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Synergistic Antimicrobial and Antibiofilm Activity of Hydroethanolic Extracts of Cinnamomum verum J. Presl and Punica granatum L. Against Multidrug-Resistant Acinetobacter baumannii and Pseudomonas aeruginosa

Thaís Cristine Pereira
Universidade Estadual Paulista (Unesp)
Patrícia Michelle Nagai de Lima
Universidade Estadual Paulista (Unesp)
Júlia Andrade de Oliveira
julia-andrade.oliveira@unesp.br
Universidade Estadual Paulista (Unesp)
Lara Steffany de Carvalho
Universidade Estadual Paulista (Unesp)
Gleiciany Rosário da Rosa
Universidade Estadual Paulista (Unesp)
Maria Cristina Marcucci
Universidade Estadual Paulista (Unesp)
Vanessa Marques Meccatti-Domiciano
Universidade Estadual Paulista (Unesp)
Claudio Antonio Talge Carvalho
Universidade Estadual Paulista (Unesp)
Amjad Abu Hasna
Universidade Estadual Paulista (Unesp)
Luciane Dias de Oliveira
Universidade Estadual Paulista (Unesp)

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Synergistic Antimicrobial and Antibiofilm Activity of Hydroethanolic Extracts of *Cinnamomum verum* J. Presl and *Punica granatum* L. Against Multidrug-Resistant *Acinetobacter baumannii* and *Pseudomonas aeruginosa.*

Thaís Cristine Pereira[1], Patrícia Michelle Nagai de Lima[1], Júlia Andrade de Oliveira[2*], Lara Steffany de Carvalho[1], Gleiciany Rosário da Rosa[1], Maria Cristina Marcucci[1], Vanessa Marques Meccatti-Domiciano[1], Claudio Antonio Talge Carvalho[2], Amjad Abu Hasna[2] and Luciane Dias de Oliveira[1].

[1] Department of Biosciences and Oral Diagnosis, Institute of Science and Technology, São Paulo State University (ICT-UNESP), São José dos Campos 12245-000, SP, Brazil. thatha.this@hotmail.com, patricia.nagai@unesp.br, vanessa.meccatti@unesp.br, larasteffany2011@gmail.com, gleiciany.rosario@unesp.br, cristina.marcucci@unesp.br, raquel.teles@unesp.br, , luciane.oliveira@unesp.br.

[2] Department of Restorative Dentistry, Endodontics Division, Institute of Science and Technology, São Paulo State University (ICT-UNESP), São José dos Campos 12245-000, SP, Brazil. d.d.s.amjad@gmail.com, julia-andrade.oliveira@unesp.br, claudio.talge@unesp.br.

***** Correspondence: Júlia Andrade de Oliveira. Department of Restorative Dentistry, Endodontics Division, Institute of Science and Technology, São Paulo State University (ICT-UNESP), São José dos Campos 12245-000, SP, Brazil. julia-andrade.oliveira@unesp.br.

Abstract

Antibiotic resistance in pathogens, such as *Acinetobacter baumannii* and *Pseudomonas aeruginosa,* represents a global health threat, highlighting the need for therapeutic alternatives. **AIMS**: Investigate the synergistic antimicrobial and antibiofilm potential of *Cinnamomum verum* and *Punica granatum* extracts against multidrug-resistant (MDR) strains of *A. baumannii* and *P. aeruginosa*, along with their phytochemical composition and antioxidant activity. **METHODS AND RESULTS:** The extracts were characterized by total phenolic and flavonoid contents, and their bioactive compounds were identified using HPLC. Antioxidant activity was evaluated by free-radical scavenging assays. The minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) were determined by microdilution method. Synergistic interactions between the extracts were assessed by the checkerboard microdilution assay. The antibiofilm activity of the extracts was evaluated by their ability to inhibit biofilm formation and to reduce preformed biofilms of MDR strains**.** Both extracts exhibited antioxidant activity and antibacterial effects, with minimum bactericidal concentrations as low as 3.3 mg/mL. *C. verum* showed 0.935 mg/mL of flavonoids and 2.069 mg/mL of phenolics, while *P. granatum* showed 0.853 mg/mL and 1.406 mg/mL, respectively. The combination of extracts showed synergistic antimicrobial interactions, with 12 synergistic combinations (FIC ≤ 0.5), particularly against *A. baumannii***.** Concentrations of *C. verum* and *P. granatum* extracts inhibited biofilm formation in *A. baumannii* and *P. aeruginosa*, with effects comparable to chlorhexidine. **CONCLUSIONS:** Extracts of *C. verum* and *P. granatum* exhibit relevant antimicrobial and antibiofilm activities, with synergistic interactions. Their combination represents a promising natural strategy to enhance antimicrobial efficacy against MDR pathogens.

Keywords: *Acinetobacter baumannii*; *Pseudomonas aeruginosa*; *Cinnamomum verum*; *Punica granatum.*

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1. Introduction

Bacterial resistance to antibiotics has progressively increased worldwide and is expected to cause more deaths in the next years. In 2024, the World Health Organization (WHO) published the updated list of priority resistant bacteria that require new antibiotics, including the species *Acinetobacter baumannii* and *Pseudomonas aeruginosa* (World Health Organization, 2024). These bacteria belong to the ESKAPE group (*Enterococcus faecium, Staphylococcus aureus, Klebsiella pneumoniae, A. baumannii, P. aeruginosa* and *Enterobacter* spp.) created in 2008. This group comprises six clinically significant pathogens associated with healthcare-related infections (HAIs) and is characterized by a high capacity to acquire multiple antimicrobial resistance mechanisms, favoring the emergence of multidrug-resistant strains (El-Mahallawy et al., 2016; Marturano,Lowery, 2019).

Such resistance represents an evolutionary response of bacteria to antimicrobial exposure (Avershina et al., 2021). In some cases, bacteria exhibit intrinsic resistance, which may result from mechanisms such as efflux pumps, alterations in the cell wall, or the presence of porins that limit drug entry (Eichenberger,Thaden, 2019). Conversely, other strains may have acquired resistance because of genetic mutations or transfers of resistance genes through conjugation (Eichenberger,Thaden, 2019; Lopatkin et al., 2021).

Pseudomonas aeruginosa is an aerobic, Gram-negative, opportunistic bacillus belonging to the Pseudomonadaceae family. It exhibits intrinsic resistance to multiple antibiotics, which is associated with the low permeability of its outer membrane, the activity of efflux pumps, and the production of antibiotic-inactivating enzymes. In addition, its ability to form biofilms and to exchange genetic material further contributes to its resistance profile (Chevalier et al., 2017; Pang et al., 2019). *P. aeruginosa* causes several diseases, ranging from acute local to chronic general infections, which can be fatal (Sadikot et al., 2005). It can cause bloodstream and respiratory infections, and to a lesser extent, urinary tract infections (Pelegrin et al., 2021). In addition to the ability to affect different human organs, when there is the presence of antibiotic resistance mechanisms, these infections become more problematic, making treatment difficult and representing a threat to susceptible patient populations (Pelegrin et al., 2021).

Acinetobacter baumannii is an opportunistic Gram-negative bacterium capable of causing various serious nosocomial infections, especially in patients admitted to intensive care units (ICUs), including ventilator-associated pneumonia, bloodstream infections, urinary tract infections, and surgical site infections (Antunes et al., 2014). Furthermore, *A. baumannii* infections are related to the limited availability of therapeutic options, inefficient infection control of hospital devices, and negligence in personal protective equipment, factors that favor the occurrence of cross-infections (De Freitas et al., 2020). There are numerous reports of *A. baumannii* resistance to various antibiotics, including carbapenems (imipenem, doripenem, and meropenem), considered drugs of choice, making its eradication particularly difficult, which reinforces the need for new therapeutic strategies for its control (Aurilio et al., 2022; Ibrahim et al., 2021; Miyakis et al., 2011).

One of the resistance mechanisms presented by these pathogens is the formation of biofilm (Roy et al., 2018). Biofilm is a structured community of microorganisms with a polymeric

extracellular matrix adhered to an inert or living surface. These structures are more resistant to antimicrobial agents; therefore, drug concentrations need to be higher for them to be effective and can be up to four times greater than those used for bacteria that do not have this mechanism (Dunne, 2002; Pokharel et al., 2022). This resistance to several antimicrobials is due to the difficulty in penetrating the extracellular matrix, as well as the accelerated rate of microbial growth and physiological changes observed in the growth of the biofilm (Donlan,Costerton, 2002; Pokharel et al., 2022).

Faced with increasing bacterial resistance to antibiotics, there is a growing need to develop new therapeutic strategies. The phytotherapy has gained notoriety for combating infections, particularly those caused by nosocomial bacteria. Plant extracts have been widely investigated due to their diverse biological properties, especially their antimicrobial activity (de Lima et al., 2024; Meccatti et al., 2023a, 2022; Dos Santos Liberato et al., 2021; Santos et al., 2023; Da Silva et al., 2022). The antimicrobial effects of medicinal plant extracts have been reported as part of integrative treatment strategies, contributing to an expanded therapeutic arsenal, such as antifungal, antibacterial, antiprotozoal and antioxidant action (DS,CS, 2009; Imran et al., 2021; Kumar et al., 2006; Mahlo et al., 2016).

Plant-derived compounds are rich in phytochemical constituents, such as flavonoids, phenolics, alkaloids, tannins, glycosides, saponins, and terpenoids, which are associated with a wide range of pharmacological effects. Given the vast diversity of plant species, these natural sources represent an important target for the discovery of new therapeutic agents. Among them, *Cinnamomum verum* and *Punica granatum* have been extensively studied for their multiple biological activities. *C. verum* has demonstrated antifungal, antimicrobial, anti-inflammatory, antidiabetic, cardioprotective, antioxidant properties and wound-healing activities (Essid et al., 2017; Mathew,Abraham, 2006; Meccatti et al., 2023a; Nawaz et al., 2023; Schink et al., 2018). Similarly, *P. granatum* is rich in bioactive compounds and exhibits antimicrobial, antioxidant, anti-inflammatory, antitumor, anti-obesity, nephroprotective, among others (Arra et al., 2024; Maphetu et al., 2022; Noreen et al., 2025). These findings reinforce the therapeutic potential of these plant species; however, despite their known antimicrobial potential, studies specifically evaluating these extracts against multidrug-resistant strains of *A. baumannii* and *P. aeruginosa* remain scarce, particularly regarding their antibiofilm activity.

Given the high antimicrobial resistance exhibited by *Acinetobacter baumannii* and *Pseudomonas aeruginosa*, the combination of antimicrobial agents, such as plant extracts, propolis, and conventional antibiotics, represents an attractive strategy. These agents contain multiple bioactive compounds that can act synergistically, enhancing antimicrobial efficacy and potentially reducing resistance development (Meccatti et al., 2023a; Sookkhee et al., 2022). This study investigated the synergistic antimicrobial and antibiofilm potential of hydroethanolic extracts of *Cinnamomum verum* J. Presl and *Punica granatum* L. against standardized and multidrug-resistant (MDR) strains of *Acinetobacter baumannii* and *Pseudomonas aeruginosa*, in addition to evaluating their phytochemical composition and antioxidant properties.

2. Materials and Methods

2.1. Preparation and phytochemical analysis of extracts

P. granatum extract was obtained from the seeds of the fruit and *C. verum* extract using the bark of the tree (cinnamon stick). To obtain the extracts, 40 g of commercially acquired plant material were placed in 400 mL of hydroethanolic solution - HE (50% distilled water and 50% absolute ethyl alcohol - Synth, Diadema-SP, Brazil) for 48 h under stirring (70 rpm, COLEMAN, TS-2000A VDRL Shaker, Brazil). Subsequently, the extracts were filtered using quantitative filter paper (J. Prolab, São José dos Pinhais- PR, Brazil) and stored in an amber bottle, duly labeled.

2.2. Determination of Soluble Solids Content in the Hydroethanolic Extracts

The soluble solids content (SSC) of the extracts, prepared as described earlier, was determined for use in the antioxidant activity assay. The procedure was as follows: 25 mL beakers were weighed, and their respective weights were recorded. Next, 5 mL of the *C. verum* and *P. granatum* hydroethanolic extracts were pipetted into each beaker, which were then placed in an oven (Fanem LTDA, São Paulo-SP, Brazil) at 60ºC until the solvent evaporated. After removal from the oven, the beakers were cooled in a desiccator. Subsequently, the samples were weighed, and the operation was repeated until a constant weight was obtained. The procedure was performed in triplicate. The amount of soluble solids content in the extracts was calculated according to the following equation:

$$\% \text{ soluble solids (m/V)} = (m - b) \times 100/Va$$

Where: b = beaker mass; m = final mass of the extract after drying; Va is the volume of 5 mL).

2.3. Determination of the total flavonoid content of extracts

A stock solution was prepared by placing 100 µL of the extract in a 10 mL volumetric flask and adding methyl alcohol (Synth, Diadema-SP, Brazil) up to the meniscus. Subsequently, the procedure was replicated three times. From the stock solution, a 200 µL aliquot was transferred to a 10 mL flask already containing 5 mL of methanol. Then, 200 µL of aluminum chloride ($AlCl_3$, Dinâmica, Brazil) was added, and the volume was adjusted to approximately 10 mL with methanol. The resulting solution was stirred and incubated in a water bath at 20 °C for 30 min. Later, the meniscus was adjusted, and the absorbance was measured at 425 nm using a spectrophotometer (Micronal B582, São Paulo, Brazil). The standard calibration curve for total flavonoids (TF) was made using a calibration curve prepared with different concentrations of quercetin (Sigma, St Louis, Missouri, USA) and the TF was calculated as mg of quercetin equivalents per mL of each (mg QE/mL). Analysis was performed in triplicate (Cristina Marcucci et al., 2021; Meccatti et al., 2023b; Santos et al., 2020).

2.4. Determination of total phenol content of extracts

A different stock solution was prepared, this time, by adding 200 µL of the extract to a 10 mL volumetric flask containing distilled water under agitation, the volume was completed with water until close to the meniscus to obtain the stock solution (20 µg/mL). Subsequently, the procedure was replicated three times. An aliquot of 200 µL of the stock solution was transferred

to a 10 mL volumetric flask (1:50) containing approximately 5 mL of distilled water, and 800 µL of the Folin–Ciocalteu (F-C) reagent (Dinâmica, Brazil) was added. The resulting solution was stirred, and within 1 to 8 min, 1.2 mL of 20% sodium carbonate (Synth, Diadema-SP, Brazil) buffer solution was added. The volume was completed with water until close to the meniscus, and the solution was kept in a water bath at 20 °C. After 2 h, the meniscus was adjusted to final volume at 20 °C, and the absorbance was measured at 760 nm on a spectrophotometer. The standard calibration curve for total phenol content (TP) was prepared with different concentrations of gallic acid (Sigma, St Louis, Missouri, USA) and the TP was calculated as mg of gallic acid equivalents per mL of each (mg GAE/mL). Analysis was performed in triplicate(Cristina Marcucci et al., 2021; Meccatti et al., 2023b; Santos et al., 2020).

2.5. High-Performance Liquid Chromatography (HPLC) Analyses of Plant Extracts

The determination of the content of markers in cinnamic aldehyde and punicalagin was carried out by HPLC-DAD, from Merck-Hitachi (Darsmtadt, Germany) model D-7000 with automatic injector. Separation was achieved on a Lichrochart column (Merck, Darmstadt, Germany) (RP-18, 12.5 x 0.4 cm, 5 particle size) using water, formic acid (HPLC grade, Merck, Darmstadt, Germany) (95:5, V/V) (solvent A) and methanol (HPLC grade, Dinâmica, Brazil) (solvent B). The elution was carried out with a linear gradient starting with 30% of B and completed with 90% of B was used, in a chromatographic run of 45 min and 5 min to equilibrate the column, with a flow rate of 1 mL/min. For both extracts, a sample injection volume of 20 µL were performed. The detection wavelengths were 280 and 340 nm. A calibration curve was made with the cinnamic aldehyde standard (Sigma, St Louis, Missouri, USA), between 4.0 to 10.0 µg/mL for its quantification in the *C. verum* extract. For α-punicalagin and β-punicalagin, two calibration curves were made, between 5.0 to 10.0 µg/mL, using punicalagin (with both isomers, from Sigma, St Louis, Missouri, USA) for its quantification in the *P. granatum* extract.

2.6. Free Radical Suppressive Activity (Antioxidant Activity) of the Hydroethanolic Extracts

This method is based on the reduction of the 2,2'-diphenyl-1-picrylhydrazyl radical (DPPH, Sigma-Aldrich, St Louis, Missouri, USA) in absolute ethyl alcohol solution, which presents maximum absorption in the range 515- 528 nm. For this, the extracts were diluted to 1% V/V, and subsequently, a dilution of 0.01% V/V was prepared. In 11 test tubes, numbering them from 0 to 10. Different volumes of ethanol (µL) 1000, 960, 920, 880, 840, 800, 760, 720, 680, 640, 600 were added respectively to each tube. Then, 0, 40, 80, 120, 160, 200, 240, 280, 320, 360, 400 µL of 0.01% extract were added to each tube. Tube number 0 was the control (100% DPPH without extract). 1000 µL of DPPH was added to it. After 60 seconds, DPPH was added to the remaining tubes every 1 minute. Therefore, the absorbance was measure at 517 nm, using a spectrophotometer after 30 min of adding DPPH in tube 0. The percentage of remaining DPPH *versus* the concentration of the extract (µg/mL) was plotted and the EC_{50} (concentration that eliminates 50% of free radicals in µg/mL) was calculated using the linear regression.

2.7. Antibacterial Activity of the Hydroethanolic Extracts Against Planktonic Cultures

Four multidrug-resistant clinical strains of *A. baumannii* (named A1, A2, A3 and A4) and four multidrug-resistant clinical strains of *P. aeruginosa* (named P1, P2, P3 and P4) were used in this study (Table 1); the strains were obtained from the clinical laboratories of the Policlin (Policlin Saúde group, São José dos Campos, SP, Brazil) and ValeClin Clinical Analysis Laboratory (ValeClin Clinical Analysis Laboratory, São José dos Campos, SP, Brazil). The resistance profile of each strain was obtained through the automated MicroSCAN AutoSCAN 4® system (Table 2). Additionally, the reference strains of *A. baumannii* (ATCC 19606) and *P. aeruginosa* (ATCC 15442) were used in this study. The bacterial strains were cultivated (37 °C/24 h) in BHI agar (Brain Heart Infusion—Kasvi, São José dos Pinhais, PR, Brazil) for subsequent preparation of microbial suspensions in sterile saline solution (0.9% NaCl).

Table 1. Strains used in this study.

Pseudomonas aeruginosa	***Acinetobacter baumannii***
#P1	#A1
#P2	#A2
#P3	#A3
#P4	#A4
ATCC 15442	ATCC 19606

Table 2. Antibiotic resistance profile of clinical strains of *A. baumannii* and *P. aeruginosa* provided by the Policlin Group Laboratory and ValeClin Clinical Analysis Laboratory.

Antibiotic	*A. baumannii* clinical strains			
	A1	A2	A3	A4
Ertapenem	Res	Res	Res	Res
Imipenem	Res	Res	Res	Res
Meropenem	Res	Res	Res	Res
	P. aeruginosa clinical strains			
	P1	P2	P3	P4
Amikacin	Res	Res	Sen	Res
Aztreonam	Res	Inter	Res	Res
Cefepime	Res	Res	Res	Sen
Ceftazidime	Res	Res	Res	Sen
Ciprofloxacin	Res	Sen	Res	Res
Colistin	Inter	Res	-	-
Gentamicin	Res	Res	Sen	Res
Imipenem	Res	Res	Inter	Res

Legend: S - Sensitive; I - Intermediate; R - Resistant; - Not evaluated.

Microdilution method was used according to Clinical and Laboratory Standards Institute (CLSI) documents M7-A9 to determine the minimum inhibitory (MIC) and minimum bactericidal

(MBC) concentrations of the extracts for each bacterial strain. Microbial suspensions were prepared in sterile saline solution (0.9% NaCl) with turbidity adjusted to 10^8 colony-forming units per milliliter (CFU/mL) in a spectrophotometer (Micronal, São Paulo, SP, Brazil). The parameters used were λ = 625 nm and optical density (OD) of 0.08 ± 0.02 for both bacteria tested. From these stock solutions, dilutions of 10^6 CFU ml^{-1} were prepared for testing in planktonic cultures. In a different 96-well microplates (TPP, Trasadingen, Switzerland) for each bacterial strain, 100 µL/well of Mueller Hinton Broth (Himedia) was added. Next, 200 µL of one of the extracts was added in the first well, where 10 serial dilutions were carried out. Then, 100 µL/well of the respective bacterial inoculum was added. The microplates were incubated for 24 h at 37 °C. The MIC of each extract was determined, by eye, in the first well without turbidity, following the well with apparent microbial growth. In addition, analyses of the vehicle (EtOH:H_2O/50:50) were performed to verify whether it interferes with the antimicrobial action of plant extracts. To determine the MBC, the contents of the wells were seeded onto BHI agar. After incubation (37 °C/24 h), the MBC was determined as the lowest concentration of the extract that inhibited total microorganism growth.

2.8. Synergistic Activity of the Hydroethanolic Extracts Combination Using Checkerboard Microdilution Assay

The cinnamic aldehyde checkerboard technique was used according to Moreno et al. (Moreno et al., 2020) with modifications, this method is based on the Microdilution method of the Clinical and Laboratory Standards Institute. A new bacterial inoculum was prepared and standardized in saline solution (1×10^6 cells/mL). Eight concentrations of each extract were prepared based on MIC value of each extract for each bacterial strain being diluted in Mueller Hinton broth. Then, 50 µL of the first extract (*C. verum* hydroethanolic extract) was added along the *x*-axis (horizontal) of the microplate, and 50 µL of the second extract (BGP hydroethanolic extract) was added to the *y*-axis (vertical) of the microplate. Saline solution and culture medium were used as control groups. Lastly, 100 µL of the standardized inoculum of each strain was added totaling 200 µL/well. The microplates were incubated at 37 °C/24 h for further visual reading. To evaluate the synergistic effect of the extracts, the fractional inhibitory concentration (FIC) index was adopted, which classifies the combinations as synergistic, additive, indifferent or antagonistic. The FIC index was calculated using the formula:

FIC index = FIC 1^{st} + FIC 2^{nd} = (MIC of the 1^{st} extract in combination / MIC of the 1^{st} extract alone) + (MIC of the 2^{nd} extract in combination / MIC of the 2^{nd} extract alone).

The combination was considered synergistic when FIC ≤ 0.5, additive when FIC > 0.5 and ≤1.0, indifferent when FIC > 1 and ≤ 4 and antagonist when FIC > 4.0. The same formula was used to calculate the fractional bactericidal concentration index (FBC), using MBC values instead of MIC.

2.9. Antimicrobial Activity of the Hydroethanolic Extracts by Inhibiting Biofilms Formation (prevention)

A total of 100 µL of pre-determined MBC or synergistic concentrations of pomegranate and cinnamon extracts were placed in all microplate wells. For this, the extracts were diluted in BHI broth at the desired concentrations. Subsequently, the inoculum of each bacterial strain was prepared, where colonies were diluted in sterile physiological solution (NaCl 0.9%), followed by standardization in a spectrophotometer (B582, Micronal, São Paulo, Brazil) obtaining a concentration of 10^7 CFU/mL. A total of 100 µL of the standardized suspensions were added to the wells containing the predetermined MBC and synergistic concentrations. BHI broth and inoculum were used as a negative control, and chlorhexidine solution (0.03%) as a positive control. The plates were incubated at 37ºC for 48 h, with treatments being changed after 24 h of incubation.

2.10. Antimicrobial Activity of the Hydroethanolic Extracts Against Biofilms (Treatment of formed biofilms)

Biofilms were formed on the bottom of 96-well microplates. 100 µL of BHI broth and 100 µL inoculum at 10^7 CFU/mL prepared in sterile physiological solution (0.9% NaCl) were added to these wells. The biofilms were incubated for 48 h, with a change of culture medium after 24 h. After the biofilm formation period, they were placed in contact with treatments (isolated extracts) and controls for 24 h in independent experiments and on different plates for each type of test. The microplate was divided into groups with n = 10 for each treatment with isolated extracts (MBC, MBC x 2, MBC x 4). As a positive control, a 0.06% chlorhexidine solution was used, and a negative control was BHI broth together with the inoculum. After treatments, the solutions were discarded, and the biofilm was washed with BHI broth twice before the tests described below were carried out.

After the biofilm incubation period with the treatments, the wells were washed with sterile physiological solution (0.9% NaCl) and the biofilm cell viability test was performed using MTT solution (3-(4,5-dimethylthiazol- 2-yl)-2,5-diphenyltetrazolium bromide) (Sigma-Aldrich, St Louis, Missouri, United States). For this, 100 µL of the MTT solution (0.5 mg/mL) were added to each well and the plate was incubated away from light for 1 h in an oven at 37ºC. After the incubation period, the solution was removed and the 100 µL of dimethyl sulfoxide (DMSO) (Synth, Diadema-SP, Brazil) were added. The plate was again incubated away from light in an oven at 37 ºC for 10 minutes and placed in the shaker under constant agitation for 10 minutes. Subsequently, the plate was read in a microplate reader at 570 nm, and the optical densities obtained were converted, using the formula below, into a percentage of reduction in microorganism viability:

% Viability Reduction = 100 - (OD Treated Group x 100) / Average OD Control Group)

2.11. Statistical analysis

Data were analyzed with normality tests: Shapiro–Wilk, Kolmogorov–Smirnov and D'Agostino and Pearson omnibus. Normal data were analyzed with ANOVA and Tukey's test, otherwise, with the Kruskal–Wallis test and Dunn's test. The GraphPad Prism 5.0 program was used, considering a significance level of 5%.

3. Results

3.1. Soluble Solids, total flavonoids, and total phenols content in the Hydroethanolic Extracts

The quantification of the soluble solids content of *C. verum* hydroethanolic extract was 26.44 mg/mL and that of *P. granatum* hydroethanolic extract was from 24.58 mg/mL. In addition, using the quercetin standard curve, the total flavonoid content in *C. verum* and *P. granatum* hydroethanolic extracts was determined, with the results summarized in Table 3.

The *C. verum* hydroethanolic extract contained 0.935 mg/mL (0.094%) of flavonoids, while the *P. granatum* hydroethanolic extract had a total flavonoid concentration of 0.853 mg/mL (0.085%). Similarly, the total phenol content was analyzed using the gallic acid standard curve, as shown in Table 3.

Table 3. Total flavonoid and total phenol content in the extracts.

Extract	Extraction solvent	Total flavonoid (mg QE/mL)	Total phenol (mg GAE/mL)
C. verum	EtOH: H_2O (50:50)	0.935 ± 0.206	2.069 ± 0.174
P. granatum	EtOH: H_2O (50:50)	0.853 ± -0.068	1.406± -0.033

Legend: EtOH:H_2O – hydroethanolic.

The *C. verum* hydroethanolic extract exhibited a total phenol content of 2.069 mg/mL (0.207%), whereas the *P. granatum* hydroethanolic extract contained 1.406 mg/mL (0.141%).

3.2. High-Performance Liquid Chromatography (HPLC) Analyses of Plant Extracts

HPLC chromatographic analysis identified cinnamic aldehyde in *C. verum* hydroethanolic extract, as shown in Figure 1A. In contrast, the *P. granatum* hydroethanolic extract contained punicalagins, represented as a mixture of α-punicalagin and β-punicalagin, which were quantified separately (Figure 1B). The chromatogram of the *C. verum* hydroethanolic extract displayed a retention time of 9.42 minutes for cinnamic aldehyde, while the chromatogram of the *P. granatum* hydroethanolic extract showed retention times of 6.5 minutes for α-punicalagin and 9.5 minutes for β-punicalagin.

Figure 1. A: Chromatogram of the *C. verum* hydroethanolic extract; B: Chromatogram of the *P. granatum* hydroethanolic extract.

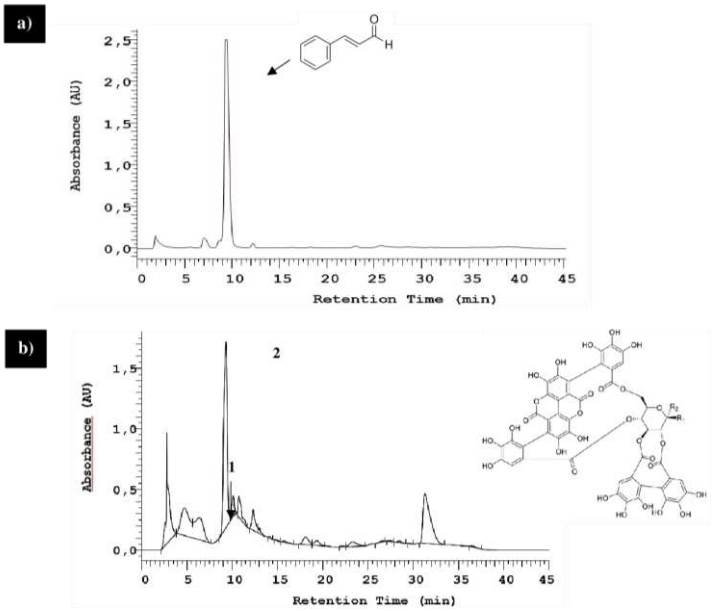


Fig. 1 a) Chromatogram of the hydroethanolic extract of *C.verum* (CHE), with the retention time of cinnamic aldehyde of 9.42 min; b) Chromatogram of the hydroethanolic extract of *P. granatum* (PHE), the retention time of α-punicalagin (**1**) is 6.5 min and of β-punicalagin (**2**), 9.5 min. Chemical structures a): cinnamic aldehyde (www.sial.com); b) punicalagin anomers α and β, α-punicalagin punicalagin α: R_1 = OH, R_2 = H (CAS # 130518-17-1) and punicalagin β: R_1 = H, R_2 = OH (CAS # 30608-10-5).

Table 4 shows the cinnamic aldehyde content in *C. verum* hydroethanolic extract and the α-punicalagin and β-punicalagin content in the *P. granatum* hydroethanolic extract, calculated using the standard calibration curve equation.

Table 4. Cinnamic aldehyde and punicalagin content in *C. verum* and *P. granatum* hydroethanolic extracts calculated by the standard calibration curve equation.

Extracts	**mg/mL**	**% (m/V)**
C. verum – Cinnamic aldehyde content	0.771	0.077
P. granatum - α punicalagin	2.246	0.225
P. granatum - β punicalagin	3.490	0.349

3.3 Free Radical Suppressive Activity (Antioxidant Activity) of the Hydroethanolic Extracts

The concentrations required to eliminate 50% of free radicals (EC_{50}) for the hydroethanolic extracts of *C. verum* and *P. granatum* were 9.11 and 27.02 µg/mL, respectively.

3.4. Antibacterial Activity of the Hydroethanolic Extracts Against Planktonic Cultures

The *C. verum* and *P. granatum* hydroethanolic extracts showed bactericidal activity against all evaluated strains of *A. baumannii* and *P. aeruginosa*, the MBC values are shown in Table 5.

Table 5. MBC (mg/mL) of *C. verum* and *P. granatum* hydroethanolic extract for ATCC and clinical strains of *A. baumannii* and *P. aeruginosa* provided by the Policlin Group Laboratory and ValeClin Clinical Analysis Laboratory.

Strains	***C. verum***	***P. granatum***
#A1	3.3	6.1
#A2	3.3	6.1
#A3	3.3	3.0
#A4	3.3	6.1
A. baumannii ATCC	3.3	1.5
#P1	6.6	3.0
#P2	6.6	6.1
#P3	6.6	6.1
#P4	6.6	6.1
P. aeruginosa ATCC	6.6	6.1

Legend: MBC – Minimum Bactericidal Concentration; A – *Acinetobacter baumannii*; P – *Pseudomonas aeruginosa*.

It was not possible to identify the Minimum Inhibitory Concentration (MIC) values due to the turbidity caused by the coloring of the extracts, which made visual reading impossible, therefore, in this study, the MBC values were considered for the next tests (*C. verum* hydroethanolic extract checkerboard and biofilms). The hydroethanolic vehicle control (50:50) did not show antibacterial action on all strains of *A. baumannii* and *P. aeruginosa* analyzed.

3.5. Synergistic Activity of the Hydroethanolic Extracts Combination Using Checkerboard Microdilution Assay

The combination of hydroethanolic extracts of *Cinnamomum verum* and *Punica granatum* showed marked synergistic interactions against four MDR strains of *Acinetobacter baumannii* (FIC ≤ 0.50), with reductions of up to 1220-fold for *P. granatum* and 4-fold for *C. verum* compared to their individual antimicrobial activities (Table 4). For the other strains (*A. baumannii* ATCC and *P. aeruginosa)* there was no inhibition of bacterial growth (Table 6).

Table 6. Values of the minimum bactericidal concentration (MBC) of isolated and associated *C. verum* and *P. granatum* hydroethanolic extract, and the synergistic effect of their combination against clinical strains of *A. baumannii*.

Strains	Isolated Extract MBC Value (mg/mL)		Combined Concentrations (mg/mL)		FBC Index	Reduction in MBC		Effect
	C. verum	*P. granatum*	*C. verum*	*P. granatum*		*C. verum*	*P. granatum*	
#A1	3.3	6.1	0.82	1.52	0.49	4x	4x	Syn
#A1	3.3	6.1	1.6	0.09	0.49	2x	67x	Syn
#A1	3.3	6.1	1.6	0.04	0.49	2x	152x	Syn

#A1	3.3	6.1	1.6	0.02	0.48	1x	305x	Syn
#A1	3.3	6.1	1.6	0.01	0.48	2x	610x	Syn
#A1	3.3	6.1	1.6	0.005	0.48	2x	1.220x	Syn
#A2	3.3	6.1	1.6	0.04	0.49	2x	1.220x	Syn
#A2	3.3	6.1	1.6	0.02	0.48	2x	305x	Syn
#A3	3.3	3.0	1.6	0.04	0.49	2x	75x	Syn
#A3	3.3	3.0	1.6	0.02	0.49	2x	150x	Syn
#A3	3.3	3.0	1.6	0.01	0.48	2x	300x	Syn
#A4	3.3	6.1	0.82	1.52	0.49	4x	4x	Syn

Legend: MBC – Minimum Bactericidal Concentration; FBC: Fractional Bactericidal Concentration Index. Values are expressed in mg/mL.

3.6. Antimicrobial Activity of the Hydroethanolic Extracts by Inhibiting Biofilms Formation (prevention)

For strain #A1, *C. verum* exhibited 95% inhibition at concentrations of 3.3 mg/mL and 6.6 mg/mL, while *P. granatum* at 6.1 mg/mL achieved 96% inhibition, both showing statistically significant differences compared to the negative control ($p < 0.0001$). For strain #A2, *C. verum* significantly inhibited biofilm formation by 91% at concentrations of 1.6 mg/mL and 3.3 mg/mL ($p < 0.0001$), and *P. granatum* at 6.1 mg/mL resulted in a 92% inhibition ($p < 0.0001$). Regarding strain #A3, *C. verum* achieved 92% and 93% inhibition at 1.6 mg/mL and 3.3 mg/mL, respectively ($p < 0.0001$). Similarly, for the standard *A. baumannii* strain ATCC 19606, *C. verum* inhibited biofilm formation by 91% and 93% at concentrations of 1.6 mg/mL and 3.3 mg/mL ($p < 0.0001$).

For clinical strain #P1, *C. verum* achieved 91.5% and 85% inhibition at concentrations of 3.3 mg/mL and 6.6 mg/mL, respectively ($p < 0.0001$). *P. granatum* demonstrated 93% inhibition of biofilm formation for clinical strain #P2 at 6.1 mg/mL ($p < 0.0001$). For strain #P3, *C. verum* at 3.3 mg/mL and 6.6 mg/mL resulted in 91% and 87% inhibition, respectively ($p < 0.0001$). Clinical strain #P4 exhibited 91% biofilm inhibition when treated with 6.1 mg/mL of *P. granatum* ($p < 0.0001$). For the standard *P. aeruginosa* strain ATCC 15442, *C. verum* showed biofilm inhibition of 93% and 86% at 3.3 mg/mL and 6.6 mg/mL, respectively ($p < 0.0001$), while *P. granatum* at 6.1 mg/mL achieved 92% inhibition ($p < 0.0001$). These results are illustrated in Figure 2.

Figure 2. *A. baumannii* and *P. aeruginosa* biofilm load reduction after treatment with the isolated extracts to prevent biofilm formation.

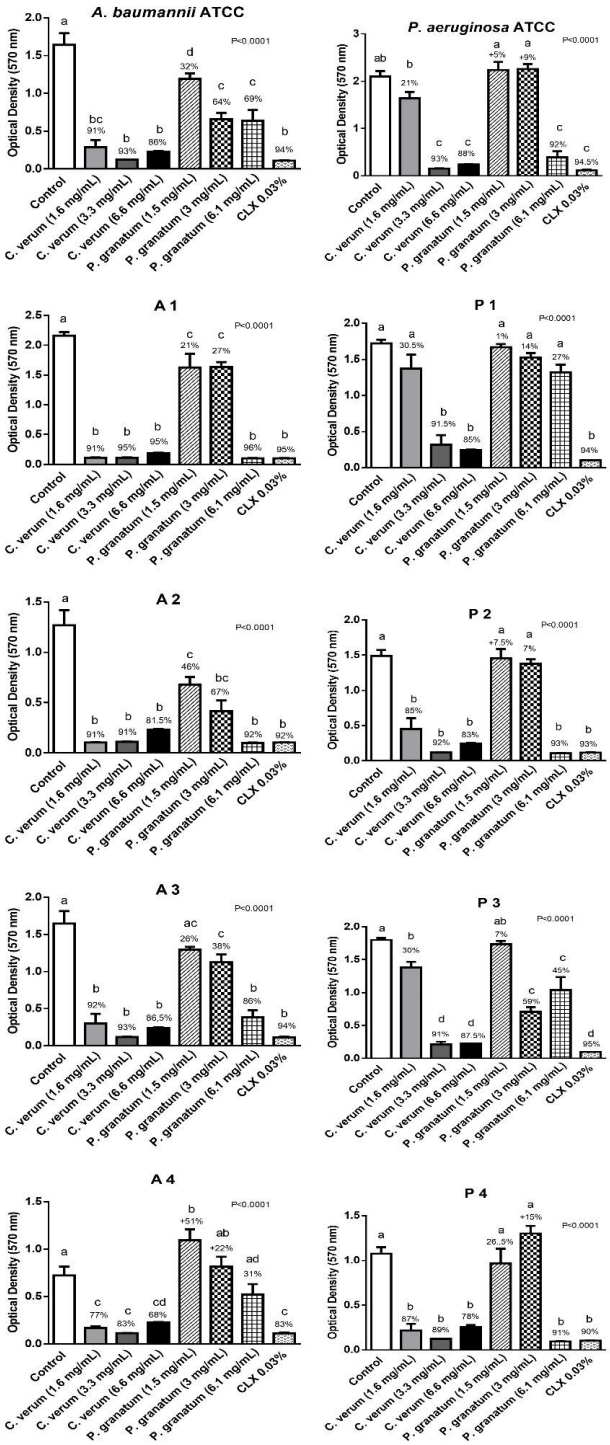


Fig. 2 CLX – chlorhexidine 0.03%. The letters "a", "b", "c" and "d" indicate a statistically significant difference among the groups ($p<0.0001$).

3.7. Antimicrobial Activity of the Hydroethanolic Extracts Against Biofilms (Treatment of formed biofilms)

For strain #A1, treatment with *C. verum* at a concentration of 13.2 mg/mL resulted in a 72% reduction in biofilm viability compared to the negative control ($p < 0.0001$). *P. granatum* at 12.2

mg/mL led to a 76% reduction ($p < 0.0001$). In clinical strain #A2, *P. granatum* at 12.2 mg/mL achieved an 81% reduction ($p < 0.0001$). For clinical strain #A3, both *C. verum* (13.2 mg/mL) and *P. granatum* (12.2 mg/mL) induced significant reductions of 72% and 76%, respectively ($p < 0.0001$). *C. verum* also caused a 60% and 74% reduction in strain #A4 at concentrations of 6.6 mg/mL and 13.2 mg/mL, respectively ($p < 0.0001$). In the case of the standard *A. baumannii* ATCC strain, both extracts significantly reduced biofilm viability compared to the negative control ($p < 0.0001$).

For *P. aeruginosa* biofilms, *C. verum* caused significant reductions of 73% and 80% at concentrations of 6.6 mg/mL and 13.2 mg/mL, respectively ($p < 0.0001$). *P. granatum* concentrations of 3 mg/mL, 6.1 mg/mL, and 12.2 mg/mL led to reductions in biofilm viability of 73%, 76%, and 71%, respectively. For strain #P2, *C. verum* at 6.6 mg/mL reduced biofilm viability by 84%, while *P. granatum* at 12.2 mg/mL reduced it by 88.5% ($p < 0.0001$). For clinical strain #P3, *P. granatum* at 12.2 mg/mL achieved a reduction similar to chlorhexidine 0.6%. In strain #P4, *P. granatum* at concentrations of 3 mg/mL, 6.1 mg/mL, and 12.2 mg/mL promoted reductions of 82%, 81%, and 86%, respectively ($p < 0.0001$). Both extracts showed significant antibiofilm effects against *P. aeruginosa* ATCC, with statistical differences compared to the negative control ($p < 0.0001$) (Figure 3).

Figure 3. *A. baumannii* and *P. aeruginosa* biofilm load reduction after treatment with the isolated extracts for 24h to reduce mature biofilm.

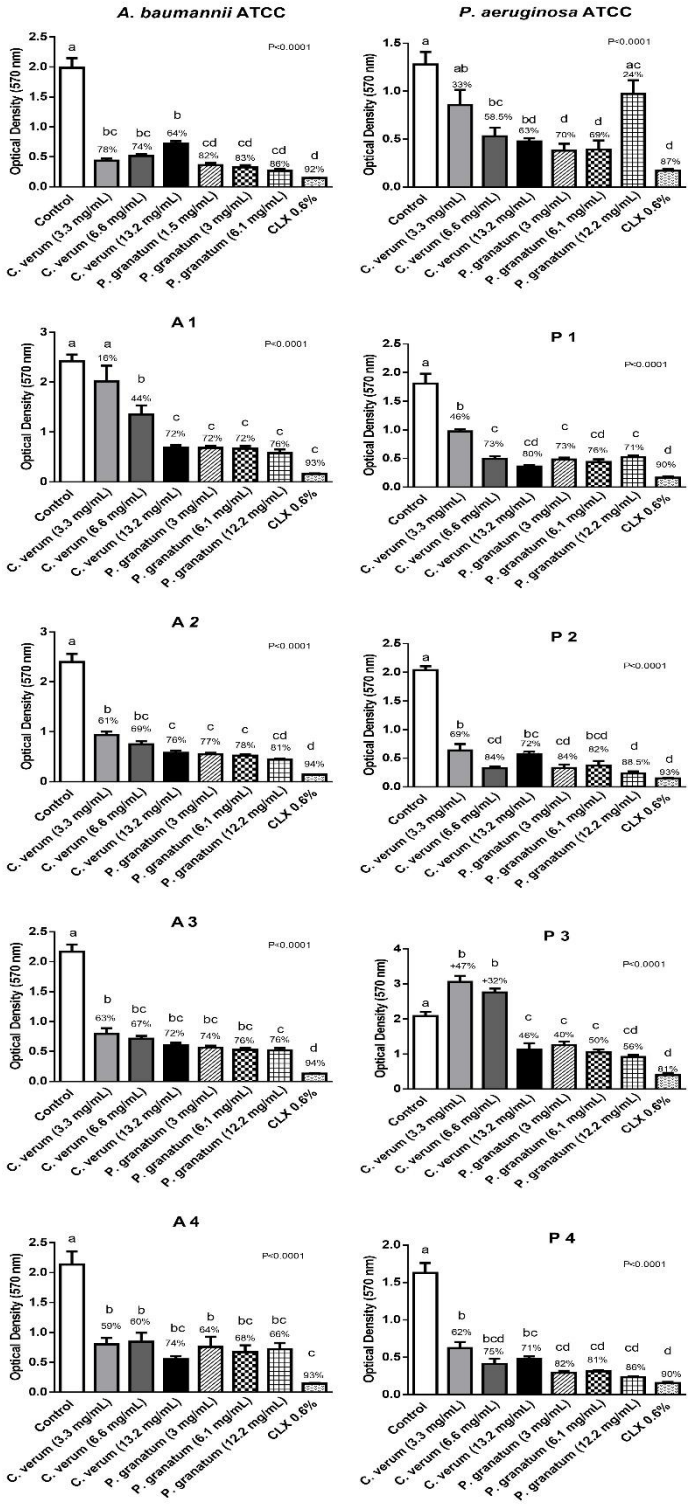


Fig. 3 CLX – chlorhexidine 0.03%. The letters "a", "b", "c" and "d" indicate a statistically significant difference among the groups (p<0.0001).

3.8. Inhibition of biofilm formation (prevention) of associated extracts on *A. baumannii* biofilms

Given the observed synergistic effect on *A. baumannii* strains, three synergistic combinations of *C. verum* and *P. granatum* were selected to assess their ability to inhibit biofilm

formation: *C. verum* 0.82 + *P. granatum* 1.52 mg/mL, *C. verum* 1.6 + *P. granatum* 0.04 mg/mL, and *C. verum* 1.6 + *P. granatum* 0.02 mg/mL. The combination of *C. verum* 1.6 + *P. granatum* 0.02 mg/mL demonstrated a 59% inhibition of biofilm formation in clinical strain #A1 ($p < 0.0001$).

For strain #A2, the combinations of *C. verum* 1.6 with 0.04 and 0.02 mg/mL of *P. granatum* inhibited biofilm formation by 52.5% and 72%, respectively ($p < 0.0001$). All selected combinations were effective in inhibiting biofilm formation in strain #A3, with inhibition values of 68%, 61%, and 72%. For strain #A4, all synergistic combinations inhibited biofilm formation, with the *C. verum* 1.6 + *P. granatum* 0.02 mg/mL combination achieving a 71% inhibition ($p < 0.0001$) (Figure 4).

Figure 4. Inhibition of biofilm formation (prevention) of associated extracts on *A. baumannii* biofilms

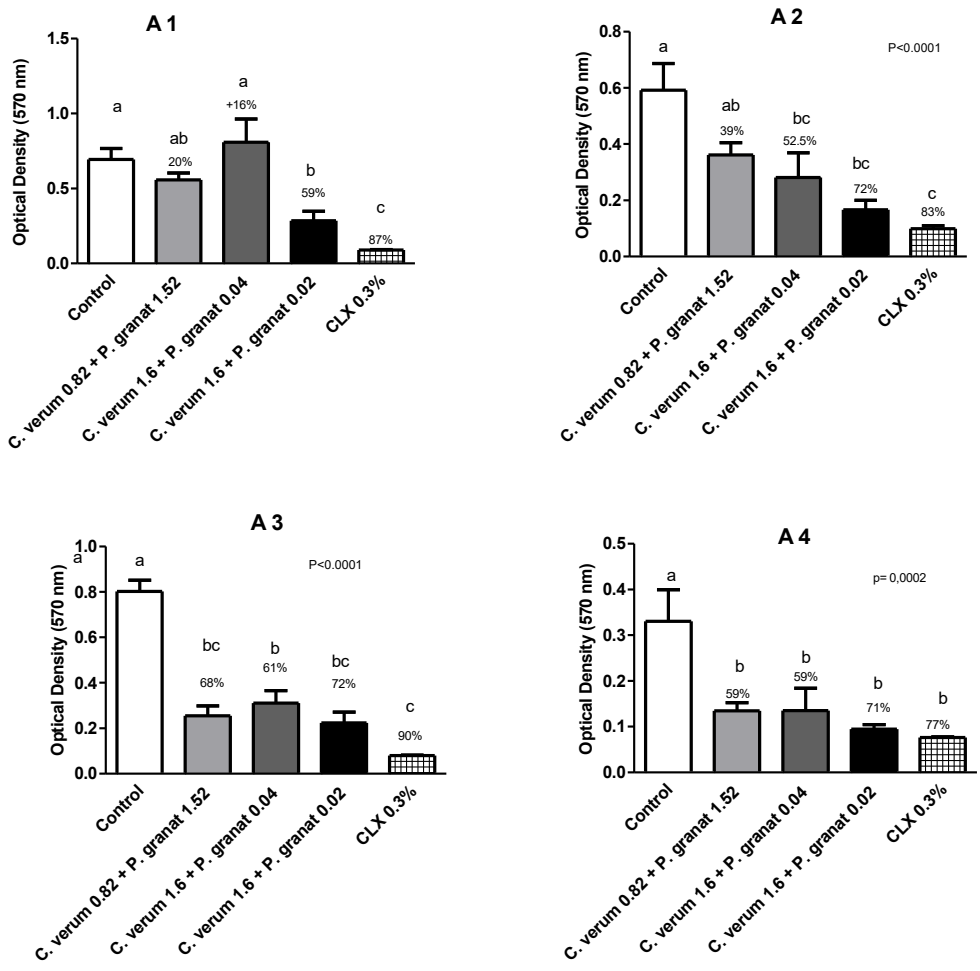


Fig. 4 CLX – chlorhexidine 0.03%. The letters "a", "b" and "c" indicate a statistically significant difference among the groups ($p<0.0001$).

4. Discussion

Given the urgent need for alternative strategies to combat antibiotic-resistant bacteria, the present study demonstrated that hydroethanolic extracts of *C. verum* and *P. granatum*

exhibit relevant antimicrobial, antibiofilm, and antioxidant activities. Notably, the combination of these extracts showed marked synergistic interactions, particularly against MDR *Acinetobacter baumannii*, highlighting the potential of these natural products as complementary therapeutic agents. The therapeutic effects observed in this study may be attributed to the presence of diverse chemical constituents in the plant extracts, including flavonoids, phenolics, alkaloids, tannins, glycosides, saponins, and terpenoids(Amsterdam JD et al., 2012; Dubale et al., 2023; Talib,Mahasneh, 2010).

This study identified the presence of total flavonoids and phenols in both *C. verum* and *P. granatum*, with flavonoid values being 0.935 and 0.853 mg/mL, respectively, and *P. granatum* values being 2.069 mg/mL for *C. verum* and 1.406 mg/mL for *P. granatum*. The amount of total phenols was quantified in another study using cinnamon, where the aqueous extract presented 0.380 mg/mL, the difference between the values found in our study and that of Singh et al. (2009) (Singh et al., 2009)can be attributed to the different extraction methods (solvent, distillation method, pressing and sublimation) and the solvent used (ethanol, water, methanol, among others), so different compounds can be extracted (Joshi et al., 2019). In the case of this study, the solvent used was hydroethanolic, as used by Singh et al. (2009) (Singh et al., 2009) was watery. The selection of the solvent is essential for the extraction of compounds, when the solvents and solutes are similar in terms of polarity, the extraction efficiency improves, in addition, the particle size also affects the result of the extraction, the finer the better the effectiveness (Zhang et al., 2018).

What also differentiates the results when using different solvents is the duration of the extraction in each time range, the higher the ratio of solvent and solute, the higher the extraction yield (Zhang et al., 2018). This can be observed when comparing the presence of different compounds in oils and hydroethanolic extracts. Normally, cinnamaldehyde can be extract in polar and unipolar solvents (Susanti et al., 2026). After HPLC analysis, the presence of cinnamaldehyde was detected in the methanolic extract from different cinnamon barks, varying from 0.17 to 18.31 mg/g of dry extract (Jayaprakasha et al., 2002; Vangalapati,Prakash, 2012). Varadarajan et al. (In Vitro Anticancer Effects of Cinnamomum verum J. Presl, Cinnamaldehyde, 4 Hydroxycinnamic Acid and Eugenol on an Oral Squamous Cell Carcinoma Cell Line - PubMed, n.d.) quantified the cinnamic aldehyde content in three different types of extracts, such as ethanolic, hydroalcoholic and aqueous extracts of *Cinnamomum verum* J. Presl (bark). In the same type of extract, we made (hydroethanolic, 70:30), they found a concentration of 0.17238% (w/w) or 1.7238 mg/g of dried extract. In the ethanolic extract, the concentration of cinnamic aldehyde found was 0.05727% (w/w) or 0.5727 mg/g. In our analysis, the *C. verum* extract showed a cinnamic aldehyde concentration of 0.771 mg/mL, the different but similar amount was due to the different polarities of the solvents.

As for *P. granatum*, the extracts obtained from the peel and flowers extracted with a mixture of water and ethanol 70:30 (V/V) demonstrated a total phenolic content of 12.34 µmol GAE/g (2.1 mg/mL) (Gigliobianco et al., 2022). Another study demonstrated phenolic compounds ranging from 1.29 to 2.17 mg GAE/g (1.29 to 2.17 mg/mL) from methanolic extracts of Chinese pomegranate seeds(Jing et al., 2012). The total phenolic content found in our study was 1.40 mg GAE/mL for the hydroethanolic extract obtained from pomegranate seeds. In all studies mentioned, the Folin-Ciocalteu method was used. Total flavonoids ranged from 1.76 to 2.11 mg

QE/mL and 5.92 to 16.92 mg/mL in the *P. granatum* methanolic extract of pomegranate seeds (Orak et al., 2012; Sabraoui et al., 2020), in our study the value found was 0.853 mg/mL in the hydroethanolic extract.

Regarding the antioxidant action of cinnamon and *P. granatum*, using the DDPH radical scavenging method, which is one of the most used to determine the antioxidant capacity of different compounds, cinnamon oil showed a strong free radical scavenging capacity with an EC_{50} of 4 .78 µg/mL (Mutlu et al., 2023). The EC_{50} values of the ethanolic and aqueous extract of cinnamon indicated potent antioxidant action (21.25 and 15.71 µg/mL, respectively). In another study, the concentration of antioxidant activity (EC50) was 29.01 µg/mL for the aqueous extract of cinnamon (de Lima et al., 2022). In our study, the EC50 value found for *C. verum* was 9.11 µg/mL, indicating potent antioxidant action. Regarding *P. granatum* extract, the EC50 values obtained were 42 and 65 µg/mL (peel) and 888 and 1,945 µg/mL (seeds) for methanolic extracts extracted from pomegranates grown in Morocco(Sabraoui et al., 2020). In another study, the methanolic extract extracted from the pomegranate peel showed 81% antioxidant activity and 23.2% when extracted from the seeds of this fruit (Singh et al., 2002).

Given the presence of compounds with diverse biological activities, it is extremely important to increasingly investigate these actions. Previous studies evaluating cinnamon extracts have reported variable antimicrobial activity depending on the extraction method and tested microorganisms. For instance, the crude extract of cinnamon leaves and its enriched fractions showed low activity against Gram-negative bacteria, including *Klebsiella pneumoniae* and multidrug-resistant *Pseudomonas aeruginosa*, with MIC values as high as 1000 mg/mL (de Lima et al., 2021). In contrast, in the present study, hydroethanolic extracts of *C. verum* exhibited considerably lower bactericidal concentrations against multidrug-resistant clinical strains of *Acinetobacter baumannii* and *P. aeruginosa*, with MBC values of 3.3 mg/mL and 6.6 mg/mL, respectively. Other studies have reported MIC values ranging from 0.25 to 0.5 mg/mL for cinnamon essential oil against MDR *A. baumannii* strains (Ganić et al., 2022), and even lower values for emulsified formulations. Although several studies have demonstrated the biological properties of cinnamon, investigations specifically focusing on hydroethanolic extracts of its seeds remain limited. This highlights the relevance of the present findings and supports the potential of this type of extract as an antimicrobial agent against multidrug-resistant pathogens.

Regarding antibiofilm activity, cinnamon essential oil had a significant effect (inhibitions of the formation and reduction of pre-formed biofilm) with values of 47–81 and 30–62%, respectively, when tested at concentrations of 0.25-2 mg/mL against *A. baumannii* strains (Ganić et al., 2022). The antibiofilm activity of cinnamon essential oil was also verified by (Wijesinghe et al., 2021), the minimum concentrations of biofilm inhibition and eradication of the formed biofilm (MBIC50 and MBEC) were 1.0 and 4.0 mg/mL for the strains *P. aeruginosa, S. aureus and K. pneumoniae*. Scanning electron microscopy (SEM) indicated cell shrinkage, cell wall damage and decreased biofilm density (Wijesinghe et al., 2021). Studies involving the antibiofilm action of *C. verum* are scarce in the literature, however our findings corroborate studies involving the essential oil against strains of *A. baumannii* and *P. aeruginosa*. In this study, after 24 h treatment of formed biofilms, viability reductions were 72% for strains #A2 and #A3 when in contact with *C. verum*. Regarding the prophylactic action in which it inhibits its formation, *C. verum* inhibited biofilm formation of strain #A1 by 95%, while *P. aeruginosa* had 87.5% inhibition.

In relation to *P. granatum* extract, their antimicrobial action has been reported in the literature (Dey et al., 2015; Trabelsi et al., 2020). The *P. granatum* methanol/water extract of pomegranate peel and its isolated constituents were active against *Mycobacterium tuberculosis* and *Klebsiella pneumoniae* producing β-lactamases.

For multidrug-resistant pathogens, total HPLC fractions of *P. granatum* showed potent antimicrobial activity when analyzing the inhibitory zones by the disk diffusion method (Dey et al., 2015). The hydroethanolic extract was able to reduce *P. aeruginosa* biofilm formation by up to 17–30%, when tested at 10–50 µg/ml. When this extract was applied to preformed biofilm, it could eradicate the biofilm by approximately 15–19%; and biofilm viability (metabolic activity measured by MTT assay) was reduced by approximately 13–22%(Joshi et al., 2019). Antibiofilm effect was also found in mouthwash formulations containing *P. granatum* bark extract, the formulation resulted in a loss of surface hardness of 34.5% and 36%, after 10 min treatment, of *S. mutans* and *C. albicans* biofilms, respectively (Fernandes et al., 2022). In our study, *P. granatum* demonstrated significant antibiofilm activity against both *A. baumannii* and *P. aeruginosa*. Biofilm formation by *A. baumannii* strain Ab1 was inhibited by up to 96%, while *P. aeruginosa* showed 93% inhibition under the same conditions. Following 24 h of treatment on pre-formed biofilms, a reduction in viability of 83% was observed for the *A. baumannii* ATCC strain, whereas *P. aeruginosa* exhibited a reduction of up to 88.5% when treated with *P. granatum*.

The combination of hydroethanolic extracts of *Cinnamomum verum* and *Punica granatum* showed marked synergistic interactions against clinical MDR strains of *Acinetobacter baumannii*, as evidenced by 12 synergistic combinations (FIC ≤ 0.5) and substantial reductions in minimum bactericidal concentrations, reaching up to a 1220-fold decrease. This pronounced effect highlights the relevance of combining plant-derived extracts as an effective strategy to enhance antimicrobial activity against multidrug-resistant pathogens.

Synergism between natural compounds has been increasingly recognized as a promising approach, as it may result from the interaction of multiple bioactive compounds targeting different bacterial pathways. Previous studies have reported that combinations of plant-derived compounds can significantly reduce MIC/MBC values; for example, associations involving punicalagin and other phytochemicals have achieved reductions of up to 128-fold against *A. baumannii*, along with antibiofilm activity (Aslan,Alim, 2025). Similarly, combinations of plant extracts have demonstrated up to a four-fold reduction in MIC values, supporting the hypothesis of multi-target interactions contributing to enhanced antimicrobial efficacy (Jeong et al., 2023). The strong synergism observed between *C. verum* and *P. granatum* extracts in the present study not only corroborates existing literature but also extends current knowledge by demonstrating a markedly higher reduction in bactericidal concentrations. This enhanced activity may allow the use of lower effective doses, which could contribute to reduced toxicity and potentially delay the development of bacterial resistance.

In summary, the hydroethanolic extracts of *C. verum* and *P. granatum* demonstrated robust antibacterial, antibiofilm, and antioxidant activities, highlighting their potential as alternative therapeutic agents against multidrug-resistant pathogens. The observed effects are likely associated with the rich phytochemical composition of the extracts, including flavonoids, phenols, cinnamic aldehyde, and punicalagin, which play key roles in disrupting microbial resistance mechanisms and biofilm formation. These findings underscore the importance of continued

research into plant-based bioactives to address the escalating challenge of antibiotic resistance, paving the way for the development of innovative and sustainable therapeutic strategies. Future studies should focus on in vivo validations, formulation optimization, and the investigation of the mechanisms underlying these synergistic interactions, aiming to bridge the gap between experimental findings and clinical applications.

5. Conclusions

Hydroethanolic extracts of *Cinnamomum verum* J. Presl and *Punica granatum* L. are rich in bioactive chemical compounds, including flavonoids and punicalagins, and exhibited significant antioxidant, antibacterial, and antibiofilm activities against multidrug-resistant strains of *Acinetobacter baumannii* and *Pseudomonas aeruginosa*. Notably, their combination showed marked synergistic interactions, particularly against *A. baumannii*, highlighting their potential as alternative strategies to combat multidrug-resistant infections.

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