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In Vitro Synergistic Effect of the CM11 Antimicrobial Peptide in Combination with Common Antibiotics Against Clinical Isolates of Six Species of Multidrug-Resistant Pathogenic Bacteria

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Mehrdad M. Moghaddam

Abstract: During the last decades, increase of antibiotic resistance among pathogenic bacteria has been considered as a global concern. Therefore, it is important to find new antimicrobial agents and/or therapeutic strategies. In previous studies we investigated antibacterial activity of the CM11 peptide against multiple drug resistant clinical isolates of six bacteria species including *Pseudomonas aeruginosa, Staphylococcus aureus, Acinetobacter baumannii, Escherichia coli*, *Klebsiella pneumoniae* and *Salmonella typhimurium*. In this study, in order to reduce treatment costs and the cytotoxic effect of CM11 peptide, was analyzed its synergic interaction with selected antibiotics. In this reason, specific antibiotics for each bacterium were selected considering the guidelines of the "Clinical and Laboratory Standards Institute". Based on the results , using a checkerboard procedure through the broth microdilution method, MICs of antibiotic agents alone and in combination with the peptide were determined. In most cases, synergistic effects between CM11 peptide and selected antibiotics against six bacteria species were observed as partial synergy. However, for *S. aureus* and *P. aeruginosa*a synergic interaction between peptide and selective antibiotics was observed with penicillin and ceftazidime, respectively. For *K. pneumoniae*, synergic effect was observed when CM11 peptide was used in combination with norfloxacin and also the combination of peptide with norfloxacin showed synergic effect against *A. baumannii*. Combination between the CM11 peptide and ciprofloxacin showed synergic effect on *E. coli* while only partial synergy was observed for *S. typhimurium* in combination with cefotaxime and ceftazidime. These results suggest that when selected antibiotic used in combination with the CM11 peptide, the dose of some antibiotics, especially the dose independent antibiotics, may be reduced for eliminating drug resistant bacteria.

Keywords: CM11 peptide, Antimicrobial peptides, Antibiotic combination, synergistic effect.

INTRODUCTION

While the bacterial pathogens acquire resistance to different antibiotics with various mechanisms, the antimicrobial efficacy of older antibiotics are ceaselessly diminishing [1-4]. So in recent years, much attention and effort has been paid to the search for new generations of antimicrobial compounds with new mechanisms of action to which bacteria cannot create resistance. Among the large number of compounds that are currently under investigation for infection treatment, antimicrobial peptides (AMPs) are promising candidates as novel therapeutic agents with a unique mechanism of action and a number of them are undergoing new efforts of clinical evaluation [5-10]. Most of AMPs are cationic in nature that can receive an amphipathic consistence; in which the positive charge has a key role in the mode of action. In Gram-negative bacteria, electrostatic interaction is started by the cationic domain with the negatively charged lipopolysaccharide (LPS) and the hydrophobic portion is supposed to allow the peptides to insert into and permeate the membrane. On the other hand this process occurs in Gram-positive bacteria through binding with surface teichoic acids located in cell membrane [11, 12]. Since some dramatic changes would be required to occur in phospholipid membrane composition and/or organization, scientists believe that this unique mode of action decreases the likelihood of emergence of resistant pathogens compared with conventional antibiotics. Other advantages are that they are able to eliminate bacteria rapidly and have broad activity spectrum against bacteria (Gram-positive and Gram-negative), fungi, enveloped viruses, parasites, and tumor cells. Also, some might neutralize bacterial endotoxins, with not many reported complications [13-17]. Cecropins and melittin are two members of the most studied cationic antimicrobial peptides. Melittin with having 26 amino acids and high antimicrobial properties, is the main alpha-helical component of honey bee venom. Cecropins originally isolated from immune haemolymph of the giant silk moth (*Hyalophora cecropia*), Cecropins are linear amphipatic peptides, active against bacterial infections [15, 18-20]. Among the different types of cecropin, type A has 37 amino acid and acts strongly against

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Gram-positive and Gram-negative bacteria without cytotoxic effect on eukaryotic cells. However, the high expenses of producing both peptides (because of their long sequences), hemolysing nature of melittin, and susceptibility to protease degradation of cecropin A makes these peptides unsuitable for antimicrobial treatments [15, 19]. A solution for these problems is to design and synthesis of small peptides with improved properties that are shorter in length with higher potential, nontoxic or decreased toxicity, and better protection against cleavage by proteases, [14, 21] CM11 is one of these small peptides that include 11-residue sequence ($WKLFKKILKVL\text{-}NH_2$) derived from cecropin A residues 2 to 8 and from melittin residues 6 to 9 that have been designed and produced to improve the properties of parent peptides [15, 20, 22]. Considering these processes, for the first time Ferre et al., (2006) investigated antibacterial effects of this peptide against economically important plant pathogenic bacteria [15, 23]. Moreover, studies already implemented by our group (2012 and 2014) showed antimicrobial effects of this peptide against seven human pathogenic bacteria [19, 20]. In the present study we focused on peptide-antibiotic synergistic effects to reduce the dosage of the peptide, selected antibiotics and subsequently to decrease their associated side effects such as cytotoxicity. In general, combinational prescription of antimicrobial agents is common for the treatment of clinical infections. This method of prescription expands the spectrum of targeted organisms, decreases the possibility of resistance emergence in organisms, decreases the toxicity through using lower doses of both agents and increases the number of candidates for antibacterial treatments [24, 25]. So in this study we investigated the synergistic effects of CM11 and common clinical antibiotics against six important multidrug resistant nosocominal bacteria including *Pseudomonas aeruginosa, Staphylococcus aureus, Acinetobacter baumannii, Escherichia coli*, *Klebsiella pneumoniae* and *Salmonella typhimurium*.

MATERIALS AND METHODS

Peptide Synthesis

Solid-phase synthesis method was utilized on a Rinkp-methylbenzhydrylamine resin for synthesis of the CM11 peptide [18]. The peptide was purified with reversed-phase semi preparative HPLC on C18 Tracer column. The purity of peptide was more than 95%. Electrospray ionization mass spectrometry was used to confirm peptide identity.

Selective Antibiotics

Specific antibiotics for each bacterium were selected according to Clinical and Laboratory Standards Institute (CLSI) guidelines [26]. All antibiotics including Ampicillin (AMP), Piperacillin (PIP), Penicillin (PEN), Oxacillin (OXA), Cephalothin (CEF), Cefotaxime (CTX), Ceftazidime (CAZ), Imipenem (IPM), Norfloxacin (NOR), Ciprofloxacin (CIP), Rifampin (RIF), Amoxiclav (Amoxicillin-clavulanic acid) (AMC), Gentamicin (GEN), Amikacin (AMK), Kanamycin (KAN) and Vancomycin (VAN) were purchased from Sigma-Aldrich (USA) as disk and powder. Antibiotic powders were dissolved in accordance with the manufacturers' recommendations before use.

Bacterial Strains and Growth Conditions

The control strains *P. aeruginosa* (ATCC 27853), *A. baumannii* (ATCC 17978), *S. aureus* subsp. *aureus* (ATCC 25923), *E. coli* (ATCC 25922), *K. pneumoniae* (ATCC 700630) and *S. typhimurium* (ATCC 14028) were obtained from the American Type Culture Collection (ATCC). Cells were grown in Luria-Bertani (LB) agar (Sigma-Aldrich, USA) at 37°C. Also 50, 50, 50, 50, 50, 35 clinical isolates of each bacteria strain, respectively, were retrieved from clinical diagnostic laboratories (In Khatamal-Anbia and Shahid Motahari, Tehran), which were confirmed with laboratory control tests in these laboratories and PCR by designed specific primers for each bacterial strain in our laboratory (Table **1**).

Selection of Multidrug-Resistant Isolates

For investigation of the antibiotic resistance isolates, agar disk diffusion test was used on Mueller–Hinton agar (Sigma-Aldrich, USA) using 0.2 ml of an initial inoculum1.5 x 10^8 CFU/mlat exponential growth phase. For each bacterial strain, antibiotics were selected according to CLSI guidelines [26]. Screening of resistant isolates was accomplished after anti-biogram test (Kirby-Bauer Method) and the antibiotic resistance was determined by measuring the diameter of inhibition zones. Among the samples, bacteria isolates with the highest antibiotic resistance level were selected.

MICs Determination

In previous studies we determined minimal inhibitory concentration (MIC) for CM11 peptide [19, 20]. For antibiotics, MICs were determined using (a) a broth micro-dilution method with cation-adjusted Mueller-Hinton broth (CAMHB; BD Diagnostic Systems, adjusted to pH 5.9) and (b) an initial inoculum of 1.5 x 10^8 CFU/ml was used in clinical settings according to the procedures outlined by the CLSI for measuring in vitro susceptibility of bacteria to antimicrobial agents. Briefly, susceptibility panel in 96-well microtiter plates were prepared with various concentrations of the test antibiotic agents in 100 µl final volumes so far the concentration of each well was twofold compared to previous well, then 100 µl of standardized numbers of bacteria were dispensed per well into 96-well microtiter plates. For susceptibility analysis, after 18 h of incubation at 37°C with shaking method, bacterial turbidity was measured by the absorbance at 600 nm of each sample using a microtiter plate reader (BioTek ELx800). The lowest concentration that inhibited bacterial growth was considered MIC. All the tests were performed in triplicate

MICs Combination Assay

MICs of antibiotic agents in combination with peptide were determined by checkerboard assay with the broth microdilution method in accordance with the CLSI protocols using cation-adjusted Mueller-Hinton broth [27]. It is noteworthy that after determination of antibiotic resistance and related MICs, for combination assay we selected isolates in each strain with extensive resistance pattern and higher MICs to related antibiotics. Briefly, a two-dimensional checkerboard with twofold dilutions of each agent was set up

Table 1. Primer sequences used for molecular detection of isolates.

Strains	F Primer	R Primer	Gene Target	Amplicon Size (bp)
P. aeruginosa	5'ATGATCGTACAAATGGTCGG3'	5'GTCATGAAACCGCCAGTC3'	*lasI*	606
A. baumannii	5'AATTTACAGTGGCACATTAGGTCCC3'	5'GCAGAGATACCAGCAGAGATACACG3'	*gltA*	722
S. aureus	5'GTAGAAATGACTGAACGTCCGATAA3'	5'CCAATTCCACATTGTTTCGGTCTAA3'	*mecA*	310
E. coli	5'GTGACAAAAGCCCGGACACCATAAATGCC3'	5'TACACTGTCATTACGTTGCGGATTTGGCG3'	*phoA*	903
K. pneumoniae	5'GCGTGGCGGTAGATCTAAGTCATA3'	5'TTCAGCTCCGCCACAAAGGTA3'	*mdh*	222
S. typhimurium	5'AACAACGGCTCCGGTAATGAGATT3'	5'ATGACAAACTCTTGATTCTGAAGATG3'	*stm*	310

so far in microplate wells, serial dilution of antibiotic agent and peptide was mixed as the column and row contained a fixed amount of antibiotic and increasing amounts of peptide or conversely. The concentration ranges were based on the MICs of each antibiotic, peptide and bacteria, with values between 4 x MIC and 0.25 x MIC. Each plate contained two bacteria growth control wells containing the medium alone and bacterial culture. After 18 h of incubation at 37°C, the MIC was defined as described above. The interaction CM11 peptide (A) with each antibiotic (B) was evaluated by the fractional inhibitory concentration index (FICI). This index is calculated according to the equation: $FICI = FIC_A + FIC_B =$ (MIC_A in combination/MIC_A alone) + (MIC_B in combination/MIC_B alone). FICI was interpreted as follows: FICI $\leq$ 0.5 synergy, 0.5 < FICI $\leq$ 1 partial synergy, 1 <FICI $\leq$ 4 additive effect or indifference, 4 < FICI antagonism. Each test was performed in triplicate [28, 29].

The Time-Kill Assay

Following CLSI guidelines, Time-kill assay was performed on selected isolates of *P. aeruginosa* and *S. aureus*, as the most important bacteria for nosocomial infections in recent years. Among *P. aeruginosa* and *S. aureus* isolates we selected one isolate which had higher MIC for peptide and antibiotics in comparison with other isolates. Related specific antibiotics and CM11 peptide alone and in combination at a concentration equal to MIC were tested against bacteria isolates. Microplate wells containing fresh Mueller–Hinton broth supplemented with antibacterial agents alone and combination, were inoculated with 1.5 x 10^8 CFU/ml of each strain and incubated at 37°C. Aliquots were diluted and cultured on Mueller–Hinton agar plates for cell counts after 0, 1, 2, 3, 4, 5, 6, 9, 12, 15 and 18 h. Synergy was defined as a $\geq$2 $\log_{10}$ decrease in colony count by the combination, compared with the most active single agent. Also, indifference was defined as a <2 $\log_{10}$ increase or decrease in colony count by the combination, compared with the most active drug alone. Antagonism was defined as a $\geq$2 $\log_{10}$ increase in colony count in an overnight period by the combination, compared with the most active drug alone [30].

Statistical Analysis

The data in each experiment was a representative of three independent experiments expressed as the mean ±standard deviation (SD). The statistical significance of the differences between the control and test values was evaluated using a one-way ANOVA t-test.

RESULTS

Multidrug-Resistant Isolates

To evaluate and select clinical isolates with the highest rates of antibiotic resistance we used anti-biogram test. Results are analyzed based on CLSI guidelines for each strain and susceptibility of all isolates to selected antibiotics are provided in Table **2**. Among all *P. aeruginosa, A. baumannii, S. aureus, E. coli, K. pneumoniae* and *S. typhimurium* samples 30, 30, 30, 30, 30 and 25 isolates, respectively, were selected with the highest antibiotic resistance pattern.

Antibiotic MICs Determination

In our previous studies, MICs of CM11 peptide for clinical isolates of seven pathogenic bacteria were demonstrated in same ranges of inhibitory values; so far MIC_{90} for all of the clinical isolates was 8 mg/L. In this study, in order to evaluate the synergistic effect of peptide in combination with antibiotic agents , we determined the MIC of antibiotics for each strain based on CLSI standards using broth microdilution method. Resistant rate of isolates for each strain and related antibiotic was obtained based on the ratio of resistant isolates to all isolates. Results are summarized in Table **3**.

Synergistic Effect Between CM11 Peptide and Antibiotics

In order to determine whether antibiotics and CM11 peptide can interact and have an enhanced antibacterial effect, we used checkerboard analysis. The CM11 peptide interaction with antibiotics was evaluated by FICI. Our results (Table **4**) showed a positive effect between peptide and most antibiotics. Generally, for *S. aureus* isolates partial synergy interaction was observed between peptide and rifampicin, ciprofloxacin, norfloxacin, oxacillin and gentamicin antibiotics while interaction between peptide and penicillin was synergic (MIC_{PEN}: 256 → 64 µg/ml and $MIC_{Peptide}$: 8 → 2 µg/ml). Also, partial synergy effect was observed against *P. aeruginosa* for ciprofloxacin and norfloxacin in combination with CM11 peptide while ceftazidime showed dual effect in different concentrations as partial synergy (MIC_{CAZ}: 2048 → 128 µg/ml and $MIC_{Peptide}$: 16 → 8 µg/ml) and synergy

Table 2. Antibiotic resistance rates of clinical isolates for each bacteria specie.

Antibiotics**	Antibiotic resistance rate of clinical isolates [a] (%)*					
	P. aeruginosa (N:50)	*A. baumannii* (N:50)	*S. aureus* (N:50)	*E. coli* (N:50)	*K. pneumoniae* (N:50)	*S. typhimurium* (N:35)
Ampicillin	NA[b]	(41) >80	NA	(46) >90	100	(18) >50
Piperacillin	(36)[c] >70	(43) >85	NA	(3) >5	(31) >60	(23) >65
Penicillin	NA	NA	(46) >90	NA	NA	NA
Oxacillin	NA	NA	(46) >90	NA	NA	NA
Cefalotin	NA	NA	NA	(48) >95	(48) >95	(8) >20
Cefotaxime	NA	(46) >90	NA	(43) >85	(41) >80	(8) >20
Ceftazidime	(31) >60	(43) >85	NA	(41) >80	(43) >85	(9) >25
Imipenem	(38) >75	(46) >90	NA	(41) >80	(41) >80	0
Norfloxacin	(41) >80	NA	(41) >80	(43) >85	(41) >80	NA
Ciprofloxacin	(41) >80	(43) >85	(38) >75	(46) >90	(43) >85	0
Rifampicin	NA	NA	(36) >70	NA	NA	NA
Amoxiclav	NA	NA	NA	(23) >45	(46) >90	(22) >60
Gentamicin	(45) 90	(43) >85	(41) >80	(37) 75	(28) >55	0
Amikacin	(33) >65	(31) >60	NA	(26) >50	(26) >50	(4) >10
Kanamycin	NA	(48) >95	(41) >80	(31) >60	NA	(20) >55
Vancomycin	NA	NA	0	NA	NA	NA

* The data in each column represents three independent experiments ($p<0.05$).

** Specific antibiotics were selected according to Clinical and Laboratory Standards Institute guidelines

[a] Isolates were selected among samples which randomize received from three clinical diagnostic laboratories.

[b] Ineffective antibiotics for each bacterium indicated with ''NA'' (not applicable).

[c] Number of isolates that were resistance to related antibiotic.

(MIC_{CAZ}: 2048 → 512 µg/ml and $MIC_{Peptide}$: 16 → 2 µg/ml). Combination of gentamicin and amikacin with peptide showed no action (activity) ($MIC_{Antibiotic}$: Up to 2048 µg/ml). For *K. pneumoniae*, synergistic effect was observed when CM11 peptide was used in combination with norfloxacin against bacterium (MIC_{NOR}: 64 → 16 µg/ml and $MIC_{Peptide}$: 8 → 2 µg/ml). This activity was as partial synergy for cefotaxime and ceftazidime while indifference action was for ciprofloxacin and ampicillin but gentamicin showed no action. Although a synergic effect against *A. baumannii* was found regarding to the combination of peptide with norfloxacin (MIC_{NOR}: 1024 → 256 µg/ml and $MIC_{Peptide}$: 8 → 2 µg/ml), but a partial synergism was found for ciprofloxacin, ceftazidime and gentamicin while ampicillin did not show any effect. Synergistic effect between CM11 peptide and selective antibiotics against *E. coli* was observed as "synergy" and "partial synergy" for ciprofloxacin (MIC_{CIP}: 1024 → 256 µg/ml and $MIC_{Peptide}$: 8 → 2 µg/ml & MIC_{CIP}: 1024 → 32 µg/ml and $MIC_{Peptide}$: 8 → 4 µg/ml), "partial synergy" for gentamicin and cefotaxime; and indifferent action with ampicillin. Our combination analyses on *S. typhimurium* also showed that peptide in combination with cefotaxime and ceftazidime provided a "partial synergy" (MIC_{CTX}: 1024 → 256 µg/ml and $MIC_{Peptide}$: 16 → 8 µg/ml & MIC_{CAZ}: 128 → 8 µg/ml and $MIC_{Peptide}$: 16 → 8 µg/ml activity while with ampicillin no synergism(effect) was not observed. However these results showed that synergistic effect between the CM11 peptide and most selected antibiotics is in the range of "partial synergism" (Table **4**).

Bacterial Killing Assay

Viable counts of clinical isolate of *P. aeruginosa* and *S. aureus* treated with specific antibiotics and CM11 peptide as alone and in combination at MIC concentrations, are displayed graphically in the Fig. **1** & **2**. It is noteworthy that peptide MICs for *P. aeruginosa* and *S. aureus* isolate were 16 and 8 mg/L, respectively. According to our previous study, the elimination process of *P. aeruginosa* and *S. aureus* by peptide was completed after a 30 min exposure period, but in FIC concentrations, peptide alone did not lead to decrease the colony counts however growth rate in time period was slow in comparison with controls. This result was observed for antibiotics on bacteria samples but gentamicin showed a reduction effect on viable counts of *S. aureus* (Fig. **2D**).

Table 3. MIC ranges of selected antibiotics against clinical isolates of six pathogenic bacteria.

Antibiotics	MIC Ranges (mg/L) *					
	P. aeruginosa	*A. baumannii*	*S. aureus*	*E. coli*	*K. pneumoniae*	*S. typhimurium*
AM	---	128 - 4096	---	64 - 2048	16 - 4096	16 - 4096
PEN	---	---	64 - 256	---	---	---
OX	---	---	4 - 2048	---	---	---
CTZ	256 - 2048	---	---	16 - 128	32 - 512	16 - 256
CTX	---	64 - 1024	---	64 - 2048	16 - 4096	8 - 1024
NOR	32 - 256	64 - 1024	16 - 128	---	16 - 64	4 - 8
CIP	32 - 128	32 - 1024	8 - 64	8 - 1024	32 - 256	4 - 8
RIF	---	---	32 - 1024	---	---	---
GEN	64 - 2048	64 - 2048	32 - 128	32 - 512	32 - 2048	---
AN	32 - 2048	---	---	---	---	---

* The data in each column represents three independent experiments ($p<0.05$).

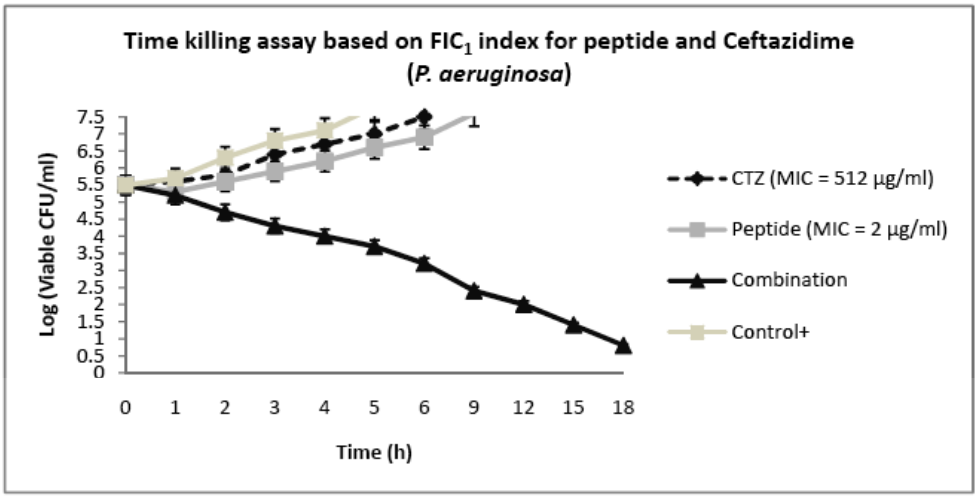
Table 4. FIC index for CM11 peptide in combination with antibiotics tested against clinical isolates of six pathogenic bacteria.

Antibiotics	The FIC index *					
	P. aeruginosa	*A. baumannii*	*S. aureus*	*E. coli*	*K. pneumoniae*	*S. typhimurium*
AM	---	Non action***	---	1	Non action	Non action
PEN	---	---	0.475	---	---	---
OX	---	---	0.75_{FIC1} 0.515_{FIC2}	---	---	---
CTZ	0.325_{FIC1} 0.562_{FIC2}	---	---	0.75	0.75	0.562
CTX	---	0.75_{FIC1} 0.625_{FIC2}	---	1	0.75_{FIC1} 0.625_{FIC2}	0.75
NOR	0.625	0.5	0.75	---	0.5	**
CIP	0.75_{CIF1} 0.625_{CIF2}	$0.75_{FIC1\&2}$	0.625	0.652_{FIC1} 0.5_{FIC2} 0.531_{FIC3}	1	**
RIF	---	---	0.531	---	---	---
GEN	Non action	$0.75_{FIC1\&2}$	0.75	0.75_{FIC1} 0.562_{FIC2}	Non action	---
AN	Non action	---	---	---	---	---

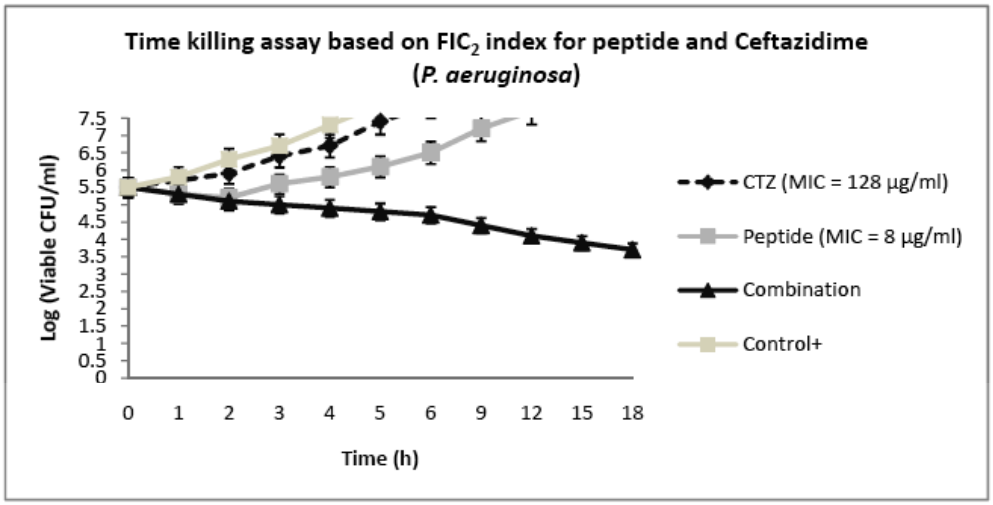
* The data in each column represents three independent experiments ($p<0.05$).

** Antibiotics with low MICs against *S. typhimurium* isolates (unsuitable for checkerboard analysis).

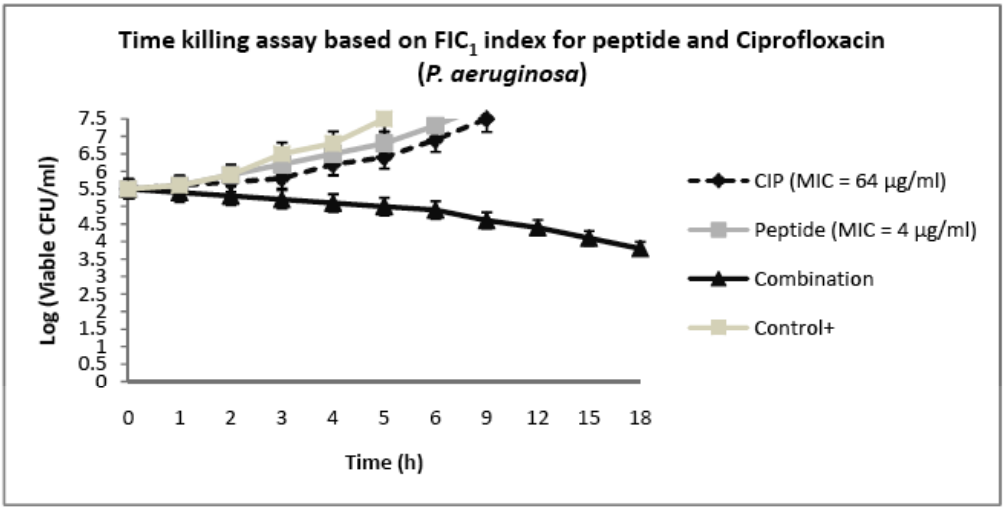
*** Non combination effect was detected in peptide and antibiotic MIC range.


Time killing assay based on FIC1 index for peptide and Ceftazidime
(P. aeruginosa)
Log (Viable CFU/ml)
Time (h)
CTZ (MIC = 512 µg/ml)
Peptide (MIC = 2 µg/ml)
Combination
Control+

1A


Time killing assay based on FIC2 index for peptide and Ceftazidime
(P. aeruginosa)
Log (Viable CFU/ml)
Time (h)
CTZ (MIC = 128 µg/ml)
Peptide (MIC = 8 µg/ml)
Combination
Control+

1B


Time killing assay based on FIC1 index for peptide and Ciprofloxacin
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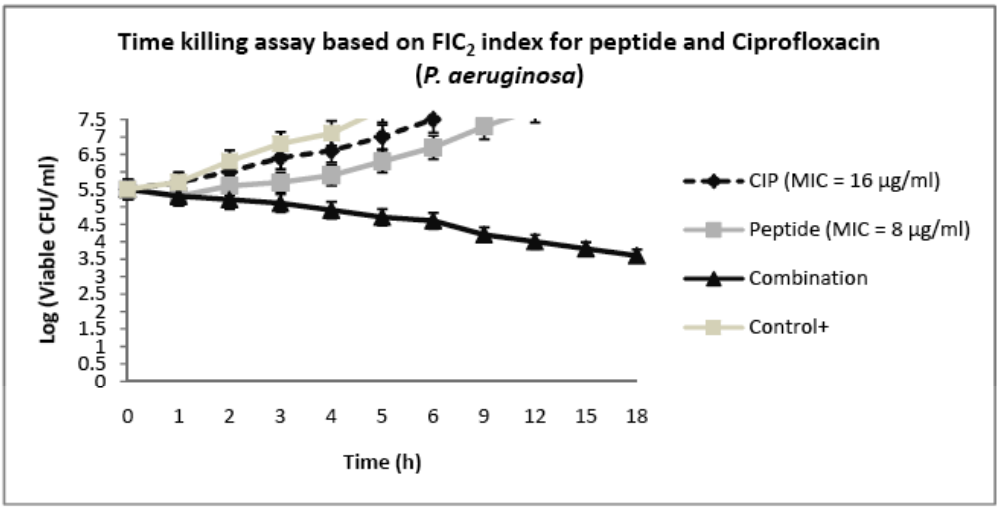
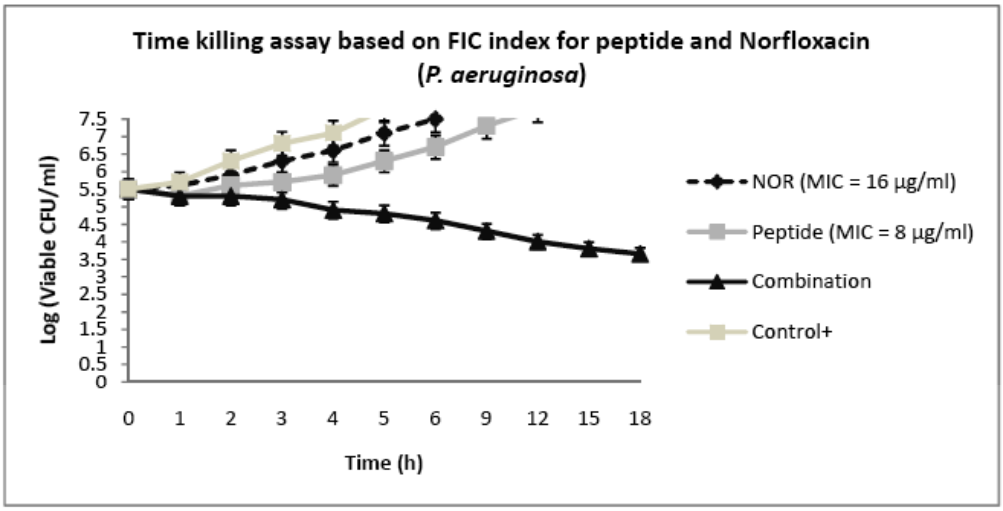
Log (Viable CFU/ml)
Time (h)
CIP (MIC = 64 µg/ml)
Peptide (MIC = 4 µg/ml)
Combination
Control+

1C



1D



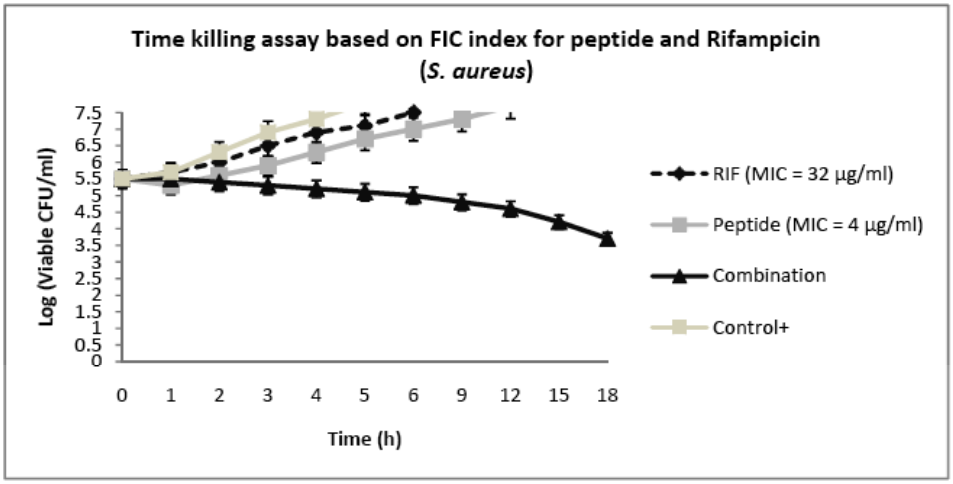
1E

Figure 1. Time-killing assessment for *Pseudomonas aeruginosa* isolate, after treatment of bacterium with Ceftazidime (**1A** & **1B**), Ciprofloxacin (**1C** & **1D**), Norfloxacin (**1E**) antibiotics and CM11 peptide alone and in combination at a concentration equal to MIC (alone) and MIC_{FIC} (in combination). For Ceftazidime and Ciprofloxacin, two FIC were observed that shown with FIC_1 and FIC_2. Synergy was defined as a ≥ 2 $\log_{10}$ decrease in colony count by the combination, compared with the most active single agent. Horizontal and vertical axes show the killing time and the percentage of bacterial survival, respectively. Survival counts were performed three times in different days, and the means and the standard deviations indicates statistically significant difference survival rates ($p<0.05$).

Time killing study by combination of peptide and antibiotics showed decline in colony counts of *P. aeruginosa* (Fig. **1**). Based on results, decrease in colony counts for ceftazidime in FIC_1 (Fig. **1A**) and FIC_2 (Fig. **1B**) were > 2 $\log_{10}$ (synergism) and < 2 $\log_{10}$ (indifference) respectively in comparison with control. Also for ciprofloxacin and norfloxacin decline in colony counts was < 2 $\log_{10}$ (Fig. **1C**, **1D** & **1E**). Time kill results for *S. aureus* showed indifference effect for combination of peptide with rifampicin (Fig. **2A**), Oxacillin (Fig. **2B** & **2C**), ciprofloxacin (Fig. **2F**), norfloxacin (Fig. **2G**) in FICs. However, combination of peptide with gentamicin (Fig. **2D**) and penicillin (Fig. **2E**) showed synergic effect on *S. aureus*.

DISCUSSION

The boundless utilization of antibiotics has prompted the emergence of various antibiotic-resistant bacteria and an urgent need for new antimicrobial agents [31]. Among the new pharmacologic products agents and as important members of the host defense system in eukaryotes with broad spectrum activity against many Gram-positive and Gram-negative bacteria, antimicrobial peptides are regarded as suitable candidates for treatment of bacterial infections. However, practical use of AMPs has its own limitations such as sensitivity to proteases and pH variations, low biological activity and so on. To overcome these limitations, there are a number of methods including the design and synthesis of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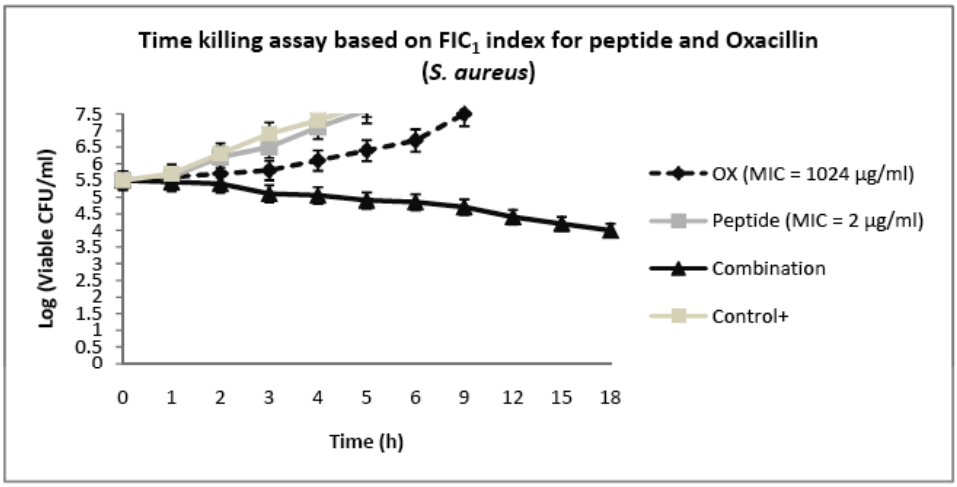
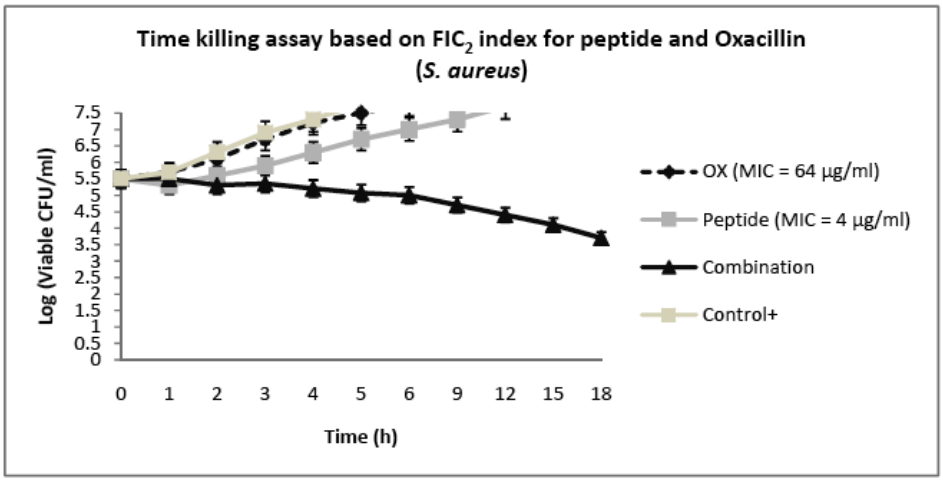
Log (Viable CFU/ml)
Time (h)
RIF (MIC = 32 µg/ml)
Peptide (MIC = 4 µg/ml)
Combination
Control+

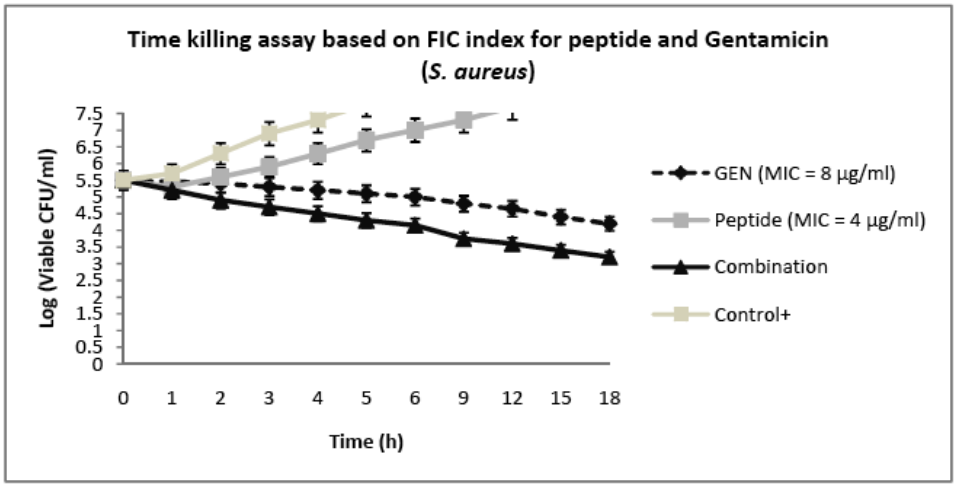
2A


Time killing assay based on FIC1 index for peptide and Oxacillin
(S. aureus)
Log (Viable CFU/ml)
Time (h)
OX (MIC = 1024 µg/ml)
Peptide (MIC = 2 µg/ml)
Combination
Control+

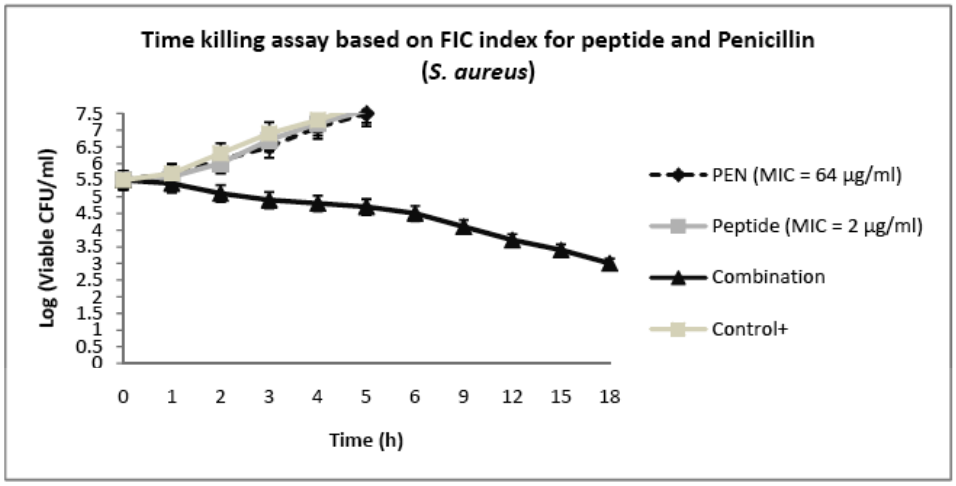
2B


Time killing assay based on FIC2 index for peptide and Oxacillin
(S. aureus)
Log (Viable CFU/ml)
Time (h)
OX (MIC = 64 µg/ml)
Peptide (MIC = 4 µg/ml)
Combination
Control+

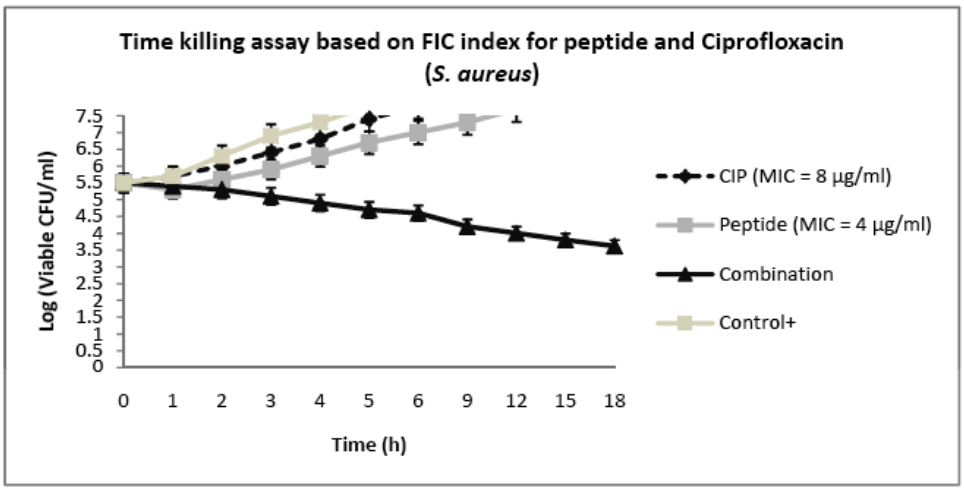
2C


Time killing assay based on FIC index for peptide and Gentamicin
(S. aureus)
Log (Viable CFU/ml)
Time (h)
GEN (MIC = 8 µg/ml)
Peptide (MIC = 4 µg/ml)
Combination
Control+

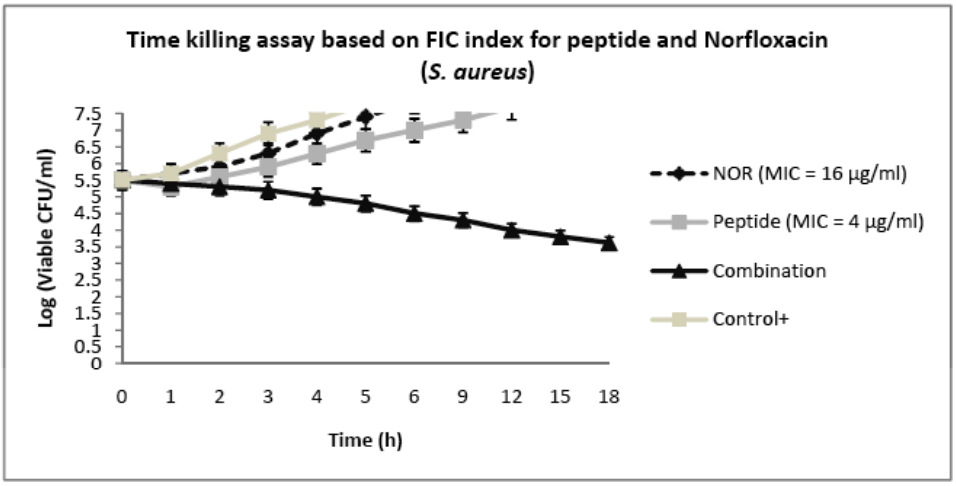
2D


Time killing assay based on FIC index for peptide and Penicillin
(S. aureus)
Log (Viable CFU/ml)
Time (h)
PEN (MIC = 64 µg/ml)
Peptide (MIC = 2 µg/ml)
Combination
Control+

2E


Time killing assay based on FIC index for peptide and Ciprofloxacin
(S. aureus)
Log (Viable CFU/ml)
Time (h)
CIP (MIC = 8 µg/ml)
Peptide (MIC = 4 µg/ml)
Combination
Control+

2F



2G

Figure 2. Time-killing assessment for *Staphylococcus aureus* isolate, after treatment of bacterium with Rifampicin (**2A**), Oxacillin (**2B** & **2C**), Gentamicin (**2D**), Penicillin (**2E**), Ciprofloxacin (**2F**), Norfloxacin (**2G**) antibiotics and CM11 peptide alone and in combination at a concentration equal to MIC (alone) and MIC_{FIC} (in combination). For Oxacillin, two FIC were observed that shown with FIC_1 and FIC_2. Synergy was defined as a ≥ 2 $\log_{10}$ decrease in colony count by the combination, compared with the most active single agent. Horizontal and vertical axes show the killing time and the percentage of bacterial survival, respectively. Survival counts were performed three times in different days, and the means and the standard deviations indicates statistically significant difference survival rates ($p<0.05$).

short AMPs with high potency and low toxicity, changes in amino acid sequence including adding, removing, replacing one or more amino acids and also modification to the amino or carboxyl termini, which may lead to a suitable peptide for therapeutic application [5]. In the meantime, synthetic and hybrid peptide analogs, such as the CM11 peptide, that are designed based on natural peptides, have been more effective with greater antimicrobial effects and low toxicity in compression with the parent peptides [21, 25, 32]. On the other hand, it is noteworthy that unlike most small-molecule antibiotics, AMPs have been refined by evolution to work synergistically within the host environment. The mechanisms of synergy are complex and specific, and in recent years some progress has been done in order to better understanding of these mechanisms. Thus, antimicrobial peptides are powerful candidates to broaden our limited therapeutic arsenal, and are very well suited to be used in synergic combinations with currently available antibiotics [33].

In general, the synergy between two antimicrobial drugs is obtained when the combination of drugs elicits a more than additive effect as opposed to each drug alone. Its clinical importance resides in providing an additional therapeutic choice in difficult-to-treat infections, and it is also beneficial when optimal concentrations of a single antibiotic cannot be reached, for example, because of the infection site or toxicity. These drug combinations have also been successfully employed to overcome antibiotic resistance in selected cases.

AMPs have been vigorously utilized as a part of collaboration studies, fundamentally on account of the very limited number of classes of new antibacterial drugs but additionally because of the presence of a number of 'old', licensed AMPs on the sidelines. Generally, many AMPs are perfectly suited for using in synergic combinations as they function on different targets than most conventional antibiotics, and in some cases they impair the activity of antimicrobial-inactivating enzymes and re-establish full activity for the complementary drug [29, 33, 34]. According to these topics in this study, we analyzed synergistic effect of the CM11 peptide in combination of prevalent antibiotics against some important hospital pathogenic isolates of bacteria. Combination studies showed that the synergism is present between peptide/penicillin and ceftazidime for *S. aureus* and *P. aeruginosa*, respectively. For *K. pneumoniae*, this effect was observed when CM11 peptide was used in combination with norfloxacin and also the combination of peptide/norfloxacin and ciprofloxacin showed synergy effect against *A. baumannii* and *E. coli*, respectively, while only partial synergy was observed for *S. typhimurium* when peptide was used in combination with cefotaxime and ceftazidime. Other combination interactions were as partial synergy and additive effect or without action.

Generally, the rising rate in antibiotic resistance makes the production of some novel antimicrobial peptides essential, however applying high dose makes most AMPs toxic. Synergistic combination of the antimicrobial peptides and the antibiotics is a method to tackle resistance and toxicity, which is used in anticancer therapy and is accepted in the treatment of infectious diseases [35]. Accordingly, a number of similar studies have been done on other cecropin or melittin hybrids, these studies indicated positive interaction between hybrid peptides and some antibiotics [18, 31, 36]. For example, Giacometti et al., (2003) [37] investigated antibacterial activity a cecropin A–melittin hybrid peptide [CA(1–7)M(2–9)NH_2] or CM15 peptide, alone and in combination with various clinically used antimicrobial agents against nosocomial isolates of *Acinetobacter baumannii*. In this study Co-amoxiclav, ceftazidime, piperacillin, imipenem,

ciprofloxacin and netilmicin were tested in combination with CM15 peptide.In case of β-lactam antibiotics combined with the peptide, the synergy was observed, while the partial synergy and the indifference combination effect were shown in case of ciprofloxacin and netilmicin, respectively. In another study Jeong et al., (2009) [30] investigated the antimicrobial activity and synergistic effect of a 20-residue hybrid peptide CA(1-8)-MA(1-12) (CAMA) incorporating residues 1-8 of cecropin A (CA) and residues 1-12 of magainin 2 (MA) and a hybrid peptide analogue (CAMA-syn) was designed with substitutions of Ile^{10} and Ser^{16} with Lys on some Gram-negative and positive bacteria. This study showed CAMA-syn has antimicrobial activity similar with CAMA but without hemolytic activity and much lower cytotoxicity. The peptide/antibiotic combination studies with selected antibiotics (Naringenin, 3, 6-dihydroxy flavones and YKAs3001) showed that CAMA and CAMA-syn have synergistic effects with synthetic compound and flavonoid (3, 6-dihydroxy flavones and YKAs3001) against *Enterococcus faecalis* and vancomycin-resistant *E. faecalis*. One mechanism of reduced antimicrobial activity and bacterial resistance to common antibiotics is low membrane permeability to antimicrobial agents, so they examined the synergistic effects of CAMA and CAMA-syn to aid membrane permeability of antimicrobial natural compounds such as flavonoids. Their results suggested that the cationic antimicrobial peptides can aid antimicrobial agents to permeate the bacterial membrane. On the other hand, narigenin showed no synergistic effects with any peptide against bacterial cells and it can be proposed that their low antimicrobial activities have no relation with membrane permeability.

Based on this topic, Iwasaki et al., studied the mechanism of interaction between antimicrobial peptides and common antibiotic. They showed that peptides increases the permeability of membrane causing antibiotics penetrates more quickly, so the permeabilization of the outer membrane, allowing access of antimicrobial agents across this barrier, might explain this positive interaction [18, 31]. In another reports, Giacometti et al., offered that the positive interaction of peptides and β-lactam antibiotics such as piperacillin, ceftazidime, meropenem may be due to the simultaneous effect of the peptide and the antibiotic on peptidoglycan. This means that the peptide might act by inserting into the cytoplasmic membrane and triggering the activity of bacterial murein hydrolases, resulting in damage or degradation of the peptidoglycan and lysis of the bacteria cell. In addition, the peptide might increase the access of the β-lactam antibiotics to the cytoplasmic membrane following breakdown of peptidoglycan [37, 38]. In general, these observations are also valuable because by reducing the concentration of antibiotic in treatment of bacterial infections cause to decrease the resistance of bacteria [39]. It should be noted that although clinical strategies often favor synergistic interactions because they maximize the rate at which the infection is cleared from an individual, but it is unclear how such interactions affect the evolution of multi-drug resistance. Based on some studies, a number of scientists propose that a lower dose of antibiotics might actually favor the development of resistance. Accordingly, synergy has dual conflicting effects: it clears the infection faster and thereby decreases the time during which resistant mutants can arise, on the other hand, increases the selective advantage of these mutants over wild-type cells. These studies suggest that the optimal strategy for suppressing multi-drug resistance is not always to maximize synergy, and that in some cases drug antagonism, despite its weaker efficacy, may better suppress the evolution of multi-drug resistance [40, 41]. Therefore, while presenting clinical knowledge generally favors drug synergistic strategies, but the possibility of associated risks such as evolution of multi-drug resistance must also be considered.

Finally, our results suggest that for some antibiotics, the dose of antibiotic in therapy of drug resistant bacteria could be significantly reduced if used in combination with this peptide and the peptides may be useful to prevent increasing occurrence of antibiotic bacterial pathogens. Furthermore, the combination approach can enable use of lower concentrations of peptide and restore the effectiveness of antibiotics.

CONFLICT OF INTEREST

The authors confirm that this article content has no conflicts of interest.

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