed to react for 12-16 hours in the dark. Then, the ABTS+ working solution was diluted with 2 mL of ethanol until an absorbance of 0.70 ± 0.05 was obtained at 750nm. In 96-well microplates, 100 µL of each *M. umbellata* ME, EAE, HE and DE extract (0.50, 0.50, 0.25, 0.25 mg.mL^{-1}, respectively) was added per well. with 100 µL of ABTS+ solution and kept at room temperature and protected from light for 10 minutes, after which the absorbance was read at 750 nm in a microplate reader. As a negative control, 100 µL of ABTS+ solution and 100 µL of ethanol were used. The antioxidant activity was calculated according to the calculation of extract dilutions (mg.L^{-1}) equivalent to 1000 µM of Trolox, equation 2: y= - ax + b, where y is the absorbance corresponding to 1000 µM of Trolox (Equation 1) and x is the sample dilution (mg.L^{-1}) equivalent to 1000 µM Trolox. From the result found (x) in equation 2, it was divided by 1000 to obtain the value in g. The final result X(g) = x / 1000 and Z = 1000 / X(g).[1] (equation 3), was calculated by dividing 1000 (µM) by the value of X(g) and multiplying by 1(g) where the final value (Z) was found, which was expressed in µM Trolox. g ext^{-1}.

2.7. Antibiofilm activity

2.7.1. Microorganisms

For the assay, different strains from the *American Type Culture Collection* (ATCC) collection were used, which are reported in the literature as excellent biofilm producers, being one gram-positive strain *S. aureus* (ATCC 25923) and two gram-negative strains *E. coli* (ATCC 25922) and *P. aeruginosa* (ATCC 27853), are the main etiological bacterial agents responsible for a number of infectious diseases and show greater resistance to antimicrobials. Brain Heart Infusion medium (BHI) supplemented with 1% glucose and inoculum of the tested strains was used as a negative control; and as a control for color interference, MH and BHI, diluted in dimethyl sulfoxide (DMSO) and BHI, were used at different concentrations. And as control of the diluent DMSO to verify if there was interference of the same in the test.

2.7.2. Bacterial biofilm activity test

The test was performed in 96-well microplates (flat bottom) with polystyrene surface. To standardize the inoculum, the strains were diluted 1:100 (10^8 CFU.mL^{-1}) in saline solution (0.85%), the turbidity of the bacterial suspension was adjusted as the reading pattern on the scale of 0.5 McFarland, then transferred an aliquot of 50 µl in 4.950 mL of BHI broth supplemented with glucose 1% resulting in a final concentration of 0.5 X 10^6 CFU.mL^{-1}. In each well of the microplates were distributed 180 µl of BHI medium supplemented with 1% glucose and 20 µl of bacterial inoculum for biofilm formation and these were incubated for 24 hours at 37± 1ºC until reaching the late exponential phase (24 hour) to allow cell fixation and biofilm formation. The evaluation of biofilm formation was performed by reading the optical density (OD) of 570 nm using an microplate reader. Based on the OD produced by biofilms, bacterial strains were classified into the following categories: OD ≤ ODc = no biofilm producer; ODc < OD ≤2X ODc = weak biofilm producer; 2X ODc < OD ≤ 4X ODc= moderate biofilm producer; 4X ODc <OD= strong biofilm producer. The plates that presented strong biofilm production in 24 hours of formation were used for the assay. After the incubation period, the culture medium was remived and the wells washed once with buffered phosphate saline solution (PBS 1X), and the added 150 µl of a new BHI medium containing EM (200 to 1.56 mg.mL^{-1}). The material was incubated again at 37 ± 1 ºC for 24 h (Stepanović et al., 2007).

2.7.3. Quantification of biofilm biomass by crystal violet

After incubation, the medium was gently aspirated, and the wells were washed three times with PBS 1X. Then, the wells received 150 µl of methanol P.A. for 20 minutes for sample fixation, after removal of methanol 150 µl of 2% crystal violet was added for 15 minutes. Then, the wells were washed in PBS 1X until the dye was completely removed. After drying, 150 µl of 95% ethanol was added and left in contact for 30 minutes, then the contents of the wells were transferred to a new plate and the optical density (OD) was read at 570 nm using a Biotek microplate reader, (Epoch model). The data were averaged into the optical density (OD) of the biofilm biomass quantified by staining with crystal violet. The percentage of biofilm eradication was determined by the equation: Percentage of eradication (%): ((OD negative control - OD experimental) / OD negative control) x 100 in Microsoft Excel 2010 program. The inhibition percentage values were classified as follows: (<50%) indicate low antibiofilm activity and (>50%) high antibiofilm activity (Christensen et al., 1985; Sandasi et al., 2008; Laskoski et al., 2022; Bandeira et al., 2022).

2.7.4. Biofilm cell viability test

The activity of the extract on the biofilm was measured by the reduction (%) of the absorbance value when compared with the negative control, since the sample tested is a biofilm producer. As a criterion to express the results of the antibiofilm potential ME of *M. umbellata*, both the biofilm biomass test by crystal violet assay and the cell viability by the 3-(4,5-dimethylthiazol2-yl)-2,5-diphenyltetrazolium bromide test (MTT) were determined from the MIC concentrations, 2X MIC and 4X MIC of each tested bacterial strain. After the methanol was removed, a 180 µl aliquot of BHI and 20 µl of MTT were added to each well, and the plates were incubated in the dark at 37ºC for 2 hours. After, the medium was removed and added 150 µl of DMSO for 15 minutes. The supernatants were then transferred to a new plate and the optical density was measured at 570 nm in Epochtype Biotek microplate reader. The cell viability data of mature biofilms were expressed in general mean OD and the percentage of cell viability (CV%) was determined by the equation: (OD experimental /OD negative control) x 100 (Famuyide et al., 2019). The values of the percentage of cell viability were classified as: (< 50%) indicate low cellular activity and (> 50%) indicate high cellular activity (Sandasi et al., 2008; Laskoski et al., 2022).

3. Results and Discussion

3.1. Yield and phytochemical prospection of plant extracts

From obtaining plant extracts of *M. umbellata* with different solvents, the following yields were obtained: ME (24.75%), EAE (9.50%), DE (7.97%) and HE (5.97%).

The phytochemical prospection of plant extracts of *M. umbellata* revealed the presence of compounds belonging to the classes of saponins, steroids, alkaloids, anthocyanins, anthocyanidins, flavonoids (flavones, flavonols, xanthones and flavanonols), and tannins. The presence of triterpenoids and coumarins was not detected in any of the extracts tested (Table 1). Secondary metabolites such as alkaloids and flavonoids were identified in all extracts. The highest diversity of compound classes was observed in the HE extracts with 9 different classes of compounds, followed by ME with 8 classes, EAE and DE with 6 classes.

Several reasons can influence the yield of different plant extracts, such as temperature, extraction time, solid-solvent ratio, among others. However, the most important variable is the choice of solvent extractor, since its complex chemical characteristics, such as different solubility and polarity, influence their yield (Fernández-Agulló et al., 2013).

Table 1. Phytochemical prospection of secondary metabolites present in different plant extracts of *M. umbellata* leaves.

Secondary metabolites	ME	EAE	HE	DE
Saponins	+	-	-	-
Free steroids	+	+	+	+
Triterpenoids	-	-	-	-
Alkaloids	+	+	+	+
Anthocyanins	-	-	+	-
Anthocyanidins	-	-	+	-
Flavones	+	+	+	+
Flavanols	+	+	+	+
Xantonas	+	+	+	+
Chalcones	-	-	-	-
Auronas	-	-	-	-
Flavanols	+	+	+	+
Condensed tannins	+	-	+	-
Coumarins	-	-	-	-

(+) presence; (-) absence. ME: methanolic extract; EAE: ethyl acetate extract; HE: hexane extract; DE: dichloromethane extract.

Table 2. Total phenolic content of different *M. umbellata* leaf extracts.

	Total Phenolic Content (μM GAE g ext. $^{-1}$)
DE	280.24 ± 0.037 ^{a}
ME	159.01 ± 0.031 ^{b}
EAE	19.10 ± 0.022 ^{c}
HE	0.99 ± 0.001 ^{d}

Average ± standard deviation. Values followed by the same letter in the columns do not differ by Tukey test (p-valor <0,05). GAE: gallic acid equivalent; DE: dichloromethane extrac; ME: methanolic extract; EAE: ethyl acetate extract; HE: hexane extract.

According to literature reports, plant extracts using hexane and ethyl acetate as solvent extractants are commonly rich in secondary metabolite, which corroborates the data found in our study (Cabana et al., 2013; Fernández-Agulló et al., 2013).

In the literature, reports were found on the phytochemical prospection of plant extracts: ethanolic and acetonic from the leaves of *M. umbellata*, revealed the presence of compounds belonging to the classes of free alkaloids, flavonoids and tannins in both extracts, and saponins only in ethanolic extract (Laskoski et al., 2022). In addition, various secondary metabolites from various species of the genus *Myrsine* have been identified and isolated, such as triterpenoids in *R. melanophloes* and *M. africana* (Ohtani and Hostettmann, 1992; Manguro et al., 1997), flavonoids in *M. seguinii* and *M. coriacea* (Zhong et al., 1997; Alves et al., 2012) terpenes in *R. guyanensis* and *R. lancifolia* (Ohtani and Hostettmann, 1992; Januário et al., 1992), tannins, saponins and phenolic compounds in *M. coriacea* (Alves et al., 2012), corroborating the results obtained in our study, since these compounds were also identified in the extracts of *M. umbellata*.

3.2. Quantitative analysis of the total content of phenolic compounds in plant extracts from leaves of M. umbellata

Quantitative analysis of the total content of phenolic compounds was performed in all extracts studied in this research. The results of *M. umbellata* leaf extracts regarding the total content of phenolic compounds are shown in Table 2.

The results of the quantitative phytochemical estimation of *M. umbellata* leaf extracts revealed a higher total phenolic content in DE (280.24 ± 0.037 μM GAE g ext. $^{-1}$) followed by ME (159.01 ± 0.031 μM GAE g ext.$^{-1}$), EAE (19.10 ± 0.022 μM GAE g ext.$^{-1}$) and HE (0.99±0.001 μM GAE g ext.$^{-1}$). In the literature, the total phenolic content of the methanolic, acetone, ethyl acetate, dichloromethane and hexane extract of *M. coriaceae* leaves is 386.7 mg GAE.g^{-1}, 303.95 mg GAE.g^{-1}, 113.47 mg GAE.g $^{-1}$, 62.96 mg GAE.g $^{-1}$ and 40.98 mg GAE.g $^{-1}$, respectively (Miranda et al., 2015).

There are several studies with plant extracts of the *Myrsine* species. Although there are already studies carried out with plant extracts from the leaves of *M. umbellata*, they are still limited. In the literature, only one study of *M. umbellata* leaves on the total phenolic content with acetonic, methanolic, ethyl acetate, dichloromethane and hexane extracts was reported with results of 446.65 μM GAE g ext.$^{-1}$, 276.07 μM GAE g ext.$^{-1}$, 108.68 μM GAE g ext.$^{-1}$, 24.77 μM GAE g ext.$^{-1}$ and 21.50 μM GAE g ext.$^{-1}$, respectively (Miranda et al., 2015), these results are similar to that of this study, differing only that the dichloromethane extract was the one with the highest phenolic content in our study, which may be due to a greater presence of flavonoids (Taiz and Zeiger, 2009). In both studies, it was observed that the phenolic components are significantly higher in polar extracts when compared to non-polar extracts (Miranda et al., 2015).

Furthermore, our results are similar to those of other species of the Myrsine genus, as the presence of flavonoids is common within the Primulaceae family in the species *M. coriaceae*, *M.seguinii*, (Alves et al., 2012; Zhong et al., 1997), *M. cuneifolia* (Burger, 2009), *M. parvifoia* (Corrêa, 2018), *M. Africana* (Arot et al., 1996). Phenolic compounds, especially flavonoids, are produced by plants in large quantities (Taiz and Zeiger, 2009).

3.3. Antimicrobial activity

Regarding antimicrobial activity, in the broth microdilution assay, all plant extracts of *M. umbellata* leaves were tested for their ability to inhibit growth (Minimum Inhibitory Concentration-MIC) or cause death (Minimum Bactericidal Concentration- MBC/ Minimum Fungicidal Concentration- MFC) of microorganisms. The MIC and MBC/MFC of plant extracts were classified according to (Araújo, 2010; Pandini et al., 2015) and the activity was classified into one of four different classes: high (<12.5 mg. mL^{-1}), moderate (12.5 to 25 mg. mL^{-1}), low (50 to 100 mg. mL^{-1}), and very low (>100 mg. mL^{-1}).

All tested plant extracts of *M. umbellata* showed antimicrobial potential when tested against the twelve pathogenic strain patterns (ATCCs) (Table 3).

The ME showed the best antimicrobial activity when compared to the other extracts, with MIC/MBC values ranging from 3.12 to 25 mg. mL^{-1}, classifying both MIC and MBC from high to moderate, highlighting its gram-positive *S. epidermidis* (MIC/CBM of 3.12/6.25 mg. mL^{-1}). This extract performed better, and its activity can be explained by the presence of saponins and tannins when compared to other extracts that did not present these classes of compounds.

Then EH shower the second-best antimicrobial activity, being classified with MIC of high, moderate and low, and MBC of moderate, low, and very low. This as well as EM, also showed efficiency against the gram-positive strain *S. epidermidis*, with MIC and MBC values of 6.25 and 12.5 mg. mL^{-1}, respectively.

Finally, EAE and DE were classified with antimicrobial potential of MIC of high, moderate, and low, and MBC of moderate, low, and very low. The gram-positive strain *S. aureus* (MIC and MBC of 3.12 and 25 mg. mL^{-1}, respectively) was the most susceptible to EAE. The ED showed the best both inhibitory and bactericidal/fungicidal activities with MIC of 6.25 mg. mL^{-1} and MBC/MFC of 25 mg. mL^{-1} for the gram-negative strains *P. aeruginosa* and yeast *C. albicans*.

Although HE is the only one to present the anthocyanins and anthocyanidins compounds, which present antimicrobial activity (due to their ability to damage cells by changing the selective permeability of the plasma membrane) and antioxidant activity (which can mitigate the oxidative stress caused by free radicals) (Cardoso et al., 2011), this extract did not show the best results regarding antimicrobial and antioxidant potential when compared to the other extracts tested This can be explained by phytochemical prospection, as it is a qualitative method that does not allow quantifying these groups, as well as their presence in low amounts, probably not enough to significantly inhibit the tested microorganisms and reduce free radicals. Furthermore, these compounds can have additive or synergistic effects when used in combination (Penteado et al., 2016).

However, data on the antimicrobial potential of *M. umbellata* plant extracts against ATCC standard strains are incipient in the literature, considering this the first report of the antimicrobial potential of these extracts from this species.

Table 3. Minimum Inhibitory Concentration (MIC) and Bactericidal Concentration (CBM) or Minimum Fungicidal Concentration (MFC) of the different leaf extracts of *M. umbellata* front different pathogenic strains.

Strains	MIC/MBC (mg. mL^{-1})		HE	DE
	ME	EAE		
Gram-positive				
Staphylococcus aureus (ATCC 25923)	25/25	3.12/25	6.25/25	50/200
Staphylococcus epidermidis (ATCC 12228)	3.12/6.25	6.25/12.5	6.25/12.5	12.5/25
Enterococcus faecalis (ATCC 19433)	6.25/12.5	50/200	50/200	50/200
Bacillus subtilis (CCC B005)	6.25/25	25/200	50/100	100/100
Gram-negative				
Salmonella enterica Typhimurium (ATCC 14028)	6.25/12.5	25/200	25/100	50/200
Salmonella enterica Enteritidis (ATCC 13076)	6.25/12.5	50/200	50/200	25/200
Salmonella enterica Abaetetuba (ATCC 35640)	12.5/25	25/100	25/100	50/200
Escherichia coli (ATCC 25922)	3.12/12.5	25/50	50/100	25/100
Klebsiella pneumoniae (ATCC 13883)	12.5/25	25/200	25/100	25/50
Proteus mirabilis (ATCC 25933)	3.12/12.5	50/200	100/200	50/100
Pseudomonas aeruginosa (ATCC 27853)	3.12/12.5	12.5/200	50/200	6.25/25
Yeast	MIC/MFC			
Candida albicans (ATCC 10231)	6.25/12.5	50/200	50/200	6.25/25

ME: methanolic extract; EAE: ethyl acetate extract; HE: hexane extract; DE: dichloromethane extract.

Studies on the antimicrobial potential of ethanolic (EE) and acetonic (AE) plant extracts from *M. umbellat*a leaves were found in the literature, with antimicrobial activity on Gram-positive strains *S. epidermidis* (AE) and Gram-negative *E. coli* strain (EE) (Laskoski et al., 2022). Furthermore, Montovani et al. (2009) reported the antimicrobial activity of hexane, ethyl acetate and methanolic extract of *Rapanea sp*. (synonymous with *Myrsine sp*.) on the *S. aureus* strain, and among the tested extracts, only the hexane extract was not able to inhibit bacterial growth.

All tested extracts showed bacteriostatic/fungistatic or bactericidal/fungicidal activity on ATCC strains, suggesting that the antimicrobial potential of *M. umbellata* plant extracts is related to its phytochemical profile, classes of flavonoids, alkaloids, and tannins. The flavonoids (flavones, flavonols, xanthones and flavanonols) present in all extracts tested are hydroxylated phenolic substances that have proven antimicrobial activity in the literature. Their mode of antimicrobial action occurs due to the formation of complexes with extracellular and soluble proteins, which bind to the bacterial wall causing irreversible damage to the cells. In addition, they can cause perforation and reduced plasma membrane fluidity, inhibition of topoisomerase, leading to inhibition of nucleic acid synthesis and/or inhibition of energy metabolism (Samy and Gopalakrishnakone, 2010; Cushnie and Lamb, 2011).

Steroids, present in all the extracts tested, are terpene compounds formed by decarboxylation of precursors of triterpene origin. They are active against several microorganisms, and although their mechanisms of action are not fully understood, they are believed to involve cell membrane rupture by lipophilic compounds (Silva et al., 2014).

Tannins were identified in ME and HE, being water and polar solvent soluble phenolic compounds provide an antifungal and antimicrobial effect by precipitating proteins. Their mode of action may be related to their ability to inhibit microbial adhesions and bacterial enzymes and may or may not be complexed with the substrates of these enzymes and proteins involved in cellular transport. In addition, they can also act on the cell membranes of microorganisms, modifying their metabolism and, finally, complex with metal ions, reducing the availability of ions essential for microbial metabolism (Loguercio et al., 2005).

All tested extracts showed the class of alkaloids, which are nitrogenous substances that inhibit the action of gram-negative bacteria, causing cell lysis and morphological changes in microorganisms (Fumagali et al., 2008). Finally, saponins were identified in ME, which act by increasing the permeability of bacterial cell membranes (Gyawali and Ibrahim, 2014).

3.4. Antioxidant activity

The antioxidant capacity of plant extracts of *M. umbellata* was determined by DPPH free radical sequestration assay. The commercial antioxidant butylated hydroxytoluene (BHT) was used at concentrations from 0.1 to 1.0 mg. mL^{-1}. In this study, different concentrations of extracts ranging from 0.1 to 15 mg. mL^{-1} were tested. The data shown in Table 4 refer to the concentration of 1.0 mg. mL^{-1}, which presented the most relevant results.

ME was the extract capable of sequestering more DPPH radicals when compared to the other extracts tested, with antioxidant activity of 92.54% and IC_{50} value of 0.50 mg. mL^{-1}, necessary to reduce the DPPH value by 50%.

While EAE, HE and DE showed the lowest percentages of free radical sequestration with antioxidant percentage of 18.78%, 11.64% and 9.16%, respectively, and IC_{50} values of 5.76 mg. mL^{-1}, 10.88 mg. mL^{-1} and 11.65 mg. mL^{-1}, respectively. These results affirm the need for higher concentrations of these extracts to sequester the same amount of DPPH radicals when compared to BHT and ME. Thus, it was possible to observe that the antioxidant activity depends on the solvent extractant, and the concentration tested.

According to the classification proposed by (Scherer and Godoy, 2009) plant extracts of *M. umbellata* showed strong antioxidant activity index (AAI) for ME (AAI 1.85) and weak index for the other HE (AAI 0.13), EAE (AAI 0.05), and DE (AAI 0.05).

Literature data on the antioxidant activity of plant extracts of *M. umbellata* are still incipient. (Laskoski et al., 2022) observed the antioxidant activity of acetonic and ethanolic plant extracts from the leaves of *M. umbellata*, which showed antioxidant potential of 89.55% and 63.05%, respectively, and strong antioxidant activity index (IAA) for AE (IAA 1.90) and moderate for EE (IAA 0.59). There are also reports of the antioxidant potential of leaf extracts of *M. coriacea*, a species belonging to the genus *Myrsine*, where ME presented a very strong antioxidant activity index (IAA) (IAA >2), with an AAI value of 5.95 (Miranda et al., 2015). This makes our results promising, as the ME of *M. umbellata* showed a higher percentage of free radical scavenging, which resulted in a strong index of antioxidant activity.

The excellent antioxidant activity of these extracts is related to the content of phenolic compounds (flavonoids) present. Flavonoids are produced in large quantities by plants and are great antioxidants, being the largest group also found in foods and having ideal structure for radical sequestration and reducing the redox potential of the medium (Jing et al., 2012).

Table 4. Percentage of root sequestration of DPPH and IC50 value by DPPH assay of *M. umbellata* leaf extracts.

Concentration (mg. mL^{-1})	BHT	ME	EAE	HE	DE
1.0	97.20±0.30	92.54±0.86	18.78±1.08*	11.64±0.88*	9.16±0.17*
IC_{50}	0.46±0.00	0.50±0.00	5.76±0.05	10.88±0.04	11.65±0.08
AAI	2.11	1.85	0.13	0.05	0.05

*Express a significant difference between the extracts when compared to BHT (Butylated Hydroxytoluene) (Tukey test ($p<0.05$). Absorbance average (%) ± standard deviation. IC_{50}: Concentration inhibiting 50% of DPPH; AAI: Antioxidant Activity Index. ME: methanolic extract; EAE: ethyl acetate extract; HE: hexane extract; DE: dichloromethane extract.

M. umbellata extracts showed antioxidant activity by scavenging the ABTS radical (Table 5). The results revealed a greater capture of the ABTS radical in the ME extract (420.31 ± 0.026 µM Trolox g ext.$^{-1}$), followed by HE (359.87 ± 0.024 µM Trolox g ext.$^{-1}$), finally the EAE extracts (161.89 ± 0.013 µM Trolox g ext.$^{-1}$) and DE (38.13 ± 0.009 µM Trolox g ext.$^{-1}$), with lower antioxidant potential compared to ABTS. When the ABTS values are compared with the values obtained by the DPPH radical inhibition methodology, the values found are more efficient, which means that a lower concentration of plant extracts is required to achieve the same antioxidant capacity.

This can be explained by the fact that the structures of the DPPH radical and the ABTS radical present stereochemical differences, which can generate actions in the reaction mechanism of different antioxidant compounds present in plant species (Pérez-Jiménez et al., 2008).

There are no reports in the literature of studies with antioxidant prospection by capturing the ABTS radical from plant extracts of leaves of *M. umbellata*. Similar results regarding the antioxidant potential against the ABTS radical of extracts of *M. africana* have been reported, it was observed that the ethanolic extract had an IC50 of 46.61 ± 0.08 µg.mL^{-1} against the free radical DPPH and a value of 122.64 ± 1.55 µg.mL^{-1} against the ABTS radical, these data support the conclusions of the present study (Gul et al., 2017).

Table 5. Determination of antioxidant activity by capture ABTS free radical of different *M. umbellata* leaf extracts.

	ABTS (µM Trolox g ext. $^{-1}$)
ME	420.31 ± 0.026 ^{a}
HE	359.87 ± 0.024 ^{b}
EAE	161.89 ± 0.013 ^{c}
DE	38.13 ± 0.009 ^{d}

Average ± standard deviation. Values followed by the same letter in the columns do not differ by Tukey test (p-valor <0,05). ABTS: (2,2'- azino-bis(3-ethylbenzo-thiazoline-6-sulfonic acid) diammoninum salt); ME: methanolic extract; HE: hexane extract; EAE: ethyl acetate extract; DE: dichloromethane extract.

3.5. Evaluation of the activity of plant extracts on bacterial biofilm

Considering that the ME extract was the best antimicrobial and antioxidant, it was chosen to evaluate the activity on standard bacterial biofilms. Statistical analysis of biomass and cell viability data was performed, ANOVA and Tukey tests when data were in normality and Kruskal-Wallis and Dunn when data were not in normal distribution.

There were no significant differences between the different concentrations tested (MIC, 2X MIC and 4X MIC), therefore, the data expressed in Tables 6 are related to the action of MIC on the biofilm.

The antibiofilm potential of ME was verified only against the biofilm of the gram-positive strain *S. aureus*, where it was able to eradicate the biomass by 92.53% (Table 6). Therefore, this extract showed better antimicrobial and antibiofilm potential against the tested gram-positive strains.

Your phytochemical compounds present in ME (saponins, free steroids, alkaloids, flavonoids, tannins) act dehydrating the cell wall, thus preventing nutrient replenishment, and break down the structure of already formed biofilms (Cushnie and Lamb, 2011; Nuño et al., 2018).

Regarding the biofilms of gram-negative strains, the ME showed low antibiofilm activity, moreover, it promoted the increase of the biofilm biomass of the strain *E. coli*.

Supposedly, this increase in biomass can be explained by the action of phytochemical compounds at lower concentrations, or low penetration of them, or increase of cell membrane efflux pumps or even the synergistic effect between them. These factors favor the increase in the biomass of the biofilm formed by *E. coli* through the activation of genes that provide excess cellular matrix (Jamal et al., 2018).

Table 6. Effect of *M. umbellata* methanolic extract on eradication and viability of biofilms of standard bacteria.

Microorganisms	ME	CV	MTT
		ERADICATION	VIABILITY
Staphylococcus aureus (ATCC 25923)	CONTROL	3.88±0.08	0.32±0.06
	MIC	0.29±0.12*	1.79±0.01*
	%	92.53	NI
Pseudomonas aeruginosa (ATCC 27853)	CONTROL	3.49±0.17	0.28±0.04
	MIC	1.93±0.09*	0.50±0.04*
	%	44.70	NI
Escherichia coli (ATCC 25922)	CONTROL	1.74±0.11	0.20±0.06
	MIC	1.88±0.02*	0.31±0.01*
	%	NI	NI

*Values differ by t test (p>0.05). % eradication = data are expressed as percentage of eradication of biofilm biomass at MIC concentration (%). Average of optical density ± standard deviation. CV= Crystal Violet; ME= methanolic extract; NI= not inhibition; MTT= 3-(4,5-dimethylthiazol-2-yl)-2,5- diphenyltetrazolyl. % Viability = data are expressed as percentage of cell viability of biofilms at MIC concentration (%).

There are no data in the literature on the antibiofilm potential of plant extracts of *M. umbellata* tested in this study. However, (Laskoski et al., 2022) observed the antibiofilm activity of *M. umbellata* leaves in two other types of extracts, EE and AE, showing that EE had the best antibiofilm activity, as it presented biofilm disruption rates for all bacterial strains tested, being more efficient against biofilm formed by *S. aureus* (84.28%). The AE, on the other hand, showed antibiofilm potential against the biofilm formed by the gram-negative strain *S.* Enteritidis, with an eradication percentage of 67.16%. These data corroborate our study and make our result auspicious, as the ME of *M. umbellata* showed better antibiofilm potential when compared to other studies.

4. Conclusion

The yield of the tested plant extracts varied according to the solvents used. The ME showed the highest yield (24.75%) when compared to the other extracts tested. Regarding the phytochemical prospection, HE presented 9 classes of phytochemical compounds, revealing the presence of saponins, steroids, alkaloids, anthocyanins, anthocyanidins, flavones, flavonols, flavanonols, xanthones (flavonoids), and tannins. The results of the quantitative phytochemical estimation revealed a higher content of total phenolics in DE (175.15 ± 0.037 µM GAE.g ext.$^{-1}$) followed by ME (159.02 ± 0.031 µM GAE.g ext.$^{-1}$).

The ME showed the best biological activities (antimicrobial, antioxidant and antibiofilm) when compared to the other extracts tested. Its antimicrobial activity was classified as high to low for the gram-positive strain *S. epidermidis* (MIC 3.12 mg. mL^{-1} and MBC 6.25 mg. mL^{-1}). Its antioxidant percentage was 92.58% against the DPPH radical and 420.44 mg Trolox g ext.$^{-1}$ against the ABTS radical and still showed antibiofilm activity against the biofilm of gram-positive strain *S. aureus*, with high inhibition (92.53%).

The results suggest that the ME from *M. umbellata* can be used as a new alternative in the production of products with plant bioactives, showing a new point of view for possible uses in different industrial sectors. Therefore, the influence of phenolic compounds (flavonoids and tannins) on the high antimicrobial, antioxidant and antibiofilm activity is evident. These results indicate the biological potential of *M. umbellata*, therefore, are a valuable contribution for future bioassays to identify and isolate the plant bioactives responsible for the high antimicrobial, antioxidant and antibiofilm potential.

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