



Original article

Synergistic activity of melittin with mupirocin: A study against methicillin-resistant *S. Aureus* (MRSA) and methicillin-susceptible *S. Aureus* (MSSA) isolatesReza Hakimi Alni^a, Fatemeh Tavasoli^b, Amirhomayoon Barati^b, Shaghayegh Shahrokhi Badarbandi^b, Zahra Salimi^b, Laleh Babaeekhou^{b,*}^a Department of Microbiology, Faculty of Medicine, Hamadan University of Medical Sciences, Hamadan, Iran^b Department of Biology, Islamshahr Branch, Islamic Azad University, Islamshahr, Iran

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ABSTRACT

Methicillin-Resistant *Staphylococcus aureus* (MRSA) biofilms are involved in various nosocomial infections, being in the limelight of academic research. The current study aimed to determine the antimicrobial effects of melittin on planktonic and biofilm forms of *S. aureus*. Following the identification of MRSA and SCCmec types (using PCR method), Minimum Inhibitory Concentration (MIC), Minimum Bactericidal Concentration (MBC), and fractional inhibitory concentration index (FICI), for melittin and mupirocin were determined by broth microdilution assay. Melittin anti-biofilm activity was determined, using a microtiter-plate test (MtP) and scanning electron microscope (SEM) methods. The quorum sensing inhibitory activity of ½ MIC melittin was examined using a quantitative real-time RT-PCR method, and melittin cytotoxicity on Vero cells was examined by tetrazolium-based colorimetric (MTT) test. The Results of our study showed that Geometric means of MIC values of the melittin and mupirocin were 4.4 and 14.22 µg/ml respectively. The geometric mean of the FICI for both melittin-mupirocin was 0.75. No *S. aureus* biofilm was formed and *hld* gene (as a biofilm regulator) expression down-regulated. It seems that melittin can be useful in the treatment of *S. aureus* infections (especially MRSA) by reducing the *hld* expression. Furthermore, synergistic growth-inhibitory effects of mupirocin with melittin could be considered as a promising approach in the treatment of MRSA isolates.

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1. Introduction

Methicillin-resistant *S. aureus* (MRSA) is the greatest concern of all health-care-associated pathogens and causes hospital and community-acquired infection disease (Gordon and Lowy 2008). Resistant Staphylococcal infections have led to a higher mortality rate and have prolonged antibiotic therapy as compared with methicillin-sensitive Staphylococcal infection. MRSA strains have

acquired a mobile genetic element into their genome which harbors the genes encoding penicillin-binding protein gene (*mecA*) and other regulatory genes such as *mecR* and *mecI*. These mobile genetic elements termed the staphylococcal cassette chromosome *mec* (SCCmec) and are seen in MRSA strains (Ito et al. 2001, Ji 2007).

A variety of antimicrobial agents such as tetracycline, daptomycin, fluoroquinolone, and linezolid are used for the treatment of *S. aureus* infections (Tiwari and Sen 2006) but due to the ability of bacteria in neutral of them and biofilm formation, antimicrobial agents soon become ineffective (Lowy 2003; Alaouadi 2015). Biofilms are a community of bacteria that attach to biotic or abiotic surfaces via the production of an extracellular matrix called exopolysaccharide (Watnick and Kolter 2000). Biofilm formation and other processes including bioluminescence, sporulation, competence, and virulence factor secretion are usually controlled by a Quorum sensing (QS) system. QS is a bacterial cell-cell communication process and releases extracellular signaling molecules (autoinducers) that increase in concentration as a function of cell

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density. Hence, the reduction in the expression of genes involved in QS can be important in *S. aureus* infection control (Moormeier and Bayles 2017).

Anti-QS compounds are known to have the ability to prohibit bacterial pathogenicity (Choo et al. 2006). In this regard, antimicrobial peptides (AMPs) as therapeutic agents are attractive solutions and are currently under evaluation to be used as QS system inhibitors against multidrug-resistant pathogens (including MRSA isolates) (Chung and Khanum 2017). However, they cannot act as the sole solution to overcome the biofilm formation ability. So, the combined use of AMPs and conventional antibiotics (such as mupirocin) would be a rationale to address bacterial infections in a superior way (Le and Otto 2015).

Among AMPs, melittin is a natural antimicrobial peptide (26 amino acid peptide) that can act against a broad range of microorganisms, including Gram-negative and -positive bacteria, and may have a good synergistic effect on killing and inhibiting biofilm formation of bacteria. Due to its amphipathic nature, melittin presents a strong binding affinity to the bacterial membrane. This feature provides its therapeutic potential for various bacterial and viral diseases (Bardbari et al. 2018).

Accordingly, the first aim of the present study was to assess the antibacterial, anti-biofilm, and anti-quorum sensing activities of melittin against strong biofilm-forming *S. aureus* and reference strains. Then, the synergic effects of melittin and mupirocin against MRSA isolates were evaluated.

2. Material and methods

2.1. Isolation and identification of *S. Aureus*

From March 2016 to February 2017, a total of 56 microbiological wound swabs were collected from patients with burn wound infection in Shahid Beheshti hospital of Hamedan province, Iran. The swabs were cultured on blood agar and mannitol salt agar media followed by incubation at 37 °C for 18–24 h. Identification of *S. aureus* was performed by common biochemical tests (included Gram staining, growth on mannitol salt agar, catalase, DNase, and coagulase tests). In the following, DNA samples were extracted using the phenol–chloroform method, and the isolates were confirmed as *S. aureus* using species-specific PCR (identification of *nuc* gene) (Table 1) (Brakstad et al. 1992).

2.2. Identification of MRSA isolates and SCCmec types

MRSA strains were determined by the presence of the *mecA* gene using the PCR technique as described previously. Also, the SCCmec type of the MRSA strains was determined using 8 sequences of primers as described by Zhang et al., 2005 (Table 1) (Zhang et al. 2005).

2.3. Biofilm assay

All bacterial isolates were evaluated for biofilm formation by end-smooth 96-cells microplates as explained by Tendolkar et al. (Tendolkar et al. 2004). In summary, 200 µl of fresh bacterial culture in TSB medium was dropped into each well of 96-cells microplates and incubated for 24 h at 37° C. The wells were emptied of TSB medium and were washed three times with PBS. In the following 100 µl of crystal violet (%1) were placed to each well. The extra dye was then removed and the cells attached to the microplate floor were dissolved using 100 µl of alcohol-acetic acid and the optical density (OD) of each well was measured at 570 nm using a microtiter plate.

Table 1

Primers used to determine the SCCmec types, MRSA isolates and expression of *hld* gene.

Primer	Sequence 5'- 3'	PCR product size (bp)	Type
Type I-F	GCTTTAAAGAGTGTCTGTTACAGG	613	SCCmecI
Type I-R	GTTCTCTCATAGTATGACGTCC		
Type II-F	CGTTGAAGATGATGAAGCG	398	SCCmecII
Type II-R	CGAAATCAATGGTTAATGGACC		
Type III-F	CCATATTGTGTACGATGCG	280	SCCmecIII
Type III-R	CCTTAGTTGTCTGTAACAGATCC		
Type IV a -F	GCCTTATTGGAAGAAACCG	776	SCCmecIVa
Type IV a R	CTACTCTTCTGAAAAGCGTCC		
Type V-F	GAACATTGTTACTTAATGAGCG	325	SCCmecV
Type V-R	TGAAAGTTGTACCTTGACACC		
<i>MecA</i> 147-F	GTGAAGATATACCAAGTGATT	147	-
<i>MecA</i> 147-R	ATGCGCTATAGATTGAAAGGAT		
<i>hld</i> -F	ATTTGTCTACTGTCTCGATAATCC	120	-
<i>hld</i> -R	GGAGTGATTCAATGGCCACAAG		
16sRNA-F	AGCCGACCTGAGAGGGTGA	187	-
16sRNA-R	TCTGGACCGTGTCTCAGTCC		
<i>nucA</i> -F	GCGATTGATGGTGATACGGTI	267	-
<i>nuc A</i> -R	AGCCAAGCCTTGACGAACCTAAAGC		

2.4. Peptide synthesis order and MIC and MBC determinations

Melittin was made by an external facility (Mimotops Co., Australia) in 98% purity using the solid-phase method. The purified Melittin injected to reversed-phase high-performance liquid chromatography (RP-HPLC) again to control the purity. The accuracy of the synthetic peptide was controlled by mass spectrometry on a triple quad LC/MS instrument (Sciex API100 LC/MS instrument, Perkin Elmer Co., Norwalk, CT, USA). The MICs for melittin and mupirocin were determined by broth microdilution assays according to the Clinical and Laboratory Standards Institute (CLSI) recommendations (CLSI, 2015). Briefly, 100 µl of the bacterial inocula (OD of the suspension was set 0.09 at 625 nm), 100 µl of melittin, or mupirocin at different concentrations (0.06 to 31.5 µl.ml⁻¹), were distributed into each well of ELISA microplates (96 well). The microplates were incubated at 37 °C for 18–24 h. The MIC was recorded as the lowest amount of the antibacterial agents producing complete inhibition of visible growth and interpreted as v/v percentage of stock solution. To determine MBC, 10 µl of last three wells with no growth bacteria, were cultured on blood-agar plates followed by incubation at 37 °C for 24 h. The MBC was considered as the lowest concentration at which no bacterial colonies were developed.

2.5. Measurement of the synergistic effects

The synergistic effects of melittin and mupirocin were assessed using the broth microdilution checkerboard method. Serial dilutions of melittin and mupirocin were added to the microtiter plates at a volume of 100 µl, then 100 µl of the bacterial suspension (corresponding to 0.5 of the McFarland) was added to each well. The fractional inhibitory concentration index (FIC_i) for melittin and mupirocin was calculated as follows: $FIC_i = \frac{(MIC_{of drug A in combination})}{(MIC_{of drug A alone})} + \frac{(MIC_{of drug B in combination})}{(MIC_{of drug B alone})}$. The FIC_is were interpreted as follows: FIC_i ≤ 0.5: synergy; FIC_i > 0.5 to 1.0: addition; FIC_i < 4.0: indifference; FIC_i ≥ 4.0: antagonism (Giacometti et al. 2003).

2.6. Biofilm inhibition assay

The effect of the melittin on the biofilm formation of *S. aureus* was assessed by Tissue Culture Plate (TCP) method as described before, with some modifications (Adukwu et al. 2012). Briefly,

100 µl of overnight cultures (0.5 MacFarland bacterial culture) was dispensed into each well of 96-well polystyrene flat-bottomed microtiter plates in the presence of 100 µl of TSB contained by different concentrations of the melittin (MIC/2, MIC/4, MIC/8, and MIC/16). The MIC value of melittin has already been calculated. After incubation for 24 h at 37 °C, the planktonic cells were removed and each well was washed with phosphate-buffered saline (PBS) and stained for 20 min with 1% (w/v) crystal violet and washed again with PBS. The stained biofilms were solubilized in 200 µl absolute ethanol and the optical density (OD) values were measured at 630 nm using UV-visible spectrophotometer (Bio-Tek, Winooski, USA). Each assay was performed in triplicates and the negative control was the bacteria in TSB without melittin or the antibiotics. Finally, the percentage of inhibition of biofilm was calculated using the following formula. Percentage of inhibition = $100 - [(OD_{630\text{ nm}}$ of the treated wells) / (mean $OD_{630\text{ nm}}$ of the negative control wells contained no antimicrobial agent) $\times 100]$ (Onsare and Arora 2015).

2.7. Effect of melittin on quorum sensing gene expression

The effect of sub-MIC concentrations (MIC/2) of melittin on the expression of quorum sensing (*hld*) genes was measured in clinical isolates (8 strains) and reference strain (*S. aureus* ATCC33591). For the synthesis of cDNA, each bacterium was grown with and without melittin (MIC/2) in tubes contained TSB medium with DMSO (0.1%) and incubated at 37 °C for 24 h. then, Total RNA was extracted by using an RNA Isolation Kit (MoBio, USA) and was quantified by BioDrop (BioDrop, UK) and immediately converted to cDNA using the cDNA Reverse Transcription Kit (SinaClon, Iran) and random primer oligonucleotides. The Quantitative real-time-PCR (qPCR) assays were carried out using a commercial SYBR Green master mix. The primers used in this study are listed in Table 1. The target genes expression levels in comparison to the internal 16 s rRNA control were evaluated with the $2^{-\Delta\Delta Ct}$ method.

2.8. Scanning electron microscope (SEM)

SEM was employed for investigating of biofilm formation and the effect of melittin (MIC/2 concentration) on the biofilm structure of *S. aureus* isolate. To do this, *S. aureus* isolate was cultured in well microtiter plates and a glass coverslip was placed in each well. The control wells contained TSB medium with DMSO and *S. aureus* (1.5 ml of an overnight culture), and the treated biofilm groups contained TSB medium with MIC/2 concentration of melittin plus *S. aureus*. After 21 h of incubation at 37 °C, the glass coverslip was fixed in a solution containing 2.5% buffered glutaraldehyde (% v/v) for 3 h. The samples were then dehydrated in a graded ethanol series and dried at room temperature. At last, the samples were examined using a JEOL JSM-840 scanning electron microscope; the acceleration tension was 15 kV.

2.9. Cytotoxicity assay

Cytotoxicity of melittin was evaluated using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay using a previously described method (Sadeghi et al. 2013).

2.10. Statistical analysis

Each experiment was performed at least three times, and the data are expressed as means \pm SD. Eventually, the results were analyzed by Student's *t*-test using SPSS software and $P < 0.05$ was considered statistically significant

3. Results

3.1. Isolation of *S. aureus*, identification of MRSA isolates and SCCmec types

In this study, 25 *S. aureus* strains were recovered from burn wound infection and methicillin resistance gene (*mecA*) was found in 19 (76%) isolates by PCR method. SCCmec typing among MRSA isolates showed that 8 (42.11%), 3 (15.8%), 2 (10.53%), and 1 (5.26%) isolates contained SCCmec type III, type I, type IV, type II, and type V, respectively; however, 5 (26.32%) isolates were nontypable.

3.2. Biofilm assay

According to Tendolkar et al method, each isolate was characterized in four groups of non-biofilm forming ($OD \leq OD_c$), weak biofilm forming ($OD_c < OD \leq 2 OD_c$), moderate biofilm-forming ($2 OD_c < OD < 4 OD_c$) and strong biofilm-forming ($OD > 4 OD_c$) bacteria. In this study, from a total of 25 *S. aureus* strains, 12 isolates (48%) were grouped as strong biofilm producers and 6 (24%), 2 (8%), and 5 (20%) isolates were grouped as moderate, weak, and non-biofilm producers respectively.

3.3. MIC and MBC determinations

A total of 8 isolates (4 MRSA isolates, 4 MSSA isolates) was tested for the effect of the melittin on the planktonic cell growth. Also, *S. aureus* ATCC33591 was used as a positive control. The MICs and MBCs of melittin for each organism are given in Table 2. The geometric mean of the MIC and MBC values of melittin for all isolates were 4.4 and 8.8 µg, respectively.

3.4. Measurement of the synergistic effect

Synergistic action of melittin and mupirocin against planktonic growth isolates was detected by the checkerboard assay. The geometric means of the FIC_i for all strains for melittin and mupirocin were calculated as 0.75, (Table 2). A paired sample *t*-test showed a significant difference between Σ FIC results of MRSA and MSSA isolates ($p < 0.05$).

3.5. Effect of the melittin on the biofilm formation

In Fig. 1, the biofilm formation of the 9 strains (mean of 8 isolates and 1 standard isolate) in the presence of different sub-MICs of melittin are presented. In MtP assay, sub-MICs concentrations of melittin (Except MIC/16) play a significant role in the inhibition of biofilm formation by all of the bacteria ($P < 0.001$).

3.6. Quantification of gene expression by qPCR

The effect of MIC/2 dose of melittin on the expression levels of the quorum-sensing gene and 16S rRNA gene were evaluated for 8 isolates of *S. aureus* and reference strain (*S. aureus* ATCC33591) and the result is shown in chart 1. As shown in Fig. 2, the expression level of quorum sensing genes of clinical isolates of *S. aureus* significantly reduced after exposure to MIC/2 dose of melittin compared with untreated *S. aureus* isolates ($P < 0.05$, Fig. 2).

3.7. Scanning electron microscopy (SEM)

SEM was the chosen method for analyzing the effect of melittin on the biofilm structure of *S. aureus* isolate. SEM results showed that the bacteria in the control slide were attached and formed a

Table 2
MIC, MBC and FIC_i of melittin and mupirocin against the isolates.

Isolates	Mupirocin (µg/ml)		Melittin (µg/ml)		FIC _i Mel-Mu
	MIC	MBC	MIC	MBC	
ATCC33591	1	2	4	8	1.25
MRSA-1, SCCmecI	4	8	2	4	0.75
MRSA-2, SCCmecII	8	16	4	8	0.50
MRSA-3, SCCmecIII	64	128	8	16	0.50
MRSA-4, SCCmecIII	32	64	8	16	0.75
MSSA-1	2	4	2	4	1.00
MSSA-2	1	2	4	8	1.25
MSSA-3	8	16	4	8	0.38
MSSA-4	8	16	4	8	0.38
Geometric mean	14.22	28.44	4.4	8.8	0.75

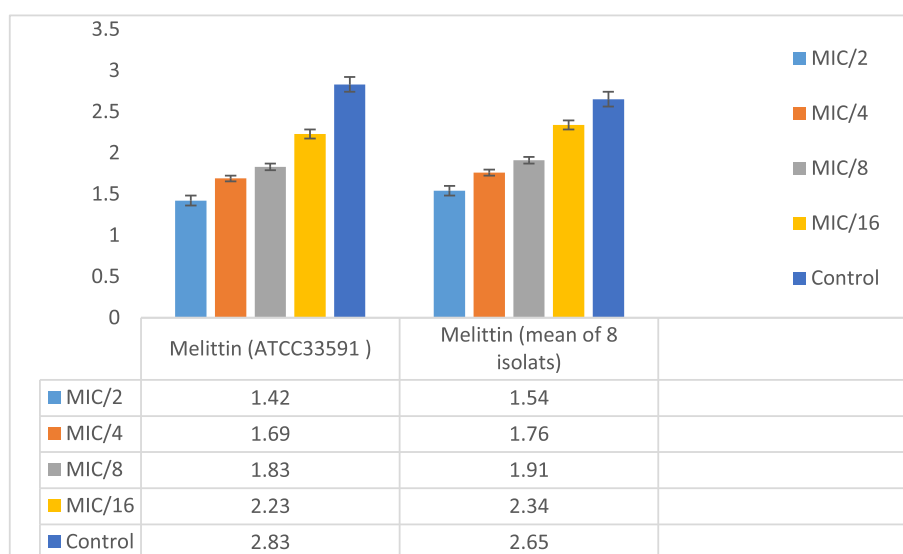


Fig 1. Effect of the melittin in different concentration on the biofilm formation.

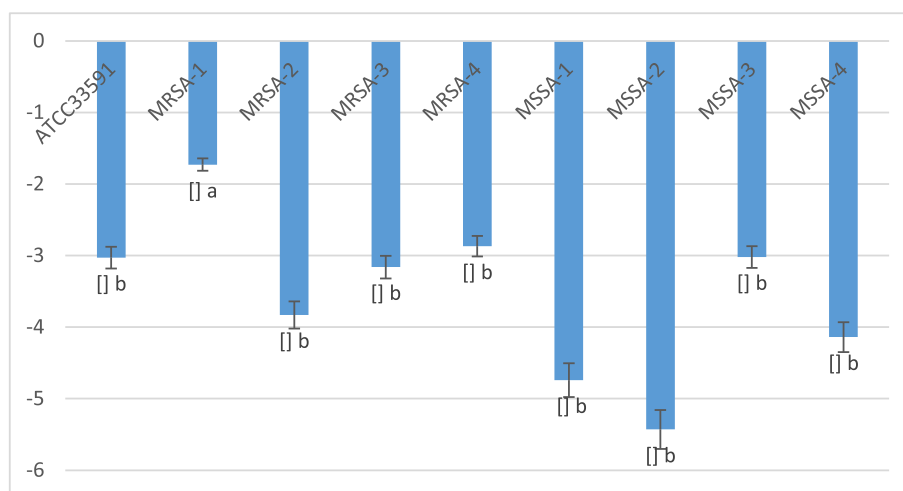


Fig 2. The expression of *hld* gene in bacterial treated with 1/2MIC of melittin a: significant downregulation; b: non-significant downregulation.

strong biofilm but in bacteria cells treated with melittin, the biofilm structure was destroyed and bacterial cells separated from the slide surface (Fig. 3).

3.8. Cytotoxicity assay

The assessment of the toxic potential of melittin by MTT test showed that this material had no significant toxic effect on the

Vero cell line. Also, The IC₅₀ values for melittin were calculated to be 33.5 µl.ml⁻¹.

4. Discussion

Antimicrobial peptides are a diverse group of molecules with 12–50 amino acids. These types of peptides have been demonstrated to kill Gram-positive and Gram-negative bacteria,

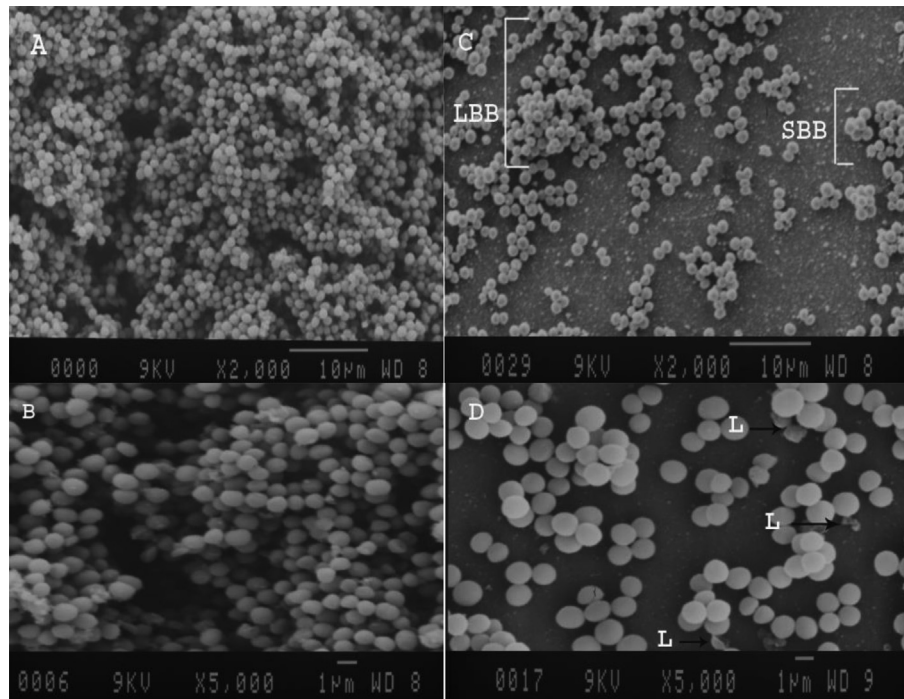


Fig 3. Effect of melittin on biofilm Formation. A and B: Untreated group. C and D treated group. LBB: Large biofilm biomass, SBB: Small biofilm biomass, L: Lysis.

enveloped viruses, fungi, and even transformed or cancerous cells (Reddy et al. 2004). In the present study, the *in vitro* antibacterial, anti-biofilm, and QSI activity of melittin were investigated against MRSA bacteria. Melittin is part of honeybee (*Apis mellifera* L.) venom which participates in the bee defenses against predators and external threats. Melittin is a small linear cytolytic peptide composed of 26 amino acids with various biological activities (Choi et al. 2015).

The MICs of melittin against *S. aureus* planktonic cells were from 2 to 8 µg.ml⁻¹, and in this issue, SCCmecIII isolates showed higher resistance to melittin and mupirocin than SCCmecI and SCCmecII. These results indicate a high intrinsic resistance of SCCmecIII isolates to antimicrobial agents and this is probably due to the existence of other resistance mechanisms in this type of *Staphylococcus aureus* isolates (Rong et al. 2017). Nevertheless, melittin has good antibacterial properties against both MRSA and MSSA bacteria. It should be mentioned that the antibacterial activities of venom against several human and animal pathogens have been reported. As melittin is the predominant component of bee venom (40–48%, w/w), most antimicrobial properties of bee venom may be related to this compound (Adade et al. 2013, Gajski and Garaj-Vrhovac 2013). Also, other toxic compounds of bee venom including PLA2, adolpanin, dopamine, and hyaluronidase may have antibacterial properties (Park et al. 2014).

Based on the structural and functional study, melittin forms pore by inserting into lipid bilayers and thus leads to the leakage of ions and molecules and the enhancement of permeability that causing cell lysis ultimately. Therefore, it can be concluded that a major part of the antimicrobial properties of melittin is related to this function (Ostroumova et al. 2015).

Encouraged by the strong biological effects of melittin some investigators have started to combine it with different antibiotics to treat MDR bacterial infections (Issam et al. 2015). Using the checkerboard method we confirmed that melittin has a synergistic or additive effect with mupirocin. Mupirocin is a novel antibacterial agent with a unique chemical structure is used in the treatment of skin impetigo caused by *S. aureus* and *Streptococcus pyogenes* (Odom 1989).

Based on previous studies the frequency of mupirocin resistance is different among clinical strains of MRSA (from 0% to 65%) (Hogue et al. 2010). Consistent with these reports, we observed that some tested *S. aureus* isolates were resistant to mupirocin (MIC >4 µg.ml⁻¹). Interestingly, in these isolates, melittin had synergistic properties with mupirocin. In a study by Issam et al., it is reported that melittin exhibits a broad-spectrum antibacterial activity against MRSA and vancomycin-resistant enterococci (anti-VRE activity) (MIC values between 6 and 800 µg/ml) (Issam et al. 2015). Moerman et al. also reported that melittin acts synergistically with amoxicillin and cefuroxime against Gram-positive bacteria and with erythromycin against Gram-negative bacteria (Moerman et al. 2002). Also, the synergistic properties of melittin is reported with β-lactam antibiotics or polymyxin B against multidrug resistant (MDR) bacteria (Giacometti et al. 2003). Due to the cell membrane degradation by melittin (Ostroumova et al. 2015), when melittin combined with antibiotics, the entry rate of antibiotics into the bacterial cell may be accelerated and facilitated.

In the second part of the study, the anti-biofilm activity of the melittin was determined using the MtP test and SEM examination. The results of the MtP test revealed that sub-MIC concentrations (MIC/2, MIC/4, and MIC/8) of melittin significantly ($P < 0.05$) inhibit biofilm formation in both clinical and standard strains (Fig. 2). Interestingly, this result was confirmed in the microscope images. In *S. aureus* like other pathogenic bacteria, biofilm formation contributes to colonization, pathogenicity, and bacterial resistance (Sharifi et al. 2018). In a study performed by Dosler et al. the 1/10 MIC concentration of two antimicrobial cationic peptides (AMPs) melittin and colistin significantly inhibited the biofilm formation of *Pseudomonas aeruginosa*, *Escherichia coli* and *Klebsiella pneumoniae* (Dosler et al. 2016). These results indicate that melittin affects biofilm formation in both Gram-positive and Gram-negative bacteria. Melittin is a phenolic compound with amphipathic properties that may alter the permeability of plasma membranes, abolish bacterial adhesion, and subsequently reduce biofilm formation (Ostroumova et al. 2015).

The anti-biofilm activities of AMPs are not completely understood. They may affect matrix disruption, binding of DNA, and altering the expression of biofilm-related genes, such as the production of pili, QS systems, cytoplasmic membrane, and flagella assembly or several of these mechanisms (Park et al. 2014, Issam et al. 2015, Galdiero et al. 2019).

According to the effects of the QS system on the various virulence factors as well as bacterial antibiotic resistance, we investigated the effect of melittin on this system. To do this, the expression of the staphylococcal QS-related gene (*hld*) in treatment with MIC/2 concentration of melittin was evaluated. Our results showed that all treated isolates with melittin had a >3-fold reduction in *hld* expression compared to the untreated cells.

It is well known that many bacterial virulence factors such as biofilm formation, motility, productions of exoenzymes, hemolysin, and toxins are under the control of the QS system. According to this, the disruption of the QS system of pathogens has been proposed as a new anti-infective strategy (Rasmussen and Givskov 2006).

In the final stage of the present study, we examined the cytotoxic effects of melittin on the Vero cell line. Our results showed that melittin at MIC and sub-MIC concentrations were not cytotoxic toward Vero cell. These results are consistent with Choi et al. findings in which it is shown that melittin, exhibits antibacterial effects with minimal toxicity against eukaryotic cells (Choi et al. 2015).

5. Conclusion

The inability of antibiotics to penetrate through the biofilm is one of the important factors. In conclusion, our results demonstrated that melittin not only can inhibit MRSA growth but also reduce cell populations in biofilm form at sub-MIC concentrations and this is probably due to the ability of melittin to penetrate the bacterial biofilm. Accordingly, antimicrobial cationic peptides like melittin seem to be a good candidate for anti-MRSA chemotherapy with their antibacterial and anti-biofilm activities as a single agent or in combination with antibiotics.

Conflicts of interest

The authors declare no conflicts of interest.

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