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Article

Star anise and oregano essential oil: A comparative evaluation of antibacterial effect

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SUMMARY

Antibiotic resistance poses a growing threat, driving the need for new antimicrobial agents. This study aimed to assess the antibacterial effects of star anise and oregano essential oils against two pathogenic bacteria, *Staphylococcus epidermidis* (gram-positive) and *Escherichia coli* (gram-negative). We used a disc diffusion assay to demonstrate the antimicrobial properties of these essential oils and compare their efficacy between bacterial types. We hypothesized that oregano oil would have a larger zone of inhibition (ZOI) than star anise oil, due to the presence of a higher number of bioactive compounds such as carvacrol and thymol in oregano oil. However, we also hypothesized that both essential oils would be less effective than the positive control antibiotic tetracycline. To test this, paper discs were impregnated with essential oils, tetracycline, or a canola oil negative control and were placed on agar plates inoculated with *E. coli* or *S. epidermidis*. The ZOI around each disc was measured. For both *S. epidermidis* and *E. coli*, oregano oil had the largest mean ZOI, which was significantly greater than the ZOIs of star anise oil and tetracycline. However, for *E. coli*, star anise oil had the second highest ZOI followed by tetracycline. In conclusion, oregano oil displayed potent antibacterial activity against both gram-positive and gram-negative species. These findings highlight the potential role of natural essential oils as alternative antimicrobial agents to combat antibiotic resistance.

INTRODUCTION

Bacterial infections remain a major global threat, with over 2.8 million antibiotic-resistant cases and 35,000 deaths annually in the U.S. alone (1). The urgency is highlighted by reports stating that antibiotic resistance caused 1.27 million deaths worldwide in 2019 (2). Antibiotic resistance occurs when bacteria evolve mechanisms that reduce or eliminate the effectiveness of antibiotics (2). Some common resistance mechanisms include bacteria expressing efflux pumps to remove antibiotics from the cell and producing enzymes that inactivate antibiotics (2). Bacteria can also acquire resistance by exchanging genetic material and gaining new resistance genes from other bacteria (2). The rapid reproduction of bacteria allows resistant strains to quickly proliferate and dominate populations (2). Over time, antibiotic use selectively allows the survival of resistant bacteria, enabling the spread of resistance traits. Ultimately, this causes antibiotic medications to lose effectiveness against bacterial targets, leaving limited treatment options. In contrast to the U.S., over 80% of the global population uses traditional plant-based treatments, a $62 billion market expected to reach $5 trillion by 2050 (1). One strategy to combat antibiotic resistance is to investigate natural compounds found in plants, as many exhibit promising antibacterial properties, and avoid issues like human toxicity from high antibiotic doses. In this context, essential oils and compounds exhibit a promising future as alternative antibacterial agents.

Essential oils are volatile aromatic compounds extracted from plants, which have been used for centuries in food preservation, pharmaceuticals, and natural therapies (3,4). Plants synthesize these organic oils to hinder predators and pathogens while attracting pollinators (4). For centuries, humans have utilized essential oils to treat skin and respiratory tract infections (4). Many essential oils also exhibit antibacterial, antifungal, antiviral, and antiprotozoal properties (3). Their primary antibacterial mechanism involves damaging the bacterial cellular membrane through the accumulation of hydrophobic compounds in the lipid bilayer, which leads to increased membrane permeability and disruption of membrane processes like electron transport, protein translocation, and ATP synthesis, further resulting in cell content leakage and lysis (3). With the rapid emergence of antibiotic resistance, scientists are revisiting the antimicrobial properties of these plant-based extracts as alternatives to traditional antibiotics (4).

Star anise essential oil is derived from the fruits of the *Illicium verum* tree, which is native to northeast Vietnam and southwest China (5,6). Used for centuries in traditional Chinese medicine to treat digestive issues, nausea, and skin infections, star anise oil displays antifungal and antibacterial properties (5). These antibacterial properties are likely due to terpenes and phenylpropanoids in the oil that damage bacterial cell membranes, inhibit cell wall and nucleic acid synthesis, and suppress virulence factors (6). Bacterial cells treated with star anise oil extract were observed to shrink and collapse under scanning electron microscopy, providing visual evidence of membrane disruption (6). The ability of star anise oil to target multiple bacterial structures and virulence mechanisms makes it a promising potential antibiotic agent.

Oregano essential oil, rich in phenolic compounds like carvacrol and thymol, is obtained from the leaves and flowers of *Origanum vulgare*, an herb native to the Mediterranean and Asian regions (7). Traditionally used in various medicinal practices for its antibacterial, antifungal, and anti-inflammatory properties, oregano oil has been studied for its

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antimicrobial effects (7). The phenols present in the oil disrupt bacterial cell membranes, as visualized by scanning electron microscopy, and induce oxidative stress (7). Specifically, carvacrol and thymol can bind to and penetrate bacterial membranes, reduce glucose uptake, inhibit biofilm formation, and suppress virulence factors, making it difficult for bacteria to develop resistance (7). Overall, oregano oil's ability to disrupt bacterial membranes makes it a promising alternative to traditional antibiotics.

To assess the antibacterial properties of essential oils, we studied their ability to inhibit the growth of the bacteria *Staphylococcus epidermidis* and *Escherichia coli. S. epidermidis* is a gram-positive, non-motile bacterium commonly found on human skin and mucous membranes (8). It is an opportunistic pathogen that can cause infections due to its ability to form antibiotic-resistant biofilms (8). *E. coli* is a gram-negative, rod-shaped bacterium present in the gut of warm-blooded animals (9). While most *E. coli* strains are harmless commensals, some pathogenic strains can cause foodborne illnesses and diarrhea through the production of virulence factors and toxins (9). The main difference between gram-positive and gram-negative bacteria lies in their cell wall structure (9). Gram-positive bacteria have a thick peptidoglycan layer, making them generally more susceptible to certain antibiotics like penicillin and cephalosporin, while gram-negative bacteria have a thin peptidoglycan layer between an outer membrane and an inner cell membrane and often develop resistance to antibiotics because the outer membrane acts as a barrier and contains efflux pumps (8,9).

Oregano and star anise essential oils are two plant extracts known for their antibacterial activities, but comprehensive comparisons of their efficacy against gram-positive and gram-negative bacteria are lacking. Oregano oil contains higher concentrations of antibacterial compounds like carvacrol and thymol, which can disrupt bacterial cell membranes and inhibit bacterial growth. Thus, our study compared the ability of oregano and star anise essential oils to inhibit growth of *S. epidermis and E. coli* compared to tetracycline, a widely used antibiotic effective against gram-positive and gram-negative bacteria. We hypothesized that oregano oil would have a higher ability to inhibit bacterial growth than star anise oil, but that tetracycline, a conventional antibiotic, would outperform both essential oils. To test this, we placed discs impregnated with star anise oil, oregano oil, and tetracycline on agar plates inoculated with either *S. epidermidis* or *E. coli.* We then measured the zone of inhibition (ZOI), which indicates the area around the disc where bacterial growth is prevented. After conducting our research, we discovered that oregano oil exhibited strong antibacterial activity against both gram-positive and gram-negative species, with larger ZOIs than star anise oil and tetracycline for both *S. epidermis and E. coli*. The larger zone of inhibition observed for oregano oil compared to star anise oil against both bacterial strains highlights the potential of certain essential oils as alternative antibacterial agents that could help combat the growing issue of antibiotic resistance.

RESULTS

We tested the antibacterial properties of oregano oil and star anise oil using the disc diffusion method, which is commonly used for antibiotic sensitivity tests (10). We tested the samples using two types of bacteria, *S. epidermidis* and *E. coli*. Positive control samples with tetracycline discs were used to demonstrate representative ZOIs for a potent antibacterial agent. Hence, we used tetracycline as a positive control to serve as a baseline for the expected ZOI(s) against *S. epidermidis* and *E. coli.* Additionally, we used canola oil as a negative control to rule out oil viscosity as a factor in inhibiting microbial growth on the agar surface.

It was observed that some ZOI(s) appeared to overlap with other quadrants on the same agar plate. This was likely due to the test samples diffusing extensively through the agar medium, resulting in larger-than-expected ZOI(s) that extended beyond their designated quadrants and into adjacent quadrants. Therefore, the ZOI(s) were measured by taking the diameter across each ZOI, regardless of any overlap into adjacent quadrants.

Antibacterial efficacy against *S. epidermidis*

In *S. epidermidis* inoculated plates, oregano oil, star anise oil, and tetracycline discs showed clear ZOI(s) diameters, while no ZOI was observed for the negative control samples (**Figure 1**). The oregano oil displayed a mean ZOI diameter of 41 mm, calculated from 10 replicate sample plates, which was significantly larger than the 23.2 mm mean ZOI diameter for star anise oil ($p<0.0001$, **Figure 2**). The mean ZOI diameter for tetracycline was 23 mm, which was not significantly different from the star anise oil ($p=0.89$), but significantly smaller than the oregano oil ($p<0.0001$). The antibacterial efficacy of star anise oil and oregano oil against *S. epidermidis* is illustrated, and the mean ZOI diameters with 2 SEM error bars are shown (**Figure 2**). The results indicate that oregano oil had significantly stronger antibacterial effects against *S. epidermidis* compared to both star anise oil and tetracycline.

Antibacterial efficacy against *E. coli*

Agar plates inoculated with *E.coli* exhibited clear ZOI diameters around the oregano oil, star anise oil, and tetracycline discs, while no ZOI was observed for the negative control samples **(Figure 3)**. Oregano oil exhibited the largest mean ZOI diameter of 38.2 mm, calculated from 10 replicate sample plates, which was significantly larger than the 28.7 mm mean ZOI diameter for star anise oil ($p<0.0001$) and the 22 mm mean ZOI diameter for tetracycline ($p<0.0001$). Additionally, star anise oil (mean ZOI diameter 28.7 mm) showed significantly more inhibition against *E. coli* growth compared to tetracycline (mean ZOI diameter 22 mm) ($p=0.0004$). The antibacterial efficacy of star anise oil and oregano oil against *E. coli* is illustrated, and the mean ZOI diameters with 2 SEM error bars are shown **(Figure 4)**. Overall, oregano oil demonstrated the most potent antibacterial efficacy against *E. coli* compared to star anise oil and tetracycline.

DISCUSSION

Our study hypothesized that oregano oil would have a larger ZOI than star anise oil against both *S. epidermidis* and *E. coli*, but that both essential oils would have smaller ZOIs than the antibiotic tetracycline. The hypothesis was partially supported. Against *S. epidermidis,* oregano oil exhibited the greatest mean ZOIs, while star anise oil had significantly lower mean ZOIs. However, the ZOI from star anise oil was not significantly different from tetracycline against these gram-positive bacteria. Against the gram-negative *E. coli*, oregano oil exhibited greater mean ZOIs compared to both star anise

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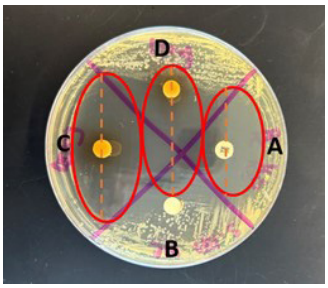

Figure 1: Representative image showing ZOIs of essential oils and tetracycline against *S. epidermidis.* The agar plate was divided into four quadrants each containing a paper disc soaked in **(A)** tetracycline (positive control), **(B)** canola oil (negative control), **(C)** oregano oil, or **(D)** star anise oil. The red ovals represent the observed zones of inhibition (ZOIs) for the experimental and control groups. The red dashed line indicates the measured diameter of the ZOI.

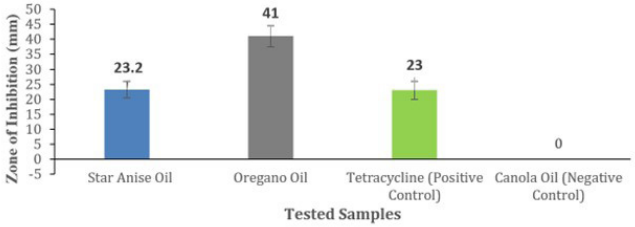

Figure 2: Antimicrobial efficacy of star anise and oregano oil against *S. epidermidis.* Oregano oil exhibited a significantly stronger antimicrobial effect compared to both star anise oil and tetracycline, as measured by their zone of inhibition (ZOI). Bars show mean ± SEM, n = 10.

oil and tetracycline. However, star anise oil also showed significantly more inhibition of *E. coli* growth compared to tetracycline.

Bunse, *et al.* argued that star anise and oregano extracts may have antibacterial effects against *S. epidermidis* and *E. coli* and the ability to prevent and eliminate biofilm formation of various bacterial strains (11). Contrary to the initial hypothesis, we found that oregano oil displayed significantly higher ZOIs against both *S. epidermidis* and *E. coli* than tetracycline. *S. epidermidis* and *E. coli* were more susceptible to oregano oil than star anise oil.

The differential effect observed, with oregano oil exhibiting better antibacterial activity against both *S. epidermidis* and *E. coli* compared to tetracycline, could be attributed to several factors. While the presence of bioactive compounds like carvacrol and thymol in oregano oil contributes to its antibacterial properties, the relative concentrations of the essential oil used in this study may have played a significant role. The study utilized concentrated pure oregano oil, which likely provided a higher concentration of active compounds than tetracycline. The ZOI is a concentration-dependent property, and higher concentrations of antibacterial agents generally lead to greater inhibition of bacterial growth (1). Therefore, the observed superior efficacy of oregano oil could be attributed to the use of concentrated pure oregano oil in this study, which likely provided a significantly higher concentration of active compounds compared to the tetracycline used (1). This differential effect may also be influenced by the varying compositions of the essential oils and their specific mechanisms of action against gram-positive and gram-negative bacteria. Oregano oil's antibacterial activity is primarily attributed to its phenolic compounds like carvacrol and thymol, which disrupt the bacterial cell membrane, leading to increased permeability, leakage of cellular contents, and ultimately cell death (7). In contrast, tetracycline inhibits bacterial protein synthesis by binding to the 30S ribosomal subunit, preventing the attachment of aminoacyl-tRNA to the ribosome complex (12). The combined effects of multiple antibacterial compounds in oregano oil could synergistically enhance its potency. Additionally, oregano oil's different mode of action may make it less affected by resistance mechanisms developed by bacterial strains against tetracycline, contributing to its better efficacy observed in the study.

Star anise exhibited a greater antibacterial effect against *E. coli* compared to *S. epidermidis*. The differences in ZOI between the gram-positive *S. epidermidis* and gram-negative *E. coli* may be due to differences in their cell wall/membrane structures. Gram-negative bacteria like *E. coli* have a more complex cell wall with a thin peptidoglycan layer (10-50%) and an outer membrane (13). Antibacterial compounds can penetrate the outer membrane of gram-negative bacteria through porins, which are hydrophilic pathways that allow the compounds to separate the phospholipids and lipopolysaccharides (13). In contrast, gram-positive bacteria like *S. epidermidis* lack an outer membrane but have a thicker

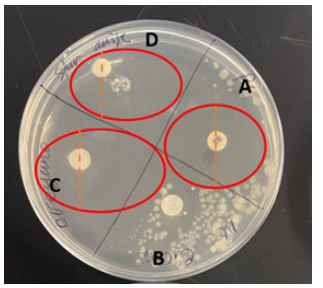

Figure 3: Representative image showing ZOIs of essential oils and tetracycline against *E. coli.* The agar plate was divided into four quadrants each containing a paper disc soaked in **(A)** tetracycline (positive control), **(B)** canola oil (negative control), **(C)** oregano oil, or **(D)** star anise oil. The red ovals represent the observed zones of inhibition (ZOIs) for the experimental and control groups. The red dashed line indicates the measured diameter of the ZOI.

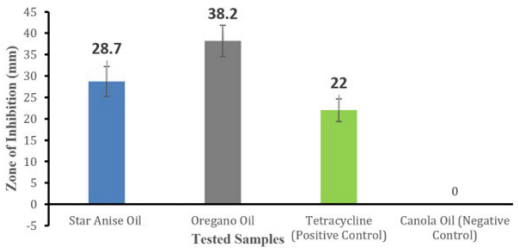

Figure 4: Antimicrobial efficacy of star anise and oregano oil against *E. coli.* Oregano oil exhibited a significantly stronger antimicrobial effect compared to both star anise oil and tetracycline, as measured by their zone of inhibition (ZOI). Bars show mean ± SEM, n = 10.

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peptidoglycan layer (approx. 90%), making it generally harder for antibacterial compounds to penetrate (13).

Our findings regarding the antibacterial effects of natural oils are promising. However, the scope of this study has been limited to a few types of bacteria, and more research is required to determine the full extent of these oils' antibacterial properties. Further investigations could explore the effectiveness of these oils against a broader range of bacteria, including those in biofilms, and in combination with other antibacterial agents. It would also be prudent to investigate the mechanisms of action by which these oils exert their antibacterial effects as well as their efficacy *in vitro* and *in vivo*. Moreover, it is essential to evaluate the toxicity of these oils to ensure their safety for use in clinical applications. Further research could also analyze the chemical compositions of these oils and optimize their formulations and delivery methods to improve their efficacy. Standardizing the oils would be crucial to support clinical translation and enable the development of novel therapies against drug-resistant bacteria. In summary, additional research could provide a deeper understanding of the antibacterial properties of natural oils and expand the data on their antibacterial spectrum, potency, and mechanisms. It would also enable the development of safe and effective clinical applications, thereby supporting the fight against drug-resistant bacteria.

MATERIALS AND METHODS

Preparation of agar media plates

Agar media plates were prepared by suspending 28 g of nutrient agar powder (Carolina Biological Supply) in 1 L distilled water and heated to boiling with mixing until fully dissolved. Then, the prepared media was allowed to cool down to around 40°C. Due to a lack of an autoclave to sterilize the media, these heating and cooling steps were repeated two more times for a total of three times. The media was then poured into sterile 100 mm Petri dishes and allowed to solidify at room temperature. Plates were stored at 4°C until use. Additionally, some prepared plates were incubated at 30-35°C to verify sterility before using them in the study.

Preparation of the test sample discs

Two sterile empty Petri dishes were labeled "star anise oil" and "oregano oil", and the respective essential oil samples (pure therapeutic grade oils, Edens Garden, 100%, Undiluted, 10 mL) were added to each dish. Using sterile forceps, 20 sterile empty paper discs (6 mm, Becton, Dickinson, and Company) were transferred into each dish and allowed to soak for approximately 1 to 2 minutes. After confirming that the discs were saturated with oil, they were transferred to separate sterile dishes to air dry and were stored individually. Similarly, 20 negative control discs were prepared by soaking in canola oil (365 by Whole Foods Market). Additionally, commercially purchased ready-to-use 30 µg tetracycline antibiotic discs (1/4" in diameter, Carolina Biological Supply) were utilized as the positive control.

Testing of samples

Twenty agar plates were removed from refrigeration and allowed to reach room temperature. Ten plates each were labeled for *S. epidermidis* ATCC 14990 suspension (Microbiologics, 1.0x108 cfu/mL) and *E. coli* ATCC 8739 suspension (Microbiologics, 1.0x108 cfu/mL) and divided into four quadrants. The respective bacterial suspensions were mixed, and 0.1 mL was transferred to each plate using a sterile pipette and spread onto the plates using a sterile spreader. Negative control discs soaked in canola oil were placed in the test negative control (TNC) quadrant of all plates using sterile forceps. Tetracycline discs were added to the positive control sample (PCS) quadrant. Sample discs prepared using oregano oil and star anise oil were placed in the "TS #1" and "TS #2" quadrants, respectively.

The plates were incubated inverted at 35-37°C for approximately 72 hours with daily observation for growth or contamination. After 72 hours of incubation, the ZOI, a clear zone without any bacterial growth, was measured with a ruler in millimeters. The ruler was placed across the ZOI from one edge to the other over the center of the disk to measure the diameter. The bacterial growth observed on the negative controls and the ZOIs observed on the positive controls served as references for determining the ZOIs observed for the oregano and star anise test samples. The zone sizes for the tested oil samples and the difference in efficacy for each oil against both bacteria were compared against the controls for statistical differences in efficacy for each bacterial strain. A student's two-tailed t-test was used to determine statistical significance, with a threshold of $p \leq 0.05$ to determine significance. The Bonferroni correction was applied to control the error rate, resulting in an adjusted significance level of 0.0083 (as there were 6 comparisons when all 4 samples were compared against each other). Additionally, the standard error of the mean was calculated to show the variability between the different test samples.

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