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Inhibition of Biofilm Formation in Bacteria by Essential Oils

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Abstract: The formation of S. epidermidis and P. aeruginosa biofilms were successfully inhibited in the presence of sub-inhibitory concentrations of S. aromaticum (clove) and L. angustifolia (lavender) essential oils. These substances achieved good in-vitro test results. On the other hand, pure and organic types of N. sativa (black seed) essential oil did not exhibit any inhibitory effects on the biofilm formed by the tested bacteria.

Although the tested essential oils may share a similar mechanism of action, ANOVA analysis showed strong statistical differences between all essential oils at their sub-MIC levels, and also identified two different trends, biofilm inhibitors, represented by S. aromaticum (clove) and L. angustifolia (lavender) oils, and biofilm stimulators, represented by both types of N. sativa (black seed) oils. A more thorough perspective on the research question could be achieved by considering the various elements that have essentially contributed to the processes and the findings within this study.

Keywords: Biofilm, S. epidermidis, P. aeruginosa, N. sativa, Clove, Lavender, Essential oils, Inhibition.

تثبيط تكون البيوفيلم في البكتيريا بواسطة الزيوت العطرية

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الملخص: تم بنجاح تثبيط تكون البيوفيلم (الغشاء الحيوي الرقيق) في وجود تركيزات شبه مثبطة من الزيوت العطرية (القرنفل، والخزامى). هذه الزيوت حققت نتائج جيدة في المختبر. من ناحية أخرى، فإن زيت الحبة السوداء العطري بنوعية النقي والعضوي لم يظهر أي آثار مثبطة للبيوفيلم الذي تشكله البكتيريا المختبرة.

وعلى الرغم من أن الزيوت العطرية التي تم اختبارها قد تشترك في آلية عمل مماثلة، إلا أن تحليل (ANOVA) الإحصائي أظهر اختلافات إحصائية قوية بين جميع الزيوت العطرية في جميع تركيزاتها (الأقل من مثبطة)، وقد تم أيضا تحديد اتجاهين مختلفين في آلية عمل الزيوت العطرية: مثبطات الأغشية الحيوية (والتي مثلها زيت القرنفل والخزامى)، ومحفزات الغشاء الحيوي (والتي مثلها زيت الحبة السوداء بنوعيه). يمكن تحقيق منظور أكثر شمولا حول سؤال البحث من خلال الأخذ بالاعتبار العناصر المختلفة التي ساهمت بشكل أساسي في العمليات والنتائج ضمن هذه الدراسة.

الكلمات المفتاحية: الغشاء الحيوي (البيوفيلم)، الحبة السوداء، القرنفل، الخزامى، الزيوت العطرية، تثبيط.

Introduction

In the world's battle against diseases, one of the biggest concerns for researchers is the increasing number of drug-resistant bacteria and scientific research has confirmed the failure of antibiotics in killing

such microorganisms that cause disease (Abdallah, 2011). Researchers are looking for alternative ways to tackle this issue; one of these alternative approaches is the use of essential oils. Bacteria have more than one way of growth; they can grow as separate cells or as colonies known as 'biofilm'. The biofilm is the most common mode of growth in several bacterial infections. Bacteria in biofilm form are in fact adherent bacteria attached to solid surfaces. They are surrounded by a protective material, which they secrete (Anusriha and Ponnarmadha, 2020).

Biofilm is simply defined as colonies of microbial cells encased in a porous slim and attached to a surface. Bacteria in a biofilm are known to be much more resistant to antimicrobial agents than free-living cells and may act as continuous sources of spoilage and pathogenic bacteria that contaminate food (Lindsay and Holy, 2006). The biofilm mode of growth tends to be the most favourable to bacteria since it can improve and enhance bacterial growth and survival rates (Oral, et al., 2010). Besides, it helps bacteria to become attached to nutrient-rich surfaces (e.g. animal tissues), and it allows bacterial cells to live in close association with each other, thus improving genetic exchange (Madigan and Martinko, 2006). The present study examines the inhibition of biofilm formation in bacteria by essential oils and the biofilm produced by two types of pathogenic bacteria: Pseudomonas aeruginosa (P. aeruginosa is a Gram-negative bacterium and one of the opportunistic pathogens in humans) and Staphylococcus epidermidis (S. epidermidis is a Gram-positive bacterium and a group member of Coagulase-Negative Staphylococci (CoNS).

Research Background

Bacteria in a biofilm are much more resistant to antibiotics than when they are in planktonic form and growing in biofilm is a major mode of growth. Biofilms are responsible for more than 60% of microbial infections (Chaieb, et al., 2011a). This has been a major contributor to the numerous hazardous and costly problems that accompany bacterial infections in numerous environments; in medical settings (device-related infections), in industrial settings (biofouling in drinking water distribution systems), in food processing settings (source of contamination, food spoilage), and human health (CF lung infections, dental plaque, middle ear infections) (Oral, et al., 2010). Several types of bacteria can produce biofilm and several factors referred to as virulence factors responsible for bacterial infections in humans and that also contribute significantly towards the development of the biofilm. These factors are displayed by pathogenic bacteria (e.g. Pseudomonas aeruginosa) (Kipni, et al., 2006).

The virulence factors in Pseudomonas aeruginosa - for instance - have been identified. One of these factors is the presence of Flagella, which allows the bacterium to bind and adhere to epithelial cells, and gives full support to the early stages of attachment of the biofilm to abiotic (e.g. glass) services (Kipnis, et al., 2006; Morisaki, et al., 1999). This gliding motility helps in spreading the biofilm on the surface for colonization (Kipnis, et al., 2006; Merz and Forest, 2002). The quorum-sensing auto-inducer has been also

considered as one of the virulence factors in Pseudomonas aeruginosa; where N-(-3-oxododecanoyl)-Lhomoserinelactone can be responsible for repressing host immune responses and it may regulate the expression of extracellular enzymes and exotoxins in infections related to P. aeruginosa (Greenberg, 1997; Kipnis, et al., 2006).

The relationship between auto-inducers and biofilm is that the former is an essential agent in cell-to-cell signalling in the biofilm formation (Davies, et al., 1998). Moreover, the production of Exotoxin A in P. aeruginosa biofilm is shown to be increased particularly when the bacterium is attacked by a sub-fatal concentration of antibiotics. It is worth noting that P. aeruginosa produces Exotoxin A during the infection of the host cell to weaken the immune responses and inhibit the protein synthesis of the host cell, therefore causing lethal ending of the infected cell of the host (Horii, et al., 2005). Another important point that should be highlighted is that conjugation in bacteria allows genetic materials to be transferred. These genetic materials (e.g. plasmid coding for antibiotic resistance), can take place in the inter-species and intra-species levels within the. This highlights the importance of the biofilm as a reservoir in passing on and distributing the antibacterial genes that are responsible for resistance in bacteria (Hall-Stoodley and Stoodley, 2004).

Research Aim

The current study mainly aims to examine the inhibition of biofilm formation in bacteria by essential oils. The main objective is supported by the following sub-objectives:

1. Identify the different sub-inhibitory concentrations of each essential oil and the extent of their effects on bacterial growth.
2. Identify the role of oil type in the bacterial growth process.
3. Identify the statistical interactions and different patterns of effect across all oils at different sub-inhibitory concentrations.

Research Significance

Biofilm is known as a thick layer of prokaryotic organisms aggregated to form a community or colony. The colony sets on a surface with a slime layer in order to protect the micro-organisms. The current study sheds the light on the several factors behind biofilms' development; factors that promote growth and the survival of the microorganisms. For many years, plant distillate and their active elements have been a primary source for rich products from nature, which have an essential role in curing and inhibiting diseases and the source to preserve balanced health for human beings. The study's significance lies in its results and final material; as in many years of research, few explosion and studies have been conducted to discover the molecules with the power to inhibit the formation of biofilms. Nevertheless, there is some significant evidence that withdraws essential fractions, plant products, phytochemicals

elements, or several natural chemicals from plants have the possibility to be created as inhibitory treatment or agents to work against the biofilm-based diseases.

The significance of this study shows the need to offer a pathway for rational experimentation in the clinical environments by confirming both the capacity and safety of these active agents of plant compounds in-vitro. The present discovery and development of modern agents aim to lower the costs and decrease the risk of creating a resistance to antibiotics by integrating only the novel molecules or infusion with current antimicrobial drugs. The current study also shows that the transformation and transport processes in biofilms, which can be affected by the biofilm's structure, and investigate this process at a microscopic level using several biochemical tests and electrodes, which can lead to various applications useful to this field of research.

Research Methodology

Essential Oils and Bacteria

Four essential oils were purchased from local mills and used in this study. The oils comprised Clove oil (Syzygium aromaticum) from (Niharti Ltd, UK), Lavender oil (Lavandula angustifolia) from (Country Harvest, UK), and two Black Seed oils (Nigella sativa) (Pure and Organic) purchased online from a specialist supplier. For the bacteria, two different types were used, Pseudomonas aeruginosa PAO1-L (Gram-negative) and Staphylococcus epidermidis 1457) (Gram-positive). Both bacteria were in the form of Microban® beads (TCS Ltd., Merseyside, UK).

Preparation Stage

To examine the targeted bacteria, Luria Bertani agar medium (LB Agar, Fisher Scientific, New Jersey, US) and Brain Heart Infusion Agar medium (BHI Agar, Oxoid, Basingstoke, UK) were prepared on an agar plate as a first step. Two beads of P. aeruginosa were sub-cultured onto LB agar, and two beads of S. epidermidis were sub-cultured onto BHI agar. The agar plates and control were incubated at 37°C for 24 hours.

Preliminary Stage

Determination of the Minimum Inhibitory Concentrations (MIC) and Minimum Bactericidal Concentrations (MBC) of essential oils

Determination of MICs and MBCs were carried out using the broth microdilution method in a 96-well microtiter plate (Sterilin®, Fisher Scientific, and the UK). In the microtiter plate, the first well of each row received 100 µl double-strength Iso-sensitest broth (Oxoid, Basingstoke, UK). The remaining rows received 100 µl single-strength Iso-sensitest broth. Following this, 100 µl of the tested oil was added to the first well of each row and mixed very well. Then, from that well, 100 µl of the solution was taken, added to

the second well, and mixed. Using a fresh pipette, another 100 µl was taken from the second well, added to the following well, and mixed. This was repeated until the last well of the row (well number 12), which the last 100 µl was discarded from it. This gave series dilutions of 1, 0.5, 0.25, 0.125, 0.0625, 0.3125, 0.016125, 0.008, 0.004, 0.002, and 0.001 mg ml^{-1}. Each well was inoculated with 10 µl of the tested bacterial culture. This means that each well-received 10^5 cells.

Each essential oil was tested in triplicate. The bacterial suspension was used as the positive control and oil in the broth was used as the negative control. Finally, the plates were incubated at 30°C or 37°C (depending on the tested bacteria) for 24 hours. Following the incubation time, each plate was visually observed and two points were recorded; turbidity (which is an indication of the bacterial growth), and pigment formation. A photometric measurement was undertaken by a microtitre plate reader, which was used to confirm the visual results. At this stage, the MIC was identified as the highest dilution or lowest concentration that inhibited visible growth (showed no turbidity) after 24 hours of incubation at 37°C.

Biofilm Formation Assay

Congo Red Agar (CRA) assay was conducted to screen for biofilm formation ability in the tested bacteria; a specific solid medium was specially prepared and supplemented with 5% Sucrose (Sigma, Life Science, USA) and Congo Red (Fisher Scientific, UK). Both bacteria were tested; this was done by preparing a medium consisting of LB (40g/L) (for *P. aeruginosa)* or BHI (47g/L) (for *S. epidermidis)*, plus Sucrose (50g/L) and Congo Red stain (0.8g/L). The LB or BHI agar was prepared, sterilized, and cooled to 55°C. Then the Congo Red stain was prepared separately. Because it comes in a powder form, a concentrated aqueous solution was prepared and autoclaved at 121°C for 15 minutes.

Pilot Stage

Studying the Inhibitory Effects of Essential Oils on Sub-MIC Levels

To study the inhibitory effects of the essential oils under investigation on a sub-MIC level, four sets (for each bacterium) were prepared accordingly. These sets consisted of 1/2, 1/4, and 1/8 MIC oils and positive and negative controls. First, the tested bacterium (*P. aeruginosa* or *S. epidermidis)* was grown overnight for 24 hours at 37°C in the suitable medium (LB or BHI), then a loopful of the bacterial colonies was taken and added to a freshly prepared, sterilized, and cooled 20 ml broth (either LB or BHI) in a conical flask. This was then incubated in the shaker incubator (G24 environmental incubator shaker, New Brunswick Scientific, Co., Inc USA) at 200rpm and 37°C for 19 hours. Following the incubation time, a bacterial suspension (unknown CFU) was obtained and ready to use for the following step.

The following step consisted of preparing one set of sub-MIC oil and positive and negative controls) for each oil, which was four sets in total for each bacterium. In each set, a six-well plate was used.

The first row of the plate (wells 1-3) included the sub-inhibitory concentrations of the tested oils MIC concentrations respectively, while the second row included the positive (no oil) and negative (no bacteria) controls (wells 4 and 5). All wells had one sterile floating glass (1×1cm) (which had been sterilized by ethanol for 20 minutes using a mini orbital shaker (Mini Orbital Shaker; SO5, Stuart Scientific, UK) and received 10 ml of broth media (LB or BHI broth), and 100µl of the 19h bacterial suspension prepared earlier, except for the well that represented the negative control. All wells in the first row received $^1/_2$, $^1/_4$ and $^1/_8$ MIC's oil concentrations respectively (as the MIC's values obtained from the preliminary stage).

After preparing four sets of all oils, they were incubated for 24 hours at 37°C. Following incubation time, each set was observed and tested. The floating glass of each well was picked up by a sterilized force and then washed in sterilized distilled water by immersion for 30 seconds (to remove non-adhering bacteria) and then placed into a sterilized 50 ml tube that contained 20 ml of freshly prepared, sterilized, and cooled broth media. Then the tube was labeled and shaken vigorously by using a test tube shaker (TopMix FB1SO13; Fisher Scientific; US) for one minute.

Statistical Analysis

Each stage of the practical experiments was repeated three times. Data obtained from the experiments are presented in detail in the following section. Those data were analyzed by the Analysis of Variance (ANOVA), using the SPSS software (Statistical Package for the Social Sciences). This parametric test is one of the most important and broadly used tests employed to investigate whether there are any significant differences between groups under interest. In the present study, the main aim of utilizing this statistical technique was to find out if there are any differences between the means of three different sub-inhibitory concentrations of four different essential oils.

The ANOVA test cannot inform us whether a specific concentration is different from others. To specify the result obtained, the post hoc test (Tukey's Honestly significant difference (HSD) and the Tamhane test) were employed (where appropriate) to determine, which specific concentration differed from each of the other concentrations, or specific essential oil differed from each of the other essential oils, and also to follow up the significant results. However, this test should only be conducted when there are overall significant differences in group means (i.e. a significant one-way ANOVA result). It is worth noting that this statistical technique attempts to control the experiment-wise error rate (alpha = 0.05).

The rationale behind employing the ANOVA test (one-way and two-way ANOVA) instead of other statistical methods (e.g. t-test) is because the nature of the present study required comparing more than two means, thus the t-test would not be appropriate to analyze the data. Analyzing the results by using the t-test could expose the findings of this study to a Type 1 error, which is usually 5%. In the case of

running this test more than once on the same data, the chance of making that error will increase subsequently; 10%, 15%... and so on.

Findings

Determination of MIC and MBC of essential oils

The results generally show that MIC values were lower (higher inhibitory effects) when using the *S. aromaticum* and *L. Angustifolia* essential oils against *P. aeruginosa* compared to *S. epidermidis*, which was found to be less sensitive to the tested oils. This was not the case with both varieties of *Nigella Sativa*, where it was found to be more effective in inhibiting the growth of *S. epidermidis* when compared to the effect of oil against *P. aeruginosa.*

Regarding the MBC of *N. sativa* oil against *P. aeruginosa*, it is notable to mention that for the first time in this study, *N. sativa* oil had not shown any killing effect at the concentrations used in this study, while *S. aromaticum* oil, followed by *L. angustifolia* oil, showed a cidal effects at -relatively- lower concentrations. Moreover, and in the case of *S. epidermidis,* the cidal effects were pronounced at lower concentrations in both *S. aromaticum* oil and (Pure) *N. sativa* oil compared to the other oils. *L. Angustifolia* oil took second place in the killing effects against *S. epidermidis*, and finally, organic *N. sativa* oil at higher concentration was found to be fatal to the same bacterium. The results collected from the antimicrobial susceptibility test of both target bacteria are shown in table 3.1.

Table (3.1) The MIC and MBC concentrations of the essential oils.

	P. aeruginosa			
	Clove (*S. aromaticum*)	Lavender (*L. angustifolia*)	Pure Black seed *N. sativa* (P)	Organic Blackseed *N. sativa* (O)
MIC (inhibitory level)	7.42 µl/ml	29.76 µl/ml	238.09 µl/ml	238.09 µl/ml
MBC (cidal level)	14.85 µl/ml	49.6 µl/ml	> 476.18 µl/ml	> 476.18 µl/ml
	S. epidermidis			
	Clove (*S. aromaticum*)	Lavender (*L. angustifolia*)	Pure Black seed *N. sativa* (P)	Organic Blackseed *N. sativa* (O)
MIC (inhibitory level)	14.85 µl/ml	19.82 µl/ml	24.79 µl/ml	198.40 µl/ml
MBC (cidal level)	24.79 µl/ml	29.76 µl/ml	24.79 µl/ml	198.40 µl/ml

Moreover, an inhibition effect of the biofilm formation (and the pigment) had been seen in MICs levels of all tested oils on both bacteria (See figure 3.1 below).

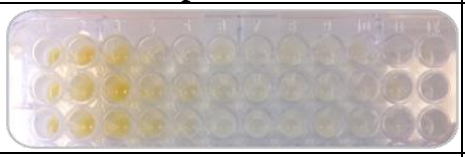
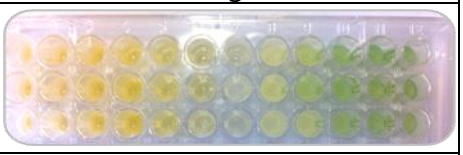
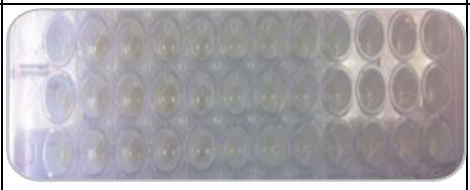
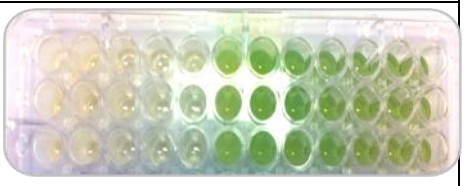
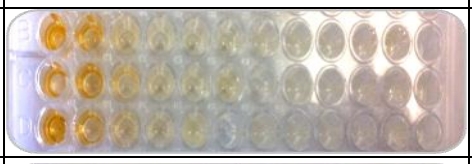
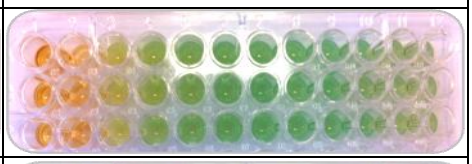
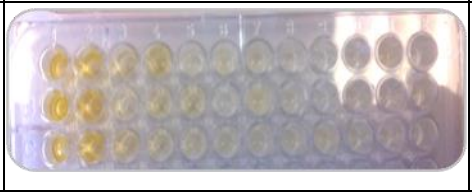
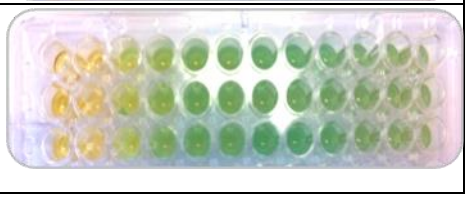
	S. epidermidis	***P. aeruginosa***
S. aromaticum Clove oil		
L. angustifolia Lavender oil		
N. sativa (P) Pure Black Seed oil		
N. sativa (O) Organic Black Seed oil		

Figure (3.1) MIC test results for all essential oils.

Detection of Biofilm Formation

Congo Red Agar Assay

The bacteria under study were tested for their ability to form a biofilm. The Congo Red Agar test revealed two different results. A positive result was obtained from *S. epidermidis* 1457 strain, which showed dark, crystalline colonies; this (according to Freeman, et al., 1989) is an indicator of the strong ability of this bacterium in the biofilm formation. On the other hand, the *P. aeruginosa* PAO1–L strain showed slimy, pink colonies, which can be an indicator of the weak ability in biofilm formation on that bacterium (see figure 3.2).

S. epidermidis 1457	*P. aeruginosa* PAO1-L	Control

Figure (3.2) CRA screening of *S. epidermidis, P. aeruginosa* PAO1-L, and control.

Studying the Inhibitory Effects of Essential Oils on Sub-MIC Levels

Several results had been obtained from testing the effects of sub-MIC concentrations (1⁄2, 1⁄4, and 1/8) of the essential oils on the biofilm formation. The percentage of reductions in the biofilm formation and bacterial growth (optical density) was computed, and is represented in table 3.2 below:

Table (3.2) Inhibition of biofilm formation and bacterial growth (% of reduction) at sub-MIC of essential oils.

Inhibition of Biofilm Formation and (Optical Density) (% of reduction)				
Bacteria	Essential oil	0.5 MIC	0.25 MIC	0.125 MIC
S. epidermidis	*S. aromaticum*	98.84 (98.03)	72.97(69.74)	16.60(15.27)
	L. angustifolia	98.07 (87.14)	70.66(65.20)	2.32(1.66)
	N. sativa (p)	83.40 (7.71)	90.73 (18.30)	94.98 (21.33)
	N. sativa (o)	+ (+)	65.64 (10.74)	76.06 (15.27)
P. aeruginosa	*S. aromaticum*	97.43(64.13)	70.08 (57.61)	53.84 (48.91)
	L. angustifolia	96.15 (58.70)	58.12 (44.57)	1.71(31.52)
	N. sativa (p)	+ (+)	14.52 (6.52)	38.03 (23.91)
	N. sativa (o)	+ (+)	+ (+)	+ (+)

Note: *S. aromaticum* (clove), *L. angustifolia* (lavender), *N. sativa* (p) (pure black seed), *N. sativa* (o) (organic black seed); + increasing in the biofilm formation and bacterial growth (stimulation effect).

The results obtained from S. epidermidis showed clear evidence that two of the tested essential oils (Clove and Lavender) in their sub-MIC levels reduced the bacterial growth, which in turn reduced the biofilm formation (see picture 3.3).

Clove oil took the lead in inhibiting the biofilm produced by S. epidermidis. Half of the MIC value reduced the biofilm to 98.84%, followed by 72.97% MIC, and 16.60% MIC compared to the control. A similar theme of results had been shown with the Lavender oil; where the biofilm formation declined by 98.07%, 70.66, and 2.32% for the concentrations of MIC respectively. It is also shown that the reductions in the biofilm formation were slightly higher than the reduction in the bacterial growth (O.D), which indicates that these oils -at their sub-MIC levels- have more effect in inhibiting the biofilm formation than inhibiting the bacterial growth.

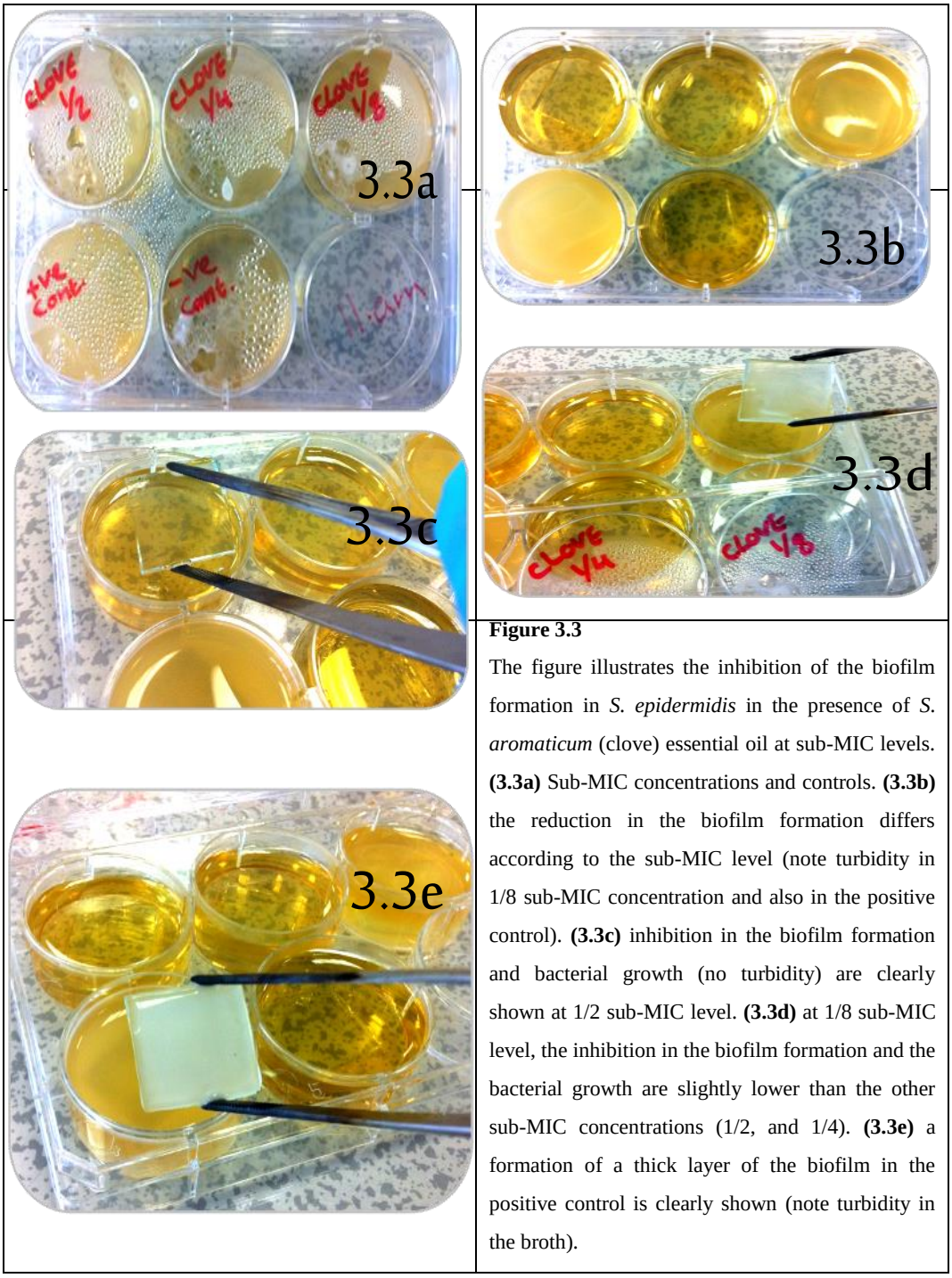


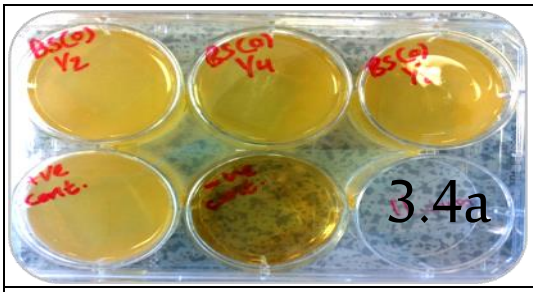
Figure 3.3

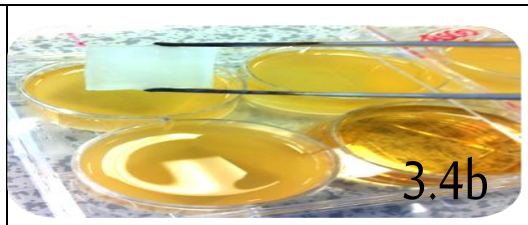
The figure illustrates the inhibition of the biofilm formation in *S. epidermidis* in the presence of *S. aromaticum* (clove) essential oil at sub-MIC levels. **(3.3a)** Sub-MIC concentrations and controls. **(3.3b)** the reduction in the biofilm formation differs according to the sub-MIC level (note turbidity in 1/8 sub-MIC concentration and also in the positive control). **(3.3c)** inhibition in the biofilm formation and bacterial growth (no turbidity) are clearly shown at 1/2 sub-MIC level. **(3.3d)** at 1/8 sub-MIC level, the inhibition in the biofilm formation and the bacterial growth are slightly lower than the other sub-MIC concentrations (1/2, and 1/4). **(3.3e)** a formation of a thick layer of the biofilm in the positive control is clearly shown (note turbidity in the broth).

Figure (3.3) The effects of sub-MIC concentrations (0.5, 0.25, & 0.125) of *S. aromaticum* (clove) essential oil on the biofilm formation of *S. epidermidis.*

Interestingly, and for the first time in this study, *Nigella sativa* showed opposite results. In its pure type, the lowest sub-MIC concentration (1/8) showed the highest percentage of biofilm reduction (94.98%), while the highest sub-MIC concentration (1/2) showed the lowest percentage of biofilm reduction (83.40%) and bacterial growth- when compared to the control. This gave a strong indication that the oil can stimulate bacterial growth; thus, biofilm formation. The organic type, on the other hand, showed very different results (see figure 3.4). The 1/2 MIC level showed a clear stimulation effect on the

biofilm formation of the bacterium (18.5%) when compared with the control, which is again an indication that this amount of oil can enhance the growth to more than that, which occurs in the positive control. However, both 1/4 and 1/8 sub-MIC levels showed an increase in the biofilm formation, but not exceeding the maximum level of growth found in the positive control.





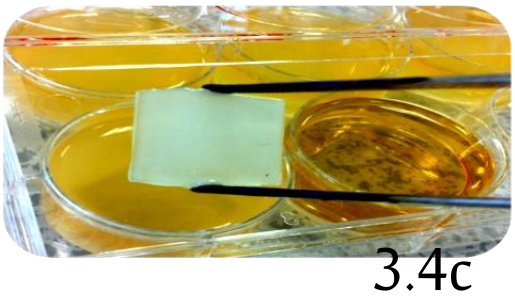


Figure 3.4
The figure clearly illustrates the enhancement of the biofilm formation in *S. epidermidis* in the presence of *N. sativa* (organic black seed) essential oil at sub-MIC levels. **(3.a)** Sub-MIC concentrations and controls (note turbidity in all wells except the negative control). **(3.4b)** A clear enhancement of the biofilm formation at 1/2 sub-MIC level (similar to positive control). **(3.4c)** A formation of a thick layer of the biofilm in the positive control is clearly shown (note turbidity in the broth).

Figure (3.4) The effects of sub-MIC concentrations (0.5, 0.25, & 0.125) of *N. sativa* (organic black seed) essential oil on the biofilm formation of *S. epidermidis.*

The case was partially similar in the case of *P. aeruginosa*; where the sub-inhibitory concentrations (1/2, 1/4, and 1/8 MIC) of Clove essential oil reduced the number of adherent bacteria to 97.43%, 70.08%, and 53.84% respectively. A similar trend was found in the Lavender oil, which reduced the bacterial growth and also the biofilm formation to 96.15%, 58.12%, and 1.71% respectively (see figure 3.5 below), but slightly lower compared to the reductions caused by Clove oil.

It is also shown that the reductions in the biofilm formation were slightly higher than the reduction in the bacterial growth, which indicates that these oils -at their sub-MIC levels- have more effect in inhibiting the biofilm formation than inhibiting the bacterial growth. Black seed oils on the other hand showed opposite findings; where the sub-inhibitory concentration showed a stimulation effect and enhancement on the bacterial growth and the biofilm formation except for the 1/4 and 1/8 sub-inhibitory concentrations of pure Black seed oil; where the adherent bacteria were reduced to 14.52% and 38.03% respectively.

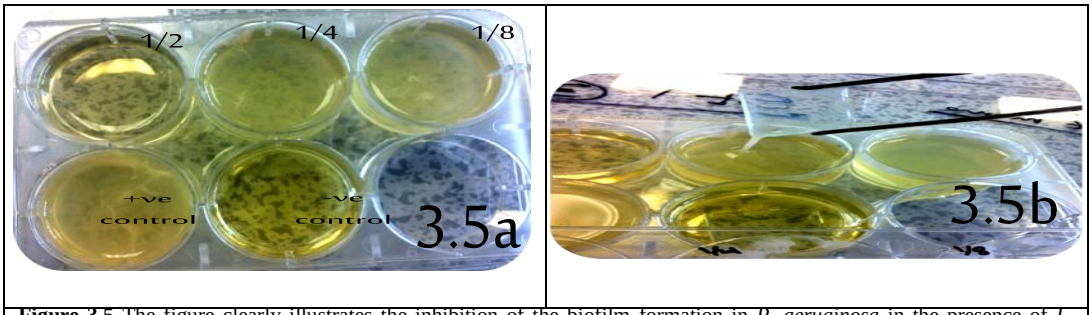


Figure 3.5 The figure clearly illustrates the inhibition of the biofilm formation in *P. aeruginosa* in the presence of *L. Angustifolia* (lavender) essential oil at sub-MIC levels. **(3.5a)** Sub-MIC concentrations and controls (note turbidity in 1/4, 1/8, and positive control). **(3.5b)** A reduction of the biofilm formation at 1/4 sub-MIC level (note the slime).

Figure (3.5) The effects of sub-MIC concentrations (0.5, 0.25, & 0.125) of *L. angustifolia* (lavender) essential oil on the biofilm formation of P. aeruginosa

Statistical Analysis by ANOVA Test

One-Way ANOVA Analysis

Table (3.4) One-Way ANOVA.

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	11602.731	3	3867.577	3.789	.019*
Within Groups	35724.500	35	1020.700		
Total	47327.231	38			

* The mean difference is significant at the.05 level.

The results show that there was a statistically significant difference between concentrations (including control) as determined by one-way ANOVA (F(3,35) = 3.789, P = 0.019. Therefore, the null hypothesis is rejected.

Two-way ANOVA

Table (3.5) Factors (Main effects and interaction effects).

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	39078.889[a]	11	3552.626	894.367	.000
Intercept	37377.778	1	37377.778	9409.790	.000
Oil	8214.000	3	2738.000	689.287	.000
Conc	3494.389	2	1747.194	439.853	.000
Oil * Conc	27370.500	6	4561.750	1148.413	.000
Error	95.333	24	3.972		
Total	76552.000	36			

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Total	39174.222	35			
a. R Squared =.998 (Adjusted R Squared =.996)					

From the table above, there is a strong statistically significant interaction effect of oil types and concentrations (F (6, 24) = 1148.413, P<0.05. There is a strong statistically significant main effect of oil types (F (3, 24) = 689.287, P<0.05, and finally, there is a strong statistically significant main effect of sub-inhibitory concentrations (F (2, 24) = 439.853, p <0.05). The interaction between oil types and sub-inhibitory concentration was plotted (see graph in figure 3.3). The interaction between oil types and sub-inhibitory concentration was plotted (see graph in figure 3.6).

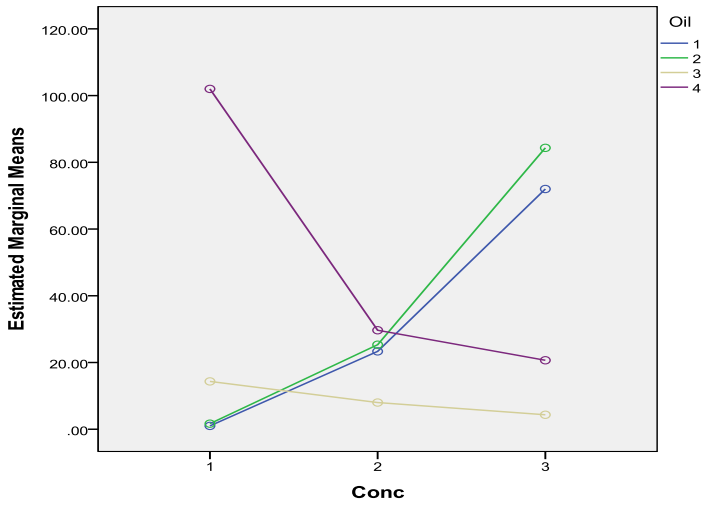


Figure (3.6): Estimated Marginal Means (sub-inhibitory concentrations*oil types).

The graph clearly illustrates that there is a strong interaction between oil types and sub-inhibitory concentrations. This interaction has two different trends. As seen from the graph above, the sub-inhibitory concentration 1/2 (conc.1) is the most effective concentrations to be used in Clove oil and Lavender oil (oils 1 and 2). In contrast, this particular concentration is shown to stimulate bacterial growth when it is used on both Black Seed oils (oils 3 and 4). However, both Black seed oils were at their best (to more inhibitory on bacterial growth) when they were used at the 1/8 sub-inhibitory concentration, which is completely different to the case with the Clove and Lavender oils. On the other hand, the second group, the 'negative group', represented by Black seed oils (pure and organic) (oils 3 and 4) suggests a decrease in bacterial growth and biofilm formation from 1/2, 1/4, and 1/8 (see figure 3.7 below):

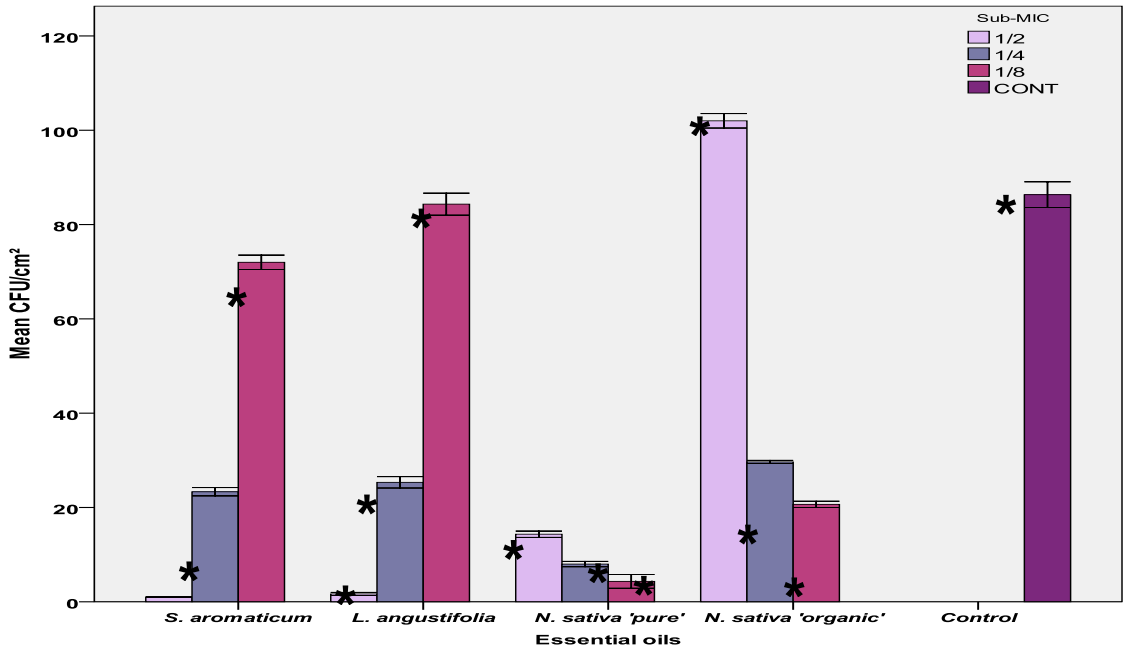


Figure (3.7) Effects of sub-MIC levels of essential oils on bacterial growth and biofilm formation in *S. epidermidis.*

The above figure illustrates the following: Mean CFU/cm2 of *S. epidermidis*. Errors bars indicate ±1 Standard Error 'SE'. Bars marked with asterisks are significantly different; * P< 0.05, **P<.01. In summary, on average, each sub-inhibitory concentration, in each oil, is significantly different from others and control. (For a detailed analysis, please refer to appendix 1).

P. Aeruginosa Results

One-way ANOVA Analysis

Table (3.6) ANOVA.

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	1300.936	3	433.645	.263	.851
Within Groups	57678.500	35	1647.957		
Total	58979.436	38			

* The mean difference is significant at the.05 level.

The results show there were no statistically significant differences overall between concentration means (including control) as determined by one-way ANOVA ($F(3,35) = 0.263$, $P = .851$). Therefore, the null hypothesis is accepted.

Two-way ANOVA

Table (3.7) Factors (Main effects and interaction effects).

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	57697.222[a]	11	5245.202	704.579	.000

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Intercept	121336.111	1	121336.111	16298.881	.000
Oil	39210.333	3	13070.111	1755.687	.000
Conc	199.389	2	99.694	13.392	.000
Oil * Conc	18287.500	6	3047.917	409.422	.000
Error	178.667	24	7.444		
Total	179212.000	36			
Corrected Total	57875.889	35			
a. R Squared =.997 (Adjusted R Squared =.995)					

From the table above, there is a strong statistically significant interaction effect of oil types and concentration; (F (6, 24) = 409.422, P<0.05. There is a strong statistically significant main effect of oil types; (F (3, 24) = 1755.687, P<0.05. and finally, there is a strong statistically significant main effect of sub-inhibitory concentrations; (F(2, 24) = 13.392, p <0.05). The interaction between oil types and sub-inhibitory concentration was plotted (see the following graph).

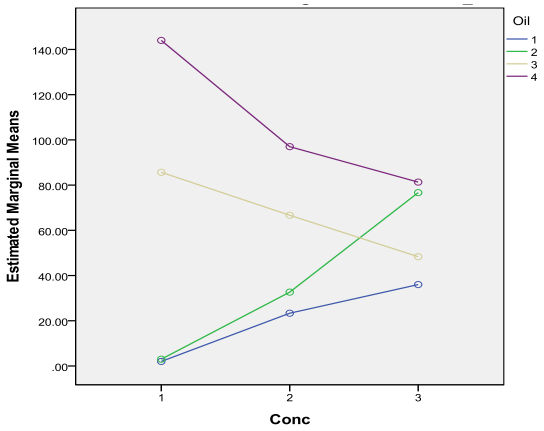


Figure (3.8) Estimated Marginal Means (sub-inhibitory concentrations*oil's type).

As seen from the graph above, the sub-inhibitory concentration 1/2 (conc.1) is the most effective one to be used in clove oil and lavender oil (oils 1 and 2). However, both black seed oils were at their best (to inhibit the bacterial growth more) when they were used at the 1/8 sub-inhibitory concentration, which is a completely different case to the clove and lavender oils. Group one, the 'positive group' is represented by two types of oil; Clove and Lavender (oils 1 and 2). Those two oils demonstrate an increase in bacterial growth, starting from 1/2, 1/4, and 1/8 (concentrations 1, 2, and 3). The second group, the 'negative group', is represented by black seed oils (pure and organic) (oils 3 and 4) suggest a decrease in the bacterial growth from 1/2, 1/4, and 1/8 (concentrations 1, 2, and 3) (see figure 3.9 below):

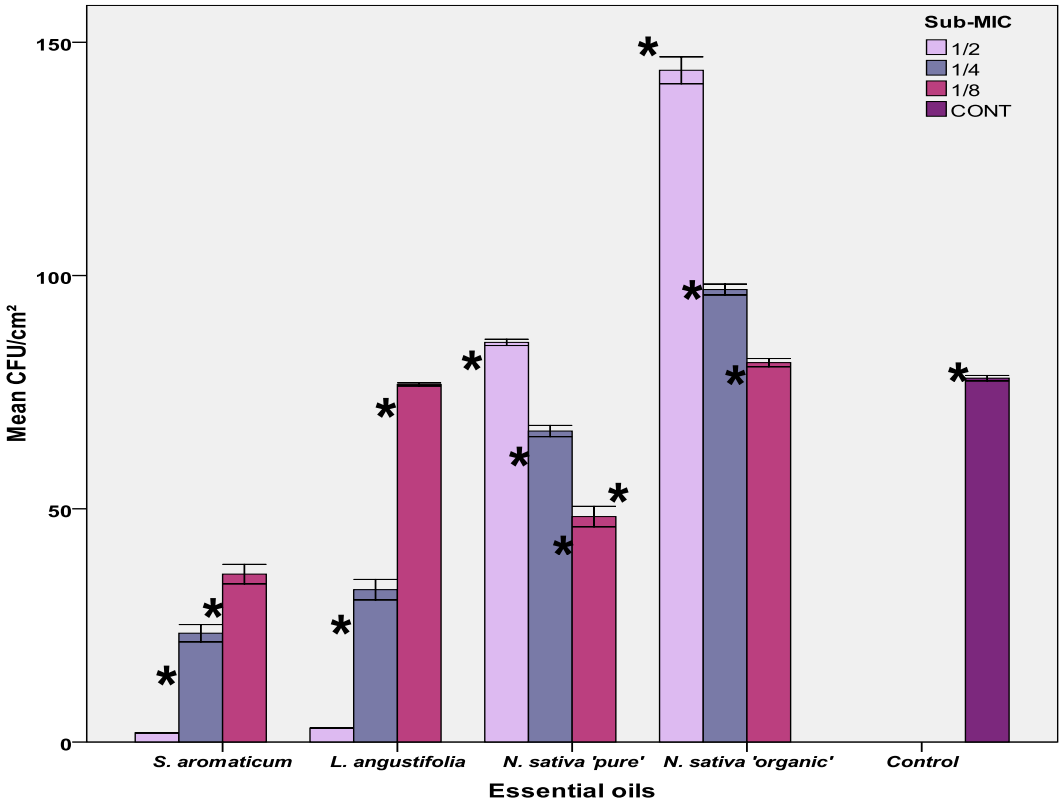


Figure (3.9) Effects of sub-MIC levels of essential oils on bacterial growth and biofilm formation in P. aeruginosa.

The above figure illustrates the following: Mean CFU/cm2 of *P. aeruginosa*. Errors bars indicate ±1 Standard Error 'SE'. Bars marked with asterisks are significantly different; * P< 0.05, **P<.01. In summary, on average, each sub-inhibitory concentration, of each oil, is significantly different from control only. The 1/2 sub-inhibitory MIC of Clove and Lavender is not significant, although they are different from control. (For a detailed analysis, please refer to appendix 1).

Discussion

The study of biofilm formation in the presence of essential oils was undertaken to offer further insight into the inhibitory properties of essential oils at sub-MIC levels. As shown in the results section, there was a statistically significant difference between essential oil types (including control) and their sub-inhibitory concentrations. From the results obtained from the MIC susceptibility test, the Gram-negative bacterium (*P. aeruginosa)* was most susceptible to *S. aromaticum* oil (clove), then to *L. angustifolia* oil (lavender), and finally less susceptible to both types of *N. sativa* oils (black seed). Gram-positive bacterium (*S. epidermidis)* was also most affected by *S. aromaticum* oil, followed by *L. Angustifolia* oil, then pure *N. sativa*, and finally the organic *N. sativa*. The overall comparison showed that only *S. aromaticum* oil has a stronger inhibitory effect on *P. aeruginosa* (Gram-negative) than *S. epidermidis* (Gram-positive). All other oils showed more inhibitory effects on the Gram-positive bacterium than on the Gram-negative one.

The findings relating to the effects of *S. aromaticum* oil are dissimilar to what has already been reported in some of the previous studies (e.g. Chaieb, et al., 2007); that Gram-negative bacteria (i.e. *P. aeruginosa*) are more resistant to essential oils. The reason for such resistance according to Gaunt and colleagues (2005) was because the outer lipopolysaccharide wall in the cell wall structure can serve as a barrier against toxic chemicals. Furthermore, the hydrophilic permeability barrier of *P. aeruginosa* can be another factor that helps the bacterium to be highly resistant to the effect of antimicrobial agents by reducing the diffusion of antimicrobial agents across the outer membrane (Parr, et al., 1987; Angus, et al., 1982).

However, this finding can be attributed to the fact that the major chemical component in *S. aromaticum is* eugenol, which is a phenolic compound. This particular component has been reported in many studies to have a pronounced antimicrobial activity (Kalemba, and Kunicka, 2003). The cell wall of *P. aeruginosa* was not resistant enough to such a toxic chemical. Moreover, the strong antibacterial effect of eugenol against *P. aeruginosa* in the present study is in agreement with a previous study conducted by El abed, et al., (2011) who found a high reduction in the biofilm of two pathogenic strains of *P. aeruginosa* because of the presence of eugenol. Moreover, Cox and Markham (2007) confirmed the susceptibility of *P. aeruginosa* to many active compounds including eugenol, which can be seen as potential efflux transporter substrates in *P. aeruginosa*. Concerning the remaining oils (*L. angustifolia* and *N. sativa*), they showed stronger effects against the Gram-positive *S. epidermidis* (compared to Gram-negative) due to their strong chemical components (linalool and thymoquinone), which have been well documented in previous studies (Chaieb, et al., 2011b; Farrag, 2000; Kim, et al., 2008; Lis-Balchin, et al., 1998; Salman, et al., 2002).

The reason for this can be explained by the fact that the cell wall structure of Gram-positive bacteria does not have that extra layer found in Gram-negatives, which greatly helps the toxic agents to damage the cell wall, disrupt the cell membrane and block the enzyme systems and ensure the progressivity of ion permeability (Lang and Buchbauer, 2011; Saei-Dehkordi, 2010). Other important points that might explain the reason for the variation in the MIC results between all tested oils is that there may be other effects involved such as altered porin expression, influences on lipopolysaccharide, or influences of the outer membrane composition (Cox and Markham, 2007).

This can also be explained by the fact that the mechanism of action of eugenol may cause some kind of disruption to the cytoplasmic membrane, which consequently caused some disturbance to the proton motive force, to the active transport, and the coagulation of cell contents (Sikkema, et al., 1995). The reduction in the bacterial growth and also the biofilm formation that occurred as a result of the sub-inhibitory concentrations in the present study provides strong evidence that *S. aromaticum* oil (clove) followed by *L. angustifolia* oil (lavender) were found to be potent antagonists of both *S. epidermidis* and *P. aeruginosa* biofilms at 1/2 sub-inhibitory concentration, followed by concentrations of 1/4 and finally

1/8. It is not surprising to observe these strong antibacterial and antibiofilm effects since both oils have high concentrations of antimicrobial compounds, mainly eugenol phenylpropene and linalool terpene alcohol, which are shown, in many cases, to exhibit antibacterial activity against a large number of bacteria, even the Gram-negative ones; where these kinds of chemicals can break up the cytoplasmic membrane, release lipopolysaccharides from the cell wall, and increase membrane permeability (Burt, 2004).

These results are consistent with other published studies (e.g. Guynot, et al., 2003), which suggested that phenolic compounds (e.g. eugenol) could be strong enough to disturb the cellular membrane and react with the active sites of enzymes. Moreover, these findings are also in agreement with other studies (Dorman, et al. 2000; Kalemba, and Kunicka, 2003) who reported that the main constituents of essential oils such as linalool and eugenol have been shown to have a broad spectrum of activities against microbes. On the contrary, both types of *N. sativa* oils (black seed) did not show any inhibition effect on the bacterial growth and the biofilm formation at sub-MIC levels, although both of them showed some degree of inhibition in the MIC test, particularly against *S. epidermidis*.

Another important point is that *P. aeruginosa* was the bacterium most affected by the organic type of *N. sativa* oil; where the bacterial growth and biofilm formation exceeded the maximum bacterial growth and biofilm production found in the positive control. This raises the following point: Why and how did such an effect occur particularly with Gram-negative bacterium, which has a naturally stronger cell wall? One of the possible explanations is that the tested oil may possess poor antagonistic activity against *P. aeruginosa* biofilm because of other factors (e.g., the method of extraction used) (El abed, et al., 2011).

The reason for the variation in the study results might be that different essential oils have differential modes of action for inhibiting biofilm formation. The extent of antibacterial activity and mode of action in the essential oils can be governed by the particular effect (additive, synergistic, or antagonistic) caused by each component (Lang and Buchbauer, 2011). The enhancement in biofilm growth seen when using sub-MIC levels of *N. sativa* is an example of one of the drawbacks of essential oils regarding their chemical compositions. Given that *N. sativa* oils consist of a variety of chemically active compounds, the antibacterial/antibiofilm activity cannot be allocated to one single component (Leonard, et al., 2010; Sandasi, et al., 2008). The activity expressed by *N. sativa* essential oil might be an outcome of a specific interaction between the chemicals, which in turn may decrease their effectiveness against the bacteria. Since the major active component of *N. sativa* is thymoquinone, it is possible that other molecules, which therefore weaken its antibacterial and antibiofilm effects, have influenced it. When we look at these findings from another angle, it appears that there might be a collaboration between the chemical components of the oil and the chemical content of the bacterial cell, which led them to enhance the bacterial growth, consequently enhancing the biofilm formation as a result.

In the present study, both *S. aromaticum* (clove) and *L. angustifolia* (lavender) had strong antibacterial effects on both tested bacteria, while *N. sativa* (black seed) oils had strong stimulation effects on both tested bacteria. It is possible that *N. sativa* had no toxicity on the cell membrane of both tested bacteria, particularly in that toxicity may not be caused by disturbing the structure of the bacterial cell membrane only; it may also need to chemically modify specific cellular targets (Cox and Markham, 2007). The anti-biofilm formation at sub-MIC levels (1/2, 1/4, and 1/8) was further analysed by ANOVA. Based on the obtained results, it can be proposed that there are two different trends regarding the sub-MIC levels.

Conclusion

The antibiofilm activities of sub-MIC levels of *S. aromaticum* and *L. angustifolia* essential oils are promising. However, the results for *N. sativa* oils contradict the findings of previous studies. The discrepancies between the findings of the present study and those within the published literature could be attributed to many factors including differences in the strains used, chemical compositions, concentration, and quality of essential oils used, time of exposure to the essential oils, the growth environment of the biofilm and the media used to grow the biofilm. The enhancement (increase in biofilm formation) achieved with *N. sativa* essential oil may need to be re-examined in a further study that investigates in more depth the mechanism in which the chemical components act as inhibitors or stimulators and identifies the factors that may play a role in such action. This study also reinforces previous suggestions about the necessity for standardized *in-vitro* methods for determining the influence of essential oils on biofilm formation and provides some exciting insights concerning the limitations as well as the effectiveness of the essential oils used in the research.

It is important to bear in mind that there are certain conditions in which the oils need to have when stored such as using airtight containers and ensuring that the oils are not exposed to light; otherwise, their effectiveness will be reduced. Bacteria are known to be capable of rapidly adapting to the chemicals and thus creating a problem of resistance (as with the antibiotic problem). Therefore, to circumvent any adaptation by bacteria that may occur, it is extremely important to apply the oils in concentrations high enough to inhibit (bacteriostatic) or to kill (bactericidal) target bacteria. It is also important that care is taken not to cause any other problems (e.g. formulation difficulties, undesirable side effects, or limitations in the effectiveness of particular essential oil.

The findings of the present study can stand as a starting point for future research, which might be needed particularly concerning *N. sativa* oil. One of the most important factors that should be emphasized by future research is how the findings can collaborate in the improvement of medicine, and thus create a platform for new products that can fight diseases caused by the bacterial biofilms of Gram-negative and Gram-positive bacteria. The study recommends conducting more in-depth studies to further investigate

the validation of the study results, particularly relating to *N. sativa* essential oil and to gain further insight into the mode of action when applied against different types of bacteria. This study also suggests conducting further research on the effects of essential oils on the internal biofilm by applying them in-vivo to understand how essential oils can be applied to internal areas such as artificial heart valves, or artificial joints, and what the most suitable concentrations are to prevent biofilm formation in such areas.

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