

Investigating the effects of *Zataria Multiflora* essential oil and silver nanoparticles on the expression of *abaI* and *pil* genes in *Acinetobacter baumannii* biofilm formation

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ABSTRACT

The aim of this research was to investigate the effect of *Zataria multiflora* and silver nanoparticles on the biofilm formation ability of *A. baumannii* and the expression of genes involved in this process. Thirty isolates of *A. baumannii* bacteria were collected and studied from the Milad Hospital of Tehran. The effects of *Zataria multiflora* essential oil and silver nanoparticles on the biofilm formation process were analyzed using the microtiter plate method. Also, the gene expressions of *pil* and *aba* after exposure to nanoparticles and essential oil were measured. Treatment the strains with *Zataria multiflora* essential oil at the MIC concentration, along with silver nanoparticles, reduced biofilm formation by the strains assessed and the expression of the genes noted. It seems that *Z. multiflora* essential oil and silver nanoparticles, can be used as candidate drugs in treatment or disinfection.

Keywords: *Acinetobacter baumannii*, biofilm, essential oil, silver nanoparticles

INTRODUCTION

Acinetobacter baumannii is a Gram-negative bacteria widely present in soil and water. This bacterium is also dispersed in

the hospital environment, where it can survive for a long time and be easily transmitted to patients [1,2]. Due to the ability of this bacterium to develop drug resistance, this bacterium is considered to

be resistant to treatment [3]. Although *A. baumannii* is considered a cause of nosocomial infections such as bacteremia, urinary tract infections, and secondary meningitis, It commonly causes infections where there is a prolonged utilization of medical devices such as CSF shunts, catheters, endotracheal tubes, and similar [4,5]. This opportunistic pathogen often does not lead to a disease in healthy people; however, it is easily transmitted between people, and this issue is critical in hospitals, which are obliged to update their infection control plans. As a nosocomial pathogen, *A. baumannii* mainly inflicts ICU patients, including those with traumas, injuries, and burns, as well as patients requiring mechanical ventilation. Moreover, all patients with immunodeficiencies or those with underlying conditions, are at risk of contracting *A. baumannii* infection [3,4]. As an opportunistic pathogen, *Acinetobacter* possesses several main pathogenic factors, among which are biofilm formation on surfaces, the ability to absorb essential nutrients such as iron, attaching to and then stimulating epithelial cells, and gelatinase production [5,6].

Biofilm is defined as a community of microorganisms irreversibly attached to a surface, produce extracellular polymeric

materials, and have a different phenotype compared to planktonic cells, particularly in terms of gene expression profile and interaction with other cells [7]. Although biofilm-forming microorganisms also have planktonic life as well, they retain a distinct biochemical profile, which, according to prior studies, can be attributed to the switching off of a group of genes and the activation of another gene group. Research in the field of biofilm formation by *Acinetobacter* shows that this phenomenon is a complex process controlled by several factors, including the existence of antibiotic resistance genes and suitable growth conditions and cellular density. Vast studies on the factors involved in biofilm formation by *A. baumannii* indicate the involvement of different genes in this event, including the *AbaI* gene, whose product (known as AbaIR) is a component of the Quorum Sensing system (QS) in *Acinetobacter*. This system regulates a wide range of *Acinetobacter* activities, including the production of virulence factors and biofilm production [8-11]. Another gene involved in biofilm formation by *A. baumannii* isolates is *Pil*, coding the main protein component of the pili in this bacterium. In addition to its role in bacterial attachment to different surfaces

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and receptors, Pili in *A. baumannii* also contribute to the attachment of bacteria to each other and the twitching movement, all of which finally participate in biofilm production [9,10]. Biofilm nurtures bacterial resistance to disinfectants and helps bacterial survival in harsh environmental conditions [12,13].

Considering that medicinal plants have antimicrobial effects and are widely propagated in Iran, it is amenable to conduct studies on these plants to divulge their antimicrobial properties. The results of such studies can be helpful in building a source of natural drugs to control and treat bacterial infections. *Zataria multiflora*, a, belongs to the Lamiaceae plant family and is endemic to the southern parts of Iran. Thymol, a phenolic compound, is the most important effective ingredient of thyme, which is soluble in alcohol and organic solvents, and its alcoholic extracts have been proven to exert antiseptic effects [11]. The essential oil of this plant has been reported to have antibacterial, antifungal, and immunostimulatory properties [13].

Nanotechnology is considered an emerging science widely contributing to the advancement of modern pharmacology. Nanoparticles, due to their high potential in

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therapeutic processes, are widely used in biological and pharmaceutical studies [14]. Nanoparticles show minimal toxicity in the life cycle and ecosystem, rendering them suitable materials for coping with pathogenic microbes. Therefore, the aim of this study was to investigate the effects of *Zataria multiflora* essential oil and silver nanoparticles on the expression of the *abaI* and *pil* genes involved in biofilm formation by *A. baumannii*.

MATERIALS AND METHODS

Isolation of bacteria

This research was studied on 148 urine samples isolated from hospitalized patients in the special care units and inpatient wards of the Milad Hospital in Tehran City. The clinical samples collected were transferred to the hospital's laboratory to isolation and confirm the organism. The samples were cultured on blood agar and EMB media and then identified using gram staining and biochemical tests such as oxidase, catalase, citrate, MRVP, SIM, TSI.

Assessment of the Isolated Strains' Biofilm Formation Ability

Initially, the Tryptic Soy Broth (TSB) culture medium containing 2 % glucose was prepared and sterilized. In order to

prepare bacterial suspensions, the strains under study were diluted in a certain volume of the TSB medium supplemented with 2 % glucose to reach 0.5 McFarland turbidity. Finally, bacterial suspensions were added to each well of a 96-well plate. Three wells were regarded as positive controls for growth of bacteria and formation of biofilm, and three wells containing no bacteria were regarded as negative controls (no growth and, therefore, no biofilm formation) in microtiter plates. Then the plates at 37 °C for 24 hours incubated. For biofilm staining, the culture media were removed from the wells, followed by washing with PBS buffer 1-2 times. Then absolute methanol was added to the wells, and incubation continued for 10 min at 25 °C temperature. Then, the methanol was eliminated, and the plate to be air-dried to completely remove the remaining alcohol. Crystal violet 1 % was added to the wells, and the plate was incubated at 25 °C temperature. The wells were rinsed with distilled water to clean residual dye. Finally, the wells were emptied, and glacial acetic acid 33 % was added to each well. After 20 min, absorption was read at 570 nm using an ELISA reader [15].

The results were analyzed using the Optical Density cut-off (ODc) method according to the below formula:

$$\text{ODc} = (\text{Mean OD}) \text{ negative control wells} + (3 \times \text{standard deviation of negative control wells})$$

When OD was $>4 \times \text{ODc}$, this was indicative formation of strong biofilm; $2 \times \text{ODc} < \text{OD} \leq 4 \times \text{ODc}$ formation of moderate biofilm; $\text{ODc} < \text{OD} \leq 2 \times \text{ODc}$ formation of weak biofilm, and $\text{OD} \leq \text{ODc}$ no biofilm formation.

Minimum Inhibitory Concentration Determination Using the Serial Dilution Method

In order to investigate MIC, the serial dilution method was employed in the Mueller Hinton Broth (MHB) culture medium in triplicates. First, 100 µL of the MHB medium was poured to all wells. Then, the wells in the first row were loaded with either 100 µL of *Z. multiflora* essential oil (code 2547-A, Golha Tab Kashan Co., Iran) or silver nanoparticles (Sigma Co., Cat. No. 730793) with the dimensions of 20 nm at the concentration of 128 µg/mL using the serial dilution method to last well. In the end, 100 µL of the bacterial suspension at a concentration of 10^6 (1/100, 0.5 McFarland turbidity) was added to all the wells. In each

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plate, a positive control well (containing the medium and suspension of bacteria) and a negative control well (the medium and essential oil) were used. The plates were incubated at 37 °C for 24 hours. Finally, turbidity at 620 nm was measured using an ELISA reader for all wells.

The Effects of Essential Oil and Silver Nanoparticles on Biofilm Formation

Initially, 0.5 McFarland bacterial suspensions from the strains studied were prepared in the TSB culture medium containing 2 % glucose. The bacterial suspensions were then poured into wells and treated with the MIC concentration of essential oil of *Z. multiflora* and silver nanoparticles to analyze biofilm formation by each Strain. The mixture was incubated at 37 °C for 24 hours. Next, conventional staining was performed, as mentioned above.

The Effects of Zataria multiflora Essential Oil and Silver Nanoparticles on the Expression of the Genes Involved in Biofilm Formation

The strains studied were into two grouped: 1) the control, in which only bacterial suspensions were used without adding essential oil or nanoparticles, and 2) the treatment group, in which bacterial strains were exposed to the MIC concentration of

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the essential oil and nanoparticles separately. In the end, bacteria in both the control and treatment groups were incubated at 37 °C for 24 hours. After this period, RNA was extracted from the samples using a kit procured from the Qiagen Company (Qiagen, USA). For cDNA synthesis, the RNA extracted was used in a reaction containing the reverse transcriptase enzyme following the instructions of the manufacturer (GeenALL, South Korea).

Table 1 demonstrates the sequences of the primers used in this study to detect the *pilA*, *pilT*, *pilD*, and *abaI* genes as the genes involved in biofilm formation. The 16S rRNA gene was used as the house keeping gene. These primers obtained from the literature and checked using BLAST software on the NCBI website. The quality of the primers (i.e., lack of stems and loops) was assessed with Oligo7 software. The primers were finally synthesized by SinaGene Co. (Iran).

In order to conduct real time-PCR, 12.5 µL of a master mix containing CyberGreen dye (United Kingdom, Applied Biosystem), 10 pMol of each of the primers, 10 ng of cDNA, and 9 µL water were used to prepare a reaction mixture in 25 µL. The

PCR reaction was performed in a thermal cycler (ABI, USA) applying the following temperature profile: 95 °C for 10 min, followed by 40 cycles of 95 °C for 20 sec, 60 °C for 40 sec, and 72 °C for 60 sec.

Statistical Analysis

The quantification of gene expression relative to the house keeping gene (16S rRNA) was performed by the $\Delta\Delta C_t$ method using REST software. Statistical analyses were conducted in SPSS ver.22 software. Correlations were calculated and the means were compared by t-test with a significance level of $p < 0.05$.

RESULTS

Bacterial isolates

Thirty *A. baumannii* strains were isolated from the clinical samples of hospitalized patients of the Milad Hospital, Tehran, and identified.

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The MICs of Zataria multiflora Essential Oil and Silver Nanoparticles

The MICs of *Z. multiflora* essential oil and silver nanoparticles for *A. baumannii* were investigated by the broth dilution method. The 0.25 and 0.125 µg/mL concentrations of *Z. multiflora* essential oil exerted the highest and lowest growth inhibitory effects, suppressing the with the frequency of 46.7 % and 3.3 % of the strains investigated, respectively (Figure 1). The 0.5 µg/mL concentration of silver nanoparticles presented the highest growth inhibition suppressing the growth of 43.3 % of the bacterial strains studied, and the concentrations of 4 and 8 µg/mL (with a respective frequency of 3 %) had the lowest growth inhibitory effects (Figure 2).

Table 1. The features of the primers used in PCR

Primers	Sequence (5'-3')	Product length
	F: ACACTGCTCGTTCTCAAA	
	R: GCTGCGGGGTATTATTC	
	F: CACCCAACTCAGCCATTT	
	R: CCGTAGCCCATACCTTCT	
	F: TATCCTCGTGGCATTGTA	
	R: ATGGTCATAGCGGTTTTTC	
	F: TTTCCCCAACTTCTC	
	R: ACACAGCCTGACTGC	
	F:TCG CTA GTA ATC GCG GAT CA	
	R:GAC GGG CGG TGT GTA CAA G	

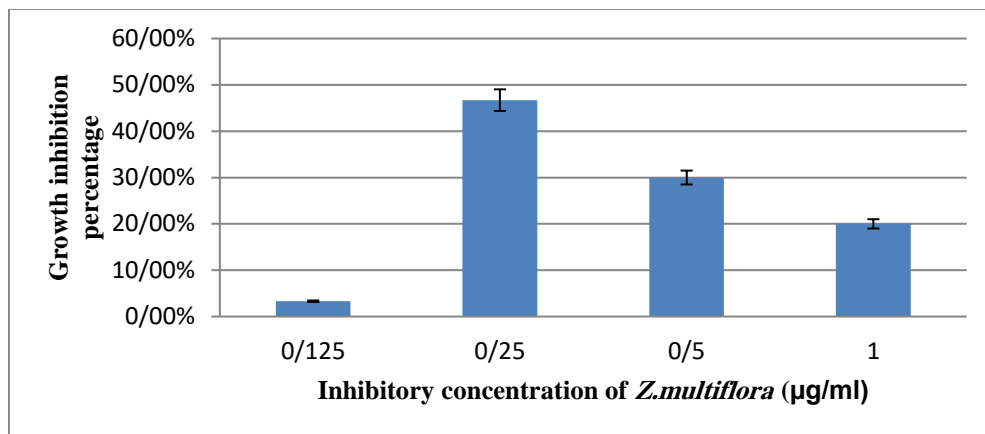


Figure 1. The Minimum inhibitory concentrations of *Zataria multiflora* essential oil against *A. baumannii*.

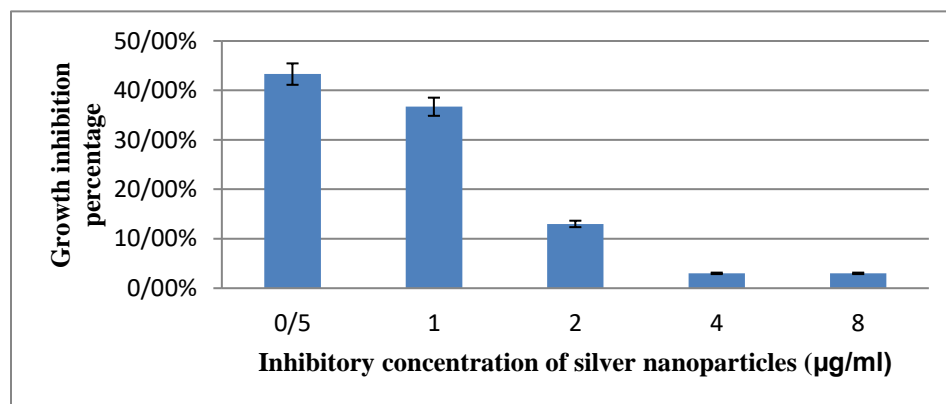


Figure 2. The Minimum inhibitory concentrations of silver nanoparticles against *A. baumannii*.

Biofilm Formation Assessment by the Microtiter Plate Method

In this study, all strains were able to form biofilm, of which 80 % formed a strong biofilm; 16.7 % of the strains formed a moderate biofilm, and 3.3 % were weak biofilm formers.

The Effects of MICs of *Z. multiflora* Essential Oil and Silver Nanoparticles on Formation of Biofilm

According to Figure 3, after treatment with the MIC concentration of *Z. multiflora* essential oil (0.25 µg/mL), 46.7 % of the strains treated formed weak biofilms, and 53.3 % produced no biofilm. Also, 56.7 %, 50 % and 33.3 % of the strains treated with silver nanoparticles at concentrations of 0.5, 1 and 2 µg/mL, respectively, formed weak biofilms, and 43.3 %, 50 % and 66.7 % produced no biofilm.

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40 %, and 3.3 % of the strains exposed to silver nanoparticles at the MIC concentration (0.5 µg/mL) produced strong, moderate, and weak biofilms, respectively.

Statistical analysis of the mean ODs obtained from the biofilm formation assay revealed a significant effect for the MIC concentration of *Z. multiflora* essential oil and silver nanoparticles on the biofilm-producing ability of the bacterial strains studied ($p=0.0001$).

Effect of MIC of Z. multiflora Essential Oil on the Gene Expression Levels of pilA, pilT, pilD, and abaI

In the present study, none of the investigated strains contained the *pilA* gene. According to Figure 5 and gene

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expression calculations described by the fold changes, 58.33 %, 50 %, and 41.66 % of the strains treated with the MIC concentration of *Z. multiflora* essential oil showed decreased expressions of the *pilT* ($p=0.007$), *pilD* ($p=0.007$), and *abaI* ($p=0.502$) genes, respectively.

Effect of MIC of Silver Nanoparticles on the Gene Expression Levels of pilT, pilD, and abaI

According to Figure 6 and gene expression calculations, 50 %, 88.33 %, and 58.33 % after the treatment of strains with the MIC concentration of silver nanoparticles showed decreases in expression of the *pilT* ($p=0.603$), *pilD* ($p=0.001$), and *abaI* ($p=0.662$) genes, respectively.

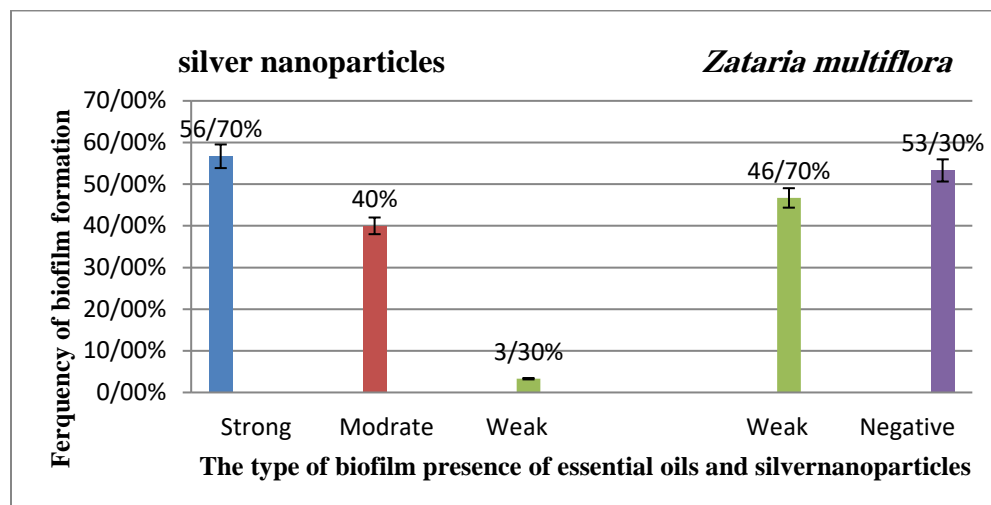


Figure 3. Biofilm formation in *A. baumannii* after treatment with essential oil and silver NP.

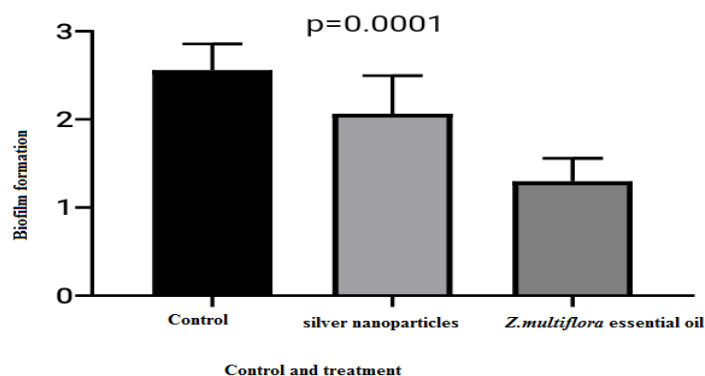


Figure 4. The biofilm formation ability of *A. baumannii* strains at MIC of essential oil and nanoparticles.

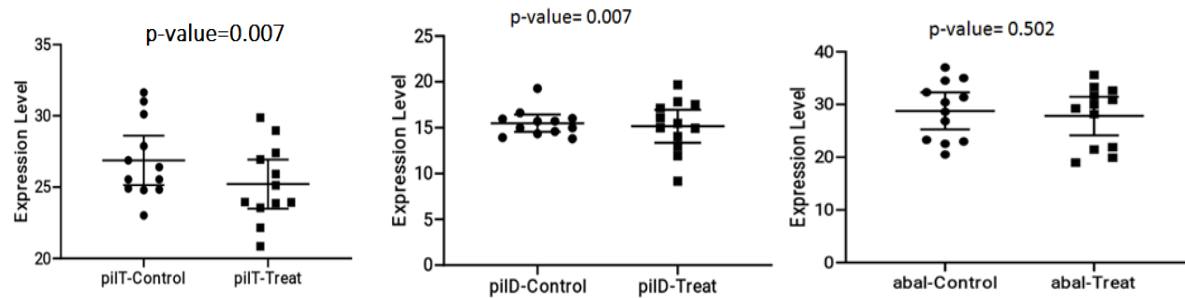


Figure 5. The expression of the *pilT*, *pilD*, and *abal* genes in the strains treated with the MIC of *Z. multiflora* essential oil.

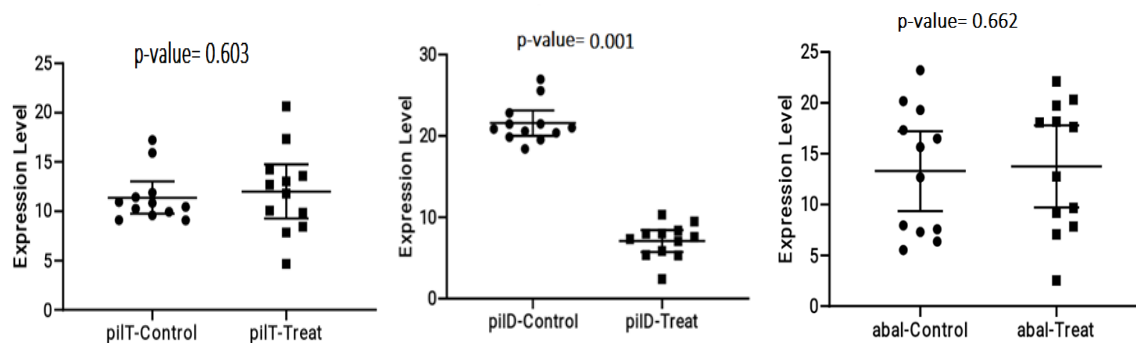


Figure 6. The expression of the *pilT*, *pilD*, and *abal* genes in the strains treated with the MIC of silver nanoparticles.

DISCUSSION

One of the clinical problems related to *A. baumannii* infections is the emergence of multi-drug resistant strains that are unresponsive to different classes of antibiotics such as beta-lactams,

aminoglycosides, fluoroquinolones, and most other types of antibacterial drugs [20]. Biofilm formation is a key element helping bacteria survive in various environments, cope with adverse conditions such as food shortages, and evade the immune system of host and antimicrobial substances. In the

host's body, macrophages can more easily phagocytize and eradicate planktonic cells. Bacteria with the ability to grow in a biofilm can easily escape from the host's immune system, causing chronic infections [21]. Bacterial biofilms substantially contribute to the development of infectious diseases, particularly chronic inflammation in the host's body [22].

Several studies have been conducted on the antibacterial effects of the essential oils of the plants belonging to the Lamiaceae family, a plant family embracing *Z. multiflora* as a member. Some important known compounds in the essential oils include carvacrol and thymol, whose antimicrobial effects have been attributed to their cell permeability ability. These compounds can destroy the cations embedded in the cell membrane, leading to its dysfunction. Thymol also suppresses the activity of the ATPase enzyme and, thereby, increases the cell membrane specific permeability. Thymol and carvacrol not only prevent the accumulation of bacteria but also increase the permeability of the cell membrane, leading to the leakage of intracellular materials, increasing the sensitivity of bacteria to antibacterial agents, and ultimately causing their death [23,24].

In this study, the antibacterial effects of *Z. multiflora* essential oil were investigated on *A. baumannii* strains. The results showed that the 0.25 µg/mL concentration of *Z. multiflora* had the strongest growth inhibitory effects, inhibiting the growth of 46.7 % of *A. baumannii* strains assessed. Our results showed the higher growth inhibitory effects of *Z. multiflora* essential oil compared to silver nanoparticles on the strains investigated. The findings of Sultan Dalal *et al.* showed that *Z. multiflora* essential oil had considerable anti-growth effects on food-derived *Staphylococcus aureus* resistant to tetracycline, erythromycin, trimethoprim-sulfamethoxazole, and methicillin [25]. In a study by Ravanshad *et al.* it was shown that *Z. multiflora* essential oil at concentrations of 1 % and 2 % had potent microbicidal effects against *Enterococcus faecalis* [26]. In another study by Ramazan Pour *et al.* in 2016, it was reported that *Z. multiflora* essential oil has the highest antibacterial effect, with a frequency of 64.2 % [27].

In this study, the antibacterial effects of silver nanoparticles against *A. baumannii* strains isolated from clinical samples were evaluated. The concentration of 0.5 µg/mL of silver nanoparticles had an inhibitory

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effect on *A. baumannii* growth with 43.3 % frequency. In the study of Minghui Chen *et al.*, who investigated the antibacterial effects of silver nanoparticles on the clinical isolates of Multi-Drug Resistant (MDR) *A. baumannii*, the results revealed that silver nanoparticles had dose-dependent antibacterial activity against MDR *A. baumannii* [28]. In 2018, Dehghan *et al.* investigated the antimicrobial effects of silver nanoparticles produced by sesame plant and declared that these nanoparticles had considerable antimicrobial activity against *Bacillus subtilis*, *Escherichia coli*, *Staphylococcus aureus*, *Saccharomyces cerevisiae*, and *Candida albicans* [29]. In the study of Habibipour *et al.* in 2015, in which the ability of silver nanoparticles to eradicate *Klebsiella pneumoniae* isolated from the industrial effluents in Hamedan City, the concentration of silver nanoparticles and the type of bacteria were evaluated as an important factors on antimicrobial properties of silver nanoparticles. Although the concentrations used in the recent study reduced the burden of bacteria, they could not completely eradicate them [30].

Nanoparticles have shown the little level of toxicity in the nature, so the use of these materials to destroy pathogenic

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microorganisms can be useful. Numerous studies have assessed the reactions occur between nanoparticles and biomacromolecules in organisms. The negative surface charge of microorganisms and the positive charge of the nanoparticle create an electromagnetic force between them, facilitating the attachment of nanoparticles to the cell surface [31]. Many of these reactions can lead to oxidation the molecules of surface and cause immediate death of microorganisms. It has been noted that nanomaterials release the ions, and proteins contain of thiol (S-H bonding) groups on the surface of bacterial cells can react with these ions. A number of these bacterial proteins are responsible for transporting materials through the bacterial cell wall, which are inactivated after binding to nanoparticles, causing the membrane to become impermeable [32] and, eventually, cell death. Also, nanomaterials delay the attachment of some bacteria to surfaces and biofilm formation, preventing them from being stabilized and then divided [33].

In this study, examining the ability of biofilm formation of bacteria using the microtiter plate method revealed that all the bacterial strains assessed had the ability to produce biofilm, of which 80 %, 16.7 %,

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and 3.3% were strong, moderate, and weak biofilm formers, respectively. When treated with *Z. multiflora* essential oil at the concentration of 250 µg/mL, 46.7 % of the *A. baumanni* strains produced weak biofilm, and 53.3% of the strains created no biofilm. None of the strains produced strong or moderate biofilm, indicating the strong anti-biofilm effects of *Z. multiflora* essential oil on *A. baumannii*. In a study by Narenji *et al.*, the researchers showed that *Z. multiflora* essential oil could inhibit biofilm formation by six *Staphylococcus aureus* strains that were able to produce stable biofilm. These findings suggest that herbal essential oils can be used to inhibit biofilm formation by pathogenic bacteria [34]. Akanksha Singh *et al.* showed that thyme essential oil (75 µM) decrease the formation of biofilm and pathogenesis of *Xanthomonas* [35]. Jiamu Kang *et al.* investigated the effects of thyme essential oil on planktonic and biofilm-forming *Bacillus cereus* and reported that the MIC of this compound on the planktonic form of this bacterium was 0.25 µg/mL. Also, the constituents of this essential oil markedly inhibited biofilm formation by this bacterium, as evidenced by Scanning Electron Microscopy (SEM) [36]. Shirdel in 2017 revealed that ZEO and SNP at MIC

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and OIC concentrations represented a strong removal ability (>70 %) on biofilm [37].

The effect of silver nanoparticles on biofilm formation of *A. baumannii* strains was weaker than *Z. multiflora* essential oil. When treated with the MIC (0.5 µg/ml) of silver nanoparticles, 56.7 %, 40 %, and 3.3 % of the strains assessed produced strong, moderate, and weak biofilms, respectively, while this treatment could not suppress biofilm formation. On the other hand, treatment with *Z. multiflora* essential oil inhibited biofilm formation in a significant ratio of the strains (53.3 %).

Richa Sing *et al.* in their study assessed the applicability of nanoparticles in controlling the infections caused by biofilm-forming *Acinetobacter* species and highlighted the importance of nanoparticles as anti-biofilm agents that could, as surface coating materials, reduce biofilm formation by *Acinetobacter* [38]. Ahmadasab *et al.* showed in a study that silver-iodide nanoparticles at the concentrations of 5 and 100 g/mL could respectively reduce and completely suppress biofilm formation by *Listeria monocytogenes*, suggesting that these nanoparticles could be considered effective antimicrobial agents in the food

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industry to inhibit biofilm formation by *L. monocytogenes* [15].

In the current study, the effect of treatment with MIC of *Z. multiflora* essential oil and silver nanoparticles was assessed on the expression of the *pilT*, *pilD*, and *abaI* genes. The results showed that treatment with silver nanoparticles induced the largest decrease in the expression of the *pilD* gene, while the lowest decline was related to the *pilT* gene. Also, in the strains treated with *Z. multiflora* essential oil, the largest and smallest decrease were observed in the expression of the *pilT* and *abaI* genes, respectively.

Piri *et al.* investigated the inhibitory effects of silver nanoparticles on the gene expression of *bap* in antibiotic-resistant *A. baumannii* strains. The results showed that exposure to the sub-MIC dose of silver nanoparticles significantly reduced the expression of the *bap* gene in all strains ($p < 0.05$) [39]. Azizi *et al.*, in other study, analyzed the expression of the genes involved in biofilm formation by MRD strains of *A. baumannii* and reported that the upregulation of the *bap* gene affected biofilm formation at low iron concentrations [40]. In an experiment by Bahador *et al.* on *A. baumannii* strains, it

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was reported that *Satureja khuzestanica* essential oil reduced the expression of the *bap* gene *in vitro*, but it had no effect on the expression of the DNA gyrase-A housekeeping gene [41]. Azizkhani showed that *Z. multiflora* essential oil in subMIC level decrease expression of *seA*, *seC*, *seE* and *agrA* genes [42].

CONCLUSION

The results of this research demonstrated that *Z. multiflora* essential oil and silver nanoparticles decreased formation of biofilm of *A. baumannii* strains. Our results also revealed that *Z. multiflora* essential oil reduced the expression of the *pilT* gene that prevent the bacteria to attach the surfaces and the initiation the formation of biofilm. Treatment with *Z. multiflora* essential oil also reduced, though to a lesser amount, the expression of the gene encoding *abaI*, which is responsible for quorum sensing. Therefore, *Z. multiflora* can probably affect the first step formation of biofilm and, and has less effect in the later steps of biofilm formation. In comparison, silver nanoparticles induced more reductions in the expression of the *pilD* and *abaI* genes, offer a role for this agent in biofilm formation and quorum sensing stages.

Based on phenotypic evidence, the effect of silver nanoparticles was less pronounced than that of *Z. multiflora* on biofilm formation. Nevertheless, data in this field are incomplete, requiring more molecular and phenotypic studies to define the exact mode of action of these agents in biofilm formation.

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