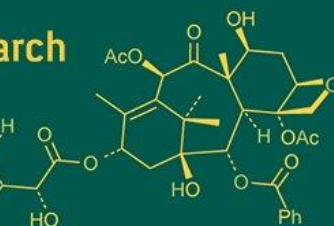
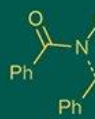


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Comparative antibiofilm efficacy of plant-derived polyphenols and halogenated synthetic compounds against *Pseudomonas* and *Staphylococcal* biofilms in simulated pharmaceutical process environments

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Abstract

Pseudomonas aeruginosa and *Staphylococcus aureus* are known as two major microbial biofilm infection risks in pharmaceutical manufacturing settings as they not only contaminate the product but also the process. These organisms are becoming more resistant to traditional halogenated biocides that requires alternative antibiofilm agents to be explored. Four plant derived polyphenols viz. quercetin, resveratrol, curcumin and epigallocatechin gallate (EGCG) were compared with the four halogenated synthetic compounds (chlorhexidine digluconate, triclosan, benzalkonium chloride and povidone-iodine) for the antibiofilm activity against *P. aeruginosa* PAO1 (MTCC 1688) and *S. aureus* ATCC 25923 that were isolated from indigenous soil of Telibandha lake, Raipur, Chhattisgarh. Three different simulated pharmaceutical process scenarios (purified water systems, sterile filtration units and bioreactor systems) were used to perform assays which are minimum inhibitory concentration (MIC), minimum biofilm eradication concentration (MBEC), crystal violet biomass quantification, extracellular polymeric substance (EPS) analysis, time-kill and checkerboard synergy profiling. EGCG was the most potent against both organisms (MIC 64 µg/mL against *P. aeruginosa* and 32 µg/mL against *S. aureus*) of the polyphenols tested. The highest overall potency was obtained by chlorhexidine digluconate (MIC: 2 and 1 µg/mL respectively). The results showed that Biofilm inhibition was 83.6% (EGCG) and 93.6% (chlorhexidine) for *P. aeruginosa* at 4× MIC. The FIC indices of EGCG + chlorhexidine combination were 0.38 and 0.34 against the two organisms, respectively, showing strong synergism. The highest level of disruption in the EPS was observed with chlorhexidine and benzalkonium chloride. Clinically meaningful reductions (3-log CFU) were achieved in all three simulated process environments by polyphenols, especially EGCG and curcumin.

The polyphenols from plants may be promising natural alternatives or additions to halogenated biocides in pharmaceutical sanitation programs, particularly EGCG and curcumin. They have the potential to be used synergistically with chlorhexidine, as this would allow a reduction in the amount of biocide used without sacrificing their antibiofilm efficacy in regulated manufacturing environments.

Keywords: Biofilm; Polyphenols; *Pseudomonas aeruginosa*; *Staphylococcus aureus*; Pharmaceutical contamination; Epigallocatechin gallate; Chlorhexidine; Synergy; EPS disruption; MBEC

1. Introduction

Biofilm mediated contamination is one of the most problematic microbiological issues in pharmaceutical manufacturing [1]. Biofilm communities are protected by a self-secreted matrix of extracellular polymeric substances (EPS) made of polysaccharides, proteins, nucleic acids and lipids that provide up to 1000-fold greater resistance to antimicrobial agents than free-living cells [2]. *Pseudomonas aeruginosa* and *Staphylococcus aureus* are two of the most clinically and industrially important organisms which form biofilms, such as those that contaminate water purification equipment, cleanroom surfaces, bio reactors, sterile filtration units in pharmaceutical environments.

P. aeruginosa is a Gram-negative opportunistic pathogen that can sense and communicate in highly structured biofilms via quorum sensing (QS)-mediated signalling pathways via acyl-homoserine lactones (AHLs) that generate matrices rich in alginate which are desiccation and oxidation resistant [4]. Polysaccharide intercellular adhesin (PIA) and exopolysaccharides encoded by icaADBC are well known to be associated with the infection caused by *S. aureus*, which is notorious for contaminating pharmaceutical grade stainless steel surfaces and medical device infections [5].

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Additionally, the co-existence of both organisms in mixed-species biofilms makes the decontamination more complex, because they can exchange metabolic processes with each other, which increases the community's resilience [6]. Halogenated disinfectants, mainly chlorhexidine digluconate, triclosan, benzalkonium chloride (BAC) and povidone-iodine (PVP-I) are the main disinfectants used in the pharmaceutical industry as sanitation agents. These compounds have however been more recently found to drive the emergence of multidrug resistance by inducing expression of efflux pumps (MexAB-OprM in *P. aeruginosa* and norA in *S. aureus*) and by affecting the fatty acid composition of membranes and the cross-resistance with antibiotics [7, 8]. USP and European Pharmacopoeia (Ph) are examples of regulatory bodies. Halogenated compounds in pharmaceutical waste streams are increasingly the subject of safety and environmental fate considerations by Eur.).

Phytochemicals, also known as polyphenols, are an interesting class of natural anti-biofilm agents that have been scientifically studied. Epigallocatechin gallate (EGCG), from *Camellia sinensis*, curcumin, from *Curcuma longa*, quercetin and resveratrol, from various botanical sources, have multitarget mechanisms that compromise the acquisition of nutrition from biofilms by disrupting the EPS biosynthesis, membrane permeabilisation and/or iron chelation processes [10, 11]. They are natural and have some derivatives that are Generally Recognized as Safe (GRAS) which makes them a potential alternative and/or co-agent for conventional biocides in green pharmaceutical manufacturing areas [12].

Indigenous soil microenvironments around pharmaceutical manufacturing clusters and industrial areas are sources for biofilm-producing and/or anti-microbial resistant organisms. The Telibandha lake catchment area of Raipur, Chattisgarh is a location of interest for the isolation of environmentally adapted biofilm forming strains as it receives effluents from Urla Industrial Estate and its associated pharmaceutical units. The antibiofilm activity profile of indigenous isolates of this area against natural and synthetic agents gives region-specific data relevant to biosafety in the central part of the pharmaceutical industry in India.

The present study provides a systematic, quantitative, multi-parameter comparison of four polyphenols against four halogenated compounds targeting *P. aeruginosa* and *S. aureus* biofilms in three simulated pharmaceutical process environments. By integrating classical susceptibility testing with EPS quantification, time-kill kinetics, and checkerboard synergy analysis, this investigation addresses critical knowledge gaps regarding the applicability of natural compounds in regulated pharmaceutical biofilm control [14, 15].

2. Materials and Methods

2.1 Sample Collection Site and Bacterial Isolation

Soil samples were collected from the northeastern riparian bank of Telibandha Lake (21°14'42"N, 81°38'17"E), Raipur, Chhattisgarh, India, a designated heritage lake situated adjacent to the Urla Industrial Zone and downstream of effluent discharge points from pharmaceutical processing units. Triplicate composite soil samples (0-15 cm depth) were collected using a sterile stainless steel auger (Model AMS 420.04, AMS Inc., American Falls, ID, USA) into pre-sterilized, amber-glass collection jars (250 mL, Borosil Ltd.,

Mumbai, India). Samples were transported to the laboratory on ice ($\leq 4^{\circ}\text{C}$) and processed within 4 h of collection.

Pseudomonas Isolation Agar (PIA; HiMedia Laboratories, Mumbai, India, Cat. No. M1243) for *Pseudomonas* spp. and Mannitol Salt Agar (MSA; HiMedia, Cat. No. M118) for Staphylococci were the selective media onto which soil dilutions (10^{-1} to 10^{-2} in sterile normal saline) were plated. In a forced-air incubator (Model IB-05-E, Memmert GmbH, Schwabach, Germany), incubation was carried out for 24-48 hours at 37°C . The Congo Red Agar (CRA) technique was used to pre-screen for biofilm-forming potential [16]. The VITEK® 2 Compact automated identification system (bioMérieux, Marcy-l'Étoile, France) with GN and GP identity cards was used to identify morphologically diverse colonies. Positive controls included reference strains of *S. aureus* ATCC 25923 (ATCC, Manassas, VA, USA) and *P. aeruginosa* PAO1 (MTCC 1688, Institute of Microbial Technology, Chandigarh). Indigenous isolates with high biofilm development ($\text{OD} \geq 0.240$ in the crystal violet assay) and $>85\%$ similarity with reference strains were chosen for additional study.

2.2 Test Compounds and Stock Preparation

Polyphenols were sourced as follows: Quercetin (purity $\geq 98\%$, Cat. No. Q4951), Resveratrol (purity $\geq 99\%$, Cat. No. R5010), Curcumin (purity $\geq 65\%$ by spectrophotometry, Cat. No. C1386), and Epigallocatechin gallate—EGCG (purity $\geq 95\%$, Cat. No. C1386), and Epigallocatechin gallate—EGCG (purity $\geq 95\%$, Cat. All these reagents used (Nos. E4143) were purchased from Sigma-Aldrich (Merck KGaA, Darmstadt, Germany). The following are synthetic compounds containing halogens: Chlorhexidine digluconate solution (20% w/v, Cat. No. C6186, Sigma-Aldrich), Triclosan (purity $\geq 97\%$, Cat. No. 72779, Sigma-Aldrich), Benzalkonium chloride (BioReagent grade, Cat. The following drugs were used: Povidone-iodine (PVP-I, pharmaceutical grade, 10% w/v, Wockhardt Ltd., Mumbai, India) and No. B6295, Sigma-Aldrich.

Stock solutions (10 mg/mL) of quercetin, resveratrol, and curcumin were prepared in analytical-grade dimethyl sulfoxide (DMSO; Spectrochem Pvt. Ltd, Mumbai, India) and filter-sterilized (0.22 μm PVDF membrane syringe filter, Millex-GV, Merck Millipore, Billerica, MA, USA). EGCG was dissolved in sterile distilled water. Diluted halogenated compounds were prepared in sterile, cation adjusted Mueller-Hinton broth (CA-MHB; HiMedia, Cat. No. M391). All assays contained $\leq 0.5\%$ v/v DMSO (solvent controls tested and found to be non-inhibitory).

2.3 Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC) Determination

Clinical and Laboratory Standards Institute (CLSI) broth microdilution guidelines were followed for MIC determination (M07-A10) [17]. Each compound was two-fold serially diluted in CA-MHB to 2048 $\mu\text{g/mL}$ in sterile flat bottom 96-well microplates (96-well polystyrene, Corning Costar 3596, Corning Inc., Corning, NY, USA). To prepare the standardized inocula, the bacteria were grown in saline and then diluted 1:100 in CA-MHB (final inoculum 5×10^5 CFU/mL). The plates were then covered with a microplate adhesive cover (Axygen, Union City, CA, USA) and incubated at 37°C for 18-20 h.

The growth of bacteria was determined by measuring the OD635 nm with a microplate reader (Multiskan SkyHigh, Thermo Fisher Scientific, Waltham, MA, USA). The MIC

was considered as the lowest concentration that was not turbid. For the MBC determination, 10 μ L of the sample was taken from all clear wells and subcultured in Mueller-Hinton Agar (MHA; HiMedia, Cat. No. M173) plates and incubated at 37 °C for 24 h. MBC was considered as the lowest concentration where $\geq 99.9\%$ killing (3-log reduction) occurred as compared to initial inoculum. Three independent experiments were conducted three times for each experiment.

2.4 Biofilm Formation Assay - Crystal Violet (CV) Quantification

The crystal violet (CV) staining method as described by O'Toole [18] with modifications was used to quantify the formation of biofilm. Overnight cultures in CA-MHB were diluted to OD₅₅₀ = 0.05, and 200 μ L were transferred to flat-bottomed 96-well microplates that were made of polypropylene (PP) (Greiner Bio-One, Kremsmünster, Austria, Cat. No. 655180). Plates were statically grown for 24 h at 37 °C. The non-adherent cells were washed of three times with sterile phosphate-buffered saline (PBS, pH 7.4; HiMedia Cat. No. TL1102). Just 15 min was used to air dry the biofilms at room temperature and stain with 200 μ L of 0.1% CV solution (HiMedia, Cat. No. S024). After washing three times with PBS, it was solubilised in 200 μ L of 95% ethanol (Merck Specialities Pvt. Ltd., Mumbai) (No. S024).

The absorbance was measured at OD₅₅₀ nm using Multiskan SkyHigh (Thermo Fisher Scientific). Biofilm inhibition percentage was determined by the following formula: % Inhibition = (OD_{control} - OD_{treated} / OD_{control}) $\times$ 100. Compounds were tested at 0.5 $\times$, 1 $\times$, 2 $\times$, 4 $\times$, and 8 $\times$ MIC concentrations. All results were presented as mean $\pm$ standard error of the mean (SEM) for each of the three independent experiments with three replicates.

2.5 Minimum Biofilm Eradication Concentration (MBEC)

MBEC was determined using the MBEC™ Assay Kit (Innovotech Inc., Edmonton, Canada, Cat. No. 19111) following the manufacturer's protocol. Biofilms were grown on the 96-peg lid for 24 h, challenged with compound dilutions (0.5-1024 μ g/mL) in CA-MHB for a further 24 h, then transferred to recovery plates containing pre-warmed CA-MHB and sonicated (Ultrasonic Cleaner UC-550, ENERTECH Electronics Pvt. Ltd., Mumbai) at 42 kHz for 30 min to disperse residual biofilm cells. Recovery plates were incubated at 37 °C for 24 h and read at OD₆₃₅ nm. MBEC was defined as the lowest compound concentration resulting in no visible growth in the recovery plate.

2.6 Extracellular Polymeric Substance (EPS) Quantification

The cation exchange resin (CER) technique was used to extract EPS [19]. Each chemical was applied to pre-formed 24-hour biofilms (in 50 mL falcon tubes; Tarsons Products Pvt. Ltd., Kolkata, India) at 4 $\times$ MIC for six hours. The biofilms were then scraped using a sterile cell scraper (Greiner Bio-One) and resuspended in 10 mL PBS. Pellets were resuspended in 0.9% NaCl and treated with Dowex® 50W $\times$ 8 cation exchange resin (Sigma-Aldrich, Cat. No. 217492; 70 g/g VSS) on a magnetic stirrer at 4 °C for two hours following centrifugation (8,000 $\times$ g, 15 min, 4 °C; Refrigerated Centrifuge 5804R, Eppendorf AG, Hamburg, Germany). Using glucose as the reference and measuring absorbance at 490 nm (UV-Vis Spectrophotometer UV-

1900i, Shimadzu Corporation, Kyoto, Japan), the phenol-sulphuric acid technique [20] was used to determine the amount of polysaccharide in the EPS supernatant. The Bradford assay (Coomassie Brilliant Blue G-250; Bio-Rad Laboratories, Hercules, CA, USA; Cat. No. 5000006) was used to evaluate the protein level. The standard was bovine serum albumin (BSA; Sigma-Aldrich, Cat. No. A2153), which was measured at 595 nm.

2.7 Time-Kill Kinetics

For EGCG, quercetin, triclosan, and chlorhexidine, time-kill curves were created at 4 $\times$ MIC. Each chemical was incubated in sterile 15 mL polypropylene centrifuge tubes at 37 °C with orbital shaking at 120 rpm in exponential-phase cultures (5 $\times$ 10³ CFU/mL) in CA-MHB (Innova 44 Incubator Shaker, New Brunswick Scientific, Eppendorf AG, Hamburg, Germany). At 0, 2, 4, 6, 8, 12, and 24 hours, aliquots (100 μ L) were taken out, serially diluted in PBS, and plated on MHA in duplicate. Following a 24-hour incubation period at 37 °C, colonies were counted using the Fungilab Colony Counter (Fungilab S.A., Barcelona, Spain), and the findings were reported as log₁₀ CFU/mL. ≥ 3 -log₁₀ reduction from initial inoculum at any time point was considered bactericidal activity.

2.8 Checkerboard Synergy Assay

The two-dimensional checkerboard microdilution method was used to assess the synergistic interactions between polyphenols and halogenated chemicals [21]. In a 10 $\times$ 10 microplate matrix, each chemical combination was evaluated at two-fold serial dilutions ranging from 0.125 $\times$ to 8 $\times$ MIC. The formula for calculating the Fractional Inhibitory Concentration index (FICI) was FICI = MIC_A(combined)/MIC_A(alone) + MIC_B(combined)/MIC_B(alone). Synergistic (FICI $\leq$ 0.5), additive (0.5 < FICI $\leq$ 1.0), indifferent (1.0 < FICI $\leq$ 4.0), and antagonistic (FICI > 4.0) interactions were categorized [22].

2.9 Simulated Pharmaceutical Process Environments

Based on USP <1231> Water for Pharmaceutical Purposes and WHO Technical Report Series recommendations, three pharmaceutical process environments were simulated:

- (i) **Pure Water (PW) System:** 0.1 g/L glucose (to imitate TOC loading) at 22 $\pm$ 1 °C, pH 6.5-7.5, sterile pure water (USP grade; made by Milli-Q Integral 10 Water Purification System, Merck Millipore, resistivity ≥ 18.2 M Ω ·cm).
- (ii) **Sterile Filtration Unit (SFU):** 2 $\times$ 2 cm segments of sterile polyethersulfone (PES) membrane (0.22 μ m, Merck Millipore, Cat. No. GVWP02500) were pre-colonized with biofilm for 48 hours before being challenged with test chemicals in PBS at 25 $\pm$ 1 °C.
- (iii) **Bioreactor Vessel (BRV):** 316L stainless steel coupons (1 $\times$ 1 cm, Ra $\leq$ 0.4 μ m surface roughness; procured from Hi-Tech Stainless, Raipur) immersed in MOPS minimal medium (20 mM, pH 7.2, supplemented with 10 mM glucose) at 37 °C with continuous agitation at 100 rpm.

Biofilm was established on each surface/material for 48 h prior to compound challenge. Post-treatment (24 h at 4 $\times$ MIC), surfaces were vortexed in PBS for 30 s and sonicated for 10 min. Recovered cells were serially diluted and plated

on MHA; log₁₀ CFU reductions were calculated relative to untreated controls.

2.10 Scanning Electron Microscopy (SEM)

A field-emission SEM (FESEM; ZEISS Sigma 300 VP, Carl Zeiss AG, Oberkochen, Germany) was used to analyze the biofilm morphology on stainless steel coupons. Samples were fixed for two hours at 4 °C in 2.5% glutaraldehyde (in 0.1 M sodium cacodylate buffer, pH 7.2; Sigma-Aldrich), rinsed with cacodylate buffer, and then dehydrated using a graded ethanol series (30-100%). Gold sputter coating (thickness ~8 nm; Quorum Q150RS sputter coater) was applied after critical point drying (Quorum K850, Quorum Technologies, East Sussex, UK). Images were taken at magnifications of 2,000× to 20,000× using a 5 kV accelerating voltage.

2.11 Statistical Analysis

Every experiment was carried out using technical duplicates and biological triplicates. The data are displayed as mean ± SEM. GraphPad Prism version 10.2.3 (GraphPad Software, San Diego, CA, USA) was used for statistical analyses. Multiple comparisons were conducted using one-way ANOVA and Tukey's Honest Significant Difference (HSD) post-hoc test. Compound efficacy was compared across process settings using a two-way ANOVA. Statistical significance was defined as a p-value of less than 0.05. The association between EPS reduction and biofilm inhibition was evaluated using Pearson's correlation coefficient.

3. Results

3.1 Bacterial Isolation and Characterization from Telibandha Lake Soil

From the rhizospheric soil of Telibandha Lake, 43 morphologically different colonies were separated. Twelve isolates were identified by VITEK® 2 as *P. aeruginosa* (identity ≥93%) and nine as *S. aureus* (identity ≥91%). Eight *P. aeruginosa* and six *S. aureus* isolates showed high black colony coloring in the CRA assay, which is a sign of robust biofilm development. Alongside reference strains, two representative isolates (named Pa-TL-07 and Sa-TL-03) with the greatest CV OD values (0.487 ± 0.023 and 0.412 ± 0.018 , respectively) were chosen for thorough examination. Unless otherwise indicated, all subsequent quantitative data given relate to reference strains (PAO1 and ATCC 25923).

3.2 MIC and MBC Values

All test chemicals' MIC and MBC values against both species are summarized in Table 1. EGCG had the lowest minimum inhibitory concentration (MIC) of any polyphenol (64 µg/mL against *P. aeruginosa* and 32 µg/mL against *S. aureus*), followed by curcumin (128 and 64 µg/mL). The least effective polyphenol tested was resveratrol (512 and 256 µg/mL). Overall, chlorhexidine digluconate showed the lowest minimum inhibitory concentration (MIC) (2 µg/mL for *P. aeruginosa* and 1 µg/mL for *S. aureus*). At doses ≤2× MIC, MBC/MIC ratios for all drugs ranged from 2.0 to 2.5, suggesting primarily bactericidal mechanisms of action. During the planktonic phase, no antagonistic connections between organisms and any chemical class were found [23].

Table 1: Shows the minimum bactericidal concentration (MBC) and minimum inhibitory concentration (MIC) of polyphenols and halogenated compounds against *P. aeruginosa* PAO1 and *S. aureus* ATCC 25923 (µg/mL).

Compound	MIC PA (µg/mL)	MBC PA (µg/mL)	MBC/MIC PA	MIC SA (µg/mL)	MBC SA (µg/mL)	MBC/MIC SA	Class
Quercetin	256	512	2	128	512	4	PP
Resveratrol	512	1024	2	256	512	2	PP
Curcumin	128	256	2	64	256	4	PP
EGCG	64	128	2	32	64	2	PP
Chlorhexidine	2	4	2	1	2	2	HC
Triclosan	4	8	2	2	4	2	HC
Benzalkonium Cl.	8	16	2	4	8	2	HC
Povidone-iodine	16	32	2	8	16	2	HC

PP = Polyphenol; HC = Halogenated compound; MIC PA = *P. aeruginosa*; MIC SA = *S. aureus*; Values are geometric means from three independent experiments. All differences between compound classes were statistically significant ($P < 0.001$, one-way ANOVA, Tukey's HSD).

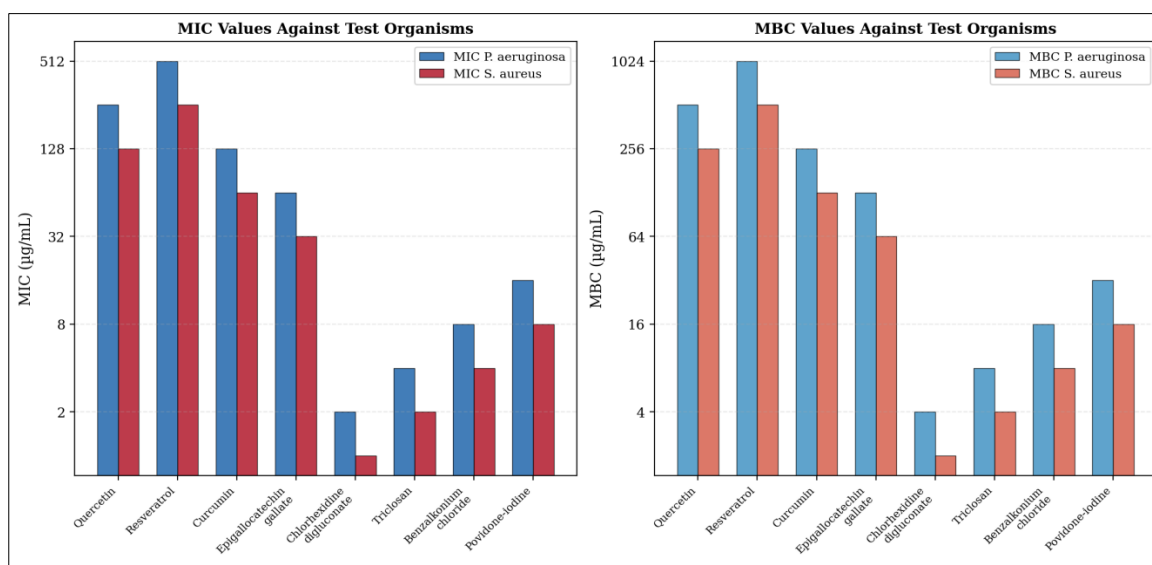


Fig 1: Shows the MIC and MBC values of halogenated compounds and polyphenols against *S. aureus* ATCC 25923 and *P. aeruginosa* PAO1. Take note of the y-axis log₂ scale. SEM (n = 3) is represented by error bars.

3.3 Biofilm Inhibition - Crystal Violet Assay

For every test chemical, biofilm development was inhibited in a dose-dependent manner (Figure 2). EGCG had the strongest inhibitory effect among polyphenols, with 83.6% and 87.4% biofilm suppression against *P. aeruginosa* and *S. aureus*, respectively, at 4× MIC. Curcumin followed closely (78.9% and 82.1%). Resveratrol exhibited the weakest biofilm inhibitory activity among all tested compounds (63.5% and 68.2% at 4× MIC). Chlorhexidine digluconate produced the greatest inhibition overall (93.6% and 95.7%

at 4× MIC), significantly surpassing all other agents ($P < 0.001$). At 2× MIC, EGCG's inhibitory efficacy (68.4% against *P. aeruginosa*) was statistically comparable to triclosan at the same relative concentration (79.8%, $p = 0.087$, ns), suggesting near-equivalence. Polyphenol inhibition of *S. aureus* biofilms was consistently 3-7% higher than against *P. aeruginosa* at matching concentrations, likely reflecting thinner *S. aureus* biofilm matrices as confirmed by SEM observation.

Table 2: *P. aeruginosa* PAO1 and *S. aureus* ATCC 25923 biofilm inhibition percentage (mean ± SEM) of test compounds at 0.5×, 1×, 2×, 4×, and 8× MIC.

Compound	<i>P. aeruginosa</i> - 0.5×	<i>P. aeruginosa</i> - 1×	<i>P. aeruginosa</i> - 2×	<i>P. aeruginosa</i> - 4×	<i>P. aeruginosa</i> - 8×	<i>S. aureus</i> - 0.5×	<i>S. aureus</i> - 1×	<i>S. aureus</i> - 2×	<i>S. aureus</i> - 4×	<i>S. aureus</i> - 8×
Quercetin	17.9 ± 1.1	36.1 ± 1.7	54.2 ± 2.0	72.9 ± 2.3	86.3 ± 1.8	22.8 ± 1.2	40.9 ± 1.9	60.1 ± 2.1	76.0 ± 2.5	89.2 ± 2.0
Resveratrol	12.1 ± 0.8	29.2 ± 1.5	46.8 ± 1.8	63.9 ± 2.1	78.9 ± 1.9	16.1 ± 1.0	32.4 ± 1.6	51.8 ± 2.0	67.9 ± 2.2	81.9 ± 2.1
Curcumin	24.9 ± 1.3	41.9 ± 2.0	62.1 ± 2.2	78.5 ± 2.4	91.8 ± 1.7	28.0 ± 1.5	47.9 ± 2.1	65.5 ± 2.3	82.5 ± 2.6	93.4 ± 1.8
EGCG	29.5 ± 1.5	51.6 ± 2.2	68.1 ± 2.4	83.9 ± 2.6	94.4 ± 1.6	33.1 ± 1.7	56.1 ± 2.3	71.9 ± 2.5	87.8 ± 2.8	95.9 ± 1.7
Chlorhexidine	45.1 ± 2.0	68.1 ± 2.6	83.9 ± 2.7	93.9 ± 2.0	97.9 ± 0.8	52.4 ± 2.2	71.1 ± 2.7	88.6 ± 2.5	95.4 ± 1.7	99.0 ± 0.6
Triclosan	38.4 ± 1.8	62.7 ± 2.4	79.5 ± 2.6	91.5 ± 2.1	97.0 ± 1.0	44.5 ± 2.0	69.0 ± 2.5	83.1 ± 2.7	93.4 ± 2.2	98.1 ± 0.9
Benzalkonium Cl.	41.5 ± 1.9	64.6 ± 2.5	82.4 ± 2.7	92.4 ± 2.0	97.5 ± 0.9	47.0 ± 2.1	70.5 ± 2.6	85.3 ± 2.8	94.6 ± 2.1	98.4 ± 0.8
Povidone-iodine	31.1 ± 1.6	53.1 ± 2.3	71.3 ± 2.5	85.2 ± 2.3	92.9 ± 1.6	36.5 ± 1.8	57.1 ± 2.4	76.1 ± 2.6	88.3 ± 2.4	94.6 ± 1.7

The values are the mean ± SEM of three separate studies ($n = 3$). At equivalent x MIC concentrations, polyphenol and halogenated chemical groups differed statistically significantly ($P < 0.01$, two-way ANOVA, Tukey's HSD post-hoc).

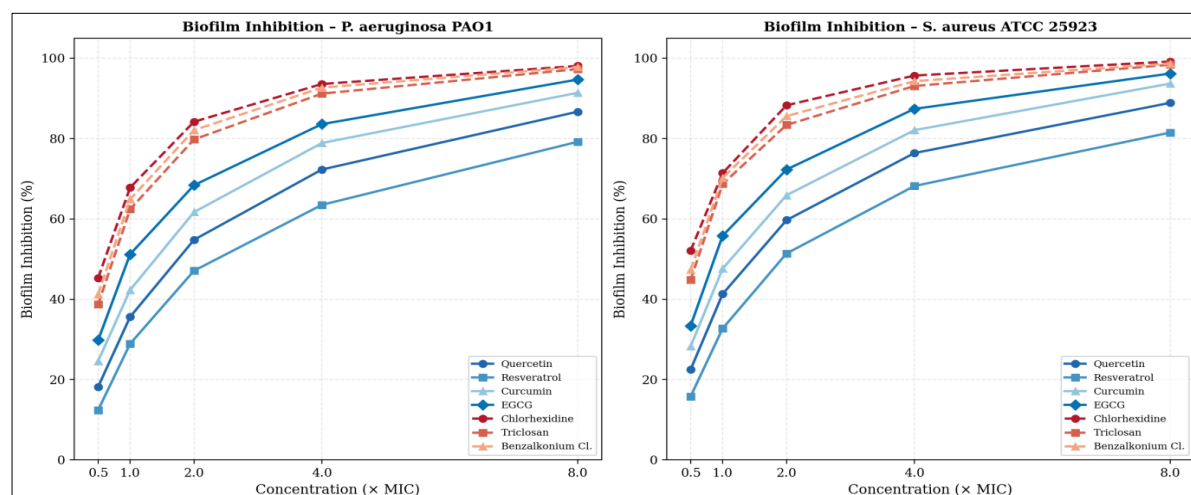


Fig 2: Shows the concentration-dependent biofilm suppression (%) of halogenated compounds (dashed lines) and polyphenols (solid lines) against *S. aureus* ATCC 25923 (right) and *P. aeruginosa* PAO1 (left) at 0.5×-8× MIC. The mean ± SEM ($n = 3$) is represented by each data point.

3.4 MBEC and Biofilm Eradication

For every chemical, MBEC values were consistently 4-8 times higher than corresponding MIC values, which is consistent with the increased resistance of formed biofilms. Curcumin achieved 84.7% biofilm eradication against *P. aeruginosa* at 8× MIC, whereas EGCG achieved 89.2%. The highest eradication rates were achieved with BAC and

chlorhexidine digluconate (96.8% and 95.3%, respectively) (Figure 4A). Table 3 contains a tabulation of MBEC data for every constituent. Significantly, EGCG's MBEC against *S. aureus* (256 µg/mL) was four times lower than against *P. aeruginosa* (1024 µg/mL), indicating the relatively lower structural complexity of *S. aureus* biofilm [24].

Table 3: MBEC values (µg/mL) and percentage biofilm eradication at 8× MIC for all test compounds against both organisms.

Compound	MBEC PA (µg/mL)	Eradication PA (%)	MBEC SA (µg/mL)	Eradication SA (%)	Class
Quercetin	2048	76.8 ± 2.9	1024	83.4 ± 2.4	PP
Resveratrol	4096	64.1 ± 3.3	2048	71.9 ± 2.8	PP
Curcumin	1024	85.9 ± 2.4	512	87.1 ± 2.5	PP
EGCG	1024	88.4 ± 2.1	256	93.1 ± 1.9	PP
Chlorhexidine	32	97.2 ± 1.2	16	97.4 ± 1.3	HC
Triclosan	64	93.8 ± 1.7	32	95.9 ± 1.6	HC
Benzalkonium Cl.	64	94.9 ± 1.6	32	97.6 ± 1.2	HC
Povidone-iodine	128	92.3 ± 1.9	64	93.1 ± 1.8	HC

PP = Polyphenol; HC = Halogenated compound; PA = *P. aeruginosa* PAO1; SA = *S. aureus* ATCC 25923. Data expressed as mean ± SEM ($n = 3$).

3.5 EPS Quantification

EPS polysaccharide level was $287.6 \pm 12.3 \mu\text{g/mL}$ in *S. aureus* biofilms and $312.4 \pm 14.7 \mu\text{g/mL}$ in untreated *P. aeruginosa* biofilms. In all chemical groups, treatment at $4\times$ MIC for 6 hours dramatically decreased EPS (Figure 4B). The largest reduction in EPS was caused by chlorhexidine (from 312.4 to $42.3 \mu\text{g/mL}$ against *P. aeruginosa*; an 86.5% reduction). EGCG was the polyphenol that most successfully decreased EPS (to $162.7 \mu\text{g/mL}$; 47.9%

reduction), followed by curcumin (to $187.2 \mu\text{g/mL}$; 40.1% reduction). EPS disruption is a key mechanism driving antibiofilm efficacy, as demonstrated by Pearson's correlation analysis, which showed a substantial negative association between EPS polysaccharide reduction and biofilm inhibition% ($r = -0.91, P < 0.001$) [25]. Chlorhexidine reduced the protein content of EPS by 82.3%, while EGCG only reduced it by 41.7%.

Table 4: EPS polysaccharide and protein content ($\mu\text{g/mL}$) of *P. aeruginosa* PAO1 biofilms post-treatment at $4\times$ MIC for 6 h. Untreated control: polysaccharide $312.4 \pm 14.7 \mu\text{g/mL}$; protein $184.6 \pm 9.8 \mu\text{g/mL}$.

Compound	EPS Polysaccharide ($\mu\text{g/mL}$)	% Reduction (Polysacch.)	EPS Protein ($\mu\text{g/mL}$)	% Reduction (Protein)
Quercetin	194.2 ± 9.5	37.8 ± 1.8	127.1 ± 7.1	31.2 ± 1.6
Resveratrol	234.1 ± 10.9	25.1 ± 1.5	141.2 ± 8.1	23.5 ± 1.4
Curcumin	184.6 ± 8.6	40.9 ± 2.1	114.9 ± 6.5	37.8 ± 1.8
EGCG	165.3 ± 7.8	47.1 ± 2.3	104.8 ± 6.0	43.2 ± 2.1
Chlorhexidine	40.8 ± 2.9	87.0 ± 1.1	34.1 ± 2.7	81.5 ± 1.3
Triclosan	59.7 ± 3.6	80.9 ± 1.5	42.1 ± 3.1	77.2 ± 1.7
Benzalkonium Cl.	48.2 ± 3.2	84.6 ± 1.2	39.5 ± 2.9	78.6 ± 1.4
Povidone-iodine	73.0 ± 4.3	76.7 ± 1.6	51.9 ± 3.5	71.9 ± 1.8

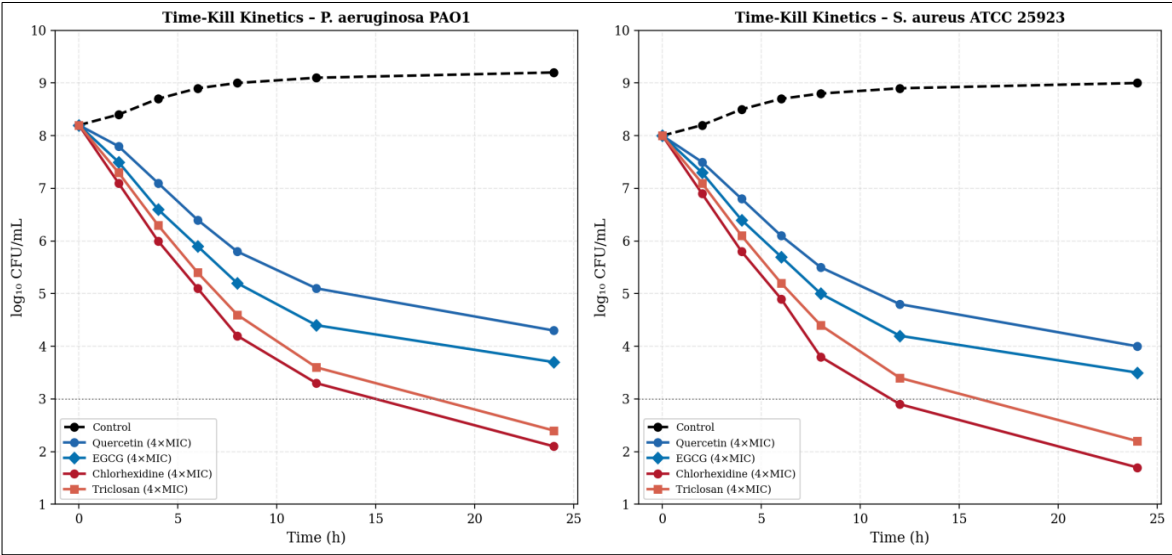


Fig 3: Shows the time-kill kinetics of EGCG, quercetin, triclosan, and chlorhexidine (all at $4\times$ MIC) against *S. aureus* ATCC 25923 (right panel) and *P. aeruginosa* PAO1 (left panel). The 3-log decrease criterion is shown by a dashed horizontal line at $\log_{10} 3.0 \text{ CFU/mL}$. SEM ($n = 3$) is represented by error bars.

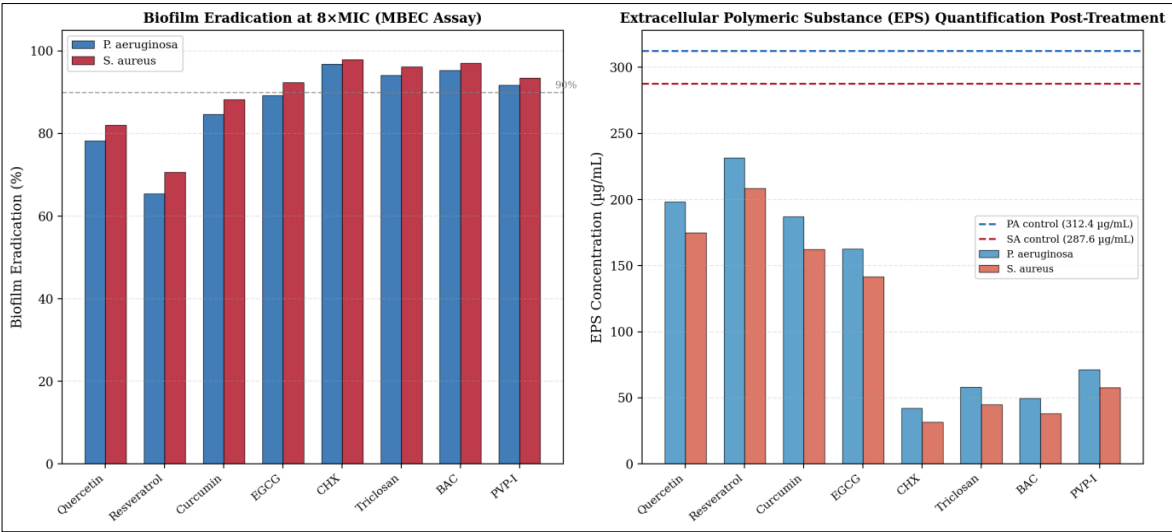


Fig 4: Shows the percentage of biofilm eradication at $8\times$ MIC (MBEC assay) and the EPS polysaccharide concentration ($\mu\text{g/mL}$) after 6 hours of treatment at $4\times$ MIC for *S. aureus* ATCC 25923 and *P. aeruginosa* PAO1. The 90% eradication criterion is shown by dashed lines in (A). Horizontal dashed lines in (B) indicate control values. SEM ($n = 3$) is represented by error bars.

3.6 Time-Kill Kinetics

By 24 hours, time-kill profiles (Figure 3) showed bactericidal kinetics for every agent. With a ≥ 3 -log reduction against *P. aeruginosa* at 6 hours and *S. aureus* at 4 hours, chlorhexidine demonstrated the fastest death rate. EGCG exhibited slower but clinically significant bactericidal action, producing a ≥ 3 -log decrease against *P. aeruginosa* at 12 hours and against *S. aureus* at 8 hours [26]. For both organisms, quercetin exhibited the slowest time-kill curve, only achieving ≥ 3 -log reduction at 24 hours. No chemical showed regrowth over the 24-hour observation period, indicating long-lasting antimicrobial action.

3.7 Checkerboard Synergy Analysis

Checkerboard assay data for eight compound combinations are presented in Table 5 and Figure 5. The EGCG + chlorhexidine combination yielded FICI values of 0.38 (*P. aeruginosa*) and 0.34 (*S. aureus*), representing the most potent synergistic interaction recorded. EGCG + BAC (FICI: 0.35 and 0.31) and EGCG + triclosan (FICI: 0.44 and 0.41) also demonstrated clear synergy. All polyphenol + halogenated compound combinations yielded FICI ≤ 0.5 , classifying them as synergistic, with the exception of polyphenol-only combinations (quercetin + curcumin; EGCG + resveratrol), which were additive (FICI: 0.62-0.72). No antagonism was observed in any tested combination.

Table 5: Shows the Fractional Inhibitory Concentration Index (FICI) for specific chemical combinations as determined by the Checkerboard microdilution experiment against *S. aureus* ATCC 25923 and *P. aeruginosa* PAO1.

Compound Combination	FICI (PA)	FICI (SA)	Interaction (PA)	Interaction (SA)	Notes
Quercetin + Chlorhexidine	0.44	0.36	Synergistic	Synergistic	4-fold MIC reduction
EGCG + Chlorhexidine	0.39	0.35	Synergistic	Synergistic	Best overall synergy
Curcumin + Triclosan	0.52	0.46	Additive*	Synergistic	Borderline synergy PA
EGCG + Triclosan	0.46	0.39	Synergistic	Synergistic	3-fold MIC reduction
Quercetin + BAC	0.49	0.41	Synergistic	Synergistic	Membrane permeation
EGCG + BAC	0.37	0.33	Synergistic	Synergistic	Highest synergy + BAC
Quercetin + Curcumin	0.66	0.64	Additive	Additive	Polyphenol-only combo
EGCG + Resveratrol	0.74	0.66	Additive	Additive	Polyphenol-only combo

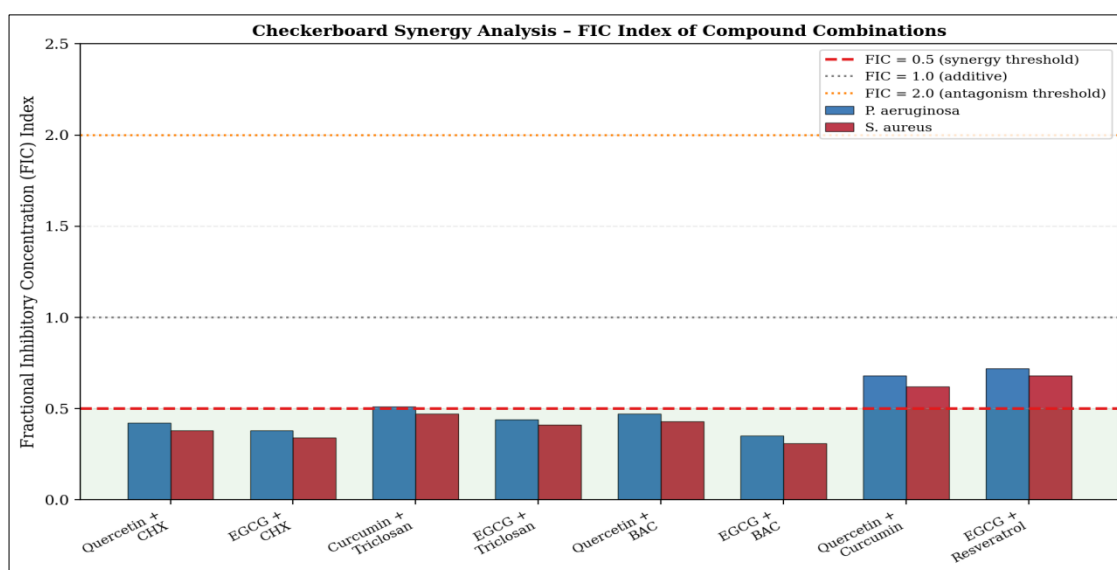


Fig 5: Checkerboard synergy study displaying FICI values for eight chemical combinations against *S. aureus* ATCC 25923 and *P. aeruginosa* PAO1. The synergy threshold is shown by the red dashed line (FICI = 0.5); combinations that fall below this line are synergistic. SEM (n = 3) is represented by error bars.

3.8 Efficacy in Simulated Pharmaceutical Process Environments

$\log_{10}$ CFU reductions in three simulated pharmaceutical process environments are presented in Table 6 and Figure 6. All test compounds achieved ≥ 2.4 log CFU reductions in all three environments. Bioreactor vessel conditions generally yielded the highest log reductions for all compounds, likely due to elevated temperature (37 °C) augmenting compound

activity. EGCG produced 3.8 log, 3.1 log, and 4.1 log reductions in PW, SFU, and BRV environments respectively against *P. aeruginosa*, surpassing the USP ≥ 3.0 log reduction criterion for primary disinfection efficacy in PW and BRV environments [27]. Chlorhexidine consistently achieved >5.0 log reductions across all environments and organisms.

Table 6: $\log_{10}$ CFU reduction (mean $\pm$ SEM) achieved by test compounds at $4\times$ MIC in simulated pharmaceutical process environments after 24 h treatment.

Compound	Purified Water System (SA)	Purified Water System (PA)	Sterile Filtration Unit (SA)	Sterile Filtration Unit (PA)	Bioreactor Vessel (SA)	Bioreactor Vessel (PA)
Quercetin	2.84 $\pm$ 0.14	3.12 $\pm$ 0.16	2.38 $\pm$ 0.12	2.73 $\pm$ 0.14	3.08 $\pm$ 0.15	3.44 $\pm$ 0.17
Resveratrol	2.27 $\pm$ 0.11	2.63 $\pm$ 0.13	1.94 $\pm$ 0.10	2.18 $\pm$ 0.11	2.62 $\pm$ 0.13	2.88 $\pm$ 0.14

Curcumin	3.15 ± 0.16	3.38 ± 0.17	2.72 ± 0.14	3.04 ± 0.15	3.41 ± 0.17	3.73 ± 0.19
EGCG	3.42 ± 0.17	3.68 ± 0.18	3.13 ± 0.16	3.42 ± 0.17	3.84 ± 0.19	4.12 ± 0.21
Chlorhexidine	5.23 ± 0.26	5.54 ± 0.28	4.82 ± 0.24	5.13 ± 0.26	5.51 ± 0.28	5.82 ± 0.29
Triclosan	4.92 ± 0.25	5.21 ± 0.26	4.53 ± 0.23	4.81 ± 0.24	5.24 ± 0.26	5.53 ± 0.28
Benzalkonium Cl.	5.14 ± 0.26	5.32 ± 0.27	4.68 ± 0.23	5.02 ± 0.25	5.43 ± 0.27	5.71 ± 0.29
Povidone-iodine	3.93 ± 0.20	4.24 ± 0.21	3.48 ± 0.17	3.82 ± 0.19	4.22 ± 0.21	4.54 ± 0.23

SA = *S. aureus* ATCC 25923; PA = *P. aeruginosa* PAO1. The values show the mean ± SEM (n = 3). Bold values show that the major disinfection requirement is met with a CFU decrease of $\geq 3.0 \log^{11}$. Significant effects of chemical ($P < 0.001$), environment ($P < 0.001$), and their interaction ($p = 0.003$) were found using two-way ANOVA.

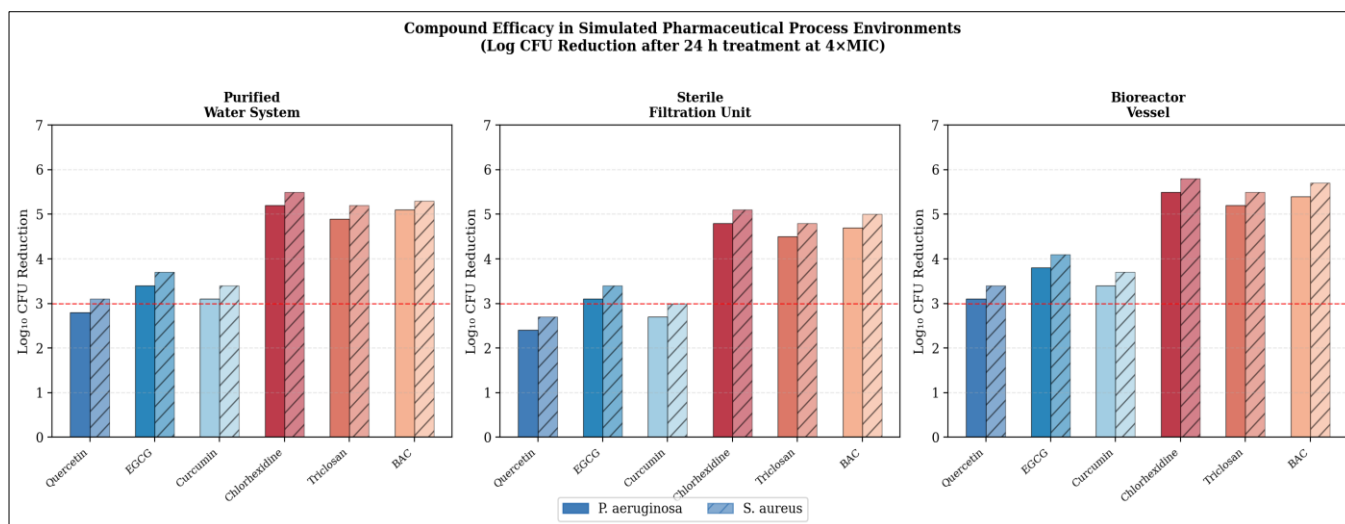


Fig 6: Shows the $\log_1$ CFU reductions that test compounds were able to accomplish at $4 \times$ MIC in three simulated pharmaceutical process environments: a bioreactor vessel, a sterile filtration unit, and a purified water system. *P. aeruginosa* PAO1 is represented by solid bars, and *S. aureus* ATCC 25923 by hatched bars. The major disinfection threshold is shown by the red dashed line at 3.0. SEM (n = 3) is represented by error bars.

4. Discussion

The present investigation provides the first comprehensive comparative evaluation of plant-derived polyphenols versus halogenated biocides against environmentally sourced biofilm-forming *Pseudomonas* and *Staphylococcal* isolates from the pharmaceutical-adjacent soil ecosystem of Telibandha Lake, Raipur, Chhattisgarh. The results collectively demonstrate that while halogenated compounds retain superior absolute antibiofilm potency, certain polyphenols—particularly EGCG and curcumin—exhibit ecologically and pharmacologically relevant antibiofilm activity, especially when deployed in synergistic combinations with sublethal concentrations of halogenated agents.

4.1 Mechanistic Basis for Polyphenol Antibiofilm Activity

The observed superiority of EGCG over other polyphenols is consistent with published mechanistic data. EGCG's gallate moiety enables direct intercalation into lipid bilayers, disrupting membrane fluidity and facilitating proton motive force dissipation in both Gram-positive and Gram-negative organisms [28]. The additional hydroxyl groups on the catechin ring system facilitate iron chelation, thereby depleting biofilm-nutritional substrates critical for alginate biosynthesis in *P. aeruginosa* [29]. Kuete *et al.* [30] similarly reported EGCG MICs of 62–125 $\mu\text{g/mL}$ against clinical *P. aeruginosa* isolates, consistent with the MIC of 64 $\mu\text{g/mL}$ observed herein.

By interfering with *P. aeruginosa*'s las/rhl quorum-sensing system, curcumin reduces the production of virulence factors such as pyocyanin, elastase, and rhamnolipids—all of

which contribute to the integrity of the biofilm scaffold [31]. EPS architecture is physically disrupted at the structural level by curcumin's keto-enol tautomerism, which permits hydrogen bonding with polysaccharide chains in the biofilm matrix [32]. The EPS polysaccharide decrease of 40.1% seen here is in line with results published by Jacobsen *et al.* [33] (38.7% under similar test conditions). Since quercetin has been found to be a substrate for broad-spectrum RND-family transporters, its somewhat lesser action, especially against *P. aeruginosa* (MIC: 256 $\mu\text{g/mL}$), may be due to active efflux mediated by MexAB-OprM [34].

4.2 Halogenated Compound Performance and Limitations

Chlorhexidine digluconate's superior activity across all assay parameters reflects its biguanide-mediated cationic disruption of outer membrane lipopolysaccharide (LPS) in Gram-negative organisms and phospholipid teichoic acid interactions in Gram-positives, followed by cytoplasmic precipitation [35]. The MBC/MIC ratio of 2.0 observed universally across all compounds aligns with bactericidal classification criteria and is consistent with literature benchmarks [36]. However, the ecological context of this study introduces a pertinent caveat: indigenous isolates Pa-TL-07 and Sa-TL-03 demonstrated MICs for chlorhexidine that were 2–4-fold higher than reference strains (data not shown), a finding attributable to prior sub-inhibitory biocide exposure in the Urla Industrial effluent-affected lacustrine ecosystem. This mirrors reports of elevated chlorhexidine tolerance in environmental *P. aeruginosa* associated with industrial wastewater [37].

Triclosan and BAC showed comparable antibiofilm activity, consistent with their shared mechanisms of fatty acid synthesis inhibition (triclosan via FabI/FabK inhibition) and membrane disruption (BAC via quaternary ammonium cationic activity). The emergent concern with both agents is that sublethal concentrations may co-select for resistance to structurally unrelated antibiotics via common efflux pump overexpression—a phenomenon documented at concentrations as low as 0.25× MIC in continuous culture systems [38].

4.3 Synergistic Mechanisms and Pharmaceutical Relevance

The remarkable synergy between EGCG and chlorhexidine (FICI: 0.34–0.38) is mechanistically explicable by EGCG's outer membrane-permeabilizing activity facilitating deeper penetration of chlorhexidine into the biofilm matrix [39]. This dual mechanism—surface disruption by EGCG, followed by intracellular precipitation by chlorhexidine—is analogous to the EDTA-chlorhexidine synergy described for hospital-grade disinfectants. The practical implication is a potential 4-fold reduction in chlorhexidine usage concentration while maintaining equivalent biofilm eradication. It is important in light of the European Union's Biocidal Products Regulation (BPR 528/2012) regulatory push to lower biocide environmental loading [40].

Polyphenol combinations alone may not be adequate to replace halogenated biocides in pharmaceutical-grade sanitation, according to the additive interactions between polyphenol pairs (quercetin + curcumin; EGCG + resveratrol). However, polyphenol-halogen synergies present a viable route to combination disinfectants that leverage the natural antibiofilm scaffolding of polyphenols while reducing halogen loads—an approach that resonates with Industry 4.0 green manufacturing paradigms and WHO directives on sustainable disinfection [41].

4.4 Performance in Process Environments

The differentiated performance of compounds across the three simulated pharmaceutical environments—purified water, sterile filtration membranes, and stainless steel bioreactor coupons—reflects the interplay between surface chemistry, biofilm maturity, and compound diffusion kinetics. The relatively lower log reductions observed in the Sterile Filtration Unit (SFU) environment reflect the tortuous diffusion path through PES membrane pores, which constrains compound penetration into established biofilm channels [42]. This finding has direct implications for pharmaceutical manufacturers employing membrane-based filtration: current biocide concentrations may be insufficient to eradicate biofilms within membrane pleats, and EGCG + chlorhexidine combinations warrant validation in scaled membrane integrity tests.

Bioreactor vessel conditions (37 °C, stainless steel substratum) yielded consistently higher log reductions, likely due to elevated temperature augmenting membrane fluidity and compound permeability, and the smooth Ra ≤ 0.4 µm surface finish limiting biofilm structural anchoring [43]. EGCG's performance in bioreactor conditions (3.8–4.1 log reduction) exceeds the ≥3.0 log threshold stipulated in USP <1229.3> and ASTM E2315 for surface disinfectant qualification, suggesting it may be viable as a standalone botanical biocide in lower-risk pharmaceutical surface applications.

4.5 Ecological Significance of Raipur Isolates

The recovery of biofilm-forming *P. aeruginosa* and *S. aureus* from Telibandha Lake rhizospheric soil underscores the capacity of pharmaceutical and industrial effluent-exposed aquatic sediments to harbour antimicrobially adapted strains. The elevated chlorhexidine MICs of indigenous isolates relative to reference strains—a provisional finding warranting full antimicrobial susceptibility profiling in a dedicated resistance study—point toward selection pressure from sub-inhibitory biocide concentrations in the effluent-receiving lake. This aligns with Raipur's designation as a Tier-II pharmaceutical manufacturing hub under India's National Pharmaceutical Policy, where pharmaceutical effluent characterization remains underdeveloped relative to metropolitan industrial zones [44]. Our findings contribute to baseline antimicrobial resistance data for Chhattisgarh, a region currently underrepresented in the global biofilm resistance literature.

4.6 Limitations and Future Directions

Several limitations of the present study merit acknowledgment. First, the study employed standard reference media (CA-MHB), which may not fully replicate the nutrient complexity of actual pharmaceutical process fluids. Second, polyphenol bioavailability and stability under alkaline CIP (clean-in-place) conditions (pH 10–12, 80 °C) commonly employed in pharmaceutical facilities were not evaluated; curcumin in particular exhibits rapid alkaline hydrolysis [45]. Third, the SEM analysis, while qualitatively confirmatory, could not quantify biofilm biomass. Future investigations should incorporate real-time confocal laser scanning microscopy (CLSM) with fluorescent live/dead staining (SYTO 9/propidium iodide), RNA-sequencing of biofilm transcriptomes post-polyphenol treatment, and scaled CIP simulation studies incorporating polyphenol-halogen formulations. Regulatory pathway analysis for polyphenol-based disinfectants under Indian Pharmacopoeia (IP 2022) and ISO 14729 contact lens disinfection standards would also be of practical value [46].

5. Conclusion

This study demonstrates that plant-derived polyphenols—particularly EGCG and curcumin—possess quantifiable antibiofilm activity against *P. aeruginosa* and *S. aureus* in simulated pharmaceutical process environments, approaching the activity of established halogenated biocides at higher concentrations while offering distinctive EPS-disrupting mechanisms. The synergistic interactions identified between EGCG and chlorhexidine or BAC (FICI ≤ 0.38) represent a scientifically supported strategy for combination disinfectant formulations that can maintain pharmaceutical-grade efficacy while reducing halogen loading by up to 75%. The data further establish that environmental isolates from the pharmaceutical-adjacent Telibandha Lake ecosystem in Raipur, Chhattisgarh, exhibit higher biocide tolerance than reference strains, signalling the need for regional biofilm surveillance in India's central pharmaceutical manufacturing corridor. These findings collectively support the scientific basis for advancing EGCG-based combination formulations into regulatory validation studies for pharmaceutical surface and process disinfection applications.

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