Trop J Nat Prod Res, March 2024; 8(3):6498-6502 **ISSN 2616-0684 (Print)**
ISSN 2616-0692 (Electronic)

Tropical Journal of Natural Product Research

Available online at https://www.tjnpr.org

Original Research Article

The Effect of Sub-Inhibitory Concentration of Clove Essential Oil on the Expression of *Pseudomonas aeruginosa* Virulence genes

Fatimah A. Awad[1], Mohammed R. Mohaisen[1,2]*, Karama T. A. Al-Taee[1]

[1]*Department of Basic Sciences, College of Dentistry, University of Anbar, Ramadi, Iraq.*
[2]*Department of Clinical Infection, Microbiology and Immunology, Institute of Infection, Veterinary, and Ecological Sciences, Faculty of Health and Life Sciences, University of Liverpool, Liverpool, L69 7TX, United Kingdom*

ARTICLE INFO

Article history:
Received 02 September 2023
Revised 29 February 2024
Accepted 06 March 2024
Published online 01 April 2024

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ABSTRACT

Clove essential oil (CEO) has been reported to be used in the treatment of bacterial, fungal, and yeast infections. It has also been reported to have positive impact on the virulence factors in some pathogenic bacteria. This study aims to investigate the growth inhibitory activity of CEO against *Pseudomonas aeruginosa* and its effect on the expression of key virulence genes for biofilm formation and protease production. The antibacterial activity in respect to the minimum inhibitory concentration (MIC) of clove oil against six multidrug-resistant *Pseudomonas aeruginosa* strains was evaluated using the broth dilution method. The cytotoxic, protease production, and biofilm inhibitory effects of CEO were evaluated following standard protocols. The effect of the oil at sub-MIC concentrations on the expression of the virulence genes; *aprA* and *pelF* was evaluated by Real-Time Polymerase Chain Reduction (RT-PCR) technique. Treatment with CEO resulted in a significant inhibition of biofilm formation and protease production in *Pseudomonas aeruginosa*. RT-PCR analysis showed a reduction in the expression of the two virulence genes; *pelF and aprA*. These findings suggest a potential for the use of CEO as alternative antibacterial agent for the treatment of infections due to multidrug-resistant *Pseudomonas aeruginosa*.

***Keywords*:** Clove oil, *Pseudomonas aeruginosa*, Wound infections, Virulence Genes, Essential oil.

Introduction

Pseudomonas aeruginosa is one of the most common opportunistic bacterial pathogens that causes nosocomial infections, especially burn and wound infections.[1] Due to their prominent virulence factors and multidrug resistance attributes, the infections due to this bacterium are very severe.[2-5] Burn deaths have been associated with *Pseudomonas* infection in over 75% of cases.[6,7] Biofilm and basal protease enzyme are the medically important virulence factors responsible for the invasiveness and disease transmission by this bacterium, especially in the resistant strains.[3,8-10] It has been reported that 65 to 80% of infectious diseases are caused by strains that have a tendency to form biofilm.[11] There is a close connection between these virulence factors and the communication of bacterial cells with small signaling molecules that regulate the expression of several genes in the bacterial cell, including the virulence genes. This communication is termed Quorum Sensing.[12,13] There are two kinds of Quorum Sensing systems in *P. aeruginosa*; Rhl AHL-based systems, as well as Las AHL-based systems. The outcomes of the later system are proteins of LasB and LasR. LasR regulates the production of LasB protein which is one of the important proteins involved in biofilm formation in the bacteria.[14] The gene that encodes the production of the *P. aeruginosa* protease enzyme is aprA gene.[15] Three polysaccharides: Algenate, Psl and Pel are involved in the formation of biofilm in bacterial cells.[16]

*Corresponding author. E mail: den.m.radhi@uoanbar.edu.iq
Tel: 009647806347795

Citation: Awad FA, Mohaisen MR, Al-Taee KTA. The Effect of Sub-Inhibitory Concentration of Clove Essential Oil on the Expression of *Pseudomonas aeruginosa* Virulence genes. Trop J Nat Prod Res. 2024; 8(3):6498-6502. https://doi.org/10.26538/tjnpr/v8i3.4

Official Journal of Natural Product Research Group, Faculty of Pharmacy, University of Benin, Benin City, Nigeria

A total of seven genes encode the proteins involved in Pel exopolysaccharide synthesis; PelA, PelB, PelC, PelD, PelE, PelF, and PelG.[17,18] The severity of the infection caused by *P. aeruginosa* is not determined only by virulence factors, but also by the presence of multidrug-resistant strains. Multidrug-resistant *P. aeruginosa* are associated with high mortality worldwide.[19] Disease such as persistent cystic fibrosis caused by multidrug-resistant *P. aeruginosa* spreads rapidly from patients to patients leading to a high possibility of an outbreak, and therefore, the need for extreme caution in the management of such infection.[20,21] The role of plants' essential oils like *Syzygium aromaticum* essential oil commercially called clove essential oil (CEO) in the treatment of bacterial infections has been emphasized in several studies.[22-27] The influence of the components of CEO on several bacterial virulence factors have been evaluated. These include factors that are controlled by Rhl and Las genes, including protease, LasB, swarming, and the production of exopolysaccharides and proteins like pyocyanins and chitinases.[28] According to the American Food and Drug Administration (FDA), CEO is harmless when used in foods and for medical treatments.[29] The present study aims to investigate the effect of clove essential oil on pelF and aprA expression in multidrug-resistant strains of *P. aeruginosa*.

Materials and Methods

Plant collection and identification

Clove (*Syzygium aromaticum*) flower buds were collected from Baghdad city national parks and gardens, from the middle of April to the end of May, 2022. The plant material was identified and authenticated by Assistant Professor Dr. Sukeyna Abaas Aliwy, College of Sciences, Department of Biology, University of Baghdad, Iraq, (Plant material's voucher number: BUH143759).

Extraction of essential oil

Clove essential oil was extracted by steam distillation using a Clevenger apparatus. The volatile components in clove flower buds were

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vaporized at about 100°C. The essential oil obtained was dried with sodium sulphate and kept at 4°C until further experiments.[30]

Collection and identification of bacteria strains

Pseudomonas aeruginosa strains were isolated from patients with wound infection at Ramadi Hospital, Iraq. Identification of the bacteria strains was done in the Microbiology laboratories, Department of Biology, College of Sciences, University of Anbar, Iraq. Bacterial samples were collected from the wounds using sterile swabs, and then transferred into sterile white tubes containing 0.5 mL of physiological fluid. The samples were cultured on Blood Agar plates and incubated at 37°C for 24 h. *P. aeruginosa* strains were identified using morphological and biochemical tests as previously described.[31,32]

Determination of MIC of clove oil

Clove oil was dissolved in dimethyl sulfoxide (DMSO) to prepare a stock essential oil (EO) solution at a final concentration of 0.1 mg/mL. The stock solution was kept in a dark place at 4°C. The EO stock solution was diluted with Mueller Hinton Broth into eight serial dilutions (0.012, 0.011, 0.01, 0.009, 0.008, 0.007, 0.006, and 0.005 mg/mL) to 2 mL final volume. The EO dilutions (1 mL each) were added to eight different tubes containing 2 mL of 9×105 cells/mL *P. aeruginosa* culture in Mueller Hinton Broth. After mixing, the tubes were incubated at 37°C. After overnight incubation, the tubes were examined for the presence of turbidity which is an indication of microbial growth. The last tube that showed no growth indicated by a clear medium was considered as the minimal inhibitory concentration of the EO. The experiment was done in duplicate. Test without the oil was used as the negative control.

Cytotoxicity test

The cytotoxicity of clove essential oil was assessed using the red blood cell haemolytic assay.[33] Eight millilitres (8 mL) of the oil was placed into a 10 mL tube containing 1 mL of 1:5 dilution of red blood cells, and then shaken slightly at 37oC for 30 min followed by centrifugation at 1000 rpm for 5 min.[33] The red blood cell hemolysis in the test sample was compared to the control tube that contained blood only without the essential oil.

Protease production inhibition test

The protease production inhibitory capacity of the oil was evaluated on skim milk agar according to the method described by Vijayaraghavan *et al*. (2013).[34] After overnight incubation at 37°C, the diameter of the clear zone surrounding each well was measured to determine the protease activity.

Biofilm inhibition assay

Microtiter plates (96-well) were used to grow biofilm with or without sub-MIC of clove oil. The plates were then read at a wavelength of 590 nm using an ELISA microplate reader.[35]

Real-time PCR analysis

Extraction of RNA

A sub-MIC concentration of clove essential oil was added to LB broth containing bacterial isolates and incubated at 37°C overnight. RNA was extracted using the Accuzol Reagent according to the manufacturer's protocol.

Real-time PCR

Primers used in the Real-time PCR experiments are listed in Table 1. These primers were specific for the genes: *gyrB, aprA*, and *pelF*. Following the manufacturer's procedure, RNA was extracted from both the clove oil-exposed bacteria, as well as from the non-oil-grown bacteria. The efficiency of each pair of primers for both samples were confirmed using a standard curve analysis. Gene expression levels for the *aprA*, *gyrB* and *pelF* genes were measured using RT-PCR under the following conditions: one cycle at 95°C for 3 min, 35 cycles at 95°C for 30 sec, 35 cycles at 54°C for 30 sec, 35 cycles at 72°C for 1 min, and finally 35 cycles at 72°C for 10 min. Housekeeping gene *gyrB* was used as an internal control.

The Ct value Calculation

The quantitative levels for the *pelF* and *aprA* genes, which resulted from Real-Time-PCR experiments was calculated using the Delta Delta Ct method.[36]

Table 1: The sequences of Real Time-PCR Primers

Primer	Sequence	Size
apr A-F	GACGTCACCAATATCCACT	341bp
apr A-R	CAGTAGCTCATCACCGAATA	
pel F–F	GTTCGAGCTGAGCAGTT	202bp
pel F–R	ATCAGCGGCACGAAGA	
gyr B –F	GCGTGGGTGTGGAAGT	191bp
gyr B –R	GGTGGCGATCTTGAACTTCT	

Results and Discussion

Essential oils are volatile, aromatic oils extracted from plants containing potentially beneficial compounds. A wide variety of essential oils are commercially available from a variety of plants.[30] There have been numerous studies evaluating the efficiency of these oils, particularly their use in the treatment of problematic multidrug-resistant microbes.[37,38] Many biochemical compounds present in the source plants contribute to the activity of these oils.[39] Chemical analysis of clove oil showed carvacrol as a principal component of the oil accounting for 93% of the essential oil, while constituents like eugenol, p-cymene, and thymol were present in smaller amount (1%, 0.8%, and 0.6%, respectively). These compounds were found to inhibit virulence factors and bacterial growth in a wide variety of bacteria.[40] The essential oil of clove, (*Syzygium aromaticum*), has old-time antimicrobial and antioxidant properties making it an important food preservative.[41] Through interactions with bacterial cells, polysaccharides, and several bacterial enzymes, including proteases, CEO has bactericidal activity against a broad spectrum of bacteria. The CEO compound was found to be effective against the lipopolysaccharide layer of the outer membrane of many Gram-negative bacteria, including *P. aeruginosa*.[42]

In the present study, clove essential oil was tested against six isolates of *Pseudomonas aeruginosa* with multidrug resistance properties. We hypothesized that clove essential oil affects two of the most important virulence genes (*aprA* and *pelF*) of *P. aeruginosa*. Bacterial protease production as well as biofilm formation is dependent on these two genes.

All isolates of *P. aeruginosa* were tested for their sensitivity to clove essential oil using eight concentrations (0.0012, 0.0011, 0.001, 0.009, 0.008, 0.007, 0.006 and 0.005 mg/mL). The MIC against these isolates is shown in Table 2. The MIC were determined on the basis of the presence or absence turbidity which is indicative of bacterial growth or inhibition. The tube with the lowest concentration that showed no growth among the eight serial dilutions was taken as the MIC. From the results as presented in table 2, the MIC of CEO against the various P. aeruginosa strains ranged from 0.009 to 0.006 mg/mL. A Similar outcome was obtained by previous studies that examined the effect of CEO on a wide spectrum of pathogenic gram-positive and gram-negative bacteria including *P. aeruginosa*.[43-45]

To examine the ability of CEO to inhibit the synthesis of bacterial protease, skim milk agar was used to culture both treated and untreated bacterial isolates. There was a significant decrease in the level of protease synthesis in the bacterial isolates that were treated with CEO compared to bacterial isolates without CEO treatment (Table 3 and Figure 1). The work of Husain *et al*. (2013), reported the significant ability of clove essential oil to inhibit the synthesis of bacterial protease, which is in agreement with the result of the present study.[28] in addition, the study by Chen *et al.* (2023).[46] further substantiated the claim that clove essential oil has an inhibitory activity against bacterial protease enzyme as demonstrated by its inhibitory effect on *Campylobacter jejuni* protease.[46]

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With respect to the effect of CEO on bacterial biofilm formation, an ELISA technique was used to test the effects of CEO on gene phenotypes. The biofilm phenotype of bacteria treated with clove oil differed significantly from the untreated bacteria. From the ELISA results, the formation of bacterial biofilm was clearly inhibited by the presence of CEO in the bacterial culture (Table 4, Figure 1). Similar outcomes were reported by previous studies on the effect of CEO on *P. aeruginosa* as well as on other bacterial species, such as Aeromonas hydrophila and Multidrug-Resistant Salmonella enteritidis.[28,45,47]
Finally, to examine what is responsible for the observed modification of the bacterial enzymes phenotypes after clove oil treatment, RT-PCR was conducted. The purpose was to determine whether clove oil affects bacterial enzyme production and biofilm formation by affecting the expression of genes involved in both processes. Two genes were targeted by Real-Time PCR experiment, *pelF* which is involved in the production of bacterial biofilm, and *apsA* which encodes the production of protease enzyme.[32,40] Clove essential oil impact on the *pelF* and *aprA* expression was investigated by Real-Time PCR analysis of the gene expression before and after clove essential oil treatment. The result showed a significant reduction in the expression of *pelF* and *aprA*, with *P* values of 0.00102 and 0.0005, respectively (Figure 1). This result confirmed the results obtained from the phenotype experiments for both biofilm formation and protease enzyme production thereby revealing the significant effect of CEO on two of the most important *P. aeruginosa* virulence genes.[45] Previous studies have shown results that are consistent with the findings from the present study. For example, the study of Jayalekshmi *et al.* (2019) confirmed the reduction of *P. aeruginosa* virulence genes expression following treatment with essential oil.[48] Therefore, on the basis of its antimicrobial properties, clove essential oil may serve as an alternative remedy for multidrug-resistant *P. aeruginosa* infections.

Conclusion

The essential oil extracted from the clove plant (*Syzygium aromaticum*), has demonstrated a significant inhibitory effect against *Pseudomonas aeruginosa* key virulence factors by reducing the expression of *pelF* and *aprA* the two virulence genes that encodes biofilm formation and protease production in *Pseudomonas aeruginosa*. When multidrug-resistant strains of *Pseudomonas aeruginosa* are treated with CEO, their ability to produce these virulence factors (biofilm formation and production of protease enzyme) is greatly hindered. This outcome indicates that CEO has a potential as alternative antibacterial agent, that could be used in the treatment of severe bacterial infections caused by *Pseudomonas aeruginosa*.

Conflict of Interest

The authors declare no conflict of interest.

Authors' Declaration

The authors hereby declare that the work presented in this article is original and that any liability for claims relating to the content of this article will be borne by them.

Acknowledgments

The authors would like to acknowledge the Microbiology laboratories, Department of Biology, College of Science, University of Anbar, Iraq, for their assistance and support in preparing the main part of this work for publication.

Table 2: Minimum Inhibitory Concentration (MIC) of clove essential oil against six strains of *P. aeruginosa*

Strain	MIC (mg/mL)	Bacterial isolates No
S.1	0.006	0.005
S.2	0.006	0.005
S.3	0.007	0.006
S.4	0.009	0.008
S.5	0.008	0.007
S.6	0.09	0.008

Table 3: *P. aeruginosa* protease phenotype before and after clove essential oil (CEO) treatment

Sample No	Inhibition zone diameter (mm) Before CEO treatment	Inhibition zone diameter (mm) After CEO treatment
S.1	13	7
S.2	10	5
S.3	25	6
S.4	15	12
S.5	14	3
S.6	24	8
Mean	16.8	6.83
S.D	6.17	3.06

Table 4: *P. aeruginosa* biofilm production phenotype before and after clove essential oil treatment

Bacterial isolates No.	Control O.D.	O.D. Before CEO treatment	O.D. After CEO treatment
S.1	0.1006	0.74	0.22
S.2	0.1006	0.199	0.103
S.3	0.1006	0.96	0.226
S.4	0.1006	0.186	0.078
S.5	0.1006	0.294	0.102
S.6	0.1006	0.168	0.09
Mean		0.423	0.135
S.D		0.338	0.066

O.D. = Optical density.

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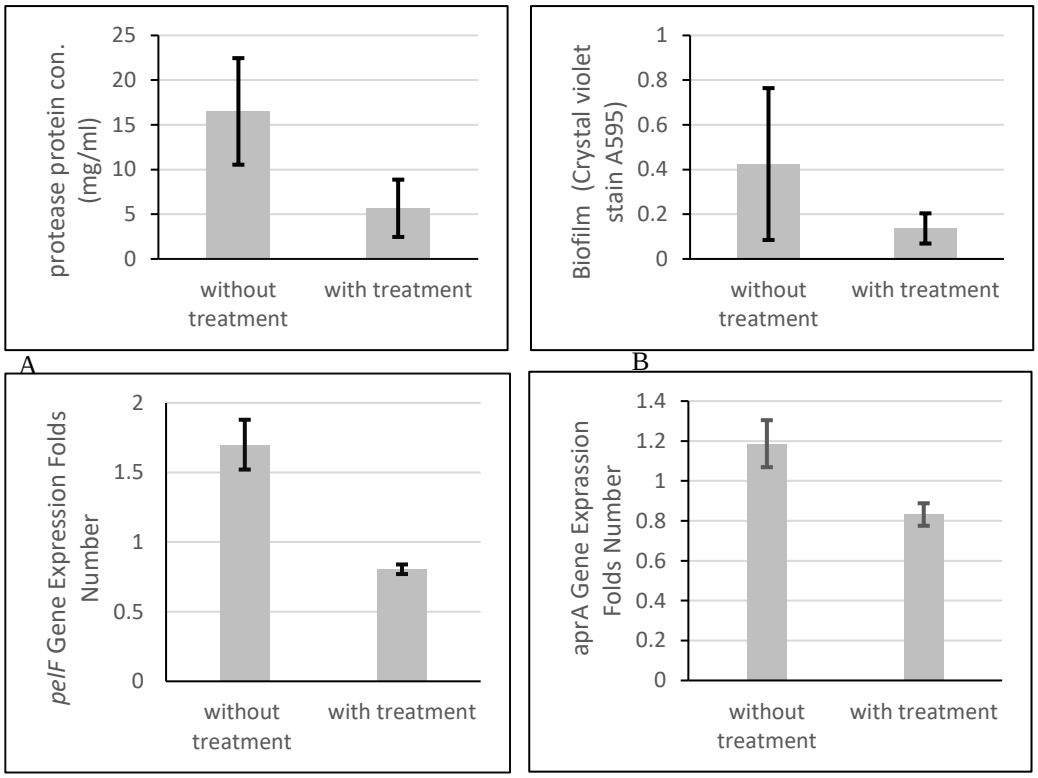

Figure 1: The *Pseudomonas aeruginosa* virulence genes' phenotypes, and the RT-PCR results of *Pseudomonas aeruginosa* virulence gene expression. A: The protease enzyme production with and without CEO treatment (P = 0.007). B: The formation of biofilm with and without CEO treatment (P = 0.02). C: The *pelF* expression with and without CEO treatment (P = 0.0005). D: The *aprA* expression with and without CEO treatment (P = 0.00101).

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