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Research Article

The Effect of *Ferula assa-foetida* L and *Carum copticum* Hydroalcoholic Extract on the Expression Levels of *Staphylococcus aureus* Genes Involved in Quorum Sensing

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Abstract

Background: Quorum sensing is a microbial cell-to-cell communication process. Quorum sensing bacteria produce and release extracellular messenger molecules called autoinducers. Gram-positive and Gram-negative, homoserine lactones, and oligopeptides are autoinducers used to communicate and regulate gene expression.
Objectives: The goal of this study was to assess the impact of subinhibitory concentrations of *Ferula assa-foetida* l oleo-gum resin and *Carum copticum* fruit on the expression of *tst* and *hld* genes of methicillin-resistant *Staphylococcus aureus* (MRSA) and methicillin-sensitive *S. aureus* (MSSA) strains.
Methods: This analytical study was performed using standard strains of MRSA (ATCC 33591) and MSSA (ATCC 29213). Suspensions of MRSA and MSSA bacteria were incubated at 37°C for 7 and 16 hours in the presence of ethanol extracts from *F. assa-foetida* and *C. copticum*. The expression of the *hld* and *tst* genes was then assessed using the real-time PCR protocol and SYBR Green Master Mix. The data analysis was carried out using the $2^{-\Delta\Delta CT}$ method.
Results: The *hld* gene expression (RNAIII) of MRSA after 7 and 16 hours of exposure to the sMIC of the *F. assa-foetida* extract showed a fold change of -1 and 0.08, respectively, in comparison with controls. After 7 and 16 hours of exposure to the sMIC of the *C. copticum* extract, the fold change was -0.23 and -0.27, respectively. After exposure to the sMIC of the *C. copticum* extract for 16 hours, the fold change in the expression of the *tst* (TSST-1) MSSA gene was 0.37 lower than that of the control sample.
Conclusions: The results indicate that sMICs of ethanol extracts from *F. assa-foetida* and *C. copticum* can be used to control the expression of virulence genes in pathogenic bacteria, such as MRSA and MSSA.

Keywords: Methicillin-resistant *Staphylococcus aureus*, Methicillin-sensitive *S. aureus*, *Ferula assa-foetida* L, *Carum copticum*

1. Background

Staphylococcus aureus is a well-known and versatile pathogen, which causes a wide variety of infections, such as cutaneous infections, furunculosis, impetigo, septic shock, scalded skin syndrome, and toxic shock syndrome (1). *Staphylococcus aureus* secretes various proteins and virulence factors, including toxins, hemolysins, and tissue-degrading enzymes, at the end of an exponential growth period, with the expression levels regulated by a network of interacting regulators in a process called quorum sensing (2). Quorum sensing is a mechanism by which a bacterial population receives input from adjacent cells and generates an appropriate response, enabling the bacteria to survive within the host (3). This regulatory system includes the SarA protein family and two-component systems, including agr and sae (4). The accessory gene regulator locus (agr) of *S. aureus* is a quorum sensing gene cluster of five genes (*hld*, *agrA*, *agrB*, *agrC*, and *agrD*) (5). The *agr* locus, which controls approximately 150 genes, is critical to *S. aureus* virulence (6). The *hld* gene encodes δ-hemolysin and a fragment of RNAIII, which acts as the effector of the *agr* system.

Quantification of the δ-toxin reflects the agr activity of *S. aureus* (7, 8). δ-hemolysin is a 26-amino-acid peptide and heat-stabile protein produced by 97% of *S. aureus* strains. The toxin is capable of causing membrane damage in a wide range of cells and organelles. It is also able to bind and exert toxic effects in immune cells (7). The *tst* gene of *S. aureus* encodes TSST-1, which is controlled

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by the agr system. TSST-1 is the key causative agent of toxic shock syndrome, which is an acute-onset and potentially life-threatening staphylococcal syndrome (9). In addition, δ-hemolysin and TSST-1 are superantigens and exotoxins. Bacterial resistance to antibiotics is a rapidly growing problem in both hospital and community settings. The need for alternative antimicrobial agents has led to increased research on the use of treatments for infections based on natural resources (10).

The genus *Ferula* belongs to the *Apiaceae* family. It is represented by about 140 species in the Mediterranean area and central Asia. Oleo-gum resin obtained from exudates of rhizomes of *Ferula assa-foetida* has commercial uses. It has also long been used as a folk phytomedicine for the treatment of different diseases, such as asthma, epilepsy, flatulence, intestinal parasites, poor digestion, and influenza (11).

Carum copticum also belongs to the *Apiaceae* family. Traditionally, *C. copticum* has been used as a treatment for various ailments, including bloating, indigestion, respiratory distress, and diarrhea. Other health benefits of *C. copticum* include antibacterial, antioxidant, and antiparasitic effects (12). Despite research on the antibacterial effects of plant extracts, little is known about the effects of these extracts on the expression of bacterial pathogenicity and virulence factors.

2. Objectives

The goal of this study was to assess the impact of subinhibitory concentrations *F. assa-foetida* oleo-gum resin and *C. copticum* fruit on the expression of *tst* and *hld* genes in methicillin-resistant *S. aureus* (MRSA) and methicillin-sensitive *S. aureus* (MSSA) strains.

3. Methods

3.1. Bacterial Strains and Reagents

MRSA (ATCC 33591) and MSSA (ATCC 29213) strains were obtained from the Pasteur institute of Iran. *F. assa-foetida* L (oleo-gum resin) plants and *C. copticum* fruit were obtained from the medicinal plants research center of Isfahan university.

3.2. Extraction of Plant Materials

Dry plant materials were ground into a fine powder using a homogenizer. Ethanolic extracts were obtained from the plant samples by soaking them in flasks containing 95% denatured EtOH in a ratio of 2.5 g of plant material to 50 mL of EtOH for 72 hours. The flasks were agitated daily. Stock concentrations of 50 mg/mL of dry extract in an excipient (DMSO or dH_2O) were prepared, sterile filtered (0.2 μm), and stored in darkness at 25°C.

3.3. Antimicrobial Susceptibility Assays

3.3.1. Determination of Minimum Inhibitory Concentrations (MICs)

The MICs of *F. assa-foetida* and *C. copticum* for MRSA and MSSA were assessed in triplicate, using the microdilution method as recommended by the clinical and laboratory standards institute (CLSI) (13). The MIC was defined as the lowest drug concentration that inhibited growth.

3.3.2. Well Diffusion Assay

The susceptibility of the bacteria to the plant extracts was first determined using a well diffusion assay, following the method described in the CLSI manual (14). Each dried plant extract sample was dissolved and prepared as described. The bacteria were cultured overnight (24 hours) on nutrient agar at 37°C for the preparation of cell suspensions. The bacterial cell suspensions were adjusted to a 0.5 McFarland standard (10^7 CFU/mL) and spread evenly over the entire surface of the agar plates using a sterile cotton swab. Using a cork borer, 6-mm wells were made on the plates. The plates were allowed to air-dry. Then, 150 μL of the *F. assa-foetida* (50 mg/mL) and *C. copticum* (25 mg/mL) extracts were added to the wells, and the same concentrations of ethanol and ampicillin (10 μg) were added as a negative control and positive control, respectively. The tests of each extract were replicated three times. The plates were then incubated at 37°C for 24 hours. The diameter of the zone of inhibition was measured in millimeters, and the results were recorded.

3.4. Bacterial Cultures

For all the experiments, an overnight culture was made from bacterial frozen stock in 5 mL of BHI medium (Lio Filchem, Italy) at 37°C. Isolated colonies were adjusted to a turbidity equivalent to that of a 0.5 McFarland standard corresponding to 10^8CFU/mL, as confirmed by bacterial counts. When the optical density reached a turbidity equivalent to that of 1.5×10^5CFU/mL, concentrations of 25 mg/mL and 12.5 mg/mL of the sMIC of each plant extract (*F. assa-foetida* and *C. copticum*, respectively) were added to glass culture tubes. Cultures with or without antibiotics (growth control) were incubated at 37°C, with shaking. Aliquots were taken 7 and 16 hours later.

3.5. RNA Extraction and cDNA Synthesis

Total RNA was extracted using an RNX-Plus solution (Cinnagen, Iran) kit based on the manufacture's protocol, with some modifications. Briefly, from each sample,

1 ml was transferred to a sterile 1.5 mL microtube and centrifuged at 6708 × g for 5 minutes. The pellet was washed three times with phosphate-buffered saline and centrifuged at 1677 × g for 5 minutes. Then, 200 μL of ice-cold RNX-Plus lysis buffer were added to each tube, homogenized by vortexing for 10 seconds, and incubated at 70°C for 30 minutes. The vortex process was repeated every 5 minutes (15). Subsequent RNA extraction steps followed the manufacturer's instructions. After extraction, each sample was treated with DNase I (Thermo Scientific, U.S.) according to the manufacturer's instructions. RNA integrity was analyzed by electrophoresis on a 1% agarose gel (Cinnagen, Iran). RNA quantification was performed by measuring the absorbance at 260 nm. Nucleic acid purity was assessed by measuring the ratio of the absorbance at 260 nm and 280 nm. Extracted RNAs were stored at -70°C until required for the experiments. The cDNA synthesis of each sample was measured using a cDNA synthesis kit (Thermo Scientific, U.S.), following the manufacturer's instructions. All the samples were stored at -20°C until used in the analysis.

3.6. Relative Quantitative Real-Time PCR

SYBR Green Real-Time PCR Master Mix (Thermo Scientific, U.S.) was used for amplification and the real-time PCR analysis, according to the manufacturer's instructions.

The primers (Pishgam, Iran) used were *tst*-F: 5′-ATTTGTTCACTGTGTCGATAATCC-3′; *tst*-R: 5′-GGAGTGATTTCAATGGCACAAG-3′; *hld*-F: 5′-CTGATGCTGCCATCTGTGTT-3′; *hld*-R: 5′-GTAAGCCCTTTGTTGCTTGC-3′; 16SrRNA-F: 5′-CTGCTGCCTCCCGTAG-3′; and 16SrRNA-R: 5′-CCGACCTGAGAGGGTGA-3′ (16-18).

The amplification was done with the Applied Biosystems StepOne (Thermo Fisher Scientific, ABI, U.S.) system and proceeded as follows: denaturation at 95°C for 5 minutes and then 40 cycles, including denaturation at 95°C for 1 minute, annealing at 55°C for 30 seconds, and 72°C for 30 seconds. The final extension was done at 72°C for 1 minute. A negative control was included in each run. All the samples were analyzed in triplicate, and the 16SrRNA housekeeping gene served as an internal control to normalize the expression levels of the samples. The relative expression levels were analyzed by the $\Delta\Delta$CT method described in the Applied Biosystems user bulletin no. 2 (19).

3.7. Statistical Analysis

The relative expression ratios were calculated by the $\Delta\Delta$Ct method, and the statistical analysis was done using a Chi-square test with SPSS vs. 16.0.

4. Results

4.1. Determination of the MIC

The MIC of the *F. assa-foetida* extract for the MSSA and MRSA strains was 25 mg/mL and 50 mg/mL, respectively. The MIC of the *C. copticum* extract for the MSSA and MRSA strains was 25 mg/mL.

4.2. Well Diffusion Assay

The inhibition zone diameter (IZD) of the *F. assa-foetida* extract for the MSSA and MRSA strains was 18 mm and 16 mm, respectively. The IZD of the *C. copticum* extract for the MSSA and MRSA strains was 13 mm and 14 mm, respectively. The IZD for the positive control (ampicillin, 10 μg) was 12 mm for MSSA and 30 mm for MRSA. Ethanol was negative in all the samples.

4.3. Effects of F. assa-foetida on the Expression of MRSA hld and tst Genes

The goal was to examine the influence of a subinhibitory concentration of *F. assa-foetida* (25 mg/mL) on *hld* and *tst* gene expression. Real time-PCR was performed to detect the relative expression levels of the *hld* and *tst* in cultures of the MRSA strain, grown with and without *F. assa-foetida* for 7 and 16 hours. The results represent the differences in *hld* mRNA levels detected in the presence of *F. assa-foetida*, relative to the *hld* mRNA level of the growth control. As expected, the transcription levels of the *hld* gene of MRSA declined significantly in response to the *F. assa-foetida* treatment, whereas no effects were observed on the gene expression of *tst*. The values presented are the means ± SD of three repeated experiments) (Table 1).

Table 1. Relative Gene Expression Levels of the *S. aureus* ATCC 29213 *hld* gene After Treatment With 12.5 mg/mL and 25 mg/mL of *C. copticum* and *F. assa-foetida*, Respectively, After 7 and 16 Hours

Gene	Product	Fold Change ± SD[a]
FII hld	δ-toxin	-1 ± 1.2
FIII hld	δ-toxin	-0.08 ± 0.43
CII hld	δ-toxin	-0.23 ± 0.37
CIII hld	δ-toxin	-0.27 ± 1.6

[a]The real-time PCR results represent the mean of three biological replicates, with three technical replicates for each gene. SD, standard deviation.

4.4. Effects of Carum copticum on the Expression of MRSA hld and tst Genes

The transcription levels of the *hld* gene were significantly decreased in the MRSA strain (ATCC 33591) upon treatment with *C. copticum* (Table 1). When the strain was

cultured with a 12.5 mg/mL of *C. copticum* (sMIC), the transcriptional levels of *hld* decreased by -0.23 $\pm$ 0.37 and -0.27 $\pm$ 1.6 after 7 and 16 h, respectively. *Carum copticum* had no effects on *tst* gene expression of MRSA strains.

4.5. Effect of F. assa-foetida and Carum copticum on the Expression of MSSA hld and tst Genes

The influence of subinhibitory concentrations of *F. assa-foetida* and *C. copticum* (25 mg/mL and 12.5 mg/mL, respectively) on *hld* and *tst* gene expression of the MSSA strain (ATCC 29213) was investigated. *Carum copticum* at a sMIC of 12.5 mg/mL inhibited *tst* gene expression of MSSA after 16 hours of exposure (fold change of -0.37 $\pm$ 0.07), but it had no effect on *hld* gene expression. *Ferula assa-foetida* had no effect on the expression of the *tst* or *hld* genes in the MSSA strain.

5. Discussion

The increasing incidence of bacterial infections due to MRSA and the resistance of *S. aureus* to many commonly used antibiotics, such as macrolides, tetracyclines, and aminoglycosides, have reduced the number of therapeutic options (20). *Staphylococcus aureus* produces virulence factors, which play an important role in the pathogenesis of infection.

Studies have shown that the use of antibiotics and medicinal plants at suboptimal concentrations can interfere with the translation of one or more virulence factors and gene products, such as exoprotein-encoding genes, in *S. aureus* (21). For example, subinhibitory concentrations of clindamycin differentially repressed the transcription of *S. aureus* exoprotein genes and acted partially through the two-component system sar (22). The rich antimicrobial content of plants has been described previously, including the quorum sensing inhibitory ability of some antimicrobials, such as eugenol (4-allyl-2-methoxyphenol), an essential oil component in plants (clove) (23). A Western blot analysis in the aforementioned study showed that eugenol reduced the production of staphylococcal enterotoxin A and toxic shock syndrome toxin-1, as well as the expression of α-hemolysin (23). Aqueous extracts from edible plants and fruits, such as Ananascomosus, Musa paradiciaca, Manilkarazapota, and Ocimum sanctum, displayed quorum sensing inhibitory ability against violacein production by *C. violaceum*, in addition to pyocyanin pigment production, staphylolytic protease production, elastase production, and biofilm formation by *P. aeruginosa* PAO1 (24).

The present study investigated the effect of *F. assa-foetida* and *C. copticum* hydroalcoholic extracts on the expression levels of *hld* and *tst* genes in both MSSA and MRSA strains. The sMIC of the *F. assa-foetida* extract for the MRSA and MSSA strains was 25 mg/mL and 12.5 mg/mL, respectively. The sMIC of the *C. copticum* extract for both MRSA and MSSA was 12.5 mg/mL. In this study, the expression of the *hld* and *tst* genes was examined after 7 and 16 hours of exposure, and the 16srRNA gene was used as an internal control (25). According to the results, at a concentration of 25 mg/mL, exposure to *F. assa-foetida* decreased the expression of the MRSA *hld* gene compared to that of the control sample. After 7 hours, the expression of the *hld* gene was greatly reduced compared with afar 16 hours of exposure. A previous study of three medicinal plants (Ballotanigra, Castaneasativa, and Sambucusebulus) reported a dose-dependent response in the production of δ-hemolysin, indicating strong antiquorum sensing activity in a pathogenic MRSA isolate (5). In the present study, the *C. copticum* extract also reduced the expression of the MRSA *hld* gene after exposure for 7 and 16 hours.

The results of the present study showed that the *F. assa-foetida* extract was more effective that the *C. copticum* extract in reducing *hld* gene expression of MRSA, but this finding was inconsistent with the microdilution results. Based on the microdilution results, *C. copticum* (MIC, 25 mg/mL) inhibited the growth of MRSA at a lower concentration than *F. assa-foetida* (MIC, 50 mg/mL). However, the *F. assa-foetida* extract was more effective than the *C. copticum* extract in decreasing the expression of the *hld* gene of MRSA. Hamamelitannin extracted from the bark of *Hamamelis virginiana* (witch hazel), in common with RIP, did not affect the growth of *Staphylococcus* spp. (26) However, it inhibited the quorum-sensing regulator RNAIII and prevented biofilm formation and cell attachment in vitro (26). In addition, Burt et al. showed that at sublethal concentrations (0.5 mM), carvacrol inhibited the formation of biofilms of *Chromobacterium violaceum* (ATCC 12472) and Salmonella enterica subsp. but did not impair the bacterial growth or survival of Typhimurium (DT104) and *S. aureus* (ATCC 0074) (27).

The results of the present study are consistent with those of Burt et al.'s study. In the current study, the expression of the MSSA *hld* gene showed no decrease following exposure to the sMIC (12.5 mg/mL) of *F. assa-foetida* and *C. copticum*. The *F. assa-foetida* extract was not effective in reducing the expression of the MSSA *tst* gene at the same exposure. However, the *C. copticum* extract decreased the expression of this gene after 16 hours of exposure compared to the control sample. As the quorum sensing system plays an important role in regulating the expression of virulence factors, sporulation, and biofilm formation, a better understanding of this system may be helpful in the control and management of diseases and antimicrobial therapy.

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Footnotes

Authors' Contribution: All the authors significantly contributed to this work. Mohammad Bagher Khalili: supervisor, bacteriologist; Gilda Eslami: acted as advisor, analyzed the data, and revised the manuscript; Najmeh Jomehpour: performed the experiments and wrote the preliminary draft.

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