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EVALUATED THE EFFECT OF A SUB-INHIBITORY CONCENTRATION OF CLOVE OIL ON THE EXPRESSION OF APRA AND PELF GENES IN PSEUDOMONAS AERUGINOSA

Fatimah Abdulazeez Awad[1], Mohammed Radhi Mohaisen[1], and Karama Tahreer Ahmed Al-Taee[1]
[1]College of Dentistry, University of Anbar, Ramadi, Iraq
Email: den.fatimah.abdulazez@uoanbar.edu.iq

ABSTRACT
The use of clove oil in the inhibition of pathogenic bacterial as well as yeast infections has been reported. The microbi-cide activity of this oil against Pseudomonas aeruginosa virulence genes was tested in this paper to estimate the effect of clove oil against the expression of Pseudomonas aeruginosa aprA and pelF genes in six wound isolates that showed highly multidrug resistance behavers. The minimum inhibitory concentrations of the oil on the bacterial isolates were identified as the six Pseudomonas isolates were treated with sub-MIC of clove oil. Then, the genes expression of aprA and pelF was detected be mean of a real-time PCR technique. The results show a significant decrease in the gene ex-pression of aprA and pelF, and their phenotypes, after treatment with clove oil.
Keywords: Pseudomonas aeruginosa, clove oil, aprA gene, pelF gene

1. INTRODUCTION
Pseudomonas aeruginosa is an opportunistic bacterial agent responsible for serious nosocomial infections including burning and wound infections. The severity of these bacteria is attributed to their outstanding virulence factors, as well as, their multidrug resistance action [1-3]. Over 75% of death cases due to burning have belonged to Pseudomonas infection [4].
The most infectious factors that P. aeruginosa use for its infection invasive are the production of basal protease enzyme as well as the biofilm formation especially in fierce strains [2, 5, 6]. 65%-80% of infectious diseases are related to biofilm formation [7].
Both of these virulence factors are closely linked to the quorum sensing (QS) phenomenon, which is bacterial cell-cell communication to regulate the expression of various genes including virulence genes by diffusion of small signalling molecules between bacterial cells [8, 9]. QS systems in P. aeruginosa are in two versions; las AHL-based system and rhl AHL-based system. The former system products are LasR and LasB proteins. LasR is a transcriptional regulator protein that targetsits chemical molecule (3-oxo-C12-HSL) and interacts the expression of the toxic and the biofilm former LasB protein [10].
Pseudomonas aeruginosa protease enzyme is encoded by aprA gene [4], while biofilm formation is depending on the production of three externally secreted polysaccharides; Pel, Psl and algenate. Proteins that are responsible for Pel exopolysaccharide biosynthesis are encoded by seven genes; pelA, pelB, pelC, pelD, pelE, pelF, and pelG [11, 12].

Pseudomonas aeruginosa virulence factors are not the only reasons behind their bacterial infection severity; multi-drug resistant (MDR) P. aeruginosa is considered a worldwide threat resulting in high mortality of people due to bacterial infections [13]. MDR P. aeruginosa is a consequence of P. aeruginosa persistent infections, especially with cystic fibrosis infections, or it could be an outcome of bacterial transmission from patients infected with resistant bacteria [14]. The patient-to-patient transmission of MDR P. aeruginosa might lead to infection outbreaks which could be a serious problem and so defecate to be solved [15].

Using of clove essential oil (Syzygium aromaticum) as an antibacterial antiseptic was reported in many studies. The effect of this oil on bacterial virulence factors has been evaluated. Clove oil has a reductional effect on rhl- and las- regulated virulence factors such as protease, LasB, swarming, exopolysaccharide synthesis and pyocyanin and chitinase synthesis [16]. Clove essential oil is considered safe when used in perfumes, medicines, and foods, as recommended by the FDA [17]. The current study aim is to estimate the clove oil effect on aprA and pelF genes expression in MDR strains of P. aeruginosa.

2. MATERIALS AND METHODS

2.1 Preparation of plant

In order to obtain the clove essential oil, dried buds was used by steam distillation technique and then it be dried at 4°C with sodium sulphate [18].

2.2 Bacteria Isolation

Initially, sterile swabs from the wounds were used to collect samples. The swab was then placed into sterile tubes containing 0.5 ml of physiological saline, followed by cultivation on Blood Agar. At 37 °C, the cultured samples were incubated for 18–24 hours. The colonies were then subjected to morphological and biochemical testing to identify the isolated strains [19, 20].

Six P. aeruginosa isolates were grown on MHA medium and left at 37oC for 24 hours after being isolated from wounds at Ramadi hospital.

2.3 Determination of MIC of clove oil

By using dimethyl sulfoxide (DMSO) as a solvent, clove oil was dissolved to prepare the stock essential oil (EO) 0.1 mg/ml concentration and kept at 4oC in dark place. Eight concentrations of oil were prepared from the EO stock (0.1 mg /ml). These concentrations are (0.005, 0,006, 0.007, 0.008, 0.009, 0.01, 0.011, and 0. 012 mg /ml) with a total volume of 2ml by adding MHB and then, from each concentration, 1 ml of clove EO were transferred to eight sterile glass tubes which were then mixed with 2 ml of MHB containing 9×105 CFU/ml of P. aeruginosa and incubated at 37°C for 24 hours. Ex-perimental tests were achieved in duplicate, with negative as well as positive controls and inoculated with bacteria in present or absent oil.

Table 1: The RT-PCR Primers and their sequences

Primer	Sequence	Outcome Size

aprA-F	GGACGTCACCAATATCCACTT	340bp
aprA-R	CCAGTAGCTCATCACCGAATAG	
pelF–F	TGTTCGAGCTGAGCAGTTG	200bp
pelF–R	GATCAGCGGCACGAAGAA	
gyrB –F	GGCGTGGGTGTGGAAGTC	190bp
gyrB –R	TGGTGGCGATCTTGAACTTCTT	

Cytotoxicity test

The cytotoxicity of clove oil was examined by placing 0.8 ml of clove oil in a clean sterile test tube and adding 0.2 ml of human red blood cells to it, to make the final volume 1 ml and incubation after shaking it slightly for 30 minutes at a temperature of 37 oC, and centrifuged for 5 minutes at a rate of 1000 cycles/min [21]. After that, hemolysis was observed by comparing it with the control treatment (test tube containing blood only) to note the difference in hemolysis.

2.5 Protease inhibition Assays

The ability of clove oil to inhibit the production of protease was tested on skim milk agar according to Vijayaraghavan, et al. (2013)[22] The activity of proteases was determined by measuring the diameter of the clearance zone surrounding each well after overnight incubation of the plate at 37oC.

2.6 Biofilm Inhibition Assay

Biofilms were grown on 96-well microtiter plates at sub-minimum inhibition concentration or in the absence of clove oil. In an infrared reader, a device that reads the results of ELISA tests, the plate was then spectrophotometrically scanned at 590 nm[23].

2.7 Real-time RT PCR

RNA extraction

After determining the minimum inhibition concentration of clove oil, isolates were grown in LB broth with clove EO with a concentration less than MIC and incubated for 24 hours at 37°C. Total amount of RNA was extracted using a commercial RNA extraction kit (Accuzol Reagent).

cDNA synthesis

Synthesis of cDNA was performed in reaction mixture with 20 µl as total volume, including 2 µg of extracted RNA, 50 ng of random Hexamer primer, 1 µl of dNTPs (10 mM), 100>U of M-MuLV reverse transcriptase and 2 µl of M-MuLV buffer (10X), with conditions of 65°C initial denaturation for 5 minutes and 42°C reverse transcription for 60 minutes (Vivantis, Malaysia). The outcome were immediately applied to Real-time PCR assays.

RT PCR experiment

All real time PCR spearmints were done according to the standard conditions of the comply cycling. The software GenScript Real-time PCR (TaqMan) Primer Construct was used to design specific primers for the aprA, pelF, and gyrB genes (Table 1).

The RNA was then extracted from both; bacteria exposed to clove oil and those without clove oil in their media ,as negative controls, using the manufacturer's methodology. For both samples,

cDNA was synthesized, and the efficiency of the primers was confirmed by standard curve analysis for each pair of primers (Copy number range =3□104 - 3□108).

Changes in the expression levels of the aprA, pelF, and gyrB, genes were detected using the RT-PCR method with the following conditions: 3 minutes at 95°C (1 cycle), 30 seconds at 95°C (35 cycles), 30 seconds at 54 degrees Fahrenheit (35 cycles), 1 minute at 72°C (35 cycles), and 10 minutes at 72°C for the ultimate extension. As an internal control, the housekeeping gene gyrB was employed.

Calculation of ct value

Resulted data of the RT-PCR were analyzed using the Delta Delta CT method to calculate the quantitative results of each gene expression for pelF and aprA genes.

3. RESULTS

One of the important biological phenomena of CEO is its antimicrobial activity. This oil has bactericidal activity ageist broad-spectrum of bacterial pathogens as a result of interaction with bacterial cells polysaccharides production as well as many other bacterial enzymes including protease. Likewise, CEO could inhibit many Gram-negative bacteria including P. aeruginosa by its significant effectiveness against the lipopolysaccharide layers in the bacterial cell outer membrane.

Our hypothesis in this study is CEO has a significant effect on two of P. aeruginosa most important virulence genes. These two genes are involved in the production of protease and biofilm formation. Six P. aeruginosa isolates were used in this study. These isolates were isolated from wound infections. All isolates were tested for their production of protease and biofilm formation. The study included the identification of clove oil's effect on aprA and pelF genes expression in P. aeruginosa.

3.1 Determination the MIC of EO

The antibacterial effect of eight concentrations (0.005, 0.006, 0.007, 0.008, 0.009, 0.001, 0.0011 and 0.0012 mg/ml) of clove oil were examined against all P. aeru-ginosa isolates. MIC results are listed in Table 2.

Table 2: The MIC of clove oil against *P. aeruginosa*

MIC (mg /ml) clove oil	Sub inhibitory concentration	No.of *P.aeruginosa* isolate
0.005	0.004	Ps1
0.006	0.005	Ps2
0.006	0.005	Ps3
0.008	0.007	Ps4
0.007	0.006	Ps5
0.01	0.009	Ps6

3.2 Virulence Phenotype Assays

Skim milk agar media were used to find out if there is any effect of clove oil on the protease enzyme produc-tion. The results showed a significant effect of clove oil on protease enzyme activity (Table 2). Same results were optioned with clove oil effect on biofilm produc-tion as there were a clear different between isolates treated with oil compere to that without treatment. (Ta-ble 4)

Table 3: Production of protease phenotype by *P.aeruginosa* on skim milk media. T test *P* value is 0.0067.

Control OD	After treatment OD	Before treatment OD	No. of isolate
0.1006	0.22	0.74	PA1
0.1006	0.103	0.199	PA2
0.1006	0.226	0.96	PA3
0.1006	0.078	0.186	PA4
0.1006	0.102	0.294	PA5
0.1006	0.09	0.168	PA6
	0.136	0.424	**Mean**
	0.067	0.339	**SD**

Table 4: Production of biofilm phenotype by *P. aeruginosa*, spectrophotometrically scanned at 590 nm. T test *p* value is 0.0067.

After treatment Inhibition zoon mm	Before treatment Inhibition zoon mm	No. of sample
6	12	Ps1
3	9	Ps2
5	24	PA3
11	16	Ps4
2	15	Ps5
7	23	Ps6
5.66	16.5	**Mean**
3.2	5.95	**SD**

3.3 Virulence genes expression

To fine out the clove oil effect on the aprA and pelF genes expression in P. aeruginosa, Real Time PCR were performed to compere the expression of both genes before and after the clove oil treatment.

Results shows that expression of both aprA and pelF genes belonged to bacterial isolates treated with clove oil were significantly less than those genes belonged to bacterial isolates without treatments, with P values 0.0010 and 0.0004, respectively (Fig.1).

Further investigation of the clove oil effect was con-ducted on the gene's expression phenotypes using the ELISA technique for both protease and biofilm. ELISA results showed a significant phenotype difference be-tween the bacterial isolates treated with clove oil and those without treatment (Fig.2).

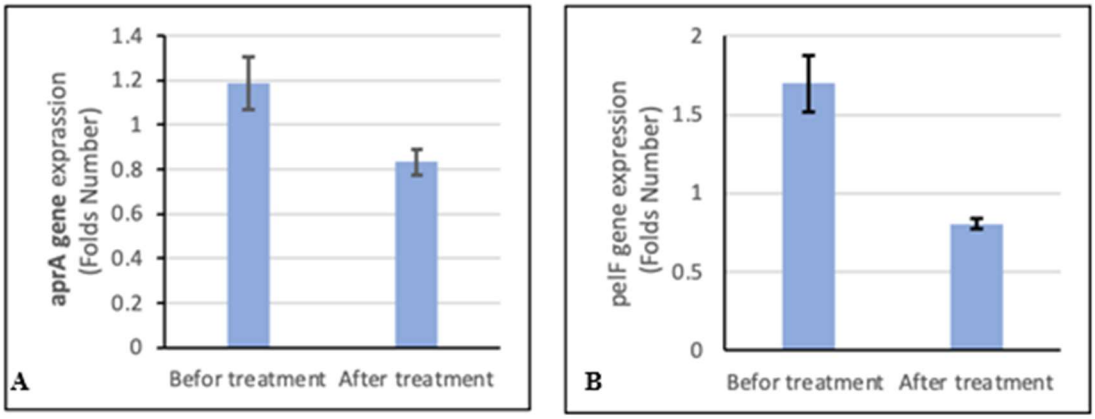

Figure 1: RT PCR results of virulence genes expressions of *Pseudomonas aeruginosa* studied in this paper. A, The different between the expression of aprA before and after clove oil treatments, *P* value is 0.0010. **B,** The different between the expression of pelF before and after clove oil treatments, *P* value is 0.0004.

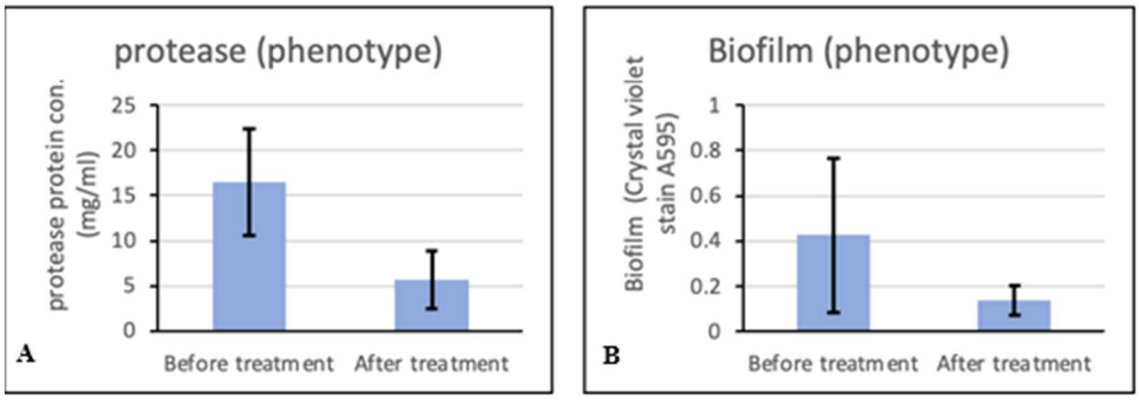

Figure 2: ELISA results of virulence genes phenotypes of Pseudomonas aeruginosa studied in this paper. A, the different between the concentration of protease enzyme before and after clove oil treatments, Pvalue is 0.0067. **B,** the different between biofilm production before and after the clove oil treatments, Pvalue is 0.024.

4. DISCUSSION

Essential oils can be defined as plant-extracted oils that have volatile aromatic activity based on a variety of fundamental chemical substances. Hundreds of differ-ent oils extracted from different plants are distributed commercially [18]. The efficiency of these oils was in-vestigated by many studies, particularly, low doses us-ing of these oils against problematic multi-drug resistant microbes [24, 25]. The activity of these oils belongs to many biochemical compounds that are present in sup-plier plants [26]. Chemical analysis studies showed the main components of these

plants include 93% car-vacrol, 1%eugenol, 0.8% P-cymene and 0.6% thymol. Most of these compounds have an inhibition ability to many bacteria and their virulence factors [27].

Likewise, clove essential oil which is belonged to Syzyg-ium aromaticum has an important value as food pre-servative and it has been used for medical applications for centuries due to its activities of antimicrobial and antioxidant [28]

In this study we used clove essential oil against six mul-tidrug resistance isolates of a medically important mi-crobe, Pseudomonas aeruginosa. The aim is to find out the clove oil effect on the bacterial production of bio-film and protease enzyme. All bacterial isolates are forming biofilm and all of them produce protease en-zyme.

The minimum inhibition concentration of clove oil showed a convergence between the isolates chosen for the study, which ranged from 0.005 to 0.01 mg/ml table 2 this result identical other study [29].

When the isolates were treated with clove oil the prote-ase production enzyme showed a significant difference between the two statements, with and without clove oil (Table 3). Ps5 isolate showed the highest affected iso-late as the diameter of protease zoon reduced from 15mm to 2mm after treatment. Other bacterial isolates showed affected also with a clove oil treatment. Husain et al. (2013) reported in their paper that clove oil has a significant effect on the production of bacterial prote-ase, confirming our results [16].

Regarding the clove oil treatment effect on biofilm formation, the result showed a significantly reduce in its formation, with variable biofilm production. This result was obtained when we used sub-inhibitory concentra-tion of clove oils and compared it to that of the control ($P<0.05$) (Table 4). Similar results were stated by Husain et al. (2013) and Islamieh et al. (2019) as they con-ducted their experiments on other types of bacteria, as well as on P. aeruginosa bacteria [16, 29].

To find out what is the reason behind these changes in the bacterial enzymatic behavior, Real Time PCR tech-nique was used. The aim is to examine the effect of clove oil on the expression of bacterial genes that in-volved in the production of protease enzyme and bio-film formation. The study target genes, PelF and AprA, are involved in biofilm production and protease en-zyme respectively [2,8]. In this work, the inhibitory im-pact of clove oil on the gene expression of the PelF and AprA was tested. The results showed significant inhibi-tion of the expression of both genes in the bacterial iso-lates treated with the essential oil compared to those isolates without treatment (Fig 1). This genotype out-come was confirmed by testing the phenotype results using ELISA technique as it gave compatible results to that obtained by Real-Time PCR (Fig. 2). This outcome stated that the clove essential oil has a great effect on the expression of important virulence genes for P. ae-ruginosa bacterial to start and produce serious infec-tions [29]. Similar results were obtained by previous studies which conformed our outcome. For example, Jayalekshmi reported that clove essential oil has positive effect on P. aeruginosa virulence genes [30]. Therefor clove essential could be used as an alternate treatment for solve problems of multi-drug resistant (MDR) P. aeruginosa infections.

CONCLUSIONS

We can conclude that the clove essential oil, which be-longed to Syzygium aromaticum, has a positive effect on two essential virulence genes within P. aeruginosa. Treating with this oil leads bacteria to lack their ability to produce protease enzyme and the formation of bio-film.

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