INVESTIGATION OF MINIMUM INHIBITORY CONCENTRATION/ MINIMUM BACTERICIDAL CONCENTRATION AND ANTIBIOFILM EFFECTS OF VARIOUS ESSENTIAL OILS ON BIOFILM-PRODUCING BACTERIA AND YEAST STRAINS

What are the Minimum Inhibitory Concentration, Minimum Bactericidal Concentration and measuring antibiofilm effects of various essential oils (*Melaleuca alternifolia*, *Eucalyptus globulus*, *Mentha piperita* and *Citrus limon*) on biofilm producing bacteria (*Staphylococcus epidermidis*) *and yeast strain (Candida parapsilosis*) by spectrophotometric method using Microplate reader?

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INTRODUCTION

Antimicrobial resistance is a significant global health problem caused by the misuse and overuse of antibiotics, leading to the emergence of resistant microorganisms which pose significant challenges to healthcare systems worldwide. It has been labeled a ‘‘silent pandemic’’ by the World Health Organization and is expected to be the most common cause of death by 2050.

Biofilm molecules produced by microorganisms contributes to the antimicrobial resistance. Biofilm is the name given to a community of living microorganisms embedded in a sticky matrix formed by microorganisms and protecting themselves from external factors such as dryness, antimicrobials and host's immune system (1,2). Biofilms are structures that allow microorganisms to attach to inanimate surfaces or human tissues. This structure is responsible for the long-term survival of microorganisms in foods, surfaces, and hospital environments, and their resistance to antimicrobials (1,2).

In order to combat both antimicrobial resistance and biofilm formation, new molecule options are necessary. Studies have shown that essential oils have antimicrobial and antibiofilm activities against various human pathogens, making them excellent candidates for new natural drug discovery (3,4).

Essential oils are aromatic mixtures of terpenoids and phenolic compounds obtained from flowers, buds, seeds, leaves, branches, barks, grasses, woods, fruits and roots (4). Thousands of essential oils are known, many of which are of commercial importance and have extensive biological properties, including antibacterial, antiviral, antifungal and antiparasitic properties; in medical applications (3).

In this study, *Melaleuca alternifolia* (tea tree oil), *Eucalyptus globulus* (eucalyptus oil), *Mentha piperita* (pepermint oil), and *Citrus limon* (lemon) will be evaluated for antimicrobial and antiadhesive activities against biofilm producing bacteria.[1]

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3. Nazzaro F, Fratianni F, De Martino L, Coppola R, De Feo V. Effect of essential oils on pathogenic bacteria. Pharmaceuticals (Basel), 2013;6(12):1451-74.
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***Melaleuca alternifolia* (tea tree oil=TTO):** TTO consists of terpene hydrocarbons and their associated alcohols. Terpenes are volatile, aromatic hydrocarbons. This essential oil is available worldwide both as a pure oil and as an active ingredient in a variety of products. Tea tree oil has been utilized for its antiseptic and anti-inflammatory effects (5).

***Eucalyptus globulus* (eucalyptus oil):** Eucalyptus extracts have been shown to have antimicrobial effects on various bacteria. Eucalyptus extract is derived from components such as cineole, citronellol, [23]citronellyl acetate, p-cymene, eukamalol, limonene, linalool, pinene, terpinene, alloochimen and aromadendrene as active ingredients (6).

***Mentha piperita* (pepermint oil):** The fresh or dried leaves pepermint are used in antiseptic mouth rinses. Peppermint consists menthol, menthone, menthyl acetate, caffeic acid, flavonoids polyphenols, carotenes, and tannins (7).

***Citrus limon* (lemon):** Lemon extract was found to possess promising antimicrobial activity. It contains many bioactive compounds such as flavonoids, carotenoids, limonoid, tannin, and terpenoids (8).

To demonstrate the effects of the selected essential oils, ***Staphylococcus epidermidis* ATCC 35984** and ***Candida parapsilosis* ATCC 22019 strains** were chosen. These are standart strains which were known as biofilm producers and most commonly were used in scientific researches (3).

***Staphylococcus epidermidis* ATCC 35984:** It is a bacterium that is a natural member of the human healthy microbiota. It can cause infections by forming biofilm in patients whose immune systems are not functioning well or in patients with prosthetic devices (9).

5. Carson CF, Hammer KA, Riley TV. Melaleuca alternifolia (Tea Tree) oil: a review of antimicrobial and other medicinal properties. Clin Microbiol Rev. 2006 Jan;19(1):50-62. doi: 10.1128/CMR.19.1.50-62.2006.
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7. Rajinder Singh, Muftah A.M. Shushni, Asma Belkheir. Antibacterial and antioxidant activities of Mentha piperita. Arabian Journal of Chemistry, Volume 8, Issue 3, 2015, Pages 322-328. doi.org/10.1016/j.arabjc.2011.01.019.
8. Junab Ali, Biswajit Das, Trideep Saikia. Antimicrobial activity of lemon peel (Citrus limon) extract. Int J Curr Pharm Res 2017;9(4):79-82
9. Stepanović, S., et al., Quantification of biyofilm in microtiter plates: overview of testing conditions and practical recommendations for assessment of biyofilm production by staphylococci. 2007. 115(8): p. 891-899.).

Candida parapsilosis* ATCC 22019*: It is a yeast that is a natural member of the human healthy microbiota. It can cause infections by forming biofilm in cancer patients or in patients with prosthetic devices (9).

These microorganisms do not cause disease in healthy people and follow the IB regulations. Laboratory stages of the study were carried out using the Biosafety Cabinet. The lids of the microorganisms in the media were kept closed and the culture plates were sterilized and discarded.

Gloves and masks were used during the study and biosafety rules were followed. The study was performed in Medical Microbiology Department of Ankara Oncology Hospital.

Minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) methods were used to investigate the antimicrobial effects of essential oils on selected standard microorganisms and their effects on biofilm formation was measured by spectrophotometric method using a microplate reader

Research Question: What are the minimum inhibitory concentration (MIC), minimum bactericidal concentration (MBC) and measuringantibiofilm effects of various essential oils (*Melaleuca alternifolia, Eucalyptus globulus, Mentha piperita* and *Citrus limon*) on biofilm producing bacteria (*Staphylococcus epidermidis*) *and* yeast strains *(Candida parapsilosis*) by spectrophotometric method using Microplate reader?

HYPOTHESIS

Essential oils derived from plants have been used in medicine for their biological properties, such as larvicidal effects, analgesic and anti-inflammatory properties, antioxidant, fungicidal, and antitumor activities, and many other reasons. Existing articles suggest that essential oils offer a potential alternative to synthetic antimicrobials, particularly given the increasing resistance of pathogenic microorganisms. Publications mention the antimicrobial effects of various essential oils. However, this effectiveness can vary depending on the microorganism. The publications also mention that although the effectiveness can vary depending on the microorganism, the differences of the effects between the essential oils will not be statistically significant. The hypothesis of this study is that various essential oils will be effective against bacteria and yeast strains known to produce biofilm but will not have a statistically significant difference between each other.

MATERIALS AND METHODS

Materials:

Sheep blood agar (90 mm diameter plate)

Mueller Hinton agar (150 mm diameter plate)

Mueller Hinton Broth (100 ml)

Brain-heart infusion broth (100 ml) x2

Dimethyl sulfoxid (DMSO) (%10 solution)

Loop (0,1 mm) x10

Test tubes (5 ml) x16

Microplate (81 u-shaped wells) x2

Devices:

McFarland Reader (STI/China) (±0,01)

Vortex mixer (Stuart/UK)

Micropipette (10-100 mikrolitre) x1 (±0,5)

Microplate Reader (BioRad/USA) (±0,0001)

Weight scale (Schimadzu, Japan) (±0,01)

Autoclave (Sanyo, Japan)

Revival of strains:

The stock bacterial cultures stored at -20 °C are thawed by waiting at room temperature and passages are made with a single colony dropping technique with a 5% sheep blood agar loop. The media are incubated in a 35 ° C oven for 18-24 hours. The plates with growth are seeded and incubated again in the same way. The purpose of the second passage is for the bacteria stored at very low temperatures to fully regain all their physiological and biochemical properties. Studies are carried out from these second passages.

Determination of Minimum Inhibitory Concentration (MIC) of Essential Fatty Acids (3):

1. Serial Dilution of Essential oils: 96-well (8x12) microtiter plates were used in the study. For this, 50 microliters of sterile %10 DMSO was added to all wells. Then, 50 microliters of essential oils was added to the first well. Starting from the second well, it was prepared with two-fold dilutions to be between 0.5-0.002 concentrations. Essential oil dilution is not applied to the last two wells. One of these wells is a growth control well, the other is a sterility control well.
2. Preparation of Isolate: Standard *S. epidermidis* ATCC 35984 and *C. parapsilosis* ATCC 22019 strains were used to demonstrate the antimicrobial activity of essential oils. Pure cultures of the standard strains used in the study were prepared. For this, the frozen isolate was passaged onto commercially available sheep blood agar and incubated for 24 hours at 35 ± 2 ^{0}C. Colonies taken from the pure culture were used to prepare a suspension adjusted to 0.5 McFarland standard (approximately 1–2 × 10^{8} CFU/mL).
3. Inoculation: 50 µL was taken from the 0.5 McFarland tube and transferred to the tube containing 4950 µL of Mueller Hinton II Broth, thus achieving a final bacterial concentration of 1x105 CFU/ml. The tube was vortexed, and 50 µL was added to all wells except the last well. The last well was used as a sterility control well to verify test performance.
4. The last two wells were left empty. One of them was prepared as a sterility control without microorganisms and the other as a growth control without essential fatty acids. If no growth is observed in the growth control at the end of the test, the test is considered invalid because the microorganism used in the test has not grown in sufficient quantities, and false susceptibility is detected. Growth in the sterility well suggests contamination, and in this case, the test is considered invalid.
5. Prepared microdilution plates were incubated in a covered oven at 35°C for 18±2 hours under aerobic conditions to prevent evaporation.
6. MIC reading: At the end of the incubation period, the plates were checked. The well with the lowest concentration that showed no growth to the naked eye was recorded as the MIC value.

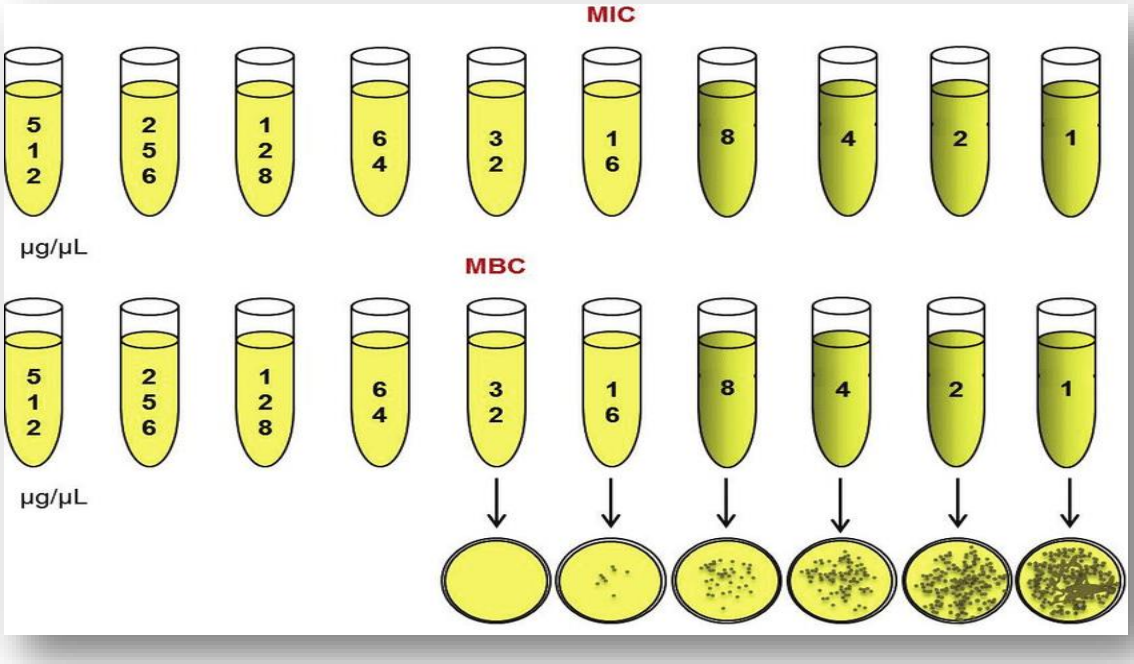

Figure 1. Schematic explanation of the test performed to determine MIC and MBC values

Figure 2. Microbroth dilution test plate for determining the minimum inhibitory concentration results of essential oils on *S. epidermidis* ATCC 35984 and *C. parapsilosis* ATCC 22019 strains

Determination of Minimal Bactericidal Concentration (3):

1. The minimum bactericidal concentration (MBC) is the lowest concentration of an antibiotic (expressed in mg/L) that under defined in vitro conditions reduces by 99.9% (3 logarithms), the number of organisms in a medium containing a defined inoculum of bacteria, within a defined period of time.
2. After the MIC value was read, all wells showing no growth were inoculated onto Mueller Hinton agar. The samples were incubated in an oven at 35°C for approximately 24 hours.
3. At the end of the period, the agars were checked. The lowest concentration that resulted in no growth was recorded as the MBC value.

Figure 3. Determination of minimum bactericidal concentration results of essential oils on *S. epidermidis* ATCC 35984 and *C. parapsilosis* ATCC 22019 strains

Detection of Biofilm Formation (9)

1. To see the effect of essential oils on biofilm production, high-level biofilm producers *S. epidermidis* ATCC 35984 and C. parapsilosis ATCC 22019 strains were used.
2. Colonies taken from the pure culture were used to prepare a suspension adjusted to 0.5 McFarland standard (approximately 1–2 × 10^8 CFU/mL).
3. Biofilm formation is determined on negatively charged polystyrene surfaces in 96-well microplates (8X12 wells).
4. Serum physiological oil and 100 µL of the tested bacterial suspension prepared in brain heart infusion broth were added.
5. 50 µL of 10% DMSO, 50 µL of essential oil and 100 µL of the tested bacterial suspension prepared in brain heart infusion broth were added to the last six wells in each row.
6. The microplate is incubated at 37 ºC for 48 hours.
7. Then, each well is washed twice with 200 µL of phosphate-buffered saline.
8. Biofilms are fixed with 200 µL of 99% methanol and stained with 2% crystal violet.
9. Finally, 160 µL of 95% ethanol is added to each well.
10. Optical density (OD) is measured 3 times at 595nm (OD 595) with SpectraMax i3x Multi-mode Microplate Reader (Moleculer Devices, USA).
11. Negative control contains only BHI. Evaluation of the results is done as described in the literature. After 3 measurements at OD 595, the mean value is calculated for all tested strains and the negative control. Then, the cut-off value (ODc) is established. ODc is defined as three standard deviations (SD) above the mean OD of the negative control:

ODc = mean of the negative control + (3 x SD of the negative control)

If the mean OD value of a tested strain is greater than ODc, it is interpreted as biofilm formation, if it is less than ODc, it is interpreted as no biofilm formation.

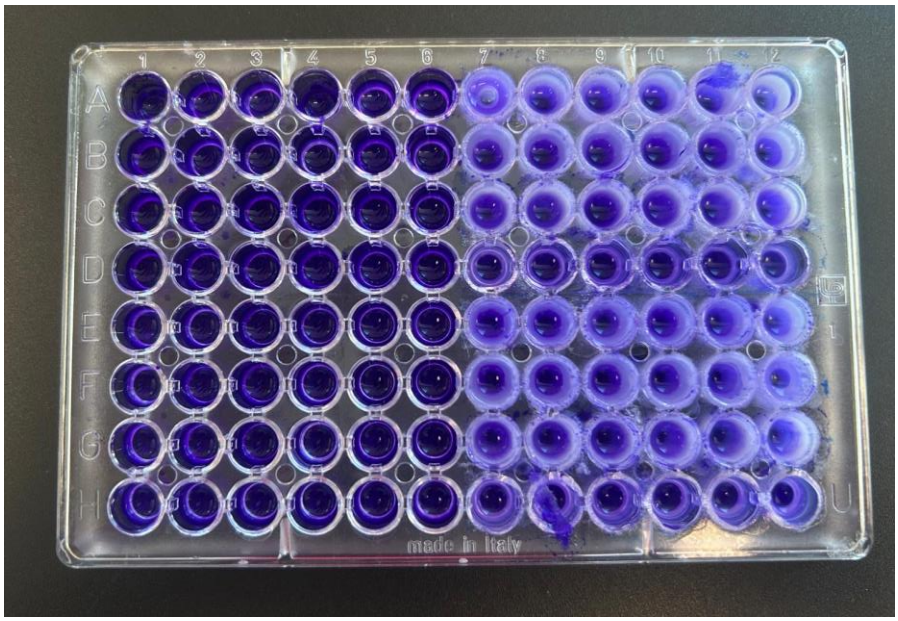

Figure 4. Determination of antibiofilm effects of essential oils on *S. epidermidis* ATCC 35984 and *C. parapsilosis* ATCC 22019 strains using spectrophotometric method

RESULTS

Table 1- Table showing the at which dillusions the MIC and MBC were recorded

MICROORGANISM	ESSENTIAL OIL	Trial 1		Trial 2		Trial 3		Trial 4		Trial 5	
		MIC 1 ±0,001	MBC 1 ±0,001	MIC 2 ±0,001	MBC 2 ±0,001	MIC 3 ±0,001	MBC 3 ±0,001	MIK 4 ±0,001	MBC 4 ±0,001	MIK 5 ±0,001	MBC 5 ±0,001
Staphylococcus epidermidis	TEA TREE OİL	0,062	0,250	0,062	0,250	0,062	0,250	0,062	0,250	0,062	0,250
	PEPPERMİNT	0,062	0,250	0,062	0,250	0,062	0,250	0,062	0,250	0,062	0,250
	OCALYPTUS	0,062	0,125	0,062	0,125	0,062	0,125	0,062	0,125	0,062	0,125
	LEMON	0,062	0,125	0,062	0,125	0,062	0,125	0,062	0,125	0,062	0,125
Candida parapsilosis	TEA TREE OİL	0,031	0,125	0,031	0,125	0,031	0,125	0,031	0,125	0,031	0,125
	PEPPERMİNT	0,062	0,250	0,062	0,250	0,062	0,250	0,062	0,250	0,062	0,250
	OCALYPTUS	0,008	0,015	0,008	0,015	0,008	0,015	0,008	0,015	0,008	0,015
	LEMON	0,008	0,031	0,008	0,031	0,008	0,031	0,008	0,031	0,008	0,031

Table 2- Table showcasing how the microplate was organized and which concentrations of essential oil were added to each plate.

DILLUTION 1	DILLUTION 2	DILLUTION 3	DILLUTION 4	DILLUTION 5	DILLUTION 6	DILLUTION 7	DILLUTION 8	DILLUTION 9	DILLUTION 10	REPRODUCTION CONTROL	STERILITY CONTROL
0,500 ±0,001	0,250 ±0,001	0,125 ±0,001	0,062 ±0,001	0,031 ±0,001	0,015 ±0,001	0,008 ±0,001	0,004 ±0,001	0,002 ±0,001	0,001 ±0,001	OBSERVED	NOT OBSERVED

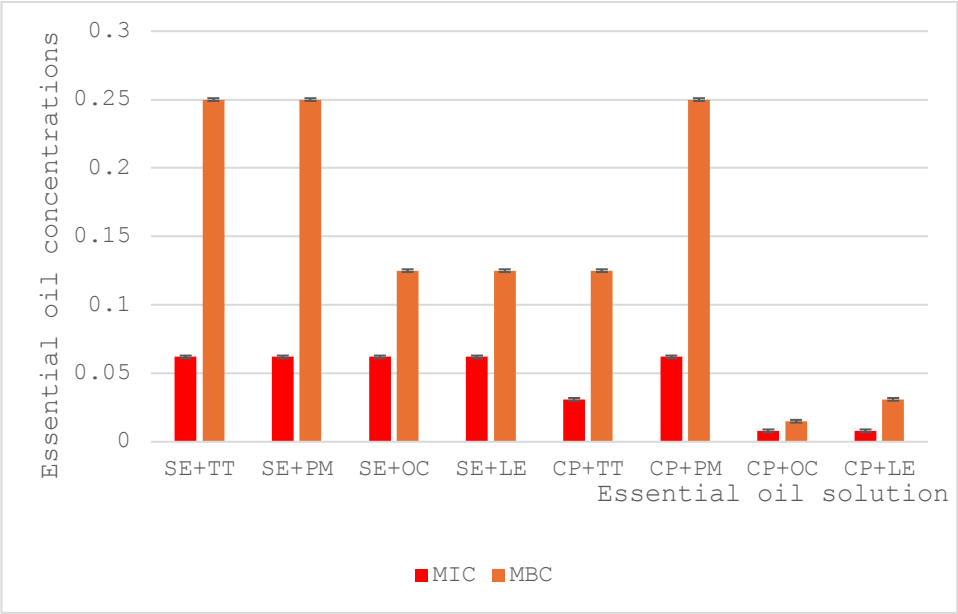

Table 3- Graph showing the concentrations at which MIC and MBC were recorded. (error bars were created using fixed error bars in Excel)

After analyzing the table, we can reach various conclusions. In the trials with the *Staphylococcus epidermidis* bacteria, we can see that the MIC of all of the essential oils were recorded at the same dillution of 0,062. Meanwhile, the MBC of the ocalyptus and lemon are recorded at a lesser concentration than that of tea tree oil and peppermint. The MBC of the ocalyptus and lemon being at a lesser concentration while the MIC of all the essential oils were same shows that although all of the essential oils were visibly equal at stopping the bacteria production, the ocalyptus and lemon essential oils were better when it came to stopping 99% of the bacteria production. When it comes to the *Candida parapsilosis* yeast strain, the peppermint oil was the least effective at stopping the yeast production as both it's MIC and MBC were recorded at higher concentrations, 0,062 and 0,250 respectively, which does not differ from the previous recordings. After that, the tea tree oil was also nearly the same as the previous ones, just differing one concentration, which is acceptable and not significant according to literature about the topic. However, when it comes to the ocalyptus and lemon essential oils, the MIC and MBC were recorded in concentrations significantly less than their previous recordings with the *Staphylococcus epidermidis* bacteria. Due to these recordings, we can conclude that in general the ocalyptus and lemon essential oils were more affective than tea tree oil and peppermint oil at stopping

the microorganisms from producing. Furthermore, they were even more effective at stopping the production of *Candida parapsilosis* yeast strain compared to the *Staphylococcus epidermidis* bacteria.

Table 4. Table showing the mean of the 3 readings of all the trials of the biofilm experiment using microplate reader, rounded to 4 decimal places

TRIAL 1-5		Trial 1 ±0.0001		Trial 2 ±0.0001		Trial 3 ±0.0001		Trial 4 ±0.0001		Trial 5 ±0.0001	
SE	SE+TT	0,6904	0,2828	0,5349	0,3373	0,5666	0,1633	0,6395	0,3125	0,8607	0,6644
SE	SE+PM	0,3818	0,1595	0,4209	0,1208	0,7576	0,4107	0,5532	0,3365	0,2638	0,1315
SE	SE+OC	0,5870	0,3312	0,2177	0,1323	0,6243	0,3028	0,6090	0,3382	0,4387	0,1171
SE	SE+LE	0,2550	0,1142	0,3794	0,2525	0,5309	0,1921	0,7563	0,3393	0,8299	0,3269
CP	CP+TT	0,6490	0,3244	0,5292	0,3444	0,3609	0,2688	0,3034	0,1441	0,5653	0,3044
CP	CP+PM	0,2676	0,0394	0,4292	0,2508	0,2916	0,1512	0,4186	0,2242	0,5189	0,1316
CP	CP+OC	0,2271	0,0719	0,6425	0,4289	0,6659	0,3464	0,5513	0,2455	0,5728	0,3308
CP	CP+LE	0,6446	0,4799	0,5331	0,1795	0,3639	0,1709	0,9896	0,2553	0,7881	0,5878

SE: *Staphylococcus epidermidis* , CP: *Candida parapsilosis*, TT: Tea tree oil, PM: Pepermint, OC: Ocalyptus, LE: Lemon

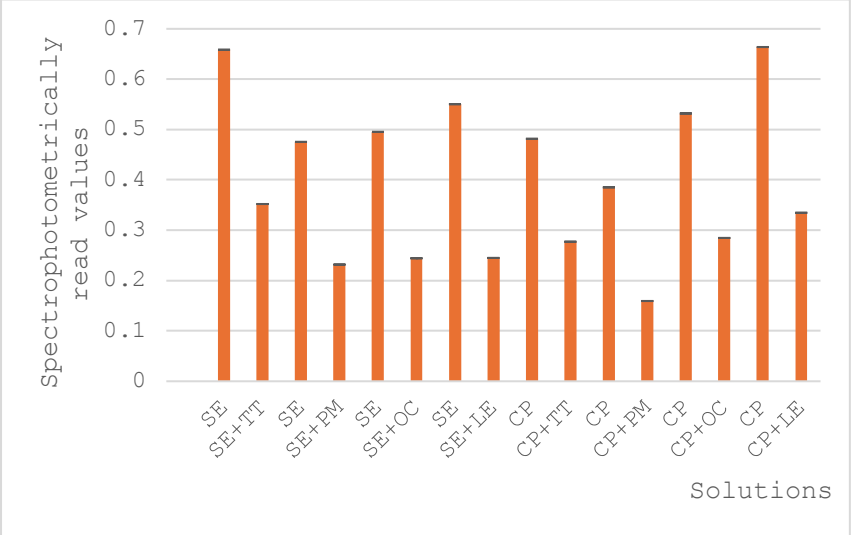

Table 5- Graph showing the mean value of the biofilm produced recorded in the 5 trials (error bars were created using fixed error bars in Excel)

Microorganism	**Microorganism+Essential oil**	**ODcutoff**±0.0001	**OD**±0.0001	**Inhibition value**±0.0001
SE	SE+TT	0,6509	0,3521	0,2989
SE	SE+PM	0,4895	0,2318	0,2577
SE	SE+OC	0,4965	0,2443	0,2522
SE	SE+LE	0,5783	0,2450	0,3333
CP	CP+TT	0,4814	0,2772	0,2042
CP	CP+PM	0,3852	0,1594	0,2257
CP	CP+OC	05319	0,2847	0,2472
CP	CP+LE	0,6639	0,3347	0,3292

Table 6- Table showing the mean ODc, OD and Inhibition values of the datas from Table-4 (the bacteria essential oil combinations were put vertically with all 5 trials together), rounded to 4 decimal places

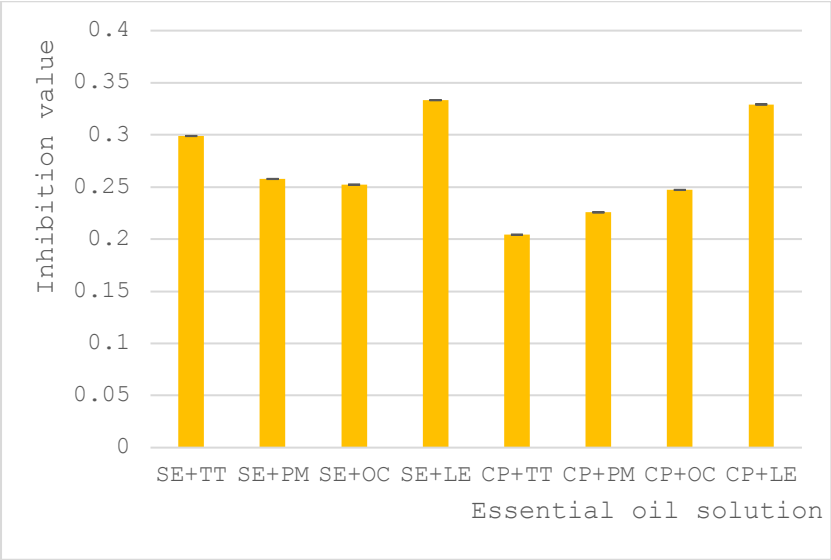

Table 7- Graph comparing the average inhibition values of al the trials of the bacteria-essential oil combinations (error bars were created using fixed error bars in Excel)

H_0: Essential oils do not have an effect at decreasing the biofilm produced by microorganisms.

H_1: Essential oils do have an effect at decreasing the biofilm produced by microorganisms.H_2: There is not a statistically significant difference between how much each essential oil decreases the biofilm production.

H_2: There is a statistically significant difference between how much each essential oil decreases the biofilm production.

H_3: There is not a statistically significant difference between how much each essential oil decreases the biofim production.

As can be seen from the graphs and tables, the OD values of the test tubes containing the essential oils alongside the bacteria are less than the ODc values derived using the test tubes only containing the bacteria. Had the OD value been equal to or more than the ODc, it would mean that the essential oils didn't have any effect on the biofilm production by the bacteria. However, the decrease from the ODc to OD, called the inhibition value, helps us conclude that the essential oils did in fact have an effect on decreasing the amount of biofilm produced. The inhibition values shown in the table goes on to falsify H_0 and prove H.

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Sample	0,01151584	1	0,01151584	0,75298299	0,3919945	4,14909745
Columns	0,05294207	3	0,01764736	1,15390263	0,34249541	2,90111958
Interaction	0,01356193	3	0,00452064	0,29558991	0,82828261	2,90111958
Within	0,48939612	32	0,01529363			
Total	0,56741596	39				

Table 8- ANOVA two factor table

In order to investigate if there is a statistically significant difference between the combinations of the 2 bacteria and the 4 groups of essential oils, ANOVA two factor analysis is performed. In the table, samples show the main effect of bacteria, columns show the main effect of oil and the interactions show the interactions between bacteria and oil. All 3 of the P-values, of samples, columns and the interactions are above the α value (0,05), which goes on to show that the differences between the effects of the essential oils on the bacteria and yeast strain are not statistically significant, although they were all successful at decreasing the amount of biofilm the bacteria produced. The sample has a P-value of 0.392, showing no detectable differences between the SE (*Staph epidermidis*) bacteria and CP (*Candida parapsilosis*) yeast strain. The p-value of the columns, 0.343, gives that there is also no detectable differences among the four oils overall. Last, the

interactions of bacteria and the yeast strain with the oils P-value, 0.828, shows us that there is no evidence that the effect of an oil changes between *Staphylococcus epidermidis* and *Candida parapsilosis*. However, ANOVA by itself is not enough to come to these conclusions, as the ANOVA test focuses on the whole experiment, rather than the individual effects of specific essential oils on the bacteria and yeast strain. Therefore, I performed Games-Howell post-hoc tests in order to see whether there is a statistically significant difference between the effects of specific essential oils between each other.

Post-Hoc Tests

Comparison	mean_A	mean_B	diff	SE	t	df_GH	q	p_GH	Holm p (GH)
LE vs TT	0.3292	0.2042	0.1250	0.1137	1.0993	5.093	1.5546	0.7051	1.0
LE vs PM	0.3292	0.2257	0.1034	0.1148	0.9011	5.258	1.2744	0.8055	1.0
OC vs TT	0.2472	0.2042	0.0050	0.0499	0.8614	7.4673	1.2182	0.8242	1.0
LE vs OC	0.3292	0.2472	0.0820	0.1107	0.7402	4.6403	1.0468	877	1.0
OC vs PM	0.2472	0.2257	0.0215	0.0524	0.41	7.1973	0.5798	975	1.0
PM vs TT	0.2257	0.2042	0.0215	0.0584	0.3688	7.9571	0.5216	0.9817	1.0

Table 9- Games Howell test on the difference of the effects of essential oils on CP (*Candida parapsilosis)*

Comparison	mean_A	mean_B	diff	SE	t	df_GH	q	p_GH	Holm p (GH)
LE vs OC	0.3333	0.2522	0.0811	0.0762	1.0639	7.1464	1.5046	0.7202	1.0
LE vs PM	0.3333	0.2577	0.0756	0.0723	1.046	6.4131	1.4793	0.7307	1.0
OC vs TT	0.2522	0.2989	-0.0467	0.0598	0.7814	7.9671	1.1051	0.8608	1.0
PM vs TT	0.2577	0.2989	-0.0412	0.0546	0.7541	7.8854	1.0664	0.8725	1.0
LE vs TT	0.3333	0.2989	0.0344	0.0747	0.4604	6.8921	651	0.9654	1.0
OC vs PM	0.2522	0.2577	-0.0055	0.0567	0.0974	7.7398	0.1377	0.9996	1.0

Table 10- Games Howell test on the difference of the effects of essential oils on SE (*Staph epidermidis*)

I ran two seperate Games Howell tests, one for the pairwise differences of essential oils on CP (*Candida parapsilosis*) and the other for the pairwise differences of essential oils on SE (*Staph epidermidis*). The first table, with the pairwise differences of essential on CP (*Candida parapsilosis*), had an Holm adjusted P-value of 1, showing that they were still not statistically significant. The table showing SE (*Staphylococcus epidermidis*), also had the same Holm adjusted P-value of 1. These post-hoc tests show that even after the post hoc tests, where the differences between spesific essential oil pairs were used rather than the whole experiment, the results still proved to be statistically insignificant, which is backed up by prior literature saying that it is supposed to be insignificant. However, the differences being insignificant doesn't show that the essential oils were ineffective at stopping the biofilm production, it just shows that all 4 essential oils have the same anti-biofilm ability. This means that none of the essential oils were better than one another at stopping the production of biofilm, even though they all were able to stop the production. In conclusion of these tests, we can approve the validity of H_3, "There is not a statistically significant difference between how much each essential oil decreases the biofim production" and falsify H_2.

DISCUSSION

Conclusion

My research question that led me to this investigation was 'What are the Minimum Inhibitory Concentration, Minimum Bactericidal Concentration and antibiofilm effects of various essential oils (*Melaleuca alternifolia, Eucalyptus globulus, Mentha piperita* and *Citrus limon*) on biofilm producing bacteria (*Staphylococcus epidermidis*) *and yeast strain (Candida parapsilosis*)?'. In order to find an answer to my question, I prepared an experiment in which I could see the effects of the essential oils with the use of microplates. First, only the microorganisms were put on the microplates and then the other wells were filled with the 8 combinations between the 2 microorganisms and 4 essential oils. Creating these groups allowed me to see the effects of each variable. To minimize errors as much as possible, I did 5 trials of the experiment and read each trial 3 times by microplate reader.

In order to determine whether the essential oils had an effect on the microorganisms the ODc and OD values found from the formulas were compared. Since the OD values were lower than the ODc, the hypothesis for my experiment was supported. Moreover, the P values of the investigation was calculated in order to find out if the effects the essential oils had were significantly different from each other, which reached to the conclusion that they were not, further supported by prior scientific investigations. Analysing both datas from the investigations showed that H_1 and H_2 ('Essential oils do not have an effect at decreasing the biofilm produced by microorganisms' and 'Essential oils do have an effect at decreasing the biofilm produced by microorganisms.') were correct hypothesises while H_o and H_3 ('There is not a statistically significant difference between how much each essential oil decreases the biofilm production.' and 'There is a statistically significant difference between how much each essential oil decreases the biofilm production.') were not.

Comparing my investigations results to previous review publications such as the 'Essential Oils as Antimicrobial Agents—Myth or Real Alternative?' (10) by the Wrocław University of Environmental and Life Sciences, which states that essential oils such as the tea tree oil aslo used in my experiment is a real alternative as an antimicrobial agent is parallel to the conclusions reached by my investigation. A scientific investigation such as this one by the Wroclaw University of Environmental and Life Sciences, supporting my investigation increases its reliability and confirms the conclusions I reached.

Moreover, leading from my research question, the dillutions of essential oils at which the MIC (Minimum Inhibitory Concentration) and MBC (Minimum Bactericidal Concentration) values seen at the microorganisms were investigated to determine the anti-microbial effects of the essential oils used. In order to determine the values, each essential oil were prepared as a variety of dillutions and combined with both the microorganisms separately. First, the determination of the MICs were done on a microplate. After that, in order to determine the MBC, each essential oil-microorganism combination were put onto Mueller Hinton agars. In light of the investigation it was seen that the essential oils were effective at stopping the production of the microorganism even thought they were dilluted. Furthermore, in correlation with antibiotics, the MBC values were only one or two dillutions different than the MIC values.

Evaluation

This investigation for my extended essay was a succes. It gave promising results that helped me see the effects of essential oils on biofilm-producing microorganisms that were also in correlation with prior publications made upon the topic. The reason *Staphylococcus epidermidis* and *Candida parapsilosis* microorganisms were chosen fort he experiment was that they were both naturally found in the human flora and were well-known high level biofilm producers, providing for both a safe experiment and easier to see biofilm production. Both of these microorganisms were approved and put under the American Type Culture Collection (ATCC) for use in scientific studies as both of the microorganisms' all genetic characteristics were known and they exhibited high biofilm production. Moreover, these strains are the most frequently used strains in scientific studies on biofilms. Therefore, even though other microorganisms could have also been investigated, these two microorganisms were selected since they would give better and more trustworthy data.

The essential oils selected to be used in the investigation were *Melaleuca alternifolia*, *Eucalyptus globulus*, *Mentha piperita* and *Citrus limon*, otherwise more commonly known as tea tree oil, eucalyptus oil, peppermint oil and lemon, respectively. A lot more essential oils other than the ones selected for this investigation could have been used for the same purpose. However, these four were selected as they are the most commonly encountered essential oils in daily life.

Concerning the limitations of this investigation, both in the MIC and MBC readings and the biofilm production readings I did 5 trials for each group in order to minimize the errors. Moreover, in the biofilm production readings, all of the groups were spectrophotometrically read 3 times using the microplate reader. However, even more trials could have been done in order to minimize the error margin as much as possible. In order to deepen the investigation, a bigger variety of microorganisms and essential oils could have been used, providing even more knowledge on the effects of various essential oils on biofilm producing microorganisms.

With the purpose of degrading the intra- and inter-personal variation for measurement, all of the devices that were used in the readings were the same. In order to make sure that the values I reached were subjective and not deceived by the human eye, best possible devices for readings were used thanks

to my access to a high-end lab. The method chosen to investigate the anti-biofilm effects of the essential oils were spectrophotometry. Spectrophotometry was chosen as it is the second most reliable detection method after electron microscopy, which was not selected as the method fort his investigation due to the high prices needed to do readings from the electron microscope. Another method that could have been used to do the readings in this investigation is the Congo Red Agar method. However, its sensitivity, the ratio in which it gives correct positives, is less than that of the microplate reader.

All equipment and devices used in the investigation were compared, tested and prepared beforehand in order to completely elliminate systematic errors. Even so, random errors could still have taken place in the investigation, such as minor errors in the preparations of dillutions, but these were accounted for by calculating uncertainties and performing several trials.

Although it is possible for me to prepare essential oil extractions by myself, comercially available ones were preferred in this study because they are more standardized, producing more reliable results.

Despite the possibilities of minor errors, the results that I was able to reach in my investigation that indicated essential oils had an anti-microbial and anti-biofilm effects on bacteria and yeast strains can be qualified as valid as the conclusion reached is in correlation with other publications made upon the topic.

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