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Effect of *Mentha Spicata* and *Rosmarinus Officinales* Volatile Oils on Proteases Activity of *Pseudomonas Aeruginosa*

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Abstract: Evaluation the ability of volatile oils to inhibit protease activity and some virulence factors of *Pseudomonas aeruginosa* isolated from different clinical sources and studying the prevalence of proteases gene among isolates. Investigate the effects of volatile oils in combination with antibiotics to treat the infections of *P. aeruginosa*. PCR assay was used to detection proteases genes. Protease, pyocyanin and hemolysin activity before and after treated with volatile oils was estimated. Checkerboard assay was used to determine the combined effects of volatile oils and antibiotics in *P. aeruginosa*. About 50 clinical isolates of *P. aeruginosa* were identified using genetic test and phenotypic test. Antibiotic susceptibility test showed high resistance against nalidixic acid, tetracycline, ampicillin, cefotaxime, amoxicillin, moderate resistance against ceftazidime, amikacin, vancomycin, augmentin, chloramphenicol, gentamicin while recorded low resistance towards imipenem. Detection of protease genes revealed that 96% of isolates were harbor lasB gene while 90% were positive for aprA and TC gene was found in 86% of isolate. The isolates showed variability in their activity of protease which ranged between 51.7- 236 U/mL. Those isolates showed differences in their ability to hemolysin production where ranged between 0-64. The results showed variance in the pyocyanin production, ranged between 0.08-0.91 at 400 nm. The volatile oils exhibited markedly antibacterial activity where showed decreased in the protease, hemolysin, pyocyanin activity. *Mentha spicata* volatile oil exhibited synergistic effect when combined with amoxicillin, ampicillin, tetracycline and additive effect with cefotaxime nalidixic acid. *Rosmarinus officinales* volatile oil showed synergistic effect with amoxicillin, tetracycline and additive effect with cefotaxime, ampicillin, nalidixic acid. We conclude that the volatile oils showed high efficiency in inhibiting the production of protease and other virulence factors. Volatile oils exhibited synergistic effect when combined with antibiotic to treat the infections resulted from drug-resistant bacteria.

Key words: *P. aeruginosa*, protease, volatile oils, synergistic effect, amoxicillin, chloramphenicol

INTRODUCTION

Pseudomonas aeruginosa is recognized as the causative agent of a remarkable spectrum of diseases in both healthy and immune-compromised patients including pulmonary infections, ear infections, burn-wound infections, folliculitis as well as urinary tract infections (Sara, 2012), pathogenicity noted for its high intrinsic resistance to antibiotics and for its ability to cause a wide spectrum of opportunistic infections (Brooks *et al*., 2007). It have many virulence factors to colonize the cells of its host, for example, adheres to tissue surfaces using its flagellum, pili and exoenzyme then it replicates to create infectious critical mass and lastly it damages tissue using its virulence factors (Pyocyanin, Protease, Hemolysin and Exotoxin A) (Filho *et al*., 2007).

P. aeruginosa produces three types of protease, alkaline protease, protease 4 and elastase which encoded by specific genes and regulates their expression in association with some other virulence factors this regulation is controlled it by quorum sensing (Seo and Darwin, 2013) these proteases are important extracellular virulence factor is shown to play an important role in the pathogenicity of *P. aeruginosa* during host infection (Yu *et al*., 2014). Proteases are enzymes that can hydrolyze peptide bonds within peptides and proteins and it is histotoxic and facilitates an invasion of the organism into the blood stream (Levinson, 2010).

Many research and studies have become uses of medicinal plants including volatile oils in the treatment of microbial infections caused by microbes that resistance to antibiotics (Wallace *et al*., 2002). The volatile oils (also

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known as essential oils) are generally liquid with an oily consistency, complex and the present compounds are volatile, synthesized by aromatic plants during secondary metabolism. These compounds have a wide spectrum of pharmacological efficiency (Saharkhiz *et al*., 2012). Volatile oils have been notified to possess a significant antibacterial, antiviral and antioxidant activities (Bassole and Juliani, 2012).

MATERIALS AND METHODS

Bacterial isolates: About 135 samples were obtained from some hospitals in Baghdad and Ramadi City which were from various sources including burns, wounds infections, keratitis, cystic fibrosis, ear infections and urinary tract infections. The identification was accomplished by cultural and biochemical tests following schematic diagram for identification suggested by Collee *et al*. (1996).

DNA extraction: Genomic DNA of bacterial isolates was extracted by using mini kits extraction, product by Geneaid company, Korea.

Identification of *P. aeruginosa* using 16SrRNA: To detect 16SrRNA gene of *P. aeruginosa* isolates the following primer was used: F (GGGGGAT CTTCGGACCTCA) R (TCCTTAGAGTGCCCACCCG) (Stover *et al*., 2000) and using the following thermal cycling conditions: an initial denaturation step (3 min at 95°C) followed by 35 cycles of amplification (denaturation for 45 sec at 94°C, annealing for 45 sec at 58°C and extension for 1.5 min at 72°C), finally, the final extension step for 7 min at 72°C.

PCR assay: Detection of lasB, aprA and TC genea (encoding protease enzyme) was performed by PCR with the specific primers listed in Table 1 (Stover *et al*., 2000; Winsor *et al*., 2008). The thermal cycling conditions for lasB amplification included an initial denaturation step (5 min at 95°C) followed by 35 cycles of amplification (denaturation for 30 sec at 95°C, annealing for 30 sec at 55°C, extension for 30 sec at 72°C) and by 1 cycle of final extension for 7 min at 72°C. The conditions for aprA amplification: initial denaturation (5 min at 95°C) followed by 30 cycles of amplification (denaturation for 30 sec at 95°C, annealing for 1 min at 65°C, extension for 2 min at 72°C) and by 1 cycle of final extension for 7 min at 72°C. The conditions for TC amplification, initial denaturation (5 min at 94°C) followed by 34 cycles of amplification (denaturation for 30 sec at 94°C, annealing for 30 sec at 60°C, extension for 2 min at 72°C) and 1 cycle of final extension for 7 min at 72°C.

Antibiotics susceptibility test: Kirby Bayer method was used to examine antibiotic sensitivity by discs-diffusion on Mueller-Hinton agar. Augmentin, amikacin, amoxicillin, ampicillin, cefotaxime, ceftazidime, chloramphenicol, imipenem, gentamicin, nalidixic acid, tetracycline and vancomycin were used. The results were compared with standard inhibition diameters of CLSI (CLSI, 2016).

Protease activity: It was determined by mixed 0.2 mL of supernatants of bacterial growth with 1.8 mL of casein substrate solution then incubated in a water bath at 37°C for 20 min. The reaction stopped by added 3 mL of 5% Trichloroacetic acid, after that centrifuged at 2500 rpm and measured at 275 nm (Whitaker, 1972).

Hemolysin activity: It was assayed by doingserial dilutions of the bacterial growth in 1 mL of sodium phosphate. After that add 80 μL of 5% RBC then incubated for 3 h at 37°C (Wilson *et al*., 1987).

Pyocyanin production: About 3 mL of the bacterial growth were filtered (by 0.20 μm). Then the absorbance was measured in a spectrophotometer at 400 nm (Wilson *et al*., 1987).

Extraction of *M. spicata* and *R. officinales* volatile oils: It was accomplished by steam distillation using clevenger apparatus. The 500 mL of distilled water were put with 100 g of leaves in a clevenger flask which the lower part was linked to a heat source and the higher part to a condenser. The procedure was completed for 2.5 h then the water vapor generated in the flask pass through the

Table 1: Sequences of PCR primers that use to detection 16SrRNA gene and proteases genes

Genes	Sequences (5'-3')	Temperature (°C)	Product size (bp)	References
aprA				
F	GTCGACCAGGCGGCGGAGCAGAATA	69.5	993	Stover *et al*. (2000)
R	GCCGAGGCCGCCGTAGAGGATGTC	71.3		
TC				
F	TATTTCGCCGACTCCCTGTA	57.3	752	Stover *et al*. (2000)
R	GAATAGACGCCGCTGAAATC	57.3		
lasB				
F	TTCTACCCGAAGGACTGATAC	57.9	153	Winsor *et al*. (2008)
R	AACACCCATGATCGCAAC	53.7		

leaves loaded with volatile oil to the condenser where it is condensed. After that, the oil separated by decantation (Lawrence and Reynolds, 1986).

Identification of active compounds of volatile oils: Oil compounds were identified using Gas Chromatography-Mass Spectrometry (GC-MS) by use Shemaza (2010) Qpplus.

Determination of MIC: It was estimated by using two fold dilutions by Resazurin Microtiter-plate Assay (REMA) as described by Janssen *et al*. (1989).

Effect of volatile oil on some virulence factors: About 100 μL of each volatile oil was added to 10 mL of nutrient broth with 100 μL of the inoculum (1×108 CFU/mL) and incubated at 37°C for 24 h. After that we used this cultural growth to an estimation of some virulence factors.

Checkerboard test: The probable existence of synergy effect between the volatile oils and some antibiotics: ampicillin, amoxicillin, tetracycline, cefotaxime and nalidixic acid was tested by the checkerboard technique as described by Langeveld *et al*. (2014) and Mutambuze (2014).

RESULTS AND DISCUSSION

The results of isolation and identification showed getting 50 isolates were belonged to *P. aeruginosa* which distributed to 15 isolates from burns, 12 isolates from wounds, 6 isolates from Cystic Fibrosis (CF), 7 isolates from ear infection, 5 isolates from keratitis and 5 isolates from urine as shown in Table 2.

Table 2: Prevalence and percentage of *P. aeruginosa* isolates

Types of specimen	No. of samples	No. of isolates	Percentage
Burn	40	15	37.50
Wound	31	12	38.70
CF	6	6	100.00
Ear	21	7	33.30
Keratitis	16	5	31.25
Urine	21	5	23.80
Total	135	50	

The results showed that burns isolates were more when compared with other sources where attained 15 isolates of *P. aeruginosa* in percentage 37.5% and this may be due to that these bacteria are of an opportunistic pathogen which opportunist occurs defect in one of the body's mechanical or immunological defenses or both to cause infection (Brooks *et al*., 2007).

Detection of 16SrRNA gene: The results of the gel electrophoresis for PCR products of the DNA sequence using the 16SrRNA primer which is a diagnostic gene for *P. aeruginosa*, showed bands with a molecular size of 956 bp for all isolates Fig. 1. This supports the results of the diagnostic by the phenotypic methods that these isolates belong to *P. aeruginosa* (Hamid *et al*., 2017).

Susceptibility to antibiotics: The present study showed high resistance towards ampicillinn (80%) and amoxicillin (74%) (Al-Marzoqi and Al Taee, 2013). The results showed that the percentage resistance this isolates for nalidixic acid was 96% (AL-Salihi *et al*., 2014). The results obtained show high resistance against cefotaxime and ceftazidime with a value of 78 and 70%, respectively (Kirecci and Kareem, 2014; Kahaleq *et al*., 2015). The results indicated that the resistance percentage to tetracycline was 82% (Ahmed, 2016). The results showed moderate resistance to amikacin and gentamycin reached 66 and 44%, respectively (Hassan, 2017; Naqvi *et al*., 2005). The results of resistance against imipenem were 8% (Kirecci and Kareem, 2014). The results showed that the resistance rate towards chloramphenicol was 46% (Hamid *et al*., 2017). Finally, the results showed 54% resistance to augmentin (Alsaimary *et al*., 2010) and showed 62% towards vancomycin (Ahmadi *et al*., 2016). The resistance of *P. aeruginosa* to those antibiotics is due to its possession of an arsenal of enzymes and virulence factors that enable them to resist these antibiotics such as •-lactamase, cephalosporinase, aminoglycoside-modifying enzymes, efflux pump systems and the other mechanisms (Lopez-Yeste *et al*., 1996; Breidenstein *et al*., 2011; Aghazadeh *et al*., 2014).

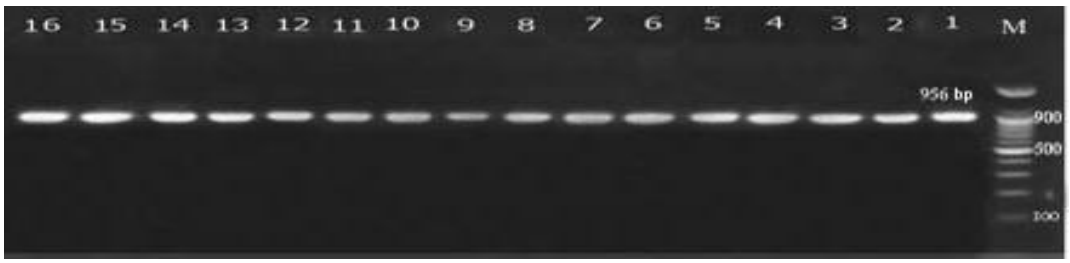

Fig. 1: Agarose gel electrophoresis (1.5% agarose, 7 V/cm^2 for 90 min) of 16SrRNA gene (956 bp). Lane M 1500 bp DNA ladder, lanes 1-16 represent bands of *P. aeruginosa* isolates

Detection of protease genes for *P. aeruginosa* isolates: lasB gene was screened by PCR technique for the all studied isolates by using specific primer. After that, the amplified products were carried on 1.5% agarose gel for 1.5 h. The results showed that 48/50 (96%) of isolates were possessed lasB gene (elastase B) where the bands appeared within the expected size of the gene (153 bp) for all positive isolates Fig. 2 (Benie *et al*., 2017). Whereas the alkaline protease encoded by the aprA gene was the second gene from proteases of *P. aeruginosa* detected by using a specific primer. Results of aprA gene prevalence revealed that 45/50 (90%) of the isolates were harbored this gene. Figure 3 shows the positive results for some isolates with amplified size approximately equal to (993 bp) (Ahmed, 2016). Finally, through PCR technique by using the specific primer (TC: 752 bp) and after electrophoresis of the outcome of PCR amplification, the bands of this gene (Protease 4) appeared in 43/50 isolates in a percentage 86% Fig. 4 (Al-Saa'edi *et al*., 2016). We also noted that all isolates possessed at least one gene of protease genes, although, some of these isolates didn't show any activity for the protease.

Analysis of volatile oils: An analysis of the volatile oils by GC-MC was conducted to determine the active compounds found in these oils. Figure 5, we observed that the compounds found in *M. spicata* volatile oil as flowing (Hawryl *et al*., 2015) (Table 3). In *R. officinales* volatile oil, we showed that the compounds which found were as folowing Table 4 (Takayama *et al*., 2016) (Fig. 6).

Determination of MIC: The results obtained showed a difference in the values of the Minimum Inhibitory Concentration (MIC) depends on the variability of bacterial isolate and volatile oil type. We chose the isolate No. 4 where its MIC value for *M. spicata* volatile oil was 32 while for *R. officinales* volatile oil was 8 as shown in Table 5, Fig. 7a and b.

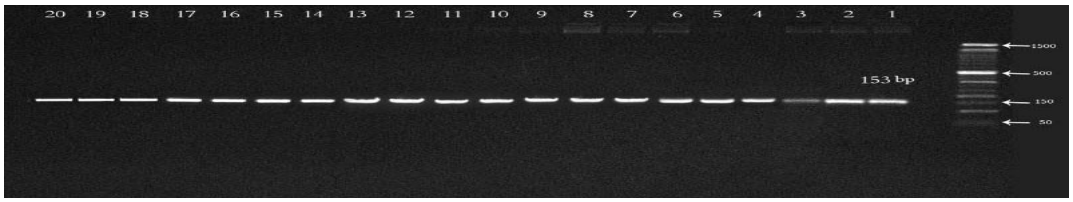

Fig. 2: Agarose gel electrophoresis (1.5% agarose, 7 V/cm^2 for 90 min) of lasB gene (153 bp). Lane M represents 1500 bp DNA ladder, lanes 1-20 represent bands of *P. aeruginosa* isolates

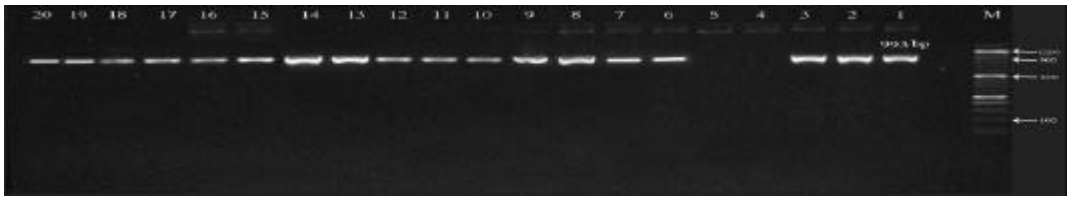

Fig. 3: Agarose gel electrophoresis (1.5% agarose, 7 V/cm^2 for 90 min) of aprA gene (993 bp). Lane M represents 1500 bp DNA ladder, lanes 1-20 represent bands of *P. aeruginosa* isolates

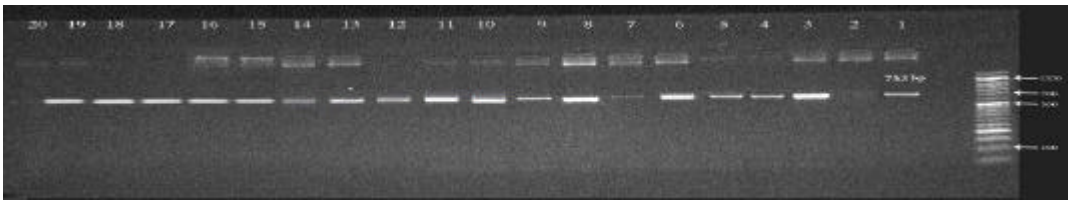

Fig. 4: Agarose gel electrophoresis (1.5% agarose, 7 V/cm^2 for 90 min) of TC gene (752 bp). Lane M represents 1500 bp DNA ladder, lanes 1-20 represent bands of *P. aeruginosa* isolates

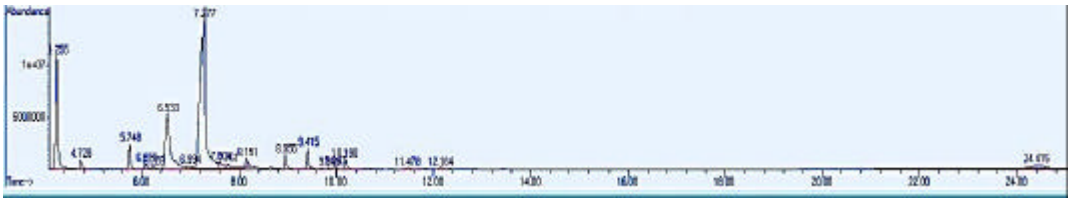

Fig. 5: GC-MS analysis of *Mentha spicata* volatile oil

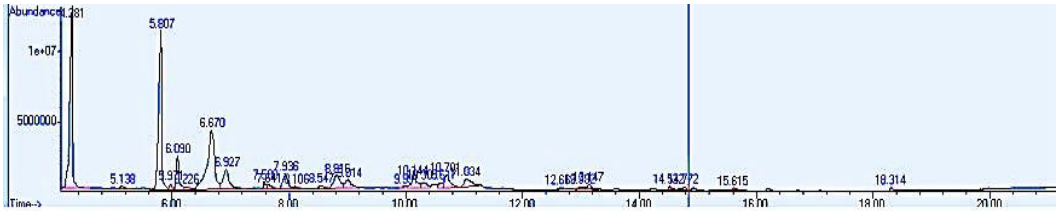

Fig. 6: GC-MS analysis of *Rosmarinus officinales* volatile oil

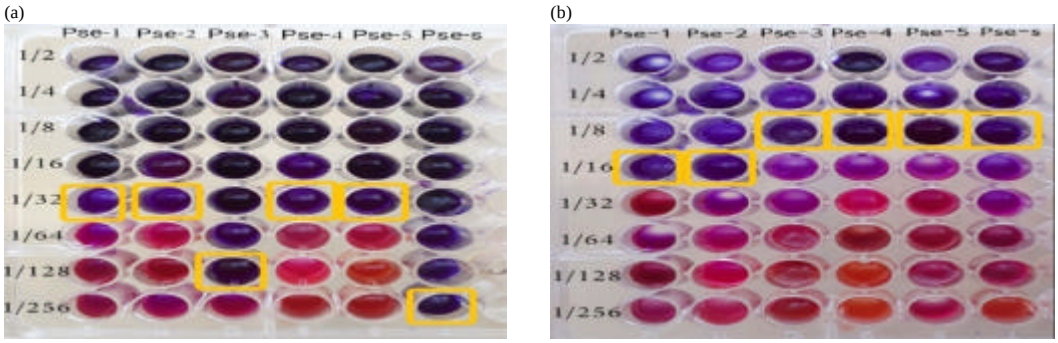

Fig. 7: Effect of volatile oils on *P. aeruginosa* growth. The numbers on the left, volatile oil concentrations. The numbers at the top, number of isolate. Pes-s: standard isolate. The yellow squares, the MICs: a) *M. spicata* volatile oil and b) *R. officinales* volatile oil

Table 3: Yield and chemical composition of *Mentha spicata* volatile oil

Pk#	Retention time	Constituents	Qual.
1	4.253	Limonene	97
2	4.727	a-terpinol	93
3	5.745	Camphor	97
4	6.076	Borneol	97
5	6.227	Terpinen-4-ol	87
6	6.536	Dihydrocarvone	89
7	6.995	Menthol	50
8	7.274	Carvone	96
9	7.606	Carvone	70
10	7.764	Carvone	60
11	8.148	Thujene	74
12	8.955	Bourbonene	94
13	9.414	Caryophyllene	99
14	9.851	Humulene	95
15	9.964	Cubebene	97
16	10.190	Germacrene D	99
17	11.479	Caryophyllene oxide	94
18	12.165	Gamma-Muurolene	90
19	24.417	Laurin	46

The ability of volatile oils in inhibiting the growth were due to phenolic compounds that disrupt the cytoplasmic membrane, disrupting the proton motive force, the flow of electrons, active transport and coagulation of cellular contents (Davidson *et al*., 2013) (Table 4 and 5).

Estimation of some virulence factors before and after treatment with volatile oils: The 20 isolates were selected for determination protease, hemolysin and pyocyanin activity. Before treated with volatile oils, protease activity of isolates were ranged between 0 and 236 U/mL (Shiny *et al*., 2016) whereas hemolysin activity was ranged between 0 and 64 where the results showed that 27/30 (90%) of isolates were producing hemolysin, Hamid *et al*. (2017) reported that the isolates were differ in

their ability to produce hemolysin. While the ability of isolates to produce pyocyanin was variable and a gradual in terms of quality and quantity where its absorbance ranged between 0.08 and 0.91 at 400 nm (Oleiwi, 2015).

Volatile oils possess important volatile compounds with diverse bioactivities including antimicrobial potential. The MICs of volatile oils were used in inhibiting the production of some virulence factors of *P. aeruginosa*. After adding the MICs, the volatile oils have been shown to have an effect on the production of these virulence factors that by reducing or stopping production it where these effect varies depending on the type of used oil and isolate. *M. spicata* volatile oil had a good effect on these virulence factors compared to *R. officinales* volatile oil. Protease and pyocyanin activities were notable inhibited as well as observed reduced in hemolysin production Table 6 in compared with virulence factors production before treatment with *M. spicata* volatile oil. This is due to the presence of active compounds in *M. spicata* volatile oil which acts to inhibit the production of these virulence factors (Mezaal, 2013).

When treated the isolates with *R. officinales* volatile oil, pyocyanin and protease production was influenced

Table 4: Yield and chemical composition of *Rosmarinus officinales* volatile oil

Pk#	Retention time	Constituents	Qual.
1	4.283	1,8-Cineol	97
2	5.142	Caryophyllene	42
3	5.805	Camphor	97
4	5.971	• -Pinene	76
5	6.091	Borneol	97
6	6.227	• -Bisabolene	95
7	6.671	Verbenone	42
8	6.928	Calamenene	91
9	7.583	Isobornyl acetate	97
10	7.644	• -Calacorene	96
11	7.937	1,Z-5,E-7-Dodecatriene	78
12	8.103	Jasmone	46
13	8.548	Caryophyllenyl alcohol	80
14	8.819	Caryophyllene oxide	60
15	9.015	Clovene	43
16	9.987	Caparratriene	64
17	10.145	• -Cadinene	83
18	10.303	tau.-Cadinol	96
19	10.620	• -Guaiene	91
20	10.703	Alloaromadendrene	91
21	11.034	Cadalene	98
22	12.670	Geranylgeraniol	25
23	12.994	Butane, 1-bromo-2-methyl-	43
24	13.144	4a(2H)-Naphthalenemethanol, octahydro-	38
25	14.523	Sclarene	89
26	14.772	Linalool	44
27	15.616	3-Octanone	42
28	18.314	Tricosane	97

Table 5: The MICs of volatile oils for the growth of *P. aeruginosa* isolates

	Minimum Inhibitor Concentration (MIC)	
No. of isolate	*R. officinales* volatile oil (Titer)	M. *spicata* volatile oil (Titer)
Pse-1	16	32
Pse-2	16	32
Pse-3	8	128
Pse-4	8	32
Pse-5	8	32
Pse-s	8	256

Table 6: Effect of M. *spicata* volatile oil on protease, hemolysin and pyocyanin activity of *P. aeruginosa* isolates

		Volatile oil of *Mentha spicata*					
		Protease activity (U/mL)		Hemolysin activity (Titer)		Pyocyanin production (absorbance at 400 nm)	
Isolates	Source of samples	Before add volatile oil	After add volatile oil	Before add volatile oil	After add volatile oil	Before add volatile oil	After add volatile oil
P-1	Burn	142.0	49.00	8	16	0.42	0.16
P-2	Burn	196.6	52.00	16	32	0.61	0.26
P-3	Burn	168.5	41.00	16	32	0.53	0.21
P-4	Burn	91.0	24.00	8	16	0.29	0.12
P-5	Burn	236.0	33.00	64	128	0.24	0.11
P-6	Wound	177.0	38.00	0	8	0.91	0.33
P-7	Wound	123.0	25.00	16	32	0.56	0.15
P-8	Wound	231.0	40.00	4	16	0.59	0.14
P-9	Wound	88.4	21.60	8	16	0.34	0.16
P-10	Wound	176.5	40.00	64	128	0.82	0.30
P-11	CF	165.0	34.75	32	64	0.66	0.28
P-12	CF	193.0	19.00	8	16	0.74	0.26
P-13	CF	63.0	13.50	0	16	0.28	0.11
P-14	Keratitis	0.0	0.00	2	16	0.08	0.01
P-15	Keratitis	29.0	9.00	8	32	0.22	0.10
P-16	Keratitis	191.4	47.00	16	32	0.35	0.18
P-17	Ear	0.0	0.00	2	8	0.11	0.06
P-18	Ear	51.7	7.00	16	32	0.76	0.30
P-19	Urine	172.0	39.50	8	32	0.45	0.25
P-20	Urine	123.6	27.00	4	16	0.33	0.21

Table 7:Effect of *R. officinales* volatile oil on protease, hemolysin and pyocyanin activity of *P. aeruginosa* isolates

		Volatile oil of *Rosmarinus officinales*					
		Protease activity (U/mL)		Hemolysin activity (Titer)		Pyocyanin production (absorbance at 400 nm)	
Isolates	Source of samples	Before add volatile oil	After add volatile oil	Before add volatile oil	After add volatile oil	Before add volatile oil	After add volatile oil
zP-1	Burn	142.0	109.20	8	16	0.42	0.230
P-2	Burn	196.6	145.50	16	16	0.61	0.320
P-3	Burn	168.5	112.00	16	32	0.53	0.290
P-4	Burn	91.0	56.00	8	16	0.29	0.130
P-5	Burn	236.0	149.00	64	64	0.24	0.180
P-6	Wound	177.0	122.00	0	4	0.91	0.405
P-7	Wound	123.0	86.60	16	32	0.56	0.300
P-8	Wound	231.0	156.00	4	8	0.59	0.209
P-9	Wound	88.4	50.75	8	16	0.34	0.150
P-10	Wound	176.5	92.60	64	64	0.82	0.380
P-11	CF	165.0	103.00	32	64	0.66	0.302
P-12	CF	193.0	105.75	8	8	0.74	0.230
P-13	CF	63.0	38.50	0	4	0.28	0.160
P-14	Keratitis	0.0	0.00	2	8	0.08	0.030
P-15	Keratitis	29.0	20.00	8	16	0.22	0.113
P-16	Keratitis	191.4	118.60	16	32	0.35	0.190
P-17	Ear	0.0	0.00	2	4	0.11	0.060
P-18	Ear	51.7	38.00	16	16	0.76	0.420
P-19	Urine	172.0	39.50	8	32	0.45	0.250
P-20	Urine	123.6	27.00	4	16	0.33	0.210

Table 8: Effect of the combination between volatile oils and antibiotics on *P. aeruginosa* growth

Antimicrobials	MIC (µg/mL)	Combined antimicrobials	Combined MIC (µg/mL)	FICI • FIC	Outcomes
Amoxicillin	62.5	*M. spicata*+Amoxicillin	0.78125 (128) titer+7.8125	0.375	Synergistic
Cefotaxime	62.5	*M. spicata*+Cefotaxime	1.5625 (64) titer+15.625	0.75	Additive
Ampicillin	125	*M. spicata*+Ampicillin	0.78125 (128) titer+31.25	0.5	Synergistic
Tetracycline	62.5	*M. spicata*+Tetracycline	0.78125 (128) titer+7.8125	0.375	Synergistic
Nalidixic acid	250	*M. spicata*+Nalidixic acid	1.5625 (64) titer+125	1	Additive
M. spicata volatile oil	3.125 (32) Titer	*R. officinales*+Amoxicillin	3.125 (32) titer+15.625	0.5	Synergistic
R. officinales volatile oil	12.5 (8) Titer	*R. officinales*+Cefotaxime	12.5 (8) titer+62.5	2	Additive
		R. officinales+Ampicillin	6.25 (16) titer+31.25	0.75	Additive
		R. officinales+Tetracycline	1.5625 (64) titer+7.8125	0.25	Synergistic
		R. officinales+Nalidixic acid	6.25 (16) titer+62.5	0.75	Additive

and decreased in most studied isolates while hemolysin production was relatively decreased excepted the isolates P-2, P-5, P-10, P-12 and P-18 which not affected Table 7. Khan *et al.* demonstrated that the inhibition of pigments production was detected in *M. spicata* essential oil while no effected on pigments inhibition was observed by *R. officinales* essential oil (Khan *et al.*, 2009). The work of essential oils lies in the destruction of the bacterial cell membrane and it is not reversible, it also stimulates of the loss the cytoplasm and cellular contents as well as the loss of energy molecules (glucose and ATP), leading to the decomposition of the bacterial cell. The addition of essential oils acts to inhibited the production of enzymes including protease and decreased toxins produce such as hemolysin, thus, leads to the clotting of the contents of the cell and its death (Bakkali *et al.*, 2008).

Quorum Sensening (QS) contributes to the regulation and production of virulence factors of *P. aeruginosa*. Therefore, the effect of volatile oils on the QS leads to reduce the production of the virulence factors (Faleiro, 2011).

Combined effects of volatile oils with antibiotics: In respect of study whether the volatile oils of *M. spicata* and *R. officinales* in combined with antibiotics produce a higher inhibition through synergistic, additive or an antagonistic effects, checkerboard technique was conducted.

Checkerboard tests resulted in synergistic and additive interactions only Table 8, Fig. 8a and b when the volatile oils were combined with selected antibiotics against *P. aeruginosa*. The MICs for the volatile oils and antibiotics and the FIC indices for their combinations are shown in Table 8.

M. spicata volatile oil with tetracycline and amoxicillin showed synergistic interactions with FICI value reached 0.375 for both. The FIC index value for combination with ampicillin was 0.5 which also classified as synergy effect. While the combination of *M. spicata* volatile oil with cefotaxime and nalidixic acid were more likely to consider additive effect than synergistic interaction with FICI values 0.75 and 1, respectively. *R. officinales* volatile oil shown synergistic

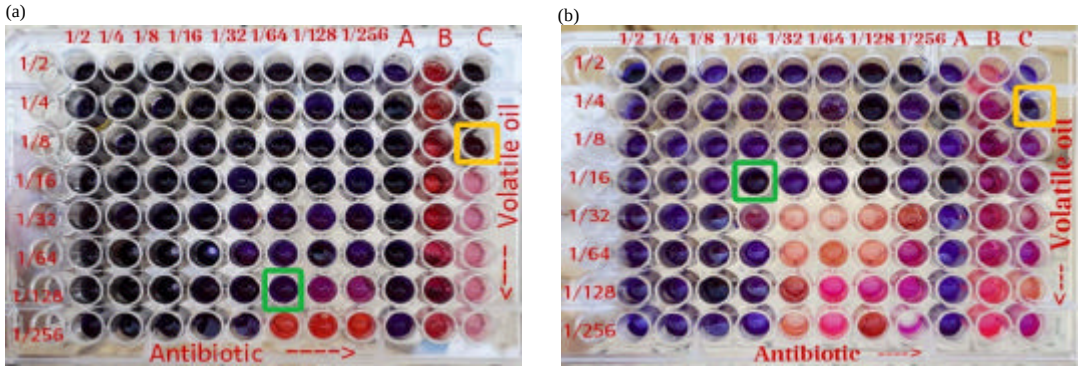

Fig. 8: Checkerboard assay for the antimicrobial combination of volatile oils+antibiotics. The numbers on the left: volatile oil concentrations. The numbers at the top: antibiotics concentrations. Yellow square: MIC of antibiotics. Green square: MIC of both antimicrobials intersect: A) only media, B) media+bacteria, C) Antibiotic+bacteria: a) *M. spicata* with amoxicillin and b) *R. officinales* with cefotaxime

effect when combined with tetracycline and amoxicillin with FICI value 0.25 and 0.5, respectively while when combined with cefotaxime, ampicillin and nalidixic acid gave additive effect against P. *aeruginosa* with FICIs reached 2 and 0.75, respectively. Veras *et al.* (2012) observed from the combination between *M. spicata* volatile oil and neomycin, synergistic effects against *P. aeruginosa* isolates whereas Van Vuuren *et al.* (2009) noted a synergic effect against *Klebsiella pneumoniae* and additive effect against *Staphylococcus aureus* from the combination between *R. officinales* volatile oil and ciprofloxacin (Fig. 8).

The phytoconstituents, like polyphenols and monoterpenes which existence in volatile oils, perhaps interacted with some antimicrobials to enhance its mechanisms of action at the target positions for which the antimicrobial was prepared, therefore, this coupled increase the antimicrobial activities for both agents (Olajuyigbe and Afolayan, 2012).

These constituents possess a strong binding affinity to different molecular structures such as protein or glycoproteins due to their large lipophilicity. Therefore, it have great affinities for cell membranes and exhibit high potential to permeate through cell walls cause disruption for membrane or translocate through the cell membrane into inside the cell to arrive their target receptor to pave ways for these antibiotics to arrive their target sites of action, leading to the leakage of cell contents (Wang *et al.*, 2012). So, the volatile oils alone and/or in combined with antibiotics may provide a promising new strategy in phytotherapy against bacterial infections.

Generally, the volatile oils inhibit the growth of microbial cells as well as inhibit the production of toxic metabolic of bacteria and inhibit act the efflux pump that leads to facilitate the access of antibiotics to its active positions in the bacterial cell (Gutierrez *et al.*, 2008).

CONCLUSION

Genetic detection showed that most *P. aeruginosa* isolates were possessed protease genes. *P. aeruginosa* distinguishes by resistant to a several antibiotics as a result of its effective mechanisms. The volatile oils of the studied plants showed high efficiency in inhibiting the production of virulence factors and that the *Mentha spicata* volatile oil was more effective in inhibiting the growth of isolates than *Rosmarinus officinales*. Combination of antibiotics with volatile oils resulted in synergic and additive effects and the checkerboard technique considers a perfect alternative strategy to treat the infections resulted from drug-resistant bacteria.

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SIGNIFICANCE STATEMENT

We observed that the volatile oils were inhibited to several of *P. aeruginosa* virulence factors as well as inhibited the growth of bacteria. Moreover, the combination of volatile oils with antibiotics resulted in synergic effects.

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