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Nanoparticles Impact the Expression of the Genes Involved in Biofilm Formation in *S. aureus*, a Model Antimicrobial-Resistant Species

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ABSTRACT

Background: Infection with resistant bacteria are still reported in hospitals despite the routine cleaning of hospital surfaces. Presence of drug-resistant microbes in the on environment of hospitals and on medical equipment is indicative of the need for control measures which could impact the emergence of such microbes. In addition, biofilms are increasingly associated with human infections and it necessitates careful considerations on usage of a diverse range of medical devices, such as catheters, implants and pacemakers in hospitals.

Methods: This study was designed to compare the effect of silver, ZnO nanoparticles and curcumin on drug-resistant Gram-positive and Gram-negative bacteria which were already isolated from different wards of the hospital. The MIC value were determined for silver, curcumin and ZnO nanoparticles. As the second step, the expression level of the genes involved in biofilm formation in *S. aureus*, including *icaA*, *icaD*, *fnbA* and *fnbB*, was studied to analyze the physiological reaction to controlled concentrations of such nanoparticles using RT-qPCR assessments.

Results: In this study, a total of 172 bacterial isolates were recovered from clinical and environmental samples (96 and 76 isolates, respectively). API-20 test revealed that these isolates belonged to 8 species. All antimicrobial resistant isolates were susceptible to the metal oxide nanoparticles. The results of q-PCR in this study showed that the expression of *icaA* and *icaD* genes in the presence of silver, curcumin and zinc nanoparticles were not significantly reduced compared to the control samples. But, exposure to nanoparticles reduced the expression of *fnbA* and *fnbB* genes from 0.46 to 0.06.

Conclusion: The results of our study showed that nanoparticles are highly effective on antibiotics-resistant isolates and these compounds can be used in the treatment of resistant bacteria. In addition, this study also demonstrates the promising potential of using nanoparticles as anti-biofilm formation agents.

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Introduction

We are still facing resistant nosocomial infections in patients, despite standard protocols for controlling the spread of infections in the hospital, which indicates pathogen resistance to disinfectants and detergents (1-3). On the other hand, bacteria are becoming resistant to antibiotics and drugs. A diverse range of studies have investigated methods to develop new antimicrobial agents in order to overcome this problem. Furthermore, over 65% of all human infections are estimated to be related to the biofilm-formation capabilities of pathogens (4). In addition, over 12 million people in the USA are reported to be affected by pathogens which have the biofilm-related infections (BRIs) every year, with an estimated annual economic burden of \$6 billion (5). Bacterial biofilms harbor organized communities of various bacteria embedded in a self-produced matrix of extracellular polymeric substances (EPS). Living in such bio-matrixes is indeed the predominant bacterial lifestyle in natural environments. Biofilms are increasingly associated with human infections, especially due to the rise in use of medical devices, such as catheters, implants and pacemakers (6). The increased host immune system evasion as well as tolerance and resistance to antimicrobials displayed by biofilms lead to failure of conventional antimicrobial therapy (7-9). Thus, biofilms cause persistent infections characterized by increased morbidity and mortality. *Staphylococcus aureus* is one of the most frequent causes of nosocomial and medical device-related biofilm infections (10).

Recently inorganic antimicrobial agents, including heavy metals, are considered as they are safe for human beings (11). Antibacterial activity of zinc oxide and silver nanoparticles are significantly considered in recent years especially by using nanotechnology to synthesize particles in nanoscale size by physical and chemical methods (12). The advantage of using nanoparticles is their

higher surface to volume ratio and therefore increased a number of active atoms at the outer surfaces (13).

The mechanism of the antibacterial effect of these nanoparticles is due to the attachment to the surface of the cell membrane and disruption of permeability and respiration functions of cells and inactivating membrane proteins. These particles can also pass through the cell wall and either the membranes of bacteria and bind to bacterial DNA and disrupt DNA replication. Furthermore, they can interfere in ribosomal function to translate mRNA into protein forms, and so they activate cytochrome b (14). However, further investigation is needed to understand the complete mechanism of the nanoparticles and their mode of action on different bacteria which differs due to the type of nanoparticles and also bacterial strains and their concentrations. There are various factors affecting the susceptibility of different microorganisms to heavy metal nanoparticles including the structure of bacterial cell wall and bacterial growth rate (15). Another antimicrobial agent which has been recently considered is curcumin, the major phytochemical of *Curcuma longa* L. and commonly known as turmeric (16).

It is commonly used in food as a spice and gives the yellow color to the foods and is majorly produced in India. It can be extracted by turmeric by different methods by solvent extraction and purified by column chromatography (17).

Many properties of this compound have been reported including antimicrobial, anti-inflammatory and antioxidant activities (18) and it has been observed that there will be an improved antibacterial activity if combined with other drugs (19). The safe dose of usage has been reported up to 8 grams daily for three months (20). The mechanism of its activity are yet to be studied but it seems that it has various activities affecting the cellular processes which lead to inhibition of the cell growth in Gram-positive and either Gram-negative bacteria (21, 22).

Studies have shown that the antibacterial effect of curcumin against *Bacillus subtilis* is by inhibiting the proliferation of bacterial cells through blocking of *FtsZ* assemblage in the Z ring (22) and for *Pseudomonas aeruginosa* it affects the virulence, quorum sensing and biofilm formation of the bacteria (23).

Therefore, this study was designed to (i) compare the effect of silver, ZnO nanoparticles and curcumin on Gram-positive and Gram-negative microorganisms resistant to antibiotics and antiseptics which were isolated from different wards of the hospital. The isolates covered a diverse range of bacterial taxonomy including: *E. coli*, *A. baumannii*, *S. epidermiditis*, *K. pneumonia*, *P. aeruginosa*, *S. aureus*, *E. faecalis*, and *E. faecium*, and (ii) RT-qPCR-measurement of the expression level of the genes involved in biofilm formation of *S. aureus* when exposed to sub-MIC concentrations of nanoparticle.

Materials and Methods

Bacterial isolates

Clinical isolates were collected from samples including sputum, blood, cutaneous lesions, nasal mucus, urine, stool, burns, catheters, bronchial secretions and wound secretions. Environmental isolates were collected from ICU and CCU wards from suction devices, monitor, incubators, beds, walls, floors and sinks in Rasoul Akram hospital, Tehran, Iran in 6 months. The swabs were inoculated on Blood Agar culture media and were incubated overnight at 37 °C. Identification of bacterial isolates was carried out by API-20E kit.

Definition of antimicrobial susceptibility

Antimicrobial resistance of the isolates was determined by disk diffusion method on Mueller Hinton Agar (Merck, Germany) according to Clinical & Laboratory Standards Institute Guidelines (CLSI, 2015). The antibiotic disks used in this study were (Mast, Germany) Ampicillin (30µg). Co-Amoxiclav (20 µg),

Ciprofloxacin (5 µg), Erythromycin (5 µg), Oxacillin (5 µg) and Cefoxitin (30 µg). Inoculation on Mueller Hinton was performed with a broth culture diluted to match a 0.5 McFarland turbidity standard, which was roughly equivalent to 150 million cells per mL. The sterile swab was immersed into the broth culture and then the Mueller-Hinton Agar was streaked to form a bacterial lawn and antibiotic disks were placed on the agar by a dispenser. Cultures were then incubated at 37 °C for 18-24 hrs. Then, the zone of inhibition was measured and compared to the world standards (CLSI) and were reported as resistant, Intermediate (I) and Sensitive (S). In order to study the susceptibility to antiseptics, disk diffusion agar method was used. Standard disks (made of Acetate Cellulose, prepared from PadTanTeb Company) with a diameter of 6 mm were suspended in the antiseptic solutions including Formaldehyde, Benzalkonium chloride, Chlorhexidine and deconex and incubated at 37 °C for 30 minutes. Based on CLSI guidelines, the diameter of the zone of inhibition measured 6 mm, 7-10 mm, 11-15 mm and more than 15 mm were considered neutral low-level, intermediate-level and high-level disinfectants, respectively. *Staphylococcus aureus* ATCC25923 and *Escherichia coli* ATCC25922 were used as standard strains. Chloramphenicol and Gentamycin were used as controls.

MIC determination

MIC and MBC concentrations were determined for nanoparticles by serial dilution method in 96-well microplates according to CLSI guideline at 25 °C. for this purpose, serial dilutions were prepared in 10 wells with concentrations of 400, 300, 200, 150, 100, 50, 25, 12.5, 6.25, and 3.12 µg/ml and two wells were positive (including culture media and microbial suspension) and negative (including culture media) controls. Then, 10 microliters of bacterial suspension matching a 0.5 McFarland turbidity standard was added to wells containing different concentrations of nanoparticles and the plates were then incubated overnight at 35 °C. MIC

and MBC were then determined. The abovementioned standard strains were also included into the assay as controls.

RT-qPCR reactions and Gene expression analysis

The effect of nanoparticles on expression of the genes involved in biofilm formation in *Staphylococcus aureus*, including *icaA*, *icaD*, *fnbA* and *fnbB* genes, was studied using RT-qPCR technique. The sub-MIC wells in 96-well microplate were collected and pelleted by centrifugation at 2500 ×g for 15 min. Total RNA was isolated using the QIAGEN RNeasy Mini kit. Extracted RNA was analyzed using a Nanodrop ND1000 and run on a denaturing 1.5% TAE-agarose gels (80 V for 1 h) to assess RNA concentration, quality, and integrity. The RNA was DNase treated with Promega RNase-free DNase (at 37 °C for 1 h). RNA was precipitated with 1 volume isopropanol and 0.1 volume of 3 M NaOAc (pH 4.6). The suspension was incubated on ice for 20 min and centrifuged at high speed for 30 min at 4 °C. The RNA pellet was dried and resuspended with RNase-free MilliQ H₂O. According to the manufacturer's instructions, 500 ng-1µg RNA was converted into cDNA using AccuPower CycleScript RT PreMix (Bioneer; Korea). Quantitative Real-Time PCR was performed in a Rotor-Gene thermal cycler (Corbett 6000; Australia) using SYBR Green method (AccuPower Green Star qPCR Master Mix; Bioneer; Korea). A total volume of 20 µl reaction containing 2 µl of cDNA, 12.5 µl SYBR Green master mix, 4.5 µl nuclease-free water and 1 µl of each primer (5 pmol) was run according to following program: an initial activation step at 94 °C for 4 minutes, 35 cycles of denaturation at 94 °C for 30 s, annealing at (Table 2 shows annealing T_m for each primer) for 30 s and extension at 72 °C for 20 s. The *gyrA* gene was also analyzed as an internal control to normalize target gene expression measurements.

Statistical Analysis

Data were analyzed using SPSS software (version 16) and T-test.

Results

In this study, a total of 172 bacterial isolates were recovered from clinical and environmental samples (96 and 76 isolates, respectively). API-20 test revealed that these isolates belonged to 8 species. Of these *Escherichia coli* was the predominant (25%); *Staphylococcus aureus* (19%), *Klebsiella pneumonia* (16%), *Staphylococcus epidermidis* (15%), *Acinetobacter baumannii* (8.5%), *Pseudomonas aeruginosa* (7.5%), *Enterococcus faecalis* (5%) and *Enterococcus faecium* (4%) were the next species.

Antimicrobial activities of antiseptics and antibiotics are presented in Table 1. *E. coli* isolates were mostly resistant to Ampicillin (84.5%) and Tetracycline (77%) while *A. baumannii* was resistant to Ciprofloxacin (73.33%) and Ampicillin (73.34%). *K. pneumoniae* was highly resistant to Tetracycline (71%) and *P. aeruginosa* was highly resistant to Ciprofloxacin (70%) and Ampicillin (70%). *E. faecalis* was mostly resistant to Benzalkonium chloride (67%) while *E. faecium* was resistant to all antiseptic agents. *S. epidermidis* was mostly resistant to Ceftriaxone and *S. aureus* was highly resistant to Tetracycline (91%), Amikacin (85%) and kanamycin (85%).

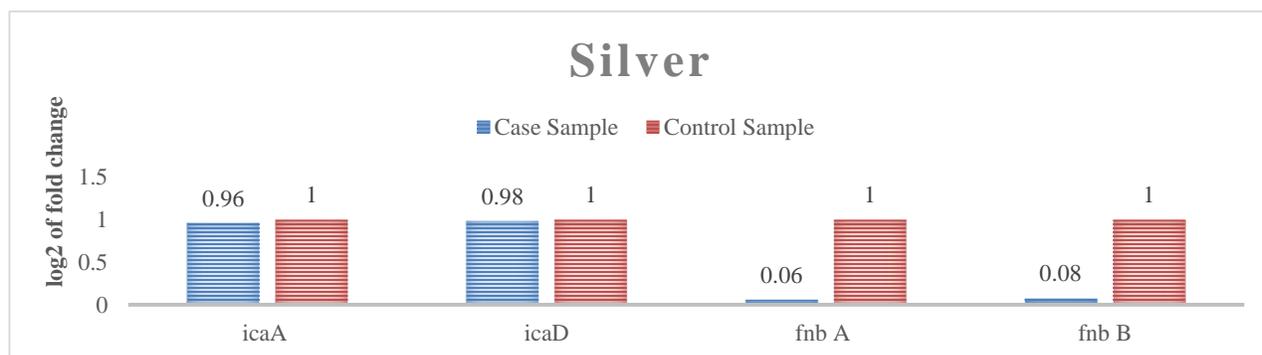
Table 1. Percentage of antimicrobial resistance of the isolated strains by Disk Diffusion Method.

	Cip	AMK	CTX	Caz	Amp	CPE	Ipm	Gen	Kan	Mem	Tcn	F	BZK	CHX	Dec
<i>E. coli</i>	28	2	28	20	84.5	456	4.5	7	32.5	2	77	14	28	33	19
<i>A. baumannii</i>	73.33	40	46.67	33.33	73.34	20	13.33	26.67	26.67	13.33	66.67	15	7	7	7
<i>K. pneumoniae</i>	30	15	52	48	48	52	19	15	30	22	71	7	11	7	4
<i>P. aeruginosa</i>	70	30	38	23	70	7	15	30	30	15	63	39	54	23	31
<i>E. faecalis</i>	44.44	55.55	22.23	22.23	33.33	22.23	-	44.44	33.23	-	22.23	56	67	44	33
<i>E. faecium</i>	57	71	0	29	57	29	-	57	29	-	29	100	100	100	100
<i>S. epidermidis</i>	23	16	70	50	57	53	-	34	27	-	57	19	23	27	12
<i>S. aureus</i>	66	85	79	6	69	50	-	41	85	-	91	6	3	3	3

Cip:Ciprofloxacin; AMK:Amikacin; CTX:Ceftriaxone; Caz:Ceftazidime; Amp:Ampicillin; CPE:Cefepime; Ipm:Imipenem; Gen:Gentamycin; Kan:Kanamycin; Mem:Meropenem; Tcn:Tetracycline; F:Formaldehyde; BZK: Benzalkonium chloride; CHX:Chlorhexidine; Dec:Deconex

Table 2. The designed primers and their corresponding target genes used in the study.

Ta Genes	Sequence (5' → 3')	TM (°C)	Amplicon size	Reference
<i>icaA</i>	F AAAGTGCAGTTGTGCGACGTT	59	161 bp	In this study
	R TTGCTTCCAAAGACCTCCCA			
<i>icaD</i>	F TGGGCATTTTCGCGATTATCA	59	80 bp	In this study
	R CGATTCTCTTCCTCTCTGCCATT			
<i>fnbA</i>	F GACCACCACCTGGGTTTGTA	59	147 bp	In this study
	R TGGATAGCGAAGCAGGTCAC			
<i>fnbB</i>	F GACCACCACCTGGGTTTGTA	59	143 bp	In this study
	R AGGTGCAGAAGGTCATGCAG			
<i>gyrA</i>	F ATTGCAGAGCTCGTTTCGTGA	59	89 bp	In this study
	R ATAACGACACGCACACCAGT			

**Figure 1.** The expression profiles of biofilm-associated genes in *S. aureus* in Sub-MIC concentration of silver nanoparticles ($P < 0.05$).

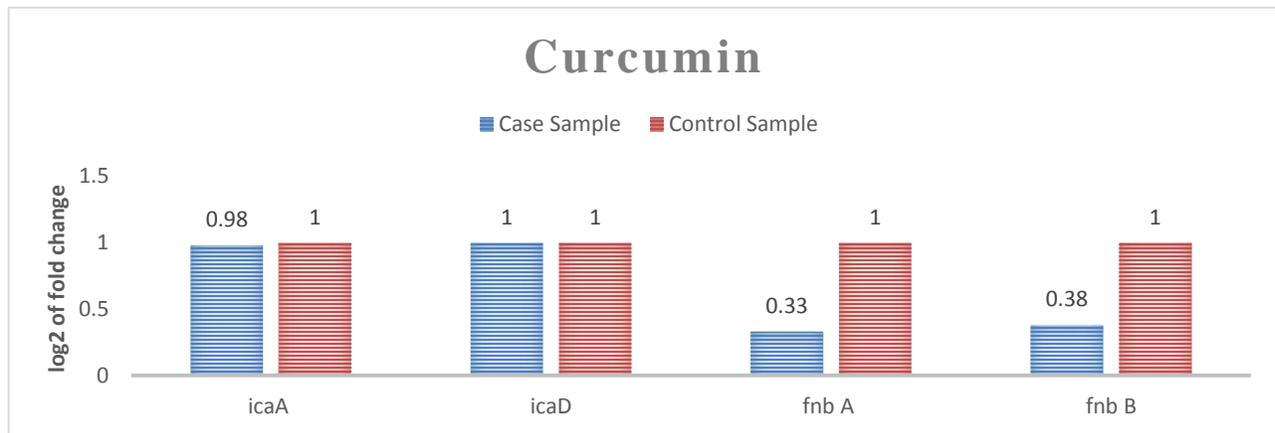


Figure 2. The expression profiles of biofilm-associated genes in *S. aureus* in Sub-MIC concentration of curcumin nanoparticles ($P < 0.05$).



Figure 3. The expression profiles of biofilm-associated genes in *S. aureus* in Sub-MIC concentration of ZnO nanoparticles ($P < 0.05$).

The range of MIC50 of zinc nanoparticles for resistant *E. coli*, *S. aureus*, *A. baumannii*, *K. pneumonia*, *P. aeruginosa*, *E. faecalis*, *E. faecium* and *S. epidermidis* strains were 3.12-12.5 µg/ml, 3.12-12.5 µg/ml, 3.12-6.25 µg/ml, 3.12-6.25 µg/ml, 3.12-25 µg/ml, 3.12-50 µg/ml, 3.12-100 µg/ml and 3.12-25 µg/ml, respectively. The range of MIC50 of silver nanoparticles for resistant *E. coli*, *S. aureus*, *A. baumannii*, *K. pneumonia*, *P. aeruginosa*, *E. faecalis*, *E. faecium* and *S. epidermidis* strains were 3.12-50 µg/ml, 3.12-25 µg/ml, 3.12-25 µg/ml, 3.12-6.25 µg/ml, 3.12-12.5 µg/ml, 3.12-25 µg/ml, 3.12-25 µg/ml and 3.12-12.5 µg/ml respectively. The range of MIC50 of curcumin nanoparticles for resistant *E. coli*, *S. aureus*, *A. baumannii*, *K. pneumonia*, *P. aeruginosa*, *E. faecalis*, *E. faecium* and *S. epidermidis* strains were 3.12-50 µg/ml, 3.12-25 µg/ml, 3.12-12.5 µg/ml, 3.12-25 µg/ml, 3.12-12.5 µg/ml, 3.12-50 µg/ml, 3.12-6.25 µg/ml and 3.12-12.5 µg/ml, respectively.

The results of q-PCR showed that the expression of *icaA* and *icaD* genes in the presence of silver, curcumin and zinc nanoparticles were not significantly reduced in comparison to the control samples, whereas the expression level of *fnbA* and *fnbB* genes in the presence of the silver nanoparticles decreased by 0.06 and 0.08 folds, respectively. Curcumin nanoparticles reduced the expression of *fnbA* and *fnbB* genes by 0.33 and 0.38 fold, respectively; the expression of the *fnbA* and *fnbB* genes by ZnO nanoparticles also decreased by 0.44 and 0.46 folds (Figures 1 to 3).

Discussion

Antimicrobial resistance is considered as a basic problem in the process of treatment and infection control (9). In recent years, bacteria have become resistant to common antibiotics, worldwide. There have been various reports considering the expansion of antibacterial resistance in different wards of the hospital and society, in different parts of the world, which could be caused by unnecessary consumption of antibacterial agents. As antibacterial resistance could be transferred by

plasmids, it is important to study the resistance of infectious bacteria in different wards of the hospital in order to devise treatment protocols and prevention strategies. Although sterilization methods and antiseptic agents are continuously developing, novel methods are not completely used in our country and traditional methods are still being used (24). Lack of efficiency in antiseptic agents could be caused by excessive dilution of the agents for economic reasons or lack of precision in their preparation (25). Currently, nanotechnology is considered as a beneficial technology in the field of Nano-antibiotics. This technology modifies the size and the scale of the particles. Surface to volume ratio of nanoparticles is high and the particle in nanoscales have different features compared to its larger scales. Silver, ZnO and curcumin nanoparticles are the products of nanotechnology (19, 26).

In the current study, 172 bacterial isolates were collected, from which 96 were isolated from different clinical samples (55.8%) including sputum, blood, cutaneous lesions, nasal mucus, urine, stool, burn wounds, catheter, bronchial secretions and wound secretions and 76 were isolated from the environment of the hospital including suction device, monitor, incubator, suture table, beds, walls, and ceiling. In this study, 74 of the isolated bacteria were Gram-positive and 98 were Gram-negative. Therefore, the prevalence of Gram-negative bacteria was higher compared to the Gram-positive bacteria. In a similar study conducted in Mashhad University of Medical Sciences, the prevalence of Gram-positive bacteria was twice the number of Gram-negative bacteria and the prevalence of non-pathogenic bacteria was higher compared to pathogens, which were not compatible with the current study. This may be in part because in this study only pathogens were identified. In the current study, *S. aureus* had the highest prevalence among Gram-positive bacteria and *E. coli* had the highest prevalence among Gram-negative bacteria. The study conducted by Mashouf et al showed that the most prevalent Gram-positive bacteria in microbial contaminations of the hospitals was *S. epidermidis*,

Micrococcus and *Bacillus subtilis* and *E. coli* and *P. aeruginosa* were the most prevalent among Gram-negative bacteria (27). These results were consistent with our study.

There have been several studies considering the antimicrobial resistance in standard and clinical bacterial strains, especially resistance to antibiotics. But resistance against antiseptic agents has not been noticed much. Anderson et al and Rotala et al showed that there is no relationship between the antibacterial resistance of hospital isolates and their susceptibility to disinfectants. Methicillin-resistant *Staphylococcus aureus* (MRSA) are considered as highly resistant bacteria against quaternary ammonium.

The current study investigated the effects of disinfectants with antimicrobial bases of Deconex, Benzalkonium chloride, Anizosin, nanocide against microbial isolates of different wards of Rasoul Akram Hospital, Tehran, Iran. In contrast to the development in contamination controls, nosocomial infection is still on increasing and *S. aureus*, *Enterococci*, and *P. aeruginosa* are considered as the most important agents to contaminate medical devices and internal and external hospital surfaces (28-32).

Nanoparticles have the lowest toxicity to the ecosystem and therefore can be a suitable choice in fighting against pathogens. Metal oxide nanoparticles display different antibacterial effects based on their surface to volume ration. Gram-positive bacteria show more resistance to metal oxide nanoparticles compared to Gram-negative bacteria, which could be related to their cell wall structure. This study showed that antimicrobial resistant bacteria were highly sensitive to silver, ZnO and curcumin nanoparticles.

Previous studies proved the antimicrobial effect of ZnO nanoparticle on Gram-positive and Gram-negative bacteria including *S. aureus*, *E. faecalis*, *S. typhimurium* and *E. aerogenes* (33). Sherry Wastava et al (2010) showed the dose-dependent antimicrobial effect of silver nanoparticles against *S. aureus* and *S. typhimurium* which is incompatible with our study (34). Elham Ansari et al (2012) studied the antibacterial effect of

curcumin nanoparticles against MRSA in pre-clinical conditions. Their results were incompatible with the current study (35).

The results of q-PCR in this study showed that expression of *icaA* and *icaD* genes in the presence of silver, curcumin and zinc nanoparticles were not significantly reduced compared to the control sample. However, the expression of *fnbA* and *fnbB* genes in the presence of nanoparticles decreased from 0.46 to 0.06 fold. The results showed that the silver nanoparticle is more effective than curcumin and ZnO nanoparticles, as well as other studies, since *icaA* and *icaD* genes do not play a role in the biofilm of MRSA strains, and biofilms in MSSA strains is known to be *ica*-dependent, therefore, the lack of effect of nanoparticles on the expression of *icaA* and *icaD* genes is reasonable (36).

The results of the study by Xiaojuan Tan et al. in 2015 Showed that the mechanism of the MSSA biofilm formation was different from that of the MRSA, due to absence of accessory gene regulator (*agr*) function (36). Also, Fitzpatrick et al. revealed that biofilm formation of the *icaADBC* operon deleted MRSA mutants was not affected, whereas biofilm formation of the *icaADBC* operon deleted MSSA mutants was impaired. This study showed that *ica*-independent biofilm formation is strain specific (37). The results of this study are consistent with the results of the current study.

Overlay, Biofilm of MSSA is formed in *ica*-dependent manner (PIA-dependent) by PIA that is encoded by *icaADBC* gene, whereas biofilm of MRSA is formed in *ica*-independent manner (PIA-independent) by surface proteins containing LPXTG anchoring domain that are anchored to peptidoglycan by sortase as a transpeptidase coded by *srtA* gene. Adherence to surfaces and intercellular aggregations of MSSA and MRSA cells are contributed by PIA in *ica*-dependent manner and surface proteins in *ica*-independent manner, respectively (37).

Conclusion

The results of our study showed that nanoparticles are highly effective on resistant

isolates to antibiotics and disinfectants and these compounds can be used in the treatment of resistant bacteria. In addition, this study also demonstrates the promising use of nanoparticles as antibacterial biofilm agents for use in the health centers. Although more studies are needed especially on animal models. Also, the possible harmful effects of these compounds should be investigated.

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Conflict of interest

The authors declare no conflict of interest.

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