The Effects of Lemongrass Essential Oil on the Biofilms of Five Bacterial Species

Responsible for Nosocomial Infections

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Abstract

Plant essential oils have been increasing in popularity as possible natural alternatives to human-made antibiotics, especially in situations where antibiotics are widely unsuccessful such as the case with biofilms and some nosocomial infections. Lemongrass essential oil (Cymbopogon flexuosus) has been shown to be an effective antimicrobial substance against planktonic bacteria in several studies, but has yet to be ascertained against biofilms. A range of concentrations of lemongrass oil solution from 0.05% to 0.25% was tested on the biofilms of five different bacteria responsible for nosocomial infections: Staphylococcus aureus, Enterococcus faecalis, Escherichia coli, Pseudomonas aeruginosa, and Proteus vulgaris. Cultures were inoculated into a 96-well plate and allowed to establish biofilms before being subjected to lemongrass oil in phosphate buffer solutions. A microtiter plate biofilm/crystal violet assay with biofilm degradation measured by optical density showed that there was a significant difference between groups in E. coli (p < 0.001), S. aureus (p < 0.001), P. vulgaris (p < 0.001), and E. faecalis (p < 0.0001) but no significant difference between groups in P. aeruginosa (p = 0.443). The minimum bactericidal concentration (MBC) of E. coli fell within the range of 0.20%-0.25%, and S. aureus fell within the range of 0.15%-0.20%. The MBCs of the other species could not be determined. The results from my study suggest that lemongrass oil can degrade the biofilms of some species with the MBC of biofilms generally double that of their planktonic counterparts, but further research is needed to determine an alternative method for species which were not significantly affected.

Introduction

Nosocomial infections, or infections contracted during hospital treatments, are an increasingly pressing issue for the healthcare community. The severity of these cases is a result
of the difficulty in treating these infections, the scarcity of prevention and sanitation procedures, and the high potential for patient mortality. According to the Centers for Disease Control and Prevention (CDC), nosocomial infections are responsible for 1.7 million infections per year, approximately 99,000 annual fatalities, and were recently considered the fourth leading cause of death in the United States (Bryers 2008). Of these infections, roughly 60-70% are associated with the implantation of biomedical devices such as prosthetic heart valves, intravascular or urinary catheters, orthopedic devices, pacemakers, cerebrospinal fluid shunts, and intrauterine contraceptive devices (Stewart and Costerton 2001; Bryers 2008). The significant contribution of implanted devices to the nosocomial infection rate is due to their unfortunate ability to provide the ideal foundation for bacterial biofilm formation.

Bacteria can inhabit their hosts in many forms such as free-floating planktonic individuals, microcolonies, and well-protected aggregates called biofilms. Biofilms can be formed by a wide variety of microbes, including some species of fungi, and have become a subject of great concern for many research and healthcare professionals. Of the many harmful organisms that have been known to cause biofilm-related nosocomial infections in their hosts, a three-year study monitoring and cataloging the United States’ nosocomial infections showed that roughly 64% were Gram-positive bacteria, 27% were Gram-negative bacteria, and 8% were fungi (Edmond et al. 1999). In this same study, infection accountability was averaged to 15.7% \textit{Staphylococcus aureus}, 11.1% \textit{Enterococcus} species, 5.7% \textit{Escherichia coli}, and 4.4% \textit{Pseudomonas} species.

The exact mechanisms as to how biofilms procure their antibiotic resistance outside of the structural integrity of their matrices are largely unknown at this point in time (Stewart and Costerton 2001; Stewart 2002). Pathogenic biofilms are somehow able to evade host defenses
and are protected from biocides, antibiotics, disinfectants, and other chemical or physical challenges. How biofilms achieve this is dissimilar to individual, planktonic bacteria in that they do not use efflux pumps, enzymes, or beneficial mutations. Hypotheses proposed by Stewart and Costerton of the Center for Biofilm Engineering and Department of Chemical Engineering of Montana State University (Stewart and Costerton 2001; Stewart 2002) include deactivation of antibiotics by the top layers of the biofilm, reduced exposure of some cells due to their relative location in the biofilm matrix, gradients of nutrients, pH, and oxygen within the biofilm, and increased stress response of biofilm bacteria. The last and most speculative hypothesis is the existence of a subpopulation within the biofilm that undergoes cell differentiation and forms a unique phenotype. These cells, dubbed persisters, use a process similar to spore formation to heavily fortify themselves against antibiotics and other stressors, but only make up a tiny percentage of the biofilm community.

Besides the apparent complication of antibiotic resistance, healthcare professionals are faced with a myriad of other difficulties stemming from the unique tendencies of biofilms. Biofilms can cause chronic inflammation, prevent wounds from healing properly, at times contain bacteria that cannot be extracted and grown in the lab for study, and can cause systemic infection when clusters break off and enter the bloodstream where they can recolonize elsewhere (Donlan 2001; Bryers 2008). Biofilm infections are rarely defeated therapeutically and often require complete surgical extraction of the colonized material (Stewart and Costerton 2001). Because of the scarcity of treatment options and the severity of symptoms, new and improved strategies to eradicate biofilms are being investigated. One such strategy is harnessing the organic antimicrobial properties of some plant essential oils.
Essential oils have been used in holistic and natural medicine to treat a variety of ailments for hundreds of years. Recently, however, they have been gaining clinical credibility as possible antibacterial, antiviral, and antifungal substances (Utchariyakiat et al. 2016). Some essential oils contain volatile and aromatic compounds which have been shown to break down the outer membranes or cell walls of a wide variety of bacterial cells which then causes them to lyse (Kavanaugh and Ribbeck 2012; Utchariyakiat et al. 2016). Besides their effectiveness in inducing cell lysis through membrane disruption, essential oils are an attractive alternative because they are relatively inexpensive, easy to obtain, have a low environmental impact, and have an extremely low risk of bacteria acquiring a genetic resistance against them (Kavanaugh and Ribbeck 2012). Of the many plant essential oils tested in various studies, extracts from members of the *Cymbopogon* genus, commonly known as lemongrass, have repeatedly proven to be potent antimicrobial substances when addressing planktonic bacteria (Naik et al. 2010; Adukwu et al. 2016; De Silva et al. 2017).

The two most common varieties of commercially available lemongrass oil are extracted from *Cymbopogon citratus*, or West Indian lemongrass, and *Cymbopogon flexuosus*, also known as East Indian lemongrass. India has been utilizing lemongrass for over 2000 years for treating ailments such as fever, colds, muscle spasms, gastrointestinal distress, anxiety, and fatigue. The antibacterial effect of lemongrass oil is due to the combined effect of the highly aromatic and volatile chemical components: the aldehyde citral and the monoterpane myrcene. The only difference between *C. citratus* and *C. flexuosus* is that *C. flexuosus* contains a significantly higher amount of myrcene than *C. citratus* (Shutes 2017).

In a previous study inspired by Kavanaugh and Ribbeck (2012) and conducted by myself (Richards 2017), I tested the biofilm degrading effects of cassia oil against the bacteria
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*Staphylococcus aureus, Enterococcus faecalis, Escherichia coli, Pseudomonas aeruginosa,* and *Proteus vulgaris.* My data showed that cassia oil was an effective biofilm degrader for some, but not all species. The motivation for my current study was to expand upon the experiment conducted by Kavanaugh and Ribbeck (2012) and my previous research to determine if lemongrass oil was capable of degrading the biofilms of these species and determine the minimal bactericidal concentration (MBC) if biofilms were affected. *Cymbopogon flexuosus* lemongrass oil was chosen because it has been proven to be successful against planktonic bacteria in multiple studies and it was an oil that was not tested in Kavanaugh and Ribbeck’s experiment. I selected *S. aureus, E. faecalis, E. coli, P. aeruginosa,* and *P. vulgaris* because they fall within the species responsible for a large number of nosocomial infections and their propensity to form biofilms make them difficult to treat and a danger to the livelihood of patients. The questions in my study are, for each species: will lemongrass oil degrade biofilms and, if so, what is the minimum bactericidal concentration? I hypothesize that lemongrass oil will degrade biofilms and my prediction is that species treated with the lemongrass oil solution will encounter greater biofilm degradation as measured by a lower optical density than species that are not treated with the oil solution which would have a higher optical density.

**Materials and Methods**

A 4 ounce bottle of NOW Essential Oils 100% Pure Lemongrass Oil was obtained from Good Earth Natural Foods Co. in Elmira Heights, New York, and 96-well Plates were ordered from Carolina Biological Supply Company’s website. Bacterial strains were obtained from the culture collection of the Microbiology lab of Mansfield University of Pennsylvania’s Department of Biology. All other supplies such as nutrient broth, phosphate buffer, crystal violet solution, acetic acid, and Eagle brand adjustable pipettors were supplied by the Microbiology lab.
of Mansfield University of Pennsylvania’s Department of Biology. All research was conducted at Mansfield University under the supervision of Dr. Jeanne Kagle in the Department of Biology’s Microbiology lab. The following methods are modified from the Basic Microtiter Plate Biofilm Assay methods of the 2011 edition of the Current Protocols in Microbiology handbook, Supplement 22. This experiment was conducted with sterile nitrile gloves and under the guidelines of the aseptic technique (Merritt et al. 2011).

**Thawing, Inoculation, and Initial Biofilm Growth**

The *Staphylococcus aureus, Enterococcus faecalis, Escherichia coli, Pseudomonas aeruginosa*, and *Proteus vulgaris* species were sampled from the -84°C culture collection freezer of the Microbiology Lab. Bacteria were streaked onto plates of nutrient agar and left to incubate for 24 hours at 37°C. When 24 hours had passed, each species was inoculated into nutrient broth tubes and left at 37°C for another 24 hours until they reached a stationary phase.

**Inoculation of 96-well Plates**

Overnight cultures were diluted 1:100 in nutrient broth before 200 μL was transferred into 36 wells of a sterile 96-well plate so the experiment could be run in triplicate. Each species had its own plate with rows A-E, columns 1-6 filled with inoculated broth dilutions.

**Inoculated 96-well Plate Incubation**

The 96-well plates were then covered and put in an incubator at 37 °C for 48 hours, the mean time needed for most bacterial biofilms to adhere to surfaces as stated in the protocol (Merritt et al. 2011). 37 °C was selected because it falls within the range of optimal growth temperature for the bacteria being tested and it is also the mean human body temperature (ABIS...
Online Encyclopedia 2007). When 48 hours passed, the 96-well plate was removed from the incubator.

**Lemongrass Oil Treatment**

The supernatant in each well was removed by pipette, leaving behind the established biofilms. I then tested a range of concentrations of lemongrass oil solute in phosphate buffer solvent based on the average minimum bactericidal concentrations (MBC) of my selected bacteria provided by previous studies (Cimanga et al. 2002; Naik et al. 2010; Adukwu et al. 2016; De Silva et al. 2017). I extended the range beyond the given MBCs to account for biofilms being sturdier than free-floating cells. Next, 200 μL of the oil solutions were pipetted into the wells as follows: A4-A6 received 0.05%, B4-B6 received 0.1%, C4-C6 received 0.15%, D4-D6 received 0.2%, and E4-E6 received 0.25%. Columns 1-3 were filled with 200 μL of phosphate buffer with no oil added to serve as a control. A depiction of this layout can be found in Figure 1.

![Figure 1. 96-well plate layout for five different species challenged against a range of concentrations of lemongrass oil/phosphate buffer solution. Inoculated wells of the control group (red) were exposed only to phosphate buffer](image-url)

while experimental groups (blue) received the concentration by row as depicted. Wells F1-F6 were uninoculated and filled with phosphate buffer only to serve as a reference value during optical density analysis.

The samples were then covered and left to incubate at room temperature for 2 hours to allow the essential oil to process in accordance with the methods described by Kavanaugh and Ribbeck (2012).

**Crystal Violet Staining**

Once the two hours had passed, 40 μL of 1% Crystal Violet Solution was added to each well before being left to stain for 10 minutes. The solution was then removed by pipette before being washed with phosphate buffer three times to ensure that the only contents remaining in the wells were the dyed biofilms. The plate was then left to air dry uncovered for 15 minutes.

**Dye Solubilizing**

After 15 minutes, 200 μL of 30% acetic acid was added to each well and then left to process, covered, for another 15 minutes at room temperature to allow the dye to solubilize.

**Read Preparation**

200 μL of phosphate buffer was added to wells F1-F6 to serve as a reference for the MBC optical density value as it is a clear liquid synonymous to the desired clear wells of a biofilm that has been completely eradicated. The plates were read by a Biotek Synergy HT Microplate Reader with the optical density measured at a wavelength of 600 nm, as stated by the Current Protocols in Microbiology handbook, to determine if biofilms had been successfully degraded and, if so, at what point the minimum bactericidal concentration was reached (Merritt et al. 2011).
**Data Analysis**

Optical density data obtained for biofilm degradation was analyzed in SPSS by One-Way ANOVA to determine if there was a difference of means between the oil concentrations of each species.

**Results**

Of the five species tested, *P. aeruginosa* was the only biofilm to have no significant difference in optical density between groups (F = 0.993, df = 5, p = 0.443; Figure 2).

*Escherichia coli* (F = 13279.659, df = 5, p < 0.001), *S. aureus* (F = 1556.970, df = 5, p < 0.001), *P. vulgaris* (F = 933.692, df = 5, p < 0.001), and *E. faecalis* (F = 32911.392, df = 5, p < 0.001) all had a significant difference in optical density data between groups and a Tukey HSD post hoc test of multiple comparisons was run to determine where this difference occurred. In *E. coli*, there was a significant difference between the control and concentrations 0.15%, 0.2%, and 0.25% (p < 0.001; Figure 3). There was no significant difference between the control and 0.05% (p = 0.989) and 0.1% (p = 0.889). *Staphylococcus aureus* had a significant difference between the control and concentrations 0.1%, 0.15%, 0.2%, and 0.25% (p < 0.001) but no significant difference between the control and 0.05% (p = 1.00; Figure 4). Both *P. vulgaris* and *E. faecalis* had a significant difference between the control and all tested concentration groups (p < 0.001) but no significant difference between the concentration groups themselves (p = 1.000; Figures 5 and 6). During the experiment, the mean optical density value of the phosphate buffer used as the MBC reference was determined to be 0.050 and optical density values that reached this threshold were considered to have biofilms completely degraded. The MBC of *E. coli* was observed at 0.25% which means the actual value fell within the range of 0.20%-0.25% and *S. aureus* was observed at 0.20% which means the actual value fell within the range of 0.15%-
0.20% (Figures 3 and 4). *Proteus vulgaris* and *E. faecalis* reached the MBC immediately at 0.05% (Figures 5 and 6) while *P. aeruginosa* never reached this value (Figure 2).

![Figure 2. Mean optical density values of Pseudomonas aeruginosa biofilms after being treated with five different concentrations of lemongrass oil/phosphate buffer solution (LGO). *Control was exposed only to phosphate buffer. REF and its corresponding dotted line is the average optical density of uninoculated, untreated phosphate buffer (clear liquid). The dotted REF line indicates the value that needed to be reached for the biofilms to be considered completely eradicated. The concentration that reached this value first is interpreted as the minimum bactericidal concentration (MBC). Brackets at the top of bars represent standard error. This description, besides species name, is applicable to all following figures.*]
Figure 3. Mean optical density values of *Escherichia coli* biofilms after being treated with five different concentrations of lemongrass oil/phosphate buffer solution (LGO). *

Figure 4. Mean optical density values of *Staphylococcus aureus* biofilms after being treated with five different concentrations of lemongrass oil/phosphate buffer solution (LGO). *
Figure 5. Mean optical density values of *Proteus vulgaris* biofilms after being treated with five different concentrations of lemongrass oil phosphate buffer solution (LGO). *

Figure 6. Mean optical density values of *Enterococcus faecalis* biofilms after being treated with five different concentrations of lemongrass oil/phosphate buffer solution (LGO). *
**Discussion**

The results of my experiment suggest that bacterial species that are susceptible to lemongrass oil treatment in planktonic form are also vulnerable to it as biofilms. A previous study on a selection of pathogens showed lemongrass oil had successful antimicrobial activity on planktonic *E. coli* and *S. aureus* (Naik et al. 2010). In this study, *E. coli*’s MBC fell at 0.12%. According to my observations, the MBC of *E. coli* fell in a range that was approximately double that of its planktonic counterpart which is a logical conclusion considering that biofilms are a much harder structure. In the same study conducted with *E. coli*, *S. aureus* reached its MBC at 0.06% while a second study reported a different MBC value at 0.13% (Naik et al. 2010; Adukwu et al. 2016).

The discrepancy between the two *S. aureus* MBCs can be explained by the study I referenced in my experimental design by Kavanaugh and Ribbeck (2012). In this study, they tested for strain-specific effects of the antimicrobial essential oils clove, red thyme, and tea tree on *Pseudomonas aeruginosa* and *Pseudomonas putida*. Their data illustrated that *P. putida* was more susceptible to oil, especially clove, than *P. aeruginosa* despite being genomically similar. They concluded that their results suggested essential oils had undetermined strain and species-specific effects depending on their differing chemical compositions. It is possible that the strain of *S. aureus* I tested was more closely related to the strain tested by Naik et al. (2010) than the one tested by Adukwu et al. (2016) as it would be unlikely that the MBC of planktonic cells would be similar if not the same as that of its stronger biofilm.

Previous studies also showed successful antimicrobial activity against *P. vulgaris* and *E. faecalis* cells when exposed to lemongrass oil (Cimanga et al. 2002; De Silva et al. 2017). It was determined that *P. vulgaris* exhibited cell death at a 0.3% concentration, but the exact rate of
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clearance and a total MBC value was not given (Cimanga et al. 2002). My observed results for
*P. vulgaris* agreed with the conclusion that lemongrass oil is a successful antibacterial substance
as it was completely cleared at the smallest concentration tested. This could mean that the *P.
vulgaris* was extremely susceptible to treatment and the MBC was smaller than the minimum
concentration. However, though my results were considered statistically significant, I do not
believe this conclusion is a reliable one. Like my previous experiment using the same bacteria, I
could not get *P. vulgaris* to establish as much biofilm as the other bacterial species (Richards
2017). This could be because I grew all of the bacteria in the same environment and said
environment might not have been as ideal for *P. vulgaris* as the others. Having less material to
work with could have been a source of error when trying to determine the MBC. *Enterococcus
faecalis* grew an amount of biofilm comparable to the other strains, but like *P. vulgaris*, showed
complete clearance at the smallest concentration. Again, this could mean that the lemongrass
was extremely effective and that the MBC was smaller than my tested range, but I feel it is more
likely that this was some sort of error on my end and that the trial should be repeated for more
conclusive results.

*Pseudomonas aeruginosa* did not show a significant difference in optical density between
groups suggesting that the lemongrass oil had no effect. This conforms with previous
lemongrass oil studies against planktonic forms of *P. aeruginosa* which also showed no
antimicrobial activity (Naik et al. 2010; De Silva et al. 2017). Some possible explanations for
this lies within the inherent behaviors of *P. aeruginosa* itself. *Pseudomonas aeruginosa* is
commonly known for its ability to quickly and skillfully form excessive amounts of biofilm in a
variety of environments where other pathogens have difficulty doing so. Because *P. aeruginosa*
made so much biofilm in my experiment, as per its nature, it is possible that the sheer mass
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prevented the oil from being effective. *Pseudomonas aeruginosa* is also known for being one of the more resistant pathogens due to its ability to rapidly develop antibiotic resistance and its utilization of multidrug efflux pumps (Lister et al. 2009). It is possible that its resistance mechanisms were too efficient for the lemongrass oil to counteract in my experiment. However, in my reference study by Kavanaugh and Ribbeck (2012) and the previous study conducted by myself (Richards 2017), *P. aeruginosa* biofilms experienced some degradation when subjected to cassia oil. This suggests that the antimicrobial properties of various essential oils could be based on the organic compounds they are comprised of, such as cinnamaldehyde for cassia and citral for lemongrass, and how they interact in different ways with the components of the cell walls of the bacteria.

During the course of my experiment, a situation arose that could have been a source of error that affected my results. In an attempt to improve my previous methods, I used a microtiter biofilm assay protocol taken directly from a recently published lab supplement that I assumed would be reliable due to its publication. Despite conducting multiple trials, this method produced results that were too light to be read by the spectrophotometer. To remedy this issue and get detectable results, I had to modify the protocol with older assay methods that the newer supplement deemed still effective but obsolete. That being said, when compared to the supplement’s protocol, there were very few differences, and these modifications should not have significantly affected my results.

Though significant results were shown for the majority of the species treated with lemongrass oil in my experiment, the understanding of how essential oils can be utilized to defeat biofilms could be expanded by challenging a greater variety of bacteria as well as other types of organisms. For instance, fungi species are responsible for 8% of nosocomial infections,
the majority of which is due to *Candida albicans* (Edmond et al. 1999). It would be interesting to see if lemongrass oil would have similar results on *C. albicans* or other fungi, thus serving as a possible alternative to antifungals. Besides lemongrass, many other promising essential oils have not been tested against biofilms to this date and deserve to be added to the analysis.

The results of my own research and future potential research done by others would greatly benefit the clinical community in which alternative methods are needed for biofilm treatment. With surgical removal being the only current reliable method of eliminating nosocomial biofilms, biofilm infected patients often have to endure lengthy, stressful, and expensive hospital stays. This is in addition to the surgery itself which can prove traumatic, invasive, and has its own list of potential side effects. Lemongrass and other antimicrobial essential oils could eliminate the need for these drastic measures. The antimicrobial components of the oils could be isolated into an oral or topical antibiotic form for bodily infections. Nosocomial infections could also be avoided altogether by using oils or oil components to disinfect surfaces commonly encountered by patients and hospital staff. My findings suggest that lemongrass essential oil is a viable method of biofilm eradication for all of my tested species of bacteria except for *P. aeruginosa* which will require a different approach and further study.

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