

Effect of *Mentha spicata* volatile oil on some virulence factors of *Pseudomonas aeruginosa* isolated from clinical samples

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Abstract

Mentha spicata volatile oil (Peppermint oil) has been used in traditional medicine because of its therapeutic value, which has antibacterial, antiviral and antioxidant activities resulting from its active compounds. *Pseudomonas aeruginosa* has several virulence factors including protease, hemolysin and pyocyanin which are important. The ability of isolates to produce protease, hemolysin and pyocyanin before and after treatment with volatile oil was investigated. The use of combinations of peppermint oil and antibiotics by using checkerboard assay is thus new approach to enhance the efficacy of its antimicrobial activity. Therefore, the Minimum Inhibitory Concentrations (MIC) of peppermint oil and antibiotics and Fractional Inhibitory Concentration Index (FICI) of their combination were determined for *P. aeruginosa*.

The volatile oil of these extracts exhibited markedly antibacterial activity and the results showed decrease in the protease activity, hemolysin activity and production of pyocyanin. The MICs of all the antibiotics ranged between 62.5 and 250 µg/ml. Peppermint oil exhibited synergistic effect when in combination with amoxicillin, ampicillin and tetracycline, while additive effect in two instances when combined with cefotaxime and nalidixic acid.

Keywords: Peppermint oil, antibiotics, virulence factors, *P. aeruginosa*, checkerboard.

Introduction

Pseudomonas aeruginosa is gram negative, facultative anaerobic rods, non-fermentative, non-sporulation, motile by one polar flagellum and the most important and opportunistic pathogen¹³ that causes a high rate of mortality and morbidity in hospitalized patients with compromised immune systems⁵⁵ and it is frequently resistant to commonly used antibiotics and disinfectants²⁸. In recent years, infections caused by this bacterium are one of the major problems in hospitals and are related to high rates of mortality which range from 18% to 61%³⁴.

The infections of this bacteria are associated with large number of virulence genes and their products are either accumulated inside the cell or released outside to growth

media. Some of these virulence factors: pili, flagella, endotoxin, exotoxins, exoenzymes, pyocyanin, hemolysin and proteases [elastase (*lasB*), protease iv (*piv*) and alkaline protease (*aprA*)] are tightly regulated by cell-to-cell signalling systems²³. Proteases are histotoxic and facilitate invasion of the organism into the bloodstream. Pyocyanin damages the cilia and mucosal cells of the respiratory tract²⁹. Hemolysin has necrotizing effect on red blood cells²¹ and has the ability to lysis lipids⁴².

The volatile oils (also known as essential oils) are usually liquid with an oily consistency, unusually colored or clear, complex and its compounds are volatile, characterized by a strong smell and synthesized by aromatic plants during secondary metabolism⁴³. These compounds have a wide spectrum of pharmacological activities⁴¹. Volatile oils have been reported to possess significant antibacterial, antioxidant and antiviral activities⁷.

Mentha spicata L. is a plant belonging to the Lamiaceae family. *M. spicata* is widely used for various purposes- in the food industry, cosmetic, industrial and in medicine due to its odor, flavoring and therapeutic properties⁴³. *M. spicata* is known to have antibacterial, antifungal and antioxidant properties²⁶. Peppermint oil (*M. spicata* volatile oil) is a natural product widely used in various fields including cosmetics, perfumes, phototherapy, aromatherapy and spices and is considered one of the most commonly and widely used essential oils, maybe because of its main components menthone and menthol¹¹. Previous studies of peppermint oil have shown significant antibacterial, antiviral, antifungal²⁶, antibiofilm formation and antioxidant activities⁴³.

An increase in bacterial resistance to antibiotics and the lack of new antibiotics introduced into the market resulted in a need to find alternative strategies⁶ in order to increase or restore antimicrobial efficacy against multidrug-resistant bacteria. Addition of volatile oils to antibiotics can induce a reduction in the antimicrobial MIC⁴⁹.

In order to study whether *M. spicata* volatile oil and some antibiotics in combination produce a higher inhibition via synergistic, additive or an antagonistic interaction and enhancing antibiotics activities against *P. aeruginosa*, checkerboard experiments were performed⁴⁹.

In this study, we aim to determine whether peppermint oil has effects on *P. aeruginosa* virulence factors production

and target to test the effect combination of peppermint oil with some antibiotics on *P. aeruginosa* growth.

Material and Methods

Collection of bacterial samples: 135 clinical samples were collected from different hospitals in Baghdad city which were from various sources including burns, wounds, Keratitis, ear infections and cystic fibrosis. These samples were collected by sterile cotton swabs while urinary tract infections (UTI) samples were collected by sterile container from Ramadi Teaching Hospital.

Bacterial isolation and identification: Bacterial isolates were subjected to a number of cultural and biochemical tests for identification of these isolates according to diagram suggested by Collee et al.⁹

Virulence Phenotype Assays

Protease production: Protease activity was assayed by mixing 1.8 ml of casein substrate solution and 0.2 ml of supernatants from overnight cultures of bacterial growth and incubated in water bath at 37°C for 20 min. The reaction was stopped by the addition 3 ml of 5% trichloroacetic acid (TCA) and then centrifuged at 2500 rpm for 20 min. The control was prepared using the same steps except the addition of TCA reagent before supernatants, then absorbance was measured at 275 nm⁵²:

$$\text{Enzyme activity (U/ml)} = \frac{\text{Absorbance at 275 nm}}{(0.001)(0.2)(20)}$$

Pyocyanin Production: 3 ml from the bacterial suspensions were filtered by 0.20 µm pore size. Then absorbance was measured at 400 nm in a Spectrophotometer. The results were calculated according to following⁵³:

Pyocyanin activity (U/ml) = Absorption of the sample test - Absorption of control (broth only).

Hemolysin production: To serial dilutions of the bacterial suspension in 1 ml of sodium phosphate buffer, add 80 µl of 5% RBC and incubate at 37°C for 3 h. Then the results can be read as following⁵³:

$$\text{Enzyme activity (titer)} = \frac{1}{\text{Higher dilution giving complete hemolysis}}$$

Extraction of Volatile oil: Volatile oil was isolated by steam distillation by using a Clevenger-type apparatus. 100 g of leaves were put in 500 ml of distilled water in 1000 ml round flask, of which the lower and higher parts were connected to a heating mantle and a condenser respectively. The extraction process was carried out for 2.5 h and the water vapour produced in the flask crosses the plant charged with volatile oil and then to the condenser, where it is

condensed. After condensation, the oil is separated from water by decantation¹.

Determination of Minimum Inhibitor Concentration

(MIC): The minimum inhibitory concentration (MIC) of the volatile oil and antibiotics was estimated by Resazurin Microtitre-plate Assay (REMA). 100 µl of Mueller-Hinton Broth (MHB) was added to the all wells of microtitre-plates, then 100 µl of (1%) volatile oil were added to the first row, thus 100 µl transferring to next wells to get a serially concentration from 1/2 to 1/256. Then 10 µl of bacterial suspension containing 1×10⁸ CFU/ml was added to each well and incubated for 18-24 h at 35±2°C.²⁰ After incubation period, 10 µl of resazurin solution (Alamar blue) was added to each well and the plate was re-incubated for 24 h. Any color change observed from purple to pink was taken as positive (growth), while the lowest concentration remains blue representing the MIC⁵⁰.

The effect of volatile oil on some virulence factors: 100 µl of volatile oil was added to tubes which contain 10 ml of nutrient broth + 100 µl of the inoculum containing 1×10⁸ CFU/ml of bacterial growth, then incubated for 24 h at 37°C. After incubating period, these cultural growths were used for detection of some virulence factors in the same methods as previously mentioned¹⁰.

The effect of the combination of volatile oil and some antibiotics on bacterial growth: The possible presence of synergy interaction between the *M. spicata* volatile oil and five antibiotic agents: amoxicillin, ampicillin, cefotaxime, tetracycline and nalidixic acid was tested by the checkerboard method in 96 well microplates²⁷.

100 µl of MICs antibiotics solutions were added to the first column, thus 100 µl transferring to next wells (horizontally). In the other microplate, 100 µl of (1%) volatile oil was added to the first row and 100 µl transferring to next wells (vertically) to get serial concentrations (1/2 to 1/256). 10 µl of bacterial suspension (1×10⁸ CFU/ml) were added to each well and incubated for 24 h at 37°C. After that 10 µl of Alamar blue was added to each well and incubate again for 24 h. The well where no colour change was considered the MIC value of the two antimicrobials in combination^{8,22,47}.

The Fractional Inhibitory Concentrations (FICs) were calculated from the MIC of the volatile oil or the antibiotic in the combination divided by the MIC of the volatile oil or the antibiotic alone and the FIC index was obtained by the sum of individual FICs. When FIC indices were ≤ 0.5, the results were interpreted as synergistic, as additive or indifferent when values were between 0.5 and 4.0 and as antagonistic when values were > 4.

Synergism is observed when the effect of the combined substances (antimicrobial agents) is greater than the sum of the individual effects. An additive combination of two drugs

produces the same effect as the combination (the equally effective concentrations) when the agents are used alone^{22,47}.

Results and Discussion

The capability of 10 isolates of *P. aeruginosa* to the production of some virulence factors in the nutrient broth was investigated. Where the susceptibility of the studied isolates varied in their productivity of protease, some isolates were showing high activity in protease production, while others were moderate in their activity, where protease activity was ranging from 51.7 to 236 U/ml (table 1). The results were compatible with Saleem⁴⁴ who found that 100% of isolates were producing of protease, while Younis et al⁵⁴ found that only (34%) of *P. aeruginosa* isolates were positive to protease.

This variation in activity might have been due to the diversity in the sources from which the bacteria were isolated, which was from different clinical sources, this indicates presence of a genetic diversity among the isolates²⁴, so it may not exhibit all isolates. Ahmed and Ghafel² attributed high activity of protease production to genes activity which is responsible for enzyme coding.

There was also a difference in the susceptibility of isolates to the production of hemolysin and pyocyanin, which ranged in intensity among high, medium and weak (tables 2 and 3), this result is in agreement with Al-Musaw³ and Shiny et al⁴⁶ who found that 100% and 93.3% respectively of isolates produced hemolysin and Olewi³⁷ observed that most isolates have the ability to produce pyocyanin but in varying degrees and some of them cannot produce it.

This difference in hemolysin activity was due to factors affecting in its production including pH, temperature, number of bacteria and incubation period³¹. Özcan and Kahrman³⁹ said that the agitation rate and temperature are two leading factors as crucial for the cell growth and pyocyanin formation, as well as the differences in nutrient needs and location of infection leads to this variability. The variation of these isolates in the production of pyocyanin is due to antimicrobial and helps *P. aeruginosa* to compete with other bacterial species and stay in their habitat³⁸.

Effect of Peppermint Oil on *P. aeruginosa* Growth: The MICs values were calculated using the Resazurin Microtitre-plate Assay (REMA). The results showed a difference in the values of the MICs with differences in bacterial isolates as shown in table 4. Figure 1 shows the MIC values of the volatile oil; blue color represented inhibition of bacterial growth, while the red color indicates the growth of bacteria¹⁸.

From the previous table, we conclude that peppermint oil has a good effect on *P. aeruginosa* isolates. And that the ability of the volatile oils in inhibiting the growth of isolates is due to the presence of the phenolic compounds which acts to disrupting of the cytoplasmic membrane, disrupting the

proton motive force, flow of electrons, active transport and coagulation of contents of the cell¹⁵. The effectiveness of peppermint oil against bacteria was due to it contains active compounds such as menthol and menthon characterized by good effectiveness as an antibacterial³³.

Our results were in agreement with those obtained by Soković et al⁴⁸ who confirmed that essential oil from *M. spicata* possessed a high antibacterial effect. Andrade et al⁴ failed to determine the MIC value of peppermint oil against *P. aeruginosa* and Kizil et al²⁶ reported that the volatile oil of *M. spicata* had no antimicrobial activity against *P. aeruginosa*.

Ignacimuthu et al¹⁹ mentioned that the effectiveness of volatile oils may be due to its hydrophobicity characteristics, thus assist it to pass through the cell membrane and enables it to the division of bacterial cell membrane and mitochondria, which make the membrane more permeable and thus lead to leaking the ions and molecules from the cell causing its death.

Effect the volatile oil on some virulence factors: After determining the MIC value for peppermint oil and observing their effect on *P. aeruginosa* growth, this volatile oil was used in inhibiting the production of some virulence factors of *P. aeruginosa*, the volatile oil has been shown to have an effect on the production of virulence factors by reducing or stopping production of it.

The peppermint oil was a good effect on this virulence factor. Protease and pyocyanin production was significantly inhibited. Decrease in the production of hemolysin was also observed. As shown in table 5, also hemolysin and pyocyanin production reduced after adding the *M. spicata* volatile oil (tables 6 and 7).

This is due to the presence of the active compounds in the volatile oil which inhibits the production of these virulence factors and the most important compounds are Menthol and Menthon. Active compounds act to prevent the synthesis of proteins and carbohydrates and decrease in the production of biofilm and Alginate³³. The addition of essential oils acts to inhibit the production of enzymes including protease and decreased toxins producing such as hemolysin lead to the clotting of the contents of the cell and its death⁵.

Mezaal³³ noted that the effect of peppermint oil was significant on protease and hemolysin. Also, Khan et al²⁵ show inhibition of pigment production detected in peppermint oil. Quorum Sensing (QS) contributes to the regulation and production of virulence factors of *P. aeruginosa*. Therefore, the effect of peppermint oil on the QS leads to reduce the production of the virulence factors¹².

Effect of the combination of volatile oil and some antibiotics on bacterial growth: In order to study whether *M. spicata* volatile oil and some antibiotics in combination produce a higher inhibition via synergistic, additive or an

antagonistic interaction, checkerboard experiments were performed.

Checkerboard assays of *P. aeruginosa* gave only additive and synergistic profiles (table 8 and figs. 2-a and b) when peppermint oil was combined with selected antibiotics at tested concentrations. Synergistic effects were observed with three pairs of the five combination against *P. aeruginosa*. The MIC values for the peppermint oil and antibiotics and the FIC indices values for their combinations are shown in table 8.

Peppermint oil with Amoxicillin and Tetracycline showed synergistic effects against *P. aeruginosa* with FICI value 0.375 for both. Results for the combination with Ampicillin showed a FIC index of 0.5 also classified as synergy interaction. In contrast, the combination with Cefotaxime and Nalidixic acid was less effective and more likely to have additive effect than synergistic with FICI value reach 0.75 and 1 respectively. Veras et al⁵¹ noted there are synergy effects against *P. aeruginosa* from combination between *M. spicata* volatile oil and Neomycin, whereas Schelz et al⁴⁵ showed that the peppermint oil has a synergic effect against *E. coli* when coupled with Oxytetracycline and additive effect when combined with Ampicillin, with FICI values 0.5 and 1.0 respectively.

In the checkerboard assay, synergy interaction is based on the increased susceptibility of the tested microbe to the presence of those antimicrobial agents which is reflected by changes in the MICs values¹⁴.

Exploitation of volatile oils in preventing bacterial resistance is believed to be more promising because volatile oils are multicomponent in nature compared to many conventional antimicrobials that only have a single target site⁴⁰. The phytoconstituents, such as Menthon and Menthol present in peppermint oil, may interact with some antibiotics to enhance its mechanisms of action at the target positions for which the antibiotic was prepared, so this may enhance the antibacterial properties for both agents³⁶.

On the other hand, the cell membrane, is the primary target for a variety of antibacterial agents. The inhibitory effects of protective enzymes were blocked by those compounds¹⁷. Thus, the peppermint oil alone or in combination with other antimicrobial agents may provide a promising new scheme in phytotherapy for bacterial infections³⁵. In general, volatile oils act to inhibit the growth of bacterial cells and also inhibit the efflux pump and the production of toxic bacterial metabolites which facilitate the access of antibiotics to its active sites in the bacterial cell¹⁴.

Table 1
Protease activity (U/ml) of *P. aeruginosa* isolates

S.N.	Source of sample	Protease activity (U/ml)	S.N.	Source of sample	Protease activity (U/ml)
P1	Burn	168.5	P6	Cystic fibrosis	176
P2	Burn	236	P7	Ear	51.7
P3	Wound	177	P8	Keratitis	67
P4	Wound	123	P9	Urine	171
P5	Cystic fibrosis	193	P10	Urine	188

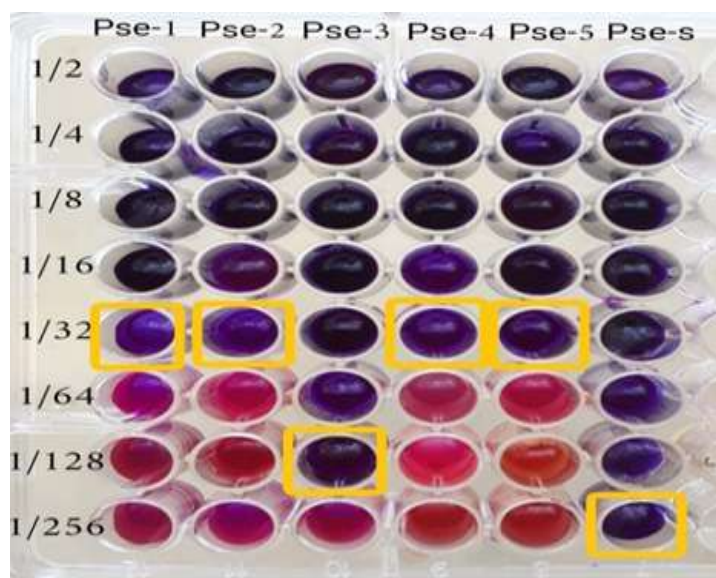
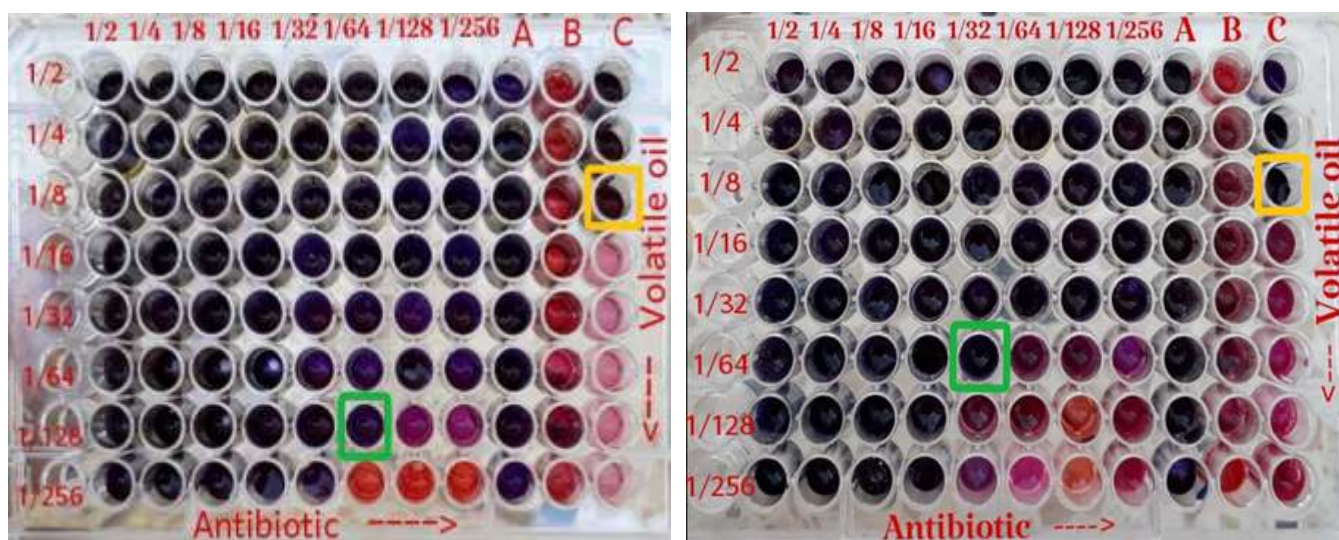


Figure 1: Effect of *M. spicata* volatile oil on *P.aeruginosa* growth where the numbers on the left show the volatile oil concentrations from 1/2 to 1/256 and the numbers at the top indicate to the number of an isolate. *Pes-s*: mean standard isolate. The yellow squares indicates to the MICs.



a: *M. spicata* with Amoxicillin.

b: *M. spicata* with Cefotaxime.

Figure 2: A representative trial of the checkerboard assay for the antimicrobial combination of *M. spicata* volatile oil + Antibiotic. The numbers on the left show the volatile oil concentrations, and the numbers at the top indicate to the antibiotics concentrations, which diluted from 1/2 to 1/256. The wells represented by yellow square indicate the point at which the MIC of antibiotics. The wells represented by the green square indicate the point at which the MIC of both antimicrobials intersect. A, B and C letters are the control wells, A) only media, B) media + bacterial growth, C) Antibiotic + bacterial growth.

Table 2
Hemolysin activity (Titer) of *P. aeruginosa* isolates

S.N.	Source of sample	Hemolysin Activity (Titer)	S.N.	Source of sample	Hemolysin Activity (Titer)
P1	Burn	32	P6	Cystic fibrosis	16
P2	Burn	64	P7	Ear	16
P3	Wound	32	P8	Keratitis	32
P4	Wound	64	P9	Urine	4
P5	Cystic fibrosis	32	P10	Urine	8

Table 3
Pyocyanin production by *P. aeruginosa* isolates

S.N.	Source of sample	Pyocyanin activity (U/ml)	S.N.	Source of sample	Pyocyanin activity (U/ml)
P1	Burn	0.61	P6	Cystic fibrosis	0.28
P2	Burn	0.87	P7	Ear	0.25
P3	Wound	0.91	P8	Keratitis	0.22
P4	Wound	0.82	P9	Urine	0.45
P5	Cystic fibrosis	0.74	P10	Urine	0.51

Table 4
The MICs values of *M. spicata* volatile oil for *P. aeruginosa*

S.N.	Minimum Inhibitor Concentration (MIC) (Titer)
Pse-1	32
Pse-2	32
Pse-3	128
Pse-4	32
Pse-5	32
Pse-s	256

Table 5
Effect of *M. spicata* volatile oil on protease activity of *P. aeruginosa*

S.N.	Source of Sample	Protease activity before add volatile oil (U/ml)	Protease activity after add volatile oil (U/ml)
P-1	Burn	236	33
P-2	Burn	104	20
P-3	Wound	177	38
P-4	Wound	231	40
P-5	Cystic fibrosis	193	19
P-6	Cystic fibrosis	176	15.75
P-7	Ear	51.7	7
P-8	Keratitis	29	9
P-9	Urine	171	41
P-10	Urine	188	63

Table 6
Effect of *M. spicata* volatile oil on Hemolysin activity of *P. aeruginosa*

S.N.	Source of Sample	Hemolysin activity before add volatile oil (U/ml) (Titer)	Hemolysin activity after add volatile oil (U/ml) (Titer)
P-1	Burn	64	128
P-2	Burn	16	64
P-3	Wound	0	8
P-4	Wound	4	16
P-5	Cystic fibrosis	8	16
P-6	Cystic fibrosis	16	32
P-7	Ear	16	32
P-8	Keratitis	8	32
P-9	Urine	4	16
P-10	Urine	0	8

Table 7
Effect of *M. spicata* volatile oil on pyocyanin production of *P. aeruginosa*

S.N.	Source of Sample	Pyocyanin activity before add volatile oil (U/ml)	Pyocyanin activity after add volatile oil (U/ml)
P-1	Burn	0.24	0.11
P-2	Burn	0.58	0.18
P-3	Wound	0.91	0.33
P-4	Wound	0.59	0.14
P-5	Cystic fibrosis	0.74	0.26
P-6	Cystic fibrosis	0.21	0.104
P-7	Ear	0.18	0.76
P-8	Keratitis	0.22	0.1
P-9	Urine	0.38	0.127
P-10	Urine	0.51	0.19

Table 8
Effect of the combination between peppermint oil and antibiotics on *P. aeruginosa* growth

Antimicrobial	MIC ($\mu\text{g/ml}$) by REMA	Combined Antimicrobials	Combined Antimicrobial MIC ($\mu\text{g/ml}$)	FICI (ΣFICI)	Interaction
Amoxicillin	62.5	<i>M. spicata</i> + Amoxicillin	0.78125 + 7.8125	0.375	Synergistic
Cefotaxime	62.5	<i>M. spicata</i> + Cefotaxime	1.5625 + 15.625	0.75	Additive
Ampicillin	125	<i>M. spicata</i> + Ampicillin	0.78125 + 31.25	0.5	Synergistic
Tetracycline	62.5	<i>M. spicata</i> + Tetracycline	0.78125 + 7.8125	0.375	Synergistic
Nalidixic acid	250	<i>M. spicata</i> + Nalidixic acid	1.5625 + 125	1	Additive
<i>M. spicata</i> volatile oil	32 titer	-	-	-	-

Conclusion

1. We conclude that the peppermint oil has good antimicrobial activity against *P. aeruginosa*, by inhibiting or decreased production.

2. The combination between peppermint oil and some antibiotics results in a synergistic effect against *P. aeruginosa*.

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