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# Prevention, inhibition, and degradation effects of melittin alone and in combination with vancomycin and rifampin against strong biofilm producer strains of methicillin-resistant *Staphylococcus epidermidis*

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ABSTRACT

Methicillin-resistant *Staphylococcus epidermidis* (MRSE) bacteria are being recognized as true pathogens as they are able to resist methicillin and commonly form biofilms. Recent studies have shown that antimicrobial peptides (AMPs) are promising agents against biofilm-associated bacterial infections. In this study, we aimed to explore the antibiofilm activity of melittin, either alone or in combination with vancomycin and rifampin, against biofilm-producing MRSE strains. Minimum biofilm preventive concentration (MBPC), minimum biofilm inhibition concentration (MBIC), and minimum biofilm eradication concentration (MBEC), as well as fractional biofilm preventive-, inhibitory-, and eradication concentrations (FBPCi, FBICi, and FBECi), were determined for the antimicrobial agents tested. Cytotoxicity and hemolytic activity of melittin at its synergistic concentration were examined on human embryonic kidney cells (HEK-293) and Red Blood Cells (RBCs), respectively. The effect of melittin on the downregulation of biofilm-associated genes was explored using Real-Time PCR. MBPC, MBIC, and MBEC values for melittin were in the range of 0.625–20, 0.625–20, and 10–40 μg/μL, respectively. Melittin showed high synergy (FBPCi, FBICi and FBECi $< 0.5$). The synergism resulted in a 64–512-fold, 2–16 and 2–8-fold reduction in melittin, rifampicin and vancomycin concentrations, respectively. The synergistic melittin concentration found to be effective did not manifest either cytotoxicity on HEK-293 or hemolytic activity on RBCs. Results showed that melittin downregulated the expression of biofilm-associated *icaA*, *aap*, and *psm* genes in all isolates tested, ranging from 0.04-folds to 2.11-folds for *icaA* and from 0.05 to 3.76-folds for *aap* and *psm*. The preventive and therapeutic indexes of melittin were improved 8-fold when combined with vancomycin and rifampin. Based on these findings, the combination of melittin with conventional antibiotics could be proposed for treating or preventing biofilm-associated MRSE infections.

*Abbreviations:* MRSE, methicillin-resistant *S. epidermidis*; MDR, multidrug-resistant; AMPs, antimicrobial peptides; TSB, Tryptic soy broth; MHB, Mueller-Hinton broth; HEK, human embryonic kidney cell; MTT, 3-(4, 5-dimethyl-2-thiazolyl)-2, 5-diphenyl-2 H-tetrazolium bromide; DMSO, dimethyl sulfoxide; DMEM, Dulbecco's Modified Eagle's medium; FBS, Fetal Bovine Serum; LC-MS, liquid chromatography-mass spectrometry; MSSE, methicillin sensitive S. epidermidis; Glu, Glucose; OD, optical density; CFU, colony forming unit; MBPC, Minimum Biofilm Preventive Concentration; MBIC, Minimum Biofilm Inhibitory Concentration; MBEC, Minimum Biofilm Eradication Concentration; FBPCi, fractional biofilm prevention concentration index; FBICi, fractional biofilm inhibitory concentration index; RBC, Red Blood Cell; FCS, Fetal-Calf Serum; CoNS, Coagulase negative staphylococci; PVE, prosthetic valve endocarditis; IE, Infective endocarditis.

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## 1. Introduction

*Staphylococcus epidermidis* is a common commensal bacterium found on human skin and mucous membranes [1]. The widespread use of indwelling medical devices, such as urinary catheters, prosthetic joints, vascular access devices and fracture fixation devices, has given *S. epidermidis* the chance to emerge as a new opportunistic pathogen [2–7]. *S. epidermidis* causes approximately 20% of all infections related to orthopedic devices, up to 50% in late-onset neonatal infections [2]. And indeed, indwelling medical devices can facilitate infection by promoting the adhesion and accumulation of *S. epidermidis* on the surface of the device and the colonization of the tissues surrounding the device [5,6].

The surface-related bacterial accumulation, the so-called biofilm, is crucial for the opportunistic pathogens that cause device-associated infections [2,8]. Biofilm is defined as an aggregate of bacteria embedded in a self-produced extracellular matrix that is attached to abiotic and biotic surfaces. Biofilm characterizes up to 80% of all bacterial diseases [9–11]. Studies show that *S. epidermidis* biofilm is recalcitrant to antibacterial drugs and is able to evade immune defenses [12]. Therefore, the treatment of *S. epidermidis* infections associated with medical devices is based on the removal of the medical device and subsequent replacement. This surgical procedure, in addition to causing severe discomfort to patients who undergo it, being painful, invasive and at high risk of adverse events, also involves a heavy increase in therapeutic costs for health services [13]. It has been estimated that 75–90% of strains of *S. epidermidis* circulating in hospitals are resistant to methicillin, known as methicillin-resistant *S. epidermidis* (MRSE) [14]. Of note, many strains of MRSE are resistant to other antibiotics and therefore are defined as multidrug-resistant (MDR) [15,16]. Of particular concern is the emergence of vancomycin-resistant strains of *S. epidermidis* and the presence of rifampicin-resistant subpopulations [15–19].

Defeating bacteria embedded in their biofilm is difficult due to the protective function of the biofilm matrix. Furthermore, bacterial cells can shift to a dormant state thus becoming *persister cells*. The bacterial dormancy significantly hinders the effectiveness of conventional antibacterial drugs, whose mechanisms of action are directed against growing and multiplying bacteria [20]. Therefore, much attention has been devoted to finding a possible solution to the phenomenon of the resistance associated with biofilm [21,22]. In this context, antimicrobial peptides (AMPs), as part of the innate immunity of organisms, are a promising class of compounds that are currently receiving considerable attention as an emerging alternative to conventional antibacterial drugs against biofilm-producing MDR pathogens [20,23–26]. AMPs also exhibit immunomodulatory activities. These compounds destroy bacteria by acting on multiple targets, a feature that explains the limited emergence of resistance to AMPs [27,28]. Currently, many AMPs are undergoing preclinical and clinical trials to assess their effectiveness against infectious diseases [29–36].

Combination treatment appears especially appealing in the case of biofilms since the diversified composition of these microbial populations requires addressing cells in various metabolic stages (e.g., actively growing cells and dormant cells) [37]. Therefore, the combination of different bioactive molecules could be a promising strategy for biofilm prevention, control, and/or eradication [38,39]. In search of new antibiofilm drugs, scientists examined several AMPs, testing them individually or in combination with conventional antibiotics, to enhance their activity [20]. Noticeably, some AMPs exhibited the ability to prevent the accumulation of biofilm in the early stages of its formation, while others even acted on formed biofilms by reducing biofilm mass, destroying biofilm matrix and killing the bacteria embedded in the matrix [20,40].

Melittin, a cationic AMP (CAMP), acts against a wide range of Gram-positive and Gram-negative bacteria and our team found that melittin, alone or in combination with conventional antibiotics, exhibits strong antibiofilm activity, as it inhibits biofilm formation and also kills biofilm-producing bacteria, such as *Acinetobacter baumannii* and *Pseudomonas aeruginosa* [23,24,40–43]. In this study, we examined the antibiofilm effects of melittin alone and its synergistic effects with vancomycin and rifampicin against methicillin-resistant, biofilm-producing isolates of *S. epidermidis*. These bacteria can be considered among the most potent and insidious biofilm producers found in clinical observation.

## 2. Materials and methods

### 2.1. Media, reagents, and drugs

Vancomycin and rifampin powders were purchased from Sigma-Aldrich (St. Louis, MO, USA). Tryptic soy broth (TSB), Blood Agar, Mueller-Hinton Agar, Mueller-Hinton broth (MHB), glucose, and Sodium chloride (NaCl) were purchased from Merck Co., USA. The human embryonic kidney cells (HEK-293, NCBI Code: C548) (National Cell Bank of Iran, Pasteur Institute of Iran, Tehran, Iran) were generously donated by Dr. Ali Teimoori (Hamadan University of Medical Sciences, Iran). 3-(4, 5-dimethyl-2-thiazolyl)-2, 5-diphenyl-2H-tetrazolium bromide (MTT), dimethyl sulfoxide (DMSO), Dulbecco's Modified Eagle's medium (DMEM), and Fetal Bovine Serum (FBS) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Crystal violet, Triton X-100, and agarose were purchased from Sigma-Aldrich (Sigma Chemical Co., USA). U-Bottom 96 Well Sterile polystyrene microplates were purchased from NEST Biotechnology Co., Ltd, China.

### 2.2. Peptide

Melittin (GIGAVLKVLTTGLPALISWIKRKRQQ) was synthesized in > 96% purity by using the solid-phase synthesis technique by DGpeptides Co., Wuhan, Hubei, China. The company surveyed the purity of synthetic peptides via reversed-phase high-performance liquid chromatography. The company performed mass spectrometry on liquid chromatography-mass spectrometry (LC-MS) equipment to confirm accurate synthesis. As previously described, the peptide content and purity were validated by using bicinchoninic acid assay (BCA) and RP-HPLC, respectively [44].

### 2.3. Staphylococcus epidermidis isolates and ATCC strains

Twenty strains of *S. epidermidis*, including clinical MRSE, MSSE, as well as ATCC strains, were used for all experiments in this work. In this regard, sixteen clinical isolates from human sources were obtained from university hospitals in Hamedan, Iran (Table 1). *S. epidermidis* ATCC 35984 was kindly provided by Dr. Eyup Dogan (Biotechnology Institute, Ankara, Turkey) and Dr. Fereshteh Saffari (Kerman University of Medical Sciences, Kerman, Iran). *S. epidermidis* DSMZ 3270 was kindly provided by Prof. Bibi Sedigheh Fazly Bazzaz (Mashhad University of Medical Sciences, Mashhad, Iran). Additionally, *S. epidermidis* ATCC 12228 was purchased from the Pasteur Culture Collection of Tehran, Iran.

### 2.4. Biofilm assay

Initial screening of the most significant parameters affecting biofilm formation was performed by reviewing the literature [12,45–47]. In this regard, four experimental factors were chosen for further study, namely glucose (0–5%), NaCl (0–7%), centrifuge speed (0–120 rpm), and time (0–48 h). In the optimization test, we found that glucose (2. 5%), NaCl (3.5%), and centrifuge speed 75 rpm and time 24 h were the significant factors affecting the amount formed biofilm.

Briefly, *S. epidermidis* ATCC 35984 fresh colonies were cultured in 5 mL TSB supplied with 2.5% Glu + 3.5% NaCl TSB overnight at 37 °C. Then, 0.5 McFarland standard suspension was prepared by measuring the absorbance of a bacterial suspension at the wavelength of 625 nm.

**Table 1**
The characterization of *Staphylococcus epidermidis* isolates.

| Strains (n = 20) | Source | MDR/ NonMDR | Van-MIC (μg/ mL) | Van-MBC (μg/ mL) | Rif-MIC (μg/ mL) | Rif-MBC (μg/ mL) | Mel-MIC (μg/ mL) | Mel-MBC (μg/ mL) |
|---|---|---|---|---|---|---|---|---|
| **ATCC 35984 Turkey (MRSE)** | – | MDR | 2 | 4 | 0.0039 | 0.0625 | 2.5 | 5 |
| **ATCC 35984 Kerman (MRSE)** | – | MDR | 2 | 4 | 0.0039 | 0.0625 | 2.5 | 5 |
| **DSMZ 3270 (MSSE)** | – | NonMDR | 2 | 4 | 0.00156 | 0.0312 | 2.5 | 5 |
| **ATCC 12228 (MSSE)** | – | NonMDR | 2 | 4 | 0.0078 | 0.0156 | 0.625 | 5 |
| **MRSE 1** | Sputum | MDR | 16 | 16 | > 1024 | > 1024 | 1.25 | 1.25 |
| **MRSE 2** | Blood | MDR | 8 | 8 | 0.25 | 2 | 0.625 | 0.625 |
| **MRSE 3** | Blood | NonMDR | 4 | 8 | 0.125 | 1 | 0.312 | 0.312 |
| **MSSE 1** | Blood | NonMDR | 4 | 4 | 0.0625 | 0.5 | 0.625 | 1.25 |
| **MRSE 4** | Catheter | NonMDR | 8 | 8 | > 1024 | > 1024 | 1.25 | 1.25 |
| **MRSE 5** | Blood | MDR | 32 | 32 | 0.25 | 2 | 1.25 | 1.25 |
| **MRSE 6** | Blood | MDR | 8 | 8 | 0.125 | 1 | 1.25 | 1.25 |
| **MRSE 7** | Urine | MDR | 8 | 8 | 0.25 | 2 | 2.5 | 5 |
| **MRSE 8** | Catheter | MDR | 8 | 8 | 0.125 | 1 | 2.5 | 2.5 |
| **MRSE 9** | Blood | MDR | 8 | 16 | 0.0625 | 0.5 | 2.5 | 2.5 |
| **MRSE 10** | Urine | MDR | 8 | 8 | 0.125 | 1 | 2.5 | 2.5 |
| **MRSE 11** | Urine | MDR | 4 | 8 | 0.0625 | 0.5 | 2.5 | 2.5 |
| **MRSE 12** | Blood | MDR | 8 | 8 | 0.25 | 2 | 2.5 | 5 |
| **MRSE 13** | Wound | NonMDR | 4 | 4 | 0.25 | 2 | 2.5 | 5 |
| **MRSE 14** | Wound | MDR | 2 | 4 | 0.125 | 1 | 2.5 | 2.5 |
| **MRSE 15** | Wound | MDR | 8 | 8 | 0.0625 | 0.5 | 2.5 | 2.5 |

Van; vancomycin; Rif, rifampin, Mel, melittin; MRSE, methicillin-resistant S. epidermidis; MSSE, Methicillin sensitive S. epidermidis; MDR, multidrug-resistant.

Based on the routine definition of 0.5 McFarland, a bacterial suspension with optical density (OD) between 0.08 and 0.1 is equal to $10^8$ colony forming units (CFU)/mL, but to avoid variations in the number of bacteria examined in this study, this parameter was set to 0.09. Afterward, 100 μL from the prepared bacterial suspension containing $10^7$ CFUs was added to 900 μL TSB with various conditions in a sterile tube, and finally 200 μL from provided suspension equal to $2 \times 10^6$ cells were added to each well of 96 Well microplates and incubated at 37 °C for various times at various rpm. The wells were washed thoroughly (three times) with normal saline after incubation and air-dried as a result. Then, 200 μL absolute methanol was added per well for biofilm fixation. After 15 min, the solution was aspirated, and the plates were allowed to dry at room temperature. The wells were stained with 200 μL of crystal violet (0.05%) for 5 min, and the solution was aspirated, and wells were washed three times with normal saline and allowed to dry at room temperature again. Finally, 200 μL of absolute ethanol was added to each well with shaking for 30 min at 37 °C. The content of each well was transferred to its equivalent well in another microplate and, the absorbance was measured at 595 nm using a Synergy™ HTX Multi-Mode Microplate Reader (BioTek Co., Winooski, VT, USA). All experiments were repeated three times. Finally, the biofilm formation assay based on optimized conditions was performed as described above for subsequent tests.

Biofilm formation was interpreted based on previous references [48]. The OD cut-off value (ODc) was established as three standard deviations (SD) above the mean of the OD of the negative control as follows: ODc = average OD of negative control + (3 × SD of negative control). Finally, the findings were divided into the four following categories according to their ODs as (1) strong biofilm producer (4 × ODc < OD); (2) medium biofilm producer (2 × ODc < OD ≤ 4 × ODc); (3) weak biofilm producer (ODc < OD ≤ 2 × ODc); and (4) non-biofilm (OD ≤ ODc).

### 2.5. Minimum biofilm preventive concentration

In this assay, we evaluated the capability of melittin or antibiotics to prevent biofilm formation as the Minimum Biofilm Preventive Concentration (MBPC) that is the lowest concentration of an antibacterial agent that completely prevents biofilm formation visually and by measuring the absorbance at 595 nm. Briefly, the fresh *S. epidermidis* colonies were cultured in 5 mL of MHB at 37 °C with shaking at 180 rpm for 24 h. The number of bacteria was adjusted to 0.5 McFarland standard by spectrophotometry at 625 nm. Ten μL of the bacterial suspension containing $10^6$ CFUs was added to the medium (990 μL, 2.5% Glu+ 3.5% NaCl TSB). Then, 100 μL of the bacterial suspension containing $10^5$ CFUs along with 100 μL of two-fold serial dilutions of the melittin from 20 to 0.019 μg/μL and the antibiotics from 256 to 0.25 μg/μL in the same medium was added into wells of 96 Well microplates and incubated at 37 °C for 24 h with shaking at 75 rpm. The quantity of biofilm formation was then determined, as mentioned above. The MBPC experiments were repeated three times for all isolates.

### 2.6. Minimum biofilm inhibitory concentration

As previously described, the inhibitory effects of melittin, vancomycin, and rifampin on 24 h pre-formed biofilm so-called Minimum Biofilm Inhibitory Concentration (MBIC) were examined [24]. In this regard, fresh *S. epidermidis* colonies were cultured in 5 mL of TSB medium (supplemented with 2.5% Glu+ 3.5% NaCl) at 37 °C with shaking at 180 rpm for 24 h. As mentioned above, the number of bacteria was adjusted to 0.5 McFarland standard by spectrophotometry at 625 nm. Then, a suspension of $2 \times 10^6$ CFUs was added to each well of 96 Well microplates and incubated at 37 °C for 24 h with shaking at 75 rpm. After overnight incubation, the content of wells was gently discarded and washed three times with normal saline solution. At the same time, melittin (from 40 to 0.039 μg/ μL) and the antibiotics (from 1024 to 1 μg/μL) which had been prepared in normal saline solution were added into the wells at the volume of 100 μL and incubated at 37 °C for 24 h. Then, the quantity of biofilm was measured as described above. Each experiment was done in triplicate and repeated at least three times for all isolates. According to the previous references [24,49,50], the MBIC was considered as the lowest amount of melittin and antibiotics that caused at least 90% inhibition in biofilm biomass compared to the untreated control via the following formula: % biofilm inhibition = [1-(OD test / OD control)] × 100.

### 2.7. Minimum biofilm eradication concentration

The Minimum Biofilm Eradication Concentration (MBEC) assay was performed using 96 Well microplates as described before. Some modifications were done to survey the biofilm degradation and biofilm embedded bacterial killing capability of melittin, vancomycin, and rifampin [24]. In summary, the examined isolates were first allowed to

form 24 h pre-formed biofilm as mentioned above [24]. Then, the contents of the wells were gently discarded and washed three times with the normal saline solution. At the same time, 100 μL serially two-fold diluted melittin (from 80 to 0.019 to μg/μL), vancomycin, and rifampin (from 1024 to 1 μg/μL) in normal saline solution were added into the wells at the volume of 100 μL and incubated at 37 °C for 24 h. Finally, after discarding the contents of wells, they were washed three times with saline solution, and 100 μL of fresh saline solution was added to wells and after scratching, and mixing, 10 μL of the contents of wells were cultured on MHA at 37 °C for 48 and the grown colonies were counted. The MBEC values for melittin, vancomycin, and rifampin were defined as the lowest amount of antibiotics required to kill 100% of the embedded bacteria.

### 2.8. Measurement of the synergistic effects

The synergistic effects of melittin, vancomycin, and rifampin were assessed using the broth microdilution checkerboard method with major modifications based on MBPC-, MBIC-, and MBEC values [23,24,51]. Accordingly, fractional indices for MBPC-, MBIC-, and MBEC were respectively designated as fractional biofilm preventive concentration index (FBPCi), fractional biofilm inhibitory concentration index (FBICi), and fractional biofilm eradication concentration index (FBECi). The selected *S. epidermidis* isolates were potent biofilm-forming MRSE 1, strong biofilm-forming MRSE 4, moderate biofilm-forming MSSE 1, moderate biofilm-forming MRSE 8, and strong biofilm-forming *S. epidermidis* ATCC 35984.

#### 2.8.1. FBPCi

Briefly, *S. epidermidis* isolates were cultured in 5 mL of MHB at 37 °C with shaking at 180 rpm for 24 h. Then, dilutions of each of melittin (from 20 to 0.019 μg/mL), rifampin (from 256 to 0.03125 μg/mL), and vancomycin (from 32 to 0.0125 μg/mL) were provided and added to the wells of 96-well microplate at a volume of 100 μL. At the same time, the bacterial solution was prepared as mentioned above, and 100 μL of the diluted bacterial solution containing $10^5$ CFUs was added to each well. The microplate was incubated at 37 °C for 24 h with shaking at 75 rpm. Afterward, the lowest value of the antibacterial agents that prevent biofilm formation entirely was considered FBPCi by visual inspection and measuring the absorbance at 595 nm. FBPCi for the two combined antibacterial agents was calculated as follows: FBPCi = (MBPC drug A in combination/ MBPC drug A alone) + (MBPC drug B in combination/ MBPC drug B alone). FBPC indices are pointed to the kind of drug interaction if the following data are established: Synergy, values $n \leq 0.5$; Partial synergy, values $0.5 < n < 1$; Additive effect, for a value $n = 1$; Indifferent effect, for values $1 < n < 4$; Antagonistic effect, for a value $4 \leq n$ [23,24,51].

#### 2.8.2. FBICi

Briefly, *S. epidermidis* isolates were cultured in 5 mL of 2.5% Glu + 3.5% NaCl TSB at 37 °C with shaking at 180 rpm for 24 h. Then, the number of bacteria was adjusted to 0.5 McFarland standard as mentioned above. A suspension of $2 \times 10^6$ CFUs as above prepared was added to each well of 96 Well microplate and incubated as discussed above. After overnight incubation, the content of wells was gently discarded, and the wells were washed three times with normal saline solution. At the same time, the dilutions of each of melittin (from 40 μg to 0.039 μg/μL), rifampin (from 1024 to 1 μg/μL), and vancomycin (from 512 to 0.5 μg/μL) were provided at a volume of 100 μL and added into the wells, and plates were incubated at 37 °C for 24 h with shaking at 75 rpm. The quantity of biofilm was then measured, as mentioned above. Afterward, MBIC was considered as discussed above, and FBICi for the two combined anti-bacterial agents was calculated as follows: FBPCi= (MBIC drug A in combination/ MBIC drug A alone) + (MBIC drug B in combination/ MBIC drug B alone). FBPC indices are pointed to the kind of drug interaction if the following data are established: Synergy, values $n \leq 0.5$; Partial synergy, values $0.5 < n < 1$; Additive effect, for a value $n = 1$; Indifferent effect, for values $1 < n < 4$; Antagonistic effect, for a value $4 \leq n$ [23,24,51].

#### 2.8.3. FBECi

Briefly, *S. epidermidis* isolates were cultured in 5 mL of 2.5% Glu+ 3.5% NaCl TSB at 37 °C with shaking at 180 rpm for 24 h. Then, the number of bacteria was adjusted to 0.5 McFarland standard as above mentioned, and a suspension of $2 \times 10^6$ CFUs as above prepared was added to each well of 96 Well microplates and incubated as discussed above, and after overnight incubation, the content of wells was gently discarded and washed three times with normal saline solution. At the same time, the dilutions of each of melittin (from 80 to 0.039 μg/μL), rifampin (from 1024 to 1 μg/μL), and vancomycin (from 1024 to 1 μg/μL) were provided at a volume of 100 μL and added to the wells and plates were incubated at 37 °C for 24 h with shaking at 75 rpm. After discarding the contents of wells, they were washed three times with saline solution, and 100 μL of the saline solution was added to the wells, and after scratching and mixing, 10 μL of contents of wells were cultured on MHA at 37 °C for 48 ', and the grown colonies were counted. The MBEC values for melittin, vancomycin, and rifampin were defined as mentioned above. Afterward, FBECi for the two combined antibacterial agents was calculated as follows: FBECi = (MBEC drug A in combination/ MBEC drug A alone) + (MBEC drug B in combination/ MBEC drug B alone). FBEC indices are pointed to the kind of drug interaction if the following data are established: Synergy, values $n \leq 0.5$; Partial synergy, values $0.5 < n < 1$; Additive effect, for a value $n = 1$; Indifferent effect, for values $1 < n < 4$; Antagonistic effect, for a value $4 \leq n$ [23,24,51].

### 2.9. Evaluation of the effect of melittin on the expression of the biofilm-associated genes in Staphylococcus epidermidis

#### 2.9.1. RNA extraction and complementary DNA (cDNA) synthesis

Biofilm forming *S. epidermidis* isolates include strong biofilm-forming MRSE 1, strong biofilm-forming MRSE 4, moderate biofilm-forming MRSE 5, moderate biofilm-forming MSSE 1, as well as strong biofilm-forming *S. epidermidis* ATCC 35984 were used for evaluation of biofilm-associated expression genes (*icaA*, *aap*, and *psmβ*) by real-time PCR. The candidate isolates were treated with sub-MIC concentrations of melittin ranging from 1.25 to 0.009 μg for 24 h, and mRNA was subsequently extracted using an mRNA extraction kit (Gene All Co., South Korea) according to the manufacturer's instructions. The RNA concentration and its purity were determined by a NanoDrop spectrophotometer (Thermo Scientific Co., USA), as well as agarose gel electrophoresis. A total of 1 μg RNA was used for cDNA synthesis using a Two-step RT-PCR kit following the manufacturer's protocol (Gene All Co., South Korea).

#### 2.9.2. Quantitative real-time PCR

Gene expression was quantified using Q-PCR Master Mix (SYBR, no ROX) (SMOBIO, Taiwan) containing 2 μL cDNA and 1 μL from each primer specific to the *icaA, aap,* and *psmβ* genes and *16 S rRNA* genes in a final volume of 20 μL on the RT-PCR instrument (LightCycler® 96 Instrument, Roche, USA). The *icaA, aap,* and *psmβ,* and *16 S rRNA* primers were used in reference to the previous study [52]. The reaction conditions were initial denaturation at 95 °C for 10 min, followed by 40 cycles of 95 °C for 15 s, annealing at 60 °C for 45 s, and extension at 72 °C for 30 s. Following PCR cycling, melting point data were collected, and a dissociation curve was examined for each well. Standard curves were generated for the *icaA, aap,* and *psmβ* genes using serial dilutions of RNA isolated from untreated ATCC 35984 to control amplification efficiency. Besides, the gene expression was calculated using the ΔΔCt method [53]. The *16 S rRNA* gene was also amplified as the internal control. And also, to monitor the fact that melittin may affect the proliferation of bacteria via binding to the *16 S rRNA* gene, the obtained Ct value for this gene was compared with that of untreated isolates.

### 2.10. Toxicity assays for melittin

To survey the safety of melittin for in vivo and future clinical trials, the cytotoxicity of this peptide at the synergistic concentrations was assessed by MTT. Also, to survey the effect of melittin when reaching into the bloodstream, the hemolysis of Red Blood Cells (RBCs) was checked too.

#### 2.10.1. MTT assay

HEK-293 cells were cultured in DMEM supplemented with 10% Fetal-Calf Serum (FCS) and antibiotics (100 U/mL streptomycin, and 100 U/mL penicillin) [23]. Then, the cells were incubated at 37 °C, with 5% $CO_2$ and 95% humidity. In brief, the cells were seeded at a density of $4 \times 10^4$ cells/well and incubated for 24 h. Melittin at the concentrations of 5, 2.5, 1.25, 0.625, 0.312, 0.156, 0.078, and 0.039 μg/μL was added to the wells and incubated at 37 °C for 24 h. Then, 20 μL of MTT solution (5 mg/mL) was added to each well and further incubated for 4 h. The supernatants were then discarded, followed by adding 100 μL of DMSO to the wells. Finally, the absorbance was measured at a wavelength of 570 nm using a microplate spectrophotometer (Synergy™ HTX Multi-Mode Microplate Reader-BioTek Co., USA). All experiments were done in triplicate. The percentage of cell survival was calculated according to the following formula: Survival percent = (OD test / OD control) × 100.

#### 2.10.2. Hemolysis assay

Besides, to survey hemolytic activity, melittin at the MIC, MBC, and the synergistic concentrations was used according to the previously described method [54]. In this regard, heparinized blood from a healthy volunteer was collected, centrifuged at 3500 × rpm for 10 min, washed with PBS three times, the supernatant was discarded, and 2% RBCs suspension was prepared with PBS, then, 100 μL of this 2% RBC suspension was transferred to a 96 Well microplate. Melittin at concentrations of 5, 2.5, 1.25, 0.625, 0.312, 0.156, 0.078, and 0.039 μg was added to the RBCs, and the microplate was incubated at 37 °C for 2 h, and the microplate was centrifuged at 3000 × rpm for 10 min. One hundred μL of supernatant from each well was moved gently to a new 96-well microplate, and the OD of liberated hemoglobin was measured at 540 nm with a microplate spectrophotometer (Synergy™ HTX Multi-Mode Microplate Reader-BioTek Co., USA). The results were compared with the positive control (100 μL RBC and 100 μL Triton X-100 1%) and negative control (100 μL RBC and 100 μL PBS), and all experiments were performed in triplicate. Finally, the percent of hemolysis for melittin was determined by the following formula [54]:

[(OD sample − OD negative control) / (OD positive control − OD negative control)] × 100.

### 2.11. Calculation of preventive index of melittin

For the first time in the field, the preventive index was defined as the ratio of the minimum hemolytic concentration to MBPC of melittin alone or in combination with antibiotics.

### 2.12. Calculation of therapeutic index of melittin

The therapeutic index was calculated according to Memariani et al. [55] with significant modification as the ratio of the minimum hemolytic concentration to MBIC and MBEC of melittin alone or in combination with antibiotics.

### 2.13. Statistical analysis

The GraphPad Prism (version 9) was used for the statistical analyses. In this regard, a paired-sample t-test was used to survey the significance between melittin and the melittin-drug combinations. Besides, a one-way analysis of variance (ANOVA) was used to compare the differences in survival percent of the HEK-293 between the various concentrations of melittin and the control, as well as to compare the differences in expression of the biofilm-associated genes between the treated samples and the control, as well as between the FBPC, FBIC, and FBEC indices. The results are generally expressed as the means ± SD unless otherwise indicated. All statistical analyses were done with a confidence level of 95%, and the p-values $< 0.05$ were considered statistically significant.

## 3. Results

### 3.1. Biofilm formation assay

The majority of isolates were able to form varying degrees of biofilm. The maximum and minimum OD values for isolates were 4.9 and 0.08, respectively. Based on the results, the biofilm production capabilities of the isolates were classified as strong, moderate, and non-biofilm producers. Further details are shown in Table 2.

### 3.2. Minimum biofilm preventive concentrations

The results showed that melittin alone prevented biofilm formation in all *S. epidermidis* isolates, with MBPC values ranging from 20 to 1.25 μg/mL. MBPC values for vancomycin and rifampin were 128–4 μg/mL, and 1024–4 μg/mL, respectively. In this regard, the geometric means for melittin, vancomycin, and rifampin were 4.15, 36.2, and 108.6 μg/mL, respectively. On the other hand, the $MBPC_{50}$ for melittin, rifampin, and vancomycin were 2.5, 4, and 16 μg/μL, and $MBPC_{90}$ for melittin rifampin, and vancomycin, were 10, 16, and 64 μg/mL, respectively. Further details and results of the MBPCs for melittin–vancomycin, and melittin–rifampin, are shown in Table 3 and Fig. 1.

### 3.3. Minimum biofilm inhibitory concentrations

The results showed that melittin inhibited the biofilm formation of all *S. epidermidis* isolates, with MBIC values ranging from 20 to 0.625 μg/mL. Besides, the MBIC results for rifampin and vancomycin were > 1024–4 μg/mL, and 128–8 μg/mL, respectively. The geometric means of the MBICs for melittin, rifampin, and vancomycin, were 4.15, 117.4, and 51.6 μg/mL, respectively. On the other hand, the $MBIC_{50}$ for melittin, rifampin, and vancomycin, were 2.5 μg/mL, 16 μg/ mL, and 32 μg/mL, respectively. Besides, the $MBIC_{90}$ for melittin, rifampin, and vancomycin, were 10 μg/mL, 32 μg/mL, and 128 μg/mL, respectively. Further details are depicted in Table 4.

**Table 2**
Biofilm production capabilities of the *S. epidermidis* isolates.

| Strains (n = 20) | Biofilm formation |
|---|---|
| ATCC 35984 T | Strong |
| ATCC 35984 K | Strong |
| DSMZ 3270 | Moderate |
| ATCC 12228 | Non-biofilm forming |
| MRSE 1 | Strong |
| MRSE 2 | Moderate |
| MRSE 3 | Moderate |
| MSSE 1 | Moderate |
| MRSE 4 | Strong |
| MRSE 5 | Moderate |
| MRSE 6 | Strong |
| MRSE 7 | Moderate |
| MRSE 8 | Moderate |
| MRSE 9 | Moderate |
| MRSE 10 | Moderate |
| MRSE 11 | Strong |
| MRSE 12 | Moderate |
| MRSE 13 | Strong |
| MRSE 14 | Strong |
| MRSE 15 | Strong |

**Table 3**
Minimum biofilm prevention concentration of vancomycin, rifampin, and melittin for *Staphylococcus epidermidis* isolates.

| Strains (n = 20) | Vancomycin-MBPC (μg/mL) | Rifampin-MBPC (μg/mL) | Melittin-MBPC (μg/mL) |
|---|---|---|---|
| **ATCC 35984 T** | 64 | 8 | 5 |
| **ATCC 35984 K** | 64 | 16 | 1.25 |
| **DSMZ 3270** | 64 | 16 | 1.25 |
| **ATCC 12228** | 8 | 4 | 0.625 |
| **MRSE 1** | 128 | 1024 | 2.5 |
| **MRSE 2** | 8 | 4 | 1.25 |
| **MRSE 3** | 16 | 4 | 1.25 |
| **MSSE 1** | 4 | 4 | 2.5 |
| **MRSE 4** | 128 | 1024 | 20 |
| **MRSE 5** | 16 | 16 | 10 |
| **MRSE 6** | 16 | 4 | 10 |
| **MRSE 7** | 8 | 4 | 5 |
| **MRSE 8** | 16 | 8 | 2.5 |
| **MRSE 9** | 16 | 4 | 1.25 |
| **MRSE 10** | 32 | 8 | 2.5 |
| **MRSE 11** | 8 | 4 | 5 |
| **MRSE 12** | 16 | 4 | 2.5 |
| **MRSE 13** | 32 | 4 | 5 |
| **MRSE 14** | 64 | 4 | 2.5 |
| **MRSE 15** | 16 | 8 | 1.25 |
| **GM-MBPC** | 36.2 | 108.6 | 4.15 |
| **MBPC 50** | 16 | 4 | 2.5 |
| **MBPC 90** | 64 | 16 | 10 |

MBPC, Minimum biofilm prevention concentration; MRSE, methicillin-resistant Staphylococcus epidermidis; GM-MBPC, geometric mean-minimum biofilm prevention concentration.

### 3.4. Minimum biofilm eradication concentrations

The results showed that melittin eradicated all *S. epidermidis* isolates with MBEC values ranging from 40 to 10 μg/mL. Besides, the MBEC results for rifampin and vancomycin were > 1024–32 μg/mL, and > 1024–16 μg/mL, respectively. The geometric means of the MBECs for melittin, rifampin, and vancomycin, were 26.5, 374.8, and 548.7 μg/mL, respectively. On the other hand, the $MBEC_{50}$ for melittin, rifampin, and vancomycin, were 20, 512, and > 1024 μg/mL, respectively. The $MBEC_{90}$ for melittin was 40 μg/mL, while rifampin and vancomycin were > 1024 μg/mL. Further details are depicted in Table 4.

### 3.5. Measurement of the synergistic effects

In the present study, we applied the modified checkerboard method to survey the synergism of antibacterial agents by calculating the FBPCi, FBICi, and FBECi, which are the interaction coefficient that indicates whether the combined antibiofilm effects of antibacterial agents are synergistic, additive, indifferent, and/or antagonistic against selected isolates. The geometric means of the FBPCi, FBICi, and FBECi at various melittin–rifampin synergistic concentrations for strong biofilm-forming MRSE 1, strong biofilm-forming MRSE 4, moderate biofilm-forming MSSE 1, moderate biofilm-forming MRSE 8, and strong biofilm-forming ATCC 35984 were calculated as '0.33, 0.1, 0.59, 0.59, and 1.19' for FBPCi and '0.32, 0.26, 0.2, 0.2, and 0.57' for FBICi, and '0.26, 0.28, 0.57, 0.53, and 0.54' for FBECi, respectively.

The geometric means of the FBPCi, FBICi, and FBECi at various melittin–vancomycin synergistic concentrations for strong biofilm-forming MRSE 1, strong biofilm-forming MRSE 4, moderate biofilm-forming MSSE 1, moderate biofilm-forming MRSE 8, and strong biofilm-forming ATCC 35984 were calculated as '0.2, 0.17, 0.59, 0.2, and 0.32' for FBPCi, and '0.32, 0.29, 0.19, 0.33, and 0.33' for FBICi and '0.15, 0.18, 0.26, 0.28, and 0.26' for FBECi, respectively (Tables 5–10).

Based on MBPC values of rifampin for the strong biofilm-forming MRSE 1, maximum synergistic effect with FBPCi = 0.26 was found for the concentration of 0.039 and 256 μg/mL for melittin, and rifampin, respectively which their MPBC was reduced 64- and > 4-fold, respectively. For the strong biofilm-forming MRSE 4, maximum synergistic effect with FBPCi = 0.064 was found at 0.039, and 64 μg/mL for melittin and rifampin their MBPC was reduced 512- and 16-fold, respectively. For the strong biofilm-forming ATCC 35984, maximum synergistic effect with FBPCi = 1.007 was found at the concentrations of 0.039 and 8 μg/mL for melittin and rifampin respectively which their MBPC was reduced 128- and 1-fold, respectively. Additionally, for the moderate biofilm-forming MSSE 1, maximum synergistic effect with FBPCi = 0.515 was found at the concentration of 0.039 and 2 μg/mL for melittin and rifampin respectively which their MBPC was reduced 64- and 2-fold, respectively. Finally, for the moderate biofilm-forming MRSE 8, maximum synergistic effect with FBPCi = 0.515 was found at the concentrations of 0.039 and 4 μg/mL for melittin and rifampin respectively which their MBPC was reduced 64- and 2-fold, respectively.

Based on MBPC values of vancomycin, for the strong biofilm-forming MRSE 1, maximum synergistic effect with FBPCi = 0.14 was found at the concentrations of 0.039 and 16 μg/mL for melittin, and vancomycin, respectively which their MBPC was reduced 64- and 8-fold, respectively. For strong biofilm-forming MRSE 4, maximum synergistic effect with FBPCi = 0.126 was found at the concentrations of 0.039 and 16 μg/mL for melittin and vancomycin, respectively, which their MBPC was reduced 512- and 8-fold, respectively. For the strong biofilm-forming ATCC 35984, maximum synergistic effect with FBPCi = 0.257 was found at the concentrations of 0.039 and 2 μg/mL for melittin, and vancomycin, respectively which their MBPC was reduced 128- and 4-fold, respectively. Additionally, for the moderate biofilm-forming MSSE 1, maximum synergistic effect with FBPCi = 0.515 was found at the concentrations of 0.039 and 2 μg/mL for melittin, and vancomycin, respectively which their MBPC was reduced 64- and 2-fold, respectively. Finally, for the moderate biofilm-forming MRSE 8, maximum synergistic effect with FBPCi= 0.14 was found at the concentrations of 0.039 and 2 μg/mL for melittin and vancomycin respectively which their MBPC was reduced 64- and 8-fold, respectively.

Besides, based on MBIC values of rifampin, for the strong biofilm-forming MRSE 1, maximum synergistic effect with FBICi = 0.25 was found at the concentrations of 0.039 and 256 μg/mL for melittin, and rifampin, respectively which their MBIC was reduced 64- and > 4-fold, respectively. For the strong biofilm-forming MRSE 4, maximum synergistic effect with FBICi = 0.25 was found at the concentrations of 0.039 and 256 μg/mL for melittin and rifampin, respectively, which their MBIC was reduced 512- and 4-fold, respectively. For the strong biofilm-forming ATCC 35984, maximum synergistic effect with FBICi = 0.5 was found at the concentration of 0.039 and 8 μg/mL for melittin and rifampin their MBIC was reduced 128- and 2-fold, respectively. Additionally, for the moderate biofilm-forming MSSE 1, maximum synergistic effect with FBICi = 0.14 was found at 0.039 and 4 μg/mL for melittin and rifampin which their MBIC was reduced 64- and 8-fold, respectively. Finally, for the moderate biofilm-forming MRSE 8, maximum synergistic effect with FBICi = 0.14 was found at the concentrations of 0.039 and 2 μg/mL for melittin, and rifampin, respectively which their MBIC was reduced 64- and 8-fold, respectively.

Based on MBIC values of vancomycin, for the strong biofilm-forming MRSE 1, maximum synergistic effect with FBICi= 0.25 was found at the concentrations of 0.039 and 32 μg/mL for melittin and vancomycin, respectively which their MBIC was reduced 64- and 4-fold, respectively. For the strong biofilm-forming MRSE 4, maximum synergistic effect with FBICi= 0.25 was found for the concentration of 0.039 and 32 μg/mL for melittin and vancomycin, respectively, which their MBIC was reduced 512- and 4-fold, respectively. For the strong biofilm-forming ATCC 35984, maximum synergistic effect with FBICi = 0.132 was found at 0.039 and 4 μg/mL for melittin and vancomycin, respectively, which their MBIC was reduced 128- and 8-fold, respectively. Additionally, for the moderate biofilm-forming MSSE 1, maximum synergistic effect with FBICi = 0.26 was found at 0.039 and 8 μg/mL for melittin

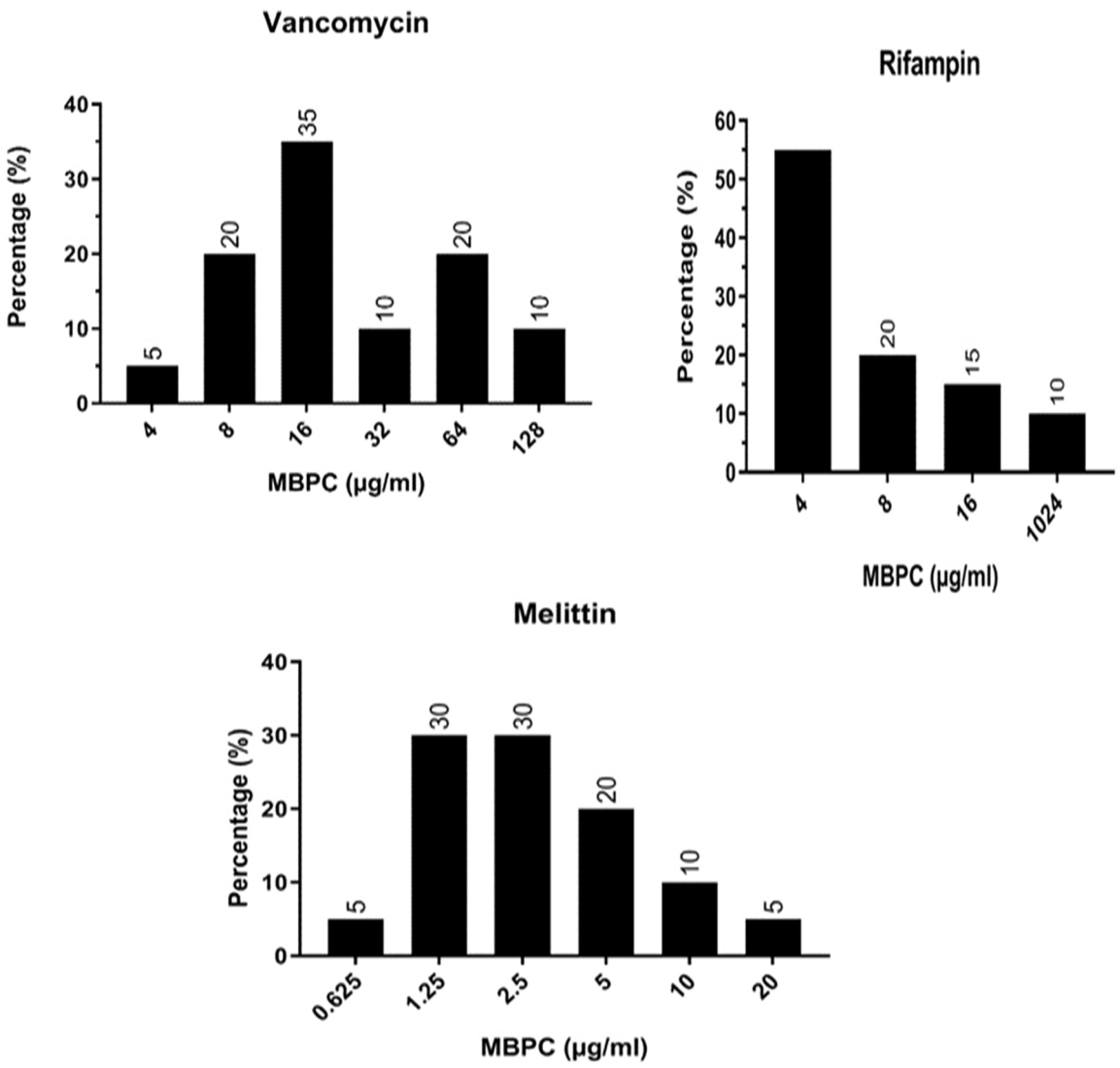


**Fig. 1.** MBPC frequency distribution in *Staphylococcus epidermidis* strains (n = 20). Reference to the results, the biofilm formation in the majority of strains was prevented at lower amounts of melittin in comparison to vancomycin, and rifampin. Melittin at the amounts of 20 μg/mL prevented the biofilm formation of all the examined strains. In contrast, vancomycin, and rifampin at the concentration of 128, and 1024 μg/mL, respectively, were able to prevent the biofilm formation of the strains. There was a major difference between the MBPC of vancomycin and melittin (p-value = 0.0006).

and vancomycin, respectively, which their MBPC was reduced 64- and 4-fold, respectively. Finally, for the moderate biofilm-forming MRSE 8, maximum synergistic effect with FBICi= 0.26 was found at 0.039 and 16 μg/mL for melittin and vancomycin, respectively, which their MBIC was reduced 64- and 4-fold, respectively.

Most importantly, based on MBEC values of rifampin, for the strong biofilm-forming MRSE 1, maximum synergistic effect with FBECi = 0.25 was found at the concentrations of 0.078 and 256 μg/mL for melittin, and rifampin, respectively which their MBEC was reduced 512- and 4-fold, respectively. For the strong biofilm-forming MRSE 4, maximum synergistic effect with FBECi = 0.25 was found at the concentrations of 0.078 and 256 μg/mL for melittin and rifampin, respectively, which their MBPC was reduced 256- and 4-fold, respectively. For the strong biofilm-forming ATCC 35984, maximum synergistic effect with FBECi = 0.5 was found at 0.078, and 64 μg/mL for melittin and rifampin their MBEC was reduced 512- and 2-fold, respectively. Additionally, for the moderate biofilm-forming MSSE 1, maximum synergistic effect with FBECi = 0.5 was found at the concentrations of 0.078 and 32 μg/mL for melittin and rifampin, respectively, which their MBEC was reduced 512- and 2-fold, respectively. Finally, for the moderate biofilm-forming MRSE 8, maximum synergistic effect with FBECi = 0.5 was found at 0.078 and 256 μg/mL for melittin and rifampin their MBEC was reduced 512- and 2-fold, respectively.

Finally, based on MBEC of vancomycin, for strong biofilm-forming MRSE 1, maximum synergistic effect with FBECi = 0.12 was found at the concentration of 0.078 and 128 μg/mL for melittin and vancomycin, respectively which their MBEC was reduced 512- and > 8-fold, respectively. For strong biofilm-forming MRSE 4, maximum synergistic effect with FBECi = 0.25 was found at the concentration of 0.078 and 128 μg/mL for melittin and vancomycin, respectively, which their MBEC was reduced 256- and > 8-fold, respectively. For the strong biofilm-forming ATCC 35984 and moderate biofilm-forming MSSE 1, maximum synergistic effect with FBECi= 0.25 was found at the concentration of 0.078 and 256 μg/mL for melittin and vancomycin, respectively which their MBPC was reduced 512- and > 4-fold, respectively. Finally, for the moderate biofilm-forming MRSE 8, maximum synergistic effect with FBECi = 0.25 was found at the concentration of 0.078 and 256 μg/mL for melittin and vancomycin respectively which their MBEC was reduced 256- and > 4-fold, respectively.

**Table 4**
Minimum biofilm inhibition concentrations and minimum biofilm eradication concentrations of vancomycin, rifampin, and melittin for *Staphylococcus epidermidis*.

| Strains (n = 20) | Van-MBIC (μg/mL) | Van-MBEC (μg/mL) | Rif-MBIC (μg/mL) | Rif-MBEC (μg/mL) | Mel-MBIC (μg/mL) | Mel-MBEC (μg/mL) |
|---|---|---|---|---|---|---|
| **ATCC 35984 T** | 32 | > 1024 | 16 | 128 | 5 | 40 |
| **ATCC 35984 K** | 64 | > 1024 | 16 | 128 | 1.25 | 40 |
| **DSMZ 3270** | 64 | > 1024 | 32 | 128 | 1.25 | 20 |
| **ATCC 12228** | 8 | 16 | 4 | 32 | 0.625 | 10 |
| **MRSE 1** | 128 | > 1024 | > 1024 | > 1024 | 2.5 | 40 |
| **MRSE 2** | 16 | 64 | 16 | > 1024 | 1.25 | 20 |
| **MRSE 3** | 32 | > 1024 | 16 | > 1024 | 1.25 | 20 |
| **MSSE 1** | 32 | > 1024 | 32 | 64 | 2.5 | 40 |
| **MRSE 4** | 128 | > 1024 | > 1024 | > 1024 | 20 | 20 |
| **MRSE 5** | 32 | > 1024 | 32 | > 1024 | 10 | 40 |
| **MRSE 6** | 32 | 512 | 16 | > 1024 | 10 | 20 |
| **MRSE 7** | 16 | 256 | 16 | > 1024 | 5 | 40 |
| **MRSE 8** | 64 | > 1024 | 16 | 512 | 2.5 | 20 |
| **MRSE 9** | 32 | > 1024 | 8 | 256 | 1.25 | 20 |
| **MRSE 10** | 64 | > 1024 | 16 | 512 | 2.5 | 20 |
| **MRSE 11** | 32 | 256 | 8 | > 1024 | 5 | 40 |
| **MRSE 12** | 32 | > 1024 | 16 | > 1024 | 2.5 | 20 |
| **MRSE 13** | 64 | 512 | 16 | > 1024 | 5 | 20 |
| **MRSE 14** | 128 | 256 | 8 | 256 | 2.5 | 20 |
| **MRSE 15** | 32 | > 1024 | 16 | 512 | 1.25 | 20 |
| **GM-MBIC** | 51.6 | – | 117.4 | – | 4.15 | – |
| **GM-MBEC** | – | – | – | – | – | 26.5 |
| **MBIC 50** | 32 | – | 16 | – | 2.5 | – |
| **MBIC 90** | 128 | – | 32 | – | 10 | – |
| **MBEC 50** | – | > 1024 | – | 512 | – | 20 |
| **MBEC 90** | – | > 1024 | – | > 1024 | – | 40 |

MBIC, Minimum biofilm inhibition concentration; MBEC, Minimum biofilm eradication concentration; MRSE, methicillin-resistant Staphylococcus epidermidis; GM-MBIC, geometric mean-Minimum biofilm inhibition concentration; GM-MBEC, geometric mean-Minimum biofilm eradication concentration.

**Table 5**
The best synergistic concentrations of Rifampin-Melittin based on MBPC against 5 selected strains.

| MRSE 1 | | MRSE 4 | | ATCC 35984 | | MSSE 1 | | MRSE 8 | |
|---|---|---|---|---|---|---|---|---|---|
| Rif+Mel (μg/mL) | FBPC indices | Rif+Mel (μg/mL) | FBPC indices | Rif+Mel (μg/mL) | FBPC indices | Rif+Mel μg/mL | FBPC indices | Rif+Mel (μg/mL) | FBPC indices |
| **256 + 0.039** | 0.265625 | **64 + 0.039** | 0.064453 | 8 + 0.039 | 1.0078125 | 2 + 0.039 | 0.515625 | 4 + 0.039 | 0.515625 |
| **256 + 0.078** | 0.28125 | **64 + 0.078** | 0.06640625 | 8 + 0.078 | 1.015625 | 2 + 0.078 | 0.53125 | 4 + 0.078 | 0.53125 |
| **256 + 0.156** | 0.3125 | **64 + 0.156** | 0.0703125 | 8 + 0.156 | 1.03125 | 2 + 0.156 | 0.5625 | 4 + 0.156 | 0.5625 |
| **254 + 0.312** | 0.373046875 | **64 + 0.312** | 0.078125 | 8 + 0.312 | 1.0625 | 2 + 0.312 | 0.625 | 4 + 0.312 | 0.625 |
| **252 + 0.625** | 0.49609375 | **64 + 0.625** | 0.09375 | 8 + 0.625 | 1.125 | 2 + 0.625 | 0.75 | 4 + 0.625 | 0.75 |
| 250 + 1.25 | 0.744140625 | **62 + 1.25** | 0.123046875 | 8 + 1.25 | 1.25 | – | – | – | – |
| – | – | **62 + 2.5** | 0.185546875 | 6 + 2.5 | 1.5 | – | – | – | – |
| – | – | **60 + 5** | 0.30859375 | 6 + 5 | 1.75 | – | – | – | – |
| – | – | 58 + 10 | 0.556640625 | – | – | – | – | – | – |

Abbreviations: MBPC, minimum biofilm prevention concentration; MRSE, methicillin-resistant S. epidermidis; ATCC, American type culture collection; MSSE, methicillin-susceptible S. epidermidis; Rif, rifampin; Mel, melittin; FBPC, fractional biofilm prevention concentration.

**Table 6**
The best synergistic concentrations of Vancomycin-Melittin based on MBPC against 5 selected strains.

| MRSE 1 | | ATCC 35984 | | MRSE 4 | | MSSE 1 | | MRSE 8 | |
|---|---|---|---|---|---|---|---|---|---|
| Van+Mel (μg/mL) | FBPC indices | Van+Mel (μg/mL) | FBPC indices | Van+Mel (μg/mL) | FBPC indices | (Van+Mel) μg/mL | FBPC indices | Van+Mel (μg/mL) | FBPC indices |
| **16 + 0.039** | 0.140625 | **2 + 0.039** | 0.2578125 | **16 + 0.039** | 0.126953125 | 2 + 0.039 | 0.515625 | **2 + 0.039** | 0.140625 |
| **16 + 0.078** | 0.15625 | **2 + 0.078** | 0.265625 | **16 + 0.078** | 0.12890625 | 2 + 0.078 | 0.53125 | **2 + 0.078** | 0.15625 |
| **16 + 0.156** | 0.1875 | **2 + 0.156** | 0.28125 | **16 + 0.156** | 0.1328125 | 2 + 0.156 | 0.5625 | **2 + 0.156** | 0.1875 |
| **16 + 0.312** | 0.25 | **2 + 0.312** | 0.3125 | **16 + 0.312** | 0.140625 | 2 + 0.312 | 0.625 | **2 + 0.312** | 0.25 |
| **16 + 0.625** | 0.375 | **2 + 0.625** | 0.375 | **16 + 0.625** | 0.15625 | 2 + 0.625 | 0.75 | **2 + 0.625** | 0.375 |
| 16 + 1.25 | 0.625 | **2 + 1.25** | 0.5 | **16 + 1.25** | 0.1875 | – | – | 2 + 1.25 | 0.625 |
| – | – | 2 + 2.5 | 0.75 | **14 + 2.5** | 0.234375 | – | – | – | – |
| – | – | – | – | **14 + 5** | 0.359375 | – | – | – | – |
| – | – | – | – | 12 + 10 | 0.59375 | – | – | – | – |

Abbreviations: Van, vancomycin; Mel, melittin.

### 3.6. Effect of melittin on the expression of the biofilm-associated genes

The effect of sub-MIC concentrations of melittin (range 1.25–0.009 μg) on the expression of the *icaA*, *aap*, *psmβ,* and *16 S rRNA* genes were evaluated for the selected *S. epidermidis* isolates. Expression of the *16 S rRNA* gene was also measured as an internal control. The expression of *icaA*, *aap*, and *psmβ* genes were downregulated in all examined isolates ranging from $0.04 \pm 0.01$–$2.11 \pm 0.22$ folds for *icaA*,

**Table 7**
The best synergistic concentrations of Rifampin-Melittin based on MBIC against 5 selected strains.

| MRSE 1 | | MRSE 4 | | ATCC 35984 | | MSSE 1 | | MRSE 8 | |
|---|---|---|---|---|---|---|---|---|---|
| Rif+Mel (μg/mL) | FBIC indices | Rif+Mel (μg/mL) | FBIC indices | Rif+Mel (μg/mL) | FBIC indices | Rif+Mel μg/mL | FBIC indices | Rif+Mel (μg/mL) | FBIC indices |
| **256 + 0.039** | 0.2578125 | **256 + 0.039** | 0.251953125 | 8 + 0.039 | 0.5078125 | **4 + 0.039** | 0.140625 | **2 + 0.039** | 0.140625 |
| **256 + 0.078** | 0.265625 | **256 + 0.078** | 0.25390625 | 8 + 0.078 | 0.515625 | **4 + 0.078** | 0.15625 | **2 + 0.078** | 0.15625 |
| **256 + 0.156** | 0.28125 | **256 + 0.156** | 0.2578125 | 8 + 0.156 | 0.53125 | **4 + 0.156** | 0.1875 | **2 + 0.156** | 0.1875 |
| **254 + 0.312** | 0.310546875 | **254 + 0.312** | 0.263671875 | 8 + 0.312 | 0.5625 | **4 + 0.312** | 0.25 | **2 + 0.312** | 0.25 |
| **252 + 0.625** | 0.37109375 | **252 + 0.625** | 0.27734375 | 8 + 0.625 | 0.625 | **4 + 0.625** | 0.375 | **2 + 0.625** | 0.375 |
| **250 + 1.25** | 0.494140625 | **250 + 1.25** | 0.306640625 | 8 + 1.25 | 0.75 | 4 + 1.25 | 0.625 | 2 + 1.25 | 0.625 |
| – | – | – | – | 8 + 2.5 | 1 | – | – | – | – |

Abbreviations: MBIC, minimum biofilm inhibitory concentration; MRSE, methicillin-resistant S. epidermidis; ATCC, American type culture collection; MSSE, methicillin-susceptible S. epidermidis; Rif, rifampin; Mel, melittin; FBIC, fractional biofilm inhibitory concentration.

**Table 8**
The best synergistic concentrations of Vancomycin-Melittin based on MBIC against 5 selected strains.

| MRSE 1 | | MRSE 4 | | ATCC 35984 | | MSSE 1 | | MRSE 8 | |
|---|---|---|---|---|---|---|---|---|---|
| Van+Mel (μg/mL) | FBIC indices | Van+Mel (μg/mL) | FBIC indices | Van+Mel (μg/mL) | FBIC indices | (Van+Mel) μg/mL | FBIC indices | Van+Mel (μg/mL) | FBIC indices |
| **32 + 0.039** | 0.2578125 | **32 + 0.039** | 0.251953125 | **4 + 0.039** | 0.1328125 | **8 + 0.039** | 0.265625 | **16 + 0.039** | 0.265625 |
| **32 + 0.078** | 0.265625 | **32 + 0.078** | 0.25390625 | **4 + 0.078** | 0.140625 | **8 + 0.078** | 0.28125 | **16 + 0.078** | 0.28125 |
| **32 + 0.156** | 0.28125 | **32 + 0.156** | 0.2578125 | **4 + 0.156** | 0.15625 | **8 + 0.156** | 0.3125 | **16 + 0.156** | 0.3125 |
| **32 + 0.312** | 0.3125 | **32 + 0.312** | 0.265625 | **4 + 0.312** | 0.1875 | **8 + 0.312** | 0.375 | **16 + 0.312** | 0.375 |
| **32 + 0.625** | 0.375 | **32 + 0.625** | 0.28125 | **4 + 0.625** | 0.25 | **8 + 0.625** | 0.5 | **16 + 0.625** | 0.5 |
| **30 + 1.25** | 0.484375 | **30 + 1.25** | 0.296875 | **4 + 1.25** | 0.375 | 8 + 1.25 | 0.75 | 16 + 1.25 | 0.75 |
| **30 + 2.5** | 0.734375 | **30 + 2.5** | 0.359375 | 4 + 2.5 | 0.625 | – | – | – | – |
| – | – | **28 + 5** | 0.46875 | – | – | – | – | – | – |
| – | – | 26 + 10 | 0.703125 | – | – | – | – | – | – |

Abbreviation: Van, vancomycin; Mel, melittin.

**Table 9**
The best synergistic concentrations of Rifampin-Melittin based on MBEC against 5 selected strains.

| MRSE 1 | | MRSE 4 | | ATCC 35984 | | MSSE 1 | | MRSE 8 | |
|---|---|---|---|---|---|---|---|---|---|
| Rif+Mel (μg/mL) | FBEC indices | Rif+Mel (μg/mL) | FBEC indices | Rif+Mel (μg/mL) | FBEC indices | Rif+Mel μg/mL | FBEC indices | Rif+Mel (μg/mL) | FBEC indices |
| **256 + 0.078** | 0.251953125 | **256 + 0.078** | 0.25390625 | 64 + 0.078 | 0.501953125 | 32 + 0.078 | 0.501953125 | 256 + 0.078 | 0.50390625 |
| **256 + 0.156** | 0.25390625 | **256 + 0.156** | 0.257813 | 64 + 0.156 | 0.50390625 | 32 + 0.156 | 0.50390625 | 256 + 0.156 | 0.5078125 |
| **256 + 0.312** | 0.2578125 | **256 + 0.312** | 0.265625 | 64 + 0.312 | 0.5078125 | 32 + 0.312 | 0.5078125 | 256 + 0.312 | 0.515625 |
| **254 + 0.625** | 0.263671875 | **254 + 0.625** | 0.279296875 | 64 + 0.625 | 0.515625 | 32 + 0.625 | 0.515625 | 254 + 0.625 | 0.52734375 |
| **252 + 1.25** | 0.27734375 | **252 + 1.25** | 0.30859375 | 62 + 1.25 | 0.515625 | 32 + 1.25 | 0.53125 | 252 + 1.25 | 0.5546875 |
| **250 + 2.5** | 0.306640625 | **250 + 2.5** | 0.369140625 | 62 + 2.5 | 0.546875 | 30 + 2.5 | 0.53125 | 250 + 2.5 | 0.61328125 |
| – | – | – | – | 60 + 5 | 0.59375 | 30 + 5 | 0.59375 | – | – |
| – | – | – | – | 58 + 10 | 0.703125 | 28 + 10 | 0.6875 | – | – |
| – | – | – | – | – | – | 26 + 20 | 0.90625 | – | – |

Abbreviations: MBEC, minimum biofilm eradication concentration; MRSE, methicillin-resistant S. epidermidis; ATCC, American type culture collection; MSSE, methicillin-susceptible S. epidermidis; Rif, rifampin; Mel, melittin; FBEC, fractional biofilm eradication concentration.

**Table 10**
The best synergistic concentrations of Vancomycin-Melittin based on MBEC against 5 selected strains.

| MRSE 1 | | ATCC 35984 and MSSE 1 | | MRSE 4 | | MRSE 8 | |
|---|---|---|---|---|---|---|---|
| Van+Mel (μg/mL) | FBEC indices | Van+Mel (μg/mL) | FBEC indices | Van+Mel (μg/mL) | FBEC indices | Van+Mel (μg/mL) | FBEC indices |
| **128 + 0.078** | 0.126953125 | **256 + 0.078** | 0.251953125 | **128 + 0.078** | 0.12890625 | **256 + 0.078** | 0.25390625 |
| **128 + 0.156** | 0.12890625 | **256 + 0.156** | 0.25390625 | **128 + 0.156** | 0.1328125 | **256 + 0.156** | 0.2578125 |
| **128 + 0.312** | 0.1328125 | **256 + 0.312** | 0.2578125 | **128 + 0.312** | 0.140625 | **256 + 0.312** | 0.265625 |
| **128 + 0.625** | 0.140625 | **254 + 0.625** | 0.263671875 | **128 + 0.625** | 0.15625 | **254 + 0.625** | 0.279296875 |
| **126 + 1.25** | 0.154296875 | **252 + 1.25** | 0.27734375 | **126 + 1.25** | 0.185546875 | **252 + 1.25** | 0.30859375 |
| **124 + 2.5** | 0.18359375 | **250 + 2.5** | 0.306640625 | **124 + 2.5** | 0.24609375 | **250 + 2.5** | 0.369140625 |
| **122 + 5** | 0.244140625 | – | – | **122 + 5** | 0.369140625 | – | – |

Abbreviation: Van, vancomycin; Mel, melittin.

0.05 ± 0.018–3.76 ± 0.17 folds for *aap*, and *psm*, respectively, in comparison to the negative control. In particular, for ATCC 35984, at 0.039–1.25 μg, the downregulation ranged from 0.04 ± 0.02–1.47 ± 0.16 for *ica*, 0.06 ± 0.03–2.47 ± 0.13 for *aap*, and 0.06 ± 0.02–2.47 ± 0.1 for *psm*, respectively. For MRSE 1, at 0.019–0.625 μg, the downregulation ranged from 0.0 to 1.23 ± 0.19 for *ica*, 0.22 ± 0.11–3.23 ± 0.3 for *aap*, and *psm*, respectively. For MSSE 1, at 0.009–0.312 μg, the downregulation ranged from

0.06 ± 0.04–1.06 ± 0.24 for *ica*, 0.11 ± 0.09–3.76 ± 0.54 for *aap*, and 0.15 ± 0.1–3.76 ± 0.3 for *psm*, respectively. Besides, for MRSE 4, at 0.009–0.312 μg, the downregulation ranged from 0.06 ± 0.027–2.11 ± 0.15 for *ica*, 0.09 ± 0.04–3.11 ± 0.25 for *aap*, and 0.09 ± 0.038–2.33 ± 0.19 for *psm*, respectively. Finally, for MRSE 5, at 0.019–0.625 μg, the downregulation ranged from 0.05 ± 0.03–1.75 ± 0.14 for *ica*, 0.05 ± 0.02–3.54 ± 0.18 for *aap*, and 0.05 ± 0.04–2.54 ± 0.1 for *psm*, respectively. In this regard, based on regression analysis, linearity was seen at the examined concentrations of melittin ranging from 0. 0.009–1.25 μg/μL. ANOVA indicated a significant difference between the test and untreated groups for all genes ($p < 0.05$). Of note, melittin had no significant effects on the *16 S rRNA* gene expression of *S. epidermidis* isolates. Further details are depicted in Fig. 2.

### 3.7. Toxicity assays

The cytotoxicity results showed that melittin at 5, 2.5, 1.25, 0.625, 0.312, 0.156,.0.078, and 0.039 μg/mL induced 85.9 ± 4%, 69.4 ± 3.7%, 45.3 ± 4.3%, 25.2 ± 3%, 10 ± 3.9%, 3.5 ± 2%, 0%, and 0% toxicity toward HEK-293, respectively (Fig. 3). The paired sample t-test showed no significant difference between the survival rate of melittin at 0.039 μg/mL and control ($p = 0.0857$). Besides, hemolytic activity of melittin at the concentration of 5, 2.5, 1.25, 0.625, 0.312, 0.156,.0.078, and 0.039 μg/mL showed 96 ± 2.1%, 80.5 ± 2.3%, 74.2 ± 2.2%, 59.5 ± 1.8%, 25 ± 1.2%, 6 ± 0.8%, 0%, and 0% hemolysis on human RBCs, respectively (Fig. 4).

### 3.8. Preventive and therapeutic indexes of melittin

The preventive and therapeutic indexes were calculated with antibiotics for melittin alone and at the best synergistic concentration. The preventive index was 0.59, 4.73, and 4.73 for melittin alone, melittin-rifampin, and melittin-vancomycin, respectively. The therapeutic index was 0.59, 4.73, and 4.73 for melittin alone, melittin-rifampin, and melittin-vancomycin, respectively.

## 4. Discussion

Chronic diseases, in particular foreign body infections, are mainly caused by biofilm-growing bacteria and are characterized by severe tissue injury, a consequence of prolonged inflammatory processes [56]. These infections survive despite antibiotic treatment and host immunological responses [56]. Mortality and morbidity caused by the production of biofilms from MRSE are one of the most serious concerns related to foreign body infections [57]. This issue is significantly associated with the ability of bacteria to survive and persist in the patient's body or hospital setting due to the formation of biofilm layers. This process is fueled by multiple molecular pathways that inhibit the absorption of antibiotics and promote antimicrobial resistance [58,59]. Over the years, various approaches have been suggested for the treatment of bacterial biofilms, including prophylaxis, weakening, disruption, killing of biofilm-embedded bacteria [60,61]. Within the narrow range of new antimicrobials under development, AMPs appear to be endowed with characteristics favorable to ensure their advancement as effective agents against biofilm-associated infections [62–64].

Furthermore, it has been shown that AMPs could be suitable candidates for the development of new antibiofilm drugs as they can act against biofilms through a variety of mechanisms, including the prevention, blocking and destruction of preformed biofilms [65]. Indeed, melittin has been found to have powerful antibacterial and antibiofilm activity [24,66–69]. In this connection, the present study aimed to examine the effect of melittin, both alone and in synergy with conventional antibiotics, against biofilm-producing MRSE bacteria, in terms of preventing biofilm formation and promoting biofilm degradation, as well as of killing the embedded bacteria.

The results of the current study of MBIC and MBEC showed the excellent activity of melittin against the biofilms of all *S. epidermidis* isolates. The MBIC values of melittin against all isolates ranged from 0.625 to 20 μg/mL, and the MBEC values ranged from 10 to 40 μg/mL, respectively. In comparison to melittin, the results of MBIC and MBEC for rifampin and vancomycin showed a weaker effect against the biofilm of *S. epidermidis* isolates. In this regard, MBIC values ranged from 4 to > 1024 μg/mL for rifampin and from 8 to 128 μg/mL for vancomycin, respectively. Additionally, MBEC values ranged from 32 to > 1024 μg/mL and 16 to > 1024 μg/mL for rifampin and vancomycin, respectively. Besides, $MBIC_{50}$, $MBIC_{90}$, $MBEC_{50}$, and $MBEC_{90}$ values were 16, 32, 512, and > 1024 μg/mL for rifampin, and 32, 128, > 1024, and > 1024 μg/mL for vancomycin, respectively. These results are higher than those reported by the previous studies [70–72]. For example, Douthit et al. [70] found that MBIC for rifampin and vancomycin was 80 ng/mL and 1 μg/mL respectively, and also, MBEC for rifampicin was 80 ng/mL, while MBEC for vancomycin was 6 μg/mL against *Staphylococcus aureus* biofilm. It should be noted that the results of MBEC in our study are in accordance with those of other studies [73,74].

Bacteria can migrate in the circulation in a planktonic state, especially in systemic diseases [75,76]. Entering the circulation from the main site of infection causes the bloodstream to spread the infection to distant organs and tissues, bacteria to adhere to additional sites and form biofilms. This occurs for example in bacterial endocarditis and septic arthritis [77]. Infective endocarditis (IE) is potentially very dangerous. The concept that a biofilm-associated infection causes IE clarifies why it is resistant to antimicrobials and why surgical destruction and biofilm elimination enhance the chances of cure [78]. *S. epidermidis* is one of the most prevalent species responsible for IE in both the artificial and native valves [79]. Coagulase negative staphylococci (CoNS) cause up to 40% of prosthetic valve endocarditis (PVE) cases [79]. Bacteria can aggregate and generate vegetations on heart valves whenever they form biofilms [79]. According to this, we defined a test called MBPC. The results of this test found the excellent activity of melittin in the prevention of biofilm formation by *S. epidermidis*. The MBPC values of melittin showed that melittin acted against all isolates ranging from 0.625 to 20 μg/mL. Besides, $MBPC_{50}$ and $MBPC_{90}$ values of melittin were 2.5 and 5 μg/mL, respectively. The results of the MBPC showed a weak effect of rifampin and vancomycin against *S. epidermidis* isolates. In this regard, the MBPC values of rifampin and vancomycin against all isolates ranged from 4-> 1024 μg/mL and 4–128 μg/mL. Besides, $MBPC_{50}$, $MBPC_{90}$ values were 4 and 16 μg/mL for rifampin and 16 and 64 μg/mL for vancomycin, respectively.

Our results confirmed that melittin could be recognized as an antibiofilm peptide (ABP). AMPs have been found to have many convergent anti-biofilm mechanisms, including degradation of the membrane of biofilm-embedded bacteria, degradation of the polysaccharide and biofilm matrix, down-regulation of the genes responsible for biofilm formation [80]. Biofilm formation in staphylococci depends on the synthesis of the polysaccharide intercellular adhesin (PIA), which is encoded by the *icaADBC* locus [81]. The results obtained using Real-Time PCR showed that the expression of the intercellular adhesion A (*icaA)*, accumulation-associated protein *(aap)*, and phenol-soluble modulin *(psm)* genes was down-regulated by melittin in all examined isolates ranging from 0.04 to 2.11-fold for *icaA*, 0.05–3.76-fold for *aap*, and *psm*, respectively. These results are in agreement with those reported by Mohammadi et al. [24], who found that administration of melittin resulted in a statistically significant decrease in *bap* mRNA expression in *A. baumannii* isolates at sub-MIC concentrations. Hence, based on these findings, in addition to its direct anti-biofilm effects, melittin can also prevent the expression of the biofilm-related genes. Thus, the development of the biofilm is prevented, as above shown by the MBPC test.

Combination treatment is especially appealing in the case of bacterial biofilm since the diverse nature of this form of development necessitates addressing cells in various metabolic stages, such as the

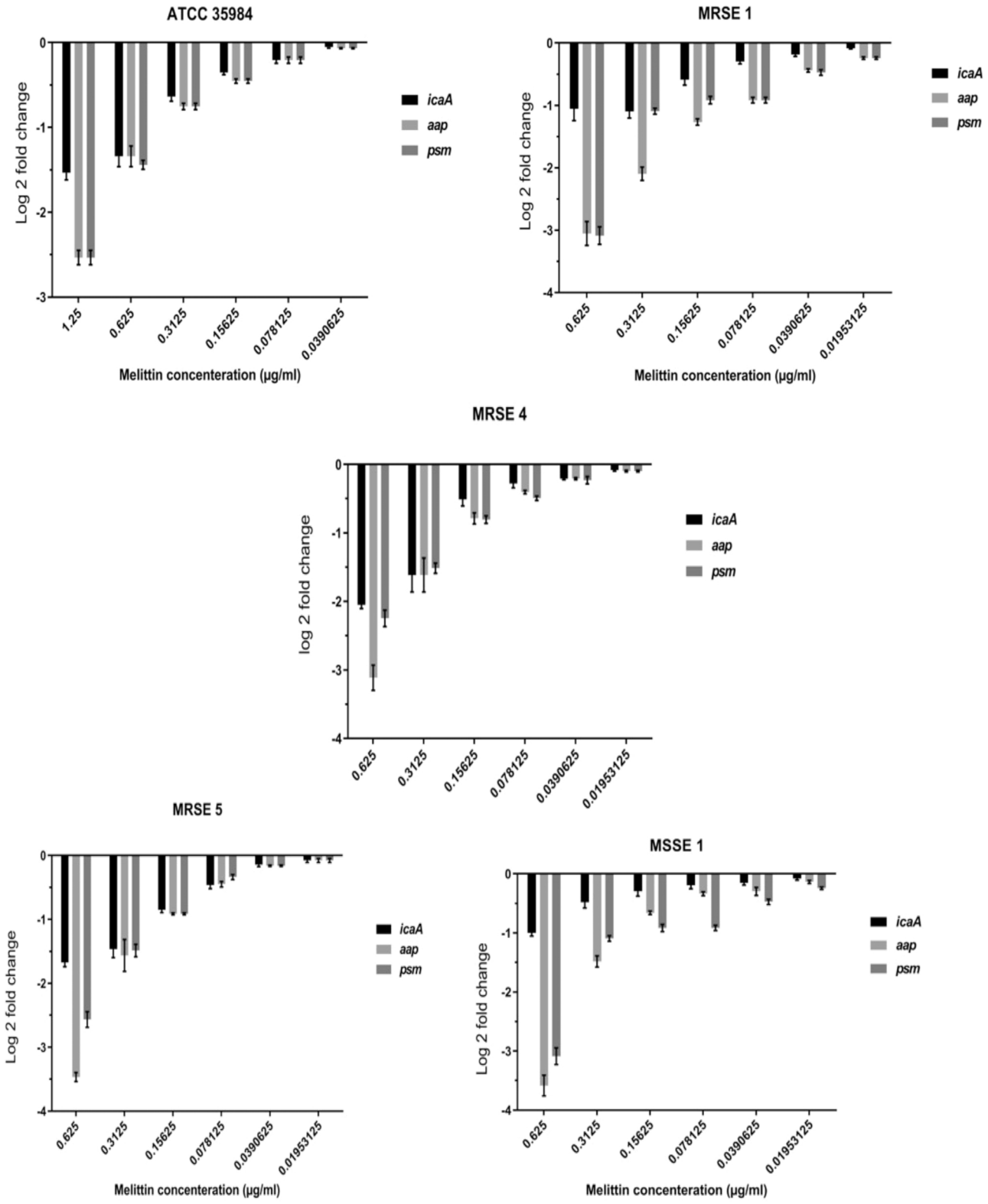


**Fig. 2.** Downregulation of biofilm-associated genes in 5 selected strains at different sub-MIC concentrations of Melittin. In this regard, for ATCC 35984, the downregulation ranged from 0.04 ± 0.02–1.47 ± 0.16 for *ica*, 0.06 ± 0.03–2.47 ± 0.13 for *aap*, and 0.06 ± 0.02–2.47 ± 0.1 for *psm*, respectively. For MRSE 1, the downregulation ranged from 0.0 to 1.23 ± 0.19 for *ica*, 0.22 ± 0.11–3.23 ± 0.3 for *aap*, and *psm*, respectively. For MSSE 1, the downregulation ranged from 0.06 ± 0.04–1.06 ± 0.24 for *ica*, 0.11 ± 0.09–3.76 ± 0.54 for *aap*, and 0.15 ± 0.1–3.76 ± 0.3 for *psm*, respectively. Besides, for MRSE 4, the downregulation ranged from 0.06 ± 0.027–2.11 ± 0.15 for *ica*, 0.09 ± 0.04–3.11 ± 0.25 for *aap*, and 0.09 ± 0.038–2.33 ± 0.19 for *psm*, respectively. Finally, for MRSE 5, the downregulation ranged from 0.05 ± 0.03–1.75 ± 0.14 for *ica*, 0.05 ± 0.02–3.54 ± 0.18 for *aap*, and 0.05 ± 0.04–2.54 ± 0.1 for *psm*, respectively. The findings are expressed as the mean ± SD. ANOVA indicated a significant difference between each of the strains and untreated groups (p-value $< 0.05$).

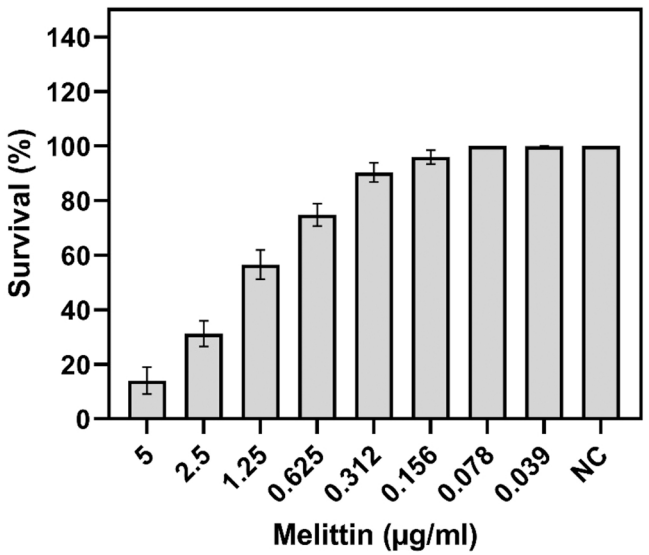


**Fig. 3.** Toxicity of melittin alone and in synergistic concentrations toward HEK-293 cells. The cytotoxicity results showed that melittin at 5, 2.5, 1.25, 0.625, 0.312, 0.156, 0.078, and 0.039 μg/mL induced 85.9 ± 4%, 69.4 ± 3.7%, 45.3 ± 4.3%, 25.2 ± 3%, 10 ± 3.9%, 3.5 ± 2%, 0%, and 0% toxicity toward HEK-293, respectively. Data are expressed as the mean ± S.D. ANOVA indicated a no significant difference between negative control and 0.078, and 0.039 concentrations (p-value = 0.3739).

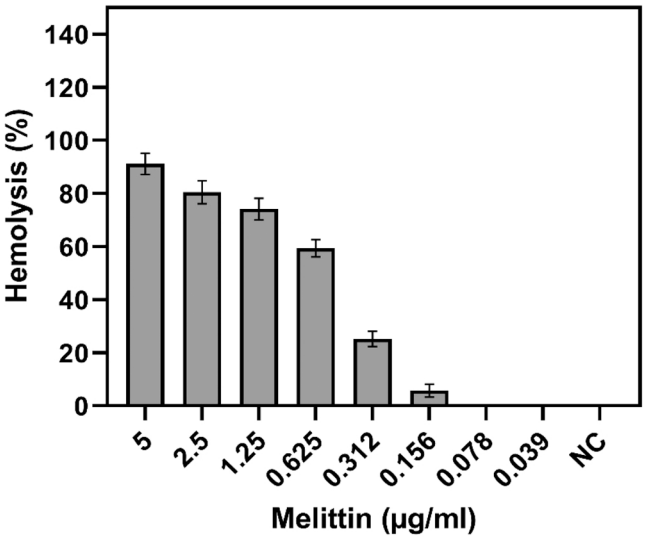


**Fig. 4.** Hemolysis of melittin alone and in synergistic concentrations toward human RBC. Hemolytic activity of melittin at the concentration of 5, 2.5, 1.25, 0.625, 0.312, 0.156, 0.078, and 0.039 μg/mL showed 96 ± 2.1%, 80.5 ± 2.3%, 74.2 ± 2.2%, 59.5 ± 1.8%, 25 ± 1.2%, 6 ± 0.8%, 0%, and 0% hemolysis on RBCs, respectively. Data are expressed as the mean ± S.D. NC, negative control. ANOVA indicated a no significant difference between negative control and 0.078, and 0.039 concentrations (p-value = 0.3739).

exponential growth phase and latent bacterial cells [82]. As a result, the combination of several molecules functioning on biofilm components can eliminate biofilm. Furthermore, it has been discovered that rifampin penetrates into *S. epidermidis* biofilms but does not efficiently destroy the bacteria within biofilm [83].

This finding supports previous research which indicated that the low penetration of antibiotics into biofilms was not an adequate explanation for the decreased sensitivity to antibiotics that bacteria exhibit when they are in a biofilm [84]. For example, resistance of *S. epidermidis* to killing by rifampicin was found to be associated with reduced bacterial growth within the biofilm. Additionally, traditional antibiotics are much less effective against biofilm bacteria than planktonic ones. In fact, biofilms covered with an external matrix of polysaccharides show greater resistance to antibiotics and a marked aptitude to evade the immune response [85,86]. Consequently, antibiotics are commonly administered in combination in order to limit the occurrence of resistance to mono antimicrobial and to exploit the synergy [87].

Some articles report that AMPs have synergistic functions against bacterial biofilms when combined with conventional antibiotics [88,89]. Teicoplanin coupled with SAAP-148 or SAAP-276 had a considerable effect on *Staphylococcus aureus* biofilms, according to Koppen et al. [88]. According to Mohammadi et al. [24], melittin has a significant potential for application in conjunction with colistin and imipenem towards MDR *A. baumannii* isolates, which are able to produce robust biofilms. As predicted, the traditional antibiotics examined had little effect on the biofilms generated by MRSE isolates, but the combination of these antibiotics with melittin showed interesting results. In the present research, the antibiotics evaluated belong to different classes with different targets; nevertheless, when coupled with melittin, they demonstrated synergistic activities against biofilm development, this implying that the peptide causes biofilm degradation and inhibition via a mechanism of action distinct from that of conventional antibiotics.

Apart from bypassing drug-resistant mutants, another aim of combination therapy is to achieve greater efficacy utilizing lower-dose combinations compared with higher-dose monotherapy [90–92]. This can potentially cause a lower risk of side effects of antibacterial drugs and a better quality of life [93]. In the present study, according to cytotoxicity results, the induced synergism between melittin, vancomycin, and rifampin led to a decrease in melittin cytotoxicity against HEK-293 cells while the peptide showed 85.9% toxicity alone. These findings are consistent with the cytotoxicity findings of Akbari et al. [23]; melittin cytotoxicity against HEK-293 cells was reduced due to its synergistic actions with traditional antibiotics against traditional antibiotics MDR strains of *A. baumannii* and *P. aeruginosa*. Most importantly, a decrease in the concentrations of vancomycin and rifampin in combination with melittin can reduce their side effects too. In the present study, the induced synergism led to 2–16-fold and 2–8-fold reduction in rifampin, and vancomycin concentration, respectively. This indicates that the combination of melittin with vancomycin or rifampin might be a promising therapy for biofilm-associated infections caused by MRSE. Besides, the present study provided insight into the hemolytic activity of melittin alone and in synergistic concentrations on human RBCs; that is, the melittin did not show hemolytic activity in synergistic concentration, whereas the peptide showed 96% hemolytic activity when used alone. These results are in accordance with the observations of Zarghami et al. [35], which found that melittin had no hemolytic effect at the low concentrations. In the end, we found that the preventive and therapeutic indexes of melittin were improved 8-fold when combined with rifampin and vancomycin. Taken together, these results appear interesting because they demonstrate that melittin in combination with antibiotics is capable of both preventing biofilm formation and degrading the preformed biofilm, while it is not toxic to host cells.

## 5. Conclusion

Today, controlling biofilm-associated infections is a challenging task for the medical sciences. In this context, antibiotic treatment alone usually fails to eradicate bacterial biofilms, requiring large amounts of antimicrobial drugs, often equally ineffective, and, in some circumstances, even prescribing them periodically, with a high risk of adverse responses and of emergence of new resistant strains. According to our results, melittin alone was effective against MRSE biofilm in terms of preventing biofilm formation but also destroying the formed biofilm and killing biofilm-embedded bacteria. Our research has also demonstrated a synergistic action of classic antibiotics in combination with melittin

against biofilm-producing MRSE. This combined treatment involves the use of standard antibiotics at lower but still effective doses, thus reducing adverse effects. Melittin is capable of degrading biofilm: this allows melittin and traditional antibiotics to reach and kill the built-in bacteria. Hence, the present study identifies in the use of combinations of AMPs with several conventional antibiotics an effective strategy against biofilm-forming pathogens. Based on these results, we propose the application of melittin in synergy with vancomycin or rifampicin against biofilm-producing MRSE as a new preventive or therapeutic perspective.

## CRediT authorship contribution statement

R.M performed all experiments and analyses and also contributed to writing the manuscript. MBPC, MBIC, MBEC, FBPCi, FBICi, and FBECi were named by R.M. M.Y.A, C.R.A, and I.S served as advisors. R.Y.M. contributed as a supervisor. K.P.B. suggested the implementation of melittin, contributed as a supervisor, and also in the writing, revision, and redaction of the manuscript. Preventive index was named by K.P.B and R.M.

## Conflict of interest statement

The authors declare no potential conflict of interest.

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