The synergistic effect of combining natural compounds 1,8-cineole (Eucalyptol) and Naringenin with 11 antibiotics of different drug classes

Grant Proposal

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Abstract

Antibiotic resistance is one of the most urgent global health threats, as common bacterial infections are becoming increasingly difficult to treat with existing antibiotics. Millions of people are dying every year from infections caused by antibiotic-resistant bacteria, highlighting the dire need for new strategies to combat antimicrobial resistance. This study looks into the potential of two natural compounds, 1,8-cineole (Eucalyptol) and Naringenin, when combined with 11 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 – Eucalyptol has demonstrated antimicrobial effects through membrane disruption — and membrane permeabilizers — Naringenin damages bacterial membranes. The aim of this research is to evaluate the synergistic effects of these natural compounds when combined with 11 antibiotics from various drug classes (eg. β-lactams, fluoroquinolones, and aminoglycosides) against Escherichia Coli. The minimum inhibitory concentrations (MICs) and optical density (OD) values will be compared for each chemical both individually and combined, using broth microdilution assays and checkerboard assays. It is hypothesized that both Eucalyptol and Naringenin will enhance antibiotic effectiveness. Results of this study could identify these natural compounds as antibiotic adjuvants that improve the treatment of bacterial infections and reduce the possibility of resistance. It holds significant implications for addressing the growing challenge of antimicrobial resistance by offering a novel approach to restoring the efficacy of existing antibiotics using new, uninvestigated natural compounds.

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

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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). This is 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 can be 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 skill specific bacteria (WHO, 2024). Another group 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 MIC values 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).

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 a lower MIC when compared to the individual MICs of the antibiotics and natural compounds. The results of this will show if the relationship is additive, synergistic, 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 the synergistic effect. Multiple assays will be performed to determine how effective the combination is. A simple broth microdilution assay will be performed to determine the individual MIC values of each natural compound and antibiotic. Then, a checkerboard assay will be performed with the natural compound and antibiotic, and MIC values from this test will be collected. MIC values from the two tests will then be compared (individual vs combined MIC values) to determine if the relationship is additive, synergistic, or antagonistic. Relationships between two compounds are additive if the combined effect is equal to the sum of the individual effects, synergistic if the combined effect is greater than the sum of the individual effects (if the combined MIC is lower than individual ones), and antagonistic if the combined effect is lower than the sum of the individual effects (if the combined MIC 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.

Section II: Specific Aims

This proposal's objective is to provide information on the relationship and benefits of two natural compounds, 1,8-cineole (Eucalyptol) and Naringenin when combined with a selection of 11 antibiotics from varying drug classes. Specifically, it aims to determine if the combination of these natural compounds with antibiotics leads to a synergistic effect, with the combined MICs being lower compared to the individual MICs. This experiment will help identify if the natural compounds perform well as antibiotic adjuvants, and if they can be used to help improve the effectiveness of existing antibiotics to help combat antibiotic resistance.

The long-term goal is to see if these natural compounds can perform well as antibiotic adjuvants, and if they can be used in real-world scenarios, being administered to patients along with existing antibiotics to enhance their performance and reduce the chance of antibiotic resistance. The central hypothesis of this proposal is that 1,8-cineole (Eucalyptol) and Naringenin have a synergistic effect when combined with 11 antibiotics of various drug classes. The rationale is that past research suggests these two natural compounds perform similar to two types of antibiotic adjuvants, efflux pump inhibitors and

membrane permeabilizers, respectively. The work proposed here is essential in the process of finding more natural compounds with strong performance as antibiotic adjuvants, to help combat the rising issue of antibiotic resistance, which is a big issue with severe potential to negatively impact global health.

Specific Aim 1: Determine best growth media for future assays.

Specific Aim 2: Evaluate the antimicrobial activity of 1,8-cineole (Eucalyptol) and Naringenin as individual compounds against E. Coli.

Specific Aim 3: Assess the synergistic effect of combining 1,8-cineole (Eucalyptol) and Naringenin with a selection of 11 antibiotics against E. Coli.

Specific Aim 4: Compare the effects of the combination of 1,8-cineole (Eucalyptol) and Naringenin with antibiotics on E. Coli to determine the optimal combinations that result in the lowest MIC values. Use this information to decide if these natural compounds are strong-performing antibiotic adjuvants that can be used to help combat the global rise in antibiotic resistance.

The expected outcome of this work is that both 1,8-cineole (Eucalyptol) and Naringenin have a synergistic effect with a smaller group selection of the 11 overall antibiotics chosen. This outcome is expected because both natural compounds demonstrate having mechanisms of action that directly relate to specific mechanisms of action of two types of antibiotic adjuvants, just on different bacterial pathogens. A similar (or better) effect is expected to occur when combined with antibiotics and tested against *E. Coli*.

Section III: Project Goals and Methodology

Relevance/Significance

This research is important because it can lead to the discovery of two new antibiotic adjuvants that can be combined with antibiotics to enhance their performance, therefore helping combat the rising global issue of antibiotic resistance.

Innovation

Although there is existing research showing antimicrobial properties of 1,8-cineole (Eucalyptol) and Naringenin, as well as their MIC values, there is no research studying their performance as antibiotic adjuvants, or if they have a synergistic effect when combined with antibiotics. This project is conducting novel research that will help determine if this combination can result in less antibiotic resistance for patients who are administered the drug combination. If successful, there will be <u>two</u> new antibiotic adjuvants discovered, which will greatly benefit researchers who are looking for new alternatives to existing antibiotics. Also, it can be a more effective solution because it is an addition to existing methods.

Methodology

Specific Aim #1:

Determine best growth media for future assays.

Justification and Feasibility. The most optimal growth media must be chosen so that bacteria can grow during assays, but while making sure the growth media does not contain too much nutrients to invalidate the results (by allowing mass growth of bacteria following their death from a compound).

Summary of Preliminary Data.

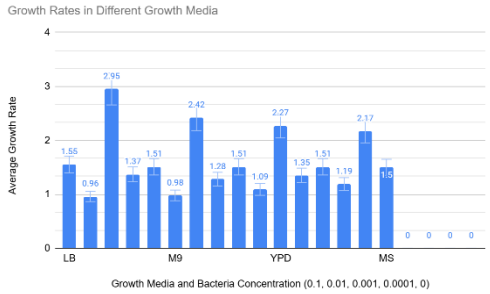

Figure 1. Growth Rates in Different Growth Media.

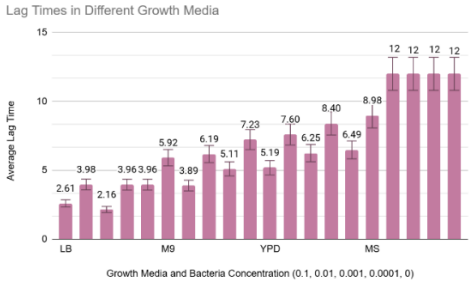

Figure 2. Lag Times in Different Growth Media.

Preliminary data collected (shown in Figures 1 and 2 above) shows comparison of growth rates and lag times of 5 different growth media at different concentrations. The graphs show that LB growth media provides the best environment for growth of *E. Coli*, as it has the highest average growth rate and lowest average lag time. M9 had the second-best growth rate and second lowest lag time and will be used for future experiments (different assays) because it provides enough nutrition for bacteria to grow without giving it too many nutrients to continue growth even after being inhibited by an added compound.

Expected Outcomes. The overall outcome of this aim is to determine best growth media. This information will later be used for future broth microdilution and checkerboard assays.

Potential Pitfalls and Alternative Strategies. M9 broth will be used for all future assays.

Specific Aim #2:

Determine the MIC values of 1,8-cineole (Eucalyptol), Naringenin, and 11 antibiotics individually. The MIC values will be found using broth microdilution assays.

Justification and Feasibility.

Compound	Minimal Inhibitory Concentration in Solid Media (μg/μL) *							
	Gram-Negative Bacteria				Gram-Positive Bacteria			
	E. cloacae	*E. coli*	*K. pneumoniae*	*P. mirabilis*	*B. cereus*	*B. coagulans*	*B. subtilis*	*S. aureus*
Naringenin (4,5,7-Trihydroxyflavanone)	2.00	4.00	>4.00	2.00	2.00	2.00	2.00	>4.00

Figure 3. MIC value of Naringenin (Echeverría et al., 2017).

Broth microdilution assays will be performed to verify the individual MICs of the chemicals (natural compounds and antibiotics) and will serve as a baseline to determine results of the project. OD values will also be collected. Past research shows that the MIC values should be around 4 μg/μL for Naringenin (Figure 1) (Echeverria et al., 2017) and 6.2 μg/ml for Eucalyptol (Wang et al, 2022).

Expected Outcomes. The overall outcome of this aim is to determine MIC values of the individual chemicals. This information will later be used for comparison.

Potential Pitfalls and Alternative Strategies. It is expected that MIC values collected in this step are similar to those found in past research.

Specific Aim #3:

Determine the MIC values of 1,8-cineole (Eucalyptol) and Naringenin when combined with antibiotics. These MIC values will be found using checkerboard assays.

Justification and Feasibility. Checkerboard assays will be performed to find the combined MICs of the chemicals (natural compounds and antibiotics) and will be used for comparison later.

Expected Outcomes. The overall outcome of this aim is to determine MIC values when each natural compound and the antibiotics are combined. This information will later be used for comparison.

Potential Pitfalls and Alternative Strategies. It is expected that combined MIC values are lower than individual MICs.

Specific Aim #4:

Compare the effects of the combination of 1,8-cineole (Eucalyptol) and Naringenin with antibiotics on E. Coli to determine the optimal combinations that result in the lowest MIC values.

Justification and Feasibility. This step is essential as this comparison will determine if Eucalyptol and Naringenin enhance the performance of a smaller group of 11 overall antibiotics, and if there is a synergistic relationship between them.

Expected Outcomes. This aim will determine the success of this project. This information will be used to decide if the natural compounds 1,8-cineole (Eucalyptol) and Naringenin are strong-performing antibiotic adjuvants.

Potential Pitfalls and Alternative Strategies. It is expected that the MIC values of the combined chemicals is lower than the MIC values of the individual chemicals. Therefore, it is expected that both Eucalyptol and Naringenin are strong-performing antibiotic adjuvants.

Section III: Resources/Equipment

Materials required for this experiment include 1,8-cineole (Eucalyptol), Naringenin, 11 antibiotics (Ciprofloxacin, Norfloxacin , Cefdinir, Aztreonam, Azithromycin, Tetracycline, Kanamycin, Trimethoprim, Sulfamethoxazole, Colistin, and Nitrofurantoin) and *Escherichia coli*, as well as materials required to prepare stock solutions and perform broth microdilution assays and checkerboard assays. Specialized equipment used in this project include an incubator, centrifuge machine, electronic 96 channel pipette and a spectrophotometer.

Section V: Ethical Considerations

There are no ethical concerns from this project, as the risk of harmful effects is minimal (many safety precautions in place), and there are no animal or human subjects involved. It is focused on laboratory testing of compounds and antibiotics, which are standard practices in microbiology.

Section VI: Timeline

Task	Duration	Time Frame
Complete initial training, learn to perform these types of assays. Determine best growth media for future assays.	1 week	Week 1
Set up broth microdilution assay for Eucalyptol, Naringenin, and 11 antibiotics, collect MIC values and OD values	1 week	Week 2
Set up checkerboard assay for combination of either Eucalyptol or Naringenin with 11 antibiotics, collected MIC values and OD values	1 week	Week 3
Perform time kill assay (if time allows)	1 week	Week 4
Data analysis	1 week	Week 5
Finalize report	1 week	Week 6

Section VIII: References

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