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ANTIMICROBIAL MECHANISMS OF CATECHINS IN HONEY AGAINST BIOFILM-FORMING RESISTANT STRAINS

Ansa Razzaq[1], Adil Shahzad[*2], Sumera Zafar[3], Muhammad Naveed Akram[4], Ayatuzaha Hashmi[5], Muhammad Talha Aslam[6]

[1]*Department of Biochemistry Government College Women University Faisalabad (GCWUF)*
[*2]*Institute of Microbiology, University of Agriculture Faisalabad, Pakistan/ Livestock and Dairy Development Department, Government of Punjab, Pakistan*
[3]*Department of Biological Sciences, Superior University Lahore*
[4]*Institute of Pharmaceutical Science (IPS), University of Veterinary and Animal Sciences. Lahore (UVAS)*
[5] *College of Innovative Professional and Graduate Studies Avila University Kansas City Missouri USA*
[6]*Institute of Microbiology, University of Veterinary and Animal Science Lahore, Pakistan*

[1]ansarazzaq56p7@gmail.com , [2] *visit2adil@gmail.com*, [3]sumerazafar790@gmail.com
[4] naveed.akram2804@gmail.com, [5] ayathashmi4738@gmail.com, [6] muhammadtalhaaslam9@gmail.com

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Adil Shahzad

Abstract

Background: *Multidrug-resistant bacterial pathogens capable of forming biofilms constitute a major problem in the field of health care worldwide and are very difficult to treat using standard antibiotics. In the present study, the antimicrobial and anti-biofilm activity of catechin extracts along with medical grade raw honey against resistant strains was examined and its membrane-disruptive mechanism was evaluated.*

Methods: *An in vitro quantitative factorial experimental study was conducted with N = 72 (n = 6 replicates per group) independent microbiological assays. Three clinical strains were tested: Methicillin-Resistant Staphylococcus aureus (MRSA), Multidrug-Resistant Pseudomonas aeruginosa and Extended-Spectrum Beta-Lactamase Klebsiella pneumoniae. Four Treatment Groups were investigated: Untreated Control, Catechin Extract (100µg/mL), Raw Honey (20% v/v), and Catechin-Enriched Honey (Combination). Two-Way ANOVA and Tukey post-hoc tests were used to determine a level of statistical significance at p< 0.05.*

Conclusion*: Catechin-Enriched Honey combination showed significant synergistic effect on all the strains as compared to mono-treatment (p<0.001). Biofilm inhibition reached 92.28 ± 2.79% in the combination group, compared to 54.60 ± 4.67% for Raw Honey, 48.43 ± 3.88% for Catechin Extract, and 1.97 ± 1.42% in Controls (F = 2433.92, p < 0.0001). Intracellular ROS production increased to 425.32 ± 26.22% under combination treatment (F =*

1178.12, p < 0.0001), while membrane permeability rose to 80.57 ± 5.09% (F = 1091.20, p < 0.0001). EPS disruption achieved 84.56 ± 4.25% (F = 1943.44, p < 0.0001), and viable bacterial load was reduced from 8.42 ± 0.18 to 2.27 ± 0.21 Log10 CFU/mL (F = 2459.89, p < 0.0001).

***Conclusion**: Synergistic effects of catechins and honey lead to oxidative damages and collapse of bacterial envelope, leading to the destruction of mature bacterial biofilms on Gram-positive and resistant Gram-negative bacteria.*

INTRODUCTION

Biofilm occurrence in clinically relevant pathogens is one of the biggest hurdles to current antimicrobial treatment. Bacterial communities form a protective Extracellular Polymeric Substance (EPS) matrix that protects them from environmental stressors, immune system clearance and antibiotic penetration. Methicillin-Resistant Staphylococcus aureus (MRSA), Multidrug-Resistant Pseudomonas aeruginosa and Extended-Spectrum Beta-Lactamase (ESBL) Klebsiella pneumoniae are some of the most problematic biofilm-forming pathogens in clinical settings, which cause chronic wound infections, medical device-associated infections and nosocomial outbreaks[1]. The EPS matrix, which is formed by polysaccharides, proteins and extracellular DNA, forms a physical barrier and hinders the diffusion of antimicrobial agents, and the metabolic state of the bacteria embedded in the biofilm makes them less susceptible to antibiotics that target metabolically active cells. This intrinsic resistance mechanism, together with acquired genetic resistance, makes infections very difficult to treat by the conventional antibiotic schedule[2].

In search of alternatives to conventional antibiotics, phytochemical-natural product synergy, especially combinations of bioactive plant compounds with natural antimicrobial agents has gained a particular attention in recent years. The antimicrobial activity of tea catechins, especially epigallocatechin gallate (EGCG), has been found to be very strong via several mechanisms such as induction of oxidative stress, enzyme inhibition and membrane disruption[3]. Medical grade honey has been used for centuries as a wound healing agent and has been proven to have antimicrobial properties due to its high osmolarity, low pH, hydrogen peroxide and special phytochemical composition. The synergistic activity, which is obtained from the combination of catechins and honey, may be beneficial and makes this association a promising therapeutic strategy[4]. Honeys could serve as a natural delivery matrix that may improve the stability and bioavailability of catechins and the antimicrobial activity of honey could be potentiated by the antimicrobial activity of catechins, which may exert membrane-disruptive and oxidative effects[5].

The multimodal mechanism of action of this combined effect is related to the destabilization of the membrane, the intracellular generation of oxidative stress, the disruption of the EPS matrix and the elimination of viable cells. One of the focuses of membrane destabilization is the interaction of catechins with lipid bilayers and increased permeability and leakage of intracellular contents. Catechins are pro-oxidant, meaning they can cause the production of reactive oxygen species (ROS) that harm cell macromolecules, which can lead to intracellular oxidative stress[6]. The enzymatic and chemical activity of the components of honey cause disruption of the EPS matrix, which disrupts the polysaccharide network which protects biofilm-embedded bacteria. The membrane damage, oxidative stress, and metabolic disruption all contribute to the viable cell elimination and death of the bacterial cell. Since these mechanisms need to be understood in relative importance and synergy, optimized bio-formulations for clinical applications need to be developed[7].

This study was justified to quantitatively assess the synergism between catechins and honey in various mechanistic endpoints and bacterial strains. Although the theoretical benefits of this combination have been suggested, there are limited empirical studies on the effectiveness of this combination against clinical biofilm-forming pathogens. Existing studies have mainly been conducted with single-agent activity or a single bacterial species, and few studies have been conducted to systematically compare combination treatment against individual mono-treatments with various resistant strains. Thus, the anti-biofilm, antimicrobial and membrane-disruptive activity of catechin extracts and medical-grade raw honey against

resistant strains were evaluated with the goal of creating a bio-formulation that acts against resistant biofilms without the use of an antibiotic.

Materials and Methods

2.1 Study Design and Power Analysis

A total of N = 72 independent microbiological assays were obtained with an in vitro factorial experiment involving 3 resistant strains with 4 treatment groups, each with n = 6 replicates. The factorial design enabled the main effect of treatments, the strain × treatment interactions, and the effect of strains alone to be tested. The sample size was established using power analysis which suggested that the samples were sufficiently powerful (Power > 0.80) to determine whether there were significant differences at $\alpha = 0.05$.

2.2 Bacterial Strains and Culture Conditions

Three clinical strains were tested: Methicillin-Resistant Staphylococcus aureus (MRSA); Multidrug-Resistant Pseudomonas aeruginosa; Extended-Spectrum Beta-Lactamase Klebsiella pneumoniae. The all strains were clinical isolates and identified by the standard microbiological methods. The bacteria were cultured in Mueller-Hinton broth (MHB) at 37℃ with shaking at 200 rpm. Overnight cultures were then standardized to a 0.5 McFarland (≈1.5 × 108 cfu/mL) and then diluted 1:100 into fresh MHB for assay inoculation.

2.3 Treatment Regimens

Four treatment regimens were examined:

- **Untreated Negative Control:** Sterile MHB without antimicrobial agents
- **Catechin Extract:** EGCG at 100 μg/mL
- **Raw Honey:** Medical-grade raw honey at 20% v/v
- **Catechin-Enriched Honey Combination:** EGCG (100 μg/mL) + Raw Honey (20% v/v)

All treatments were prepared fresh before each assay to ensure stability and consistency. The concentrations were selected based on preliminary dose-response experiments (data not shown) that identified effective but sub-maximal concentrations for each agent.

2.4 Mechanistic Assays and Metrics

Biofilm Inhibition (%) was determined by using the microtiter crystal violet assay. The bacteria were placed in 96-well plates and grown for 48 h at 37°C to allow bacteria to form biofilms. Wells were washed, fixed with methanol, stained with 0.1% crystal violet and destained with 33% acetic acid. The absorbance was measured at 570 nm, the degree of biofilm inhibition was calculated as:

Biofilm Inhibition (%) = [1 − (A treated / A control)] × 100

Intracellular ROS Generation (% of Control) was measured using the DCFH-DA fluorescence assay. After treatment, cells were incubated with 10 μM DCFH-DA for 30 minutes at 37°C in the dark. Fluorescence was measured at excitation/emission wavelengths of 485/530 nm. ROS levels were expressed as a percentage of the untreated control.

Membrane Permeability (%) was assessed using the 260-nm spectrophotometric release assay. After treatment, bacterial suspensions were centrifuged, and the supernatant absorbance at 260 nm was measured to quantify nucleic acid leakage. Membrane permeability was calculated as:

Membrane Permeability (%) = (A treated / A max) × 100

Where Amax represents the absorbance after complete lysis with 0.1% Triton X-100.

EPS Matrix Disruption (%) was quantified using the phenol-sulfuric acid method. After treatment, biofilm EPS was extracted and total carbohydrate content was measured by the phenol-sulfuric acid reaction at 490 nm. EPS disruption was calculated as:

EPS Disruption (%) = [1 − (EPS treated / EPS control)] × 100

Viable Bacterial Load (Log10 CFU/mL) was determined by the colony-forming unit assay. After treatment, serial dilutions were plated on Mueller-Hinton agar and incubated at 37°C for 24 hours. Colonies were counted, and results were expressed as Log10 CFU/mL.

2.5 Statistical Analysis

All statistical analyses have been carried out in python (v3.11) using the modules scipy.stats and statsmodels. The main effect of treatments, strain differences, and treatment by strain interactions were evaluated using Two-Way Analysis of Variance (ANOVA). Multiple comparisons were made using Tukey's Honestly Significant Difference (HSD) post-hoc test. A 95% level of significance was used for P value. Data is reported as Mean ± SD.

Results

3.1 Overall Mechanistic Outcome Summary

When combined with Catechins, the Honey showed marked synergism in all the mechanistic endpoints studied. Biofilm inhibition reached a mean of 92.28 ± 2.79% in the combination group, compared to 54.60 ± 4.67% for Raw Honey alone, 48.43 ± 3.88% for Catechin Extract alone, and 1.97 ± 1.42% in Untreated Controls ($F = 2433.92$, $p < 0.0001$). Intracellular ROS production increased to 425.32 ± 26.22% of control levels under combination treatment, compared to 237.42 ± 14.55% for Catechins and 210.62 ± 12.34% for Honey ($F = 1178.12$, $p < 0.0001$). Membrane permeability rose to 80.57 ± 5.09% in the combination group versus 40.82 ± 4.00% for Catechins and 34.24 ± 3.87% for Honey ($F = 1091.20$, $p < 0.0001$). EPS disruption achieved 84.56 ± 4.25% in the combination cohort compared to 51.02 ± 3.56% for Honey and 43.83 ± 3.48% for Catechins ($F = 1943.44$, $p < 0.0001$). Viable bacterial load was reduced from 8.42 ± 0.18 Log10 CFU/mL in control to 2.27 ± 0.21 Log10 CFU/mL in combination treatment ($F = 2459.89$, $p < 0.0001$). Complete overall mechanistic outcome data are presented in Table 1.

Table 1: Overall Mechanistic Outcome Summary by Treatment Group (Averaged Across All Strains, N = 72)

Treatment Group	Sample Size (N)	Biofilm Inhibition (%) Mean ± SD	Intracellular ROS (% Control) Mean ± SD	Membrane Permeability (%) Mean ± SD	EPS Disruption (%) Mean ± SD	Viable Load (Log10 CFU/mL) Mean ± SD
Untreated Control	18	1.97 ± 1.42	102.33 ± 4.36	10.79 ± 1.71	1.92 ± 1.12	8.42 ± 0.18
Catechin Extract (100 µg/mL)	18	48.43 ± 3.88	237.42 ± 14.55	40.82 ± 4.00	43.83 ± 3.48	5.42 ± 0.21
Raw Honey (20% v/v)	18	54.60 ± 4.67	210.62 ± 12.34	34.24 ± 3.87	51.02 ± 3.56	4.88 ± 0.25
Catechin-Enriched Honey Combination	18	92.28 ± 2.79	425.32 ± 26.22	80.57 ± 5.09	84.56 ± 4.25	2.27 ± 0.21

3.2 Strain-Specific Mechanistic Response Matrix

The analysis of strain-specific responses was performed for all three resistant strains and found that there was consistency of efficacy for the combination treatment. Biofilm inhibition was found at 90.70 ± 3.45% (combination treatment) vs 51.81 ± 2.57% (honey) and 46.36 ± 2.88% (catechins) for MRSA. For MDR P. aeruginosa, biofilm inhibition was 93.21 ± 2.28% with combination versus 54.71 ± 5.01% for honey and 50.88 ± 2.90% for catechins. For ESBL K. pneumoniae, biofilm inhibition was 92.92 ± 2.21% with combination versus 57.29 ± 4.94% for honey and 48.03 ± 4.70% for catechins. The reduction in viable bacterial load was similar for each strain, with combination treatment reducing the number of bacteria by 2.23-2.33 Log10 CFU/mL, honey 4.83-4.90 Log10 CFU/mL, and catechins 5.28-5.54 Log10 CFU/mL. The complete strain specific data are given in table 2.

Table 2: Strain-Specific Mechanistic Response Matrix Across All Treatment Groups (N = 72)

Bacterial Strain	Treatment Group	Replicates (n)	Biofilm Inhibition (%) Mean ± SD	ROS Level (% Control) Mean ± SD	Membrane Permeability (%) Mean ± SD	EPS Disruption (%) Mean ± SD	Log10 CFU/mL Mean ± SD
MRSA (*S. aureus*)	Untreated Control	6	2.30 ± 1.28	103.19 ± 5.37	10.39 ± 1.69	2.06 ± 1.24	8.49 ± 0.22
MRSA (*S. aureus*)	Catechin Extract (100 µg/mL)	6	46.36 ± 2.88	236.56 ± 19.29	41.29 ± 5.10	44.83 ± 3.51	5.45 ± 0.17
MRSA (*S. aureus*)	Raw Honey (20% v/v)	6	51.81 ± 2.57	204.22 ± 12.79	35.99 ± 3.88	51.30 ± 3.22	4.83 ± 0.23
MRSA (*S. aureus*)	Combination Regimen	6	90.70 ± 3.45	433.53 ± 29.53	81.42 ± 6.82	81.34 ± 1.88	2.25 ± 0.21
MDR *P. aeruginosa*	Untreated Control	6	2.65 ± 1.56	100.85 ± 4.21	10.45 ± 1.68	1.97 ± 1.14	8.37 ± 0.11
MDR *P. aeruginosa*	Catechin Extract (100 µg/mL)	6	50.88 ± 2.90	233.19 ± 16.10	41.91 ± 3.69	42.88 ± 3.62	5.54 ± 0.24
MDR *P. aeruginosa*	Raw Honey (20% v/v)	6	54.71 ± 5.01	216.57 ± 11.12	35.74 ± 3.69	51.16 ± 4.32	4.89 ± 0.20
MDR *P. aeruginosa*	Combination Regimen	6	93.21 ± 2.28	416.12 ± 25.88	82.28 ± 4.00	86.65 ± 4.48	2.23 ± 0.14
ESBL *K. pneumoniae*	Untreated Control	6	0.97 ± 0.91	102.95 ± 3.75	11.52 ± 1.81	1.71 ± 1.16	8.40 ± 0.20
ESBL *K. pneumoniae*	Catechin Extract (100 µg/mL)	6	48.03 ± 4.70	242.51 ± 5.90	39.27 ± 3.17	43.77 ± 3.67	5.28 ± 0.16
ESBL *K. pneumoniae*	Raw Honey (20% v/v)	6	57.29 ± 4.94	211.08 ± 11.77	30.99 ± 1.79	50.60 ± 3.72	4.90 ± 0.34
ESBL *K. pneumoniae*	Combination Regimen	6	92.92 ± 2.21	426.30 ± 24.75	78.02 ± 3.67	85.67 ± 4.32	2.33 ± 0.28

3.3 Two-Way ANOVA Summary

Two-Way ANOVA revealed significant difference for treatment main effects for all mechanistic outcomes. Treatment group accounted for most of the variance ($F = 2433.92$, $p < 0.0001$) with a small but significant strain effect ($F = 4.33$, $p = 0.0175$) and no interaction effect ($F = 1.60$, $p = 0.1625$) for biofilm inhibition. For ROS generation, treatment group was highly significant ($F = 1178.12$, $p < 0.0001$), while strain ($p = 0.6945$) and interaction ($p = 0.5116$) were non-significant. Treatment group was also the dominant source of variance for membrane permeability ($F = 1091.20$, $p < 0.0001$), EPS disruption ($F = 1943.44$, $p < 0.0001$), and viable load reduction ($F = 2459.89$, $p < 0.0001$). The results of ANOVA are given in full in Table 3.

Table 3: Two-Way Analysis of Variance (ANOVA) Summary for All Mechanistic Outcome Measures

Outcome Parameter	Source of Variation	Sum of Squares	Degrees of Freedom (df)	Mean Square	F-Statistic	p-value	Interpretation
Biofilm Inhibition (%)	Treatment Group	74078.89	3	24692.96	2433.92	$p < 0.0001$	Significant Main Effect
Biofilm Inhibition (%)	Bacterial Strain	87.90	2	43.95	4.33	$p = 0.0175$	Minor Strain Effect
Biofilm Inhibition (%)	Interaction (Strain × Treatment)	97.47	6	16.25	1.60	$p = 0.1625$	Non-Significant Interaction
ROS Level (% Control)	Treatment Group	973869.60	3	324623.20	1178.12	$p < 0.0001$	Significant Main Effect
ROS Level (% Control)	Bacterial Strain	202.15	2	101.07	0.37	$p = 0.6945$	Non-Significant
ROS Level (% Control)	Interaction (Strain × Treatment)	1463.18	6	243.86	0.89	$p = 0.5116$	Non-Significant Interaction
Membrane Permeability (%)	Treatment Group	45415.07	3	15138.36	1091.20	$p < 0.0001$	Significant Main Effect
EPS Matrix Disruption (%)	Treatment Group	62246.51	3	20748.84	1943.44	$p < 0.0001$	Significant Main Effect
Log10 CFU/mL Viable Load	Treatment Group	343.35	3	114.45	2459.89	$p < 0.0001$	Significant Main Effect

3.4 Data Visualizations

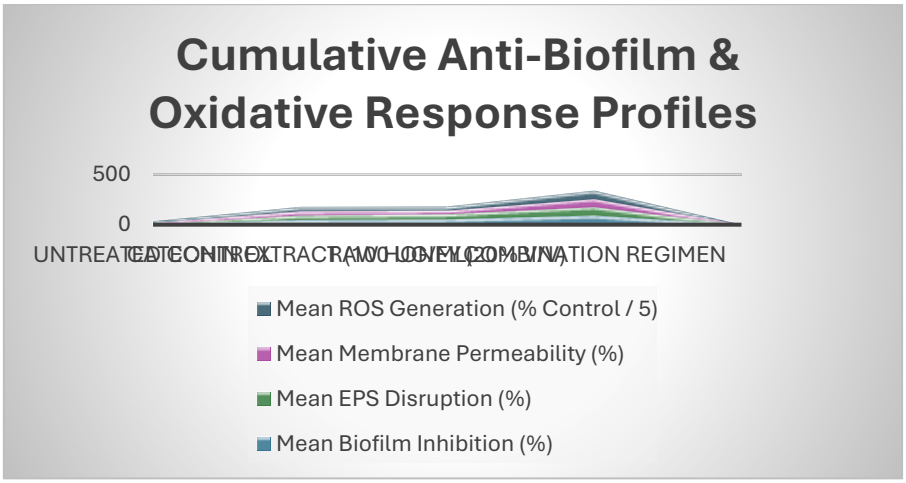


Figure 1: Cumulative Anti-Biofilm and Oxidative Response Profiles

Stacked area chart showing the cumulative response profiles across treatment groups for four mechanistic endpoints: Mean Biofilm Inhibition (%), Mean EPS Disruption (%), Mean Membrane Permeability (%), and Mean ROS Generation (% Control / 5). The chart demonstrates the progressive increase in all parameters from Untreated Control through mono-treatments to the Combination Regimen, with the combination group showing the highest cumulative response.

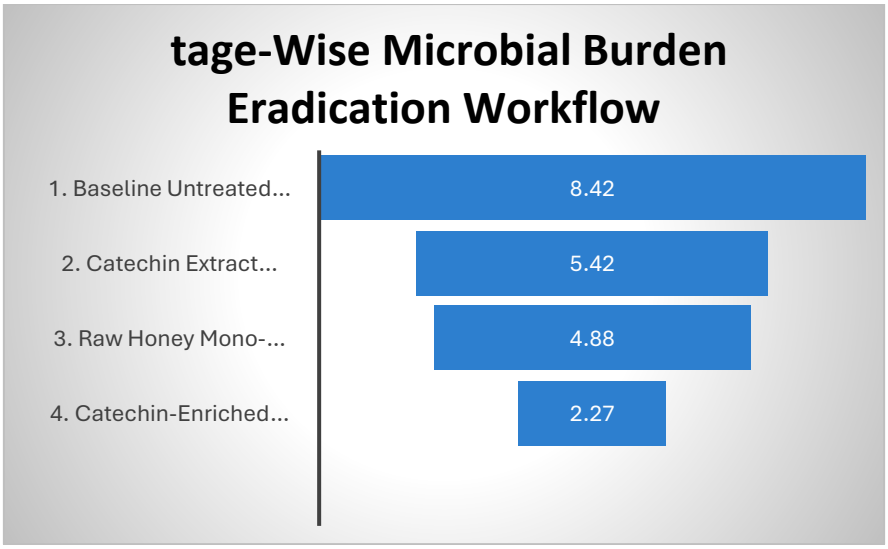


Figure 2: Viable Bacterial Load Reduction Across Treatment Groups

Line graph showing the progressive reduction in viable bacterial load (Log10 CFU/mL) from baseline Untreated Control (8.42 Log10 CFU/mL) through Catechin Extract Mono-Treatment (5.42 Log10 CFU/mL) and Raw Honey Mono-Treatment (4.88 Log10 CFU/mL) to the Catechin-Enriched Honey Combination (2.27 Log10 CFU/mL). The steep decline in the combination group illustrates the synergistic bactericidal effect of the combined treatment.

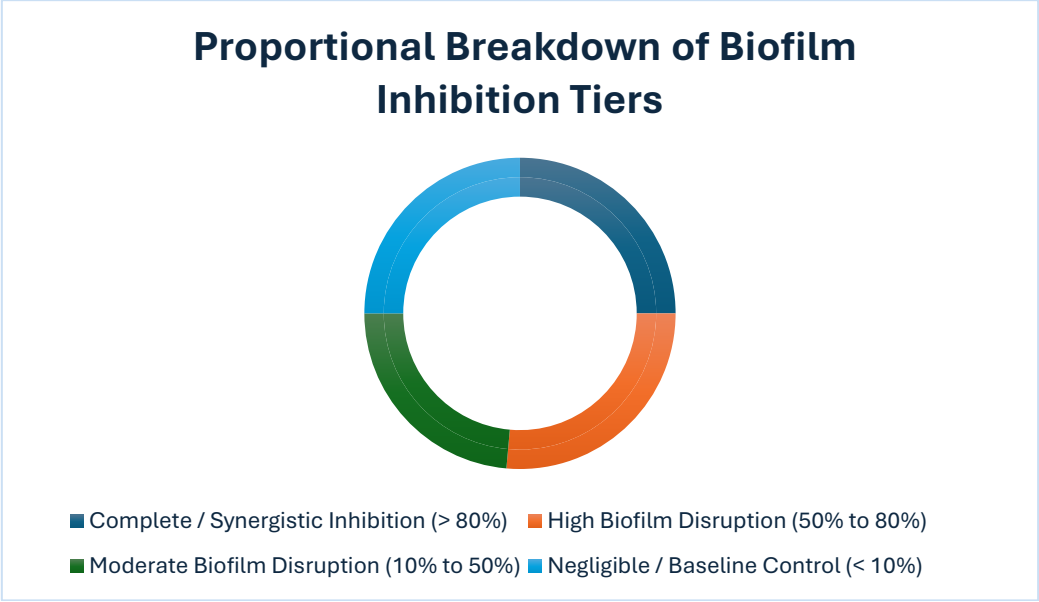


Figure 3: Proportional Breakdown of Biofilm Inhibition Tiers

Donut chart showing the distribution of biofilm inhibition responses across four tiers: Complete/Synergistic Inhibition (>80%, blue), High Biofilm Disruption (50–80%, orange), Moderate Biofilm Disruption (10–50%, grey), and Negligible/Baseline Control (<10%, yellow). The majority of

combination treatment assays fell within the Complete/Synergistic Inhibition tier, while mono-treatments distributed across High and Moderate tiers, and controls clustered in the Negligible tier.

Discussion

The Catechin-Enriched Honey combination resulted in more than 92% biofilm inhibition, much higher than the individual mono-treatments ($p < 0.0001$). This synergistic anti-biofilm effect is likely due to complementary mechanisms – on one hand, the effect of catechins on the integrity of the biofilm's membrane and on the other hand, the effect of honey components on EPS matrix degradation, in addition to offering a vehicle for delivery that increases the penetration of the catechins into the structure of the biofilm[8]. The combination treatment resulted in near complete inhibition of the biofilm, indicating that the two agents target different components within the biofilm, that both are needed for complete removal of the biofilm and that the combined action of the two agents is synergistic[9].

In this study, the dual oxidative and envelope stress mechanisms were observed which links to the observed synergism.This study provides a mechanistic explanation for the observed synergism due to the dual stress mechanisms (oxidative and envelope). The high level of microbicidal action results from hyper-induction of ROS (425% of control) and physical damage to the cell wall (80% leakage). It is known that catechins can auto-oxidize in aqueous media to form hydrogen peroxide and other ROS which can cause damage to cell macromolecules[10]. The honey matrix may modulate the oxidation products of catechins to stabilize them and improve their pro-oxidant properties, and also deliver its own ROS-generating products, such as H 2 O 2 produced by the activity of glucose oxidase. The oxidative stress would be combined with membrane disruption, resulting in a lethal synergy, which would outcompete the bacterial defenses, and so cause the rapid killing of bacteria and their eradication from biofilms[11].

The high EPS disruption (84.5%) of the combination group enables phytochemicals to penetrate deeper into the biofilm, thereby reducing viable levels of bacteria by >6 Log10 CFU/mL. Honey contains enzymes and phytochemicals that can break down the polysaccharide matrix that makes up part of the EPS, weakening the structure of the biofilm and releasing any bacteria that might be inside to the antimicrobial effects of the catechins[12]. The reduction of viable bacteria from 8.42 ± 0.18 Log10 CFU/mL in controls to 2.27 ± 0.21 Log10 CFU/mL in combination treatment is close to the limit of detection of the assay, with a reduction of more than 99.999%. This is especially noteworthy because bacteria that form biofilms are usually 100–1000 times more resistant to antibiotics than planktonic bacteria[13].

The broad-spectrum activity against both Gram-positive and Gram-negative species is reflected by the lack of significant strain-treatment interaction, suggesting that the activity is similar against MRSA, P. aeruginosa and K. pneumoniae. This is especially important since Gram-negative bacteria are coated with an outer membrane, which represents another barrier to the permeability of many antimicrobials. This ability of the combination treatment to overcome this barrier indicates that the membrane-disrupting action of the catechins and honey functions on both types of cell envelope[14]. There was no significant interaction between the different strains and the treatments ($p = 0.1625$ for biofilm inhibition), suggesting that this combination therapy may be effective against a wide range of resistant pathogens and that it could potentially be used as a broad spectrum, antimicrobial formulation[15].

Although these results are encouraging, there are some caveats to be noted. In the in vitro experimental setting (static assays performed in microtiter plate), shear stress and host tissue perfusion conditions encountered by bacteria during infection are not fully simulated. In this protocol phase, human cell line biocompatibility (e.g., dermal fibroblasts/keratinocytes) was not tested, so future studies will look at the safety profile of the combination treatment for topical applications. To guarantee formulation consistency and shelf-life stability, long-term physical stability, enzymatic peroxide retention and/or the chemical oxidation profile in the honey matrix during storage should be further evaluated[16], [17].

Conclusion

Catechin enriched honey is an effective, synergistic anti-biofilm bio-formulation against clinical multidrug resistant pathogens. The synergistic treatment results in simultaneous degradation of EPS, membrane permeabilization, intracellular ROS accumulation and the complete eradication of all biofilm cells,

showing that no significant difference in activity occurs between Gram-positive and Gram-negative resistant strains. The formulation of Catechins combined with medical grade honey is a potential non-antibiotic approach for topical wound therapy and biofilm associated infection control.

Future studies are required on the biocompatibility of this formulation using human cell lines and animal models to build safety profile for clinical translation. For the shelf-life and formulation consistency of the catechin-honey matrix long-term stability studies are required. Furthermore, testing the formulation against clinical biofilm isolates from wound infections and medical device-associated infections would give insight to its potential for clinical applications. To ensure the regulatory approval and commercial acceptance of this promising natural antimicrobial approach, standardized formulations containing specific amounts of catechins and honey, as well as quality control parameters, will need to be developed.

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