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RESEARCH ARTICLE

Generating a Non-toxic, Multi-pathway Targeted Combination Treatment to Inhibit *Pseudomonas* Biofilm *In-vitro*

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ABSTRACT: Eighty percent of chronic microbial infections are associated with antibiotic-resistant bacterial biofilm. This project devised combination therapy of substances with Food and Drug Administration (FDA) approved concentrations, which targeted three major biofilm formation pathways: pyocyanin production and proliferation of eDNA using N-Acetyl cysteine (NAC), biofilm matrix using DNase, and Quorum Sensing using Carvacrol. *Pseudomonas fluorescens* were individually grown with treatments of NAC at 10% and 20%, Carvacrol at 0.15% and 0.75%, and DNase at 10µg/ml and 20µg/ml; biofilm formation was measured using Resazurin assay. Concentrations exhibiting greater efficacy were tested further on *Pseudomonas* to determine the best condition for biofilm inhibition. Results indicated that the control (no treatment) had a Relative Fluorescence Unit (RFU) of 3.92. NAC 20% was the most effective individual treatment with RFU 0.973 and inhibition efficacy of 75%. NAC 20% - DNase 20µg/ml was the most effective dual-combination treatment, with RFU 0.579 and inhibition efficacy of 85%. Finally, the triple-combination treatment exhibited remarkable effectiveness, with RFU 0.208 and inhibition efficacy of 95%. Statistical significance was shown using ANOVA test. Cytotoxicity assay on *C. elegans* confirmed the treatment's safety *in-vitro*. These findings can lead to development of novel adjuvants to deliver the treatment *in-vivo*, thus reducing the morbidity and mortality from chronic biofilm-related infections.

KEYWORDS: Microbiology; Antibiotic resistance; Bacterial biofilm; eDNA; Pyocyanin.

Introduction

Biofilm refers to the complex, sessile communities of microbes found either attached to a surface or buried firmly in an extracellular matrix as aggregates. A biofilm matrix is a self-produced polymer consisting of polysaccharide, protein, and DNA.[1,2] The process of biofilm formation consists of bacterial adherence to hardware or tissue, followed by multiplication and aggregation of exopolysaccharides; over time, microcolonies of bacteria encased in glycocalyx merge to form biofilm. Bacteria also secrete stringy sugars, proteins, and DNA fragments into this defensive mesh (extracellular polymeric substances (EPS) and makes them tolerant of harsh conditions and resistant to antibacterial treatments.[3]

The Harms of Bacterial Biofilm:

Bacterial biofilms are known for causing severe medical infections. Eighty percent of chronic microbial infections in the body are caused by bacteria growing as a biofilm, accounting for tens of thousands of deaths across the North American continent annually.[3,4] Examples of infections associated with biofilms in the human body are often in the form of chronic urinary tract infections, catheter infections, middle-ear infections, dental plaque, gingivitis, endocarditis, infections in cystic fibrosis patients, and infections of permanent indwelling devices such as joint prostheses, heart valves, and intervertebral discs. By far the most prominent example of bacterial biofilms, which most broadly contextualizes their severe detriment to human health, is chronic lung infections in cystic fibrosis patients caused by biofilm-growing mucoid strains of *Pseudomonas aeruginosa*.[3,5] Furthermore, the Agency for Healthcare Research and Quality declares that hospital-acquired infections are in the top ten leading causes of death in the United States and are responsible for nearly hundred thousand deaths per year.[6]

Antibiotic Resistance:

According to the National Center for Biotechnology Information, biofilm microorganisms are one hundred thousand times less susceptible to antibiotics than their planktonic counterparts.[3] Due to their inaccessibility and recalcitrance toward antibiotics, internal biofilms have become nearly impossible to eradicate. Bacteria found deep within the biofilm are metabolically inactive and grow slowly, making them less susceptible to antibiotics and protected from host defenses. Biofilm also acts as a mechanical shield preventing antibiotic penetration. Once the biofilm forms, delivery of nutrients to the cells is dependent on diffusion through the Extracellular Polymeric Substances (EPS), which act as a diffusion barrier, either by limiting the rate of molecular transport to the biofilm interior or by chemically reacting with the molecules themselves. Furthermore, the negatively charged EPS restricts permeation of several positively charged molecules of antibiotics by chemical interaction or molecular binding.[4]

Current Treatments and Limitations:

Various strategies to eradicate and inhibit internal bacterial biofilm have already been employed in practical settings to attempt to eradicate biofilm; however, these methods have several limitations in terms of efficacy and practicality.[4] Methods of targeting the adhesion stage of biofilm development such as coating medical equipment with hydrophilic coatings using Poly-N-vinylpyrrolidone or Hyaluronic acid has proven to be insufficient in terms of efficiency and safety.[5] Other

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methods such as UV radiation, chlorination, and flushing have proven to be either too expensive for practical and widespread use, toxic to epithelial cells, or ineffective at inhibiting robust biofilm.[4,5] The limited capacity of current treatments in targeting and inhibiting bacterial biofilm strongly suggest the need for new and novel methods of inhibiting bacterial biofilm aggregation. Thus, the goal was to discover a treatment composed of non-toxic substances to disrupt the biofilm matrix and inhibit biofilm formation while minimizing side effects.

Purpose:

Our research goal was to find Food and Drug Administration (FDA) approved concentrations of non-toxic substances to specifically target the three main pathways of *Pseudomonas* biofilm formation. These include N-acetylcysteine (NAC) to target pyocyanin production, DNase to disrupt the biofilm matrix, and Carvacrol to suppress quorum sensing. This experiment focuses on *Pseudomonas aeruginosa*, which several studies have touted as one of the most potent biofilm forming bacteria.[7,9] Experimental trials were caried out on *P. fluorescens*, which is a biosafety level (BSL) 1 organism sharing the same characteristics of *P. aeruginosa*. Our research is among the first of its kind to attempt to develop a relatively inexpensive and non-toxic therapy for inhibiting bacterial biofilm *in-vitro.* Furthermore, my research is among the first to suggest targeting various pathways of bacterial biofilm formation.

Pseudomonas aeruginosa:

The bacteria used in this investigation is *P. aeruginosa*, an opportunistic Gram-negative bacterium that is primarily responsible for infections related to cystic fibrosis (CF) airways, burn wounds, urinary tract infections, and HIV-related illness.[10,12] According to the National Center for Biotechnology Information, *P. aeruginosa* is among the most virulent of opportunistic pathogens and is a leading cause of a variety of acute and chronic infections. It is one of the most notorious biofilm producers and is a major cause of nosocomial infections which affect more than 2 million patients every year and accounts for around 90,000 deaths annually.[13] Thus, the treatment targets three main pathways of biofilm formation.

Pathway 1: Pyocyanin Production :

P. aeruginosa predominantly synthesizes pyocyanin [5-methyl-1(5H)-phenazinone], which is a secondary metabolite.[14] Nearly 95% of *P. aeruginosa* strains produce pyocyanin, a potent virulence factor causing cell death in infected cystic fibrosis patients and is associated with high mortality. Pyocyanin strongly binds with extracellular DNA (eDNA) and dictates the establishment of *P. aeruginosa* biofilm and thus is a key player in the formation of *P. aeruginosa* biofilm. eDNA is a key constituent in the construction and structural integrity of the biofilm matrix and acts as a scaffold for the biofilm by binding with other biomolecules such as peptides, enzymes, proteins, and polysaccharides. Pyocyanin triggers tissue damage mainly by its redox cycling and induction of reactive oxygen species (ROS). Pyocyanin also enables the maintenance of a basal rate of respiration for energy harvesting and maintains cytoplasmic redox homeostasis.[14,15] eDNA is one of the main biofilm-promoting factors, whereas pyocyanin is considered as a secondary metabolite essential for the persistence of *P. aeruginosa* cells in highly dense biofilm. Pyocyanin intercalates with the nitrogenous bases of DNA and creates structural perturbation on the double-helix structure. Pyocyanin-eDNA binding significantly influences *P. aeruginosa* cell surface hydrophobicity. This tremendously influences the physiochemical interactions that facilitate bacterial cell-to-cell interaction or aggregation and ultimately, facilitates robust biofilm formation.[15]

Use of N-acetylcysteine (NAC):

Studies have shown that glutathione (GSH) or N-acetylcysteine (precursor to glutathione) is the most effective antioxidant for removing reactive oxygen species (ROS) in cells.[16,19] Studies have also shown that many pulmonary diseases such as cystic fibrosis are associated with the lower levels of glutathione in the epithelial lining fluid (ELF) than those in healthy individuals.[20,21] In CF patients, pyocyanin-mediated ROS oxidize host intracellular and extracellular reduced glutathione (GSH) to form glutathione disulfide or oxidized glutathione, thus depleting host GSH levels. This leads to widespread epithelial cell death and subsequent lung damage, respiratory failure, and mortality.[21] Past literature has found that in *P. aeruginosa* biofilms, GSH directly interacts with pyocyanin and modulates its structure to inhibit pyocyanin intercalation with DNA and biofilm integration, thus disrupting the biofilm matrix and enhancing antibiotic efficiency.[22,24] Oxidative injury inflicted by *P. aeruginosa* is one of the major causes to aggravating cystic fibrosis disease,[24] so these findings may bring more insights into the molecular pathogenesis of *P. aeruginosa* infections and lead to novel therapeutic intervention for inhibiting *P. aeruginosa* infections.

Pathway 2: Quorum Sensing:

Quorum sensing (QS) has been shown to be a cell signaling process that allows bacterial cells to respond to the needs related to cell density through the expression of specific genes.[25] It is a bacterial cell to cell communication process that involves the production, detection, and response to extracellular signaling molecules called autoinducers (AIs). AIs accumulate in the environment as the bacterial population density increases, and bacteria monitor this information to track changes in their cell numbers and collectively alter gene expression. QS systems among planktonic bacterial cells use a transcriptional activator protein that acts in concert with a small AI signaling molecule to stimulate expression of target genes. The concentration of AIs increases as the bacterial cell population increases. Once a significant number of AIs have accumulated, they bind to the transcriptional activator, enabling it to induce expression of target genes.[25,26] In the case of *P. aeruginosa*, two well-studied QS systems are las and rhl, and they play a significant role in the proliferation of biofilm. QS is largely responsible for biofilm development as it regulates several bacterial virulence factors and is essential to intra-bacterial communication.[26,27] Finding new ways to target QS in bacteria is an acknowledged strategy in the scientific community for discovering and developing new antibiotics.[28] Using specific molecules to interfere with QS has been declared as a promising strategy to prevent e

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biofilm formation. However, current inhibitors to target QS are highly expensive and are not proven to be safe to use in humans due to their associated toxicities.[27,28].

Use of Phenolic Compounds: Carvacrol :

Several studies have corroborated that carvacrol is a promising antioxidant in inhibiting biofilm formation through the disruption of QS and can be safely administered in humans at regulated concentrations.[29,31] Carvacrol, a phenolic compound and one of the major antimicrobial components of oregano oil, inhibits QS due to its ability to interact with cell membrane and protein receptors involved in biofilm formation. Phenolic compounds comprise one or more aromatic rings with attached hydroxyl groups in their structures. These substances protect against free radicals and toxins due to their capacity to scavenge oxidatively generated free radicals. Carvacrol is a phenolic compound that is a natural monoterpene derivative of cymene. It is able to diffuse through the cytoplasmic membrane, then becoming deprotonated by binding to a monovalent cation such as potassium. Carvacrol can diffuse out of the cytoplasm where it again takes up a proton from the external environment, therefore acting as a transmembrane carrier of monovalent cations.[32] The compound can act as a binding mode to inhibit QS and prevent the production of the virulence factor pyocyanin in *P. aeruginosa*, as one study found that pyocyanin production by *P. aeruginosa* reduced up to 60% at 3.9 mM of carvacrol.[31] Through this method, carvacrol may interfere with the QS signaling through reduction of bacterial virulence, thus also reducing the capacity for biofilm formation.

Pathway 3: Biofilm Matrix (Extracellular Polymeric Substances) :

The following method aims to target the external structure of Extracellular Polymeric Substances (EPS). Extracellular DNA (eDNA) has been shown to be one of the primary components of the biofilm matrix and plays an instrumental role in biofilm's resistance to conventional antibiotics by enhancing biofilm cohesion, cation chelation, biocide resistance, and genetic exchange.[33] eDNA in the biofilm matrix is typically what makes it difficult for large antibiotic particles to penetrate the biofilm and significantly contributes to bacterial biofilm's overall antibiotic resistance. Not only that, but eDNA has shown to play a major role in *P. aeruginosa* biofilm formation, as it acts as the binding site for pyocyanin which proliferates the biofilm.[34] Thus, targeting this component of the biofilm is crucial in inhibiting overall biofilm growth.

Use of DNase :

An enzyme with great promise is deoxyribonuclease I (DNase I). DNase is an enzyme that catalyzes the hydrolytic cleavage of phosphodiester linkages in the DNA backbone, thus degrading DNA. DNase I is able to cleave DNA in the extracellular space down to an average of tetranucleotides with 5′ monophosphate and 3′ hydroxyl DNA ends. In humans, DNase I is largely responsible for the nucleolytic activity on DNA in serum and the degradation of the majority of circulating DNA derived from apoptotic and necrotic cell death. DNase has been proven to degrade the extracellular DNA (e-DNA) present in the matrix, rendering the matrix weak and susceptible to antimicrobials.[35,36] However, DNase also can cleave bacterial DNA, making it a potential inhibitor of the proliferation of extracellular polymeric substances outside the biofilm. In one study conducted by Sharma and Singh, inclusion of Mg^{2+} alongside DNase I post-treatment at a concentration of 5 µg/ml resulted in 90% reduction in biofilm within only 5 min of contact time and irrespective of the age of biofilm.[37] In another study conducted by Tetz and Tetz, bacterial biofilm formed in the presence of DNase I (5.0 µg/ml) displayed reduced biofilm biomass, total bacterial biomass, decreased viability of bacteria, and decreased tolerance to antibiotics.[36] Through this method, DNase has tremendous potential in cleaving the eDNA and nucleotides present within the biofilm matrix. Our research is among the first of its kind to attempt to develop a relatively inexpensive and non-toxic therapy for inhibiting bacterial biofilm in-vitro. Furthermore, our research is among the first to suggest targeting various pathways of bacterial biofilm formation.

Methods

First, Food and Drug Administration (FDA) approved concentrations of NAC, Carvacrol, and DNase were selected for the experiment. It was concluded that DNase could be safely used at FDA approved concentrations from 10 µg/ml to 20 µg/ml.[35], NAC from 10-20%,[38] and Carvacrol from 0.15% to 0.75%.[39] The experiment was conducted in four phases: Resazurin Assay (Phase One), Individual Treatments (Phase Two), Combination Treatments (Phase Three), and Cytotoxicity Assay (Phase Four).

Phase One: Resazurin Assay:

Phase One was carried out by first streaking out *Pseudomonas flourescens* on an agar plate and letting it grow overnight at room temperature. 3 ml of liquid broth was then pipetted into a sterile borosilicate tube and inoculated with a small scoop of the enriched Pseudomonas sample. This solution was gently vortexed and incubated at room temperature for three days. After three days, the liquid broth and free-floating planktonic cells were flushed from the borosilicate tube, and 600 µl of clean liquid broth was added to the borosilicate tubes and gently vortexed to harvest the biofilm. Five 10-fold dilutions of the biofilm solution were then created. 100 µl of the biofilm solution from each dilution was pipetted into separate agar plates, and the CFU/ml on each agar plate was calculated after four hours. Meanwhile, 200 µl of solution from each dilution was pipetted into each well of a column in a 96-plate. Each well was then treated with 20 µl of alamarBlue™ reagent (Resazurin) and incubated for four hours. Finally, the fluorescence read-out of the 96-well plate was measured using a fluorescence reader. An output curve correlating fluorescence read-out and the cfu/ml was developed.

Phase Two: Individual Treatments:

Phase Two was carried out by first creating a stock *Pseudomonas flourescens* bacterial solution similar to that of the first phase. Then, stock solutions for each experimental group (NAC 10%, NAC 20%, Carvacrol 0.15%, Carvacrol 0.75%, DNase 10 µg/ml, and DNase 20 µg/ml) were created. 3 ml of each stock solution and 0.3 ml of the inoculum were pipetted each into three separate borosilicate tubes. Three trials of

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control group consisting solely of 3 ml of broth and 0.3 ml of inoculum were also created. Each experimental group had three trials. Immediately after pipetting the stock solution and inoculum, all 21 borosilicate tubes were incubated for three days at 37° Celsius. After three days, the biofilm of each borosilicate tube was harvested and 200 µl sample of each tube was pipetted into each cell of a 96-well plate to conduct a Resazurin assay. Each cell was treated with 20 µl of alamarBlue™ reagent (Resazurin) and incubated for four hours before the fluorescence output was recorded using a fluorescence reader with excitation at 550 nM and emission at 600 nM.

Phase Three: Combination Treatments:

Phase Three was conducted in the same manner as Phase Two, except the experimental groups were in dual and a triple combination (NAC 20% & Carvacrol 0.75%, NAC 20% & DNase 20 µg/ml, Carvacrol 0.75% & DNase 20 µg/ml, and NAC 20% & Carvacrol 0.75% & DNase 20 µg/ml)

Phase Four: Cytotoxicity Assay using Caenorhabditis elegans:

Phase Four was conducted by first obtaining 100 ml of sterile, commercially prepared nematode growth medium (consisting of NaCl, peptone, cholesterol, $CaCl_2$, $MgSO_4$, and KPO_4 in appropriate concentrations). Half of this media was then cooled into two separate agar plates, acting as the control group. The rest of the nematode growth medium was combined with appropriate concentrations of the triple combination therapy and cooled into two separate agar plates. *Escherichia coli* was then cultured on one plate with no treatment and one plate with treatment. The four plates used in this phase were: No treatment - No *E. coli*; No treatment - With *E. coli*; Treatment - No *E. coli*, Treatment - With *E. coli*. Finally, approximately ten C. elegans were transported into each plate and incubated at room temperature. At three separate time stamps (24 hours, 48 hours, and 72 hours), the *C. elegans* were observed for motility and growth using a digital microscope. High-resolution images and videos were also taken using a digital microscope. Trials were carried out with and without *E. coli* to ensure that the cocktail treatment did not alter the results of the assay due to its potential lethal effects on the *E. coli* (an essential nutrient for *C. elegans*).

Results and Discussion

The Resazurin assay resulted in the correlation curve between CFU/ml and Relative Fluorescence Units (RFU). The line had an equation of y = 10255x - 1136.3 (Figure 1), with y equaling the CFU/ml and x equaling the RFU. This experiment ensured that there were viable bacterial cells in the biofilm tested in the experiment. Through this equation, the CFU/ml of the biofilm formed under experimental conditions may also be extrapolated (i.e., any RFU values obtained in the latter half of the experiment could be substituted into the equation to obtain the CFU/ml of the remaining viable bacteria). The Resazurin assay not only ensured bacterial biofilm cell viability but also allowed the development of a curve correlating CFU/ml and RFU, demonstrating that the RFU decreases with decrease in the viable bacterial cells.

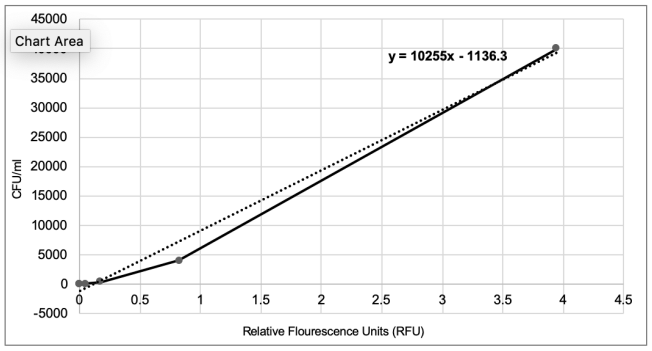

Figure 1: CFU/ml vs relative fluorescence units (RFU) of *Pseudomonas fluorescens* bacterial biofilm proves bacterial viability within the tested biofilm.

A lower RFU (Relative Fluorescence Unit) value indicates a lower number of viable bacteria in the biofilm as demonstrated in the Resazurin assay. Therefore, a lower RFU valuable indicates less viable biofilm, suggesting that the treatment was effective in inhibiting biofilm formation. Results showed that the control group with no treatment had an RFU value of 3.92. NAC 20% was the most effective individual treatment in inhibiting biofilm formation, with an RFU value of 0.973 and inhibition efficacy of 75% (Table 2). NAC 10% followed closely behind, with an RFU value of 1.19 and inhibition efficacy of 70% (Table 2). DNase 20 µg/ml and DNase 10 µg/ml were the second most effective individual treatments with RFU values of 1.27 and 1.54 and inhibition efficacies of 68% and 61% respectively (Table 2). Finally, Carvacrol 0.75% and Carvacrol 0.15% were the least effective individual treatments with RFU values of 1.93 and 2.10 and inhibition efficacies of 51% and 46% respectively (Table 2). Biofilm inhibition was clearly demonstrated using individual treatments of DNase, NAC, and carvacrol. Higher concentrations of each substances exhibited greater biofilm inhibition, likely because greater quantities

Table 1: Colony forming units (CFU)/ml and relative fluorescence units (RFU) of five dilutions of *Pseudomonas fluorescens* bacterial biofilm proves bacterial viability within the tested biofilm.

Dilution	CFU/ml	Relative Fluorescence Units (RFU)
Stock Solution	40000 (TNTC)	3.94
10^{-1}	4000 (TNTC)	0.823
10^{-2}	440 (TNTC)	0.175
10^{-3}	40	0.054
10^{-4}	0	0.005
10^{-5}	0	0.001

Table 2: Average relative fluorescence units (RFU) and inhibition efficacy (%) of various concentrations of carvacrol, DNase, and NAC against *Pseudomonas fluorescens* suggests biofilm inhibition efficacy of individual treatments.

Individual Treatment and Concentration	Average Relative Fluorescence Units (RFU)	Inhibition Efficacy (%)
Control	3.94	0%
Carvacrol 0.15%	2.10	46%
Carvacrol 0.75%	1.93	51%
DNase 10 µg/ml	1.54	61%
DNase 20 µg/ml	1.27	68%
NAC 10%	1.19	70%
NAC 20%	0.973	75%

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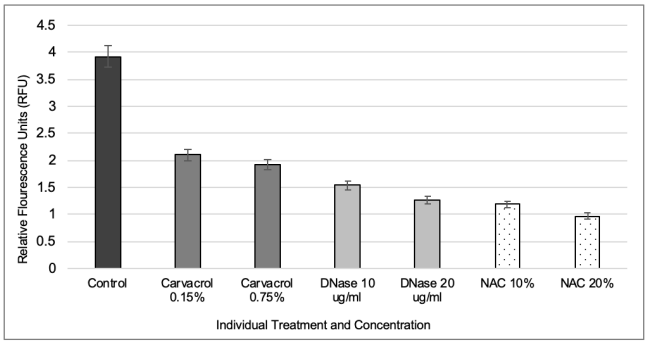

Figure 2: Average relative fluorescence units (RFU) of various concentrations of Carvacrol, DNase, and NAC proves biofilm inhibition efficacy of individual treatments.

Of the dual combination treatments, the NAC 20% - DNase 20 µg/ml combination was the most effective, with an RFU value of 0.579 and inhibition efficacy of 85% (Table 3). NAC 20% - Carvacrol 0.75% and DNase 20 µg/ml - Carvacrol 0.75% followed closely behind, with RFU values of 0.749 and 0.860 and inhibition efficacies of 81% and 78% respectively (Table 3). The triple combination treatment exhibited remarkable effectiveness, with an RFU value of 0.208 and inhibition efficacy of 95% respectively (Table 3). Biofilm inhibition was clearly demonstrated using combination treatments of DNase, NAC, and Carvacrol. Further objectives would include testing the biofilm-inhibiting triple combination therapy with various antibiotics to possibly achieve a near 100% biofilm inhibition efficacy. This procedure could also be tested on various other ESKAPE bacteria (*Enterococcus faecium, Staphylococcus aureus, Klebsiella pneumoniae, Acinetobacter baumannii, Pseudomonas aeruginosa, and Enterobacter species*) to determine the efficacy of triple combination therapy. The experimental trials may also be tested on *P. aeruginosa*.

Table 3: Average relative fluorescence units (RFU), average CFU/ml, and inhibition efficacy (%) of various combinations of Carvacrol, DNase, and NAC proves biofilm inhibition efficacy of combination treatments.

Combination Treatment	Average Relative Fluorescence Units (RFU)	Inhibition Efficacy (%)
Carvacrol + DNase	0.860	78%
Carvacrol + NAC	0.749	81%
DNase + NAC	0.579	85%
Carvacrol + DNase + NAC	0.208	95%

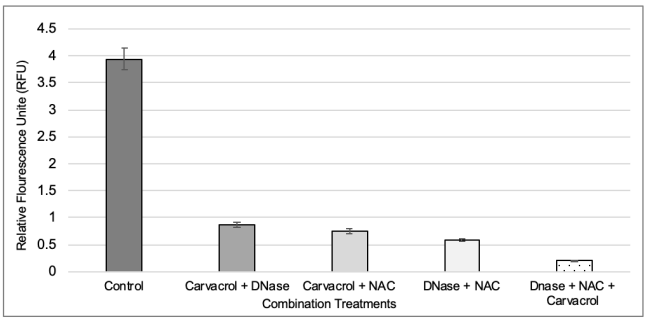

Figure 3: Average relative fluorescence units (RFU) of various combinations of Carvacrol, DNase, and NAC proves biofilm inhibition efficacy of combination treatments.

After 24, 48, and 72 hours, *C. elegans* motility was not significantly hindered in the treatment group compared with *C. elegans* with no treatment (control group). After 24 hours, 100% (10 out of 10) *C. elegans* in the treatment group exhibited full motility, with no change in size or number, as did *C. elegans* in the control group without treatment (Table 4). After 48 hours, 90% (9 out of 10) of *C. elegans* in the treatment group exhibited full motility, with slight increase in their size compared to 100% (10 out of 10) of *C. elegans* in the control group, which also increased in size (Table 4). After 72 hours, 90% (27 out of 30) of *C. elegans* in the treatment group exhibited full motility compared to 93% (28 out of 30) of *C. elegans* in the control group. All organisms in both groups exhibited increased size and a tripled sample population (Table 4). In the plates without *E. coli*, after 24 hours, 60% (6 out of 10) of *C. elegans* in the treatment group exhibited full motility, compared to 50% (5 out of 10) of *C. elegans* without treatment (Table 4). This suggests that the treatment may have harmed some of the *E. coli* in the experimental and control groups in rows 1 and 2 (Table 4). These results further imply that the treatment is potentially safe for mammalian cells.

Table 4: Motility of *C. elegans* grown with and without treatment and *E. coli* after 24, 48, and 72 hours alludes to non-toxicity and potential in-vivo safety.

Treatment Group	24 hours	48 hours	72 hours
with treatment, with *E. coli*	10/10 motile, no change in size or number	9/10 motile, bigger in size, no change in number	27/30 motile, bigger in size, tripled in number
without treatment, with *E. coli*	10/10 motile, no change in size or number	10/10 motile, bigger in size, no change in number	28/30 motile, bigger in size, tripled in number
with treatment, without *E. coli*	6/10 motile, no change in size	All non-motile, shrunken	All non-motile, shrunken
without treatment, without *E. coli*	5/10 motile, no change in size	All non-motile, shrunken	All non-motile, shrunken

Statistical Analysis:

A one-way ANOVA test was conducted to ensure that the differences between the means of the independent groups are statistically significant. The null hypothesis (H_0) was determined to be that there was no statistical difference between the RFU values of the independent groups. The alternative hypothesis (H_A) was determined to be that there was statistical difference between the RFU values of the independent groups, and each variation of the treatment had some effect on bacterial biofilm inhibition. The results of the statistical analysis test are shown below

Table 5: One-way ANOVA test proves statistical significance of results.

SUMMARY

Groups	Count	Sum	Average	Variance
Control	6	23.519	3.919833	0.000192967
Carvacrol 0.15%	6	12.625	2.104167	0.000323767
Carvacrol 0.75%	6	10.744	1.790667	0.116320267
DNase 10 ug/ml	6	9.236	1.539333	0.000150667
DNase 20 ug/ml	6	7.615	1.269167	0.000234167
NAC 10%	6	7.111	1.185167	0.000310967
NAC 20%	6	5.835	0.9725	0.0009387
Carvacrol 0.75% + Dnase 20 ug/ml	6	5.162	0.860333	0.000191467
Carvacrol 0.75% + NAC 20%	6	4.496	0.749333	0.000249467
NAC 20% + Dnase 20 ug/ml	6	3.476	0.579333	6.02667E-05
NAC 20% + Dnase 20 ug/ml + Carvacrol 0.75%	6	1.251	0.2085	0.0001347

ANOVA

Source of Variation	SS	df	MS	F	P-value	F crit
Between Groups	60.40066948	10	6.040067	557.8220701	1.67798E-51	2.007791769
Within Groups	0.595537	55	0.010828			
Total	60.99620648	65				

Conclusion

Overall, the treatment with NAC, Carvacrol, and DNase showed promising efficacy in inhibiting the growth of *P. fluorescens* biofilm, with a near 95% inhibition efficacy. The cytoto

xicity assay further suggested that the triple combination treatment could be non-toxic for human use. These findings can be translated into the development of novel methods in treating patients with chronic biofilm-related microbial infections such as pulmonary infections in cystic fibrosis, urinary tract infections, endocarditis, chronic wounds, and infections in in dwelling catheters. Newer methodologies can be developed to deliver this treatment *in-vivo* through inhalation, intravenous or oral medications in conjunction with antibiotics, thus reducing morbidity and mortality from chronic microbial infections that account for millions of deaths across the world and saving billions of dollars in product contamination.

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