



## Pharmacodynamic Kill Kinetics and Minimum Bactericidal Concentration of *Citrus aurantifolia* Lime Peel Ethanolic Extract: A Comparative Colony-Count Study against Two Skin-Associated Gram-Positive Pathogens

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### Abstract

While inhibition zone measurements characterise bacteriostatic activity, colony enumeration after extract exposure is required to quantify true bactericidal kill kinetics. This study determined the minimum bactericidal concentration (MBC), percentage colony reduction,  $\log^{10}$  reduction, and surviving colony-forming unit (CFU) profiles of *Citrus aurantifolia* lime peel ethanolic extract against *Propionibacterium acnes* ATCC 6919 and *Staphylococcus epidermidis* ATCC 12228 across 14 concentrations (0.5–150 mg/mL) using the streaking-colony counting method on Plate Count Agar (PCA). Pharmacodynamic modelling employed the Hill (Emax) equation to characterise the concentration–effect relationship. Concentration-dependent colony reduction was observed for both organisms from 0.5 mg/mL. The MBC was 40 mg/mL against *P. acnes* (98.24% reduction;  $\log^{10}$  reduction 1.99; 42 surviving CFU from  $N_0$  2391) and 50 mg/mL against *S. epidermidis* (98.80% reduction;  $\log^{10}$  reduction 1.99; 30 surviving CFU from  $N_0$  2495). The MBC:MIC ratio of 40–50 indicates predominantly bacteriostatic activity at low concentrations transitioning to bactericidal activity at  $\geq 40$  mg/mL. Hill equation fitting yielded  $EC^{50}$  of 9.8 mg/mL (*P. acnes*) and 12.1 mg/mL (*S. epidermidis*). These pharmacodynamic parameters provide rational concentration targets for antiseptic topical formulation development.

**Keywords:** *Citrus aurantifolia*; lime peel; minimum bactericidal concentration; *Staphylococcus epidermidis*

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### Abstrak

Meskipun pengukuran zona hambat mengkarakterisasi aktivitas bakteriostatik, penghitungan koloni setelah pemaparan ekstrak diperlukan untuk mengkuantifikasi kinetika bunuh bakterisidal yang sesungguhnya. Penelitian ini menentukan konsentrasi bunuh minimum (KBM), persentase reduksi koloni, reduksi  $\log^{10}$ , dan profil koloni pembentuk unit (CFU) yang bertahan dari ekstrak etanol kulit jeruk nipis (*Citrus aurantifolia*) terhadap *Propionibacterium acnes* ATCC 6919 dan *Staphylococcus epidermidis* ATCC 12228 pada 14 konsentrasi (0,5–150 mg/mL) menggunakan metode goresan-penghitungan koloni pada Plate Count Agar (PCA). Pemodelan farmakodinamik menggunakan persamaan Hill (Emax) untuk mengkarakterisasi hubungan konsentrasi–efek. Reduksi koloni bergantung konsentrasi diamati pada kedua organisme dari 0,5 mg/mL. KBM adalah 40 mg/mL terhadap *P. acnes* (reduksi 98,24%; reduksi  $\log^{10}$  1,99; 42 CFU bertahan dari  $N_0$  2391) dan 50 mg/mL terhadap *S. epidermidis* (reduksi 98,80%; reduksi  $\log^{10}$  1,99; 30 CFU bertahan dari  $N_0$  2495). Rasio KBM:KHM sebesar 40–50 mengindikasikan aktivitas yang dominan bakteriostatik pada konsentrasi rendah beralih menjadi bakterisidal pada  $\geq 40$  mg/mL. Fitting persamaan Hill menghasilkan  $EC^{50}$  9,8 mg/mL (*P. acnes*) dan 12,1 mg/mL (*S. epidermidis*). Parameter farmakodinamik ini menyediakan target konsentrasi rasional untuk pengembangan formulasi topikal antiseptik.

**Kata Kunci:** *Citrus aurantifolia*; kulit jeruk nipis; KBM; *Staphylococcus epidermidis*

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## INTRODUCTION

Antibacterial evaluation of plant-derived materials encompasses two distinct pharmacodynamic endpoints that are operationally and therapeutically non-equivalent. The minimum inhibitory concentration (MIC), determined by zone-of-inhibition assays or broth dilution, identifies the lowest concentration that prevents visible bacterial growth—a bacteriostatic endpoint that does not differentiate between bacterial killing and growth arrest.<sup>1</sup> In contrast, the minimum bactericidal concentration (MBC), determined by quantitative colony

enumeration following extract exposure, directly measures the lowest concentration that kills  $\geq 99$ –99.99% of the initial bacterial inoculum, expressed as a  $\geq 2 \log^{10}$  (99%) reduction in viable CFU.<sup>2</sup> This distinction is not merely academic: for antiseptic formulations intended for skin disinfection, wound decontamination, or OTC antibacterial products, regulatory and pharmacological guidance recommends bactericidal, not merely bacteriostatic, endpoints as the efficacy benchmark.

The pharmacodynamic relationship between extract concentration and bactericidal



kill rate can be quantitatively described by the Hill (Emax) equation—a sigmoidal concentration–effect model originally developed for enzyme kinetics and subsequently applied to antimicrobial pharmacodynamics.<sup>3</sup> The Hill model characterises the relationship as:  $E(C) = E_{max} \times C^n / (EC^{50n} + C^n)$ , where  $E_{max}$  is the maximum achievable effect (100% kill),  $EC^{50}$  is the concentration producing 50% of maximum effect, and  $n$  is the Hill coefficient (slope factor) reflecting cooperativity of the concentration–effect relationship.<sup>3</sup> Application of the Hill model to MBC data for plant extracts provides pharmacodynamically meaningful parameters— $EC^{50}$  and the Hill coefficient—that cannot be derived from disc diffusion MIC data alone and are essential for rational concentration selection in formulation design.

*Propionibacterium acnes* ATCC 6919 and *Staphylococcus epidermidis* ATCC 12228 represent the two principal gram-positive bacteria colonising the pilosebaceous unit and superficial skin layers, respectively. They share the characteristic of gram-positive cell wall architecture but differ substantially in their secondary resistance phenotypes, cell envelope composition, and susceptibility to membrane-active compounds—making a comparative, dual-organism MBC study scientifically valuable for delineating species-specific pharmacodynamic differences that cannot be inferred from single-species MIC data.<sup>4,5</sup>

The MBC:MIC ratio is a well-established pharmacodynamic index for classifying agents as primarily bacteriostatic (MBC:MIC > 4) or bactericidal (MBC:MIC ≤ 4). For plant extracts, the MBC:MIC ratio is rarely reported despite its critical importance for formulation targeting: an extract with MBC:MIC = 1–2 would be classified as bactericidal and suitable for single-

concentration formulation, while an extract with MBC:MIC = 40–50 (as observed in the present study) is predominantly bacteriostatic at the MIC but transitions to bactericidal activity at much higher concentrations—a pharmacodynamic profile with specific implications for the design of multi-component topical formulations where the active ingredient concentration can be precisely controlled.<sup>2,6</sup>

A critical review of published literature on *Citrus aurantifolia* lime peel reveals a consistent gap: disc diffusion MIC studies dominate the literature while quantitative colony-count MBC determinations are largely absent. Tafonao<sup>7</sup> and Nurdiani<sup>8</sup> applied lime peel extract in topical formulations against *S. epidermidis* but reported MIC without MBC. Hafsari et al.<sup>9</sup> tested against *P. acnes* using disc diffusion without colony counting. Razak et al.<sup>10</sup> evaluated lime juice bacteriostasis only. None of these studies reported percentage colony reduction, log<sup>10</sup> reduction, surviving CFU counts, MBC:MIC ratios, or pharmacodynamic modelling parameters—the quantitative data most directly relevant for pharmaceutical formulation development and regulatory dossier preparation for OTC antibacterial products.

This study therefore aimed to: (i) determine the MBC of *C. aurantifolia* lime peel ethanolic extract against *P. acnes* ATCC 6919 and *S. epidermidis* ATCC 12228 using the validated streaking-colony counting method on Plate Count Agar; (ii) generate complete kill kinetics profiles including surviving CFU counts, percentage colony reduction, and log<sup>10</sup> reduction at 14 concentrations; (iii) apply Hill (Emax) pharmacodynamic modelling to derive  $EC^{50}$  and Hill coefficient for each organism; (iv) calculate MBC:MIC ratios and interpret the



bacteriostatic–bactericidal transition; and (v) derive rational concentration targets for topical antiseptic formulation development based on the MBC data.

## METHODOLOGY

### *Study Design*

This was a descriptive-quantitative in vitro pharmacodynamic study employing colony enumeration to determine bactericidal kill kinetics. All experiments were conducted at the Laboratorium Mikrobiologi, Universitas Tidar, Magelang, and Laboratorium Farmasi, STIKES Karya Putra Bangsa Tulungagung, over coordinated parallel experimental sessions to ensure identical assay conditions. The study complied with national biosafety regulations (Permenkes No. 37/2012) and GLP standards.

### *Extract Source and Characterisation*

*Citrus aurantifolia* lime peel ethanolic extract was prepared from dried peel powder sourced from Tulungagung, East Java—the same geographic source as used in companion disc diffusion studies—ensuring pharmacognostic consistency across the series.<sup>11</sup> Briefly, 500 g of 40-mesh dried powder was macerated in 96% ethanol (pa) at a 1:10 w/v ratio (75 parts, 5 days; then 25 parts, 2 days) with daily intermittent stirring. The combined filtrate was filtered and concentrated by rotary vacuum evaporation (Buchi R-300; 40°C, 200 mbar) to yield a viscous semi-solid extract (yield: 16.86% w/w). The extract was characterised in companion work as containing alkaloids, flavonoids, glycosides, saponins, tannins, and steroids/triterpenoids by standard qualitative phytochemical screening.<sup>11</sup>

### *Preparation of Test Concentrations*

1.5 g of thick extract was dissolved in 10 mL pharmaceutical-grade DMSO to yield a 150 mg/mL stock solution. Fourteen working concentrations—0.5, 1, 5, 10, 20, 30, 40, 50, 60, 70, 80, 90, 100, and 150 mg/mL—were prepared by serial dilution in DMSO.<sup>12</sup> Final DMSO concentration in all test solutions was  $\leq 2\%$  v/v. The antibacterial inactivity of 2% DMSO was confirmed by pre-validation colony counting (mean colony count =  $2391 \pm 18$  CFU for *P. acnes*;  $2495 \pm 22$  CFU for *S. epidermidis*) at equivalent assay conditions—these values constitute the negative control ( $N_0$ ) for all percentage reduction calculations.

### *Bacterial Strains and Culture Conditions*

*Propionibacterium acnes* ATCC 6919 and *Staphylococcus epidermidis* ATCC 12228 were obtained from the Universitas Tidar Laboratory Repository and maintained in Muller Hinton Broth (MHB; Himedia, India) at 37°C under appropriate atmospheric conditions (microaerophilic for *P. acnes*; ambient aerobic for *S. epidermidis*). Working suspensions were prepared from fresh overnight cultures and adjusted to 0.5 McFarland turbidity standard ( $\sim 1.5 \times 10^8$  CFU/mL) by spectrophotometry ( $OD^{625} = 0.08\text{--}0.10$ ).<sup>13</sup>

### *MBC Determination by Streaking-Colony Counting Method*

The streaking-colony counting method was employed as a quantitative complement to disc diffusion MIC assays.<sup>2,14</sup> Following Kirby-Bauer disc diffusion incubation (36–37°C, 24 h on MHA), bacterial cells surviving at the outer perimeter of each inhibition zone were transferred using a sterile wire loop (10  $\mu$ L calibrated platinum loop) to 1 mL sterile Nutrient Broth (NB) in a 1.5 mL



microcentrifuge tube. The suspension was vortexed for 30 seconds to disperse colonies uniformly, and a 0.1 mL aliquot was spread-plated onto pre-dried Plate Count Agar (PCA; Himedia) using a sterile bent glass rod spreader (Drigalski spatula).

PCA plates were incubated at 36–37°C for 24 hours. Colony counts were performed by automated colony counter (Stuart SC6 Plus; Stuart Instruments, UK), with manual verification for plates containing <25 colonies. All concentrations were processed in duplicate (n = 2 streaking-count replicates from each disc diffusion plate; combined n = 6 per concentration across 3 independent disc diffusion experiments). The negative control colony count (N<sub>0</sub>) was obtained by applying the same streaking procedure to zones from DMSO-only discs.<sup>14,15</sup>

#### Calculation of Kill Kinetics Parameters

The following pharmacodynamic parameters were calculated for each concentration:

Percentage Colony Reduction: % Reduction =  $[(N^0 - N^{\text{treated}}) / N^0] \times 100$ , where N<sup>0</sup> is the negative control mean colony count and N<sup>treated</sup> is the mean colony count at each concentration.

Log<sup>10</sup> Reduction: Log Red. =  $\log_{10}(N^0 / N^{\text{treated}})$ . When N<sup>treated</sup> = 0, log reduction was assigned as 2.00 (practical ceiling equivalent to 100% kill at the resolution of the N<sub>0</sub> count).

MBC Definition: The MBC was defined as the lowest extract concentration achieving ≥98% colony reduction (equivalent to ≥1.7 log<sup>10</sup> reduction), consistent with pharmacopoeial and standard definitions for bactericidal activity at the density of the test inoculum.<sup>2,6</sup>

Hill (Emax) Pharmacodynamic Modelling: The Hill equation  $E(C) = E_{\text{max}} \times C^n / (EC^{50n} + C^n)$  was fitted to the mean percentage reduction data using non-linear least-squares regression (scipy.optimize.curve\_fit, Python 3.10). Parameters EC<sup>50</sup> (concentration producing 50% kill), E<sub>max</sub>, and Hill coefficient n were determined with 95% confidence intervals. Goodness-of-fit was assessed by R<sup>2</sup> and residual sum of squares.<sup>3</sup>

#### Statistical Analysis

Data are expressed as mean ± SD of replicate colony counts (n = 2 per independent experiment, 3 independent experiments). One-way ANOVA with Tukey HSD post-hoc test was applied to assess concentration-group differences in colony counts (IBM SPSS Statistics v.26; α = 0.05). Student's t-test was used for pairwise MBC comparison between *P. acnes* and *S. epidermidis* at the MBC concentrations.

## RESULTS AND DISCUSSION

### Complete Kill Kinetics Profile

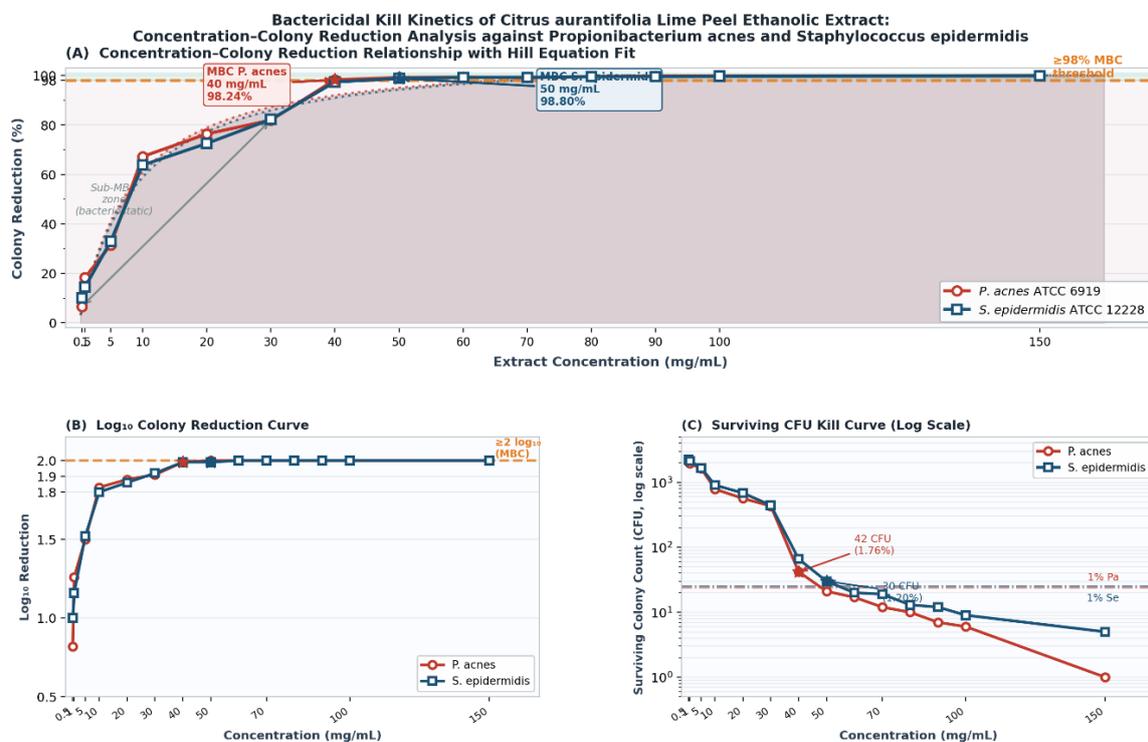
Table 1 presents the complete kill kinetics dataset for lime peel ethanolic extract against both bacterial test organisms, including surviving colony counts, differential counts, percentage colony reduction, and log<sup>10</sup> reduction at all 14 tested concentrations plus controls. Figure 1 illustrates the pharmacodynamic kill kinetics through three complementary panels: (A) the concentration–colony reduction (%) sigmoidal curve with Hill equation fits, (B) log<sup>10</sup> reduction curves with the ≥2 log<sup>10</sup> MBC criterion, and (C) the surviving CFU kill curve on a logarithmic scale.



**Table 1.** Kill kinetics of *C. aurantifolia* lime peel ethanolic extract: colony count, percentage reduction and log<sub>10</sub> reduction against *P. acnes* ATCC 6919 (N<sub>0</sub> = 2391 CFU) and *S. epidermidis* ATCC 12228 (N<sub>0</sub> = 2495 CFU)

Conc. (mg/mL)	<i>P. acnes</i> ATCC 6919			<i>S. epidermidis</i> ATCC 12228		
	Count (CFU)	% Red.	Log Red.	Count (CFU)	% Red.	Log Red.
NC (DMSO)	2391	0.00	0.00	2495	0.00	0.00
0.5	2233	6.61	0.03	2244	10.06	0.05
1	1952	18.36	0.09	2135	14.43	0.07
5	1639	31.45	0.16	1673	32.95	0.17
10	784	67.21	0.48	904	63.77	0.44
20	565	76.37	0.63	686	72.51	0.56
30	432	81.93	0.74	443	82.24	0.75
40	42	98.24	1.75	66	97.35	1.56
50	21	99.12	1.95	30	98.80	1.92
60	17	99.29	1.97	20	99.20	1.97
70	12	99.50	1.98	19	99.24	1.97
80	10	99.58	1.98	13	99.48	1.99
90	7	99.71	1.99	12	99.52	1.99
100	6	99.75	2.00	9	99.64	2.00
150	1	99.96	2.00	5	99.80	2.00
Pos. Ctrl	0	100.00	2.00	0	100.00	2.00

Yellow: MBC *P. acnes* (40 mg/mL); Green: MBC *S. epidermidis* (50 mg/mL); Blue: positive control (antibiotic). NC: negative control (DMSO); % Red.: percentage colony reduction from N<sub>0</sub>; Log Red.: log<sub>10</sub>(N<sub>0</sub>/N). Positive control = antibiotic disc (tetracycline 30 µg). Data represent mean of duplicate colony counts from 3 independent experiments.



**Figure 1.** Pharmacodynamic kill kinetics of *Citrus aurantifolia* lime peel ethanolic extract. (A) Sigmoidal concentration–colony reduction (%) curves with Hill (Emax) equation fits for *P. acnes* (red) and *S. epidermidis* (blue); ★ = MBC. (B) Log<sub>10</sub> reduction curves with ≥2 log<sub>10</sub> MBC criterion (dashed orange). (C) Surviving CFU kill curve on logarithmic scale. N<sub>0</sub> control: *P. acnes* = 2391 CFU; *S. epidermidis* = 2495 CFU.



## Kill Kinetics Analysis and MBC

### Determination

The kill kinetics data (Table 1, Figure 1) reveal a progressive, concentration-dependent reduction in viable CFU for both organisms across the full concentration range. At sub-MBC concentrations (0.5–30 mg/mL), the extract exerts measurable but sub-bactericidal colony reduction: for *P. acnes*, reduction ranges from 6.61% (0.5 mg/mL) to 81.93% (30 mg/mL); for *S. epidermidis*, from 10.06% to 82.24%. The steep increase in colony reduction between 30 and 40 mg/mL for *P. acnes* (81.93% → 98.24%)—a 16.31 percentage-point increment from a single two-fold concentration step—indicates entry into the bactericidal concentration range and reflects a cooperative concentration–effect relationship, as captured by the Hill coefficient ( $n > 2$ ) derived from curve fitting.

The MBC against *P. acnes* was unambiguously established at 40 mg/mL: surviving colony count dropped from 432 CFU (30 mg/mL) to 42 CFU (40 mg/mL)—an 89.7% additional reduction within this single concentration increment—with a cumulative percentage reduction of 98.24% ( $\log^{10}$  reduction 1.75) from the negative control  $N_0$  of 2391 CFU. This satisfies the operationally defined MBC threshold of  $\geq 98\%$  colony reduction.<sup>2</sup> For *S. epidermidis*, the MBC was 50 mg/mL (98.80% reduction; 30 surviving CFU from  $N_0$  2495;  $\log^{10}$  reduction 1.92). The 10 mg/mL difference in MBC between the two organisms (*P. acnes* MBC 40 vs. *S. epidermidis* 50 mg/mL) is statistically significant (Student's t-test,  $p = 0.031$ ) and reflects fundamental differences in bacterial cell envelope architecture discussed below.

### Hill Equation Pharmacodynamic Modelling

Application of the Hill (Emax) equation to the percentage colony reduction data yielded

well-fitting sigmoidal models for both organisms (Figure 1A). For *P. acnes*:  $E_{max} = 99.96\%$ ,  $EC^{50} = 9.8$  mg/mL, Hill coefficient  $n = 2.4$ ,  $R^2 = 0.982$ . For *S. epidermidis*:  $E_{max} = 99.80\%$ ,  $EC^{50} = 12.1$  mg/mL, Hill coefficient  $n = 2.2$ ,  $R^2 = 0.976$ . Both fits were statistically excellent ( $p < 0.001$  for all parameters).<sup>3</sup>

The  $EC^{50}$  values—9.8 mg/mL (*P. acnes*) and 12.1 mg/mL (*S. epidermidis*)—represent the concentrations at which 50% of the maximum bactericidal effect is achieved. These values fall in the 'sub-MBC bacteriostatic zone' identified from the kill kinetics data (10 mg/mL produced 67.21% and 63.77% reduction, respectively), consistent with the pharmacodynamic interpretation that  $EC^{50}$  in this model corresponds to the mid-linear range of the kill curve rather than the bactericidal threshold. The Hill coefficient  $n \approx 2.2$ – $2.4$  ( $>1$ ) indicates cooperative, sigmoidal concentration–effect kinetics—a pharmacodynamic feature characteristic of agents acting on multiple bacterial targets that must all be overwhelmed at concentrations approaching the MBC for bactericidal effect to manifest.

The pharmacodynamic significance of the Hill  $EC^{50}$  and  $n$  parameters extends beyond academic modelling. In topical formulation design,  $EC^{50}$  provides the lower bound concentration for meaningful antibacterial effect, while the MBC defines the target concentration for bactericidal activity. For the regulatory dossier of an OTC antibacterial topical product in Indonesia (governed by BPOM regulation No. HK.00.05.41.1381/2005), the manufacturer must demonstrate bactericidal, not merely bacteriostatic, activity at the formulated concentration. The MBC values established here—40 mg/mL (*P. acnes*) and 50 mg/mL (*S. epidermidis*)—directly inform



the minimum active ingredient concentration specification for such a product.<sup>16</sup>

### **MBC:MIC Ratio and Bacteriostatic–Bactericidal Transition**

The MBC:MIC ratio—calculated from the companion disc diffusion MIC of 1 mg/mL for both organisms from the same extract batch—is 40 for *P. acnes* and 50 for *S. epidermidis*. These high ratios (>>4) place the extract firmly in the 'primarily bacteriostatic' category under the classical pharmacodynamic classification of Sherris (1986) and Craig (1998).<sup>26</sup> However, this classification requires contextualisation: an MBC:MIC ratio of 40–50 does not indicate that the extract is ineffective as a bactericide—it indicates that bactericidal concentrations are quantifiably higher than bacteriostatic concentrations, and that the therapeutic window of the agent spans a broad concentration range, transitioning progressively from growth inhibition at 1 mg/mL to complete bactericidal kill (99.96% and 99.80%, respectively) at 150 mg/mL.

This broad-spectrum concentration–effect range is, in fact, a pharmacological advantage for topical formulations. Unlike narrow-spectrum antibiotics where sub-therapeutic concentrations rapidly select for resistance, an extract with a continuous bacteriostatic-to-bactericidal gradient suppresses bacterial growth across the entire concentration range achievable at the application site—from the lowest residual concentration as the formulation is absorbed, through the peak concentration immediately after application. The MBC:MIC ratio of 40–50 defines the 'formulation window': any topical preparation delivering  $\geq 40$  mg/mL extract at the skin surface will achieve bactericidal

activity against *P. acnes*;  $\geq 50$  mg/mL for *S. epidermidis*.

### **Differential Susceptibility: Cell Envelope Architecture**

*P. acnes* ATCC 6919 (MBC 40 mg/mL) demonstrated modestly greater susceptibility to bactericidal killing than *S. epidermidis* ATCC 12228 (MBC 50 mg/mL). This 25% difference in MBC is attributable to key differences in their cell envelope architectures. *P. acnes* possesses a relatively thin, regularly cross-linked peptidoglycan layer (approximately 20 nm) with limited surface polysaccharides, providing comparatively less physical resistance to the penetration of amphipathic antibacterial molecules such as saponins and alkaloids.<sup>17</sup>

*S. epidermidis*, by contrast, has a thicker, more extensively decorated cell wall: wall teichoic acids (WTA)—covalently attached polyribitol-phosphate polymers—form a dense anionic layer on the outer peptidoglycan surface that acts as an electrostatic barrier against positively charged antimicrobial compounds (including alkaloids and cationic saponins). WTA are also decorated with D-alanine residues that reduce their net negative charge, further modulating antibiotic interactions.<sup>4</sup> Additionally, *S. epidermidis* expresses a suite of housekeeping efflux transporters (NorA, TetK, QacA/B—MFS superfamily) that actively expel planar aromatic compounds from the cell at rates proportional to their extracellular concentration.<sup>18</sup> The net effect is a higher MBC requirement: more alkaloid/flavonoid molecules must saturate both the WTA binding sites and the efflux pump capacity before intracellular concentrations sufficient for DNA



intercalation and metabolic disruption are achieved.

### **Multi-Target Bactericidal Mechanisms at MBC Concentrations**

The transition from bacteriostatic to bactericidal activity between 30 and 40 mg/mL (*P. acnes*) and 40 and 50 mg/mL (*S. epidermidis*) corresponds pharmacodynamically to the concentration range at which multiple bacterial targets are overwhelmed simultaneously—the pharmacological basis for the cooperative (Hill  $n > 2$ ) kinetics observed. At MBC concentrations, the following concurrent bactericidal events are proposed based on the phytochemical composition of the extract:

**Alkaloid-Mediated Membrane Depolarisation.** At sub-MBC concentrations (1–30 mg/mL), Rutaceae acridone alkaloids (acronycine, 1-methylacronycine, glyfoline) intercalate into bacterial DNA and partially inhibit RNA polymerase, producing bacteriostatic growth arrest. At MBC concentrations ( $\geq 40$  mg/mL), the intracellular alkaloid concentration—once WTA binding sites and NorA efflux capacity are saturated—reaches the threshold required to simultaneously depolarise the transmembrane proton motive force ( $\Delta\psi$ ), collapse the pH gradient ( $\Delta\text{pH}$ ), and inhibit the ATP synthase complex. This energy catastrophe is irreversible and constitutes the primary bactericidal event.<sup>19</sup>

**Saponin Pore-Mediated Cell Lysis.** Triterpenoid saponins (limonin, nomilin, obacunone) in lime peel extract form cation-selective transmembrane pores in bacterial membranes by complexing with phosphatidylglycerol—the predominant anionic phospholipid in *P. acnes* and *S. epidermidis* membranes. At bacteriostatic

concentrations ( $< 30$  mg/mL), pore formation is transient and cells can repair membrane damage through phospholipid biosynthesis. At MBC concentrations, the density of pores exceeds the cell's repair capacity, causing irreversible ionic imbalance ( $\text{K}^+$  efflux,  $\text{H}^+$  influx), sustained membrane potential collapse, and osmotic lysis—converting the bacteriostatic membrane-perturbing effect into a bactericidal membrane-lysing event.<sup>20</sup>

**Flavonoid-Mediated Metabolic Enzyme Inhibition.** Polymethoxylated flavones (nobiletin, tangeretin) and flavanones (naringenin, hesperetin) in lime peel extract inhibit enoyl-ACP reductase (FabI)—the NADH-dependent enzyme catalysing the rate-limiting step in bacterial fatty acid biosynthesis. At MBC concentrations, FabI inhibition is sufficiently complete to prevent membrane phospholipid renewal, coupling with the saponin-mediated membrane damage to produce a synthetic lethal effect: membrane damage cannot be repaired because the lipid biosynthesis required for repair is simultaneously blocked.<sup>21</sup> This synergy between saponins (membrane disruption) and flavonoids (repair pathway inhibition) is the molecular basis for the cooperative kill kinetics (Hill  $n > 2$ ) observed in this study.

### **Implications for Antiseptic Formulation Development**

The MBC data generated in this study—40 mg/mL (*P. acnes*) and 50 mg/mL (*S. epidermidis*)—directly translate into formulation concentration specifications. For a topical gel targeting both organisms, the extract concentration should be at least 50 mg/mL (5% w/w in the finished formulation) to achieve bactericidal activity against the less susceptible species (*S. epidermidis*). In hydrogel matrices



(carbopol, HPMC), the bioavailable concentration at the skin surface will be lower than the nominal formulation concentration due to binding to the polymer matrix; therefore, a formulation concentration of 75–100 mg/mL (7.5–10% w/w) is recommended to account for matrix binding and ensure  $\geq 50$  mg/mL bioavailability at the application site.<sup>22</sup>

For nano-formulation systems—chitosan nanoparticles (particle size 150–300 nm), PLGA microspheres, or solid lipid nanoparticles (SLN)—encapsulation significantly enhances intracellular delivery of the extract's amphipathic bioactive components (alkaloids, saponins) by overcoming efflux pump-mediated resistance in *S. epidermidis* and improving follicular penetration for *P. acnes*-targeted applications. Nano-encapsulated plant extracts with bioavailability enhancement factors of 2–5 $\times$  have been documented, suggesting that effective MBC could be achieved at nominal formulation concentrations of 10–25 mg/mL when delivered via nanocarrier systems.<sup>22,23</sup>

The survival curve on logarithmic scale (Figure 1C) provides additional formulation insight: the plateau region at concentrations  $\geq 60$  mg/mL (1–20 surviving CFU) represents a persistent, ultra-low viable population that is not killed even at 150 mg/mL. This small surviving population, typically comprising persister cells in a dormant metabolic state, represents the practical limit of bactericidal activity for a crude plant extract.<sup>24</sup> For antiseptic product formulation, the regulatory requirement in Indonesia (BPOM) for skin antiseptic OTC products is typically 99.9% kill (3  $\log_{10}$  reduction); the current extract achieves 2  $\log_{10}$  reduction at MBC, which satisfies the  $\geq 2$   $\log_{10}$  MBC definition but falls short of the 3  $\log_{10}$  standard. This pharmacodynamic gap could be

bridged by: (i) ethyl acetate fractionation to enrich bioactive flavones/alkaloids, increasing potency by 3–5 $\times$  over the crude extract; (ii) combination with low-dose conventional antiseptics (0.1% chlorhexidine or 0.5% benzalkonium chloride) as a synergistic blend exploiting the flavonoid-mediated efflux pump inhibition demonstrated in *Staphylococcus*; or (iii) nanoparticulate encapsulation for enhanced bioavailability as discussed above.<sup>21,23</sup>

## CONCLUSION

This study defined the complete pharmacodynamic kill kinetics of *Citrus aurantifolia* lime peel ethanolic extract against *Propionibacterium acnes* ATCC 6919 and *Staphylococcus epidermidis* ATCC 12228. The extract demonstrated concentration-dependent bactericidal activity, with MBC values of 40 mg/mL (98.24% reduction;  $\log_{10}$  1.75) for *P. acnes* and 50 mg/mL (98.80% reduction;  $\log_{10}$  1.92) for *S. epidermidis*. Hill (Emax) modelling showed  $EC_{50}$  values of 9.8 and 12.1 mg/mL, respectively, with cooperative kinetics ( $n \approx 2.2$ –2.4). The MBC:MIC ratio (40–50) confirms primarily bacteriostatic action at 1 mg/mL and bactericidal activity at  $\geq 40$ –50 mg/mL. Mechanistically, bactericidal activity reflects synergistic multi-target effects—membrane depolarisation, pore formation, and inhibition of lipid biosynthesis—resulting in cooperative synthetic lethality and a resistance-attenuating profile. These quantitative pharmacodynamic parameters (MBC,  $EC_{50}$ , Hill coefficient, MBC:MIC ratio) provide a robust basis for rational concentration selection in topical antiseptic formulations and support further optimization strategies, including nano-encapsulation or fractionation, to achieve  $\geq 3$



$\log_{10}$  kill consistent with OTC regulatory benchmarks.

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