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Phytochemical characterization and evaluation of antimicrobial activity of *Eugenia* sp.: antibacterial, antibiofilm and ampicillin-potentiating activities against resistant *Staphylococcus aureus* and *Staphylococcus epidermidis*

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This study investigated the phytochemical composition, antibacterial, antibiofilm, synergistic, and cytotoxic effects of hydroethanolic extracts and fractions from *Eugenia astringens* and *Eugenia mattosii*. HPLC–ESI-MS/MS analysis revealed the presence of nine compounds were identified in *E. astringens*, which was characterized by a high content of gallic acid (542.4 ± 8.2 μg g^{-1}), whereas *E. mattosii* contained eleven identified compounds, with epicatechin (533.2 ± 90.4 μg g^{-1}) and catechin (359.8 ± 10.8 μg g^{-1}) as major compounds. Gallic acid, protocatechuic acid, syringic acid, *p*-coumaric acid, scopoletin, *p*-anisic acid, abscisic acid, quercetin, and galangin were identified for the first time in *E. astringens*, while epicatechin, vanillic acid, and ferulic acid were reported for the first time in *E. mattosii*. The extracts exhibited activity against Gram-positive bacteria, with *E. astringens* showing the highest potency (MIC = 15.62 μg mL^{-1} against *Staphylococcus aureus* and MRSA; 7.81 μg mL^{-1} against *Staphylococcus epidermidis*), whereas *E. mattosii* showed MIC values of 125 μg mL^{-1} against all strains. No relevant activity was observed against the Gram-negative bacteria tested. Active extracts and fractions inhibited biofilm formation at 7.81–15.62 μg mL^{-1}, while mature biofilm eradication was observed mainly against MRSA, with EBH ≥125 μg mL^{-1} and DCM ≥62.5 μg mL^{-1}. Growth kinetics confirmed sustained inhibition by selected fractions. Checkerboard assays revealed synergistic interactions between ampicillin and *E. astringens* against MRSA, (fractional inhibitory concentration index (FICI) = 0.24–0.28), and against *S. epidermidis* (FICI = 0.35–0.36). These findings are relevant because potentiation of ampicillin activity may enhance β-

lactam efficacy against resistant *Staphylococcus*. SYTOX green assays demonstrated that the active samples, especially DCM from *E. astringens* and FAE from *E. mattosii* increased bacterial membrane permeability, suggesting membrane disruption as one of the mechanisms underlying their antibacterial activity. Cytotoxicity assays in RAW 264.7 macrophages demonstrated low toxicity at MIC concentrations. Collectively, the potent anti-staphylococcal activity, inhibition of biofilm formation, synergistic interactions with ampicillin, membrane permeabilization, and low cytotoxicity at MIC concentrations highlight *E. astringens* as a promising source of antibacterial compounds and antibiotic adjuvants. These findings support further investigation of *E. astringens* against resistant Gram-positive pathogens.

KEYWORDS

antibacterial activity, biofilm, *Eugenia astringens*, *Eugenia mattosii*, MRSA, phenolic compounds, synergism

1 Introduction

Antimicrobial resistance is recognized as one of the most pressing threats to global public health, reducing the effectiveness of currently available antimicrobial agents and complicating the treatment of infections caused by pathogenic bacteria. In 2019, an estimated 4.95 million deaths were associated with antimicrobial-resistant bacterial infections (Murray et al., 2022). Beyond the growing resistance to conventional antimicrobials, virulence factors such as biofilm formation play a crucial role in the persistence of infections, increasing bacterial tolerance and contributing to therapeutic failure. Biofilm formation is considered one of the major bacterial virulence factors because it protects microorganisms from host immune defenses and antimicrobial agents, contributing to chronic, recurrent, and device-associated infections (Flemming et al., 2016).

Given this scenario, the search for novel antimicrobial agents has become increasingly important.

Despite advances in antimicrobial development, current discovery pipelines remain insufficient to overcome the increasing burden of antimicrobial resistance (WHO, 2024). Natural products have long played a central role in drug discovery, serving as a rich source of structurally diverse and biologically active compounds. The relevance of natural products in antibacterial drug discovery is evident from the analysis of FDA-approved antibacterial agents between 1981 and 2019, in which 78 of the 162 approved agents (48%) were classified as natural products or natural product derivatives, underscoring the significant contribution of natural scaffolds to the development of clinically useful antibacterials. In particular, plant-derived secondary metabolites, including terpenoids, flavonoids and other phenolic compounds, have exhibited promising antimicrobial properties against a broad spectrum of microorganisms. These effects are mediated through multiple mechanisms, such as disruption of membrane integrity, inhibition of key enzymatic pathways, modulation of virulence factors, and suppression of biofilm formation (Newman and Cragg, 2020; Das and Ruhal, 2025). Moreover, several of these metabolites have been reported to act synergistically with conventional antimicrobial agents, thereby enhancing their efficacy against resistant pathogens (Hemaiswarya et al., 2008).

The genus *Eugenia* (Myrtaceae) comprises 407 species in Brazil and is widely distributed throughout the Atlantic Forest, Amazon Rainforest, and Cerrado biomes. Species of this genus are predominantly shrubs or trees and represent an important component of Brazilian biodiversity (Jardim Botânico do Rio de Janeiro, 2026). Beyond their ecological significance, *Eugenia* species are also recognized for their economic value. Several species produce edible fruits rich in nutrients and bioactive compounds, which are consumed fresh or processed into juices, pulps, and other food products. Notable examples include pitanga (*Eugenia uniflora*), uvaia (*Eugenia pyriformis*), cagaita (*E. dysenterica*), and araçá-boi (*E. stipitata*) (de Araujo et al., 2019).

Species of this genus are recognized for producing flavonoids, tannins, triterpenes, phenolic acids, and essential oils rich in monoterpenes and sesquiterpenes, metabolites that have been associated with a wide range of biological activities, including antibacterial and antifungal properties (Apel et al., 2002; Magina et al., 2009; Mendes et al., 2018; da Silva et al., 2022). Extracts, fractions, and essential oils obtained from species such as *E. astringens*, *E. brasiliensis*, *E. involucrata* and *E. uniflora* have shown antibacterial activity against different bacterial pathogens (Faqueti et al., 2015; Pereira et al., 2017; Toledo et al., 2023). Despite the promising antimicrobial properties reported for several *Eugenia* species, studies addressing their effects on biofilm formation and their synergistic interactions with clinically relevant antimicrobial agents remain limited. Moreover, knowledge regarding their activity against clinically important microorganisms, underlying mechanisms of action, and therapeutic potential is still insufficient for many Brazilian species of this genus. These knowledge gaps highlight the need for further investigations aimed at exploring the antimicrobial potential of *Eugenia*-derived natural products and their possible applications in combating resistant infections.

Therefore, the present study aimed to comprehensively investigate the antimicrobial potential of extracts and fractions obtained from *Eugenia* species against clinically relevant bacterial pathogens, as well as to characterize the phenolic compounds present in the crude hydroethanolic extracts of the most active species. In addition, the interactions of these samples with clinically used antimicrobial agents, their ability to inhibit biofilm formation, cytotoxicity, and potential mechanisms underlying their antimicrobial effects were investigated. The selected *Eugenia* species are endemic to Brazil and occur in the Atlantic Forest biome of Santa Catarina state, yet they remain poorly investigated regarding their phytochemical composition and

antimicrobial properties, highlighting their potential for bioprospecting studies. Unlike previous investigations on *Eugenia* species that have primarily focused on antimicrobial screening or isolated chemical characterization, the present study combines phytochemical profiling, antibiofilm activity, bacterial growth kinetics, assessment of membrane permeability, synergistic interactions with clinically relevant antibiotics, and cytotoxicity evaluation, providing a comprehensive assessment of their antibacterial potential and contributing to the elucidation of their mechanisms of action.

2 Materials and methods

2.1 Plant material collection and identification

Plant material from *Eugenia mattosii* D. Legrand, *Eugenia astringens* Cambess., *Eugenia catharinensis* D. Legrand, *Eugenia involucrata* DC., *Eugenia brasiliensis* Lam. and *Eugenia platysema* O. Berg were collected as described in Supplementary Table S1. Botanical identification was performed by Dr. André Luis de Gasper, and voucher specimens were deposited in the Roberto Miguel Klein Herbarium (FURB). Access to the Brazilian genetic heritage was registered in the National System for the Management of Genetic Heritage and Associated Traditional Knowledge (SISGEN) under registration number A7519AA.

2.2 Preparation of crude extracts and fractions

The plant materials were dried at room temperature and ground using a knife mill. The powdered samples were extracted by maceration in 70% hydroethanolic solutions for 7 days. Subsequently, the extracts were filtered and concentrated under reduced pressure using a rotary evaporator to obtain the crude hydroethanolic extract (EBH). After preparation, the extracts were stored at −20 °C until use.

The crude hydroethanolic extracts of the most active species were resuspended in 20% hydroethanolic solution, and stored under refrigeration for 12 h. The resulting precipitate was separated by filtration to obtain the insoluble fraction (FI). The supernatant was subsequently subjected to liquid–liquid partitioning using solvents of increasing polarity, yielding dichloromethane (DCM), ethyl acetate (FAE), and aqueous (FA) fractions. The resulting fractions were concentrated under reduced pressure and stored under refrigeration until further analyses.

2.3 Chemical characterization of extracts and fractions of *E. astringens* and *E. mattosii*

2.3.1 Phenolic compound analysis by HPLC-ESI-MS/MS

The HPLC-ESI-MS/MS analysis was performed using the sample pre-treatment, chromatographic and mass spectrometer parameters previously described by Borges et al. (2024). The eluent was formed by mixing solvents A (MeOH/H_2O in ratio of 95:5, v/v) and B (H_2O/formic acid 0.1%) as follows: 1st stage – 10% solvent A and 90% B (isocratic mode) for 5 min; 2nd stage–linear gradient of solvents A and B (from 10% to 90% of A) for 2 min; 3rd stage – 90% solvent A and 10% B (isocratic mode) for 3 min; 4th stage–linear gradient of solvents A and B (from 90% to 10% of A) for 7 min with a flow rate of 250 µL/min of mobile phase. In all analyses, the injected volume was 5 uL.

EBH of *E. astringens* and *E. mattosii* was prepared by dissolving 50 mg of dried material in a 5 mL solution of hydrochloric acid at pH 2. This solution was extracted three times with 2 mL of ethyl ether and the ethereal extract resulting of three extractions were combined, dried and stored in a sealed container at −20 °C. Prior to analysis, the dried material was dissolved in 1 mL of MeOH and centrifuged at 14,000 rpm for 5 min, being dissolved in 10 parts of ultrapure water.

For the identification and quantification, 39 standard phenolic compounds were analyzed under the same conditions described above, being them: abscisic acid, 4-aminobenzoic acid, 4-hydroxymethylbenzoic acid, apigenin, caffeic acid, catechin, chlorogenic acid, chrysin, cinnamic acid, coniferaldehyde, eriodictyol, ferulic acid, fustin, gallic acid, hispidulin, isoorientin, isoquercetin, mandelic acid, methoxyphenylacetic acid, naringerin, p-anisic acid, p-coumaric acid, pinocembrin, protocatechuic acid, resveratrol, rosmarinic acid, rutin, salicylic acid, scopoletin, sinapaldehyde, sinapic acid, syringaldehyde, syringic acid, taxifolin, umbelliferone, 4-methylumbelliferone, vanillic acid, vanillin and vitexin dissolved in MeOH (0.02–6 µg mL^{-1}).

2.4 Microorganisms

Reference strains obtained from the American Type Culture Collection (ATCC) and clinical isolates were used in this study. The bacterial strains evaluated were *Staphylococcus aureus* ATCC 25923, *Escherichia coli* ATCC 25922, *Klebsiella pneumoniae* ATCC 4352, *Pseudomonas aeruginosa* ATCC 27853, *Salmonella* sp. (PNCQ), methicillin-resistant *S. aureus* (MRSA, clinical isolate), and oxacillin-resistant *Staphylococcus epidermidis* (clinical isolate). The clinical isolates were kindly provided and identified by the Verner Willrich Laboratory (Blumenau, Santa Catarina, Brazil).

2.5 Determination of minimum inhibitory concentration (MIC)

The antibacterial activity of the extracts and fractions was evaluated by the broth microdilution method according to the guidelines of the Clinical and Laboratory Standards Institute (CLSI, 2012). Bacterial strains were cultured on Brain Heart Infusion (BHI) or Mueller–Hinton (MH) agar for 24 h at 37 °C. Inocula were prepared in sterile saline solution and adjusted to a turbidity equivalent to the 0.5 McFarland standard.

The extracts and fractions were dissolved in dimethyl sulfoxide (DMSO) at an initial concentration of 20 mg mL^{-1} and subjected to two-fold serial dilutions (1000–7.81 µg mL^{-1}, final concentrations) in Mueller–Hinton broth. Subsequently, 10 µL of the bacterial inoculum was added to each well of a sterile 96-well microplate.

Gentamicin (40–0.31 µg mL^{-1}) was used as the positive control. Growth and sterility controls were also included. Following incubation for 24 h at 37 °C, 10 µL of a MeOH

solution of 2,3,5-triphenyltetrazolium chloride (TTC; 5 mg mL^{-1}) was added to each well. The plates were reincubated for 2 h, and the minimum inhibitory concentration (MIC) was defined as the lowest concentration capable of preventing the development of the red coloration indicative of bacterial growth.

2.6 Antibiofilm assays

2.6.1 Evaluation of biofilm formation by bacterial strains

The biofilm-forming ability of the bacterial strains was assessed according to the method described by Stepanović et al. (2004). Flat-bottom 96-well microplates were prepared with 180 µL of BHI broth and 20 µL of a bacterial suspension adjusted to the 0.5 McFarland standard. The plates were incubated at 37 °C for 48 h to promote biofilm development. After incubation, the contents of the wells were removed, and the wells were washed three times with sterile saline solution (0.9%). The adhered biofilms were fixed with methanol and subsequently stained with 0.1% crystal violet for 15 min. Excess stain was removed by washing with sterile saline solution, and the bound dye was solubilized with a solution of 33% of acetic acid. Absorbance was measured at 595 nm using a microplate reader. Wells containing only culture medium were used as the negative control (NC).

Based on the optical density (OD) values obtained from the bacterial biofilms, strains were classified as follows: OD ≤ ODNC, non-biofilm producer; ODNC < OD ≤ 2 × ODNC, weak biofilm producer; 2 × ODNC < OD ≤ 4 × ODNC, moderate biofilm producer; and OD > 4 × ODNC, strong biofilm producer. Bacterial strains classified as biofilm producers were subsequently used in the biofilm inhibition and biofilm eradication assays.

2.6.2 Eradication of biofilm formation

The effects of *E. astringens* and *E. mattosii* extracts and fractions on preformed biofilms were evaluated according to the method described by Teanpaisan et al. (2017), with slight modifications. Biofilm formation was performed as previously described in the biofilm-forming ability assay. Briefly, bacterial suspensions standardized to the 0.5 McFarland standard were incubated in BHI broth at 37 °C for 48 h to allow biofilm development.

After incubation, the culture medium containing planktonic cells was carefully removed, and the wells were washed three times with sterile saline solution (0.9%) to eliminate non-adherent cells. The preformed biofilms were then exposed to the extracts and fractions diluted in 200 µL of BHI broth at final concentrations ranging from 7.81 to 1,000 µg mL^{-1}. The plates were further incubated at 37 °C for 24 h.

Following incubation, the contents of the wells were removed, and the wells were washed three times with sterile saline solution (0.9%). The remaining biofilms were fixed with methanol and stained with 0.1% crystal violet for 15 min. Excess stain was removed by washing with sterile saline solution, the plates were air-dried, and the bound dye was solubilized with a solution of 33% of acetic acid. Wells containing DMSO instead of the test samples were used as untreated controls. Sample blanks were prepared using culture medium and the corresponding concentrations of the extracts or fractions, without bacterial inoculation. Biofilm was quantified by measuring absorbance at 595 nm using a microplate reader. The percentage of biofilm eradication was calculated according to the following equation: % eradication = $[1 - (Abs_{595}$ sample/Abs_{595} untreated control)] × 100.

2.6.3 Inhibition of biofilm formation

The effects of *E. astringens* and *E. mattosii* extracts and fractions on biofilm formation were evaluated according to the method described by Teanpaisan et al. (2017), with slight modifications. Briefly, two-fold serial dilutions of the samples were prepared to obtain final concentrations ranging from 7.81 to 1,000 µg mL^{-1}.

Bacterial suspensions were prepared in Brain Heart Infusion (BHI) broth and adjusted to the 0.5 McFarland standard (approximately 1 × 10^8 CFU mL^{-1}). Subsequently, 20 µL of the bacterial suspension, 20 µL of the test sample, and 180 µL of BHI broth were added to each well of a sterile 96-well microplate. Wells containing bacterial suspension and culture medium without sample were used as untreated controls. The plates were incubated at 37 °C for 48 h to allow biofilm formation. After incubation, the contents of the wells were carefully removed, and the wells were washed three times with sterile saline solution (0.9%) to remove non-adherent cells. The remaining biofilms were fixed with methanol and stained with 0.1% crystal violet for 15 min. Excess stain was removed by washing with sterile saline solution, and the bound dye was subsequently solubilized with a solution of 33% of acetic acid. Biofilm was quantified by measuring absorbance at 595 nm using a microplate reader. The percentage of biofilm inhibition was calculated according to the following equation: % inhibition = $[1 - (Abs_{595}$ sample /Abs_{595} untreated control)] × 100.

2.7 Time-kill assay

Bacterial growth kinetics were evaluated according to the methodology described by Bianco et al. (2017), with minor modifications. Samples exhibiting minimum inhibitory concentration (MIC) values lower than 125 µg mL^{-1} were selected for this assay. The selected samples were tested at concentrations corresponding to the MIC and 2 × MIC, dissolved in DMSO.

Bacterial suspensions were adjusted to the 0.5 McFarland standard and subsequently diluted in MH broth to obtain a final inoculum of approximately 1 × 10^5 cells mL^{-1}. Flat-bottom 96-well microplates were prepared by adding 190 µL of MH broth containing the bacterial inoculum and 10 µL of the test sample. Wells containing DMSO instead of the test sample were used as negative controls. The assay was performed in triplicate, and the plates were incubated at 37 °C.

Bacterial growth was monitored by measuring the optical density at 600 nm (OD_{600}) after 0, 2, 4, 6, 12, and 24 h of incubation. The percentage of bacterial growth inhibition was calculated according to the following equation: % Inhibition = $[1 - ((OD_{600}$ S 24 h − OD_{600} S 0 h)/(OD_{600} NC 24 h − OD_{600} NC 0 h))] × 100, where OD_{600} S represents the optical density of the samples and OD_{600} NC represents the optical density of the negative control.

2.8 Evaluation of synergistic effects by the checkerboard assay

The interaction between extracts, fractions, and antimicrobial agents was evaluated using the checkerboard microdilution method, as described by Kumar et al. (2012). Stock solutions of the antimicrobial agents and test samples were prepared in DMSO. Sample concentrations were established based on the minimum inhibitory concentration (MIC) values previously determined for each microorganism and were tested at final concentrations ranging from 0.25 × MIC to 4 × MIC. Ampicillin and vancomycin were evaluated at concentrations ranging from 3.125 to 50 μg mL^{-1}.

Bacterial suspensions were prepared from fresh cultures grown on Mueller–Hinton agar. Isolated colonies were transferred to sterile saline solution and adjusted to the 0.5 McFarland standard, corresponding to approximately 1 × 10^8 CFU mL^{-1}. The assays were performed in sterile 96-well microplates using MH broth as the culture medium. Additional wells were included for determination of the MIC of the antimicrobial agent alone, the MIC of the test sample alone, sterility control, and bacterial growth control. The plates were incubated at 37 °C for 24 h. Subsequently, 10 μL of a MeOH solution of 2,3,5-triphenyltetrazolium chloride (TTC; 5 mg mL^{-1}) was added to each well to assess bacterial growth. The plates were reincubated at 35 °C until the development of a red coloration in wells containing viable cells.

The MIC was defined as the lowest concentration capable of preventing visible bacterial growth, as indicated by the absence of red coloration following TTC addition. The interaction between the test samples and antimicrobial agents was assessed by calculating the fractional inhibitory concentration index (FICI) according to the following equation: FICI = (MIC of the sample in combination/MIC of the sample alone) + (MIC of the antimicrobial agent in combination/MIC of the antimicrobial agent alone). Based on the FICI values, interactions were classified as synergistic (FICI ≤0.5), additive (0.5 < FICI ≤1.0), indifferent (1.0 < FICI ≤2.0), or antagonistic (FICI >2.0) (Kumar et al., 2012).

2.9 Evaluation of bacterial membrane permeability by the SYTOX green fluorescence assay

Bacterial membrane permeability was evaluated using the DNA-intercalating fluorescent dye SYTOX Green. Bacterial inocula were prepared and adjusted to an OD_{600} of 0.3. The inocula (50 μL) were distributed into black 96-well microplates. EBH, FI, DCM, FAE and FA from *E. astringens* and *E. mattosii* were evaluated. All samples were tested at a subinhibitory concentration of 2 μg mL^{-1}. Polymyxin B was used as a positive control at concentrations of 100 and 200 μg mL^{-1}, while phosphate-buffered saline (PBS) was used as the negative control.

The plates were incubated for 1 h, after which 100 μL of SYTOX Green was added to each well to achieve a final concentration of 1 μM. Following an additional 1h incubation period, fluorescence was measured using a Cytation 1 microplate reader (BioTek®, Winooski, VT, United States) and Gen5™ 3.11 software. Fluorescence was recorded using excitation and emission wavelengths of 485 and 528 nm, respectively.

To correct background fluorescence and the intrinsic fluorescence of the extracts and fractions, fluorescence measurements were performed before SYTOX Green addition and subsequently subtracted from the final readings. All experiments were performed in triplicate and fluorescence values were expressed as mean ± standard deviation of three independent experiments. according to the methodology described by Yuen et al. (2023).

2.10 Evaluation of cytotoxic effects on RAW 264.7 macrophages

The cytotoxicity of the extracts and fractions was evaluated using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) reduction assay, according to Chen (2011). Murine macrophage RAW 264.7 cells were cultured in Dulbecco's Modified Eagle Medium (DMEM) containing high glucose and supplemented with 10% fetal bovine serum (FBS), gentamicin sulfate (50 mg mL^{-1}), and amphotericin B (25 μg mL^{-1}). Cells were maintained at 37 °C in a humidified atmosphere containing 5% CO_2.

For the cytotoxicity assay, cells were seeded into sterile 96-well plates at a density of 1 × 10^5 cells per well and incubated for 4 h to allow cell adhesion. After this period, the cells were treated with the extracts and fractions at concentrations ranging from 500 to 7.8 μg mL^{-1}. DMSO was used as cytotoxic control, whereas cells maintained in culture medium alone served as cell viability control.

Following 21 h of treatment, 10 μL of MTT solution was added to each well to achieve a final concentration of 0.5 mg mL^{-1}. The plates were incubated for an additional 3 h at 37 °C in a humidified atmosphere containing 5% CO_2 to allow formazan crystal formation. Subsequently, the supernatant was carefully removed, and the resulting formazan crystals were dissolved in 200 μL of DMSO. Absorbance was measured at 600 nm using a microplate reader. Cell viability was expressed as a percentage relative to the untreated control cells, which were considered 100% viable.

2.11 Statistical analysis

Statistical analyses were performed using GraphPad Prism software version 9.0 (GraphPad Software, San Diego, CA, United States). Differences among experimental groups were evaluated by one-way analysis of variance (ANOVA), followed by Tukey or Dunnet *post hoc* tests. Results were considered statistically significant when $p < 0.05$.

3 Results and discussion

The antimicrobial activity of the EBH of *Eugenia* species evaluated in this study is summarized in Supplementary Table S2. Overall, the hydroethanolic extracts exhibited selective antibacterial activity against Gram-positive bacteria, while showing limited or no activity against the Gram-negative strains tested. This selectivity may be associated with the intrinsic differences in the cell envelope architecture of Gram-negative bacteria, whose outer membrane, rich in lipopolysaccharides, constitutes an additional permeability barrier that restricts the uptake of several bioactive compounds, including plant-derived

TABLE 1 Chromatographic parameters and concentrations of compounds identified in the hydroethanolic crude extracts (EBH; μg g^{-1}) of *Eugenia astringens* and *Eugenia mattosii* by HPLC-ESI-MS/MS.

Compound	*Rt*	Equation	R^2	*E. astringens* (μg g^{-1})	*E. mattosii* (μg g^{-1})
Gallic acid	3.03	y = 139215x + 26417	0.9929	542.40 ± 8.20	76.80 ± 17.40
Protocatechuic acid	4.42	y = 177242x + 8240.6	0.9942	13.00 ± 5.00	n.d.
Epicatechin	7.39	y = 23530x + 2594.8	0.9979	n.d.	533.20 ± 90.40
Catechin	7.50	y = 36330x + 930.64	0.9978	n.d.	359.80 ± 10.80
Vanillic acid	8.29	y = 10887x + 2480.2	0.9923	n.d.	90.40 ± 1.00
Chlorogenic acid	8.32	y = 335434x − 7383.5	0.9936	n.d.	< LOQ
Syringic acid	8.94	y = 29298x + 562.97	0.9976	< LOQ	< LOQ
p-Coumaric acid	9.36	y = 650368x + 18135	0.9908	< LOQ	16.80 ± 1.20
Ferulic acid	9.77	y = 288143x + 8279	0.9910	n.d.	14.00 ± 0.20
Scopoletin	10.05	y = 175205x + 1035.1	0.9940	< LOQ	n.d.
p-Anisic acid	10.32	y= 44220x + 688	1.0000	< LOQ	n.d.
Abscisic acid	10.36	y = 95507x + 912.24	0.9989	80.40 ± 1.00	n.d.
Quercetin	11.02	y = 1888988x − 11214	0.9989	88.40 ± 9.00	270.00 ± 54.40
Pinocembrin	12.13	y = 360840x + 3149.6	0.9929	n.d.	45.60 ± 1.20
Galangin	12.41	y = 13843x + 1624.2	0.9912	< LOQ	144.00 ± 16.00

Rt, Retention time; LOQ, limit of quantification.

compounds. Moreover, the presence of efficient efflux systems can further decrease the intracellular accumulation of these metabolites, contributing to the reduced susceptibility of Gram-negative bacteria to natural products (Hemaiswarya et al., 2008; Silhavy et al., 2010). Among the species investigated, *E. astringens* was the most active, presenting the lowest MIC values against *S. aureus*, methicillin-resistant *S. aureus* (MRSA), and *S. epidermidis*. Similarly, *E. mattosii* displayed relevant antibacterial activity against Gram-positive bacteria. These findings suggest that the antibacterial potential of the evaluated *Eugenia* species is primarily directed toward Gram-positive pathogens.

The higher susceptibility of Gram-positive bacteria to the plant extracts is consistent with previous reports and can be largely explained by differences in bacterial cell wall architecture (Hemaiswarya et al., 2008; Silhavy et al., 2010). Gram-positive bacteria possess a thick peptidoglycan layer but lack an outer membrane, facilitating the diffusion of bioactive compounds to intracellular and membrane-associated targets. Conversely, Gram-negative bacteria are protected by an additional outer membrane enriched with lipopolysaccharides (LPS), which act as an efficient permeability barrier and restricts the entry of hydrophobic molecules and larger secondary metabolites (Hemaiswarya et al., 2008). Accordingly, the limited activity observed against *E. coli*, *K. pneumoniae*, *P. aeruginosa*, and *Salmonella* sp. Reinforces the importance of the outer membrane as a key determinant of intrinsic resistance to plant-derived antimicrobial compounds (Hemaiswarya et al., 2008; Silhavy et al., 2010).

Based on their antibacterial activity in the preliminary screening assays, *E. astringens* and *E. mattosii* were selected for further investigation. Chemical characterization of EBH by LC–MS/MS (Supplementary Figure S1) was performed to identify the major metabolites present in the extracts, followed by fractionation and evaluation of antibacterial activity against the most susceptible bacterial strains. The compounds identified in EBH by LC–MS/MS are listed in Table 1.

The chromatographic profile of EBH from *E. mattosii* revealed the presence of 11 phenolic compounds, including three simple phenolic acid derivatives, three hydroxycinnamic acid derivatives, three flavonoids, and two flavan-3-ols. Notably, the flavan-3-ols catechin and epicatechin were the major constituents identified in the extract, representing the most abundant class of compounds detected in *E. mattosii*.

Previous phytochemical investigations of this species have reported the occurrence of protocatechuic acid, *p*-coumaric acid, pinocembrin, galangin, eriodictyol, gallic acid, catechin, ellagic acid, chlorogenic acid, quercetin, hispidulin, myricitrin, epigallocatechin gallate, isoquercetin, and rutin (Tenfen et al., 2021), as well as pinostrobin and cryptostrobin (Vechi et al., 2021). The present results are in agreement with these previous reports, confirming the occurrence of several phenolic compounds and flavonoids already described for the species.

In addition, the present study expands the phytochemical knowledge of *E. mattosii* by reporting, for the first time in this species, the presence of epicatechin, vanillic acid, and ferulic acid. These compounds have been widely associated with biological activities, including antioxidant, anti-inflammatory, and antimicrobial effects, and may contribute to the antibacterial activity observed for *E. mattosii*. The identification of these metabolites further highlights the chemical diversity of the species and reinforces its potential as a source of bioactive phenolic compounds.

TABLE 2 Minimum inhibitory concentration (μg mL^{-1}) of the EBH and fractions obtained from *Eugenia astringens* and *Eugenia mattosii* against Gram-positive bacterial strains.

Strains	EBH	FI	DCM	FAE	FA	Gentamicin*	Ampicillin*	Vancomycin*
Eugenia astringens								
S. aureus 25923	15.62	7.81	15.62	62.5	31.25	31.25	0.125	3.12
S. aureus MRSA	15.62	7.81	15.62	62.5	31.25	>1000	500	1.56
S. epidermidis	7.81	15.62	15.62	31.25	7.81	>1000	15.62	6.25
Eugenia mattosii								
S. aureus 25923	125	62.5	1000	125	125	31.25	0.125	3.12
S. aureus MRSA	125	125	1000	62.5	125	>1000	500	1.56
S. epidermidis	125	125	1000	125	125	>1000	15.62	6.25

EBH, crude hydroethanolic extract; FAE, ethyl acetate fraction; DCM, dichloromethane fraction; FI, insoluble fraction; FA, aqueous fraction.
*Positive controls.

The chromatographic profile of EBH from *E. astringens* revealed the presence of nine compounds, including four simple phenolic acid derivatives, one hydroxycinnamic acid derivative, one coumarin, two flavonoids, and one terpenoid. Gallic acid was the major constituent identified in the extract. Previous phytochemical investigations of *E. astringens* have predominantly reported the occurrence of terpenoid and meroterpenoid constituents. Goldoni et al. (2019) identified the phloroglucinol-derived meroterpenoids eugenial C, eugenial D, and eugenial E in leaf extracts of the species. In the fruits, six major anthocyanins were identified, namely delphinidin 3-O-β-glucopyranoside, cyanidin 3-O-β-glucopyranoside, petunidin 3-O-β-glucopyranoside, pelargonidin 3-O-β-glucopyranoside, peonidin 3-O-β-glucopyranoside, and malvidin 3-O-β-glucopyranoside (Kuskoski et al., 2003). Studies on the leaves also reported the occurrence of the triterpenes taraxerol, α-amyrin, β-amyrin, betulin, and betulinic acid (Meyre-Silva et al., 2009). More recently, analyses of the hexane fraction of the leaves identified the sesquiterpenes caryophyllene oxide and β-selinene (Santiago-Carvalho et al., 2022), as well as α-guaiene and italicene (Almeida et al., 2025). Taken together, previous phytochemical studies on *E. astringens* have mainly reported terpenoids, meroterpenoids, and anthocyanins. In contrast, none of the phenolic compounds identified in the present study were found in the available literature for this species, expanding the current knowledge of its phytochemical composition.

The antibacterial activities of the fractions of *E. astringens* and *E. mattosii* are presented in Table 2.

Following the fractionation of *E. astringens*, an increase in antibacterial activity was observed in some fractions to *S. aureus* strains, particularly the less polar fractions. This finding suggests that the metabolites responsible for the antibacterial activity exhibit affinity for solvents of low to intermediate polarity and became concentrated during the partitioning process. Previous phytochemical studies have reported the presence of meroterpenoids in *E. astringens*, consisting of a phloroglucinol moiety coupled to a sesquiterpene skeleton. Among these compounds, eugenial C and eugenial D were isolated from the dichloromethane fraction of the fruits and exhibited potent antibacterial activity against *S. aureus* and methicillin-resistant *S. aureus* (MRSA) (Faqueti et al., 2015). These compounds were also identified in the methanolic extract of *E. astringens* leaves (Goldoni et al., 2019). The antibacterial potential of *E. astringens* is further supported by previous studies reporting the antimycobacterial activity of its n-hexane fraction. This fraction was able to inhibit the growth of mycobacterial strains both in culture media and within infected macrophages, highlighting the promising antimicrobial potential of this species against pathogenic bacteria, including *Mycobacterium* spp. (Santiago-Carvalho et al., 2022). The low MIC values observed for the less polar fractions may indicate that fractionation promoted the enrichment of active constituents, thereby enhancing antibacterial activity. However, FAE and FA fractions also exhibited relevant antibacterial effects, suggesting that more polar metabolites may additionally contribute to the observed activity. These findings indicate that the antibacterial properties of *E. astringens* are likely associated with multiple classes of bioactive compounds distributed across fractions of different polarities.

Several studies have reported that more polar plant-derived metabolites, especially phenolic compounds and flavonoids, exhibit greater antibacterial activity against Gram-positive bacteria, largely due to the absence of the protective outer membrane found in Gram-negative organisms. These compounds can act through multiple mechanisms, including disruption of membrane structure and function, increased cellular permeability, inhibition of key enzymatic pathways, and interference with essential metabolic processes, ultimately impairing bacterial viability and proliferation (Cushnie and Lamb, 2011; Liu et al., 2025). Thus, the occurrence of compounds belonging to different chemical classes highlights the chemical complexity of *E. astringens* and suggests that the antibacterial activity observed may result from the combined action of multiple bioactive metabolites.

3.1 Antibiofilm assays

Staphylococcus aureus, Methicillin-resistant *S. aureus* (MRSA), and *S. epidermidis* were the most susceptible strains in the antibacterial assays, thus, their ability to form biofilms was further investigated. *Staphylococcus aureus* exhibited the highest biofilm-forming capacity and was classified as a strong biofilm

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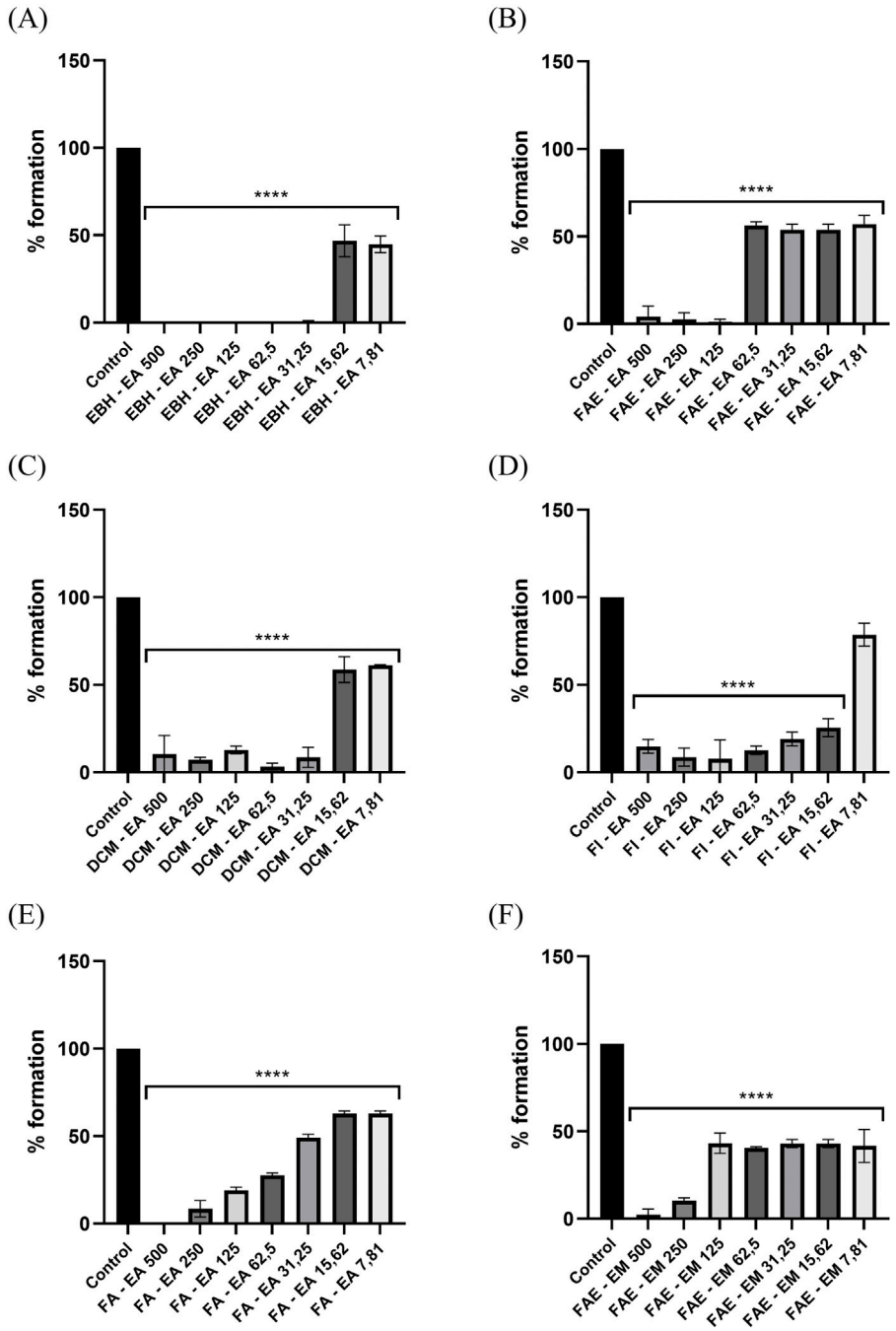


FIGURE 1
Percentage inhibition of MRSA biofilm formation following exposure to extracts and fractions from *E. astringens* and *E. mattosii* at different concentrations (μg mL^{-1}). **(A)** *E. astringens* HCE, **(B)** *E. astringens* EA, **(C)** *E. astringens* DCM, **(D)** *E. astringens* IF, **(E)** *E. astringens* FA, and **(F)** *E. mattosii* EAF. Data are expressed as mean ± standard deviation (SD). Significant differences compared with the control group were determined by one-way ANOVA followed by Tukey's multiple comparison test (*p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001).

producer (OD = 0.379), whereas *S. epidermidis* was classified as a moderate biofilm producer (OD = 0.207). The MRSA isolate displayed weaker biofilm formation, with an OD value of 0.099, and was therefore classified as a weak biofilm producer (negative control = 0.071). Based on their susceptibility to the extracts and fractions, *S. aureus*, MRSA, and *S. epidermidis* were selected for further biofilm studies. Samples exhibiting MIC values below 125 μg mL^{-1} were subsequently evaluated for their ability to inhibit biofilm formation and eradicate preformed biofilms. The results of the biofilm inhibition assays are shown in Figures 1–3, while the effects on preformed biofilm eradication are presented in Figures 4–6.

The biofilm inhibition assays demonstrated that most extracts and fractions were effective at low concentrations

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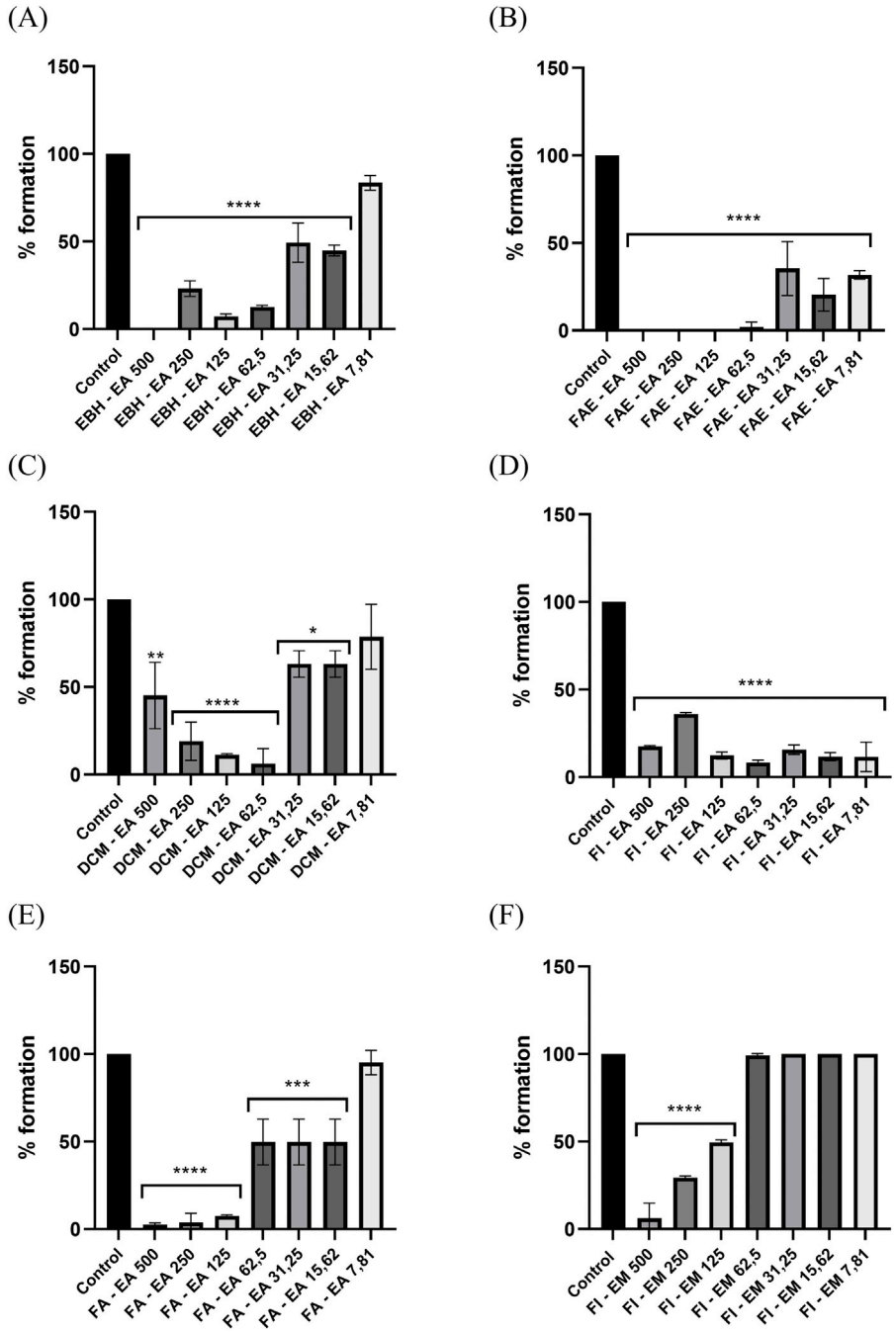


FIGURE 2
Percentage inhibition of *Staphylococcus aureus* biofilm formation following exposure to extracts and fractions from *E. astringens* and *E. mattosii* at different concentrations (μg mL^{-1}). **(A)** *E. astringens* EBH, **(B)** *E. astringens* EAF, **(C)** *E. astringens* DCM, **(D)** *E. astringens* IF, **(E)** *E. astringens* FA, and **(F)** *E. mattosii* IF. Data are expressed as mean ± standard deviation (SD). Significant differences compared with the control group were determined by one-way ANOVA followed by Tukey's multiple comparison test (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$).

against the evaluated staphylococcal strains (Figures 1–3). For MRSA, all tested samples significantly inhibited biofilm formation at concentrations as low as 7.81 μg mL^{-1}, with the exception of the FI of *E. astringens*, which became effective from 15.62 μg mL^{-1}. A similar pattern was observed for *S. epidermidis*, for which all samples inhibited biofilm formation from 7.81 μg mL^{-1}, except for the FA of *E. astringens*, which required 15.62 μg mL^{-1} to produce a significant effect. For *S. aureus*, most fractions of *E. astringens* inhibited biofilm formation from 15.62 μg mL^{-1}, whereas the FAE and FI fractions displayed greater activity, showing significant inhibition at 7.81 μg mL^{-1}. In contrast, the insoluble fraction of *E. mattosii* was less active, requiring a concentration of 125 μg mL^{-1} to significantly inhibit biofilm formation. Overall, the results indicate that the evaluated extracts and fractions possess a pronounced ability to prevent biofilm

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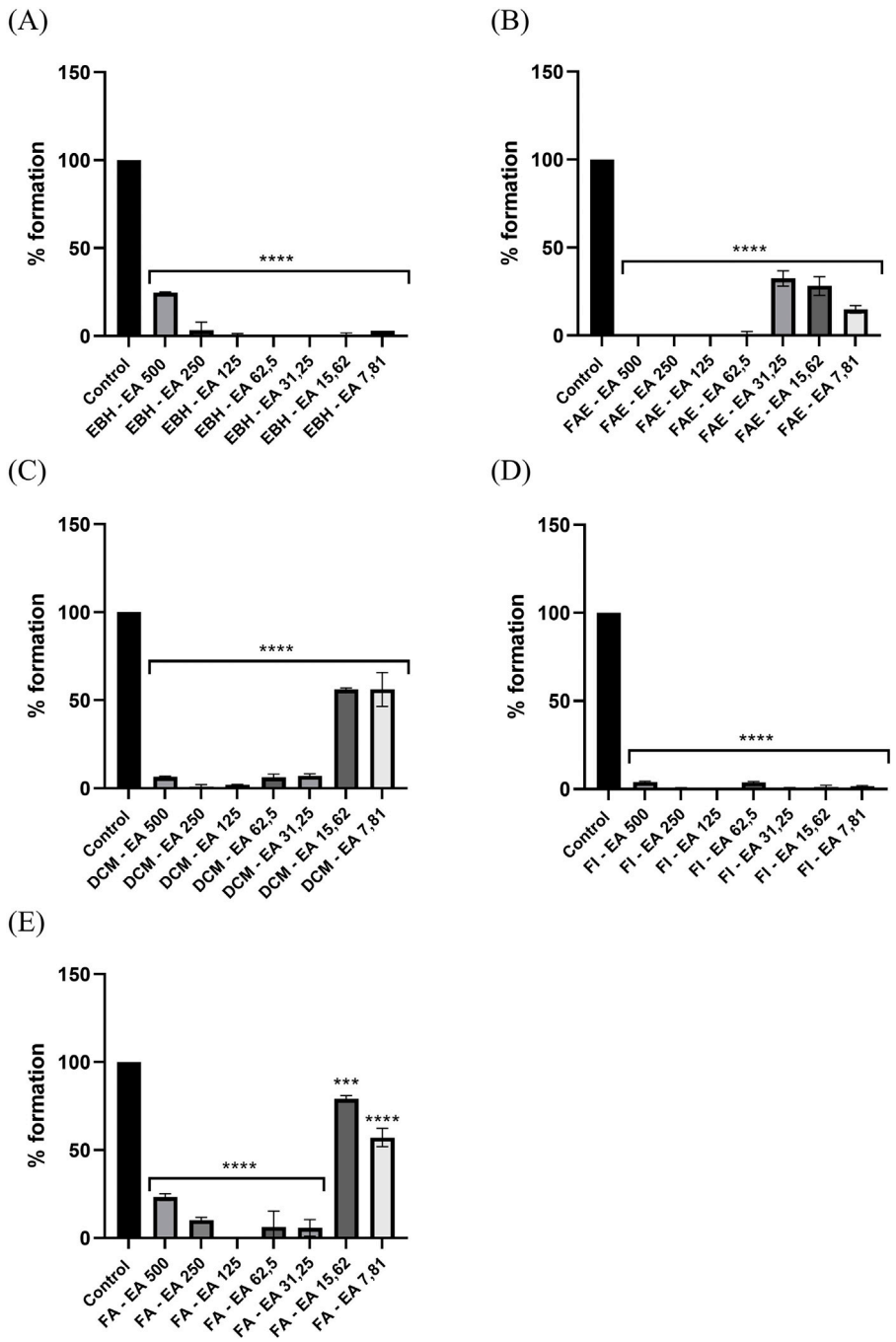


FIGURE 3
Percentage inhibition of *Staphylococcus epidermidis* biofilm formation following exposure to extracts and fractions from *E. astringens* at different concentrations (μg mL^{-1}). **(A)** *E. astringens* EBH, **(B)** *E. astringens* EAF, **(C)** *E. astringens* DCM, **(D)** *E. astringens* IF, and **(E)** *E. astringens* FA. Data are expressed as mean ± standard deviation (SD). Significant differences compared with the control group were determined by one-way ANOVA followed by Tukey's multiple comparison test (*p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001).

establishment, particularly against MRSA and *S. epidermidis*, with inhibitory effects observed at concentrations lower than those required to inhibit bacterial growth.

In the mature biofilm eradication assays, the most promising results were observed against MRSA (Figures 4–6). Among the tested samples, the hydroethanolic crude extract (EBH) of *E. astringens* significantly reduced biofilm biomass at concentrations of 125 μg mL^{-1} and above, while the dichloromethane fraction (DCM) was effective from 62.5 μg mL^{-1}. The ethyl acetate fraction (FAE) also exhibited antibiofilm activity, although only at the highest tested concentration (500 μg mL^{-1}). In contrast, no significant biofilm eradication was observed against *S. aureus* or *S. epidermidis*. Likewise, the fractions obtained from *E. mattosii*

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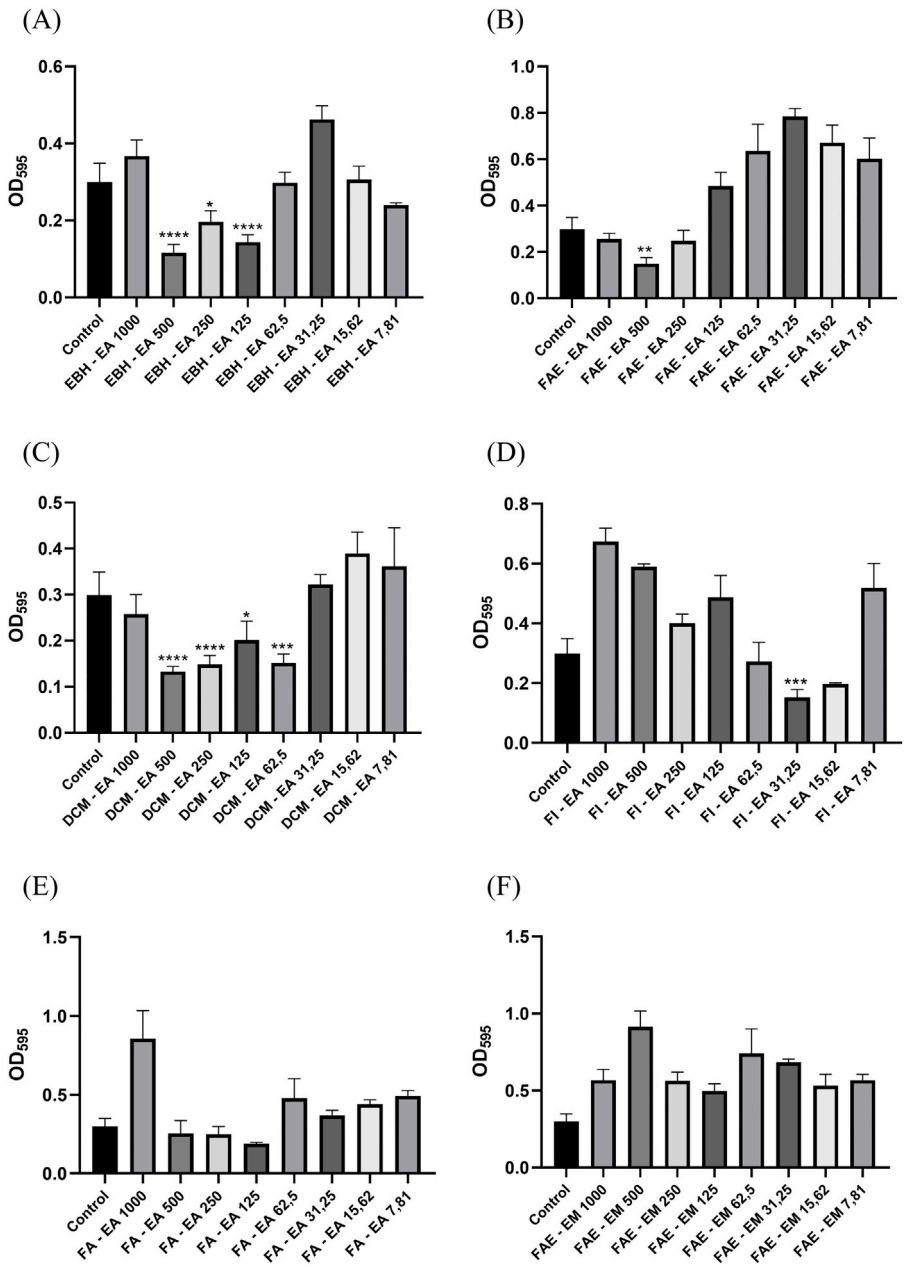


FIGURE 4
Eradication of MRSA biofilms following exposure to extracts and fractions from *E. astringens* and *E. mattosii* at different concentrations (μg mL^{-1}). **(A)** *E. astringens* (EBH), **(B)** *E. astringens* EAF, **(C)** *E. astringens* (DCM), **(D)** of *E. astringens* (FI), **(E)** *E. astringens* (FA), and **(F)** *E. mattosii* FAE. Biofilm eradication was assessed by measuring absorbance at 595 nm (OD_{595}). Data are expressed as mean ± standard deviation (SD). Significant differences compared with the control group were determined by one-way ANOVA followed by Tukey's multiple comparison test (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$).

did not produce relevant effects on mature biofilms under the conditions evaluated.

The greater susceptibility of MRSA biofilms observed in the present study may be related to the physicochemical properties of the phenolic compounds present in the extracts and fractions. According to Silva et al. (2016), phenolic compounds exhibiting higher antibiofilm activity against MRSA generally possess an enhanced ability to interact with lipophilic environments, although they do not necessarily fully penetrate the bacterial membrane. This behavior suggests that such compounds may partition into the lipid bilayer, altering its physicochemical properties and compromising membrane integrity. These interactions can affect membrane function and cellular homeostasis, thereby contributing to the increased susceptibility of MRSA biofilms to phenolic-rich plant extracts.

Interestingly, some extract and fraction concentrations resulted in an increase in the adhered biomass relative to the untreated control, whereas others promoted partial biofilm reduction. This variable response suggests that the antibiofilm activity is strongly influenced by the chemical composition of each extract and fraction, as well as by the susceptibility of the target microorganism.

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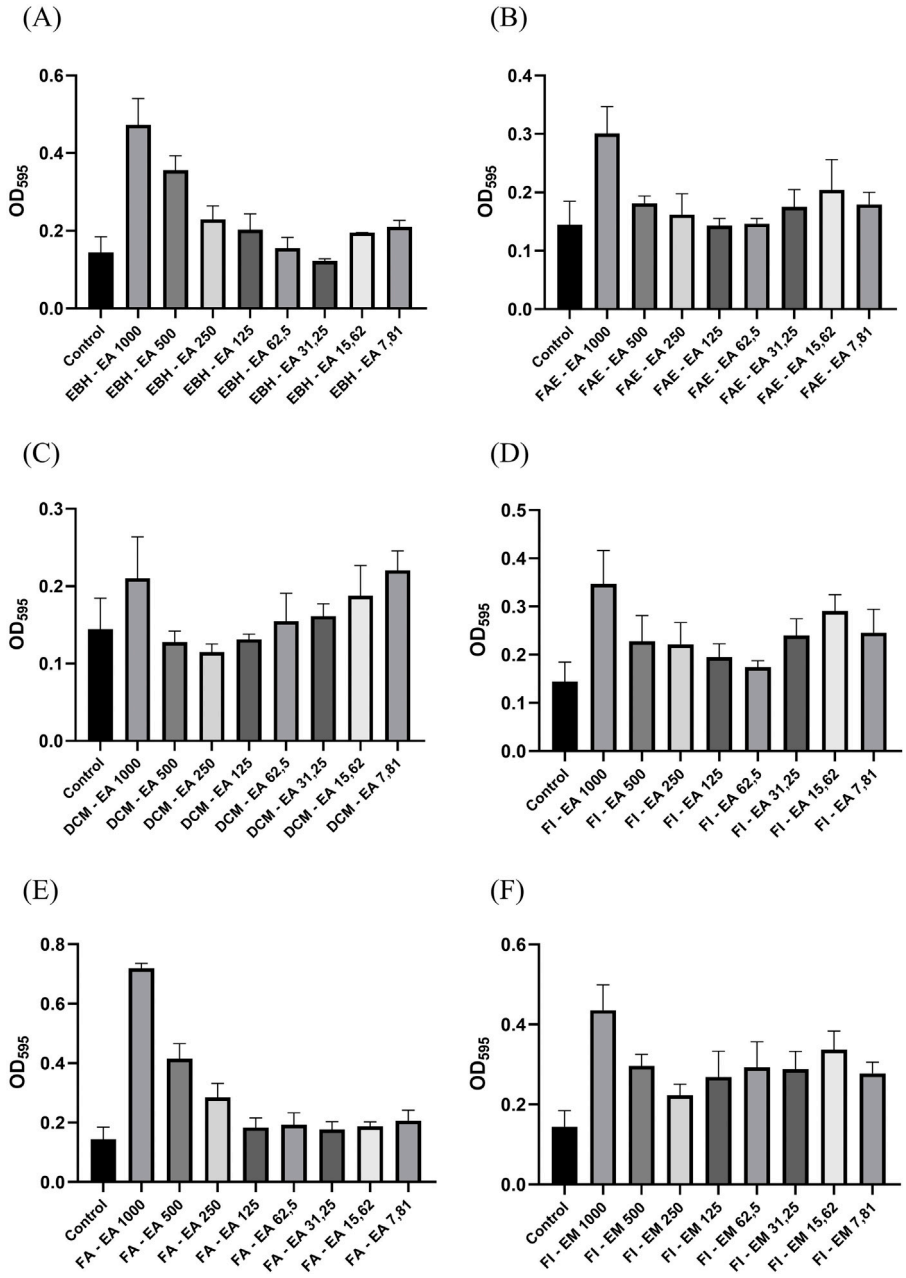


FIGURE 5
Eradication of *Staphylococcus aureus* biofilms following exposure to extracts and fractions from *E. astringens* and *E. mattosii* at different concentrations (μg mL^{-1}). **(A)** *E. astringens* EBH, **(B)** *E. astringens* FAE, **(C)** *E. astringens* DCM, **(D)** *E. astringens* FI, **(E)** *E. astringens* FA, and **(F)** *E. mattosii* FI. Biofilm eradication was assessed by measuring absorbance at 595 nm (OD_{595}). Data are expressed as mean ± standard deviation (SD). Significant differences compared with the control group were determined by one-way ANOVA followed by Tukey's multiple comparison test (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$).

Extracellular polymeric substances (EPS) constitute the extracellular matrix that surrounds microbial cells within biofilms, promoting adhesion, structural stability, and protection against antimicrobial agents. The extracellular polysaccharides present in EPS contribute to water retention and the formation of a highly hydrated matrix. As a result, hydrophilic compounds may interact with these matrix components, weakening the EPS network, compromising biofilm architecture, and facilitating the disruption of mature biofilms (Biharee et al., 2020). However, the increase in adhered biomass observed at certain concentrations may reflect adaptive responses of the biofilm to antimicrobial stress. Exposure to subinhibitory concentrations of bioactive compounds can induce structural reorganization and increased production of extracellular polymeric substances (EPS), resulting in enhanced biofilm formation and protection of embedded cells. This phenomenon has been described as a survival strategy that contributes to biofilm persistence under antimicrobial challenge (Lima-e-Silva et al., 2017; Salzer et al., 2020).

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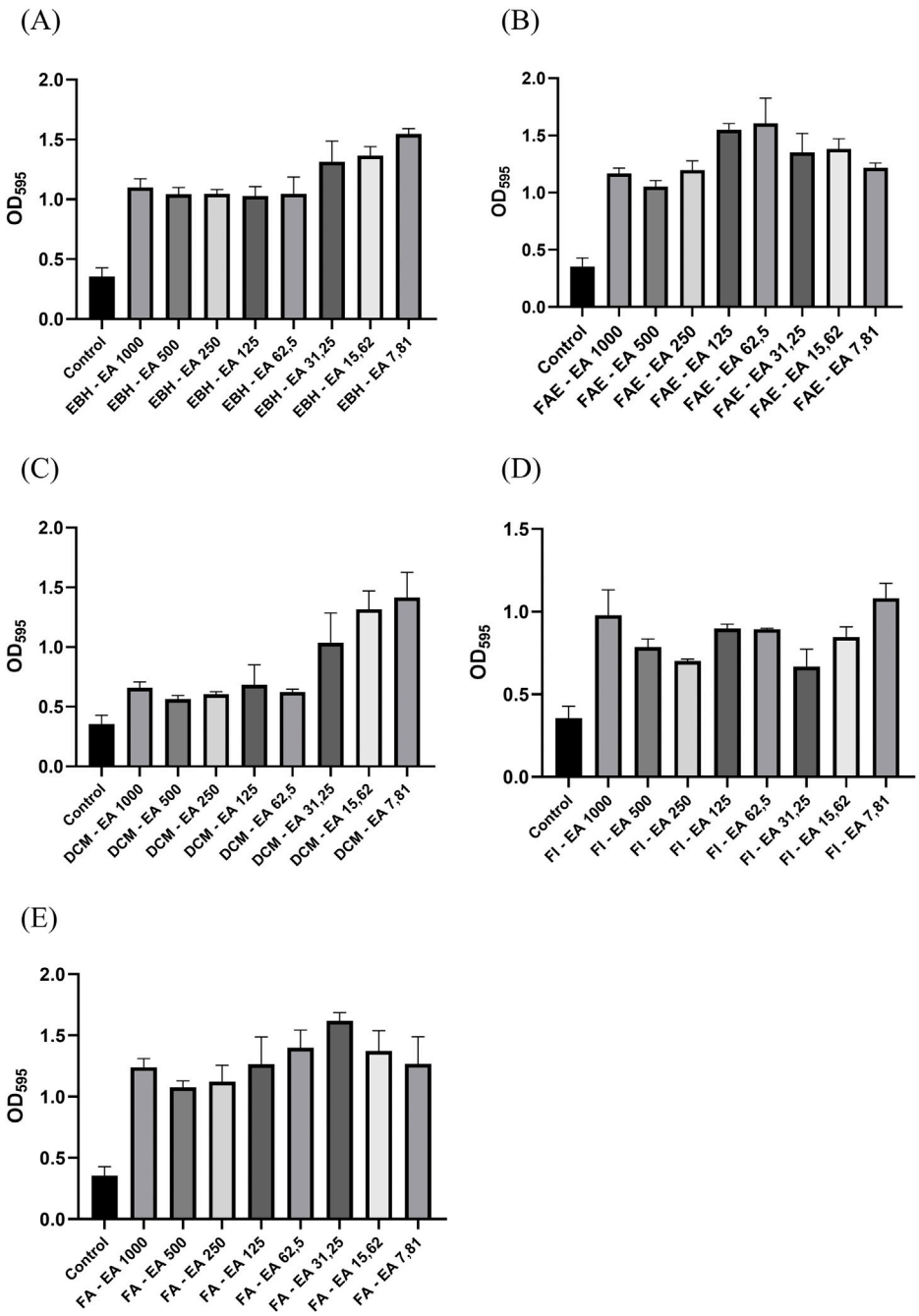


FIGURE 6
Eradication of *Staphylococcus epidermidis* biofilms following exposure to extracts and fractions from *E. astringens* at different concentrations (μg mL^{-1}). **(A)** *E. astringens* EBH, **(B)** *E. astringens* FAE, **(C)** *E. astringens* DCM, **(D)** *E. astringens* FI, and **(E)** *E. astringens* FA. Biofilm eradication was assessed by measuring absorbance at 595 nm (OD_{595}). Data are expressed as mean ± standard deviation (SD). Significant differences compared with the control group were determined by one-way ANOVA followed by Tukey's multiple comparison test (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$).

The ability of the extracts and fractions to reduce mature biofilms was observed predominantly against MRSA, suggesting a greater susceptibility of this strain to the bioactive compounds present in the samples. Phenolic compounds such as quercetin and gallic acid, which were detected in relevant amounts in *E. astringens*, have been reported to interfere with key mechanisms involved in biofilm formation and maintenance. These compounds are capable of reducing the expression of *sarA*, a major regulator of biofilm formation in *Staphylococcus* spp., decreasing the production of extracellular polymeric substances (EPS), and compromising the structural integrity of the biofilm matrix (Liu et al., 2024; Sang et al., 2024). The modulation of this global regulatory system suggests that phenolic compounds may act not only through direct antibacterial effects but also by interfering with molecular pathways associated with cell adhesion, extracellular matrix synthesis, and biofilm organization. Such mechanisms may

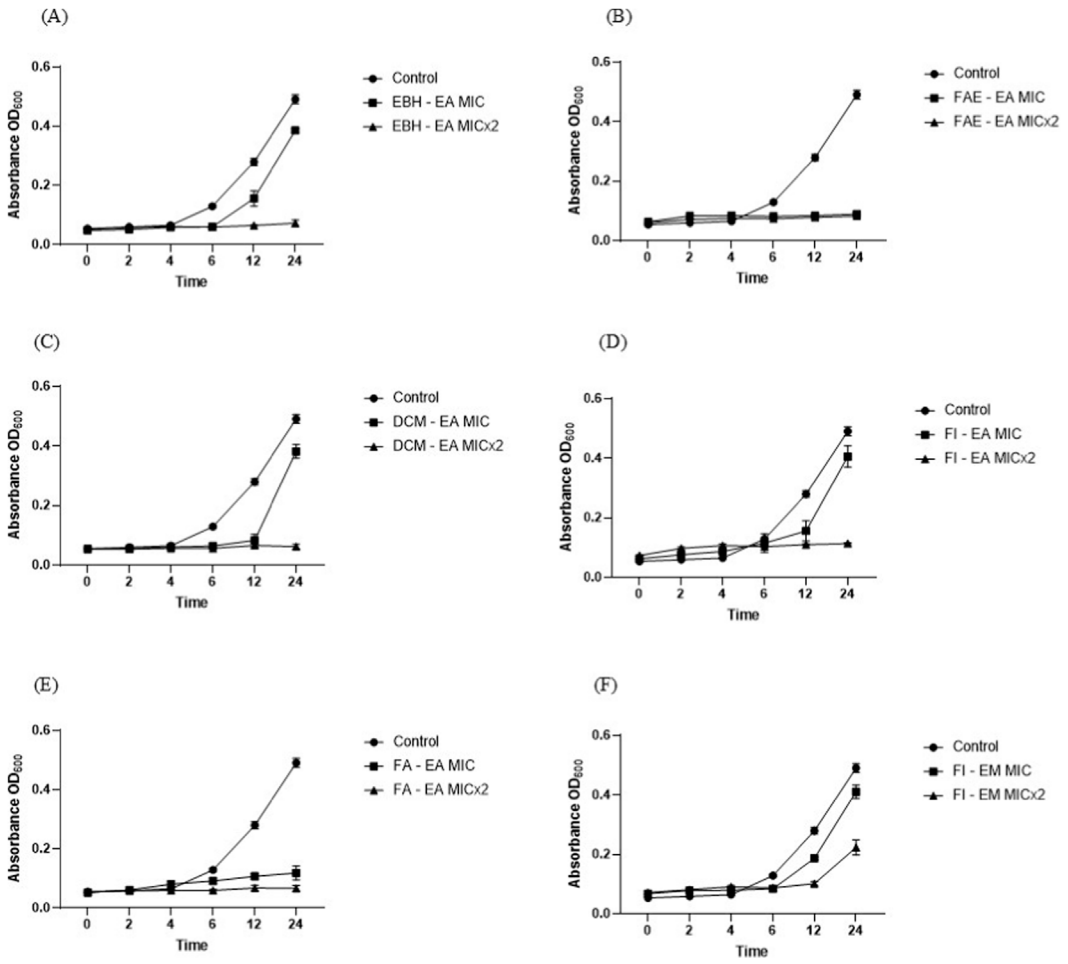


FIGURE 7
Bacterial growth curves (time–kill assay) of *Staphylococcus aureus* ATCC 25923 following exposure to *E. astringens* and *E. mattosii*. **(A)** *E. astringens* EBH, **(B)** *E. astringens* FAE, **(C)** *E. astringens* DCM, **(D)** *E. astringens* FI, **(E)** *E. astringens* FA, and **(F)** *E. mattosii* FI. Bacterial growth was assessed by measuring absorbance at 600 nm (OD_{600}) over a 24-h period, comparing the growth control with treatments at MIC and 2 × MIC concentrations. Data are expressed as mean ± standard deviation (SD).

contribute to the greater susceptibility of MRSA biofilms observed in the present study.

Otherwise, Badra et al. (2022) reported that, at higher concentrations, gallic acid was able to increase biofilm formation despite reducing the number of planktonic cells, suggesting that bacterial exposure to this compound may trigger adaptive stress responses. Under such conditions, subinhibitory antimicrobial effects and environmental stress can activate bacterial survival mechanisms, leading to enhanced production of extracellular matrix components and, consequently, increased biofilm formation. These findings highlight the complex relationship between phenolic compounds and biofilm development, in which the biological response may vary according to the concentration and exposure conditions of the microorganism (Badra et al., 2022).

3.2 Time-kill assays

Following the antibiofilm assays, the selected strains were evaluated for their growth kinetics in the presence of extracts and fractions from *E. astringens* and *E. mattosii*. Only samples exhibiting MIC values ≤125 μg mL^{-1} were included in this assay. Bacterial growth was monitored over a 24-h period by measuring optical density (OD_{600}), allowing the assessment of the effects of the samples on the growth dynamics of the tested microorganisms. The growth kinetics curves obtained for the evaluated strains are presented in Figures 7–9.

The growth kinetics assays revealed that, at 2 × MIC, all evaluated extracts and fractions inhibited the growth of *S. aureus* throughout the experimental period. A similar pattern was observed for MRSA, with the exception of the *E. mattosii* FAE, which did not maintain growth inhibition under the tested conditions. For *S. epidermidis*, only the low- and medium-polarity fractions of *E. astringens* (FAE, DCM, and FI) were able to consistently inhibit bacterial growth throughout the evaluated time intervals.

This effect was particularly evident for the DCM and FI fractions, which maintained reduced OD_{600} values over the entire incubation period. The growth curves obtained for these fractions showed limited bacterial recovery after the initial exposure phase, suggesting that fractionation promoted the concentration of

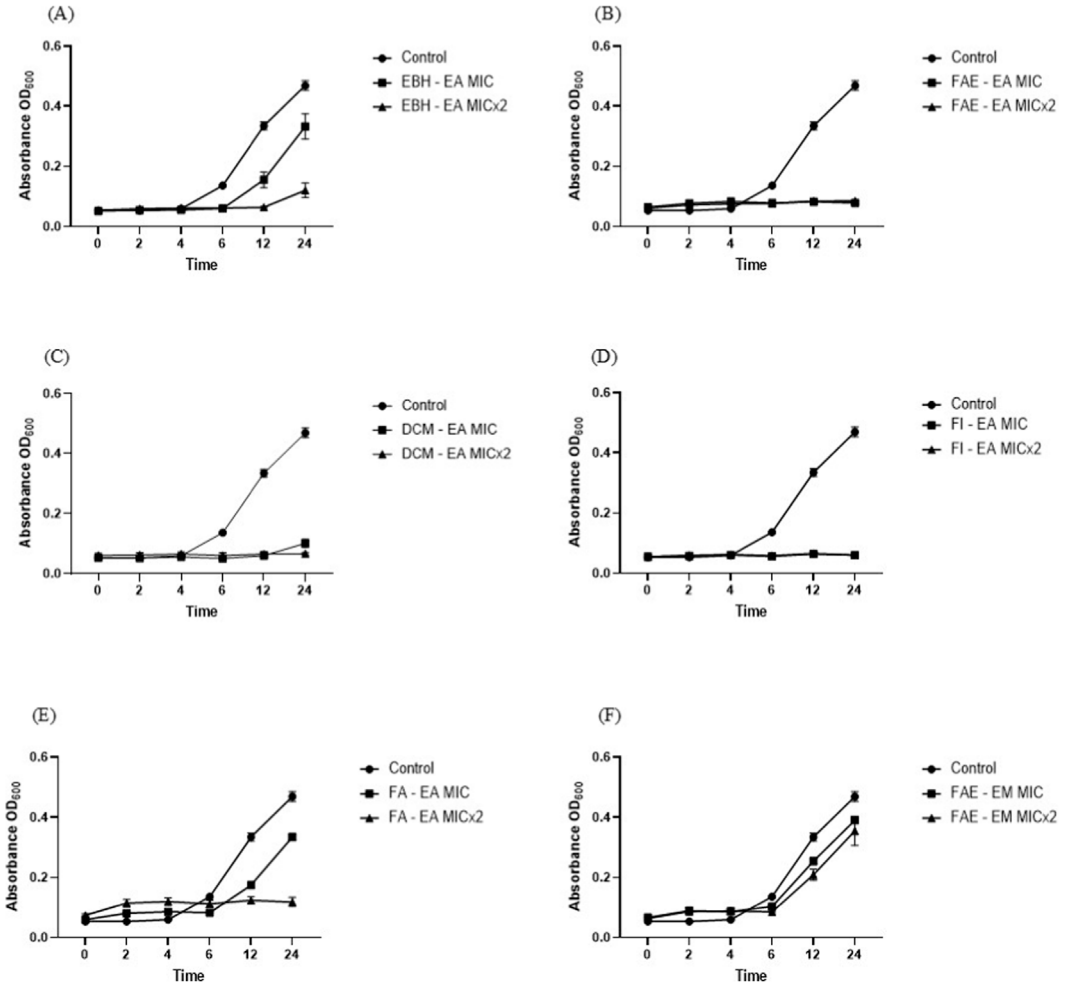


FIGURE 8
Bacterial growth curves (time–kill assay) of MRSA following exposure to *E. astringens* and *E. mattosii*. **(A)** *E. astringens* EBH, **(B)** *E. astringens* FAE, **(C)** *E. astringens* DCM, **(D)** *E. astringens* FI, **(E)** *E. astringens* FA, and **(F)** *E. mattosii* FAE. Bacterial growth was assessed by measuring absorbance at 600 nm (OD_{600}) over a 24-h period, comparing the growth control with treatments at MIC and 2 × MIC concentrations. Data are expressed as mean ± standard deviation (SD).

biologically active metabolites capable of exerting a more persistent inhibitory effect. The enhanced activity observed for these fractions may be associated with the enrichment of less polar compounds, which are frequently reported to affect bacterial viability through disruption of the cytoplasmic membrane, increased membrane permeability, and leakage of essential intracellular constituents (Bagatini et al., 2023). Terpenes, for example, exhibit a high affinity for lipid bilayers and may induce membrane disorganization, loss of cellular integrity, and alterations in bacterial homeostasis (Han et al., 2019; Kachur and Suntres, 2020; Sundaramoorthy et al., 2019).

The activity observed for the FAE fraction suggests an additional contribution from phenolic compounds of intermediate polarity, such as flavonoids, phenolic acids and hydrolyzable tannins, which have previously been reported in the investigated *Eugenia* species. These metabolites exhibit multitarget mechanisms of action, including membrane disruption, enzyme inhibition, and interference with biofilm formation (Keyvani-Ghamsari et al., 2023; Sang et al., 2024). Furthermore, flavonoids can interact with cell wall and membrane proteins, impairing essential cellular processes involved in bacterial growth and replication (Donadio et al., 2021). The combined presence of these metabolites may therefore contribute to the sustained antibacterial effects observed during the growth kinetics assays.

3.3 Evaluation of synergistic effects by the checkerboard assay

The potential interactions between extracts and fractions from *E. astringens* and *E. mattosii* and the antimicrobial agents ampicillin and vancomycin were evaluated using the checkerboard assay. The resulting fractional inhibitory concentration index (FICI) values were used to classify the interactions as synergistic (FICI ≤0.5), additive (0.5 < FICI ≤1.0), indifferent (1.0 < FICI ≤2.0), or antagonistic (FICI >2.0) (Kumar et al., 2012). The checkerboard assay revealed that the interactions between the extracts, fractions, and conventional antibiotics varied according to both the bacterial strain and the antimicrobial agent evaluated (Table 3).

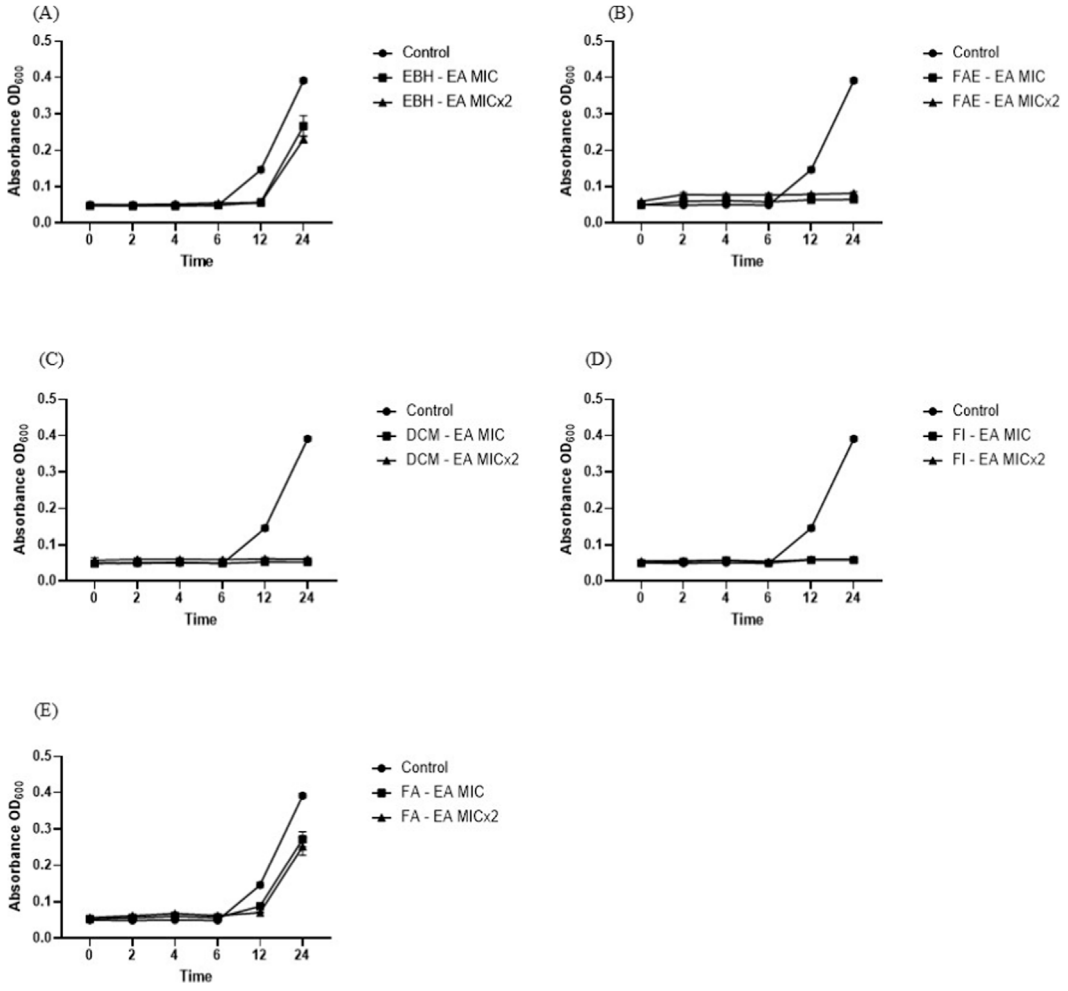


FIGURE 9
Bacterial growth curves (time–kill assay) of *Staphylococcus epidermidis* following exposure to *E. astringens*. **(A)** *E. astringens* EBH, **(B)** *E. astringens* FAE, **(C)** *E. astringens* DCM, **(D)** *E. astringens* FI, and **(E)** *E. astringens* FA. Bacterial growth was assessed by measuring absorbance at 600 nm (OD_{600}) over a 24-h period, comparing the growth control with treatments at MIC and 2 × MIC concentrations. Data are expressed as mean ± standard deviation (SD).

When combined with ampicillin, the most promising results were observed against MRSA. All fractions from *E. astringens* exhibited synergistic or additive effects, with the strongest interactions observed for EBH, FAE, DCM, and FI (FICI = 0.24–0.28), indicating marked potentiation of ampicillin activity. The FAE fraction also displayed synergistic interactions against *S. epidermidis* (FICI = 0.35), while DCM showed a similar effect (FICI = 0.36). In contrast, antagonistic interactions were observed against *S. aureus* for EBH, FI, and FA, indicating that the response to combination therapy was strain dependent.

The combinations with vancomycin showed a different profile. Most interactions were classified as indifferent, indicating that the extracts and fractions generally did not interfere with vancomycin activity. Nevertheless, some additive effects were observed, particularly for DCM against *S. aureus* (FICI = 0.61) and for FI and FA against MRSA (FICI = 1.00 and 0.97, respectively). Antagonistic interactions were restricted to a few combinations, including EBH and FI against *S. epidermidis* and FA against *S. aureus*.

Overall, the results indicate that the greatest potential for combination therapy was observed with ampicillin, especially against MRSA and *S. epidermidis*. The predominance of synergistic and additive interactions suggests that compounds present in the extracts and fractions may enhance antimicrobial efficacy through complementary mechanisms of action, potentially increasing bacterial susceptibility to β-lactam antibiotics.

The favorable interactions observed between the extracts and β-lactam antibiotics against resistant strains may be associated with the ability of phenolic compounds to modulate bacterial resistance mechanisms. According to Borges et al. (2013), gallic acid, the major compound identified in EBH of *E. astringens*, can induce irreversible damage to the bacterial cell membrane, causing alterations in surface charge, pore formation, and increased membrane permeability. Such effects may facilitate the access of ampicillin to its targets, the penicillin-binding proteins (PBPs), thereby enhancing its antibacterial activity. Furthermore, changes in cell surface charge may reduce electrostatic repulsion toward β-lactam molecules, while membrane damage may compromise physical barriers that restrict antibiotic penetration. In addition, molecular docking studies performed by Saini et al. (2024) demonstrated a strong interaction between quercetin and PBP3, suggesting that

TABLE 3 Fractional inhibitory concentration index (FICI) values calculated for combinations of ampicillin and vancomycin with extracts and fractions from *E. astringens* and *E. mattosii*.

Sample	*S. aureus*	MRSA	*S. epidermidis*
Ampicillin			
EBH (EA)	3 (A)	0.24 (S)	0.73 (AD)
FAE (EA)	1.5 (I)	0.24 (S)	0.35 (S)
DCM (EA)	1.24 (I)	0.24 (S)	0.36 (S)
FI (EA)	3 (A)	0.28 (S)	0.57 (AD)
FA (EA)	2.5 (A)	0.56 (AD)	0.54 (AD)
FAE (EM)	NT	0.75 (AD)	NT
FI (EM)	1 (AD)	n.t.	n.t.
Vancomycin			
EBH (EA)	1.12 (I)	1.5 (I)	3 (A)
FAE (EA)	1.02 (I)	1.03 (I)	1.01 (I)
DCM (EA)	0.61 (AD)	1.01 (I)	1.03 (I)
FI (EA)	1.02 (I)	1 (AD)	2.01 (A)
FA (EA)	2.12 (A)	0.97 (AD)	1.24 (I)
FAE (EM)	n.t.	1.03 (I)	NT
FI (EM)	1.12 (I)	n.t.	n.t.

EBH, crude hydroethanolic extract; FAE, ethyl acetate fraction; DCM, dichloromethane fraction; FI, insoluble fraction; FA, aqueous fraction. EA, eugenia astringens; EM, Eugenia mattosii. A, antagonism; AD, additive effect; I, indifferent interaction; S, synergistic interaction.

phenolic compounds may also contribute to the modulation of proteins involved in β-lactam resistance. The presence of gallic acid and quercetin in the extracts of *E. astringens* may therefore partially explain the synergistic effects observed in combination with ampicillin, particularly against MRSA.

Additional evidence supporting the modulation of β-lactam resistance by *Eugenia*-derived metabolites has been reported for essential oils from *Eugenia brejoensis*, *E. pohliana*, *E. gracillima*, and *E. stictopetala*. Silva et al. (2024) demonstrated, through molecular docking analyses, that compounds present in these essential oils were able to interact with PBP1 and PBP2a. The authors suggested that these metabolites may bind to an allosteric site of PBP2a, inducing conformational changes that modulate the active site of the protein and restore the bactericidal activity of β-lactam antibiotics against resistant strains. These findings reinforce the hypothesis that metabolites from *Eugenia* species may act as resistance-modifying agents, enhancing the efficacy of conventional antibiotics through mechanisms complementary to their intrinsic antibacterial activity.

3.4 Evaluation of bacterial membrane permeability

The effects of the extracts and fractions on bacterial membrane permeability were evaluated using the SYTOX Green assay. In *S. aureus* ATCC 25923, several samples significantly increased fluorescence intensity compared with the negative control, indicating membrane permeabilization. Among the *E. astringens* samples, the highest increases were observed for DCM (9.8%) and FA (8.8%), whereas in *E. mattosii* the greatest effect was detected for FAE (13.6%), followed by FA (9.6%) and EBH (8.5%). As expected, polymyxin B induced a more pronounced increase in fluorescence, reaching 22.7% and 37.2% at concentrations of 200 and 100 μg mL^{-1}, respectively (Figure 10).

A similar pattern was observed for the multidrug-resistant *S. aureus* strain, although the effects were generally more pronounced. The DCM fraction of *E. astringens* increased fluorescence by 38.5%, while the FAE fraction of *E. mattosii* promoted the highest increase among all tested samples (47.6%). Additional significant increases were observed for FA and EBH from both species. Polymyxin B increased fluorescence by 61.5% and 77.4% at 200 and 100 μg mL^{-1}, respectively (Figure 11).

In *S. epidermidis*, the strongest membrane-permeabilizing effects were again observed for DCM from *E. astringens* (39.5%) and FAE from *E. mattosii* (45.5%). Significant increases were also detected for FA, EBH, and FI from both species. The positive control increased fluorescence by 25.5% and 71.0% at 200 and 100 μg mL^{-1}, respectively (Figure 12).

SYTOX Green is a fluorescent nucleic acid stain that is unable to penetrate intact cell membranes but readily enters cells with compromised membranes, where it binds to DNA and emits fluorescence. Therefore, increases in fluorescence intensity are indicative of membrane permeabilization or membrane damage. This mechanism is commonly observed for membrane-active antimicrobial agents, such as polymyxin B, which induce marked increases in fluorescence compared with untreated cells (Johnson and Criss, 2013; McKenzie et al.,

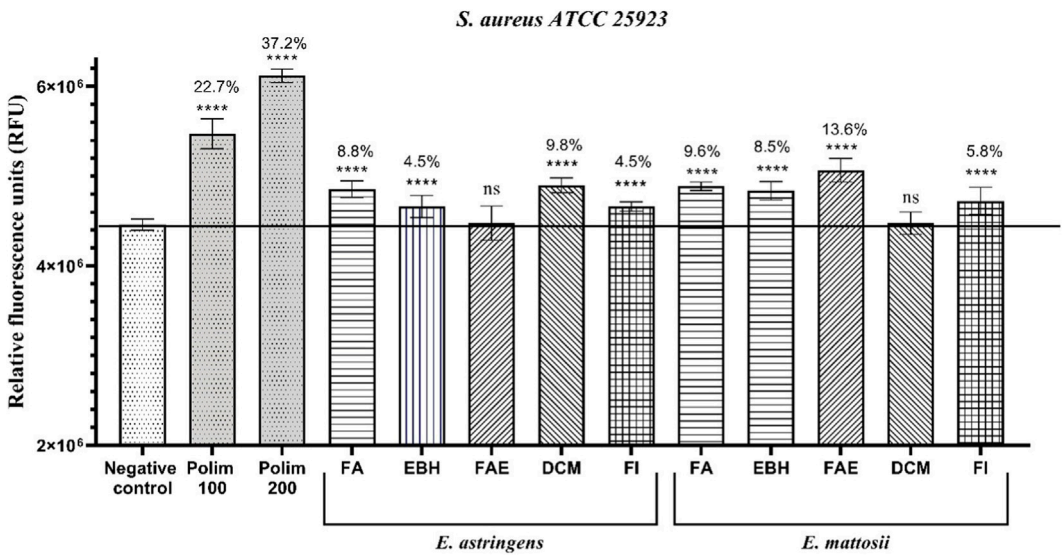


FIGURE 10
Evaluation of the action of extracts and fractions obtained from *E. astringens* and *E. mattosii* on the permeability of the bacterial membrane of *Staphylococcus aureus* ATCC 25923. Aqueous fraction (FA), crude extract (EBH), ethyl acetate fraction (FAE), dichloromethane fraction (DCM), and insoluble fraction (FI). ****$p < 0.0001$ vs. negative control; ns = not significative. Results are expressed as mean fluorescence intensity. Data are expressed as mean ± standard deviation (SD).

2016). The results obtained in the present study demonstrate that extracts and fractions from *E. astringens* and *E. mattosii* promote membrane permeabilization in susceptible strains, suggesting that disruption of membrane integrity represents one of the mechanisms contributing to their antibacterial activity. The membrane permeabilization observed in the SYTOX Green assay may contribute to explaining the synergistic interaction observed with ampicillin, as membrane damage could facilitate antibiotic access to its target penicillin-binding proteins (PBPs). However, a direct causal relationship between membrane permeabilization and synergism was not established in the present study. Since no specific assays were performed to investigate other resistance mechanisms, the involvement of additional pathways, such as efflux pump inhibition or other complementary mechanisms, cannot be excluded. Therefore, further studies are required to elucidate the precise mechanisms underlying the observed synergistic effects.

3.5 Evaluation of cytotoxic effects on RAW 264.7 macrophages

The cell viability assays (Supplementary Figures 1–2) demonstrated that, in general, the extracts and fractions exhibited low cytotoxicity toward RAW 264.7 macrophages. Reductions in cell viability were observed only for a limited number of samples and primarily at the highest concentration tested (500 μg mL^{-1}). At 125 μg mL^{-1}, the highest MIC value observed, none of the samples reduced cell viability below 75% (Supplementary Table S2). These findings indicate a favorable safety profile at concentrations close to those associated with antibacterial activity. These results are consistent with those reported by Santiago-Carvalho et al. (2022), who evaluated the cytotoxicity of *E. astringens* ethanol extracts and fractions in RAW 264.7 macrophages and Vero epithelial cells and observed low toxicity toward mammalian cells. Similar results have been described for other species of the genus *Eugenia*. In contrast, Vechi et al. (2021) reported higher cytotoxicity for extracts and fractions of *E. mattosii* against HeLa cells, with LD_{50} values ranging from 46.7 to 93.5 μg mL^{-1}. These differences may be related to the distinct cellular models employed, since tumor-derived cell lines such as HeLa cells are known to exhibit different susceptibility profiles. Overall, the low cytotoxicity observed in RAW 264.7 macrophages reinforces the potential of the evaluated extracts and fractions as sources of bioactive compounds with promising antibacterial properties.

4 Conclusion

This study demonstrated that *E. astringens* and *E. mattosii* are sources of bioactive metabolites with promising antibacterial activity against Gram-positive bacteria. Chemical characterization revealed distinct phenolic profiles for the two species, with gallic acid and quercetin among the major compounds identified in *E. astringens*, whereas catechin, epicatechin, and quercetin predominated in *E. mattosii*. Moreover, the identification of phenolic compounds not previously reported in the available literature for *E. astringens* expands the current phytochemical knowledge of this species. The extracts and fractions exhibited antibacterial activity against *S. aureus*, MRSA, and *S. epidermidis*, with enhanced activity observed after fractionation, particularly in fractions of low and intermediate polarity. The samples were highly effective in preventing biofilm formation, whereas disruption of mature biofilms was more limited and predominantly observed against MRSA. Growth kinetics assays further confirmed the

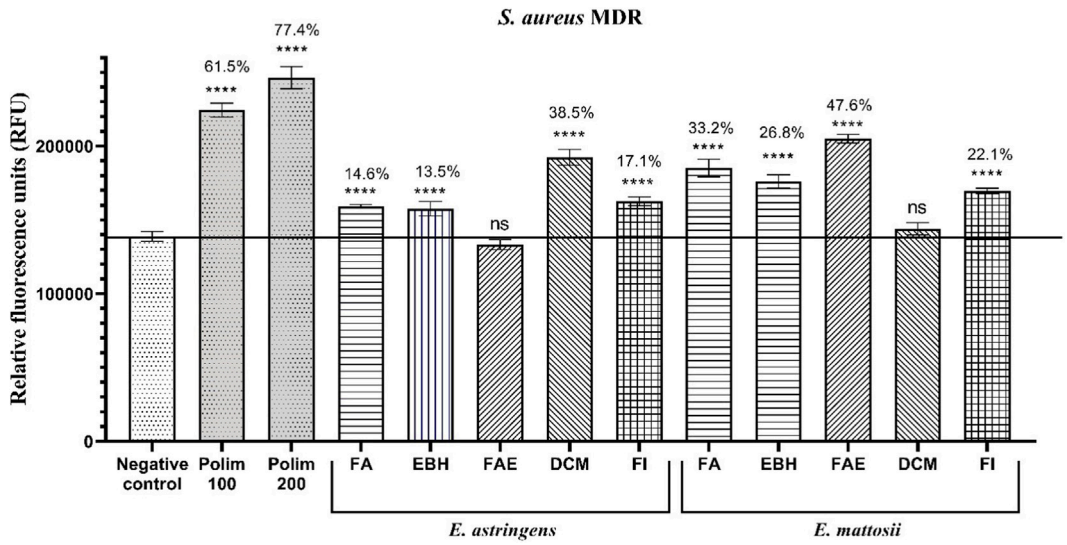


FIGURE 11
Evaluation of extracts and fractions obtained from *E. astringens* and *E. mattosii* on the permeability of the bacterial membrane of *Staphylococcus aureus* multidrug resistance. Aqueous fraction (FA), crude extract (EBH), ethyl acetate fraction (FAE), dichloromethane fraction (DCM), and insoluble fraction (FI). ****$p < 0.0001$ vs. negative control; ns = not significative. Results are expressed as mean fluorescence intensity. Data are expressed as mean ± standard deviation (SD).

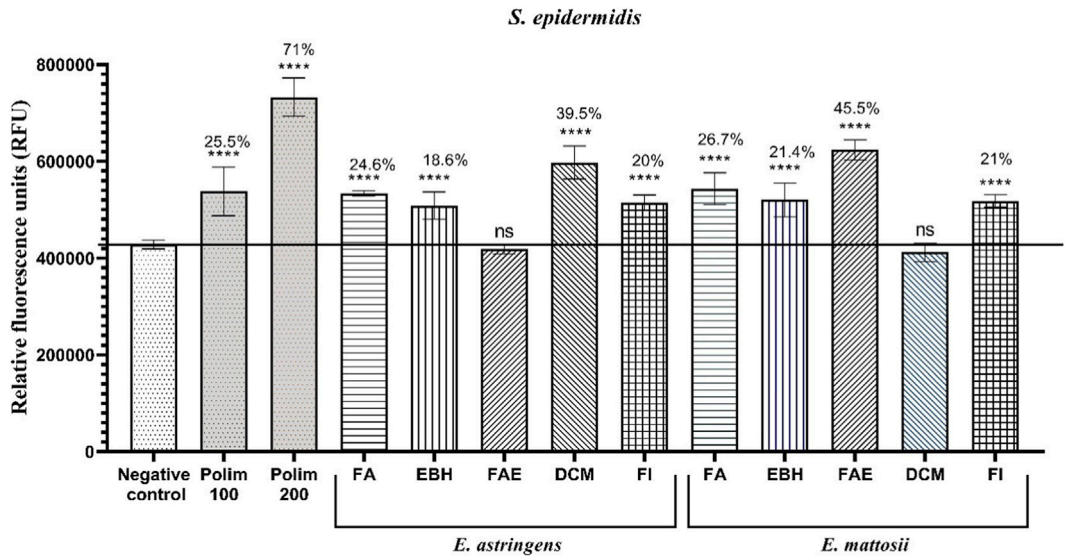


FIGURE 12
Evaluation of extracts and fractions obtained from *E. astringens* and *E. mattosii* on the permeability of the bacterial membrane of *Staphylococcus epidermidis*. Aqueous fraction (FA), crude extract (EBH), ethyl acetate fraction (FAE), dichloromethane fraction (DCM), and insoluble fraction (FI). ****$p < 0.0001$ vs. negative control; ns = not significative. Results are expressed as mean fluorescence intensity. Data are expressed as mean ± standard deviation (SD).

sustained inhibitory effects of selected fractions from *E. astringens*. In addition, assays demonstrated that several active fractions promoted bacterial membrane permeabilization, suggesting that alterations in membrane integrity may represent one of the mechanisms contributing to their antibacterial effects. Among the evaluated biological properties, the most promising findings were the pronounced anti-MRSA activity, inhibition of biofilm formation, membrane permeabilization effects, and the synergistic interactions observed between several extracts and fractions and ampicillin. These effects, combined with the low cytotoxicity observed in RAW 264.7 macrophages at MIC concentrations, highlight the potential of *E. astringens* and *E. mattosii* as sources of antibacterial compounds and potential antibiotic adjuvants

capable of enhancing the activity of conventional antibiotics against resistant staphylococcal infections.

Data availability statement

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

Ethics statement

Ethical approval was not required for the studies on animals in accordance with the local legislation and institutional requirements because only commercially available established cell lines were used.

Author contributions

PD: Conceptualization, Data curation, Methodology, Software, Writing – original draft. LB: Data curation, Investigation, Methodology, Writing – original draft. ABG: Data curation, Investigation, Methodology, Writing – original draft. TR: Data curation, Investigation, Methodology, Writing – original draft. NZ: Data curation, Investigation, Methodology, Writing – original draft. MD: Conceptualization, Data curation, Investigation, Methodology, Writing – original draft. ML: Conceptualization, Data curation, Investigation, Methodology, Writing – original draft. ST: Conceptualization, Data curation, Investigation, Methodology, Writing – original draft. TB: Data curation, Funding acquisition, Investigation, Methodology, Writing – original draft. DS: Conceptualization, Data curation, Investigation, Methodology, Writing – original draft, Writing – review and editing. LV: Conceptualization, Data curation, Funding acquisition, Investigation, Methodology, Writing – original draft. CO-T: Conceptualization, Data curation, Funding acquisition, Investigation, Methodology, Writing – original draft, Writing – review and editing. IM: Data curation, Funding acquisition, Investigation, Methodology, Writing – original draft. MA: Conceptualization, Funding acquisition, Investigation, Project administration, Resources, Supervision, Writing – original draft, Writing – review and editing.

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Conflict of interest

The author(s) declared that this work was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fntpr.2026.1920078/full#supplementary-material

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