REPUBLIC OF TURKEY

ACIBADEM MEHMET ALİ AYDINLAR UNIVERSITY

INSTITUTE OF HEALTH SCIENCES

DEVELOPMENT OF PROTEASE RESISTANT ANTIMICROBIAL PEPTIDES

BETÜL ZEHRA KARAKUŞ

MASTER THESIS

DEPARTMENT of MEDICAL BIOTECHNOLOGY

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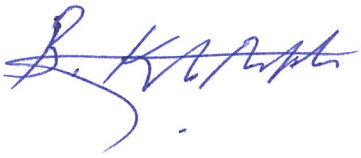
DECLARATION

I hereby declare that, this thesis has been written by me based on the data obtained in line with the scientific rules and ethical principles of responsible conduct of research. All information, data, comments, analyses have been collected and processed through scientific, academic writing style, and literature used have been duly shown by giving reference to the original sources in accordance with the publication ethics. I also announce and emphasize that I have not violated any rules secured by patent and copyrights whilst the conduct and writing of this research.

07.08.2020

Betül Zehra KARAKUŞ



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LIST OF ABBREVIATIONS AND SYMBOLS

°C	Celsius Degree
µl	Microliter
AMP	Antimicrobial Peptide
Arg	Arginin
$CaCl_2$	Calcium Chloride
CO_2	Carbon Dioxide
CPP	Cell-Penetrating Peptides
Cys	Cysteine
DCM	Diisopropylcarbodiimide
DMF	Dimethylformamide
DNA	Deoxyribonucleic Acid
FBS	Fetal Bovine Serum
g	Gram
GPA	Glycopeptide Antibiotics
Ile	Isoleucine
kDa	Kilodaltons
Leu	Leucine
LSU	Large Subunit
M	Molarite
mg	Milligram

MIC	Minimal Inhibitory Concentration
ml	Milliliter
mRNA	Messenger RNA
MS	Mass Spectrometry
NaCl	Sodium Chloride
PBS	Phosphate Buffered Saline
PG	Peptidoglycan
pH	Power of Hydrogen
Pro	Prolin
RNA	Ribonucleic Acid
SEM	Scanning Electron Microscope
SPPS	Solid Phase Peptide Synthesis
SSU	Small Subunit
TEM	Transmission Electron Microscope
TFA	Trifluoroacetic Acid
TIS	Triisopropylsilane
UPLC	Ultra Performance Liquid Chromatography
α	Alpha
β	Beta
θ	Theta

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SUMMARY

When antibiotics entered our lives, they extended the life expectancy. However, new resistance mechanisms developed by microorganisms, in the last few decades, enabled them to create lethal infections that cannot be treated by any type of antibiotic. Natural antimicrobial peptides produced by many organisms, including humans, in which, microorganisms cannot develop resistance but are sensitive to proteases, are the subject of many studies. In this study, we have developed derivatives of these peptides, resistant to proteases. Peptides called TN1, TN3 and TN6 were synthesized with D amino acids. D-Amino acids made peptides resistant to proteases and caused changes in efficacy and toxicity values. Peptides synthesized with L and D amino acids were treated with proteinase K at the same conditions. In HPLC analysis, it was seen that peptides synthesized by D amino acids did not break down and came out from the column at the same retention time as their protease untreated form. Peptides synthesized with L amino acid molecules, were found to be cleaved and fragments came out from the HPLC column at different retention times. After protease treatment, the biological activities of the peptides were also studied. L-peptides were found to lose activity while D-peptides remained active. In scanning and transmission electron microscopy scans performed with three different concentrations with D-TN6, this antimicrobial peptide was found to disrupt bacterial cell membranes. In the new designs, replacing the amino acid of the leucine with isoleucine and valine caused a decrease in the activity of the peptides. With this study, it was possible to develop protease resistant antimicrobial peptides similar to natural peptide antibiotics.

Keywords: Antibiotic Resistance, Antimicrobial Activity, Antimicrobial Peptide, Protease Resistance, Synthetic Peptide

ÖZET

Proteazlara Dirençli Antimikrobiyal Peptitlerin Geliştirilmesi

Antibiyotikler hayatımıza girdiğinde insan yaşam süresini uzattılar. Bununla birlikte, mikroorganizmalarda son birkaç on yılda ortaya çıkan yeni direnç mekanizmaları hiç bir antibiyotik ile tedavi edilemeyen ölümcül enfeksiyonların oluşturmalarına neden oldu. Mikroorganizmaların direnç geliştiremediği ancak proteazlara duyarlı olan, insanlar da dahil olmak üzere birçok organizma tarafından üretilen doğal antimikrobiyal peptitler, bir çok çalışmanın konusudur. Bu çalışmada, bu peptitlerin proteazlara dirençli türevlerini geliştirdik. TN1, TN3 ve TN6 adı verilen peptitler, D amino asitlerle sentezlendi. D-Amino asitler peptitleri proteazlara karşı dirençli hale getirdi, etkinlik ve toksisite değerlerinde değişikliklere neden oldu. L ve D amino asitleri ile sentezlenen peptitler, aynı koşullarda proteinaz K ile muamele edildi. HPLC incelemesinde, D amino asitleri tarafından sentezlenen peptitlerin parçalanmadığı ve aynı alıkonma zamanında kolondan çıktığı görüldü. L amino asit molekülleri ile sentezlenen peptitlerin parçalandığı ve oluşan parçaların HPLC kolonundan farklı alıkonma sürelerinde çıktığı bulundu. Proteaz muamelesinden sonra peptitlerin biyolojik aktiviteleri incelendi. D-peptidler aktif kalırken L-peptidlerin aktivitesini kaybettiği belirlendi. D-TN6 ile üç farklı konsantrasyonda gerçekleştirilen taramalı ve geçirimli elektron mikroskobi incelemelerinde, bu antimikrobiyal peptidin bakteri hücre zarını bozduğu gözlendi. Lösin amino asidinin izolösin ve valin ile değiştirilmesi, yeni tasarlanan peptitlerin aktivitesinde azalmaya neden oldu. Bu çalışma ile doğal peptit antibiyotiklerine benzer proteaza dirençli antimikrobiyal peptitler geliştirildi.

Anahtar Sözcükler: Antibiyotik Direnci, Antimikrobiyal Aktivite, Antimikrobiyal Peptit, Proteaz Direnci, Sentetik Peptitler

1. BACKGROUND AND AIM OF STUDY

Since antibiotics have been discovered, it has facilitated human life and increased the life span. Today, antibiotic use has increased considerably and has become a part of human life. Antibiotics lose their effect when microorganisms develop resistance. For this reason, researchers are working on developing antibiotics that microorganisms cannot develop resistance.

There are many and diverse microorganisms in our environment. Organisms developed many defense mechanisms against the invasion of microorganisms. Antimicrobial peptides (AMPs) in a wide range of living creatures are important molecules of innate immunity (1, 2).

The most common feature of AMPs is their amphiphilic characters. According to this feature, they can penetrate the cell membranes of pathological microorganisms. The presence of negatively charged groups in the bacterial cell membrane creates electrostatic interaction with positively charged amphipathic groups of AMPs. The hydrophilic groups in AMP structure enables the AMP to allign correctly within the cell membrane of the pathogenic microorganism (3,4).

The main advantages of AMPs are their large spectrum of antimicrobial activity and low induced pathogen resistance. However, the high expense of synthesis and sensitivity to proteases are the main disadvantages of AMPs.

Members of the family of defensin peptides such as cathelicidin, are produced by mammals. Cathelicidins are positively charged peptides. They interact

electrostatically with microorganisms with cell membranes and kill microorganisms directly by forming pores along the cell membrane (5). In humans, only LL-37, the derivative of a cathelicidin, is produced. LL-37 is an alpha-helix peptide and acts by disturbing the bacterial cell membrane (6).

Protease is an enzyme that breaks down proteins by hydrolyzing peptide bonds. Peptide molecules are inhibited by proteases at in vivo conditions and lose their effectiveness. Pathogenic bacteria produce proteases that inactivate AMPs (7).

In a previous study, the conserved amino acid sequences of AMPs were mimicked and helical peptides similar to cathelidine LL-37, called TN peptides, were developed by Tanil Kocagoz and Nihan Unubol (8). The most important disadvantage of TN peptides was their protease susceptibility. The main purpose of this study was to render these AMPs resistant to proteases. We also aimed to improve these peptides to increase their efficacy.

2. INTRODUCTION

2.1Antibiotics

Antibiotics that show selective toxicity on bacteria act without damaging the cells of the host organism. Antibiotics are able to inhibit the growth of the bacterial cell (bacteriostatic), or directly kill bacteria (bactericidal) (9). Since the introduction of the first antimicrobials (in 1911), antibiotics not only had an impact on the cure of infectious diseases, but also had an impact on lowering the morbidity and mortality of the community (10).

Arsphenamine (called Salvarsan) was the first sulfa drug introduced in 1910. Until the 1940s, it was the most commonly used antimicrobial drug. However, the mechanism of action was indefinite (11,12). Prontosil, another sulfa antibiotic, was introduced in 1932 and the first to be widely known (13). Sulfonamides are analogues of paraminobenzoic acid (PABA), bacteriostatic antimetabolite drugs. They inhibit folate synthesis. Depletion of folate store causes DNA, RNA and protein synthesis to cease (10,14). Sulfonamides have activity against, both Gram negative and positive.

Antimicrobial therapy revolutionized with penicillin (in 1928), one of the most important drugs of the last century, discovered by Alexander Fleming (15). Fleming extracted the penicillin from cultures of *Penicillium notatum* (10). Penicillin class members consist of a beta-lactam ring structure (Figure 1), necessary for antimicrobial activity. Penicilins inhibit peptidoglycan crosslinking in the bacterial cell wall. They bind to penicillin binding proteins (PBP), thus activating cell wall autolysis (16). The treatment of syphilis and rheumatic fever were two most

impressive effects of the penicillin usage (17). Penicillins are classified based on additional chemical groups found in their side chains. Additional groups mostly stimulate differences in the spectrum of activity and the bioavailability (10).

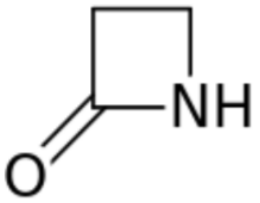

Figure 1 Chemical structure of beta lactam ring (18)

The first aminoglycoside, Streptomycin, was isolated in 1943 (19). Streptomycin (Figure 2) is a protein synthesis inhibitor that binds to the small 16S rRNA of the 30S subunit of bacterial ribosome, important to inhibition of protein synthesis and codon misreading (20). Streptomycin have an activity against, both Gram positive and Gram negative bacteria (21).

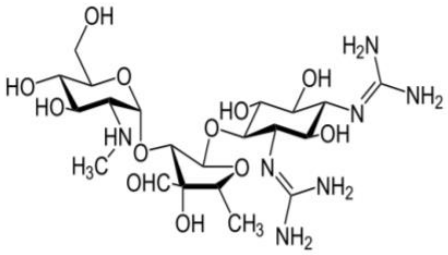

Figure 2 Chemical structure of Aminoglycoside (Streptomycin)

The cephalosporins are the second-class beta-lactam antibiotics discovered at 1945 after the penicillins. The cephalosporins have a six-member dihydrothiazine ring instead of five-member thiazolidine ring different from penicilins. After first

cephalosporin was used for treatment, more than twenty cephalosporin have been released (22). The cephalosporins, as beta-lactam antibiotics, are bactericidal, and inhibit bacterial cell wall synthesis (23).

Chlorotetracycline (Aureomycin), the first member of the tetracycline class, was discovered in 1948. Antibiotics in this class contain four hydrocarbon rings in their structure (Figure 3). They have activity against both Gram negative and Gram positive bacteria (24). The drugs bind to 30S subunits of bacterial ribosomes and block the binding of aminoacyl-t-RNA to the ribosomal acceptor site during protein synthesis (24,25).

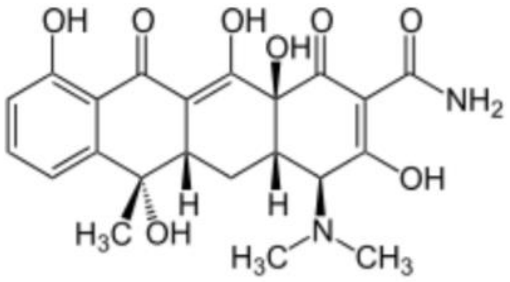

Figure 3 Chemical structure of tetracycline (26)

In 1952, the first antibiotic of macrolide class was isolated as a metabolic product of a soil. Macrolides are often administered to patients who have allergy to penicillin. They have a broader spectrum of activity than penicillins. Macrolides inhibit bacterial protein synthesis by binding to bacterial ribosome (27).

Nalidixic acid is the first antibiotic discovered in the quinolone class. Quinolones interfere with transcription and DNA replication in bacteria (28). Structure of quinolones generally contains two rings. Lastest generations of

quinolones have an added ring structure which admit them to broaden their spectrum of antimicrobial activity to anaerobic bacteria that were resistant to quinolones (28).

In the late 1960's, emergence of beta-lactamase in bacteria caused resistance in bacteria against penicillin. Carbapenems were discovered in 1976 which are not sensitive to the hydrolytic action of the beta-lactamase enzymes (29). However emergence of new class of beta-lactamases called carbapenemases, rendered these drugs ineffective in infection caused by bacteria producing these enzymes (30).

Glycopeptide antibiotics (GPAs) were discovered as natural products. The last 20 years, semi-synthetic GPAs derivatives with improved activity were developed. GPAs are used to cure Gram-positive bacterial infections (31). Vancomycin is the first glycopeptide antibiotic that was developed in the 1950s (32). GPAs are natural glycopeptides consisting of cyclic peptide core, where two amino sugars bind (Figure 4) (33).

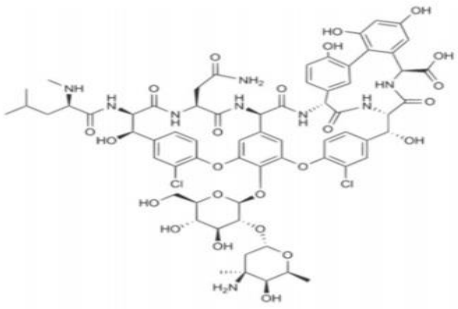

Figure 4 Chemical structure of Vancomycin (33)

2.1.1 Antibiotics Mechanism of Action

2.1.1.1 Inhibition of Bacterial Cell Wall Synthesis

Bacterial cell wall, which is built from the polymer peptidoglycan (PG), surrounds most bacterial cells. Beta-lactams inactivate penicillin-binding protein (PBPs) enzymes, which are responsible in cross-linking the PG chains and thus inhibit cell wall synthesis (34). Glycopeptides inhibit bacterial cell wall synthesis binding themselves peptide side chains of PG units (35).

2.1.1.2 Inhibition of Bacterial Protein Synthesis

This group of antimicrobials acts by inhibiting protein synthesis in bacterial ribosomes. Some of them combine with bacterial ribosomes and disrupt protein synthesis managed by mRNA. Ribosomes in mammalian cells have 80S units and bacterial ribosomes have 70S unit and this 70S ribosome is divided into small subunit (30S) and large subunit (50S). Therefore they do not disrupt mammalian ribosomes (36,37). Antibiotics that inhibit protein synthesis can be divided into two subclasses: the small subunit inhibitors and large subunit inhibitors (37). Aminoglycosides, tetracyclines and macrolides are antibiotics that inhibit protein synthesis.

2.1.1.3 Inhibition of Bacterial DNA Synthesis

Degradation of nucleic acid synthesis is harmful to both the posterity and survival of bacteria. This class of antibiotics interfere with nucleic acid synthesis by stopping transcription or blocking replication (38). Quinolones, that inhibits nucleic acid synthesis, target topoisomerase IV and topoisomerase II. Rifamycins inhibit RNA polymerase and stop RNA synthesis (18).

2.1.1.4 Disruption of the Bacterial Cell Membrane Function or Structure

Antimicrobial agents that affect the cell membrane increase the permeability of the cytoplasmic membrane, causing the small molecules to escape the cytoplasm. This action leads to the death of the microorganism (36).

2.1.1.5 Blockage of Key Metabolic Pathways

Antimetabolites are mimics of substrates that are needed for cellular mechanisms and compete with them for active sites on enzymes (36). Unlike mammals that obtain folate from food, bacteria produce the folate themselves. Tetrahydrofolate, which is required for folic acid synthesis, is mimicked by sulfonamides. Tetrahydrofolate derivatives, which are the cofactors of enzymes that supply the production of purine bases and thymidine, cannot be produced and DNA and RNA synthesis disrupted in bacteria (18,36).

2.1.2 Antibiotic Resistance

The resistance of a bacterial species to a certain antibiotic is indicative of its ability to resist the effects of that antibiotic. In nature, naturally resistant bacteria to some antibiotics can be found. Also, they can get resistance to antibiotics via by horizontal gene transfer and mutations in chromosomal genes (39,40).

Intrinsic (natural) resistance is a type of resistance that is not inherited. The absence of a target molecule to which the antimicrobial agent is effective by binding and natural barrier that prevent the drug from reaching the target, are responsible for this type of resistance. Metabolically inactive microorganisms, such as bacterial spores or dormant mycobacteria, may be resistant to drugs, but replicating bacteria are susceptible to drugs (36). Many genes were identified which are responsible for natural resistance to antibiotics, including aminoglycosides, β-lactams, and fluoroquinolones (41).

Bacteria may not be affected by an antibacterial, due to changes in a genetic trait (42). As a result of changes in the bacterial genome, resistant generations emerge and spread. This resistance of genetic origin is under the control of chromosomal and extrachromosomal elements (36). Chromosomal resistance appear as a result of spontaneous mutations in the bacterial chromosome (42). Extrachromosomal resistance is due to plasmids, transposons and integron, which are transmitted in several ways.

2.1.2.1 Mechanisms of Resistance to Antibiotics

2.1.2.1.1 Changing the Target Molecule

Antibiotics can bind to different targets, such as ribosomes and various enzymes (42). The interaction of antibiotics with target molecules is very specific. Small changes in the target molecule can have important effects on the binding of the antibiotic. Bacteria can have resistance for beta-lactam antibiotics via modification in or lack of the PBPs that are the receptor sites for beta-lactam antibiotics (43,44).

2.1.2.1.2 Enzymatic Resistance

Both Gram negative and positive bacteria, synthesize enzymes that inactivate many antibiotics. This is the most widely used mechanism in antibiotic resistance. Beta-lactamases hydrolyze the β-lactam group antibiotics leading to the development of resistance (42).

2.1.2.1.3 Inhibition of the Drug to Reach Its Target

Antibiotics enter the cells through the porins in Gram-negative bacteria and the peptidoglycan layer in Gram-positive bacteria. The outer membrane structure provides an advantage to Gram-negative bacteria. Due to changes in the structure of porin, they can evolve resistance to more than one antibiotic class. *P. aeruginosa* decreases the number of porins and thus develops resistance to imipenem (45).

Bacterial membranes contain efflux pumps, which pump out antibiotics. In this way, the antibiotic cannot reach sufficient concentration in the cell (44). This type of resistance is under plasmid or chromosome control and was determined 20 years ago for tetracyclines (42).

2.1.2.1.4 Using an Alternative Metabolic Pathway

Some bacteria develop a new metabolic pathway that eliminates the need for a drug-sensitive target (42). For instance, instead of synthesizing folate, they acquire the ability to take it from the environment (43).

2.2 Antimicrobial Peptides (AMP)

The resistance to existing antibiotics has led to the search for new types of antibiotic molecules. Antibiotics based on antimicrobial peptides (AMPs) are being investigated for this purpose (3). All living organisms from insects to humans produce AMPs. These are part of the natural immune system to prevent microorganisms from multiplying in their environment or to inhibit their harmful activity (46). Almost all known AMPs share three characteristics feature; (i) small size with 10-25 amino acids and molecular weight from 1kDa to 5 kDa, (ii) tendency to admit amphipathic structures in non-polar media, (iii) have cationic character with net positive charge. This physicochemical properties gives AMPs a tendency to fuse with negatively charged microbial membranes and surfaces (47). A variety of peptide with different sizes and structures are associated with antimicrobial activity in eukaryotic hosts (1).

AMPs exhibit viricidal, bactericidal, tumoricidal and fungicidal properties. They are effective at a low concentration, and bacterial resistance to AMP is less likely to develop (48). Positively charged AMP groups create electrostatic interaction with negatively charged groups in the bacterial cell membrane. The hydrophilic area in the AMP structure ensures that the peptide is properly aligned within the cell membrane of the pathogenic microorganism. The majority of AMPs compose amphiphilic helixes, facilitating the incorporation and disruption of the membrane (3,4). In order for a microorganism to develop a resistance to AMPs, whose target is the bacterial membrane, it will have to redesign its membrane. For many microorganisms, changing the composition their lipids is an inconvenient solution (49,50). AMPs cover the external membrane of Gram-negative bacteria, then pass through the inner membrane and the PG layer, reaching the cytoplasm of the bacteria (51,52).

AMPs have both advantages and disadvantages. Among its advantages rapid action of killing, low levels of induced pathogen resistance, broad-spectrum activity, broad anti-inflammatory activity can be stated. Systemic and local toxicity, decrease of the activity based due to serum, according to pH and salt concentration, sensitization, pharmacokinetic issues, allergy after repeated application, susceptibility to proteolysis, intrinsic resistance, confounding biological functions, high producing costs are AMPs disadvantages (53).

The classification based on chemical-structural criteria of AMPs defines two broad groups, cyclic and linear structures. Linear AMPs have two subgroups; (i) to admit a-helical amphipathic conformation and (ii) unusual composition, rich in amino acids such as Arg, Pro, or Trp. Cyclic AMPs, encompassing all cysteine-containing peptides, have two subgroups according to single or multiple disulfide structures (1,3,46).

2.2.1 Post Translational Modifications of AMPs

Post-translational changes, which can significantly change AMPs' activity, occur on the amino acids. It has been observed that the change in the C-terminal of AMPs can stabilize the secondary structure and increase the affinity of the AMP towards the membrane (54).

Glycosylation increases the peptide variety and expands their range of functionality. Four different types of glycosylation are known; O-, C-, N-and S-glycosylation (55,56). It has been observed that the mechanisms of action of glycosylated peptides are not membrane permeability (3).

There are simple modifications in the amino acid sequence of eukaryotic cell peptides, consisting largely of disulfide bonds (57). As with other postlational modifications, disulfide bond modifications do not have an only function and in some peptides display opposite effects depending on the peptide and activity. For instance, reducing disulfide bonds in natural peptides have little or no effect on activity. Structures varying from one to five disulfides have been reported (58).

The most common posttranslational modification in AMPs is amidation (59). The peptide structure is known to be important in binding to the target bacterial membrane. It has been referred that the amidized peptide model $CONH_2$ is due to improved helical stability at the membrane interface, which backing a higher helicity compared to COOH (60). In some cases, C-terminal amidized peptide analogs show increased antimicrobial activity compared to non-amidized peptide (3).

N-terminal acetylation, seen only in a small group of natural AMPs from bacteria and worms, is a frequent modification in synthetic peptides. Another N-terminal modification is that glutamine residues transform to pyroglutamate. This change has been detected in AMPs isolated from spiders, plants, amphibians, reptiles, and insects (61).

Halogenation is a strategy that is used to modulate the characteristics of biologically active molecules. Studies, focusing on the effects of halogenation on the protease resistance and cytotoxicity of AMPs, are being conducted (62).

Except for Gly, each of the 20 amino acids is chiral and can be found in two stereoisomeric structures: D-form and L-form. L-aminoacids are usually found in much higher abundance than D-aminoacids. Though, recent research has show both abundant and potency of D-amino acids in nature. D-Amino acids are typically observed in environmental samples associated with microbiota (63,64). D-Amino acids were first observed in bombines from amphibians (65). The sensitivity of AMPs to proteases limits their application. AMPs can be synthesized from D-amino acids instead of L-amino acids to make AMPs resistant to protease breakage. Antimicrobial peptides synthesized in both L-form and D-form have similar antimicrobial activity (66,67). D-Form AMPs are more stable than L-form AMPs in the presence of human serum albumin, $CaCl_2$ or NaCl at physiological concentrations (68).

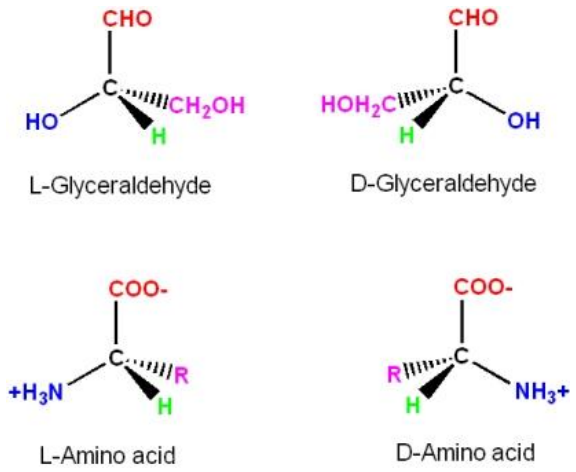

Figure 5 L- and D- form of aminoacids (69)

2.2.2. Mechanism of action of AMPs

AMPs, that inhibit DNA, RNA synthesis and proteins, disrupting membrane integrity or interacting with specific intracellular targets, kill cells (70).

Even if AMP's target is intracellular, it must first interact with the cell membrane. Most membrane active AMPs that have both hydrophobic and cationic surfaces, are amphipathic. (71). Positively charged amphipathic AMP groups create electrostatic interaction with negatively charged groups in the bacterial cell membrane. The hydrophilic area in the AMP structure ensures that the peptide is properly aligned within the cell membrane of the pathogenic microorganism (3,4).

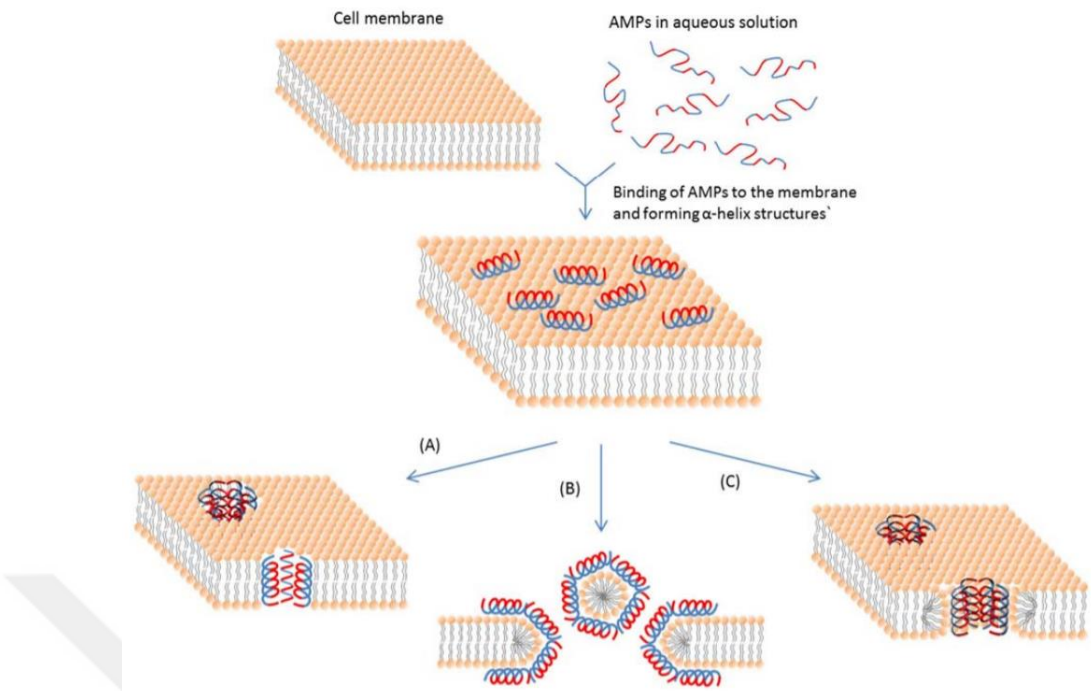

Figure 6 Representation of action mechanisms of some membrane-active AMPs (72)

(A) Barrel-Stave model, (B) Carpet model, (C) Toroidal pore model. The blue color represents the hydrophobic portions of AMPs, while the red color represents the hydrophilic parts of the AMPs.

The effect of AMP can be attributed to a first stage of membrane surface coverage, until charge neutralization on the membrane surface is achieved. Next, the AMP orientation changes to the membrane surface perpendicularly, followed by pore creation and membrane degradation (73).

The most commonly expressed phenomenological model is called "carpet model". These AMPs are directed parallel to the lipid membrane surface, leaving the hydrophobic edges of the pores inward, forming a large layer or carpet. Then AMPs penetrate the two layers of the lipid, leaving holes behind the carpet formation (72,74).

In the Barrel-Stave model, peptides are first attached to the outer leaflet of the bacterial membrane by electrostatic binding parallel to the cell membrane. Then they are grouped into barrel, as bundles that cover the pores of the amphipathic membrane. The non-polar side chains of AMP join with fatty acid tails in the phospholipid double layer, and the hydrophilic side-chains are directed to inside of the pore (72,75).

In the toroidal pore model, AMPs are inserted inside the membrane after binding to phospholipid head groups and then clustered in the membrane. These bundles, together with water molecules make channels permitting the leak of ions and bigger molecules across the membrane. Unlike other models, this model allows AMPs to cross the membrane without causing membrane depolarization by creating short-lived transmembrane channels (47).

2.2.3 Mechanisms of Resistance to AMPs

Bacteria develop resistance strategies against AMP, normally controlled by coordinated stress responses organized by various operons (76). Resistance mechanisms could aim to (i) modify the target of AMP to make it less sensitive, (ii) extinction of AMPs, (iii) remove AMPs from their action domains. (77). Pathogens exposed to antimicrobial peptides basically use two different resistance strategies: Constitutive (passive) and inducible (adaptive) resistance. Constitutive resistance mechanisms refer to the natural properties of microorganism, which provide resistance and are normally created even in the lack of peptide. Inducible resistance is mechanism that occur in response to the AMP or the target cell stress that AMPs cause (77).

AMP resistance mechanisms include capture of peptides, removal from the cell via active transport, degradation by proteases, and structural modification of the cell surface to prevent interaction with AMPs (78). AMPs interact first with the bacterial cell membrane. The extracellular inactivation of AMP is the first line of protection to decrease harm to bacteria. Bacteria often make modifications to cell surface components to resist the action of AMPs. Some of these modifications are the decrease negative charge of the cell, change the fluidity of the membrane or make direct changes to AMP targets. (79).

Mechanisms for removal of AMP are the most extensive mechanism of AMP resistance in Gram-positive bacteria. These include the two main mechanisms, modification of the bacterial cell surface composition and efflux pumps (80).

Proteolytic digestion is a resistance mechanism found in many Gram-positive bacterial species, including *Staphylococcus aureus*, *Enterococcus faecalis*. The proteases that cause the digestion of AMPs are usually secreted by bacteria into the extracellular environment (81,82).

2.2.4 Classification of Antimicrobial Peptides

AMPs can obtain different molecular forms. The linear peptides produced in animals, plants, and insects are the most common ones. Bacteria produce polycyclic peptides, plants produce circular peptides containing cyclotites and animals produce θ-defensins (83,84).

AMPs are divided into five groups, according to the organisms in which they are produced, as bacterial AMPs, plant AMPs, amphibious AMPs, mammalian AMPs and insect AMPs.

AMPs produced in multicellular organisms have a wide spectrum. AMPs produced in bacteria have selective toxicity. They are toxic for bacteria which do not produce that AMP (85). AMPs produced by bacteria are called "bacteriocin". Bacteriocins have an extremely strong bactericidal effect. Bacteriocin resistance is independent of antibiotic resistance and there has not been identified any cross resistance so far (86,87). One of the strategies developed by bacteria to gain resistance against bacteriocins is to convert some D-alanines into teichoic acid to decrease the negative charge of the cell membrane. In this way, the primary electrostatic interactions are weakened, and the binding efficacy of bacteriocins, which are positively charged, to cells is reduced (86,88).

Plant AMPs have common features with AMPs found in bacteria, insects and animals, such as their amphipathic structures, molecular forms, and positive charges. AMPs in plants affect membrane permeability for killing pathogens. Some AMPs in plants contain cell-penetrating peptides (CPPs) that interact with phospholipids in the membrane (89,90). Cell-penetrating peptides (CPP) can penetrate into the cell at low concentrations without causing any apparent membrane damage and using any receptors. According to these properties of CPPs, they can be useful substances in drug delivery applications (91,92). Despite significant differences between the amino acid sequences of plant AMPs, significant similarities can be seen in their tertiary structure. Structural basic features of AMPs include high Cys or Gly content and presence of disulfide bridges (93).

Amphibians, which are very rich as AMP sources in their skin, have very different peptide sequences. Many amphibian peptides show wide spectrum antimicrobial activity against fungi, bacteria, and protozoa. There are amphibian AMP variants that do not show hemolytic activity at effective antimicrobial concentrations (94). Magainin, a new class of AMP, is isolated from the skin of the African claw frog *Xenopus laevis* (95). The presence of D-amino acids in amphibious AMPs is an important feature. This modification, which is thought to occur posttranslationally in AMPs, appears to increase the resistance of the peptide to proteases rather than its antimicrobial effect (96).

Insects are found in many different ecological niches in the world, they encounter a lot of pathogenic bacteria, viruses and fungi. They have developed highly effective defense mechanisms against all these potential pathogens (97). There is no acquired immune system in invertebrates such as insects. Innate defense systems and peptide antibiotics in the defense system act as preservatives (98). AMPs expressed in insects are structurally divided into four main groups; (a) amphipathic α-helix forming peptides, (b) β-peptides with disulfide bridges, (c) prolin-rich AMP, (d) glycine-rich polypeptides.

Mammals have acquired and innate immune systems. Mammalian AMPs have similar properties with the ones found in other organisms. Mammalian AMPs have a wide spectrum including fungi, bacteria, and some parasites. Mammalian AMPs are encoded by genes and can be found in granules of mucosal cells, neutrophils, or skin secretions of epithelial cells, as degradation products of proteins (1). The most widely studied mammalian AMPs are defensins and catelicidins (99). Defensins have three disulfide bonds in their structure. They have α-, β -, and θ- subgroups, high content of arginine and six cysteines in their structure (99,100). Mammalian AMPs are cationic and amphipathic that support interactions with biological cell membranes. Arginines in their structure extend equatorially across the hydrophobic

surface. With this sequence of arginines, the peptides form multimeric pores in the lipid-layer and thus show antimicrobial activity (4,101,102).

2.2.4.1 AMPs in Humans

AMPs increase their effect by stimulating the immune system to overcome infections. Therefore, AMPs may have a dual role in host cell defense: rapid microbial killing and immune regulation (98). AMPs have been identified in surfaces or tissues such as eyes, skin, airways, ears, lung, intestines, and mouth in humans (103). Human AMPs also have other properties such as immune regulation, wound healing, and apoptosis (104). The lengths of human AMPs are between 10 and 149 amino acids. Net charges of peptides range from -3 to +20. The sequence variety of human AMPs determines their functional and structural properties (98). In 1922, human lysozyme, discovered by Alexander Fleming in saliva, also found in tears and intestine, as the first antimicrobial protein (98,105).

Human AMPs have five large family: Defensins, histatins, cathelicidins, dermicidines and hepcidin (98). Defensins, cathelicidins and histamines are important peptide antibiotics of the oral cavity. Newborns have human cathelicidin LL-37 on their skin, and older individuals have human beta-defensin 2 in their skin (103,106).

Histatins are a family of AMPs rich in histidine (His), discovered in the early 1970s. They are small, cationic AMPs found in human saliva (107–109). Histatins are part of the innate immune system. With their bactericidal and fungicidal properties, they have an important role in preserving oral health (107). The histatin family is made up of several members. Histatin 1, 3 and 5 are the most important ones, but only histatin 1 and 3 are encoded genetically. Among all histatins, histatin 5

has the strongest antimicrobial activity and has also strong antifungal activity against pathogenic fungal species (110).

In 1985, the term "defensins" was used for the human neutrophil peptide 13 (HNP13), an AMP that is three homologous cysteine-rich peptides, isolated from human neutrophils. Defensins are found in bone marrow, spleen, thymus, leukocytes and human epithelial cells. In mammals, defensins are subclassified according to their structure as α-, β- and θ-defensins (111). There are six different variations of α-defensins and they are all produce in human cells. Human β-defensins were discovered in the 1990s, unlike α-defensins, β-defensins have three disulfide bonds and have a lightly longer amino acid sequence to permit for an additional helical region (112).

Dermcidin is an anionic defensive peptide released by human sweat glands on the surface of the skin for antimicrobial activity. Dermcidin is also known as proteolysis-inducing factor (PIF). They demonstrate antimicrobial activity by forming an ion channel in the target cell or as a result of protelitic activity (113).

Hepcidins was discovered in human blood in 2000. It is particularly rich in Cys (32%) and contains four disulfide bonds (114).

2.2.4.1.1 Cathelicidins

Cathelicidins are the family of AMP derived from proteins containing a precursor region with a highly conserved signal sequence, a cathepsin L inhibitor that is highly homologous to the cathelin (115). The N-terminal of the AMP sequence

contains the region of the cathelin that provides resistance to proteases. Cathelicidins are helical peptides rich in proline and arginine (116). In humans, only the variant of a cathelid called LL-37 is characterized. LL-37 is derived with proteolysis from the C-terminal of hCAP18 protein, human CAP18 (117). LL-37 which begins with a pair of leucine and consisting of 37 amino acids, has linear structure since it does not contain Cys (98). LL-37 is expressed in leukocytes such as T cells, NK, and B cells, neutrophils, monocytes, as well as in epithelial cells of the testicles, skin, and gastrointestinal tract (118). LL-37 has antimicrobial activity against both Gram positive and negative bacteria. It also has antitumor activity (119).

LL-37 peptide has chemotactic activity on circulating host immune cells, enables pro-inflammatory signaling molecules to be secreted from epithelial or circulating cells and modulate pro-inflammatory activities of microbial components (120).

2.3 Proteases

Proteases are enzymes that break down proteins by hydrolyzing peptide bonds. Hydrolysis reactions catalyzed by proteases take place in cell metabolism and are very important for living organisms (121). Proteases are not specific to a particular substrate, unlike other enzymes, but specific to the particular structural forms of the peptide chain. Proteases from plants and animals were obtained in the 1940s. Studies on microbial proteases started to be studied in the 1950s and the first microbial proteases were obtained in crystalline form (121,122).

2.3.1 Classification of Proteases

We can classify proteases according to their sources, the type of reaction that they catalyze and the region on the proteins that they catalyze.

2.3.1.1 Classification of Proteases According to Their Sources

Proteases are found in all living organisms as they are necessary in physiological functions. They have a large spectrum of sources, like animals, plants, and microorganisms.

Proteases are found in the parts of plants such as stem, root, leaf, fruit, flower, seed and resin. Various factors, such as the suitability of the soil for planting and climatic conditions affect the use plants as a source of protease (123,124). Papain, keratinases, bromelain, and ficin represent proteases of plant origin. The best-known plant protease is papain. Carica is extracted from papaya fruits. Papain is active between pH 5.0 - 9.0 and can show activity up to 80-90 ºC. It has wide usage in industry; widely used as milk coagulant, meat softener and also has other uses in the pharmaceutical, detergent, and food industries (124).

As examples to animal origin proteases, trypsin, chymotrypsin, pepsin, and rennin can be given. Pepsin is an acidic protease, exhibiting optimum efficacy between pH 1.0 and 2.0, found in the stomachs of about all vertebrates. It catalyzes the hydrolysis of peptide bonds between two hydrophobic amino acids. It is inactivated above pH 6.0. Trypsin is a serine protease that hydrolyses food proteins. It contributes to the hydrolysis of Lys and Arg residues of carboxyl groups. It has

limited administration in the food industry because of the bitter taste of trypsin hydrolysates (124).

Interest in microbial proteases increased with the insufficiency of proteases obtained from plants and animals. Microorganisms are suitable for biotechnological applications because they allow easy genetic manipulations and their properties can be changed in the desired direction. Generation times of microorganisms are very short and large amounts of product can be obtained. The fact that they can be grown in artificial culture media makes them cheaper. In addition, they can be obtained more purely than vegetable and animal proteases (123).

2.3.1.2 Classification of Proteases According to Reaction Type of Catalysed

All proteases catalyze the hydrolysis reaction of peptide bonds. They show selectivity based on the location of the peptide bonds in the substrate, the amino acid residues surrounding it, and the characteristic features of the substrates (125). Proteases are classified into two major classes, exopeptidases and endopeptidases, based on their action site.

Endopeptidases hydrolyze internal alpha-peptide bonds in a polypeptide chain. They do not hydrolyze peptide bonds present at the amino or carboxyl ends. The presence of the free carboxyl or amino group negatively affects endopeptidase activity. Endopeptidases initiate digestion of food proteins, providing the formation of the new N- and C-terminus, which will be substrates of exopeptidases. Endopeptidases, called oligoproteases, can only act on substrates smaller than proteins (126,127). Endopeptidases are subdivided into Ser, Cys, Asp, Thr and glutamic type endopeptidases (128).

Exopeptidases hydrolyze the peptide bonds at the amino (- NH2) or carboxyl (-COOH) end near the ends of the polypeptide chain. They are subclassified as amino and carboxy peptidases (129). Aminopeptidase is an exopeptidase, which hydrolyze peptide bonds at the amino end of the protein. Aminopeptidases are intracellular enzymes found in various microbial species, including fungi and bacteria (124,129). Exopeptidases that hydrolyze peptide bonds at the carboxyl end are called carboxypeptidases. Dipeptidases hydrolyze dipeptides, and omegapeptidases catalyze the hydrolysis of peptide bonds at the carboxyl or amino ends of amino acids bound to the prosthetic group (129).

2.3.1.3 Classification of Proteases According to Mechanism of Action

According to presence of the main catalytic amino acid residue in its active sites, proteases are divided into 4 main groups: cysteine proteinases, aspartic proteinases, serine proteinases and metalloproteases.

Serine proteases perform a two-step reaction for hydrolysis. After the amino acid fragment is removed, a covalently linked enzyme-peptide intermediate is formed (130). This acylation phase is followed by deacylation. A nucleophilic attack is carried out with water on the intermediate formed and results in the hydrolysis of the peptide (131). Serine proteases are the most widely used enzymes in the industry because they are activated under alkaline pH conditions and maintain their stability (124). Serine proteases are found in bacteria, eukaryotes and viruses, as they are essential for organisms (132). Serine endopeptidases are divided into three groups according to substrate preferences: (i) trypsin-like, which breaks down after positively charged residues; (ii) elastase-like, which cleaves after small hydrophobic residues; (iii) chymotrypsin-like, which cleaves after large hydrophobic residues

(131). Serine proteases are irreversibly inhibited by 3,4-dichloroisocouminal (3,4-DCI), phenylmethylsulphonyl fluoride (PMSF), and diisopropylfluorophosphate (DFP) (132).

Cysteine proteases are known as thiol proteases. They are distinguished by the presence of trio of cysteine (Cys), aspartic acid (Asp) and histidine (His) in their active centers. Cysteine is an important amino acid in the enzyme since it complexes with the substrate and is inhibited by heavy metal and oxidizing molecules due to the -SH group it contains in its active center (133). Cysteine proteases are found in all organisms, eukaryotes and prokaryotes. The activity of Cys proteases depends on histidine or cysteine. Some of the cysteine proteases act at neutral and some at acidic pH. Papain is the well known Cys protease (124).

Aspartic proteases, known as acidic proteases, are dependent on aspartic acid residues for their catalytic functions. The catalytic domains of aspartic proteases contain two aspartic acids and these proteases generally show activity at low pH values (134). Aspartic proteases are generally inhibited by pepstatin. Examples of aspartic protease include pepsin A, which is involved in the digestive system of mammals, cathepsin D, which is involved in breast cancer metastasis, pepsin, which is responsible for defence against bacterial and fungal pathogens (135).

The catalytic effect of metallo proteases is different from other proteases. They need divalent metals, especially zinc, for their activities. They are inhibited by chelating agents like EDTA (136).

3. MATERIALS AND METHODS

3.1 Materials

The equipment used during the study is given in the Table 1.

Table 1. Equipments using in experiments

Equipment	Brand
Peptide Synthesizer	CEM
Peptide Razor	CEM
HPLC	Agilent
Xevo G2-XS QTof (MS)	Waters Coorp
Precision Scales	Shimadzu
Vortex	VWR
Pure Water Device	Advantage Milli-Q
CO_2 Incubator	Thermo Scientific
Biosafety Cabinets	Thermo Scientific
pH meter	Thermo Scientific
Centrifuge for 15-50mL falcons	Thermo Scientific
Centrifuge for Eppendorf	Thermo Scientific
Magnetic stirrer	Thermo Scientific
Autoclave	Nüve / Witeg
Sonicator	Isolab
Light microscope	ZeissLab
Scanning Electron Microscope	Thermo Scientific- Quattro
Transmission Electron Microscope	Thermo Scientific- Talos
-20 Freezers	Kirsch Froster
-80 Freezers	GFL
Microplate Reader	Thermo Scientific Varioskan
Water bath	Thermo Digital
Freeze Dryer (lyophilizer)	Labconco
C18 Analytical HPLC Column	Agilent
C18 Semi Preparative HPLC Column	Agilent

The chemicals used during the study are given in the Table 2.

Table 2 Chemicals using in experiments

Chemical	Brand
Fmoc-L- aminoacids	Sigma-Aldrich
Fmoc-D- aminoacids	Novabiochem
Oxyma	Sigma-Aldrich
Rink Amide Resin	Sigma-Aldrich
Acetonitrile HPLC Grade	Merck
Dichloromethane	Carlo Erba
Diethyl ether	Carlo Erba
N,N-Dimethylformamide(DMF)	abcr
N,N'-Diisopropylcarbodiimide(DCM)	Merck
Piperidine	Merck
Triisopropylsilane (TIS)	Across
Trifluoroacetic acid (TFA) (HPLC grade)	Sigma
Dulbecco's Modified Eagle Medium (DMEM)	Sigma
Fetal Bovine Serum (FBS)	Thermo Sci
Penicillin-Streptomycin	Thermo Sci
Phosphate Buffered Saline (PBS)	GeneMarkBio
Triton X-100	Merck
DMSO	Merck
Trypsin EDTA %25	Thermo Sci
NaCl	Merck
Mueller Hinton (MH) agar	Merck
Mueller Hinton (MH) broth	Merck
Tris base	Sigma
Osmium(VIII)	EMS
Ampicillin	GenemarkBio
SRB cytotoxicity kit	Biovissio
Methanol	Merck
Proteinase K	GeneMark
Tris Buffered Saline (TBS)	Sigma Aldrich

3.2 General Procedure for Solid Phase Peptide Synthesis and Purification

Peptides were synthesized peptide synthesizer (CEM, Liberty ™ Blue and CEM Discover ™) using standard Fmoc protocol at Acibadem University. Liberty ™ Blue has 20 positions for natural aminoacids and 5 positions for unnatural aminoacids. The temperature in the reaction vessel of the device was kept at 50 °C during the synthesis. It is possible to control temperature with microwave energy.

In Solid Phase Peptide Synthesis (SPPS), peptides were synthesized on resin from the C-terminal to the N-terminal (137). The resins used in this study are as follow: Rink Amide (0.70 mmol/g loading capacity) resin for the synthesis of amide end peptides and Cl-TCP (Cl) ProTide resin (0.42 mmol/g) for the synthesis of carboxyl end peptides. In SPPS, peptides were synthesized to extend from the C-terminal to the N-terminal. Amino acids used in this reaction have protecting groups to prevent unwanted reactions.

The resins were swollen in DMF for 1 hour before synthesis. In the first step of the synthesis cycle, first amino acid (0.2 M in DMF) was coupled to resin with its C-terminal (Figure 7). To bind the second amino acid, piperidine (20% in DMF) was used to remove the Fmoc protecting group of the first amino acid on the N-terminal. The carboxyl group of the second amino acid was activated using Oxyma® (1.0 M in DMF) and DIC (0.5 M in DMF). In the final stage of the synthesis cycle, the N terminal protecting group of the last amino acid was removed as described previously.

Cleavage

When the synthesis finished, the resin was taken to the Razor (Rapid Peptide Cleavage System) device and washed three times with DCM before cleavage. Peptides were separated from the resin by TFA / H_2O / TIS (95 / 2.5 / 2.5, v / v / v) solution at 37°C. Side chain protecting groups of amino acids were also removed at the same time at cleavage conditions at 37°C. The cleavage time varied between 30-45 minutes according to the number of arginine present in the peptide. Cleavage time for three or less arginine was 30 minutes. After three arginines, extra 5 minutes were added for each arginine up to 45 minutes. The cleaved peptides were collected with cold diethyl ether (-20 °C) and centrifuged for 3min at 3500 rpm. Thereby, the crude peptide was precipitated and obtained as free of chemicals. The precitipation step was repeated (3x). The precipitated peptides were dried under nitrogen for further purification.

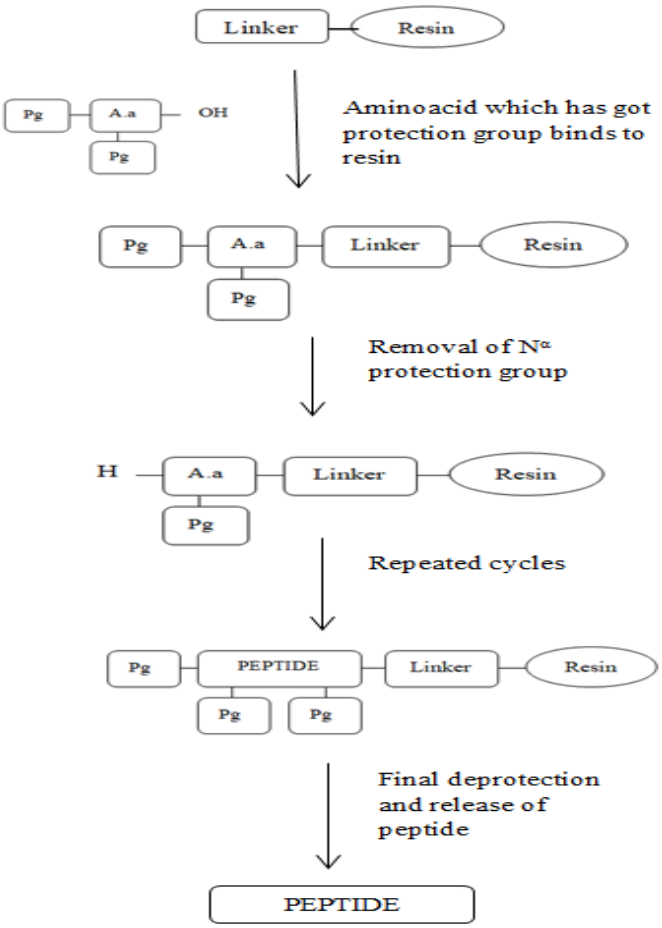

Figure 7 General Procedure for Solid Phase Peptide Synthesis

Purification

The peptides were purified using Agilent technologies 1260 Infinity. The UV-absorption was detected at 214 nm and 280nm. The columns used to analyze and purify the peptides were as follows:

Analytical HPLC: Agilent 675950-902 AdvanceBio Peptide Plus, 2.1 x 150 mm, 2.7 µm, LC column.

Semi-preparative HPLC: C-18 column Agilent Technologies VariTide RPC 250x10mm ID column

Analytical HPLC was performed at flow rate of 0.2 ml/min, semi preparative HPLC at flow rate of 1 ml/min. Two different mobile phases were used; Eluent A: 0.05% TFA in H_2O and eluent B: 0.25% TFA in acetonitrile. Synthesized peptides were first analyzed in 30 minutes in a range of 5-80% B with linear gradient. Afterwards, purification was done, by creating a new method, according to the retention % B. So the gradient of the solvent was chosen appropriate for each compound. Peptides were separated by peak based on HPLC and were frozen at -80 degrees and then lyophilized.

Table 3 Properties of synthesized peptides

Peptide	Sequence	Molecular Weight (g/mol)	Net charge at pH 7.0	Isoelectric Point	%Hydrophilic Residues
TN1	RLLRLLLLRLLR	1547.06	5.0	Non Isoelectric Point*	33 %
D-TN1	RLLRLLLLRLLR	1547.06	5.0	Non Isoelectric Point*	33 %
TN3-carboxy	RLLRLLRLLL	1278.69	3.0	12.8	30 %
D-TN3-carboxy	RLLRLLRLLL	1278.69	3.0	12.8	30 %
TN3V1	RVLRVLRVLL	1235.63	4.0	Non Isoelectric Point*	30 %
TN3V9	RVVRVVRVVV	1179.52	4.0	Non Isoelectric Point*	30 %
TN6	RLLRLLLRLLR	1433.90	5.0	Non Isoelectric Point*	36 %
D-TN6	RLLRLLLRLLR	1433.90	5.0	Non Isoelectric Point*	36 %
TN6I1	RIIRIIIRIIR	1433.90	5.0	Non Isoelectric Point*	36 %
TN6I2	RILRILIRLIR	1433.90	5.0	Non Isoelectric Point*	36 %

* Always positively charged, even at pH 0.

3.3 Examination of Structure-Activity Properties Using Physicochemical Techniques

Physicochemical characterization of synthetic peptides was done by Dr. Ahmet Emin Atik with analytical devices in Turgut İlaclari Biotechnology Development Center. Characterization of peptides synthesized by general metod for solid-phase peptide synthesis was studied by high-resolution mass spectrometry. Electrospray (ESI) mass spectrometry (MS) coupled to Waters H-Class Bio ultra performance liquid chromatography (UPLC) was used for characterization of peptides. The UPLC parameters used in the experiment is given in Table 5 and mass spectrometry parameters are given in Table 6.

Table 4 Experimental Parameters UPLC

UPLC Parameters					
Mobile Phase A	%100 Ultra Pure Water				
Mobile Phase B	%100 acetonitrile				
Mobile Phase C	%1 Formic acid				
Column Feature	ACQUITY UPLC Peptide CSH C18, 130 Å, 1.7 µm, 2.1 × 100 mm, Waters, 186006937				
Injection Volume	10 µL				
Column Temperature	40 °C				
Sampler Temperature	10 °C				
UV Wavelength	214 nm				
Analysis time	25 min				
Gradient Table and Flow Rates	Time (min)	%A	%B	%C	Flow rate (mL/min)
	0	85	5	10	0.200
	2	85	5	10	0.200
	9	70	20	10	0.200
	12	50	40	10	0.200
	16	20	70	10	0.200
	18	0	90	10	0.200
	20	0	90	10	0.200
	21	85	5	10	0.200
	25	85	5	10	0.200

Table 5 Experimental Parameters of Mass Spectrometry Used in Analysis

Mass Spectrometry Parameters	
Mass Spectrometry	Xevo G2-XS QToF
Software	UNIFI v1.8.2
ESI Polarity	Positive
Analysis Mode	Sensitivity
Source Parameters	Capillary Voltage (kV): 2.0 Kon Voltage (V): 50 Source Temperature (°C): 100 Desolvation Temperature (°C): 300 Con Gas Flow Rate (L/h): 50.0 Desolvation Gas Flow Rate (L/h): 600.0
Scan Time	0.500 sec
Low Collision Energy	4 eV
Collision Energy Range	25 eV – 30 eV
m/z Mass Range	50 – 2000 Da
Data Collection Time	0 – 25 min

Sequences of the analyzed peptides and theoretical monoisotopic mass (da) information are given in Table 3.

3.4 Determination of Antimicrobial Activity

The Minimal Inhibitory Concentration (MIC) is a test to determine the lowest drug concentration at which the growth of microorganism is inhibited completely. *Staphylococcus aureus (S. aureus)* ATCC 29213, which is Gram positive cocci, *Escherichia coli (E. Coli)* ATCC 25922 for fermentative Gram negative bacilli, *Pseudomonas aeruginosa (P. aeruginosa)* ATCC 27853 for non-fermentative Gram negative bacilli and *Candida albicans (C.albicans)* ATCC 10231 for fungi were used as a members belonging to different type of organisms, in MIC assays. The bacterial strains to be used were inoculated into the MH agar and incubated overnight at 37° C. The bacteria grown the next day were suspended to be 1 x 10^6 CFU / ml in the MH broth for use in the MIC experiment. AMPs were dissolved in the appropriate solution and the initial doses were adjusted to 1024µg/ml using the Pierce™

Quantitative Fluorometric Peptide Assay (Catalog number: 23290) kit. Eleven dilutions were made for each peptide in 96 well plates. Bacterial suspensions were added on prepared dilutions. Ampicillin, known for its effectiveness on bacteria, was used as a control. Results were evaluated after overnight incubation (138).

3.5 Determination of Hemolytic Activity (HC50)

The hemolytic activity test is a measurement of the increase in free plasma hemoglobin level as a result of contact of the substance with blood causing erythrocyte loss / damage. Fresh human blood was used in the experiments. 30 µl of fresh blood was suspended with autoclaved 10 mL TRIS Saline and centrifuged for 5 min at 1500 rpm (3x). The supernatant was removed and the pellet was resuspended with 10 ml TRIS Saline. Peptide concentrations were prepared with the Pierce™ Quantitative Fluorometric Peptide Assay kit and serial dilution was performed with TRIS Saline. 100µl of the solution prepared with blood cells was distributed to every well of the 96-well plate. Serial diluted peptide concentrations were also added as 100µl and incubated for 30 minutes at 37°C. Three replicates were performed for each dose of the peptides. 20% Triton-X 100 / DMSO, known to perform 100% hemolysis, was used as a positive control in experiments. At the end of the incubation time, the 96-well plate was centrifuged at 1500 rpm for 10 min. The supernatant in the wells was transferred to another plate for spectrophotometric measurement. Spectrophotometric reading was done at 414nm. The HC50s of the peptides were calculated with the formula below (138).

$$\% \text{ lysis} = OD_{414} - OD_{414}\,(\text{blank}) / OD_{414}\,(\text{total lysis} - \text{blank}) \times 100$$

3.6 Determination of Cytotoxicity

In order to evaluate the toxicity level of AMPs, in vitro cytotoxicity tests were performed in eukaryotic cells in cell culture medium. In this study, we used the CytoScan™ SRB Cell Cytotoxicity Assay kit based on staining the quantitative cellular proteins with Sulforodamine B (Biovision) to assess the damage that peptides can cause in eukaryotic cells. For cytotoxicity studies, It was decided to use the HaCaT cell line in the study because of its physical similarity with normal human keratinocyte cells and its unlimited growth feature (139). Tests were performed on multiple cell lines to analyze the cytotoxic effect of molecules on different cell types. In addition, Henrita Lacks Cell (HeLa) were used for this test. Firstly, to prepare the cell lines for experiment, we have incubated cells in a 5% CO_2 incubator at 37°C with DMEM consisting of 10% FBS, 1% penicillin-streptomycin. Cell lines were incubated until 80-85% adhesion to T75 culture flasks occured. After cell growth, we washed once with 1x phosphate buffered saline (PBS) without removing the cells. Cells were removed with 0.25% Trypsin-EDTA solution. Some cells were stained with trypan blue to perform cell count in the hemocytometer. Cells were inoculated in 96-well plates, with 10,000 cells in 200 µl of DMEM without FBS in each well. The plates were incubated 24 hours in an incubator with 5% CO_2 at 37°C. Peptide concentrations were prepared with the Pierce™ Quantitative Fluorometric Peptide Assay kit and serial dilution was performed with DMEM without FBS (140). Each peptide concentration was added to the cells as three replicates. Cells incubated under the same conditions without adding peptides, were used as controls. Cells that were incubated overnight with peptide containing media were stained by following the SRB kit protocol and analyzed by reading with a microplate reader at 565 nm wavelength(141).

3.7 Protease Resistance Assay

The peptide solution was prepared at a concentration of 1 mg/ml in tris-buffered saline (TBS buffer, pH = 7.6, Aldrich) with 10% DMSO. Proteinase K solution was prepared in TBS buffer that the stock solution was 1 mg/ml (enzyme activity = 30U/mg). 100 µl of the enzyme solution was added to 1 ml of peptide solution. It was incubated overnight at 37°C with shaking. The reaction was blocked by adding 1% TFA (100 µl) in water (142).

HPLC analyzes of peptide solutions with and without proteinase K enzyme were performed. Peptide solutions were first analyzed in 30 minutes in a range of 5-80% B with Linear gradient, with an analytical C18 Agilent technologies AdvanceBio Peptide Plus, 2.1 x 150mm, and 2.7µm, column. Two different mobile phases were used: A: 0.05% TFA / H20, B: 0.25% TFA / Acetonitrile.

MIC experiments were repeated to check biological activities after treating peptides with proteinase K.

3.8 Sample Preparation for SEM and TEM

For scanning electron microscope (SEM) and transmission electron microscope (TEM) evaluation, *E. coli* ATCC 25922 samples with D-TN6 peptide solution applied in 3 different concentrations (4 µg/ml, 8 µg/ml and 16 µg/ml) were studied. *E. coli* ATCC 25922 samples without D-TN6 peptide solution were used as a control. *E. coli* ATCC 25922 was treated with D-TN6 solution for 4 hours. After this application, samples were prepared for examination under TEM and SEM microscopes. These studies were conducted by Dr. Merve Acikel Elmas, a member

of Acibadem Mehmet Ali Aydinlar University, Department of Histology and Embryology, at Acıbadem Mehmet Ali Aydınlar University.

Preparation of cells for TEM. 20ml of prepared *E. coli* ATCC 25922 solutions were centrifuged at 4500 rpm for 10 minutes. The pellet was dissolved with 1ml of MH medium. 500µl solution was centrifuged at 10000 rpm for 5 minutes. The pellet was dissolved again in 1ml of MH broth. 500µl was taken and 6 different dilutions were made. Bacterial samples were fixed in 2.5% 0.1 M PBS buffered (pH 7.2) glutaraldehyde fixative for 3 hours. Samples, washed with PBS, were embedded in agar agar. Then, the samples were post-treated for 1 hour with 1% osmium tetroxide. Bacterial samples were dehydrated by rising with alcohol series of 50%, 70%, 90%, 96%, and 100%. Thin sections, approximately 60 nm thickness, were obtained from the agar blocks, which were passed through propylene oxide and embedded in epon 812 and polymerized for 1 night in an oven at 60°C. Sections were contrasted with a 3% uranyl acetate solution and analyzed by transmission electron microscopy (Thermo Scientific- Talos L120C) (143).

Preparation of cells for SEM. 10µl of bacterial solutions treated with different concentrations of D-TN6 were dropped to double-sided carbon tape. Bacterial samples were fixed in 2.5% 0.1 M PBS buffered (pH 7.2) glutaraldehyde fixative for overnight. After washing with buffer, post fixation was performed with 1% osmium tetroxide for 1 hour. It was dehydrated by passing through rinsing alcohol series (70%, 90%, 96%, and 100%). Following dehydration, 3/1, 1/1 and 3/3 alcohol / amylasetate series, the samples placed in pure amylasetate, were coated with gold after drying in the critical point dryer and examined under a scanning electron microscope (Thermo Scientific-Quattro SEM) (143).

4. RESULTS

4.1 Design of Synthesized Peptides

TN peptides developed by Dr. Nihan Unubol and Prof. Dr. Tanil Kocagöz formed the basis of this study. Peptides are designed as an alpha helix structure containing hydrophobic amino acids and positively charged amino acids that target the phospholipids of the bacterial cell membrane. While designing the peptides, the C-terminal region of the cathelicidin LL-37 of, known to have antimicrobial activity, was used as basis structure.

TN1, TN3 and TN6 peptides contain the longest and largest positively charged side chain arginine (Arg) and the longest hydrophophobic side chain leucine (Leu) amino acids. It is thought that the positively charged Arg will interact with negatively charged phosphate groups of the phospholipid membrane, while hydrophobic Leu will be embedded in the membrane. While designing the peptides, the PEPFOLD program was used to see its three-dimensional structures. Pepfold images show that the designed peptides retain their alpha helix structure (Figure 8).

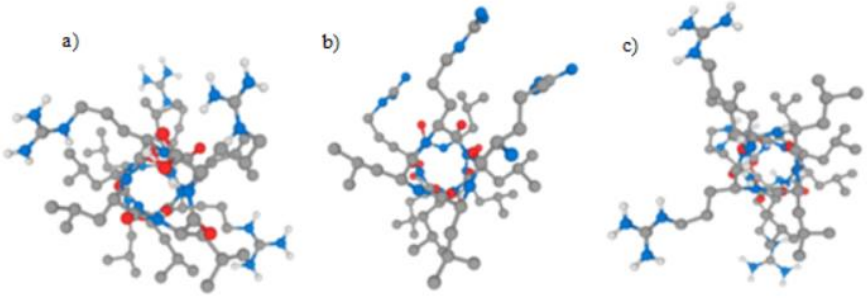

Figure 8 Three dimensional structures of a) TN1 b) TN3 c) TN6

In this study, TN1, TN3 and TN6 peptides, designed in the preliminary study and determined to have antimicrobial effects, were synthesized in D-form to make them protease resistant. In addition, new pepetides with valine (Val) and isoleucine (Ile) amino acids, which are also hydrophobic, replacing Leu amino acids, were designed and synthesized.

Based on the Arg amino acid sequence of the TN3 peptide, two new peptides have been designed by replacing the amino acid Leu and Val (Figure 9). Val amino acid has been chosen because it has a hydrophobic structure.

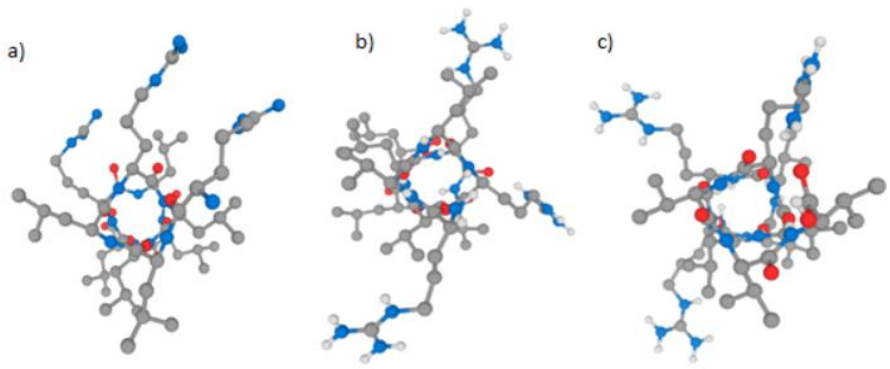

Figure 9 Three dimensional structure of a) TN3 b) TN3V1 c) TN3V9

Based on the Arg amino acid sequence of the TN6 peptide, TN6I1 and TN6I2 peptides were designed as a result of combinations of the hydrophobic Ile amino acid, which replaced Leu (Figure 10).

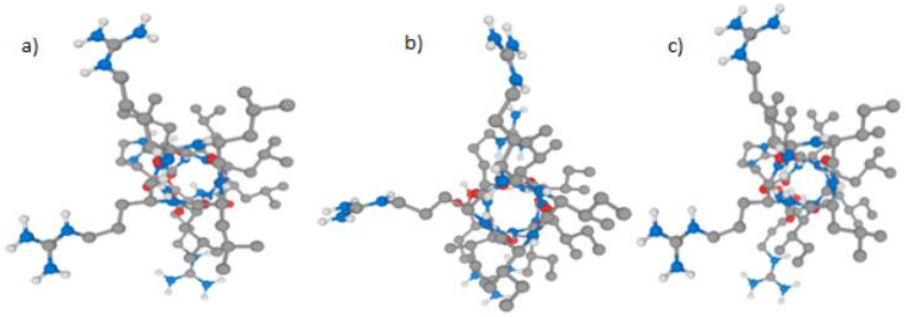

Figure 10 Three dimensional structure of a) TN6 b) TN6I1 c) TN6I2

Amino acid sequences and the features of the designed AMPs are given in Table 4.

4.2 Purification of Synthesized Peptides

The first analysis of peptides, synthesized by the SPPS method, was made by RP-HPLC. Chromotogram analyzes of the synthesized peptides are given below. Retention times and according to percentage of B solution of peptides were determined by linnear gradient, then purification of peptides were done with focus gradient by narrow the scanned area of B%.

HPLC chromatograms of TN1 peptides are shown in Figure 11. The retention time L-Form TN1 was 29.7 min and D-TN1 was 25.45 min. The appearance of one main peak in these chromatograms, indicated that the peptides contained a single type of molecule. During the purification process, molecules belonging to only main peaks were collected, small fluctuations were also eliminated and 100% purity peptides were obtained.

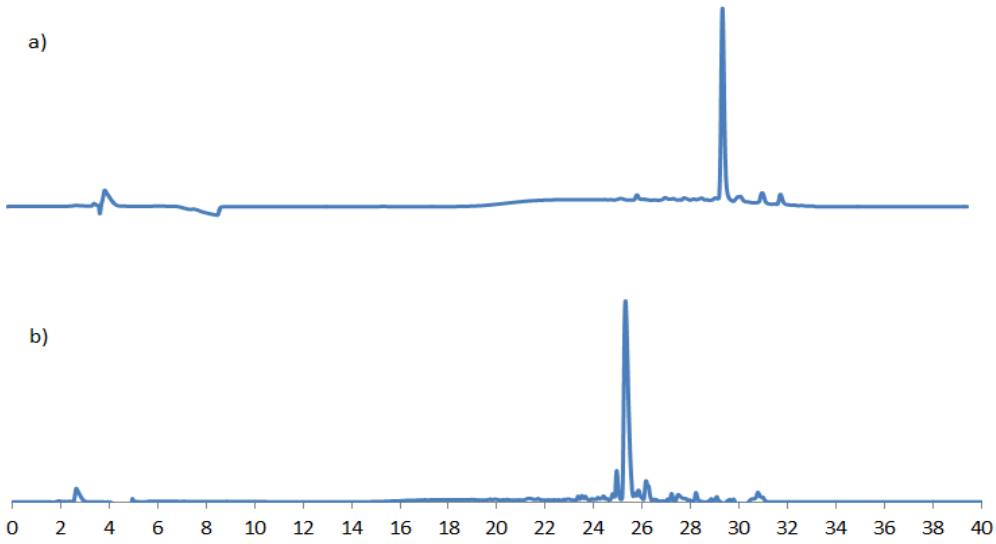

Figure 11 HPLC chromatograms of TN1 peptides

a) L form TN1, b) D form TN1

HPLC chromatograms of TN3 peptides are shown in Figure 12. The retention time L-Form of TN3 was 29.6 min and D-TN3 was 29.8 min. The appearance of one main peak in these chromatograms, indicated that the peptides contain a single type of molecule. During the purification process, molecules belonging to only main peaks were collected, small fluctuations were also eliminated and 100% pure peptides, was obtained.

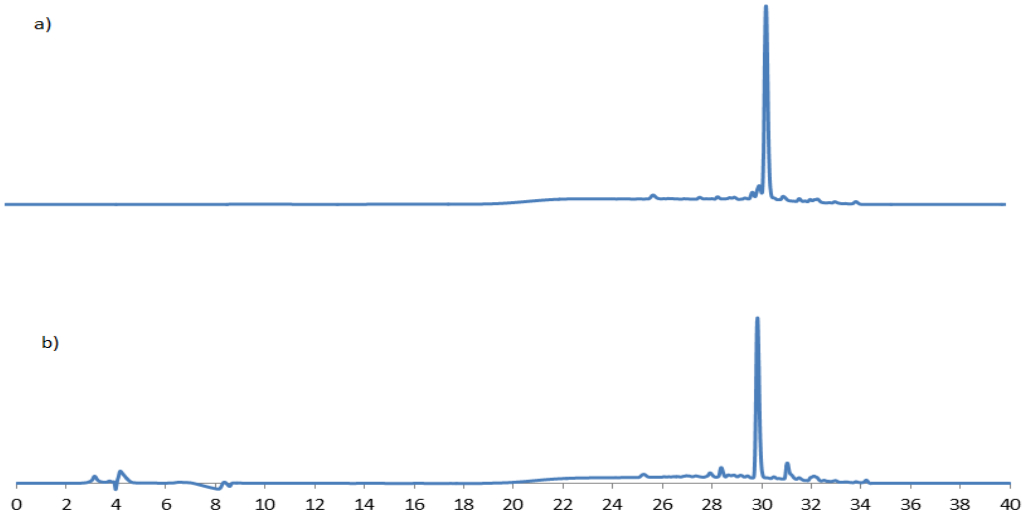

Figure 12 HPLC chromatograms of TN3 peptides

a) L form TN3 b) D form TN3

HPLC chromatograms of TN6 peptides are shown in Figure 13. The retention time of L-Form TN6 was 16.28 min and D-TN6 was 16.28 min. The appearance of one main peak in these chromatograms, indicated that the peptides contain a single molecule. During the purification process, molecules belonging to only main peaks were collected, small fluctuations were also eliminated and 100% purity peptides were obtained.

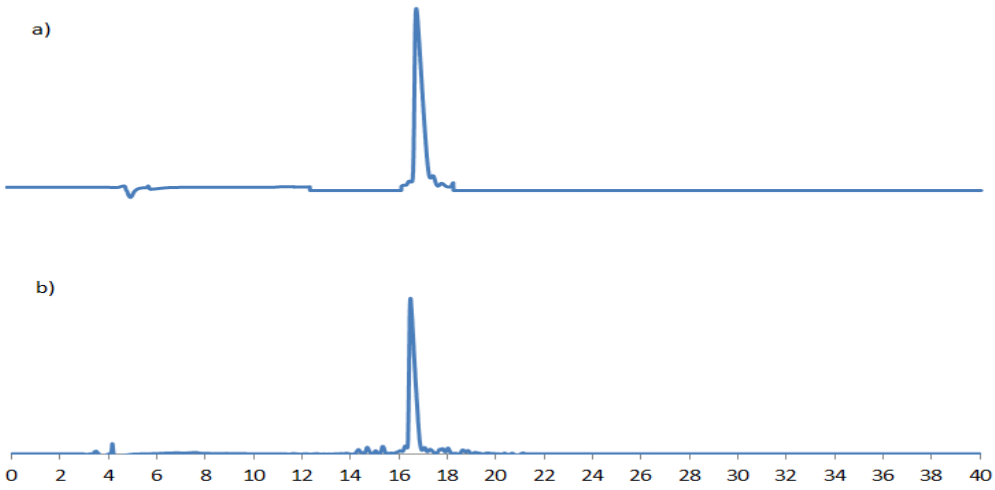

Figure 13 HPLC chromatograms of TN6 peptides

A)L form TN6 B) D form TN6

HPLC chromatograms of TN3V1 and TN3V9 peptides are shown in Figure 14. The retention time of TN3V1 was 18.2 min and TN3V9 was 29. min. The appearance of one main peak in these chromatograms, indicated that the peptides contain a single molecule. During the purification process, molecules belonging to only main peaks were collected, small fluctuations were also eliminated and 100% purity peptides were obtained.

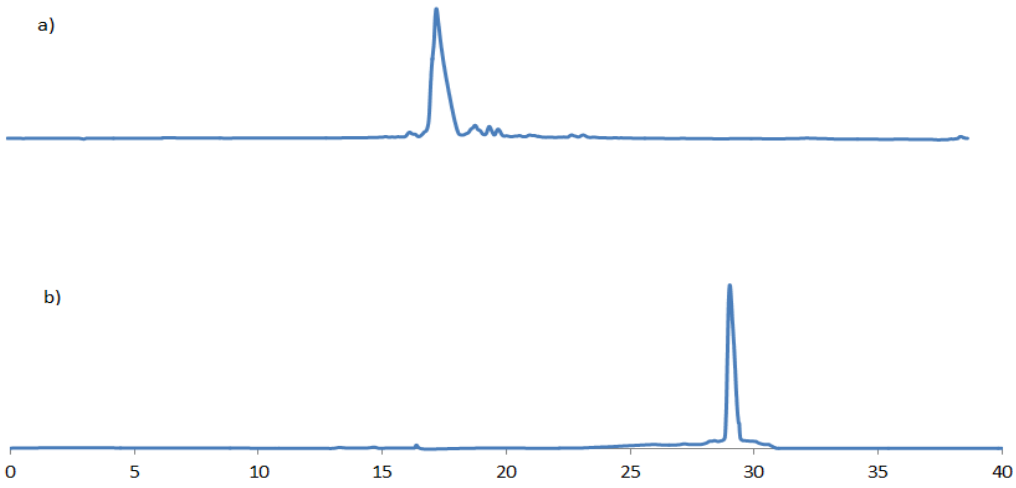

Figure 14 HPLC chromatograms of a) TN3V1 and b) TN3V9 peptides

HPLC chromatograms of TN6I1 and TN6I2 peptides are shown in Figure 15. The retention time of TN6I2 was 19.05 min. The appearance of one main peak in TN6I2 chromatogram indicates that the peptides contain a single type of molecules. During the purification process, only molecules belonging to main peak were collected, small fluctuations were also eliminated and 100% purity of peptides was obtained. TN6I2 chromogram showed two peaks, although LC-MS / MS studies have shown that these two peaks belong to molecules with the same amino acid sequence.

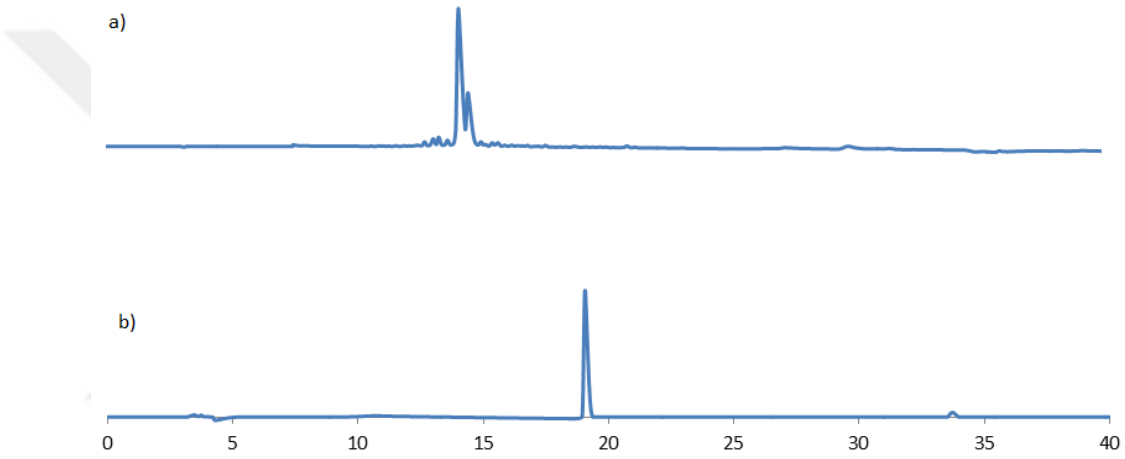

Figure 15 HPLC chromatograms of a) TN6I1 and b) TN6I2 peptides

4.3 Examination of Structure-Activity Properties of AMPs Using Physicochemical Techniques

LC-MS / MS Analysis Results of TN1 Peptide

The synthesized peptide contains twelve amino acids and the C-terminus of the peptide is in amide form. The sequence of the peptide is RLLRLLLLRLLR-NH_2. Peptide peak was detected in UPLC system with UV detector in 14.45 minutes (Figure 16).

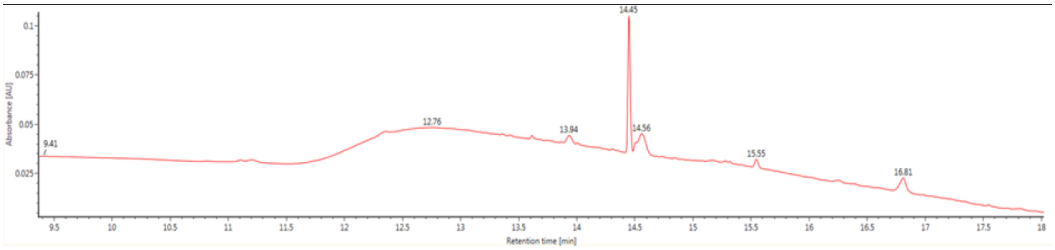

Figure 16 LC-UV Chromatogram of TN1 Peptide

At 14.45th minute the mass spectrum of the peak from the column indicated that two proton-acquired peptide ions ([M + 2H] $^{2+}$) at *m / z* 774.0670 and three proton-acquired peptide ions ([M + 3H] $^{3+}$) m / z 516.3776 (Figure 17).

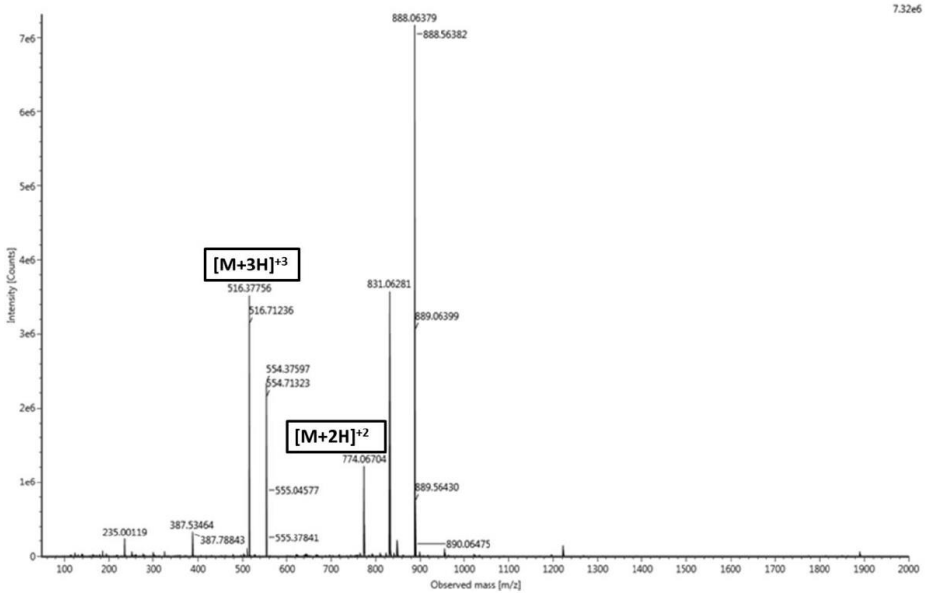

Figure 17 Mass Spectrum of TN1 Peptide

The mass of the [M + H] $^{+}$ ion of the peptide with the UNIFI software was calculated as *m / z* 1547.1125. The theoretically calculated monoisotopic [M + H] $^{+}$ is 1547.1108 and the margin of error in mass measurement is 1.1 ppm. The MS / MS spectrum of the peak at 14.45 minutes confirmed that the desired peptide was synthesized (Figure 18).

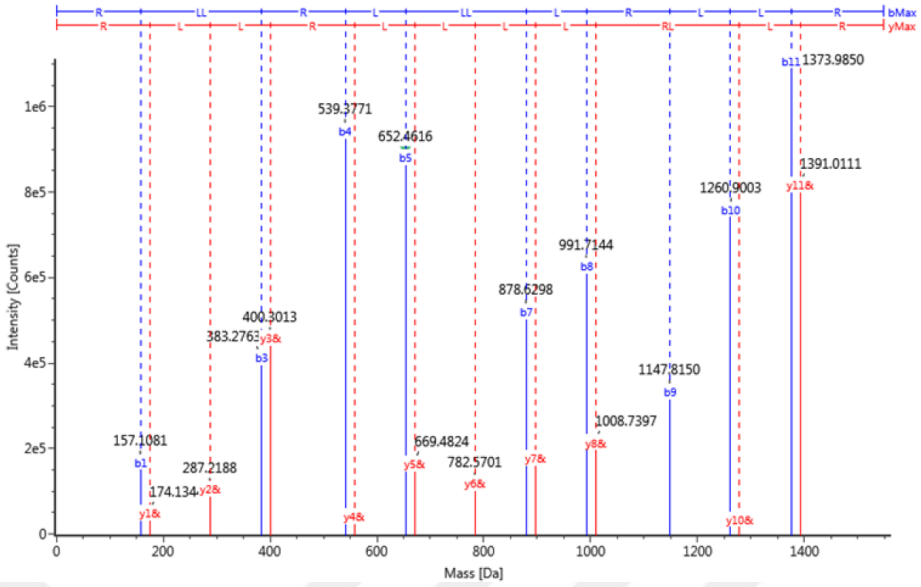

Figure 18 MS / MS Mass Spectrum of TN1 Peptide

LC-MS / MS Analysis Results of TN3 Peptide

The synthesized peptide contains ten amino acids and the C-terminus of the peptide is in carboxy form. The peptide sequence is RLLRLLRLLL-COOH. Peptide peak was detected in UPLC system with UV detector at 14.52 minutes (Figure 19)

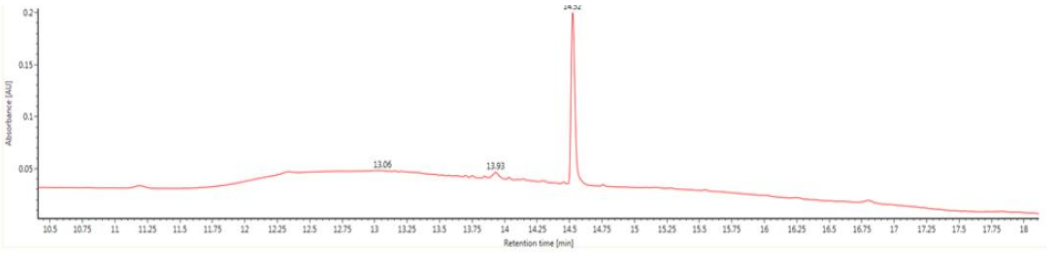

Figure 19 LC-UV Chromatogram of TN3 Peptide

At 14.52nd minute the mass spectrum of the peak from the column indicated, two proton-acquired peptide ions ([M + 2H] $^{2+}$) at m / z 639.9610 and three proton-acquired peptide ions ([M + 3H] $^{3+}$) m / z 426.9743 (Figure 20).

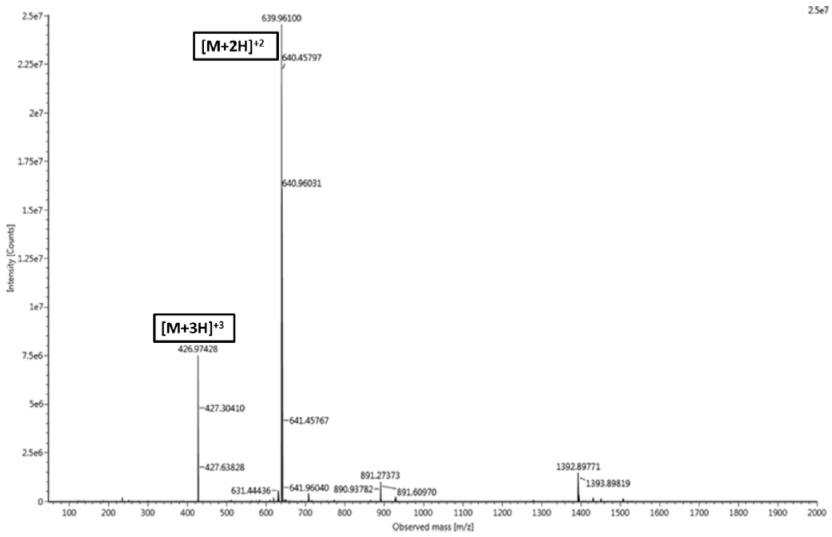

Figure 20 Mass Spectrum of TN3 Peptide

The observed mass of [M + H] $^{+}$ ion was calculated as *m / z* 1278.9116. The theoretically calculated monoisotopic [M + H] $^{+}$ is 1278.9096 and the margin of error in mass measurement was calculated as 1.5 ppm. The MS / MS spectrum of the peak at 14.52 minutes confirmed that the desired peptide was synthesized (Figure 21).

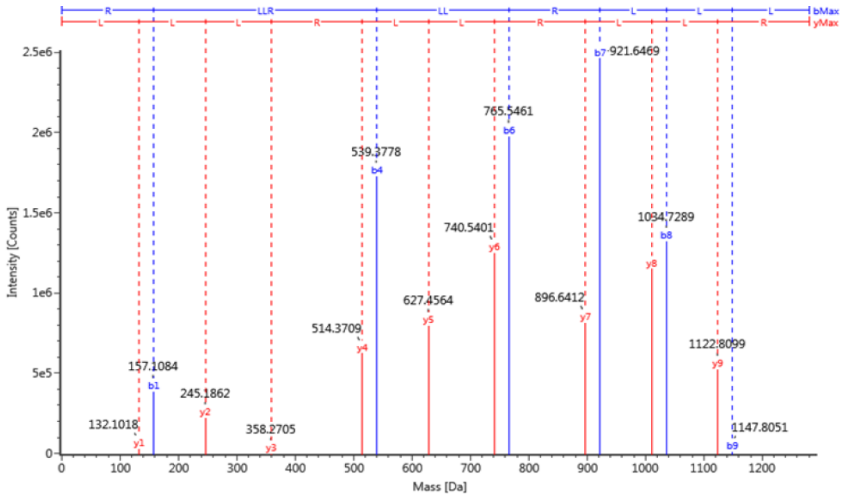

Figure 21 MS / MS Mass Spectrum of TN3 Peptide

LC-MS / MS Analysis Results of TN6 Peptide

The synthesized peptide contains eleven amino acids and the C-terminus of the peptide is in amide form. The peptide sequence is RLLRLLLRLLR-NH_2. Peptide peak was detected in UPLC system with UV detector at 11.64 minutes (Figure 22).

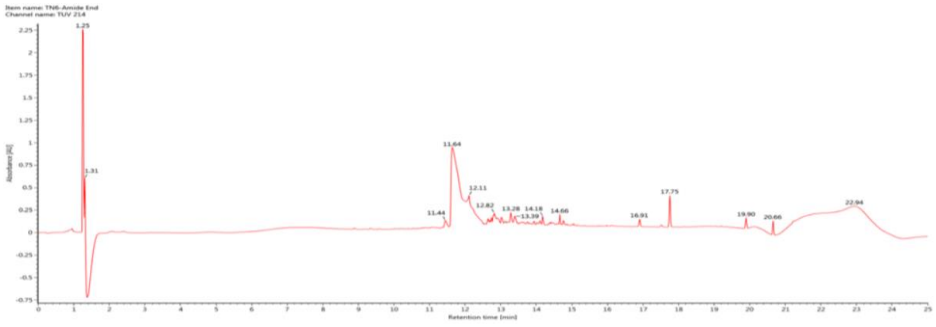

Figure 22 LC-UV Chromatogram of TN6 Peptide

At 11.64th minute the mass spectrum of the peak from the column indicated, two proton-acquired peptide ions ($[M + 2H]^{2+}$) at *m / z* 717.5122 and three proton-acquired peptide ions ($[M + 3H]^{3+}$) at *m /z* 478.6799 (Figure 23)

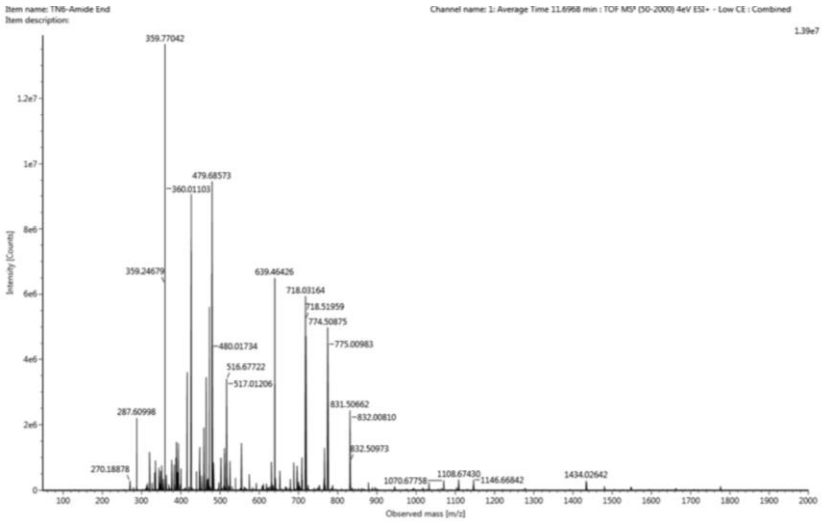

Figure 23 Mass Spectrum of TN6 Peptide

Fragment ions (b and y ions) obtained as a result of gas-phase cleavage of peptides (MS / MS) yielded the amino acid sequence of the AMP (Figure 24). All these obtained data showed that the desired peptide was synthesized correctly.

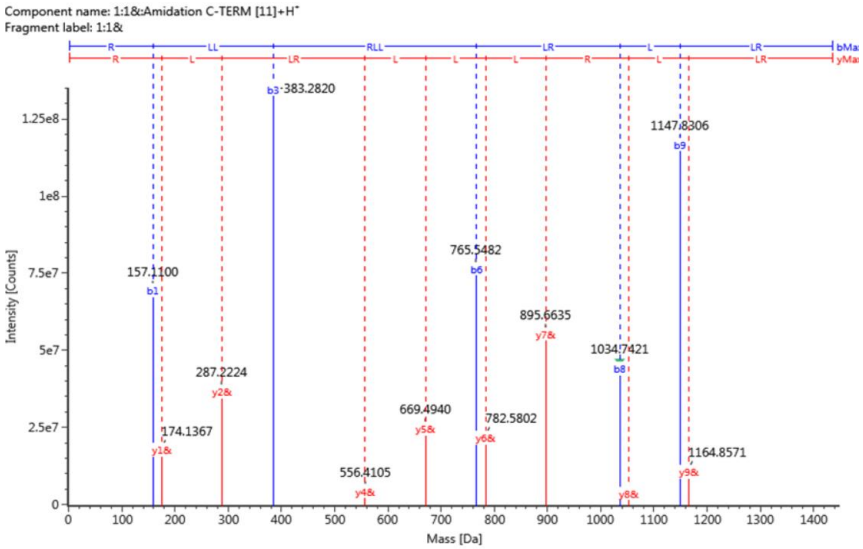

Figure 24 MS / MS Mass Spectrum of TN6 Peptide

LC-MS / MS Analysis Results of TN1 D-Form Peptide

The synthesized peptide contains twelve amino acids and the C-terminus of the AMP is in amide form. The sequence of the AMP is RLLRLLLLRLLR-NH2. Peptide peak was detected in the UV detector of UPLC system at 11.87 minutes (Figure 25).

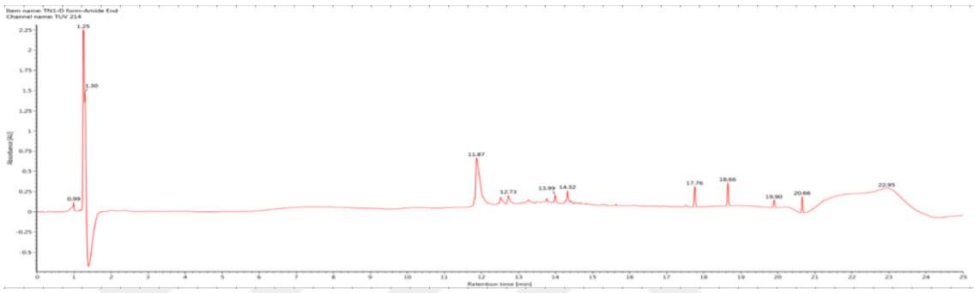

Figure 25 LC-UV Chromatogram of TN1 D-Form Peptide

At 11.87th minute the mass spectrum of the peak from the column indicated, three proton-acquired peptide ions ($[M+3H]^{3+}$) at *m/z* 516.3751 and four proton-acquired peptide ions ($[M+4H]^{4+}$) at *m/z* 387.5328 (Figure 26).

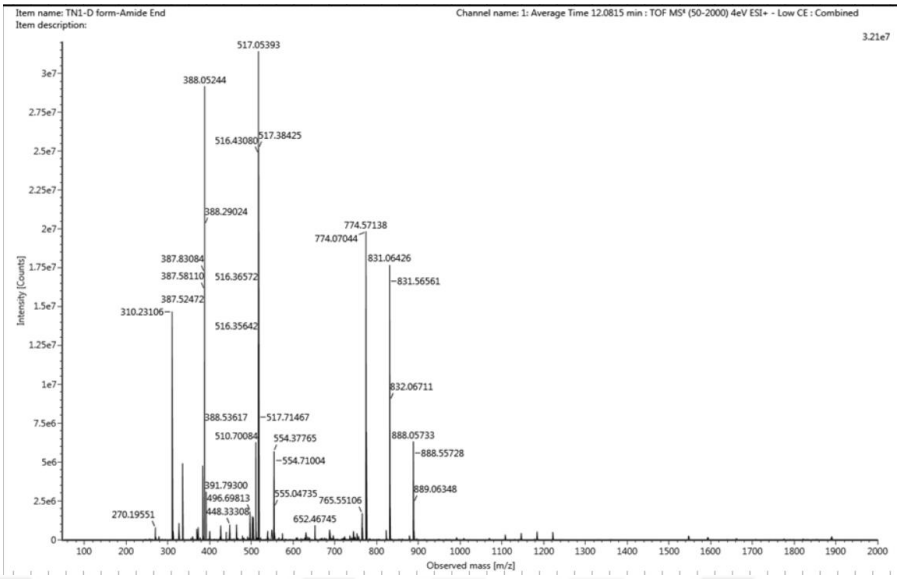

Figure 26 Mass Spectrum of TN1 D-Form Peptide

The fragmentation ions (b and y ions) obtained as a result of gas-phase cleavage of the peptides (MS / MS) yielded the amino acid sequence of the peptide (Figure 27). All these data obtained showed that the desired peptide was synthesized correctly.

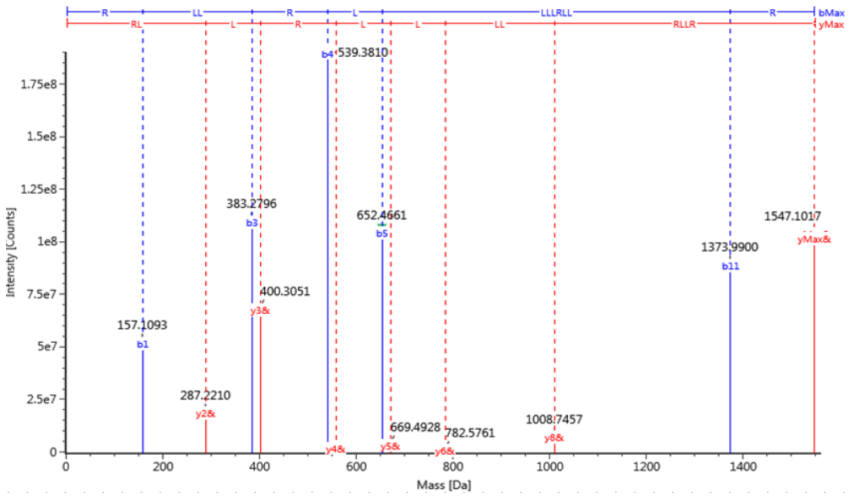

Figure 27 MS / MS Mass Spectrum of TN1 D-Form Peptide

LC-MS / MS Analysis Results of TN3 D-Form Peptide

The synthesized peptide contains amino acids ten D-molecules and the C-terminus of the peptide is in the form of carboxyl acid. The peptide sequence is RLLRLLRLLL-COOH. Peptide peak was detected in 14.54 minutes in UPLC system with UV detector (Figure 28).

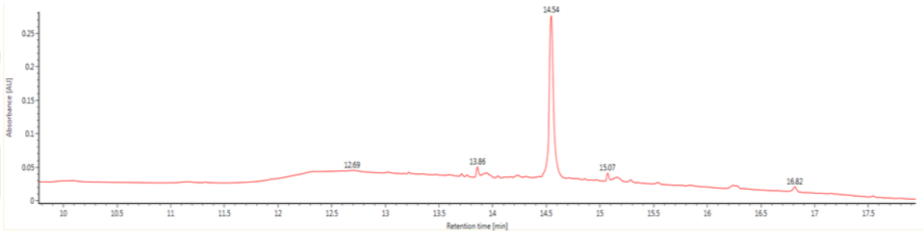

Figure 28 LC-UV Chromatogram of TN3 D-Form Peptide

When the 15.54th minute examines the mass spectrum of the peak from the column, two proton-acquired peptide ions ($[M + 2H]^{2+}$) at *m / z* 639.9662 and three proton-acquired peptide ions ($[M + 3H]^{3+}$) at *m /z* 426.9743 were determined (Figure 29).

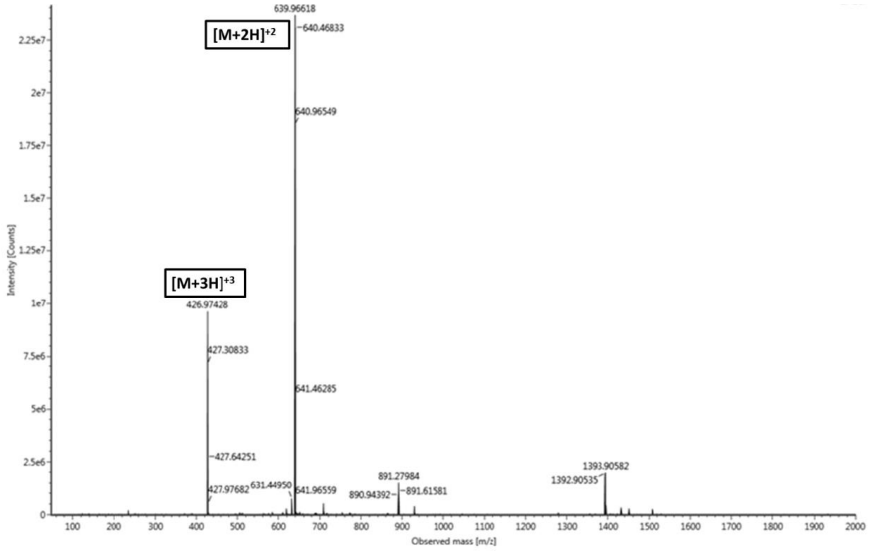

Figure 29 Mass Spectrum of TN3 D-Form Peptide

The observed mass of [M + H] + ion was calculated as m / z 1278.9107. The theoretically calculated monoisotopic [M + H] + is 1278.9096 and the tolerans of error in mass measurement was calculated as 0.8 ppm. The MS / MS spectrum of the peak at 14.54 minutes confirmed that the desired peptide was synthesized (Figure 30).

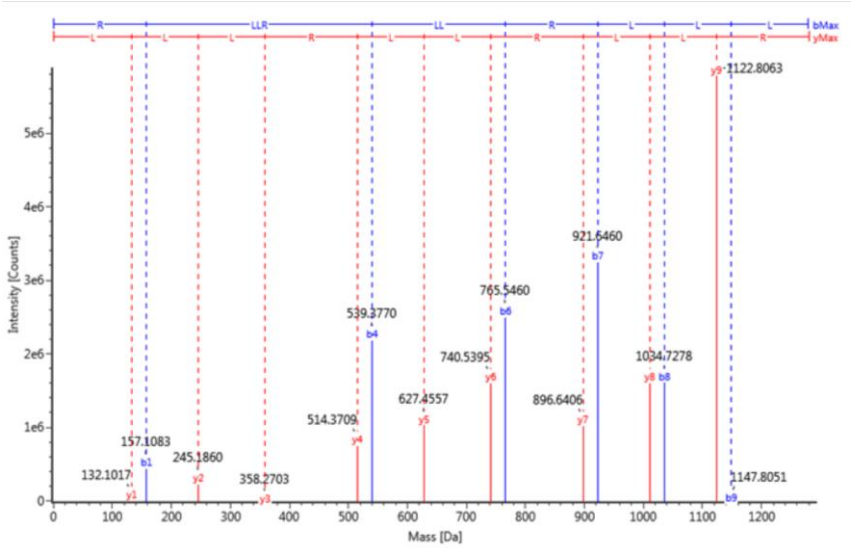

Figure 30 MS / MS Mass Spectrum of TN3 D-Form Peptide

LC-MS / MS Analysis Results of TN6 D-Form Peptide

The synthesized peptide contains amino acids eleven D-molecules and the C-terminus of the AMP is in the form of carboxyl acid. The peptide sequence is RLLRLLLRLLR-NH_2. Peptide peak was detected in 11.69 minutes in UPLC system with UV detector (Figure 31).

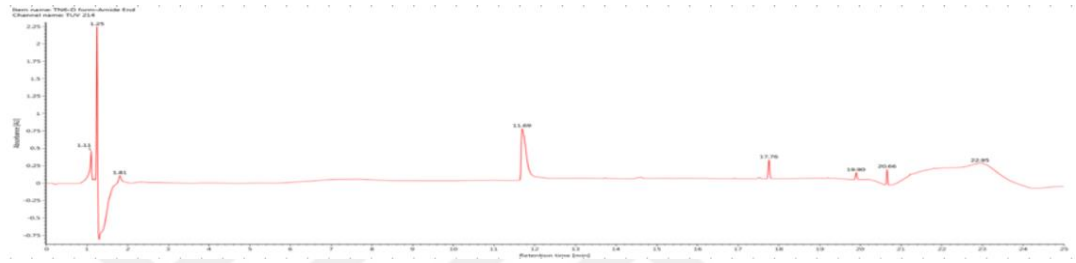

Figure 31 LC-UV Chromatogram of TN6 D-Form Peptide

When the 11.69th minute examines the mass spectrum of the peak from the column, three proton-acquired peptide ($[M+3H]^{3+}$) at *m/z* 478.6811 and four proton-acquired peptide ions ($[M+4H]^{4+}$) at *m/z* 359.2625 were determined (Figure 32).

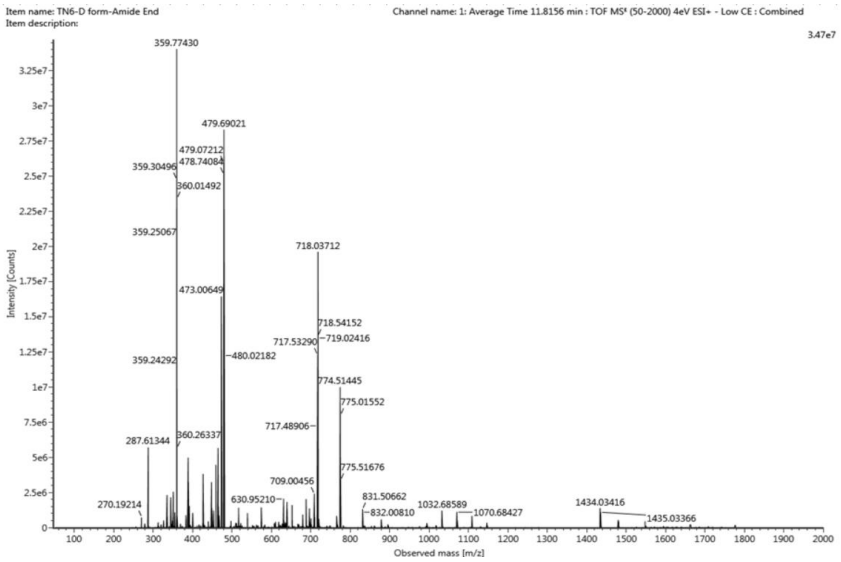

Figure 32 Mass Spectrum of TN6 D-Form Peptide

The fragmentation ions (b and y ions) obtained as a result of gas-phase cleavage of the peptides (MS / MS) yielded the amino acid sequence of the AMP (Figure 33). All these data obtained showed that the desired peptide was synthesized correctly.

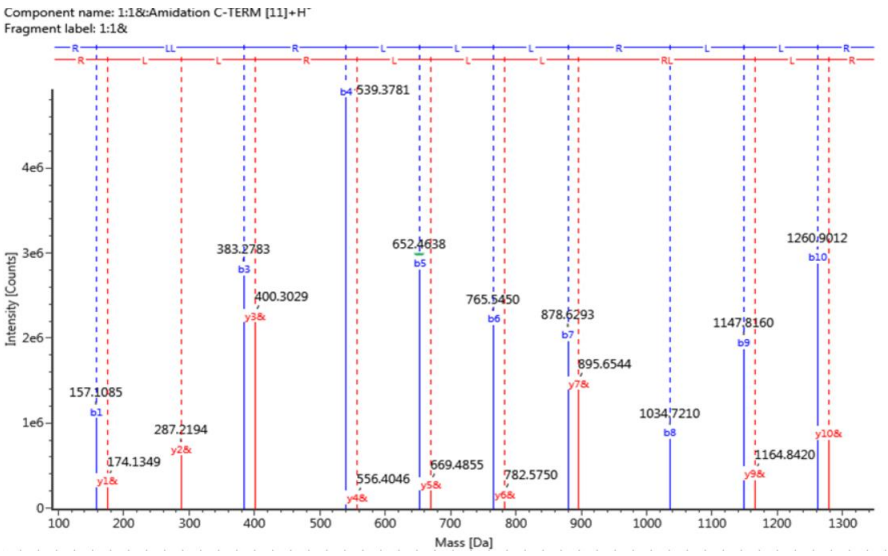

Figure 33 MS / MS Mass Spectrum of TN6 D-Form Peptide

UPLC Analysis Results of TN6I1 Peptide Peak 1

HPLC chromatogram of TN6I1 peptide is shown in Figure 15 A. When this chromatogram is examined, it is seen that there are two peaks of TN6I1 peptide solution. UPLC analyzes were performed by collecting both peaks from the HPLC system.

The synthesized peptide contains eleven amino acids and the C-terminal of the AMP is in amide form. The sequence of the peptide is RIIRIIIRIIR- NH_2. Peptide peak was at 9.12 min in UPLC device with UV detector (Figure 34).

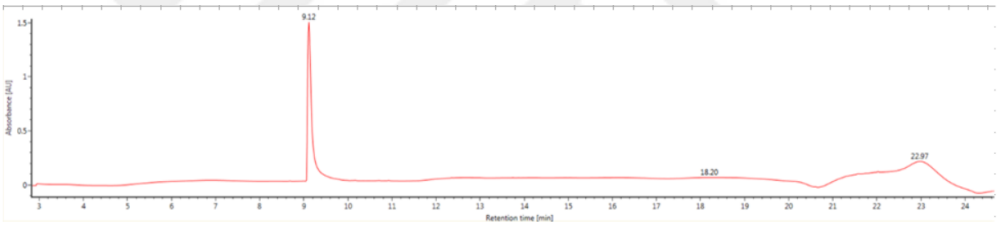

Figure 34 LC-UV Chromatogram of TN6I1-Peak 1 Peptide

At 9.12nd minute examines the mass spectrum of the peak from the column indicate, two proton-acquired peptide ($[M+2H]^{2+}$) at *m/z* 717.5162 and three proton-acquired peptide ions ($[M+3H]^{3+}$) at *m/z* 478.6828.

UPLC Analysis Results of TN6I1 Peptide Peak 2

The synthesized peptide contains eleven amino acids and the C-terminal of the AMP is in amide form. The sequence of the peptide is RIIRIIIRIIR- NH_2. Peptide peak was at 10.59 min in UPLC device with UV detector (Figure 35).

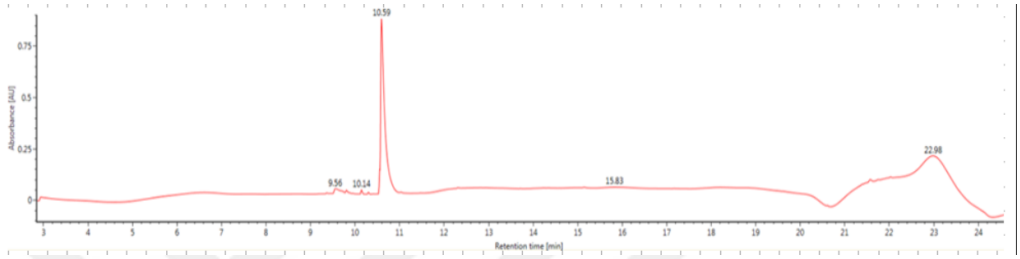

Figure 35 LC-UV Chromatogram of TN6I1-Peak 1 Peptide

At 10.59th minute the mass spectrum of the peak from the column indicated, three proton-acquired peptide ($[M+3H]^{3+}$) at *m/z* 478.6811 and four proton-acquired peptide ions ($[M+4H]^{4+}$) at *m/z* 359.2625.

When UPLC results were examined, it was seen that both peak images referred to the same amino acid sequence.

LC-MS / MS Analysis Results of TN6I2 Peptide

The synthesized AMP contains 11 amino acids and the C-terminal of the AMP is in amide form. The sequence of the peptide is RILRILIRLIR-NH_2. Peptide peak was at 10.34 min in UPLC device with UV detector (Figure 36).

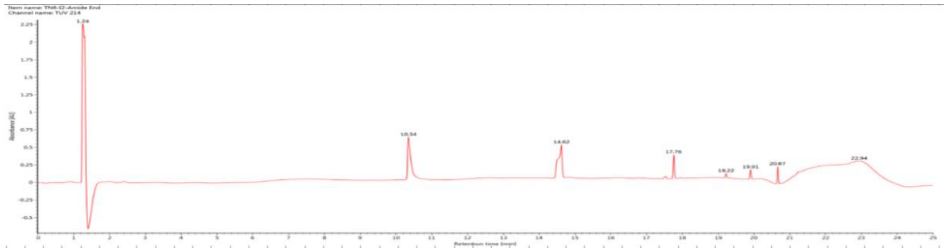

Figure 36 LC-UV Chromatogram of TN6I2 Peptide

At 10.34th minute the mass spectrum of the peak from the column indicated, three proton-acquired peptide ions ($[M+3H]^{3+}$) and at *m/z* 478.6812 two proton-acquired peptide ($[M+2H]^{2+}$) at *m/z* 717.5182 (Figure 37).

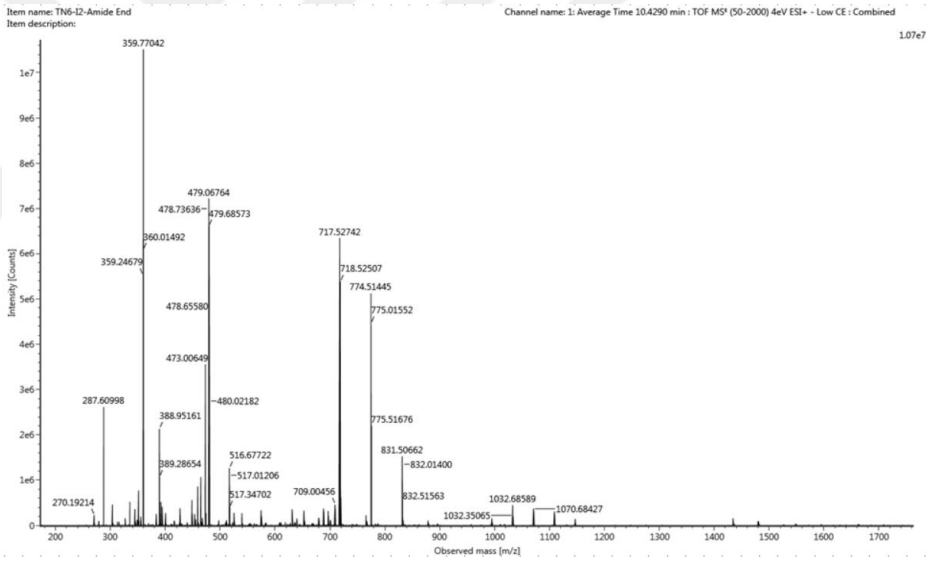

Figure 37 Mass Spectrum of TN6I2 Peptide

The fragmentation ions (b and y ions) obtained as a result of gas-phase cleavage of the peptides (MS / MS) yielded the amino acid sequence of the peptide (Figure 38). All these data obtained showed that the desired peptide was synthesized correctly.

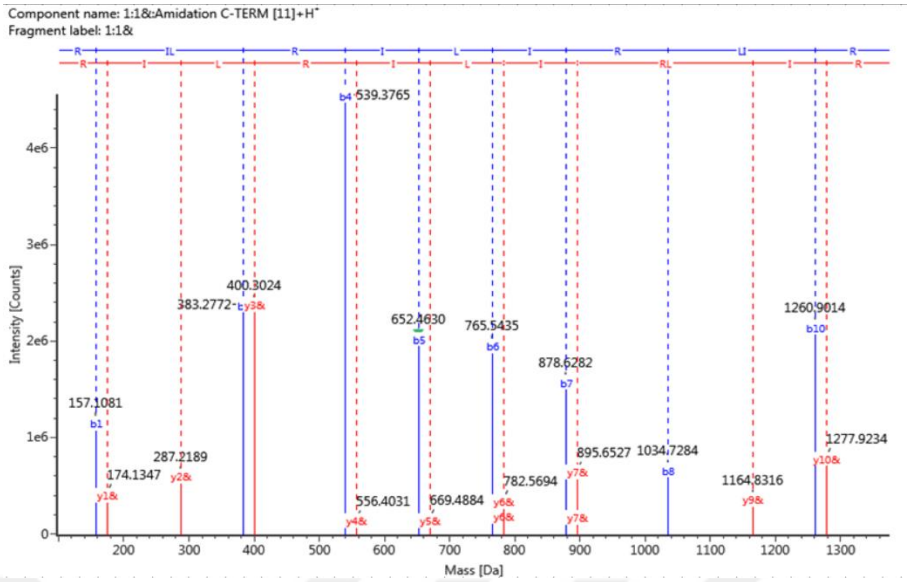

Figure 38 MS / MS Mass Spectrum of TN6I2 Peptide

4.4 Antimicrobial Efficacy Results of the Synthesized AMPs

MIC results of the designed peptides on *S. aureus* ATCC 29213, *E. coli* ATCC 25922, *P. aeruginosa* ATCC 27853 and *Candida albican* ATCC 10231 strains are given in Table 7. Some of the designed peptides have shown better efficacy than ampicillin. For *E. coli* ATCC 25922, ampicillin has a MIC value of 8 µg/ml while the MIC value of the TN1 is 2 µg/ml, TN3 peptide is 4 µg/ml, and the TN6 peptide is 2 µg/ml. TN6-D-form peptide synthesized in D-form was found to be 2 times more effective than L form for *E. coli* ATCC 25922, and MIC value of TN3-D-form peptide was 2 times more effective than L form. When the TN1 peptide was synthesized in D-form, its efficacy decreased 4 times.

When the efficacy of TN1, TN3 and TN6 peptides on strain *S. aureus* ATCC 29213 were examined, it was seen that the most effective one was the TN6 peptide, which contained arg at both ends, and three leu in the middle. TN3, which contains arg only at one end, is 8 times less active than TN6 peptide. While TN1 activity

remained the same in D-form synthesized derivative D-TN1, D-TN3 and D-TN6 activity increased 2-fold compared to L forms.

When the activity of TN1, TN3 and TN6 peptides on *P. aeruginosa* ATCC 27853 strain were compared, the activity of TN3 and TN1 were found to be significantly reduced, while the activity of TN6 peptide was determined as 8μg/ml. It was found that synthesizing peptides in D-form increased their activity. The most active peptide was determined as TN6-D-form peptide.

Among all peptides synthesized in this study, TN1, TN3 and TN6 had highest activity on *Candida albicans* ATCC 10231 in both D and L forms.

Based on the arg positions of the TN3, TN3V1 and TN3V9 peptides were designed by the replacement of leu with val. Val replacement increased 2-fold the activity of the TN3V1 peptide on *S. aureus* ATCC 29213. However, its activity on *E. coli* ATCC 25922 and *Candida albicans* ATCC 10231 decreased 4-fold while its activity on *P. aeruginosa* ATCC 27853 remained the same. On the other hand, the activity of TN3V9 peptide, remained the same compared to TN3, on *S. aureus* ATCC 29213, while decreasing on other species of bacteria.

Based on the arg positions of the TN6, TN6I2 and TN6I2 peptides, designed by replacing leu with ile amino acids. Compared to TN6, the activity of TN6I2 on *S. aureus* ATCC 29213 remained the same. However, the activity of TN6I1 and TN6I2 on all other species of bacteria tested, decreased.

Further studies with TN6I1, TN3V1 and TN3V9, whose MIC values were not satisfactory, have not been performed.

Table 6 MIC (µg / ml) results of synthesized AMPs

Antimicrobial Peptides	*S. aureus* ATCC 29213	*E. coli* ATCC 25922	*P.aeruginosa* ATCC 27853	*Candida albicans* ATCC 10231
TN6 – amide end RLLRLLLRLLR-NH_2	2 µg/ml	2 µg/ml	8 µg/ml	<1 µg/ml
D-TN6 –D form RLLRLLLRLLR-NH_2	1 µg/ml	<1 µg/ml	2 µg/ml	< 1µg/ml
D-TN1 –D form- RLLRLLLLRLLR-NH_2	4 µg/ml	8 µg/ml	4 µg/ml	1 µg/ml
D-TN3 –D form RLLRLLRLLL-COOH	8 µg/ml	2 µg/ml	4 µg/ml	<1 µg/ml
TN6I1– amide end RIIRIIIRIIR-NH_2	16 µg/ml	32 µg/ml	128 µg/ml	128 µg/ml
TN6I2– amide end RILRILIRLIR-NH_2	2 µg/ml	16 µg/ml	32 µg/ml	128 µg/ml
TN3V1– amide end RVLRVLRVLL-NH_2	8 µg/ml	16 µg/ml	32 µg/ml	4 µg/ml
TN3V9– amide end RVVRVVRVVV-NH_2	16 µg/ml	64 µg/ml	256 µg/ml	128 µg/ml
TN3- RLLRLLRLLL-COOH	16 µg/ml	4 µg/ml	32 µg/ml	1 µg/ml
TN1- RLLRLLLLRLLR-NH_2	4 µg/ml	2 µg/ml	32 µg/ml	<1 µg/ml

4.5 Hemolytic Activity (HC50) Results of Synthesized AMPs

Percentage lysis values of red blood by synthesized AMPs cells betweeen the concentration range of 1µg/ml and 128µg/ml are shown in Figure 39. The MIC values of these peptides on different organisms are shown in Table 6.

Among the peptides we have developed, D-TN6 had highest activity on the *P.aeruginosa* with an MIC of 2 µg / ml. D-TN6 peptide did not reach HC_{50} at a concentration of 128µg/ml, which is 64 times the MIC value (Figure 35).

D-TN1 and D-TN3 peptides reached HC_{50} at a concentration of 16µg/ml. This concentration is 2 times the highest MIC value of D-TN1 and D-TN3 peptides. (Figure 35).

TN6 has the highest MIC for *P.aeruginosa* which is 8µg/ml. The HC_{50} of TN6 is higher than its MIC. The highest MIC of TN6I2 peptide, which was 32µg/ml, was for *P.aeruginosa* and 128µg/ml for *Candida albicans* ATCC 10231. The HC_{50} of this peptide exceeded 128 µg/ml.

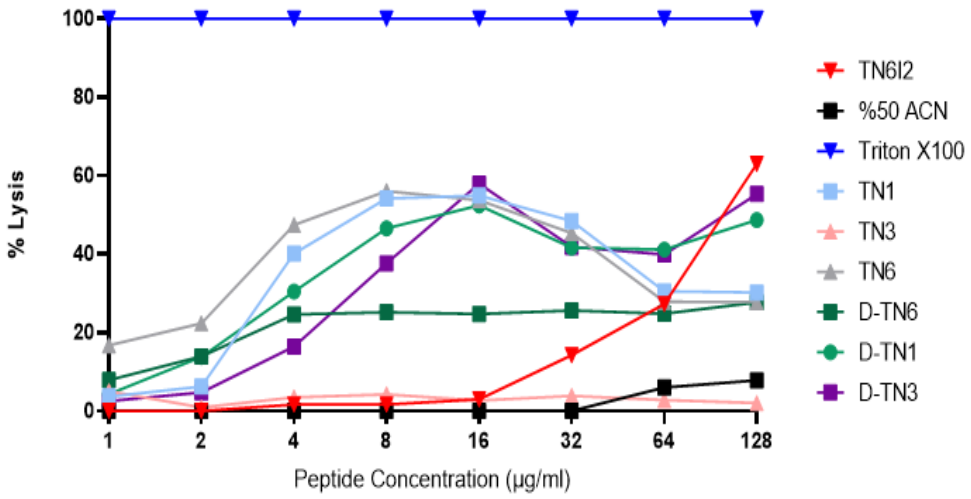

Figure 39 % Lysis results of synthesized peptides

4.6 Cytotoxicity Results of Synthesized AMPs

Toxicity profiles of peptides designed and produced by chemical production on eukaryotic cells were compared with the Magainin peptide.

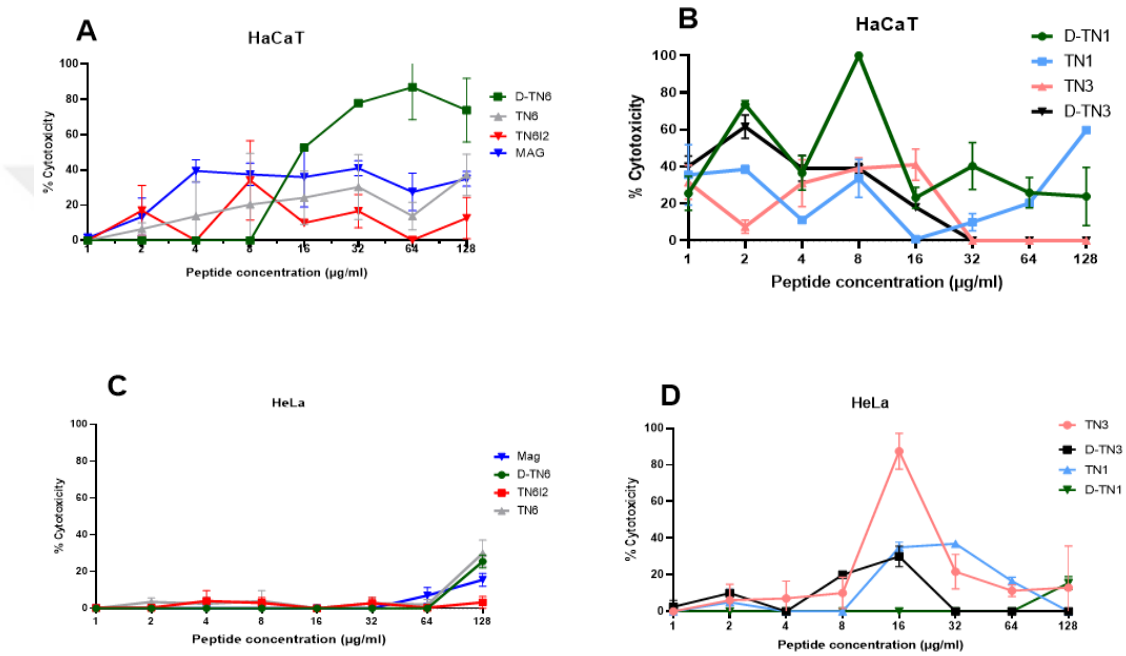

Figure 40 % Cytotoxicity Results of Synthesized AMPs

Evaluation of designed peptides at different peptide concentrations with Magain cytotoxicity results. **A)** HaCaT cell line cytotoxicity results of D-TN6, TN6, TN6I2, Mag peptides **B)** HaCaT cell line cytotoxicity results of D-TN1, TN1, TN3, D-TN3 **C)** HeLa cell line cytotoxicity results of D-TN6, TN6, TN6I2, Mag peptides **D)** HeLa cell line cytotoxicity results of D-TN1, TN1, TN3, D-TN3

According to the results given in Figure 36, peptides were found to be less toxic than magainin, especially in the HaCaT cell line. The toxicity of the high concentration D-TN6 peptide was found to be more than 50%. According to the results given in Table 7, the MIC value of the D-TN6 peptide was 1µg/ml, while the peptide started to become toxic at a concentration of 16µg/ml. However, for example, TN3 peptide has been found to be non-toxic to the HaCaT cell line at high peptide concentrations, but becomes toxic to the cell at low concentrations. While

cytotoxicity values were 0% at high concentrations, it started to become toxic to the cell at 32μg/ml peptide concentration. It was found that peptides in the HeLa cell line were more toxic than magainine but all peptides, except TN3 peptide, had below 50% toxicity up to a concentration of 128μg/ml.

4.7 Results of Protease Resistance

HPLC Analysis of Peptides after Proteinase K Treatment

TN1, TN3 and TN6 peptides synthesized in D and L form were used in protease resistance assays. The analytical HPLC examination results of the peptides after treated with proteinase K, comparing the D and L forms, are given in the following figures 41, 42 and 43. In the HPLC chromatograms of the protease resistance assays, the peaks in the first 5 minutes belong to the TBS buffer and DMSO used in the experiment.

The retention time in HPLC of the L-form TN1 peptide not incubated with the proteinase K is 29.7 minutes in the given conditions above (Figure 11 A). The chromatograms in Figure 41 A, it is seen that the peptide disappeared at 29.7 minutes after incubation with proteinase K and appeared in different sizes at different retention times. It was observed that the retention time of the D-form TN1 peptide did not change before and after incubation (25.3 min at Figure 11 and Figure 41 B).

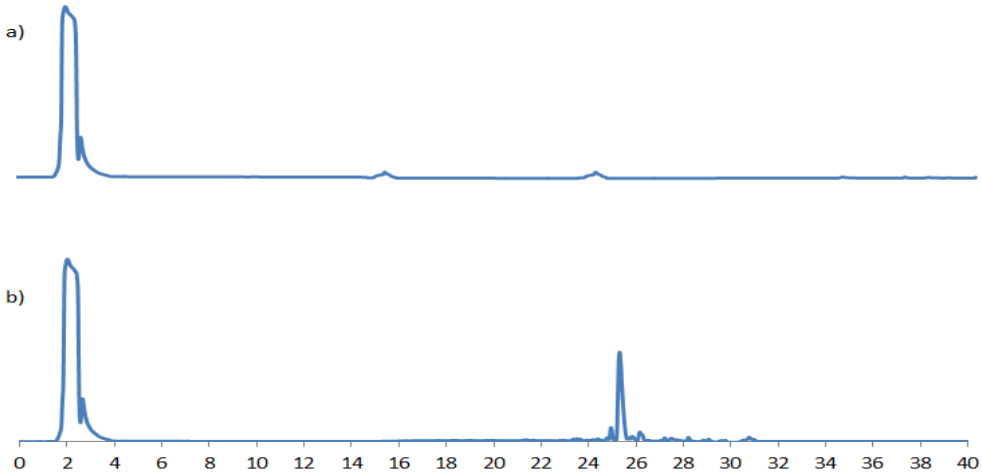

Figure 41 HPLC Chromatogram of TN1 Peptides After Protease Treatment

A) L-TN1 B) D-TN1

The retention time in HPLC of the L-form TN3 peptide not incubated with the proteinase K is 30.4 minutes in the given conditions above (Figure 12 A). The chromatograms in Figure 42 A, it is seen that the peptide disappeared at 30.4 minutes after incubation with proteinase K and three new peaks appeared. It was observed that the retention time of the D-form TN3 peptide did not change before and after incubation (29.8 min at Figure 12 B and Figure 42 B).

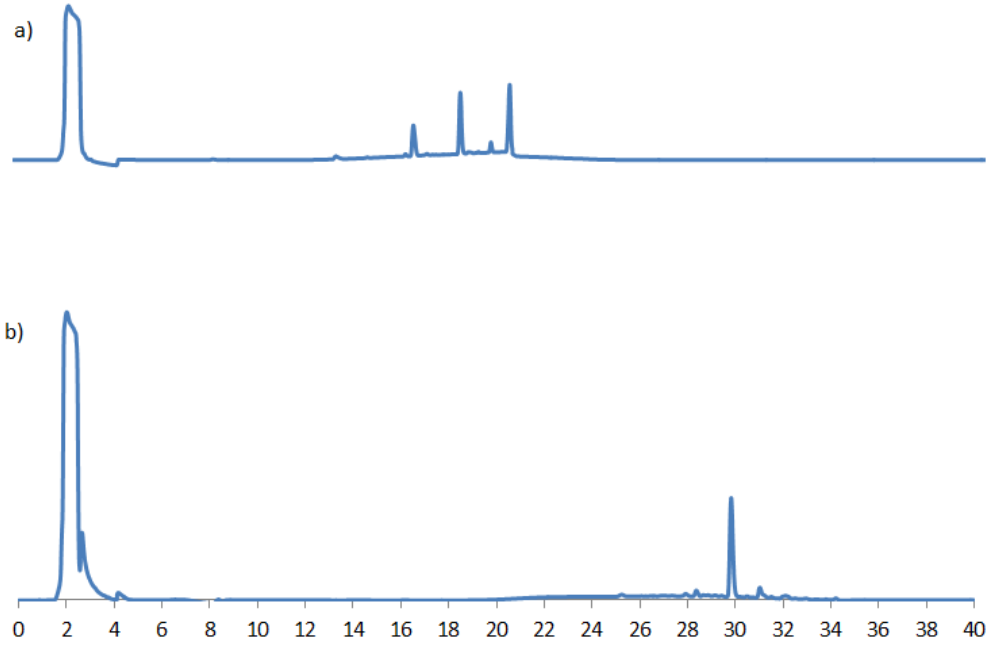

Figure 42 HPLC Chromatogram of TN3 Peptides After Protease Treatment

A)L-form TN3 B) D-form TN3

The retention time in HPLC of the L-form TN6 peptide not incubated with the proteinase K is 16.28 minutes in the given conditions above (Figure 13 A). The chromatograms in Figure 43 A, it is seen that the peptide disappeared at 16.28 minutes after incubation with Proteinase K and appeared in different sizes at different retention times. It was observed that the retention time of the D-form TN6 peptide did not change before and after incubation (16.8 min at Figure 13 B and Figure 43 B).

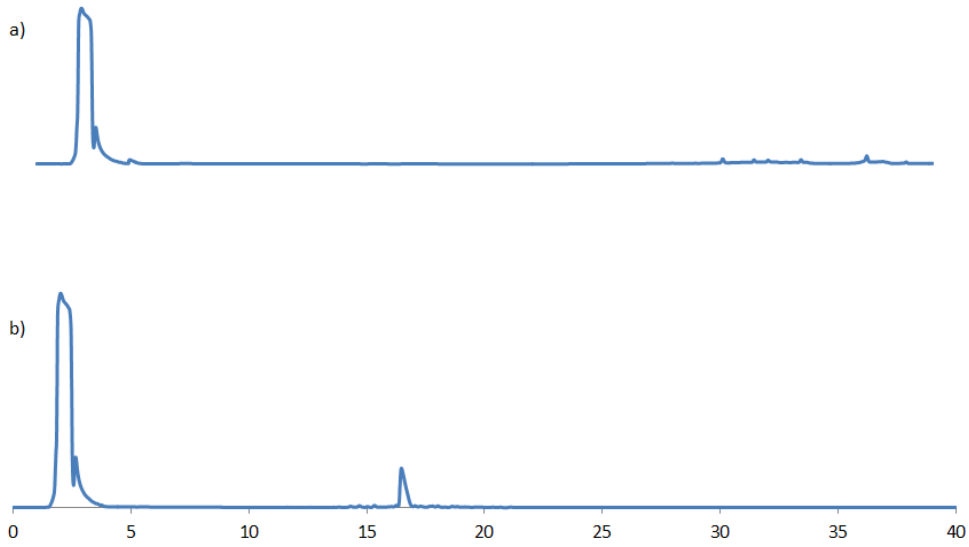

Figure 43 HPLC Chromatogram of TN6 Peptides After Protease Treatment

A) L form TN6 B) D form TN6

Biological Activity of Peptides after Proteinase K Treatment

The highest peptide concentration used in this study was 256 µg/mL. The buffer (TBS with 10% DMSO) without any peptide, used for proteinase assay, did not have any inhibitory effect on microorganisms.

MIC values for D-TN1, TN1, D-TN6 and TN6 peptides are given in Table 7. It was observed that the activities of D-TN1, D-TN3 and D-TN6 peptides, treated with Proteinase K, overnight were the same as the MIC values indicated in Table 7. This proved that biological activity is preserved in D-form peptides. For TN1, TN3 and TN6 peptides in L form, proteinase K-treated peptides lost their activity on *E. coli* (ATCC 25922), *S. aureus* (ATCC 29213) and *P.aeruginosa* (ATCC 27853). Peptides in L form, treated with Proteinase K were unable to inhibit bacterial growth at the highest concentration of 256 µg/ml.

4.8 Evaluation of the Effects of AMPs by SEM and TEM

Evaluation by SEM

SEM micrographs of *E. coli* ATCC 25922 samples without D-TN6 applied have a normal morphological structure. Bacteria belonging to this group have a long, smooth and robust bacterial surface structure (Figure 44 A-F).

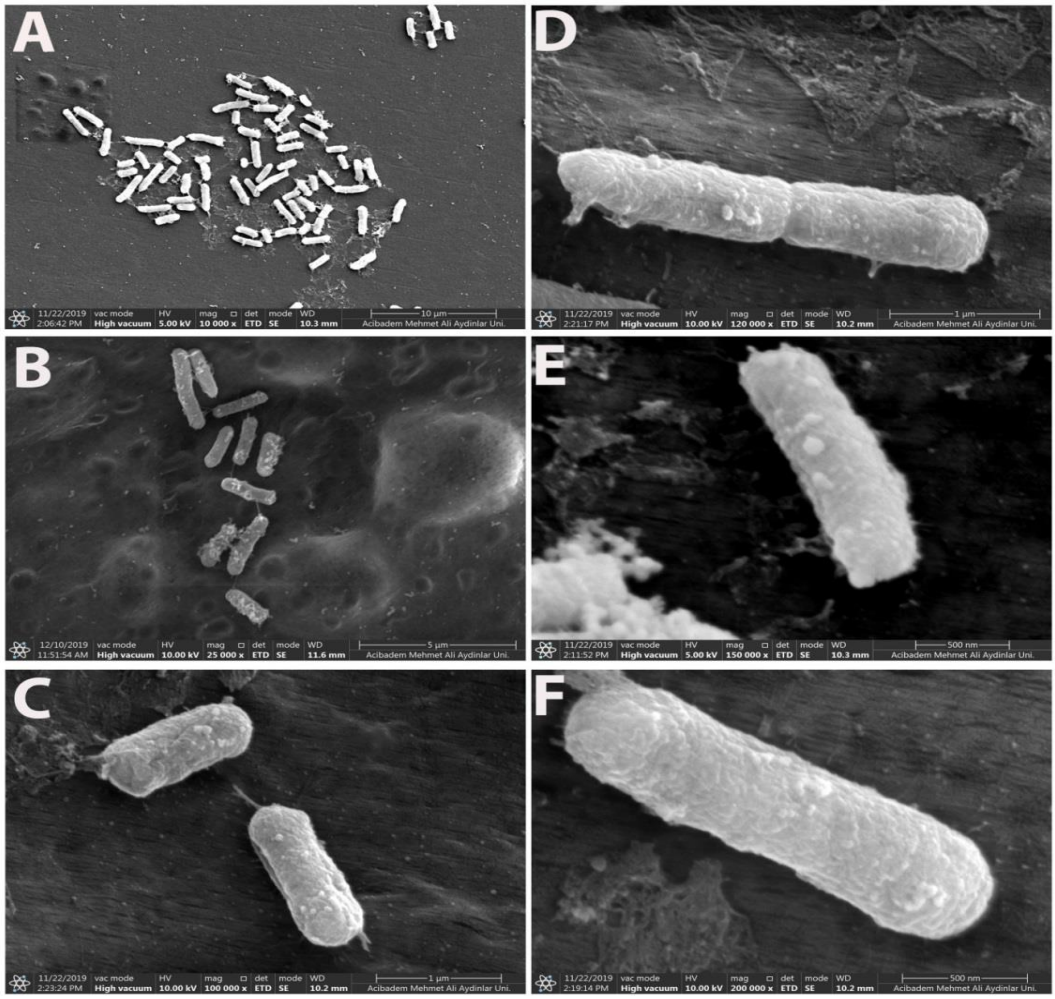

Figure 44 SEM micrographs of *E.coli* bacteria without D-TN6 application

Bacterial images taken at **A.** Low magnification (10.000) **B.** 25,000 magnifications **C.** 100.000 magnification **D.** Dividing bacteria at 120.000 magnifications **E.** 150,000 magnifications **F.** 200.000 magnifications.

In the samples of *E.coli* treated with 4 µg / ml D-TN6 peptide, bubble formation (Figure 45 B and D) and small depressions were observed on the surface of the cells (Figure 45 C, E, F). However, it was shown that the ordinary length of the bacteria remained unchanged.

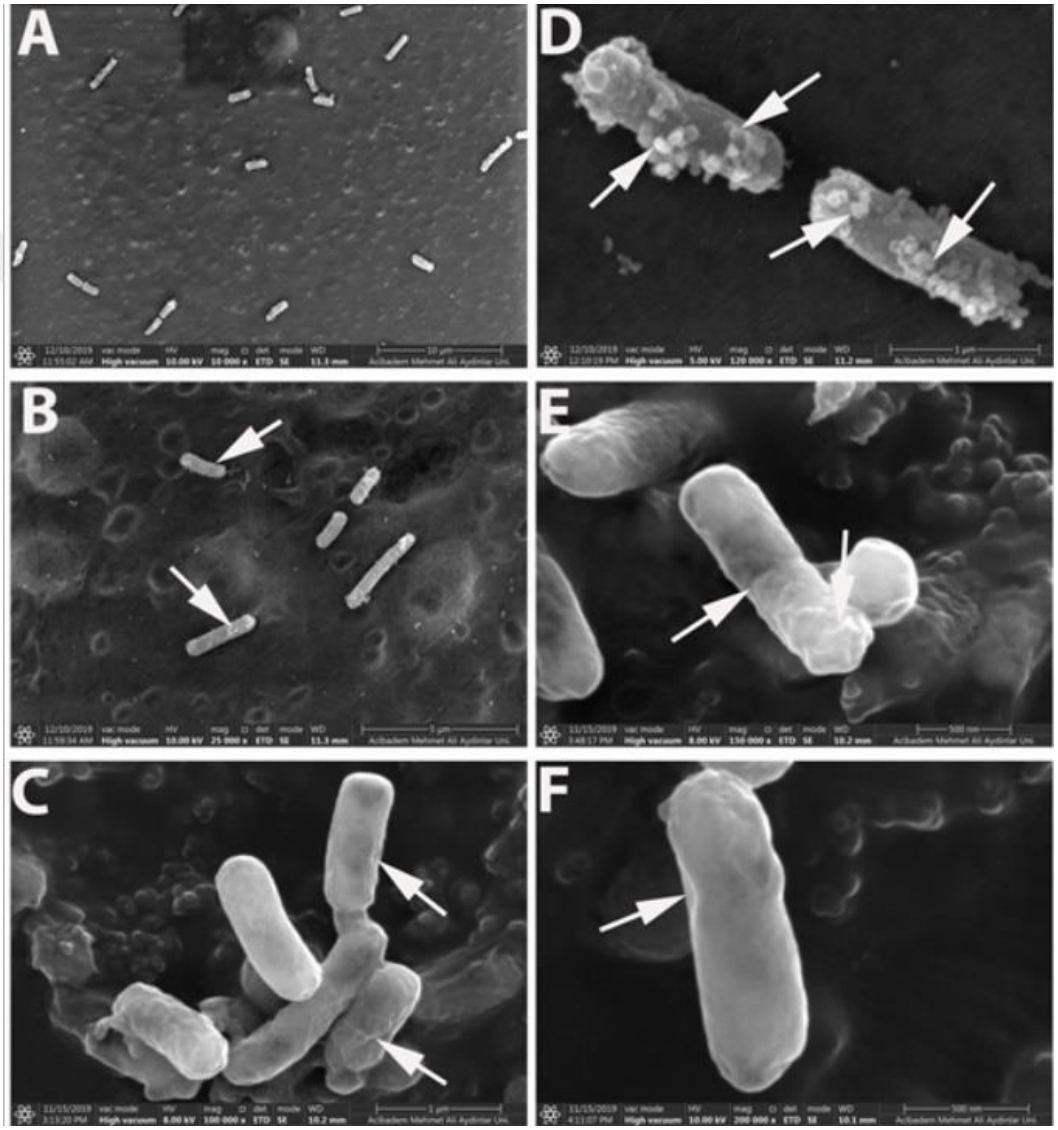

Figure 45 SEM micrographs of *E.coli* bacteria treated with 4 µg/ml D-TN6

Bacterial images taken at **A.** Low magnification (10.000) **B.** 25,000 magnifications, the arrow shows the bubble structure on the bacterial surface. **C.** 100.000 magnification, the arrow shows the hole structure on the bacterial surface. **D.** 120.000 magnification, arrows show the bubble structure on the bacterial surfaces **E.** 150,000 magnification, arrows show hole structures on the bacterial surfaces. **F.** 200.000 magnification arrows show hole structures on the bacterial surfaces.

Small holes (Figure 46 B) and bubble-like formations (Figure 46 B-F) were also observed in samples of *E.coli* bacteria treated with 8 µg / ml concentration of D-TN6. Bacteria with shorter lengths were detected in the bacteria samples belonging to this group.

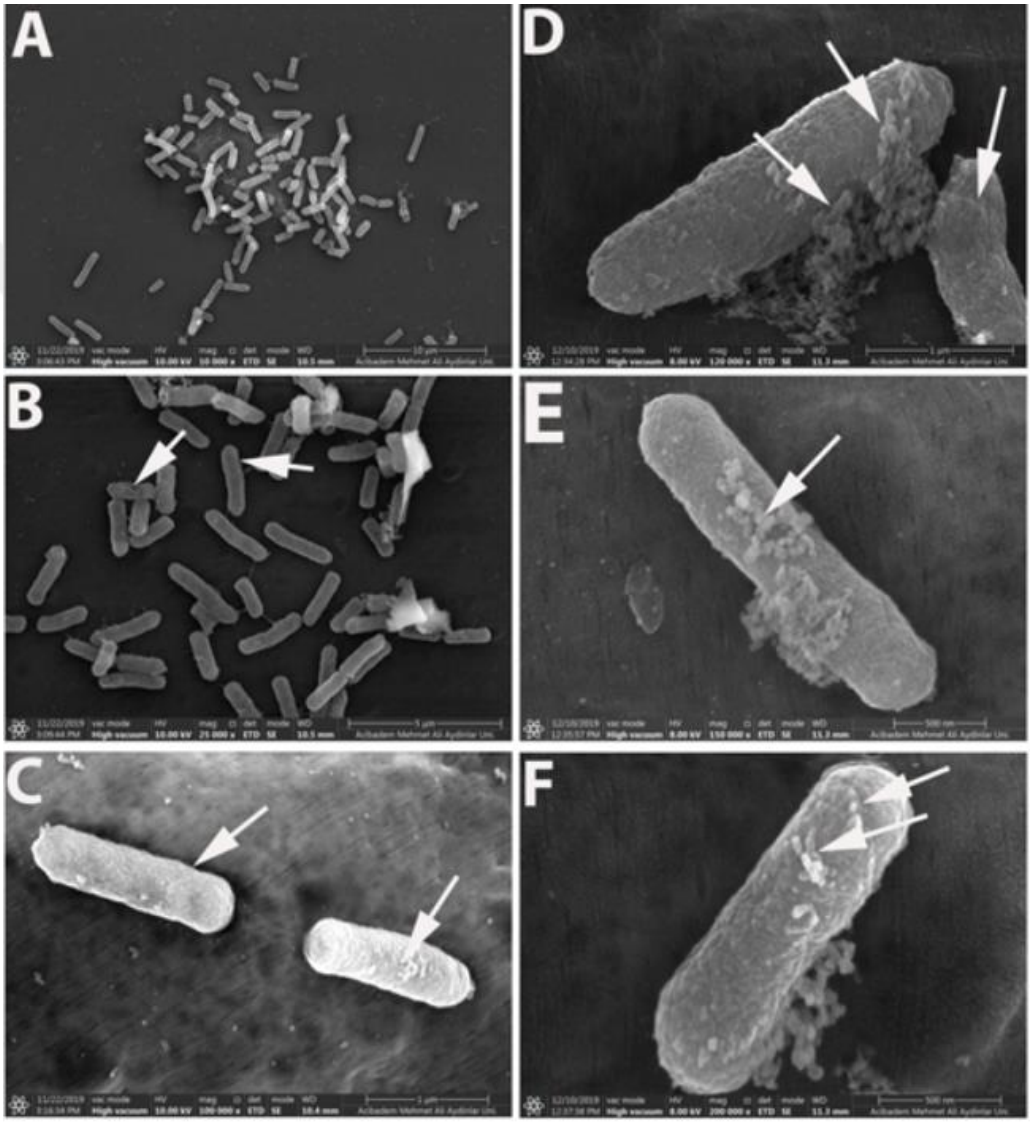

Figure 46 SEM micrographs of *E.coli* bacteria treated with 8 µg/ml D-TN6 application

Bacterial images taken at **A**. Low magnification (10.000) **B.** 25,000 magnifications, the arrow shows the hole structure on the bacterial surface. **C.** 100.000 magnification, the arrows show hole and bubble structures on the bacterial surface. **D.** 120.000 magnification, arrows show bubble structures on the bacterial surfaces **E.** 150,000 magnification, arrows show the bubble structures on the bacterial surfaces. **F.** 200.000 magnification arrows show hole and bubble structures on the bacterial surfaces.

In the samples of *E.coli* treated with 16 µg / ml D-TN6 applied, small hole-formations (Figure 47 E and F) and bubble-like structures (Figure 47 B-F) were observed. It appears that as the applied peptide concentration increases, the bubble-formations also increase.

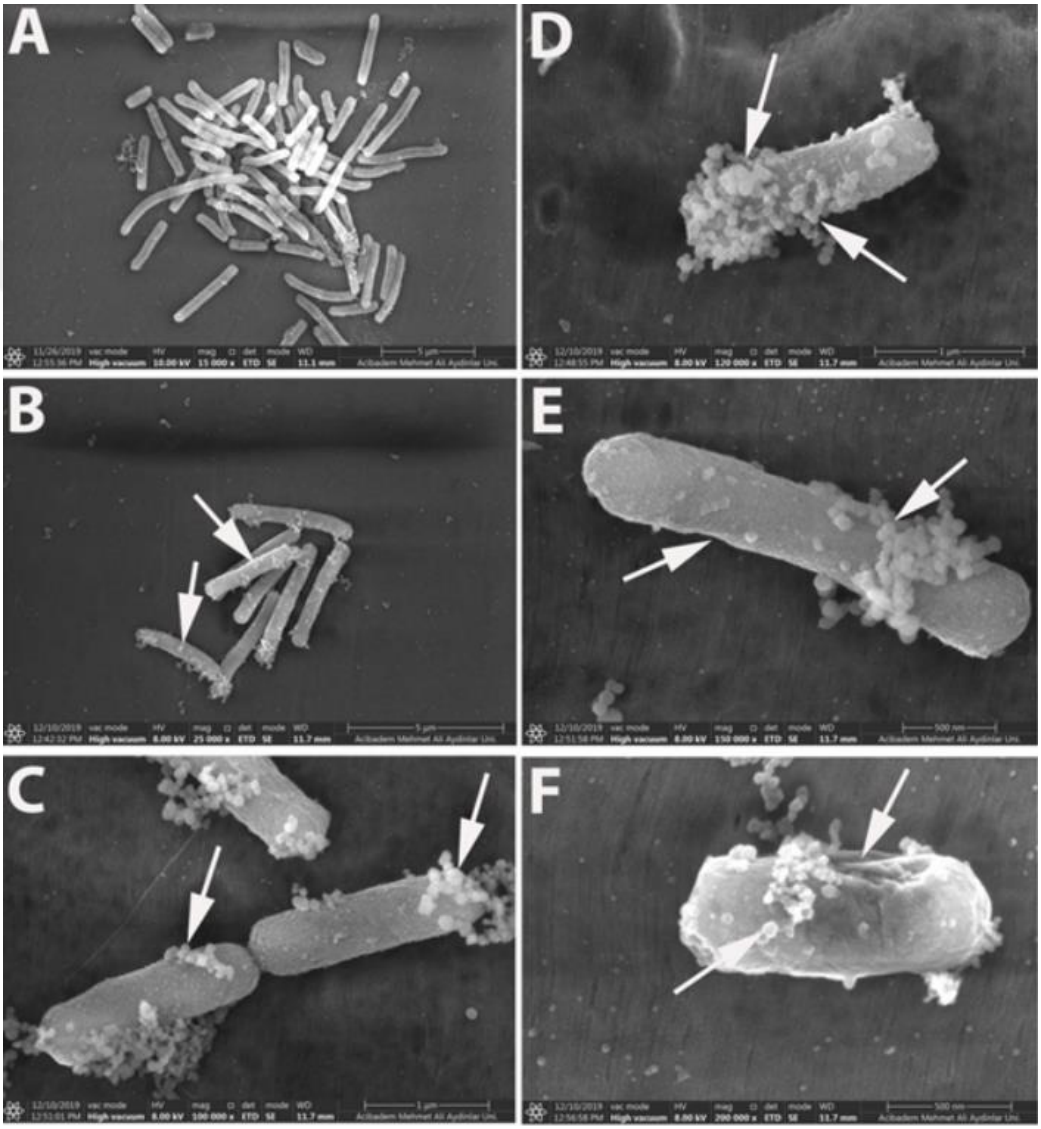

Figure 47 SEM micrographs of *E.coli* bacteria treated with 16 µg/ml D-TN6

Bacterial images taken at **A**. Low magnification (10.000) **B.** 25,000 magnification, arrows show bubble structures on the bacterial surface. **C.** 100.000 magnification, arrow shows bubble structures on the bacterial surface. **D.** 120.000 magnification, arrows show bubble structures on the bacterial surfaces **E.** 150,000 magnification, arrows show hole and bubble structures on the bacterial surfaces. **F.** 200.000 magnification arrows show hole and bubble structures on the bacterial surfaces.

Evaluation by TEM

TEM micrographs of bacterial samples without D-TN6 application also showed normal morphological structure. It has been determined that the inner and outer membranes maintain their integrity. Normal morphology was observed in the plasma membrane and cell wall (Figure 48 A-D).

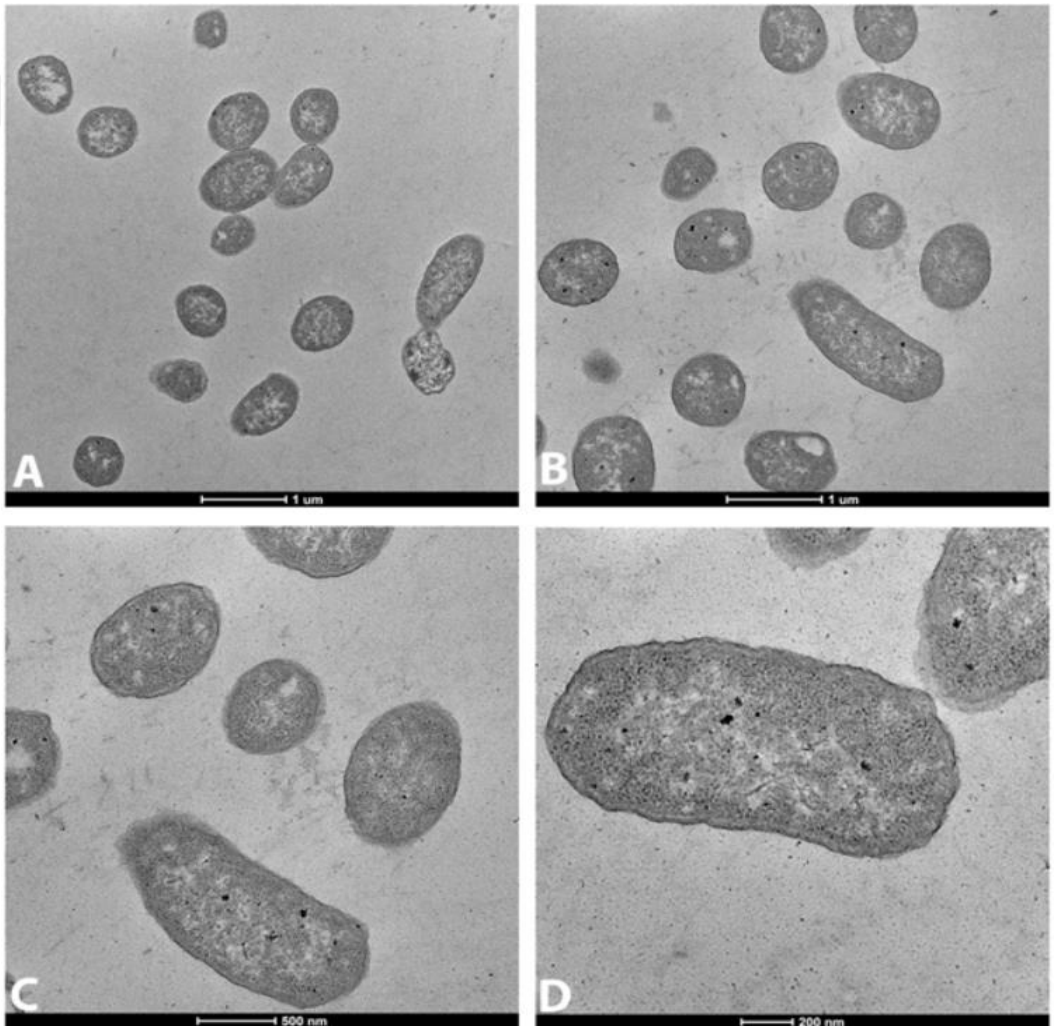

Figure 48 TEM micrographs taken of *E.coli* without D-TN6 application.

Normal morphology is observed in the samples of bacteria belonging to this group. A. A group of bacterial cells at low magnification. B. Normal morphology is observed in bacterial cells. C. Bacterial samples show normal structure and cytoplasmic integrity on the cell wall. D. Bacterial samples show normal morphology in the cell wall, cell membrane and cytoplasm.

In some samples of *E.coli* trated with 4µg/ml D-TN6, cell morphology appears to be impaired (Figure 49 A-D). In some bacteria, the cell membrane structure (Figure 49 B and C) separated from the cell wall and the electron-dense protrusion structure (Figure 49 C and D) on the cell surface are observed in the periplasmic space.

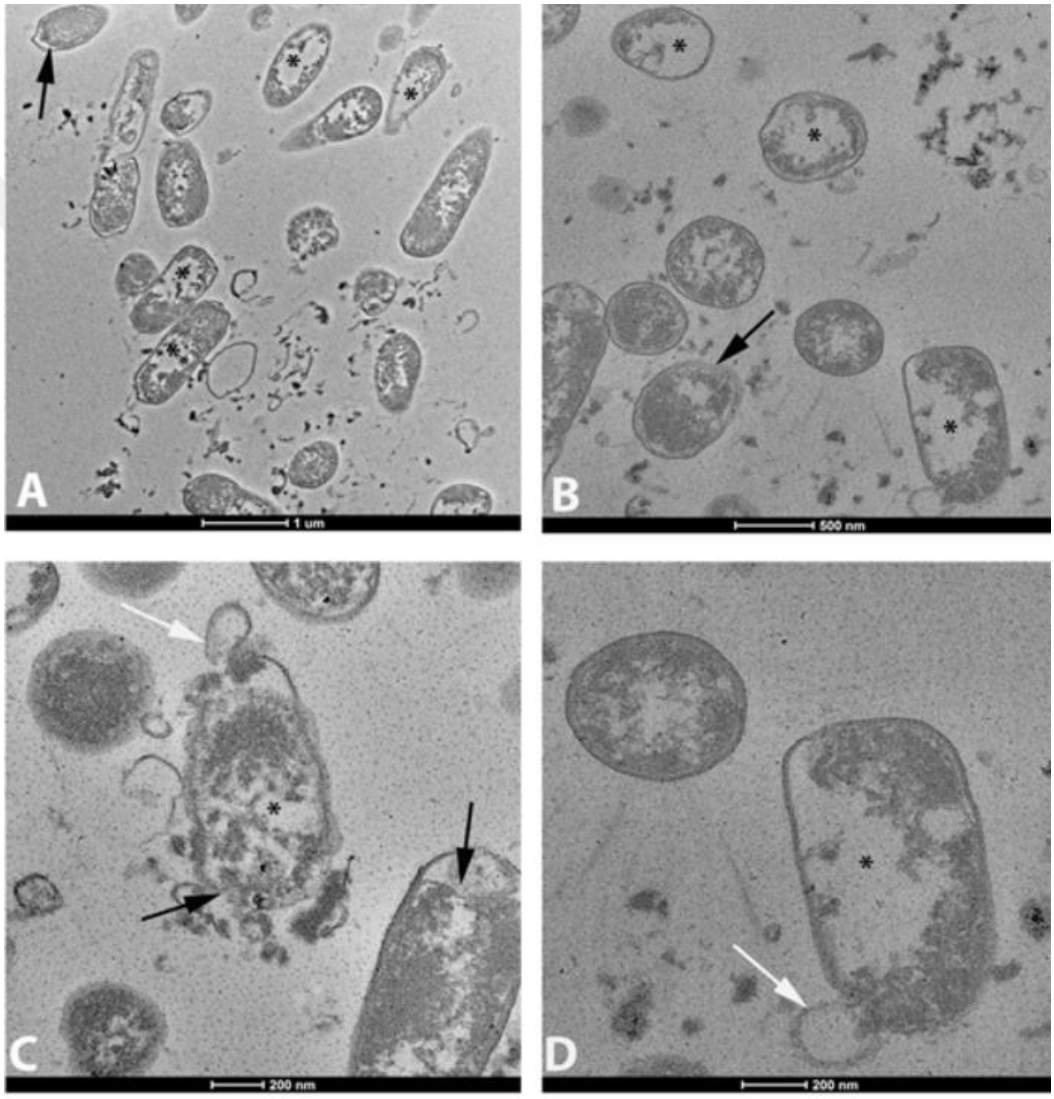

Figure 49 TEM micrographs taken of *E.coli* bacteria with 4 µg / ml D-TN6 application.

A. Impaired cytoplasmic structure (star) in bacterial cells and cell membrane structure (arrow) separated from the cell wall. **B.** Disrupted cytoplasmic structure (star) in bacterial cells and membrane separated from cell wall (arrow). **C.** Electronically dense protrusion (white arrow) on the cell surface in the periplasmic space, membrane structure (arrow) separated from the cell wall, and disrupted cytoplasmic structure (star). **D.** Electronically dense protrusion (arrow) and impaired cytoplasm (star) on the cell surface in the bacterial cell, periplasmic space at large magnification.

In the samples of *E.coli* treated with 8 µg/ml D-TN6 applied, impaired morphological structure is observed (Figure 50 A-D). Some bacteria have impaired membrane structure (Figure 50 B and D), cell membrane structure separated from the cell wall (Figure 50 B and C), and impaired cytoplasmic structure is observed (Figure 50 A-D).

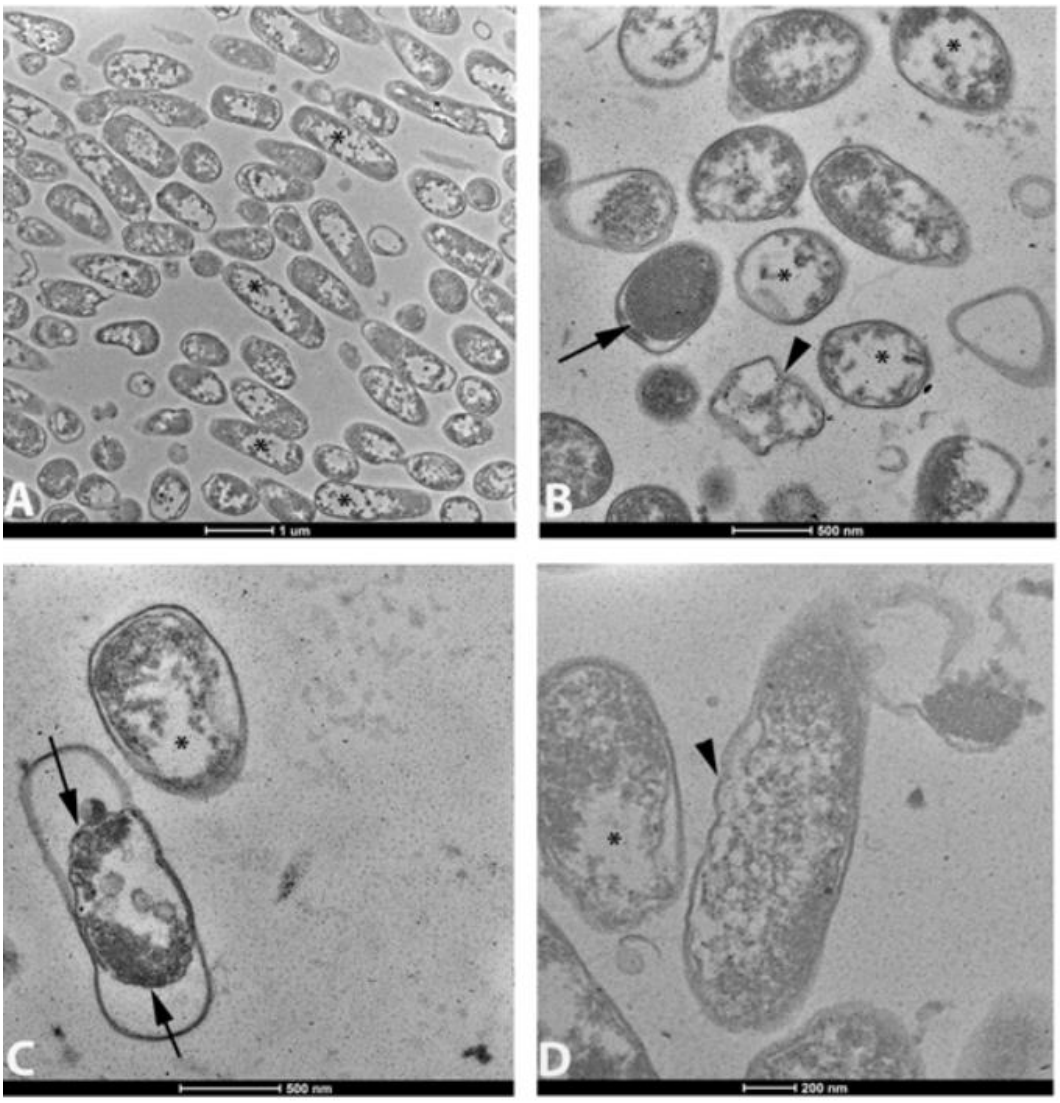

Figure 50 TEM micrographs of *E.coli* bacteria treated with 8 µg/ml D-TN6**.**

A.Disrupted cytoplasmic structure (star) is seen in bacterial cells. **B.** Impaired cytoplasmic structure (star) in bacterial cells, membrane structure separated from cell wall (arrow), ondulation observed in thickened and intermittent cell membrane structure (arrowhead). **C.** The membrane structure separated from the cell wall (arrow) and the disrupted cytoplasmic structure (star). **D.** Less dense of electron, thickened and intermittent appearance in the cell membrane structure, undulation (arrowhead) and impaired cytoplasm (star).

In the samples of E.coli treated with 16µg/ml D-TN6, disrupted morphological structure is observed (Figure 51 A-D). In some bacteria, a corrugated appearance with a thickening and discontinuous appearance in the cell membrane is observed (Figure 51 B), while in some bacteria, there are ballooned filamentous structures extending from the cytosol to the periplasmic area (Figure 51 B and D).

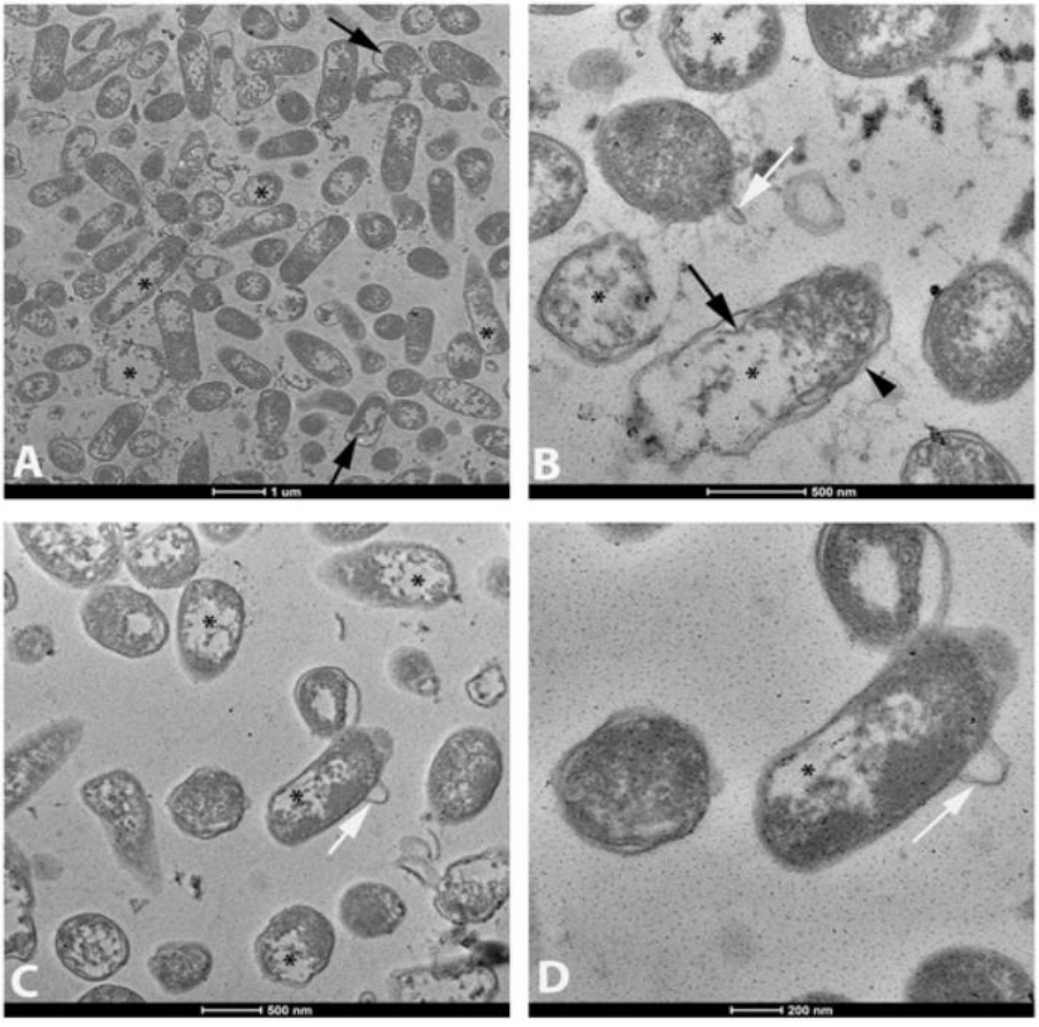

Figure 51. TEM micrographs of *E.coli* treated with 16µg/ml D-TN6.

A.Disrupted cytoplasmic structure (star) and membrane structure (arrow) separated from the cell wall is seen in bacterial cells. **B.** Impaired cytoplasmic structure (star) in bacterial cells, thickened and intermittent appearance in the membrane structure (arrowhead) and ballooned filamentous structure extending towards the cell surface (white arrow). **C.** Distrupted cytoplasmic structure (star) and ballooned filamentous structure (white arrow) extending towards the cell surface are seen. **D.** Large magnification shows disrupted cytoplasm structure (star) and ballooned filamentous structure (white arrow) extending towards the cell surface.)

5. DISCUSSION AND CONCLUSION

New antibiotic resistance mechanisms that have emerged globally continue to spread. According to the World Health Organization, wide spread of antibiotic resistance restricts our capability to control infectious diseases. This restriction results in prolonged illness, disability and death. Antimicrobial resistance occurs naturally over time with genetic changes. Recently, the abuse and overuse of increasing antimicrobials cause this process to accelerate. Antimicrobial resistant microorganisms are found in environmental sources, humans and animals. They can pass from animals to humans through nutrition and spread among humans. Improper use of food causes antimicrobial resistance to spread with poor health conditions. Antibiotic resistance occurs and spreads much faster than effective new antimicrobial agents enter clinical use. Rapid development of antibiotic resistance reduced the interest of the pharmaceutical industry in antimicrobial production. New researches have focused on new antimicrobial agents found in nature that microorganisms cannot develop resistance. Antimicrobial peptides are agents that are found in almost all living things in nature since millions of years, and microorganisms still do not develop resistance. AMPs, unlike many antibiotics, show bactericidal action instead of bacteriostatic action. AMPs mechanisms of action depend on damaging the cell membrane and disturb cytoplasmic components. Microorganisms need to change the lipid structure in the membrane layers in order to develop resistance to this target mechanism. This large-scale change is very difficult to achieve by the microorganism, also it can be harmful to the organism.

In 2015, Kim Lewis and his colleagues discovered the Eleftheria terrae Gram-negative soil bacteria with iChip technology and managed to grow in vitro. They reported that they isolated a new cell wall inhibitor antimicrobial molecule from this species of bacteria. This molecule called "teixobactin" contains enduracididine, methylphenylalanine, and four D-amino acids. Teixobactin has been found to have very good activity against bacteria such as *Streptococcus pneumoniae,*

Mycobacterium tuberculosis, which are known to develop antibiotic resistance. All developments in this direction offer important possibilities for the struggle against antimicrobial resistance of AMPs (144).

TN peptides which have alpha helical structure, designed by Unubol and friends, contain hydrophobic and positively charged amino acids. TN1, TN3 and TN6 sequences, which are designed to be rich in arginine and leucine, have high activity on microorganisms and low toxicity to cells of human origin. However, they have been found to be sensitive to proteases (8). In this study, we have developed protease resistant forms of these three peptides and modified their structure to increase their activity.

Pujals et al., synthesized the SAP peptide with D-amino acids by standard solid phase peptide synthesis method. They showed that the secondary structure of D-SAP is mirror image compared to the main peptide (145). The interaction between a protein receptor and its ligand is known to be a stereospecific. Thus, proteases cannot bind to D-amino acids, the enantiomer of the ligand. D-TN1, D-TN3 and D-TN6 peptides, which we synthesized with D-Aminoacids by standard solid phase peptide synthesis method, have been found to be resistant to proteases. Changing the amino acids from L form to D form changed their activity and toxicity.

TN1 peptide has positively charged arginine amino acid at the beginning and at the end of the AMP. When the TN1 peptide was synthesized with D-amino acids, its activity on *S. aureus* ATCC 29213 remained the same, while its activity on *E. coli* ATCC 25922 decreased 4 fold and its activity on *P. aeruginosa* ATCC 27853 increased 8 fold. When TN1's cytotoxic activity on HaCaT cells was investigated, it was observed that D-TN1 showed very high toxicity at 8μg/ml, however toxicity decreased as its concentration increased. TN1 peptide exceeded 50% toxic effect at

128µg/ml. When TN1's cytotoxic activity on HeLa cells was investigated, D-Tn1 had low toxicity even at a concentration of 128µg/ml. The lower cytotoxicity of the TN peptides to mammalian cells at higher concentration may be due to the load distribution and aggregation ability of the peptides to adhere to each other. In addition, in cytotoxicity experiments with SRB method, it is thought that there may be losses of cells in the washing steps although it is assumed that they are fixed to the plate however they may be liberated by the action of antimicrobial peptides and washed away. It was observed that the cytotoxic activities of both peptides to red blood cells, exceeded HC_{50} at a concentration of 8µg/ml. TN1 peptides which have MIC of 8µg/ml to *S. aureus* ATCC 29213, were toxic to HaCaT cells and red blood cells at this concentration. When these peptides were treated with proteinase K, which is an enzyme that digests proteins after hydrophobic amino acids and analysed by HPLC, it was observed that the retention time of D-TN1 stayed the same. However, the TN1 peptide did not arrive at the retention time of intact molecule, but produced small peaks at different retention times. This proved that the L-peptide was cleaved while the D-peptide remained intact by protease treatment.

Unlike TN1, the TN3 peptide contains arginine at only one end. It was observed that MIC values decreased 2 to 4 times against different bacteria by removing an arginine. When the toxic effects were compared, it was observed that toxic concentration to HaCaT cells varied. Toxicity exceeded 50% value in HeLa cells at a concentration of 16µg/ml. But at higher concentrations, it was obderved similarly that they had lower toxicity. They did not have an any toxic effect on red blood cells even at a concentration of 128µg/ml. This situation, which was also observed in TN1 and D-TN1 peptides, supported the idea that peptides may have been aggregating at higher concentration. D-TN3, the form of TN3 synthesized with D-amino acids, has better activity than TN3. The MIC values were at least 2 times lower. However, its toxic effects on mammalian cells and red blood cells were similar. Considering the MIC results, D-TN3 was found to be a more effective antimicrobial candidate than TN3. While it was observed that D-TN3 was resistant to protease, TN3 synthesized

with L-amino acids has been fragmented into three different size molecule in HPLC analysis.

TN6 is a peptide that contains arginine at both ends and contains less leucine than TN3. It has been shown to be very active against different strains of bacteria. When TN6 was synthesized with D-Amino acids, its activity was observed to increase at least twice. D-TN6 had lower activity against *P. aeruginosa* ATCC 27853, compared to other species of bacteria, having an MIC value of 8μg/ml. When the cytotoxic effects were examined, it was observed that toxicity of magainin to both forms of HeLa cell lines were similar. However, the toxicity of D-TN6 peptide in the HaCaT cell line increased above 50% at a concentration of 16μg/ml. The MIC of D-TN6 is 1μg/ml for *S. aureus* ATCC 29213 and *E. coli* ATCC 25922. So the toxic concentration of D-TN6 is 16 times the MIC value. In red blood cells, the toxicity of D-TN6 peptide remained below the HC_{50} value at a concentration of 128μg/ml. The toxicity of TN6 peptide increased above the HC_{50} at a concentration of 8μg/ml, but showed lower toxicity at higher concentrations. In protease treatment studies, D-TN6 peptide has been shown to be resistant to proteinase K.

New peptides have been synthesized by redesigning TN6 and TN3 peptides. They have been synthesized by replacing hydrophobic amino acids leucine with other hydrophobic amino acids Ile or val. TN3V1 and TN3V9 peptides have been designed using valine amino acid. TN3V1 peptide, designed with the changes of leucine and valine, while the arginines of the TN3 peptide remained constant. This change increased the activity on *S. aureus* ATCC 29213 by 2 folds, while its activity on other bacterial strains decreased. TN3V1 peptide, have been designed by replacement all leucines with valines, while the arginines of the TN3 peptide were kept constant. With this change the activity of the peptide on *S. aureus* ATCC 29213 remained the same, however, it became at least 4-fold less active against other species of bacteria. With these new designs of the peptides it was not possible to increase their activity. We assessed that leucine could better adhere to the membrane

than the valine and it looks wiser to continue developing AMPs with leucine containing peptides.

TN6I1 and TN6I2 peptides were synthesized by redesigning of the TN6 peptide with isoleucines instead of leucines. The TN6I1 peptide was created by replacing all leucines with isoleucines. The TN6I2 peptide was created by succeeding leucine and isoleucine amino acids. In the TN6I1 peptide, activity has been shown to decrease against all bacteria. In TN6I2 peptide, it has been observed that the activity against *S. aureus* ATCC 29213 remained the same, while the activity against other bacteria decreased. This has shown that leucine amino acid is a better choice than isoleucine, similar to valine.

The D-TN6 peptide was the most active AMP in this study. It was resistant to proteinase K, which is a very strong protease, which is an important feature that makes this peptide a strong antimicrobial drug candidate. In SEM and TEM scans with D-TN6, it was possible to observe the effects of this peptide on bacteria. In the examinations, it was observed that as Pajals and Lewis showed in their AMP studies and similar to the other information in literature AMP disrupted the bacterial membranes.

The AMPs, produced in this study and made resistant to protease, are promising drug candidate with their high antimicrobial activity and comparably low mammalian cell toxicity. The activity of these AMPs was tested with bacteria used as standard in antimicrobial activity experiments. These bacteria are known to be capable of developing resistance to the antibiotics used for the treatment. In addition, it will be an important to investigate the efficacy of these AMPs on strains known to be resistant to other antibiotics. Also, cytotoxicity experiments with mammalian cells should be repeated with different methods to understand why these peptides have

lower toxicity to mammalian cells at higher concentrations. Further studies in animal infection models will be crucial to lead the way to human clinical studies to turn the peptides developed in this study, to useful antimicrobial drugs.

6. REFERENCES

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7. CURRICULUM VITAE

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	Quantitative	**Equally Weighted**	**Verbal**
ALES Note	84,65	78,42	81,74

Publications

- Betul Zehra Karakus, İlknur Korkmaz, Kubra Demirci, Kadir Sinan Arslan, Ozge Unlu, Tunc Catal, Development of Bio-pesticide for Organic Farming, Turkish Patent and Trademark Office Turkish Patent and Trademark Office, PT2018-00785.

- Karakus, B.Z., Korkmaz, İ., Demirci, K., Arslan, K.S., Unlu, O., Catal, T., A Combined Treatment Using Ethylmethane Sulfonate and Ultraviolet Light to Compare Amylase Production by Three Bacillus sp. Isolates, Preparative Biochemistry and Biotechnology, 2018 doi: 10.1080/10826068.2018.1509088.

- Cebecioglu, R., Yaman, B., Karakus, B.Z., Demirci, K., Karakadioglu, G., Korkmaz, I., Bermek, H., Catal**, T**. Microbial Biotechnology: From Energy to Enzymes, Researches on Science and Art in 21st Century Turkey, Publisher: Gece Kitapligi, 2017, Vol 2. 2267.

Awards

- KKB 'HAYAL EDİN GERÇEKLEŞTİRELİM' Social Responsibility Project Competition 2nd prize in Social Category

Projects

- Establishment of Istanbul Protein Research Development and Innovation Center, Uskudar University, ISTKA, 2018,

- Development of Antimicrobial Peptides and Mimicking Molecules Through Inspiration from Nature, Acibadem University, TUBITAK, 2019

- Development of Neutralizing Antibodies Against COVID-19, TUBITAK, 2020