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Production of a phenolic-rich extract of aroeira honey and characterization of its antimicrobial, antitumoral and antioxidant activities

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Summary

Objective: a phenolic-rich extract of *Astronium urundeuva* honey ($PhEA^H$) was produced and its pharmacological proprieties were determined. **Method:** $PhEA^H$ was prepared using a solid-phase extraction column. Next, antibacterial and antifungal activities were evaluated by broth microdilution method and the antioxidant effect was investigated using 2,2-Diphenyl-1-picrylhydrazyl (DPPH) assay. Urethral catheter sensitized with $PhEA^H$ were produced and its anti-adhesive and anti-biofilm effect determined. Finally, antitumoral and antiviral activities were studded using 3-(4.5-dimethylthiazol-2-yl)-2.5-diphenyltetrazolium bromide (MTT) test. **Results:** $PhEA^H$ showed an elevated total phenol concentration ($PhEA^H$: 18.7±0.4 mg GA/g

vs. fresh honey: 0.99±0.005 mg GA/g). Although PhEA^{H} did not show significant antifungal and antiviral effects, it was moderately active against Gram-negative bacilli (*Klebsiella aerogenes*, *K. pneumoniae*, *Proteus mirabilis*, *Pseudomonas aeruginosa* and *Enterobacter cloacae*) and showed increased antibacterial activity against salmonellosis pathogens (*Salmonella* Typhimurium and *Salmonella* Enteritidis). PhEA^{H}-impregnated urethral catheters inhibited the growth of various pathogenic bacteria and impaired the ability of *P. aeruginosa* to colonize and adhere to it. In addition to its antimicrobial activity, PhEA^{H} presented antioxidant properties and reduced the viability of human glioblastoma cells. **Conclusion:** in conclusion, our study shows that PhEA^{H} contains large amounts of phenolic compounds, which are associated with its antibacterial, anti-adhesive, antioxidant, and antitumor effects.

Keywords: Astronium urundeuva (M. Allemão) engl.; *salmonella*, glioblastoma, oxidative stress, arbovirus, coronavirus.

Resumen

Producción de un extracto rico en fenoles de miel de aroeira y caracterización de sus actividades antimicrobiana, antitumoral y antioxidante

Objetivo: se produjo un extracto rico en fenoles de miel de *Astronium urundeuva* (PhEAH) y se determinaron sus propiedades farmacológicas. **Métodos:** PhEAH se preparó utilizando una columna de extracción en fase sólida. Luego, se evaluaron las actividades antibacteriana y antifúngica mediante el método de microdilución en caldo y se investigó el efecto antioxidante mediante el ensayo de 2,2-difenil-1-picrilhidrazilo (DPPH). Se fabricaron catéteres uretrales sensibilizados con PhEAH y se determinó su efecto antiadhesivo y antibiofilm. Finalmente, las actividades antitumoral y antiviral se evaluaron mediante la prueba de bromuro de 3-(4,5-dimetiltiazol-2-il)-2,5-difeniltetrazolio (MTT). **Resultados:** PhEAH mostró una alta concentración de fenoles totales (PhEAH: 18,7±0,4 mg AG/g vs. miel fresca: 0,99±0,005 mg AG/g). Aunque PhEAH no mostró efectos antifúngicos y antivirales significativos, fue moderadamente activo contra los bacilos Gram-negativos (Klebsiella aerogenes, *K. pneumoniae*, *Proteus mirabilis*, *Pseudomonas aeruginosa* y *Enterobacter cloacae*) y mostró una mayor actividad antibacteriana contra los patógenos de la salmonelosis (*Salmonella Typhimurium* y *Salmonella enteriditis*). Los catéteres uretrales impregnados con PhEAH inhibieron el crecimiento de varias bacterias patógenas y afectaron la capacidad de *P. aeruginosa* para colonizar y adherirse a ella. Además de su actividad antimicrobiana,

PhEAH mostró propiedades antioxidantes y redujo la viabilidad de las células de glioblastoma humano. **Conclusión:** en conclusión, nuestro estudio muestra que PhEAH contiene grandes cantidades de compuestos fenólicos, los cuales están asociados con sus efectos antibacterianos, antiadhesivos, antioxidantes y antitumorales.

Palabras claves: Astronium urundeuva (M. Allemão) engl.; *Salmonella*; glioblastoma; estrés oxidativo; arbovírus; coronavírus.

Resumo

Produção de um extrato rico em fenólicos do mel de aroeira e caracterização de suas atividades antimicrobiana, antitumoral e antioxidante

Objetivos: um extrato rico em fenólicos do mel de *Astronium urundeuva* (PhEAH) foi produzido e suas propriedades farmacológicas foram determinadas. **Métodos:** o PhEAH foi preparado usando uma coluna de extração em fase sólida. Em seguida, as atividades antibacteriana e antifúngica foram avaliadas pelo método de microdiluição em caldo e o efeito antioxidante foi investigado pelo ensaio de 2,2-difenil-1-picrilhidrazil (DPPH). Cateteres uretrais sensibilizados com PhEAH foram produzidos e seu efeito antiadesivo e antibiofilme determinado. Finalmente, as atividades antitumoral e antiviral foram avaliadas usando o teste de brometo de 3-(4,5-dimetiltiazol-2-il)-2,5-difeniltetrazólio (MTT). **Resultados:** PhEAH mostrou uma concentração de fenol total elevada (PhEAH: 18,7±0,4 mg GA/g vs. mel fresco: 0,99±0,005 mg GA/g). Embora o PhEAH não tenha apresentado efeitos antifúngicos e antivirais significativos, foi moderadamente ativo contra bacilos Gram-negativos (*Klebsiella aerogenes, K. pneumoniae*, *Proteus mirabilis*, *Pseudomonas aeruginosa* e *Enterobacter cloacae*) e mostrou atividade antibacteriana aumentada contra patógenos de salmonelose (*Salmonella Typhimurium* e *Salmonella Enteritidis*). Cateteres uretrais impregnados com PhEAH inibiram o crescimento de várias bactérias patogênicas e prejudicaram a capacidade de *P. aeruginosa* de colonizar e aderir a ela. Além de sua atividade antimicrobiana, PhEAH apresentou propriedades antioxidantes e reduziu a viabilidade de células de glioblastoma humano. **Conclusão:** em conclusão, nosso estudo mostra que o PhEAH contém grandes quantidades de compostos fenólicos, que estão associados aos seus efeitos antibacteriano, antiadesivo, antioxidante e antitumoral.

Palavras-chaves: Astronium urundeuva (M. Allemão) engl.; *salmonella*; glioblastoma; estresse oxidativo; arbovírus; coronavírus.

Introduction

Honey is a product consumed as a food all over the world. It is a rich source of various carbohydrates, mineral salts, and proteins, and also contain other compounds from the nectar collected by the bee. Indeed, honey contains large amounts of active compounds, such as flavonoids and polyphenols, which can act as antioxidant, antitumor, and antimicrobial agents [1, 2]. In this context, honey obtained during the flowering of *Astronium urundeuva* (M. Allemão) Engl. (Anacardiaceae) (formerly *Myracrodruon urundeuva* and popularly known as "aroeira-do-sertão") is a promising source of phenolic compounds that can be associated with its antimicrobial and antitumor activities. Aroeira honey is a product derived from the dry forest of northern Minas Gerais (Brazil), where only *A. urundeuva* flowers. It is known for its significant amounts of phenolic compounds [3].

Aroeira honey is moderately active against *Escherichia coli*, *Staphylococcus aureus* and *Helicobacter pylori* [3]. However, the required concentration of Aroeira honey to inhibit the growth of these pathogens is considerably high, limiting its clinical use as a therapeutic agent. Therefore, the use of chromatographic techniques to concentrate phenolic compounds in this honey is a promising alternative [4-7]. In this study, we prepared and characterized a phenolic-rich extract of *Astronium urundeuva* (M. Allemão) Engl. (Anacardiaceae) honey ($PhEA^{H}$) and determined its antimicrobial, antitumor, and antioxidant activities. In addition, we produced an anti-biofilm urethral catheter sensitized with $PhEA^{H}$ to prevent catheter-associated urinary tract infections.

Materials and methods

Microorganisms and cells

The microorganisms used in the study were originated from the American Type Culture Collection (ATCC) and kindly provided by the Reference Microorganisms Laboratory of the Oswaldo Cruz Foundation (FIOCRUZ; Rio de Janeiro, RJ, Brazil). The antibacterial activity was investigated against *Enterococcus faecalis* ATCC 51299, *E. faecium* ATCC 700221, *S. pyogenes* ATCC 19615, *M. luteus* ATCC 10240, *S. aureus* ATCC 16530, Methicillin-resistant *S. aureus* (MRSA) USA300, *S. saprophyticus* ATCC 15305, *S. epidermidis* ATCC 12228, *Klebsiella pneumoniae* ATCC 700603, *K. pneumoniae* ATCC 43816, *K. aerogenes* ATCC 30048, *K. oxytoca* ATCC 13182, *Acinetobacter baumannii* ATCC 19606, *Pseudomonas aeruginosa* ATCC 15442, *Enterobacter cloacae* ATCC 23355, *Escherichia coli* ATCC 8739, *Proteus mirabilis* ATCC 15290, *P. vulgaris* ATCC 13315, *Corynebacterium diphtheriae* ATCC 13812,

Serratia marcencens ATCC 14756, *Citrobacter freundii* ATCC 8090, *Stenotrophomonas maltophilia* ATCC 13637, *Burkholderia cepacia* ATCC 17759, *Salmonella enterica* subsp. *enterica* serovar Typhimurium ATCC 14028, *Salmonella enterica* subsp. *enterica* serovar Enteritidis ATCC 13076, *Listeria monocytogenes* ATCC 15313, *Bacillus cereus* ATCC 11778, *B. subtilis* ATCC 6051, *Shigella flexneri* ATCC 12022, *Shigella sonnei* ATCC 12022, and *Aeromonas hydrophila* IOC 11036. In addition, eight clinical isolates of multidrug-resistant (MDR) bacteria were included: Vancomycin-intermediate *S. aureus* (VISA), Carbapenem-resistant *A. baumannii* AC37, Uropathogenic *E. coli* (ESBL positive), Uropathogenic *E. coli* (GyrA mutation and *qnrS1* positive), Carbapenem-resistant *P. mirabilis, Cephalosporin-resistant S. marcencens,* Enterohemorrhagic *E. coli* (EHEC), and Enteroinvasive *E. coli* (EIEC). All isolates were identified as previously described [8-10].

The antifungal activity was determined against *Candida albicans* ATCC 10231, *C. krusei* ATCC 34135, *C. glabrata* ATCC 2001, *C. parapsilosis* ATCC 22019, *C. tropicalis* ATCC 28707, *Cryptococcus neoformans* ATCC 32045, *C. gattii* ATCC 24065, *Rhodotorula mucilaginosa* ATCC 32763, and *R. glutinis* ATCC 26207. According to the ATCC specifications, *C. albicans* 10231 is resistant to anidulafungin, voriconazole, itraconazole, fluconazole, and ketoconazole [11].

The antiviral assays were performed against three arboviruses (i.e., Dengue virus serotype 2 (DENV-2), Zika virus (ZIKV), and Mayaro virus (MAYV)) and one betacoronavirus (Mouse hepatitis virus (MHV)). DENV-2 and ZIKV were kindly provided by Ph.D. Erna Geessien Kroon (Universidade Federal de Minas Gerais, Belo Horizonte, MG, Brazil) and Ph.D. Eurico de Arruda Neto (Universidade de São Paulo, Ribeirão Preto, SP, São Paulo), respectively. MAYV was kindly provided by Ph.D. Maurício Lacerda Nogueira (Faculdade de Medicina de São José do Rio Preto, São José do Rio Preto, SP, Brazil). MHV was kindly provided by Ph.D. Mauro Martins Teixeira (Universidade Federal de Minas Gerais, Belo Horizonte, MG, Brazil). All arbovirus were initially propagated in C6/36 cells (Aedes albopictus clone C6/36 cells, ATCC CRL-1660) and later titrated in Vero cells (African green monkey kidney, ATCC CCL-81) by Dulbecco's plaque formation assay [12]. MHV was propagated and titrated in L929 cells (Mouse fibroblast cell line, ATCC CCL-1). Both cells (Vero and L9-29) were cultured in Dulbecco's modified minimal essential medium supplemented with 5% fetal bovine serum supplemented with antimicrobials (100 U mL^{-1} of penicillin, 100 $\mu g \cdot mL^{-1}$ of streptomycin and 2.5 $\mu g \cdot mL^{-1}$ of amphotericin B), and maintained at 37 °C in 5% CO_2 (Thermo Scientific™ Forma™ Series II 3110 Water-Jacketed CO_2 Incubator, Waltham, USA).

The antitumoral activity was assessed against a neuroblast cell from human neural tissue (SH-SY5Y, ATCC CRL-2266). Cells were cultured in Dulbecco's Modified

Eagle Medium: Nutrient Mixture F-12 (DMEM/F12, Gibco) supplemented with 10% of fetal bovine serum, antimicrobial solution (100 U mL^{-1} of penicillin, 100 $\mu g \cdot mL^{-1}$ of streptomycin and 2.5 $\mu g \cdot mL^{-1}$ of amphotericin B), non-essential amino acids solution and sodium pyruvate (1 mM). Cells were maintained at 37 °C in 5% CO_2 (Thermo Scientific™ Forma™ Series II 3110 Water-Jacketed CO2 Incubator, Waltham, USA).

Reagents

Honey samples originated from the Seasonally Dry Tropical Forest in Janaúba region, Minas Gerais, Brazil (43 ° 31 ′ 06 ″ W, 15 ° 40 ′ 34 ″ S) with annual precipitation mean of 730.46 mm and an annual temperature ranging from 15.2 to 40.2 °C (INMET 2018). Meropenem, colistin, gentamycin (Inlab, São Paulo, SP, Brazil), chloramphenicol, amphotericin B, penicillin, streptomycin (Sigma-Aldrich, San Francisco, CA, USA), vancomycin, miconazole (Pharma Nostra, Rio de Janeiro, RJ, Brazil), fluconazole (Fragon, São Paulo, SP, Brazil), dimethyl sulphoxide (DMSO), methanol, sodium chloride (NaCl) (Synth, São Paulo, SP, Brazil), trypsin, fetal bovine serum (Gibco, ThermoFisher ScintificScientificrt, HE, Germany), glucose (Inlab, São Paulo, SP, Brazil), 2,2-Diphenyl-1-picrylhydrazyl (DPPH), 3-(4.5-dimethylthiazol-2-yl)-2.5-diphenyltetrazolium bromide (MTT), methanol, gallic acid, and trifluoroacetic acid (Sigma-Aldrich, San Louis, MO, USA) were purchased from commercial suppliers and used without additional purification. Sabouraud-Dextrose broth (SDB), Sabouraud-Dextrose agar (SDA), Mueller–Hinton broth (MHB), Mueller–Hinton agar (MHA), and Nutrient agar were purchased from Kasvi (São José do Pinhais, PR, Brazil). Dulbecco's Modified Eagle Medium (DMEM) was purchased from CultLab (São Paulo, SP, Brazil) and solid-phase extraction (SPE) column from Supelco-LC18 (Milan, NO, Italy).

Production of PhEA^{H}

Approximately 5 g of crude honey sample was weighed and added to 100 mL acidic solution (water and TFA 0.1%). Then, the sample was continuously stirred, and the resulting mixture was filtered twice using Whatman nº.4 and filter paper. The filtrate was applied to a solid-phase extraction (SPE) column (Supelco-LC18, Milan, Italy). Elution of phenolic compounds from the column was done with methanol and concentrated in a rotary evaporator [13].

Total phenolic compounds dosage

Total phenolic compounds of honey and extract samples were quantified using the Folin-Ciocalteu's method [14], with modifications. One g of honey and 10 mg of the PhEA^{H} were weighed and placed in a 10 mL volumetric flask, and the volume was

completed with Milli-Q water. This solution was then filtered and transferred to test tubes in triplicate. 2.5 mL of 0.2 N Folin & Ciocalteu's Phenol (Sigma) solution was added to the test tubes, which were homogenized and then allowed to rest for 5 minutes. 2 mL of 75 $g{\cdot}L^{-1}$ sodium carbonate solution was added to the samples, which were homogenized again and allowed to rest for 2 h. Then, the absorbance was measured using a UV-Vis spectrophotometer (Shimadzu) at 760 nm, using ultrapure water as the blank. A calibration curve for the total phenolic compound was generated from a 1 $g{\cdot}L^{-1}$ standard solution of gallic acid. The standard curve presented an R^2 value of 99.8%.

Antibacterial and antifungal activity

Inoculum preparation: Microorganisms were maintained in nutrient agar and cultured after 24 (for bacteria) and 48 (for fungi) h of incubation at 37 °C. The microorganisms were collected aseptically using an inoculation loop and suspended in 10 mL of sterile saline (0.9% NaCl) to obtain the inoculum. The suspension was homogenized for approximately 10 seconds with a vortex (Bunzl Saúde, Osasco, SP, Brazil), and the optical density (OD) was measured at 530 nm (yeast) or 625 nm (bacteria) and adjusted to 0.190-0.210 in a spectrophotometer (Nova Instruments, Sao Paulo, SP, Brazil). The resulting suspensions were diluted in SDB (1:1000) to obtain an end inoculum containing 10^3 colony forming units (CFU)/mL of fungal cells or in MHB (1:100) to obtain an inoculum containing 10^6 CFU/mL of bacterial cells.

Minimum inhibitory concentration (MIC): The antibacterial and antifungal activities were evaluated by determining the MIC using the broth microdilution method according to Clinical and Laboratory Standards Institute (CLSI) documents M07 [15] and M27 [16], respectively. Briefly, 100 µL of a microbial inoculum (10^3 CFU/mL for fungi and 10^6 CFU/mL for bacteria) was added to sterile microplates containing twofold serial dilutions (2-1,024 µg/mL) of $PhEA^H$ in MHB (for bacteria) or SDB (for fungi). Plates were then incubated for 24 h (for bacteria) or 48 h (for fungi) at 37 °C. The MIC was defined as the lowest concentration of extract that inhibited the visible growth of microorganisms. Miconazole and fluconazole were used as positive controls (1-64 µg/mL) in the antifungal assay. Meropenem, colistin, gentamycin, chloramphenicol or vancomycin were used as positive controls (1-64 µg/mL) for the antibacterial test.

Microbicidal action: The minimum bactericidal concentration (MBC) and minimum fungicidal concentration (MFC) of $PhEA^H$ were determined as previously described by Lima *et al.* [17]. Briefly, 10 µL samples were collected from each well where no visible growth was detected in the MIC assay and plated on MHA (for bacteria) or SDA (for fungi). After incubation at 37 °C for 24 h (for bacteria) or 48 h (for fungi), the

MBC and MFC were defined as the lowest concentration of extract or controls that kills at least 99% of the inoculum compared to the untreated control.

Anti-biofilm activity in urethral catheter

Catheter sensibilization: One-centimeter (1 cm) sterile polyvinyl chloride urethral catheters (Embramed®, Cravinhos, SP, Brazil) were used. PhEA^{H} was first diluted in a solution of formic acid (79%) and orthophosphoric acid (8%). After the extract was dissolved, glycerol (18%) was added to the coating solution as a plasticizer. The catheter segments were then added to the PhEA^{H} solution at concentrations of 10 mg/mL, 100 mg/mL, and 500 mg/mL. The catheters were immersed in the coating at 45 °C for 60 minutes with agitation to ensure homogeneous coating. Then, the segments were placed in an oven at 37 °C for 48 h to dry. Finally, the catheters were washed with sterile distilled water to remove unbound substances and subjected to a new drying cycle at 37 °C for 24h.

Agar diffusion assay: The *in vitro* antimicrobial activities of 1 cm segments of all three testing concentrations of PhEA^{H} (10 mg/mL, 100 mg/mL, and 500 mg/mL) were evaluated by determining the zone of inhibition against bacteria and fungi using a modified Kirby-Bauer method [18]. Each microorganism was sub-cultured in nutrient agar for 24 h (for bacteria) or 48 h (for fungi), and a microbial suspension of 0.5 McFarland in 0.9% saline was prepared using spectrophotometric methods. Individual MHA and SDA plates were then inoculated with each bacterial and fungal suspension, respectively, so that the entire plate was covered. Segments of urethral catheters from each test group were then placed in the center of the inoculated agar plate and incubated at 37 °C for 48 h. The zones of inhibition were determined by measuring the diameter of the zones around the segments minus 10 mm (the outer diameter of each segment).

Anti-biofilm assay: The three uropathogenic microorganisms that were susceptible to PhEA^{H} in the agar diffusion assay (i.e. *Klebsiella pneumoniae* ATCC 700603, methicillin-resistant *S. aureus* (MRSA) USA300 and *Pseudomonas aeruginosa* ATCC 15442) were evaluated for their ability to form viable biofilms on previously functionalized urethral catheters. Biofilm formation on sterile catheter segments treated with PhEA^{H} at 500 mg/mL was performed according to de Sousa *et al.* [19], with modifications. Briefly, the segments were immersed in tubes containing a microbial suspension (10^{6} CFU/mL) in TBS supplemented with 1 mM glucose. All tubes were incubated at 37 °C for 24 h to allow biofilm formation and adhesion to the catheter surface. Bacterial cultures were then discarded, and the catheter segments were placed in tubes containing 5 mL of sterile saline (0.9% NaCl) and shaken for 30 minutes. The segments were removed using sterilized dissecting forceps, transferred to another tube containing 5 mL of sterile saline, and sonicated at 40 KHz for 5 minutes (Soniclean, New York, NY, USA). Samples were then homogenized, and 100 µL was aliquoted, serially diluted

(10^{-1} - 10^{-6}) in sterile saline and plated on nutrient agar. Plates were incubated at 37 °C for 24 h, and CFU/cm of the catheter was determined by colony counting.

Antiviral activity

Cytotoxicity assay: The 50% cytotoxic concentration (CC_{50}) was assessed by the MTT colorimetric assay [20]. Vero cells were cultured in 96-well plates (10^6 cells/well) and treated with various concentrations of $PhEA^H$ (2-1,024 μg/mL), followed by incubation at 37°C for 48 h. MTT solution (5 mg/mL) was then added to each well, and the plates were incubated at 37°C for 90 minutes. Then, 100 μL DMSO was added to each well to dissolve the formazan crystals. The absorbance was measured in a microplate reader (Bio-Tek Instruments®, Winooski, VT, USA) at 540 nm, and the CC_{50} was calculated [21].

Antiviral assay: The antiviral activity of $PhEA^H$ was evaluated as described by dos Santos *et al.* [22]. Anti-arboviral assays were performed with confluent cultures of Vero cells in 96-well plates infected with DENV-2, ZIKV, or MAYV at an infection multiplicity (MOI) of 0.1. Anti-coronavirus assays were performed in confluent cultures of L929 infected with MHV at MOI of 0.01. After infection, all plates were incubated for 1 h at 37 °C in a humidified atmosphere containing 5% CO_2 (Thermo Scientific™ Forma™ Series II 3110 Water-Jacketed CO_2 Incubator, Waltham, USA). Then, the infected cells were treated with a twofold dilution of $PhEA^H$ prepared in DMEM (6.25-400 μg/mL) and incubated at 37 °C and 5% CO_2 for 48 h. Finally, MTT solution was added to each well, and cellular viability was determined as described above. The concentration that protected 50% of the infected and $PhEA^H$ treated cells (EC_{50}) was calculated as described by Santos *et al.* [23].

Antioxidant activity

The antioxidant activity was determined by the DPPH method, as previously described [24]. DPPH solution at 0.002% w/v in 80% methanol was used for this assay. DPPH solution (150 mL) was added to each well of a microplate, followed by 75 mL of $PhEA^H$, resulting in five concentrations: 1, 10, 100, 250 and 500 μg/mL. The plate was then covered and incubated at room temperature (25 °C), protected from light. After 30 minutes, the absorbance was measured at 517 nm using a spectrophotometer (Bio-Tek Instruments®, Winooski, VT, USA). The absorbance of the DPPH solution prepared at time zero was measured after incubation for 30 minutes, protected from light. Pure solvent was used as the blank. The percentage inhibition of DPPH (or percentage of antioxidant activity) was calculated using the following equation:

$$\% \text{ inhibition of DPPH} = [1 - (A_s/A_b)] \times 100$$

where A_s = absorbance of the sample and A_b = absorbance of DPPH solution.

Finally, the effective concentration for the 50% discoloration of the DPPH solution (EC_{50}) was calculated using the probit analysis method, as described by de Teixeira de Oliveira *et al.* [24].

Antitumoral activity

The inhibitory concentration to 50% of cancer cells (IC_{50}) was evaluated using the MTT colorimetric assay, as described above. SH-SY5Y cells were cultured in 96-well plates (105 cells/well) and treated with different concentrations of $PhEA^H$ (12.5-800 μg/mL) or Irinotecan hydrochloride (10 – 0.156 μg/mL), followed by incubation at 37 °C for 48 h. Next, a MTT solution (5 mg/mL) was added to each well and the plates were incubated at 37 °C for 90 minutes. Then, 100 μL of DMSO was added to each well to solubilize formazan crystals. The absorbance was measured in a microplate reader (Bio-Tek Instruments®, Winooski, VT, USA) at 540 nm, and the CC_{50} was calculated [21].

Statistical analysis

All *in vitro* tests were performed in triplicate, and the results are reported as mean ± standard deviation of these three experiments. Data regarding the anti-biofilm effect of urethral catheters sensitized with $PhEA^H$ were analyzed using Student's T-test. The statistical analyzes and graphical representations were performed using GraphPad Prism version 8.0 for Windows (GraphPad Software, La Jolla California, Los Angeles, CA, USA).

Results

First, we applied a chromatographic method to extract phenolic compounds from the honey of *Astronium urundeuva*. The method used in this study allowed us to obtain a phenolic-rich honey extract ($PhEA^H$). The total phenolic concentration of the $PhEA^H$ was 18.7±0.4 mg GA/g sample, which was almost 20 times higher than the total phenolic concentration of *in natura* honey (0.99±0.005 mg GA/g sample).

After extracting $PhEA^H$, we evaluated its therapeutic potential as an antimicrobial, antitumor, and antioxidant agent. The antibacterial activity was evaluated by determining the minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) by the broth microdilution method. As shown in **Table 1**, $PhEA^H$ was active against the Gram-negative bacilli *Klebsiella aerogenes* (MIC: 32 μg/mL; MBC: 256 μg/mL), *Klebsiella pneumoniae* (MIC and MBC: 64-128 μg/mL), *Proteus mirabilis* (MIC and MBC: 64-128 μg/mL), *Pseudomonas aeruginosa*

(MIC and MBC: 256 μg/mL) and *Enterobacter cloacae* (MIC: 256 μg/mL; MBC: 512 μg/mL). Notably, the extract was active against an isolate of *P. mirabilis* that was highly resistant to carbapenems, suggesting that $PhEA^{H}$ exerts its activity even in multidrug-resistant pathogens (table 1). $PhEA^{H}$ was also tested against some important foodborne pathogens. According to table 2, the extract was effective against *Salmonella* Typhimurium (MIC: 16 μg/mL; MBC: 32 μg/mL) and *Salmonella* Enteritidis (MIC and MBC: 8 μg/mL). In contrast, $PhEA^{H}$ not showed antifungal (table 3) or antiviral (table 4) activity against the species included in this study.

Table 1. Minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) of phenolic-rich extract from Aroeira honey ($PhEA^{H}$) against pathogenic bacteria.

Microrganisms	$PhEA^{H}$ (μg/mL)		Reference Drug (μg/mL)	
	MIC	CBM	MIC	CBM
Enterococcus faecalis ATCC 51299	>1,024	-	16#	32#
Enterococcus faecium ATCC 700221	>1,024	-	32*	32*
Streptococcus pyogenes ATCC 19615	>1,024	-	0.06#	0.25#
Micrococcus luteus ATCC 10240	>1,024	-	64#	64#
Staphylococcus aureus ATCC 16530	1,024	1,024	0.25*	0.25*
Methicillin-resistant *Staphylococcus aureus* (MRSA) USA300	>1,024	-	0.5**	0.5**
Vancomycin-intermediate *Staphylococcus aureus* (VISA)[CI]	>1,024	-	8**	32**
Staphylococcus saprophyticus ATCC 15305	>1,024	-	1**	1**
Staphylococcus epidermidis ATCC 12228	512	512	0.5**	1**
Klebsiella pneumoniae ATCC 700603	128	128	0.06*	2*
Klebsiella pneumoniae ATCC 43816	64	64	4***	>64***
Crabapenem-resistant *Acinetobacter baumannii* AC37[CI]	>1,024	-	0.5****	0.5****
Acinetobacter baumannii ATCC 19606	1,024	1,024	0.5****	4****
Pseudomonas aeruginosa ATCC 15442	256	256	8*	8*
Enterobacter cloacae ATCC 23355	256	512	0.12*	0.12*
Klebsiella aerogenes ATCC 30048	32	256	0.25*	0.25*
Klebsiella oxytoca ATCC 13182	>1,024	-	0.25#	0.50#
Escherichia coli ATCC 8739	>1,024	-	1****	1****
Uropathogenic *Escherichia coli* (ESBL positive)[CI]	>1,024	-	0.5****	0.5****
Uropathogenic *Escherichia coli* (GyrA mutation and *qnrS1* positive)[CI]	>1,024	-	1****	1****

(*Continue*)

Microrganisms	PhEA^{H} (µg/mL)		Reference Drug (µg/mL)	
	MIC	CBM	MIC	CBM
Carbapenem-resistant *Proteus mirabilis*^{CI}	64	128	0.25*	0.25*
Proteus mirabilis ATCC 15290	128	128	8*	8*
Proteus vulgaris ATCC 13315	1,024	1,024	0.12$^{\#}$	0.25$^{\#}$
Corynebacterium diphtheriae ATCC 13812	>1,024	-	1$^{\#}$	1$^{\#}$
Serratia marcencens ATCC 14756	>1,024	-	8*	32*
Serratia marcencens^{CI}	>1,024	-	64*	>64*
Citrobacter freundii ATCC 8090	>1,024	-	4$^{\#}$	4$^{\#}$
Stenotrophomonas maltophilia ATCC 13637	>1,024	-	1$^{\#}$	1$^{\#}$
Burkholderia cepacia ATCC 17759	>1,024	-	0.25$^{\#}$	0.25$^{\#}$

Reference drug: *Meropenem; **Vancomycin; ***Chloramphenicol; ****Colistin; $^{\#}$Gentamycin

Table 2. Minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) of phenolic-rich extract from Aroeira honey (PhEAH) against relevants foodborne pathogens.

Microorganism	PhEA^{H} (µg/mL)		Reference Drug (µg/mL)	
	MIC	CBM	MIC	CBM
Enterohemorrhagic Escherichia coli (EHEC)	>1,024	-	0.25**	0.50**
Enteroinvasive Escherichia coli (EIEC)	>1,024	-	1**	1**
Salmonella enterica subsp. *enterica* serovar Typhimurium ATCC 14028	16	32	≥0.06*	0.12*
Salmonella enterica subsp. *enterica* serovar Enteritidis ATCC 13076	8	8	≥0.06*	0.06*
Listeria monocytogenes ATCC 15313	>1,024	-	0.5*	0.5*
Bacillus cereus ATCC 11778	>1,024	-	0.03**	0.03**
Bacillus subtilis ATCC 6051	>1,024	-	0.125**	0.125**
Shigella flexneri ATCC 12022	>1,024	-	1**	1**
Shigella sonnei ATCC 11060	>1,024	-	0.5**	2**
Aeromonas hydrophila IOC 11036	>1,024	-	1**	1**

Reference drug: *Meropenem; **Gentamycin

Table 3. Minimum inhibitory concentration (MIC) and minimum fungicidal concentration (MFC) of phenolic rich extract from Aroeira honey (PhEAH) against pathogenic yeast.

Microrganismo	PhEA^{H} (μg/mL)		Reference Drug (μg/mL)	
	MIC	MFC	MIC	MFC
Candida albicans ATCC 10231	>1,024	-	8*	8*
Candida dubliniensis IC1	>1,024	-	8*	32*
Candida krusei ATCC 34135	>1,024	-	4*	4*
Candida glabrata ATCC 2001	>1,024	-	0.25*	0.25*
Candida parapsilosis ATCC 22019	>1,024	-	0.5***	0.5***
Candida tropicalis ATCC 28707	>1,024	-	0.12*	0.25*
Cryptococcus neoformans ATCC 32045	>1,024	-	2**	2**
Cryptococcus gattii ATCC 24065	>1,024	-	1***	1***
Rhodotorula mucilaginosa ATCC 32763	>1,024	-	1***	1***
Rhodotorula glutinis ATCC 26207	>1,024	-	1***	1***

Reference drugs: *Miconazole; **Fluconazole

Table 4. Antiviral activity of a phenolic-rich extract from Aroeira honey (PhEA^{H}).

Virus	Cell lineage	CC_{50} (μg/mL)	EC_{50} (μg/mL)
Dengue virus sorotipo 2	Vero	668.3 ±258.6	>600
Zika virus	Vero	668.3 ±258.6	>600
Mayaro virus	Vero	668.3 ±258.6	>600
Mice Hepatitis Virus	L929	467.2 ±92.6	>600

CC_{50}: Concentration cytotoxic to 50% of cells; EC_{50}: Concentration that protected 50% of the infected cells

To evaluate the potential application of PhEA^{H} against biofilms, we investigated the ability of this extract to inhibit colonization/biofilm formation on the surface of the urethral catheter. The results shows that PhEA^{H}-impregnated catheters inhibited the growth of most bacteria tested, but no yeast was sensitive to the functionalized catheters (figure S1, supplementary material). As shown in fable 5, the anti-biofilm effect of PhEA^{H} was concentration-dependent, and the catheters exposed to the highest concentration of PhEA^{H} (500 mg/mL) presented the biggest zones of inhibition in the agar diffusion assay. The Gram-negative bacilli *K. pneumoniae* (23.00±1.41 mm) and *P. aeruginosa* (23.00±1.41 mm), and the Gram-positive cocci methicillin-resistant *S. aureus* (23.50±2.12 mm) were highly susceptible to PhEA^{H} in the diffusion test. Therefore, we investigated the ability of the PhEA^{H}-impregnated catheter to inhibit

biofilm formation by these species *in vitro*. figure 1 shows that sensitization with $PhEA^{H}$ impaired the colonization of the urethral catheter by *P. aeruginosa* ($PhEA^{H}$-impregnated catheter: 4.30±0.08 UFC/cm *vs.* Control catheter: 5.01±1.00 UFC/cm; p-value < 0.001). To our knowledge, this is the first study using urethral catheters impregnated with phenolic extracts of honey against uropathogens.

Table 5. Zones of inhibition (mm) produced by phenolic-rich extract from Aroeira honey ($PhEA^{H}$)-impregnated catheter segments in Mueller-Hinton agar.

Microorganism	$PhEA^{H}$		
	10 mg/mL	100 mg/mL	500 mg/mL
Escherichia coli K 12	-	-	13.00±1.41
ESBL-producing and quinolone-resistant	15.50±3.54	13.50±0.71	14.00±0.00
Enteroinvasive Escherichia coli (EIEC)	18.00±7.07	18.00±4.24	15.00±2.83
Klebsiella pneumoniae ATCC 700603	16.00±2.83	13.50±0.71	23.00±1.41
Klebsiella aerogenes ATCC 30048	18.00±5.66	13.50±3.54	19.00±0.00
Klebsiella oxytoca ATCC 13182	10.00±0.00	11.00±1.41	16.50±3.54
Enterococcus faecalis ATCC 51299	-	-	-
Proteus mirabilis ATCC 15290	-	15.00±0.00	14.00±0.00
Carbapenem-resistant *Proteus mirabilis*	22.00±4.24	21.50±2.12	20.00±0.00
Proteus vulgaris ATCC 13315	23.50±0.71	26.00±0.00	22.50±0.71
Pseudomonas aeruginosa ATCC 15442	20.50±0.71	17.00±1.41	23.00±0.00
Staphylococcus aureus ATCC 16530	-	-	16.50±2.12
Methicillin-resistant *S. aureus* (MRSA USA 300)	18.50±0.71	18.50±2.12	23.50±2.12
Vancomycin-intermediate *S. aureus* (VISA)	22.00±4.24	27.50±9.19	19.50±0.71
Staphylococcus saprophyticus ATCC 15305	25.00±8.49	15.00±0.00	20.00±0.00
Staphylococcus epidermidis ATCC 12228	-	-	12.50±0.71
Streptococcus agalactiae ATCC 12386	14.00±0.00	15.00±0.00	22.00±0.00
Streptococcus pyogenes ATCC 19615	-	11.50±2.12	16.00±0.00
Acinetobacter baumannii ATCC 19606	20.00±2.83	16.00±4.24	16.00±2.83
Carbapenem-resistant *Acinetobacter baumannii*	16.00±7.07	13.50±2.12	16.00±2.83
Burkholderia cepacia ATCC 17759	-	-	10.00±4.24
Stenotrophomonas maltophilia ATCC 13637	21.50±2.12	17.50±3.54	19.50±0.71
Serratia marcencens ATCC 14756	-	-	15.00±0.00
Listeria monocytogenes ATCC 15313	23.00±7.07	14.00±1.41	23.00±4.24
Bacillus cereus ATCC 11778	19.50±7.78	12.50±0.71	18.50±0.71
Bacillus subtilis ATCC 6051	22.00±1.41	15.00±1.41	20.00±4.24
Shigella flexneri ATCC 12022	22.50±3.54	27.50±0.71	23.00±0.00
Shigella sonnei ATCC 11060	22.00±4.24	26.00±0.00	22.50±3.54
Aeromonas hydrophila IOC 11036	-	10.50±0.71	13.50±0.71

(*Continue*)

Microorganism	PhEA^{H}		
	10 mg/mL	**100 mg/mL**	**500 mg/mL**
Corynebacterium diphtheriae ATCC 13812	19.50±0.71	19.50±4.95	19.00±0.00
Citrobacter freundii ATCC 8090	19.00±5.66	14.50±2.12	20.50±0.71
Enterobacter cloacae ATCC 23355	-	-	-
Micrococcus luteus ATCC 10240	-	-	12.00±0.00
Fungal			
Candida albicans ATCC 18804	-	-	-
Azole-resistant *Candida albicans* ATCC 10231	-	-	-
Candida krusei ATCC 34135	-	-	-
Candida glabrata ATCC 2001	-	-	-
Candida tropicalis ATCC 28707	-	-	-

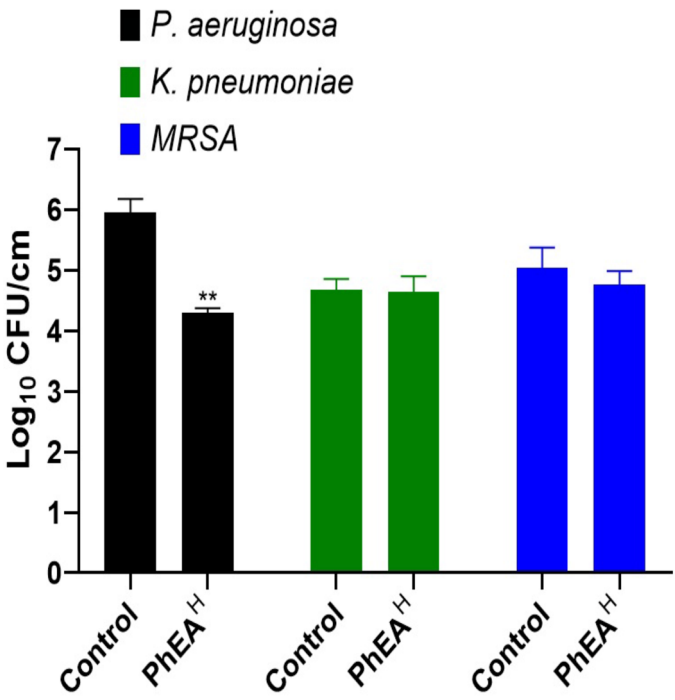

Figure 1. Anticolonization activity of phenolic-rich extract from *Astronium urundeuva* (M. Allemão) Engl. (PhEA^{H})-impregnated catheter segments exposed to different bacterial suspensions. Error bars indicate standard errors of the means (SEM) for bacterial colony counts per centimeter of catheter. An asterisk indicates a significant difference in mean bacterial colony counts ($P < 0.001$) between control and PhEA^{H} devices.

In addition to their known antimicrobial activity, phenolic extracts have shown the ability to scavenge reactive oxygen species (ROS) *in vitro* [24]. Therefore, we investigated the potential of $PhEA^{H}$ to scavenge free radicals using the DPPH assay, which showed moderate antioxidant activity (EC_{50} 701.5±12.00 μg/mL). Since one of the main complications of oxidative stress is carcinogenesis [25], we also investigated the antitumor effects of $PhEA^{H}$ against glioblastoma *in vitro*. The results show that, at the highest concentrations tested, the extract reduced tumor cell viability, showing EC_{50} of 666.31±42.8 μg/mL.

Discussion

Honey has numerous therapeutic activities, many of which are directly linked to the presence of plant-derived phenolic compounds [1, 2]. However, the concentration of these phenolic compounds is usually low from *in natura* honey. Thus, to produce the desired therapeutic effects, a significant amount of honey would need to be used [4-7]. In this sense, our study aimed to develop a methodology for the concentration of phenolic compounds from monofloral aroeira honey, which has shown promising pharmaceutical activity [3].

Using chromatographic methods was produced a phenolic-rich extract from aroeira honey, which showed a 20 times higher concentration of phenolic compounds than *in natura* honey. Similar results were shown by Ferreira *et al.* [26] in which phenolic-rich extracts from three monofloral honeys from northeast Portugal (*Rosmarinus officinalis*, *Echium vulgare* and *Erica australis*) were obtained by chromatography in a column with Amberlite XAD-2. Using the same chromatographic column, Lianda *et al.* [5] produced phenolic-rich extracts from nine Brazilian honeys (four multifloral and five monofloral – *Citrus* sp.), which had total phenolic contents ranging from 127.2 to 766.5 mg GA/g for the extract and 0.34 to 0.78 mg GA/g for *in natura* honey. Moreover, a flavonoid-rich fraction was obtained from 33 types of honey (11 multifloral and 22 monofloral) obtained by combining chromatography on Amberlite XAD-2 and liquid-liquid extraction using diethyl ether as solvent [4].

The evaluation of the antibacterial activity of aroeira honey extract ($PhEA^{H}$) showed that it has significant antimicrobial action against Gram-negative bacteria, including to isolates that are highly resistant to antibiotics. Gram-negative bacilli cause several infections in healthcare settings, such as pneumonia, bloodstream infections, wound infections, surgical site infections, and meningitis [26, 27]. Moreover, Gram-negative bacteria are resistant to several drugs and are becoming increasingly resistant to most available antibiotics [28]. Therefore, severe infections caused by Gram-negative bacilli

have become a clinical challenge. In this context, this study shows that PhEA^{H} could be a promising source of new antimicrobial agents against these pathogens.

Another important clinical challenge is foodborne diseases, which account for 48 million cases in the United States each year. It means that 1 in 6 Americans become ill from contaminated food, resulting in an estimated 128 000 hospitalizations and 3,000 deaths annually [29]. Our study reveals that the PhEA^{H} had a potent antibacterial effect against *Salmonella* spp., one of the main foodborne pathogens. Organisms belonging to the genus *Salmonella* are flagellated rod-shaped Gram-negative facultative anaerobes that can cause non-invasive non-typhoidal salmonellosis, invasive non-typhoidal salmonellosis, and typhoid fever in humans [30]. The different types of salmonellosis are associated with more than 1,200,000 illnesses annually. Of these, at least 100,000 infections are due to antibiotic-resistant *Salmonella* [31]. The high antibacterial activity observed against the genus *Salmonella* highlights PhEA^{H} as a good option for developing effective therapies to combat salmonellosis, especially those caused by antibiotic-resistant strains.

Fungi are increasingly recognized as the cause not only of superficial infections that affect many people and are relatively easy to treat but also of invasive and disseminated infections [32]. Fungal spores contribute to significant reactive airway respiratory illness in more than 10 million people. In addition, it is estimated that more than 300 million people worldwide suffer from severe fungal infections each year, of which 1.5 million die [33]. Therefore, we investigated the antifungal activity of PhEA^{H} but no significant antifungal effect was obtained for PhEA^{H}. Viral diseases, such as dengue, Zika [34], and coronavirus [17], have affected millions of people worldwide, with significant implications to human health around the world. In this study, we have shown that PhEA^{H} is also not active against arbovirus (dengue, zika, and Mayaro virus) and beta-coronavirus (MHV).

Biofilm formation represents a protected growth mode that makes bacterial and yeast cells less susceptible to antimicrobial agents and killing by the host immune system, allowing pathogens to survive in hostile environments and spread and colonize new niches [35, 36]. The most important disease in hospital settings is catheter-associated urinary tract infection (CA-UTI), in which microorganisms form biofilms in the urinary catheter and cause recurrent or chronic urinary tract infections in hospitalized patients and the elderly in nursing homes [37]. CA-UTI is currently considered the most common type of nosocomial infection and is responsible for over 1 million cases annually [38] and over 40% of all nosocomial infections in hospitals worldwide [39]. Interestingly, our study showed that PVC-composed urethral catheter sensitized with the PhEA^{H} inhibit the growth of uropathogenic bacteria. Furthermore, these bioactive

catheters prevent adhesion and biofilm formation by important uropathogens, such as *P. aeruginosa*. These results highlight the urethral catheter sensitized with the $PhEA^{H}$ as a potential product in prevention of CA-UTI.

Finally, we showed that the $PhEA^{H}$ has a potent antioxidant effect. The biological response induced by ROS is directly related to chronic diseases that have a major impact on human health, such as cancer, neurodegenerative diseases, cancer, diabetes, metabolic disorders, atherosclerosis, and cardiovascular diseases [40]. Thus, in addition to being useful in the treatment of infectious diseases, especially caused by Gram-negative bacilli, the extract can be useful in the prophylaxis of chronic non-communicable diseases due to its antioxidant effect. Confirming this proposition, glioblastoma cells, a tumor whose etiology is known to be linked to the generation of ROS [41], were drastically affected after exposure to the $PhEA^{H}$.

Conclusion

In conclusion, $PhEA^{H}$ has proven to be a promising source of novel antibacterial agents against fermenters (*K. aerogenes*, *K. pneumoniae*, *P. mirabilis*, *E. cloacae, S.* Typhimurium, and *S.* Enteritidis) and non-fermenters (*P. aeruginosa*) Gram-negative bacilli. It also presented relevant antioxidant and antitumor activities. Furthermore, we showed that $PhEA^{H}$-impregnated catheters could be useful in the prophylaxis of CA-UTI by inhibiting the adhesion and colonization of important uropathogens.

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Compliance with ethical standards

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Credit author statement

W.G.L. developed the concept, design, definition of intellectual content, literature search, experimental studies, data acquisition, and data analysis of experiments related to antibacterial and antifungal activity of extract, as well as participated in manuscript preparation. V.O.F. and J.C.M.B. produced, characterized and purified the phenolic-rich extract of Aroeira honey. F.R.S.S., J.M.S.F. and L.P.S.C. contributed to the experimental studies and data acquisition related to antiviral and antitumoral activity of extract. NA.S. and M.C.P. contributed to the experimental studies, data acquisition, and data analysis related to anti-adhesive and antibiofilm effects of extract. D.C.S.A. and W.S.C.N. contributed with manuscript editing and manuscript review.

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Production of a phenolic-rich extract of roeira honey and characterization of its antimicrobial, antitumoral and antioxidant activities

Supplementary material

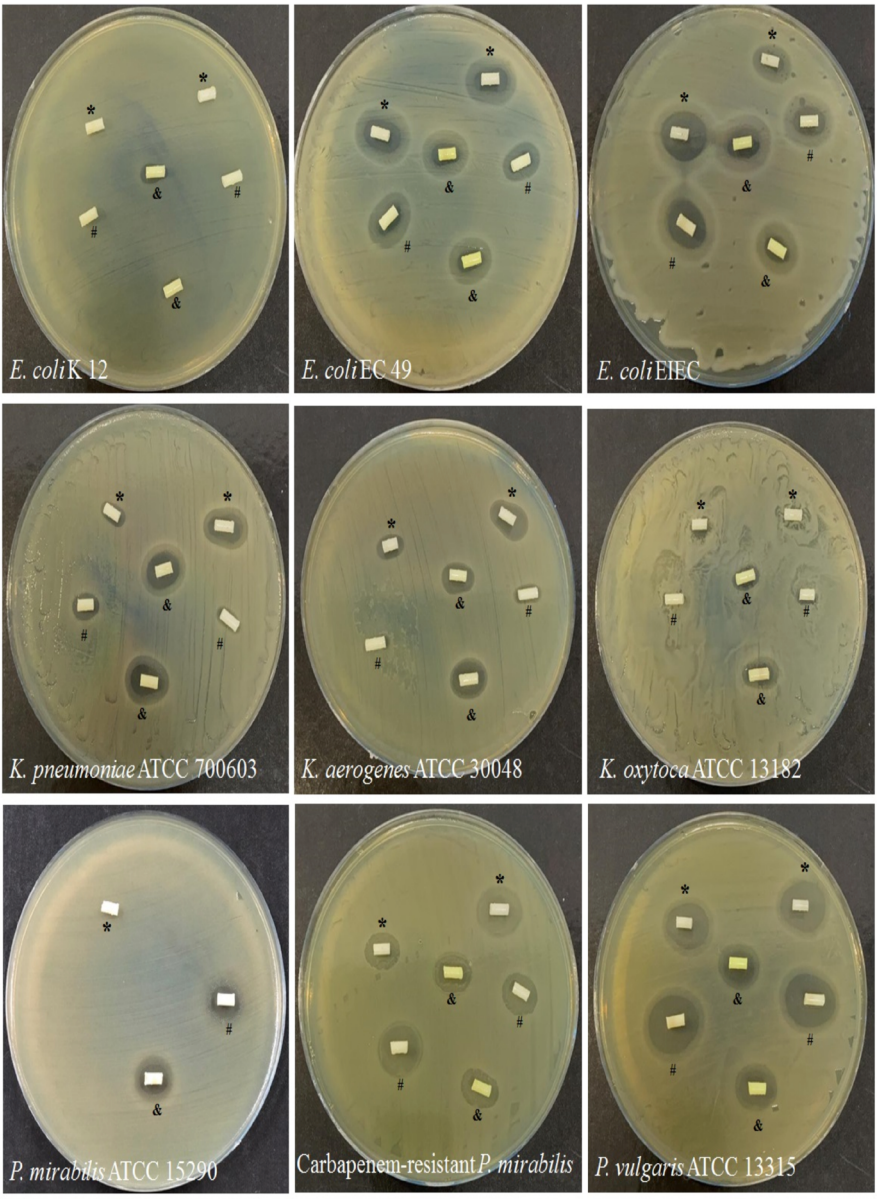


Figure S1. Zones of inhibition produced by a phenolic-rich extract from *Astronium urundeuva* (M. Allemão) Engl. ($PhEA^{H}$)-impregnated catheter segments.

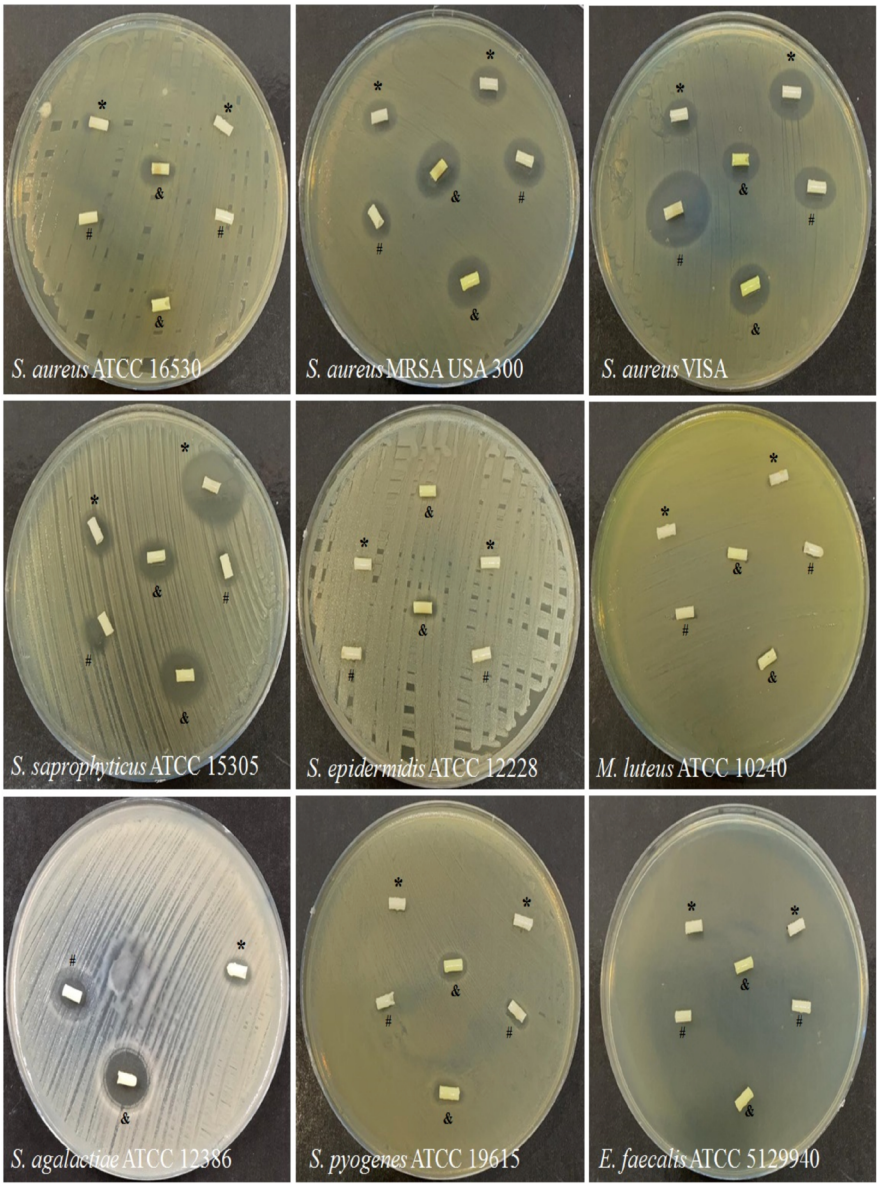
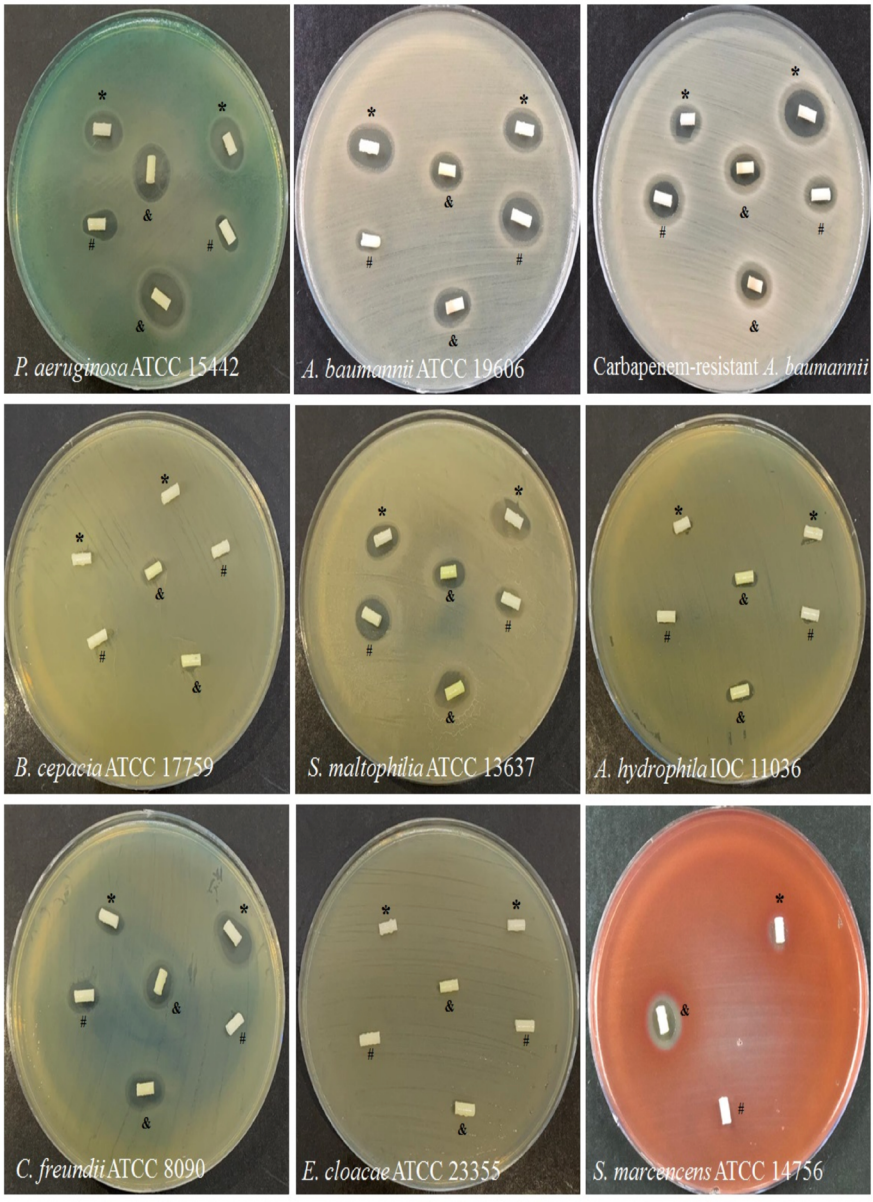


Figure S1. Zones of inhibition produced by a phenolic-rich extract from *Astronium urundeuva* (M. Allemão) Engl. (PhEA^{H})-impregnated catheter segments. (Continue).



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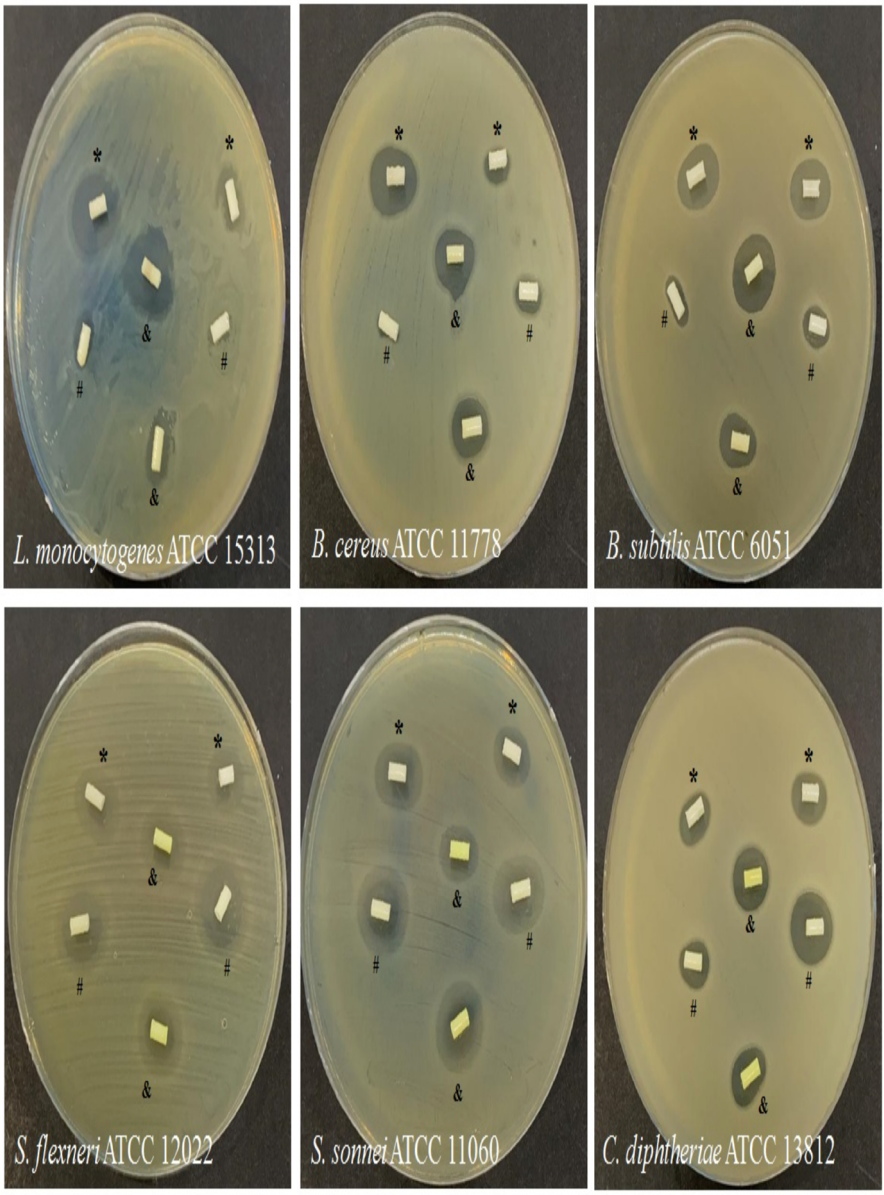

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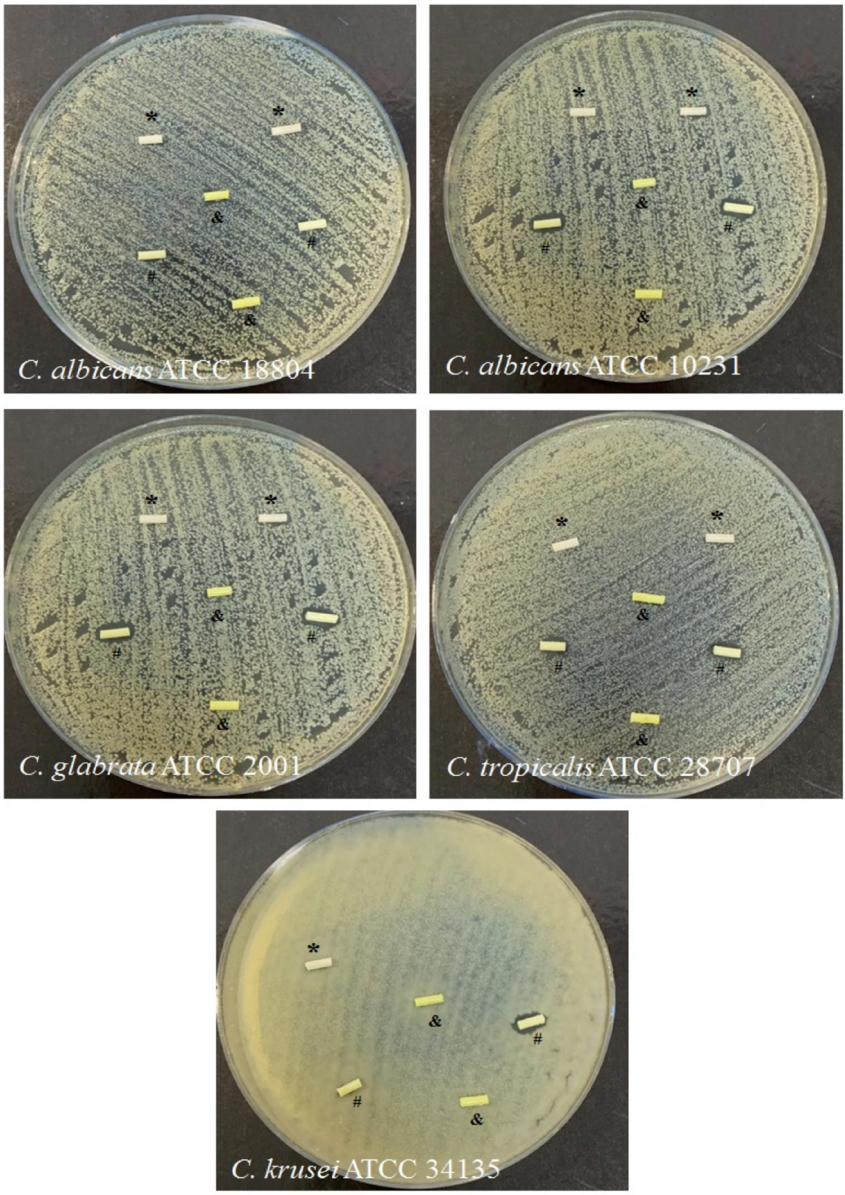

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