The Synergistic Effect of Combining Natural Compounds 1,8-cineole (Eucalyptol) and Naringenin with 11 Antibiotics of Different Drug Classes

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ABSTRACT

Antibiotic resistance represents a critical global health threat, as increasingly prevalent bacterial infections become more challenging to treat with existing antibiotics. Millions of lives are lost to infections caused by antibiotic-resistant bacteria, highlighting the urgent need for innovative strategies to combat this resistance. This study investigates the efficacy of two natural compounds, 1,8-Cineole (Eucalyptol) and Naringenin, in combination with eleven antibiotics of various drug classes, as antibiotic adjuvants to enhance the efficacy of existing antibiotics to combat resistance. Both compounds exhibit antimicrobial properties and are known to have mechanisms of action similar to efflux pump inhibitors and membrane permeabilizers. Eucalyptol has demonstrated antimicrobial effects through membrane disruption, and Naringenin damages bacterial membranes. The aim of this research is to evaluate the synergistic effects of these natural compounds against *Escherichia coli*, as there is no current research on the potential effects of combining these specific compounds with any antibiotics. Using broth microdilution assays and a derivative of checkerboard assays, Minimum Inhibitory Concentration (MIC) values, Area Under Growth (AUC) values, and Inhibitory Concentration 50% (IC50) values were compared to identify any potential synergistic interactions. It is hypothesized that both Eucalyptol and Naringenin will enhance antibiotic effectiveness. Results of this study demonstrate a synergistic effect occurred when Eucalyptol was combined with Azithromycin. In contrast, antagonistic interactions were found when Eucalyptol was combined with Tetracycline and Kanamycin, and when Naringenin was combined with Trimethoprim. This research holds significant implications for addressing the growing challenge of antimicrobial resistance by identifying novel compound combinations for restoring the efficacy of existing antibiotics and by expanding potential for new combinations for other applications.

KEYWORDS

1,8-Cineole, Eucalyptol, Naringenin, antibiotics, antibiotic resistance, efflux pump inhibitors, membrane permeabilizers

NOTE

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INTRODUCTION

Imagine a world where common infections, at one point easily treatable with antibiotics, could once again become deadly. In 2019 alone, over 1.27 million people died due to infections that could no longer be treated with existing antibiotics (Antimicrobial Resistance Collaborators, 2022). Antibiotics are one of the most essential medical breakthroughs of the 20th century, with their invention allowing for the treatment of infectious diseases, various modern medical procedures, such as cancer treatment and organ transplants, and preventing the reproduction and spread of bacteria (Patel et al., 2023). They are crucial in modern medicine and have played a central role in reducing mortality due to common bacterial infections, such as tuberculosis, salmonella, and whooping cough. They can also be used in combination therapies, which utilize both antibiotics and other methods of treatment to treat co-infections (Kapoor et al., 2017). Overall, they are important for public health and epidemic control, as timely antibiotic treatment can prevent large-scale outbreaks and fatalities.

In recent years, there has been a gradual decrease in antibiotic discovery and development of new antibiotics, as pharmaceutical scientists state it is more difficult to find new chemical combinations that are safe and effective for use as antibiotics (WHO, 2022). Only 12 new antibiotics have been developed since 2017, 10 of which are part of classes to which bacteria are very resistant (WHO, 2022), leading to concerns from public health officials who believe that this can lead to a drastic increase in antibiotic resistance. Antibiotic resistance is when bacteria evolve and become resistant to drugs that once killed them or inhibited their growth (Habboush & Guzman, 2023). This ineffectiveness will occur because bacteria are constantly evolving, and when exposed to an antibiotic, most bacteria are killed, except for a small number that may have genetic mutations or traits that make them less susceptible to the drug. These resistant bacteria gain exposure to the selective pressure and then can survive, multiply, and pass on their resistance traits to new bacteria (Habboush & Guzman, 2023), making infections caused by them more difficult to treat. Also, when antibiotics are overprescribed or incorrectly prescribed, or when patients do not use them long enough or in the correct dosage amounts, bacteria have more opportunities to evolve resistance, which overall will cause drastic increases in death rates and healthcare costs if solutions are not found.

Currently, scientists are looking into new methods against resistance, as there is a high need for innovations that can be used to help treat bacterial infections. Researchers are studying various methods to combat drug resistance, including bacteriocins, antimicrobial peptides produced by bacteria that inhibit or kill other bacteria (Darbandi et al., 2021), and bacteriophages, viruses that target and kill specific bacteria (WHO, 2024). Another group of compounds being researched is antibiotic adjuvants, nonantibiotic compounds that can be used in combination with antibiotics to improve their performance (Dhanda et al., 2024). Currently, researchers are looking into various natural compounds to see if they can synergize with antibiotics to minimize bacterial resistance and help conserve antibiotic activity. Many of the current antibiotic adjuvants being investigated in pharmacodynamics target bacterial molecules or cellular structures central to mechanisms of resistance (Dhanda et al., 2024), but there are still many compounds left to test. There are three main types of antibiotic adjuvants: β-lactamase inhibitors, efflux pump inhibitors, and outer membrane permeabilizers. They have different mechanisms of action, but all have been shown to be effective when combined with antibiotics. β-lactamase inhibitors are designed to inhibit the action of enzymes called beta-lactamases that break down the beta-lactam ring in antibiotics, protecting them from degradation (Khanna & Gerriets, 2022). The inhibitors do this by binding to the enzymes and

blocking their ability to hydrolyze the beta-lactam ring, thus preserving the antibiotic's activity against pathogens (Khanna & Gerriets, 2022). Efflux pump inhibitors are compounds that work against efflux pumps, which are membrane-bound proteins that actively transport a wide range of antimicrobial agents, such as antibiotics, out of bacterial cells, thus reducing the drug's effectiveness (Sharma et al., 2019). The inhibitors do this by binding to the pump or interfering with its ability to use energy (such as ATP), preventing it from effectively removing antibiotics from the bacterial cell and restoring susceptibility to the antibiotic (Sharma et al., 2019). Lastly, outer membrane permeabilizers are compounds that enhance the ability of antibiotics to penetrate the outer membrane of Gram-negative bacteria (eg. *Escherichia coli*, or *E. coli*), since this membrane is typically more protective and restricts the entrance of many antibiotics, making Gram-negative bacteria more difficult to treat than Gram-positive bacteria (Delcour, 2010). The permeabilizers work by disrupting or destabilizing the outer membrane, interacting with the lipid components (lipopolysaccharide layer), and causing pores or channels to form in the membrane for antibiotics to enter the cell (Delcour, 2010). Past research has investigated all of these types of antibiotic adjuvants to see which compounds have the best synergistic effect once combined with antibiotics. One adjuvant, β-lactamase inhibitor Clavulanic Acid, has been shown to significantly improve performance of antibiotic Amoxicillin, and is a commonly prescribed drug today (Evans et al., 2024). Another β-lactamase inhibitor adjuvant of Ampicillin, Sulbactam, showed significant improvements in the MIC value when combining the two substances together (Lamp & Vickers, 1998).

When looking at natural compounds with a higher chance of having good performance as antibiotic adjuvants, it is essential that the compound displays antimicrobial properties and low MIC values when tested on bacteria. This project has chosen to focus on two natural compounds, 1,8-cineole (Eucalyptol) and Naringenin, due to their promise of strong results when combined with various antibiotics. Eucalyptol is a naturally occurring, major component of the essential oils of eucalyptus, rosemary, camphor laurel, and several other plants (Hoch et al., 2023). It has gained attention for its potent antimicrobial properties, demonstrating effectiveness against many pathogens. Its antimicrobial action is primarily due to its ability to disrupt microbial cell membranes, leading to a leakage of cellular contents and cell death (Hoch et al., 2023). Studies show that the MIC of Eucalyptol with *E. coli* is 6.2 µg/ml (Wang et al., 2022), highlighting its effectiveness at relatively low concentrations. Due to these various properties, Eucalyptol is commonly used in various applications, such as a preservative in cosmetics and food, and in therapeutic settings for its antimicrobial, anti-inflammatory, antioxidant, bronchodilatory, analgesic, and pro-apoptotic effects (Hoch et al., 2023). Other current applications of this natural compound include management for various medical conditions such as Alzheimer's disease and cancer, showing its strong health advantages and potential for strong performance when combined with antibiotics. Past research suggests that Eucalyptol is an efflux pump inhibitor, as a study by Verma et al. (2022) showed that it targets the AdeABC efflux pump of MDR Acinetobacter baumannii, thus leading to efflux inhibitory activity, among other mechanisms of action observed against the pathogen.

Naringenin is a plant compound and flavonoid primarily found in citrus fruits, such as grapefruits. It is a bioactive compound known for its antimicrobial, antioxidant, anti-inflammatory, and anti-cancer properties (Salehi et al., 2019). This compound is primarily used for dietary supplements aimed at boosting the immune system and fighting off potential infections, skin care products (protects skin from damage caused by oxidative stress and inflammation), and is considered a potential therapeutic agent (Salehi et al., 2019). Past research shows that it has a

MIC value of 4.00 µg/µL against *E. coli* (Echeverria et al., 2017), making it a great potential antibiotic adjuvant. Past research suggests that Naringenin is a membrane permeabilizer as it disrupts membrane integrity and damages it, creating pores or channels within the outer membrane layer (Merghni et al., 2023). This is because the antibiotic is not

Though current studies show that both natural compounds demonstrate antimicrobial properties on their own and have lower MIC values (which indicates that a smaller amount of the antimicrobial agent is required to inhibit the growth of the microorganism – *E. coli* – making it a better choice since the microorganism is more susceptible to the compound), there is no current research showing the synergistic effect of these two compounds when combined with various antibiotics. The aim of this research project is to study this combination and see if it leads to lower Inhibitory Concentration 50% (IC50) values when compared to the individual MICs of the antibiotics and natural compounds. The results of this will show if the relationship is synergistic, antagonistic, or neither.

There will be 11 antibiotics combined with Eucalyptol and Naringenin, all from different drug classes, which will allow the effects of the natural compounds to be tested against antibiotics with different mechanisms of action against pathogenic bacteria. These antibiotics are sorted based on their chemical structure, mechanism of action, and the spectrum of bacteria (Gram-positive or Gram-negative) targeted. This project will use Ciprofloxacin, Norfloxacin, Cefdinir, Aztreonam, Azithromycin, Tetracycline, Kanamycin, Trimethoprim, Sulfamethoxazole, Colistin, and Nitrofurantoin. These belong to various classes, allowing the interactions of natural compounds Eucalyptol and Naringenin to be studied across antibiotics with various mechanisms of action to kill bacteria. Although there is past research showing antimicrobial properties and MIC values of the two natural compounds, there is no research showing the combination of those compounds with these antibiotics, and no research studies showing if synergistic or antagonistic effects occur from the combination. Multiple assays will be performed to determine how effective the combination is. A simple broth microdilution assay will be performed to determine individual MIC values of each natural compound and antibiotics. This will help determine concentrations of each compound to use in the following assay, which is a derivative of a checkerboard assay that will be performed with the natural compound and antibiotic. In a typical checkerboard assay, two compounds are diluted in the same plate, with one being diluted “across” the plate (meaning in the same column, every well has the same concentration of Compound A), and the other being diluted down the plate (in the same row, every well has the same concentration of Compound B). In this experiment, only the antibiotic will be diluted (two-fold dilution down the plate), and the same concentration of the natural compound will be used in all wells of the plate. IC50 values will be calculated from this assay, based on Area Under Curve (AUC) values from growth curves. Then, IC50 values from the derivative of checkerboard assay will be compared (individual vs combined IC50 values) to determine if the relationship is synergistic, antagonistic, or neither. Relationships between two compounds are synergistic if the combined effect is greater than the sum of the individual effects (if the combined IC50 is lower than individual ones), and antagonistic if the combined effect is lower than the sum of the individual effects (if the combined IC50 is greater than individual ones) (Fong et al., 2017). Using this information, it will be determined whether or not natural compounds 1,8-cineole (Eucalyptol) and Naringenin have a synergistic effect when combined with 11 antibiotics of various drug classes.

METHODS

Equipment and Materials

During all experimentation, *Escherichia coli* BW 25113 (Non-Pathogenic) was used, along with M9 as growth media. Important equipment used includes Integra Mini-96 electronic pipette machine, centrifuge, incubator, spectrophotometer, Tecan Microplate Reader, and BioTek LogPhase 600 Microbiology Reader machine.

Technique 1

To determine the MIC values of natural compounds and antibiotics, bacterial cultures are first grown and prepared under sterile conditions. Drug solutions are diluted to create a range of concentrations. These dilutions are added to microplates designed for broth microdilution assays. The bacterial culture is then normalized to a standard concentration and added to each well, except for control wells. Plates are incubated under appropriate conditions, and bacterial growth is assessed after incubation to determine the lowest drug concentration that inhibits visible growth, representing the MIC value.

From Technique 1 (used to find MIC values of individual natural compounds and antibiotics), scans of each plate were taken, and MATLAB scripts were run to determine the MIC values from each plate. Averages were taken from three replicates, for the final MIC values of each individual substance.

Technique 2

Bacterial cultures are first prepared by inoculating colonies into liquid growth media and incubating under controlled conditions. Drug and natural compound solutions are diluted to create a range of concentrations, which are added to 96-well plates in accordance with a predefined layout. A serial dilution is performed to achieve varying concentrations across the plate. The bacterial culture is normalized to a consistent optical density and added to each well. Automated pipetting equipment is used to ensure precise and consistent dispensing. Plates are then incubated with shaking at the appropriate temperature, and bacterial growth is later analyzed to assess the effects of each treatment condition.
From Technique 2, the machine collected absorbance values every 20 minutes. These values were taken and inputted into MATLAB scripts, creating growth curves for each well in each plate. Area Under Curve (AUC) values were calculated from these growth curves, and those AUC values were used to calculate IC50 values. IC50 values of individual antibiotics and antibiotics combined with natural compounds were compared using a One-tailed Two-Sample t-Test assuming unequal variances.

RESULTS

Test One (MICs of Antibiotics)

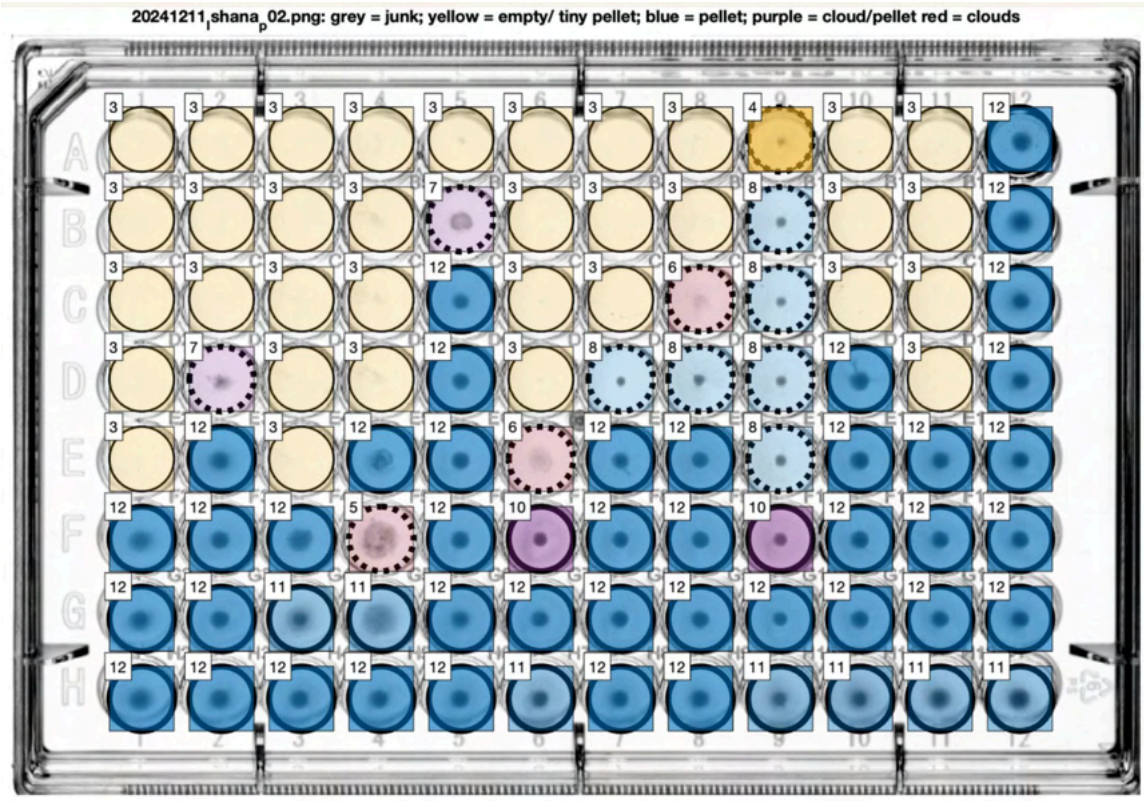

Figure 1. Replicate 2. Analyzed Broth Microdilution plate for MIC values of antibiotics. Each column 1-11 contains different concentrations of the same antibiotic. Upon analysis of bacteria pellets in each well, colors were assigned: yellow indicated no/minor bacteria presence, blue indicated full bacteria pellet, purple indicated cloudy bacteria pellet, red indicated cloudy well, and grey indicated unidentifiable object in the well. Using this information, MIC values were chosen based on the first concentration of antibiotic where there was a yellow well.

	MIC				
Drug	Rep 1	Rep 2	Rep 3	averages	stdev
Ciprofloxacin	0.024	0.024	0.024	0.024	0.0
Norfloxacin	0.391	0.391	0.391	0.39	0.0
Cefdinir	0.391	0.391	0.391	0.39	0.0
Aztreonam	0.098	0.098	0.098	0.10	0.0
Azithromycin	15.63	15.63	15.63	15.63	0.0
Tetracycline HCl	8.00	8.00	8.00	8.00	0.0
Kanamycin	7.81	15.63	7.81	10.42	4.5
Trimethoprim	7.81	7.81	7.81	7.81	0.0
Sulfamethoxazole	31.3	63	63	52.08	18.0
Colistin	3.13	3.13	3.13	3.13	0.0
Nitrofurantoin	31.25	31.25	31.25	31.25	0.0

Figure 2. MIC values for 11 antibiotics, calculated by taking an average of three replicates.

After performing initial broth microdilution assays, plates were scanned and ran through MATLAB scripts, which analyzed bacteria pellets in each well and marked them with certain colors, depending on the size/shape of bacteria. Figure 1 is an example of an analyzed plate. Looking at this, Minimum Inhibitory Concentration (MIC) values were chosen based on the first concentration of antibiotic where there was a yellow well, indicating that the concentration of antibiotic is strong enough to kill all bacteria in the well. Three replicates of this experiment were completed, and averages were taken to determine final MIC values of the antibiotics. Final MIC values from this experiment are shown in Figure 2.

Test Two (MICs of Natural Compounds)

During MIC experimentation for natural compounds Eucalyptol and Naringenin (repeating the same process used for finding MICs of the antibiotics), a MIC value could not be found. The highest concentration tested for Eucalyptol was 11 Ug/mL, and the highest concentration tested for Naringenin was 200 Ug/mL. Since MIC value could not be found, the next technique was adjusted.

Derivative of Checkerboard Assays (Combined Assays)

plate 4	compound A	no	no	no	yes	yes	yes	no	no	no	yes	yes	yes
	A	62.5	62.5	62.5	62.5	62.5	62.5	31.24	31.24	31.24	31.24	31.24	31.24
	B	31.25	31.25	31.25	31.25	31.25	31.25	15.62	15.62	15.62	15.62	15.62	15.62
	C	15.63	15.63	15.63	15.63	15.63	15.63	7.81	7.81	7.81	7.81	7.81	7.81
	D	7.81	7.81	7.81	7.81	7.81	7.81	1.95	1.95	1.95	1.95	1.95	1.95
	E	3.91	3.91	3.91	3.91	3.91	3.91	0.98	0.98	0.98	0.98	0.98	0.98
	F	1.953	1.953	1.953	1.953	1.953	1.953	0.488	0.488	0.488	0.488	0.488	0.488
	G	0.977	0.977	0.977	0.977	0.977	0.977	0.244	0.244	0.244	0.244	0.244	0.244
	H	0	0	0	0	0	0	0	0	0	0	0	0
				Kanamycin						Trimethoprim			

Figure 3. Plate map showing concentrations of Kanamycin and Trimethoprim in combined assay with Eucalyptol. “No” indicates wells where natural compound was not added, and “yes” indicates wells where the compound was added.

Instead of performing a typical checkerboard assay, an alternative assay was developed. The basic layout of this assay is shown in a plate map in Figure 3. The same concentration of the natural compound was used in every well that contained the compound (highest concentration tested in the MIC broth microdilution assays), and the antibiotics had a two-fold dilution down the plate, with the MIC value in the third or four well down each column. This made sure that when combined, the effects were being tested at both above the antibiotic MIC value and below the antibiotic MIC value. After running scripts on each combined plate, which created growth curves based on absorbance values taken every 20 minutes, calculated Area Under Curve (AUC) values from the curves, and calculated IC50 values based on AUC values, these results were analyzed and compared.

Experiment Set One (with Eucalyptol)

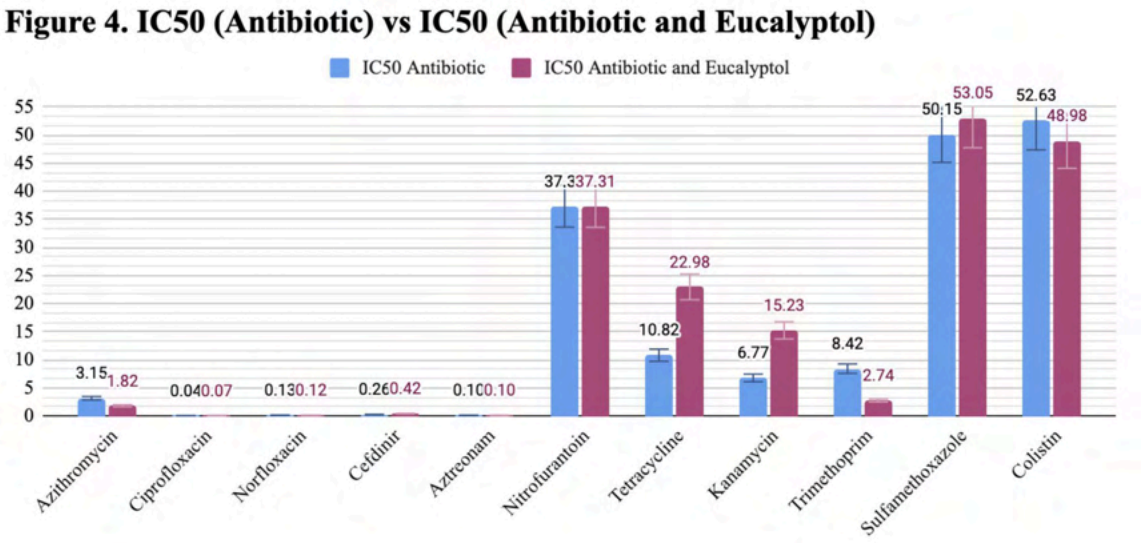

Figure 4. IC50 values of individual antibiotics vs IC50 values of combined wells (antibiotics and Eucalyptol).

The first round of experiments was completed with Eucalyptol. Figure 4 shows a comparison of IC50 values of each individual antibiotic vs IC50 values of combined wells, which contained both an antibiotic and Eucalyptol.

Experiment Set Two (with Naringenin)

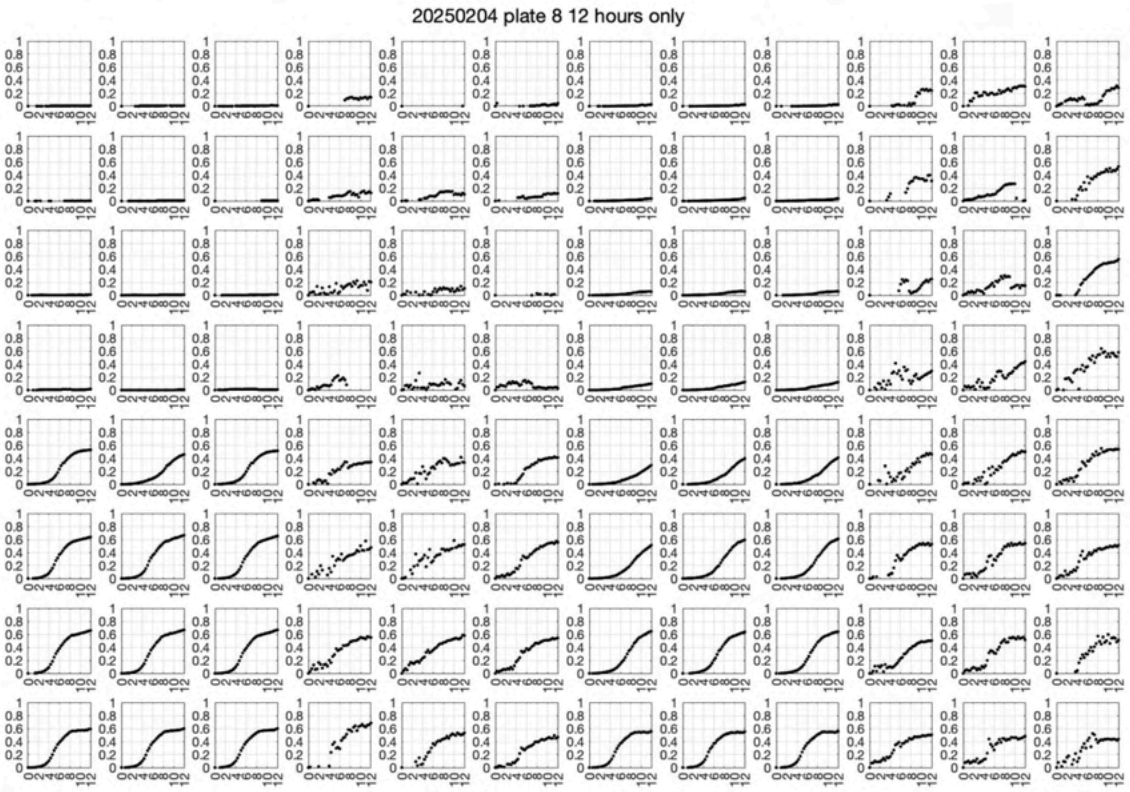

Figure 5. Growth curves from February 4, 2025. Plate contained Naringenin, Kanamycin, and trimethoprim.

When performing the same experiment again, using Naringenin, the combined plates were placed in the Logphase 600 machine, exactly like in the first round of experiments. However, growth curves had lots of noise, as shown in Figure 5.

Experiment Set Three (repetition experiments with Naringenin)

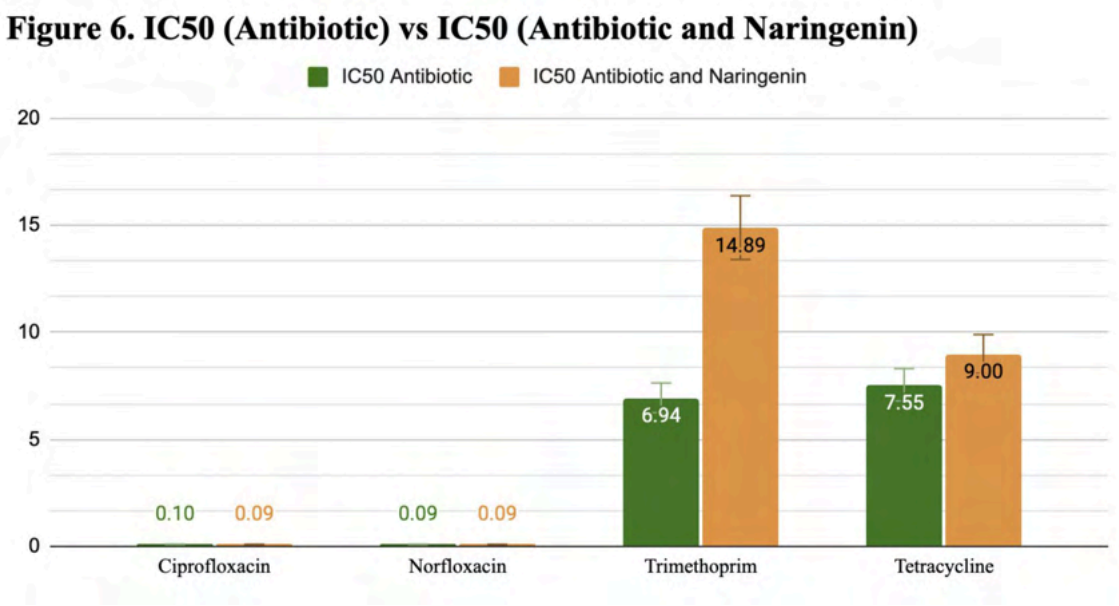

Figure 6. IC50 values of individual antibiotics vs IC50 values of combined wells (antibiotic and Naringenin)

Only two combinations tested in the second round of experiments, with Naringenin, did not contain noise in the growth curves. These combinations were fully run through the rest of the MATLAB scripts, calculating AUC values and IC50 values. Then, the rest of the growth curves were analyzed by eye, to determine which combinations appeared to have some sort of difference between the individual antibiotic and the combined wells. Two such combinations were found, and the second experiment was repeated with these specific antibiotics, and the combined plate was placed in the Tecan plate reader. Figure 6 shows a comparison of IC50 values of each individual antibiotic vs IC50 values of combined wells, which contained both an antibiotic and Eucalyptol.

MATLAB Script

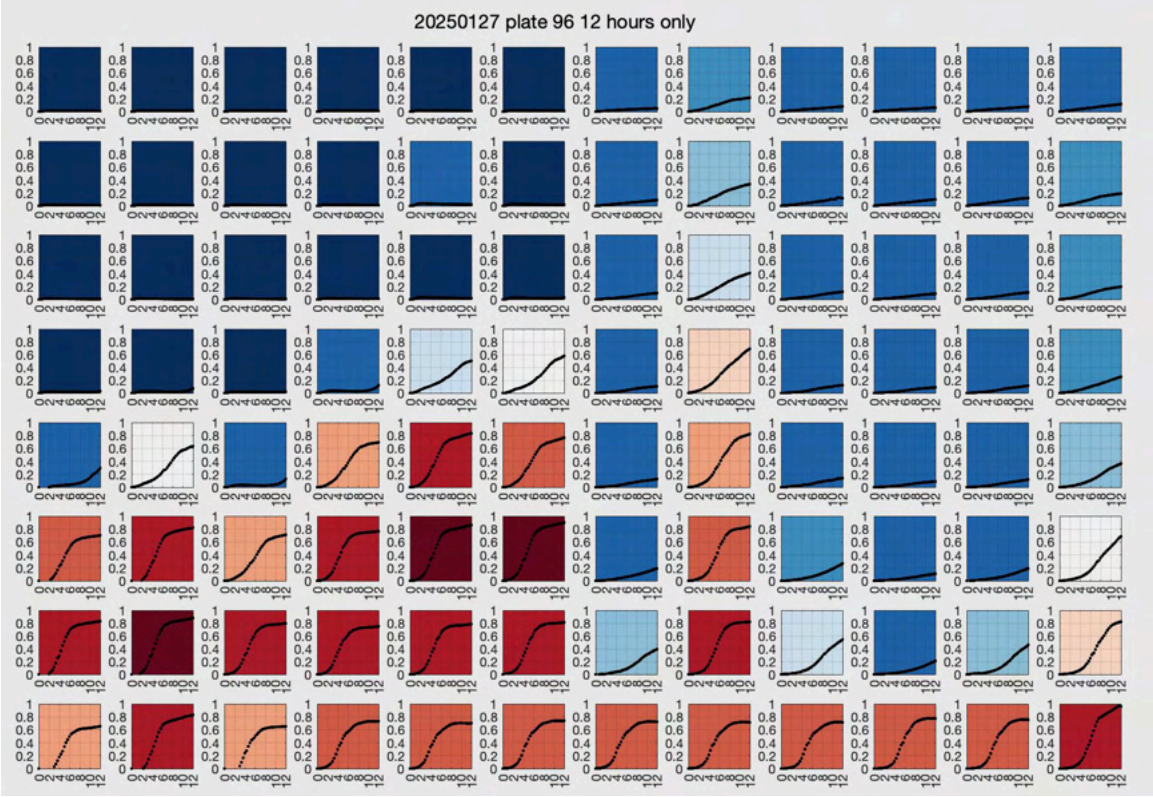

Figure 7. Growth curves of combined plate containing Eucalyptol, Kanamycin, and Trimethoprim after 12 hours of growth in Logphase 600 machine.

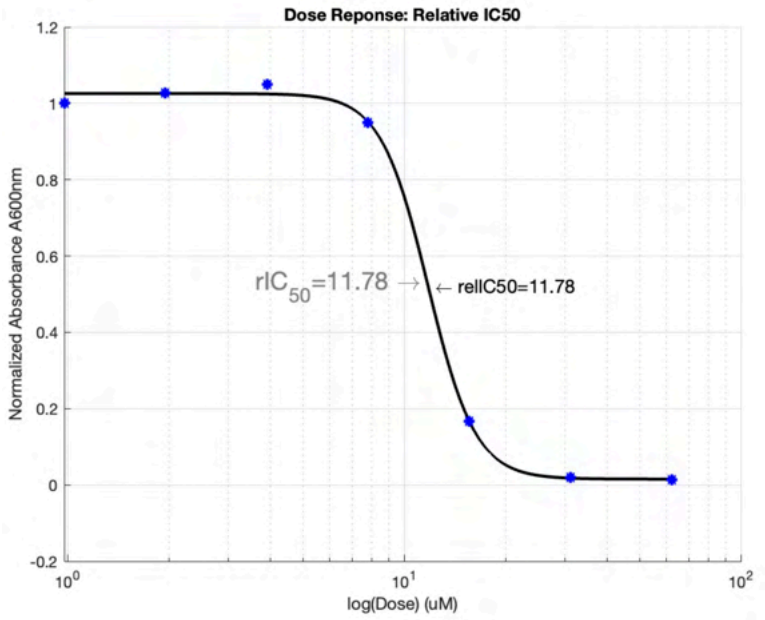

Figure 8. Dose-response curve showing how different concentrations of Eucalyptol combined with Kanamycin affect growth of *E. coli*. Highlights point at relative IC50 value.

Once the final experiment was complete, growth curves, AUC values, and IC50 values were generated. The microplate with the "best" comparison of growth curves is shown in Figure 7. Also, the most "normal" IC50 curve is shown in Figure 8.

One-tailed Two Sample t-Test assuming Unequal Variances

	Azithromycin	Ciprofloxacin	Norfloxacin	Cefdinir	Aztreonam	Nitrofurantoin	Tetracycline	Kanamycin	Trimethoprim	Sulfamethoxazole	Colistin
Eucalyptol	0.0069	0.221	0.191	0.157	0.222	0.49	0.048	0.018	0.23	0.443	0.432
Naringenin	-	0.365	0.374	-	-	-	0.419	-	0.00062	-	-

Table 1. P-values from One-tailed Two-Sample t-Test assuming Unequal Variances between IC50 values of antibiotics vs antibiotics with natural compounds. Boxes highlighted with pink indicate a synergistic relationship, while boxes highlighted in yellow indicate an antagonistic relationship.

For final analysis, a One-tailed Two-Sample t-Test assuming Unequal Variances was performed, to determine if differences between IC50 values of individual antibiotics and combined wells were statistically significant. All collected P-values are contained in Table 1.

DISCUSSION

All MIC values found for antibiotics in the first Broth Microdilution assays were significantly close to those reported in literature. These MIC values are contained in Figure 2.

However, such MIC values could not be found for the natural compounds. This occurred due to large differences in solubility levels that could be reached vs reported MIC values of the compounds. When preparing initial stocks of natural compounds Eucalyptol (liquid) and Naringenin (powder), due to limitation in materials that the researcher had access to in the lab, and safety concerns, a high enough concentration of each natural compound in their solvent (DMSO) could not be reached due to the amount of original solution/powder that could be fully dissolved. Thus, the original plan to perform checkerboard assays was changed. Instead of testing different concentrations of both the antibiotic and natural compound, only the antibiotic concentration was changed in this new assay. The concentration used for natural compounds in this second assay type was the highest concentration tested in the Broth Microdilution assays for MIC, and was given to all wells in every plate. The basic layout of these “combined” plates is shown in Figure 3.

In order for a relationship to be considered synergistic, the combined IC50 value must be lower than the IC50 value of individual antibiotic, indicating that the combination of the potential antibiotic adjuvant with the original antibiotic killed more bacteria. If the opposite scenario occurs, where the combined IC50 value is higher than that of individual antibiotic, this indicates that the combination of the natural compound with the original antibiotic helps increase growth of bacteria, making the relationship antagonistic.

In this research, the relative IC50 value was used instead of absolute IC50 value. Relative IC50 is the concentration at which 50% of maximal inhibition occurs, with a lower IC50 value indicating a more effective drug or drug combination. Absolute IC50 is the concentration at which a response is reduced to 50% of the control, and this value provokes a response halfway between the blank and positive control. Looking at all IC50 curves generated (most were not saved since they are not useful or provide much information visually for this specific research) many times the absolute IC50 could not be calculated because this point was never reached. Therefore, relative IC50 values were used for comparison. Some reasons why this point could not be reached include the dose-response curve not reaching both the baseline (0% inhibition) and the maximum inhibition level (100%). Also, it is highly likely that the combination being tested may only be capable of reducing a measured response to 60% of its blank value. If the combination can’t fully suppress the target response, meaning the dose response curve never crosses the 50% inhibition line, then the absolute IC50 is undefined, so concentration of the substance(s) cannot achieve that 50% inhibition level; this was the case for many trials during experimentation.

Figure 4 shows a comparison of IC50 values between individual antibiotics and antibiotics combined with Eucalyptol. Comparing average IC50 values by eye, there appears to be some potential synergistic relationships, with notably lower IC50 values occurring when combining Eucalyptol with Azithromycin, Trimethoprim, or Colistin. Additionally, there appeared to be notable increases in IC50 values when combining Eucalyptol with Tetracycline, Kanamycin, or Sulfamethoxazole, making these potential antagonistic relationships. Some of these relationships are also noticeable by eye when looking at the growth curves. For example, in the first six columns of wells in Figure 7, growth curves of Eucalyptol and Eucalyptol combined with Kanamycin are shown. Looking at row 4, it is clear that with just the antibiotic present, no bacteria was able to grow (indicated by a flat growth line, and dark blue square). However, in the combined wells, bacteria is starting to grow. This is a very noticeable difference,

and the trend continues in row 5, with Kanamycin now showing small growth rates, and the combined wells showing steep growth curves with the stationary phase at a much higher value. Overall, however, t-Tests must be performed on all 11 combinations to confirm any relationships, which was done later.

When observing growth curves from the second set of combined plate experiments with Naringenin, there appeared to be lots of noise in the curves, as observed in Figure 5. This occurred because precipitation occurred in the wells where Naringenin was combined with antibiotics. This can be confirmed by comparing the first three columns of wells, which just contained Naringenin, with the second three columns, which had both Naringenin and Kanamycin, and again with the combination of Naringenin and Trimethoprim in the last three columns. It is clear this occurred when the natural compound is combined with antibiotics, confirming that the noise observed in growth curves was due to precipitation and not machine errors. However, this did not occur when combining Naringenin with Ciprofloxacin or Norfloxacin, so those IC50 values were analyzed as normal. For the rest of the combinations, growth curves were analyzed by eye to determine possible synergistic combinations, by comparing individual antibiotic growth curves with combined well growth curves, to determine if there was some difference noticeable by eye. Such a difference was seen between Trimethoprim and Tetracycline, so these antibiotics were selected for the final experiment with Naringenin. The initial Naringenin experiments were performed in a Logphase 600 machine, which takes absorbance readings at a single spot in the well every 20 minutes. Since precipitation occurred in the combined wells, the machine could not take accurate readings, leading to the observed noise. To combat this issue in the two selected combinations, the Tecan plate reader was used. The Tecan machine takes multiple absorbance readings at multiple spots in the well and then averages them, so this machine was used for the last experiment. Once this experiment was completed, individual IC50 values vs combined IC50 values were graphed, shown in Figure 6. Analyzing this graph by eye, there appears to be two potential antagonistic relationships when Naringenin was combined with Trimethoprim or Tetracycline. Only IC50 values for Ciprofloxacin, Norfloxacin, Trimethoprim, and Tetracycline were graphed, since these values could not be calculated for the rest of drug combinations (since there was noise, an accurate AUC value could not be calculated, so IC50 values could not be determined).

To determine if synergistic or antagonistic relationships observed by eye were true, One-tailed Two Sample t-Tests assuming Unequal Variances were performed between individual antibiotic IC50 values and combined IC50 values. This test was particularly suitable for analyzing the impact of natural compounds on antibiotic efficacy, as the IC50 values for different conditions could have different levels of variability. The one-tailed approach was used because the primary research question focused on whether the natural compounds significantly enhanced or reduced antibiotic efficacy, rather than simply identifying any difference in either direction. This statistical method ensured a rigorous assessment of whether the observed changes in antibiotic effectiveness were statistically significant, supporting the study's conclusions regarding synergistic and antagonistic interactions. This would also help determine if there were relationships not noticeable by eye. Results of the t-Tests are shown in Table 1. There were four statistically significant relationships, shown when Eucalyptol was combined with Azithromycin**, Tetracycline*, or Kanamycin*, and when Naringenin was combined with Trimethoprim***. Since IC50 values of combined Azithromycin and Eucalyptol were lower than individual Azithromycin IC50 values, this relationship is synergistic. Since Tetracycline and

Kanamycin had higher IC50 values when combined with Eucalyptol, their relationships are antagonistic. Also, Trimethoprim and Naringenin have an antagonistic relationship.

Overall, objectives of this project were accomplished. The hypothesis was proven true, as a novel synergistic relationship was found through this research, and three other, unexpected, novel antagonistic relationships were also found.

The synergistic effect of azithromycin and eucalyptol when combined may be due to their complementary mechanisms in combating bacterial infections. Azithromycin, a macrolide antibiotic, works by inhibiting bacterial protein synthesis, preventing the growth and replication of bacteria. Eucalyptol, on the other hand, has been shown to exhibit mild antimicrobial properties, potentially disrupting bacterial cell membranes and enhancing the permeability of bacterial cell walls. This could allow azithromycin to penetrate bacterial cells more effectively, improving its ability to target and kill bacteria. Furthermore, eucalyptol's ability to disrupt bacterial biofilms—protective layers that often make bacteria more resistant to antibiotics—could make the bacteria more susceptible to the action of azithromycin. Together, these combined actions may enhance the antimicrobial potency of both agents, leading to a more effective eradication of bacterial infections. On the other hand, Eucalyptol may have an antagonistic relationship with certain antibiotics, such as tetracycline and kanamycin, due to its effects on bacterial cell membranes and drug permeability. Eucalyptol can alter the integrity of bacterial membranes, potentially disrupting the uptake and efficacy of these antibiotics, which rely on specific cellular mechanisms to reach their targets and inhibit bacterial growth. This disruption could reduce the effectiveness of tetracycline, which targets protein synthesis, and kanamycin, which affects ribosomal function. Similarly, naringenin, a flavonoid known for its antioxidant and antimicrobial properties, could have an antagonistic effect when combined with trimethoprim. Naringenin may interfere with the folate synthesis pathway targeted by trimethoprim, either by altering cell membrane permeability or by interacting with enzymes involved in the drug’s mechanism of action, thereby reducing the antibiotic's effectiveness. In both cases, the changes to bacterial cell membranes and interference with drug absorption or activity can undermine the intended therapeutic outcomes of these antibiotics. More research is required to confirm this.

To address the limitation of the number and type of antibiotics tested, this study carefully selected representative antibiotics from different classes to ensure a diverse range of mechanisms were examined. Although only a subset of antibiotics could be tested, the findings provide a foundation for future studies that can expand on this work by including additional antibiotic classes. The limited number of natural compounds tested was addressed by selecting compounds with previously reported antimicrobial properties, increasing the likelihood of meaningful interactions. Future studies can explore a broader range of natural compounds to further validate and expand upon these findings. To mitigate the effects of a limited number of replicates, strict methodological consistency was maintained across experiments to reduce variability. Standardized bacterial inoculum concentrations, incubation times, and antibiotic preparation methods were implemented to improve reliability. Additionally, statistical analyses were performed to assess significance despite the limited replicates. Time constraints were managed by prioritizing key assays that provided the most direct insights into antibiotic-compound interactions. Although gene analysis could not be performed in this study, the data collected serves as a basis for future genetic studies. Further research incorporating

transcriptomic and proteomic analyses will provide a deeper understanding of the molecular mechanisms underlying these interactions.

One potential confounding variable is the variability in bacterial response due to environmental factors such as nutrient availability, incubation time, and inoculum density. These variables were controlled by maintaining standardized experimental conditions across all assays. Additionally, the natural compounds used in this study may contain impurities or secondary metabolites that could contribute to observed effects, necessitating further purification and analysis. Another confounding variable is the potential for off-target effects of natural compounds, which could influence bacterial growth independently of antibiotic interactions. To minimize this, appropriate controls were included in all experiments to ensure that observed effects were specifically due to the antibiotic-compound interactions rather than individual compound toxicity.

This study represents the first investigation into the interactions between Eucalyptol and Azithromycin, uncovering both synergistic and antagonistic relationships. Prior research has primarily focused on examining the efficacy of individual antibiotics or their mechanisms of resistance. Studies have extensively explored how antibiotics function in isolation, often neglecting the broader context in which natural compounds might influence their performance. However, our research takes a unique approach by exploring the potential of natural compounds, specifically Eucalyptol, as modulators of antibiotic activity. This is a departure from conventional antibiotic research, where the emphasis has typically been placed on the development of new antibiotics or enhancing the activity of existing ones against resistant strains.

The findings of this study contribute to a growing body of knowledge on the role that natural compounds play in influencing antibiotic efficacy. In particular, our discovery that Eucalyptol can act as a potential antibiotic adjuvant opens up new avenues for treatment strategies, especially in light of the global antibiotic resistance crisis. By identifying both synergistic and antagonistic interactions between Eucalyptol and Azithromycin, our research broadens the understanding of how non-antibiotic compounds might affect antibiotic performance. While previous studies have largely focused on the independent antimicrobial properties of plant-derived compounds, this work dives deeper into the combinatorial effects of such compounds in tandem with traditional antibiotics.

The research is distinct in its emphasis on the interactions between a widely used antibiotic and a natural compound, highlighting the potential for improving antibiotic treatments without the need for developing entirely new classes of antibiotics. This approach is not only cost-effective but also offers a novel strategy to overcome resistance, particularly as bacteria continue to evolve mechanisms to evade existing treatments. By showcasing how Eucalyptol modulates the activity of Azithromycin, our study suggests that a re-evaluation of current treatment regimens may be necessary, incorporating natural compounds as potential adjuncts to enhance therapeutic outcomes.

Furthermore, this study opens up possibilities for future research that could focus on optimizing antibiotic formulations to counteract resistance more effectively. By combining antibiotics with natural compounds in targeted ways, researchers may be able to design more robust drug regimens that are not only more effective in the short term but also contribute to

long-term strategies for controlling bacterial growth and resistance. As the field moves toward more personalized medicine and more effective multi-drug strategies, this research lays the groundwork for further investigation into how the strategic use of natural compounds could revolutionize antibiotic therapy.

This work also paves the way for the development of synergistic drug combinations that could be tailored for specific bacterial infections, potentially reducing the reliance on single-drug treatments that often contribute to resistance. By providing a deeper understanding of the interaction dynamics between antibiotics and natural compounds, this study advances the scientific community's knowledge of how to integrate these substances more effectively into clinical settings. These findings have significant implications not only for the treatment of infections but also for the broader field of drug development, offering insights into how we might enhance the efficacy of existing treatments without creating additional pressure on the environment or healthcare systems.

CONCLUSION

This study presents novel findings on the interactions between natural compounds and antibiotics, identifying both synergistic and antagonistic relationships that have not been previously reported. The primary objective was to evaluate whether Eucalyptol and Naringenin could enhance or inhibit antibiotic efficacy. The discovery that Eucalyptol enhances the efficacy of Azithromycin suggests its potential as an antibiotic adjuvant, offering a new approach to improving treatment effectiveness and potentially combating antibiotic resistance. In contrast, the observed antagonistic interactions—Eucalyptol with Tetracycline, Eucalyptol with Kanamycin, and Naringenin with Trimethoprim—highlight the complex ways in which natural compounds can influence bacterial response to antibiotics. While antagonistic effects are generally undesirable in clinical applications, they open new avenues for research into bacterial growth modulation in industrial and research settings.

This study utilized Broth Microdilution assays to determine the Minimum Inhibitory Concentrations (MIC) of antibiotics and natural compounds, ensuring accuracy by comparing results with published literature. However, due to solubility limitations, the MIC values for natural compounds could not be reliably determined, leading to a modification in the experimental approach. Instead of performing checkerboard assays, a revised method was implemented where only the antibiotic concentration was varied, while the highest testable concentration of each natural compound (Eucalyptol or Naringenin) was maintained across all wells. Growth curves were analyzed to assess bacterial response, with IC50 values used as a comparative metric. Relative IC50 values were calculated due to inconsistencies in absolute IC50 determination caused by incomplete inhibition in some trials. To improve measurement accuracy, different plate readers were used. The Logphase 600 was initially employed but generated noise due to precipitation in Naringenin combination assays. Consequently, a Tecan plate reader was used for the final experiments, as it takes multiple readings across each well, providing more reliable data. Statistical analysis was performed using one-tailed two-sample t-tests assuming unequal variances to determine whether observed changes in IC50 values were statistically significant. This method was selected to account for variability between sample

groups and to specifically assess whether natural compounds enhanced or reduced antibiotic efficacy. These statistical tests confirmed both synergistic and antagonistic interactions between antibiotics and natural compounds.

The findings of this study contribute to the broader field of antibiotic research by providing the first evidence of these specific interactions. Unlike previous studies that have focused primarily on the independent antimicrobial properties of plant-derived compounds, this research demonstrates how they interact with existing antibiotics, expanding our understanding of combination therapies. The use of rigorous statistical analysis ensured the reliability of the results, despite inherent limitations such as the number of antibiotics and compounds tested, time constraints, and the inability to perform gene-level analyses.

Future research should build upon these findings by conducting gene expression studies to elucidate the molecular mechanisms driving these interactions. Additionally, expanding the range of antibiotics and natural compounds tested, as well as employing alternative assays such as time-kill studies, will further validate and refine these conclusions. Ultimately, this research lays the groundwork for future investigations into the potential of natural compounds in antibiotic therapies, offering new strategies to enhance antibiotic efficacy and address the growing challenge of resistance.

FUTURE WORK

Future research should focus on elucidating the molecular mechanisms underlying these synergistic and antagonistic effects. Gene analysis studies could help determine the specific genetic pathways influenced by these natural compounds when combined with antibiotics. Transcriptomic and proteomic studies could reveal bacterial gene expression changes in response to these treatments, shedding light on resistance mechanisms or synergistic enhancement pathways. Additionally, more experiments using different types of assays, such as time-kill assays, should be conducted to further assess the performance of these natural compounds in combination with antibiotics. Expanding the range of antibiotics tested, particularly within the same drug classes that demonstrated significant interactions, could provide further insights into the generalizability of these findings. Overall, this study highlights the potential of natural compounds as antibiotic adjuvants and underscores the complexity of antibiotic interactions. Continued research in this area could contribute to the development of novel therapeutic strategies aimed at overcoming antibiotic resistance and improving the effectiveness of existing treatments.

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