



# Synthetic Short Cryptic Antimicrobial Peptides as Templates for the Development of Novel Biotherapeutics Against WHO Priority Pathogen

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## Abstract

**Background** The emergence of multidrug-resistant pathogens through excessive and indiscriminate use of antibiotics, together with the lack of highly efficient treatment options for bacterial infections, has raised the development of novel antimicrobial agents to top priority. In this context, cryptic host defense peptides (cHDPs) are being explored as a novel class of antimicrobial agents. In this study, short peptides were designed from the long nonantibacterial protein ToAP2 and analysed for their positive net charge, hydrophobicity, hydrophobic moment, hydrophobic and hydrophilic planes.

**Methods** From the designed fragments, five 15 amino acid fragments were synthesised by solid-phase peptide synthesis (SPPS) and analysed for antimicrobial activity against ESKAPE pathogens. All the peptides were subject to cytotoxicity, mode of action, structure and function studies to find the best template for further optimisation.

**Results** Among them, two peptides, FKL15 and SKL15, showed better efficiency in killing *P. aeruginosa* under physiological salt and plasma conditions with no cytotoxicity issues. Further, the peptides destroyed the bacterial membranes and adopted a random coil structure in the presence of the bacteria.

**Conclusions** The data indicates that FKL15 and SKL15 are promising antimicrobial peptides against antibiotic-resistant bacteria with great potential to develop as drugs with high economic value.

**Keywords** Host defense peptides · Eskape pathogens · *P. aeruginosa* · Peptide templates · Cell membrane · Toxins · Scorpion

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## Introduction

Antimicrobial agents have made a significant impact on human life, and their success is being threatened due to the emergence of antimicrobial resistance (AMR) in pathogens. Among all, AMR in the ESKAPE pathogens (*Enterococcus faecium*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Acinetobacter baumannii*, *Pseudomonas aeruginosa*, and *Enterobacter spp*) is of great concern for clinicians around the world, as they are part of the normal microflora and opportunistic pathogens (e.g., illness, surgery, transplantation, cancer) (Chang et al. 2022; Serapide et al. 2022). Given the high incidence of AMR in the ESKAPE pathogens around the globe, the World Health Organisation has declared them as critical priority pathogens against whom a new class of antibacterials are needed. The current therapeutic regime, to treat ESKAPE pathogen-associated infections involves the use of antibiotic combinations at high concentrations (Das et al. 2022; Oliveira et al. 2020). However, this excessive or improper use of antibiotics has further led to the development of multidrug-resistant pathogens.

Currently, there is renewed interest in discovering antimicrobial agents, from natural sources, as these molecules are designed and optimised by natural selection (Li et al. 2022a; Rocha and Ferreira 2022; Sarkar et al. 2021). Among the vast repertoire of natural products, toxins and venoms represent a unique group consisting of a variety of bioactive substances ranging from peptides, lipids, enzymes, amines, etc. (Bosso et al. 2020; Luo et al. 2022; Roque-Borda et al. 2022).

Recently, some researchers have started to explore the unique proteins and peptides in the toxins, which have the potential to inhibit and control the menace created by antimicrobial resistance pathogens (Sup Table 1). Interestingly, Scorpion venom's major bioactive protein components share physicochemical properties, such as cationic nature and structural flexibility, with HDPs (Amorim-Carmo et al. 2022). HDPs are short (~50 amino acids), amphipathic, hydrophobic, evolutionary conserved molecules found in all living organisms with a net cationic charge of +2 to +9. HDPs are expressed either constitutively or during the inflammatory response to infection in the host. HDPs act by permeabilizing bacterial membranes and simultaneously blocking the over-activation of inflammatory molecules by binding to factors like LPS, and exotoxins (Greve and Cowan 2022; Valenti et al. 2022).

Given this, we decided to further explore the short sequence variant of peptide ToAP2 (FFGTLFKLGSKLIP-GVMKLFSKKKER) obtained from the venomous glands of scorpion *Tityus obscurus*. ToAP2 is a 26 amino acid long peptide, having a +6 net positive charge, 42% hydrophobicity, and a propensity to form an  $\alpha$ -helical structure. ToAP2

and its sequence similar C<sub>ss</sub>54 (FFGSLLSLGSKLLPS-VFKLFQRKKE) demonstrated antifungal activity (Guilhelmelli et al. 2016; Park et al. 2020, 2024).

Interestingly, we and others have reported that proteolytic cleavage of the homeostasis-related protein leads to the generation of cryptic host defense peptides (De Oliveira Costa and Franco 2020; Horam et al. 2019; Lata et al. 2023; Papareddy et al. 2010; Pizzo et al. 2018; Raj et al. 2021). Strangely, cryptic HDPs also demonstrate functions similar to those of the natural ones like antimicrobial, antiviral, immunomodulatory etc. (De Oliveira Costa and Franco 2020; Pizzo et al. 2018). Given their bioactive nature, flexibility for modification with natural and unnatural amino acids, cryptic HDPs are regarded as one of the most promising alternative sources for novel agents.

In this work, we have tried to develop a novel 15 amino acids long cryptic peptide from the scorpion *Tityus obscurus* toxin for potential broad-spectrum antimicrobial activity against the ESKAPE pathogens and assessed the shorter peptides antibacterial activity, toxicity, proteolytic stability, and mechanism of action against 4 bacterial species of the ESKAPE group. Among them, FKL15 and SKL15, shorter than parent ToAP2, exhibited antibacterial activity against Gram-negative bacteria *P. aeruginosa* without any significant cytotoxicity towards Macrophages (THP-1) and Microglia (BV-2) cells. We here go on to demonstrate the antibacterial potential of the short variant of scorpion venom peptide, ToPA2 for the first time.

## Methodology

### Materials

All the peptides and LL-37 (LLGDFFRKSKEKIGKEFKRIVQRIKDFLRNLPVPTES) used in this work were synthesized by Fmoc solid-phase synthesis protocol and obtained from Genscript, USA. The purity (>95%) of the peptides was confirmed by mass spectral analysis (MALDI-TOF) before diluting in H<sub>2</sub>O (5 mM stock). This stock solution was stored at -20° C, until use. The microbes used in the present study were *K. pneumoniae* ATCC 27,736, *A. baumannii* ATCC 1605, *P. aeruginosa* ATCC 25,668 and *S. aureus* ATCC 29,213 were obtained from ATCC, USA.

### In Silico Analysis of Peptides

The peptide sequence of ToAP2, a scorpion (*Tityus obscurus*) toxin was obtained from the Antimicrobial Peptide Database (APD, <https://aps.unmc.edu/>). The amino acid framework of this peptide was then used to generate 15 amino acid long cryptic analogues, which correspond to the

average AMP length. For each designed fragment the positive net charge, hydrophobicity, and hydrophobic moment were calculated using APD's prediction tools. Further, the distribution pattern of the amino acids in hydrophobic and hydrophilic planes was deduced by preparing helical wheel projections of peptides using HeliQuest (<https://heliquet.ipmc.cnrs.fr/cgi-bin/ComputParams.py>) and their secondary structures was modelled using the PEP-FOLD3 server (<https://bioserv.rpbs.univ-paris-diderot.fr/services/PEP-FOLD3/>).

### Radial Diffusion Assay (RDA)

RDA assay was carried out essentially as described elsewhere (Horam et al. 2019; Setty et al. 2017). Briefly, test bacteria were grown to the mid-logarithmic phase in 10 mL of 3% w/v of tryptic soy broth (TSB). Bacteria were centrifuged at 10,000 RPM for 10 min, the supernatant was discarded and the pellet was resuspended in 10 mM Tris-HCl pH 7.4 to get  $1-2 \times 10^9$  CFU/mL of the bacterial suspension. After washing, bacteria were added to 15 mL of underlay agarose gel (0.03% (w/v) TSB, 1% (w/v) low electroendosmosis (EEO) type agarose and 0.02% (v/v) Tween 20) such that the final concentration of the bacteria reaches  $4 \times 10^6$  CFU/mL and poured into a 140 mm Petri plate. After solidification, 4 mm diameter holes were punched, followed by the addition of 6  $\mu$ L peptide solution of different concentrations (25, 50, and 100  $\mu$ M). Petri plates were incubated for 3 h to allow diffusion of peptides into the gel, followed by pouring of 15 ml of overlay gel. Further petri plate with bacteri and peptide incubation was done for 16–24 h at 37 °C. The antimicrobial activity of peptide was visualized as a zone of inhibition around each well and was measured in mm.

### Minimum Inhibitory Concentration (MIC)

Peptides were screened for their in vitro antibacterial activity against reported Gram-positive and Gram-negative pathogenic bacterial strains by broth micro-dilution method as per CLSI guidelines (formerly known as NCLS) as reported elsewhere (Wiegand et al. 2008). Bacteria were grown overnight in 3% TSB to mid-logarithmic phase, centrifuged and washed two times in 10 mM Tris buffer, and re-suspended in 2X of cation adjusted MHB broth (Becton Dickinson, USA) to get  $1 \times 10^5$  CFU/mL. Serially diluted peptide concentration (160  $\mu$ M to 0.31  $\mu$ M) was added to each corresponding well of a 96-well microtiter plate (polypropylene, Costar Corp.) followed by the addition of 50  $\mu$ L of 2X of cation-adjusted MHB broth containing  $1 \times 10^5$  CFU/mL bacteria and incubated at 37° C for 16 h. MIC values were determined by mere visual inspection of plates and that value

was considered where growth inhibition observed was greater than 90%. MIC determination and the assay were performed in the triplicate. In each assay, a negative control lacking peptide addition is included, alongside LL-37 peptide serving as the positive control.

### Viable Count Assay (VCA)

VCA was done as described previously (Horam et al. 2019; Lata et al. 2023). Bacteria of interest were grown in 10 mL of TSB and incubated at 37 °C until the mid-logarithmic phase. After washing with 10mM Tris HCl buffer, the bacteria pellet was re-suspend in 10mM Tris HCl buffer containing 10 mM glucose to get  $1 \times 10^9$  CFU/mL bacterial suspension. The culture was further diluted to 1000 X in 10mM Tris HCl containing 10 mM glucose, and incubated in the presence of peptides at the indicated concentration for 2 h at 37 °C. After treatment with the peptides, the samples were serially diluted and plated on Todd Hewitt (TH) medium and incubated for 16–18 h at 37 °C. To assess peptide activity under physiological conditions,  $1 \times 10^9$  CFU/mL bacterial suspension was added to 10 mM Tris HCl + 10 mM glucose + 0.15 M sodium chloride or 10 mM Tris HCl + 10 mM glucose + 20% citrate human plasma. Samples without peptide treatment were considered to represent 100% survival under same test conditions. Mean  $\pm$  SD values from three biological replicates were used for drawing conclusions.

### Hemolysis Assay

For the hemolysis assay, blood was collected from a human volunteer in a vacutainer containing anticoagulant EDTA (BD Vacutainer® EDTA Tubes, 367864) and centrifuged at 800 g for 10 min (Raj et al. 2021). The plasma and buffy coat were removed and erythrocytes were then washed three times in PBS, pH 7.4 and re-suspended in the same (5% suspension) after which they were incubated with end-over-end rotation for 1 h at 37° C in the presence of peptides (50 and 100  $\mu$ M). The samples were then centrifuged at 800 g for 10 min. The hemoglobin released in the supernatant was measured at 540 nm by transferring 100  $\mu$ L of supernatant in 96 well plate and expressed as % of Triton X-100 induced hemolysis. 2% Triton X-100 (Sigma-Aldrich, St. Louis, USA) was taken as the positive control.

The use of human blood was approved by the CSIR-CDRI human ethical Committee (CDRI/IEC/2019/A1).

Hemolysis (%) was calculated according to the following equation:

$$\text{Hemolysis (\%)} = \frac{[(\text{treated sample} - \text{blank}) / (\text{triton X100} - \text{blank})] \times 100}{100}$$

## Protease Sensitivity Assay

To investigate the stability of peptides against the protease of both bacterial as well as the human origin, Protease stability studies were done as described elsewhere (Setty et al. 2017). Briefly, test peptides (1 µg) were incubated at 37 °C with V8 protease (0.1 µg 25,000 units/mg), Proteinase K and Human neutrophil elastase (0.4 µg, 29 units/mg), in a total volume of 30 µl for 3 h. After the incubation, the reaction was terminated by heating the sample in 4X lammelic buffer at 100 °C for 5 min, and the degradation of the peptide was analyzed on 16.5% precast SDS-PAGE Tris-Tricine gels after staining with Coomassie Blue R-250, followed by destaining.

## MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide) Assay

To assess the effect of peptides on the cells, MTT assay was performed (Gondru et al. 2021; Raj et al. 2021). BV-2 and THP-1, 3000 cells/well were grown in presence of 10% FBS till the 80% confluence stage in 96 well plates, followed by peptide (50 and 100 µM) addition. After 24 h of incubation at 5% CO<sub>2</sub> and 37 °C, 20 µL of filter-sterilized MTT (5 mg/mL in PBS) was added to each well and plates were further incubated for 4 h. The MTT-containing medium was removed by aspiration and blue formazan was dissolved in 100 µl of DMSO. Then plates were swirled gently for 10 min at room temperature to completely dissolve formazan followed by measuring the absorbance at 550 nm. The results given here represent Mean ± SD values from triplicate measurements.

## Lactate Dehydrogenase (LDH) Assay

To complement the MTT results, an LDH assay was done (Horam et al. 2019; Lata et al. 2023), with minor modification, after the treatment of BV-2 and THP-1 cells with the desired peptide concentration (50 and 100 µM) for 24 h, the supernatant was collected by centrifugation at 2500 RPM and mixed with LDH-based Roche cytotoxicity kit reagents as per the manufacturer's instructions. The LDH release from 2% Triton X-100 was considered as the positive control (100% LDH release). The results represented here is the mean + SD from three independent experiments.

## Liposome Preparation and Leakage Assay

Large unilamellar vesicles (LUV's) were prepared using the freeze-thaw method. Anionic lipids phosphatidylglycerol (PG), phosphatidylserine (PE) in 3:1 ratio or zwitterionic phosphatidylcholine (PC) (1:4). Subsequently, 10 mM Tris

buffer, pH 8 was added together with calcein (8 mg) and left on a shaker overnight. After five freeze-thaw cycles with liquid nitrogen and heating up to 60 °C in the water bath, lipid suspensions were extruded 15–20 times through 100 µm polycarbonate membranes mounted in a Lipofast mini extruder (Avestin, Ottawa, Canada) at 25 °C. Untrapped calcein was removed by passing sample from Sephadex G-25 (Sigma) column three times at 25 °C with 10 mM Tris buffer, pH 8 as eluent. Calcein released from the Liposomes treated with peptides was measured by monitoring the emitted fluorescence at 520 nm (excitation at 470 nm). An absolute leakage scale was obtained by disrupting liposomes at the end of the experiment through 0.1% Triton X-100. Measurements were performed in triplicate at 25 °C by using a Tecan Infinite M Nano + spectrometer.

## CD Spectroscopy

To find out the secondary structure of peptides, circular dichroism (CD) spectra of peptides were obtained on a Jasco J-1500 Spectropolarimeter as reported else where (Horam et al. 2019; Lata et al. 2023). All measurements were performed in triplicate in a quartz cuvette (1 mm path length) at 37 °C with water, buffer and peptide. Liposomes at a concentration of 0.1 mM were monitored in the wavelength range of 190–250 nm. The background value, where no peptide signal is present was subtracted, to account for instrumental differences between measurements.

## Flow Cytometry

*K. pneumoniae* ATCC 27,736, *A. baumannii* ATCC 1605, *P. aeruginosa* ATCC 25,668 and *S. aureus* ATCC 29,213 were grown to midlogarithmic phase in 3% TSB medium, centrifuged, and washed with buffer (10 mM Tris buffer, pH 7.4) and resuspended in the same buffer to obtain  $1 \times 10^9$  CFU/mL of the bacterial suspension. The bacterial suspension was incubated with the peptide (100 µM) at 37 °C for 1 h. The bacteria were stained with 200 µl of propidium iodide (10 µM/mL) for 15 min at room temperature in dark under shaking conditions, followed by repeated washing with buffer. The pellet was resuspended in 500 µl of 10 mM Tris buffer, pH 7.4 + 10 mM glucose. For the data acquisition, FACS caliber flow cytometer was used with a gate setting for the intact bacterial cells population and data processing was done in the logarithmic mode as done earlier (Lata et al. 2023).

## Scanning Electron Microscopy

Scanning electron microscopy was done to see the membrane damaging effects of the peptides on *K. pneumoniae*

ATCC 27,736, *A. baumannii* ATCC 1605, *P. aeruginosa* ATCC 25,668 and *S. aureus* ATCC 29,213. The processing was done as stated elsewhere (Horam et al. 2019; Raj et al. 2021). Bacteria were grown, washed and treated with the peptide as stated above, After treatment with peptides, bacteria were washed with PBS three times before fixing them, with 2.5% (w/v) glutaraldehyde at 4° C overnight. After the fixing bacteria were washed twice with PBS to remove the fixative and dehydrated through a graded ethanol series (50%, 70%, 90%, and 100%) for 15 min each. The samples were dried using a critical point dryer. The dried bacterial specimens were sputtered with gold using a sputter coater, then visualized under a scanning electron microscope (FEI Quanta 250, Netherlands). The images were obtained at 20 kV using a SE detector.

## Statistics

The mean and standard deviation values obtained from the triplicate biological repeat experiments are reported here. The significance was considered at  $P < 0.05$  and the ANOVA or student t-Test was used as indicated in the figure legends. Statistical significance was done using the SigmaStat; SPSS Inc., Chicago, IL Version 14 software.

## Results

### Synthetic Peptide Design and Characterization

Shorter peptide fragments, with better or broad-spectrum activity than the parent molecule, is of great interest for developing novel and innovative bio-therapies (Ramesh et al. 2016). Usually, peptides of very large size and length are not preferred for development as potential pharmacological candidates due to their high susceptibility to proteolytic degradation, high cytotoxicity, low bioavailability, probable antigenicity, and high cost associated with their synthesis (Fosgerau and Hoffmann 2015; Zane et al. 2021). On the contrary, short peptides with reduced cytotoxicity and higher antimicrobial activity are preferred for designing variants with modifications in charge, hydrophobicity and helicity.

In this work, we aimed to develop 15 mer shorter variants from 26 amino acids long parent peptide that was previously isolated from the venomous gland of the scorpion (*Tityus obscurus*) (Guilhelmelli et al. 2016). It is demonstrated that continuous stretch of more than 3 hydrophobic amino acids leads to aggregation or gelling of peptides in the solution phase (Matsui et al. 2017; Mueller et al. 2020). Our previous studies have shown that peptides containing less than 12 amino acids demonstrate lower antimicrobial activity

(Pasupuleti et al. 2009). To avoid both issues, we decided to keep the size of the peptides to 15 amino acids in length. By using the QSAR guided *in silico* analysis as reported previously (Lata et al. 2023; Raj et al. 2021), 5 different peptide sequences of length 15 amino acids were selected based on net positive charge (+4 to +6), hydrophobicity (H), and hydrophobic movement ( $\mu$ H) and ease to form alpha-helical structure (Fig. 1A & Sup Table 2). Next, we investigated the evolutionary conservation of the hit molecules in the scorpion toxin and found that peptides FKL15, LGS15 & SKL15 sequence are highly conserved in the scorpion *sp*. It is interesting to observe that even though the species of scorpions are geographically separated (Sup Table 3), certain amino acids are highly conserved, e.g., the positively charged amino acids like lysine (Fig. 1B).

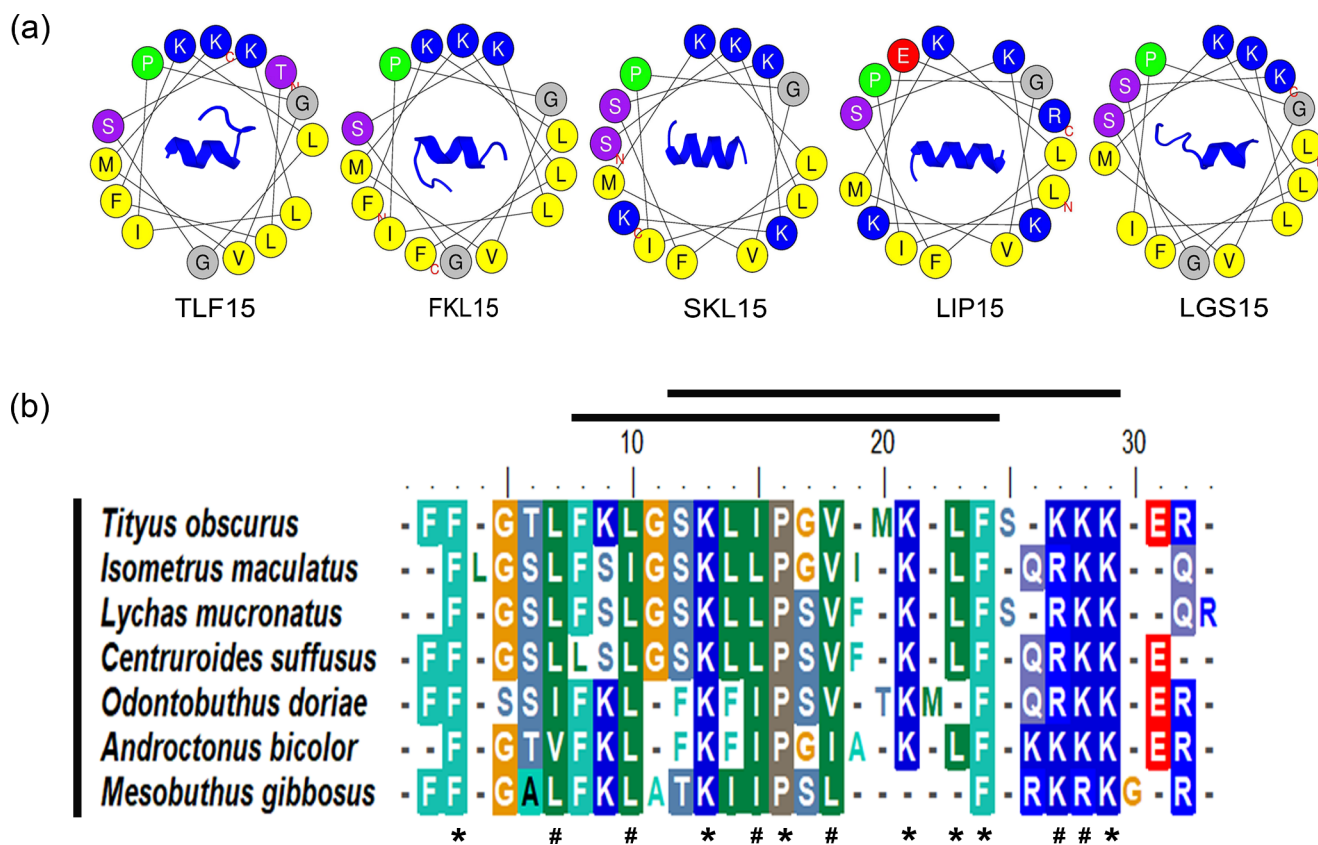
Our initial antimicrobial studies of the peptides against, *S. aureus*, *K. pneumoniae*, *A. baumannii*, and *P. aeruginosa*, showed that all the shortlisted peptides have antimicrobial activity (Fig. 2A, ). Among the peptides investigated, all of them exhibited broad-spectrum activity, in viable count assay at  $>6 \mu\text{M}$  (Fig. 2B, upper panel). To understand further the antimicrobial activity, MIC assay was done as prescribed by the NCCLS guidelines, the peptides show activity at higher concentrations (Sup Table 2) indicating that salts might antagonize the antimicrobial activity.

It is well reported in the literature that the HDPs antimicrobial activity is dependent on the microenvironment and tend to lose their activity partially or completely in the presence of high salt or plasma (Chen et al. 2021; Maharjan et al. 2022; Roshanak et al. 2021; Wang et al. 2021; Zhao et al. 2021). We, therefore, examined the influence of physiological conditions on the antimicrobial activity of the peptides. As shown in Fig. 2, peptides displayed 100% killing at 10 times higher concentrations (30  $\mu\text{M}$ ) in the presence of salt and plasma (Fig. 2B, middle and lower panel). As expected, we observed variability in the sensitivities towards the bacteria by different peptides and our results are consistent with the previous results obtained with other peptides in the presence of plasma or salt (Horam et al. 2019; Raj et al. 2021). Interestingly, LL-37 also showed the same phenomenon, i.e., an increase in the concentration. Nevertheless, more convincing data are emerging that show that the AMPs contribute to enhanced bacterial killing under *in vivo* conditions, likely through synergism or immune modifiers (Li et al. 2022b; Sandhu et al. 2022).

### Cytotoxic Activities of Peptide

Armed with this information, cytotoxicity of the peptides against THP-1 (human macrophage cell line), human erythrocytes, and BV-2 (brain microglia) were performed at 50 and 100  $\mu\text{M}$ . Results indicated that cells are healthy with a





**Fig. 1** Helical Wheel Projection Of The Peptides. **(a)** In helical wheel projections hydrophobic residues are represented as yellow, negatively charged residues are red, positively charged residues are blue, and the particular polar residue is violet. A molecular model of the peptide is

present in the center of the wheel. **(b)** Alignment of ToAP2 peptide sequence: conserved amino acids are represented with \* and identical amino acids are represented with #

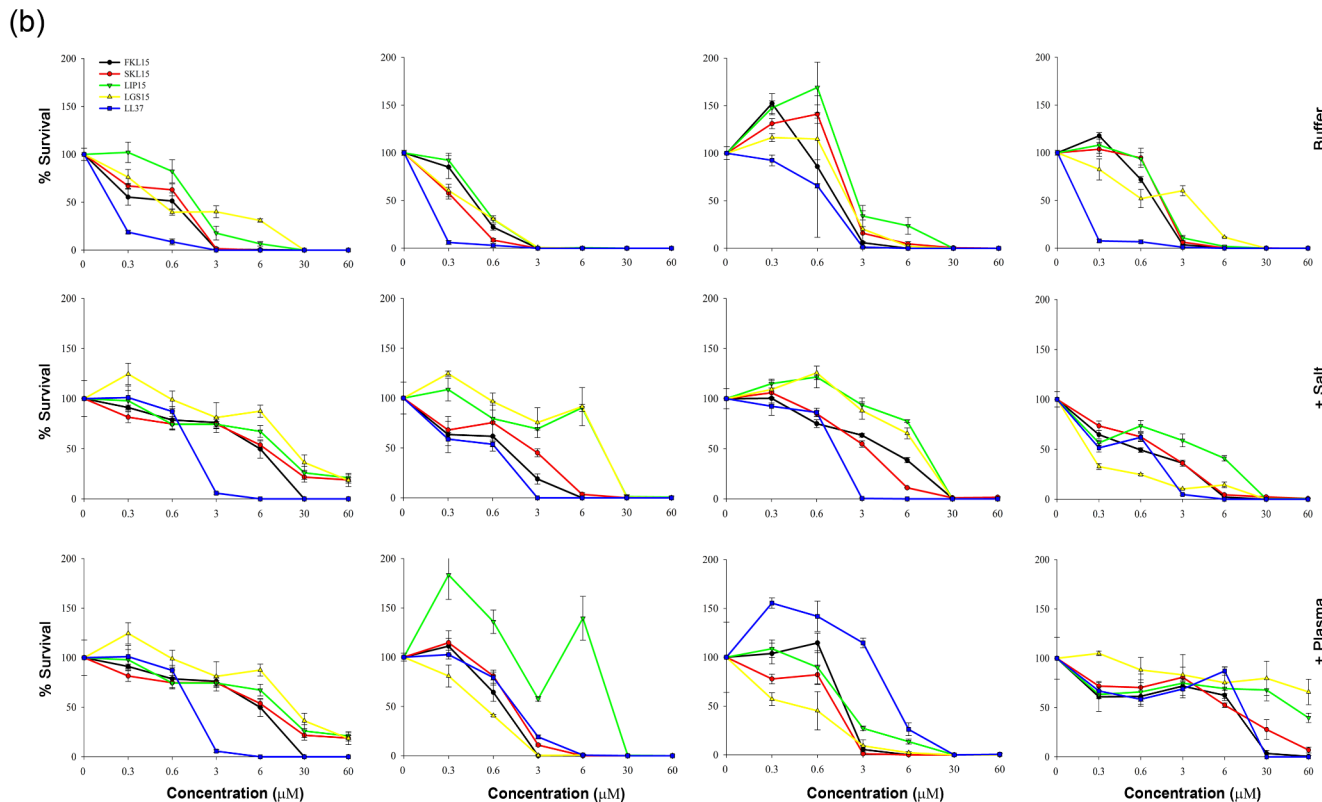
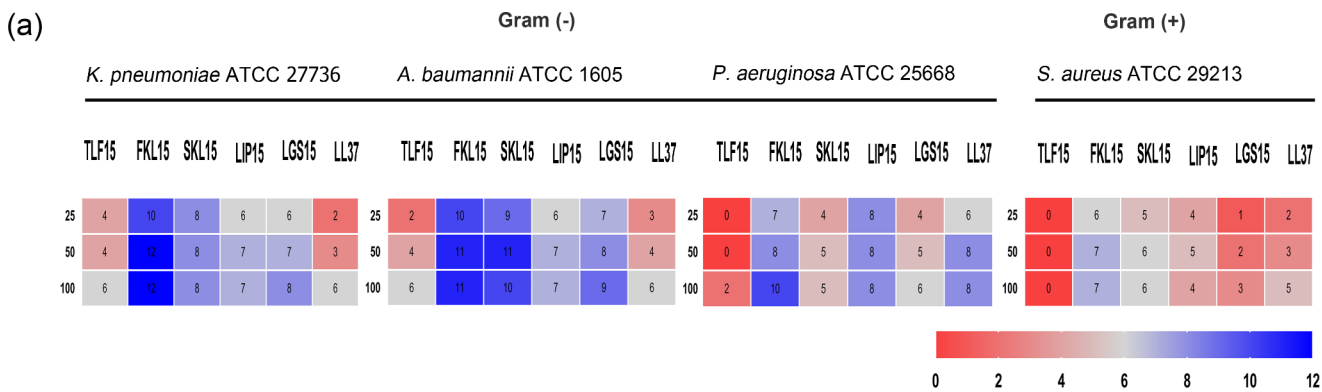
high percent (> 80%) of survival and no significant (< 10%) cytotoxicity was detected for our peptides in MTT assay (Fig. 3A, B and C). Based on the antimicrobial activity results in the presence of plasma, it can be argued that the observed lack of peptide cytotoxicity could be due to binding to the components or degradation by proteases present in the plasma. To shed light on this aspect, protease stability studies were performed using protease of bacterial origin with broad and narrow specificity. Results showed that the tested peptides were stable up to 3 h in the presence of the protease, except proteinase K which has a broad specificity (Fig. 3D). This contrasted with the benchmark peptide LL37 which was cleaved by all the bacterial proteases.

### Mode of Action Studies

Scanning electron microscopy, flow cytometry and membrane permeabilization investigations were carried out to gain deeper insights into the mode of action. Our EM studies clearly showed that the tested peptides could damage the membrane and induce ghost-like structures on the membranes, with the release of intracellular contents (Fig. 4).

Notably, shorter cryptic antimicrobial peptides from various proteins also showed ghost-like appearances on the membranes with extracellular extrusions. Although all the bacteria showed signs of membrane damage, the degree of the damage induced varied among the peptides probably due to architectural differences between the bacteria membrane components. e.g. capsule of *K. pneumoniae*, outer cell walls in *A. baumannii*. Never the less flow cytometry analysis on the test bacteria confirmed the EM results that membranes were damaged, thus accessible to the propidium iodide (Fig S1).

Our VCA assay in the presence of the physiological conditions showed that the peptides partially inhibition antimicrobial activity at low concentration probably due to binding to salts or plasma (Fig. 2, lower row) (Maharjan et al. 2022; Roshanak et al. 2021). To rule out the role of plasma in the absence of cytotoxicity, liposome leakage assay was performed to demonstrate the ability of peptides to differentiate between the prokaryotic and eukaryotic membrane mimics. Results of calcein leakage from liposome mimicking the bacterial membrane, i.e., PE/PG demonstrated that at 1  $\mu$ M concentration 80% of release occurred within 10 min of



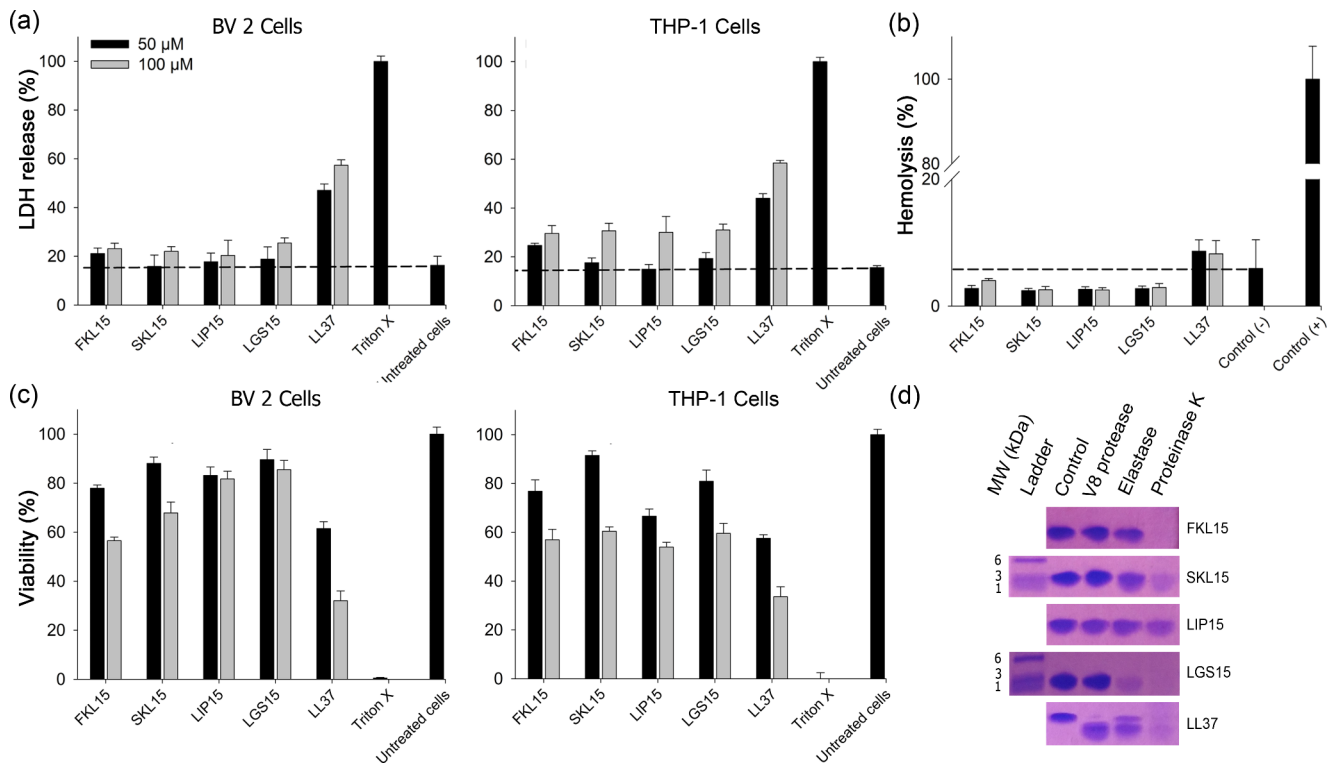
**Fig. 2** Antibacterial activity of ToAP2 peptide analogs. (a) The activity of peptides as assessed by radial diffusion assay (RDA) against *S. aureus* ATCC 29,213, *P. aeruginosa* ATCC 25,668 *A. baumannii* ATCC 1605, and *K. pneumoniae* ATCC 27,736 (bottom to top). The clear zones corresponding to the inhibitory effect of each peptide were measured after incubation at 37 °C for 16–24 h. The color of the cell indicates the zone of inhibition (in mm) observed after the peptide treatment. The values (25, 50, 100 µM ) indicate the tested concentrations.

peptide addition (Fig. 5A). The sole purpose of using mimic membranes was to quantitatively monitor the membrane damage effects of the peptides. On contrary, except FKL15, all the other peptides failed to induce membrane damage in the liposome mimicking the eukaryotic membranes, i.e., PC (Fig. 5B). These results confirm our observation that the peptides are highly specific for bacterial membranes.

We next investigated the peptide’s ability to form helical structures in the presence of membranes. As exemplified in

Fig. 5D, E and F peptides showed the random structure in the presence of 10 mM Tris buffer, PE/PG, and PC except for LL-3, which adopts a perfect  $\alpha$ -helical structure in all conditions. The CD-structures results indicate that peptides assume random structure in the presence of membranes. Based on our and other results, we are of the opinion that the peptides might have used electrostatic attraction and hydrophobicity for antimicrobial activity, rather than having a helical structure for the antimicrobial activity (Duan

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**Fig. 3** Cytotoxicity assay of peptides. **(a)** C57/BL6 murine-derived microglial cell, BV2 and human-derived monocyte, THP-1 were incubated with peptides at 50 and 100  $\mu$ M and then LDH enzyme released was measured **(b)** hemolytic effects of the peptides on human erythrocytes was analysed at 50 and 100  $\mu$ M. Hemoglobin released was measured at 540 nm and is expressed as % of Triton X-100 induced hemolysis. **(c)** On the contrary, the survival of BV2 and THP-1 cells

in the presence of peptides was measured by using the live-cell ability to convert MTT to blue formazan as an indicator. All the values (mean + SD) represented here are from three biological repeats. **(d)** Protease sensitivity assay. Peptides (200  $\mu$ g) were incubated with V8 protease, Proteinase K, and Human neutrophil elastase at 37  $^{\circ}$ C for 3 h and analyzed on 16.5% precast Tris-tricine SDS PAGE, faint or no staining indicates partial or complete cleavage

et al. 2021; Horam et al. 2019; Krishnamoorthy et al. 2023; Lata et al. 2023; Theansungnoen et al. 2022).

## Discussion

The central conceptually novel finding in this study is the identification of short cryptic epitopes from the toxic protein with antimicrobial activity. ESKAPE pathogens, which account for 70% of bacterial infections in human, have an unprecedented ability to inactivate the small molecule based antimicrobial agents by hydrolysis or modification; alteration or bypassing of drug targets, permeation barriers, and removal of antibiotics out of the cell via efflux pump (Oliveira et al. 2020; Denissen et al. 2022). The discovery and development of a novel class, broad-spectrum antimicrobial agents that act on the evolutionary conserved, unique, and hard-to-mutate targets should be the utmost priority. In this regard, host defense peptides (HDPs) have captured the attention of researchers in recent years because of their efficiency as the first line of defense against

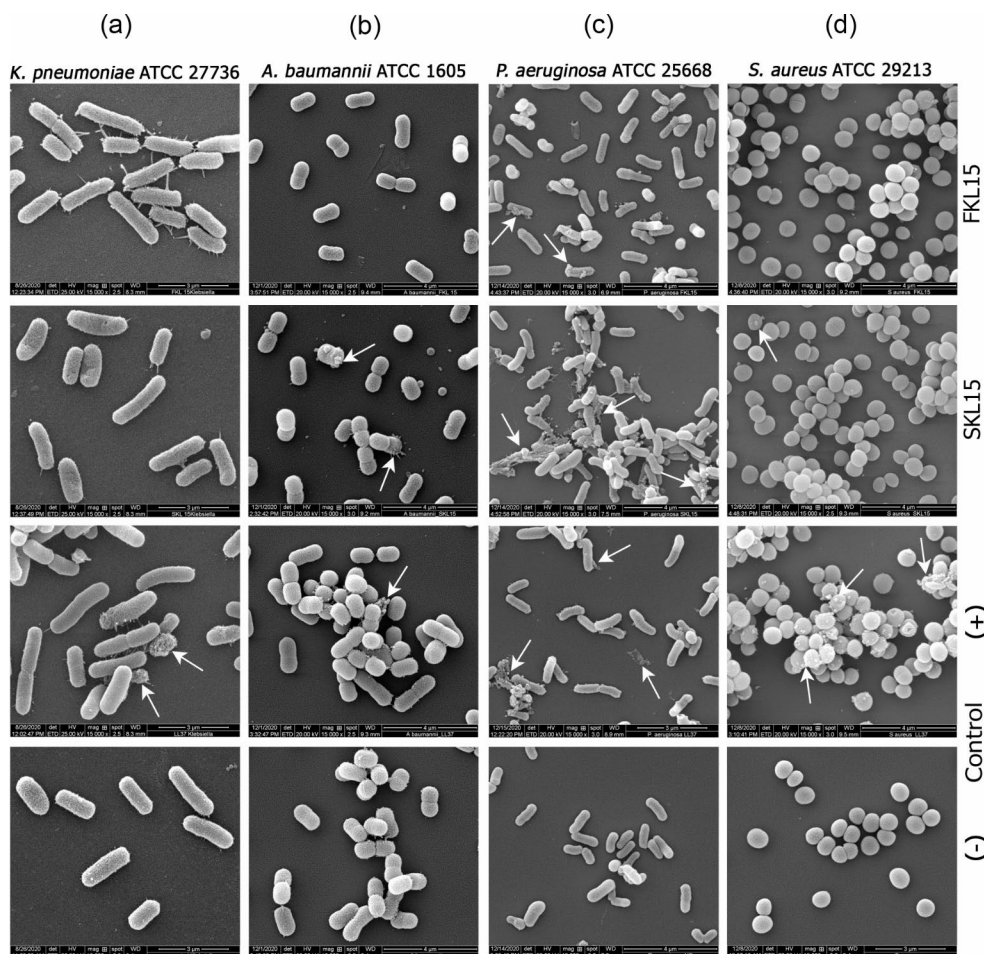
multi-drug-resistant pathogens (Heselpoth et al. 2022; Pasupuleti et al. 2012; Sarkar et al. 2021).

In the present investigation, five new synthetic peptides were designed TFL15, FKL15, SKL15, LIP15, LGS15, and analyzed for their antimicrobial and hemolytic activities. The peptide sequences were deduced from the parent ToAP2. Even though the investigated peptides have different amino acid sequences, they share similar biophysical parameters which allow them to interact with the membranes (Sup Table 2). All the peptides were 15 amino acids long with a net positive charge in the range of +4 to +6 and rich in basic amino acids. The peptides showed activity at higher concentrations illustrating the fact that a blend of cationicity, amphipathicity, and hydrophobicity are essential for the activity, especially at physiological concentrations. Our data is consistent with the previous reports from our lab and others (Apostolopoulos et al. 2021; Horam et al. 2019; Ko et al. 2020; Raj et al. 2021).

It is well reported that metal cations like  $\text{Na}^+$ ,  $\text{Mg}^{2+}$ , and  $\text{Fe}^{3+}$  decrease the antimicrobial activity of the HDPs by masking the electrostatic charges on the peptides as well as the membranes leading to lower peptide-membrane



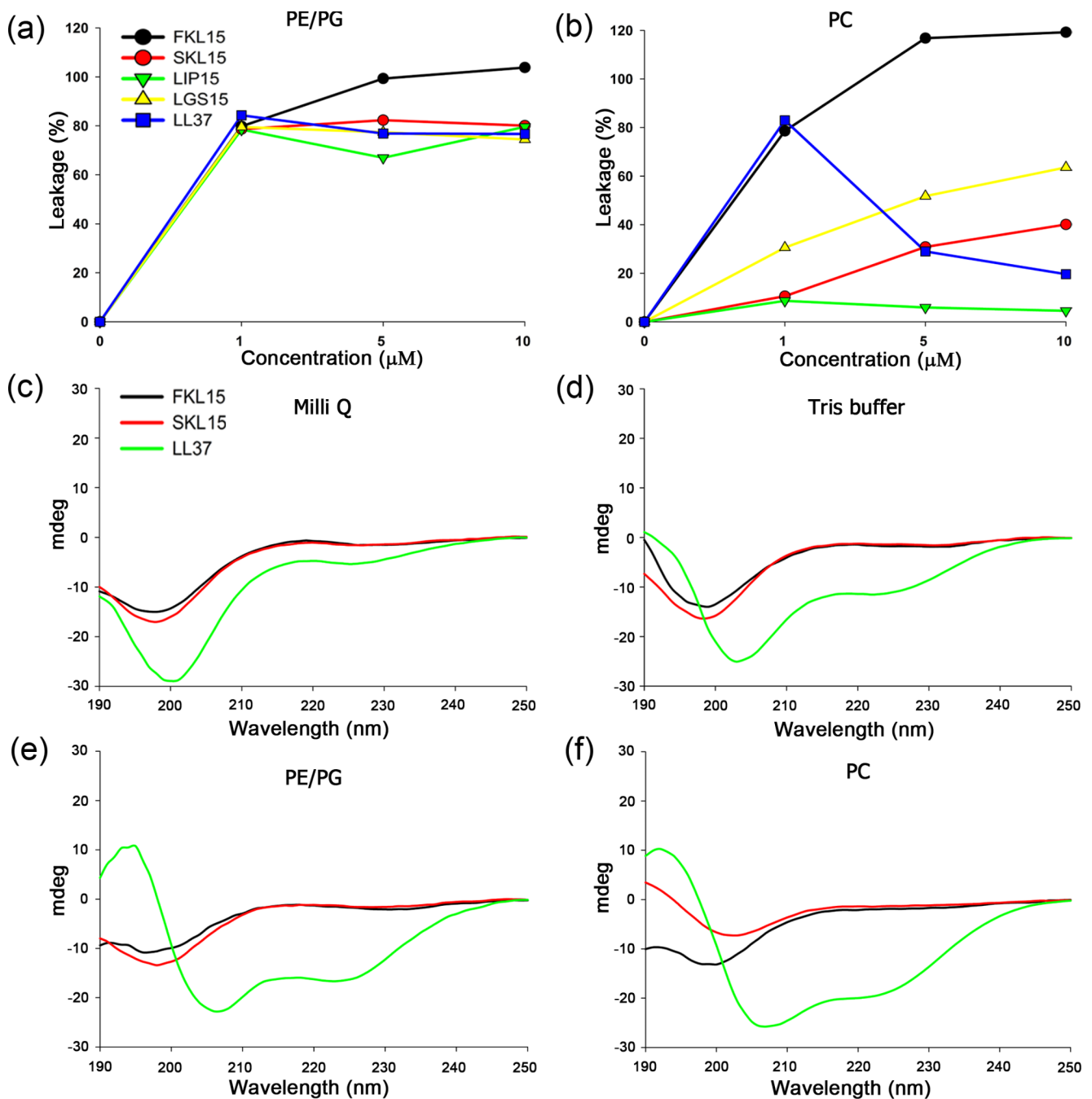
**Fig. 4** Scanning electron microscopy (SEM) images of bacteria treated with peptides. Peptides (100  $\mu$ M) were treated with (a) *K. pneumoniae* ATCC 27,736, (b) *A. baumannii* ATCC 1605, (c) *P. aeruginosa* ATCC 25,668 and (d) *S. aureus* ATCC 29,213 and analyzed by scanning electron microscopy (FEI Quanta 250, Netherlands). Peptide-treated bacterial cells showed bursting and leakage of intracellular contents. LL37 was represented as positive (+) control and while cells untreated with peptide served as the negative control



interactions (Ko et al. 2020). As reported, peptides showed varying degrees of antibacterial activity in physiological buffers and plasma conditions. We believe that the loss of activity at lower concentrations in the plasma could be due to the loss of initial electrostatic interactions or binding to the plasma components. Our data show that the inhibition of peptide's antimicrobial activity in the plasma is more than in the presence of physiological salt alone. Studies by Su Jin Ko et al. with melectin peptide as a template showed that divalent cations inhibit the peptide more in comparison to mono or trivalent ions (Ko et al. 2020). At high cations concentrations, the membrane rigidity also increases due to the decrease in the electrostatic interactions between the negatively charged phospholipids. The lower antimicrobial activity in the plasma could be due to the high concentration of  $\text{Na}^+$  and  $\text{Mg}^{2+}$  as they have been shown to hamper the initial electrostatic interaction of the peptides and binding with membrane. Probably, peptides LIP15 and LGS15 activity are more affected by divalent cation than the FKL15 and SKL15, as those lost their activity in the salt and plasma significantly. On the contrary, FKL15 higher hydrophobicity might have helped in deeper penetration into membranes and subsequent cell damage (Edwards et al. 2016; Tan et

al. 2021). Nevertheless, there is now enough evidence that demonstrates that the HDPs do have antibacterial activity under physiological conditions, and work in synergism with other natural HDPs present in the host as well as by their immunomodulatory activities (De San Nicolas et al. 2022; Ridyard et al. 2023; Sharma and Bisht 2023; Xia et al. 2023).

Our toxicity as well as the liposome leakage data demonstrate that the peptides are selective towards the prokaryotic membranes. Bacterial membranes are rich in acidic phospholipids like phosphatidylglycerol (PG), phosphatidylserine (PE) and cardiolipin, which are negatively charged surfaces. On the contrary, eukaryotic membranes are rich in mostly zwitterionic lipids (e.g., phosphatidylcholine), and sterols which are mostly neutral in charge. This electrostatic difference might play a role in the interaction of the peptide with the membranes (Miyazaki and Shinoda 2022; Utterstrom et al. 2022). Furthermore, it is very clear that in addition to binding to the membranes, the peptides have to undergo oligomerization and assembly on the surface before inducing the membrane rupture. Likely, all these mechanisms, together with the high electronegativity of *P. aeruginosa* LPS might explain the herein noted higher



**Fig. 5** Liposome leakage assay and CD studies. (a, b) The membrane disruption effect of the peptides was measured by monitoring the emitted fluorescence of calcein released from anionic bacterial membrane (PE/PG) or zwitterionic eukaryotic membrane (PC). (c, d, e, f) For CD studies, CD spectra of peptides were obtained in Milli Q, 10 mM Tris

buffer pH 7.4, PE/PG, and PC liposomes. Readings were taken in triplicate under stirring conditions at 100  $\mu\text{M}$  at 37  $^{\circ}\text{C}$ . The background value (where peptide was not present) was subtracted to nullify the instrumental differences between measurements

activity of the SKL15 and FKL15 peptides. Probably, the capsular nature of *K. pneumoniae* may explain why there is a loss of activity or higher MIC values. However, further in depth investigations are needed to clearly understand the lack of high activity.

Helical wheel projections of peptides showed both hydrophilic and hydrophobic faces and the *in silico* modeling indicated that the peptides assume a helical structure (Fig. 1A). On the contrary, CD analysis of the peptides suggests that peptides assume random structure in the presence of membranes and buffer. Given this, we are opinion of

that our lead peptides resemble other classical linear HDPs with regularly interspersed hydrophobic residues like those derived from GST, pneumolysin, and growth factors (Horam et al. 2019; Malmsten et al. 2007; Raj et al. 2021; Svensson et al. 2010). Our previous works have demonstrated that a combination of helicity and net charge is required for cytotoxicity (Pasupuleti et al. 2008). Thus it is likely that lack of helicity might contribute to a lack of cytotoxicity against BV-2, THP-1, and erythrocytes.

One of the drawbacks of this study is that we did not try to increase the peptide's activity by increasing the charge or hydrophobicity or helicity other parameters which influence the activity. Even though, there are many reported methods of enhancing the activity in the literature (Han et al. 2021; Kang et al. 2022; Mwangi et al. 2023). For valid reasons, we refrained from proceeding in this line. For example, the replacement of each position in tripeptide would generate around 35 billion individual peptide sequences. Given the practical feasibility issues, it cannot be explored in this work. The other method is to develop a novel HDP sequence by *de novo* method, based upon our years of experience and literature reports. However, we believe that each peptide sequence is a unique and simple replacement of amino acids at any position will not work as positioning charged or hydrophobic amino acids at the strategic position is very crucial for activity. We in future studies wish to explore this option as carry out such kind of studies is beyond the scope of this paper.

It is now very clear that the HDPs are widely distributed in life forms, and they are not just antimicrobial molecules but have diverse functions such as cell signaling, wound healing, immunomodulatory, antiviral, angiogenesis, and controlling both the adaptive and innate immune systems. The results obtained in this study will have implications in our understanding of toxic proteins and multifunctionality of the HDPs. It will be logical to use naturally occurring peptides as a test sequence and apply the acquired rational drug design knowledge (structural prerequisites, helix stabilization, QSAR modelling, etc.) for further refinements to obtain a better bioactive and protease resistant molecule.

## Conclusion

In light of this, our findings indicate that a strategy based on the selection of peptides from toxic proteins may prove to be a starting point for the generation of new variants with enhanced activities or discovering additional novel sequences as lead candidates for the development of AMPs. Further short antimicrobial peptide templates with extended broad-spectrum antimicrobial activities can be identified.

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**Author Contributions** ML, VT, PG and GP planned, carried out the experiments and analyzed the results. MP, MK and JA conceived planned, supervised and interpreted the results. ML devised the proof outline and wrote the manuscript. MP and JA contributed to the final version of the manuscript. All authors discussed the results and contributed to the final manuscript.

## Declarations

**Ethical Approval** The study was approved by the Human ethics committee as per CSIR-CDRI human ethical permission (CDRI/IEC/2019/A1).

**Competing Interests** The authors declare no competing interests.

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