Synthesis and Characterization of Antimicrobial Peptides for Medical and Dental Applications

By

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ABSTRACT

Epidemically, infectious diseases had always remained one of the leading causes of mortality and therefore are identified as a public health issue all around the world. It has been reported that one of the major cause of bacterial pathogens increasing at an alarming rate are due to over-prescription, general, aimless and pervasive use of antibiotics for both medical and non-medical means and generating multi drug resistance due to these pathogenic bacteria. The situation has been worsened due to serious decline in the level and quality of industrial research aimed for developing novel antimicrobial drugs.

We interrogating synthetic human defensins antimicrobial peptides analogs by chemical synthesis will provide intuition of its architecture, function, and antimicrobial properties. In this project, six peptide sequences were selected from α- and β human defensins, synthesized by solid phase peptide synthesis (SPPS) and purified by high pressure liquid chromatography (HPLC). Mass spectrometry was used to analyze the mass of synthetic peptides and their purity. Minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) was used to measure antimicrobial spectrum against *Streptococcus epidermidis* and *Pseudomonas aeruginosa*. MIC were also used for evaluation of their turgidity in presence of microorganisms. MBC were used for quantifying concentration of antimicrobial peptide against

microorganisms. The main outcome of this work was producing antimicrobial peptides sequences.

Dedicated to my Parents

&

My wife

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ABBREVIATIONS

A, (Alanine)	Alanine
AMPs	Antimicrobial peptides
Arg	Arginine
Asp	Aspartic Acid
Asn	Asparagine
Cys	Cysteine
°C	Degree Celsius
CFU	Colony forming unit
DCM	Dichloromethane
DIPEA	N, N-Diisopropylethylamine
DMF	N, N-Dimethylformamide
Fmoc	Fluorenylmethyloxycarbonyl
Glu	Glutamic acid
Gly	Glycine
His	Histidine
HBTU	O-(Benotriazol-1-yl)-N, N, N', N'-Tetramethyluronium Hexa fluorophosphate
HPLC	High pressure liquid chromatography
Ile	Isoleucine
Leu	Leucine
MBC	Minimum bactericidal concentration
Met	Methionine
mg	Milligram
MIC	Minimum Inhibitory concentration
min (s)	Minutes

mL	Milliliter
mm	Millimeter
m/z	Mass to charge
Pro	Proline
rpm	Round per minute
Ser	Serine
SPPS	Solid phase peptide synthesis
Thr	Threonine
TFA	Trifluoroacetic acid
TIPS	Triisopropylsilane
Trp	Tryptophan
Tyr	Tyrosine
µL	Microliter
Val	Valine
wt %	Weight percent

Chapter 1: Literature Review

1.1. Protein and Peptides

Proteins are captivating bio-molecular devices present in nature. They can receive or send information and have certain catastrophic functions such as divergence, recruitment and mixing/matching of domains. Divergence is change in sequence and structure, lead to change in specificity and nature of the reaction catalyst. One protein adapted without any structural change for second function is called recruitment. During the 'mixing and matching of domains, mass scale structural changes occur in the surrounding environment [1]. Dr Francis Crick proposed the idea of protein formation in his statement called *Central Dogma* of molecular biology in 1958 [2]. This statement brought a revolution in the proteins and proteomics world.

Proteins are made up of polymer chains of amino acids e.g. polysaccharides. They are the main essential energetic agents in molecular biochemistry; in the absence of them no metabolic processes can happen [3]. The 3D structure and function of proteins depend strongly on the sequence of the different amino acids (Figure-1) [4].

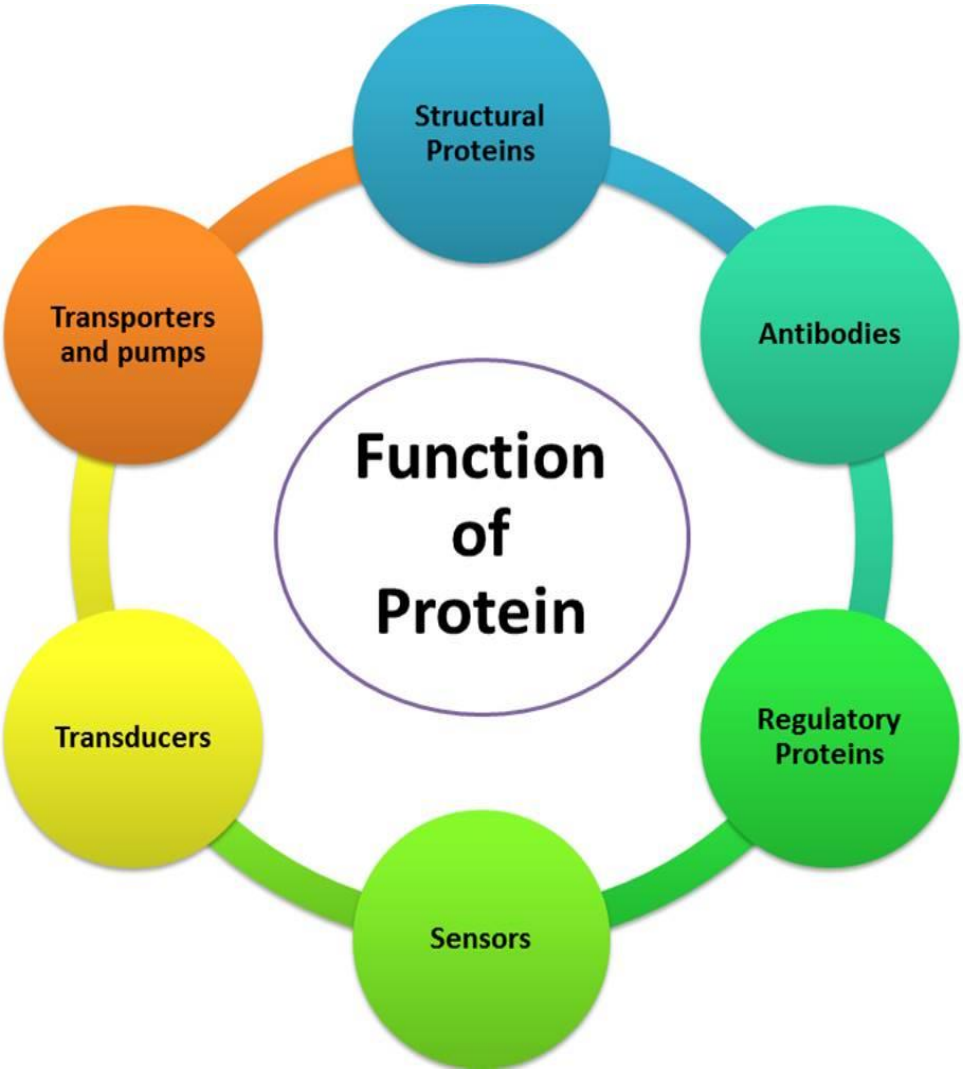

Figure 1: Illustration representing the diverse function of proteins.

Amino acids have different physicochemical properties and structures. For example some are polarized or non-polarized, charged or neutral, hydrophobic or hydrophilic, basic or acidic [5]. These differences are characterized by a central carbon atom to which a hydrogen atom, a carboxyl group (—COO^-), an amino group (—NH_3^+) and a side chain (R) are attached. These amino acids are named as the building bricks of proteins. The names of amino acids are not systematic; they are abbreviated by their first three letters

except of Asparagine (Asn), Glutamine (Gln), Isoleucine (Ile) and Tryptophan (Trp) (Table 1) [6].

Table 1: Molecular weight, abbreviation, charge, and nature of amino acids

Amino acid	Molecular weight	Three Letters	Charges	Nature	One Letter
Alanine	311.33 g/mole	Ala	Uncharged	Hydrophobic	A
Valine	339.39 g/mole	Val	Neutral	Hydrophobic	V
Arginine	648.77 g/mole	Arg	Positive +	Hydrophilic	R
glutamic acid	526.54 g/mole	Glu	Negative -	Hydrophilic	E
Leucine	353.41 g/mole	Leu	Neutral	Hydrophobic	L
Proline	337.37 g/mole	Pro	Uncharged	Hydrophobic	P
Tyrosine	403.43 g/mole	Tyr	Neutral	Hydrophobic	Y
Threonine	397.46 g/mole	Thr	Uncharged	Hydrophilic	T
Serine	327.33 g/mole	Ser	Uncharged	Hydrophilic	S
Phenylalanine	387.43 g/mole	Phe	Neutral	Hydrophobic	F
Lysine	468.54 g/mole	Lys	Positive +	Hydrophilic	K
Isoleucine	353.41 g/mole	Ile	Neutral	Hydrophobic	I
Histidine	619.71 g/mole	His	Positive +	Hydrophilic	H
Glycine	297.31 g/mole	Gly	Uncharged	Hydrophilic	G
Glutamine	368.38 g/mole	Gln	Uncharged	Hydrophilic	Q
Cysteine	585.71 g/mole	Cys	Uncharged	Hydrophilic	C
Aspartic Acid	411.45g/mole	Asp	Negative -	Hydrophilic	D
Asparagine	580.63g/mole	Asn	Uncharged	Hydrophilic	N
Methionine	371.45 g/mole	Met	Neutral	Hydrophobic	M
Tryptophan	426.46 g/mole	Trp	Neutral	Hydrophobic	W

Biochemically, the presence of amino acids in the human body is divided as e.g. Essential, Non-essential and Conditional amino acids. The essential amino acids (Leucine, Isoleucine, Lysine, Threonine, Methionine, Phenylalanine, Valine and Tryptophan) are not synthesized in the human body but are supplied by food. Although there are many classifications proposed for amino acids, Table 2 below presents a widely accepted classification.

Table 2: Classification of amino acids according to nature

CLASS	NAME OF AMINO ACIDS
Aliphatic	Leucine, Isoleucine, Glycine, Valine, and Alanine
Neutral	Asparagine, Serine, Threonine, and Glutamine
Acidic	Glutamic acid and Aspartic acid
Basic	Arginine and Lysine
Aromatic	Phenylalanine, Tryptophan, and Tyrosine

In Figure 2, it is clear that all amino acids have different structural architecture due to differences in side chains. Some side chains are charged for example lysine/arginine are positively charged and glutamic acid/aspartic acid are negatively charged. These charged atoms are located at or near the end of flexible and long side chains. A few amino acids have polar side chains e.g., threonine, tyrosine and serine contain hydroxyl groups whereas glutamine and asparagine contain amide groups and the remaining side chains are electrically neutral and because of thermodynamically undesirable interactions of hydrocarbons with water, they are called 'hydrophobic' residues [7]. During polymerized, 50 or less amino acids form short chains that are joined by peptide bonds to formed then called *peptides*. When the peptide chains stretch or extend over 50 amino acids then *polypeptides* are formed. Similarly, proteins are formed by multiple polypeptides joined together. Small adjustments in the amino acids sequence can switch the nature of proteins [7].

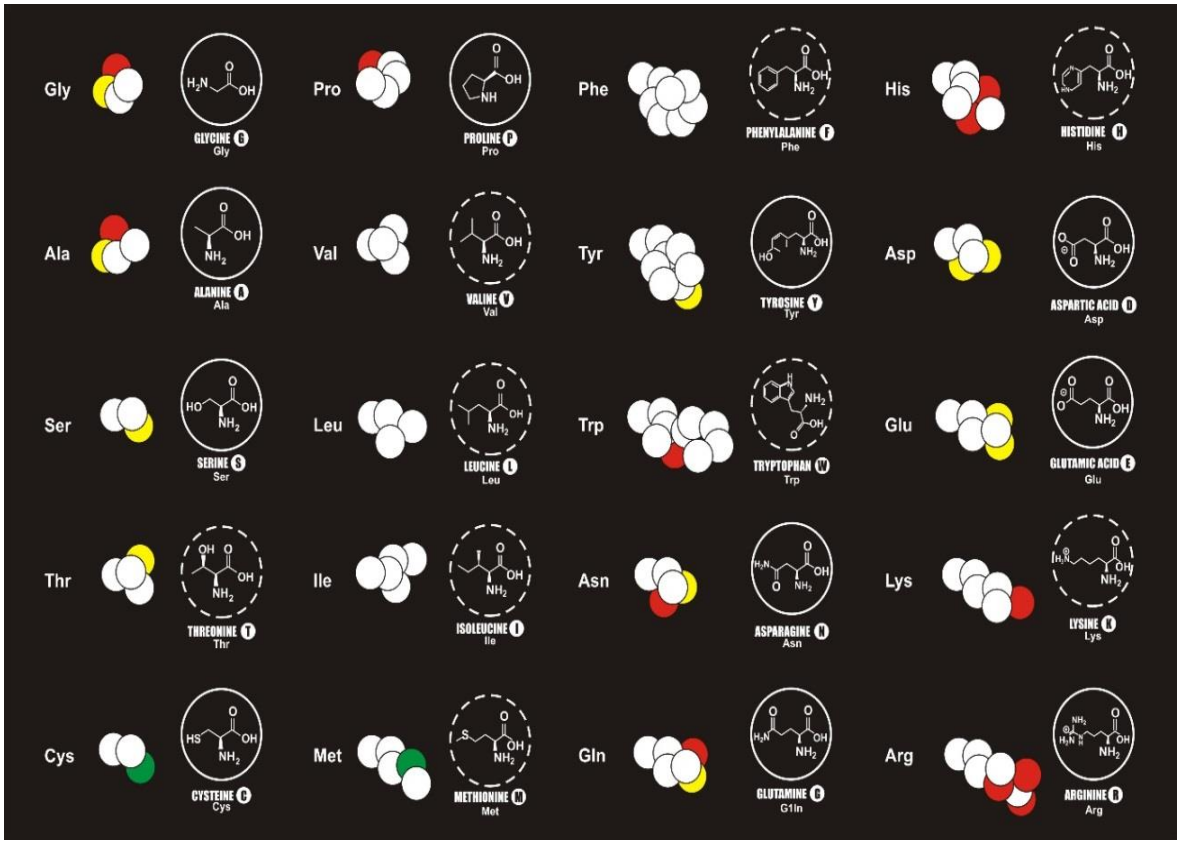

Figure 2: Structural architecture of natural amino acids [1].

Primary, secondary and tertiary levels of protein structure were described by *Dr. Kaj. Ulrik Linderstrøm-Lang* (Danish protein chemist) and the quaternary level was proposed by Dr. John. D. Bernal in 1934. The primary structure is a basic structure, which is determined by the amino acids sequence plus the intra/interchain cross-links. Some rotational movements, lengthening of the amino acids chain and hydrogen bonding (H-bonds) will shape the secondary structure. These polypeptides are flexible and can exhibit α- Helix and β-sheets structure, representing the secondary structure. The α-helix is formed by a single sequential set of amino acids sequence or creation of hydrogen bonding within the same chain e.g. myoglobin. In β- sheets, the main chains

interact by lateral hydrogen bonding. The establishment of new proteins by combining or interaction of helices and sheets will lead to unusual 3-dimensional spatial arrangements of chains that form *tertiary structures* or *folding pattern. Quaternary structure* form when the subunits or polypeptides of the same or different subunits merge together by secondary forces e.g., Hydrogen bonds, disulfide bonds, van der Waals forces and electrostatic forces. The three different structures of proteins are presented in Figure 3 below [8].

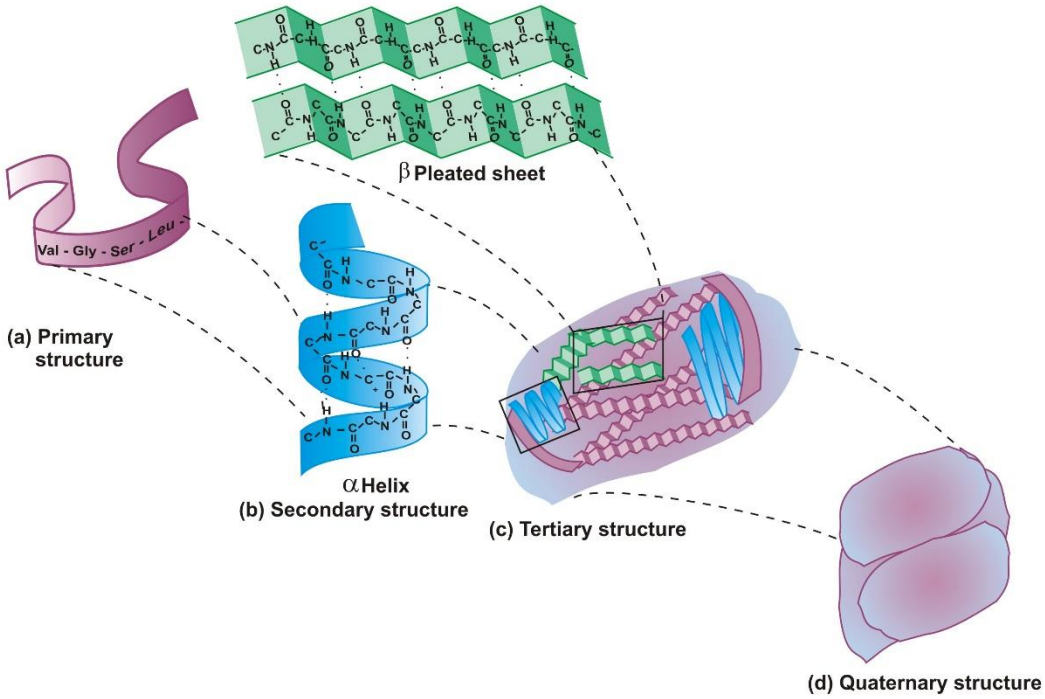

Figure 3: Illustration of the three different structures of proteins [8].

After the completion of protein structure, they are genetically coded for specific functions in the body e.g. regulation, defence, membrane transportation etc. Any interference in the production of proteins or

prevention of binding at target active sites will cause loss of function and diseases such as Alzheimer's and spongiform encephalopathy [9].

1.2. Antimicrobial Peptides

In nature, antimicrobial peptides (AMPs) are evolutionary weapons of multicellular organisms [10]. They are widely distributed in plant and animal kingdoms (vertebrates and invertebrates) as a weapon for offenses and defences. Pioneering studies discovering these peptides led to various host defence peptides, e.g., Defensins, cathelicidins, cecropins, histatins and magainins [11].

A variety of AMPs have been discovered and isolated from different species including plants, animals, microorganisms, fungi and insects in the past 10 years and these are identified for therapeutic functions [12]. Antimicrobial peptides are fascinating novel antibiotic molecules especially these days where antibiotic resistance is becoming a very serious problem [13]. Advantages of peptides include accessibility, practicality and simplicity [14]. AMPs are mostly located in the epithelial and non-epithelial surfaces in humans where they aid to maintain natural barriers and protect against microbial intervention. The AMPs activity begins when they come in contact with the target organism electrostatically. AMPs pass through the cell membrane by binding it and causing the alteration in structure following which they enter the cell and interact with the targets. They initiate the

activity of autolytic enzymes and cease the cell wall biosynthesis, synthesis of DNA, RNA and protein. They possess immunomodulation functions; cause clearance of infection, promote wound healing, and modulate the responses of dendritic cells and cells of the adaptive immune response [15]. The classification of antimicrobial peptides is divided in groups-I, II, III and IV according to their composition and their three-dimensional structure.

a) Group I:

They are the most extensively studied AMPs containing α- helical peptides without cysteines [16]. They adopt disturbed or disordered structures in water and fold into α-helical conformation upon interaction with hydrophobic solvents or lipid surfaces e.g., magainins [17]. Magainins secreted from African clawed frog (*Xenopus laevis*) contains 23 amino acids [18]. These α- helical peptides are absorbed or inserted into the membrane as a cluster of helical bundles and often are found as amphipathic with selective toxicity for microbes [19].

b) Group II:

In comparison to group I, these peptides contain cysteine residues linked by disulfide bridges e.g., human defensins [20], protegrin [21], lectoferricin [22], tachyplesins [23], gramicidin S [24], polymyxin B [25], and tyrocidines [26]. These peptides form β- sheets which are stabilized by interaction with lipid surfaces [27].

c) Group III:

These AMPs have unusually high proportion of one specific or two amino acids sequence for example histatin. Histatin is highly rich in histamine (His) and is produced in saliva, it acts as potent antifungal and antibacterial having a significant wound healing activity [28]. Other examples of this group are cathelicidins [29], tritrpticin (VRRFPWWWPFLRR) [30] and indolicidin (ILPWKWPWWPWRR-amide) [31] . In this group of peptide translocate them across the yeast membrane and targets to the mitochondria of microorganisms [32].

d) Group IV:

These peptides contain a looped structure; they are short in size and can be easily synthesized with proteolytic stability upon binding to lipid membranes. This group of peptides contains Lantibiotics (Nisin) and have the capacity to fight against present and emerging infectious diseases [33].

In the literature, another classification considers the presence of four large families of peptides; α, β, αβ, and non- αβ peptides [34]. Peptides belonging to α- family exhibit α-helical structure e.g., histatins, dermicidin, granulysin and human cathelicidins LL-37. These included in β-family have at least one pair of two β-strands in their structures e.g., human α-defensins and hepcidins. Peptides of the αβ-family contain both α- and β- structures compared to the non-αβ family that do not have any

structure and are called as extended structured peptides [35]. On the basis of structural homology motifs, a different classification of families of antimicrobial peptides can be generated that reflects the relationship between family members. In humans, peptide antibiotics of three families have been identified: the defensins, cathelicidins, and histatins (Table 3).

Table 3: Different types of antimicrobial peptides [36].

Peptide family	Features
Defensins	They are greatly explicit and demonstrate bactericidal, antifungal and antiviral activity and are primarily found in cells and tissues of host defence. According to the position of the cysteins, defensins have been classified as α, β- and θ-defensins.
Cathelicidins	This family structurally diverse and located at carboxyl terminus. They are synthesized and stored in cells as two- domain proteins and on demand break in to cathelin protein or antimicrobial peptide.
Histatins	They are small, cationic, histidine-rich peptides. They are secreted by the oral salivary gland (parotid, submandibular and sublingual glands) and show a potent bactericidal and also fungicidal activity. Therefore play an important role in maintaining oral health by limiting infections in the oral cavity.

1.2.1. Mechanism of Action

There are extensive studies on antimicrobial peptides killing mechanism. Their amino acids compositions, cationic charges, amphipathicity and size allow them to attract and attach to bacterial lipid bilayer to form pores by '*carpet*', '*barrel-stave'* or '*toroidal-pore'* models (table -4) [37].

Table 4: This represents the different models of antimicrobial peptide mechanism of action against microbes.

Model's	Description
Carpet model	Attracted or attached peptides aggregates and penetrate into the membrane bilayer. The hydrophilic peptide domain form the interior region of the pore and hydrophobic peptide domain align with the lipid core region.
Barrel-Stave Model	Peptides position themselves around the cell membrane for binding and this leads to conversion to a bilayer membrane with peptide aggregation. In this way, hydrophobic peptides attach to the lipid side, whereas hydrophilic peptides attach to the interior part of the cell membrane.
Toroidal Model	In this peptide helices put into the membrane and induce the lipid monolayers to bend constantly through the pore so that water core is lined by equally inserted peptides and the lipid head groups.

Despite the fact that all these models are very beneficial for defining the mechanism of antimicrobial peptide activity, their pertinence to how peptide destroy or kill microorganisms needs still some justifications. In recent time, it has been contemplated that pore formation is not only for killing microorganisms. Many remarks come from different research groups for example; translocation of peptides from the cytoplasmic membrane to alter it, inhibition of synthesis (Nucleic acids, Protein and enzymatic activity) [37]. Different studies proved different mechanisms of action in antimicrobial peptides. *Lehrer et.al* observed that *E. coli* bacteria were killed in 15 minutes by DEFB118 forming membranous blebs and therefore decreasing the viability of the bacteria [38]. *Carol et al.* published data on the cationic capability of antimicrobial peptides in comparison to customary bactericidal antibiotics. They observed that the minimum inhibitory concentration and the

minimum bactericidal concentration of antimicrobial peptides are higher than conventional antibiotics and AMPs can destroy bacteria faster compared to antibiotics (Figure 4) [39].

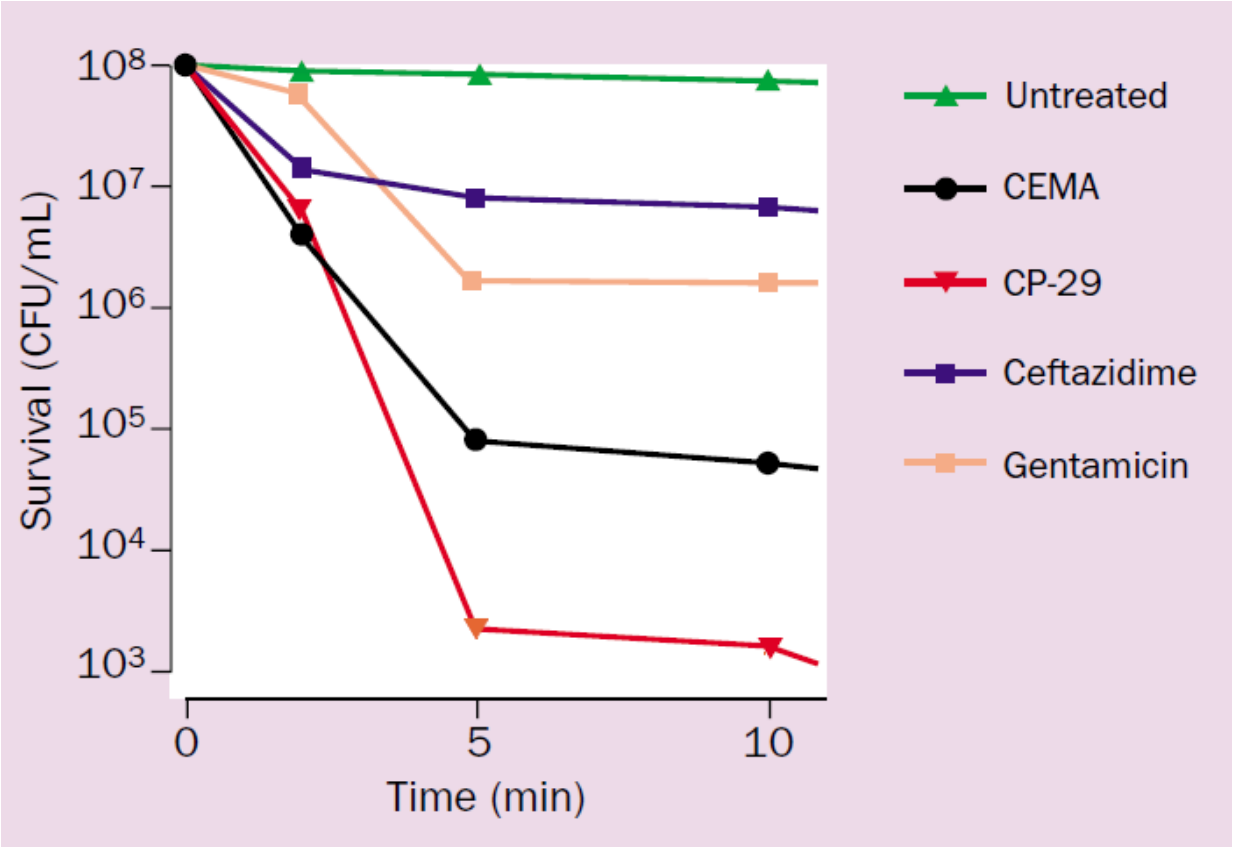

Figure 4: Comparison of AMPs with conventional antibiotics in Mueller-Hilton broth. Ceftazidime at 2 µg/mL, gentamicin at 0.5 µg/mL, CEMA at 4 µg/mL and cationic peptide (CP-29) at 2 µg/mL, published data by Friedrich et al. [39].

The characteristics of AMPs are very important for killing microorganisms e.g. size, sequence, charge, structure, hydrophobicity and amphipathicity [40]. Magainins are 21- 23 peptide residues without cysteine and their activity

against gram negative organisms is 10 times less than cecropins, that have a strongly basic N-terminal and are linked to neutral C-terminal by a pliable glycine-proline chain [41, 42]. The activity of amphipathic α-helical peptides is stronger than those peptides with less defined secondary structures [43]. AMPs are initially attracted toward the bacterial membrane by a mechanism of electrostatic bonding between anionic or cationic peptides. Then they attach to the membrane resulting in thinning of the membrane, formation of pores, creation of holes, chaotic distribution of lipids between the bilayer and finally membrane disruption [10, 44, 45]. Figure 5 shows a proposed model for the mechanism of antimicrobial peptides against bacteria by Shai-Matsuzaki-Huang (SMH). This model represents the mechanism through which antimicrobial peptides attack and destroy bacterial cells when they come in contact. Peptides bind to the outer surface of the bacterial cell membrane to alter in the architecture of the lipid bilayer. Lipids are displaced leading to the thinning of the bilayer and increase in the local surface tension causing the development of pores in the cell membrane thus, allowing penetration of peptides into the bacterial cell and causing their death [46].

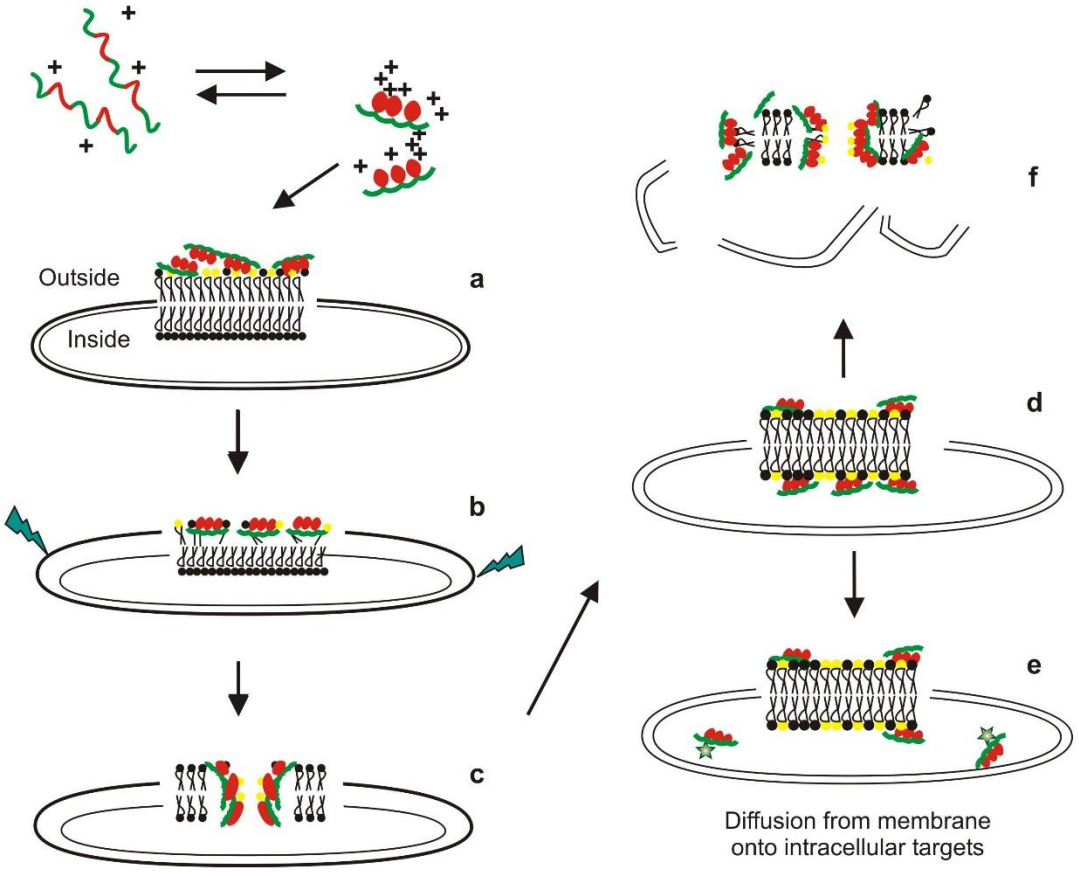

Figure 5: Model proposed by Shai-Matsuzaki-Huang (SMH) for the mechanism of action of antimicrobial peptides. (a) Wrapping of the outer leaflet with peptides. (b) Segregation of the peptide into the membrane and thinning of the outer leaflet. The surface area of the outer leaflet expands relative to the inner leaflet resulting in strain within the bilayer (jagged arrows). (c) Phase transition and `wormhole' formation. Transient pores form at this stage. (d) Transport of lipids and peptides into the inner leaflet. (e) Diffusion of peptides onto intracellular targets (in some cases). (f) Collapse of the membrane into fragments and physical disruption of the target cell's membrane. Lipids with yellow head groups are acidic, or negatively charged. Lipids with black head groups have no net charge [10].

1.2.2. Application in Medicine and Dentistry

Antimicrobial peptides are competent to kill various infectious microbes and play a role as immunomodulatory therefore substantial efforts have been made to use them as potential therapeutic agents. The first antimicrobial peptide based therapeutic application was reported in 1944 by the Russian scientist *Dr. Georgii F. Gause.* He isolated Gramicidins (AMP) from *Bacillus brevis*. These peptides exhibit activity against a broad range of gram-positive bacteria both in vitro and in vivo. Their first clinical use was to successfully treat infectious wounds of guinea-pig skin [47]. After this phenomenal discovery, many other research groups started working on different microorganisms, plants, animals and elicited new antimicrobial peptide families [10, 42, 48]. Table-5 presents a peptides discovery list, sources and activities of synthetic antimicrobial peptides.

Table 5: Discovery, sources and activity of synthetic antimicrobial peptides

Name	Year	Sources	Activity	References
Gramicidins	1944	*Bacillus brevis*	Gram- positive	[47]
Lactoferrin	1960	Milk	Gram – negative and positive	[49]
Bombinin	1962	Frog	Kill bacteria and haemolytic activity	[50-52]
Purothionin	1970s	Wheat endosperm(Plant)	*Pseudomonas solanacearum* and *Xanthomonas campestris*	[53]
Cecropins	1981	*Hylophora cecropia*	*Bacillus subtilis and Enterobacter cloacae*	[54]
Magainins -2	1987	*Xenopus laevis*	*Candida albicans*	[42]
Tachylepsin	1988	Horseshoe crab	Gram-negative R-type bacteria, Gram-negative R-type bacteria	[23]
Protegrin	1991	Porcine leukocytes	bacteria, fungi, and	[55]

			some enveloped viruses	
Cathelicidins	1995	Mammalian myeloid cell	Bacteria killing and promotion of wound healing	[56, 57]
Histatin	1987	Human parotid secretion	Fungistatic effects	[28, 58]

Denture stomatitis is a commonly reported disease of immune-compromised old patients due to poly methyl methacrylate (PMMA) denture base material and the adhesion of *Candida albicans* and biofilm formation. *Yoshinari et al.* synthesised chemically histatins (Hst)-5; antimicrobial peptides secreted by the parotid gland. AMPs were loaded in poly methyl methacrylate (PMMA) surfaces leading to a decrease in the formation of *Candida albicans* biofilm and less colonisation of *C. albicans* [59]. *Pusateri et al.* loaded histatins (Hst)-5, β-Defensins (HBD)-3 and Chlorohexidine in preconditioned acrylic denture base samples in 500 µl saliva and concluded that the action of histatins (Hst)-5 as antimicrobials was very effective [60]. Oral candidiasis is another disorder caused by dry mouth syndrome, immunocompromised and denture wearing patients [61]. In order to treat the above infection, Xanthan (artificial saliva) with cationic antimicrobial peptides have been used effectively [62].

Currently, during clinical use of biomaterials such as in contact lenses, tooth filling materials, bone cements, implanted cardiac valves, urinary catheters, endotracheal tubes, coronary stents and hip/dental implants, infections have been developed and manufacturers are keen to develop multifunctional

devices with improved bioactivity, antimicrobial and anti-infective behaviour [63-65]. These approaches are described in detail with the use of examples in Table 6.

Table 6: Approaches used to make antimicrobial biomaterials for tackling microbial infections.

	Approach	References
1	Modification of the biomaterial surface to confer anti-adhesive properties; - Coatings based on hydrophilic polymeric brushes - Based on poly (ethylene glycol) (PEG) and/or poly (ethylene oxide) (PEO) - Bio-surfactants - Polyamidoamine based dendrimers	[66-68]
2	Doping the material with antimicrobial substances - Antibiotic loaded biomaterials - Loading with disinfectants and bactericidal substances (e.g. NO^{+}, Ag^{+}, Zn^{2+}, Cu^{2+}, Chlorohexidine, lysozyme, metal nanoparticles) - Immobilized antimicrobial peptides	[69-71]
3	Combining anti-adhesive and antimicrobial coatings - Multilayer film constructed by assembling layer-by-layer heparin and chitosan - Covalent conjugation of antimicrobial peptides immobilized onto a hydrophilic polymer	[67, 72, 73]
4	Materials able to oppose biofilm formation and, at the same time, to support tissue integration - Silver containing hydroxyapatite coatings - Poly(L-lysine)-grafted-poly(ethylene glycol) functionalized with adhesive peptides such as RGD - Bio-glasses doped with gold nanoparticles	[74-76]

A successful *in-vitro* drug release, cytotoxicity and antimicrobial study was reported by *Mehdi et al.* In this study, *in-vitro* bone growth (osteo-conductive) was achieved by using calcium phosphate coatings on medical grade titanium surfaces loaded with antimicrobial peptides (HHC36:

KRWWKWWRR and Tet213: KRWWKWWRRC). The surfaces were successfully tested against *Staphylococcus aureus* and *Pseudomonas aeruginosa* [77]. *Wilmes* and *Sahl et al* reported very recently in their review about human defensins, the fascinating role of host defense peptide (HDP) in anti-infective drugs [78]. The presence of disulfide bridges in defensins raised the value of resistance against proteolytic degradation [79]. Human 1-4 β-defensins play a key role in wound healing by migration and proliferating epidermal keratinocytes to promote healing [80, 81]. Antimicrobial peptides are suitable candidates to combat microbial colonies and inhibit biofilm formations [82, 83].

1.3. Design of Antimicrobial Peptides

There is currently huge demand for the use of alternative antimicrobial approaches that can lead to a decrease in antimicrobial resistance [43]. Peptides can be derived from three sources (a) Natural or bioactive peptides (produced by plants, animals or humans), (b) Use of genetic or recombinant technology for isolating peptides and (c) Use of chemical libraries for discovering peptides [40]. There are few estates modulated by peptide design and modification like increasing activity against microbial cells, heighten susceptibility against proteolytic degradation and reduction in immunological reactions against human cells [40, 48]. Reported data on designing antimicrobials include peptide analogues or hybridization through amino acid

deletion of inactive or combining active motifs together for increased antimicrobial activity. Other methods include the chemical synthesis of antimicrobial peptides mimicking natural antimicrobial peptides and screening peptide structural-activity relationship from combinatorial libraries for better understanding [84]. Usually, the amount and size of the peptide sequence determines the method of their production e.g., Chemical synthesis, soluble fusion expression method, recombinant DNA technology, cell-free expression system, plant or enzymatic method [85]. Especially, synthetic therapeutic peptides production has become possible for pharmaceutical companies or biotech laboratories with the introduction of solid-phase peptide synthesis (SPPS) by Merrifield and his work on three principle sequential synthesis, convergent synthesis and chemical ligations [86, 87]. Very recently *R. Jiang* and *S.S.J. Leong et al.* reported a new method "Intein based bioprocess" for synthesizing AMP as an alternative to solid-phase peptide synthesis (SPPS); this method is cheaper, eco-friendly to manufacture for 11-mer synthetic antimicrobial peptides [88].

1.4. Human Defensins

Defensins are a large family of antimicrobial peptides with molecular weight of 4-5 kDa and more extensively studied as well. Defensins are short peptides (less than 50 amino acids) with positive net charge, cationic, and meaningful proportion of hydrophobic residues that permit to adopt amphipathic

structures in membrane mimicking environments [89]. These peptides contain 6-8 cysteine residues which form 3-4 disulfide bonds with arginine and aromatic residues. These cysteines are linked in 1-6, 2-4 and 3-5 positions. This family is subdivided into α-, β- and θ-defensins on the basis of molecular architecture and connectivity of disulfide-bonds. During the last decades, sequences of endogenous α- , β- and θ-defensins were identified [78]. Their sources and history are presented in Table-7. Both α & β- defensins were found in breast milk, marking the role of defensins in protecting infants from infections.

Table 7: History and sources of defensins peptides.

Name	Discovery	Source	Reference
α- Defensins (1-4)	1980s	Human neutrophil granules	[90]
α- Defensins (5-6)	1992-1993	Small intestinal Paneth cell	[90]
β-Defensins-1	1995	Purified from human plasma but also found in epithelial cells originate from the kidney, respiratory tract, pancreas, oral tissues (like parotid gland, buccal mucosa and tongue) and female reproductive tract.	[91, 92]
β- Defensins-2	1997	Extracted from lesional scales from psoriasis patient and is greatly found in skin, lung and trachea, also in gingival mucosa.	[91, 92]
β- Defensins-3	2000	It's also extracted from lesional scales from patients suffering from psoriasis and also detected in the placenta, adult heart, skeletal muscles, thymus, oesophagus and trachea.	[93]
β- Defensins-4	2001	Present in high levels in the gastric antrum and testis. It's also expressed in uterus, lungs and kidneys.	[90, 91]
β- Defensins-5 & 6	2002	Human epididymis	[94]

β- Defensins-25 to 27	2003	Their transcripts only exist in few organ .e.g. male genital tracts.	[94]
θ- Defensins (RTD1),(RDT2) and (RDT3)	1999	Isolated from leukocytes of Rhesus macaques.	[95]

1.4.1. Role of disulfide connectivity on the antimicrobial behaviour of human defensins

Defensins are cysteine-rich and they have a β- sheet structure which is stabilized by three disulfide linkages. This chemical linkage is covalent and is derived by coupling of the two thiols groups. In α–defensins the disulfide bridges form between the first and the sixth cysteine residues (Cys^1-Cys^6), Cys^2-Cys^4, and Cys^3-Cys^5 , while in β–defensins, the cysteine bridge form in Cys^1-Cys^5, Cys^2-Cys^4, and Cys^3-Cys^6 residues [96]. It has been suggested that the antimicrobial activity of defensins depends upon this disulfide bridging. θ-Defensins are macrocyclic structures with disulfide linkages in between Cys^1-Cys^6, Cys^2-Cys^5, and Cys^3-Cys^4 residues [84]. Different disulfide linkages is believed to show variation in molecular structure and antimicrobial function but as Miguel et al. reported, the antimicrobial activity of human β- defensin-3 is independent of disulfide bridging. Only the chemotactic property is dependent on the disulfide bond formation [97]. In nature these disulfide bonds are taken care by the protease inhibitors and proteolysis [98]. In a molecular dynamic study (MDR) of human defensins (HNP-3 and HBD-1) by *Nagaraj R. et al.* it was reported, that non-native

disulfide bonds are biologically active against bacteria in comparison with native disulfide bridged human defensins (HNP-3 and HBD-1) [99].

1.4.2. Mechanism of antimicrobial action of human defensins

The meticulous antimicrobial mechanism of defensins has not yet been clarified. It has been suggested that they can permeate the cell membrane through the formation of multimeric pores. The cationic nature and amphipathic characteristics enable binding to the cell membrane lipid bilayer leading to leakage of the internal content of the bacterial cells [78]. It has been reported that all defensin precursors were established on the basis of similarities in architecture, sequences, mode of action, and inter-physicochemical functionality [100] e.g., Myxobacteria (*Anaeromyxobacter dehalogenans* and *Stigmatella aurantiaca*) showed strong resemblance in sequences and construction to fungal defensins [101]. The human β-defensins have been widely studied and their role has been widely recognised as the main contributors of innate immunity for direct action against gram-positive and gram-negative bacteria [102], viruses [15], fungi and parasites [99].

1.4.3. Types of Human Defensins

1.4.3.1. α- Defensins

In 1980s *Lehrer et al.* observed a biological molecule 29 to 35 amino acids long with cationic capability and expressed by human neutrophils [103].

Human α-defensins-1,-2,-3 and -4 are mainly synthesized by bone marrow neutrophil precursor cells (promyelocytes) and these mature peptide is stored in neutrophil granules [36] but human neutrophil peptide (HNP)-5 & -6 are emancipated as a propeptide which processed extracellularly [104]. *Selsted et al.* proposed a canonical sequence of α-defensins as $x_{1-2}CxCRx_{2-3}Cx_3Ex_3GxCx_3Gx_5CCx_{1-4}$, where x symbolises any amino acid residue [105]. The structural homology of HNP 1-3 is highly similar to HNP-4 and the peptides have been studied as closed group and few studies revealed their antimicrobial and immunomodulatory functional distinctions [106]. *Lehrer et.al* demonstrated the direct action of HNP-1, HNP-2, and HNP-3 against herpes simplex virus (HSV) type 1 and 2, cytomegalovirus, vesicular stomatitis virus, and influenza virus. HNP-1 has a more potent neutralizing effect against HSV-2 however, represented no activity against non-enveloped viruses [107].

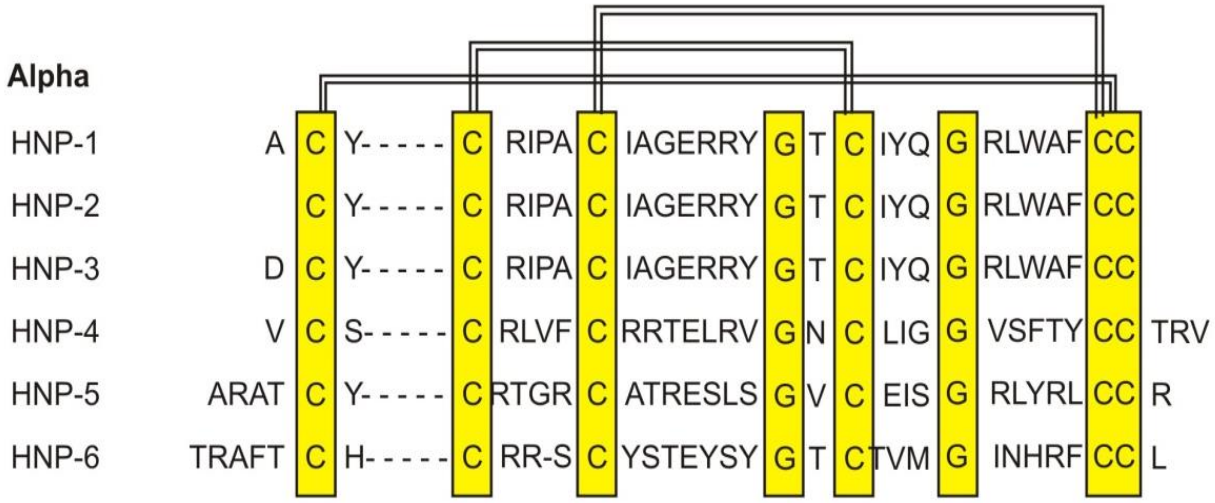

Figure 6: The amino acid sequence of α- human neutrophil peptides with cysteine bonding.

1.4.3.2. β- Defensins

In 1993, *Selsted.et.al* and co-workers isolated and purified bovine tracheal cells and observed 38-42 amino acid peptides, six-cysteine motifs creating three disulfide bonds [108]. Up-to-date six human β-defensins have been identified [89] and five gene-based searches (HBD-25 to HBD-29) reported by *Peng Jia, Hong, et al* and *Yashwanth, et al* [109, 110]. It is strongly evidenced by Hancock and Scott (2000), that β-defensins play a key role in the defence of mucosal and body epithelial surfaces [111]. The canonical sequence of β-defensins is presented by *Selsted.et.al* as $x_{2\text{-}10}Cx_{5\text{-}6}(G/A)_xCX_{3\text{-}4}Cx_{9\text{-}13}Cx_{4\text{-}7}CCx_n$ [108]. The highest endeavoured members of β –defensins are HBD-1, HBD-2 and HBD-3 because of highly effective killing capability for gram negative in comparison to α-defensins [112] and their expression

identified in epithelial surface of external skin [102], oral mucosa [113], trachea, lungs [114] and uterus [115].

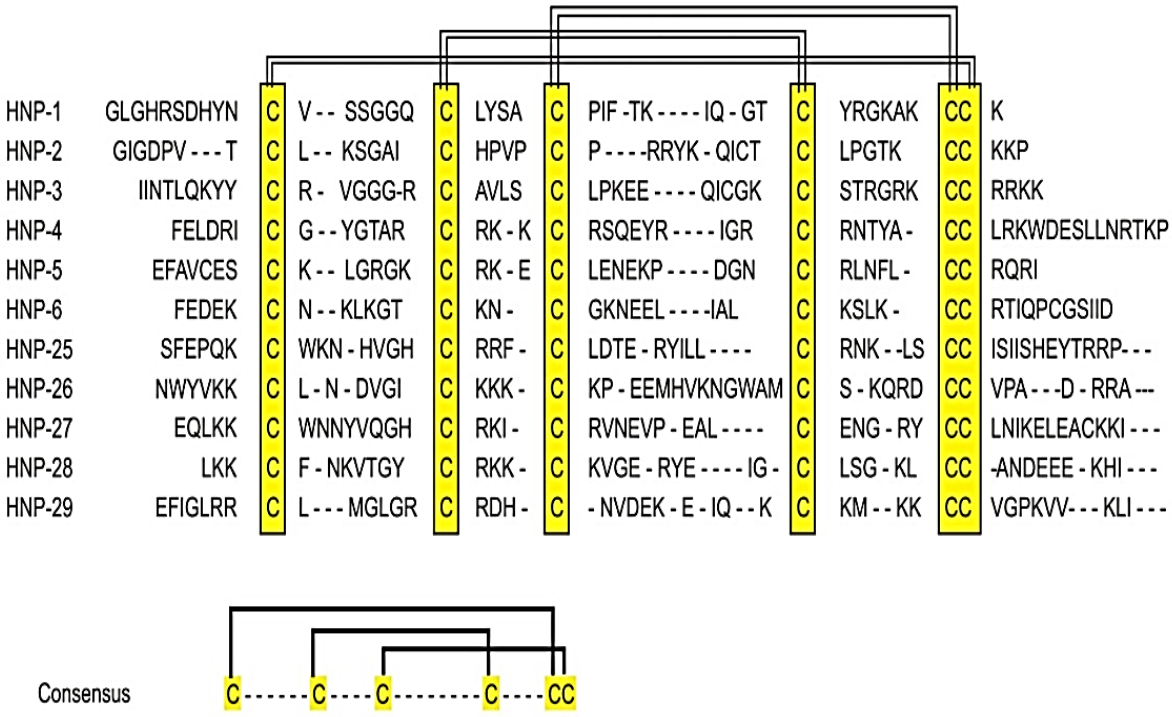

Figure 7: Structures of β- Defensins.

14.3.3. θ- Defensins

In recent years, a new family of lectin-like cyclic octapeptides are explicit from polymorph nuclear leukocytes (PMNs) of old world monkey (macaques and baboons) but not new world monkeys (gorillas and chimpanzees) [95]. They exit as RDT-1 to RDT-6 [116]. Selsted et al. suggested that the antibacterial activity of θ–defensins is greater compared to human α – defensins and the RDT-1 ,-2 and -3 have similar antimicrobial activity [117]. θ-defensins may be conjugated from different head-to-tail splicing of non-

peptides from the similar pro-θ-defensins precursor (Figure 8) [116]. *Cole et al*. identified a pseudogene from the human bone marrow that is encoded as antimicrobial peptide analogous to rhesus monkey mini-defensins (Circular peptides) with potent ability to restrain pro-viral DNA formations [118]. Previous study on synthetic retrocyclin-1 has shown effect on human immuno deficiency virus, type 1 (HIV-1) by inhibiting envelop mediated fusion at concentration of 4 to 6 µM [119].

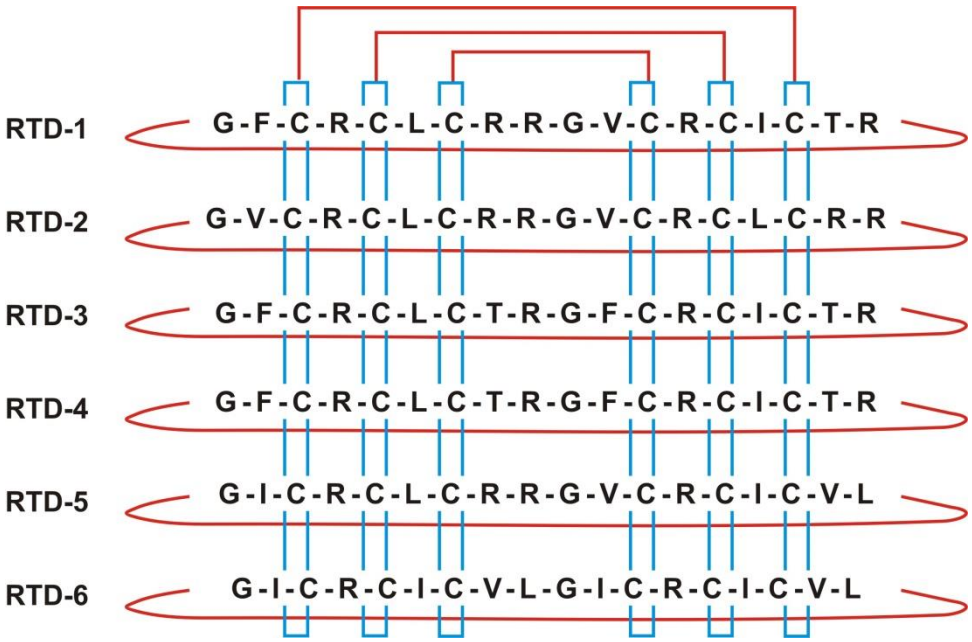

Figure 8: Description of θ -defensin sequences. The blue line represents disulphide linkages and the red circular lines show the circular folding.

1.5. SOLID PHASE PEPTIDE SYNTHESIS (SPPS)

As discussed in the previous section about the synthesis of proteins or peptides, here in this section a detailed description of solid phase peptide synthesis is provided. This method was introduced by Robert Bruce Merrifield in 1959 and in his work published in the Journal of the American

Chemical Society [87] he reported that during "solid phase peptide synthesis" "*Polypeptides are joined with one and other by amide bond or peptide bond*". These bonds form between amine groups of one amino acid (N-terminal) to carboxylic acid (C-terminal) of a second amino acid (Figure 9).

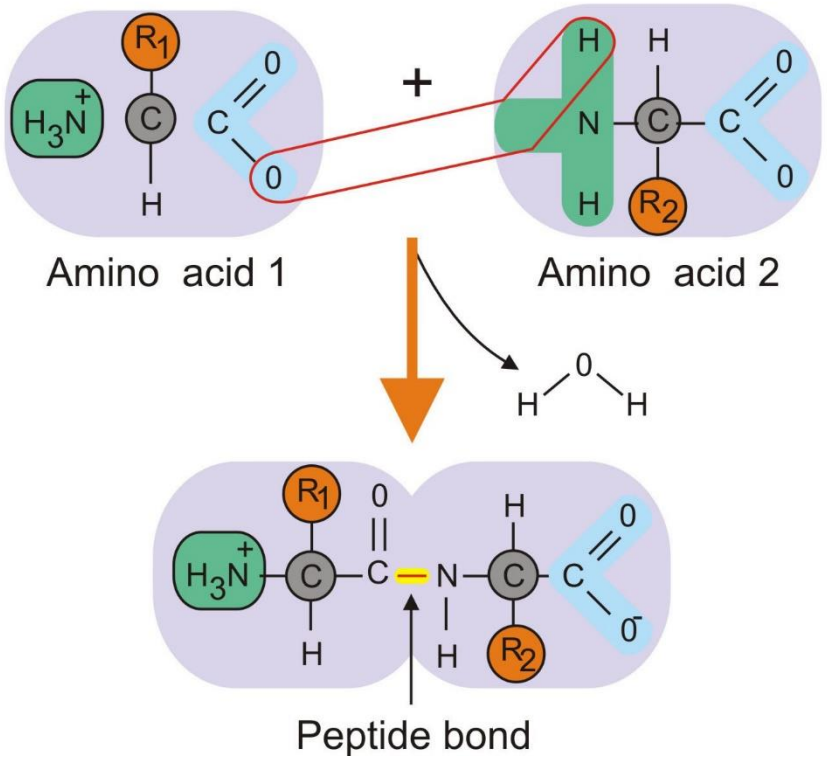

Figure 9: Representation of peptide bond formation between C-terminal of first amino acid and the N-terminal of the second amino acid.

Peptide chains have two terminals; the N-terminal and C-terminal). The C-terminal is attached to polymer beads by a linking agent. Commonly used linking agents are di- and tri-substituted benzenes. The amino acids also have their amino group protected for the prevention of acid reaction with each other. The protective molecule is 9-fluorenylmethoxycarbonyl (Fmoc).The

Fmoc protected amino acids also give high purity and yield as well. The conventional solid phase peptide synthesis (SPPS) cycle is illustrated in Figure 10.

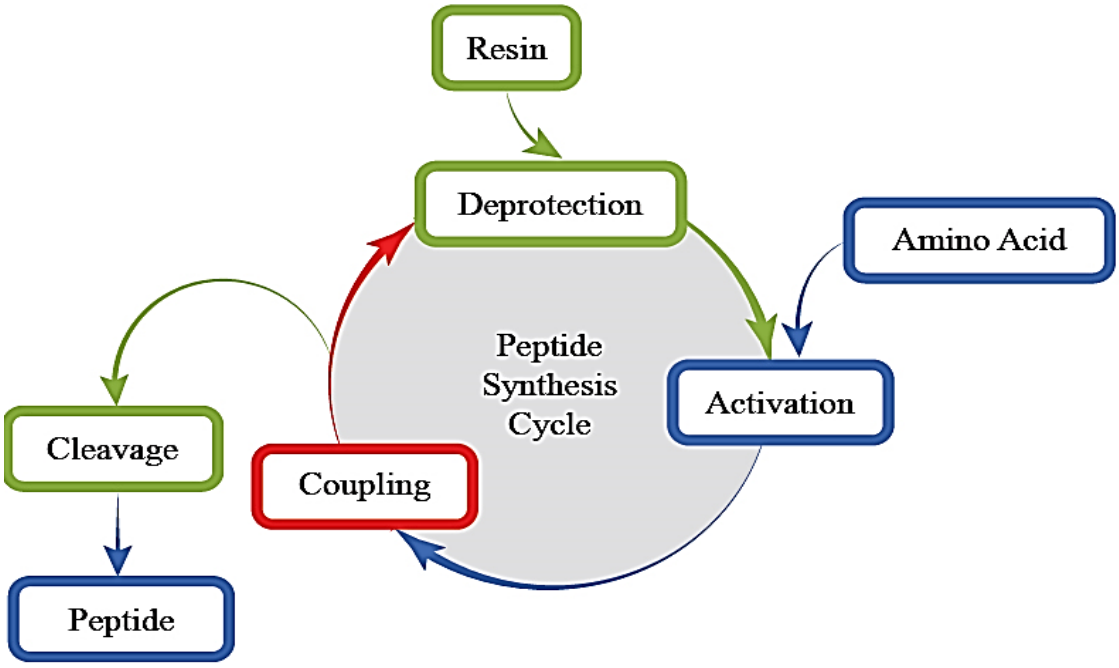

Figure 10: illustration of the Solid-phase peptide synthesis (SPPS) cycle.

1.6. RESEARCH AIMS & OBJECTIVES

The main aim of this project is to develop, identify, and characterise antimicrobial peptide sequences inspired by the antimicrobial structural motifs of human defensins. The peptide sequences that were synthesized by the SPPS method are shown in Table 8. The purity of peptides is very important to create novelty in the peptide sequences. This purity will give good antimicrobial properties.

Table 8: Description of all peptide sequences with their molecular weight and nature.

Sequence Code	Peptide Sequence	Length of Amino Acids	Molecular Weight	Peptides Status	Natural Source
DLAMP-1	PACIAGERRYG	11	1192.37 g/mole	Basic	Defensins Human Neutrophil peptide 1 & 2
DLAMP-2	CATRESLSGVC	11	1125.29 g/mole	Neutral	Human α-Defensins 5
DLAMP-3	CRVRGGRCA	9	977.18 g/mole	Basic	Human β-Defensin 3
DLAMP-4	GTCIYQRLNAF	11	1285.49 g/mole	Basic	Defensins Human Neutrophil peptide 2 & 3
DLAMP-5	GTCGLPGTKCC	11	1039.27 g/mole	Basic	Human β-Defensin 2
DLAMP-6	CISEKTTDGHC	11	1193.32 g/mole	Neutral	Nad1 From Nicotiana Alata (plant protein)

DLAPM (Defensin-like antimicrobial peptides).

CHAPTER 2: MATERIALS AND METHODS

2.1. Materials

For the synthesis reaction Aldrich® System 45™ vessels with cap and fritted disc were purchased from Sigma-Aldrich, UK. All amino acids were N-terminal Fluorenyl-methyloxy-carbonyl (Fmoc) protected with purity of >98%. Amino acid preloaded Wang resin beads (loading 0.7 - 5.3 mmol) and O-benzotriazole-N, N, N', N'-tetramethyl-uroniumhexafluoro- phosphate (HBTU) used in Solid Peptide Phase Synthesis (SPPS) were also purchased from Sigma-Aldrich, UK. The amino acids and resin beads used are listed below: Fmoc-Ala-OH, Fmoc-Ala-Wang resin (loading 0.33 mmol/g), Fmoc-Arg(Pbf)-OH, Fmoc-Asp(OtBu)-OH, Fmoc-L-Asparagine(DOD) , Fmoc-Cys(Trt)-OH, Fmoc-Cys(Trt)-Wang resin (loading 0.7 mmol/g), Fmoc-Glu(OtBu)-OH, Fmoc-Gln(Trt)-OH, Fmoc-Gly-OH, Fmoc-Gly-Wang (loading 0.36 mmol/g) , Fmoc-His(Trt)-OH, Fmoc-Ile-OH, Fmoc-Phe-OH, Fmoc-Phe-Wang resin (loading 0.07 mmol/g), Fmoc-Ser-OH, Fmoc-Thr(tBu)-OH, Fmoc-Trp-OH, Fmoc-Val-OH, Fmoc-Leu-OH, Fmoc-Lys(Boc)-OH and Fmoc-Pro-OH. N,N-Dimethylformamide (DMF), Dichloromethane (DCM), N,N-diisopropylethylamine (DIPEA) and piperidine were used as solvent and were all purchased from AGTC Bioproducts. Ninhydrin for Kaiser Test was purchased from Sigma-Aldrich,

UK. DL-Dithiothreitol (DTT) was used for inhibiting formation of Disulfide bonds and was purchased from Sigma-Aldrich, UK. Trifluoroacetic acid (TFA) and triisopropylsilane (TIPS) of HPLC grade and purity of >99% were purchased from Sigma-Aldrich, UK. MILLEX®HA filter paper and Acetonitrile [high-performance liquid chromatography (HPLC) grade] were also supplied by Sigma-Aldrich, UK.

For the antimicrobial testing, Wheaton narrow mouth bottles with caps, Nunc® inoculating loops, Petri dishes (Polystyrene) supplied by Sigma Aldrich, UK were used. For the cultivation of bacteria, Oxoid Ltd., Basingstoke, UK supplied the tryptic soy broth (TSB) containing 0.25% glucose.

In this project, the sensitivity of the peptide bond is very important, so the protocol is modified as resin deprotection, activation of first amino acid, coupling, then double coupling of same amino acid and this step continues till last amino acid of the sequence.

2.2 Solid-Phase Peptide Synthesis

In this research project, the fluorenyl-methyloxy-carbonyl (Fmoc) protecting group was used. For the deprotection of N-Fmoc a short treatment of 20% piperidine in DMF for 30 min with shaking at 300 rpm in the tightly closed reaction vessel was used. The liquid was drained and the resin beads were washed 3 times with DMF (2mL), 3 times with DCM (2mL) and again 3

times with DMF (2mL) and for every wash, the solution was discarded by vacuum filtration. Coupling of the second amino acid was done by Fmoc-AA2 and HBTU dissolved in DMF (1.25 mL) followed by addition of DIPEA (0.0016 mL).The solution was mixed and added to the washed resin beads. This reaction usually takes 2 hours at 250 rpm to complete except for cysteine and arginine. Then it was filtered off by vacuum filtration and the resin beads were washed again. Each washed solution was discarded by vacuum filtration. After this, the coupling progress was monitored by the Kaiser Test or Ninhydrin test. This test was performed by adding 10 resin beads to the solution of ninhydrin in water (5/10, w: v, 1 mL) allowing them to boil for 3 minutes. If the test was positive [blue/purple resin beads], coupling was incomplete. In this case, the coupling solution was discarded and new coupling solution was added with the use of the same amino acid. Negative test was indicated by pale yellow/brown colour. The complete reaction was followed by deprotection, wash and coupling with the next Fmoc-AA in the sequence.

This cycle continued until all of the amino acids were coupled on the resin beads. The final deprotection of the Fmoc group was performed and again resin beads were washed 3 times with DMF (2mL) followed by 3 times with DCM (2 mL) and then 3 times with DMF (2 mL) followed by another 2 times

with DCM (2mL) and dried finally in vacuum. The dried peptides were then transferred to a bottle and left open in desiccator for 24 hours.

2.2.1. Deprotection of Fmoc Group from the N-Terminal of Amino Acid

Resin beads were swollen by 2mL DMF for 30 minutes using an orbital shaker (300 rpm). Then, piperidine was used for the cleavage of the Fmoc protection group and the mechanism is illustrated in Figure- 12. A mixture of piperidine in DMF (2mL, 1:5 v: v) was added in the synthesis flask and was tightly closed. The flask was fit in an orbital shaker for the reaction. The process continued for 30 minutes with a frequency of 250 rpm. Then the reaction solution was removed by vacuum filtration and reaction vessel walls as well as the resin beads were washed three times with DMF (2mL), three times with DCM (2mL) and finally again three time with DMF (2mL) for ensuring that no piperidine residue was left in the reaction vessels.

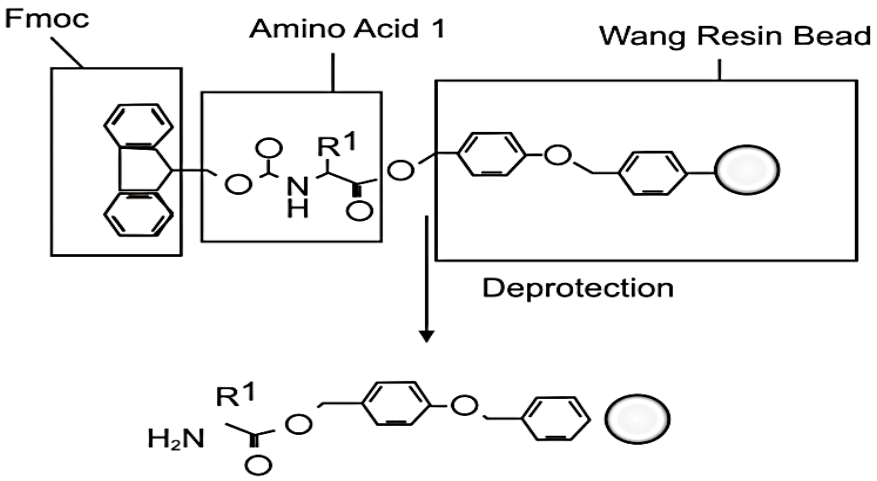

Figure 11: Representation of the Fmoc group deprotection from an amino acid.

2.2.2. Cleavage of peptide from the resin

The most crucial step of solid phase peptide synthesis (SPPS) is the cleavage of peptides from the resin. Serious care should be taken here to complete the reaction. Few amino acids have problems with trifluoroacetic acid (TFA) cleavage cocktail mentioned in Table 1. In this stipulation, protected side-chain groups produce stabilized carbo-cations, which react with electron charged side chains of amino acids e.g. cysteine, methionine, tyrosine, threonine, serine and tryptophan and release unwanted by-products. Scavengers added in the cleavage cocktail to trap these carbocations. In this project, the cleavage cocktail consisted of 88% trifluoroacetic acid (TFA) , 5% phenol crystals , 5% triisopropylsilane (TIPS) and 2% deionised water (88/5/5/2). The sequence of added chemicals should be like this: crystals of phenol, H_2O, TIPS and TFA. After preparing the cleavage cocktail, the resin was added in the flask. 5mL of the cleavage cocktail for 0.5 g of resin was enough to saturate and swell the resin. The reaction time varied according to the presence of amino acids e.g. one arginine may take 4 hours, two arginine can take 8 hours and one cysteine can take two hours etc. The mixture was left for 2-8 hours stirred with the help of a magnetic stirrer. On completion of reaction, the cleavage mixture and the resin beads were filtered and then added drop by drop in to ice cold diethyl ether. Figure-13 shows the cleavage reaction.

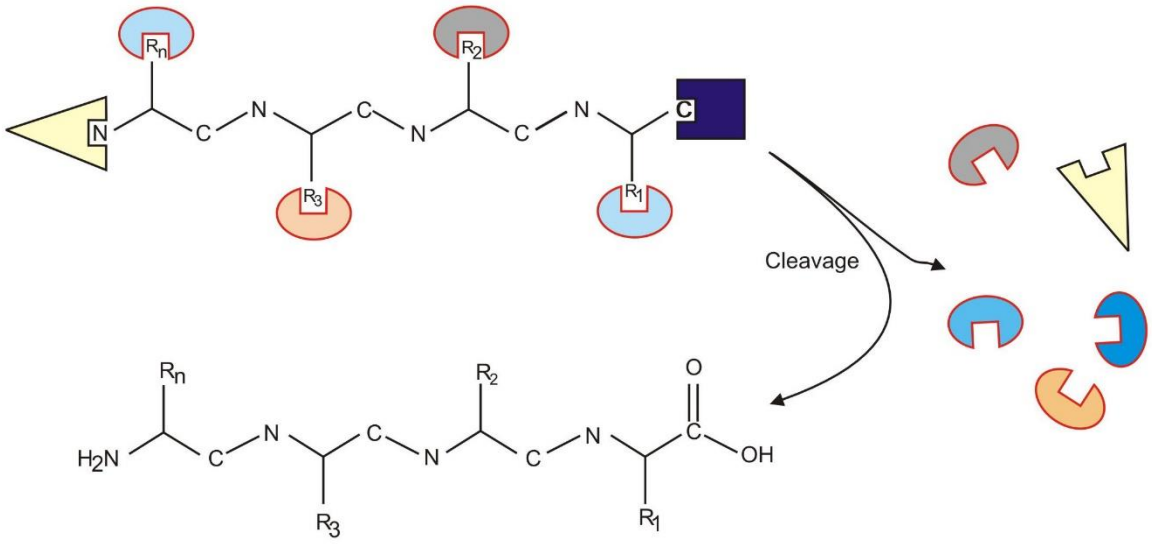

Figure 12: Illustration of the cleavage mechanism.

2.2.3. Precipitation of Peptides

The peptide solution was filtered in a clean round flask with the help of a filter paper. The peptide solution was added drop wise in ice cold diethyl ether for precipitation and the precipitate was left in -20˚C in a freezer overnight. Then the precipitate was transferred in an Eppendorf tube and centrifuged at 13,500 rpm for 4 minutes. Finally, the supernatant was carefully discarded in a waste bottle and the Eppendorf with the peptides were dried in vacuum for 2 hours and were then transferred in a freezer at -20˚C for storage.

2.3. Characterisation of Antimicrobial Peptides

2.3.1 High Pressure Liquid Chromatography (HPLC)

For the analysis and purification of all six peptide sequences a Dionex Summit High Pressure Liquid Chromatography (HPLC) system was used. The use of this technique for the evaluation of proteins and proteomics is very useful since ages. In this machine the synthetic molecule is separated on the basis of its hydrophobicity by using different columns (C4, C18) and a stationary phase (sorbent). The component of the analytical solvent passes through the column at different velocities which are recorded according to the chemical nature of each component (both stationary phase and mobile phase). The time at which a particular analyte elutes from the column is called retention time and this will characterise the features of the analyte. In this case, the analytical Dionex Summit equipment was used to study the purity of the peptides. A Dionex Summit Preparative HPLC was used also for the crude peptide purification.

For the analytical HPLC, precipitated peptides were dissolved in 1ml Deionized water and 0.0018g Dithiothreitol (DTT). Use of DTT in all sequences helped to prevent disulphide bonding between cysteines. Ready peptide solution was injected into the column and run for 60 minutes; during this linear solvent gradient were 100% inorganic solvent and 0% organic solvent. The amount of organic solvent was increased for the elution of

hydrophobic molecules from the column to 40% water and 60% acetonitrile. Finally the calculation of the initial purity was based on merging the peak areas.

After collecting suitable peaks a similar procedure was performed using the preparative HPLC. The peptides were filtered by MILLEX®HA filter paper and then were put through reverse phase-high pressure liquid chromatography (RP-HPLC). Different peaks represent different components. In order to purify the peptides, a particular component flows out of the column and is collected. The collected peptides were identified by mass spectrometry.

2.3.2. Mass spectrometry

Peptide sequences were analysed by chemical ionization-mass spectrometry in the School of Chemistry, University of Birmingham. This is an analytical technique that characterises molecules by means of measuring the masses of their ions. When liquid, gas or solid ionized when bombarded by electrons, charged fragments form [120]. The sequence of fragmentation is; ionization, acceleration, deflection and detection [121]. In the mass-spectra the X-axis represents the detected ion molecular weight divided by the charge of the ion (m/z) and the Y-axis represents the relative intensity.

2.3.3. Anti-Microbial Study

Minimum inhibitory concentration and minimum bactericidal concentration were measured by using methods delineated previously by *Wiegand et al.*

Staphylococcus epidermidis (ATCC) and *Pseudomonas aeruginosa* (ATCC) stains were provided by Dr Izabela Radecka of the School of Biology, Chemistry and Forensic Sciences at the University of Wolverhampton.

2.3.3.1. Minimum Inhibitory Concentration (MIC)

500µL of tryptic soya broth (TSB) were added in all 9 sterilized bottles (see Figure 14). Three bottles were used for control group; one with only TSB, second bottle contained TSB with peptide, and third contained TSB with microorganism. The other five bottles contained different peptides concentration (35mg/mL, 17.5 mg/mL, 8.75 mg/mL, 4.375 mg/mL, 2.187 mg/mL and 1.09 mg/mL). The bacterial culture was diluted in TSB 1:100 [10-8 CFU per mL] and 10µL of bacterial culture were added in all bottles except the bottles that contained TSB and TSB with peptides. After adding bacterial culture the bottles were incubated for 24 hours at 37˚C. The sample's turgidity was recorded as low turgidity (+), medium turgidity (++) and high turgidity (+++). After taking turgidity records, the bottles were incubated again for additional 48 hours and additional readings were taken.

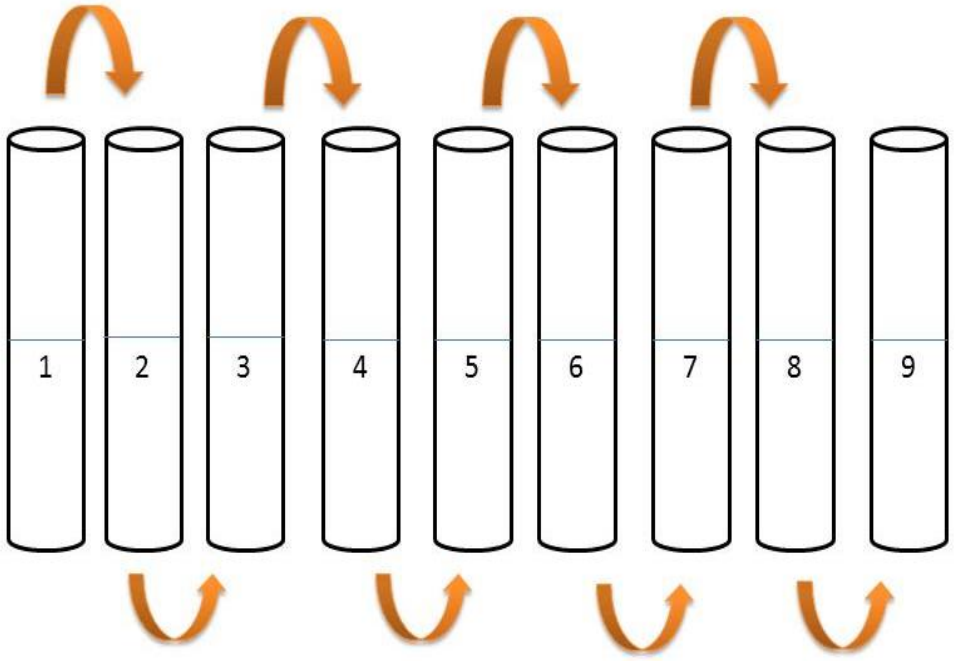

Figure 13: Dilution test.

2.3.3.2. Minimum Bactericidal Concentration (MBC)

For minimum bactericidal concentration, preparation of bacteria free agar plates to culture all samples for confirmation of bacterial growth was performed. Agar plates were divided into four parts (three parts for peptides and one for penicillin as a control group) for spreading bacteria culture. A sterilised Inocular was used to spread bacterial culture and all samples were incubated in 37˚C for 24 hours (Figure 15). *P. aeruginosa* growth was different and had a green colour, whereas *S.epidermidis* was opaque.

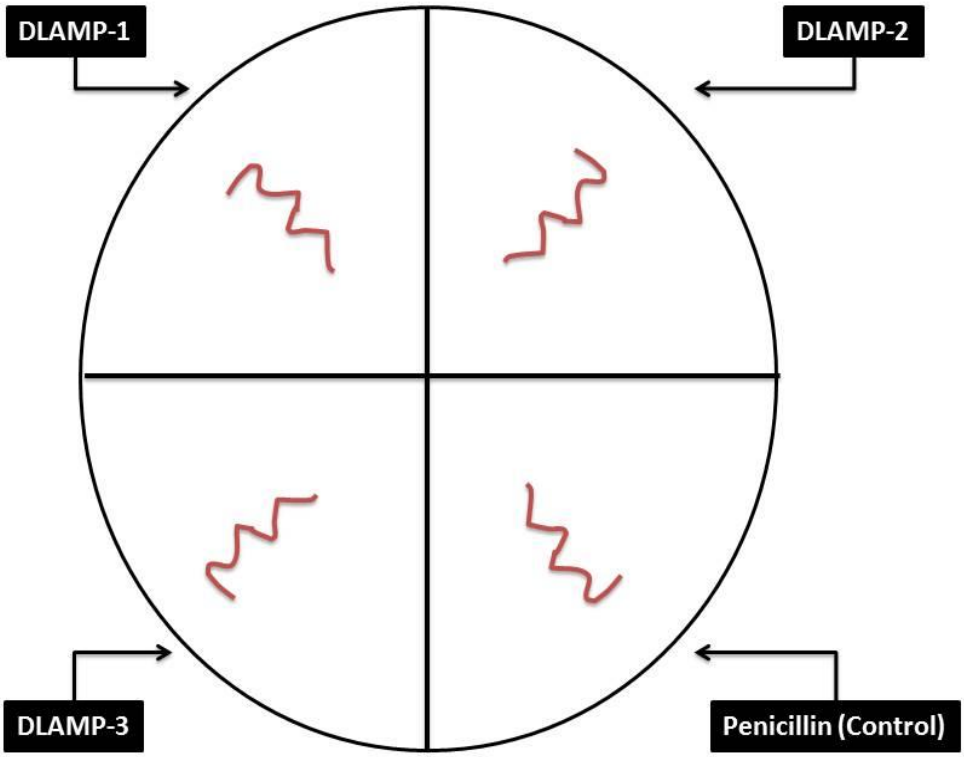

Figure 14: Agar plate test for measuring the minimum bactericidal concentration (MBC).

Chapter 3: RESULTS AND DISCUSSION

3.1. Fluorenyl-methyloxy-carbonyl (Fmoc) Solid Phase Peptide Synthesis

The human defensins-like peptide structures were synthesized by fluorenyl-methyloxy-carbonyl (Fmoc) solid phase peptide synthesis (SPPS) and for better peptide linkage between amino acids, double coupling of peptides was performed without labelling to avoid the influence of the dye on the antibacterial properties [122]. Although the fluorenyl-methyloxy-carbonyl (Fmoc) solid phase peptide synthesis method is commonly performed to synthesise peptides, many complex reactions are still performed using the original tert-butyl-oxy-carbony (Boc) chemistry. The original Boc method is performed when un-natural amino acids or other derivatives that are base sensitive are incorporated into the sequence. In this project, peptides have different sequences, number of amino acids, charges and molar masses (Table 9). In order to confirm the desired amino acids sequence and purity of peptides, high pressure liquid chromatography (HPLC) and mass spectrometry were used as previously described [122, 123].

Table 9: Amino acid sequences, charges, and theoretical molar mass of synthesised human defensins-like AMPs.

Codes	Peptide Sequence	No. of Amino acids	Charges	Theoretical Molar Masses
DLAMP-1	PACIAGERRYG	11	1	1192.37 g/mole
DLAMP-2	CATRESLSGVC	11	0	1125.29 g/mole
DLAMP-3	CRVRGGRCA	9	3	977.18 g/mole
DLAMP-4	GTCIYQRLNAF	11	1	1285.49 g/mole
DLAMP-5	GTCGLPGTKCC	11	1	1039.27 g/mole
DLAMP-6	CISEKTTDGHC	11	0	1193.32 g/mole

3.2. High Pressure Liquid Chromatography

Analytical reverse-phase high pressure liquid chromatography (RP-HPLC) was performed on a Shimadzu operating system located in the School of Chemistry, University of Birmingham. The partial purification of synthetic defensin-like antimicrobial peptide-1 (DLAMP-1) using reverse phase high performance liquid chromatography (RP-HPLC) is shown in Figure 16. The major peak of around 500 mAU was obtained at 21.76 minutes. Some other smaller peaks are also eluted but the absorbance of all these peaks was below 100 mAU. The LC program was binary with general water methanol mobile phase system. The gradient was set as 0 - 100% B in 40 minutes, then 100% B for 9 minutes and then back to initial condition of 0% B in 50 minutes. The absorbance was read at 210 nm. The flow rate was 1ml/minutes.

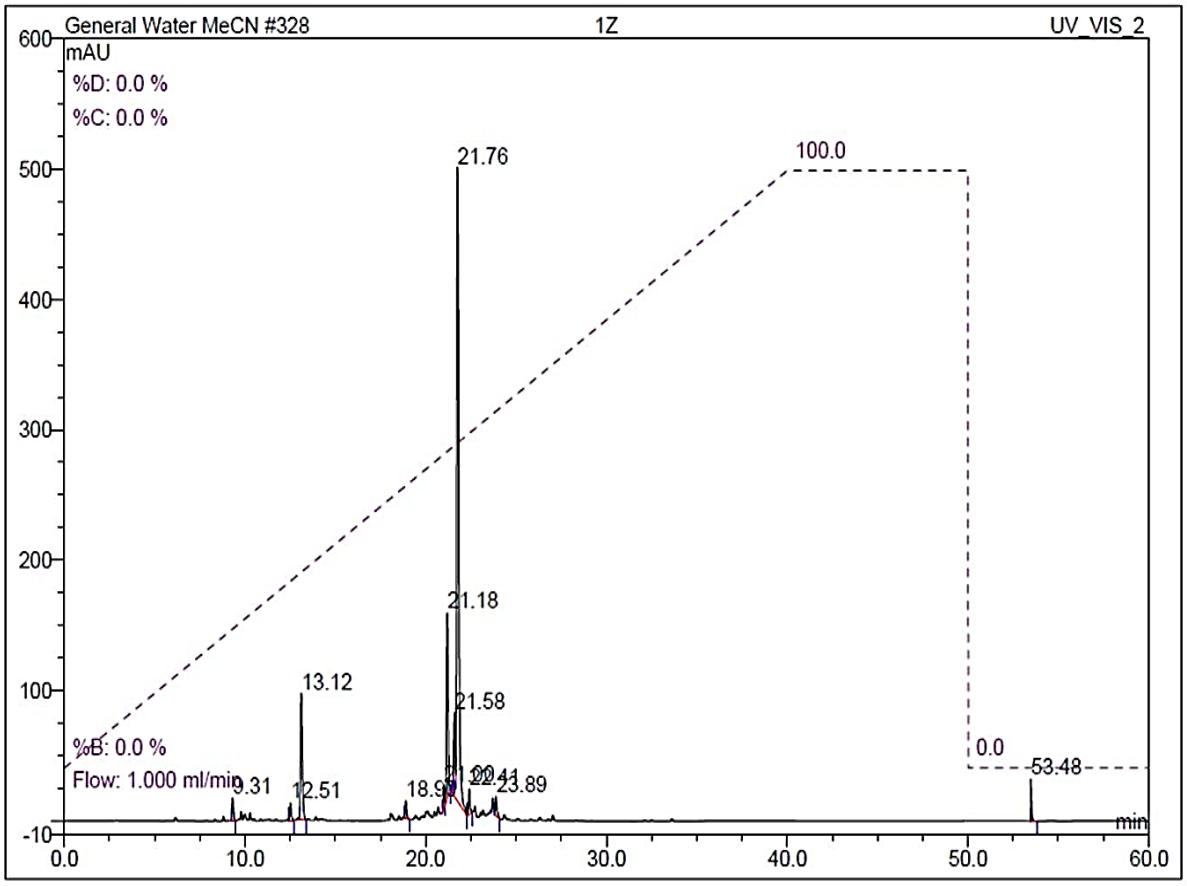

Figure 15: Reverse phase-High pressure liquid chromatography (RP-HPLC) of Defensins - like antimicrobial peptide–1 (DLAMP-1).

The purification of synthetic defensin-like antimicrobial peptide - 1 (DLAMP-1) collected from the first HPLC run was again applied on reverse phase high performance liquid chromatography (RP-HPLC) and is shown in Figure 17. The major peak at around 475 mAU was obtained at 21.35 minutes. The LC program was binary with general water methanol mobile phase system. The gradient was set as 100% B at 40 minutes, then 100% B for 9 minutes and then reach to 0% B in 50 minutes. The absorbance was read at 210 nm. The flow rate was 1ml/minutes.

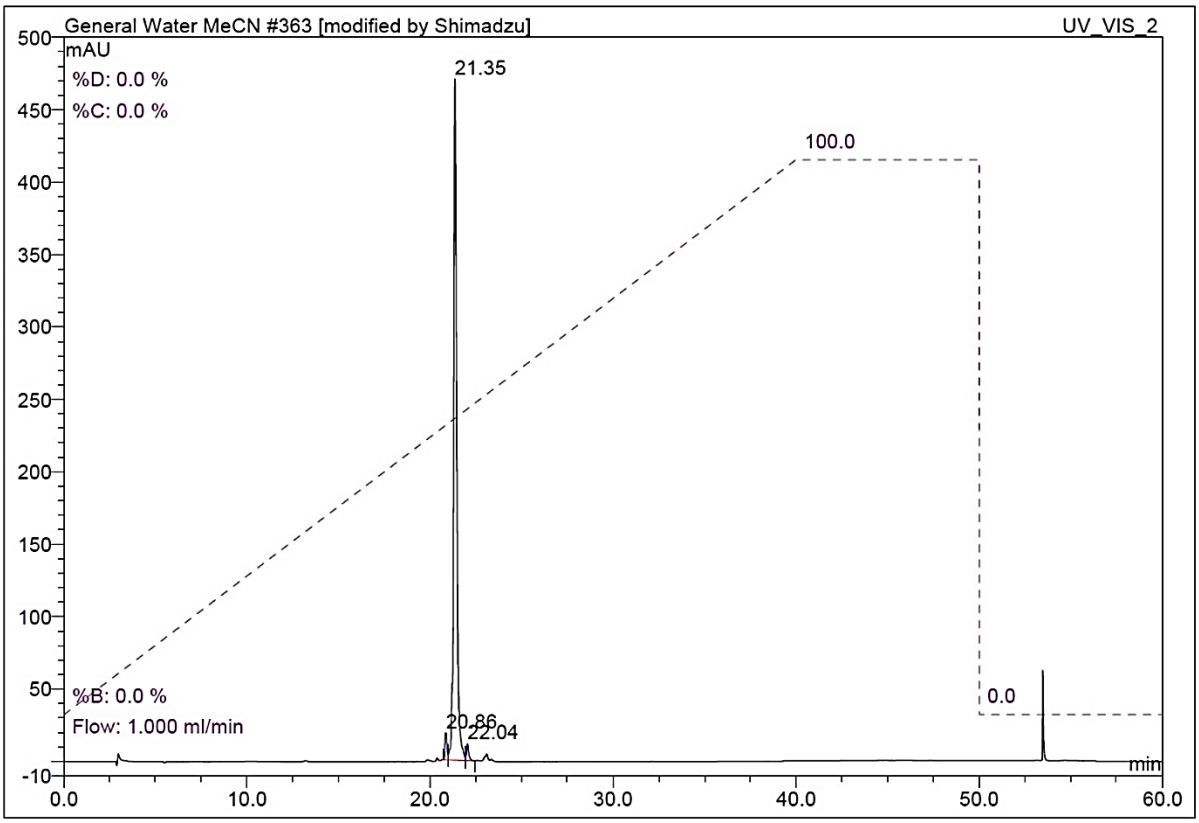

Figure 16: High pressure liquid chromatography (HPLC) of purified collected peaks of Defensins -like antimicrobial peptide–1 (DLAMP-1).

The partial purification of synthetic defensin-like antimicrobial peptide-2 (DLAMP-2) on reverse phase high performance liquid chromatography (RP-HPLC) has been shown in Figure 18. The major peak at around 225 mAU was obtained at 19.30 minutes together with some other minor peaks but the mAU of all of those were below 120. The liquid chromatography (LC) program was binary with general water methanol mobile phase system. The gradient is set as 100% B in 40 minutes then 100% B for 9 minutes and then reach to 0% B at 50 min. The absorbance was read at 210nm at a flow rate of 1ml/minutes.

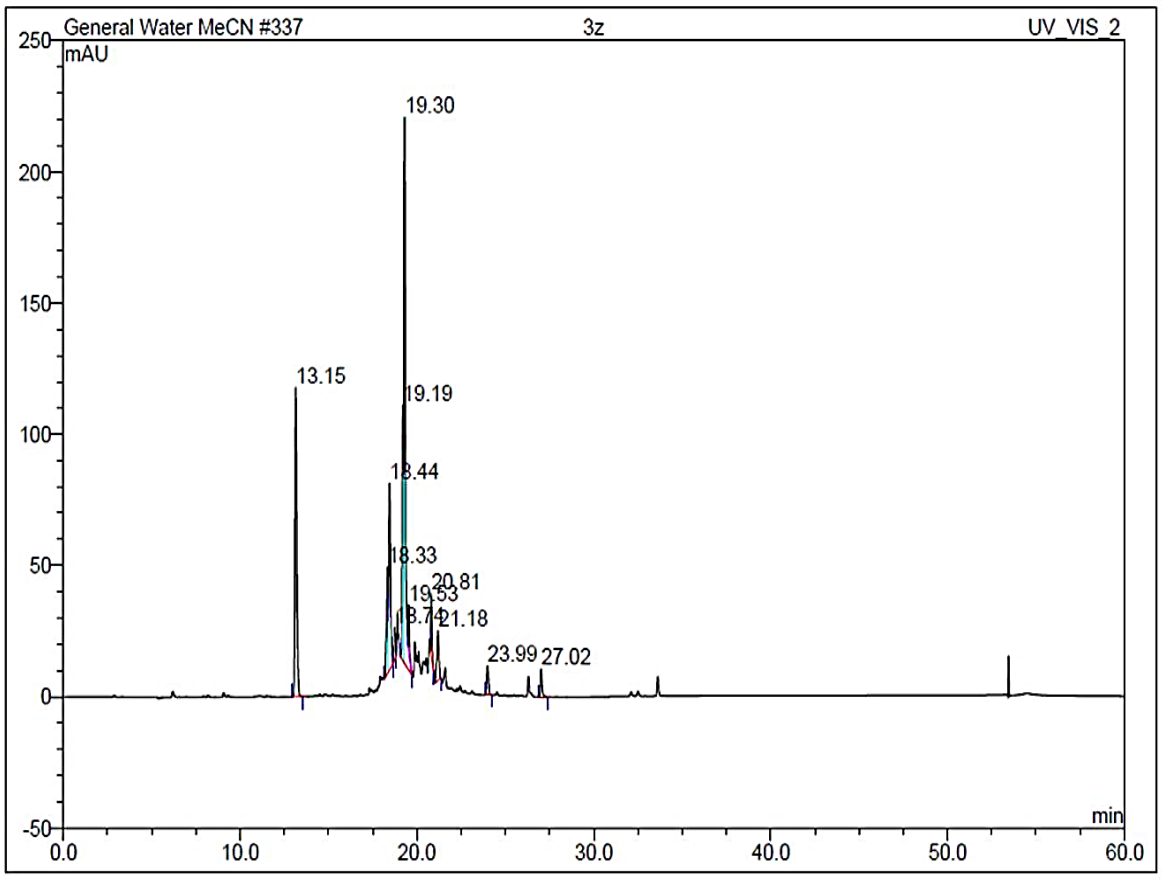

Figure 17: Reverse phase-High pressure liquid chromatography (RP-HPLC) of Defensins - like antimicrobial peptide – 2 (DLAMP-2).

For final purification; the collected defensin-like antimicrobial peptide - 2 (DLAMP-2) on (RP-HPLC) has been shown in Figure 19. The major peak at around 250 mAU was obtained at 19.05 minutes. The liquid chromatography (LC) program was binary with general water methanol mobile phase system. The gradient is set as 100% B in 40 minutes then 100% B for 9 min and then reach to 0% B at 50 minutes. The absorbance was read at 210nm at flow rate of 1ml/minutes.

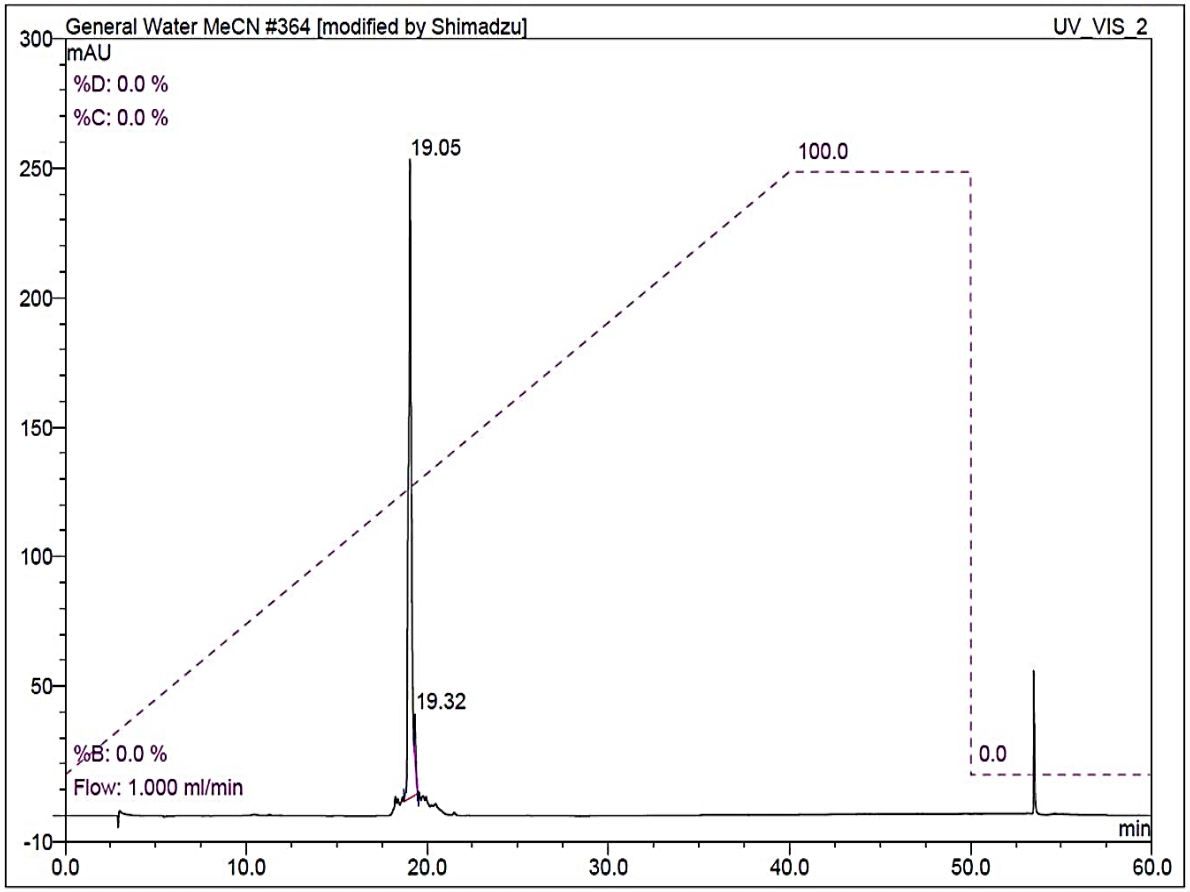

Figure 18: High pressure liquid chromatography (HPLC) of purified collected peaks of Defensins -like antimicrobial peptide–2 (DLAMP-2).

The partial purification of synthetic Defensin-like antimicrobial peptide-3 (DLAMP-3) on (RP-HPLC) has been shown in Figure 20. The major peak at around 155 mAU was obtained at 17.32 minutes. Some other smaller peaks at lower than 30 mAU were also obtained. . The LC program was binary with general water methanol mobile phase system. The gradient is set as 100% B in 40 minutes then 100% B for 9 minutes and then reach to 0% B at 50 minutes. The absorbance was read at 210 nm at a flow rate of 1ml/minutes.

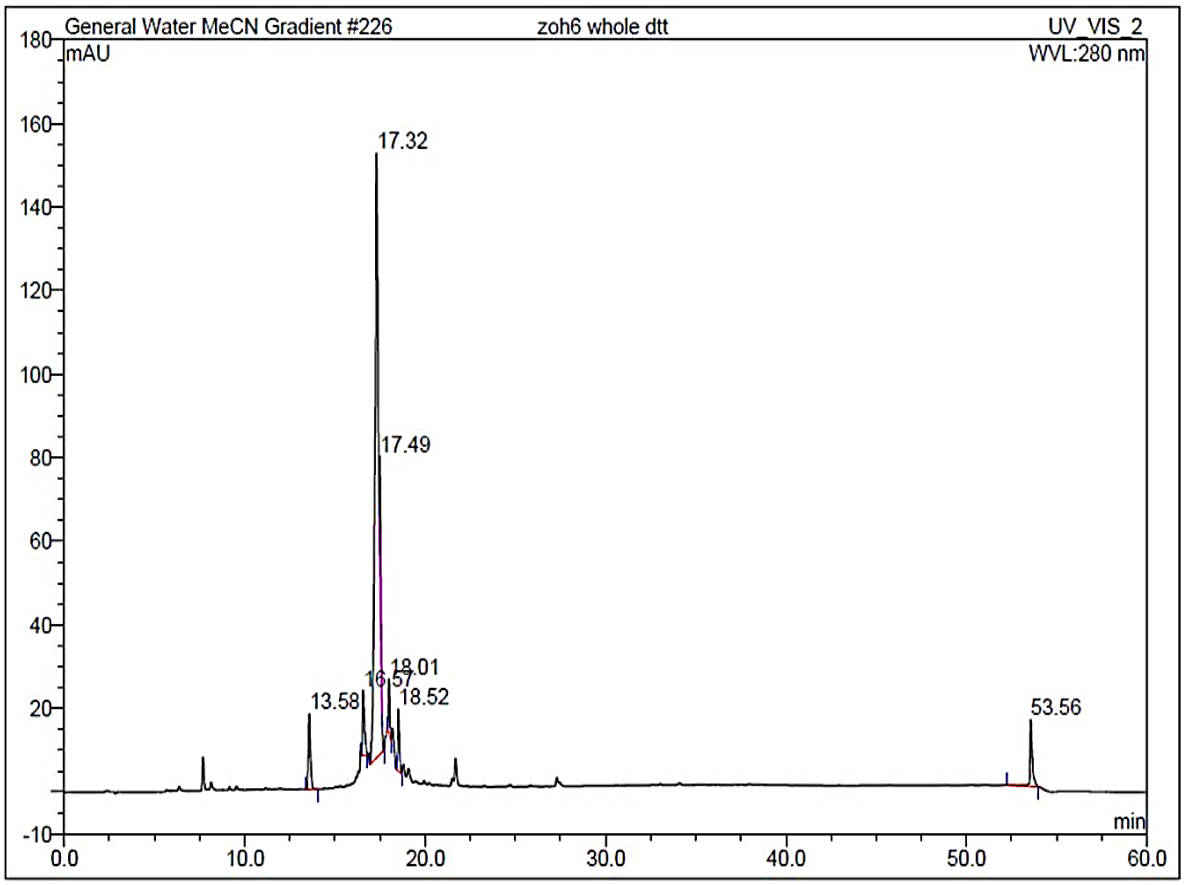

Figure 19: Reverse-phase high pressure liquid chromatography (HPLC) of Defensins -like antimicrobial peptide–3 (DLAMP-3).

The purification of synthetic Defensin-like antimicrobial peptide - 3 (DLAMP-3) on (RP-HPLC) has been shown in figure-21. The major peak at around 600 mAU was obtained at 17.37 minutes. The LC program was binary with general water methanol mobile phase system. The gradient was set as 100% B in 40 minutes then 100% B for 9 minutes and then reach to 0% B at 50 minutes. The absorbance was read at 210nm at a flow rate of 1ml/minutes.

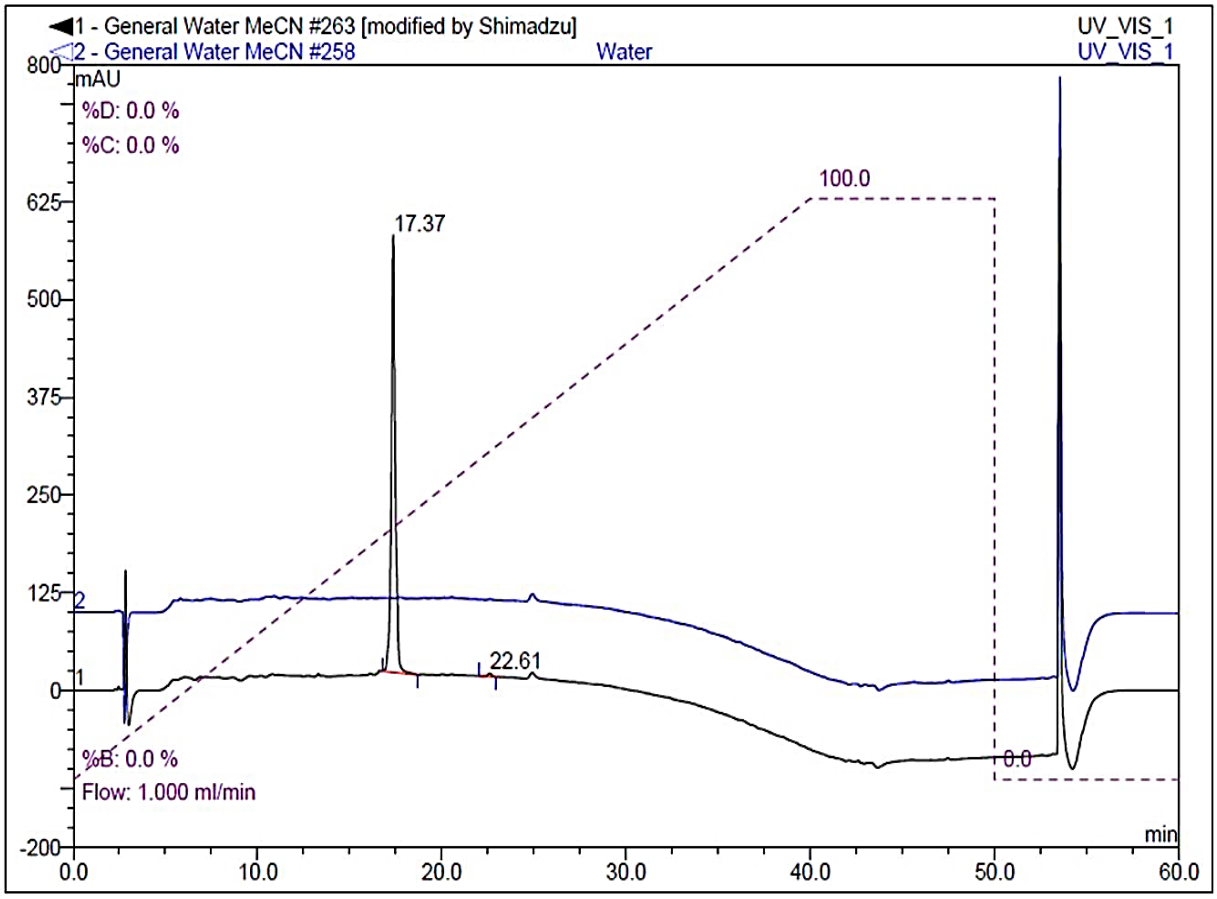

Figure 20: High pressure liquid chromatography (HPLC) of collected peaks of Defensins - like antimicrobial peptide–3 (DLAMP-3).

The RP-HPLC of synthetic Defensin-like antimicrobial peptide - 4 (DLAMP-4) is shown in Figure 22. The chromatogram showed two early eluting peaks at 3.3 and 5.9 minutes and some broad peaks with shoulders. Broad peaks were expected due to aggregation of peptides or degradation of peptides into smaller fragments. The gradient program and flow rate used was same as described in Figure 16.

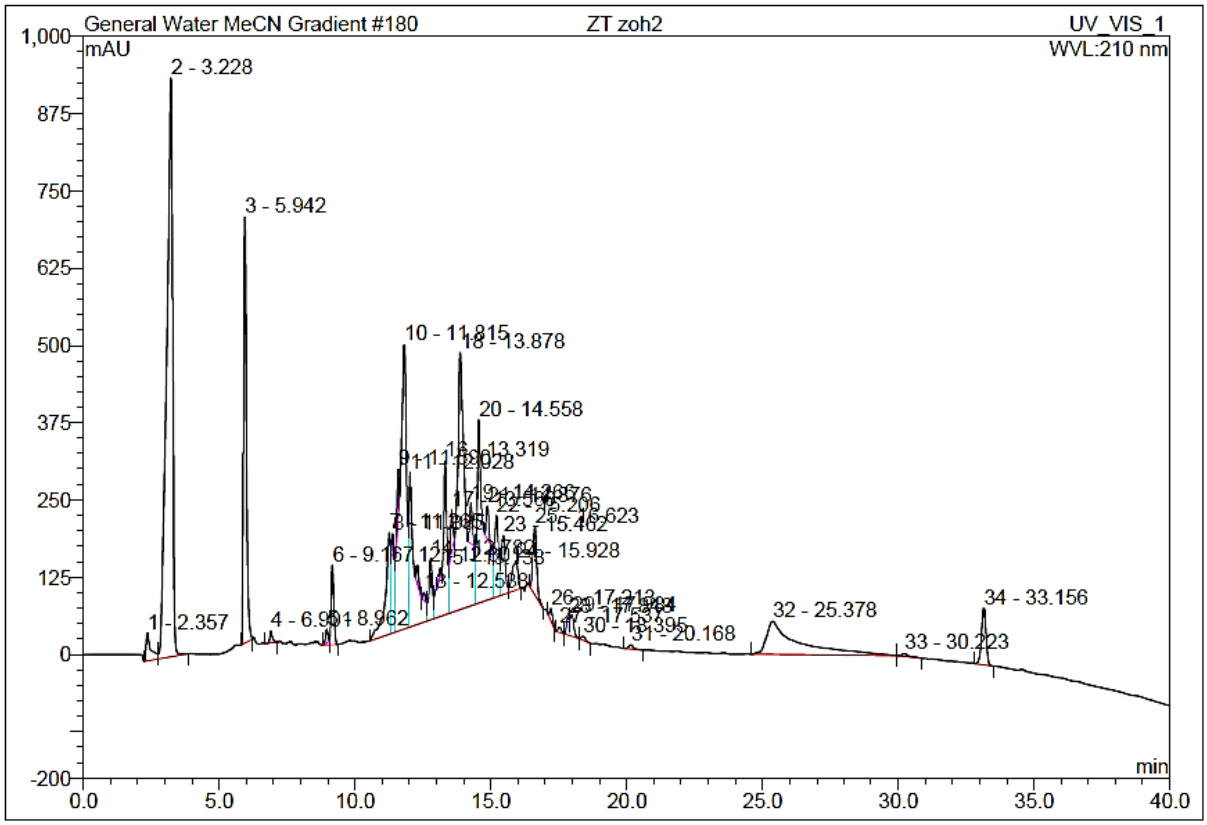

Figure 21: Reverse phase-High pressure liquid chromatography (RP-HPLC) of Defensins - like antimicrobial peptide – 4 (DLAMP-4).

The RP-HPLC of synthetic Defensin-like antimicrobial peptide - 5 (DLAMP-5) is shown in Figure 23. The chromatogram showed some high intensity base line peaks at 5.7 and 8.02 minutes and some broad peaks with shoulders. Broad peaks were expected due to degradation of product or aggregation of peptide. The gradient program and flow rate used was the same as described in Figure 16.

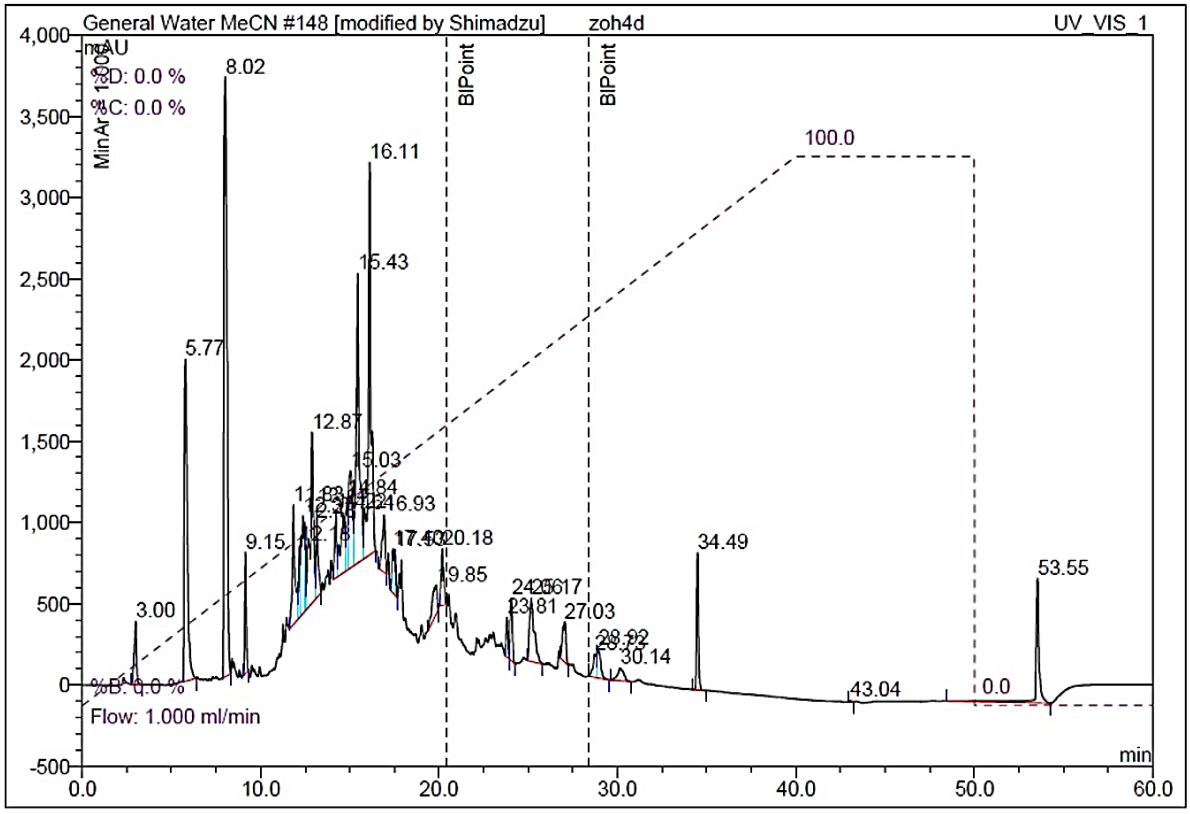

Figure 22: Reverse phase-High pressure liquid chromatography (RP-HPLC) of Defensins - like antimicrobial peptide – 5 (DLAMP-5).

The RP-HPLC of synthetic Defensin-like antimicrobial peptide - 6 (DLAMP-6) is shown in Figure 24. The chromatogram showed some high intensity base line peaks at 3.14, 5.79 and 7.99 minutes and some broad peaks with shoulders. Broad peaks were expected due to aggregation of peptides. The gradient program and flow rate used was the same as described in Figure 16.

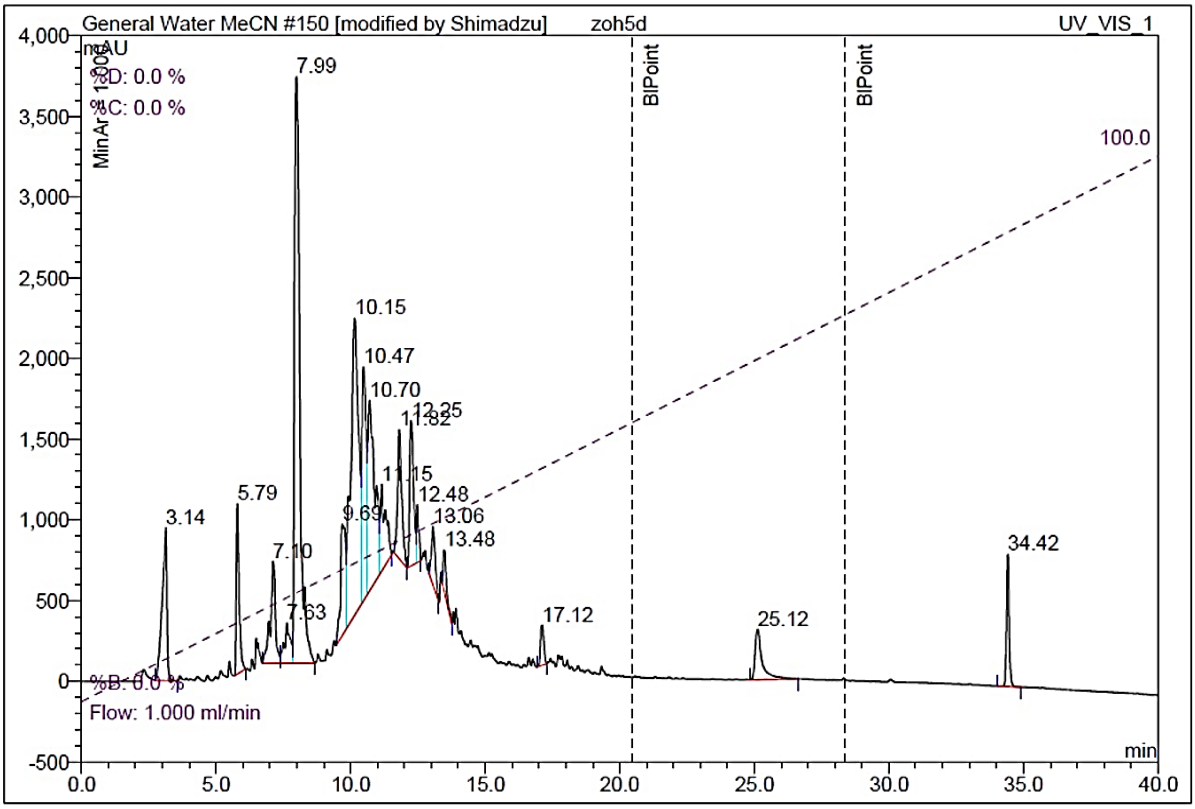

Figure 23: Reverse phase-High pressure liquid chromatography (RP-HPLC) of Defensins - like antimicrobial peptide – 6 (DLAMP-6).

3.3. Mass Spectrometry

Time of flight mass spectrometer with electron spray ionization (TOF MS ESI) source in School of Chemistry, University of Birmingham were used to find the experimental mass of synthetic peptide DLAMP-1 in positive mode. The spectra contain mass to charge ratio (m/z) on x-axis and percentage on y-axis. In this two major ion peaks are obtained. The first peak having 400 mass with 100% intensity. The second peak showed the mass of 600 with approximately 30% intensity and 342.5 and 489.2 are mass of fragmented peptides. After calculations it was found that the peaks possess +3 (400.6mz) and +2 (600.3mz) charge respectively. The sum of experimental mass is about 1200 Da which is absolutely near to theoretical mass i.e., 1192 Da. This may be indicative of difference in sequence of peptide that might occurred during synthesis.

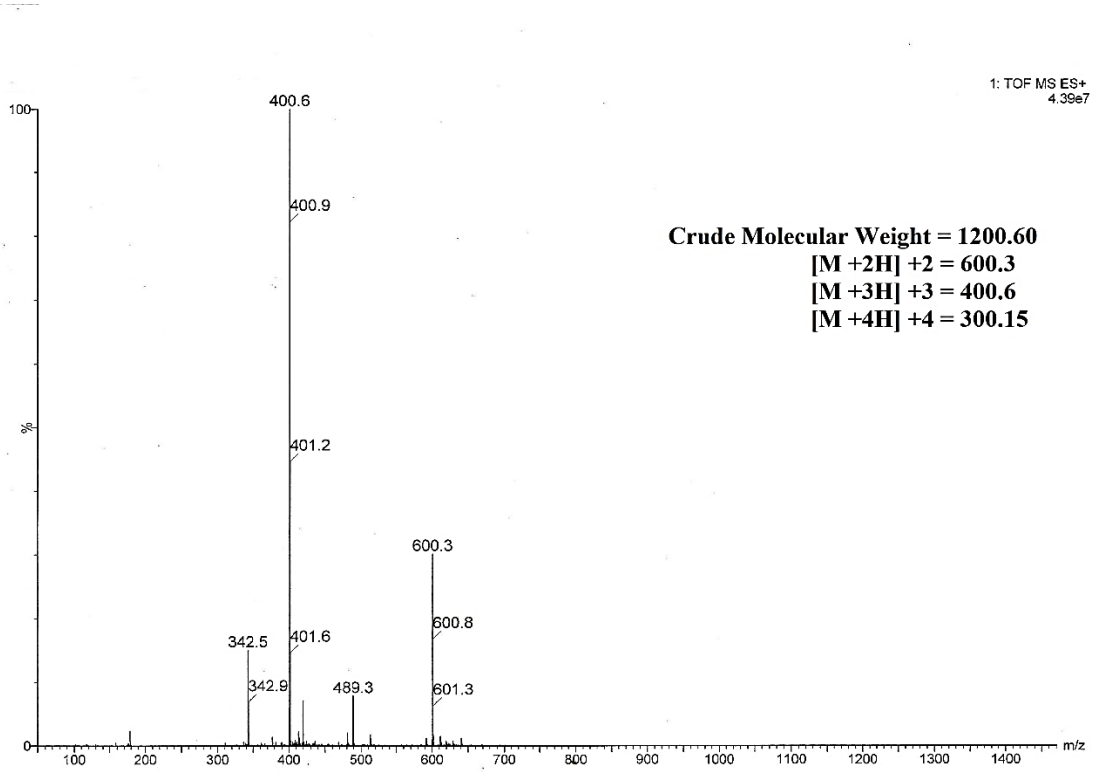

Figure 24: Mass-spectrometry of purified peptide (DLAMP-1) after freeze dried.

Time of flight mass spectrometer with electron spray ionization (TOF MS ESI) source was used to find the experimental mass of synthetic peptide DLAMP-2 in positive mode. The spectra contain mass to charge ratio (m/z) on x-axis and percentage on y-axis. In this case only one major ion peak was obtained with 100% intensity having mass of 716.9 Da. The experimental mass was not in accordance with theoretical mass which was 1125.29 Da. The peak obtained was found to be +2 charged. This may be indicative of difference in sequence of peptide that might occurred during synthesis.

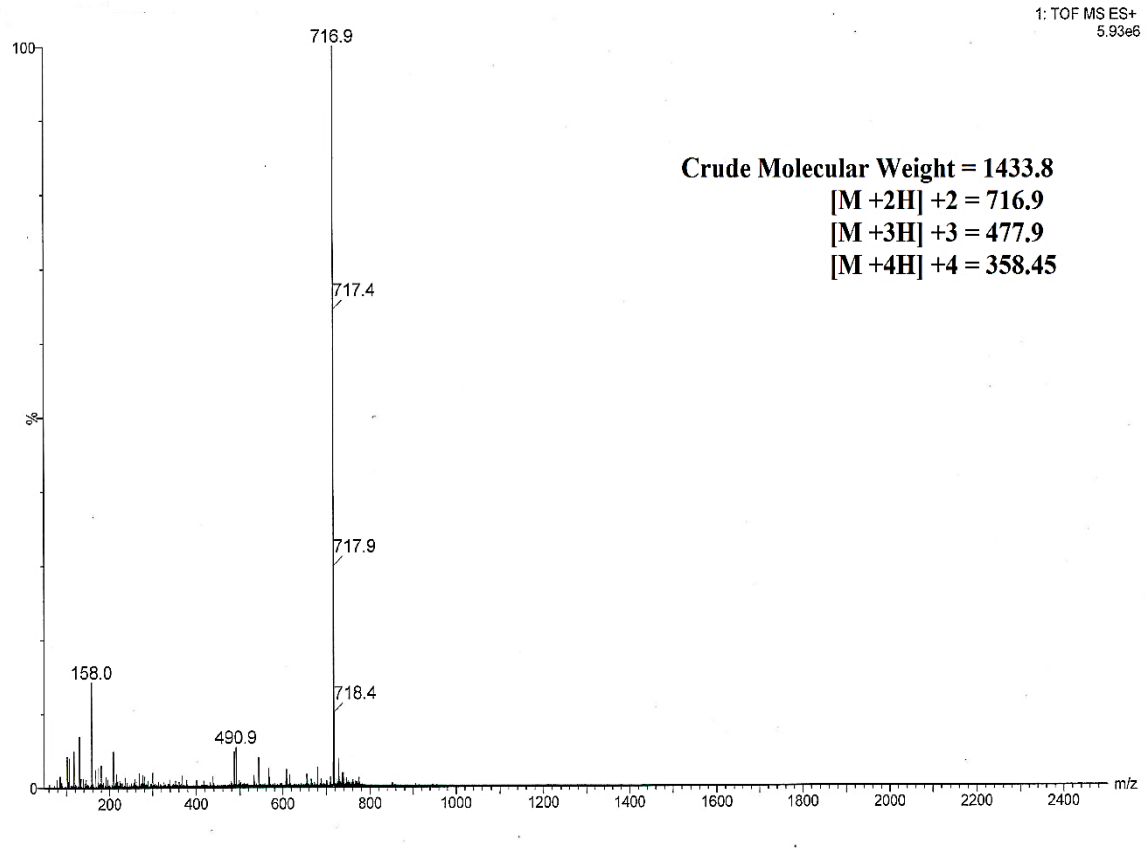

Figure 25: Mass-spectrometry of purified peptide (DLAMP-2) after freeze dried.

Time of flight mass spectrometer with electron spray ionization (TOF MS ESI) source was used to find the experimental mass of synthetic peptide DLAMP-3 in positive mode. The spectra contain mass to charge ratio (m/z) on x-axis and percentage on y-axis. In this case only one major ion peak is obtained with 100% intensity having mass of 707.9 Da. The experimental mass is not in accordance with theoretical mass which is 977.18 Da. The peak obtained was found to be +2 charged.

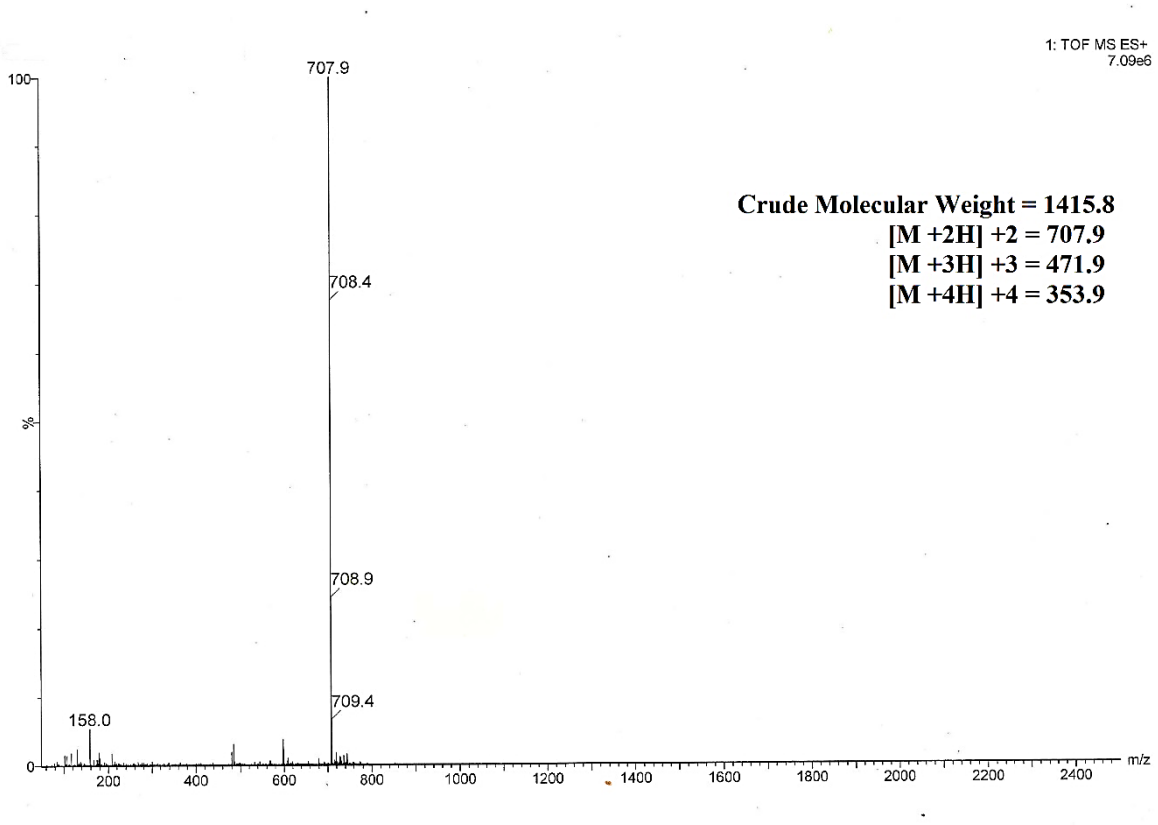

Figure 26: Mass-spectrometry of purified peptide (DLAMP-3) after freeze dried.

3.4. Antimicrobial Study (MIC/MBC)

To sum up all observations, we concluded that all three peptides have different antibacterial activity due to different amino acids, charges and polarity. In this study, high arginine peptide sequence showed better results than others. In clinical microbiology; use of antibiotics to inhibit or kill bacteria at different concentration and for this combination minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) were used. In this research dilution test (serial solution test) was used [124]. For minimum inhibitory concentration (MIC) of synthetic peptides

reading were measured on the basis of turbidity of solution e.g. Low turbidity (+), medium turbidity (++), high turbidity (+++) and no turbidity (Clear). At low DLAPM-1, 2 and 3 concentration of less than equal to 2.187 mg/mL, the viability of *S. epidermidis* and *P. aeruginosa* were more than 98%. Even when concentration was increased to 8.75 mg/mL of DLAMP (1, 2 and 3), the viability of *S. epidermidis* and *P. aeruginosa* were still 91.95% and 95.95% respectively and all assays were performed twice. At concentration 17.5mg/mL of DLAMP-3 and DLAMP-2 showed medium turbidity against *S.epidermidis* not for *P. aeruginosa* medium. Similarly at concentration of 17.5 mg/mL of DLAMP-1 demonstrated no or minor bactericidal effect against *S. epidermidis* and *P. aeruginosa*. In control groups, we observed no bacterial growth reflecting the non-contaminated environment. All work was conducted in a fume cupboard to control contamination. Tables 10 and 11 show all concentration of peptides and microorganisms. DLAMP-3 maintained their effect against *S.epidermidis* and *P. aeruginosa* medium from 35 mg/mL to 4.375 mg/mL of concentrations. The reason was their short amino acid architecture (9 amino acids), net charge and high arginine (R) content as compared to other synthetic peptides. As previously described by *Zou et al.* about the effect of introducing arginine (R) in α-defensins in contrast to lysine (K) [125]. Another study reported that replacing arginine (R) affects the antimicrobial activity as well as the salt sensitivity in analogs of Human β- defensins [126]. For antimicrobial peptide, higher net charge

and arginine residues are required is reported in Antimicrobial Peptide Database (http://aps.unmc.edu/AP/main.php).

Table 10: Peptides concentration against *S. epidermidis*.

Groups	DLAMP- 1	DLAMP-2	DLAMP-3
TSB	Clear	Clear	Clear
TSB + Peptides	Clear	Clear	Clear
TSB + Peptides +D_2O	+++	+++	+++
TSB + 35mg/mL	+	+	+
TSB + 17.5mg/mL	+++	+	+
TSB + 8.75mg/mL	+++	++	++
TSB + 4.375mg/mL	+++	+++	++
TSB + 2.187mg/mL	+++	+++	+++
TSB + 1.09mg/mL	+++	+++	+++

*Tryptic soya broth (TSB), **+** [low turbidity] = visible growth and high cell death of microorganisms, **++** [Medium turbidity] = visible growth, low cell death and slightly opaque solution, **+++** [High turbidity] = visible growth, no cell death and totally opaque solution, Clear [no turbidity].*

Table 11: Peptides concentration against *P. aeruginosa*.

Groups	DLAMP- 1	DLAMP- 2	DLAMP- 3
TSB	Clear	Clear	Clear
TSB + Peptides	Clear	Clear	Clear
TSB + Peptides +D_2O	+++	+++	+++
TSB + 35mg/mL	++	+++	+
TSB + 17.5mg/mL	+++	+++	++
TSB + 8.75mg/mL	+++	+++	++
TSB + 4.375mg/mL	+++	+++	++
TSB + 2.187mg/mL	+++	+++	+++
TSB + 1.09mg/mL	+++	+++	+++

*Tryptic soya broth (TSB), **+** [low turbidity] = visible growth and high cell death of microorganisms, **++** [Medium turbidity] = visible growth, low cell death and slightly opaque solution, **+++** [High turbidity] = visible growth, no cell death and totally opaque solution and Clear [no turbidity].*

The minimum bactericidal concentration (MBC) dose of antibiotics to kill bacterial cells was identified. In this study the control group drug was

ampicillin for both selected bacteria and a good killing zone was observed as compared to DLAMP-3 against *S. epidermis* and *P. aeruginosa* (Figure 28). DLAMP-1 also shows few micrometre bacterial killing zones against *S.epidermidis*. DLAPM-2 have no or less zone of killing *S. epidermis* and *P. aeruginosa.*

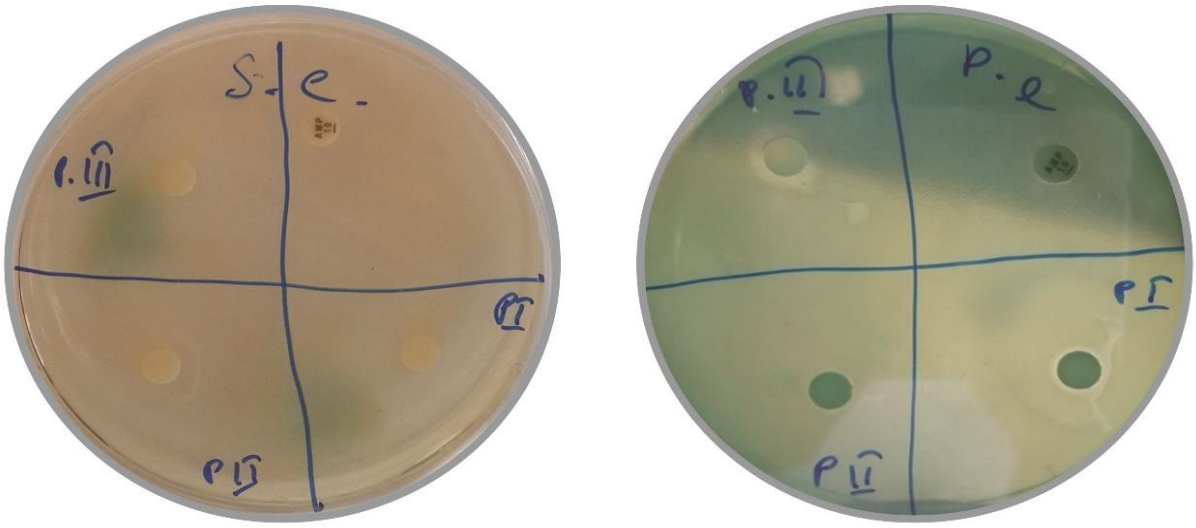

Figure 27: Represents all peptides with Ampicillin against *S. epidermidis* and *P.aeroginosa*. In peptide 3 clearly visible zone of inhibition and in peptide 1 few micron zones are visible.

Our defensins-like antimicrobial peptide (DLAPM) -1, 2, and 3 have arginine and cysteine in their design so they gave antibacterial activity during MIC/MBC. The coupling in remaining sequences (DLAMP-4, 5, and 6) was most likely due to aggregation or formation of unwanted products due to long sequences or due to poor motifs from Human defensins is still questionable. Host defense antimicrobial peptides (AMPs) are important component of innate immunity of both vertebrates and invertebrates [127]. One group of

researchers reported the total synthesis of Human α- defensins 4, 5, and 6 and they mimicked Human neutrophil peptides-4, Human defensins-5 and 6, in which they used oxidative folding with disulfide bridges protocol and received high yield purity (10% to 16%) and culture them in colony forming methods LD50, LD90 of *E. coli* ATCC 252922 and *S. aureus* ATCC 29213 [128]. *Hoover Et al.* and his co-workers worked on linear peptides with native disulfide bridge and without bridge to evaluate the antibacterial and antifungal activity of human defensins peptide-3, with the protocol of tert-butyloxycarbonyl (Boc) solid-phase peptide synthesis although experiment is different from this project but the design and concept is same to create human defensins nature based cores [129]. Developing short defensins with disulfide bridge help in stabilizing role in inhibiting of microorganisms [79]. Very recently *Wang et al.* evaluated antimicrobial activity of human β-defensin-1 (hBD-1), human β-defensin-2 (hBD-2) and β-defensin-3 (hBD-3) against three common probiotic strains of lactic acid bacterium (*Bifidobacterium longum* JDM301, *Bifidobacterium lactis* HN019 and *Lactobacillus rhamnosus GG* (LGG), but viability of lactic acid bacteria were very high at concentration of 10µg hBD/mL. Then they introduced carbonyl cyanide 3-chlorophenylhydrazone (CCCP) to inhibit efflux pumps as previously described, showed potent activity against *B. longum* JDM301, and *B. lactis* HN019 not for *Lactobacillus rhamnosus GG* (LGG) [130].

According to the antimicrobial data base (http://aps.unmc.edu/AP/main.php) reportedly cationic peptides are positively charged due to the presence of lysine (K) and arginine (R) residues also histidine (H) in their sequences. Human defensins are with high net charge (positive) and have high antibacterial, antifungal, antiviral or anticancer capabilities. The attraction between lipid membranes and peptides, obviously with high positive charged antimicrobial peptides attracted with negative charged membranes [131]. The reason behind this is too high positive charge of peptides will decrease the membrane activity and create strong electrostatic interactions with lipid head group region. Another possibilities are repulsions between positives charges of side chains or may be due to more negative charges on membrane lips attracts positive charged peptides [46]. In Table 12, a description of human β- defensins peptides with their name, sequence, net-charge and references is presented.

Table 12: Description of Human Beta (β) - Defensins sequences and net charges with references reported in the last decade.

Name	Sequence	Net-Charge	References
HBD-1	NH_2-DHYNC—VSSGGQCLYSACPIFTKIQGTCYRGKAKCCK-COOH	+4	[132]
HBD-2	GIGDPVTC--LKSGAICHPVFCPRRYKQIGTCGLPGTKCCKKP	+6	[133]
HBD-3	GIINTLQKYYC--RVRGGRCAVLSCLPKEEQIGKCSTRGRKCCRRKK	+11	[134]
HBD-4	ELDRIC—GYGTARCR—KKCRSQEYRIGRCPN-TYACCLRK	+7	[135]
HBD-27	EQLKKCWNNYVQGHCRK-ICRVNEVPEALC-ENGRYCCLNIK	+3	[136]
HBD-28	ARLKKC-FNKVTGYCRK-KCKVGERYEIGC-LSGKLCCAN	+8	[136]

CHAPTER 4: CONCLUSIONS AND FUTURE WORK

4.1. Conclusions

We concluded novel human defensins antimicrobial peptides analogs or motifs for making antibiotics and antimicrobial biomaterial surfaces. To this aim, we mimicked natural Human defensins namely defensins human Neutrophil peptide 1-2, human α-defensins-5, human β- defensin-3, defensins human neutrophil peptide 2-3, human β-defensin 2 and Nad1 From Nicotiana Alata (plant protein) by solid-phase peptide synthesis (SPPS). In this experiment design is linear and short peptides to evaluate the purity percentage, minimum inhibitory concentration and minimum bactericidal concentration of all six sequences but unfortunately we achieved only three peptides in pure percentage around 98% and amount for antibacterial testing. DLAMP-1 (PACIAGERRYG), DLAMP-2 (CATRESLSGVC) and DLAMP-3 (CRVRGGRCA) showed purity of 98% and they give inhibitory effect against *S. epidermidis* and *P. aeruginosa*. DLAMP-3 (CRVRGGRCA) is more active compared to DLAMP-1 (PACIAGERRYG) and DLAPM-2 (CATRESLSGVC) because of its net charge, presence of arginine (R) and cysteine in its design. We used different concentration of 35 mg/mL, 17.5 mg/mL, 8.75 mg/mL, 4.375 mg/mL, 2.187 mg/mL and 1.09 mg/mL with tryptic soy broth (TSB) of all samples to measure the dose concentration at which inhibition or killing of microorganisms occurs. DLAMP-3

(CRVRGGRCA) showed high inhibitory effect on *S. epidermidis* at 4.37 mg/mL. For *P. aeruginosa* only DLAMP-3 (CRVRGGRCA) showed an effect at 35 mg/mL and other peptides had no or little effect. The minimum bactericidal concentration (MBC) test showed that an inhibitory zone in Ampicillin (control group), DLAMP-3 and DLAMP-1 was observed.

4.2. Future Work

With the successful synthesis of Human defensins peptides and plant defensins, work can begin on determining molecular principles and structural properties against infection creating organisms. Once it will fully understand will be used as synthetic drug for coating in medical and dental implants as well as incorporate in bone cement materials to create antibacterial capability. A list of work reported in last few years on creating or developing antimicrobial medical and dental implants is shown in Table 13.

Table 13: Antimicrobial peptide based medical and dental implants.

Years	Title of Reported research	Conclusion	References
2010	Controlling the release of peptide antimicrobial agents from surfaces	Successful incorporation and release of an AMPs, ponericin G1, from hydrolytically degradable LbL assembled thin films and successful Layer-by-layer assembling.	[137]
2011	The biocompatibility and biofilm resistance of implant coatings based on hydrophilic polymer brushes conjugated with antimicrobial peptides	Successful creation of a biofilm resistant, non-toxic hydrophilic polymer coatings having broad spectrum antimicrobial peptides on surfaces.	[68]

2012	Drug release and bone growth studies of antimicrobial peptide-loaded calcium phosphate coating on titanium	Capability of an antimicrobial coating while maintaining osseointegration, AMP-CaP coating can effectively kill S. aureus, and P. aeruginosa bacteria and in vivo test no involvement of peptides coating in bone growth.	[77]
2013	Bio-inspired stable antimicrobial peptide coatings for dental applications	Functionalized titanium with GL13K, a novel bio-inspired coating, antimicrobial against P. gingivalis and a putative pathogen of peri-implantitis	[138]
2014	Covalent immobilization of hLf1-11 peptide on a titanium surface reduces bacterial adhesion and biofilm formation	Successfully immobilize the antimicrobial peptide, the covalent attachment of the hLf1-11 peptide to titanium surfaces and antimicrobial activity.	[139]
2015	Peptide-functionalized zirconia and new zirconia/titanium biocermets for dental applications	Successfully developed functional bio-coatings on zirconia and biocermets made of oligopeptides.	[140]

Also investigate the reason for not purifying peak in DLAMP-4 (GTCIYQRLNAF), DLAMP-5 (GTCGLPGTKCC) and DLAMP-6 (CISEKTTDGHC) after synthesis same as other peptides sequences. May be design of sequence affect, number of amino acids or charge of the sequence on the purification. There is need for larger amounts of peptides with antimicrobial properties. The more amount will open the gate for culture them with other microorganisms. It has been already reported that Human defensins antimicrobial peptides have capability to inhibit bacteria (Gram -ve and Gram +ve), fungal colonies and viral activities [78]. They can be

cooperated in denture base materials as an antifungal substitute. Hip implants are failed most commonly by microbial infection, need for modification their surface with antibacterial agents and these synthesized peptides are good candidates for this opportunity [141].

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