Investigating the Effects of Penicillin and Essential Oils on the Viability of *Staphylococcus aureus*

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ABSTRACT

The emergence of *Staphylococcus aureus* related illnesses is posing an alarmingly dangerous lifestyle for individuals with compromised immune systems. The increasing resistance of bacterial strains and microorganisms to conventional antibiotics and treatments is a serious problem that has alerted scientists to identify new approaches to prevent their emergence. Essential oils contain properties that are capable of inhibiting or slowing the growth of bacteria. The combination of antibiotics and essential oils are representing a new development in combating antibiotic resistance. The aim of this study was to investigate the efficacy of essential oils and penicillin in decreasing the viability of *S. aureus*. The disk diffusion assay was utilized to examine the effects of penicillin and the essential oils, lavender and tea tree oil, individually and in combination. All the treatments decreased *S. aureus* viability to different extents, by exhibiting moderate zones of inhibition. Penicillin was observed to be more potent than the essential oils. In addition, the efficacy of the combination of penicillin and the essential oils was investigated. Interestingly, the results portrayed the combinations had a lower potency than the sum of the individual treatments. The results demonstrated that the essential oils of lavender and tea tree can be used as potential antibacterial agents against *S. aureus* infections and penicillin in combination with essential oils has an antagonist effect.

INTRODUCTION

Antibiotics are the most common form of treatment in fighting infectious disease and have tremendously enhanced the health of individuals since their creation. However, the incidents of antibiotic resistant infections are alarmingly on the rise. The emergence and spread of antibiotic resistance have occurred due to many factors. The main factor influencing the emergence is the excessive bacterial exposure to antibiotics with the intensive use in the community. Bacterial strains are becoming resistant to multiple antibiotics that is fueling the rampant spread. The persistence of antibiotic resistance urges the need of finding new therapies against the multi-antibiotic resistant bacteria.

The model organism under investigation is *Staphylococcus aureus*. *S. aureus* is among the bacterial species of high interest in research. *S. aureus* is a commensal bacterium and human pathogen that causes clinical infections and bacteremia (Tong et al, 2015). Bacteremia can lead to sepsis, death and both incidence and mortality from *S. aureus*- associated bacteremia are on the rise (Van Hal et al, 2012). Infectious strains of *S. aureus* are commonly found in hospital settings; however, they are now major community-based pathogens (Knox et al, 2015). Approximately 30% of the human population is colonized with *S. aureus* due to its prevalence in household environments (Tong et al, 2015). *S. aureus* can be found on the skin and mucous membranes, allowing humans to be major reservoirs (Tong et al, 2019). These strains become pathogenic when introduced to a debilitated immune system (Tong et al, 2015). Due to their prevalence, *S. aureus* are becoming resistant to standard treatments, such as penicillin and treatments, such as topical antibacterial (Charles & Simon, 1990). The emergence of Methicillin resistant *Staphylococcus aureus* (MRSA) is becoming a significant health concern, not only in clinical settings, but also in homes (Charles & Simon, 1990). Therefore, it is important to identify potential ways to enhance the efficacy of common antibiotics that can also be safely used in household environments against *S. aureus*.

The standard treatment for infections relating to *S. aureus* is penicillin. Penicillin functions by bursting the bacterial cell wall by acting directly on peptidoglycans (Penicillin, 2019). However, there are strains of *S. aureus* that have evolved resistance to penicillin through a variety of mechanisms. Resistance to antibiotics becomes difficult when treating *S. aureus* and requires continual development of new drugs to combat the rising concern for infections in clinical and household environments. Combinatorial therapies have been shown to be effective at reducing the adaptive responses that lead to antibiotic resistance (Penicillin, 2019). However, implementation of these approaches in household settings has not been thoroughly investigated and is important as more individuals are turning towards alternative methods like essential oils.

Essential oils have been of great interest for exhibiting broad biological properties. They are aromatic liquids derived from plants that have demonstrated therapeutic, antimicrobial, and antioxidant properties (Dhifi et al, 2016). Due to these properties, essential oils are becoming a desirable alternative ingredient in cosmetics, food, beverages, and medicinal applications (Dhifi et al, 2016). Importantly, studies have recognized that essential oils have properties that can eliminate bacterial growth and enhance the bactericidal effects of beta-lactams. Many studies focus on selected essential oils to provide an explanation to their mechanisms. Furthermore, studies have shown the synergistic effect of antibiotics and essential oils against various human pathogens. Recently, it was shown that, geranium oil enhanced the action of piperacillin at a lower dose compared to piperacillin alone (Orchard, 2017). Piperacillin and penicillin are members of the same drug class, suggesting that combinatorial treatments may be feasible against *S. aureus*. However, few studies have investigated the combination of antibiotics with essential oils. Therefore, it is important to investigate if the standard treatment of penicillin will portray these mechanisms with the essential oils. With the combination of penicillin and similar drugs with essential oils, there is the potential to enhance bactericidal effects thus decreasing resistance.

The essential oils, lavender and tea tree, were selected for the experimentation. Lavender oil is utilized in perfumes, household cleaners, and bath products due its antimicrobial properties (Kavanaugh et al, 2012). Lavender oil is one of the most common essential oils used in household environments. Studies have identified that they can help enhance bacterial elimination. However, they cannot completely eradicate the bacteria (Kavanaugh et al, 2012). Similarly, tea tree oil contains antibacterial and immune stimulant properties and has shown to rapidly decrease bacterial strains, such as *Klebsiella spp.* and *S. maltophilia* (May et al, 2000). Lavender and tea tree oil exhibit antibacterial properties and can decrease bacteria. However, they cannot completely eradicate those bacterial strains. Recent studies have discovered that thyme oil and lemon oil are active against *S. aureus* and other bacterial strains (Man et al, 2019). The essential oils selected for this study have similar structures and mechanisms to the oils used in previous studies, indicating that lavender and tea tree oil should be potent in eliminating bacteria.

The study has been conducted to investigate how the addition of essential oils to penicillin will affect the viability of *S. aureus* due to a lack of research of this combination. It was hypothesized that the addition of lavender and tea tree oils to the penicillin treatment would decrease viability in *S. aureus*. The study aimed to determine the individual potencies of the essential oils and penicillin. It was hypothesized that the individual treatments would decrease the viability but not completely eradicate the bacteria. The study focused on determining the combinatorial effects of penicillin and the essential oils. It was hypothesized that the combination would have a higher potency than the individual treatments following the data seen in previous studies.

MATERIALS AND METHODS

Essential Oil and Penicillin Material

The oils, Tea tree pure essential oil therapeutic grade and lavender pure essential oil were acquired from Amazon. Penicillin G (potassium salt) 5g was utilized in the experiment.

Bacterial Strain

The bacterial strain, *Staphylococcus aureus Subsp. aureus* Rosenbach (ATCC ® 25904), was used to test the essential oil and penicillin treatments. The bacterial strain was inoculated in trypticase- soy media.

Turbidity Assay

There is no clear standardization of the methodology to evaluate the activity of essential as well-established as antibiotics. Therefore, in the beginning of the experiment, the turbidity assay was used to investigate the individual effects of essential oils and penicillin on the viability of *S. aureus* and to identify the standard growth curve for the bacterial strain.. In order to complete the assay, bacterial pellets were isolated by centrifugation at 3000g for 15 minutes. The assay required a 0.9% NaCl solution that was used to resuspend each bacterial pellet. The assay was performed using a turbidity assay sensor, in which each absorbance was recorded. After generating and interpreting data, the methodology was switched to disk diffusion assay due to issues with the solubility of the essential oils.

Mueller Hinton Agar Plates

The plates were made by adding 38g of Mueller Hinton Agar media to 1L of water. Next the agar was placed on a hot plate with frequent agitation and boiled for one minute to completely dissolve the medium. Once that was complete, the medium was placed in an autoclave at 121ºC for 15 minutes. Finally, the media was poured into plates till the bottom was completely covered. Plates were solidified at room temperature and stored in a refrigerator for future use.

Disk Diffusion Assay

Investigating the Individual Effects of Essential Oils and Penicillin

The disk diffusion assay was utilized to determine the individual effects of essential oils and penicillin on the viability of *S. aureus*. Disk diffusion assay is one of the most methods used for susceptibility testing. In the experiment, each disk contained a different treatment and were placed on Mueller Hinton Agar Plates. For individual treatments, each trial contained 41 disks: a ten- fold dilution of lavender oil, tea tree oil, DMSO (negative control), and penicillin were completed to acquire data from broad concentrations. For the essential oil and DMSO treatments, the first disk contained solely the lavender oil, tea tree oil or penicillin. The second concentration was 1 mL of the selected oil in 9 mL of broth. This was then diluted as seen in Figure 1. For penicillin, the initial concentration had 0.1g/ml penicillin in 9 mL of broth. Figure 2 shows the concentration was diluted to complete the ten-fold dilution. With the serial dilution, it allowed for the comparison of the effect of the essential oils and penicillin against the viability of S. *aureus*. The ten-fold dilution allowed for ten different concentrations to be tested to observe the differing effects they individually had on the viability and if there was a relationship with the dosages. The DMSO was used to identify if the oils could have a masking effect, such as promoting the growth of *S. aureus.* In addition, there was a positive control of chloroform to eliminate all the bacteria and a negative control without treatment to be able to analyze the viability of *S. aureus*. Before the placement of the disks, a lawn of bacteria was grown by adding 20µL of *S. aureus* to each plate. The lawn was created by distributing the bacterial inoculation across the plate using a cell spreader. 20µL of treatment were added to each disk of each concentration and applied onto the agar plate. For penicillin, one disk was placed per plate. The essential oils and DMSO disks were placed three disks to a plate, containing the same concentration. Plates were then placed in the incubator for 24 hours. The zone of inhibition was measured using the program, Image J, to compare the efficiency of each individual treatment and to identify the IC50 for penicillin.

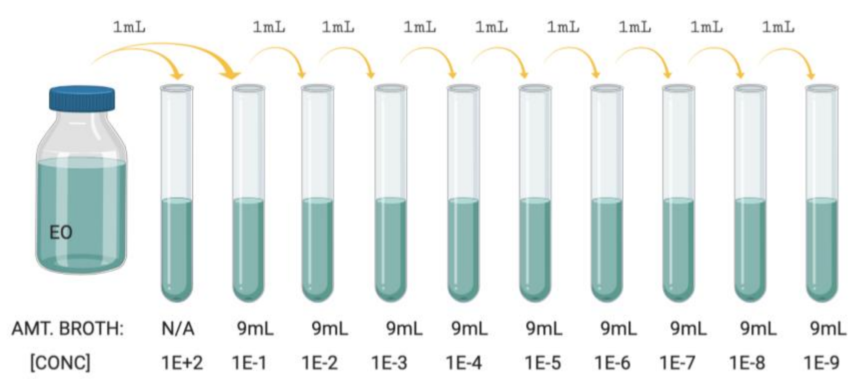

Figure 1. Serial Dilution for Essential Oil Treatments

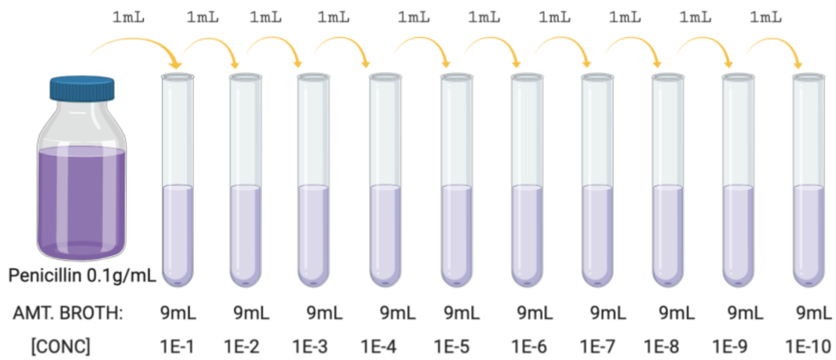

Figure 2. Serial Dilution for Penicillin Treatments

Investigating the Combinatorial Effects of Essential Oils and Penicillin

The disk diffusion assay was also used to determine the combinatorial effects of essential oils and penicillin. For each trial, 23 plates were made and used. The treatment groups used were the combination of penicillin and lavender oil and the combination of penicillin and tea tree oil to try and answer the main question. In addition, there were treatment groups of the lavender oil, tea tree oil, and the IC50 of penicillin. These treatment groups were tested in order to normalize the data and ensure that the results could be interpreted correctly. These trials also used chloroform as the positive control and had a negative control with no treatment. On each plate, a lawn of bacteria was placed using the exact methodology above. The treatment disks with the individual essential oils and the IC50 of penicillin were made using the methodology above. For these trials, only the positive dilutions were placed on the agar plates. The combination treatment contained the IC50 of penicillin with the differing essential oil concentrations in a 1:1 ratio and added to broth as seen in Table 1. 20µL of treatment were added to each disk of each concentration and applied onto the agar plate. Each trial was placed in the incubator for 24 hours and had the zone of inhibitions measured using image J.

Combination Penicillin + EO
IC50 + 1E+2
IC50 + 1E-2
IC50 + 1E-4
IC50 + 1E-6
IC50 + 1E-8

Table 1. Combinatorial Treatments

RESULTS

DETERMINING THE INDIVIDUAL EFFECTS OF ESSENTIAL OILS & PENICILLIN

Essential oils are potent in decreasing the viability of *S. aureus*.

Different concentrations of tea tree oil and lavender oil were tested against a strain of *Staphylococcus aureus* using a disk diffusion assay to determine their efficacy. It was hypothesized that the individual essential oil treatments would decrease the viability. The zone of inhibition sizes measured varied depending on the oils and concentrations used as seen in Figure 3. Figure 3 demonstrates results acquired from the disk diffusion assay and portrays how the zones vary by the treatment group. Figure 4 demonstrates the effect of the essential oils selected on the growth of *S. aureus* using an inhibition percentage relative to the positive control, chloroform. The essential oils showed potency against the tested bacterial strain and portrayed a dosage dependent relationship. The zone of inhibition decreased as the treatment concentration became more dilute. Figure 4 depicts how the essential oils have a higher potency when they are less dilute; however, they are not potent enough to eliminate the bacterial strain entirely. The highest potency of lavender oil and tea tree oil was observed at their beginning dilution (1 E+2). The graph depicts the tea tree oil eliminated 47% of the bacterial strain while lavender oil eliminated 45% at this concentration. Compared to the positive control, the essential oils are not as potent. However, they are capable of decreasing the viability of *S. aureus*. Compared to the negative control, the essential oils can eliminate more than ten percent of the bacterial strain. Throughout the diluted concentrations, lavender and tea tree oil portray a similar potency until the fourth dilution. From the fourth to seventh dilution, there was a larger inhibition depicted in tea tree oil.

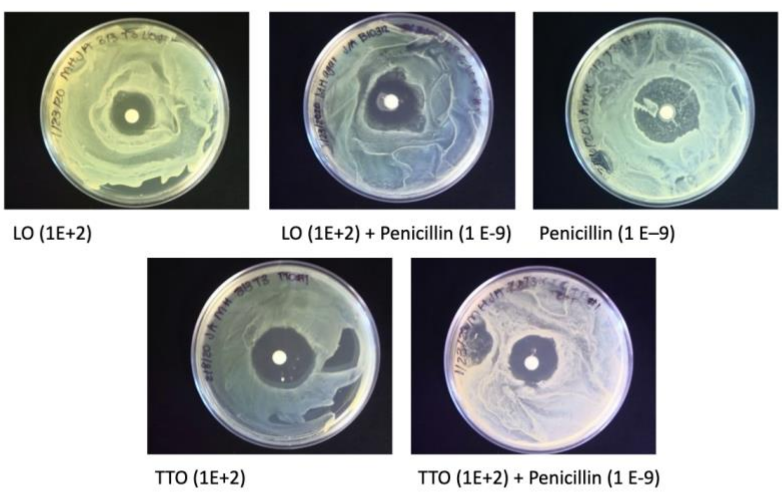

Figure 3. Representative Images of Disk Diffusion Assays

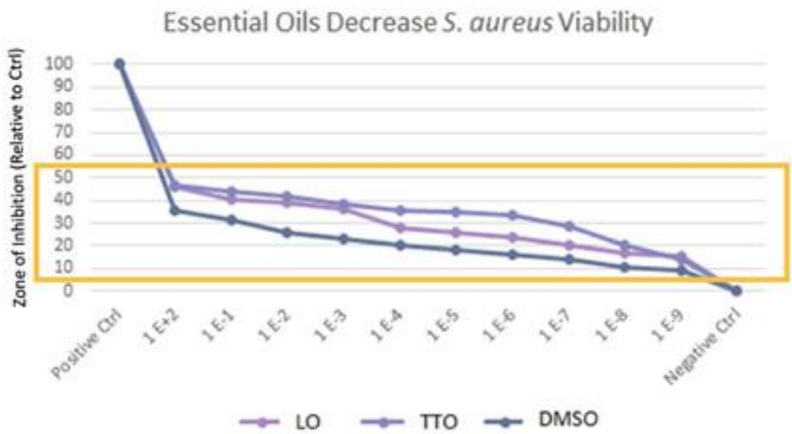

Figure 4. Potency of Essential oils in Decreasing *S. aureus* Viability

<u>Penicillin is potent in decreasing *S. aureus* viability.</u>

In addition, different concentrations of penicillin were tested in a disk diffusion assay to determine its efficacy and to investigate the IC50 to use in the combinatorial treatment. It was hypothesized that penicillin would be potent and decrease the viability of the bacterial strain. Figure 5 depicts that penicillin also had a dosage dependent relationship. Compared to the positive control, the concentrations tested eliminated less, but they had a higher potency than the lavender and tea tree oil. Compared to the negative control, the concentrations tested were much more potent by differing in a forty to eighty five percent difference between them. Figure 5 shows how the first nine concentrations could eliminate more than 50% of the bacterial strain when normalized to the controls. The first concentration (0.1g/ml) had the highest potency and eliminated 85% of the bacterial strain. The IC50 is defined as the concentration of penicillin at which half of the bacterial culture is eliminated. Figure 5 shows that the ideal concentration in the target effective range to acquire the IC50 is the ninth penicillin concentration (1 E-9 g). This concentration was used to investigate the combinatorial effects of the essential oils and penicillin.

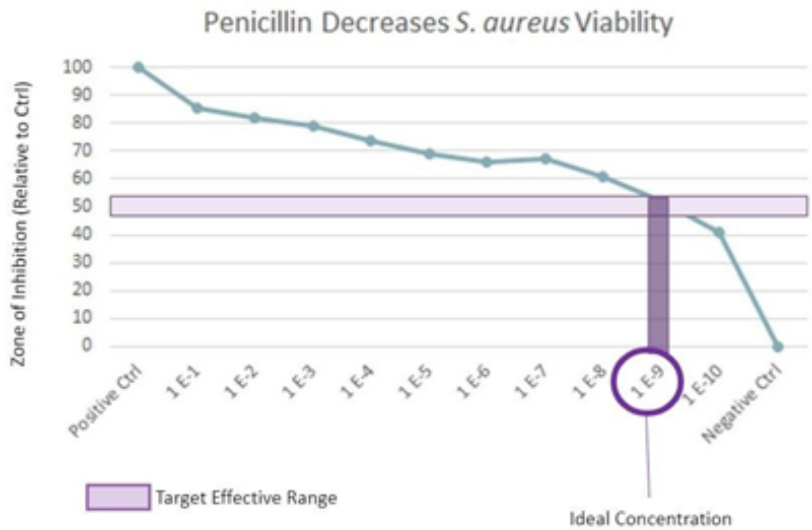

Figure 5. Potency of Penicillin in Decreasing *S. aureus* Viability

DETERMINING THE COMBINATORIAL EFFECTS OF ESSENTIAL OILS & PENICILLIN

<u>Lavender and tea tree oil exhibit antagonistic activity in combination with penicillin.</u>

The study also focused upon analyzing the combinatorial effects from applying treatment disks with different essential oil and penicillin treatments. It was hypothesized that the addition of the essential oils to penicillin would decrease the viability of *S.* aureus. Each of the positive concentrations were used in combination with the IC50 of penicillin at a ratio of 1:1. Analysis of the results in Figure 6 showed significant differences from what was expected. It was estimated that the combination of the essential oils and penicillin would depict an additive effect. An additive effect describes how a combined effect will be produced by the action of two or more agents. It was expected that the combination of penicillin and essential oils would eliminate more bacteria than the individual treatments. Figure 6 shows how the combinatorial treatments were not very active and were less potent than penicillin alone for majority of the combinations. The combination that used the first dilution of the essential oil (1 E+2) had the highest potency and had a slightly higher efficacy than the target range of the penicillin. The lavender oil and penicillin combination had a 28% inhibition while the tea tree oil combination had a 42% inhibition. Similar to the individual effects of the essential oils, the treatments with tea tree oil had a higher potency than the treatments with lavender oil. The other combinations had a lower zone of inhibition than target range, but they were higher than the zones for the individual essential oil treatments. Figure 6 does not portray an additive effect; the combinatorial treatments rather show an antagonist relationship. An antagonist effect depicts how the effect of the two treatments is less than the sum of the individual treatments. The combinations overall portray an inhibition that is less than sum of the target effective range, penicillin, and the essential oils.

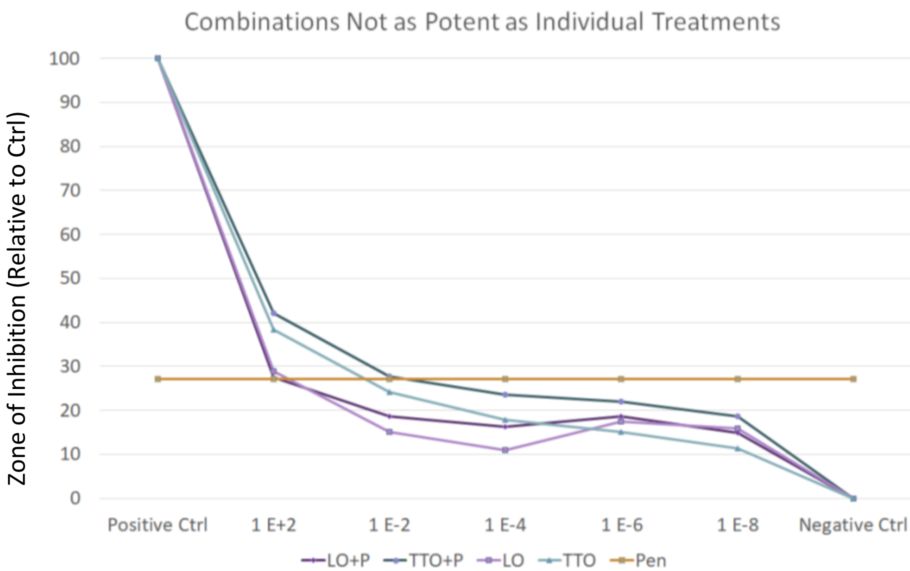

Figure 6. Potency of Combinations in Decreasing *S. aureus* Viability

DISCUSSION

The number of scientific studies on the activity of antibiotics and essential oils has grown over the years; however, the use of many susceptibility testing and different definitions of efficacy make it difficult to fully comprehend the potency of their combinations. Many studies focus on selected essential oils to provide an explanation to their mechanisms, but few studies have investigated the combination of antibiotics with essential oils. This research was conducted to investigate the efficacy of the essential oils and penicillin on one of the most common pathogenic species, *S. aureus,* as seen in Figure 7. *S. aureus* causes diseases, such as MRSA and bacteremia. The study revealed that tea tree oil, lavender oil, and penicillin have potency to eliminate *S. aureus* to. However, individually, there are not able to completely eradicate the bacteria. The results showed that the tested treatments reduced the growth of the bacterial strain in similar pattern. The individual and combinatorial treatment depicted that there was a dosage dependent relationship, in which the zone of inhibition decreased as the concentration became more dilute. Out of the two essential oils, tea tree oil exhibited a higher potency at all the tested concentrations. At the lowest concentration, it had an 18% inhibition, while at the higher concentration, *S. aureus* growth was reduced, with a 47% inhibition. These findings over the individual effects are consistent with results seen in previous studies that have investigated other essential oils. Previous studies have discovered that thyme oil and lemon oil are active against *S. aureus* and other bacterial strains. The essential oils selected for this study have similar structures and mechanisms to the oils used in previous studies, indicating that lavender and tea tree oil should be potent in eliminating bacteria. Occasionally, essential oils have been found to synergistic or additive when used in combination with an antibiotic. Studies have depicted the potential for essential oils to act synergistically with antibiotics due to essential oils inhibiting efflux pumps that can restore the activity of the antibiotics. Studies have showed that the combination of ampicillin and eucalyptus oil against *S. aureus* portrayed a synergistic effect. The synergistic effect exceeds the sum of their individual presentation while the additive sums the individual effects. However, the findings acquired were not consistent with previous research. Although the essential oils and penicillin showed moderate antibacterial effects alone, the interactions between the antibiotic and essential oils were not synergistic or additive against *S. aureus*. It has been examined that the combination of essential oils and penicillin possess bactericidal activity that is slightly higher than the potency of the oils alone. However, results indicate that lavender and tea tree oil exhibit antagonistic activity when in combination with penicillin, which is opposite of what was expected and observed in previous studies. The zone of inhibition is less than the sum of the two individual effects. Observations of the disk diffusion assay plates included a foul aroma being released that indicated there could have been an issue with the glycerol stocks used in the third trial. In order to investigate the issue, it would have been imperative to complete further trials with new glycerol stocks. This was not possible due to a restraint in lab availability and time. However, the foul aroma only arose in half of the disk diffusion assay plates in the trial. There was no correlation in the plates that had the aroma. While analyzing the first two trials and taking these observations, it can be estimated that the glycerol stocks were not the issue since it did not affect all the plates used. In addition, the treatments were synthesized in the same order and every time before the disk diffusion assay was completed. It is possible that this was the source of error that induced the foul aroma and the difference in the relationship observed with the combinations. However, all the trials completed were consistent with the results of an antagonistic relationship with the addition of essential oils to penicillin. Further research would have investigated the source of the issue to ensure that the combination of the penicillin and essential oils truly demonstrate an antagonistic effect. In order to answer this question, disk diffusion assays would be completed with new glycerol stocks and new agar plates. If the same observations persisted, there would be a revision over the completion of the treatments and their application. If after these revisions were completed and the combination portrayed an antagonistic relationship, it would be beneficial to test these combinations against multiple bacterial strains to investigate if the combination itself is antagonist or if it only occurs against *S. aureus*. The data collected may contribute to the ongoing scientific investigation regarding the application of essential oils as a prospective source of alternative antimicrobial agents. The emergence of antibiotic resistance indicates that new alternatives must be created to help prevent the prevalence of these emerging bacterial strains. Additionally, the optimal ratio should be explored for higher efficacy.

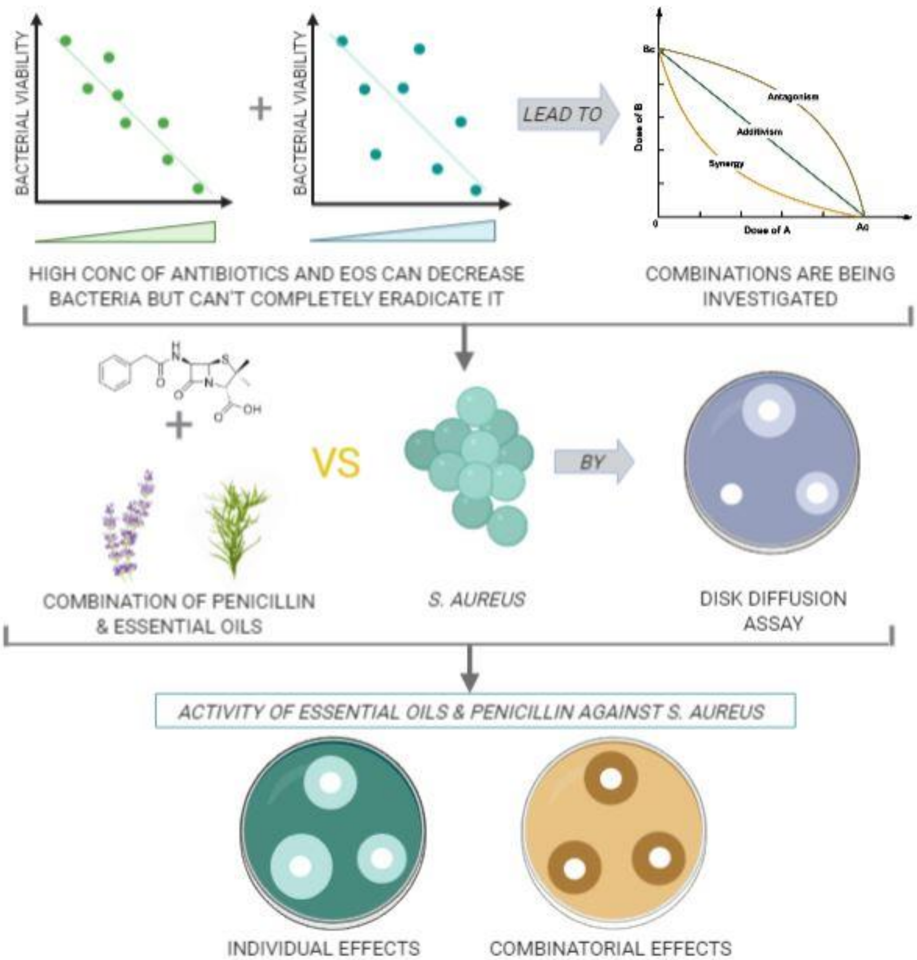

Figure 7. Graphical Summarization of Research

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