



## **Folding Behaviour and Antibacterial Activity of Ionic Complementary Peptide EAK-16**

**Abdul Majid<sup>1\*</sup>, Farah Naz<sup>1</sup>, Hatim Ali Jamro<sup>1</sup>, Sham Lal<sup>2</sup>, Inayatullah Soomro<sup>3</sup>,  
Sanaullah Abbasi<sup>1</sup> and Safdar Ali Ujjan<sup>4</sup>**

<sup>1</sup>Department of Biochemistry, Shah Abdul Latif University Khairpur, Post Code: 66020, Sindh, Pakistan.

<sup>2</sup>Institute of Microbiology, Shah Abdul Latif University, Khairpur, Post Code: 66020, Sindh, Pakistan.

<sup>3</sup>Department of Mathematics, Shah Abdul Latif University, Khairpur, Post Code: 66020, Sindh, Pakistan.

<sup>4</sup>Department of Zoology, Shah Abdul Latif University, Khairpur, Post Code: 66020, Sindh, Pakistan.

### **Authors' contributions**

*This work was carried out in collaboration among all authors. Author AM designed the study, author SA performed the statistical analysis, author FN wrote the protocol and authors FN and SL wrote the first draft of the manuscript. Authors HAU and SAU managed the analyses of the study. Author IS managed the literature searches. All authors read and approved the final manuscript.*

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### **ABSTRACT**

**Aim:** A major challenge in the development of new antibiotics is the biocompatibility within biological environment. Ionic complementary peptide (EAK-16) from amyloid protein, have the ability to adopt secondary structure conformation at membrane interfaces. This study aimed to investigate the effect of membrane on EAK-16 peptide folding and their antibacterial applications.

**Methodology:** We studied secondary structural conformation of EAK-16 using circular dichroism (CD) spectroscopy in an aqueous environment and at membrane bilayers interfaces. Initially, the antibacterial efficacy was investigated against both Gram-positive and Gram-negative bacteria. Membrane mimicking models were synthesised with dimyristoylphosphatidylcholine (DMPC) and dimyristoylphosphatidylserine (DMPS) lipid vesicles using calcein leakage assay.

**Results:** EAK-16 showed transition in secondary structural conformation. In aqueous environment,

\*Corresponding author: E-mail: [amajid.ansari@salu.edu.pk](mailto:amajid.ansari@salu.edu.pk);

it was predominantly  $\beta$ -sheets and at membrane interfaces, it was mainly  $\alpha$ -helical. EAK-16 peptide was highly active against bacteria (at minimum concentration applied) and membrane leakage was found to be > 60%. This effect was confirmed with both anionic lipids (DMPS) and neutral lipids (DMPC). The helical transition of EAK-16 could be a major factor to disrupt the membrane and bacterial death

**Conclusion:** The secondary structural conformation and calcein leakage data suggest that EAK-16 has potential to kill bacteria by adopting helical tilted conformation and membrane perturbation *via* lysis. This study revealed structure-function relationship of peptide and lipid bilayers to further investigate the mode of pore formation and mode of action of EAK-16 in membrane perturbation and antibacterial efficacy.

**Keywords:** Antibacterial peptide; ionic complementary peptide; peptide lipid interactions; secondary structure conformation; membrane leakage.

## 1. INTRODUCTION

The widespread increase in bacterial resistance is an emerging problem for majority of antibiotics. The success in broad spectrum antibiotics provided astonishing efficacy but has also been compromised due to multidrug resistant microbes that are currently resistant to almost all available drugs [1]. This has presented a unique challenge to scientists. Thus, to design and to develop alternate antimicrobial agents for multidrug resistant microbes with new mode of action is pressing than ever [2]. Microorganism develops antibiotic resistance commonly from evolutionary adaptation *via* various mechanisms. These include; gene mutation, targets site modification and efflux pumps regulations [3]. Environment overuse can also produce resistance [4]. For example *Pseudomonas aeruginosa* pathogen is multidrug resistant to beta-lactams, carbapenems, aminoglycosides and fluoroquinolones and colonises the lungs of cystic fibrosis patients [5]. In such cases, higher salinity of the bronchopulmonary fluids is known to reduce antibiotics efficacy. Moreover, in certain conditions hypersalinity facilitate the bacterial colonisation and resistance has been reported especially in bronchitis [6]. The infections are further compounded due to inactivation of antimicrobial peptides such as defensins. Antimicrobial peptides are expressed in almost organisms, such as insect, plants, animals and humans and are key elements of the natural defence system [7]. Commonly, these antimicrobial peptides are small (<10 kDa), ionic and amphiphilic with different sequences, length and structural conformations. These show diversity of activities against fungal, viruses, bacteria, tumor cells along immunomodulator system [8]. Antimicrobial peptides are usually expressed on epithelial cells of lungs and are the first line of defence to pulmonary infections. In

lungs diseases, such as cystic fibrosis, antimicrobial peptides could provide an active defence if their inactivation to hypersalinity could be overcome [9]. In fact, antimicrobial peptides are found to have increased efficacy against variety of microbes in lower salt environment, even for microbes having drug resistance phenotypes. There are still a limited number of studies available on the effect of secondary structure on antimicrobial peptides efficacy, though the conformational sensitivity to antimicrobial peptides activity remains a major obstacle to their applications [10]. Hence, it is important to enhance the antimicrobial efficacy of antimicrobial peptides by investigating the key elements affecting the various antimicrobial properties.

Electrostatic interactions are one of key structural element driving the peptide folding and always identify as prerequisite for antimicrobial peptides efficacy [10]. This property reflects the cluster formation of residues forming hydrophobic and ionic faces on the secondary structure periphery, enabling the antimicrobial peptides to bind with ionic head groups of lipid bilayers through electrostatic interactions. This is followed by penetration of hydrophobic residues into hydrophobic leaflet of bilayers, eventually destabilising the membrane integrity [11]. Keeping this property, naturally occurring peptide are modified by altering the sequences of residues to achieve perfect amphiphilic structures and ionic configurations. Alteration of polar residue with hydrophobic face or residue contributes to enhanced antimicrobial activity, decreased haemolysis and preferably pore formation [12].

According to these observations,  $\alpha$ -helical antimicrobial peptides may be a new candidate for development of novel treatment against

bacterial diseases [9]. Antimicrobial peptides are extensively studied, though most of them have exhibited membrane interactive properties, however some antimicrobial peptides attacks on intracellular organelles [13]. In most cases, primary killing mechanism of antimicrobial peptides is the destabilisation of lipid bilayers [11]. Despite extensive studies on their mode of action, there is still a need of clear understanding of antimicrobial peptides structure-function relationship. In majority of cases, it is observed that ionic interactions of peptide are interacting with respective charges on lipid bilayers [9]. The impact of exposure of salts might be a key factor on the microbial resistance while initiating the ionic interactions derived from environment.

First ionic complementary peptide EAK-16 was originally isolated from the zuotin, a Z-DNA binding protein from yeast [14]. EAK-16 is linear peptide consisting 16 subunits (AEAEAEAEAKAKAKAK) amino acids in alternate hydrophobic (alanine) and hydrophilic (glutamic acid and lysine) fashion. EAK-16 have shown membrane interactive properties with model lipid vesicle. Halocidin antimicrobial peptide derive positive charges from histidine residues, which was a major factor for antimicrobial peptides to retain their efficacy [15]. In this study, the folding behaviour of EAK-16 was investigated to get insight on membrane interactions and peptide folding with various membrane model systems and in presence of aqueous environment and membrane mimicking conditions. These studies can further relate the peptide to be potential candidate for the treatment of infections, especially against multidrug resistant bacteria.

## 2. MATERIALS AND METHODS

### 2.1 Materials

EAK-16 was synthesised using solid-state method as described by [10] with a Liberty microwave peptide synthesizer (CEM Co., Matthews, NC, USA). The purification of EAK-16 was determined with RP-HPLC on C<sub>18</sub> column. These were further confirmed with laser desorption ionisation mass spectrometry (Shimadzu, Japan). Phospholipids including; dimyristoylphosphoserine (DMPS) and dimyristoylphosphocholine (DMPC) were purchased from Avanti Polar Lipids (USA). Ethanol, trifluoro ethanol (TFE) were purchased from Sigma (UK). Buffers and chemical used for CD spectroscopy and leakage studies were prepared in Milli-Q water.

### 2.1.1 Microbial strains

All microbial strains were obtained from the American Type Culture Collection (ATCC), these include; *Listeria monocytogenes* (ATCC 35152), *Staphylococcus aureus* (ATCC 29213), *Pseudomonas aeruginosa* (ATCC 15442), and *Escherichia coli* (ATCC 8739).

### 2.2 Primary Structure Analysis

The primary structure of EAK-16 was analysed according to hydrophobic moment plot method. The mean hydrophobic moment ( $\mu_H$ ) and mean hydrophobicity ( $H_0$ ) were calculated on the assumption of periodicity residue of 100° using 11-residue window based on Eisenberg's normalised hydrophobicity scale [16]. The residue helical wheel summation was calculated using 'HMOMENT' (<https://heliquet.ipmc.cnrs.fr/cgi-bin/ComputParams.py>), where ( $\mu_H$ ) represents overall amphiphilicity of EAK-16. The 3-D structural projection was simulated using online application (<http://mobyle.rpbs.univ-paris-diderot.fr/cgi-bin/portal.py>).

### 2.3 Vesicle Preparation and Calcein Leakage Assay

Membrane mimetic lipid bilayers were prepared as previously described [10]. DMPC or DMPS was dissolved in chloroform; the solvent was then evaporated under nitrogen gas influx. The lipid film was then dried overnight in vacuum desiccator. Lipid film was then hydrated with PBS (pH 7.4), subsequently 100 mM calcein was entrapped in the lipids. The resulting suspension was sonicated and extruded 10 times using extruder (0.05  $\mu$ m polycarbonate filter from Avanti Polar, USA). Untrapped calcein was filtered out using Sephadex G75 gel (Sigma). Calcein entrapped vesicles were rehydrated in 5.0 mM HEPES, 100 mM and 1.0 mM EDTA overnight.

The calcein leakage was studied as previously described [10]. Calcein fluorescence intensity was monitored at 490 nm (excitation wavelength) and 520 nm (emission wavelength) using RF-6000 spectrofluorometer (Shimadzu, Japan). Triton X-100 was used for maximum calcein leakage. The percentage calcein leakage was calculated using equation:

$$= \% L = \frac{(I - I_0)}{I_t - I_0} \quad (1)$$

Where  $I_0$  represents initial intensity of vesicles,  $I$  represent intensity in presence of EAK-16 and  $I_t$  is for total intensity with Triton X-100.

## 2.4 Antibacterial Activity Assay

Gram-positive and Gram-negative bacterial strains were grown according to protocols of ATCC. Antibacterial activities were measured as previously described [17]. The fresh cultures from log phase (8 hours incubated) were diluted with Mueller–Hinton broth (MHB), supplemented with 150 mM NaCl (pH 7.4), to a final concentration of  $3 \times 10^5$  CFU mL<sup>-1</sup>. The EAK-16 concentrations (ranging from 3  $\mu$ M to 30  $\mu$ M) were prepared in PBS (pH 7.4) and 0.25 % bovine serum albumin (BSA). Bacteria in mid-logarithmic phase were then incubated with EAK-16. The cultures were incubated for 24-48 hours at 37 °C and growth was monitored based on OD<sub>600</sub>. Growth inhibition of bacteria was calculated, where maximum bacterial growth was inhibited with minimum use of peptide concentration.

## 2.5 Secondary Structure of EAK-16

Far-UV CD spectra were recorded on J-810 spectropolarimeter (Jasco, Japan) at 20 °C as previously described [10]. CD spectra of EAK-16 were obtained in PBS (pH, 7.4, 6.4 and 8.0), TFE and at temperature up to 90 °C. Membrane mimetic bilayers were prepared by dissolving either DMPC or DMPS in chloroform and dried under influx of nitrogen gas. The dried film was rehydrated with PBS (pH 7.4) and sonicated until solution became clear. Data were collected over wavelength ranges 260 nm to 189 nm at 0.1 nm intervals, band width 1 nm with five scan and scan speed of 50 nm/min for each sample. 10 mm path-length cell was used.

## 2.6 Statistical Analysis

All experiments were conducted in triplicates. Results are depicted as mean  $\pm$  standard deviation (SD  $\pm$  03). Data of lipid membrane models were analysed using Student's *t* test. Antibacterial efficacy was compared using the ANOVA with SAS 9.3 software. Mean differences at a value of  $p < 0.05$  were considered significant.

## 3. RESULTS AND DISCUSSION

### 3.1 Theoretical Analysis of EAK-16

EAK-16 sequence on hydrophobic moment analysis showed a  $\mu_H = 0.113$  and  $H = 0.345$ .

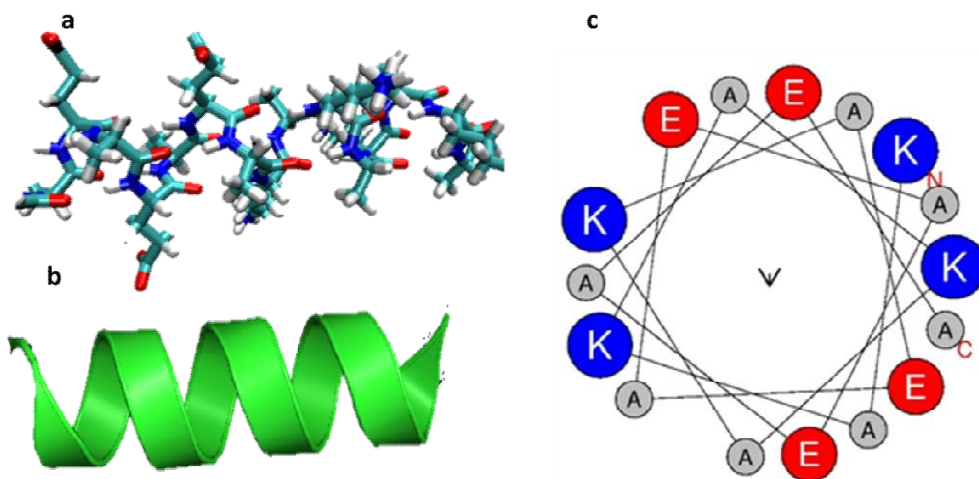
The sequence was plotted on Cartesian plane, where these values depicted the putative helical confirmation in oblique-orientation fashion [17]. Hence, EAK-16 is possibly a candidate among oblique-oriented helical peptides having biological activity. Peptide was designed and synthesised by addition of Alanine (A) residue in alternate configuration with glutamate (E) and lysine (K) residues (Fig. 1 a). Negatively charged glutamate (helix promoter) and positively charged lysine (a cation) are facilitators for helix formation (Fig. 1 a and b). Alanine was added due to its known membrane perturbation tendency. Lysine and glutamate were substituted on ionic amphiphilic motif in hydrophilic face, while alanine in hydrophobic face. The hydrophobic moment for EAK-16 was (0.113) higher than other than antimicrobial peptides [18]. The helix wheel diagram shows residue position within  $\alpha$ -helices structure confirmation (Fig. 1 c).

### 3.2 Antimicrobial Activity and Calcein Leakage Analysis

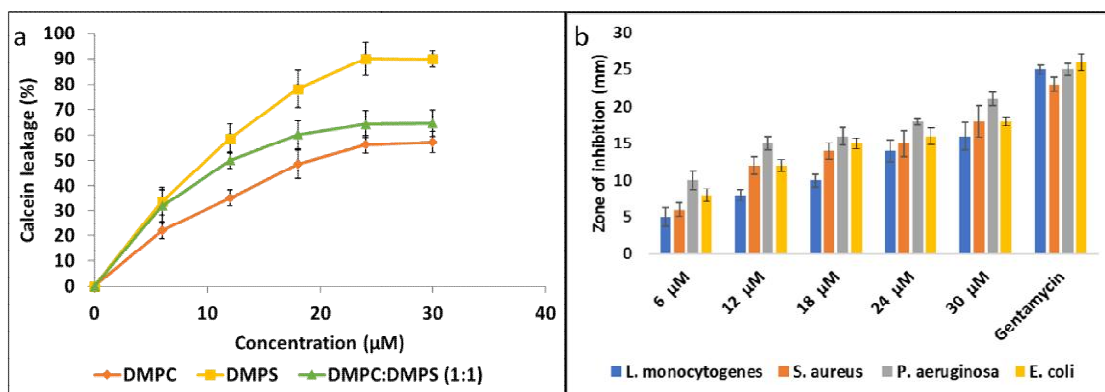
Ionic complementary amphiphilic peptides show broad-range antibacterial activity [19]. EAK-16 were found to have strong activity against Gram-positive and Gram-negative bacteria, where it did not show the antibacterial activity preference over against Gram-positive and Gram-negative bacteria. Fig. 2 (b) showed antimicrobial activity of EAK-16 against both Gram-positive and Gram-negative bacterial strains at various concentration ranges from 6.0  $\mu$ M to 30  $\mu$ M. EAK-16 in both type of bacteria depicted inhibition  $> 6 \mu$ M. Preference was not confirmed with statistical analysis that depicted the significance level ( $F_{2,21} = 33.61$ ;  $p > 0.05$ ) for both strains of Gram-positive and Gram-negative bacteria. Such indicated observations for antibacterial peptides are consistent with reported data [19]. Calcein release assay further confirmed EAK-16 lysis of membrane bilayer mimics of DMPC and DMPS, where in both membrane models showed ( $> 60\%$ ) lysis at 18  $\mu$ M and 12  $\mu$ M, respectively. (Fig. 2 (a)). Fig. 2 (b) shows the calcein leakage from membrane mimetic vesicles induced with EAK-16, we could not find any significant difference on membrane lysis of either DMPC or DMPS ( $T = -0.40$ ;  $p = 0.72$ ). Meanwhile, we could not found decrease in calcein release from either type of bilayers (DMPC and DMPS) was seen in both investigated pH 6.4 ( $T = -2.95$ ;  $p = 0.06$ ) and pH 7.4 ( $T = 2.87$ ;  $p = 0.065$ ).

Membrane leakage data clearly suggested that EAK-16 has greater lytic efficacy against both membrane models (DMPC and DMPS). The correlation in efficacy of antibacterial results and leakage data with MIC revealed that EAK-16 most like kill the bacteria via membrane disruption mode of action. In addition, this also suggested the broad spectrum of peptide for lysis of both Gram-positive and Gram-negative bacterial classes. Membrane composition of *L. monocytogenes* and *S. aureus* are mostly DMPC and phosphatidylethanolamine (PE), whereas, *E. coli* and *P. aeruginosa* membrane composition is

predominantly DMPS and phosphatidylglycerol (PG). EAK-16 induced leakage for DMPC and DMPS was 80.5% and 58.3%, respectively. For comparison of this study, EAK-16 has not been investigated for antimicrobial applications, so far as per knowledge. Other antimicrobial peptide depicted preference in varying ability to interact with charged membrane lipids. This further confirm our observation that EAK-16 directly induces the membrane disruption of bacterial membranes to endorse this mode of antibacterial efficacy.



**Fig. 1. Ionic potential of the EAK-16 peptide.**(a) three dimensional side chains of EAK-16 possessing lysine basic residues, glutamate acidic residues and alanine hydrophobic and neutral residues(b) predicted amphiphilic helical conformation that are linked with intervening alanine residues (modelled using Pymol) (c)  $\alpha$ -helical conformation of EAK-16 peptide AEAEAEAKAKAKAK depicting defined amphiphilicity with segregation of polar and non-polar residues.



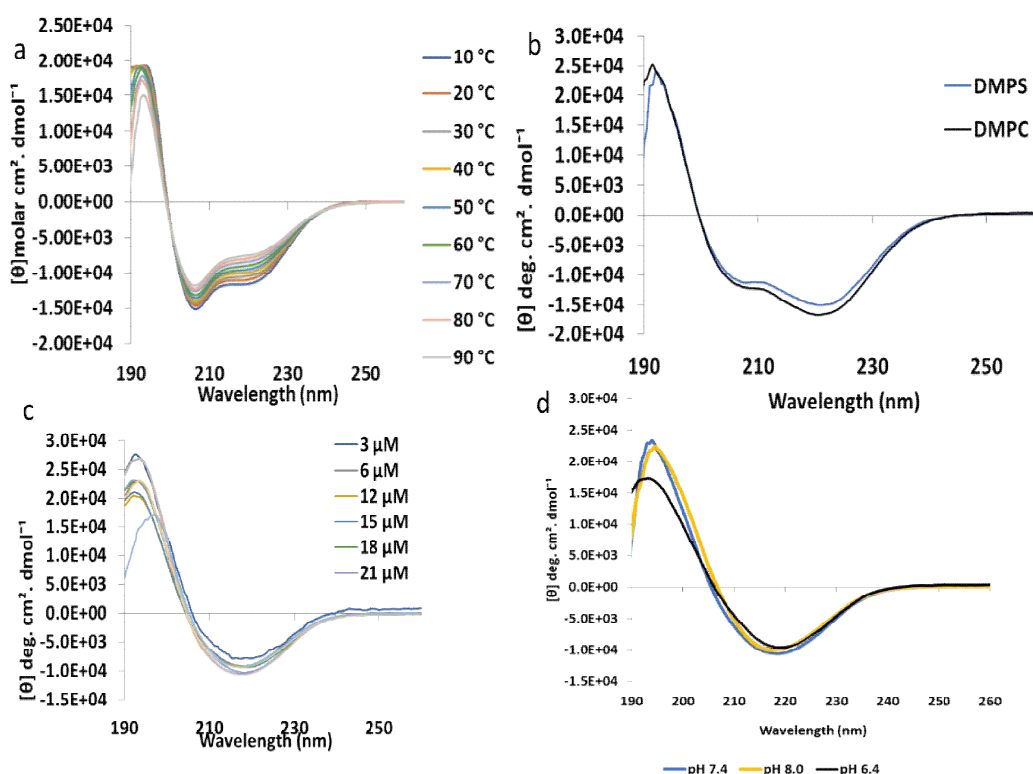
**Fig. 2. Membrane model perturbation with EAK-16** (a) Antimicrobial activity of EAK-16 against both Gram-positive and Gram-negative bacterial strains at various concentration ranges (b). The data are the mean of three replicates and error bars are the standard deviation

### 3.3 Secondary Structure Conformational Behaviour

CD spectroscopy depicted < 15% of  $\alpha$ -helices, whereas it predominantly formed  $\beta$ -sheets (Fig. 3). Previous studies also reported under similar conditions, EAK-16 adopts  $\beta$ -sheets in aqueous environment [14]. At lipid membrane interfaces, EAK-16 adopted predominantly  $\alpha$ -helical conformation, similar to membrane mimicking solvents such as TFE, PBS (Fig. 3). EAK-16 also adopted  $\alpha$ -helical conformation in bacterial membrane mimicking lipid vesicles. CD spectroscopy also revealed that EAK-16 peptide is mainly active and adopts  $\alpha$ -helical structure in amphiphilic interfacial conditions. The adaptation of  $\alpha$ -helices was also similar in both DMPC and DMPs membrane vesicles (55% and 53%), respectively. Such differences in helicity prediction were not significant upon the statistical analysis ( $p > 0.5$ ). Previous literature reported that the level of helicity is strongly correlated with membrane disruption activity [19]. Increasing

helicity of antimicrobial peptides enhances their amphiphilic surfaces, thereby increasing their efficacy to disrupt and penetrate the membranes. The membrane leakage results, and adaptation of helicity are key factors for inducing the membrane disruption of bacteria.

EAK -16 displayed as potential antibacterial agent at micromolar concentration that is appropriate to therapeutic development, where it showed the broad-spectrum applications (Fig. 2). This pattern of antibacterial efficacy of EAK-16 resembles with halocidin [15]. This also supported the view that disulphide linkage (in Rana box) is not key for antibacterial efficacy of peptide [20]. Other amphiphilic antimicrobial peptide also supports our results, recently [21]. Rana box of certain antimicrobial peptides may serve other important biofunctions compared to only factor responsible for antibacterial efficacy. It has also reported that disulphide bond in Gaegurin-6 stimulate insulin secretion in  $\beta$  cells of pancreas [22].



**Fig. 3.** EAK-16 adopted predominantly secondary structure conformation, (a) EAK-16 in TFE shows predominant  $\alpha$ -helical conformation (b) membrane model mimics shows the  $\alpha$ -helical conformation (c) EAK-16 in water shows predominant  $\beta$ -sheets and (d) shows resistance of EAK-16 at different pH (7.4, 6.4 and 8.0)

EAK-16 depicted secondary structure transition according to behaviour and integrity of lipid membranes, such property is in consistent with proposal for peptide having ability to kill bacteria that involves with permeabilisation to membranes. An experimentally studies, mechanism of pore formation E2EM peptide, which may be similar to membrane disruption of EAK-16 peptide [23]. This study depicted for first the antimicrobial response of EAK-16 with help of lipid peptide interaction and membrane perturbation. In preliminary stages, EAK-16 is mainly  $\beta$ -sheets in aqueous environment, adopting helical conformation at lipid membrane interfaces, this may be facilitated by amphiphilic environment of both lipid membranes and peptide. Helical architecture promotes the partitioning into bilayer via electrostatic interactions of glutamate and lysine with head groups of bilayers and hydrophobic association of alanine with acyl chain of membrane tails. In second stage of this model, localisation of helical EAK-16 leads to reorient the peptide in membranes to induce the membrane disruption and in results the killing of bacteria. Previous studies of E2EM peptide proposed the membrane destabilisation by pore formation either barrel stave pores or toroidal pores [23]. In either cases, helical conformation defined by N-terminal amino acids and C-terminal amino acids, both are associated with alanine residues [23].

The role of DMPC and DMPS in leakage action of EAK-16 is still not clear but this study has established that both (DMPC and DMPS) are able to induce different membrane behaviours that promotes the antibacterial action of EAK-16. However, there could be another mode of action that rely on non-permeabilisation at membrane interfaces and such mode of action of antimicrobial peptides have been reported [24]. For example, antimicrobial peptide revealed high degree of activity against *B. subtilis* employing of different mode of action such as influencing the cellular metabolism and homeostasis rather than lysis of membrane that induced cell death [24]. DMPC facilitate to adopt helical conformation that enhance the behaviour of tilted peptide to disturb and penetrate membranes, as proposed for peptides when interacted with *E. coli* [24]. This seems feasible for EAK-16 peptide against *E. coli* and *P. aeruginosa* bacteria.

#### 4. CONCLUSION

EAK-16 depicts the transition in secondary structure and potential efficacy against Gram-

positive and Gram-negative bacteria. After biocompatible studies of EAK-16, this could be potential candidate for development of broad-spectrum antibacterial agent. Such bacteria are main cause of clinical manifestation due to their ability with different pathologies, especially those with multidrug resistance. The antibacterial efficacy of EAK-16 could further support the thermostable role as antibacterial agent in food and nutrition industry. Several bacterial strains have been reported to tolerate high temperature are already known to spoil food industry.

#### DISCLAIMER

There is absolutely no conflict of interest between the authors and we do not intend to use research as an avenue for any litigation but for the advancement of knowledge. Also, the research was not funded by any organisation rather it was funded by personal efforts of the authors.

#### CONSENT

It is not applicable.

#### ETHICAL APPROVAL

It is not applicable.

#### COMPETING INTERESTS

Authors have declared that no competing interests exist.

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