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Evaluating of the Synergistic Effect of three Essential Oils on the Virulence Factors (resistance gene) of *Pseudomonas aeruginosa* and *Serratia fonticola* Isolated from Wounds

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Abstract

Depending on the high resistance to antibiotics, five isolates of *Pseudomonas aeruginosa* and 7 isolates of *Serratia fonticola* were selected out of 150 bacterial isolates from burn wards in Baghdad hospitals, which were later identified by VITEK2. A susceptibility test was done by using 15 antibiotics. The results showed that all the selected isolates were resistant to antibiotics: AMP, CTX, CAZ, GEN, PIP, TIC and TMP especially, while they were sensitive to IPE. The essential oils of *Aloysia citrodora* (Family: Verbenaceae), *Rosmarinus officinalis* (Family: Lamiaceae) and *Thymus vulgaris* (Family: Lamiaceae) were extracted by the Clevenger. The synergistic effect of essential oils of these plants on bacterial growth were studied by determining the MIC of essential oils for *P. aeruginosa* and *S. fonticola*. The MIC concentration of *P. aeruginosa* was: 2500 ppm of *A. citrodora*, 2500 ppm of *R. officinalis*, 2500 ppm of *T. vulgaris*, whilst the synergistic effect of each two plants was:1250 ppm of *A. citrodora* with 2500 ppm of *R. officinalis*, 1250 ppm of *A. citrodora* with 312.5 ppm of *T. vulgaris*, 1250 ppm of *R. officinalis* with 156.25 ppm of *T. vulgaris*, whereas the synergistic effect of three plants was: 156.25 ppm of *A. citrodora* with 156.25 ppm of *R. officinalis* and 39.0625 ppm *T. vulgaris*. While the MIC concentration of *S. fonticola* was: 1250 ppm of *A. citrodora*, 5000 ppm of *R. officinalis*, 1250 ppm of *T. vulgaris*, whilst the synergistic effect of each two plants was: 1250 ppm of *A. citrodora* with 2500 ppm of *R. officinalis*, 1250 ppm of *A. citrodora* with 312.5 ppm of *T. vulgaris*, 1250 ppm of *R. officinalis* with 156.25 ppm of *T. vulgaris*, whereas the synergistic effect of three plants was: 312.5 ppm of *A. citrodora* with 312.5 ppm of *R. officinalis* and 78.125 ppm *T. vulgaris*. The results of bacterial gene expression showed that three isolates of *P. aeruginosa* had a *mexB* gene expression that ranged between 2.5 - 0.4 (concentration 1) and 2.8 to 0.6 (concentration 2). It increased in two isolates and decreased in one isolate. The melting point ranged between 90.91 - 91.28^{0}C compared with 89.89 to 90.33^{0}C for *fbp* gene. Whereas *S. fonticola* did not have bla_{KPc} gene, instead it had bla_{TEM} gene with the gene expression ranging between 2.4 - 0.1 (concentration 1) and 4.5 - 0.2 (concentration 2). The gene expression increased in two isolates and decreased in five isolates. The melting point ranged between 83.63 - 84.20^{0}C compared with 75.82 - 85.56^{0}C for *gyrB* gene.

Keywords: Essential oils, Virulence factors, Resistance gene, *Pseudomonas*, *Serratia*.

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تقييم التأثير التآزري لثلاثة زيوت عطرية على عوامل الضراوة (جين المقاومة) لبكتيريا *Pseudomonas* و *Serratia* المعزولتين من الجروح

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الخلاصة

اعتمادا على مقاومتها العالية للمضادات الحيوية، تم انتخاب 5 عزلات من بكتيريا *Pseudomonas aeruginosa* و 7 عزلات من بكتيريا *Serratia fonticola* من اصل 150 عزلة بكتيرية تم عزلها من ردهات الحروق في مستشفيات بغداد، وبعد تمييزها بجهاز الفايتك، تم اجراء اختبار الحساسية عليها باستخدام ١٥ مضاد حيوي من المستخدمة عادة في المستشفيات. بينت النتائج ان كل العزلات المنتخبة مقاومة للمضادات الحيوية، خاصة مضاد AMP, CTX, CAZ, GEN, PIP, TIC, TMP بينما كانت حساسة لمضاد IPE . تم استخلاص الزيوت العطرية لنباتات اكليل الجبل (العائلة الشفوية)، الزعتر (العائلة الشفوية) واللويزة الليمونية (العائلة اللويزية)لدراسة تاثير هذه الزيوت على النمو البكتيري، وقد تم تحديد التركيز المثبط الادنى لكل زيت عطري وكانت النتائج لبكتيريا*P. Aeruginosa*اللويزة الليمونية 2500 ppm، اكليل الجبل 2500 ppm، الزعتر 2500 ppm.

اما بكتيريا *S. fonticola* فكان مع اللويزة الليمونية 1250 ppm، اكليل الجبل 5000 ppm، الزعتر 1250 ppm.ثم تم تحديد التركيز المثبط الادنى التآزري لكل نباتين معا وكانت النتائج لبكتيريا*P. aeruginosa*: اللويزة الليمونية 1250 ppmواكليل الجبل 2500 ppm، اكليل الجبل 1250 ppm والزعتر 156.25 ppm، اللويزة الليمونية 1250 ppm والزعتر 312.5 ppm، لبكتيريا *S. fonticola*: بإستخدام اللويزة الليمونية 1250 ppm واكليل الجبل 2500 ppm، اكليل الجبل 1250 ppm والزعتر 156.25 ppm، اللويزة الليمونية 1250 ppm والزعتر 312.5 ppm. ثم تم تحديد التركيز المثبط الادنى وبيان التاثير التازري للنباتات الثلاثة وكانت النتائج لبكتيريا *P. aeruginosa* اللويزة الليمونية 156.25 ppm واكليل الجبل 156.25 ppm والزعتر 39.0625 ppm، اما بكتيريا *S. fonticola* ، مع اللويزة الليمونية 312.5 ppm، اكليل الجبل 312.5 ppm والزعتر 78.125 ppm.كما اظهرت نتائج التعبير الجيني البكتيري ان ثلاث عزلات من بكتيريا *P. aeruginosa*حاملة لجين *MexB* وان التعبير الجيني ازداد في عزلتين ونقص في عزلة واحدة،وكان يتراوح من 2.5 الى 0.4 للتركيز الاول ومن 2.8 الى 0.6 للتركيز الثاني، ونقطة الانصهار تتراوح من 90.91 الى 91.28 م^0 مقارنة مع جينات *fbp* التي تراوحت فيها من 89.89 م^{0}الى 90.33 م^0. بينما كانت بكتيريا *S.fonticola* غير حاملة لجين bla_{KPC} وحاملة لجين bla_{TEM} وان التعبير الجيني قد ازداد في عزلتين ونقص في خمسة عزلات، وكان يتراوح من 2.4 الى 0.1 للتركيز الاول ومن 4.5 الى 0.2 للتركيز الثاني، ونقطة الانصهار تتراوح من 83.63 م^0 الى 84.20 م^0 مقارنة مع جينات *gyrB* التي تراوحت من 75.82 م^0 الى 85.56 م^0.

Introduction

Burn wound injuries are the main health problems throughout the world as these wounds appear as a favorable region for opportunistic colonization of microorganisms with exogenous and endogenous source [1]. Initially, burn wounds become colonized and infecting with gram positive bacteria, which are later replaced by gram negative bacteria during the second week [2]. Burn wound patients are incubators for different types of aerobic bacteria and average of isolation of these organisms increase with rise in total body surface area [3]. Results of burn wound infections by bacteria end in more than 75% of deaths. Burn injury infection is associated with both the depth and the size of the wound, and the chances of higher infection

are correlated with the longer the wound remains open [4]. Gram-positive bacteria colonize the injury surface within the first 48 h if topical antimicrobial agents are not used in time, that outruns the thermal damage be located deep within sweat glands and hair follicles [5]. *Streptococcus pyogenes* was the most common microorganism that infected burn wounds before the antibiotics era [6]. However, instantly after the discovery of penicillin. *Staphylococcus aureus* became the most common microorganism contaminating of burn wounds [7]. According to recent studies *Pseudomonas aeruginosa* has become the predominant pathogen implicated in burn wound infection [5]. Virulence factors, like pili, flagella, adhesins, lipopolysaccharide, alginate, coagulase, hemolysins, proteases, exotoxin A, leukocidins, elastase, iron-binding proteins, exoenzyme S, and super antigens, are important in the pathogenesis of invasive infections. A series of processes that interfers with virulence factors, that could cause burn wounds infection, involves bloodstream invasion, nutrient acquest, leukocyte homicide, adhesion, tissue destruction, and evasion of the immune system [8]. There are three widespread types of mechanisms of antimicrobial resistance in bacteria: modifying the target location, changing the antibiotic absorption and antibiotic obstruction. The acquired antibiotic resistance takes place through two genetic processes: spontaneous mutations and the genes acquired from exogenous sources [9]. Approximately 25% of the recent drugs used in treatments are derived from plants. Majority of these remedies were detected as a direct outcome of plant chemical studies focused on the extraction of active materials that could be utilized in conventional medicine [10]. Medicinal plants are of great significance for peoples and societies health. The medical importance of these plants is in some chemical matters that produce a specified physiological effect on the human body [11]. Secondary metabolites are composed of great and diverse group of organic compounds which are synthesized in small amount, do not have explicit function in primary processes like photosynthesis, nutrient assimilation, respiration, solute transport, and synthesis of carbohydrates, lipids, and protein [12]. Secondary metabolites play important role in plant defence against pathogens and predators, essentially acting as allelopathic factors that affect the growth, reproduction, and survival of other plants, attract pollinators; confrontation, overcome and adaptation of sudden changes in drought, humidity, light intensity and temperature [13]. All these processes and others appear in plants due to chemical conversions even though most activities are unknown [14]. The secondary plants metabolites are formed in a small amount with distinct bioactivities [15]. Essential oils are redolent and volatile composites that involve about 10% of plant kingdom [16], and are called ethereal or volatile oils as they evaporate at high temperatures in contrast with fixed oils [17]. Plants essential oils content production is little and rarely overrides 1% [18]. However, in a few cases it may reach 10% or more. Essential oils density is less than water density. They are hydrophobic and fluid mostly, slightly dissolvable in water and completely in alcohol and non-polar or weak polar dissolvents [19]. Due to their molecular structures, essential oils are easily oxidizable by air, heat and light [20]. Compounds of essential oils can penetrate the skin membranes and reach all target cells as their lipids are dissolvable [21].

Materials and Methods

Instruments

Autoclave, balance, centrifuge, theClevenger, electrical blender, electrical oven, gel electrophoresis, incubator, McFarland, quantus fluorometer, water bath and 96 well U bottom plate.

Source of Plant Material

Dried leaves of 25 kg *Aloysia citrodora*, 5 kg each of*Rosmarinus officinalis* and *Thymus vulgaris* were taken from medicinal herbs store.

Extraction of Plants Oils

In a round-bottomed flask, 250g of dried leaves were placed, and then subordinated to hydro distillation for 3 hours by heating to 60ºC using the Clevenger apparatus. The aqueous stage was divided into two layers: the under layer that included water was neglected, while the top layer involved the oils gathered. Later the separated oil was stored in a refrigerator in glass vials which were locked and marked until used [22].

Preparation of Different Concentrations of Plant Extracts

The concentrated oil extract was utilized as stock solution which was prepared by utilizing Dimethyl sulfoxide (DMSO) to dilute the oil. Next different concentrations were prepared by mixing a specific volume of the stock with a specific volume of DMSO, applying the following equation [23]:

$C_1V_1=C_2V_2$

C_1= Stock solution concentration.

V_1= Volume obtained from stock solution.

C_2= Final concentration.

V_2= Final volume.

The minimum inhibitory concentration (MIC) of each essential oil was lethal to bacterial growth in a certain concentration. A concentration of 40000 ppm was determined as the stock. Serial dilutions were made as following: 20000, 10000, 5000, 2500, 1250 and 625 ppm. Whereas a concentration of 10000 ppm was the stock solution for synergistic effect determination. The serial dilutions were made as follows: 5000, 2500, 1250, 625, 312.5, 156.25, 78.125 and 39.0625 ppm.

Preparation of Laboratory Media

All media were prepared in their containers on the bases of the scientific recommendation by the manufacturer manual of company instructions.

Standard Turbidity Solution Preparation

McFarland standard solution is the turbidity standard solution that is to a large degree utilized in methods for inoculum preparation or standardization, particularly the McFarland No.0.5 standard solution.

Bacterial Isolates Cultivation

All the collected swabs specimens were streaked, inoculated and plated onto a differential agar medium. Chromogenic agar medium acts as characterizing the bacterial enzymatic activity and can give bacterial identification, and so it would be possible to recognize different bacterial species and genera by the colonies color.

Determination of Minimal Inhibitory Concentration of Antibiotic (MIC)

micro-titer plates of 96 wells were utilized according to the method laid down by Wiegand *et al.* [24] to determine the following:

1. Isolates most resistant to antibiotics were inoculated in nutrient broth and incubated at 30°C for 24 hours.
2. Muller-Hinton broth was prepared and used as diluent.
3. Micro-titer plates of 96 flat wells were used in this method.
4. All wells of the plate were winded with 100 µL of Muller-Hinton broth in the first horizontal line, except the 12th well which was also the last well.
5. Extracts of three plants (volatile or essential oils) were used.

6. Extracts of three plants with a serial dilution having range of 40000, 20000, 10000, 5000, 2500, and 1250 µg/ml concentrations were prepared.
7. Plants extracts were prepared as stock solutions [25].
8. One hundred µL of stock solution was put down in the first well only. with all contents mixed well.
9. One hundred µL was taken from the first well and transferred to the second one, and then all contents were mixed well.
10. Same process, as in previous step, was repeated for the wells from 3 to 10.
11. One hundred µL was taken from the 10th well and then discarded so that the final volume in all tubes was 100 µL.
12. One hundred µL of distilled water was added to the 11th well, and then mixed well with Muller-Hinton broth before transferring 100 µL of it to the 12th well.
13. Inoculums were prepared by transferring 3-5 colonies into a 5 ml tube of normal saline to gain culture with 1.5 × 108CFU/ml. After modifying of turbidity standard of McFarland 0.5, suspensions were utilized within 30 minutes of preparation.
14. Ten µL of bacterial suspension from the first well was added to all wells, except for the negative control wells, until we reached the 11th well.
15. The eleventh well was the positive control, whereas 12th well was the negative control.
16. Sixteen µL of resazurin pigment was added to all wells.
17. Micro-titer plates were incubated at 37°C for 18–24 hours.
The minimal inhibitory concentration (MIC) for each bacterial isolate was defined as the lowest concentration of bacterial agent that showed no visible growth and inhibits of bacteria.

Molecular Assay

For molecular study, two sub MICs for (312.5, 312.5 and 78.125 ppm) and (625, 625 and 156.25 ppm) concentration of volatile oil for *A. citrodora*, *R. officinalis*, *T. vulgaris* respectively, were used against 5 isolates of *P. aeruginosa* and 7 isolates of *S. fonticola.* In addition, 5 and 7 bacterial isolates, that were not treated with any volatile oil, were used as controls to study and compare bla_{TEM} and bla_{KPC} gene expression for *S. fonticola* and *mexB* gene for *P. aeruginosa* when treated with volatile oil.

Extraction of DNA: Optimization of primer

Extraction of bacterial genome was done for all isolates by determining the concentrations by quantus fluorometer, which was 25 ng/L, and then using gel electrophoresis which confirmed the presence of intact DNA bands. To check the primer optimum annealing temperature, DNA template was magnified with the same primer pair, forward and reverse, at annealing temperatures of 55, 58, 60, 63 and 65°C [26].

DNA Concentration Estimation

By utilizing a nano drop spectrophotometer, DNA samples concentrations were determined ranged between 10-100 ng/µl [26].

Polymerase Chain Reaction Technique (PCR)

Conventional polymerase chain reaction (PCR) was implemented to amplify various fragments of interesting genes to reveal bacteria and the genes associated with antibiotic resistance [27].

Extraction of RNA

RNA was extracted from the selected isolates of *P. aeruginosa* and *S. fonticola* which grew overnight in nutrient broth as a control. In addition, isolates were treated with volatile oils of

sub-MICs for (312.5, 312.5 and 78.125 ppm) and (625, 625 and 156.25 ppm) concentrations. Total RNA of samples was extracted by utilizing TRIzol reagent. The concentrations ranged between 10 - 50.4 ng/µl. The gene expression levels of one or more genes depended on the analysis and calculation of RNA/miRNA concentration after its conversion to cDNA [28].

Purification of RNA

RNA was isolated from the samples by following TRIzol™ reagent protocol.

Analysis of Gene Expression by Utilizing Livak Method: Relative quantification

Folding = 2-ΔΔCT
ΔCT = CT gene - CT housekeeping gene
ΔΔCT = ΔCT treated or control - ΔCT control

Statistical Analysis

To compare diverse groups with each other, the gained data was subjected to analysis of variance test "ANOVA". Statistical Package for the Social Sciences (SPSS) sofware was employed for all tests [29].

Results

Fifteen antibiotics that are usually used in hospitals, were tested in this study (Table 1).

Table 1: Antibiotics were used for sensitivity testing

No.	Antibiotics	Symbol	Potency (µg/disc)
1	Amikacin	AK	10
2	Ampicillin	Am	10
3	Azithromycin	AZM	15
4	Carbenicillin	CB	25
5	Cefotaxime	CTX	30
6	Ceftazidime	CAZ	10
7	Ceftriaxone	CRO	10
8	Ciprofloxacin	CIP	10
9	Gentamycin	GM	10
10	Imipenem	IPE	10
11	Levofloxacin	LEV	5
12	Meropenem	MEM	10
13	Piperacillin	PIP	10
14	Ticarcillin	TIC	10
15	Trimethoprim	TMP	5

The results revealed that all the isolates were resistant to antibiotics, with each isolate being resistant to 12 or 13 antibiotics (Table 2).

Table 2: Resistance of bacterial isolates to antibiotics

No.	Bacterial species	Antibiotics sensitivity test														
		AK	AMP	AZM	CB	CTX	CAZ	CRO	CIP	GEN	IPE	LEV	MEM	PIP	TIC	TMP
57	*P. aeruginosa* 1	S	R	R	R	R	R	R	R	R	R	S	S	R	R	R
60	*P. aeruginosa* 2	S	R	R	R	R	R	R	R	R	S	S	R	R	R	R
66	*P. aeruginosa* 3	R	R	S	R	R	R	R	R	R	S	R	S	R	R	R

75	*P. aeruginosa* 4	R	R	R	R	R	R	R	R	R	S	R	S	R	R	R
100	*P. aeruginosa* 5	R	R	S	R	R	R	R	S	R	S	R	R	R	R	R
39	*S. fonticola* 1	R	R	R	R	R	R	R	S	R	S	S	R	R	R	R
64	*S. fonticola* 2	S	R	R	R	R	R	R	R	R	S	R	R	R	R	R
90	*S. fonticola* 3	R	R	S	R	R	R	R	R	R	S	R	R	R	R	R
99	*S. fonticola* 4	R	R	S	R	R	R	S	R	R	S	R	R	R	R	R
101	*S. fonticola* 5	R	R	S	R	R	R	R	R	R	S	R	R	R	R	R
124	*S. fonticola* 6	R	R	R	S	R	R	R	S	R	S	R	R	R	R	R
131	*S. fonticola* 7	R	R	R	R	R	R	R	R	R	S	R	S	R	R	R

R= Resistant/ S= Susceptible

The concentration of essential oils (volatile oil) of plants were as follows: 20000, 10000, 5000, 2500, 1250 and 625 ppm. MIC of EOs against *P. aeruginosa* were 2500 ppm for *A. citrodora*, *R. officinalis* and *T. vulgaris* was, while MIC of EOs against *S. fonticola* were 1250 ppm for *A. citrodora* and *T. vulgaris*, and 5000 ppm for *R. officinalis*(Table 3).

Table 3: MIC of *A. citrodora*, *R. officinalis* and *T. vulgaris*

No.	Bacteria Isolates	A (ppm)	R (ppm)	T (ppm)
57	*P. aeruginosa* 1	2500	2500	2500
60	*P. aeruginosa* 2	1250	1250	1250
66	*P. aeruginosa* 3	2500	2500	2500
75	*P. aeruginosa* 4	1250	5000	2500
100	*P. aeruginosa* 5	5000	5000	1250
39	*S. fonticola* 1	1250	1250	1250
64	*S. fonticola* 2	1250	1250	1250
90	*S. fonticola* 3	1250	5000	2500
99	*S. fonticola* 4	1250	5000	1250
101	*S. fonticola* 5	5000	10000	1250
124	*S. fonticola* 6	10000	10000	1250
131	*S. fonticola* 7	10000	20000	5000

A: *A. citrodora*, R: *R, officinalis*, T: *T, vulgaris*
l.s.d.: 193.7X min.rep, 1189.2 max-min, 1184.6 max.rep
MIC of synergistic effects of two plants against *P. aeruginosa* and *S. Fonticola* were 1250 ppm of *A. citrodora* with 2500 ppm of *R. officinalis*, 1250 ppm of *A. citrodora* with 312.5 ppm of *T. vulgaris*, 1250 ppm of *R. officinalis* with 156.25 ppm of *T. vulgaris*. (Table 4).

Table 4: MIC of synergistic effect of two plants for *A. citrodora* with *R. officinalis*, *A. citrodora* with *T. vulgaris*, and *R. officinalis* with *T. vulgaris*

No.	Bacteria Isolates	AR(ppm)		AT(ppm)		RT(ppm)	
		A	**R**	**A**	**T**	**R**	**T**
57	*P. aeruginosa* 1	1250	2500	625	156.25	312.5	39.0625
60	*P. aeruginosa* 2	1250	2500	1250	312.5	625	78.125
66	*P. aeruginosa* 3	1250	2500	2500	625	625	78.125
75	*P. aeruginosa* 4	2500	5000	156.25	39.0625	1250	156.25
100	*P. aeruginosa* 5	1250	2500	156.25	39.0625	1250	156.25
39	*S. fonticola* 1	1250	2500	156.25	39.0625	312.5	39.0625

64	*S. fonticola* 2	1250	2500	1250	312.5	1250	156.25
90	*S. fonticola* 3	2500	5000	312.5	78.125	1250	156.25
99	*S. fonticola* 4	2500	5000	312.5	78.125	1250	156.25
101	*S. fonticola* 5	1250	2500	1250	312.5	625	78.125
124	*S. fonticola* 6	1250	2500	625	156.25	1250	156.25
131	*S. fonticola* 7	1250	2500	1250	312.5	2500	312.5

l.s.d. of A. & R: 204.6X min.rep, 203.8 max-min, 203.0 max.rep
l.s.d. of A. & T.: 233.4X min.rep, 232.5 max-min, 231.6 max.rep
l.s.d. of R. & T.: 219.0X min.rep, 218.2 max-min, 217.3 max.rep
Depending on the MIC, 10 concentrations were tested as factorial design by serial dilutions which was considered as stock solution to study the synergistic effect (Tables 5 and 6).

Table 5: Ten stock solutions of selected essential oils tested for *P. aeruginosa*

No.	*A. citrodora*(ppm)	*R. officinalis*(ppm)	*T. vulgaris*(ppm)
1	2500	2500	2500
2	2500	2500	1250
3	5000	5000	2500
4	2500	2500	5000
5	2500	5000	2500
6	5000	2500	2500
7	1250	1250	2500
8	1250	2500	1250
9	2500	1250	1250
10	1250	2500	2500

Table 6: Ten stock solutions of selected essential oils tested for *S. fonticola*

No.	*A. citrodora*(ppm)	*R. officinalis*(ppm)	*T. vulgaris*(ppm)
1	1250	5000	1250
2	1250	2500	1250
3	1250	10000	1250
4	2500	5000	2500
5	1250	5000	2500
6	2500	5000	1250
7	1250	5000	625
8	625	5000	1250
9	625	2500	625
10	1250	1250	1250

There were significant differences between the concentrations of synergistic effect. The results of MIC against *P. aeruginosa* were 156.25 ppm of *A. citrodora* with 156.25 ppm of *R. officinalis* and 39.0625 ppm of *T. Vulgaris*. Whereas, MIC against *S. fonticola* were 312.5 ppm of *A. citrodora* with 312.5 ppm of *R. officinalis* and 78.125 ppm of *T. vulgaris*, as shown in Table 7 (a) and (b).

Table 7 (a): MIC of synergistic effect for *A. citrodora*, *R. officinalis* and *T. vulgaris* for 5 different concentrations

N0.	Bacteria isolates	ART1			ART2			ART3			ART4			ART5		
		A	R	T	A	R	T	A	R	T	A	R	T	A	R	T
57	*P. aeruginosa* 1	156.25	312.5	39.0625	156.25	156.25	39.0625	312.5	78.125	78.125	78.125	78.125	78.125	78.125	312.5	78.125
60	*P. aeruginosa* 2	156.25	312.5	39.0625	156.25	156.25	39.0625	625	156.25	156.25	312.5	312.5	312.5	39.0625	156.25	39.0625
66	*P. aeruginosa* 3	156.25	312.5	39.0625	156.25	156.25	39.0625	312.5	78.125	78.125	78.125	78.125	78.125	78.125	312.5	78.125
75	*P. aeruginosa* 4	156.25	312.5	39.0625	156.25	156.25	39.0625	312.5	78.125	78.125	78.125	78.125	78.125	78.125	312.5	78.125
100	*P. aeruginosa* 5	156.25	312.5	39.0625	625	625	156.25	312.5	78.125	78.125	78.125	78.125	78.125	39.0625	156.25	39.0625
39	*S. fonticola* 1	312.5	625	78.125	156.25	156.25	39.0625	156.25	39.0625	39.0625	39.0625	39.0625	39.0625	39.0625	156.25	39.0625
64	*S. fonticola* 2	156.25	312.5	39.0625	156.25	156.25	39.0625	312.5	78.125	78.125	78.125	78.125	78.125	78.125	312.5	78.125
90	*S. fonticola* 3	156.25	312.5	39.0625	312.5	312.5	78.125	312.5	78.125	78.125	78.125	78.125	78.125	39.0625	156.25	39.0625
99	*S. fonticola* 4	156.25	312.5	39.0625	625	625	156.25	312.5	78.125	78.125	78.125	78.125	78.125	78.125	312.5	78.125
101	*S. fonticola* 5	156.25	312.5	39.0625	312.5	312.5	78.125	312.5	78.125	78.125	78.125	78.125	78.125	78.125	312.5	78.125
124	*S. fonticola* 6	156.25	312.5	39.0625	156.25	156.25	39.0625	156.25	39.0625	39.0625	78.125	78.125	78.125	78.125	312.5	78.125
131	*S. fonticola* 7	312.5	625	78.125	312.5	312.5	78.125	312.5	78.125	78.125	78.125	78.125	78.125	78.125	312.5	78.125

l.s.d. of ART1: 47.68X min.rep, 47.50 max-min, 47.32 max.rep/ l.s.d. of ART2: 20.01X min.rep, 19.94 max-min, 19.86 max.rep/ l.s.d.of ART3: 18.46X min.rep, 18.39 max-min, 18.32 max.rep/ l.s.d. of ART4: Non-significant */ l.s.d. of ART5: 19.81X min.rep, 19.74 max-min, 19.66 max.rep

Table 7 (b): MIC of synergistic effect for *A. citrodora*, *R. officinalis* and *T. vulgaris* for 5 different concentrations

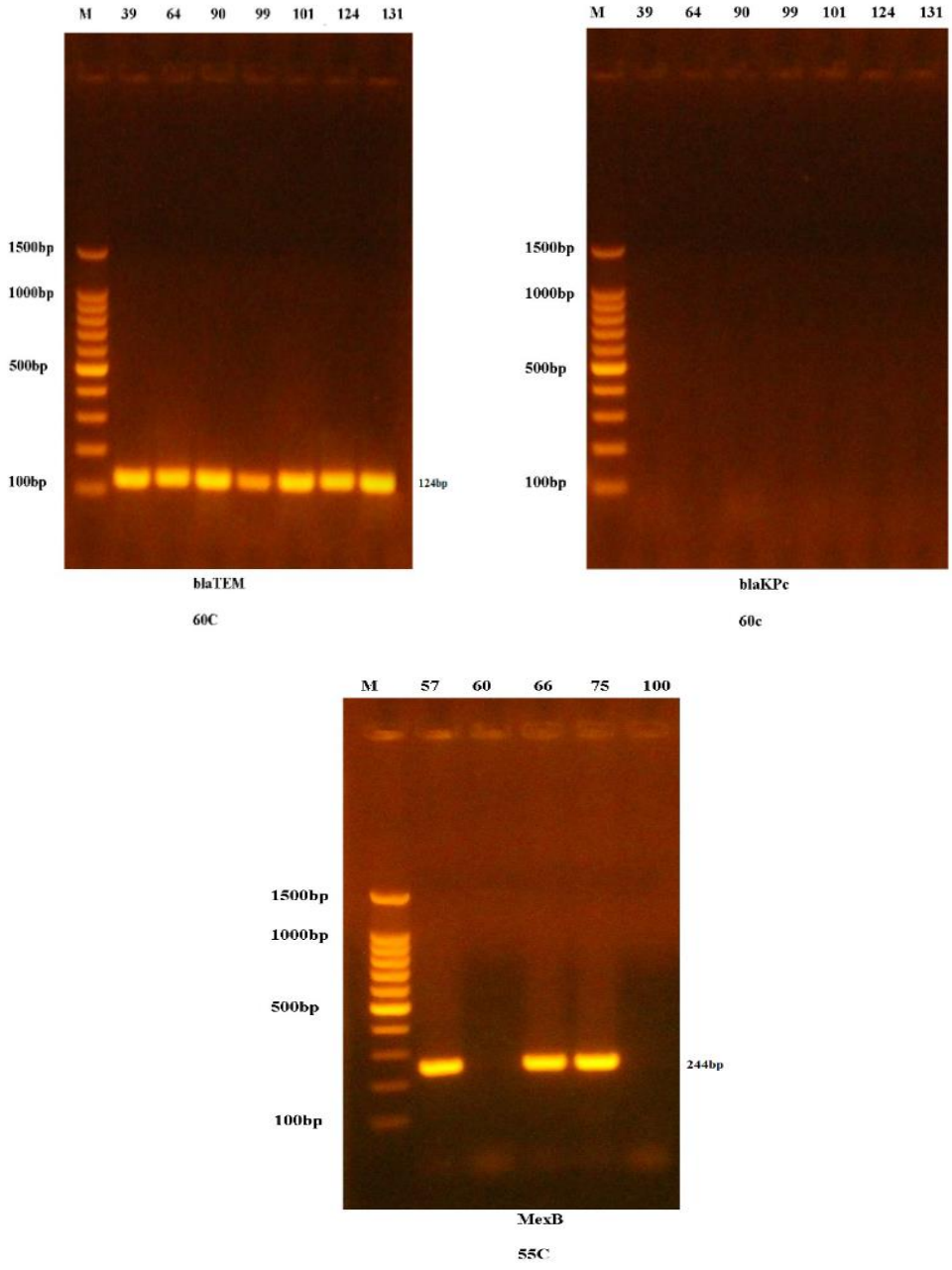
N0.	Bacteria isolates	ART6			ART7			ART8			ART9			ART10		
		A	R	T	A	R	T	A	R	T	A	R	T	A	R	T
57	*P. aeruginosa* 1	78.125	625	78.125	625	78.125	78.125	156.25	156.25	625	1250	625	156.25	625	625	78.125
60	*P. aeruginosa* 2	78.125	625	78.125	625	78.125	78.125	156.25	156.25	625	625	312.5	78.125	312.5	312.5	39.0625
66	*P. aeruginosa* 3	78.125	625	78.125	625	78.125	78.125	312.5	312.5	1250	312.5	156.25	39.0625	312.5	312.5	39.0625
75	*P. aeruginosa* 4	39.0625	312.5	39.0625	625	78.125	78.125	625	625	2500	312.5	156.25	39.0625	312.5	312.5	39.0625
100	*P. aeruginosa* 5	78.125	625	78.125	312.5	39.0625	39.0625	312.5	312.5	1250	312.5	156.25	39.0625	625	625	78.125
39	*S. fonticola* 1	78.125	625	78.125	312.5	39.0625	39.0625	312.5	312.5	1250	625	312.5	78.125	312.5	312.5	39.0625
64	*S. fonticola* 2	78.125	625	78.125	625	78.125	78.125	312.5	312.5	1250	312.5	156.25	39.0625	312.5	312.5	39.0625

90	*S. fonticola* 3	39.0625	312.5	39.0625	625	78.125	78.125	156.25	156.25	625	312.5	156.25	39.0625	625	625	78.125
99	*S. fonticola* 4	78.125	625	78.125	625	78.125	78.125	156.25	156.25	625	312.5	156.25	39.0625	312.5	312.5	39.0625
101	*S. fonticola* 5	78.125	625	78.125	312.5	39.0625	39.0625	625	625	2500	1250	625	156.25	625	625	78.125
124	*S. fonticola* 6	78.125	625	78.125	625	78.125	78.125	78.125	78.125	312.5	312.5	156.25	39.0625	312.5	312.5	39.0625
131	*S. fonticola* 7	156.25	1250	156.25	625	78.125	78.125	39.0625	39.0625	156.25	1250	625	156.25	625	625	78.125

l.s.d.of ART6: 60.8X min.rep, 60.6 max-min, 60.4 max.rep/ l.s.d. of ART7: 41.91X min.rep, 41.76 max-min, 41.60 max.rep/ l.s.d. of ART8: 106.9X min.rep, 106.5 max-min, 106.1 max.rep/ l.s.d. of ART9: 52.7X min.rep, 52.5 max-min, 52.3 max.rep/ l.s.d. of ART10: 42.49X min.rep, 42.33 max-min, 42.16 max.rep

Detection of *bla*$_{TEM}$, *bla*$_{KPC}$ and *mexB*

PCR was utilized to amplify the constitutional genes utilizing primers for mexB and *fbp* (housekeeping gene) of 5 isolates of *P. aeruginosa*, and *blaTEM*, *gyrB* (housekeeping gene) and *bla*$_{KPC}$ gene of 7 isolates of *S. fonticola*. The bands were confirmed by utilizing gel electrophoresis. The results revealed that 3 isolates of *P. aeruginosa* contained *mexB* gene (244 bp) and *fbp* gene (115 bp). Whereas all isolates of *S. fonticola*, appeared to have *bla*$_{TEM}$ gene (124 bp) and *sefA_gyrB* gene (258 bp). However, *bla*$_{KPC}$ gene didnot appear in any isolates of *S. fonticola* (Figure 1).



1 2 3

Figure 1: (1, 2): Results of the amplification of *bla*$_{TEM}$ and *bla*$_{KPC}$ gene of *S. fonticola*species were fractionated on 1.5% agarose gel electrophoresis stained with Eth.Br. M: 100bp ladder marker. Lanes 39-131 resembled PCR products. 3: Results of the amplification of *mexB* gene of *P. aeruginosa* species were fractionated on 1.5% agarose gel electrophoresis stained with Eth.Br. M: 100bp ladder marker. Lanes 57-100 resembled PCR products.

Real Time-qPCR

Effects of Volatile Oils on the mexB and blaTEM Expression

In order to determine the synergistic effects of sub-MIC *A. citrodora*, *R. officinalis*, *T. vulgaris* volatile oils on the expression of the *mexB* gene in *P. aeruginosa* isolates and bla_{TEM} in *S. fonticola* isolates, quantitative real-time PCR was performed in a one-step RT-PCR technique. The results showed a decrease in *mexB* and bla_{TEM} gene expression levels in some isolates and increase in other isolates under the synergistic volatile oil effects. Pomari *et al.* demonstrated a stimulation of gene expression [30] and then measured relative changes in gene expression in real-time quantitative PCR experiments. The qRT- PCR results were revealed existence of volatile oils at sub-MIC concentration (A: 5000, R:10000 and T: 1250 ppm). The *mexB* gene expression ranged between 2.5 - 0.4, whereas the bla_{TEM} gene expression ranged between 2.4 - 0.1. Whereas at sub-MIC concentration (A: 5000, R: 5000 and T: 1250 ppm) the bla_{TEM} gene expression at sub-MIC concentration (A: 5000, R: 5000 and T: 1250 ppm), the *mexB* gene expression ranged between 2.8 - 0.6, whereas the bla_{TEM} gene expression ranged betwen 4.5 - 0.2, compared with control samples as shown in Figures (2), (3), (4), (5), (6) and (7).

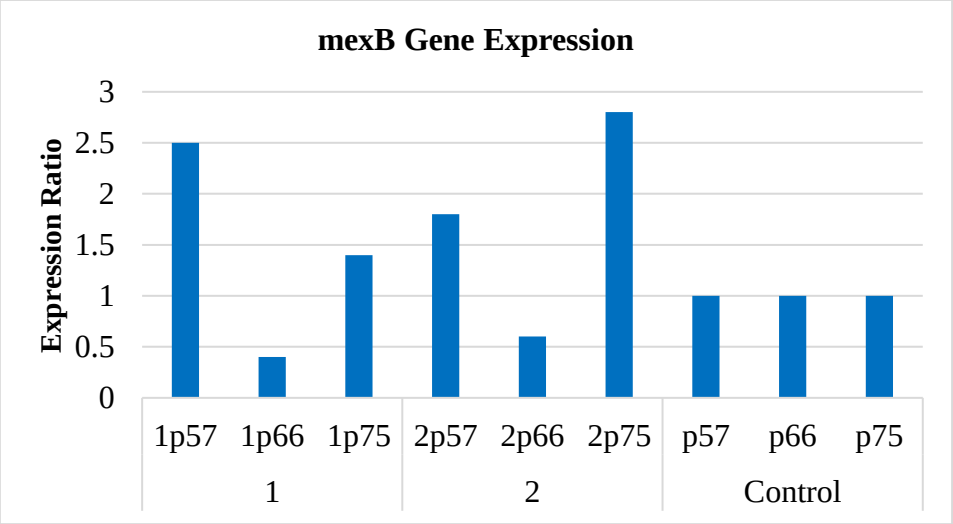


Figure 2: Fold change of mexB gene in *P. aeruginosa* with sub-MIC of volatile oil, Group 1 (A: 5000, R: 5000, and T: 1250 ppm), Group 2 (A:5000, R: 10000 and T: 1250 ppm)

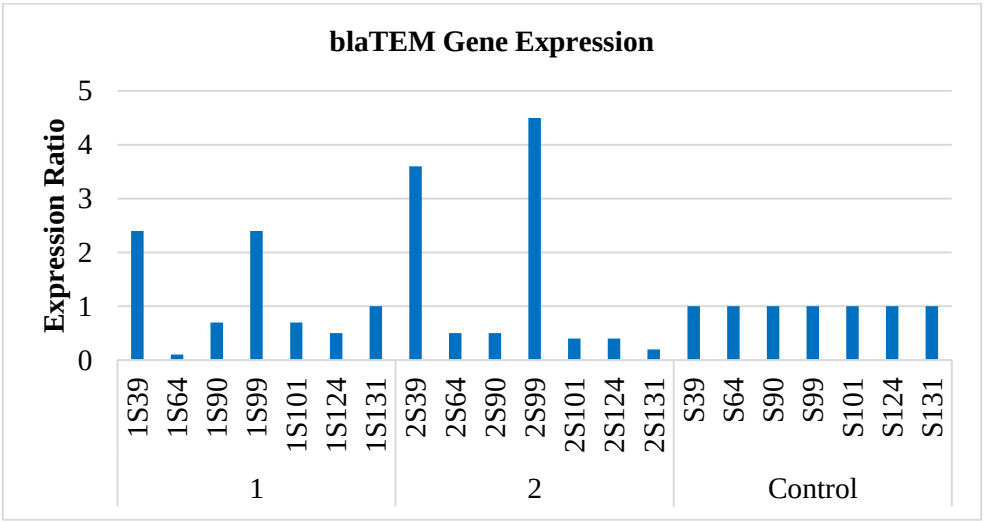


Figure 3: Fold change of bla_{TEM} gene in *S. fonticola* with sub-MIC of volatile oils, Group 1 (A: 5000, R:5000 and T:1250 ppm), Group 2 (A: 5000, R:10000 and T:1250 ppm)

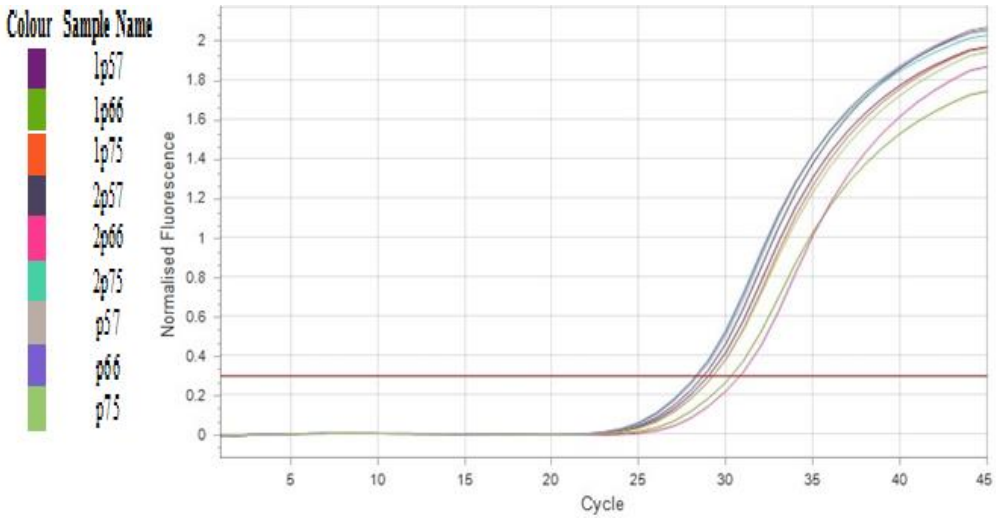


Figure 4: The amplification curve of *mexB* gene in *P. aeruginosa* with sub-MIC of volatile oil, Group 1 (A: 5000, R:5000 and T:1250 ppm), Group 2 (A: 5000, R:10000 and T:1250 ppm)

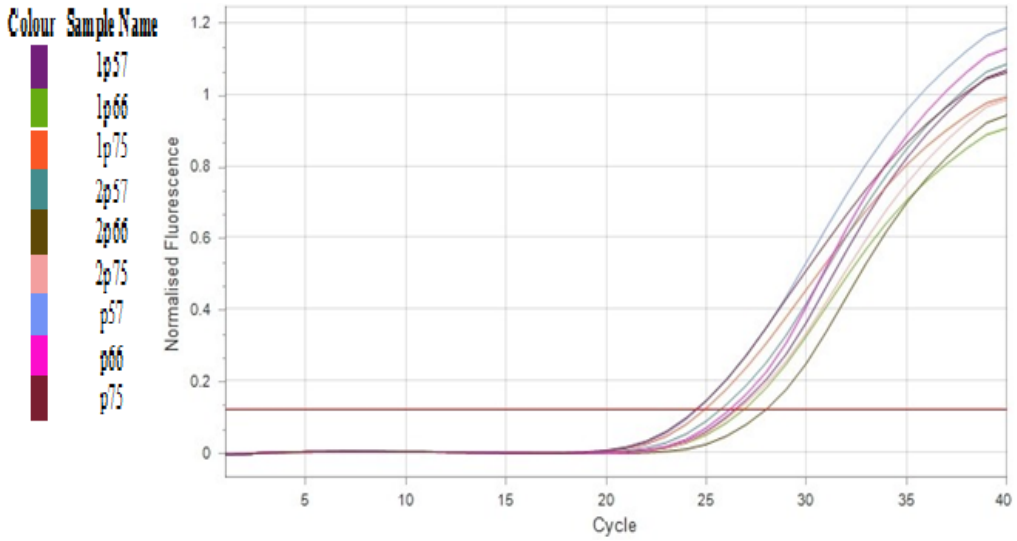


Figure 5: The amplification curve of housekeeping gene (*fbp*) in *P. aeruginosa* with sub-MIC of volatile oil, Group 1 (A: 5000, R:5000 and T:1250 ppm), Group 2 (A: 5000, R:10000 and T:1250 ppm)

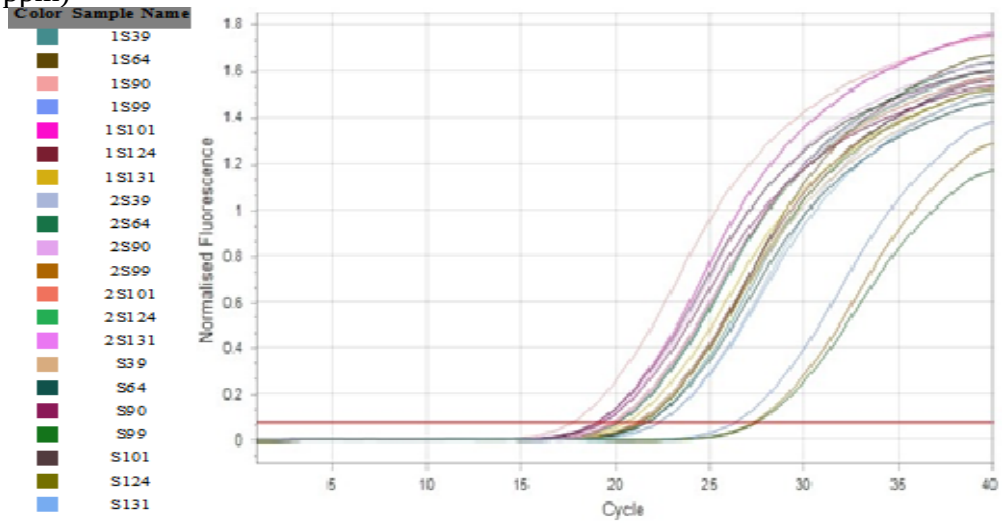


Figure 6: The amplification curve of *bla*$_{TEM}$ gene in *S. fonticola* with sub-MIC of volatile oil, Group 1 (A: 5000, R:5000 and T:1250 ppm), Group 2 (A: 5000, R:10000 and T:1250 ppm)

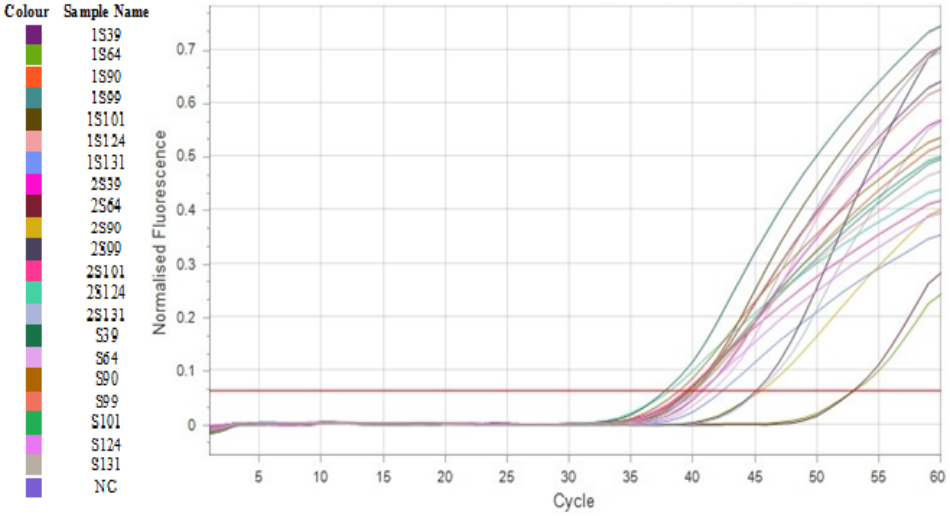


Figure 7: The amplification curve of housekeeping gene (*gyrB*) in *S. fonticola* with sub-MIC of volatile oil, Group 1 (A: 5000, R:5000 and T:1250 ppm), Group 2 (A: 5000, R:10000 and T:1250 ppm)

The melting point for *mexB* gene ranged between 90.91^{0}C - 91.28^{0}C. Whereas the melting point for *fbp* gene ranged between 89.89^{0}C - 90.33 ^{0}C. Melting point for *bla*$_{TEM}$ gene ranged between 83.63^{0}C - 84.20^{0}C. Melting point for *gyrB* gene it ranged between 75.82^{0}C - 85.56^{0}C. The genes were pure in all isolates because melting point curve showed one peak of melting point in all processes as shown in Figures (8), (9), (10) and (11). The results revealed there was a relationship between the phenotypic and gene expressions of virulence factors genes.

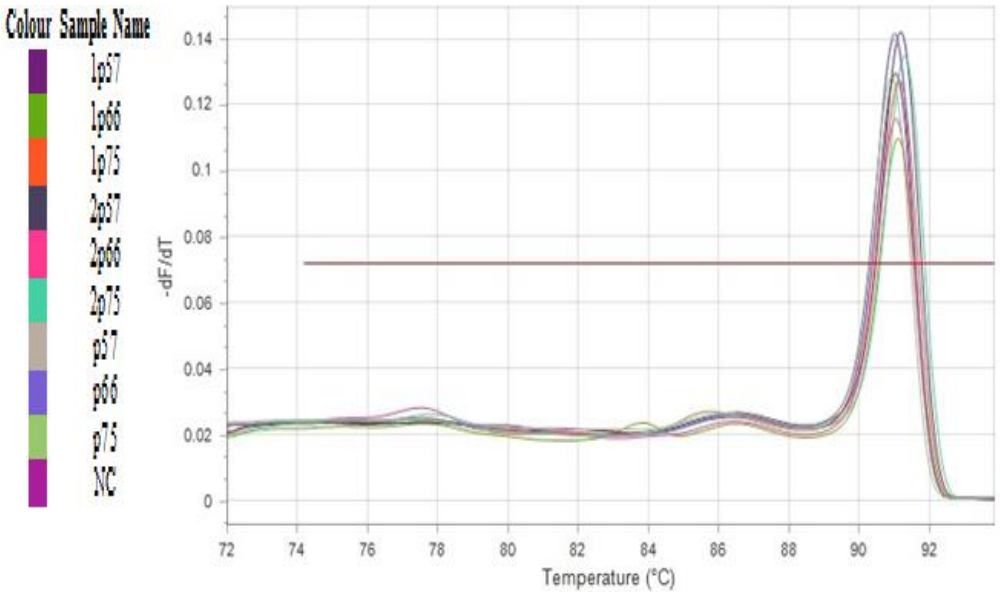


Figure 8: The Melting curve of *mexB* gene in *P. aeruginosa* with sub-MIC of volatile oil, Group 1 (A: 5000, R:5000 and T:1250 ppm), Group 2 (A: 5000, R:10000 and T:1250 ppm)

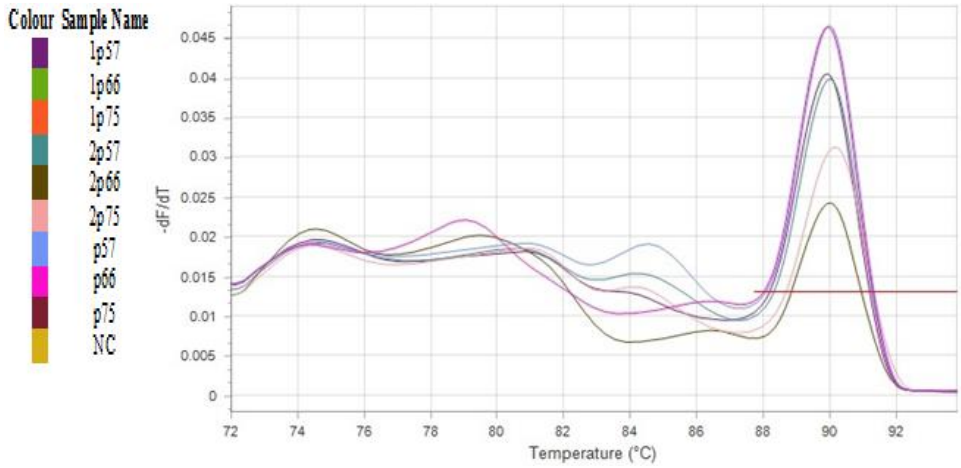


Figure 9: The Melting curve of housekeeping gene (*fbp*) in *P. aeruginosa* with sub-MIC of volatile oil, Group 1 (A: 5000, R:5000 and T:1250 ppm), Group 2 (A: 5000, R:10000 and T:1250 ppm)

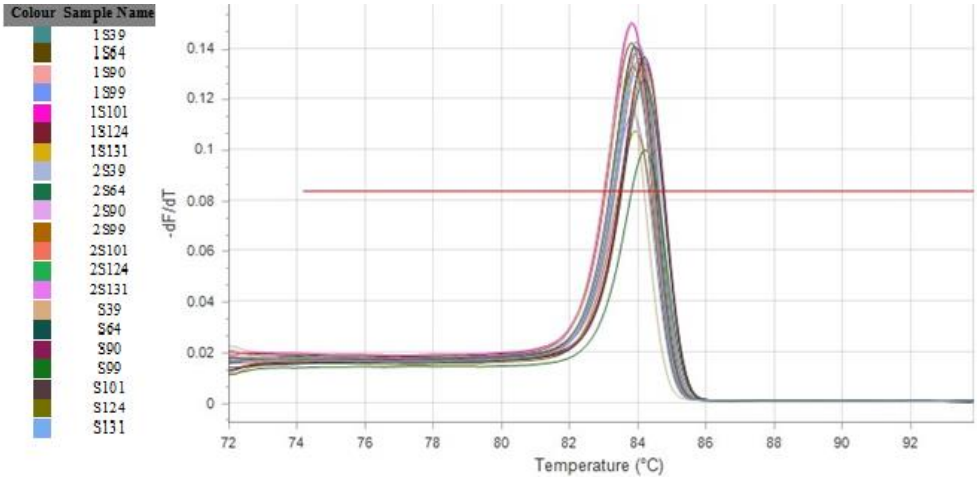


Figure 10: Melting curve of *bla*$_{TEM}$ gene in *S. fonticola* with sub-MIC of volatile oil, Group 1 (A: 5000, R:5000 and T:1250 ppm), Group 2 (A: 5000, R:10000 and T:1250 ppm)

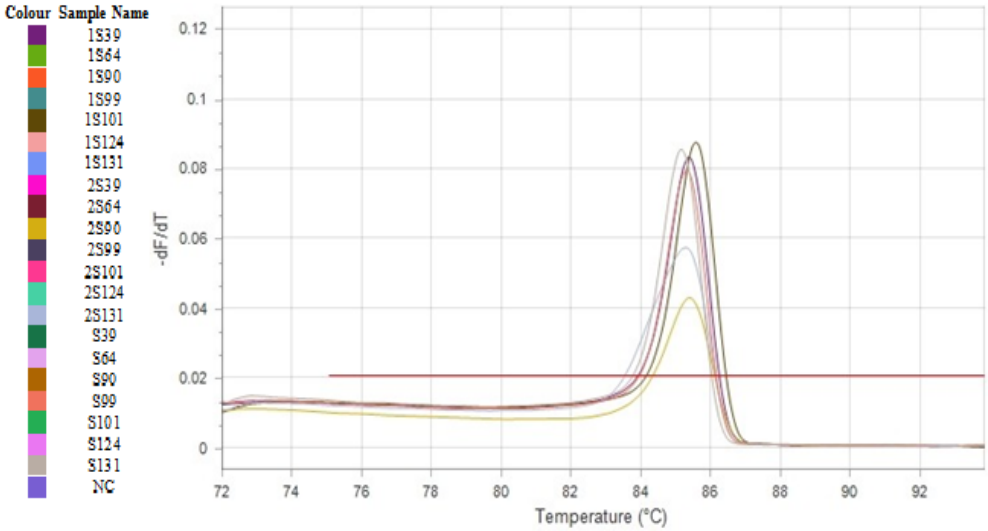


Figure 11: Melting curve of housekeeping gene (*gyrB*) in *S. fonticola* with sub-MIC of volatile oil, Group 1 (A: 5000, R:5000 and T:1250 ppm), Group 2 (A: 5000, R:10000 and T:1250 ppm)

Discussion

The virulence factors are adhesion or exotoxin agents. So, the clinical activeness of antimicrobial drugs used to remedy *P. aeruginosa* and *S. fonticola* contagions is not dependent on the characteristics of antibiotic bacteriostatic or bactericidal only. It is dependent on targeting bacterial virulence factors by alternative strategy. Antimicrobial resistance is a difficul issue. It can occur by gaining resistance genes via horizontal gene convey or mutations in genes usually existing in the genome [31]. Antimicrobial activities occur due to the chemical ingredients of plant extracts. The extracts are rich in polyphenols compounds, which have antimicrobial activities. While monophenolic compounds do not have bacterial effects [32]. The chemical ingredients of a plant extracts may vary from region to region, depending on climatic conditions and plant varieties [33]. Observation and study of the potential synergistic effects among various plant extracts is highly recommended because the results of many studies have shown diverse outcomes and different conclusions. So, further studies are required to interpret the mechanisms underlying antimicrobial activity in order to understand the synergism of volatile oil components. Knowing the synergistic effective concentrations of plant extract compounds can probably be taken as decrease the adverse side effects and toxicity correlating with the use of one plant extract or high concentrations [34]. Synergistic effectives are very significant factors because they promote the antimicrobial activities by using the effectiveness of associated agents and lowering the possibility concentrations [35]. Suitable concentrations and ingredients combination are significant agents that must be studied for acquiring maximum synergistic effects [36]. The mechanism of synergistic effects is due to the plant extract containing many types of compounds because of the structural complexity which acts combined with different biochemical processes in microorganisms [37]. Since environmental conditions are very diverse, making pathogenic bacteria have many mechanisms which sense and respond to all signals and changes that occur in their external surroundings, particularly treatment with antibiotics, as regulate their functions and activities to arrange the virulence factors appropriate with the abnormal conditions. The main regulatory mechanisms, that are responsible for the gene expression of virulence factors in pathogenic bacteria, making them able to have defence strategies to evade making them resistant to various treatments is also very important.

Conclusion

Essential oils play important role in bacterial growth inhibition. and are considered as well alternative to antibiotics that are resistant by many bacterial species. The synergistic effect has more significant influence than essential oils. The bacterial gene expression decreased in 8 bacterial isolates and decreased in 4 others.

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