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An Investigation on Biofilm Formation and Expression of the *icaA* Gene among *Staphylococcus aureus* Strains Treated with Garlic Extract and Ciprofloxacin

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Staphylococcus aureus is a bacterial pathogen that is responsible for a broad range of human infections. Infections caused by drug-resistant *S. aureus* are a global health threat. Biofilm formation capability of *S. aureus*, which is an important factor in infection establishment and drug resistance and thus therapeutic failure, is mainly mediated by *ica* operon, which is responsible for the production of polysaccharide intercellular adhesions. Inhibition of bacterial biofilm as a promising antibacterial approach has gained attention recently. In addition, several natural compounds, especially of plant origin, have displayed anti-biofilm potential against bacteria. Allicin is the major bioactive compound found in garlic (*Allium sativum*) and is responsible for its antimicrobial activity. Thus, this study aimed to evaluate the effects of garlic extract in combination with ciprofloxacin on the biofilm formation of pathogenic *S. aureus*. Antimicrobial effects and minimum inhibitory concentration (MIC) of either the agent alone or in combination were determined, and the bacterial biofilm formation and expression of the *icaA* gene were investigated. The results showed that garlic extract in combination with ciprofloxacin had a strong biofilm inhibitory potential and reduced biofilm formation by 54-61% compared to the control. The reduction of bacterial biofilm can be attributed to the reduction of the *icaA* gene, which was reduced by 59-65%. Thus, this study showed that garlic extract can be used as a promising anti-biofilm agent in combination with antibiotics and can be a good candidate to combat bacterial infections.

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Introduction

Staphylococcus aureus is a Gram-positive bacteria that is responsible for a significant number of nosocomial and community-acquired infections in humans throughout the world (Sharifi-Rad et al., 2014). Pathogenicity of *S.*

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aureus is attributed to several factors including bacterial virulence factors, drug resistance mechanisms, and biofilm formation.

Biofilm is a community of bacterial cells that adhere to each other and surfaces and are encased in self-produced extracellular polymeric substances (EPS) (Wu et al., 2015). Biofilm is formed in two steps including primary attachment using bacterial adhesion factors and then, immobilization using bacterial EPS (Kalia, 2013). Biofilm formation not only is involved in infection establishment but also causes higher resistance to antibacterial drugs. In other words, bacteria embedded in biofilm gain resistance to antibiotics due to the poor drug penetration into the biofilm as well as the easier exchange of drug resistance genes (Stewart and Costerton, 2001). In addition, the slower metabolic state of bacteria in the biofilm mode of growth results in lower efficacy of the growth-dependent antibacterial agents. Thus, biofilm formation capability is an important factor influencing bacterial establishment, survival, and infection development.

Ciprofloxacin, an antibiotic belonging to the fluoroquinolone class of antibacterial drugs, is commonly prescribed against bacterial infections both in hospitals and out of hospital settings (Gao et al., 2018). This is among the most common drug for the treatment of infections caused by *S. aureus*. However, growing resistance to fluoroquinolones has been observed and thus there has been a focus to improve the efficacy of these drugs (Ocheretyaner and Park, 2018).

Combined therapy by natural antimicrobial agents including medicinal herbs could be a good approach to improving the efficacy of antibiotics against bacterial infection. Garlic (*Allium sativum*) has been traditionally used for its medicinal properties including antimicrobial, antioxidant, and anticancer effects. Previous research has shown that garlic extract can efficiently inhibit bacterial growth and biofilm formation (Pérez-Giraldo et al., 2003; Cai et al., 2007; Mohsenipour and Hassanshahian, 2015). Antimicrobial properties of garlic extract are attributed to the organosulfur compounds and mainly allicin, a precursor of sulfur compounds. Allicin is converted into diallydissulphinate, which has antimicrobial activity (Venâncio et al., 2017). The synergistic effect of garlic extract or its derivatives with different antibiotics, especially β-lactamse, has been demonstrated against bacterial infections (Cai et al., 2007; Reuter et al., 1995; Sohn et al., 2009). However, very few studies concerned simultaneous use of allicin and other garlic derivatives with antibiotics to combat biofilm formation of *S. aureus*.

The significance of biofilm formation in infection development and drug resistance has provoked interest in finding novel anti-biofilm agents, especially from natural origin. In addition, the combination of anti-biofilm agents with antibiotics could result in better bacterial eradication and infection control. Thus, this study aimed to evaluate the effect of garlic extract alone or in combination with ciprofloxacin on biofilm formation ability and expression of the *icaA* gene among pathogenic *S. aureus* strains.

Materials and Methods

S. aureus strains

The study used pathogenic *S. aureus* strains, isolated from clinical specimens including blood, wounds, and urine samples. Bacterial identification was performed using conventional biochemical and molecular assays. Then, bacterial antibiotic susceptibility was evaluated using the disk diffusion method according to the CLSI guideline (CLSI, 2017) (data not shown). Finally, two *S. aureus* strains with the highest resistance to different antibiotics, as well as *S. aureus* ATCC 25923 as standard strain, were selected for further experiments (Table 1).

Table. 1 Sequence of the primers used in this study

Gene	Primer Sequences (5'-3')	Anealing temperature	Amplicon size (bp)	Reference
icaA-F	GAGGTAAAGCCAACGCACTC	60^{0}C	151 bp	Atshan et al. 2013
icaA-R	CCTGTAACCGCACCAAGTTT			
16s rRNA-F	GGGACCCGCACAAGCGGTGG	60^{0}C	191 bp	Atshan et al. 2013
16s rRNA-R	GGGTTGCGCTCGTTGCGGGA			

Antibacterial susceptibility testing

Bacterial susceptibility to ciprofloxacin and allicin was determined using the disk diffusion method. At first, suspensions of bacterial strains from a fresh bacterial culture with an approximate population of 1.5×10^8 cfu/ml were prepared in distilled water and uniformly inoculated on the surface of Mueller Hinton Agar (HiMedia). Then, blank disks impregnated with 50 µl of different

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agents including ciprofloxacin solution (1 mg/ml) and garlic extract solutions (1.25 and 2.5 mg/ml) (GolDaru pharmaceutical Co, Iran) were placed on the medium. The plates were incubated for 24 h at 37°C, and the inhibition zones formed around each disk were measured. The assay was performed in triplicates.

Minimum inhibitory concentration (MIC)

The MIC of different agents including ciprofloxacin, garlic extract, and ciprofloxacin-garlic extract solutions was determined using the broth macro-dilution assay (CLSI, 2017). In brief, two-fold serial dilution of different agents (1-1/32) was prepared in Mueller Hinton broth and they were inoculated with fresh bacterial solution (7.5×10^6 cfu/ml). The tubes were incubated at 37°C for 24 h and the bacterial growth was evaluated. The MIC was determined as the minimum concentration of each agent that inhibited bacterial growth. Mueller Hinton broth was used in the control tubes.

Biofilm formation assay

Biofilm formation capability of bacterial strains in the presence of different agents was explored in 96-well micro-titer flat-bottom plates. At first, ciprofloxacin, garlic extract, and ciprofloxacin-garlic extract solutions with a concentration of ½ MIC were prepared, and the wells were inoculated with fresh bacterial suspension with an approximate population of 1.5×10^6 cfu/ml. The plates were incubated at 37°C for 48 h without shaking. After the incubation period, the medium was removed and the wells were rinsed using distilled water and then, 100 µl of ethanol 70% (Merck) was added for 10 min. In the next step, the wells were stained with 0.2% crystal violet for 10 min and washed using distilled water. Finally, the dye was solubilized using 30% acetic acid solution, and biofilm formation was quantified at 570 nm. The measured optical density was correlated with biofilm formation in each well (Stepanović et al., 2000).

Expression of the icaA gene

In order to investigate the effect of different agents on the expression of the *icaA* gene, bacterial strains were grown in the presence of sub-MIC concentrations of different agents. Based on the results of the MIC assay, 100 µL of bacterial strains (7.5×10^6 cfu/mL) were inoculated to the tubes containing Mueller Hinton Broth (HiMedia) with sub-MIC concentration of different agents. The bacteria were grown for 24 h at 37°C and were then harvested using centrifugation (3500 g, 10 min). The bacteria grown in the medium lacking antimicrobial agents were used as control.

Bacterial RNA was extracted using a Bioneer RNA extraction kit according to the manufacturer's protocol. The quality and quantity of the extracted RNA were evaluated using a nano-drop system, and the first strand cDNA was synthesized using a Bioneer cDNA synthesis kit according to the instruction.

Quantitative Real-Time Polymerase Chain Reaction (qRT-PCR) was used to determine the expression of the *icaA* gene among bacteria from different treatment groups. SYBR Green pre-mix was prepared according to the manufacturer's protocol, and the master mix, primers, and synthesized cDNA were mixed in a microtube with a final reaction volume of 20 µl. The temperature protocol was determined based on the primers Tm and characteristics of different stages of PCR. The 16s rRNA gene was used as the reference gene, and the $2^{-\Delta\Delta CT}$ method was used to analyze the data (Pfaffl, 2001).

Statistical analyses

The assays were performed in triplicates. Data analysis was conducted by SPSS 16.0 (SPSS Inc., Chicago, IL). P values <0.05 were regarded to be statistically significant.

Results

Disk diffusion assay

Antibacterial activity of different agents including ciprofloxacin (1 mg/ml) and garlic extract solutions (2.5 and 1.25 mg/ml) against *S. aureus* strains was evaluated. Based on the results, ciprofloxacin and garlic extract solutions with a concentration of 2.5 mg/ml inhibited bacterial growth with a zone of inhibition (ZOI) of 22-24 mm and 24-26 mm, respectively depending on bacterial strains. However, the garlic extract solution with a final concentration of 1.25 mg/ml did not show satisfactory antimicrobial potential with a ZOI of 6-9 mm (Table 2; Figure 1).

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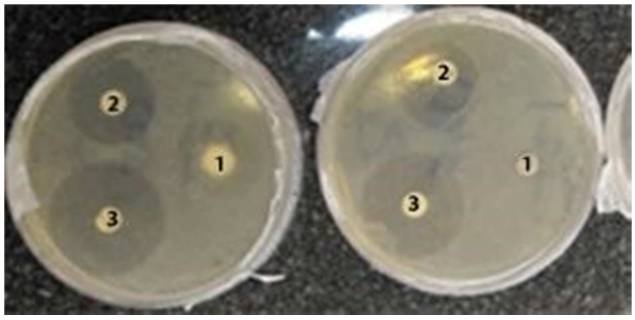


Fig. 1- Antibacterial potential of different agents against *S. aureus* strains. 1, garlic extract (1.25 mg/ml); 2, ciprofloxacin (1 mg/ml); 3, garlic extract (2.5 mg/ml).

MIC of different agents

The MIC value of different agents including ciprofloxacin, garlic extract, and ciprofloxacin-garlic extract solutions was determined using the broth macro-dilution assay. Based on the results, the MIC of ciprofloxacin against different strains was recorded in the range of 250-500 µg/ml, while it was in the range of 156-625 µg/ml for garlic extract solution. In addition, the MIC of ciprofloxacin-garlic extract solution against all strains was determined to be 250 µg/ml (Table 2). Based on the results of the MIC assay, the concentration of ½ MIC of each solution was selected for molecular analyses.

Biofilm inhibition assay

The anti-biofilm effect of different agents against *S. aureus* strains was determined. The results showed that all agents could decrease the biofilm formation of *S. aureus* strains. The highest biofilm inhibition was observed in the solution containing garlic extract + ciprofloxacin with an inhibition range of 54-61% compared with the control. The minimum anti-biofilm effect was observed in garlic extract solution with an inhibitory effect of 13-56% depending on the bacterial strains. The anti-biofilm effects of different agents against *S. aureus* strains are displayed in Figures 2 and 3.

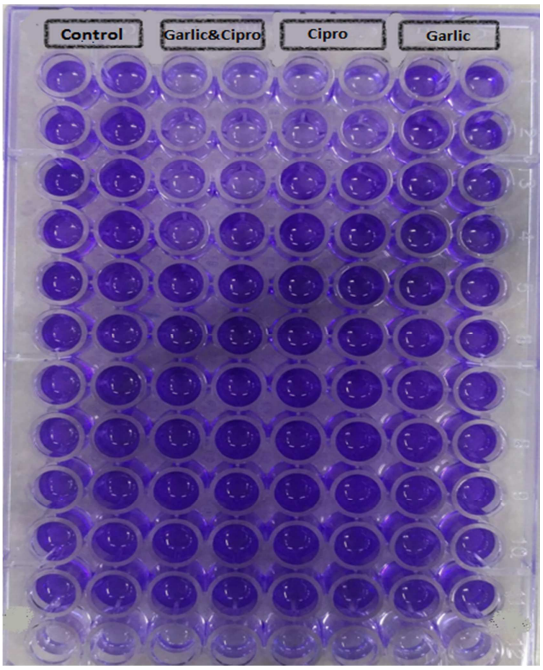


Fig. 2- Biofilm test results in microplate with garlic extract and ciprofloxacin treatments.

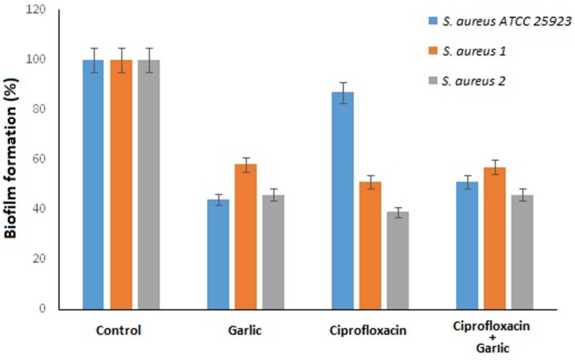


Fig. 3- Biofilm formation of *S. aureus* strains in the presence of different agents

Amplification of icaA

PCR was performed to prove the presence of the *icaA* gene in the studied strains using primers designed for this gene under optimal temperature conditions. The PCR products of 151 bp were expected, and a band close to this size was observed in all three strains of *S. aureus* (Figure 4).

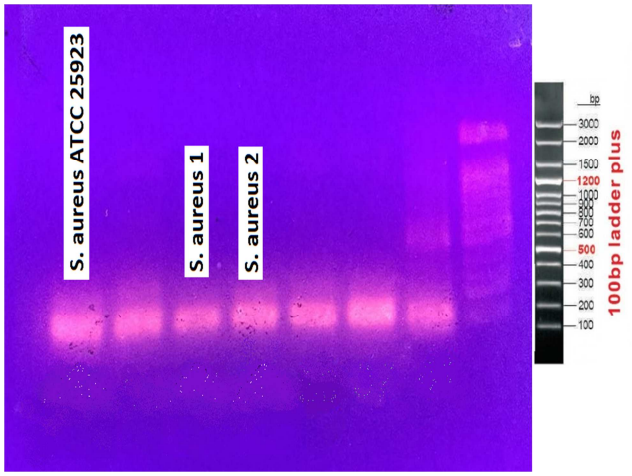


Fig. 4- PCR product bands of *icaA* gene in the studied strains on 1% agarose gel (expected size: 151 bp)

Expression of the icaA gene

The effect of garlic extract, ciprofloxacin, and ciprofloxacin + garlic extract at a concentration of ½ MIC on the expression of the *icaA* gene among *S. aureus* strains was determined using qRT-PCR. The results showed that the expression of the *icaA* gene among *S. aureus* in the presence of all agents decreased. The highest reduction of the *icaA* gene was observed among the bacteria treated with ciprofloxacin combined with garlic extract, while the solution containing garlic extract alone caused the lowest reduction of the mentioned gene. The effects of different agents on the expression of the *icaA* gene among different strains of *S. aureus* are presented in Table 3 and figure 5. Ciprofloxacin alone and in combination with garlic extract significantly reduced the *icaA* gene compared to the cells exposed to garlic extract alone. Although there was not a significant difference between the cells treated with ciprofloxacin alone and ciprofloxacin combined with garlic extract, a higher reduction was observed for the cells treated with both agents.

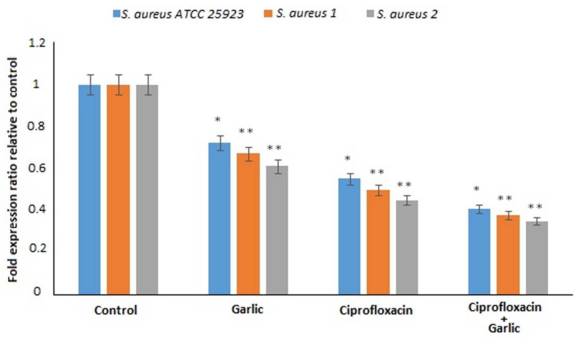


Fig. 5- Expression of *icaA* gene among *S. aureus* strains treated with different agents. Statistical differences were marked using *, (p value <0.05)**

Discussion

Garlic (*Allium sativum*), which has been traditionally known for strong antibacterial and antifungal properties, contains a variety of sulfur compounds. Among them, allicin is recognized as the major bioactive component of garlic with a broad spectrum antimicrobial activity against different pathogenic bacteria, including *S. aureus* (Sharifi-Rad et al., 2014). This species is an opportunistic pathogen causing a variety of infections from simple skin infections to life-threatening ones. Infection with drug-resistant *S. aureus* strains is a global health threat. Biofilm formation by bacteria is an important approach for infection establishment and drug resistance and thus, therapeutic failure. Finding anti-biofilm agents to combat bacterial infection is a novel approach that has gained attention recently. Several studies have reported the anti-biofilm potential of garlic derivatives including allicin (Pérez-Giraldo et al., 2003; Wu et al., 2015). However, very few studies have investigated the effect of allicin in combination with antibiotics on the biofilm formation of pathogenic bacteria. In this study, we investigated the effect of garlic extract and ciprofloxacin, alone or in combination, on the biofilm formation and the expression of the *icaA* gene among *S. aureus* strains.

This research showed that garlic extract at a concentration of 2.5 mg/ml obviously inhibited bacterial growth with a ZOI higher than that of ciprofloxacin. However, no significant difference was observed for the ZOI between ciprofloxacin and garlic extract (2.5 mg/ml). In addition, garlic

Caferahoglu

solution with a concentration of 1.25 mg/ml did not cause bacterial inhibition. Several reasons have been reported for the antimicrobial activity of allicin including the alteration of structure and integrity of the bacterial outer surface and the reduction of total lipid content (Chen et al., 2018). In addition, it has been reported that allicin is readily diffused through membranes and reaches target sites where it can inhibit bacterial enzymes and DNA (Ratthawongjirakul et al., 2016).

We observed that there was a synergism between garlic extract and ciprofloxacin against biofilm formation by *S. aureus*. Based on our results, the combination of garlic extract with ciprofloxacin not only reduced biofilm formation but also significantly decreased the expression of the *icaA* gene. The antibiofilm characteristic of allicin against several bacteria has been reported previously (Pérez-Giraldo et al., 2003; Ratthawongjirakul et al., 2016). Ratthawongjirakul et al. (2016) reported that garlic extract interfered with bacterial adherence to the surfaces that was the first step in biofilm formation. The reduction of biofilm in the presence of allicin observed in this study is in accordance with their report. However, we observed that the expression of the *icaA* gene, the gene involved in the second step of biofilm formation, was also reduced. *icaA* is a gene located within *ica* operon and is responsible for the production of polysaccharide intercellular adhesion (Elkhashab et al., 2018). These molecules mediate cell aggregation and are thus involved in biofilm maturation. Thus, reduced biofilm formation caused by garlic extract among the strains treated with garlic + ciprofloxacin not only could be associated with the inhibition of bacterial primary adhesion but also could be associated with the inhibition of the *icaA* gene.

Bacterial biofilm is regulated by quorum sensing (QS). QS is a cell-to-cell communication system that is dependent on cell density and controls the expression of virulence genes including the genes associated with biofilm (Kalia, 2013). Several studies have reported that allicin has an anti-quorum sensing property (Bjarnsholt et al. 2005; Bodini et al. 2009). Thus, the reduction of the *icaA* gene could be associated with the inhibition of bacterial QS by allicin. On the other hand, ciprofloxacin as a drug belongs to the fluoroquinolones and acts against bacteria by inhibiting bacterial topoisomerases, the enzymes that are necessary for bacterial transcription (Hooper, 2001). As mentioned above, in the presence of allicin, the integrity of the bacterial membrane is decreased. Higher permeability of bacterial cells causes efficient penetration of antibiotic into the bacterial cell. Inside the cells, ciprofloxacin reduces bacterial transcription by inhibiting topoisomerases. Thus, two approaches are involved in the reduced expression of the *icaA* gene: first, the anti-QS effect of allicin inhibits the expression of the biofilm-associated genes such as *icaA*, and second, the facilitated diffusion of ciprofloxacin into the bacterial cells caused by allicin decreases bacterial transcription.

Conclusions

In the present study, a synergism was observed between allicin, a bioactive compound from garlic, and ciprofloxacin against *S. aureus* biofilm. Our study demonstrated that the combination of garlic extract with ciprofloxacin could inhibit bacterial biofilm at molecular level, which could be used as an efficient approach against infections caused by drug-resistant *S. aureus*. So far, the effects of garlic extract with ciprofloxacin on the expression of the genes involved in biofilm production have not been investigated and this research can be the beginning of further research in this field. However, further experiments must be performed to characterize their antimicrobial and anti-QS potential.

Acknowledgments

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Current Research

Table 2. Antibacterial potential of different agents against *S. aureus* strains

Bacterial strain	ZOI of different agents (mm)			MIC (µg/mL)		
	Ciprofloxac (1 mg/mL)	Garlic extract (1.25 mg/mL)	Garlic extract (2.5 mg/mL)	Ciprofloxac	Garlic extract	Cipro+ garlic
S. aureus 1	22	9	24	250	312	250
S. aureus 2	24	6	26	500	156	250
S. aureus 25923	24	6	6	250	625	250

ZOI: Zone of inhibition; MIC: Minimum inhibitory concentration

Table 3. Expression of *icaA* gene among *S. aureus* strains treated with different agents.

Strains of *S. aureus*	Treatments	*icaA* gene expression levels
S. aureus ATCC 25923	Control (No treatment)	0.99 ± 0.65 a
	Garlic extract	0.72 ± 0.36 a
	Ciprofloxacin	0.55 ± 0.35 b
	Garlic + Ciprofloxacin	0.41 ± 0.44 c
S. aureus 1	Control (No treatment)	1.00 ± 0.71 a
	Garlic extract	0.67 ± 0.32 b
	Ciprofloxacin	0.5 ± 0.28 c
	Garlic + Ciprofloxacin	0.38 ± 0.21 c
S. aureus 2	Control (No treatment)	1.01 ± 0.66 a
	Garlic extract	0.61 ± 0.3 b
	Ciprofloxacin	0.45 ± 0.23 c
	Garlic + Ciprofloxacin	0.35 ± 0.15 c

Statistical differences were marked using a,b,c (p value <0.05)

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